

I. PREFACE

The European Pharmacopoeia was inaugurated in 1964 through the Convention on the Elaboration of a European Pharmacopoeia. The present Fifth Edition of the European Pharmacopoeia is therefore published at the time where the 40th Anniversary of the Pharmacopoeia can be celebrated. The work on the Pharmacopoeia has gone through a remarkable development since the first difficult years. Elaboration and approval of monographs and other texts proceed by an effective and smoothly running process producing public quality standards that keep pace with scientific progresses. The work is remarkable because of its volume - the Fifth Edition presents close to 2000 monographs and other texts - and because all technical requirements have to be adopted by the European Pharmacopoeia Commission by unanimous decision. The monographs of the Pharmacopoeia are legally enforced in the countries being signatories to the Convention on the Elaboration of a European Pharmacopoeia. In addition to the 31 European countries and the European Union now being parties to the Convention, the work on the Pharmacopoeia is followed by 16 European and non-European countries and the WHO as observers. The quality standards of the European Pharmacopoeia have, therefore, an impact on the quality of medicines, which goes far beyond the European region.

The Fifth Edition of the European Pharmacopoeia will become effective on 1st January 2005. Like the Fourth Edition, the present main volumes will be added to by three annual supplements implementing the decisions of each of the three annual Sessions of the European Pharmacopoeia Commission. The presentation of the Pharmacopoeia in a main volume and three annual supplements was initiated by the publication of the Fourth Edition. The intention was to increase the flexibility of the publication scheme and, in particular, to shorten the time span between adoption and enforcement. The shortening of the time span, which has indeed been successful, is possible only thanks to a very flexible attitude by those countries that make national translations of the European Pharmacopoeia monographs. A very low number of rapid revisions implemented in the past three years is another result of the new publication scheme. The Fourth Edition is completed with the publication of Supplement 4.8 since it is impracticable to work with more than the eight supplements. The Commission decided therefore to proceed to the Fifth Edition by consolidation of the Fourth Edition after three years, only. The change from First to Second Edition was caused by major changes in the general methods, while the change from Second to Third Edition was due to the wish to consolidate the work achieved and to change the form of presentation from a loose-leaf format into a main volume followed by annual supplements. The change from Fourth Edition to Fifth Edition continues the work of making the publication of the Pharmacopoeia as user-friendly as possible. It is assumed that the publication of this Fifth Edition will proceed by publication of supplements over the next three years.

The eight founder countries of the Convention realised in 1964 that manufacturing and quality control standards for medicinal products on the European market had to be harmonised for reasons of public health and to facilitate the free movement of medicines. Since 1964 the world has changed and the market for medicinal products has become global. Accordingly, international harmonisation among the three major pharmacopoeias of the world, the European Pharmacopoeia, the Japanese Pharmacopoeia and the United States Pharmacopoeia, has been in progress since

1990 when the Pharmacopoeial Discussion Group was set up to co-ordinate the harmonisation work. In the first years, the work was focused on the harmonisation of monographs on widely used excipients. In the absence of harmonised general methods this was a difficult work, which has now been speeded up by 'harmonisation by attribute' meaning that there may be tests that cannot be fully harmonised before the concerned general method is harmonised. At the stage where the monographs are harmonised, detailed information will be provided in the monograph and in a chapter of the Pharmacopoeia devoted to information on international harmonisation. In recent years, harmonisation of a wide range of general methods has been in progress, partly because of an impact from the International Conference on Harmonisation (ICH). Implementation in the Pharmacopoeia of harmonised general methods, for example for a dosage form specification, needs careful consideration because the specification must be met by products already on the market as well as new products submitted to the regulatory process.

The European Pharmacopoeia Commission supports strongly the international harmonisation. It is not the harmonisation work itself that gives rise to the greatest problems, rather the implementation, which has to be decided in mutual agreement with the registration authorities. The links between the European Pharmacopoeia Commission and European regulators have been steadily strengthened during the years, as have the links with the pharmaceutical manufacturers and their associations.

The new European Directives 2001/82/EC and 2001/83/EC on medicines for human use and veterinary use maintain the mandatory character of the European Pharmacopoeia monographs in the preparation of dossiers for marketing authorisation of medicines, which was instituted in the first directive 75/318/EEC in 1975. It means that the monographs of the European Pharmacopoeia must therefore be updated to keep pace with products on the market, with scientific progress, and with regulatory developments. In the field of active pharmaceutical substances, the European Pharmacopoeia Commission decided at its March 2002 Session that the principles and terminology of the revised ICH Q3A impurity testing guideline *Impurities in new drug substances* should as far as possible be implemented in the monographs on active substances, both new and already published. A change in terminology has been introduced in the Impurities section of monographs published in Supplement 4.6 and later where the term 'specified impurities' is used for impurities that have a defined individual acceptance criterion. A revision of the general monograph *Substances for pharmaceutical use (2034)* was also presented in Supplement 4.6 to implement the threshold values for reporting, identification and qualification of organic impurities in active substances of the revised ICH guideline. For the Fifth Edition a new chapter, *5.10. Control of impurities in substances for pharmaceutical use* has been developed with great assistance by the chairs of the chemical Groups of Experts and other experts from the Commission, and by consultations of the Groups of Experts. The next step will be revision of monographs to ensure that they contain related substances tests and lists on specified and other detectable impurities. Monographs containing a related substances test based on TLC will be considered for revision. Major revision work will thus proceed during the coming years. Hopefully, these revisions can be completed with the publication of the Sixth Edition. In the meantime, users of the Pharmacopoeia must consult the new Chapter 5.10

VISIT...

LANZAROTE
Caliente.COM

on impurity control for the interpretation of monographs published in the past and therefore adapted to a style that has now been changed as described above. Users can in addition find information on representative chromatograms, reagents and columns used in drafting the monographs on the EDQM web site.

The aim of the revision is to ensure that the related substances test and impurity lists reflect the purity of pharmaceutical substances being authorised for the European market. The goal cannot be met without close collaboration with the registration authorities and consultations regarding the specifications for impurities. A procedure for co-operation with the CPMP/CVMP Quality Working Party has been established. It will certainly contribute to ensure the validity of the European Pharmacopoeia monographs. The Certification of Suitability of Monographs of the European Pharmacopoeia might be a valuable source of information on the purity of pharmaceutical substances. The procedure is, however, confidential and will be kept so. In cases where a new impurity is present and calls for revision of the monograph, this can be done only when the manufacturer provides the concerned Group of Experts with the information required for updating.

The growing number of monographs on pharmaceutical substances and the need to keep them updated means a great workload on the Groups of Experts. In 2001, the number of chemical groups was increased and some reallocations of experts between the groups took place. There is, however, still a need for more experts with access to experimental facilities as permanent members of the Groups of Experts or as members on an *ad hoc* basis. In addition to the reorganisation of the system of Groups of Experts and Working Parties the working procedures for the elaboration of monographs have been expanded. In addition to Procedure 1, the traditional elaboration by a Group of Experts, and Procedure 2, adaptation of national monographs, which is now considered almost complete, Procedures 3 and 4 have been established in recent years. Procedure 3 applies to substances produced by only one manufacturer and which are close to patent expiry. The manufacturer and the national pharmacopoeia authority of the country where the substance is produced carry out the preliminary drafting stages and check the requirements experimentally. The draft is reviewed by the working party also responsible for the adaptation procedure and then processed in the usual way by public inquiry. Procedure 3 has proved successful. The Commission decided in 2002 to establish a modified version, Procedure 4. This procedure implies collaboration between the manufacturer of the substance and the EDQM on the draft monograph and experimental checking by the EDQM laboratory and laboratories of national pharmacopoeia authorities before publication for public inquiry. At present, Procedure 4 is run as a pilot project supervised by members of the European Pharmacopoeia Commission. It is the aim of the Commission to have a full coverage of monographs on substances no longer subject to a patent and being present on more than one European market. It requires the collaboration with the innovators and manufacturers of active substances, which has been established during recent years.

The Fifth Edition of the European Pharmacopoeia has a number of excipient monographs containing a non-mandatory section on functionality-related characteristics. The aim is to provide users with a list of physical and physicochemical characteristics that are critical to the typical uses of the concerned excipient, and to provide the general methods required to assess

these characteristics. The section does not necessarily give acceptance criteria for the concerned properties; this is usually left as an option for labelling by the manufacturers and where specified, the values are indicative only. This is a new development which is in agreement with the policy of the European Pharmacopoeia Commission to make monographs and other texts appropriate to the needs of regulatory authorities and manufacturers of starting materials and medicinal products. The intention is to provide manufacturers of excipient materials and manufacturers of medicinal products a 'common language', to facilitate the establishment of product-specific specifications, and to provide regulators with data generated by methods that have been independently assessed.

It is the intention of the European Pharmacopoeia Commission to continue the work by drafting sections on functionality-related characteristics in monographs on excipients available in more than one physical grade. Introduction of the concept of functionality-related characteristics presupposes that the relevant general methods are available in the Pharmacopoeia. The European Pharmacopoeia Commission has therefore established a Working Party on synthetic polymers to investigate the need for general methods for polymers and a Working Party on powder characterisation methods. The provision of the needed general methods, for example in the field of powder characterisation, is also included in international harmonisation among the pharmacopoeias.

The achievements of the European Pharmacopoeia Commission during the past three years would not have been possible without the participation of the great number of experts from industry, academia and national authorities, who have given their time and expertise to the work of Groups of Experts and Working Parties. The Commission is indebted to all these experts whose work is given on a voluntary basis. The Commission is equally indebted to the Chairs of the Groups and Working Parties who have the responsibility of guiding the work through and bringing it to term according to tight time limits. The Chairs are thanked for their contributions within the Groups and also for their advice and counsel to the Commission itself.

The work of the European Pharmacopoeia Commission is strongly dependent on an effective Secretariat. The role of the Secretariat is to obtain and process all the information and reports needed for the Groups of Experts, Working Parties and for the Commission, to undertake laboratory work to support the experts and to ensure the availability of all the reference standards needed to allow the requirements in the monographs to be tested. The prompt publication of the Pharmacopoeia main volumes and Supplements and the on-line electronic version is possible, only, because of dedicated and hard work by the staff at the Secretariat.

Along with the growing volume of the European Pharmacopoeia and its adjustment to the regulatory process, the use of the Pharmacopoeia and its interpretation has become rather complex. The journal of the European Pharmacopoeia, *Pharmeuropa*, is a valuable source of information. General chapters for information will appear in the Pharmacopoeia during the publication of the Fifth Edition as a result of the international harmonisation, and because the European Pharmacopoeia Commission has agreed on the elaboration of other chapters for information. During the past two years, the staff at the EDQM have offered training courses to users of the Pharmacopoeia. The Commission is grateful to the EDQM for having taken this initiative, which also strengthens the role of the Pharmacopoeia and the links to its users. The links to users of the Pharmacopoeia are also strengthened by

the frequent workshops and conferences organised by the EDQM. This activity is highly valued by the Commission as it gives the opportunity to Commission members to exchange viewpoints and to discuss new developments with experts from authorities, industry and academia. The EDQM web site is another valuable source for information on the work programme and other activities of the Commission, its Groups and the EDQM.

During the past three years I have had the honour to serve the European Pharmacopoeia Commission as its elected chair. The task has been challenging but, certainly, rewarding because of the insight it has given me into the many quality aspects of the development, manufacture and marketing of medicinal products. I wish to thank members of the European Pharmacopoeia Commission for their support and collaborative spirit within and in between the Sessions of

the Commission. The two vice-chairs of the Commission are thanked for good collaboration and support during the years we have joined the Presidium. I will also thank the staff at the EDQM, in particular the secretaries to the Groups, for their kindness, enthusiasm and hard work for the benefit of the Pharmacopoeia. Finally, I wish to express warm thanks to the Director of EDQM, Dr. Agnes Artiges, and her deputy as secretary to the European Pharmacopoeia Commission, Mr. Peter Castle. I have appreciated our collaboration during the three years and wish to express heartfelt thanks to both for their support to the chair and for the tremendous work they are doing to develop the European Pharmacopoeia and its role in the European regulatory system.

Professor, Dr. Henning G. Kristensen

Chair of the European Pharmacopoeia Commission

II. INTRODUCTION

The European Pharmacopoeia is prepared under the auspices of the Council of Europe in accordance with the terms of the *Convention on the elaboration of a European Pharmacopoeia* (European Treaty Series No. 50) as amended by the Protocol to the Convention (European Treaty Series No. 134), signed by the Governments of Austria, Belgium, Bosnia and Herzegovina, Croatia, Cyprus, the Czech Republic, Denmark, Estonia, Finland, France, Germany, Greece, Hungary, Iceland, Ireland, Italy, Latvia, Luxembourg, the Netherlands, Norway, Portugal, Romania, Serbia and Montenegro, Slovak Republic, Slovenia, Spain, Sweden, Switzerland, “the Former Yugoslav Republic of Macedonia”, Turkey, the United Kingdom, and by the European Community.

The preparation of the Pharmacopoeia is the responsibility of the *European Pharmacopoeia Commission* (“the Commission”), appointed in accordance with Article 5 of the above-mentioned Convention. It is composed of delegations appointed by the Contracting Parties. Each delegation consists of not more than 3 members chosen for their competence in matters within the functions of the Commission.

Observers from non-Member States and international organisations are admitted to Sessions of the Commission in accordance with the Rules of Procedures. Observers are at present admitted from: Albania, Algeria, Australia, Bulgaria, Canada, China, Georgia, Lithuania, Malaysia, Malta, Morocco, Poland, Senegal, Syria, Tunisia, Ukraine, and the World Health Organisation.

The functions of the Commission established by Article 6 of the Convention as amended by the Protocol are:

Article 6

“Subject to the provision of Article 4 of the present Convention, the functions of the Commission shall be:

- (a) to determine the general principles applicable to the elaboration of the European Pharmacopoeia;
- (b) to decide upon methods of analysis for that purpose;
- (c) to arrange for the preparation of and to adopt monographs to be included in the European Pharmacopoeia and;
- (d) to recommend the fixing of the time limits within which its decisions of a technical character relating to the European Pharmacopoeia shall be implemented within the territories of the Contracting Parties.”

In accordance with the terms of the Convention, the Contracting Parties undertake to take the necessary measures to ensure that the monographs of the European Pharmacopoeia shall become the official standards applicable within their respective territories.

PURPOSE OF THE EUROPEAN PHARMACOPOEIA

The purpose of the European Pharmacopoeia is to promote public health by the provision of recognised common standards for use by health-care professionals and others concerned with the quality of medicines. Such standards are to be of appropriate quality as a basis for the safe use of medicines by patients and consumers. Their existence:

- facilitates the free movement of medicinal products in Europe;
- ensures the quality of medicinal products exported from Europe.

European Pharmacopoeia monographs and other texts are designed to be appropriate to the needs of:

- regulatory authorities;
- those engaged in the control of quality;
- manufacturers of starting materials and medicinal products.

The European Pharmacopoeia is widely used internationally. It is the intention of the Commission to work closely with users of the Pharmacopoeia in order to satisfy better their needs and facilitate their co-operation. To this end improved procedures are being developed for obtaining advice on priorities for elaborating new monographs and enhancing the quality of the Pharmacopoeia.

TECHNICAL SECRETARIAT AND LABORATORY

The European Pharmacopoeia Commission has a Technical Secretariat with scientific and administrative staff, situated in Strasbourg. The European Pharmacopoeia Laboratory is situated within the Secretariat and, amongst other duties, is in charge of the establishment and monitoring of all reference substances, preparations and spectra needed for the monographs of the Pharmacopoeia. The Technical Secretariat is an administrative division of the European Directorate for the Quality of Medicines (EDQM) of the Council of Europe.

GENERAL PRINCIPLES

General rules for interpretation of the texts of the Pharmacopoeia are given in the General Notices. The following information should also be noted.

The general principles applied in the elaboration of monographs of the European Pharmacopoeia are laid down in technical guides. The *Technical Guide for the Elaboration of Monographs*, which deals mainly with monographs on chemical substances, is available as a special issue of *Pharmeuropa* (see below under Publications). Other technical guides are being prepared to deal with aspects specific to monographs on other groups of products. The principles applied are revised from time to time without complete retrospective application so that monographs published already may not always follow the latest recommendations, but wherever an issue with impact on public health is identified, monographs are revised.

The procedures for the tests and assays published in the individual monographs have been validated, according to current practice at the time of their elaboration, for the purpose for which they are intended.

It is recognised that general chapters are used elsewhere than in the monographs of the Pharmacopoeia; in these circumstances users are recommended to consult the Technical Guide which gives extensive information on the application of many of the methods.

General monographs. The standards of the European Pharmacopoeia are represented by general and specific monographs. The use of general monographs has developed in recent years to provide standards that best fulfil the aims stated above and meet the needs of users. It is now usually necessary to apply one or more general monographs along with any specific monograph. Since it is not practically possible to include in each specific monograph a cross-reference to applicable or potentially applicable general monographs, cross-referencing has been discontinued except where it is necessary to avoid ambiguity.

A list of general monographs is included in each new edition and supplement to aid users in identifying those that are needed for use with a specific monograph.

Use of animals. In accordance with the *European Convention on the protection of animals used for experimental and other scientific purposes (1986)*, the Commission is committed to the reduction of animal usage, wherever possible, in pharmacopoeia testing and encourages those associated with its work to seek alternative procedures. An alternative or modified method is adopted by the Commission once it has been clearly demonstrated that it offers satisfactory control for pharmacopoeial purposes. Considerable progress was made in this area while the 4th Edition was in force and while the 5th Edition was being prepared.

Hydrates. With the publication of the 4th Edition, the policy on monograph titles for hydrated forms was changed. For all monographs published for the first time in the 4th Edition or subsequent editions, the degree of hydration, where applicable, is indicated in the monograph title. In previous editions, the policy was to indicate the degree of hydration only where several forms exist. If a monograph on both an anhydrous and a hydrated form of a given substance are published, then “anhydrous” will be included in the title of the relevant form. In order to avoid placing an unnecessary burden on manufacturers for relabelling, this policy will not be applied retrospectively to monographs published already, unless there is reason to believe that this is justified as a public health measure, notably for safety reasons where the substance contains a large proportion of water.

Chiral substances. Monographs on chiral substances that describe a particular enantiomer have a test to confirm enantiomeric purity, usually by measurement of optical rotation. Monographs that describe racemates are, in this respect, heterogeneous because of changes of policy during the 3rd Edition. Older monographs do not always have a test to show racemic character. During the course of the 3rd Edition, a test for racemic character was included in all new and revised monographs on racemates, using measurement of optical rotation. When it was shown that in many cases a test for optical rotation, even with narrow limits around zero rotation, was not necessarily sufficiently discriminating because of the low specific optical rotation of the enantiomers, the Commission modified the policy applied. A test for racemic character using optical rotation is now included only if there is information on the specific optical rotation of the enantiomers that indicates that such a test would be discriminating in terms of enantiomeric purity. If other techniques, such as circular dichroism, can serve the intended purpose, they will be prescribed instead of optical rotation.

Polymorphism. Where a substance shows polymorphism, this is usually stated under Characters. In general, no particular crystalline form is required in monographs; exceptionally, in a few monographs, the crystalline form required is specified, for example, via an infrared absorption spectrophotometric identification test where the spectrum is required to be recorded using the substance in the solid state without recrystallisation, the chemical reference substance provided being of the required crystalline form. However, for substances other than these exceptional cases, depending on the use of a given substance in a dosage form, it may be necessary for a manufacturer to ensure that a particular crystalline form is used. The information given under Characters is intended to alert users to the need to evaluate this aspect during the development of a dosage form. The monograph on *Substances for pharmaceutical use (2034)* and 5.9. *Polymorphism* should also be consulted.

Specificity of assays. For the elaboration of monographs on chemical substances, the approach generally preferred by the Commission is to provide control of impurities via a well designed Tests section rather than by the inclusion of an assay that is specific for the active moiety. It is therefore the full set of requirements of a monograph that is designed to ensure that the product is of suitable quality.

Impurities. Following a review of policy on control of impurities, a new general chapter 5.10. *Control of impurities in substances for pharmaceutical use* has been included in the 5th Edition. Together with the general monograph *Substances for pharmaceutical use (2034)*, it describes the policy of controlling impurities in specific monographs and provides explanations on how the limits in the related substances test should be understood. Currently the test is a limit test (comparison of peaks areas). In the future (next Edition) and in order to be in line with licensing practice and international collaboration, this test will progressively be changed to utilise a quantitative acceptance criterion. At present, some of the current monographs already satisfy this approach.

Except where required for the application of the monograph, in which case the name is followed by “CRS”, impurities are not provided as reference substances nor can they be provided for experimental purposes.

Chromatographic columns. As an aid to users, information is made available via the web site www.pheur.org on chromatographic columns that have been found satisfactory during development of monographs and general methods. Information is also given on other equipment and reagents where this is considered useful. This information is given without warranty and does not imply that other columns, equipment or reagents than those specified are not suitable.

Residual solvents. The requirements for residual solvents are given in the monograph *Substances for pharmaceutical use (2034)* together with the general chapters 2.4.24. *Identification and control of residual solvents* and 5.4. *Residual solvents*. Thus all active substances and excipients are subject to relevant control of residual solvents, even where no test is specified in the individual monograph. The requirements have been aligned with the ICH guideline on this topic.

Reference substances, reference preparations and reference spectra. Where necessary for application of a monograph, reference substances, reference preparations and reference spectra are established and provided to users. They are chosen for their suitability for the purposes stated in the monograph and are not necessarily suitable for other uses. Any necessary information for proper use is given, for example a declared content, but no complete certificate of analysis is provided since this is not relevant for the intended use. No expiry date is attributed to reference substances and preparations, which are subjected to regular periodic monitoring to ensure their continued suitability. Where an assigned value for a given attribute, for example chemical content, is provided, no uncertainty for the assigned value is indicated. The reference substances, preparations and spectra are provided to enable the analyst to determine compliance or otherwise with a monograph. The uncertainty of an assigned value is not to be taken into account when judging compliance, since the uncertainty is already allowed for in the prescribed limits.

Medical devices. All editions of the Pharmacopoeia have contained monographs on articles that are regarded as medical devices. For Member States of the European Union, a unified framework for standardisation of medical devices is now provided by a Directive (93/42/EEC). Following

an agreement between the various parties involved, the Commission has decided that the monographs on medical devices will be deleted once standards have been developed as foreseen by the Directive. Specifications included in the section on containers will be adapted to take account of future standards developed within the framework of the Directive. The monographs on surgical sutures remain in the Pharmacopoeia but they have been modified to conform to the requirements of the Directive and are now to be seen as standards of the type foreseen there. This adaptation of the monographs has involved deletion of some monographs on specific types of sutures in favour of a more general approach.

Homoeopathic preparations. A general monograph on homoeopathic preparations was added to the Pharmacopoeia during the 2nd Edition. A number of monographs on substances used in homoeopathic preparations are now also included and further monographs are in preparation. All of these texts have been grouped in a separate section. It is understood that when the same substance is used in both homoeopathic and other preparations then the monograph in the main body of the Pharmacopoeia applies.

Patents. The description in the Pharmacopoeia of articles subject to protection by patent does not confer or imply any right to the use of such patents by any person or persons other than the proprietors of the patents concerned.

Protected species. Monographs, notably those on herbal drugs, may cover material obtained from protected species. Inclusion of these monographs is without prejudice to the provisions for protection of these species by national and international law.

CERTIFICATION PROCEDURE

A procedure for the certification of suitability of monographs of the Pharmacopoeia with respect to control of the purity of a product from a given source has been established [see Public Health Committee (Partial Agreement) Resolution AP-CSP (99) 4 or any subsequent revision available from EDQM and on the web site (www.pheur.org)] as an aid to the use of monographs in applications for marketing authorisation. The certification procedure also applies to herbal drugs, herbal drug preparations and transmissible spongiform encephalopathy (TSE) risk. Certificates may be granted with respect to published monographs. Details of the operation of this scheme are

available from the Secretariat and on the EDQM web site. A daily updated list of certificates granted is available on-line on the EDQM web site. A list of voided or suspended certificates is also published in *Pharmeuropa*.

PUBLICATIONS

The European Pharmacopoeia is available in English and French versions in the form of a book with 3 supplements per year, and in electronic form (internet and CD-ROM).

Pharmeuropa, the European Pharmacopoeia Forum, is published 4 times per year as an aid in the elaboration of monographs and as a vehicle for information on pharmacopoeial and related matters. It is available on subscription from EDQM.

Web site. Information on activities and many other aspects of the European Pharmacopoeia is to be found on the EDQM web site (www.pheur.org).

Implementation. The date on which monographs are to be implemented is fixed by a resolution of the Public Health Committee (Partial Agreement) of the Council of Europe, following a recommendation by the Commission. This date is usually about 6 months after publication. Where a monograph is to be implemented at a date earlier than the next publication date of the Pharmacopoeia or a supplement, a Resolution of the Public Health Committee gives the full text to be implemented. The text is also published in *Pharmeuropa* for information and posted on the web site as part of the Resolution.

Revision programme. Monographs and other texts of the Pharmacopoeia are revised as necessary following a decision of the Commission. Revision proposals are published in *Pharmeuropa*.

INTERNATIONAL HARMONISATION

The European Pharmacopoeia is engaged in a process of harmonisation with the Japanese Pharmacopoeia and the United States Pharmacopoeia, within an informal structure referred to as the Pharmacopoeial Discussion Group (PDG). The activities are developed in co-ordination with those of the International Conference on Harmonisation (ICH). Information on the status of harmonised texts is given in chapter 5.8. *Pharmacopoeial harmonisation*. Harmonised general chapters have a preliminary statement indicating interchangeability with the other two pharmacopoeias.

III. EUROPEAN PHARMACOPOEIA COMMISSION

COMPOSITION OF THE COMMISSION, LIST OF EXPERTS AND OF THE SECRETARIAT AS OF 30 NOVEMBER 2003

CHAIR AND VICE-CHAIRS OF THE COMMISSION

Chair	Henning G.	KRISTENSEN	Hungary	Hilda Jozsef J.	KÖSZEGI-SZALAI LIPTAK
Vice-chairs	Dries Liisa	DE KASTE TURAKKA	Iceland	Gudrun Ingolf J.	BALDURSDOTTIR PETERSEN

MEMBERS OF THE COMMISSION

Austria	Kristof Andreas Christian	LISZKA MAYRHOFER NOE	Italy	Maurizio Anna Graziella	CIGNITTI FARINA OREFICI
Belgium	Luc Jos Paule	ANGENOT HOOGMARTENS JACQMAIN	Latvia	Janis	OZOLINS
Bosnia and Herzegovina	Aida	MEHMEDAGIC	Luxembourg	Jacqueline Jean-Louis	GENOUX-HAMES ROBERT
Croatia	Dragica Ivana Laila	BEGIC STARESINIC-SERNHORST STEFANINI ORESIC	Netherlands	Dries Jan Willem Pieter H.	DE KASTE DORPEMA VREE
Cyprus	Louis	PANAYI	Norway	Gunhild Valborg Randi	BRUGAARD HOLTEN WINSNES
Czech Republic	Jiri M.	PORTYCH TRAVNICKOVA	Portugal	José Manuel Rui	CORREIA NEVES SOUSA LOBO MORGADO
Denmark	Kirsten Steen Honoré Eva	BRØNNUM-HANSEN HANSEN SANDBERG	Romania	Daniele	ENACHE
Estonia	Signe	LEITO	Serbia and Montenegro	Marija Stana	MASKOVIC MICIC
Finland	Jussi Kaarina Liisa	HOLMALAHTI SINIVUO TURAKKA	Slovak Republic	N. Ruzena J.	CHALABALA MARTINCOVA SLANY
France	Jean-Paul An Alain	FOURNIER LÊ NICOLAS	Slovenia	Martina Evgen Uros	CVELBAR TOMAZIN URLEB
Germany	Ulrike Dietrich D.	HOLZGRABE KRÜGER SCHNÄDELBACH	Spain	Franco Jordi Alexandra	FERNANDEZ GONZALEZ RUIZ COMBALIA VARDULAKI
Greece	Michael A. Alexandra	KOUPPARIS TSOKA	Sweden	Lennart Marianne Christina	AKERBLOM EK GRAFFNER

Switzerland	Werner Silvia Helena	ERNI WEBER BRUNNER WINDEMANN	Slovak Republic	Daniel Ladislav	GRANCAI SOVIK
“The Former Yugoslav Republic of Macedonia”	Aneta Tatjana	DIMITROVSKA PERUSEVSKA	Slovenia	Maja Barbara Ales	LUSIN RAZINGER-MIHOVEC ROTAR
Turkey	Orhan Yilmaz Hayriye	CANBOLAT CAPAN MIHCAK	Sweden	Torbjörn	ARVIDSSON
United Kingdom	Derek H. Gerard A.David	CALAM LEE WOOLFSON	Switzerland	Andreas Uwe Eugen	BRUTSCHE VÖLKER WACHBERGER
European Commission	Nicolas	ROSSIGNOL	United Kingdom	Aileen M.T. John M.	LEE MIDGLEY
EMEA	Emer	COOKE			

ALTERNATE MEMBERS

Austria	J. Josef	KURZ TRENKER	Susan	AGERHOLM
Belgium	Jacques Luc Arnold J.	DE BEER DELATTRE VLIETINCK	Maqbool	AHMED
Denmark	Sven Lars J Lene	FRØKJAER HUSAGER THOMSEN	Jean-Marc	AIACHE
Estonia	Juhan	RUUT	Lennart	AKERBLOM
Finland	Hannele	SALOMIES	Ferhan	AKTAN
France	Thierry Hendrick Jan Caroline	BOURQUIN DE JONG VILAIN	Concepcion	ALONSO VERDURAS
Germany	Gerhard Rainer Rainer	FRANZ MOHR SEITZ	Hansruedy	ALTORFER
Greece	Evangelos A.	PETRODASKALAKIS TSANTILI-KAKOULIDOU	Julio	ALVAREZ-BUILLA
Hungary	Tamas L.	PAÁL	Hanspeter	AMSTUTZ
Ireland	Edward	BOURKE	Cyrille	ANDRES
Italy	Massimo Agostino Loredana	DI MUZIO MACRI NICOLETTI	Luc	ANGENOT
Luxembourg	Mariette	BACKES-LIES	Gunnar	ANTONI
Netherlands	Ellen Peter M.J.M. Jan-Anton	DE ROOIJ-LAMME JONGEN NORDER	Astrid	ARBIN
			Jean-Claude	ARGOUD
			Torbjörn	ARVIDSSON
			Wilfried	ARZ
			Nataliya Nikolaevna	ASMOLOVA
			Jérôme	AUCOUTURIER
			Sylvie	AUDOLY
			Paolo	AURELI
			Teresa	AZCONA LLANEZA
			Kenneth	BÄCKSTRÖM
			Elizabeth Ann	BAKER
			K. Hüsnü Can	BASER
			Michel	BAUER
			Alain	BAYOL
			Françoise	BEAUJEAN
			Denis	BELLENOT
			Leandro	BELLENTANI
			David N.	BENTLEY
			Brita	BERGH
			Kathrin	BERNARD-SUMMMATTER
			Serge	BESSET
			Pietro	BIANCHINI
			Jean-Pierre	BINDER
			Hanno	BINDER
			Mikael	BISRAT
			Johannes	BLÜMEL
			Giovanni	BOCCARDI
			Colin	BOOTH
			Nicole	BORNSTEIN
			Thierry	BOURQUIN
			Bernard	BOUYSSIÈRE

EXPERTS

Brigitte	BRAKE	Maria Helena	DOS ANJOS RODRIGUES AMARAL
Harald	BREIVIK	Robert	DRILLIEN
Per O.	BREMER	Anil	DUDANI
Einar M.	BREVIK	Siegfried	EBEL
Adrian F.	BRISTOW	Erling	EHRIN
Kirsten	BRØNNUM-HANSEN	Marianne	EK
Lukas	BRUCKNER	Torben	ELHAUGE
Peter	BRUEGGER	Ulrich	ENGEL
Volker	BUEHLER	Magnus	ERICKSON
Marian	BUKOVSKY	Bengt	ERLANDSSON
Rosario	BULLIDO	Jean-Pierre	ETCHEGARAY
Jörg	BUND	Øystein	EVENSEN
Roger	BURGENER	Bernard M.	EVERETT
Peter R.	BYRON	Charles J.	FALLAIS
Derek H.	CALAM	Gemma L.M.	FEENSTRA-BIELDERS
Kandemir	CANEFE	Peter	FEIGENWINTER
Salvador	CANIGUERAL	Rainer	FENDT
François	CANO	V'Iain	FENTON-MAY
Gunnar	CARLIN	Øystein	FODSTAD
Sergio	CAROLI	Ton	FÖRCH
A.J.	CAWS	Ulf	FORSMAN
Richard	CAWTHORNE	Lucien	FOSSE
Pierre	CHAMINADE	Isabelle	FOURASTÉ
Xavier	CHENIVESSE	Jean-Paul	FOURNIER
Vivienne	CHRIST	Bruno	FRANK
Maurizio	CIANFRIGLIA	Gerhard	FRANZ
Klaus	CICHUTEK	Florence	FUCHS
Juan	CLARAMUNT CAMPANA	Nicola	FUZZATI
Giovanni	COLLI	Rose E.	GAINES DAS
Laurence	COLLIERE	Maria Cristina	GALLI
Pierre-Albert	COMPAGNON	Andreas	GARDI
Stéphane	CORNEN	Jesus	GARICANO AISA
Giordano Bruno	CORSI	Didier	GARONNAT
Klaus	CUSSLER	Andrea	GAZZANIGA
Gérard	DAMIEN	Olga	GELDOF
Jacques-Christian	DARBORD	Nicole	GIBELIN
Alastair	DAVIDSON	Michel	GIRARD
Jacqueline	DAYAN-KENIGSBERG	Chris T.	GODDARD
Jacques	DE BEER	Marcel	GOVERDE
Josep M.	DE CIURANA i GAY	Christina	GRAFFNER
Hendrick Jan	DE JONG	Tatjana	GRAFNETTEROVA
Dries	DE KASTE	Daniel	GRANCAI
Carmen	DE LA MORENA CRIADO	Marta	GRANSTRÖM
Anna	DE PASQUALE	Norbert	GREIDZIAK
Ellen	DE ROOIJ-LAMME	Gerhard	GROHMANN
Paul	DECLERCK	Peter	GRONSKI
Clemens	DECRISTOFORO	Kjell-Olov	GRÖNVIK
Louis H.T.	DEDEREN	Jean Louis	GROSSIORD
Luc	DELATTRE	Emanuel	GUADAGNINO
Reto	DELLA CASA	Giuseppe	GUALANDI
Joseph	DEMEESTER	Michèle	GUITTET
Jan	DEN HARTIGH	Robert	GURNY
S.	DENYER	Sylvie	GUYOMARD-DEVANLAY
Jan J.	DIJKSTERHUIS	Klaus	HABERER
Roland	DOBBELAER	Lilian	HAMILTON
Johannes	DODT	Franz-Josef	HAMMERSCHMIDT
Eric	DOELKER	Steen Honoré	HANSEN
Erik	DOEVENDANS	Paul	HARGREAVES
Milada	DOLEZALOVA	Goetz	HARNISCHFEGER
Thomas	DOLL	Vassiliki	HARTOFYLAX
Jan Willem	DORPEMA	Kaare	HASLOV

Heribert	HÄUSLER	Marie-Frédérique	LE POTIER
Mary Alice	HEFFORD	Aileen M.T.	LEE
Ingrid	HEGGER	Eva	LEMBERKOVICS
Irmtraud	HELD	Ulla	LENNMARKER
Keith	HELLIWELL	Franck	LEVEILLER
Peter	HENRYS	Silvano	LONARDI
Jaakko-Juhani	HIMBERG	Céline	LORTEAU-SOURGEN
B.	HINSCH	Patrick	LOUIS
François	HIRSCH	Heiner	LUDWIG
Rikke	HOFF-JØRGENSEN	Bruce	MADSEN
Valborg	HOLTEN	Antonio	MANES ARMENGOL
Ulrike	HOLZGRABE	Warren C.	MANN
Ronald	HOOGERBRUGGE	Georges	MANSVELD
Ernö	HORVATH	Carla	MARCHIORO
Rolf	HOVIK	Ana Maria	MARQUES GONCALVES
Anthony R.	HUBBARD	Andreas	MARTI
Ronny	HÜBINETTE	Alessandro	MARTINI
Lars J	HUSAGER	D.L.	MASSART
Herwig	IGEL	Jos H.A.	MATHÔT
Marianne	INZELT-KOVACS	Andreas	MAYRHOFER
Miia	JAKAVA-VILJANEN	Bernard	MAZIERE
M.B.	JAMES	Geerd J.	MEYER
Jana	JERABKOVA	Jacques	MICHAUD
Christa	JERSCH	John M.	MIDGLEY
Mats E.	JOHANSSON	Giovanni	MIGLIACCIO
Edgar	JOHN	Marianne	MIKAELSSON
Robert	JOHNSON	Michael	MILCHARD
Peter M.J.M.	JONGEN	Miquel	MIR
Karl-Henrik	JÖNSSON	Tony	MOFFAT
Juan Ignacio	JORQUERA NIETO	Birgitte	MØLLGAARD
Mats	JOSEFSON	Thomas	MONTAG-LESSING
Jan	KARLSEN	Patrick	MONTENOISE
Hans	KEEGSTRA	Manfred	MOOS
Ernst	KELLER	Jacques	MOREL
Lawrence	KELLY	Laurence	MOUILLOT
Isabelle	KEMPF	Carl	MROZ
Damien	KERLOC'H	Zdenka	MRVOVA
Marylène	KOBISCH	B.W.	MÜLLER
Claus	KOCH	Hartwig	MÜLLER
Brigitte	KOPP	Michael	MUTZ
Frans	KORSE	Vera	MYSLIVCOVA
Hilda	KÖSZEGI-SZALAI	Philip S.	NEWLANDS
Katjusa	KREFT	Ria	NIBBELING
Henning G.	KRISTENSEN	Robin A.J.	NICHOLAS
Nikolaus G.	KRIZ	Steven C.	NICHOLS
Burt H.	KROES	Alain	NICOLAS
M.	KROON	Loredana	NICOLETTI
Michael	KRÜGER	Vittorio	NISTRIO
Ursula	KUKRAL	Jochen	NORWIG
Harry V.	KUPPERS	Dagmar	NOVA
Francesco	LA TORRE	Ningur	NOYANALPAN
Fritz	LACKNER	Werner	OBEXER
Reinhard	LANGE	Hok Liang	OEI
Mervi	LANKINEN	Edgar	OHST
Christophe	LAROCHE	Rose-Marie	ÖLANDER
Daniel	LARZUL	Bo	OLSSON
Annie	LASSERRE	Graziella	OREFICI
Maria Grazia	LAVAGGI	Carsten	OVERBALLE-PETERSEN
John	LAVDIOTIS	Inge	OVERBY JENSEN
Joyce	LAWRENCE	R.A.	PACKER
An	LÊ	A.	PADILLA

Béatrice	PANTERNE	Theres	SCHNEIDER
Roya	PARKER	Henrik	SCHULTZ
Berit Smestad	PAULSEN	Dieter	SCHULZ
Maurice	PENSAERT	Harald	SCHULZ
Teresa Caroline	PEPPER	Volker	SCHULZE
J.M.	PERSON	Michael	SCHWANIG
Skevos	PHILIANOS	Roland	SEGONDS
Geoffrey F.	PHILLIPS	Gerhard	SEIPKE
Jean-Paul	PICAULT	Rainer	SEITZ
Roger D.	PICKETT	Carlo	SERAFINI
Aude	PLANTEFEVE	Dorothea	SESARDIC
Wolfgang	POHLER	Carlo	SESSA
Bertrand	POIRIER	Hanfried	SEYFARTH
Gilles	PONCHEL	Ekrem	SEZIK
Stephen	POOLE	François	SIMONDET
Poly	POPOVA	Kaarina	SINIVUO
Agustín	PORTELA MOREIRA	Carl Einar	SJØGREN
Juhani	POSTI	Wenche	SKARE
Roberto	POZZI	Mikael	SKOOG
Brian	PRIESTLY	Jan W.H.	SMEETS
Bernard	PRILLEUX	William Henry	SMITH
Miluse	PSEIDLOVA	Glenn	SMITH
Ivan	PSIKAL	Robert	SOUSSAIN
Martin	PUNZENGRUBER	Axel	STAHLBOM
Ain	RAAL	Juerg	STALDER
Jean	RABIAN	Roland	STERN
Alain	RAGON	Otto	STICHER
H.H.T.	RAYMAKERS	Borut	STRUKELJ
Alessandro	REGOLA	Rainer	SUCHI
Eike	REICH	Adriana	SUPPA
Franz	REIGEL	Maryse	SURGOT
Ascensao Maria	RIBEIRO FARINHA	Karl Gustav	SVENSSON
Markus	RICHTER	Lennart	SVENSSON
Valérie	RIDOUX	Pierre-Cyril	TCHORELOFF
Therese	RINGBOM	Robin C.	THORPE
Hans Peter	RINIKER	Maria	TOLLIS
Jean-Louis	ROBERT	Keith G.	TRUMAN
Yves	ROCHÉ	Liisa	TURAKKA
Eugène	ROETS	Michael	TÜRCK
Joachim	RÖHMEL	Peter	TURECEK
Véronique	ROSILIO	Michel	ULMSCHNEIDER
Michael	RÖSSLER	Uros	URLEB
Ales	ROTAR	Miguel Angel	USERA
D.	RUDD	Lars	VAELDS FREDERIKSEN
Maria-Sol	RUIZ	Luisa	VALVO
Jordi	RUIZ COMBALIA	Anja	VAN ARKENS
Christoph	SAAL	F.J.	VAN DE VAART
Alain	SABOURAUD	Willem G.	VAN DER SLUIS
Michael	SAENGER	Heim	VAN DER VELDE
Corinne	SAINT-REQUIER	Cees	VAN DER VLIES
Piero A.	SALVADORI	Hans	VAN DOORNE
Eva	SANDBERG	Hans P.	VAN EGMOND
Magali	SAUTEL	Bernard M.M.	VAN GENUGTEN
Gabriele	SCHÄFFNER	Daniel	VAN GYSEGEM
Marjolijn	SCHALK	Jos	VAN ROMPAY
J.J.C.	SCHEFFER	Philippe	VANNIER
Jeannot	SCHELCHER	Alexandra	VARDULAKI
Martin	SCHIESTL	Paul	VARLEY
Ernst	SCHLÄFLI	Michel	VEILLARD
Heinz	SCHMITTER	Alfons	VERBRUGGEN
D.	SCHNÄDELBACH	Geert	VERDONK

Christopher H.	VERMAAT
Michel	VERT
Peep	VESKI
Philippe	VILLATTE
Eva	VITKOVA
Arnold J.	VLIETINCK
Pieter H.	VREE
Claude	WASTIEL
Stephen	WATERS
Silvia	WEBER BRUNNER
Marjolein	WEDA
Volker	WESSELY
A.J.	WEST
Elisabeth M.	WILLIAMSON
Randi	WINSNES
Maria	WIRZ
Bengt	WITTGREN
Bernhard	WOLF
David	WOOD
A.David	WOOLFSON
Kaskashan	ZAIDI
Giorgio	ZANNI
Max	ZELLER
Tatjana	ZEUGIN MISEV
Stéphan	ZIENTARA
Ange	ZOLA

SECRETARIAT OF THE EUROPEAN PHARMACOPOEIA COMMISSION

Director (European Directorate for the Quality of Medicines)

Agnès ARTIGES

Scientific Officers (Technical Secretariat, Laboratory and Biological Standardisation):

Peter	CASTLE (Secretary to the Commission)
John	MILLER
Jean-Marc	SPIESER
Stefan	ALMELING
Anne-Sophie	BOUIN
Karl-Heinz	BUCHHEIT
Catherine	CHAMBERLIN
Emmanuelle	CHARTON
Arnold	DAAS
Marie-Emmanuelle	BEHR-GROSS

Brigitte	JACQUEL
Andrea	LODI
Isabelle	MERCIER
Catherine	MILNE
Ellen	PEL
Pascale	POUKENS-RENWART
Guy	RAUTMANN
Ulrich	ROSE
Monica	SORINAS-JIMENO
Laure	TACONET
Michael	WIERER

Publication

Claude	COUNE
Hans-Joachim	BIGALKE
Stephan	BREHIN
Lynne	HENDERSON
Valérie	MÉAU-BOUDES
Catherine	NICOLAS
Alice	ROBERTS
Rachel	TURNER

Quality Assurance

Vincent	EGLOFF
Pierre	LEVEAU

Translation

Michelle	BACQUE
Benoît	BERNARD
Rex	HUISH

Public relations

Caroline	LARSEN-LE TARNEC
----------	------------------

Expert consultants

Syed Laik	ALI
Raymond	BOUDET-DALBIN

The European Pharmacopoeia Commission and the European Directorate for the Quality of Medicines also wishes to thank the Secretariat for their contribution towards the publication:

Isabelle	BYLINSKI
Anne	ESPIN
Sandra	FROMWEILER
Carole	KNAUP

IV. CONTENTS OF THE 5th EDITION

The 5th Edition consists of all texts published in the 4th Edition, which may subsequently have been revised or corrected, and new texts.

For the information of the reader, lists are given below of monographs and general chapters that are new, or that have been revised, corrected or deleted, and texts whose title has been changed for the 5th Edition.

The version date (01/2005 for volume 5.0) and the reference number (four digits for monographs and five digits for general chapters) are specified above the title of each text (monographs and general chapters). The version date makes it possible to identify the successive versions of revised texts in different volumes of the 5th Edition. Corrections that are indicated by the word “corrected” under the version date are to be taken into account from the publication date of the volume.

For the 5th Edition, the following systematic modifications have been made to the texts of the Pharmacopoeia.

- The term “specified impurities” has replaced “qualified impurities” in the Impurities section of monographs

in accordance with the texts on *Substances for pharmaceutical use (2034)* and *5.10. Control of impurities in substances for pharmaceutical use*. This term, which is compliant with the ICH guidelines, applies to impurities for which a specific acceptance criterion has been defined.

- In cases covered by the general monograph on *Substances for pharmaceutical use (2034)*, the test for sterility and the corresponding information in the Labelling section are no longer included in specific monographs.
- Chromatograms published for information no longer appear in the Pharmacopoeia; they are now available on the Internet site: www.pheur.org.
- A reference to available biological reference preparations has been added to the monographs concerned.
- The solubility in ether has been deleted from the Characters section and from the reagent descriptions.
- The reference to storage in a cool place has been deleted from the monographs and reagent descriptions.

A vertical line in the margin indicates where part of a text has been revised or corrected. A horizontal line in the margin indicates where part of a text has been deleted. It is to be emphasized that these indications, which are not necessarily exhaustive, are given for information and do not form an official part of the texts. Editorial changes are not indicated.

Individual copies of texts will not be supplied.

NEW TEXTS INCLUDED IN THE 5th EDITION

GENERAL CHAPTERS

- 2.4.30. Ethylene glycol and diethylene glycol in ethoxylated substances
- 2.6.24. Avian viral vaccines: tests for extraneous agents in seed lots (*previously texts 2.6.3, 2.6.4, 2.6.5 and 2.6.6*)
- 2.6.25. Avian live virus vaccines: tests for extraneous agents in batches of finished product (*previously texts 2.6.3, 2.6.4, 2.6.5 and 2.6.6*)
- 5.9. Polymorphism
- 5.10. Control of impurities in substances for pharmaceutical use
- 5.11. Characters section in monographs

MONOGRAPHS

The monographs below appear for the first time in the European Pharmacopoeia. They will be implemented on 1 January 2005 at the latest.

Monographs

- Botulinum toxin type A for injection (2113)
- Ciprofibrate (2013)
- Clioquinol (2111)

- Clofazimine (2054)
- Closantel sodium dihydrate for veterinary use (1716)
- Colestyramine (1775)
- Coriander oil (1820)
- Dipivefrine hydrochloride (1719)
- Dodecyl gallate (2078)
- Edrophonium chloride (2106)
- Formoterol fumarate dihydrate (1724)
- Human coagulation factor VIII (rDNA) (1643)
- Hydromorphone hydrochloride (2099)
- Insulin aspart (2084)
- Insulin lispro (2085)
- Isradipine (2110)
- Lactobionic acid (1647)
- Lysine acetate (2114)
- Mitomycin (1655)
- Octyl gallate (2057)
- Oleyl alcohol (2073)
- Oxeladin hydrogen citrate (1761)
- Propylene glycol dicaprylocaprate (2122)

Pyrantel embonate (1680)
 Ribavirin (2109)
 Salmon oil, farmed (1910)
 Tiamulin for veterinary use (1660)
 Tiamulin hydrogen fumarate for veterinary use (1659)
 Vinorelbine tartrate (2107)

Vaccines for human use

Meningococcal group C conjugate vaccine (2112)

Vaccines for veterinary use

Avian viral tenosynovitis vaccine (live) (1956)
 Bovine leptospirosis vaccine (inactivated) (1939)
 Infectious chicken anaemia vaccine (live) (2038)

Radiopharmaceutical preparations

Sodium fluoride (¹⁸F) injection (2100)
 Sodium iodide (¹³¹I) solution for radiolabelling (2121)

REVISED TEXTS IN THE 5th EDITION

GENERAL CHAPTERS

1. General notices
- 2.2.1. Clarity and degree of opalescence of liquids
- 2.2.3. Potentiometric determination of pH
- 2.2.5. Relative density
- 2.2.24. Absorption spectrophotometry, infrared
- 2.2.34. Thermal analysis
- 2.2.40. Near-infrared spectrophotometry
- 2.2.46. Chromatographic separation techniques
- 2.4.8. Heavy metals
- 2.6.9. Abnormal toxicity
- 2.6.14. Bacterial endotoxins
- 2.7.16. Assay of pertussis vaccine (acellular)
- 2.9.11. Test for methanol and 2-propanol
- 3.2.1. Glass containers for pharmaceutical use
4. Reagents
- 5.2.8. Minimising the risk of transmitting animal spongiform encephalopathy agents via human and veterinary medicinal products

MONOGRAPHS

The monographs below have been technically revised since their last publication. They will be implemented on 1 January 2005.

General monographs

Substances for pharmaceutical use (2034)

Monographs

Adrenaline tartrate (0254)
 Ammonium glycyrrhizate (1772)
 Bitter-fennel fruit oil (1826)
 Buserelin (1077)
 Cefapirin sodium (1650)
 Codergocrine mesilate (2060)
 Dexpanthenol (0761)
 Doxycycline hyclate (0272)
 Epirubicin hydrochloride (1590)
 Estradiol benzoate (0139)

Etilefrine hydrochloride (1205)
 Eucalyptus oil (0390)
 Famotidine (1012)
 Goserelin (1636)
 Human anti-D immunoglobulin (0557)
 Hyoscine butylbromide (0737)
 Hyoscine hydrobromide (0106)
 Hyoscyamine sulphate (0501)
 Insulin, human (0838)
 Iohexol (1114)
 Iopamidol (1115)
 Josamycin propionate (1982)
 Ketoprofen (0922)
 Lactose, anhydrous (1061)
 Lactose monohydrate (0187)
 Lavender oil (1338)
 Paraffin, light liquid (0240)
 Paraffin, liquid (0239)
 Penbutolol sulphate (1461)
 Povidone, iodinated (1142)
 Primidone (0584)
 Propyl gallate (1039)
 Propylene glycol (0430)
 Protirelin (1144)
 Risperidone (1559)
 Sulfamethoxazole (0108)
 Thiamazole (1706)

Vaccines for human use

BCG for immunotherapy (1929)
 BCG vaccine, freeze-dried (0163)
 Diphtheria and tetanus vaccine (adsorbed) (0444)
 Diphtheria and tetanus vaccine (adsorbed) for adults and adolescents (0647)
 Diphtheria, tetanus and hepatitis B (rDNA) vaccine (adsorbed) (2062)
 Diphtheria, tetanus and pertussis vaccine (adsorbed) (0445)

Diphtheria, tetanus, pertussis and poliomyelitis (inactivated) vaccine (adsorbed) (2061)

Diphtheria, tetanus, pertussis, poliomyelitis (inactivated) and haemophilus type b conjugate vaccine (adsorbed) (2066)

Diphtheria vaccine (adsorbed) (0443)

Diphtheria vaccine (adsorbed) for adults and adolescents (0646)

Haemophilus type b conjugate vaccine (1219)

Meningococcal polysaccharide vaccine (0250)

Tetanus vaccine (adsorbed) (0452)

Typhoid polysaccharide vaccine (1160)

Vaccines for veterinary use

Avian infectious bronchitis vaccine (live) (0442)

Avian infectious bursal disease vaccine (live) (0587)

Avian infectious encephalomyelitis vaccine (live) (0588)

Avian infectious laryngotracheitis vaccine (live) (1068)

Canine leptospirosis vaccine (inactivated) (0447)

Duck viral hepatitis type I vaccine (live) (1315)

Fowl-pox vaccine (live) (0649)

Marek's disease vaccine (live) (0589)

Newcastle disease vaccine (live) (0450)

CORRECTED TEXTS IN THE 5th EDITION

The texts below from the 4th Edition have been modified and specify "01/2005:XXXX-corrected" above the title. These modifications are to be taken into account from the publication date of the 5th Edition.

GENERAL CHAPTERS

2.4.18. Free formaldehyde

2.4.24. Identification and control of residual solvents

2.5.34. Acetic acid in synthetic peptides

2.6.15. Prekallikrein activator

2.6.21. Nucleic acid amplification techniques

2.7.6. Assay of diphtheria vaccine (adsorbed)

2.7.11. Assay of human coagulation factor IX

2.7.14. Assay of hepatitis A vaccine

2.7.15. Assay of hepatitis B vaccine (rDNA)

5.3. Statistical analysis of results of biological assays and tests

MONOGRAPHS

General monographs

Vaccines for veterinary use (0062)

Monographs

Acetylcholine chloride (1485)

Acetylcystein (0967)

Acitretin (1385)

Air, medicinal (1238)

Almagate (2010)

Alteplase for injection (1170)

Amantadine hydrochloride (0463)

Amikacin sulphate (1290)

Amoxicillin sodium (0577)

Ampicillin sodium (0578)

Anise oil (0804)

Aspartame (0973)

Azelastine hydrochloride (1633)

Azithromycin (1649)

Benfluorex hydrochloride (1601)

Benzyl alcohol (0256)

Calcium hydrogen phosphate, anhydrous (0981)

Calcium hydroxide (1078)

Carbidopa (0755)

Castor oil, hydrogenated (1497)

Castor oil, virgin (0051)

Cefaclor (0986)

Cefadroxil monohydrate (0813)

Cefamandole nafate (1402)

Cefixime (1188)

Cefotaxime sodium (0989)

Cefradine (0814)

Chlorhexidine digluconate solution (0658)

Ciclosporin (0994)

Cilazapril (1499)

Ciprofloxacin (1089)

Clarithromycin (1651)

Clobazam (1974)

Clobetasone butyrate (1090)

Detomidine hydrochloride for veterinary use (1414)

Dihydralazine sulphate, hydrated (1310)

Diphenhydramine hydrochloride (0023)

Disodium phosphate, anhydrous (1509)

Domperidone maleate (1008)

Doxepin hydrochloride (1096)

Ebastine (2015)

Econazole (2049)

Econazole nitrate (0665)

Eleutherococcus (1419)	Papaverine hydrochloride (0102)
Erythropoietin concentrated solution (1316)	Pefloxacin mesilate dihydrate (1460)
Fibrin sealant kit (0903)	Pepsin powder (0682)
Fish oil, rich in omega-3-acids (1912)	Phenoxymethylpenicillin (0148)
Foscarnet sodium hexahydrate (1520)	Phenoxymethylpenicillin potassium (0149)
Framycetin sulphate (0180)	Piroxicam (0944)
Gentamicin sulphate (0331)	Polymyxin B sulphate (0203)
Glycerol (0496)	Polysorbate 20 (0426)
Glycerol (85 per cent) (0497)	Potassium acetate (1139)
Glycine (0614)	Potassium clavulanate, diluted (1653)
Goldenrod (1892)	Povidone (0685)
Goldenrod, European (1893)	Pravastatin sodium (2059)
Gonadorelin acetate (0827)	Primaquine diphosphate (0635)
Guaifenesin (0615)	Propanol (2036)
Halofantrine hydrochloride (1979)	Propranolol hydrochloride (0568)
Heparins, low-molecular-mass (0828)	Propylene glycol dilaurate (2087)
Heptaminol hydrochloride (1980)	Propylene glycol monolaurate (1915)
Human anti-D immunoglobulin for intravenous administration (1527)	Pseudoephedrine hydrochloride (1367)
Human plasma for fractionation (0853)	Rifabutin (1657)
Human plasma (pooled and treated for virus inactivation) (1646)	Sodium alendronate (1564)
Hyaluronidase (0912)	Sodium hyaluronate (1472)
Hydroxycarbamide (1616)	Sodium polystyrene sulphonate (1909)
Ifosfamide (1529)	Somatropin (0951)
Imipramine hydrochloride (0029)	Somatropin bulk solution (0950)
Insulin, bovine (1637)	Somatropin for injection (0952)
Insulin, porcine (1638)	Sorbitol, liquid (crystallising) (0436)
Interferon alfa-2 concentrated solution (1110)	Sorbitol, liquid (non-crystallising) (0437)
Interferon gamma-1b concentrated solution (1440)	Spiramycin (0293)
Ioxaglic acid (2009)	Star anise oil (2108)
Isoflurane (1673)	Sucrose (0204)
Ivermectin (1336)	Tamoxifen citrate (1046)
Lemon oil (0620)	Thiamine hydrochloride (0303)
Lisinopril dihydrate (1120)	Ticlopidine hydrochloride (1050)
Lobeline hydrochloride (1988)	<i>DL</i> - α -Tocopheryl hydrogen succinate (1258)
Lomustine (0928)	<i>RRR</i> - α -Tocopheryl hydrogen succinate (1259)
Magaldrate (1539)	Tramazoline hydrochloride monohydrate (1597)
Maltitol, liquid (1236)	Triacetin (1106)
Methylprednisolone hydrogen succinate (1131)	Vancomycin hydrochloride (1058)
Methylthioninium chloride (1132)	Vinblastine sulphate (0748)
Minoxidil (0937)	Vincristine sulphate (0749)
Mometasone furoate (1449)	Water for injections (0169)
Netilmicin sulphate (1351)	Water, highly purified (1927)
Nifedipine (0627)	Water, purified (0008)
Nifuroxazide (1999)	Willow bark (1583)
Omega-3-acid ethyl esters 60 (2063)	Xylose (1278)
	Zopiclone (1060)

Vaccines for human use

Measles, mumps and rubella vaccine (live) (1057)

Measles vaccine (live) (0213)

Mumps vaccine (live) (0538)

Pertussis vaccine (acellular, co-purified, adsorbed) (1595)

Rubella vaccine (live) (0162)

Vaccines for veterinary use

Clostridium novyi (type B) vaccine for veterinary use (0362)

Clostridium perfringens vaccine for veterinary use (0363)

Clostridium septicum vaccine for veterinary use (0364)

Equine influenza vaccine (inactivated) (0249)

Foot-and-mouth disease (ruminants) vaccine (inactivated) (0063)

Swine erysipelas vaccine (inactivated) (0064)

Tetanus vaccine for veterinary use (0697)

Radiopharmaceutical preparationsIobenguane (¹²³I) injection (1113)L-Methionine ([¹¹C]methyl) injection (1617)**Homoeopathic preparations**

Iron for homoeopathic preparations (2026)

TEXTS WHOSE TITLE HAS CHANGED FOR THE 5th EDITION*The titles of the following texts have been changed in the 5th Edition.***GENERAL CHAPTERS**2.2.34. Thermal analysis (*previously Thermogravimetry*)2.7.4. Assay of human coagulation factor VIII (*previously Assay of blood coagulation factor VIII*)3.1.4. Polyethylene without additives for containers for parenteral preparations and for ophthalmic preparations (*previously Polyethylene without additives for containers for preparations for parenteral use and for ophthalmic preparations*)3.1.5. Polyethylene with additives for containers for parenteral preparations and for ophthalmic preparations (*previously Polyethylene with additives for containers for preparations for parenteral use and for ophthalmic preparations*)3.1.6. Polypropylene for containers and closures for parenteral preparations and ophthalmic preparations (*previously Polypropylene for containers and closures for preparations for parenteral and ophthalmic use*)5.2.8. Minimising the risk of transmitting animal spongiform encephalopathy agents via human and veterinary medicinal products (*previously Minimising the risk of transmitting animal spongiform encephalopathy agents via medicinal products*)**MONOGRAPHS****Monographs**Amfetamine sulphate (0368) (*previously Amphetamine sulphate*)Riboflavin (0292) (*previously Riboflavine*)Riboflavin sodium phosphate (0786) (*previously Riboflavine sodium phosphate*)Tosylchloramide sodium (0381) (*previously Chloramine*)Triacetin (1106) (*previously Glycerol triacetate*)**Vaccines for veterinary use**Avian infectious bronchitis vaccine (live) (0442) (*previously Avian infectious bronchitis vaccine (live), freeze-dried*)Avian infectious bursal disease vaccine (live) (0587) (*previously Avian infectious bursal disease (Gumboro disease) vaccine (live), freeze-dried*)Avian infectious laryngotracheitis vaccine (live) (1068) (*previously Avian infectious laryngotracheitis vaccine (live) for chickens*)Canine leptospirosis vaccine (inactivated) (0447) (*previously Leptospira vaccine for veterinary use*)Duck viral hepatitis type I vaccine (live) (1315) (*previously Duck viral hepatitis vaccine (live)*)Fowl-pox vaccine (live) (0649) (*previously Fowl-pox vaccine (live), freeze-dried*)Newcastle disease vaccine (live) (0450) (*previously Newcastle disease vaccine (live), freeze-dried*)**TEXTS DELETED FOR THE 5th EDITION***The following texts were deleted on 1 January 2005.***GENERAL CHAPTERS**

2.6.3. Tests for extraneous viruses using fertilised eggs

2.6.4. Test for leucosis viruses

2.6.5. Test for extraneous viruses using cell cultures

2.6.6. Test for extraneous agents using chicks

MONOGRAPHS

Carbenicillin sodium (0812)

CONTENTS

VOLUME 1

I. PREFACE	i
II. INTRODUCTION	v
III. EUROPEAN PHARMACOPOEIA COMMISSION	ix
IV. CONTENTS OF THE FIFTH EDITION	xv
GENERAL CHAPTERS	
1. General Notices	1
2. Methods of Analysis	13
2.1. Apparatus	15
2.2. Physical and physicochemical methods	21
2.3. Identification	93
2.4. Limit tests	101
2.5. Assays	125
2.6. Biological tests	143
2.7. Biological assays	185
2.8. Methods in Pharmacognosy	213
2.9. Pharmaceutical technical procedures	223
3. Materials for Containers, and Containers	265
3.1. Materials used for the manufacture of containers	267
3.2. Containers	301
4. Reagents	319
5. General Texts	441
GENERAL MONOGRAPHS	567
MONOGRAPHS ON DOSAGE FORMS	597
MONOGRAPHS ON VACCINES FOR HUMAN USE	633
MONOGRAPHS ON VACCINES FOR VETERINARY USE	713
MONOGRAPHS ON IMMUNOSERA FOR HUMAN USE	799
MONOGRAPHS ON IMMUNOSERA FOR VETERINARY USE	807
MONOGRAPHS ON RADIOPHARMACEUTICAL PREPARATIONS	815
MONOGRAPHS ON SUTURES FOR HUMAN USE	871
MONOGRAPHS ON SUTURES FOR VETERINARY USE	883
MONOGRAPHS ON HOMOEOPATHIC PREPARATIONS	891

VOLUME 2

MONOGRAPHS	903
INDEX	2739

Note : on the first page of each chapter/section there is a list of contents.

01/2005:10100

1.1. GENERAL STATEMENTS

The General Notices apply to all monographs and other texts of the European Pharmacopoeia.

The official texts of the European Pharmacopoeia are published in English and French. Translations in other languages may be prepared by the signatory States of the European Pharmacopoeia Convention. In case of doubt or dispute, the English and French versions are alone authoritative.

In the texts of the European Pharmacopoeia, the word “Pharmacopoeia” without qualification means the European Pharmacopoeia. The official abbreviation Ph. Eur. may be used to indicate the European Pharmacopoeia.

The use of the title or the subtitle of a monograph implies that the article complies with the requirements of the relevant monograph. Such references to monographs in the texts of the Pharmacopoeia are shown using the monograph title and reference number in *italics*.

A preparation must comply throughout its period of validity; a distinct period of validity and/or specifications for opened or broached containers may be decided by the competent authority. The subject of any other monograph must comply throughout its period of use. The period of validity that is assigned to any given article and the time from which that period is to be calculated are decided by the competent authority in the light of experimental results of stability studies.

Unless otherwise indicated in the General Notices or in the monographs, statements in monographs constitute mandatory requirements. General chapters become mandatory when referred to in a monograph, unless such reference is made in a way that indicates that it is not the intention to make the text referred to mandatory but rather to cite it for information.

The active ingredients (medicinal substances), excipients (auxiliary substances), pharmaceutical preparations and other articles described in the monographs are intended for human and veterinary use (unless explicitly restricted to one of these uses). An article is not of Pharmacopoeia quality unless it complies with all the requirements stated in the monograph. This does not imply that performance of all the tests in a monograph is necessarily a prerequisite for a manufacturer in assessing compliance with the Pharmacopoeia before release of a product. The manufacturer may obtain assurance that a product is of Pharmacopoeia quality from data derived, for example, from validation studies of the manufacturing process and from in-process controls. Parametric release in circumstances deemed appropriate by the competent authority is thus not precluded by the need to comply with the Pharmacopoeia.

The tests and assays described are the official methods upon which the standards of the Pharmacopoeia are based. With the agreement of the competent authority, alternative methods of analysis may be used for control purposes, provided that the methods used enable an unequivocal decision to be made as to whether compliance with the standards of the monographs would be achieved if the official methods were used. In the event of doubt or dispute, the methods of analysis of the Pharmacopoeia are alone authoritative.

Certain materials that are the subject of a pharmacopoeial monograph may exist in different grades suitable for different purposes. Unless otherwise indicated in the monograph, the requirements apply to all grades of the material. In some monographs, particularly those on excipients, a list of

functionality-related characteristics that are important for the use of the substance may be appended to the monograph for information. Test methods for determination of one or more of these characteristics may be given, also for information.

General monographs. Substances and preparations that are the subject of an individual monograph are also required to comply with relevant, applicable general monographs. Cross-references to applicable general monographs are not normally given in individual monographs.

General monographs apply to all substances and preparations within the scope of the Definition section of the general monograph, except where a preamble limits the application, for example to substances and preparations that are the subject of a monograph of the Pharmacopoeia.

General monographs on dosage forms apply to all preparations of the type defined. The requirements are not necessarily comprehensive for a given specific preparation and requirements additional to those prescribed in the general monograph may be imposed by the competent authority.

Conventional terms. The term “competent authority” means the national, supranational or international body or organisation vested with the authority for making decisions concerning the issue in question. It may, for example, be a national pharmacopoeia authority, a licensing authority or an official control laboratory.

The expression “unless otherwise justified and authorised” means that the requirements have to be met, unless the competent authority authorises a modification or an exemption where justified in a particular case.

Statements containing the word “should” are informative or advisory.

In certain monographs or other texts, the terms “suitable” and “appropriate” are used to describe a reagent, micro-organism, test method etc.; if criteria for suitability are not described in the monograph, suitability is demonstrated to the satisfaction of the competent authority.

Interchangeable methods. Certain general chapters contain a statement that the text in question is harmonised with the corresponding text of the Japanese Pharmacopoeia and/or the United States Pharmacopoeia and that these texts are interchangeable. This implies that if a substance or preparation is found to comply with a requirement using an interchangeable method from one of these pharmacopoeias it complies with the requirements of the European Pharmacopoeia. In the event of doubt or dispute, the text of the European Pharmacopoeia is alone authoritative.

01/2005:10200

1.2. OTHER PROVISIONS APPLYING TO GENERAL CHAPTERS AND MONOGRAPHS

Quantities. In tests with numerical limits and assays, the quantity stated to be taken for examination is approximate. The amount actually used, which may deviate by not more than 10 per cent from that stated, is accurately weighed or measured and the result is calculated from this exact quantity. In tests where the limit is not numerical, but usually depends upon comparison with the behaviour of a reference in the same conditions, the stated quantity is taken for examination. Reagents are used in the prescribed amounts. Quantities are weighed or measured with an accuracy commensurate with the indicated degree of precision. For weighings, the precision corresponds to plus or minus 5 units

after the last figure stated (for example, 0.25 g is to be interpreted as 0.245 g to 0.255 g). For the measurement of volumes, if the figure after the decimal point is a zero or ends in a zero (for example, 10.0 ml or 0.50 ml), the volume is measured using a pipette, a volumetric flask or a burette, as appropriate; otherwise, a graduated measuring cylinder or a graduated pipette may be used. Volumes stated in microlitres are measured using a micropipette or microsyringe.

It is recognised, however, that in certain cases the precision with which quantities are stated does not correspond to the number of significant figures stated in a specified numerical limit. The weighings and measurements are then carried out with a sufficiently improved accuracy.

Apparatus and procedures. Volumetric glassware complies with Class A requirements of the appropriate International Standard issued by the International Organisation for Standardisation.

Unless otherwise prescribed, analytical procedures are carried out at a temperature between 15 °C and 25 °C.

Unless otherwise prescribed, comparative tests are carried out using identical tubes of colourless, transparent, neutral glass with a flat base; the volumes of liquid prescribed are for use with tubes having an internal diameter of 16 mm but tubes with a larger internal diameter may be used provided the volume of liquid used is adjusted (2.1.5). Equal volumes of the liquids to be compared are examined down the vertical axis of the tubes against a white background, or if necessary against a black background. The examination is carried out in diffuse light.

Any solvent required in a test or assay in which an indicator is to be used is previously neutralised to the indicator, unless a blank test is prescribed.

Water-bath. The term “water-bath” means a bath of boiling water unless water at another temperature is indicated. Other methods of heating may be substituted provided the temperature is near to but not higher than 100 °C or the indicated temperature.

Drying and ignition to constant mass. The terms “dried to constant mass” and “ignited to constant mass” mean that 2 consecutive weighings do not differ by more than 0.5 mg, the second weighing following an additional period of drying or of ignition respectively appropriate to the nature and quantity of the residue.

Where drying is prescribed using one of the expressions “in a desiccator” or “*in vacuo*”, it is carried out using the conditions described under 2.2.32. *Loss on drying*.

REAGENTS

The proper conduct of the analytical procedures described in the Pharmacopoeia and the reliability of the results depend, in part, upon the quality of the reagents used. The reagents are described in general chapter 4. It is assumed that reagents of analytical grade are used; for some reagents, tests to determine suitability are included in the specifications.

SOLVENTS

Where the name of the solvent is not stated, the term “solution” implies a solution in water.

Where the use of water is specified or implied in the analytical procedures described in the Pharmacopoeia or for the preparation of reagents, water complying with the requirements of the monograph on *Purified water (0008)* is used, except that for many purposes the requirements for bacterial endotoxins (*Purified water in bulk*) and microbial

contamination (*Purified water in containers*) are not relevant. The term “distilled water” indicates purified water prepared by distillation.

The term “ethanol” without qualification means anhydrous ethanol. The term “alcohol” without qualification means ethanol (96 per cent). Other dilutions of ethanol are indicated by the term “ethanol” or “alcohol” followed by a statement of the percentage by volume of ethanol (C₂H₆O) required.

EXPRESSION OF CONTENT

In defining content, the expression “per cent” is used according to circumstances with one of two meanings:

- per cent *m/m* (percentage, mass in mass) expresses the number of grams of substance in 100 grams of final product,
- per cent *V/V* (percentage, volume in volume) expresses the number of millilitres of substance in 100 millilitres of final product.

The expression “parts per million (ppm)” refers to mass in mass, unless otherwise specified.

TEMPERATURE

Where an analytical procedure describes temperature without a figure, the general terms used have the following meaning:

- in a deep-freeze: below –15 °C,
- in a refrigerator: 2 °C to 8 °C,
- cold or cool: 8 °C to 15 °C,
- room temperature: 15 °C to 25 °C.

01/2005:10300

1.3. GENERAL CHAPTERS

CONTAINERS

Materials used for containers are described in general chapter 3.1. General names used for materials, particularly plastic materials, each cover a range of products varying not only in the properties of the principal constituent but also in the additives used. The test methods and limits for materials depend on the formulation and are therefore applicable only for materials whose formulation is covered by the preamble to the specification. The use of materials with different formulations, and the test methods and limits applied to them, are subject to agreement by the competent authority.

The specifications for containers in general chapter 3.2 have been developed for general application to containers of the stated category but in view of the wide variety of containers available and possible new developments, the publication of a specification does not exclude the use, in justified circumstances, of containers that comply with other specifications, subject to agreement by the competent authority.

Reference may be made within the monographs of the Pharmacopoeia to the definitions and specifications for containers provided in chapter 3.2. *Containers*. The general monographs for pharmaceutical dosage forms may, under the heading Definition/Production, require the use of certain types of container; certain other monographs may, under the heading Storage, indicate the type of container that is recommended for use.

01/2005:10400

1.4. MONOGRAPHS

TITLES

Monograph titles are in English and French in the respective versions and there is a Latin subtitle.

RELATIVE ATOMIC AND MOLECULAR MASSES

The relative atomic mass (A_r) or the relative molecular mass (M_r) is shown, as and where appropriate, at the beginning of each monograph. The relative atomic and molecular masses and the molecular and graphic formulae do not constitute analytical standards for the substances described.

DEFINITION

Statements under the heading Definition constitute an official definition of the substance, preparation or other article that is the subject of the monograph.

Limits of content. Where limits of content are prescribed, they are those determined by the method described under Assay.

Vegetable drugs. In monographs on vegetable drugs, the definition indicates whether the subject of the monograph is, for example, the whole drug or the drug in powdered form. Where a monograph applies to the drug in several states, for example both to the whole drug and the drug in powdered form, the definition states this.

PRODUCTION

Statements under the heading Production draw attention to particular aspects of the manufacturing process but are not necessarily comprehensive. They constitute instructions to manufacturers. They may relate, for example, to source materials, to the manufacturing process itself and its validation and control, to in-process testing or to testing that is to be carried out by the manufacturer on the final article either on selected batches or on each batch prior to release. These statements cannot necessarily be verified on a sample of the final article by an independent analyst. The competent authority may establish that the instructions have been followed, for example, by examination of data received from the manufacturer, by inspection of manufacture or by testing appropriate samples.

The absence of a section on Production does not imply that attention to features such as those referred to above is not required. A product described in a monograph of the Pharmacopoeia is manufactured in accordance with a suitable quality system in accordance with relevant international agreements and supranational and national regulations governing medicinal products for human or veterinary use.

Where in the section under the heading Production a monograph on a vaccine defines the characteristics of the vaccine strain to be used, any test methods given for confirming these characteristics are provided for information as examples of suitable methods. Similarly, test methods for choice of vaccine composition are provided for information as examples of suitable methods.

CHARACTERS

The statements under the heading Characters are not to be interpreted in a strict sense and are not requirements.

Solubility. In statements of solubility in the section headed Characters, the terms used have the following significance referred to a temperature between 15 °C and 25 °C.

Descriptive term	Approximate volume of solvent in millilitres per gram of solute			
Very soluble	less than	1		
Freely soluble	from	1	to	10
Soluble	from	10	to	30
Sparingly soluble	from	30	to	100
Slightly soluble	from	100	to	1000
Very slightly soluble	from	1000	to	10 000
Practically insoluble	more than			10 000

The term “partly soluble” is used to describe a mixture where only some of the components dissolve. The term “miscible” is used to describe a liquid that is miscible in all proportions with the stated solvent.

IDENTIFICATION

The tests given in the identification section are not designed to give a full confirmation of the chemical structure or composition of the product; they are intended to give confirmation, with an acceptable degree of assurance, that the article conforms to the description on the label.

Certain monographs have subdivisions entitled “First identification” and “Second identification”. The test or tests that constitute the “First identification” may be used for identification in all circumstances. The test or tests that constitute the “Second identification” may be used for identification provided it can be demonstrated that the substance or preparation is fully traceable to a batch certified to comply with all the other requirements of the monograph.

TESTS AND ASSAYS

Scope. The requirements are not framed to take account of all possible impurities. It is not to be presumed, for example, that an impurity that is not detectable by means of the prescribed tests is tolerated if common sense and good pharmaceutical practice require that it be absent. See also below under Impurities.

Calculation. Where the result of a test or assay is required to be calculated with reference to the dried or anhydrous substance or on some other specified basis, the determination of loss on drying, water content or other property is carried out by the method prescribed in the relevant test in the monograph. The words “dried substance” or “anhydrous substance” etc. appear in parenthesis after the result.

Limits. The limits prescribed are based on data obtained in normal analytical practice; they take account of normal analytical errors, of acceptable variations in manufacture and compounding and of deterioration to an extent considered acceptable. No further tolerances are to be applied to the limits prescribed to determine whether the article being examined complies with the requirements of the monograph. In determining compliance with a numerical limit, the calculated result of a test or assay is first rounded to the number of significant figures stated, unless otherwise prescribed. The last figure is increased by one when the part rejected is equal to or exceeds one half-unit, whereas it is not modified when the part rejected is less than a half-unit.

Indication of permitted limit of impurities. For comparative tests, the approximate content of impurity tolerated, or the sum of impurities, may be indicated for information only. Acceptance or rejection is determined on the basis

of compliance or non-compliance with the stated test. If the use of a reference substance for the named impurity is not prescribed, this content may be expressed as a nominal concentration of the substance used to prepare the reference solution specified in the monograph, unless otherwise described.

Vegetable drugs. For vegetable drugs, the sulphated ash, total ash, water-soluble matter, alcohol-soluble matter, water content, content of essential oil and content of active principle are calculated with reference to the drug that has not been specially dried, unless otherwise prescribed in the monograph.

Equivalents. Where an equivalent is given, for the purposes of the Pharmacopoeia only the figures shown are to be used in applying the requirements of the monograph.

STORAGE

The information and recommendations given under the heading Storage do not constitute a pharmacopoeial requirement but the competent authority may specify particular storage conditions that must be met.

The articles described in the Pharmacopoeia are stored in such a way as to prevent contamination and, as far as possible, deterioration. Where special conditions of storage are recommended, including the type of container (see *1.3. General chapters*) and limits of temperature, they are stated in the monograph.

The following expressions are used in monographs under Storage with the meaning shown.

In an airtight container means that the product is stored in an airtight container (3.2). Care is to be taken when the container is opened in a damp atmosphere. A low moisture content may be maintained, if necessary, by the use of a desiccant in the container provided that direct contact with the product is avoided.

Protected from light means that the product is stored either in a container made of a material that absorbs actinic light sufficiently to protect the contents from change induced by such light or in a container enclosed in an outer cover that provides such protection or stored in a place from which all such light is excluded.

LABELLING

In general, labelling of medicines is subject to supranational and national regulation and to international agreements. The statements under the heading Labelling therefore are not comprehensive and, moreover, for the purposes of the Pharmacopoeia only those statements that are necessary to demonstrate compliance or non-compliance with the monograph are mandatory. Any other labelling statements are included as recommendations. When the term "label" is used in the Pharmacopoeia, the labelling statements may appear on the container, the package, a leaflet accompanying the package or a certificate of analysis accompanying the article, as decided by the competent authority.

WARNINGS

Materials described in monographs and reagents specified for use in the Pharmacopoeia may be injurious to health unless adequate precautions are taken. The principles of good quality control laboratory practice and the provisions of any appropriate regulations are to be observed at all times. Attention is drawn to particular hazards in certain monographs by means of a warning statement; absence of such a statement is not to be taken to mean that no hazard exists.

IMPURITIES

A list of all known and potential impurities that have been shown to be detected by the tests in a monograph may be given for information. See also *5.10. Control of impurities in substances for pharmaceutical use*.

FUNCTIONALITY-RELATED CHARACTERISTICS

A list of functionality-related characteristics that are not the subject of official requirements but which are nevertheless important for the use of a substance may be appended to a monograph, for information (see also above *1.1. General statements*).

REFERENCE SUBSTANCES, REFERENCE PREPARATIONS AND REFERENCE SPECTRA

Certain monographs require the use of a reference substance, a reference preparation or a reference spectrum. These are chosen with regard to their intended use as prescribed in the monographs of the Pharmacopoeia and are not necessarily suitable in other circumstances. The European Pharmacopoeia Commission does not accept responsibility for any errors arising from use other than as prescribed.

The reference substances, the reference preparations and the reference spectra are established by the European Pharmacopoeia Commission and may be obtained from the Technical Secretariat. They are the official standards to be used in cases of arbitration. A list of reference substances, reference preparations and reference spectra may be obtained from the Technical Secretariat.

Local standards may be used for routine analysis, provided they are calibrated against the standards established by the European Pharmacopoeia Commission.

Any information necessary for proper use of the reference substance or reference preparation is given on the label or in the accompanying leaflet or brochure. Where no drying conditions are stated in the leaflet or on the label, the substance is to be used as received. No certificate of analysis or other data not relevant to the prescribed use of the product are provided. No expiry date is indicated: the products are guaranteed to be suitable for use when dispatched. The stability of the contents of opened containers cannot be guaranteed.

Chemical Reference Substances. The abbreviation CRS indicates a Chemical Reference Substance established by the European Pharmacopoeia Commission. Some Chemical Reference Substances are used for the microbiological assay of antibiotics and their activity is stated, in International Units, on the label or on the accompanying leaflet and defined in the same manner as for Biological Reference Preparations.

Biological Reference Preparations. The majority of the primary biological reference preparations referred to in the European Pharmacopoeia are the appropriate International Standards and Reference Preparations established by the World Health Organisation. Because these reference materials are usually available only in limited quantities, the Commission has established Biological Reference Preparations (indicated by the abbreviation BRP) where appropriate. Where applicable, the potency of the Biological Reference Preparations is expressed in International Units. For some Biological Reference Preparations, where an international standard or reference preparation does not exist, the potency is expressed in European Pharmacopoeia Units.

Reference spectra. The reference spectrum is accompanied by information concerning the conditions used for sample preparation and recording the spectrum.

01/2005:10500

1.5. ABBREVIATIONS AND SYMBOLS

<i>A</i>	Absorbance
$A_{1\text{ cm}}^{1\text{ per cent}}$	Specific absorbance
A_r	Relative atomic mass
$[\alpha]_{\text{D}}^{20}$	Specific optical rotation
bp	Boiling point
BRP	Biological Reference Preparation
CRS	Chemical Reference Substance
d_{20}^{20}	Relative density
IU	International Unit
λ	Wavelength
M	Molarity
M_r	Relative molecular mass
mp	Melting point
n_{D}^{20}	Refractive index
Ph. Eur. U.	European Pharmacopoeia Unit
ppm	Parts per million
R	Substance or solution defined under 4. <i>Reagents</i>
R_f	Used in chromatography to indicate the ratio of the distance travelled by a substance to the distance travelled by the solvent front
R_{st}	Used in chromatography to indicate the ratio of the distance travelled by a substance to the distance travelled by a reference substance
RV	Substance used as a primary standard in volumetric analysis (chapter 4.2.1)
Abbreviations used in the monographs on immunoglobulins, immunosera and vaccines	
LD ₅₀	The statistically determined quantity of a substance that, when administered by the specified route, may be expected to cause the death of 50 per cent of the test animals within a given period
MLD	Minimum lethal dose
L+/10 dose	The smallest quantity of a toxin that, in the conditions of the test, when mixed with 0.1 IU of antitoxin and administered by the specified route, causes the death of the test animals within a given period
L+ dose	The smallest quantity of a toxin that, in the conditions of the test, when mixed with 1 IU of antitoxin and administered by the specified route, causes the death of the test animals within a given period
lr/100 dose	The smallest quantity of a toxin that, in the conditions of the test, when mixed with 0.01 IU of antitoxin and injected intracutaneously causes a characteristic reaction at the site of injection within a given period

Lp/10 dose	The smallest quantity of toxin that, in the conditions of the test, when mixed with 0.1 IU of antitoxin and administered by the specified route, causes paralysis in the test animals within a given period
Lo/10 dose	The largest quantity of a toxin that, in the conditions of the test, when mixed with 0.1 IU of antitoxin and administered by the specified route, does not cause symptoms of toxicity in the test animals within a given period
Lf dose	The quantity of toxin or toxoid that flocculates in the shortest time with 1 IU of antitoxin
CCID ₅₀	The statistically determined quantity of virus that may be expected to infect 50 per cent of the cell cultures to which it is added
EID ₅₀	The statistically determined quantity of virus that may be expected to infect 50 per cent of fertilised eggs into which it is inoculated
ID ₅₀	The statistically determined quantity of a virus that may be expected to infect 50 per cent of the animals into which it is inoculated
PD ₅₀	The statistically determined dose of a vaccine that, in the conditions of the tests, may be expected to protect 50 per cent of the animals against a challenge dose of the micro-organisms or toxins against which it is active
ED ₅₀	The statistically determined dose of a vaccine that, in the conditions of the tests, may be expected to induce specific antibodies in 50 per cent of the animals for the relevant vaccine antigens
PFU	Pock-forming units or plaque-forming units
SPF	Specified-pathogen-free.

Collections of micro-organisms

ATCC	American Type Culture Collection 10801 University Boulevard Manassas, Virginia 20110-2209, USA
C.I.P.	Collection de Bactéries de l'Institut Pasteur B.P. 52, 25 rue du Docteur Roux 75724 Paris Cedex 15, France
IMI	International Mycological Institute Bakeham Lane Surrey TW20 9TY, Great Britain
I.P.	Collection Nationale de Culture de Microorganismes (C.N.C.M.) Institut Pasteur 25, rue du Docteur Roux 75724 Paris Cedex 15, France
NCIMB	National Collection of Industrial and Marine Bacteria Ltd 23 St Machar Drive Aberdeen AB2 1RY, Great Britain

NCPF	National Collection of Pathogenic Fungi London School of Hygiene and Tropical Medicine Keppel Street London WC1E 7HT, Great Britain
NCTC	National Collection of Type Cultures Central Public Health Laboratory Colindale Avenue London NW9 5HT, Great Britain
NCYC	National Collection of Yeast Cultures AFRC Food Research Institute Colney Lane Norwich NR4 7UA, Great Britain
S.S.I.	Statens Serum Institut 80 Amager Boulevard, Copenhagen, Denmark

The derived units may be formed by combining the base units according to the algebraic relationships linking the corresponding quantities. Some of these derived units have special names and symbols. The SI units used in the European Pharmacopoeia are shown in Table 1.6-2.

Some important and widely used units outside the International System are shown in Table 1.6-3.

The prefixes shown in Table 1.6-4 are used to form the names and symbols of the decimal multiples and submultiples of SI units.

NOTES

1. In the Pharmacopoeia, the Celsius temperature is used (symbol *t*). This is defined by the equation:

$$t = T - T_0$$

where $T_0 = 273.15$ K by definition. The Celsius or centigrade temperature is expressed in degree Celsius (symbol °C). The unit “degree Celsius” is equal to the unit “kelvin”.

2. The practical expressions of concentrations used in the Pharmacopoeia are defined in the General Notices.
3. The radian is the plane angle between two radii of a circle which cut off on the circumference an arc equal in length to the radius.
4. In the Pharmacopoeia, conditions of centrifugation are defined by reference to the acceleration due to gravity (*g*):

$$g = 9.806\,65\,m \cdot s^{-2}$$

5. Certain quantities without dimensions are used in the Pharmacopoeia: relative density (2.2.5), absorbance (2.2.25), specific absorbance (2.2.25) and refractive index (2.2.6).
6. The microkatal is defined as the enzymic activity which, under defined conditions, produces the transformation (e.g. hydrolysis) of 1 micromole of the substrate per second.

01/2005:10600

1.6. UNITS OF THE INTERNATIONAL SYSTEM (SI) USED IN THE PHARMACOPOEIA AND EQUIVALENCE WITH OTHER UNITS

INTERNATIONAL SYSTEM OF UNITS (SI)

The International System of Units comprises 3 classes of units, namely base units, derived units and supplementary units⁽¹⁾. The base units and their definitions are set out in Table 1.6-1.

Table 1.6-1. – SI base units

Quantity		Unit		Definition
Name	Symbol	Name	Symbol	
Length	<i>l</i>	metre	m	The metre is the length of the path travelled by light in a vacuum during a time interval of 1/299 792 458 of a second.
Mass	<i>m</i>	kilogram	kg	The kilogram is equal to the mass of the international prototype of the kilogram.
Time	<i>t</i>	second	s	The second is the duration of 9 192 631 770 periods of the radiation corresponding to the transition between the two hyperfine levels of the ground state of the caesium-133 atom.
Electric current	<i>I</i>	ampere	A	The ampere is that constant current which, maintained in two straight parallel conductors of infinite length, of negligible circular cross-section and placed 1 metre apart in vacuum would produce between these conductors a force equal to 2×10^{-7} newton per metre of length.
Thermodynamic temperature	<i>T</i>	kelvin	K	The kelvin is the fraction 1/273.16 of the thermodynamic temperature of the triple point of water.
Amount of substance	<i>n</i>	mole	mol	The mole is the amount of substance of a system containing as many elementary entities as there are atoms in 0.012 kilogram of carbon-12 [*] .
Luminous intensity	<i>I_v</i>	candela	cd	The candela is the luminous intensity in a given direction of a source emitting monochromatic radiation with a frequency of 540×10^{12} hertz and whose energy intensity in that direction is 1/683 watt per steradian.
* When the mole is used, the elementary entities must be specified and may be atoms, molecules, ions, electrons, other particles or specified groups of such particles.				

(1) The definitions of the units used in the International System are given in the booklet “Le Système International d’Unités (SI)” published by the Bureau International des Poids et Mesures, Pavillon de Breteuil, F-92310 Sèvres.

Table 1.6.-2. – SI units used in the European Pharmacopoeia and equivalence with other units

Quantity		Unit				Conversion of other units into SI units
Name	Symbol	Name	Symbol	Expression in SI base units	Expression in other SI units	
Wave number	ν	one per metre	1/m	m^{-1}		
Wavelength	λ	micrometre nanometre	μm nm	10^{-6}m 10^{-9}m		
Area	A, S	square metre	m^2	m^2		
Volume	V	cubic metre	m^3	m^3		1 ml = 1 cm ³ = 10 ⁻⁶ m ³
Frequency	ν	hertz	Hz	s^{-1}		
Density	ρ	kilogram per cubic metre	kg/m ³	kg·m ⁻³		1 g/ml = 1 g/cm ³ = 10 ³ kg·m ⁻³
Velocity	v	metre per second	m/s	m·s ⁻¹		
Force	F	newton	N	m·kg·s ⁻²		1 dyne = 1 g·cm·s ⁻² = 10 ⁻⁵ N 1 kp = 9.806 65 N
Pressure	p	pascal	Pa	m ⁻¹ ·kg·s ⁻²	N·m ⁻²	1 dyne/cm ² = 10 ⁻¹ Pa = 10 ⁻¹ N·m ⁻² 1 atm = 101 325 Pa = 101.325 kPa 1 bar = 10 ⁵ Pa = 0.1 MPa 1 mm Hg = 133.322 387 Pa 1 Torr = 133.322 368 Pa 1 psi = 6.894 757 kPa
Dynamic viscosity	η	pascal second	Pas	m ⁻¹ ·kg·s ⁻¹	N·s·m ⁻²	1 P = 10 ⁻¹ Pas = 10 ⁻¹ N·s·m ⁻² 1 cP = 1 mPas
Kinematic viscosity	ν	square metre per second	m ² /s	m ² ·s ⁻¹	Pas·m ³ ·kg ⁻¹ N·m·s·kg ⁻¹	1 St = 1 cm ² ·s ⁻¹ = 10 ⁻⁴ m ² ·s ⁻¹
Energy	W	joule	J	m ² ·kg·s ⁻²	N·m	1 erg = 1 cm ² ·g·s ⁻² = 1 dynecm = 10 ⁻⁷ J 1 cal = 4.1868 J
Power	P	watt	W	m ² ·kg·s ⁻³	N·m·s ⁻¹	1 erg/s = 1 dynecm·s ⁻¹ =
Radiant flux					J·s ⁻¹	10 ⁻⁷ W = 10 ⁻⁷ N·m·s ⁻¹ = 10 ⁻⁷ J·s ⁻¹
Absorbed dose (of radiant energy)	D	gray	Gy	m ² ·s ⁻²	J·kg ⁻¹	1 rad = 10 ⁻² Gy
Electric potential, electromotive force	U	volt	V	m ² ·kg·s ⁻³ ·A ⁻¹	W·A ⁻¹	
Electric resistance	R	ohm	Ω	m ² ·kg·s ⁻³ ·A ⁻²	V·A ⁻¹	
Quantity of electricity	Q	coulomb	C	As		
Activity of a radionuclide	A	becquerel	Bq	s^{-1}		1 Ci = 37·10 ⁹ Bq = 37·10 ⁹ s ⁻¹
Concentration (of amount of substance), molar concentration	c	mole per cubic metre	mol/m ³	mol·m ⁻³		1 mol/l = 1M = 1 mol/dm ³ = 10 ³ mol·m ⁻³
Mass concentration	ρ	kilogram per cubic metre	kg/m ³	kg·m ⁻³		1 g/l = 1 g/dm ³ = 1 kg·m ⁻³

Table 1.6.-3. – Units used with the International System

Quantity	Unit		Value in SI units
	Time	Symbol	
Time	minute	min	1 min = 60 s
	hour	h	1 h = 60 min = 3600 s
	day	d	1 d = 24 h = 86 400 s
Plan angle	degree	°	1° = (π/180) rad
Volume	litre	l	1 l = 1 dm ³ = 10 ⁻³ m ³
Mass	tonne	t	1 t = 10 ³ kg
Rotational frequency	revolution per minute	r/min	1 r/min = (1/60) s ⁻¹

Table 1.6.-4. – Decimal multiples and sub-multiples of units

Factor	Prefix	Symbol	Factor	Prefix	Symbol
10 ¹⁸	exa	E	10 ⁻¹	deci	d
10 ¹⁵	peta	P	10 ⁻²	centi	c
10 ¹²	tera	T	10 ⁻³	milli	m
10 ⁹	giga	G	10 ⁻⁶	micro	μ
10 ⁶	mega	M	10 ⁻⁹	nano	n
10 ³	kilo	k	10 ⁻¹²	pico	p
10 ²	hecto	h	10 ⁻¹⁵	femto	f
10 ¹	deca	da	10 ⁻¹⁸	atto	a

2.1. APPARATUS

01/2005:20102

01/2005:20101

2.1.1. DROPPERS

The term “drops” means standard drops delivered from a standard dropper as described below.

Standard droppers (Figure 2.1.1-1) are constructed of practically colourless glass. The lower extremity has a circular orifice in a flat surface at right angles to the axis.

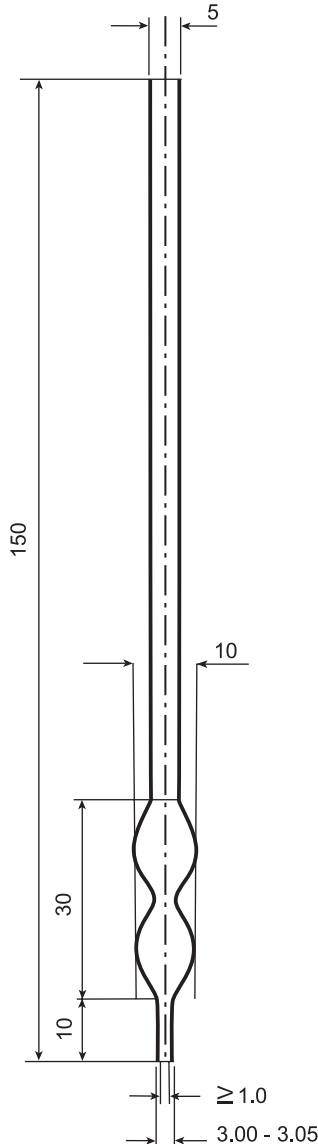


Figure 2.1.1-1. – Standard dropper
Dimensions in millimetres

Other droppers may be used provided they comply with the following test.

Twenty drops of *water R* at 20 ± 1 °C flowing freely from the dropper held in the vertical position at a constant rate of one drop per second weighs 1000 ± 50 mg.

The dropper must be carefully cleaned before use. Carry out three determinations on any given dropper. No result may deviate by more than 5 per cent from the mean of the three determinations.

(1) The given limits are only approximate.

(2) The European Pharmacopoeia has adopted the system proposed by the International Organisation for Standardisation (ISO).

2.1.2. COMPARATIVE TABLE OF POROSITY OF SINTERED-GLASS FILTERS⁽¹⁾

Table 2.1.2-1

Porosity number (Ph. Eur.) ⁽²⁾	Maximum diameter of pores in micrometres	Germany	France	United Kingdom
1.6	less than 1.6	5f	–	–
–	1 - 2.5	5	–	5
4	1.6 - 4	–	–	–
–	4 - 6	–	5	–
10	4 - 10	4f	–	4
16	10 - 16	4	4	–
40	16 - 40	3	3	3
–	40 - 50	–	–	2
100	40 - 100	2	2	–
–	100 - 120	–	–	1
160	100 - 160	1	1	–
–	150 - 200	0	0	–
250	160 - 250	–	–	–
–	200 - 500	–	00	–

Special Uses

Diameters in micrometres

< 2.5	Bacteriological filtration
4 - 10	Ultra-fine filtration, separation of micro-organisms of large diameter
10 - 40	Analytical filtration, very fine filtration of mercury, very fine dispersion of gases
40 - 100	Fine filtration, filtration of mercury, fine dispersion of gases
100 - 160	Filtration of coarse materials, dispersion and washing of gases, support for other filter materials
160 - 500	Filtration of very coarse materials, dispersion and washing of gases.

01/2005:20103

2.1.3. ULTRAVIOLET RAY LAMPS FOR ANALYTICAL PURPOSES

Mercury vapour in quartz lamps is used as the source of ultraviolet light. A suitable filter may be fitted to eliminate the visible part of the spectrum emitted by the lamp. When the Pharmacopoeia prescribes in a test the use of ultraviolet light of wavelength 254 nm or 365 nm, an instrument consisting of a mercury vapour lamp and a filter which gives an emission band with maximum intensity at about 254 nm or 365 nm is used. The lamp used should be capable of revealing without doubt a standard spot of sodium salicylate with a diameter of about 5 mm on a support of *silica gel G R*, the spot being examined while in a position normal to the radiation.

For this purpose apply 5 µl of a 0.4 g/l solution of *sodium salicylate R* in *alcohol R*⁽³⁾ for lamps of maximum output at 254 nm and 5 µl of a 2 g/l solution in *alcohol R*⁽¹⁾ for lamps of maximum output at 365 nm. The distance between the lamp and the chromatographic plate under examination used in a pharmacopoeial test should never exceed the distance used to carry out the above test.

Maximum tolerance⁽⁴⁾ for an aperture (+ *X*): no aperture size shall exceed the nominal size by more than *X*, where:

$$X = \frac{2(w^{0.75})}{3} + 4(w^{0.25})$$

w = width of aperture.

Tolerance for mean aperture (± *Y*): the average aperture size shall not depart from the nominal size by more than ± *Y*, where:

$$Y = \frac{w^{0.98}}{27} + 1.6$$

Intermediary tolerance (+ *Z*): not more than 6 per cent of the total number of apertures shall have sizes between “nominal + *X*” and “nominal + *Z*”, where:

$$Z = \frac{X + Y}{2}$$

2.1.4. SIEVES

Sieves are constructed of suitable materials with square meshes. For purposes other than analytical procedures, sieves with circular meshes may be used, the internal diameters of which are 1.25 times the aperture of the square mesh of the corresponding sieve size. There must be no reaction between the material of the sieve and the substance being sifted. Degree of comminution is prescribed in the monograph using the sieve number, which is the size of the mesh in micrometres, given in parenthesis after the name of the substance (Table 2.1.4.-1).

Wire diameter *d*: the wire diameters given in Table 2.1.4.-1 apply to woven metal wire cloth mounted in a frame. The nominal sizes of the wire diameters may depart from these values within the limits *d*_{max} and *d*_{min}. The limits define a permissible range of choice ± 15 per cent of the recommended nominal dimensions. The wires in a test sieve shall be of a similar diameter in warp and weft directions.

Table 2.1.4.-1 (values in micrometers)

Sieve numbers (Nominal dimensions of apertures)	Tolerances for apertures			Wire diameters		
	Maximum tolerance for an aperture	Tolerance for mean aperture	Intermediary tolerance	Recommended nominal dimensions	Admissible limits	
	+ <i>X</i>	± <i>Y</i>	+ <i>Z</i>	<i>d</i>	<i>d</i> _{max}	<i>d</i> _{min}
11 200	770	350	560	2500	2900	2100
8000	600	250	430	2000	2300	1700
5 600	470	180	320	1600	1900	1300
4000	370	130	250	1400	1700	1200
2 800	290	90	190	1120	1300	950
2000	230	70	150	900	1040	770
1 400	180	50	110	710	820	600
1000	140	30	90	560	640	480
710	112	25	69	450	520	380
500	89	18	54	315	360	270
355	72	13	43	224	260	190
250	58	9.9	34	160	190	130
180	47	7.6	27	125	150	106
125	38	5.8	22	90	104	77
90	32	4.6	18	63	72	54
63	26	3.7	15	45	52	38
45	22	3.1	13	32	37	27
38	–	–	–	30	35	24

(3) The *alcohol R* used must be free from fluorescence.

(4) See the International Standard ISO 3310/1 (1975).

01/2005:20105

2.1.5. TUBES FOR COMPARATIVE TESTS

Tubes used for comparative tests are matched tubes of colourless glass with a uniform internal diameter. The base is transparent and flat.

A column of the liquid is examined down the vertical axis of the tube against a white background, or if necessary, against a black background. The examination is carried out in diffused light.

It is assumed that tubes with an internal diameter of 16 mm will be used. Tubes with a larger internal diameter may be used instead but the volume of liquid examined must then be increased so that the depth of liquid in the tubes is not less than where the prescribed volume of liquid and tubes 16 mm in internal diameter are used.

01/2005:20106

2.1.6. GAS DETECTOR TUBES

Gas detector tubes are cylindrical, sealed tubes consisting of an inert transparent material and are constructed to allow the passage of gas. They contain reagents adsorbed onto inert substrates that are suitable for the visualisation of the substance to be detected and, if necessary, they also contain preliminary layers and/or adsorbent filters to eliminate substances that interfere with the substance to be detected. The layer of indicator contains either a single reagent for the detection of a given impurity or several reagents for the detection of several substances (monolayer tube or multilayer tube).

The test is carried out by passing the required volume of the gas to be examined through the indicator tube. The length of the coloured layer or the intensity of a colour change on a graduated scale gives an indication of the impurities present. The calibration of the detector tubes is verified according to the manufacturer's instructions.

Operating conditions. Examine according to the manufacturer's instructions or proceed as follows:

The gas supply is connected to a suitable pressure regulator and needle valve. Connect the flexible tubing fitted with a Y-piece to the valve and adjust the flow of gas to be examined to purge the tubing in order to obtain an appropriate flow (Figure 2.1.6.-1). Prepare the indicator tube and fit to the metering pump, following the manufacturer's instructions. Connect the open end of the indicator tube to the short leg of the tubing and operate the pump by the appropriate number of strokes to pass a suitable volume of gas to be examined through the tube. Read the value corresponding to the length of the coloured layer or the intensity of the colour on the graduated scale. If a negative result is achieved, indicator tubes can be verified with a calibration gas containing the appropriate impurity.

In view of the wide variety of available compressor oils, it is necessary to verify the reactivity of the oil detector tubes for the oil used. Information on the reactivity for various oils is

given in the leaflet supplied with the tube. If the oil used is not cited in the leaflet, the tube manufacturer must verify the reactivity and if necessary provide a tube specific for this oil.

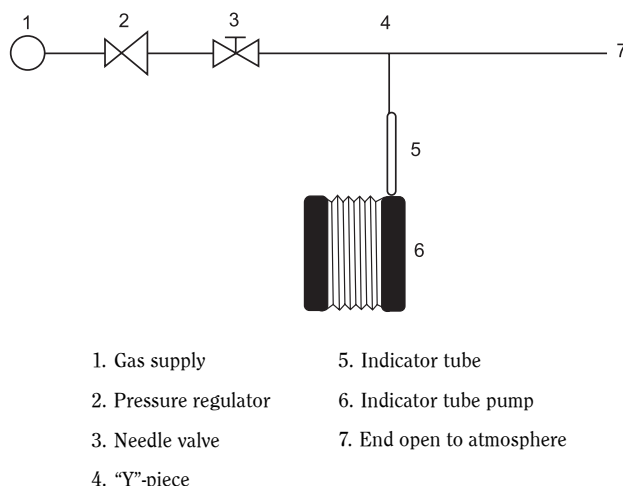


Figure 2.1.6.-1. – Apparatus for gas detector tubes

Carbon dioxide detector tube. Sealed glass tube containing adsorbent filters and suitable supports for hydrazine and crystal violet indicators. The minimum value indicated is 100 ppm with a relative standard deviation of at most ± 15 per cent.

Sulphur dioxide detector tube. Sealed glass tube containing adsorbent filters and suitable supports for the iodine and starch indicator. The minimum value indicated is 0.5 ppm with a relative standard deviation of at most ± 15 per cent.

Oil detector tube. Sealed glass tube containing adsorbent filters and suitable supports for the sulphuric acid indicator. The minimum value indicated is 0.1 mg/m³ with a relative standard deviation of at most ± 30 per cent.

Nitrogen monoxide and nitrogen dioxide detector tube. Sealed glass tube containing adsorbent filters and suitable supports for an oxidising layer (Cr(VI) salt) and the diphenylbenzidine indicator. The minimum value indicated is 0.5 ppm with a relative standard deviation of at most ± 15 per cent.

Carbon monoxide detector tube. Sealed glass tube containing adsorbent filters and suitable supports for di-iodine pentoxide, selenium dioxide and fuming sulphuric acid indicators. The minimum value indicated is 5 ppm or less, with a relative standard deviation of at most ± 15 per cent.

Hydrogen sulphide detector tube. Sealed glass tube containing adsorbent filters and suitable supports for an appropriate lead salt indicator. The minimum value indicated is 1 ppm or less, with a relative standard deviation of at most ± 10 per cent.

Water vapour detector tube. Sealed glass tube containing adsorbent filters and suitable supports for the magnesium perchlorate indicator. The minimum value indicated is 67 ppm or less, with a relative standard deviation of at most ± 20 per cent.

2.2. PHYSICAL AND PHYSICOCHEMICAL METHODS

01/2005:20201

2.2.1. CLARITY AND DEGREE OF OPALESCENCE OF LIQUIDS

VISUAL METHOD

Using identical test tubes of colourless, transparent, neutral glass with a flat base and an internal diameter of 15-25 mm, compare the liquid to be examined with a reference suspension freshly prepared as described below, the depth of the layer being 40 mm. Compare the solutions in diffused daylight 5 min after preparation of the reference suspension, viewing vertically against a black background. The diffusion of light must be such that reference suspension I can readily be distinguished from *water R*, and that reference suspension II can readily be distinguished from reference suspension I.

A liquid is considered *clear* if its clarity is the same as that of *water R* or of the solvent used when examined under the conditions described above, or if its opalescence is not more pronounced than that of reference suspension I.

Hydrazine sulphate solution. Dissolve 1.0 g of *hydrazine sulphate R* in *water R* and dilute to 100.0 ml with the same solvent. Allow to stand for 4-6 h.

Hexamethylenetetramine solution. In a 100 ml glass-stoppered flask, dissolve 2.5 g of *hexamethylenetetramine R* in 25.0 ml of *water R*.

Primary opalescent suspension (formazin suspension). To the solution of hexamethylenetetramine in the flask add 25.0 ml of hydrazine sulphate solution. Mix and allow to stand for 24 h. This suspension is stable for 2 months, provided it is stored in a glass container free from surface defects. The suspension must not adhere to the glass and must be well mixed before use.

Standard of opalescence. Dilute 15.0 ml of the primary opalescent suspension to 1000.0 ml with *water R*. This suspension is freshly prepared and may be stored for at most 24 h.

Reference suspensions. Prepare the reference suspensions according to Table 2.2.1-1. Mix and shake before use.

Table 2.2.1-1

	I	II	III	IV
Standard of opalescence	5.0 ml	10.0 ml	30.0 ml	50.0 ml
<i>Water R</i>	95.0 ml	90.0 ml	70.0 ml	50.0 ml

Turbidity standard. The formazin suspension prepared by mixing equal volumes of the hydrazine sulphate solution and the hexamethylenetetramine solution is defined as a 4000 NTU (nephelometric turbidity units) primary reference standard. Reference suspensions I, II, III and IV have values of 3 NTU, 6 NTU, 18 NTU and 30 NTU respectively. Stabilised formazin suspensions that can be used to prepare stable, diluted turbidity standards are available commercially and may be used after comparison with the standards prepared as described.

Formazin has several desirable characteristics that make it an excellent turbidity standard. It can be reproducibly prepared from assayed raw materials. The physical characteristics make it a desirable light-scatter calibration standard. The

formazin polymer consists of chains of different lengths, which fold into random configurations. This results in a wide assay of particle shapes and sizes, which analytically fits the possibility of different particle sizes and shapes that are found in the real samples. Due to formazin's reproducibility, scattering characteristics and traceability, instrument calibration algorithms and performance criteria are mostly based on this standard.

INSTRUMENTAL METHODS

INTRODUCTION

The degree of opalescence may also be determined by instrumental measurement of the light absorbed or scattered on account of submicroscopic optical density inhomogeneities of opalescent solutions and suspensions. 2 such techniques are nephelometry and turbidimetry. For turbidity measurement of coloured samples, ratio turbidimetry and nephelometry with ratio selection are used.

The light scattering effect of suspended particles can be measured by observation of either the transmitted light (turbidimetry) or the scattered light (nephelometry). Ratio turbidimetry combines the principles of both nephelometry and turbidimetry. Turbidimetry and nephelometry are useful for the measurement of slightly opalescent suspensions. Reference suspensions produced under well-defined conditions must be used. For quantitative measurements the construction of calibration curves is essential, since the relationship between the optical properties of the suspension and the concentration of the dispersed phase is at best semi-empirical.

The determination of opalescence of coloured liquids is done with ratio turbidimeters or nephelometers with ratio selection since colour provides a negative interference, attenuating both incident and scattered light and lowering the turbidity value. The effect is so great for even moderately coloured samples that conventional nephelometers cannot be used.

The instrumental assessment of clarity and opalescence provides a more discriminatory test that does not depend on the visual acuity of the analyst. Numerical results are more useful for quality monitoring and process control, especially in stability studies. For example, previous numerical data on stability can be projected to determine whether a given batch of dosage formulation or active pharmaceutical ingredient will exceed shelf-life limits prior to the expiry date.

NEPHELOMETRY

When a suspension is viewed at right angles to the direction of the incident light, the system appears opalescent due to the reflection of light from the particles of the suspension (Tyndall effect). A certain portion of the light beam entering a turbid liquid is transmitted, another portion is absorbed and the remaining portion is scattered by the suspended particles. If measurement is made at 90° to the light beam, the light scattered by the suspended particles can be used for the determination of their concentration, provided the number and size of particles influencing the scattering remain constant. The reference suspension must maintain a constant degree of turbidity and the sample and reference suspensions must be prepared under identical conditions. The Tyndall effect depends both upon the number of particles and their size. Nephelometric measurements are more reliable in low turbidity ranges, where there is a linear relationship between nephelometric turbidity unit (NTU) values and relative detector signals. As the degree of turbidity increases, not all the particles are exposed to the incident light and the scattered radiation of other particles is hindered on its way to the detector. The maximum nephelometric values at which reliable measurements can

be made lie between 1750-2000 NTU. Linearity must be demonstrated by constructing a calibration curve using at least 4 concentrations.

TURBIDIMETRY

The optical property expressed as turbidity is the interaction between light and suspended particles in liquid. This is an expression of the optical property that causes light to be scattered and absorbed rather than transmitted in a straight line through the sample. The quantity of a solid material in suspension can be determined by the measurement of the transmitted light. A linear relationship between turbidity and concentration is obtained when the particle sizes are uniform and homogeneous in the suspension. This is true only in very dilute suspensions containing small particles. Linearity between turbidity and concentration must be established by constructing a calibration curve using at least 4 concentrations.

RATIO TURBIDIMETRY

In ratio turbidimetry the relationship of the transmission measurement to the 90° scattered light measurement is determined. This procedure compensates for the light that is diminished by the colour of the sample. The influence of colour of the sample may also be eliminated by using an infrared light-emitting diode (IR LED) at 860 nm as light source of the instrument. The instrument's photodiode detectors receive and measure scattered light at a 90° angle from the sample as well as measuring the forward scatter (light reflected) in front of the sample along with the measurement of light transmitted directly through the sample. The measuring results are given in NTU(ratio) and are obtained by calculating the ratio of the 90° angle scattered light measured to the sum of the components of forward scattered and transmitted light values. In ratio turbidimetry the influence of stray light becomes negligible. Nephelometers are used for measurements of the degree of opalescence of colourless liquids.

Measurements of reference suspensions I-IV with a ratio turbidimeter show a linear relationship between the concentrations and measured NTU values. Reference suspensions I-IV (Ph. Eur.) may be used as calibrators for the instrument.

Table 2.2.1-2

Formazin suspensions	Opalescent values (NTU)
Reference suspension I	3
Reference suspensions II	6
Reference suspension III	18
Reference suspension IV	30
Standard of opalescence	60
Primary opalescent suspension	4000

INSTRUMENTAL DETERMINATION OF OPALESCENCE

Requirements in monographs are expressed in terms of the visual examination method with the defined reference suspensions. Instrumental methods may also be used for determining compliance with monograph requirements once the suitability of the instrument as described below has been established and calibration with reference suspensions I-IV and with *water R* or the solvent used has been performed.

Apparatus. Ratio turbidimeters or nephelometers with selectable ratio application use as light source a tungsten lamp with spectral sensitivity at about 550 nm operating at a filament colour temperature of 2700 K or IR LED having an emission maximum at 860 nm with a 60 nm spectral bandwidth. Other suitable light sources may also be used. Silicon photodiodes and photomultipliers are commonly

used as detectors and record changes in light scattered or transmitted by the sample. The light scattered at $90 \pm 2.5^\circ$ is detected by the primary detector. Other detectors are those to detect back and forward scatter as well as transmitted light. The instruments used are calibrated against standards of known turbidity and are capable of automatic determination of turbidity. The test results expressed in NTU units are obtained directly from the instrument and compared to the specifications in the individual monographs.

Instruments complying with the following specifications are suitable.

- **Measuring units:** NTU. NTU is based on the turbidity of a primary reference standard of formazin. FTU (Formazin Turbidity Units) or FNU (Formazin Nephelometry Units) are also used which are equivalent to NTU in low regions (up to 40 NTU). These units are used in all 3 instrumental methods, nephelometry, turbidimetry and ratio turbidimetry.
- **Measuring range:** 0.01-1100 NTU.
- **Resolution:** 0.01 NTU within the range of 0-10 NTU, 0.1 NTU within the range of 10-100 NTU and 1 NTU for the range > 100 NTU. The instrument is calibrated and controlled with reference standards of formazin.
- **Accuracy:** 0-10 NTU: ± 0.01 NTU. 10-1000 NTU: ± 5 per cent.
- **Repeatability:** 0-10 NTU: ± 0.01 NTU. 10-1000 NTU: ± 2 per cent of the measured value.
- **Calibration:** with 4 reference suspensions of formazin in the range of interest. Reference suspensions described in this chapter or suitable reference standards calibrated against the primary reference suspensions may be used.
- **Stray light:** this is a significant source of error in low level turbidimetric measurement; stray light reaches the detector of an optical system, but does not come from the sample < 0.15 NTU for the range 0-10 NTU, < 0.5 NTU for the range 10-1000 NTU.

Instruments complying with the above characteristics and verified using the reference suspensions described under Visual method may be used instead of visual examination for determination of compliance with monograph requirements. Instruments with range or resolution, accuracy and repeatability capabilities other than those mentioned above may be used provided they are sufficiently validated and are capable for the intended use. The test methodology for the specific substance/product to be analysed must also be validated to demonstrate its analytical capability. The instrument and methodology should be consistent with the attributes of the product to be tested.

01/2005:20202

2.2.2. DEGREE OF COLORATION OF LIQUIDS

The examination of the degree of coloration of liquids in the range brown-yellow-red is carried out by one of the 2 methods below, as prescribed in the monograph.

A solution is *colourless* if it has the appearance of *water R* or the solvent or is not more intensely coloured than reference solution B₉.

METHOD I

Using identical tubes of colourless, transparent, neutral glass of 12 mm external diameter, compare 2.0 ml of the liquid to be examined with 2.0 ml of *water R* or of the solvent or of the reference solution (see Tables of reference

solutions) prescribed in the monograph. Compare the colours in diffused daylight, viewing horizontally against a white background.

METHOD II

Using identical tubes of colourless, transparent, neutral glass with a flat base and an internal diameter of 15 mm to 25 mm, compare the liquid to be examined with *water R* or the solvent or the reference solution (see Tables of reference solutions) prescribed in the monograph, the depth of the layer being 40 mm. Compare the colours in diffused daylight, viewing vertically against a white background.

REAGENTS

Primary solutions

Yellow solution. Dissolve 46 g of *ferric chloride R* in about 900 ml of a mixture of 25 ml of *hydrochloric acid R* and 975 ml of *water R* and dilute to 1000.0 ml with the same mixture. Titrate and adjust the solution to contain 45.0 mg of $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ per millilitre by adding the same acidic mixture. Protect the solution from light.

Titration. Place in a 250 ml conical flask fitted with a ground-glass stopper, 10.0 ml of the solution, 15 ml of *water R*, 5 ml of *hydrochloric acid R* and 4 g of *potassium iodide R*, close the flask, allow to stand in the dark for 15 min and add 100 ml of *water R*. Titrate the liberated iodine with 0.1 M *sodium thiosulphate*, using 0.5 ml of *starch solution R*, added towards the end of the titration, as indicator.

1 ml of 0.1 M *sodium thiosulphate* is equivalent to 27.03 mg of $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$.

Red solution. Dissolve 60 g of *cobalt chloride R* in about 900 ml of a mixture of 25 ml of *hydrochloric acid R* and 975 ml of *water R* and dilute to 1000.0 ml with the same mixture. Titrate and adjust the solution to contain 59.5 mg of $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ per millilitre by adding the same acidic mixture.

Titration. Place in a 250 ml conical flask fitted with a ground-glass stopper, 5.0 ml of the solution, 5 ml of *dilute hydrogen peroxide solution R* and 10 ml of a 300 g/l solution of *sodium hydroxide R*. Boil gently for 10 min, allow to cool and add 60 ml of *dilute sulphuric acid R* and 2 g of *potassium iodide R*. Close the flask and dissolve the precipitate by shaking gently. Titrate the liberated iodine with 0.1 M *sodium thiosulphate*, using 0.5 ml of *starch solution R*, added towards the end of the titration, as indicator. The end-point is reached when the solution turns pink.

1 ml of 0.1 M *sodium thiosulphate* is equivalent to 23.79 mg of $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$.

Blue primary solution. Dissolve 63 g of *copper sulphate R* in about 900 ml of a mixture of 25 ml of *hydrochloric acid R* and 975 ml of *water R* and dilute to 1000.0 ml with the same mixture. Titrate and adjust the solution to contain 62.4 mg of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ per millilitre by adding the same acidic mixture.

Titration. Place in a 250 ml conical flask fitted with a ground-glass stopper, 10.0 ml of the solution, 50 ml of *water R*, 12 ml of *dilute acetic acid R* and 3 g of *potassium*

iodide R. Titrate the liberated iodine with 0.1 M *sodium thiosulphate*, using 0.5 ml of *starch solution R*, added towards the end of the titration, as indicator. The end-point is reached when the solution shows a slight pale brown colour.

1 ml of 0.1 M *sodium thiosulphate* is equivalent to 24.97 mg of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$.

Standard solutions

Using the 3 primary solutions, prepare the 5 standard solutions as follows:

Table 2.2.2.-1

Standard solution	Volume in millilitres			
	Yellow solution	Red solution	Blue solution	Hydrochloric acid (10 g/l HCl)
B (brown)	3.0	3.0	2.4	1.6
BY (brownish-yellow)	2.4	1.0	0.4	6.2
Y (yellow)	2.4	0.6	0.0	7.0
GY (greenish-yellow)	9.6	0.2	0.2	0.0
R (red)	1.0	2.0	0.0	7.0

Reference solutions for Methods I and II

Using the 5 standard solutions, prepare the following reference solutions.

Table 2.2.2.-2. - Reference solutions B

Reference solution	Volumes in millilitres	
	Standard solution B	Hydrochloric acid (10 g/l HCl)
B ₁	75.0	25.0
B ₂	50.0	50.0
B ₃	37.5	62.5
B ₄	25.0	75.0
B ₅	12.5	87.5
B ₆	5.0	95.0
B ₇	2.5	97.5
B ₈	1.5	98.5
B ₉	1.0	99.0

Table 2.2.2.-3. - Reference solutions BY

Reference solution	Volumes in millilitres	
	Standard solution BY	Hydrochloric acid (10 g/l HCl)
BY ₁	100.0	0.0
BY ₂	75.0	25.0
BY ₃	50.0	50.0
BY ₄	25.0	75.0
BY ₅	12.5	87.5
BY ₆	5.0	95.0
BY ₇	2.5	97.5

Table 2.2.2.4. - Reference solutions Y

Reference solution	Volumes in millilitres	
	Standard solution Y	Hydrochloric acid (10 g/l HCl)
Y ₁	100.0	0.0
Y ₂	75.0	25.0
Y ₃	50.0	50.0
Y ₄	25.0	75.0
Y ₅	12.5	87.5
Y ₆	5.0	95.0
Y ₇	2.5	97.5

Table 2.2.2.5. - Reference solutions GY

Reference solution	Volumes in millilitres	
	Standard solution GY	Hydrochloric acid (10 g/l HCl)
GY ₁	25.0	75.0
GY ₂	15.0	85.0
GY ₃	8.5	91.5
GY ₄	5.0	95.0
GY ₅	3.0	97.0
GY ₆	1.5	98.5
GY ₇	0.75	99.25

Table 2.2.2.6. - Reference solutions R

Reference solution	Volumes in millilitres	
	Standard solution R	Hydrochloric acid (10 g/l HCl)
R ₁	100.0	0.0
R ₂	75.0	25.0
R ₃	50.0	50.0
R ₄	37.5	62.5
R ₅	25.0	75.0
R ₆	12.5	87.5
R ₇	5.0	95.0

Storage

For Method I, the reference solutions may be stored in sealed tubes of colourless, transparent, neutral glass of 12 mm external diameter, protected from light.

For Method II, prepare the reference solutions immediately before use from the standard solutions.

01/2005:20203

2.2.3. POTENTIOMETRIC DETERMINATION OF pH

The pH is a number which represents conventionally the hydrogen ion concentration of an aqueous solution. For practical purposes, its definition is an experimental one. The pH of a solution to be examined is related to that of a reference solution (pH_s) by the following equation:

$$\text{pH} = \text{pH}_s - \frac{E - E_s}{k}$$

in which E is the potential, expressed in volts, of the cell containing the solution to be examined and E_s is the potential, expressed in volts, of the cell containing the solution of known pH (pH_s), k is the change in potential per unit change in pH expressed in volts, and calculated from the Nernst equation.

Table 2.2.3.1. - Values of k at different temperatures

Temperature (°C)	k (V)
15	0.0572
20	0.0582
25	0.0592
30	0.0601
35	0.0611

The potentiometric determination of pH is made by measuring the potential difference between 2 appropriate electrodes immersed in the solution to be examined: 1 of these electrodes is sensitive to hydrogen ions (usually a glass electrode) and the other is the reference electrode (for example, a saturated calomel electrode).

Apparatus. The measuring apparatus is a voltmeter with an input resistance at least 100 times that of the electrodes used. It is normally graduated in pH units and has a sensitivity such that discrimination of at least 0.05 pH unit or at least 0.003 V may be achieved.

Method. Unless otherwise prescribed in the monograph, all measurements are made at the same temperature (20-25 °C). Table 2.2.3.2 shows the variation of pH with respect to temperature of a number of reference buffer solutions used for calibration. For the temperature correction, when necessary, follow the manufacturer's instructions. The apparatus is calibrated with the buffer solution of potassium hydrogen phthalate (primary standard) and 1 other buffer solution of different pH (preferably one shown in Table 2.2.3.2). The pH of a third buffer solution of intermediate pH read off on the scale must not differ by more than 0.05 pH unit from the value corresponding to this solution. Immerse the electrodes in the solution to be examined and take the reading in the same conditions as for the buffer solutions.

When the apparatus is in frequent use, checks must be carried out regularly. If not, such checks should be carried out before each measurement.

All solutions to be examined and the reference buffer solutions must be prepared using *carbon dioxide-free water R*.

PREPARATION OF REFERENCE BUFFER SOLUTIONS

Potassium tetraoxalate 0.05 M. Dissolve 12.61 g of C₄H₃KO₈·2H₂O in *carbon dioxide-free water R* and dilute to 1000.0 ml with the same solvent.

Potassium hydrogen tartrate, saturated at 25 °C. Shake an excess of C₄H₅KO₆ vigorously with *carbon dioxide-free water R* at 25 °C. Filter or decant. Prepare immediately before use.

Potassium dihydrogen citrate 0.05 M. Dissolve 11.41 g of C₆H₇KO₇ in *carbon dioxide-free water R* and dilute to 1000.0 ml with the same solvent. Prepare immediately before use.

Potassium hydrogen phthalate 0.05 M. Dissolve 10.13 g of C₈H₅KO₄, previously dried for 1 h at 110 ± 2 °C, in *carbon dioxide-free water R* and dilute to 1000.0 ml with the same solvent.

Table 2.2.3.-2. – pH of reference buffer solutions at various temperatures

Temperature (°C)	Potassium tetraoxalate 0.05 M	Potassium hydrogen tartrate saturated at 25 °C	Potassium dihydrogen citrate 0.05 M	Potassium hydrogen phthalate 0.05 M	Potassium dihydrogen phosphate 0.025 M + disodium hydrogen phosphate 0.025 M	Potassium dihydrogen phosphate 0.0087 M + disodium hydrogen phosphate 0.0303 M	Disodium tetraborate 0.01 M	Sodium carbonate 0.025 M + sodium bicarbonate 0.025 M	Calcium hydroxide, saturated at 25 °C
	C ₄ H ₃ KO ₈ ·2H ₂ O	C ₄ H ₅ KO ₆	C ₆ H ₇ KO ₇	C ₈ H ₅ KO ₄	KH ₂ PO ₄ + Na ₂ HPO ₄	KH ₂ PO ₄ + Na ₂ HPO ₄	Na ₂ B ₄ O ₇ · 10H ₂ O	Na ₂ CO ₃ + NaHCO ₃	Ca(OH) ₂
15	1.67		3.80	4.00	6.90	7.45	9.28	10.12	12.81
20	1.68		3.79	4.00	6.88	7.43	9.23	10.06	12.63
25	1.68	3.56	3.78	4.01	6.87	7.41	9.18	10.01	12.45
30	1.68	3.55	3.77	4.02	6.85	7.40	9.14	9.97	12.29
35	1.69	3.55	3.76	4.02	6.84	7.39	9.10	9.93	12.13
$\frac{\Delta pH^{(1)}}{\Delta t}$	+ 0.001	– 0.0014	– 0.0022	+ 0.0012	– 0.0028	– 0.0028	– 0.0082	– 0.0096	– 0.034

(1) pH variation per degree Celsius.

Potassium dihydrogen phosphate 0.025 M + disodium hydrogen phosphate 0.025 M. Dissolve 3.39 g of KH₂PO₄ and 3.53 g of Na₂HPO₄, both previously dried for 2 h at 120 ± 2 °C, in *carbon dioxide-free water R* and dilute to 1000.0 ml with the same solvent.

Potassium dihydrogen phosphate 0.0087 M + disodium hydrogen phosphate 0.0303 M. Dissolve 1.18 g of KH₂PO₄ and 4.30 g of Na₂HPO₄, both previously dried for 2 h at 120 ± 2 °C, in *carbon dioxide-free water R* and dilute to 1000.0 ml with the same solvent.

Disodium tetraborate 0.01 M. Dissolve 3.80 g of Na₂B₄O₇·10H₂O in *carbon dioxide-free water R* and dilute to 1000.0 ml with the same solvent. Store protected from atmospheric carbon dioxide.

Sodium carbonate 0.025 M + sodium hydrogen carbonate 0.025 M. Dissolve 2.64 g of Na₂CO₃ and 2.09 g of NaHCO₃ in *carbon dioxide-free water R* and dilute to 1000.0 ml with the same solvent. Store protected from atmospheric carbon dioxide.

Calcium hydroxide, saturated at 25 °C. Shake an excess of *calcium hydroxide R* with *carbon dioxide-free water R* and decant at 25 °C. Store protected from atmospheric carbon dioxide.

STORAGE

Store buffer solutions in suitable chemically resistant, tight containers, such as type I glass bottles or plastic containers suitable for aqueous solutions.

Table 2.2.4.-1

Reaction	pH	Indicator	Colour
Alkaline	> 8	<i>Litmus paper red R</i>	Blue
		<i>Thymol blue solution R</i> (0.05 ml)	Grey or violet-blue
Slightly alkaline	8.0 – 10.0	<i>Phenolphthalein solution R</i> (0.05 ml)	Colourless or pink
		<i>Thymol blue solution R</i> (0.05 ml)	Grey
Strongly alkaline	> 10	<i>Phenolphthalein paper R</i>	Red
		<i>Thymol blue solution R</i> (0.05 ml)	Violet-blue
Neutral	6.0 – 8.0	<i>Methyl red solution R</i>	Yellow
		<i>Phenol red solution R</i> (0.05 ml)	
Neutral to methyl red	4.5 – 6.0	<i>Methyl red solution R</i>	Orange-red
Neutral to phenolphthalein	< 8.0	<i>Phenolphthalein solution R</i> (0.05 ml)	Colourless; pink or red after adding 0.05 ml of 0.1 M base
Acid	< 6	<i>Methyl red solution R</i>	Orange or red
		<i>Bromothymol blue solution R1</i>	Yellow
Faintly acid	4.0 – 6.0	<i>Methyl red solution R</i>	Orange
		<i>Bromocresol green solution R</i>	Green or blue
Strongly acid	< 4	<i>Congo red paper R</i>	Green or blue

01/2005:20204

01/2005:20205

2.2.4. RELATIONSHIP BETWEEN REACTION OF SOLUTION, APPROXIMATE pH AND COLOUR OF CERTAIN INDICATORS

To 10 ml of the solution to be examined, add 0.1 ml of the indicator solution, unless otherwise prescribed in Table 2.2.4.-1.

2.2.5. RELATIVE DENSITY

The relative density $d_{t_2}^{t_1}$ of a substance is the ratio of the mass of a certain volume of a substance at temperature t_1 to the mass of an equal volume of water at temperature t_2 . Unless otherwise indicated, the relative density d_{20}^{20} is used. Relative density is also commonly expressed as d_4^{20} . Density ρ_{20} , defined as the mass of a unit volume of the substance at 20 °C may also be used, expressed in kilograms per cubic metre or grams per cubic centimetre

($1 \text{ kg}\cdot\text{m}^{-3} = 10^{-3} \text{ g}\cdot\text{cm}^{-3}$). These quantities are related by the following equations where density is expressed in grams per cubic centimetre:

$$\rho_{20} = 0.998203 \times d_{20}^{20} \text{ or } d_{20}^{20} = 1.00180 \times \rho_{20}$$

$$\rho_{20} = 0.999972 \times d_4^{20} \text{ or } d_4^{20} = 1.00003 \times \rho_{20}$$

$$d_4^{20} = 0.998230 \times d_{20}^{20}$$

Relative density or density are measured with the precision to the number of decimals prescribed in the monograph using a density bottle (solids or liquids), a hydrostatic balance (solids), a hydrometer (liquids) or a digital density meter with an oscillating transducer (liquids and gases). When the determination is made by weighing, the buoyancy of air is disregarded, which may introduce an error of 1 unit in the 3rd decimal place. When using a density meter, the buoyancy of air has no influence.

Oscillating transducer density meter. The apparatus consists of:

- a U-shaped tube, usually of borosilicate glass, which contains the liquid to be examined;
- a magneto-electrical or piezo-electrical excitation system that causes the tube to oscillate as a cantilever oscillator at a characteristic frequency depending on the density of the liquid to be examined;
- a means of measuring the oscillation period (T), which may be converted by the apparatus to give a direct reading of density, or used to calculate density using the constants A and B described below.

The resonant frequency (f) is a function of the spring constant (c) and the mass (m) of the system:

$$f^2 = \frac{1}{T^2} = \frac{c}{m} \times \frac{1}{4\pi^2}$$

Hence:

$$T^2 = \left(\frac{M}{c} + \frac{\rho \times V}{c} \right) \times 4\pi^2$$

M = mass of the tube,

V = inner volume of the tube.

Introduction of 2 constants $A = c/(4\pi^2 \times V)$ and $B = M/V$, leads to the classical equation for the oscillating transducer:

$$\rho = A \times T^2 - B$$

The constants A and B are determined by operating the instrument with the U-tube filled with 2 different samples of known density, for example, degassed *water R* and air. Control measurements are made daily using degassed *water R*. The results displayed for the control measurement using degassed *water R* shall not deviate from the reference value ($\rho_{20} = 0.998203 \text{ g}\cdot\text{cm}^{-3}$, $d_{20}^{20} = 1.000000$) by more than its specified error. For example, an instrument specified to $\pm 0.0001 \text{ g}\cdot\text{cm}^{-3}$ shall display $0.9982 \pm 0.0001 \text{ g}\cdot\text{cm}^{-3}$ in order to be suitable for further measurement. Otherwise a re-adjustment is necessary. Calibration with certified reference materials is carried out regularly. Measurements are made using the same procedure as for calibration. The liquid to be examined is equilibrated in a thermostat at 20°C before introduction into the tube, if necessary, to avoid the formation of bubbles and to reduce the time required for measurement.

Factors affecting accuracy include:

- temperature uniformity throughout the tube,
- non-linearity over a range of density,
- parasitic resonant effects,
- viscosity, whereby solutions with a higher viscosity than the calibrant have a density that is apparently higher than the true value.

The effects of non-linearity and viscosity may be avoided by using calibrants that have density and viscosity close to those of the liquid to be examined (± 5 per cent for density, ± 50 per cent for viscosity). The density meter may have functions for automatic viscosity correction and for correction of errors arising from temperature changes and non-linearity.

Precision is a function of the repeatability and stability of the oscillator frequency, which is dependent on the stability of the volume, mass and spring constant of the cell.

Density meters are able to achieve measurements with an error of the order of $1 \times 10^{-3} \text{ g}\cdot\text{cm}^{-3}$ to $1 \times 10^{-5} \text{ g}\cdot\text{cm}^{-3}$ and a repeatability of $1 \times 10^{-4} \text{ g}\cdot\text{cm}^{-3}$ to $1 \times 10^{-6} \text{ g}\cdot\text{cm}^{-3}$.

01/2005:20206

2.2.6. REFRACTIVE INDEX

The refractive index of a medium with reference to air is equal to the ratio of the sine of the angle of incidence of a beam of light in air to the sine of the angle of refraction of the refracted beam in the given medium.

Unless otherwise prescribed, the refractive index is measured at $20 \pm 0.5^\circ\text{C}$, with reference to the wavelength of the D-line of sodium ($\lambda = 589.3 \text{ nm}$); the symbol is then n_D^{20} .

Refractometers normally determine the critical angle. In such apparatus the essential part is a prism of known refractive index in contact with the liquid to be examined. Calibrate the apparatus using certified reference materials.

When white light is used, the refractometer is provided with a compensating system. The apparatus gives readings accurate to at least the third decimal place and is provided with a means of operation at the temperature prescribed. The thermometer is graduated at intervals of 0.5°C or less.

01/2005:20207

2.2.7. OPTICAL ROTATION

Optical rotation is the property displayed by chiral substances of rotating the plane of polarisation of polarised light.

Optical rotation is considered to be positive (+) for dextrorotatory substances (i.e. those that rotate the plane of polarisation in a clockwise direction) and negative (−) for laevorotatory substances.

The specific optical rotation $[\alpha_m]_\lambda^t$ is the rotation, expressed in radians (rad), measured at the temperature t and at the wavelength λ given by a 1 m thickness of liquid or a solution containing $1 \text{ kg}/\text{m}^3$ of optically active substance. For practical reasons the specific optical rotation $[\alpha_m]_\lambda^t$ is normally expressed in milliradians metre squared per kilogram ($\text{mrad}\cdot\text{m}^2\cdot\text{kg}^{-1}$).

The Pharmacopoeia adopts the following conventional definitions.

The *angle of optical rotation* of a neat liquid is the angle of rotation α , expressed in degrees ($^\circ$), of the plane of polarisation at the wavelength of the D-line of sodium ($\lambda = 589.3 \text{ nm}$) measured at 20°C using a layer of 1 dm; for a solution, the method of preparation is prescribed in the monograph.

The *specific optical rotation* $[\alpha]_{\text{D}}^{20}$ of a liquid is the angle of rotation α , expressed in degrees ($^{\circ}$), of the plane of polarisation at the wavelength of the D-line of sodium ($\lambda = 589.3 \text{ nm}$) measured at 20°C in the liquid substance to be examined, calculated with reference to a layer of 1 dm and divided by the density expressed in grams per cubic centimetre.

The *specific optical rotation* $[\alpha]_{\text{D}}^{20}$ of a substance in solution is the angle of rotation α , expressed in degrees ($^{\circ}$), of the plane of polarisation at the wavelength of the D-line of sodium ($\lambda = 589.3 \text{ nm}$) measured at 20°C in a solution of the substance to be examined and calculated with reference to a layer of 1 dm containing 1 g/ml of the substance. The specific optical rotation of a substance in solution is always expressed with reference to a given solvent and concentration.

In the conventional system adopted by the Pharmacopoeia the specific optical rotation is expressed by its value without units; the actual units, degree millilitres per decimetre gram $[(^{\circ})\text{ml}\cdot\text{dm}^{-1}\cdot\text{g}^{-1}]$ are understood.

The conversion factor from the International System to the Pharmacopoeia system is the following:

$$[\alpha_m]_{\lambda}^t = [\alpha]_{\lambda}^t \times 0.1745$$

In certain cases specified in the monograph the angle of rotation may be measured at temperatures other than 20°C and at other wavelengths.

The polarimeter must be capable of giving readings to the nearest 0.01° . The scale is usually checked by means of certified quartz plates. The linearity of the scale may be checked by means of sucrose solutions.

Method. Determine the zero of the polarimeter and the angle of rotation of polarised light at the wavelength of the D-line of sodium ($\lambda = 589.3 \text{ nm}$) at $20 \pm 0.5^{\circ}\text{C}$. Measurements may be carried out at other temperatures only where the monograph indicates the temperature correction to be made to the measured optical rotation. Determine the zero of the apparatus with the tube closed; for liquids the zero is determined with the tube empty and for solids filled with the prescribed solvent.

Calculate the specific optical rotation using the following formulae.

For neat liquids:

$$[\alpha]_{\text{D}}^{20} = \frac{\alpha}{l \cdot \rho_{20}}$$

For substances in solution:

$$[\alpha]_{\text{D}}^{20} = \frac{1000\alpha}{l \cdot c}$$

where c is the concentration of the solution in g/l.

Calculate the content c in g/l or the content c' in per cent m/m of a dissolved substance using the following formulae:

$$c = \frac{1000\alpha}{l \cdot [\alpha]_{\text{D}}^{20}} \quad c' = \frac{1000\alpha}{l \cdot [\alpha]_{\text{D}}^{20} \cdot \rho_{20}}$$

α = angle of rotation in degrees read at $20 \pm 0.5^{\circ}\text{C}$,

l = length in decimetres of the polarimeter tube,

ρ_{20} = density at 20°C in grams per cubic centimetre. For the purposes of the Pharmacopoeia, density is replaced by relative density (2.2.5),

c = concentration of the substance in g/l,

c' = content of the substance in per cent m/m .

01/2005:20208

2.2.8. VISCOSITY

The *dynamic viscosity* or *viscosity coefficient* η is the tangential force per unit surface, known as *shearing stress* τ and expressed in pascals, necessary to move, parallel to the sliding plane, a layer of liquid of 1 square metre at a rate (v) of 1 metre per second relative to a parallel layer at a distance (x) of 1 metre.

The ratio dv/dx is a speed gradient giving the *rate of shear* D expressed in reciprocal seconds (s^{-1}), so that $\eta = \tau/D$.

The unit of dynamic viscosity is the pascal second (Pa·s). The most commonly used submultiple is the millipascal second (mPa·s).

The *kinematic viscosity* ν , expressed in square metres per second, is obtained by dividing the dynamic viscosity η by the density ρ expressed in kilograms per cubic metre, of the liquid measured at the same temperature, i.e. $\nu = \eta/\rho$. The kinematic viscosity is usually expressed in square millimetres per second.

A capillary viscometer may be used for determining the viscosity of Newtonian liquids and a rotating viscometer for determining the viscosity of Newtonian and non-Newtonian liquids. Other viscometers may be used provided that the accuracy and precision is not less than that obtained with the viscometers described below.

01/2005:20209

2.2.9. CAPILLARY VISCOMETER METHOD

The determination of viscosity using a suitable capillary viscometer is carried out at a temperature of $20 \pm 0.1^{\circ}\text{C}$, unless otherwise prescribed. The time required for the level of the liquid to drop from one mark to the other is measured with a stop-watch to the nearest one-fifth of a second. The result is valid only if two consecutive readings do not differ by more than 1 per cent. The average of not fewer than three readings gives the flow time of the liquid to be examined.

Calculate the dynamic viscosity η (2.2.8) in millipascal seconds using the formula:

$$\eta = k\rho t$$

k = constant of the viscometer, expressed in square millimetres per second squared,

ρ = density of the liquid to be examined expressed in milligrams per cubic millimetre, obtained by multiplying its relative density (d_{20}^{20}) by 0.9982,

t = flow time, in seconds, of the liquid to be examined.

The constant k is determined using a suitable viscometer calibration liquid.

To calculate the kinematic viscosity ($\text{mm}^2\cdot\text{s}^{-1}$), use the following formula: $\nu = kt$.

The determination may be carried out with an apparatus (Figure 2.2.9.-1) having the specifications described in Table 2.2.9.-1⁽¹⁾:

Table 2.2.9.-1

Size number	Nominal constant of viscometer	Kinematic viscosity range	Internal diameter of tube <i>R</i>	Volume of bulb <i>C</i>	Internal diameter of tube <i>N</i>
	mm ² ·s ⁻²	mm ² ·s ⁻¹	mm (± 2 %)	ml (± 5 %)	mm
1	0.01	3.5 to 10	0.64	5.6	2.8 to 3.2
1A	0.03	6 to 30	0.84	5.6	2.8 to 3.2
2	0.1	20 to 100	1.15	5.6	2.8 to 3.2
2A	0.3	60 to 300	1.51	5.6	2.8 to 3.2
3	1.0	200 to 1000	2.06	5.6	3.7 to 4.3
3A	3.0	600 to 3000	2.74	5.6	4.6 to 5.4
4	10	2000 to 10 000	3.70	5.6	4.6 to 5.4
4A	30	6000 to 30 000	4.07	5.6	5.6 to 6.4
5	100	20 000 to 100 000	6.76	5.6	6.8 to 7.5

Method. Fill the viscometer through tube (*L*) with a sufficient quantity of the liquid to be examined, previously brought to 20 °C unless otherwise prescribed, to fill bulb (*A*) but ensuring that the level of liquid in bulb (*B*) is below the exit to ventilation tube (*M*). Immerse the viscometer in the bath of water at 20 ± 0.1 °C, unless otherwise prescribed, maintain it in the upright position and allow to stand for not less than 30 min to allow the temperature to reach equilibrium. Close tube (*M*) and raise the level of the liquid in tube (*N*) up to a level about 8 mm above mark (*E*). Keep the liquid at this level by closing tube (*N*) and opening tube (*M*). Open tube (*N*) and measure, with a stop-watch to the nearest one-fifth of a second, the time required for the level of the liquid to drop from mark (*E*) to (*F*).

01/2005:20210

2.2.10. ROTATING VISCOMETER METHOD

Commonly used types of rotating viscometers are based on the measurement of shearing forces in a liquid medium placed between two coaxial cylinders, one of which is driven by a motor and the other is made to revolve by the rotation of the first. Under these conditions, the viscosity (or apparent viscosity) becomes a measurement (*M*) of the angle of deflection of the cylinder made to revolve, which corresponds to a moment of force expressed in Newton metres.

For laminar flow, the dynamic viscosity η expressed in pascal seconds is given by the formula:

$$\eta = \frac{1}{\omega} \left(\frac{M}{4\pi \cdot h} \right) \cdot \left(\frac{1}{R_A^2} - \frac{1}{R_B^2} \right)$$

where *h* is the height of immersion in metres of the cylinder made to revolve in the liquid medium, *R_A* and *R_B* are the radii in metres of the cylinders, *R_A* being smaller than *R_B*, and ω is the angular velocity in radians per second. The constant *k* of the apparatus⁽²⁾ may be determined at various speeds of rotation using a suitable viscometer calibration liquid. The viscosity then corresponds to the formula:

$$\eta = k \frac{M}{\omega}$$

Method. Measure the viscosity according to the instructions for the operation of the rotating viscometer. The temperature for measuring the viscosity is indicated in the monograph. For pseudoplastic and other non-Newtonian systems, the monograph indicates the type of viscometer to be used and the angular velocity or the shear rate at which the measurement is made. If it is impossible to obtain the indicated shear rate exactly, use a shear rate slightly higher and a shear rate slightly lower and interpolate.

01/2005:20211

2.2.11. DISTILLATION RANGE

The distillation range is the temperature interval, corrected for a pressure of 101.3 kPa (760 Torr), within which a liquid, or a specified fraction of a liquid, distils in the following conditions.

Apparatus. The apparatus (see Figure 2.2.11.-1) consists of a distillation flask (*A*), a straight tube condenser (*B*) which fits on to the side arm of the flask and a plain-bend adaptor (*C*) attached to the end of the condenser. The lower end of the condenser may, alternatively, be bent to replace the

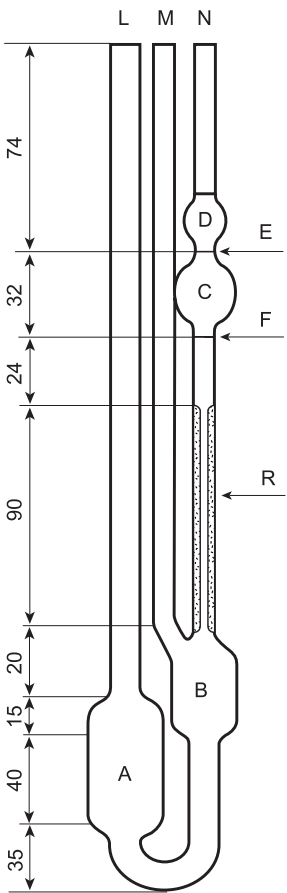


Figure 2.2.9.-1. – Suspended level viscometer
Dimensions in millimetres

The minimum flow time should be 350 s for size no. 1 and 200 s for all other sizes.

(1) The European Pharmacopoeia describes the system proposed by the International Organisation for Standardisation (ISO).

(2) Commercially available apparatus is supplied with tables giving the constants of the apparatus in relation to the surface area of the cylinders used and their speed of rotation.

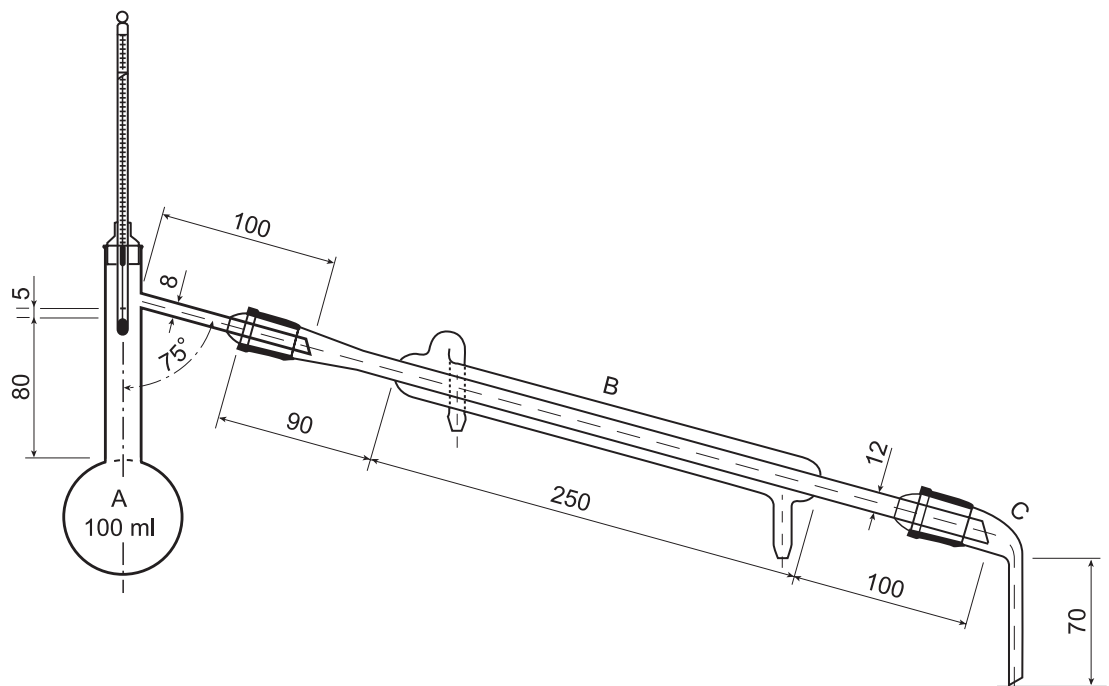


Figure 2.2.11.-1. – Apparatus for the determination of distillation range
Dimensions in millimetres

adaptor. A thermometer is inserted in the neck of the flask so that the upper end of the mercury reservoir is 5 mm lower than the junction of the lower wall of the lateral tube. The thermometer is graduated at 0.2 °C intervals and the scale covers a range of about 50 °C. During the determination, the flask, including its neck, is protected from draughts by a suitable screen.

Method. Place in the flask (A) 50.0 ml of the liquid to be examined and a few pieces of porous material. Collect the distillate in a 50 ml cylinder graduated in 1 ml. Cooling by circulating water is essential for liquids distilling below 150 °C. Heat the flask so that boiling is rapidly achieved and note the temperature at which the first drop of distillate falls into the cylinder. Adjust the heating to give a regular rate of distillation of 2-3 ml/min and note the temperature when the whole or the prescribed fraction of the liquid, measured at 20 °C, has distilled.

Table 2.2.11.-1. – Temperature correction in relation to the pressure

Distillation temperature	Correction factor <i>k</i>
up to 100 °C	0.30
above 100 °C up to 140 °C	0.34
above 140 °C up to 190 °C	0.38
above 190 °C up to 240 °C	0.41
above 240 °C	0.45

Correct the observed temperatures for barometric pressure by means of the formula:

$$t_1 = t_2 + k(101.3 - b)$$

*t*₁ = the corrected temperature,
*t*₂ = the observed temperature, at the barometric pressure *b*,

k = the correction factor taken from Table 2.2.11.-1 unless the factor is given,
b = the barometric pressure, expressed in kilopascals, during the distillation.

01/2005:20212

2.2.12. BOILING POINT

The boiling point is the corrected temperature at which the vapour pressure of a liquid is equal to 101.3 kPa.

Apparatus. The apparatus is that used for Distillation Range (2.2.11) with the exception that the thermometer is inserted in the neck of the flask so that the lower end of the mercury reservoir is level with the lower end of the neck of the distillation flask and that the flask is placed on a plate of isolating material pierced by a hole 35 mm in diameter.

Method. Place in the flask (A) 20 ml of the liquid to be examined and a few pieces of porous material. Heat the flask so that boiling is rapidly achieved and record the temperature at which liquid runs from the side-arm into the condenser.

Correct the observed temperature for barometric pressure by means of the formula:

$$t_1 = t_2 + k(101.3 - b)$$

*t*₁ = the corrected temperature,
*t*₂ = the observed temperature at barometric pressure *b*,
k = the correction factor as shown in Table 2.2.11.-1 under Distillation Range,
b = the barometric pressure, in kilopascals, at the time of the determination.

01/2005:20213

2.2.13. DETERMINATION OF WATER BY DISTILLATION

The apparatus (see Figure 2.2.13-1) consists of a glass flask (A) connected by a tube (D) to a cylindrical tube (B) fitted with a graduated receiving tube (E) and reflux condenser (C). The receiving tube (E) is graduated in 0.1 ml. The source of heat is preferably an electric heater with rheostat control or an oil bath. The upper portion of the flask and the connecting tube may be insulated.

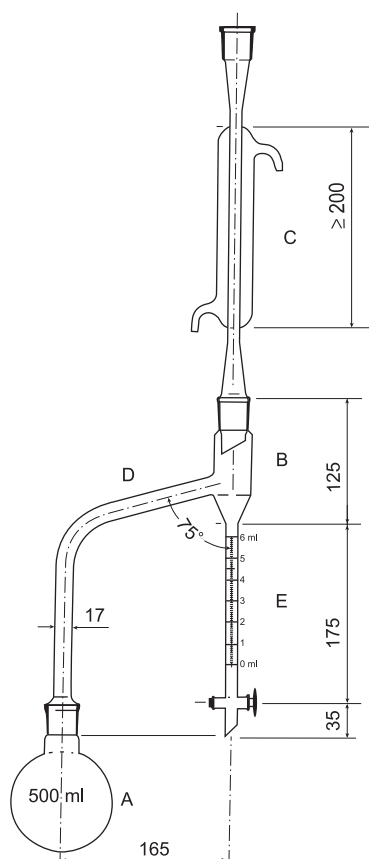


Figure 2.2.13-1. – Apparatus for the determination of water by distillation

Dimensions in millimetres

Method. Clean the receiving tube and the condenser of the apparatus, thoroughly rinse with water, and dry.

Introduce 200 ml of *toluene R* and about 2 ml of *water R* into the dry flask. Distil for 2 h, then allow to cool for about 30 min and read the water volume to the nearest 0.05 ml. Place in the flask a quantity of the substance, weighed with an accuracy of 1 per cent, expected to give about 2 ml to 3 ml of water. If the substance has a pasty consistency, weigh it in a boat of metal foil. Add a few pieces of porous material and heat the flask gently for 15 min. When the toluene begins to boil, distil at the rate of about two drops per second until most of the water has distilled over, then increase the rate of distillation to about four drops per second. When the water has all distilled over, rinse the inside of the condenser tube with *toluene R*. Continue the distillation for 5 min, remove the heat, allow the receiving tube to cool to room

temperature and dislodge any droplets of water which adhere to the walls of the receiving tube. When the water and toluene have completely separated, read the volume of water and calculate the content present in the substance as millilitre per kilogram, using the formula:

$$\frac{1000 (n_2 - n_1)}{m}$$

- m = the mass in grams of the substance to be examined,
 n_1 = the number of millilitres of water obtained in the first distillation,
 n_2 = the total number of millilitres of water obtained in the 2 distillations.

01/2005:20214

2.2.14. MELTING POINT - CAPILLARY METHOD

The melting point determined by the capillary method is the temperature at which the last solid particle of a compact column of a substance in a tube passes into the liquid phase.

When prescribed in the monograph, the same apparatus and method are used for the determination of other factors, such as meniscus formation or melting range, that characterise the melting behaviour of a substance.

Apparatus. The apparatus consists of:

- a suitable glass vessel containing a liquid bath (for example, water, liquid paraffin or silicone oil) and fitted with a suitable means of heating,
- a suitable means of stirring, ensuring uniformity of temperature within the bath,
- a suitable thermometer with graduation at not more than 0.5 °C intervals and provided with an immersion mark. The range of the thermometer is not more than 100 °C,
- alkali-free hard-glass capillary tubes of internal diameter 0.9 mm to 1.1 mm with a wall 0.10 mm to 0.15 mm thick and sealed at one end.

Method. Unless otherwise prescribed, dry the finely powdered substance *in vacuo* and over *anhydrous silica gel R* for 24 h. Introduce a sufficient quantity into a capillary tube to give a compact column 4 mm to 6 mm in height. Raise the temperature of the bath to about 10 °C below the presumed melting point and then adjust the rate of heating to about 1 °C/min. When the temperature is 5 °C below the presumed melting point, correctly introduce the capillary tube into the instrument. For the apparatus described above, immerse the capillary tube so that the closed end is near the centre of the bulb of the thermometer, the immersion mark of which is at the level of the surface of the liquid. Record the temperature at which the last particle passes into the liquid phase.

Calibration of the apparatus. The apparatus may be calibrated using melting point reference substances such as those of the World Health Organisation or other appropriate substances.

01/2005:20215

2.2.15. MELTING POINT - OPEN CAPILLARY METHOD

For certain substances, the following method is used to determine the melting point (also referred to as slip point and rising melting point when determined by this method).

Use glass capillary tubes open at both ends, about 80 mm long, having an external diameter of 1.4 mm to 1.5 mm and an internal diameter of 1.0 mm to 1.2 mm.

Introduce into each of 5 capillary tubes a sufficient amount of the substance, previously treated as described, to form in each tube a column about 10 mm high and allow the tubes to stand for the appropriate time and at the prescribed temperature.

Unless otherwise prescribed, substances with a waxy consistency are carefully and completely melted on a water-bath before introduction into the capillary tubes. Allow the tubes to stand at 2-8 °C for 2 h.

Attach one of the tubes to a thermometer graduated in 0.5 °C so that the substance is close to the bulb of the thermometer. Introduce the thermometer with the attached tube into a beaker so that the distance between the bottom of the beaker and the lower part of the bulb of the thermometer is 1 cm. Fill the beaker with water to a depth of 5 cm. Increase the temperature of the water gradually at a rate of 1 °C/min.

The temperature at which the substance begins to rise in the capillary tube is regarded as the melting point.

Repeat the operation with the other 4 capillary tubes and calculate the result as the mean of the 5 readings.

01/2005:20216

2.2.16. MELTING POINT - INSTANTANEOUS METHOD

The instantaneous melting point is calculated using the expression:

$$\frac{t_1 + t_2}{2}$$

in which t_1 is the first temperature and t_2 the second temperature read under the conditions stated below.

Apparatus. The apparatus consists of a metal block resistant to the substance to be examined, of good heat-conducting capacity, such as brass, with a carefully polished plane upper surface. The block is uniformly heated throughout its mass by means of a micro-adjustable gas heater or an electric heating device with fine adjustment. The block has a cylindrical cavity, wide enough to accommodate a thermometer, which should be maintained with the mercury column in the same position during the calibration of the apparatus and the determination of the melting point of the substance to be examined. The cylindrical cavity is parallel to the upper polished surface of the block and about 3 mm from it. The apparatus is calibrated using appropriate substances of known melting point.

Method. Heat the block at a suitably rapid rate to a temperature about 10 °C below the presumed melting temperature, then adjust the heating rate to about 1 °C/min.

At regular intervals drop a few particles of powdered and, where appropriate, dried substance, prepared as for the capillary tube method, onto the block in the vicinity of the thermometer bulb, cleaning the surface after each test. Record the temperature t_1 at which the substance melts instantaneously for the first time in contact with the metal. Stop the heating. During cooling drop a few particles of the substance at regular intervals on the block, cleaning the surface after each test. Record the temperature t_2 at which the substance ceases to melt instantaneously when it comes in contact with the metal.

Calibration of the apparatus. The apparatus may be calibrated using melting point reference substances such as those of the World Health Organisation or other appropriate substances.

01/2005:20217

2.2.17. DROP POINT

The drop point is the temperature at which the first drop of the melting substance to be examined falls from a cup under defined conditions.

Apparatus. The apparatus (see Figure 2.2.17-1)

consists of 2 metal sheaths (A) and (B) screwed together. Sheath (A) is fixed to a mercury thermometer. A metal cup (F) is loosely fixed to the lower part of sheath (B) by means of 2 tightening bands (E). Fixed supports (D) 2 mm long determine the exact position of the cup in addition to which they are used to centre the thermometer. A hole (C) pierced in the wall of sheath (B) is used to balance the pressure. The draining surface of the cup must be flat and the edges of the outflow orifice must be at right angles to it. The lower part of the mercury thermometer has the form and size shown in the Figure; it covers a range from 0 °C to 110 °C and on its scale a distance of 1 mm represents a difference of 1 °C. The mercury reservoir of the thermometer has a diameter of 3.5 ± 0.2 mm and a height of 6.0 ± 0.3 mm. The apparatus is placed in the axis of a tube about 200 mm long and with an external diameter of about 40 mm. It is fixed to the test-tube by means of a stopper through which the thermometer passes, and is provided with a side groove. The opening of the cup is placed about 15 mm from the bottom of the test-tube. The whole device is immersed in a beaker with a capacity of about 1 litre, filled with water. The bottom of the test-tube is placed about 25 mm from the bottom of the beaker. The water level reaches the upper part of sheath (A). A stirrer is used to ensure that the temperature of the water remains uniform.

Method. Fill the cup to the brim with the substance to be examined, without melting it, unless otherwise prescribed. Remove the excess substance at the 2 ends of the cup with a spatula. When sheaths (A) and (B) have been assembled press the cup into its housing in sheath (B) until it touches the supports. Remove with a spatula the substance pushed out by the thermometer. Place the apparatus in the water-bath as described above. Heat the water-bath and when the temperature is at about 10 °C below the presumed drop point, adjust the heating rate to about 1 °C/min. Note the temperature at the fall of the first drop. Carry out at least 3 determinations, each time with a fresh sample of the substance. The difference between the readings must not exceed 3 °C. The mean of three readings is the drop point of the substance.

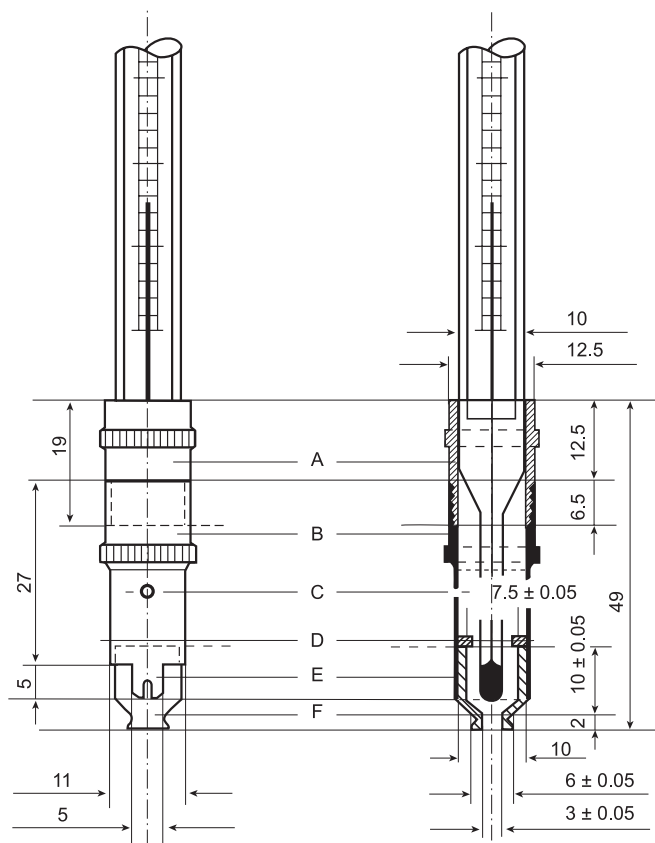


Figure 2.2.17-1. – Apparatus for the determination of drop point
Dimensions in millimetres

01/2005:20218

2.2.18. FREEZING POINT

The freezing point is the maximum temperature occurring during the solidification of a supercooled liquid.

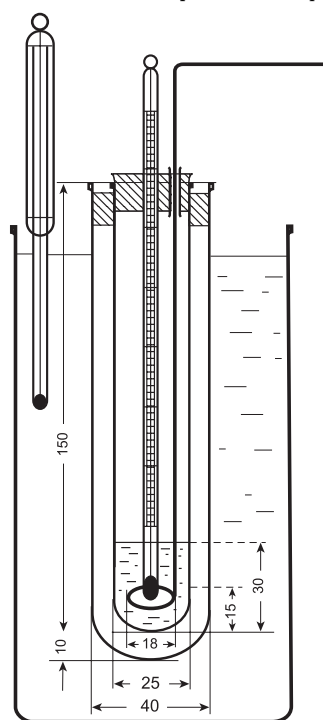


Figure 2.2.18-1. – Apparatus for the determination of
freezing point
Dimensions in millimetres

Apparatus. The apparatus (see Figure 2.2.18-1) consists of a test-tube about 25 mm in diameter and 150 mm long placed inside a test-tube about 40 mm in diameter and 160 mm long. The inner tube is closed by a stopper which carries a thermometer about 175 mm long and graduated in 0.2 °C fixed so that the bulb is about 15 mm above the bottom of the tube. The stopper has a hole allowing the passage of the stem of a stirrer made from a glass rod or other suitable material formed at one end into a loop of about 18 mm overall diameter at right angles to the rod. The inner tube with its jacket is supported centrally in a 1 litre beaker containing a suitable cooling liquid to within 20 mm of the top. A thermometer is supported in the cooling bath.

Method. Place in the inner tube sufficient quantity of the liquid or previously melted substance to be examined, to cover the thermometer bulb and determine the approximate freezing point by cooling rapidly. Place the inner tube in a bath about 5 °C above the approximate freezing point until all but the last traces of crystals are melted. Fill the beaker with water or a saturated solution of sodium chloride, at a temperature about 5 °C lower than the expected freezing point, insert the inner tube into the outer tube, ensuring that some seed crystals are present, and stir thoroughly until solidification takes place. Note the highest temperature observed during solidification.

01/2005:20219

2.2.19. AMPEROMETRIC TITRATION

In amperometric titration the end-point is determined by following the variation of the current measured between 2 electrodes (either one indicator electrode and one reference

electrode or 2 indicator electrodes) immersed in the solution to be examined and maintained at a constant potential difference as a function of the quantity of titrant added.

The potential of the measuring electrode is sufficient to ensure a diffusion current for the electroactive substance.

Apparatus. The apparatus comprises an adjustable voltage source and a sensitive microammeter; the detection system generally consists of an indicator electrode (for example, a platinum electrode, a dropping-mercury electrode, a rotating-disc electrode or a carbon electrode) and a reference electrode (for example, a calomel electrode or a silver-silver chloride electrode).

A three-electrode apparatus is sometimes used, consisting of an indicator electrode, a reference electrode and a polarised auxiliary electrode.

Method. Set the potential of the indicator electrode as prescribed and plot a graph of the initial current and the values obtained during the titration as functions of the quantity of titrant added. Add the titrant in not fewer than 3 successive quantities equal to a total of about 80 per cent of the theoretical volume corresponding to the presumed equivalence point. The 3 values must fall on a straight line. Continue adding the titrant beyond the presumed equivalence point in not fewer than 3 successive quantities. The values obtained must fall on a straight line. The point of intersection of the 2 lines represents the end-point of the titration.

For amperometric titration with 2 indicator electrodes, the whole titration curve is recorded and used to determine the end-point.

01/2005:20220

2.2.20. POTENTIOMETRIC TITRATION

In a potentiometric titration the end-point of the titration is determined by following the variation of the potential difference between 2 electrodes (either one indicator electrode and one reference electrode or 2 indicator electrodes) immersed in the solution to be examined as a function of the quantity of titrant added.

The potential is usually measured at zero or practically zero current.

Apparatus. The apparatus used (a simple potentiometer or electronic device) comprises a voltmeter allowing readings to the nearest millivolt.

The indicator electrode to be used depends on the substance to be determined and may be a glass or metal electrode (for example, platinum, gold, silver or mercury). The reference electrode is generally a calomel or a silver-silver chloride electrode.

For acid-base titrations and unless otherwise prescribed, a glass-calomel or glass-silver-silver chloride electrode combination is used.

Method. Plot a graph of the variation of potential difference as a function of the quantity of the titrant added, continuing the addition of the titrant beyond the presumed equivalence point. The end-point corresponds to a sharp variation of potential difference.

01/2005:20221

2.2.21. FLUORIMETRY

Fluorimetry is a procedure which uses the measurement of the intensity of the fluorescent light emitted by the substance to be examined in relation to that emitted by a given standard.

Method. Dissolve the substance to be examined in the solvent or mixture of solvents prescribed in the monograph, transfer the solution to the cell or the tube of the fluorimeter and illuminate it with an excitant light beam of the wavelength prescribed in the monograph and as near as possible monochromatic.

Measure the intensity of the emitted light at an angle of 90° to the excitant beam, after passing it through a filter which transmits predominantly light of the wavelength of the fluorescence. Other types of apparatus may be used provided that the results obtained are identical.

For quantitative determinations, first introduce into the apparatus the solvent or mixture of solvents used to dissolve the substance to be examined and set the instrument to zero. Introduce the standard solution and adjust the sensitivity of the instrument so that the reading is greater than 50. If the second adjustment is made by altering the width of the slits, a new zero setting must be made and the intensity of the standard must be measured again. Finally introduce the solution of unknown concentration and read the result on the instrument. Calculate the concentration c_x of the substance in the solution to be examined, using the formula:

$$c_x = \frac{I_x c_s}{I_s}$$

c_x = concentration of the solution to be examined,

c_s = concentration of the standard solution,

I_x = intensity of the light emitted by the solution to be examined,

I_s = intensity of the light emitted by the standard solution.

If the intensity of the fluorescence is not strictly proportional to the concentration, the measurement may be effected using a calibration curve.

In some cases, measurement can be made with reference to a fixed standard (for example a fluorescent glass or a solution of another fluorescent substance). In such cases, the concentration of the substance to be examined must be determined using a previously drawn calibration curve under the same conditions.

01/2005:20222

2.2.22. ATOMIC EMISSION SPECTROMETRY

Atomic emission spectrometry is a method for determining the concentration of an element in a substance by measuring the intensity of one of the emission lines of the atomic vapour of the element generated from the substance. The determination is carried out at the wavelength corresponding to this emission line.

Apparatus. This consists essentially of an atomic generator of the element to be determined (flame, plasma, arc, etc.), a monochromator and a detector. If the generator is a flame, *water R* is the solvent of choice for preparing test and reference solutions, although organic solvents may also be used if precautions are taken to ensure that the solvent does not interfere with the stability of the flame.

Method. Operate an atomic emission spectrometer in accordance with the manufacturer's instructions at the prescribed wavelength setting. Introduce a blank solution into the atomic generator and adjust the instrument reading to zero. Introduce the most concentrated reference solution and adjust the sensitivity to obtain a suitable reading.

Determinations are made by comparison with reference solutions with known concentrations of the element to be determined either by the Method of Direct Calibration (Method I) or the Method of Standard Additions (Method II).

METHOD I - DIRECT CALIBRATION

Prepare the solution of the substance to be examined (test solution) as prescribed. Prepare not fewer than 3 reference solutions of the element to be determined the concentrations of which span the expected value in the test solution. Any reagents used in the preparation of the test solution are added to the reference solutions at the same concentration. Introduce the test solution and each reference solution into the instrument at least 3 times and record the steady reading. Rinse the apparatus with blank solution each time and ascertain that the reading returns to its initial blank value. Prepare a calibration curve from the mean of the readings obtained with the reference solutions and determine the concentration of the element in the test solution from the curve so obtained.

METHOD II - STANDARD ADDITIONS

Add to at least 3 similar volumetric flasks equal volumes of the solution of the substance to be examined (test solution) prepared as prescribed. Add to all but one of the flasks progressively larger volumes of a reference solution containing a known concentration of the element to be determined to produce a series of solutions containing steadily increasing concentrations of that element known to give responses in the linear part of the curve. Dilute the contents of each flask to volume with solvent.

Introduce each of the solutions into the instrument at least 3 times and record the steady reading. Rinse the apparatus with solvent each time and ascertain that the reading returns to its initial blank value.

Calculate the linear equation of the graph using a least-squares fit, and derive from it the concentration of the element to be determined in the test solution.

Alternatively, plot on a graph the mean of readings against the added quantity of the element to be determined. Extrapolate the line joining the points on the graph until it meets the concentration axis. The distance between this point and the intersection of the axes represents the concentration of the element to be determined in the test solution.

If a solid sampling technique is required, full details of the procedure to be followed are provided in the monograph.

ensure that the solvent does not interfere with the stability of the flame. When a furnace is used, substances may be introduced dissolved in *water R* or an organic solvent, but with this technique, solid sampling is also possible.

The atomic vapour may also be generated outside the spectrometer, for example, the cold vapour method for mercury or certain hydrides. For mercury, atoms are generated by chemical reduction and the atomic vapour is swept by a stream of an inert gas into an absorption cell mounted in the optical path of the instrument. Hydrides are either mixed with the gas feeding the burner or swept by an inert gas into a heated cell in which they are dissociated into atoms.

Method. Operate an atomic absorption spectrometer in accordance with the manufacturer's instructions at the prescribed wavelength setting. Introduce a blank solution into the atomic generator and adjust the instrument reading so that it indicates maximum transmission. Introduce the most concentrated reference solution and adjust the sensitivity to obtain a suitable absorbance reading.

Determinations are made by comparison with reference solutions with known concentrations of the element to be determined either by the Method of Direct Calibration (Method I) or the Method of Standard Additions (Method II).

METHOD I - DIRECT CALIBRATION

Prepare the solution of the substance to be examined (test solution) as prescribed. Prepare not fewer than 3 reference solutions of the element to be determined the concentrations of which span the expected value in the test solution. Any reagents used in the preparation of the test solution are added to the reference and blank solutions at the same concentration.

Introduce the test solution and each reference solution into the instrument at least 3 times and record the steady reading. Rinse the apparatus with the blank solution each time and ascertain that the reading returns to its initial blank value.

If a furnace is being used, it is fired between readings.

Prepare a calibration curve from the mean of the readings obtained with the reference solutions and determine the concentration of the element to be determined in the test solution from the curve so obtained.

If a solid sampling technique is required, full details of the procedure to be followed are provided in the monograph.

METHOD II - STANDARD ADDITIONS

Add to at least 3 similar volumetric flasks equal volumes of the solution of the substance to be examined (test solution) prepared as prescribed. Add to all but one of the flasks progressively larger volumes of a reference solution containing a known concentration of the element to be determined to produce a series of solutions containing increasing concentrations of that element known to give responses in the linear part of the curve. Dilute the contents of each flask to volume with solvent.

Introduce each of the solutions into the instrument at least 3 times and record the steady reading. Rinse the apparatus with solvent each time and ascertain that the reading returns to its initial blank value.

If a furnace is being used, it is fired between readings.

Calculate the linear equation of the graph using a least-squares fit, and derive from it the concentration of the element to be determined in the test solution.

Alternatively, plot on a graph the mean of readings against the added quantity of the element to be determined. Extrapolate the line joining the points on the graph until it meets the concentration axis. The distance between

01/2005:20223

2.2.23. ATOMIC ABSORPTION SPECTROMETRY

Atomic absorption spectrometry is a method for determining the concentration of an element in a substance by measuring the absorption of radiation by the atomic vapour of the element generated from the substance. The determination is carried out at the wavelength of one of the absorption lines of the element concerned.

Apparatus. This consists essentially of a source of radiation, an atomic generator of the element to be determined (flame, furnace, etc.), a monochromator and a detector.

The method of introducing the substance to be analysed depends on the type of atomic generator used. If it is a flame, substances are nebulised and *water R* is the solvent of choice for preparing test and reference solutions although organic solvents may also be used if precautions are taken to

this point and the intersection of the axes represents the concentration of the element to be determined in the test solution.

If a solid sampling technique is required, full details of the procedure to be followed are provided in the monograph.

01/2005:20224

2.2.24. ABSORPTION SPECTROPHOTOMETRY, INFRARED

Infrared spectrophotometers are used for recording spectra in the region of 4000-650 cm⁻¹ (2.5-15.4 µm) or in some cases down to 200 cm⁻¹ (50 µm).

APPARATUS

Spectrophotometers for recording spectra consist of a suitable light source, monochromator or interferometer and detector.

Fourier transform spectrophotometers use polychromatic radiation and calculate the spectrum in the frequency domain from the original data by Fourier transformation. Spectrophotometers fitted with an optical system capable of producing monochromatic radiation in the measurement region may also be used. Normally the spectrum is given as a function of transmittance, the quotient of the intensity of the transmitted radiation and the incident radiation. It may also be given in absorbance.

The absorbance (*A*) is defined as the logarithm to base 10 of the reciprocal of the transmittance (*T*):

$$A = \log_{10} \left(\frac{1}{T} \right) = \log_{10} \left(\frac{I_0}{I} \right)$$

$$T = \frac{I}{I_0},$$

*I*₀ = intensity of incident radiation,

I = intensity of transmitted radiation.

PREPARATION OF THE SAMPLE

FOR RECORDING BY TRANSMISSION OR ABSORPTION
Prepare the substance by one of the following methods.

Liquids. Examine a liquid either in the form of a film between 2 plates transparent to infrared radiation, or in a cell of suitable path length, also transparent to infrared radiation.

Liquids or solids in solution. Prepare a solution in a suitable solvent. Choose a concentration and a path length of the cell which give a satisfactory spectrum. Generally, good results are obtained with concentrations of 10-100 g/l for a path length of 0.5-0.1 mm. Absorption due to the solvent is compensated by placing in the reference beam a similar cell containing the solvent used. If an FT-IR instrument is used, the absorption is compensated by recording the spectra for the solvent and the sample successively. The solvent absorbance, corrected by a compensation factor, is subtracted using calculation software.

Solids. Examine solids dispersed in a suitable liquid (mull) or in a solid (halide disc), as appropriate. If prescribed in the monograph, make a film of a molten mass between 2 plates transparent to infrared radiation.

A. Mull

Triturate a small quantity of the substance to be examined with the minimum quantity of *liquid paraffin R* or other suitable liquid; 5-10 mg of the substance to be examined

is usually sufficient to make an adequate mull using one drop of *liquid paraffin R*. Compress the mull between 2 plates transparent to infrared radiation.

B. Disc

Triturate 1-2 mg of the substance to be examined with 300-400 mg, unless otherwise specified, of finely powdered and dried *potassium bromide R* or *potassium chloride R*. These quantities are usually sufficient to give a disc of 10-15 mm diameter and a spectrum of suitable intensity. If the substance is a hydrochloride, it is recommended to use *potassium chloride R*. Carefully grind the mixture, spread it uniformly in a suitable die, and submit it to a pressure of about 800 MPa (8 tcm⁻²). For substances that are unstable under normal atmospheric conditions or are hygroscopic, the disc is pressed *in vacuo*. Several factors may cause the formation of faulty discs, such as insufficient or excessive grinding, humidity or other impurities in the dispersion medium or an insufficient reduction of particle size. A disc is rejected if visual examination shows lack of uniform transparency or when transmittance at about 2000 cm⁻¹ (5 µm) in the absence of a specific absorption band is less than 60 per cent without compensation, unless otherwise prescribed.

Gases. Examine gases in a cell transparent to infrared radiation and having an optical path length of about 100 mm. Evacuate the cell and fill to the desired pressure through a stopcock or needle valve using a suitable gas transfer line between the cell and the container of the gas to be examined.

If necessary adjust the pressure in the cell to atmospheric pressure using a gas transparent to infrared radiation (for example *nitrogen R* and *argon R*). To avoid absorption interferences due to water, carbon dioxide or other atmospheric gases, place in the reference beam, if possible, an identical cell that is either evacuated or filled with the gas transparent to infrared radiation.

FOR RECORDING BY DIFFUSE REFLECTANCE

Solids. Triturate a mixture of the substance to be examined with finely powdered and dried *potassium bromide R* or *potassium chloride R*. Use a mixture containing approximately 5 per cent of the substance, unless otherwise specified. Grind the mixture, place it in a sample cup and examine the reflectance spectrum.

The spectrum of the sample in absorbance mode may be obtained after mathematical treatment of the spectra by the Kubelka-Munk function.

FOR RECORDING BY ATTENUATED TOTAL REFLECTION

Attenuated total reflection (including multiple reflection) involves light being reflected internally by a transmitting medium, typically for a number of reflections. However, several accessories exist where only one reflection occurs. Prepare the substance as follows. Place the substance to be examined in close contact with an internal reflection element (IRE) such as diamond, germanium, zinc selenide, thallium bromide-thallium iodide (KRS-5) or another suitable material of high refractive index. Ensure close and uniform contact between the substance and the whole crystal surface of the internal reflection element, either by applying pressure or by dissolving the substance in an appropriate solvent, then covering the IRE with the obtained solution and evaporating to dryness. Examine the attenuated total reflectance (ATR) spectrum.

IDENTIFICATION USING REFERENCE SUBSTANCES

Prepare the substance to be examined and the reference substance by the same procedure and record the spectra between 4000-650 cm⁻¹ (2.5-15.4 µm) under the same operational conditions. The transmission minima (absorption

maxima) in the spectrum obtained with the substance to be examined correspond in position and relative size to those in the spectrum obtained with the reference substance (CRS).

When the spectra recorded in the solid state show differences in the positions of the transmission minima (absorption maxima), treat the substance to be examined and the reference substance in the same manner so that they crystallise or are produced in the same form, or proceed as prescribed in the monograph, then record the spectra.

IDENTIFICATION USING REFERENCE SPECTRA

Control of resolution performance. For instruments having a monochromator, record the spectrum of a polystyrene film approximately 35 µm in thickness. The difference x (see Figure 2.2.24.-1) between the percentage transmittance at the transmission maximum A at 2870 cm⁻¹ (3.48 µm) and that at the transmission minimum B at 2849.5 cm⁻¹ (3.51 µm) must be greater than 18. The difference y between the percentage transmittance at the transmission maximum C at 1589 cm⁻¹ (6.29 µm) and that at the transmission minimum D at 1583 cm⁻¹ (6.32 µm) must be greater than 10.

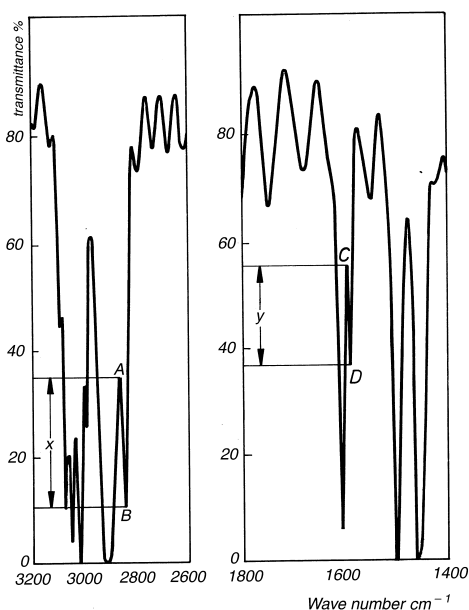


Figure 2.2.24.-1. – Typical spectrum of polystyrene used to verify the resolution performance

For Fourier-transform instruments, use suitable instrument resolution with the appropriate apodisation prescribed by the manufacturer. The resolution is checked by suitable means, for example by recording the spectrum of a polystyrene film approximately 35 µm in thickness. The difference between the absorbances at the absorption minimum at 2870 cm⁻¹ and the absorption maximum at 2849.5 cm⁻¹ is greater than 0.33. The difference between the absorbances at the absorption minimum at 1589 cm⁻¹ and the absorption maximum at 1583 cm⁻¹ is greater than 0.08.

Verification of the wave-number scale. The wave-number scale may be verified using a polystyrene film, which has transmission minima (absorption maxima) at the wave numbers (in cm⁻¹) shown in Table 2.2.24.-1.

Table 2.2.24.-1. – Transmission minima and acceptable tolerances of a polystyrene film

Transmission minima (cm ⁻¹)	Acceptable tolerance (cm ⁻¹)	
	Monochromator instruments	Fourier-transform instruments
3060.0	± 1.5	± 1.0
2849.5	± 2.0	± 1.0
1942.9	± 1.5	± 1.0
1601.2	± 1.0	± 1.0
1583.0	± 1.0	± 1.0
1154.5	± 1.0	± 1.0
1028.3	± 1.0	± 1.0

Method. Prepare the substance to be examined according to the instructions accompanying the reference spectrum/reference substance. Using the operating conditions that were used to obtain the reference spectrum, which will usually be the same as those for verifying the resolution performance, record the spectrum of the substance to be examined.

The positions and the relative sizes of the bands in the spectrum of the substance to be examined and the reference spectrum are concordant in the 2 spectra.

Compensation for water vapour and atmospheric carbon dioxide. For Fourier-transform instruments, spectral interference from water vapour and carbon dioxide is compensated using suitable algorithms according to the manufacturer's instructions. Alternatively, spectra can be acquired using suitable purged instruments or ensuring that sample and background single beam spectra are acquired under exactly the same conditions.

IMPURITIES IN GASES

For the analysis of impurities, use a cell transparent to infrared radiation and of suitable optical path length (for example, 1-20 m). Fill the cell as prescribed under Gases. For detection and quantification of the impurities, proceed as prescribed in the monograph.

01/2005:20225

2.2.25. ABSORPTION SPECTROPHOTOMETRY, ULTRAVIOLET AND VISIBLE

Determination of absorbance. The absorbance (A) of a solution is defined as the logarithm to base 10 of the reciprocal of the transmittance (T) for monochromatic radiation:

$$A = \log_{10} \left(\frac{1}{T} \right) = \log_{10} \left(\frac{I_0}{I} \right)$$

$$T = I/I_0,$$

$$I_0 = \text{intensity of incident monochromatic radiation,}$$

$$I = \text{intensity of transmitted monochromatic radiation.}$$

In the absence of other physico-chemical factors, the measured absorbance (A) is proportional to the path length (b) through which the radiation passes and to the concentration (c) of the substance in solution in accordance with the equation:

$$A = \varepsilon \cdot c \cdot b$$

ε = molar absorptivity, if b is expressed in centimetres and c in moles per litre.

The expression $A_{1\text{ cm}}^{1\text{ per cent}}$ representing the specific absorbance of a dissolved substance refers to the absorbance of a 10 g/l solution in a 1 cm cell and measured at a defined wavelength so that:

$$A_{1\text{ cm}}^{1\text{ per cent}} = \frac{10\varepsilon}{M_r}$$

Unless otherwise prescribed, measure the absorbance at the prescribed wavelength using a path length of 1 cm and at $20 \pm 1^\circ\text{C}$. Unless otherwise prescribed, the measurements are carried out with reference to the same solvent or the same mixture of solvents. The absorbance of the solvent measured against air and at the prescribed wavelength shall not exceed 0.4 and is preferably less than 0.2. Plot the absorption spectrum with absorbance or function of absorbance as ordinate against wavelength or function of wavelength as abscissa.

Where a monograph gives a single value for the position of an absorption maximum, it is understood that the value obtained may differ by not more than ± 2 nm.

Apparatus. Spectrophotometers suitable for measuring in the ultraviolet and visible range of the spectrum consist of an optical system capable of producing monochromatic radiation in the range of 200 nm to 800 nm and a device suitable for measuring the absorbance.

Control of wavelengths. Verify the wavelength scale using the absorption maxima of *holmium perchlorate solution R*, the line of a hydrogen or deuterium discharge lamp or the lines of a mercury vapour shown in Table 2.2.25-1. The permitted tolerance is ± 1 nm for the ultraviolet range and ± 3 nm for the visible range.

Table 2.2.25-1. – Absorption maxima for control of wavelength scale

241.15 nm (Ho)	404.66 nm (Hg)
253.7 nm (Hg)	435.83 nm (Hg)
287.15 nm (Ho)	486.0 nm (D β)
302.25 nm (Hg)	486.1 nm (H β)
313.16 nm (Hg)	536.3 nm (Ho)
334.15 nm (Hg)	546.07 nm (Hg)
361.5 nm (Ho)	576.96 nm (Hg)
365.48 nm (Hg)	579.07 nm (Hg)

Control of absorbance. Check the absorbance using suitable filters or a solution of *potassium dichromate R* at the wavelengths indicated in Table 2.2.25-2 which gives for each wavelength the exact values and the permitted limits of the specific absorbance. The tolerance for the absorbance is ± 0.01 .

For the control of absorbance, use a solution of potassium dichromate prepared as follows. Dissolve 57.0 mg to 63.0 mg of *potassium dichromate R*, previously dried to constant mass at 130°C , in 0.005 M sulphuric acid and dilute to 1000.0 ml with the same acid.

Table 2.2.25-2

Wavelength (nm)	Specific absorbance $A_{1\text{ cm}}^{1\text{ per cent}}$	Maximum tolerance
235	124.5	122.9 to 126.2
257	144.5	142.8 to 146.2
313	48.6	47.0 to 50.3
350	107.3	105.6 to 109.0

Limit of stray light. Stray light may be detected at a given wavelength with suitable filters or solutions: for example the absorbance of a 12 g/l solution of *potassium chloride R* in a 1 cm cell increases steeply between 220 nm and 200 nm and is greater than 2 at a wavelength between 198 nm and 202 nm when compared with water as compensation liquid.

Resolution (for qualitative analysis). When prescribed in a monograph, measure the resolution of the apparatus as follows: record the spectrum of a 0.02 per cent V/V solution of *toluene R* in *hexane R*. The minimum ratio of the absorbance at the maximum at 269 nm to that at the minimum at 266 nm is stated in the monograph.

Spectral slit-width (for quantitative analysis). To avoid errors due to spectral slit-width, when using an instrument on which the slit-width is variable at the selected wavelength, the slit-width must be small compared with the half-width of the absorption band but it must be as large as possible to obtain a high value of I_0 . Therefore, a slit-width is chosen such that further reduction does not result in a change in absorbance reading.

Cells. The tolerance on the path length of the cells used is ± 0.005 cm. When filled with the same solvent, the cells intended to contain the solution to be examined and the compensation liquid must have the same transmittance. If this is not the case, an appropriate correction must be applied.

The cells must be cleaned and handled with care.

DERIVATIVE SPECTROPHOTOMETRY

Derivative spectrophotometry involves the transformation of absorption spectra (zero-order) into first-, second- or higher-order-derivative spectra.

A *first-derivative spectrum* is a plot of the gradient of the absorption curve (rate of change of the absorbance with wavelength, $dA/d\lambda$) against wavelength.

A *second-derivative spectrum* is a plot of the curvature of the absorption spectrum against wavelength ($d^2A/d\lambda^2$). The second derivative at any wavelength λ is related to concentration by the following equations:

$$\frac{d^2A}{d\lambda^2} = \frac{d^2A_{1\text{ cm}}^{1\text{ per cent}}}{d\lambda^2} \times \frac{c'b}{10} = \frac{d^2A\varepsilon}{d\lambda^2} \times \frac{cb}{10}$$

c' = concentration of the absorbing solute, in grams per litre.

Apparatus. Use a spectrophotometer complying with the requirements prescribed above and equipped with an analogue resistance-capacitance differentiation module or a digital differentiator or other means of producing derivative spectra. Some methods of producing second-derivative spectra produce a wavelength shift relative to the zero-order spectrum and this is to be taken into account where applicable.

Resolution power. When prescribed in a monograph, record the second-derivative spectrum of a 0.2 g/l solution of *toluene R* in *methanol R*, using *methanol R* as the compensation liquid. The spectrum shows a small negative extremum located between 2 large negative extrema at 261 nm and 268 nm, respectively, as shown in Figure 2.2.25.-1. Unless otherwise prescribed in the monograph, the ratio A/B (see Figure 2.2.25.-1) is not less than 0.2.

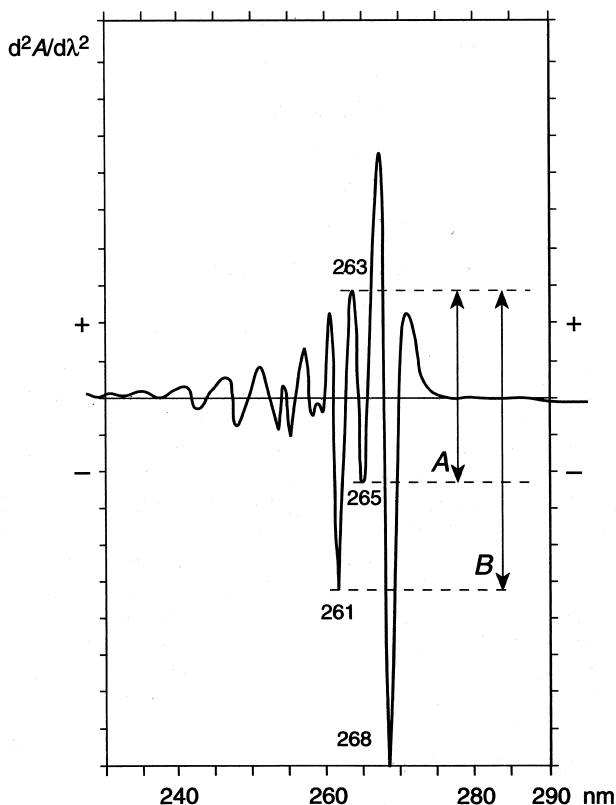


Figure 2.2.25.-1

Procedure. Prepare the solution of the substance to be examined, adjust the various instrument settings according to the manufacturer's instructions and calculate the amount of the substance to be determined as prescribed in the monograph.

01/2005:20226

2.2.26. PAPER CHROMATOGRAPHY

ASCENDING PAPER CHROMATOGRAPHY

Apparatus. The apparatus consists of a glass tank of suitable size for the chromatographic paper used, ground at the top to take a closely fitting lid. In the top of the tank is a device which suspends the chromatographic paper and is capable of being lowered without opening the chamber. In the bottom of the tank is a dish to contain the mobile phase into which the paper may be lowered. The chromatographic paper consists of suitable filter paper, cut into strips of sufficient length and not less than 2.5 cm wide; the paper is cut so that the mobile phase runs in the direction of the grain of the paper.

Method. Place in the dish a layer 2.5 cm deep of the mobile phase prescribed in the monograph. If prescribed in the monograph, pour the stationary phase between the walls of the tank and the dish. Close the tank and allow to stand for 24 h at 20 °C to 25 °C. Maintain the tank at this temperature throughout the subsequent procedure. Draw a fine pencil

line horizontally across the paper 3 cm from one end.

Using a micro pipette, apply to a spot on the pencil line the volume of the solution prescribed in the monograph. If the total volume to be applied would produce a spot more than 10 mm in diameter, apply the solution in portions allowing each to dry before the next application. When more than one chromatogram is to be run on the same strip of paper, space the solutions along the pencil line at points not less than 3 cm apart. Insert the paper into the tank, close the lid and allow to stand for 1 h 30 min. Lower the paper into the mobile phase and allow elution to proceed for the prescribed distance or time. Remove the paper from the tank and allow to dry in air. Protect the paper from bright light during the elution process.

DESCENDING PAPER CHROMATOGRAPHY

Apparatus. The apparatus consists of a glass tank of suitable size for the chromatographic paper used, ground at the top to take a closely fitting glass lid. The lid has a central hole about 1.5 cm in diameter closed by a heavy glass plate or a stopper. In the upper part of the tank is suspended a solvent trough with a device for holding the chromatographic paper. On each side of the trough, parallel to and slightly above its upper edges, are two glass guide rods to support the paper in such a manner that no part of it is in contact with the walls of the tank. The chromatographic paper consists of suitable filter paper, cut into strips of sufficient length, and of any convenient width between 2.5 cm and the length of the trough; the paper is cut so that the mobile phase runs in the direction of the grain of the paper.

Method. Place in the bottom of the tank a layer 2.5 cm deep of the solvent prescribed in the monograph, close the tank and allow to stand for 24 h at 20 °C to 25 °C. Maintain the tank at this temperature throughout the subsequent procedure. Draw a fine pencil line horizontally across the paper at such a distance from one end that when this end is secured in the solvent trough and the remainder of the paper is hanging freely over the guide rod, the line is a few centimetres below the guide rod and parallel with it. Using a micro-pipette, apply on the pencil line the volume of the solution prescribed in the monograph. If the total volume to be applied would produce a spot more than 10 mm in diameter, apply the solution in portions, allowing each to dry before the next application. When more than one chromatogram is to be run on the same strip of paper, space the solutions along the pencil line at points not less than 3 cm apart. Insert the paper in the tank, close the lid, and allow to stand for 1 h 30 min. Introduce into the solvent trough, through the hole in the lid, a sufficient quantity of the mobile phase, close the tank and allow elution to proceed for the prescribed distance or time. Remove the paper from the tank and allow to dry in air. The paper should be protected from bright light during the elution process.

01/2005:20227

2.2.27. THIN-LAYER CHROMATOGRAPHY

Thin-layer chromatography is a separation technique in which a stationary phase consisting of an appropriate material is spread in a uniform thin layer on a support (plate) of glass, metal or plastic. Solutions of analytes are deposited on the plate prior to development. The separation is based on adsorption, partition, ion-exchange or on combinations of these mechanisms and is carried out by migration (development) of solutes (solutions of analytes) in a solvent or a suitable mixture of solvents (mobile phase) through the thin-layer (stationary phase).

APPARATUS

Plates. The chromatography is carried out using pre-coated plates as described under *Reagents (4.1.1)*.

Preconditioning of the plates. It may be necessary to wash the plates prior to separation. This can be done by migration of an appropriate solvent. The plates may also be impregnated by procedures such as development, immersion or spraying. At the time of use, the plates may be activated, if necessary, by heating in an oven at 100-105 °C for 1 h.

A chromatographic tank with a flat bottom or twin trough, of inert, transparent material, of a size suitable for the plates used and provided with a tightly fitting lid. For horizontal development the tank is provided with a trough for the mobile phase and it additionally contains a device for directing the mobile phase to the stationary phase.

Micropipettes, microsyringes, calibrated disposable capillaries or other application devices suitable for the proper application of the solutions.

Fluorescence detection device to measure direct fluorescence or the inhibition of fluorescence.

Visualisation reagents to detect the separated spots by spraying, exposure to vapour or immersion.

METHOD

Vertical development. Line the walls of the chromatographic tank with filter paper. Pour into the chromatographic tank a sufficient quantity of the mobile phase for the size of the tank to give after impregnation of the filter paper a layer of appropriate depth related to the dimension of the plate to be used. For saturation of the chromatographic tank, replace the lid and allow to stand at 20-25 °C for 1 h. Unless otherwise indicated, the chromatographic separation is performed in a saturated tank.

Apply the prescribed volume of the solutions in sufficiently small portions to obtain bands or circular spots at an appropriate distance from the lower edge and from the sides of the plate. Apply the solutions on a line parallel to the lower edge of the plate with an interval of at least 10 mm between the spots.

When the solvent has evaporated from the applied solutions, place the plate in the chromatographic tank, ensuring that the plate is as vertical as possible and that the spots or bands are above the surface of the mobile phase. Close the chromatographic tank, maintain it at 20-25 °C and protect from sunlight. Remove the plate when the mobile phase has moved over the prescribed distance. Dry the plate and visualise the chromatograms as prescribed.

For two-dimensional chromatography, dry the plates after the first development and carry out a second development in a direction perpendicular to that of the first development.

Horizontal development. Apply the prescribed volume of the solutions in sufficiently small portions to obtain circular spots 1 mm to 2 mm in diameter, or bands 5 mm to 10 mm by 1 mm to 2 mm, at an appropriate distance from the lower edge and from the sides of the plate. Apply the solutions on a line parallel to the lower edge of the plate with an interval of at least 5 mm between the spots. When the solvent has evaporated from the applied solutions, introduce a sufficient quantity of the mobile phase into the trough of the chamber using a syringe or pipette, place the plate horizontally in the chamber and connect the mobile phase direction device according to the manufacturer's instructions. If prescribed, develop the plate starting simultaneously at both ends. Close the chamber and maintain it at 20-25 °C. Remove the

plate when the mobile phase has moved over the distance prescribed in the monograph. Dry the plate and visualise the chromatograms as prescribed.

For two-dimensional chromatography, dry the plates after the first development and carry out a second development in a direction perpendicular to that of the first development.

VISUAL ESTIMATION

Identification. The principal spot in the chromatogram obtained with the test solution is visually compared to the corresponding spot in the chromatogram obtained with the reference solution by comparing the colour, the size and the retention factor (R_f) of both spots.

The retention factor (R_f) is defined as the ratio of the distance from the point of application to the centre of the spot and the distance travelled by the solvent front from the point of application.

Verification of the separating power for identification. Normally the performance given by the suitability test described in *Reagents (4.1.1)* is sufficient. Only in special cases an additional performance criterion is prescribed in the monograph.

Related substances test. The secondary spot(s) in the chromatogram obtained with the test solution is (are) visually compared to either the corresponding spot(s) in the chromatogram obtained with the reference solution containing the impurity(ies) or the spot in the chromatogram obtained with the reference solution prepared from a dilution of the test solution.

Verification of the separating power. The requirements for the verification of the separating power are prescribed in the monographs concerned.

Verification of the detecting power. The detecting power is satisfactory if a spot or band is clearly visible in the chromatogram obtained with the most dilute reference solution.

QUANTITATIVE MEASUREMENT

The requirements for resolution and separation are prescribed in the monographs concerned.

Substances separated by thin-layer chromatography and responding to UV-Vis irradiation can be determined directly on the plate, using appropriate instrumentation. While moving the plate or the measuring device, examine the plate by measuring the reflectance or transmittance of the incident light. Similarly, fluorescence may be measured using an appropriate optical system. Substances containing radionuclides can be quantified in three ways: either directly by moving the plate alongside a suitable counter or vice versa (see *Radiopharmaceutical preparations (0125)*), by cutting the plates into strips and measuring the radioactivity on each individual strip using a suitable counter or by scraping off the stationary phase, dissolving it in a suitable scintillation cocktail and measuring the radioactivity using a liquid scintillation counter.

Apparatus. The apparatus for direct measurement on the plate consists of:

- a device for exact positioning and reproducible dispensing of the amount of substances onto the plate,
- a mechanical device to move the plate or the measuring device along the x -axis or the y -axis,
- a recorder and a suitable integrator or a computer,
- *for substances responding to UV-Vis irradiation:* a photometer with a source of light, an optical device able to generate monochromatic light and a photo cell of adequate sensitivity are used for the measurement of reflectance or transmittance. In the case where

fluorescence is measured, a monochromatic filter is required in addition, to select a particular spectral region of the emitted light,

- *for substances containing radionuclides*: a suitable counter for radioactivity. The linearity range of the counting device is to be verified.

Method. Prepare the solution of the substance to be examined (test solution) as prescribed in the monograph and, if necessary, prepare the reference solutions of the substance to be determined using the same solvent as in the test solution. Apply the same volume of each solution to the plate and develop.

Substances responding to UV-Vis irradiation: Prepare and apply not fewer than three reference solutions of the substance to be examined, the concentrations of which span the expected value in the test solution (about 80, 100 and 120 per cent). Spray with the prescribed reagent, if necessary, and record the reflectance, the transmittance or fluorescence in the chromatograms obtained with the test and reference solutions. Use the measured results for the calculation of the amount of substance in the test solution.

Substances containing radionuclides: Prepare and apply a test solution containing about 100 per cent of the expected value. Determine the radioactivity as a function of the path length and report the radioactivity in each resulting peak as a percentage of the total amount of radioactivity.

Criteria for assessing the suitability of the system are described in the chapter on *Chromatographic separation techniques* (2.2.46). The extent to which adjustments of parameters of the chromatographic system can be made to satisfy the criteria of system suitability are also given in this chapter.

01/2005:20228

2.2.28. GAS CHROMATOGRAPHY

Gas chromatography (GC) is a chromatographic separation technique based on the difference in the distribution of species between two non-miscible phases in which the mobile phase is a carrier gas moving through or passing the stationary phase contained in a column. It is applicable to substances or their derivatives which are volatilised under the temperatures employed.

GC is based on mechanisms of adsorption, mass distribution or size exclusion.

APPARATUS

The apparatus consists of an injector, a chromatographic column contained in an oven, a detector and a data acquisition system (or an integrator or a chart recorder). The carrier gas flows through the column at a controlled rate or pressure and then through the detector.

The chromatography is carried out either at a constant temperature or according to a given temperature programme.

INJECTORS

Direct injections of solutions are the usual mode of injection, unless otherwise prescribed in the monograph. Injection may be carried out either directly at the head of the column using a syringe or an injection valve, or into a vaporisation chamber which may be equipped with a stream splitter.

Injections of vapour phase may be effected by static or dynamic head-space injection systems.

Dynamic head-space (purge and trap) injection systems include a sparging device by which volatile substances in solution are swept into an absorbent column maintained at a low temperature. Retained substances are then desorbed into the mobile phase by rapid heating of the absorbent column.

Static head-space injection systems include a thermostatically controlled sample heating chamber in which closed vials containing solid or liquid samples are placed for a fixed period of time to allow the volatile components of the sample to reach equilibrium between the non-gaseous phase and the vapour phase. After equilibrium has been established, a predetermined amount of the head-space of the vial is flushed into the gas chromatograph.

STATIONARY PHASES

Stationary phases are contained in columns which may be:

- a capillary column of fused-silica whose wall is coated with the stationary phase,
- a column packed with inert particles impregnated with the stationary phase,
- a column packed with solid stationary phase.

Capillary columns are 0.1 mm to 0.53 mm in internal diameter ($\emptyset$) and 5 m to 60 m in length. The liquid or stationary phase, which may be chemically bonded to the inner surface, is a film 0.1 μ m to 5.0 μ m thick.

Packed columns, made of glass or metal, are usually 1 m to 3 m in length with an internal diameter ($\emptyset$) of 2 mm to 4 mm. Stationary phases usually consist of porous polymers or solid supports impregnated with liquid phase.

Supports for analysis of polar compounds on columns packed with low-capacity, low-polarity stationary phase must be inert to avoid peak tailing. The reactivity of support materials can be reduced by silanising prior to coating with liquid phase. Acid-washed, flux-calcinated diatomaceous earth is often used. Materials are available in various particle sizes, the most commonly used particles are in the ranges of 150 μ m to 180 μ m and 125 μ m to 150 μ m.

MOBILE PHASES

Retention time and peak efficiency depend on the carrier gas flow rate; retention time is directly proportional to column length and resolution is proportional to the square root of the column length. For packed columns, the carrier gas flow rate is usually expressed in millilitres per minute at atmospheric pressure and room temperature. Flow rate is measured at the detector outlet, either with a calibrated mechanical device or with a bubble tube, while the column is at operating temperature. The linear velocity of the carrier gas through a packed column is inversely proportional to the square root of the internal diameter of the column for a given flow volume. Flow rates of 60 ml/min in a 4 mm internal diameter column and 15 ml/min in a 2 mm internal diameter column, give identical linear velocities and thus similar retention times.

Helium or nitrogen are usually employed as the carrier gas for packed columns, whereas commonly used carrier gases for capillary columns are nitrogen, helium and hydrogen.

DETECTORS

Flame-ionisation detectors are usually employed but additional detectors which may be used include: electron-capture, nitrogen-phosphorus, mass spectrometric, thermal conductivity, Fourier transform infrared spectrophotometric, and others, depending on the purpose of the analysis.

METHOD

Equilibrate the column, the injector and the detector at the temperatures and the gas flow rates specified in the monograph until a stable baseline is achieved. Prepare the test solution(s) and the reference solution(s) as prescribed. The solutions must be free from solid particles.

Criteria for assessing the suitability of the system are described in the chapter on *Chromatographic separation techniques* (2.2.46). The extent to which adjustments of parameters of the chromatographic system can be made to satisfy the criteria of system suitability are also given in this chapter.

Static head-space gas chromatography

Static head-space gas chromatography is a technique particularly suitable for separating and determining volatile compounds present in solid or liquid samples. The method is based on the analysis of the vapour phase in equilibrium with the solid or liquid phase.

APPARATUS

The apparatus consists of a gas chromatograph provided with a device for introducing the sample that may be connected to a module that automatically controls the pressure and the temperature. If necessary, a device for eliminating solvents can be added.

The sample to be analysed is introduced into a container fitted with a suitable stopper and a valve-system which permits the passage of the carrier gas. The container is placed in a thermostatically controlled chamber at a temperature set according to the substance to be examined. The sample is held at this temperature long enough to allow equilibrium to be established between the solid or liquid phase and the vapour phase.

The carrier gas is introduced into the container and, after the prescribed time, a suitable valve is opened so that the gas expands towards the chromatographic column taking the volatilised compounds with it.

Instead of using a chromatograph specifically equipped for the introduction of samples, it is also possible to use airtight syringes and a conventional chromatograph. Equilibration is then carried out in a separate chamber and the vapour phase is carried onto the column, taking the precautions necessary to avoid any changes in the equilibrium.

METHOD

Using the reference preparations, determine suitable instrument settings to produce an adequate response.

DIRECT CALIBRATION

Separately introduce into identical containers the preparation to be examined and each of the reference preparations, as prescribed in the monograph, avoiding contact between the sampling device and the samples.

Close the containers hermetically and place in the thermostatically controlled chamber set to the temperature and pressure prescribed in the monograph; after equilibration, carry out the chromatography under the prescribed conditions.

STANDARD ADDITIONS

Add to a set of identical suitable containers equal volumes of the preparation to be examined. Add to all but one of the containers, suitable quantities of a reference preparation containing a known concentration of the substance to be determined so as to produce a series of preparations containing steadily increasing concentrations of the substance.

Close the containers hermetically and place in the thermostatically controlled chamber set to the temperature and pressure prescribed in the monograph; after equilibration, carry out the chromatography under the prescribed conditions.

Calculate the linear equation of the graph using a least-squares fit, and derive from it the concentration of the substance to be determined in the preparation to be examined.

Alternatively, plot on a graph the mean of readings against the added quantity of the substance to be determined. Extrapolate the line joining the points on the graph until it meets the concentration axis. The distance between this point and the intersection of the axes represents the concentration of the substance to be determined in the preparation to be examined.

SUCCESSIVE WITHDRAWALS (MULTIPLE HEAD-SPACE EXTRACTION)

If prescribed, the successive withdrawal method is fully described in the monograph.

01/2005:20229

2.2.29. LIQUID CHROMATOGRAPHY

Liquid chromatography (LC) is a method of chromatographic separation based on the difference in the distribution of species between two non-miscible phases, in which the mobile phase is a liquid which percolates through a stationary phase contained in a column.

LC is mainly based on mechanisms of adsorption, mass distribution, ion exchange, size exclusion or stereochemical interaction.

APPARATUS

The apparatus consists of a pumping system, an injector, a chromatographic column (a column temperature controller may be used), a detector and a data acquisition system (or an integrator or a chart recorder). The mobile phase is supplied from one or several reservoirs and flows through the column, usually at a constant rate, and then through the detector.

PUMPING SYSTEMS

LC pumping systems are required to deliver the mobile phase at a constant flow rate. Pressure fluctuations are to be minimised, e.g. by passing the pressurised solvent through a pulse-dampening device. Tubing and connections are capable of withstanding the pressures developed by the pumping system. LC pumps may be fitted with a facility for "bleeding" the system of entrapped air bubbles.

Microprocessor controlled systems are capable of accurately delivering a mobile phase of either constant (isocratic elution) or varying composition (gradient elution), according to a defined programme. In the case of gradient elution, pumping systems which deliver solvent(s) from several reservoirs are available and solvent mixing can be achieved on either the low or high-pressure side of the pump(s).

INJECTORS

The sample solution is introduced into the flowing mobile phase at or near the head of the column using an injection system which can operate at high pressure. Fixed-loop and variable volume devices operated manually or by an auto-sampler are used. Manual partial filling of loops may lead to poorer injection volume precision.

STATIONARY PHASES

There are many types of stationary phases employed in LC, including:

- silica, alumina or porous graphite, used in normal-phase chromatography, where the separation is based on differences in adsorption and/or mass distribution,
- resins or polymers with acid or basic groups, used in ion-exchange chromatography, where separation is based on competition between the ions to be separated and those in the mobile phase,
- porous silica or polymers, used in size-exclusion chromatography, where separation is based on differences between the volumes of the molecules, corresponding to steric exclusion,
- a variety of chemically modified supports prepared from polymers, silica or porous graphite, used in reversed-phase LC, where the separation is based principally on partition of the molecules between the mobile phase and the stationary phase,
- special chemically modified stationary phases, e.g. cellulose or amylose derivatives, proteins or peptides, cyclodextrins etc., for the separation of enantiomers (chiral chromatography).

Most separations are based upon partition mechanisms utilising chemically modified silica as the stationary phase and polar solvents as the mobile phase. The surface of the support, e.g. the silanol groups of silica, is reacted with various silane reagents to produce covalently bound silyl derivatives covering a varying number of active sites on the surface of the support. The nature of the bonded phase is an important parameter for determining the separation properties of the chromatographic system.

Commonly used bonded phases are shown below:

octyl	= Si-[CH ₂] ₇ -CH ₃	C ₈
octadecyl	= Si-[CH ₂] ₁₇ -CH ₃	C ₁₈
phenyl	= Si-[CH ₂] _n -C ₆ H ₅	C ₆ H ₅
cyanopropyl	= Si-[CH ₂] ₃ -CN	CN
aminopropyl	= Si-[CH ₂] ₃ -NH ₂	NH ₂
diol	= Si-[CH ₂] ₃ -O-CH(OH)-CH ₂ -OH	

Unless otherwise stated by the manufacturer, silica based reversed-phase columns are considered to be stable in mobile phases having an apparent pH in the range 2.0 to 8.0. Columns containing porous graphite or particles of polymeric materials such as styrene-divinylbenzene copolymer are stable over a wider pH range.

Analysis using normal-phase chromatography with unmodified silica, porous graphite or polar chemically modified silica, e.g. cyanopropyl or diol, as the stationary phase with a non-polar mobile phase is applicable in certain cases.

For analytical separations, the particle size of the most commonly used stationary phases varies between 3 µm and 10 µm. The particles may be spherical or irregular, of varying porosity and specific surface area. These parameters contribute to the chromatographic behaviour of a particular stationary phase. In the case of reversed phases, the nature of the stationary phase, the extent of bonding, e.g. expressed as the carbon loading, and whether the stationary phase is end-capped (i.e. residual silanol groups are silylated) are additional determining factors. Tailing of peaks, particularly of basic substances, can occur when residual silanol groups are present.

Columns, made of stainless steel unless otherwise prescribed in the monograph, of varying length and internal diameter (Ø) are used for analytical chromatography. Columns with internal diameters of less than 2 mm are often referred to as microbore columns. The temperature of the mobile phase and the column must be kept constant during an analysis. Most separations are performed at room temperature, but columns may be heated to give higher efficiency. It is recommended that columns not be heated above 60 °C because of the potential for stationary phase degradation or changes occurring to the composition of the mobile phase.

MOBILE PHASES

For normal-phase chromatography, less polar solvents are employed. The presence of water in the mobile phase is to be strictly controlled to obtain reproducible results. In reversed-phase LC, aqueous mobile phases, with or without organic modifiers, are employed.

Components of the mobile phase are usually filtered to remove particles greater than 0.45 µm. Multicomponent mobile phases are prepared by measuring the required volumes (unless masses are specified) of the individual components, followed by mixing. Alternatively, the solvents may be delivered by individual pumps controlled by proportioning valves by which mixing is performed according to the desired proportion. Solvents are normally degassed before pumping by sparging with helium, sonication or using on-line membrane/vacuum modules to avoid the creation of gas bubbles in the detector cell.

Solvents for the preparation of the mobile phase are normally free of stabilisers and are transparent at the wavelength of detection, if an ultraviolet detector is employed. Solvents and other components employed are to be of appropriate quality. Adjustment of the pH, if necessary, is effected using only the aqueous component of the mobile phase and not the mixture. If buffer solutions are used, adequate rinsing of the system is carried out with a mixture of water and the organic modifier of the mobile phase (5 per cent V/V) to prevent crystallisation of salts after completion of the chromatography.

Mobile phases may contain other components, e.g. a counter-ion for ion-pair chromatography or a chiral selector for chromatography using an achiral stationary phase.

DETECTORS

Ultraviolet/visible (UV/Vis) spectrophotometers, including diode array detectors, are the most commonly employed detectors. Fluorescence spectrophotometers, differential refractometers, electrochemical detectors, mass spectrometers, light scattering detectors, radioactivity detectors or other special detectors may also be used.

METHOD

Equilibrate the column with the prescribed mobile phase and flow rate, at room temperature or at the temperature specified in the monograph, until a stable baseline is achieved. Prepare the solution(s) of the substance to be examined and the reference solution(s) required. The solutions must be free from solid particles.

Criteria for assessing the suitability of the system are described in the chapter on *Chromatographic separation techniques* (2.2.46). The extent to which adjustments of parameters of the chromatographic system can be made to satisfy the criteria of system suitability are also given in this chapter.

01/2005:20230

2.2.30. SIZE-EXCLUSION CHROMATOGRAPHY

Size-exclusion chromatography is a chromatographic technique which separates molecules in solution according to their size. With organic mobile phases, the technique is known as *gel-permeation chromatography* and with aqueous mobile phases, the term *gel-filtration chromatography* has been used. The sample is introduced into a column, which is filled with a gel or a porous particle packing material, and is carried by the mobile phase through the column. The size separation takes place by repeated exchange of the solute molecules between the solvent of the mobile phase and the same solvent in the stagnant liquid phase (stationary phase) within the pores of the packing material. The pore-size range of the packing material determines the molecular-size range within which separation can occur.

Molecules small enough to penetrate all the pore spaces elute at the *total permeation volume* (V_t). On the other hand, molecules apparently larger than the maximum pore size of the packing material migrate along the column only through the spaces between the particles of the packing material without being retained and elute at the *exclusion volume* (V_0 void volume). Separation according to molecular size occurs between the exclusion volume and the total permeation volume, with useful separation usually occurring in the first two thirds of this range.

Apparatus. The apparatus consists essentially of a chromatographic column of varying length and internal diameter ($\emptyset$), if necessary temperature-controlled, packed with a separation material that is capable of fractionation in the appropriate range of molecular sizes and through which the eluent is passed at a constant rate. One end of the column is usually fitted with a suitable device for applying the sample such as a flow adapter, a syringe through a septum or an injection valve and may also be connected to a suitable pump for controlling the flow of the eluent. Alternatively the sample may be applied directly to the drained bed surface or, where the sample is denser than the eluent, it may be layered beneath the eluent. The outlet of the column is usually connected to a suitable detector fitted with an automatic recorder which enables the monitoring of the relative concentrations of separated components of the sample. Detectors are usually based on photometric, refractometric or luminescent properties. An automatic fraction collector may be attached, if necessary.

The packing material may be a soft support such as a swollen gel or a rigid support composed of a material such as glass, silica or a solvent-compatible, cross-linked organic polymer. Rigid supports usually require pressurised systems giving faster separations. The mobile phase is chosen according to sample type, separation medium and method of detection. Before carrying out the separation, the packing material is treated, and the column is packed, as described in the monograph, or according to the manufacturer's instructions. Criteria for assessing the suitability of the system are described in the chapter on *Chromatographic separation techniques* (2.2.46). The extent to which adjustments of parameters of the chromatographic system can be made to satisfy the criteria of system suitability are also given in this chapter.

DETERMINATION OF RELATIVE COMPONENT COMPOSITION OF MIXTURES

Carry out the separation as stated in the monograph. If possible, monitor the elution of the components continuously and measure the corresponding peak areas. If the sample

is monitored by a physico-chemical property to which all the components of interest exhibit equivalent responses (for example if they have the same specific absorbance), calculate the relative amount of each component by dividing the respective peak area by the sum of the peak areas of all the components of interest. If the responses to the property used for detection of the components of interest are not equivalent, calculate the content by means of calibration curves obtained with the calibration standards prescribed in the monograph.

DETERMINATION OF MOLECULAR MASSES

Size-exclusion chromatography may be used to determine molecular masses by comparison with appropriate calibration standards specified in the monograph. The retention volumes of the calibration standards may be plotted against the logarithm of their molecular masses. The plot usually approximates a straight line within the exclusion and total permeation limits for the separation medium used. From the calibration curve, molecular masses may be estimated. The molecular-mass calibration is valid only for the particular macromolecular solute/solvent system used under the specified experimental conditions.

DETERMINATION OF MOLECULAR SIZE DISTRIBUTION OF POLYMERS

Size-exclusion chromatography may be used to determine the distribution of the molecular size of polymers. However, sample comparison may be valid only for results obtained under the same experimental conditions. The reference substances used for the calibration and the methods for determination of the distribution of molecular sizes of polymers are specified in the monograph.

01/2005:20231

2.2.31. ELECTROPHORESIS

GENERAL PRINCIPLE

Under the influence of an electrical field, charged particles dissolved or dispersed in an electrolyte solution migrate in the direction of the electrode bearing the opposite polarity. In gel electrophoresis, the movements of the particles are retarded by interactions with the surrounding gel matrix, which acts as a molecular sieve. The opposing interactions of the electrical force and molecular sieving result in differential migration rates according to sizes, shapes and charges of particles. Because of their different physico-chemical properties, different macromolecules of a mixture will migrate at different speeds during electrophoresis and will thus be separated into discrete fractions. Electrophoretic separations can be conducted in systems without support phases (e.g. free solution separation in capillary electrophoresis) and in stabilising media such as thin-layer plates, films or gels.

FREE OR MOVING BOUNDARY ELECTROPHORESIS

This method is mainly used for the determination of mobility, the experimental characteristics being directly measurable and reproducible. It is chiefly employed with substances of high relative molecular mass and low diffusibility. The boundaries are initially located by a physical process such as refractometry or conductimetry. After applying a given electric field for an accurately measured time, the new boundaries and their respective positions are observed. The operating conditions must be such as to make it possible to determine as many boundaries as there are components.

ZONE ELECTROPHORESIS USING A SUPPORTING MEDIUM

This method requires the use of small samples only.

The nature of the support, such as paper, agar gel, cellulose acetate, starch, agarose, methacrylamide, mixed gel, introduces a number of additional factors modifying the mobility:

- owing to channelling in the supporting medium, the apparent distance covered is less than the real distance,
- some supporting media are not electrically neutral. As the medium is a stationary phase it may sometimes give rise to a considerable electro-endosmotic flow,
- any heating due to the joule effect may cause some evaporation of the liquid from the supporting medium which, by capillarity, causes the solution to move from the ends towards the centre. The ionic strength therefore tends to increase gradually.

The rate of migration then depends on four main factors: the mobility of the charged particle, the electro-endosmotic flow, the evaporation flow, and the field strength. Hence it is necessary to operate under clearly defined experimental conditions and to use, wherever possible, reference substances.

An *apparatus* for electrophoresis consists of:

- a *generator supplying direct current* whose voltage can be controlled and, preferably, stabilised,
- an *electrophoresis chamber*. This is usually rectangular and made of glass or rigid plastic, with two separate compartments, the anodic and the cathodic, containing the electrolyte solution. In each compartment is immersed an electrode, for example of platinum or graphite. These are connected by means of an appropriately isolated circuit to the corresponding terminal of the power supply to form the anode and the cathode. The level of the liquid in the two compartments is kept equal to prevent siphoning.

The electrophoresis chamber is fitted with an airtight lid which maintains a moisture-saturated atmosphere during operation and reduces evaporation of the solvent. A safety device may be used to cut off the power when the lid is removed. If the electrical power measured across the strip exceeds 10 W, it is preferable to cool the support.

- a *support-carrying device*:

Strip electrophoresis. The supporting strip, previously wetted with the same conducting solution and dipped at each end into an electrode compartment is appropriately tightened and fixed on to a suitable carrier designed to prevent diffusion of the conducting electrolyte, such as a horizontal frame, inverted-V stand or a uniform surface with contact points at suitable intervals.

Gel electrophoresis. The device consists essentially of a glass plate (for example, a microscope slide) over the whole surface of which is deposited a firmly adhering layer of gel of uniform thickness. The connection between the gel and the conducting solution is effected in various ways according to the type of apparatus used. Precautions must be taken to avoid condensation of moisture or drying of the solid layer.

- measuring *device or means of detection*.

Method. Introduce the electrolyte solution into the electrode compartments. Place the support suitably impregnated with electrolyte solution in the chamber under the conditions prescribed for the type of apparatus used. Locate the starting line and apply the sample. Apply the electric current for the prescribed time. After the current has been switched off, remove the support from the chamber, dry and visualise.

POLYACRYLAMIDE ROD GEL ELECTROPHORESIS

In polyacrylamide rod gel electrophoresis, the stationary phase is a gel which is prepared from a mixture of acrylamide and *N,N'*-methylenebisacrylamide. Rod gels are prepared in tubes 7.5 cm long and 0.5 cm in internal diameter, one solution being applied to each rod.

Apparatus. This consists of two buffer solution reservoirs made of suitable material such as poly(methyl methacrylate) and mounted vertically one above the other. Each reservoir is fitted with a platinum electrode. The electrodes are connected to a power supply allowing operation either at constant current or at constant voltage. The apparatus has in the base of the upper reservoir a number of holders equidistant from the electrode.

Method. The solutions should usually be degassed before polymerisation and the gels used immediately after preparation. Prepare the gel mixture as prescribed and pour into suitable glass tubes, stoppered at the bottom, to an equal height in each tube and to about 1 cm from the top, taking care to ensure that no air bubbles are trapped in the tubes. Cover the gel mixture with a layer of *water R* to exclude air and allow to set. Gel formation usually takes about 30 min and is complete when a sharp interface appears between the gel and the water layer. Remove the water layer. Fill the lower reservoir with the prescribed buffer solution and remove the stoppers from the tubes. Fit the tubes into the holders of the upper reservoir and adjust so that the bottom of the tubes are immersed in the buffer solution in the lower reservoir. Carefully fill the tubes with the prescribed buffer solution. Prepare the test and reference solutions containing the prescribed marker dye and make them dense by dissolving in them *sucrose R*, for example. Apply the solutions to the surface of a gel using a different tube for each solution. Add the same buffer to the upper reservoir. Connect the electrodes to the power supply and allow electrophoresis to proceed at the prescribed temperature and using the prescribed constant voltage or current. Switch off the power supply when the marker dye has migrated almost into the lower reservoir. Immediately remove each tube from the apparatus and extrude the gel. Locate the position of the bands in the electropherogram as prescribed.

SODIUM DODECYL SULPHATE POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

Scope. Polyacrylamide gel electrophoresis is used for the qualitative characterisation of proteins in biological preparations, for control of purity and quantitative determinations.

Purpose. Analytical gel electrophoresis is an appropriate method with which to identify and to assess the homogeneity of proteins in pharmaceutical preparations. The method is routinely used for the estimation of protein subunit molecular masses and for determining the subunit compositions of purified proteins.

Ready-to-use gels and reagents are widely available on the market and can be used instead of those described in this text, provided that they give equivalent results and that they meet the validity requirements given below under Validation of the test.

CHARACTERISTICS OF POLYACRYLAMIDE GELS

The sieving properties of polyacrylamide gels are established by the three-dimensional network of fibres and pores which is formed as the bifunctional bisacrylamide cross-links adjacent polyacrylamide chains. Polymerisation is catalysed by a free radical-generating system composed of ammonium persulphate and tetramethylethylenediamine.

As the acrylamide concentration of a gel increases, its effective pore size decreases. The effective pore size of a gel is operationally defined by its sieving properties; that is, by the resistance it imparts to the migration of macromolecules. There are limits on the acrylamide concentrations that can be used. At high acrylamide concentrations, gels break much more easily and are difficult to handle. As the pore size of a gel decreases, the migration rate of a protein through the gel decreases. By adjusting the pore size of a gel, through manipulating the acrylamide concentration, the resolution of the method can be optimised for a given protein product. Thus, a given gel is physically characterised by its respective composition in acrylamide and bisacrylamide.

In addition to the composition of the gel, the state of the protein is an important component to the electrophoretic mobility. In the case of proteins, the electrophoretic mobility is dependent on the pK value of the charged groups and the size of the molecule. It is influenced by the type, concentration and pH of the buffer, by the temperature and the field strength as well as by the nature of the support material.

DENATURING POLYACRYLAMIDE GEL ELECTROPHORESIS

The method cited as an example is limited to the analysis of monomeric polypeptides with a mass range of 14 000 to 100 000 daltons. It is possible to extend this mass range by various techniques (e.g. gradient gels, particular buffer system) but those techniques are not discussed in this chapter.

Denaturing polyacrylamide gel electrophoresis using sodium dodecyl sulphate (SDS-PAGE) is the most common mode of electrophoresis used in assessing the pharmaceutical quality of protein products and will be the focus of the example method. Typically, analytical electrophoresis of proteins is carried out in polyacrylamide gels under conditions that ensure dissociation of the proteins into their individual polypeptide subunits and that minimise aggregation. Most commonly, the strongly anionic detergent sodium dodecyl sulphate (SDS) is used in combination with heat to dissociate the proteins before they are loaded on the gel. The denatured polypeptides bind to SDS, become negatively charged and exhibit a consistent charge-to-mass ratio regardless of protein type. Because the amount of SDS bound is almost always proportional to the molecular mass of the polypeptide and is independent of its sequence, SDS-polypeptide complexes migrate through polyacrylamide gels with mobilities dependent on the size of the polypeptide.

The electrophoretic mobilities of the resultant detergent-polypeptide complexes all assume the same functional relationship to their molecular masses. Migration of SDS complexes is toward the anode in a predictable manner, with low-molecular-mass complexes migrating faster than larger ones. The molecular mass of a protein can therefore be estimated from its relative mobility in calibrated SDS-PAGE and the occurrence of a single band in such a gel is a criterion of purity.

Modifications to the polypeptide backbone, such as *N*- or *O*-linked glycosylation, however, have a significant impact on the apparent molecular mass of a protein since SDS does not bind to a carbohydrate moiety in a manner similar to a polypeptide. Thus, a consistent charge-to-mass ratio is not maintained. The apparent molecular mass of proteins having undergone post-translational modifications is not a true reflection of the mass of the polypeptide chain.

Reducing conditions. Polypeptide subunits and three-dimensional structure is often maintained in proteins by the presence of disulphide bonds. A goal of SDS-PAGE

analysis under reducing conditions is to disrupt this structure by reducing disulphide bonds. Complete denaturation and dissociation of proteins by treatment with 2-mercaptoethanol or dithiothreitol (DTT) will result in unfolding of the polypeptide backbone and subsequent complexation with SDS. In these conditions, the molecular mass of the polypeptide subunits can be calculated by linear regression in the presence of suitable molecular-mass standards.

Non-reducing conditions. For some analyses, complete dissociation of the protein into subunit peptides is not desirable. In the absence of treatment with reducing agents such as 2-mercaptoethanol or DTT, disulphide covalent bonds remain intact, preserving the oligomeric form of the protein. Oligomeric SDS-protein complexes migrate more slowly than their SDS-polypeptide subunits. In addition, non-reduced proteins may not be completely saturated with SDS and, hence, may not bind the detergent in a constant mass ratio. This makes molecular-mass determinations of these molecules by SDS-PAGE less straightforward than analyses of fully denatured polypeptides, since it is necessary that both standards and unknown proteins be in similar configurations for valid comparisons. However, the staining of a single band in such a gel is a criterion of purity.

CHARACTERISTICS OF DISCONTINUOUS BUFFER SYSTEM GEL ELECTROPHORESIS

The most popular electrophoretic method for the characterisation of complex mixtures of proteins involves the use of a discontinuous buffer system consisting of two contiguous, but distinct gels: a resolving or separating (lower) gel and a stacking (upper) gel. The two gels are cast with different porosities, pH, and ionic strengths. In addition, different mobile ions are used in the gel and electrode buffers. The buffer discontinuity acts to concentrate large volume samples in the stacking gel, resulting in improved resolution. When power is applied, a voltage drop develops across the sample solution which drives the proteins into the stacking gel. Glycinate ions from the electrode buffer follow the proteins into the stacking gel. A moving boundary region is rapidly formed with the highly mobile chloride ions in the front and the relatively slow glycinate ions in the rear. A localised high-voltage gradient forms between the leading and trailing ion fronts, causing the SDS-protein complexes to form into a thin zone (stack) and migrate between the chloride and glycinate phases. Within broad limits, regardless of the height of the applied sample, all SDS-proteins condense into a very narrow region and enter the resolving gel as a well-defined, thin zone of high protein density. The large-pore stacking gel does not retard the migration of most proteins and serves mainly as an anticonvective medium. At the interface of the stacking and resolving gels, the proteins experience a sharp increase in retardation due to the restrictive pore size of the resolving gel. Once in the resolving gel, proteins continue to be slowed by the sieving of the matrix. The glycinate ions overtake the proteins, which then move in a space of uniform pH formed by the tris(hydroxymethyl)aminomethane and glycine. Molecular sieving causes the SDS-polypeptide complexes to separate on the basis of their molecular masses.

PREPARING VERTICAL DISCONTINUOUS BUFFER SDS POLYACRYLAMIDE GELS

Assembling of the gel moulding cassette. Clean the two glass plates (size: e.g. 10 cm × 8 cm), the polytetrafluoroethylene comb, the two spacers and the silicone rubber tubing (diameter e.g. 0.6 mm × 35 cm) with mild detergent and rinse extensively with water. Dry all the items with a paper towel or tissue. Lubricate the spacers and the tubing with non-silicone grease. Apply the spacers along each of the two short sides of the glass plate 2 mm away from the edges

and 2 mm away from the long side corresponding to the bottom of the gel. Begin to lay the tubing on the glass plate by using one spacer as a guide. Carefully twist the tubing at the bottom of the spacer and follow the long side of the glass plate. While holding the tubing with one finger along the long side twist again the tubing and lay it on the second short side of the glass plate, using the spacer as a guide. Place the second glass plate in perfect alignment and hold the mould together by hand pressure. Apply two clamps on each of the two short sides of the mould. Carefully apply four clamps on the longer side of the gel mould thus forming the bottom of the gel mould. Verify that the tubing is running along the edge of the glass plates and has not been extruded while placing the clamps. The gel mould is now ready for pouring the gel.

Preparation of the gel. In a discontinuous buffer SDS polyacrylamide gel, it is recommended to pour the resolving gel, let the gel set, and then pour the stacking gel since the composition of the two gels in acrylamide-bisacrylamide, buffer and pH are different.

Preparation of the resolving gel. In a conical flask, prepare the appropriate volume of solution containing the desired concentration of acrylamide for the resolving gel, using the values given in Table 2.2.31-1. Mix the components in the order shown. Where appropriate, before adding the ammonium persulphate solution and the tetramethylethylenediamine (TEMED), filter the solution if necessary under vacuum through a cellulose acetate membrane (pore diameter 0.45 µm); keep the solution under vacuum by swirling the filtration unit until no more bubbles are formed in the solution. Add appropriate amounts of ammonium persulphate solution and TEMED as indicated in Table 2.2.31-1, swirl and pour immediately into the gap

between the two glass plates of the mould. Leave sufficient space for the stacking gel (the length of the teeth of the comb plus 1 cm). Using a tapered glass pipette, carefully overlay the solution with water-saturated isobutanol. Leave the gel in a vertical position at room temperature to allow polymerisation.

Preparation of the stacking gel. After polymerisation is complete (about 30 min), pour off the isobutanol and wash the top of the gel several times with water to remove the isobutanol overlay and any unpolymerised acrylamide. Drain as much fluid as possible from the top of the gel, and then remove any remaining water with the edge of a paper towel.

In a conical flask, prepare the appropriate volume of solution containing the desired concentration of acrylamide, using the values given in Table 2.2.31-2. Mix the components in the order shown. Where appropriate, before adding the ammonium persulphate solution and the TEMED, filter the solution if necessary under vacuum through a cellulose acetate membrane (pore diameter: 0.45 µm); keep the solution under vacuum by swirling the filtration unit until no more bubbles are formed in the solution. Add appropriate amounts of ammonium persulphate solution and TEMED as indicated in Table 2.2.31-2, swirl and pour immediately into the gap between the two glass plates of the mould directly onto the surface of the polymerised resolving gel. Immediately insert a clean polytetrafluoroethylene comb into the stacking gel solution, being careful to avoid trapping air bubbles. Add more stacking gel solution to fill the spaces of the comb completely. Leave the gel in a vertical position and allow to polymerise at room temperature.

Table 2.2.31-1. – Preparation of resolving gel

Solution components	Component volumes (ml) per gel mould volume of							
	5 ml	10 ml	15 ml	20 ml	25 ml	30 ml	40 ml	50 ml
6 per cent acrylamide								
Water R	2.6	5.3	7.9	10.6	13.2	15.9	21.2	26.5
Acrylamide solution ⁽¹⁾	1.0	2.0	3.0	4.0	5.0	6.0	8.0	10.0
1.5 M Tris (pH 8.8) ⁽²⁾	1.3	2.5	3.8	5.0	6.3	7.5	10.0	12.5
100 g/l SDS ⁽³⁾	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
100 g/l APS ⁽⁴⁾	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
TEMED ⁽⁵⁾	0.004	0.008	0.012	0.016	0.02	0.024	0.032	0.04
8 per cent acrylamide								
Water R	2.3	4.6	6.9	9.3	11.5	13.9	18.5	23.2
Acrylamide solution ⁽¹⁾	1.3	2.7	4.0	5.3	6.7	8.0	10.7	13.3
1.5 M Tris (pH 8.8) ⁽²⁾	1.3	2.5	3.8	5.0	6.3	7.5	10.0	12.5
100 g/l SDS ⁽³⁾	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
100 g/l APS ⁽⁴⁾	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
TEMED ⁽⁵⁾	0.003	0.006	0.009	0.012	0.015	0.018	0.024	0.03
10 per cent acrylamide								
Water R	1.9	4.0	5.9	7.9	9.9	11.9	15.9	19.8
Acrylamide solution ⁽¹⁾	1.7	3.3	5.0	6.7	8.3	10.0	13.3	16.7
1.5 M Tris (pH 8.8) ⁽²⁾	1.3	2.5	3.8	5.0	6.3	7.5	10.0	12.5
100 g/l SDS ⁽³⁾	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
100 g/l APS ⁽⁴⁾	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
TEMED ⁽⁵⁾	0.002	0.004	0.006	0.008	0.01	0.012	0.016	0.02

Solution components	Component volumes (ml) per gel mould volume of							
	5 ml	10 ml	15 ml	20 ml	25 ml	30 ml	40 ml	50 ml
12 per cent acrylamide								
Water R	1.6	3.3	4.9	6.6	8.2	9.9	13.2	16.5
Acrylamide solution ⁽¹⁾	2.0	4.0	6.0	8.0	10.0	12.0	16.0	20.0
1.5 M Tris (pH 8.8) ⁽²⁾	1.3	2.5	3.8	5.0	6.3	7.5	10.0	12.5
100 g/l SDS ⁽³⁾	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
100 g/l APS ⁽⁴⁾	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
TEMED ⁽⁵⁾	0.002	0.004	0.006	0.008	0.01	0.012	0.016	0.02
14 per cent acrylamide								
Water R	1.4	2.7	3.9	5.3	6.6	8.0	10.6	13.8
Acrylamide solution ⁽¹⁾	2.3	4.6	7.0	9.3	11.6	13.9	18.6	23.2
1.5 M Tris (pH 8.8) ⁽²⁾	1.2	2.5	3.6	5.0	6.3	7.5	10.0	12.5
100 g/l SDS ⁽³⁾	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
100 g/l APS ⁽⁴⁾	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
TEMED ⁽⁵⁾	0.002	0.004	0.006	0.008	0.01	0.012	0.016	0.02
15 per cent acrylamide								
Water R	1.1	2.3	3.4	4.6	5.7	6.9	9.2	11.5
Acrylamide solution ⁽¹⁾	2.5	5.0	7.5	10.0	12.5	15.0	20.0	25.0
1.5 M Tris (pH 8.8) ⁽²⁾	1.3	2.5	3.8	5.0	6.3	7.5	10.0	12.5
100 g/l SDS ⁽³⁾	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
100 g/l APS ⁽⁴⁾	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
TEMED ⁽⁵⁾	0.002	0.004	0.006	0.008	0.01	0.012	0.016	0.02

(1) Acrylamide solution: 30 per cent acrylamide/bisacrylamide(29:1) solution R.

(2) 1.5 M Tris (pH 8.8): 1.5 M tris-hydrochloride buffer solution pH 8.8 R.

(3) 100 g/l SDS: a 100 g/l solution of sodium dodecyl sulphate R.

(4) 100 g/l APS: a 100 g/l solution of ammonium persulphate R. Ammonium persulphate provides the free radicals that drive polymerisation of acrylamide and bisacrylamide. Since ammonium persulphate solution decomposes slowly, fresh solutions must be prepared weekly.

(5) TEMED: tetramethylethylenediamine R.

Mounting the gel in the electrophoresis apparatus and electrophoretic separation. After polymerisation is complete (about 30 min), remove the polytetrafluoroethylene comb carefully. Rinse the wells immediately with water or with the *SDS-PAGE running buffer R* to remove any unpolymerised acrylamide. If necessary, straighten the teeth of the stacking gel with a blunt hypodermic needle attached to a syringe. Remove the clamps on one short side, carefully pull out the tubing and replace the clamps. Proceed similarly on the other short side. Remove the tubing from the bottom part of

the gel. Mount the gel in the electrophoresis apparatus. Add the electrophoresis buffers to the top and bottom reservoirs. Remove any bubbles that become trapped at the bottom of the gel between the glass plates. This is best done with a bent hypodermic needle attached to a syringe. Never pre-run the gel before loading the samples, since this will destroy the discontinuity of the buffer systems. Before loading the sample carefully rinse the slot with *SDS-PAGE running buffer R*. Prepare the test and reference solutions in the recommended sample buffer and treat as specified in the

Table 2.2.31-2. – Preparation of stacking gel

Solution components	Component volumes (ml) per gel mould volume of							
	1 ml	2 ml	3 ml	4 ml	5 ml	6 ml	8 ml	10 ml
Water R	0.68	1.4	2.1	2.7	3.4	4.1	5.5	6.8
Acrylamide solution ⁽¹⁾	0.17	0.33	0.5	0.67	0.83	1.0	1.3	1.7
1.0 M Tris (pH 6.8) ⁽²⁾	0.13	0.25	0.38	0.5	0.63	0.75	1.0	1.25
100 g/l SDS ⁽³⁾	0.01	0.02	0.03	0.04	0.05	0.06	0.08	0.1
100 g/l APS ⁽⁴⁾	0.01	0.02	0.03	0.04	0.05	0.06	0.08	0.1
TEMED ⁽⁵⁾	0.001	0.002	0.003	0.004	0.005	0.006	0.008	0.01

(1) Acrylamide solution: 30 per cent acrylamide/bisacrylamide (29:1) solution R.

(2) 1.0 M Tris (pH 6.8): 1 M tris-hydrochloride buffer solution pH 6.8 R.

(3) 100 g/l SDS: a 100 g/l solution of sodium dodecyl sulphate R.

(4) 100 g/l APS: a 100 g/l solution of ammonium persulphate R. Ammonium persulphate provides the free radicals that drive polymerisation of acrylamide and bisacrylamide. Since ammonium persulphate solution decomposes slowly, fresh solutions must be prepared weekly.

(5) TEMED: tetramethylethylenediamine R.

individual monograph. Apply the appropriate volume of each solution to the stacking gel wells. Start the electrophoresis using the conditions recommended by the manufacturer of the equipment. Manufacturers of SDS-PAGE equipment may provide gels of different surface area and thickness. Electrophoresis running time and current/voltage may need to vary as described by the manufacturer of the apparatus in order to achieve optimum separation. Check that the dye front is moving into the resolving gel. When the dye is reaching the bottom of the gel, stop the electrophoresis. Remove the gel assembly from the apparatus and separate the glass plates. Remove the spacers, cut off and discard the stacking gel and immediately proceed with staining.

DETECTION OF PROTEINS IN GELS

Coomassie staining is the most common protein staining method with a detection level of the order of 1 µg to 10 µg of protein per band. Silver staining is the most sensitive method for staining proteins in gels and a band containing 10 ng to 100 ng can be detected.

All of the steps in gel staining are done at room temperature with gentle shaking (e.g. on an orbital shaker platform) in any convenient container. Gloves must be worn when staining gels, since fingerprints will stain.

Coomassie staining. Immerse the gel in a large excess of *Coomassie staining solution R* and allow to stand for at least 1 h. Remove the staining solution.

Destain the gel with a large excess of *destaining solution R*. Change the destaining solution several times, until the stained protein bands are clearly distinguishable on a clear background. The more thoroughly the gel is destained, the smaller is the amount of protein that can be detected by the method. Destaining can be speeded up by including a few grams of anion-exchange resin or a small sponge in the *destaining solution R*.

NOTE: the acid-alcohol solutions used in this procedure do not completely fix proteins in the gel. This can lead to losses of some low-molecular-mass proteins during the staining and destaining of thin gels. Permanent fixation is obtainable by allowing the gel to stand in a mixture of 1 volume of trichloroacetic acid *R*, 4 volumes of methanol *R* and 5 volumes of water *R* for 1 h before it is immersed in the *Coomassie staining solution R*.

Silver staining. Immerse the gel in a large excess of *fixing solution R* and allow to stand for 1 h. Remove the fixing solution, add fresh fixing solution and incubate either for at least 1 h or overnight, if convenient. Discard the fixing solution and wash the gel in a large excess of water *R* for 1 h. Soak the gel for 15 min in a 1 per cent V/V solution of *glutaraldehyde R*. Wash the gel twice for 15 min in a large excess of water *R*. Soak the gel in fresh *silver nitrate reagent R* for 15 min, in darkness. Wash the gel three times for 5 min in a large excess of water *R*. Immerse the gel for about 1 min in *developer solution R* until satisfactory staining has been obtained. Stop the development by incubation in the *blocking solution R* for 15 min. Rinse the gel with water *R*.

DRYING OF STAINED SDS POLYACRYLAMIDE GELS

Depending on the staining method used, gels are treated in a slightly different way. For Coomassie staining, after the destaining step, allow the gel to stand in a 100 g/l solution of *glycerol R* for at least 2 h (overnight incubation is possible). For silver staining, add to the final rinsing a step of 5 min in a 20 g/l solution of *glycerol R*.

Immerse two sheets of porous cellulose film in water *R* and incubate for 5 min to 10 min. Place one of the sheets on a drying frame. Carefully lift the gel and place it on the cellulose film. Remove any trapped air bubbles and pour a

few millilitres of water *R* around the edges of the gel. Place the second sheet on top and remove any trapped air bubbles. Complete the assembly of the drying frame. Place in an oven or leave at room temperature until dry.

MOLECULAR-MASS DETERMINATION

Molecular masses of proteins are determined by comparison of their mobilities with those of several marker proteins of known molecular weight. Mixtures of proteins with precisely known molecular masses blended for uniform staining are available for calibrating gels. They are obtainable in various molecular mass ranges. Concentrated stock solutions of proteins of known molecular mass are diluted in the appropriate sample buffer and loaded on the same gel as the protein sample to be studied.

Immediately after the gel has been run, the position of the bromophenol blue tracking dye is marked to identify the leading edge of the electrophoretic ion front. This can be done by cutting notches in the edges of the gel or by inserting a needle soaked in India ink into the gel at the dye front. After staining, measure the migration distances of each protein band (markers and unknowns) from the top of the resolving gel. Divide the migration distance of each protein by the distance travelled by the tracking dye. The normalised migration distances so obtained are called the relative mobilities of the proteins (relative to the dye front) and conventionally denoted as R_f . Construct a plot of the logarithm of the relative molecular masses (M_r) of the protein standards as a function of the R_f values. Note that the graphs are slightly sigmoid. Unknown molecular masses can be estimated by linear regression analysis or interpolation from the curves of $\log M_r$ against R_f as long as the values obtained for the unknown samples are positioned along the linear part of the graph.

VALIDATION OF THE TEST

The test is not valid unless the proteins of the molecular mass marker are distributed along 80 per cent of the length of the gel and over the required separation range (e.g. the range covering the product and its dimer or the product and its related impurities) the separation obtained for the relevant protein bands shows a linear relationship between the logarithm of the molecular mass and the R_f . Additional validation requirements with respect to the solution under test may be specified in individual monographs.

QUANTIFICATION OF IMPURITIES

Where the impurity limit is specified in the individual monograph, a reference solution corresponding to that level of impurity should be prepared by diluting the test solution. For example, where the limit is 5 per cent, a reference solution would be a 1:20 dilution of the test solution. No impurity (any band other than the main band) in the electropherogram obtained with the test solution may be more intense than the main band obtained with the reference solution.

Under validated conditions impurities may be quantified by normalisation to the main band using an integrating densitometer. In this case, the responses must be validated for linearity.

01/2005:20232

2.2.32. LOSS ON DRYING

Loss on drying is the loss of mass expressed as per cent m/m .

Method. Place the prescribed quantity of the substance to be examined in a weighing bottle previously dried under the conditions prescribed for the substance to be examined. Dry the substance to constant mass or for the prescribed

time by one of the following procedures. Where the drying temperature is indicated by a single value rather than a range, drying is carried out at the prescribed temperature $\pm 2^\circ\text{C}$.

- "in a desiccator": the drying is carried out over *diphosphorus pentoxide R* at atmospheric pressure and at room temperature;
- "*in vacuo*": the drying is carried out over *diphosphorus pentoxide R*, at a pressure of 1.5 kPa to 2.5 kPa at room temperature;
- "*in vacuo* within a specified temperature range": the drying is carried out over *diphosphorus pentoxide R*, at a pressure of 1.5 kPa to 2.5 kPa within the temperature range prescribed in the monograph;
- "in an oven within a specified temperature range": the drying is carried out in an oven within the temperature range prescribed in the monograph;
- "under high vacuum": the drying is carried out over *diphosphorus pentoxide R* at a pressure not exceeding 0.1 kPa, at the temperature prescribed in the monograph.

If other conditions are prescribed, the procedure to be used is described in full in the monograph.

01/2005:20233

2.2.33. NUCLEAR MAGNETIC RESONANCE SPECTROMETRY

Nuclear magnetic resonance (NMR) spectrometry is based on the fact that nuclei such as ^1H , ^{13}C , ^{19}F , ^{31}P possess a permanent nuclear magnetic moment. When placed in an external magnetic field (main field), they take certain well-defined orientations with respect to the direction of this field which correspond to distinct energy levels. For a given field value, transitions between neighbouring energy levels take place due to absorption of electromagnetic radiation of characteristic wavelengths at radio frequencies.

The determination of these frequencies may be made either by sequential search of the resonance conditions (continuous-wave spectrometry) or by simultaneous excitation of all transitions with a multifrequency pulse followed by computer analysis of the free-induction decay of the irradiation emitted as the system returns to the initial state (pulsed spectrometry).

A *proton* magnetic resonance spectrum appears as a set of signals which correspond to protons and are characteristic of their nuclear and electronic environment within the molecule. The separation between a given signal and that of a reference compound is called a chemical shift (δ) and is expressed in parts per million (ppm); it characterises the kind of proton in terms of electronic environment. Signals are frequently split into groups of related peaks, called doublets, triplets, multiplets; this splitting is due to the presence of permanent magnetic fields emanating from adjacent nuclei, particularly from other protons within two to five valence bonds. The intensity of each signal, determined from the area under the signal, is proportional to the number of equivalent protons.

Apparatus. A nuclear magnetic resonance spectrometer for continuous-wave spectrometry consists of a magnet, a low-frequency sweep generator, a sample holder, a radio-frequency transmitter and receiver, a recorder and an electronic integrator. A pulsed spectrometer is additionally equipped with a pulse transmitter and a computer for the acquisition, storage and mathematical transformation of the data into a conventional spectrum.

Use a nuclear magnetic resonance spectrometer operating at not less than 60 MHz for ^1H . Unless otherwise prescribed, follow the instructions of the manufacturer.

Before recording the spectrum, verify that:

- The resolution is equal to 0.5 Hz or less by measuring the peak width at half-height using an adequate scale expansion of:

- either the band at δ 7.33 ppm or at δ 7.51 ppm of the symmetrical multiplet of a 20 per cent V/V solution of *dichlorobenzene R* in *deuterated acetone R*,
- or the band at δ 0.00 ppm of a 5 per cent V/V solution of *tetramethylsilane R* in *deuterated chloroform R*.

- The signal-to-noise ratio (S/N), measured over the range from δ 2 ppm to δ 5 ppm on the spectrum obtained with a 1 per cent V/V solution of *ethylbenzene R* in *deuterated chloroform R*, is at least 25:1. This ratio is calculated as the mean of five successive determinations from the expression:

$$\frac{S}{N} = 2.5 \frac{A}{H}$$

A = amplitude, measured in millimetres, of the largest peak of the methylene quartet of ethylbenzene centred at δ 2.65 ppm. The amplitude is measured from a base line constructed from the centre of the noise on either side of this quartet and at a distance of at least 1 ppm from its centre.

H = peak to peak amplitude of the base line noise measured in millimetres obtained between δ 4 ppm and δ 5 ppm.

- The amplitude of spinning side bands is not greater than 2 per cent of the sample peak height in a tube rotating at a speed appropriate for the spectrometer used.

- For quantitative measurements verify the repeatability of the integrator responses, using a 5 per cent V/V solution of *ethylbenzene R* in *deuterated chloroform R*. Carry out five successive scans of the protons of ethyl groups and determine the mean of the values obtained. None of the individual values differs by more than 2.5 per cent from the mean.

Method. Dissolve the substance to be examined as prescribed and filter; the solution must be clear. Use a chemical shift internal reference compound, which, unless otherwise prescribed, is a solution containing 0.5 per cent V/V to 1.0 per cent V/V of *tetramethylsilane R* (TMS) in deuterated organic solvents or 5 g/l to 10 g/l of *sodium tetradeuteriodimethylsilapentanoate acid R* (TSP) in *deuterium oxide R*. Take the necessary quantity and record the spectrum.

CONTINUOUS-WAVE SPECTROMETRY

Adjust the spectrometer so that it is operating as closely as possible in the pure absorption mode and use a radio-frequency setting which avoids saturation of the signals. Adjust the controls of the spectrometer so that the strongest peak in the spectrum of the substance to be examined occupies almost the whole of the scale on the recorder chart and that the signal of the internal reference compound corresponds to a chemical shift of δ 0.00 ppm. Record the spectrum over the prescribed spectral width and, unless otherwise specified, at a sweep rate of not more than 2 Hz per second. Record the integral spectrum over the same spectral width and at a suitable sweep rate according to the instrument used. When quantitative measurements are required, these should be obtained as prescribed.

PULSED SPECTROMETRY

Set the spectrometer controls, e.g. pulse flip angle, pulse amplitude, pulse interval, spectral width, number of data points (resolution) and data acquisition rate, as indicated in the manufacturer's instructions and collect the necessary number of free induction decays. After mathematical transformation of the data by the computer, adjust the phase control in order to obtain as far as possible a pure absorption spectrum and calibrate the spectrum relative to the resonance frequency of the chemical shift internal reference compound. Display the spectrum stored in the computer on a suitable output device and, for quantitative measurements, process the integral according to the facility of the instrument.

01/2005:20234

2.2.34. THERMAL ANALYSIS

Thermal analysis is a group of techniques in which the variation of a physical property of a substance is measured as a function of temperature. The most commonly used techniques are those which measure changes of mass or changes in energy of a sample of a substance.

THERMOGRAVIMETRY

Thermogravimetry is a technique in which the mass of a sample of a substance is recorded as a function of temperature according to a controlled temperature programme.

Apparatus. The essential components of a thermobalance are a device for heating or cooling the substance according to a given temperature program, a sample holder in a controlled atmosphere, an electrobalance and a recorder. In some cases the instrument may be coupled to a device permitting the analysis of volatile products.

Temperature verification. Check the temperature scale using a suitable material according to the manufacturer's instructions.

Calibration of the electrobalance. Place a suitable quantity of a suitable certified reference material in the sample holder and record the mass. Set the heating rate according to the manufacturer's instructions and start the temperature increase. Record the thermogravimetric curve as a graph with temperature, or time, on the abscissa, increasing from left to right, and mass on the ordinate, increasing upwards. Stop the temperature increase at about 230 °C. Measure the difference on the graph between the initial and final mass-temperature plateaux, or mass-time plateaux, which corresponds to the loss of mass. The declared loss of mass for the certified reference material is stated on the label.

Method. Apply the same procedure to the substance to be examined, using the conditions prescribed in the monograph. Calculate the loss of mass of the substance to be examined from the difference measured in the graph obtained. Express the loss of mass as per cent $\Delta m/m$.

If the apparatus is in frequent use, carry out temperature verification and calibration regularly. Otherwise, carry out such checks before each measurement.

Since the test atmosphere is critical, the following parameters are noted for each measurement: pressure or flow rate, composition of the gas.

DIFFERENTIAL SCANNING CALORIMETRY

Differential Scanning Calorimetry (DSC) is a technique that can be used to demonstrate the energy phenomena produced during heating (or cooling) of a substance (or a mixture of substances) and to determine the changes in enthalpy and specific heat and the temperatures at which these occur.

The technique is used to determine the difference in the flow of heat (with reference to the temperature) evolved or absorbed by the test sample compared with the reference cell, as a function of the temperature. Two types of DSC apparatuses are available, those using power compensation to maintain a null temperature difference between sample and reference and those that apply a constant rate of heating and detect temperature differential as a difference in heat flow between sample and reference.

Apparatus. The apparatus for the power compensation DSC consists of a furnace containing a sample holder with a reference cell and a test cell. The apparatus for the heat flow DSC consists of a furnace containing a single cell with a sample holder for the reference crucible and the test crucible. A temperature-programming device, thermal detector(s) and a recording system which can be connected to a computer are attached. The measurements are carried out under a controlled atmosphere.

Calibration of the apparatus. Calibrate the apparatus for temperature and enthalpy change, using indium of high purity or any other suitable certified material, according to the manufacturer's instructions. A combination of 2 metals, e.g. indium and zinc may be used to control linearity.

Operating procedure. Weigh in a suitable crucible an appropriate quantity of the substance to be examined; place it in the sample holder. Set the initial and final temperatures, and the heating rate according to the operating conditions prescribed in the monograph.

Begin the analysis and record the differential thermal analysis curve, with the temperature or time on the abscissa (values increasing from left to right) and the energy change on the ordinate (specify whether the change is endothermic or exothermic).

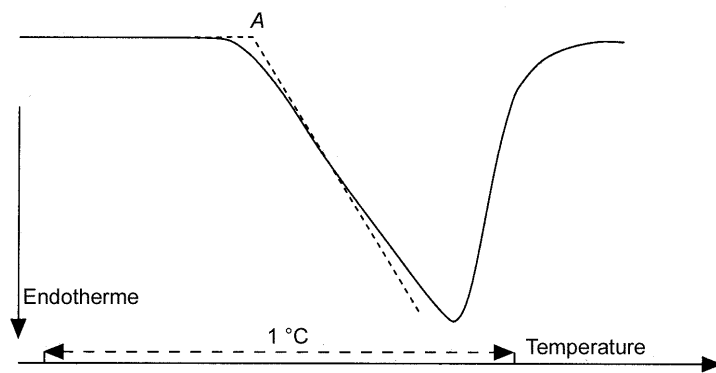


Figure 2.2.34-1. - Thermogram

The temperature at which the phenomenon occurs (the onset temperature) corresponds to the intersection (A) of the extension of the baseline with the tangent at the point of greatest slope (inflexion point) of the curve (see Figure 2.2.34-1). The end of the thermal phenomenon is indicated by the peak of the curve.

The enthalpy of the phenomenon is proportional to the area under the curve limited by the baseline; the proportionality factor is determined from the measurement of the heat of fusion of a known substance (e.g., indium) under the same operating conditions.

Each thermogram may be accompanied by the following data: conditions employed, record of last calibration, sample size and identification (including thermal history), container, atmosphere (identity, flow rate, pressure), direction and rate of temperature change, instrument and recorder sensitivity.

Applications

Phase changes. Determination of the temperature, heat capacity change and enthalpy of phase changes undergone by a substance as a function of temperature.

solid - solid transition:	allotropy - polymorphism glass transition desolvation amorphous-crystalline
solid - liquid transition:	melting
solid - gas transition:	sublimation
liquid - solid transition:	freezing recrystallisation
liquid - gas transition:	evaporation

Changes in chemical composition. Measurement of heat and temperatures of reaction under given experimental conditions, so that, for example, the kinetics of decomposition or of desolvation can be determined.

Application to phase diagrams. Establishment of phase diagrams for solid mixtures. The establishment of a phase diagram may be an important step in the preformulation and optimisation of the freeze-drying process.

Determination of purity. The measurement of the heat of fusion and the melting point by DSC enables the impurity content of a substance to be determined from a single

thermal diagram, requiring the use of only a few milligrams of sample with no need for repeated accurate measurements of the true temperature.

In theory, the melting of an entirely crystalline, pure substance at constant pressure is characterised by a heat of fusion ΔH_f in an infinitely narrow range, corresponding to the melting point T_0 . A broadening of this range is a sensitive indicator of impurities. Hence, samples of the same substance, whose impurity contents vary by a few tenths of a per cent, give thermal diagrams that are visually distinct (see Figure 2.2.34-2).

The determination of the molar purity by DSC is based on the use of a mathematical approximation of the integrated form of the Van't Hoff equation applied to the concentrations (not the activities) in a binary system [$\ln(1 - x_2) = -x_2$ and $T \times T_0 = T_0^2$]:

$$T = T_0 - \frac{RT_0^2}{\Delta H_f} \times x_2 \quad (1)$$

x_2 = mole fraction of the impurity i.e. the number of molecules of the impurity divided by the total number of molecules in the liquid phase (or molten phase) at temperature T (expressed in kelvins),

T_0 = melting point of the chemically pure substance, in kelvins,

ΔH_f = molar heat of fusion of the substance, in joules,

R = gas constant for ideal gases, in $\text{joules} \cdot \text{kelvin}^{-1} \cdot \text{mole}^{-1}$.

Hence, the determination of purity by DSC is limited to the detection of impurities forming a eutectic mixture with the principal compound and present at a mole fraction of less than 2 per cent in the substance to be examined.

This method cannot be applied to:

- amorphous substances,
- solvates or polymorphic compounds that are unstable within the experimental temperature range,
- impurities forming solid solutions with the principal substance,
- impurities that are insoluble in the liquid phase or in the melt of the principal substance.

During the heating of the substance to be examined, the impurity melts completely at the temperature of the eutectic mixture. Above this temperature, the solid phase contains

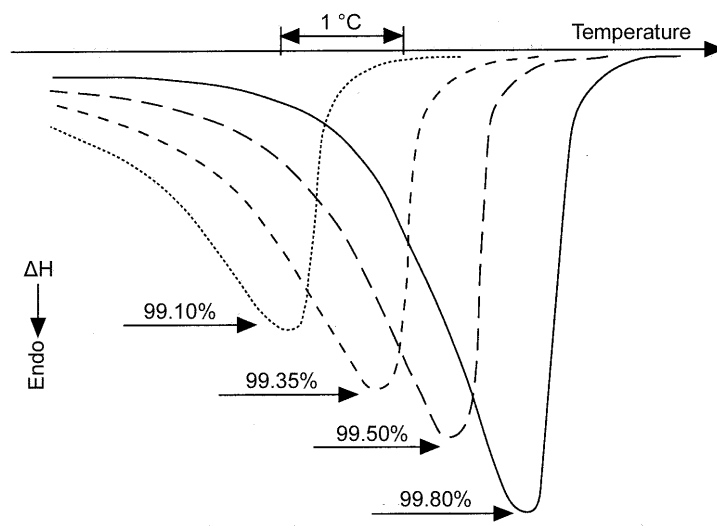


Figure 2.2.34-2. – Thermal diagrams according to purity

only the pure substance. As the temperature increases progressively from the temperature of the eutectic mixture to the melting point of the pure substance, the mole fraction of impurity in the liquid decreases constantly, since the quantity of liquified pure substance increases constantly. For all temperatures above the eutectic point:

$$x_2 = \frac{1}{F} \times x_2^* \quad (2)$$

F = molten fraction of the analysed sample,

x_2^* = mole fraction of the impurity in the analysed sample.

When the entire sample has melted, $F = 1$ and $x_2 = x_2^*$.

If equation (2) is combined with equation (1), the following equation is obtained:

$$T = T_0 - \frac{x_2^* RT_0^2}{\Delta H_f} \times \frac{1}{F}$$

The value of the heat of fusion is obtained by integrating the melting peak.

The melting point T_0 of the pure substance is extrapolated from the plot of $1/F$ versus the temperature expressed in kelvins. The slope α of the curve, obtained after linearisation, if necessary, corresponding to $RT_0^2 \frac{x_2^*}{\Delta H_f}$ allows x_2^* to be evaluated.

The fraction x_2^* , multiplied by 100 gives the mole fraction in per cent for the total eutectic impurities.

THERMOMICROSCOPY

Phase changes may be visualised by thermomicroscopy, a method which enables a sample subjected to a programmed temperature change to be examined, in polarised light, under a microscope.

The observations made in thermomicroscopy allow the nature of the phenomena detected using thermogravimetry and differential thermal analysis to be clearly identified.

Apparatus. The apparatus consists of a microscope fitted with a light polariser, a hot plate, a temperature and heating rate and/or cooling rate programmer and a recording system for the transition temperatures. A video camera and video recorder may be added.

m = molality of the solution, that is the number of moles of solute per kilogram of solvent,

Φ = molal osmotic coefficient which takes account of the interactions between ions of opposite charge in the solution. It is dependent on the value of m . As the complexity of solutions increases, Φ becomes difficult to measure.

The unit of osmolality is osmole per kilogram (osmol/kg), but the submultiple milliosmole per kilogram (mosmol/kg) is usually used.

Unless otherwise prescribed, osmolality is determined by measurement of the depression of freezing point. The following relationship exists between the osmolality and the depression of freezing point ΔT :

$$\xi_m = \frac{\Delta T}{1.86} \times 1000 \text{ mosmol/kg}$$

Apparatus. The apparatus (osmometer) consists of:

- a means of cooling the container used for the measurement,
- a system for measuring temperature consisting of a resistor sensitive to temperature (thermistor), with an appropriate current or potential-difference measurement device that may be graduated in temperature depression or directly in osmolality,
- a means of mixing the sample is usually included.

Method. Prepare reference solutions as described in Table 2.2.35-1, as required. Determine the zero of the apparatus using *water R*. Calibrate the apparatus using the reference solutions: introduce 50 μl to 250 μl of sample into the measurement cell and start the cooling system. Usually, the mixing device is programmed to operate at a temperature below that expected through cryoscopic depression to prevent supercooling. A suitable device indicates attainment of equilibrium. Before each measurement, rinse the measurement cell with the solution to be examined.

Table 2.2.35-1. – Reference solutions for osmometer calibration

Mass in grams of sodium chloride <i>R</i> per kilogram of <i>water R</i>	Real osmolality (mosmol/kg)	Ideal osmolality (mosmol/kg)	Molal osmotic coefficient	Cryoscopic depression (°C)
3.087	100	105.67	0.9463	0.186
6.260	200	214.20	0.9337	0.372
9.463	300	323.83	0.9264	0.558
12.684	400	434.07	0.9215	0.744
15.916	500	544.66	0.9180	0.930
19.147	600	655.24	0.9157	1.116
22.380	700	765.86	0.9140	1.302

01/2005:20235

2.2.35. OSMOLALITY

Osmolality is a practical means of giving an overall measure of the contribution of the various solutes present in a solution to the osmotic pressure of the solution.

An acceptable approximation for the osmolality ξ_m of a given aqueous solution is given by:

$$\xi_m = \nu m \Phi$$

If the solute is not ionised, $\nu = 1$; otherwise ν is the total number of ions already present or formed by solvolysis from one molecule of solute.

Carry out the same operations with the test sample. Read directly the osmolality or calculate it from the measured depression of freezing point. The test is not valid unless the value found is within two values of the calibration scale.

01/2005:20236

Table 2.2.36-1. - Values of k at different temperatures

Temperature (°C)	k
20	0.0582
25	0.0592
30	0.0602

2.2.36. POTENTIOMETRIC DETERMINATION OF IONIC CONCENTRATION USING ION-SELECTIVE ELECTRODES

Ideally, the potential E of an ion-selective electrode varies linearly with the logarithm of the activity a_i of a given ion, as expressed by the Nernst equation:

$$E = E_0 + 2.303 \frac{RT}{z_i F} \log a_i$$

- E_0 = part of the constant potential due to the apparatus used,
 R = gas constant,
 T = absolute temperature,
 F = Faraday's number,
 z_i = charge number of the ion including its sign.

At a constant ionic strength, the following holds:

$$E = E_0 + \frac{k}{z_i} \log f C_i$$

- C_i = molar concentration of the ion,
 f = the activity coefficient ($a_i = f C_i$),
 $k = \frac{RT}{F}$
 If: $E_0 + \frac{k}{z_i} \log f = E'_0$ and $S = \frac{k}{z_i}$

S = slope of the calibration curve of the electrode,

the following holds: $E = E'_0 + S \log C_i$

and for $-\log C_i = \text{p}C_i$: $E = E'_0 - S \text{p}C_i$.

The potentiometric determination of the ion concentration is carried out by measuring the potential difference between two suitable electrodes immersed in the solution to be examined; the indicator electrode is selective for the ion to be determined and the other is a reference electrode.

Apparatus. Use a voltmeter allowing measurements to the nearest 0.1 millivolt and whose input impedance is at least one hundred times greater than that of the electrodes used.

Ion-selective electrodes may be primary electrodes with a crystal or non-crystal membrane or with a rigid matrix (for example, glass electrodes), or electrodes with charged (positive or negative) or uncharged mobile carriers, or sensitised electrodes (enzymatic-substrate electrodes, gas-indicator electrodes). The reference electrode is generally a silver-silver chloride electrode or a calomel electrode, with suitable junction liquids producing no interference.

Procedure. Carry out each measurement at a temperature constant to ± 0.5 °C, taking into account the variation of the slope of the electrode with temperature (see Table 2.2.36-1). Adjust the ionic strength and possibly the pH of the solution to be analysed using the buffer reagent described in the monograph and equilibrate the electrode by immersing it in the solution to be analysed, under slow and uniform stirring, until a constant reading is obtained.

If the electrode system is used frequently, check regularly the repeatability and the stability of responses, and the linearity of the calibration curve or the calculation algorithm in the range of concentrations of the test solution; if not, carry out the test before each set of measurements. The response of the electrode may be regarded as linear if the slope S of the calibration curve is approximately equal to k/z_i , per unit of $\text{p}C_i$.

METHOD I (DIRECT CALIBRATION)

Measure at least three times in succession the potential of at least three reference solutions spanning the expected concentration of the test solution. Calculate the calibration curve, or plot on a chart the mean potential E obtained against the concentration of the ion to be determined expressed as $-\log C_i$ or $\text{p}C_i$.

Prepare the test solution as prescribed in the monograph; measure the potential three times and, from the mean potential, calculate the concentration of the ion to be determined using the calibration curve.

METHOD II (MULTIPLE STANDARD ADDITIONS)

Prepare the test solution as prescribed in the monograph. Measure the potential at equilibrium E_T of a volume V_T of this solution of unknown concentration C_T of the ion to be determined. Make at least three consecutive additions of a volume V_S negligible compared to V_T ($V_S \leq 0.01 V_T$) of a reference solution of a concentration C_S known to be within the linear part of the calibration curve. After each addition, measure the potential and calculate the difference of potential ΔE between the measured potential and E_T . ΔE is related to the concentration of the ion to be determined by the equation:

$$\Delta E = S \log \left(1 + \frac{C_S V_S}{C_T V_T} \right)$$

or

$$10^{\frac{\Delta E}{S}} = 1 + \frac{C_S V_S}{C_T V_T}$$

- V_T = volume of the test solution,
 C_T = concentration of the ion to be determined in the test solution,
 V_S = added volume of the reference solution,
 C_S = concentration of the ion to be determined in the reference solution,
 S = slope of the electrode determined experimentally, at constant temperature, by measuring the difference between the potentials obtained with two reference solutions whose concentrations differ by a factor of ten and are situated within the range where the calibration curve is linear.

Plot on a graph $10 \frac{\Delta E}{S}$ (y -axis) against V_S (x -axis) and extrapolate the line obtained until it intersects the x -axis. At the intersection, the concentration C_T of the test solution in the ion to be determined is given by the equation:

$$C_T = \frac{C_S V_S}{V_T}$$

METHOD III (SINGLE STANDARD ADDITION)

To a volume V_T of the test solution prepared as prescribed in the monograph, add a volume V_S of a reference solution containing an amount of the ion to be determined known to give a response situated in the linear part of the calibration curve. Prepare a blank solution in the same conditions. Measure at least three times the potentials of the test solution and the blank solution, before and after adding the reference solution. Calculate the concentration C_T of the ion to be analysed using the following equation and making the necessary corrections for the blank:

$$C_T = \frac{C_S V_S}{10 \frac{\Delta E}{S} (V_T + V_S) - V_T}$$

- V_T = volume of the test solution or the blank,
 C_T = concentration of the ion to be determined in the test solution,
 V_S = added volume of the reference solution,
 C_S = concentration of the ion to be determined in the reference solution,
 ΔE = difference between the average potentials measured before and after adding V_S ,
 S = slope of the electrode determined experimentally, at constant temperature, by measuring the difference between the potentials obtained from two reference solutions whose concentrations differ by a factor of ten and are situated within the range where the calibration curve is linear.

01/2005:20237

2.2.37. X-RAY FLUORESCENCE SPECTROMETRY⁽³⁾

Wavelength dispersive X-ray fluorescence spectrometry is a procedure that uses the measurement of the intensity of the fluorescent radiation emitted by an element having an atomic number between 11 and 92 excited by a continuous primary X-ray radiation. The intensity of the fluorescence produced by a given element depends on the concentration of this element in the sample but also on the absorption by the matrix of the incident and fluorescent radiation. At trace levels, where the calibration curve is linear, the intensity of the fluorescent radiation emitted by an element in a given matrix, at a given wavelength, is proportional to the concentration of this element and inversely proportional to the mass absorption coefficient of the matrix at this wavelength.

Method. Set and use the instrument in accordance with the instructions given by the manufacturer. Liquid samples are placed directly in the instrument; solid samples are first compressed into pellets, sometimes after mixing with a suitable binder.

To determine the concentration of an element in a sample, it is necessary to measure the net impulse rate produced by one or several standard preparations containing known amounts of this element in given matrices and to calculate or measure the mass absorption coefficient of the matrix of the sample to be analysed.

Calibration. From a calibration solution or a series of dilutions of the element to be analysed in various matrices, determine the slope of the calibration curve b_0 from the following equation:

$$b_0 \frac{1}{\mu_M} = \frac{I_C^N}{C}$$

- μ_M = absorption coefficient of the matrix M, calculated or measured,
 I_C^N = net impulse rate,
 C = concentration of the element to be assayed in the standard preparation.

Mass absorption coefficient of the matrix of the sample. If the empirical formula of the sample to be analysed is known, calculate its mass absorption coefficient from the known elemental composition and the tabulated elemental mass absorption coefficients. If the elemental composition is unknown, determine the mass absorption coefficient of the sample matrix by measuring the intensity of the scattered X-radiation I_U (Compton scattering) from the following equation:

$$\frac{1}{\mu_{MP}} = a + b I_U$$

- μ_{MP} = mass absorption coefficient of the sample,
 I_U = scattered X-radiation.

Determination of the net pulse rate of the element to be determined in the sample. Calculate the net impulse rate I_{EP}^N of the element to be determined from the measured intensity of the fluorescence line and the intensity of the background line(s), allowing for any tube contaminants present.

Calculation of the trace content. If the concentration of the element is in the linear part of the calibration curve, it can be calculated using the following equation:

$$C = \frac{I_{EP}^N}{b_0 \frac{1}{\mu_{MP}}} \times f$$

- f = dilution factor.

01/2005:20238

2.2.38. CONDUCTIVITY

The conductivity of a solution (κ) is, by definition, the reciprocal of resistivity (ρ). Resistivity is defined as the quotient of the electric field and the density of the current. The resistance R (Ω) of a conductor of cross-section S (cm^2) and length L (cm) is given by the expression:

$$R = \rho \frac{L}{S}$$

(3) G. Andermann & M.W. Kemp, Analytical Chemistry 30 1306 (1958). Z.H. Kalman & L. Heller, Analytical Chemistry 34 946 (1962). R.C. Reynolds, Jr., The American Mineralogist 46 1133 (1963). R.O. Müller, Spectrochimica Acta 20 143 (1964). R.O. Müller, Spectrochemische Analyse mit Röntgenfluoreszenz, R. Oldenburg München-Wien (1967).

$$\text{thus: } R = \frac{1}{\kappa} \cdot \frac{L}{S} \text{ or } \kappa = \frac{1}{R} \cdot \frac{L}{S}$$

The unit of conductivity in the International System is the siemens per metre (S m^{-1}). In practice, the electrical conductivity of a solution is expressed in siemens per centimetre (S cm^{-1}) or in microsiemens per centimetre ($\mu\text{S cm}^{-1}$). The unit of resistivity in the International System is the ohm-metre (Ωm). The resistivity of a solution is generally expressed in ohm-centimetres (Ωcm). Unless otherwise prescribed, the reference temperature for the expression of conductivity or resistivity is 20 °C.

APPARATUS

The apparatus used (conductivity meter or resistivity meter) measures the resistance of the column of liquid between the electrodes of the immersed measuring device (conductivity cell). The apparatus is supplied with alternating current to avoid the effects of electrode polarisation. It is equipped with a temperature compensation device or a precision thermometer.

The conductivity cell contains two parallel platinum electrodes coated with platinum black, each with a surface area S , and separated from the other by a distance L . Both are generally protected by a glass tube that allows good exchange between the solution and the electrodes.

The cell constant C of the conductivity cell is given in cm^{-1} according to the equation:

$$C = \alpha \frac{L}{S}$$

α = a dimensionless numerical coefficient, which is characteristic of the cell design.

REAGENTS

Prepare three standard solutions of *potassium chloride R* containing 0.7455 g, 0.0746 g and 0.0149 g, respectively, of *potassium chloride R* per 1000.0 g of solution, using *carbon dioxide-free water R*, prepared from *distilled water R* whose conductivity does not exceed $2 \mu\text{S cm}^{-1}$.

The conductivity and resistivity of these three solutions at 20 °C are given below:

Table 2.2.38.-1. - *Conductivity and resistivity of potassium chloride solutions*

Concentration in g per 1000.0 g of solution	Conductivity $\mu\text{S cm}^{-1}$	Resistivity Ωcm
0.7455	1330	752
0.0746	133.0	7519
0.0149	26.6	37594

If the determination cannot be made at the temperature of 20 °C, use the following equation to correct the conductivity of the potassium chloride solutions indicated in the table. This equation is valid only for temperatures in the range 20 ± 5 °C.

$$C_T = C_{20} [1 + 0.021 (T - 20)]$$

T = measurement temperature prescribed in the monograph,

C_T = Conductivity of the solution at T °C,

C_{20} = Conductivity of the solution at 20 °C.

OPERATING PROCEDURE

Determination of the cell constant

Choose a conductivity cell that is appropriate for the conductivity of the solution to be examined. The higher the expected conductivity, the higher the cell constant that must be chosen (low ρ) so that the value R measured is as large as possible for the apparatus used. Commonly used conductivity cells have cell constants of the order of 0.1 cm^{-1} , 1 cm^{-1} and 10 cm^{-1} . Use a standard solution of *potassium chloride R* that is appropriate for the measurement. Rinse the cell several times with *carbon dioxide-free water R* prepared from *distilled water R* and at least twice with the potassium chloride solution used for the determination of the cell constant of the conductivity cell. Measure the resistance of the conductivity cell using the potassium chloride solution at 20 ± 0.1 °C or at the temperature prescribed in the monograph. The constant C (in cm^{-1}) of the conductivity cell is given by the expression:

$$C = R_{\text{KCl}} \cdot \kappa_{\text{KCl}}$$

R_{KCl} = measured resistance, expressed in mega-ohms,

κ_{KCl} = conductivity of the standard solution of *potassium chloride R* used, expressed in $\mu\text{S cm}^{-1}$.

The measured constant C of the conductivity cell must be within 5 per cent of the given value.

Determination of the conductivity of the solution to be examined

After calibrating the apparatus with one of the standard solutions, rinse the conductivity cell several times with *carbon dioxide-free water R* prepared from *distilled water R* and at least twice with the aqueous solution to be examined at 20 ± 0.1 °C or at the temperature prescribed in the monograph. Carry out successive measurements as described in the monograph.

01/2005:20239

2.2.39. MOLECULAR MASS DISTRIBUTION IN DEXTRANS

Examine by size-exclusion chromatography (2.2.30).

Test solution. Dissolve 0.200 g of the substance to be examined in the mobile phase and dilute to 10 ml with the mobile phase.

Marker solution. Dissolve 5 mg of *glucose R* and 2 mg of *dextran V₀ CRS* in 1 ml of the mobile phase.

Calibration solutions. Dissolve separately in 1 ml of the mobile phase 15 mg of *dextran 4 for calibration CRS*, 15 mg of *dextran 10 for calibration CRS*, 20 mg of *dextran 40 for calibration CRS*, 20 mg of *dextran 70 for calibration CRS* and 20 mg of *dextran 250 for calibration CRS*.

System suitability solution. Dissolve either 20 mg of *dextran 40 for performance test CRS* (for dextran 40) or 20 mg of *dextran 60/70 for performance test CRS* (for dextran 60 and dextran 70) in 1 ml of the mobile phase.

The chromatographic procedure may be carried out using:

- a column 0.3 m long and 10 mm in internal diameter, packed with *cross-linked agarose for chromatography R* or a series of columns, 0.3 m long and 10 mm in internal diameter, packed with *polyether hydroxylated gel for chromatography R*,
- as the mobile phase, at a flow rate of 0.5-1 ml/min, kept constant to ± 1 per cent per hour, a solution containing 7 g of *anhydrous sodium sulphate R* and 1 g of *chlorobutanol R* in 1 litre of *water R*,

- as detector a differential refractometer,
 - a 100 µl to 200 µl loop injector,
- maintaining the system at a constant temperature (± 0.1 °C).

Calibration by calculation of the curve. Calculate from equations (2) and (3) below, using a suitable method⁽⁴⁾, values for b_1 , b_2 , b_3 , b_4 and b_5 that give values of M_w within 5 per cent of the declared values of each of the dextrans for calibration and 180 ± 2 for glucose:

$$M_i = b_5 + e^{(b_4 + b_1 K_i + b_2 K_i^2 + b_3 K_i^3)} \quad (2)$$

$$\overline{M}_w = \frac{\sum_{i=1}^p (y_i M_i)}{\sum_{i=1}^p y_i} \quad (3)$$

- p = number of sections dividing the chromatograms,
 y_i = height of the chromatographic line above the baseline in section i ,
 M_i = molecular mass in section i .

CALIBRATION OF THE CHROMATOGRAPHIC SYSTEM

Carry out replicate injections of the chosen volume of the marker solution. The chromatogram shows 2 peaks, the first of which corresponds to *dextran* V_0 CRS and the second of which corresponds to *dextrose* R. From the elution volume of the peak corresponding to dextran V_0 , calculate the void volume V_0 and from the peak corresponding to dextrose, calculate the total volume V_t .

Inject the chosen volume of each of the calibration solutions. Draw carefully the baseline of each of the chromatograms. Divide each chromatogram into p (at least 60) equal vertical sections (corresponding to equal elution volumes). In each section i , corresponding to an elution volume V_i measure the height (y_i) of the chromatogram line above the baseline and calculate the coefficient of distribution K_i using the expression:

$$\frac{(V_i - V_0)}{(V_t - V_0)} \quad (1)$$

- V_0 = void volume of the column, determined using the peak corresponding to *dextran* V_0 CRS in the chromatogram obtained with the marker solution,
 V_t = total volume of the column, determined using the peak corresponding to glucose in the chromatogram obtained with the marker solution,
 V_i = elution volume of section i in the chromatogram obtained with each of the calibration solutions.

Carry out the calibration using either of the following methods.

Calibration by plotting of the curve. For each of the dextrans for calibration calculate the coefficient of distribution $K_{\max}$ corresponding to the maximum height of the chromatographic line, using expression (1). Plot on semilogarithmic paper the values of $K_{\max}$ (on the x-axis) against the declared molecular mass at the maximum height of the chromatographic line ($M_{\max}$) of each of the dextrans for calibration and glucose. Draw a first calibration curve through the points obtained, extrapolating it from the point $K_{\max}$ obtained with *dextran* 250 for calibration CRS to the lowest K value obtained for this CRS (Figure 2.2.39.-1). Using this first calibration curve, transform, for each chromatogram, all K_i values into the corresponding molecular mass M_i , thus obtaining the molecular mass distribution. Calculate for each dextran for calibration the average molecular mass M_w using equation (3) below. If the calculated values for M_w do not differ by more than 5 per cent from those declared for each of the dextrans for calibration and the mean difference is within ± 3 per cent, the calibration curve is approved. If not, move the calibration curve along the y-axis and repeat the procedure above until the calculated and the declared values for M_w do not differ by more than 5 per cent.

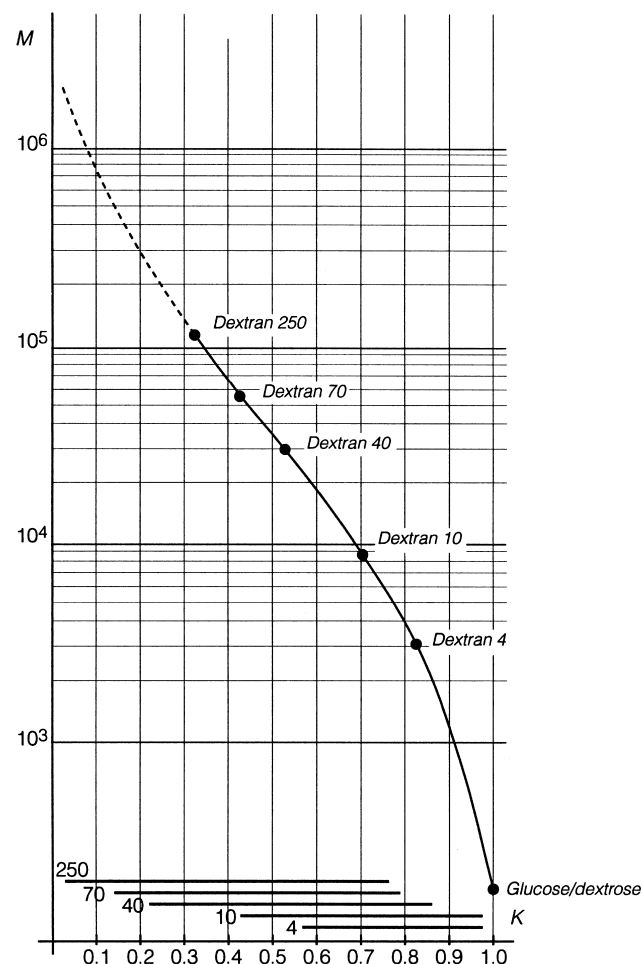


Figure 2.2.39.-1. - Example of a calibration curve.

The dotted line corresponds to the part of the curve that is extrapolated. Horizontal lines at the bottom of the figure represent the width and the position of the chromatographic line obtained with each of the dextrans for calibration.

(4) An iterative method such as the Gauss-Newton method modified by Hartley is suitable (see O. Hartley, *Tecnometrics*, 3 (1961) and G. Nilsson and K. Nilsson, *J. Chromat.* 101, 137 (1974)). A curve-fitting programme for microcomputers, capable of non-linear regression, may be used.

SYSTEM SUITABILITY

Inject the chosen volume of the appropriate system suitability solution.

Average molecular mass of dextran for performance test CRS.

Calculate the average molecular mass M_w as indicated under Calibration of the chromatographic system, using either the plotted calibration curve or the values obtained above for b_1 , b_2 , b_3 , b_4 and b_5 . The test is not valid unless M_w is:

- 41 000 to 47 000 (*dextran 40 for performance test CRS*),
- 67 000 to 75 000 (*dextran 60/70 for performance test CRS*).

Average molecular mass of the 10 per cent high-fraction dextran. Calculate M_w for the 10 per cent high-fraction dextran eluted through section n using the equation:

$$M_w = \frac{\sum_{i=1}^n (y_i M_i)}{\sum_{i=1}^n y_i} \quad (4)$$

in which n is defined by the expressions:

$$\sum_{i=1}^n y_i \leq 0.1 \left(\sum_{i=1}^p y_i \right) \quad (5)$$

$$\sum_{i=1}^{n+1} y_i > 0.1 \left(\sum_{i=1}^p y_i \right) \quad (6)$$

p = number of sections dividing the chromatograms,

y_i = height of the chromatographic line above the baseline in section i ,

M_i = molecular mass in section i .

The test is not valid unless M_w of the 10 per cent high fraction dextran is:

- 110 000 to 130 000 (*dextran 40 for performance test CRS*),
- 190 000 to 230 000 (*dextran 60/70 for performance test CRS*).

Average molecular mass of the 10 per cent low-fraction dextran.

Calculate M_w for the 10 per cent low-fraction dextran eluted in and after section m using the expression:

$$M_w = \frac{\sum_{i=m}^p (y_i M_i)}{\sum_{i=m}^p y_i} \quad (7)$$

in which m is defined by the expressions:

$$\sum_{i=m}^p y_i \leq 0.1 \left(\sum_{i=1}^p y_i \right) \quad (8)$$

$$\sum_{i=m-1}^p y_i > 0.1 \left(\sum_{i=1}^p y_i \right) \quad (9)$$

p = number of sections dividing the chromatograms,

y_i = height of the chromatographic line above the baseline in section i ,

M_i = molecular mass in section i .

The test is not valid unless M_w of the 10 per cent low-fraction dextran is:

- 6000 to 8500 (*dextran 40 for performance test CRS*),

- 7000 to 11 000 (*dextran 60/70 for performance test CRS*).

MOLECULAR MASS DISTRIBUTION OF THE DEXTRAN TO BE ANALYSED

Inject the chosen volume of the test solution and calculate M_w of the total molecular mass distribution, M_w of the 10 per cent high-fraction dextran and M_w of the 10 per cent low-fraction dextran as indicated under System suitability.

01/2005:20240

2.2.40. NEAR-INFRARED SPECTROPHOTOMETRY

Near-infrared (NIR) spectrophotometry is a technique with wide and varied applications in pharmaceutical analysis. The NIR spectral range extends from about 780 nm to about 2500 nm (from about 12 800 cm⁻¹ to about 4000 cm⁻¹). In some cases the most useful information is found in the spectral range from about 1700 nm to about 2500 nm (from about 6000 cm⁻¹ to 4000 cm⁻¹). NIR spectra are dominated by C-H, N-H, O-H and S-H overtone resonances and combinations of fundamental vibrational modes; they have a high informative character if the information is extracted by suitable chemometric algorithms. NIR bands are much weaker than the fundamental mid-IR vibrations from which they originate. Because molar absorptivities in the NIR range are low, radiation typically penetrates several millimeters into materials, including solids. Furthermore, many materials such as glass are relatively transparent in this region.

Measurements can be made directly on *in situ* samples, in addition to standard sampling and testing procedures. Physical as well as chemical information, both qualitative and quantitative, is available from NIR spectra. However, direct comparison of the spectrum obtained with the substance being examined with a reference spectrum of a chemical reference substance, as used in infrared absorption spectrophotometry, is not appropriate. Suitable validated mathematical treatment of the data is required.

NIR spectrophotometry has a wide variety of applications for both chemical and physical analysis, for example:

chemical analysis

- identification of active substances, excipients, dosage forms, manufacturing intermediates, chemical raw materials and packaging materials,
- quantification of active substances and excipients, determination of chemical values such as hydroxyl value, iodine value, acid value, determination of water content, determination of degree of hydroxylation, control of solvent content,
- process control.

physical analysis

- crystalline form and crystallinity, polymorphism, pseudopolymorphism, particle size,
- dissolution behaviour, disintegration pattern, hardness,
- examination of film properties,
- process control, for example monitoring of blending and granulation.

Measurements in the NIR region are influenced by many chemical and physical factors as described below; reproducibility and relevance of results depend on control of these factors and measurements are usually valid only for a defined calibration model.

APPARATUS

NIR spectrophotometers are used for recording spectra in the region of about 780 nm to about 2500 nm (about $12\,800\text{ cm}^{-1}$ to about 4000 cm^{-1}). All NIR measurements are based on passing light through or into a sample and measuring the attenuation of the emerging (transmitted, scattered or reflected) beam. Spectrophotometers for measurement in the NIR region consist of a suitable light source, a monochromator or interferometer. Common monochromators are acousto-optical tuneable filters (AOTF), gratings or prisms. High intensity light sources such as quartz or tungsten lamps or similar are used. The tungsten lamp light source can be highly stabilised. Therefore many NIR instruments have the single-beam design. Silicon, lead sulphide, indium arsenide, indium gallium arsenide, mercury cadmium telluride (MCT) and deuterated triglycine sulphate are commonly used detector materials. Conventional cuvette sample holders, fibre-optic probes, transmission dip cells and spinning or traversing sample holders are a few common sampling devices. The selection is based on the intended application, paying particular attention to the suitability of the sampling system for the type of sample to be analysed. Suitable data processing and evaluation units are usually part of the system.

MEASUREMENT METHODS

Transmission mode. Transmittance (T) is a measure of the decrease in radiation intensity at given wavelengths when radiation is passed through the sample. The sample is placed in the optical beam between the source and detector. The arrangement is analogous to that in many conventional spectrophotometers and the result can be presented directly in terms of transmittance (T) or/and absorbance (A).

$$T = \frac{I}{I_0},$$

I_0 = intensity of incident radiation,

I = intensity of transmitted radiation,

$$A = -\log_{10} T = \log_{10} \left(\frac{1}{T} \right) = \log_{10} \left(\frac{I_0}{I} \right).$$

Diffuse reflection mode. The diffuse reflection mode gives a measure of reflectance (R), the ratio of the intensity of light reflected from the sample (I) to that reflected from a background or reference reflective surface (I_r). NIR radiation can penetrate a substantial distance into the sample, where it can be absorbed by vibrational combinations and overtone resonances of the analyte species present in the sample. Non-absorbed radiation is reflected back from the sample to the detector. NIR reflectance spectra are typically obtained by calculating and plotting $\log(1/R)$ versus the wavelength or wavenumbers.

$$R = \frac{I}{I_r},$$

I = intensity of light diffusively reflected from the sample,

I_r = intensity of light reflected from the background or reference reflective surface,

$$A_R = \log_{10} \left(\frac{1}{R} \right) = \log_{10} \left(\frac{I_r}{I} \right).$$

Transflection mode. This mode is a combination of transmittance and reflectance. In the measurement of transfectance (T^*) a mirror or a diffuse reflectance surface

is used to reflect the radiation transmitted through the sample a second time and thus doubling the pathlength. Non-absorbed radiation is reflected back from the sample to the detector.

$$T^* = \frac{I}{I_T},$$

I_T = intensity of transflected radiation, without sample

I = intensity of transmitted and reflected radiation measured with the sample,

$$A^* = \log_{10} \left(\frac{1}{T^*} \right).$$

SAMPLE PREPARATION/PRESENTATION

Transmission mode. The measurement of transmittance (T) is dependent on a background transmittance spectrum for its calculation. A background reference can be air, an empty cell, and a solvent blank or in special cases a reference sample. The method generally applies to liquids, diluted or undiluted, dispersions, solutions and solids. For transmittance measurements of solids, a suitable sample accessory is to be used. The samples are examined in a cell of suitable pathlength (generally 0.5-4 mm), transparent to NIR radiation, or by immersion of a fibre optic probe of a suitable configuration, which yields a spectrum situated in a zone of transmission compatible with the specifications of the apparatus and appropriate for the intended purpose.

Diffuse reflection mode. This method generally applies to solids. The sample is examined in a suitable device. Care must be taken to make the measuring conditions as reproducible as possible from one sample to another. When immersing a fibre optic probe in the sample, care must be taken in the positioning of the probe to ensure that it remains stationary during the acquisition of the spectra and that the measuring conditions are as reproducible as possible from one sample to another. The reflected radiation of a background reference is scanned to obtain the baseline, and then the reflectance of one or more analytical samples is measured. Common reflectance references are ceramic tiles, perfluorinated polymers and gold. Other suitable materials may be used. Only spectra measured against a background possessing the same optical properties can be directly compared with one another. The particle size, water of hydration and state of solvation must be taken into consideration.

Transflection mode. A reflector is placed behind the sample so as to double the pathlength. This configuration can be adopted to share the same instrument geometry with reflectance and fibre optic probe systems where the source and the detector are on the same side of the sample. The sample is examined in a cell with a mirror or a suitable diffusive reflector, made either of metal or of an inert substance (for example titanium dioxide) not absorbing in the NIR region.

FACTORS AFFECTING SPECTRAL RESPONSE

Sample temperature. This parameter is important for aqueous solutions and many liquids, where a difference of a few degrees can result in substantial spectral changes. Temperature is also an important parameter for solids and powders containing water.

Moisture and solvent residues. Moisture and solvent residues present in the samples will contribute significant absorption bands in the NIR region.

Sample thickness. Sample thickness is a known source of spectral variability and must be understood and/or controlled. For example, in a reflection measurement the sample may be “infinitely” thick, or thinner samples of constant thickness must have a stable, diffusively reflecting backing material of constant, and preferably high reflectivity.

Sample optical properties. In solids, both surface and bulk scattering properties of samples must be taken into account. Spectra of physically, chemically or optically heterogeneous samples may require sample averaging by increasing the beam size or examining multiple samples or spinning the probe. Certain factors such as differing degree of compaction or particle size in powdered materials and surface finish can cause significant spectral differences.

Polymorphism. The variations in crystalline structure (polymorphism) influence the spectra. Hence different crystalline forms as well as the amorphous form of a solid may be distinguished from one another on the basis of their NIR spectra. Where multiple crystalline forms are present, care must be taken to ensure that the calibration standards have a distribution of forms relevant to the intended application.

Age of samples. Samples may exhibit changes in their chemical, physical or optical properties over time. Care must be taken to ensure that samples for NIR analysis are representative of those used for calibration. If samples of different age are to be analysed, potential differences in the properties must be accounted for.

CONTROL OF INSTRUMENT PERFORMANCE

Use the apparatus according to the manufacturer's instructions and carry out the prescribed verification at regular intervals, according to the use of the apparatus and the substances to be tested.

Verification of the wavelength scale (except for filter apparatus). Verify the wavelength scale employed, generally in the region between about 780 nm and about 2500 nm (about 12 800 cm⁻¹ to about 4000 cm⁻¹) or in the intended spectral range using one or more suitable wavelength standards which have characteristic maxima or minima within the range of wavelengths to be used. For example, methylene chloride or a mixture of rare-earth oxides are suitable reference materials. Take one spectrum with the same spectral resolution used to obtain the certified value, and measure the position of at least 3 peaks distributed over the range used. Acceptable tolerances are ± 1 nm at 1200 nm, ± 1 nm at 1600 nm and ± 1.5 nm at 2000 nm (± 8 cm⁻¹ at 8300 cm⁻¹, ± 4 cm⁻¹ at 6250 cm⁻¹ and ± 4 cm⁻¹ at 5000 cm⁻¹). For the reference material used, apply the tolerance for the nearest wavelength (wavenumber) from the above for each peak used. For FT instruments, the calibration of the wavenumber scale may be performed using a narrow water-vapour line at 7299.86 cm⁻¹ or a narrow line from a certified material. For rare-earth oxides, NIST 1920 (a) is the most appropriate reference.

Measurement in transmission mode. *Methylene chloride R* may be used at an optical pathlength of 1.0 mm. Methylene chloride has characteristic sharp bands at 1155 nm, 1366 nm, 1417 nm, 1690 nm, 1838 nm, 1894 nm, 2068 nm and 2245 nm. The bands at 1155 nm, 1417 nm, 1690 nm and 2245 nm are used for calibration. Other suitable standards may also be used.

Measurement in diffuse reflection (reflectance) mode. A mixture of dysprosium, holmium and erbium oxides (1+1+1 by mass) or other certified material may be used. This reference material exhibits characteristic peaks at 1261 nm, 1681 nm and 1935 nm. If it is not possible to use external

solid standards and if measurements of diffuse reflection are carried out in cells or if fibre optic probes are used, a suspension of 1.2 g of *titanium dioxide R* in about 4 ml of *methylene chloride R*, vigorously shaken, is used directly in the cell or probe. The spectrum is recorded after 2 min. Titanium dioxide has no absorption in the NIR range. Spectra are recorded with a maximum nominal instrument bandwidth of 10 nm at 2500 nm (16 cm⁻¹ at 4000 cm⁻¹). Measurement is made of the position of at least 3 peaks distributed over the range used. The acceptance tolerances are given under Verification of the wavelength scale. For the reference material used, apply the tolerance for the nearest wavelength (wavenumber) for each peak used.

Verification of the wavelength repeatability (except for filter apparatus). Verify the wavelength repeatability using suitable standards. The standard deviation of the wavelength is consistent with the specifications of the instrument manufacturer.

Verification of photometric linearity and response stability. Verification of photometric linearity is demonstrated with a set of transmission or reflection standards with known values of transmittance or reflectance in percentage. For reflectance measurements, carbon-doped polymer standards are available. At least 4 reference standards in the range of 10-90 per cent such as 10 per cent, 20 per cent, 40 per cent and 80 per cent with respective absorbance values of 1.0, 0.7, 0.4 and 0.1 are used. If the system is used for analytes with absorbances higher than 1.0, a 2 per cent and/or 5 per cent standard is added to the set. Plot the observed absorbance values against the reference absorbance values and perform a linear regression. Acceptable tolerances are 1.00 ± 0.05 for the slope and 0.00 ± 0.05 for the intercept.

Spectra obtained from reflectance standards are subject to variability due to the difference between the experimental conditions under which they were factory-calibrated and those under which they are subsequently put to use. Hence, the percentage reflectance values supplied with a set of calibration standards may not be useful in the attempt to establish an “absolute” calibration for a given instrument. But as long as the standards do not change chemically or physically and the same reference background is used as was used to obtain the certified values, subsequent measurements of the same standards under identical conditions including precise sample positioning give information on long-term stability of the photometric response. A tolerance of ± 2 per cent is acceptable for long-term stability; this is only necessary if spectra are used without pre-treatment.

Verification of photometric noise. Determine the photometric noise using a suitable reflectance standard, for example white reflective ceramic tiles or reflective thermoplastic resins (for example, PTFE). Scan the reflection standard over a suitable wavelength/wavenumber range in accordance with the manufacturer's recommendation and calculate the photometric noise as peak-to-peak noise. The value is approximately twice the standard deviation. The photometric noise is consistent with the specification of the spectrophotometer.

IDENTIFICATION AND CHARACTERISATION (QUALITATIVE ANALYSIS)

Establishment of a spectral reference library. Record the spectra of a suitable number of batches of the substance which have been fully tested according to established specifications and which exhibit the variation typical for the substance to be analysed (for example, manufacturer, physical form, particle size). The set of spectra represents the information for identification and characterisation that defines the similarity border for that substance and is the

entry for that substance in the spectral library used to identify the substance. The number of substances in the library depends on the specific application, but libraries that are too big can cause some difficulties in discriminating between different materials and in validation. All spectra in the library used have the same:

- spectral range and number of data points,
- technique of measurement,
- data pre-treatment.

If sub-groups (libraries) are created, the above criteria are applied independently for each group. The collection of spectra in the library may be represented in different ways defined by the mathematical technique used for identification. These may be:

- all individual spectra representing the substance,
- a mean spectrum of each batch of substance,
- if necessary, a description of the variability within the substance spectra.

Electronic raw data for the preparation of the spectral library must be archived.

Pre-treatment of data. In many cases, and particularly for reflection mode spectra, some form of mathematical pretreatment of the spectrum may be useful before the development of a classification or calibration model. The aim can be, for example, to reduce baseline variations, to reduce the impact of known variations that are interfering in the subsequent mathematical models, or to compress data before use. Typical methods are multiplicative scatter correction (MSC), the Kubelka-Munk transforms, spectral compression techniques that may include windowing and noise reduction and the numerical calculation of the first- or second-order derivative of the spectrum. Higher-order derivatives are not recommended. In some cases spectra may also be normalised, for example against the maximum absorbance, the mean absorbance or the integrated absorbance area under the spectrum.

Caution must be exercised when performing any mathematical transformation, as artefacts can be introduced or essential information (important with qualification methods) can be lost. An understanding of the algorithm is required and in all cases the rationale for the use of transform must be documented.

Data evaluation. Direct comparison of the spectrum of the substance under investigation is made with the individual or mean reference spectra of all substances in the database on the basis of their mathematical correlation or other suitable algorithms. A set of known reference mean spectra and the variability around this mean can be used with an algorithm for classification. There are different algorithms based on principal component analysis (PCA) combined with cluster analysis, SIMCA (soft independent modelling by class analogy), COMPARE functions using filters or UNEQ (unequal dispersed class) and others used in the software of NIR instruments or supplied as third-party software. The reliability of the algorithm chosen for a particular application has to be validated. For example, correlation coefficient, the sum of squared residuals or the distance using cluster analysis must comply with the acceptance limits defined in the validation procedure.

Validation of the database

Specificity. The selectivity of the classification using database spectra for positive identification of a given material and adequate discrimination against other materials in the database is to be established during the validation procedure. Acceptance thresholds are established. High

thresholds achieve a higher discriminatory power, but may cause some errors due to the own variability of materials. Lower thresholds solve these problems, but could produce ambiguous results. Potential challenges must be addressed to the spectral database. These can be materials received on site that are similar to database members in visual appearance, chemical structure or by name. This challenge must fail identification. Independent samples of materials represented in the database, but not used to create it (i.e. different batches, blends) must give positive identification when analysed.

Robustness. The robustness of the qualitative procedure must also be challenged to test the effect of minor changes to normal operating conditions on the analysis. There must be no changes to pre-processing and calibration algorithm parameters. Typical challenges are:

- effect of differences across operators on variations in environmental conditions (for example, temperature and humidity in the laboratory),
- effect of sample temperature, sample positioning on the optical window and probe depth and compression/packing of material,
- replacement of instrument parts or sampling presentation devices.

QUANTITATIVE ANALYSIS

Establishment of a spectral reference library for a calibration model. Calibration is the process of constructing a mathematical model to relate the response from an analytical instrument to the properties of the samples. Any calibration algorithm that can be clearly defined in an exact mathematical expression and gives suitable results can be used. Record spectra of a suitable number of samples with known values of the content throughout the range to be measured (for example, content of water). Wavelengths used in the calibration model can be compared to the known bands of the analyte and those of the matrix to verify that the bands of the analyte of interest are being used by the calibration. Establish the calibration model with about two-thirds of the measured samples. Compare the remaining one-third of the measured samples with the database. All samples must give quantitative results within a precision interval as defined by the intended purpose of the method. Correct quantification must be demonstrated in the presence of variations in the matrix within the specified range. Multiple linear regression (MLR), partial least squares (PLS) and principal component regression (PCR) are commonly used. For PLS or PCR calibrations, the coefficients or the loadings can be plotted and the regions of large coefficients compared with the spectrum of the analyte. Raw data for the preparation of the calibration model must be archived, without data pretreatment.

Pre-treatment of data. Data pre-treatment can be defined as the mathematical transformation of the NIR spectral data to enhance spectral features and/or remove or reduce unwanted sources of variation prior to the development of the calibration model. Many suitable algorithms for data pre-treatment and calibration exist. The selection is based on the suitability for the intended use. Wavelength selection may enhance the efficiency of calibration models such as MLR (for example, in particle-size determination). It is useful to delete certain ranges of the wavelength scale in some cases, for example in the determination of water of hydration. Wavelength compression may be applied to the data.

Validation parameters. Analytical performance characteristics to be considered for demonstrating the validation of NIR methods are similar to those required

for any analytical procedure. Specific acceptance criteria for each validation parameter must be consistent with the intended use of the method.

Specificity. The relative discriminatory power and selectivity for quantitative determination must be similar to those mentioned under Qualitative analysis. The extent of specificity testing is dependent on the application and the risks being controlled. Variations in matrix concentrations within the operating range of the method must not affect the quantitative measurement significantly.

Linearity. The validation of linearity involves the correlation of NIR results calculated from NIR responses within the used algorithms to reference method results distributed throughout the defined range of the calibration model. Actual NIR responses that are non-linear may still be valid.

Range. The range of analyte reference values defines the range of the NIR method and quantitation limits of the method. Controls must be in place to ensure that results outside the validated range are not accepted.

Accuracy. This can be determined by comparison with the validation method or with known samples (samples of blank and added amounts of tested substance). Accuracy can be indicated by the standard error of prediction (SEP) of the NIR method that should be in close agreement with the data of the validated method. The SEP is the standard deviation of the residuals obtained from comparing the NIR results with analytical reference data for the specified samples. It is demonstrated by correlation of NIR results with analytical reference data, by comparison of the SEP to the reference method used for validation. Alternatively statistical comparison methods may be used to compare NIR results with reference values (paired *t*-test, bias evaluation).

Precision. This expresses the closeness of agreement between a series of measurements under the prescribed conditions. It is assessed by a minimum of 6 measurements performed according to the developed analytical method. Precision may be considered at 2 levels, repeatability (replicate measurements of the same sample with or without variation in sample positioning) and intermediate precision (replicate measurements by different analysts, different days of measurements).

Robustness. This includes the effects of variations of temperature, humidity, sample handling and the influence of instrument changes.

Outliers. Outlier results from NIR measurements of a sample containing an analyte outside the calibration range indicates that further testing is required. If further testing of the sample by an appropriate analytical method gives the analyte content within the specifications, this may be accepted and considered to have met the specifications. Thus an outlier result generated by NIR measurements of the sample may still meet specifications for the analyte of interest.

ONGOING MODEL EVALUATION

NIR models validated for use are subjected to ongoing performance evaluation and monitoring of validation parameters. If discrepancies are found, corrective action will be necessary. The degree of revalidation required depends on the nature of the changes. Revalidation of a qualitative model will be necessary when a new material is added to the reference library and may be necessary when changes in the physical properties of the material occur and when changes in the source of supply take place. Revalidation of a quantitative model is required on account of changes in the composition of the finished product, in the manufacturing process and in sources/grades of raw materials.

TRANSFER OF DATABASES

When databases are transferred to another instrument, spectral range, number of data points, spectral resolution and other parameters have to be taken into consideration. Further procedures and criteria must be applied to demonstrate that the model remains valid with the new database or new instrument.

DATA STORAGE

Store the electronic NIR spectra, libraries and data according to the current regulations.

Store the NIR spectra with the necessary data pre-treatment for the special use (for example identification, particle size analysis, content of water etc.) according to the current specifications.

01/2005:20241

2.2.41. CIRCULAR DICHROISM

The difference in absorbance of optically active substances within an absorption band for left and right circularly polarised light is referred to as circular dichroism.

Direct measurement gives a mean algebraic value:

$$\Delta A = A_L - A_R$$

ΔA = circular dichroic absorbance,

A_L = absorbance of left circularly polarised light,

A_R = absorbance of right circularly polarised light.

Circular dichroism is calculated using the equation:

$$\Delta \varepsilon = \varepsilon_L - \varepsilon_R = \frac{\Delta A}{c \times l}$$

$\Delta \varepsilon$ = molar circular dichroism or molar differential dichroic absorptivity expressed in litre-mole⁻¹cm⁻¹,

ε_L = molar absorptivity (2.2.25) of left circularly polarised light,

ε_R = molar absorptivity of right circularly polarised light,

c = concentration of the test solution in mole-litre⁻¹,

l = optical path of the cell in centimetres.

The following units may also be used to characterise circular dichroism:

Dissymmetry factor:

$$g = \frac{\Delta \varepsilon}{\varepsilon}$$

ε = molar absorptivity (2.2.25).

Molar ellipticity:

Certain types of instruments display directly the value of ellipticity Θ , expressed in degrees. When such instruments are used, the molar ellipticity $[\Theta]$ may be calculated using the following equation:

$$[\Theta] = \frac{\Theta \times M}{c \times l \times 10}$$

$[\Theta]$ = molar ellipticity, expressed in degrees-cm²decimole⁻¹,

Θ = value of ellipticity given by the instrument,

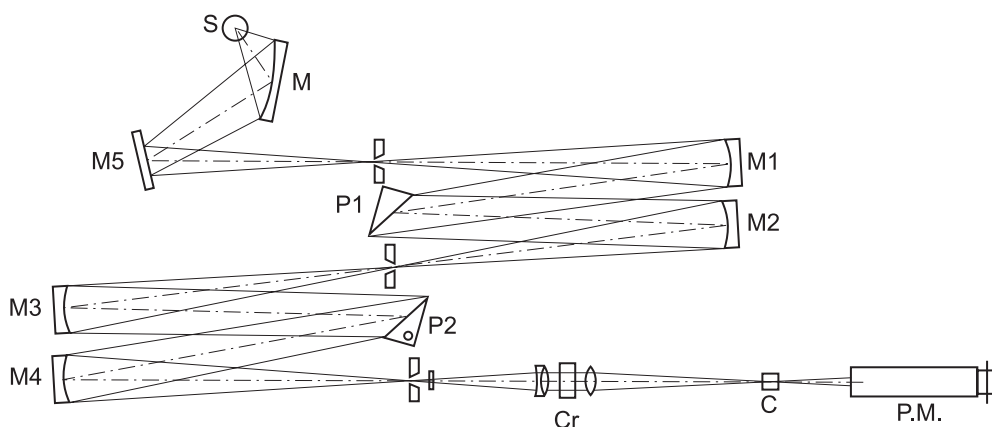


Figure 2.2.41.-1. – Optical scheme of a dichrograph

- M = relative molecular mass of the substance to be examined,
 c = concentration of the solution to be examined in g/ml,
 l = optical path of the cell in centimetres.

Molar ellipticity is also related to molar circular dichroism by the following equation:

$$[\Theta] = 2.303\Delta\epsilon \frac{4500}{\pi} \approx 3300\Delta\epsilon$$

Molar ellipticity is often used in the analysis of proteins and nucleic acids. In this case, molar concentration is expressed in terms of monomeric residue, calculated using the expression:

$$\frac{\text{molecular mass}}{\text{number of amino acids}}$$

The mean relative molecular mass of the monomeric residue is 100 to 120 (generally 115) for proteins and about 330 for nucleic acids (as the sodium salt).

Apparatus. The light source (S) is a xenon lamp (Figure 2.2.41.-1); the light passes through a double monochromator (M) equipped with quartz prisms (P1, P2).

The linear beam from the first monochromator is split into 2 components polarised at right angles in the second monochromator. The exit slit of the monochromator eliminates the extraordinary beam.

The polarised and monochromatic light passes through a birefringent modulator (Cr): the result is alternating circularly polarised light.

The beam then passes through the sample to be examined (C) and reaches a photomultiplier (PM) followed by an amplifier circuit which produces 2 electrical signals: one is a direct current V_c and the other is an alternating current at the modulation frequency V_{ac} characteristic of the sample to be examined. The phase gives the sign of the circular dichroism. The ratio V_{ac}/V_c is proportional to the differential absorption ΔA which created the signal. The region of wavelengths normally covered by a dichrograph is 170 nm to 800 nm.

Calibration of the apparatus

Accuracy of absorbance scale. Dissolve 10.0 mg of *isoandrosterone R* in *dioxan R* and dilute to 10.0 ml with the same solvent. Record the circular dichroism spectrum of the solution between 280 nm and 360 nm. Measured at the maximum at 304 nm, $\Delta\epsilon$ is + 3.3.

The solution of *(1S)-(+)-10-camphorsulphonic acid R* may also be used.

Linearity of modulation. Dissolve 10.0 mg of *(1S)-(+)-10-camphorsulphonic acid R* in *water R* and dilute to 10.0 ml with the same solvent. Determine the exact concentration of camphorsulphonic acid in the solution by ultraviolet spectrophotometry (2.2.25), taking the specific absorbance to be 1.49 at 285 nm.

Record the circular dichroism spectrum between 185 nm and 340 nm. Measured at the maximum at 290.5 nm, $\Delta\epsilon$ is + 2.2 to + 2.5. Measured at the maximum at 192.5 nm, $\Delta\epsilon$ is - 4.3 to - 5.

(1S)-(+)- or antipodal *(1R)-(-)-ammonium 10-camphorsulphonate R* can also be used.

01/2005:20242

2.2.42. DENSITY OF SOLIDS

The density of solids corresponds to their average mass per unit volume and typically is expressed in grams per cubic centimetre (g/cm³) although the International Unit is the kilogram per cubic meter (1 g/cm³ = 1000 kg/m³).

Unlike gases and liquids whose density depends only on temperature and pressure, the density of a solid particle also depends on its molecular assembly and therefore varies with the crystal structure and degree of crystallinity.

When a solid particle is amorphous or partially amorphous, its density may further depend upon the history of preparation and treatment.

Therefore, unlike fluids, the densities of two chemically equivalent solids may be different, and this difference reflects a difference in solid-state structure. The density of constituent particles is an important physical characteristic of pharmaceutical powders.

The density of a solid particle can assume different values depending on the method used to measure the volume of the particle. It is useful to distinguish three levels of expression of density:

- the *crystal density* which only includes the solid fraction of the material; the crystal density is also called *true density*;
- the *particle density* which also includes the volume due to intraparticulate pores,
- the *bulk density* which further includes the interparticulate void volume formed in the powder bed; the bulk density is also called *apparent density*.

CRYSTAL DENSITY

The crystal density of a substance is the average mass per unit volume, exclusive of all voids that are not a fundamental part of the molecular packing arrangement. It is an intrinsic property of the substance, and hence should

be independent of the method of determination. The crystal density can be determined either by calculation or by simple measurement.

- A. The *calculated crystal density* is obtained using crystallographic data (size and composition of the unit cell) of a perfect crystal, from for example X-ray diffraction data, and the molecular mass of the substance.
- B. The *measured crystal density* is the mass to volume ratio after measuring the monocystal mass and volume.

PARTICLE DENSITY

The particle density takes into account both the crystal density and the intraparticulate porosity (sealed and/or open pores). Thus, particle density depends on the value of the volume determined which in turn depends on the method of measurement. The particle density can be determined using one of the two following methods.

- A. The *pycnometric density* is determined by measuring the volume occupied by a known mass of powder which is equivalent to the volume of gas displaced by the powder using a gas displacement pycnometer (2.9.23). In pycnometric density measurements, the volume determined includes the volume occupied by open pores; however, it excludes the volume occupied by sealed pores or pores inaccessible to the gas. Due to the high diffusivity of helium, which is the preferred choice of gas, most open pores are accessible to the gas. Therefore, the pycnometric density of a finely milled powder is generally not very different from the crystal density.
- B. The *mercury porosimeter density* is also called *granular density*. With this method the volume determined also excludes contributions from sealed pores; however, it includes the volume only from open pores larger than some size limit. This pore size limit or minimal access diameter depends on the maximal mercury intrusion pressure applied during the measurement and under normal operating pressures the mercury does not penetrate the finest pores accessible to helium. Various granular densities can be obtained from one sample since, for each applied mercury intrusion pressure, a density can be determined that corresponds to the pore size limit at that pressure.

BULK AND TAPPED DENSITY

The bulk density of a powder includes the contribution of interparticulate void volume. Hence, the bulk density depends on both the density of powder particles and the space arrangement of particles in the powder bed.

The bulk density of a powder is often very difficult to measure since the slightest disturbance of the bed may result in a new density. Thus, it is essential in reporting bulk density to specify how the determination was made.

- A. The *bulk density* is determined by measuring the volume of a known mass of powder, that has been passed through a screen, into a graduated cylinder (2.9.15).
- B. The *tapped density* is achieved by mechanically tapping a measuring cylinder containing a powder sample. After observing the initial volume, the cylinder is mechanically tapped, and volume readings are taken until little further volume change is observed (2.9.15).

01/2005:20243

2.2.43. MASS SPECTROMETRY

Mass spectrometry is based on the direct measurement of the ratio of the mass to the number of positive or negative elementary charges of ions (m/z) in the gas phase obtained

from the substance to be analysed. This ratio is expressed in atomic mass units (1 a.m.u. = one twelfth the mass of ^{12}C) or in daltons (1 Da = the mass of the hydrogen atom).

The ions, produced in the ion *source* of the apparatus, are accelerated and then separated by the *analyser* before reaching the *detector*. All of these operations take place in a chamber where a pumping system maintains a vacuum of 10^{-3} to 10^{-6} Pa.

The resulting spectrum shows the relative abundance of the various ionic species present as a function of m/z . The signal corresponding to an ion will be represented by several peaks corresponding to the statistical distribution of the various isotopes of that ion. This pattern is called the *isotopic profile* and (at least for small molecules) the peak representing the most abundant isotopes for each atom is called the *monoisotopic peak*.

Information obtained in mass spectrometry is essentially qualitative (determination of the molecular mass, information on the structure from the fragments observed) or quantitative (using internal or external standards) with limits of detection ranging from the picomole to the femtomole.

INTRODUCTION OF THE SAMPLE

The very first step of an analysis is the introduction of the sample into the apparatus without overly disturbing the vacuum. In a common method, called *direct liquid introduction*, the sample is placed on the end of a cylindrical rod (in a quartz crucible, on a filament or on a metal surface). This rod is introduced into the spectrometer after passing through a vacuum lock where a primary intermediate vacuum is maintained between atmospheric pressure and the secondary vacuum of the apparatus.

Other introduction systems allow the components of a mixture to be analysed as they are separated by an appropriate apparatus connected to the mass spectrometer.

Gas chromatography/mass spectrometry. The use of suitable columns (capillary or semi-capillary) allows the end of the column to be introduced directly into the source of the apparatus without using a separator.

Liquid chromatography/mass spectrometry. This combination is particularly useful for the analysis of polar compounds, which are insufficiently volatile or too heat-labile to be analysed by gas chromatography coupled with mass spectrometry. This method is complicated by the difficulty of obtaining ions in the gas phase from a liquid phase, which requires very special interfaces such as:

- *direct liquid introduction*: the mobile phase is nebulised, and the solvent is evaporated in front of the ion source of the apparatus,
- *particle-beam interface*: the mobile phase, which may flow at a rate of up to 0.6 ml/min, is nebulised in a desolvation chamber such that only the analytes, in neutral form, reach the ion source of the apparatus; this technique is used for compounds of relatively low polarity with molecular masses of less than 1000 Da,
- *moving-belt interface*: the mobile phase, which may flow at a rate of up to 1 ml/min, is applied to the surface of a moving belt; after the solvent evaporates, the components to be analysed are successively carried to the ion source of the apparatus where they are ionised; this technique is rather poorly suited to very polar or heat-labile compounds.

Other types of coupling (electrospray, thermospray, atmospheric-pressure chemical ionisation) are considered to be ionisation techniques in their own right and are described in the section on modes of ionisation.

Supercritical fluid chromatography/mass spectrometry.

The mobile phase, usually consisting of supercritical carbon dioxide enters the gas state after passing a heated restrictor between the column and the ion source.

Capillary electrophoresis/mass spectrometry. The eluent is introduced into the ion source, in some cases after adding another solvent so that flow rates of the order of a few microlitres per minute can be attained. This technique is limited by the small quantities of sample introduced and the need to use volatile buffers.

MODES OF IONISATION

Electron impact. The sample, in the gas state, is ionised by a beam of electrons whose energy (usually 70 eV) is greater than the ionisation energy of the sample. In addition to the molecular ion M^+ , fragments characteristic of the molecular structure are observed. This technique is limited mainly by the need to vaporise the sample. This makes it unsuited to polar, heat-labile or high molecular mass compounds. Electron impact is compatible with the coupling of gas chromatography to mass spectrometry and sometimes with the use of liquid chromatography.

Chemical ionisation. This type of ionisation involves a reagent gas such as methane, ammonia, nitrogen oxide, nitrogen dioxide or oxygen. The spectrum is characterised by ions of the $(M + H)^+$ or $(M - H)^-$ types, or adduct ions formed from the analyte and the gas used. Fewer fragments are produced than with electron impact. A variant of this technique is used when the substance is heat-labile: the sample, applied to a filament, is very rapidly vaporised by the Joule-Thomson effect (desorption chemical ionisation).

Fast-atom bombardment (FAB) or fast-ion bombardment ionisation (liquid secondary-ion mass spectrometry LSIMS).

The sample, dissolved in a viscous matrix such as glycerol, is applied to a metal surface and ionised by a beam of neutral atoms such as argon or xenon or high-kinetic-energy caesium ions. Ions of the $(M + H)^+$ or $(M - H)^-$ types or adduct ions formed from the matrix or the sample are produced. This type of ionisation, well suited to polar and heat-labile compounds, allows molecular masses of up to 10 000 Da to be obtained. The technique can be combined with liquid chromatography by adding 1 per cent to 2 per cent of glycerol to the mobile phase; however, the flow rates must be very low (a few microlitres per minute). These ionisation techniques also allow thin-layer chromatography plates to be analysed by applying a thin layer of matrix to the surface of these plates.

Field desorption and field ionisation. The sample is vaporised near a tungsten filament covered with microneedles (*field ionisation*) or applied to this filament (*field desorption*). A voltage of about 10 kV, applied between this filament and a counter-electrode, ionises the sample. These two techniques mainly produce molecular ions M^+ , and $(M + H)^+$ ions and are used for low polarity and/or heat-labile compounds.

Matrix-assisted laser desorption ionisation (MALDI).

The sample, in a suitable matrix and deposited on a metal support, is ionised by a pulsed laser beam whose wavelength may range from UV to IR (impulses lasting from a picosecond to a few nanoseconds). This mode of ionisation plays an essential role in the analysis of very high molecular mass compounds (more than 100 000 Da) but is limited to time-of-flight analysers (see below).

Electrospray. This mode of ionisation is carried out at atmospheric pressure. The samples, in solution, are introduced into the source through a capillary tube, the end of which has a potential of the order of 5 kV. A gas can be

used to facilitate nebulisation. Desolvation of the resulting microdroplets produces singly or multiply charged ions in the gas phase. The flow rates vary from a few microlitres per minute to 1 ml/min. This technique is suited to polar compounds and to the investigation of biomolecules with molecular masses of up to 100 000 Da. It can be coupled to liquid chromatography or capillary electrophoresis.

Atmospheric-pressure chemical ionisation (APCI).

Ionisation is carried out at atmospheric pressure by the action of an electrode maintained at a potential of several kilovolts and placed in the path of the mobile phase, which is nebulised both by thermal effects and by the use of a stream of nitrogen. The resulting ions carry a single charge and are of the $(M + H)^+$ type in the positive mode and of the $(M - H)^-$ type in the negative mode. The high flow rates that can be used with this mode of ionisation (up to 2 ml/min) make this an ideal technique for coupling to liquid chromatography.

Thermospray. The sample, in the mobile phase consisting of water and organic modifiers and containing a volatile electrolyte (generally ammonium acetate) is introduced in nebulised form after having passed through a metal capillary tube at controlled temperature. Acceptable flow rates are of the order of 1 ml/min to 2 ml/min. The ions of the electrolyte ionise the compounds to be analysed. This ionisation process may be replaced or enhanced by an electrical discharge of about 800 volts, notably when the solvents are entirely organic. This technique is compatible with the use of liquid chromatography coupled with mass spectrometry.

ANALYSERS

Differences in the performance of analysers depend mainly on two parameters:

- the range over which m/z ratios can be measured, ie, the *mass range*,
- their *resolving power* characterised by the ability to separate two ions of equal intensity with m/z ratios differing by ΔM , and whose overlap is expressed as a given percentage of valley definition; for example, a resolving power ($M/\Delta M$) of 1000 with 10 per cent valley definition allows the separation of m/z ratios of 1000 and 1001 with the intensity returning to 10 per cent above baseline. However, the resolving power may in some cases (time-of-flight analysers, quadrupoles, ion-trap analysers) be defined as the ratio between the molecular mass and peak width at half height (50 per cent valley definition).

Magnetic and electrostatic analysers. The ions produced in the ion source are accelerated by a voltage V , and focused towards a magnetic analyser (magnetic field B) or an electrostatic analyser (electrostatic field E), depending on the configuration of the instrument. They follow a trajectory of radius r according to Laplace's law:

$$\frac{m}{z} = \frac{B^2 r^2}{2V}$$

Two types of scans can be used to collect and measure the various ions produced by the ion source: a scan of B holding V fixed or a scan of V with constant B . The magnetic analyser is usually followed by an electric sector that acts as a kinetic energy filter and allows the resolving power of the instrument to be increased appreciably. The maximum resolving power of such an instrument (double sector) ranges from 10 000 to 150 000 and in most cases allows the value of m/z ratios to be calculated accurately enough to determine the elemental composition of the corresponding ions. For monocharged ions, the mass range is from 2000 Da to 15 000 Da. Some ions may decompose spontaneously (metastable transitions) or by colliding with a gas (collision-activated dissociation

(CAD)) in field-free regions between the ion source and the detector. Examination of these decompositions is very useful for the determination of the structure as well as the characterisation of a specific compound in a mixture and involves tandem mass spectrometry. There are many such techniques depending on the region where these decompositions occur:

- *daughter-ion mode* (determination of the decomposition ions of a given parent ion): $B/E = \text{constant}$, *MIKES* (*Mass-analysed Ion Kinetic Energy Spectroscopy*),
- *parent-ion mode* (determination of all ions which by decomposition give an ion with a specific m/z ratio): $B^2/E = \text{constant}$,
- *neutral-loss mode* (detection of all the ions that lose the same fragment):
 $B/E(1 - E/E_0)^{1/2} = \text{constant}$, where E_0 is the basic voltage of the electric sector.

Quadrupoles. The analyser consists of four parallel metal rods, which are cylindrical or hyperbolic in cross-section. They are arranged symmetrically with respect to the trajectory of the ions; the pairs diagonally opposed about the axis of symmetry of rods are connected electrically. The potentials to the two pairs of rods are opposed. They are the resultant of a constant component and an alternating component. The ions produced at the ion source are transmitted and separated by varying the voltages applied to the rods so that the ratio of continuous voltage to alternating voltage remains constant. The quadrupoles usually have a mass range of 1 a.m.u. to 2000 a.m.u., but some may range up to 4000 a.m.u. Although they have a lower resolving power than magnetic sector analysers, they nevertheless allow the monoisotopic profile of single charged ions to be obtained for the entire mass range. It is possible to obtain spectra using three quadrupoles arranged in series, Q_1 , Q_2 , Q_3 (Q_2 serves as a collision cell and is not really an analyser; the most commonly used collision gas is argon).

The most common types of scans are the following:

- *daughter-ion mode*: Q_1 selects an m/z ion whose fragments obtained by collision in Q_2 are analysed by Q_3 ,
- *parent-ion mode*: Q_3 filters only a specific m/z ratio, while Q_1 scans a given mass range. Only the ions decomposing to give the ion selected by Q_3 are detected,
- *neutral loss mode*: Q_1 and Q_3 scan a certain mass range but at an offset corresponding to the loss of a fragment characteristic of a product or family of compounds.

It is also possible to obtain spectra by combining quadrupole analysers with magnetic or electrostatic sector instruments; such instruments are called *hybrid mass spectrometers*.

Ion-trap analyser. The principle is the same as for a quadrupole, this time with the electric fields in three dimensions. This type of analyser allows product-ion spectra over several generations (MS^n) to be obtained.

Ion-cyclotron resonance analysers. Ions produced in a cell and subjected to a uniform, intense magnetic field move in circular orbits at frequencies which can be directly correlated to their m/z ratio by applying a Fourier transform algorithm. This phenomenon is called ion-cyclotron resonance. Analysers of this type consist of superconducting magnets and are capable of very high resolving power (up to 1000 000 and more) as well as MS^n spectra. However, very low pressures are required (of the order of 10^{-7} Pa).

Time-of-flight analysers. The ions produced at the ion source are accelerated at a voltage V of 10 kV to 20 kV. They pass through the analyser, consisting of a field-free tube, 25 cm

to 1.5 m long, generally called a *flight tube*. The time (t) for an ion to travel to the detector is proportional to the square root of the m/z ratio. Theoretically the mass range of such an analyser is infinite. In practice, it is limited by the ionisation or desorption method. Time-of-flight analysers are mainly used for high molecular mass compounds (up to several hundred thousand daltons). This technique is very sensitive (a few picomoles of product are sufficient). The accuracy of the measurements and the resolving power of such instruments may be improved considerably by using an electrostatic mirror (reflectron).

SIGNAL ACQUISITION

There are essentially three possible modes.

Complete spectrum mode. The entire signal obtained over a chosen mass range is recorded. The spectrum represents the relative intensity of the different ionic species present as a function of m/z . The results are essentially qualitative. The use of spectral reference libraries for more rapid identification is possible.

Fragmentometric mode (Selected-ion monitoring). The acquired signal is limited to one (single-ion monitoring (SIM)) or several (multiple-ion monitoring (MIM)) ions characteristic of the substance to be analysed. The limit of detection can be considerably reduced in this mode. Quantitative or semiquantitative tests can be carried out using external or internal standards (for example, deuterated standards). Such tests cannot be carried out with time-of-flight analysers.

Fragmentometric double mass spectrometry mode (multiple reaction monitoring (MRM)). The unimolecular or bimolecular decomposition of a chosen precursor ion characteristic of the substance to be analysed is followed specifically. The selectivity and the highly specific nature of this mode of acquisition provide excellent sensitivity levels and make it the most appropriate for quantitative studies using suitable internal standards (for example, deuterated standards). This type of analysis can be performed only on apparatus fitted with three quadrupoles in series, ion-trap analysers or cyclotron-resonance analysers.

CALIBRATION

Calibration allows the corresponding m/z value to be attributed to the detected signal. As a general rule, this is done using a reference substance. This calibration may be external (acquisition file separate from the analysis) or internal (the reference substance(s) are mixed with the substance to be examined and appear on the same acquisition file). The number of ions or points required for reliable calibration depends on the type of analyser and on the desired accuracy of the measurement, for example, in the case of a magnetic analyser where the m/z ratio varies exponentially with the value of the magnetic field, there should be as many points as possible.

SIGNAL DETECTION AND DATA PROCESSING

Ions separated by an analyser are converted into electric signals by a detection system such as a photomultiplier or an electron multiplier. These signals are amplified before being re-converted into digital signals for data processing, allowing various functions such as calibration, reconstruction of spectra, automatic quantification, archiving, creation or use of libraries of mass spectra. The various physical parameters required for the functioning of the apparatus as a whole are controlled by computer.

01/2005:20244

2.2.44. TOTAL ORGANIC CARBON IN WATER FOR PHARMACEUTICAL USE

Total organic carbon (TOC) determination is an indirect measure of organic substances present in water for pharmaceutical use. TOC determination can also be used to monitor the performance of various operations in the preparation of medicines.

A variety of acceptable methods is available for determining TOC. Rather than prescribing a given method to be used, this general chapter describes the procedures used to qualify the chosen method and the interpretation of results in limit tests. A standard solution is analysed at suitable intervals, depending on the frequency of measurements; the solution is prepared with a substance that is expected to be easily oxidisable (for example, sucrose) at a concentration adjusted to give an instrument response corresponding to the TOC limit to be measured. The suitability of the system is determined by analysis of a solution prepared with a substance expected to be oxidisable with difficulty (for example, 1,4-benzoquinone).

The various types of apparatus used to measure TOC in water for pharmaceutical use have in common the objective of completely oxidising the organic molecules in the sample water to produce carbon dioxide followed by measurement of the amount of carbon dioxide produced, the result being used to calculate the carbon concentration in the water.

The apparatus used must discriminate between organic and inorganic carbon, the latter being present as carbonate. The discrimination may be effected either by measuring the inorganic carbon and subtracting it from the total carbon, or by purging inorganic carbon from the sample before oxidation. Purging may also entrain organic molecules, but such purgeable organic carbon is present in negligible quantities in water for pharmaceutical use.

Apparatus. Use a calibrated instrument installed either on-line or off-line. Verify the system suitability at suitable intervals as described below. The apparatus must have a limit of detection specified by the manufacturer of 0.05 mg or less of carbon per litre.

TOC water. Use highly purified water complying with the following specifications:

- conductivity: not greater than $1.0 \mu\text{S cm}^{-1}$ at 25°C ,
- total organic carbon: not greater than 0.1 mg/l .

Depending on the type of apparatus used, the content of heavy metals and copper may be critical. The manufacturer's instructions should be followed.

Glassware preparation. Use glassware that has been scrupulously cleaned by a method that will remove organic matter. Use TOC water for the final rinse of glassware.

Standard solution. Dissolve sucrose R, dried at 105°C for 3 h in TOC water to obtain a solution containing 1.19 mg of sucrose per litre (0.50 mg of carbon per litre).

Test solution. Using all due care to avoid contamination, collect water to be tested in an airtight container leaving minimal head-space. Examine the water with minimum delay to reduce contamination from the container and its closure.

System suitability solution. Dissolve 1,4-benzoquinone R in TOC water to obtain a solution having a concentration of 0.75 mg of 1,4-benzoquinone per litre (0.50 mg of carbon per litre).

TOC water control. Use TOC water obtained at the same time as that used to prepare the standard solution and the system suitability solution.

Control solutions. In addition to the TOC water control, prepare suitable blank solutions or other solutions needed for establishing the baseline or for calibration adjustments following the manufacturer's instructions; run the appropriate blanks to zero the instrument.

System suitability. Run the following solutions and record the responses: TOC water (r_w); standard solution (r_s); system suitability solution (r_{ss}). Calculate the percentage response efficiency using the expression:

$$\frac{r_{ss} - r_w}{r_s - r_w} \times 100$$

The system is suitable if the response efficiency is not less than 85 per cent and not more than 115 per cent of the theoretical response.

Procedure. Run the test solution and record the response (r_u). The test solution complies with the test if r_u is not greater than $r_s - r_w$.

The method can also be applied using on-line instrumentation that has been adequately calibrated and shown to have acceptable system suitability. The location of instrumentation must be chosen to ensure that the responses are representative of the water used.

01/2005:20245

2.2.45. SUPERCRITICAL FLUID CHROMATOGRAPHY

Supercritical fluid chromatography (SFC) is a method of chromatographic separation in which the mobile phase is a fluid in a supercritical or a subcritical state. The stationary phase, contained in a column, consists of either finely divided solid particles, such as a silica or porous graphite, a chemically modified stationary phase, as used in liquid chromatography, or, for capillary columns, a cross-linked liquid film evenly coated on the walls of the column.

SFC is based on mechanisms of adsorption or mass distribution.

APPARATUS

The apparatus usually consists of a cooled pumping system, an injector, a chromatographic column, contained in an oven, a detector, a pressure regulator and a data acquisition device (or an integrator or a chart recorder).

Pumping system

Pumping systems are required to deliver the mobile phase at a constant flow rate. Pressure fluctuations are to be minimised, e.g. by passing the pressurised solvent through a pulse-damping device. Tubing and connections are capable of withstanding the pressures developed by the pumping system.

Microprocessor controlled systems are capable of accurately delivering a mobile phase in either constant or varying conditions, according to a defined programme. In the case of gradient elution, pumping systems which deliver solvent(s) from several reservoirs are available and solvent mixing can be achieved on either the low or high-pressure side of the pump(s).

Injectors

Injection may be carried out directly at the head of the column using a valve.

Stationary phases

Stationary phases are contained in columns which have been described in the chapters on *Liquid chromatography* (2.2.29) (packed columns) and *Gas chromatography* (2.2.28)

(capillary columns). A capillary column has a maximum internal diameter ($\emptyset$) of 100 μm .

Mobile phases

Usually the mobile phase is carbon-dioxide which may contain a polar modifier such as methanol, 2-propanol or acetonitrile. The composition, pressure (density), temperature and flow rate of the prescribed mobile phase may either be constant throughout the whole chromatographic procedure (isocratic, isodense, isothermic elution) or may vary according to a defined programme (gradient elution of the modifier, pressure (density), temperature or flow rate).

Detectors

Ultraviolet/visible (UV/Vis) spectrophotometers and flame ionisation detectors are the most commonly employed detectors. Light scattering detectors, infrared absorption spectrophotometers, thermal conductivity detectors or other special detectors may be used.

METHOD

Prepare the test solution(s) and the reference solution(s) as prescribed. The solutions must be free from solid particles.

Criteria for assessing the suitability of the system are described in the chapter on *Chromatographic separation techniques* (2.2.46). The extent to which adjustments of parameters of the chromatographic system can be made to satisfy the criteria of system suitability are also given in this chapter.

01/2005:20246

2.2.46. CHROMATOGRAPHIC SEPARATION TECHNIQUES

Chromatographic separation techniques are multi-stage separation methods in which the components of a sample are distributed between 2 phases, one of which is stationary, while the other is mobile. The stationary phase may be a solid or a liquid supported on a solid or a gel. The stationary phase may be packed in a column, spread as a layer, or distributed as a film, etc. The mobile phase may be gaseous or liquid or supercritical fluid. The separation may be based on adsorption, mass distribution (partition), ion exchange, etc., or may be based on differences in the physico-chemical properties of the molecules such as size, mass, volume, etc.

This chapter contains definitions and calculations of common parameters and generally applicable requirements for system suitability. Principles of separation, apparatus and methods are given in the following general methods:

- paper chromatography (2.2.26),
- thin-layer chromatography (2.2.27),
- gas chromatography (2.2.28),
- liquid chromatography (2.2.29),
- size-exclusion chromatography (2.2.30),
- supercritical fluid chromatography (2.2.45).

DEFINITIONS

The following definitions have been used to calculate the limits in monographs.

With some equipment, certain parameters, such as the signal-to-noise ratio, can be calculated using software provided by the manufacturer. It is the responsibility of the user to ensure that the calculation methods used in the software are compatible with the requirements of the European Pharmacopoeia. If not, the necessary corrections must be made.

Chromatogram

A chromatogram is a graphical or other representation of detector response, effluent concentration or other quantity used as a measure of effluent concentration, versus time, volume or distance. Idealised chromatograms are represented as a sequence of gaussian peaks on a baseline.

RETENTION DATA

Retention time and retention volume

Retention measurements in elution chromatography may be given as the retention time (t_R) directly defined by the position of the maximum of the peak in the chromatogram. From the retention time, the retention volume (V_R) may be calculated.

$$V_R = v \times t_R$$

t_R = retention time or distance along the baseline from the point of injection to the perpendicular dropped from the maximum of the peak corresponding to the component,

v = flow rate of the mobile phase.

Mass distribution ratio

The mass distribution ratio (D_m) (also known as the capacity factor k' or retention factor k) is defined as:

$$D_m = \frac{\text{amount of solute in stationary phase}}{\text{amount of solute in mobile phase}} = K_C \frac{V_S}{V_M}$$

K_C = equilibrium distribution coefficient (also known as distribution constant),

V_S = volume of the stationary phase,

V_M = volume of the mobile phase.

The mass distribution ratio of a component may be determined from the chromatogram using the expression:

$$D_m = \frac{t_R - t_M}{t_M}$$

t_R = retention time (or volume) or distance along the baseline from the point of injection to the perpendicular dropped from the maximum of the peak corresponding to the component,

t_M = hold-up time (or volume): time (or volume) or distance along the baseline from the point of injection to the perpendicular dropped from the maximum of the peak corresponding to an unretained component.

Distribution coefficient

The elution characteristics of a component in a particular column, in size-exclusion chromatography, may be given by

the distribution coefficient (K_o) which is calculated from the expression:

$$K_o = \frac{t_R - t_o}{t_t - t_o}$$

- t_R = retention time (or volume) or distance along the baseline from the point of injection to the perpendicular dropped from the maximum of the peak corresponding to the component,
- t_o = hold-up time (or volume): time (or volume) or distance along the baseline from the point of injection to the perpendicular dropped from the maximum of the peak corresponding to an unretained component,
- t_t = retention time (or volume) or distance along the baseline from the point of injection to the perpendicular dropped from the maximum of the peak corresponding to a component which has full access to the pores of the stationary phase.

Retardation factor

The retardation factor (R_F) (also known as retention factor R_f), used in planar chromatography, is the ratio of the distance from the point of application to the centre of the spot and the distance travelled by the solvent front from the point of application.

$$R_F = \frac{b}{a}$$

- b = migration distance of the analyte,
- a = migration distance of the solvent front.

CHROMATOGRAPHIC DATA

The peak may be defined by the *peak area* (A) or the *peak height* (h) and the *peak width at half-height* (w_h) or the *peak height* (h) and the *peak width between the points of inflection* (w_i). In gaussian peaks (Figure 2.2.46-1) there is the relationship:

$$w_h = 1.18w_i$$

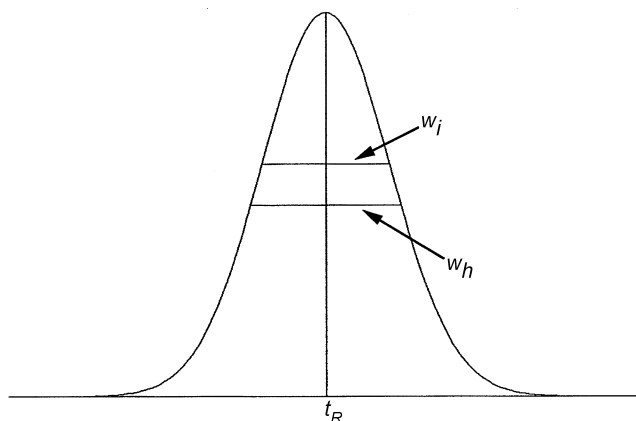


Figure 2.2.46-1.

Symmetry factor

The symmetry factor (A_s) (or tailing factor) of a peak (Figure 2.2.46-2) is calculated from the expression:

$$A_s = \frac{w_{0.05}}{2d}$$

$w_{0.05}$ = width of the peak at one-twentieth of the peak height,

d = distance between the perpendicular dropped from the peak maximum and the leading edge of the peak at one-twentieth of the peak height.

A value of 1.0 signifies complete (ideal) symmetry.

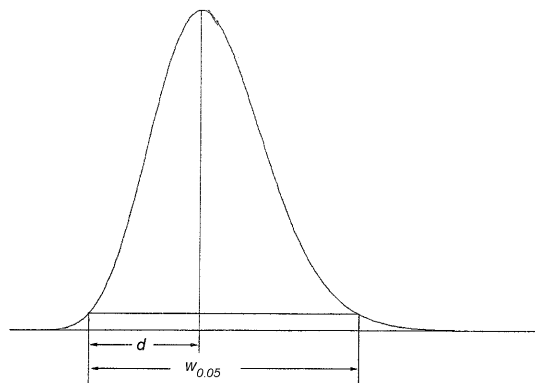


Figure 2.2.46-2.

Column performance and apparent number of theoretical plates

The column performance (apparent efficiency) may be calculated from data obtained under either isothermal, isocratic or isodense conditions, depending on the technique, as the apparent number of theoretical plates (N) from the following expression, where the values of t_R and w_h have to be expressed in the same units (time, volume or distance).

$$N = 5.54 \left(\frac{t_R}{w_h} \right)^2$$

t_R = retention time (or volume) or distance along the baseline from the point of injection to the perpendicular dropped from the maximum of the peak corresponding to the component,

w_h = width of the peak at half-height.

The apparent number of theoretical plates varies with the component as well as with the column and the retention time.

SEPARATION DATA

Resolution

The resolution (R_s) between peaks of 2 components may be calculated from the expression:

$$R_s = \frac{1.18 (t_{R2} - t_{R1})}{w_{h1} + w_{h2}}$$

$$t_{R2} > t_{R1}$$

t_{R1} and t_{R2} = retention times or distances along the baseline from the point of injection to the perpendiculars dropped from the maxima of 2 adjacent peaks,

w_{h1} and w_{h2} = peak widths at half-height.

A resolution of greater than 1.5 corresponds to baseline separation.

The expression given above may not be applicable if the peaks are not baseline separated.

In quantitative planar chromatography, the migration distances are used instead of retention times and the resolution may be calculated using the expression:

$$R_s = \frac{1.18 a (R_{F2} - R_{F1})}{w_{h1} + w_{h2}}$$

R_{F1} and R_{F2} = ratios of the distances from the point of application to the centres of the spots and the distance travelled by the solvent front from the point of application (retardation factor),

w_{h1} and w_{h2} = peak widths at half-height,

a = migration distance of the solvent front.

Peak-to-valley ratio

The peak-to-valley ratio (p/v) may be employed as a system suitability requirement in a test for related substances when baseline separation between 2 peaks is not reached (Figure 2.2.46.3).

$$p/v = \frac{H_p}{H_v}$$

H_p = height above the extrapolated baseline of the minor peak,

H_v = height above the extrapolated baseline at the lowest point of the curve separating the minor and major peaks.

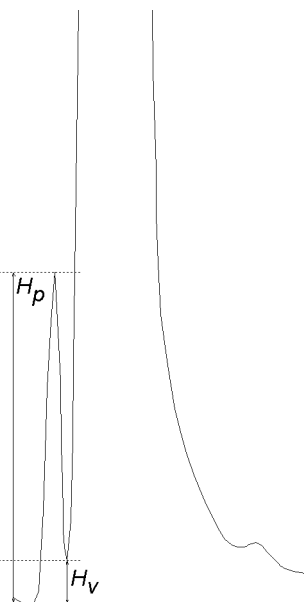


Figure 2.2.46.3.

Relative retention

The relative retention (r) is calculated as an estimate from the expression:

$$r = \frac{t_{R2} - t_M}{t_{R1} - t_M}$$

t_{R2} = retention time of the peak of interest,

t_{R1} = retention time of the reference peak (usually the peak corresponding to the substance to be examined),

t_M = hold-up time: time or distance along the baseline from the point of injection to the perpendicular dropped from the maximum of the peak corresponding to an unretained component.

The unadjusted relative retention (r_G) is calculated from the expression:

$$r_G = \frac{t_{R2}}{t_{R1}}$$

Unless otherwise indicated, values for relative retention stated in monographs correspond to unadjusted relative retention.

In planar chromatography, the retardation factors R_{F2} and R_{F1} are used instead of t_{R2} and t_{R1} .

PRECISION OF QUANTIFICATION

Signal-to-noise ratio

The signal-to-noise ratio (S/N) influences the precision of quantification and is calculated from the equation:

$$S/N = \frac{2H}{h}$$

H = height of the peak (Figure 2.2.46.4) corresponding to the component concerned, in the chromatogram obtained with the prescribed reference solution, measured from the maximum of the peak to the extrapolated baseline of the signal observed over a distance equal to 20 times the width at half-height,

h = range of the background noise in a chromatogram obtained after injection or application of a blank, observed over a distance equal to 20 times the width at half-height of the peak in the chromatogram obtained with the prescribed reference solution and, if possible, situated equally around the place where this peak would be found.

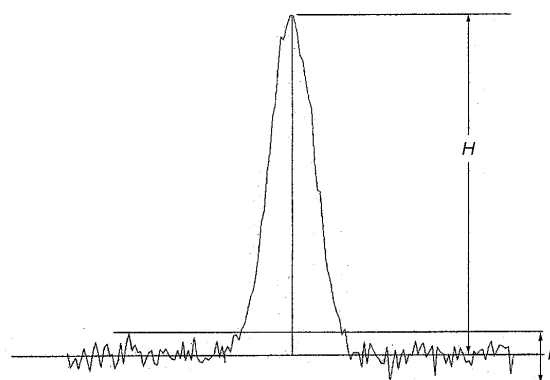


Figure 2.2.46.4.

Repeatability

The repeatability of response is expressed as an estimated percentage relative standard deviation ($RSD_{\%}$) of a consecutive series of measurement of injections or

applications of a reference solution and is calculated from the expression:

$$RSD\% = \frac{100}{\bar{y}} \sqrt{\frac{\sum (y_i - \bar{y})^2}{n - 1}}$$

y_i = individual values expressed as peak area, peak height, or ratio of areas by the internal standardisation method,

$\bar{y}$ = mean of individual values,

n = number of individual values.

The maximal permitted relative standard deviation ($RSD_{\max}$) is calculated for a series of injections of the reference solution for defined limits using the following expression:

$$RSD_{\max} = \frac{KB\sqrt{n}}{t_{90\%,n-1}}$$

K = constant (0.349), obtained from the expression $K = \frac{0.6}{\sqrt{2}} \times \frac{t_{90\%,5}}{\sqrt{6}}$ in which $\frac{0.6}{\sqrt{2}}$ represents the required RSD after 6 injections for $B = 1.0$,

B = upper limit given in the definition of the individual monograph minus 100 per cent,

n = number of replicate injections of the reference solution ($3 \leq n \leq 6$),

$t_{90\%,n-1}$ = Student's t at the 90 per cent probability level (double sided) with $n - 1$ degrees of freedom.

SYSTEM SUITABILITY

The system suitability tests represent an integral part of the method and are used to ensure adequate performance of the chromatographic system. Apparent efficiency, mass distribution ratio, resolution, relative retention and the symmetry factor are the parameters which are usually employed in assessing the performance of the column. Factors which may affect the chromatographic behaviour include the composition, ionic strength, temperature and apparent pH of the mobile phase, flow rate, column length, temperature and pressure, and stationary phase characteristics including porosity, particle size, type of particles, specific surface area and, in the case of reverse-phase supports, the extent of chemical modification (as expressed by end-capping, carbon loading etc.).

The various components of the equipment employed must be qualified and be capable of achieving the precision required to conduct the test or assay.

The following requirements are to be fulfilled unless otherwise stated in the monograph.

- The symmetry factor of the principal peak is to be between 0.8 and 1.5 unless otherwise stated in the monograph. This requirement has general applicability to tests or assays described in the monographs.
- Maximal permitted relative standard deviation for replicate injections of the prescribed reference solution do not exceed the values given in Table 2.2.46-1. This requirement is applicable to assays for content only and does not apply to the test for related substances.
- The limit of detection of the peak (corresponding to a signal-to-noise ratio of 3) is below the disregard limit of the test for related substances.
- The limit of quantitation of the peak (corresponding to a signal-to-noise ratio of 10) is equal to or less than the disregard limit of the test for related substances.

Table 2.2.46-1. – *Repeatability requirements*

B (per cent)	Number of individual injections			
	3	4	5	6
2.0	0.41	0.59	0.73	0.85
2.5	0.52	0.74	0.92	1.06
3.0	0.62	0.89	1.10	1.27

ADJUSTMENT OF CHROMATOGRAPHIC CONDITIONS

The extent to which the various parameters of a chromatographic test may be adjusted to satisfy the system suitability criteria without fundamentally modifying the methods are listed below for information. The chromatographic conditions described have been validated during the elaboration of the monograph. The system suitability tests are included to ensure the separation required for satisfactory performance of the test or assay. Nonetheless, since the stationary phases are described in a general way and there is such a variety available commercially, with differences in chromatographic behaviour, some adjustments of the chromatographic conditions may be necessary to achieve the prescribed system suitability requirements. With reverse-phase liquid chromatographic methods, in particular, adjustment of the various parameters will not always result in satisfactory chromatography. In that case, it may be necessary to replace the column with another of the same type (e.g. octadecylsilyl silica gel) which exhibits the desired chromatographic behaviour.

For critical parameters the adjustments are defined clearly in the monograph to ensure the system suitability.

Multiple adjustments which may have a cumulative effect in the performance of the system are to be avoided.

Thin-layer chromatography and paper chromatography

Composition of the mobile phase: the amount of the minor solvent component may be adjusted by ± 30 per cent relative or ± 2 per cent absolute, whichever is the larger; for a minor component at 10 per cent of the mobile phase, a 30 per cent relative adjustment allows a range of 7-13 per cent whereas a 2 per cent absolute adjustment allows a range of 8-12 per cent, the relative value being therefore the larger; for a minor component at 5 per cent of the mobile phase, a 30 per cent relative adjustment allows a range of 3.5-6.5 per cent whereas a 2 per cent absolute adjustment allows a range of 3-7 per cent, the absolute value being in this case the larger. No other component is altered by more than 10 per cent absolute.

pH of the aqueous component of the mobile phase: ± 0.2 pH, unless otherwise stated in the monograph, or ± 1.0 pH when neutral substances are to be examined.

Concentration of salts in the buffer component of a mobile phase: ± 10 per cent.

Application volume: 10-20 per cent of the prescribed volume if using fine particle size plates (2-10 μm).

Migration distance of the solvent front is to be not less than 50 mm or 30 mm on high-performance plates.

Liquid chromatography

Composition of the mobile phase: the amount of the minor solvent component may be adjusted by ± 30 per cent relative or ± 2 per cent absolute, whichever is the larger (see example above). No other component is altered by more than 10 per cent absolute.

pH of the aqueous component of the mobile phase: ± 0.2 pH, unless otherwise stated in the monograph, or ± 1.0 pH when neutral substances are to be examined.

Concentration of salts in the buffer component of a mobile phase: ± 10 per cent.

Detector wavelength: no adjustment permitted.

Stationary phase:

- *column length:* ± 70 per cent,
- *column internal diameter:* ± 25 per cent,
- *particle size:* maximal reduction of 50 per cent, no increase permitted.

Flow rate: ± 50 per cent. When in a monograph the retention time of the principle peak is indicated, the flow rate has to be adjusted if the column internal diameter has been changed. No decrease of flow rate is permitted if the monograph uses apparent number of theoretical plates in the qualification section.

Temperature: ± 10 per cent, to a maximum of $60\text{ }^{\circ}\text{C}$.

Injection volume: may be decreased, provided detection and repeatability of the peak(s) to be determined are satisfactory.

Gradient elution: the configuration of the equipment employed may significantly alter the resolution, retention time and relative retentions described in the method. Should this occur, it may be due to excessive dwell volume which is the volume between the point at which the 2 eluants meet and the top of the column.

Gas chromatography

Stationary phase:

- *column length:* ± 70 per cent,
- *column internal diameter:* ± 50 per cent,
- *particle size:* maximal reduction of 50 per cent, no increase permitted,
- *film thickness:* -50 per cent to $+100$ per cent.

Flow rate: ± 50 per cent.

Temperature: ± 10 per cent.

Injection volume: may be decreased, provided detection and repeatability are satisfactory.

Supercritical fluid chromatography

Composition of the mobile phase: for packed columns, the amount of the minor solvent component may be adjusted by ± 30 per cent relative or ± 2 per cent absolute, whichever is the larger. No adjustment is permitted for a capillary column system.

Detector wavelength: no adjustment permitted.

Stationary phase:

- *column length:* ± 70 per cent,
- *column internal diameter:*
 - ± 25 per cent (packed columns),
 - ± 50 per cent (capillary columns),
- *particle size:* maximal reduction of 50 per cent, no increase permitted (packed columns).

Flow rate: ± 50 per cent.

Temperature: ± 10 per cent.

Injection volume: may be decreased, provided detection and repeatability are satisfactory.

QUANTIFICATION

- *Detector response.* The detector sensitivity is the signal output per unit concentration or unit mass of a substance in the mobile phase entering the detector. The relative detector response factor, commonly referred to as *response factor*, expresses the sensitivity of a detector relative to a standard substance. The *correction factor* is the reciprocal of the response factor.
- *External standard method.* The concentration of the component(s) to be analysed is determined by comparing the response(s) (peak(s)) obtained with the test solution to the response(s) (peak(s)) obtained with a reference solution.
- *Internal standard method.* Equal amounts of a component that is resolved from the substance to be examined (the internal standard) is introduced into the test solution and a reference solution. The internal standard should not react with the substance to be examined; it must be stable and must not contain impurities with a retention time similar to that of the substance to be examined. The concentration of the substance to be examined is determined by comparing the ratio of the peak areas or peak heights due to the substance to be examined and the internal standard in the test solution with the ratio of the peak areas or peak heights due to the substance to be examined and the internal standard in the reference solution.
- *Normalisation procedure.* The percentage content of one or more components of the substance to be examined is calculated by determining the area of the peak or peaks as a percentage of the total area of all the peaks, excluding those due to solvents or any added reagents and those below the disregard limit.
- *Calibration procedure.* The relationship between the measured or evaluated signal (y) and the amount (concentration, mass, etc.) of substance (x) is determined and the calibration function is calculated. The analytical results are calculated from the measured signal or evaluated signal of the analyte by means of the inverse function.

For assays and for quantitative determination of components the external standard method, the internal standard method or the calibration procedure may be described in the monograph, and the normalisation procedure is not normally applied. In tests for related substances, either the external standard method with a single reference solution or the normalisation procedure is generally applied. However, with both the normalisation procedure or the external standard method, when a dilution of the test solution is used for comparison, the responses of the related substances are similar to the substance itself (response factor of 0.8 to 1.2), otherwise correction factors are included in the text.

When the related substances test prescribes the summation of impurities or there is quantitative determination of an impurity, it is important to choose an appropriate threshold setting and appropriate conditions for the integration of the peak areas. In such tests the *disregard limit*, e.g. the areas of peaks whose areas are below the limit are not taken into account, is generally 0.05 per cent. Thus, the threshold setting of the data collection system corresponds to, at least, half of the disregard limit. Integration of the peak areas of the impurities, which are not completely separated from the main peak, are preferably performed by valley-to-valley extrapolation (tangential skim). Peaks due to the solvent(s) used to dissolve the sample are also to be disregarded.

01/2005:20247

2.2.47. CAPILLARY ELECTROPHORESIS

GENERAL PRINCIPLES

Capillary electrophoresis is a physical method of analysis based on the migration, inside a capillary, of charged analytes dissolved in an electrolyte solution, under the influence of a direct-current electric field.

The migration velocity of an analyte under an electric field of intensity E , is determined by the electrophoretic mobility of the analyte and the electro-osmotic mobility of the buffer inside the capillary. The electrophoretic mobility of a solute (μ_{ep}) depends on the characteristics of the solute (electric charge, molecular size and shape) and those of the buffer in which the migration takes place (type and ionic strength of the electrolyte, pH, viscosity and additives). The electrophoretic velocity (v_{ep}) of a solute, assuming a spherical shape, is given by the equation:

$$v_{ep} = \mu_{ep} \times E = \left(\frac{q}{6\pi\eta r} \right) \times \left(\frac{V}{L} \right)$$

q = effective charge of the solute,

η = viscosity of the electrolyte solution,

r = Stoke's radius of the solute,

V = applied voltage,

L = total length of the capillary.

When an electric field is applied through the capillary filled with buffer, a flow of solvent is generated inside the capillary, called electro-osmotic flow. The velocity of the electro-osmotic flow depends on the electro-osmotic mobility (μ_{eo}) which in turn depends on the charge density on the capillary internal wall and the buffer characteristics. The electro-osmotic velocity (v_{eo}) is given by the equation:

$$v_{eo} = \mu_{eo} \times E = \left(\frac{\varepsilon\zeta}{\eta} \right) \times \left(\frac{V}{L} \right)$$

ε = dielectric constant of the buffer,

ζ = zeta potential of the capillary surface.

The velocity of the solute (v) is given by:

$$v = v_{ep} + v_{eo}$$

The electrophoretic mobility of the analyte and the electro-osmotic mobility may act in the same direction or in opposite directions, depending on the charge of the solute. In normal capillary electrophoresis, anions will migrate in the opposite direction to the electro-osmotic flow and their velocities will be smaller than the electro-osmotic velocity. Cations will migrate in the same direction as the electro-osmotic flow and their velocities will be greater than the electro-osmotic velocity. Under conditions in which there is a fast electro-osmotic velocity with respect to the electrophoretic velocity of the solutes, both cations and anions can be separated in the same run.

The time (t) taken by the solute to migrate the distance (l) from the injection end of the capillary to the detection point (capillary effective length) is given by the expression:

$$t = \frac{l}{v_{ep} + v_{eo}} = \frac{l \times L}{(\mu_{ep} + \mu_{eo}) \times V}$$

In general, uncoated fused-silica capillaries above pH 3 have negative charge due to ionised silanol groups in the inner wall. Consequently, the electro-osmotic flow is from anode to cathode. The electro-osmotic flow must remain constant from run to run if good reproducibility is to be obtained in the migration velocity of the solutes. For some applications, it may be necessary to reduce or suppress the electro-osmotic flow by modifying the inner wall of the capillary or by changing the concentration, composition and/or pH of the buffer solution.

After the introduction of the sample into the capillary, each analyte ion of the sample migrates within the background electrolyte as an independent zone, according to its electrophoretic mobility. Zone dispersion, that is the spreading of each solute band, results from different phenomena. Under ideal conditions the sole contribution to the solute-zone broadening is molecular diffusion of the solute along the capillary (longitudinal diffusion). In this ideal case the efficiency of the zone, expressed as the number of theoretical plates (N), is given by:

$$N = \frac{(\mu_{ep} + \mu_{eo}) \times V \times l}{2 \times D \times L}$$

D = molecular diffusion coefficient of the solute in the buffer.

In practice, other phenomena such as heat dissipation, sample adsorption onto the capillary wall, mismatched conductivity between sample and buffer, length of the injection plug, detector cell size and unlevelled buffer reservoirs can also significantly contribute to band dispersion.

Separation between 2 bands (expressed as the resolution, R_s) can be obtained by modifying the electrophoretic mobility of the analytes, the electro-osmotic mobility induced in the capillary and by increasing the efficiency for the band of each analyte, according to the equation:

$$R_s = \frac{\sqrt{N}(\mu_{epb} - \mu_{epa})}{4(\bar{\mu}_{ep} + \mu_{eo})}$$

μ_{epa} and μ_{epb} = electrophoretic mobilities of the 2 analytes separated,

$\bar{\mu}_{ep}$ = mean electrophoretic mobility of the 2 analytes $\bar{\mu}_{ep} = \frac{1}{2}(\mu_{epb} + \mu_{epa})$.

APPARATUS

An apparatus for capillary electrophoresis is composed of:

- a high-voltage, controllable direct-current power supply;
- 2 buffer reservoirs, held at the same level, containing the prescribed anodic and cathodic solutions;
- 2 electrode assemblies (the cathode and the anode), immersed in the buffer reservoirs and connected to the power supply;
- a separation capillary (usually made of fused-silica) which, when used with some specific types of detectors, has an optical viewing window aligned with the detector. The ends of the capillary are placed in the buffer reservoirs. The capillary is filled with the solution prescribed in the monograph;
- a suitable injection system;
- a detector able to monitor the amount of substances of interest passing through a segment of the separation capillary at a given time; it is usually based on absorption spectrophotometry (UV and visible) or fluorimetry, but conductimetric, amperometric or mass spectrometric

detection can be useful for specific applications; indirect detection is an alternative method used to detect non-UV-absorbing and non-fluorescent compounds;

- a thermostatic system able to maintain a constant temperature inside the capillary is recommended to obtain a good separation reproducibility;
- a recorder and a suitable integrator or a computer.

The definition of the injection process and its automation are critical for precise quantitative analysis. Modes of injection include gravity, pressure or vacuum injection and electrokinetic injection. The amount of each sample component introduced electrokinetically depends on its electrophoretic mobility, leading to possible discrimination using this injection mode.

Use the capillary, the buffer solutions, the preconditioning method, the sample solution and the migration conditions prescribed in the monograph of the considered substance. The employed electrolytic solution is filtered to remove particles and degassed to avoid bubble formation that could interfere with the detection system or interrupt the electrical contact in the capillary during the separation run. A rigorous rinsing procedure should be developed for each analytical method to achieve reproducible migration times of the solutes.

CAPILLARY ZONE ELECTROPHORESIS

PRINCIPLE

In capillary zone electrophoresis, analytes are separated in a capillary containing only buffer without any anticonvective medium. With this technique, separation takes place because the different components of the sample migrate as discrete bands with different velocities. The velocity of each band depends on the electrophoretic mobility of the solute and the electro-osmotic flow in the capillary (see General Principles). Coated capillaries can be used to increase the separation capacity of those substances adsorbing on fused-silica surfaces.

Using this mode of capillary electrophoresis, the analysis of both small ($M_r < 2000$) and large molecules ($2000 < M_r < 100\,000$) can be accomplished. Due to the high efficiency achieved in capillary zone electrophoresis, separation of molecules having only minute differences in their charge-to-mass ratio can be effected. This separation mode also allows the separation of chiral compounds by addition of chiral selectors to the separation buffer.

OPTIMISATION

Optimisation of the separation is a complex process where several separation parameters can play a major role. The main factors to be considered in the development of separations are instrumental and electrolytic solution parameters.

Instrumental parameters

Voltage. A Joule heating plot is useful in optimising the applied voltage and capillary temperature. Separation time is inversely proportional to applied voltage. However, an increase in the voltage used can cause excessive heat production, giving rise to temperature and, as a result thereof, viscosity gradients in the buffer inside the capillary. This effect causes band broadening and decreases resolution.

Polarity. Electrode polarity can be normal (anode at the inlet and cathode at the outlet) and the electro-osmotic flow will move toward the cathode. If the electrode polarity is reversed, the electro-osmotic flow is away from the outlet and only charged analytes with electrophoretic mobilities greater than the electro-osmotic flow will pass to the outlet.

Temperature. The main effect of temperature is observed on buffer viscosity and electrical conductivity, and therefore on migration velocity. In some cases, an increase in capillary temperature can cause a conformational change in proteins, modifying their migration time and the efficiency of the separation.

Capillary. The dimensions of the capillary (length and internal diameter) contribute to analysis time, efficiency of separations and load capacity. Increasing both effective length and total length can decrease the electric fields (working at constant voltage) which increases migration time. For a given buffer and electric field, heat dissipation, and hence sample band-broadening, depend on the internal diameter of the capillary. The latter also affects the detection limit, depending on the sample volume injected and the detection system employed.

Since the adsorption of the sample components on the capillary wall limits efficiency, methods to avoid these interactions should be considered in the development of a separation method. In the specific case of proteins, several strategies have been devised to avoid adsorption on the capillary wall. Some of these strategies (use of extreme pH and adsorption of positively charged buffer additives) only require modification of the buffer composition to prevent protein adsorption. In other strategies, the internal wall of the capillary is coated with a polymer, covalently bonded to the silica, that prevents interaction between the proteins and the negatively charged silica surface. For this purpose, ready-to-use capillaries with coatings consisting of neutral-hydrophilic, cationic and anionic polymers are available.

Electrolytic solution parameters

Buffer type and concentration. Suitable buffers for capillary electrophoresis have an appropriate buffer capacity in the pH range of choice and low mobility to minimise current generation.

Matching buffer-ion mobility to solute mobility, whenever possible, is important for minimising band distortion. The type of sample solvent used is also important to achieve on-column sample focusing, which increases separation efficiency and improves detection.

An increase in buffer concentration (for a given pH) decreases electro-osmotic flow and solute velocity.

Buffer pH. The pH of the buffer can affect separation by modifying the charge of the analyte or additives, and by changing the electro-osmotic flow. In protein and peptide separation, changing the pH of the buffer from above to below the isoelectric point (pI) changes the net charge of the solute from negative to positive. An increase in the buffer pH generally increases the electro-osmotic flow.

Organic solvents. Organic modifiers (methanol, acetonitrile, etc.) may be added to the aqueous buffer to increase the solubility of the solute or other additives and/or to affect the degree of ionisation of the sample components. The addition of these organic modifiers to the buffer generally causes a decrease in the electro-osmotic flow.

Additives for chiral separations. For the separation of optical isomers, a chiral selector is added to the separation buffer. The most commonly used chiral selectors are cyclodextrins, but crown ethers, polysaccharides and proteins may also be used. Since chiral recognition is governed by the different interactions between the chiral selector and each of the enantiomers, the resolution achieved for the chiral compounds depends largely on the type of chiral selector used. In this regard, for the development of a given separation it may be useful to test cyclodextrins having a different cavity size (α -, β -, or γ -cyclodextrin) or modified

cyclodextrins with neutral (methyl, ethyl, hydroxyalkyl, etc.) or ionisable (aminomethyl, carboxymethyl, sulphobutyl ether, etc.) groups. When using modified cyclodextrins, batch-to-batch variations in the degree of substitution of the cyclodextrins must be taken into account since it will influence the selectivity. Other factors controlling the resolution in chiral separations are concentration of chiral selector, composition and pH of the buffer and temperature. The use of organic additives, such as methanol or urea can also modify the resolution achieved.

CAPILLARY GEL ELECTROPHORESIS

PRINCIPLE

In capillary gel electrophoresis, separation takes place inside a capillary filled with a gel that acts as a molecular sieve. Molecules with similar charge-to-mass ratios are separated according to molecular size since smaller molecules move more freely through the network of the gel and therefore migrate faster than larger molecules. Different biological macromolecules (for example, proteins and DNA fragments), which often have similar charge-to-mass ratios, can thus be separated according to their molecular mass by capillary gel electrophoresis.

CHARACTERISTICS OF GELS

2 types of gels are used in capillary electrophoresis: permanently coated gels and dynamically coated gels. Permanently coated gels, such as cross-linked polyacrylamide, are prepared inside the capillary by polymerisation of the monomers. They are usually bonded to the fused-silica wall and cannot be removed without destroying the capillary. If the gels are used for protein analysis under reducing conditions, the separation buffer usually contains sodium dodecyl sulphate and the samples are denatured by heating in a mixture of sodium dodecyl sulphate and 2-mercaptoethanol or dithiothreitol before injection. When non-reducing conditions are used (for example, analysis of an intact antibody), 2-mercaptoethanol and dithiothreitol are not used. Separation in cross-linked gels can be optimised by modifying the separation buffer (as indicated in the capillary zone electrophoresis section) and controlling the gel porosity during the gel preparation. For cross-linked polyacrylamide gels, the porosity can be modified by changing the concentration of acrylamide and/or the proportion of cross-linker. As a rule, a decrease in the porosity of the gel leads to a decrease in the mobility of the solutes. Due to the rigidity of these gels, only electrokinetic injection can be used.

Dynamically coated gels are hydrophilic polymers, such as linear polyacrylamide, cellulose derivatives, dextran, etc., which can be dissolved in aqueous separation buffers giving rise to a separation medium that also acts as a molecular sieve. These separation media are easier to prepare than cross-linked polymers. They can be prepared in a vial and filled by pressure in a wall-coated capillary (with no electro-osmotic flow). Replacing the gel before every injection generally improves the separation reproducibility. The porosity of the gels can be increased by using polymers of higher molecular mass (at a given polymer concentration) or by decreasing the polymer concentration (for a given polymer molecular mass). A reduction in the gel porosity leads to a decrease in the mobility of the solute for the same buffer. Since the dissolution of these polymers in the buffer gives low viscosity solutions, both hydrodynamic and electrokinetic injection techniques can be used.

CAPILLARY ISOELECTRIC FOCUSING

PRINCIPLE

In isoelectric focusing, the molecules migrate under the influence of the electric field, so long as they are charged, in a pH gradient generated by ampholytes having pI values in a wide range (poly-aminocarboxylic acids), dissolved in the separation buffer.

The three basic steps of isoelectric focusing are loading, focusing and mobilisation.

Loading step. Two methods may be employed:

- loading in one step: the sample is mixed with ampholytes and introduced into the capillary either by pressure or vacuum;
- sequential loading: a leading buffer, then the ampholytes, then the sample mixed with ampholytes, again ampholytes alone and finally the terminating buffer are introduced into the capillary. The volume of the sample must be small enough not to modify the pH gradient.

Focusing step. When the voltage is applied, ampholytes migrate toward the cathode or the anode, according to their net charge, thus creating a pH gradient from anode (lower pH) to cathode (higher pH). During this step the components to be separated migrate until they reach a pH corresponding to their isoelectric point (pI) and the current drops to very low values.

Mobilisation step. If mobilisation is required for detection, use one of the following methods.

- in the first method, mobilisation is accomplished during the focusing step under the effect of the electro-osmotic flow; the electro-osmotic flow must be small enough to allow the focusing of the components;
- in the second method, mobilisation is accomplished by applying positive pressure after the focusing step;
- in the third method, mobilisation is achieved after the focusing step by adding salts to the cathode reservoir or the anode reservoir (depending on the direction chosen for mobilisation) in order to alter the pH in the capillary when the voltage is applied. As the pH is changed, the proteins and ampholytes are mobilised in the direction of the reservoir which contains the added salts and pass the detector.

The separation achieved, expressed as ΔpI , depends on the pH gradient (dpH/dx), the number of ampholytes having different pI values, the molecular diffusion coefficient (D), the intensity of the electric field (E) and the variation of the electrophoretic mobility of the analyte with the pH ($-d\mu/dpH$):

$$\Delta pI = 3 \times \sqrt{\frac{D (dpH/dx)}{E (-d\mu/dpH)}}$$

OPTIMISATION

The main parameters to be considered in the development of separations are:

Voltage. Capillary isoelectric focusing utilises very high electric fields, 300 V/cm to 1000 V/cm in the focusing step.

Capillary. The electro-osmotic flow must be reduced or suppressed depending on the mobilisation strategy (see above). Coated capillaries tend to reduce the electro-osmotic flow.

Solutions. The anode buffer reservoir is filled with a solution with a pH lower than the pI of the most acidic ampholyte and the cathode reservoir is filled with a solution with a pH

higher than the pI of the most basic ampholyte. Phosphoric acid for the anode and sodium hydroxide for the cathode are frequently used.

Addition of a polymer, such as methylcellulose, in the ampholyte solution tends to suppress convective forces (if any) and electro-osmotic flow by increasing the viscosity. Commercial ampholytes are available covering many pH ranges and may be mixed if necessary to obtain an expanded pH range. Broad pH ranges are used to estimate the isoelectric point whereas narrower ranges are employed to improve accuracy. Calibration can be done by correlating migration time with isoelectric point for a series of protein markers.

During the focusing step precipitation of proteins at their isoelectric point can be prevented, if necessary, using buffer additives such as glycerol, surfactants, urea or zwitterionic buffers. However, depending on the concentration, urea denatures proteins.

MICELLAR ELECTROKINETIC CHROMATOGRAPHY (MEKC)

PRINCIPLE

In micellar electrokinetic chromatography, separation takes place in an electrolyte solution which contains a surfactant at a concentration above the critical micellar concentration (*cmc*). The solute molecules are distributed between the aqueous buffer and the pseudo-stationary phase composed of micelles, according to the partition coefficient of the solute. The technique can therefore be considered as a hybrid of electrophoresis and chromatography. It is a technique that can be used for the separation of both neutral and charged solutes, maintaining the efficiency, speed and instrumental suitability of capillary electrophoresis. One of the most widely used surfactants in MEKC is the anionic surfactant sodium dodecyl sulphate, although other surfactants, for example cationic surfactants such as cetyltrimethylammonium salts, are also used.

The separation mechanism is as follows. At neutral and alkaline pH, a strong electro-osmotic flow is generated and moves the separation buffer ions in the direction of the cathode. If sodium dodecyl sulphate is employed as the surfactant, the electrophoretic migration of the anionic micelle is in the opposite direction, towards the anode. As a result, the overall micelle migration velocity is slowed down compared to the bulk flow of the electrolytic solution. In the case of neutral solutes, since the analyte can partition between the micelle and the aqueous buffer, and has no electrophoretic mobility, the analyte migration velocity will depend only on the partition coefficient between the micelle and the aqueous buffer. In the electropherogram, the peaks corresponding to each uncharged solute are always between that of the electro-osmotic flow marker and that of the micelle (the time elapsed between these two peaks is called the separation window). For electrically charged solutes, the migration velocity depends on both the partition coefficient of the solute between the micelle and the aqueous buffer, and on the electrophoretic mobility of the solute in the absence of micelle.

Since the mechanism in MEKC of neutral and weakly ionised solutes is essentially chromatographic, migration of the solute and resolution can be rationalised in terms of the retention factor of the solute (*k*), also referred to as mass distribution ratio (*D_m*), which is the ratio of the number of moles of solute in the micelle to those in the mobile phase. For a neutral compound, *k* is given by:

$$k = \frac{t_R - t_0}{t_0 \times \left(1 - \frac{t_R}{t_{mc}}\right)} = K \times \frac{V_S}{V_M}$$

- t_R = migration time of the solute,
- t_0 = analysis time of an unretained solute (determined by injecting an electro-osmotic flow marker which does not enter the micelle, for instance methanol),
- t_{mc} = micelle migration time (measured by injecting a micelle marker, such as Sudan III, which migrates while continuously associated in the micelle),
- K = partition coefficient of the solute,
- V_S = volume of the micellar phase,
- V_M = volume of the mobile phase.

Likewise, the resolution between 2 closely-migrating solutes (R_s) is given by:

$$R_s = \frac{\sqrt{N}}{4} \times \frac{\alpha - 1}{\alpha} \times \frac{k_b}{k_b + 1} \times \frac{1 - \left(\frac{t_0}{t_{mc}}\right)}{1 + k_a \times \left(\frac{t_0}{t_{mc}}\right)}$$

- N = number of theoretical plates for one of the solutes,
- α = selectivity,
- k_a and k_b = retention factors for both solutes, respectively ($k_b > k_a$).

Similar, but not identical, equations give *k* and R_s values for electrically charged solutes.

OPTIMISATION

The main parameters to be considered in the development of separations by MEKC are instrumental and electrolytic solution parameters.

Instrumental parameters

Voltage. Separation time is inversely proportional to applied voltage. However, an increase in voltage can cause excessive heat production that gives rise to temperature gradients and viscosity gradients of the buffer in the cross-section of the capillary. This effect can be significant with high conductivity buffers such as those containing micelles. Poor heat dissipation causes band broadening and decreases resolution.

Temperature. Variations in capillary temperature affect the partition coefficient of the solute between the buffer and the micelles, the critical micellar concentration and the viscosity of the buffer. These parameters contribute to the migration time of the solutes. The use of a good cooling system improves the reproducibility of the migration time for the solutes.

Capillary. As in capillary zone electrophoresis, the dimensions of the capillary (length and internal diameter) contribute to analysis time and efficiency of separations. Increasing both effective length and total length can decrease the electric fields (working at constant voltage), increase migration time and improve the separation efficiency. The internal diameter controls heat dissipation (for a given buffer and electric field) and consequently the sample band broadening.

Electrolytic solution parameters

Surfactant type and concentration. The type of surfactant, in the same way as the stationary phase in chromatography, affects the resolution since it modifies separation selectivity.

Also, the log k of a neutral compound increases linearly with the concentration of surfactant in the mobile phase. Since resolution in MEKC reaches a maximum when k approaches the value of $\sqrt{t_{mc}/t_0}$, modifying the concentration of surfactant in the mobile phase changes the resolution obtained.

Buffer pH. Although pH does not modify the partition coefficient of non-ionised solutes, it can modify the electro-osmotic flow in uncoated capillaries. A decrease in the buffer pH decreases the electro-osmotic flow and therefore increases the resolution of the neutral solutes in MEKC, resulting in a longer analysis time.

Organic solvents. To improve MEKC separation of hydrophobic compounds, organic modifiers (methanol, propanol, acetonitrile, etc.) can be added to the electrolytic solution. The addition of these modifiers usually decreases migration time and the selectivity of the separation. Since the addition of organic modifiers affects the critical micellar concentration, a given surfactant concentration can be used only within a certain percentage of organic modifier before the micellisation is inhibited or adversely affected, resulting in the absence of micelles and, therefore, in the absence of partition. The dissociation of micelles in the presence of a high content of organic solvent does not always mean that the separation will no longer be possible; in some cases the hydrophobic interaction between the ionic surfactant monomer and the neutral solutes forms solvophobic complexes that can be separated electrophoretically.

Additives for chiral separations. For the separation of enantiomers using MEKC, a chiral selector is included in the micellar system, either covalently bound to the surfactant or added to the micellar separation electrolyte. Micelles that have a moiety with chiral discrimination properties include salts of *N*-dodecanoyl-L-amino acids, bile salts, etc. Chiral resolution can also be achieved using chiral discriminators, such as cyclodextrins, added to the electrolytic solutions which contain micellised achiral surfactants.

Other additives. Several strategies can be carried out to modify selectivity, by adding chemicals to the buffer. The addition of several types of cyclodextrins to the buffer can also be used to reduce the interaction of hydrophobic solutes with the micelle, thus increasing the selectivity for this type of compound.

The addition of substances able to modify solute-micelle interactions by adsorption on the latter, is used to improve the selectivity of the separations in MEKC. These additives may be a second surfactant (ionic or non-ionic) which gives rise to mixed micelles or metallic cations which dissolve in the micelle and form co-ordination complexes with the solutes.

QUANTIFICATION

Peak areas must be divided by the corresponding migration time to give the corrected area in order to:

- compensate for the shift in migration time from run to run, thus reducing the variation of the response,
- compensate for the different responses of sample constituents with different migration times.

Where an internal standard is used, verify that no peak of the substance to be examined is masked by that of the internal standard.

CALCULATIONS

From the values obtained, calculate the content of the component or components being examined. When prescribed, the percentage content of one or more components of the sample to be examined is calculated by determining the corrected area(s) of the peak(s) as a

percentage of the total of the corrected areas of all peaks, excluding those due to solvents or any added reagents (normalisation procedure). The use of an automatic integration system (integrator or data acquisition and processing system) is recommended.

SYSTEM SUITABILITY

In order to check the behaviour of the capillary electrophoresis system, system suitability parameters are used. The choice of these parameters depends on the mode of capillary electrophoresis used. They are: retention factor (k) (only for micellar electrokinetic chromatography), apparent number of theoretical plates (N), symmetry factor (A_s) and resolution (R_s). In previous sections, the theoretical expressions for N and R_s have been described, but more practical equations that allow these parameters to be calculated from the electropherograms are given below.

APPARENT NUMBER OF THEORETICAL PLATES

The apparent number of theoretical plates (N) may be calculated using the expression:

$$N = 5.54 \times \left(\frac{t_R}{w_h} \right)^2$$

- t_R = migration time or distance along the baseline from the point of injection to the perpendicular dropped from the maximum of the peak corresponding to the component,
- w_h = width of the peak at half-height.

RESOLUTION

The resolution (R_s) between peaks of similar height of 2 components may be calculated using the expression:

$$R_s = \frac{1.18 \times (t_{R2} - t_{R1})}{w_{h1} + w_{h2}}$$

$$t_{R2} > t_{R1}$$

- t_{R1} and t_{R2} = migration times or distances along the baseline from the point of injection to the perpendiculars dropped from the maxima of two adjacent peaks,
- w_{h1} and w_{h2} = peak widths at half-height.

When appropriate, the resolution may be calculated by measuring the height of the valley (H_v) between 2 partly resolved peaks in a standard preparation and the height of the smaller peak (H_p) and calculating the peak-to-valley ratio:

$$\frac{p}{v} = \frac{H_p}{H_v}$$

SYMMETRY FACTOR

The symmetry factor (A_s) of a peak may be calculated using the expression:

$$A_s = \frac{w_{0.05}}{2d}$$

- $w_{0.05}$ = width of the peak at one-twentieth of the peak height,
- d = distance between the perpendicular dropped from the peak maximum and the leading edge of the peak at one-twentieth of the peak height.

Tests for area repeatability (standard deviation of areas or of the area/migration-time ratio) and for migration time repeatability (standard deviation of migration time) are introduced as suitability parameters. Migration time repeatability provides a test for the suitability of the capillary

washing procedures. An alternative practice to avoid the lack of repeatability of the migration time is to use migration time relative to an internal standard.

A test for the verification of the signal-to-noise ratio for a standard preparation (or the determination of the limit of quantification) may also be useful for the determination of related substances.

SIGNAL-TO-NOISE RATIO

The detection limit and quantification limit correspond to signal-to-noise ratios of 3 and 10 respectively. The signal-to-noise ratio (S/N) is calculated using the expression:

$$\frac{S}{N} = \frac{2H}{h}$$

- H = height of the peak corresponding to the component concerned, in the electropherogram obtained with the prescribed reference solution, measured from the maximum of the peak to the extrapolated baseline of the signal observed over a distance equal to twenty times the width at half-height,
- h = range of the background in an electropherogram obtained after injection of a blank, observed over a distance equal to twenty times the width at the half-height of the peak in the electropherogram obtained with the prescribed reference solution and, if possible, situated equally around the place where this peak would be found.

01/2005:20248

2.2.48. RAMAN SPECTROMETRY

Raman spectrometry (inelastic light scattering) is a light-scattering process in which the specimen under examination is irradiated with intense monochromatic light (usually laser light) and the light scattered from the specimen is analysed for frequency shifts.

Raman spectrometry is complementary to infrared spectrometry in the sense that the two techniques both probe the molecular vibrations in a material. However, Raman and infrared spectrometry have different relative sensitivities for different functional groups. Raman spectrometry is particularly sensitive to non-polar bonds (e.g. C-C single or multiple bonds) and less sensitive to polar bonds. Hence, water, which has a strong infrared absorption spectrum, is a weak Raman scatterer and is thus well suited as a solvent for Raman spectrometry.

Apparatus: Spectrometers for recording Raman spectra typically consist of the following components:

- a monochromatic light source, typically a laser, with a wavelength in the ultraviolet, visible or near-infrared region,
- suitable optics (lens, mirrors or optical-fibre assembly) which directs the irradiating light to and collects the scattered light from the sample,
- an optical device (monochromator or filter) that transmits the frequency-shifted Raman scattering and prevents the intense incident frequency (Rayleigh scattering) from reaching the detector,
- a dispersing device (grating or prism monochromator) combined with wavelength-selecting slits and a detector (usually a photomultiplier tube),

or:

- a dispersing device (grating or prism) combined with a multichannel detector (usually a charge-coupled device (CCD)),
- or:
- an interferometer with a detector that records the intensity of the scattered light over time, and a data-handling device that converts the data to the frequency or wavenumber domain by a Fourier-transform calculation.

PREPARATION OF THE SAMPLE

Raman spectra can be obtained from solids, liquids and gases either directly, or in glass containers or tubes, generally without prior sample preparation or dilution.

A major limitation of Raman spectrometry is that impurities may cause fluorescence that interferes with the detection of the much weaker Raman signal. Fluorescence may be avoided by choosing a laser source with a longer wavelength, for example in the near infrared, as the exciting line. The intensity of certain Raman lines may be enhanced in a number of ways, for instance in Resonance Raman (RR) and by Surface Enhanced Raman Spectrometry (SERS).

Due to the narrow focus of the irradiating laser beam, the spectrum is typically obtained from only a few microlitres of sample. Hence, sample inhomogeneities must be considered, unless the sample volume is increased, for example by rotation of the sample.

IDENTIFICATION AND QUANTITATION USING REFERENCE SUBSTANCES

Prepare the substance to be examined and the reference substance by the same procedure and record the spectra under the same operational conditions. The maxima in the spectrum obtained with the substance to be examined correspond in position and relative intensity to those in the spectrum obtained with the reference substance (CRS).

When the spectra recorded in the solid state show differences in the positions of the maxima, treat the substance to be examined and the reference substance in the same manner so that they crystallise or are produced in the same form, or proceed as described in the monograph, then record the spectra.

While Beer-Lambert's law is not valid for Raman spectrometry, Raman intensity is directly proportional to the concentration of the scattering species. As for other spectroscopic techniques, quantitation can be performed using known amounts or concentrations of reference substances. Owing to the small spatial resolution of the technique, care must be taken to ensure representative samples of standards and unknowns, for example by making sure that they are in the same physical state or by using an internal standard for liquid samples.

IDENTIFICATION AND QUANTITATION USING SPECTRAL LIBRARIES AND STATISTICAL METHODS FOR CLASSIFICATION AND CALIBRATION

Control of instrument performance. Use the apparatus according to the manufacturer's instructions and carry out the prescribed calibrations and system performance tests at regular intervals, depending on the use of the apparatus and the substances to be examined. When using Raman spectrometry for quantitative determinations, or when setting up spectral reference libraries for (chemometric) classification or calibration, particular care should be taken to ensure that corrections are made or measures are taken to control the variability in wavenumber and response-intensity of the instrumentation.

Verification of the wavenumber scale. Verify the wavenumber scale of the Raman shift (normally expressed in reciprocal centimetres) using a suitable standard which has characteristic maxima at the wavenumbers under investigation, for example, an organic substance, an Ne lamp or Ar⁺ plasma lines from an argon-ion laser.

The calibration measurement should be matched to the sample type, i.e. a solid calibration sample should be used for solid samples and a liquid calibration sample for liquid samples. Choose a suitable substance (e.g. indene, cyclohexane or naphthalene) for which accurate wavenumber shifts have been established. The indene sample can favourably be placed in an NMR tube, evacuated and sealed under inert gas, and stored cool in the dark to avoid degradation of the sample.

Table 2.2.48-1. — *Wavenumber shifts (and acceptable tolerances) of cyclohexane, indene and naphthalene.*

cyclohexane ^A	indene ^B	naphthalene ^A
		3056.4 (± 1.5)
2938.3 (± 1.5)		
2923.8 (± 1.5)		
2852.9 (± 1.5)		
	1609.7 (± 1.0)	1576.6 (± 1.0)
1444.4 (± 1.0)	1552.6 (± 1.0)	1464.5 (± 1.0)
1266.4 (± 1.0)	1205.2 (± 1.0)	1382.2 (± 1.0)
1157.6 (± 1.0)		1147.2 (± 1.0)
1028.3 (± 1.0)	1018.6 (± 1.0)	1021.6 (± 1.0)
801.3 (± 1.0)	730.5 (± 1.0)	763.8 (± 1.0)
	533.9 (± 1.0)	513.8 (± 1.0)

^A Standard guide for Raman shift standards for spectrometer calibration (American Society for Testing and Materials ASTM E 1840).

^B D. A. Carter, W. R. Thompson, C. E. Taylor and J. E. Pemberton, *Applied Spectroscopy*, 1995, 49 (11), 1561-1576.

Verification of the response-intensity scale. The absolute and relative intensities of the Raman bands are affected by several factors including:

- the state of polarisation of the irradiating light,
- the state of polarisation of the collection optics,
- the intensity of the irradiating light,
- differences in instrument response,
- differences in focus and geometry at sample,
- differences in packing density for solid samples.

Appropriate acceptance criteria will vary with the application but a day-to-day variation of ± 10 per cent in relative band intensities is achievable in most cases.

Establishment of a spectral reference library. Record the spectra of a suitable number of materials which have been fully tested (e.g. as prescribed in a monograph) and which exhibit the variation (manufacturer, batch, crystal modification, particle size, etc.) typical of the material to be analysed. The set of spectra represents the information that defines the similarity border or quantitative limits, which may be used, e.g. to identify the substance or control the amount formed in a manufacturing process. The number of substances in the database depends on the specific application. The collection of spectra in the database may be represented in different ways defined by the mathematical technique used for classification or quantitation.

The selectivity of the database which makes it possible to identify positively a given material and distinguish it adequately from other materials in the database is to be

established during the validation procedure. This selectivity must be challenged on a regular basis to ensure ongoing validity of the database; this is especially necessary after any major change in a substance (e.g. change in supplier or in the manufacturing process of the material) or in the set-up of the Raman instrument (e.g. verification of the wavenumber and response repeatability of the spectrometer).

This database is then valid for use only with the originating instrument, or with a similar instrument, provided the transferred database has been demonstrated to remain valid.

Method. Prepare and examine the sample in the same manner as for the establishment of the database. A suitable mathematical transformation of the Raman spectrum may be calculated to facilitate spectrum comparison or quantitative prediction.

Comparison of the spectra or transforms of the spectra or quantitative prediction of properties or amounts in the material in question may involve the use of a suitable chemometric or statistical classification or calibration technique.

01/2005:20249

2.2.49. FALLING BALL VISCOMETER METHOD

The determination of dynamic viscosity of Newtonian liquids using a suitable falling ball viscometer is performed at 20 ± 0.1 °C, unless otherwise prescribed in the monograph. The time required for a test ball to fall in the liquid to be examined from one ring mark to the other is determined. If no stricter limit is defined for the equipment used the result is valid only if 2 consecutive measures do not differ by more than 1.5 per cent.

Apparatus. The falling ball viscometer consists of: a glass tube enclosed in a mantle, which allow precise control of temperature; six balls made of glass, nickel-iron or steel with different densities and diameters. The tube is fixed in such a way that the axis is inclined by 10 ± 1° with regard to the vertical. The tube has 2 ring marks which define the distance the ball has to roll. Commercially available apparatus is supplied with tables giving the constants, the density of the balls and the suitability of the different balls for the expected range of viscosity.

Method. Fill the clean, dry tube of the viscometer, previously brought to 20 ± 0.1 °C, with the liquid to be examined, avoiding bubbles. Add the ball suitable for the range of viscosity of the liquid so as to obtain a falling time not less than 30 s. Close the tube and maintain the solution at 20 ± 0.1 °C for at least 15 min. Let the ball run through the liquid between the 2 ring marks once without measurement. Let it run again and measure with a stop-watch, to the nearest one-fifth of a second, the time required for the ball to roll from the upper to the lower ring mark. Repeat the test run at least 3 times.

Calculate the dynamic viscosity η in millipascal seconds using the formula:

$$\eta = k(\rho_1 - \rho_2) \times t$$

k = constant, expressed in millimeter squared per second squared,

ρ_1 = density of the ball used, expressed in grams per cubic centimetre,

- ρ_2 = density of the liquid to be examined, expressed in grams per cubic centimetre, obtained by multiplying its relative density d_{20}^{20} by 0.9982,
- t = falling time of the ball, in seconds.

01/2005:20254

2.2.54. ISOELECTRIC FOCUSING

GENERAL PRINCIPLES

Isoelectric focusing (IEF) is a method of electrophoresis that separates proteins according to their isoelectric point. Separation is carried out in a slab of polyacrylamide or agarose gel that contains a mixture of amphoteric electrolytes (ampholytes). When subjected to an electric field, the ampholytes migrate in the gel to create a pH gradient. In some cases gels containing an immobilised pH gradient, prepared by incorporating weak acids and bases to specific regions of the gel network during the preparation of the gel, are used. When the applied proteins reach the gel fraction that has a pH that is the same as their isoelectric point (pI), their charge is neutralised and migration ceases. Gradients can be made over various ranges of pH, according to the mixture of ampholytes chosen.

THEORETICAL ASPECTS

When a protein is at the position of its isoelectric point, it has no net charge and cannot be moved in a gel matrix by the electric field. It may, however, move from that position by diffusion. The pH gradient forces a protein to remain in its isoelectric point position, thus concentrating it; this concentrating effect is called "focusing". Increasing the applied voltage or reducing the sample load result in improved separation of bands. The applied voltage is limited by the heat generated, which must be dissipated. The use of thin gels and an efficient cooling plate controlled by a thermostatic circulator prevents the burning of the gel whilst allowing sharp focusing. The separation is estimated by determining the minimum pI difference (ΔpI), which is necessary to separate 2 neighbouring bands:

$$\Delta pI = 3 \times \sqrt{\frac{D (dpH/dx)}{E (-d\mu/dpH)}}$$

- D = diffusion coefficient of the protein,
- $\frac{dpH}{dx}$ = pH gradient,
- E = intensity of the electric field, in volts per centimetre,
- $-\frac{d\mu}{dpH}$ = variation of the solute mobility with the pH in the region close to the pI.

Since D and $-\frac{d\mu}{dpH}$ for a given protein cannot be altered, the separation can be improved by using a narrower pH range and by increasing the intensity of the electric field. Resolution between protein bands on an IEF gel prepared with carrier ampholytes can be quite good. Improvements in resolution may be achieved by using immobilised pH gradients where the buffering species, which are analogous to carrier ampholytes, are copolymerised within the gel matrix. Proteins exhibiting pIs differing by as little as 0.02 pH units may be resolved using a gel prepared with carrier ampholytes while immobilised pH gradients can resolve proteins differing by approximately 0.001 pH units.

PRACTICAL ASPECTS

Special attention must be paid to sample characteristics and/or preparation. Having salt in the sample can be problematic and it is best to prepare the sample, if possible, in deionised water or 2 per cent ampholytes, using dialysis or gel filtration if necessary.

The time required for completion of focusing in thin-layer polyacrylamide gels is determined by placing a coloured protein (e.g. haemoglobin) at different positions on the gel surface and by applying the electric field: the steady state is reached when all applications give an identical band pattern. In some protocols the completion of the focusing is indicated by the time elapsed after the sample application.

The IEF gel can be used as an identity test when the migration pattern on the gel is compared to a suitable standard preparation and IEF calibration proteins, the IEF gel can be used as a limit test when the density of a band on IEF is compared subjectively with the density of bands appearing in a standard preparation, or it can be used as a quantitative test when the density is measured using a densitometer or similar instrumentation to determine the relative concentration of protein in the bands subject to validation.

APPARATUS

An apparatus for IEF consists of:

- a controllable generator for constant potential, current and power; potentials of 2500 V have been used and are considered optimal under a given set of operating conditions; a supply of up to 30 W of constant power is recommended;
- a rigid plastic IEF chamber that contains a cooled plate, of suitable material, to support the gel;
- a plastic cover with platinum electrodes that are connected to the gel by means of paper wicks of suitable width, length and thickness, impregnated with solutions of anodic and cathodic electrolytes.

ISOELECTRIC FOCUSING IN POLYACRYLAMIDE GELS: DETAILED PROCEDURE

The following method is a detailed description of an IEF procedure in thick polyacrylamide slab gels, which is used unless otherwise stated in the monograph.

PREPARATION OF THE GELS

Mould. The mould (see Figure 2.2.54.-1) is composed of a glass plate (A) on which a polyester film (B) is placed to facilitate handling of the gel, one or more spacers (C), a second glass plate (D) and clamps to hold the structure together.

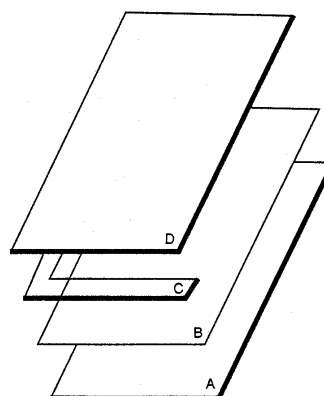


Figure 2.2.54.-1 – Mould

7.5 per cent polyacrylamide gel. Dissolve 29.1 g of *acrylamide R* and 0.9 g of *methylenabisacrylamide R* in 100 ml of *water R*. To 2.5 volumes of this solution, add the mixture of ampholytes specified in the monograph and dilute to 10 volumes with *water R*. Mix carefully and degas the solution.

Preparation of the mould. Place the polyester film on the lower glass plate, apply the spacer, place the second glass plate and fit the clamps. Before use, place the solution on a magnetic stirrer and add 0.25 volumes of a 100 g/l solution of *ammonium persulphate R* and 0.25 volumes of *tetramethylethylenediamine R*. Immediately fill the space between the glass plates of the mould with the solution.

METHOD

Dismantle the mould and, making use of the polyester film, transfer the gel onto the cooled support, wetted with a few millilitres of a suitable liquid, taking care to avoid forming air bubbles. Prepare the test solutions and reference solutions as specified in the monograph. Place strips of paper for sample application, about 10 mm × 5 mm in size, on the gel and impregnate each with the prescribed amount of the test and reference solutions. Also apply the prescribed quantity of a solution of proteins with known isoelectric points as pH markers to calibrate the gel. In some protocols the gel has pre-cast slots where a solution of the sample is applied instead of using impregnated paper strips. Cut 2 strips of paper to the length of the gel and impregnate them with the electrolyte solutions: acid for the anode and alkaline for the cathode. The compositions of the anode and cathode solutions are given in the monograph. Apply these paper wicks to each side of the gel several millimetres from the edge. Fit the cover so that the electrodes are in contact with the wicks (respecting the anodic and cathodic poles). Proceed with the isoelectric focusing by applying the electrical parameters described in the monograph. Switch off the current when the migration of the mixture of standard proteins has stabilised. Using forceps, remove the sample application strips and the 2 electrode wicks. Immerse the gel in *fixing solution for isoelectric focusing in polyacrylamide gel R*. Incubate with gentle shaking at room temperature for 30 min. Drain off the solution and add 200 ml of *destaining solution R*. Incubate with shaking for 1 h. Drain the gel, add *coomassie staining solution R*. Incubate for 30 min. Destain the gel by passive diffusion with *destaining solution R* until the bands are well visualised against a clear background. Locate the position and intensity of the bands in the electropherogram as prescribed in the monograph.

VARIATIONS TO THE DETAILED PROCEDURE (SUBJECT TO VALIDATION)

Where reference to the general method on isoelectric focusing is made, variations in methodology or procedure may be made subject to validation. These include:

- the use of commercially available pre-cast gels and of commercial staining and destaining kits,
- the use of immobilised pH gradients,
- the use of rod gels,
- the use of gel cassettes of different dimensions, including ultra-thin (0.2 mm) gels,
- variations in the sample application procedure, including different sample volumes or the use of sample application masks or wicks other than paper,
- the use of alternate running conditions, including variations in the electric field depending on gel dimensions and equipment, and the use of fixed migration times rather than subjective interpretation of band stability,

- the inclusion of a pre-focusing step,
- the use of automated instrumentation,
- the use of agarose gels.

VALIDATION OF ISO-ELECTRIC FOCUSING PROCEDURES

Where alternative methods to the detailed procedure are employed they must be validated. The following criteria may be used to validate the separation:

- formation of a stable pH gradient of desired characteristics, assessed for example using coloured pH markers of known isoelectric points,
- comparison with the electropherogram provided with the chemical reference substance for the preparation to be examined,
- any other validation criteria as prescribed in the monograph.

SPECIFIED VARIATIONS TO THE GENERAL METHOD

Variations to the general method required for the analysis of specific substances may be specified in detail in monographs. These include:

- the addition of urea in the gel (3 M concentration is often satisfactory to keep protein in solution but up to 8 M can be used): some proteins precipitate at their isoelectric point; in this case, urea is included in the gel formulation to keep the protein in solution; if urea is used, only fresh solutions should be used to prevent carbamylation of the protein;
- the use of alternative staining methods;
- the use of gel additives such as non-ionic detergents (e.g. octylglucoside) or zwitterionic detergents (e.g., CHAPS or CHAPSO), and the addition of ampholyte to the sample, to prevent proteins from aggregating or precipitating.

POINTS TO CONSIDER

Samples can be applied to any area on the gel, but to protect the proteins from extreme pH environments samples should not be applied close to either electrode. During method development the analyst can try applying the protein in 3 positions on the gel (i.e. middle and both ends); the pattern of a protein applied at opposite ends of the gel may not be identical.

A phenomenon known as cathodic drift, where the pH gradient decays over time, may occur if a gel is focused too long. Although not well understood, electroendosmosis and absorption of carbon dioxide may be factors that lead to cathodic drift. Cathodic drift is observed as focused protein migrating off the cathode end of the gel. Immobilised pH gradients may be used to address this problem.

Efficient cooling (approximately 4 °C) of the bed that the gel lies on during focusing is important. High field strengths used during isoelectric focusing can lead to overheating and affect the quality of the focused gel.

01/2005:20255

2.2.55. PEPTIDE MAPPING

Peptide mapping is an identity test for proteins, especially those obtained by rDNA technology. It involves the chemical or enzymatic treatment of a protein resulting in the formation of peptide fragments followed by separation and identification of these fragments in a reproducible manner. It is a powerful test that is capable of identifying almost any single amino acid changes resulting from events such as errors in the reading of complementary DNA

(cDNA) sequences or point mutations. Peptide mapping is a comparative procedure because the information obtained, compared to a reference substance similarly treated, confirms the primary structure of the protein, is capable of detecting whether alterations in structure have occurred, and demonstrates process consistency and genetic stability. Each protein presents unique characteristics which must be well understood so that the scientific and analytical approaches permit validated development of a peptide map that provides sufficient specificity.

This chapter provides detailed assistance in the application of peptide mapping and its validation to characterise the desired protein, to evaluate the stability of the expression construct of cells used for recombinant DNA products and to evaluate the consistency of the overall process, to assess product stability as well as to ensure the identity of the protein, or to detect the presence of protein variant.

Peptide mapping is not a general method, but involves developing specific maps for each unique protein. Although the technology is evolving rapidly, there are certain methods that are generally accepted. Variations of these methods will be indicated, when appropriate, in specific monographs.

A peptide map may be viewed as a fingerprint of a protein and is the end product of several chemical processes that provide a comprehensive understanding of the protein being analysed. 4 principal steps are necessary for the development of the procedure: isolation and purification of the protein, if the protein is part of a formulation; selective cleavage of the peptide bonds; chromatographic separation of the peptides; and analysis and identification of the peptides. A test sample is digested and assayed in parallel with a reference substance. Complete cleavage of peptide bonds is more likely to occur when enzymes such as endoproteases (e.g., trypsin) are used, instead of chemical cleavage reagents. A map must contain enough peptides to be meaningful. On the other hand, if there are too many fragments, the map might lose its specificity because many proteins will then have the same profiles.

ISOLATION AND PURIFICATION

Isolation and purification are necessary for analysis of bulk drugs or dosage forms containing interfering excipients and carrier proteins and, when required, will be specified in the monograph. Quantitative recovery of protein from the dosage form must be validated.

SELECTIVE CLEAVAGE OF PEPTIDE BONDS

The selection of the approach used for the cleavage of peptide bonds will depend on the protein under test. This selection process involves determination of the type of cleavage to be employed, enzymatic or chemical, and the type of cleavage agent within the chosen category. Several cleavage agents and their specificity are shown in Table 2.2.55.-1. This list is not all-inclusive and will be expanded as other cleavage agents are identified.

Pretreatment of sample. Depending on the size or the configuration of the protein, different approaches in the pretreatment of samples can be used. If trypsin is used as a cleavage agent for proteins with a molecular mass greater than 100 000 Da, lysine residues must be protected by citraconylation or maleylation; otherwise, too many peptides will be generated.

Pretreatment of the cleavage agent. Pretreatment of cleavage agents, especially enzymatic agents, might be necessary for purification purposes to ensure reproducibility of the map. For example, trypsin used as a cleavage agent will have to be treated with tosyl-L-phenylalanine chloromethyl ketone to inactivate chymotrypsin. Other methods, such as purification of trypsin by high performance liquid chromatography (HPLC) or immobilisation of enzyme on a gel support, have been successfully used when only a small amount of protein is available.

Pretreatment of the protein. Under certain conditions, it might be necessary to concentrate the sample or to separate the protein from added substances and stabilisers used in formulation of the product, if these interfere with the mapping procedure. Physical procedures used for pretreatment can include ultrafiltration, column chromatography and lyophilization. Other pretreatments, such as the addition of chaotropic agents (e.g. urea) can be used to unfold the protein prior to mapping. To allow the enzyme to have full access to cleavage sites and permit some unfolding of the protein, it is often necessary to reduce and alkylate the disulphide bonds prior to digestion.

Digestion with trypsin can introduce ambiguities in the peptide map due to side reactions occurring during the digestion reaction, such as non-specific cleavage, deamidation, disulphide isomerisation, oxidation of methionine residues, or formation of pyroglutamic groups created from the deamidation of glutamine at the *N*-terminal side of a peptide. Furthermore, peaks may be produced by

Table 2.2.55.-1. – Examples of cleavage agents

Type	Agent	Specificity
Enzymatic	Trypsin (EC 3.4.21.4)	C-terminal side of Arg and Lys
	Chymotrypsin (EC 3.4.21.1)	C-terminal side of hydrophobic residues (e.g. Leu, Met, Ala, aromatics)
	Pepsin (EC 3.4.23.1 and 2)	Non-specific digest
	Lysyl endopeptidase (Lys-C endopeptidase) (EC 3.4.21.50)	C-terminal side of Lys
	Glutamyl endopeptidase (from <i>S. aureus</i> strain V8) (EC 3.4.21.19)	C-terminal side of Glu and Asp
	Peptidyl-Asp metallo-endopeptidase (endoproteinase Asp-N)	<i>N</i> -terminal side of Asp
	Clostripain (EC 3.4.22.8)	C-terminal side of Arg
Chemical	Cyanogen bromide	C-terminal side of Met
	2-Nitro-5-thio-cyanobenzoic acid	<i>N</i> -terminal side of Cys
	<i>O</i> -Iodosobenzoic acid	C-terminal side of Trp and Tyr
	Dilute acid	Asp and Pro
	BNPS-skatole	Trp

autohydrolysis of trypsin. Their intensities depend on the ratio of trypsin to protein. To avoid autohydrolysis, solutions of proteases may be prepared at a pH that is not optimal (e.g. at pH 5 for trypsin), which would mean that the enzyme would not become active until diluted with the digest buffer.

Establishment of optimal digestion conditions. Factors that affect the completeness and effectiveness of digestion of proteins are those that could affect any chemical or enzymatic reactions.

pH of the reaction milieu. The pH of the digestion mixture is empirically determined to ensure the optimisation of the performance of the given cleavage agent. For example, when using cyanogen bromide as a cleavage agent, a highly acidic environment (e.g. pH 2, formic acid) is necessary; however, when using trypsin as a cleavage agent, a slightly alkaline environment (pH 8) is optimal. As a general rule, the pH of the reaction milieu must not alter the chemical integrity of the protein during the digestion and must not change during the course of the fragmentation reaction.

Temperature. A temperature between 25 °C and 37 °C is adequate for most digestions. The temperature used is intended to minimise chemical side reactions. The type of protein under test will dictate the temperature of the reaction milieu, because some proteins are more susceptible to denaturation as the temperature of the reaction increases. For example, digestion of recombinant bovine somatotropin is conducted at 4 °C, because at higher temperatures it will precipitate during digestion.

Time. If sufficient sample is available, a time course study is considered in order to determine the optimum time to obtain a reproducible map and avoid incomplete digestion. Time of digestion varies from 2 h to 30 h. The reaction is stopped by the addition of an acid which does not interfere in the map or by freezing.

Amount of cleavage agent used. Although excessive amounts of cleavage agent are used to accomplish a reasonably rapid digestion time (i.e. 6-20 hours), the amount of cleavage agent is minimised to avoid its contribution to the chromatographic map pattern. A protein to protease ratio between 20:1 and 200:1 is generally used. It is recommended that the cleavage agent is added in 2 or more stages to optimise cleavage. Nonetheless, the final reaction volume remains small enough to facilitate the next step in peptide mapping, the separation step. To sort out digestion artifacts that might interfere with the subsequent analysis, a blank determination is performed, using a digestion control with all the reagents, except the test protein.

CHROMATOGRAPHIC SEPARATION

Many techniques are used to separate peptides for mapping. The selection of a technique depends on the protein being mapped. Techniques that have been successfully used for separation of peptides are shown in Table 2.2.55-2. In this section, a most widely used reversed-phase HPLC method is described as one of the procedures of chromatographic separation.

The purity of solvents and mobile phases is a critical factor in HPLC separation. HPLC-grade solvents and water that are commercially available, are recommended for reversed-phase HPLC. Dissolved gases present a problem in gradient systems where the solubility of the gas in a solvent may be less in a mixture than in a single solvent. Vacuum degassing and agitation by sonication are often used as useful degassing procedures. When solid particles in the solvents are drawn into the HPLC system, they can damage the sealing of pump valves or clog the top of the chromatographic column. Both pre- and post-pump filtration is also recommended.

Table 2.2.55-2. – *Techniques used for the separation of peptides*

Reversed-phase high performance liquid chromatography (HPLC)
Ion-exchange chromatography (IEC)
Hydrophobic interaction chromatography (HIC)
Polyacrylamide gel electrophoresis (PAGE), non-denaturing
Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)
Capillary electrophoresis (CE)
Paper chromatography-high voltage (PCHV)
High voltage-paper electrophoresis (HVPE)

Chromatographic column. The selection of a chromatographic column is empirically determined for each protein. Columns with 10 nm or 30 nm pore size with silica support can give optimal separation. For smaller peptides, *octylsilyl silica gel for chromatography R* (3-10 µm) and *octadecylsilyl silica gel for chromatography R* (3-10 µm) column packings are more efficient than *butylsilyl silica gel for chromatography R* (5-10 µm).

Solvent. The most commonly used solvent is water with acetonitrile as the organic modifier to which not more than 0.1 per cent trifluoroacetic acid is added. If necessary, add propyl alcohol or isopropyl alcohol to solubilise the digest components, provided that the addition does not unduly increase the viscosity of the components.

Mobile phase. Buffered mobile phases containing phosphate are used to provide some flexibility in the selection of pH conditions, since shifts of pH in the 3.0-5.0 range enhance the separation of peptides containing acidic residues (e.g. glutamic and aspartic acids). Sodium or potassium phosphates, ammonium acetate, phosphoric acid at a pH between 2 and 7 (or higher for polymer-based supports) have also been used with acetonitrile gradients. Acetonitrile containing trifluoroacetic acid is used quite often.

Gradient. Gradients can be linear, nonlinear, or include step functions. A shallow gradient is recommended in order to separate complex mixtures. Gradients are optimised to provide clear resolution of 1 or 2 peaks that will become "marker" peaks for the test.

Isocratic elution. Isocratic HPLC systems using a single mobile phase are used on the basis of their convenience of use and improved detector responses. Optimal composition of a mobile phase to obtain clear resolution of each peak is sometimes difficult to establish. Mobile phases for which slight changes in component ratios or in pH significantly affect retention times of peaks in peptide maps must not be used in isocratic HPLC systems.

Other parameters. Temperature control of the column is usually necessary to achieve good reproducibility. The flow rates for the mobile phases range from 0.1-2.0 ml/min, and the detection of peptides is performed with a UV detector at 200-230 nm. Other methods of detection have been used (e.g. post-column derivatisation), but they are not as robust or versatile as UV detection.

Validation. This section provides an experimental means for measuring the overall performance of the test method. The acceptance criteria for system suitability depend on the identification of critical test parameters that affect data interpretation and acceptance. These critical parameters are also criteria that monitor peptide digestion and peptide analysis. An indicator that the desired digestion endpoint has been achieved is shown by comparison with a reference standard, which is treated in the same manner as the test protein. The use of a reference substance in parallel with the

test protein is critical in the development and establishment of system suitability limits. In addition, a specimen chromatogram is included with the reference substance for additional comparison purposes. Other indicators may include visual inspection of protein or peptide solubility, the absence of intact protein, or measurement of responses of a digestion-dependent peptide. The critical system suitability parameters for peptide analysis will depend on the particular mode of peptide separation and detection and on the data analysis requirements.

When peptide mapping is used as an identification test, the system suitability requirements for the identified peptides cover selectivity and precision. In this case, as well as when identification of variant protein is done, the identification of the primary structure of the peptide fragments in the peptide map provides both a verification of the known primary structure and the identification of protein variants by comparison with the peptide map of the reference substance for the specified protein. The use of a digested reference substance for a given protein in the determination of peptide resolution is the method of choice. For an analysis of a variant protein, a characterised mixture of a variant and a reference substance can be used, especially if the variant peptide is located in a less-resolved region of the map. The index of pattern consistency can be simply the number of major peptides detected. Peptide pattern consistency can be best defined by the resolution of peptide peaks. Chromatographic parameters, such as peak-to-peak resolution, maximum peak width, peak area, peak tailing factors, and column efficiency, may be used to define peptide resolution. Depending on the protein under test and the method of separation used, single peptide or multiple peptide resolution requirements may be necessary.

The replicate analysis of the digest of the reference substance for the protein under test yields measures of precision and quantitative recovery. Recovery of the identified peptides is generally ascertained by the use of internal or external peptide standards. The precision is expressed as the relative standard deviation (RSD). Differences in the recovery and precision of the identified peptides are to be expected; therefore, the system suitability limits will have to be established for both the recovery and the precision of the identified peptides. These limits are unique for a given protein and will be specified in the individual monograph.

Visual comparison of the relative retentions, the peak responses (the peak area or the peak height), the number of peaks, and the overall elution pattern is completed initially. It is then complemented and supported by mathematical analysis of the peak response ratios and by the chromatographic profile of a 1:1 (V/V) mixture of sample and reference substance digest. If all peaks in the sample digest and in the reference substance digest have the same relative retentions and peak response ratios, then the identity of the sample under test is confirmed.

If peaks that initially eluted with significantly different relative retentions are then observed as single peaks in the 1:1 mixture, the initial difference would be an indication of system variability. However, if separate peaks are observed in the 1:1 mixture, this would be evidence of the nonequivalence of the peptides in each peak. If a peak in the 1:1 mixture is significantly broader than the corresponding peak in the sample and reference substance digest, it may indicate the presence of different peptides. The use of computer-aided pattern recognition software for the analysis of peptide mapping data has been proposed and applied, but issues related to the validation of the computer software preclude its use in a compendial test in the near future. Other automated approaches have been

used that employ mathematical formulas, models, and pattern recognition. Such approaches are, for example, the automated identification of compounds by IR spectroscopy and the application of diode-array UV spectral analysis for identification of peptides. These methods have limitations due to inadequate resolutions, co-elution of fragments, or absolute peak response differences between reference substance and sample digest fragments.

The numerical comparison of the peak retention times and peak areas or peak heights can be done for a selected group of relevant peaks that have been correctly identified in the peptide maps. Peak areas can be calculated using 1 peak showing relatively small variation as an internal reference, keeping in mind that peak area integration is sensitive to baseline variation and likely to introduce error in the analysis. Alternatively, the percentage of each peptide peak height relative to the sum of all peak heights can be calculated for the sample under test. The percentage is then compared to that of the corresponding peak of the reference substance. The possibility of auto-hydrolysis of trypsin is monitored by producing a blank peptide map, that is, the peptide map obtained when a blank solution is treated with trypsin.

The minimum requirement for the qualification of peptide mapping is an approved test procedure that includes system suitability as a test control. In general, early in the regulatory process, qualification of peptide mapping for a protein is sufficient. As the regulatory approval process for the protein progresses, additional qualifications of the test can include a partial validation of the analytical procedure to provide assurance that the method will perform as intended in the development of a peptide map for the specified protein.

ANALYSIS AND IDENTIFICATION OF PEPTIDES

This section gives guidance on the use of peptide mapping during development in support of regulatory applications.

The use of a peptide map as a qualitative tool does not require the complete characterisation of the individual peptide peaks. However, validation of peptide mapping in support of regulatory applications requires rigorous characterisation of each of the individual peaks in the peptide map. Methods to characterise peaks range from *N*-terminal sequencing of each peak followed by amino acid analysis to the use of mass spectroscopy (MS).

For characterisation purposes, when *N*-terminal sequencing and amino acids analysis are used, the analytical separation is scaled up. Since scale-up might affect the resolution of peptide peaks, it is necessary, using empirical data, to assure that there is no loss of resolution due to scale-up. Eluates corresponding to specific peptide peaks are collected, vacuum-concentrated, and chromatographed again, if necessary. Amino acid analysis of fragments may be limited by the peptide size. If the *N*-terminus is blocked, it may need to be cleared before sequencing. *C*-terminal sequencing of proteins in combination with carboxypeptidase and matrix-assisted laser desorption ionisation coupled to time-of-flight analyser (MALDI-TOF) can also be used for characterisation purposes.

The use of MS for characterisation of peptide fragments is by direct infusion of isolated peptides or by the use of on-line LC-MS for structure analysis. In general, it includes electrospray and MALDI-TOF-MS, as well as fast-atom bombardment (FAB). Tandem MS has also been used to sequence a modified protein and to determine the type of amino acid modification that has occurred. The comparison of mass spectra of the digests before and after reduction provides a method to assign the disulphide bonds to the various sulphydryl-containing peptides.

If regions of the primary structure are not clearly demonstrated by the peptide map, it might be necessary to develop a secondary peptide map. The goal of a validated method of characterisation of a protein through peptide mapping is to reconcile and account for at least 95 per cent of the theoretical composition of the protein structure.

01/2005:20256

2.2.56. AMINO ACID ANALYSIS

Amino acid analysis refers to the methodology used to determine the amino acid composition or content of proteins, peptides, and other pharmaceutical preparations. Proteins and peptides are macromolecules consisting of covalently bonded amino acid residues organised as a linear polymer. The sequence of the amino acids in a protein or peptide determines the properties of the molecule. Proteins are considered large molecules that commonly exist as folded structures with a specific conformation, while peptides are smaller and may consist of only a few amino acids. Amino acid analysis can be used to quantify proteins and peptides, to determine the identity of proteins or peptides based on their amino acid composition, to support protein and peptide structure analysis, to evaluate fragmentation strategies for peptide mapping, and to detect atypical amino acids that might be present in a protein or peptide. It is necessary to hydrolyse a protein/peptide to its individual amino acid constituents before amino acid analysis. Following protein/peptide hydrolysis, the amino acid analysis procedure can be the same as that practiced for free amino acids in other pharmaceutical preparations. The amino acid constituents of the test sample are typically derivatised for analysis.

APPARATUS

Methods used for amino acid analysis are usually based on a chromatographic separation of the amino acids present in the test sample. Current techniques take advantage of the automated chromatographic instrumentation designed for analytical methodologies. An amino acid analysis instrument will typically be a low-pressure or high-pressure liquid chromatograph capable of generating mobile phase gradients that separate the amino acid analytes on a chromatographic column. The instrument must have post-column derivatisation capability, unless the sample is analysed using precolumn derivatisation. The detector is usually an ultraviolet/visible or fluorescence detector depending on the derivatisation method used. A recording device (e.g., integrator) is used for transforming the analogue signal from the detector and for quantitation. It is preferred that instrumentation be dedicated particularly for amino acid analysis.

GENERAL PRECAUTIONS

Background contamination is always a concern for the analyst in performing amino acid analysis. High purity reagents are necessary (e.g., low purity hydrochloric acid can contribute to glycine contamination). Analytical reagents are changed routinely every few weeks using only high-pressure liquid chromatography (HPLC) grade solvents. Potential microbial contamination and foreign material that might be present in the solvents are reduced by filtering solvents before use, keeping solvent reservoirs covered, and not placing amino acid analysis instrumentation in direct sunlight.

Laboratory practices can determine the quality of the amino acid analysis. Place the instrumentation in a low traffic area of the laboratory. Keep the laboratory clean. Clean and

calibrate pipets according to a maintenance schedule. Keep pipet tips in a covered box; the analysts may not handle pipet tips with their hands. The analysts may wear powder-free latex or equivalent gloves. Limit the number of times a test sample vial is opened and closed because dust can contribute to elevated levels of glycine, serine, and alanine.

A well-maintained instrument is necessary for acceptable amino acid analysis results. If the instrument is used on a routine basis, it is to be checked daily for leaks, detector and lamp stability, and the ability of the column to maintain resolution of the individual amino acids. Clean or replace all instrument filters and other maintenance items on a routine schedule.

REFERENCE MATERIAL

Acceptable amino acid standards are commercially available for amino acid analysis and typically consist of an aqueous mixture of amino acids. When determining amino acid composition, protein or peptide standards are analysed with the test material as a control to demonstrate the integrity of the entire procedure. Highly purified bovine serum albumin has been used as a protein standard for this purpose.

CALIBRATION OF INSTRUMENTATION

Calibration of amino acid analysis instrumentation typically involves analysing the amino acid standard, which consists of a mixture of amino acids at a number of concentrations, to determine the response factor and range of analysis for each amino acid. The concentration of each amino acid in the standard is known. In the calibration procedure, the analyst dilutes the amino acid standard to several different analyte levels within the expected linear range of the amino acid analysis technique. Then, replicates at each of the different analyte levels can be analysed. Peak areas obtained for each amino acid are plotted versus the known concentration for each of the amino acids in the standard dilution. These results will allow the analyst to determine the range of amino acid concentrations where the peak area of a given amino acid is an approximately linear function of the amino acid concentration. It is important that the analyst prepare the samples for amino acid analysis so that they are within the analytical limits (e.g., linear working range) of the technique employed in order to obtain accurate and repeatable results.

4 to 6 amino acid standard levels are analysed to determine a response factor for each amino acid. The response factor is calculated as the average peak area or peak height per nanomole of amino acid present in the standard. A calibration file consisting of the response factor for each amino acid is prepared and used to calculate the concentration of each amino acid present in the test sample. This calculation involves dividing the peak area corresponding to a given amino acid by the response factor for that amino acid to give the nanomoles of the amino acid. For routine analysis, a single-point calibration may be sufficient; however, the calibration file is updated frequently and tested by the analysis of analytical controls to ensure its integrity.

REPEATABILITY

Consistent high quality amino acid analysis results from an analytical laboratory require attention to the repeatability of the assay. During analysis of the chromatographic separation of the amino acids or their derivatives, numerous peaks can be observed on the chromatogram that correspond to the amino acids. The large number of peaks makes it necessary to have an amino acid analysis system that can repeatedly identify the peaks based on retention time and integrate the peak areas for quantitation. A typical repeatability evaluation involves preparing a standard amino acid solution and analysing many replicates (e.g., 6 analyses or more)

of the same standard solution. The relative standard deviation (RSD) is determined for the retention time and integrated peak area of each amino acid. An evaluation of the repeatability is expanded to include multiple assays conducted over several days by different analysts. Multiple assays include the preparation of standard dilutions from starting materials to determine the variation due to sample handling. The amino acid composition of a standard protein (e.g., bovine serum albumin) is often analysed as part of the repeatability evaluation. By evaluating the replicate variation (i.e., RSD), the laboratory can establish analytical limits to ensure that the analyses from the laboratory are under control. It is desirable to establish the lowest practical variation limits to ensure the best results. Areas to focus on to lower the variability of the amino acid analysis include sample preparation, high background spectral interference due to quality of reagents and/or laboratory practices, instrument performance and maintenance, data analysis and interpretation, and analyst performance and habits. All parameters involved are fully investigated in the scope of the validation work.

SAMPLE PREPARATION

Accurate results from amino acid analysis require purified protein and peptide samples. Buffer components (e.g., salts, urea, detergents) can interfere with the amino acid analysis and are removed from the sample before analysis. Methods that utilise post-column derivatisation of the amino acids are generally not affected by buffer components to the extent seen with pre-column derivatisation methods. It is desirable to limit the number of sample manipulations to reduce potential background contamination, to improve analyte recovery, and to reduce labour. Common techniques used to remove buffer components from protein samples include the following methods: (1) injecting the protein sample onto a reversed-phase HPLC system, removing the protein with a volatile solvent containing a sufficient organic component, and drying the sample in a vacuum centrifuge; (2) dialysis against a volatile buffer or water; (3) centrifugal ultrafiltration for buffer replacement with a volatile buffer or water; (4) precipitating the protein from the buffer using an organic solvent (e.g., acetone); (5) gel filtration.

INTERNAL STANDARDS

It is recommended that an internal standard be used to monitor physical and chemical losses and variations during amino acid analysis. An accurately known amount of internal standard can be added to a protein solution prior to hydrolysis. The recovery of the internal standard gives the general recovery of the amino acids of the protein solution. Free amino acids, however, do not behave in the same way as protein-bound amino acids during hydrolysis, whose rates of release or destruction are variable. Therefore, the use of an internal standard to correct for losses during hydrolysis may give unreliable results. It will be necessary to take this point into consideration when interpreting the results. Internal standards can also be added to the mixture of amino acids after hydrolysis to correct for differences in sample application and changes in reagent stability and flow rates. Ideally, an internal standard is an unnaturally occurring primary amino acid that is commercially available and inexpensive. It should also be stable during hydrolysis, its response factor should be linear with concentration, and it needs to elute with a unique retention time without overlapping other amino acids. Commonly used amino acid standards include norleucine, nitrotyrosine, and α -aminobutyric acid.

PROTEIN HYDROLYSIS

Hydrolysis of protein and peptide samples is necessary for amino acid analysis of these molecules. The glassware used for hydrolysis must be very clean to avoid erroneous results. Glove powders and fingerprints on hydrolysis tubes may cause contamination. To clean glass hydrolysis tubes, boil tubes for 1 h in 1 M hydrochloric acid or soak tubes in concentrated nitric acid or in a mixture of equal volumes of concentrated hydrochloric acid and nitric acid. Clean hydrolysis tubes are rinsed with high-purity water followed by a rinse with HPLC grade methanol, dried overnight in an oven, and stored covered until use. Alternatively, pyrolysis of clean glassware at 500 °C for 4 h may also be used to eliminate contamination from hydrolysis tubes. Adequate disposable laboratory material can also be used.

Acid hydrolysis is the most common method for hydrolysing a protein sample before amino acid analysis. The acid hydrolysis technique can contribute to the variation of the analysis due to complete or partial destruction of several amino acids: tryptophan is destroyed; serine and threonine are partially destroyed; methionine might undergo oxidation; and cysteine is typically recovered as cystine (but cystine recovery is usually poor because of partial destruction or reduction to cysteine). Application of adequate vacuum (less than 200 μ m of mercury or 26.7 Pa) or introduction of an inert gas (argon) in the headspace of the reaction vessel can reduce the level of oxidative destruction. In peptide bonds involving isoleucine and valine the amido bonds of Ile-Ile, Val-Val, Ile-Val, and Val-Ile are partially cleaved; and asparagine and glutamine are deamidated, resulting in aspartic acid and glutamic acid, respectively. The loss of tryptophan, asparagine, and glutamine during an acid hydrolysis limits quantitation to 17 amino acids. Some of the hydrolysis techniques described are used to address these concerns. Some of the hydrolysis techniques described (i.e., Methods 4-11) may cause modifications to other amino acids. Therefore, the benefits of using a given hydrolysis technique are weighed against the concerns with the technique and are tested adequately before employing a method other than acid hydrolysis.

A time-course study (i.e., amino acid analysis at acid hydrolysis times of 24 h, 48 h and 72 h) is often employed to analyse the starting concentration of amino acids that are partially destroyed or slow to cleave. By plotting the observed concentration of labile amino acids (e.g., serine and threonine) versus hydrolysis time, the line can be extrapolated to the origin to determine the starting concentration of these amino acids. Time-course hydrolysis studies are also used with amino acids that are slow to cleave (e.g., isoleucine and valine). During the hydrolysis time course, the analyst will observe a plateau in these residues. The level of this plateau is taken as the residue concentration. If the hydrolysis time is too long, the residue concentration of the sample will begin to decrease, indicating destruction by the hydrolysis conditions.

An acceptable alternative to the time-course study is to subject an amino acid calibration standard to the same hydrolysis conditions as the test sample. The amino acid in free form may not completely represent the rate of destruction of labile amino acids within a peptide or protein during the hydrolysis. This is especially true for peptide bonds that are slow to cleave (e.g., Ile-Val bonds). However, this technique will allow the analyst to account for some residue destruction. Microwave acid hydrolysis has been used and is rapid but requires special equipment as well as special precautions. The optimal conditions for microwave hydrolysis must be investigated for each individual protein/peptide sample. The microwave hydrolysis

technique typically requires only a few minutes, but even a deviation of one minute may give inadequate results (e.g., incomplete hydrolysis or destruction of labile amino acids). Complete proteolysis, using a mixture of proteases, has been used but can be complicated, requires the proper controls, and is typically more applicable to peptides than proteins.

During initial analyses of an unknown protein, experiments with various hydrolysis time and temperature conditions are conducted to determine the optimal conditions.

METHOD 1

Acid hydrolysis using hydrochloric acid containing phenol is the most common procedure used for protein/peptide hydrolysis preceding amino acid analysis. The addition of phenol to the reaction prevents the halogenation of tyrosine.

Hydrolysis solution. 6 M hydrochloric acid containing 0.1 per cent to 1.0 per cent of phenol.

Procedure

Liquid phase hydrolysis. Place the protein or peptide sample in a hydrolysis tube, and dry (the sample is dried so that water in the sample will not dilute the acid used for the hydrolysis). Add 200 µl of hydrolysis solution per 500 µg of lyophilised protein. Freeze the sample tube in a dry ice-acetone bath, and flame seal *in vacuo*. Samples are typically hydrolysed at 110 °C for 24 h *in vacuo* or in an inert atmosphere to prevent oxidation. Longer hydrolysis times (e.g., 48 h and 72 h) are investigated if there is a concern that the protein is not completely hydrolysed.

Vapour phase hydrolysis. This is one of the most common acid hydrolysis procedures, and it is preferred for microanalysis when only small amounts of the sample are available. Contamination of the sample from the acid reagent is also minimised by using vapour phase hydrolysis. Place vials containing the dried samples in a vessel that contains an appropriate amount of hydrolysis solution. The hydrolysis solution does not come in contact with the test sample. Apply an inert atmosphere or vacuum (less than 200 µm of mercury or 26.7 Pa) to the headspace of the vessel, and heat to about 110 °C for a 24 h hydrolysis time. Acid vapour hydrolyses the dried sample. Any condensation of the acid in the sample vials is to be minimised. After hydrolysis, dry the test sample *in vacuo* to remove any residual acid.

METHOD 2

Tryptophan oxidation during hydrolysis is decreased by using mercaptoethanesulfonic acid as the reducing acid.

Hydrolysis solution. 2.5 M mercaptoethanesulfonic acid solution.

Vapour phase hydrolysis. Dry about 1 µg to 100 µg of the protein/peptide under test in a hydrolysis tube. Place the hydrolysis tube in a larger tube with about 200 µl of the hydrolysis solution. Seal the larger tube *in vacuo* (about 50 µm of mercury or 6.7 Pa) to vaporise the hydrolysis solution. Heat the hydrolysis tube to 170-185 °C for about 12.5 min. After hydrolysis, dry the hydrolysis tube *in vacuo* for 15 min to remove the residual acid.

METHOD 3

Tryptophan oxidation during hydrolysis is prevented by using thioglycolic acid (TGA) as the reducing acid.

Hydrolysis solution. 7 M hydrochloric acid containing 1 per cent of phenol, 10 per cent of trifluoroacetic acid and 20 per cent of thioglycolic acid.

Vapour phase hydrolysis. Dry about 10 µg to 50 µg of the protein/peptide under test in a sample tube. Place the sample tube in a larger tube with about 200 µl of the hydrolysis solution. Seal the larger tube *in vacuo* (about 50 µm of mercury or 6.7 Pa) to vaporise the TGA. Heat the

sample tube to 166 °C for about 15-30 min. After hydrolysis, dry the sample tube *in vacuo* for 5 min to remove the residual acid. Recovery of tryptophan by this method may be dependent on the amount of sample present.

METHOD 4

Cysteine/cystine and methionine oxidation is performed with performic acid before the protein hydrolysis.

Oxidation solution. Use performic acid freshly prepared by mixing 1 volume of hydrogen peroxide solution (30 per cent) and 9 volumes of anhydrous formic acid and incubating at room temperature for 1 h.

Procedure. Dissolve the protein/peptide sample in 20 µl of anhydrous formic acid and heat at 50 °C for 5 min; then add 100 µl of the oxidation solution. Allow the oxidation to proceed for 10-30 min. In this reaction, cysteine is converted to cysteic acid and methionine is converted to methionine-sulphone. Remove the excess reagent from the sample in a vacuum centrifuge. The oxidised protein can then be acid hydrolysed using Method 1 or Method 2. This technique may cause modifications to tyrosine residues in the presence of halides.

METHOD 5

Cysteine/cystine oxidation is accomplished during the liquid phase hydrolysis with sodium azide.

Hydrolysis solution. To 6 M hydrochloric acid containing 0.2 per cent of phenol, add sodium azide to obtain a final concentration of 2 g/l. The added phenol prevents halogenation of tyrosine.

Liquid phase hydrolysis. Conduct the protein/peptide hydrolysis at about 110 °C for 24 h. During the hydrolysis, the cysteine/cystine present in the sample is converted to cysteic acid by the sodium azide present in the hydrolysis solution. This technique allows better tyrosine recovery than Method 4, but it is not quantitative for methionine. Methionine is converted to a mixture of the parent methionine and its 2 oxidative products, methionine-sulphoxide and methionine-sulphone.

METHOD 6

Cysteine/cystine oxidation is accomplished with dimethyl sulphoxide (DMSO).

Hydrolysis solution. To 6 M hydrochloric acid containing 0.1 per cent to 1.0 per cent of phenol, add dimethyl sulphoxide to obtain a final concentration of 2 per cent V/V.

Vapour phase hydrolysis. Conduct the protein/peptide hydrolysis at about 110 °C for 24 h. During the hydrolysis, the cysteine/cystine present in the sample is converted to cysteic acid by the DMSO present in the hydrolysis solution. As an approach to limit variability and compensate for partial destruction, it is recommended to evaluate the cysteic acid recovery from oxidative hydrolysis of standard proteins containing 1-8 mol of cysteine. The response factors from protein/peptide hydrolysates are typically about 30 per cent lower than those for non-hydrolysed cysteic acid standards. Because histidine, methionine, tyrosine, and tryptophan are also modified, a complete compositional analysis is not obtained with this technique.

METHOD 7

Cysteine/cystine reduction and alkylation is accomplished by a vapour phase pyridylethylation reaction.

Reducing solution. Transfer 83.3 µl of pyridine, 16.7 µl of 4-vinylpyridine, 16.7 µl of tributylphosphine, and 83.3 µl of water to a suitable container and mix.

Procedure. Add the protein/peptide (between 1 and 100 µg) to a hydrolysis tube, and place in a larger tube. Transfer the reducing solution to the large tube, seal *in vacuo* (about

50 µm of mercury or 6.7 Pa), and heat at about 100 °C for 5 min. Then remove the inner hydrolysis tube, and dry it in a vacuum desiccator for 15 min to remove residual reagents. The pyridylethylated sample can then be acid hydrolysed using previously described procedures. The pyridylethylation reaction is performed simultaneously with a protein standard sample containing 1-8 mol of cysteine to evaluate the pyridylethyl-cysteine recovery. Longer incubation times for the pyridylethylation reaction can cause modifications to the α-amino terminal group and the ε-amino group of lysine in the protein.

METHOD 8

Cysteine/cystine reduction and alkylation is accomplished by a liquid phase pyridylethylation reaction.

Stock solutions. Prepare and filter 3 solutions: 1 M Tris-hydrochloride pH 8.5 containing 4 mM disodium edetate (stock solution A), 8 M guanidine hydrochloride (stock solution B), and 10 per cent of 2-mercaptoethanol (stock solution C).

Reducing solution. Prepare a mixture of 1 volume of stock solution A and 3 volumes of stock solution B to obtain a buffered solution of 6 M guanidine hydrochloride in 0.25 M tris-hydrochloride.

Procedure. Dissolve about 10 µg of the test sample in 50 µl of the reducing solution, and add about 2.5 µl of stock solution C. Store under nitrogen or argon for 2 h at room temperature in the dark. To achieve the pyridylethylation reaction, add about 2 µl of 4-vinylpyridine to the protein solution, and incubate for an additional 2 h at room temperature in the dark. Desalt the protein/peptide by collecting the protein/peptide fraction from a reversed-phase HPLC separation. The collected sample can be dried in a vacuum centrifuge before acid hydrolysis.

METHOD 9

Cysteine/cystine reduction and alkylation is accomplished by a liquid phase carboxymethylation reaction.

Stock solutions. Prepare as directed for Method 8.

Carboxymethylation solution. Prepare a 100 g/l solution of iodoacetamide in alcohol.

Buffer solution. Use the reducing solution, prepared as described for Method 8.

Procedure. Dissolve the test sample in 50 µl of the buffer solution, and add about 2.5 µl of stock solution C. Store under nitrogen or argon for 2 h at room temperature in the dark. Add the carboxymethylation solution in a ratio 1.5 fold per total theoretical content of thiols, and incubate for an additional 30 min at room temperature in the dark. If the thiol content of the protein is unknown, then add 5 µl of 100 mM iodoacetamide for every 20 nmol of protein present. The reaction is stopped by adding excess of 2-mercaptoethanol. Desalt the protein/peptide by collecting the protein/peptide fraction from a reversed-phase HPLC separation. The collected sample can be dried in a vacuum centrifuge before acid hydrolysis. The S-carboxyamidomethyl-cysteine formed will be converted to S-carboxymethyl-cysteine during acid hydrolysis.

METHOD 10

Cysteine/cystine is reacted with dithiodiglycolic acid or dithiodipropionic acid to produce a mixed disulphide. The choice of dithiodiglycolic acid or dithiodipropionic acid depends on the required resolution of the amino acid analysis method.

Reducing solution. A 10 g/l solution of dithiodiglycolic acid (or dithiodipropionic acid) in 0.2 M sodium hydroxide.

Procedure. Transfer about 20 µg of the test sample to a hydrolysis tube, and add 5 µl of the reducing solution. Add 10 µl of isopropyl alcohol, and then remove all of the sample liquid by vacuum centrifugation. The sample is then hydrolysed using Method 1. This method has the advantage that other amino acid residues are not derivatised by side reactions, and that the sample does not need to be desalted prior to hydrolysis.

METHOD 11

Asparagine and glutamine are converted to aspartic acid and glutamic acid, respectively, during acid hydrolysis. Asparagine and aspartic acid residues are added and represented by *Asx*, while glutamine and glutamic acid residues are added and represented by *Glx*. Proteins/peptides can be reacted with bis(1,1-trifluoroacetoxy)iodobenzene (BTI) to convert the asparagine and glutamine residues to diaminopropionic acid and diaminobutyric acid residues, respectively, upon acid hydrolysis. These conversions allow the analyst to determine the asparagine and glutamine content of a protein/peptide in the presence of aspartic acid and glutamic acid residues.

Reducing solutions. Prepare and filter 3 solutions: a solution of 10 mM trifluoroacetic acid (Solution A), a solution of 5 M guanidine hydrochloride and 10 mM trifluoroacetic acid (Solution B), and a freshly prepared solution of dimethylformamide containing 36 mg of BTI per millilitre (Solution C).

Procedure. In a clean hydrolysis tube, transfer about 200 µg of the test sample, and add 2 ml of Solution A or Solution B and 2 ml of Solution C. Seal the hydrolysis tube *in vacuo*. Heat the sample at 60 °C for 4 h in the dark. The sample is then dialysed with water to remove the excess reagents. Extract the dialysed sample 3 times with equal volumes of butyl acetate, and then lyophilise. The protein can then be acid hydrolysed using previously described procedures. The α,β-diaminopropionic and α,γ-diaminobutyric acid residues do not typically resolve from the lysine residues upon ion-exchange chromatography based on amino acid analysis. Therefore, when using ion-exchange as the mode of amino acid separation, the asparagine and glutamine contents are the quantitative difference in the aspartic acid and glutamic acid content assayed with underivatised and BTI-derivatised acid hydrolysis. The threonine, methionine, cysteine, tyrosine, and histidine assayed content can be altered by BTI derivatisation; a hydrolysis without BTI will have to be performed if the analyst is interested in the composition of these other amino acid residues of the protein/peptide.

METHODOLOGIES OF AMINO ACID ANALYSIS: GENERAL PRINCIPLES

Many amino acid analysis techniques exist, and the choice of any one technique often depends on the sensitivity required from the assay. In general, about one-half of the amino acid analysis techniques employed rely on the separation of the free amino acids by ion-exchange chromatography followed by post-column derivatisation (e.g., with ninhydrin or *o*-phthalaldehyde). Post-column derivatisation techniques can be used with samples that contain small amounts of buffer components, (such as salts and urea) and generally require between 5 µg and 10 µg of protein sample per analysis. The remaining amino acid techniques typically involve pre-column derivatisation of the free amino acids (e.g., phenyl isothiocyanate; 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate or *o*-phthalaldehyde; (dimethylamino)azobenzenesulphonyl chloride; 9-fluorenylmethyl chloroformate; and 7-fluoro-4-nitrobenzo-2-oxa-1,3-diazole) followed by reversed-phase HPLC. Pre-column derivatisation techniques

are very sensitive and usually require between 0.5 µg and 1.0 µg of protein sample per analysis but may be influenced by buffer salts in the samples. Pre-column derivatisation techniques may also result in multiple derivatives of a given amino acid, which complicates the result interpretation. Post-column derivatisation techniques are generally influenced less by performance variation of the assay than pre-column derivatisation techniques.

The following methods may be used for quantitative amino acid analysis. Instruments and reagents for these procedures are available commercially. Furthermore, many modifications of these methodologies exist with different reagent preparations, reaction procedures, chromatographic systems, etc. Specific parameters may vary according to the exact equipment and procedure used. Many laboratories will use more than one amino acid analysis technique to exploit the advantages offered by each. In each of these methods, the analogue signal is visualised by means of a data acquisition system, and the peak areas are integrated for quantification purposes.

METHOD 1 - POST-COLUMN NINHYDRIN DERIVATISATION

Ion-exchange chromatography with post-column ninhydrin derivatisation is one of the most common methods employed for quantitative amino acid analysis. As a rule, a lithium-based cation-exchange system is employed for the analysis of the more complex physiological samples, and the faster sodium-based cation-exchange system is used for the more simplistic amino acid mixtures obtained with protein hydrolysates (typically containing 17 amino acid components). Separation of the amino acids on an ion-exchange column is accomplished through a combination of changes in pH and cation strength. A temperature gradient is often employed to enhance separation.

When the amino acid reacts with ninhydrin, the reactant has a characteristic purple or yellow colour. Amino acids, except imino acid, give a purple colour, and show an absorption maximum at 570 nm. The imino acids such as proline give a yellow colour, and show an absorption maximum at 440 nm. The post-column reaction between ninhydrin and amino acids eluted from the column is monitored at 440 nm and 570 nm, and the chromatogram obtained is used for the determination of amino acid composition.

The detection limit is considered to be 10 pmol for most of the amino acid derivatives, but 50 pmol for the proline derivative. Response linearity is obtained in the range of 20-500 pmol with correlation coefficients exceeding 0.999. To obtain good composition data, samples larger than 1 µg before hydrolysis are best suited for this amino acid analysis of protein/peptide.

METHOD 2 - POST-COLUMN OPA DERIVATISATION

o-Phthalaldehyde (OPA) reacts with primary amines in the presence of thiol compound, to form highly fluorescent isoindole products. This reaction is used for the post-column derivatisation in analysis of amino acids by ion-exchange chromatography. The rule of the separation is the same as Method 1.

Although OPA does not react with secondary amines (imino acids such as proline) to form fluorescent substances, the oxidation with sodium hypochlorite or chloramine T allows secondary amines to react with OPA. The procedure employs a strongly acidic cation-exchange column for separation of free amino acids followed by post-column oxidation with sodium hypochlorite or chloramine T and post-column derivatisation using OPA and a thiol compound such as

N-acetyl-L-cysteine or 2-mercaptoethanol. The derivatisation of primary amino acids is not noticeably affected by the continuous supply of sodium hypochlorite or chloramine T.

Separation of the amino acids on an ion-exchange column is accomplished through a combination of changes in pH and cation strength. After post-column derivatisation of eluted amino acids with OPA, the reactant passes through the fluorometric detector. Fluorescence intensity of OPA-derivatised amino acids are monitored with an excitation wavelength of 348 nm and an emission wavelength of 450 nm.

The detection limit is considered to be a few tens of picomole level for most of the OPA-derivatised amino acids. Response linearity is obtained in the range of a few picomole level to a few tens of nanomole level. To obtain good compositional data, samples larger than 500 ng of protein/peptide before hydrolysis are recommended.

METHOD 3 - PRE-COLUMN PITC DERIVATISATION

Phenylisothiocyanate (PITC) reacts with amino acids to form phenylthiocarbamyl (PTC) derivatives which can be detected with high sensitivity at 254 nm. Therefore, pre-column derivatisation of amino acids with PITC followed by a reversed-phase HPLC separation with UV detection is used to analyse the amino acid composition.

After the reagent is removed under vacuum, the derivatised amino acids can be stored dry and frozen for several weeks with no significant degradation. If the solution for injection is kept cold, no noticeable loss in chromatographic response occurs after 3 days.

Separation of the PTC-amino acids on a reversed-phase HPLC with an octadecylsilyl (ODS) column is accomplished through a combination of changes in concentrations of acetonitrile and buffer ionic strength. PTC-amino acids eluted from the column are monitored at 254 nm.

The detection limit is considered to be 1 pmol for most of the PTC-amino acids. Response linearity is obtained in the range of 20-500 pmol with correlation coefficients exceeding 0.999. To obtain good compositional data, samples larger than 500 ng of protein/peptide before hydrolysis are recommended.

METHOD 4 - PRE-COLUMN AQC DERIVATISATION

Pre-column derivatisation of amino acids with 6-aminoquinolyl-*N*-hydroxysuccinimide carbamate (AQC) followed by reversed-phase HPLC separation with fluorometric detection is used.

AQC reacts with amino acids to form stable, fluorescent unsymmetric urea derivatives (AQC-amino acids) which are readily amenable to analysis by reversed-phase HPLC. Therefore, pre-column derivatisation of amino acids with AQC followed by reversed-phase HPLC separation with fluorimetric detection is used to analyse the amino acid composition.

Separation of the AQC-amino acids on a reversed-phase HPLC with an ODS column is accomplished through a combination of changes in concentrations of acetonitrile and buffer ionic strength. Selective fluorescence detection of the derivatives with an excitation wavelength at 250 nm and an emission wavelength at 395 nm allows for the direct injection of the reaction mixture with no significant interference from the only major fluorescent reagent by-product, 6-aminoquinoline. Excess reagent is rapidly hydrolysed ($t_{1/2} < 15$ s) to yield 6-aminoquinoline, *N*-hydroxysuccinimide and carbon dioxide, and after 1 min no further derivatisation can take place.

Peak areas for AQC-amino acids are essentially unchanged for at least 1 week at room temperature. Therefore AQC-amino acids have more than sufficient stability to allow for overnight automated chromatographic analysis.

The detection limit is considered to range from about 40 fmol to 320 fmol for each amino acid, except for cysteine. The detection limit for cysteine is approximately 800 fmol. Response linearity is obtained in the range of 2.5-200 µM with correlation coefficients exceeding 0.999. Good compositional data can be obtained from the analysis of derivatised protein hydrolysates derived from as little as 30 ng of protein/peptide.

METHOD 5 - PRE-COLUMN OPA DERIVATISATION

Pre-column derivatisation of amino acids with *o*-phthalaldehyde (OPA) followed by reversed-phase HPLC separation with fluorometric detection is used. This technique does not detect amino acids that exist as secondary amines (e.g., proline).

OPA in conjunction with a thiol reagent reacts with primary amine groups to form highly fluorescent isoindole products. 2-Mercaptoethanol or 3-mercaptopropionic acid can be used as the thiol. OPA itself does not fluoresce and consequently produces no interfering peaks. In addition, its solubility and stability in aqueous solution, along with the rapid kinetics for the reaction, make it amenable to automated derivatisation and analysis using an autosampler to mix the sample with the reagent. However, lack of reactivity with secondary amino acids has been a predominant drawback. This method does not detect amino acids that exist as secondary amines (e.g., proline). To compensate for this drawback, this technique may be combined with another technique described in Method 7 or Method 8.

Pre-column derivatisation of amino acids with OPA is followed by a reversed-phase HPLC separation. Because of the instability of the OPA-amino acid derivative, HPLC separation and analysis are performed immediately following derivatisation. The liquid chromatograph is equipped with a fluorometric detector for the detection of derivatised amino acids. Fluorescence intensity of OPA-derivatised amino acids is monitored with an excitation wavelength of 348 nm and an emission wavelength of 450 nm.

Detection limits as low as 50 fmol via fluorescence have been reported, although the practical limit of analysis remains at 1 pmol.

METHOD 6 - PRE-COLUMN DABS-Cl DERIVATISATION

Pre-column derivatisation of amino acids with (dimethylamino)azobenzenesulphonyl chloride (DABS-Cl) followed by reversed-phase HPLC separation with visible light detection is used.

DABS-Cl is a chromophoric reagent employed for the labelling of amino acids. Amino acids labelled with DABS-Cl (DABS-amino acids) are highly stable and show an absorption maximum at 436 nm.

DABS-amino acids, all naturally occurring amino acid derivatives, can be separated on an ODS column of a reversed-phase HPLC by employing gradient systems consisting of acetonitrile and aqueous buffer mixture. Separated DABS-amino acids eluted from the column are detected at 436 nm in the visible region.

This method can analyse the amino acids such as proline together with the amino acids at the same degree of sensitivity. DABS-Cl derivatisation method permits the simultaneous quantification of tryptophan residues by previous hydrolysis of the protein/peptide with sulphonic acids such as mercaptoethanesulphonic acid, *p*-toluenesulphonic acid or methanesulphonic acid

described in Method 2 under Protein hydrolysis. The other acid-labile residues, asparagine and glutamine, can also be analysed by previous conversion into diaminopropionic acid and diaminobutyric acid, respectively, by treatment of protein/peptide with BTI described in Method 11 under Protein hydrolysis.

The non-proteinogenic amino acid norleucine cannot be used as an internal standard in this method as this compound is eluted in a chromatographic region crowded with peaks of primary amino acids. Nitrotyrosine can be used as an internal standard because it is eluted in a clean region.

The detection limit of DABS-amino acid is about 1 pmol. As little as 2-5 pmol of an individual DABS-amino acid can be quantitatively analysed with reliability, and only 10-30 ng of the dabsylated protein hydrolysate is required for each analysis.

METHOD 7 - PRE-COLUMN FMOC-Cl DERIVATISATION

Pre-column derivatisation of amino acids with 9-fluorenylmethyl chloroformate (FMOC-Cl) followed by reversed-phase HPLC separation with fluorometric detection is used.

FMOC-Cl reacts with both primary and secondary amino acids to form highly fluorescent products. The reaction proceeds under mild conditions in aqueous solution and is completed in 30 s. The derivatives are stable, only the histidine derivative showing any breakdown. Although FMOC-Cl is fluorescent itself, the reagent excess and fluorescent side-products can be eliminated without loss of FMOC-amino acids.

FMOC-amino acids are separated by a reversed-phase HPLC using an ODS column. The separation is carried out by gradient elution varied linearly from a mixture of 10 volumes of acetonitrile, 40 volumes of methanol and 50 volumes of acetic acid buffer to a mixture of 50 volumes of acetonitrile and 50 volumes of acetic acid buffer and 20 amino acid derivatives are separated in 20 min. Each derivative eluted from the column is monitored by a fluorometric detector set at an excitation wavelength of 260 nm and an emission wavelength of 313 nm.

The detection limit is in the low femtomole range. A linearity range of 0.1-50 µM is obtained for most of the amino acids.

METHOD 8 - PRE-COLUMN NBD-F DERIVATISATION

Pre-column derivatisation of amino acids with 7-fluoro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-F) followed by reversed-phase HPLC separation with fluorometric detection is used.

NBD-F reacts with both primary and secondary amino acids to form highly fluorescent products. Amino acids are derivatised with NBD-F by heating to 60 °C for 5 min.

NBD-amino acid derivatives are separated on an ODS column of a reversed-phase HPLC by employing a gradient elution system consisting of acetonitrile and aqueous buffer mixture, and 17 amino acid derivatives are separated in 35 min. ϵ -Aminocaproic acid can be used as an internal standard, because it is eluted in a clean chromatographic region. Each derivative eluted from the column is monitored by a fluorometric detector set at an excitation wavelength of 480 nm and an emission wavelength of 530 nm.

The sensitivity of this method is almost the same as for the pre-column OPA derivatisation method (Method 5), excluding proline to which OPA is not reactive, and might be advantageous for NBD-F against OPA. The detection limit for each amino acid is about 10 fmol. Profile analysis can be achieved with about 1.5 mg of protein hydrolysates in the pre-column reaction mixture.

DATA CALCULATION AND ANALYSIS

When determining the amino acid content of a protein/peptide hydrolysate, it should be noted that the acid hydrolysis step destroys tryptophan and cysteine. Serine and threonine are partially destroyed by acid hydrolysis, while isoleucine and valine residues may be only partially cleaved. Methionine can undergo oxidation during acid hydrolysis, and some amino acids (e.g., glycine and serine) are common contaminants. Application of adequate vacuum (less than 200 µm of mercury or 26.7 Pa) or introduction of inert gas (argon) in the headspace of the reaction vessel during vapour phase hydrolysis can reduce the level of oxidative destruction. Therefore, the quantitative results obtained for cysteine, tryptophan, threonine, isoleucine, valine, methionine, glycine, and serine from a protein/peptide hydrolysate may be variable and may warrant further investigation and consideration.

Amino Acid Mole Percent. This is the number of specific amino acid residues per 100 residues in a protein. This result may be useful for evaluating amino acid analysis data when the molecular mass of the protein under investigation is unknown. This information can be used to corroborate the identity of a protein/peptide and has other applications. Carefully identify and integrate the peaks obtained as directed for each procedure. Calculate the mole percent for each amino acid present in the test sample using the formula:

$$\frac{100r_U}{r}$$

in which r_U is the peak response, in nanomoles, of the amino acid under test; and r is the sum of peak responses, in nanomoles, for all amino acids present in the test sample. Comparison of the mole percent of the amino acids under test to data from known proteins can help establish or corroborate the identity of the sample protein.

Unknown Protein Samples. This data analysis technique can be used to estimate the protein concentration of an unknown protein sample using the amino acid analysis data. Calculate the mass, in micrograms, of each recovered amino acid using the formula:

$$\frac{mM_r}{1000}$$

in which m is the recovered quantity, in nanomoles, of the amino acid under test; and M_r is the average molecular mass for that amino acid, corrected for the mass of the water molecule that was eliminated during peptide bond formation. The sum of the masses of the recovered amino acids will give an estimate of the total mass of the protein analysed after appropriate correction for partially and completely destroyed amino acids. If the molecular mass of the unknown protein is available (i.e., by SDS-PAGE analysis or mass spectroscopy), the amino acid composition of the unknown protein can be predicted. Calculate the number of residues of each amino acid using the formula:

$$\frac{m}{\left(\frac{1000M}{M_{rt}}\right)}$$

in which m is the recovered quantity, in nanomoles, of the amino acid under test; M is the total mass, in micrograms, of the protein; and M_{rt} is the molecular mass of the unknown protein.

Known protein samples. This data analysis technique can be used to investigate the amino acid composition and protein concentration of a protein sample of known molecular mass and amino acid composition using the amino acid analysis data. When the composition of the protein being analysed is known, one can exploit the fact that some amino acids are recovered well, while other amino acid recoveries may be compromised because of complete or partial destruction (e.g., tryptophan, cysteine, threonine, serine, methionine), incomplete bond cleavage (i.e., for isoleucine and valine) and free amino acid contamination (i.e., by glycine and serine).

Because those amino acids that are recovered best represent the protein, these amino acids are chosen to quantify the amount of protein. Well-recovered amino acids are, typically, aspartate-asparagine, glutamate-glutamine, alanine, leucine, phenylalanine, lysine, and arginine. This list can be modified based on experience with one's own analysis system. Divide the quantity, in nanomoles, of each of the well-recovered amino acids by the expected number of residues for that amino acid to obtain the protein content based on each well-recovered amino acid. Average the protein content results calculated. The protein content determined for each of the well-recovered amino acids should be evenly distributed about the mean. Discard protein content values for those amino acids that have an unacceptable deviation from the mean. Typically greater than 5 per cent variation from the mean is considered unacceptable. Recalculate the mean protein content from the remaining values to obtain the protein content of the sample. Divide the content of each amino acid by the calculated mean protein content to determine the amino acid composition of the sample by analysis.

Calculate the relative compositional error, in percentage, using the formula:

$$\frac{100m}{m_s}$$

in which m is the experimentally determined quantity, in nanomoles per amino acid residue, of the amino acid under test; and m_s is the known residue value for that amino acid. The average relative compositional error is the average of the absolute values of the relative compositional errors of the individual amino acids, typically excluding tryptophan and cysteine from this calculation. The average relative compositional error can provide important information on the stability of analysis run over time. The agreement in the amino acid composition between the protein sample and the known composition can be used to corroborate the identity and purity of the protein in the sample.

2.3. IDENTIFICATION

01/2005:20301

2.3.1. IDENTIFICATION REACTIONS OF IONS AND FUNCTIONAL GROUPS

ACETATES

- a) Heat the substance to be examined with an equal quantity of *oxalic acid R*. Acid vapours with the characteristic odour of acetic acid are liberated, showing an acid reaction (2.2.4).
- b) Dissolve about 30 mg of the substance to be examined in 3 ml of *water R* or use 3 ml of the prescribed solution. Add successively 0.25 ml of *lanthanum nitrate solution R*, 0.1 ml of 0.05 M *iodine* and 0.05 ml of *dilute ammonia R2*. Heat carefully to boiling. Within a few minutes a blue precipitate is formed or a dark blue colour develops.

ACETYL

In a test-tube about 180 mm long and 18 mm in external diameter, place about 15 mg of the substance to be examined, or the prescribed quantity, and 0.15 ml of *phosphoric acid R*. Close the tube with a stopper through which passes a small test-tube about 100 mm long and 10 mm in external diameter containing *water R* to act as a condenser. On the outside of the smaller tube, hang a drop of *lanthanum nitrate solution R*. Except for substances hydrolysable only with difficulty, place the apparatus in a water-bath for 5 min, then take out the smaller tube. Remove the drop and mix it with 0.05 ml of 0.01 M *iodine* on a tile. Add at the edge 0.05 ml of *dilute ammonia R2*. After 1 min to 2 min, a blue colour develops at the junction of the two drops; the colour intensifies and persists for a short time.

For *substances hydrolysable only with difficulty* heat the mixture slowly to boiling over an open flame and then proceed as prescribed above.

ALKALOIDS

Dissolve a few milligrams of the substance to be examined, or the prescribed quantity, in 5 ml of *water R*, add *dilute hydrochloric acid R* until an acid reaction occurs (2.2.4), then 1 ml of *potassium iodobismuthate solution R*. An orange or orange-red precipitate is formed immediately.

ALUMINIUM

Dissolve about 15 mg of the substance to be examined in 2 ml of *water R* or use 2 ml of the prescribed solution. Add about 0.5 ml of *dilute hydrochloric acid R* and about 0.5 ml of *thioacetamide reagent R*. No precipitate is formed. Add dropwise *dilute sodium hydroxide solution R*. A gelatinous white precipitate is formed which dissolves on further addition of *dilute sodium hydroxide solution R*. Gradually add *ammonium chloride solution R*. The gelatinous white precipitate is re-formed.

AMINES, PRIMARY AROMATIC

Acidify the prescribed solution with *dilute hydrochloric acid R* and add 0.2 ml of *sodium nitrite solution R*. After 1 min to 2 min, add 1 ml of β -*naphthol solution R*. An intense orange or red colour and usually a precipitate of the same colour are produced.

AMMONIUM SALTS

To the prescribed solution add 0.2 g of *magnesium oxide R*. Pass a current of air through the mixture and direct the gas that escapes just beneath the surface of a mixture of 1 ml of 0.1 M *hydrochloric acid* and 0.05 ml of *methyl red*

solution R. The colour of the indicator changes to yellow. On addition of 1 ml of a freshly prepared 100 g/l solution of *sodium cobaltinitrite R* a yellow precipitate is formed.

AMMONIUM SALTS AND SALTS OF VOLATILE BASES

Dissolve about 20 mg of the substance to be examined in 2 ml of *water R* or use 2 ml of the prescribed solution. Add 2 ml of *dilute sodium hydroxide solution R*. On heating, the solution gives off vapour that can be identified by its odour and by its alkaline reaction (2.2.4).

ANTIMONY

Dissolve with gentle heating about 10 mg of the substance to be examined in a solution of 0.5 g of *sodium potassium tartrate R* in 10 ml of *water R* and allow to cool: to 2 ml of this solution, or to 2 ml of the prescribed solution, add *sodium sulphide solution R* dropwise; an orange-red precipitate is formed which dissolves on addition of *dilute sodium hydroxide solution R*.

ARSENIC

Heat 5 ml of the prescribed solution on a water-bath with an equal volume of *hypophosphorous reagent R*. A brown precipitate is formed.

BARBITURATES, NON-NITROGEN SUBSTITUTED

Dissolve about 5 mg of the substance to be examined in 3 ml of *methanol R*, add 0.1 ml of a solution containing 100 g/l of *cobalt nitrate R* and 100 g/l of *calcium chloride R*. Mix and add, with shaking, 0.1 ml of *dilute sodium hydroxide solution R*. A violet-blue colour and precipitate are formed.

BENZOATES

- a) To 1 ml of the prescribed solution add 0.5 ml of *ferric chloride solution R1*. A dull-yellow precipitate, soluble in *ether R*, is formed.
- b) Place 0.2 g of the substance to be examined, treated if necessary as prescribed, in a test-tube. Moisten with 0.2 ml to 0.3 ml of *sulphuric acid R*. Gently warm the bottom of the tube. A white sublimate is deposited on the inner wall of the tube.
- c) Dissolve 0.5 g of the substance to be examined in 10 ml of *water R* or use 10 ml of the prescribed solution. Add 0.5 ml of *hydrochloric acid R*. The precipitate obtained, after crystallisation from warm *water R* and drying *in vacuo*, has a melting point (2.2.14) of 120 °C to 124 °C.

BISMUTH

- a) To 0.5 g of the substance to be examined add 10 ml of *dilute hydrochloric acid R* or use 10 ml of the prescribed solution. Heat to boiling for 1 min. Cool and filter if necessary. To 1 ml of the solution obtained add 20 ml of *water R*. A white or slightly yellow precipitate is formed which on addition of 0.05 ml to 0.1 ml of *sodium sulphide solution R* turns brown.
- b) To about 45 mg of the substance to be examined add 10 ml of *dilute nitric acid R* or use 10 ml of the prescribed solution. Boil for 1 min. Allow to cool and filter if necessary. To 5 ml of the solution obtained add 2 ml of a 100 g/l solution of *thiourea R*. A yellowish-orange colour or an orange precipitate is formed. Add 4 ml of a 25 g/l solution of *sodium fluoride R*. The solution is not decolorised within 30 min.

BROMIDES

- a) Dissolve in 2 ml of *water R* a quantity of the substance to be examined equivalent to about 3 mg of bromide (Br⁻) or use 2 ml of the prescribed solution. Acidify with *dilute nitric acid R* and add 0.4 ml of *silver nitrate solution R1*.

Shake and allow to stand. A curdled, pale yellow precipitate is formed. Centrifuge and wash the precipitate with three quantities, each of 1 ml, of *water R*. Carry out this operation rapidly in subdued light disregarding the fact that the supernatant solution may not become perfectly clear. Suspend the precipitate obtained in 2 ml of *water R* and add 1.5 ml of *ammonia R*. The precipitate dissolves with difficulty.

b) Introduce into a small test-tube a quantity of the substance to be examined equivalent to about 5 mg of bromide (Br^-) or the prescribed quantity. Add 0.25 ml of *water R*, about 75 mg of *lead dioxide R*, 0.25 ml of *acetic acid R* and shake gently. Dry the inside of the upper part of the test-tube with a piece of filter paper and allow to stand for 5 min. Prepare a strip of suitable filter paper of appropriate size. Impregnate it by capillarity, by dipping the tip in a drop of *decolorised fuchsin solution R* and introduce the impregnated part immediately into the tube. Starting from the tip, a violet colour appears within 10 s that is clearly distinguishable from the red colour of fuchsin, which may be visible on a small area at the top of the impregnated part of the paper strip.

CALCIUM

a) To 0.2 ml of a neutral solution containing a quantity of the substance to be examined equivalent to about 0.2 mg of calcium (Ca^{2+}) per millilitre or to 0.2 ml of the prescribed solution add 0.5 ml of a 2 g/l solution of *glyoxal-hydroxynil R* in *alcohol R*, 0.2 ml of *dilute sodium hydroxide solution R* and 0.2 ml of *sodium carbonate solution R*. Shake with 1 ml to 2 ml of *chloroform R* and add 1 ml to 2 ml of *water R*. The chloroform layer is coloured red.

b) Dissolve about 20 mg of the substance to be examined or the prescribed quantity in 5 ml of *acetic acid R*. Add 0.5 ml of *potassium ferrocyanide solution R*. The solution remains clear. Add about 50 mg of *ammonium chloride R*. A white, crystalline precipitate is formed.

CARBONATES AND BICARBONATES

Introduce into a test-tube 0.1 g of the substance to be examined and suspend in 2 ml of *water R* or use 2 ml of the prescribed solution. Add 3 ml of *dilute acetic acid R*. Close the tube immediately using a stopper fitted with a glass tube bent twice at right angles. The solution or the suspension becomes effervescent and gives off a colourless and odourless gas. Heat gently and collect the gas in 5 ml of *barium hydroxide solution R*. A white precipitate is formed that dissolves on addition of an excess of *hydrochloric acid R1*.

CHLORIDES

a) Dissolve in 2 ml of *water R* a quantity of the substance to be examined equivalent to about 2 mg of chloride (Cl^-) or use 2 ml of the prescribed solution. Acidify with *dilute nitric acid R* and add 0.4 ml of *silver nitrate solution R1*. Shake and allow to stand. A curdled, white precipitate is formed. Centrifuge and wash the precipitate with three quantities, each of 1 ml, of *water R*. Carry out this operation rapidly in subdued light, disregarding the fact that the supernatant solution may not become perfectly clear. Suspend the precipitate in 2 ml of *water R* and add 1.5 ml of *ammonia R*. The precipitate dissolves easily with the possible exception of a few large particles which dissolve slowly.

b) Introduce into a test-tube a quantity of the substance to be examined equivalent to about 15 mg of chloride (Cl^-) or the prescribed quantity. Add 0.2 g of *potassium dichromate R* and 1 ml of *sulphuric acid R*. Place a filter-paper strip impregnated with 0.1 ml of *diphenylcarbazide solution R*

over the opening of the test-tube. The paper turns violet-red. The impregnated paper must not come into contact with the potassium dichromate.

CITRATES

Dissolve in 5 ml of *water R* a quantity of the substance to be examined equivalent to about 50 mg of citric acid or use 5 ml of the prescribed solution. Add 0.5 ml of *sulphuric acid R* and 1 ml of *potassium permanganate solution R*. Warm until the colour of the permanganate is discharged. Add 0.5 ml of a 100 g/l solution of *sodium nitroprusside R* in *dilute sulphuric acid R* and 4 g of *sulphamic acid R*. Make alkaline with *concentrated ammonia R*, added dropwise until all the sulphamic acid has dissolved. Addition of an excess of *concentrated ammonia R* produces a violet colour, turning to violet-blue.

ESTERS

To about 30 mg of the substance to be examined or the prescribed quantity add 0.5 ml of a 70 g/l solution of *hydroxylamine hydrochloride R* in *methanol R* and 0.5 ml of a 100 g/l solution of *potassium hydroxide R* in *alcohol R*. Heat to boiling, cool, acidify with *dilute hydrochloric acid R* and add 0.2 ml of *ferric chloride solution R1* diluted ten times. A bluish-red or red colour is produced.

IODIDES

a) Dissolve a quantity of the substance to be examined equivalent to about 4 mg of iodide (I^-) in 2 ml of *water R* or use 2 ml of the prescribed solution. Acidify with *dilute nitric acid R* and add 0.4 ml of *silver nitrate solution R1*. Shake and allow to stand. A curdled, pale-yellow precipitate is formed. Centrifuge and wash with three quantities, each of 1 ml, of *water R*. Carry out this operation rapidly in subdued light disregarding the fact that the supernatant solution may not become perfectly clear. Suspend the precipitate in 2 ml of *water R* and add 1.5 ml of *ammonia R*. The precipitate does not dissolve.

b) To 0.2 ml of a solution of the substance to be examined containing about 5 mg of iodide (I^-) per millilitre, or to 0.2 ml of the prescribed solution, add 0.5 ml of *dilute sulphuric acid R*, 0.1 ml of *potassium dichromate solution R*, 2 ml of *water R* and 2 ml of *chloroform R*. Shake for a few seconds and allow to stand. The chloroform layer is coloured violet or violet-red.

IRON

a) Dissolve a quantity of the substance to be examined equivalent to about 10 mg of iron (Fe^{2+}) in 1 ml of *water R* or use 1 ml of the prescribed solution. Add 1 ml of *potassium ferricyanide solution R*. A blue precipitate is formed that does not dissolve on addition of 5 ml of *dilute hydrochloric acid R*.

b) Dissolve a quantity of the substance to be examined equivalent to about 1 mg of iron (Fe^{3+}) in 30 ml of *water R*. To 3 ml of this solution or to 3 ml of the prescribed solution, add 1 ml of *dilute hydrochloric acid R* and 1 ml of *potassium thiocyanate solution R*. The solution is coloured red. Take two portions, each of 1 ml, of the mixture. To one portion add 5 ml of *isoamyl alcohol R* or 5 ml of *ether R*. Shake and allow to stand. The organic layer is coloured pink. To the other portion add 2 ml of *mercuric chloride solution R*. The red colour disappears.

c) Dissolve a quantity of the substance to be examined equivalent to not less than 1 mg of iron (Fe^{3+}) in 1 ml of *water R* or use 1 ml of the prescribed solution. Add 1 ml of *potassium ferrocyanide solution R*. A blue precipitate is formed that does not dissolve on addition of 5 ml of *dilute hydrochloric acid R*.

LACTATES

Dissolve a quantity of the substance to be examined equivalent to about 5 mg of lactic acid in 5 ml of *water R* or use 5 ml of the prescribed solution. Add 1 ml of *bromine water R* and 0.5 ml of *dilute sulphuric acid R*. Heat on a water-bath until the colour is discharged, stirring occasionally with a glass rod. Add 4 g of *ammonium sulphate R* and mix. Add dropwise and without mixing 0.2 ml of a 100 g/l solution of *sodium nitroprusside R* in *dilute sulphuric acid R*. Still without mixing add 1 ml of *concentrated ammonia R*. Allow to stand for 30 min. A dark green ring appears at the junction of the two liquids.

LEAD

a) Dissolve 0.1 g of the substance to be examined in 1 ml of *acetic acid R* or use 1 ml of the prescribed solution. Add 2 ml of *potassium chromate solution R*. A yellow precipitate is formed that dissolves on addition of 2 ml of *strong sodium hydroxide solution R*.

b) Dissolve 50 mg of the substance to be examined in 1 ml of *acetic acid R* or use 1 ml of the prescribed solution. Add 10 ml of *water R* and 0.2 ml of *potassium iodide solution R*. A yellow precipitate is formed. Heat to boiling for 1 min to 2 min. The precipitate dissolves. Allow to cool. The precipitate is re-formed as glistening, yellow plates.

MAGNESIUM

Dissolve about 15 mg of the substance to be examined in 2 ml of *water R* or use 2 ml of the prescribed solution. Add 1 ml of *dilute ammonia R1*. A white precipitate is formed that dissolves on addition of 1 ml of *ammonium chloride solution R*. Add 1 ml of *disodium hydrogen phosphate solution R*. A white crystalline precipitate is formed.

MERCURY

a) Place about 0.1 ml of a solution of the substance to be examined on well-scraped copper foil. A dark-grey stain that becomes shiny on rubbing is formed. Dry the foil and heat in a test-tube. The spot disappears.

b) To the prescribed solution add *dilute sodium hydroxide solution R* until strongly alkaline (2.2.4). A dense yellow precipitate is formed (mercuric salts).

NITRATES

To a mixture of 0.1 ml of *nitrobenzene R* and 0.2 ml of *sulphuric acid R*, add a quantity of the powdered substance equivalent to about 1 mg of nitrate (NO_3^-) or the prescribed quantity. Allow to stand for 5 min. Cool in iced water and add slowly and with mixing 5 ml of *water R*, then 5 ml of *strong sodium hydroxide solution R*. Add 5 ml of *acetone R*. Shake and allow to stand. The upper layer is coloured deep violet.

PHOSPHATES (ORTHOPHOSPHATES)

a) To 5 ml of the prescribed solution, neutralised if necessary, add 5 ml of *silver nitrate solution R1*. A yellow precipitate is formed whose colour is not changed by boiling and which dissolves on addition of *ammonia R*.

b) Mix 1 ml of the prescribed solution with 2 ml of *molybdovanadic reagent R*. A yellow colour develops.

POTASSIUM

a) Dissolve 0.1 g of the substance to be examined in 2 ml of *water R* or use 2 ml of the prescribed solution. Add 1 ml of *sodium carbonate solution R* and heat. No precipitate is formed. Add to the hot solution 0.05 ml of *sodium sulphide solution R*. No precipitate is formed. Cool in iced water and add 2 ml of a 150 g/l solution of *tartaric acid R*. Allow to stand. A white crystalline precipitate is formed.

b) Dissolve about 40 mg of the substance to be examined in 1 ml of *water R* or use 1 ml of the prescribed solution. Add 1 ml of *dilute acetic acid R* and 1 ml of a freshly prepared 100 g/l solution of *sodium cobaltinitrite R*. A yellow or orange-yellow precipitate is formed immediately.

SALICYLATES

a) To 1 ml of the prescribed solution add 0.5 ml of *ferric chloride solution R1*. A violet colour is produced that persists after the addition of 0.1 ml of *acetic acid R*.

b) Dissolve 0.5 g of the substance to be examined in 10 ml of *water R* or use 10 ml of the prescribed solution. Add 0.5 ml of *hydrochloric acid R*. The precipitate obtained, after recrystallisation from hot *water R* and drying *in vacuo*, has a melting point (2.2.14) of 156 °C to 161 °C.

SILICATES

Mix the prescribed quantity of the substance to be examined in a lead or platinum crucible by means of a copper wire with about 10 mg of *sodium fluoride R* and a few drops of *sulphuric acid R* to give a thin slurry. Cover the crucible with a thin, transparent plate of plastic under which a drop of *water R* is suspended and warm gently. Within a short time a white ring is rapidly formed around the drop of water.

SILVER

Dissolve about 10 mg of the substance to be examined in 10 ml of *water R* or use 10 ml of the prescribed solution. Add 0.3 ml of *hydrochloric acid R1*. A curdled, white precipitate is formed that dissolves on addition of 3 ml of *dilute ammonia R1*.

SODIUM

a) Dissolve 0.1 g of the substance to be examined in 2 ml of *water R* or use 2 ml of the prescribed solution. Add 2 ml of a 150 g/l solution of *potassium carbonate R* and heat to boiling. No precipitate is formed. Add 4 ml of *potassium pyroantimonate solution R* and heat to boiling. Allow to cool in iced water and if necessary rub the inside of the test-tube with a glass rod. A dense white precipitate is formed.

b) Dissolve a quantity of the substance to be examined equivalent to about 2 mg of sodium (Na^+) in 0.5 ml of *water R* or use 0.5 ml of the prescribed solution. Add 1.5 ml of *methoxyphenylacetic reagent R* and cool in ice-water for 30 min. A voluminous, white, crystalline precipitate is formed. Place in water at 20 °C and stir for 5 min. The precipitate does not disappear. Add 1 ml of *dilute ammonia R1*. The precipitate dissolves completely. Add 1 ml of *ammonium carbonate solution R*. No precipitate is formed.

SULPHATES

a) Dissolve about 45 mg of the substance to be examined in 5 ml of *water R* or use 5 ml of the prescribed solution. Add 1 ml of *dilute hydrochloric acid R* and 1 ml of *barium chloride solution R1*. A white precipitate is formed.

b) To the suspension obtained during reaction (a), add 0.1 ml of 0.05 M *iodine*. The suspension remains yellow (distinction from sulphites and dithionites), but is decolorised by adding dropwise *stannous chloride solution R* (distinction from iodates). Boil the mixture. No coloured precipitate is formed (distinction from selenates and tungstates).

TARTRATES

a) Dissolve about 15 mg of the substance to be examined in 5 ml of *water R* or use 5 ml of the prescribed solution. Add 0.05 ml of a 10 g/l solution of *ferrous sulphate R* and 0.05 ml of *dilute hydrogen peroxide solution R*. A transient

01/2005:20302

yellow colour is produced. After the colour has disappeared add *dilute sodium hydroxide solution R* dropwise. An intense blue colour is produced.

b) To 0.1 ml of a solution of the substance to be examined containing the equivalent of about 15 mg of tartaric acid per millilitre or to 0.1 ml of the prescribed solution add 0.1 ml of a 100 g/l solution of *potassium bromide R*, 0.1 ml of a 20 g/l solution of *resorcinol R* and 3 ml of *sulphuric acid R*. Heat on a water-bath for 5 min to 10 min. A dark-blue colour develops. Allow to cool and pour the solution into *water R*. The colour changes to red.

XANTHINES

To a few milligrams of the substance to be examined or the prescribed quantity add 0.1 ml of *strong hydrogen peroxide solution R* and 0.3 ml of *dilute hydrochloric acid R*. Heat to dryness on a water-bath until a yellowish-red residue is obtained. Add 0.1 ml of *dilute ammonia R2*. The colour of the residue changes to violet-red.

ZINC

Dissolve 0.1 g of the substance to be examined in 5 ml of *water R* or use 5 ml of the prescribed solution. Add 0.2 ml of *strong sodium hydroxide solution R*. A white precipitate is formed. Add a further 2 ml of *strong sodium hydroxide solution R*. The precipitate dissolves. Add 10 ml of *ammonium chloride solution R*. The solution remains clear. Add 0.1 ml of *sodium sulphide solution R*. A flocculent white precipitate is formed.

2.3.2. IDENTIFICATION OF FATTY OILS BY THIN-LAYER CHROMATOGRAPHY

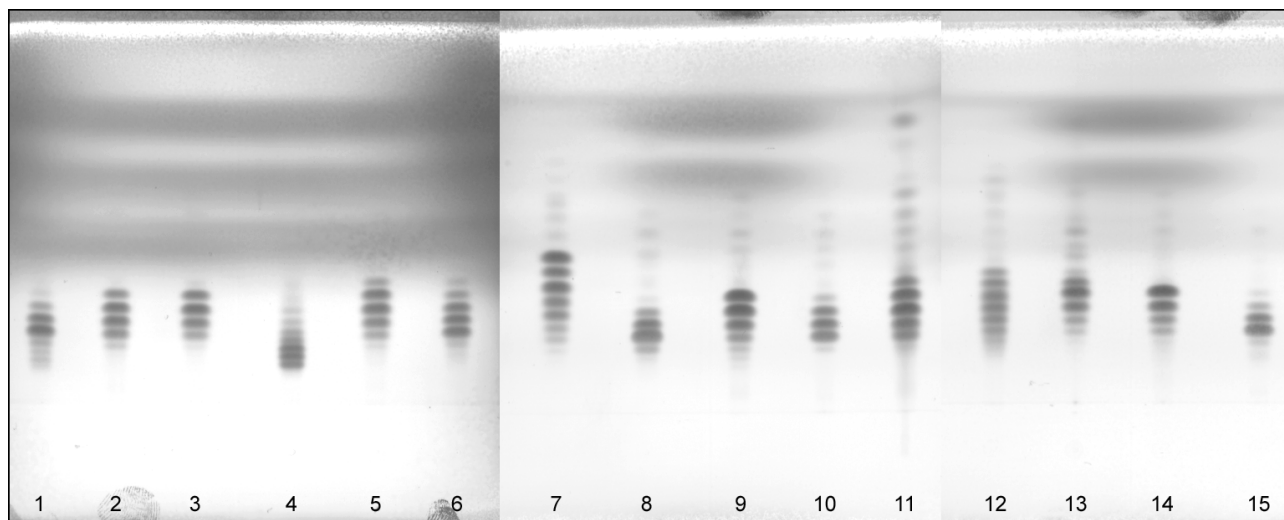
Examine by thin-layer chromatography (2.2.27), using as the coating substance a suitable octadecylsilyl silica gel for high performance thin-layer chromatography.

Test solution. Unless otherwise prescribed, dissolve about 20 mg (1 drop) of the fatty oil in 3 ml of *methylene chloride R*.

Reference solution. Dissolve about 20 mg (1 drop) of *maize oil R* in 3 ml of *methylene chloride R*.

Apply separately to the plate 1 µl of each solution. Develop twice over a path of 0.5 cm using *ether R*. Develop twice over a path of 8 cm using a mixture of 20 volumes of *methylene chloride R*, 40 volumes of *glacial acetic acid R* and 50 volumes of *acetone R*. Allow the plate to dry in air and spray with a 100 g/l solution of *phosphomolybdic acid R* in *alcohol R*. Heat the plate at 120 °C for about 3 min and examine in daylight.

The chromatogram obtained typically shows spots comparable to those in Figure 2.3.2.-1.



- | | | |
|------------------|------------------------------------|-----------------------------|
| 1. arachis oil | 6. rapeseed oil (erucic acid-free) | 11. wheat-germ oil |
| 2. sesame oil | 7. linseed oil | 12. borage oil |
| 3. maize oil | 8. olive oil | 13. evening primrose oil |
| 4. rapeseed oil | 9. sunflower oil | 14. safflower oil (type I) |
| 5. soya-bean oil | 10. almond oil | 15. safflower oil (type II) |

Figure 2.3.2.-1. – Chromatograms for the identification of fatty oils

01/2005:20303

2.3.3. IDENTIFICATION OF PHENOTHIAZINES BY THIN-LAYER CHROMATOGRAPHY

Examine by thin-layer chromatography (2.2.27) using *kieselguhr G R* as the coating substance. Impregnate the plate by placing it in a closed tank containing the necessary quantity of the impregnation mixture composed of a solution containing 10 per cent V/V of *phenoxyethanol R* and 50 g/l of *macrogol 300 R* in *acetone R* so that the plate dips about 5 mm beneath the surface of the liquid. When the impregnation mixture has risen at least 17 cm from the lower edge of the plate, remove the plate and use immediately for chromatography. Carry out the chromatography in the same direction as the impregnation.

Test solution. Dissolve 20 mg of the substance to be examined in *chloroform R* and dilute to 10 ml with the same solvent.

Reference solution. Dissolve 20 mg of the corresponding chemical reference substance (CRS) in *chloroform R* and dilute to 10 ml with the same solvent.

Apply separately to the plate 2 µl of each solution and develop in the dark over a path of 15 cm using a mixture of 50 ml of *light petroleum R* and 1 ml of *diethylamine R* saturated with *phenoxyethanol R* (i.e. add about 3 ml to 4 ml of *phenoxyethanol R* to the above mixture of solvents to give a persistent cloudiness on shaking, decant, and use the supernatant liquid, even if it is cloudy). After development place the plate under ultraviolet light at 365 nm and examine after a few minutes. The spot in the chromatogram obtained with the test solution is similar in position, fluorescence and size to the spot in the chromatogram obtained with the reference solution. Spray with a 10 per cent V/V solution of *sulphuric acid R* in *alcohol R*. The spot in the chromatogram obtained with the test solution is of the same colour as that in the chromatogram obtained with the reference solution and has similar stability over a period of at least 20 min.

01/2005:20304

2.3.4. ODOUR

On a watch-glass 6 cm to 8 cm in diameter, spread in a thin layer 0.5 g to 2.0 g of the substance to be examined. After 15 min, determine the odour or verify the absence of odour.

2.4. LIMIT TESTS

01/2005:20401

2.4.1. AMMONIUM

Unless otherwise prescribed, use method A.

METHOD A

Dissolve the prescribed quantity of the substance to be examined in 14 ml of *water R* in a test-tube, make alkaline if necessary by the addition of *dilute sodium hydroxide solution R* and dilute to 15 ml with *water R*. To the solution add 0.3 ml of *alkaline potassium tetraiodomercurate solution R*. Prepare a standard by mixing 10 ml of *ammonium standard solution (1 ppm NH₄) R* with 5 ml of *water R* and 0.3 ml of *alkaline potassium tetraiodomercurate solution R*. Stopper the test-tubes.

After 5 min, any yellow colour in the test solution is not more intense than that in the standard.

METHOD B

In a 25 ml jar fitted with a cap, place the prescribed quantity of the finely powdered substance to be examined and dissolve or suspend in 1 ml of *water R*. Add 0.30 g of *heavy magnesium oxide R*. Close immediately after placing a piece of *silver manganese paper R* 5 mm square, wetted with a few drops of *water R*, under the polyethylene cap. Swirl, avoiding projections of liquid, and allow to stand at 40 °C for 30 min. If the silver manganese paper shows a grey colour, it is not more intense than that of a standard prepared at the same time and in the same manner using the prescribed volume of *ammonium standard solution (1 ppm NH₄) R*, 1 ml of *water R* and 0.30 g of *heavy magnesium oxide R*.

01/2005:20402

2.4.2. ARSENIC

METHOD A

The apparatus (see Figure 2.4.2-1) consists of a 100 ml conical flask closed with a ground-glass stopper through which passes a glass tube about 200 mm long and of internal diameter 5 mm. The lower part of the tube is drawn to an internal diameter of 1.0 mm, and 15 mm from its tip is a lateral orifice 2 mm to 3 mm in diameter. When the tube is in position in the stopper, the lateral orifice should be at least 3 mm below the lower surface of the stopper. The upper end of the tube has a perfectly flat, ground surface at right angles to the axis of the tube. A second glass tube of the same internal diameter and 30 mm long, with a similar flat ground surface, is placed in contact with the first, and is held in position by two spiral springs. Into the lower tube insert 50 mg to 60 mg of *lead acetate cotton R*, loosely packed, or a small plug of cotton and a rolled piece of *lead acetate paper R* weighing 50 mg to 60 mg. Between the flat surfaces of the tubes place a disc or a small square of *mercuric bromide paper R* large enough to cover the orifice of the tube (15 mm × 15 mm).

In the conical flask dissolve the prescribed quantity of the substance to be examined in 25 ml of *water R*, or in the case of a solution adjust the prescribed volume to 25 ml with *water R*. Add 15 ml of *hydrochloric acid R*, 0.1 ml of *stannous chloride solution R* and 5 ml of *potassium iodide solution R*, allow to stand for 15 min and introduce 5 g of

activated zinc R. Assemble the two parts of the apparatus immediately and immerse the flask in a bath of water at a temperature such that a uniform evolution of gas is maintained. Prepare a standard in the same manner, using 1 ml of *arsenic standard solution (1 ppm As) R*, diluted to 25 ml with *water R*.

After not less than 2 h the stain produced on the mercuric bromide paper in the test is not more intense than that in the standard.

METHOD B

Introduce the prescribed quantity of the substance to be examined into a test-tube containing 4 ml of *hydrochloric acid R* and about 5 mg of *potassium iodide R* and add 3 ml of *hypophosphorous reagent R*. Heat the mixture on a water-bath for 15 min, shaking occasionally. Prepare a standard in the same manner, using 0.5 ml of *arsenic standard solution (10 ppm As) R*.

After heating on the water-bath, any colour in the test solution is not more intense than that in the standard.

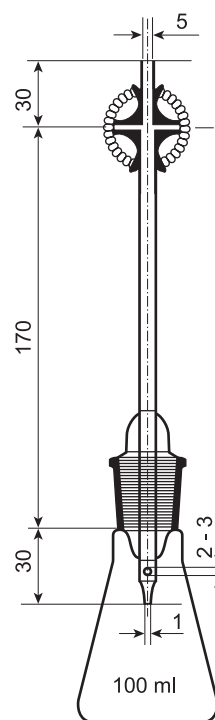


Figure 2.4.2-1. - Apparatus for limit test A for arsenic
Dimensions in millimetres

01/2005:20403

2.4.3. CALCIUM

All solutions used for this test should be prepared with distilled water R.

To 0.2 ml of *alcoholic calcium standard solution (100 ppm Ca) R*, add 1 ml of *ammonium oxalate solution R*. After 1 min, add a mixture of 1 ml of *dilute acetic acid R* and 15 ml of a solution containing the prescribed quantity of the substance to be examined and shake. Prepare a standard in the same manner using a mixture of 10 ml of *aqueous calcium standard solution (10 ppm Ca) R*, 1 ml of *dilute acetic acid R* and 5 ml of *distilled water R*.

After 15 min, any opalescence in the test solution is not more intense than that in the standard.

2.4.4. CHLORIDES

To 15 ml of the prescribed solution add 1 ml of *dilute nitric acid R* and pour the mixture as a single addition into a test-tube containing 1 ml of *silver nitrate solution R2*. Prepare a standard in the same manner using 10 ml of *chloride standard solution (5 ppm Cl) R* and 5 ml of *water R*. Examine the tubes laterally against a black background.

After standing for 5 min protected from light, any opalescence in the test solution is not more intense than that in the standard.

01/2005:20404

phenolphthalein solution R. Maintain a constant volume (20 ml) in the tube during distillation and ensure that the distillate remains alkaline, adding 0.1 M *sodium hydroxide* (test solution). Prepare a standard in the same manner by distillation, using 5 ml of *fluoride standard solution (10 ppm F) R* instead of the substance to be examined. Into two glass-stoppered cylinders introduce 20 ml of the test solution and 20 ml of the standard and 5 ml of *aminomethylalizarindiacetic acid reagent R*.

After 20 min, any blue colour in the test solution (originally red) is not more intense than that in the standard.

01/2005:20406

2.4.5. FLUORIDES

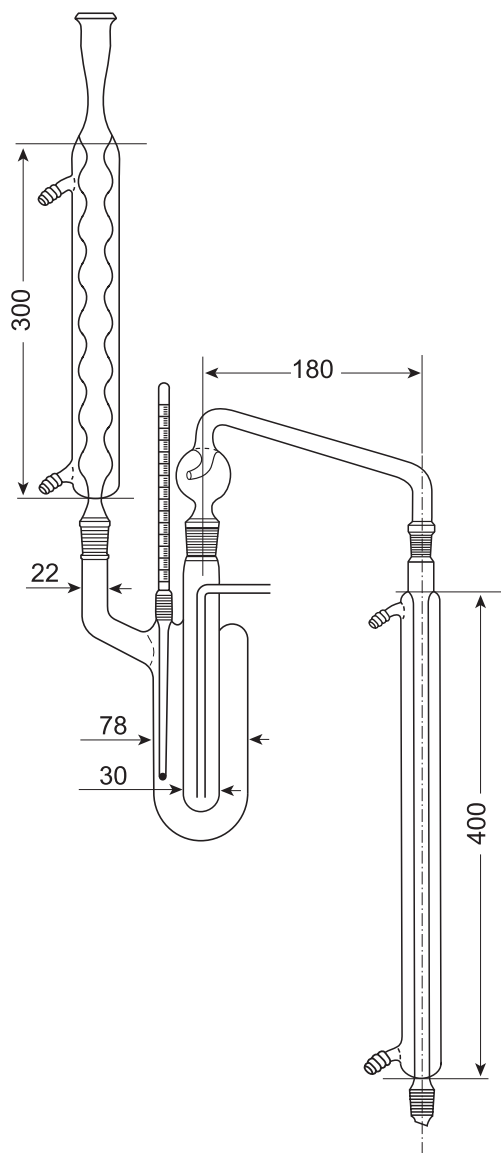


Figure 2.4.5.-1. – Apparatus for limit test for fluorides
Dimensions in millimetres

Introduce into the inner tube of the apparatus (see Figure 2.4.5.-1) the prescribed quantity of the substance to be examined, 0.1 g of acid-washed *sand R* and 20 ml of a mixture of equal volumes of *sulphuric acid R* and *water R*. Heat the jacket containing *tetrachloroethane R* maintained at its boiling point (146 °C). Heat the steam generator and distil, collecting the distillate in a 100 ml volumetric flask containing 0.3 ml of 0.1 M *sodium hydroxide* and 0.1 ml of

2.4.6. MAGNESIUM

To 10 ml of the prescribed solution add 0.1 g of *disodium tetraborate R*. Adjust the solution, if necessary, to pH 8.8 to pH 9.2 using *dilute hydrochloric acid R* or *dilute sodium hydroxide solution R*. Shake with 2 quantities, each of 5 ml, of a 1 g/l solution of *hydroxyquinoline R* in *chloroform R*, for 1 min each time. Allow to stand. Separate and discard the organic layer. To the aqueous solution add 0.4 ml of *butylamine R* and 0.1 ml of *triethanolamine R*. Adjust the solution, if necessary, to pH 10.5 to pH 11.5. Add 4 ml of the solution of hydroxyquinoline in chloroform, shake for 1 min, allow to stand and separate. Use the lower layer for comparison. Prepare a standard in the same manner using a mixture of 1 ml of *magnesium standard solution (10 ppm Mg) R* and 9 ml of *water R*.

Any colour in the solution obtained from the substance to be examined is not more intense than that in the standard.

01/2005:20407

2.4.7. MAGNESIUM AND ALKALINE-EARTH METALS

To 200 ml of *water R* add 0.1 g of *hydroxylamine hydrochloride R*, 10 ml of *ammonium chloride buffer solution pH 10.0 R*, 1 ml of 0.1 M *zinc sulphate* and about 15 mg of *mordant black 11 triturate R*. Heat to about 40 °C. Titrate with 0.01 M *sodium edetate* until the violet colour changes to full blue. To the solution add the prescribed quantity of the substance to be examined dissolved in 100 ml of *water R* or use the prescribed solution. If the colour of the solution changes to violet, titrate with 0.01 M *sodium edetate* until the full blue colour is again obtained.

The volume of 0.01 M *sodium edetate* used in the second titration does not exceed the prescribed quantity.

01/2005:20408

2.4.8. HEAVY METALS

The methods described below require the use of *thioacetamide reagent R*. As an alternative, *sodium sulphide solution R1* (0.1 ml) is usually suitable. Since tests prescribed in monographs have been developed using *thioacetamide reagent R*, if *sodium sulphide solution R1* is used instead, it is necessary to include also for methods A and B a monitor solution, prepared from the quantity of the substance to be examined prescribed for the test, to which has been added the volume of lead standard solution prescribed for preparation of the reference solution. The test is invalid if the monitor solution is not comparable with the reference solution.

METHOD A

Test solution. 12 ml of the prescribed aqueous solution of the substance to be examined.

Reference solution (standard). A mixture of 10 ml of *lead standard solution (1 ppm Pb) R* or *lead standard solution (2 ppm Pb) R*, as prescribed, and 2 ml of the prescribed aqueous solution of the substance to be examined.

Blank solution. A mixture of 10 ml of *water R* and 2 ml of the prescribed aqueous solution of the substance to be examined.

To each solution, add 2 ml of *buffer solution pH 3.5 R*. Mix. Add 1.2 ml of *thioacetamide reagent R*. Mix immediately. Examine the solutions after 2 min. The test is invalid if the reference solution does not show a slight brown colour compared to the blank solution. The substance to be examined complies with the test if any brown colour in the test solution is not more intense than that in the reference solution.

If the result is difficult to judge, filter the solutions through a membrane filter (pore size 3 µm; see Figure 2.4.8-1, without the prefilter). Carry out the filtration slowly and uniformly, applying moderate and constant pressure to the piston. Compare the spots on the filters obtained with the different solutions.

METHOD B

Test solution. 12 ml of the prescribed solution of the substance to be examined prepared using an organic solvent containing a minimum percentage of water (for example, dioxan containing 15 per cent of water or acetone containing 15 per cent of water).

Reference solution (standard). A mixture of 10 ml of lead standard solution (1 or 2 ppm Pb), as prescribed, and 2 ml of the prescribed solution of the substance to be examined in an organic solvent. Prepare the lead standard solution (1 or 2 ppm Pb) by dilution of *lead standard solution (100 ppm Pb) R* with the solvent used for the substance to be examined.

Blank solution. A mixture of 10 ml of the solvent used for the substance to be examined and 2 ml of the prescribed solution of the substance to be examined in an organic solvent.

To each solution, add 2 ml of *buffer solution pH 3.5 R*. Mix. Add 1.2 ml of *thioacetamide reagent R*. Mix immediately. Examine the solutions after 2 min. The test is invalid if the reference solution does not show a slight brown colour compared to the blank solution. The substance to be examined complies with the test if any brown colour in the test solution is not more intense than that in the reference solution.

If the result is difficult to judge, filter the solutions through a membrane filter (pore size 3 µm; see Figure 2.4.8-1, without the prefilter). Carry out the filtration slowly and uniformly, applying moderate and constant pressure to the piston. Compare the spots on the filters obtained with the different solutions.

METHOD C

Test solution. Place the prescribed quantity (not more than 2 g) of the substance to be examined in a silica crucible with 4 ml of a 250 g/l solution of *magnesium sulphate R* in *dilute sulphuric acid R*. Mix using a fine glass rod. Heat cautiously. If the mixture is liquid, evaporate gently to dryness on a water-bath. Progressively heat to ignition and continue heating until an almost white or at most greyish residue is obtained. Carry out the ignition at a temperature not exceeding 800 °C. Allow to cool. Moisten the residue with a few drops of *dilute sulphuric acid R*. Evaporate, ignite again and allow to cool. The total period of ignition must not exceed 2 h. Take up the residue in 2 quantities, each of 5 ml, of *dilute hydrochloric acid R*. Add 0.1 ml of *phenolphthalein solution R*, then *concentrated ammonia R* until a pink colour is obtained. Cool, add *glacial acetic acid R* until the solution is decolorised and add 0.5 ml in excess. Filter if necessary and wash the filter. Dilute to 20 ml with *water R*.

Reference solution (standard). Prepare as described for the test solution, using the prescribed volume of *lead standard solution (10 ppm Pb) R* instead of the substance to be examined. To 10 ml of the solution obtained add 2 ml of the test solution.

Monitor solution. Prepare as described for the test solution, adding to the substance to be examined the volume of *lead standard solution (10 ppm Pb) R* prescribed for preparation of the reference solution. To 10 ml of the solution obtained add 2 ml of the test solution.

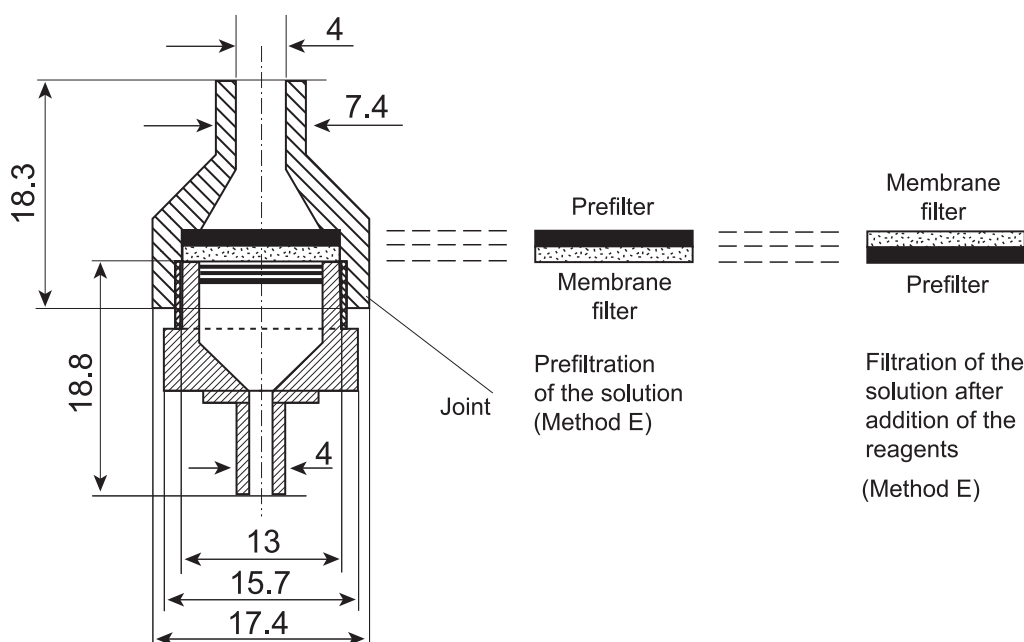


Figure 2.4.8-1. – Apparatus for the test for heavy metals
Dimensions in millimetres

Blank solution. A mixture of 10 ml of *water R* and 2 ml of the test solution.

To 12 ml of each solution, add 2 ml of *buffer solution pH 3.5 R*. Mix. Add 1.2 ml of *thioacetamide reagent R*. Mix immediately. Examine the solutions after 2 min. The test is invalid if the reference solution does not show a slight brown colour compared to the blank solution or if the monitor solution is not comparable with the reference solution. The substance to be examined complies with the test if any brown colour in the test solution is not more intense than that in the reference solution.

If the result is difficult to judge, filter the solutions through a membrane filter (pore size 3 µm; see Figure 2.4.8-1, without the prefilter). Carry out the filtration slowly and uniformly, applying moderate and constant pressure to the piston. Compare the spots on the filters obtained with the different solutions.

METHOD D

Test solution. In a silica crucible, mix thoroughly the prescribed quantity of the substance to be examined with 0.5 g of *magnesium oxide R1*. Ignite to dull redness until a homogeneous white or greyish-white mass is obtained. If after 30 min of ignition the mixture remains coloured, allow to cool, mix using a fine glass rod and repeat the ignition. If necessary repeat the operation. Heat at 800 °C for about 1 h. Take up the residue in 2 quantities, each of 5 ml, of a mixture of equal volumes of *hydrochloric acid R1* and *water R*. Add 0.1 ml of *phenolphthalein solution R* and then *concentrated ammonia R* until a pink colour is obtained. Cool, add *glacial acetic acid R* until the solution is decolorised and add 0.5 ml in excess. Filter if necessary and wash the filter. Dilute to 20 ml with *water R*.

Reference solution (standard). Prepare as described for the test solution using the prescribed volume of *lead standard solution (10 ppm Pb) R* instead of the substance to be examined and drying in an oven at 100-105 °C. To 10 ml of the solution obtained add 2 ml of the test solution.

Monitor solution. Prepare as described for the test solution, adding to the substance to be examined the volume of *lead standard solution (10 ppm Pb) R* prescribed for preparation of the reference solution and drying in an oven at 100-105 °C. To 10 ml of the solution obtained add 2 ml of the test solution.

Blank solution. A mixture of 10 ml of *water R* and 2 ml of the test solution.

To 12 ml of each solution, add 2 ml of *buffer solution pH 3.5 R*. Mix. Add 1.2 ml of *thioacetamide reagent R*. Mix immediately. Examine the solutions after 2 min. The test is invalid if the reference solution does not show a slight brown colour compared to the blank solution or if the monitor solution is not comparable with the reference solution. The substance to be examined complies with the test if any brown colour in the test solution is not more intense than that in the reference solution.

If the result is difficult to judge, filter the solutions through a membrane filter (pore size 3 µm; see Figure 2.4.8-1, without the prefilter). Carry out the filtration slowly and uniformly, applying moderate and constant pressure to the piston. Compare the spots on the filters obtained with the different solutions.

METHOD E

Test solution. Dissolve the prescribed quantity of the substance to be examined in 30 ml of *water R* or the prescribed volume.

Reference solution (standard). Unless otherwise prescribed, dilute the prescribed volume of *lead standard solution (1 ppm Pb) R* to the same volume as the test solution.

Prepare the filtration apparatus by adapting the barrel of a 50 ml syringe without its piston to a support containing, on the plate, a membrane filter (pore size 3 µm) and above it a prefilter (Figure 2.4.8-1).

Transfer the test solution into the syringe barrel, put the piston in place and then apply an even pressure on it until the whole of the liquid has been filtered. In opening the support and removing the prefilter, check that the membrane filter remains uncontaminated with impurities. If this is not the case replace it with another membrane filter and repeat the operation under the same conditions.

To the prefiltrate or to the prescribed volume of the prefiltrate add 2 ml of *buffer solution pH 3.5 R*. Add to 1.2 ml of *thioacetamide reagent R*. Mix and allow to stand for 10 min and again filter as described above, but inverting the order of the filters, the liquid passing first through the membrane filter before passing through the prefilter (Figure 2.4.8-1). The filtration must be carried out slowly and uniformly by applying moderate and constant pressure to the piston of the syringe. After complete filtration, open the support, remove the membrane filter, and dry using filter paper.

In parallel, treat the reference solution in the same manner as the test solution.

The colour of the spot obtained with the test solution is not more intense than that obtained with the reference solution.

METHOD F

Test solution. Place the prescribed quantity or volume of the substance to be examined in a clean, dry, 100 ml long-necked combustion flask (a 300 ml flask may be used if the reaction foams excessively). Clamp the flask at an angle of 45°. If the substance to be examined is a solid, add a sufficient volume of a mixture of 8 ml of *sulphuric acid R* and 10 ml of *nitric acid R* to moisten the substance thoroughly; if the substance to be examined is a liquid, add a few millilitres of a mixture of 8 ml of *sulphuric acid R* and 10 ml of *nitric acid R*. Warm gently until the reaction commences, allow the reaction to subside and add additional portions of the same acid mixture, heating after each addition, until a total of 18 ml of the acid mixture has been added. Increase the amount of heat and boil gently until the solution darkens. Cool, add 2 ml of *nitric acid R* and heat again until the solution darkens. Continue the heating, followed by the addition of *nitric acid R* until no further darkening occurs, then heat strongly until dense, white fumes are produced. Cool, cautiously add 5 ml of *water R*, boil gently until dense, white fumes are produced and continue heating to reduce to 2-3 ml. Cool, cautiously add 5 ml of *water R* and examine the colour of the solution. If the colour is yellow, cautiously add 1 ml of *strong hydrogen peroxide solution R* and again evaporate until dense, white fumes are produced and reduce to a volume of 2-3 ml. If the solution is still yellow in colour, repeat the addition of 5 ml of *water R* and 1 ml of *strong hydrogen peroxide solution R* until the solution is colourless. Cool, dilute cautiously with *water R* and rinse into a 50 ml colour comparison tube, ensuring that the total volume does not exceed 25 ml. Adjust the solution to pH 3.0-4.0, using short range pH indicator paper as external indicator, with *concentrated ammonia R1* (*dilute ammonia R1* may be used, if desired, as the specified range is approached), dilute with *water R* to 40 ml and mix. Add 2 ml of *buffer solution pH 3.5 R* and 1.2 ml of *thioacetamide reagent R*. Mix immediately. Dilute to 50 ml with *water R* and mix.

Reference solution (standard). Prepare at the same time and in the same manner as the test solution, using the prescribed volume of *lead standard solution (10 ppm Pb) R*.

Monitor solution. Prepare as described for the test solution, adding to the substance to be examined the volume of *lead standard solution (10 ppm Pb) R* prescribed for the preparation of the reference solution.

Blank solution. Prepare as described for the test solution, omitting the substance to be examined.

Examine the solutions vertically against a white background. After 2 min, any brown colour in the test solution is not more intense than that in the reference solution.

The test is invalid if the reference solution does not show a brown colour compared to the blank solution or if the monitor solution is not comparable with the reference solution.

If the result is difficult to judge, filter the solutions through a membrane filter (pore size 3 µm; see Figure 2.4.8-1, without the prefilter). Carry out the filtration slowly and uniformly, applying moderate and constant pressure to the piston. Compare the spots on the filters obtained with the different solutions.

METHOD G

CAUTION: when using high-pressure digestion vessels the safety precautions and operating instructions given by the manufacturer must be followed. The digestion cycles have to be elaborated depending on the type of microwave oven to be used (for example, energy-controlled microwave ovens, temperature-controlled microwave ovens or high-pressure ovens). The cycle must be conform to the manufacturer's instructions. The digestion cycle is suitable if a clear solution is obtained.

Test solution. Place the prescribed amount of the substance to be examined (not more than 0.5 g) in a suitable, clean beaker. Add successively 2.7 ml of *sulphuric acid R*, 3.3 ml of *nitric acid R* and 2.0 ml of *strong hydrogen peroxide solution R* using a magnetic stirrer. Allow the substance to react with a reagent before adding the next one. Transfer the mixture to a dry high-pressure-resistant digestion vessel (fluoropolymer or quartz glass).

Reference solution (standard). Prepare as described for the test solution, using the prescribed volume of *lead standard solution (10 ppm Pb) R* instead of the substance to be examined.

Monitor solution. Prepare as prescribed for the test solution, adding to the substance to be examined the volume of *lead standard solution (10 ppm Pb) R* prescribed for the preparation of the reference solution.

Blank solution. Prepare as described for the test solution, omitting the substance to be examined.

Close the vessels and place in a laboratory microwave oven. Digest using a sequence of 2 separate suitable programmes. Design the programmes in several steps in order to control the reaction, monitoring pressure, temperature or energy depending on the type of microwave oven available. After the first programme allow the digestion vessels to cool before opening. Add to each vessel 2.0 ml of *strong hydrogen peroxide solution R* and digest using the second programme. After the second programme allow the digestion vessels to cool before opening. If necessary to obtain a clear solution, repeat the addition of *strong hydrogen peroxide solution R* and the second digestion programme.

Cool, dilute cautiously with *water R* and rinse into a flask, ensuring that the total volume does not exceed 25 ml.

Using short-range pH indicator paper as external indicator, adjust the solutions to pH 3.0-4.0 with *concentrated ammonia R1* (*dilute ammonia R1* may be used as the specified range is approached). To avoid heating of the solutions use an ice-bath and a magnetic stirrer. Dilute to 40 ml with *water R* and mix. Add 2 ml of *buffer solution pH 3.5 R* and 1.2 ml of *thioacetamide reagent R*. Mix immediately. Dilute to 50 ml with *water R*, mix and allow to stand for 2 min.

Filter the solutions through a membrane filter (pore size 3 µm; see Figure 2.4.8-1, without the prefilter). Carry out the filtration slowly and uniformly, applying moderate and constant pressure to the piston. Compare the spots on the filters obtained with the different solutions.

Examine the spots on the filters. The brown colour from the spot of the test solution is not more intense than that from the reference solution.

The test is invalid if the reference solution spot does not show a brown colour compared to the blank spot, or if the spot from the monitor solution is not comparable with the spot from the reference solution.

01/2005:20409

2.4.9. IRON

Dissolve the prescribed quantity of the substance to be examined in *water R* and dilute to 10 ml with the same solvent or use 10 ml of the prescribed solution. Add 2 ml of a 200 g/l solution of *citric acid R* and 0.1 ml of *thioglycollic acid R*. Mix, make alkaline with *ammonia R* and dilute to 20 ml with *water R*. Prepare a standard in the same manner, using 10 ml of *iron standard solution (1 ppm Fe) R*.

After 5 min, any pink colour in the test solution is not more intense than that in the standard.

01/2005:20410

2.4.10. LEAD IN SUGARS

Determine the lead by atomic absorption spectrometry (2.2.23, *Method II*).

Test solution. Dissolve 20.0 g of the substance to be examined in a mixture of equal volumes of *dilute acetic acid R* and *water R* and dilute to 100.0 ml with the same mixture of solvents. Add 2.0 ml of a clear 10 g/l solution of *ammonium pyrrolidinedithiocarbamate R* and 10.0 ml of *methyl isobutyl ketone R* and then shake for 30 s protected from bright light. Allow the layers to separate and use the methyl isobutyl ketone layer.

Reference solutions. Prepare 3 reference solutions in the same manner as the test solution but adding 0.5 ml, 1.0 ml and 1.5 ml respectively of *lead standard solution (10 ppm Pb) R* in addition to the 20.0 g of the substance to be examined.

Set the zero of the instrument using *methyl isobutyl ketone R* treated as described for the test solution without the substance to be examined. Measure the absorbance at 283.3 nm using a lead hollow-cathode lamp as source of radiation and an air-acetylene flame.

The substance to be examined contains not more than 0.5 ppm of lead, unless otherwise prescribed.

01/2005:20411

01/2005:20415

2.4.11. PHOSPHATES

To 100 ml of the solution prepared and, if necessary, neutralised as prescribed add 4 ml of *sulphomolybdic reagent R3*. Shake and add 0.1 ml of *stannous chloride solution R1*. Prepare a standard in the same manner using 2 ml of *phosphate standard solution (5 ppm PO₄) R* and 98 ml of *water R*. After 10 min, compare the colours using 20 ml of each solution.

Any colour in the test solution is not more intense than that in the standard.

01/2005:20412

2.4.12. POTASSIUM

To 10 ml of the prescribed solution add 2 ml of a freshly prepared 10 g/l solution of *sodium tetraphenylborate R*. Prepare a standard in the same manner using a mixture of 5 ml of *potassium standard solution (20 ppm K) R* and 5 ml of *water R*.

After 5 min, any opalescence in the test solution is not more intense than that in the standard.

01/2005:20413

2.4.13. SULPHATES

All solutions used for this test must be prepared with distilled water R.

Add 3 ml of a 250 g/l solution of *barium chloride R* to 4.5 ml of *sulphate standard solution (10 ppm SO₄) R1*. Shake and allow to stand for 1 min. To 2.5 ml of this solution, add 15 ml of the solution to be examined and 0.5 ml of *acetic acid R*. Prepare a standard in the same manner using 15 ml of *sulphate standard solution (10 ppm SO₄) R* instead of the solution to be examined.

After 5 min, any opalescence in the test solution is not more intense than that in the standard.

01/2005:20414

2.4.14. SULPHATED ASH

Ignite a suitable crucible (silica, platinum, porcelain or quartz) at 600 ± 50 °C for 30 min, allow to cool in a desiccator over silica gel and weigh. Place the prescribed amount of the substance to be examined in the crucible and weigh. Moisten the substance to be examined with a small amount of *sulphuric acid R* (usually 1 ml) and heat gently at as low a temperature as practicable until the sample is thoroughly charred. After cooling, moisten the residue with a small amount of *sulphuric acid R*, heat gently until white fumes are no longer evolved and ignite at 600 ± 50 °C until the residue is completely incinerated. Ensure that flames are not produced at any time during the procedure. Allow the crucible to cool in a desiccator over silica gel, weigh it again and calculate the mass of the residue.

If the mass of the residue so obtained exceeds the prescribed limit, repeat the moistening with *sulphuric acid R* and ignition, as previously, to constant mass, unless otherwise prescribed.

2.4.15. NICKEL IN POLYOLS

Determine the nickel by atomic absorption spectrometry (2.2.23, *Method II*).

Test solution. Dissolve 20.0 g of the substance to be examined in a mixture of equal volumes of *dilute acetic acid R* and *water R* and dilute to 100.0 ml with the same mixture of solvents. Add 2.0 ml of a saturated solution of *ammonium pyrrolidinedithiocarbamate R* (about 10 g/l) and 10.0 ml of *methyl isobutyl ketone R* and then shake for 30 s protected from bright light. Allow the layers to separate and use the methyl isobutyl ketone layer.

Reference solutions. Prepare 3 reference solutions in the same manner as the test solution but adding 0.5 ml, 1.0 ml and 1.5 ml respectively of *nickel standard solution (10 ppm Ni) R* in addition to the 20.0 g of the substance to be examined.

Set the zero of the instrument using *methyl isobutyl ketone R* treated as described for preparation of the test solution omitting the substance to be examined. Measure the absorbance at 232.0 nm using a nickel hollow-cathode lamp as source of radiation and an air-acetylene flame.

The substance to be examined contains not more than 1 ppm of nickel, unless otherwise prescribed.

01/2005:20416

2.4.16. TOTAL ASH

Heat a silica or platinum crucible to redness for 30 min, allow to cool in a desiccator and weigh. Unless otherwise prescribed, evenly distribute 1.00 g of the substance or the powdered vegetable drug to be examined in the crucible. Dry at 100 °C to 105 °C for 1 h and ignite to constant mass in a muffle furnace at 600 ± 25 °C, allowing the crucible to cool in a desiccator after each ignition. Flames should not be produced at any time during the procedure. If after prolonged ignition the ash still contains black particles, take up with hot water, filter through an ashless filter paper and ignite the residue and the filter paper. Combine the filtrate with the ash, carefully evaporate to dryness and ignite to constant mass.

01/2005:20417

2.4.17. ALUMINIUM

Place the prescribed solution in a separating funnel and shake with 2 quantities, each of 20 ml, and then with one 10 ml quantity of a 5 g/l solution of *hydroxyquinoline R* in *chloroform R*. Dilute the combined chloroform solutions to 50.0 ml with *chloroform R* (test solution).

Prepare a standard in the same manner using the prescribed reference solution.

Prepare a blank in the same manner using the prescribed blank solution.

Measure the intensity of the fluorescence (2.2.21) of the test solution (I_1), of the standard (I_2) and of the blank (I_3) using an excitant beam at 392 nm and a secondary filter with a transmission band centred on 518 nm or a monochromator set to transmit at this wavelength.

The fluorescence ($I_1 - I_3$) of the test solution is not greater than that of the standard ($I_2 - I_3$).

01/2005:20418
corrected

01/2005:20419

2.4.18. FREE FORMALDEHYDE

Use method A, unless otherwise prescribed. Method B is suitable for vaccines where sodium metabisulphite has been used to neutralise excess formaldehyde.

METHOD A

For vaccines for human use, prepare a 1 in 10 dilution of the vaccine to be examined. For bacterial toxoids for veterinary use, prepare a 1 in 25 dilution of the vaccine to be examined.

To 1 ml of the dilution, add 4 ml of *water R* and 5 ml of *acetylacetone reagent R1*. Place the tube in a water-bath at 40 °C for 40 min. Examine the tubes down their vertical axes. The solution is not more intensely coloured than a standard, prepared at the same time and in the same manner, using 1 ml of a dilution of *formaldehyde solution R* containing 20 µg of formaldehyde (CH₂O) per millilitre, instead of the dilution of the vaccine to be examined.

METHOD B

Test solution. Prepare a 1 in 200 dilution of the vaccine to be examined with *water R*. If the vaccine is an emulsion, prepare an equivalent dilution using the aqueous phase separated by a suitable procedure (see below). If one of the methods described below is used for separation of the aqueous phase, a 1 in 20 dilution of the latter is used.

Reference solutions. Prepare solutions containing 0.25 g/l, 0.50 g/l, 1.00 g/l and 2.00 g/l of CH₂O by dilution of *formaldehyde solution R* with *water R*. Prepare a 1 in 200 dilution of each solution with *water R*.

To 0.5 ml of the test solution and of each of the reference solutions in test-tubes, add 5.0 ml of a freshly prepared 0.5 g/l solution of *methylbenzothiazolone hydrazone hydrochloride R*. Close the tubes, shake and allow to stand for 60 min. Add 1 ml of *ferric chloride-sulphamic acid reagent R* and allow to stand for 15 min. Measure the absorbance (2.2.25) of the solutions at 628 nm. Calculate the content of formaldehyde in the vaccine to be examined from the calibration curve established using the reference solutions. The test is invalid if the correlation coefficient (*r*) of the calibration curve is less than 0.97.

Emulsions. If the vaccine to be examined is an emulsion, the aqueous phase is separated using a suitable procedure and used for preparation of the test solution. The following procedures have been found suitable.

(a) Add 1.0 ml of the vaccine to be examined to 1.0 ml of *isopropyl myristate R* and mix. Add 1.3 ml of 1 M *hydrochloric acid*, 2.0 ml of *chloroform R* and 2.7 ml of a 9 g/l solution of *sodium chloride R*. Mix thoroughly. Centrifuge at 15 000 *g* for 60 min. Transfer the aqueous phase to a 10 ml volumetric flask and dilute to volume with *water R*. If this procedure fails to separate the aqueous phase, add 100 g/l of *polysorbate 20 R* to the sodium chloride solution and repeat the procedure but centrifuge at 22 500 *g*.

(b) Add 1.0 ml of the vaccine to be examined to 1.0 ml of a 100 g/l solution of *sodium chloride R* and mix. Centrifuge at 1000 *g* for 15 min. Transfer the aqueous phase to a 10 ml volumetric flask and dilute to volume with *water R*.

(c) Add 1.0 ml of the vaccine to be examined to 2.0 ml of a 100 g/l solution of *sodium chloride R* and 3.0 ml of *chloroform R* and mix. Centrifuge at 1000 *g* for 5 min. Transfer the aqueous phase to a 10 ml volumetric flask and dilute to volume with *water R*.

2.4.19. ALKALINE IMPURITIES IN FATTY OILS

In a test-tube mix 10 ml of recently distilled *acetone R* and 0.3 ml of *water R* and add 0.05 ml of a 0.4 g/l solution of *bromophenol blue R* in *alcohol R*. Neutralise the solution if necessary with 0.01 M *hydrochloric acid* or 0.01 M *sodium hydroxide*. Add 10 ml of the oil to be examined, shake and allow to stand. Not more than 0.1 ml of 0.01 M *hydrochloric acid* is required to change the colour of the upper layer to yellow.

01/2005:20421

2.4.21. FOREIGN OILS IN FATTY OILS BY THIN-LAYER CHROMATOGRAPHY

Examine by thin-layer chromatography (2.2.27) using *kieselguhr G R* as the coating substance. Impregnate a plate by placing it in a chromatographic tank containing the necessary quantity of a mixture of 10 volumes of *liquid paraffin R* and 90 volumes of *light petroleum R* so that the plate dips about 5 mm beneath the surface of the liquid. When the impregnation mixture has risen by at least 12 cm from the lower edge of the plate, remove the plate and allow the solvent to evaporate for 5 min. Carry out the chromatography in the same direction as the impregnation.

Preparation of the mixture of fatty acids. Heat 2 g of the oil with 30 ml of 0.5 M *alcoholic potassium hydroxide* under a reflux condenser for 45 min. Add 50 ml of *water R*, allow to cool, transfer to a separating funnel and extract with three quantities, each of 50 ml, of *ether R*. Discard the ether extracts, acidify the aqueous layer with *hydrochloric acid R* and extract with three quantities, each of 50 ml, of *ether R*. Combine the ether extracts and wash with three quantities, each of 10 ml, of *water R*; discard the washings, dry the ether over *anhydrous sodium sulphate R* and filter. Evaporate the ether on a water-bath. Use the residue to prepare the test solution. The fatty acids may also be obtained from the soap solution prepared during the determination of the unsaponifiable matter.

Test solution. Dissolve 40 mg of the mixture of fatty acids obtained from the substance to be examined in 4 ml of *chloroform R*.

Reference solution. Dissolve 40 mg of the mixture of fatty acids obtained from a mixture of 19 volumes of *maize oil R* and 1 volume of *rapeseed oil R* in 4 ml of *chloroform R*.

Apply to the plate 3 µl of each solution. Develop over a path of 8 cm using a mixture of 10 volumes of *water R* and 90 volumes of *glacial acetic acid R*. Dry the plate at 110 °C for 10 min. Allow to cool and, unless otherwise prescribed, place the plate in a chromatographic chamber, with a tightly fitting lid, that has previously been saturated with iodine vapour by placing *iodine R* in an evaporating dish at the bottom of the chamber. After some time brown or yellowish-brown spots become visible. Remove the plate and allow to stand for a few minutes. When the brown background colour has disappeared, spray with *starch solution R*. Blue spots appear which may become brown on drying and again become blue after spraying with *water R*. The chromatogram obtained with the test solution always shows a spot with an *R_f* of about 0.5 (oleic acid) and a spot with an *R_f* of about 0.65 (linoleic acid) corresponding to the spots in the chromatogram obtained with the reference solution. With some oils a spot with an *R_f* of about 0.75 may be present (linolenic acid). By comparison with the spot in

the chromatogram obtained with the reference solution, verify the absence in the chromatogram obtained with the test solution of a spot with an R_f of about 0.25 (erucic acid).

01/2005:20422

2.4.22. COMPOSITION OF FATTY ACIDS BY GAS CHROMATOGRAPHY

The test for foreign oils is carried out on the methyl esters of the fatty acids contained in the oil to be examined by gas chromatography (2.2.28).

METHOD A

This method is not applicable to oils that contain glycerides of fatty acids with an epoxy-, hydroepoxy-, cyclopropyl or cyclopropenyl group, or those that contain a large proportion of fatty acids of chain length less than 8 carbon atoms or to oils with an acid value greater than 2.0.

Test solution. When prescribed in the monograph, dry the oil to be examined before the methylation step. Weigh 1.0 g of the oil into a 25 ml round-bottomed flask with a ground-glass neck fitted with a reflux condenser and a gas port into the flask. Add 10 ml of *anhydrous methanol R* and 0.2 ml of a 60 g/l solution of *potassium hydroxide R* in *methanol R*. Attach the reflux condenser, pass *nitrogen R* through the mixture at a rate of about 50 ml/min, shake and heat to boiling. When the solution is clear (usually after about 10 min), continue heating for a further 5 min. Cool the flask under running water and transfer the contents to a separating funnel. Rinse the flask with 5 ml of *heptane R* and transfer the rinsings to the separating funnel and shake. Add 10 ml of a 200 g/l solution of *sodium chloride R* and shake vigorously. Allow to separate and transfer the organic layer to a vial containing *anhydrous sodium sulphate R*. Allow to stand, then filter.

Reference solution (a). Prepare 0.50 g of the mixture of calibrating substances with the composition described in one of the tables 2.4.22, as prescribed in the individual monograph (if the monograph does not mention a specific solution, use the composition described in Table 2.4.22-1). Dissolve in *heptane R* and dilute to 50.0 ml with the same solvent.

Reference solution (b). Dilute 1.0 ml of reference solution (a) to 10.0 ml with *heptane R*.

Reference solution (c). Prepare 0.50 g of a mixture of fatty acid methyl esters⁽¹⁾, which corresponds in composition to the mixture of fatty acids indicated in the monograph of the substance to be examined. Dissolve in *heptane R* and dilute to 50.0 ml with the same solvent. Commercially available mixtures of fatty acid methyl esters may also be used.

Column:

- **material:** fused silica, glass or quartz,
- **size:** $l = 10\text{--}30$ m, $\varnothing = 0.2\text{--}0.8$ mm,
- **stationary phase:** *poly[(cyanopropyl)(methyl)](phenyl)(methyl)siloxane R* or *macrogol 20 000 R* (film thickness 0.1–0.5 μm) or some other suitable stationary phase.

Carrier gas: *helium for chromatography R* or *hydrogen for chromatography R*.

Flow rate: 1.3 ml/min (for a column $\varnothing = 0.32$ mm).

Split ratio: 1:100 or less, according to the internal diameter of the column used (1:50 when $\varnothing = 0.32$ mm).

Temperature:

- **column:** 160–200 °C, according to the length and type of column used (200 °C for a column 30 m long and coated with a layer of *macrogol 20 000 R*); if necessary, or where prescribed, raise the temperature of the column at a rate of 3 °C/min from 170 °C to 230 °C (for the *macrogol 20 000 R* column),
- **injection port:** 250 °C,
- **detector:** 250 °C.

Detection: flame ionisation.

Injection: 1 μl .

Sensitivity: the height of the principal peak in the chromatogram obtained with reference solution (a) is 50 per cent to 70 per cent of the full scale of the recorder.

System suitability when using the mixture of calibrating substances in Table 2.4.22-1 or 2.4.22-3:

- **resolution:** minimum 1.8 between the peaks due to methyl oleate and methyl stearate in the chromatogram obtained with reference solution (a),
- **signal-to-noise ratio:** minimum 5 for the peak due to methyl myristate in the chromatogram obtained with reference solution (b),
- **number of theoretical plates:** minimum 30 000 calculated for the peak due to methyl stearate in the chromatogram obtained with reference solution (a).

System suitability when using the mixture of calibrating substances in Table 2.4.22-2:

- **resolution:** minimum 4.0 between the peaks due to methyl caprylate and methyl caprate in the chromatogram obtained with reference solution (a),
- **signal-to-noise ratio:** minimum 5 for the peak due to methyl caproate in the chromatogram obtained with reference solution (b),
- **number of theoretical plates:** minimum 15 000 calculated for the peak due to methyl caprate in the chromatogram obtained with reference solution (a).

ASSESSMENT OF CHROMATOGRAMS

Avoid working conditions tending to give masked peaks (presence of constituents with small differences between retention times, for example linolenic acid and arachidic acid).

Qualitative analysis. Identify the peaks in the chromatogram obtained with reference solution (c); the peaks may also be identified by drawing calibration curves using the chromatogram obtained with reference solution (a) and the information given in Tables 2.4.22-1, 2.4.22-2 and 2.4.22-3:

a) using isothermal operating conditions giving the logarithms of reduced retention times as a function of the number of carbon atoms of the fatty acid; identify the peaks by means of the straight line thus obtained and the “equivalent chain lengths” of the different peaks. The calibration curve of the saturated acids is a straight line. The logarithms of reduced retention times of unsaturated acids are situated on this line at points corresponding to non-integer values of carbon atoms known as “equivalent chain lengths”;

b) using linear temperature programming giving the retention time according to the number of carbon atoms of the fatty acid; identify by reference to the calibration curve.

Quantitative analysis. In general, the normalisation procedure is used in which the sum of the areas of the peaks in the chromatogram, except that of the solvent, is set at 100 per cent. The content of a constituent is calculated

(1) The fatty acid methyl esters used show a quality at least as good as that guaranteed by the BCR (Community Bureau of Reference) of the European Union.

by determining the area of the corresponding peak as a percentage of the sum of the areas of all the peaks. Disregard any peak with an area less than 0.05 per cent of the total area.

In certain cases, for example in the presence of fatty acids with 12 or less carbon atoms, correction factors can be prescribed in the individual monograph to convert peak areas in per cent *m/m*.

METHOD B

This method is not applicable to oils that contain glycerides of fatty acids with an epoxy-, hydroepoxy-, cyclopropyl or cyclopropenyl group or to oils with an acid value greater than 2.0.

Test solution. Introduce 0.100 g of the substance to be examined in a 10 ml centrifuge tube with a screw cap. Dissolve with 1 ml of *heptane R* and 1 ml of *dimethyl carbonate R* and mix vigorously under gentle heating (50–60 °C). Add, while still warm, 1 ml of a 12 g/l solution of *sodium R* in *anhydrous methanol R*, prepared with the necessary precautions and mix vigorously for about 5 min. Add 3 ml of *distilled water R* and mix vigorously for about 30 s. Centrifuge for 15 min at 1500 *g*. Inject 1 µl of the organic phase.

Reference solutions and assessment of chromatograms. Without specific prescription in the individual monograph, proceed as described under Method A.

Column:

- **material:** fused silica,
- **size:** *l* = 30 m, Ø = 0.25 mm,
- **stationary phase:** *macrogol 20 000 R* (film thickness 0.25 µm),

Carrier gas: *helium for chromatography R*.

Flow rate: 0.9 ml/min.

Split ratio: 1:100.

Temperature:

	Time (min)	Temperature (°C)
Column	0 - 15	100
	15 - 36	100 → 225
	36 - 61	225
Injection port		250
Detector		250

Detection: flame ionisation.

Injection: 1 µl.

METHOD C

This method is not applicable to oils that contain glycerides of fatty acids with epoxy-, hydroperoxy-, aldehyde, ketone, cyclopropyl and cyclopropenyl groups, and conjugated polyunsaturated and acetylenic compounds because of partial or complete destruction of these groups.

Test solution. Dissolve 0.10 g of the substance to be examined in 2 ml of a 20 g/l solution of *sodium hydroxide R* in *methanol R* in a 25 ml conical flask and boil under a reflux condenser for 30 min. Add 2.0 ml of *boron trifluoride-methanol solution R* through the condenser and boil for 30 min. Add 4 ml of *heptane R* through the condenser and boil for 5 min. Cool and add 10.0 ml of *saturated sodium chloride solution R*, shake for about 15 s and add a quantity of *saturated sodium chloride solution R*

such that the upper phase is brought into the neck of the flask. Collect 2 ml of the upper phase, wash with 3 quantities, each of 2 ml, of *water R* and dry over *anhydrous sodium sulphate R*.

Reference solutions, chromatographic procedure and assessment of chromatograms. Without specific prescription in the individual monograph, proceed as described under Method A.

Table 2.4.22-1. – *Mixture of calibrating substances*⁽²⁾

Mixture of the following substances	Equivalent chain length ⁽³⁾	Composition (per cent <i>m/m</i>)	
		Iso-thermal	Linear temperature programme
<i>Methyl laurate R</i>	12.0	5	10
<i>Methyl myristate R</i>	14.0	5	15
<i>Methyl palmitate R</i>	16.0	10	15
<i>Methyl stearate R</i>	18.0	20	20
<i>Methyl arachidate R</i>	20.0	40	20
<i>Methyl oleate R</i>	18.3	20	20

Table 2.4.22-2. – *Mixture of calibrating substances*⁽²⁾

Mixture of the following substances	Equivalent chain length ⁽³⁾	Composition (per cent <i>m/m</i>)	
		Iso-thermal	Linear temperature programme
<i>Methyl caproate R</i>	6.0	5	10
<i>Methyl caprylate R</i>	8.0	5	35
<i>Methyl caprate R</i>	10.0	10	35
<i>Methyl laurate R</i>	12.0	20	10
<i>Methyl myristate R</i>	14.0	40	10

Table 2.4.22-3. – *Mixture of calibrating substances*⁽²⁾

Mixture of the following substances	Equivalent chain length ⁽³⁾	Composition (per cent <i>m/m</i>)	
		Iso-thermal	Linear temperature programme
<i>Methyl myristate R</i>	14.0	5	15
<i>Methyl palmitate R</i>	16.0	10	15
<i>Methyl stearate R</i>	18.0	15	20
<i>Methyl arachidate R</i>	20.0	20	15
<i>Methyl oleate R</i>	18.3	20	15
<i>Methyl eicosenoate R</i>	20.2	10	10
<i>Methyl behenate R</i>	22.0	10	5
<i>Methyl lignocerate R</i>	24.0	10	5

01/2005:20423

2.4.23. STEROLS IN FATTY OILS

SEPARATION OF THE STEROL FRACTION

Prepare the unsaponifiable matter and then isolate the sterol fraction of the fatty oil by thin-layer chromatography (2.2.27), using *silica gel G R* in a 0.3 mm to 0.5 mm layer as the coating substance.

(2) For GC with capillary column and split inlet system, it is recommended that the component with the longest chain length of the mixture to be examined be added to the calibration mixture, when the qualitative analysis is done using calibration curves.

(3) This value, which is to be calculated using calibration curves, is given as an example for a column of *macrogol 20 000 R*.

Test solution (a). In a 150 ml flask fitted with a reflux condenser, place a volume of a 2 g/l solution of *betulin R* in *methylene chloride R* containing betulin corresponding to about 10 per cent of the sterol content of the sample used for the determination (e.g. in the case of olive oil add 500 µl, in the case of other vegetable oils add 1500 µl of the betulin solution). If the monograph requires the content of the individual sterols as a percentage of the sterol fraction, the addition of betulin may be omitted. Evaporate to dryness under a current of *nitrogen R*. Add 5.00 g (*m g*) of the substance to be examined. Add 50 ml of 2 *M alcoholic potassium hydroxide R* and heat on a water-bath for 1 h, swirling frequently. Cool to a temperature below 25 °C and transfer the contents of the flask to a separating funnel with 100 ml of *water R*. Shake the liquid carefully with 3 quantities, each of 100 ml, of *peroxide-free ether R*. Combine the ether layers in another separating funnel containing 40 ml of *water R*, shake gently for a few minutes, allow to separate and reject the aqueous phase. Wash the ether phase with several quantities, each of 40 ml, of *water R*, until the aqueous phase is no longer alkaline to phenolphthalein. Transfer the ether phase to a tared flask, washing the separating funnel with *peroxide-free ether R*. Distil off the ether with suitable precautions and add 6 ml of *acetone R*. Carefully remove the solvent in a current of *nitrogen R*. Dry to constant mass at 100 °C to 105 °C. Allow to cool in a desiccator and weigh. Dissolve the residue in a minimal volume of *methylene chloride R*.

Test solution (b). Treat 5.00 g of *rapeseed oil R* as prescribed for the substance to be examined, beginning at the words "Add 50 ml of 2 *M alcoholic potassium hydroxide R*".

Test solution (c). Treat 5.00 g of *sunflower oil R* as prescribed for the substance to be examined, beginning at the words "Add 50 ml of 2 *M alcoholic potassium hydroxide R*".

Reference solution. Dissolve 25 mg of *cholesterol R* and 10 mg of *betulin R* in 1 ml of *methylene chloride R*.

Use a separate plate for each test solution. Apply separately as a band 20 mm by 3 mm 20 µl of the reference solution and as a band 40 mm by 3 mm 0.4 ml of test solution (a), test solution (b) or test solution (c). Develop over a path of 18 cm using a mixture of 35 volumes of *ether R* and 65 volumes of *hexane R*. Dry the plates in a current of *nitrogen R*. Spray the plates with a 2 g/l solution of *dichlorofluorescein R* in *ethanol R* and examine in ultraviolet light at 254 nm. The chromatogram obtained with the reference solution shows bands corresponding to cholesterol and betulin. The chromatograms obtained with the test solutions show bands with similar *R_f* values due to sterols. From each of the chromatograms, remove an area of coating corresponding to the area occupied by the sterol bands and additionally the area of the zones 2 mm to 3 mm above and below the visible zones corresponding to the reference solution. Place separately in three 50 ml flasks. To each flask add 15 ml of hot *methylene chloride R* and shake. Filter each solution through a sintered-glass filter (40) or suitable filter paper and wash each filter with three quantities, each of 15 ml, of *methylene chloride R*. Place the combined filtrate and washings from each filter separately in three tared flasks, evaporate to dryness under a stream of *nitrogen R* and weigh.

DETERMINATION OF THE STEROLS

Examine by gas chromatography (2.2.28). Carry out the operations protected from humidity and prepare the solutions immediately before use.

Test solution. To the sterols separated from the substance to be examined by thin-layer chromatography add, per milligram of residue, 0.02 ml of a freshly prepared mixture of 1 volume of *chlorotrimethylsilane R*, 3 volumes of *hexamethyldisilazane R* and 9 volumes of *anhydrous pyridine R*. Shake carefully until the sterols are completely dissolved. Allow to stand in a desiccator over *diphosphorus pentoxide R* for 30 min. Centrifuge if necessary and use the supernatant liquid.

Reference solution (a). To 9 parts of the sterols separated from *rapeseed oil R* by thin-layer chromatography add 1 part of *cholesterol R*. To the mixture add, per milligram of residue, 0.02 ml of a freshly prepared mixture of 1 volume of *chlorotrimethylsilane R*, 3 volumes of *hexamethyldisilazane R* and 9 volumes of *anhydrous pyridine R*. Shake carefully until the sterols are completely dissolved. Allow to stand in a desiccator over *diphosphorus pentoxide R* for 30 min. Centrifuge if necessary and use the supernatant liquid.

Reference solution (b). To the sterols separated from *sunflower oil R* by thin-layer chromatography add, per milligram of residue, 0.02 ml of a freshly prepared mixture of 1 volume of *chlorotrimethylsilane R*, 3 volumes of *hexamethyldisilazane R* and 9 volumes of *anhydrous pyridine R*. Shake carefully until the sterols are completely dissolved. Allow to stand in a desiccator over *diphosphorus pentoxide R* for 30 min. Centrifuge if necessary and use the supernatant liquid.

The chromatographic procedure may be carried out using:

- a fused-silica column 20 m to 30 m long and 0.25 mm to 0.32 mm in internal diameter, coated with *poly[methyl(95)phenyl(5)]siloxane R* or of *poly[methyl(94)phenyl(5) vinyl(1)]siloxane R* (film thickness 0.25 µm),
- *hydrogen for chromatography R* at a velocity of 30 cm to 50 cm per second or *helium for chromatography R* at a velocity of 20 cm to 35 cm per second as the carrier gas. Measure the velocity as follows: maintaining the indicated operating conditions for the determination of the sterols, inject 1 µl to 3 µl of methane or propane. Measure the time in seconds required by the gas to pass through the column from the moment of the injection to the appearance of the peak (*t_M*). The velocity is given by *L/t_M*, where *L* is the length of the column in centimetres,
- a flame-ionisation detector,
- a split injector (1:50 or 1:100),

maintaining the temperature of the column at 260 °C, that of the injection port at 280 °C and that of the detector at 290 °C.

Inject 1 µl of each solution.

The chromatogram obtained with reference solution (a) shows 4 principal peaks corresponding to cholesterol, brassicasterol, campesterol and β-sitosterol and the chromatogram obtained with reference solution (b) shows 4 principal peaks corresponding to campesterol, stigmasterol, β-sitosterol and Δ⁷-stigmastenol. The retention times of the sterols relative to β-sitosterol are given in Table 2.4.23.-1.

The peak of the internal standard (betulin) must be clearly separated from the peaks of the sterols to be determined.

Table 2.4.23-1. – Retention times of sterols relative to β -sitosterol for 2 different columns

	Poly[methyl(95)-phenyl(5)]siloxane	Poly[methyl(94)-phenyl(5)vinyl(1)]siloxane
Cholesterol	0.63	0.67
Brassicasterol	0.71	0.73
24-Methylenecholesterol	0.80	0.82
Campesterol	0.81	0.83
Campestanol	0.82	0.85
Stigmasterol	0.87	0.88
Δ 7-Campesterol	0.92	0.93
Δ 5,23-Stigmastadienol	0.95	0.95
Clerosterol	0.96	0.96
β -Sitosterol	1	1
Sitostanol	1.02	1.02
Δ 5-Avenasterol	1.03	1.03
Δ 5,24-Stigmastadienol	1.08	1.08
Δ 7-Stigmastenol ⁽¹⁾	1.12	1.12
Δ 7-Avenasterol	1.16	1.16
Betulin	1.4	1.6

(1) This sterol may also be referred to as Δ 7-stigmasterol in literature.

For the chromatogram obtained with the test solution, identify the peaks and calculate the percentage content of each sterol in the sterol fraction of the substance to be examined using the following expression:

$$\frac{A}{S} \times 100$$

A = area of the peak corresponding to the component to be determined,

S = sum of the areas of the peaks corresponding to the components indicated in Table 2.4.23-1.

If required in the monograph, calculate the content of each sterol in milligrams per 100 grams of the substance to be examined using the following expression:

$$\frac{A \times m_S \times 100}{A_S \times m}$$

A = area of the peak corresponding to the component to be determined,

A_S = area of the peak corresponding to betulin,

m = mass of the sample of the substance to be examined in grams,

m_S = mass of *betulin R* added in milligrams.

01/2005:20424
corrected

2.4.24. IDENTIFICATION AND CONTROL OF RESIDUAL SOLVENTS

The test procedures described in this general method may be used:

i. for the identification of the majority of Class 1 and Class 2 residual solvents in an active substance, excipient or medicinal product when the residual solvents are unknown;

ii. as a limit test for Class 1 and Class 2 solvents when present in an active substance, excipient or medicinal product;

iii. for the quantification of Class 2 solvents when the limits are greater than 1000 ppm (0.1 per cent) or for the quantification of Class 3 solvents when required.

Class 1, Class 2 and Class 3 residual solvents are listed in general chapter 5.4. *Residual solvents*.

Three diluents are described for sample preparation and the conditions to be applied for head-space injection of the gaseous sample onto the chromatographic system. Two chromatographic systems are prescribed but System A is preferred whilst System B is employed normally for confirmation of identity. The choice of sample preparation procedure depends on the solubility of the substance to be examined and in certain cases the residual solvents to be controlled.

The following residual solvents are not readily detected by the head-space injection conditions described: formamide, 2-ethoxyethanol, 2-methoxyethanol, ethylene glycol, *N*-methylpyrrolidone and sulfolane. Other appropriate procedures should be employed for the control of these residual solvents.

When the test procedure is applied quantitatively to control residual solvents in a substance, then it must be validated.

PROCEDURE

Examine by gas chromatography with static head-space injection (2.2.28).

Sample preparation 1. This is intended for the control of residual solvents in water-soluble substances.

Sample solution (1). Dissolve 0.200 g of the substance to be examined in *water R* and dilute to 20.0 ml with the same solvent.

Sample preparation 2. This is intended for the control of residual solvents in water-insoluble substances.

Sample solution (2). Dissolve 0.200 g of the substance to be examined in *N,N*-dimethylformamide *R* (DMF) and dilute to 20.0 ml with the same solvent.

Sample preparation 3. This is intended for the control of *N,N*-dimethylacetamide and/or *N,N*-dimethylformamide, when it is known or suspected that one or both of these substances are present in the substance to be examined.

Sample solution (3). Dissolve 0.200 g of the substance to be examined in *1,3-dimethyl-2-imidazolidinone R* (DMI) and dilute to 20.0 ml with the same solvent.

In some cases none of the above sample preparation procedures are appropriate, in which case the diluent to be used for the preparation of the sample solution and the static head-space conditions to be employed must be demonstrated to be suitable.

Solvent solution (a). To 1.0 ml of *Class 1 residual solvent solution CRS*, add 9 ml of *dimethyl sulphoxide R* and dilute to 100.0 ml with *water R*. Dilute 1.0 ml of this solution to 100 ml with *water R*. Dilute 1.0 ml of this solution to 10.0 ml with *water R*.

The reference solutions correspond to the following limits:

- benzene: 2 ppm,
- carbon tetrachloride: 4 ppm,
- 1,2-dichloroethane: 5 ppm,
- 1,1-dichloroethene: 8 ppm,
- 1,1,1-trichloroethane: 10 ppm.

Solvent solution (b). Dissolve appropriate quantities of the Class 2 residual solvents in *dimethyl sulphoxide R* and dilute to 100.0 ml with *water R*. Dilute to give a concentration of 1/20 of the limits stated in Table 2 (see 5.4. *Residual solvents*).

Solvent solution (c). Dissolve 1.00 g of the solvent or solvents present in the substance to be examined in *dimethyl sulphoxide R* or *water R*, if appropriate, and dilute to 100.0 ml with *water R*. Dilute to give a concentration of 1/20 of the limit(s) stated in Table 1 or 2 (see 5.4. *Residual solvents*).

Blank solution. Prepare as described for solvent solution (c) but without the addition of solvent(s) (used to verify the absence of interfering peaks).

Test solution. Introduce 5.0 ml of the sample solution and 1.0 ml of the blank solution into an injection vial.

Reference solution (a) (Class 1). Introduce 1.0 ml of solvent solution (a) and 5.0 ml of the appropriate diluent into an injection vial.

Reference solution (a₁) (Class 1). Introduce 5.0 ml of the sample solution and 1.0 ml of solvent solution (a) into an injection vial.

Reference solution (b) (Class 2). Introduce 1.0 ml of solvent solution (b) and 5.0 ml of the appropriate diluent into an injection vial.

Reference solution (c). Introduce 5.0 ml of the sample solution and 1.0 ml of solvent solution (c) into an injection vial.

Reference solution (d). Introduce 1.0 ml of the blank solution and 5.0 ml of the appropriate diluent into an injection vial.

Close the vials with a tight rubber membrane stopper coated with polytetrafluoroethylene and secure with an aluminium crimped cap. Shake to obtain a homogeneous solution.

The following static head-space injection conditions may be used:

Operating parameters	Sample preparation procedure		
	1	2	3
Equilibration temperature (°C)	80	105	80
Equilibration time (min)	60	45	45
Transfer-line temperature (°C)	85	110	105
Carrier gas: <i>Nitrogen for chromatography R</i> or <i>Helium for chromatography R</i> at an appropriate pressure			
Pressurisation time (s)	30	30	30
Injection volume (ml)	1	1	1

The chromatographic procedure may be carried out using:

SYSTEM A

- a fused-silica capillary or wide-bore column 30 m long and 0.32 mm or 0.53 mm in internal diameter coated with cross-linked 6 per cent polycyanopropylphenylsiloxane and 94 per cent polydimethylsiloxane (film thickness: 1.8 µm or 3 µm),
 - *nitrogen for chromatography R* or *helium for chromatography R* as the carrier gas, split ratio 1:5 with a linear velocity of about 35 cm/s,
 - a flame-ionisation detector (a mass spectrometer may also be used or an electron-capture detector for the chlorinated residual solvents of Class 1),
- maintaining the temperature of the column at 40 °C for 20 min, then raising the temperature at a rate of 10 °C per min to 240 °C and maintaining it at 240 °C for 20 min

and maintaining the temperature of the injection port at 140 °C and that of the detector at 250 °C, or, where there is interference from the matrix, use:

SYSTEM B

- a fused-silica capillary or wide-bore column 30 m long and 0.32 mm or 0.53 mm in internal diameter coated with *macrogol 20 000 R* (film thickness: 0.25 µm),
 - *nitrogen for chromatography R* or *helium for chromatography R* as the carrier gas, split ratio 1:5 with a linear velocity of about 35 cm/s.
 - a flame-ionisation detector (a mass spectrophotometer may also be used or an electron-capture detector for the chlorinated residual solvents of Class 1),
- maintaining the temperature of the column at 50 °C for 20 min, then raising the temperature at a rate of 6 °C per min to 165 °C and maintaining it at 165 °C for 20 min and maintaining the temperature of the injection port at 140 °C and that of the detector at 250 °C.

Inject 1 ml of the gaseous phase of reference solution (a) onto the column described in System A and record the chromatogram under such conditions that the signal-to-noise ratio for 1,1,1-trichloroethane can be measured. The signal-to-noise ratio must be at least five. A typical chromatogram is shown in Figure 2.4.24-1.

Inject 1 ml of the gaseous phase of reference solution (a₁) onto the column described in System A. The peaks due to the Class 1 residual solvents are still detectable.

Inject 1 ml of the gaseous phase of reference solution (b) onto the column described in System A and record the chromatogram under such conditions that the resolution between acetonitrile and methylene chloride can be determined. The system is suitable if the chromatogram obtained resembles the chromatogram shown in Figure 2.4.24-2 and the resolution between acetonitrile and methylene chloride is at least 1.0.

Inject 1 ml of the gaseous phase of the test solution onto the column described in System A. If in the chromatogram obtained, there is no peak which corresponds to one of the residual solvent peaks in the chromatograms obtained with reference solution (a) or (b), then the substance to be examined meets the requirements of the test. If any peak in the chromatogram obtained with the test solution corresponds to any of the residual solvent peaks obtained with reference solution (a) or (b) then System B is to be employed.

Inject 1 ml of the gaseous phase of reference solution (a) onto the column described in System B and record the chromatogram under such conditions that the signal-to-noise ratio for benzene can be measured. The signal-to-noise ratio must be at least five. A typical chromatogram is shown in Figure 2.4.24-3.

Inject 1 ml of the gaseous phase of reference solution (a₁) onto the column described in System B. The peaks due to the Class I residual solvents are still detectable.

Inject 1 ml of the gaseous phase of reference solution (b) onto the column described in System B and record the chromatogram under such conditions that the resolution between acetonitrile and trichloroethene can be determined. The system is suitable if the chromatogram obtained resembles the chromatogram shown in Figure 2.4.24-4 and the resolution between acetonitrile and trichloroethene is at least 1.0.

Inject 1 ml of the gaseous phase of the test solution onto the column described in System B. If in the chromatogram obtained, there is no peak which corresponds to any of the residual solvent peaks in the chromatogram obtained with the reference solution (a) or (b), then the substance

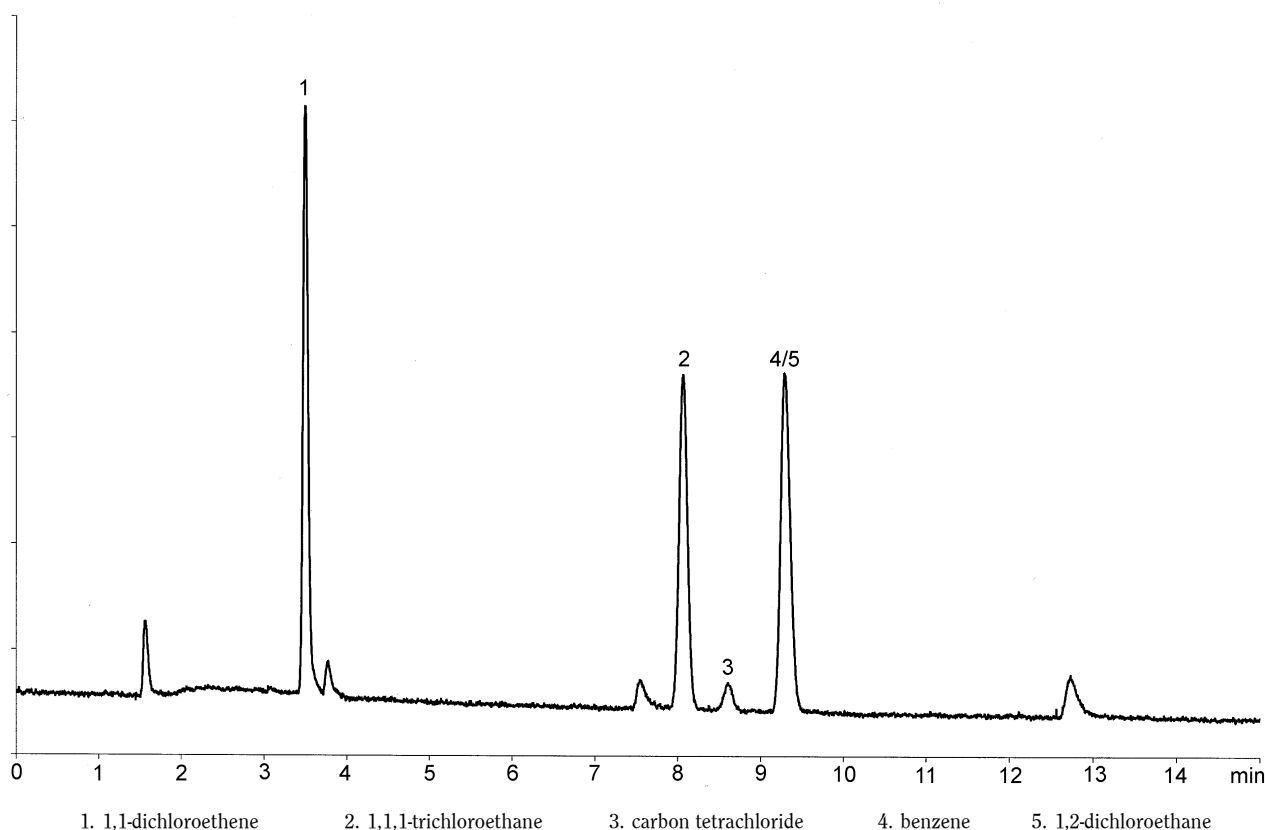


Figure 2.4.24-1. – Typical chromatogram of class 1 solvents using the conditions described for System A and Procedure 1. Flame-ionisation detector.

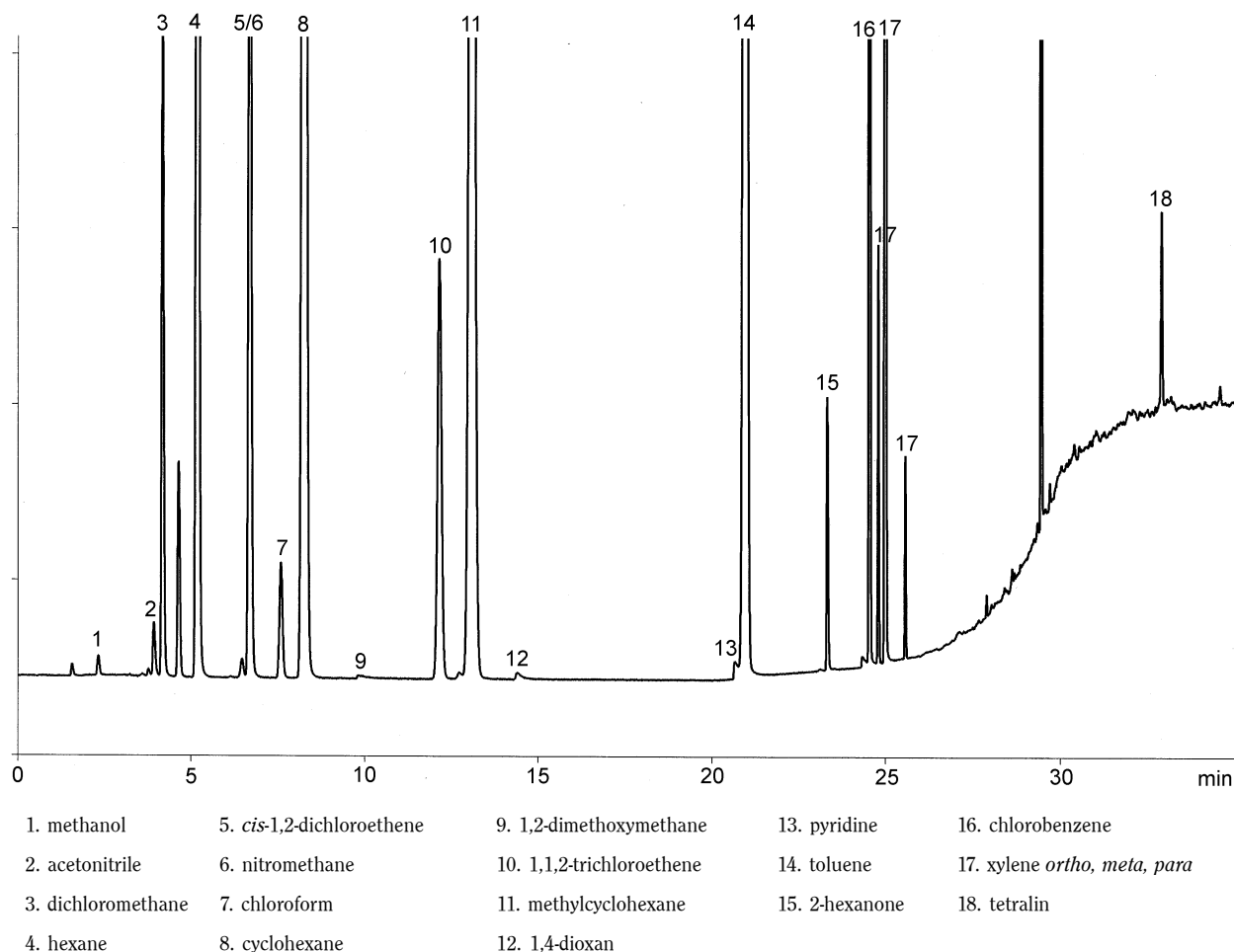


Figure 2.4.24-2. – Chromatogram of Class 2 solvents using the conditions described for System A and Procedure 1. Flame-ionisation detector.

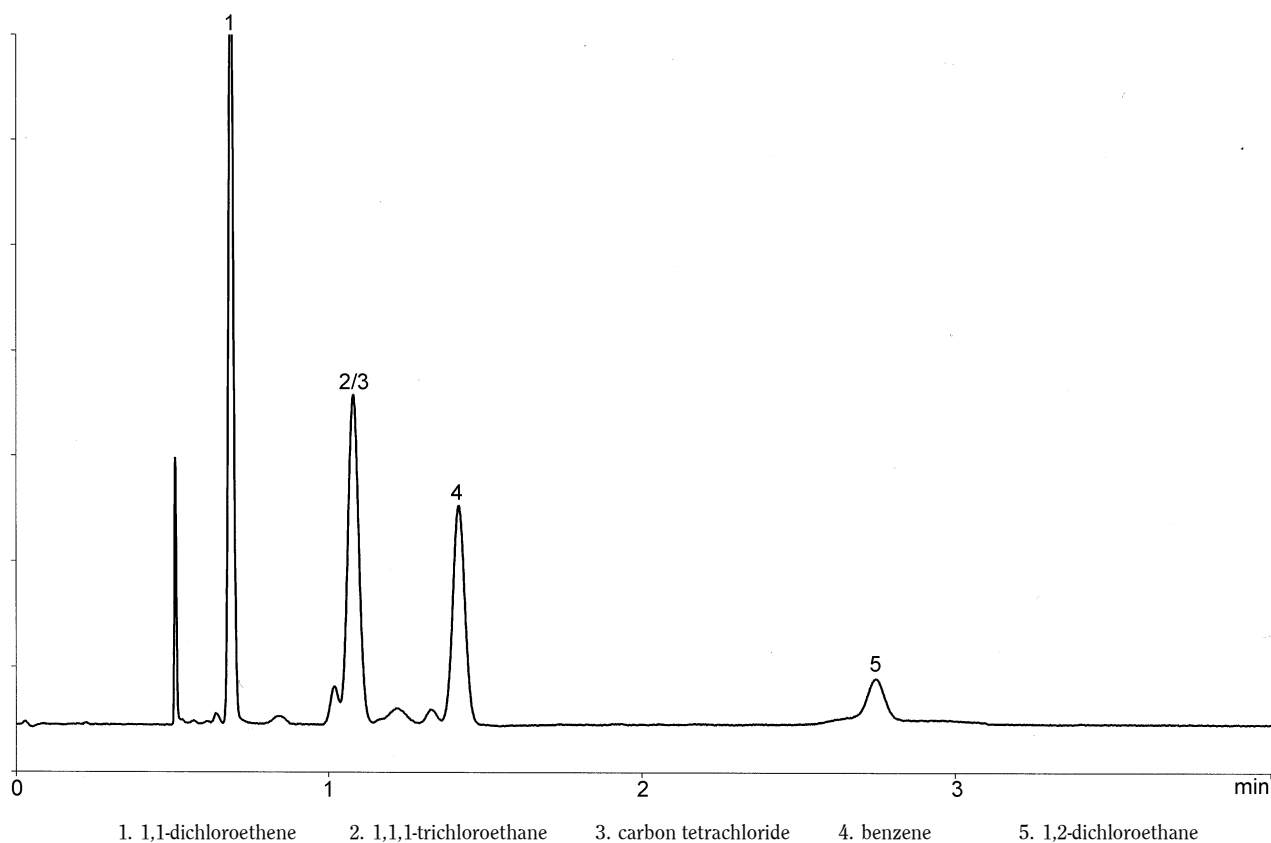


Figure 2.4.24-3. – Chromatogram of Class 1 residual solvents using the conditions described for System B and Procedure 1. Flame-ionisation detector.

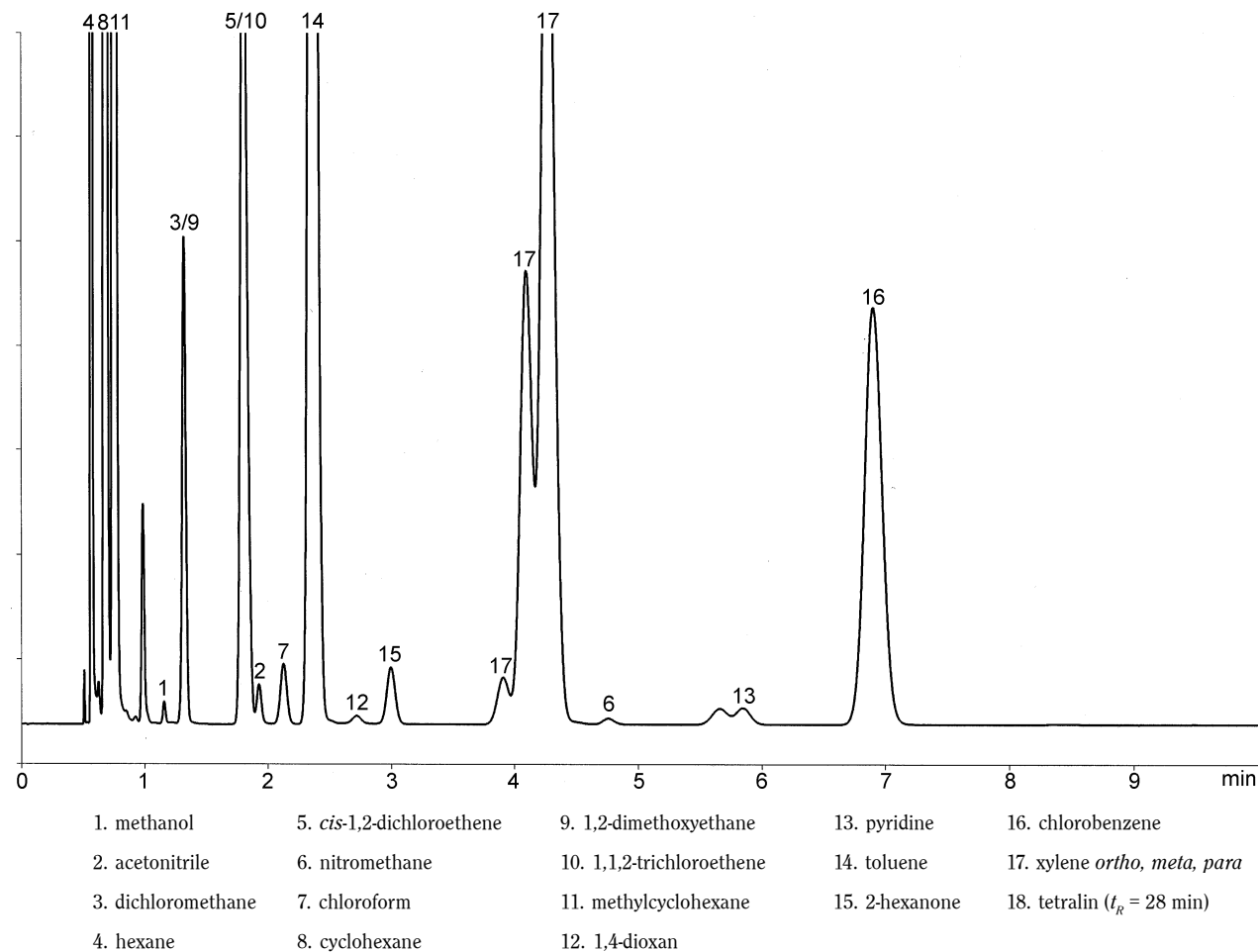


Figure 2.4.24-4. – Typical chromatogram of class 2 residual solvents using the conditions described for System B and Procedure 1. Flame-ionisation detector.

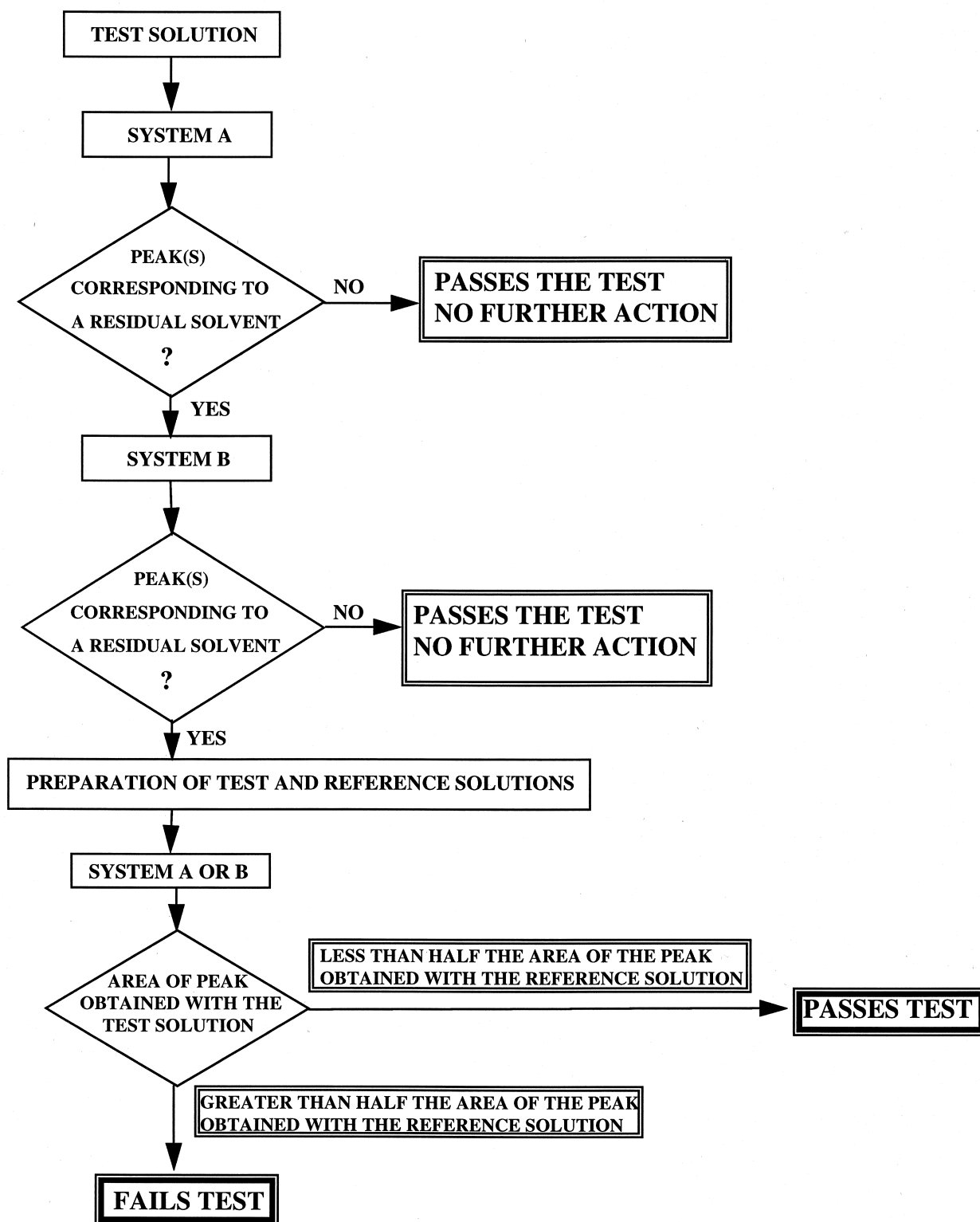


Figure 2.4.24-5. – Diagram relating to the identification of residual solvents and the application of limit tests

to be examined meets the requirements of the test. If any peak in the chromatogram obtained with the test solution corresponds to any of the residual solvent peaks obtained with reference solution (a) or (b) and confirms the correspondence obtained when using System A, then proceed as follows.

Inject 1 ml of the gaseous phase of reference solution (c) onto the column described for System A or System B. If necessary, adjust the sensitivity of the system so that the height of the peak corresponding to the identified residual solvent(s) is at least 50 per cent of the full scale of the recorder.

Inject 1 ml of the gaseous phase of reference solution (d) onto the column. No interfering peaks should be observed.

Inject 1 ml of the gaseous phase of the test solution and 1 ml of the gaseous phase of reference solution (c) on to the column. Repeat these injections twice more.

The mean area of the peak of the residual solvent(s) in the chromatograms obtained with the test solution is not greater than half the mean area of the peak of the corresponding residual solvent(s) in the chromatograms obtained with reference solution (c). The test is not valid unless the relative standard deviation of the differences in areas between the analyte peaks obtained from three replicate paired injections of reference solution (c) and the test solution, is at most 15 per cent.

A flow diagram of the procedure is shown in Figure 2.4.24.-5.

When a residual solvent (Class 2 or Class 3) is present at a level of 0.1 per cent or greater then the content may be quantitatively determined by the method of standard additions.

01/2005:20425

2.4.25. ETHYLENE OXIDE AND DIOXAN

The test is intended for the determination of residual ethylene oxide and dioxan in samples soluble in water or dimethylacetamide. For substances that are insoluble or insufficiently soluble in these solvents, the preparation of the sample solution and the head-space conditions to be employed are given in the individual monograph.

Examine by head-space gas chromatography (2.2.28).

- A. For samples soluble in or miscible with water, the following procedure may be used.

Test solution. Weigh 1.00 g (M_T) of the substance to be examined in a 10 ml vial (other sizes may be used depending on the operating conditions) and add 1.0 ml of *water R*. Close and mix to obtain a homogeneous solution. Allow to stand at 70 °C for 45 min.

Reference solution (a). Weigh 1.00 g (M_R) of the substance to be examined into an identical 10 ml vial, add 0.50 ml of *ethylene oxide solution R3* and 0.50 ml of *dioxan solution R1*. Close and mix to obtain a homogeneous solution. Allow to stand at 70 °C for 45 min.

Reference solution (b). To 0.50 ml of *ethylene oxide solution R3* in a 10 ml vial add 0.1 ml of a freshly prepared 10 mg/l solution of *acetaldehyde R* and 0.1 ml of *dioxan solution R1*. Close and mix to obtain a homogeneous solution. Allow to stand at 70 °C for 45 min.

- B. For samples soluble in or miscible with dimethylacetamide, the following procedure may be used.

Test solution. Weigh 1.00 g (M_T) of the substance to be examined in a 10 ml vial (other sizes may be used depending on the operating conditions) and add 1.0 ml of *dimethylacetamide R* and 0.20 ml of *water R*. Close and mix to obtain a homogeneous solution. Allow to stand at 90 °C for 45 min.

Reference solution (a). Weigh 1.00 g (M_R) of the substance to be examined into a 10 ml vial, add 1.0 ml of *dimethylacetamide R*, 0.10 ml of *dioxan solution R* and 0.10 ml of *ethylene oxide solution R2*. Close and mix to obtain a homogeneous solution. Allow to stand at 90 °C for 45 min.

Reference solution (b). To 0.10 ml of *ethylene oxide solution R2* in a 10 ml vial, add 0.1 ml of a freshly prepared 10 mg/l solution of *acetaldehyde R* and 0.10 ml of *dioxan solution R*. Close and mix to obtain a homogeneous solution. Allow to stand at 70 °C for 45 min.

The following static head-space injection conditions may be used:

- equilibration temperature: 70 °C (90 °C for solutions in dimethylacetamide),
- equilibration time: 45 min,
- transfer-line temperature: 75 °C (150 °C for solutions in dimethylacetamide),
- carrier gas: *helium for chromatography R*,
- pressurisation time: 1 min,
- injection time: 12 s.

The chromatographic procedure may be carried out using:

- a capillary glass or quartz column 30 m long and 0.32 mm in internal diameter the inner surface of which is coated with a 1.0 µm thick layer of *poly(dimethyl)siloxane R*,
- *helium for chromatography R* or *nitrogen for chromatography R* as the carrier gas with a linear velocity of about 20 cm/s and a split ratio of 1:20,
- a flame-ionisation detector,

maintaining the temperature of the column at 50 °C for 5 min, then raising the temperature at a rate of 5 °C per minute to 180 °C and then raising the temperature at a rate of 30 °C per minute to 230 °C and maintaining at 230 °C for 5 min; maintaining the temperature of the injection port at 150 °C and that of the detector at 250 °C.

Inject a suitable volume, for example 1.0 ml, of the gaseous phase of reference solution (b). Adjust the sensitivity of the system so that the heights of the peaks due to ethylene oxide and acetaldehyde in the chromatogram obtained are at least 15 per cent of the full scale of the recorder. The test is not valid unless the resolution between the peaks corresponding to acetaldehyde and ethylene oxide is at least 2.0 and the peak of dioxan is detected with a signal-to-noise ratio of at least 5.

Inject separately suitable volumes, for example 1.0 ml (or the same volume used for reference solution (b)), of the gaseous phases of the test solution and reference solution (a). Repeat the procedure twice more.

Verification of precision

For each pair of injections, calculate for ethylene oxide and for dioxan the difference in area between the peaks obtained with the test solution and reference solution (a). The test is not valid unless the relative standard deviation of the 3 values obtained for ethylene oxide is not greater than 15 per cent and the relative standard deviation of the 3 values obtained for dioxan is not greater than 10 per cent. If the weighings used for the test solution and reference solution differ from 1.00 g by more than 0.5 per cent, the appropriate corrections must be made.

The content of ethylene oxide or dioxan in parts per million is calculated from the expressions:

$$\frac{A_T \times C}{(A_R \times M_T) - (A_T \times M_R)}$$

A_T = area of the peak corresponding to ethylene oxide in the chromatogram obtained with the test solution,

A_R = area of the peak corresponding to ethylene oxide in the chromatogram obtained with reference solution (a),

M_T = mass of the substance to be examined in the test solution, in grams,

- M_R = mass of the substance to be examined in the reference solution, in grams,
 C = the amount of ethylene oxide added to reference solution (a), in micrograms.

$$\frac{D_T \times C}{(D_R \times M_T) - (D_T \times M_R)}$$

- D_T = area of the peak corresponding to dioxan in the chromatogram obtained with the test solution,
 D_R = area of the peak corresponding to dioxan in the chromatogram obtained with reference solution (a),
 C = the amount of dioxan added to reference solution (a) in micrograms.

01/2005:20426

2.4.26. *N,N*-DIMETHYLANILINE

METHOD A

Examine by gas chromatography (2.2.28), using *N,N*-diethylaniline *R* as the internal standard.

Internal standard solution. Dissolve 50 mg of *N,N*-diethylaniline *R* in 4 ml of 0.1 *M* hydrochloric acid and dilute to 50 ml with water *R*. Dilute 1 ml of this solution to 100 ml with water *R*.

Test solution. Dissolve in a ground-glass-stoppered tube 0.50 g of the substance to be examined in 30.0 ml of water *R*. Add 1.0 ml of the internal standard solution. Adjust the solution to a temperature of 26 °C to 28 °C. Add 1.0 ml of strong sodium hydroxide solution *R* and mix until completely dissolved. Add 2.0 ml of trimethylpentane *R*. Shake for 2 min and allow the phases to separate. Use the upper layer.

Reference solution. Dissolve 50.0 mg of *N,N*-dimethylaniline *R* in 4.0 ml of 0.1 *M* hydrochloric acid and dilute to 50.0 ml with water *R*. Dilute 1.0 ml of this solution to 100.0 ml with water *R*. Dilute 1.0 ml of this solution to 30.0 ml with water *R*. Add 1.0 ml of the internal standard solution and 1.0 ml of strong sodium hydroxide solution *R*. Add 2.0 ml of trimethylpentane *R*. Shake for 2 min and allow the phases to separate. Use the upper layer.

The chromatographic procedure may be carried out using:

- a fused-silica capillary column 25 m long and 0.32 mm in internal diameter coated with cross-linked polymethylphenylsiloxane *R* (film thickness 0.52 µm),
- helium for chromatography *R* as the carrier gas with a split ratio 1:20, a column head pressure of 50 kPa and a split vent of 20 ml/min,
- a flame-ionisation detector,
- a split-liner consisting of a column about 1 cm long packed with diatomaceous earth for gas chromatography *R* impregnated with 10 per cent *m/m* of poly(dimethyl)siloxane *R*,

maintaining the temperature of the column at 150 °C for 5 min, then raising the temperature at a rate of 20 °C per min to 275 °C and maintaining it at 275 °C for 3 min and maintaining the temperature of the detector at 300 °C and that of the injection port at 220 °C.

The retention times are: *N,N*-dimethylaniline about 3.6 min, *N,N*-diethylaniline about 5.0 min.

Inject 1 µl of the test solution and 1 µl of the reference solution.

METHOD B

Examined by gas chromatography (2.2.28), using naphthalene *R* as the internal standard.

Internal standard solution. Dissolve 50 mg of naphthalene *R* in cyclohexane *R* and dilute to 50 ml with the same solvent. Dilute 5 ml of this solution to 100 ml with cyclohexane *R*.

Test solution. To 1.00 g of the substance to be examined in a ground-glass-stoppered tube add 5 ml of 1 *M* sodium hydroxide and 1.0 ml of the internal standard solution. Stopper the tube and shake vigorously for 1 min. Centrifuge if necessary and use the upper layer.

Reference solution. To 50.0 mg of *N,N*-dimethylaniline *R* add 2 ml of hydrochloric acid *R* and 20 ml of water *R*, shake to dissolve and dilute to 50.0 ml with water *R*. Dilute 5.0 ml of this solution to 250.0 ml with water *R*. To 1.0 ml of the latter solution in a ground-glass-stoppered tube add 5 ml of 1 *M* sodium hydroxide and 1.0 ml of the internal standard solution. Stopper the tube and shake vigorously for 1 min. Centrifuge if necessary and use the upper layer.

The chromatographic procedure may be carried out using:

- a glass column 2 m long and 2 mm in internal diameter packed with silanised diatomaceous earth for gas chromatography *R* impregnated with 3 per cent *m/m* of polymethylphenylsiloxane *R*,
- nitrogen for chromatography *R* as the carrier gas at a flow rate of 30 ml/min,
- a flame-ionisation detector,

maintaining the temperature of the column at 120 °C and that of the injection port and of the detector at 150 °C.

Inject 1 µl of the test solution and 1 µl of the reference solution.

01/2005:20427

2.4.27. HEAVY METALS IN HERBAL DRUGS AND FATTY OILS

Examine by atomic absorption spectrometry (2.2.23).

CAUTION: when using closed high-pressure digestion vessels and microwave laboratory equipment, be familiar with the safety and operating instructions given by the manufacturer.

APPARATUS

The apparatus typically consists of the following:

- as digestion flasks, polytetrafluoroethylene flasks with a volume of about 120 ml, fitted with an airtight closure, a valve to adjust the pressure inside the container and a polytetrafluoroethylene tube to allow release of gas,
- a system to make flasks airtight, using the same torsional force for each of them,
- a microwave oven, with a magnetron frequency of 2450 MHz, with a selectable output from 0 to 630 ± 70 W in 1 per cent increments, a programmable digital computer, a polytetrafluoroethylene-coated microwave cavity with a variable speed exhaust fan, a rotating turntable drive system and exhaust tubing to vent fumes,
- an atomic absorption spectrometer, equipped with hollow-cathode lamps as source of radiation and a deuterium lamp as background corrector; the system is fitted with:
 - (a) a graphite furnace as atomisation device for cadmium, copper, iron, lead, nickel and zinc.
 - (b) an automated continuous-flow hydride vapour generation system for arsenic and mercury.

METHOD

In case alternative apparatus is used, an adjustment of the instrument parameters may be necessary.

Clean all the glassware and laboratory equipment with a 10 g/l solution of *nitric acid R* before use.

Test solution. In a digestion flask place the prescribed quantity of the substance to be examined (about 0.50 g of powdered drug (1400) or 0.50 g of fatty oil). Add 6 ml of *heavy metal-free nitric acid R* and 4 ml of *heavy metal-free hydrochloric acid R*. Make the flask airtight.

Place the digestion flasks in the microwave oven. Carry out the digestion in 3 steps according to the following programme, used for 7 flasks each containing the test solution: 80 per cent power for 15 min, 100 per cent power for 5 min, 80 per cent power for 20 min.

At the end of the cycle allow the flasks to cool in air and to each add 4 ml of *heavy metal-free sulphuric acid R*. Repeat the digestion programme. After cooling in air, open each digestion flask and introduce the clear, colourless solution obtained into a 50 ml volumetric flask. Rinse each digestion flask with 2 quantities, each of 15 ml, of *water R* and collect the rinsings in the volumetric flask. Add 1.0 ml of a 10 g/l solution of *magnesium nitrate R* and 1.0 ml of a 100 g/l solution of *ammonium dihydrogen phosphate R* and dilute to 50.0 ml with *water R*.

Blank solution. Mix 6 ml of *heavy metal-free nitric acid R* and 4 ml of *heavy metal-free hydrochloric acid R* in a digestion flask. Carry out the digestion in the same manner as for the test solution.

CADMIUM, COPPER, IRON, LEAD, NICKEL AND ZINC
Measure the content of cadmium, copper, iron, lead, nickel and zinc by the standard additions method (2.2.23, *Method II*), using reference solutions of each heavy metal and the instrumental parameters described in Table 2.4.27-1.

The absorbance value of the blank solution is automatically subtracted from the value obtained with the test solution.

Table 2.4.27-1

		Cd	Cu	Fe	Ni	Pb	Zn
Wavelength	nm	228.8	324.8	248.3	232	283.5	213.9
Slit width	nm	0.5	0.5	0.2	0.2	0.5	0.5
Lamp current	mA	6	7	5	10	5	7
Ignition temperature	°C	800	800	800	800	800	800
Atomisation temperature	°C	1800	2300	2300	2500	2200	2000
Background corrector		on	off	off	off	off	off
Nitrogen flow	l/min	3	3	3	3	3	3

ARSENIC AND MERCURY

Measure the content of arsenic and mercury in comparison with the reference solutions of arsenic or mercury at a known concentration by direct calibration (2.2.23, *Method I*) using an automated continuous-flow hydride vapour generation system.

The absorbance value of the blank solution is automatically subtracted from the value obtained with the test solution.

Arsenic

Sample solution. To 19.0 ml of the test solution or of the blank solution as prescribed above, add 1 ml of a 200 g/l solution of *potassium iodide R*. Allow the test solution to

stand at room temperature for about 50 min or at 70 °C for about 4 min.

Acid reagent. *Heavy metal-free hydrochloric acid R*.

Reducing reagent. A 6 g/l solution of *sodium tetrahydroborate R* in a 5 g/l solution of *sodium hydroxide R*.

The instrumental parameters in Table 2.4.27-2 may be used.

Mercury

Sample solution. Test solution or blank solution, as prescribed above.

Acid reagent. A 515 g/l solution of *heavy metal-free hydrochloric acid R*.

Reducing reagent. A 10 g/l solution of *stannous chloride R* in *dilute heavy metal-free hydrochloric acid R*.

The instrumental parameters in Table 2.4.27-2 may be used.

Table 2.4.27-2

		As	Hg
Wavelength	nm	193.7	253.7
Slit width	nm	0.2	0.5
Lamp current	mA	10	4
Acid reagent flow rate	ml/min	1.0	1.0
Reducing reagent flow rate	ml/min	1.0	1.0
Sample solution flow rate	ml/min	7.0	7.0
Absorption cell		Quartz (heated)	Quartz (unheated)
Background corrector		off	off
Nitrogen flow rate	l/min	0.1	0.1

01/2005:20428

2.4.28. 2-ETHYLHEXANOIC ACID

Examine by gas chromatography (2.2.28), using *3-cyclohexylpropionic acid R* as the internal standard.

Internal standard solution. Dissolve 100 mg of *3-cyclohexylpropionic acid R* in *cyclohexane R* and dilute to 100 ml with the same solvent.

Test solution. To 0.300 g of the substance to be examined, add 4.0 ml of a 33 per cent *V/V* solution of *hydrochloric acid R*. Shake vigorously for 1 min with 1.0 ml of the internal standard solution. Allow the phases to separate (if necessary, centrifuge for a better separation). Use the upper layer.

Reference solution. Dissolve 75.0 mg of *2-ethylhexanoic acid R* in the internal standard solution and dilute to 50.0 ml with the same solution. To 1.0 ml of the solution add 4.0 ml of a 33 per cent *V/V* solution of *hydrochloric acid R*. Shake vigorously for 1 min. Allow the phases to separate (if necessary, centrifuge for a better separation). Use the upper layer.

The chromatographic procedure may be carried out using:

- a wide-bore fused-silica column 10 m long and 0.53 mm in internal diameter coated with *macrogol 20 000 2-nitroterephthalate R* (film thickness 1.0 µm),
- *helium for chromatography R* as the carrier gas at a flow rate of 10 ml/min,
- a flame-ionisation detector,

with the following temperature programme:

	Time (min)	Temperature (°C)	Rate (°C/min)	Comment
Column	0 - 2	40	–	isothermal
	2 - 7.3	40 → 200	30	linear gradient
	7.3 - 10.3	200	–	isothermal
Injection port		200		
Detector		300		

Inject 1 µl of the test solution and 1 µl of the reference solution.

The test is not valid unless the resolution between the peaks corresponding to 2-ethylhexanoic acid (first peak) and the internal standard is at least 2.0.

Calculate the percentage content of 2-ethylhexanoic acid from the expression:

$$\frac{A_T \times I_R \times m_R \times 2}{A_R \times I_T \times m_T}$$

- A_T = area of the peak corresponding to 2-ethylhexanoic acid in the chromatogram obtained with the test solution,
- A_R = area of the peak corresponding to 2-ethylhexanoic acid in the chromatogram obtained with the reference solution,
- I_T = area of the peak corresponding to the internal standard in the chromatogram obtained with the test solution,
- I_R = area of the peak corresponding to the internal standard in the chromatogram obtained with the reference solution,
- m_T = mass of the substance to be examined in the test solution, in grams,
- m_R = mass of 2-ethylhexanoic acid in the reference solution, in grams.

01/2005:20429

2.4.29. COMPOSITION OF FATTY ACIDS IN OILS RICH IN OMEGA-3-ACIDS

The assay may be used for quantitative determination of the EPA and DHA content in omega-3-containing products of fish oil in different concentrations. The method is applicable to triglycerides or ethyl esters and the results are expressed as triglycerides or ethyl esters, respectively.

EPA AND DHA

Gas chromatography (2.2.28). Carry out the operations as rapidly as possible, avoiding exposure to actinic light, oxidising agents, oxidation catalysts (for example, copper and iron) and air.

The assay is carried out on the methyl or ethyl esters of (all-*Z*)-eicosa-5,8,11,14,17-pentaenoic acid (EPA; 20:5 n-3) and (all-*Z*)-docosa-4,7,10,13,16,19-hexaenoic acid (DHA; 22:6 n-3) in the substance to be examined.

Internal standard. Methyl tricosanoate R.

Test solution (a)

- A. Dissolve the mass of sample to be examined according to Table 2.4.29.-1 and about 70.0 mg of the internal standard in a 50 mg/l solution of *butylhydroxytoluene R* in *trimethylpentane R* and dilute to 10.0 ml with the same solution.

Table 2.4.29.-1.

Approximative sum EPA + DHA (per cent)	Amount sample to be weighed (grams)
30 - 50	0.4 - 0.5
50 - 70	0.3
70 - 90	0.25

Ethyl esters are now ready for analysis. For triglycerides continue as described in step B.

- B. Introduce 2.0 ml of the solution obtained into a quartz tube and evaporate the solvent with a gentle current of *nitrogen R*. Add 1.5 ml of a 20 g/l solution of *sodium hydroxide R* in *methanol R*, cover with *nitrogen R*, cap tightly with a polytetrafluoroethylene-lined cap, mix and heat on a water-bath for 7 min. Allow to cool. Add 2 ml of *boron trichloride-methanol solution R*, cover with *nitrogen R*, cap tightly, mix and heat on a water-bath for 30 min. Cool to 40-50 °C, add 1 ml of *trimethylpentane R*, cap and shake vigorously for at least 30 s. Immediately add 5 ml of a *saturated sodium chloride solution R*, cover with *nitrogen R*, cap and shake thoroughly for at least 15 s. Transfer the upper layer to a separate tube. Shake the methanol layer once more with 1 ml of *trimethylpentane R*. Wash the combined trimethylpentane extracts with 2 quantities, each of 1 ml, of *water R* and dry over *anhydrous sodium sulphate R*. Prepare 3 solutions for each sample.

Test solution (b). Dissolve 0.300 g of the sample to be examined in a 50 mg/l solution of *butylhydroxytoluene R* in *trimethylpentane R* and dilute to 10.0 ml with the same solution. Proceed as described for test solution (a).

Reference solution (a). Dissolve 60.0 mg of *docosahexaenoic acid ethyl ester CRS*, about 70.0 mg of the internal standard and 90.0 mg of *eicosapentaenoic acid ethyl ester CRS* in a 50 mg/l solution of *butylhydroxytoluene R* in *trimethylpentane R* and dilute to 10.0 ml with the same solution. Proceed as described for test solution (a) step A when analysing ethyl esters. For analysis of triglycerides, continue with step B in the same manner as for test solution (a). Prepare 3 solutions for each sample.

Reference solution (b). Into a 10 ml volumetric flask dissolve 0.3 g of *methyl palmitate R*, 0.3 g of *methyl stearate R*, 0.3 g of *methyl arachidate R* and 0.3 g of *methyl behenate R*, in a 50 mg/l solution of *butylhydroxytoluene R* in *trimethylpentane R* and dilute to 10.0 ml with the same solution.

Reference solution (c). Into a 10 ml volumetric flask dissolve a sample containing about 55.0 mg of *docosahexaenoic acid methyl ester R* and about 5.0 mg of *tetracos-15-enoic acid methyl ester R* in a 50 mg/l solution of *butylhydroxytoluene R* in *trimethylpentane R* and dilute to 10.0 ml with the same solution.

Column:

- material: fused silica,
- dimensions: l = at least 25 m, $\varnothing$ = 0.25 mm,
- stationary phase: bonded *macrogol 20 000 R* (film thickness 0.2 µm).

Carrier gas: *hydrogen for chromatography R* or *helium for chromatography R*.

Split ratio: 1:200, alternatively splitless with temperature control (sample solutions need to be diluted 1/200 with a 50 mg/l solution of *butylhydroxytoluene R* in *trimethylpentane R* before injection).

Temperature:

	Time (min)	Temperature (°C)
Column	0 - 2	170
	2 - 25.7	170 → 240
	25.7 - 28	240
Injection port		250
Detector		270

Detection: flame ionisation.

Injection: 1 µl, twice.

System suitability:

- in the chromatogram obtained with reference solution (b), the area per cent composition increases in the following order: methyl palmitate, methyl stearate, methyl arachidate, methyl behenate; the difference between the percentage area of methyl palmitate and that of methyl behenate is less than 2 area per cent units,
- *resolution*: minimum of 1.2 between the peaks due to docosahexaenoic acid methyl ester and to tetracos-15-enoic acid methyl ester in the chromatogram obtained with reference solution (c),
- in the chromatogram obtained with test solution (a), the peaks due to methyl tricosanoate and any heneicosapentaenoic acid methyl ester or ethyl ester (C21:5) present when compared with the chromatogram obtained with test solution (b) are clearly separated (if not, a correction factor has to be used),
- in the chromatogram obtained with test solution (a) the recovery for the added *eicosapentaenoic acid ethyl ester CRS* and *docosahexaenoic acid ethyl ester CRS* is greater than 95 per cent when due consideration has been given to the correction by the internal standard, and the standard addition method is used.

Calculate the percentage content of EPA and DHA using the following expression and taking into account the assigned value of the reference substances:

$$A_x \times \frac{A_3}{m_3} \times \frac{m_1}{A_1} \times \frac{m_{x,r}}{A_{x,r}} \times \frac{1}{m_2} \times C \times 100$$

- m_1 = mass of the internal standard in test solution (a), in milligrams,
- m_2 = mass of the sample to be examined in test solution (a), in milligrams,
- m_3 = mass of the internal standard in reference solution (a), in milligrams,
- $m_{x,r}$ = mass of *eicosapentaenoic acid ethyl ester CRS* or *docosahexaenoic acid ethyl ester CRS* in reference solution (a), in milligrams,
- A_x = area of the peak due to eicosapentaenoic acid ester or docosahexaenoic acid ester in the chromatogram obtained with test solution (a),
- $A_{x,r}$ = area of the peak due to eicosapentaenoic acid ester or docosahexaenoic acid ester in the chromatogram obtained with reference solution (a),
- A_1 = area of the peak due to the internal standard in the chromatogram obtained with test solution (a),

A_3 = area of the peak due to the internal standard in the chromatogram obtained with reference solution (a),

C = conversion factor between ethyl ester and triglycerides,

$C = 1.00$ for ethyl esters,

$C = 0.954$ for EPA,

$C = 0.957$ for DHA.

TOTAL OMEGA-3-ACIDS

From the assay for EPA and DHA, calculate the percentage content of the total omega-3-acids using the following expression and identifying the peaks from the chromatograms:

$$EPA + DHA + \frac{A_{n-3} (EPA + DHA)}{A_{EPA} + A_{DHA}}$$

EPA = percentage content of EPA,

DHA = percentage content of DHA,

A_{n-3} = sum of the areas of the peaks due to C18:3 n-3, C18:4 n-3, C20:4 n-3, C21:5 n-3 and C22:5 n-3 methyl esters in the chromatogram obtained with test solution (b),

A_{EPA} = area of the peak due to EPA ester in the chromatogram obtained with test solution (b),

A_{DHA} = area of the peak due to DHA ester in the chromatogram obtained with test solution (b).

01/2005:20430

2.4.30. ETHYLENE GLYCOL AND DIETHYLENE GLYCOL IN ETHOXYLATED SUBSTANCES

Ethoxylated substances may contain varied amounts of ethylene glycol and diethylene glycol, as a result of the manufacturing process. The following method may be used for the quantitative determination of these substances, in particular in the case of the following surfactants: macrogolglycerol ricinoleate, macrogolglycerol hydroxystearate, macrogol 15 hydroxystearate, nonoxinol 9 and macrogol cetostearyl ether.

Gas chromatography (2.2.28).

Internal standard solution. Dissolve 30.0 mg of *1,2-pentanediol R* in *acetone R* and dilute to 30.0 ml with the same solvent. Dilute 1.0 ml of this solution to 20.0 ml with *acetone R*.

Test solution. Dissolve 0.500 g of the substance to be examined in the internal standard solution and dilute to 10.0 ml with the same solution.

Reference solution (a). Mix 30.0 mg of *ethylene glycol R* with *acetone R* and dilute to 100.0 ml with the same solvent. Dilute 1.0 ml to 10.0 ml with the internal standard solution.

Reference solution (b). Prepare a solution of *diethylene glycol R* with a concentration corresponding to the prescribed limit and using the same solvents as for the preparation of reference solution (a).

Column:

- *material*: fused silica,
- *size*: $l = 30$ m, $\varnothing = 0.53$ mm,

– *stationary phase*: *macrogol 20 000 R* (film thickness 1 µm).
Carrier gas: *helium for chromatography R*.
Flow rate: 30 ml/min.
Split ratio: 1:3.
Temperature:

Detection: flame ionisation.
Injection: 2 µl.
Relative retentions with reference to 1,2-pentanediol (retention time = about 19 min): ethylene glycol = about 0.7; diethylene glycol = about 1.3.

	Time (min)	Temperature (°C)
Column	0 - 40	80 → 200
	40 - 45	200 → 230
	45 - 65	230
Injection port		250
Detector		250

2.5. ASSAYS

01/2005:20501

2.5.1. ACID VALUE

The acid value I_A is the number that expresses in milligrams the quantity of potassium hydroxide required to neutralise the free acids present in 1 g of the substance.

Dissolve 10.00 g of the substance to be examined, or the quantity prescribed (m g) in 50 ml of a mixture of equal volumes of *alcohol R* and *ether R*, previously neutralised with 0.1 M *potassium hydroxide*, unless otherwise specified, using 0.5 ml of *phenolphthalein solution R1* as indicator. When the substance to be examined has dissolved, titrate with 0.1 M *potassium hydroxide* until the pink colour persists for at least 15 s (n ml of 0.1 M *potassium hydroxide*).

$$I_A = \frac{5.610n}{m}$$

01/2005:20502

2.5.2. ESTER VALUE

The ester value I_E is the number that expresses in milligrams the quantity of potassium hydroxide required to saponify the esters present in 1 g of the substance. It is calculated from the saponification value I_S and the acid value I_A :

$$I_E = I_S - I_A$$

01/2005:20503

2.5.3. HYDROXYL VALUE

The hydroxyl value I_{OH} is the number that expresses in milligrams the quantity of potassium hydroxide required to neutralise the acid combined by acylation in 1 g of the substance.

METHOD A

Introduce the quantity of the substance to be examined shown in Table 2.5.3.-1 (m g) into a 150 ml acetylation flask fitted with an air condenser, unless another quantity is prescribed in the monograph. Add the quantity of *acetic anhydride solution R1* stated in Table 2.5.3.-1 and attach the air condenser.

Table 2.5.3.-1

Presumed value I_{OH}	Quantity of sample (g)	Volume of acetylating reagent (ml)
10 - 100	2.0	5.0
100 - 150	1.5	5.0
150 - 200	1.0	5.0
200 - 250	0.75	5.0
250 - 300	0.60 or 1.20	5.0 or 10.0
300 - 350	1.0	10.0
350 - 700	0.75	15.0
700 - 950	0.5	15.0

Heat the flask in a water-bath for 1 h keeping the level of the water about 2.5 cm above the level of the liquid in the flask. Withdraw the flask and allow to cool. Add 5 ml of *water R* through the upper end of the condenser. If a cloudiness appears add sufficient *pyridine R* to clear it, noting the volume added. Shake the flask and replace in the water-bath

for 10 min. Withdraw the flask and allow to cool. Rinse the condenser and the walls of the flask with 5 ml of *alcohol R*, previously neutralised to *phenolphthalein solution R1*. Titrate with 0.5 M *alcoholic potassium hydroxide* using 0.2 ml of *phenolphthalein solution R1* as indicator (n_1 ml of 0.5 M *alcoholic potassium hydroxide*). Carry out a blank test under the same conditions (n_2 ml of 0.5 M *alcoholic potassium hydroxide*).

$$I_{OH} = \frac{28.05(n_2 - n_1)}{m} + I_A$$

METHOD B

Introduce the prescribed quantity of the substance to be examined (m g) into a perfectly dry 5 ml conical flask fitted with a ground-glass or suitable plastic stopper and add 2.0 ml of *propionic anhydride reagent R*. Close the flask and shake gently to dissolve the substance. Allow to stand for 2 h unless otherwise prescribed. Remove the stopper and transfer the flask and its contents into a wide-mouthed 500 ml conical flask containing 25.0 ml of a 9 g/l solution of *aniline R* in *cyclohexane R* and 30 ml of *glacial acetic acid R*. Swirl the contents of the flask, allow to stand for 5 min, add 0.05 ml of *crystal violet solution R* and titrate with 0.1 M *perchloric acid* until an emerald-green colour is obtained (n_1 ml of 0.1 M *perchloric acid*). Carry out a blank test under the same conditions (n_2 ml of 0.1 M *perchloric acid*).

$$I_{OH} = \frac{5.610(n_1 - n_2)}{m}$$

To take account of any water present, determine this (y per cent) by the semi-micro determination of water (2.5.12).

The hydroxyl value is then given by the equation:

$$I_{OH} = (\text{hydroxyl value as determined}) - 31.1y$$

01/2005:20504

2.5.4. IODINE VALUE

The iodine value I_I is the number that expresses in grams the quantity of halogen, calculated as iodine, that can be fixed in the prescribed conditions by 100 g of the substance.

When the monograph does not specify the method to be used, method A is applied. Any change from method A to method B is validated.

METHOD A

Unless otherwise prescribed, use the following quantities (Table 2.5.4.-1) for the determination.

Table 2.5.4.-1

Presumed value I_I	Quantity of sample (g)
less than 20	1.0
20 - 60	0.5 - 0.25
60 - 100	0.25 - 0.15
more than 100	0.15 - 0.10

Introduce the prescribed quantity of the substance to be examined (m g) into a 250 ml flask fitted with a ground-glass stopper and previously dried or rinsed with *glacial acetic acid R*, and dissolve it in 15 ml of *chloroform R* unless otherwise prescribed. Add very slowly 25.0 ml of *iodine bromide solution R*. Close the flask and keep it in the dark for 30 min unless otherwise prescribed, shaking frequently. Add 10 ml of a 100 g/l solution of *potassium iodide R* and 100 ml of *water R*. Titrate with 0.1 M *sodium*

thiosulphate, shaking vigorously until the yellow colour is almost discharged. Add 5 ml of *starch solution R* and continue the titration adding the 0.1 M *sodium thiosulphate* dropwise until the colour is discharged (n_1 ml of 0.1 M *sodium thiosulphate*). Carry out a blank test under the same conditions (n_2 ml of 0.1 M *sodium thiosulphate*).

$$I_1 = \frac{1.269 (n_2 - n_1)}{m}$$

METHOD B

Unless otherwise prescribed, use the following quantities (Table 2.5.4-2) for the determination.

Table 2.5.4-2

Presumed value I_1	Mass (g) (corresponding to an excess of 150 per cent ICl)	Mass (g) (corresponding to an excess of 100 per cent ICl)	Iodine chloride solution (ml)
<3	10	10	25
3	8.4613	10.5760	25
5	5.0770	6.3460	25
10	2.5384	3.1730	20
20	0.8461	1.5865	20
40	0.6346	0.7935	20
60	0.4321	0.5288	20
80	0.3173	0.3966	20
100	0.2538	0.3173	20
120	0.2115	0.2644	20
140	0.1813	0.2266	20
160	0.1587	0.1983	20
180	0.1410	0.1762	20
200	0.1269	0.1586	20

The mass of the sample is such that there will be an excess of *iodine chloride solution R* of 50 per cent to 60 per cent of the amount added, i.e. 100 per cent to 150 per cent of the amount absorbed.

Introduce the prescribed quantity of the substance to be examined (m g) into a 250 ml flask fitted with a ground-glass stopper and previously rinsed with *glacial acetic acid R* or dried, and dissolve it in 15 ml of a mixture of equal volumes of *cyclohexane R* and *glacial acetic acid R*, unless otherwise prescribed. If necessary, melt the substance before dissolution (melting point greater than 50 °C). Add very slowly the volume of *iodine chloride solution R* stated in Table 2.5.4-2. Close the flask and keep it in the dark for 30 min, unless otherwise prescribed, shaking frequently. Add 10 ml of a 100 g/l solution of *potassium iodide R* and 100 ml of *water R*. Titrate with 0.1 M *sodium thiosulphate*, shaking vigorously until the yellow colour is almost discharged. Add 5 ml of *starch solution R* and continue the titration adding the 0.1 M *sodium thiosulphate* dropwise until the colour is discharged (n_1 ml of 0.1 M *sodium thiosulphate*). Carry out a blank test under the same conditions (n_2 ml of 0.1 M *sodium thiosulphate*).

$$I_1 = \frac{1.269 (n_2 - n_1)}{m}$$

2.5.5. PEROXIDE VALUE

The peroxide value I_p is the number that expresses in milliequivalents of active oxygen the quantity of peroxide contained in 1000 g of the substance, as determined by the methods described below.

When the monograph does not specify the method to be used, method A is applied. Any change from method A to method B is validated.

METHOD A

Place 5.00 g of the substance to be examined (m g) in a 250 ml conical flask fitted with a ground-glass stopper. Add 30 ml of a mixture of 2 volumes of *chloroform R* and 3 volumes of *glacial acetic acid R*. Shake to dissolve the substance and add 0.5 ml of *saturated potassium iodide solution R*. Shake for exactly 1 min then add 30 ml of *water R*. Titrate with 0.01 M *sodium thiosulphate*, adding the titrant slowly with continuous vigorous shaking, until the yellow colour is almost discharged. Add 5 ml of *starch solution R* and continue the titration, shaking vigorously, until the colour is discharged (n_1 ml of 0.01 M *sodium thiosulphate*). Carry out a blank test under the same conditions (n_2 ml of 0.01 M *sodium thiosulphate*). The volume of 0.01 M *sodium thiosulphate* used in the blank titration must not exceed 0.1 ml.

$$I_p = \frac{10 (n_1 - n_2)}{m}$$

METHOD B

Carry out the operations avoiding exposure to actinic light.

Place 50 ml of a mixture of 2 volumes of *trimethylpentane R* and 3 volumes of *glacial acetic acid R* in a conical flask and replace the stopper. Swirl the flask until the substance to be examined (m g; see Table 2.5.5-1) has dissolved. Using a suitable volumetric pipette, add 0.5 ml of *saturated potassium iodide solution R* and replace the stopper. Allow the solution to stand for 60 ± 1 s, thoroughly shaking the solution continuously, then add 30 ml of *water R*.

Table 2.5.5-1

Expected peroxide value I_p	Mass of substance to be examined (g)
0 to 12	2.00 to 5.00
12 to 20	1.20 to 2.00
20 to 30	0.80 to 1.20
30 to 50	0.500 to 0.800
50 to 90	0.300 to 0.500

Titrate the solution with 0.01 M *sodium thiosulphate* (V_1 ml), adding it gradually and with constant, vigorous shaking, until the yellow iodine colour has almost disappeared. Add about 0.5 ml of *starch solution R1* and continue the titration, with constant shaking especially near the end-point, to liberate all of the iodine from the solvent layer. Add the sodium thiosulphate solution dropwise until the blue colour just disappears.

Depending on the volume of 0.01 M *sodium thiosulphate* used, it may be necessary to titrate with 0.1 M *sodium thiosulphate*.

NOTE: there is a 15 s to 30 s delay in neutralising the starch indicator for peroxide values of 70 and greater, due to the tendency of trimethylpentane to float on the surface of the aqueous medium and the time necessary to adequately mix

the solvent and the aqueous titrant, thus liberating the last traces of iodine. It is recommended to use *0.1 M sodium thiosulphate* for peroxide values greater than 150. A small amount (0.5 per cent to 1.0 per cent (*m/m*)) of high HLB emulsifier (for example polysorbate 60) may be added to the mixture to retard the phase separation and decrease the time lag in the liberation of iodine.

Carry out a blank determination (V_0 ml). If the result of the blank determination exceeds 0.1 ml of titration reagent, replace the impure reagents and repeat the determination.

$$I_p = \frac{1000 (V_1 - V_0) c}{m}$$

c = concentration of the sodium thiosulphate solution in moles, per litre.

01/2005:20506

2.5.6. SAPONIFICATION VALUE

The saponification value I_s is the number that expresses in milligrams the quantity of potassium hydroxide required to neutralise the free acids and to saponify the esters present in 1 g of the substance.

Unless otherwise prescribed, use the quantities indicated in Table 2.5.6.-1 for the determination.

Table 2.5.6.-1

Presumed value I_s	Quantity of sample (g)
<3	20
3 to 10	12 to 15
10 to 40	8 to 12
40 to 60	5 to 8
60 to 100	3 to 5
100 to 200	2.5 to 3
200 to 300	1 to 2
300 to 400	0.5 to 1

Introduce the prescribed quantity of the substance to be examined (m g) into a 250 ml borosilicate glass flask fitted with a reflux condenser. Add 25.0 ml of *0.5 M alcoholic potassium hydroxide* and a few glass beads. Attach the condenser and heat under reflux for 30 min, unless otherwise prescribed. Add 1 ml of *phenolphthalein solution R1* and titrate immediately (while still hot) with *0.5 M hydrochloric acid* (n_1 ml of *0.5 M hydrochloric acid*). Carry out a blank test under the same conditions (n_2 ml of *0.5 M hydrochloric acid*).

$$I_s = \frac{28.05 (n_2 - n_1)}{m}$$

01/2005:20507

2.5.7. UNSAPONIFIABLE MATTER

The term “unsaponifiable matter” is applied to the substances non-volatile at 100-105 °C obtained by extraction with an organic solvent from the substance to be examined after it has been saponified. The result is calculated as per cent *m/m*.

Use *ungreased ground-glass glassware*.

Introduce the prescribed quantity of the substance to be examined (m g) into a 250 ml flask fitted with a reflux condenser. Add 50 ml of *2 M alcoholic potassium*

hydroxide R and heat on a water-bath for 1 h, swirling frequently. Cool to a temperature below 25 °C and transfer the contents of the flask to a separating funnel with the aid of 100 ml of *water R*. Shake the liquid carefully with 3 quantities, each of 100 ml, of *peroxide-free ether R*. Combine the ether layers in another separating funnel containing 40 ml of *water R*, shake gently for a few minutes, allow to separate and reject the aqueous phase. Wash the ether phase with 2 quantities, each of 40 ml, of *water R* then wash successively with 40 ml of a 30 g/l solution of *potassium hydroxide R* and 40 ml of *water R*; repeat this procedure 3 times. Wash the ether phase several times, each with 40 ml of *water R*, until the aqueous phase is no longer alkaline to phenolphthalein. Transfer the ether phase to a tared flask, washing the separating funnel with *peroxide-free ether R*. Distil off the ether with suitable precautions and add 6 ml of *acetone R* to the residue. Carefully remove the solvent in a current of air. Dry to constant mass at 100-105 °C. Allow to cool in a desiccator and weigh (a g).

$$\text{Unsaponifiable matter} = \frac{100a}{m} \text{ per cent}$$

Dissolve the residue in 20 ml of *alcohol R*, previously neutralised to *phenolphthalein solution R* and titrate with *0.1 M ethanolic sodium hydroxide*. If the volume of *0.1 M ethanolic sodium hydroxide* used is greater than 0.2 ml, the separation of the layers has been incomplete; the residue weighed cannot be considered as “unsaponifiable matter”. In case of doubt, the test must be repeated.

01/2005:20508

2.5.8. DETERMINATION OF PRIMARY AROMATIC AMINO-NITROGEN

Dissolve the prescribed quantity of the substance to be examined in 50 ml of *dilute hydrochloric acid R* or in another prescribed solvent and add 3 g of *potassium bromide R*. Cool in ice-water and titrate by slowly adding *0.1 M sodium nitrite* with constant stirring.

Determine the end-point electrometrically or by the use of the prescribed indicator.

01/2005:20509

2.5.9. DETERMINATION OF NITROGEN BY SULPHURIC ACID DIGESTION

SEMI-MICRO METHOD

Place a quantity of the substance to be examined (m g) containing about 2 mg of nitrogen in a combustion flask, add 4 g of a powdered mixture of 100 g of *dipotassium sulphate R*, 5 g of *copper sulphate R* and 2.5 g of *selenium R*, and three glass beads. Wash any adhering particles from the neck into the flask with 5 ml of *sulphuric acid R*, allowing it to run down the sides of the flask, and mix the contents by rotation. Close the mouth of the flask loosely, for example by means of a glass bulb with a short stem, to avoid excessive loss of sulphuric acid. Heat gradually at first, then increase the temperature until there is vigorous boiling with condensation of sulphuric acid in the neck of the flask; precautions should be taken to prevent the upper part of the flask from becoming overheated. Continue the heating for 30 min, unless otherwise prescribed. Cool, dissolve the solid material by cautiously adding to the mixture 25 ml of *water R*, cool again and place in a steam-distillation apparatus. Add 30 ml of *strong sodium hydroxide solution R*

and distil immediately by passing steam through the mixture. Collect about 40 ml of distillate in 20.0 ml of 0.01 M hydrochloric acid and enough water R to cover the tip of the condenser. Towards the end of the distillation, lower the receiver so that the tip of the condenser is above the surface of the acid. Take precautions to prevent any water on the outer surface of the condenser from reaching the contents of the receiver. Titrate the distillate with 0.01 M sodium hydroxide, using methyl red mixed solution R as indicator (n_1 ml of 0.01 M sodium hydroxide).

Repeat the test using about 50 mg of glucose R in place of the substance to be examined (n_2 ml of 0.01 M sodium hydroxide).

$$\text{Content of nitrogen} = \frac{0.01401 (n_2 - n_1)}{m} \text{ per cent}$$

01/2005:20510

2.5.10. OXYGEN-FLASK METHOD

Unless otherwise prescribed the combustion flask is a conical flask of at least 500 ml capacity of borosilicate glass with a ground-glass stopper fitted with a suitable carrier for the sample, for example in platinum or platinum-iridium.

Finely grind the substance to be examined, place the prescribed quantity in the centre of a piece of filter paper measuring about 30 mm by 40 mm provided with a small strip about 10 mm wide and 30 mm long. If paper impregnated with lithium carbonate is prescribed, moisten the centre of the paper with a saturated solution of lithium carbonate R and dry in an oven before use. Envelop the substance to be examined in the paper and place it in the sample carrier. Introduce into the flask water R or the prescribed solution designed to absorb the combustion products, displace the air with oxygen by means of a tube having its end just above the liquid, moisten the neck of the flask with water R and close with its stopper. Ignite the paper strip by suitable means with the usual precautions. Keep the flask firmly closed during the combustion. Shake the flask vigorously to completely dissolve the combustion products. Cool and after about 5 min, unless otherwise prescribed, carefully unstopper the flask. Wash the ground parts and the walls of the flask, as well as the sample carrier, with water R. Combine the combustion products and the washings and proceed as prescribed in the monograph.

01/2005:20511

2.5.11. COMPLEXOMETRIC TITRATIONS

ALUMINIUM

Introduce 20.0 ml of the prescribed solution into a 500 ml conical flask, add 25.0 ml of 0.1 M sodium edetate and 10 ml of a mixture of equal volumes of a 155 g/l solution of ammonium acetate R and dilute acetic acid R. Boil for 2 min, then cool. Add 50 ml of ethanol R and 3 ml of a freshly prepared 0.25 g/l solution of dithizone R in ethanol R. Titrate the excess of sodium edetate with 0.1 M zinc sulphate until the colour changes from greenish-blue to reddish-violet.

1 ml of 0.1 M sodium edetate is equivalent to 2.698 mg of Al.

BISMUTH

Introduce the prescribed solution into a 500 ml conical flask. Dilute to 250 ml with water R and then, unless otherwise prescribed, add dropwise, with shaking, concentrated

ammonia R until the mixture becomes cloudy. Add 0.5 ml of nitric acid R. Heat to about 70 °C until the cloudiness disappears completely. Add about 50 mg of xylenol orange triturate R and titrate with 0.1 M sodium edetate until the colour changes from pinkish-violet to yellow.

1 ml of 0.1 M sodium edetate is equivalent to 20.90 mg of Bi.

CALCIUM

Introduce the prescribed solution into a 500 ml conical flask, and dilute to 300 ml with water R. Add 6.0 ml of strong sodium hydroxide solution R and about 15 mg of calconecarboxylic acid triturate R. Titrate with 0.1 M sodium edetate until the colour changes from violet to full blue.

1 ml of 0.1 M sodium edetate is equivalent to 4.008 mg of Ca.

MAGNESIUM

Introduce the prescribed solution into a 500 ml conical flask and dilute to 300 ml with water R. Add 10 ml of ammonium chloride buffer solution pH 10.0 R and about 50 mg of mordant black 11 triturate R. Heat to about 40 °C then titrate at this temperature with 0.1 M sodium edetate until the colour changes from violet to full blue.

1 ml of 0.1 M sodium edetate is equivalent to 2.431 mg of Mg.

LEAD

Introduce the prescribed solution into a 500 ml conical flask and dilute to 200 ml with water R. Add about 50 mg of xylenol orange triturate R and hexamethylenetetramine R until the solution becomes violet-pink. Titrate with 0.1 M sodium edetate until the violet-pink colour changes to yellow.

1 ml of 0.1 M sodium edetate is equivalent to 20.72 mg of Pb.

ZINC

Introduce the prescribed solution into a 500 ml conical flask and dilute to 200 ml with water R. Add about 50 mg of xylenol orange triturate R and hexamethylenetetramine R until the solution becomes violet-pink. Add 2 g of hexamethylenetetramine R in excess. Titrate with 0.1 M sodium edetate until the violet-pink colour changes to yellow.

1 ml of 0.1 M sodium edetate is equivalent to 6.54 mg of Zn.

01/2005:20512

2.5.12. WATER: SEMI-MICRO DETERMINATION

The titration vessel, of about 60 ml capacity, is fitted with 2 platinum electrodes, a nitrogen inlet tube, a stopper which accommodates the burette tip, and a vent-tube protected by a desiccant. The substance to be examined is introduced through a side-arm which can be closed by a ground stopper. Stirring is effected magnetically or by means of a stream of dried nitrogen passed through the solution during the titration.

The end-point is determined by amperometry. A suitable circuit consists of a potentiometer of about 2000 Ω connected across a 1.5 V battery to supply a variable potential. This potential is adjusted so that an initial low current passes through the platinum electrodes connected in series with a microammeter. On adding the reagent, the needle of the microammeter shows a deflection but returns immediately to its starting position. At the end of the reaction, a deflection is obtained which persists for not less than 30 s.

Use the iodosulphurous reagent R after determination of the water equivalent (4.1.1). The reagents and solutions used must be kept anhydrous and precautions must be taken throughout to prevent exposure to atmospheric moisture.

The *iodosulphurous reagent R* is protected from light, preferably stored in a bottle to which is fitted an automatic burette.

The composition of commercially available iodosulphurous reagents often differs from that of iodosulphurous reagent R by the replacement of pyridine with various other basic compounds. The use of these reagents must previously be validated, in order to verify, in each individual case, the stoichiometry and the absence of incompatibility between the substance under test and the reagent (1.1. General Notices).

Unless otherwise prescribed, use Method A.

Method A. Add about 20 ml of *anhydrous methanol R* or the solvent prescribed in the monograph to the titration vessel and titrate to the amperometric end-point with the *iodosulphurous reagent R*. Quickly transfer the prescribed amount of the substance to be examined to the titration vessel. Stir for 1 min and titrate again to the amperometric end-point using *iodosulphurous reagent R*.

Method B. Add about 10 ml of *anhydrous methanol R* or the solvent prescribed in the monograph to the titration vessel and titrate to the amperometric end-point with *iodosulphurous reagent R*. Quickly transfer the prescribed amount of the substance to be examined in a suitable state of division followed by an accurately measured volume of *iodosulphurous reagent R*, sufficient to give an excess of about 1 ml or the volume prescribed in the monograph. Allow the stoppered flask to stand protected from light for 1 min or the time prescribed in the monograph, stirring from time to time. Titrate the excess of *iodosulphurous reagent R* until the initial low current is again obtained, using *anhydrous methanol R* or the solvent prescribed in the monograph, to which has been added an accurately known amount of *water R* equivalent to about 2.5 g/l.

01/2005:20513

2.5.13. ALUMINIUM IN ADSORBED VACCINES

Homogenise the preparation to be examined and transfer a suitable quantity, presumed to contain 5 mg to 6 mg of aluminium, to a 50 ml combustion flask. Add 1 ml of *sulphuric acid R*, 0.1 ml of *nitric acid R* and some glass beads. Heat the solution until thick, white fumes are evolved. If there is charring at this stage add a few more drops of *nitric acid R* and continue boiling until the colour disappears. Allow to cool for a few minutes, carefully add 10 ml of *water R* and boil until a clear solution is obtained. Allow to cool, add 0.05 ml of *methyl orange solution R* and neutralise with *strong sodium hydroxide solution R* (6.5 ml to 7 ml). If a precipitate forms dissolve it by adding, dropwise, sufficient *dilute sulphuric acid R*. Transfer the solution to a 250 ml conical flask, rinsing the combustion flask with 25 ml of *water R*. Add 25.0 ml of 0.02 M *sodium edetate*, 10 ml of *acetate buffer solution pH 4.4 R* and a few glass beads and boil gently for 3 min. Add 0.1 ml of *pyridylazonaphthol solution R* and titrate the hot solution with 0.02 M *copper sulphate* until the colour changes to purplish-brown. Carry out a blank titration omitting the vaccine.

1 ml of 0.02 M *sodium edetate* is equivalent to 0.5396 mg of Al.

01/2005:20514

2.5.14. CALCIUM IN ADSORBED VACCINES

All solutions used for this test must be prepared using water R.

Determine the calcium by atomic emission spectrometry (2.2.22, Method I). Homogenise the preparation to be examined. To 1.0 ml add 0.2 ml of *dilute hydrochloric acid R* and dilute to 3.0 ml with *water R*. Measure the absorbance at 620 nm.

01/2005:20515

2.5.15. PHENOL IN IMMUNOSERA AND VACCINES

Homogenise the preparation to be examined. Dilute an appropriate volume with *water R* so as to obtain a solution presumed to contain 15 µg of phenol per millilitre. Prepare a series of reference solutions with *phenol R* containing 5 µg, 10 µg, 15 µg, 20 µg and 30 µg of phenol per millilitre respectively. To 5 ml of the solution to be examined and to 5 ml of each of the reference solutions respectively, add 5 ml of *buffer solution pH 9.0 R*, 5 ml of *aminopyrazolone solution R* and 5 ml of *potassium ferricyanide solution R*. Allow to stand for 10 min and measure the intensity of colour at 546 nm.

Plot the calibration curve and calculate the phenol content of the preparation to be examined.

01/2005:20516

2.5.16. PROTEIN IN POLYSACCHARIDE VACCINES

Test solution. Use a volumetric flask with a suitable volume for preparation of a solution containing about 5 mg per millilitre of dry polysaccharide. Transfer the contents of a container quantitatively to the flask and dilute to volume with *water R*. Place 1 ml of the solution in a glass tube and add 0.15 ml of a 400 g/l solution of *trichloroacetic acid R*. Shake, allow to stand for 15 min, centrifuge for 10 min at 5000 r/min and discard the supernatant. Add 0.4 ml of 0.1 M *sodium hydroxide* to the centrifugation residue.

Reference solutions. Dissolve 0.100 g of *bovine albumin R* in 100 ml of 0.1 M *sodium hydroxide* (stock solution containing 1 g of protein per litre). Dilute 1 ml of the stock solution to 20 ml with 0.1 M *sodium hydroxide* (working dilution 1: 50 mg of protein per litre). Dilute 1 ml of the stock solution to 4 ml with 0.1 M *sodium hydroxide* (working dilution 2: 250 mg of protein per litre). Place in 6 glass tubes 0.10 ml, 0.20 ml and 0.40 ml of working dilution 1 and 0.15 ml, 0.20 ml and 0.25 ml of working dilution 2. Make up the volume in each tube to 0.40 ml using 0.1 M *sodium hydroxide*.

Prepare a blank using 0.40 ml of 0.1 M *sodium hydroxide*. Add 2 ml of *cupri-tartaric solution R3* to each tube, shake and allow to stand for 10 min. Add to each tube 0.2 ml of a mixture of equal volumes of *phosphomolybdotungstic reagent R* and *water R*, prepared immediately before use. Stopper the tubes, mix by inverting and allow to stand in the dark for 30 min. The blue colour is stable for 60 min. If necessary, centrifuge to obtain clear solutions.

Measure the absorbance (2.2.25) of each solution at 760 nm using the blank as the compensation liquid. Draw a calibration curve from the absorbances of the 6 reference solutions and the corresponding protein contents and read from the curve the content of protein in the test solution.

01/2005:20517

2.5.17. NUCLEIC ACIDS IN POLYSACCHARIDE VACCINES

Test solution. Use a volumetric flask with a suitable volume for preparation of a solution containing about 5 mg per millilitre of dry polysaccharide. Transfer the contents of a container quantitatively to the flask and dilute to volume with *water R*.

Dilute the test solution if necessary to obtain an absorbance value suitable for the instrument used. Measure the absorbance (2.2.25) at 260 nm using *water R* as the compensation liquid.

The absorbance of a 1 g/l solution of nucleic acid at 260 nm is 20.

01/2005:20518

2.5.18. PHOSPHORUS IN POLYSACCHARIDE VACCINES

Test solution. Use a volumetric flask with a suitable volume for preparation of a solution containing about 5 mg per millilitre of dry polysaccharide. Transfer the contents of a container quantitatively to the flask and dilute to volume with *water R*. Dilute the solution so that the volume used in the test (1 ml) contains about 6 µg of phosphorus. Transfer 1.0 ml of the solution to a 10 ml ignition tube.

Reference solutions. Dissolve 0.2194 g of *potassium dihydrogen phosphate R* in 500 ml of *water R* to give a solution containing the equivalent of 0.1 mg of phosphorus per millilitre. Dilute 5.0 ml of the solution to 100.0 ml with *water R*. Introduce 0.5 ml, 1.0 ml and 2.0 ml of the dilute solution into 3 ignition tubes.

Prepare a blank solution using 2.0 ml of *water R* in an ignition tube.

To all the tubes add 0.2 ml of *sulphuric acid R* and heat in an oil bath at 120 °C for 1 h and then at 160 °C until white fumes appear (about 1 h). Add 0.1 ml of *perchloric acid R* and heat at 160 °C until the solution is decolorised (about 90 min). Cool and add to each tube 4 ml of *water R* and 4 ml of *ammonium molybdate reagent R*. Heat in a water-bath at 37 °C for 90 min and cool. Adjust the volume to 10.0 ml with *water R*. The blue colour is stable for several hours.

Measure the absorbance (2.2.25) of each solution at 820 nm using the blank solution as the compensation liquid. Draw a calibration curve with the absorbances of the 3 reference solutions as a function of the quantity of phosphorus in the solutions and read from the curve the quantity of phosphorus in the test solution.

01/2005:20519

2.5.19. O-ACETYL IN POLYSACCHARIDE VACCINES

Test solution. Use a volumetric flask with a suitable volume for preparation of a solution containing about 5 mg per millilitre of dry polysaccharide. Transfer the contents of a

container quantitatively to the flask and dilute to volume with *water R*. Dilute the solution so that the volumes used in the test contain 30 µg to 600 µg of acetylcholine chloride (*O*-acetyl). Introduce 0.3 ml, 0.5 ml and 1.0 ml in duplicate into 6 tubes (3 reaction solutions and 3 correction solutions).

Reference solutions. Dissolve 0.150 g of *acetylcholine chloride R* in 10 ml of *water R* (stock solution containing 15 g of acetylcholine chloride per litre). Immediately before use, dilute 1 ml of the stock solution to 50 ml with *water R* (working dilution 1: 300 µg of acetylcholine chloride per millilitre). Immediately before use, dilute 1 ml of the stock solution to 25 ml with *water R* (working dilution 2: 600 µg of acetylcholine chloride per millilitre). Introduce 0.1 ml and 0.4 ml of working dilution 1 in duplicate (reaction and correction solutions) in 4 tubes and 0.6 ml and 1.0 ml of working dilution 2 in duplicate (reaction and correction solutions) in another 4 tubes.

Prepare a blank using 1 ml of *water R*.

Make up the volume in each tube to 1 ml with *water R*. Add 1.0 ml of 4 M *hydrochloric acid* to each of the correction tubes and to the blank. Add 2.0 ml of *alkaline hydroxylamine solution R* to each tube. Allow the reaction to proceed for exactly 2 min and add 1.0 ml of 4 M *hydrochloric acid* to each of the reaction tubes. Add 1.0 ml of a 100 g/l solution of *ferric chloride R* in 0.1 M *hydrochloric acid* to each tube, stopper the tubes and shake vigorously to remove bubbles.

Measure the absorbance (2.2.25) of each solution at 540 nm using the blank as the compensation liquid. For each reaction solution, subtract the absorbance of the corresponding correction solution. Draw a calibration curve from the corrected absorbances for the 4 reference solutions and the corresponding content of acetylcholine chloride and read from the curve the content of acetylcholine chloride in the test solution for each volume tested. Calculate the mean of the 3 values.

1 mole of acetylcholine chloride (181.7 g) is equivalent to 1 mole of *O*-acetyl (43.05 g).

01/2005:20520

2.5.20. HEXOSAMINES IN POLYSACCHARIDE VACCINES

Test solution. Use a volumetric flask with a suitable volume for preparation of a solution containing about 5 mg per millilitre of dry polysaccharide. Transfer the contents of a container quantitatively to the flask and dilute to volume with *water R*. Dilute the solution so that the volumes used in the test contain 125 µg to 500 µg of glucosamine (hexosamine). Introduce 1.0 ml of the diluted solution into a graduated tube.

Reference solutions. Dissolve 60 mg of *glucosamine hydrochloride R* in 100 ml of *water R* (stock solution containing 0.500 g of glucosamine per litre). Introduce 0.25 ml, 0.50 ml, 0.75 ml, and 1.0 ml of the working dilution into 4 graduated tubes.

Prepare a blank using 1 ml of *water R*.

Make up the volume in each tube to 1 ml with *water R*. Add 1 ml of a solution of *hydrochloric acid R* (292 g/l) to each tube. Stopper the tubes and place in a water-bath for 1 h. Cool to room temperature. Add to each tube 0.05 ml of a 5 g/l solution of *thymolphthalein R* in *alcohol R*; add a solution of *sodium hydroxide R* (200 g/l) until a blue colour is obtained and then 1 M *hydrochloric acid* until the solution is colourless. Dilute the volume in each tube to 10 ml with *water R* (neutralised hydrolysates).

In a second series of 10 ml graduated tubes, place 1 ml of each neutralised hydrolysate. Add 1 ml of acetylacetone reagent (a mixture, prepared immediately before use, of 1 volume of *acetylacetone R* and 50 volumes of a 53 g/l solution of *anhydrous sodium carbonate R*) to each tube. Stopper the tubes and place in a water-bath at 90 °C for 45 min. Cool to room temperature. Add to each tube 2.5 ml of *alcohol R* and 1.0 ml of dimethylaminobenzaldehyde solution (immediately before use dissolve 0.8 g of *dimethylaminobenzaldehyde R* in 15 ml of *alcohol R* and add 15 ml of *hydrochloric acid R*) and dilute the volume in each tube to 10 ml with *alcohol R*. Stopper the tubes, mix by inverting and allow to stand in the dark for 90 min. Measure the absorbance (2.2.25) of each solution at 530 nm using the blank as the compensation liquid.

Draw a calibration curve from the absorbances for the 4 reference solutions and the corresponding content of hexosamine and read from the curve the quantity of hexosamine in the test solution.

01/2005:20521

2.5.21. METHYLPENTOSE IN POLYSACCHARIDE VACCINES

Test solution. Use a volumetric flask with a suitable volume for preparation of a solution containing about 5 mg per millilitre of dry polysaccharide. Transfer the contents of a container quantitatively to the flask and dilute to volume with *water R*. Dilute the solution so that the volumes used in the test contain 2 µg to 20 µg of rhamnose (methylpentoses). Introduce 0.25 ml, 0.50 ml and 1.0 ml of the diluted solution into 3 tubes.

Reference solutions. Dissolve 0.100 g of *rhamnose R* in 100 ml of *water R* (stock solution containing 1 g of methylpentose per litre). Immediately before use, dilute 1 ml of the stock solution to 50 ml with *water R* (working dilution: 20 mg of methylpentose per litre). Introduce 0.10 ml, 0.25 ml, 0.50 ml, 0.75 ml and 1.0 ml of the working dilution into 5 tubes.

Prepare a blank using 1 ml of *water R*.

Make up the volume in each tube to 1 ml with *water R*. Place the tubes in iced water and add dropwise and with continuous stirring to each tube 4.5 ml of a cooled mixture of 1 volume of *water R* and 6 volumes of *sulphuric acid R*. Warm the tubes to room temperature and place in a water-bath for a few minutes. Cool to room temperature. Add to each tube 0.10 ml of a 30 g/l solution of *cysteine hydrochloride R*, prepared immediately before use. Shake and allow to stand for 2 h.

Measure the absorbance (2.2.25) of each solution at 396 nm and at 430 nm using the blank as compensation liquid. For each solution, calculate the difference between the absorbance measured at 396 nm and that measured at 430 nm. Draw a calibration curve from the absorbance differences for the 5 reference solutions and the corresponding content of methylpentose and read from the curve the quantity of methylpentose in the test solution for each volume tested. Calculate the mean of the 3 values.

01/2005:20522

2.5.22. URONIC ACIDS IN POLYSACCHARIDE VACCINES

Test solution. Use a volumetric flask with a suitable volume for preparation of a solution containing about 5 mg per millilitre of dry polysaccharide. Transfer the contents of a

container quantitatively to the flask and dilute to volume with *water R*. Dilute the solution so that the volumes used in the test contain 4 µg to 40 µg of glucuronic acid (uronic acids). Introduce 0.25 ml, 0.50 ml and 1.0 ml of the diluted solution into 3 tubes.

Reference solutions. Dissolve 50 mg of *sodium glucuronate R* in 100 ml of *water R* (stock solution containing 0.4 g of glucuronic acid per litre). Immediately before use, dilute 5 ml of the stock solution to 50 ml with *water R* (working dilution: 40 mg of glucuronic acid per litre). Introduce 0.10 ml, 0.25 ml, 0.50 ml, 0.75 ml, and 1.0 ml of the working dilution into 5 tubes.

Prepare a blank using 1 ml of *water R*.

Make up the volume in each tube to 1 ml with *water R*. Place the tubes in iced water and add dropwise and with continuous stirring to each tube 5.0 ml of *borate solution R*. Stopper the tubes and place in a water-bath for 15 min. Cool to room temperature. Add 0.20 ml of a 1.25 g/l solution of *carbazole R* in *ethanol R* to each tube. Stopper the tubes and place in a water-bath for 15 min. Cool to room temperature. Measure the absorbance (2.2.25) of each solution at 530 nm using the blank as the compensation liquid.

Draw a calibration curve from the absorbances for the 5 reference solutions and the corresponding content of glucuronic acid and read from the curve the quantity of glucuronic acid in the test solution for each volume tested. Calculate the mean of the 3 values.

01/2005:20523

2.5.23. SIALIC ACID IN POLYSACCHARIDE VACCINES

Test solution. Transfer quantitatively the contents of one or several containers to a volumetric flask of a suitable volume that will give a solution with a known concentration of about 250 µg per millilitre of polysaccharide and dilute to volume with *water R*. Using a syringe, transfer 4.0 ml of this solution to a 10 ml ultrafiltration cell suitable for the passage of molecules of relative molecular mass less than 50 000. Rinse the syringe twice with *water R* and transfer the rinsings to the ultrafiltration cell. Carry out the ultrafiltration, with constant stirring, under *nitrogen R* at a pressure of about 150 kPa. Refill the cell with *water R* each time the volume of liquid in it has decreased to 1 ml and continue until 200 ml has been filtered and the remaining volume in the cell is about 2 ml. Using a syringe, transfer this residual liquid to a 10 ml volumetric flask. Wash the cell with 3 quantities, each of 2 ml, of *water R*, transfer the washings to the flask and dilute to 10.0 ml with *water R* (test solution). In each of 2 test-tubes place 2.0 ml of the test solution.

Reference solutions. Use the reference solutions prescribed in the monograph.

Prepare 2 series of 3 test-tubes, place in the tubes of each series 0.5 ml, 1.0 ml and 1.5 ml respectively, of the reference solution corresponding to the type of vaccine to be examined and adjust the volume in each tube to 2.0 ml with *water R*. Prepare blank solutions using 2.0 ml of *water R* in each of 2 test-tubes.

To all the tubes add 5.0 ml of *resorcinol reagent R*. Heat at 105 °C for 15 min, cool in cold water and transfer the tubes to a bath of iced water. To each tube add 5 ml of *isoamyl alcohol R* and mix thoroughly. Place in the bath of iced water for 15 min. Centrifuge the tubes and keep them in the bath of iced water until the examination by absorption spectrophotometry. Measure the absorbance (2.2.25) of each supernatant solution at 580 nm and 450 nm using *isoamyl*

alcohol R as the compensation liquid. For each wavelength, calculate the absorbance as the mean of the values obtained with 2 identical solutions. Subtract the mean value for the blank solution from the mean values obtained for the other solutions.

Draw a graph showing the difference between the absorbances at 580 nm and 450 nm of the reference solutions as a function of the content of *N*-acetylneuraminic acid and read from the graph the quantity of *N*-acetylneuraminic acid (sialic acid) in the test solution.

01/2005:20525

2.5.25. CARBON MONOXIDE IN GASES

METHOD I

Apparatus. The apparatus (see Figure 2.5.25.-1) consists of the following parts connected in series:

- a U-tube (U_1) containing *anhydrous silica gel R* impregnated with *chromium trioxide R*,
- a wash bottle (F_1) containing 100 ml of a 400 g/l solution of *potassium hydroxide R*,
- a U-tube (U_2) containing pellets of *potassium hydroxide R*,
- a U-tube (U_3) containing *diphosphorus pentoxide R* dispersed on previously granulated, fused pumice,
- a U-tube (U_4) containing 30 g of *recrystallised iodine pentoxide R* in granules, previously dried at 200 °C and kept at a temperature of 120 °C (T) during the test. The iodine pentoxide is packed in the tube in 1 cm columns separated by 1 cm columns of glass wool to give an effective length of 5 cm,
- a reaction tube (F_2) containing 2.0 ml of *potassium iodide solution R* and 0.15 ml of *starch solution R*.

Method. Flush the apparatus with 5.0 litres of *argon R* and, if necessary, discharge the blue colour in the iodide solution by adding the smallest necessary quantity of freshly prepared 0.002 M *sodium thiosulphate*. Continue flushing until not more than 0.045 ml of 0.002 M *sodium thiosulphate* is required after passage of 5.0 litres of *argon R*. Pass the gas to be examined from the cylinder through the apparatus, using the prescribed volume and the flow rate. Flush the last traces of liberated iodine into the reaction tube by passing through the apparatus 1.0 litre of *argon R*. Titrate the liberated iodine with 0.002 M *sodium thiosulphate*. Carry out a blank test, using the prescribed volume of *argon R*. The difference between the volumes of 0.002 M *sodium thiosulphate* used in the titrations is not greater than the prescribed limit.

01/2005:20524

2.5.24. CARBON DIOXIDE IN GASES

Carbon dioxide in gases is determined using an infrared analyser (see Figure 2.5.24.-1).

The infrared analyser comprises 2 generators of identical infrared beams. The generators are equipped with reflectors and coils electrically heated to low red heat. One beam crosses a sample cell and the other beam crosses a reference cell. The sample cell receives a stream of the gas to be analysed and the reference cell contains *nitrogen R1*. The 2 chambers of the detector are filled with *carbon dioxide R1* and the radiation is automatically received selectively. The absorption of this radiation produces heat and differential expansion of the gas in the 2 chambers, owing to absorption of some of the emitted radiation by the carbon dioxide in the gas to be examined. The pressure difference between the 2 chambers of the detector causes distension of the metal diaphragm that separates them. This diaphragm is part of a capacitor, whose capacitance varies with the pressure difference, which itself depends on the carbon dioxide content in the gas to be examined. Since the infrared beams are periodically blocked by a rotating chopper, the electric signal is frequency modulated.

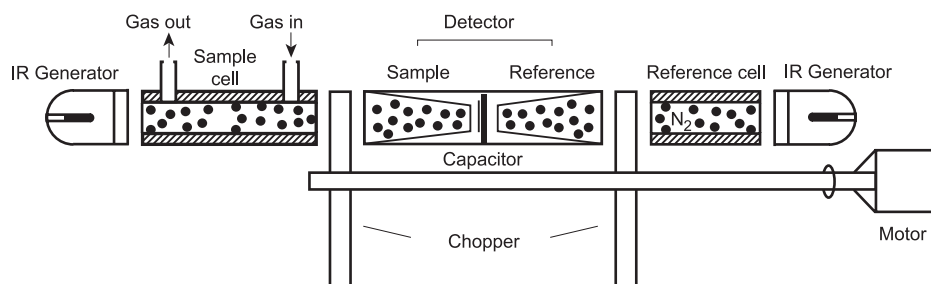


Figure 2.5.24.-1. – Infrared analyser

METHOD II

01/2005:20526

Carbon monoxide in gases may also be determined using an infrared analyser (see Figure 2.5.25.-2).

The infrared analyser comprises 2 generators of identical infrared beams. The generators are equipped with reflectors and coils electrically heated to low red heat. One beam crosses a sample cell and the other beam crosses a reference cell. The sample cell receives a current of the gas to be analysed and the reference cell contains *nitrogen R1*. The 2 chambers of the detector are filled with *carbon monoxide R* and the radiation is automatically received selectively. The absorption of this radiation produces heat and differential expansion of the gas in the two chambers, owing to absorption of some of the emitted radiation by the carbon monoxide in the gas to be examined. The pressure difference between the two chambers of the detector causes distension of the metal diaphragm that separates them. This diaphragm is part of a capacitor, whose capacitance varies with the pressure difference, which itself depends on the carbon monoxide content in the gas to be examined. Since the infrared beams are periodically blocked by a rotating chopper, the electric signal is frequency modulated.

2.5.26. NITROGEN MONOXIDE AND NITROGEN DIOXIDE IN GASES

Nitrogen monoxide and nitrogen dioxide in gases are determined using a chemiluminescence analyser (Figure 2.5.26.-1).

The apparatus consists of the following:

- a device for filtering, checking and controlling the flow of the gas to be examined,
- a converter that reduces nitrogen dioxide to nitrogen monoxide, to determine the combined content of nitrogen monoxide and nitrogen dioxide. The efficiency of the converter has to be verified prior to use,
- a controlled-flow-rate ozone generator; the ozone is produced by high-voltage electric discharges across two electrodes; the ozone generator is supplied with pure oxygen or with dehydrated ambient air and the concentration of ozone obtained must greatly exceed the maximum content of any detectable nitrogen oxides,
- a chamber in which nitrogen monoxide and ozone can react,
- a system for detecting light radiation emitted at a wavelength of 1.2 μm , consisting of a selective optical filter and a photomultiplier tube.

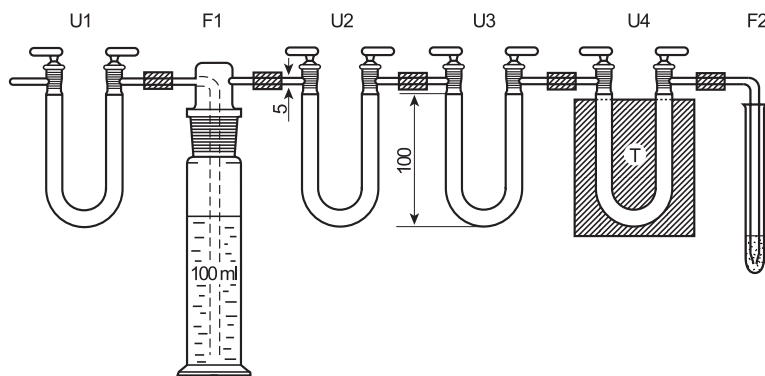


Figure 2.5.25.-1. – Apparatus for the determination of carbon monoxide
Dimensions in millimetres

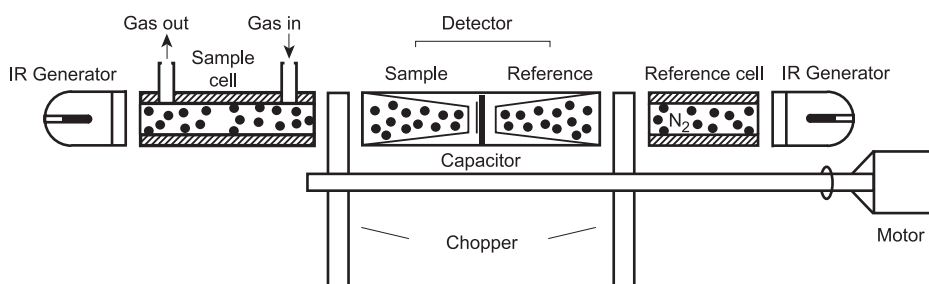


Figure 2.5.25.-2. – Infrared analyser

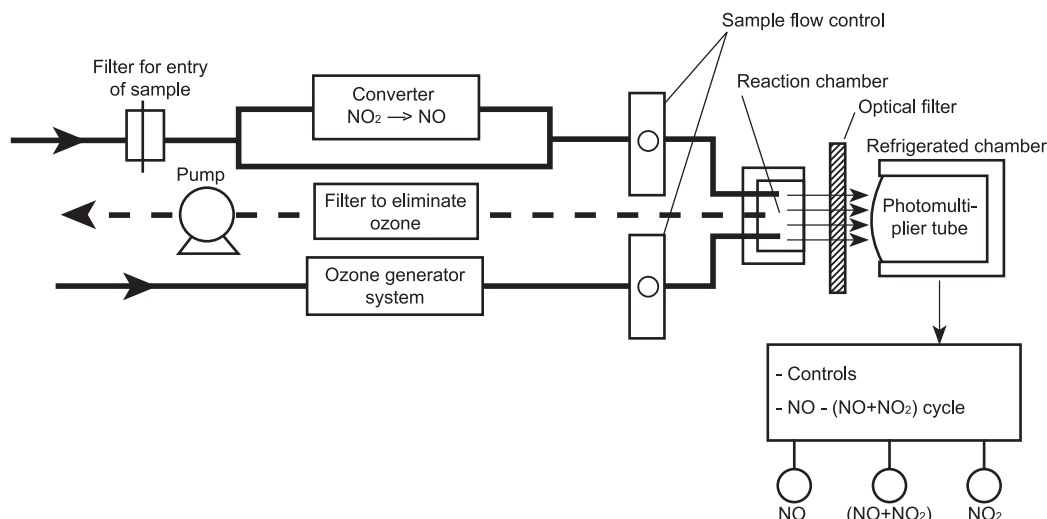


Figure 2.5.26.-1. – Chemiluminescence analyser

2.5.27. OXYGEN IN GASES

Oxygen in gases is determined using a paramagnetic analyser.

The principle of the method is based on the high paramagnetic sensitivity of the oxygen molecule. Oxygen exerts a strong interaction on magnetic fields, which is measured electronically, amplified and converted to a reading of oxygen concentration. The measurement of oxygen concentration is dependent upon the pressure and temperature and, if the analyser is not automatically compensated for variations in temperature and pressure, it must be calibrated immediately prior to use. As the paramagnetic effect of oxygen is linear the instrument must have a suitable range with a readability of 0.1 per cent or better.

Calibration of the instrument. Make the setting in the following manner:

- set the zero by passing *nitrogen R1* through the instrument at a suitable flow rate until a constant reading is obtained. It should be set to zero according to the manufacturer's instructions;
- set the appropriate limit by passing air (20.9 per cent V/V O₂) through the instrument at a suitable flow rate until a constant reading is obtained. The limit should be set to 20.9 per cent V/V in accordance with the manufacturer's instructions.

Assay. Pass the gas to be examined through the instrument at a constant flow rate until a suitable reading is obtained.

01/2005:20528

2.5.28. WATER IN GASES

Water in gases is determined using an electrolytic hygrometer, described below.

The measuring cell consists of a thin film of diphosphorus pentoxide, between 2 coiled platinum wires which act as electrodes. The water vapour in the gas to be examined

01/2005:20527

is absorbed by the diphosphorus pentoxide, which is transformed to phosphoric acid, an electrical conductor. A continuous voltage applied across the electrodes produces electrolysis of the water and the regeneration of the diphosphorus pentoxide. The resulting electric current, which is proportional to the water content in the gas to be examined, is measured. This system is self-calibrating since it obeys Faraday's law.

Take a sample of the gas to be examined. Allow the gas to stabilise at room temperature. Purge the cell continuously until a stable reading is obtained. Measure the water content in the gas to be examined, making sure that the temperature is constant throughout the device used to introduce the gas into the apparatus.

01/2005:20529

2.5.29. SULPHUR DIOXIDE

Introduce 150 ml of *water R* into the flask (A) (see Figure 2.5.29.-1) and pass *carbon dioxide R* through the whole system for 15 min at a rate of 100 ml/min. To 10 ml of *dilute hydrogen peroxide solution R* add 0.15 ml of a 1 g/l solution of *bromophenol blue R* in *alcohol (20 per cent V/V) R*. Add 0.1 M *sodium hydroxide* until a violet-blue colour is obtained, without exceeding the end-point. Place the solution in the test-tube (D). Without interrupting the stream of carbon dioxide, remove the funnel (B) and introduce through the opening into the flask (A) 25.0 g of the substance to be examined (*m* g) with the aid of 100 ml of *water R*. Add through the funnel 80 ml of *dilute hydrochloric acid R* and boil for 1 h. Open the tap of the funnel and stop the flow of carbon dioxide and also the heating and the cooling water. Transfer the contents of the test-tube with the aid of a little *water R* to a 200 ml wide-necked, conical flask. Heat on a water-bath for 15 min and allow to cool. Add 0.1 ml of a 1 g/l solution of *bromophenol blue R* in *alcohol (20 per cent V/V) R* and titrate with 0.1 M *sodium hydroxide* until the colour changes from yellow to violet-blue (*V*₁ ml). Carry out a blank titration (*V*₂ ml).

01/2005:20531

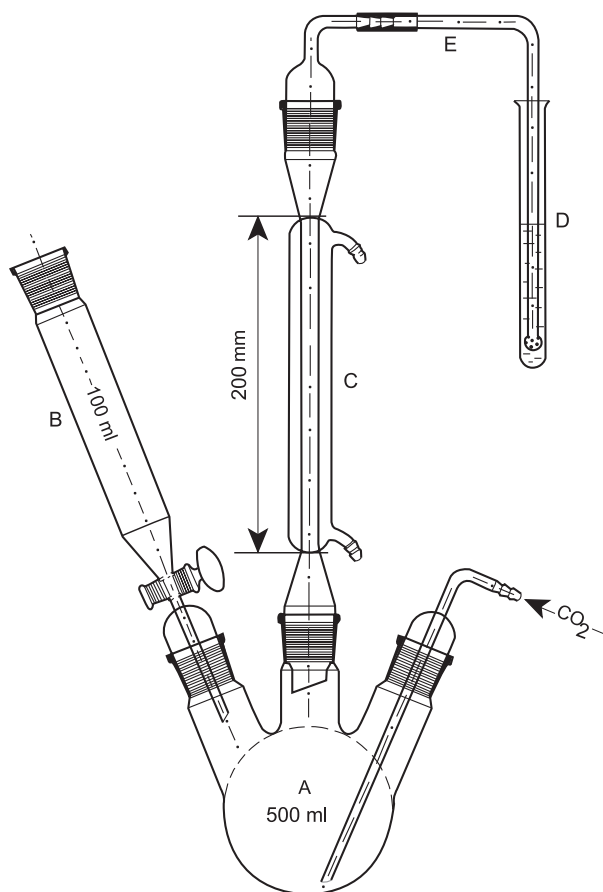


Figure 2.5.29.-1.- Apparatus for the determination of sulphur dioxide

Calculate the content of sulphur dioxide in parts per million from the expression:

$$32\,030 \times (V_1 - V_2) \times \frac{n}{m}$$

n = molarity of the sodium hydroxide solution used as titrant.

01/2005:20530

2.5.30. OXIDISING SUBSTANCES

Transfer 4.0 g to a glass-stoppered, 125 ml conical flask and add 50.0 ml of *water R*. Insert the stopper and swirl for 5 min. Transfer to a glass-stoppered 50 ml centrifuge tube and centrifuge. Transfer 30.0 ml of the clear supernatant liquid to a glass-stoppered 125 ml conical flask. Add 1 ml of *glacial acetic acid R* and 0.5 g to 1.0 g of *potassium iodide R*. Insert the stopper, swirl, and allow to stand for 25 min to 30 min in the dark. Add 1 ml of *starch solution R* and titrate with 0.002 M *sodium thiosulphate* until the starch-iodine colour disappears. Carry out a blank determination. Not more than 1.4 ml of 0.002 M *sodium thiosulphate* is required (0.002 per cent, calculated as H_2O_2).

1 ml of 0.002 M *sodium thiosulphate* is equivalent to 34 µg of oxidising substances, calculated as hydrogen peroxide.

2.5.31. RIBOSE IN POLYSACCHARIDE VACCINES

Test solution. Use a volumetric flask with a suitable volume for preparation of a solution containing about 5 mg per millilitre of dry polysaccharide. Transfer the contents of a container quantitatively to the flask and dilute to volume with *water R*. Dilute the solution so that the volumes used in the test contain 2.5 µg to 25 µg of ribose. Introduce 0.20 ml and 0.40 ml of the diluted solution into tubes in triplicate.

Reference solutions. Dissolve 25 mg of *ribose R* in *water R* and dilute to 100.0 ml with the same solvent (stock solution containing 0.25 g/l of ribose). Immediately before use, dilute 1 ml of the stock solution to 10.0 ml with *water R* (working dilution: 25 mg/l of ribose). Introduce 0.10 ml, 0.20 ml, 0.40 ml, 0.60 ml, 0.80 ml and 1.0 ml of the working dilution into 6 tubes.

Prepare a blank using 2 ml of *water R*.

Make up the volume in each tube to 2 ml with *water R*. Shake. Add 2 ml of a 0.5 g/l solution of *ferric chloride R* in *hydrochloric acid R* to each tube. Shake. Add 0.2 ml of a 100 g/l solution of *orcinol R* in *ethanol R*. Place the tubes in a water-bath for 20 min. Cool in iced water. Measure the absorbance (2.2.25) of each solution at 670 nm using the blank as the compensation liquid. Draw a calibration curve from the absorbance readings for the 6 reference solutions and the corresponding content of ribose and read from the curve the quantity of ribose in the test solution for each volume tested. Calculate the mean of the 3 values.

01/2005:20532

2.5.32. WATER: MICRO DETERMINATION

PRINCIPLE

The coulometric titration of water is based upon the quantitative reaction of water with sulphur dioxide and iodine in an anhydrous medium in the presence of a base with sufficient buffering capacity. In contrast to the volumetric method described under (2.5.12), iodine is produced electrochemically in the reaction cell by oxidation of iodide. The iodine produced at the anode reacts immediately with the water and the sulphur dioxide contained in the reaction cell. The amount of water in the substance is directly proportional to the quantity of electricity up until the titration end-point. When all of the water in the cell has been consumed, the end-point is reached and thus an excess of iodine appears. 1 mole of iodine corresponds to 1 mole of water, a quantity of electricity of 10.71 C corresponds to 1 mg of water.

Moisture is eliminated from the system by pre-electrolysis. Individual determinations can be carried out successively in the same reagent solution, under the following conditions:

- each component of the test mixture is compatible with the other components,
- no other reactions take place,
- the volume and the water capacity of the electrolyte reagent are sufficient.

Coulometric titration is restricted to the quantitative determination of small amounts of water, a range of 10 µg up to 10 mg of water is recommended.

Accuracy and precision of the method are predominantly governed by the extent to which atmospheric moisture is excluded from the system. Control of the system must be monitored by measuring the amount of baseline drift.

APPARATUS

The apparatus consists of a reaction cell, electrodes and magnetic stirrer. The reaction cell consists of a large anode compartment and a smaller cathode compartment. Depending on the design of the electrode, both compartments can be separated by a diaphragm. Each compartment contains a platinum electrode. Liquid or solubilised samples are introduced through a septum, using a syringe. Alternatively, an evaporation technique may be used in which the sample is heated in a tube (oven) and the water is evaporated and carried into the cell by means of a stream of dry inert gas. The introduction of solid samples into the cell should in general be avoided. However, if it has to be done it is effected through a sealable port; appropriate precautions must be taken to avoid the introduction of moisture from air, such as working in a glove box in an atmosphere of dry inert gas. The analytical procedure is controlled by a suitable electronic device, which also displays the results.

METHOD

Fill the compartments of the reaction cell with *electrolyte reagent for the micro determination of water R* according to the manufacturer's instructions and perform the coulometric titration to a stable end-point. Introduce the prescribed amount of the substance to be examined into the reaction cell, stir for 30 s, if not otherwise indicated in the monograph, and titrate again to a stable end-point. In case an oven is used, the prescribed sample amount is introduced into the tube and heated. After evaporation of the water from the sample into the titration cell, the titration is started. Read the value from the instrument's output and calculate if necessary the percentage or amount of water that is present in the substance. When appropriate to the type of sample and the sample preparation, perform a blank titration.

VERIFICATION OF THE ACCURACY

Between two successive sample titrations, introduce an accurately weighed amount of water in the same order of magnitude as the amount of water in the sample, either as *water R* or in the form of *standard solution for the micro determination of water R*, and perform the coulometric titration. The recovery rate is within the range from 97.5 per cent to 102.5 per cent for an addition of 1000 µg of H₂O and in the range from 90.0 per cent to 110.0 per cent for the addition of 100 µg of H₂O.

01/2005:20533

2.5.33. TOTAL PROTEIN

Many of the assay methods described in this chapter can be performed using kits from commercial sources.

METHOD 1

Protein in solution absorbs ultraviolet light at a wavelength of 280 nm, due to the presence of aromatic amino acids, mainly tyrosine and tryptophan, in the protein structure. This property can be used for assay purposes. If the buffer used to dissolve the protein has a high absorbance relative to that of water, an interfering substance is present. This interference may be obviated by using the buffer as compensation liquid but if the interfering substance produces a high absorbance, the results may nevertheless be compromised. At low concentrations, protein adsorbed onto the cell may

significantly reduce the content in solution. This can be prevented by preparing samples at higher concentration or by using a non-ionic detergent in the preparation.

Test solution. Dissolve a suitable quantity of the substance to be examined in the prescribed buffer to obtain a solution having a protein concentration between 0.2 mg/ml and 2 mg/ml.

Reference solution. Prepare a solution of a suitable reference substance for the protein to be determined, in the same buffer and at the same protein concentration as the test solution.

Procedure. Keep the test solution, the reference solution and the compensation liquid at the same temperature during the performance of this test. Determine the absorbances (2.2.25) of the test solution and the reference solution in quartz cells at 280 nm, using the prescribed buffer as the compensation liquid. The response must be linear in the range of protein concentrations to be assayed to obtain accurate results.

Light scattering. The accuracy of the determination of protein can be diminished by the scattering of light by the test sample. If the proteins in solution exist as particles comparable in size to the wavelength of the measuring light (250 nm to 300 nm), scattering of the light beam results in an apparent increase in absorbance of the test sample. To calculate the absorbance at 280 nm due to light scattering, determine the absorbances of the test solution at wavelengths of 320 nm, 325 nm, 330 nm, 335 nm, 340 nm, 345 nm and 350 nm. Plot the logarithm of the observed absorbance against the logarithm of the wavelength and determine the standard curve best fitting the plotted points by linear regression. Extrapolate the curve to determine the logarithm of the absorbance at 280 nm. The antilogarithm of this value is the absorbance attributed to light scattering. Correct the observed values by subtracting the absorbance attributed to light scattering from the total absorbance at 280 nm to obtain the absorbance value of the protein in solution. Filtration with a 0.2 µm filter that does not adsorb protein or clarification by centrifugation may be performed to reduce the effect of light scattering, especially if the solution is noticeably turbid.

Calculations. Use corrected values for the calculations. Calculate the concentration of protein in the test solution (C_U) from the following equation:

$$C_U = C_S (A_U/A_S)$$

where C_S is the concentration of protein in the reference solution and A_U and A_S are the corrected absorbances of the test solution and the reference solution, respectively.

METHOD 2

This method (commonly referred to as the Lowry assay) is based on the reduction by protein of the phosphomolybdotungstic mixed acid chromogen in the phosphomolybdotungstic reagent, which results in an absorbance maximum at 750 nm. The phosphomolybdotungstic reagent reacts primarily with tyrosine residues in the protein. Colour development reaches a maximum in 20 min to 30 min at room temperature, after which there is a gradual loss of colour. Because the method is sensitive to interfering substances, a procedure for precipitation of the protein from the test sample may be used. Most interfering substances cause a lower colour yield; however, some detergents cause a slight increase in colour. A high salt concentration may cause a precipitate to form. Because different protein species may give different colour response intensities, the reference substance and test protein must be the same. Where separation of interfering

substances from the protein in the test sample is necessary, proceed as directed below for interfering substances prior to preparation of the test solution. The effect of interfering substances may be minimised by dilution, provided the concentration of the test protein remains sufficient for accurate measurement.

Use *distilled water R* to prepare all buffers and reagents used for this method.

Test solution. Dissolve a suitable quantity of the substance to be examined in the prescribed buffer to obtain a solution having a concentration within the range of the standard curve. A suitable buffer will produce a solution of pH 10.0 to 10.5.

Reference solutions. Dissolve the reference substance for the protein to be determined in the prescribed buffer. Dilute portions of this solution with the same buffer to obtain not fewer than five reference solutions having protein concentrations evenly spaced over a suitable range situated between 5 µg/ml and 100 µg/ml.

Blank. Use the buffer used to prepare the test solution and the reference solutions.

Copper sulphate reagent. Dissolve 100 mg of *copper sulphate R* and 0.2 g of *sodium tartrate R* in *distilled water R* and dilute to 50 ml with the same solvent. Dissolve 10 g of *anhydrous sodium carbonate R* in *distilled water R* and dilute to 50 ml with the same solvent. Slowly pour the sodium carbonate solution into the copper sulphate solution with mixing. Use within 24 h.

Alkaline copper reagent. Mix 1 volume of copper sulphate reagent, 2 volumes of a 50 g/l solution of *sodium dodecyl sulphate R* and 1 volume of a 32 g/l solution of *sodium hydroxide R*. Store at room temperature and use within 2 weeks.

Diluted phosphomolybdotungstic reagent. Mix 5 ml of *phosphomolybdotungstic reagent R* with 55 ml of *distilled water R*. Store in an amber bottle, at room temperature.

Procedure. To 1.0 ml of each reference solution, of the test solution and of the blank, add 1.0 ml of alkaline copper reagent and mix. Allow to stand for 10 min. Add 0.5 ml of the diluted phosphomolybdotungstic reagent, mix and allow to stand at room temperature for 30 min. Determine the absorbances (2.2.25) of the solutions at 750 nm, using the solution from the blank as compensation liquid.

Calculations. The relationship of absorbance to protein concentration is non-linear; however, if the range of concentrations used to prepare the standard curve is sufficiently small, the latter will approach linearity. Plot the absorbances of the reference solutions against the protein concentrations and use linear regression to establish the standard curve. From the standard curve and the absorbance of the test solution, determine the concentration of protein in the test solution.

Interfering substances. In the following procedure, deoxycholate-trichloroacetic acid is added to a test sample to remove interfering substances by precipitation of proteins before determination; this technique can also be used to concentrate proteins from a dilute solution.

Add 0.1 ml of a 1.5 g/l solution of *sodium deoxycholate R* to 1 ml of a solution of the substance to be examined. Mix using a vortex mixer and allow to stand at room temperature for 10 min. Add 0.1 ml of a 720 g/l solution of *trichloroacetic acid R* and mix using a vortex mixer. Centrifuge at 3000 g for 30 min, decant the liquid and remove any residual liquid with a pipette. Redissolve the protein pellet in 1 ml of alkaline copper reagent.

METHOD 3

This method (commonly referred to as the Bradford assay) is based on the absorption shift from 470 nm to 595 nm observed when the acid blue 90 dye binds to protein. The acid blue 90 dye binds most readily to arginine and lysine residues in the protein which can lead to variation in the response of the assay to different proteins. The protein used as reference substance must therefore be the same as the protein to be determined. There are relatively few interfering substances, but it is preferable to avoid detergents and ampholytes in the test sample. Highly alkaline samples may interfere with the acidic reagent.

Use *distilled water R* to prepare all buffers and reagents used for this method.

Test solution. Dissolve a suitable quantity of the substance to be examined in the prescribed buffer to obtain a solution having a concentration within the range of the standard curve.

Reference solutions. Dissolve the reference substance for the protein to be determined in the prescribed buffer. Dilute portions of this solution with the same buffer to obtain not fewer than five reference solutions having protein concentrations evenly spaced over a suitable range situated between 0.1 mg/ml and 1 mg/ml.

Blank. Use the buffer used to prepare the test solution and the reference solutions.

Acid blue 90 reagent. Dissolve 0.10 g of *acid blue 90 R* in 50 ml of *alcohol R*. Add 100 ml of *phosphoric acid R*, dilute to 1000 ml with *distilled water R* and mix. Filter the solution and store in an amber bottle at room temperature. Slow precipitation of the dye occurs during storage. Filter the reagent before using.

Procedure. Add 5 ml of acid blue 90 reagent to 0.100 ml of each reference solution, of the test solution and of the blank. Mix by inversion. Avoid foaming, which will lead to poor reproducibility. Determine the absorbances (2.2.25) of the standard solutions and of the test solution at 595 nm, using the blank as compensation liquid. Do not use quartz (silica) spectrophotometer cells because the dye binds to this material.

Calculations. The relationship of absorbance to protein concentration is non-linear; however, if the range of concentrations used to prepare the standard curve is sufficiently small, the latter will approach linearity. Plot the absorbances of the reference solutions against protein concentrations and use linear regression to establish the standard curve. From the standard curve and the absorbance of the test solution, determine the concentration of protein in the test solution.

METHOD 4

This method (commonly referred to as the bicinchoninic acid or BCA assay) is based on reduction of the cupric (Cu²⁺) ion to cuprous (Cu¹⁺) ion by protein. The bicinchoninic acid reagent is used to detect the cuprous ion. Few substances interfere with the reaction. When interfering substances are present their effect may be minimised by dilution, provided that the concentration of the protein to be determined remains sufficient for accurate measurement. Alternatively, the protein precipitation procedure given in Method 2 may be used to remove interfering substances. Because different protein species may give different colour response intensities, the reference protein and protein to be determined must be the same.

Use *distilled water R* to prepare all buffers and reagents used for this method.

Test solution. Dissolve a suitable quantity of the substance to be examined in the prescribed buffer to obtain a solution having a concentration within the range of the concentrations of the reference solutions.

Reference solutions. Dissolve the reference substance for the protein to be determined in the prescribed buffer. Dilute portions of this solution with the same buffer to obtain not fewer than five reference solutions having protein concentrations evenly spaced over a suitable range situated between 10 µg/ml and 1200 µg/ml.

Blank. Use the buffer used to prepare the test solution and the reference solutions.

BCA reagent. Dissolve 10 g of *disodium bicinehoninate R*, 20 g of *sodium carbonate monohydrate R*, 1.6 g of *sodium tartrate R*, 4 g of *sodium hydroxide R*, and 9.5 g of *sodium hydrogen carbonate R* in *distilled water R*. Adjust, if necessary, to pH 11.25 with a solution of *sodium hydroxide R* or a solution of *sodium hydrogen carbonate R*. Dilute to 1000 ml with *distilled water R* and mix.

Copper-BCA reagent. Mix 1 ml of a 40 g/l solution of *copper sulphate R* and 50 ml of BCA reagent.

Procedure. Mix 0.1 ml of each reference solution, of the test solution and of the blank with 2 ml of the copper-BCA reagent. Incubate the solutions at 37 °C for 30 min, note the time and allow the mixtures to cool to room temperature. Within 60 min of the end of incubation, determine the absorbances (2.2.29) of the reference solutions and of the test solution in quartz cells at 562 nm, using the blank as compensation liquid. After the solutions have cooled to room temperature, the colour intensity continues to increase gradually.

Calculations. The relationship of absorbance to protein concentration is non-linear; however, if the range of concentrations used to prepare the standard curve is sufficiently small, the latter will approach linearity. Plot the absorbances of the reference solutions against protein concentrations and use linear regression to establish the standard curve. From the standard curve and the absorbance of the test solution, determine the concentration of protein in the test solution.

METHOD 5

This method (commonly referred to as the biuret assay) is based on the interaction of cupric (Cu²⁺) ion with protein in alkaline solution and resultant development of absorbance at 545 nm. This test shows minimal difference between equivalent IgG and albumin samples. Addition of the sodium hydroxide and the biuret reagent as a combined reagent, insufficient mixing after the addition of the sodium hydroxide, or an extended time between the addition of the sodium hydroxide solution and the addition of the biuret reagent will give IgG samples a higher response than albumin samples. The trichloroacetic acid method used to minimise the effects of interfering substances also can be used to determine the protein content in test samples at concentrations below 500 µg/ml.

Use *distilled water R* to prepare all buffers and reagents used for this method.

Test solution. Dissolve a suitable quantity of the substance to be examined in a 9 g/l solution of *sodium chloride R* to obtain a solution having a concentration within the range of the concentrations of the reference solutions.

Reference solutions. Dissolve the reference substance for the protein to be determined in a 9 g/l solution of *sodium chloride R*. Dilute portions of this solution with a 9 g/l solution of *sodium chloride R* to obtain not fewer than three

reference solutions having protein concentrations evenly spaced over a suitable range situated between 0.5 mg/ml and 10 mg/ml.

Blank. Use a 9 g/l solution of *sodium chloride R*.

Biuret reagent. Dissolve 3.46 g of *copper sulphate R* in 10 ml of hot *distilled water R*, and allow to cool (Solution A). Dissolve 34.6 g of *sodium citrate R* and 20.0 g of *anhydrous sodium carbonate R* in 80 ml of hot *distilled water R*, and allow to cool (Solution B). Mix solutions A and B and dilute to 200 ml with *distilled water R*. Use within 6 months. Do not use the reagent if it develops turbidity or contains any precipitate.

Procedure. To one volume of the test solution add an equal volume of a 60 g/l solution of *sodium hydroxide R* and mix. Immediately add biuret reagent equivalent to 0.4 volumes of the test solution and mix rapidly. Allow to stand at a temperature between 15 °C and 25 °C for not less than 15 min. Within 90 min of addition of the biuret reagent, determine the absorbances (2.2.29) of the reference solutions and of the test solution at the maximum at 545 nm, using the blank as compensation liquid. Any solution that develops turbidity or a precipitate is not acceptable for calculation of protein concentration.

Calculations. The relationship of absorbance to protein concentration is approximately linear within the indicated range of protein concentrations for the reference solutions. Plot the absorbances of the reference solutions against protein concentrations and use linear regression to establish the standard curve. Calculate the correlation coefficient for the standard curve. A suitable system is one that yields a line having a correlation coefficient not less than 0.99. From the standard curve and the absorbance of the test solution, determine the concentration of protein in the test solution.

Interfering substances. To minimise the effect of interfering substances, the protein can be precipitated from the test sample as follows: add 0.1 volumes of a 500 g/l solution of *trichloroacetic acid R* to 1 volume of a solution of the test sample, withdraw the supernatant layer and dissolve the precipitate in a small volume of 0.5 M *sodium hydroxide*. Use the solution obtained to prepare the test solution.

METHOD 6

This fluorimetric method is based on the derivatisation of the protein with *o*-phthalaldehyde, which reacts with the primary amines of the protein (*N*-terminal amino acid and the ε-amino group of lysine residues). The sensitivity of the assay can be increased by hydrolysing the protein before adding *o*-phthalaldehyde. Hydrolysis makes the α-amino group of the constituent amino acids available for reaction with the phthalaldehyde reagent. The method requires very small quantities of the protein. Primary amines, such as tris(hydroxymethyl)aminomethane and amino acid buffers, react with phthalaldehyde and must be avoided or removed. Ammonia at high concentrations reacts with phthalaldehyde. The fluorescence obtained when amine reacts with phthalaldehyde can be unstable. The use of automated procedures to standardise this procedure may improve the accuracy and precision of the test.

Use *distilled water R* to prepare all buffers and reagents used for this method.

Test solution. Dissolve a suitable quantity of the substance to be examined in a 9 g/l solution of *sodium chloride R* to obtain a solution having a concentration within the range of the concentrations of the reference solutions. Adjust the solution to pH 8 to 10.5 before addition of the phthalaldehyde reagent.

Reference solutions. Dissolve the reference substance for the protein to be determined in a 9 g/l solution of *sodium chloride R*. Dilute portions of this solution with a 9 g/l solution of *sodium chloride R* to obtain not fewer than five reference solutions having protein concentrations evenly spaced over a suitable range situated between 10 µg/ml and 200 µg/ml. Adjust the solutions to pH 8 to 10.5 before addition of the phthalaldehyde reagent.

Blank solution. Use a 9 g/l solution of *sodium chloride R*.

Borate buffer solution. Dissolve 61.83 g of *boric acid R* in *distilled water R* and adjust to pH 10.4 with a solution of *potassium hydroxide R*. Dilute to 1000 ml with *distilled water R* and mix.

Phthalaldehyde stock solution. Dissolve 1.20 g of *phthalaldehyde R* in 1.5 ml of *methanol R*, add 100 ml of borate buffer solution and mix. Add 0.6 ml of a 300 g/l solution of *macrogol 23 lauryl ether R* and mix. Store at room temperature and use within 3 weeks.

Phthalaldehyde reagent. To 5 ml of phthalaldehyde stock solution add 15 µl of *2-mercaptoethanol R*. Prepare at least 30 min before use. Use within 24 h.

Procedure. Mix 10 µl of the test solution and of each of the reference solutions with 0.1 ml of phthalaldehyde reagent and allow to stand at room temperature for 15 min. Add 3 ml of 0.5 M *sodium hydroxide* and mix. Determine the fluorescent intensities (2.2.21) of solutions from the reference solutions and from the test solution at an excitation wavelength of 340 nm and an emission wavelength between 440 and 455 nm. Measure the fluorescent intensity of a given sample only once, since irradiation decreases the fluorescence intensity.

Calculations. The relationship of fluorescence to protein concentration is linear. Plot the fluorescent intensities of the reference solutions against protein concentrations and use linear regression to establish the standard curve. From the standard curve and the fluorescent intensity of the test solution, determine the concentration of protein in the test solution.

METHOD 7

This method is based on nitrogen analysis as a means of protein determination. Interference caused by the presence of other nitrogen-containing substances in the test sample can affect the determination of protein by this method. Nitrogen analysis techniques destroy the test sample during the analysis but are not limited to protein presentation in an aqueous environment.

Procedure A. Proceed as prescribed for the determination of nitrogen by sulphuric acid digestion (2.5.9) or use commercial instrumentation for Kjeldahl nitrogen assay.

Procedure B. Commercial instrumentation is available for nitrogen analysis. Most nitrogen analysis instruments use pyrolysis (i.e. combustion of the sample in oxygen at temperatures approaching 1000 °C), which produces nitric oxide (NO) and other oxides of nitrogen (NO_x) from the nitrogen present in the substance to be examined. Some instruments convert the nitric oxides to nitrogen gas, which is quantified using a thermal-conductivity detector. Other instruments mix nitric oxide (NO) with ozone (O₃) to produce excited nitrogen dioxide (NO₂^{*}), which emits light when it decays and can be quantified with a chemiluminescence detector. A protein reference material that is relatively pure and is similar in composition to the test proteins is used to optimise the injection and pyrolysis parameters and to evaluate consistency in the analysis.

Calculations. The protein concentration is calculated by dividing the nitrogen content of the sample by the known nitrogen content of the protein. The known nitrogen content of the protein can be determined from the chemical composition of the protein or by comparison with a suitable reference substance.

01/2005:20534
corrected

2.5.34. ACETIC ACID IN SYNTHETIC PEPTIDES

Examine by liquid chromatography (2.2.29).

Test solution. Prepare as described in the monograph. The concentration of peptide in the solution may be adapted, depending on the expected amount of acetic acid in the sample.

Reference solution. Prepare a 0.10 g/l solution of *glacial acetic acid R* in a mixture of 5 volumes of mobile phase B and 95 volumes of mobile phase A.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4.6 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (5 µm),
- as mobile phase at a flow rate of 1.2 ml/min:

Mobile phase A. Dilute 0.7 ml of *phosphoric acid R* to 1000 ml with *water R*; adjust the pH to 3.0 with *strong sodium hydroxide solution R*,

Mobile phase B. *Methanol R2*,

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)
0 - 5	95	5
5 - 10	95 → 50	5 → 50
10 - 20	50	50
20 - 22	50 → 95	50 → 5
22 - 30	95	5

- as detector a spectrophotometer set at 210 nm.

Inject 10 µl of the reference solution and 10 µl of the test solution. In the chromatograms obtained, the peak corresponding to acetic acid has a retention time of 3-4 min. The baseline presents a steep rise after the start of the linear gradient, which corresponds to the elution of the peptide from the column. Determine the content of acetic acid in the peptide.

01/2005:20535

2.5.35. NITROUS OXIDE IN GASES

Nitrous oxide in gases is determined using an infrared analyser (see Figure 2.5.35.-1).

The infrared analyser comprises 2 generators of identical infrared beams. The generators are equipped with reflectors and coils electrically heated to low red heat. One beam crosses a sample cell and the other beam crosses a reference cell. The sample cell receives a stream of the gas to be examined and the reference cell contains *nitrogen R1*. The 2 chambers of the detector are filled with *nitrous oxide R* and the radiation is automatically received selectively. The absorption of this radiation produces heat and differential expansion of the gas in the 2 chambers, owing to absorption of some of the emitted radiation by the nitrous oxide in the gas to be examined. The pressure difference between the

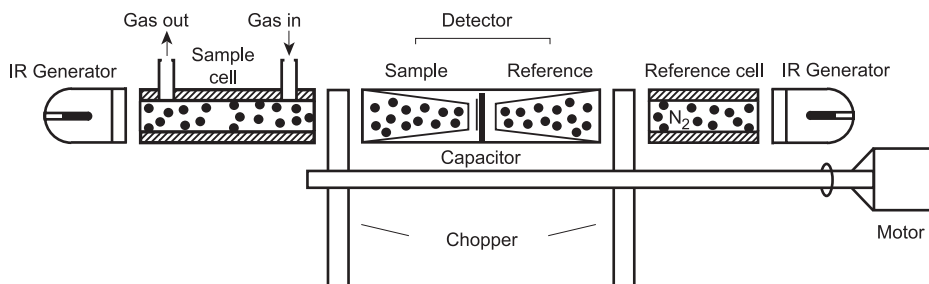


Figure 2.5.35-1. – Infrared analyser

2 chambers of the detector causes distension of the metal diaphragm that separates them. This diaphragm is part of a capacitor, whose capacitance varies with the pressure difference, which itself depends on the nitrous oxide content in the gas to be examined. Since the infrared beams are periodically blocked by a rotating chopper, the electric signal is frequency modulated.

01/2005:20536

2.5.36. ANISIDINE VALUE

The anisidine value is defined as 100 times the optical density measured in a 1 cm cell of a solution containing 1 g of the substance to be examined in 100 ml of a mixture of solvents and reagents according to the following method.

Carry out the operations as rapidly as possible, avoiding exposure to actinic light.

Test solution (a). Dissolve 0.500 g of the substance to be examined in *trimethylpentane R* and dilute to 25.0 ml with the same solvent.

Test solution (b). To 5.0 ml of test solution (a) add 1.0 ml of a 2.5 g/l solution of *p-anisidine R* in *glacial acetic acid R*, shake and store protected from light.

Reference solution. To 5.0 ml of *trimethylpentane R* add 1.0 ml of a 2.5 g/l solution of *p-anisidine R* in *glacial acetic acid R*, shake and store protected from light.

Measure the absorbance (2.2.25) of test solution (a) at the maximum at 350 nm using *trimethylpentane R* as the compensation liquid. Measure the absorbance of test solution (b) at 350 nm exactly 10 min after its preparation, using the reference solution as the compensation liquid.

Calculate the anisidine value from the expression:

$$\frac{25 \times (1.2A_1 - A_2)}{m}$$

A_1 = absorbance of test solution (b) at 350 nm,

A_2 = absorbance of test solution (a) at 350 nm,

m = mass of the substance to be examined in test solution (a), in grams.

2.6. BIOLOGICAL TESTS

01/2005:20601

2.6.1. STERILITY

The test is applied to substances, preparations or articles which, according to the Pharmacopoeia, are required to be sterile. However, a satisfactory result only indicates that no contaminating micro-organism has been found in the sample examined in the conditions of the test. Guidance for using the test for sterility is given at the end of this text.

PRECAUTIONS AGAINST MICROBIAL CONTAMINATION

The test for sterility is carried out under aseptic conditions. In order to achieve such conditions, the test environment has to be adapted to the way in which the sterility test is performed. The precautions taken to avoid contamination are such that they do not affect any micro-organisms which are to be revealed in the test. The working conditions in which the tests are performed are monitored regularly by appropriate sampling of the working area and by carrying out appropriate controls (such as those indicated in the appropriate European Community Directives and associated guidance documents on GMP).

CULTURE MEDIA AND INCUBATION TEMPERATURES

Media for the test may be prepared as described below, or equivalent commercial media may be used provided that they comply with the growth promotion test.

The following culture media have been found to be suitable for the test for sterility. Fluid thioglycollate medium is primarily intended for the culture of anaerobic bacteria; however, it will also detect aerobic bacteria. Soya-bean casein digest medium is suitable for the culture of both fungi and aerobic bacteria.

Other media may be used provided that they pass the growth promotion and the validation tests.

Fluid thioglycollate medium

L-Cystine	0.5 g
Agar, granulated (moisture content not in excess of 15 per cent)	0.75 g
Sodium chloride	2.5 g
Glucose monohydrate/anhydrous	5.5 g/5.0 g
Yeast extract (water-soluble)	5.0 g
Pancreatic digest of casein	15.0 g
Sodium thioglycollate or	0.5 g
Thioglycollic acid	0.3 ml
Resazurin sodium solution (1 g/l of resazurin sodium), freshly prepared	1.0 ml
Water R	1000 ml

pH of the medium after sterilisation 7.1 ± 0.2

Mix the L-cystine, agar, sodium chloride, glucose, water-soluble yeast extract and pancreatic digest of casein with the *water R* and heat until solution is effected. Dissolve the sodium thioglycollate or thioglycollic acid in the solution and, if necessary, add *1 M sodium hydroxide* so that, after sterilisation, the solution will have a pH of 7.1 ± 0.2 . If filtration is necessary, heat the solution again without boiling and filter while hot through moistened filter paper. Add the resazurin sodium solution, mix and place the medium in suitable vessels which provide a ratio of surface to depth of medium such that not more than the upper half of the

medium has undergone a colour change indicative of oxygen uptake at the end of the incubation period. Sterilise using a validated process. If the medium is stored, store at $2-25^\circ\text{C}$ in a sterile, airtight container. If more than the upper third of the medium has acquired a pink colour, the medium may be restored once by heating the containers in a water-bath or in free-flowing steam until the pink colour disappears and cooling quickly, taking care to prevent the introduction of non-sterile air into the container. Do not use the medium for a longer storage period than has been validated.

Fluid thioglycollate medium is to be incubated at $30-35^\circ\text{C}$.

Soya-bean casein digest medium

Pancreatic digest of casein	17.0 g
Papaic digest of soya-bean meal	3.0 g
Sodium chloride	5.0 g
Dipotassium hydrogen phosphate	2.5 g
Glucose monohydrate/anhydrous	2.5 g/2.3 g
Water R	1000 ml

pH of the medium after sterilisation 7.3 ± 0.2

Dissolve the solids in *water R*, warming slightly to effect solution. Cool the solution to room temperature. Add *1 M sodium hydroxide*, if necessary, so that after sterilisation the medium will have a pH of 7.3 ± 0.2 . Filter, if necessary, to clarify, distribute into suitable vessels and sterilise using a validated process. Store at $2-25^\circ\text{C}$ in a sterile well-closed container, unless it is intended for immediate use. Do not use the medium for a longer storage period than has been validated.

Soya-bean casein digest medium is to be incubated at $20-25^\circ\text{C}$.

The media used comply with the following tests, carried out before or in parallel with the test on the product to be examined.

Sterility. Incubate portions of the media for 14 days. No growth of micro-organisms occurs.

Growth promotion test of aerobes, anaerobes and fungi.

Test each batch of ready-prepared medium and each batch of medium prepared either from dehydrated medium or from the ingredients. Suitable strains of micro-organisms are indicated in Table 2.6.1-1.

Inoculate portions of fluid thioglycollate medium with a small number (not more than 100 CFU) of the following micro-organisms, using a separate portion of medium for each of the following species of micro-organism: *Clostridium sporogenes*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*. Inoculate portions of soya-bean casein digest medium with a small number (not more than 100 CFU) of the following micro-organisms, using a separate portion of medium for each of the following species of micro-organism: *Aspergillus niger*, *Bacillus subtilis*, *Candida albicans*. Incubate for not more than 3 days in the case of bacteria and not more than 5 days in the case of fungi.

Seed lot culture maintenance techniques (seed-lot systems) are used so that the viable micro-organisms used for inoculation are not more than 5 passages removed from the original master seed-lot.

The media are suitable if a clearly visible growth of the micro-organisms occurs.

VALIDATION TEST

Carry out a test as described below under Test for sterility of the product to be examined using exactly the same methods except for the following modifications.

Table 2.6.1-1 – *Strains of the test micro-organisms suitable for use in the Growth Promotion Test and the Validation Test*

Aerobic bacteria	
<i>Staphylococcus aureus</i>	ATCC 6538, CIP 4.83, NCTC 10788, NCIMB 9518
<i>Bacillus subtilis</i>	ATCC 6633, CIP 52.62, NCIMB 8054
<i>Pseudomonas aeruginosa</i>	ATCC 9027, NCIMB 8626, CIP 82.118
Anaerobic bacterium	
<i>Clostridium sporogenes</i>	ATCC 19404, CIP 79.3, NCTC 532 or ATCC 11437
Fungi	
<i>Candida albicans</i>	ATCC 10231, IP 48.72, NCPF 3179
<i>Aspergillus niger</i>	ATCC 16404, IP 1431.83, IMI 149007

Membrane filtration. After transferring the contents of the container or containers to be tested to the membrane add an inoculum of a small number of viable micro-organisms (not more than 100 CFU) to the final portion of sterile diluent used to rinse the filter.

Direct inoculation. After transferring the contents of the container or containers to be tested (for catgut and other surgical sutures for veterinary use: strands) to the culture medium add an inoculum of a small number of viable micro-organisms (not more than 100 CFU) to the medium.

In both cases use the same micro-organisms as those described above under Growth promotion test of aerobes, anaerobes and fungi. Perform a growth promotion test as a positive control. Incubate all the containers containing medium for not more than 5 days.

If clearly visible growth of micro-organisms is obtained after the incubation, visually comparable to that in the control vessel without product, either the product possesses no antimicrobial activity under the conditions of the test or such activity has been satisfactorily eliminated. The test for sterility may then be carried out without further modification.

If clearly visible growth is not obtained in the presence of the product to be tested, visually comparable to that in the control vessels without product, the product possesses antimicrobial activity that has not been satisfactorily eliminated under the conditions of the test. Modify the conditions in order to eliminate the antimicrobial activity and repeat the validation test.

This validation is performed:

- when the test for sterility has to be carried out on a new product,
- whenever there is a change in the experimental conditions of the test.

The validation may be performed simultaneously with the test for sterility of the product to be examined.

TEST FOR STERILITY OF THE PRODUCT TO BE EXAMINED

The test may be carried out using the technique of membrane filtration or by direct inoculation of the culture media with the product to be examined. Appropriate negative controls are included. The technique of membrane filtration is used whenever the nature of the product permits, that is, for filterable aqueous preparations, for alcoholic or oily preparations and for preparations miscible with or soluble in aqueous or oily solvents provided these solvents do not have an antimicrobial effect in the conditions of the test.

Membrane filtration. Use membrane filters having a nominal pore size not greater than 0.45 µm whose effectiveness to retain micro-organisms has been established. Cellulose nitrate filters, for example, are used for aqueous, oily and

weakly alcoholic solutions and cellulose acetate filters, for example, for strongly alcoholic solutions. Specially adapted filters may be needed for certain products, e.g. for antibiotics. The technique described below assumes that membranes about 50 mm in diameter will be used. If filters of a different diameter are used the volumes of the dilutions and the washings should be adjusted accordingly. The filtration apparatus and membrane are sterilised by appropriate means. The apparatus is designed so that the solution to be examined can be introduced and filtered under aseptic conditions; it permits the aseptic removal of the membrane for transfer to the medium or it is suitable for carrying out the incubation after adding the medium to the apparatus itself.

Aqueous solutions. If appropriate, transfer a small quantity of a suitable, sterile diluent such as a 1 g/l neutral solution of meat or casein peptone pH 7.1 ± 0.2 onto the membrane in the apparatus and filter. The diluent may contain suitable neutralising substances and/or appropriate inactivating substances for example in the case of antibiotics.

Transfer the contents of the container or containers to be tested to the membrane or membranes, if necessary after diluting to the volume used in the validation test with the chosen sterile diluent but in any case using not less than the quantities of the product to be examined prescribed in Table 2.6.1-2. Filter immediately. If the product has antimicrobial properties, wash the membrane not less than 3 times by filtering through it each time the volume of the chosen sterile diluent used in the validation test. Do not exceed a washing cycle of 5 times 200 ml, even if during validation it has been demonstrated that such a cycle does not fully eliminate the antimicrobial activity. Transfer the whole membrane to the culture medium or cut it aseptically into 2 equal parts and transfer one half to each of 2 suitable media. Use the same volume of each medium as in the validation test. Alternatively, transfer the medium onto the membrane in the apparatus. Incubate the media for not less than 14 days.

Soluble solids. Use for each medium not less than the quantity prescribed in Table 2.6.1-2 of the product dissolved in a suitable solvent such as a 1 g/l neutral solution of meat or casein peptone and proceed with the test as described above for aqueous solutions using a membrane appropriate to the chosen solvent.

Oils and oily solutions. Use for each medium not less than the quantity of the product prescribed in Table 2.6.1-2. Oils and oily solutions of sufficiently low viscosity may be filtered without dilution through a dry membrane. Viscous oils may be diluted as necessary with a suitable sterile diluent such as isopropyl myristate shown not to have antimicrobial activity in the conditions of the test. Allow the oil to penetrate the membrane by its own weight then filter, applying the pressure or suction gradually. Wash the membrane at least

Table 2.6.1-2 – Minimum quantity to be used for each medium

Quantity per container	Minimum quantity to be used for each medium unless otherwise justified and authorised
<i>Liquids</i>	
– less than 1 ml	The whole contents of each container
– 1-40 ml	Half the contents of each container but not less than 1 ml
– greater than 40 ml and not greater than 100 ml	20 ml
– greater than 100 ml	10 per cent of the contents of the container but not less than 20 ml
<i>Antibiotic liquids</i>	1 ml
<i>Other preparations soluble in water or in isopropyl myristate</i>	The whole contents of each container to provide not less than 200 mg
<i>Insoluble preparations, creams and ointments to be suspended or emulsified</i>	The whole contents of each container to provide not less than 200 mg
<i>Solids</i>	
– less than 50 mg	The whole contents of each container
– 50 mg or more but less than 300 mg	Half the contents of each container but not less than 50 mg
– 300 mg to 5 g	150 mg
– greater than 5 g	500 mg
<i>Catgut and other surgical sutures for veterinary use</i>	3 sections of a strand (each 30 cm long)

3 times by filtering through it each time about 100 ml of a suitable sterile solution such as 1 g/l neutral meat or casein peptone containing a suitable emulsifying agent at a concentration shown to be appropriate in the validation of the test, for example polysorbate 80 at a concentration of 10 g/l. Transfer the membrane or membranes to the culture medium or media or vice versa as described above for aqueous solutions, and incubate at the same temperatures and for the same times.

Ointments and creams. Use for each medium not less than the quantities of the product prescribed in Table 2.6.1-2. Ointments in a fatty base and emulsions of the water-in-oil type may be diluted to 1 per cent in isopropyl myristate as described above, by heating, if necessary, to not more than 40 °C. In exceptional cases it may be necessary to heat to not more than 44 °C. Filter as rapidly as possible and proceed as described above for oils and oily solutions.

Direct inoculation of the culture medium. Transfer the quantity of the preparation to be examined prescribed in Table 2.6.1-2 directly into the culture medium so that the volume of the product is not more than 10 per cent of the volume of the medium, unless otherwise prescribed.

If the product to be examined has antimicrobial activity, carry out the test after neutralising this with a suitable neutralising substance or by dilution in a sufficient quantity of culture medium. When it is necessary to use a large volume of the product it may be preferable to use a concentrated culture medium prepared in such a way that it takes account of the subsequent dilution. Where appropriate, the concentrated medium may be added directly to the product in its container.

Oily liquids. Use media to which have been added a suitable emulsifying agent at a concentration shown to be appropriate in the validation of the test, for example polysorbate 80 at a concentration of 10 g/l.

Ointments and creams. Prepare by diluting to about 1 in 10 by emulsifying with the chosen emulsifying agent in a suitable sterile diluent such as a 1 g/l neutral solution of meat or casein peptone. Transfer the diluted product to a medium not containing an emulsifying agent.

Incubate the inoculated media for not less than 14 days. Observe the cultures several times during the incubation period. Shake cultures containing oily products gently each day. However when thioglycollate medium or other

similar medium is used for the detection of anaerobic micro-organisms keep shaking or mixing to a minimum in order to maintain anaerobic conditions.

Catgut and other surgical sutures for veterinary use. Use for each medium not less than the quantities of the product prescribed in Table 2.6.1-2. Open the sealed package using aseptic precautions and remove 3 sections of the strand for each culture medium. Carry out the test on 3 sections, each 30 cm long, cut off from the beginning, the centre and the end of the strand. Use whole strands from freshly opened cassette packs. Transfer each section of the strand to the selected medium. Use sufficient medium to cover adequately the material to be tested (20 ml to 150 ml).

OBSERVATION AND INTERPRETATION OF RESULTS

At intervals during the incubation period and at its conclusion, examine the media for macroscopic evidence of microbial growth. If the material being tested renders the medium turbid so that the presence or absence of microbial growth cannot be readily determined by visual examination, 14 days after the beginning of incubation transfer portions (each not less than 1 ml) of the medium to fresh vessels of the same medium and then incubate the original and transfer vessels for not less than 4 days.

If no evidence of microbial growth is found, the product to be examined complies with the test for sterility. If evidence of microbial growth is found the product to be examined does not comply with the test for sterility, unless it can be clearly demonstrated that the test was invalid for causes unrelated to the product to be examined. The test may be considered invalid only if one or more of the following conditions are fulfilled:

- the data of the microbiological monitoring of the sterility testing facility show a fault,
- a review of the testing procedure used during the test in question reveals a fault,
- microbial growth is found in the negative controls,
- after determination of the identity of the micro-organisms isolated from the test, the growth of this species or these species may be ascribed unequivocally to faults with respect to the material and/or the technique used in conducting the sterility test procedure.

If the test is declared to be invalid it is repeated with the same number of units as in the original test.

If no evidence of microbial growth is found in the repeat test the product examined complies with the test for sterility.

If microbial growth is found in the repeat test the product examined does not comply with the test for sterility.

APPLICATION OF THE TEST TO PARENTERAL PREPARATIONS, OPHTHALMIC AND OTHER NON-INJECTABLE PREPARATIONS REQUIRED TO COMPLY WITH THE TEST FOR STERILITY

When using the technique of membrane filtration, use, whenever possible, the whole contents of the container, but not less than the quantities indicated in Table 2.6.1-2, diluting where necessary to about 100 ml with a suitable sterile solution, such as 1 g/l neutral meat or casein peptone.

When using the technique of direct inoculation of media, use the quantities shown in Table 2.6.1-2, unless otherwise justified and authorised. The tests for bacterial and fungal sterility are carried out on the same sample of the product to be examined. When the volume or the quantity in a single container is insufficient to carry out the tests, the contents of 2 or more containers are used to inoculate the different media.

GUIDELINES FOR USING THE TEST FOR STERILITY

The purpose of the test for sterility, as that of all pharmacopoeial tests, is to provide an independent control analyst with the means of verifying that a particular material meets the requirements of the European Pharmacopoeia. A manufacturer is neither obliged to carry out such tests nor precluded from using modifications of, or alternatives to, the stated method, provided he is satisfied that, if tested by the official method, the material in question would comply with the requirements of the European Pharmacopoeia.

Precautions against microbial contamination. Aseptic conditions for performance of the test can be achieved using, for example, a class A laminar-air-flow cabinet located within a class B clean-room, or an isolator.

Guidance to manufacturers. The level of assurance provided by a satisfactory result of a test for sterility (the absence of contaminated units in the sample) as applied to the quality of

the batch is a function of the homogeneity of the batch, the conditions of manufacture and the efficiency of the adopted sampling plan. Hence for the purpose of this text a batch is defined as a homogeneous collection of sealed containers prepared in such a manner that the risk of contamination is the same for each of the units contained therein.

In the case of terminally sterilised products, physical proofs, biologically based and automatically documented, showing correct treatment throughout the batch during sterilisation are of greater assurance than the sterility test. The circumstances in which parametric release may be considered appropriate are described under Methods of preparation of sterile products (5.1.1). The method of media-fill runs may be used to evaluate the process of aseptic production. Apart from that the sterility test is the only analytical method available for products prepared under aseptic conditions and furthermore it is, in all cases, the only analytical method available to the authorities who have to examine a specimen of a product for sterility.

The probability of detecting micro-organisms by the test for sterility increases with their number present in the sample tested and varies according to the readiness of growth of micro-organism present. The probability of detecting very low levels of contamination even when it is homogenous throughout the batch is very low. The interpretation of the results of the test for sterility rests on the assumption that the contents of every container in the batch, had they been tested, would have given the same result. Since it is manifest that every container cannot be tested, an appropriate sampling plan should be adopted. In the case of aseptic production, it is recommended to include samples filled at the beginning and at the end of the batch and after significant intervention.

Guidance on the minimum number of items recommended to be tested in relation to the size of the batch is given in Table 2.6.1-3. The application of the recommendations must have regard to the volume of preparation per container, to the validation of the sterilisation method and to any other special considerations concerning the intended sterility of the product.

Table 2.6.1-3. — *Minimum number of items to be tested*

Number of items in the batch	Minimum number of items to be tested for each medium, unless otherwise justified and authorised*
<i>Parenteral preparations</i>	
– Not more than 100 containers	10 per cent or 4 containers, whichever is the greater
– More than 100 but not more than 500 containers	10 containers
– More than 500 containers	2 per cent or 20 containers, whichever is less
<i>Ophthalmic and other non-injectable preparations</i>	
– Not more than 200 containers	5 per cent or 2 containers, whichever is the greater
– More than 200 containers	10 containers
– If the product is presented in the form of single-dose containers, apply the scheme shown above for preparations for parenteral use	
<i>Catgut and other surgical sutures for veterinary use</i>	2 per cent or 5 packages whichever is the greater, up to a maximum total of 20 packages
<i>Bulk solid products</i>	
– Up to 4 containers	Each container
– More than 4 containers but not more than 50 containers	20 per cent or 4 containers, whichever is the greater
– More than 50 containers	2 per cent or 10 containers, whichever is the greater
<i>Pharmacy bulk packages of antibiotics (greater than 5 g)</i>	6 containers
*If the contents of one container are enough to inoculate the two media, this column gives the number of containers needed for both the media together.	

Observation and interpretation of results. Conventional microbiological/biochemical techniques are generally satisfactory for identification of micro-organisms recovered from a sterility test. However, if a manufacturer wishes to use condition (d) as the sole criterion for invalidating a sterility test, it may be necessary to employ sensitive typing techniques to demonstrate that a micro-organism isolated from the product test is identical to a micro-organism isolated from the test materials and/or the testing environment. While routine microbiological/biochemical identification techniques can demonstrate that 2 isolates are not identical, these methods may not be sufficiently sensitive or reliable enough to provide unequivocal evidence that two isolates are from the same source. More sensitive tests, for example, molecular typing with RNA/DNA homology, may be necessary to determine that micro-organisms are clonally related and have a common origin.

01/2005:20602

2.6.2. MYCOBACTERIA

If the sample to be examined may be contaminated by micro-organisms other than mycobacteria, treat it with a suitable decontamination solution, such as acetylcysteine-sodium hydroxide solution or sodium laurilsulfate solution.

Inoculate 0.2 ml of the sample in triplicate onto each of 2 suitable solid media (Löwenstein-Jensen medium and Middlebrook 7H10 medium are considered suitable). Inoculate 0.5 ml in triplicate into a suitable liquid medium. Incubate all media at 37 °C for 56 days.

Establish the fertility of the media in the presence of the preparation to be examined by inoculation of a suitable strain of a *Mycobacterium* sp. such as BCG and if necessary use a suitable neutralising substance.

If contaminating micro-organisms develop during the first 8 days of incubation, repeat the test and carry out at the same time a bacteriological sterility test.

If at the end of the incubation time no growth of mycobacteria occurs in any of the test media, the preparation complies with the test.

01/2005:20607

2.6.7. MYCOPLASMAS

Where the test for mycoplasmas is prescribed for a master cell bank, for a working cell bank, for a virus seed lot or for control cells, both the culture method and the indicator cell culture method are used. Where the test for mycoplasmas is prescribed for a virus harvest, for a bulk vaccine or for the final lot (batch), the culture method is used. The indicator cell culture method may also be used, where necessary, for screening of media.

CULTURE METHOD

CHOICE OF CULTURE MEDIA

The test is carried out using a sufficient number of both solid and liquid media to ensure growth in the chosen incubation conditions of small numbers of mycoplasmas that may be present in the product to be examined. Liquid media must contain phenol red. The range of media chosen is shown to have satisfactory nutritive properties for at least the organisms shown below. The nutritive properties of each new batch of medium are verified for the appropriate organisms in the list.

Acholeplasma laidlawii (vaccines for human and veterinary use where an antibiotic has been used during production)

Mycoplasma gallisepticum (where avian material has been used during production or where the vaccine is intended for use in poultry)

Mycoplasma hyorhinis (non-avian veterinary vaccines)

Mycoplasma orale (vaccines for human and veterinary use)

Mycoplasma pneumoniae (vaccines for human use) or other suitable species of D-glucose fermenter

Mycoplasma synoviae (where avian material has been used during production or where the vaccine is intended for use in poultry).

The test strains are field isolates having undergone not more than fifteen subcultures and are stored frozen or freeze-dried. After cloning the strains are identified as being of the required species by a suitable method, by comparison with type cultures, for example:

<i>A. laidlawii</i>	NCTC 10116	CIP 75.27	ATCC 23206
<i>M. gallisepticum</i>	NCTC 10115	CIP 104967	ATCC 19610
<i>M. hyorhinis</i>	NCTC 10130	CIP 104968	ATCC 17981
<i>M. orale</i>	NCTC 10112	CIP 104969	ATCC 23714
<i>M. pneumoniae</i>	NCTC 10119	CIP 103766	ATCC 15531
<i>M. synoviae</i>	NCTC 10124	CIP 104970	ATCC 25204

INCUBATION CONDITIONS

Divide inoculated media into two equal parts and incubate one in aerobic conditions and the other in microaerophilic conditions; for solid media maintain an atmosphere of adequate humidity to prevent desiccation of the surface. For aerobic conditions, incubate in an atmosphere of air containing, for solid media, 5 to 10 per cent of carbon dioxide. For microaerophilic conditions, incubate in an atmosphere of nitrogen containing, for solid media, 5 to 10 per cent of carbon dioxide.

NUTRITIVE PROPERTIES

Carry out the test for nutritive properties for each new batch of medium. Inoculate the chosen media with the appropriate test organisms; use not more than 100 CFU (colony-forming units) per 60 mm plate containing 9 ml of solid medium and not more than 40 CFU per 100 ml container of the corresponding liquid medium; use a separate plate and container for each species of organism. Incubate the media in the conditions that will be used for the test of the product to be examined (aerobically, microaerophilically or both, depending on the requirements of the test organism). The media comply with the test for nutritive properties if there is adequate growth of the test organisms accompanied by an appropriate colour change in liquid media.

INHIBITORY SUBSTANCES

Carry out the test for nutritive properties in the presence of the product to be examined. If growth of the test organisms is notably less than that found in the absence of the product to be examined, the latter contains inhibitory substances that must be neutralised (or their effect otherwise countered, for example, by dilution) before the test for mycoplasmas is carried out. The effectiveness of the neutralisation or other process is checked by repeating the test for inhibitory substances after neutralisation.

TEST FOR MYCOPLASMAS IN THE PRODUCT TO BE EXAMINED

For solid media, use plates 60 mm in diameter and containing 9 ml of medium. Inoculate each of not fewer than two plates of each solid medium with 0.2 ml of the product

to be examined and inoculate 10 ml per 100 ml of each liquid medium. Incubate at 35 °C to 38 °C, aerobically and microaerophilically, for 21 days and at the same time incubate an uninoculated 100 ml portion of each liquid medium for use as a control. If any significant pH change occurs on addition of the product to be examined, restore the liquid medium to its original pH value by the addition of a solution of either sodium hydroxide or hydrochloric acid. On the first, second or third day after inoculation subculture each liquid culture by inoculating each of two plates of each solid medium with 0.2 ml and incubating at 35 °C to 38 °C aerobically and microaerophilically for not less than 21 days. Repeat the procedure on the sixth, seventh or eighth day and again on the thirteenth or fourteenth day of the test. Observe the liquid media every 2 or 3 days and if any colour change occurs subculture immediately. Observe solid media once per week.

If the liquid media show bacterial or fungal contamination, repeat the test. If, not earlier than 7 days after inoculation, not more than one plate at each stage of the test is accidentally contaminated with bacteria or fungi, or broken, that plate may be ignored provided that on immediate examination it shows no evidence of mycoplasmal growth. If, at any stage of the test, more than one plate is accidentally contaminated with bacteria or fungi, or broken, the test is invalid and must be repeated.

Include in the test positive controls prepared by inoculating not more than 100 CFU of suitable species such as *M. orale* and *M. pneumoniae*.

At the end of the incubation periods, examine all the inoculated solid media microscopically for the presence of mycoplasmas. The product passes the test if growth of mycoplasmas has not occurred in any of the inoculated media. If growth of mycoplasmas has occurred, the test may be repeated once using twice the amount of inoculum, media and plates; if growth of mycoplasmas does not occur, the product complies with the test. The test is invalid if the positive controls do not show growth of the relevant test organism.

INDICATOR CELL CULTURE METHOD

Cell cultures are stained with a fluorescent dye that binds to DNA. Mycoplasmas are detected by their characteristic particulate or filamentous pattern of fluorescence on the cell surface and, if contamination is heavy, in surrounding areas.

VERIFICATION OF THE SUBSTRATE

Using a Vero cell culture substrate, pretest the procedure using an inoculum of not more than 100 CFU (colony-forming units) of a strain growing readily in liquid or solid medium and demonstrate its ability to detect potential mycoplasma contaminants such as suitable strains of *Mycoplasma hyorhinis* and *Mycoplasma orale*. A different cell substrate may be used, for example the production cell line, if it has been demonstrated that it will provide at least equal sensitivity for the detection of potential mycoplasma contaminants.

Test method

Take not less than 1 ml of the product to be examined and use it to inoculate in duplicate, as described under Procedure, indicator cell cultures representing not less than 25 cm² of cell culture area at confluence.

Include in the test a negative (non-infected) control and two positive mycoplasma controls, such as *M. hyorhinis* and *M. orale*. Use an inoculum of not more than 100 CFU for the positive controls.

If for viral suspensions the interpretation of results is affected by marked cytopathic effects, the virus may be neutralised using a specific antiserum that has no inhibitory effects on mycoplasmas or a cell culture substrate that does not allow growth of the virus may be used. To demonstrate the absence of inhibitory effects of serum, carry out the positive control tests in the presence and absence of the antiserum.

Procedure

1. Seed culture at a regular density (2×10^4 to 2×10^5 cells/ml, 4×10^3 to 2.5×10^4 cells/cm²) and incubate at 36 ± 1 °C for at least 2 days. Inoculate the product to be examined and incubate for at least 2 days; make not fewer than one subculture. Grow the last subculture on coverslips in suitable containers or on some other surface suitable for the test procedure. Do not allow the last subculture to reach confluence since this would inhibit staining and impair visualisation of mycoplasmas.

2. Remove and discard the medium.

3. Rinse the monolayer with *phosphate buffered saline pH 7.4 R*, then with a mixture of equal volumes of *phosphate buffered saline pH 7.4 R* and a suitable fixing solution and finally with the fixing solution; when *bisbenzimidazole R* is used for staining, a freshly prepared mixture of 1 volume of *glacial acetic acid R* and 3 volumes of *methanol R* is a suitable fixing solution.

4. Add the fixing solution and allow to stand for 10 min.

5. Remove the fixing solution and discard.

6. If the monolayer is to be stained later, dry it completely. (Particular care is needed for staining of the slides after drying because of artefacts that may be produced.)

7. If the monolayer is to be stained directly, wash off the fixing solution twice with sterile water and discard the wash.

8. Add *bisbenzimidazole working solution R* or some other suitable DNA staining agent and allow to stand for 10 min.

9. Remove the stain and rinse the monolayer with water.

10. Mount each coverslip, where applicable, with a drop of a mixture of equal volumes of *glycerol R* and *phosphate-citrate buffer solution pH 5.5 R*; blot off surplus mountant from the edge of the coverslip.

11. Examine by epifluorescence (330 nm/380 nm excitation filter, LP 440 nm barrier filter) at 100-400 × magnification or greater.

12. Compare the microscopic appearance of the test cultures with that of the negative and positive controls, examining for extranuclear fluorescence. Mycoplasmas give pinpoints or filaments over the cytoplasm and sometimes in intercellular spaces.

The product to be examined complies with the test if there is no evidence of the presence of mycoplasmas in the test cultures inoculated with it. The test is invalid if the positive controls do not show the presence of the appropriate test organisms.

The following section is published for information.

RECOMMENDED MEDIA FOR THE CULTURE METHOD

The following media are recommended. Other media may be used providing their ability to sustain the growth of mycoplasmas has been demonstrated on each batch in the presence and absence of the product to be examined.

RECOMMENDED MEDIA FOR THE DETECTION OF MYCOPLASMA GALLISEPTICUM

Liquid medium

Beef heart infusion broth (1)	90.0 ml
Horse serum (unheated)	20.0 ml

Yeast extract (250 g/l)	10.0 ml	Solid medium	
Thallium acetate (10 g/l solution)	1.0 ml	Hanks' balanced salt solution (modified) (4)	200 ml
Phenol red (0.6 g/l solution)	5.0 ml	DEAE-dextran	200 mg
Penicillin (20 000 IU/ml)	0.25 ml	Ionagar (3)	15.65 mg
Deoxyribonucleic acid (2 g/l solution)	1.2 ml	Mix well and sterilise by autoclaving. Cool to 100 °C. Add to 1740 ml of liquid medium as described above.	
Adjust to pH 7.8.		(1) Beef heart infusion broth	
Solid medium		Beef heart (for preparation of the infusion)	500 g
Prepare as described above replacing beef heart infusion broth by beef heart infusion agar containing 15 g/l of agar.		Peptone	10 g
RECOMMENDED MEDIA FOR THE DETECTION OF MYCOPLASMA SYNOVIAE		Sodium chloride	5 g
Liquid medium		Distilled water to	1000 ml
Beef heart infusion broth (1)	90.0 ml	Sterilise by autoclaving.	
Essential vitamins (2)	0.025 ml	(2) Essential vitamins	
Glucose monohydrate (500 g/l solution)	2.0 ml	Biotin	100 mg
Swine serum (inactivated at 56 °C for 30 min)	12.0 ml	Calcium pantothenate	100 mg
β-Nicotinamide adenine dinucleotide (10 g/l solution)	1.0 ml	Choline chloride	100 mg
Cysteine hydrochloride (10 g/l solution)	1.0 ml	Folic acid	100 mg
Phenol red (0.6 g/l solution)	5.0 ml	l-Inositol	200 mg
Penicillin (20 000 IU/ml)	0.25 ml	Nicotinamide	100 mg
Mix the solutions of β-nicotinamide adenine dinucleotide and cysteine hydrochloride and after 10 min add to the other ingredients. Adjust to pH 7.8.		Pyridoxal hydrochloride	100 mg
Solid medium		Riboflavin	10 mg
Beef heart infusion broth (1)	90.0 ml	Thiamine hydrochloride	100 mg
Ionagar (3)	1.4 g	Distilled water to	1000 ml
Adjust to pH 7.8, sterilise by autoclaving then add:		(3) Ionagar	
Essential vitamins (2)	0.025 ml	A highly refined agar for use in microbiology and immunology prepared by an ion-exchange procedure which results in a product having superior purity, clarity and gel strength.	
Glucose monohydrate (500 g/l solution)	2.0 ml	It contains about:	
Swine serum (unheated)	12.0 ml	Water	12.2 per cent
β-Nicotinamide adenine dinucleotide (10 g/l solution)	1.0 ml	Ash	1.5 per cent
Cysteine hydrochloride (10 g/l solution)	1.0 ml	Acid-insoluble ash	0.2 per cent
Phenol red (0.6 g/l solution)	5.0 ml	Chlorine	0
Penicillin (20 000 IU/ml)	0.25 ml	Phosphate (calculated as P ₂ O ₅)	0.3 per cent
RECOMMENDED MEDIA FOR THE DETECTION OF NON-AVIAN MYCOPLASMAS		Total nitrogen	0.3 per cent
Liquid medium		Copper	8 ppm
Hanks' balanced salt solution (modified) (4)	800 ml	Iron	170 ppm
Distilled water	67 ml	Calcium	0.28 per cent
Brain heart infusion (5)	135 ml	Magnesium	0.32 per cent
PPLO Broth (6)	248 ml	(4) Hanks' balanced salt solution (modified)	
Yeast extract (170 g/l)	60 ml	Sodium chloride	6.4 g
Bacitracin	250 mg	Potassium chloride	0.32 g
Meticillin	250 mg	Magnesium sulphate heptahydrate	0.08 g
Phenol red (5 g/l)	4.5 ml	Magnesium chloride hexahydrate	0.08 g
Thallium acetate (56 g/l)	3 ml	Calcium chloride, anhydrous	0.112 g
Horse serum	165 ml	Disodium hydrogen phosphate dihydrate	0.0596 g
Swine serum	165 ml	Potassium dihydrogen phosphate, anhydrous	0.048 g
Adjust to pH 7.4 - 7.45.		Distilled water to	800 ml

(5) Brain heart infusion

Calf-brain infusion	200 g
Beef-heart infusion	250 g
Proteose peptone	10 g
Glucose monohydrate	2 g
Sodium chloride	5 g
Disodium hydrogen phosphate, anhydrous	2.5 g
Distilled water to	1000 ml

(6) PPLO broth

Beef-heart infusion	50 g
Peptone	10 g
Sodium chloride	5 g
Distilled water to	1000 ml

01/2005:20608

2.6.8. PYROGENS

The test consists of measuring the rise in body temperature evoked in rabbits by the intravenous injection of a sterile solution of the substance to be examined.

Selection of animals. Use healthy, adult rabbits of either sex weighing not less than 1.5 kg, fed a complete and balanced diet not containing antibiotics, and not showing loss of body mass during the week preceding the test. A rabbit is not be used in a pyrogen test:

- if it has been used in a negative pyrogen test in the preceding 3 days, or
- if it has been used in the preceding 3 weeks in a pyrogen test in which the substance under examination failed to pass the test.

Animals' quarters. Keep the rabbits individually in a quiet area with a uniform appropriate temperature. Withhold food from the rabbits overnight and until the test is completed; withhold water during the test. Carry out the test in a quiet room where there is no risk of disturbance exciting the animals and in which the room temperature is within 3 °C of that of the rabbits' living quarters, or in which the rabbits have been kept for at least 18 h before the test.

Materials. Glassware, syringes and needles. Thoroughly wash all glassware, syringes and needles with water for injections and heat in a hot-air oven at 250 °C for 30 min or at 200 °C for 1 h.

Retaining boxes. The retaining boxes for rabbits whose temperature is being measured by an electrical device are made in such a way that the animals are retained only by loosely fitting neck-stocks; the rest of the body remains relatively free so that the rabbits may sit in a normal position. They are not restrained by straps or other similar methods which may harm the animal. The animals are put into the boxes not less than 1 h before the first record of the temperature and remain in them throughout the test.

Thermometers. Use a thermometer or electrical device which indicates the temperature with a precision of 0.1 °C and insert into the rectum of the rabbit to a depth of about 5 cm. The depth of insertion is constant for any one rabbit in any one test. When an electrical device is used it may be left in position throughout the test.

Preliminary test. After selection of the animals, one to three days before testing the product to be examined, treat those animals that have not been used during the previous 2 weeks by intravenous injection of 10 ml per kilogram of body mass of a pyrogen-free 9 g/l solution of *sodium chloride R* warmed to about 38.5 °C. Record the temperatures of the animals, beginning at least 90 min before injection and continuing for 3 h after the injection of the solution. Any animal showing a temperature variation greater than 0.6 °C is not used in the main test.

Main test. Carry out the test using a group of three rabbits. Preparation and injection of the product. Warm the liquid to be examined to approximately 38.5 °C before the injection. The product to be examined may be dissolved in, or diluted with, a pyrogen-free 9 g/l solution of *sodium chloride R* or another prescribed liquid. Inject the solution slowly into the marginal vein of the ear of each rabbit over a period not exceeding 4 min, unless otherwise prescribed in the monograph. The amount of the product to be injected varies according to the product to be examined and is prescribed in the monograph. The volume injected is not less than 0.5 ml per kilogram and not more than 10 ml per kilogram of body mass.

Determination of the initial and maximum temperatures. The "initial temperature" of each rabbit is the mean of two temperature readings recorded for that rabbit at an interval of 30 min in the 40 min immediately preceding the injection of the product to be examined. The "maximum temperature" of each rabbit is the highest temperature recorded for that rabbit in the 3 h after the injection. Record the temperature of each rabbit at intervals of not more than 30 min, beginning at least 90 min before the injection of the product to be examined and continuing 3 h after the injection. The difference between the maximum temperature and the initial temperature of each rabbit is taken to be its response. When this difference is negative, the result is counted as a zero response.

Rabbits showing a temperature variation greater than 0.2 °C between two successive readings in the determination of the initial temperature are withdrawn from the test. In any one test, only rabbits having initial temperatures which do not differ from one another by more than 1 °C are used. All rabbits having an initial temperature higher than 39.8 °C or less than 38.0 °C are withdrawn from the test.

Interpretation of results. Having carried out the test first on a group of three rabbits, repeat if necessary on further groups of three rabbits to a total of four groups, depending on the results obtained. If the summed response of the first group does not exceed the figure given in the second column of the Table 2.6.8-1, the substance passes the test. If the summed response exceeds the figure given in the second column of the table but does not exceed the figure given in the third column of the table, repeat the test as indicated above. If the summed response exceeds the figure given in the third column of the table, the product fails the test.

Table 2.6.8-1

Number of rabbits	Product passes if summed response does not exceed	Product fails if summed response exceeds
3	1.15 °C	2.65 °C
6	2.80 °C	4.30 °C
9	4.45 °C	5.95 °C
12	6.60 °C	6.60 °C

Rabbits used in a test for pyrogens where the mean rise in the rabbits' temperature has exceeded 1.2 °C are permanently excluded.

01/2005:20609

2.6.9. ABNORMAL TOXICITY

GENERAL TEST

Inject intravenously into each of 5 healthy mice, weighing 17 g to 22 g, the quantity of the substance to be examined prescribed in the monograph, dissolved in 0.5 ml of *water for injections R* or of a 9 g/l sterile solution of *sodium chloride R*. Inject the solution over a period of 15 s to 30 s, unless otherwise prescribed.

The substance passes the test if none of the mice die within 24 h or within such time as is specified in the individual monograph. If more than one animal dies the preparation fails the test. If one of the animals dies, repeat the test. The substance passes the test if none of the animals in the second group die within the time interval specified.

IMMUNOSERA AND VACCINES FOR HUMAN USE

Unless otherwise prescribed, inject intraperitoneally 1 human dose but not more than 1.0 ml into each of 5 healthy mice, weighing 17 g to 22 g. The human dose is that stated on the label of the preparation to be examined or on the accompanying leaflet. Observe the animals for 7 days.

The preparation passes the test if none of the animals shows signs of ill health. If more than one animal dies, the preparation fails the test. If one of the animals dies or shows signs of ill health, repeat the test. The preparation passes the test if none of the animals in the second group die or shows signs of ill health in the time interval specified.

The test must also be carried out on 2 healthy guinea-pigs weighing 250 g to 350 g. Inject intraperitoneally into each animal 1 human dose but not more than 5.0 ml. The human dose is that stated on the label of the preparation to be examined or on the accompanying leaflet. Observe the animals for 7 days.

The preparation passes the test if none of the animals shows signs of ill health. If more than one animal dies the preparation fails the test. If one of the animals dies or shows signs of ill health, repeat the test. The preparation passes the test if none of the animals in the second group die or shows signs of ill health in the time interval specified.

01/2005:20610

2.6.10. HISTAMINE

Kill a guinea-pig weighing 250 g to 350 g that has been deprived of food for the preceding 24 h. Remove a portion of the distal small intestine 2 cm in length and empty the isolated part by rinsing carefully with solution B described below using a syringe. Attach a fine thread to each end and make a small transverse incision in the middle of the piece of intestine. Place it in an organ bath with a capacity of 10 ml to 20 ml, containing solution B maintained at a constant temperature (34 °C to 36 °C) and pass through the solution a current of a mixture of 95 parts of oxygen and 5 parts of carbon dioxide. Attach one of the threads near to the bottom of the organ bath. Attach the other thread to an isotonic myograph and record the contractions of the organ on a kymograph or other suitable means of giving a permanent record. If a lever is used, its length is such that the movements of the organ are amplified about 20 times. The tension on the intestine should be about 9.8 mN (1 g) and it should be adjusted to the sensitivity of the organ. Flush out the organ bath with solution B. Allow it to stand for 10 min. Flush 2 or 3 times more with

solution B. Stimulate a series of contractions by the addition of measured volumes between 0.2 ml and 0.5 ml of a solution of *histamine dihydrochloride R* having a strength which produces reproducible submaximal responses. This dose is termed the "high dose". Flush the organ bath (preferably by overflow without emptying the bath) 3 times with solution B before each addition of histamine. The successive additions should be made at regular intervals allowing a complete relaxation between additions (about 2 min). Add equal volumes of a weaker dilution of *histamine dihydrochloride R* which produces reproducible responses approximately half as great as the "high dose". This dose is termed the "low dose". Continue the regular additions of "high" and "low" doses of histamine solution as indicated above, and alternate each addition with an equal volume of a dilution of the solution to be examined, adjusting the dilution so that the contraction of the intestine, if any, is smaller than that due to the "high dose" of histamine. Determine whether the contraction, if any, is reproducible and that the responses to the "high" and "low" doses of histamine are unchanged. Calculate the activity of the substance to be examined in terms of its equivalent in micrograms of histamine base from the dilution determined as above.

The quantity so determined does not exceed the quantity prescribed in the monograph.

If the solution to be examined does not produce a contraction, prepare a fresh solution adding a quantity of histamine corresponding to the maximum tolerated in the monograph and note whether the contractions produced by the preparation with the added histamine correspond to the amount of histamine added. If this is not the case, or if the contractions caused by the substance to be examined are not reproducible or if subsequent responses to "high" and "low" doses of histamine are diminished, the results of the tests are invalid and the test for depressor substances (2.6.11) must be carried out.

Solution A

Sodium chloride	160.0 g
Potassium chloride	4.0 g
Calcium chloride, anhydrous	2.0 g
Magnesium chloride, anhydrous	1.0 g
Disodium hydrogen phosphate dodecahydrate	0.10 g
<i>Water for injections R</i> sufficient to produce	1000 ml

Solution B

Solution A	50.0 ml
Atropine sulphate	0.5 mg
Sodium hydrogen carbonate	1.0 g
Glucose monohydrate	0.5 g
<i>Water for injections R</i> sufficient to produce	1000 ml

Solution B should be freshly prepared and used within 24 h.

01/2005:20611

2.6.11. DEPRESSOR SUBSTANCES

Carry out the test on a cat weighing not less than 2 kg and anaesthetised with chloralose or with a barbiturate that allows the maintenance of uniform blood pressure. Protect the animal from loss of body heat and maintain it so that the rectal temperature remains within physiological limits. Introduce a cannula into the trachea. Insert a cannula filled with a heparinised 9 g/l solution of sodium chloride into the common carotid artery and connect it to a device capable of

giving a continuous record of the blood pressure. Insert into the femoral vein another cannula, filled with a heparinised 9 g/l solution of sodium chloride, through which can be injected the solutions of histamine and of the substance to be examined. Determine the sensitivity of the animal to histamine by injecting intravenously at regular intervals, doses of *histamine solution R* corresponding to 0.1 µg and 0.15 µg of histamine base per kilogram of body mass. Repeat the lower dose at least 3 times. Administer the second and subsequent injections not less than 1 min after the blood pressure has returned to the level it was at immediately before the previous injection. The animal is used for the test only if a readily discernible decrease in blood pressure that is constant for the lower dose is obtained and if the higher dose causes greater responses. Dissolve the substance to be examined in sufficient of a 9 g/l solution of sodium chloride or other prescribed solvent, to give the prescribed concentration. Inject intravenously per kilogram of body mass 1.0 ml of *histamine solution R*, followed by 2 successive injections of the prescribed amount of the solution to be examined and, finally, 1.0 ml of *histamine solution R*. The second, third and fourth injections are given not less than 1 min after the blood pressure has returned to the level it was at immediately before the preceding injection. Repeat this series of injections twice and conclude the test by giving 1.5 ml of *histamine solution R* per kilogram of body mass.

If the response to 1.5 ml of *histamine solution R* per kilogram of body mass is not greater than that to 1.0 ml the test is invalid. The substance to be examined fails the test if the mean of the series of responses to the substance is greater than the mean of the responses to 1.0 ml of *histamine solution R* per kilogram of body mass or if any one dose of the substance causes a greater depressor response than the concluding dose of the histamine solution. The test animal must not be used in another test for depressor substances if the second criterion applies or if the response to the high dose of histamine given after the administration of the substance to be examined is less than the mean response to the low doses of histamine previously injected.

01/2005:20612

2.6.12. MICROBIOLOGICAL EXAMINATION OF NON-STERILE PRODUCTS (TOTAL VIABLE AEROBIC COUNT)

The tests described hereafter will allow quantitative enumeration of mesophilic bacteria and fungi which may grow under aerobic conditions.

The tests are designed primarily to determine whether or not a substance that is the subject of a monograph in the Pharmacopoeia complies with the microbiological requirements specified in the monograph in question. When used for such purposes follow the instructions given below, including the number of samples to be taken and interpret the results as stated below. The tests may also be used for the test for *Efficacy of antimicrobial preservation* (5.1.3) as described in the Pharmacopoeia. They may furthermore be used for monitoring raw material quality and may be used in association with guidelines on *Microbiological quality of pharmaceutical preparations* (5.1.4). When used for such purposes, for example by a manufacturer for raw materials and/or finished product monitoring or for process validation, the conduct of the tests including the number of samples to be taken and the interpretation of the results are matters for agreement between the manufacturer and the competent authority.

Carry out the determination under conditions designed to avoid accidental contamination of the product to be examined. The precautions taken to avoid contamination must be such that they do not affect any micro-organisms which are revealed in the test. If the product to be examined has antimicrobial activity this must be adequately neutralised. If inactivators are used for this purpose their efficacy and non-toxicity versus micro-organisms are demonstrated.

Determine the total viable aerobic count by the membrane filtration method, or the plate-count method as prescribed in the monograph.

The Most Probable Number (MPN) method is reserved for bacterial counts when no other method is available. The choice of a method may be based on factors such as the nature of the product and the expected number of micro-organisms. Any method which is chosen must be properly validated.

When used in conjunction with chapter 5.1.3 or 5.1.4, the pour-plate method, the surface-spread method and the membrane filtration method may be used.

PREPARATION OF THE SAMPLE

Sampling plan. Sampling of the product must follow a well-defined sampling plan. The sampling plan will be dependent on factors such as batch size, health hazard associated with unacceptably highly contaminated products, the characteristics of the product and the expected level of contamination. Unless otherwise prescribed, use sample(s) of 10 g or 10 ml of the substance or preparation to be examined taken with the precautions referred to above. Select the sample(s) at random from the bulk material or from the available containers of the preparation. If necessary, to obtain the required quantity, mix the contents of a sufficient number of containers to provide each sample, depending on the nature of the substance or preparation to be examined.

An example of a sampling plan applicable to products where homogeneity with respect to the distribution of micro-organisms may be a problem, is the three-class sampling plan. In this case five samples from each batch are drawn and investigated separately. The three recognised classes are:

- (i) acceptable samples, i.e. samples containing less than m CFU (colony-forming units) per gram or millilitre, where m is the limit specified in the relevant monograph;
- (ii) marginal samples, i.e. with more than m CFU, but less than $10m$ CFU per gram or millilitre;
- (iii) defective samples, i.e. containing more than $10m$ CFU per gram or millilitre.

Water-soluble products. Dissolve or dilute 10 g or 10 ml of the product to be examined in buffered sodium chloride-peptone solution pH 7.0 or in another suitable liquid. In general a one in ten dilution is prepared. However, the characteristics of the product, or the required sensitivity may necessitate the use of other ratios. If the product is known to have antimicrobial activity, an inactivating agent may be added to the diluent. If necessary adjust the pH to about pH 7 and prepare further serial tenfold dilutions using the same diluent.

Non-fatty products insoluble in water. Suspend 10 g or 10 ml of the product to be examined in buffered sodium chloride-peptone solution pH 7.0 or in another suitable liquid. In general a one in ten suspension is prepared, but the characteristics of some products may necessitate the use of larger volumes. A suitable surface-active agent such as 1 g/l of polysorbate 80 may be added to assist the suspension of poorly wettable substances. If the product is known to have antimicrobial activity, an inactivating agent

may be added to the diluent. If necessary adjust the pH to about pH 7 and prepare further serial tenfold dilutions using the same diluent.

Fatty products. Homogenise 10 g or 10 ml of the product to be examined with not more than half its weight of sterile polysorbate 80 or another suitable sterile surface-active agent, heated if necessary to not more than 40 °C, in exceptional cases to not more than 45 °C. Mix carefully and if necessary maintain the temperature in a water-bath or in an incubator. Add sufficient pre-warmed buffered sodium chloride-peptone solution pH 7.0 to make a one in ten dilution of the original product. Mix carefully whilst maintaining the temperature for the shortest time necessary for the formation of an emulsion and in any case for not more than 30 min. Further serial tenfold dilutions may be prepared using buffered sodium chloride-peptone solution pH 7.0 containing a suitable concentration of sterile polysorbate 80 or another sterile surface-active agent.

Transdermal patches. Remove the protective cover sheets ("release liner") of ten patches of the transdermal preparation by using sterile forceps and place them, the adhesive side upwards, on sterile glass or plastic trays. Cover the adhesive surface with sterile gauze (or woven-filter type monofilament polymer grid), if necessary, and transfer the ten patches to a minimum volume of 500 ml of buffered sodium chloride-peptone solution pH 7.0 containing suitable inactivators such as polysorbate 80 and/or lecithin. Shake vigorously the preparation for at least 30 min (preparation A). Prepare another ten patches in the same way, place them in a minimum volume of 500 ml of broth medium D and shake vigorously for at least 30 min (preparation B).

EXAMINATION OF THE SAMPLE

Membrane filtration. Use membrane filters having a nominal pore size not greater than 0.45 µm and whose effectiveness to retain bacteria has been established. The type of filter material is chosen in such a way that the bacteria retaining efficiency is not affected by the components of the sample to be investigated. Cellulose nitrate filters, for example, may be used for aqueous, oily and weakly alcoholic solutions and cellulose acetate filters, for example, for strongly alcoholic solutions. The filtration apparatus is designed to allow the transfer of the filter to the culture medium.

Transfer a suitable amount of the sample prepared as described in the section Preparation of the sample (preferably representing 1 g of the product, or less if large numbers of colony-forming units are expected) to each of two membrane filters and filter immediately. Wash each filter with three quantities, each of about 100 ml of a suitable liquid such as buffered sodium chloride-peptone solution pH 7.0. To this solution, surface-active agents such as polysorbate 80, or inactivators of antimicrobial agents may be added. If validated, less than three washes may be applied. Transfer one of the membrane filters, intended primarily for the enumeration of bacteria, to the surface of a suitable agar medium, such as medium B and the other, intended primarily for the enumeration of fungi, to the surface of a suitable agar medium, such as medium C. Incubate the plate of agar medium B at 30 °C to 35 °C, and the plate of agar medium C at 20 °C to 25 °C for five days, unless a reliable count is obtained in a shorter time. Select plates with the highest number less than 100 colonies and calculate the number of colony-forming units per gram or millilitre of product.

When examining transdermal patches, filter 50 ml of preparation A separately through each of two sterile filter membranes. Place one membrane to agar medium B for total aerobic microbial count, the other membrane to agar medium C for the count of fungi.

PLATE-COUNT METHODS

a. Pour-plate method. Using Petri dishes 9 cm in diameter, add to each dish 1 ml of the sample prepared as described in the section Preparation of the sample and 15 ml to 20 ml of a liquefied agar medium suitable for the cultivation of bacteria (such as medium B), or 15 ml to 20 ml of a liquefied agar medium suitable for the cultivation of fungi (such as medium C) at not more than 45 °C. If larger Petri dishes are used the amount of agar is increased accordingly. Prepare for each medium at least two Petri dishes for each level of dilution. Incubate the plates at 30 °C to 35 °C (20 °C to 25 °C for fungi) for five days, unless a reliable count is obtained in a shorter time. Select the plates corresponding to one dilution and showing the highest number of colonies less than 300 (100 colonies for fungi). Take the arithmetic average of the counts and calculate the number of colony-forming units per gram or millilitre.

b. Surface-spread method. Using Petri dishes 9 cm in diameter, add 15 ml to 20 ml of a liquefied agar medium suitable for the cultivation of bacteria (such as medium B) or a liquefied agar medium suitable for the cultivation of fungi (such as medium C) at about 45 °C to each Petri dish and allow to solidify. If larger Petri dishes are used, the volume of the agar is increased accordingly. Dry the plates, for example in a LAF bench or in an incubator. Spread a measured volume of not less than 0.1 ml of the sample prepared as described in the section Preparation of the sample over the surface of the medium. Use at least two Petri dishes for each medium and each level of dilution. For incubation and calculation of the number of colony-forming units proceed as described for the pour-plate method.

MOST-PROBABLE-NUMBER METHOD

The precision and accuracy of the most-probable-number method (MPN) is less than that of the membrane filtration method or the plate-count methods. Unreliable results are obtained particularly for the enumeration of moulds. For these reasons the MPN method is reserved for the enumeration of bacteria in situations where no other method is available. If the use of the method is justified, proceed as follows.

Prepare a series of at least three subsequent tenfold dilutions of the product as described in the section Preparation of the sample. From each level of dilution three aliquots of 1 g or 1 ml are used to inoculate three tubes with 9 ml to 10 ml of a suitable liquid medium (such as broth medium A). If necessary a surface-active agent such as polysorbate 80, or an inactivator of antimicrobial agents may be added to the medium. Thus, if three levels of dilution are prepared nine tubes are inoculated. Incubate all tubes for five days at 30 °C to 35 °C. Record for each level of dilution the number of tubes showing microbial growth. If the reading of the results is difficult or uncertain owing to the nature of the product to be examined, subculture in the same broth, or on a suitable agar medium (such as agar medium B), for 18 h to 24 h at the same temperature and use these results. Determine the most probable number of bacteria per gram or millilitre of the product to be examined from Table 2.6.12-1.

Table 2.6.12-1. – *Most-probable-number values of bacteria*

Three tubes at each level of dilution							
Number of positive tubes			MPN per gram	Category*		95 per cent confidence limits	
0.1 g	0.01 g	0.001 g		1	2		
0	0	0	< 3			–	–
0	1	0	3		x	< 1	17
1	0	0	3	x		1	21
1	0	1	7		x	2	27
1	1	0	7	x		2	28
1	2	0	11		x	4	35
2	0	0	9	x		2	38
2	0	1	14		x	5	48
2	1	0	15	x		5	50
2	1	1	20		x	8	61
2	2	0	21	x		8	63
3	0	0	23	x		7	129
3	0	1	38	x		10	180
3	1	0	43	x		20	210
3	1	1	75	x		20	280
3	2	0	93	x		30	390
3	2	1	150	x		50	510
3	2	2	210		x	80	640
3	3	0	240	x		100	1400
3	3	1	460	x		200	2400
3	3	2	1100	x		300	4800
3	3	3	> 1100			–	–
Category 1: Normal results, obtained in 95 per cent of the cases.							
Category 2: Less likely results, obtained in only 4 per cent of cases. These are not to be used for important decisions. Results that are even less likely than those of category 2 are not mentioned and are always unacceptable.							

EFFECTIVENESS OF CULTURE MEDIA AND VALIDITY OF THE COUNTING METHOD

Grow the bacterial test strains separately in containers containing a suitable liquid medium (such as broth medium A) at 30 °C to 35 °C for 18 h to 24 h. Grow the fungal test strains separately on a suitable agar medium (such as medium C without antibiotics) at 20 °C to 25 °C for 48 h for *Candida albicans* and at 20 °C to 25 °C for 7 days for *Aspergillus niger*.

<i>Staphylococcus aureus</i>	such as ATCC 6538 (NCIMB 9518, CIP 4.83)
<i>Escherichia coli</i>	such as ATCC 8739 (NCIMB 8545, CIP 53.126)
<i>Bacillus subtilis</i>	such as ATCC 6633 (NCIMB 8054, CIP 52.62)
<i>Candida albicans</i>	such as ATCC 10231 (NCPF 3179, IP 48.72)
<i>Aspergillus niger</i>	such as ATCC 16404 (IMI 149007, IP 1431.83)

Use buffered sodium chloride-peptone solution pH 7.0 to make reference suspensions containing about 100 colony-forming units per millilitre. Use the suspension of each of the micro-organisms separately as a control of the counting methods, in the presence and absence of the product to be examined. When testing the membrane filtration method or the plate-count method, a count of any of the test organisms differing by not more than a factor of

five from the calculated value from the inoculum is to be obtained. When testing the most-probable-number method the calculated value from the inoculum is to be within the 95 per cent confidence limits of the results obtained. To test the sterility of the medium and of the diluent and the aseptic performance of the test, carry out the method using sterile sodium chloride-peptone solution pH 7.0 as the test preparation. There must be no growth of micro-organisms.

INTERPRETATION OF THE RESULTS

The bacterial count will be considered to be equal to the average number of colony-forming units found on agar medium B. The fungal count will be considered to be equal to the average number of colony-forming units on agar medium C. The total viable aerobic count is the sum of the bacterial count and the fungal count as described above. If there is evidence that the same types of micro-organisms grow on both media this may be corrected. If the count is carried out by the most-probable-number method the calculated value is the bacterial count.

When a limit is prescribed in a monograph it is interpreted as follows:

10² micro-organisms: maximum acceptable limit: 5 × 10²,
10³ micro-organisms: maximum acceptable limit: 5 × 10³,
and so forth.

If a sampling plan such as the three-class sampling plan for example, is used, proceed as follows:

Calculate the total viable aerobic count separately for each of the five samples. The substance or preparation passes the test if the following conditions are fulfilled:

- none of the individual total viable aerobic counts exceeds the prescribed limit by a factor of ten or more (i.e. no “unacceptable samples”),
- and not more than two of the individual total viable aerobic counts are between the prescribed limit and ten times this limit (i.e. no more than two “marginal samples”).

The solutions and culture mediums recommended are described in the general chapter 2.6.13.

01/2005:20613

2.6.13. MICROBIOLOGICAL EXAMINATION OF NON-STERILE PRODUCTS (TEST FOR SPECIFIED MICRO-ORGANISMS)

In this general method the use of certain selective media is proposed. A feature common to all selective media is that sub-lethally injured organisms are not detected. As sub-lethally injured organisms are relevant for the quality of the product a resuscitation must be included in examination procedures that rely on selective media.

If the product to be examined has antimicrobial activity this must be adequately neutralised.

Enterobacteria and certain other gram-negative bacteria

Although the test has been designed to detect bacteria belonging to the family of Enterobacteriaceae, it is recognised that other types of organisms (e.g. *Aeromonas*, *Pseudomonas*) may be recovered.

Detection of bacteria. Prepare the product to be examined as described in the general method 2.6.12, but using broth medium D in place of buffered sodium chloride-peptone solution pH 7.0, homogenise and incubate at 35–37 °C for a time sufficient to revive the bacteria but not sufficient to encourage multiplication of the organisms (usually 2 h but not more than 5 h). Shake the container, transfer the

quantity of the contents (homogenate A) corresponding to 1 g or 1 ml of the product to 100 ml of enrichment medium E and incubate at 35-37 °C for 18-48 h. Subculture on plates of agar medium F. Incubate at 35-37 °C for 18-24 h. The product passes the test if there is no growth of colonies of gram-negative bacteria on any plate.

Quantitative evaluation. Inoculate suitable quantities of enrichment broth medium E with homogenate A and/or dilutions of it containing respectively 0.1 g, 0.01 g and 0.001 g (or 0.1 ml, 0.01 ml and 0.001 ml) of the product to be examined. Incubate at 35-37 °C for 24-48 h. Subculture each of the cultures on a plate of agar medium F to obtain selective isolation. Incubate at 35-37 °C for 18-24 h. Growth of well-developed colonies, generally red or reddish, of gram-negative bacteria constitutes a positive result. Note the smallest quantity of the product which gives a positive result and the largest quantity that gives a negative result. Determine from Table 2.6.13-1 the probable number of bacteria.

Table 2.6.13-1

Results for each quantity of product			Probable number of bacteria per gram of product
0.1 g or 0.1 ml	0.01 g or 0.01 ml	0.001 g or 0.001 ml	
+	+	+	More than 10 ³
+	+	–	Less than 10 ³ and more than 10 ²
+	–	–	Less than 10 ² and more than 10
–	–	–	Less than 10

When testing transdermal patches, filter 50 ml of preparation B as described in the general method 2.6.12 through a sterile filter membrane, place the membrane in 100 ml of enrichment broth medium E and incubate at 35-37 °C for 18-24 h. After incubation, spread on agar medium F for the detection of Enterobacteria and other gram-negative micro-organisms.

Escherichia coli

Prepare the product to be examined as described in the general method 2.6.12 and use 10 ml or the quantity corresponding to 1 g or 1 ml to inoculate 100 ml of broth medium A, homogenise and incubate at 35-37 °C for 18-48 h. Shake the container, transfer 1 ml to 100 ml of broth medium G and incubate at 43-45 °C for 18-24 h. Subculture on plates of agar medium H at 35-37 °C for 18-72 h. Growth of red, non-mucoid colonies of gram-negative rods indicates the possible presence of *E. coli*. This is confirmed by suitable biochemical tests, such as indole production. The product passes the test if such colonies are not seen or if the confirmatory biochemical tests are negative.

Salmonella

Prepare the product to be examined as described in the general method 2.6.12, but using broth medium A in place of buffered sodium chloride-peptone solution pH 7.0, homogenise and incubate at 35-37 °C for 18-24 h. Transfer 1 ml of the enrichment culture to 10 ml of broth medium I and incubate at 41-43 °C for 18-24 h. Subculture on at least 2 different agar media chosen from agar medium J, agar medium K and agar medium L. Incubate at 35-37 °C for 18-72 h. The probable presence of salmonellae is indicated by the growth of cultures having the following appearance:

- agar medium J: well-developed, colourless colonies,
- agar medium K: well-developed, red colonies, with or without black centres,
- agar medium L: small, transparent, colourless or pink or opaque-white colonies, often surrounded by a pink or red zone.

Transfer separately a few of the suspect colonies to agar medium M in tubes, using surface and deep inoculation. The presence of salmonellae is provisionally confirmed if in the deep inoculation but not in the surface culture there is a change of colour from red to yellow and usually a formation of gas, with or without production of hydrogen sulphide in the agar. Precise confirmation may be carried out by appropriate biochemical and serological tests. The product passes the test if colonies of the type described do not appear or if the confirmatory biochemical and serological tests are negative.

Pseudomonas aeruginosa

Prepare the product to be examined as described in the general method 2.6.12 and use 10 ml or the quantity corresponding to 1 g or 1 ml to inoculate 100 ml of broth medium A, homogenise and incubate at 35-37 °C for 18-48 h. Subculture on a plate of agar medium N and incubate at 35-37 °C for 18-72 h. If no growth of micro-organisms is detected, the product passes the test. If growth of gram-negative rods occurs, transfer some material of morphologically different, isolated colonies to broth medium A and incubate at 41-43 °C for 18-24 h. The product passes the test if no growth occurs at 41-43 °C.

When testing transdermal patches, filter 50 ml of preparation A as described in the general method 2.6.12 through a sterile filter membrane and place in 100 ml of broth medium A and incubate at 35-37 °C for 18-48 h. After incubation spread on agar medium N.

Staphylococcus aureus

Prepare the product to be examined as described in the general method 2.6.12 and use 10 ml or the quantity corresponding to 1 g or 1 ml to inoculate 100 ml of broth medium A, homogenise and incubate at 35-37 °C for 18-48 h. Subculture on a plate of agar medium O and incubate at 35-37 °C for 18-72 h. Black colonies of gram-positive cocci, surrounded by a clear zone indicate the presence of *S. aureus*. Confirmation may be effected by suitable biochemical tests such as the coagulase test and the deoxyribonuclease test. The product passes the test if colonies of the type described do not appear on agar medium O or if the confirmatory biochemical tests are negative.

When testing transdermal patches, filter 50 ml of preparation A as described in the general method 2.6.12 through a sterile filter membrane and place in 100 ml of broth medium A and incubate at 35-37 °C for 18-48 h. After incubation spread on agar medium O.

Nutritive and selective properties of the media and validity of the test

The tests described hereafter must be performed at least on each lot of dehydrated media.

Proceed as follows. Grow the following test strains separately, in tubes containing suitable media such as those indicated, at 30-35 °C for 18-24 h:

<i>Staphylococcus aureus</i>	such as ATCC 6538 (NCIMB 9518, CIP 4.83): broth medium A,
<i>Pseudomonas aeruginosa</i>	such as ATCC 9027 (NCIMB 8626, CIP 82.118): broth medium A,

Escherichia coli such as ATCC 8739 (NCIMB 8545, CIP 53.126): broth medium A,

Salmonella typhimurium no strain number is recommended (a salmonella not pathogenic for man, such as *Salmonella abony* (NCTC 6017, CIP 80.39), may also be used): broth medium A.

Dilute portions of each of the cultures using buffered sodium chloride-peptone solution pH 7.0 to make test suspensions containing about 1000 viable micro-organisms per millilitre. Mix equal volumes of each suspension and use 0.4 ml (approximately 100 micro-organisms of each strain) as an inoculum in tests for *S. aureus*, *P. aeruginosa*, *E. coli* and *Salmonellae* in the presence and in the absence of the product to be examined. A positive result for the respective micro-organisms must be obtained.

Clostridia

The tests described below are intended for distinct purposes. The first method is intended for products where exclusion of pathogenic clostridia is essential and it is necessary to test for their absence. The products generally have a low total count. The second method is a semi-quantitative test for *Clostridium perfringens* and is intended for products where the level of this species is a criterion of quality.

1. Test for *Clostridia*

Prepare the product to be examined as described in the general method 2.6.12. Take 2 equal portions corresponding to 1 g or 1 ml of the product to be examined. Heat 1 portion to 80 °C for 10 min and cool rapidly. Do not heat the other portion. Transfer 10 ml of each of the homogenised portions to 2 containers (38 mm × 200 mm) or other suitable containers containing 100 ml of medium P. Incubate under anaerobic conditions at 35-37 °C for 48 h. After incubation, make subcultures from each tube on medium Q to which gentamicin has been added and incubate under anaerobic conditions at 35-37 °C for 48 h. If no growth of micro-organisms is detected, the product passes the test.

Where growth occurs, subculture each distinct colony form on culture medium Q, without gentamicin, and incubate in both aerobic and anaerobic conditions. The occurrence of only anaerobic growth of gram-positive bacilli (with or without endospores) giving a negative catalase reaction indicates the presence of *Clostridium spp.* Compare, if necessary, colony morphology on the 2 plates and apply the catalase test to eliminate aerobic and facultatively anaerobic *Bacillus spp.* which give a positive catalase reaction. This test may be applied to discrete colonies on agar, or indirectly following transfer to a glass slide, by application of a drop of dilute hydrogen peroxide solution R. The formation of gas bubbles indicates a positive catalase reaction.

2. Count of *Clostridium perfringens*

Prepare the product to be examined as described in the general method 2.6.12, and prepare 1:100 and 1:1000 dilutions in buffered sodium chloride-peptone solution pH 7.0. Determine the most probable number of bacteria as described under total viable aerobic count 2.6.12, using culture medium R in tubes or other suitable containers with a small Durham tube. Mix with minimum shaking and incubate at 45.5-46.5 °C for 24-48 h. The containers showing a blackening due to iron sulphide and abundant formation of gas in the Durham tube (at least 1/10 of the volume) indicate the presence of *Cl. perfringens*. Estimate the most probable number of *Cl. perfringens* by means of Table 2.6.13-2.

Table 2.6.13-2. – Most-probable-number (MPN) values of bacteria

3 tubes at each level of dilution							
Number of positive tubes			MPN per gram	Category*		95 per cent confidence limits	
0.1 g	0.01 g	0.001 g		1	2		
0	0	0	< 3			–	–
0	1	0	3		x	< 1	17
1	0	0	3	x		1	21
1	0	1	7		x	2	27
1	1	0	7	x		2	28
1	2	0	11		x	4	35
2	0	0	9	x		2	38
2	0	1	14		x	5	48
2	1	0	15	x		5	50
2	1	1	20		x	8	61
2	2	0	21	x		8	63
3	0	0	23	x		7	129
3	0	1	38	x		10	180
3	1	0	43	x		20	210
3	1	1	75	x		20	280
3	2	0	93	x		30	390
3	2	1	150	x		50	510
3	2	2	210		x	80	640
3	3	0	240	x		100	1400
3	3	1	460	x		200	2400
3	3	2	1100	x		300	4800
3	3	3	> 1100			–	–

* Category 1: normal results, obtained in 95 per cent of cases.
Category 2: less likely results, obtained in only 4 per cent of cases. These are not to be used for important decisions. Results that are even less likely than those of category 2 are not mentioned and are always unacceptable.

Controls

Use the following test strains:

For method 1: *Clostridium sporogenes*, e.g. ATCC 19404 (NCTC 532) or CIP 79.3,

For method 2: *Clostridium perfringens*, e.g. ATCC 13124 (NCIMB 6125, NCTC 8237, CIP 103 409).

If necessary combine with *Cl. sporogenes* to check selectivity and anaerobic conditions.

The following section is published for information.

RECOMMENDED SOLUTION AND CULTURE MEDIA

The following solution and culture media have been found satisfactory for the purposes for which they are prescribed in the test for microbial contamination in the Pharmacopoeia. Other media may be used if they have similar nutritive and selective properties for the micro-organisms to be tested for.

Buffered sodium chloride-peptone solution pH 7.0

Potassium dihydrogen phosphate	3.6 g
Disodium hydrogen phosphate dihydrate	7.2 g, equivalent to 0.067 M phosphate
Sodium chloride	4.3 g
Peptone (meat or casein)	1.0 g
Purified water	1000 ml

To this solution surface-active agents or inactivators of antimicrobial agents may be added, such as:

Polysorbate 80 1 g/l to 10 g/l

Sterilise by heating in an autoclave at 121 °C for 15 min.

Broth medium A (Casein soya bean digest broth)

Pancreatic digest of casein	17.0 g
Papaic digest of soya bean	3.0 g
Sodium chloride	5.0 g
Dipotassium hydrogen phosphate	2.5 g
Glucose monohydrate	2.5 g
Purified water	1000 ml

Adjust the pH so that after sterilisation it is 7.3 ± 0.2 .

Sterilise by heating in an autoclave at 121 °C for 15 min.

Agar medium B (Casein soya bean digest agar)

Pancreatic digest of casein	15.0 g
Papaic digest of soya bean	5.0 g
Sodium chloride	5.0 g
Agar	15.0 g
Purified water	1000 ml

Adjust the pH so that after sterilisation it is 7.3 ± 0.2 .

Sterilise by heating in an autoclave at 121 °C for 15 min.

Agar medium C (Sabouraud-glucose agar with antibiotics)

Peptones (meat and casein)	10.0 g
Glucose monohydrate	40.0 g
Agar	15.0 g
Purified water	1000 ml

Adjust the pH so that after sterilisation it is 5.6 ± 0.2 . Sterilise by heating in an autoclave at 121 °C for 15 min. Immediately before use, add 0.10 g of benzylpenicillin sodium and 0.10 g of tetracycline per litre of medium as sterile solutions or, alternatively, add 50 mg of chloramphenicol per litre of medium before sterilisation.

Broth medium D (Lactose monohydrate broth)

Beef extract	3.0 g
Pancreatic digest of gelatin	5.0 g
Lactose monohydrate	5.0 g
Purified water	1000 ml

Adjust the pH so that after sterilisation it is 6.9 ± 0.2 .

Sterilise by heating in an autoclave at 121 °C for 15 min and cool immediately.

Enrichment broth medium E (Enterobacteria enrichment broth-Mossel)

Pancreatic digest of gelatin	10.0 g
Glucose monohydrate	5.0 g
Dehydrated ox bile	20.0 g
Potassium dihydrogen phosphate	2.0 g
Disodium hydrogen phosphate dihydrate	8.0 g
Brilliant green	15 mg
Purified water	1000 ml

Adjust the pH so that after heating it is 7.2 ± 0.2 . Heat at 100 °C for 30 min and cool immediately.

Agar medium F (Crystal violet, neutral red, bile agar with glucose)

Yeast extract	3.0 g
Pancreatic digest of gelatin	7.0 g
Bile salts	1.5 g
Lactose monohydrate	10.0 g
Sodium chloride	5.0 g
Glucose monohydrate	10.0 g
Agar	15.0 g
Neutral red	30 mg
Crystal violet	2 mg
Purified water	1000 ml

Adjust the pH so that after heating it is 7.4 ± 0.2 . Heat to boiling; do not heat in an autoclave.

Broth medium G (MacConkey broth)

Pancreatic digest of gelatin	20.0 g
Lactose monohydrate	10.0 g
Dehydrated ox bile	5.0 g
Bromocresol purple	10 mg
Purified water	1000 ml

Adjust the pH so that after sterilisation it is 7.3 ± 0.2 .

Sterilise by heating in an autoclave at 121 °C for 15 min.

Agar medium H (MacConkey agar)

Pancreatic digest of gelatin	17.0 g
Peptones (meat and casein)	3.0 g
Lactose monohydrate	10.0 g
Sodium chloride	5.0 g
Bile salts	1.5 g
Agar	13.5 g
Neutral red	30.0 mg
Crystal violet	1 mg
Purified water	1000 ml

Adjust the pH so that after sterilisation it is 7.1 ± 0.2 . Boil for 1 min with constant shaking then sterilise by heating in an autoclave at 121 °C for 15 min.

Broth medium I (Tetrathionate bile brilliant green broth)

Peptone	8.6 g
Ox bile, dried	8.0 g
Sodium chloride	6.4 g
Calcium carbonate	20.0 g
Potassium tetrathionate	20.0 g
Brilliant green	70 mg
Purified water	1000 ml

Adjust the pH so that after heating it is 7.0 ± 0.2 . Heat just to boiling. Do not re-heat.

Agar medium J (Deoxycholate citrate agar)

Beef extract	10.0 g
Meat peptone	10.0 g
Lactose monohydrate	10.0 g
Sodium citrate	20.0 g

Ferric citrate	1.0 g	Agar	12.0 g
Sodium deoxycholate	5.0 g	Purified water	1000 ml
Agar	13.5 g	Heat to boiling for 1 min with shaking. Adjust the pH so that after sterilisation it is 7.4 ± 0.2 . Fill into tubes to one-third of their height, sterilise by heating in an autoclave at 121°C for 15 min and allow to cool in a position that gives a deep portion and a sloping surface.	
Neutral red	20 mg		
Purified water	1000 ml		

Adjust the pH so that after heating it is 7.3 ± 0.2 . Heat gently to boiling and boil for 1 min, cool to 50°C and pour into Petri dishes. Do not heat in an autoclave.

Agar medium K (Xylose, lysine, deoxycholate agar)

Xylose	3.5 g	Pancreatic digest of gelatin	20.0 g
L-Lysine	5.0 g	Magnesium chloride	1.4 g
Lactose monohydrate	7.5 g	Dipotassium sulphate	10.0 g
Sucrose	7.5 g	Cetrimide	0.3 g
Sodium chloride	5.0 g	Agar	13.6 g
Yeast extract	3.0 g	Purified water	1000 ml
Phenol red	80 mg	Glycerol	10.0 ml
Agar	13.5 g	Heat to boiling for 1 min with shaking. Adjust the pH so that after sterilisation it is 7.2 ± 0.2 . Sterilise by heating in an autoclave at 121°C for 15 min.	
Sodium deoxycholate	2.5 g		
Sodium thiosulphate	6.8 g		
Ferric ammonium citrate	0.8 g	Agar medium O (Baird-Parker agar)	
Purified water	1000 ml		

Adjust the pH so that after heating it is 7.4 ± 0.2 . Heat just to boiling, cool to 50°C and pour into Petri dishes. Do not heat in an autoclave.

Agar medium L (Brilliant green, phenol red, lactose monohydrate, sucrose agar)

Peptones (meat and casein)	10.0 g	Pancreatic digest of casein	10.0 g
Yeast extract	3.0 g	Beef extract	5.0 g
Sodium chloride	5.0 g	Yeast extract	1.0 g
Lactose monohydrate	10.0 g	Lithium chloride	5.0 g
Sucrose	10.0 g	Agar	20.0 g
Agar	20.0 g	Glycine	12.0 g
Phenol red	80 mg	Sodium pyruvate	10.0 g
Brilliant green	12.5 mg	Purified water	950 ml
Purified water	1000 ml	Heat to boiling for 1 min with shaking. Adjust the pH so that after sterilisation it is 6.8 ± 0.2 . Sterilise by heating in an autoclave at 121°C for 15 min, cool to $45\text{--}50^\circ\text{C}$ and add 10 ml of a sterile 10 g/l solution of potassium tellurite and 50 ml of egg-yolk emulsion.	

Heat to boiling for 1 min. Adjust the pH so that after sterilisation it is 6.9 ± 0.2 . Immediately before use, sterilise by heating in an autoclave at 121°C for 15 min, cool to 50°C and pour into Petri dishes.

Agar medium M (Triple sugar, iron agar)

Beef extract	3.0 g	Beef extract	10.0 g
Yeast extract	3.0 g	Peptone	10.0 g
Peptones (casein and beef)	20.0 g	Yeast extract	3.0 g
Sodium chloride	5.0 g	Soluble starch	1.0 g
Lactose monohydrate	10.0 g	Glucose monohydrate	5.0 g
Sucrose	10.0 g	Cysteine hydrochloride	0.5 g
Glucose monohydrate	1.0 g	Sodium chloride	5.0 g
Ferric ammonium citrate	0.3 g	Sodium acetate	3.0 g
Sodium thiosulphate	0.3 g	Agar	0.5 g
Phenol red	25 mg	Purified water	1000 ml
		Hydrate the agar, dissolve by heating to boiling with continuous stirring. If necessary, adjust the pH so that after sterilisation it is about 6.8. Sterilise by heating in an autoclave at 121°C for 15 min.	

Medium Q (Columbia agar)

Pancreatic digest of casein	10.0 g
Meat peptic digest	5.0 g
Heart pancreatic digest	3.0 g
Yeast extract	5.0 g

Maize starch	1.0 g
Sodium chloride	5.0 g
Agar, according to gelling power	10.0 g to 15.0 g
Purified water	1000 ml

Hydrate the agar, dissolve by heating to boiling with continuous stirring. If necessary, adjust the pH so that after sterilisation it is 7.3 ± 0.2 . Sterilise by heating in an autoclave at 121 °C for 15 min. Allow to cool to 45–50 °C; add, where necessary, gentamicin sulphate corresponding to 20 mg of gentamicin base and pour into Petri dishes.

Medium R (Lactose monohydrate sulphite medium)

Pancreatic digest of casein	5.0 g
Yeast extract	2.5 g
Sodium chloride	2.5 g
Lactose monohydrate	10.0 g
Cysteine hydrochloride	0.3 g
Purified water	1000 ml

Dissolve, adjust to pH 7.1 ± 0.1 and fill to 8 ml in 16 mm × 160 mm tubes containing a small Durham tube. Sterilise by heating in an autoclave at 121 °C for 15 min and store at 4 °C.

Before use, heat the medium for 5 min in a water-bath and cool. Add to each tube 0.5 ml of a 12 g/l solution of *sodium metabisulphite R* and 0.5 ml of a 10 g/l solution of ferric ammonium citrate, both solutions being freshly prepared and filtered through membranes (pore size: 0.45 µm).

Agar medium S (R2A)

Yeast extract	0.5 g
Proteose peptone	0.5 g
Casein hydrolysate	0.5 g
Glucose	0.5 g
Starch	0.5 g
Dipotassium hydrogen phosphate	0.3 g
Magnesium sulphate, anhydrous	0.024 g
Sodium pyruvate	0.3 g
Agar	15.0 g
Purified water	1000 ml

Adjust the pH so that after sterilisation it is 7.2 ± 0.2 . Sterilise by heating in an autoclave at 121 °C for 15 min.

NEUTRALISING AGENTS

Neutralising agents may be used to neutralise the activity of antimicrobial agents. They may be added to buffered sodium chloride-peptone solution pH 7.0, preferably before sterilisation. If utilised their efficacy and non-toxicity towards micro-organisms are demonstrated.

A typical neutralising fluid has the following composition:

Polysorbate 80	30 g
Lecithin (egg)	3 g
Histidine hydrochloride	1 g
Peptone (meat or casein)	1 g
Sodium chloride	4.3 g
Potassium dihydrogen phosphate	3.6 g
Disodium hydrogen phosphate dihydrate	7.2 g
Purified water	1000 ml

Sterilise by heating in an autoclave at 121 °C for 15 min.

If the solution has insufficient neutralising capacity the concentration of polysorbate 80 or lecithin may be increased. Alternatively, the neutralisers mentioned in Table 2.6.13-3 may be added.

Table 2.6.13-3. – *Inactivators for antimicrobial agents to be added to buffered sodium chloride-peptone solution pH 7.0*

Type of antimicrobial agent	Inactivator	Concentration	Comment
Phenolics	Sodium laurilsulfate	4 g/l	Add after sterilisation of buffered sodium chloride-peptone solution pH 7.0
	Polysorbate 80 and lecithin	30 g/l and 3 g/l	
	Egg yolk	5 ml/l - 50 ml/l	
Organo-mercurals	Sodium thioglycolate	0.5 g/l - 5 g/l	
Halogens	Sodium thiosulphate	5 g/l	
Quaternary ammonium compounds	Egg yolk	5 ml/l - 50 ml/l	Add after sterilisation of buffered sodium chloride-peptone solution pH 7.0

01/2005:20614

2.6.14. BACTERIAL ENDOTOXINS

The test for bacterial endotoxins is used to detect or quantify endotoxins of gram-negative bacterial origin using amoebocyte lysate from horseshoe crab (*Limulus polyphemus* or *Tachypleus tridentatus*). There are 3 techniques for this test: the gel-clot technique, which is based on gel formation; the turbidimetric technique, based on the development of turbidity after cleavage of an endogenous substrate; and the chromogenic technique, based on the development of colour after cleavage of a synthetic peptide-chromogen complex.

The following 6 methods are described in the present chapter:

- Method A. Gel-clot method: limit test
- Method B. Gel-clot method: semi-quantitative test
- Method C. Turbidimetric kinetic method
- Method D. Chromogenic kinetic method
- Method E. Chromogenic end-point method
- Method F. Turbidimetric end-point method

Proceed by any of the 6 methods for the test. In the event of doubt or dispute, the final decision is made based upon method A unless otherwise indicated in the monograph.

The test is carried out in a manner that avoids endotoxin contamination.

Apparatus

Depyrogenate all glassware and other heat-stable apparatus in a hot-air oven using a validated process. A commonly used minimum time and temperature is 30 minutes at 250 °C. If employing plastic apparatus, such as microtitre plates and pipette tips for automatic pipettors, use apparatus shown to be free of detectable endotoxin and of interfering effects for the test.

NOTE: In this chapter, the term 'tube' includes all types of receptacles, for example microtitre plate wells.

Preparation of the standard endotoxin stock solution

The standard endotoxin stock solution is prepared from an endotoxin reference standard that has been calibrated against the International Standard, for example *endotoxin standard BRP*.

Endotoxin is expressed in International Units (IU). The equivalence in IU of the International Standard is stated by the World Health Organisation.

NOTE: One International Unit (IU) of endotoxin is equal to one Endotoxin Unit (E.U.).

Follow the specifications in the package leaflet and on the label for preparation and storage of the standard endotoxin stock solution.

Preparation of the standard endotoxin solutions

After vigorously mixing the standard endotoxin stock solution, prepare appropriate serial dilutions of this solution using water for bacterial endotoxins test (water for BET).

Use the solutions as soon as possible to avoid loss of activity by adsorption.

Preparation of the test solutions

Prepare the test solutions by dissolving or diluting active substances or medicinal products using water for BET. Some substances or preparations may be more appropriately dissolved or diluted in other aqueous solutions. If necessary, adjust the pH of the test solution (or dilution thereof) so that the pH of the mixture of the lysate and test solution falls within the pH range specified by the lysate manufacturer. This usually applies to a product with a pH in the range of 6.0 to 8.0. The pH may be adjusted by the use of acid, base or a suitable buffer, as recommended by the lysate manufacturer. Acids and bases may be prepared from concentrates or solids with water for BET in containers free of detectable endotoxin. Buffers must be validated to be free of detectable endotoxin and interfering factors.

Determination of the Maximum Valid Dilution

The Maximum Valid Dilution (MVD) is the maximum allowable dilution of a sample at which the endotoxin limit can be determined. Determine the MVD using the following formulae:

$$\text{MVD} = \frac{\text{endotoxin limit} \times \text{concentration of test solution}}{\lambda}$$

Endotoxin limit: the endotoxin limit for active substances administered parenterally, defined on the basis of dose, is equal to:

$$\frac{K}{M}$$

K = threshold pyrogenic dose of endotoxin per kilogram of body mass in a single hour period,

M = maximum recommended dose of product per kilogram of body mass in a single hour period.

The endotoxin limit for active substances administered parenterally is specified in units such as IU/ml, IU/mg, IU/Unit of biological activity, etc., in monographs.

Concentration of test solution:

– in mg/ml if the endotoxin limit is specified by mass (IU/mg),

– in Units/ml if the endotoxin limit is specified by unit of biological activity (IU/Unit),

– in ml/ml if the endotoxin limit is specified by volume (IU/ml).

λ = the labelled lysate sensitivity in the gel-clot technique (IU/ml) or the lowest point used in the standard curve of the turbidimetric or chromogenic techniques.

GEL-CLOT TECHNIQUE (METHODS A AND B)

The gel-clot technique allows detection or quantification of endotoxins and is based on clotting of the lysate in the presence of endotoxins. The concentration of endotoxins required to cause the lysate to clot under standard conditions is the labelled lysate sensitivity. To ensure both the precision and validity of the test, confirm the labelled lysate sensitivity and perform the test for interfering factors as described under 1. *Preparatory testing*.

1. PREPARATORY TESTING**(i) Confirmation of the labelled lysate sensitivity**

Confirm in 4 replicates the labelled sensitivity λ , expressed in IU/ml, of the lysate solution prior to use in the test. Confirmation of the lysate sensitivity is carried out when a new batch of lysate is used or when there is any change in the experimental conditions which may affect the outcome of the test.

Prepare standard solutions of at least 4 concentrations equivalent to 2λ , λ , 0.5λ and 0.25λ by diluting the standard endotoxin stock solution with water for BET.

Mix a volume of the lysate solution with an equal volume of 1 of the standard solutions (such as 0.1 ml aliquots) in each tube. When single test vials or ampoules containing lyophilised lysate are employed, add solutions directly to the vial or ampoule. Incubate the reaction mixture for a constant period according to the recommendations of the lysate manufacturer (usually at $37 \pm 1^\circ\text{C}$ for 60 ± 2 min), avoiding vibration. Test the integrity of the gel: for tubes, take each tube in turn directly from the incubator and invert it through approximately 180° in one smooth motion. If a firm gel has formed that remains in place upon inversion, record the result as positive. A result is negative if an intact gel is not formed.

The test is not valid unless the lowest concentration of the standard solutions shows a negative result in all replicate tests.

The end-point is the last positive result in the series of decreasing concentrations of endotoxin. Calculate the mean value of the logarithms of the end-point concentrations and then the antilogarithm of the mean value using the following expression:

$$\text{Geometric mean end-point concentration} = \text{antilog} \frac{\sum e}{f}$$

$\sum e$ = sum of the log end-point concentrations of the dilution series used,

f = number of replicates.

The geometric mean end-point concentration is the measured sensitivity of the lysate solution (IU/ml). If this is not less than 0.5λ and not more than 2λ , the labelled sensitivity is confirmed and is used in the tests performed with this lysate.

(ii) Test for interfering factors

Prepare solutions A, B, C and D as shown in Table 2.6.14.-1, and use the test solutions at a dilution less than the MVD, not containing any detectable endotoxins, operating as described under 1. *Preparatory testing*, (i) Confirmation of the labelled lysate sensitivity.

Table 2.6.14.-1

Solution	Endotoxin concentration/ Solution to which endotoxin is added	Diluent	Dilution factor	Initial endotoxin concentration	Number of replicates
A	None/Test solution	-	-	-	4
B	2λ/Test solution	Test solution	1	2λ	4
			2	1λ	4
			4	0.5λ	4
			8	0.25λ	4
C	2λ/Water for BET	Water for BET	1	2λ	2
			2	1λ	2
			4	0.5λ	2
			8	0.25λ	2
D	None/Water for BET	-	-	-	2

Solution A = solution of the preparation being examined that is free of detectable endotoxins.

Solution B = test for interference.

Solution C = control of the labelled lysate sensitivity.

Solution D = negative control (water for BET).

The geometric mean end-point concentrations of solutions B and C are determined using the expression described in 1. Preparatory testing, (i) Confirmation of the labelled lysate sensitivity.

The test for interfering factors is repeated when any changes are made to the experimental conditions that are likely to influence the result of the test.

The test is not valid unless all replicates of solutions A and D show no reaction and the result of solution C confirms the labelled lysate sensitivity.

If the sensitivity of the lysate determined with solution B is not less than 0.5λ and not greater than 2λ, the test solution does not contain interfering factors under the experimental conditions used. Otherwise, the solution interferes with the test.

If the preparation being examined interferes with the test at a dilution less than the MVD, repeat the test for interfering factors using a greater dilution, not exceeding the MVD. The use of a more sensitive lysate permits a greater dilution of the preparation being examined and this may contribute to the elimination of interference.

Interference may be overcome by suitable treatment, such as filtration, neutralisation, dialysis or heat treatment. To establish that the treatment chosen effectively eliminates interference without loss of endotoxins, repeat the test for interfering factors using the preparation being examined to which the standard endotoxin has been added and which has then been submitted to the chosen treatment.

2. LIMIT TEST (METHOD A)

(i) Procedure

Prepare solutions A, B, C and D as shown in Table 2.6.14.-2, and perform the test on these solutions following the procedure described under 1. Preparatory testing, (i) Confirmation of the labelled lysate sensitivity.

Table 2.6.14.-2

Solution	Endotoxin concentration/ Solution to which endotoxin is added	Number of replicates
A	None/Diluted test solution	2
B	2λ/Diluted test solution	2
C	2λ/Water for BET	2
D	None/Water for BET	2

Prepare solution A and solution B (positive product control) using a dilution not greater than the MVD and treatments as described in 1. Preparatory testing, (ii) Test for interfering factors. Solutions B and C (positive controls) contain the

standard endotoxin at a concentration corresponding to twice the labelled lysate sensitivity. Solution D (negative control) consists of water for BET.

(ii) Interpretation

The test is not valid unless both replicates of the 2 positive control solutions B and C are positive and those of the negative control solution D are negative.

The preparation being examined complies with the test when a negative result is found for both replicates of solution A.

When a positive result is found for both replicates of solution A:

- if the preparation being examined is diluted to the MVD, it does not comply with the test,
- if the preparation being examined is diluted to a dilution less than the MVD, the test is repeated at a dilution not greater than the MVD.

Repeat the test if a positive result is found for one replicate of solution A and a negative result is found for the other. The preparation being examined complies with the test if a negative result is found for both replicates of solution A in the repeat test.

3. SEMI-QUANTITATIVE TEST (METHOD B)

(i) Procedure

The test quantifies bacterial endotoxins in the test solution by titration to an end-point. Prepare solutions A, B, C and D as shown in Table 2.6.14.-3, and test these solutions according to the procedure described under 1. Preparatory testing, (i) Confirmation of the labelled lysate sensitivity.

(ii) Calculation and interpretation

The test is not valid unless the following 3 conditions are met:

- (a) both replicates of solution D (negative control) are negative,
- (b) both replicates of solution B (positive product control) are positive,
- (c) the geometric mean end-point concentration of solution C is in the range of 0.5λ to 2λ.

To determine the endotoxin concentration of solution A, calculate the end-point concentration for each replicate series of dilutions by multiplying each end-point dilution factor by λ.

The endotoxin concentration in the test solution is the geometric mean end-point concentration of the replicates (see the expression given under 1. Preparatory testing, (i) Confirmation of the labelled lysate sensitivity). If the

test is conducted with a diluted test solution, calculate the concentration of endotoxin in the original solution by multiplying the result by the dilution factor.

If none of the dilutions of the test solution is positive in a valid test, record the endotoxin concentration as less than λ (or, if a diluted sample was tested, as less than $\lambda \times$ the lowest dilution factor of the sample). If all dilutions are positive, the endotoxin concentration is recorded as equal to or greater than the greatest dilution factor multiplied by λ (e.g. in Table 2.6.14.-3, the initial dilution factor $\times 8 \times \lambda$).

The preparation meets the requirements of the test if the endotoxin concentration is less than that specified in the individual monograph.

PHOTOMETRIC TECHNIQUES (METHODS C, D, E AND F)

1. TURBIDIMETRIC TECHNIQUE (METHODS C AND F)

This technique is a photometric test to measure the increase in turbidity. Based on the test principle employed, this technique is classified as being the end-point-turbidimetric test or the kinetic-turbidimetric test.

The end-point-turbidimetric test (Method F) is based on the quantitative relationship between the endotoxin concentration and the turbidity (absorbance or transmission) of the reaction mixture at the end of an incubation period.

The kinetic-turbidimetric test (Method C) is a method to measure either the time (onset time) needed for the reaction mixture to reach a predetermined absorbance, or the rate of turbidity development.

The test is carried out at the incubation temperature recommended by the lysate manufacturer (usually $37 \pm 1^\circ\text{C}$).

2. CHROMOGENIC TECHNIQUE (METHODS D AND E)

This technique is used to measure the chromophore released from a suitable chromogenic peptide by the reaction of endotoxins with the lysate. Depending on the test principle employed, this technique is classified as being the end-point-chromogenic test or the kinetic-chromogenic test.

The end-point-chromogenic test (Method E) is based on the quantitative relationship between the endotoxin concentration and the quantity of chromophore released at the end of an incubation period.

The kinetic-chromogenic test (Method D) measures either the time (onset time) needed for the reaction mixture to reach a predetermined absorbance, or the rate of colour development.

The test is carried out at the incubation temperature recommended by the lysate manufacturer (usually $37 \pm 1^\circ\text{C}$).

3. PREPARATORY TESTING

To assure the precision or validity of the turbidimetric and chromogenic tests, preparatory tests are conducted to assure that the criteria for the standard curve are satisfied and that the test solution does not interfere with the test.

Validation of the test method is required when any changes are made to the experimental conditions that are likely to influence the result of the test.

(i) Assurance of criteria for the standard curve

Using the standard endotoxin solution, prepare at least 3 endotoxin concentrations to generate the standard curve. Perform the test using at least 3 replicates of each standard endotoxin solution as recommended by the lysate manufacturer (volume ratios, incubation time, temperature, pH, etc.).

If the desired range is greater than 2 log in the kinetic methods, additional standards must be included to bracket each log increase in the range of the standard curve.

The absolute value of the correlation coefficient, $|r|$, must be greater than or equal to 0.980, for the range of endotoxin concentrations indicated by the lysate manufacturer.

(ii) Test for interfering factors

Select an endotoxin concentration at or near the middle of the endotoxin standard curve.

Prepare solutions A, B, C and D as shown in Table 2.6.14.-4. Perform the test on at least 2 replicates of these solutions as recommended by the lysate manufacturer (volume of test solution and lysate solution, volume ratio of test solution to lysate solution, incubation time, etc.).

Table 2.6.14.-3

Solution	Endotoxin concentration/ Solution to which endotoxin is added	Diluent	Dilution factor	Initial endotoxin concentration	Number of replicates
A	None/Test solution	Water for BET	1	-	2
			2	-	2
			4	-	2
			8	-	2
B	2λ /Test solution		1	2λ	2
C	2λ /Water for BET	Water for BET	1	2λ	2
			2	λ	2
			4	0.5λ	2
			8	0.25λ	2
D	None/Water for BET	-	-	-	2

Solution A = test solution at the dilution, not exceeding the MVD, with which the test for interfering factors was carried out. Subsequent dilution of the test solution must not exceed the MVD. Use water for BET to make two dilution series of 1, 1/2, 1/4 and 1/8, relative to the dilution with which the test for interfering factors was carried out. Other dilutions may be used as appropriate.

Solution B = solution A containing standard endotoxin at a concentration of 2λ (positive product control).

Solution C = 2 series of water for BET containing the standard endotoxin at concentrations of 2λ , λ , 0.5λ and 0.25λ .

Solution D = water for BET (negative control).

Table 2.6.14.4.

Solution	Endotoxin concentration	Solution to which endotoxin is added	Number of replicates
A	None	Test solution	Not less than 2
B	Middle concentration of the standard curve	Test solution	Not less than 2
C	At least 3 concentrations (lowest concentration is designated λ)	Water for BET	Each concentration not less than 2
D	None	Water for BET	Not less than 2

Solution A = test solution, that may be diluted not to exceed the MVD.

Solution B = preparation to be examined at the same dilution as solution A, containing added endotoxin at a concentration equal to or near the middle of the standard curve.

Solution C = standard endotoxin solution at the concentrations used in the validation of the method as described under 3. Preparatory testing, (i) Assurance of criteria for the standard curve (positive controls).

Solution D = water for BET (negative control).

Calculate the mean recovery of the added endotoxin by subtracting the mean endotoxin concentration in the solution (if any) from that in the solution containing the added endotoxin.

The test solution is considered free of interfering factors if under the conditions of the test, the measured concentration of the endotoxin added to the test solution is within 50-200 per cent of the known added endotoxin concentration, after subtraction of any endotoxin detected in the solution without added endotoxin.

When the endotoxin recovery is out of the specified ranges, the interfering factors must be removed as described in the section Gel-clot technique, under 1. Preparatory testing, (ii) Test for interfering factors. The efficiency of the treatment is verified by repeating the test for interfering factors.

4. TEST

(i) Procedure

Follow the procedure described in 3. Preparatory testing, (ii) Test for interfering factors.

(ii) Calculation

Calculate the endotoxin concentration of each replicate of solution A using the standard curve generated by the series of positive controls, solution C.

The test is not valid unless the following 3 requirements are met:

- (a) the result obtained with solution D (negative control) does not exceed the limit of the blank value required in the description of the lysate employed,
- (b) the results obtained with the series of positive controls, solution C, comply with the requirements for validation defined under 3. Preparatory testing, (i) Assurance of criteria for the standard curve,
- (c) the endotoxin recovery, calculated from the endotoxin concentration found in solution B after subtracting the endotoxin concentration found in solution A, is within the range of 50-200 per cent.

(iii) Interpretation

The preparation being examined complies with the test if the mean endotoxin concentration of the replicates of solution A, after correction for dilution and concentration, is less than the endotoxin limit for the product.

5. REAGENTS

(i) Lysate solution

Dissolve amoebocyte lysate in water for BET or in a buffer, as recommended by the lysate manufacturer, by gentle stirring. Store the reconstituted lysate, refrigerated or frozen, as indicated by the manufacturer.

(ii) Amoebocyte lysate

Amoebocyte lysate is a lyophilised product obtained from amoebocyte lysate from Horseshoe Crab (*Limulus polyphemus* or *Tachypleus tridentatus*). This reagent refers only to a product manufactured in accordance with the regulations of the competent authority.

Amoebocyte lysate reacts with some β -glucans in addition to endotoxins. Amoebocyte lysate preparations which do not react with glucans are available; they are prepared by removing from amoebocyte lysate the G factor, which reacts with glucans, or by inhibiting the G factor reacting system of amoebocyte lysate. These preparations may be used for endotoxin testing in the presence of glucans.

(iii) Water for BET (water for bacterial endotoxins test)

Water for BET is *water for injections R* or water produced by other procedures that shows no reaction with the lysate employed at the detection limit of the reagent.

The following section is published for information.

Test for bacterial endotoxins: guidelines

1. INTRODUCTION

Endotoxins from gram-negative bacteria are the most common cause of toxic reactions resulting from contamination of pharmaceutical products with pyrogens; their pyrogenic activity is much higher than that of most other pyrogenic substances. These endotoxins are lipo-polysaccharides. Although there are a small number of pyrogens which possess a different structure, the conclusion is generally justified that the absence of bacterial endotoxins in a product implies the absence of pyrogenic components, provided the presence of non-endotoxin pyrogenic substances can be ruled out.

The presence of endotoxins in a product may be masked by factors interfering with the reaction between the endotoxins and the amoebocyte lysate. Hence, the analyst who wishes to replace the rabbit pyrogen test required in a pharmacopoeial monograph by a test for bacterial endotoxins has to demonstrate that a valid test can be carried out on the product concerned; this may entail a procedure for removing interfering factors.

As indicated in the test for bacterial endotoxins, information must be available on the 2 following aspects before a test on a sample can be regarded as valid.

1.1. The suitability of the material to be used for the test has to be established. The absence of endotoxins in the water for BET and in the other reagents must be assured and the sensitivity of the amoebocyte lysate must be checked to confirm the sensitivity declared by the manufacturer.

1.2. As the product to be examined may interfere with the test, the sensitivity of the amoebocyte lysate is determined in the presence and in the absence of the product under examination. There must be no significant difference between the 2 sensitivity values.

The test for bacterial endotoxins (2.6.14) indicates methods for removing interfering factors; in the case of interference, another test must be carried out after such a method has been applied to check whether the interference has indeed been neutralised or removed.

This annex explains the reasons for the requirements in the test for bacterial endotoxins, then deals with the reading and interpretation of the results.

Substitution of the rabbit pyrogen test required in a pharmacopoeial monograph by an amoebocyte lysate test constitutes the use of an alternative method of analysis and hence requires validation; some guidance on how to proceed is given in section 11.

The reference method for bacterial endotoxins is stated in the monograph on a given product; where no method is stated, method A is the reference method. If a method other than the reference method is to be used, the analyst must demonstrate that the method is appropriate for this product and gives a result consistent with that obtained with the reference method (see also Section 13).

2. METHOD

The addition of endotoxins to amoebocyte lysate may result in turbidity, precipitation or gelation (gel-clot); only the gel-clot method was used in the Pharmacopoeia as an evaluation criterion in the first type of test for bacterial endotoxins. The advantage was the simplicity of basing the decision to pass or fail the product under examination on the absence or presence of a gel-clot, visible with the naked eye. The quantitative methods described as methods C, D, E and F were developed later: they require more instrumentation, but they are easier to automate for the regular testing of large numbers of samples of the same product.

Endotoxins may be adsorbed onto the surface of tubes or pipettes made from certain plastics or types of glass. Interference may appear due to the release of substances from plastic materials. Hence, the materials used should be checked; subsequent batches of tubes or pipettes may have a slightly different composition, and therefore the analyst is advised to repeat such tests on starting with new batches of materials.

The decision to use the test for bacterial endotoxins as a limit test implies first, that a threshold endotoxin concentration must be defined for the product to be tested and second, that the objective of the test is to know whether the endotoxin concentration in the product under examination is below or above this threshold. The quantitative methods C, D, E and F make it possible to determine the endotoxin concentration in the sample under examination, but for compliance with the Pharmacopoeia and in routine quality control the final question is whether or not this concentration exceeds a defined limit.

In setting a threshold concentration of endotoxin for the product to be tested, due attention should be paid to the dose of the product: the threshold should be set so as to ensure that as long as the endotoxin concentration in the product remains below this threshold even the maximal dose administered by the intended route per hour does not contain sufficient endotoxin to cause a toxic reaction.

When the endotoxin concentration in the product exactly equals the threshold value, gelation will occur, as is the case when the endotoxin concentration is much higher, and the product will fail the test, because the all-or-none character of the test makes it impossible to differentiate between a concentration exactly equal to the threshold concentration and one that is higher. It is only when no gelation occurs that the analyst may conclude that the endotoxin concentration is below the threshold concentration.

For products in the solid state, this threshold concentration of endotoxin per mass unit or per International Unit (IU) of product has to be translated into a concentration of endotoxin per millilitre of solution to be tested, as the test can only be carried out on a solution. The case of products that already exist in the liquid state (such as infusion fluids) is discussed below.

Endotoxin limit: the endotoxin limit for active substances administered parenterally, defined on the basis of dose, is equal to, where:

$$\frac{K}{M}$$

K = threshold pyrogenic dose of endotoxin per kilogram of body mass in a single hour period,

M = maximum recommended dose of product per kilogram of body mass in a single hour period.

The endotoxin limit depends on the product and its route of administration and is stated in monographs. Values for K are suggested in Table 2.6.14.-5.

For other routes, the acceptance criterion for bacterial endotoxins is generally determined on the basis of results obtained during the development of the preparation.

Table 2.6.14.-5

Route of administration	K (IU of endotoxin per kilogram of body mass per hour)
Intravenous	5.0
Intravenous, for radiopharmaceuticals	2.5
Intrathecal	0.2

Which dilution of the product is to be used in the test to obtain maximal assurance that a negative result means that the endotoxin concentration of the product is less than the endotoxin limit and that a positive result means that the lysate detected an endotoxin concentration equal to or greater than the endotoxin limit? This dilution depends on the endotoxin limit and on the sensitivity of the lysate: it is called the Maximum Valid Dilution (MVD) and its value may be calculated as follows:

$$\text{MVD} = \frac{\text{endotoxin limit} \times \text{concentration of test solution}}{\lambda}$$

Concentration of test solution:

- in mg/ml if the endotoxin limit is specified by mass (IU/mg),
- in Units/ml if the endotoxin limit is specified by unit of biological activity (IU/Unit),
- in ml/ml if the endotoxin limit is specified by volume (IU/ml).

λ = the labelled lysate sensitivity in the gel-clot technique (IU/ml) or the lowest point used in the standard curve of the turbidimetric or chromogenic techniques.

When the value of the maximum valid dilution is not a whole number, a convenient whole number smaller than the MVD may be used for routine purposes (which means preparing a solution of the product which is less diluted than the MVD indicates). In this case, a negative result indicates that the endotoxin concentration of the product lies below the limit value. However, when the endotoxin concentration of the product in such a test is less than the endotoxin limit but high enough to make the reaction with the lysate result in a clot, the test may be positive under these conditions. Hence, when a test with this 'convenient' dilution factor is positive, the product should be diluted to the MVD and the test should be repeated. In any case of doubt or dispute the MVD must be used.

This stresses the importance of the confirmation of the sensitivity of the lysate.

Example

A 50 mg/ml solution of phenytoin sodium (intended for intravenous injection) has to be tested. Determine the MVD, given the following variables:

- M = maximum human dose = 15 mg per kilogram of body mass per hour,
 c = 50 mg/ml,
 K = 5 IU of endotoxin per kilogram of body mass per hour,
 λ = 0.4 IU of endotoxin per millilitre.

$$\text{MVD} = \frac{5 \times 50}{15} \times \frac{1}{0.4} = 41.67$$

For routine tests on this product, it may be expedient to dilute 1 ml of the solution to be tested to 20 ml (MVD/2 rounded to the next lower whole number). However, if this test result is positive the analyst will have to dilute 1 ml to 41.67 ml and repeat the test. A dilution to 41.67 ml is also necessary when the test is performed to settle a dispute.

3. REFERENCE MATERIAL

Endotoxin standard BRP is intended for use as the reference preparation. It has been assayed against the WHO International Standard for Endotoxin and its potency is expressed in International Units of endotoxin per ampoule. The International Unit of endotoxin is defined as the specific activity of a defined mass of the International Standard.

For routine purposes, another preparation of endotoxin may be used, provided it has been assayed against the International Standard for Endotoxin or the BRP and its potency is expressed in International Units of endotoxin.

NOTE: 1 International Unit (IU) of endotoxin is equal to 1 Endotoxin Unit (E.U.).

4. WATER FOR BET

Testing the absence of endotoxin in this reagent by a technique derived from the rabbit pyrogen test was rejected for practical and theoretical reasons:

- 4.1. The rabbit test is not sensitive enough to detect endotoxin in water for BET intended for tests on products with a very low endotoxin limit.
 4.2. The relatively low precision of the rising temperature response in rabbits would call for many replications in rabbits.
 4.3. The terms 'pyrogens' and 'endotoxins' denote groups of entities that do not coincide completely.

The text of the test for bacterial endotoxins indicates that methods other than triple distillation may be used to prepare water for BET. Reverse osmosis has been used with good results; some analysts may prefer to distil the water more than three times. Whatever method is used, the resultant product must be free of detectable endotoxins.

5. pH OF THE MIXTURE

In the test for bacterial endotoxins, optimum gel-clot occurs for a mixture at pH 6.0 to 8.0. However, the addition of the lysate to the sample may result in a lowering of the pH.

6. VALIDATION OF THE LYSATE

It is important to follow the manufacturer's instructions for the preparation of the solutions of the lysate.

The positive end-point dilution factors in the gel-clot methods A and B are converted to logarithms. The reason is that if the frequency distribution of these logarithmic values is plotted, it usually approaches a normal distribution curve much more

closely than the frequency distribution of the dilution factors themselves; in fact it is so similar that it is acceptable to use the normal frequency distribution as a mathematical model and to calculate confidence limits with Student's *t*-test.

7. PRELIMINARY TEST FOR INTERFERING FACTORS

Some products cannot be tested directly for the presence of endotoxins because they are not miscible with the reagents, they cannot be adjusted to pH 6.0 to 8.0 or they inhibit or activate gel formation. Therefore a preliminary test is required to check for the presence of interfering factors; when these are found the analyst must demonstrate that the procedure to remove them has been effective.

The object of the preliminary test is to test the null hypothesis that the sensitivity of the lysate in the presence of the product under examination does not differ significantly from the sensitivity of the lysate in the absence of the product. A simple criterion is used in methods A and B: the null hypothesis is accepted when the sensitivity of the lysate in the presence of the product is at least 0.5 times and not more than twice the sensitivity of the lysate by itself.

A classical approach would have been to calculate the means of the log dilution factor for the lysate sensitivity with and without the product and to test the difference between the two means with Student's *t*-test.

The test for interfering factors in gel-clot methods A and B requires the use of a sample of the product in which no endotoxins are detectable. This presents a theoretical problem when an entirely new product has to be tested. Hence, a different approach was designed for quantitative methods C, D, E and F.

8. REMOVAL OF INTERFERING FACTORS

The procedures to remove interfering factors must not increase or decrease (for example, by adsorption) the amount of endotoxin in the product under examination. The correct way of checking this is to apply the procedures to a spiked sample of the product, that is, a sample to which a known amount of endotoxin has been added, and then to measure the recovery of the endotoxin.

Methods C and D. If the nature of the product to be analysed shows interference which cannot be removed by classical methods, it may be possible to carry out the standard curve in the same type of product freed from endotoxins by appropriate treatment or by dilution of the product. The endotoxins test is then carried out by comparison with this standard curve.

Ultrafiltration with cellulose triacetate asymmetric membrane filters has been found to be suitable in most cases. The filters should be properly validated, because under some circumstances cellulose derivatives (β -D-glucans) can cause false positive results.

Polysulphone filters have been found to be unsuitable because false positive results had been obtained by some users.

9. THE PURPOSE OF THE CONTROLS

The purpose of the control made up with water for BET and the reference preparation of endotoxin at twice the concentration of the labelled lysate sensitivity is to verify the activity of the lysate at the time and under the conditions of the test. The purpose of the negative control is to verify the absence of a detectable concentration of endotoxin in water for BET.

The positive control, which contains the product to be examined at the concentration used in the test, is intended to show the absence of inhibiting factors at the time and under the conditions of the test.

10. READING AND INTERPRETATION OF THE RESULTS

Minute amounts of endotoxin in the water for BET, or in any other reagent or material to which the lysate is exposed during the test, may escape detection as long as they do not reach the sensitivity limit of the lysate. However, they may raise the amount of endotoxin in the solution containing the product under examination to just above the sensitivity limit and cause a positive reaction.

The risk of this happening may be reduced by testing the water for BET and the other reagents and materials with the most sensitive lysate available, or at least one that is more sensitive than the one used in the test on the product. Even then, the risk of such a 'false positive result' cannot be ruled out completely. It should be realised, however, that in this respect the test design is 'fail-safe' in contrast to a test design permitting a false negative result, which could lead to the release of an unsatisfactory product, thus endangering the patient's health.

11. REPLACEMENT OF THE RABBIT PYROGEN TEST BY A TEST FOR BACTERIAL ENDOTOXINS

Monographs on pharmaceutical products intended for parenteral use that may contain toxic amounts of bacterial endotoxins require either a test for bacterial endotoxins or a rabbit pyrogen test. As a general policy:

11.1. In any individual monograph, when a test is required, only one test is included, either that for pyrogens or that for bacterial endotoxins.

11.2. In the absence of evidence to the contrary, the test for bacterial endotoxins is preferred over the test for pyrogens, since it is usually considered to provide equal or better protection to the patient.

11.3. Before including a test for bacterial endotoxins in a monograph, evidence is required that one of the tests described in chapter 2.6.14 can be applied satisfactorily to the product in question.

11.4. The necessary information is sought from manufacturers. Companies are invited to provide any validation data that they have concerning the applicability of the test for bacterial endotoxins to the substances and formulations of interest. Such data include details of sample preparation and of any procedures necessary to eliminate interfering factors. In addition, any available parallel data for rabbit pyrogen testing that would contribute to an assurance that the replacement of a rabbit pyrogen test by the test for bacterial endotoxin is appropriate, must be provided.

Additional requirements are defined in the following sections.

12. USE OF A DIFFERENT BACTERIAL ENDOTOXIN TEST FROM THAT PRESCRIBED IN THE MONOGRAPH

When a test for bacterial endotoxins is prescribed in a monograph and none of the six methods (A to F) described in chapter 2.6.14 is specified, then method A, the gel-clot method limit test, has been validated for this product. If one of the other methods (B to F) is specified, this is the one which has been validated for this product.

13. VALIDATION OF ALTERNATIVE METHODS

Replacement of a rabbit pyrogen test by a bacterial endotoxin test, or replacement of a stated or implied method for bacterial endotoxins by another method, is to be regarded as the use of an alternative method in the replacement of a pharmacopoeial test, as described in the General Notices:

"The test and assays described are the official methods upon which the standards of the Pharmacopoeia are based. With the agreement of the competent authority, alternative methods of analysis may be used for control

purposes, provided that the methods used enable an unequivocal decision to be made as to whether compliance with the standards of the monographs would be achieved if the official methods were used. In the event of doubt or dispute, the methods of analysis of the Pharmacopoeia are alone authoritative."

The following procedures are suggested for validating a method for bacterial endotoxins other than the one implied or indicated in the monograph.

13.1. The procedure and the materials and reagents used in the method should be validated as described for the test concerned.

13.2. The presence of interfering factors (and, if needed, the procedure for removing them) should be tested on samples of at least three production batches. It should be borne in mind that methods D and E, using a chromogenic peptide, require reagents that are absent in methods A, B, C and F, and hence compliance of methods A, B, C or F with the requirements for interfering factors cannot be extrapolated to method D or method E without further testing.

14. VALIDATION OF THE TEST FOR NEW PRODUCTS

The procedures described under 13.1 and 13.2 should be applied to all new products intended for parenteral use that have to be tested for the presence of bacterial endotoxins according to the requirements of the Pharmacopoeia.

01/2005:20615
corrected

2.6.15. PREKALLIKREIN ACTIVATOR

Prekallikrein activator (PKA) activates prekallikrein to kallikrein and may be assayed by its ability to cleave a chromophore from a synthetic peptide substrate so that the rate of cleavage can be measured spectrophotometrically and the concentration of PKA calculated by comparison with a reference preparation calibrated in International Units.

The International Unit is the activity of a stated amount of the International Standard which consists of freeze-dried prekallikrein activator. The equivalence in International Units of the International Standard is stated by the World Health Organisation.

Prekallikrein activator in albumin BRP is calibrated in International Units by comparison with the International Standard.

PREPARATION OF PREKALLIKREIN SUBSTRATE

To avoid coagulation activation, blood or plasma used for the preparation of prekallikrein must come into contact only with plastics or silicone-treated glass surfaces.

Draw 9 volumes of human blood into 1 volume of anticoagulant solution (ACD, CPD or 38 g/l sodium citrate R) to which 1 mg/ml of hexadimethrine bromide R has been added. Centrifuge the mixture at 3600 g for 5 min. Separate the plasma and centrifuge again at 6000 g for 20 min to sediment platelets. Separate the platelet-poor plasma and dialyse against 10 volumes of buffer A for 20 h. Apply the dialysed plasma to a chromatography column containing agarose-DEAE for ion exchange chromatography R which has been equilibrated in buffer A and is equal to twice the volume of the plasma. Elute from the column with buffer A at 20 ml/cm²/h. Collect the eluate in fractions and record the absorbance at 280 nm (2.2.25). Pool the fractions containing the first protein peak so that the volume of the pool is about 1.2 times the volume of the platelet-poor plasma.

Test the substrate pool for absence of kallikrein activity by mixing 1 part with 20 parts of the pre-warmed chromogenic substrate solution to be used in the assay and incubate at 37 °C for 2 min. The substrate is suitable if the increase in absorbance is less than 0.001 per minute. Add to the pooled solution 7 g/l of *sodium chloride R* and filter using a membrane filter (porosity 0.45 µm). Freeze the filtrate in portions and store at –25 °C; the substrate may be freeze-dried before storage.

Carry out all procedures from the beginning of the chromatography to freezing in portions during a single working day.

ASSAY

The assay is preferably carried out using an automated enzyme analyser at 37 °C, with volumes, concentration of substrates and incubation times adjusted so that the reaction rate is linear at least up to 35 IU/ml. Standards, samples and prekallikrein substrate may be diluted as necessary using buffer B.

Incubate diluted standards or samples with prekallikrein substrate for 10 min such that the volume of the undiluted sample does not exceed 1/10 of the total volume of the incubation mixture to avoid errors caused by variation in ionic strength and pH in the incubation mixture. Incubate the mixture or a part thereof with at least an equal volume of a solution of a suitable synthetic chromogenic substrate, known to be specific for kallikrein (for example, *N*-benzoyl-L-prolyl-L-phenylalanyl-L-arginine 4-nitroanilide acetate *R* or *D*-prolyl-L-phenylalanyl-L-arginine-4-nitroanilide-dihydrochloride *R*), dissolved in buffer B. Record the rate of change in absorbance per minute for 2 min to 10 min at the wavelength specific for the substrate used. Prepare a blank for each mixture of sample or standard using buffer B instead of prekallikrein substrate.

Correct $\Delta A/\text{min}$ by subtracting the value obtained for the corresponding blank. Plot a calibration curve using the values thus obtained for the reference preparation and the respective concentrations; use the curve to determine the PKA activity of the preparation to be examined.

Buffer A

<i>Tris(hydroxymethyl)aminomethane R</i>	6.055 g
<i>Sodium chloride R</i>	1.17 g
<i>Hexadimethrine bromide R</i>	50 mg
<i>Sodium azide R</i>	0.100 g

Dissolve the ingredients in *water R*, adjust to pH 8.0 with 2 M *hydrochloric acid* and dilute to 1000 ml with *water R*.

Buffer B

<i>Tris(hydroxymethyl)aminomethane R</i>	6.055 g
<i>Sodium chloride R</i>	8.77 g

Dissolve the ingredients in *water R*, adjust to pH 8.0 with 2 M *hydrochloric acid* and dilute to 1000 ml with *water R*.

01/2005:20616

2.6.16. TESTS FOR EXTRANEIOUS AGENTS IN VIRAL VACCINES FOR HUMAN USE

In those tests that require prior neutralisation of the virus, use specific antibodies of non-human, non-simian origin; if the virus has been propagated in avian tissues, the antibodies must also be of non-avian origin. To prepare antiserum, use an immunising antigen produced in cell culture from a

species different from that used for the production of the vaccine and free from extraneous agents. Where the use of SPF eggs is prescribed, the eggs are obtained from a flock free from specified pathogens (5.2.2).

VIRUS SEED LOT

Take samples of the virus seed lot at the time of harvesting and, if they are not tested immediately, keep them at a temperature below –40 °C.

Adult mice. Inoculate each of at least ten adult mice, each weighing 15 g to 20 g, intracerebrally with 0.03 ml and intraperitoneally with 0.5 ml of the virus seed lot. Observe the mice for at least 21 days. Carry out an autopsy of all mice that die after the first 24 h of the test or that show signs of illness and examine for evidence of viral infection, both by direct macroscopical observation and by subinoculation of appropriate tissue suspensions by the intracerebral and intraperitoneal routes into at least five additional mice which are observed for 21 days. The virus seed lot complies with the test if no mouse shows evidence of infection attributable to the seed lot. The test is not valid unless at least 80 per cent of the original inoculated mice survive the observation period.

Suckling mice. Inoculate each of at least twenty mice, less than 24 h old, intracerebrally with 0.01 ml and intraperitoneally with at least 0.1 ml of the virus seed lot. Observe the mice daily for at least 14 days. Carry out an autopsy of all mice that die after the first 24 h of the test or that show signs of illness and examine for evidence of viral infection, both by direct macroscopical observation and by subinoculation of appropriate tissue suspensions by the intracerebral and intraperitoneal routes into at least five additional suckling mice which are observed daily for 14 days. The virus seed lot passes the test if no mouse shows evidence of infection attributable to the seed lot. The test is not valid unless at least 80 per cent of the original inoculated mice survive the observation period.

Guinea-pigs. Inoculate intraperitoneally into each of at least five guinea pigs, each weighing 350 g to 450 g, 5.0 ml of the virus seed lot. Observe the animals for at least 42 days for signs of disease. Carry out an autopsy of all guinea-pigs that die after the first 24 h of the test, or that show signs of illness and examine macroscopically; examine the tissues both microscopically and culturally for evidence of infection. Kill animals that survive the observation period and examine in a similar manner. The virus seed lot passes the test if no guinea-pig shows evidence of infection attributable to the seed lot. The test is not valid unless at least 80 per cent of the guinea-pigs survive the observation period.

VIRUS SEED LOT AND VIRUS HARVESTS

Take samples at the time of harvesting and, if not tested immediately, keep them at a temperature below –40 °C.

Bacterial and fungal sterility. A 10 ml sample complies with the test for sterility (2.6.1).

Mycoplasmas. A 10 ml sample complies with the test for mycoplasmas (2.6.7).

Mycobacteria (2.6.2). A 5 ml sample is tested for the presence of *Mycobacterium spp.* by culture methods known to be sensitive for the detection of these organisms.

Test in cell culture for other extraneous agents. Neutralised samples equivalent, unless otherwise prescribed, to 500 human doses of vaccine or 50 ml, whichever is the greater, are tested for the presence of extraneous agents by inoculation into continuous simian kidney and human cell cultures. If the virus is grown in human diploid cells, the neutralised virus harvest is also tested on a separate

culture of the diploid cells. If the vaccine virus is grown in a cell system other than simian or human, cells of that species, from a separate batch, are also inoculated. The cells are incubated at 36 ± 1 °C and observed for a period of 14 days. The virus seed lot or harvest passes the tests if none of the cell cultures shows evidence of the presence of any extraneous agents not attributable to accidental contamination. The test is not valid unless at least 80 per cent of the cell cultures remain viable.

Avian viruses (only required for virus propagated in avian tissues). Neutralise a sample equivalent to 100 human doses or 10 ml, whichever is the greater. Using 0.5 ml per egg, inoculate a group of fertilised SPF eggs, 9 to 11 days old, by the allantoic route and a second group, 5 to 7 days old, into the yolk sac. Incubate for 7 days. The virus seed lot or harvest complies with the test if the allantoic and yolk sac fluids show no sign of the presence of any haemagglutinating agent and if all embryos and chorio-allantoic membranes, examined for gross pathology, are normal. The test is not valid unless at least 80 per cent of the inoculated eggs survive for 7 days.

PRODUCTION CELL CULTURE: CONTROL CELLS

Examine the control cells microscopically for freedom from any virus causing cytopathic degeneration throughout the time of incubation of the inoculated production cell cultures or for not less than 14 days beyond the time of inoculation of the production vessels, whichever is the longer. The test is not valid unless at least 80 per cent of the control cell cultures survive to the end of the observation period.

At 14 days or at the time of the last virus harvest, whichever is the longer, carry out the tests described below.

Test for haemadsorbing viruses. Examine not fewer than 25 per cent of the control cultures for the presence of haemadsorbing viruses by the addition of guinea-pig red blood cells. If the guinea-pig red blood cells have been stored, they shall have been stored at 5 ± 3 °C for not more than 7 days. Read half of the cultures after incubation at 5 ± 3 °C for 30 min and the other half after incubation at 20 °C to 25 °C for 30 min. No evidence of haemadsorbing agents is found.

Tests in cell cultures for other extraneous agents. Pool the supernatant fluids from the control cells and examine for the presence of extraneous agents by inoculation of simian kidney and human cell cultures. If the vaccine virus is grown in a cell system other than simian or human, cells of that species, but from a separate batch, are also inoculated. In each cell system, at least 5 ml is tested. Incubate the inoculated cultures at a temperature of 36 ± 1 °C and observe for a period of 14 days. No evidence of extraneous agents is found.

If the production cell culture is maintained at a temperature different from 36 ± 1 °C, a supplementary test for extraneous agents is carried out at the production temperature using the same type of cells as used for growth of the virus.

Avian leucosis viruses (required only if the virus is propagated in avian tissues). Carry out a test for avian leucosis viruses using 5 ml of the supernatant fluid from the control cells.

CONTROL EGGS

Haemagglutinating agents. Examine 0.25 ml of the allantoic fluid from each egg for haemagglutinating agents by mixing directly with chicken red blood cells and after a passage in SPF eggs carried out as follows: inoculate a 5 ml sample of the pooled amniotic fluids from the control eggs in 0.5 ml volumes into the allantoic cavity and into the amniotic cavity

of SPF eggs. The control eggs comply with the test if no evidence of the presence of haemagglutinating agents is found in either test.

Avian leucosis viruses. Use a 10 ml sample of the pooled amniotic fluids from the control eggs. Carry out amplification by five passages in leucosis-free chick-embryo cell cultures; carry out a test for avian leucosis using cells from the fifth passage. The control eggs comply with the test if no evidence of the presence of avian leucosis viruses is found.

Other extraneous agents. Inoculate 5 ml samples of the pooled amniotic fluids from the control eggs into human and simian cell cultures. Observe the cell cultures for 14 days. The control eggs comply with the test if no evidence of the presence of extraneous agents is found. The test is not valid unless 80 per cent of the inoculated cultures survive to the end of the observation period.

01/2005:20617

2.6.17. TEST FOR ANTICOMPLEMENTARY ACTIVITY OF IMMUNOGLOBULIN

For the measurement of anticomplementary activity (ACA) of immunoglobulin, a defined amount of test material (10 mg of immunoglobulin) is incubated with a defined amount of guinea-pig complement (20 CH₅₀) and the remaining complement is titrated; the anticomplementary activity is expressed as the percentage consumption of complement relative to the complement control considered as 100 per cent.

The haemolytic unit of complement activity (CH₅₀) is the amount of complement that, in the given reaction conditions, will produce the lysis of 2.5×10^8 out of a total of 5×10^8 optimally sensitised red blood cells.

Magnesium and calcium stock solution. Dissolve 1.103 g of calcium chloride R and 5.083 g of magnesium chloride R in water R and dilute to 25 ml with the same solvent.

Barbital buffer stock solution. Dissolve 207.5 g of sodium chloride R and 25.48 g of barbital sodium R in 4000 ml of water R and adjust to pH 7.3 using 1 M hydrochloric acid. Add 12.5 ml of magnesium and calcium stock solution and dilute to 5000 ml with water R. Filter through a membrane filter (pore size 0.22 µm). Store at 4 °C in glass containers.

Gelatin solution. Dissolve 12.5 g of gelatin R in about 800 ml of water R and heat to boiling in a water-bath. Cool to 20 °C and dilute to 10 litres with water R. Filter through a membrane filter (pore size: 0.22 µm). Store at 4 °C. Use clear solutions only.

Citrate solution. Dissolve 8.0 g of sodium citrate R, 4.2 g of sodium chloride R and 20.5 g of glucose R in 750 ml of water R. Adjust to pH 6.1 using a 100 g/l solution of citric acid R and dilute to 1000 ml with water R.

Gelatin barbital buffer solution. Add 4 volumes of gelatin solution to 1 volume of barbital buffer stock solution and mix. Adjust to pH 7.3, if necessary, using 1 M sodium hydroxide or 1 M hydrochloric acid. Maintain at 4 °C. Prepare fresh solutions daily.

Stabilised sheep blood. Collect one volume of sheep blood into one volume of citrate solution and mix. Store at 4 °C for not less than 7 days and not more than 28 days. (Stabilised sheep blood and sheep red blood cells are available from a number of commercial sources.)

Haemolysin. Antiserum against sheep red blood cells prepared in rabbits. (Such antisera are available from a number of commercial sources.)

Guinea-pig complement. Prepare a pool of serum from the blood of not fewer than ten guinea-pigs. Separate the serum from the clotted blood by centrifugation at about 4 °C. Store the serum in small amounts below –70 °C.

METHOD

Preparation of standardised 5 per cent sheep red blood cell suspension. Separate sheep red blood cells by centrifuging an appropriate volume of stabilised sheep blood and wash the cells at least three times with gelatin barbital buffer solution and prepare as a 5 per cent V/V suspension in the same solution. Measure the cell density of the suspension as follows: add 0.2 ml to 2.8 ml of *water R* and centrifuge the lysed solution for 5 min at 1000 *g*; the cell density is suitable if the absorbance (2.2.25) of the supernatant liquid at 541 nm is 0.62 ± 0.01. Correct the cell density by adding gelatin barbital buffer solution according to the formula:

$$V_f = \frac{V_i \times A}{0.62}$$

V_f = final adjusted volume,

V_i = the initial volume,

A = absorbance of the original suspension at 541 nm.

The adjusted suspension contains about 1 × 10⁹ cells/ml.

Haemolysin titration

Prepare haemolysin dilutions as shown in Table 2.6.17-1.

Table 2.6.17-1

Required dilution of haemolysin	Prepared using		
	Gelatin barbital buffer solution		Haemolysin
	Volume (millilitres)	Dilution (1: ...)	Volume (millilitres)
7.5	0.65	undiluted	0.1
10	0.90	undiluted	0.1
75	1.80	7.5	0.2
100	1.80	10	0.2
150	1.00	75	1.0
200	1.00	100	1.0
300	1.00	150	1.0
400	1.00	200	1.0
600	1.00	300	1.0
800	1.00	400	1.0
1200	1.00	600	1.0
1600	1.00	800	1.0
2400	1.00	1200	1.0
3200*	1.00	1600	1.0
4800*	1.00	2400	1.0

* discard 1.0 ml of the mixture.

Add 1.0 ml of 5 per cent sheep red blood cell suspension to each tube of the haemolysin dilution series, starting at the 1:75 dilution, and mix. Incubate at 37 °C for 30 min.

Transfer 0.2 ml of each of these incubated mixtures to new tubes and add 1.10 ml of gelatin barbital buffer solution and 0.2 ml of diluted guinea-pig complement (for example, 1:150). Perform this in duplicate.

As the unhaemolysed cell control, prepare three tubes with 1.4 ml of gelatin barbital buffer solution and 0.1 ml of 5 per cent sheep red blood cell suspension.

As the fully haemolysed control, prepare three tubes with 1.4 ml of *water R* and 0.1 ml of 5 per cent sheep red cell suspension.

Incubate all tubes at 37 °C for 60 min and centrifuge at 1000 *g* for 5 min. Measure the absorbance (2.2.25) of the supernatants at 541 nm and calculate the percentage degree of haemolysis in each tube using the expression:

$$\frac{A_a - A_1}{A_b - A_1} \times 100$$

A_a = absorbance of tubes with haemolysin dilution,

A_b = mean absorbance of the three tubes with full haemolysis,

A_1 = mean absorbance of the three tubes with no haemolysis.

Plot the percentage degree of haemolysis as the ordinate against the corresponding reciprocal value of the haemolysin dilution as the abscissa on linear graph paper. Determine the optimal dilution of the haemolysin from the graph by inspection. Select a dilution such that further increase in the amount of haemolysin does not cause appreciable change in the degree of haemolysis. This dilution is defined as one minimal haemolytic unit (1 MHU) in 1.0 ml. The optimal haemolytic haemolysin dilution for preparation of sensitised sheep red blood cells contains 2 MHU/ml.

The haemolysin titration is not valid unless the maximum degree of haemolysis is 50 per cent to 70 per cent. If the maximum degree of haemolysis is not in this range, repeat the titration with more or less diluted complement solution.

Preparation of optimised sensitised sheep red blood cells (haemolytic system)

Prepare an appropriate volume of diluted haemolysin containing 2 MHU/ml and an equal volume of standardised 5 per cent sheep red blood cell suspension. Add the haemolysin dilution to the standardised cell suspension and mix. Incubate at 37 °C for 15 min, store at 2 °C to 8 °C and use within 6 h.

Titration of complement

Prepare an appropriate dilution of complement (for example, 1:250) with gelatin barbital buffer solution and perform the titration in duplicate as shown in Table 2.6.17-2.

Add 0.2 ml of sensitised sheep red blood cells to each tube, mix well and incubate at 37 °C for 60 min. Cool the tubes in an ice-bath and centrifuge at 1000 *g* for 5 min. Measure the absorbance of the supernatant liquid at 541 nm and calculate the degree of haemolysis (Y) using the expression:

$$\frac{A_c - A_1}{A_b - A_1}$$

A_c = absorbance of tubes 1 to 12,

A_b = mean absorbance of tubes with 100 per cent haemolysis,

A_1 = mean absorbance of cell controls with 0 per cent haemolysis.

Plot $Y/(1-Y)$ as the abscissa against the amount of diluted complement in millilitres as the ordinate on log-log graph paper. Fit the best line to the points and determine the

ordinate for the 50 per cent haemolytic complement dose where $Y/(1-Y) = 1.0$. Calculate the activity in haemolytic units (CH_{50}/ml) from the expression:

$$\frac{C_d}{C_a \times 5}$$

- C_d = reciprocal value of the complement dilution,
 C_a = volume of diluted complement in millilitres resulting in 50 per cent haemolysis,
 5 = scaling factor to take account of the number of red blood cells.

The test is not valid unless the plot is a straight line between 15 per cent and 85 per cent haemolysis and the slope is 0.15 to 0.40, and preferably 0.18 to 0.30.

Table 2.6.17-2

Tube Number	Volume of diluted complement in millilitres (for example 1:250)	Volume of gelatin barbital buffer solution in millilitres
1	0.1	1.2
2	0.2	1.1
3	0.3	1.0
4	0.4	0.9
5	0.5	0.8
6	0.6	0.7
7	0.7	0.6
8	0.8	0.5
9	0.9	0.4
10	1.0	0.3
11	1.1	0.2
12	1.2	0.1
Three tubes as cell control at 0 per cent haemolysis	—	1.3
Three tubes at 100 per cent haemolysis	—	1.3 ml of water

Test for anticomplementary activity

Prepare a complement dilution having 100 CH_{50}/ml by diluting titrated guinea-pig complement with gelatin barbital buffer solution. If necessary, adjust the immunoglobulin to be examined to pH 7. Prepare incubation mixtures as follows for an immunoglobulin containing 50 mg/ml:

Table 2.6.17-3

	Immunoglobulin to be examined	Complement control (in duplicate)
Immunoglobulin (50 mg/ml)	0.2 ml	—
Gelatin barbital buffer	0.6 ml	0.8 ml
Complement	0.2 ml	0.2 ml

Carry out the test on the immunoglobulin to be examined and prepare ACA negative and positive controls using *human immunoglobulin BRP*, as indicated in the leaflet accompanying the reference preparation. Higher or lower volumes of sample and of gelatin barbital buffer solution are added if the immunoglobulin concentration varies from 50 mg/ml; for example, 0.47 ml of gelatin barbital buffer solution is added to 0.33 ml of immunoglobulin containing 30 mg/ml to give 0.8 ml. Close the tubes and incubate at 37 °C for 60 min. Add 0.2 ml of each incubation mixture to 9.8 ml of gelatin barbital buffer solution to dilute the complement. Perform complement titrations as described

above on each tube to determine the remaining complement activity (Table 2.6.17-2). Calculate the anticomplementary activity of the preparation to be examined relative to the complement control considered as 100 per cent, from the expression:

$$\frac{a - b}{a} \times 100$$

- a = mean complement activity (CH_{50}/ml) of complement control,
 b = complement activity (CH_{50}/ml) of tested sample.

The test is not valid unless:

- the anticomplementary activities found for ACA negative control and ACA positive control are within the limits stated in the leaflet accompanying the reference preparation,
- the complement activity of the complement control (a) is in the range 80 to 120 CH_{50}/ml .

01/2005:20618

2.6.18. TEST FOR NEUROVIRULENCE OF LIVE VIRUS VACCINES

For each test, use not fewer than ten monkeys that are seronegative for the virus to be tested. For each monkey, inject not more than 0.5 ml of the material to be examined into the thalamic region of each hemisphere, unless otherwise prescribed. The total amount of virus inoculated in each monkey must be not less than the amount contained in the recommended single human dose of the vaccine. As a check against the introduction of wild neurovirulent virus, keep a group of not fewer than four control monkeys as cage-mates or in the immediate vicinity of the inoculated monkeys. Observe the inoculated monkeys for 17 to 21 days for symptoms of paralysis and other evidence of neurological involvement; observe the control monkeys for the same period plus 10 days. Animals that die within 48 h of injection are considered to have died from non-specific causes and may be replaced. The test is not valid if: more than 20 per cent of the inoculated monkeys die from nonspecific causes; serum samples taken from the control monkeys at the time of inoculation of the test animals and 10 days after the latter are killed show evidence of infection by wild virus of the type to be tested or by measles virus. At the end of the observation period, carry out autopsy and histopathological examinations of appropriate areas of the brain for evidence of central nervous system involvement. The material complies with the test if there is no unexpected clinical or histopathological evidence of involvement of the central nervous system attributable to the inoculated virus.

01/2005:20619

2.6.19. TEST FOR NEUROVIRULENCE OF POLIOMYELITIS VACCINE (ORAL)

Monkeys used in the neurovirulence test comply with the requirements given in the monograph on *Poliomyelitis vaccine oral (0215)* and weigh not less than 1.5 kg. The pathogenicity for *Macaca* or *Cercopithecus* monkeys is tested in comparison with that of a reference virus preparation for neurovirulence testing by inoculation into the lumbar region of the central nervous system after sedation with a suitable substance, for example, ketamine hydrochloride. A sample of serum taken before

the injection shall be shown not to contain neutralising antibody at a dilution of 1:4 when tested against not more than 1000 CCID₅₀ of each of the three types of poliovirus.

Number of monkeys. The vaccine and the appropriate homotypic reference virus are tested concurrently in the same group of monkeys. Equal numbers of animals are inoculated with the vaccine to be examined and the reference preparation. The animals are allocated randomly to treatment groups and cages and their identity is coded so that the treatment received by each animal is concealed from the observers and the evaluators of the sections. The number of monkeys inoculated is such that in the evaluation of both the vaccine and the reference preparation not fewer than eleven positive monkeys are included for type 1 and type 2 virus and not fewer than eighteen positive monkeys for type 3 virus (positive monkeys are those that show specific neuronal lesions of poliovirus in the central nervous system). More than one batch of vaccine may be tested with the same homotypic reference. Monkeys from the same quarantine group are used wherever possible, otherwise monkeys from two groups are used and equal numbers from each group are treated with the vaccine and the reference preparation. If the test is carried out on two working days, an equal number of monkeys from each group are inoculated on each day with the vaccine and the homotypic reference preparation.

Virus content. The virus contents of the vaccine and the homotypic reference preparation are adjusted so as to be as near as possible equal and between 10^{5.5} and 10^{6.5} CCID₅₀/0.1 ml.

Observation. All monkeys are observed for 17 to 22 days for signs of poliomyelitis or other virus infection. Monkeys that survive the first 24 h but die before the 11th day after inoculation are autopsied to determine whether poliomyelitis was the cause of death. Animals that die from causes other than poliomyelitis are excluded from the evaluation. Animals that become moribund or are severely paralysed are killed and autopsied. All animals that survive until the end of the observation period are autopsied. The test is not valid if more than 20 per cent of the animals show intercurrent infection during the observation period.

Number of sections examined. The lumbar cord, the cervical cord, the lower and upper medulla oblongata, the midbrain, the thalamus and the motor cortex of each monkey, as a minimum, are subjected to histological examination. Sections are cut with a thickness of 15 µm and stained with gallocyenin. The minimum number of sections examined is as follows:

- 12 sections representative of the whole of the lumbar enlargement,
- 10 sections representative of the whole of the cervical enlargement,
- 2 sections from the medulla oblongata,
- 1 section from the pons and cerebellum,
- 1 section from the midbrain,
- 1 section from the left and the right of the thalamus,
- 1 section from the left and the right motor cerebral cortex.

Scoring of virus activity. For the evaluation of virus activity in the hemisections of the spinal cord and brain-stem, a score system for the severity of lesions is used, differentiating cellular infiltration and destruction of neurons as follows:

1. Cellular infiltration only (the monkey is not counted as positive),
2. Cellular infiltration with minimal neuronal damage,
3. Cellular infiltration with extensive neuronal damage,

4. Massive neuronal damage with or without cellular infiltration.

The scores are recorded on a standard form⁽¹⁾. A monkey with neuronal lesions in the sections but that shows no needle tract is counted as positive. A monkey showing a needle tract in the sections, but no neuronal lesions is not regarded as positive. A section that shows damage from trauma but no specific virus lesions is not included in the score.

Severity scores are based on hemisection readings of the lumbar (L), cervical (C) and brain (B) histological sections. The lesion score (LS) for each positive monkey is calculated as follows:

$$LS = \frac{\left[\begin{array}{c} \text{Sum of} \\ \text{L score} \\ \text{Number of} \\ \text{hemisections} \end{array} \right] + \left[\begin{array}{c} \text{Sum of} \\ \text{C score} \\ \text{Number of} \\ \text{hemisections} \end{array} \right] + \left[\begin{array}{c} \text{Sum of} \\ \text{B score} \\ \text{Number of} \\ \text{hemisections} \end{array} \right]}{3}$$

A mean lesion score is calculated for each group of positive monkeys.

Evaluation. The comparison of the virus activity in the vaccine and the reference preparation is based on the activity in the lumbar enlargement of the cord and the degree of spread of activity from this region to the cervical enlargement and the brain. Acceptance or rejection is based on the total score of all the test animals. Individual animals showing evidence of unusually high activity, either in the lumbar region or as the result of spread from this region, are also taken into consideration in the final evaluation. The monovalent bulk passes the test if the required number of animals is positive and if none of the clinical and histopathological examinations shows a significant difference in pathogenicity between the vaccine virus and the reference material. Criteria for acceptance are given below.

Criteria. A suitable number of neurovirulence qualifying tests (for example, four tests) is carried out on each reference vaccine (types 1, 2 and 3) to provide data on the activity of such vaccines that will serve as the basis of the criteria for vaccines to be tested. The overall mean lesion score (*M*) for the replicate tests on each reference virus is calculated together with the pooled estimate of the within-test variance (*s*²) and the within-test deviation (*s*).

Validity criteria for the results of a test on a reference preparation are established on the basis of the cumulative data from the qualifying tests. No generally applicable criteria can be given; for laboratories with limited experience, the following empirical method for setting acceptable limits for the mean lesion score for the reference preparation (*X*_{ref}) may be helpful (see Table 2.6.19-1):

Table 2.6.19-1

	Lower limit	Upper limit
Types 1 and 2	$M - s$	$M + s$
Type 3	$M - s/2$	$M + s$

If the mean lesion score for the vaccine to be tested is *X*_{test} and *C*₁, *C*₂ and *C*₃ are constants determined as described below, then:

the vaccine is not acceptable if:

$$X_{\text{test}} - X_{\text{ref}} > C_1$$

the vaccine may be retested once if:

$$C_1 < X_{\text{test}} - X_{\text{ref}} < C_2$$

(1) A suitable form is shown in the Requirements for Poliomyelitis Vaccine (Oral) (Requirements for Biological Substances No. 7, World Health Organization).

If the vaccine is retested, the means of the lesion scores for the vaccine to be tested and the reference vaccine are recalculated. The vaccine is not acceptable if:

$$\frac{X_{(\text{test } 1 + \text{test } 2)} - X_{(\text{ref } 1 + \text{ref } 2)}}{2} > C_3$$

The constants C_1 , C_2 and C_3 are calculated from the expressions:

$$C_1 = 2.3 \sqrt{\frac{2s^2}{N_1}}$$

$$C_2 = 2.6 \sqrt{\frac{2s^2}{N_1}}$$

$$C_3 = 1.6 \sqrt{\frac{2s^2}{N_1}}$$

N_1 = number of positive monkeys per vaccine test,

N_2 = number of positive monkeys in the two tests,

2.3 = normal deviate at the 1 per cent level,

2.6 = normal deviate at the 0.5 per cent level,

1.6 = normal deviate at the 5 per cent level.

A neurovirulence test in which the mean lesion score for the reference (X_{ref}) is not compatible with previous experience is not used for assessing a test vaccine. If the test is valid, the mean lesion score for the vaccine to be tested (X_{test}) is calculated and compared with that of the homotypic reference vaccine.

01/2005:20620

2.6.20. ANTI-A AND ANTI-B HAEMAGGLUTININS (INDIRECT METHOD)

Prepare in duplicate serial dilutions of the preparation to be examined in a 9 g/l solution of *sodium chloride R*. To each dilution of one series add an equal volume of a 5 per cent V/V suspension of group A₁ red blood cells previously washed three times with the sodium chloride solution. To each dilution of the other series add an equal volume of a 5 per cent V/V suspension of group B red blood cells previously washed three times with the sodium chloride solution. Incubate the suspensions at 37 °C for 30 min then wash the cells three times with the sodium chloride solution. Leave the cells in contact with a polyvalent anti-human globulin reagent for 30 min. Without centrifuging, examine each suspension for agglutination under a microscope.

01/2005:20621
corrected

2.6.21. NUCLEIC ACID AMPLIFICATION TECHNIQUES

1. INTRODUCTION

Nucleic acid amplification techniques are based on two different approaches:

1. amplification of a target nucleic acid sequence using, for example, polymerase chain reaction (PCR), ligase chain reaction (LCR), or isothermal ribonucleic acid (RNA) amplification,

2. amplification of a hybridisation signal using, for example, for deoxyribonucleic acid (DNA), the branched DNA (bDNA) method. In this case signal amplification is achieved without subjecting the nucleic acid to repetitive cycles of amplification.

In this general chapter, the PCR method is described as the reference technique. Alternative methods may be used, if they comply with the quality requirements described below.

2. SCOPE

This section establishes the requirements for sample preparation, *in vitro* amplification of DNA sequences and detection of the specific PCR product. With the aid of PCR, defined DNA sequences can be detected. RNA sequences can also be detected following reverse transcription of the RNA to complementary DNA (cDNA) and subsequent amplification.

3. PRINCIPLE OF THE METHOD

PCR is a procedure that allows specific *in vitro* amplification of segments of DNA or of RNA after reverse transcription into cDNA.

Following denaturation of double-stranded DNA into single-stranded DNA, two synthetic oligonucleotide primers of opposite polarity, anneal to their respective complementary sequences in the DNA to be amplified. The short double-stranded regions which form as a result of specific base pairing between the primers and the complementary DNA sequence, border the DNA segment to be amplified and serve as starting positions for *in vitro* DNA synthesis by means of a heat-stable DNA polymerase.

Amplification of the DNA occurs in cycles consisting of:

- heat denaturation of the nucleic acid (target sequence) into two single strands;
- specific annealing of the primers to the target sequence under suitable reaction conditions;
- extension of the primers, which are bound to both single strands, by DNA polymerase at a suitable temperature (DNA synthesis).

Repeated cycles of heat denaturation, primer annealing and DNA synthesis results in an exponential amplification of the DNA segment limited by the primers.

The specific PCR product known as an amplicon can be detected by a variety of methods of appropriate specificity and sensitivity.

4. TEST MATERIAL

Because of the high sensitivity of PCR, the samples must be optimally protected against external contamination with target sequences. Sampling, storage and transport of the test material are performed under conditions that minimise degradation of the target sequence. In the case of RNA target sequences, special precautions are necessary since RNA is highly sensitive to degradation by ribonucleases. Care must be taken since some added reagents, such as anticoagulants or preservatives, may interfere with the test procedure.

5. TEST METHOD

5.1. Prevention of contamination

The risk of contamination requires a strict segregation of the areas depending on the material handled and the technology used. Points to consider include movement of personnel, gowning, material flow and air supply and decontamination procedures.

The system should be sub-divided into compartments such as:

- master-mix area (area where exclusively template-free material is handled, e.g. primers, buffers, etc.),

- pre-PCR (area where reagents, samples and controls are handled),
- PCR amplification (amplified material is handled in a closed system),
- post-PCR detection (the only area where the amplified material is handled in an open system).

5.2. Sample preparation

When preparing samples, the target sequence to be amplified needs to be efficiently extracted or liberated from the test material in a reproducible manner and in such a way that amplification under the selected reaction conditions is possible. A variety of physico-chemical extraction procedures and/or enrichment procedures may be employed.

Additives present in test material may interfere with PCR. The procedures described under 7.3.2. must be used as a control for the presence of inhibitors originating from the test material.

In the case of RNA-templates, care must be taken to avoid ribonuclease activity.

5.3. Amplification

PCR amplification of the target sequence is conducted under optimised cycling conditions (temperature profile for denaturation of double-stranded DNA, annealing and extension of primers; incubation times at selected temperatures; ramp rates). These depend on various parameters such as:

- the length and base composition of primer and target sequences;
- the type of DNA polymerase, buffer composition and reaction volume used for the amplification;
- the type of thermocycler used and the thermal conductivity rate between the apparatus, reaction tube and reaction fluid.

5.4. Detection

The amplicon generated by PCR may be identified by size, sequence, chemical modification or a combination of these parameters. Characterisation by size may be achieved by gel electrophoresis (using agarose or polyacrylamide slab gels or capillary electrophoresis) or column chromatography (for example, HPLC). Characterisation by sequence composition may be achieved by the specific hybridisation of probes having a sequence complementary to the target sequence or by cleavage of the amplified material reflecting target-specific restriction-enzyme sites. Characterisation by chemical modification may be achieved, for example, by incorporation of a fluorophore into the amplicons and subsequent detection of fluorescence following excitation.

Detection of amplicons may also be achieved by using probes labelled to permit a subsequent radioisotopic or immuno-enzyme-coupled detection.

6. EVALUATION AND INTERPRETATION OF RESULTS

A valid result is obtained within a test only if the positive control(s) is unambiguously positive and the negative control(s) is unambiguously negative. Due to the very high sensitivity of the PCR method and the inherent risk of contamination, it is necessary to confirm positive results by repeating the complete test procedure in duplicate, where possible on a new aliquot of the sample. The sample is considered positive if at least one of the repeat tests gives a positive result.

7. QUALITY ASSURANCE

7.1. Validation of the PCR assay system

The validation programme must include validation of instrumentation and the PCR method employed. Reference should be made to the *ICH guidelines* (topic Q2B) Validation of Analytical Method: Methodology.

Appropriate official working reference preparations or in-house reference preparations calibrated against International Standards for the target sequences for which the test system will be used are indispensable for validation of a PCR test.

During validation the positive cut-off point must be determined. The positive cut-off point is defined as the minimum number of target sequences per volume sample which can be detected in 95 per cent of test runs. The positive cut-off point depends on interrelated factors such as the volume of the sample extracted and the efficacy of the extraction methodology, the transcription of the target RNA into cDNA, the amplification process and the detection.

To define the detection limit of the assay system, reference must be made to the positive cut-off point for each target sequence and the test performance above and below the positive cut-off point.

7.2. Quality control of reagents

All reagents crucial for the methodology used have to be controlled prior to use in routine applications. Their acceptance/withdrawal is based on pre-defined quality criteria.

Primers are a crucial component of the PCR assay and as such their design, purity and the validation of their use in a PCR assay require careful attention. Each new batch of primers is tested for specificity, amplification efficiency and absence of inhibitory impurities before acceptance. Primers may be modified (for example, by conjugation with a fluorophore or antigen) in order to permit a specific method of detection of the amplicon, provided such modifications do not inhibit accurate and efficient amplification of the target sequence.

7.3. Run controls

7.3.1. External controls

In order to minimise the risk of contamination and to ensure adequate sensitivity, the following external controls are included in each PCR test:

- positive control: this contains a defined number of target-sequence copies, the number being determined individually for each assay system and indicated as a multiple of the positive cut-off value of the test system;
- negative control: a sample of the same matrix already proven to be free of the target sequences;

7.3.2. Internal control

Internal controls are defined nucleic acid sequences containing the primer binding sites. Internal controls must be amplified with similar efficacy as the target sequence to be tested, but the amplicons must be clearly discernible. Internal controls must be of the same type of nucleic acid (DNA/RNA) as the material to be tested. The internal control is preferably added to the test material before isolating the nucleic acid and therefore acts as an overall control (extraction, reverse transcription, amplification, detection).

7.4. External quality assessment

Participation in external quality assessment programmes is an important PCR quality assurance procedure for each laboratory and each operator.

The following section is published for information.

Validation of nucleic acid amplification techniques (NAT) for the detection of hepatitis C virus (HCV) RNA in plasma pools: guidelines

1. SCOPE

The majority of nucleic acid amplification analytical procedures are qualitative (quantal) tests for the presence of nucleic acid with some quantitative tests (either in-house or commercial) being available. For the detection of HCV RNA contamination of plasma pools, qualitative tests are adequate and may be considered to be a limit test for the control of impurities as described in the *Pharmeuropa* Technical Guide for the elaboration of monographs, December 1999, Chapter III "Validation of analytical procedures". These guidelines describe methods to validate only qualitative nucleic acid amplification analytical procedures for assessing HCV RNA contamination of plasma pools. Therefore, the two characteristics regarded as the most important for validation of the analytical procedure are the specificity and the detection limit. In addition, the robustness of the analytical procedure should be evaluated.

However, this document may also be used as a basis for the validation of nucleic acid amplification in general.

For the purpose of this document, an analytical procedure is defined as the complete procedure from extraction of nucleic acid to detection of the amplified products.

Where commercial kits are used for part of or the complete analytical procedure, documented validation points already covered by the kit manufacturer can substitute for the validation by the user. Nevertheless, the performance of the kit with respect to its intended use has to be demonstrated by the user (e.g. detection limit, robustness, cross contamination).

2. SPECIFICITY

Specificity is the ability to unequivocally assess nucleic acid in the presence of components which may be expected to be present.

The specificity of nucleic acid amplification analytical procedures is dependent on the choice of primers, the choice of probe (for analysis of the final product) and the stringency of the test conditions (for both the amplification and detection steps).

When designing primers and probes, the specificity of the primers and probes to detect only HCV RNA should be investigated by comparing the chosen sequences with sequences in published data banks. For HCV, primers (and probes) will normally be chosen from areas of the 5' non-coding region of the HCV genome which are highly conserved for all genotypes.

The amplified product should be unequivocally identified by using one of a number of methods such as amplification with nested primers, restriction enzyme analysis, sequencing or hybridisation with a specific probe.

In order to validate the specificity of the analytical procedure, at least 100 HCV RNA-negative plasma pools should be tested and shown to be non-reactive. Suitable samples of non-reactive pools are available from the European Directorate for the Quality of Medicines.

The ability of the analytical procedure to detect all HCV genotypes will again depend on the choice of primers, probes and method parameters. This ability should be demonstrated using characterised reference panels. However, in view of

the difficulty in obtaining samples of some genotypes (e.g. genotype 6), the most prevalent genotypes (e.g. genotype 1 and 3 in Europe) should be detected at a suitable level.

3. DETECTION LIMIT

The detection limit of an individual analytical procedure is the lowest amount of nucleic acid in a sample which can be detected but not necessarily quantitated as an exact value.

The nucleic acid amplification analytical procedure used for the detection of HCV RNA in plasma pools usually yields qualitative results. The number of possible results is limited to two, either positive or negative. Although the determination of the detection limit is recommended, for practical purposes, a positive cut-off point should be determined for the nucleic acid amplification analytical procedure. The positive cut-off point (as defined in the General Chapter (2.6.21)) is the minimum number of target sequences per volume sample which can be detected in 95 per cent of test runs. This positive cut-off point is influenced by the distribution of viral genomes in the individual samples being tested and by factors such as enzyme efficiency and can result in different 95 per cent cut-off values for individual analytical test runs.

In order to determine the positive cut-off point, a dilution series of a working reagent or of the *hepatitis C virus RNA for NAT testing BRP*, which has been calibrated against the WHO HCV International Standard 96/790, should be tested on different days to examine variation between test runs. At least 3 independent dilution series should be tested with a sufficient number of replicates at each dilution to give a total number of 24 test results for each dilution to enable a statistical analysis of the results.

For example, a laboratory could test 3 dilution series on different days with 8 replicates for each dilution, 4 dilution series on different days with 6 replicates for each dilution, or 6 dilution series on different days with 4 replicates for each dilution. In order to keep the number of dilutions at a manageable level, a preliminary test (using, for example, log dilutions of the plasma pool sample) should be done in order to obtain a preliminary value for the positive cut-off point (i.e. the highest dilution giving a positive signal). The range of dilutions can then be chosen around the predetermined preliminary cut-off point (using, for example, a dilution factor of 0.5 log or less and a negative plasma pool for the dilution matrix). The concentration of HCV RNA which can be detected in 95 per cent of test runs can then be calculated using an appropriate statistical evaluation.

These results may also serve to demonstrate the intra-assay variation and the day-to-day variation of the analytical procedure.

4. ROBUSTNESS

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small but deliberate variations in method parameters and provides an indication of its reliability during normal usage.

The evaluation of robustness should be considered during the development phase. It should show the reliability of the analytical procedure with respect to deliberate variations in method parameters. For NAT, small variations in the method parameters can be crucial. However, the robustness of the method can be demonstrated during its development when small variations in the concentrations of reagents (e.g. MgCl₂, primers or dNTP) are tested. To demonstrate robustness, at least 20 HCV RNA negative plasma pools (selected at random) spiked with HCV RNA to a final concentration of 3 times the previously determined 95 per cent cut-off value should be tested and found positive.

Problems with robustness may also arise with methods which use an initial ultracentrifugation step prior to extraction of the viral RNA. Therefore, to test the robustness of such methods, at least 20 plasma pools containing varying levels of HCV RNA, but lacking HCV specific antibodies, should be tested and found positive.

Cross contamination prevention should be demonstrated by the accurate detection of a panel of at least 20 samples consisting of alternate samples of negative plasma pools and negative plasma pools spiked with high concentrations of HCV (at least 10^2 times the 95 per cent cut-off value or at least 10^4 IU/ml).

Human plasma pools for NAT validation BRP are suitable for use as a negative control.

5. QUALITY ASSURANCE

For biological tests such as NAT, specific problems may arise which may influence both the validation and interpretation of results. The test procedures must be described precisely in the form of standard operating procedures (SOPs). These should cover:

- the mode of sampling (type of container, etc.),
- the preparation of mini-pools (where appropriate),
- the conditions of storage before analysis,
- the exact description of the test conditions, including precautions taken to prevent cross contamination or destruction of the viral RNA, reagents and reference preparations used,
- the exact description of the apparatus used,
- the detailed formulae for calculation of results, including statistical evaluation.

The use of a suitable run control (for example, an appropriate dilution of *hepatitis C virus RNA for NAT testing BRP* or plasma spiked with an HCV sample calibrated against the WHO HCV International Standard 96/790) can be considered a satisfactory system suitability check and ensures that the reliability of the analytical procedure is maintained whenever used.

Technical qualification: an appropriate installation and operation qualification programme should be implemented for each critical piece of the equipment used. Confirmation of analytical procedure performance after change of critical equipment (e.g. thermocyclers) should be documented by conducting a parallel test on 8 replicate samples of a plasma pool spiked with HCV RNA to a final concentration of 3 times the previously determined 95 per cent cut-off value. All results should be positive.

Operator qualification: an appropriate qualification programme should be implemented for each operator involved in the testing. To confirm successful training each operator should test at least 8 replicate samples of a plasma pool spiked with HCV RNA to a final concentration of 3 times the previously determined 95 per cent cut-off value. This test (8 replicate samples) should be repeated twice on two separate days, i.e. a total of 24 tests performed on three different days. All results should be positive.

01/2005:20622

2.6.22. ACTIVATED COAGULATION FACTORS

Where applicable, determine the amount of heparin present (2.7.12) and neutralise the heparin by addition of *protamine sulphate R* (10 µg of protamine sulphate neutralises 1 IU of heparin). Prepare 1 to 10 and 1 to 100 dilutions of the preparation to be examined using

tris(hydroxymethyl)aminomethane buffer solution

pH 7.5 R. Place a series of polystyrene tubes in a water-bath at 37 °C and add to each tube 0.1 ml of *platelet-poor plasma R* and 0.1 ml of a suitable dilution of *cephalin R* or *platelet substitute R*. Allow to stand for 60 s. Add to each tube either 0.1 ml of one of the dilutions or 0.1 ml of the buffer solution (control tube). To each tube add immediately 0.1 ml of a 3.7 g/l solution of *calcium chloride R* (previously warmed to 37 °C) and measure, within 30 min of the original dilution, the time that elapses between addition of the calcium chloride solution and the formation of a clot. The test is not valid unless the coagulation time measured for the control tube is 200 s to 350 s.

01/2005:20624

2.6.24. AVIAN VIRAL VACCINES: TESTS FOR EXTRANEEOUS AGENTS IN SEED LOTS

GENERAL PROVISIONS

- a) In the following tests, chickens and/or chicken material such as eggs and cell cultures shall be derived from chicken flocks free from specified pathogens (SPF) (5.2.2).
- b) Cell cultures for the testing of extraneous agents comply with the requirements for the master cell seed of chapter 5.2.4. *Cell cultures for the production of veterinary vaccines*, with the exception of the karyotype test and the tumorigenicity test, which do not have to be carried out.
- c) In tests using cell cultures, precise specifications are given for the number of replicates, monolayer surface areas and minimum survival rate of the cultures. Alternative numbers of replicates and cell surface areas are possible as well, provided that a minimum of 2 replicates are used, the total surface area and the total volume of test substance applied are not less than that prescribed here and the survival rate requirements are adapted accordingly.
- d) For a freeze-dried preparation, reconstitute using a suitable liquid. Unless otherwise stated or justified, the test substance must contain a quantity of virus equivalent to at least 10 doses of vaccine in 0.1 ml of inoculum.
- e) If the virus of the seed lot would interfere with the conduct and sensitivity of the test, neutralise the virus in the preparation with a monospecific antiserum.
- f) Monospecific antiserum and serum of avian origin used for cell culture or any other purpose, in any of these tests, shall be free of antibodies against and free from inhibitory effects on the organisms listed hereafter under 7. Antibody specifications for sera used in extraneous agents testing.
- g) Where specified in a monograph or otherwise justified, if neutralisation of the virus of the seed lot is required but difficult to achieve, the *in vitro* tests described below are adapted, as required, to provide the necessary guarantees of freedom from contamination with an extraneous agent.
- h) Other types of tests than those indicated may be used provided they are at least as sensitive as those indicated and of appropriate specificity. Nucleic acid amplification techniques (2.6.21) give specific detection for many agents and can be used after validation for sensitivity and specificity.

1. TEST FOR EXTRANEEOUS AGENTS USING EMBRYONATED HENS' EGGS

Use a test substance, diluted if necessary, containing a quantity of neutralised virus equivalent to at least 10 doses of vaccine in 0.2 ml of inoculum. Suitable antibiotics may be added. Inoculate the test substance into 3 groups of 10 embryonated hens' eggs as follows:

- group 1: 0.2 ml into the allantoic cavity of each 9- to 11-day-old embryonated egg,
- group 2: 0.2 ml onto the chorio-allantoic membrane of each 9- to 11-day-old embryonated egg,
- group 3: 0.2 ml into the yolk sac of each 5- to 6-day-old embryonated egg.

Candle the eggs in groups 1 and 2 daily for 7 days and the eggs in group 3 for 12 days. Discard embryos that die during the first 24 h as non-specific deaths; the test is not valid unless at least 6 embryos in each group survive beyond the first 24 h after inoculation. Examine macroscopically for abnormalities all embryos which die more than 24 h after inoculation, or which survive the incubation period. Examine also the chorio-allantoic membranes of these eggs for any abnormality and test the allantoic fluids for the presence of haemagglutinating agents.

Carry out a further embryo passage. Pool separately material from live and from the dead and abnormal embryos. Inoculate each pool into 10 eggs for each route as described above, chorio-allantoic membrane material being inoculated onto chorio-allantoic membranes, allantoic fluids into the allantoic cavity and embryo material into the yolk sac. For eggs inoculated by the allantoic and chorio-allantoic routes, candle the eggs daily for 7 days, proceeding and examining the material as described above. For eggs inoculated by the yolk sac route, candle the eggs daily for 12 days, proceeding and examining the material as described above.

The seed lot complies with the test if no test embryo shows macroscopic abnormalities or dies from causes attributable to the seed lot and if examination of the chorio-allantoic membranes and testing of the allantoic fluids show no evidence of the presence of any extraneous agent.

2. TEST IN CHICKEN KIDNEY CELLS

Prepare 7 monolayers of chicken kidney cells, each monolayer having an area of about 25 cm². Maintain 2 monolayers as negative controls and treat these in the same way as the 5 monolayers inoculated with the test substance, as described below. Remove the culture medium when the cells reach confluence. Inoculate 0.1 ml of test substance onto each of the 5 monolayers. Allow adsorption for 1 h, add culture medium and incubate the cultures for a total of at least 21 days, subculturing at 4- to 7-day intervals. Each passage is made with pooled cells and fluids from all 5 monolayers after carrying out a freeze-thaw cycle. Inoculate 0.1 ml of pooled material onto each of 5 recently prepared monolayers of about 25 cm² each, at each passage. For the last subculture, grow the cells also on a suitable substrate so as to obtain an area of about 10 cm² of cells from each of the monolayers for test A. The test is not valid if less than 80 per cent of the monolayers survive after any passage.

Examine microscopically all the cell cultures frequently throughout the entire incubation period for any signs of cytopathic effect or other evidence of the presence of contaminating agents in the test substance. At the end of the total incubation period, carry out the following procedures.

- A. Fix and stain (with Giemsa or haematoxylin and eosin) about 10 cm² of confluent cells from each of the 5 monolayers. Examine the cells microscopically for any cytopathic effect, inclusion bodies, syncytial formation, or any other evidence of the presence of contaminating agents from the test substance.
- B. Drain and wash about 25 cm² of cells from each of the 5 monolayers. Cover these cells with a 0.5 per cent suspension of washed chicken erythrocytes (using at least 1 ml of suspension for each 5 cm² of cells). Incubate

the cells at 4 °C for 20 min and then wash gently in phosphate buffered saline pH 7.4. Examine the cells microscopically for haemadsorption attributable to the presence of a haemadsorbing agent in the test substance.

- C. Test pooled cell culture fluids using chicken erythrocytes for haemagglutination attributable to the presence of a haemagglutinating agent in the test substance.

The test is not valid if there are any signs of extraneous agents in the negative control cultures. The seed lot complies with the test if there is no evidence of the presence of any extraneous agent.

3. TEST FOR AVIAN LEUCOSIS VIRUSES

Prepare at least 13 replicate monolayers of primary or secondary chick embryo fibroblasts from the tissues of 9- to 11-day-old embryos that are known to be genetically susceptible to subgroups A, B and J of avian leucosis viruses and that support the growth of exogenous but not endogenous avian leucosis viruses (cells from C/E strain chickens are suitable). Each replicate shall have an area of about 50 cm².

Remove the culture medium when the cells reach confluence. Inoculate 0.1 ml of the test substance onto each of 5 of the replicate monolayers. Allow adsorption for 1 h, and add culture medium. Inoculate 2 of the replicate monolayers with subgroup A avian leucosis virus (not more than 10 CCID₅₀ in 0.1 ml), 2 with subgroup B avian leucosis virus (not more than 10 CCID₅₀ in 0.1 ml) and 2 with subgroup J avian leucosis virus (not more than 10 CCID₅₀ in 0.1 ml) as positive controls. Maintain not fewer than 2 non-inoculated replicate monolayers as negative controls.

Incubate the cells for a total of at least 9 days, subculturing at 3- to 4-day intervals. Retain cells from each passage level and harvest the cells at the end of the total incubation period. Wash cells from each passage level from each replicate and resuspend the cells at 10⁷ cells per millilitre in barbital-buffered saline for subsequent testing by a Complement Fixation for Avian Leucosis (COFAL) test or in phosphate buffered saline for testing by Enzyme-Linked Immunosorbent Assay (ELISA). Then, carry out 3 cycles of freezing and thawing to release any group-specific antigen and perform a COFAL test or an ELISA test on each extract to detect group-specific avian leucosis antigen if present.

The test is not valid if group-specific antigen is detected in fewer than 5 of the 6 positive control replicate monolayers or if a positive result is obtained in any of the negative control monolayers, or if the results for both of the 2 negative control monolayers are inconclusive. If the results for more than 1 of the test replicate monolayers are inconclusive, then further subcultures of reserved portions of the fibroblast monolayers shall be made and tested until an unequivocal result is obtained. If a positive result is obtained for any of the test monolayers, then the presence of avian leucosis virus in the test substance has been detected.

The seed lot complies with the test if there is no evidence of the presence of any avian leucosis virus.

4. TEST FOR AVIAN RETICULOENDOTHELIOSIS VIRUS

Prepare 11 monolayers of primary or secondary chick embryo fibroblasts from the tissues of 9- to 11-day old chick embryos or duck embryo fibroblasts from the tissues of 13- to 14-day-old embryos, each monolayer having an area of about 25 cm².

Remove the culture medium when the cells reach confluence. Inoculate 0.1 ml of the test substance onto each of 5 of the monolayers. Allow adsorption for 1 h, and add culture medium. Inoculate 4 of the monolayers with avian

reticuloendotheliosis virus as positive controls (not more than 10 CCID₅₀ in 0.1 ml). Maintain 2 non-inoculated monolayers as negative controls.

Incubate the cells for a total of at least 10 days, subculturing twice at 3- to 4-day intervals. The test is not valid if fewer than 3 of the 4 positive controls or fewer than 4 of the 5 test monolayers or neither of the 2 negative controls survive after any passage.

For the last subculture, grow the fibroblasts on a suitable substrate so as to obtain an area of about 10 cm² of confluent fibroblasts from each of the original 11 monolayers for the subsequent test: test about 10 cm² of confluent fibroblasts derived from each of the original 11 monolayers by immunostaining for the presence of avian reticuloendotheliosis virus. The test is not valid if avian reticuloendotheliosis virus is detected in fewer than 3 of the 4 positive control monolayers or in any of the negative control monolayers, or if the results for both of the 2 negative control monolayers are inconclusive. If the results for more than 1 of the test monolayers are inconclusive then further subcultures of reserved portions of the fibroblast monolayers shall be made and tested until an unequivocal result is obtained.

The seed lot complies with the test if there is no evidence of the presence of avian reticuloendotheliosis virus.

5. TEST FOR CHICKEN ANAEMIA VIRUS

Prepare eleven 20 ml suspensions of the MDCC-MSBI cell line or another cell line of equivalent sensitivity in 25 ml flasks containing about 5 × 10⁵ cells/ml. Inoculate 0.1 ml of test substance into each of 5 flasks. Inoculate 4 of the suspensions with 10 CCID₅₀ chicken anaemia virus as positive controls. Maintain not fewer than 2 non-inoculated suspensions. Maintain all the cell cultures for a total of at least 24 days, subculturing 8 times at 3- to 4-day intervals. During the subculturing the presence of chicken anaemia virus may be indicated by a metabolic colour change in the infected cultures, the culture fluids become red in comparison with the control cultures. Examine the cells microscopically for cytopathic effect. At this time or at the end of the incubation period, centrifuge the cells from each flask at low speed and resuspend at about 10⁶ cells/ml and place 25 µl in each of 10 wells of a multi-well slide. Examine the cells by immunostaining.

The test is not valid if chicken anaemia virus is detected in fewer than 3 of the 4 positive controls or in any of the non-inoculated controls. If the results for more than 1 of the test suspensions are inconclusive, then further subcultures of reserved portions of the test suspensions shall be made and tested until an unequivocal result is obtained.

The seed lot complies with the test if there is no evidence of the presence of chicken anaemia virus.

6. TEST FOR EXTRANEIOUS AGENTS USING CHICKS

Inoculate each of at least 10 chicks, with the equivalent of 100 doses of vaccine by the intramuscular route and with the equivalent of 10 doses by eye-drop. Chicks that are 2 weeks of age are used in the test except that if the seed virus is pathogenic for birds of this age, older birds may be used, if required and justified. In exceptional cases, for inactivated vaccines, the virus may be neutralised by specific antiserum if the seed virus is pathogenic for birds at the age of administration. Repeat these inoculations 2 weeks later. Observe the chicks for a period of 5 weeks from the day of the first inoculation. No antimicrobial agents shall be administered to the chicks during the test period. The test is not valid if fewer than 80 per cent of the chicks survive to the end of the test period.

Collect serum from each chick at the end of the test period. Test each serum sample for antibodies against each of the agents listed below (with the exception of the virus type of the seed lot) using one of the methods indicated for testing for the agent.

A. Standard tests

AGENT	TYPE OF TEST
Avian adenoviruses, group 1	SN, EIA, AGP
Avian encephalomyelitis virus	AGP, EIA
Avian infectious bronchitis virus	EIA, HI
Avian infectious laryngotracheitis virus	SN, EIA, IS
Avian leucosis viruses	SN, EIA
Avian nephritis virus	IS
Avian reoviruses	IS, EIA
Avian reticuloendotheliosis virus	AGP, IS, EIA
Chicken anaemia virus	IS, EIA, SN
Egg drop syndrome virus	HI, EIA
Avian infectious bursal disease virus	AGP, EIA
Influenza A virus	AGP, EIA
Marek's disease virus	AGP
Newcastle disease virus	HI, EIA
Turkey rhinotracheitis virus	EIA
<i>Salmonella pullorum</i>	Agg

Agg: agglutination

AGP: agar gel precipitation

EIA: enzyme immunoassay (e.g. ELISA)

IS: immunostaining (e.g. fluorescent antibody)

HI: haemagglutination inhibition

SN: serum neutralisation

B. Additional tests for turkey extraneous agents

If the seed virus is of turkey origin or was propagated in turkey substrates, tests for antibodies against the following agents are also carried out.

AGENT	TYPE OF TEST
<i>Chlamydia</i> spp.	EIA
Avian infectious haemorrhagic enteritis virus	AGP
Avian paramyxovirus 3	HI
Avian infectious bursal disease virus type 2	SN

A test for freedom from turkey lympho-proliferative disease virus is carried out by intraperitoneal inoculation of twenty 4-week-old turkey poults. Observe the poults for 40 days. The test is not valid if more than 20 per cent of the poults die from non-specific causes. The seed lot complies with the test if sections of spleen and thymus taken from 10 poults 2 weeks after inoculation show no macroscopic or microscopic lesions (other than those attributable to the seed lot virus) and no poult dies from causes attributable to the seed lot.

C. Additional tests for duck extraneous agents

If the seed virus is of duck origin or was propagated in duck substrates, tests for antibodies against the following agents are also carried out.

AGENT	TYPE OF TEST
<i>Chlamydia</i> spp.	EIA
Duck and goose parvoviruses	SN
Duck enteritis virus	SN
Duck hepatitis virus type I	SN

The seed lot complies with the test if there is no evidence of the presence of any extraneous agent.

The test is not valid if antibodies are detected in the chicks to any of the test agents before inoculation.

Clinical signs of disease in the chicks during the test period (other than signs attributable to the virus of the seed lot) and the detection of antibodies in the chicks after inoculation, (with the exception of antibodies to the virus of the seed lot) are classed as evidence of the presence of an extraneous agent in the seed lot.

It is recommended that sera from these birds is retained so that additional testing may be carried out if requirements change.

7. ANTIBODY SPECIFICATIONS FOR SERA USED IN EXTRANEEOUS AGENTS TESTING

All batches of serum to be used in extraneous agents testing either to neutralise the vaccine virus (seed lot or batch of finished product) and all batches of avian serum used as a supplement for culture media used for tissue culture propagation, shall be shown to be free of antibodies against and free from inhibitory effects on the following micro-organisms by suitably sensitive tests:

Avian adenoviruses

Avian encephalomyelitis virus

Avian infectious bronchitis viruses

Avian infectious bursal disease virus types 1 and 2

Avian infectious haemorrhagic enteritis virus

Avian infectious laryngotracheitis virus

Avian leucosis viruses

Avian nephritis virus

Avian paramyxoviruses 1 to 9

Avian reoviruses

Avian reticuloendotheliosis virus

Chicken anaemia virus

Duck enteritis virus

Duck hepatitis virus type I

Egg drop syndrome virus

Fowl pox virus

Influenza viruses

Marek's disease virus

Turkey herpesvirus

Turkey rhinotracheitis virus

Non-immune serum for addition to culture media can be assumed to be free of antibodies against any of these viruses if the agent is known not to infect the species of origin of the serum and it is not necessary to test the serum for such antibodies. Monospecific antisera for virus neutralisation can be assumed to be free of the antibodies against any of these viruses if it can be shown that the immunising antigen could not have been contaminated with antigens derived from that virus and if the virus is known not to infect the species of origin of the serum; it is not necessary to test the serum for such antibodies. It is not necessary to retest sera obtained from birds from SPF chicken flocks (5.2.2).

Batches of sera prepared for neutralising the vaccine virus must not be prepared from any passage level derived from the virus isolate used to prepare the master seed lot or from an isolate cultured in the same cell line.

01/2005:20625

2.6.25. AVIAN LIVE VIRUS VACCINES: TESTS FOR EXTRANEEOUS AGENTS IN BATCHES OF FINISHED PRODUCT

GENERAL PROVISIONS

a) In the following tests, chickens and/or chicken material such as eggs and cell cultures shall be derived from chicken flocks free from specified pathogens (SPF) (5.2.2).

b) Cell cultures for the testing of extraneous agents comply with the requirements for the master cell seed of chapter 5.2.4. *Cell cultures for the production of veterinary vaccines*, with the exception of the karyotype test and the tumorigenicity test, which do not have to be carried out.

c) In tests using cell cultures, precise specifications are given for the number of replicates, monolayer surface areas and minimum survival rate of the cultures. Alternative numbers of replicates and cell surface areas are possible as well, provided that a minimum of 2 replicates are used, the total surface area and the total volume of vaccine test applied are not less than that prescribed here and the survival rate requirements are adapted accordingly.

d) In these tests, use the liquid vaccine or reconstitute a quantity of the freeze-dried preparation to be tested with the liquid stated on the label or another suitable diluent such as water for injections. Unless otherwise stated or justified, the test substance contains the equivalent of 10 doses in 0.1 ml of inoculum.

e) If the vaccine virus would interfere with the conduct and sensitivity of the test, neutralise the virus in the preparation with a monospecific antiserum.

f) Where specified in a monograph or otherwise justified, if neutralisation of the vaccine virus is required but difficult to achieve, the *in vitro* tests described below are adapted, as required, to provide the necessary guarantees of freedom from contamination with an extraneous agent. Alternatively, or in addition to *in vitro* tests conducted on the batch, a test for extraneous agents may be conducted on chick sera obtained from testing the batch of vaccine, as described under 6. Test for extraneous agents using chicks of chapter 2.6.24. *Test for extraneous agents in seed lots*.

g) Monospecific antiserum and serum of avian origin used for cell culture and any other purpose, in any of these tests, shall be free of antibodies against and free from inhibitory effects on the organisms listed under 7. Antibody specifications for sera used in extraneous agents testing (2.6.24).

h) Other types of tests than those indicated may be used provided they are at least as sensitive as those indicated and of appropriate specificity. Nucleic acid amplification techniques (2.6.21) give specific detection for many agents and can be used after validation for sensitivity and specificity.

1. TEST FOR EXTRANEEOUS AGENTS USING EMBRYONATED HENS' EGGS

Prepare the test vaccine, diluted if necessary, to contain neutralised virus equivalent to 10 doses of vaccine in 0.2 ml of inoculum. Suitable antibiotics may be added. Inoculate the test vaccine into 3 groups of 10 embryonated hens' eggs as follows:

- group 1: 0.2 ml into the allantoic cavity of each 9- to 11-day-old embryonated egg,

- group 2: 0.2 ml onto the chorio-allantoic membrane of each 9- to 11-day-old embryonated egg,
- group 3: 0.2 ml into the yolk sac of each 5- to 6-day-old embryonated egg.

Candle the eggs in groups 1 and 2 daily for 7 days and the eggs in group 3 for 12 days. Discard embryos that die during the first 24 h as non-specific deaths; the test is not valid unless at least 6 embryos in each group survive beyond the first 24 h after inoculation. Examine macroscopically for abnormalities all embryos which die more than 24 h after inoculation, or which survive the incubation period. Examine also the chorio-allantoic membranes of these eggs for any abnormality and test the allantoic fluids for the presence of haemagglutinating agents.

Carry out a further embryo passage. Pool separately material from live and from the dead and abnormal embryos. Inoculate each pool into 10 eggs for each route as described above, chorio-allantoic membrane material being inoculated onto chorio-allantoic membranes, allantoic fluids into the allantoic cavity and embryo material into the yolk sac. For eggs inoculated by the allantoic and chorio-allantoic routes, candle the eggs daily for 7 days, proceeding and examining the material as described above. For eggs inoculated by the yolk sac route, candle the eggs daily for 12 days, proceeding and examining the material as described above.

The batch of vaccine complies with the test if no test embryo shows macroscopic abnormalities or dies from causes attributable to the vaccine and if examination of the chorio-allantoic membranes and testing of the allantoic fluids show no evidence of the presence of extraneous agents.

2. TEST IN CHICKEN EMBRYO FIBROBLAST CELLS

Prepare 7 monolayers of primary or secondary chicken embryo fibroblasts, from the tissues of 9- to 11-day-old embryos, each monolayer having an area of about 25 cm². Maintain 2 monolayers as negative controls and treat these in the same way as the 5 monolayers inoculated with the test vaccine, as described below. Remove the culture medium when the cells reach confluence. Inoculate 0.1 ml of test vaccine onto each of 5 of the monolayers. Allow adsorption for 1 h and add culture medium. Incubate the cultures for a total of at least 21 days, subculturing at 4- to 5-day intervals. Each passage is made with pooled cells and fluids from all 5 monolayers after carrying out a freeze-thaw cycle. Inoculate 0.1 ml of pooled material onto each of 5 recently prepared monolayers of chicken embryo fibroblast cells, each monolayer having an area of about 25 cm² each as before. For the last subculture, grow the cells also on a suitable substrate so as to obtain an area of about 10 cm² of cells from each of the monolayers, for test A. The test is not valid if less than 80 per cent of the test monolayers, or neither of the 2 negative control monolayers survive after any passage.

Examine microscopically all the cell cultures frequently throughout the entire incubation period for any signs of cytopathic effect or other evidence of the presence of contaminating agents in the test vaccine. At the end of the total incubation period, carry out the following procedures.

- A. Fix and stain (with Giemsa or haematoxylin and eosin) about 10 cm² of confluent cells from each of the 5 original monolayers. Examine the cells microscopically for any cytopathic effect, inclusion bodies, syncytial formation, or any other evidence of the presence of a contaminating agent from the test vaccine.
- B. Drain and wash about 25 cm² of cells from each of the 5 monolayers. Cover these cells with a 0.5 per cent suspension of washed chicken red blood cells (using at least 1 ml of suspension for each 5 cm² of cells). Incubate

the cells at 4 °C for 20 min and then wash gently in phosphate buffered saline pH 7.4. Examine the cells microscopically for haemadsorption attributable to the presence of a haemadsorbing agent in the test vaccine.

- C. Test pooled cell culture fluids using chicken red blood cells for haemagglutination attributable to the presence of a haemagglutinating agent in the test vaccine.

The test is not valid if there are any signs of extraneous agents in the negative control cultures. The batch of vaccine complies with the test if there is no evidence of the presence of any extraneous agent.

3. TEST FOR EGG DROP SYNDROME VIRUS

Prepare 11 monolayers of chicken embryo liver cells, from the tissues of 14- to 16-day-old embryos, each monolayer having an area of about 25 cm². Remove the culture medium when the cells reach confluence. Inoculate 0.1 ml of test vaccine onto each of 5 of the monolayers (test monolayers). Allow adsorption for 1 h, add culture medium. Inoculate 4 of the monolayers with a suitable strain of egg drop syndrome virus (not more than 10 CCID₅₀ in 0.1 ml) to serve as positive control monolayers. Maintain 2 non-inoculated monolayers as negative control monolayers.

Incubate the cells for a total of at least 21 days, subculturing every 4-5 days. Each passage is made as follows: carry out a freeze-thaw cycle; prepare separate pools of the cells plus fluid from the test monolayers, from the positive control monolayers and from the negative control monolayers; inoculate 0.1 ml of the pooled material onto each of 5, 4 and 2 recently prepared monolayers of chicken embryo liver cells, each monolayer having an area of about 25 cm² as before. The test is not valid if fewer than 4 of the 5 test monolayers or fewer than 3 of the 4 positive controls or neither of the 2 negative control monolayers survive after any passage.

Examine microscopically all the cell cultures at frequent intervals throughout the entire incubation period for any signs of cytopathic effect or other evidence of the presence of a contaminating agent in the test vaccine. At the end of the total incubation period, carry out the following procedure: test separately, cell culture fluid from the test monolayers, positive control monolayers and negative control monolayers, using chicken red blood cells, for haemagglutination attributable to the presence of haemagglutinating agents.

The test is not valid if egg drop syndrome virus is detected in fewer than 3 of the 4 positive control monolayers or in any of the negative control monolayers, or if the results for both of the 2 negative control monolayers are inconclusive. If the results for more than 1 of the test monolayers are inconclusive then further subcultures of reserved portions of the monolayers shall be made and tested until an unequivocal result is obtained.

The batch of vaccine complies with the test if there is no evidence of the presence of egg drop syndrome virus or any other extraneous agent.

4. TEST FOR MAREK'S DISEASE VIRUS

Prepare 11 monolayers of primary or secondary chick embryo fibroblasts from the tissues of 9- to 11-day-old embryos, each monolayer having an area of about 25 cm². Remove the culture medium when the cells reach confluence. Inoculate 0.1 ml of test vaccine onto each of 5 of the monolayers (test monolayers). Allow adsorption for 1 h, and add culture medium. Inoculate 4 of the monolayers with a suitable strain of Marek's disease virus (not more than 10 CCID₅₀ in 0.1 ml) to serve as positive controls. Maintain 2 non-inoculated monolayers as negative controls.

Incubate the cultures for a total of at least 21 days, subculturing at 4- to 5-day intervals. Each passage is made as follows: trypsinise the cells, prepare separate pools of the cells from the test monolayers, from the positive control monolayers and from the negative control monolayers. Mix an appropriate quantity of each with a suspension of freshly prepared primary or secondary chick embryo fibroblasts and prepare 5, 4 and 2 monolayers, as before. The test is not valid if fewer than 4 of the 5 test monolayers or fewer than 3 of the 4 positive controls or neither of the 2 negative control monolayers survive after any passage.

Examine microscopically all the cell cultures frequently throughout the entire incubation period for any signs of cytopathic effect or other evidence of the presence of a contaminating agent in the test vaccine.

For the last subculture, grow the cells on a suitable substrate so as to obtain an area of about 10 cm² of confluent cells from each of the original 11 monolayers for the subsequent test: test about 10 cm² of confluent cells derived from each of the original 11 monolayers by immunostaining for the presence of Marek's disease virus. The test is not valid if Marek's disease virus is detected in fewer than 3 of the 4 positive control monolayers or in any of the negative control monolayers, or if the results for both of the 2 negative control monolayers are inconclusive.

The batch of vaccine complies with the test if there is no evidence of the presence of Marek's disease virus or any other extraneous agent.

5. TESTS FOR TURKEY RHINOTRACHEITIS VIRUS

A. In chicken embryo fibroblasts

NOTE: this test can be combined with Test 2 by using the same test monolayers and negative controls, for all stages up to the final specific test for turkey rhinotracheitis virus on cells prepared from the last subculture.

Prepare 11 monolayers of primary or secondary chick embryo fibroblasts from the tissues of 9- to 11-day-old embryos, each monolayer having an area of about 25 cm². Remove the culture medium when the cells reach confluence. Inoculate 0.1 ml of test vaccine onto each of 5 of the monolayers (test monolayers). Allow adsorption for 1 h, and add culture medium. Inoculate 4 of the monolayers with a suitable strain of turkey rhinotracheitis virus as positive controls (not more than 10 CCID₅₀ in 0.1 ml). Maintain 2 non-inoculated monolayers as negative controls.

Incubate the cultures for a total of at least 21 days, subculturing at 4- to 5-day intervals. Each passage is made as follows: carry out a freeze-thaw cycle; prepare separate pools of the cells plus fluid from the test monolayers, from the positive control monolayers and from the negative control monolayers; inoculate 0.1 ml of the pooled material onto each of 5, 4 and 2 recently prepared monolayers of chicken embryo fibroblasts cells, each monolayer having an area of about 25 cm² as before. The test is not valid if fewer than 4 of the 5 test monolayers or fewer than 3 of the 4 positive controls or neither of the 2 negative control monolayers survive after any passage.

For the last subculture, grow the cells on a suitable substrate so as to obtain an area of about 10 cm² of confluent cells from each of the original 11 monolayers for the subsequent test: test about 10 cm² of confluent cells derived from each of the original 11 monolayers by immunostaining for the presence of turkey rhinotracheitis virus. The test is not valid if turkey rhinotracheitis virus is detected in fewer than 3 of the 4 positive control

monolayers or in any of the negative control monolayers, or if the results for both of the 2 negative control monolayers are inconclusive. If the results for both of the 2 test monolayers are inconclusive then further subcultures of reserved portions of the fibroblasts shall be made and tested until an unequivocal result is obtained.

The batch of vaccine complies with the test if there is no evidence of the presence of turkey rhinotracheitis virus or any other extraneous agent.

B. In Vero cells

Prepare 11 monolayers of Vero cells, each monolayer having an area of about 25 cm². Remove the culture medium when the cells reach confluence. Inoculate 0.1 ml of test vaccine onto each of 5 of the monolayers (test monolayers). Allow adsorption for 1 h, and add culture medium. Inoculate 4 of the monolayers with a suitable strain of turkey rhinotracheitis virus (not more than 10 CCID₅₀ in 0.1 ml) to serve as positive controls. Maintain 2 non-inoculated monolayers as negative controls.

Incubate the cultures for a total of at least 21 days, subculturing at 4- to 5-day intervals. Each passage is made as follows: carry out a freeze-thaw cycle. Prepare separate pools of the cells plus fluid from the test monolayers, from the positive control monolayers and from the negative control monolayers. Inoculate 0.1 ml of the pooled material onto each of 5, 4 and 2 recently prepared monolayers of Vero cells, each monolayer having an area of about 25 cm² as before. The test is not valid if fewer than 4 of the 5 test monolayers or fewer than 3 of the 4 positive controls or neither of the 2 negative controls survive after any passage.

For the last subculture, grow the cells on a suitable substrate so as to obtain an area of about 10 cm² of confluent cells from each of the original 11 monolayers for the subsequent test: test about 10 cm² of confluent cells derived from each of the original 11 monolayers by immunostaining for the presence of turkey rhinotracheitis virus. The test is not valid if turkey rhinotracheitis virus is detected in fewer than 3 of the 4 positive control monolayers or in any of the negative control monolayers, or if the results for both of the 2 negative control monolayers are inconclusive. If the results for more than 1 of the test monolayers are inconclusive then further subcultures of reserved portions of the monolayers shall be made and tested until an unequivocal result is obtained.

The batch of vaccine complies with the test if there is no evidence of the presence of turkey rhinotracheitis virus or any other extraneous agent.

6. TEST FOR CHICKEN ANAEMIA VIRUS

Prepare eleven 20 ml suspensions of the MDCC-MSBI cell line or another cell line of equivalent sensitivity in 25 ml flasks containing about 5×10^5 cells/ml. Inoculate 0.1 ml of test vaccine into each of 5 of these flasks. Inoculate 4 other suspensions with 10 CCID₅₀ chicken anaemia virus as positive controls. Maintain not fewer than 2 non-inoculated suspensions. Maintain all the cell cultures for a total of at least 24 days, subculturing 8 times at 3- to 4-day intervals. During the subculturing the presence of chicken anaemia virus may be indicated by a metabolic colour change in the infected cultures, the culture fluids becoming red in comparison with the control cultures. Examine the cells microscopically for cytopathic effect. At this time or at the end of the incubation period, centrifuge the cells from each flask at low speed, resuspend at about 10^6 cells per millilitre and place 25 µl in each of 10 wells of a multi-well slide. Examine the cells by immunostaining.

The test is not valid if chicken anaemia virus is detected in fewer than 3 of the 4 positive controls or in any of the non-inoculated controls. If the results for more than 1 of the test suspensions are inconclusive then further subcultures of reserved portions of the test suspensions shall be made and tested until an unequivocal result is obtained.

The batch of vaccine complies with the test if there is no evidence of the presence of chicken anaemia virus.

7. TEST FOR DUCK ENTERITIS VIRUS

This test is carried out for vaccines prepared on duck or goose substrates.

Prepare 11 monolayers of primary or secondary Muscovy duck embryo liver cells, from the tissues of 21- or 22-day-old embryos, each monolayer having an area of about 25 cm². Remove the culture medium when the cells reach confluence. Inoculate 0.1 ml of test vaccine onto each of 5 of the monolayers (test monolayers). Allow adsorption for 1 h and add culture medium. Inoculate 4 of the monolayers with a suitable strain of duck enteritis virus (not more than 10 CCID₅₀ in 0.1 ml) to serve as positive controls. Maintain 2 non-inoculated monolayers as negative controls.

Incubate the cultures for a total of at least 21 days, subculturing at 4- to 5-day intervals. Each passage is made as follows: trypsinise the cells and prepare separate pools of the cells from the test monolayers, from the positive control monolayers and from the negative control monolayers. Mix a portion of each with a suspension of freshly prepared primary or secondary Muscovy duck embryo liver cells to prepare 5, 4 and 2 monolayers, as before. The test is not valid if fewer than 4 of the 5 test monolayers or fewer than 3 of the 4 positive controls or neither of the 2 negative controls survive after any passage.

For the last subculture, grow the cells on a suitable substrate so as to obtain an area of about 10 cm² of confluent cells from each of the original 11 monolayers for the subsequent test: test about 10 cm² of confluent cells derived from each of the original 11 monolayers by immunostaining for the presence of duck enteritis virus. The test is not valid if duck enteritis virus is detected in fewer than 3 of the 4 positive control monolayers or in any of the negative control monolayers, or if the results for both of the 2 negative control monolayers are inconclusive. If the results for more than 1 of the test monolayers are inconclusive then further subcultures of reserved portions of the monolayers shall be made and tested until an unequivocal result is obtained.

The batch of vaccine complies with the test if there is no evidence of the presence of duck enteritis virus or any other extraneous agent.

8. TEST FOR DUCK AND GOOSE PARVOVIRUSES

This test is carried out for vaccines prepared on duck or goose substrates.

Prepare a suspension of sufficient primary or secondary Muscovy duck embryo fibroblasts from the tissues of 16- to 18-day-old embryos, to obtain not fewer than 11 monolayers, each having an area of about 25 cm². Inoculate 0.5 ml of test vaccine into an aliquot of cells for 5 monolayers and seed into 5 replicate containers to form 5 test monolayers. Inoculate 0.4 ml of a suitable strain of duck parvovirus (not more than 10 CCID₅₀ in 0.1 ml) into an aliquot of cells for 4 monolayers and seed into 4 replicate containers to form 4 positive control monolayers. Prepare 2 non-inoculated monolayers as negative controls.

Incubate the cultures for a total of at least 21 days, subculturing at 4- to 5-day intervals. Each passage is made as follows: carry out a freeze-thaw cycle. Prepare separate pools of the cells plus fluid from the test monolayers, from the positive control monolayers and from the negative control monolayers. Inoculate 0.5 ml, 0.4 ml and 0.2 ml of the pooled materials into aliquots of a fresh suspension of sufficient primary or secondary Muscovy duck embryo fibroblast cells to prepare 5, 4 and 2 monolayers, as before. The test is not valid if fewer than 4 of the 5 test monolayers or fewer than 3 of the 4 positive controls or neither of the 2 negative controls survive after any passage.

For the last subculture, grow the cells on a suitable substrate so as to obtain an area of about 10 cm² of confluent cells from each of the original 11 monolayers for the subsequent test: test about 10 cm² of confluent cells derived from each of the original 11 monolayers by immunostaining for the presence of duck or goose parvovirus. The test is not valid if duck parvovirus is detected in fewer than 3 of the 4 positive control monolayers or in any of the negative control monolayers, or if the results for both of the 2 negative control monolayers are inconclusive.

The batch of vaccine complies with the test if there is no evidence of the presence of duck (or goose) parvovirus or any other extraneous agent.

2.7. BIOLOGICAL ASSAYS

01/2005:20701

2.7.1. IMMUNOCHEMICAL METHODS

Immunochemical methods are based on the selective, reversible and non-covalent binding of antigens by antibodies. These methods are employed to detect or quantify either antigens or antibodies. The formation of an antigen-antibody complex may be detected, and the amount of complex formed may be measured by a variety of techniques. The provisions of this general method apply to immunochemical methods using labelled or unlabelled reagents, as appropriate.

The results of immunochemical methods depend on the experimental conditions and the nature and quality of the reagents used. It is essential to standardise the components of an immunoassay and to use, wherever available, international reference preparations for immunoassays.

The reagents necessary for many immunochemical methods are available as commercial assay kits, that is, a set including reagents (particularly the antigen or the antibody) and materials intended for the *in vitro* estimation of a specified substance as well as instructions for their proper use. The kits are used in accordance with the manufacturers' instructions; it is important to ascertain that the kits are suitable for the analysis of the substance to be examined, with particular reference to selectivity and sensitivity. Guidance concerning immunoassay kits is provided by the World Health Organisation, Technical Report Series 658 (1981).

METHODS IN WHICH A LABELLED ANTIGEN OR A LABELLED ANTIBODY IS USED

Methods using labelled substances may employ suitable labels such as enzymes, fluorophores, luminophores and radioisotopes. Where the label is a radioisotope, the method is described as a "radio-immunoassay". The recommendations for the measurement of radioactivity given in the monograph on *Radiopharmaceutical Preparations (0125)* are applicable to immunoassays involving radioisotopes. All work with radioactive materials must be carried out in conformity with national legislation and internationally accepted codes of practice for protection against radiation hazards.

METHODS IN WHICH AN UNLABELLED ANTIGEN OR ANTIBODY IS USED

Immunoprecipitation methods

Immunoprecipitation methods include flocculation and precipitation reactions. When a solution of an antigen is mixed with its corresponding antibody under suitable conditions, the reactants form flocculating or precipitating aggregates. The ratio of the reactants which gives the shortest flocculation time or the most marked precipitation is called the optimal ratio, and is usually produced by equivalent amounts of antigen and antibody. Immunoprecipitation can be assessed visually or by light-scattering techniques (nephelometric or turbidimetric assay). An increase in sensitivity can be obtained by using antigen- or antibody-coated particles (e.g. latex) as reactants. In flocculation methods, stepwise dilutions of one of the reactants is usually used whereas, in immunodiffusion (ID) methods, the dilution is obtained by diffusion in a gel medium: concentration gradients of one or both of the reactants are obtained, thus creating zones in the gel medium where the ratio of the reactants favours precipitation. While flocculation methods are performed in

tubes, immunodiffusion methods may be performed using different supports such as tubes, plates, slides, cells or chambers.

Where the immunoprecipitating system consists of one antigen combining with its corresponding antibody, the system is referred to as *simple*; when it involves related but not serologically identical reactants, the system is *complex* and where several serologically unrelated reactants are involved, the system is *multiple*.

In *simple diffusion methods*, a concentration gradient is established for only one of the reactants diffusing from an external source into the gel medium containing the corresponding reactant at a comparatively low concentration.

Single radial immunodiffusion (SRID) is a simple quantitative immunodiffusion technique. When the equilibrium between the external and the internal reactant has been established, the circular precipitation area, originating from the site of the external reactant, is directly proportional to the amount of the antigen applied and inversely proportional to the concentration of the antibody in the gel.

In *double diffusion methods*, concentration gradients are established for both reactants. Both antigen and antibody diffuse from separate sites into an initially immunologically neutral gel.

Comparative double diffusion methods are used for qualitatively comparing various antigens versus a suitable antibody or vice versa. The comparison is based on the presence or absence of interaction between the precipitation patterns. Reactions of identity, non-identity or partial identity of antigens/antibodies can be distinguished.

Immuno-electrophoretic methods

Immuno-electrophoresis (IE) is a qualitative technique combining 2 methods: gel electrophoresis followed by immunodiffusion.

Crossed immuno-electrophoresis is a modification of the IE method. It is suitable both for qualitative and quantitative analysis. The first part of the procedure is an ordinary gel electrophoresis, after which a longitudinal gel strip, containing the separated fractions to be determined, is cut out and transferred to another plate. The electrophoresis in the second direction is carried out perpendicular to the previous electrophoretic run in a gel containing a comparatively low concentration of antibodies corresponding to the antigens. For a given antibody concentration and gel thickness, the relationship between the area of the respective precipitation peaks and the amount of the corresponding antigen is linear.

Electro-immunoassay, often referred to as *rocket immuno-electrophoresis* is a rapid quantitative method for determining antigens with a charge differing from that of the antibodies or vice versa. The electrophoresis of the antigen to be determined is carried out in a gel containing a comparatively lower concentration of the corresponding antibody. The test material and dilutions of a standard antigen used for calibration are introduced into different wells in the gel. During electrophoresis, migrating peak-shaped precipitation zones originating from the wells are developed. The front of the precipitate becomes stationary when the antigen is no longer in excess. For a given antibody concentration, the relationship between the distance travelled by the precipitate and the amount of antigen applied is linear.

Counter-immuno-electrophoresis is a rapid quantitative method allowing concentration gradients of external antigen and external antibody to be established in an electric field depending on the different charges. Dilutions of a

standard for calibration and dilutions of the test material are introduced into a row of wells in a gel and a fixed amount of the corresponding reactant is introduced into an opposite row of wells. The titre of the test material may be determined as the highest dilution showing a precipitation line.

A number of modifications of crossed immunoelectrophoresis and electroimmunoassay methods exist.

Other techniques combine separation of antigens by molecular size and serological properties.

Visualisation and characterisation of immunoprecipitation lines

These may be performed by selective or non-selective stains, by fluorescence, by enzyme or isotope labelling or other relevant techniques. Selective staining methods are usually performed for characterisation of non-protein substances in the precipitates.

In translucent gels such as agar or agarose, the precipitation line becomes clearly visible in the gel, provided that the concentration of each of the reactants is appropriate.

VALIDATION OF THE METHOD

Validation criteria

A quantitative immunochemical method is not valid unless:

- 1) The antibody or antigen does not significantly discriminate between the test and standard. For a labelled reactant, the corresponding reactant does not significantly discriminate between the labelled and unlabelled compound,
- 2) The method is not affected by the assay matrix, that is, any component of the test sample or its excipients, which can vary between samples. These may include high concentrations of other proteins, salts, preservatives or contaminating proteolytic activity,
- 3) The limit of quantitation is below the acceptance criteria stated in the individual monograph,
- 4) The precision of the assay is such that the variance of the results meets the requirements stated in the individual monographs,
- 5) The order in which the assay is performed does not give rise to systematic errors.

Validation methods

In order to verify these criteria, the validation design includes the following elements:

- 1) The assay is performed at least in triplicate,
- 2) The assay includes at least 3 different dilutions of the standard preparation and 3 dilutions of sample preparations of presumed activity similar to the standard preparation,
- 3) The assay layout is randomised,
- 4) If the test sample is presented in serum or formulated with other components, the standard is likewise prepared,
- 5) The test includes the measurement of non-specific binding of the labelled reactant,
- 6) For displacement immunoassay:
 - (a) maximum binding (zero displacement) is determined,
 - (b) dilutions cover the complete response range from values close to non-specific binding to maximum binding, preferably for both standard and test preparations.

STATISTICAL CALCULATION

To analyse the results, response curves for test and standard may be analysed by the methods described in 5.3. *Statistical Analysis of Results of Biological Assays and Tests*.

Significant non-parallelism indicates that the antibody or antigen discriminates between test and standard, and the results are not valid.

In displacement immunoassays, the values for non-specific binding and maximum displacement at high test or standard concentration must not be significantly different. Differences may indicate effects due to the matrix, either inhibition of binding or degradation of tracer.

01/2005:20702

2.7.2. MICROBIOLOGICAL ASSAY OF ANTIBIOTICS

The potency of an antibiotic is estimated by comparing the inhibition of growth of sensitive micro-organisms produced by known concentrations of the antibiotic to be examined and a reference substance.

The reference substances used in the assays are substances whose activity has been precisely determined with reference to the corresponding international standard or international reference preparation.

The assay must be designed in a way that will permit examination of the validity of the mathematical model on which the potency equation is based. If a parallel-line model is chosen, the 2 log dose-response (or transformed response) lines of the preparation to be examined and the reference preparation must be parallel; they must be linear over the range of doses used in the calculation. These conditions must be verified by validity tests for a given probability, usually $P = 0.05$. Other mathematical models, such as the slope ratio model, may be used provided that proof of validity is demonstrated.

Unless otherwise stated in the monograph, the confidence limits ($P = 0.95$) of the assay for potency are not less than 95 per cent and not more than 105 per cent of the estimated potency.

Carry out the assay by method A or method B.

A. DIFFUSION METHOD

Liquefy a medium suitable for the conditions of the assay and inoculate it at a suitable temperature, for example 48 °C to 50 °C for vegetative forms, with a known quantity of a suspension of micro-organisms sensitive to the antibiotic to be examined, such that clearly defined zones of inhibition of suitable diameter are produced with the concentrations of the antibiotic used for the assay. Immediately pour into Petri dishes or large rectangular dishes a quantity of the inoculated medium to form a uniform layer 2 mm to 5 mm thick. Alternatively, the medium may consist of 2 layers, only the upper layer being inoculated.

Store the dishes so that no appreciable growth or death of the micro-organisms occurs before the dishes are used and so that the surface of the medium is dry at the time of use.

Using the solvent and the buffer solution indicated in Table 2.7.2.-1, prepare solutions of the reference substance and of the antibiotic to be examined having known concentrations and presumed to be of equal activity. Apply the solutions to the surface of the medium, for example, in sterile cylinders of porcelain, stainless steel or other suitable material, or in cavities prepared in the agar. The same volume of solution must be added to each cylinder or cavity. Alternatively, use sterile absorbent paper discs of suitable quality; impregnate the discs with the solutions of the reference substance or the solutions of the antibiotic to be examined and place on the surface of the agar.

In order to assess the validity of the assay, use not fewer than 3 doses of the reference substance and 3 doses of the antibiotic to be examined having the same presumed activity as the doses of the reference substance. It is preferable to use a series of doses in geometric progression.

In routine assays when the linearity of the system has been demonstrated over an adequate number of experiments using a three-point assay, a two-point assay may be sufficient, subject to agreement by the competent authority. However, in all cases of dispute, a three-point assay as described above must be applied.

Table 2.7.2.-1. – Diffusion assay

Antibiotic	Reference substance	Solvent to be used in preparing the stock solution	Buffer solution (pH)	Micro-organism	Medium and final pH (± 0.1 pH unit)	Incubation temperature
Amphotericin B	<i>Amphotericin B CRS</i>	<i>Dimethyl sulphoxide R</i>	pH 10.5 (0.2 M)	<i>Saccharomyces cerevisiae</i> ATCC 9763 IP 1432-83	F - pH 6.1	35-37 °C
Bacitracin zinc	<i>Bacitracin zinc CRS</i>	<i>0.01 M hydrochloric acid</i>	pH 7.0 (0.05 M)	<i>Micrococcus luteus</i> NCTC 7743 CIP 53.160 ATCC 10240	A - pH 7.0	35-39 °C
Bleomycin sulphate	<i>Bleomycin sulphate CRS</i>	<i>Water R</i>	pH 6.8 (0.1 M)	<i>Mycobacterium smegmatis</i> ATCC 607	G - pH 7.0	35-37 °C
Colistimethate sodium	<i>Colistimethate sodium CRS</i>	<i>Water R</i>	pH 6.0 (0.05 M)	<i>Bordetella bronchiseptica</i> NCTC 8344 CIP 53.157 ATCC 4617 <i>Escherichia coli</i> NCIB 8879 CIP 54.127 ATCC 10536	B - pH 7.3	35-39 °C
Dihydrostreptomycin sulphate	<i>Dihydrostreptomycin sulphate CRS</i>	<i>Water R</i>	pH 8.0 (0.05 M)	<i>Bacillus subtilis</i> NCTC 8236 CIP 1.83 <i>Bacillus subtilis</i> NCTC 10400 CIP 52.62 ATCC 6633	A - pH 7.9 A - pH 7.9	30-37 °C 30-37 °C
Erythromycin estolate	<i>Erythromycin CRS</i>	<i>Methanol R</i> (see the monographs)	pH 8.0 (0.05 M)	<i>Bacillus pumilus</i> NCTC 8241 CIP 76.18 <i>Bacillus subtilis</i> NCTC 10400 CIP 52.62 ATCC 6633	A - pH 7.9	30-37 °C
Framycetin sulphate	<i>Framycetin sulphate CRS</i>	<i>Water R</i>	pH 8.0 (0.05 M)	<i>Bacillus subtilis</i> NCTC 10400 CIP 52.62 ATCC 6633 <i>Bacillus pumilus</i> NCTC 8241 CIP 76.18	E - pH 7.9 E - pH 7.9	30-37 °C 30-37 °C
Gentamicin sulphate	<i>Gentamicin sulphate CRS</i>	<i>Water R</i>	pH 8.0 (0.05 M)	<i>Bacillus pumilus</i> NCTC 8241 CIP 76.18 <i>Staphylococcus epidermidis</i> NCIB 8853 CIP 68.21 ATCC 12228	A - pH 7.9 A - pH 7.9	35-39 °C 35-39 °C
Josamycin	<i>Josamycin CRS</i>	<i>Methanol R</i> (see the monograph)	pH 5.6	<i>Bacillus subtilis</i> CIP 52.62 ATCC 6633 NCTC 10400	A - pH 6.6	35-37 °C
Josamycin propionate	<i>Josamycin propionate CRS</i>	<i>Methanol R</i> (see the monograph)	pH 5.6	<i>Bacillus subtilis</i> CIP 52.62 ATCC 6633 NCTC 10400	A - pH 6.6	35-37 °C

Antibiotic	Reference substance	Solvent to be used in preparing the stock solution	Buffer solution (pH)	Micro-organism	Medium and final pH (± 0.1 pH unit)	Incubation temperature
Kanamycin monosulphate	<i>Kanamycin monosulphate CRS</i>	<i>Water R</i>	pH 8.0 (0.05 M)	<i>Bacillus subtilis</i> NCTC 10400 CIP 52.62 ATCC 6633	A - pH 7.9	30-37 °C
Kanamycin acid sulphate				<i>Staphylococcus aureus</i> NCTC 7447 CIP 53.156 ATCC 6538 P	A - pH 7.9	35-39 °C
Neomycin sulphate	<i>Neomycin sulphate for microbiological assay CRS</i>	<i>Water R</i>	pH 8.0 (0.05 M)	<i>Bacillus pumilus</i> NCTC 8241 CIP 76.18 <i>Bacillus subtilis</i> NCTC 10400 CIP 52.62 ATCC 6633	E - pH 7.9 E - pH 7.9	30-37 °C 30-37 °C
Netilmicin sulphate	<i>Netilmicin sulphate CRS</i>	<i>Water R</i>	pH 8.0 $\pm$ 0.1	<i>Staphylococcus aureus</i> ATCC 6538P CIP 53.156	A - pH 7.9	32-35 °C
Nystatin	<i>Nystatin CRS</i>	<i>Dimethylformamide R</i>	pH 6.0 (0.05 M) containing 5 per cent V/V of dimethylformamide R	<i>Candida tropicalis</i> CIP 1433-83 NCYC 1393 <i>Saccharomyces cerevisiae</i> NCYC 87 CIP 1432-83 ATCC 9763	F - pH 6.0 F - pH 6.0	30-37 °C 30-32 °C
Rifamycin sodium	<i>Rifamycin sodium CRS</i>	<i>Methanol R</i>	pH 7.0 (0.05 M)	<i>Micrococcus luteus</i> NCTC 8340 CIP 53.45 ATCC 9341	A - pH 6.6	35-39 °C
Spiramycin	<i>Spiramycin CRS</i>	<i>Methanol R</i>	pH 8.0 (0.05 M)	<i>Bacillus subtilis</i> NCTC 10400 CIP 52.62 ATCC 6633	A - pH 7.9	30-32 °C
Streptomycin sulphate	<i>Streptomycin sulphate CRS</i>	<i>Water R</i>	pH 8.0 (0.05 M)	<i>Bacillus subtilis</i> NCTC 8236 CIP 1.83 <i>Bacillus subtilis</i> NCTC 10400 CIP 52.62 ATCC 6633	A - pH 7.9 A - pH 7.9	30-37 °C 30-37 °C
Tylosin for veterinary use Tylosin tartrate for veterinary use	<i>Tylosin CRS</i>	2.5 per cent V/V solution of <i>methanol R</i> in 0.1 M phosphate buffer solution pH 7.0 R	A mixture of 40 volumes of <i>methanol R</i> and 60 volumes of 0.1 M phosphate buffer solution pH 8.0 R	<i>Micrococcus luteus</i> NCTC 8340 CIP 53.45 ATCC 9341	A - pH 8.0	32-35 °C
Vancomycin hydrochloride	<i>Vancomycin hydrochloride CRS</i>	<i>Water R</i>	pH 8.0	<i>Bacillus subtilis</i> NCTC 8236 CIP 52.62 ATCC 6633	A - pH 8.0	37-39 °C

Arrange the solutions on each Petri dish or on each rectangular dish according to a statistically suitable design, except for small Petri dishes that cannot accommodate more than 6 solutions, arrange the solutions of the antibiotic to be examined and the solutions of the reference substance in an alternate manner to avoid interaction of the more concentrated solutions.

Incubate at a suitable temperature for about 18 h. A period of diffusion prior to incubation, usually 1 h to 4 h, at room temperature or at about 4 °C, as appropriate, may be used

to minimise the effects of the variation in time between the application of the solutions and to improve the regression slope.

Measure the diameters with a precision of at least 0.1 mm or the areas of the circular inhibition zones with a corresponding precision and calculate the potency using appropriate statistical methods.

Use in each assay the number of replications per dose sufficient to ensure the required precision. The assay may be repeated and the results combined statistically to obtain the

required precision and to ascertain whether the potency of the antibiotic to be examined is not less than the minimum required.

B. TURBIDIMETRIC METHOD

Inoculate a suitable medium with a suspension of the chosen micro-organism having a sensitivity to the antibiotic to be examined such that a sufficiently large inhibition of microbial growth occurs in the conditions of the test. Use a known quantity of the suspension chosen so as to obtain a readily measurable opacity after an incubation period of about 4 h.

Use the inoculated medium immediately after its preparation.

Using the solvent and the buffer solution indicated in Table 2.7.2.-2 prepare solutions of the reference substance and of the antibiotic to be examined having known concentrations presumed to be of equal activity.

In order that the validity of the assay may be assessed, use not fewer than 3 doses of the reference substance and 3 doses of the antibiotic to be examined having the same presumed activity as the doses of the reference substance. It is preferable to use a series of doses in geometric progression. In order to obtain the required linearity, it may be necessary to select from a large number 3 consecutive doses, using corresponding doses for the reference substance and the antibiotic to be examined.

Distribute an equal volume of each of the solutions into identical test-tubes and add to each tube an equal volume of inoculated medium (for example, 1 ml of the solution and 9 ml of the medium). For the assay of tyrothricin add 0.1 ml of the solution to 9.9 ml of inoculated medium.

Prepare at the same time 2 control tubes without antibiotic, both containing the inoculated medium and to one of which is added immediately 0.5 ml of *formaldehyde R*. These tubes are used to set the optical apparatus used to measure the growth.

Place all the tubes, randomly distributed or in a Latin square or randomised block arrangement, in a water-bath or other suitable apparatus fitted with a means of bringing all the tubes rapidly to the appropriate incubation temperature and maintain them at that temperature for 3 h to 4 h, taking precautions to ensure uniformity of temperature and identical incubation time.

After incubation, stop the growth of the micro-organisms by adding 0.5 ml of *formaldehyde R* to each tube or by heat treatment and measure the opacity to 3 significant figures using suitable optical apparatus. Alternatively use a method which allows the opacity of each tube to be measured after exactly the same period of incubation.

Calculate the potency using appropriate statistical methods.

Linearity of the dose-response relationship, transformed or untransformed, is often obtained only over a very limited range. It is this range which must be used in calculating the activity and it must include at least 3 consecutive doses in order to permit linearity to be verified. In routine assays when the linearity of the system has been demonstrated over an adequate number of experiments using a three-point assay, a two-point assay may be sufficient, subject to agreement by the competent authority. However, in all cases of dispute, a three-point assay must be applied.

Use in each assay the number of replications per dose sufficient to ensure the required precision. The assay may be repeated and the results combined statistically to obtain the required precision and to ascertain whether the potency of the antibiotic to be examined is not less than the minimum required.

Table 2.7.2.-2. – Turbidimetric assay

Antibiotic	Reference substance	Solvent to be used in preparing the stock solution	Buffer solution (pH)	Micro-organism	Medium and final pH (± 0.1 pH unit)	Incubation temperature
Colistimethate sodium	<i>Colistimethate sodium CRS</i>	<i>Water R</i>	pH 7.0	<i>Escherichia coli</i> NCIB 8666 CIP 2.83 ATCC 9637	C - pH 7.0	35-37 °C
Dihydrostreptomycin sulphate	<i>Dihydrostreptomycin sulphate CRS</i>	<i>Water R</i>	pH 8.0	<i>Klebsiella pneumoniae</i> NCTC 7427 CIP 53.153 ATCC 10031	C - pH 7.0	35-37 °C
Erythromycin estolate	<i>Erythromycin CRS</i>	<i>Methanol R</i> (see the monographs)	pH 8.0	<i>Klebsiella pneumoniae</i> NCTC 7427 CIP 53.153 ATCC 10031	D - pH 7.0	35-37 °C
Erythromycin ethylsuccinate				<i>Staphylococcus aureus</i> NCTC 7447 CIP 53.156 ATCC 6538 P	C - pH 7.0	35-37 °C
Framycetin sulphate	<i>Framycetin sulphate CRS</i>	<i>Water R</i>	pH 8.0	<i>Staphylococcus aureus</i> NCTC 7447 CIP 53.156 ATCC 6538 P	C - pH 7.0	35-37 °C

Antibiotic	Reference substance	Solvent to be used in preparing the stock solution	Buffer solution (pH)	Micro-organism	Medium and final pH (± 0.1 pH unit)	Incubation temperature
Gentamicin sulphate	<i>Gentamicin sulphate CRS</i>	<i>Water R</i>	pH 7.0	<i>Staphylococcus aureus</i> NCTC 7447 CIP 53.156 ATCC 6538 P	C - pH 7.0	35-37 °C
Gramicidin	<i>Gramicidin CRS</i>	<i>Methanol R</i>	pH 7.0*	<i>Enterococcus hirae</i> CIP 58.55 ATCC 10541 <i>Staphylococcus aureus</i> ATCC 6538 P	C - pH 7.0	35-37 °C
	*Addition of a detergent may be necessary to avoid adsorption on the material during the dilutions, for example 0.1 mg/ml of <i>polysorbate 80 R</i>					
Josamycin	<i>Josamycin CRS</i>	<i>Methanol R</i> (see the monograph)	pH 5.6	<i>Staphylococcus aureus</i> CIP 53.156 ATCC 6538 P NCTC 7447	C - pH 8.0	35-37 °C
Josamycin propionate	<i>Josamycin propionate CRS</i>	<i>Methanol R</i> (see the monograph)	pH 5.6	<i>Staphylococcus aureus</i> CIP 53.156 ATCC 6538 P NCTC 7447	C - pH 8.0	35-37 °C
Kanamycin monosulphate Kanamycin acid sulphate	<i>Kanamycin monosulphate CRS</i>	<i>Water R</i>	pH 8.0	<i>Staphylococcus aureus</i> NCTC 7447 CIP 53.156 ATCC 6538 P	C - pH 7.0	35-37 °C
Neomycin sulphate	<i>Neomycin sulphate for microbiological assay CRS</i>	<i>Water R</i>	pH 8.0	<i>Staphylococcus aureus</i> NCTC 7447 CIP 53.156 ATCC 6538 P	C - pH 7.0	35-37 °C
Rifamycin sodium	<i>Rifamycin sodium CRS</i>	<i>Methanol R</i>	pH 7.0	<i>Escherichia coli</i> NCIB 8879 CIP 54.127 ATCC 10536	C - pH 7.0	35-37 °C
Spiramycin	<i>Spiramycin CRS</i>	<i>Methanol R</i>	pH 7.0	<i>Staphylococcus aureus</i> NCTC 7447 CIP 53.156 ATCC 6538 P	C - pH 7.0	35-37 °C
Streptomycin sulphate	<i>Streptomycin sulphate CRS</i>	<i>Water R</i>	pH 8.0	<i>Klebsiella pneumoniae</i> NCTC 7427 CIP 53.153 ATCC 10031	C - pH 7.0	35-37 °C
Tylosin for veterinary use Tylosin tartrate for veterinary use	<i>Tylosin CRS</i>	2.5 per cent V/V solution of <i>methanol R</i> in 0.1 M phosphate buffer solution pH 7.0 R	pH 7.0	<i>Staphylococcus aureus</i> NCTC 6571 ATCC 9144 CIP 53.154	C - pH 7.0	37 °C
Tyrothricin	<i>Gramicidin CRS</i>	<i>Alcohol R</i>	<i>Alcohol R</i>	<i>Enterococcus hirae</i> ATCC 10541	C - pH 7.0	37 °C
Vancomycin hydrochloride	<i>Vancomycin hydrochloride CRS</i>	<i>Water R</i>	pH 8.0	<i>Staphylococcus aureus</i> CIP 53.156 ATCC 6538 P	C - pH 7.0	37-39 °C

The following section is published for information.

RECOMMENDED MICRO-ORGANISMS

The following text details the recommended micro-organisms and the conditions of use. Other micro-organisms may be used provided that they are shown to be sensitive to the

antibiotic to be examined and are used in appropriate media and appropriate conditions of temperature and pH. The concentrations of the solutions used should be chosen so as to ensure that a linear relationship exists between the logarithm of the dose and the response in the conditions of the test.

Preparation of inocula. *Bacillus cereus* var. *mycoides*; *Bacillus subtilis*; *Bacillus pumilus*. Spore suspensions of the organisms to be used as inocula are prepared as follows.

Grow the organism at 35-37 °C for 7 days on the surface of a suitable medium to which has been added 0.001 g/l of *manganese sulphate R*. Using sterile *water R*, wash off the growth, which consists mainly of spores. Heat the suspension at 70 °C for 30 min and dilute to give an appropriate concentration of spores, usually 10×10^6 to 100×10^6 per millilitre. The spore suspensions may be stored for long periods at a temperature not exceeding 4 °C.

Alternatively, spore suspensions may be prepared by cultivating the organisms in medium C at 26 °C for 4-6 days, then adding, aseptically, sufficient *manganese sulphate R* to give a concentration of 0.001 g/l and incubating for a further 48 h. Examine the suspension microscopically to ensure that adequate spore formation has taken place (about 80 per cent) and centrifuge. Re-suspend the sediment in sterile *water R* to give a concentration of 10×10^6 to 100×10^6 spores per millilitre, and then heat to 70 °C for 30 min. Store the suspension at a temperature not exceeding 4 °C.

Bordetella bronchiseptica. Grow the test organism on medium B at 35-37 °C for 16-18 h. Wash off the bacterial growth with sterile *water R* and dilute to a suitable opacity.

Staphylococcus aureus; *Klebsiella pneumoniae*; *Escherichia coli*; *Micrococcus luteus*; *Staphylococcus epidermidis*. Prepare as described above for *B. bronchiseptica* but using medium A and adjusting the opacity to one which has been shown to produce a satisfactory dose-response relationship in the turbidimetric assay, or to produce clearly defined zones of inhibition of convenient diameter in the diffusion assay, as appropriate.

Saccharomyces cerevisiae; *Candida tropicalis*. Grow the test organism on medium F at 30-37 °C for 24 h. Wash off the growth with a sterile 9 g/l solution of *sodium chloride R*. Dilute to a suitable opacity with the same solution.

Buffer solutions. Buffer solutions having a pH between 5.8 and 8.0 are prepared by mixing 50.0 ml of 0.2 M *potassium dihydrogen phosphate R* with the quantity of 0.2 M *sodium hydroxide* indicated in Table 2.7.2.-3. Dilute with freshly prepared *distilled water R* to produce 200.0 ml.

Table 2.7.2.-3.

pH	0.2 M Sodium hydroxide (ml)
5.8	3.72
6.0	5.70
6.2	8.60
6.4	12.60
6.6	17.80
6.8	23.65
7.0	29.63
7.2	35.00
7.4	39.50
7.6	42.80
7.8	45.20
8.0	46.80

These buffer solutions are used for all microbiological assays shown in Table 2.7.2.-1 with the exception of bleomycin sulphate and amphotericin B.

For bleomycin sulphate, prepare the buffer solution pH 6.8 as follows: dissolve 6.4 g of *potassium dihydrogen phosphate R* and 18.9 g of *disodium hydrogen phosphate R* in *water R* and dilute to 1000 ml with *water R*.

For amphotericin B, prepare the 0.2 M phosphate buffer solution pH 10.5 as follows: dissolve 35 g of *dipotassium hydrogen phosphate R* in 900 ml of *water R*, add 20 ml of 1 M *sodium hydroxide* and dilute to 1000.0 ml with *water R*.

Culture media. The following media or equivalent media may be used.

Medium A

Peptone	6 g
Pancreatic digest of casein	4 g
Beef extract	1.5 g
Yeast extract	3 g
Glucose monohydrate	1 g
Agar	15 g
Water to produce	1000 ml

Medium B

Pancreatic digest of casein	17 g
Papaic digest of soya bean	3 g
Sodium chloride	5 g
Dipotassium hydrogen phosphate	2.5 g
Glucose monohydrate	2.5 g
Agar	15 g
Polysorbate 80	10 g
Water to produce	1000 ml

The polysorbate 80 is added to the hot solution of the other ingredients after boiling, and immediately before adjusting to volume.

Medium C

Peptone	6 g
Beef extract	1.5 g
Yeast extract	3 g
Sodium chloride	3.5 g
Glucose monohydrate	1 g
Dipotassium hydrogen phosphate	3.68 g
Potassium dihydrogen phosphate	1.32 g
Water to produce	1000 ml

Medium D

Heart extract	1.5 g
Yeast extract	1.5 g
Peptone-casein	5 g
Glucose monohydrate	1 g
Sodium chloride	3.5 g
Dipotassium hydrogen phosphate	3.68 g
Potassium dihydrogen phosphate	1.32 g
Potassium nitrate	2 g
Water to produce	1000 ml

Medium E

Peptone	5 g
Meat extract	3 g
Disodium hydrogen phosphate, 12H ₂ O	26.9 g
Agar	10 g
Water to produce	1000 ml

The disodium hydrogen phosphate is added as a sterile solution after sterilisation of the medium.

Medium F

Peptone	9.4 g
Yeast extract	4.7 g
Beef extract	2.4 g
Sodium chloride	10.0 g
Glucose monohydrate	10.0 g
Agar	23.5 g
Water to produce	1000 ml

Medium G

Glycerol	10 g
Peptone	10 g
Meat extract	10 g
Sodium chloride	3 g
Agar	15 g
Water to produce	1000 ml

pH 7.0 ± 0.1 after sterilisation.

01/2005:20704

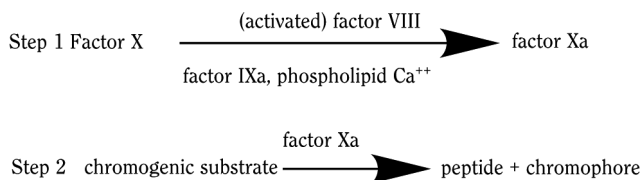
2.7.4. ASSAY OF HUMAN COAGULATION FACTOR VIII

Human coagulation factor VIII is assayed by its biological activity as a cofactor in the activation of factor X by activated factor IX (factor IXa) in the presence of calcium ions and phospholipids. The potency of a factor VIII preparation is estimated by comparing the quantity necessary to achieve a certain rate of factor Xa formation in a test mixture containing the substances that take part in the activation of factor X, and the quantity of the International Standard, or of a reference preparation calibrated in International Units, required to produce the same rate of factor Xa formation.

The International Unit is the factor VIII activity of a stated amount of the International Standard which consists of a freeze-dried human coagulation factor VIII concentrate. The equivalence in International Units of the International Standard is stated by the World Health Organisation.

Human coagulation factor VIII BRP is calibrated in International Units by comparison with the International Standard.

The chromogenic assay method consists of two consecutive steps: the factor VIII-dependent activation of factor X in a coagulation-factor reagent composed of purified components, and the enzymatic cleavage of a chromogenic factor Xa substrate to yield a chromophore that can be quantified spectrophotometrically. Under appropriate assay conditions, there is a linear relation between the rate of factor Xa formation and the factor VIII concentration. The assay is summarised by the following scheme:



Both steps employ reagents that may be obtained commercially from a variety of sources. Although the composition of individual reagents may be subject to some variation, their essential features are described in the following specification. Deviations from this description may be permissible provided that it has been shown, using the International Standard for Human Blood Coagulation Factor VIII concentrate as the standard, that the results obtained do not differ significantly.

Commercial assay kits are to be used in accordance with the manufacturers' instructions; it is important to ascertain the suitability for the assay of the kit used.

REAGENTS

The coagulation factor reagent comprises purified proteins derived from human or bovine sources. These include factor X, factor IXa, and a factor VIII activator, usually thrombin. These proteins are partly purified, preferably to at least 50 per cent, and do not contain impurities that interfere with the activation of factor VIII or factor X. Factor X is present in amounts giving a final concentration during the first step of the assay of 10-350 nmol/l, preferably 15-30 nmol/l. Factor IXa is prepared by activating purified factor IX to factor IXa β using factor XIa, and by subsequent purification of factor IXa β from the reaction mixture. Its final concentration during factor Xa generation is less than 30 per cent of the factor X concentration, usually 1-100 nmol/l, preferably 1-10 nmol/l. Thrombin may be present in its precursor form prothrombin, provided that its activation in the reagent is sufficiently rapid to give almost instantaneous, complete activation of factor VIII in the assay. Phospholipids may be obtained from natural sources such as bovine brain or spinal cord or soya-bean extract, or synthetically prepared, and must consist to a substantial extent, usually 15 per cent to 35 per cent, of the species phosphatidylserine. The final phospholipid concentration during factor Xa generation is 1-50 μ mol/l, preferably 10-35 μ mol/l. The reagent contains calcium ions to give a final concentration of 5-15 mmol/l. The final factor Xa generation is performed in a solution containing at least 1 mg/ml of human or bovine albumin which is appropriately buffered, at a pH of 7.3-8.0. The components of the complete reagent are usually divided into at least two separate reagents each lacking the ability to generate factor Xa on its own. After reconstitution, these may be combined provided that no substantial amounts of factor Xa are generated in the absence of factor VIII. In the final incubation mixture, factor VIII must be the only rate-limiting component.

The second step comprises the quantification of the formed factor Xa employing a chromogenic substrate that is specific for factor Xa. Generally this consists of a derivatised short peptide of between three and five amino acids, joined to a chromophore group. On cleavage of this group from the peptide substrate, its chromophoric properties shift to a wavelength allowing its spectrophotometric quantification. The substrate is usually dissolved in water and used at a final concentration of 0.2-2 mmol/l. The substrate may further contain appropriate inhibitors to stop further factor Xa generation and to suppress thrombin activity, thereby improving selectivity for factor Xa.

ASSAY PROCEDURE

01/2005:20705

Reconstitute the entire contents of one ampoule of the reference preparation and the preparation to be examined by adding the appropriate quantity of *water R*; use immediately. Add sufficient prediluent to the reconstituted preparations to produce solutions containing between 0.5 IU/ml and 2.0 IU/ml.

The prediluent consists of plasma from a patient with severe haemophilia A, or of an artificially prepared reagent that gives results that do not differ significantly from those obtained employing haemophilic plasma and the same reference and test preparations. The prediluted materials must be stable beyond the time required for the assay, for at least 30 min at 20 °C and must be used within 15 min.

Prepare further dilutions of reference and test preparations using an isotonic non-chelating buffer containing 1 per cent of human or bovine albumin and for example, tris(hydroxy-methyl)aminomethane or imidazole, buffered preferably between pH 7.3 and 8.0. Prepare at least three separate, independent dilutions for each material, preferably with each one prepared in duplicate. Prepare the dilutions such that the final factor VIII concentration is below 0.03 IU/ml, and preferably below 0.01 IU/ml, during the step of factor Xa generation.

Prepare a control solution that includes all components except factor VIII.

Prepare all dilutions in plastic tubes and use without delay.

Step 1. Mix prewarmed dilutions of the factor VIII reference preparation and the preparation to be examined with an appropriate volume of the prewarmed coagulation factor reagent or a combination of its separate constituents, and incubate the mixture in plastic tubes or microplate wells at 37 °C. The concentrations of the various components during the factor Xa generation must be as specified above under the description of the reagents. Allow the activation of factor X to proceed for a suitable time, preferably terminating the reaction before the factor Xa concentration has reached its maximal level in order to obtain a satisfactory linear dose-response relationship. The activation time is also chosen to achieve linear production of factor Xa in time. Appropriate activation times are usually between 2 min and 5 min, but deviations are permissible if better linearity of the dose-response relationship is thus obtained.

Step 2. Terminate the activation by addition of a prewarmed reagent containing a chromogenic substrate. Quantify the rate of substrate cleavage, which must be linear with the concentration of factor Xa formed, by measuring the absorbance change at an appropriate wavelength using a spectrophotometer, either monitoring the absorbance continuously, thus allowing the initial rate of substrate cleavage to be calculated, or terminating the hydrolysis reaction after a suitable interval by lowering the pH by addition of a suitable reagent, such as acetic acid (50 per cent V/V $C_2H_4O_2$) or a citrate solution (1 mol/l) at pH 3. Adjust the hydrolysis time to achieve a linear development of chromophore in time. Appropriate hydrolysis times usually are between 3 min and 15 min, but deviations are permissible if better linearity of the dose-response relationship is thus obtained.

Check the validity of the assay and calculate the potency of the test preparation by the usual statistical methods (for example, 5.3. *Statistical analysis of results of biological assays and tests*).

2.7.5. ASSAY OF HEPARIN

The anticoagulant activity of heparin is determined *in vitro* by comparing its ability in given conditions to delay the clotting of recalcified citrated sheep plasma with the same ability of a reference preparation of heparin calibrated in International Units.

The International Unit is the activity contained in a stated amount of the International Standard, which consists of a quantity of freeze-dried heparin sodium from pork intestinal mucosa. The equivalence in International Units of the International Standard is stated by the World Health Organisation.

Heparin sodium BRP is calibrated in International Units by comparison with the International Standard by means of the assay given below.

Carry out the assay using one of the following methods for determining the onset of clotting and using tubes and other equipment appropriate to the chosen method:

- direct visual inspection, preferably using indirect illumination and viewing against a matt black background;
- spectrophotometric recording of the change in optical density at a wavelength of approximately 600 nm;
- visual detection of the change in fluidity on manual tilting of the tubes;
- mechanical recording of the change in fluidity on stirring, care being taken to cause the minimum disturbance of the solution during the earliest phase of clotting.

ASSAY PROCEDURE

The volumes in the text are given as examples and may be adapted to the apparatus used provided that the ratios between the different volumes are respected.

Dilute *heparin sodium BRP* with a 9 g/l solution of *sodium chloride R* to contain a precisely known number of International Units per millilitre and prepare a similar solution of the preparation to be examined which is expected to have the same activity. Using a 9 g/l solution of *sodium chloride R*, prepare from each solution a series of dilutions in geometric progression such that the clotting time obtained with the lowest concentration is not less than 1.5 times the blank recalcification time, and that obtained with the highest concentration is such as to give a satisfactory log dose-response curve, as determined in a preliminary test.

Place 12 tubes in a bath of iced water, labelling them in duplicate: T_1 , T_2 and T_3 for the dilutions of the preparation to be examined and S_1 , S_2 and S_3 for the dilutions of the reference preparation. To each tube add 1.0 ml of thawed *plasma substrate RI* and 1.0 ml of the appropriate dilution of the preparation to be examined or the reference preparation. After each addition, mix but do not allow bubbles to form. Treating the tubes in the order S_1 , S_2 , S_3 , T_1 , T_2 , T_3 , transfer each tube to a water-bath at 37 °C, allow to equilibrate at 37 °C for about 15 min and add to each tube 1 ml of a dilution of *cephalin R* to which has been added an appropriate activator such as kaolin so that a suitable blank recalcification time not exceeding 60 s is obtained. When kaolin is used, prepare just before use, a mixture of equal volumes of *cephalin R* and a 4 g/l suspension of *light kaolin R* in a 9 g/l solution of *sodium chloride R*. After exactly 2 min add 1 ml of a 3.7 g/l solution of *calcium chloride R* and record as the clotting time the interval in seconds between this last addition and the onset of clotting determined by the chosen technique. Determine the blank recalcification time at the beginning and at the end of the

procedure in a similar manner, using 1 ml of a 9 g/l solution of *sodium chloride R* in place of one of the heparin dilutions; the 2 blank values obtained should not differ significantly. Transform the clotting times to logarithms, using the mean value for the duplicate tubes. Repeat the procedure using fresh dilutions and carrying out the incubation in the order $T_1, T_2, T_3, S_1, S_2, S_3$. Calculate the results by the usual statistical methods.

Carry out not fewer than 3 independent assays. For each such assay prepare fresh solutions of the reference preparation and the preparation to be examined and use another, freshly thawed portion of plasma substrate.

Calculate the potency of the preparation to be examined by combining the results of these assays by the usual statistical methods. When the variance due to differences between assays is significant at $P = 0.01$ a combined estimate of potency may be obtained by calculating the non-weighted mean of potency estimates.

01/2005:20706
corrected

2.7.6. ASSAY OF DIPHTHERIA VACCINE (ADSORBED)

The potency of diphtheria vaccine (adsorbed) is determined by comparing the dose of the vaccine required to protect guinea-pigs from the effects of either an erythrogenic dose of diphtheria toxin administered intradermally or a lethal dose of diphtheria toxin administered subcutaneously with the dose of a reference preparation, calibrated in International Units, needed to give the same protection.

The International Unit is the activity contained in a stated amount of the International Standard which consists of a quantity of diphtheria toxoid adsorbed on aluminium hydroxide. The equivalence in International Units of the International Standard is stated by the World Health Organisation.

Diphtheria vaccine (adsorbed) BRP is suitable for use as a reference preparation.

The design of the assay described below follows a parallel-line model with 3 dilutions for the test and reference preparations. Once the analyst has sufficient experience with this method for a given vaccine, it is possible to apply a simplified model using a single dilution for both test and reference preparations. Such a model enables the analyst to determine whether the potency of the test preparation is significantly higher than the minimum required but does not give information on linearity, parallelism and the dose-response curve. The simplified model leads to a considerable reduction in the number of experimental animals required and must be considered by each analyst in accordance with the provisions of the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes.

METHOD OF INTRADERMAL CHALLENGE

Selection and distribution of the test animals. Use in the test, healthy, white guinea-pigs from the same stock and of a size suitable for the prescribed number of challenge sites, the difference in body mass between the heaviest and the lightest animal being not greater than 100 g. Distribute the guinea-pigs in not fewer than 6 equal groups; use groups containing a number of animals sufficient to obtain results that fulfil the requirements for a valid assay prescribed

below. If the challenge toxin to be used has not been shown to be stable or has not been adequately standardised, include 5 guinea-pigs as unvaccinated controls. Use guinea-pigs of the same sex or with males and females equally distributed between the groups.

Selection of the challenge toxin. Select a preparation of diphtheria toxin containing 67 to 133 lr/100 in 1 Lf and 25 000 to 50 000 minimal reacting doses for guinea-pig skin in 1 Lf. If the challenge toxin preparation has been shown to be stable, it is not necessary to verify the activity for every assay.

Preparation of the challenge toxin solution. Immediately before use, dilute the challenge toxin with a suitable diluent to obtain a challenge toxin solution containing about 0.0512 Lf in 0.2 ml. Prepare from this a further series of 5 four-fold dilutions containing about 0.0128, 0.0032, 0.0008, 0.0002 and 0.00005 Lf in 0.2 ml.

Determination of potency of the vaccine. Using a 9 g/l solution of *sodium chloride R*, prepare dilutions of the vaccine to be examined and of the reference preparation, such that for each, the dilutions form a series differing by not more than 2.5-fold steps and in which the intermediate dilutions, when injected subcutaneously at a dose of 1.0 ml per guinea-pig, will result in an intradermal score of approximately 3 when the animals are challenged. Allocate the dilutions 1 to each of the groups of guinea-pigs and inject subcutaneously 1.0 ml of each dilution into each guinea-pig in the group to which that dilution is allocated. After 28 days, shave both flanks of each guinea-pig and inject 0.2 ml of each of the 6 toxin dilutions intradermally into 6 separate sites on each of the vaccinated guinea-pigs in such a way as to minimise interference between adjacent sites.

Determination of the activity of the challenge toxin. If necessary, inject the unvaccinated control animals with dilutions containing 80, 40, 20, 10 and 5 millionths of an Lf of the challenge toxin.

Reading and interpretation of results. Examine all injection sites 48 h after injection of the challenge toxin and record the incidence of specific diphtheria erythema. Record also the number of sites free from such reactions as the intra-dermal challenge score. Tabulate together the intradermal challenge scores for all the animals receiving the same dilution of vaccine and use those data with a suitable transformation, such as $(\text{score})^2$ or $\arcsin((\text{score}/6)^2)$, to obtain an estimate of the relative potency for each of the test preparations by parallel-line quantitative analysis.

Requirements for a valid assay. The test is not valid unless:

- for both the vaccine to be examined and the reference preparation, the mean score obtained at the lowest dose level is less than 3 and the mean score at the highest dose level is more than 3,
- if applicable, the toxin dilution that contains 40 millionths of an Lf gives a positive erythema in at least 80 per cent of the control guinea-pigs and the dilution containing 20 millionths of an Lf gives a positive erythema in less than 80 per cent of the guinea-pigs (if these criteria are not met a different toxin has to be selected),
- the confidence limits ($P = 0.95$) are not less than 50 per cent and not more than 200 per cent of the estimated potency,
- the statistical analysis shows no deviation from linearity and parallelism.

The test may be repeated but when more than 1 test is performed the results of all valid tests must be combined in the estimate of potency.

METHOD OF LETHAL CHALLENGE

Selection and distribution of the test animals. Use in the test healthy guinea-pigs from the same stock, each weighing 250 g to 350 g. Distribute the guinea-pigs in not fewer than 6 equal groups; use groups containing a number of animals sufficient to obtain results that fulfil the requirements for a valid assay prescribed below. If the challenge toxin to be used has not been shown to be stable or has not been adequately standardised, include 4 further groups of 5 guinea-pigs as unvaccinated controls. Use guinea-pigs of the same sex or with males and females equally distributed between the groups.

Selection of the challenge toxin. Select a preparation of diphtheria toxin containing not less than 100 LD₅₀ per millilitre. If the challenge toxin preparation has been shown to be stable, it is not necessary to verify the lethal dose for every assay.

Preparation of the challenge toxin solution. Immediately before use, dilute the challenge toxin with a suitable diluent to obtain a challenge toxin solution containing approximately 100 LD₅₀ per millilitre. If necessary, dilute portions of the challenge toxin solution 1 in 32, 1 in 100 and 1 in 320 with the same diluent.

Determination of potency of the vaccine. Using a 9 g/l solution of *sodium chloride R*, prepare dilutions of the vaccine to be examined and of the reference preparation, such that for each, the dilutions form a series differing by not more than 2.5-fold steps and in which the intermediate dilutions, when injected subcutaneously at a dose of 1.0 ml per guinea-pig, protect approximately 50 per cent of the animals from the lethal effects of the subcutaneous injection of the quantity of diphtheria toxin prescribed for this test. Allocate the dilutions 1 to each of the groups of guinea-pigs and inject subcutaneously 1.0 ml of each dilution into each guinea-pig in the group to which that dilution is allocated. After 28 days, inject subcutaneously into each animal 1.0 ml of the challenge toxin solution (100 LD₅₀).

Determination of the activity of the challenge toxin. If necessary, allocate the challenge toxin solution and the 3 dilutions made from it, 1 to each of the 4 groups of 5 guinea-pigs and inject subcutaneously 1.0 ml of each solution into each guinea-pig in the group to which that solution is allocated.

Reading and interpretation of results. Count the number of surviving guinea-pigs 4 days after injection of the challenge toxin. Calculate the potency of the vaccine to be examined relative to the potency of the reference preparation on the basis of the proportion of animals surviving in each of the groups of vaccinated guinea-pigs, using the usual statistical methods.

Requirements for a valid assay. The test is not valid unless:

- for the vaccine to be examined and the reference preparation the 50 per cent protective dose lies between the largest and smallest doses of the preparations given to the guinea-pigs,
- if applicable, the number of animals that die in the 4 groups of 5 injected with the challenge toxin solution and its dilutions indicates that the challenge dose was approximately 100 LD₅₀,
- the confidence limits ($P = 0.95$) are not less than 50 per cent and not more than 200 per cent of the estimated potency,

- the statistical analysis shows no deviation from linearity and parallelism.

The test may be repeated but when more than 1 test is performed the results of all valid tests must be combined in the estimate of potency.

01/2005:20707

2.7.7. ASSAY OF PERTUSSIS VACCINE

The potency of pertussis vaccine is determined by comparing the dose necessary to protect mice against the effects of a lethal dose of *Bordetella pertussis*, administered intracerebrally, with the quantity of a reference preparation, calibrated in International Units, needed to give the same protection.

The International Unit is the activity contained in a stated amount of the International Standard which consists of a quantity of dried pertussis vaccine. The equivalence in International Units of the International Standard is stated by the World Health Organisation.

Selection and distribution of the test animals. Use in the test, healthy mice less than 5 weeks old of a suitable strain from the same stock, the difference in mass between the heaviest and the lightest being not greater than 5 g. Distribute the mice in 6 groups of not fewer than 16 and 4 groups of 10. The mice must all be of the same sex or the males and females should be distributed equally between the groups.

Selection of the challenge strain and preparation of the challenge suspension. Select a suitable strain of *B. pertussis* capable of causing the death of mice within 14 days of intracerebral injection. If more than 20 per cent of the mice die within 48 h of the injection the strain is not suitable. Make one subculture from the strain and suspend the harvested *B. pertussis* in a solution containing 10 g/l of *casein hydrolysate R* and 6 g/l of *sodium chloride R* and having a pH of 7.0 to 7.2 or in another suitable solution. Determine the opacity of the suspension. Prepare a series of dilutions in the same solution and allocate each dilution to a group of ten mice. Inject intracerebrally into each mouse a dose (0.02 ml or 0.03 ml) of the dilution allocated to its group. After 14 days, count the number of mice surviving in each group. From the results, calculate the expected opacity of a suspension containing 100 LD₅₀ in each challenge dose. For the test of the vaccine to be examined make a fresh subculture from the same strain of *B. pertussis* and prepare a suspension of the harvested organisms with an opacity corresponding to about 100 LD₅₀ in each challenge dose. Prepare 3 dilutions of the challenge suspension.

Determination of potency. Prepare 3 serial dilutions of the vaccine to be examined and 3 similar dilutions of the reference preparation such that in each the intermediate dilution may be expected to protect about 50 per cent of the mice from the lethal effects of the challenge dose of *B. pertussis*. Suggested doses are 1/8, 1/40 and 1/200 of the human dose of the vaccine to be examined and 0.5 IU, 0.1 IU and 0.02 IU of the reference preparation, each dose being contained in a volume not exceeding 0.5 ml. Allocate 6 dilutions one to each of the groups of not fewer than 16 mice and inject intraperitoneally into each mouse one dose of the dilution allocated to its group. After 14 to 17 days inject intracerebrally into each animal in the groups of not fewer than 16, one dose of the challenge suspension. Allocate the challenge suspension and the 3 dilutions made from it one to each of the groups of 10 mice and inject intracerebrally one dose of each suspension into each mouse in the group to which that suspension is allocated.

Exclude from consideration any mice that die within 48 h of challenge. Count the number of mice surviving in each of the groups after 14 days. Calculate the potency of the vaccine to be examined relative to the potency of the reference preparation on the basis of the numbers of animals surviving in each of the groups of not fewer than 16.

The test is not valid unless:

- for both the vaccine to be examined and the reference preparation, the 50 per cent protective dose lies between the largest and the smallest doses given to the mice;
- the number of animals which die in the four groups of ten injected with the challenge suspension and its dilutions indicates that the challenge dose is approximately 100 LD₅₀;
- and the statistical analysis shows no deviation from linearity or parallelism.

The test may be repeated but when more than one test is performed the results of all valid tests must be combined.

01/2005:20708

2.7.8. ASSAY OF TETANUS VACCINE (ADSORBED)

The potency of tetanus vaccine is determined by administration of the vaccine to animals (guinea-pigs or mice) followed either by challenge with tetanus toxin (method A or B) or by determination of the titre of antibodies against tetanus toxoid in the serum of the guinea-pigs (method C). In both cases the potency of the vaccine is calculated by comparison with a reference vaccine, calibrated in International Units. For methods A and B, in countries where the paralysis method is not obligatory the LD₅₀ method may be used. For the LD₅₀ method, the number of animals and the procedure are identical with those described for the paralysis method but the end-point is the death of the animal rather than paralysis.

The International Unit is the activity contained in a stated amount of the International Standard for tetanus toxoid (adsorbed). The equivalence in International Units of the International Standard is stated by the World Health Organisation.

Tetanus vaccine (adsorbed) BRP is calibrated in International Units with reference to the International Standard.

The method chosen for assay of tetanus vaccine (adsorbed) depends on the intended purpose. Method A or B is used:

1. during development of a vaccine, to assay batches produced to validate the production;
2. wherever revalidation is needed following a significant change in the manufacturing process.

Method A or B may also be used for routine assay of batches of vaccine but in the interests of animal welfare, method C is used wherever possible.

Method C may be used, except as specified under 1 and 2 above, after verification of the suitability of the method for the product. For this purpose, a suitable number of batches (usually 3) are assayed by method C and method A or B. Where different vaccines (monovalent or combinations) are prepared from tetanus toxoid of the same origin, suitability demonstrated for the combination with the highest number of components can be assumed to be valid for combinations with fewer components and for monovalent vaccine. For combinations with a whole-cell pertussis component, a separate demonstration of equivalence must be made for the highest combination.

The design of the assays described below uses multiple dilutions for the test and reference preparations. Based on the potency data obtained in multidilution assays, it may be possible to decrease the number of animals needed to obtain a statistically significant result by applying a simplified model using a single dilution for both test and reference preparations. Such a model enables the analyst to determine whether the potency of the test preparation is significantly higher than the minimum required but does not give information on the dose-response curves and their linearity, parallelism and significant slope. The simplified model may lead to a considerable reduction in the number of animals required and its use must be considered in accordance with the provisions of the European Convention for the protection of vertebrate animals used for experimental and other scientific purposes.

Where a single-dilution assay is used, production and test consistency over time are monitored via suitable indicators and by carrying out a full multiple-dilution assay periodically, for example every 2 years. For serological assays, suitable indicators to monitor test consistency are:

- mean and standard deviation of relative antitoxin titres or scores of the serum samples obtained after administration of a fixed dose of the vaccine reference preparation,
- antitoxin titres or scores of run controls (positive and negative serum samples),
- ratio of antitoxin titres or scores for the positive serum control and the serum samples corresponding to the reference vaccine.

METHOD A. CHALLENGE TEST IN GUINEA-PIGS SELECTION AND DISTRIBUTION OF THE TEST ANIMALS

Use in the test healthy guinea-pigs from the same stock, each weighing 250-350 g. Distribute the guinea-pigs in not fewer than 6 equal groups; use groups containing a number of animals sufficient to obtain results that fulfil the requirements for a valid assay prescribed below. If the activity of the challenge toxin has to be determined, include 3 further groups of 5 guinea-pigs as unvaccinated controls. Use guinea-pigs of the same sex or with the males and females equally distributed between the groups.

SELECTION OF THE CHALLENGE TOXIN

Select a preparation of tetanus toxin containing not less than 50 times the 50 per cent paralytic dose per millilitre. If the challenge toxin preparation has been shown to be stable, it is not necessary to verify the paralytic dose for every assay.

PREPARATION OF THE CHALLENGE TOXIN SOLUTION

Immediately before use, dilute the challenge toxin with a suitable diluent (for example, peptone buffered saline solution pH 7.4) to obtain a stable challenge toxin solution containing approximately 50 times the 50 per cent paralytic dose per millilitre. If necessary, use portions of the challenge toxin solution diluted 1 to 16, 1 to 50 and 1 to 160 with the same diluent to determine the activity of the toxin.

DILUTION OF THE TEST AND REFERENCE PREPARATIONS

Using a 9 g/l solution of *sodium chloride R*, prepare dilutions of the vaccine to be examined and of the reference preparation, such that for each, the dilutions form a series differing by not more than 2.5-fold steps and in which the intermediate dilutions, when injected subcutaneously at a dose of 1.0 ml per guinea-pig, protect approximately 50 per cent of the animals from the paralytic effects of the subcutaneous injection of the quantity of tetanus toxin prescribed for this test.

IMMUNISATION AND CHALLENGE

Allocate the dilutions, 1 to each of the groups of guinea-pigs and inject subcutaneously, 1.0 ml of each dilution into each guinea-pig in the group to which that dilution is allocated. After 28 days, inject subcutaneously into each animal 1.0 ml of the challenge toxin solution (containing 50 times the 50 per cent paralytic dose).

DETERMINATION OF THE ACTIVITY OF THE CHALLENGE TOXIN

If necessary, allocate the 3 dilutions made from the challenge toxin solution, 1 to each of the 3 groups of 5 guinea-pigs, and inject subcutaneously 1.0 ml of each solution into each guinea-pig in the group to which that solution is allocated. The activity and stability of the challenge toxin are determined by carrying out a suitable number of determinations of the 50 per cent paralytic dose. It is then not necessary to repeat the determination for each assay.

READING AND INTERPRETATION OF RESULTS

Examine the guinea-pigs twice daily. Remove and humanely kill all animals showing definite signs of tetanus paralysis. Count the number of guinea-pigs without paralysis 5 days after injection of the challenge toxin. Calculate the potency of the vaccine to be examined relative to the potency of the reference preparation on the basis of the proportion of challenged animals without paralysis in each of the groups of vaccinated guinea-pigs, using the usual statistical methods.

REQUIREMENTS FOR A VALID ASSAY

The test is not valid unless:

- for both the vaccine to be examined and the reference preparation the 50 per cent protective dose lies between the largest and smallest doses of the preparations given to the guinea-pigs,
- if applicable, the number of paralysed animals in the 3 groups of 5 injected with the dilutions of the challenge toxin solution indicates that the challenge was approximately 50 times the 50 per cent paralytic dose,
- the confidence limits ($P = 0.95$) are not less than 50 per cent and not more than 200 per cent of the estimated potency,
- the statistical analysis shows significant slope and no deviation from linearity and parallelism of the dose-response lines (chapter 5.3 describes possible alternatives if significant deviations are observed).

The test may be repeated but when more than 1 test is performed the results of all valid tests must be combined in the estimate of potency.

METHOD B. CHALLENGE TEST IN MICE**SELECTION AND DISTRIBUTION OF THE TEST ANIMALS**

Use in the test healthy mice from the same stock, about 5 weeks old and from a strain shown to be suitable. Distribute the mice in not fewer than 6 equal groups; use groups containing a number of animals sufficient to obtain results that fulfil the requirements for a valid assay prescribed below. If the challenge toxin to be used has not been shown to be stable or has not been adequately standardised, include 3 groups of not fewer than 5 mice to serve as unvaccinated controls. Use mice of the same sex or with males and females equally distributed between the groups.

SELECTION OF THE CHALLENGE TOXIN

Select a preparation of tetanus toxin containing not less than 100 times the 50 per cent paralytic dose per millilitre. If the challenge toxin preparation has been shown to be stable, it is not necessary to verify the paralytic dose for every assay.

PREPARATION OF THE CHALLENGE TOXIN SOLUTION

Immediately before use, dilute the challenge toxin with a suitable diluent (for example, peptone buffered saline solution pH 7.4) to obtain a stable challenge toxin solution containing approximately 50 times the 50 per cent paralytic dose in 0.5 ml. If necessary, use portions of the challenge toxin solution diluted 1 to 16, 1 to 50 and 1 to 160 with the same diluent to determine the activity of the toxin.

DILUTION OF THE TEST AND REFERENCE PREPARATIONS

Using a 9 g/l solution of *sodium chloride R*, prepare dilutions of the vaccine to be examined and of the reference preparation, such that for each, the dilutions form a series differing by not more than 2.5-fold steps and in which the intermediate dilutions, when injected subcutaneously at a dose of 0.5 ml per mouse, protect approximately 50 per cent of the animals from the paralytic effects of the subcutaneous injection of the quantity of tetanus toxin prescribed for this test.

IMMUNISATION AND CHALLENGE

Allocate the dilutions, 1 to each of the groups of mice and inject subcutaneously 0.5 ml of each dilution into each mouse in the group to which that dilution is allocated. After 28 days, inject subcutaneously into each animal 0.5 ml of the challenge toxin solution (containing 50 times the 50 per cent paralytic dose).

DETERMINATION OF THE ACTIVITY OF THE CHALLENGE TOXIN

If necessary, allocate the 3 dilutions made from the challenge toxin solution, 1 to each of the 3 groups of not fewer than 5 mice and inject subcutaneously 0.5 ml of each solution into each mouse in the group to which that solution is allocated.

READING AND INTERPRETATION OF RESULTS

Examine the mice twice daily. Remove and humanely kill all animals showing definite signs of tetanus paralysis. Count the number of mice without paralysis 4 days after injection of the challenge toxin. Calculate the potency of the vaccine to be examined relative to the potency of the reference preparation on the basis of the proportion of challenged animals without paralysis in each group of vaccinated mice, using the usual statistical methods.

REQUIREMENTS FOR A VALID ASSAY

The test is not valid unless:

- for both the vaccine to be examined and the reference preparation the 50 per cent protective dose lies between the largest and smallest doses of the preparations given to the mice,
- if applicable, the number of paralysed animals in the 3 groups of not fewer than 5 injected with the dilutions of the challenge toxin solution, indicates that the challenge dose was approximately 50 times the 50 per cent paralytic dose,
- the confidence limits ($P = 0.95$) are not less than 50 per cent and not more than 200 per cent of the estimated potency,
- the statistical analysis shows a significant slope and no deviation from linearity and parallelism of the dose-response lines (chapter 5.3 describes possible alternatives if significant deviations are observed).

The test may be repeated but when more than 1 test is performed the results of all valid tests must be combined in the estimate of potency.

METHOD C. DETERMINATION OF ANTIBODIES IN GUINEA-PIGS

SELECTION AND DISTRIBUTION OF THE TEST ANIMALS

Use in the test healthy guinea-pigs from the same stock, each weighing 250-350 g. Use guinea-pigs of the same sex or with males and females equally distributed between groups. Distribute the guinea-pigs in not fewer than 6 equal groups; use groups containing a number of animals sufficient to obtain results that fulfil the requirements for a valid assay. Use a further group of non-vaccinated guinea-pigs of the same origin to provide a negative serum control. If test consistency has been demonstrated, a reference negative serum control may be used.

REFERENCE PREPARATION

Use a suitable reference preparation such as *tetanus vaccine (adsorbed) BRP* or a batch of vaccine shown to be effective in clinical studies, or a batch representative thereof, and which has been calibrated in International Units with reference to *tetanus vaccine (adsorbed) BRP* or the International Standard for tetanus toxoid (adsorbed).

DILUTION OF THE TEST AND REFERENCE PREPARATIONS

Using a 9 g/l solution of *sodium chloride R* as diluent, prepare serial dilutions of the vaccine to be examined and the reference preparation; series differing by 2.5- to 5-fold steps have been found suitable. Use not fewer than 3 dilutions within the range for example 0.5-16 IU/ml for each series. Use dilutions for immunisation preferably within 1 h of preparation. Allocate 1 dilution to each group of guinea-pigs.

IMMUNISATION

Inject subcutaneously in the nape of each guinea-pig 1.0 ml of the dilution allocated to its group.

BLOOD SAMPLING

35-42 days after immunisation, take a blood sample from each vaccinated and control guinea-pig using a suitable method.

PREPARATION OF SERUM SAMPLES

Avoid frequent freezing and thawing of serum samples. To avoid microbial contamination, it is preferable to carry out manipulations in a laminar-flow cabinet.

DETERMINATION OF ANTIBODY TITRE

Determine the relative antibody titre or score of each serum sample by a suitable immunochemical method (2.7.1). The methods shown below (enzyme-linked immunosorbent assay (ELISA) and toxin-binding inhibition (ToBI)) have been found suitable.

CALCULATION OF POTENCY

Calculate the potency of the vaccine to be examined in International Units relative to the reference preparation, using the usual statistical methods (for example 5.3).

NOTE: *International Units of potency refer to the reference vaccine and not to the International Units of antitoxin of the reference guinea-pig serum.*

Requirements for a valid assay. The test is not valid unless:

- the confidence limits ($P = 0.95$) are not less than 50 per cent and not more than 200 per cent of the estimated potency,
- the statistical analysis shows significant slope and no deviation from linearity and parallelism of the dose-response lines (chapter 5.3 describes possible alternatives if significant deviations are observed).

The test may be repeated but when more than 1 test is performed the results of all valid tests must be combined in the estimate of potency.

Assay of tetanus vaccine (adsorbed): guidelines

METHOD A. CHALLENGE TEST IN GUINEA-PIGS

READING AND INTERPRETATION OF RESULTS

In order to minimise suffering in the test animals, it is recommended to note the degree of paralysis on a scale such as that shown below. The scale gives typical signs when injection of the challenge toxin is made mid-ventrally directly behind the sternum with the needle pointing towards the neck of the guinea-pig. Grade T3 is taken as the end-point, but with experience grade T2 can be used instead. Tetanus toxin produces in at least 1 of the forelimbs paralysis that can be recognised at an early stage. The tetanus grades in guinea-pigs are characterised by the following signs:

- T1: slight stiffness of 1 forelimb, but difficult to observe;
- T2: paresis of 1 forelimb which still can function;
- T3: paralysis of 1 forelimb. The animal moves reluctantly, the body is often slightly banana-shaped owing to scoliosis;
- T4: the forelimb is completely stiff and the toes are immovable. The muscular contraction of the forelimb is very pronounced and usually scoliosis is observed;
- T5: tetanus seizures, continuous tonic spasm of muscles;
- D: death.

METHOD B. CHALLENGE TEST IN MICE

READING AND INTERPRETATION OF RESULTS

In order to minimise suffering in the test animals, it is recommended to note the degree of paralysis on a scale such as that shown below. The scale gives typical signs when injection of the challenge toxin is made in the dorsal region, close to one of the hind legs. Grade T3 is taken as the end-point, but with experience grade T2 can be used instead. Tetanus toxin produces in the toxin-injected hind leg paresis followed by paralysis that can be recognised at an early stage. The tetanus grades in mice are characterised by the following signs:

- T1: slight stiffness of toxin-injected hind leg, only observed when the mouse is lifted by the tail;
- T2: paresis of the toxin-injected hind leg, which still can function for walking;
- T3: paralysis of the toxin-injected hind leg, which does not function for walking;
- T4: the toxin-injected hind leg is completely stiff with immovable toes;
- T5: tetanus seizures, continuous tonic spasm of muscles;
- D: death.

METHOD C. DETERMINATION OF ANTIBODIES IN GUINEA-PIGS

PREPARATION OF SERUM SAMPLES

For preparation of serum samples, the following technique has been found suitable. Invert the tubes containing blood samples 6 times and allow to stand at 37 °C for 2 h, then at 4 °C for 2 h. Centrifuge at room temperature at 800 g for 20 min. Transfer the serum to sterile tubes and store at a temperature below –20 °C. At least 40 per cent yield of serum is obtained by this procedure.

DETERMINATION OF ANTIBODY TITRE

The ELISA and ToBI tests shown below are given as examples of immunochemical methods that have been found suitable for the determination of antibody titre.

Determination of antibody titre in guinea-pig serum by enzyme-linked immunosorbent assay (ELISA). Dilutions of test and reference sera are made on ELISA plates coated with tetanus toxoid. A positive guinea-pig serum control and a negative guinea-pig serum control are included on each plate to monitor the assay performance. Peroxidase-conjugated rabbit or goat antibody directed against guinea-pig-IgG is added followed by a peroxidase substrate. Optical density is measured and the relative antibody titre is calculated using the usual statistical methods (for example 5.3).

Reagents and equipment

- ELISA plates: 96 wells, columns 1-12, rows A-H.
- *Clostridium tetani* guinea-pig antiserum (for vaccines-human use) BRP (positive control serum).
- Peroxidase conjugate. Peroxidase-conjugated rabbit or goat antibody directed against guinea-pig IgG.
- Tetanus toxoid.
- Carbonate coating buffer pH 9.6. Dissolve 1.59 g of *anhydrous sodium carbonate R* and 2.93 g of *sodium hydrogen carbonate R* in 1000 ml of *water R*. Distribute into 150 ml bottles and sterilise by autoclaving at 121 °C for 15 min.
- Phosphate buffered saline pH 7.4 (PBS). Dissolve with stirring 80.0 g of *sodium chloride R*, 2.0 g of *potassium dihydrogen phosphate R*, 14.3 g of *disodium hydrogen phosphate dihydrate R* and 2.0 g of *potassium chloride R* in 1000 ml of *water R*. Store at room temperature to prevent crystallisation. Dilute to 10 times its volume with *water R* before use.
- Citric acid solution. Dissolve 10.51 g of *citric acid R* in 1000 ml of *water R* and adjust the solution to pH 4.0 with a 400 g/l solution of *sodium hydroxide R*.
- Washing buffer. PBS containing 0.5 g/l of *polysorbate 20 R*.
- Diluent block buffer. PBS containing 0.5 g/l of *polysorbate 20 R* and 25 g/l of dried skimmed milk.
- Peroxidase substrate. Shortly before use, dissolve 10 mg of *diammonium 2,2'-azinobis(3-ethylbenzothiazoline-6-sulphonate) R* (ABTS) in 20 ml of citric acid solution. Immediately before use add 5 µl of *strong hydrogen peroxide solution R*.

Method

The description below is given as an example of a suitable plate lay-out but others may be used. Wells 1A-H are for negative control serum and wells 2A-H and 3A-H are for positive control serum for assay monitoring. Wells 4-12A-H are for test samples.

Coat each well of the ELISA plates with 100 µl of tetanus toxoid solution (0.5 Lf/ml in carbonate coating buffer). Allow to stand overnight at 4 °C in a humid atmosphere. To avoid interference from temperature gradient, do not stack more than 4 plates high. On the following day, wash the plates thoroughly with washing buffer. Block the plates by addition of 100 µl of diluent block buffer to each well. Incubate in a humid atmosphere at 37 °C for 1 h. Wash the plates thoroughly with washing buffer. Place 100 µl of diluent block buffer in each well of the plates, except those of row A. Prepare suitable dilutions of negative control serum, positive control serum (from about 0.01 IU/ml) and test sera. Allocate the negative control serum to column 1, positive control serum to columns 2 and 3 and test sera to columns 4-12 and add 100 µl of each serum to the first 2 wells of the column to which it is allocated. Using a multichannel micropipette, make twofold serial dilutions from row B down the plate to row H by transferring 100 µl to the following well. Discard 100 µl from the last row so that all wells

contain 100 µl. Incubate at 37 °C for 2 h. Wash thoroughly with washing buffer. Prepare a suitable dilution (a 1 in 2000 dilution has been found suitable) of peroxidase conjugate in diluent block buffer and add 100 µl to each well. Incubate at 37 °C in a humid atmosphere for 1 h. Wash the plates thoroughly with washing buffer. Add 100 µl of peroxidase substrate to each well. Allow to stand at room temperature, protected from light, for 30 min. Read the plates at 405 nm in the same order as addition of substrate was made.

Determination of antibody titre in guinea-pig serum by toxin- or toxoid-binding inhibition (ToBI). Tetanus toxin or toxoid is added to serial dilutions of test and reference sera; the serum/antigen mixtures are incubated overnight. To determine unbound toxin or toxoid, the mixtures are transferred to an ELISA plate coated with tetanus antitoxin. Peroxidase-conjugated equine anti-tetanus IgG is added followed by a peroxidase substrate. Optical density is measured and the antibody titre is calculated using the usual statistical methods (for example 5.3). A positive control serum and a negative control serum are included on each plate to monitor assay performance.

Reagents and equipment

- Round-bottomed, rigid polystyrene microtitre plates.
- Flat-bottomed ELISA plates.
- Tetanus toxin or tetanus toxoid.
- *Clostridium tetani* guinea-pig antiserum (for vaccines-human use) BRP.
- Equine anti-tetanus IgG.
- Peroxidase-conjugated equine anti-tetanus IgG.
- Carbonate buffer pH 9.6. Dissolve 1.5 g of *anhydrous sodium carbonate R*, 2.39 g of *sodium hydrogen carbonate R* and 0.2 g of *sodium azide R* in 1000 ml of *water R*, adjust to pH 9.6 and autoclave at 121 °C for 20 min.
- Sodium acetate buffer pH 5.5. Dissolve 90.2 g of *anhydrous sodium acetate R* in 900 ml of *water R*, adjust to pH 5.5 using a saturated solution of *citric acid monohydrate R* and dilute to 1000 ml with *water R*.
- Phosphate buffered saline pH 7.2 (PBS). Dissolve 135.0 g of *sodium chloride R*, 20.55 g of *disodium hydrogen phosphate dihydrate R* and 4.80 g of *sodium dihydrogen phosphate monohydrate R* in *water R* and dilute to 15 litres with the same solvent. Autoclave at 100 °C for 60 min.
- Diluent buffer. PBS containing 5 g/l of *bovine albumin R* and 0.5 g/l of *polysorbate 80 R*.
- Block buffer. PBS containing 5 g/l of *bovine albumin R*.
- Tetramethylbenzidine solution. 6 g/l solution of *tetramethylbenzidine R* in *alcohol R*. The substance dissolves within 30-40 min at room temperature.
- Peroxidase substrate. Mix 90 ml of *water R*, 10 ml of sodium acetate buffer pH 5.5, 1.67 ml of tetramethylbenzidine solution and 20 µl of *strong hydrogen peroxide solution R*.
- Washing solution. Tap water containing 0.5 g/l of *polysorbate 80 R*.

Method

Block the round-bottomed polystyrene microtitre plates by placing in each well 150 µl of block buffer. Cover the plates with a lid or sealer. Incubate in a humid atmosphere at 37 °C for 1 h. Wash the plates thoroughly with washing solution. Place 100 µl of PBS in each well. Place 100 µl of reference guinea-pig tetanus antitoxin in the first well of a row. Place 100 µl of undiluted test sera in the first well of the required number of rows. Using a multichannel micropipette, make

twofold serial dilutions across the plate (up to column 10), by transfer of 100 µl to the following well. Discard 100 µl from the last column so that all wells contain 100 µl. Prepare a 0.1 Lf/ml solution of tetanus toxin or toxoid using PBS as diluent. Add 40 µl of this solution to all wells except those of column 12. The wells of row 11 are a positive control. Add 40 µl of PBS to the wells of column 12 (negative control). Shake the plates gently and cover them with lids. Coat the ELISA plates: immediately before use make a suitable dilution of equine anti-tetanus IgG in carbonate buffer pH 9.6 and add 100 µl to all wells. Incubate the 2 series of plates overnight in a humid atmosphere at 37 °C. To avoid temperature gradient effects, do not stack more than 4 plates high. Cover the plates with lids. On the following day, wash the ELISA plates thoroughly with washing solution. Block the plates by placing in each well 125 µl of block buffer. Incubate at 37 °C in a humid atmosphere for 1 h. Wash the plates thoroughly with washing solution. Transfer 100 µl of the pre-incubation mixture from the polystyrene plates to the corresponding wells of the ELISA plates, starting with column 12 and then from 1 to 11. Cover the plates with a lid. Incubate at 37 °C in a humid atmosphere for 2 h. Wash the ELISA plates thoroughly with washing solution. Make a suitable dilution (a 1 in 4000 dilution has been found suitable) of the peroxidase-conjugated equine anti-tetanus IgG in diluent buffer. Add 100 µl of the dilution to each well and cover the plates with a lid. Incubate at 37 °C in a humid atmosphere for 1.5 h. Wash the ELISA plates thoroughly with washing solution. Add 100 µl of peroxidase substrate to each well. A blue colour develops. Incubate the plates at room temperature. Stop the reaction at a given time (within 10 min) by the addition of 100 µl of 2 M sulphuric acid to each well in the same order as the addition of substrate. The colour changes from blue to yellow. Measure the absorbance at 450 nm immediately after addition of the sulphuric acid or maintain the plates in the dark until reading.

01/2005:20709

2.7.9. TEST FOR Fc FUNCTION OF IMMUNOGLOBULIN

Stabilised human blood. Collect group O human red blood into ACD anticoagulant solution. Store the stabilised blood at 4 °C for not more than 3 weeks.

Phosphate buffered saline pH 7.2. Dissolve 1.022 g of anhydrous disodium hydrogen phosphate R, 0.336 g of anhydrous sodium dihydrogen phosphate R and 8.766 g of sodium chloride R in 800 ml of water R and dilute to 1000 ml with the same solvent.

Magnesium and calcium stock solution. Dissolve 1.103 g of calcium chloride R and 5.083 g of magnesium chloride R in water R and dilute to 25 ml with the same solvent.

Barbital buffer stock solution. Dissolve 207.5 g of sodium chloride R and 25.48 g of barbital sodium R in 4000 ml of water R and adjust to pH 7.3 using 1 M hydrochloric acid. Add 12.5 ml of magnesium and calcium stock solution and dilute to 5000 ml with water R. Filter through a membrane filter (pore size 0.22 µm). Store at 4 °C in glass containers.

Albumin barbital buffer solution. Dissolve 0.150 g of bovine albumin R in 20 ml of barbital buffer stock solution and dilute to 100 ml with water R.

Tannic acid solution. Dissolve 10 mg of tannic acid R in 100 ml of phosphate-buffered saline pH 7.2. Prepare immediately before use.

Guinea-pig complement. Prepare a pool of serum from the blood of not fewer than 10 guinea-pigs. Separate the serum from the clotted blood by centrifugation at about 4 °C. Store

the serum in small amounts below –70 °C. Immediately before starting complement-initiated haemolysis, dilute to 125–200 CH₅₀ per millilitre with albumin barbital buffer solution and store in an ice-bath during the test.

Rubella antigen. Suitable rubella antigen for haemagglutination-inhibition titre (HIT). Titre > 256 HA units.

Preparation of tanned human red blood cells. Separate human red blood cells by centrifuging an appropriate volume of stabilised human blood and wash the cells at least 3 times with phosphate-buffered saline pH 7.2 and suspend at 2 per cent V/V in phosphate-buffered saline pH 7.2. Dilute 0.1 ml of tannic acid solution to 7.5 ml with phosphate-buffered saline pH 7.2 (final concentration 1.3 mg/l). Mix 1 volume of the freshly prepared dilution with 1 volume of human red blood cell suspension and incubate at 37 °C for 10 min. Collect the cells by centrifugation (400–800 g for 10 min), discard the supernatant and wash the cells once with phosphate-buffered saline pH 7.2. Resuspend the tanned cells at 1 per cent V/V in phosphate-buffered saline pH 7.2.

Antigen coating of tanned human red blood cells. Take a suitable volume (V_s) of tanned cells, add 0.2 ml of rubella antigen per 1.0 ml of tanned cells and incubate at 37 °C for 30 min. Collect the cells by centrifugation (400–800 g for 10 min) and discard the supernatant, leaving a volume of 200 µl. Add a volume of albumin barbital buffer solution equivalent to the discarded supernatant, resuspend and collect the cells as described and repeat the washing procedure. Make up the remaining 200 µl to three-quarters of V_s , thereby obtaining the initial volume (V_i). Mix 900 µl of albumin barbital buffer solution with 100 µl of V_i , which is thereby reduced to the residual volume (V_r), and determine the initial absorbance at 541 nm (A). Dilute V_r by a factor equal to A using albumin barbital buffer solution, thereby obtaining the final adjusted volume $V_f = V_r \times A$ of sensitised human red blood cells and adjusting A to 1.0 ± 0.1 for a tenfold dilution.

Antibody binding of antigen-coated tanned human red blood cells. Prepare the following solutions in succession and in duplicate, using for each solution a separate half-micro cuvette (for example, disposable type) or test-tube:

(1) **Test solutions.** If necessary, adjust the immunoglobulin to be examined to pH 7, for example by addition of 1 M sodium hydroxide. Dilute volumes of the preparation to be examined containing 30 mg and 40 mg of immunoglobulin with albumin barbital buffer solution and adjust the volume to 900 µl.

(2) **Reference solutions.** Prepare as for the test solutions using human immunoglobulin BRP.

(3) **Complement control.** 900 µl of albumin barbital buffer solution.

Add to each cuvette/test-tube 100 µl of sensitised human red blood cells and mix well.

Incubate at room temperature for 15 min, add 1000 µl of albumin barbital buffer solution, collect the cells by centrifugation (1000 g for 10 min) of the cuvette/test-tube and remove 1900 µl of the supernatant. Replace the 1900 µl with albumin barbital buffer solution and repeat the whole of the washing procedure, finally leaving a volume of 200 µl. Test samples may be stored in sealed cuvette/test-tubes at 4 °C for 24 h.

Complement-initiated haemolysis. To measure haemolysis, add 600 µl of albumin barbital buffer solution warmed to 37 °C to the test sample, resuspend the cells carefully by repeated pipetting (not fewer than 5 times) and place the cuvette in the thermostatted cuvette holder of a spectrophotometer. After 2 min, add 200 µl of diluted guinea-pig complement (125–200 CH₅₀/ml), mix thoroughly

by pipetting twice and start immediately after the second pipetting the time-dependent recording of absorbance at 541 nm, using albumin barbital buffer solution as the compensation liquid. Stop the measurement if absorbance as a function of time has clearly passed the inflexion point.

Evaluation. Determine the slope (S) of the haemolysis curve at the approximate inflexion point by segmenting the steepest section in suitable time intervals Δt (for example, $\Delta t = 1$ min) and calculate S between adjacent intersection points, expressed as ΔA per minute. The largest value for S serves as (S_{exp}). In addition, determine the absorbance at the start of measurement (A_s) by extrapolating the curve, which is almost linear and parallel to the time axis within the first few minutes. Correct (S_{exp}) using the expression:

$$S' = \frac{S_{\text{exp}}}{A_s}$$

Calculate the arithmetic mean of the values of S' for each preparation. Calculate the index of Fc function (I_{Fc}) from the expression:

$$I_{\text{Fc}} = \frac{100 \times (\overline{S'} - \overline{S'_c})}{\overline{S'_s} - \overline{S'_c}}$$

$\overline{S'}$ = arithmetic mean of the corrected slope for the preparation to be examined,

$\overline{S'_s}$ = arithmetic mean of the corrected slope for the reference preparation,

$\overline{S'_c}$ = arithmetic mean of the corrected slope for the complement control.

Calculate the index of Fc function for the preparation to be examined: the value is not less than that stated in the leaflet accompanying the reference preparation.

01/2005:20710

2.7.10. ASSAY OF HUMAN COAGULATION FACTOR VII

Human coagulation factor VII is assayed by its biological activity as a factor VIIa-tissue factor complex in the activation of factor X in the presence of calcium ions and phospholipids. The potency of a factor VII preparation is estimated by comparing the quantity necessary to achieve a certain rate of factor Xa formation in a test mixture containing the substances that take part in the activation of factor X, and the quantity of the International Standard, or of a reference preparation calibrated in International Units, required to produce the same rate of factor Xa formation.

The International Unit is the factor VII activity of a stated amount of the International Standard which consists of freeze-dried plasma. The equivalence in International Units of the International Standard is stated by the World Health Organisation.

The chromogenic assay method consists of two consecutive steps: the factor VII-dependent activation of factor X reagent mixture containing tissue factor, phospholipids and calcium ion, followed by enzymatic cleavage of a chromogenic factor Xa substrate into a chromophore that can be quantified spectrophotometrically. Under appropriate assay conditions, there is a linear relation between the rate of factor Xa formation and the factor VII concentration. The assay is summarised in Figure 2.7.10.-1.

Step 1

a) Factor VII $\xrightarrow{\text{Tissue Factor} + \text{Ca}^{++}}$ Factor VIIa

b) Factor X $\xrightarrow{\text{Factor VIIa} + \text{Ca}^{++} + \text{Tissue factor/Phospholipid}}$ Factor Xa

Step 2

Chromogenic substrate $\xrightarrow{\text{Factor Xa}}$ Peptide + chromophore

Figure 2.7.10.-1. – Schematic representation of the assay of human coagulation factor VII

Both steps employ reagents that may be obtained commercially from a variety of sources. Although the composition of individual reagents may be subject to some variation, their essential features are described in the following specification.

REAGENTS

The coagulation factor reagent comprises purified proteins derived from human or bovine sources. These include factor X and thromboplastin tissue factor/phospholipid as factor VII activator. These proteins are partly purified and do not contain impurities that interfere with the activation of factor VII or factor X. Factor X is present in amounts giving a final concentration during the first step of the assay of 10 nmol/litre to 350 nmol/litre, preferably 14 nmol/litre to 70 nmol/litre. Thromboplastin from natural sources (bovine or rabbit brain) or synthetic preparations may be used as the tissue factor/phospholipid component. Thromboplastin suitable for use in prothrombin time determination is diluted 1:5 to 1:50 in buffer such that the final concentration of Ca^{2+} is 15 mmol/litre to 25 mmol/litre. The final factor Xa generation is performed in a solution containing human or bovine albumin at a concentration such that adsorption losses do not occur and which is appropriately buffered at pH 7.3 to 8.0. In the final incubation mixture, factor VII must be the only rate-limiting component and each reagent component must lack the ability to generate factor Xa on its own.

The second step comprises the quantification of the formed factor Xa employing a chromogenic substrate that is specific for factor Xa. Generally this consists of a short peptide of between three and five amino acids, bound to a chromophore group. On cleavage of this group from the peptide substrate, its absorption maximum shifts to a wavelength allowing its spectrophotometric quantification. The substrate is usually dissolved in *water R* and used at a final concentration of 0.2 mmol/litre to 2 mmol/litre. The substrate may also contain appropriate inhibitors to stop further factor Xa generation (addition of edetate).

ASSAY PROCEDURE

Reconstitute the entire contents of one ampoule of the reference preparation and the preparation to be examined by adding the appropriate quantity of *water R*; use within 1 h. Add sufficient prediluent to the reconstituted preparations to produce solutions containing between 0.5 IU and 2.0 IU of factor VII per millilitre.

Prepare further dilutions of reference and test preparations using an isotonic non-chelating buffer containing 1 per cent of bovine or human albumin, buffered preferably between pH 7.3 and 8.0. Prepare at least three separate, independent dilutions for each material, preferably in duplicate. Prepare the dilutions such that the final factor VII concentration is below 0.005 IU/ml.

Prepare a control solution that includes all components except factor VII.

Prepare all dilutions in plastic tubes and use within 1 h.

Step 1. Mix dilutions of the factor VII reference preparation and the preparation to be examined with an appropriate volume of the prewarmed coagulation factor reagent or a combination of its separate constituents, and incubate the mixture in plastic tubes or microplate wells at 37 °C. The concentrations of the various components during the factor Xa generation must be as specified above under the description of the reagents.

Allow the activation of factor X to proceed for a suitable time, usually terminating the reaction before the factor Xa concentration has reached its maximal level in order to obtain a satisfactory linear dose-response relationship. The activation time is also chosen to achieve linear production of factor Xa in time. Appropriate activation times are usually between 2 min and 5 min, but deviations are permissible if acceptable linearity of the dose-response relationship is thus obtained.

Step 2. Terminate the activation by the addition of a prewarmed reagent containing a chromogenic substrate. Quantify the rate of substrate cleavage, which must be linear with the concentration of factor Xa formed, by measuring the absorbance change at an appropriate wavelength using a spectrophotometer, either monitoring the absorbance continuously, thus allowing the initial rate of substrate cleavage to be calculated, or terminating the hydrolysis reaction after a suitable interval by lowering the pH by the addition of a suitable reagent, such as acetic acid (500 g/l C₂H₄O₂) or a citrate solution (1 mol/l) at pH 3. Adjust the hydrolysis time to achieve a linear development of chromophore with time. Appropriate hydrolysis times are usually between 3 min and 15 min, but deviations are permissible if better linearity of the dose-response relationship is thus obtained.

Check the validity of the assay and calculate the potency of the test preparation by the usual statistical methods (for example, 5.3. *Statistical analysis of results of biological assays and tests*).

01/2005:20711
corrected

2.7.11. ASSAY OF HUMAN COAGULATION FACTOR IX

The potency is determined by comparing the quantity of the preparation to be examined necessary to reduce the coagulation time of a test mixture containing the substances, other than factor IX, that take part in the coagulation of blood and the quantity of a reference preparation, calibrated in International Units, required to produce the same effect.

The International Unit is the activity of a stated amount of the International Standard, which consists of a freeze-dried concentrate of human coagulation factor IX. The equivalence in International Units of the International Standard is stated by the World Health Organisation.

Human coagulation factor IX concentrate BRP is calibrated in International Units by comparison with the International Standard.

Reconstitute separately the preparation to be examined and the reference preparation as stated on the label and use immediately. Where applicable, determine the amount of heparin present (2.7.12) and neutralise the heparin by addition of *protamine sulphate R* (10 µg of protamine sulphate neutralises 1 IU of heparin). Dilute the preparation

to be examined and the reference preparation with a sufficient quantity of *imidazole buffer solution pH 7.3 R* to produce solutions containing 0.5 IU to 2.0 IU per millilitre. Prepare twofold dilutions in the range 1 to 10 to 1 to 80 using a mixture of 1 volume of a 38 g/l solution of *sodium citrate R* and 5 volumes of *imidazole buffer solution pH 7.3 R*. Make these dilutions accurately and use immediately.

Use, for example, incubation tubes maintained in a water-bath at 37 °C. Place in each tube 0.1 ml of *plasma substrate R2* and 0.1 ml of one of the dilutions of the reference preparation or of the preparation to be examined. Add to each tube 0.1 ml of a suitable dilution of *cephalin R* or *platelet substitute R* and 0.1 ml of a suspension of 0.5 g of *light kaolin R* in 100 ml of a 9 g/l solution of *sodium chloride R* and allow to stand for about 10 min, tilting the tubes regularly. To each tube, add 0.1 ml of a 7.4 g/l solution of *calcium chloride R*. Using a timer, measure the coagulation time, i.e. the interval between the moment of the addition of the calcium chloride and the first indication of the formation of fibrin, which may be observed visually or by the use of a suitable apparatus. Calculate the potency using the usual statistical methods (for example, 5.3. *Statistical analysis of results of biological assays and tests*).

To ensure that there is no appreciable contamination of *plasma substrate R2* by factor IX, carry out a blank test using, instead of the preparation to be examined, a corresponding volume of a mixture of 1 volume of a 38 g/l solution of *sodium citrate R* and 5 volumes of *imidazole buffer solution pH 7.3 R*. The test is not valid unless the coagulation time measured in the blank test is 100 s to 200 s.

01/2005:20712

2.7.12. ASSAY OF HEPARIN IN COAGULATION FACTORS

Heparin is assayed as a complex with antithrombin III (AT) via its inhibition of coagulation factor Xa (anti-Xa activity). An excess of AT is maintained in the reaction mixture to ensure a constant concentration of the heparin-AT complex. Factor Xa is neutralised by the heparin-AT complex and the residual factor Xa hydrolyses a specific chromogenic peptide substrate to release a chromophore. The quantity of chromophore is inversely proportional to the activity of the heparin.

Factor Xa chromogenic substrate. Specific chromogenic substrate for factor Xa such as: *N*-benzoyl-L-isoleucyl-L-glutamyl-glycyl-L-arginine-4-nitroanilide hydrochloride. Reconstitute according to the manufacturer's instructions.

Dilution buffer. 6.05 g/l solution of *tris(hydroxymethyl)aminomethane R*. Adjust to pH 8.4 if necessary using *hydrochloric acid R*.

Test solution. Dilute the preparation to be examined with dilution buffer to obtain a solution expected to contain 0.1 IU of heparin per millilitre.

Reference solution. Dilute the heparin reference preparation with dilution buffer to obtain a solution containing 0.1 IU of heparin per millilitre.

The following working conditions apply to microtitre plates. If the assay is carried out in tubes, the volumes are adjusted while maintaining the proportions in the mixture.

Warm all solutions to 37 °C in a water-bath shortly before the test.

Distribute in a series of wells, 20 µl of normal human plasma and 20 µl of *antithrombin III solution R1*. Add to the wells a series of volumes (20 µl, 60 µl, 100 µl and 140 µl) of the test

solution or the reference solution and make up the volume in each well to 200 µl using dilution buffer (0.02-0.08 IU of heparin per millilitre in the final reaction mixture).

End-point method. Transfer 40 µl from each well to a second series of wells, add 20 µl of *bovine factor Xa solution R* and incubate at 37 °C for 30 s. Add 40 µl of a 1 mmol/l solution of factor Xa chromogenic substrate and incubate at 37 °C for 3 min. Terminate the reaction by lowering the pH by the addition of a suitable reagent, such as a 20 per cent *V/V* solution of *glacial acetic acid R* and measure the absorbance at 405 nm (2.2.25). Appropriate reaction times are usually between 3 min and 15 min, but deviations are permissible if better linearity of the dose-response relationship is thus obtained.

Kinetic method. Transfer 40 µl from each well to a second series of wells, add 20 µl of *bovine factor Xa solution R* and incubate at 37 °C for 30 s. Add 40 µl of a 2 mmol/l solution of factor Xa chromogenic substrate, incubate at 37 °C and measure the rate of substrate cleavage by continuous measurement of the absorbance change at 405 nm (2.2.25), thus allowing the initial rate of substrate cleavage to be calculated. This rate must be linear with the concentration of residual factor Xa.

Check the validity of the assay and calculate the heparin activity of the test preparation by the usual statistical methods for a slope-ratio assay (for example, 5.3. *Statistical analysis of results of biological assays and tests*).

01/2005:20713

2.7.13. ASSAY OF HUMAN ANTI-D IMMUNOGLOBULIN

METHOD A

The potency of human anti-D immunoglobulin is determined by comparing the quantity necessary to produce agglutination of D-positive red blood cells with the quantity of a reference preparation, calibrated in International Units, required to produce the same effect.

The International Unit is the activity contained in a stated amount of the International Reference Preparation. The equivalence in International Units of the International Reference Preparation is stated by the World Health Organisation.

Human anti-D immunoglobulin BRP is calibrated in International Units by comparison with the International Standard and intended for use in the assay of human anti-D immunoglobulin.

Use pooled D-positive red blood cells, collected not more than 7 days earlier and suitably stored, obtained from not fewer than 4 group O R₁R₁ donors. To a suitable volume of the cells, previously washed 3 times with a 9 g/l solution of *sodium chloride R*, add an equal volume of *bromelains solution R*, allow to stand at 37 °C for 10 min, centrifuge, remove the supernatant liquid and wash 3 times with a 9 g/l solution of *sodium chloride R*. Suspend 20 volumes of the red blood cells in a mixture of 15 volumes of inert serum, 20 volumes of a 300 g/l solution of *bovine albumin R* and 45 volumes of a 9 g/l solution of *sodium chloride R*. Stand the resulting suspension in iced water, stirring continuously.

Using a calibrated automated dilutor, prepare suitable dilutions of the preparation to be examined and of the reference preparation using as diluent a solution containing 5 g/l of *bovine albumin R* and 9 g/l of *sodium chloride R*. Use a suitable apparatus for automatic continuous analysis. The following protocol is usually suitable: maintain the temperature in the manifold, except for the incubation coils,

at 15.0 °C. Pump into the manifold of the apparatus the red blood cell suspension at a rate of 0.1 ml/min and a 3 g/l solution of *methylcellulose 450 R* at a rate of 0.05 ml/min. Introduce the dilutions of the preparation to be examined and the reference preparation at a rate of 0.1 ml/min for 2 min, followed by the diluent solution at a rate of 0.1 ml/min for 4 min before the next dilution is introduced.

Introduce air at a rate of 0.6 ml/min. Incubate at 37 °C for 18 min and then disperse the rouleaux by introducing at a rate of 1.6 ml/min a 9 g/l solution of *sodium chloride R* containing a suitable wetting agent (for example, *polysorbate 20 R* at a final concentration of 0.2 g/l) to prevent disruption of the bubble pattern. Allow the agglutinates to settle and decant twice, first at 0.4 ml/min and then at 0.6 ml/min. Lyse the unagglutinated red blood cells with a solution containing 5 g/l of *octoxinol 10 R*, 0.2 g/l of *potassium ferricyanide R*, 1 g/l of *sodium hydrogen carbonate R* and 0.05 g/l of *potassium cyanide R* at a rate of 2.5 ml/min. A ten-minute delay coil is introduced to allow for conversion of the haemoglobin. Continuously record the absorbance (2.2.25) of the haemolysate at a wavelength between 540 nm and 550 nm. Determine the range of antibody concentrations over which there is a linear relationship between concentration and the resultant change in absorbance (ΔA). From the results, prepare a standard curve and use the linear portion of the curve to determine the activity of the preparation to be examined.

Calculate the potency of the preparation to be examined using the usual statistical methods (5.3).

METHOD B

The potency of human anti-D immunoglobulin is determined by competitive enzyme-linked immunoassay on erythrocyte-coated microtitre plates. The method is based on the competitive binding between a polyclonal anti-D immunoglobulin preparation and a biotinylated monoclonal anti-D antibody directed against a D-antigen specific epitope. The activity of the preparation to be examined is compared with a reference preparation calibrated in International Units.

The International Unit is the activity of a stated amount of International Reference Preparation. The equivalence in International Units of the International Reference Preparation is stated by the World Health Organisation.

Human anti-D immunoglobulin BRP is calibrated in International Units by comparison with the International Standard and intended for use in the assay of human anti-D immunoglobulin.

MATERIALS

Reagents not specified are of analytical grade.

PBS (Phosphate-buffered saline). Dissolve 8.0 g of *sodium chloride R*, 0.76 g of *anhydrous disodium hydrogen phosphate R*, 0.2 g of *potassium chloride R*, 0.2 g of *potassium dihydrogen phosphate R* and 0.2 g of *sodium azide R* in *water R* and dilute to 1000 ml with the same solvent.

TBS (Tris-buffered saline). Dissolve 8.0 g of *sodium chloride R* and 0.6 g of *tris(hydroxymethyl) aminomethane R* in *water R*. Adjust to pH 7.2 (2.2.3) with 1 M *hydrochloric acid* and dilute to 1000 ml with the same solvent.

Papain solution. Prepare a solution by stirring 1 g of *papain R* at 37 °C for 30 min in 10 ml of 0.067 M *phosphate buffer solution pH 5.4 R*, centrifuge at 10 000 g for 5 min and filter through a membrane with a pore size of 0.22 µm. To activate, combine 1 ml of the filtrate with 1 ml of a 48.44 g/l solution of *L-cysteine R* and 1 ml of a 3.72 g/l

solution of *sodium edetate R* and dilute to 10 ml with 0.067 M phosphate buffer solution pH 5.4 *R*. Freeze in aliquots at -20°C or below.

Red blood cells. Use pooled D-positive red blood cells obtained from not fewer than 3 group O R_2R_2 donors. Wash the cells 4 times with PBS. Centrifuge the cells at 1800 *g* for 5 min, mix a suitable volume of prewarmed packed cells with a suitable volume of prewarmed papain solution (2 volumes to 1 volume has been found suitable) and incubate at 37°C for 10 min. Wash the cells 4 times with PBS. Store at 4°C in an appropriate stabiliser for up to 1 week.

Biotinylated Brad-5. Use according to instructions.

Alkaline phosphatase-conjugated avidin/streptavidin reagent. Preferably modified to combine high specific activity with low non-specific binding. Use according to instructions.

Substrate solution. Use *para*-nitrophenyl phosphate according to instructions.

Cell fixation buffer. Dissolve 18.02 g of *glucose R*, 4.09 g of *sodium chloride R*, 1.24 g of *boric acid R*, 10.29 g of *sodium citrate R* and 0.74 g of *sodium edetate R* in *water R*. Adjust to pH 7.2-7.3 (2.2.3) using 1 M *sodium hydroxide* or 1 M *hydrochloric acid*, and dilute to 1000 ml with *water R*. Use directly from storage at 4°C .

Glutaraldehyde solution. Immediately before use, add 90 μl of a 250 g/l solution of *glutaraldehyde R* to 24 ml of cold PBS.

Microtitre plates. Plates to be coated with red blood cells are flat-bottomed polystyrene plates with surface properties optimised for enzyme immunoassay and high protein-binding capacity. Plates used to prepare immunoglobulin dilutions are U or V-bottomed polystyrene or poly(vinyl chloride) plates.

METHOD

Prepare a 0.1 per cent (V/V) suspension of papain-treated red blood cells in cold cell fixation buffer. Pipette 50 μl into each well of the flat-bottomed microtitre plate.

Centrifuge the plate at 350 *g* for 3 min, preferably at 4°C . Without removing the supernatant, gently add 100 μl of glutaraldehyde solution to each well and leave for 10 min.

Drain the wells by quickly inverting the plate and wash 3 times with 250-300 μl of PBS. This may be done manually or using a suitable automated plate washer. Either carry out the assay as described below, or store the plate at 4°C after draining off the PBS and adding 100 μl of cell fixation buffer per well and sealing with plastic film. Plates can be stored at 4°C for up to 1 month.

Test solutions. For freeze-dried preparations, reconstitute as stated on the label. Prepare 4 independent replicates of 5 serial two-fold dilutions starting with 30 IU/ml in PBS containing 10 g/l of *bovine albumin R*. If necessary, adjust the starting dilution to obtain responses falling in the linear portion of the dose-response curve.

Reference solutions. Reconstitute the reference preparation according to instructions. Prepare 4 independent replicates of 5 serial two-fold dilutions starting with 30 IU/ml in PBS containing 10 g/l of *bovine albumin R*.

Using U or V-bottomed microtitre plates, add 35 μl of each of the dilutions of the test solution or reference solution to each of a series of wells. To each well add 35 μl of biotinylated Brad-5 at 250 ng/ml.

Empty the wells of the red cell-coated plate by inverting and draining on a paper towel. Add 250 μl of PBS containing 20 g/l of *bovine albumin R* and leave at room temperature for 30 min.

Empty the wells of the red cell-coated plate by inverting and draining on a paper towel and transfer 50 μl from each of the dilutions of the test solution or reference solution containing biotinylated Brad-5 into the wells. Use 50 μl of PBS containing 10 g/l of *bovine albumin R* as negative control. Seal the plate with plastic film and incubate at room temperature for 1 h.

Remove liquid from the wells of the red cell-coated plate and wash 3 times with 250-300 μl of TBS.

Dilute the alkaline phosphatase-conjugated avidin/streptavidin reagent in TBS containing 10 g/l of *bovine albumin R* and add 50 μl to each well. Incubate for 30 min at room temperature.

Remove liquid from the wells of the red cell-coated plate and wash 3 times with 250-300 μl of TBS.

Add 100 μl of substrate solution to each of the wells and incubate at room temperature for 10 min in the dark. To stop the reaction, add 50 μl of 3 M *sodium hydroxide* to each of the wells.

Measure the absorbances at 405 nm. and subtract the negative control reading. Use the absorbance values in the linear range of the titration curve to estimate the potency of the preparation to be examined by the usual statistical methods (5.3).

METHOD C

The potency of human anti-D immunoglobulin is determined by flow cytometry in a microtitre plate format. The method is based on the specific binding between anti-D immunoglobulin and D-positive red blood cells. The activity of the preparation to be examined is compared with a reference preparation calibrated in International Units.

The International Unit is the activity of a stated amount of International Reference Preparation. The equivalence in International Units of the International Reference preparation is stated by the World Health Organisation.

Human anti-D immunoglobulin BRP is calibrated in International Units by comparison with the International Standard and intended for use in the assay of human anti-D immunoglobulin.

MATERIALS

Reagents not specified are of analytical grade.

PBS. Dissolve 8.0 g of *sodium chloride R*, 0.76 g of *disodium hydrogen phosphate R*, 0.2 g of *potassium chloride R* and 0.2 g of *potassium dihydrogen phosphate R* in *water R* and dilute to 1000 ml with the same solvent.

PBS-BSA solution. PBS containing 10.0 g/l of *bovine albumin R*.

Red blood cells. Use D-positive red blood cells obtained from a group O R_1R_1 donor within 2 weeks of collection. Store if necessary in an appropriate stabiliser at 4°C . Wash the cells at least twice with PBS-BSA solution and prepare a suspension containing 1×10^4 cells per microlitre but not more than 5×10^4 cells per microlitre in PBS-BSA solution.

Use D-negative red blood cells obtained from a group O rr donor and prepared similarly.

Secondary antibody. Use a suitable fluorescent dye conjugated anti-IgG antibody-fragment specific for human IgG or parts of it. Store and use according to the manufacturer's instructions.

Microtitre plates. Use flat-bottomed plates without surface treatment for enzyme immunoassays.

METHOD

Test solutions. For freeze-dried preparations, reconstitute as stated on the label. Prepare at least 3 independent replicates of at least 3 serial 1.5 or two-fold dilutions starting

with a concentration in the range of 1.2-0.15 IU/ml using PBS/BSA solution as diluent. If necessary, adjust the starting dilution to obtain responses falling in the linear portion of the dose-response curve.

Reference solutions. Reconstitute the reference preparation according to instructions. Prepare at least 3 independent replicates of at least 3 serial 1.5 or two-fold dilutions starting with a concentration in the range of 1.2-0.15 IU/ml using PBS-BSA solution as diluent. If necessary, adjust the starting dilution to obtain responses falling in the linear portion of the dose-response curve.

Distribute 50 µl of the D-positive red blood cells into each well of a microtitre plate. Add 50 µl of each of the dilutions of the test solution or reference solution to each of a series of wells. Use 50 µl of PBS-BSA solution as negative control. Distribute 50 µl of the D-negative red blood cells into 4 wells of the same microtitre plate and add 50 µl of the lowest dilution of the test preparation. To monitor spurious reactions distribute 50 µl of the D-positive red blood cells into 4 wells of the same microtitre plate and add 50 µl of PBS-BSA solution. Seal with plastic film and incubate at 37 °C for 40 min.

Centrifuge the plates at 50 *g* for 3 min, discard the supernatant and wash the cells with 200-250 µl of PBS-BSA solution. Repeat this at least once.

Centrifuge the plates at 50 *g* for 3 min, discard the supernatant and add 50 µl of the secondary antibody diluted with PBS-BSA solution to a suitable protein concentration. Seal with plastic film and incubate, protected from light, at room temperature for 20 min.

Centrifuge the plates at 50 *g* for 3 min, discard the supernatant and wash the cells with 200-250 µl of PBS-BSA solution. Repeat this at least once.

Centrifuge the plates at 50 *g* for 3 min, resuspend the cells into 200-250 µl of PBS. Transfer the cell suspension into a tube suitable for the flow cytometry equipment available and further dilute by adding PBS to allow a suitable flow rate.

Proceed immediately with measurement of the median fluorescence intensity in a flow cytometer. Record at least 10 000 events without gating but excluding debris.

Use the median fluorescence intensity in the linear range of the dose response curve to estimate the potency of the preparation to be examined by the usual statistical methods, (5.3).

01/2005:20714
corrected

2.7.14. ASSAY OF HEPATITIS A VACCINE

The assay of hepatitis A vaccine is carried out either *in vivo*, by comparing in given conditions its capacity to induce specific antibodies in mice with the same capacity of a reference preparation, or *in vitro*, by an immunochemical determination of antigen content.

IN VIVO ASSAY

The test in mice shown below is given as an example of a method that has been found suitable for a given vaccine; other validated methods may also be used.

Selection and distribution of the test animals. Use in the test healthy mice from the same stock, about 5 weeks old and from a strain shown to be suitable. Use animals of the same sex. Distribute the animals in at least 7 equal groups of a number suitable for the requirements of the assay.

Determination of potency of the vaccine to be examined.

Using a 9 g/l solution of *sodium chloride R* containing the aluminium adjuvant used for the vaccine, prepare at least three dilutions of the vaccine to be examined and matching dilutions of the reference preparation. Allocate the dilutions one to each of the groups of animals and inject subcutaneously not more than 1.0 ml of each dilution into each animal in the group to which that dilution is allocated. Maintain a group of unvaccinated controls, injected subcutaneously with the same volume of diluent. After 28 to 32 days, anaesthetise and bleed all animals, keeping the individual sera separate. Assay the individual sera for specific antibodies against hepatitis A virus by a suitable immunochemical method (2.7.1).

Calculations. Carry out the calculations by the usual statistical methods for an assay with a quantal response (5.3).

From the distribution of reaction levels measured on all the sera in the unvaccinated group, determine the maximum reaction level that can be expected to occur in an unvaccinated animal for that particular assay. Any response in vaccinated animals that exceeds this level is by definition a seroconversion.

Make a suitable transformation of the percentage of animals showing seroconversion in each group (for example, a probit transformation) and analyse the data according to a parallel-line log dose-response model. Determine the potency of the test preparation relative to the reference preparation.

Validity conditions. The test is not valid unless:

- for both the test and the reference vaccine, the ED₅₀ lies between the smallest and the largest doses given to the animals,
- the statistical analysis shows no significant deviation from linearity or parallelism,
- the confidence limits ($P = 0.95$) are not less than 33 per cent and not more than 300 per cent of the estimated potency.

Potency requirement. The upper confidence limit ($P = 0.95$) of the estimated relative potency is not less than 1.0.

IN VITRO ASSAY

Carry out an immunochemical determination (2.7.1) of antigen content with acceptance criteria validated against the *in vivo* test. The acceptance criteria are approved for a given reference preparation by the competent authority in the light of the validation data.

Hepatitis A vaccine (inactivated, adsorbed) type A BRP, hepatitis A vaccine (inactivated, adsorbed) type B BRP and hepatitis A vaccine (inactivated, adsorbed) type C BRP are suitable for the *in vitro* assay of certain vaccines as described in the accompanying leaflet.

01/2005:20715
corrected

2.7.15. ASSAY OF HEPATITIS B VACCINE (rDNA)

The assay of hepatitis B vaccine (rDNA) is carried out either *in vivo*, by comparing in given conditions its capacity to induce specific antibodies against hepatitis B surface antigen (HBsAg) in mice or guinea-pigs with the same capacity of a reference preparation, or *in vitro*, by an immunochemical determination of the antigen content.

IN VIVO ASSAY

Selection and distribution of the test animals. Use in the test healthy mice from the same stock, about 5 weeks old. The strain of mice used for this test must give a significant slope for the dose-response curve to the antigen; mice with haplotype H-2^a or H-2^d are suitable. Healthy guinea-pigs weighing 300 g to 350 g (about 7 weeks old) from the same stock are also suitable. Use animals of the same sex. Distribute the animals in at least 7 equal groups of a number appropriate to the requirements of the assay.

Determination of potency of the vaccine to be examined.

Using a 9 g/l solution of *sodium chloride R* containing the aluminium adjuvant used for the vaccine or another appropriate diluent, prepare at least three dilutions of the vaccine to be examined and matching dilutions of the reference preparation. Allocate the dilutions one to each of the groups of animals and inject intraperitoneally not more than 1.0 ml of each dilution into each animal in the group to which that dilution is allocated. One group of animals remains unvaccinated and is injected intraperitoneally with the same volume of diluent. After an appropriate time interval (for example, 4 to 6 weeks), anaesthetise and bleed the animals, keeping the individual sera separate. Assay the individual sera for specific antibodies against HBsAg by a suitable immunochemical method (2.7.1).

Calculations. Calculations are carried out by the usual statistical methods for an assay with a quantal response (5.3).

From the distribution of reaction levels measured on all the sera in the unvaccinated group, the maximum reaction level that can be expected to occur in an unvaccinated animal for that particular assay is determined. Any response in vaccinated animals that exceeds this level is by definition a seroconversion.

Make a suitable transformation of the percentage of animals showing seroconversion in each group (for example, a probit transformation) and analyse the data according to a parallel-line log dose-response model. Determine the potency of the test preparation relative to the reference preparation.

Validity conditions. The test is not valid unless:

- for both the test and the reference vaccine, the ED₅₀ lies between the smallest and the largest doses given to the animals,
- the statistical analysis shows no significant deviation from linearity or parallelism,
- the confidence limits ($P = 0.95$) are not less than 33 per cent and not more than 300 per cent of the estimated potency.

Potency requirement. The upper confidence limit ($P = 0.95$) of the estimated relative potency is not less than 1.0.

IN VITRO ASSAY

Carry out an immunochemical determination (2.7.1) of antigen content with acceptance criteria validated against the *in vivo* test.

Enzyme-linked immunosorbent assay (ELISA) and radio-immunoassay (RIA) using monoclonal antibodies specific for protection-inducing epitopes of HBsAg have been shown to be suitable. Suitable numbers of dilutions of the vaccine to be examined and the reference preparation are used and a parallel-line model is used to analyse the data which may be suitably transformed. Kits for measuring HBsAg *in vitro* are commercially available and it is possible to adapt their test procedures for use as an *in vitro* potency assay.

The acceptance criteria are approved for a given reference preparation by the competent authority in the light of the validation data.

Hepatitis B vaccine (rDNA) method A BRP and *hepatitis B vaccine (rDNA) method B BRP* are suitable for the *in vitro* assay of certain vaccines as described in the accompanying leaflet.

01/2005:20716

2.7.16. ASSAY OF PERTUSSIS VACCINE (ACELLULAR)

The capacity of the vaccine to induce the formation of specific antibodies is compared with the same capacity of a reference preparation examined in parallel; antibodies are determined using suitable immunochemical methods (2.7.1) such as enzyme-linked immunosorbent assay (ELISA). The test in mice shown below uses a three-point model but, after validation, for routine testing a single-dilution method may be used.

Reference vaccine. A batch of vaccine shown to be effective in clinical trials or a batch representative thereof is used as a reference vaccine. For the preparation of a representative batch, strict adherence to the production process used for the batch tested in clinical trials is necessary. The stability of the reference vaccine shall be documented.

Reference antiserum. *Bordetella pertussis* mouse antiserum BRP is suitable for use as a reference antiserum.

Requirement. The capacity of the vaccine to induce antibodies is not significantly ($P = 0.95$) less than that of the reference vaccine.

The following test model is given as an example of a method that has been found to be satisfactory.

Selection and distribution of test animals. Use in the test healthy mice (for example, CD1 strain) of the same stock, about 5 weeks old. Distribute the animals in 6 groups of a number appropriate to the requirements of the assay. Use 3 dilutions of the vaccine to be examined and 3 dilutions of a reference preparation and attribute each dilution to a group of mice. Inject intraperitoneally or subcutaneously into each mouse 0.5 ml of the dilution attributed to its group.

Collection of serum samples. 4 to 5 weeks after vaccination, bleed the mice individually under anaesthesia. Store the sera at $-20\text{ }^{\circ}\text{C}$ until tested for antibody content.

Antibody determination. Assay the individual sera for content of specific antibodies to each component using a validated method such as the ELISA test shown below.

ELISA test. Microtitre plates (poly(vinyl chloride) or polystyrene as appropriate for the specific antigen) are coated with the purified antigen at a concentration of 100 ng per well. After washing, unreacted sites are blocked by incubating with a solution of bovine serum albumin and then washed. Two-fold dilutions of sera from mice immunised with test or reference vaccines are made on the plates. After incubation at $22\text{--}25\text{ }^{\circ}\text{C}$ for 1 h, the plates are washed. A suitable solution of anti-mouse IgG enzyme conjugate is added to each well and incubated at $22\text{--}25\text{ }^{\circ}\text{C}$ for 1 h. After washing, a chromogenic substrate is added from which the bound enzyme conjugate liberates a chromophore which can be quantified by measurement of absorbance (2.2.25). The test conditions are designed to obtain a linear response for absorbance with respect to antibody content over the range of measurement used and absorbance values within the range 0.1 to 2.0.

A reference antiserum of assigned potency is used in the test and serves as the basis for calculation of the antibody levels in test sera. A standardised control serum is also included in the test.

The test is not valid if:

- the value found for the control serum differs by more than 2 standard deviations from the assigned value,
- the confidence limits ($P = 0.95$) are less than 50 per cent or more than 200 per cent of the estimated potency.

Calculations. The antibody titres in the sera of mice immunised with reference and test vaccines are calculated and from the values obtained the potency of the test vaccine in relation to the reference vaccine is calculated by the usual statistical methods (5.3).

01/2005:20717

2.7.17. ASSAY OF HUMAN ANTITHROMBIN III

The antithrombin III content of the preparation to be examined is determined by comparing its ability to inactivate thrombin in the presence of an excess of heparin with the same ability of a reference preparation of human antithrombin III concentrate calibrated in International Units. Varying quantities of the preparation to be examined are mixed with a given quantity of thrombin and the remaining thrombin activity is determined using a suitable chromogenic substrate.

The International Unit is the activity of a stated amount of the International Standard for human antithrombin III concentrate. The equivalence in International Units of the International Standard is stated by the World Health Organisation.

Method. Prepare 2 independent series of 3 or 4 dilutions in the range 1/75 to 1/200 from 1 IU/ml, for both the preparation to be examined and the reference preparation, using *tris-EDTA BSA buffer solution pH 8.4 R* containing 15 IU of heparin per millilitre.

Warm 200 µl of each dilution at 37 °C for 1-2 min. Add to each dilution 200 µl of a solution of *bovine thrombin R* containing 2 IU/ml in *tris-EDTA BSA buffer solution pH 8.4 R*. Mix and maintain at 37 °C for exactly 1 min. Add 500 µl of a suitable chromogenic substrate (for example, D-phenylalanyl-L-pipecolyl-L-arginine-4-nitroanilide, reconstituted in *water R* to give a solution containing 4 mmol/l and further diluted to a concentration suitable for the assay using *tris-EDTA BSA buffer solution pH 8.4 R* without albumin). Immediately start measurement of the change in absorbance at 405 nm (2.2.25), continuing the measurement for at least 30 s. Calculate the rate of change of absorbance ($\Delta A/\text{min}$). (Alternatively, an end-point assay may be used by stopping the reaction with acetic acid and measuring the absorbance at 405 nm.)

The rate of change of absorbance ($\Delta A/\text{min}$) is inversely proportional to antithrombin III activity.

Check the validity of the assay and calculate the potency of the test preparation by the usual statistical methods (5.3.).

01/2005:20718

2.7.18. ASSAY OF HUMAN COAGULATION FACTOR II

Human coagulation factor II is assayed following specific activation to form factor IIa. Factor IIa is estimated by comparing its activity in cleaving a specific chromogenic

peptide substrate with the same activity of the International Standard or of a reference preparation calibrated in International Units.

The International Unit is the factor II activity of a stated amount of the International Standard which consists of a freeze-dried concentrate of human blood coagulation factor II. The equivalence in International Units of the International Standard is stated by the World Health Organisation.

The chromogenic assay method consists of 2 steps: snake venom-dependent activation of factor II, followed by enzymatic cleavage of a chromogenic factor IIa substrate to form a chromophore that can be quantified spectrophotometrically. Under appropriate assay conditions, there is a linear relation between factor IIa activity and the cleavage of the chromogenic substrate.

REAGENTS

Viper venom specific factor II activator (Ecarin). A protein derived from the venom of the saw-scaled viper (*Echis carinatus*) which specifically activates factor II. Reconstitute according to the manufacturer's instructions. Store the reconstituted preparation at 4 °C and use within 1 month.

Factor IIa chromogenic substrate. Specific chromogenic substrate for factor IIa such as: *H*-D-phenylalanyl-L-pipecolyl-L-arginine-4-nitroanilide dihydrochloride, 4-toluenesulphonyl-glycyl-prolyl-L-arginine-4-nitroanilide, *H*-D-cyclohexylglycyl- α -aminobutyryl-L-arginine-4-nitroanilide, D-cyclohexylglycyl-L-alanyl-L-arginine-4-nitroanilide diacetate. Reconstitute according to the manufacturer's instructions.

Dilution buffer. Solution containing 6.06 g/l of *tris(hydroxymethyl)aminomethane R*, 17.53 g/l of *sodium chloride R*, 2.79 g/l of *(ethylenedinitrilo)tetra-acetic acid R* and 1 g/l of *bovine albumin R* or *human albumin R*. Adjust to pH 8.4 if necessary, using *hydrochloric acid R*.

METHOD

Test solution. Dilute the preparation to be examined with dilution buffer to obtain a solution containing 0.015 IU of factor II per millilitre. Prepare at least 3 further dilutions in dilution buffer.

Reference solution. Dilute the reference preparation to be examined with dilution buffer to obtain a solution containing 0.015 IU of factor II per millilitre. Prepare at least 3 further dilutions in dilution buffer.

Warm all solutions to 37 °C in a water-bath shortly before the test.

The following working conditions apply to microtitre plates. If the assay is carried out in tubes, the volumes are adjusted while maintaining the proportions in the mixture.

Using a microtitre plate maintained at 37 °C, add 25 µl of each dilution of the test solution or the reference solution to each of a series of wells. To each well add 125 µl of dilution buffer, then 25 µl of ecarin and incubate for exactly 2 min. To each well add 25 µl of factor IIa chromogenic substrate.

Read the rate of change of absorbance (2.2.25) at 405 nm continuously over a period of 3 min and obtain the mean rate of change of absorbance ($\Delta A/\text{min}$). If continuous monitoring is not possible, read the absorbance at 405 nm at suitable consecutive intervals, for instance 40 s, plot the absorbances against time on a linear graph and calculate $\Delta A/\text{min}$ as the slope of the line. From the $\Delta A/\text{min}$ values of each individual dilution of standard and test preparations, calculate the potency of the preparation to be examined and check the validity of the assay by the usual statistical methods (5.3).

01/2005:20719

2.7.19. ASSAY OF HUMAN COAGULATION FACTOR X

Human coagulation factor X is assayed following specific activation to form factor Xa. Factor Xa is estimated by comparing its activity in cleaving a specific chromogenic peptide substrate with the same activity of the International Standard or of a reference preparation calibrated in International Units.

The International Unit is the factor X activity of a stated amount of the International Standard which consists of a freeze-dried concentrate of human coagulation factor X. The equivalence in International Units of the International Standard is stated by the World Health Organisation.

The chromogenic assay method consists of 2 steps: snake venom-dependent activation of factor X, followed by enzymatic cleavage of a chromogenic factor Xa substrate to form a chromophore that can be quantified spectrophotometrically. Under appropriate assay conditions, there is a linear relation between factor Xa activity and the cleavage of the chromogenic substrate.

REAGENTS

Russell's viper venom specific factor X activator (RVV). A protein derived from the venom of Russell's viper (*Vipera russelli*) which specifically activates factor X. Reconstitute according to the manufacturer's instructions. Store the reconstituted preparation at 4 °C and use within 1 month.

Factor Xa chromogenic substrate. Specific chromogenic substrate for factor Xa such as: *N*- α -benzyloxycarbonyl-D-arginyl-L-glycyl-L-arginine-4-nitroanilide dihydrochloride, *N*-benzoyl-L-isooleucyl-L-glutamyl-glycyl-L-arginine-4-nitroanilide hydrochloride, methanesulphonyl-D-leucyl-glycyl-L-arginine-4-nitroanilide, methoxycarbonyl-D-cyclohexylalanyl-glycyl-L-arginine-4-nitroanilide acetate. Reconstitute according to the manufacturer's instructions.

Dilution buffer. Solution containing 3.7 g/l of *tris(hydroxymethyl)aminomethane* R, 18.0 g/l of *sodium chloride* R, 2.1 g/l of *imidazole* R, 0.02 g/l of *hexadimethrine bromide* R and 1 g/l of *bovine albumin* R or *human albumin* R. Adjust to pH 8.4 if necessary using *hydrochloric acid* R.

METHOD

Test solution. Dilute the preparation to be examined with dilution buffer to obtain a solution containing 0.18 IU of factor X per millilitre. Prepare at least 3 further dilutions in dilution buffer.

Reference solution. Dilute the reference preparation to be examined with dilution buffer to obtain a solution containing 0.18 IU of factor X per millilitre. Prepare at least 3 further dilutions in dilution buffer.

Warm all solutions to 37 °C in a water-bath shortly before the test.

The following working conditions apply to microtitre plates. If the assay is carried out in tubes, the volumes are adjusted while maintaining the proportions in the mixture.

Using a microtitre plate maintained at 37 °C, add 12.5 μ l of each dilution of the test solution or the reference solution to each of a series of wells. To each well add 25 μ l of RVV and incubate for exactly 90 s. To each well add 150 μ l of factor Xa chromogenic substrate, diluted 1 in 6 in dilution buffer.

Read the rate of change of absorbance (2.2.25) (at 405 nm continuously over a period of 3 min and obtain the mean rate of change of absorbance ($\Delta A/\text{min}$). If continuous monitoring is not possible, read the absorbance at 405 nm at suitable consecutive intervals, for instance 40 s, plot the absorbances against time on a linear graph and calculate $\Delta A/\text{min}$ as the slope of the line. From the $\Delta A/\text{min}$ values of each individual dilution of standard and test preparations, calculate the potency of the preparation to be examined and check the validity of the assay by the usual statistical methods (5.3).

01/2005:20720

2.7.20. *IN VIVO* ASSAY OF POLIOMYELITIS VACCINE (INACTIVATED)

The capacity of the vaccine to induce the formation of neutralising antibodies is determined, *in vivo* by one of the following methods.

TEST IN CHICKS OR GUINEA-PIGS

Prepare a suitable series of not fewer than 3 dilutions of the vaccine to be examined using a suitable buffered saline solution. Distribute either guinea-pigs weighing 250-350 g or 3-week-old chicks into groups of 10, and allocate a group to each dilution of the vaccine. Inject intramuscularly into each animal 0.5 ml of the dilution intended for its group. Bleed the animals after 5-6 days and separate the sera. Examine the sera for the presence of neutralising antibodies, at a dilution of 1 in 4, to each of the human polioviruses 1, 2 and 3. Mix 100 CCID₅₀ of virus with the dilution of serum and incubate at 37 °C for 4.5-6 h. Keep at 5 $\pm$ 3 °C for 12-18 h where necessary for consistency of results. Inoculate the mixtures into cell cultures for the detection of unneutralised virus and read the results up to 7 days after inoculation. For each group of animals, note the number of sera which have neutralising antibodies and calculate the dilution of the vaccine giving an antibody response in 50 per cent of the animals. Carry out in parallel a control test using a suitable reference preparation. The vaccine complies with the test if a dilution of 1 in 100 or more produces an antibody response for each of the 3 types of virus in 50 per cent of the animals.

TEST IN RATS

A suitable *in vivo* assay method consists of intramuscular injection into the hind limb(s) of not fewer than 3 dilutions of the vaccine to be examined and a reference vaccine, using for each dilution a group of 10 specific pathogen-free rats of a suitable strain. Use of 4 dilutions is often necessary to obtain valid results for all 3 serotypes. The number of animals per group must be sufficient to obtain results that meet the validity criteria; groups of 10 rats are usually sufficient although valid results may be obtained with fewer animals per group. If animals of different sex are used, males and females are evenly distributed between all groups. A weight range of 175-250 g has been found suitable. An inoculum of 0.5 ml per rat is used. The dose range is chosen such that a dose response to all 3 poliovirus types is obtained. Bleed the animals after 20-22 days. Neutralising titres against all 3 poliovirus types are measured separately using 100 CCID₅₀ of the Sabin strains as challenge viruses, Vero or Hep2 as indicator cells, and neutralisation conditions of 3 h at 35-37 °C followed by 18 h at 2-8 °C where necessary for consistency of results. Results are read following fixation and staining after 7 days of incubation at 35 °C. For a valid antibody assay, the titre of each challenge virus must be shown to be within the range 10 CCID₅₀ to 1000 CCID₅₀.

and the neutralising antibody titre of a control serum must be within 2 twofold dilutions of the geometric mean titre of the serum. The potency is calculated by comparison of the proportion of responders for the vaccine to be examined and the reference vaccine by the probit method or, after validation, using a parallel-line model. For the probit method it is necessary to establish a cut-off neutralising antibody titre for each poliovirus type to define a responder. Due to interlaboratory variation, it is not possible to define cut-off values that could be applied by all laboratories. Rather, the cut-off values are determined for each laboratory based on a minimum series of 3 tests with the reference vaccine. The mid-point on a log₂ scale of the minimum and maximum geometric mean titres of the series of 3 or more tests is used as the cut-off value. For each of the 3 poliovirus types, the potency of the vaccine is not significantly less than that of the reference preparation. The test is not valid unless:

- for both the test and reference vaccines the ED₅₀ lies between the smallest and the largest doses given to the animals,
- the statistical analysis shows no significant deviation from linearity or parallelism,
- the confidence limits ($P = 0.95$) are not less than 25 per cent and not more than 400 per cent of the estimated potency.

01/2005:20721

2.7.21. ASSAY OF HUMAN VON WILLEBRAND FACTOR

The potency of human von Willebrand factor is determined by comparing, in given conditions its activity in collagen binding or as ristocetin cofactor with the same activity of a reference preparation calibrated against the International Standard, in International Units where applicable.

The International Unit is the activity of a stated amount of the International Standard for von Willebrand factor in human blood coagulation factor VIII concentrate. The equivalence in International Units of the International Standard is stated by the World Health Organisation.

COLLAGEN-BINDING ASSAY

Collagen-binding is determined by an enzyme-linked immunosorbent assay on collagen-coated microtitre plates. The method is based on the specific binding of von Willebrand factor to collagen fibrils and the subsequent binding of polyclonal anti-von Willebrand factor antibody conjugated to an enzyme, which on addition of a chromogenic substrate yields a product that can be quantitated spectrophotometrically. Under appropriate conditions, there is a linear relationship between von Willebrand factor collagen-binding and absorbance.

MATERIALS

Collagen. Use native equine or human fibrils of collagen type I or III. For the ease of handling, collagen solutions may be used.

Collagen diluent. Dissolve 50 g of *glucose R* in *water R*, adjust to pH 2.7-2.9 with 1 M *hydrochloric acid* and dilute to 1000 ml with *water R*.

Phosphate-buffered saline solution (PBS). Dissolve 8.0 g of *sodium chloride R*, 1.05 g of *disodium hydrogen phosphate dihydrate R*, 0.2 g of *sodium dihydrogen phosphate R* and 0.2 g of *potassium chloride R* in *water R*. Adjust to pH 7.2 using 1 M *sodium hydroxide* or 1 M *hydrochloric acid* and dilute to 1000 ml with *water R*.

Washing buffer. PBS containing 1 g/l of *polysorbate 20 R*.

Blocking reagent. PBS containing 1 g/l of *polysorbate 20 R* and 10 g/l of *bovine serum albumin R*.

Dilution buffer. PBS containing 1 g/l of *polysorbate 20 R* and 50 g/l of *bovine serum albumin R*.

Conjugate. Rabbit anti-human von Willebrand factor serum horse-radish peroxidase conjugate. Use according to the manufacturer's instructions.

Substrate solution. Immediately before use, dissolve a tablet of *o*-phenylenediamine dihydrochloride and a tablet of urea hydrogen peroxide in 20 ml of *water R* or use a suitable volume of hydrogen peroxide. Protect from light.

Microtitre plates. Flat-bottomed polystyrene plates with surface properties optimised for enzyme immunoassay and high protein-binding capacity.

METHOD

Test solutions. Reconstitute the preparation to be examined as stated on the label. Dilute with dilution buffer to produce a solution containing approximately 1 IU of von Willebrand factor. Prepare 2 independent series of not fewer than 3 dilutions using dilution buffer.

Reference solutions. Reconstitute the reference preparation as directed. Dilute with dilution buffer to produce a solution containing approximately 1 IU of von Willebrand factor. Prepare 2 independent series of not fewer than 3 dilutions using dilution buffer.

Allow the solution of collagen to warm to room temperature. Dilute with collagen diluent to obtain a solution containing 30-75 µg/ml of collagen, mix gently to produce a uniform suspension of collagen fibrils. Pipette 100 µl into each well of the microtitre plate. Cover the plate with plastic film and incubate at 37 °C overnight. Empty the wells of the collagen-coated plate by inverting and draining on a paper towel. Add 250 µl of washing buffer. Empty the wells of the plate by inverting and draining on a paper towel. Repeat this operation 3 times. Add 250 µl of blocking reagent to each well, cover the plate with plastic film and incubate at 37 °C for 1 h. Empty the wells of the plate by inverting and draining on a paper towel. Add 250 µl of washing buffer. Empty the wells of the plate by inverting and draining on a paper towel. Repeat this operation 3 times.

Add 100 µl each of the test solutions or reference solutions to the wells. Add 100 µl of dilution buffer to a series of wells to serve as negative control. Cover the plate with plastic film and incubate at 37 °C for 2 h. Empty the wells of the plate by inverting and draining on a paper towel. Add 250 µl of washing buffer. Empty the wells of the plate by inverting and draining on a paper towel. Repeat this operation 3 times.

Prepare a suitable dilution of the conjugate with PBS containing 5 g/l of *bovine serum albumin R* and add 100 µl to each well. Cover the plate with plastic film and incubate at 37 °C for 2 h. Empty the wells of the plate by inverting and draining on a paper towel. Add 250 µl of washing buffer. Empty the wells of the plate by inverting and draining on a paper towel. Repeat this operation 3 times.

Add 100 µl of substrate solution to each of the wells and incubate at room temperature for 20 min in the dark. Add 100 µl of 1 M *hydrochloric acid* to each of the wells.

Measure the absorbance at 492 nm. Use the absorbance values to estimate the potency of the preparation to be examined by the usual statistical methods (5.3).

The assay is invalid if the absorbances measured for the negative controls are greater than 0.05.

RISTOCETIN COFACTOR ASSAY

Carry out appropriate dilutions of the preparation to be examined and of the reference preparation using as diluent a solution containing 9 g/l of *sodium chloride R* and 10-50 g/l

of *human albumin R*. Add to each dilution suitable amounts of a von Willebrand reagent containing stabilised human platelets and ristocetin A. Mix on a glass slide by moving it gently in circles for 1 min. Allow to stand for a further 1 min and read the result against a dark background with side lighting. The last dilution which clearly shows visible agglutination indicates the ristocetin cofactor titre of the sample. Use diluent as a negative control.

01/2005:20722

2.7.22. ASSAY OF HUMAN COAGULATION FACTOR XI

The potency of human coagulation factor XI is determined by comparing the quantity of the preparation to be examined necessary to reduce the coagulation time of a test mixture containing the substances other than factor XI that take part in the coagulation of blood, and the quantity of human normal plasma required to produce the same effect. 1 unit of factor XI is equal to the activity of 1 ml of human normal plasma.

Reconstitute separately the preparation to be examined and the reference preparation as stated on the label and use without delay. Where applicable, determine the amount of heparin present (2.7.12) and neutralise the heparin by addition of *protamine sulphate R* (10 µg of protamine

sulphate neutralises 1 IU of heparin). Dilute the preparation to be examined and the reference preparation with a sufficient quantity of *imidazole buffer solution pH 7.3 R* containing 1 per cent of albumin to produce solutions containing 0.5 units/ml to 2.0 units/ml. Prepare twofold dilutions in the range 1 to 10 to 1 to 80 using *imidazole buffer solution pH 7.3 R*. Make these dilutions accurately and use without delay.

Use, for example, incubation tubes maintained in a water-bath at 37 °C. Place in each tube 0.1 ml of *plasma substrate R3* and 0.1 ml of one of the dilutions of the reference preparation or of the preparation to be examined. Add to each tube 0.1 ml of a suitable dilution of *cephalin R* or *platelet substitute R* and 0.1 ml of a suspension of 0.5 g of *light kaolin R* in 100 ml of a 3.7 g/l solution of *sodium chloride R* and allow to stand for about 10 min, tilting the tubes regularly. To each tube, add 0.1 ml of a 7.4 g/l solution of *calcium chloride R*. Using a timer, measure the coagulation time, i.e. the interval between the moment of the addition of the calcium chloride and the first indication of the formation of fibrin, which may be observed visually or by the use of a suitable apparatus. Calculate the potency using the usual statistical methods (5.3.).

To ensure that there is no appreciable contamination of *plasma substrate R3* by factor XI, carry out a blank test using, instead of the preparation to be examined, a corresponding volume of *imidazole buffer solution pH 7.3 R*. The test is not valid unless the coagulation time measured in the blank test is 100 s to 200 s.

2.8. METHODS IN PHARMACOGNOSY

01/2005:20801

2.8.1. ASH INSOLUBLE IN HYDROCHLORIC ACID

Ash insoluble in hydrochloric acid is the residue obtained after extracting the sulphated or total ash with hydrochloric acid, calculated with reference to 100 g of drug.

To the crucible containing the residue from the determination of sulphated or total ash, add 15 ml of *water R* and 10 ml of *hydrochloric acid R*, cover with a watch-glass, boil the mixture gently for 10 min and allow to cool. Filter through an ashless filter, wash the residue with hot *water R* until the filtrate is neutral, dry, ignite to dull redness, allow to cool in a desiccator and weigh. Reheat until the difference between 2 consecutive weighings is not more than 1 mg.

01/2005:20802

2.8.2. FOREIGN MATTER

Vegetable drugs should be free from moulds, insects and other animal contamination.

Unless otherwise prescribed, the amount of foreign matter is not more than 2 per cent *m/m*.

Foreign matter is material consisting of any or all of the following:

- 1) *Foreign organs*: matter coming from the source plant but not defined as the drug,
- 2) *Foreign elements*: matter not coming from the source plant and either of vegetable or mineral origin.

DETERMINATION OF FOREIGN MATTER

Weigh 100 g to 500 g of the substance to be examined, or the minimum quantity prescribed in the monograph, and spread it out in a thin layer. Examine for foreign matter by inspection with the unaided eye or by use of a lens (6 ×). Separate foreign matter and weigh it and calculate the percentage present.

01/2005:20803

2.8.3. STOMATA AND STOMATAL INDEX

STOMATA

There are several types of stomata (see Figure 2.8.3.-1), distinguished by the form and arrangement of the surrounding cells:

- (1) The *anomocytic* (irregular-celled) type: the stoma is surrounded by a varying number of cells in no way differing from those of the epidermis generally,

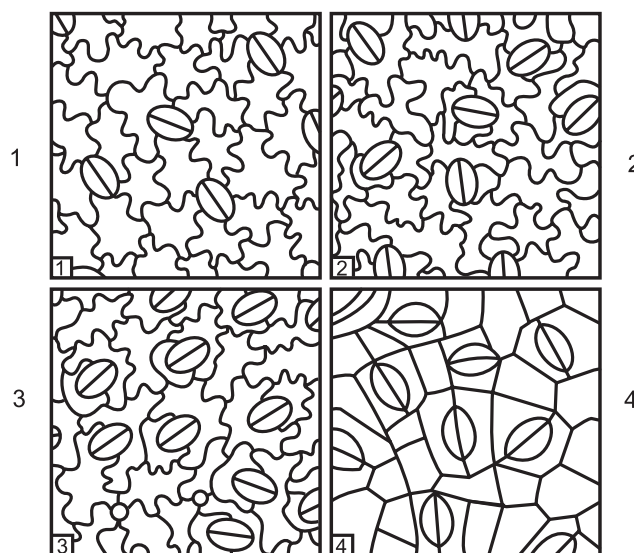


Figure 2.8.3.-1

- (2) The *anisocytic* (unequal-celled) type: the stoma is usually surrounded by 3 subsidiary cells, of which one is markedly smaller than the others,

- (3) The *diacytic* (cross-celled) type: the stoma is accompanied by 2 subsidiary cells, whose common wall is at right angles to the guard cells,

- (4) The *paracytic* (parallel-celled) type: the stoma has on each side one or more subsidiary cells parallel to the long axis of the pore and guard cells.

STOMATAL INDEX

$$\text{Stomatal Index} = \frac{100 \times S}{E + S}$$

- S = the number of stomata in a given area of leaf,
E = the number of epidermal cells (including trichomes) in the same area of leaf.

For each sample of leaf, make not fewer than 10 determinations and calculate the mean.

01/2005:20804

2.8.4. SWELLING INDEX

The swelling index is the volume in millilitres occupied by 1 gram of a drug, including any adhering mucilage, after it has swollen in an aqueous liquid for 4 h.

In a 25 ml ground-glass stoppered cylinder graduated over a height of 125 ± 5 mm in 0.5 ml divisions, place 1.0 g of the drug, whole or of the degree of comminution prescribed in the monograph. Unless otherwise prescribed, moisten the drug with 1.0 ml of *alcohol R*, add 25 ml of *water R* and close the cylinder. Shake vigorously every 10 min for 1 h. Allow to stand for 3 h. At 90 min after the beginning of the test, release any large volumes of liquid retained in the layer of the drug and any particles of the drug floating at the surface of the liquid by rotating the cylinder about a vertical axis. Measure the volume occupied by the drug, including any adhering mucilage. Carry out 3 tests at the same time.

The swelling index is given by the mean of the 3 tests.

2.8.5. WATER IN ESSENTIAL OILS

Mix 10 drops of the essential oil with 1 ml of *carbon disulphide R*. The solution remains clear on standing.

01/2005:20805

Method. Weigh the evaporating dish after having heated it on the water-bath for 1 h and cooled it in the desiccator. Weigh into the evaporating dish 5.00 g of the essential oil, unless otherwise prescribed. Heat the oil on the vigorously boiling water-bath in a draught-free atmosphere for the prescribed time. Allow to cool in the desiccator and weigh. During the test, the level of water in the bath is maintained about 50 mm beneath the level of the cover.

01/2005:20806

2.8.6. FOREIGN ESTERS IN ESSENTIAL OILS

Heat 1 ml of the essential oil for 2 min on a water-bath with 3.0 ml of a freshly prepared 100 g/l solution of *potassium hydroxide R* in *alcohol R*. No crystals are formed within 30 min, even after cooling.

01/2005:20807

2.8.7. FATTY OILS AND RESINIFIED ESSENTIAL OILS IN ESSENTIAL OILS

Allow 1 drop of the essential oil to fall onto filter paper. The drop evaporates completely within 24 h without leaving any translucent or greasy spot.

01/2005:20808

2.8.8. ODOUR AND TASTE OF ESSENTIAL OILS

Mix 3 drops of the essential oil with 5 ml of 90 per cent V/V *alcohol R* and stir in 10 g of powdered *sucrose R*. The odour and taste are similar to that of the plant or parts of the plant from which the essential oil has been obtained.

01/2005:20809

2.8.9. RESIDUE ON EVAPORATION OF ESSENTIAL OILS

The residue on evaporation of an essential oil is the percentage by mass of the oil which remains after evaporation on a water-bath under the conditions specified below.

Apparatus. The apparatus (see Figure 2.8.9-1) consists of:

- Water-bath with a cover having holes of 70 mm diameter,
- Evaporating dish of heat-resistant glass which is inert to the contents,
- Desiccator.

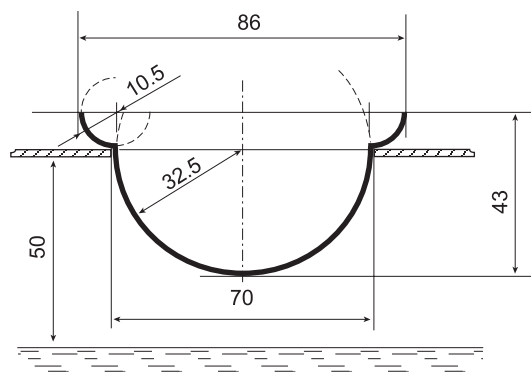


Figure 2.8.9-1.

Dimensions in millimetres

2.8.10. SOLUBILITY IN ALCOHOL OF ESSENTIAL OILS

Place 1.0 ml of the essential oil in a 25 ml or 30 ml glass-stoppered cylinder. Place in a constant temperature device, maintained at a temperature of 20 ± 0.2 °C. Using a burette of at least 20 ml capacity, add the alcohol of the strength prescribed in the monograph by increments of 0.1 ml until solution is complete and then continue adding by increments of 0.5 ml to a total of 20 ml, shaking frequently and vigorously. Record the volume of alcohol added when a clear solution has been obtained and, if the solution becomes cloudy or opalescent before 20 ml of alcohol has been added, record the volume added when the cloudiness or opalescence appears and, where applicable, the volume added when the cloudiness or opalescence disappears.

If a clear solution has not been obtained when 20 ml of alcohol of the prescribed strength has been added, repeat the test using the next highest concentration of alcohol.

An essential oil is said to be “soluble in n volumes or more of alcohol of given strength t ” when the clear solution in n volumes remains clear when compared with the undiluted oil after further addition of alcohol of the same strength up to a total of 20 volumes of alcohol.

An essential oil is said to be “soluble in n volumes of alcohol of given strength t , becoming cloudy when diluted” when the clear solution in n volumes becomes cloudy in n_1 volumes (n_1 less than 20) and stays so after further gradual addition of alcohol of the same strength up to a total of 20 volumes of alcohol.

An essential oil is said to be “soluble in n volumes of alcohol of given strength t with cloudiness between n_1 and n_2 volumes” when the clear solution in n volumes becomes cloudy in n_1 volumes (n_1 less than 20) and stays so after further gradual addition of alcohol of the same strength up to a total of n_2 volumes of alcohol and then becomes clear (n_2 less than 20).

An essential oil is said to be “soluble with opalescence” when the alcoholic solution shows a bluish tinge, similar to that of a standard of opalescence freshly prepared as follows: mix 0.5 ml of *silver nitrate solution R2* and 0.05 ml of *nitric acid R*; add 50 ml of a 12 mg/l solution of *sodium chloride R*; mix and allow to stand protected from light for 5 min.

01/2005:20811

2.8.11. ASSAY OF 1,8-CINEOLE IN ESSENTIAL OILS

Weigh 3.00 g of the oil, recently dried with *anhydrous sodium sulphate R*, into a dry test-tube and add 2.10 g of melted *cresol R*. Place the tube in the apparatus for the determination of freezing point (2.2.18) and allow to cool, stirring continuously. When crystallisation takes place there is a small rise in temperature. Note the highest temperature reached (t_1).

Remelt the mixture on a water-bath at a temperature that does not exceed t_1 by more than 5 °C and place the tube in the apparatus, maintained at a temperature 5 °C below t_1 . When crystallisation takes place, or when the temperature of the mixture has fallen 3 °C below t_1 , stir continuously. Note the highest temperature at which the mixture crystallises (t_2). Repeat the operation until 2 highest values obtained for t_2 do not differ by more than 0.2 °C. If supercooling occurs, induce crystallisation by adding a small crystal of the complex consisting of 3.00 g of *cineole* *R* and 2.10 g of melted *cresol* *R*. If t_2 is below 27.4 °C, repeat the determination after the addition of 5.10 g of the complex.

The content of cineole corresponding to the highest temperature observed (t_2) is given in Table 2.8.11-1. If 5.10 g of the complex has been added, calculate the cineole content per cent m/m from the expression:

$$2(A - 50)$$

where A is the value found in Table 2.8.11-1.

The content of cineole, corresponding to the highest temperature observed (t_2), is obtained, where necessary, by interpolation.

Table 2.8.11.-1

t_2 °C	cincole per cent m/m	t_2 °C	cincole per cent m/m	t_2 °C	cincole per cent m/m	t_2 °C	cincole per cent m/m
24	45.5	32	56.0	40	67.0	48	82.0
25	47.0	33	57.0	41	68.5	49	84.0
26	48.5	34	58.5	42	70.0	50	86.0
27	49.5	35	60.0	43	72.5	51	88.5
28	50.5	36	61.0	44	74.0	52	91.0
29	52.0	37	62.5	45	76.0	53	93.5
30	53.5	38	63.5	46	78.0	54	96.0
31	54.5	39	65.0	47	80.0	55	99.0

01/2005:20812

2.8.12. DETERMINATION OF ESSENTIAL OILS IN VEGETABLE DRUGS

The determination of essential oils in vegetable drugs is carried out by steam distillation in a special apparatus in the conditions described below. The distillate is collected in the graduated tube, using xylene to take up the essential oil; the aqueous phase is automatically returned to the distillation flask.

Apparatus. The apparatus comprises the following parts:

- (a) a suitable round-bottomed flask with a short, ground-glass neck having an internal diameter of about 29 mm at the wide end;
- (b) a condenser assembly (see Figure 2.8.12-1) that closely fits the flask, the different parts being fused into one piece; the glass used has a low coefficient of expansion:
- the stopper K' is vented and the tube K has an orifice of diameter about 1 mm that coincides with the vent; the wide end of the tube K is of ground-glass and has an internal diameter of 10 mm;
 - a pear-shaped swelling, J , of 3 ml capacity;

- the tube JL is graduated in 0.01 ml;
 - the bulb-shaped swelling L has a capacity of about 2 ml;
 - M is a three-way tap;
 - the junction B is at a level 20 mm higher than the uppermost graduation;
- (c) a suitable heating device, allowing a fine control;
- (d) a vertical support with a horizontal ring covered with insulating material.

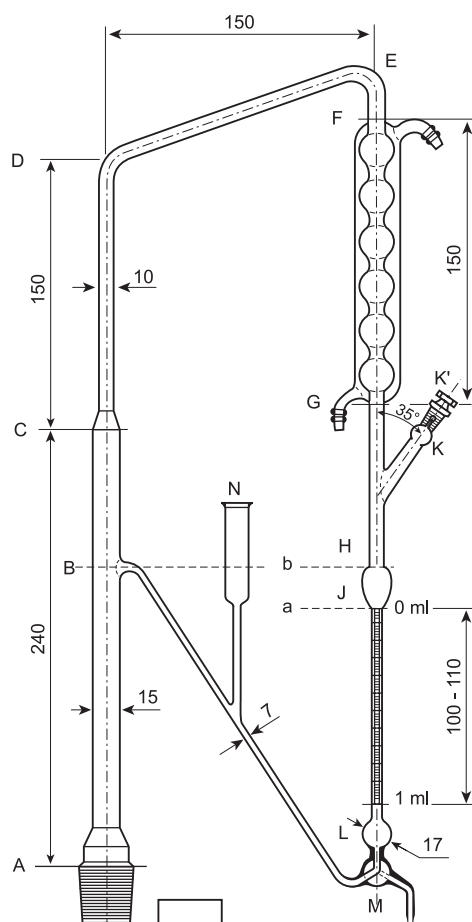


Figure 2.8.12-1. - *Apparatus for the determination of essential oils in vegetable drugs*

Dimensions in millimetres

Method. Use a thoroughly cleaned apparatus. Carry out the assay according to the nature of the drug to be examined. Place the prescribed volume of distillation liquid in the flask, add a few pieces of porous porcelain and attach the condenser assembly. Introduce *water R* through the filling funnel *N* until it is at the level *B*. Remove the stopper *K'* and introduce the prescribed quantity of *xylene R*, using a pipette with its tip at the bottom of the tube *K*. Replace the stopper *K'* and ensure that the orifice coincides with the vent. Heat the liquid in the flask to boiling and adjust the distillation rate to 2-3 ml/min, unless otherwise prescribed.

To determine the rate of distillation, during distillation lower the level of the water by means of the three-way tap until the meniscus is at the level of the lower mark (a) (see Figure 2.8.12.-2). Close the tap and measure the time taken for the liquid to reach the upper mark (b). Open the tap and continue the distillation, modifying the heat to regulate the distillation rate. Distil for 30 min. Stop the heating and after at least 10 min read off the volume of xylene in the graduated tube.

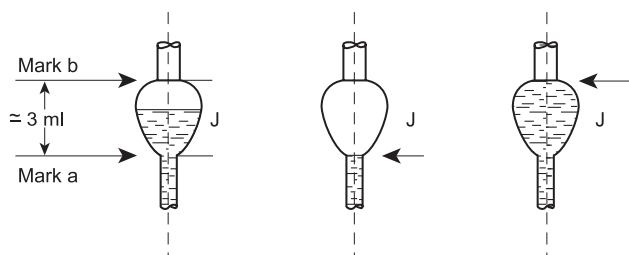


Figure 2.8.12-2

Introduce into the flask the prescribed quantity of the drug and continue the distillation as described above for the time and at the rate prescribed. Stop the heating and after 10 min read the volume of liquid collected in the graduated tube and subtract the volume of xylene previously noted. The difference represents the quantity of essential oil in the mass of the drug taken. Calculate the result as millilitres per kilogram of drug.

When the essential oil is to be used for other analytical purposes, the water-free mixture of xylene and essential oil may be recovered as follows: remove the stopper *K'* and introduce 0.1 ml of a 1 g/l solution of *sodium fluoresceinate R* and 0.5 ml of *water R*. Lower the mixture of xylene and essential oil into the bulb-shaped swelling *L* by means of the three-way tap, allow to stand for 5 min and lower the mixture slowly until it just reaches the level of the tap *M*. Open the tap anti-clockwise so that the water flows out of the connecting tube *BM*. Wash the tube with *acetone R* and with a little *toluene R* introduced through the filling funnel *N*. Turn the tap anti-clockwise in order to recover the mixture of xylene and essential oil in an appropriate flask.

01/2005:20813

2.8.13. PESTICIDE RESIDUES

Definition. For the purposes of the Pharmacopoeia, a pesticide is any substance or mixture of substances intended for preventing, destroying or controlling any pest, unwanted species of plants or animals causing harm during or otherwise interfering with the production, processing, storage, transport or marketing of vegetable drugs. The item includes substances intended for use as growth-regulators, defoliants or desiccants and any substance applied to crops either before or after harvest to protect the commodity from deterioration during storage and transport.

Limits. Unless otherwise indicated in the monograph, the drug to be examined at least complies with the limits indicated in Table 2.8.13-1. The limits applying to pesticides that are not listed in the table and whose presence is suspected for any reason comply with the limits set by European Community directives 76/895 and 90/642, including their annexes and successive updates. Limits for pesticides that are not listed in Table 2.8.13-1 nor in EC directives are calculated using the following expression:

$$\frac{ADI \times M}{MDD \times 100}$$

ADI = acceptable daily intake, as published by FAO-WHO, in milligrams per kilogram of body mass,

M = body mass in kilograms (60 kg),

MDD = daily dose of the drug, in kilograms.

If the drug is intended for the preparation of extracts, tinctures or other pharmaceutical forms whose preparation method modifies the content of pesticides in the finished product, the limits are calculated using the following expression:

$$\frac{ADI \times M \times E}{MDD \times 100}$$

E = extraction factor of the method of preparation, determined experimentally.

Higher limits can also be authorised, in exceptional cases, especially when a plant requires a particular cultivation method or has a metabolism or a structure that gives rise to a higher than normal content of pesticides.

The competent authority may grant total or partial exemption from the test when the complete history (nature and quantity of the pesticides used, date of each treatment during cultivation and after the harvest) of the treatment of the batch is known and can be checked precisely.

Sampling

Method. For containers up to 1 kg, take one sample from the total content, thoroughly mixed, sufficient for the tests. For containers between 1 kg and 5 kg, take three samples, equal in volume, from the upper, middle and lower parts of the container, each being sufficient to carry out the tests. Thoroughly mix the samples and take from the mixture an amount sufficient to carry out the tests. For containers of more than 5 kg, take three samples, each of at least 250 g from the upper, middle and lower parts of the container. Thoroughly mix the samples and take from the mixture an amount sufficient to carry out the tests.

Size of sampling. If the number (*n*) of containers is three or fewer, take samples from each container as indicated above under Method. If the number of containers is more than three, take $\sqrt{n} + 1$ samples from containers as indicated under Method, rounding up to the nearest unit if necessary.

The samples are to be analysed immediately to avoid possible degradation of the residues. If this is not possible, the samples are stored in airtight containers suitable for food contact, at a temperature below 0 °C, protected from light.

Reagents. All reagents and solvents are free from any contaminants, especially pesticides, that might interfere with the analysis. It is often necessary to use special quality solvents or, if this is not possible, solvents that have recently been re-distilled in an apparatus made entirely of glass. In any case, suitable blank tests must be carried out.

Apparatus. Clean the apparatus and especially glassware to ensure that they are free from pesticides, for example, soak for at least 16 h in a solution of phosphate-free detergent, rinse with large quantities of *distilled water R* and wash with acetone and hexane or heptane.

Qualitative and quantitative analysis of pesticide residues.

The analytical procedures used are validated according to the regulations in force. In particular, they satisfy the following criteria:

- the chosen method, especially the purification steps, are suitable for the combination pesticide residue/substance to be analysed, and not susceptible to interference from co-extractives; the limits of detection and quantification are measured for each pesticide-matrix combination to be analysed,
- between 70 per cent to 110 per cent of each pesticide is recovered,
- the repeatability of the method is not less than the values indicated in Table 2.8.13-2,

- the reproducibility of the method is not less than the values indicated in Table 2.8.13.-2,
- the concentration of test and reference solutions and the setting of the apparatus are such that a linear response is obtained from the analytical detector.

Table 2.8.13.-1

Substance	Limit (mg/kg)
Alachlor	0.02
Aldrin and Dieldrin (sum of)	0.05
Azinphos-methyl	1.0
Bromopropylate	3.0
Chlordane (sum of <i>cis</i> -, <i>trans</i> - and Oxythlordane)	0.05
Chlorfenvinphos	0.5
Chlorpyrifos	0.2
Chlorpyrifos-methyl	0.1
Cypermethrin (and isomers)	1.0
DDT (sum of <i>p,p'</i> -DDT, <i>o,p'</i> -DDT, <i>p,p'</i> -DDE and <i>p,p'</i> -TDE)	1.0
Deltamethrin	0.5
Diazinon	0.5
Dichlorvos	1.0
Dithiocarbamates (as CS ₂)	2.0
Endosulfan (sum of isomers and Endosulfan sulphate)	3.0
Endrin	0.05
Ethion	2.0
Fenitrothion	0.5
Fenvalerate	1.5
Fonofos	0.05
Heptachlor (sum of Heptachlor and Heptachlorepoxyde)	0.05
Hexachlorobenzene	0.1
Hexachlorocyclohexane isomers (other than γ)	0.3
Lindane (γ -Hexachlorocyclohexane)	0.6
Malathion	1.0
Methidathion	0.2
Parathion	0.5
Parathion-methyl	0.2
Permethrin	1.0
Phosalone	0.1
Piperonyl butoxide	3.0
Pirimiphos-methyl	4.0
Pyrethrins (sum of)	3.0
Quintozene (sum of quintozene, pentachloroaniline and methyl pentachlorophenyl sulphide)	1.0

Table 2.8.13.-2

Concentration of the pesticide (mg/kg)	Repeatability (difference, $\pm$ mg/kg)	Reproducibility (difference, $\pm$ mg/kg)
0.010	0.005	0.01
0.100	0.025	0.05
1.000	0.125	0.25

The following section is published for information.

Test for pesticides

ORGANOCHLORINE, ORGANOPHOSPHORUS AND PYRETHROID INSECTICIDES

The following methods may be used, in connection with the general method above. Depending on the substance being examined, it may be necessary to modify, sometimes extensively, the procedure described hereafter. In any case, it may be necessary to use, in addition, another column with a different polarity or another detection method (mass spectrometry...) or a different method (immunochemical methods...) to confirm the results obtained.

This procedure is valid only for the analysis of samples of vegetable drugs containing less than 15 per cent of water. Samples with a higher content of water may be dried, provided it has been shown that the drying procedure does not affect significantly the pesticide content.

1. EXTRACTION

To 10 g of the substance being examined, coarsely powdered, add 100 ml of *acetone R* and allow to stand for 20 min. Add 1 ml of a solution containing 1.8 µg/ml of *carbophenothion R* in *toluene R*. Homogenise using a high-speed blender for 3 min. Filter and wash the filter cake with two quantities, each of 25 ml, of *acetone R*. Combine the filtrate and the washings and heat using a rotary evaporator at a temperature not exceeding 40 °C until the solvent has almost completely evaporated. To the residue add a few millilitres of *toluene R* and heat again until the acetone is completely removed. Dissolve the residue in 8 ml of *toluene R*. Filter through a membrane filter (45 µm), rinse the flask and the filter with *toluene R* and dilute to 10.0 ml with the same solvent (solution A).

2. PURIFICATION

2.1. Organochlorine, organophosphorus and pyrethroid insecticides. Examine by size-exclusion chromatography (2.2.30).

The chromatographic procedure may be carried out using:

- a stainless steel column 0.30 m long and 7.8 mm in internal diameter packed with *styrene-divinylbenzene copolymer R* (5 µm),
- as mobile phase *toluene R* at a flow rate of 1 ml/min.

Performance of the column. Inject 100 µl of a solution containing 0.5 g/l of *methyl red R* and 0.5 g/l of *oracet blue 2R R* in *toluene R* and proceed with the chromatography. The column is not suitable unless the colour of the eluate changes from orange to blue at an elution volume of about 10.3 ml. If necessary calibrate the column, using a solution containing, in *toluene R*, at a suitable concentration, the insecticide to be analysed with the lowest molecular mass (for example, dichlorvos) and that with the highest molecular mass (for example, deltamethrin). Determine which fraction of the eluate contains both insecticides.

Purification of the test solution. Inject a suitable volume of solution A (100 µl to 500 µl) and proceed with the chromatography. Collect the fraction as determined above (solution B). Organophosphorus insecticides are usually eluted between 8.8 ml and 10.9 ml. Organochlorine and pyrethroid insecticides are usually eluted between 8.5 ml and 10.3 ml.

2.2. Organochlorine and pyrethroid insecticides. In a chromatography column, 0.10 m long and 5 mm in internal diameter, introduce a piece of defatted cotton and 0.5 g of silica gel treated as follows: heat *silica gel for chromatography R* in an oven at 150 °C for at least 4 h.

Allow to cool and add dropwise a quantity of *water R* corresponding to 1.5 per cent of the mass of silica gel used; shake vigorously until agglomerates have disappeared and continue shaking for 2 h using a mechanical shaker. Condition the column using 1.5 ml of *hexane R*. Prepacked columns containing about 0.50 g of a suitable silica gel may also be used provided they are previously validated.

Concentrate solution B in a current of *helium for chromatography R* or *oxygen-free nitrogen R* almost to dryness and dilute to a suitable volume with *toluene R* (200 µl to 1 ml according to the volume injected in the preparation of solution B). Transfer quantitatively onto the column and proceed with the chromatography using 1.8 ml of *toluene R* as the mobile phase. Collect the eluate (solution C).

3. QUANTITATIVE ANALYSIS

3.1. Organophosphorus insecticides. Examine by gas chromatography (2.2.28), using *carbophenothion R* as internal standard. It may be necessary to use a second internal standard to identify possible interference with the peak corresponding to carbophenothion.

Test solution. Concentrate solution B in a current of *helium for chromatography R* almost to dryness and dilute to 100 µl with *toluene R*.

Reference solution. Prepare at least three solutions in *toluene R* containing the insecticides to be determined and carbophenothion at concentrations suitable for plotting a calibration curve.

The chromatographic procedure may be carried out using:

- a fused-silica column 30 m long and 0.32 mm in internal diameter the internal wall of which is covered with a layer 0.25 µm thick of *poly(dimethyl)siloxane R*,
- *hydrogen for chromatography R* as the carrier gas. Other gases such as *helium for chromatography R* or *nitrogen for chromatography R* may also be used provided the chromatography is suitably validated.
- a phosphorus-nitrogen flame-ionisation detector or a atomic emission spectrometry detector,

maintaining the temperature of the column at 80 °C for 1 min, then raising it at a rate of 30 °C/min to 150 °C, maintaining at 150 °C for 3 min, then raising the temperature at a rate of 4 °C/min to 280 °C and maintaining at this temperature for 1 min, and maintaining the temperature of the injector port at 250 °C and that of the detector at 275 °C. Inject the chosen volume of each solution. When the chromatograms are recorded in the prescribed conditions, the relative retention times are approximately those listed in Table 2.8.13-3. Calculate the content of each insecticide from the peak areas and the concentrations of the solutions.

3.2. Organochlorine and pyrethroid insecticides. Examine by gas chromatography (2.2.28), using carbophenothion as the internal standard. It may be necessary to use a second internal standard to identify possible interference with the peak corresponding to carbophenothion

Test solution. Concentrate solution C in a current of *helium for chromatography R* or *oxygen-free nitrogen R* almost to dryness and dilute to 500 µl with *toluene R*.

Reference solution. Prepare at least three solutions in *toluene R* containing the insecticides to be determined and carbophenothion at concentrations suitable for plotting a calibration curve.

Table 2.8.13-3

Substance	Relative retention times
Dichlorvos	0.20
Fonofos	0.50
Diazinon	0.52
Parathion-methyl	0.59
Chlorpyrifos-methyl	0.60
Pirimiphos-methyl	0.66
Malathion	0.67
Parathion	0.69
Chlorpyrifos	0.70
Methidathion	0.78
Ethion	0.96
Carbophenothion	1.00
Azinphos-methyl	1.17
Phosalon	1.18

The chromatographic procedure may be carried out using:

- a fused silica column 30 m long and 0.32 mm in internal diameter the internal wall of which is covered with a layer 0.25 µm thick of *poly(dimethyl)(diphenyl)siloxane R*,
- *hydrogen for chromatography R* as the carrier gas. Other gases such as *helium for chromatography R* or *nitrogen for chromatography R* may also be used, provided the chromatography is suitably validated,
- an electron-capture detector,
- a device allowing direct cold on-column injection, maintaining the temperature of the column at 80 °C for 1 min, then raising it at a rate of 30 °C/min to 150 °C, maintaining at 150 °C for 3 min, then raising the temperature at a rate of 4 °C/min to 280 °C and maintaining at this temperature for 1 min, and maintaining the temperature of the injector port at 250 °C and that of the detector at 275 °C. Inject the chosen volume of each solution. When the chromatograms are recorded in the prescribed conditions, the relative retention times are approximately those listed in Table 2.8.13-4. Calculate the content of each insecticide from the peak areas and the concentrations of the solutions.

Table 2.8.13-4

Substance	Relative retention times
α-Hexachlorocyclohexane	0.44
Hexachlorobenzene	0.45
β-Hexachlorocyclohexane	0.49
Lindane	0.49
δ-Hexachlorocyclohexane	0.54
ε-Hexachlorocyclohexane	0.56
Heptachlor	0.61
Aldrin	0.68
cis-Heptachlor-epoxide	0.76
<i>o,p</i> -DDE	0.81
α-Endosulfan	0.82
Dieldrin	0.87
<i>p,p</i> -DDE	0.87
<i>o,p</i> -DDD	0.89
Endrin	0.91

Substance	Relative retention times
β-Endosulfan	0.92
<i>o,p'</i> -DDT	0.95
Carbophenothion	1.00
<i>p,p'</i> -DDT	1.02
<i>cis</i> -Permethrin	1.29
<i>trans</i> -Permethrin	1.31
Cypermethrin*	1.40
Fenvalerate*	1.47 and 1.49
Deltamethrin	1.54

* The substance shows several peaks.

01/2005:20814

2.8.14. DETERMINATION OF TANNINS IN HERBAL DRUGS

Carry out all the extraction and dilution operations protected from light.

In the case of a herbal drug or a dry extract, to the stated amount of the powdered drug (180) or the extract in a 250 ml round-bottomed flask add 150 ml of *water R*. Heat on a water-bath for 30 min. Cool under running water and transfer quantitatively to a 250 ml volumetric flask. Rinse the round-bottomed flask and collect the washings in the volumetric flask, then dilute to 250.0 ml with *water R*. Allow the solids to settle and filter the liquid through a filter paper 125 mm in diameter. Discard the first 50 ml of the filtrate.

In the case of a liquid extract or a tincture, dilute the stated amount of the liquid extract or tincture to 250.0 ml with *water R*. Filter the mixture through a filter paper 125 mm in diameter. Discard the first 50 ml of the filtrate.

Total polyphenols. Dilute 5.0 ml of the filtrate to 25.0 ml with *water R*. Mix 2.0 ml of this solution with 1.0 ml of *phosphomolybdotungstic reagent R* and 10.0 ml of *water R* and dilute to 25.0 ml with a 290 g/l solution of *sodium carbonate R*. After 30 min measure the absorbance (2.2.25) at 760 nm (A_1), using *water R* as the compensation liquid.

Polyphenols not adsorbed by hide powder. To 10.0 ml of the filtrate, add 0.10 g of *hide powder CRS* and shake vigorously for 60 min. Filter and dilute 5.0 ml of the filtrate to 25.0 ml with *water R*. Mix 2.0 ml of this solution with 1.0 ml of *phosphomolybdotungstic reagent R* and 10.0 ml of *water R* and dilute to 25.0 ml with a 290 g/l solution of *sodium carbonate R*. After 30 min measure the absorbance (2.2.25) at 760 nm (A_2), using *water R* as the compensation liquid.

Standard. Dissolve immediately before use 50.0 mg of *pyrogallol R* in *water R* and dilute to 100.0 ml with the same solvent. Dilute 5.0 ml of the solution to 100.0 ml with *water R*. Mix 2.0 ml of this solution with 1.0 ml of *phosphomolybdotungstic reagent R* and 10.0 ml of *water R* and dilute to 25.0 ml with a 290 g/l solution of *sodium carbonate R*. After 30 min measure the absorbance (2.2.25) at 760 nm (A_3), using *water R* as the compensation liquid. Calculate the percentage content of tannins expressed as pyrogallol from the expression:

$$\frac{62.5 (A_1 - A_2) m_2}{A_3 \times m_1}$$

m_1 = mass of the sample to be examined, in grams,
 m_2 = mass of pyrogallol, in grams.

01/2005:20815

2.8.15. BITTERNESS VALUE

The bitterness value is the reciprocal of the dilution of a compound, a liquid or an extract that still has a bitter taste. It is determined by comparison with quinine hydrochloride, the bitterness value of which is set at 200 000.

Determination of the correction factor

A taste panel comprising at least 6 persons is recommended. The mouth must be rinsed with *water R* before tasting.

To correct for individual differences in tasting bitterness amongst the panel members it is necessary to determine a correction factor for each panel member.

Stock solution. Dissolve 0.100 g of *quinine hydrochloride R* in *water R* and dilute to 100.0 ml with the same solvent. Dilute 1.0 ml of this solution to 100.0 ml with *water R*.

Reference solutions. Prepare a series of dilutions by placing in a first tube 3.6 ml of the stock solution and increasing the volume by 0.2 ml in each subsequent tube to a total of 5.8 ml; dilute the contents of each tube to 10.0 ml with *water R*.

Determine as follows the dilution with the lowest concentration that still has a bitter taste. Take 10.0 ml of the weakest solution into the mouth and pass it from side to side over the back of the tongue for 30 s. If the solution is not found to be bitter, spit it out and wait for 1 min. Rinse the mouth with *water R*. After 10 min, use the next dilution in order of increasing concentration.

Calculate the correction factor k for each panel member from the expression:

$$k = \frac{n}{5.00}$$

n = number of millilitres of the stock solution in the dilution of lowest concentration that is judged to be bitter.

Persons who are unable to taste any bitterness when using the reference solution prepared from 5.8 ml of stock solution have to be excluded from the panel.

Sample preparation

If necessary, reduce the sample to a powder (710). To 1.0 g of sample add 100 ml of boiling *water R*. Heat on a water-bath for 30 min, stirring continuously. Allow to cool and dilute to 100 ml with *water R*. Shake vigorously and filter, discarding the first 2 ml of the filtrate. The filtrate is labelled C-1 and has a dilution factor (DF) of 100.

If liquids have to be examined, 1 ml of the liquid is diluted with a suitable solvent to 100 ml and designated C-1.

Determination of the bitterness value

Test solutions:

10.0 ml of C-1 is diluted with <i>water R</i> to 100 ml: C-2	(DF = 1000)
10.0 ml of C-2 is diluted with <i>water R</i> to 100 ml: C-3	(DF = 10 000)
20.0 ml of C-3 is diluted with <i>water R</i> to 100 ml: C-3A	(DF = 50 000)
10.0 ml of C-3 is diluted with <i>water R</i> to 100 ml: C-4	(DF = 100 000)

Starting with dilution C-4 each panel member determines the dilution which still has a bitter taste. This solution is designated D. Note the DF of solution D is Y.

Starting with solution D prepare the following sequence of dilutions:

Solution D (ml)	1.2	1.5	2.0	3.0	6.0	8.0
<i>water R</i> (ml)	8.8	8.5	8.0	7.0	4.0	2.0

Determine the number of millilitres of solution D which, when diluted to 10.0 ml with *water R*, still has a bitter taste (X).

Calculate the bitterness value for each panel member from the expression:

$$\left(\frac{Y \times k}{X \times 0.1} \right)$$

Calculate the bitterness value of the sample to be examined as the average value for all panel members.

01/2005:20816

2.8.16. DRY RESIDUE OF EXTRACTS

In a flat-bottomed dish about 50 mm in diameter and about 30 mm in height, introduce rapidly 2.00 g or 2.0 ml of the extract to be examined. Evaporate to dryness on a water-bath and dry in an oven at 100-105 °C for 3 h. Allow to cool in a desiccator over *diphosphorus pentoxide R* or *anhydrous silica gel R* and weigh. Calculate the result as a mass percentage or in grams per litre.

01/2005:20817

2.8.17. LOSS ON DRYING OF EXTRACTS

In a flat-bottomed dish about 50 mm in diameter and about 30 mm in height, weigh rapidly 0.50 g of the extract to be examined, finely powdered. Dry in an oven at 100-105 °C for 3 h. Allow to cool in a desiccator over *diphosphorus pentoxide R* or *anhydrous silica gel R* and weigh. Calculate the result as a mass percentage.

2.9. PHARMACEUTICAL TECHNICAL PROCEDURES

01/2005:20901

2.9.1. DISINTEGRATION OF TABLETS AND CAPSULES

The disintegration test determines whether tablets or capsules disintegrate within the prescribed time when placed in a liquid medium in the experimental conditions prescribed below.

Disintegration is considered to be achieved when:

- no residue remains on the screen, or
- if there is a residue, it consists of a soft mass having no palpably firm, unmoistened core, or
- only fragments of coating (tablets) or only fragments of shell (capsules) remain on the screen; if a disc has been used (capsules), fragments of shell may adhere to the lower surface of the disc.

Use apparatus A for tablets and capsules that are not greater than 18 mm long. For larger tablets or capsules use apparatus B.

TEST A - TABLETS AND CAPSULES OF NORMAL SIZE

Apparatus. The main part of the apparatus (Figure 2.9.1-1) is a rigid basket-rack assembly supporting 6 cylindrical transparent tubes 77.5 ± 2.5 mm long, 21.5 mm in internal diameter, and with a wall thickness of about 2 mm. Each tube is provided with a cylindrical disc 20.7 ± 0.15 mm in diameter and 9.5 ± 0.15 mm thick, made of transparent plastic with a relative density of 1.18 to 1.20 or weighing 3.0 ± 0.2 g. Each disc is pierced by 5 holes 2 mm in diameter, 1 in the centre and the other 4 spaced equally on a circle of radius 6 mm from the centre of the disc. On the lateral surface of the disc, 4 equally spaced grooves are cut in such a way that at the upper surface of the disc they are 9.5 mm wide and 2.55 mm deep and at the lower surface 1.6 mm square. The tubes are held vertically by 2 separate and superimposed rigid plastic plates 90 mm in diameter and 6 mm thick with 6 holes. The holes are equidistant from the centre of the plate and equally spaced. Attached to the under side of the lower plate is a piece of woven gauze made from stainless steel wire 0.635 mm in diameter and having mesh apertures of 2.00 mm. The plates are held rigidly in position and 77.5 mm apart by vertical metal rods at the periphery, a metal rod is also fixed to the centre of the upper plate to enable the assembly to be attached to a mechanical device

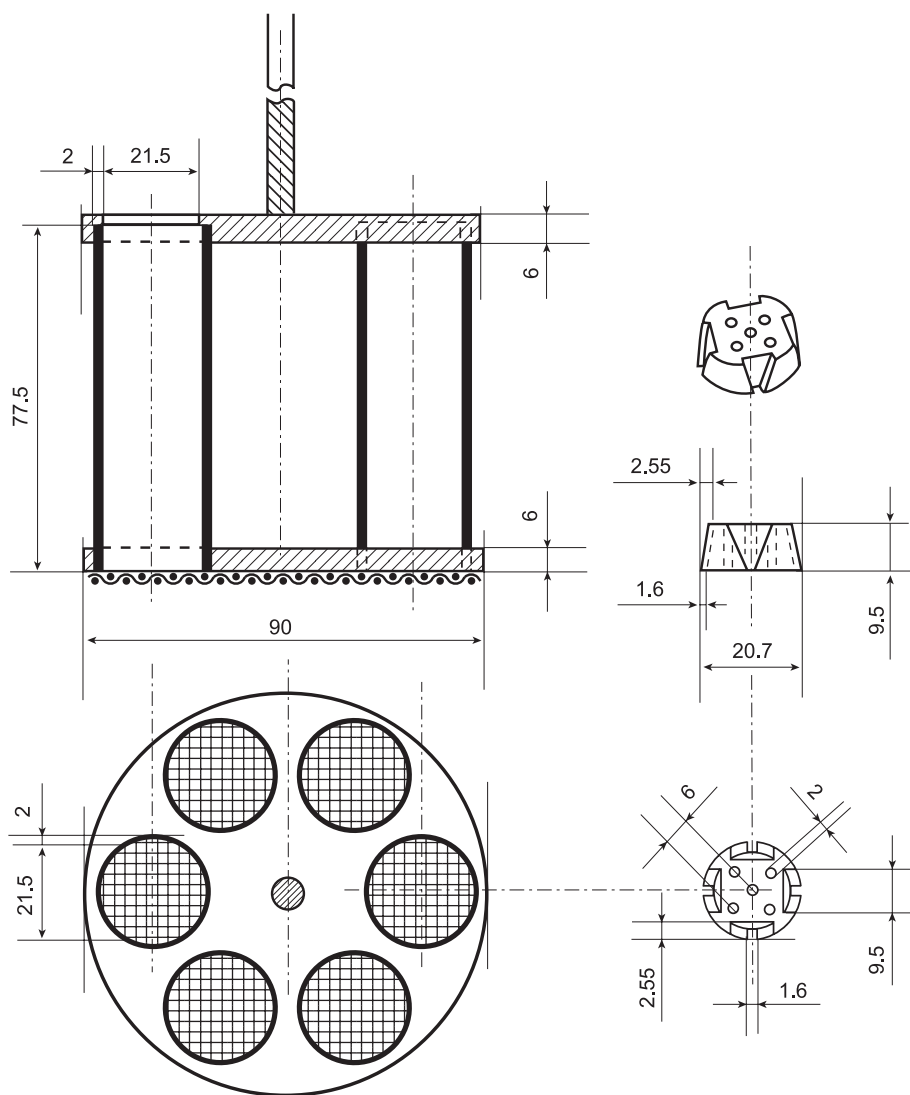


Figure 2.9.1-1. – Apparatus A
Dimensions in millimetres

capable of raising and lowering it smoothly at a constant frequency between 29 and 32 cycles per minute, through a distance of 50 mm to 60 mm.

The assembly is suspended in the specified liquid in a suitable vessel, preferably a 1 litre beaker. The volume of the liquid is such that when the assembly is in the highest position the wire mesh is at least 15 mm below the surface of the liquid, and when the assembly is in the lowest position the wire mesh is at least 25 mm above the bottom of the beaker and the upper open ends of the tubes remain above the surface of the liquid. A suitable device maintains the temperature of the liquid at 35-39 °C.

The design of the basket-rack assembly may be varied provided the specifications for the tubes and wire mesh are maintained.

Method. In each of the 6 tubes, place one tablet or capsule and, if prescribed, add a disc; suspend the assembly in the beaker containing the specified liquid. Operate the apparatus for the prescribed period, withdraw the assembly and examine the state of the tablets or capsules. To pass the test, all the tablets or capsules must have disintegrated.

TEST B – LARGE TABLETS AND LARGE CAPSULES

Apparatus. The main part of the apparatus (Figure 2.9.1-2) is a rigid basket-rack assembly supporting 3 cylindrical transparent tubes 77.5 ± 2.5 mm long, $33.0 \text{ mm} \pm 0.5$ mm in internal diameter, and with a wall thickness of 2.5 ± 0.5 mm. Each tube is provided with a cylindrical disc 31.4 ± 0.13 mm in diameter and 15.3 ± 0.15 mm thick, made of transparent plastic with a relative density of 1.18 to 1.20 or weighing 13.0 ± 0.2 g. Each disc is pierced by 7 holes, each 3.15 ± 0.1 mm in diameter, 1 in the centre and the other 6 spaced equally on a circle of radius 4.2 mm from the centre of the disc. The tubes are held vertically by 2 separate and superimposed rigid plastic plates 97 mm in diameter and 9 mm thick, with 3 holes. The holes are equidistant from the centre of the plate and equally spaced. Attached to the under side of the lower plate is a piece of woven gauze made from stainless steel wire 0.63 ± 0.03 mm in diameter and having mesh apertures of 2.0 ± 0.2 mm. The plates are held rigidly in position and 77.5 mm apart by vertical metal rods at the periphery, a metal rod is also fixed to the centre of the upper plate to enable the assembly to be attached

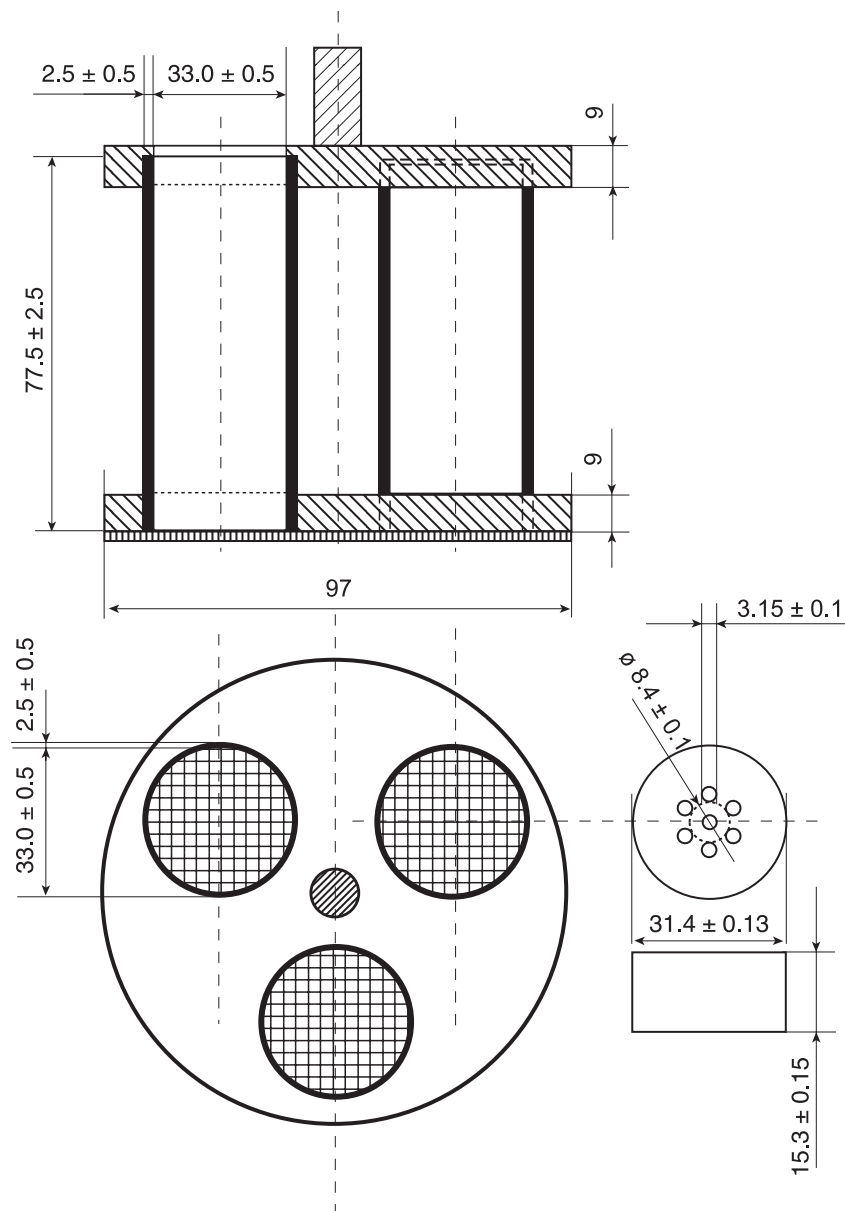


Figure 2.9.1-2. – Apparatus B
Dimensions in millimetres

to a mechanical device capable of raising and lowering it smoothly at constant frequency between 29 and 32 cycles per minute, through a distance of 55 ± 2 mm.

The assembly is suspended in the specified liquid medium in a suitable vessel, preferably a 1 litre beaker. The volume of the liquid is such that when the assembly is in the highest position the wire mesh is at least 15 mm below the surface of the liquid, and when the assembly is in the lowest position the wire mesh is at least 25 mm above the bottom of the beaker and the upper open ends of the tubes remain above the surface of the liquid. A suitable device maintains the temperature of the liquid at $35\text{--}39$ °C.

The design of the basket-rack assembly may be varied provided the specifications for the tubes and wire mesh are maintained.

Method. Test 6 tablets or capsules either by using 2 basket-rack assemblies in parallel or by repeating the procedure. In each of the 3 tubes, place one tablet or capsule and, if prescribed, add a disc; suspend the assembly in the beaker containing the specified liquid. Operate the apparatus for the prescribed period, withdraw the assembly and examine the state of the tablets or capsules. To pass the test, all 6 of the tablets or capsules must have disintegrated.

01/2005:20902

2.9.2. DISINTEGRATION OF SUPPOSITORIES AND PESSARIES

The disintegration test determines whether the suppositories or pessaries soften or disintegrate within the prescribed time when placed in a liquid medium in the experimental conditions described below.

Disintegration is considered to be achieved when:

- dissolution is complete,
- the components of the suppository or pessary have separated: melted fatty substances collect on the surface of the liquid, insoluble powders fall to the bottom and soluble components dissolve, depending on the type of preparation, the components may be distributed in one or more of these ways,
- there is softening of the sample that may be accompanied by appreciable change of shape without complete separation of the components, the softening is such that the suppository or pessary no longer has a solid core offering resistance to pressure of a glass rod,
- rupture of the gelatin shell of rectal or vaginal capsules occurs allowing release of the contents,
- no residue remains on the perforated disc or if a residue remains, it consists only of a soft or frothy mass having no solid core offering resistance to pressure of a glass rod (vaginal tablets).

Apparatus. The apparatus (Figure 2.9.2.-1) consists of a sleeve of glass or suitable transparent plastic, of appropriate thickness, to the interior of which is attached by means of three hooks a metal device consisting of two perforated stainless steel discs each containing 39 holes 4 mm in diameter; the diameter of the discs is similar to that of the interior of the sleeve; the discs are about 30 mm apart. The test is carried out using three such apparatuses each containing a single sample. Each apparatus is placed in a beaker with a capacity of at least 4 litres filled with water

maintained at $36\text{--}37$ °C, unless otherwise prescribed. The apparatuses may also be placed together in a vessel with a capacity of at least 12 litres. The beaker is fitted with a slow stirrer and a device that will hold the cylinders vertically not less than 90 mm below the surface of the water and allow them to be inverted without emerging from the water.

Method. Use three suppositories or pessaries. Place each one on the lower disc of a device, place the latter in the sleeve and secure. Invert the apparatuses every 10 min. Examine the samples after the period prescribed in the monograph. To pass the test all the samples must have disintegrated.

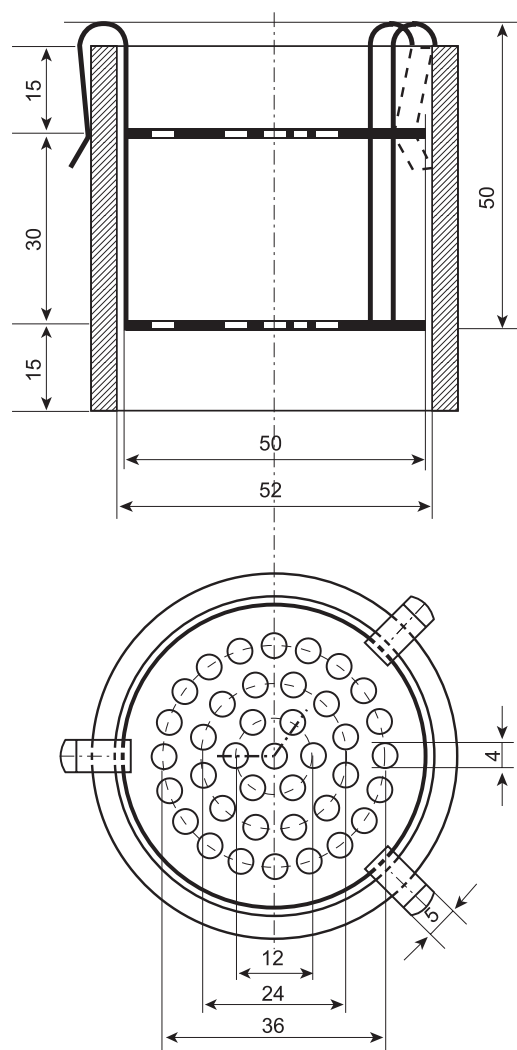
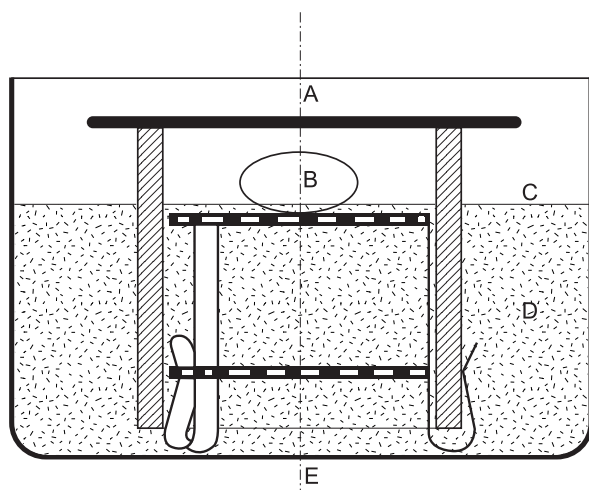


Figure 2.9.2.-1. – Apparatus for disintegration of suppositories and pessaries
Dimensions in millimetres

METHOD OF OPERATION FOR VAGINAL TABLETS

Use the apparatus described above, arranged so as to rest on the hooks (see Figure 2.9.2.-2). Place it in a beaker of suitable diameter containing water maintained at $36\text{--}37$ °C with the level just below the upper perforated disc. Using a pipette, adjust the level with water at $36\text{--}37$ °C until a uniform film covers the perforations of the disc. Use three vaginal tablets. Place each one on the upper plate of an apparatus and cover the latter with a glass plate to maintain appropriate conditions of humidity. Examine the state of the samples after the period prescribed in the monograph. To pass the test all the samples must have disintegrated.



A. glass plate
B. vaginal tablet
C. water surface
D. water
E. dish, beaker

Figure 2.9.2-2.

01/2005:20903

2.9.3. DISSOLUTION TEST FOR SOLID DOSAGE FORMS

The test is used to determine the dissolution rate of the active ingredients of solid dosage forms (for example, tablets, capsules and suppositories).

Unless otherwise justified and authorised, either the paddle apparatus or the basket apparatus or in special cases, the flow-through cell apparatus may be used.

The following are to be prescribed for each preparation to which the dissolution test is applied:

- the apparatus to be used, including in those cases where the flow-through cell apparatus is prescribed, which flow-through cell (Figures 2.9.3-4/5/6) is to be used,
- the composition, the volume and the temperature of the dissolution medium,
- the rotation speed or the flow rate of the dissolution medium,
- the time, the method and the amount for sampling of the test solution or the conditions for continuous monitoring,
- the method of analysis,
- the quantity or quantities of active ingredients required to dissolve within a prescribed time.

APPARATUS

The choice of the apparatus to be used depends on the physico-chemical characteristics of the dosage form. All parts of the apparatus that may come into contact with the preparation or the dissolution medium are chemically inert and do not adsorb or react or interfere with the test sample. All metal parts of the apparatus that may come into contact with the preparation or the dissolution medium must be made from a suitable stainless steel or coated with a suitable material to ensure that such parts do not react or interfere with the preparation or the dissolution medium. No part of the assembly or its environment contributes significant motion, agitation or vibration beyond that resulting from the smoothly rotating element or from the flow-through system.

An apparatus that permits observation of the preparation to be examined and the stirrer during the test is preferable.

Paddle apparatus. The apparatus (see Figure 2.9.3-1) consists of:

- a cylindrical vessel of borosilicate glass or other suitable transparent material with a hemispherical bottom and a nominal capacity of 1000 ml; a cover is fitted to retard evaporation; the cover has a central hole to accommodate the shaft of the stirrer and other holes for the thermometer and the devices used to withdraw liquid;
- a stirrer consisting of a vertical shaft to the lower end of which is attached a blade having the form of that part of a circle subtended by 2 parallel chords; the blade passes through the diameter of the shaft so that the bottom of the blade is flush with the bottom of the shaft; the shaft is placed so that its axis is within 2 mm of the axis of the vessel and the bottom of the blade is 25 ± 2 mm from the inner bottom of the vessel; the upper part of the shaft is connected to a motor provided with a speed regulator; the stirrer rotates smoothly without significant wobble;
- a water-bath that will maintain the dissolution medium at 37 ± 0.5 °C.

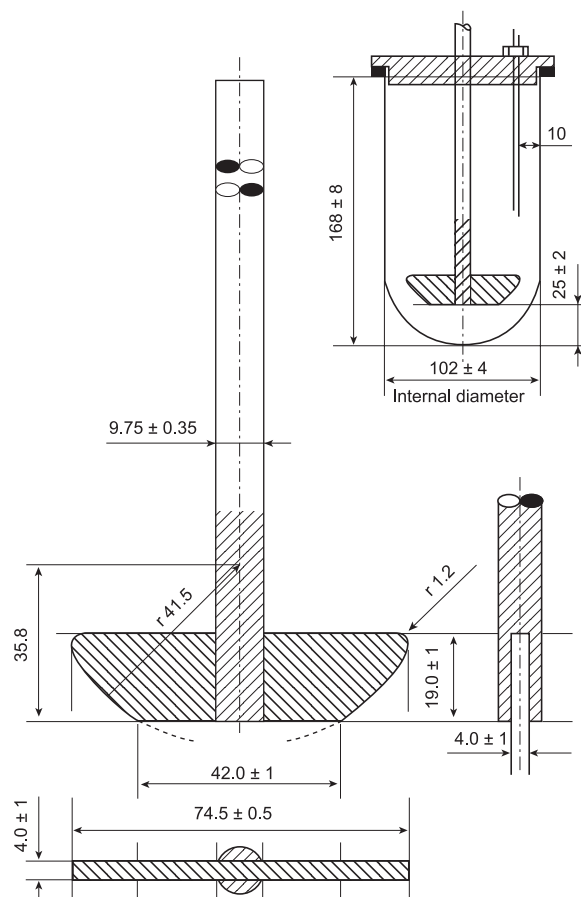


Figure 2.9.3-1. – Paddle apparatus
Dimensions in millimetres

Basket apparatus. The apparatus (see Figure 2.9.3-2) consists of:

- a vessel identical with that described for the paddle apparatus;
- a stirrer consisting of a vertical shaft to the lower part of which is attached a cylindrical basket; the basket has 2 parts: the upper part, with a 2 mm vent, is welded to the shaft and has 3 spring clips or other suitable device that allows removal of the lower part of the basket for introduction of the preparation to be examined and firmly

holds the lower part concentric with the axis of the vessel during rotation; the lower part of the basket is made of welded-seam cloth formed into a cylinder with a narrow rim of sheet metal around the top and bottom; unless otherwise prescribed, the cloth has a wire thickness of 0.254 mm in diameter and 0.381 mm square openings; a basket with a gold coating 2.5 µm thick may be used for tests carried out in dilute acid medium; the bottom of the basket is 25 ± 2 mm from the inner bottom of the vessel during the test; the upper part of the shaft is connected to a motor provided with a speed regulator; the stirrer rotates smoothly without significant wobble;

- a water-bath that will maintain the dissolution medium at 37 ± 0.5 °C.

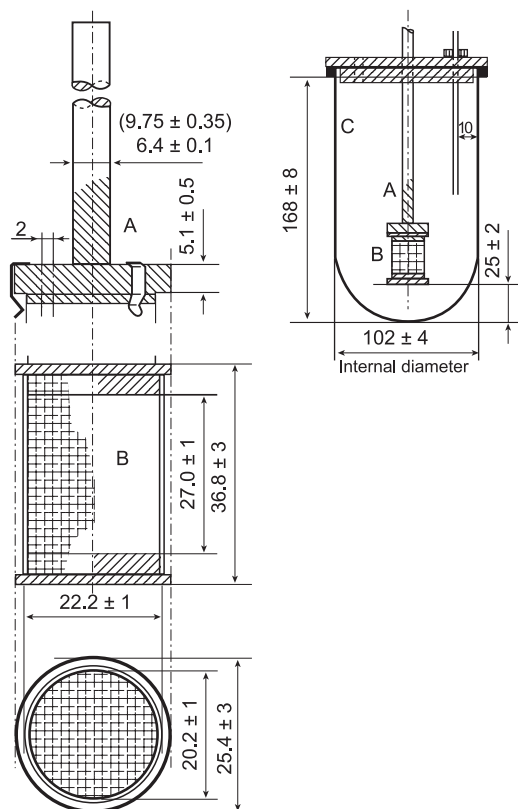


Figure 2.9.3-2. – Basket apparatus
Dimensions in millimetres

Flow-through apparatus. The apparatus (see Figure 2.9.3-3) consists of:

- a reservoir for the dissolution medium;
- a pump that forces the dissolution medium upwards through the flow-through cell;
- a flow-through cell (see Figures 2.9.3-4/5/6) of transparent material mounted vertically with a filter system preventing escape of undissolved particles.

The flow-through cell shown in Figure 2.9.3-6 is specifically intended for lipophilic solid dosage forms such as suppositories and soft capsules. It consists of 3 transparent parts which fit into each other. The lower part (1) is made up of 2 adjacent chambers connected to an overflow device.

The dissolution medium passes through chamber A and is subjected to an upwards flow. The flow in chamber B is downwards directed to a small-size bore exit which leads upwards to a filter assembly. The middle part (2) of the cell has a cavity designed to collect lipophilic excipients which float on the dissolution medium. A metal grill serves as a rough filter. The upper part (3) holds a filter unit for paper, glass fibre or cellulose filters.

- a water-bath that will maintain the dissolution medium at 37 ± 0.5 °C.

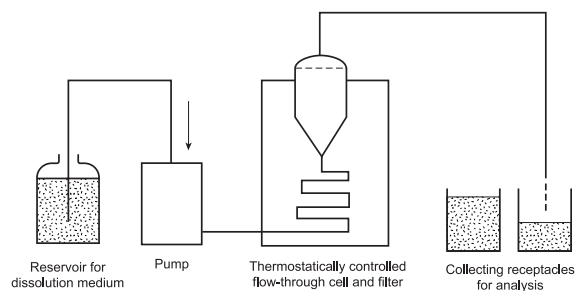


Figure 2.9.3-3. – Flow-through apparatus

Dissolution medium. If the dissolution medium is buffered, adjust its pH to within ± 0.05 units of the prescribed value. Remove any dissolved gases from the dissolution medium before the test since they can cause the formation of bubbles that significantly affect the results.

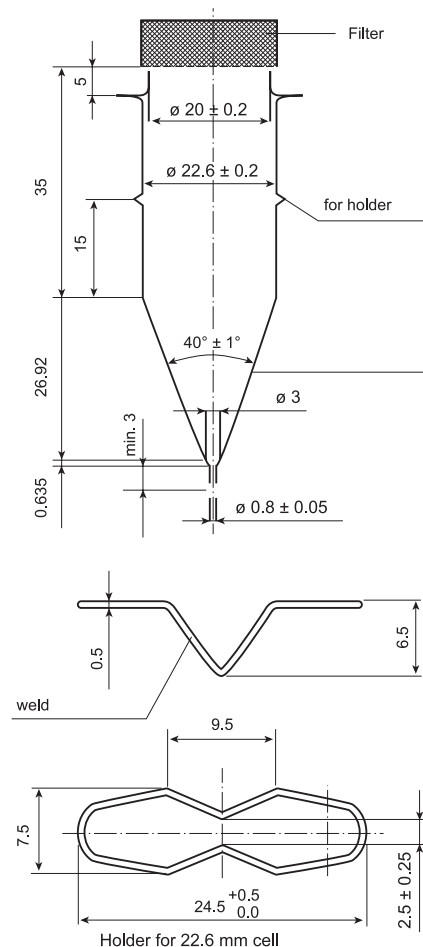


Figure 2.9.3-4. – Flow-through cell
Dimensions in millimetres

METHOD

Paddle and basket apparatus

Place the prescribed volume of dissolution medium in the vessel, assemble the apparatus, warm the dissolution medium to 37 ± 0.5 °C and remove the thermometer.

Place one unit of the preparation to be examined in the apparatus. For the paddle apparatus, place the preparation at the bottom of the vessel before starting rotation of the blade; dosage forms that would otherwise float are kept horizontal at the bottom of the vessel using a suitable device, such as a wire or glass helix.

For the basket apparatus, place the preparation in a dry basket and lower into position before starting rotation.

Take care to avoid the presence of air bubbles on the surface of the preparation. Start the rotation of the apparatus immediately at the prescribed rate (± 4 per cent).

Flow-through apparatus

- Cells (see Figures 2.9.3-4/5)

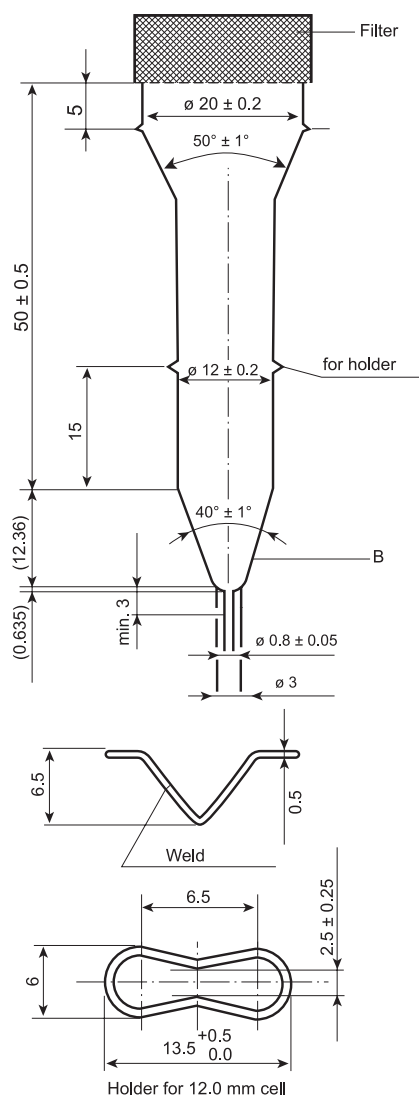


Figure 2.9.3-5. – Flow-through cell
Dimensions in millimetres

Place 1 bead of 5 mm (± 0.5 mm) diameter at the bottom of the cone to protect the fluid entry of the tube and then glass beads of suitable size, preferably 1 mm (± 0.1 mm) diameter. Introduce 1 unit of the preparation in the cell on or within the layer of glass beads, by means of a holder. Assemble the filter head. Heat the dissolution medium to 37 ± 0.5 °C. Using a suitable pump, introduce the dissolution medium through the bottom of the cell to obtain a suitable continuous flow through an open or closed circuit at the prescribed rate (± 5 per cent).

- Cell (Figure 2.9.3-6)

Place 1 unit of the preparation to be examined in chamber A. Close the cell with the prepared filter assembly. At the beginning of the test, chamber A requires air removal via a small orifice connected to the filter assembly. Heat the dissolution medium to an appropriate temperature taking

the melting point of the preparation into consideration. Using a suitable pump, introduce the warmed dissolution medium through the bottom of the cell to obtain a suitable continuous flow through an open or closed circuit at the prescribed rate (± 5 per cent). When the dissolution medium reaches the overflow, air starts to escape through the capillary and chamber B fills with the dissolution medium. The preparation spreads through the dissolution medium according to its physico-chemical properties.

In justified and authorised cases, representative fractions of large volume suppositories may be tested.

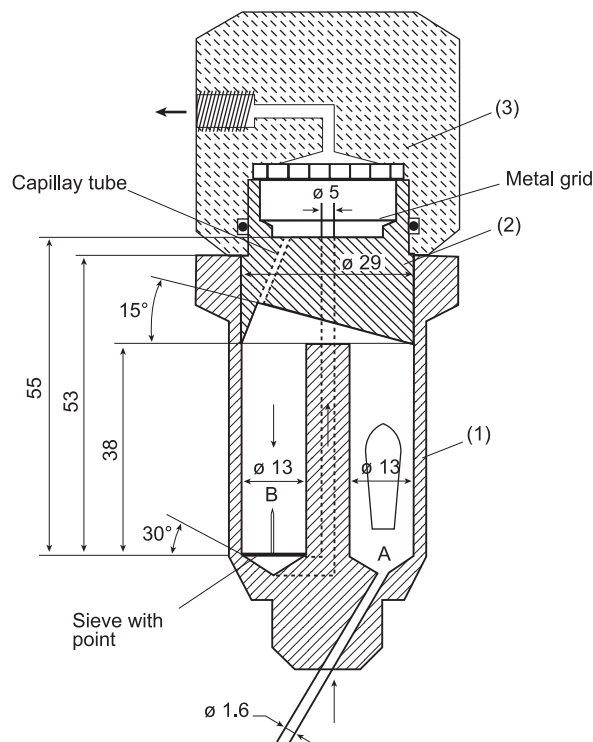


Figure 2.9.3-6. – Flow-through cell
Dimensions in millimetres

SAMPLING AND EVALUATION

In the case of the paddle apparatus and the basket apparatus, withdraw at the prescribed time, or at the prescribed intervals or continuously, the prescribed volume or volumes from a position midway between the surface of the dissolution medium and the top of the basket or blade and not less than 10 mm from the vessel wall.

In the case of the flow-through apparatus, samples are always collected at the outlet of the cell, irrespective of whether the circuit is opened or closed.

Except where continuous measurement is used with the paddle or basket method (the liquid removed being returned to the vessel) or where a single portion of liquid is removed, add a volume of dissolution medium equal to the volume of liquid removed or compensate by calculation.

Filter the liquid removed using an inert filter of appropriate pore size that does not cause significant adsorption of the active ingredient from the solution and does not contain substances extractable by the dissolution medium that would interfere with the prescribed analytical method. Proceed with analysis of the filtrate as prescribed.

The quantity of the active ingredient dissolved in a specified time is expressed as a percentage of the content stated on the label.

01/2005:20904

2.9.4. DISSOLUTION TEST FOR TRANSDERMAL PATCHES

This test is used to determine the dissolution rate of the active ingredients of transdermal patches.

1. DISK ASSEMBLY METHOD

Equipment. Use the paddle and vessel assembly from the paddle apparatus described in the dissolution test for solid oral dosage forms (2.9.3) with the addition of a stainless steel disk assembly (SSDA) in the form of a net with an aperture of 125 µm (see Figure 2.9.4.-1).

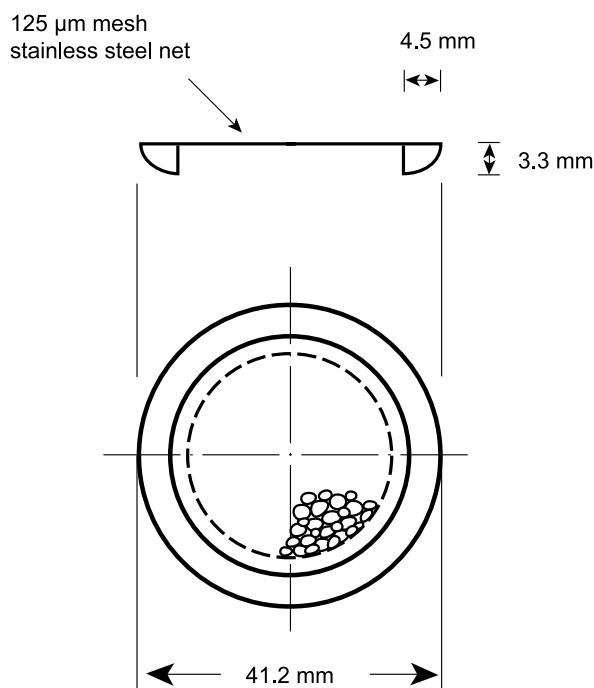


Figure 2.9.4.-1. – Disk assembly

The SSDA holds the system at the bottom of the vessel and is designed to minimise any dead volume between the SSDA and the bottom of the vessel. The SSDA holds the patch flat, with the release surface uppermost and parallel to the bottom of the paddle blade. A distance of 25 ± 2 mm between the bottom of the paddle blade and the surface of the SSDA is maintained during the test (see Figure 2.9.4.-2). The temperature is maintained at 32 ± 0.5 °C. The vessel may be covered during the test to minimise evaporation.

Procedure. Place the prescribed volume of the dissolution medium in the vessel and equilibrate the medium to the prescribed temperature. Apply the patch to the SSDA, ensuring that the release surface of the patch is as flat as possible. The patch may be attached to the SSDA by a

prescribed adhesive or by a strip of a double-sided adhesive tape. The adhesive or tape are previously tested for the absence of interference with the assay and of adsorption of the active ingredient(s). Press the patch, release surface facing up, onto the side of the SSDA made adhesive. The applied patch must not overlap the borders of the SSDA. For this purpose and provided that the preparation is homogeneous and uniformly spread on the outer covering, an appropriate and exactly measured piece of the patch may be cut and used for testing the dissolution rate. This procedure may also be necessary to achieve appropriate sink conditions. This procedure must not be applied to membrane-type patches. Place the patch mounted on the SSDA flat at the bottom of the vessel with the release surface facing upwards. Immediately rotate the paddle at 100 r/min, for example. At predetermined intervals, withdraw a sample from the zone midway between the surface of the dissolution medium and the top of the blade, not less than 1 cm from the vessel wall.

Perform the assay on each sample, correcting for any volume losses, as necessary. Repeat the test with additional patches.

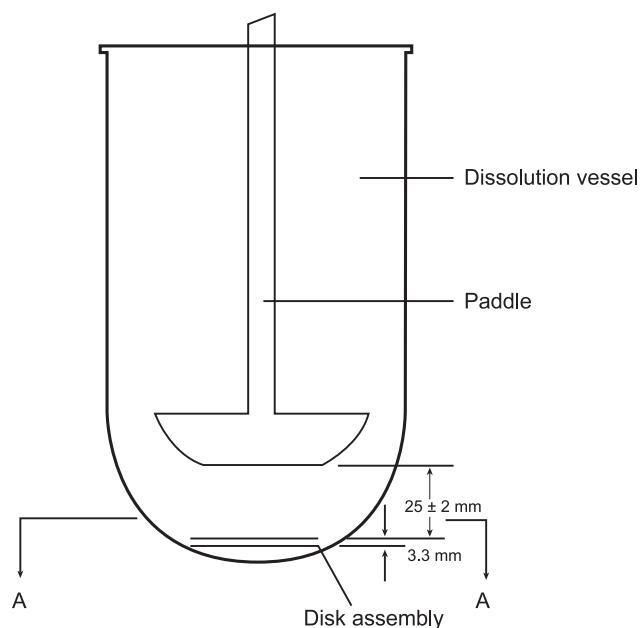


Figure 2.9.4.-2. – Paddle and disk

2. CELL METHOD

Equipment. Use the paddle and vessel assembly from the paddle apparatus described in the dissolution test for solid oral dosage forms (2.9.3) with the addition of the extraction cell (*cell*).

The *cell* is made of chemically inert materials and consists of a *support*, a *cover* and, if necessary, a *membrane* placed on the patch to isolate it from the medium that may modify or adversely affect the physico-chemical properties of the patch (see Figure 2.9.4.-3).

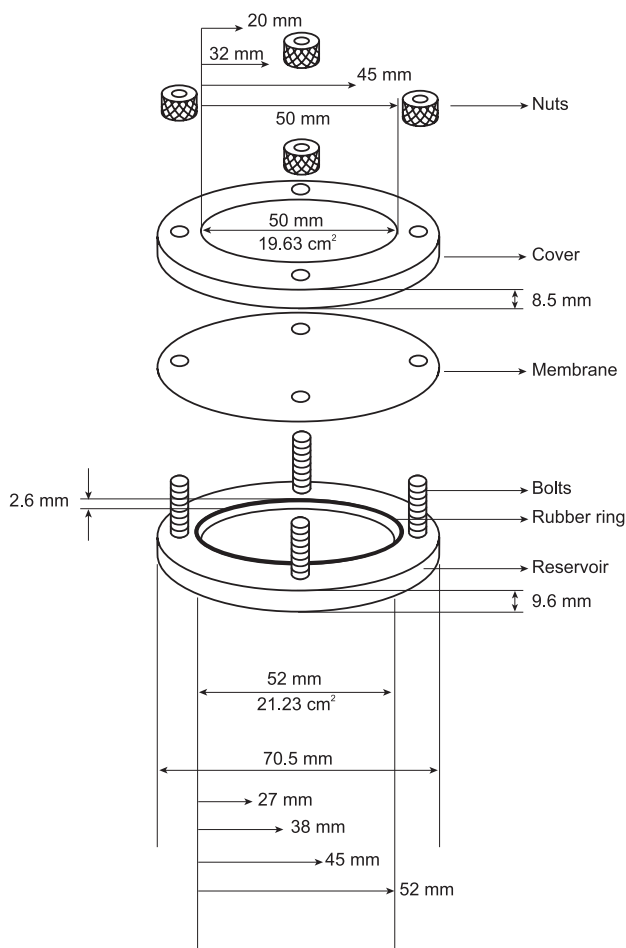


Figure 2.9.4.3. – Extraction cell

Support. The central part of the support forms a cavity intended to hold the patch. The cavity has a depth of 2.6 mm and a diameter that is appropriate to the size of the patch to be examined. The following diameters can be used: 27 mm, 38 mm, 45 mm, 52 mm, corresponding to volumes of 1.48 ml, 2.94 ml, 4.13 ml, 5.52 ml, respectively.

Cover. The cover has a central opening with a diameter selected according to the size of the patch to be examined. The patch can thus be precisely centred, and its releasing surface limited. The following diameters may be used: 20 mm, 32 mm, 40 mm, 50 mm corresponding to areas of 3.14 cm², 8.03 cm², 12.56 cm², 19.63 cm², respectively. The cover is held in place by nuts screwed onto bolts projecting from the support. The cover is sealed to the support by a rubber ring set on the reservoir.

Extraction cell. The cell holds the patch flat, with the release surface uppermost and parallel to the bottom of the paddle blade. A distance of 25 ± 2 mm is maintained between the paddle blade and the surface of the patch (see Figure 2.9.4.4). The temperature is maintained at 32 ± 0.5 °C. The vessel may be covered during the test to minimise evaporation.

Procedure. Place the prescribed volume of the dissolution medium in the vessel and equilibrate the medium to the prescribed temperature. Precisely centre the patch in the cell with the releasing surface uppermost. Close the cell, if necessary applying a hydrophobic substance (for example, petrolatum) to the flat surfaces to ensure the seal, and ensure that the patch stays in place. Introduce the cell flat into the bottom of the vessel with the cover facing upwards. Immediately rotate the paddle, at 100 r/min for example. At

predetermined intervals, withdraw a sample from the zone midway between the surface of the dissolution medium and the top of the paddle blade, not less than 1 cm from the vessel wall.

Perform the assay on each sample, correcting for any volume losses, as necessary. Repeat the test with additional patches.

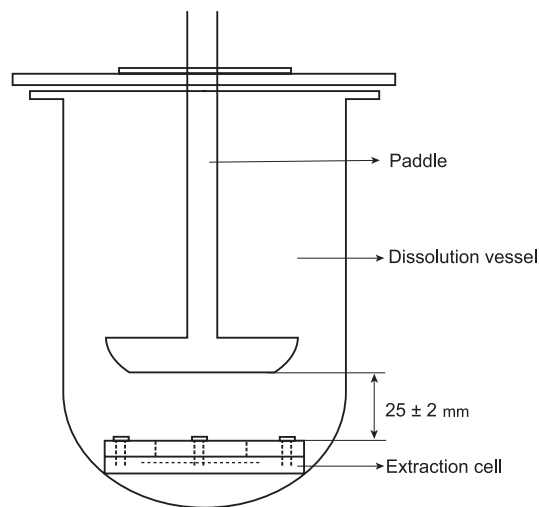


Figure 2.9.4.4. – Paddle over extraction cell

3. ROTATING CYLINDER METHOD

Equipment. Use the assembly of the paddle apparatus described in the dissolution test for solid oral dosage forms (2.9.3). Replace the paddle and shaft with a stainless steel cylinder stirring element (*cylinder*) (see Figure 2.9.4.5). The patch is placed on the *cylinder* at the beginning of each test. The distance between the inside bottom of the vessel and the *cylinder* is maintained at 25 ± 2 mm during the test. The temperature is maintained at 32 ± 0.5 °C. The vessel is covered during the test to minimise evaporation.

Procedure. Place the prescribed volume of the dissolution medium in the vessel and equilibrate the medium to the prescribed temperature. Remove the protective liner from the patch and place the adhesive side on a piece of suitable inert porous membrane that is at least 1 cm larger on all sides than the patch. Place the patch on a clean surface with the membrane in contact with this surface. Two systems for adhesion to the *cylinder* may be used:

- apply a suitable adhesive to the exposed membrane borders and, if necessary, to the back of the patch,
- apply a double-sided adhesive tape to the external wall of the *cylinder*.

Using gentle pressure, carefully apply the non-adhesive side of the patch to the *cylinder*, so that the release surface is in contact with the dissolution medium and the long axis of the patch fits around the circumference of the *cylinder*.

The system for adhesion used is previously tested for absence of interference with the assay and of adsorption of the active ingredient(s).

Place the *cylinder* in the apparatus, and immediately rotate the *cylinder* at 100 r/min, for example. At determined intervals, withdraw a sample of dissolution medium from a zone midway between the surface of the dissolution medium and the top of the rotating *cylinder*, and not less than 1 cm from the vessel wall.

Perform the assay on each sample as directed in the individual monograph, correcting for any volume withdrawn, as necessary. Repeat the test with additional patches.

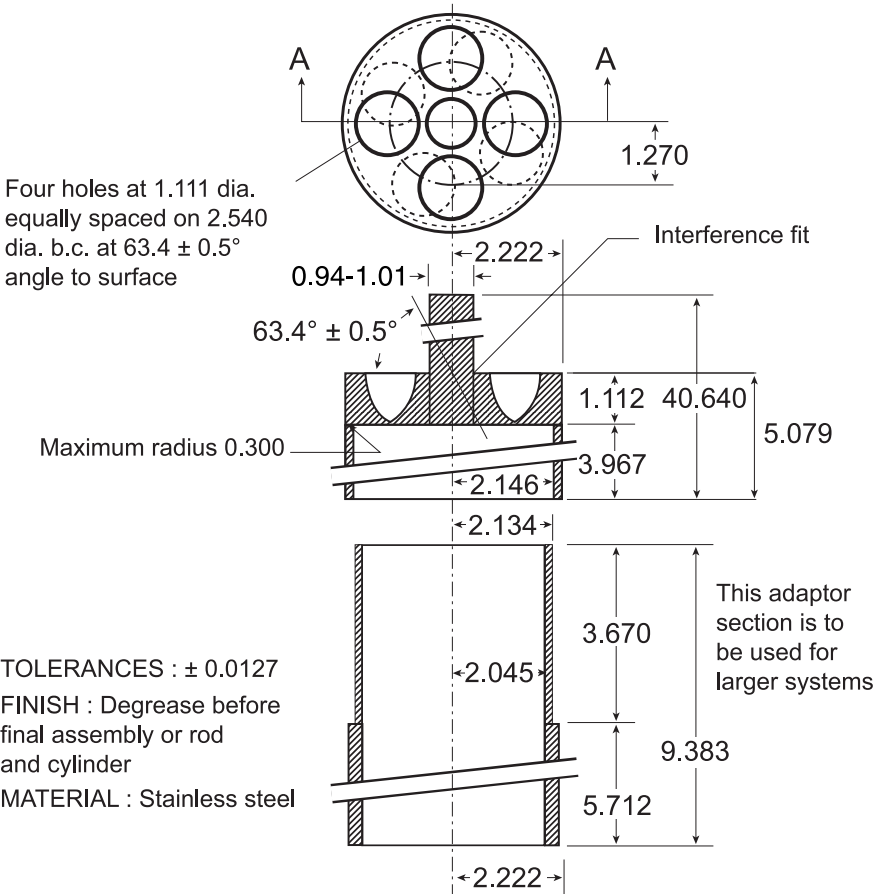


Figure 2.9.4-5. – *Cylinder stirring element*
Dimensions in centimetres

Interpretation. The requirements are met if the quantity of active ingredient(s) released from the patch, expressed as the amount per surface area per time unit, is within the prescribed limits at the defined sampling times.

01/2005:20905

2.9.5. UNIFORMITY OF MASS OF SINGLE-DOSE PREPARATIONS

Weigh individually 20 units taken at random or, for single-dose preparations presented in individual containers, the contents of 20 units, and determine the average mass. Not more than 2 of the individual masses deviate from the average mass by more than the percentage deviation shown in Table 2.9.5-1 and none deviates by more than twice that percentage.

For capsules and powders for parenteral use, proceed as described below.

CAPSULES

Weigh an intact capsule. Open the capsule without losing any part of the shell and remove the contents as completely as possible. For soft shell capsules, wash the shell with a suitable solvent and allow to stand until the odour of the solvent is no longer perceptible. Weigh the shell. The mass of the contents is the difference between the weighings. Repeat the procedure with another 19 capsules.

Table 2.9.5-1

Pharmaceutical Form	Average Mass	Percentage deviation
Tablets (uncoated and film-coated)	80 mg or less	10
	More than 80 mg and less than 250 mg	7.5
	250 mg or more	5
Capsules, granules (uncoated, single-dose) and powders (single-dose)	Less than 300 mg	10
	300 mg or more	7.5
Powders for parenteral use* (single-dose)	More than 40 mg	10
Suppositories and pessaries	All masses	5
Powders for eye-drops and powders for eye lotions (single-dose)	Less than 300 mg	10
	300 mg or more	7.5

* When the average mass is equal to or below 40 mg, the preparation is not submitted to the test for uniformity of mass but to the test for uniformity of content of single-dose preparations (2.9.6).

POWDERS FOR PARENTERAL USE

Remove any paper labels from a container and wash and dry the outside. Open the container and without delay weigh the container and its contents. Empty the container as completely as possible by gentle tapping, rinse it if necessary with *water R* and then with *alcohol R* and dry at 100-105 °C for 1 h, or, if the nature of the container precludes heating at this temperature, dry at a lower temperature to constant mass. Allow to cool in a desiccator and weigh. The mass of the contents is the difference between the weighings. Repeat the procedure with another 19 containers.

01/2005:20906 TEST C

2.9.6. UNIFORMITY OF CONTENT OF SINGLE-DOSE PREPARATIONS

The test for uniformity of content of single-dose preparations is based on the assay of the individual contents of active substance(s) of a number of single-dose units to determine whether the individual contents are within limits set with reference to the average content of the sample.

The test is not required for multivitamin and trace-element preparations and in other justified and authorised circumstances.

Method. Using a suitable analytical method, determine the individual contents of active substance(s) of 10 dosage units taken at random.

Apply the criteria of test A, test B or test C as specified in the monograph for the dosage form in question.

TEST A

Tablets, powders for parenteral use, ophthalmic inserts, suspensions for injection. The preparation complies with the test if each individual content is between 85 per cent and 115 per cent of the average content. The preparation fails to comply with the test if more than one individual content is outside these limits or if one individual content is outside the limits of 75 per cent to 125 per cent of the average content.

If one individual content is outside the limits of 85 per cent to 115 per cent but within the limits of 75 per cent to 125 per cent, determine the individual contents of another 20 dosage units taken at random. The preparation complies with the test if not more than one of the individual contents of the 30 units is outside 85 per cent to 115 per cent of the average content and none is outside the limits of 75 per cent to 125 per cent of the average content.

TEST B

Capsules, powders other than for parenteral use, granules, suppositories, pessaries. The preparation complies with the test if not more than one individual content is outside the limits of 85 per cent to 115 per cent of the average content and none is outside the limits of 75 per cent to 125 per cent of the average content. The preparation fails to comply with the test if more than 3 individual contents are outside the limits of 85 per cent to 115 per cent of the average content or if one or more individual contents are outside the limits of 75 per cent to 125 per cent of the average content.

If 2 or 3 individual contents are outside the limits of 85 per cent to 115 per cent but within the limits of 75 per cent to 125 per cent, determine the individual contents of another 20 dosage units taken at random. The preparation complies with the test if not more than 3 individual contents of the 30 units are outside the limits of 85 per cent to 115 per cent of the average content and none is outside the limits of 75 per cent to 125 per cent of the average content.

Transdermal patches. The preparation complies with the test if the average content of the 10 dosage units is between 90 per cent and 110 per cent of the content stated on the label and if the individual content of each dosage unit is between 75 per cent and 125 per cent of the average content.

01/2005:20907

2.9.7. FRIABILITY OF UNCOATED TABLETS

This test is intended to determine, under defined conditions, the friability of uncoated tablets, the phenomenon whereby tablet surfaces are damaged and/or show evidence of lamination or breakage when subjected to mechanical shock or attrition.

APPARATUS

Use a drum with an internal diameter between 283 and 291 mm and a depth between 36 mm and 40 mm, made of a transparent synthetic polymer with polished internal surfaces and not subject to static build-up (see Figure 2.9.7-1). One side of the drum is removable. The tablets are tumbled at each turn of the drum by a curved projection with an inside radius between 75.5 mm and 85.5 mm that extends from the middle of the drum to the outer wall. The drum is attached to the horizontal axis of a device that rotates at 25 ± 1 r/min. Thus, at each turn the tablets roll or slide and fall onto the drum wall or onto each other.

METHOD

For tablets weighing up to 0.65 g each, take a sample of twenty tablets; for tablets weighing more than 0.65 g each, take ten tablets. Place the tablets on a sieve no. 1000 and remove any loose dust with the aid of air pressure or a soft brush. Accurately weigh the tablet sample and place the tablets in the drum. Rotate the drum 100 times and remove the tablets. Remove any loose dust from the tablets as before. If no tablets are cracked, split or broken, weigh the tablets to the nearest milligram.

Generally the test is run once. If the results are doubtful or if the mass loss is greater than 1 per cent, repeat the test twice and determine the mean of the 3 tests. A maximum loss of 1 per cent of the mass of the tablets tested is considered to be acceptable for most products.

For tablets having a diameter of 13 mm or greater, problems of reproducibility may be encountered due to frequent irregular tumbling. In such cases, adjust the drum so that the tablets may fall freely and do not bind together when lying next to each other, adjusting the drum so that the axis forms a 10° angle with the base is usually satisfactory.

EXPRESSION OF THE RESULTS

The friability is expressed as the loss of mass and it is calculated as a percentage of the initial mass.

Indicate the number of tablets used.

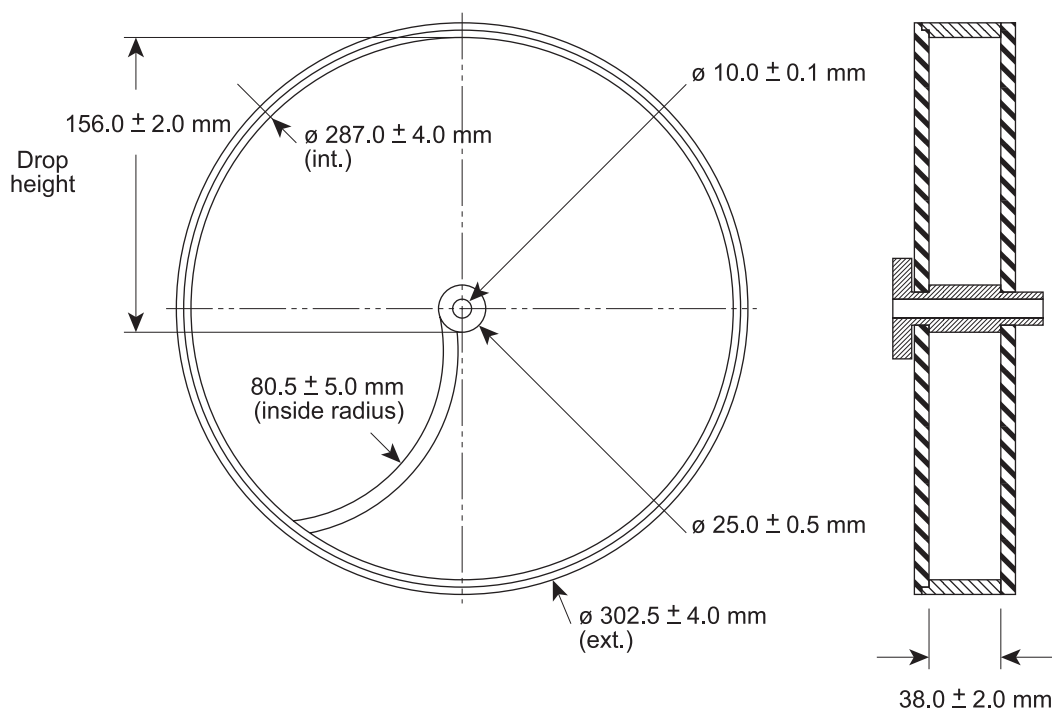


Figure 2.9.7-1. – Tablet friability apparatus

01/2005:20908 This procedure does not apply when fully automated equipment is used.

2.9.8. RESISTANCE TO CRUSHING OF TABLETS

This test is intended to determine, under defined conditions, the resistance to crushing of tablets, measured by the force needed to disrupt them by crushing.

APPARATUS

The apparatus consists of 2 jaws facing each other, one of which moves towards the other. The flat surfaces of the jaws are perpendicular to the direction of movement. The crushing surfaces of the jaws are flat and larger than the zone of contact with the tablet. The apparatus is calibrated using a system with a precision of 1 newton.

OPERATING PROCEDURE

Place the tablet between the jaws, taking into account, where applicable, the shape, the break-mark and the inscription; for each measurement orient the tablet in the same way with respect to the direction of application of the force. Carry out the measurement on 10 tablets, taking care that all fragments of tablets have been removed before each determination.

EXPRESSION OF RESULTS

Express the results as the mean, minimum and maximum values of the forces measured, all expressed in newtons.

Indicate the type of apparatus and, where applicable, the orientation of the tablets.

01/2005:20909

2.9.9. MEASUREMENT OF CONSISTENCY BY PENETROMETRY

This test is intended to measure, under determined and validated conditions, the penetration of an object into the product to be examined in a container with a specified shape and size.

APPARATUS

The apparatus consists of a penetrometer made up of a stand and a penetrating object. A suitable apparatus is shown in Figure 2.9.9-1.

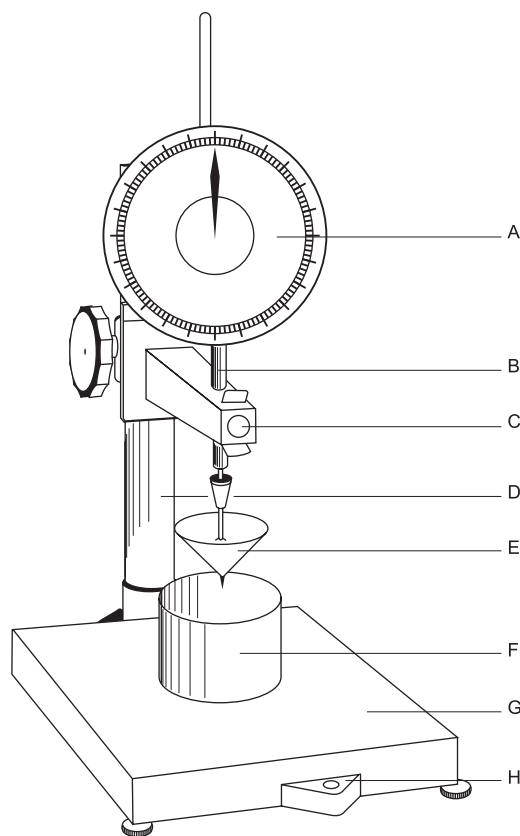


Figure 2.9.9-1. – Penetrometer

- A. Scale showing the depth of penetration, graduated in tenths of millimetres.
- B. Vertical shaft to maintain and guide the penetrating object.
- C. Device to retain and to release the penetrating object automatically and for a constant time.
- D. Device to ensure that the penetrating object is vertical and that the base is horizontal.
- E. Penetrating object (see Figures 2.9.9-2 and 3).
- F. Container.

G. Horizontal base.

H. Control for the horizontal base.

The stand is made up of:

- a vertical shaft to maintain and guide the penetrating object,
- a horizontal base,
- a device to ensure that the penetrating object is vertical,
- a device to check that the base is horizontal,
- a device to retain and release the penetrating object,
- a scale showing the depth of penetration, graduated in tenths of a millimetre.

The penetrating object, made of a suitable material, has a smooth surface, and is characterised by its shape, size and mass.

Suitable penetrating objects are shown in Figures 2.9.9-2 and 2.9.9-3.

PROCEDURE

Prepare the test samples by one of the following procedures:

- A. Carefully and completely fill three containers, without forming air bubbles. Level if necessary to obtain a flat surface. Store the samples at $25 \pm 0.5^\circ\text{C}$ for 24 h, unless otherwise prescribed.
- B. Store three samples at $25 \pm 0.5^\circ\text{C}$ for 24 h. Apply a suitable shear to the samples for 5 min. Carefully and completely fill three containers, without forming air bubbles, and level if necessary to obtain a flat surface.
- C. Melt three samples and carefully and completely fill three containers, without forming air bubbles. Store the samples at $25 \pm 0.5^\circ\text{C}$ for 24 h, unless otherwise prescribed.

Determination of penetration. Place the test sample on the base of the penetrometer. Verify that its surface is perpendicular to the vertical axis of the penetrating object. Bring the temperature of the penetrating object to $25 \pm 0.5^\circ\text{C}$ and then adjust its position such that its tip just touches the surface of the sample. Release the penetrating object and hold it free for 5 s. Clamp the penetrating object and measure the depth of penetration. Repeat the test with the two remaining containers.

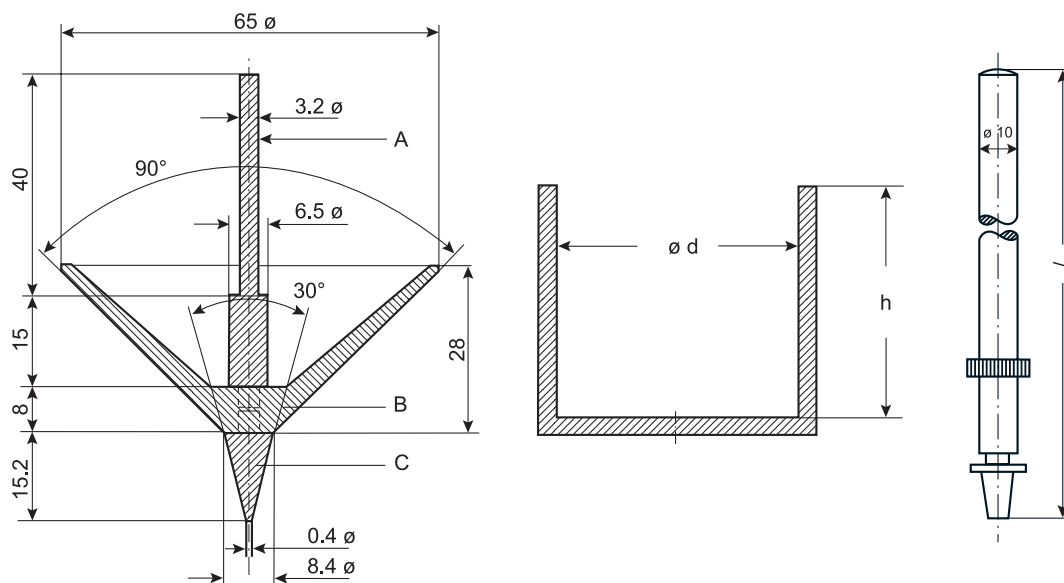


Figure 2.9.9-2. – Cone ($m = 102.5\text{ g}$), suitable container ($d = 102\text{ mm}$ or 75 mm , $h \geq 62\text{ mm}$) and shaft ($l = 162\text{ mm}$; $m = 47.5\text{ g}$).

Dimensions in millimetres

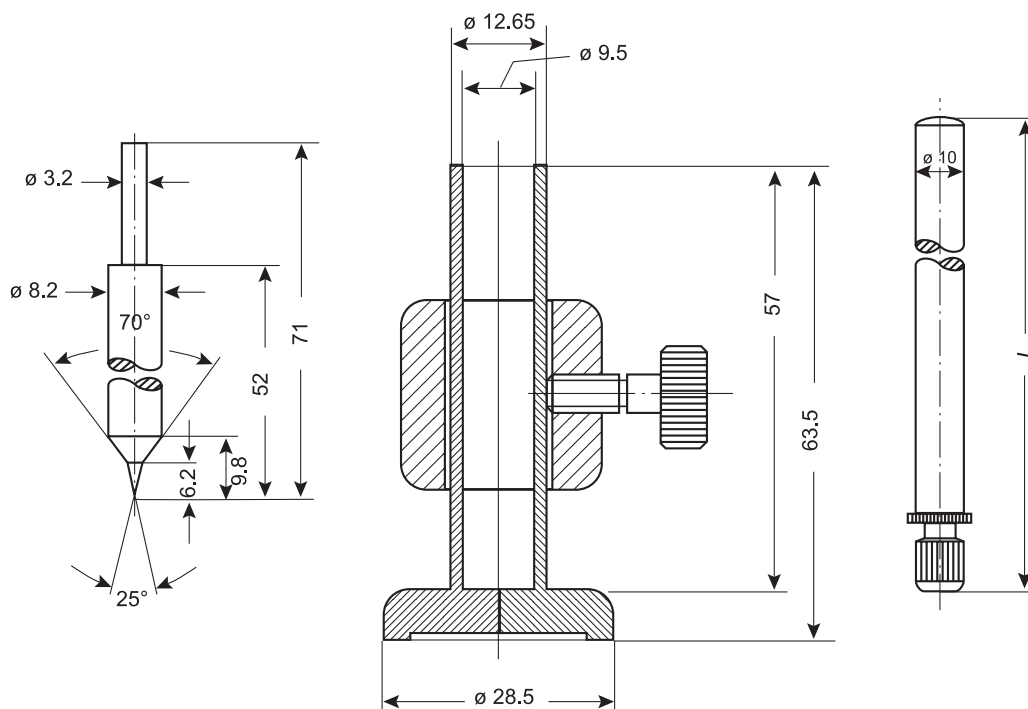


Figure 2.9.9-3 – Micro-cone ($m = 7.0$ g), suitable container and shaft ($l = 116$ mm; $m = 16.8$ g)

Dimensions in millimetres

EXPRESSION OF THE RESULTS

The penetration is expressed in tenths of a millimetre as the arithmetic mean of the three measurements. If any of the individual results differ from the mean by more than 3 per cent, repeat the test and express the results of the six measurements as the mean and the relative standard deviation.

01/2005:20910

2.9.10. ETHANOL CONTENT AND ALCOHOLIMETRIC TABLES

This method is intended only for the examination of liquid pharmaceutical preparations containing ethanol. These preparations also contain dissolved substances which must be separated from the ethanol to be determined by distillation. When distillation would distil volatile substances other than ethanol and water the appropriate precautions are stated in the monograph.

The ethanol content of a liquid is expressed as the number of volumes of ethanol contained in 100 volumes of the liquid, the volumes being measured at 20 ± 0.1 °C. This is known as the “percentage of ethanol by volume” (per cent V/V). The content may also be expressed in grams of ethanol per 100 g of the liquid. This is known as the “percentage of ethanol by mass” (per cent m/m).

The relation between the density at 20 ± 0.1 °C, the relative density (corrected to vacuum) and the ethanol content of a mixture of water and ethanol is given in the tables of the International Organisation for Legal Metrology (1972), International Recommendation No. 22.

Apparatus. The apparatus (see Figure 2.9.10-1) consists of a round-bottomed flask (A) fitted with a distillation head (B) with a steam trap and attached to a vertical condenser (C). The latter is fitted at its lower part with a tube (D) which carries the distillate into the lower part of a 100 ml or 250 ml volumetric flask. The volumetric flask is immersed in a mixture of ice and water (E) during the distillation. A disc having a circular aperture 6 cm in diameter is placed under flask (A) to reduce the risk of charring of any dissolved substances.

Method

Pycnometer method. Transfer 25.0 ml of the preparation to be examined, measured at 20 ± 0.1 °C, to the distillation flask. Dilute with 100 ml of *distilled water R* and add a few pieces of pumice. Attach the distillation head and condenser. Distil and collect not less than 90 ml of distillate in a 100 ml volumetric flask. Adjust the temperature to 20 ± 0.1 °C and dilute to 100.0 ml with *distilled water R* at 20 ± 0.1 °C. Determine the relative density at 20 ± 0.1 °C using a pycnometer.

The values indicated in Table 2.9.10-1, column 3, are multiplied by four to obtain the percentage of ethanol by volume (V/V) contained in the preparation. After calculation of the ethanol content using the Table, round off the result to one decimal place.

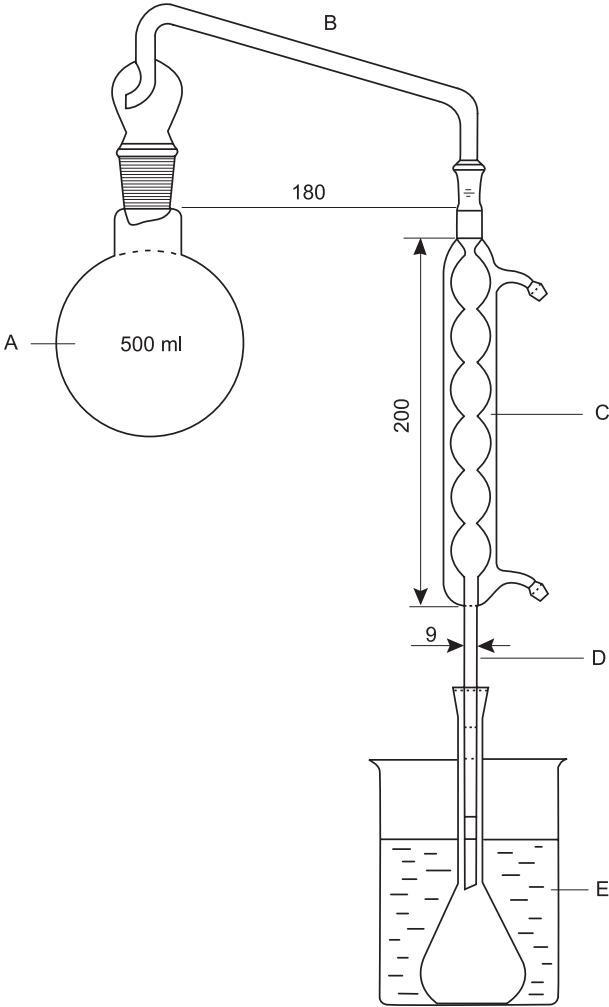


Figure 2.9.10-1. – Apparatus for the determination of ethanol content

Dimensions in millimetres

Table 2.9.10-1. - Relationship between density, relative density and ethanol content

ρ_{20} ($\text{kg}\cdot\text{m}^{-3}$)	Relative density of the distillate measured in air d_{20}^{20}	Ethanol content in per cent V/V at 20 °C
968.0	0.9697	25.09
968.5	0.9702	24.64
969.0	0.9707	24.19
969.5	0.9712	23.74
970.0	0.9717	23.29
970.5	0.9722	22.83
971.0	0.9727	22.37
971.5	0.9733	21.91
972.0	0.9738	21.45
972.5	0.9743	20.98
973.0	0.9748	20.52
973.5	0.9753	20.05
974.0	0.9758	19.59
974.5	0.9763	19.12
975.0	0.9768	18.66
975.5	0.9773	18.19

ρ_{20} ($\text{kg}\cdot\text{m}^{-3}$)	Relative density of the distillate measured in air d_{20}^{20}	Ethanol content in per cent V/V at 20 °C
976.0	0.9778	17.73
976.5	0.9783	17.25
977.0	0.9788	16.80
977.5	0.9793	16.34
978.0	0.9798	15.88
978.5	0.9803	15.43
979.0	0.9808	14.97
979.5	0.9813	14.52
980.0	0.9818	14.07
980.5	0.9823	13.63
981.0	0.9828	13.18
981.5	0.9833	12.74
982.0	0.9838	12.31
982.5	0.9843	11.87
983.0	0.9848	11.44
983.5	0.9853	11.02
984.0	0.9858	10.60
984.5	0.9863	10.18
985.0	0.9868	9.76
985.5	0.9873	9.35
986.0	0.9878	8.94
986.5	0.9883	8.53
987.0	0.9888	8.13
987.5	0.9893	7.73
988.0	0.9898	7.34
988.5	0.9903	6.95
989.0	0.9908	6.56
989.5	0.9913	6.17
990.0	0.9918	5.79
990.5	0.9923	5.42
991.0	0.9928	5.04
991.5	0.9933	4.67
992.0	0.9938	4.30
992.5	0.9943	3.94
993.0	0.9948	3.58
993.5	0.9953	3.22
994.0	0.9958	2.86
994.5	0.9963	2.51
995.0	0.9968	2.16
995.5	0.9973	1.82
996.0	0.9978	1.47
996.5	0.9983	1.13
997.0	0.9988	0.80
997.5	0.9993	0.46
998.0	0.9998	0.13

Hydrometer method. Transfer 50.0 ml of the preparation to be examined, measured at 20 ± 0.1 °C, to the distillation flask, add 200 ml to 300 ml of *distilled water R* and distil, as described above, into a volumetric flask until at least 180 ml has been collected. Adjust the temperature to 20 ± 0.1 °C and dilute to 250.0 ml with *distilled water R* at 20 ± 0.1 °C.

Transfer the distillate to a cylinder whose diameter is at least 6 mm wider than the bulb of the hydrometer. If the volume is insufficient, double the quantity of the sample and dilute the distillate to 500.0 ml with *distilled water R* at 20 ± 0.1 °C.

Multiply the strength by five to allow for the dilution during the determination. After calculation of the ethanol content using the Table 2.9.10.-1 round off the result to one decimal place.

01/2005:20911

2.9.11. TEST FOR METHANOL AND 2-PROPANOL

Examine by gas chromatography (2.2.28).

Internal standard solution. Prepare a solution containing 2.5 per cent V/V of *propanol R* in *ethanol R1*.

Test solution (a). To a certain amount of the distillate add 2.0 ml of the internal standard solution; adjust the ethanol content (2.9.10) to 10.0 per cent V/V by dilution to 50 ml with *water R* or addition of *ethanol R1*.

Test solution (b). Adjust the ethanol content (2.9.10) of a certain amount of the distillate to 10.0 per cent V/V by dilution to 50 ml with *water R* or addition of *ethanol R1*.

Reference solution (a). Prepare 50 ml of a solution containing 2.0 ml of the internal standard solution, 3.0 ml of *ethanol R1*, 0.05 per cent V/V of *2-propanol R* and sufficient *anhydrous methanol R* to give a total of 0.05 per cent V/V of methanol taking into account the methanol content of *ethanol R1*.

Reference solution (b). Prepare a 10.0 per cent V/V solution of *ethanol R1* containing 0.0025 per cent V/V of each *methanol R* and *2-propanol R*.

Column:

- **material:** fused silica,
- **size:** $l = 30$ m, $\varnothing = 0.53$ mm,
- **stationary phase:** *poly(cyanopropyl)(7)(phenyl)(7)(methyl)(86)siloxane R* (film thickness 3 µm).

Carrier gas: *helium for chromatography R*.

Flow rate: 2 ml/min.

Split ratio: 1:10.

Temperature:

	Time (min)	Temperature (°C)
Column	0 - 5	35
	5 - 15	35 - 85
Injection port		250
Detector		250

Detection: flame ionisation.

Injection: 1.0 µl.

System suitability:

- **propanol:** there is no peak corresponding to propanol in the chromatogram obtained with test solution (b),
- **peak-to-valley ratio:** minimum 15, where H_p = height above the baseline of the peak due to 2-propanol and H_v = height above the baseline of the lowest point of

the curve separating this peak from the peak due to ethanol in the chromatogram obtained with the reference solution (a),

- **signal-to-noise ratio:** minimum 10 for the peaks due to methanol and 2-propanol in the chromatogram obtained with reference solution (b).

The content of methanol and 2-propanol is calculated with reference to the original sample.

01/2005:20912

2.9.12. SIEVE TEST

The degree of fineness of a powder may be expressed by reference to sieves that comply with the specifications for non-analytical sieves (2.1.4).

Where the degree of fineness of powders is determined by sieving, it is defined in relation to the sieve number(s) used either by means of the following terms or, where such terms cannot be used, by expressing the fineness of the powder as a percentage m/m passing the sieve(s) used.

The following terms are used in the description of powders:

Coarse powder. Not less than 95 per cent by mass passes through a number 1400 sieve and not more than 40 per cent by mass passes through a number 355 sieve.

Moderately fine powder. Not less than 95 per cent by mass passes through a number 355 sieve and not more than 40 per cent by mass passes through a number 180 sieve.

Fine powder. Not less than 95 per cent by mass passes through a number 180 sieve and not more than 40 per cent by mass passes through a number 125 sieve.

Very fine powder. Not less than 95 per cent by mass passes through a number 125 sieve and not more than 40 per cent by mass passes through a number 90 sieve.

If a single sieve number is given, not less than 97 per cent of the powder passes through the sieve of that number, unless otherwise prescribed.

Assemble the sieves and operate in a suitable manner until sifting is practically complete. Weigh the separated fractions of the powder.

01/2005:20913

2.9.13. LIMIT TEST OF PARTICLE SIZE BY MICROSCOPY

Weigh a suitable quantity of the powder to be examined (for example 10 mg to 100 mg) and suspend it in 10.0 ml of a suitable medium in which the powder does not dissolve, adding, if necessary, a wetting agent. Introduce a portion of the homogeneous suspension into a suitable counting cell and scan under a microscope an area corresponding to not less than 10 µg of the powder to be examined. Count all the particles having a maximum dimension greater than the prescribed size limit. The size limit and the permitted number of particles exceeding the limit are stated in the monograph.

01/2005:20914

2.9.14. SPECIFIC SURFACE AREA BY AIR PERMEABILITY

The test is intended for the determination of the specific surface area of dry powders expressed in square metres per gram in the sub-sieve region. The effect of molecular flow ("slip flow") which may be important when testing powders

consisting of particles less than a few micrometres is not taken into account in the equation used to calculate the specific surface area.

APPARATUS

The apparatus consists of the following parts:

(a) a *permeability cell* (see Figure 2.9.14-1), which consists of a cylinder with an inner diameter of 12.6 ± 0.1 mm (A), constructed of glass or non-corroding metal. The bottom of the cell forms an airtight connection (for example, via an adapter) with the manometer (Figure 2.9.14-2). A ledge 0.5 mm to 1 mm in width is located 50 ± 15 mm from the top of the cell. It is an integral part of the cell or firmly fixed so as to be airtight. It supports a perforated metal disk (B), constructed of non-corroding metal. The disk has a thickness of 0.9 ± 0.1 mm and is perforated with thirty to forty holes 1 mm in diameter evenly distributed over this area.

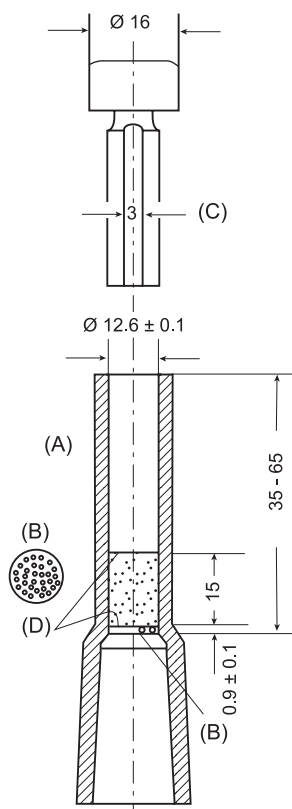


Figure 2.9.14-1. – *Permeability cell*
Dimensions in millimetres

The plunger (C) is made of non-corroding metal and fits into the cell with a clearance of not more than 0.1 mm. The bottom of the plunger has sharp square edges at right angles to the principal axis. There is an air vent 3 mm long and 0.3 mm deep on one side of the plunger. The top of the plunger has a collar such that when the plunger is placed in the cell and the collar is brought into contact with the top of the cell, the distance between the bottom of the plunger and the top of the perforated disk (B) is 15 ± 1 mm.

The filter paper disks (D) have smooth edges and the same diameter as the inside of the cell.

(b) a *U-tube manometer* (E) (Figure 2.9.14-2) is made of nominal 9 mm outer diameter and 7 mm inner diameter glass tubing with standard walls. The top of one arm of the manometer forms an airtight connection with the permeability cell (F). The manometer arm connected to the permeability cell has a line etched around the tube at 125 mm to 145 mm below the top of the side outlet and three other lines at distances of 15 mm, 70 mm and 110 mm

above that line (G). The side outlet 250 mm to 305 mm above the bottom of the manometer is used to evacuate the manometer arm connected to the permeability cell. A tap is provided on the side outlet not more than 50 mm from the manometer arm.

The manometer is mounted firmly in such a manner that the arms are vertical. It is filled to the lowest mark with *dibutyl phthalate R* containing a lipophilic dye.

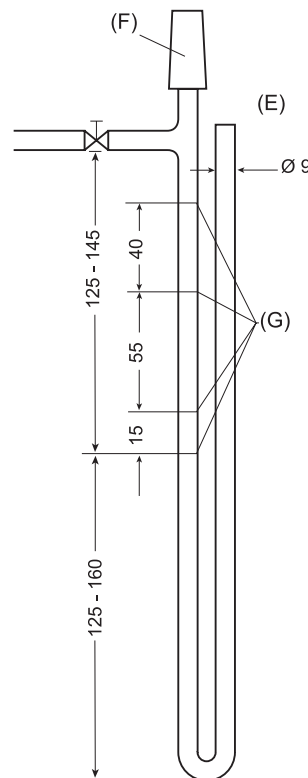


Figure 2.9.14-2. – *Manometer*
Dimensions in millimetres

METHOD

If prescribed, dry the powder to be examined and sift through a suitable sieve (for example no. 125) to disperse agglomerates. Calculate the mass (M) of the powder to be used from the following expression:

$$M = V \times \rho \times (1 - \varepsilon) \quad (1)$$

V = bulk volume of the compacted bed of powder,

ρ = density of the substance to be examined in grams per millilitre,

ε = porosity of the compacted bed of powder.

Assume first a porosity of 0.5 and introduce this value in Eq. 1 to calculate the mass (M) of the powder to be examined.

Place a filter paper disk on top of the perforated metal disk (B). Weigh the calculated mass (M) of the powder to be examined to the nearest 1 mg. Carefully transfer the powder into the cleaned, tared permeability cell and carefully tap the cell so that the surface of the powder bed is level and cover it with a second filter paper disk. Slowly compact the powder by means of the plunger, avoiding rotary movement. Maintain the pressure until the plunger is completely inserted into the permeability cell. If this is not possible, decrease the quantity of the powder used. If, on the contrary, there is not enough resistance, increase the quantity of the powder. In this case calculate the porosity again. After at least 10 s, remove the plunger.

Attach the permeability cell to the tube of the manometer by means of an airtight connection. Evacuate the air from the manometer by means of a rubber bulb until the level of the coloured liquid is at the highest mark. Close the tap and check that the apparatus is airtight by closing the upper end of the cell, for example with a rubber stopper. Remove the stopper and, using a timer, measure the time taken for the liquid to fall from the second to the third mark.

Using the measured flow time, calculate the specific surface area (S), expressed in square metres per gram, from the following expression:

$$S = \frac{K \times \sqrt{\varepsilon^3} \times \sqrt{t}}{\rho \times (1 - \varepsilon) \times \sqrt{\eta}} \quad (2)$$

- t = flow time in seconds,
 η = dynamic viscosity of air in millipascal seconds (see Table 2.9.14.-1),
 K = apparatus constant determined according to Equation (4),
 ρ = density of the substance to be examined in grams per millilitre,
 ε = porosity of the compacted bed of powder.

CALIBRATION OF THE APPARATUS

The bulk volume of the compacted bed of powder is determined by the mercury displacement method as follows:

Place two filter paper disks in the permeability cell, pressing down the edges with a rod slightly smaller than the cell diameter until the filter disks lie flat on the perforated metal disk; fill the cell with mercury, removing any air bubbles adhering to the wall of the cell and wipe away the excess to create a plane surface of mercury at the top of the cell. If the cell is made of material that will amalgamate, grease the cell and the metal disk first with a thin layer of liquid paraffin. Pour out the mercury into a tared beaker and determine the mass (M_A) and the temperature of the mercury.

Make a compacted bed using the reference powder and again fill the cell with mercury with a planar surface at the top of the cell. Pour out the mercury in a tared beaker and again determine the mass of the mercury (M_B). Calculate the bulk volume (V) of the compacted bed of powder from the following expression:

$$V = \frac{M_A - M_B}{\rho_{\text{Hg}}} \quad (3)$$

- $M_A - M_B$ = difference between the determined masses of mercury in grams,
 ρ_{Hg} = density of mercury at the determined temperature in grams per millilitre.

Repeat the procedure twice, changing the powder each time; the range of values for the calculated volume (V) is not greater than 0.01 ml. Use the mean value of the three determined volumes for the calculations.

The apparatus constant K is determined using a reference powder with known specific surface area and density as follows:

Calculate the required quantity of the reference powder to be used (Eq. 1) using the stated density and the determined volume of the compacted powder bed (Eq. 3).

Homogenise and loosen up the powder by shaking it for 2 min in a 100 ml bottle. Prepare a compacted powder bed and measure the flow time of air as previously described. Calculate the apparatus constant (K) from the following expression:

$$K = \frac{S_{\text{sp}} \times \rho \times (1 - \varepsilon) \times \sqrt{\eta}}{\sqrt{\varepsilon^3} \times \sqrt{t}} \quad (4)$$

- S_{sp} = stated specific surface area of the reference powder,
 ρ = density of the substance to be examined in grams per millilitre,
 ε = porosity of the compacted bed of powder,
 t = flow time in seconds,
 η = dynamic viscosity of air in millipascal seconds (see Table 2.9.14.-1).

The density of mercury and the viscosity of air over a range of temperatures are shown in Table 2.9.14.-1.

Table 2.9.14.-1.

Temperature (°C)	Density of mercury (g/ml)	Viscosity of air (η) (mPa·s)	$\sqrt{\eta}$
16	13.56	0.01800	0.1342
17	13.56	0.01805	0.1344
18	13.55	0.01810	0.1345
19	13.55	0.01815	0.1347
20	13.55	0.01819	0.1349
21	13.54	0.01824	0.1351
22	13.54	0.01829	0.1353
23	13.54	0.01834	0.1354
24	13.54	0.01839	0.1356

01/2005:20915

2.9.15. APPARENT VOLUME

The test for apparent volume is intended to determine under defined conditions the apparent volumes, before and after settling, the ability to settle and the apparent densities of divided solids (for example, powders, granules).

APPARATUS

The apparatus (see Figure 2.9.15.-1) consists of the following:

- a settling apparatus capable of producing in 1 min 250 ± 15 taps from a height of 3 ± 0.2 mm. The support for the graduated cylinder, with its holder, has a mass of 450 ± 5 g;
- a 250 ml graduated cylinder (2 ml intervals) with a mass of 220 ± 40 g.

METHOD

Into the dry cylinder, introduce without compacting 100.0 g (m g) of the substance to be examined. If this is not possible, select a test sample with an apparent volume between 50 ml and 250 ml and specify the mass in the expression of results. Secure the cylinder in its holder. Read the unsettled apparent volume V_0 to the nearest millilitre. Carry out 10, 500 and 1250 taps and read the corresponding volumes V_{10} , V_{500} and V_{1250} to the nearest millilitre. If the difference between V_{500} and V_{1250} is greater than 2 ml, carry out another 1250 taps.

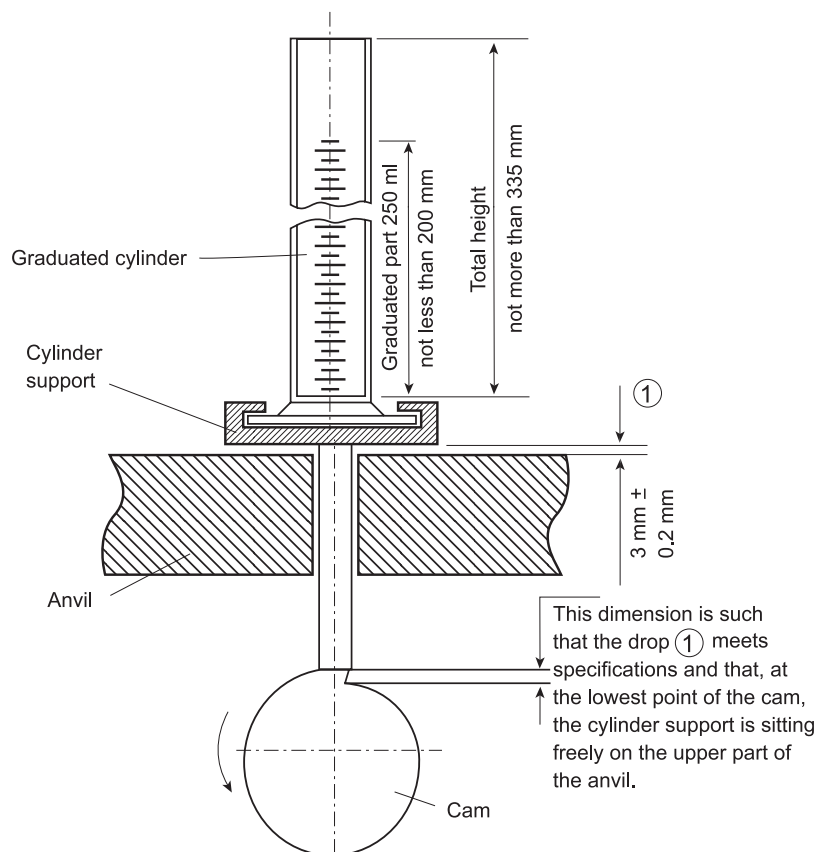


Figure 2.9.15-1

EXPRESSION OF THE RESULTS

a) Apparent volumes:

- apparent volume before settling or bulk volume: V_0 ml.
- apparent volume after settling or settled volume: V_{1250} ml or V_{2500} ml.

b) Ability to settle: difference V_{10} ml – V_{500} ml.

c) Apparent densities:

The apparent densities are expressed as follows:

- apparent density before settling or density of bulk product: m/V_0 (grams per millilitre) (poured density).
- apparent density after settling or density of settled product: m/V_{1250} or m/V_{2500} (grams per millilitre) (tapped density).

in Figures 2.9.16-1 and 2.9.16-2. The funnel is maintained upright by a suitable device. The assembly must be protected from vibrations.

METHOD

Into a dry funnel, whose bottom opening has been blocked by suitable means, introduce without compacting a test sample weighed with 0.5 per cent accuracy. The amount of the sample depends on the apparent volume and the apparatus used. Unblock the bottom opening of the funnel and measure the time needed for the entire sample to flow out of the funnel. Carry out three determinations.

EXPRESSION OF RESULTS

The flowability is expressed in seconds and tenths of seconds, related to 100 g of sample.

The results depend on the storage conditions of the material to be tested.

The results can be expressed as the following:

- the mean of the determinations, if none of the individual values deviates from the mean value by more than 10 per cent;
- as a range, if the individual values deviate from the mean value by more than 10 per cent;
- as a plot of the mass against the flow time;
- as an infinite time, if the entire sample fails to flow through.

01/2005:20916

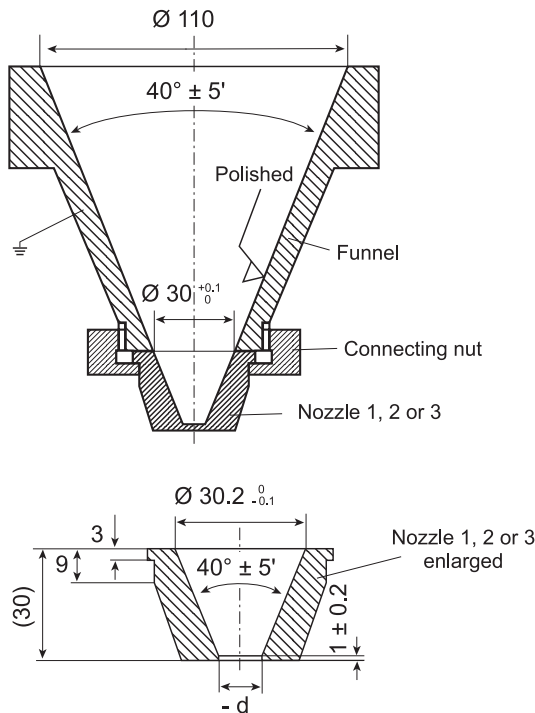
2.9.16. FLOWABILITY

The test for flowability is intended to determine the ability of divided solids (for example, powders and granules) to flow vertically under defined conditions.

APPARATUS

According to the flow properties of the material to be tested, funnels with or without stem, with different angles and orifice diameters are used. Typical apparatuses are shown

01/2005:20917



Nozzle	Diameter (d) of the outflow opening (millimetres)
1	10 ± 0.01
2	15 ± 0.01
3	25 ± 0.01

Figure 2.9.16-1. — Flow funnel and nozzle. Nozzle is made of stainless, acid-resistant steel (V4A, CrNi)

Dimensions in millimetres

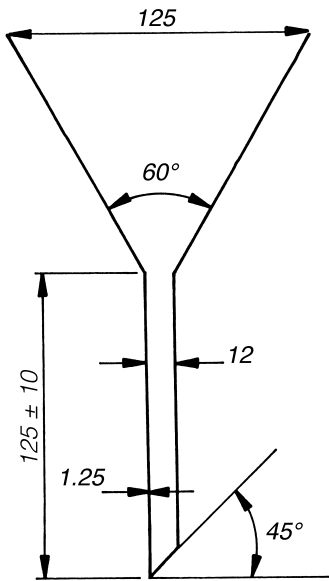


Figure 2.9.16-2

Dimensions in millimetres

2.9.17. TEST FOR EXTRACTABLE VOLUME OF PARENTERAL PREPARATIONS

Injections may be supplied in single-dose containers such as ampoules, cartridges or prefilled syringes filled with a volume of injection which is sufficient to permit administration of the nominal volume declared on the label.

Compliance with the requirements for extractable volume is assured by filling with a volume in slight excess of the nominal volume to be withdrawn. The excess volume is determined by the characteristics of the product. The single-dose container does not hold a quantity relative to the nominal volume that would present a risk should the whole contents be administered.

Suspensions and emulsions are shaken before withdrawal of the contents and before the determination of the density. Oily and viscous preparations may be warmed according to the instructions on the label, if necessary, and thoroughly shaken immediately before removing the contents. The contents are then cooled to 25 °C before measuring the volume.

SINGLE-DOSE CONTAINERS

Select one container if the nominal volume is 10 ml or more, 3 containers if the nominal volume is more than 3 ml and less than 10 ml, or 5 containers if the nominal volume is 3 ml or less. Take up individually the total contents of each container selected into a dry hypodermic syringe of a capacity not exceeding 3 times the volume to be measured, and fitted with a 21-gauge needle not less than 2.5 cm in length. Expel any air bubbles from the syringe and needle, then discharge the contents of the syringe without emptying the needle into a standardised dry cylinder (graduated to contain rather than to deliver the designated volumes) of such size that the volume to be measured occupies at least 40 per cent of its graduated volume. Alternatively, the volume of the contents in millilitres may be calculated as the mass in grams divided by the density.

The contents of 2 or 3 containers with a nominal volume of 2 ml or less may be pooled for the measurement provided that a separate, dry syringe assembly is used for each container. The contents of containers holding 10 ml or more may be determined by opening them and emptying the contents directly into the graduated cylinder or tared beaker.

The volume is not less than the nominal volume in the case of containers examined individually, or, in the case of containers with a nominal volume of 2 ml or less, is not less than the sum of the nominal volumes of the containers taken collectively.

MULTI-DOSE CONTAINERS

For injections in multidose containers labelled to yield a specific number of doses of a stated volume, select one container and proceed as directed for single-dose containers using the same number of separate syringe assemblies as the number of doses specified.

The volume is such that each syringe delivers not less than the stated dose.

CARTRIDGES AND PREFILLED SYRINGES

Select one container if the nominal volume is 10 ml or more, 3 containers if the nominal volume is more than 3 ml and less than 10 ml, or 5 containers if the nominal volume is 3 ml or less. If necessary, fit the containers with the accessories

required for their use (needle, piston, syringe) and transfer the entire contents of each container without emptying the needle into a dry tared beaker by slowly and constantly depressing the piston. Determine the volume in millilitres calculated as the mass in grams divided by the density. The volume measured for each of the containers is not less than the nominal volume.

PARENTERAL INFUSIONS

Select one container. Transfer the contents into a dry measuring cylinder of such a capacity that the volume to be determined occupies at least 40 per cent of the nominal volume of the cylinder. Measure the volume transferred. The volume is not less than the nominal volume.

01/2005:20918

2.9.18. PREPARATIONS FOR INHALATION: AERODYNAMIC ASSESSMENT OF FINE PARTICLES

This test is used to determine the fine particle characteristics of the aerosol clouds generated by preparations for inhalation. Unless otherwise justified and authorised, one of the following apparatus and test procedures is used.

APPARATUS A - GLASS IMPINGER

The apparatus is shown in Figure 2.9.18-1 (see also Table 2.9.18-1).

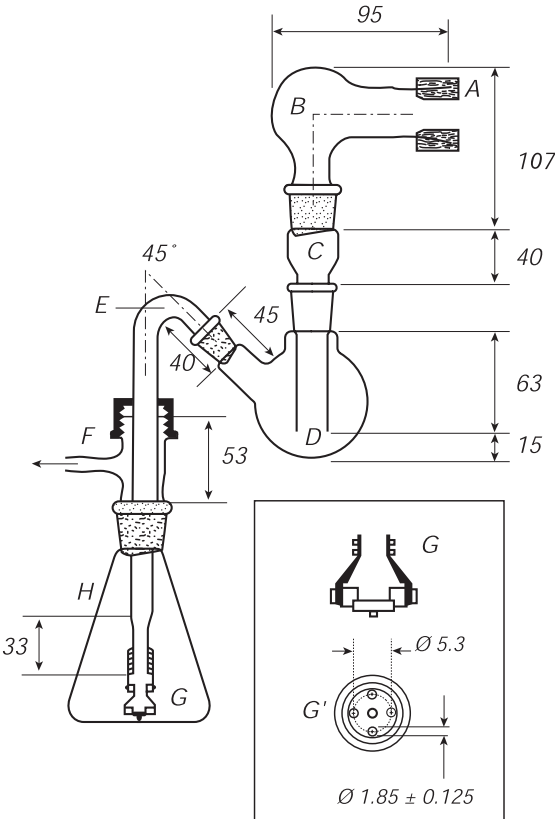


Figure 2.9.18-1. – Apparatus A for the aerodynamic assessment of fine particles
Dimensions in millimetres
(tolerances ± 1 mm unless otherwise prescribed)

Table 2.9.18-1. – Component specification for Figure 2.9.18-1

Code	Item	Description	Dimensions*
A	Mouthpiece adaptor	Moulded rubber adapter for actuator mouthpiece	
B	Throat	Modified round-bottomed flask <i>ground-glass inlet socket</i> <i>ground-glass outlet cone</i>	50 ml 29/32 24/29
C	Neck	Modified glass adapter <i>ground-glass inlet socket</i> <i>ground-glass outlet cone</i> Lower outlet section of precision-bore glass tubing <i>bore diameter</i> Selected bore light-wall glass tubing <i>external diameter</i>	24/29 24/29 14 17
D	Upper impingement chamber	Modified round-bottomed flask <i>ground-glass inlet socket</i> <i>ground-glass outlet cone</i>	100 ml 24/29 24/29
E	Coupling tube	Medium-wall glass tubing <i>ground-glass cone</i> Bent section and upper vertical section <i>external diameter</i> Lower vertical section <i>external diameter</i>	14/23 13 8
F	Screwthread, side-arm adaptor	Plastic screw cap Silicone rubber ring PTFE washer Glass screwthread, <i>thread size</i> Side-arm outlet to vacuum pump, <i>minimum bore diameter</i>	28/13 28/11 28/11 28 5
G	Lower jet assembly	Modified polypropylene filter holder connected to lower vertical section of coupling tube by PTFE tubing Acetal circular disc with the centres of four jets arranged on a projected circle of diameter 5.3 mm with an integral jet spacer peg <i>peg diameter</i> <i>peg protrusion</i>	see Figure 2.9.18-1 10 2 2
H	Lower impingement chamber	Conical flask <i>ground-glass inlet socket</i>	250 ml 24/29

* Dimensions in millimetres, unless otherwise stated.

Procedure for nebulisers

Introduce 7 ml and 30 ml of a suitable solvent into the upper and lower impingement chambers, respectively. Connect all the component parts, ensure that the assembly is vertical and adequately supported and that the jet spacer peg of the lower jet assembly just touches the bottom of the lower impingement chamber. Connect a suitable pump fitted with a filter (of suitable pore size) to the outlet of the apparatus and adjust the air flow through the apparatus, as measured at the inlet to the throat, to 60 ± 5 litres/min. Introduce the liquid preparation for inhalation into the reservoir of the nebuliser. Fit the mouthpiece and connect it by means of an adapter to the device. Switch on the pump of the apparatus and after 10 s switch on the nebuliser. After 60 s, unless otherwise justified, switch off the nebuliser, wait for about 5 s and then switch off the pump of the apparatus. Dismantle the apparatus and wash the inner surface of the upper impingement chamber collecting the washings in a volumetric flask. Wash the inner surface of

the lower impingement chamber collecting the washings in a second volumetric flask. Finally, wash the filter preceding the pump and its connections to the lower impingement chamber and combine the washings with those obtained from the lower impingement chamber. Determine the amount of active ingredient collected in each of the two flasks. Express the results for each of the two parts of the apparatus as a percentage of the total amount of active ingredient.

Procedure for pressurised inhalers

Place the actuator adapter in position at the end of the throat so that the mouthpiece end of the actuator, when inserted to a depth of about 10 mm, lines up along the horizontal axis of the throat and the open end of the actuator, which accepts the pressurised container, is uppermost and in the same vertical plane as the rest of the apparatus.

Introduce 7 ml and 30 ml of a suitable solvent into the upper and lower impingement chambers, respectively.

Connect all the component parts and ensure that the assembly is vertical and adequately supported and that the lower jet-spacer peg of the lower jet assembly just touches the bottom of the lower impingement chamber. Connect a suitable pump to the outlet of the apparatus and adjust the air flow through the apparatus, as measured at the inlet to the throat, to 60 ± 5 litres/min.

Prime the metering valve by shaking for 5 s and discharging once to waste; after not less than 5 s, shake and discharge again to waste. Repeat a further three times.

Shake for about 5 s, switch on the pump to the apparatus and locate the mouthpiece end of the actuator in the adapter, discharge once immediately. Remove the assembled inhaler from the adapter, shake for not less than 5 s, relocate the mouthpiece end of the actuator in the adapter and discharge again. Repeat the discharge sequence for a further eight times, shaking between actuations. After discharging the tenth delivery, wait for not less than 5 s and then switch off the pump. Dismantle the apparatus.

Wash the inner surface of the inlet tube to the lower impingement chamber and its outer surface that projects into the chamber with a suitable solvent collecting the washings in the lower impingement chamber. Determine the content of active ingredient in this solution. Calculate the amount of active ingredient collected in the lower impingement chamber per actuation of the valve and express the results as a percentage of the dose stated on the label.

Procedure for powder inhalers

Introduce 7 ml and 30 ml of a suitable solvent into the upper and lower impingement chambers, respectively.

Connect all the component parts and ensure that the assembly is vertical and adequately supported and that the jet spacer peg of the lower jet assembly just touches the bottom of the lower impingement chamber. Without the inhaler in place, connect a suitable pump to the outlet of the apparatus and adjust the air flow through the apparatus, as measured at the inlet to the throat, to 60 ± 5 litres/min.

Prepare the inhaler for use and locate the mouthpiece in the apparatus by means of a suitable adapter. Switch on the pump for 5 s. Switch off the pump and remove the inhaler. Repeat for a further nine discharges. Dismantle the apparatus.

Wash the inner surface of the inlet tube to the lower impingement chamber and its outer surface that projects into the chamber with a suitable solvent, collecting the washings in the lower impingement chamber. Determine the content of active ingredient in this solution. Calculate

the amount of active ingredient collected in the lower impingement chamber per discharge and express the results as a percentage of the dose stated on the label.

APPARATUS B - METAL IMPINGER

The apparatus is shown in Figures 2.9.18-2/3.

Procedure for nebulisers

The deposition of emitted droplets is tested using the impingement apparatus, which is connected to the filled nebuliser by means of a suitable adapter. The outlet of the apparatus to the pump is fitted with a suitable filter (for example pores of $0.25 \mu\text{m}$).

In this procedure, the impingement chamber is used dry. Connect all the component parts and ensure that the base of the impinger is placed on a flat, horizontal and adequately supported surface. Connect a suitable pump to the outlet of the apparatus and adjust the air flow through the apparatus, as measured at the inlet to the throat, to 60 ± 5 litres/min.

Introduce the liquid preparation for inhalation into the reservoir of a suitable nebuliser. Fit the mouth piece and connect it to the adapter of the device.

Switch on the pump of the apparatus and after 10 s, switch on the nebuliser.

After 60 s, unless otherwise justified, switch off the nebuliser, wait for about 5 s and then switch off the pump of the apparatus. Dismantle the apparatus. Wash the inner surface of the throat, the top and bottom portions of the chamber collecting the washings in a volumetric flask. Wash the filter assembly by passing a suitable solvent through the filter and collecting in a suitable graduated flask. Determine the content of active ingredient in these solutions. Express the results for each of the two parts of the apparatus as a percentage of the total amount of active ingredient.

Procedure for pressurised inhalers

Place the actuator adapter in position at the end of the throat so that the mouthpiece end of the actuator, when inserted, lines up along the horizontal axis of the throat and the open end of the actuator, which accepts the pressurised container, is uppermost and in the same vertical plane as the rest of the apparatus.

In this procedure, the impingement chamber is used dry. Connect all the component parts and ensure that the base of the impinger is placed on a flat, horizontal and adequately supported surface so that the open end of the actuator, which accepts the pressurised container is in a vertical position. Connect a suitable pump to the outlet of the apparatus and adjust the air flow through the apparatus, as measured at the inlet to the throat, to 60 ± 5 litres/min.

Prime the metering valve by shaking for 5 s and discharging once to waste; after not less than 5 s, shake and discharge again to waste. Repeat a further three times.

Shake for about 5 s, switch on the pump to the apparatus and locate the mouthpiece end of the actuator in the adapter and discharge once immediately. Remove the assembled inhaler from the adapter, shake for about 5 s, relocate the mouthpiece end of the actuator in the adapter and discharge again.

Repeat the discharge sequence for a further eight times shaking between actuations. After discharging the tenth delivery, wait for about 5 s and then switch off the pump. Dismantle the apparatus.

Wash the filter assembly by passing a suitable solvent through the filter. Determine the content of active ingredient in this solution. Calculate the amount of active ingredient

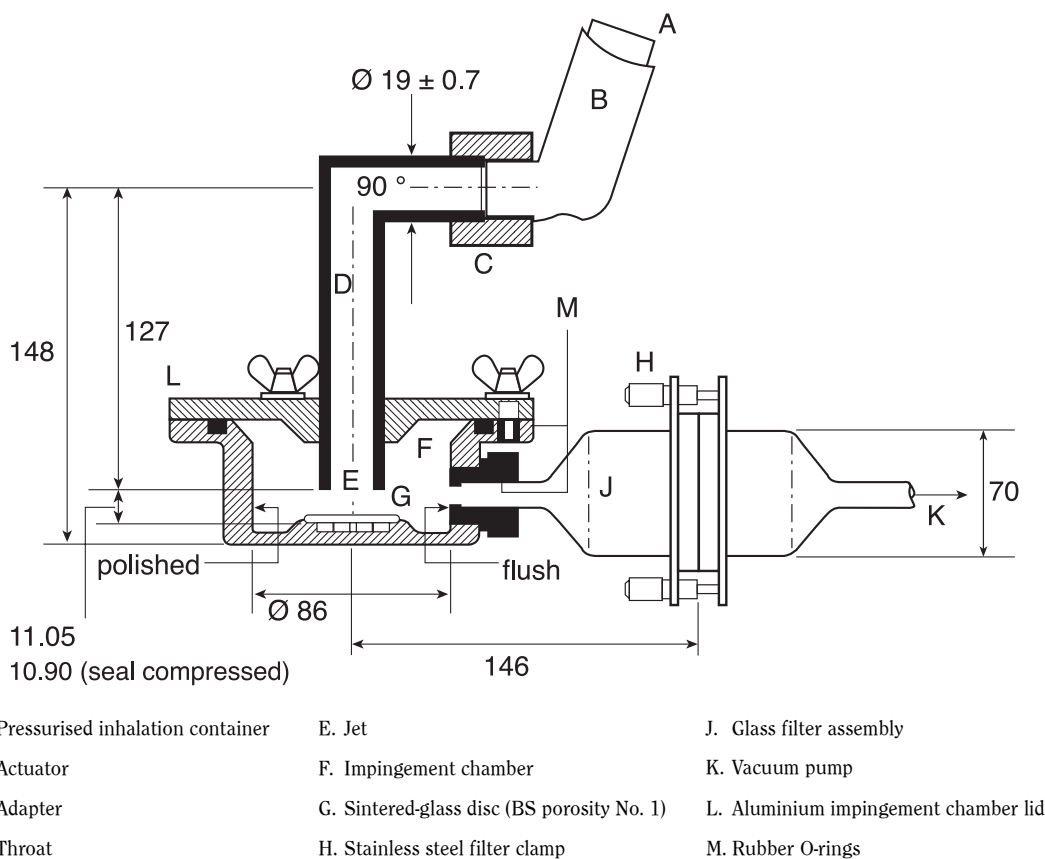


Figure 2.9.18-2. – Apparatus B for the aerodynamic assessment of fine particles
 Dimensions in millimetres

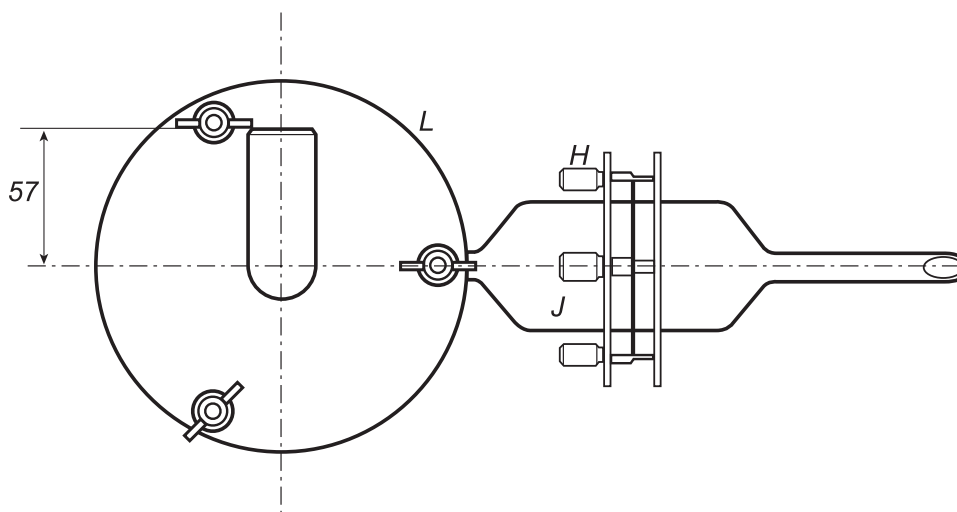


Figure 2.9.18-3. – Apparatus B for the aerodynamic assessment of fine particles (top elevation)
 Dimensions in millimetres

collected on the filter assembly per actuation of the valve and express the results as a percentage of the dose stated on the label.

Procedure for powder inhalers

Introduce 25 ml amounts of a suitable solvent into the impingement chamber so as to cover the sintered-glass disk. Connect all the component parts and ensure that the base of the impinger is placed on a flat, horizontal and adequately supported surface. Without the inhaler in place, connect a suitable pump to the outlet of the apparatus and adjust the air flow through the apparatus, as measured at the inlet to the throat, to 60 ± 5 litres/min.

Prepare the inhaler for use and locate the mouthpiece in the apparatus by means of a suitable adapter. Switch on the pump for 5 s. Switch off the pump and remove the inhaler. Repeat the discharge for a further nine discharges. Dismantle the apparatus.

Wash the filter assembly by passing a suitable solvent through the filter. Determine the content of active ingredient in this solution. Calculate the amount of active ingredient collected on the filter assembly per discharge and express the results as a percentage of the dose stated on the label.

Fine particle dose and particle size distribution

APPARATUS C – MULTI-STAGE LIQUID IMPINGER

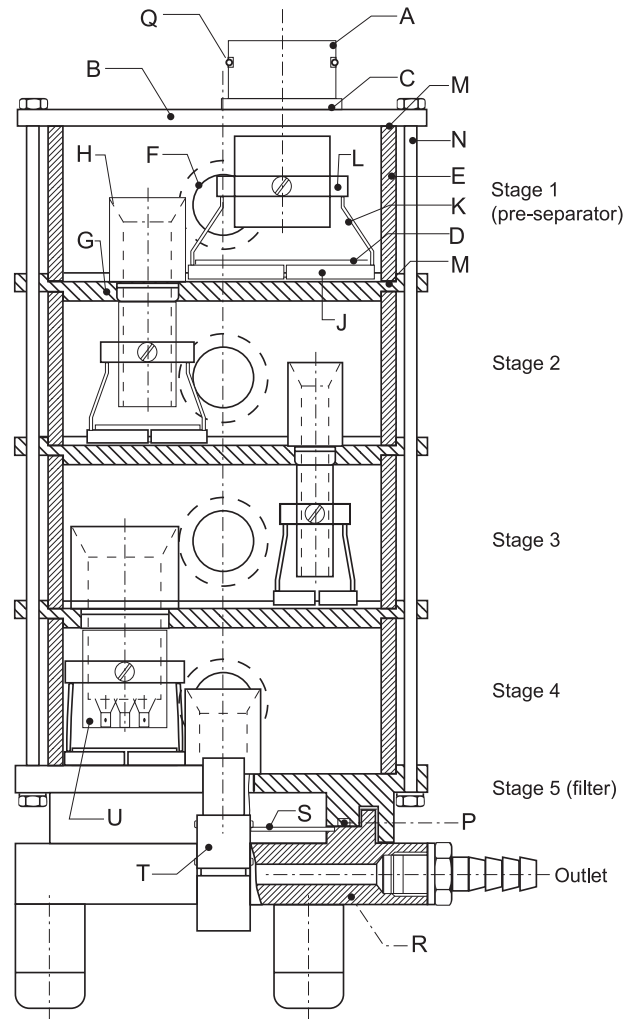


Figure 2.9.18-4. – *The Multi-stage Liquid Impinger*

Table 2.9.18-2. – *Component specification for
Figures 2.9.18-4 to -6*

Code*	Item	Description	Dimensions**
A,H	Jet tube	Metal tube screwed onto partition wall sealed by gasket (C), polished inner surface	see Figure 2.9.18-5
B,G	Partition wall	Circular metal plate - thickness - diameter - thickness	120 see Figure 2.9.18-5
C	Gasket	e.g. PTFE	to fit jet tube
D	Impaction plate	Porosity 0 sintered-glass disk, diameter	see Figure 2.9.18-5
E	Glass cylinder	Plane polished cut glass tube - height, including gaskets - outer diameter - wall thickness - sampling port (F) diameter - stopper in sampling port	46 100 3.5 18 ISO 24/25

Code*	Item	Description	Dimensions**
J	Metal frame	L-profiled circular frame with slit - inner diameter - height - thickness of horizontal section - thickness of vertical section	to fit impaction plate 4 0.5 2
K	Wire	Steel wire interconnecting metal frame and sleeve (two for each frame) - diameter	1
L	Sleeve	Metal sleeve secured on jet tube by screw - inner diameter - height - thickness	to fit jet tube 6 5
M	Gasket	e.g. silicone	to fit glass cylinder
N	Bolt	Metal bolt with nut (six pairs) - length - diameter	205 4
P	O-ring	Rubber O-ring, diameter × thickness	66.34 × 2.62
Q	O-ring	Rubber O-ring, diameter × thickness	29.1 × 1.6
R	Filter holder	Metal housing with stand and outlet	see Figure 2.9.18-6
S	Filter support	Perforated sheet metal - diameter - hole diameter - distance between holes (centre-points)	65 3 4
T	Snap-locks		
U	Multi-jet tube	Jet tube (H) ending in multi-jet arrangement	see inserts Figure 2.9.18-5

* Refers to Figure 2.9.18-4.

** Measures in mm with tolerances according to ISO 2768-m unless otherwise stated.

The Multi-stage Liquid Impinger consists of impaction stages 1 (pre-separator), 2, 3 and 4 and an integral filter stage (stage 5), see Figures 2.9.18-4 to -6. An impaction stage comprises an upper horizontal metal partition wall (B) through which a metal inlet jet tube (A) with its impaction plate (D) is protruding, a glass cylinder (E) with sampling port (F) forming the vertical wall of the stage, and a lower horizontal metal partition wall (G) through which the tube (H) connects to the next lower stage. The tube into stage 4 (U) ends in a multi-jet arrangement. The impaction plate (D) is secured in a metal frame (J) which is fastened by two wires (K) to a sleeve (L) secured on the jet tube (C). The horizontal face of the collection plate is perpendicular to the axis of the jet tube and centrally aligned. The upper surface of the impaction plate is slightly raised above the edge of the metal frame. A recess around the perimeter of the horizontal partition wall guides the position of the glass cylinder. The glass cylinders are sealed against the horizontal partition walls with gaskets (M) and clamped together by six bolts (N). The sampling ports are sealed by stoppers. The bottom-side of the lower partition wall of stage 4 has a concentric protrusion fitted with a rubber O-ring (P) which seals against the edge of a filter placed in the filter holder. The filter holder (R) is constructed as a basin with a concentric recess in which a perforated filter support (S) is flush-fitted. The filter holder is dimensioned for 76 mm diameter filters.

The assembly of impaction stages is clamped onto the filter holder by two snap-locks (T). Connect a right-angle bend metal tube induction port according to Figure 2.9.18-7 onto the stage 1 inlet jet tube of the impinger. A rubber O-ring on the jet tube provides an airtight connection to the induction port. A suitable mouthpiece adapter should be used to provide an airtight seal between the inhaler and the induction port. The front face of the inhaler mouthpiece must be flush with the front face of the induction port.

Table 2.9.18-3. – *Dimensions⁽¹⁾ of jet tube with impaction plate*

Type	Code ⁽²⁾	Stage 1	Stage 2	Stage 3	Stage 4	Filter (stage 5)
Distance	1	9.5 (-0+5)	5.5 (-0+5)	4.0 (-0+5)	6.0 (-0+5)	n.a.
Distance	2	26	31	33	30.5	0
Distance	3	8	5	5	5	5
Distance	4	3	3	3	3	n.a.
Distance	5	0	3	3	3	3
Distance	6 ⁽³⁾	20	25	25	25	25
Distance	7	n.a.	n.a.	n.a.	8.5	n.a.
Diameter	c	25	14	8.0(±.1)	21	14
Diameter	d	50	30	20	30	n.a.
Diameter	e	27.9	16.5	10.5	23.9	n.a.
Diameter	f	31.75 (-0+5)	22	14	31	22
Diameter	g	25.4	21	13	30	21
Diameter	h	n.a.	n.a.	n.a.	2.70 (±.5)	n.a.
Diameter	j	n.a.	n.a.	n.a.	6.3	n.a.
Diameter	k	n.a.	n.a.	n.a.	12.6	n.a.
Radius ⁽⁴⁾	r	16	22	27	28.5	0
Radius	s	46	46	46	46	n.a.
Radius	t	n.a.	50	50	50	50

Type	Code ⁽²⁾	Stage 1	Stage 2	Stage 3	Stage 4	Filter (stage 5)
Angle	w	10°	53°	53°	53°	53°
Angle	u	n.a.	n.a.	n.a.	45°	n.a.
Angle	v	n.a.	n.a.	n.a.	60°	n.a.

(1) Measures in mm with tolerances according to ISO 2768-m unless otherwise stated

(2) Refer to Figure 2.9.18-5

(3) Including gasket

(4) Relative centreline of stage compartment

n.a. = not applicable

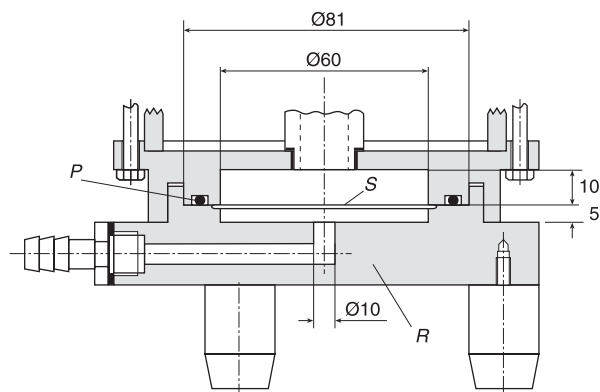


Figure 2.9.18-6. – *Details of the filter stage (stage 5). Numbers refer to dimensions (Ø = diameter). Uppercase letters refer to Table 2.9.18-2.*

Procedure for pressurised metered-dose preparations for inhalation. Dispense 20 ml of a solvent, capable of dissolving the active ingredient into each of stages 1 to 4 and replace the stoppers. Tilt the apparatus to wet the stoppers, thereby neutralising electrostatic charge. Place a suitable filter capable of quantitatively collecting the active ingredient in stage 5 and assemble the apparatus. Place a suitable mouthpiece adapter in position at the end of the induction

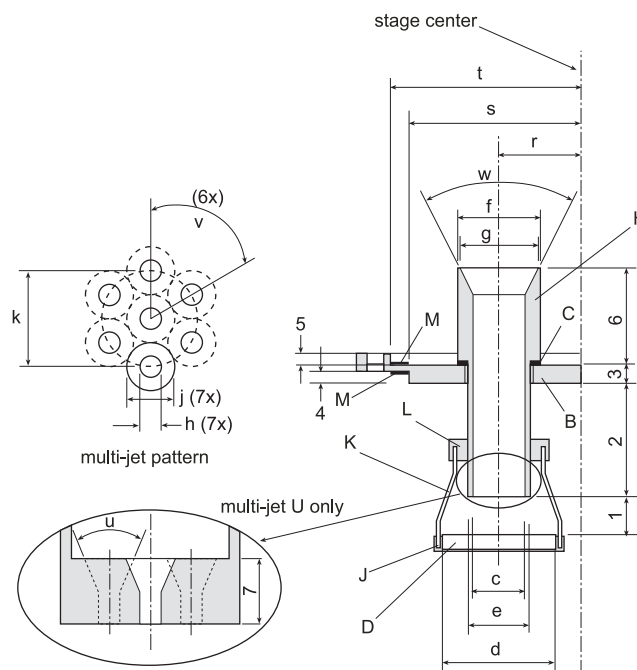
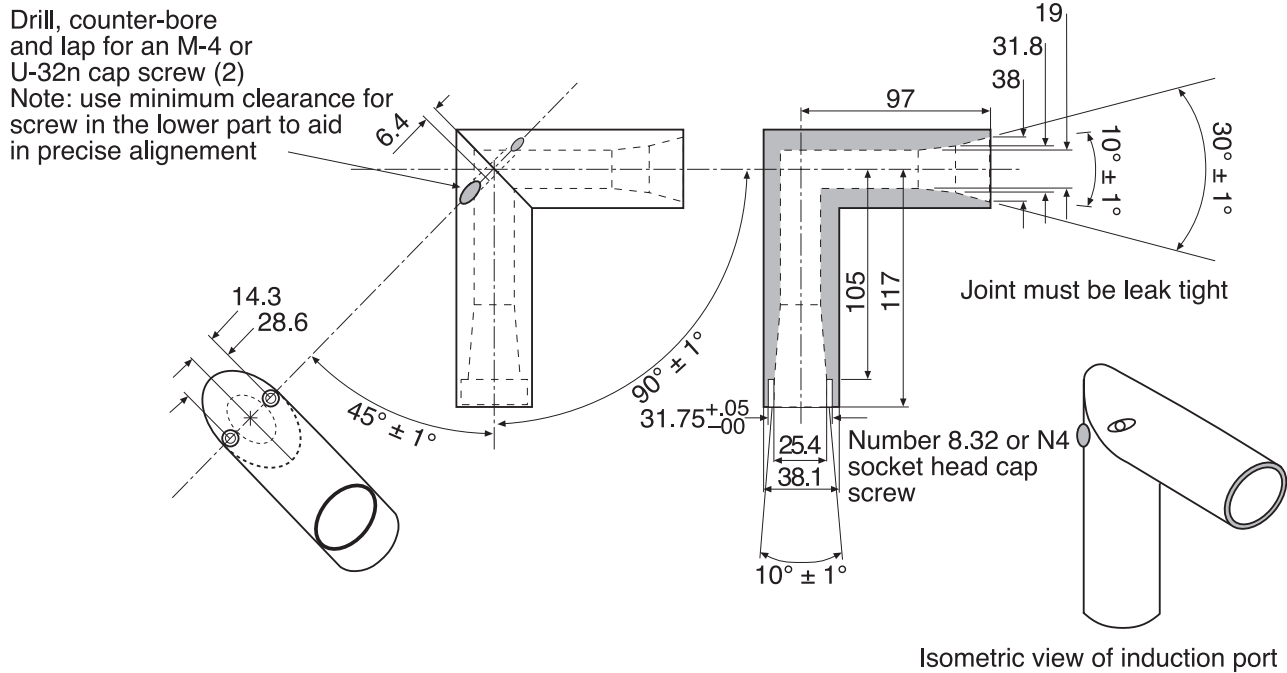


Figure 2.9.18-5. – *Details of jet tube and impaction plate. Inserts show end of multi-jet tube U leading to stage 4 (numbers and lowercase letters refer to Table 2.9.18-3 and uppercase letters refer to Figure 2.9.18-4).*



- Note
- (1) Material may be aluminium or stainless steel.
 - (2) Machine from 38 mm (1.5") bar stock.
 - (3) Bore 19 mm hole through bar.
 - (4) Cut tube to exact 45° as shown.
 - (5) The inner bores and tapers should be smooth – approximate 0.40 µm (16 µm) finish.
 - (6) Mill joining cads of stock to provide a liquid tight leak-free seal.
 - (7) Set up a holding fixture for aligning the inner 19 mm bore and for drilling and tapping M4 × 0.7 or 8-32 threads. There must be virtually no mismatch of the inner bores in the miter joint.

Figure 2.9.18-7. – Induction port
Dimensions in millimetres unless otherwise stated

port so that the mouthpiece end of the actuator, when inserted, lines up along the horizontal axis of the induction port and the inhaler is positioned in the same orientation intended for use. Connect a suitable vacuum pump to the outlet of the apparatus and adjust the air flow through the apparatus, as measured at the inlet to the induction port, to 30 ± 1.5 litres/min. Switch off the air flow.

Unless otherwise prescribed in the patient instructions, shake the inhaler for 5 s and discharge one delivery to waste. Switch on the pump to the apparatus, locate the mouthpiece end of the actuator in the adapter and discharge the inhaler into the apparatus, depressing the valve for a sufficient time to ensure complete discharge. Remove the assembled inhaler from the adapter. Repeat the procedure. The number of discharges should be minimised and typically would not be greater than ten. The number of discharges should be sufficient to ensure an accurate and precise determination of the fine particle dose. After the final discharge, wait for 5 s and then switch off the pump.

Dismantle the filter stage of the apparatus. Carefully remove the filter and extract the active ingredient into an aliquot of the solvent. Remove the induction port and mouthpiece adapter from the apparatus and extract the active ingredient into an aliquot of the solvent. If necessary, rinse the inside of the inlet jet tube to stage 1 with solvent, allowing the solvent to flow into the stage. Extract the active ingredient from the inner walls and the collection plate of each of the four upper

stages of the apparatus into the solution in the respective stage by carefully tilting and rotating the apparatus, observing that no liquid transfer occurs between the stages. Using a suitable method of analysis, determine the quantity of active ingredient contained in each of the six volumes of solvent.

Calculate the fine particle dose (see below).

Procedure for powder inhalers. Place a suitable low resistance filter capable of quantitatively collecting the drug in stage 5 and assemble the apparatus. Connect the apparatus to a flow system according to the scheme specified in Figure 2.9.18-8. Unless otherwise defined, conduct the test at the flow rate, Q, used in the test for uniformity of delivered dose, drawing 4 litres of air through the apparatus.

Table 2.9.18-4. – Component specification for Figure 2.9.18-8

Code	Item	Description
A	Connector	ID ≥ 8 mm, e.g., short metal coupling, with low-diameter branch to P3.
B	Vacuum tubing	8 ± 0.5 mm ID × 50 ± 10 cm length, e.g., silicon tubing with an OD of 14 mm and an ID of 8 mm.
C	Two-way solenoid valve	Minimum airflow resistance orifice having an internal diameter of ≥ 8 mm and a maximum response time 100 milliseconds (e.g. type 256-A08, Bürkert GmbH, D-74653 Ingelfingen), or equivalent.

Code	Item	Description
D	Vacuum pump	Pump must be capable of drawing the required flow rate through the assembled apparatus with the dry powder inhaler in the mouthpiece adapter (e.g. product type 1023, 1423 or 2565, Gast Manufacturing Inc., Benton Harbor, MI 49022), or equivalent. Connect the pump to the solenoid valve using short and/or wide (≥ 10 mm ID) vacuum tubing and connectors to minimise pump capacity requirements.
E	Timer	Timer capable to drive the solenoid valve for the required duration (e.g. type G814, RS Components International, Corby, NN17 9RS, UK), or equivalent.
P2 P3	Pressure measurements	Determine under steady-state flow condition with an absolute pressure transducer.
F	Flow control valve	Adjustable regulating valve with maximum $C_v \geq 1$, (e.g. type 8FV12LNSS, Parker Hannifin plc., Barnstaple, EX31 1NP, UK), or equivalent.

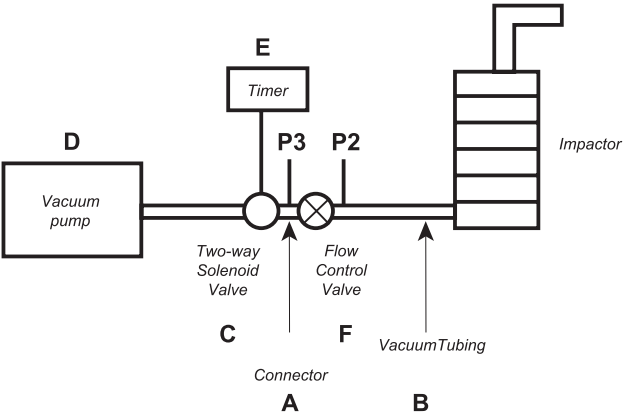


Figure 2.9.18-8. – Experimental set-up for testing powders for inhalation

Connect a flow meter, calibrated for the volumetric flow leaving the meter, to the induction port. Adjust the flow control valve to achieve steady flow through the system at the required rate, Q (± 5 per cent). Switch off the flow.

Ensure that critical flow occurs in the flow control valve by the following procedure. With the inhaler in place and the test flow rate established, measure the absolute pressure on both sides of the control valve (pressure reading points P2 and P3 in Figure 2.9.18-8). A ratio $P3/P2 \leq 0.5$ indicates critical flow. Switch to a more powerful pump and re-measure the test flow rate if critical flow is not indicated.

Dispense 20 ml of a solvent, capable of dissolving the active ingredient into each of the four upper stages of the apparatus and replace the stoppers. Tilt the apparatus to wet the stoppers, thereby neutralising electrostatic charge. Place a suitable mouthpiece adapter in position at the end of the induction port.

Prepare the dry-powder inhaler for use according to patient instructions. With the pump running and the two-way valve closed, locate the mouthpiece of the inhaler in the mouthpiece adapter. Discharge the powder into the apparatus by opening the valve for the required time, T (± 5 per cent). Repeat the procedure. The number of discharges should be minimised and typically would not be greater than ten. The number of discharges should be sufficient to ensure an accurate and precise determination of fine particle dose. After the final discharge, wait for 5 s and then switch off the pump.

Dismantle the filter stage of the apparatus. Carefully remove the filter and extract the active ingredient into an aliquot of the solvent. Remove the induction port and mouthpiece adapter from the apparatus and extract the active ingredient into an aliquot of the solvent. If necessary, rinse the inside of the inlet jet tube to stage 1 with solvent, allowing the solvent to flow into the stage. Extract the active ingredient from the inner walls and the collection plate of each of the four upper stages of the apparatus into the solution in the respective stage by carefully tilting and rotating the apparatus, observing that no liquid transfer occurs between the stages.

Using a suitable method of analysis, determine the amount of active ingredient contained in each of the six volumes of solvent.

Calculate the fine particle dose (see below).

APPARATUS D – “ANDERSEN” SIZING SAMPLER

The “Andersen” 1 ACFM non-viable ambient sizing sampler consists of 8 aluminium stages together with a final filter. The stages are clamped together and sealed with O-rings. In the configuration used for pressurised inhalers, Figure 2.9.18-9, the entry cone of the sampler is connected to a right-angle bend metal induction port defined in Figure 2.9.18-7. A suitable mouthpiece adapter should be used to provide an airtight seal between the inhaler and the induction port. The front face of the inhaler mouthpiece must be flush with the front face of the induction port. In the configuration for powder inhalers, a pre-separator is placed above the top stage to collect large masses of non-respirable powder. It is connected to the induction port as shown in Figure 2.9.18-10. To accommodate high flow rates through the sampler, the outlet nipple, used to connect the sampler to the vacuum system is enlarged to have an internal diameter ≥ 8 mm.

Procedure for pressurised inhalers. Assemble the “Andersen” Sampler with a suitable filter in place and ensure that the system is airtight. Place a suitable mouthpiece adapter in position at the end of the induction port so that the mouthpiece end of the actuator, when inserted, lines up along the horizontal axis of the induction port and the inhaler unit is positioned in the same orientation as the intended use. Connect a suitable pump to the

Table 2.9.18-5. – Calculations for Apparatus C. Use $q_1 = \sqrt{(60/Q)}$, where Q is the flow rate in litres per minute

Cut-off diameter (μm)	Mass of active ingredient deposited per discharge	Cumulative mass of active ingredient deposited per discharge	Cumulative fraction of active ingredient (per cent)
$d_4 = 1.7q_1$	mass from stage 5, m_5^*	$c_4 = m_5$	$f_4 = (c_4/c) \cdot 100$
$d_3 = 3.1q_1$	mass from stage 4, m_4	$c_3 = c_4 + m_4$	$f_3 = (c_3/c) \cdot 100$
$d_2 = 6.8q_1$	mass from stage 3, m_3	$c_2 = c_3 + m_3$	$f_2 = (c_2/c) \cdot 100$
	mass from stage 2, m_2	$c = c_2 + m_2$	100

* stage 5 is the filter stage

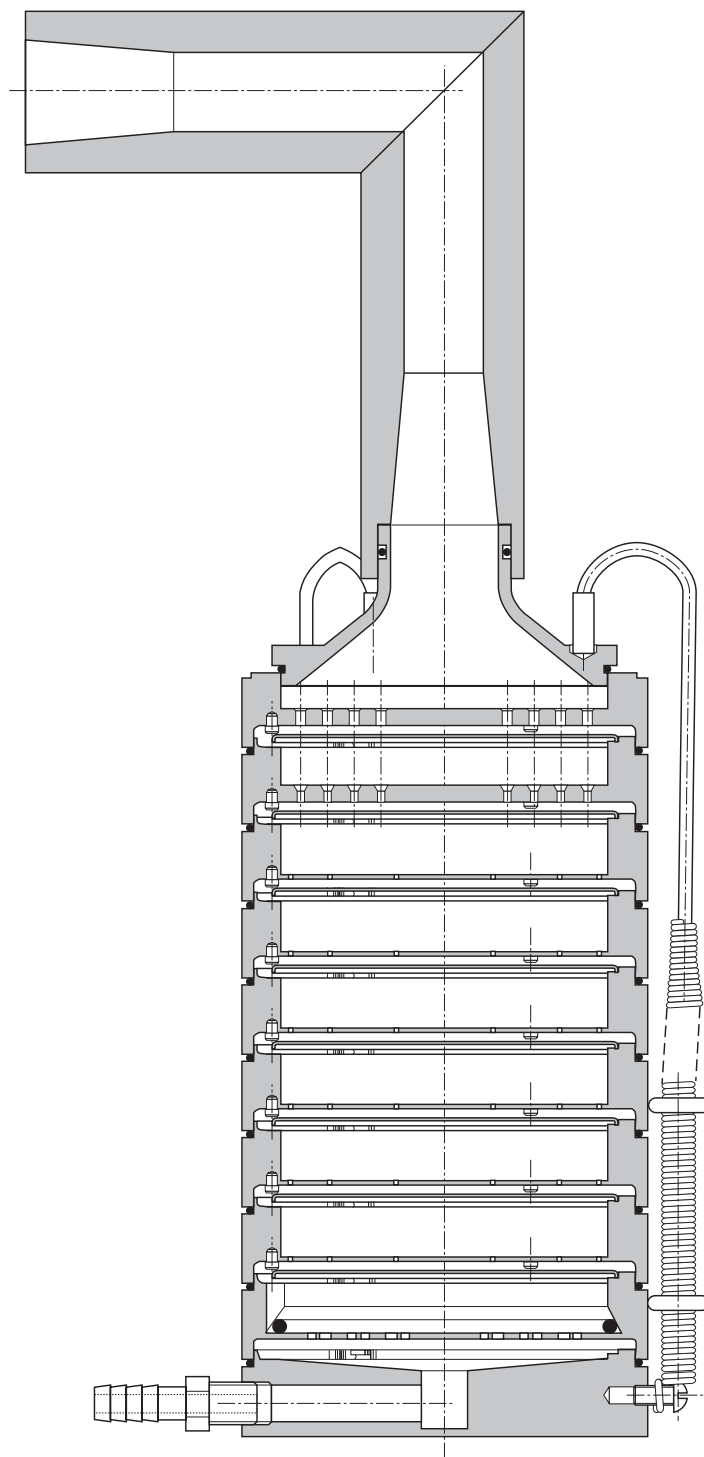


Figure 2.9.18-9. – “Andersen” sizing sampler adapted for pressurised inhalers

outlet of the apparatus and adjust the air flow through the apparatus, as measured at the inlet to the induction port, to 28.3 ± 1.5 litres/min. Switch off the air flow.

Unless otherwise prescribed in the patient instructions, shake the inhaler for 5 s and discharge one delivery to waste. Switch on the pump to the apparatus, locate the mouthpiece end of the actuator in the adapter and discharge the inverted inhaler into the apparatus, depressing the valve for a sufficient time to ensure complete discharge. Remove the assembled inhaler from the adapter. Repeat the procedure. The number of discharges should be minimised and typically would not be greater than ten. The number of discharges should be sufficient to ensure an accurate and precise determination of the fine particle dose. After the final discharge, wait for 5 s and then switch off the pump.

Dismantle the apparatus. Carefully remove the filter and extract the drug into an aliquot of the solvent. Remove the induction port and mouthpiece adapter from the apparatus and extract the drug into an aliquot of the solvent. Extract the drug from the inner walls and the collection plate of each of the stages of the apparatus into aliquots of solvent.

Using a suitable method of analysis, determine the quantity of drug contained in each of the nine volumes of solvent.

Calculate the fine particle dose (see below).

Procedure for powder inhalers. In order to assess powder inhalers, the “Andersen” Sampler may be used at flow rates other than 28.3 litres/min. However, no general calibration data is presently available. In the absence of published data,

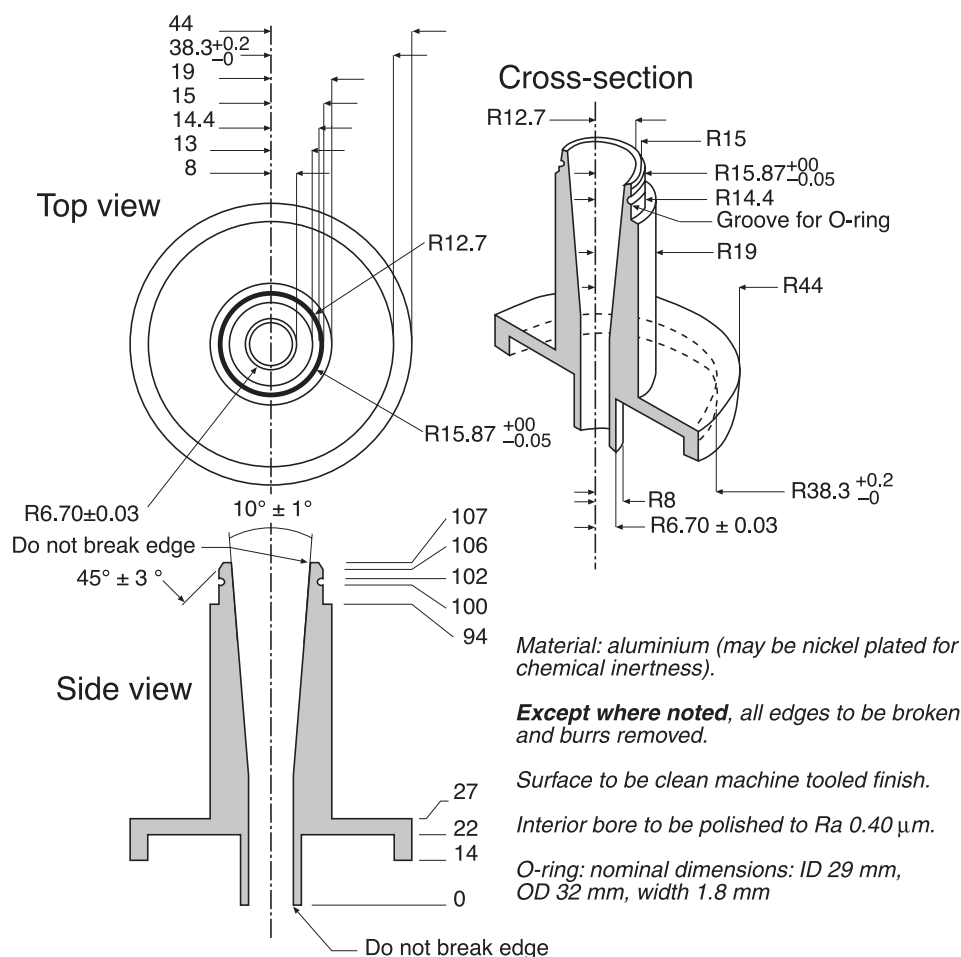


Figure 2.9.18-10. – Connection of the induction port to the preseparator of the Andersen sizing sampler

users must, therefore, validate the use of the sampler in the chosen conditions. The following experimental procedure may then be adopted.

Assemble the “Andersen” Sampler with the pre-separator and a suitable filter in place and ensure that the system is airtight. To ensure efficient particle capture, coat each plate with glycerol or similar high viscosity liquid deposited from a volatile solvent. The pre-separator should be coated in the same way or should contain 10 ml of a suitable solvent. Connect the apparatus to a flow system according to the scheme specified in Figure 2.9.18-8.

Unless otherwise defined conduct the test at the flow rate used in the test for uniformity of delivered dose drawing 4 litres of air through the apparatus. At high flow rates it may be necessary to remove the lowest stages from the stack. Connect a flow meter, calibrated for the volumetric flow leaving the meter, to the induction port. Adjust the flow control valve to achieve steady flow through the system at the required rate, Q (± 5 per cent). Ensure that critical flow occurs in the flow control valve by the procedure described for Apparatus C. Switch off the airflow.

Prepare the dry-powder inhaler for use according to the patient instructions. With the pump running and the two-way valve closed, locate the mouthpiece of the inhaler in the mouthpiece adapter. Discharge the powder into the apparatus by opening the valve for the required time, T (± 5 per cent). Repeat the discharge sequence. The number

of discharges should be minimised and typically would not be greater than ten. The number of discharges should be sufficient to ensure an accurate and precise determination of fine particle dose. After the final discharge, wait for 5 s and then switch off the pump.

Dismantle the apparatus. Carefully remove the filter and extract the active ingredient into an aliquot of the solvent. Remove the pre-separator, induction port and mouthpiece adapter from the apparatus and extract the drug into an aliquot of the solvent. Extract the active ingredient from the inner walls and the collection plate of each of the stages of the apparatus into aliquots of solvent.

Using a suitable method of analysis, determine the quantity of active ingredient contained in each of the nine volumes of solvent.

Calculate the fine particle dose (see below).

Calculations

From the analyses of the solutions, calculate the mass of active ingredient deposited on each stage per discharge and the mass of active ingredient per discharge deposited in the induction port, mouthpiece adapter and where used the pre-separator. The total mass of the active ingredient is not less than 75 per cent and not more than 125 per cent of the average delivered dose determined during testing for uniformity of delivered dose. If the total mass is outside this range the test must be repeated.

Table 2.9.18.6. – Calculations for Apparatus D when used at 28.3 litres/min

Cut-off diameter (µm)	Mass of active ingredient deposited per discharge	Cumulative mass of active ingredient deposited per discharge	Cumulative fraction of active ingredient (per cent)
$d_7 = 0.4$	mass from stage 8, m_8	$c_7 = m_8$	$f_7 = (c_7/c) \cdot 100$
$d_6 = 0.7$	mass from stage 7, m_7	$c_6 = c_7 + m_7$	$f_6 = (c_6/c) \cdot 100$
$d_5 = 1.1$	mass from stage 6, m_6	$c_5 = c_6 + m_6$	$f_5 = (c_5/c) \cdot 100$
$d_4 = 2.1$	mass from stage 5, m_5	$c_4 = c_5 + m_5$	$f_4 = (c_4/c) \cdot 100$
$d_3 = 3.3$	mass from stage 4, m_4	$c_3 = c_4 + m_4$	$f_3 = (c_3/c) \cdot 100$
$d_2 = 4.7$	mass from stage 3, m_3	$c_2 = c_3 + m_3$	$f_2 = (c_2/c) \cdot 100$
$d_1 = 5.8$	mass from stage 2, m_2	$c_1 = c_2 + m_2$	$f_1 = (c_1/c) \cdot 100$
$d_0 = 9.0$	mass from stage 1, m_1	$c_0 = c_1 + m_1$	$f_0 = (c_0/c) \cdot 100$
	mass from stage 0, m_0	$c = c_0 + m_0$	100

Starting at the filter, derive a cumulative mass vs. cut-off diameter of the respective stages (see Table 2.9.18.5 for Apparatus C or Table 2.9.18.6 for Apparatus D). Calculate by interpolation the mass of active ingredient less than 5 µm. This is the Fine Particle Dose (FPD).

If necessary, and where appropriate, plot the cumulative fraction of active ingredient versus cut-off diameter (see Tables 2.9.18.5/6) on log probability paper, and use this plot to determine values for the Mass Median Aerodynamic Diameter (MMAD) and the Geometric Standard Deviation (GSD), as appropriate. Appropriate computational methods may also be used.

01/2005:20919

2.9.19. PARTICULATE CONTAMINATION: SUB-VISIBLE PARTICLES

Particulate contamination of injections and infusions consists of extraneous, mobile undissolved particles, other than gas bubbles, unintentionally present in the solutions.

For the determination of particulate contamination 2 procedures, Method 1 (Light Obscuration Particle Count Test) and Method 2 (Microscopic Particle Count Test), are specified hereinafter. When examining injections and infusions for sub-visible particles, Method 1 is preferably applied. However, it may be necessary to test some preparations by the light obscuration particle count test followed by the microscopic particle count test to reach a conclusion on conformance to the requirements.

Not all parenteral preparations can be examined for sub-visible particles by one or both of these methods. When Method 1 is not applicable, e.g. in case of preparations having reduced clarity or increased viscosity, the test is carried out according to Method 2. Emulsions, colloids, and liposomal preparations are examples. Similarly, products that produce air or gas bubbles when drawn into the sensor may also require microscopic particle count testing. If the viscosity of the preparation to be tested is sufficiently high so as to preclude its examination by either test method, a quantitative dilution with an appropriate diluent may be made to decrease viscosity, as necessary, to allow the analysis to be performed.

The results obtained in examining a discrete unit or group of units for particulate contamination cannot be extrapolated with certainty to other units that remain untested. Thus, statistically sound sampling plans must be developed if

valid inferences are to be drawn from observed data to characterise the level of particulate contamination in a large group of units.

METHOD 1. LIGHT OBSCURATION PARTICLE COUNT TEST

Use a suitable apparatus based on the principle of light blockage which allows an automatic determination of the size of particles and the number of particles according to size.

The apparatus is calibrated using suitable certified reference materials consisting of dispersions of spherical particles of known sizes between 10 µm and 25 µm. These standard particles are dispersed in *particle-free water R*. Care must be taken to avoid aggregation of particles during dispersion.

General precautions

The test is carried out under conditions limiting particulate contamination, preferably in a laminar-flow cabinet.

Very carefully wash the glassware and filtration equipment used, except for the membrane filters, with a warm detergent solution and rinse with abundant amounts of water to remove all traces of detergent. Immediately before use, rinse the equipment from top to bottom, outside and then inside, with *particle-free water R*.

Take care not to introduce air bubbles into the preparation to be examined, especially when fractions of the preparation are being transferred to the container in which the determination is to be carried out.

In order to check that the environment is suitable for the test, that the glassware is properly cleaned and that the water to be used is particle-free, the following test is carried out: determine the particulate contamination of 5 samples of *particle-free water R*, each of 5 ml, according to the method described below. If the number of particles of 10 µm or greater size exceeds 25 for the combined 25 ml, the precautions taken for the test are not sufficient. The preparatory steps must be repeated until the environment, glassware and water are suitable for the test.

Method

Mix the contents of the sample by slowly inverting the container 20 times successively. If necessary, cautiously remove the sealing closure. Clean the outer surfaces of the container opening using a jet of *particle-free water R* and remove the closure, avoiding any contamination of the contents. Eliminate gas bubbles by appropriate measures such as allowing to stand for 2 min or sonicating.

For large-volume parenterals, single units are tested. For small-volume parenterals less than 25 ml in volume, the contents of 10 or more units are combined in a cleaned

container to obtain a volume of not less than 25 ml; where justified and authorised, the test solution may be prepared by mixing the contents of a suitable number of vials and diluting to 25 ml with *particle-free water R* or with an appropriate solvent without contamination of particles when *particle-free water R* is not suitable. Small-volume parenterals having a volume of 25 ml or more may be tested individually.

Powders for parenteral use are reconstituted with *particle-free water R* or with an appropriate solvent without contamination of particles when *particle-free water R* is not suitable.

The number of test specimens must be adequate to provide a statistically sound assessment. For large-volume parenterals or for small-volume parenterals having a volume of 25 ml or more, fewer than 10 units may be tested, based on an appropriate sampling plan.

Remove 4 portions, each of not less than 5 ml, and count the number of particles equal to or greater than 10 µm and 25 µm. Disregard the result obtained for the first portion, and calculate the mean number of particles for the preparation to be examined.

Evaluation

For preparations supplied in containers with a nominal volume of more than 100 ml, apply the criteria of test 1.A.

For preparations supplied in containers with a nominal volume of less than 100 ml, apply the criteria of test 1.B.

For preparations supplied in containers with a nominal volume of 100 ml, apply the criteria of test 1.B

If the average number of particles exceeds the limits, test the preparation by the microscopic particle count test.

Test 1.A – Solutions for infusion or solutions for injection supplied in containers with a nominal content of more than 100 ml

The preparation complies with the test if the average number of particles present in the units tested does not exceed 25 per millilitre equal to or greater than 10 µm and does not exceed 3 per millilitre equal to or greater than 25 µm.

Test 1.B – Solutions for infusion or solutions for injection supplied in containers with a nominal content of less than 100 ml

The preparation complies with the test if the average number of particles present in the units tested does not exceed 6000 per container equal to or greater than 10 µm and does not exceed 600 per container equal to or greater than 25 µm.

METHOD 2. MICROSCOPIC PARTICLE COUNT TEST

Use a suitable binocular microscope, filter assembly for retaining particulate contamination and membrane filter for examination.

The microscope is equipped with an ocular micrometer calibrated with an objective micrometer, a mechanical stage capable of holding and traversing the entire filtration area of the membrane filter, 2 suitable illuminators to provide episcopic illumination in addition to oblique illumination, and is adjusted to 100 ± 10 magnifications.

The ocular micrometer is a circular diameter graticule (see Figure 2.9.19.-1) and consists of a large circle divided by crosshairs into quadrants, transparent and black reference circles 10 µm and 25 µm in diameter at 100 magnifications, and a linear scale graduated in 10 µm increments. It is calibrated using a stage micrometer that is certified by either a domestic or international standard institution. A relative error of the linear scale of the graticule within ± 2 per cent is acceptable. The large circle is designated the graticule field of view (GFOV).

2 illuminators are required. One is an episcopic brightfield illuminator internal to the microscope, the other is an external, focusable auxiliary illuminator adjustable to give reflected oblique illumination at an angle of $10-20^\circ$.

The filter assembly for retaining particulate contamination consists of a filter holder made of glass or other suitable material, and is equipped with a vacuum source and a suitable membrane filter.

The membrane filter is of suitable size, black or dark grey in colour, non-gridded or gridded, and 1.0 µm or finer in nominal pore size.

General precautions

The test is carried out under conditions limiting particulate contamination, preferably in a laminar-flow cabinet.

Very carefully wash the glassware and filter assembly used, except for the membrane filter, with a warm detergent solution and rinse with abundant amounts of water to remove

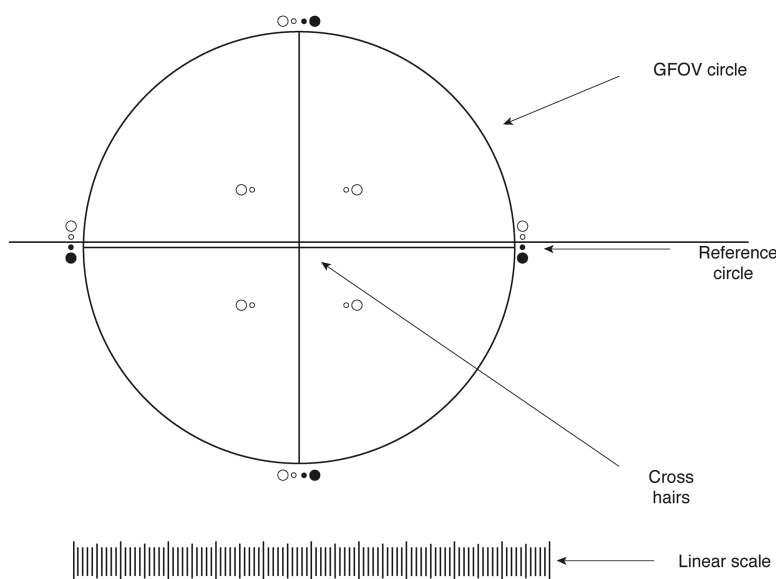


Figure 2.9.19.-1. – Circular diameter graticule

all traces of detergent. Immediately before use, rinse both sides of the membrane filter and the equipment from top to bottom, outside and then inside, with *particle-free water R*.

In order to check that the environment is suitable for the test, that the glassware and the membrane filter are properly cleaned and that the water to be used is particle-free, the following test is carried out: determine the particulate contamination of a 50 ml volume of *particle-free water R* according to the method described below. If more than 20 particles 10 µm or larger in size or if more than 5 particles 25 µm or larger in size are present within the filtration area, the precautions taken for the test are not sufficient. The preparatory steps must be repeated until the environment, glassware, membrane filter and water are suitable for the test.

Method

Mix the contents of the samples by slowly inverting the container 20 times successively. If necessary, cautiously remove the sealing closure. Clean the outer surfaces of the container opening using a jet of *particle-free water R* and remove the closure, avoiding any contamination of the contents.

For large-volume parenterals, single units are tested. For small-volume parenterals less than 25 ml in volume, the contents of 10 or more units are combined in a cleaned container; where justified and authorised, the test solution may be prepared by mixing the contents of a suitable number of vials and diluting to 25 ml with *particle-free water R* or with an appropriate solvent without contamination of particles when *particle-free water R* is not suitable. Small-volume parenterals having a volume of 25 ml or more may be tested individually.

Powders for parenteral use are constituted with *particle-free water R* or with an appropriate solvent without contamination of particles when *particle-free water R* is not suitable.

The number of test specimens must be adequate to provide a statistically sound assessment. For large-volume parenterals or for small-volume parenterals having a volume of 25 ml or more, fewer than 10 units may be tested, based on an appropriate sampling plan.

Wet the inside of the filter holder fitted with the membrane filter with several millilitres of *particle-free water R*. Transfer to the filtration funnel the total volume of a solution pool or of a single unit, and apply vacuum. If needed, add stepwise a portion of the solution until the entire volume is filtered. After the last addition of solution, begin rinsing the inner walls of the filter holder by using a jet of *particle-free water R*. Maintain the vacuum until the surface of the membrane filter is free from liquid. Place the filter in a Petri dish and allow the filter to air-dry with the cover slightly ajar. After the filter has been dried, place the Petri dish on the stage of the microscope, scan the entire membrane filter under the reflected light from the illuminating device, and count the number of particles that are equal to or greater than 10 µm and the number of particles that are equal to or greater than 25 µm. Alternatively, partial filter count and determination of the total filter count by calculation is allowed. Calculate the mean number of particles for the preparation to be examined.

The particle sizing process with the use of the circular diameter graticule is carried out by transforming mentally the image of each particle into a circle and then comparing it to the 10 µm and 25 µm graticule reference circles. Thereby the particles are not moved from their initial locations within the graticule field of view and are not superimposed on the reference circles for comparison. The inner diameter of the

transparent graticule reference circles is used to size white and transparent particles, while dark particles are sized by using the outer diameter of the black opaque graticule reference circles.

In performing the microscopic particle count test do not attempt to size or enumerate amorphous, semi-liquid, or otherwise morphologically indistinct materials that have the appearance of a stain or discoloration on the membrane filter. These materials show little or no surface relief and present a gelatinous or film-like appearance. In such cases the interpretation of enumeration may be aided by testing a sample of the solution by the light obscuration particle count test.

Evaluation

For preparations supplied in containers with a nominal volume of more than 100 ml, apply the criteria of test 2.A.

For preparations supplied in containers with a nominal volume of less than 100 ml, apply the criteria of test 2.B.

For preparations supplied in containers with a nominal volume of 100 ml, apply the criteria of test 2.B.

Test 2.A – Solutions for infusion or solutions for injection supplied in containers with a nominal content of more than 100 ml

The preparation complies with the test if the average number of particles present in the units tested does not exceed 12 per millilitre equal to or greater than 10 µm and does not exceed 2 per millilitre equal to or greater than 25 µm.

Test 2.B – Solutions for infusion or solutions for injection supplied in containers with a nominal content of less than 100 ml

The preparation complies with the test if the average number of particles present in the units tested does not exceed 3000 per container equal to or greater than 10 µm and does not exceed 300 per container equal to or greater than 25 µm.

01/2005:20920

2.9.20. PARTICULATE CONTAMINATION: VISIBLE PARTICLES

Particulate contamination of injections and infusions consists of extraneous, mobile undissolved particles, other than gas bubbles, unintentionally present in the solutions.

The test is intended to provide a simple procedure for the visual assessment of the quality of parenteral solutions as regards visible particles. Other validated methods may be used.

APPARATUS

The apparatus (see Figure 2.9.20.-1) consists of a viewing station comprising:

- a matt black panel of appropriate size held in a vertical position,
- a non-glare white panel of appropriate size held in a vertical position next to the black panel,
- an adjustable lampholder fitted with a suitable, shaded, white-light source and with a suitable light diffuser (a viewing illuminator containing two 13 W fluorescent tubes, each 525 mm in length, is suitable). The intensity of illumination at the viewing point is maintained between 2000 lux and 3750 lux, although higher values are preferable for coloured glass and plastic containers.

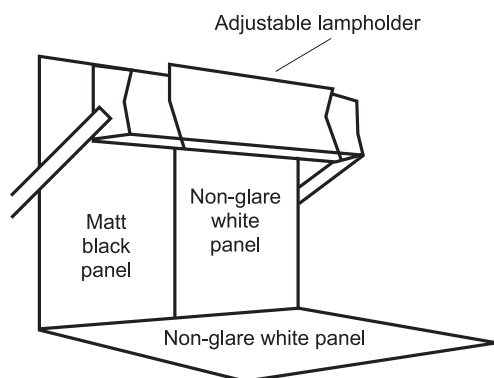


Figure 2.9.20.-1. – Apparatus for visible particles

METHOD

Remove any adherent labels from the container and wash and dry the outside. Gently swirl or invert the container, ensuring that air bubbles are not introduced, and observe for about 5 s in front of the white panel. Repeat the procedure in front of the black panel. Record the presence of any particles.

the glass tube until the metal needle touches the flat end of the suppository. Put the cover on the tube (beginning of time measurement). Note the time which elapses until the rod sinks down to the bottom of the glass tube and the mark reaches the upper level of the plastic cover.

APPARATUS B

The apparatus (see Figure 2.9.22.-2) consists of a water-bath (B) into which an inner tube (A) is inserted and fixed with a stopper. The inner tube is closed by a stopper at the bottom. The apparatus is fitted with a thermometer. 2 insets are available:

- a glass rod (C1) in the form of a tube sealed at both ends, carrying a rim at its lower end weighed with lead shot, which has a weight of 30 ± 0.4 g,
- a penetration inset (C2) consisting of a rod (7.5 ± 0.1 g) in a tube which has an enlargement for the suppository, both made of stainless steel.

Method. Pour 5 ml of water at 36.5 ± 0.5 °C into the inner tube (A), introduce a suppository with the tip downwards and onto that, place the inset (C1 or C2). Note the time which elapses between this moment and the moment when the lower, rimmed end of the glass rod (C1) or the steel rod (C2) reaches the narrowed part of the inner glass tube. Melting or dissolution is then considered as complete.

01/2005:20922

2.9.22. SOFTENING TIME DETERMINATION OF LIPOPHILIC SUPPOSITORIES

The test is intended to determine, under defined conditions, the time which elapses until a suppository maintained in water softens to the extent that it no longer offers resistance when a defined weight is applied.

APPARATUS A

The apparatus (see Figure 2.9.22.-1) consists of a glass tube 15.5 mm in internal diameter with a flat bottom and a length of about 140 mm. The tube is closed by a removable plastic cover having an opening 5.2 mm in diameter. The apparatus comprises a rod 5.0 mm in diameter which becomes wider towards the lower end, reaching a diameter of 12 mm. A metal needle 2 mm in length and 1 mm in diameter is fixed on the flat underside.

The rod consists of 2 parts, a lower part made of plastic material and an upper part made of plastic material or metal with a weight disk. The upper and lower parts are either fitted together (manual version) or separate (automated version). The weight of the entire rod is 30 ± 0.4 g. The upper part of the rod carries a sliding mark ring. When the rod is introduced into the glass tube so that it touches the bottom, the mark ring is adjusted to coincide with the upper level of the plastic cover.

Method. Place the glass tube containing 10 ml of water in a water-bath and equilibrate at 36.5 ± 0.5 °C. Fix the glass tube vertically and immerse to a depth of at least 7 cm below the surface but without touching the bottom of the water-bath. Introduce a suppository, tip first, into the tube followed by the rod with the free gliding plastic cover into

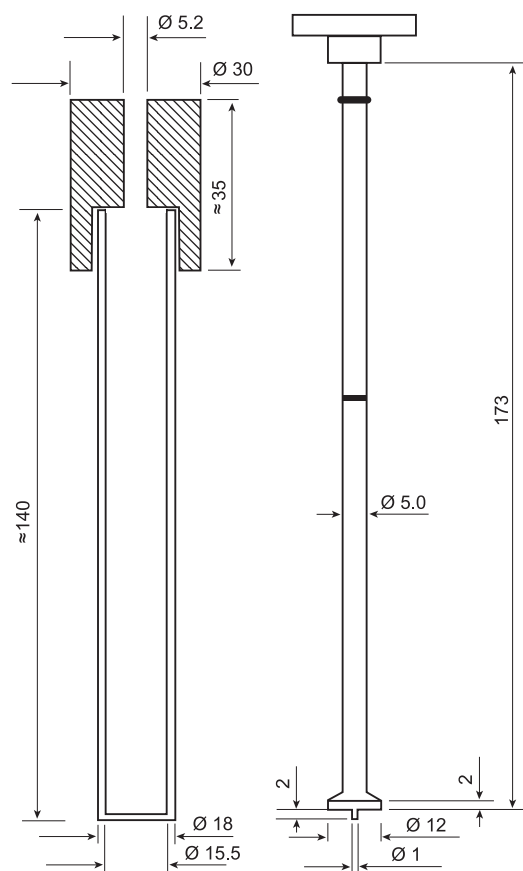


Figure 2.9.22.-1. – Apparatus A for measuring the softening time of lipophilic suppositories

Dimensions in millimetres

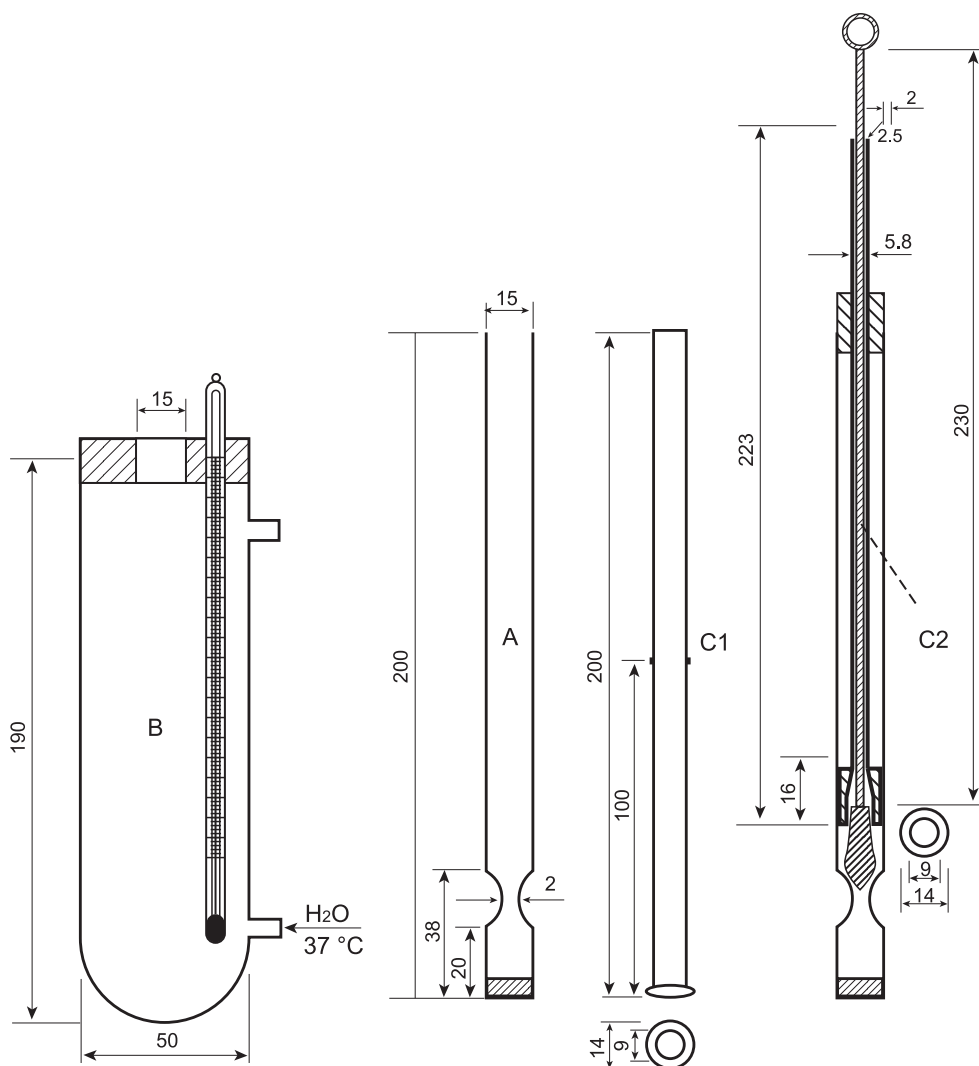


Figure 2.9.22-2. – Apparatus B for measuring the softening time of lipophilic suppositories
Dimensions in millimetres

01/2005:20923

2.9.23. PYCNOMETRIC DENSITY OF SOLIDS

The test for pycnometric density of solids is intended to determine the volume occupied by a known mass of powder by measuring the volume of gas displaced under defined conditions. Hence, its pycnometric density is calculated.

APPARATUS

The apparatus (see Figure 2.9.23-1) consists of the following:

- a sealed test cell, with an empty cell volume (V_c), connected through a valve to a reference cell, with a reference volume (V_r),
- a system capable of pressurising the test cell with the measurement gas until a defined pressure (P) indicated by a manometer,
- the system is connected to a source of measurement gas, which is preferably helium, unless another gas is specified⁽¹⁾.

- V_r = reference volume
 V_c = cell volume
 V_s = sample volume
 M = manometer

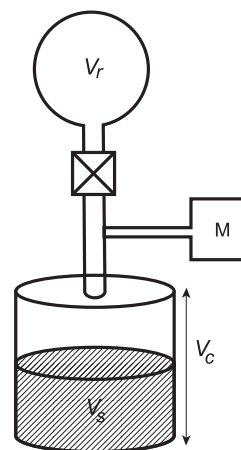


Figure 2.9.23-1. – Schematic diagram of a gas pycnometer

(1) If gases other than helium are used, it would not be surprising to obtain values different from those obtained with helium, since the penetration of the gas is dependent on the size of the pore as well as the cross-sectional area of the penetrating molecule. For example, the pycnometric density of porous materials will be overestimated by a measure using nitrogen by comparison with helium.

The temperature of the gas pycnometer is between 15 °C and 30 °C and must not vary by more than 2 °C during the course of measurement.

The apparatus is calibrated which means that the volumes (V_c) and (V_r) are determined, using calibrated, polished steel balls having a total volume (around 6 cm³) known to the nearest 0.001 cm³. The procedure described below is followed in two runs. Firstly, with an empty test cell and secondly with the steel balls placed in the test cell. The volumes (V_c) and (V_r) are calculated using the equation for the sample volume taking into account that the volume is zero in the first run.

METHOD

Weigh the test cell of the pycnometer and record the mass. Fill the test cell with a given mass of powder of the substance to be examined. Seal the test cell in the pycnometer. Remove volatile contaminants in the powder by degassing the powder under a constant purge of gas; occasionally, powders may initially have to be degassed under vacuum. Record the system reference pressure (P_r) as indicated by the manometer while the valve that connects the reference cell with the test cell is open. Close the valve to separate the reference cell from the test cell. Pressurise the test cell with the gas to an initial pressure (P_i) and record the value obtained. Open the valve to connect the reference cell with the test cell. Record the final pressure (P_f). Repeat the measurement sequence for the same powder sample until consecutive measurements of the sample volume (V_s) agree to within 0.5 per cent. The sample volume is expressed in cubic centimetres. Unload the test cell and measure the final powder mass (m) expressed in grams.

EXPRESSION OF THE RESULTS

The sample volume (V_s) is given by the expression:

$$V_s = V_c - \frac{V_r}{\frac{P_i - P_r}{P_f - P_r} - 1}$$

The density (ρ) is given by the equation:

$$\rho = \frac{m}{V_s}$$

01/2005:20924

2.9.24. RESISTANCE TO RUPTURE OF SUPPOSITORIES AND PESSARIES

This test determines, under defined conditions, the resistance to rupture of suppositories and pessaries measured by the mass needed to rupture them by crushing.

This test applies to suppositories and pessaries based on fatty excipients. It is not suited to suppositories and pessaries based on hydrophilic excipients such as a gelatin-glycerol mixture.

Apparatus. The apparatus (see Figures 2.9.24.-1/2) consists of:

- a thermostatted chamber closed in front by a glass window and containing a device that is to hold the suppository or pessary,
- two opposed jaws, the upper jaw descending vertically towards the lower jaw. The crushing surfaces of the jaws are flat, perpendicular to the direction of movement and larger than the zone of contact with the suppository or pessary. A plastic sample holder is fixed in the centre of the jaws (half a holder on each jaw). The upper jaw (top pressure block) is connected to a suspension to which discs can be added, each of which weighs 200 g. The initial mass of the device is 600 g. Crushing of the sample is carried out by successively adding 200 g discs to the initial mass of 600 g.

Method. Check that the apparatus is vertical. Heat the thermostatted chamber to 25 °C.

The dosage form to be tested has been maintained for at least 24 h at the required measuring temperature. Place the suppository or pessary vertically between the jaws in the sample holder with the point upwards. The top pressure block of the suspension loading rod is carefully positioned and the test chamber is closed with its glass window; for each determination, position the suppository or pessary in the same manner with respect to the direction of the force applied.

Wait for 1 min and add the first 200 g disc. Again wait for 1 min and add another disc. Repeat the operation until the suppository or pessary collapses.

The mass required to crush the suppository or pessary is calculated by the sum of the masses weighing on the suppository or pessary when it collapses (including the initial mass of the device) assessed as follows:

- if the suppository or pessary collapses within 20 s of placing the last disc, do not take this mass into account,
- if the suppository or pessary collapses between 20 s and 40 s of placing the last disc, use only half of this mass in the calculation, i.e. 100 g,
- if the suppository or pessary remains uncrushed for more than 40 s after the last disc is placed, use all the mass in the calculation.

Carry out each measurement on ten suppositories or ten pessaries, making sure that no residue remains before each determination.

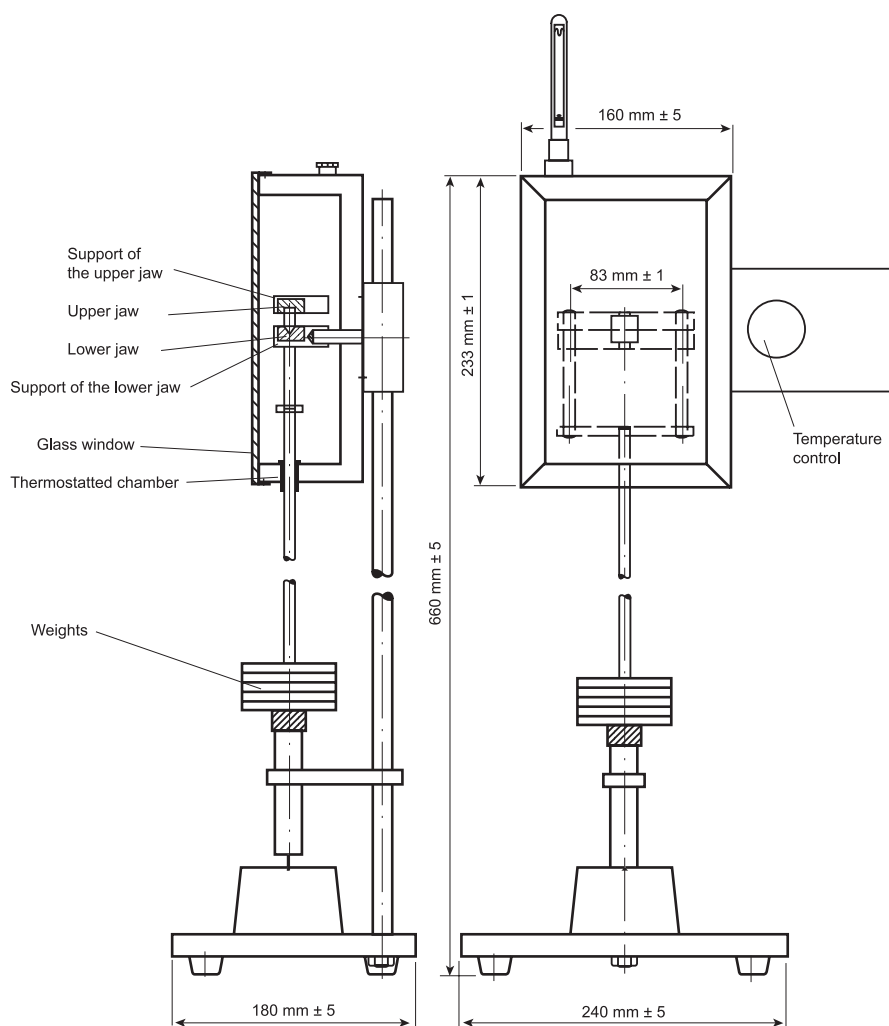


Figure 2.9.24-1. – Apparatus for the determination of the resistance to rupture of suppositories and pessaries

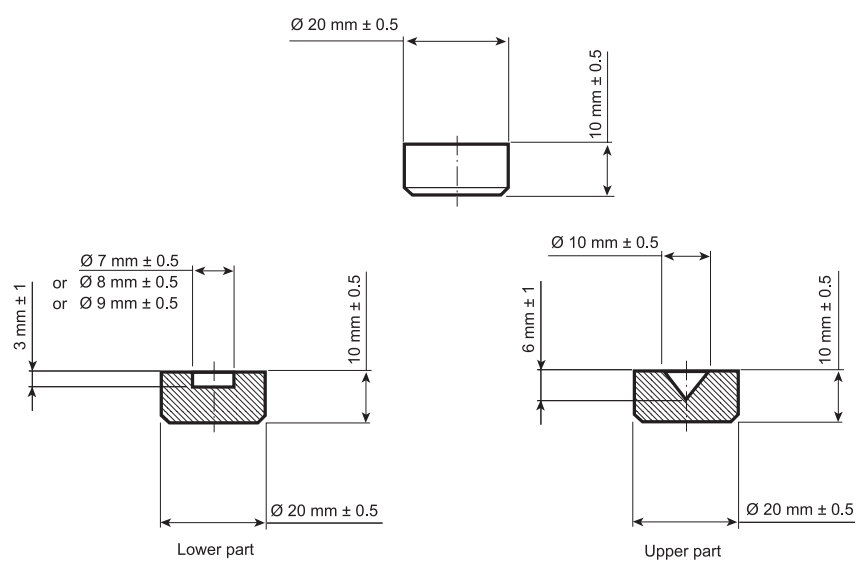


Figure 2.9.24-2. – Lower and upper jaws

01/2005:20925 PROCEDURE

**2.9.25. CHEWING GUM, MEDICATED,
DRUG RELEASE FROM****PRINCIPLE**

The drug release from medicated chewing gum is determined by applying a mechanical kneading procedure to a piece of gum placed in a small chewing chamber containing a known volume of buffer solution.

APPARATUS

The chewing apparatus (see Figure 2.9.25.-1) comprises a chewing chamber of approximately 40 ml in which the gum is artificially chewed by two horizontal pistons; the pistons operate together at a constant speed. At the end of a chew, the pistons can rotate around their own axes in opposite directions to each other. In this way, the gum is subjected to maximum chewing. A third vertical piston ("tongue") operates alternately with the two horizontal pistons and makes sure that the gum stays in the right place between chews. The pistons are driven by compressed air and their mutual movements are controlled. All materials are composed of stainless steel.

Adjust the temperature of the chewing chamber (37 ± 0.5 °C) and the speed of the pistons. Add 20 ml of buffer (generally close to pH 6) into the chewing chamber. Start the machine and let it run for 2 min with buffer, but no chewing gum. Remove all the buffer by pipette and replace it by 20 ml of fresh buffer. The buffer which has been removed is analysed as a control for the cleaning procedure. Weigh precisely a piece of chewing gum, put it into the chewing chamber and start the machine. At specified time intervals, remove samples from the reservoir to determine the drug release. The chewing frequency is usually set at 60 cycles/min. Sometimes the chewing residue is taken out and placed in a bag for later analysis.

01/2005:20926

**2.9.26. SPECIFIC SURFACE AREA BY
GAS ADSORPTION****I. INTRODUCTION**

The specific surface area of a powder is determined by physical adsorption of a gas on the surface of the solid and by measuring the amount of adsorbate gas corresponding to

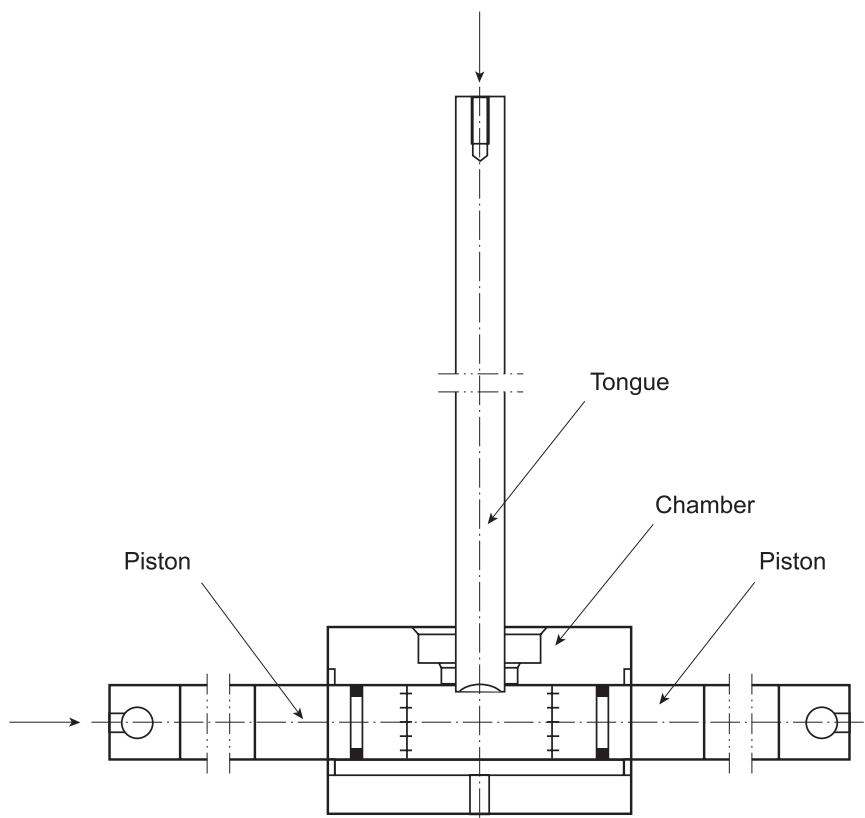


Figure 2.9.25.-1. – Apparatus for the determination of drug release from medicated chewing gum

01/2005:20925 PROCEDURE

**2.9.25. CHEWING GUM, MEDICATED,
DRUG RELEASE FROM****PRINCIPLE**

The drug release from medicated chewing gum is determined by applying a mechanical kneading procedure to a piece of gum placed in a small chewing chamber containing a known volume of buffer solution.

APPARATUS

The chewing apparatus (see Figure 2.9.25.-1) comprises a chewing chamber of approximately 40 ml in which the gum is artificially chewed by two horizontal pistons; the pistons operate together at a constant speed. At the end of a chew, the pistons can rotate around their own axes in opposite directions to each other. In this way, the gum is subjected to maximum chewing. A third vertical piston ("tongue") operates alternately with the two horizontal pistons and makes sure that the gum stays in the right place between chews. The pistons are driven by compressed air and their mutual movements are controlled. All materials are composed of stainless steel.

Adjust the temperature of the chewing chamber (37 ± 0.5 °C) and the speed of the pistons. Add 20 ml of buffer (generally close to pH 6) into the chewing chamber. Start the machine and let it run for 2 min with buffer, but no chewing gum. Remove all the buffer by pipette and replace it by 20 ml of fresh buffer. The buffer which has been removed is analysed as a control for the cleaning procedure. Weigh precisely a piece of chewing gum, put it into the chewing chamber and start the machine. At specified time intervals, remove samples from the reservoir to determine the drug release. The chewing frequency is usually set at 60 cycles/min. Sometimes the chewing residue is taken out and placed in a bag for later analysis.

01/2005:20926

**2.9.26. SPECIFIC SURFACE AREA BY
GAS ADSORPTION****I. INTRODUCTION**

The specific surface area of a powder is determined by physical adsorption of a gas on the surface of the solid and by measuring the amount of adsorbate gas corresponding to

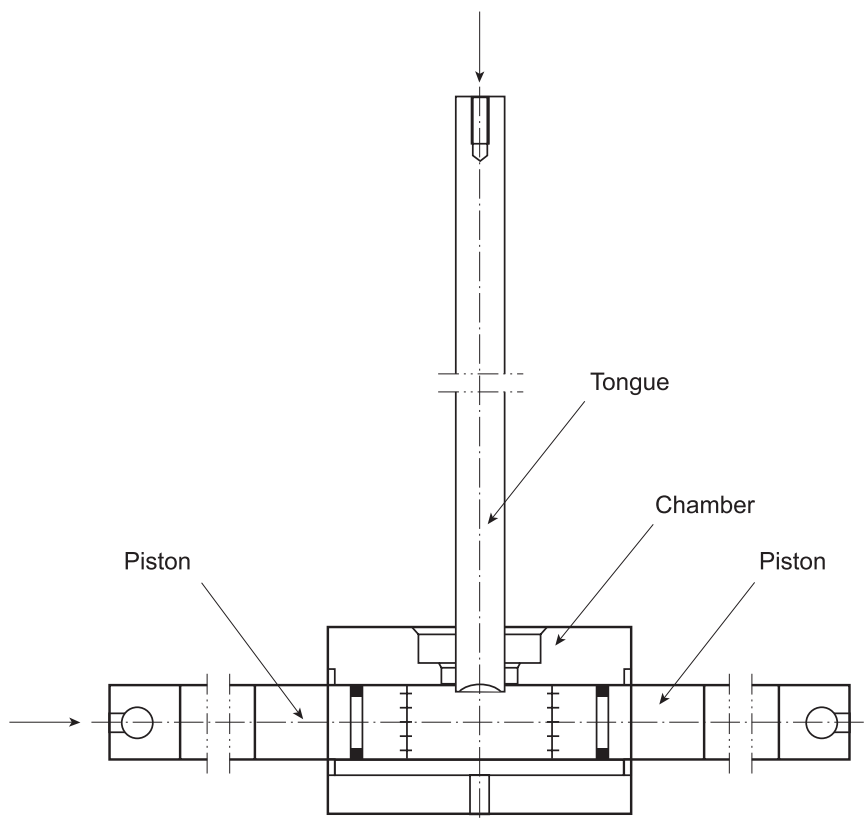


Figure 2.9.25.-1. – Apparatus for the determination of drug release from medicated chewing gum

a monomolecular layer on the surface. Physical adsorption results from relatively weak forces (van der Waals forces) between the adsorbate gas molecules and the adsorbent surface of the test powder. The amount of gas adsorbed can be measured by a gravimetric, volumetric or continuous flow procedure.

II. BRUNAUER, EMMETT AND TELLER (BET) THEORY AND SPECIFIC SURFACE AREA DETERMINATION

II.1. MULTI-POINT MEASUREMENT

The data are treated according to the Brunauer, Emmett and Teller (BET) adsorption isotherm equation:

$$P \left[\frac{1}{V_a \left(\frac{P_o}{P} - 1 \right)} \right] = \frac{C-1}{V_m C} \times \frac{P}{P_o} + \frac{1}{V_m C} \quad (1)$$

P = partial vapour pressure of adsorbate gas in equilibrium with the surface at -196°C , in pascals,
 P_o = saturated pressure of adsorbate gas, in pascals,
 V_a = volume of gas adsorbed at standard temperature and pressure (STP) [273.15 K and atmospheric pressure (1.013×10^5 Pa)], in millilitres,
 V_m = volume of gas adsorbed at STP to produce an apparent monolayer on the sample surface, in millilitres,
 C = dimensionless constant that is related to the enthalpy of adsorption of the adsorbate gas on the powder sample.

A value of V_a is measured at each of not less than three values of P/P_o .

Then the BET value

$$\frac{1}{\left[V_a \left(\frac{P_o}{P} - 1 \right) \right]}$$

is plotted against P/P_o according to equation (1). This plot should yield a straight line usually in the approximate relative pressure range 0.05 to 0.3. The data are considered acceptable if the correlation coefficient, r , of the linear regression is not less than 0.9975; that is, r^2 is not less than 0.995. From the resulting linear plot, the slope, which is equal to $(C-1)/V_m C$, and the intercept, which is equal to $1/V_m C$, are evaluated by linear regression analysis. From these values, V_m is calculated as $1/(\text{slope} + \text{intercept})$, while C is calculated as $(\text{slope}/\text{intercept}) + 1$. From the value of V_m so determined, the specific surface area, S , in m^2g^{-1} , is calculated by the equation:

$$S = \frac{V_m \times N \times a}{m \times 22400} \quad (2)$$

- N = Avogadro constant ($6.023 \times 10^{23} \text{ mol}^{-1}$),
 a = effective cross-sectional area of one adsorbate molecule, in square metres (0.162 nm^2 for nitrogen and 0.195 nm^2 for krypton),
 m = mass of test powder, in grams,
22400 = volume, in millilitres, occupied by the adsorbate gas at STP allowing for minor departures from the ideal.

II.2. SINGLE-POINT MEASUREMENT

Normally, at least three measurements of V_a each at different values of P/P_o are required for the determination of specific surface area by the dynamic flow gas adsorption technique

(*Method I*) or by volumetric gas adsorption (*Method II*). However, under certain circumstances described below, it may be acceptable to determine the specific surface area of a powder from a single value of V_a measured at a single value of P/P_o such as 0.300 (corresponding to 0.300 mole of nitrogen or 0.001038 mole fraction of krypton), using the following equation for calculating V_m :

$$V_m = V_a \left(1 - \frac{P}{P_o} \right) \quad (3)$$

The specific surface area is then calculated from the value of V_m by equation (2) given above.

The single-point method may be employed directly for a series of powder samples of a given material for which the material constant C is much greater than unity. These circumstances may be verified by comparing values of specific surface area determined by the single-point method with that determined by the multiple-point method for the series of powder samples. Close similarity between the single-point values and multiple-point values suggests that $1/C$ approaches zero.

The single-point method may be employed indirectly for a series of very similar powder samples of a given material for which the material constant C is not infinite but may be assumed to be invariant. Under these circumstances, the error associated with the single-point method can be reduced or eliminated by using the multiple-point method to evaluate C for one of the samples of the series from the BET plot, from which C is calculated as $(1 + \text{slope}/\text{intercept})$. Then V_m is calculated from the single value of V_a measured at a single value of P/P_o by the equation:

$$V_m = V_a \left(\frac{P_o}{P} - 1 \right) \left[\frac{1}{c} + \frac{c-1}{c} \times \left(\frac{P}{P_o} \right) \right] \quad (4)$$

The specific surface area is calculated from V_m by equation (2) given above.

III. EXPERIMENTAL TECHNIQUES

This section describes the methods to be used for the sample preparation, the dynamic flow gas adsorption technique (*Method I*) and the volumetric gas adsorption technique (*Method II*).

III.1. SAMPLE PREPARATION

III.1.1. Outgassing

Before the specific surface area of the sample can be determined, it is necessary to remove gases and vapours that may have become physically adsorbed onto the surface after manufacture and during treatment, handling and storage. If outgassing is not achieved, the specific surface area may be reduced or may be variable because an intermediate area of the surface is covered with molecules of the previously adsorbed gases or vapours. The outgassing conditions are critical for obtaining the required precision and accuracy of specific surface area measurements on pharmaceuticals because of the sensitivity of the surface of the materials.

Conditions. The outgassing conditions must be demonstrated to yield reproducible BET plots, a constant weight of test powder, and no detectable physical or chemical changes in the test powder.

The outgassing conditions defined by the temperature, pressure and time should be so chosen that the original surface of the solid is reproduced as closely as possible. Outgassing of many substances is often achieved by applying a vacuum or by purging the sample in a flowing stream of a non-reactive, dry gas. In either case, elevated temperatures are sometimes applied to increase the rate at which the

contaminants leave the surface. Outgassing by heating the powder sample may change the nature of the surface and should be avoided, unless specifically indicated.

If heating is employed, the recommended temperature and time of outgassing are as low as possible so as to achieve reproducibly high measures of specific surface area within an acceptable time span. For outgassing sensitive samples, other outgassing methods such as the desorption-adsorption cycling method may be employed.

III.1.2. Adsorbate

The standard technique is the adsorption of nitrogen at liquid nitrogen temperature.

For powders of low specific surface area ($< 1 \text{ m}^2\text{g}^{-1}$) the proportion adsorbed is low, the use of krypton at liquid nitrogen temperature is preferred in such cases since the low vapour pressure exerted by this gas greatly reduces the error.

All gases used must be free from moisture.

III.1.3. Quantity of sample

Accurately weigh a quantity of the test powder such that the total surface of the sample is at least 1 m^2 when adsorbate is nitrogen and 0.5 m^2 when the adsorbate is krypton.

III.2. MEASUREMENTS

Since the amount of gas adsorbed under a given pressure tends to increase on decreasing the temperature, adsorption measurements are usually made at a low temperature. Measurement is performed at -196°C , the boiling point of liquid nitrogen.

III.2.1. Method I: the dynamic flow method

III.2.1.1. Principle of the method

In the dynamic flow method (see Figure 2.9.26-1), the recommended adsorbate gas is dry nitrogen or krypton, while helium is employed as a diluent gas, which is not adsorbed under the recommended conditions.

A minimum of three mixtures of the appropriate adsorbate gas with helium are required within the P/P_0 range 0.05 to 0.30.

The gas detector-integrator should provide a signal that is approximately proportional to the volume of the gas passing through it under defined conditions of temperature and pressure. For this purpose, a thermal conductivity detector with an electronic integrator is one among various suitable types. A minimum of three data points within the recommended range of 0.05 to 0.30 for P/P_0 is to be determined.

III.2.1.2. Procedure

A known mixture of the gases, usually nitrogen and helium, is passed through a thermal conductivity cell, through the sample again through the thermal conductivity cell and then to a recording potentiometer.

Immerse the sample cell in liquid nitrogen, then the sample adsorbs nitrogen from the mobile phase. This unbalances the thermal conductivity cell, and a pulse is generated on a recorder chart.

Remove from the coolant; this gives a desorption peak equal in area and in the opposite direction to the adsorption peak. Since this is better defined than the adsorption peak, it is the one used for the determination.

To effect the calibration, inject sufficient air into the system to give a peak of similar magnitude to the desorption peak and obtain the proportion of gas adsorbed per unit peak area (air can be used instead of nitrogen since it has the same thermal conductivity).

Use a nitrogen/helium mixture for a single-point determination and several such mixtures or premixing two streams of gas for a multiple-point determination.

Calculation is essentially the same as for the volumetric method.

III.2.2. Method II: the volumetric method

III.2.2.1. Principle of the method

In the volumetric method (see Figure 2.9.26-2), the recommended adsorbate gas is nitrogen which is admitted into the evacuated space above the previously outgassed powder sample to give a defined equilibrium pressure, P , of

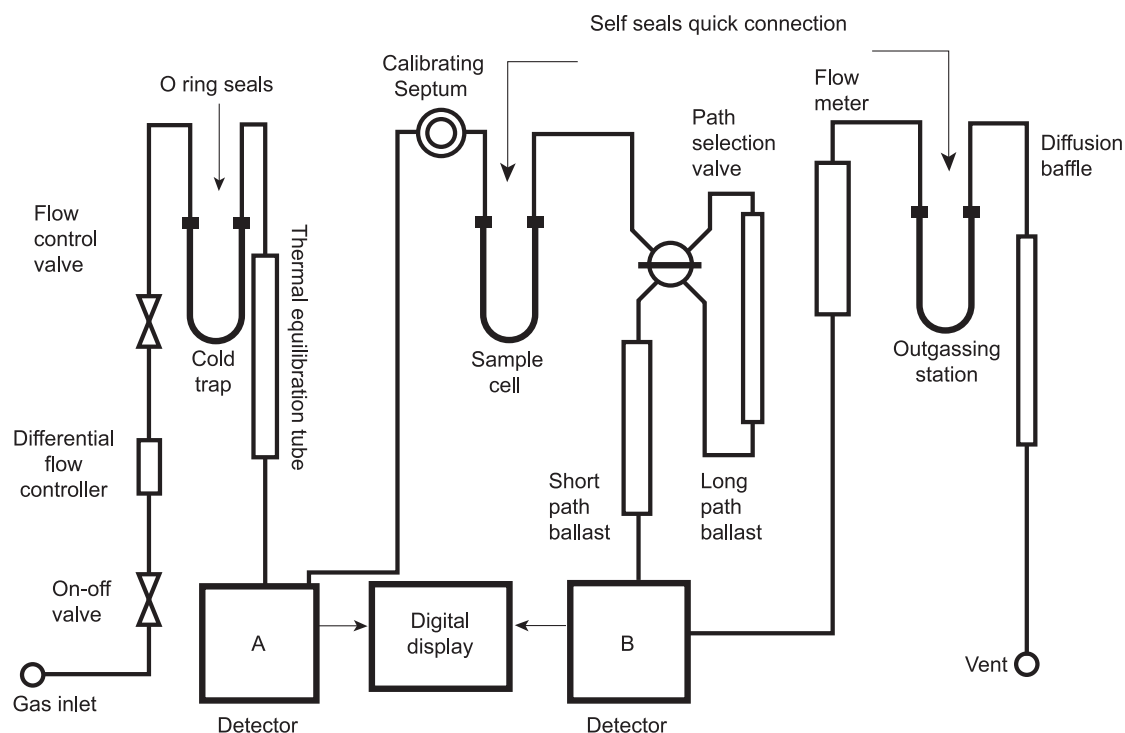


Figure 2.9.26-1. – Schematic diagram of the dynamic flow method apparatus

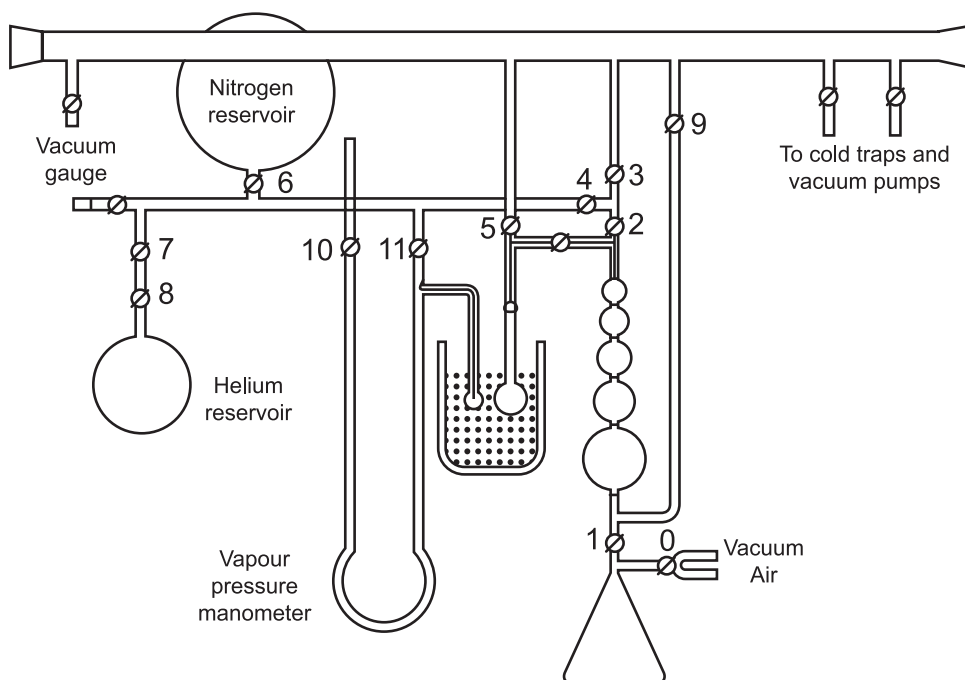


Figure 2.9.26.-2. – Schematic diagram of the volumetric method apparatus

the gas. The use of a diluent gas, such as helium, is therefore unnecessary, although helium may be employed for other purposes, such as to measure the void volume.

Since only pure adsorbate gas, instead of a gas mixture, is employed, interfering effects of thermal diffusion are avoided in this method.

III.2.2.2. Procedure

Admit a small amount of dry nitrogen into the sample tube to prevent contamination of the clean surface, remove the sample tube, insert the stopper, and weigh it. Calculate the weight of the sample. Attach the sample tube to the volumetric apparatus. Cautiously evacuate the sample down to a pressure of 2.66 Pa or less.

If the principle of operation of the instrument requires the determination of the void volume in the sample tube, for example, by the admission of a non-adsorbed gas, such as helium, this procedure is carried out at this point, followed by evacuation of the sample down to 2.66 Pa or less. The adsorption of nitrogen gas is then measured as described below.

Raise a Dewar vessel containing liquid nitrogen at $-196\text{ }^{\circ}\text{C}$ up to a defined point on the sample cell. Admit a sufficient volume of nitrogen gas to give a relative pressure, P/P_0 , equal to 0.10 ± 0.02 . Measure the volume adsorbed, V_a . Repeat the measurement of V_a at P/P_0 values of 0.20 ± 0.02 and 0.30 ± 0.02 .

A minimum of three data points is required. Additional measurements may be carried out, especially on those rare occasions when non-linearity is obtained at a P/P_0 value close to 0.3. Since non-linearity is often obtained at P/P_0 or below 0.05, values in this region are not recommended. The test for linearity, the treatment of the data, and the calculation of the specific surface area of the sample are described above.

IV. REFERENCE MATERIALS

Periodically verify the functioning of the apparatus using appropriate reference materials of known surface area which should have a specific surface area similar to that of the sample to be examined.

01/2005:20927

2.9.27. UNIFORMITY OF MASS OF DELIVERED DOSES FROM MULTIDOSE CONTAINERS

The following test is intended for oral dosage forms such as granules, powders for oral use and liquids for oral use, which are supplied in multidose containers provided at manufacture with a measuring device.

Weigh individually 20 doses taken at random from one or more containers with the measuring device provided and determine the individual and average masses. Not more than 2 of the individual masses deviate from the average mass by more than 10 per cent and none deviates by more than 20 per cent.

01/2005:20928

2.9.28. TEST FOR DELIVERABLE MASS OR VOLUME OF LIQUID AND SEMI-SOLID PREPARATIONS

The test applies to liquid (solutions, emulsions and suspensions) and semi-solid preparations supplied in single-dose containers where only part of the contents is used.

LIQUID PREPARATIONS

Empty as completely as possible the contents of one container and determine the mass or volume of the contents as appropriate. In the case of emulsions and suspensions, shake the container before the determination. The mass or volume is not less than the amount stated on the label.

SEMI-SOLID PREPARATIONS

Empty as completely as possible the contents of one container. The mass of the contents is not less than that which is stated on the label.

01/2005:30100

3.1. MATERIALS USED FOR THE MANUFACTURE OF CONTAINERS

The materials described in this chapter are used for the manufacture of containers for pharmaceutical use. Their use may also be considered for the manufacture of part or all of objects used for medico-surgical purposes.

Materials and polymers other than those described in the Pharmacopoeia may be used subject to approval in each case by the competent authority responsible for the licensing for sale of the preparation in the container.

01/2005:30101

3.1.1. MATERIALS FOR CONTAINERS FOR HUMAN BLOOD AND BLOOD COMPONENTS

NOTE: for materials based on plasticised poly(vinyl chloride) for containers for aqueous solutions for intravenous infusion, see text 3.1.14.

Plastic containers for the collection, storage, processing and administration of blood and its components may be manufactured from one or more polymers, if necessary with certain additives.

If all or part of the container consists of a material described in a text of the Pharmacopoeia, the quality of the material is controlled by the methods indicated in that text. (See 3.1.1.1. *Materials based on plasticised poly(vinyl chloride) for containers for human blood and blood components*).

In normal conditions of use the materials and containers made from such materials do not release monomers, or other substances, in amounts likely to be harmful nor do they lead to any abnormal modifications of the blood or blood components.

01/2005:90001

3.1.1.1. MATERIALS BASED ON PLASTICISED POLY(VINYL CHLORIDE) FOR CONTAINERS FOR HUMAN BLOOD AND BLOOD COMPONENTS

DEFINITION

Materials based on plasticised poly(vinyl chloride) contain not less than 55 per cent of poly(vinyl chloride) and contain various additives, in addition to the high-molecular-mass polymer obtained by polymerisation of vinyl chloride.

Materials based on plasticised poly(vinyl chloride) for containers for human blood and blood components are defined by the nature and the proportions of the substances used in their manufacture.

PRODUCTION

Materials based on plasticised poly(vinyl chloride) are produced by polymerisation methods which guarantee a residual vinyl chloride content of less than 1 ppm. The production method used is validated in order to demonstrate that the product complies with the following test:

Vinyl chloride. Not more than 1 ppm, determined by head-space gas chromatography (2.2.28), using *ether R* as the internal standard.

Internal standard solution. Using a microsyringe, inject 10 µl of *ether R* into 20.0 ml of *dimethylacetamide R*, immersing the tip of the needle in the solvent. Immediately before use, dilute the solution to 1000 times its volume with *dimethylacetamide R*.

Test solution. Place 1.000 g of the material to be examined in a 50 ml vial and add 10.0 ml of the internal standard solution. Close the vial and secure the stopper. Shake, avoiding contact between the stopper and the liquid. Place the vial in a water-bath at $60 \pm 1^\circ\text{C}$ for 2 h.

Vinyl chloride primary solution. Prepare under a ventilated hood. Place 50.0 ml of *dimethylacetamide R* in a 50 ml vial, stopper the vial, secure the stopper and weigh to the nearest 0.1 mg. Fill a 50 ml polyethylene or polypropylene syringe with gaseous *vinyl chloride R*, allow the gas to remain in contact with the syringe for about 3 min, empty the syringe and fill again with 50 ml of gaseous *vinyl chloride R*. Fit a hypodermic needle to the syringe and reduce the volume of gas in the syringe from 50 ml to 25 ml. Inject the remaining 25 ml of vinyl chloride slowly into the vial shaking gently and avoiding contact between the liquid and the needle. Weigh the vial again; the increase in mass is about 60 mg (1 µl of the solution thus obtained contains about 1.2 µg of vinyl chloride). Allow to stand for 2 hours. Keep the primary solution in a refrigerator.

Vinyl chloride standard solution. To 1 volume of the vinyl chloride primary solution add 3 volumes of *dimethylacetamide R*.

Reference solutions. Place 10.0 ml of the internal standard solution in each of six 50 ml vials. Close the vials and secure the stoppers. Inject 1 µl, 2 µl, 3 µl, 5 µl and 10 µl, respectively, of the vinyl chloride standard solution into five of the vials. The six solutions thus obtained contain, respectively, 0 µg, about 0.3 µg, 0.6 µg, 0.9 µg, 1.5 µg and 3 µg of vinyl chloride. Shake, avoiding contact between the stopper and the liquid. Place the vials in a water-bath at $60 \pm 1^\circ\text{C}$ for 2 h.

The chromatographic procedure may be carried out using:

- a stainless steel column 3 m long and 3 mm in internal diameter packed with *silanised diatomaceous earth for gas chromatography R* impregnated with 5 per cent *m/m* of *dimethylstearylamine R* and 5 per cent *m/m* of *macrogol 400 R*,
 - *nitrogen for chromatography R* as the carrier gas at a flow rate of 30 ml/min,
 - a flame-ionisation detector,
- maintaining the temperature of the column at 45°C , that of the injection port at 100°C and that of the detector at 150°C .

Inject 1 ml of the head-space of each vial. Calculate the content of vinyl chloride.

Additives

A certain number of additives are added to the polymers to optimise their chemical, physical and mechanical properties in order to adapt them for the intended use. All these additives are chosen from the following list which specifies for each product the maximum allowable content:

- not more than 40 per cent of di(2-ethylhexyl)phthalate (plastic additive 01),
- not more than 1 per cent of zinc octanoate (zinc 2-ethylhexanoate) (plastic additive 02),
- not more than 1 per cent of calcium stearate or zinc stearate or 1 per cent of a mixture of the two,

- not more than 1 per cent of *N,N'*-diacylethylenediamines (plastic additive 03),
- not more than 10 per cent of one of the following epoxidised oils or 10 per cent of a mixture of the two:
 - epoxidised soya oil (plastic additive 04), of which the oxiran oxygen content is 6 per cent to 8 per cent and the iodine value is not greater than 6,
 - epoxidised linseed oil (plastic additive 05), of which the oxiran oxygen content is not greater than 10 per cent and the iodine value is not greater than 7.

Very low amounts of antioxidants added to the vinyl chloride monomer may be detected in the polymer.

No antioxidant additive may be added to the polymer.

Ultramarine blue is the only colouring material permitted to be added.

The supplier of the material must be able to demonstrate that the qualitative and quantitative composition of the type sample is satisfactory for each production batch.

CHARACTERS

Colourless or pale yellow powder, beads, granules or, after transformation, translucent sheets of varying thickness or containers, with a slight odour. On combustion it gives off dense, black smoke.

IDENTIFICATION

If necessary, before use, cut the samples of the material to be examined into pieces of maximum dimension on a side of not greater than 1 cm.

To 2.0 g of the material to be examined add 200 ml of *peroxide-free ether R* and heat under a reflux condenser for 8 h. Separate the residue B and the solution A by filtration.

Evaporate solution A to dryness under reduced pressure in a water-bath at 30 °C. Dissolve the residue in 10 ml of *toluene R* (solution A1). Dissolve the residue B in 60 ml of *ethylene chloride R*, heating on a water-bath under a reflux condenser. Filter. Add the solution dropwise and with vigorous shaking to 600 ml of *heptane R* heated almost to boiling. Separate the coagulum B1 and the organic solution by hot filtration. Allow the latter to cool; separate the precipitate B2 that forms and filter through a tared sintered-glass filter (40).

A. Dissolve the coagulum B1 in 30 ml of *tetrahydrofuran R* and add, in small volumes with shaking, 40 ml of *ethanol R*. Separate the precipitate B3 by filtration and dry *in vacuo* at a temperature not exceeding 50 °C over *diphosphorus pentoxide R*. Dissolve a few milligrams of precipitate B3 in 1 ml of *tetrahydrofuran R*, place a few drops of the solution obtained on a sodium chloride plate and evaporate to dryness in an oven at 100 °C to 105 °C. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *poly(vinyl chloride) CRS*.

B. Examine the residue C obtained in the test for plastic additives 01, 04 and 05 by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *plastic additive 01 CRS*.

TESTS

If necessary, before use, cut samples of the material to be examined into pieces of maximum dimension on a side of not greater than 1 cm.

Solution S1. Place 5.0 g of the material to be examined in a combustion flask. Add 30 ml of *sulphuric acid R* and heat until a black, syrupy mass is obtained. Cool and add carefully 10 ml of *strong hydrogen peroxide solution R*. Heat gently. Allow to cool and add 1 ml of *strong hydrogen*

peroxide solution R; repeat by alternating evaporation and addition of hydrogen peroxide solution until a colourless liquid is obtained. Reduce the volume to about 10 ml. Cool and dilute to 50.0 ml with *water R*.

Solution S2. Place 25 g of the material to be examined in a borosilicate-glass flask. Add 500 ml of *water for injections R* and cover the neck of the flask with a borosilicate-glass beaker. Heat in an autoclave at 121 ± 2 °C for 20 min. Allow to cool and decant the solution. Make the volume up to 500 ml.

Appearance of solution S2. Solution S2 is clear (2.2.1) and colourless (2.2.2, *Method II*).

Acidity or alkalinity. To 100 ml of solution S2, add 0.15 ml of *BRP indicator solution R*. Not more than 1.5 ml of 0.01 M *sodium hydroxide* is required to change the colour of the indicator to blue. To 100 ml of solution S2 add 0.2 ml of *methyl orange solution R*. Not more than 1.0 ml of 0.01 M *hydrochloric acid* is required to initiate the colour change of the indicator from yellow to orange.

Absorbance (2.2.25). Evaporate 100.0 ml of solution S2 to dryness. Dissolve the residue in 5.0 ml of *hexane R*. From 250 nm to 310 nm the absorbance is not greater than 0.25.

Reducing substances. Carry out the test within 4 h of preparation of solution S2. To 20.0 ml of solution S2 add 1 ml of *dilute sulphuric acid R* and 20.0 ml of 0.002 M *potassium permanganate*. Boil under a reflux condenser for 3 min and cool immediately. Add 1 g of *potassium iodide R* and titrate immediately with 0.01 M *sodium thiosulphate*, using 0.25 ml of *starch solution R* as indicator. Carry out a blank titration using 20 ml of *water for injections R*. The difference between the two titration volumes is not more than 2.0 ml.

Primary aromatic amines. To 2.5 ml of solution A1 obtained during the identification, add 6 ml of *water R* and 4 ml of 0.1 M *hydrochloric acid*. Shake vigorously and discard the upper layer. To the aqueous layer add 0.4 ml of a freshly prepared 10 g/l solution of *sodium nitrite R*. Mix and allow to stand for 1 min. Add 0.8 ml of a 25 g/l solution of *ammonium sulphamate R*, allow to stand for 1 min and add 2 ml of a 5 g/l solution of *naphthylethylenediamine dihydrochloride R*. After 30 min, any colour in the solution is not more intense than that in a standard prepared at the same time in the same manner replacing the aqueous layer with a mixture of 1 ml of a 0.01 g/l solution of *naphthylamine R* in 0.1 M *hydrochloric acid*, 5 ml of *water R* and 4 ml of 0.1 M *hydrochloric acid* instead of the aqueous layer (20 ppm).

Plastic additives 01, 04 and 05. Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel GF₂₅₄ plate R* (1 mm thick).

Reference solutions. Prepare 0.1 mg/ml solutions of *plastic additive 01 CRS*, *plastic additive 04 CRS* and *plastic additive 05 CRS*, respectively, in *toluene R*.

Apply to the plate as a band 30 mm by 3 mm, 0.5 ml of solution A1 obtained during the identification. Apply to the plate 5 µl of each reference solution. Develop over a path of 15 cm using *toluene R*. Dry the plate carefully. Examine in ultraviolet light at 254 nm and locate the zone corresponding to plastic additive 01 (R_f about 0.4). Remove the area of silica gel corresponding to this zone and shake with 40 ml of *ether R* for 1 min. Filter, rinse with two quantities, each of 10 ml of *ether R*, add the rinsings to the filtrate and evaporate to dryness. The residue C weighs not more than 40 mg.

Expose the plate to iodine vapour for 5 min. Examine the chromatogram and locate the band corresponding to plastic additives 04 and 05 (R_f = 0). Remove the area of

silica gel corresponding to this zone. Similarly remove a corresponding area of silica gel as a blank reference. Separately shake both samples for 15 min with 40 ml of *methanol R*. Filter, rinse with two quantities, each of 10 ml of *methanol R*, add the rinsings to the filtrate and evaporate to dryness. The difference between the masses of both residues is not more than 10 mg.

Plastic additive 03. Wash precipitate B2 obtained during the identification and contained in the tared sintered-glass filter (40) with *ethanol R*. Dry to constant mass over *diphosphorus pentoxide R* and weigh the filter. The precipitate weighs not more than 20 mg.

Examine the residue by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *plastic additive 03 CRS*.

Barium. Not more than 5 ppm of Ba, examined by atomic emission spectrometry in an argon plasma (2.2.22, *Method I*).

Test solution. Ignite 1.0 g of the substance to be examined in a silica crucible. Take up the residue with 10 ml of *hydrochloric acid R* and evaporate to dryness on a water-bath. Take up the residue with 20 ml of 0.1 M *hydrochloric acid*.

Reference solution. A solution containing 0.25 ppm of barium prepared by dilution of *barium standard solution (50 ppm Ba) R* with 0.1 M *hydrochloric acid*.

Carry out the determination using the emission of barium at 455.40 nm, the spectral background being taken at 455.30 nm.

Verify the absence of barium in the hydrochloric acid used.

Cadmium. Not more than 0.6 ppm of Cd, determined by atomic absorption spectrometry (2.2.23, *Method I*).

Test solution. Evaporate 10 ml of solution S1 to dryness. Take up the residue using 5 ml of a 1 per cent V/V solution of *hydrochloric acid R*, filter and dilute the filtrate to 10.0 ml with the same acid solution.

Reference solutions. Prepare the reference solutions using *cadmium standard solution (0.1 per cent Cd) R*, diluted with a 1 per cent V/V solution of *hydrochloric acid R*.

Measure the absorbance at 228.8 nm using a cadmium hollow-cathode lamp as the source of radiation and an air-acetylene flame.

Verify the absence of cadmium in the hydrochloric acid used.

Calcium. Not more than 0.07 per cent of Ca, examined by atomic emission spectrometry in an argon plasma (2.2.22, *Method I*).

Test solution. Use the test solution prepared for the determination of barium.

Reference solution. A solution containing 50.0 ppm of calcium prepared by dilution of *calcium standard solution (400 ppm Ca) R* with 0.1 M *hydrochloric acid*.

Carry out the determination using the emission of calcium at 315.89 nm, the spectral background being taken at 315.60 nm.

Verify the absence of calcium in the hydrochloric acid used.

Tin. Not more than 20 ppm of Sn, examined by atomic emission spectrometry in an argon plasma (2.2.22, *Method I*).

Test solution. Dilute solution S1 ten times with *water R* immediately before use.

Reference solution. Introduce 2 ml of *tin standard solution (5 ppm (Sn) R)* into a 50 ml flask containing 5 ml of a 20 per cent V/V solution of *sulphuric acid R* and dilute to 50 ml with *water R* immediately before use.

Carry out the determination using the emission of tin at 189.99 nm, the spectral background being taken at 190.10 nm.

Verify the absence of tin in the sulphuric acid used.

Zinc. Not more than 0.2 per cent of Zn, determined by atomic absorption spectrometry (2.2.23, *Method I*).

Test solution. Dilute solution S1 100 times with 0.1 M *hydrochloric acid*.

Reference solutions. Prepare the reference solutions using *zinc standard solution (100 ppm Zn) R*, diluted with 0.1 M *hydrochloric acid*.

Measure the absorbance at 213.9 nm using a zinc hollow-cathode lamp as the source of radiation and an air-acetylene flame.

Verify the absence of zinc in the hydrochloric acid used.

Heavy metals (2.4.8). To 10 ml of solution S1 add 0.5 ml of *phenolphthalein solution R* and then *strong sodium hydroxide solution R* until a pale pink colour is obtained. Dilute to 25 ml with *water R*. 12 ml of the solution complies with limit test A (50 ppm). Prepare the standard using *lead standard solution (2 ppm Pb) R*.

Water extractable substances. Evaporate 50 ml of solution S2 to dryness on a water-bath and dry in an oven at 100-105 °C to constant mass. Carry out a blank test with 50.0 ml of *water for injections R*. The residue weighs not more than 7.5 mg (0.3 per cent) taking into account the blank test.

ASSAY

Carry out the oxygen-flask method (2.5.10) using 50.0 mg. Absorb the combustion products in 20 ml of 1 M *sodium hydroxide*. To the solution obtained add 2.5 ml of *nitric acid R*, 10.0 ml of 0.1 M *silver nitrate*, 5 ml of *ferric ammonium sulphate solution R2* and 1 ml of *dibutyl phthalate R*. Titrate with 0.05 M *ammonium thiocyanate* until a reddish-yellow colour is obtained. Carry out a blank test.

1 ml of 0.1 M *silver nitrate* is equivalent to 6.25 mg of poly(vinyl chloride).

In addition, the following tests are carried out on the sterile and empty containers.

Solution S3. If the container to be examined contains an anticoagulant solution, empty the container and wash the inside with 250 ml of *water for injections R* at 20 ± 1 °C and discard the washings before the preparation of solution S3. Introduce into the container a volume of *water for injections R* corresponding to the volume of solution. Close the container and heat in an autoclave so that the temperature of the liquid is maintained at 110 °C for 30 min. After cooling, fill the container with *water for injections R* to its nominal volume and homogenise.

Reference solution. Heat *water for injections R* in a borosilicate-glass flask in an autoclave at 110 °C for 30 min.

Reducing substances. Immediately after preparation of solution S3, transfer to a borosilicate-glass flask a volume corresponding to 8 per cent of the nominal volume of the container. At the same time, prepare a blank using an equal volume of the freshly prepared reference solution in another borosilicate-glass flask. To each solution add 20.0 ml of 0.002 M *potassium permanganate* and 1 ml of *dilute sulphuric acid R*. Allow to stand protected from light for 15 min. To each solution add 0.1 g of *potassium iodide R*. Allow to stand protected from light for 5 min and titrate immediately with 0.01 M *sodium thiosulphate*, using 0.25 ml of *starch solution R* as indicator. The difference between the two titrations is not more than 2.0 ml.

Acidity or alkalinity. To a volume of solution S3 corresponding to 4 per cent of the nominal capacity of the container add 0.1 ml of *phenolphthalein solution R*. The solution remains colourless. Add 0.4 ml of 0.01 M sodium hydroxide. The solution is pink. Add 0.8 ml of 0.01 M hydrochloric acid and 0.1 ml of *methyl red solution R*. The solution is orange-red or red.

Chlorides (2.4.4). 15 ml of solution S3 complies with the limit test for chlorides (0.4 ppm). Prepare the standard using a mixture of 1.2 ml of *chloride standard solution (5 ppm Cl) R* and 13.8 ml of *water R*.

Ammonium (2.4.1). Dilute 5 ml of solution S3 to 14 ml with *water R*. The solution complies with limit test A (2 ppm).

Water extractable substances. Evaporate 100 ml of solution S3 to dryness on a water-bath. Dry in an oven to constant mass at 100-105 °C. Carry out a blank test using 100 ml of the reference solution. The residue from solution S3 weighs not more than 3 mg, taking into account the blank test.

Absorbance (2.2.25). Measure the absorbance of solution S3 from 230 nm to 360 nm, using the reference solution as compensation liquid. At wavelengths from 230 nm to 250 nm, the absorbance is not greater than 0.30. At wavelengths from 251 nm to 360 nm, the absorbance is not greater than 0.10.

Extractable plastic additive 01. Use as the extraction solvent, *alcohol R* diluted with *water R* to have a relative density (2.2.5) of 0.9389 to 0.9395, measured with a densimeter.

Stock solution. Dissolve 0.100 g of *plastic additive 01 CRS* in the extraction solvent and dilute to 100.0 ml with the same solvent.

Standard solutions:

- (a) Dilute 20.0 ml of the stock solution to 100.0 ml with the extraction solvent,
- (b) Dilute 10.0 ml of the stock solution to 100.0 ml with the extraction solvent,
- (c) Dilute 5.0 ml of the stock solution to 100.0 ml with the extraction solvent,
- (d) Dilute 2.0 ml of the stock solution to 100.0 ml with the extraction solvent,
- (e) Dilute 1.0 ml of the stock solution to 100.0 ml with the extraction solvent.

Measure the absorbances (2.2.25) of the standard solutions at the maximum at 272 nm, using the extraction solvent as compensation liquid and plot a curve of absorbance against the concentration of plastic additive 01.

Extraction procedure. Using the donor tubing and the needle or adapter, fill the empty container with a volume equal to half the nominal volume with the extraction solvent, previously heated to 37 °C in a well-stoppered flask. Expel the air completely from the container and seal the donor tubing. Immerse the filled container in a horizontal position in a water-bath maintained at 37 ± 1 °C for 60 ± 1 min without shaking. Remove the container from the water-bath, invert it gently ten times and transfer the contents to a glass flask. Immediately measure the absorbance at the maximum at 272 nm, using the extraction solvent as compensation liquid.

Determine the concentration of plastic additive 01 in milligrams per 100 ml of extract from the calibration curve. The concentration does not exceed:

- 10 mg per 100 ml for containers of nominal volume greater than 300 ml but not greater than 500 ml;
- 13 mg per 100 ml for containers of nominal volume greater than 150 ml but not greater than 300 ml;

- 14 mg per 100 ml for containers of nominal volume up to 150 ml.

Where containers contain an anticoagulant solution, this solution complies with the monograph on *Anticoagulant and preservative solutions for human blood (0209)* and the following additional test.

Absorbance (2.2.25). Measure the absorbance of the anticoagulant solution from the container between 250 nm and 350 nm, using as the compensation liquid an anticoagulant solution of the same composition that has not been in contact with a plastic material. The absorbance at the maximum at 280 nm is not greater than 0.5.

01/2005:90002

3.1.1.2. MATERIALS BASED ON PLASTICISED POLY(VINYL CHLORIDE) FOR TUBING USED IN SETS FOR THE TRANSFUSION OF BLOOD AND BLOOD COMPONENTS

DEFINITION

Materials based on plasticised poly(vinyl chloride) for transfusion of blood and blood components contain not less than 55 per cent of poly(vinyl chloride) with di(2-ethylhexyl) phthalate (plastic additive 01) as plasticiser.

PRODUCTION

Materials based on plasticised poly(vinyl chloride) are produced by polymerisation methods which guarantee a residual vinyl chloride content of less than 1 ppm. The production method used is validated in order to demonstrate that the product complies with the following test:

Vinyl chloride. Not more than 1 ppm, determined by head-space gas chromatography (2.2.28), using *ether R* as the internal standard.

Internal standard solution. Using a microsyringe, inject 10 µl of *ether R* into 20.0 ml of *dimethylacetamide R*, immersing the tip of the needle in the solvent. Immediately before use, dilute the solution to 1000 times its volume with *dimethylacetamide R*.

Test solution. Place 1.000 g of the material to be examined in a 50 ml vial and add 10.0 ml of the internal standard solution. Close the vial and secure the stopper. Shake, avoiding contact between the stopper and the liquid. Place the vial in a water-bath at 60 ± 1 °C for 2 h.

Vinyl chloride primary solution. Prepare under a ventilated hood. Place 50.0 ml of *dimethylacetamide R* in a 50 ml vial, stopper the vial, secure the stopper and weigh to the nearest 0.1 mg. Fill a 50 ml polyethylene or polypropylene syringe with gaseous *vinyl chloride R*, allow the gas to remain in contact with the syringe for about 3 min, empty the syringe and fill again with 50 ml of gaseous *vinyl chloride R*. Fit a hypodermic needle to the syringe and reduce the volume of gas in the syringe from 50 ml to 25 ml. Inject the remaining 25 ml of vinyl chloride slowly into the vial shaking gently and avoiding contact between the liquid and the needle. Weigh the vial again; the increase in mass is about 60 mg (1 µl of the solution thus obtained contains about 1.2 µg of vinyl chloride). Allow to stand for 2 h. Keep the primary solution in a refrigerator.

Vinyl chloride standard solution. To 1 volume of the vinyl chloride primary solution add 3 volumes of *dimethylacetamide R*.

Reference solutions. Place 10.0 ml of the internal standard solution in each of six 50 ml vials. Close the vials and secure the stoppers. Inject 1 µl, 2 µl, 3 µl, 5 µl and 10 µl, respectively, of the vinyl chloride standard solution into five of the vials. The six solutions thus obtained contain respectively, 0 µg, about 0.3 µg, 0.6 µg, 0.9 µg, 1.5 µg and 3 µg of vinyl chloride. Shake, avoiding contact between the stopper and the liquid. Place the vials in a water-bath at 60 ± 1 °C for 2 h.

The chromatographic procedure may be carried out using:

- a stainless steel column 3 m long and 3 mm in internal diameter packed with *silanised diatomaceous earth for gas chromatography R* impregnated with 5 per cent *m/m* of *dimethylstearylamine R* and 5 per cent *m/m* of *macrogol 400 R*,
- *nitrogen for chromatography R* as the carrier gas at a flow rate of 30 ml/min,
- a flame-ionisation detector,

maintaining the temperature of the column at 45 °C, that of the injection port at 100 °C and that of the detector at 150 °C.

Inject 1 ml of the head-space of each vial. Calculate the content of vinyl chloride.

The supplier of the material must be able to demonstrate that the qualitative and quantitative composition of the type sample is satisfactory for each production batch.

CHARACTERS

Almost colourless or pale-yellow material in the form of powder, beads, granules or, after transformation, tubes with a slight odour. On combustion it gives off dense, black smoke.

IDENTIFICATION

If necessary, cut samples of the material to be examined into pieces of maximum dimension on a side of not greater than 1 cm.

- A. To 0.5 g add 30 ml of *tetrahydrofuran R*. Heat with stirring on a water-bath under a hood for 10 min. The material dissolves completely. Add *methanol R* dropwise with stirring. A granular precipitate is formed. Filter the precipitate and dry at 60 °C. Examine the precipitate by infrared absorption spectrophotometry (2.2.24). Dissolve 50 mg in 2 ml of *tetrahydrofuran R* and pour on a glass slide. Dry in an oven at 80 °C, remove the film and fix on a suitable mount. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *poly(vinyl chloride) CRS*.
- B. Examine the residue obtained in the test Plastic additive 01 by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *plastic additive 01 CRS*.

TESTS

If necessary, cut the material to be examined into pieces with a maximum dimension on a side of not greater than 1 cm.

Solution S1. Place 5.0 g of the material to be examined in a combustion flask. Add 30 ml of *sulphuric acid R* and heat until a black, syrupy mass is obtained. Cool and add carefully 10 ml of *strong hydrogen peroxide solution R*. Heat gently. Allow to cool and add 1 ml of *strong hydrogen peroxide solution R*; repeat by alternating evaporation and addition of hydrogen peroxide solution until a colourless liquid is obtained. Reduce the volume to about 10 ml. Cool and dilute to 50.0 ml with *water R*.

Solution S2. Place 25 g of the material to be examined in a borosilicate-glass flask. Add 500 ml of *water R* and cover the neck of the flask with a borosilicate-glass beaker. Heat in an autoclave at 121 ± 2 °C for 20 min. Allow to cool and decant the solution and make up to a volume of 500 ml.

Appearance of solution S2. Solution S2 is clear (2.2.1) and colourless (2.2.2, *Method II*).

Plastic additive 01. Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel G plate R*.

Test solution. To 2.0 g of the material to be examined add 200 ml of *peroxide-free ether R* and heat under a reflux condenser for 8 h. Separate the residue and the solution by filtration and evaporate the solution to dryness under reduced pressure in a water-bath at 30 °C. Dissolve the residue in 10 ml of *toluene R*.

Reference solution. Dissolve 0.8 g of *plastic additive 01 CRS* in *toluene R* and dilute to 10 ml with the same solvent.

Apply separately to the plate as a band 30 mm by 3 mm 0.5 ml of the test solution and 5 µl of the reference solution. Develop over a path of 15 cm using *toluene R*. Dry the plate carefully. Examine the chromatogram obtained in ultraviolet light at 254 nm and locate the zone corresponding to plastic additive 01. Remove the area of silica gel corresponding to this zone and shake with 40 ml of *ether R*. Filter without loss and evaporate to dryness. The residue weighs not more than 40 mg.

Barium. Not more than 5 ppm of Ba, examined by atomic emission spectrometry in an argon plasma (2.2.22, *Method I*).

Test solution. Ignite 1.0 g of the substance to be examined in a silica crucible. Take up the residue with 10 ml of *hydrochloric acid R* and evaporate to dryness on a water-bath. Take up the residue with 20 ml of 0.1 M *hydrochloric acid*.

Reference solution. A solution containing 0.25 ppm of barium prepared by dilution of *barium standard solution (50 ppm Ba) R* with 0.1 M *hydrochloric acid*.

Carry out the determination using the emission of barium at 455.40 nm, the spectral background being taken at 455.30 nm.

Verify the absence of barium in the hydrochloric acid used.

Cadmium. Not more than 0.6 ppm of Cd, determined by atomic absorption spectrophotometry (2.2.23, *Method I*).

Test solution. Evaporate 10.0 ml of solution S1 to dryness. Take up the residue using 5 ml of a 1 per cent V/V solution of *hydrochloric acid R*, filter and dilute the filtrate to 10.0 ml with the same acid.

Reference solutions. Prepare the reference solutions using *cadmium standard solution (0.1 per cent Cd) R*, diluted with a 1 per cent V/V solution of *hydrochloric acid R*.

Measure the absorbance at 228.8 nm using a cadmium hollow-cathode lamp as source of radiation and an air-acetylene flame.

Verify the absence of cadmium in the hydrochloric acid used.

Tin. Not more than 20 ppm of Sn, examined by atomic emission spectrometry in an argon plasma (2.2.22, *Method I*).

Test solution. Dilute solution S1 ten times with *water R* immediately before use.

Reference solution. Introduce 2 ml of *tin standard solution (5 ppm Sn) R* into a 50 ml flask containing 5 ml of a 20 per cent V/V solution of *sulphuric acid R* and dilute to 50 ml with *water R* immediately before use.

Carry out the determination using the emission of tin at 189.99 nm, the spectral background being taken at 190.10 nm.

Verify the absence of tin in the sulphuric acid used.

01/2005:30103

Heavy metals (2.4.8). To 10 ml of solution S1 add 0.5 ml of *phenolphthalein solution R* and then *strong sodium hydroxide solution R* until a pale pink colour is obtained. Dilute to 25 ml with *water R*. 12 ml of the solution complies with limit test A for heavy metals (50 ppm). Prepare the standard using *lead standard solution (2 ppm Pb) R*.

ASSAY

To 0.500 g add 30 ml of *tetrahydrofuran R* and heat with stirring on a water-bath under a hood for 10 min. The material dissolves completely. Add 60 ml of *methanol R* dropwise with stirring. A granular precipitate of poly(vinyl chloride) is formed. Allow to stand for a few minutes. Continue addition of *methanol R* until no further precipitation is observed. Transfer to a sintered-glass filter (40), using three small quantities of *methanol R* to aid transfer and to wash the precipitate. Dry the filter and the precipitate to constant mass at 60 °C and weigh.

In addition, carry out the following tests on sterilised sets.

Solution S3. Make a closed circulation system from three sets and a 300 ml borosilicate-glass vessel. Fit to the vessel a thermostat device that maintains the temperature of the liquid in the vessel at 37 ± 1 °C. Circulate 250 ml of *water for injections R* through the system in the direction used for transfusion for 2 h at a rate of 1 litre per hour (for example using a peristaltic pump applied to as short a piece of suitable silicone tubing as possible). Collect the whole of the solution and allow to cool.

Appearance of solution. Solution S3 is clear (2.2.1) and colourless (2.2.2, *Method II*).

Acidity or alkalinity. To 25 ml of solution S3 add 0.15 ml of *BRP indicator solution R*. Not more than 0.5 ml of 0.01 M *sodium hydroxide* is required to change the colour of the indicator to blue. To 25 ml of solution S3 add 0.2 ml of *methyl orange solution R*. Not more than 0.5 ml of 0.01 M *hydrochloric acid* is required to initiate the colour change of the indicator from yellow to orange.

Absorbance (2.2.25). Examined from 230 nm to 250 nm, solution S3 shows no absorbance greater than 0.30. Examined from 251 nm to 360 nm, solution S3 shows no absorbance greater than 0.15.

Reducing substances. Carry out the test within 4 h of preparation of solution S3. To 20.0 ml of solution S3 add 1 ml of *dilute sulphuric acid R* and 20.0 ml of 0.002 M *potassium permanganate*. Boil for 3 min and cool immediately. Add 1 g of *potassium iodide R* and titrate with 0.01 M *sodium thiosulphate* using 0.25 ml of *starch solution R* as indicator. Carry out a blank test using 20 ml of *water for injections R*. The difference between the titration volumes is not greater than 2.0 ml.

Water extractable substances. Evaporate 50.0 ml of solution S3 to dryness on a water-bath and dry to constant mass in an oven at 100 °C to 105 °C. Carry out a blank test using 50.0 ml of *water for injections R*. The residue obtained with solution S3 is not greater than 1.5 mg, taking account of the blank test.

3.1.3. POLYOLEFINES

DEFINITION

Polyolefines are obtained by polymerisation of ethylene or propylene or by copolymerisation of these substances with not more than 25 per cent of higher homologues (C_4 to C_{10}) or of carboxylic acids or of esters. Certain materials may be mixtures of polyolefines.

PRODUCTION

A certain number of additives are added to the polymer in order to optimise their chemical, physical and mechanical properties in order to adapt them for the intended use. All of these additives are chosen from the appended list which specifies for each product the maximum allowable content. They may contain at most 3 antioxidants, one or several lubricants or antiblocking agents as well as titanium dioxide as an opacifying agent when the material must provide protection from light.

- butylhydroxytoluene (plastic additive 07) (not more than 0.125 per cent),
- pentaerythrityl tetrakis[3-(3,5-di-*tert*-butyl-4-hydroxyphenyl)propionate] (plastic additive 09) (not more than 0.3 per cent),
- 1,3,5-tris(3,5-di-*tert*-butyl-4-hydroxybenzyl)-s-triazine-2,4,6(1*H*,3*H*,5*H*)-trione, (plastic additive 13) (not more than 0.3 per cent),
- octadecyl 3-(3,5-di-*tert*-butyl-4-hydroxyphenyl)propionate (plastic additive 11) (not more than 0.3 per cent),
- ethylene bis[3,3-bis[3-(1,1-dimethylethyl)-4-hydroxyphenyl]butanoate] (plastic additive 08) (not more than 0.3 per cent),
- dioctadecyl disulphide (plastic additive 15) (not more than 0.3 per cent),
- 4,4',4''-(2,4,6-trimethylbenzene-1,3,5-triyltrismethylene)trio[2,6-bis(1,1-dimethylethyl)phenol] (plastic additive 10) (not more than 0.3 per cent),
- 2,2'-bis(octadecyloxy)-5,5'-spirobi[1,3,2-dioxaphosphinane] (plastic additive 14) (not more than 0.3 per cent),
- didodecyl 3,3'-thiodipropionate (plastic additive 16) (not more than 0.3 per cent),
- dioctadecyl 3,3'-thiodipropionate (plastic additive 17) (not more than 0.3 per cent),
- tris[2,4-bis(1,1-dimethylethyl)phenyl] phosphite (plastic additive 12) (not more than 0.3 per cent),
- plastic additive 18 (not more than 0.1 per cent),
- copolymer of dimethyl succinate and (4-hydroxy-2,2,6,6-tetramethylpiperidin-1-yl)ethanol (plastic additive 22) (not more than 0.3 per cent).

The total of antioxidant additives listed above does not exceed 0.3 per cent.

- hydrotalcite (not more than 0.5 per cent),
- alkanamides (not more than 0.5 per cent),
- alkenamides (not more than 0.5 per cent),
- sodium silico-aluminate (not more than 0.5 per cent),
- silica (not more than 0.5 per cent),
- sodium benzoate (not more than 0.5 per cent),
- fatty acid esters or salts (not more than 0.5 per cent),
- trisodium phosphate (not more than 0.5 per cent),
- liquid paraffin (not more than 0.5 per cent),
- zinc oxide (not more than 0.5 per cent),

- talc (not more than 0.5 per cent),
- magnesium oxide (not more than 0.2 per cent),
- calcium stearate or zinc stearate or a mixture of both (not more than 0.5 per cent),
- titanium dioxide (not more than 4 per cent).

The supplier of the material must be able to demonstrate that the qualitative and quantitative composition of the type sample is satisfactory for each production batch.

CHARACTERS

Powder, beads, granules or, after transformation, sheets of varying thickness or containers. They are practically insoluble in water, soluble in hot aromatic hydrocarbons, practically insoluble in ethanol, in hexane and in methanol. They soften at temperatures between 65 °C and 165 °C. They burn with a blue flame.

IDENTIFICATION

If necessary, cut samples of the material to be examined into pieces of maximum dimension on a side of not greater than 1 cm.

- To 0.25 g add 10 ml of *toluene R* and boil under a reflux condenser for about 15 min. Place a few drops of the solution obtained on a sodium chloride slide and evaporate the solvent in an oven at 80 °C. Examine by infrared absorption spectrophotometry (2.2.24). The spectrum of the material to be examined shows maxima in particular at some of the following wave-numbers: 2920 cm⁻¹, 2850 cm⁻¹, 1475 cm⁻¹, 1465 cm⁻¹, 1380 cm⁻¹, 1170 cm⁻¹, 735 cm⁻¹, 720 cm⁻¹; the spectrum obtained is identical to the spectrum obtained with the material selected for the type sample. If the material to be examined is in the form of sheets, the identification may be determined directly on a cut piece of suitable size.
- It complies with the supplementary tests corresponding to the additives present.
- In a platinum crucible, mix about 20 mg with 1 g of *potassium hydrogen sulphate R* and heat until completely melted. Allow to cool and add 20 ml of *dilute sulphuric acid R*. Heat gently. Filter the resulting solution. To the filtrate add 1 ml of *phosphoric acid R* and 1 ml of *strong hydrogen peroxide solution R*. If the substance is opacified with titanium dioxide, an orange-yellow colour develops.

TESTS

If necessary, cut samples of the material to be examined into pieces of maximum dimension on a side of not greater than 1 cm.

Solution S1. Use solution S1 within 4 h of preparation. Place 25 g in a borosilicate-glass flask with a ground-glass neck. Add 500 ml of *water for injections R* and boil under a reflux condenser for 5 h. Allow to cool and decant. Reserve a portion of the solution for the test for appearance of solution S1 and filter the rest through a sintered-glass filter (16).

Solution S2. Place 2.0 g in a conical borosilicate-glass flask with a ground-glass neck. Add 80 ml of *toluene R* and boil under a reflux condenser with constant stirring for 90 min. Allow to cool to 60 °C and add with continued stirring 120 ml of *methanol R*. Filter the solution through a sintered-glass filter (16). Rinse the flask and the filter with 25 ml of a mixture of 40 ml of *toluene R* and 60 ml of *methanol R*, add the rinsings to the filtrate and dilute to 250 ml with the same mixture of solvents. Prepare a blank solution.

Solution S3. Place 100 g in a conical borosilicate-glass flask with a ground-glass neck. Add 250 ml of 0.1 M *hydrochloric acid* and boil under a reflux condenser with constant stirring for 1 h. Allow to cool and decant the solution.

Appearance of solution S1. Solution S1 is clear (2.2.1) and colourless (2.2.2, *Method II*).

Acidity or alkalinity. To 100 ml of solution S1, add 0.15 ml of *BRP indicator solution R*. Not more than 1.5 ml of 0.01 M *sodium hydroxide* is required to change the colour of the indicator to blue. To 100 ml of solution S1 add 0.2 ml of *methyl orange solution R*. Not more than 1 ml of 0.01 M *hydrochloric acid* is required to initiate the colour change of the indicator from yellow to orange.

Absorbance (2.2.25). At wavelengths from 220 nm to 340 nm, the absorbance of solution S1 is not greater than 0.2.

Reducing substances. To 20 ml of solution S1 add 1 ml of *dilute sulphuric acid R* and 20 ml of 0.002 M *potassium permanganate*. Boil under a reflux condenser for 3 min and cool immediately. Add 1 g of *potassium iodide R* and titrate immediately with 0.01 M *sodium thiosulphate*, using 0.25 ml of *starch solution R* as indicator. Carry out a blank titration. The difference between the titration volumes is not more than 3.0 ml.

Substances soluble in hexane. Place 10 g in a 250 ml conical borosilicate-glass flask with a ground-glass neck. Add 100 ml of *hexane R* and boil under a reflux condenser for 4 h, stirring constantly. Cool in iced water and filter rapidly (the filtration time must be less than 5 min; if necessary the filtration may be accelerated by applying pressure to the solution) through a sintered-glass filter (16) maintaining the solution at about 0 °C. Evaporate 20 ml of the filtrate in a tared borosilicate-glass dish on a water-bath. Dry the residue in an oven at 100-105 °C for 1 h. The mass of the residue obtained must be within 10 per cent of that of the residue obtained with the type sample and does not exceed 5 per cent.

Extractable aluminium. Not more than 1 ppm of extractable Al, determined by atomic emission spectrometry in an argon plasma (2.2.22, *Method I*).

Test solution. Use solution S3.

Reference solutions. Prepare the reference solutions using *aluminium standard solution (200 ppm Al) R*, diluted with 0.1 M *hydrochloric acid*.

Carry out the determination using the emission of aluminium at 396.15 nm, the spectral background being taken as 396.25 nm.

Verify the absence of aluminium in the hydrochloric acid used.

Extractable titanium. Not more than 1 ppm of extractable Ti, determined by atomic emission spectrometry in an argon plasma (2.2.22, *Method I*).

Test solution. Use solution S3.

Reference solutions. Prepare the reference solutions using *titanium standard solution (100 ppm Ti) R*, diluted with 0.1 M *hydrochloric acid*.

Carry out the determination using the emission of titanium at 336.12 nm, the spectral background being taken as 336.16 nm.

Verify the absence of titanium in the hydrochloric acid used.

Extractable zinc. Not more than 1 ppm of extractable Zn, determined by atomic absorption spectrometry (2.2.23, *Method I*).

Test solution. Use solution S3.

Reference solutions. Prepare the reference solutions using *zinc standard solution (10 ppm Zn) R*, diluted with 0.1 M *hydrochloric acid*.

Measure the absorbance at 213.9 nm using a zinc hollow-cathode lamp as a source of radiation and an air-acetylene flame.

Verify the absence of zinc in the hydrochloric acid used.

Extractable heavy metals (2.4.8). Evaporate 50 ml of solution S3 to about 5 ml on a water-bath and dilute to 20.0 ml with *water R*. 12 ml of the solution complies with limit test A for heavy metals (2.5 ppm). Prepare the standard using 2.5 ml of *lead standard solution (10 ppm Pb) R*.

Sulphated ash (2.4.14). Not more than 1.0 per cent, determined on 5.0 g. This limit does not apply to material that has been opacified with titanium dioxide.

SUPPLEMENTARY TESTS

These tests are to be carried out, in whole or in part, only if required by the stated composition or the use of the material.

Phenolic antioxidants. Examine by liquid chromatography (2.2.29).

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4.6 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (5 µm),
- as mobile phase one of the 4 following mixtures:
 - Mobile phase 1 at a flow rate of 2 ml/min: 30 volumes of *water R*, 70 volumes of *acetonitrile R*,
 - Mobile phase 2 at a flow rate of 1.5 ml/min: 10 volumes of *water R*, 30 volumes of *tetrahydrofuran R*, 60 volumes of *acetonitrile R*,
 - Mobile phase 3 at a flow rate of 1.5 ml/min: 5 volumes of *water R*, 45 volumes of *2-propanol R*, 50 volumes of *methanol R*,
 - Mobile phase 4 at a flow rate of 1.5 ml/min: 20 volumes of *tetrahydrofuran R*, 80 volumes of *acetonitrile R*,
- as detector a spectrophotometer set at 280 nm for mobile phases 1 to 3, and set at 270 nm for mobile phase 4.

The chromatographic system must ensure the following:

- a resolution of not less than 8.0 between the peaks corresponding to plastic additive 07 and plastic additive 08, with mobile phase 1,
- a resolution of not less than 2.0 between the peaks corresponding to plastic additive 09 and plastic additive 10, with mobile phase 2,
- a resolution of not less than 2.0 between the peaks corresponding to plastic additive 11 and plastic additive 12, with mobile phase 3,
- a resolution of not less than 6.0 between the 2 principal peaks (approximate retention times of 3.5 and 5.8) in the chromatogram obtained with plastic additive 18, with mobile phase 4.

Test solution S21. Evaporate 50 ml of solution S2 to dryness *in vacuo* at 45 °C. Dissolve the residue in 5.0 ml of a mixture of equal volumes of *acetonitrile R* and *tetrahydrofuran R*. Prepare a blank solution from the blank solution corresponding to solution S2.

Test solution S22. Evaporate 50 ml of solution S2 to dryness *in vacuo* at 45 °C. Dissolve the residue with 5.0 ml of *methylene chloride R*. Prepare a blank solution from the blank solution corresponding to solution S2.

Test solution S23. Evaporate 50 ml of solution S2 to dryness *in vacuo* at 45 °C. Dissolve the residue in 5.0 ml of a mixture of equal volumes of *acetonitrile R* and a 10 g/l solution of

tert-butylhydroperoxide R in *tetrahydrofuran R*. Close the flask and allow to stand for 1 h. Prepare a blank solution using the blank of solution S2.

Of the following reference solutions, prepare only those that are necessary for the analysis of the phenolic antioxidants stated in the composition of the substance to be examined.

Reference solution (a). Dissolve 25.0 mg of *butylhydroxytoluene CRS* (plastic additive 07) and 60.0 mg of *plastic additive 08 CRS* in 10.0 ml of a mixture of equal volumes of *acetonitrile R* and *tetrahydrofuran R*. Dilute 2.0 ml to 50.0 ml with a mixture of equal volumes of *acetonitrile R* and *tetrahydrofuran R*.

Reference solution (b). Dissolve 60.0 mg of *plastic additive 09 CRS* and 60.0 mg of *plastic additive 10 CRS* in 10.0 ml of a mixture of equal volumes of *acetonitrile R* and *tetrahydrofuran R*. Dilute 2.0 ml to 50.0 ml with a mixture of equal volumes of *acetonitrile R* and *tetrahydrofuran R*.

Reference solution (c). Dissolve 60.0 mg of *plastic additive 11 CRS* and 60.0 mg of *plastic additive 12 CRS* in 10.0 ml of *methylene chloride R*. Dilute 2.0 ml to 50.0 ml with *methylene chloride R*.

Reference solution (d). Dissolve 25.0 mg of *plastic additive 07 CRS* in 10.0 ml of a mixture of equal volumes of *acetonitrile R* and *tetrahydrofuran R*. Dilute 2.0 ml to 50.0 ml with a mixture of equal volumes of *acetonitrile R* and *tetrahydrofuran R*.

Reference solution (e). Dissolve 60.0 mg of *plastic additive 08 CRS* in 10.0 ml of a mixture of equal volumes of *acetonitrile R* and *tetrahydrofuran R*. Dilute 2.0 ml to 50.0 ml with a mixture of equal volumes of *acetonitrile R* and *tetrahydrofuran R*.

Reference solution (f). Dissolve 60.0 mg of *plastic additive 13 CRS* in 10.0 ml of a mixture of equal volumes of *acetonitrile R* and *tetrahydrofuran R*. Dilute 2.0 ml to 50.0 ml with a mixture of equal volumes of *acetonitrile R* and *tetrahydrofuran R*.

Reference solution (g). Dissolve 60.0 mg of *plastic additive 09 CRS* in 10.0 ml of a mixture of equal volumes of *acetonitrile R* and *tetrahydrofuran R*. Dilute 2.0 ml to 50.0 ml with a mixture of equal volumes of *acetonitrile R* and *tetrahydrofuran R*.

Reference solution (h). Dissolve 60.0 mg of *plastic additive 10 CRS* in 10.0 ml of a mixture of equal volumes of *acetonitrile R* and *tetrahydrofuran R*. Dilute 2.0 ml to 50.0 ml with a mixture of equal volumes of *acetonitrile R* and *tetrahydrofuran R*.

Reference solution (i). Dissolve 60.0 mg of *plastic additive 11 CRS* in 10.0 ml of *methylene chloride R*. Dilute 2.0 ml to 50.0 ml with *methylene chloride R*.

Reference solution (j). Dissolve 60.0 mg of *plastic additive 12 CRS* in 10.0 ml of *methylene chloride R*. Dilute 2.0 ml to 50.0 ml with *methylene chloride R*.

Reference solution (k). Dissolve 20.0 mg of *plastic additive 18 CRS* in 10.0 ml of a mixture of equal volumes of *acetonitrile R* and a 10 g/l solution of *tert-butylhydroperoxide R* in *tetrahydrofuran R*. Allow to stand in a closed container for 1 h. Dilute 2.0 ml of the solution to 50.0 ml with a mixture of equal volumes of *acetonitrile R* and *tetrahydrofuran R*.

If the substance to be examined contains plastic additive 07 and/or plastic additive 08, use mobile phase 1 and inject 20 µl of test solution S21, 20 µl of the corresponding blank solution, 20 µl of reference solution (a), and either 20 µl each of reference solutions (d) or (e) or 20 µl each of reference solutions (d) and (e).

If the substance to be examined contains one or more of the following antioxidants:

- plastic additive 09,
- plastic additive 10,
- plastic additive 11,
- plastic additive 12,
- plastic additive 13,

use mobile phase 2 and inject 20 µl of test solution S21, 20 µl of the corresponding blank solution, 20 µl of reference solution (b) and 20 µl of each of the reference solutions of the antioxidants on the list above that are stated in the composition.

If the substance to be examined contains plastic additive 11 and/or plastic additive 12, use mobile phase 3 and inject 20 µl of test solution S22, 20 µl of the corresponding blank solution, 20 µl of reference solution (c), and either 20 µl of reference solution (i) or (j) or 20 µl of reference solutions (i) and (j).

If the substance to be examined contains plastic additive 18, use mobile phase 4 and inject 20 µl of test solution S23, 20 µl of the corresponding blank solution, and 20 µl of reference solution (k).

In all cases, record the chromatogram for 30 min; the chromatograms corresponding to test solutions S21, S22 and S23 only show peaks due to antioxidants stated in the composition and minor peaks that also appear in the chromatograms corresponding to the blank solutions. The areas of the peaks corresponding to test solutions S21, S22 and S23 are less than the corresponding areas of the peaks in the chromatograms obtained with reference solutions (d) to (k).

Non-phenolic antioxidants. Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel GF₂₅₄ plate R*.

Test solution S24. Evaporate 100 ml of solution S2 to dryness *in vacuo* at 45 °C. Dissolve the residue in 2 ml of *acidified methylene chloride R*.

Reference solution (l). Dissolve 60 mg of *plastic additive 14 CRS* in 10 ml of *methylene chloride R*. Dilute 2 ml of the solution to 10 ml with *acidified methylene chloride R*.

Reference solution (m). Dissolve 60 mg of *plastic additive 15 CRS* in 10 ml of *methylene chloride R*. Dilute 2 ml of the solution to 10 ml with *acidified methylene chloride R*.

Reference solution (n). Dissolve 60 mg of *plastic additive 16 CRS* in 10 ml of *methylene chloride R*. Dilute 2 ml of the solution to 10 ml with *acidified methylene chloride R*.

Reference solution (o). Dissolve 60 mg of *plastic additive 17 CRS* in 10 ml of *methylene chloride R*. Dilute 2 ml of the solution to 10 ml with *acidified methylene chloride R*.

Reference solution (p). Dissolve 60 mg of *plastic additive 16 CRS* and 60 mg of *plastic additive 17 CRS* in 10 ml of *methylene chloride R*. Dilute 2 ml of the solution to 10 ml with *acidified methylene chloride R*.

Apply separately to the plate 20 µl of test solution S24, 20 µl of reference solution (p) and 20 µl of each of the reference solutions corresponding to all the phenolic and non-phenolic antioxidants mentioned in the type composition of the material to be examined.

Develop over a path of 18 cm using *hexane R*. Allow the plate to dry. Develop a second time over a path of 17 cm using *methylene chloride R*. Allow the plate to dry and

examine in ultraviolet light at 254 nm. Spray with *alcoholic iodine solution R* and examine in ultraviolet light at 254 nm after 10-15 min. Any spots in the chromatogram obtained with test solution S24 are not more intense than the spots in the corresponding positions in the chromatograms obtained with the reference solutions. The test is not valid unless the chromatogram obtained with reference solution (p) shows 2 clearly separated spots.

Plastic additive 22. Examine by liquid chromatography (2.2.29).

Test solution. Evaporate 25 ml of solution S2 to dryness *in vacuo* at 45 °C. Dissolve the residue in 10 ml of *toluene R* and 10 ml of a 10 g/l solution of *tetrabutylammonium hydroxide R* in a mixture of 35 volumes of *toluene R* and 65 volumes of *ethanol R*. Boil under a reflux condenser for 3 h. Allow to cool and filter if necessary.

Reference solution. Dissolve 30 mg of *plastic additive 22 CRS* in 50 ml of *toluene R*. Add 1 ml of this solution to 25 ml of blank solution S2 and evaporate to dryness *in vacuo* at 45 °C. Dissolve the residue in 10 ml of *toluene R* and 10 ml of a 10 g/l solution of *tetrabutylammonium hydroxide R* in a mixture of 35 volumes of *toluene R* and 65 volumes of *ethanol R*. Boil under a reflux condenser for 3 h. Allow to cool and filter if necessary.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4.6 mm in internal diameter packed with *aminopropylsilyl silica gel for chromatography R* (5 µm),
- as mobile phase at a flow rate of 2 ml/min a mixture of 11 volumes of *ethanol R* and 89 volumes of *hexane R*,
- as detector a spectrophotometer set at 227 nm.

Inject 20 µl of each solution. Record the chromatograms for 10 min. When the chromatograms are recorded in the prescribed conditions the resolution between the peaks corresponding respectively to the “diol” and diluent of the reference solution is at least 7.

In the chromatogram obtained with the test solution, the area of the peak corresponding to the “diol” component from plastic additive 22 is less than the corresponding peak in the chromatogram obtained with the reference solution.

Amides and stearates. Examine by thin-layer chromatography (2.2.27), using 2 plates of the *TLC silica gel GF₂₅₄ plate R* type.

Test solution. Use test solution S24 described in the test for non-phenolic antioxidants.

Reference solution (q). Dissolve 20 mg of stearic acid (*plastic additive 19 CRS*) in 10 ml of *methylene chloride R*.

Reference solution (r). Dissolve 40 mg of oleamide (*plastic additive 20 CRS*) in 20 ml of *methylene chloride R*.

Reference solution (s). Dissolve 40 mg of erucamide (*plastic additive 21 CRS*) in 20 ml of *methylene chloride R*.

Apply to the 2 plates 10 µl of test solution S24. Apply 10 µl of reference solution (q) to the first plate and 10 µl each of reference solutions (r) and (s) to the second plate.

Develop the first plate over a path of 10 cm using a mixture of 25 volumes of *ethanol R* and 75 volumes of *trimethylpentane R*. Allow the plate to dry in air. Spray with a 2 g/l solution of *dichlorophenolindophenol, sodium salt R* in *ethanol R* and heat in an oven at 120 °C for a few minutes to intensify the spots. Any spot corresponding to plastic additive 19 in the chromatogram obtained with test solution S24 is identical in position to (*R_f* about 0.5) but not more intense than the spot in the chromatogram obtained with reference solution (q).

Develop the second plate over a path of 13 cm using *hexane R*. Allow the plate to dry in air. Develop a second time over a path of 10 cm using a mixture of 5 volumes of *methanol R* and 95 volumes of *methylene chloride R*. Allow the plate to dry. Spray with a 40 g/l solution of *phosphomolybdic acid R* in *ethanol R*. Heat in an oven at 120 °C until spots appear. Any spots corresponding to plastic additive 20 or plastic additive 21 in the chromatogram obtained with test solution S24 are identical in position to (R_f about 0.2) but not more intense than the corresponding spots in the chromatograms obtained with reference solutions (r) and (s).

01/2005:30104

3.1.4. POLYETHYLENE WITHOUT ADDITIVES FOR CONTAINERS FOR PARENTERAL PREPARATIONS AND FOR OPHTHALMIC PREPARATIONS

DEFINITION

Polyethylene without additives is obtained by the polymerisation of ethylene under high pressure in the presence of oxygen or free-radical-forming initiators as catalyst.

CHARACTERS

Beads, granules, powder or, after transformation, translucent sheets of varying thickness or containers, practically insoluble in water, soluble in hot aromatic hydrocarbons, practically insoluble in ethanol, in hexane and in methanol. It softens at temperatures above 65 °C.

The relative density (2.2.5) of the material is 0.910 to 0.937.

IDENTIFICATION

If necessary, cut the material to be examined into pieces of maximum dimension on a side of not greater than 1 cm.

- A. To 0.25 g add 10 ml of *toluene R* and boil under a reflux condenser for about 15 min. Place a few drops of the solution on a sodium chloride disc and evaporate the solvent in an oven at 80 °C. Examine by infrared absorption spectrophotometry (2.2.24). The spectrum of the substance to be examined shows maxima in particular at some of the following wave-numbers: 2920 cm⁻¹, 2850 cm⁻¹, 1465 cm⁻¹, 730 cm⁻¹, 720 cm⁻¹; the spectrum obtained is identical to that obtained with the material selected for the type sample. If the material to be examined is in the form of sheets, the identification may be performed directly on a cut piece of suitable size.
- B. The substance to be examined complies with the test for additives (see Tests).

TESTS

If necessary, cut the material to be examined into pieces of maximum dimension on a side of not greater than 1 cm.

Solution S1. Place 25 g in a borosilicate-glass flask with a ground-glass neck. Add 500 ml of *water for injections R* and heat under a reflux condenser for 5 h. Allow to cool and decant. Keep part of the solution for the test for appearance of solution. Filter the rest through a sintered glass filter (16). *Use solution S1 within 4 h of preparation.*

Solution S2. Place 2.0 g in a conical borosilicate-glass flask with a ground-glass neck. Add 80 ml of *toluene R* and boil under a reflux condenser with constant stirring for 1 h 30 min. Allow to cool to 60 °C and add with continued stirring 120 ml of *methanol R*. Filter the solution through

a sintered-glass filter (16). Rinse the flask and the filter with 25 ml of a mixture of 40 ml of *toluene R* and 60 ml of *methanol R*, add the rinsings to the filtrate and dilute to 250 ml with the same mixture of solvents. Prepare a blank solution.

Solution S3. Place 100 g in a conical borosilicate-glass flask with a ground-glass neck. Add 250 ml of 0.1 M *hydrochloric acid* and boil under a reflux condenser with constant stirring for 1 h. Allow to cool and decant the solution.

Appearance of solution. Solution S1 is clear (2.2.1) and colourless (2.2.2, *Method II*).

Acidity or alkalinity. To 100 ml of solution S1 add 0.15 ml of *BRP indicator solution R*. Not more than 1.5 ml of 0.01 M *sodium hydroxide* is required to change the colour of the indicator to blue. To 100 ml of solution S1 add 0.2 ml of *methyl orange solution R*. Not more than 1.0 ml of 0.01 M *hydrochloric acid* is required to reach the beginning of the colour change of the indicator from yellow to orange.

Absorbance (2.2.25). At wavelengths from 220 nm to 340 nm, the absorbance of solution S1 is not greater than 0.2.

Reducing substances. To 20 ml of solution S1 add 1 ml of *dilute sulphuric acid R* and 20 ml of 0.002 M *potassium permanganate*. Boil under a reflux condenser for 3 min and cool immediately. Add 1 g of *potassium iodide R* and titrate immediately with 0.01 M *sodium thiosulphate*, using 0.25 ml of *starch solution R* as indicator. Carry out a blank titration. The difference between the titration volumes is not more than 0.5 ml.

Substances soluble in hexane. Place 10 g in a 250 ml conical borosilicate-glass flask with a ground-glass neck. Add 100 ml of *hexane R* and boil under a reflux condenser for 4 h, stirring constantly. Cool in iced water and filter rapidly through a sintered-glass filter (16) maintaining the solution at 0 °C (the filtration time must be less than 5 min; if necessary the filtration may be accelerated by applying pressure to the solution). Evaporate 20 ml of the filtrate in a tared glass dish on a water-bath. Dry the residue in an oven at 100-105 °C for 1 h. The mass of the residue obtained is within 10 per cent of the residue obtained with the type sample and does not exceed 5 per cent.

Additives. Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel G plate R*.

Test solution. Evaporate 50 ml of solution S2 to dryness *in vacuo* at 45 °C. Dissolve the evaporation residue with 5 ml of *methylene chloride R*. Prepare a blank solution from the blank solution corresponding to solution S2.

Reference solution. Dissolve 20 mg of *plastic additive 15 CRS* and 20 mg of *plastic additive 08 CRS* in *methylene chloride R* and dilute to 10 ml with the same solvent.

Apply to the plate 10 µl of each solution. Develop over a path of 13 cm using *hexane R*. Allow the plate to dry in air. Carry out a second development over a path of 10 cm using a mixture of 5 volumes of *methanol R* and 95 volumes of *methylene chloride R*. Allow the plate to dry in air, spray with a 40 g/l solution of *phosphomolybdic acid R* in *alcohol R* and heat at 120 °C until the spots appear in the chromatogram obtained with the reference solution. No spot appears in the chromatogram obtained with the test solution, except for a spot which may be at the solvent front from the first development and which corresponds to oligomers. Disregard any spots corresponding to those obtained in the chromatogram with the blank solution. The chromatogram obtained with the reference solution shows two distinct spots.

Extractable heavy metals (2.4.8). Evaporate 50 ml of solution S3 to about 5 ml on a water-bath and dilute to 20 ml with *water R*. 12 ml of solution complies with limit test A for heavy metals (2.5 ppm). Prepare the standard using 2.5 ml of *lead standard solution (10 ppm Pb) R*.

Sulphated ash (2.4.14). Not more than 0.02 per cent, determined on 5.0 g.

01/2005:30105

3.1.5. POLYETHYLENE WITH ADDITIVES FOR CONTAINERS FOR PARENTERAL PREPARATIONS AND FOR OPHTHALMIC PREPARATIONS

DEFINITION

Polyethylene with additives is obtained by the polymerisation of ethylene under pressure in the presence of a catalyst or by copolymerisation of ethylene with not more than 25 per cent of higher alkene homologues (C₃ to C₁₀).

PRODUCTION

A certain number of additives are added to the polymer in order to optimise their chemical, physical and mechanical properties in order to adapt them for the intended use. All these additives are chosen from the appended list which specifies for each product the maximum allowable content. They may contain at most three antioxidants, one or several lubricants or antiblocking agents as well as titanium dioxide as an opacifying agent when the material must provide protection from light.

- butylhydroxytoluene (plastic additive 07) (not more than 0.125 per cent),
- pentaerythrityl tetrakis[3-(3,5-di-*tert*-butyl-4-hydroxyphenyl)propionate] (plastic additive 09) (not more than 0.3 per cent),
- 1,3,5-tris(3,5-di-*tert*-butyl-4-hydroxybenzyl)-s-triazine-2,4,6-(1*H*,3*H*,5*H*)-trione (plastic additive 13) (not more than 0.3 per cent),
- octadecyl 3-(3,5-di-*tert*-butyl-4-hydroxyphenyl)propionate, (plastic additive 11) (not more than 0.3 per cent),
- ethylene bis[3,3-bis[3-(1,1-dimethylethyl)-4-hydroxyphenyl]butanoate] (plastic additive 08) (not more than 0.3 per cent),
- dioctadecyl disulphide (plastic additive 15) (not more than 0.3 per cent),
- 4,4',4''-(2,4,6-trimethylbenzene-1,3,5-triyltris(methylene)tris[2,6-bis(1,1-dimethylethyl)phenol]) (plastic additive 10) (not more than 0.3 per cent),
- 2,2'-bis(octadecyloxy)-5,5'-spirobi[1,3,2-dioxaphosphinane] (plastic additive 14) (not more than 0.3 per cent),
- didodecyl 3,3'-thiodipropionate (plastic additive 16) (not more than 0.3 per cent),
- dioctadecyl 3,3'-thiodipropionate (plastic additive 17) (not more than 0.3 per cent),
- tris [2,4-bis(1,1-dimethylethyl)phenyl] phosphite (plastic additive 12) (not more than 0.3 per cent).

The total of antioxidant additives listed above does not exceed 0.3 per cent.

- hydrotalcite (not more than 0.5 per cent),
- alkanamides (not more than 0.5 per cent),
- alkenamides (not more than 0.5 per cent),

- sodium silico-aluminate (not more than 0.5 per cent),
- silica (not more than 0.5 per cent),
- sodium benzoate (not more than 0.5 per cent),
- fatty acid esters or salts (not more than 0.5 per cent),
- trisodium phosphate (not more than 0.5 per cent),
- liquid paraffin (not more than 0.5 per cent),
- zinc oxide (not more than 0.5 per cent),
- magnesium oxide (not more than 0.2 per cent),
- calcium stearate or zinc stearate or a mixture of both (not more than 0.5 per cent),
- titanium dioxide (not more than 4 per cent) only for materials for containers for ophthalmic use.

The supplier of the material must be able to demonstrate that the qualitative and quantitative composition of the type sample is satisfactory for each production batch.

CHARACTERS

Powder, beads, granules or, after transformation, translucent sheets of varying thicknesses or containers. It is practically insoluble in water, soluble in hot aromatic hydrocarbons, practically insoluble in ethanol, in hexane and in methanol. It softens at temperatures between 70 °C and 140 °C.

The relative density (2.2.5) of the material is 0.890 to 0.965.

IDENTIFICATION

If necessary, cut the material to be examined into pieces of maximum dimension on a side of not greater than 1 cm.

- A. To 0.25 g add 10 ml of *toluene R* and boil under a reflux condenser for about 15 min. Place a few drops of the solution on a sodium chloride disc and evaporate the solvent in an oven at 80 °C. Examine by infrared absorption spectrophotometry (2.2.24). The spectrum of the material to be examined shows maxima in particular at some of the following wave-numbers: 2920 cm⁻¹, 2850 cm⁻¹, 1465 cm⁻¹, 1375 cm⁻¹, 1170 cm⁻¹, 730 cm⁻¹, 720 cm⁻¹; the spectrum obtained is identical to the spectrum obtained with the material selected for the type sample. If the material to be examined is in the form of sheets, the identification may be performed directly on a cut piece of suitable size.
- B. It complies with the supplementary tests corresponding to the additives present (see Tests).
- C. In a platinum crucible, mix about 20 mg with 1 g of *potassium hydrogen sulphate R* and heat until completely melted. Allow to cool and add 20 ml of *dilute sulphuric acid R*. Heat gently. Filter the resulting solution. To the filtrate add 1 ml of *phosphoric acid R* and 1 ml of *strong hydrogen peroxide solution R*. If the substance is opacified with titanium dioxide, an orange-yellow colour develops.

TESTS

If necessary, cut the material to be examined into pieces of maximum dimension on a side of not greater than 1 cm.

Solution S1. Place 25 g in a borosilicate-glass flask with a ground-glass neck. Add 500 ml of *water for injections R* and boil under a reflux condenser for 5 h. Allow to cool and decant. Reserve a portion of the solution for the test for appearance of solution and filter the rest through a sintered-glass filter (16). *Use within 4 h of preparation.*

Solution S2. Place 2.0 g in a conical borosilicate-glass flask with a ground-glass neck. Add 80 ml of *toluene R* and boil under a reflux condenser with constant stirring for 90 min. Allow to cool to 60 °C and add with continued stirring 120 ml of *methanol R*. Filter the solution through a sintered-glass filter (16). Rinse the flask and the filter with 25 ml of a

mixture of 40 ml of *toluene R* and 60 ml of *methanol R*, add the rinsings to the filtrate and dilute to 250.0 ml with the same mixture of solvents. Prepare a blank solution.

Solution S3. Place 100 g in a conical borosilicate-glass flask with a ground-glass neck. Add 250 ml of 0.1 M hydrochloric acid and boil under a reflux condenser with constant stirring for 1 h. Allow to cool and decant the solution.

Appearance of solution. Solution S1 is clear (2.2.1) and colourless (2.2.2, Method II).

Acidity or alkalinity. To 100 ml of solution S1 add 0.15 ml of *BRP indicator solution R*. Not more than 1.5 ml of 0.01 M sodium hydroxide is required to change the colour of the indicator to blue. To 100 ml of solution S1 add 0.2 ml of *methylo orange solution R*. Not more than 1.0 ml of 0.01 M hydrochloric acid is required to reach the beginning of the colour change of the indicator from yellow to orange.

Absorbance (2.2.25). At wavelengths from 220 nm to 340 nm, the absorbance of solution S1 is not greater than 0.2.

Reducing substances. To 20 ml of solution S1 add 1 ml of dilute sulphuric acid R and 20 ml of 0.002 M potassium permanganate. Boil under a reflux condenser for 3 min and cool immediately. Add 1 g of potassium iodide R and titrate immediately with 0.01 M sodium thiosulphate, using 0.25 ml of starch solution R as indicator. Carry out a blank titration. The difference between the titration volumes is not more than 0.5 ml.

Substances soluble in hexane. Place 10 g in a 250 ml conical borosilicate-glass flask with a ground-glass neck. Add 100 ml of hexane R and boil under a reflux condenser for 4 h, stirring constantly. Cool in iced water and filter rapidly through a sintered-glass filter (16) maintaining the solution at 0 °C (the filtration time must be less than 5 min; if necessary the filtration may be accelerated by applying pressure to the solution). Evaporate 20 ml of the filtrate in a tared borosilicate-glass dish on a water-bath. Dry the residue in an oven at 100–105 °C for 1 h. The mass of the residue obtained must be within 10 per cent of the residue obtained with the type sample and does not exceed 5 per cent.

Extractable aluminium. Not more than 1 ppm of extractable Al, determined by atomic emission spectrometry in an argon plasma (2.2.22, Method I).

Test solution. Use solution S3.

Reference solutions. Prepare the reference solutions using aluminium standard solution (200 ppm Al) R, diluted with 0.1 M hydrochloric acid.

Carry out the determination using the emission of aluminium at 396.15 nm, the spectral background being taken as 396.25 nm.

Verify the absence of aluminium in the hydrochloric acid used.

Extractable chromium. Not more than 0.05 ppm of extractable Cr, determined by atomic emission spectrometry in an argon plasma (2.2.22, Method I).

Test solution. Use solution S3.

Reference solutions. Prepare the reference solutions using chromium standard solution (100 ppm Cr) R, diluted with a mixture of 2 volumes of hydrochloric acid R and 8 volumes of water R.

Carry out the determination using the emission of chromium at 205.55 nm, the spectral background being taken as 205.50 nm.

Verify the absence of chromium in the hydrochloric acid used.

Extractable titanium. Not more than 1 ppm of extractable Ti, determined by atomic emission spectrometry in an argon plasma (2.2.22, Method I).

Test solution. Use solution S3.

Reference solutions. Prepare the reference solutions using titanium standard solution (100 ppm Ti) R, diluted with 0.1 M hydrochloric acid.

Carry out the determination using the emission of titanium at 336.12 nm, the spectral background being taken as 336.16 nm.

Verify the absence of titanium in the hydrochloric acid used.

Extractable vanadium. Not more than 0.1 ppm of extractable V, determined by atomic emission spectrometry in an argon plasma (2.2.22, Method I).

Test solution. Use solution S3.

Reference solutions. Prepare the reference solutions using vanadium standard solution (1 g/l V) R, diluting with a mixture of 2 volumes of hydrochloric acid R and 8 volumes of water R.

Carry out the determination using the emission of vanadium at 292.40 nm, the spectral background being taken as 292.35 nm.

Verify the absence of vanadium in the hydrochloric acid used.

Extractable zinc. Not more than 1 ppm of extractable Zn, determined by atomic absorption spectrometry (2.2.23, Method I).

Test solution. Use solution S3.

Reference solutions. Prepare the reference solutions using zinc standard solution (10 ppm Zn) R, diluted with 0.1 M hydrochloric acid.

Measure the absorbance at 213.9 nm using a zinc hollow-cathode lamp as a source of radiation and an air-acetylene flame.

Extractable zirconium. Not more than 0.1 ppm of extractable Zr, determined by atomic emission spectrometry in an argon plasma (2.2.22, Method I).

Test solution. Use solution S3.

Reference solutions. Prepare the reference solutions using zirconium standard solution (1 g/l Zr) R, diluted with a mixture of 2 volumes of hydrochloric acid R and 8 volumes of water R.

Carry out the determination using the emission of zirconium at 343.82 nm, the spectral background being taken as 343.92 nm.

Verify the absence of zirconium in the hydrochloric acid used.

Extractable heavy metals (2.4.8). Evaporate 50 ml of solution S3 to about 5 ml on a water-bath and dilute to 20.0 ml with water R. 12 ml of solution complies with limit test A for heavy metals (2.5 ppm). Prepare the standard using 2.5 ml of lead standard solution (10 ppm Pb) R.

Sulphated ash (2.4.14). Not more than 1.0 per cent, determined on 5.0 g. This limit does not apply to material opacified with titanium dioxide.

SUPPLEMENTARY TESTS

These tests are to be carried out, in whole or in part, only if required by the stated composition of the material.

Phenolic antioxidants. Examine by liquid chromatography (2.2.29).

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4.6 mm in internal diameter packed with octadecylsilyl silica gel for chromatography R (5 µm),

- as mobile phase one of the 3 following mixtures:

Mobile phase 1 at a flow rate of 2 ml/min: 30 volumes of *water R*, 70 volumes of *acetonitrile R*,

Mobile phase 2 at a flow rate of 1.5 ml/min: 10 volumes of *water R*, 30 volumes of *tetrahydrofuran R*, 60 volumes of *acetonitrile R*,

Mobile phase 3 at a flow rate of 1.5 ml/min: 5 volumes of *water R*, 45 volumes of *2-propanol R*, 50 volumes of *methanol R*,

- as detector a spectrophotometer set at 280 nm.

The chromatographic system must ensure the following:

- a resolution of not less than 8.0 between the peaks corresponding respectively to plastic additive 07 and plastic additive 08, with mobile phase 1,
- a resolution of not less than 2.0 between the peaks corresponding respectively to plastic additive 09 and plastic additive 10, with mobile phase 2,
- a resolution of not less than 2.0 between the peaks corresponding respectively to plastic additive 11 and plastic additive 12, with mobile phase 3.

Test solution S21. Evaporate 50 ml of solution S2 to dryness in vacuo at 45 °C. Dissolve the residue with 5.0 ml of a mixture of equal volumes of *acetonitrile R* and *tetrahydrofuran R*. Prepare a blank solution from the blank solution corresponding to solution S2.

Test solution S22. Evaporate 50 ml of solution S2 to dryness in vacuo at 45 °C. Dissolve the residue with 5.0 ml of *methylene chloride R*. Prepare a blank solution from the blank solution corresponding to solution S2.

Of the following reference solutions, only prepare those that are necessary for the analysis of the phenolic antioxidants stated in the composition of the substance to be examined.

Reference solution (a). Dissolve 25.0 mg of *butylhydroxytoluene CRS* (plastic additive 07) and 60.0 mg of *plastic additive 08 CRS* in 10.0 ml of a mixture of equal volumes of *acetonitrile R* and *tetrahydrofuran R*. Dilute 2.0 ml of the solution to 50.0 ml with a mixture of equal volumes of *acetonitrile R* and *tetrahydrofuran R*.

Reference solution (b). Dissolve 60.0 mg of *plastic additive 09 CRS* and 60.0 mg of *plastic additive 10 CRS* in 10.0 ml of a mixture of equal volumes of *acetonitrile R* and *tetrahydrofuran R*. Dilute 2.0 ml of the solution to 50.0 ml with a mixture of equal volumes of *acetonitrile R* and *tetrahydrofuran R*.

Reference solution (c). Dissolve 60.0 mg of *plastic additive 11 CRS* and 60.0 mg of *plastic additive 12 CRS* in 10.0 ml of *methylene chloride R*. Dilute 2.0 ml of the solution to 50.0 ml with *methylene chloride R*.

Reference solution (d). Dissolve 25.0 mg of *butylhydroxytoluene CRS* (plastic additive 07) in 10.0 ml of a mixture of equal volumes of *acetonitrile R* and *tetrahydrofuran R*. Dilute 2.0 ml of the solution to 50.0 ml with a mixture of equal volumes of *acetonitrile R* and *tetrahydrofuran R*.

Reference solution (e). Dissolve 60.0 mg of *plastic additive 08 CRS* in 10.0 ml of a mixture of equal volumes of *acetonitrile R* and *tetrahydrofuran R*. Dilute 2.0 ml of the solution to 50.0 ml with a mixture of equal volumes of *acetonitrile R* and *tetrahydrofuran R*.

Reference solution (f). Dissolve 60.0 mg of *plastic additive 13 CRS* in 10.0 ml of a mixture of equal volumes of *acetonitrile R* and *tetrahydrofuran R*. Dilute 2.0 ml of the solution to 50.0 ml with a mixture of equal volumes of *acetonitrile R* and *tetrahydrofuran R*.

Reference solution (g). Dissolve 60.0 mg of *plastic additive 09 CRS* in 10.0 ml of a mixture of equal volumes of *acetonitrile R* and *tetrahydrofuran R*. Dilute 2.0 ml of the solution to 50.0 ml with a mixture of equal volumes of *acetonitrile R* and *tetrahydrofuran R*.

Reference solution (h). Dissolve 60.0 mg of *plastic additive 10 CRS* in 10.0 ml of a mixture of equal volumes of *acetonitrile R* and *tetrahydrofuran R*. Dilute 2.0 ml of the solution to 50.0 ml with a mixture of equal volumes of *acetonitrile R* and *tetrahydrofuran R*.

Reference solution (i). Dissolve 60.0 mg of *plastic additive 11 CRS* in 10.0 ml of *methylene chloride R*. Dilute 2.0 ml of the solution to 50.0 ml with *methylene chloride R*.

Reference solution (j). Dissolve 60.0 mg of *plastic additive 12 CRS* in 10.0 ml of *methylene chloride R*. Dilute 2.0 ml of the solution to 50.0 ml with *methylene chloride R*.

If the substance to be examined contains plastic additive 07 and/or plastic additive 08, use mobile phase 1 and inject 20 µl of test solution S21, 20 µl of the corresponding blank solution, 20 µl of reference solution (a), and either 20 µl of reference solution (d) or (e), or 20 µl of reference solutions (d) and (e).

If the substance to be examined contains one or more of the following antioxidants:

- plastic additive 09,
- plastic additive 10,
- plastic additive 11,
- plastic additive 12,
- plastic additive 13,

use mobile phase 2 and inject 20 µl of test solution S21, 20 µl of the corresponding blank solution, 20 µl of reference solution (b) and 20 µl of the reference solutions of the antioxidants on the list above that are stated in the composition.

If the substance to be examined contains plastic additive 11 and/or plastic additive 12, use mobile phase 3 and inject 20 µl of test solution S22, 20 µl of the corresponding blank solution, 20 µl of reference solution (c), and either 20 µl of reference solution (i) or (j), or 20 µl of reference solutions (i) and (j).

In all cases record the chromatograms for 30 min; the chromatograms corresponding to test solutions S21 and S22 only show peaks due to antioxidants stated in the composition and minor peaks that also appear in the chromatograms corresponding to the blank solutions. The areas of the peaks of test solutions S21 and S22 are less than the areas of the corresponding peaks in the chromatograms obtained with reference solutions (d) to (j).

Non-phenolic antioxidants. Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel GF₂₅₄ plate R*.

Test solution S23. Evaporate 100 ml of solution S2 to dryness in vacuo at 45 °C. Dissolve the residue in 2 ml of *acidified methylene chloride R*.

Reference solution (k). Dissolve 60 mg of *plastic additive 14 CRS* in *methylene chloride R* and dilute to 10 ml with the same solvent. Dilute 2 ml of the solution to 10 ml with *acidified methylene chloride R*.

Reference solution (l). Dissolve 60 mg of *plastic additive 15 CRS* in *methylene chloride R* and dilute to 10 ml with the same solvent. Dilute 2 ml of the solution to 10 ml with *acidified methylene chloride R*.

Reference solution (m). Dissolve 60 mg of *plastic additive 16 CRS* in *methylene chloride R* and dilute to 10 ml with the same solvent. Dilute 2 ml of the solution to 10 ml with *acidified methylene chloride R*.

Reference solution (n). Dissolve 60 mg of *plastic additive 17 CRS* in *methylene chloride R* and dilute to 10 ml with the same solvent. Dilute 2 ml of the solution to 10 ml with *acidified methylene chloride R*.

Reference solution (o). Dissolve 60 mg of *plastic additive 16 CRS* and 60 mg of *plastic additive 17 CRS* in *methylene chloride R* and dilute to 10 ml with the same solvent. Dilute 2 ml of the solution to 10 ml with *acidified methylene chloride R*.

Apply separately to the plate 20 µl of test solution S23, 20 µl of reference solution (o) and 20 µl of the reference solutions corresponding to all the phenolic and non-phenolic antioxidants mentioned in the type composition of the material to be examined.

Develop over a path of 18 cm using *hexane R*. Allow the plate to dry. Develop a second time over a path of 17 cm using *methylene chloride R*. Allow the plate to dry and examine in ultraviolet light at 254 nm. Spray with *alcoholic iodine solution R* and examine in ultraviolet light at 254 nm after 10-15 min. Any spots in the chromatogram obtained with test solution S23 are not more intense than the spots in the same locations in the chromatograms obtained with the reference solutions. The test is not valid unless the chromatogram obtained with reference solution (o) shows two clearly separated spots.

Amides and stearates. Examine by thin-layer chromatography (2.2.27), using 2 plates of the *TLC silica gel GF₂₅₄ plates R* type.

Test solution. Use test solution S23 described in the test for non-phenolic antioxidants.

Reference solution (p). Dissolve 20 mg of *stearic acid CRS* (plastic additive 19) in *methylene chloride R* and dilute to 10 ml with the same solvent.

Reference solution (q). Dissolve 40 mg of *plastic additive 20 CRS* in *methylene chloride R* and dilute to 20 ml with the same solvent.

Reference solution (r). Dissolve 40 mg of *plastic additive 21 CRS* in *methylene chloride R* and dilute to 20 ml with the same solvent.

Apply to each of the 2 plates 10 µl of test solution S23. Apply 10 µl of reference solution (p) to the first and 10 µl of reference solutions (q) and (r) to the second. Develop the first plate over a path of 10 cm using a mixture of 25 volumes of *ethanol R* and 75 volumes of *trimethylpentane R*. Allow the plate to dry in air. Spray with a 2 g/l solution of *dichlorophenolindophenol sodium salt R* in *ethanol R* and heat in an oven at 120 °C for a few minutes to intensify the spots. Any spot corresponding to plastic additive 19 in the chromatogram obtained with test solution S23 is identical in position (R_f about 0.5) but not more intense than the spot in the same location in the chromatogram obtained with reference solution (p).

Develop the second plate over a path of 13 cm using *hexane R*. Allow the plate to dry in air. Develop a second time over a path of 10 cm using a mixture of 5 volumes of *methanol R* and 95 volumes of *methylene chloride R*. Allow the plate to dry. Spray with a 40 g/l solution of *phosphomolybdic acid R* in *ethanol R*. Heat in an oven at 120 °C until spots appear. Any spots corresponding to plastic additive 20 or plastic additive 21 in the chromatogram obtained with test solution S23 are identical in position

(R_f about 0.2) but not more intense than the corresponding spots in the chromatograms obtained with reference solutions (q) and (r).

01/2005:30106

3.1.6. POLYPROPYLENE FOR CONTAINERS AND CLOSURES FOR PARENTERAL PREPARATIONS AND OPHTHALMIC PREPARATIONS

DEFINITION

Polypropylene consists of the homopolymer of propylene or of a copolymer of propylene with not more than 25 per cent of ethylene or of a mixture (alloy) of polypropylene with not more than 25 per cent of polyethylene. It may contain additives.

PRODUCTION

A certain number of additives are added to the polymer in order to optimise their chemical, physical and mechanical properties in order to adapt them for the intended use. All these additives are chosen from the appended list which specifies for each product the maximum allowable content.

They may contain at most three antioxidants, one or several lubricants or antiblocking agents as well as titanium dioxide as opacifying agent when the material must provide protection from light.

- butylhydroxytoluene (plastic additive 07) (not more than 0.125 per cent),
- pentaerythrityl tetrakis[3-(3,5-di-*tert*-butyl-4-hydroxyphenyl)propionate] (plastic additive 09) (not more than 0.3 per cent),
- 1,3,5-tris(3,5-di-*tert*-butyl-4-hydroxybenzyl)-s-triazine-2,4,6(1*H*,3*H*,5*H*)-trione (plastic additive 13) (not more than 0.3 per cent),
- octadecyl 3-(3,5-di-*tert*-butyl-4-hydroxyphenyl)propionate, (plastic additive 11) (not more than 0.3 per cent),
- ethylene bis[3,3-bis[3-(1,1-dimethylethyl)-4-hydroxyphenyl]butanoate] (plastic additive 08) (not more than 0.3 per cent),
- dioctadecyl disulphide (plastic additive 15) (not more than 0.3 per cent),
- 2,2',2'',6,6',6''-hexa-*tert*-butyl-4,4',4''-[(2,4,6-trimethyl-1,3,5-benzenetriyl)trismethylene]triphenol (plastic additive 10) (not more than 0.3 per cent),
- 2,2'-bis(octadecyloxy)-5,5'-spirobi[1,3,2-dioxaphosphinane] (plastic additive 14) (not more than 0.3 per cent),
- didodecyl 3,3'-thiodipropionate (plastic additive 16) (not more than 0.3 per cent),
- dioctadecyl 3,3'-thiodipropionate (plastic additive 17) (not more than 0.3 per cent),
- tris(2,4-di-*tert*-butylphenyl) phosphite (plastic additive 12) (not more than 0.3 per cent),

The total of antioxidant additives listed above does not exceed 0.3 per cent.

- hydrotalcite (not more than 0.5 per cent),
- alkanamides (not more than 0.5 per cent),
- alkenamides (not more than 0.5 per cent),
- sodium silico-aluminate (not more than 0.5 per cent),
- silica (not more than 0.5 per cent),
- sodium benzoate (not more than 0.5 per cent),
- fatty acid esters or salts (not more than 0.5 per cent),

- trisodium phosphate (not more than 0.5 per cent),
- liquid paraffin (not more than 0.5 per cent),
- zinc oxide (not more than 0.5 per cent),
- talc (not more than 0.5 per cent),
- magnesium oxide (not more than 0.2 per cent),
- calcium stearate or zinc stearate or a mixture of both (not more than 0.5 per cent),
- titanium dioxide (not more than 4 per cent) only for materials for containers for ophthalmic use.

The supplier of the material must be able to demonstrate that the qualitative and quantitative composition of the type sample is satisfactory for each production batch.

CHARACTERS

Powder, beads, granules or, after transformation, translucent sheets of varying thicknesses or containers. It is practically insoluble in water, soluble in hot aromatic hydrocarbons, practically insoluble in ethanol, in hexane and in methanol. It softens at temperatures above about 120 °C.

IDENTIFICATION

If necessary, cut the material to be examined into pieces of maximum dimension on a side of not greater than 1 cm.

- To 0.25 g add 10 ml of *toluene R* and boil under a reflux condenser for about 15 min. Place a few drops of the hot solution on a sodium chloride disc and evaporate the solvent in an oven at 80 °C. Examine by infrared absorption spectrophotometry (2.2.24). The spectrum obtained with the material to be examined presents a certain number of maxima, in particular at 1375 cm⁻¹, 1170 cm⁻¹, 995 cm⁻¹ and 970 cm⁻¹. The spectrum obtained is identical to the spectrum obtained with the material selected for the type sample. If the material to be examined is in the form of sheets, the identification may be performed directly on a cut piece of suitable size.
- It complies with the supplementary tests corresponding to the additives present (see Tests).
- In a platinum crucible, mix about 20 mg with 1 g of *potassium hydrogen sulphate R* and heat until completely melted. Allow to cool and add 20 ml of *dilute sulphuric acid R*. Heat gently. Filter the resulting solution. To the filtrate add 1 ml of *phosphoric acid R* and 1 ml of *strong hydrogen peroxide solution R*. If the substance is opacified with titanium dioxide, an orange-yellow colour develops.

TESTS

If necessary, cut the material to be examined into pieces of maximum dimension on a side of not greater than 1 cm.

Solution S1. Use solution S1 within 4 h of preparation.

Place 25 g in a borosilicate-glass flask with a ground-glass neck. Add 500 ml of *water for injections R* and boil under a reflux condenser for 5 h. Allow to cool and decant. Reserve a portion of the solution for the test for appearance of solution and filter the rest through a sintered-glass filter (16).

Solution S2. Place 2.0 g in a conical borosilicate-glass flask with a ground-glass neck. Add 80 ml of *toluene R* and boil under a reflux condenser with constant stirring for 1 h 30 min. Allow to cool to 60 °C and add with continued stirring 120 ml of *methanol R*. Filter the solution through a sintered-glass filter (16). Rinse the flask and the filter with 25 ml of a mixture of 40 ml of *toluene R* and 60 ml of *methanol R*, add the rinsings to the filtrate and dilute to 250.0 ml with the same mixture of solvents. Prepare a blank solution.

Solution S3. Place 100 g in a conical borosilicate-glass flask with a ground-glass neck. Add 250 ml of 0.1 M *hydrochloric acid* and boil under a reflux condenser with constant stirring for 1 h. Allow to cool and decant the solution.

Appearance of solution. Solution S1 is not more opalescent than reference suspension II (2.2.1) and is colourless (2.2.2, *Method II*).

Acidity or alkalinity. To 100 ml of solution S1 add 0.15 ml of *BRP indicator solution R*. Not more than 1.5 ml of 0.01 M *sodium hydroxide* is required to change the colour of the indicator to blue. To 100 ml of solution S1 add 0.2 ml of *methyl orange solution R*. Not more than 1.0 ml of 0.01 M *hydrochloric acid* is required to reach the beginning of the colour change of the indicator from yellow to orange.

Absorbance (2.2.25). At wavelengths from 220 nm to 340 nm, the absorbance of solution S1 is not greater than 0.2.

Reducing substances. To 20 ml of solution S1 add 1 ml of *dilute sulphuric acid R* and 20 ml of 0.002 M *potassium permanganate*. Boil under a reflux condenser for 3 min and cool immediately. Add 1 g of *potassium iodide R* and titrate immediately with 0.01 M *sodium thiosulphate*, using 0.25 ml of *starch solution R* as indicator. Carry out a blank titration. The difference between the titration volumes is not more than 0.5 ml.

Substances soluble in hexane. Place 10 g in a 250 ml conical borosilicate-glass flask with a ground-glass neck. Add 100 ml of *hexane R* and boil under a reflux condenser for 4 h, stirring constantly. Cool in iced water and filter rapidly through a sintered-glass filter (16) maintaining the solution at 0 °C (the filtration time must be less than 5 min; if necessary the filtration may be accelerated by applying pressure to the solution). Evaporate 20 ml of the filtrate in a tared glass dish on a water-bath. Dry the residue in an oven at 100 °C to 105 °C for 1 h. The mass of the residue obtained must be within 10 per cent of the residue obtained with the type sample and does not exceed 5 per cent.

Extractable aluminium. Not more than 1 ppm of extractable Al, determined by atomic emission spectrometry in an argon plasma (2.2.22, *Method I*).

Test solution. Use solution S3.

Reference solutions. Prepare the reference solutions using *aluminium standard solution (200 ppm Al) R*, diluted with 0.1 M *hydrochloric acid*.

Carry out the determination using the emission of aluminium at 396.15 nm, the spectral background being taken as 396.25 nm.

Verify the absence of aluminium in the hydrochloric acid used.

Extractable chromium. Not more than 0.05 ppm of extractable Cr, determined by atomic emission spectrometry in an argon plasma (2.2.22, *Method I*).

Test solution. Use solution S3.

Reference solutions. Prepare the reference solutions using *chromium standard solution (100 ppm Cr) R*, diluting with a mixture of 2 volumes of *hydrochloric acid R* and 8 volumes of *water R*.

Carry out the determination using the emission of chromium at 205.55 nm, the spectral background being taken as 205.50 nm.

Verify the absence of chromium in the hydrochloric acid used.

Extractable titanium. Not more than 1 ppm of extractable Ti, determined by atomic emission spectrometry in an argon plasma (2.2.22, *Method I*).

Test solution. Use solution S3.

Reference solutions. Prepare the reference solutions using *titanium standard solution (100 ppm Ti) R*, diluted with 0.1 M hydrochloric acid.

Carry out the determination using the emission of titanium at 336.12 nm, the spectral background being taken as 336.16 nm.

Verify the absence of titanium in the hydrochloric acid used.

Extractable vanadium. Not more than 0.1 ppm of extractable V, determined by atomic emission spectrometry in an argon plasma (2.2.22, *Method I*).

Test solution. Use solution S3.

Reference solutions. Prepare the reference solutions using *vanadium standard solution (1 g/l V) R*, diluted with a mixture of 2 volumes of *hydrochloric acid R* and 8 volumes of *water R*.

Carry out the determination using the emission of vanadium at 292.40 nm, the spectral background being taken as 292.35 nm.

Verify the absence of vanadium in the hydrochloric acid used.

Extractable zinc. Not more than 1 ppm of extractable Zn, determined by atomic absorption spectrometry (2.2.23, *Method I*).

Test solution. Use solution S3.

Reference solutions. Prepare the reference solutions using *zinc standard solution (10 ppm Zn) R*, diluted with 0.1 M hydrochloric acid.

Measure the absorbance at 213.9 nm using a zinc hollow-cathode lamp as a source of radiation and an air-acetylene flame.

Verify the absence of zinc in the hydrochloric acid used.

Extractable heavy metals (2.4.8). Concentrate 50 ml of solution S3 to about 5 ml on a water-bath and dilute to 20.0 ml with *water R*. 12 ml of the solution complies with limit test A for heavy metals (2.5 ppm). Prepare the standard using 2.5 ml of *lead standard solution (10 ppm Pb) R*.

Sulphated ash (2.4.14). Not more than 1.0 per cent, determined on 5.0 g. This limit does not apply to material that has been opacified with titanium dioxide.

SUPPLEMENTARY TESTS

These tests are to be carried out, in whole or in part, only if required by the stated composition of the material.

Phenolic antioxidants. Examine by liquid chromatography (2.2.29).

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4.6 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (5 µm),
- as mobile phase one of the three following mixtures:

Mobile phase 1 at a flow rate of 2 ml/min: 30 volumes of *water R*, 70 volumes of *acetonitrile R*,

Mobile phase 2 at a flow rate of 1.5 ml/min: 10 volumes of *water R*, 30 volumes of *tetrahydrofuran R*, 60 volumes of *acetonitrile R*,

Mobile phase 3 at a flow rate of 1.5 ml/min: 5 volumes of *water R*, 45 volumes of *2-propanol R*, 50 volumes of *methanol R*,

- as detector a spectrophotometer set at 280 nm.

The chromatographic system must ensure the following:

- a resolution of not less than 8.0 between the peaks corresponding respectively to plastic additive 07 and plastic additive 08, with mobile phase 1,
- a resolution of not less than 2.0 between the peaks corresponding respectively to plastic additive 09 and plastic additive 10, with mobile phase 2,
- a resolution of not less than 2.0 between the peaks corresponding respectively to plastic additive 11 and plastic additive 12, with mobile phase 3.

Test solution S21. Evaporate 50 ml of solution S2 to dryness *in vacuo* at 45 °C. Dissolve the residue with 5.0 ml of a mixture of equal volumes of *acetonitrile R* and *tetrahydrofuran R*. Prepare a blank solution from the blank solution corresponding to solution S2.

Test solution S22. Evaporate 50 ml of solution S2 to dryness *in vacuo* at 45 °C. Dissolve the residue with 5.0 ml of *methylene chloride R*. Prepare a blank solution from the blank solution corresponding to solution S2.

Of the following reference solutions, only prepare those that are necessary for the analysis of the phenolic antioxidants stated in the composition of the substance to be examined.

Reference solution (a). Dissolve 25.0 mg of *butylhydroxytoluene CRS* (plastic additive 07) and 60.0 mg of *plastic additive 08 CRS* in 10.0 ml of a mixture of equal volumes of *acetonitrile R* and *tetrahydrofuran R*. Dilute 2.0 ml of the solution to 50.0 ml with a mixture of equal volumes of *acetonitrile R* and *tetrahydrofuran R*.

Reference solution (b). Dissolve 60.0 mg of *plastic additive 09 CRS* and 60.0 mg of *plastic additive 10 CRS* in 10.0 ml of a mixture of equal volumes of *acetonitrile R* and *tetrahydrofuran R*. Dilute 2.0 ml of the solution to 50.0 ml with a mixture of equal volumes of *acetonitrile R* and *tetrahydrofuran R*.

Reference solution (c). Dissolve 60.0 mg of *plastic additive 11 CRS* and 60.0 mg of *plastic additive 12 CRS* in 10 ml of *methylene chloride R*. Dilute 2.0 ml of the solution to 50.0 ml with *methylene chloride R*.

Reference solution (d). Dissolve 25.0 mg of *butylhydroxytoluene CRS* (plastic additive 07) in 10.0 ml of a mixture of equal volumes of *acetonitrile R* and *tetrahydrofuran R*. Dilute 2.0 ml of the solution to 50.0 ml with a mixture of equal volumes of *acetonitrile R* and *tetrahydrofuran R*.

Reference solution (e). Dissolve 60.0 mg of *plastic additive 08 CRS* in 10.0 ml of a mixture of equal volumes of *acetonitrile R* and *tetrahydrofuran R*. Dilute 2.0 ml of the solution to 50.0 ml with a mixture of equal volumes of *acetonitrile R* and *tetrahydrofuran R*.

Reference solution (f). Dissolve 60.0 mg of *plastic additive 13 CRS* in 10.0 ml of a mixture of equal volumes of *acetonitrile R* and *tetrahydrofuran R*. Dilute 2.0 ml of the solution to 50.0 ml with a mixture of equal volumes of *acetonitrile R* and *tetrahydrofuran R*.

Reference solution (g). Dissolve 60.0 mg of *plastic additive 09 CRS* in 10.0 ml of a mixture of equal volumes of *acetonitrile R* and *tetrahydrofuran R*. Dilute 2.0 ml of the solution to 50.0 ml with a mixture of equal volumes of *acetonitrile R* and *tetrahydrofuran R*.

Reference solution (h). Dissolve 60.0 mg of *plastic additive 10 CRS* in 10.0 ml of a mixture of equal volumes of *acetonitrile R* and *tetrahydrofuran R*. Dilute 2.0 ml of the solution to 50.0 ml with a mixture of equal volumes of *acetonitrile R* and *tetrahydrofuran R*.

Reference solution (i). Dissolve 60.0 mg of *plastic additive 11 CRS* in 10.0 ml of *methylene chloride R*. Dilute 2.0 ml of the solution to 50.0 ml with *methylene chloride R*.

Reference solution (j). Dissolve 60.0 mg of *plastic additive 12 CRS* in 10.0 ml of *methylene chloride R*. Dilute 2.0 ml of the solution to 50.0 ml with *methylene chloride R*.

If the substance to be examined contains plastic additive 07 and/or plastic additive 08, use mobile phase 1 and inject 20 µl of test solution S21, 20 µl of the corresponding blank solution and 20 µl of reference solution (a), and either 20 µl of reference solution (d) or (e), or 20 µl of reference solutions (d) and (e).

If the substance to be examined contains one or more of the following antioxidants:

- plastic additive 09,
- plastic additive 10,
- plastic additive 11,
- plastic additive 12,
- plastic additive 13,

use mobile phase 2 and inject 20 µl of test solution S21, 20 µl of the corresponding blank solution, 20 µl of reference solution (b) and 20 µl of the reference solutions of the antioxidants on the list above that are stated in the composition.

If the substance to be examined contains plastic additive 11 and/or plastic additive 12, use mobile phase 3 and inject 20 µl of test solution S22, 20 µl of the corresponding blank solution, 20 µl of reference solution (c), and either 20 µl of reference solution (i) or (j), or 20 µl of reference solutions (i) and (j).

In all cases record the chromatogram for 30 min; the chromatograms corresponding to test solutions S21 and S22 only show peaks due to antioxidants stated in the composition and minor peaks that also appear in the chromatograms corresponding to the blank solutions. The areas of the peaks of test solutions S21 and S22 are less than the areas of the corresponding peaks in the chromatograms obtained with reference solutions (d) to (j).

Non-phenolic antioxidants. Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel GF₂₅₄ plate R*.

Test solution S23. Evaporate 100 ml of solution S2 to dryness *in vacuo* at 45 °C. Dissolve the residue with 2 ml of *acidified methylene chloride R*.

Reference solution (k). Dissolve 60 mg of *plastic additive 14 CRS* in *methylene chloride R* and dilute to 10 ml with the same solvent. Dilute 2 ml of the solution to 10 ml with *acidified methylene chloride R*.

Reference solution (l). Dissolve 60 mg of *plastic additive 15 CRS* in *methylene chloride R* and dilute to 10 ml with the same solvent. Dilute 2 ml of the solution to 10 ml with *acidified methylene chloride R*.

Reference solution (m). Dissolve 60 mg of *plastic additive 16 CRS* in *methylene chloride R* and dilute to 10 ml with the same solvent. Dilute 2 ml of the solution to 10 ml with *acidified methylene chloride R*.

Reference solution (n). Dissolve 60 mg of *plastic additive 17 CRS* in *methylene chloride R* and dilute to 10 ml with the same solvent. Dilute 2 ml of the solution to 10 ml with *acidified methylene chloride R*.

Reference solution (o). Dissolve 60 mg of *plastic additive 16 CRS* and 60 mg of *plastic additive 17 CRS* in *methylene chloride R* and dilute to 10 ml with the same solvent. Dilute 2 ml of the solution to 10 ml with *acidified methylene chloride R*.

Apply separately to the plate 20 µl of test solution S23, 20 µl of reference solution (o) and 20 µl of the reference solutions corresponding to all the phenolic and non-phenolic antioxidants mentioned in the type composition of the material to be examined. Develop over a path of 18 cm using *hexane R*. Allow the plate to dry. Develop a second time over a path of 17 cm using *methylene chloride R*. Allow the plate to dry and examine in ultraviolet light at 254 nm. Spray with *alcoholic iodine solution R* and examine in ultraviolet light at 254 nm after 10 min to 15 min. Any spots in the chromatogram obtained with test solution S23 are not more intense than the spots in the same locations in the chromatograms obtained with the reference solutions. The test is not valid unless the chromatogram obtained with reference solution (o) shows two clearly separated spots.

Amides and stearates. Examine by thin-layer chromatography (2.2.27), using 2 plates of the *TLC silica gel GF₂₅₄ plate R* type.

Test solution. Use solution S23 described in the test for non-phenolic antioxidants.

Reference solution (p). Dissolve 20 mg of *stearic acid CRS* (plastic additive 19) in *methylene chloride R* and dilute to 10 ml with the same solvent.

Reference solution (q). Dissolve 40 mg of *plastic additive 20 CRS* in *methylene chloride R* and dilute to 20 ml with the same solvent.

Reference solution (r). Dissolve 40 mg of *plastic additive 21 CRS* in *methylene chloride R* and dilute to 20 ml with the same solvent.

Apply to each of the two plates 10 µl of solution S23. Apply 10 µl of reference solution (p) to the first and 10 µl each of reference solutions (q) and (r) to the second. Develop the first plate over a path of 10 cm using a mixture of 25 volumes of *ethanol R* and 75 volumes of *trimethylpentane R*. Allow the plate to dry in air. Spray with a 2 g/l solution of *dichlorophenolindophenol sodium salt R* in *ethanol R* and heat in an oven at 120 °C for a few minutes to intensify the spots. Any spot corresponding to plastic additive 19 in the chromatogram obtained with test solution S23 is identical in position (*R_f* about 0.5) but not more intense than the spot in the same position in the chromatogram obtained with reference solution (p).

Develop the second plate over a path of 13 cm using *hexane R*. Allow the plate to dry in air. Develop a second time over a path of 10 cm using a mixture of 5 volumes of *methanol R* and 95 volumes of *methylene chloride R*. Allow the plate to dry.

Spray with a 40 g/l solution of *phosphomolybdic acid R* in *ethanol R*. Heat in an oven at 120 °C until spots appear. Any spots corresponding to plastic additive 20 or plastic additive 21 in the chromatogram obtained with test solution S23 are identical in position (*R_f* about 0.2) but not more intense than the corresponding spots in the chromatograms obtained with reference solutions (q) and (r).

01/2005:30107

3.1.7. POLY(ETHYLENE - VINYL ACETATE) FOR CONTAINERS AND TUBING FOR TOTAL PARENTERAL NUTRITION PREPARATIONS

DEFINITION

Poly(ethylene - vinyl acetate), complying with the following requirements, is suitable for the manufacture of containers and tubing for total parenteral nutrition preparations.

Poly(ethylene - vinyl acetate) is obtained by copolymerisation of mixtures of ethylene and vinyl acetate. This copolymer contains a defined quantity of not more than 25 per cent of vinyl acetate for material to be used for containers and not more than 30 per cent for material to be used for tubing.

PRODUCTION

A certain number of additives are added to the polymer in order to optimise their chemical, physical and mechanical properties in order to adapt them for the intended use. All these additives are chosen from the appended list which specifies for each product the maximum allowable content.

Poly(ethylene - vinyl acetate) may contain not more than three of the following antioxidants:

- butylhydroxytoluene (plastic additive 07) (not more than 0.125 per cent),
- pentaerythrityl tetrakis[3-(3,5-di-*tert*-butyl-4-hydroxyphenyl)propionate] (plastic additive 09) (not more than 0.2 per cent),
- octadecyl 3-(3,5-di-*tert*-butyl-4-hydroxyphenyl)propionate (plastic additive 11) (not more than 0.2 per cent),
- tris(2,4-di-*tert*-butylphenyl) phosphite (plastic additive 12) (not more than 0.2 per cent),
- 2,2',2'',6,6',6''-hexa-*tert*-butyl-4,4',4''-[(2,4,6-trimethyl-1,3,5-benzenetriyl)trismethylene]triphenol (plastic additive 10) (not more than 0.2 per cent).

It may also contain:

- oleamide (plastic additive 20) (not more than 0.5 per cent),
- erucamide (plastic additive 21) (not more than 0.5 per cent),
- calcium stearate or zinc stearate or a mixture of both (not more than 0.5 per cent),
- calcium carbonate or potassium hydroxide (not more than 0.5 per cent of each),
- colloidal silica (not more than 0.2 per cent).

The supplier of the material must be able to demonstrate that the qualitative and quantitative composition of the type sample is satisfactory for each production batch.

CHARACTERS

Beads, granules or, after transformation, translucent sheets or tubing of varying thickness or samples of finished objects, practically insoluble in water, soluble in hot aromatic hydrocarbons, practically insoluble in ethanol, in methanol and in hexane, which dissolves, however, low molecular mass polymers. It burns with a blue flame. The temperature at which the substance softens changes with the vinyl acetate content; it decreases from about 100 °C for contents of a few per cent to about 70 °C for contents of 30 per cent.

IDENTIFICATION

If necessary, cut the material to be examined into pieces of maximum dimension on a side of not greater than 1 cm.

To 0.25 g add 10 ml of *toluene R* and boil under a reflux condenser for about 15 min. Place a few drops of the solution obtained on a disc of sodium chloride and evaporate the solvent in an oven at 80 °C. Examine by infrared absorption spectrophotometry (2.2.24). The spectrum obtained shows absorption maxima corresponding to vinyl acetate at the following positions: 1740 cm⁻¹, 1375 cm⁻¹, 1240 cm⁻¹, 1020 cm⁻¹, 610 cm⁻¹, and maxima corresponding to ethylene at the following positions: 2920 cm⁻¹ to 2850 cm⁻¹, 1470 cm⁻¹, 1460 cm⁻¹, 1375 cm⁻¹, 730 cm⁻¹, 720 cm⁻¹. The spectrum obtained is, in addition, identical to the spectrum obtained with the type sample provided by the manufacturer.

If the material to be examined is in the form of sheets, the spectrum may be determined directly on a cut piece of suitable size.

TESTS

If necessary, cut the material to be examined into pieces of maximum dimension on a side of not greater than 1 cm.

Solution S1. Place 2.0 g in a borosilicate-glass flask with a ground-glass neck. Add 80 ml of *toluene R* and heat under a reflux condenser with constant agitation for 90 min. Allow to cool to 60 °C and add 120 ml of *methanol R* to the flask with constant stirring. Filter the solution through a sintered-glass filter (16). Rinse the flask and the filter with 25 ml of a mixture of 40 ml of *toluene R* and 60 ml of *methanol R*, add the rinsing mixture to the filtrate and dilute to 250 ml with the same mixture of solvents.

Solution S2. *Use within 4 h of preparation.* Place 25 g in a borosilicate-glass flask with a ground-glass neck. Add 500 ml of *water for injections R* and boil under a reflux condenser for 5 h. Allow to cool and decant. Reserve a portion of the solution for the test for appearance of solution S2 and filter the rest through a sintered-glass filter (16).

Appearance of solution S2. Solution S2 is clear (2.2.1) and colourless (2.2.2, *Method II*).

Acidity or alkalinity. To 100 ml of solution S2 add 0.15 ml of *BRP indicator solution R*. Not more than 1.0 ml of 0.01 M *sodium hydroxide* is required to change the colour of the indicator to blue. To 100 ml of solution S2 add 0.2 ml of *methyl orange solution R*. Not more than 1.5 ml of 0.01 M *hydrochloric acid* is required to reach the beginning of the colour change of the indicator from yellow to orange.

Absorbance (2.2.25). At wavelengths from 220 nm to 340 nm, the absorbance of solution S2 is not greater than 0.2.

Reducing substances. To 20 ml of solution S2 add 1 ml of *dilute sulphuric acid R* and 20 ml of 0.002 M *potassium permanganate*. Boil under a reflux condenser for 3 min and cool immediately. Add 1 g of *potassium iodide R* and titrate immediately with 0.01 M *sodium thiosulphate*, using 0.25 ml of *starch solution R* as indicator. Carry out a blank titration. The difference between the titration volumes is not more than 0.5 ml.

Amides and stearic acid. Examine by thin-layer chromatography (2.2.27), using 2 plates of the *TLC silica gel GF₂₅₄ plate R* type.

Test solution. Evaporate 100 ml of solution S1 to dryness *in vacuo* at 45 °C. Dissolve the residue in 2 ml of *acidified methylene chloride R*.

Reference solution (a). Dissolve 20 mg of *stearic acid CRS* (plastic additive 19) in 10 ml of *methylene chloride R*.

Reference solution (b). Dissolve 40 mg of *plastic additive 20 CRS* in 10 ml of *methylene chloride R*. Dilute 1 ml of the solution to 5 ml with *methylene chloride R*.

Reference solution (c). Dissolve 40 mg of *plastic additive 21 CRS* in 10 ml of *methylene chloride R*. Dilute 1 ml of the solution to 5 ml with *methylene chloride R*.

Apply separately 10 µl of each solution to the two plates.

Develop the first plate over a path of 10 cm using a mixture of 25 volumes of *ethanol R* and 75 volumes of *trimethylpentane R*. Allow the plate to dry. Spray with a 2 g/l solution of *dichlorophenolindophenol sodium salt R* in *ethanol R* and heat in an oven at 120 °C for a few minutes to intensify the spots. Any spot corresponding to plastic additive 19 in the chromatogram obtained with the test solution is not more intense than the spot in the chromatogram obtained with reference solution (a).

Develop the second plate over a path of 13 cm using *hexane R*. Allow the plate to dry. Develop a second time over a path of 10 cm using a mixture of 5 volumes of *methanol R* and 95 volumes of *methylene chloride R*. Allow the plate to dry. Spray with a 40 g/l solution of *phosphomolybdic acid R* in *ethanol R*. Heat in an oven at 120 °C until spots appear. Any spots corresponding to plastic additive 21 or plastic additive 20 in the chromatogram obtained with the test solution are not more intense than the spots in the chromatograms obtained with reference solutions (b) and (c) respectively.

Phenolic antioxidants. Examine by liquid chromatography (2.2.29).

Test solution (a). Evaporate 50 ml of solution S1 to dryness *in vacuo* at 45 °C. Dissolve the residue in 5.0 ml of a mixture of equal volumes of *acetonitrile R* and *tetrahydrofuran R*.

Test solution (b). Evaporate 50 ml of solution S1 to dryness *in vacuo* at 45 °C. Dissolve the residue in 5.0 ml of *methylene chloride R*.

Reference solution (a). Dissolve 25 mg of *butylhydroxytoluene CRS* (plastic additive 07), 40 mg of *plastic additive 10 CRS*, 40 mg of *plastic additive 09 CRS* and 40 mg of *plastic additive 11 CRS* in 10 ml of a mixture of equal volumes of *acetonitrile R* and *tetrahydrofuran R*. Dilute 2 ml to 50.0 ml with a mixture of equal volumes of *acetonitrile R* and *tetrahydrofuran R*.

Reference solution (b). Dissolve 40 mg of *plastic additive 11 CRS* and 40 mg of *plastic additive 12 CRS* in 10 ml of *methylene chloride R*. Dilute 2 ml to 50.0 ml with *methylene chloride R*.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4.6 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (5 µm),
- as mobile phase at a flow rate of 1.5 ml/min one of the two following mixtures:
Mobile phase 1: 10 volumes of *water R*, 30 volumes of *tetrahydrofuran R*, 60 volumes of *acetonitrile R*,
Mobile phase 2: 5 volumes of *water R*, 45 volumes of *2-propanol R*, 50 volumes of *methanol R*,
- as detector a spectrophotometer set at 280 nm.

Using mobile phase 1, inject 20 µl of test solution (a) and 20 µl of reference solution (a). The chromatogram obtained with test solution (a) shows only principal peaks corresponding to the peaks in the chromatogram obtained with reference solution (a) with a retention time greater than 2 min.

The areas of the peaks in the chromatogram obtained with test solution (a) are not greater than those of the corresponding peaks in the chromatogram obtained with reference solution (a), except for the last peak eluted in the chromatogram obtained with reference solution (a).

The test is not valid unless, with mobile phase 1, the number of theoretical plates calculated for the peak corresponding to plastic additive 07 is at least 2500 and the resolution between the peaks corresponding to plastic additive 09 and plastic additive 10 is not less than 2.0.

If the chromatogram obtained with test solution (a) shows a peak with the same retention time as the last antioxidant eluted from reference solution (a), use mobile phase 2 as follows.

Inject 20 µl of test solution (b) and 20 µl of reference solution (b). The chromatogram obtained with test solution (b) shows only principal peaks corresponding to the peaks in the chromatogram obtained with reference solution (b) with a retention time greater than 3 min.

The areas of the peaks in the chromatogram obtained with test solution (b) are not greater than those of the corresponding peaks in the chromatogram obtained with reference solution (b).

The test is not valid unless the resolution between the peaks corresponding to plastic additive 11 and plastic additive 12 is at least 2.0.

Substances soluble in hexane. Place 5 g in a borosilicate-glass flask with a ground-glass neck. Add 50 ml of *hexane R*, fit a condenser and boil under reflux on a water-bath with constant stirring for 4 h. Cool in iced-water; a gel may form. Adapt a cooling jacket filled with iced water to a sintered-glass filter (16) fitted with a device allowing pressure to be applied during filtration. Allow the filter to cool for 15 min. Filter the hexane solution applying a gauge pressure of 27 kPa and without washing the residue; the filtration time must not exceed 5 min. Evaporate 20 ml of the solution to dryness on a water-bath. Dry at 100 °C for 1 h. The mass of the residue is not greater than 40 mg (2 per cent) for copolymer to be used for containers and not greater than 0.1 g (5 per cent) for copolymer to be used for tubing.

Sulphated ash (2.4.14). Not more than 1.2 per cent, determined on 5.0 g.

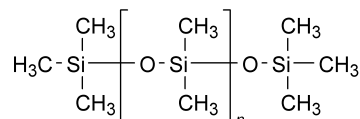
ASSAY

Introduce 0.250 g to 1.000 g of the substance to be examined, according to the vinyl acetate content of the copolymer to be examined, into a 300 ml conical flask with a ground-glass neck containing a magnetic stirrer. Add 40 ml of *xylene R*. Boil under a reflux condenser with stirring for 4 h. Stirring continuously, allow to cool until precipitation begins before slowly adding 25.0 ml of *alcoholic potassium hydroxide solution R1*. Boil again under a reflux condenser with stirring for 3 h. Allow to cool with continued stirring, rinse the condenser with 50 ml of *water R* and add 30.0 ml of 0.05 M *sulphuric acid* to the flask. Transfer the contents of the flask into a 400 ml beaker; rinse the flask with two quantities, each of 50 ml, of a 200 g/l solution of *anhydrous sodium sulphate R* and three quantities, each of 20 ml, of *water R* and add all the rinsings to the beaker containing the initial solution. Titrate the excess sulphuric acid with 0.1 M *sodium hydroxide*, determining the end-point potentiometrically (2.2.20). Carry out a blank titration.

1 ml of 0.05 M *sulphuric acid* is equivalent to 8.609 mg of vinyl acetate.

01/2005:30108

3.1.8. SILICONE OIL USED AS A LUBRICANT



DEFINITION

Silicone oil used as a lubricant is a poly(dimethylsiloxane) obtained by hydrolysis and polycondensation of dichlorodimethylsilane and chlorotrimethylsilane. Different grades exist which are characterised by a number indicating the nominal viscosity placed after the name.

Silicone oil used as lubricants have a degree of polymerisation ($n = 400$ to 1200) such that their kinematic viscosities are nominally between 1000 mm²s⁻¹ and 30 000 mm²s⁻¹.

CHARACTERS

Clear, colourless liquids of various viscosities, practically insoluble in water and in methanol, miscible with ethyl acetate, with methyl ethyl ketone and with toluene, very slightly soluble in ethanol.

IDENTIFICATION

- A. It is identified by its kinematic viscosity at 25 °C (see Tests).
- B. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *silicone oil CRS*. The region of the spectrum from 850 cm⁻¹ to 750 cm⁻¹ is not taken into account since it may show slight differences depending on the degree of polymerisation.
- C. Heat 0.5 g in a test-tube over a small flame until white fumes begin to appear. Invert the tube over a second tube containing 1 ml of a 1 g/l solution of *chromotropic acid, sodium salt R* in *sulphuric acid R* so that the fumes reach the solution. Shake the second tube for about 10 s and heat on a water-bath for 5 min. The solution is violet.
- D. In a platinum crucible, prepare the sulphated ash (2.4.14) using 50 mg. The residue is a white powder that gives the reaction of silicates (2.3.1).

TESTS

Acidity. To 2.0 g add 25 ml of a mixture of equal volumes of *ethanol R* and *ether R*, previously neutralised to 0.2 ml of *bromothymol blue solution R1* and shake. Not more than 0.15 ml of 0.01 M *sodium hydroxide* is required to change the colour of the solution to blue.

Viscosity (2.2.10). Determine the dynamic viscosity at 25 °C. Calculate the kinematic viscosity taking the relative density to be 0.97. The kinematic viscosity is not less than 95 per cent and not more than 105 per cent of the nominal viscosity stated on the label.

Mineral oils. Place 2 ml in a test-tube and examine in ultraviolet light at 365 nm. The fluorescence is not more intense than that of a solution containing 0.1 ppm of *quinine sulphate R* in 0.005 M *sulphuric acid* examined in the same conditions.

Phenylated compounds. The refractive index (2.2.6) is not greater than 1.410.

Heavy metals. Mix 1.0 g with *methylene chloride R* and dilute to 20 ml with the same solvent. Add 1.0 ml of a freshly prepared 0.02 g/l solution of *dithizone R* in *methylene chloride R*, 0.5 ml of *water R* and 0.5 ml of a mixture of 1 volume of *dilute ammonia R2* and 9 volumes of a 2 g/l solution of *hydroxylamine hydrochloride R*. At the same time, prepare a standard as follows: to 20 ml of *methylene chloride R* add 1.0 ml of a freshly prepared 0.02 g/l solution of *dithizone R* in *methylene chloride R*, 0.5 ml of *lead standard solution* (10 ppm Pb) *R* and 0.5 ml of a mixture of 1 volume of *dilute ammonia R2* and 9 volumes of a 2 g/l solution of *hydroxylamine hydrochloride R*. Immediately shake each solution vigorously for 1 min. Any red colour in the test solution is not more intense than that in the standard (5 ppm).

Volatile matter. Not more than 2.0 per cent, determined on 2.00 g by heating in an oven at 150 °C for 24 h. Carry out the test using a dish 60 mm in diameter and 10 mm deep.

LABELLING

The label indicates the nominal viscosity by a number placed after the name of the product. The label also states that the contents are to be used as a lubricant.

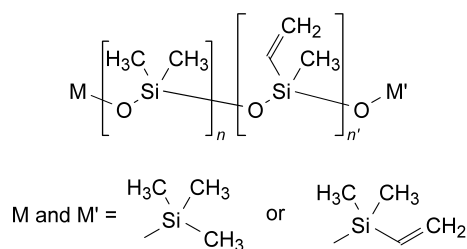
01/2005:30109

3.1.9. SILICONE ELASTOMER FOR CLOSURES AND TUBING

DEFINITION

Silicone elastomer complying with the following requirements is suitable for the manufacture of closures and tubing.

Silicone elastomer is obtained by cross-linking a linear polysiloxane constructed mainly of dimethylsiloxane units with small quantities of methylvinylsiloxane groups; the chain ends are blocked by trimethylsiloxane or dimethylvinylsiloxane groups. The general formula of the polysiloxane is:



The cross-linking is carried out in the hot state either with:

- 2,4-dichlorobenzoyl peroxide for extruded products,
- 2,4-dichlorobenzoyl peroxide or dicumyl peroxide or *OO*-(1,1-dimethylethyl) *O*-isopropyl monoperoxycarbonate or 2,5-bis[(1,1-dimethylethyl)dioxy]-2,5-dimethylhexane for moulded products,
- or
- by hydrosilylation by means of polysiloxane with -SiH groups using platinum as a catalyst.

In all cases, appropriate additives are used such as silica and sometimes small quantities of organosilicon additives (α,ω -dihydroxypolydimethylsiloxane).

CHARACTERS

A transparent or translucent material, practically insoluble in organic solvents, some of which, for example cyclohexane, hexane and methylene chloride, cause a reversible swelling of the material.

IDENTIFICATION

- A. Examine by infrared absorption spectrophotometry recording the spectrum by the multiple reflection method for solids (2.2.24), comparing with the spectrum obtained with *silicone elastomer CRS*.
- B. Heat 1.0 g in a test-tube over a small flame until white fumes begin to appear. Invert the tube over a second tube containing 1 ml of a 1 g/l solution of *chromotropic acid, sodium salt R* in *sulphuric acid R* so that the fumes reach the solution. Shake the second tube for about 10 s and heat on a water-bath for 5 min. The solution is violet.
- C. 50 mg of the residue of combustion gives the reaction of silicates (2.3.1).

TESTS

If necessary, cut the material into pieces of maximum dimension on a side of not greater than 1 cm.

Solution S. Place 25 g in a borosilicate-glass flask with a ground-glass neck. Add 500 ml of *water R* and boil under a reflux condenser for 5 h. Allow to cool and decant the solution.

Appearance of solution. Solution S is clear (2.2.1).

Acidity or alkalinity. To 100 ml of solution S add 0.15 ml of *bromothymol blue solution R1*. Not more than 2.5 ml of 0.01 M sodium hydroxide is required to change the colour of the indicator to blue. To a further 100 ml of solution S, add 0.2 ml of *methyl orange solution R*. Not more than 1.0 ml of 0.01 M hydrochloric acid is required to reach the beginning of the colour change of the indicator from yellow to orange.

Relative density (2.2.5). 1.05 to 1.25, determined using a density bottle with *ethanol R* as the immersion liquid.

Reducing substances. To 20 ml of solution S add 1 ml of *dilute sulphuric acid R* and 20 ml of 0.002 M *potassium permanganate*. Allow to stand for 15 min. Add 1 g of *potassium iodide R* and titrate immediately with 0.01 M *sodium thiosulphate* using 0.25 ml of *starch solution R* as indicator. Carry out a blank titration using 20 ml of *water R* instead of solution S. The difference between the titration volumes is not more than 1.0 ml.

Substances soluble in hexane. Evaporate 25 ml of the solution obtained in the test for phenylated compounds in a glass evaporating dish on a water-bath and dry in an oven at 100 °C to 105 °C for 1 h. The residue weighs not more than 15 mg (3 per cent).

Phenylated compounds. Place 2.0 g in a borosilicate-glass flask with a ground-glass neck and add 100 ml of *hexane R*. Boil under a reflux condenser for 4 h. Cool, then filter rapidly through a sintered-glass filter (16). Collect the filtrate and close the container immediately to avoid evaporation. At wavelengths from 250 nm to 340 nm, the absorbance (2.2.25) is not greater than 0.4.

Mineral oils. Place 2 g in a 100 ml conical flask containing 30 ml of a mixture of 5 volumes of *ammonia R* and 95 volumes of *pyridine R*. Allow to stand for 2 h, shaking frequently. Decant the pyridine solution and examine in ultraviolet light at 365 nm. The fluorescence is not greater than that of a solution containing 1 ppm of *quinine sulphate R* in 0.005 M *sulphuric acid* examined in the same conditions.

Volatile matter. Weigh 10.0 g of the substance previously stored for 48 h in a desiccator over *anhydrous calcium chloride R*. Heat in an oven at 200 °C for 4 h, allow to cool in a desiccator and weigh again. For silicone elastomer prepared using peroxides, the volatile matter is not greater than 0.5 per cent. For silicone elastomer prepared using platinum, the volatile matter is not greater than 2.0 per cent.

Silicone elastomer prepared using peroxides complies with the following additional test:

Residual peroxides. Place 5 g in a borosilicate-glass flask, add 150 ml of *methylene chloride R* and close the flask. Stir with a mechanical stirrer for 16 h. Filter rapidly, collecting the filtrate in a flask with a ground-glass neck. Replace the air in the container with *oxygen-free nitrogen R*, introduce 1 ml of a 200 g/l solution of *sodium iodide R* in *anhydrous acetic acid R*, close the flask, shake thoroughly and allow to stand protected from light for 30 min. Add 50 ml of *water R* and titrate immediately with 0.01 M *sodium thiosulphate*, using 0.25 ml of *starch solution R* as indicator. Carry out a blank titration. The difference between the titration volumes is not greater than 2.0 ml (0.08 per cent calculated as dichlorobenzoyl peroxide).

Silicone elastomer prepared using platinum complies with the following additional test:

Platinum. In a quartz crucible, ignite 1.0 g of the material to be examined, raising the temperature gradually until a white residue is obtained. Transfer the residue to a graphite crucible. To the quartz crucible add 10 ml of a

freshly prepared mixture of 1 volume of *nitric acid R* and 3 volumes of *hydrochloric acid R*, heat on a water-bath for 1 min to 2 min and transfer to the graphite crucible. Add 5 mg of *potassium chloride R* and 5 ml of *hydrofluoric acid R* and evaporate to dryness on a water-bath. Add 5 ml of *hydrofluoric acid R* and evaporate to dryness again; repeat this operation twice. Dissolve the residue in 5 ml of 1 M *hydrochloric acid*, warming on a water-bath. Allow to cool and add the solution to 1 ml of a 250 g/l solution of *stannous chloride R* in 1 M *hydrochloric acid*, rinse the graphite crucible with a few millilitres of 1 M *hydrochloric acid* and dilute to 10.0 ml with the same acid. Prepare simultaneously a standard as follows: to 1 ml of a 250 g/l solution of *stannous chloride R* in 1 M *hydrochloric acid* add 1.0 ml of *platinum standard solution (30 ppm Pt) R* and dilute to 10.0 ml with 1 M *hydrochloric acid*. The colour of the test solution is not more intense than that of the standard (30 ppm).

LABELLING

The label states whether the material was prepared using peroxides or platinum.

01/2005:30110

3.1.10. MATERIALS BASED ON NON-PLASTICISED POLY(VINYL CHLORIDE) FOR CONTAINERS FOR NON-INJECTABLE, AQUEOUS SOLUTIONS

DEFINITION

Materials based on non-plasticised poly(vinyl chloride) that comply with the following specifications are suitable for the manufacture of containers for non-injectable aqueous solutions. They may also be used for solid forms for oral administration and in some cases, subject to special studies on the compatibility of the container with its contents, these materials may be suitable for the preparation of containers for suppositories. They consist of one or more poly(vinyl chloride/vinyl acetate) or of a mixture of poly(vinyl chloride) and poly(vinyl acetate) or of poly(vinyl chloride).

They contain not more than 1 ppm of vinyl chloride.

The chlorine content expressed in poly(vinyl chloride) is not less than 80 per cent.

They may contain not more than 15 per cent of copolymers based on acrylic and/or methacrylic acids and/or their esters, and/or on styrene and/or butadiene.

PRODUCTION

Materials based on non-plasticised poly(vinyl chloride) are produced by polymerisation methods which guarantee a residual vinyl chloride content of less than 1 ppm. The production method used is validated in order to demonstrate that the product complies with the following test:

Vinyl chloride. Not more than 1 ppm, determined by head-space gas chromatography (2.2.28), using *ether R* as the internal standard.

Internal standard solution. Using a microsyringe, inject 10 µl of *ether R* into 20.0 ml of *dimethylacetamide R*, immersing the tip of the needle in the solvent. Immediately before use, dilute the solution to 1000 times its volume with *dimethylacetamide R*.

Test solution. Place 1.000 g of the material to be examined in a 50 ml vial and add 10.0 ml of the internal standard solution. Close the vial and secure the stopper. Shake, avoiding contact between the stopper and the liquid. Place the vial in a water-bath at 60 ± 1 °C for 2 h.

Vinyl chloride primary solution. Prepare under a ventilated hood. Place 50.0 ml of *dimethylacetamide R* in a 50 ml vial, stopper the vial, secure the stopper and weigh to the nearest 0.1 mg. Fill a 50 ml polyethylene or polypropylene syringe with gaseous *vinyl chloride R*, allow the gas to remain in contact with the syringe for about 3 min, empty the syringe and fill again with 50 ml of gaseous *vinyl chloride R*. Fit a hypodermic needle to the syringe and reduce the volume of gas in the syringe from 50 ml to 25 ml. Inject these 25 ml of vinyl chloride slowly into the vial, shaking gently and avoiding contact between the liquid and the needle. Weigh the vial again; the increase in mass is about 60 mg (1 µl of the solution thus obtained contains about 1.2 µg of vinyl chloride). Allow to stand for 2 h. Keep the primary solution in a refrigerator.

Vinyl chloride standard solution. To 1 volume of the vinyl chloride primary solution add 3 volumes of *dimethylacetamide R*.

Reference solutions. Place 10.0 ml of the internal standard solution in each of six 50 ml vials. Close the vials and secure the stoppers. Inject 1 µl, 2 µl, 3 µl, 5 µl and 10 µl, respectively, of the vinyl chloride standard solution into five of the vials. The six solutions thus obtained contain respectively, 0 µg, about 0.3 µg, 0.6 µg, 0.9 µg, 1.5 µg and 3 µg of vinyl chloride. Shake, avoiding contact between the stopper and the liquid. Place the vials in a water-bath at 60 ± 1 °C for 2 h.

The chromatographic procedure may be carried out using:

- a stainless steel column 3 m long and 3 mm in internal diameter packed with *silanised diatomaceous earth for gas chromatography R* impregnated with 5 per cent *m/m* of *dimethylstearylamine R* and 5 per cent *m/m* of *macrogol 400 R*,
- *nitrogen for chromatography R* as the carrier gas at a flow rate of 30 ml/min,
- a flame-ionisation detector,

maintaining the temperature of the column at 45 °C, that of the injection port at 100 °C and that of the detector at 150 °C.

Inject 1 ml of the head-space of each vial. Calculate the content of vinyl chloride.

In order to obtain the required mechanical and stability characteristics, materials based on non-plasticised poly(vinyl chloride) may contain:

- not more than 8 per cent of epoxidised soya oil of which the oxiran oxygen content is 6 per cent to 8 per cent and the iodine value is not greater than 6,
- not more than 1.5 per cent of calcium salt or zinc salts of aliphatic fatty acids with more than seven carbon atoms or not more than 1.5 per cent of their mixture,
- not more than 1.5 per cent of liquid paraffin,
- not more than 1.5 per cent of waxes,
- not more than 2 per cent of hydrogenated oils or esters of aliphatic fatty acids,
- not more than 1.5 per cent of macrogol esters,
- not more than 1.5 per cent of sorbitol,
- not more than 1 per cent of 2,4-dinonylphenyl phosphite, or di(4-nonylphenyl) phosphite or tris(nonylphenyl) phosphite.

They may contain one of the following groups of stabilisers:

- not more than 0.25 per cent of tin as di(isooctyl) 2,2'-[(dioctylstannylene)bis(thio)]diacetate containing about 27 per cent of tri(isooctyl) 2,2',2''-[(monooctylstannilydyne)tris(thio)]triacetate,
- not more than 0.25 per cent of tin as a mixture containing not more than 76 per cent of di(isooctyl) 2,2'-[(dimethylstannylene)bis(thio)]diacetate and not more than 85 per cent of tri(isooctyl) 2,2',2''-[(monomethylstannilydyne)tris(thio)]triacetate; (isooctyl is e.g. 2-ethylhexyl),
- not more than 1 per cent of 1-phenyleicosane-1,3-dione (benzoylstearyl methane) or 2-(4-dodecylphenyl)indole or didodecyl 1,4-dihydropyridine-2,6-dimethyl-3,5-dicarboxylate or 1 per cent of a mixture of two of these.

They may contain a colorant or pigment.

They may be opacified by titanium dioxide.

The supplier of the material must be able to demonstrate that the qualitative and quantitative composition of the type sample is satisfactory for each production batch.

CHARACTERS

Powder, beads, granules, sheets of varying thicknesses or samples taken from finished objects, insoluble in water, soluble in tetrahydrofuran, slightly soluble in methylene chloride, insoluble in ethanol. They burn with an orange-yellow flame edged with green, giving off thick black smoke.

IDENTIFICATION

Dissolve the residue (A) (see Tests: solution S2) in 5 ml of *tetrahydrofuran R*. Apply a few drops of the solution to a sodium chloride plate and evaporate to dryness in an oven at 100 °C to 105 °C. Examine by infrared absorption spectrophotometry (2.2.24). The material to be examined shows absorption maxima at 2975 cm⁻¹, 2910 cm⁻¹, 2865 cm⁻¹, 1430 cm⁻¹, 1330 cm⁻¹, 1255 cm⁻¹, 690 cm⁻¹, 615 cm⁻¹. In addition, the spectrum obtained is identical to that of the material selected for the type sample.

TESTS

If necessary, cut the material into pieces with a maximum dimension on a side of not greater than 1 cm.

Solution S1. Place 25 g in a borosilicate-glass flask. Add 500 ml of *water R* and cover the neck of the flask with aluminium foil or a borosilicate-glass beaker. Heat in an autoclave for 121 ± 2 °C for 20 min. Allow to cool and allow the solids to settle.

Solution S2. Dissolve 5.0 g in 80 ml of *tetrahydrofuran R* and dilute to 100 ml with the same solvent. Filter if necessary (the solution may remain opalescent). Dilute 20 ml of the solution and add dropwise with gentle shaking 70 ml of *alcohol R*. Cool in ice for 1 h. Filter or centrifuge. Wash the residue A with *alcohol R* and add the washings to the filtrate or the centrifugation liquid. Dilute to 100 ml with *alcohol R*.

Solution S3. Place 5 g in a borosilicate-glass flask with a ground-glass neck. Add 100 ml of 0.1 M *hydrochloric acid* and boil under a reflux condenser for 1 h. Allow to cool and allow the solids to settle.

Appearance of solution S1. Solution S1 is not more opalescent than reference suspension II (2.2.1) and is colourless (2.2.2, *Method II*).

Absorbance of solution S1 (2.2.25). Evaporate to dryness 100 ml of solution S1. Dissolve the residue in 5 ml of *hexane R*. Filter if necessary through a filter previously

rinsed with *hexane R*. At wavelengths from 250 nm to 310 nm, the absorbance of the filtrate is not greater than 0.25.

Absorbance of solution S2 (2.2.25). At wavelengths from 250 nm to 330 nm, the absorbance of solution S2 is not greater than 0.2 for tin-stabilised materials or 0.4 for other materials.

Extractable barium. Examine by atomic emission spectrometry in an argon plasma (2.2.22, *Method I*).

Test solution. Solution S3.

Reference solution. A solution containing 0.1 ppm of barium prepared by dilution of *barium standard solution (50 ppm Ba) R* with 0.1 M hydrochloric acid.

Carry out the determination using the emission of barium at 455.40 nm, the spectral background being taken at 455.30 nm.

Verify the absence of barium in the hydrochloric acid used.

Examined at 455.40 nm, the emission of the test solution is not greater than that of the reference solution (2 ppm).

Extractable cadmium. Examine by atomic absorption spectrometry (2.2.23, *Method I*).

Test solution. Solution S3.

Reference solution. A solution containing 0.03 ppm of cadmium prepared by diluting *cadmium standard solution (0.1 per cent Cd) R* with 0.1 M hydrochloric acid.

Verify the absence of cadmium in the hydrochloric acid used.

Examined at 228.8 nm, the absorbance of the test solution is not greater than that of the reference solution (0.6 ppm).

Tin-stabilised materials. To 0.10 ml of solution S2 in a test tube add 0.05 ml of 1 M hydrochloric acid, 0.5 ml of *potassium iodide solution R* and 5 ml of *alcohol R*. Mix thoroughly and wait for 5 min. Add 9 ml of *water R* and 0.1 ml of a 5 g/l solution of *sodium sulphite R* and mix thoroughly. Add 1.5 ml of *dithizone solution R* freshly diluted one-hundred-fold with *methylene chloride R*, shake for 15 s and allow to stand for 2 min. At the same time prepare a reference solution in the same manner using 0.1 ml of tin standard solution.

Any violet colour in the lower layer obtained with solution S2 is not more intense than that obtained with the reference solution (0.25 per cent of Sn). The greenish-blue colour of dithizone solution turns pink in the presence of tin.

Tin stock solution. Dilute 81 mg of *plastic additive 23 CRS* in a 100 ml volumetric flask to 100 ml with *tetrahydrofuran R*.

Tin standard solution. Dilute 20 ml of tin stock solution in a 100 ml volumetric flask to 100 ml with *alcohol R*.

Non-tin stabilised materials. To 5 ml of solution S2 in a test tube add 0.05 ml of 1 M hydrochloric acid and 0.5 ml of *potassium iodide solution R*. Mix thoroughly and wait for 5 min. Add 9 ml of *water R* and 0.1 ml of a 5 g/l solution of *sodium sulphite R* and mix thoroughly. If the solution obtained is not colourless, add the sodium sulphite solution in 0.05 ml fractions. Add 1.5 ml of *dithizone solution R* freshly diluted one hundred times with *methylene chloride R*, shake for 15 s and allow to stand for 2 min. At the same time prepare a standard in the same manner using 0.05 ml of tin standard solution.

Any violet colour in the lower layer obtained with solution S2 is not more intense than that obtained with the reference solution (25 ppm of Sn).

Extractable heavy metals (2.4.8). 12 ml of solution S3 complies with limit test A for heavy metals (20 ppm). Prepare the standard using 10 ml of *lead standard solution (1 ppm Pb) R*.

Extractable zinc. Examine by atomic absorption spectrometry (2.2.23, *Method I*).

Test solution. Solution S3 diluted ten times with *water R*.

Reference solution. A solution containing 0.50 ppm of zinc prepared by dilution of *zinc standard solution (5 mg/ml Zn) R* with 0.01 M hydrochloric acid.

Verify the absence of zinc in the hydrochloric acid used.

Examined at 214.0 nm, the absorbance of the test solution is not greater than that of the reference solution (100 ppm).

Sulphated ash (2.4.14). Not more than 1.0 per cent, determined on 1.0 g. When the materials are opacified using titanium dioxide, the content of sulphated ash does not exceed 4.0 per cent.

ASSAY

Carry out the oxygen-flask method (2.5.10) using 50.0 mg of the substance to be examined. Absorb the combustion products in 20 ml of 1 M sodium hydroxide. To the solution obtained add 2.5 ml of *nitric acid R*, 10.0 ml of 0.1 M *silver nitrate*, 5 ml of *ferric ammonium sulphate solution R2* and 1 ml of *dibutyl phthalate R*. Titrate with 0.05 M *ammonium thiocyanate* until a reddish-yellow colour is obtained. Carry out a blank titration.

1 ml of 0.1 M *silver nitrate* is equivalent to 6.25 mg of poly(vinyl chloride).

01/2005:30111

3.1.11. MATERIALS BASED ON NON-PLASTICISED POLY(VINYL CHLORIDE) FOR CONTAINERS FOR DRY DOSAGE FORMS FOR ORAL ADMINISTRATION

DEFINITION

Materials based on non-plasticised poly(vinyl chloride) for containers for dry dosage forms for oral administration are suitable for the manufacture of sheets or containers.

They consist of one or more poly(vinyl chloride/vinyl acetate) or of a mixture of poly(vinyl chloride) and poly(vinyl acetate) or of poly(vinyl chloride).

They contain not more than 1 ppm of vinyl chloride.

The chlorine content expressed in poly(vinyl chloride) is not less than 80 per cent.

They may contain not more than 15 per cent of copolymers based on acrylic and/or methacrylic acids and/or their esters, and/or on styrene and/or butadiene.

PRODUCTION

Materials based on non-plasticised poly(vinyl chloride) are produced by polymerisation methods which guarantee a residual vinyl chloride content of less than 1 ppm. The production method used is validated in order to demonstrate that the product complies with the following test for vinyl chloride.

Vinyl chloride. Not more than 1 ppm, determined by head-space gas chromatography (2.2.28), using *ether R* as the internal standard.

Internal standard solution. Using a microsyringe, inject 10 µl of *ether R* into 20.0 ml of *dimethylacetamide R*, immersing the tip of the needle in the solvent. Immediately before use, dilute the solution to 1000 times its volume with *dimethylacetamide R*.

Test solution. Place 1.000 g of the material to be examined in a 50 ml vial and add 10.0 ml of the internal standard solution. Close the vial and secure the stopper. Shake, avoiding contact between the stopper and the liquid. Place the vial in a water-bath at 60 ± 1 °C for 2 h.

Vinyl chloride primary solution. Prepare under a ventilated hood. Place 50.0 ml of *dimethylacetamide R* in a 50 ml vial, stopper the vial, secure the stopper and weigh to the nearest 0.1 mg. Fill a 50 ml polyethylene or polypropylene syringe with gaseous *vinyl chloride R*, allow the gas to remain in contact with the syringe for about 3 min, empty the syringe and fill again with 50 ml of gaseous *vinyl chloride R*. Fit a hypodermic needle to the syringe and reduce the volume of gas in the syringe from 50 ml to 25 ml. Inject these 25 ml of vinyl chloride slowly into the vial, shaking gently and avoiding contact between the liquid and the needle. Weigh the vial again; the increase in mass is about 60 mg (1 µl of the solution thus obtained contains about 1.2 µg of vinyl chloride). Allow to stand for 2 h. Keep the primary solution in a refrigerator.

Vinyl chloride standard solution. To 1 volume of the vinyl chloride primary solution add 3 volumes of *dimethylacetamide R*.

Reference solutions. Place 10.0 ml of the internal standard solution in each of six 50 ml vials. Close the vials and secure the stoppers. Inject 1 µl, 2 µl, 3 µl, 5 µl and 10 µl, respectively, of the vinyl chloride standard solution into 5 of the vials. The 6 solutions thus obtained contain respectively, 0 µg, about 0.3 µg, 0.6 µg, 0.9 µg, 1.5 µg and 3 µg of vinyl chloride. Shake, avoiding contact between the stopper and the liquid. Place the vials in a water-bath at 60 ± 1 °C for 2 h.

The chromatographic procedure may be carried out using:

- a stainless steel column 3 m long and 3 mm in internal diameter packed with *silanised diatomaceous earth for gas chromatography R* impregnated with 5 per cent *m/m* of *dimethylstearylamide R* and 5 per cent *m/m* of *macrogol 400 R*,
- *nitrogen for chromatography R* as the carrier gas at a flow rate of 30 ml/min,
- a flame-ionisation detector,

maintaining the temperature of the column at 45 °C, that of the injection port at 100 °C and that of the detector at 150 °C.

Inject 1 ml of the head-space of each vial. Calculate the content of vinyl chloride.

Additives

In order to obtain the required mechanical and stability characteristics, materials based on non-plasticised poly(vinyl chloride) may contain:

- not more than 2 per cent of epoxidised soya oil of which the oxiran oxygen content is 6 per cent to 8 per cent and the iodine value is not greater than 6 for tin-stabilised materials,
- not more than 3 per cent of epoxidised soya oil of which the oxiran oxygen content is 6 per cent to 8 per cent and the iodine value is not greater than 6 for non-tin-stabilised materials,
- not more than 1.5 per cent of calcium, magnesium or zinc salts of aliphatic fatty acids with more than 7 carbon atoms or not more than 1.5 per cent of their mixture,
- not more than 4 per cent of waxes,
- not more than 1.5 per cent of liquid paraffin,
- not more than 2 per cent of hydrogenated oils or esters of aliphatic fatty acids,

- not more than 4 per cent for the percentage sum of the 3 lubricants above,
- not more than 1.5 per cent of macrogol esters,
- not more than 1.5 per cent of sorbitol,
- not more than 1 per cent of 2,4-dinonylphenyl phosphite, or di(4-nonylphenyl) phosphite or tris(nonylphenyl) phosphite,
- not more than 1 per cent of calcium carbonate,
- not more than 1 per cent of silica.

They may contain one of the following groups of stabilisers:

- not more than 0.25 per cent of tin as di(isooctyl) 2,2'-[(dioctylstannylene)bis(thio)]diacetate containing about 27 per cent of tri(isooctyl) 2,2',2''-[(monooctylstannylidyne)tris(thio)]triacetate,
- not more than 0.25 per cent of tin as a mixture containing not more than 76 per cent of di(isooctyl) 2,2'-[(dimethylstannylene)bis(thio)]diacetate and not more than 85 per cent of tri(isooctyl) 2,2',2''-[(monomethylstannylidyne)tris(thio)]triacetate; (isooctyl is e.g. 2-ethylhexyl),
- not more than 1 per cent of 1-phenyleicosane-1,3-dione (benzoylstearoylmethane).

They may contain a colorant or pigment.

They may be opacified by titanium dioxide.

The supplier of the material must be able to demonstrate that the qualitative and quantitative composition of the type sample is satisfactory for each production batch.

CHARACTERS

Powder, beads, granules, sheets of varying thicknesses or samples taken from finished objects, insoluble in water, soluble in tetrahydrofuran, slightly soluble in methylene chloride, insoluble in ethanol. They burn with an orange-yellow flame edged with green, giving off thick black smoke.

IDENTIFICATION

Dissolve residue A (see Tests: solution S2) in 5 ml of *tetrahydrofuran R*. Apply a few drops of the solution to a sodium chloride plate and evaporate to dryness in an oven at 100-105 °C. Examine by infrared absorption spectrophotometry (2.2.24). The material to be examined shows absorption maxima at 2975 cm⁻¹, 2910 cm⁻¹, 2865 cm⁻¹, 1430 cm⁻¹, 1330 cm⁻¹, 1255 cm⁻¹, 690 cm⁻¹, 615 cm⁻¹. In addition, the spectrum obtained is identical to that of the material selected for the type sample.

TESTS

If necessary, cut the material into pieces with a maximum dimension on a side of not greater than 1 cm.

Solution S1. Place 25 g in a borosilicate glass flask. Add 500 ml of *water R* and cover the neck of the flask with aluminium foil or a borosilicate glass beaker. Heat in an autoclave for 121 ± 2 °C for 20 min. Allow to cool and allow the solids to settle.

Solution S2. Dissolve 5.0 g in 80 ml of *tetrahydrofuran R* and dilute to 100 ml with the same solvent. Filter if necessary (the solution may remain opalescent). Dilute 20 ml of the solution and add dropwise with gentle shaking 70 ml of *alcohol R*. Cool in ice for 1 h. Filter or centrifuge (residue A). Wash residue A with *alcohol R* and add the washings to the filtrate or the centrifugation liquid. Dilute to 100 ml with *alcohol R*.

Solution S3. Place 5 g in a borosilicate-glass flask with a ground-glass neck. Add 100 ml of 0.1 M hydrochloric acid and boil under a reflux condenser for 1 h. Allow to cool and allow the solids to settle.

Appearance of solution S1. Solution S1 is not more opalescent than reference suspension II (2.2.1) and is colourless (2.2.2, Method II).

Absorbance of solution S1 (2.2.25). Evaporate to dryness 100 ml of solution S1. Dissolve the residue in 5 ml of hexane R. Filter if necessary through a filter previously rinsed with hexane R. At wavelengths from 250 nm to 310 nm, the absorbance of the filtrate is not greater than 0.3.

Absorbance of solution S2 (2.2.25). For material that does not contain 1-phenyleicosane-1,3-dione, at wavelengths from 250 nm to 330 nm, the absorbance of solution S2 is not greater than 0.5. For material that contains 1-phenyleicosane-1,3-dione, at wavelengths from 250 nm to 330 nm, the absorbance of a tenfold dilution of solution S2 in alcohol R is not greater than 0.4.

Tin-stabilised materials. To 0.10 ml of solution S2 in a test tube add 0.05 ml of 1 M hydrochloric acid, 0.5 ml of potassium iodide solution R and 5 ml of alcohol R. Mix thoroughly and wait for 5 min. Add 9 ml of water R and 0.1 ml of a 5 g/l solution of sodium sulphite R and mix thoroughly. Add 1.5 ml of dithizone solution R freshly diluted one-hundred-fold with methylene chloride R, shake for 15 s and allow to stand for 2 min. At the same time prepare a reference solution in the same manner using 0.1 ml of tin standard solution.

Any violet colour in the lower layer obtained with solution S2 is not more intense than that obtained with the reference solution (0.25 per cent Sn). The greenish-blue colour of dithizone solution turns pink in the presence of tin.

Tin stock solution. Dilute 81 mg of plastic additive 23 CRS in a 100 ml volumetric flask to 100 ml with tetrahydrofuran R.

Tin standard solution. Dilute 20 ml of tin stock solution in a 100 ml volumetric flask to 100 ml with alcohol R.

Non tin-stabilised materials. To 5 ml of solution S2 in a test tube add 0.05 ml of 1 M hydrochloric acid and 0.5 ml of potassium iodide solution R. Mix thoroughly and wait for 5 min. Add 9 ml of water R and 0.1 ml of a 5 g/l solution of sodium sulphite R and mix thoroughly. If the solution obtained is not colourless, add the sodium sulphite solution in 0.05 ml fractions. Add 1.5 ml of dithizone solution R freshly diluted 100 times with methylene chloride R, shake for 15 s and allow to stand for 2 min. At the same time prepare a standard in the same manner using 0.05 ml of tin standard solution.

Any violet colour in the lower layer obtained with solution S2 is not more intense than that obtained with the reference solution (25 ppm of Sn).

Extractable heavy metals (2.4.8). 12 ml of solution S3 complies with limit test A (20 ppm). Prepare the standard using 10 ml of lead standard solution (1 ppm Pb) R.

Extractable zinc. Examine by atomic absorption spectrometry (2.2.23, Method I).

Test solution. Solution S3 diluted 10 times with water R.

Reference solution. A solution containing 0.50 ppm of zinc prepared by dilution of zinc standard solution (5 mg/ml Zn) R with 0.01 M hydrochloric acid.

Verify the absence of zinc in the hydrochloric acid used.

Examined at 214.0 nm, the absorbance of the test solution is not greater than that of the reference solution (100 ppm).

Sulphated ash (2.4.14). Not more than 1.0 per cent, determined on 1.0 g. When the materials are opacified using titanium dioxide, the content of sulphated ash does not exceed 4.0 per cent.

ASSAY

Carry out the oxygen-flask method (2.5.10) using 50.0 mg of the substance to be examined. Absorb the combustion products in 20 ml of 1 M sodium hydroxide. To the solution obtained add 2.5 ml of nitric acid R, 10.0 ml of 0.1 M silver nitrate, 5 ml of ferric ammonium sulphate solution R2 and 1 ml of dibutyl phthalate R. Titrate with 0.05 M ammonium thiocyanate until a reddish-yellow colour is obtained. Carry out a blank titration.

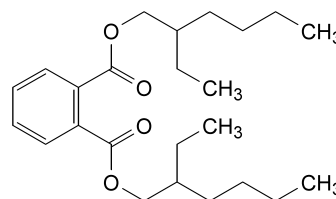
1 ml of 0.1 M silver nitrate is equivalent to 6.25 mg of poly(vinyl chloride).

01/2005:30113

3.1.13. PLASTIC ADDITIVES

NOTE: the nomenclature given first is according to the IUPAC rules. The synonym given in bold corresponds to the name given in the texts of Chapter 3. The synonym corresponding to the rules of the texts of "Chemical Abstracts" is also given.

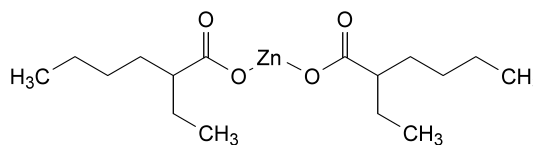
add01. C₂₄H₃₈O₄. [117-81-7]. PM RN 74640.



(2*RS*)-2-ethylhexyl benzene-1,2-dicarboxylate

synonyms: — **di(2-ethylhexyl) phthalate**,
— 1,2-benzenedicarboxylic acid,
bis(2-ethylhexyl) ester.

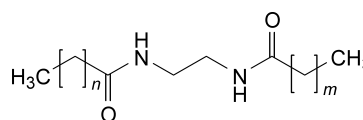
add02. C₁₆H₃₀O₄Zn. [136-53-8]. PM RN 54120.



zinc (2*RS*)-2-ethylhexanoate

synonyms: — **zinc octanoate**,
— 2-ethylhexanoic acid, zinc salt (2:1),
— zinc 2-ethylcaproate.

add03. [05518-18-3]/[00110-30-5]. PM RN 53440/53520.



N,N'-ethylenedialcanamide (with *n* and *m* = 14 or 16)

synonyms: — ***N,N'*-diacylethylenediamines**,
— *N,N'*-diacylethylenediamine (in this context
acyl means in particular palmitoyl and stearoyl).

add04. [8013-07-8]. PM RN 88640.

epoxidised soya oil

01/2005:30200

3.2. CONTAINERS

A container for pharmaceutical use is an article which contains or is intended to contain a product and is, or may be, in direct contact with it. The closure is a part of the container.

The container (see 1.3. *General Chapters*) is so designed that the contents may be removed in a manner appropriate to the intended use of the preparation. It provides a varying degree of protection depending on the nature of the product and the hazards of the environment, and minimises the loss of constituents. The container does not interact physically or chemically with the contents in a way that alters their quality beyond the limits tolerated by official requirements.

Single-dose container. A single-dose container holds a quantity of the preparation intended for total or partial use as a single administration.

Multidose container. A multidose container holds a quantity of the preparation suitable for two or more doses.

Well-closed container. A well-closed container protects the contents from contamination with extraneous solids and liquids and from loss of contents under ordinary conditions of handling, storage and transport.

Airtight container. An airtight container is impermeable to solids, liquids and gases under ordinary conditions of handling, storage and transport. If the container is intended to be opened on more than one occasion, it must be so designed that it remains airtight after re-closure.

Sealed container. A sealed container is a container closed by fusion of the material of the container.

Tamper-proof container. A tamper-proof container is a closed container fitted with a device that reveals irreversibly whether the container has been opened.

Child-proof container. A container that is fitted with a closure that prevents opening by children.

01/2005:30201

3.2.1. GLASS CONTAINERS FOR PHARMACEUTICAL USE

Glass containers for pharmaceutical use are glass articles intended to come into direct contact with pharmaceutical preparations.

Colourless glass is highly transparent in the visible spectrum.

Coloured glass is obtained by the addition of small amounts of metal oxides, chosen according to the desired spectral absorbance.

Neutral glass is a borosilicate glass containing significant amounts of boric oxide, aluminium oxide alkali and/or alkaline earth oxides. Due to its composition neutral glass has a high hydrolytic resistance and a high thermal shock resistance.

Soda-lime-silica glass is a silica glass containing alkali metal oxides, mainly sodium oxide and alkaline earth oxides, mainly calcium oxide. Due to its composition soda-lime-silica glass has only a moderate hydrolytic resistance.

The hydrolytic stability of glass containers for pharmaceutical use is expressed by the resistance to the release of soluble mineral substances into water under the prescribed conditions of contact between the inner surface of the

container or glass grains and water. The hydrolytic resistance is evaluated by titrating released alkali. According to their hydrolytic resistance, glass containers are classified as follows:

- Type I glass containers: neutral glass, with a high hydrolytic resistance due to the chemical composition of the glass itself,
- Type II glass containers: usually of soda-lime-silica glass with a high hydrolytic resistance resulting from suitable treatment of the surface,
- Type III glass containers: usually of soda-lime-silica glass with only moderate hydrolytic resistance.

The following italicised statements constitute general recommendations concerning the type of glass container that may be used for different types of pharmaceutical preparations. The manufacturer of a pharmaceutical product is responsible for ensuring the suitability of the chosen container.

Type I glass containers are suitable for most preparations whether or not for parenteral use.

Type II glass containers are suitable for most acidic and neutral, aqueous preparations whether or not for parenteral use.

Type III glass containers are in general suitable for non-aqueous preparations for parenteral use, for powders for parenteral use (except for freeze-dried preparations) and for preparations not for parenteral use.

Glass containers with a hydrolytic resistance higher than that recommended above for a particular type of preparation may generally also be used.

The container chosen for a given preparation shall be such that the glass material does not release substances in quantities sufficient to affect the stability of the preparation or to present a risk of toxicity. In justified cases, it may be necessary to have detailed information on the glass composition, so that the potential hazards can be assessed.

Preparations for parenteral use are normally presented in colourless glass, but coloured glass may be used for substances known to be light-sensitive. Colourless or coloured glass is used for the other pharmaceutical preparations. It is recommended that all glass containers for liquid preparations and for powders for parenteral use permit the visual inspection of the contents.

The inner surface of glass containers may be specially treated to improve hydrolytic resistance, to confer water-repellancy, etc. The outer surface may also be treated, for example to reduce friction and to improve resistance to abrasion. The outer treatment is such that it does not contaminate the inner surface of the container.

Except for type I glass containers, glass containers for pharmaceutical preparations are not to be re-used. Containers for human blood and blood components must not be re-used.

Glass containers for pharmaceutical use comply with the relevant test or tests for hydrolytic resistance. When glass containers have non-glass components, the tests apply only to the glass part of the container.

To define the quality of glass containers according to the intended use, one or more of the following tests are necessary.

Tests for hydrolytic resistance are carried out to define the type of glass (I, II or III) and to control its hydrolytic resistance.

In addition, containers for aqueous parenteral preparations are tested for arsenic release and coloured glass containers are tested for spectral transmission.

HYDROLYTIC RESISTANCE

Table 3.2.1-1. – *Types of glass*

Type of container	Test to be performed
Type I and Type II glass containers (to distinguish from Type III glass containers)	Test A (surface test)
Type I glass containers (to distinguish from Type II and Type III glass containers)	Test B (glass grains test) or test C (etching test)
Type I and Type II glass containers where it is necessary to determine whether the high hydrolytic resistance is due to the chemical composition or to the surface treatment	Tests A and B, or tests A and C

The test is carried out by titration of the extract solutions obtained under the conditions described for tests A, B and C.

EQUIPMENT

- an autoclave capable of maintaining a temperature of $121\text{ }^{\circ}\text{C} \pm 1\text{ }^{\circ}\text{C}$, equipped with a thermometer or a calibrated thermocouple recorder, a pressure gauge, a vent cock and a tray, of sufficient capacity to accommodate above the water level the number of containers needed to carry out the test; *clean the autoclave vessel and all ancillary equipment thoroughly before use with water R*;
- burettes with a suitable capacity;
- one-mark volumetric flasks, with a capacity of 1000 ml;
- pipettes and beakers;
- conical flasks with a capacity of 100 ml and 250 ml;
- a water-bath;
- a metal foil (e.g. aluminium, stainless steel).

Flasks and beakers shall have been already used for the test or have been filled with *water R* and kept in an autoclave at $121\text{ }^{\circ}\text{C}$ at least for 1 h before being used.

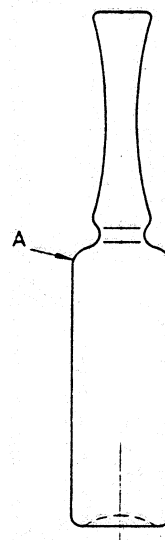
DETERMINATION OF THE FILLING VOLUME

The filling volume is the volume of water to be filled in the container for the purpose of the test. For vials and bottles the filling volume is 90 per cent of the brimful capacity. For ampoules it is the volume up to the height of the shoulder.

Vials and bottles. Select, at random, 6 containers from the sample lot, or 3 if their capacity exceeds 100 ml, and remove any dirt or debris. Weigh the empty containers with an accuracy of 0.1 g. Place the containers on a horizontal surface and fill them with *distilled water R* until about the rim edge, avoiding overflow and introduction of air bubbles. Adjust the liquid levels to the brimful line. Weigh the filled containers to obtain the mass of the water expressed to 2 decimal places for containers having a nominal volume less or equal to 30 ml, and expressed to 1 decimal place for containers having a nominal volume greater than 30 ml. Calculate the mean value of the brimful capacity in millilitres and multiply it by 0.9. This volume, expressed to 1 decimal place, is the filling volume for the particular container lot.

Ampoules. Place at least 6 dry ampoules on a flat, horizontal surface and fill them with *distilled water R* from a burette, until the water reaches point A, where the body of the ampoule declines to the shoulder (see Figure 3.2.1-1). Read the capacities (expressed to 2 decimal places) and calculate

the mean value. This volume, expressed to 1 decimal place, is the filling volume for the particular ampoule lot. The filling volume may also be determined by weighing.

Figure 3.2.1-1. – *Filling volume of ampoules (up to point A)*

TEST A. HYDROLYTIC RESISTANCE OF THE INNER SURFACES OF GLASS CONTAINERS (SURFACE TEST)

The determination is carried out on unused containers. The volumes of the test liquid necessary for the final determination are indicated in Table 3.2.1-2.

Table 3.2.1-2. – *Volume of test liquid and number of titrations*

Filling volume (ml)	Volume of test liquid for one titration (ml)	Number of titrations
Up to 3	25.0	1
Above 3 and up to 30	50.0	2
Above 30 and up to 100	100.0	2
Above 100	100.0	3

Cleaning. Remove any debris or dust. Shortly before the test, rinse each container carefully at least twice with *water R* and allow to stand. Immediately before testing empty the containers, rinse once with *water R* then with *water R1* and allow to drain. Complete the cleaning procedure from the first rinsing in not less than 20 min and not more than 25 min.

Heat closed ampoules on a water-bath or in an air-oven at about $50\text{ }^{\circ}\text{C}$ for approximately 2 min before opening; do not rinse before testing.

Filling and heating. The containers are filled with *water R1* up to the filling volume. Containers in the form of cartridges or prefilled syringes are closed in a suitable manner with material that does not interfere with the test. Each container including ampoules shall be loosely capped with an inert material such as a dish of neutral glass or aluminium foil previously rinsed with *water R*. Place the containers on the tray of the autoclave. Place the tray in the autoclave containing a quantity of *water R* such that the tray remains clear of the water. Close the autoclave and carry out the following operations:

- heat the autoclave to $100\text{ }^{\circ}\text{C}$ and allow the steam to issue from the vent cock for 10 min;
- close the ventcock and raise the temperature from $100\text{ }^{\circ}\text{C}$ to $121\text{ }^{\circ}\text{C}$ at a rate of $1\text{ }^{\circ}\text{C}$ per min;
- maintain the temperature at $121 \pm 1\text{ }^{\circ}\text{C}$ for 60 ± 1 min;

- lower the temperature from 121 °C to 100 °C at a rate of 0.5 °C per min, venting to prevent vacuum;
- do not open the autoclave before it has cooled down to 95 °C;
- remove the containers from the autoclave using normal precautions, place them in a water-bath at 80 °C, and run cold tap water, taking care that the water does not contact the loose foil caps to avoid contamination of the extraction solution;
- cooling time does not exceed 30 min.

The extraction solutions are analysed by titration according to the method described below.

Method. Carry out the titration within 1 h of removal of the containers from the autoclave. Combine the liquids obtained from the containers and mix. Introduce the prescribed volume (Table 3.2.1-2) into a conical flask. Place the same volume of *water R1* into a second similar flask as a blank. Add to each flask 0.05 ml of *methyl red solution R* for each 25 ml of liquid. Titrate the blank with 0.01 M *hydrochloric acid*. Titrate the test liquid with the same acid until the colour of the resulting solution is the same as that obtained for the blank. Subtract the value found for the blank titration from that found for the test liquid and express the results in millilitres of 0.01 M *hydrochloric acid* per 100 ml. Express titration values of less than 1.0 ml to 2 decimal places and titration values of more than or equal to 1.0 ml to 1 decimal place.

Limits. The results, or the average of the results if more than one titration is performed, is not greater than the values stated in Table 3.2.1-3.

Table 3.2.1-3. – Limit values in the test for surface hydrolytic resistance

Maximum volume of 0.01 M HCl per 100 ml of test liquid (ml)		
Glass containers		
Filling volume (ml)	Types I and II	Type III
Up to 1	2.0	20.0
Above 1 and up to 2	1.8	17.6
Above 2 and up to 5	1.3	13.2
Above 5 and up to 10	1.0	10.2
Above 10 and up to 20	0.80	8.1
Above 20 and up to 50	0.60	6.1
Above 50 and up to 100	0.50	4.8
Above 100 and up to 200	0.40	3.8
Above 200 and up to 500	0.30	2.9
Above 500	0.20	2.2

TEST B. HYDROLYTIC RESISTANCE OF GLASS GRAINS (GLASS GRAINS TEST)

Check that the articles as received have been annealed to a commercially acceptable quality.

The test may be performed on the canes used for the manufacture of tubing glass containers or on the containers.

Equipment

- a mortar, pestle (see Figure 3.2.1-2) and hammer in tempered, magnetic steel,
- a set of 3 square-mesh sieves of stainless steel, mounted on frames of the same material and consisting of the following:
 - (a) sieve no. 710,
 - (b) sieve no. 425,

- (c) sieve no. 300;
- a permanent magnet;
- a metal foil (e.g. aluminium, stainless steel);
- a hot-air oven, capable of maintaining a temperature of 140 ± 5 °C;
- a balance, capable of weighing up to 500 g with an accuracy of 0.005 g;
- a desiccator;
- an ultrasonic bath.

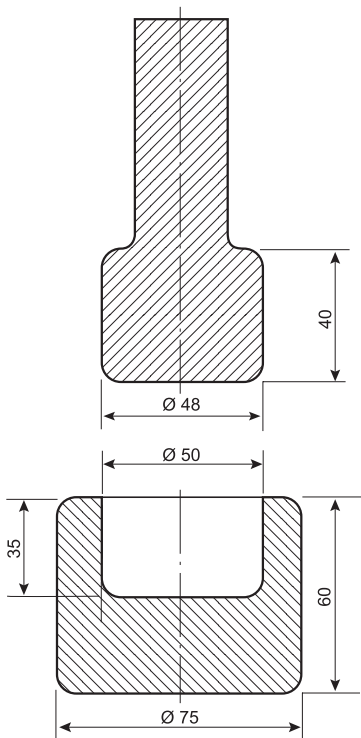


Figure 3.2.1-2. – Apparatus for glass grains method (dimensions in millimetres)

Method. Rinse the containers to be tested with *water R* and dry in the oven. Wrap at least 3 of the glass articles in clean paper and crush to produce 2 samples of about 100 g each in pieces not more than 30 mm across. Place 30-40 g of the pieces between 10-30 mm across taken from 1 of the samples in the mortar, insert the pestle and strike it heavily once only with the hammer. Transfer the contents of the mortar, to the coarsest sieve (a) of the set. Repeat the operation until all fragments have been transferred to the sieve. Shake the set of sieves a short time by hand and remove the glass which remains on sieves (a) and (b). Submit these portions to further fracture, repeating the operation until about 10 g of glass remains on sieve (a). Reject this portion and the portion which passes through sieve (c). Reassemble the set of sieves and shake for 5 min. Transfer to a weighing bottle those glass grains which passed through sieve (b) and are retained on sieve (c). Repeat the crushing and sieving procedure with the other glass sample and thus 2 samples of grains, each of which shall be in excess of 10 g, are obtained. Spread each sample on a piece of clean glazed paper and remove any iron particles by passing the magnet over them. Transfer each sample into a beaker for cleaning. Add to the grains in each beaker 30 ml of *acetone R* and scour the grains by suitable means, such as a rubber or plastic-coated glass rod. After scouring the grains, allow to settle and decant as much acetone as possible. Add another 30 ml of *acetone R*, swirl, decant again and add a new portion of *acetone R*.

Fill the bath of the ultrasonic vessel with water at room temperature, then place the beaker in the rack and immerse

it until the level of the acetone is at the level of the water; apply the ultrasound for 1 min. Swirl the beaker, allow to settle and decant the acetone as completely as possible and then repeat the ultrasonic cleaning operation. If a fine turbidity persists, repeat the ultrasonic cleaning and acetone washing until the solution remains clear. Swirl and decant the acetone then dry the grains, first by putting the beaker on a warm plate to remove excess acetone and then by heating at 140 °C for 20 min in the drying oven. Transfer the dried grains from each beaker into separate weighing bottles, insert the stoppers and cool in the desiccator. Weigh 10.00 g of the cleaned and dried grains into 2 separate conical flasks. Add 50 ml of *water R1* into each by means of a pipette (test solutions). Pipette 50 ml of *water R1* into a third conical flask which will serve as a blank. Distribute the grains evenly over the flat bases of the flasks by gentle shaking. Close the flasks with neutral glass dishes or aluminium foil rinsed with *water R* or with inverted beakers so that the inner surface of the beakers fit snugly down onto the top rims of the flasks. Place all 3 flasks in the rack in the autoclave containing the water at ambient temperature, and ensure that they are held above the level of the water in the vessel. Carry out the autoclaving procedure in a similar manner to that described under test A, but maintain the temperature of 121 ± 1 °C only for 30 ± 1 min. Do not open the autoclave until it has cooled to 95 °C. Remove the hot samples from the autoclave and cool the flasks in running tap water as soon as possible, avoiding thermal shock. To each of the 3 flasks add 0.05 ml of *methyl red solution R*. Titrate the blank solution immediately with 0.02 M *hydrochloric acid* then titrate the test solutions until the colour matches that obtained with the blank solution. Subtract the titration volume for the blank solution from that for the test solutions.

NOTE: where necessary to obtain a sharp end-point, the clear solution is to be decanted into a separate 250 ml flask. Rinse the grains with 3 quantities, each of 15 ml, of *water R1* by swirling and add the washings to the main solution. Add 0.05 ml of the *methyl red solution R*. Titrate and calculate as described below. In this case also add 45 ml of *water R1* and 0.05 ml of *methyl red solution R* to the blank solution.

Calculate the mean value of the results in millilitres of 0.02 M *hydrochloric acid* per gram of the sample and if required its equivalent in alkali extracted, calculated as micrograms of sodium oxide per gram of glass grains.

1 ml of 0.02 M *hydrochloric acid* is equivalent to 620 µg of sodium oxide.

Repeat the test if the highest and lowest observed values differ by more than 20 per cent.

Limits. Type I glass containers require not more than 1.0 ml of 0.02 M *hydrochloric acid* (equivalent to 62 µg of Na₂O per gram of glass), Type II and Type III glass containers require not more than 8.5 ml of 0.02 M *hydrochloric acid* (equivalent to 527 µg of Na₂O per gram of glass).

TEST C. TO DETERMINE WHETHER THE CONTAINERS HAVE BEEN SURFACE-TREATED (ETCHING TEST)

When it is necessary to determine if a container has been surface-treated, and/or distinguish between Type I and Type II glass containers, test C is used in addition to test A. Alternatively, test A and B may be used. Test C may be carried out either on unused samples or on samples previously tested for test A.

Vials and bottles. The volumes of test liquid required are shown in Table 3.2.1-2.

Rinse the containers twice with *water R* and fill to the brimful point with a mixture of 1 volume of *hydrofluoric acid R* and 9 volumes of *hydrochloric acid R* and allow to

stand for 10 min. Empty the containers and rinse carefully 5 times with *water R*. Immediately before the test, rinse once again with *water R*. Submit the containers thus prepared to the same autoclaving and determination procedure as described in test A for surface hydrolytic resistance. If the results are considerably higher than those obtained from the original surfaces (by about a factor of 5 to 10), the samples have been surface-treated.

Ampoules

NOTE: ampoules made from glass tubing are not normally subjected to internal surface treatment because their high chemical resistance is dependent upon the chemical composition of the glass as a material.

Apply the test method as described above for vials and bottles. If the ampoules are not surface-treated, the new values are slightly lower than those obtained in previous tests.

Distinction between Type I and Type II glass containers

The results obtained in Test C are compared to those obtained in Test A. The interpretation of the result is shown in Table 3.2.1-4.

Table 3.2.1-4. – Distinction between Types I and II glass containers

Type I	Type II
The values are closely similar to those found in the test for surface hydrolytic resistance for Type I glass containers.	The values greatly exceed those found in the test for surface hydrolytic resistance and are similar but not larger than those for Type III glass containers.

ARSENIC

The test applies to glass containers for aqueous parenteral preparations.

Hydride generation atomic absorption spectrometry (2.2.23, Method I).

Test solution. Use the extract solution obtained from containers of Types I and II, after autoclaving at 121 °C for 1 h as described under test A for surface hydrolytic resistance. Transfer 10.0 ml to a 100 ml volumetric flask. Add 10 ml of *hydrochloric acid R* and 5 ml of a 200 g/l solution of *potassium iodide R*. Heat on a water-bath at 80 °C for 20 min, allow to cool and dilute to 100.0 ml with *water R*.

Reference solutions. Prepare the reference solutions using *arsenic standard solution (1 ppm As) R*. Add 10 ml of *hydrochloric acid R* and 5 ml of a 200 g/l solution of *potassium iodide R*. Heat on a water-bath at 80 °C for 20 min, allow to cool and dilute to 100.0 ml with *water R*. The concentration range of the reference solutions is typically 0.005 ppm to 0.015 ppm of As.

Acid reservoir. *Hydrochloric acid R*.

Reducing reservoir. *Sodium tetrahydroborate reducing solution R*.

Use a hydride generation device to introduce the test solution into the cuvette of an atomic absorption spectrometer. Establish and standardise instrumental operating conditions according to the manufacturer's instructions, optimise the uptake rate of the peristaltic pump tubings, then connect tubings to the acid reservoir, the reducing reservoir and the test solution.

Source: hollow-cathode lamp.

Wavelength: 193.7 nm.

Atomisation device: air-acetylene flame.

Limit: maximum 0.1 ppm of As.

SPECTRAL TRANSMISSION FOR COLOURED GLASS CONTAINERS

Equipment. A UV-VIS spectrophotometer, equipped with a photodiode detector or equipped with a photomultiplier tube coupled with an integrating sphere.

Preparation of the specimen. Break the glass container or cut it with a circular saw fitted with a wet abrasive wheel, such as a carborundum or a bonded-diamond wheel. Select sections representative of the wall thickness and trim them as suitable for mounting in a spectrophotometer. If the specimen is too small to cover the opening in the specimen holder, mask the uncovered portion with opaque paper or tape, provided that the length of the specimen is greater than that of the slit. Before placing in the holder, wash, dry and wipe the specimen with lens tissue. Mount the specimen with the aid of wax, or by other convenient means, taking care to avoid leaving fingerprints or other marks.

Method. Place the specimen in the spectrophotometer with its cylindrical axis parallel to the slit and in such a way that the light beam is perpendicular to the surface of the section and that the losses due to reflection are at a minimum. Measure the transmission of the specimen with reference to air in the spectral region of 290-450 nm, continuously or at intervals of 20 nm.

Limits. The observed spectral transmission for coloured glass containers for preparations that are not for parenteral use does not exceed 10 per cent at any wavelength in the range of 290 nm to 450 nm, irrespective of the type and the capacity of the glass container. The observed spectral transmission in coloured glass containers for parenteral preparations does not exceed the limits given in Table 3.2.1-5.

Table 3.2.1-5. – *Limits of spectral transmission for coloured glass containers for parenteral preparations*

Filling volume (ml)	Maximum percentage of spectral transmission at any wavelength between 290 nm and 450 nm	
	Flame-sealed containers	Containers with closures
Up to 1	50	25
Above 1 and up to 2	45	20
Above 2 and up to 5	40	15
Above 5 and up to 10	35	13
Above 10 and up to 20	30	12
Above 20	25	10

Annex - test for surface hydrolytic resistance - determination by flame atomic absorption spectrometry (faas)

The surface hydrolytic resistance of glass of Types I and II may be determined by analysis of the leaching solution by flame atomic absorption spectrometry. A number of elements that, when present as oxides in glass, contribute to the alkalinity of the solution, are determined and used to express an alkali equivalent. The spectrometric method has the advantage of allowing the use of a much smaller sample of extract so that it can be applied to small individual containers. This enables an evaluation of the uniformity of the containers in a given batch where this is critical. The results of this measurement are not equivalent to those of titrimetry and the 2 methods cannot be considered interchangeable. A correlation between the 2 is dependent on the type of glass and the size and shape of the container.

The titrimetric method is the reference method of the Pharmacopoeia; the spectrometric method may be used in justified and authorised cases.

A method suitable for this type of analysis is shown below.

The determination is carried out on unused containers. The number of containers to be examined is indicated in Table 3.2.1-6.

Table 3.2.1-6. – *Number of containers to be examined for the spectrometric method*

Filling volume (ml)	Number of containers to be measured separately	Additional containers for preliminary measurements
Up to 2	20	2
Above 2 and up to 5	15	2
Above 5 and up to 30	10	2
Above 30 and up to 100	5	1
Above 100	3	1

Instructions on determination of the filling volume, cleaning of the containers, filling and heating are given above under Hydrolytic resistance and Test A. Hydrolytic resistance of the inner surfaces of glass containers.

SOLUTIONS

Spectrochemical buffer solution. Dissolve 80 g of *caesium chloride R* in about 300 ml of *water R1*, add 10 ml of 6 M *hydrochloric acid* and transfer to a 1000 ml volumetric flask. Dilute to volume with *water R1* and mix.

Stock solutions:

- sodium oxide, $c(\text{Na}_2\text{O}) = 1 \text{ mg/ml}$,
- potassium oxide, $c(\text{K}_2\text{O}) = 1 \text{ mg/ml}$,
- calcium oxide, $c(\text{CaO}) = 1 \text{ mg/ml}$.

Commercially available stock solutions may also be used.

Standard solutions. Prepare standard solutions by diluting the stock solutions with *water R1* to obtain concentrations suitable for establishing the reference solutions in appropriate manner, e.g. with concentrations of 20 µg/ml of sodium oxide, potassium oxide and calcium oxide, respectively. Commercially available standard solutions may also be used.

Reference solutions. Prepare the reference solutions for establishing the calibration graph (set of calibration solutions) by diluting suitable concentrated standard solutions with *water R1*, so that the normal working ranges of the specific elements are covered, taking into account the instrument used for the measurement. Typical concentration ranges of the reference solutions are:

- for determination by atomic emission spectrometry of sodium oxide and potassium oxide: up to 10 µg/ml,
- for determination by atomic absorption spectrometry of sodium oxide and potassium oxide: up to 3 µg/ml,
- for determination by atomic absorption spectrometry of calcium oxide: up to 7 µg/ml.

Use reference solutions containing 5 per cent V/V of the spectrochemical buffer solution.

METHOD

Carry out preliminary measurements of the potassium oxide and calcium oxide concentrations on one of the extraction solutions. If, for one container type, the concentration of potassium oxide is less than 0.2 µg/ml and if the concentration of calcium oxide is less than 0.1 µg/ml, the remaining extraction solutions of this container type need not be analysed for these ions. Aspirate the extraction solution from each sample directly into the flame of the atomic absorption or atomic emission instrument and

01/2005:30202

determine the approximate concentrations of sodium oxide (and potassium oxide and calcium oxide, if present) by reference to calibration graphs produced from the reference solutions of suitable concentration.

FINAL DETERMINATION

If dilution is unnecessary add to each container a volume of the spectrochemical buffer solution equivalent to 5 per cent of the filling volume, mix well and determine sodium oxide, calcium oxide and potassium oxide, if present, by reference to calibration graphs. For the determination of the calcium oxide concentration by flame atomic spectrometry, the nitrous oxide/acetylene flame shall be used.

If dilution is necessary, determine sodium oxide, calcium oxide and potassium oxide, if present, following the procedures as described above. The measuring solutions shall contain 5 per cent V/V of the spectrochemical buffer solution. Concentration values less than 1.0 µg/ml are expressed to 2 decimal places, values greater than or equal to 1.0 µg/ml to 1 decimal place. Correct the result for the buffer addition and for dilution, if any.

CALCULATION

Calculate the mean value of the concentration of individual oxides found in each of the samples tested, in micrograms of the oxide per millilitre of the extraction solution and calculate the sum of the individual oxides, expressed as micrograms of sodium oxide per millilitre of the extraction solution using the following mass conversion factors:

- 1 µg of potassium oxide corresponds to 0.658 µg of sodium oxide,
- 1 µg of calcium oxide corresponds to 1.105 µg of sodium oxide.

Limits. For each container tested, the result is not greater than the value given in Table 3.2.1-7.

Table 3.2.1-7. – *Limit values in the test for surface hydrolytic resistance by flame atomic absorption spectrometry*

Filling volume (ml)	Maximum values for the concentration of oxides, expressed as sodium oxide (µg/ml)
	Glass containers
	Types I and II
Up to 1	5.00
Above 1 and up to 2	4.50
Above 2 and up to 5	3.20
Above 5 and up to 10	2.50
Above 10 and up to 20	2.00
Above 20 and up to 50	1.50
Above 50 and up to 100	1.20
Above 100 and up to 200	1.00
Above 200 and up to 500	0.75
Above 500	0.50

3.2.2. PLASTIC CONTAINERS AND CLOSURES FOR PHARMACEUTICAL USE

A plastic container for pharmaceutical use is a plastic article which contains or is intended to contain a pharmaceutical product and is, or may be, in direct contact with it. The closure is a part of the container.

Plastic containers and closures for pharmaceutical use are made of materials in which may be included certain additives; these materials do not include in their composition any substance that can be extracted by the contents in such quantities as to alter the efficacy or the stability of the product or to present a risk of toxicity.

The most commonly used polymers are polyethylene (with and without additives), polypropylene, poly(vinyl chloride), poly(ethylene terephthalate) and poly(ethylene-vinyl acetate).

The nature and amount of the additives are determined by the type of the polymer, the process used to convert the polymer into the container and the intended purpose of the container. Additives may consist of antioxidants, stabilisers, plasticisers, lubricants, colouring matter and impact modifiers. Antistatic agents and mould-release agents may be used only for containers for preparations for oral use or for external use for which they are authorised. Acceptable additives are indicated in the type specification for each material described in the Pharmacopoeia. Other additives may be used provided they are approved in each case by the competent authority responsible for the licensing for sale of the preparation.

For selection of a suitable plastic container, it is necessary to know the full manufacturing formula of the plastic, including all materials added during formation of the container so that the potential hazards can be assessed. The plastic container chosen for any particular preparation should be such that:

- the ingredients of the preparation in contact with the plastic material are not significantly adsorbed on its surface and do not significantly migrate into or through the plastic,
- the plastic material does not release substances in quantities sufficient to affect the stability of the preparation or to present a risk of toxicity.

Using material or materials selected to satisfy these criteria, a number of identical type samples of the container are made by a well-defined procedure and submitted to practical testing in conditions that reproduce those of the intended use, including, where appropriate, sterilisation. In order to confirm the compatibility of the container and the contents and to ensure that there are no changes detrimental to the quality of the preparation, various tests are carried out such as verification of the absence of changes in physical characteristics, assessment of any loss or gain through permeation, detection of pH changes, assessment of changes caused by light, chemical tests and, where appropriate, biological tests.

The method of manufacture is such as to ensure reproducibility for subsequent bulk manufacture and the conditions of manufacture are chosen so as to preclude the possibility of contamination with other plastic materials or their ingredients. The manufacturer of the product must ensure that containers made in production are similar in every respect to the type samples.

For the results of the testing on type samples to remain valid, it is important that:

- there is no change in the composition of the material as defined for the type samples,
- there is no change in the manufacturing process as defined for the type samples, especially as regards the temperatures to which the plastic material is exposed during conversion or subsequent procedures such as sterilisation,
- scrap material is not used.

Recycling of excess material of well-defined nature and proportions may be permitted after appropriate validation.

Subject to satisfactory testing for compatibility of each different combination of container and contents, the materials described in the Pharmacopoeia are recognised as being suitable for the specific purposes indicated, as defined above.

01/2005:90003

3.2.2.1. PLASTIC CONTAINERS FOR AQUEOUS SOLUTIONS FOR PARENTERAL INFUSION

DEFINITION

Plastic containers for aqueous solutions for parenteral infusion are manufactured from one or more polymers, if necessary with additives. The containers described in this section are not necessarily suitable for emulsions. The polymers most commonly used are polyethylene, polypropylene and poly(vinyl chloride). The specifications of this text are to be read in conjunction with section 3.2.2. *Plastic containers and closures for pharmaceutical use.*

The containers may be bags or bottles. They have a site suitable for the attachment of an infusion set designed to ensure a secure connection. They may have a site that allows an injection to be made at the time of use. They usually have a part that allows them to be suspended and which will withstand the tension occurring during use. The containers must withstand the sterilisation conditions to which they will be submitted. The design of the container and the method of sterilisation chosen are such that all parts of the containers that may be in contact with the infusion are sterilised. The containers are impermeable to micro-organisms after closure. The containers are such that after filling they are resistant to damage from accidental freezing which may occur during transport of the final preparation. The containers are and remain sufficiently transparent to allow the appearance of the contents to be examined at any time, unless otherwise justified and authorised.

The empty containers display no defects that may lead to leakage and the filled and closed containers show no leakage.

For satisfactory storage of some preparations, the container has to be enclosed in a protective envelope. The initial evaluation of storage has then to be carried out using the container enclosed in the envelope.

TESTS

Solution S. Use solution S within 4 h of preparation. Fill a container to its nominal capacity with *water R* and close it, if possible using the usual means of closure; otherwise close using a sheet of pure aluminium. Heat in an autoclave so that a temperature of $121 \pm 2^\circ\text{C}$ is reached within 20 min

to 30 min and maintain at this temperature for 30 min. If heating at 121°C leads to deterioration of the container, heat at 100°C for 2 h.

Blank. Prepare a blank by heating *water R* in a borosilicate-glass flask closed by a sheet of pure aluminium at the temperature and for the time used for the preparation of solution S.

Appearance of solution S. Solution S is clear (2.2.1) and colourless (2.2.2, *Method II*).

Acidity or alkalinity. To a volume of solution S corresponding to 4 per cent of the nominal capacity of the container add 0.1 ml of *phenolphthalein solution R*. The solution is colourless. Add 0.4 ml of *0.01 M sodium hydroxide*. The solution is pink. Add 0.8 ml of *0.01 M hydrochloric acid* and 0.1 ml of *methyl red solution R*. The solution is orange-red or red.

Absorbance (2.2.25). Measure the absorbance of solution S from 230 nm to 360 nm, using the blank (see solution S) as the compensation liquid. At these wavelengths, the absorbance is not greater than 0.20.

Reducing substances. To 20.0 ml of solution S add 1 ml of *dilute sulphuric acid R* and 20.0 ml of *0.002 M potassium permanganate*. Boil for 3 min. Cool immediately. Add 1 g of *potassium iodide R* and titrate immediately with *0.01 M sodium thiosulphate*, using 0.25 ml of *starch solution R* as indicator. Carry out a titration using 20.0 ml of the blank. The difference between the titration volumes is not greater than 1.5 ml.

Transparency. Fill a container previously used for the preparation of solution S with a volume equal to the nominal capacity of the primary opalescent suspension (2.2.1) diluted 1 in 200 for a container made from polyethylene or polypropylene and 1 in 400 for other containers. The cloudiness of the suspension is perceptible when viewed through the container and compared with a similar container filled with *water R*.

LABELLING

The label accompanying a batch of empty containers includes a statement of:

- the name and address of the manufacturer,
- a batch number which enables the history of the container and of the plastic material of which it is manufactured to be traced.

01/2005:30203

3.2.3. STERILE PLASTIC CONTAINERS FOR HUMAN BLOOD AND BLOOD COMPONENTS

Plastic containers for the collection, storage, processing and administration of blood and its components are manufactured from one or more polymers, if necessary with additives. The composition and the conditions of manufacture of the containers are registered by the appropriate competent authorities in accordance with the relevant national legislation and international agreements. When the composition of the materials of the different parts of the containers correspond to the appropriate specifications, their quality is controlled by the methods indicated in those specifications (see 3.1. *Materials used for the manufacture of containers* and subsections).

Materials other than those described in the Pharmacopoeia may be used provided that their composition is authorised by the competent authority and that the containers

manufactured from them comply with the requirements prescribed for Sterile Plastic Containers for Human Blood and Blood Components.

In normal conditions of use the materials do not release monomers, or other substances, in amounts likely to be harmful nor do they lead to any abnormal modifications of the blood.

The containers may contain anticoagulant solutions, depending on their intended use, and are supplied sterile.

Each container is fitted with attachments suitable for the intended use. The container may be in the form of a single unit or the collecting container may be connected by one or more tubes to one or more secondary containers to allow separation of the blood components to be effected within a closed system.

The outlets are of a shape and size allowing for adequate connection of the container with the blood-giving equipment. The protective coverings on the blood-taking needle and on the appendages must be such as to ensure the maintenance of sterility. They must be easily removable but must be tamper-proof.

The capacity of the containers is related to the nominal capacity prescribed by the national authorities and to the appropriate volume of anticoagulant solution. The nominal capacity is the volume of blood to be collected in the container. The containers are of a shape such that when filled they may be centrifuged.

The containers are fitted with a suitable device for suspending or fixing which does not hinder the collection, storage, processing or administration of the blood.

The containers are enclosed in sealed, protective envelopes.

CHARACTERS

The container is sufficiently transparent to allow adequate visual examination of its contents before and after the taking of the blood and is sufficiently flexible to offer minimum resistance during filling and emptying under normal conditions of use. The container contains not more than 5 ml of air.

TESTS

Solution S₁. Fill the container with 100 ml of a sterile, pyrogen-free 9 g/l solution of *sodium chloride R*. Close the container and heat it in an autoclave so that the contents are maintained at 110 °C for 30 min.

If the container to be examined contains an anticoagulant solution, first empty it, rinse the container with 250 ml of *water for injections R* at 20 ± 1 °C and discard the rinsings.

Solution S₂. Introduce into the container a volume of *water for injections R* corresponding to the intended volume of anticoagulant solution. Close the container and heat it in an autoclave so that the contents are maintained at 110 °C for 30 min. After cooling, add sufficient *water for injections R* to fill the container to its nominal capacity.

If the container to be examined contains an anticoagulant solution, first empty it and rinse it as indicated above.

Resistance to centrifugation. Introduce into the container a volume of *water R*, acidified by the addition of 1 ml of *dilute hydrochloric acid R*, sufficient to fill it to its nominal capacity. Envelop the container with absorbent paper impregnated with a 1 in 5 dilution of *bromophenol blue*

solution R1 or other suitable indicator and then dried. Centrifuge at 5000 *g* for 10 min. No leakage perceptible on the indicator paper and no permanent distortion occur.

Resistance to stretch. Introduce into the container a volume of *water R*, acidified by the addition of 1 ml of *dilute hydrochloric acid R*, sufficient to fill it to its nominal capacity. Suspend the container by the suspending device at the opposite end from the blood-taking tube and apply along the axis of this tube an immediate force of 20 N (2.05 kgf). Maintain the traction for 5 s. Repeat the test with the force applied to each of the parts for filling and emptying. No break and no deterioration occur.

Leakage. Place the container which has been submitted to the stretch test between two plates covered with absorbent paper impregnated with a 1 in 5 dilution of *bromophenol blue solution R1* or other suitable indicator and then dried. Progressively apply force to the plates to press the container so that its internal pressure (i.e. the difference between the applied pressure and atmospheric pressure) reaches 67 kPa within 1 min. Maintain the pressure for 10 min. No signs of leakage are detectable on the indicator paper or at any point of attachment (seals, joints, etc.).

Vapour permeability. For a container containing an anticoagulant solution, fill with a volume of a 9 g/l solution of *sodium chloride R* equal to the volume of blood for which the container is intended.

For an empty container, fill with the same mixture of anticoagulant solution and sodium chloride solution. Close the container, weigh it and store it at 5 ± 1 °C in an atmosphere with a relative humidity of (50 ± 5) per cent for 21 days. At the end of this period the loss in mass is not greater than 1 per cent.

Emptying under pressure. Fill the container with a volume of *water R* at 5 ± 1 °C equal to the nominal capacity. Attach a transfusion set without an intravenous cannula to one of the connectors. Compress the container so as to maintain throughout the emptying an internal pressure (i.e. the difference between the applied pressure and atmospheric pressure) of 40 kPa. The container empties in less than 2 min.

Speed of filling. Attach the container by means of the blood-taking tube fitted with the needle to a reservoir containing a suitable solution having a viscosity equal to that of blood, such as a 335 g/l solution of *sucrose R* at 37 °C. Maintain the internal pressure of the reservoir (i.e. the difference between the applied pressure and atmospheric pressure) at 9.3 kPa with the base of the reservoir and the upper part of the container at the same level. The volume of liquid which flows into the container in 8 min is not less than the nominal capacity of the container.

Resistance to temperature variations. Place the container in a suitable chamber having an initial temperature of 20 °C to 23 °C. Cool it rapidly in a deep-freeze to –80 °C and maintain it at this temperature for 24 h. Raise the temperature to 50 °C and maintain for 12 h. Allow to cool to room temperature. The container complies with the tests for resistance to centrifugation, resistance to stretch, leakage, vapour permeability emptying under pressure and speed of filling prescribed above.

Transparency. Fill the empty container with a volume equal to its nominal capacity of the primary opalescent suspension (2.2.1) diluted so as to have an absorbance (2.2.25) at 640 nm of 0.37 to 0.43 (dilution factor about 1 in 16). The cloudiness of the suspension must be perceptible when viewed through the bag, as compared with a similar container filled with *water R*.

Extractable matter. Tests are carried out by methods designed to simulate as far as possible the conditions of contact between the container and its contents which occur in conditions of use.

The conditions of contact and the tests to be carried out on the eluates are prescribed, according to the nature of the constituent materials, in the particular requirements for each type of container.

Haemolytic effects in buffered systems

Stock buffer solution. Dissolve 90.0 g of *sodium chloride R*, 34.6 g of *disodium hydrogen phosphate R* and 2.43 g of *sodium dihydrogen phosphate R* in *water R* and dilute to 1000 ml with the same solvent.

Buffer solution A₀. To 30.0 ml of stock buffer solution add 10.0 ml of *water R*.

Buffer solution B₀. To 30.0 ml of stock buffer solution add 20.0 ml of *water R*.

Buffer solution C₀. To 15.0 ml of stock buffer solution add 85.0 ml of *water R*.

Introduce 1.4 ml of solution S₂ into each of three centrifuge tubes. To tube I add 0.1 ml of buffer solution A₀, to tube II add 0.1 ml of buffer solution B₀ and to tube III add 0.1 ml of buffer solution C₀. To each tube add 0.02 ml of fresh, heparinised human blood, mix well and warm on a water-bath at 30 ± 1 °C for 40 min. Use blood collected less than 3 h previously or blood collected into an anticoagulant citrate-phosphate-dextrose solution (CPD) less than 24 h previously.

Prepare three solutions containing, respectively:

3.0 ml of buffer solution A₀ and 12.0 ml of *water R* (solution A₁),

4.0 ml of buffer solution B₀ and 11.0 ml of *water R* (solution B₁),

4.75 ml of buffer solution B₀ and 10.25 ml of *water R* (solution C₁).

To tubes I, II and III add, respectively, 1.5 ml of solution A₁, 1.5 ml of solution B₁ and 1.5 ml of solution C₁. At the same time and in the same manner, prepare three other tubes, replacing solution S₂ by *water R*. Centrifuge simultaneously the tubes to be examined and the control tubes at exactly 2500 *g* in the same horizontal centrifuge for 5 min. After centrifuging, measure the absorbances (2.2.25) of the liquids at 540 nm using the stock buffer solution as compensation liquid. Calculate the haemolytic value as a percentage from the expression:

$$\frac{A_{exp}}{A_{100}} \times 100$$

A₁₀₀ = absorbance of tube III,

A_{exp} = absorbance of tube I or II or of the corresponding control tubes.

The solution in tube I gives a haemolytic value not greater than 10 per cent and the haemolytic value of the solution in tube II does not differ by more than 10 per cent from that of the corresponding control tube.

Sterility (2.6.1). The containers comply with the test for sterility. Introduce aseptically into the container 100 ml of a sterile 9 g/l solution of sodium chloride and shake the container to ensure that the internal surfaces have been entirely wetted. Filter the contents of the container through a membrane filter and place the membrane in the appropriate culture medium, as prescribed in the test for sterility.

Pyrogens (2.6.8). Solution S₁ complies with the test for pyrogens. Inject 10 ml of the solution per kilogram of the rabbit's mass.

Abnormal toxicity (2.6.9). Solution S₁ complies with the test for abnormal toxicity. Inject 0.5 ml of the solution into each mouse.

PACKAGING

The containers are packed in protective envelopes.

On removal from its protective envelope the container shows no leakage and no growth of micro-organisms. The protective envelope is sufficiently robust to withstand normal handling. The protective envelope is sealed in such a manner that it cannot be opened and re-closed without leaving visible traces that the seal has been broken.

LABELLING

The labelling complies with the relevant national legislation and international agreements. The label states:

- the name and address of the manufacturer,
- a batch number which enables the history of the container and of the plastic material of which it is manufactured to be traced.

A part of the label is reserved for:

- the statement of the blood group, the reference number and all other information required by national legislation or international agreements, and an empty space is provided for the insertion of supplementary labelling.

The label of the *protective envelope* or the *label* on the container, visible through the envelope, states:

- the expiry date,
- that, once withdrawn from its protective envelope, the container must be used within 10 days.

The ink or other substance used to print the labels or the writing must not diffuse into the plastic material of the container and must remain legible up to the time of use.

01/2005:30204

3.2.4. EMPTY STERILE CONTAINERS OF PLASTICISED POLY(VINYL CHLORIDE) FOR HUMAN BLOOD AND BLOOD COMPONENTS

Unless otherwise authorised as described under *Sterile Plastic Containers for Human Blood and Blood Components* (3.2.3), the nature and composition of the material from which the containers are made comply with the requirements for *Materials based on Plasticised Poly(vinyl chloride) for Containers for Human Blood and Blood Components and for Containers for aqueous solutions for intravenous infusion* (3.1.1).

TESTS

They comply with the tests prescribed for *Sterile Plastic Containers for Human Blood and Blood Components* (3.2.3) and with the following tests to detect extractable matter.

Reference solution. Heat *water for injections R* in a borosilicate-glass flask in an autoclave at 110 °C for 30 min.

Oxidisable substances. Immediately after preparation of solution S₂ (see 3.2.3), transfer to a borosilicate-glass flask a quantity corresponding to 8 per cent of the nominal capacity of the container. At the same time, prepare a blank using an equal volume of the freshly prepared reference solution in another borosilicate-glass flask. To each solution add

20.0 ml of 0.002 M *potassium permanganate* and 1 ml of *dilute sulphuric acid R*. Allow to stand protected from light for 15 min. To each solution add 0.1 g of *potassium iodide R*. Allow to stand protected from light for 5 min and titrate immediately with 0.01 M *sodium thiosulphate*, using 0.25 ml of *starch solution R* as indicator. The difference between the two titrations is not more than 2.0 ml.

Acidity or alkalinity. To a volume of solution S₂ corresponding to 4 per cent of the nominal capacity of the container add 0.1 ml of *phenolphthalein solution R*. The solution remains colourless. Add 0.4 ml of 0.01 M *sodium hydroxide*. The solution is pink. Add 0.8 ml of 0.01 M *hydrochloric acid* and 0.1 ml of *methyl red solution R*. The solution is orange-red or red.

Chlorides (2.4.4). 15 ml of solution S₂ complies with the limit test for chlorides (0.4 ppm). Prepare the standard using a mixture of 1.2 ml of *chloride standard solution (5 ppm Cl) R* and 13.8 ml of *water R*.

Ammonium (2.4.1). Dilute 5 ml of solution S₂ to 14 ml with *water R*. The solution complies with the limit test for ammonium (2 ppm).

Residue on evaporation. Evaporate to dryness 100 ml of solution S₂ in a borosilicate-glass beaker of appropriate capacity, previously heated to 105 °C. Evaporate to dryness in the same conditions 100 ml of the reference solution (blank test). Dry to constant mass at 100 °C to 105 °C. The residue from solution S₂ weighs not more than 3 mg, allowing for the blank test.

Absorbance (2.2.25). Measure the absorbance of solution S₂ from 230 nm to 360 nm, using the reference solution as compensation liquid. At wavelengths from 230 nm to 250 nm, the absorbance is not greater than 0.30. At wavelengths from 251 nm to 360 nm, the absorbance is not greater than 0.10.

Extractable di(2-ethylhexyl) phthalate. Extraction solvent, *alcohol R* diluted with *water R* to have a relative density (2.2.5) of 0.9389 to 0.9395, measured with a pycnometer.

Stock solution. Dissolve 0.100 g of *di(2-ethylhexyl) phthalate R* in the extraction solvent and dilute to 100.0 ml with the same solvent.

Standard solutions

- Dilute 20.0 ml of stock solution to 100.0 ml with extraction solvent.
- Dilute 10.0 ml of stock solution to 100.0 ml with extraction solvent.
- Dilute 5.0 ml of stock solution to 100.0 ml with extraction solvent.
- Dilute 2.0 ml of stock solution to 100.0 ml with extraction solvent.
- Dilute 1.0 ml of stock solution to 100.0 ml with extraction solvent.

Measure the absorbances (2.2.25) of the standard solutions at the maximum at 272 nm, using the extraction solvent as compensation liquid and plot a curve of absorbance against the concentration of di(2-ethylhexyl) phthalate.

Extraction procedure. Using the donor tubing and the needle or adaptor, fill the empty container with a volume equal to half the nominal volume with the extraction solvent, previously heated to 37 °C in a well-stoppered flask. Expel the air completely from the container and seal the donor tube. Immerse the filled container in a horizontal position in a water-bath maintained at 37 ± 1 °C for 60 ± 1 min without shaking. Remove the container from the water-bath, invert it

gently ten times and transfer the contents to a glass flask. Immediately measure the absorbance at the maximum at 272 nm, using the extraction solvent as compensation liquid. Determine the concentration of di(2-ethylhexyl) phthalate in milligrams per 100 ml of extract from the calibration curve. The concentration does not exceed:

- 10 mg per 100 ml for containers of nominal volume greater than 300 ml but not greater than 500 ml;
- 13 mg per 100 ml for containers of nominal volume greater than 150 ml but not greater than 300 ml;
- 14 mg per 100 ml for containers of nominal volume up to 150 ml.

PACKAGING

See *Sterile Plastic Containers for Human Blood and Blood Components (3.2.3)*.

LABELLING

See *Sterile Plastic Containers for Human Blood and Blood Components (3.2.3)*.

01/2005:30205

3.2.5. STERILE CONTAINERS OF PLASTICISED POLY (VINYL CHLORIDE) FOR HUMAN BLOOD CONTAINING ANTICOAGULANT SOLUTION

Sterile plastic containers containing an anticoagulant solution complying with the monograph on *Anticoagulant and Preservative Solutions for Human Blood (0209)* are used for the collection, storage and administration of blood. Before filling they comply with the description and characters given under *Empty Sterile Containers of Plasticised Poly (vinyl chloride) for Human Blood and Blood Components (3.2.4)*.

Unless otherwise authorised as described under *Sterile Plastic Containers for Human Blood and Blood Components (3.2.3)*, the nature and composition of the material from which the containers are made should comply with the requirements prescribed for *Materials based on Plasticised Poly (vinyl chloride) for Containers for Human Blood and Blood Components and for containers for aqueous solutions for intravenous infusion (3.1.1)*.

TESTS

They comply with the tests prescribed for *Sterile Plastic Containers for Human Blood and Blood Components (3.2.3)* and with the following tests to measure the volume of anticoagulant solution and to detect extractable matter.

Volume of anticoagulant solution. Empty the container, collecting the anticoagulant solution in a graduated cylinder. The volume does not differ by more than ± 10 per cent from the stated volume.

Spectrophotometric examination (2.2.25). Measure the absorbance of the anticoagulant solution from the container between 250 nm and 350 nm, using as the compensation liquid an anticoagulant solution of the same composition that has not been in contact with a plastic material. The absorbance at the maximum at 280 nm is not greater than 0.5.

Extractable di(2-ethylhexyl) phthalate. Carefully remove the anticoagulant solution by means of the flexible transfer tube. Using a funnel fitted to the tube, completely fill the

container with *water R*, leave in contact for 1 min squeezing the container gently, then empty completely. Repeat the rinsing.

The container, so emptied and rinsed, complies with the test for extractable di(2-ethylhexyl) phthalate prescribed for *Empty Sterile Plastic Containers of Plasticised Poly (vinyl chloride) for Human Blood and Blood Components (3.2.4)*.

PACKAGING AND LABELLING

See *Sterile Plastic Containers for Human Blood and Blood Components (3.2.3)*.

01/2005:30206

3.2.6. SETS FOR THE TRANSFUSION OF BLOOD AND BLOOD COMPONENTS

Sets for the transfusion of blood and blood components consist principally of plastic tubing to which are fitted the parts necessary to enable the set to be used for transfusion in the appropriate manner. Sets include a closure-piercing device, a blood filter, a drip chamber, a flow regulator, a Luer connector and, usually, a site that allows an injection to be made at the time of use. When the sets are to be used with containers requiring an air-filter, this may be incorporated in the closure-piercing device or a separate air-inlet device may be used. The chamber enclosing the blood filter, the drip chamber and the main tubing are transparent. The materials chosen and the design of the set are such as to ensure absence of haemolytic effects. The sets comply with current standards regarding dimensions and performance.

All parts of the set that may be in contact with blood and blood components are sterile and pyrogen-free. Each set is presented in an individual package that maintains the sterility of the contents. The sets are not to be re-sterilised or re-used.

Sets for the transfusion of blood and blood components are manufactured in accordance with the rules of good manufacturing practice for medical devices and any relevant national regulations.

TESTS

Carry out the tests on sterilised sets.

Solution S. Make a closed circulation system from three sets and a 300 ml borosilicate-glass vessel. Fit to the vessel a thermostat device that maintains the temperature of the liquid in the vessel at 37 ± 1 °C. Circulate 250 ml of *water for injections R* through the system in the direction used for transfusion for 2 h at a rate of 1 litre/h (for example using a peristaltic pump applied to as short a piece of suitable silicone tubing as possible). Collect the whole of the solution and allow to cool.

Appearance of solution S. Solution S is clear (2.2.1) and colourless (2.2.2, *Method II*).

Acidity or alkalinity. To 25 ml of solution S add 0.15 ml of *BRP indicator solution R*. Not more than 0.5 ml of 0.01 M *sodium hydroxide* is required to change the colour of the indicator to blue. To 25 ml of solution S add 0.2 ml of *methyl orange solution R*. Not more than 0.5 ml of 0.01 M *hydrochloric acid* is required to reach the beginning of the colour change of the indicator.

Absorbance (2.2.25). Examined from 230 nm to 250 nm, solution S shows no absorbance greater than 0.30. Examined from 251 nm to 360 nm, solution S shows no absorbance greater than 0.15.

Ethylene oxide. If the label states that ethylene oxide has been used for sterilisation, the content of ethylene oxide, determined by the method prescribed below, is not greater than 10 ppm. Examine by gas chromatography (2.2.28).

The chromatographic procedure may be carried out using:

- a stainless steel column 1.5 m long and 6.4 mm in internal diameter packed with *silanised diatomaceous earth for gas chromatography R* impregnated with *macrogol 1500 R* (3 g per 10 g),
- *helium for chromatography R* as the carrier gas at a flow rate of 20 ml/min,
- a flame-ionisation detector,

maintaining the temperature of the column at 40 °C, that of the injector at 100 °C and that of the detector at 150 °C. Verify the absence of peaks interfering with the ethylene oxide peak by carrying out the test using an unsterilised set or using a different chromatographic system such as:

- a stainless steel column 3 m long and 3.2 mm in internal diameter packed with *silanised diatomaceous earth for gas chromatography R* impregnated with *triscyanoethoxypropane R* (2 g per 10 g),
- *helium for chromatography R* as the carrier gas at a flow rate of 20 ml/min,
- a flame-ionisation detector,

maintaining the temperature of the column at 60 °C, that of the injector at 100 °C and that of the detector at 150 °C.

Ethylene oxide solution. Prepare under a ventilated hood. Place 50.0 ml of *dimethylacetamide R* in a 50 ml vial, stopper, secure the stopper and weigh to the nearest 0.1 mg. Fill a 50 ml polyethylene or polypropylene syringe with gaseous *ethylene oxide R*, allow the gas to remain in contact with the syringe for about 3 min, empty the syringe and fill again with 50 ml of gaseous *ethylene oxide R*. Fit a hypodermic needle to the syringe and reduce the volume of gas in the syringe from 50 ml to 25 ml. Inject these 25 ml of ethylene oxide slowly into the vial, shaking gently and avoiding contact between the needle and the liquid. Weigh the vial again: the increase in mass is 45 mg to 60 mg and is used to calculate the exact concentration of the solution (about 1 g/l).

Test. Weigh the set after removing the package. Cut the set into pieces of maximum dimension 1 cm and place the pieces in a 250 ml to 500 ml vial containing 150 ml of *dimethylacetamide R*. Close the vial with a suitable stopper and secure the stopper. Place the vial in an oven at 70 ± 1 °C for 16 h. Remove 1 ml of the hot gas from the vial and inject it onto the column. From the calibration curve and the height of the peak obtained, calculate the mass of ethylene oxide in the vial.

Calibration curve. In a series of seven vials of the same type as that used for the test and each containing 150 ml of *dimethylacetamide R*, place respectively 0 ml, 0.05 ml, 0.10 ml, 0.20 ml, 0.50 ml, 1.00 ml and 2.00 ml of the ethylene oxide solution, i.e. about 0 µg, 50 µg, 100 µg, 200 µg, 500 µg, 1000 µg and 2000 µg of ethylene oxide. Stopper the vials, secure the stoppers and place the vials in an oven at 70 ± 1 °C for 16 h. Inject 1 ml of the hot gas from each vial onto the column and draw a calibration curve from the heights of the peaks and the mass of ethylene oxide in each flask.

Reducing substances. Carry out the test within 4 h of preparation of solution S. To 20.0 ml of solution S add 1 ml of *dilute sulphuric acid R* and 20.0 ml of 0.002 M *potassium permanganate*. Boil for 3 min and cool immediately. Add 1 g of *potassium iodide R* and titrate with 0.01 M *sodium thiosulphate* using 0.25 ml of *starch solution R* as indicator.

01/2005:30208

Carry out a blank test using 20 ml of *water for injections R*. The difference between the titration volumes is not greater than 2.0 ml.

Extraneous particles. Fill the set via the normal inlet with a 0.1 g/l solution of *sodium laurilsulfate R*, previously filtered through a sintered-glass filter (16) and heated to 37 °C. Collect the liquid via the normal outlet. When examined under suitable conditions of visibility, the liquid is clear and practically free from visible particles and filaments (it is assumed that particles and filaments with a diameter equal to or greater than 50 µm are visible to the naked eye).

Flow rate. Pass through a complete set with the flow regulator fully open 50 ml of a solution having a viscosity of 3 mPas (3 cP) (for example a 33 g/l solution of *macrogol 4000 R* at 20 °C) under a static head of 1 m. The time required for passage of 50 ml of the solution is not greater than 90 s.

Resistance to pressure. Make tight the extremities of the set and any air-inlet device. Connect the set to a compressed air outlet fitted with a pressure regulator. Immerse the set in a tank of water at 20 °C to 23 °C. Apply progressively an excess pressure of 100 kPa and maintain for 1 min. No air bubble escapes from the set.

Transparency. Use as reference suspension the primary opalescent suspension (2.2.1) diluted 1 in 8 for sets having tubing with an external diameter less than 5 mm and diluted 1 in 16 for sets having tubing with an external diameter of 5 mm or greater. Circulate the reference suspension through the set and compare with a set from the same batch filled with *water R*. The opalescence and presence of bubbles are discernible.

Residue on evaporation. Evaporate 50.0 ml of solution S to dryness on a water-bath and dry to constant mass in an oven at 100 °C to 105 °C. Carry out a blank test using 50.0 ml of *water for injections R*. The difference between the masses of the residues is not greater than 1.5 mg.

Sterility (2.6.1). The sets comply with the test for sterility. If the sets are stated to be sterile only internally, pass 50 ml of buffered sodium chloride-peptone solution pH 7.0 (2.6.12) through the set and use to carry out the test by the membrane-filtration method.

If the sets are stated to be sterile both internally and externally, open the package with the necessary aseptic precautions and:

- for the direct inoculation method, place the set or its components in a suitable container containing a sufficient quantity of the culture medium to ensure complete immersion;
- for the membrane filtration method, place the set or its components in a suitable container containing a sufficient quantity of buffered sodium chloride-peptone solution pH 7.0 (2.6.12) to allow total rinsing for 10 min.

Pyrogens (2.6.8). Connect together five sets and pass through the assembly at a flow rate not exceeding 10 ml/min 250 ml of a sterile, pyrogen-free 9 g/l solution of *sodium chloride R*. Collect the solution aseptically in a pyrogen-free container. The solution complies with the test for pyrogens. Inject 10 ml per kilogram of the rabbit's mass.

LABELLING

The label states, where applicable, that the set has been sterilised using ethylene oxide.

3.2.8. STERILE SINGLE-USE PLASTIC SYRINGES

Sterile single-use plastic syringes are medical devices intended for immediate use for the administration of injectable preparations. They are supplied sterile and pyrogen-free and are not to be re-sterilised or re-used. They consist of a syringe barrel and a piston which may have an elastomer sealing ring; they may be fitted with a needle which may be non-detachable. Each syringe is presented with individual protection for maintaining sterility.

The barrel of the syringe is sufficiently transparent to permit dosages to be read without difficulty and allow air bubbles and foreign particles to be discerned.

The plastics and elastomer materials of which the barrel and piston are made comply with the appropriate specification or with the requirements of the competent authority. The most commonly used materials are polypropylene and polyethylene. The syringes comply with current standards regarding dimensions and performance.

Silicone oil (3.1.8) may be applied to the internal wall of the barrel to assist in the smooth operation of the syringe but there remains no excess capable of contaminating the contents at the time of use.

The inks, glues and adhesives for the marking on the syringe or on the package and, where necessary, the assembly of the syringe and its package, do not migrate across the walls.

TESTS

Solution S. Prepare the solution in a manner that avoids contamination by foreign particles. Using a sufficient number of syringes to produce 50 ml of solution, fill the syringes to their nominal volume with *water for injections R* and maintain at 37 °C for 24 h. Combine the contents of the syringes in a suitable borosilicate-glass container.

Appearance of solution. Solution S is clear (2.2.1) and colourless (2.2.2, *Method II*) and is practically free from foreign solid particles.

Acidity or alkalinity. To 20 ml of solution S add 0.1 ml of *bromothymol blue solution R1*. Not more than 0.3 ml of 0.01 M *sodium hydroxide* or 0.01 M *hydrochloric acid* is required to change the colour of the indicator.

Absorbance (2.2.25). Measure the absorbance of solution S from 220 nm to 360 nm. The absorbance does not exceed 0.40.

Ethylene oxide. If the label states that ethylene oxide has been used for sterilisation, the content of ethylene oxide, determined by the method described below, is not greater than 10 ppm. Examine by gas chromatography (2.2.28).

The chromatographic procedure may be carried out using:

- a stainless steel column 1.5 m long and 6.4 mm in internal diameter packed with *silanised diatomaceous earth for gas chromatography R* impregnated with *macrogol 1500 R* (3 g per 10 g),
- *helium for chromatography R* as the carrier gas at a flow rate of 20 ml/min,
- a flame-ionisation detector,

maintaining the temperature of the column at 40 °C, that of the injector at 100 °C and that of the detector at 150 °C. Verify the absence of peaks interfering with the ethylene oxide peak, either by carrying out the test using an unsterilised syringe or using a different chromatographic system such as:

- a stainless-steel column 3 m long and 3.2 mm in internal diameter packed with *silanised diatomaceous earth for gas chromatography R* impregnated with *triscyanoethoxypropane R* (2 g per 10 g),
- *helium for chromatography R* as carrier gas at a flow rate of 20 ml/min,
- a flame-ionisation detector,

maintaining the temperature of the column at 60 °C, that of the injector at 100 °C and that of the detector at 150 °C.

Ethylene oxide solution. Prepare under a ventilated hood. Place 50.0 ml of *dimethylacetamide R* in a 50 ml vial, stopper, secure the stopper and weigh to the nearest 0.1 mg. Fill a 50 ml polyethylene or polypropylene syringe with gaseous *ethylene oxide R*, allow the gas to remain in contact with the syringe for about 3 min, empty the syringe and fill again with 50 ml of gaseous *ethylene oxide R*. Fit a hypodermic needle to the syringe and reduce the volume of gas in the syringe from 50 ml to 25 ml. Inject these 25 ml of ethylene oxide slowly into the vial, shaking gently and avoiding contact between the needle and the liquid. Weigh the vial again: the increase in mass is 45 mg to 60 mg and is used to calculate the exact concentration of the solution (about 1 g/l).

Calibration curve. In a series of seven vials of the same type as that used for the test and each containing 150 ml of *dimethylacetamide R*, place respectively 0 ml, 0.05 ml, 0.10 ml, 0.20 ml, 0.50 ml, 1.00 ml and 2.00 ml of the ethylene oxide solution, i.e. about 0 µg, 50 µg, 100 µg, 200 µg, 500 µg, 1000 µg and 2000 µg of ethylene oxide. Stopper the vials, secure the stoppers and place the vials in an oven at 70 ± 1 °C for 16 h. Inject 1 ml of the hot gas from each vial onto the column and draw a calibration curve from the heights of the peaks and the mass of ethylene oxide in each flask.

Test. Weigh the syringe after removing the package. Cut the syringe into pieces of maximum dimension 1 cm and place the pieces in a 250 ml to 500 ml vial containing 150 ml of *dimethylacetamide R*. Close the vial with a suitable stopper and secure the stopper. Place the vial in an oven at 70 ± 1 °C for 16 h. Remove 1 ml of the hot gas from the vial and inject it onto the column. From the calibration curve and the height of the peak obtained, calculate the mass of ethylene oxide in the vial.

Silicone oil. Calculate the internal surface area of a syringe in square centimetres using the expression:

$$2\sqrt{V \cdot \pi \cdot h}$$

V = nominal volume of the syringe, in cubic centimetres,

h = height of the graduation, in centimetres.

Take a sufficient number of syringes to give an internal surface area of 100 cm² to 200 cm². Aspirate into each syringe a volume of *methylene chloride R* equal to half the nominal volume and make up to the nominal volume

with air. Rinse the internal surface corresponding to the nominal volume with the solvent by inverting the syringe ten times in succession with the needle fitting closed by a finger covered by a plastic film inert to methylene chloride. Expel the extracts into a tared dish and repeat the operation. Evaporate the combined extracts to dryness on a water-bath. Dry at 100 °C to 105 °C for 1 h. The residue weighs not more than 0.25 mg per square centimetre of internal surface area.

Examine the residue by infrared absorption spectrophotometry (2.2.24). It shows absorption bands typical of silicone oil at 805 cm⁻¹, 1020 cm⁻¹, 1095 cm⁻¹, 1260 cm⁻¹ and 2960 cm⁻¹.

Reducing substances. To 20.0 ml of solution S add 2 ml of *sulphuric acid R* and 20.0 ml of 0.002 M *potassium permanganate*. Boil for 3 min. Cool immediately. Add 1 g of *potassium iodide R* and titrate immediately with 0.01 M *sodium thiosulphate* using 0.25 ml of *starch solution R* as indicator. Carry out a blank titration using 20.0 ml of *water for injections R*. The difference between the titration volumes is not greater than 3.0 ml.

Transparency. Fill a syringe with *water R* (blank) and fill another with a 1 in 10 dilution of primary opalescent suspension (2.2.1). Use primary opalescent suspension that has been allowed to stand at 20 ± 2 °C for 24 h before use. Compare with the naked eye in diffused light against a dark background. The opalescence of the suspension is detectable when compared with the blank.

Sterility (2.6.1). *Syringes stated to be sterile comply with the test for sterility carried out as follows.* Using aseptic technique, open the package, withdraw the syringe, separate the components and place each in a suitable container containing sufficient culture media to cover the part completely. Use both the recommended media (2.6.1).

Syringes stated to be sterile only internally comply with the test for sterility carried out as follows. Use 50 ml of inoculation medium for each test syringe. Using aseptic technique, remove the needle protector and submerge the needle in the culture medium. Flush the syringe five times by withdrawing the plunger to its fullest extent.

Pyrogens (2.6.8). Syringes with a nominal volume equal to or greater than 15 ml comply with the test for pyrogens. Fill a minimum of three syringes to their nominal volume with a pyrogen-free 9 g/l solution of *sodium chloride R* and maintain at a temperature of 37 °C for 2 h. Combine the solutions aseptically in a pyrogen-free container and carry out the test immediately using for each rabbit 10 ml of the solution per kilogram of body mass.

LABELLING

The label on the *package* states:

- the batch number,
- a description of the syringe,
- that the syringe is for single-use only.

The label on the outer *package* states:

- the method of sterilisation,
- that the syringe is sterile or that it is sterile only internally,
- the identity of the manufacturer,
- that the syringe is not to be used if the packaging is damaged or the sterility protector is loose.

01/2005:30209

3.2.9. RUBBER CLOSURES FOR CONTAINERS FOR AQUEOUS PARENTERAL PREPARATIONS, FOR POWDERS AND FOR FREEZE-DRIED POWDERS

Rubber closures for containers for aqueous parenteral preparations for powders and for freeze-dried powders are made of materials obtained by vulcanisation (cross-linking) of macromolecular organic substances (elastomers), with appropriate additives. The specification also applies to closures for containers for powders and freeze-dried products to be dissolved in water immediately before use. The specification does not apply to closures made from silicone elastomer (which are dealt with in 3.1.9. *Silicone elastomer for closures and tubing*), to laminated closures or to lacquered closures. The elastomers are produced from natural or synthetic substances by polymerisation, polyaddition or polycondensation. The nature of the principal components and of the various additives (for example vulcanisers, accelerators, stabilisers, pigments) depends on the properties required for the finished article.

Rubber closures may be classified in 2 types: type I closures are those which meet the strictest requirements and which are to be preferred; type II closures are those which, having mechanical properties suitable for special uses (for example, multiple piercing), cannot meet requirements as severe as those for the first category because of their chemical composition.

The closures chosen for use with a particular preparation are such that:

- the components of the preparation in contact with the closure are not adsorbed onto the surface of the closure and do not migrate into or through the closure to an extent sufficient to affect the preparation adversely,
- the closure does not yield to the preparation substances in quantities sufficient to affect its stability or to present a risk of toxicity.

The closures are compatible with the preparation for which they are used throughout its period of validity.

The manufacturer of the preparation must obtain from the supplier an assurance that the composition of the closure does not vary and that it is identical to that of the closure used during compatibility testing. When the supplier informs the manufacturer of the preparation of changes in the composition, compatibility testing must be repeated, totally or partly, depending on the nature of the changes.

The closures are washed and may be sterilised before use.

CHARACTERS

Rubber closures are elastic; they are translucent or opaque and have no characteristic colour, the latter depending on the additives used. They are practically insoluble in tetrahydrofuran, in which, however, a considerable reversible swelling may occur. They are homogeneous and practically free from flash and adventitious materials (for example fibres, foreign particles, waste rubber).

Identification of the type of rubber used for the closures is not within the scope of this specification. The identification test given below distinguishes elastomer and non-elastomer closures but does not differentiate the various types of rubber. Other identity tests may be carried out with the aim

of detecting differences in a batch compared to the closures used for compatibility testing. One or more of the following analytical methods may be applied for this purpose:

determination of relative density, determination of sulphated ash, determination of sulphur content, thin-layer chromatography carried out on an extract, ultraviolet absorption spectrophotometry of an extract, infrared absorption spectrophotometry of a pyrolysate.

IDENTIFICATION

- The elasticity is such that a strip of material with a cross-section of 1 mm² to 5 mm² can be stretched by hand to at least twice its original length. Having been stretched to twice its length for 1 min, it contracts to less than 1.2 times its original length within 30 s.
- Heat 1 g to 2 g in a heat-resistant test-tube over an open flame to dry the sample and continue heating until pyrolysate vapours are condensed near the top edge of the test-tube. Deposit a few drops of the pyrolysate on a potassium bromide disc and examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with the type sample.
- The total ash (2.4.16) is within ± 10 per cent of the result obtained with the type sample.

TESTS

The samples to be analysed may be washed and sterilised before use.

Solution S. Introduce a number of uncut closures corresponding to a surface area of about 100 cm² in a suitable glass container, cover with *water for injections R*, boil for 5 min and rinse 5 times with cold *water for injections R*. Place the washed closures in a wide-necked flask (glass type I, 3.2.1), add 200 ml of *water for injections R* and weigh. Cover the mouth of the flask with a borosilicate-glass beaker. Heat in an autoclave so that a temperature of 121 ± 2 °C is reached within 20 min to 30 min and maintain at this temperature for 30 min. Cool to room temperature over about 30 min. Make up to the original mass with *water for injections R*. Shake and immediately separate the solution from the rubber by decantation. Shake solution S before each test

Blank. Prepare a blank in the same manner using 200 ml of *water for injections R*.

Appearance of solution. Solution S is not more opalescent than reference suspension II for type I closures and is not more opalescent than reference suspension III for type II closures (2.2.1). Solution S is not more intensely coloured than reference solution GY₅ (2.2.2, *Method II*).

Acidity or alkalinity. To 20 ml of solution S add 0.1 ml of *bromothymol blue solution R1*. Not more than 0.3 ml of 0.01 M sodium hydroxide or 0.8 ml of 0.01 M hydrochloric acid is required to obtain either a blue or a yellow colour, respectively.

Absorbance. Carry out the test within 5 h of preparation of solution S. Filter solution S on a membrane filter having approximately 0.45 µm pores rejecting the first few millilitres of filtrate. Measure the absorbance (2.2.25) of the filtrate at wavelengths from 220 nm to 360 nm using the blank (see solution S) as compensation liquid. At these wavelengths, the absorbance does not exceed 0.2 for type I closures or 4.0 for type II closures. If necessary, dilute the filtrate before measurement of the absorbance and correct the result for the dilution.

Reducing substances. Carry out the test within 4 h of preparation of solution S. To 20.0 ml of solution S add 1 ml of dilute sulphuric acid R and 20.0 ml of 0.002 M

potassium permanganate. Boil for 3 min. Cool. Add 1 g of *potassium iodide R* and titrate immediately with 0.01 M *sodium thiosulphate*, using 0.25 ml of *starch solution R* as indicator. Carry out a titration using 20.0 ml of the blank. The difference between the titration volumes is not greater than 3.0 ml for type I closures and 7.0 ml for type II closures.

Ammonium (2.4.1): maximum 2 ppm.

Dilute 5 ml of solution S to 14 ml with *water R*. The solution complies with limit test A.

Extractable zinc: maximum of 5 µg of extractable Zn per millilitre of solution S.

Atomic absorption spectrophotometry (2.2.23, *Method I*).

Test solution. Dilute 10.0 ml of solution S to 100 ml with 0.1 M *hydrochloric acid*.

Reference solutions. Prepare the reference solutions using *zinc standard solution (10 ppm Zn) R* diluted with 0.1 M *hydrochloric acid*.

Source: zinc hollow-cathode lamp.

Wavelength: 213.9 nm.

Flame: air-acetylene.

Extractable heavy metals (2.4.8): maximum 2 ppm.

Solution S complies with limit test A. Prepare the standard using *lead standard solution (2 ppm Pb) R*.

Residue on evaporation. Evaporate 50.0 ml of solution S to dryness on a water-bath and dry at 100 °C to 105 °C. The residue weighs not more than 2.0 mg for type I rubber and not more than 4.0 mg for type II rubber.

Volatile sulphides. Place closures, cut if necessary, with a total surface area of $20 \pm 2 \text{ cm}^2$ in a 100 ml conical flask and add 50 ml of a 20 g/l solution of *citric acid R*. Place a piece of *lead acetate paper R* over the mouth of the flask and maintain the paper in position by placing over it an inverted weighing bottle. Heat in an autoclave at $121 \pm 2 \text{ °C}$ for 30 min. Any black stain on the paper is not more intense than that of a standard prepared at the same time in the same manner using 0.154 mg of *sodium sulphide R* and 50 ml of a 20 g/l solution of *citric acid R*.

For the tests for penetrability, fragmentation and self-sealing, use the closures treated as described for the preparation of solution S and allowed to dry.

Penetrability. For closures intended to be pierced by a hypodermic needle, carry out the following test. Fill 10 suitable vials to the nominal volume with *water R*, fit the closures to be examined and secure with a cap. Using for each closure a new, lubricated long-bevel⁽¹⁾ (bevel angle $12 \pm 2^\circ$) hypodermic needle with an external diameter of 0.8 mm, pierce the closures with the needle perpendicular to the surface. The force required for piercing, determined with an accuracy of $\pm 0.25 \text{ N}$ (25 gf), is not greater than 10 N (1 kgf) for each closure.

Fragmentation. For closures intended to be pierced by a hypodermic needle, carry out the following test. If the closures are to be used for aqueous preparations, place in 12 clean vials a volume of *water R* corresponding to the nominal volume minus 4 ml, close the vials with the closures to be examined, secure with a cap and allow to stand for 16 h. If the closures are to be used with dry preparations, close 12 clean vials with the closures to be examined. Using a lubricated long-bevel⁽¹⁾ (bevel angle $12 \pm 2^\circ$) hypodermic needle with an external diameter of 0.8 mm fitted to a clean syringe, inject into the vial 1 ml of *water R* and remove 1 ml of air; carry out this operation 4 times for each closure, piercing each time at a different site. Use a new needle for each closure and check that the needle is not blunted during the test. Pass the liquid in the vials through a filter having approximately 0.5 µm pores. Count the fragments of rubber visible to the naked eye. The total number of fragments does not exceed 5. This limit is based on the assumption that fragments with a diameter equal to or greater than 50 µm are visible to the naked eye; in cases of doubt or dispute, the fragments are examined with a microscope to verify their nature and size.

Self-sealing test. For closures intended to be used with multidose containers, carry out the following test. Fill 10 suitable vials to the nominal volume with *water R*, fit the closures to be examined and secure with a cap. Using for each closure a new hypodermic needle with an external diameter of 0.8 mm, pierce each closure 10 times, piercing each time at a different site. Immerse the vials upright in a 1 g/l solution of *methylene blue R* and reduce the external pressure by 27 kPa for 10 min. Restore atmospheric pressure and leave the vials immersed for 30 min. Rinse the outside of the vials. None of the vials contains any trace of coloured solution.

(1) See ISO 7864 "Sterile hypodermic needles for single use".

4. REAGENTS

Additional information for reagents that can only be fully identified by a trademark or whose availability is limited may be found on the EDQM website at the following address: <http://www.pheur.org/knowledge.htm>. This information is given only to make it easier to obtain such reagents and this does not suggest in any way that the mentioned suppliers are especially recommended or certified by the European Pharmacopoeia Commission or the Council of Europe. It is therefore acceptable to use reagents from another source provided that they comply with the standards of the Pharmacopoeia.

01/2005:40000 **Acebutolol hydrochloride.** 1148900. [34381-68-5].
See *Acebutolol hydrochloride (0871)*.

Acetal. $C_6H_{14}O_2$. (M_r 118.2). 1112300. [105-57-7].
Acetaldehyde diethyl acetal. 1,1-Diethoxyethane.

A clear, colourless, volatile liquid, miscible with water and with alcohol.

d_{20}^{20} : about 0.824.

n_D^{20} : about 1.382.

bp: about 103 °C.

Acetaldehyde. C_2H_4O . (M_r 44.1). 1000200. [75-07-0].
Ethanal.

A clear, colourless flammable liquid, miscible with water and with alcohol.

d_{20}^{20} : about 0.788.

n_D^{20} : about 1.332.

bp: about 21 °C.

Acetaldehyde ammonia trimer trihydrate.

$C_6H_{15}N_3 \cdot 3H_2O$. (M_r 183.3). 1133500. [76231-37-3].
2,4,6-Trimethylhexahydro-1,3,5-triazine trihydrate.

mp: 95 °C to 97 °C.

Acetic acid, anhydrous. $C_2H_4O_2$. (M_r 60.1). 1000300.
[64-19-7].

Content: minimum 99.6 per cent *m/m* of $C_2H_4O_2$.

A colourless liquid or white, shining, fern-like crystals, miscible with or very soluble in water, in alcohol, in glycerol (85 per cent), and in most fatty and essential oils.

d_{20}^{20} : 1.052 to 1.053.

bp: 117 °C to 119 °C.

A 100 g/l solution is strongly acid (2.2.4).

A 5 g/l solution neutralised with *dilute ammonia R2* gives reaction (b) of acetates (2.3.1).

Freezing point (2.2.18): minimum 15.8 °C.

Water (2.5.12): maximum 0.4 per cent. If the water content is more than 0.4 per cent it may be adjusted by adding the calculated amount of *acetic anhydride R*.

Storage: protected from light.

Acetic acid, glacial. $C_2H_4O_2$. (M_r 60.1). 1000400. [64-19-7].
See *Acetic acid, glacial (0590)*.

Acetic acid. 1000401.

Content: 290 g/l to 310 g/l of $C_2H_4O_2$ (M_r 60.1).

Dilute 30 g of *glacial acetic acid R* to 100 ml with *water R*.

Acetic acid, dilute. 1000402.

Content: 115 g/l to 125 g/l of $C_2H_4O_2$ (M_r 60.1).

Dilute 12 g of *glacial acetic acid R* to 100 ml with *water R*.

Acetic anhydride. $C_4H_6O_3$. (M_r 102.1). 1000500. [108-24-7].
Content: minimum 97.0 per cent *m/m* of $C_4H_6O_3$.

A clear, colourless liquid.

bp: 136 °C to 142 °C.

Assay. Dissolve 2.00 g in 50.0 ml of 1 M sodium hydroxide in a ground-glass-stoppered flask and boil under a reflux condenser for 1 h. Titrate with 1 M hydrochloric acid, using 0.5 ml of *phenolphthalein solution R* as indicator. Calculate the number of millilitres of 1 M sodium hydroxide required for 1 g (n_1). Dissolve 2.00 g in 20 ml of *cyclohexane R* in a ground-glass-stoppered flask, cool in ice and add a cold mixture of 10 ml of *aniline R* and 20 ml of *cyclohexane R*. Boil the mixture under a reflux condenser for 1 h, add 50.0 ml of 1 M sodium hydroxide and shake vigorously. Titrate with 1 M hydrochloric acid, using 0.5 ml of *phenolphthalein*

01/2005:40100

4.1. REAGENTS, STANDARD SOLUTIONS, BUFFER SOLUTIONS

Where the name of substance or a solution is followed by the letter R (the whole in italics), this indicates a reagent included in the following list. The specifications given for reagents do not necessarily guarantee their quality for use in medicines.

Within the description of each reagent there is a seven-figure reference code in italics (for example, 1002501). This number, which will remain unchanged for a given reagent during subsequent revisions of the list, is used for identification purposes by the Secretariat, and users of the Pharmacopoeia may also find it useful, for example in the management of reagent stocks. The description may also include a CAS number (Chemical Abstract Service Registry Number) recognisable by its typical format, for example 9002-93-1.

Some of the reagents included in the list are toxic and should be handled in conformity with good quality control laboratory practice.

Reagents in aqueous solution are prepared using *water R*. Where a reagent solution is described using an expression such as "hydrochloric acid (10 g/l HCl)", the solution is prepared by an appropriate dilution with *water R* of a more concentrated reagent solution specified in this chapter. Reagent solutions used in the limit tests for barium, calcium and sulphates are prepared using *distilled water R*. Where the name of the solvent is not stated, an aqueous solution is intended.

The reagents and reagent solutions are to be stored in well-closed containers. The labelling should comply with the relevant national legislation and international agreements.

01/2005:40101

4.1.1. REAGENTS

Acacia. 1000100.

See *Acacia (0307)*.

Acacia solution. 1000101.

Dissolve 100 g of *acacia R* in 1000 ml of *water R*. Stir with a mechanical stirrer for 2 h. Centrifuge at about 2000 *g* for 30 min to obtain a clear solution.

Storage: in polyethylene containers of about 250 ml capacity at 0 °C to –20 °C.

solution R as indicator. Calculate the number of millilitres of 1 M sodium hydroxide required for 1 g (n_2). Calculate the percentage of $C_4H_6O_3$ from the expression:

$$10.2 (n_1 - n_2)$$

Acetic anhydride solution R1. 1000501.

Dissolve 25.0 ml of *acetic anhydride R* in *anhydrous pyridine R* and dilute to 100.0 ml with the same solvent.

Storage: protected from light and air.

Acetic anhydride - sulphuric acid solution. 1000502.

Carefully mix 5 ml of *acetic anhydride R* with 5 ml of *sulphuric acid R*. Add dropwise and with cooling to 50 ml of *ethanol R*.

Prepare immediately before use.

Acetone. 1000600. [67-64-1].

See *Acetone* (0872).

Acetonitrile. C_2H_3N . (M_r 41.05). 1000700. [75-05-8]. Methyl cyanide. Ethanenitrile.

A clear, colourless liquid, miscible with water, with acetone and with methanol.

d_{20}^{20} : about 0.78.

n_D^{20} : about 1.344.

A 100 g/l solution is neutral to litmus paper.

Distillation range (2.2.11). Not less than 95 per cent distils between 80 °C and 82 °C.

Acetonitrile used in spectrophotometry complies with the following additional requirement.

Minimum transmittance (2.2.25): 98 per cent from 255 nm to 420 nm, using *water R* as compensation liquid.

Acetonitrile for chromatography. 1000701.

See *Acetonitrile R*.

Acetonitrile used in chromatography complies with the following additional requirements.

Minimum transmittance (2.2.25): 98 per cent from 240 nm, using *water R* as compensation liquid.

Minimum purity (2.2.28): 99.8 per cent.

Acetonitrile R1. 1000702.

Complies with the requirements prescribed for *acetonitrile R* and with the following additional requirements.

Content: minimum 99.9 per cent of C_2H_3N .

Absorbance (2.2.25). The absorbance at 200 nm using *water R* as the compensation liquid is not more than 0.10.

Acetylacetamide. $C_4H_7NO_2$. (M_r 101.1). 1102600. [5977-14-0]. 3-Oxobutanamide.

mp: 53 °C to 56 °C.

Acetylacetone. $C_5H_8O_2$. (M_r 100.1). 1000900. [123-54-6]. 2,4-Pentanedione.

A colourless or slightly yellow, easily flammable liquid, freely soluble in water, miscible with acetone, with alcohol and with glacial acetic acid.

n_D^{20} : 1.452 to 1.453.

bp: 138 °C to 140 °C.

Acetylacetone reagent R1. 1000901.

To 100 ml of *ammonium acetate solution R* add 0.2 ml of *acetylacetone R*.

N-Acetyl-ε-caprolactam. $C_8H_{13}NO_2$. (M_r 155.2). 1102700. [1888-91-1]. *N*-Acetylhexane-6-lactam.

Colourless liquid, miscible with ethanol.

d_{20}^{20} : about 1.100.

n_D^{20} : about 1.489.

bp: about 135 °C.

Acetyl chloride. C_2H_3ClO . (M_r 78.5). 1000800. [75-36-5].

A clear, colourless liquid, flammable, decomposes in contact with water and with alcohol, miscible with ethylene chloride.

d_{20}^{20} : about 1.10.

Distillation range (2.2.11). Not less than 95 per cent distils between 49 °C and 53 °C.

Acetylcholine chloride. $C_7H_{16}ClNO_2$. (M_r 181.7). 1001000. [60-31-1].

A crystalline powder, very soluble in cold water and in alcohol; it decomposes in hot water and in alkalis.

Storage: at -20 °C.

Acetylenol. $C_{12}H_{14}O_3$. (M_r 206.2). 1100700. [93-28-7]. 2-Methoxy-4-(2-propenyl)phenylacetate.

A yellow coloured, oily liquid, freely soluble in alcohol, practically insoluble in water.

n_D^{20} : about 1.521.

bp: 281 °C to 282 °C.

Acetylenol used in gas chromatography complies with the following additional test.

Assay. Examine by gas chromatography (2.2.28) as prescribed in the monograph on *Clove oil* (1091) using the substance to be examined as the test solution.

The area of the principal peak is not less than 98.0 per cent of the total area of the peaks.

N-Acetylglucosamine. $C_8H_{15}NO_6$. (M_r 221.2). 1133600. [7512-17-6]. 2-(Acetylamino)-2-deoxy-D-glucopyranose.

mp: about 202 °C.

N-Acetylneuraminic acid. $C_{11}H_{19}NO_9$. (M_r 309.3). 1001100. [131-48-6]. *O*-Sialic acid.

White acicular crystals, soluble in water and in methanol, slightly soluble in ethanol, practically insoluble in acetone.

$[\alpha]_D^{20}$: about -36, determined on a 10 g/l solution.

mp: about 186 °C, with decomposition.

N-Acetyltryptophan. $C_{13}H_{14}N_2O_3$. (M_r 246.3). 1102800. [1218-34-4]. 2-Acetylamino-3-(indol-3-yl)propanoic acid.

A white or almost white powder or colourless crystals, slightly soluble in water. It dissolves in dilute solutions of alkali hydroxides.

mp: about 205 °C.

Assay. Dissolve 10.0 mg in a mixture of 10 volumes of *acetonitrile R* and 90 volumes of *water R* and dilute to 100.0 ml with the same mixture of solvents. Examine as prescribed in the monograph on *Tryptophan* (1272) under "1,1'-Ethylidenebis(tryptophan) and other related substances". The area of the principal peak in the chromatogram obtained is not less than 99.0 per cent of the areas of all the peaks.

Acetyltyrosine ethyl ester. $C_{13}H_{17}NO_4 \cdot H_2O$. (M_r 269.3). 1001200. [36546-50-6]. *N*-Acetyl-L-tyrosine ethyl ester monohydrate. Ethyl (*S*)-2-acetamido-3-(4-hydroxyphenyl)propionate monohydrate.

A white, crystalline powder suitable for the assay of chymotrypsin.

$[\alpha]_{\text{D}}^{20}$: + 21 to + 25, determined on a 10 g/l solution in *alcohol R*.

$A_{1\text{ cm}}^{1\%}$: 60 to 68, determined at 278 nm in *alcohol R*.

Acetyltyrosine ethyl ester 0.2 M. 1001201.

Dissolve 0.54 g of *acetyltyrosine ethyl ester R* in *alcohol R* and dilute to 10.0 ml with the same solvent.

Acid blue 83. $\text{C}_{45}\text{H}_{44}\text{N}_3\text{NaO}_7\text{S}_2$. (M_r 826). 1012200. [6104-59-2].

Colour Index No. 42660.

Brilliant blue R. Coomassie brilliant blue R 250.

Brown powder insoluble in cold water, slightly soluble in boiling water and in ethanol, soluble in sulphuric acid, glacial acetic acid and in dilute solutions of alkali hydroxides.

Acid blue 90. $\text{C}_{47}\text{H}_{48}\text{N}_3\text{NaO}_7\text{S}_2$. (M_r 854). 1001300. [6104-58-1].

Colour Index No. 42655.

Sodium [4-[[4-[(4-ethoxyphenyl)amino]phenyl][4-(ethyl)(3-sulphonatobenzyl)amino]phenyl]methylene]cyclo-hexa-2,5-dien-1-ylidene[(ethyl)-(3-sulphonatobenzyl)ammonium].

A dark brown powder, with a violet sheen and some particles having a metallic lustre, soluble in water and in ethanol.

$A_{1\text{ cm}}^{1\%}$: greater than 500, determined at 577 nm using a 0.01 g/l solution in buffer solution pH 7.0 and calculated with reference to the dried substance.

Loss on drying (2.2.32): maximum 5.0 per cent, determined on 0.500 g by drying in an oven at 100-105 °C.

Acid blue 92. $\text{C}_{26}\text{H}_{16}\text{N}_3\text{Na}_3\text{O}_{10}\text{S}_3$. (M_r 696). 1001400. [3861-73-2].

Colour Index No. 13390.

Coomassie blue. Anazoline sodium. Trisodium 8-hydroxy-4'-(phenylamino)azonaphthalene-3,5',6-trisulphonate.

Dark blue crystals slightly soluble in alcohol, soluble in water, in acetone and in ethylene glycol monoethylether.

Acid blue 92 solution. 1001401.

Dissolve 0.5 g of *acid blue 92 R* in a mixture of 10 ml of *glacial acetic acid R*, 45 ml of *alcohol R* and 45 ml of *water R*.

Acid blue 93. $\text{C}_{37}\text{H}_{27}\text{N}_3\text{Na}_2\text{O}_9\text{S}_3$. (M_r 800). 1134200. [28983-56-4].

Colour Index No. 42780.

Methyl blue. Poirrier blue.

Mixture of triphenylrosaniline di- and trisulfonate and of triphenylpararosaniline.

Dark blue powder.

Colour change: pH 9.4 to pH 14.0.

Acid blue 93 solution. 1134201.

Dissolve 0.2 g of *acid blue 93 R* in *water R* and dilute to 100 ml with the same solvent.

Acrylamide. $\text{C}_3\text{H}_5\text{NO}$. (M_r 71.1). 1001500. [79-06-1]. Propenamide.

Colourless or white flakes or a white or almost white, crystalline powder, very soluble in water and in methanol, freely soluble in ethanol.

mp: about 84 °C.

30 per cent acrylamide/bisacrylamide (29:1) solution. 1001501.

Prepare a solution containing 290 g of *acrylamide R* and 10 g of *methylenebisacrylamide R* per litre of *water R*. Filter.

30 per cent acrylamide/bisacrylamide (36.5:1) solution. 1001502.

Prepare a solution containing 292 g of *acrylamide R* and 8 g of *methylenebisacrylamide R* per litre of *water R*. Filter.

Acrylic acid. $\text{C}_3\text{H}_4\text{O}_2$. (M_r 72.1). 1133700. [79-10-7]. Prop-2-enoic acid. Vinylformic acid.

Content: minimum 99 per cent of $\text{C}_3\text{H}_4\text{O}_2$.

It is stabilised with 0.02 per cent of hydroquinone monomethyl ether.

Corrosive liquid, miscible with water and alcohol. It polymerises readily in the presence of oxygen.

d_{20}^{20} : about 1.05.

n_{D}^{20} : about 1.421.

bp: about 141 °C.

mp: 12 °C to 15 °C.

Acteoside. $\text{C}_{29}\text{H}_{36}\text{O}_{15}$. (M_r 624.6). 1145100.

[61276-17-3]. 2-(3,4-Dihydroxyphenyl)ethyl 3-O-(6-deoxy- α -L-mannopyranosyl)-4-O-[(2E)-3-(3,4-dihydroxyphenyl)prop-2-enoyl]- β -D-glucopyranoside.

Light yellowish powder, freely soluble in water and in methanol.

mp: about 140 °C, with decomposition.

Adenosine. $\text{C}_{10}\text{H}_{13}\text{N}_5\text{O}_4$. (M_r 267.2). 1001600. [58-61-7]. 6-Amino-9- β -D-ribofuranosyl-9H-purine.

A white, crystalline powder, slightly soluble in water, practically insoluble in acetone and in alcohol. It dissolves in dilute solutions of acids.

mp: about 234 °C.

Adipic acid. $\text{C}_6\text{H}_{10}\text{O}_4$. (M_r 146.1). 1095600. [124-04-9].

Prisms, freely soluble in methanol, soluble in acetone, practically insoluble in light petroleum.

mp: about 152 °C.

Adrenaline. $\text{C}_9\text{H}_{13}\text{NO}_3$. (M_r 183.2). 1155000. [51-43-4].

(1R)-1-(3,4-Dihydroxyphenyl)-2-(methylamino)ethanol. 4-[(1R)-1-hydroxy-2-(methylamino)ethyl]benzene-1,2-diol.

White or almost white powder, gradually becoming brown on exposure to light and air, very slightly soluble in water and in ethanol (96 per cent), insoluble in acetone. It dissolves in dilute solutions of mineral acids and alkali hydroxides.

mp: about 215 °C.

Adrenalone hydrochloride. $\text{C}_9\text{H}_{12}\text{ClNO}_3$. (M_r 217.7).

1155100. [62-13-5]. 1-(3,4-Dihydroxyphenyl)-2-(methylamino)ethanone hydrochloride. 3',4'-Dihydroxy-2-(methylamino)acetophenone hydrochloride.

Pale yellow crystals, freely soluble in water, soluble in ethanol (96 per cent).

mp: about 244 °C.

Aescin. 1001700. [11072-93-8].

A mixture of related saponins obtained from the seeds of *Aesculus hippocastanum* L.

A fine, almost white or slightly reddish or yellowish, amorphous powder.

Chromatography. Examine as prescribed in the monograph on *Senega root* (0202) but apply 20 μ l of the solution. After spraying with *anisaldehyde solution R* and heating, the chromatogram shows a principal band with an R_f of about 0.4.

Agarose/cross-linked polyacrylamide. 1002200.

Agarose trapped within a cross-linked polyacrylamide network; it is used for the separation of globular proteins with relative molecular masses of 2×10^4 to 35×10^4 .

Agarose-DEAE for ion-exchange chromatography. 1002100. [57407-08-6].

Cross-linked agarose substituted with diethylaminoethyl groups, presented as beads.

Agarose for chromatography. 1001800. [9012-36-6].

Swollen beads 60 μm to 140 μm in diameter presented as a 4 per cent suspension in *water R*. It is used in size-exclusion chromatography for the separation of proteins with relative molecular masses of 6×10^4 to 20×10^6 and of polysaccharides with relative molecular masses of 3×10^3 to 5×10^6 .

Agarose for chromatography, cross-linked. 1001900. [61970-08-9].

Prepared from agarose by reaction with 2,3-dibromopropanol in strongly alkaline conditions.

It occurs as swollen beads 60 μm to 140 μm in diameter and is presented as a 4 per cent suspension in *water R*. It is used in size-exclusion chromatography for the separation of proteins with relative molecular masses of 6×10^4 to 20×10^6 and of polysaccharides with relative molecular masses of 3×10^3 to 5×10^6 .

Agarose for chromatography, cross-linked R1. 1001901. [65099-79-8].

Prepared for agarose by reaction with 2,3-dibromopropanol in strongly alkaline conditions.

It occurs as swollen beads 60 μm to 140 μm in diameter and is presented as a 4 per cent suspension in *water R*. It is used in size-exclusion chromatography for the separation of proteins with relative molecular masses of 7×10^4 to 40×10^6 and of polysaccharides with relative molecular masses of 1×10^5 to 2×10^7 .

Agarose for electrophoresis. 1002000. [9012-36-6].

A neutral, linear polysaccharide, the main component of which is derived from agar.

A white or almost white powder, practically insoluble in cold water, very slightly soluble in hot water.

Alanine. 1102900. [56-41-7].

See *Alanine* (0752).

 β -Alanine. 1004500. [107-95-9].

See *3-aminopropionic acid R*.

Albumin, bovine. 1002300. [9048-46-8].

Bovine serum albumin containing about 96 per cent of protein.

A white to light-yellowish-brown powder.

Water (2.5.12): maximum 3.0 per cent, determined on 0.800 g.

Bovine albumin used in the assay of tetracosactide should be pyrogen-free, free from proteolytic activity, when examined by a suitable means, for example using chromogenic substrate, and free from corticosteroid activity determined by measurement of fluorescence as described in the biological assay of Tetracosactide (0644).

Albumin, human. 1133800.

Human serum albumin containing not less than 96 per cent of albumin.

Albumin solution, human. 1002400. [9048-46-8].

See *Human albumin solution* (0255).

Albumin solution, human R1. 1002401.

Dilute *human albumin solution R* with a 9 g/l solution of *sodium chloride R* to a concentration of 1 g/l of protein. Adjust the pH to 3.5-4.5 with *glacial acetic acid R*.

Alcohol. 1002500. [64-17-5].

See *Ethanol* (96 per cent) *R*.

Alcohol (x per cent V/V). 1002502.

See *Ethanol* (x per cent V/V) *R*.

Alcohol, aldehyde-free. 1002501.

Mix 1200 ml of *alcohol R* with 5 ml of a 400 g/l solution of *silver nitrate R* and 10 ml of a cooled 500 g/l solution of *potassium hydroxide R*. Shake, allow to stand for a few days and filter. Distil the filtrate immediately before use.

Aldehyde dehydrogenase. 1103000.

Enzyme obtained from baker's yeast which oxidises acetaldehyde to acetic acid in the presence of nicotinamide-adenine dinucleotide, potassium salts and thiols, at pH 8.0.

Aldehyde dehydrogenase solution. 1103001.

Dissolve in *water R* a quantity of *aldehyde dehydrogenase R*, equivalent to 70 units and dilute to 10 ml with the same solvent. This solution is stable for 8 h at 4 °C.

Aldrin. $\text{C}_{12}\text{H}_8\text{Cl}_6$. (M_r 364.9). 1123100. [309-00-2].

bp: about 145 °C.

mp: about 104 °C.

A suitable certified reference solution (10 ng/ μl in cyclohexane) may be used.

Aleuritic acid. $\text{C}_{16}\text{H}_{32}\text{O}_5$. (M_r 304.4). 1095700. [533-87-9].

(9*RS*,10*SR*)-9,10,16-Trihydroxyhexadecanoic acid.

A white powder, greasy to the touch, soluble in methanol.

mp: about 101 °C.

Alizarin S. $\text{C}_{14}\text{H}_7\text{NaO}_7\text{S}\cdot\text{H}_2\text{O}$. (M_r 360.3). 1002600. [130-22-3].

Schultz No. 1145.

Colour Index No. 58005.

Sodium 1,2-dihydroxyanthraquinone-3-sulphonate monohydrate. Sodium 3,4-dihydroxy-9,10-dioxo-9,10-dihydroanthracene-2-sulphonate monohydrate.

An orange-yellow powder, freely soluble in water and in alcohol.

Alizarin S solution. 1002601.

A 1 g/l solution.

Test for sensitivity. If alizarin S solution is used for the standardisation of 0.05 *M* barium perchlorate, it shows a colour change from yellow to orange-red when it is tested according to the standardisation of 0.05 *M* barium perchlorate (4.2.2).

Colour change: pH 3.7 (yellow) to pH 5.2 (violet).

Aluminium. Al. (A_r 26.98). 1118200. [7429-90-5].

A white, malleable, flexible, bluish metal, available as bars, sheets, powder, strips or wire. In moist air an oxide film forms which protects the metal from corrosion.

Analytical grade.

Aluminium chloride. $\text{AlCl}_3 \cdot 6\text{H}_2\text{O}$. (M_r 241.4). 1002700. [7784-13-6]. Aluminium chloride hexahydrate.

Content: minimum 98.0 per cent of $\text{AlCl}_3 \cdot 6\text{H}_2\text{O}$.

A white to slightly yellowish, crystalline powder, hygroscopic, freely soluble in water and in alcohol.

Storage: in an airtight container.

Aluminium chloride reagent. 1002702.

Dissolve 2.0 g of *aluminium chloride R* in 100 ml of a 5 per cent V/V solution of *glacial acetic acid R* in *methanol R*.

Aluminium chloride solution. 1002701.

Dissolve 65.0 g of *aluminium chloride R* in *water R* and dilute to 100 ml with the same solvent. Add 0.5 g of *activated charcoal R*, stir for 10 min, filter and add to the filtrate, with continuous stirring, sufficient of a 10 g/l solution of *sodium hydroxide R* (about 60 ml) to adjust the pH to about 1.5.

Aluminium nitrate. $\text{Al}(\text{NO}_3)_3 \cdot 9\text{H}_2\text{O}$. (M_r 375.1). 1002800. [7784-27-2]. Aluminium nitrate nonahydrate.

Crystals, deliquescent, very soluble in water and alcohol, very slightly soluble in acetone.

Storage: in an airtight container.

Aluminium oxide, anhydrous. 1002900. [1344-28-1].

An aluminium oxide, consisting of $\gamma\text{-Al}_2\text{O}_3$, dehydrated and activated by heat treatment. Particle size 75 μm to 150 μm .

Aluminium oxide, basic. 1118300.

A basic grade of *anhydrous aluminium oxide R* suitable for column chromatography.

pH (2.2.3). Shake 1 g with 10 ml of *carbon dioxide-free water R* for 5 min. The pH of the suspension is 9 to 10.

Aluminium oxide, neutral. Al_2O_3 . (M_r 102.0). 1118400.

See *Aluminium oxide, hydrated* (0311).

Aluminium potassium sulphate. 1003000. [7784-24-9].

See *Alum* (0006).

Amido black 10B. $\text{C}_{22}\text{H}_{14}\text{N}_6\text{Na}_2\text{O}_9\text{S}_2$. (M_r 617). 1003100. [1064-48-8].

Schultz No. 299.

Colour Index No. 20470.

Disodium 5-amino-4-hydroxy-6-[(4-nitrophenyl)azo]-3-(phenylazo)naphthalene-2,7-disulphonate.

A dark-brown to black powder, sparingly soluble in water, soluble in alcohol.

Amido black 10B solution. 1003101.

A 5 g/l solution of *amido black 10B R* in a mixture of 10 volumes of *acetic acid R* and 90 volumes of *methanol R*.

Aminoazobenzene. $\text{C}_{12}\text{H}_{11}\text{N}_3$. (M_r 197.2). 1003200. [60-09-3].

Colour Index No. 11000.

4-(Phenylazo)aniline.

Brownish-yellow needles with a bluish tinge, slightly soluble in water, freely soluble in alcohol.

mp: about 128 °C.

2-Aminobenzoic acid. $\text{C}_7\text{H}_7\text{NO}_2$. (M_r 137.1). 1003400. [118-92-3]. Anthranilic acid.

A white to pale-yellow, crystalline powder, sparingly soluble in cold water, freely soluble in hot water, in alcohol and in glycerol. Solutions in alcohol or in ether and, particularly, in glycerol show a violet fluorescence.

mp: about 145 °C.

3-Aminobenzoic acid. $\text{C}_7\text{H}_7\text{NO}_2$. (M_r 137.1). 1147400. [99-05-8].

White or almost white crystals. An aqueous solution turns brown on standing in air.

mp: about 174 °C.

Storage: in an airtight container, protected from light.

4-Aminobenzoic acid. $\text{C}_7\text{H}_7\text{NO}_2$. (M_r 137.1). 1003300. [150-13-0].

A white, crystalline powder, slightly soluble in water, freely soluble in alcohol, practically insoluble in light petroleum.

mp: about 187 °C.

Chromatography. Examine as prescribed in the monograph on *Procaine hydrochloride* (0050); the chromatogram shows only one principal spot.

Storage: protected from light.

4-Aminobenzoic acid solution. 1003301.

Dissolve 1 g of *4-aminobenzoic acid R* in a mixture of 18 ml of *anhydrous acetic acid R*, 20 ml of *water R* and 1 ml of *phosphoric acid R*. Immediately before use, mix 2 volumes of the solution with 3 volumes of *acetone R*.

N-(4-Aminobenzoyl)-L-glutamic acid. $\text{C}_{12}\text{H}_{14}\text{N}_2\text{O}_5$. (M_r 266.3). 1141700. [4271-30-1]. ABGA.

(2S)-2-[(4-Aminobenzoyl)amino]pentanedioic acid.

White or almost white, crystalline powder.

mp: about 175 °C, with decomposition.

4-Aminobutanoic acid. $\text{C}_4\text{H}_9\text{NO}_2$. (M_r 103.1). 1123200. [56-12-2]. γ -Aminobutyric acid. GABA.

Leaflets from methanol and ether, needles from water and alcohol. Freely soluble in water, practically insoluble or slightly soluble in other solvents.

mp: about 202 °C (decreases on rapid heating).

Aminobutanol. $\text{C}_4\text{H}_{11}\text{NO}$. (M_r 89.1). 1003500. [5856-63-3]. 2-Aminobutanol.

Oily liquid, miscible with water, soluble in alcohol.

d_{20}^{20} : about 0.94.

n_D^{20} : about 1.453.

bp: about 180 °C.

Aminochlorobenzophenone. $\text{C}_{13}\text{H}_9\text{ClNO}$. (M_r 231.7). 1003600. [719-59-5]. 2-Amino-5-chlorobenzophenone.

A yellow, crystalline powder, practically insoluble in water, freely soluble in acetone, soluble in alcohol.

mp: about 97 °C.

Chromatography. Examine as prescribed in the monograph on *Chlordiazepoxide hydrochloride* (0474) but apply 5 μl of a 0.5 g/l solution in *methanol R*; the chromatogram shows only one principal spot, at an R_f of about 0.9.

Storage: protected from light.

6-Aminohexanoic acid. $\text{C}_6\text{H}_{13}\text{NO}_2$. (M_r 131.2). 1103100. [60-32-2].

Colourless crystals, freely soluble in water, sparingly soluble in methanol, practically insoluble in ethanol.

mp: about 205 °C.

Aminohippuric acid. $C_9H_{10}N_2O_3$. (M_r 194.2). 1003700. [61-78-9]. (4-Aminobenzamido)acetic acid.

A white or almost white powder, sparingly soluble in water, soluble in alcohol.

mp: about 200 °C.

Aminohippuric acid reagent. 1003701.

Dissolve 3 g of *phthalic acid R* and 0.3 g of *aminohippuric acid R* in *alcohol R* and dilute to 100 ml with the same solvent.

Aminohydroxynaphthalenesulphonic acid. $C_{10}H_9NO_4S$. (M_r 239.3). 1112400. [116-63-2]. 4-Amino-3-hydroxynaphthalene-1-sulphonic acid.

White or grey needles, turning pink on exposure to light, especially when moist, practically insoluble in water and in alcohol, soluble in solutions of alkali hydroxides and in hot solutions of sodium metabisulphite.

Storage: protected from light.

Aminohydroxynaphthalenesulphonic acid solution. 1112401.

Mix 5.0 g of *anhydrous sodium sulphite R* with 94.3 g of *sodium hydrogensulphite R* and 0.7 g of *aminohydroxynaphthalenesulphonic acid R*. Dissolve 1.5 g of the mixture in *water R* and dilute to 10.0 ml with the same solvent. Prepare the solution daily.

Aminomethylalizarindiacetic acid. $C_{19}H_{15}NO_8 \cdot 2H_2O$. (M_r 421.4). 1003900. [3952-78-1]. 2,2'-[(3,4-dihydroxy-anthraquinon-3-yl)methylenenitrilo]diacetic acid dihydrate. Alizarin complexone dihydrate.

A fine, pale brownish-yellow to orange-brown powder, practically insoluble in water, soluble in solutions of alkali hydroxides.

mp: about 185 °C.

Loss on drying (2.2.32): maximum 10.0 per cent, determined on 1.000 g.

Aminomethylalizarindiacetic acid reagent. 1003901.

Solution I. Dissolve 0.36 g of *cerous nitrate R* in *water R* and dilute to 50 ml with the same solvent.

Solution II. Suspend 0.7 g of *aminomethylalizarindiacetic acid R* in 50 ml of *water R*. Dissolve with the aid of about 0.25 ml of *concentrated ammonia R*, add 0.25 ml of *glacial acetic acid R* and dilute to 100 ml with *water R*.

Solution III. Dissolve 6 g of *sodium acetate R* in 50 ml of *water R*, add 11.5 ml of *glacial acetic acid R* and dilute to 100 ml with *water R*.

To 33 ml of *acetone R* add 6.8 ml of solution III, 1.0 ml of solution II and 1.0 ml of solution I and dilute to 50 ml with *water R*.

Test for sensitivity. To 1.0 ml of *fluoride standard solution (10 ppm F) R* add 19.0 ml of *water R* and 5.0 ml of the aminomethylalizarindiacetic acid reagent. After 20 min, the solution assumes a blue colour.

Storage: use within 5 days.

Aminomethylalizarindiacetic acid solution. 1003902.

Dissolve 0.192 g of *aminomethylalizarindiacetic acid R* in 6 ml of freshly prepared 1 M *sodium hydroxide*. Add 750 ml of *water R*, 25 ml of *succinate buffer solution pH 4.6 R* and, dropwise, 0.5 M *hydrochloric acid* until the colour changes from violet-red to yellow (pH 4.5 to 5). Add 100 ml of *acetone R* and dilute to 1000 ml with *water R*.

Aminonitrobenzophenone. $C_{13}H_{10}N_2O_3$. (M_r 242.2). 1004000. [1775-95-7]. 2-Amino-5-nitrobenzophenone.

A yellow, crystalline powder, practically insoluble in water, soluble in tetrahydrofuran, slightly soluble in methanol.

mp: about 160 °C.

$A_{1\text{ cm}}^{1\%}$: 690 to 720, determined at 233 nm using a 0.01 g/l solution in *methanol R*.

Aminophenazone. $C_{13}H_{17}N_3O$. (231.3). 1133900. [58-15-1]. 4-(Dimethylamino)-1,5-dimethyl-2-phenyl-1,2-dihydro-3H-pyrazol-3-one.

White, crystalline powder or colourless crystals, soluble in water, freely soluble in alcohol.

mp: about 108 °C.

2-Aminophenol. C_6H_7NO . (M_r 109.1). 1147500. [95-55-6].

Pale yellowish-brown crystals which rapidly become brown, sparingly soluble in water, soluble in alcohol.

mp: about 172 °C.

Storage: in an airtight container, protected from light.

3-Aminophenol. C_6H_7NO . (M_r 109.1). 1147600. [591-27-5].

Pale yellowish-brown crystals, sparingly soluble in water.

mp: about 122 °C.

4-Aminophenol. C_6H_7NO . (M_r 109.1). 1004300. [123-30-8].

Content: minimum 95 per cent of C_6H_7NO .

A white or slightly coloured, crystalline powder, becoming coloured on exposure to air and light, sparingly soluble in water, soluble in ethanol.

mp: about 186 °C, with decomposition.

Storage: protected from light.

Aminopolyether. $C_{18}H_{36}N_2O_6$. (M_r 376.5). 1112500.

[23978-09-8]. 4,7,13,16,21,24-hexaoxa-1,10-diazabicyclo[8,8,8]hexacosane.

mp: 70 °C to 73 °C.

3-Aminopropanol. C_3H_9NO . (M_r 75.1). 1004400. [156-87-6].

3-Aminopropan-1-ol. Propanolamine.

A clear, colourless, viscous liquid.

d_{20}^{20} : about 0.99.

n_D^{20} : about 1.461.

mp: about 11 °C.

3-Aminopropionic acid. $C_3H_7NO_2$. (M_r 89.1). 1004500.

[107-95-9]. β -Alanine.

Content: minimum 99 per cent of $C_3H_7NO_2$.

A white, crystalline powder, freely soluble in water, slightly soluble in alcohol, practically insoluble in acetone.

mp: about 200 °C, with decomposition.

Aminopyrazolone. $C_{11}H_{13}N_3O$. (M_r 203.2). 1004600.

[83-07-8]. 4-Amino-2,3-dimethyl-1-phenylpyrazolin-5-one.

Light-yellow needles or powder, sparingly soluble in water, freely soluble in alcohol.

mp: about 108 °C.

Aminopyrazolone solution. 1004601.

A 1 g/l solution in *buffer solution pH 9.0 R*.

Ammonia, concentrated. 1004700.

See *Concentrated ammonia solution (0877)*.

Ammonia. 1004701.

Content: 170 g/l to 180 g/l of NH_3 (M_r 17.03).

Dilute 67 g of *concentrated ammonia R* to 100 ml with *water R*.

d_{20}^{20} : 0.931 to 0.934.

When used in the limit test for iron, *ammonia R* complies with the following additional requirement. Evaporate 5 ml of ammonia to dryness on a water-bath, add 10 ml of *water R*, 2 ml of a 200 g/l solution of *citric acid R* and 0.1 ml of *thioglycollic acid R*. Make alkaline by adding *ammonia R* and dilute to 20 ml with *water R*. No pink colour develops.

Storage: protected from atmospheric carbon dioxide, at a temperature below 20 °C.

Ammonia, dilute R1. 1004702.

Content: 100 g/l to 104 g/l of NH_3 (M_r 17.03).

Dilute 41 g of *concentrated ammonia R* to 100 ml with *water R*.

Ammonia, dilute R2. 1004703.

Content: 33 g/l to 35 g/l of NH_3 (M_r 17.03).

Dilute 14 g of *concentrated ammonia R* to 100 ml with *water R*.

Ammonia, dilute R3. 1004704.

Content: 1.6 g/l to 1.8 g/l of NH_3 (M_r 17.03).

Dilute 0.7 g of *concentrated ammonia R* to 100 ml with *water R*.

Ammonia, lead-free. 1004705.

Complies with the requirements prescribed for *dilute ammonia R1* and with the following additional test: to 20 ml of lead-free ammonia, add 1 ml of *lead-free potassium cyanide solution R*, dilute to 50 ml with *water R* and add 0.10 ml of *sodium sulphide solution R*. The solution is not more intensely coloured than a reference solution prepared without sodium sulphide.

Ammonia, concentrated R1. 1004800.

Content: minimum 32.0 per cent *m/m* of NH_3 (M_r 17.03).

A clear, colourless liquid.

d_{20}^{20} : 0.883 to 0.889.

Assay. Weigh accurately a ground-glass-stoppered flask containing 50.0 ml of 1 *M* hydrochloric acid. Introduce 2 ml of the concentrated ammonia and weigh again. Titrate the solution with 1 *M* sodium hydroxide, using 0.5 ml of *methyl red mixed solution R* as indicator.

1 ml of 1 *M* hydrochloric acid is equivalent to 17.03 mg of NH_3 .

Storage: protected from atmospheric carbon dioxide, at a temperature below 20 °C.

Ammonium acetate. $\text{C}_2\text{H}_7\text{NO}_2$. (M_r 77.1). 1004900. [631-61-8].

Colourless crystals, very deliquescent, very soluble in water and in alcohol.

Storage: in an airtight container.

Ammonium acetate solution. 1004901.

Dissolve 150 g of *ammonium acetate R* in *water R*. Add 3 ml of *glacial acetic acid R* and dilute to 1000 ml with *water R*.

Storage: use within 1 week.

Ammonium and cerium nitrate. $(\text{NH}_4)_2\text{Ce}(\text{NO}_3)_6$. (M_r 548.2). 1005000. [16774-21-3].

An orange-yellow, crystalline powder, or orange transparent crystals, soluble in water.

Ammonium and cerium sulphate. $(\text{NH}_4)_4\text{Ce}(\text{SO}_4)_4 \cdot 2\text{H}_2\text{O}$. (M_r 633). 1005100. [10378-47-9].

Orange-yellow, crystalline powder or crystals, slowly soluble in water.

(1R)-(-)-Ammonium 10-camphorsulphonate. $\text{C}_{10}\text{H}_{19}\text{NO}_4\text{S}$. (M_r 249.3). 1103200.

Content: minimum 97.0 per cent of (1R)-(-)-ammonium 10-camphorsulphonate.

$[\alpha]_{\text{D}}^{20}$: -18 ± 2 (50 g/l solution in *water R*).

Ammonium carbonate. 1005200. [506-87-6]. A mixture of varying proportions of ammonium hydrogen carbonate (NH_4HCO_3 , M_r 79.1) and ammonium carbamate ($\text{NH}_2\text{COONH}_4$, M_r 78.1).

A white translucent mass, slowly soluble in about 4 parts of water. It is decomposed by boiling water. Ammonium carbonate liberates not less than 30 per cent *m/m* of NH_3 (M_r 17.03).

Assay. Dissolve 2.00 g in 25 ml of *water R*. Slowly add 50.0 ml of 1 *M* hydrochloric acid, titrate with 1 *M* sodium hydroxide, using 0.1 ml of *methyl orange solution R* as indicator.

1 ml of 1 *M* hydrochloric acid is equivalent to 17.03 mg of NH_3 .

Storage: at a temperature below 20 °C.

Ammonium carbonate solution. 1005201.

A 158 g/l solution.

Ammonium chloride. 1005300. [12125-02-9].

See *Ammonium chloride* (0007).

Ammonium chloride solution. 1005301.

A 107 g/l solution.

Ammonium citrate. $\text{C}_6\text{H}_{14}\text{N}_2\text{O}_7$. (M_r 226.2). 1103300. [3012-65-5]. Diammonium hydrogen citrate.

A white, crystalline powder or colourless crystals, freely soluble in water, slightly soluble in alcohol.

pH (2.2.3): about 4.3 for a 22.6 g/l solution.

Ammonium dihydrogen phosphate. $(\text{NH}_4)_2\text{H}_2\text{PO}_4$. (M_r 115.0). 1005400. [7722-76-1]. Monobasic ammonium phosphate.

A white, crystalline powder or colourless crystals, freely soluble in water.

pH (2.2.3): about 4.2 for a 23 g/l solution.

Ammonium formate. CH_5NO_2 . (M_r 63.1). 1112600. [540-69-2].

Deliquescent crystals or granules, very soluble in water, soluble in alcohol.

mp: 119 °C to 121 °C.

Storage: in an airtight container.

Ammonium hexafluorogermanate (IV). $(\text{NH}_4)_2\text{GeF}_6$. (M_r 222.7). 1134000. [16962-47-3].

White crystals, freely soluble in water.

Ammonium hydrogen carbonate. NH_4HCO_3 . (M_r 79.1). 1005500. [1066-33-7].

Content: minimum 99 per cent of NH_4HCO_3 .

Ammonium molybdate. $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$. (M_r 1236). 1005700. [12054-85-2].

Colourless or slightly yellow or greenish crystals, soluble in water, practically insoluble in alcohol.

Ammonium molybdate reagent. 1005701.

Mix, in the given order, 1 volume of a 25 g/l solution of *ammonium molybdate R*, 1 volume of a 100 g/l solution of *ascorbic acid R* and 1 volume of *sulphuric acid R* (294.5 g/l H₂SO₄). Add 2 volumes of *water R*.

Storage: use within 1 day.

Ammonium molybdate reagent R1. 1005706.

Mix 10 ml of a 60 g/l solution of *disodium arsenate R*, 50 ml of *ammonium molybdate solution R*, 90 ml of *dilute sulphuric acid R* and dilute to 200 ml in *water R*.

Storage: in amber flasks at 37 °C for 24 h.

Ammonium molybdate reagent R2. 1005708.

Dissolve 50 g of *ammonium molybdate R* in 600 ml of *water R*. To 250 ml of cold *water R* add 150 ml of *sulphuric acid R* and cool. Mix the 2 solutions together.

Storage: use within 1 day.

Ammonium molybdate solution. 1005702.

A 100 g/l solution.

Ammonium molybdate solution R2. 1005703.

Dissolve 5.0 g of *ammonium molybdate R* with heating in 30 ml of *water R*. Cool, adjust the pH to 7.0 with *dilute ammonia R2* and dilute to 50 ml with *water R*.

Ammonium molybdate solution R3. 1005704.

Solution I. Dissolve 5 g of *ammonium molybdate R* in 20 ml of *water R* with heating.

Solution II. Mix 150 ml of *alcohol R* with 150 ml of *water R*. Add with cooling 100 ml of *sulphuric acid R*.

Immediately before use add 80 volumes of solution II to 20 volumes of solution I.

Ammonium molybdate solution R4. 1005705.

Dissolve 1.0 g of *ammonium molybdate R* in *water R* and dilute to 40 ml with the same solvent. Add 3 ml of *hydrochloric acid R* and 5 ml of *perchloric acid R* and dilute to 100 ml with *acetone R*.

Storage: protected from light; use within 1 month.

Ammonium molybdate solution R5. 1005707.

Dissolve 1.0 g of *ammonium molybdate R* in 40.0 ml of a 15 per cent V/V solution of *sulphuric acid R*. Prepare the solution daily.

Ammonium molybdate solution R6. 1005709.

Slowly add 10 ml of *sulphuric acid R* to about 40 ml of *water R*. Mix and allow to cool. Dilute to 100 ml with *water R* and mix. Add 2.5 g of *ammonium molybdate R* and 1 g of *cerium sulphate R*, and shake for 15 min to dissolve.

Ammonium nitrate. NH₄NO₃. (M_r 80.0). 1005800. [6484-52-2].

A white, crystalline powder or colourless crystals, hygroscopic, very soluble in water, freely soluble in methanol, soluble in alcohol.

Storage: in an airtight container.

Ammonium nitrate R1. 1005801. [6484-52-2].

Complies with the requirements prescribed for *ammonium nitrate R* and with the following additional requirements.

Acidity. The solution of the substance is faintly acid (2.2.4).

Chlorides (2.4.4). 0.50 g complies with the limit test for chlorides (100 ppm).

Sulphates (2.4.13). 1.0 g complies with the limit test for sulphates (150 ppm).

Sulphated ash (2.4.14): maximum 0.05 per cent, determined on 1.0 g.

Ammonium oxalate. C₂H₈N₂O₄·H₂O. (M_r 142.1). 1005900. [6009-70-7].

Colourless crystals, soluble in water.

Ammonium oxalate solution. 1005901.

A 40 g/l solution.

Ammonium persulphate. (NH₄)₂S₂O₈. (M_r 228.2). 1006000. [7727-54-0].

White, crystalline powder or granular crystals, freely soluble in water.

Ammonium phosphate. (NH₄)₂HPO₄. (M_r 132.1). 1006100. [7783-28-0]. Diammonium hydrogen phosphate.

White crystals or granules, hygroscopic, very soluble in water, practically insoluble in alcohol.

pH (2.2.3): about 8 for a 200 g/l solution.

Storage: in an airtight container.

Ammonium pyrrolidinedithiocarbamate. C₅H₁₂N₂S₂. (M_r 164.3). 1006200. [5108-96-3]. Ammonium 1-pyrrolidinyl-dithioformate.

A white to pale yellow, crystalline powder, sparingly soluble in water, very slightly soluble in alcohol.

Storage: in a bottle containing a piece of ammonium carbonate in a muslin bag.

Ammonium reineckate. NH₄[Cr(NCS)₄(NH₃)₂],H₂O. (M_r 354.4). 1006300. [13573-16-5]. Ammonium diamine-tetrakis(isothiocyanato)chromate(III) monohydrate.

Red powder or crystals, sparingly soluble in cold water, soluble in hot water and in alcohol.

Ammonium reineckate solution. 1006301.

A 10 g/l solution. Prepare immediately before use.

Ammonium sulphamate. NH₂SO₃NH₄. (M_r 114.1). 1006400. [7773-06-0].

A white, crystalline powder or colourless crystals, hygroscopic, very soluble in water, slightly soluble in alcohol. mp: about 130 °C.

Storage: in an airtight container.

Ammonium sulphate. (NH₄)₂SO₄. (M_r 132.1). 1006500. [7783-20-2].

Colourless crystals or white granules, very soluble in water, practically insoluble in acetone and in alcohol.

pH (2.2.3): 4.5 to 6.0 for a 50 g/l solution in *carbon dioxide-free water R*.

Sulphated ash (2.4.14): maximum 0.1 per cent.

Ammonium sulphide solution. 1123300.

Saturate 120 ml of *dilute ammonia R1* with *hydrogen sulphide R* and add 80 ml of *dilute ammonia R1*. Prepare immediately before use.

Ammonium thiocyanate. NH₄SCN. (M_r 76.1). 1006700. [1762-95-4].

Colourless crystals, deliquescent, very soluble in water, soluble in alcohol.

Storage: in an airtight container.

Ammonium thiocyanate solution. 1006701.

A 76 g/l solution.

Ammonium vanadate. NH_4VO_3 . (M_r 117.0). 1006800. [7803-55-6]. Ammonium trioxovanadate(V).

A white to slightly yellowish, crystalline powder, slightly soluble in water, soluble in *dilute ammonia R1*.

Ammonium vanadate solution. 1006801.

Dissolve 1.2 g of *ammonium vanadate R* in 95 ml of *water R* and dilute to 100 ml with *sulphuric acid R*.

Amoxicillin trihydrate. 1103400.

See *Amoxicillin trihydrate* (0260).

α -Amylase. 1100800. 1,4- α -D-glucane-glucanohydrolase (EC 3.2.1.1).

A white to light brown powder.

α -Amylase solution. 1100801.

A solution of *α -amylase R* with an activity of 800 FAU/g.

β -Amyrin. $\text{C}_{30}\text{H}_{50}\text{O}$. (M_r 426.7). 1141800. [559-70-6]. Olean-12-en-3 β -ol.

White or almost white powder.

mp: 187 °C to 190 °C.

Anethole. $\text{C}_{10}\text{H}_{12}\text{O}$. (M_r 148.2). 1006900. [4180-23-8]. 1-Methoxy-4-(propen-1-yl)benzene.

A white, crystalline mass up to 20 °C to 21 °C, liquid above 23 °C, practically insoluble in water, freely soluble in ethanol, soluble in ethyl acetate and in light petroleum.

n_D^{25} : about 1.56.

bp: about 230 °C.

Anethole used in gas chromatography complies with the following test.

Assay. Examine by gas chromatography (2.2.28) under the conditions described in the monograph on *Anise oil* (0804) using the substance to be examined as the test solution.

The area of the principal peak, corresponding to *trans*-anethole, with a retention time of about 41 min, is not less than 99.0 per cent of the total area of the peaks.

***cis*-Anethole.** $\text{C}_{10}\text{H}_{12}\text{O}$. (M_r 148.2). 1007000. (*Z*)-1-Methoxy-4-(propen-1-yl)benzene.

A white, crystalline mass up to 20 °C to 21 °C, liquid above 23 °C, practically insoluble in water, freely soluble in ethanol, soluble in ethyl acetate and in light petroleum.

n_D^{25} : about 1.56.

bp: about 230 °C.

cis-Anethole used in gas chromatography complies with the following test.

Assay. Examine by gas chromatography (2.2.28) in the conditions described in the monograph on *Anise oil* (0804) using the substance to be examined as the test solution.

The area of the principal peak is not less than 92.0 per cent of the total area of the peaks.

Aniline. $\text{C}_6\text{H}_7\text{N}$. (M_r 93.1). 1007100. [62-53-3]. Benzeneamine.

A colourless or slightly yellowish liquid, soluble in water, miscible with alcohol.

d_{20}^{20} : about 1.02.

bp: 183 °C to 186 °C.

Storage: protected from light.

Aniline hydrochloride. $\text{C}_6\text{H}_8\text{ClN}$. (M_r 129.6). 1147700. [142-04-1]. Benzenamine hydrochloride.

Crystals. It darkens on exposure to air and light.

mp: about 198 °C.

Storage: protected from light.

Anion exchange resin. 1007200.

A resin in chlorinated form containing quaternary ammonium groups $[\text{CH}_2\text{N}^+(\text{CH}_3)_3]$ attached to a polymer lattice consisting of polystyrene cross-linked with 2 per cent of divinylbenzene. It is available as spherical beads and the particle size is specified in the monograph.

Wash the resin with 1 M *sodium hydroxide* on a sintered-glass filter (40) until the washings are free from chloride, then wash with *water R* until the washings are neutral. Suspend in freshly prepared *ammonium-free water R* and protect from atmospheric carbon dioxide.

Anion exchange resin R1. 1123400.

A resin containing quaternary ammonium groups $[\text{CH}_2\text{N}^+(\text{CH}_3)_3]$ attached to a lattice consisting of methacrylate.

Anion exchange resin R2. 1141900.

A conjugate of homogeneous 10 μm hydrophilic polyether particles, and a quaternary ammonium salt, providing a matrix suitable for strong anion-exchange chromatography of proteins.

Anion exchange resin for chromatography, strongly basic. 1112700.

A resin with quaternary amine groups attached to a lattice of latex cross linked with divinylbenzene.

Anion exchange resin, strongly basic. 1026600.

A gel-type resin in hydroxide form containing quaternary ammonium groups $[\text{CH}_2\text{N}^+(\text{CH}_3)_3]$, type 1] attached to a polymer lattice consisting of polystyrene cross-linked with 8 per cent of divinylbenzene.

Brown transparent beads.

Particle size: 0.2-1.0 mm.

Moisture content: about 50 per cent.

Total exchange capacity: minimum 1.2 meq/ml.

Anion exchange resin, weak. 1146700.

A resin with diethylaminoethyl groups attached to a lattice consisting of poly(methyl methacrylate).

Anisaldehyde. $\text{C}_8\text{H}_8\text{O}_2$. (M_r 136.1). 1007300. [123-11-5]. 4-Methoxybenzaldehyde.

An oily liquid, very slightly soluble in water, miscible with alcohol.

bp: about 248 °C.

Anisaldehyde used in gas chromatography complies with the following test.

Assay. Examine by gas chromatography (2.2.28) in the conditions described in the monograph on *Anise oil* (0804) the substance to be examined as the test solution.

The area of the principal peak is not less than 99.0 per cent of the total area of the peaks.

Anisaldehyde solution. 1007301.

Mix in the following order, 0.5 ml of *anisaldehyde R*, 10 ml of *glacial acetic acid R*, 85 ml of *methanol R* and 5 ml of *sulphuric acid R*.

Anisaldehyde solution R1. 1007302.

To 10 ml of *anisaldehyde R* add 90 ml of *alcohol R*, mix, add 10 ml of *sulphuric acid R* and mix again.

***p*-Anisidine.** $\text{C}_7\text{H}_9\text{NO}$. (M_r 123.2). 1103500. [104-94-9]. 4-Methoxyaniline.

White crystals, sparingly soluble in water, soluble in ethanol.

Content: minimum 97.0 per cent of $\text{C}_7\text{H}_9\text{NO}$.

Caution: skin irritant, sensitiser.

Storage: protected from light, at 0 °C to 4 °C.

On storage, *p*-anisidine tends to darken as a result of oxidation. A discoloured reagent can be reduced and decolorised in the following way: dissolve 20 g of *p*-anisidine *R* in 500 ml of *water R* at 75 °C. Add 1 g of *sodium sulphite R* and 10 g of *activated charcoal R* and stir for 5 min. Filter, cool the filtrate to about 0 °C and allow to stand at this temperature for at least 4 h. Filter, wash the crystals with a small quantity of *water R* at about 0 °C and dry the crystals in vacuum over *diphosphorus pentoxide R*.

Anolyte for isoelectric focusing pH 3 to 5. 1112800.

0.1 M *Glutamic acid*, 0.5 M *phosphoric acid*.

Dissolve 14.71 g of *glutamic acid R* in *water R*. Add 33 ml of *phosphoric acid R* and dilute to 1000 ml with *water R*.

Anthracene. C₁₄H₁₀. (*M_r* 178.2). 1007400. [120-12-7].

A white, crystalline powder, practically insoluble in water, slightly soluble in chloroform.

mp: about 218 °C.

Anthrone. C₁₄H₁₀O. (*M_r* 194.2). 1007500. [90-44-8]. 9(10*H*)-Anthracenone.

A pale yellow, crystalline powder.

mp: about 155 °C.

Antimony potassium tartrate. C₄H₄KO₇Sb_{1/2}H₂O. (*M_r* 333.9). 1007600. Potassium aqua[tartrato(4-)-*O*¹,*O*²,*O*³]-antimoniate(III) hemihydrate.

A white, granular powder or colourless, transparent crystals, soluble in water and in glycerol, freely soluble in boiling water, practically insoluble in alcohol. The aqueous solution is slightly acid.

Antimony trichloride. SbCl₃. (*M_r* 228.1). 1007700. [10025-91-9].

Colourless crystals or a transparent crystalline mass, hygroscopic, freely soluble in ethanol. Antimony trichloride is hydrolysed by water.

Storage: in an airtight container, protected from moisture.

Antimony trichloride solution. 1007701.

Rapidly wash 30 g of *antimony trichloride R* with two quantities, each of 15 ml, of *ethanol-free chloroform R*, drain off the washings, and dissolve the washed crystals immediately in 100 ml of *ethanol-free chloroform R*, warming slightly.

Storage: over a few grams of *anhydrous sodium sulphate R*.

Antimony trichloride solution R1. 1007702.

Solution I. Dissolve 110 g of *antimony trichloride R* in 400 ml of *ethylene chloride R*. Add 2 g of *anhydrous aluminium oxide R*, mix and filter through a sintered-glass filter (40). Dilute to 500.0 ml with *ethylene chloride R* and mix. The absorbance (2.2.25) of the solution, determined at 500 nm in a 2 cm cell, is not greater than 0.07.

Solution II. Under a hood, mix 100 ml of freshly distilled *acetyl chloride R* and 400 ml of *ethylene chloride R*.

Mix 90 ml of solution I and 10 ml of solution II.

Storage: in brown ground-glass-stoppered bottle for 7 days. Discard any reagent in which colour develops.

Antithrombin III. 1007800. [90170-80-2].

Antithrombin III is purified from human plasma by heparin agarose chromatography and should have a specific activity of at least 6 IU/mg.

Antithrombin III solution R1. 1007801.

Reconstitute *antithrombin III R* as directed by the manufacturer and dilute with *tris(hydroxymethyl)aminomethane sodium chloride buffer solution pH 7.4 R* to 1 IU/ml.

Antithrombin III solution R2. 1007802.

Reconstitute *antithrombin III R* as directed by the manufacturer and dilute with *tris(hydroxymethyl)aminomethane sodium chloride buffer solution pH 7.4 R* to 0.5 IU/ml.

Apigenin. C₁₅H₁₀O₅. (*M_r* 270.2). 1095800. [520-36-5]. 4',5,7-Trihydroxyflavone.

Light yellowish powder; practically insoluble in water, sparingly soluble in alcohol.

mp: about 310 °C, with decomposition.

Chromatography. Examine as prescribed in the monograph on *Roman chamomile flower (0380)*, applying 10 µl of a 0.25 g/l solution in *methanol R*. The chromatogram shows in the upper third a principal zone of yellowish-green fluorescence.

Apigenin 7-glucoside. C₂₁H₂₀O₁₀. (*M_r* 432.4). 1095900. [578-74-5]. Apigenin. 7-(β-D-Glucopyranosyloxy)-5-hydroxy-2-(4-hydroxyphenyl)-4*H*-1-benzopyran-4-one.

Light yellowish powder, practically insoluble in water, sparingly soluble in alcohol.

mp: 198 °C to 201 °C.

Chromatography. Examine as prescribed in the monograph on *Roman chamomile flower (0380)*, applying 10 µl of a 0.25 g/l solution in *methanol R*. The chromatogram shows in the middle third a principal zone of yellowish fluorescence. *Apigenin-7-glucoside used in liquid chromatography complies with the following additional test.*

Assay. Examine by liquid chromatography (2.2.29) as prescribed in the monograph on *Matricaria flower (0404)*.

Test solution. Dissolve 10.0 mg in *methanol R* and dilute to 100.0 ml with the same solvent.

The content of apigenin-7-glucoside is not less than 95.0 per cent, calculated by the normalisation procedure.

Aprotinin. 1007900. [9087-70-1].

See *Aprotinin (0580)*.

Arabinose. C₅H₁₀O₅. (*M_r* 150.1). 1008000. [87-72-9]. L-(+)-Arabinose.

A white, crystalline powder, freely soluble in water.

[α]_D²⁰: + 103 to + 105, determined on a 50 g/l solution in *water R* containing about 0.05 per cent of NH₃.

Arachidyl alcohol. C₂₀H₄₂O. (*M_r* 298.5). 1156300. [629-96-9]. 1-Eicosanol.

mp: about 65 °C.

Content: minimum 96 per cent of C₂₀H₄₂O.

Arbutin. C₁₂H₁₆O₇. (*M_r* 272.3). 1008100. [497-76-7]. Arbutoside. 4-Hydroxyphenyl-β-D-glucopyranoside.

Fine, white, shiny needles, freely soluble in water, very soluble in hot water, soluble in alcohol.

[α]_D²⁰: about – 64, determined on a 20 g/l solution.

mp: about 200 °C.

Chromatography. Examine by thin-layer chromatography (2.2.27) as prescribed in the monograph *Bearberry leaf (1054)*; the chromatogram shows only one principal spot.

Arbutin used in the arbutin assay in the monograph Bearberry leaf (1054) complies with the following additional requirement.

Assay. Examine by liquid chromatography (2.2.29) as prescribed in the monograph *Bearberry leaf (1054)*.

The content of arbutin is not less than 95 per cent, calculated by the normalisation procedure.

Arginine. 1103600. [74-79-3].

See *Arginine (0806)*.

Argon. Ar. (A_r 39.95). 1008200. [7440-37-1].

Content: minimum 99.995 per cent V/V of Ar.

Carbon monoxide. When used as described in the test for carbon monoxide in medicinal gases (*Method I*, 2.5.25), after passage of 10 litres of argon *R* at a flow rate of 4 litres per hour, not more than 0.05 ml of 0.002 *M* sodium thiosulphate is required for the titration (0.6 ppm V/V).

Aromadendrene. $C_{15}H_{24}$. (M_r 204.4). 1139100. [489-39-4]. (1*R*,2*S*,4*R*,8*R*,11*R*)-3,3,11-Trimethyl-7-methylenetricyclo-[6.3.0.0^{2,4}]undecane.

Clear, almost colourless liquid.

d_4^{20} : about 0.911.

n_D^{20} : about 1.497.

$[\alpha]_D^{20}$: about + 12.

bp: : about 263 °C.

Aromadendrene used in gas chromatography complies with the following additional test.

Assay. Examine by gas chromatography (2.2.28) as prescribed in the monograph on *Tea tree oil (1837)*.

The content is not less than 92 per cent, calculated by the normalisation procedure.

Arsenious trioxide. As_2O_3 . (M_r 197.8). 1008300. [1327-53-3]. Arsenious anhydride. Diarsenic trioxide.

A crystalline powder or a white mass, slightly soluble in water, soluble in boiling water.

Arsenite solution. 1008301.

Dissolve 0.50 g of arsenious trioxide *R* in 5 ml of dilute sodium hydroxide solution *R*, add 2.0 g of sodium hydrogen carbonate *R* and dilute to 100.0 ml with water *R*.

Ascorbic acid. 1008400. [50-81-7].

See *Ascorbic acid (0253)*.

Ascorbic acid solution. 1008401.

Dissolve 50 mg in 0.5 ml of water *R* and dilute to 50 ml with dimethylformamide *R*.

Asiaticoside. $C_{48}H_{78}O_{19}$. (M_r 959). 1123500. [16830-15-2]. *O*-6-Deoxy- α -L-mannopyranosyl-(1 $\rightarrow$ 4)-*O*- β -D-glucopyranosyl-(1 $\rightarrow$ 6)- β -D-glucopyranosyl-2 α ,3 β ,23-trihydroxy-4 α -urs-12-en-28-oate.

A white powder, hygroscopic, soluble in methanol, slightly soluble in ethanol, insoluble in acetonitrile.

mp: about 232 °C, with decomposition.

Water (2.5.12): 6.0 per cent.

Storage: protected from humidity.

Asiaticoside used in liquid chromatography complies with the following additional test.

Assay. Examine by liquid chromatography (2.2.29) as prescribed in the monograph on *Centella (1498)*.

The content is not less than 97.0 per cent calculated by the normalisation procedure.

Aspartic acid. 1134100. [56-84-8].

See *Aspartic acid (0797)*.

L-Aspartyl-L-phenylalanine. $C_{13}H_{16}N_2O_5$. (M_r 280.3). 1008500. [13433-09-5]. (S)-3-Amino-N-[(S)-1-carboxy-2-phenylethyl]-succinamic acid.

A white powder.

mp: about 210 °C, with decomposition.

Aucubin. $C_{15}H_{22}O_9$. (M_r 346.3). 1145200. [479-98-1]. [1*S*,4*aR*,5*S*,7*aS*]-5-Hydroxy-7-(hydroxymethyl)-1,4*a*,5,7*a*-tetrahydrocyclopenta[*c*]pyran-1-yl β -D-glucopyranoside. Crystals, soluble in water, in alcohol and in methanol, practically insoluble in light petroleum.

$[\alpha]_D^{20}$: about - 163.

mp: about 181 °C.

Azomethine H. $C_{17}H_{12}NNaO_8S_2$. (M_r 445.4). 1008700. [5941-07-1]. Sodium hydrogen-4-hydroxy-5-(2-hydroxybenzylideneamino)-2,7-naphthalenedisulphonate.

Azomethine H solution. 1008701.

Dissolve 0.45 g of azomethine *H R* and 1 g of ascorbic acid *R* with gentle heating in water *R* and dilute to 100 ml with the same solvent.

Barbaloin. $C_{21}H_{22}O_9 \cdot H_2O$. (M_r 436.4). 1008800. [1415-73-2]. Aloin. 1,8-Dihydroxy-3-hydroxymethyl-10- β -D-glucopyranosyl-10*H*-anthracen-9-one.

A yellow to dark-yellow, crystalline powder, or yellow needles, darkening on exposure to air and light, sparingly soluble in water and in alcohol, soluble in acetone, in ammonia and in solutions of alkali hydroxides.

$A_{1\%}^{1\text{cm}}$: about 192 at 269 nm, about 226 at 296.5 nm, about 259 at 354 nm, determined on a solution in methanol *R* and calculated with reference to the anhydrous substance.

Chromatography. Examine as prescribed in the monograph on *Frangula bark (0025)*; the chromatogram shows only one principal spot.

Barbital. 1008900. [57-44-3].

See *Barbital (0170)*.

Barbital sodium. $C_8H_{11}N_2NaO_3$. (M_r 206.2). 1009000. [144-02-5].

Content: minimum 98.0 per cent of the sodium derivative of 5,5-diethyl-1*H*,3*H*,5*H*-pyrimidine-2,4,6-trione.

A white, crystalline powder or colourless crystals, freely soluble in water, slightly soluble in alcohol.

Barbituric acid. $C_4H_4N_2O_3$. (M_r 128.1). 1009100. [67-52-7]. 1*H*,3*H*,5*H*-Pyrimidine-2,4,6-trione.

A white or almost white powder, slightly soluble in water, freely soluble in boiling water and in dilute acids.

mp: about 253 °C.

Barium carbonate. $BaCO_3$. (M_r 197.3). 1009200. [513-77-9].

A white powder or friable masses, practically insoluble in water.

Barium chloride. $BaCl_2 \cdot 2H_2O$. (M_r 244.3). 1009300. [10326-27-9]. Barium dichloride.

Colourless crystals, freely soluble in water, slightly soluble in alcohol.

Barium chloride solution R1. 1009301.

A 61 g/l solution.

Barium chloride solution R2. 1009302.

A 36.5 g/l solution.

Barium hydroxide. $\text{Ba}(\text{OH})_2 \cdot 8\text{H}_2\text{O}$. (M_r 315.5). 1009400. [12230-71-6]. Barium dihydroxide.

Colourless crystals, soluble in water.

Barium hydroxide solution. 1009401.

A 47.3 g/l solution.

Barium sulphate. 1009500. [7727-43-7].

See *Barium sulphate* (0010).

Benzaldehyde. $\text{C}_7\text{H}_6\text{O}$. (M_r 106.1). 1009600. [100-52-7].

A colourless or slightly yellow liquid, slightly soluble in water, miscible with alcohol.

d_{20}^{20} : about 1.05.

n_D^{20} : about 1.545.

Distillation range (2.2.11). Not less than 95 per cent distils between 177 °C and 180 °C.

Storage: protected from light.

Benzene. C_6H_6 . (M_r 78.1). 1009800. [71-43-2].

A clear, colourless, flammable liquid, practically insoluble in water, miscible with alcohol.

bp: about 80 °C.

Benzethonium chloride. $\text{C}_{27}\text{H}_{42}\text{ClNO}_2 \cdot \text{H}_2\text{O}$. (M_r 466.1). 1009900. [121-54-0]. Benzyl dimethyl[2-[2-[4-(1,1,3,3-tetramethylbutyl)phenoxy]ethoxy]ethyl]ammonium chloride monohydrate.

A fine, white powder or colourless crystals, soluble in water and in alcohol.

mp: about 163 °C.

Storage: protected from light.

Benzidine. $\text{C}_{12}\text{H}_{12}\text{N}_2$. (M_r 184.2). 1145300. [92-87-5]. Biphenyl-4,4'-diamine.

Content: minimum 95 per cent of $\text{C}_{12}\text{H}_{12}\text{N}_2$.

White or slightly yellowish or reddish powder, darkening on exposure to air and light.

mp: about 120 °C.

Storage: protected from light.

Benzil. $\text{C}_{14}\text{H}_{10}\text{O}_2$. (M_r 210.2). 1117800. [134-81-6]. Diphenylethanedione.

A yellow, crystalline powder, practically insoluble in water, soluble in alcohol, ethyl acetate and toluene.

mp: 95 °C.

Benzocaine. $\text{C}_9\text{H}_{11}\text{NO}_2$. (M_r 165.2). 1123600. [94-09-7].

See *Benzocaine* (0011).

Benzoic acid. 1010100. [65-85-0].

See *Benzoic acid* (0066).

Benzoin. $\text{C}_{14}\text{H}_{12}\text{O}_2$. (M_r 212.3). 1010200. [579-44-2]. 2-Hydroxy-1,2-diphenylethanone.

Slightly yellowish crystals, very slightly soluble in water, freely soluble in acetone, soluble in hot alcohol.

mp: about 137 °C.

Benzophenone. $\text{C}_{13}\text{H}_{10}\text{O}$. (M_r 182.2). 1010300. [119-61-9]. Diphenylmethanone.

Prismatic crystals, practically insoluble in water, freely soluble in alcohol.

mp: about 48 °C.

1,4-Benzoquinone. $\text{C}_6\text{H}_4\text{O}_2$. (M_r 108.1). 1118500. [106-51-4]. Cyclohexa-2,5-diene-1,4-dione.

Content: minimum 98.0 per cent of $\text{C}_6\text{H}_4\text{O}_2$.

Benzoylarginine ethyl ester hydrochloride.

$\text{C}_{15}\text{H}_{23}\text{ClN}_4\text{O}_3$. (M_r 342.8). 1010500. [2645-08-1].

N-Benzoyl-L-arginine ethyl ester hydrochloride. Ethyl (S)-2-benzamido-5-guanidinovalerate hydrochloride.

A white, crystalline powder, very soluble in water and in ethanol.

$[\alpha]_D^{20}$: -15 to -18, determined on a 10 g/l solution.

mp: about 129 °C.

$A_{1\text{cm}}^{1\%}$: 310 to 340, determined at 227 nm using a 0.01 g/l solution.

Benzoyl chloride. $\text{C}_7\text{H}_5\text{ClO}$. (M_r 140.6). 1010400. [98-88-4].

A colourless, lachrymatory liquid, decomposed by water and by alcohol.

d_{20}^{20} : about 1.21.

bp: about 197 °C.

***N*-Benzoyl-L-prolyl-L-phenylalanyl-L-arginine 4-nitroanilide acetate.** $\text{C}_{35}\text{H}_{42}\text{N}_8\text{O}_8$. (M_r 703). 1010600.

2-Benzoylpyridine. $\text{C}_{12}\text{H}_9\text{NO}$. (M_r 183.2). 1134300.

[91-02-1]. Phenyl(pyridin-2-yl)methanone.

Colourless crystals, soluble in alcohol.

mp: about 43 °C.

Benzyl alcohol. 1010700. [100-51-6].

See *Benzyl alcohol* (0256).

Benzyl benzoate. 1010800. [120-51-4].

See *Benzyl benzoate* (0705).

Chromatography. Examine as prescribed in the monograph on *Peru balsam* (0754) applying 20 µl of a 0.3 per cent V/V solution in *ethyl acetate R*. After spraying and heating, the chromatogram shows a principal band with an R_f of about 0.8.

Benzyl cinnamate. $\text{C}_{16}\text{H}_{14}\text{O}_2$. (M_r 238.3). 1010900.

[103-41-3]. Benzyl 3-phenylprop-2-enoate.

Colourless or yellowish crystals, practically insoluble in water, soluble in alcohol.

mp: about 39 °C.

Chromatography. Examine as prescribed in the monograph on *Peru balsam* (0754) applying 20 µl of a 3 g/l solution in *ethyl acetate R*. After spraying and heating, the chromatogram shows a principal band with an R_f of about 0.6.

Benzyl ether. $\text{C}_{14}\text{H}_{14}\text{O}$. (M_r 198.3). 1140900. [103-50-4].

Dibenzyl ether.

Clear, colourless liquid, practically insoluble in water, miscible with acetone and with ethanol.

d_{20}^{20} : about 1.043.

n_D^{20} : about 1.562.

bp: about 296 °C, with decomposition.

Benzylpenicillin sodium. 1011000. [69-57-8].

See *Benzylpenicillin sodium* (0114).

2-Benzylpyridine. $\text{C}_{12}\text{H}_{11}\text{N}$. (M_r 169.2). 1112900. [101-82-6].

Content: minimum 98.0 per cent of $\text{C}_{12}\text{H}_{11}\text{N}$.

A yellow liquid.

mp: 13 °C to 16 °C.

Benzyltrimethylammonium chloride. $\text{C}_{10}\text{H}_{16}\text{ClN}$. (M_r 185.7).

1155700. [56-93-9]. *N,N,N*-Trimethylphenylmethanaminium chloride. *N,N,N*-Trimethylbenzenemethanaminium chloride.

White powder, soluble in water.

mp: about 230 °C, with decomposition.

Berberine chloride. $C_{20}H_{18}ClNO_4 \cdot 2H_2O$. (M_r 407.8). 1153400. [5956-60-5]. 9,10-Dimethoxy-5,6-dihydrobenzo[*g*]-1,3-benzodioxolo[5,6-*a*]quinolizinium chloride.

Yellow crystals, slightly soluble in water, practically insoluble in alcohol.

mp: 204 °C to 206 °C.

Berberine chloride used in liquid chromatography complies with the following additional requirement.

Assay. Examine by liquid chromatography (2.2.29) as prescribed in the monograph on *Golden seal rhizome* (1831). The content is not less than 95 per cent, calculated by the normalisation procedure.

Bergapten. $C_{12}H_8O_4$. (M_r 216.2). 1103700. [484-20-8]. 5-Methoxypsoralen.

Colourless crystals, practically insoluble in water, sparingly soluble in alcohol and slightly soluble in glacial acetic acid.

mp: about 188 °C.

Betulin. $C_{30}H_{50}O_2$. (M_r 442.7). 1011100. [473-98-3]. Lup-20(39)-ene-3 β ,28-diol.

A white, crystalline powder.

mp: 248 °C to 251 °C.

Bibenzyl. $C_{14}H_{14}$. (M_r 182.3). 1011200. [103-29-7]. 1,2-Diphenylethane.

A white, crystalline powder, practically insoluble in water, very soluble in methylene chloride, freely soluble in acetone, soluble in alcohol.

mp: 50 °C to 53 °C.

Biphenyl-4-ol. $C_{12}H_{10}O$. (M_r 170.2). 1011300. [90-43-7]. 4-Phenylphenol.

A white, crystalline powder, practically insoluble in water.

mp: 164 °C to 167 °C.

Bisbenzimidide. $C_{25}H_{27}Cl_3N_6O_5 \cdot 5H_2O$. (M_r 624). 1103800. [23491-44-3]. 4-[5-[5-(4-Methylpiperazin-1-yl)benzimidazol-2-yl]benzimidazol-2-yl]phenol trihydrochloride pentahydrate.

Bisbenzimidide stock solution. 1103801.

Dissolve 5 mg of *bisbenzimidide R* in *water R* and dilute to 100 ml with the same solvent.

Storage: in the dark.

Bisbenzimidide working solution. 1103802.

Immediately before use, dilute 100 μ l of *bisbenzimidide stock solution R* to 100 ml with *phosphate-buffered saline pH 7.4 R*.

Bismuth subnitrate. $[4BiNO_3(OH)_2 \cdot BiO(OH)]$. (M_r 1462). 1011500. [1304-85-4].

A white powder, practically insoluble in water.

Bismuth subnitrate R1. 1011501.

Content: 71.5 per cent to 74.0 per cent of bismuth (Bi), and 14.5 per cent to 16.5 per cent of nitrate, calculated as nitrogen pentoxide (N_2O_5).

Bismuth subnitrate solution. 1011502.

Dissolve 5 g of *bismuth subnitrate R1* in a mixture of 8.4 ml of *nitric acid R* and 50 ml of *water R* and dilute to 250 ml with *water R*. Filter if necessary.

Acidity. To 10 ml add 0.05 ml of *methyl orange solution R*. 5.0 ml to 6.25 ml of 1 M *sodium hydroxide* is required to change the colour of the indicator.

Biuret. $C_2H_5N_3O_2$. (M_r 103.1). 1011600. [108-19-0].

White crystals, hygroscopic, soluble in water, sparingly soluble in alcohol.

mp: 188 °C to 190 °C, with decomposition.

Storage: in an airtight container.

Biuret reagent. 1011601.

Dissolve 1.5 g of *copper sulphate R* and 6.0 g of *sodium potassium tartrate R* in 500 ml of *water R*. Add 300 ml of a carbonate-free 100 g/l solution of *sodium hydroxide R*, dilute to 1000 ml with the same solution and mix.

Blocking solution. 1122400.

A 10 per cent V/V solution of *acetic acid R*.

Blue dextran 2000. 1011700. [9049-32-5].

Prepared from dextran having an average relative molecular mass of 2×10^6 by introduction of a polycyclic chromophore that colours the substance blue. The degree of substitution is 0.017. It is freeze-dried and dissolves rapidly and completely in water and aqueous saline solutions.

A 1 g/l solution in a *phosphate buffer solution pH 7.0 R* shows an absorption maximum (2.2.25) at 280 nm.

Boldine. $C_{19}H_{21}NO_4$. (M_r 327.3). 1118800. [476-70-0]. 1,10-Dimethoxy-6 α -aporphine-2,9-diol.

A white crystalline powder, very slightly soluble in water, soluble in alcohol and in dilute solutions of acids.

$[\alpha]_D^{25}$: about + 127, determined on a 1 g/l solution in *ethanol R*.

mp: about 163 °C.

Chromatography. Examined as prescribed in the monograph on *Boldo leaf* (1396) the chromatogram shows a single principal spot.

Assay. Examine by liquid chromatography (2.2.29) under the conditions described in the monograph on *Boldo leaf* (1396) using the substance to be examined as the test solution.

The area of the principal peak is not less than 99.0 per cent of the total area of the peaks.

Boric acid. 1011800. [10043-35-3].

See *Boric acid* (0001).

Boric acid solution, saturated, cold. 1011801.

To 3 g of *boric acid R* add 50 ml of *water R* and shake for 10 min. Place the solution for 2 h in the refrigerator.

Borneol. $C_{10}H_{18}O$. (M_r 154.3). 1011900. [507-70-0]. *endo*-1,7,7-Trimethylbicyclo[2.2.1]heptan-2-ol.

Colourless crystals, readily sublimes, practically insoluble in water, freely soluble in alcohol and in light petroleum.

mp: about 208 °C.

Chromatography. Examine by thin-layer chromatography (2.2.27), using *silica gel G R* as the coating substance. Apply to the plate 10 μ l of a 1 g/l solution in *toluene R*. Develop over a path of 10 cm using *chloroform R*. Allow the plate to dry in air, spray with *anisaldehyde solution R*, using 10 ml for a plate 200 mm square, and heat at 100 °C to 105 °C for 10 min. The chromatogram obtained shows only one principal spot.

Bornyl acetate. $C_{12}H_{20}O_2$. (M_r 196.3). 1012000. [5655-61-8]. *endo*-1,7,7-Trimethylbicyclo[2.2.1]hept-2-yl acetate.

Colourless crystals or a colourless liquid, very slightly soluble in water, soluble in alcohol.

mp: about 28 °C.

Chromatography. Examine by thin-layer chromatography (2.2.27), using *silica gel G R* as the coating substance. Apply to the plate 10 µl of a 2 g/l solution in *toluene R*. Develop over a path of 10 cm using *chloroform R*. Allow the plate to dry in air, spray with *anisaldehyde solution R*, using 10 ml for a plate 200 mm square, and heat at 100 °C to 105 °C for 10 min. The chromatogram obtained shows only one principal spot.

Boron trichloride. BCl_3 . (M_r 117.2). 1112000. [10294-34-5].

Colourless gas. Reacts violently with water. Available as solutions in suitable solvents (2-chloroethanol, methylene chloride, hexane, heptane, methanol).

n_D^{20} : about 1.420.

bp: about 12.6 °C.

Caution: toxic, corrosive.

Boron trichloride-methanol solution. 1112001.

A 120 g/l solution of BCl_3 in *methanol R*.

Storage: protected from light at –20 °C, preferably in sealed tubes.

Boron trifluoride. BF_3 . (M_r 67.8). 1012100. [7637-07-2].

Colourless gas.

Boron trifluoride-methanol solution. 1012101.

A 140 g/l solution of *boron trifluoride R* in *methanol R*.

Brilliant blue. 1012200. [6104-59-2].

See *acid blue 83 R*.

Bromelains. 1012300. [37189-34-7].

A concentrate of proteolytic enzymes derived from *Ananas comosus* Merr.

A dull-yellow powder.

Activity. 1 g liberates about 1.2 g of amino-nitrogen from a solution of *gelatin R* in 20 min at 45 °C and pH 4.5.

Bromelains solution. 1012301.

A 10 g/l solution of *bromelains R* in a mixture of 1 volume of *phosphate buffer solution pH 5.5 R* and 9 volumes of a 9 g/l solution of *sodium chloride R*.

Bromine. Br_2 . (M_r 159.8). 1012400. [7726-95-6].

A brownish-red fuming liquid, slightly soluble in water, soluble in alcohol.

d_{20}^{20} : about 3.1.

Bromine solution. 1012401.

Dissolve 30 g of *bromine R* and 30 g of *potassium bromide R* in *water R* and dilute to 100 ml with the same solvent.

Bromine water. 1012402.

Shake 3 ml of *bromine R* with 100 ml of *water R* to saturation.

Storage: over an excess of *bromine R*, protected from light.

Bromine water R1. 1012403.

Shake 0.5 ml of *bromine R* with 100 ml of *water R*.

Storage: protected from light; use within 1 week.

Bromocresol green. $C_{21}H_{14}Br_4O_5S$. (M_r 698). 1012600. [76-60-8]. 3',3'',5',5''-Tetrabromo-*m*-cresol-sulfonphthalein. 4,4'-(3*H*-2,1-Benzoxathiol-3-ylidene)bis(2,6-dibromo-3-methylphenol)-S,S-dioxide.

A brownish-white powder, slightly soluble in water, soluble in alcohol and in dilute solutions of alkali hydroxides.

Bromocresol green-methyl red solution. 1012602.

Dissolve 0.15 g of *bromocresol green R* and 0.1 g of *methyl red R* in 180 ml of *ethanol R* and dilute to 200 ml with *water R*.

Bromocresol green solution. 1012601.

Dissolve 50 mg of *bromocresol green R* in 0.72 ml of 0.1 *M sodium hydroxide* and 20 ml of *alcohol R* and dilute to 100 ml with *water R*.

Test for sensitivity. To 0.2 ml of the bromocresol green solution add 100 ml of *carbon dioxide-free water R*. The solution is blue. Not more than 0.2 ml of 0.02 *M hydrochloric acid* is required to change the colour to yellow.

Colour change: pH 3.6 (yellow) to pH 5.2 (blue).

Bromocresol purple. $C_{21}H_{16}Br_2O_5S$. (M_r 540.2). 1012700.

[115-40-2]. 3',3''-Dibromo-*o*-cresolsulfonphthalein. 4,4'-(3*H*-2,1-Benzoxathiol-3-ylidene)bis(2-bromo-6-methylphenol)-S,S-dioxide.

A pinkish powder, practically insoluble in water, soluble in alcohol and in dilute solutions of alkali hydroxides.

Bromocresol purple solution. 1012701.

Dissolve 50 mg of *bromocresol purple R* in 0.92 ml of 0.1 *M sodium hydroxide* and 20 ml of *alcohol R* and dilute to 100 ml with *water R*.

Test for sensitivity. To 0.2 ml of the bromocresol purple solution add 100 ml of *carbon dioxide-free water R* and 0.05 ml of 0.02 *M sodium hydroxide*. The solution is bluish-violet. Not more than 0.2 ml of 0.02 *M hydrochloric acid* is required to change the colour to yellow.

Colour change: pH 5.2 (yellow) to pH 6.8 (bluish-violet).

5-Bromo-2'-deoxyuridine. $C_9H_{11}BrN_2O_5$. (M_r 307.1). 1012500. [59-14-3]. 5-Bromo-1-(2-deoxy-β-*D*-erythro-pentofuranosyl)-1*H*,3*H*-pyrimidine-2,4-dione.

mp: about 194 °C.

Chromatography. Examine as prescribed in the monograph on *Idoxuridine* (0669), applying 5 µl of a 0.25 g/l solution. The chromatogram obtained shows only one principal spot.

Bromophenol blue. $C_{19}H_{10}Br_4O_5S$. (M_r 670). 1012800. [115-39-9]. 3',3'',5',5''-Tetrabromophenolsulfonphthalein. 4,4'-(3*H*-2,1-Benzoxathiol-3-ylidene)bis(2,6-dibromophenol)-S,S-dioxide.

A light orange-yellow powder, very slightly soluble in water, slightly soluble in alcohol, freely soluble in solutions of alkali hydroxides.

Bromophenol blue solution. 1012801.

Dissolve 0.1 g of *bromophenol blue R* in 1.5 ml of 0.1 *M sodium hydroxide* and 20 ml of *alcohol R* and dilute to 100 ml with *water R*.

Test for sensitivity. To 0.05 ml of the bromophenol blue solution add 20 ml of *carbon dioxide-free water R* and 0.05 ml of 0.1 *M hydrochloric acid*. The solution is yellow. Not more than 0.1 ml of 0.1 *M sodium hydroxide* is required to change the colour to bluish-violet.

Colour change: pH 2.8 (yellow) to pH 4.4 (bluish-violet).

Bromophenol blue solution R1. 1012802.

Dissolve 50 mg of *bromophenol blue R* with gentle heating in 3.73 ml of 0.02 M sodium hydroxide and dilute to 100 ml with *water R*.

Bromophenol blue solution R2. 1012803.

Dissolve with heating 0.2 g of *bromophenol blue R* in 3 ml of 0.1 M sodium hydroxide and 10 ml of *alcohol R*. After solution is effected, allow to cool and dilute to 100 ml with *alcohol R*.

Bromophos. C₈H₈BrCl₂O₃PS. (*M_r* 366.0). 1123700. [2104-96-3].

A suitable certified reference solution (10 ng/μl in iso-octane) may be used.

Bromophos-ethyl. C₁₀H₁₂BrCl₂O₃PS. (*M_r* 394.0). 1123800. [4824-78-6].

A suitable certified reference solution (10 ng/μl in iso-octane) may be used.

Bromothymol blue. C₂₇H₂₈Br₂O₅S. (*M_r* 624). 1012900. [76-59-5]. 3',3''-Dibromothymolsulfonphthalein. 4,4'-(3*H*-2,1-Benzoxathiol-3-ylidene)bis(2-bromo-6-isopropyl-3-methylphenol) S,S-dioxide.

A reddish-pink or brownish powder, practically insoluble in water, soluble in alcohol and in dilute solutions of alkali hydroxides.

Bromothymol blue solution R1. 1012901.

Dissolve 50 mg of *bromothymol blue R* in a mixture of 4 ml of 0.02 M sodium hydroxide and 20 ml of *alcohol R* and dilute to 100 ml with *water R*.

Test for sensitivity. To 0.3 ml of *bromothymol blue solution R1* add 100 ml of carbon dioxide-free *water R*. The solution is yellow. Not more than 0.1 ml of 0.02 M sodium hydroxide is required to change the colour to blue.

Colour change: pH 5.8 (yellow) to pH 7.4 (blue).

Bromothymol blue solution R2. 1012902.

A 10 g/l solution in *dimethylformamide R*.

Bromothymol blue solution R3. 1012903.

Warm 0.1 g of *bromothymol blue R* with 3.2 ml of 0.05 M sodium hydroxide and 5 ml of *alcohol (90 per cent V/V) R*. After solution is effected, dilute to 250 ml with *alcohol (90 per cent V/V) R*.

BRP indicator solution. 1013000.

Dissolve 0.1 g of *bromothymol blue R*, 20 mg of *methyl red R* and 0.2 g of *phenolphthalein R* in *alcohol R* and dilute to 100 ml with the same solvent. Filter.

Brucine. C₂₃H₂₆N₂O₄·2H₂O. (*M_r* 430.5). 1013100. [357-57-3]. 10,11-Dimethoxystrychnine.

Colourless crystals, slightly soluble in water, freely soluble in alcohol.

mp: about 178 °C.

Butanal. C₄H₈O. (*M_r* 72.1). 1134400. [123-72-8]. Butyraldehyde.

*d*₂₀²⁰: 0.806.

*n*_D²⁰: 1.380.

bp: 75 °C.

Butanol. C₄H₁₀O. (*M_r* 74.1). 1013200. [71-36-3]. *n*-Butanol. 1-Butanol.

A clear, colourless liquid, miscible with alcohol.

*d*₂₀²⁰: about 0.81.

bp: 116 °C to 119 °C.

2-Butanol R1. C₄H₁₀O. (*M_r* 74.1). 1013301. [78-92-2]. *sec*-Butyl alcohol.

Content: minimum 99.0 per cent of C₄H₁₀O.

A clear, colourless liquid, soluble in water, miscible with alcohol.

*d*₂₀²⁰: about 0.81.

Distillation range (2.2.11). Not less than 95 per cent distils between 99 °C and 100 °C.

Assay. By gas chromatography as described in the monograph on *Isopropyl alcohol (0970)*.

Butyl acetate. C₆H₁₂O₂. (*M_r* 116.2). 1013400. [123-86-4].

A clear, colourless liquid, flammable, slightly soluble in water, miscible with alcohol.

*d*₂₀²⁰: about 0.88.

*n*_D²⁰: about 1.395.

Distillation range (2.2.11). Not less than 95 per cent distils between 123 °C and 126 °C.

Butyl acetate R1. 1013401.

A clear, colourless liquid, flammable, slightly soluble in water, miscible with alcohol.

*d*₂₀²⁰: about 0.883.

*n*_D²⁰: about 1.395.

Butanol: maximum 0.2 per cent, determined by gas chromatography.

n-Butyl formate: maximum 0.1 per cent, determined by gas chromatography.

n-Butyl propionate: maximum 0.1 per cent, determined by gas chromatography.

Water: maximum 0.1 per cent.

Assay: minimum 99.5 per cent of C₆H₁₂O₂, determined by gas chromatography.

Butylamine. C₄H₁₁N. (*M_r* 73.1). 1013600. [109-73-9]. 1-Butanamine.

Distil and use within one month.

A colourless liquid, miscible with water, with alcohol.

*n*_D²⁰: about 1.401.

bp: about 78 °C.

tert-Butylamine. 1100900. [75-64-9].

See 1,1-dimethylethylamine *R*.

Butylated hydroxytoluene. 1013800. [128-37-0].

See *Butylhydroxytoluene R*.

Butylboronic acid. C₄H₁₁BO₂. (*M_r* 101.9). 1013700. [4426-47-5].

Content: minimum 98 per cent of C₄H₁₁BO₂.

mp: 90 °C to 92 °C.

tert-Butylhydroperoxide. C₄H₁₀O₂. (*M_r* 90.1). 1118000. [75-91-2]. 1,1-Dimethylethylhydroperoxide.

Flammable liquid, soluble in organic solvents.

*d*₂₀²⁰: 0.898.

*n*_D²⁰: 1.401.

bp: 35 °C.

Butylhydroxytoluene. 1013800. [128-37-0].

See *Butylhydroxytoluene (0581)*.

Butyl methacrylate. $C_8H_{14}O_2$. (M_r 142.2). 1145400. [97-88-1]. Butyl 2-methylpropenoate.

Clear, colourless solution.

d_4^{20} : about 0.894.

n_D^{20} : about 1.424.

bp: about 163 °C.

tert-Butyl methyl ether. 1013900. [1634-04-4].

See 1,1-dimethylethyl methyl ether R.

Butyl parahydroxybenzoate. 1103900. [94-26-8].

See Butyl parahydroxybenzoate (0881).

Butyric acid. $C_4H_8O_2$. (M_r 88.1). 1014000. [107-92-6]. Butanoic acid.

Content: minimum 99.0 per cent of $C_4H_8O_2$.

An oily liquid, miscible with water and with alcohol.

d_{20}^{20} : about 0.96.

n_D^{20} : about 1.398.

bp: about 163 °C.

Butyrolactone. $C_4H_6O_2$. (M_r 86.1). 1104000. [96-48-0]. Dihydro-2(3H)-furanone. γ -Butyrolactone.

Oily liquid, miscible with water, soluble in methanol.

n_D^{25} : about 1.435.

bp: about 204 °C.

Cadmium. Cd. (A_r 112.4). 1014100. [10108-64-2].

A silvery-white, lustrous metal, practically insoluble in water, freely soluble in nitric acid and in hot hydrochloric acid.

Caesium chloride. CsCl. (M_r 168.4). 1014200. [7647-17-8].

A white powder, very soluble in water, freely soluble in methanol, practically insoluble in acetone.

Caffeic acid. $C_9H_8O_4$. (M_r 180.2). 1014300. [331-39-5]. (E)-3-(3,4-Dihydroxyphenyl)propenoic acid.

White or almost white crystals or plates, freely soluble in hot water and in alcohol, sparingly soluble in cold water.

mp: about 225 °C, with decomposition.

A freshly prepared solution at pH 7.6 shows 2 absorption maxima (2.2.25), at 293 nm and 329 nm.

Caffeine. 1014400. [58-08-2].

See Caffeine (0267).

Calcium carbonate. 1014500. [471-34-1].

See Calcium carbonate (0014).

Calcium carbonate R1. 1014501.

It complies with the requirements of calcium carbonate R and with the following additional requirement:

Chlorides (2.4.4): maximum 50 ppm.

Calcium chloride. 1014600. [10035-04-8].

See Calcium chloride (0015).

Calcium chloride solution. 1014601.

A 73.5 g/l solution.

Calcium chloride solution 0.01 M. 1014602.

Dissolve 0.147 g of calcium chloride R in water R and dilute to 100.0 ml with the same solvent.

Calcium chloride solution 0.02 M. 1014603.

Dissolve 2.94 g of calcium chloride R in 900 ml of water R, adjust to pH 6.0 to 6.2 and dilute to 1000.0 ml with water R.

Storage: at 2 °C to 8 °C.

Calcium chloride R1. $CaCl_2 \cdot 4H_2O$. (M_r 183.1). 1014700. Calcium chloride tetrahydrate.

Content: maximum 0.05 ppm of Fe.

Calcium chloride, anhydrous. $CaCl_2$. (M_r 111.0). 1014800. [10043-52-4].

Content: minimum 98.0 per cent of $CaCl_2$, calculated with reference to the dried substance.

White granules, deliquescent, very soluble in water, freely soluble in alcohol and in methanol.

Loss on drying (2.2.32): maximum 5.0 per cent, determined by drying in an oven at 200 °C.

Storage: in an airtight container, protected from moisture.

Calcium hydroxide. $Ca(OH)_2$. (M_r 74.1). 1015000. [1305-62-0]. Calcium dihydroxide.

A white powder, almost completely soluble in 600 parts of water.

Calcium hydroxide solution. 1015001.

A freshly prepared saturated solution.

Calcium lactate. 1015100. [41372-22-9].

See Calcium lactate pentahydrate (0468).

Calcium sulphate. $CaSO_4 \cdot \frac{1}{2}H_2O$. (M_r 145.1). 1015200. [10034-76-1]. Calcium sulphate hemihydrate.

A white powder, soluble in about 1500 parts of water, practically insoluble in alcohol. When mixed with half its mass of water it rapidly solidifies to a hard and porous mass.

Calcium sulphate solution. 1015201.

Shake 5 g of calcium sulphate R with 100 ml of water R for 1 h and filter.

Calcone-carboxylic acid. $C_{21}H_{14}N_2O_7S \cdot 3H_2O$. (M_r 492.5). 1015300. [3737-95-9]. 2-Hydroxy-1-(2-hydroxy-4-sulpho-1-naphthylazo)naphthalene-3-carboxylic acid.

A brownish-black powder, slightly soluble in water, very slightly soluble in acetone and in alcohol, sparingly soluble in dilute solutions of sodium hydroxide.

Calcone-carboxylic acid triturate. 1015301.

Mix 1 part of calcone-carboxylic acid R with 99 parts of sodium chloride R.

Test for sensitivity. Dissolve 50 mg of calcone-carboxylic acid triturate in a mixture of 2 ml of strong sodium hydroxide solution R and 100 ml of water R. The solution is blue but becomes violet on addition of 1 ml of a 10 g/l solution of magnesium sulphate R and 0.1 ml of a 1.5 g/l solution of calcium chloride R and turns pure blue on addition of 0.15 ml of 0.01 M sodium edetate.

Camphene. $C_{10}H_{16}$. (M_r 136.2). 1139200. [79-92-5]. 2,2-Dimethyl-3-methylenebicyclo[2.2.1]heptane.

Camphene used in gas chromatography complies with the following additional test.

Assay. Examine by gas chromatography (2.2.28) as prescribed in the monograph on Rosemary Oil (1846).

The content is not less than 90 per cent calculated by the normalisation procedure.

Camphor. 1113000. [76-22-2]. See Camphor, racemic (0655).

Camphor used in gas chromatography complies with the following additional test.

Assay. Examine by gas chromatography (2.2.28) as prescribed in the monograph on Lavender oil (1338).

Test solution. A 10 g/l solution of the substance to be examined in hexane R.

The area of the principal peak is not less than 98.0 per cent of the area of all the peaks in the chromatogram obtained. Disregard the peak due to hexane.

(1S)-(+)-10-Camphorsulphonic acid. $C_{10}H_{16}O_4S$. (M_r 232.3). 1104100. [3144-16-9]. (1S,4R)-(+)-2-Oxo-10-bornenesulphonic acid. [(1S)-7,7-Dimethyl-2-oxobicyclo[2.2.1]heptan-1-yl]methanesulphonic acid. Reychler's acid.

Prismatic crystals, hygroscopic, soluble in water.

Content: minimum 99.0 per cent of (1S)-(+)-10-camphorsulphonic acid.

$[\alpha]_D^{20}$: $+20 \pm 1$ (43 g/l solution in water R).

mp: about 194 °C, with decomposition.

ΔA (2.2.41): 10.2×10^3 determined at 290.5 nm on a 1.0 g/l solution.

Capric acid. $C_{10}H_{20}O_2$. (M_r 172.3). 1142000. [334-48-5]. Decanoic acid.

Crystalline solid, very slightly soluble in water, soluble in ethanol.

bp: about 270 °C.

mp: about 31.4 °C.

Capric acid used in the assay of total fatty acids in Saw palmetto fruit (1848) complies with the following additional requirement.

Assay. Examine by gas chromatography (2.2.28) as prescribed in the monograph on *Saw palmetto fruit (1848)*.

The content of capric acid is not less than 98 per cent, calculated by the normalisation procedure.

Capric alcohol. 1024700.

See *Decanol R*.

Caproic acid. $C_6H_{12}O_2$. (M_r 116.2). 1142100. [142-62-1]. Hexanoic acid.

Oily liquid, sparingly soluble in water.

d_4^{20} : about 0.926.

n_D^{20} : about 1.417.

bp: about 205 °C.

Caproic acid used in the assay of total fatty acids in Saw palmetto fruit (1848) complies with the following additional requirement.

Assay. Examine by gas chromatography (2.2.28) as prescribed in the monograph on *Saw palmetto fruit (1848)*.

The content of caproic acid is not less than 98 per cent, calculated by the normalisation procedure.

ε-Caprolactam. $C_6H_{11}NO$. (M_r 113.2). 1104200. [105-60-2]. Hexane-6-lactam.

Hygroscopic flakes, freely soluble in water, in ethanol and in methanol.

mp: about 70 °C.

Caprylic acid. $C_8H_{16}O_2$. (M_r 144.2). 1142200. [124-07-2]. Octanoic acid.

Slightly yellow, oily liquid.

d_4^{20} : about 0.910.

n_D^{20} : about 1.428.

bp: about 239.7 °C.

mp: about 16.7 °C.

Caprylic acid used in the assay of total fatty acids in Saw palmetto fruit (1848) complies with the following additional requirement.

Assay. Examine by gas chromatography (2.2.28) as prescribed in the monograph on *Saw palmetto fruit (1848)*.

The content of caprylic acid is not less than 98 per cent, calculated by the normalisation procedure.

Capsaicin. $C_{18}H_{27}NO_3$. (M_r 305.4). 1147900. [404-86-4]. (E)-N-[(4-Hydroxy-3-methoxyphenyl)methyl]-8-methylnon-6-enamide.

White, crystalline powder, practically insoluble in water, freely soluble in ethanol.

mp: about 65 °C.

Capsaicin used in the assay in Capsicum (1859) complies with the following additional requirement.

Assay. Examine by liquid chromatography (2.2.29) as prescribed in the monograph on *Capsicum (1859)*. The content of capsaicin is not less than 95.0 per cent, calculated by the normalisation procedure.

Carbazole. $C_{12}H_9N$. (M_r 167.2). 1015400. [86-74-8]. Dibenzopyrrole.

Crystals, practically insoluble in water, freely soluble in acetone, slightly soluble in ethanol.

mp: about 245 °C.

Carbomer. 1015500. [9007-20-9].

A cross-linked polymer of acrylic acid; it contains a large proportion (56 per cent to 68 per cent) of carboxylic acid (CO_2H) groups after drying at 80 °C for 1 h. Average relative molecular mass about 3×10^6 .

pH (2.2.3): about 3 for a 10 g/l suspension.

Carbon dioxide. 1015600. [124-38-9].

See *Carbon dioxide (0375)*.

Carbon dioxide R1. CO_2 . (M_r 44.01). 1015700.

Content: minimum 99.995 per cent V/V of CO_2 .

Carbon monoxide: less than 5 ppm.

Oxygen: less than 25 ppm.

Nitric oxide: less than 1 ppm.

Carbon dioxide R2. CO_2 . (M_r 44.01). 1134500.

Content: minimum 99 per cent V/V of CO_2 .

Carbon disulphide. CS_2 . (M_r 76.1). 1015800. [75-15-0].

A colourless or yellowish, flammable liquid, practically insoluble in water, miscible with ethanol.

d_{20}^{20} : about 1.26.

bp: 46 °C to 47 °C.

Carbon for chromatography, graphitised. 1015900.

Carbon chains having a length greater than C_9 with a particle size of 400 µm to 850 µm.

Relative density: 0.72.

Surface area: 10 m²/g.

Do not use at a temperature higher than 400 °C.

Carbon for chromatography, graphitised R1. 1153500.

Porous spherical carbon particles comprised of flat sheets of hexagonally arranged carbon atoms.

Particle size: 5-7 µm.

Pore volume: 0.7 cm³/g.

Carbon monoxide. CO . (M_r 28.01). 1016000. [630-08-0].

Content: minimum 99.97 per cent V/V of CO .

Carbon monoxide R1. CO . (M_r 28.01). 1134600. [630-08-0].

Content: minimum 99 per cent V/V of CO .

Carbon tetrachloride. CCl_4 . (M_r 153.8). 1016100. [56-23-5]. Tetrachloromethane.

A clear, colourless liquid, practically insoluble in water, miscible with alcohol.

d_{20}^{20} : 1.595 to 1.598.

bp: 76 °C to 77 °C.

Carbophenothion. $\text{C}_{11}\text{H}_{16}\text{ClO}_2\text{PS}_3$. (M_r 342.9). 1016200. [786-19-6]. *O,O*-Diethyl *S*-[[[(4-chlorophenyl)thio]methyl]-phosphorodithioate.

Yellowish liquid, practically insoluble in water, miscible with organic solvents.

d_4^{25} : about 1.27.

For the monograph *Wool Fat* (0134), a suitable certified reference solution (10 ng/μl in iso-octane) may be used.

Car-3-ene. $\text{C}_{10}\text{H}_{16}$. (M_r 136.2). 1124000. [498-15-7]. 3,7,7-Trimethylbicyclo[4.1.0]hept-3-ene. 4,7,7-Trimethyl-3-norcarene.

A liquid with a pungent odour, slightly soluble in water, soluble in organic solvents.

d_{20}^{20} : about 0.864.

n_D^{20} : 1.473 to 1.474.

$[\alpha]_D^{20}$: + 15 to + 17.

bp: 170 °C to 172 °C.

Car-3-ene used in gas chromatography complies with the following additional test.

Assay. Examine by gas chromatography (2.2.28) as prescribed in the monograph on *Nutmeg oil* (1552).

The content is not less than 95.0 per cent, calculated by the normalisation procedure.

Carminic acid. $\text{C}_{22}\text{H}_{20}\text{O}_{13}$. (M_r 492.4). 1156700. [1260-17-9]. 7- α -D-Glucopyranosyl-3,5,6,8-tetrahydroxy-1-methyl-9,10-dioxo-9,10-dihydroanthracene-2-carboxylic acid.

Dark red powder, very slightly soluble in water, soluble in dimethyl sulphoxide, very slightly soluble in ethanol (96 per cent).

Carob bean gum. 1104500.

The ground endosperm of the fruit kernels of *Ceratonia siliqua* L. Taub.

A white powder containing 70 per cent to 80 per cent of a water-soluble gum consisting mainly of galactomannoglycone.

Carvacrol. $\text{C}_{10}\text{H}_{14}\text{O}$. (M_r 150.2). 1016400. [499-75-2]. 5-Isopropyl-2-methylphenol.

Brownish liquid, practically insoluble in water, very soluble in alcohol.

d_{20}^{20} : about 0.975.

n_D^{20} : about 1.523.

bp: about 237 °C.

Carvacrol used in gas chromatography complies with the following additional test.

Assay. Examine by gas chromatography (2.2.28) as prescribed in the monograph on *Peppermint oil* (0405).

Test solution. Dissolve 0.1 g in about 10 ml of *acetone R*.

The area of the principal peak is not less than 95.0 per cent of the area of all the peaks in the chromatogram obtained. Disregard the peak due to acetone.

Carvone. $\text{C}_{10}\text{H}_{14}\text{O}$. (M_r 150.2). 1016500. [2244-16-8]. (*S*)-*p*-Mentha-6,8-dien-2-one. (+)-2-Methyl-5-(1-methylethenyl)-cyclohex-2-enone.

A liquid, practically insoluble in water, miscible with alcohol.

d_{20}^{20} : about 0.965

n_D^{20} : about 1.500.

$[\alpha]_D^{20}$: about + 61.

bp: about 230 °C.

Carvone used in gas chromatography complies with the following additional test.

Assay. Examine by gas chromatography (2.2.28) as prescribed in the monograph on *Peppermint oil* (0405) using the substance to be examined as the test solution.

The area of the principal peak is not less than 98.0 per cent of the total area of the peaks.

β -Caryophyllene. $\text{C}_{15}\text{H}_{24}$. (M_r 204.4). 1101000. [87-44-5]. (*E*)-(1*R*,9*S*)-4,11,11-Trimethyl-8-methylenebicyclo[7.2.0]undec-4-ene.

An oily liquid, practically insoluble in water, miscible with alcohol.

d_4^{17} : about 0.905.

n_D^{20} : about 1.492.

$[\alpha]_D^{15}$: about -5.2.

bp₁₄: 129 °C to 130 °C.

β -Caryophyllene used in gas chromatography complies with the following additional test.

Assay. Examine by gas chromatography (2.2.28) as prescribed in the monograph on *Clove oil* (1091) using the substance to be examined as the test solution.

The area of the principal peak is not less than 98.5 per cent of the total area of the peaks.

Caryophyllene oxide. $\text{C}_{15}\text{H}_{24}\text{O}$. (M_r 220.4). 1149000. [1139-30-6]. (-)- β -Caryophyllene epoxide. (1*R*,4*R*,6*R*,10*S*)-4,12,12-Trimethyl-9-methylene-5-oxatricyclo[8.2.0.0^{4,6}]dodecane.

Colourless, fine crystals with lumps.

mp: 62 °C to 63 °C.

Caryophyllene oxide used in gas chromatography complies with the following additional test.

Assay. Examine by gas chromatography (2.2.28) as prescribed in the monograph on *Turpentine oil, Pinus pinaster type* (1627).

The content is not less than 99.0 per cent, calculated by the normalisation procedure.

Casein. 1016600. [9000-71-9].

A mixture of related phosphoproteins obtained from milk.

White, amorphous powder or granules, very slightly soluble in water and in non-polar organic solvents. It dissolves in concentrated hydrochloric acid giving a pale-violet solution. It forms salts with acids and bases. Its isoelectric point is at about pH 4.7. Alkaline solutions are laevorotatory.

Catalpol. $\text{C}_{15}\text{H}_{22}\text{O}_{10}$. (M_r 362.3). 1142300. [2415-24-9]. (1*aS*, 1*bS*, 2*S*, 5*aR*, 6*S*, 6*aS*)-6-Hydroxy-1*a*-(hydroxymethyl)-1*a*, 1*b*, 2, 5*a*, 6, 6*a*-hexahydrooxireno[4,5]cyclopenta[1,2-*c*]pyran-2-yl β -D-glucopyranoside.

mp: 203 °C to 205 °C.

Catechin. $\text{C}_{15}\text{H}_{14}\text{O}_6 \cdot x\text{H}_2\text{O}$. (M_r 290.3 for the anhydrous substance). 1119000. [154-23-4]. (+)-(2*R*,3*S*)-2-(3,4-Dihydroxyphenyl)-3,4-dihydro-2*H*-chromene-3,5,7-triol. Catechol. Cianidanol. Cyanidol.

Catholyte for isoelectric focusing pH 3 to 5. 1113100. 0.1 M β -Alanine.

Dissolve 8.9 g of *β -alanine R* in *water R* and dilute to 1000 ml with the same solvent.

Cation exchange resin. 1016700.

A resin in protonated form with sulphonic acid groups attached to a polymer lattice consisting of polystyrene cross-linked with 8 per cent of divinylbenzene. It is available as beads and the particle size is specified after the name of the reagent in the tests where it is used.

Cation exchange resin R1. 1121900.

A resin in protonated form with sulphonic acid groups attached to a polymer lattice consisting of polystyrene cross-linked with 4 per cent of divinylbenzene. It is available as beads and the particle size is specified after the name of the reagent in the tests where it is used.

Cation-exchange resin, strong. 1156800.

A strong cation-exchange resin in protonated form with sulphonic acid groups attached to a polymer lattice consisting of polystyrene cross-linked with divinylbenzene. The particle size is specified after the name of the reagent in the tests where it is used.

Cation exchange resin (calcium form), strong. 1104600.

A resin in calcium form with sulphonic acid groups attached to a polymer lattice consisting of polystyrene cross-linked with 8 per cent of divinylbenzene. The particle size is specified after the name of the reagent in the tests where it is used.

Cellulose for chromatography. 1016800. [9004-34-6].

A fine, white, homogeneous powder with an average particle size less than 30 µm.

Preparation of a thin layer. Suspend 15 g in 100 ml of *water R* and homogenise in an electric mixer for 60 s. Coat carefully cleaned plates with a layer 0.1 mm thick using a spreading device. Allow to dry in air.

Cellulose for chromatography R1. 1016900.

Microcrystalline cellulose. A fine, white homogeneous powder with an average particle size less than 30 µm.

Preparation of a thin layer. Suspend 25 g in 90 ml of *water R* and homogenise in an electric mixer for 60 s. Coat carefully cleaned plates with a layer 0.1 mm thick using a spreading device. Allow to dry in air.

Cellulose for chromatography F₂₅₄. 1017000.

Microcrystalline cellulose F₂₅₄. A fine, white, homogeneous powder with an average particle size less than 30 µm, containing a fluorescent indicator having an optimal intensity at 254 nm.

Preparation of a thin layer. Suspend 25 g in 100 ml of *water R* and homogenise using an electric mixer for 60 s. Coat carefully cleaned plates with a layer 0.1 mm thick using a spreading device. Allow to dry in air.

Cephalin. 1017200.

To 0.5 g to 1 g of *acetone-dried ox brain R* add 20 ml of *acetone R* and allow to stand for 2 h. Centrifuge at 500 *g* for 2 min and decant the supernatant liquid. Dry the residue under reduced pressure and, to the dried material, add 20 ml of *chloroform R* and allow to stand for 2 h, shaking frequently. Remove the solid material by filtration or centrifugation and evaporate the chloroform under reduced pressure. Suspend the residue in 5 ml to 10 ml of a 9 g/l solution of *sodium chloride R*.

Solvents used to prepare the reagent should contain a suitable antioxidant, for example, 0.02 g/l of butylated hydroxyanisole.

Storage: frozen or freeze-dried for 3 months.

Cerium sulphate. Ce(SO₄)₂·4H₂O. (*M_r* 404.3). 1017300. [123333-60-8]. Cerium(IV) sulphate. Ceric sulphate.

Yellow or orange-yellow, crystalline powder or crystals, very slightly soluble in water, slowly soluble in dilute acids.

Cerous nitrate. Ce(NO₃)₃·6H₂O. (*M_r* 434.3). 1017400. [10294-41-4]. Cerium trinitrate hexahydrate.

A colourless or pale yellow, crystalline powder, freely soluble in water and in alcohol.

Cetostearyl alcohol. 1017500. [67762-27-0].

See *Cetostearyl alcohol (0702)*.

Cetrimide. 1017600. [8044-71-1].

See *Cetrimide (0378)*.

Cetyltrimethylammonium bromide. C₁₉H₄₂BrN. (*M_r* 364.5). 1017700. [57-09-0]. Cetrinonium bromide. *N*-Hexadecyl-*N,N,N*-trimethylammonium bromide.

A white, crystalline powder, soluble in water, freely soluble in alcohol.

mp: about 240 °C.

Chamazulene. C₁₄H₁₆. (*M_r* 184.3). 1148000. [529-05-5]. 7-Ethyl-1,4-dimethylazulene.

A blue liquid, very slightly soluble in water, soluble in alcohol, miscible with fatty oils, with essential oils and with liquid paraffin, soluble with discolouration in phosphoric acid (85 per cent *m/m*) and sulphuric acid (50 per cent *V/V*).

Appearance of solution. 50 mg is soluble in 2.5 ml of *hexane R*. The blue solution is clear in a thin-layer obtained by tilting the test-tube.

Chamazulene used for gas chromatography complies with the following additional test.

Assay. Examine by gas chromatography (2.2.28) as prescribed in the monograph on *Matricaria oil (1836)*, using a 4 g/l solution in *cyclohexane R*.

The content of chamazulene is not less than 95.0 per cent, calculated by the normalisation procedure.

Charcoal, activated. 1017800. [64365-11-3].

See *Activated charcoal (0313)*.

Chloral hydrate. 1017900. [302-17-0].

See *Choral hydrate (0265)*.

Chloral hydrate solution. 1017901.

A solution of 80 g in 20 ml of *water R*.

Chloramine. 1018000. [7080-50-4].

See *Chloramine (0381)*.

Chloramine solution. 1018001.

A 20 g/l solution. Prepare immediately before use.

Chloramine solution R1. 1018002.

A 0.1 g/l solution of *chloramine R*. Prepare immediately before use.

Chloramine solution R2. 1018003.

A 0.2 g/l solution. Prepare immediately before use.

Chlordane. C₁₀H₆Cl₈. (*M_r* 409.8). 1124100. [12789-03-6].

bp: about 175 °C.

mp: about 106 °C.

A suitable certified reference solution of technical grade (10 ng/µl in iso-octane) may be used.

Chlordiazepoxide. 1113200. [58-25-3].

See *Chlordiazepoxide (0656)*.

Chlorfenvinphos. $\text{C}_{12}\text{H}_{14}\text{Cl}_3\text{O}_4\text{P}$. (M_r 359.6). 1124200. [470-90-6].

A suitable certified reference solution (10 ng/ μl in cyclohexane) may be used.

Chloroacetanilide. $\text{C}_8\text{H}_8\text{ClNO}$. (M_r 169.6). 1018100. [539-03-7]. 4'-Chloroacetanilide.

Content: minimum 95 per cent of $\text{C}_8\text{H}_8\text{ClNO}$.

A crystalline powder, practically insoluble in water, soluble in alcohol.

mp: about 178 °C.

Chloroacetic acid. $\text{C}_2\text{H}_3\text{ClO}_2$. (M_r 94.5). 1018200. [79-11-8]. Colourless or white crystals, deliquescent, very soluble in water, soluble in alcohol.

Storage: in an airtight container.

Chloroaniline. $\text{C}_6\text{H}_6\text{ClN}$. (M_r 127.6). 1018300. [106-47-8]. 4-Chloroaniline.

Crystals, soluble in hot water, freely soluble in alcohol.

mp: about 71 °C.

4-Chlorobenzenesulphonamide. $\text{C}_6\text{H}_6\text{ClNO}_2\text{S}$. (M_r 191.6). 1097400. [98-64-6].

White powder.

mp: about 145 °C.

2-Chlorobenzoic acid. $\text{C}_7\text{H}_5\text{ClO}_2$. (M_r 156.6). 1139300. [118-91-2].

Soluble in water, slightly soluble in ethanol.

bp: about 285 °C.

mp: about 140 °C.

Chlorobutanol. 1018400. [57-15-8].

See *Anhydrous chlorobutanol* (0382).

2-Chloro-2-deoxy-D-glucose. $\text{C}_6\text{H}_{11}\text{ClO}_5$. (M_r 198.6). 1134700. [14685-79-1].

A white crystalline, very hygroscopic powder, soluble in water and in dimethyl sulphoxide, practically insoluble in alcohol.

2-Chloroethanol. $\text{C}_2\text{H}_5\text{ClO}$. (M_r 80.5). 1097500. [107-07-3]. Colourless liquid, soluble in alcohol.

d_{20}^{20} : about 1.197.

n_D^{20} : about 1.442.

bp: about 130 °C.

mp: about -89 °C.

2-Chloroethanol solution. 1097501.

Dissolve 125 mg of 2-chloroethanol *R* in 2-propanol *R* and dilute to 50 ml with the same solvent. Dilute 5 ml of the solution to 50 ml with 2-propanol *R*.

Chloroethylamine hydrochloride. $\text{C}_2\text{H}_7\text{Cl}_2\text{N}$. (M_r 116.0). 1124300. [870-24-6]. 2-Chloroethanamine hydrochloride.

mp: about 145 °C.

(2-Chloroethyl)diethylamine hydrochloride. $\text{C}_6\text{H}_{15}\text{Cl}_2\text{N}$. (M_r 172.1). 1018500. [869-24-9].

A white, crystalline powder, very soluble in water and in methanol, freely soluble in methylene chloride, practically insoluble in hexane.

mp: about 211 °C.

Chloroform. CHCl_3 . (M_r 119.4). 1018600. [67-66-3]. Trichloromethane.

A clear, colourless liquid, slightly soluble in water, miscible with alcohol.

d_{20}^{20} : 1.475 to 1.481.

bp: about 60 °C.

Chloroform contains 0.4 per cent *m/m* to 1.0 per cent *m/m* of ethanol.

Ethanol. Introduce 1.00 g (*m g*) into a ground-glass-stoppered flask. Add 15.0 ml of *nitrochromic reagent R*, close the flask, shake vigorously for 2 min and allow to stand for 15 min. Add 100 ml of *water R* and 5 ml of a 200 g/l solution of *potassium iodide R*. After 2 min titrate with 0.1 *M sodium thiosulphate*, using 1 ml of *starch solution R* as indicator, until a light green colour is obtained (n_1 ml of 0.1 *M sodium thiosulphate*). Carry out a blank assay (n_2 ml of 0.1 *M sodium thiosulphate*). Calculate the percentage of ethanol using the expression:

$$\frac{(n_2 - n_1) 0.115}{m}$$

Chloroform, acidified. 1018601.

To 100 ml of *chloroform R* add 10 ml of *hydrochloric acid R*. Shake, allow to stand and separate the 2 layers.

Chloroform, ethanol-free. 1018602.

Shake 200 ml of *chloroform R* with four quantities, each of 100 ml, of *water R*. Dry over 20 g of *anhydrous sodium sulphate R* for 24 h. Distil the filtrate over 10 g of *anhydrous sodium sulphate R*. Discard the first 20 ml of distillate. Prepare immediately before use.

Chloroform stabilised with amylene. CHCl_3 . (M_r 119.4). 1018700.

A clear, colourless liquid, slightly soluble in water, miscible with alcohol.

Water: maximum 0.05 per cent.

Residue on evaporation: maximum 0.001 per cent.

Minimum transmittance (2.2.25), determined using *water R* as compensation liquid: 50 per cent at 255 nm, 80 per cent at 260 nm, 98 per cent at 300 nm.

Assay: minimum 99.8 per cent of CHCl_3 , determined by gas chromatography.

Chlorogenic acid. $\text{C}_{16}\text{H}_{18}\text{O}_9$. (M_r 354.3). 1104700. [327-97-9]. (1*S*,3*R*,4*R*,5*R*)-3-[(3,4-Dihydroxycinnamoyl)oxy]-1,4,5-trihydroxycyclohexanecarboxylic acid.

A white, crystalline powder or white needles, freely soluble in boiling water, in acetone and in ethanol.

$[\alpha]_D^{26}$: about -35.2.

mp: about 208 °C.

Chromatography. Examined under the conditions described on Identification A in the monograph on *Belladonna leaf dry extract, standardised* (1294), the chromatogram shows only one principal zone.

3-Chloro-2-methylaniline. $\text{C}_7\text{H}_8\text{ClN}$. (M_r 141.6). 1139400. [87-60-5]. 6-Chloro-2-toluidine.

Not miscible with water, slightly soluble in ethanol.

d_{20}^{20} : : about 1.171.

n_D^{20} : : about 1.587.

bp: about 115 °C.

mp: about 2 °C.

2-Chloro-4-nitroaniline. $\text{C}_6\text{H}_5\text{ClN}_2\text{O}_2$. (M_r 172.6). 1018800. [121-87-9].

A yellow, crystalline powder, freely soluble in methanol.

mp: about 107 °C.

Storage: protected from light.

Chlorophenol. C_6H_5ClO . (M_r 128.6). 1018900. [106-48-9]. 4-Chlorophenol.

Colourless or almost colourless crystals, slightly soluble in water, very soluble in alcohol and in solutions of alkali hydroxides.

mp: about 42 °C.

Chloroplatinic acid. $H_2Cl_6Pt_6 \cdot 6H_2O$. (M_r 517.9). 1019000. [18497-13-7]. Hydrogen hexachloroplatinate(IV) hexahydrate.

Content: minimum 37.0 per cent *m/m* of platinum (A_r 195.1).

Brownish-red crystals or a crystalline mass, very soluble in water, soluble in alcohol.

Assay. Ignite 0.200 g to constant mass at 900 °C and weigh the residue (platinum).

Storage: protected from light.

3-Chloropropane-1,2-diol. $C_3H_7ClO_2$. (M_r 110.5). 1097600. [96-24-2].

Colourless liquid, soluble in water and alcohol.

d_{20}^{20} : about 1.322.

n_D^{20} : about 1.480

bp: about 213 °C.

5-Chloroquinolin-8-ol. C_9H_6ClNO . (M_r 179.6). 1156900. [130-16-5]. 5-Chlorooxine.

Sparingly soluble in cold dilute hydrochloric acid.

mp: about 123 °C.

Content: minimum 95.0 per cent of C_9H_6ClNO .

5-Chlorosalicylic acid. $C_7H_5ClO_3$. (M_r 172.6). 1019100. [321-14-2].

A white or almost white, crystalline powder, soluble in methanol.

mp: about 173 °C.

Chlorothiazide. 1112100. [58-94-6].

See *Chlorothiazide* (0385).

Chlorotrimethylsilane. C_3H_9ClSi . (M_r 108.6). 1019300. [75-77-4].

A clear, colourless liquid, fuming in air.

d_{20}^{20} : about 0.86.

n_D^{20} : about 1.388.

bp: about 57 °C.

Chlorpyrifos. $C_9H_{11}Cl_3NO_3PS$. (M_r 350.6). 1124400. [2921-88-2].

bp: about 200 °C.

mp: 42 °C to 44 °C.

A suitable certified reference solution (10 ng/μl in cyclohexane) may be used.

Chlorpyrifos-methyl. $C_7H_7Cl_3NO_3PS$. (M_r 322.5). 1124500. [5598-13-0].

mp: 45 °C to 47 °C.

A suitable certified reference solution (10 ng/μl in cyclohexane) may be used.

Chlortetracycline hydrochloride. 1145500.

See *Chlortetracycline hydrochloride* (0173).

Cholesterol. 1019400. [57-88-5].

See *Cholesterol* (0993).

Choline chloride. $C_5H_{14}ClNO$. (M_r 139.6). 1019500.

[67-48-1]. (2-Hydroxyethyl)trimethylammonium chloride.

Deliquescent crystals, very soluble in water and in alcohol.

Chromatography. Examine as prescribed in the monograph *Suxamethonium chloride* (0248), applying 5 μl of a 0.2 g/l solution in *methanol R*. The chromatogram shows one principal spot.

Storage: in an airtight container.

Chromazurol S. $C_{23}H_{13}Cl_2Na_3O_9S$. (M_r 605). 1019600. [1667-99-8].

Schultz No. 841.

Colour Index No. 43825.

Trisodium 5-[(3-carboxylato-5-methyl-4-oxocyclohexa-2,5-dien-1-ylidene)(2,6-dichloro-3-sulphonatophenyl)methyl]-2-hydroxy-3-methylbenzoate.

A brownish-black powder, soluble in water, slightly soluble in alcohol.

Chromic acid cleansing mixture. 1019700.

A saturated solution of *chromium trioxide R* in *sulphuric acid R*.

Chromic potassium sulphate. $CrK(SO_4)_2 \cdot 12H_2O$. (M_r 499.4). 1019800. [7788-99-0]. Chrome alum.

Large, violet-red to black crystals, freely soluble in water, practically insoluble in alcohol.

Chromium(III) trichloride hexahydrate. $[Cr(H_2O)_4Cl_2]Cl \cdot 2H_2O$. (M_r 266.5). 1104800. [10060-12-5].

A dark green crystalline powder, hygroscopic.

Storage: protected from humidity and oxidising agents.

Chromium trioxide. CrO_3 . (M_r 100.0). 1019900. [1333-82-0].

Dark brownish-red needles or granules, deliquescent, very soluble in water.

Storage: in an airtight glass container.

Chromophore substrate R1. 1020000.

Dissolve *N*-α-benzyloxycarbonyl-D-arginyl-L-glycyl-L-arginine-4-nitroanilide dihydrochloride in *water R* to give a 0.003 M solution. Dilute in *tris(hydroxymethyl)aminomethane-EDTA buffer solution pH 8.4 R* to 0.0005 M before use.

Chromophore substrate R2. 1020100.

Dissolve D-phenylalanyl-L-pipecolyl-L-arginine-4-nitroanilide dihydrochloride in *water R* to give a 0.003 M solution. Dilute before use in titrating in *tris(hydroxymethyl)aminomethane-EDTA buffer solution pH 8.4 R* to give a 0.0005 M solution.

Chromophore substrate R3. 1149100.

Dissolve D-valyl-leucyl-lysyl-4-nitroanilide dihydrochloride in *water R* to give a 0.003 M solution.

Chromotrope II B. $C_{16}H_9N_3Na_2O_{10}S_2$. (M_r 513.4). 1020200. [548-80-1].

Schultz No. 67.

Colour Index No. 16575.

Disodium 4,5-dihydroxy-3-(4-nitrophenylazo)naphthalene-2,7-disulphonate.

A reddish-brown powder, soluble in water giving a yellowish-red colour, practically insoluble in alcohol.

Chromotrope II B solution. 1020201.

A 0.05 g/l solution in *sulphuric acid R*.

Chromotropic acid, sodium salt. $C_{10}H_6Na_2O_8S_2 \cdot 2H_2O$. (M_r 400.3). 1020300. [5808-22-0].

Schultz No. 1136.

Disodium 4,5-dihydroxynaphthalene-2,7-disulphonate dihydrate. Disodium 1,8-dihydroxynaphthalene-3,6-disulphonate dihydrate.

A yellowish-white powder, soluble in water, practically insoluble in alcohol.

Chromotropic acid, sodium salt solution. 1020301.

Dissolve 0.60 g of *chromotropic acid, sodium salt R* in about 80 ml of *water R* and dilute to 100 ml with the same solvent. Use this solution within 24 h.

Chrysanthemin. $C_{21}H_{21}ClO_{11}$. (M_r 485.5). 1134800. [7084-24-4]. Kuromanin chloride. 2-(3,4-Dihydroxyphenyl)-3-(β -D-glucopyranosyl)oxy-5,7-dihydroxy-1-benzopyrylium chloride.

A reddish-brown crystalline powder, soluble in water and in alcohol.

Absorbance (2.2.25). A 0.01 g/l solution in a mixture of 1 volume of *hydrochloric acid R* and 999 volumes of *methanol R* shows a maximum at 528 nm.

α -Chymotrypsin for peptide mapping. 1142400.

α -Chymotrypsin of high purity, treated to eliminate tryptic activity.

Cinchonidine. $C_{19}H_{22}N_2O$. (M_r 294.4). 1020400. [485-71-2]. (*R*)-(Quinol-4-yl)[(2*S*,4*S*,5*R*)-5-vinylquinuclidin-2-yl]methanol.

A white, crystalline powder, very slightly soluble in water and in light petroleum, soluble in alcohol.

$[\alpha]_D^{20}$: -105 to -110, determined on a 50 g/l solution in *alcohol R*.

mp: about 208 °C, with decomposition.

Storage: protected from light.

Cinchonine. $C_{19}H_{22}N_2O$. (M_r 294.4). 1020500. [118-10-5]. (*S*)-(Quinol-4-yl)[(2*R*,4*S*,5*R*)-5-vinylquinuclidin-2-yl]methanol.

A white, crystalline powder, very slightly soluble in water, sparingly soluble in alcohol and in methanol.

$[\alpha]_D^{20}$: +225 to +230, determined on a 50 g/l solution in *alcohol R*.

mp: about 263 °C.

Storage: protected from light.

Cineole. $C_{10}H_{18}O$. (M_r 154.3). 1020600. [470-82-6].

1,8-Cineole. Eucalyptol. 1,8-Epoxy-*p*-menthane.

A colourless liquid, practically insoluble in water, miscible with ethanol.

d_{20}^{20} : 0.922 to 0.927.

n_D^{20} : 1.456 to 1.459.

Freezing point (2.2.18): 0 °C to 1 °C.

Distillation range (2.2.11): 174 °C to 177 °C.

Phenol. Shake 1 g with 20 ml of *water R*. Allow to separate and add to 10 ml of the aqueous layer 0.1 ml of *ferric chloride solution R1*. No violet colour develops.

Turpentine oil. Dissolve 1 g in 5 ml of *alcohol (90 per cent V/V) R*. Add dropwise freshly prepared *bromine water R*. Not more than 0.5 ml is required to give a yellow colour lasting for 30 min.

Residue on evaporation: maximum 0.05 per cent. To 10.0 ml add 25 ml of *water R*, evaporate on a water-bath and dry the residue to constant mass at 100-105 °C.

Cineole used in gas chromatography complies with the following additional test.

Assay. Examine by gas chromatography (2.2.28) as prescribed in the monograph on *Peppermint oil (0405)* using the substance to be examined as the test solution.

The area of the principal peak is not less than 98.0 per cent of the total area of the peaks.

1,4-Cineole. $C_{10}H_{18}O$. (M_r 154.3). 1142500. [470-67-7].

1-Methyl-4-(1-methylethyl)-7-oxabicyclo[2.2.1]heptane.

1-Isopropyl-4-methyl-7-oxabicyclo[2.2.1]heptane.

A colourless liquid.

d_4^{20} : about 0.900.

n_D^{20} : about 1.445.

bp: about 173 °C.

Cinnamamide. C_9H_9NO . (M_r 147.2). 1154800. [621-79-4].

(*E*)-3-Phenylprop-2-enamide.

White or almost white powder.

mp: about 149 °C.

Cinnamic aldehyde. C_9H_8O . (M_r 132.2). 1020700. [104-55-2].

3-Phenylpropenal.

A yellowish to greenish-yellow, oily liquid, slightly soluble in water, very soluble in alcohol.

d_{20}^{20} : 1.048 to 1.051.

n_D^{20} : about 1.620.

Storage: protected from light.

trans-Cinnamic aldehyde. C_9H_8O . (M_r 132.2). 1124600.

[14371-10-9]. (*E*)-3-Phenylprop-2-enal.

trans-Cinnamic aldehyde used in gas chromatography complies with the following additional test.

Assay. Examine by gas chromatography (2.2.28) as prescribed in the monograph on *Cassia oil (1496)*.

The content is not less than 99.0 per cent, calculated by the normalisation procedure.

Cinnamyl acetate. $C_{11}H_{12}O_2$. (M_r 176.2). 1124700.

[103-54-8]. 3-Phenylprop-2-en-1-yl acetate.

n_D^{20} : about 1.542.

bp: about 262 °C.

Cinnamyl acetate used in gas chromatography complies with the following additional test.

Assay. Examine by gas chromatography (2.2.28) as prescribed in the monograph on *Cassia oil (1496)*.

The content is not less than 99.0 per cent, calculated by the normalisation procedure.

Citral. $C_{10}H_{16}O$. (M_r 152.2). 1020800. [5392-40-5]. Mixture of (2*E*)- and (2*Z*)-3,7-Dimethylocta-2,6-dienal.

A light yellow liquid, practically insoluble in water, miscible with alcohol and with glycerol.

Chromatography. Examine by thin-layer chromatography (2.2.27), using *silica gel GF₂₅₄ R* as the coating substance. Apply to the plate 10 μ l of a 1 g/l solution in *toluene R*. Develop over a path of 15 cm using a mixture of 15 volumes of *ethyl acetate R* and 85 volumes of *toluene R*. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. The chromatogram obtained shows only one principal spot.

Citral used in gas chromatography complies with the following additional test.

Assay. Examine by gas chromatography (2.2.28) as prescribed in the monograph on *Citronella oil (1609)*.

The content of citral (neral + geranial) is not less than 95.0 per cent calculated by the normalisation procedure.

Citrated rabbit plasma. 1020900.

Collect blood by intracardiac puncture from a rabbit kept fasting for 12 h, using a plastic syringe with a No. 1 needle containing a suitable volume of 38 g/l solution of *sodium citrate R* so that the final volume ratio of citrate solution to blood is 1: 9. Separate the plasma by centrifugation at 1500 g to 1800 g at 15 °C to 20 °C for 30 min.

Storage: at 0 °C to 6 °C; use within 4 h of collection.

Citric acid. 1021000. [5949-29-1]. See *Citric acid monohydrate* (0456).

When used in the limit test for iron, it complies with the following additional requirement.

Dissolve 0.5 g in 10 ml of *water R*, add 0.1 ml of *thioglycollic acid R*, mix and make alkaline with *ammonia R*. Dilute to 20 ml with *water R*. No pink colour appears in the solution.

Citric acid, anhydrous. 1021200. [77-92-9].

See *Anhydrous citric acid* (0455).

Citronellal. C₁₀H₁₈O. (*M_r* 154.3). 1113300. [106-23-0]. 3,7-Dimethyl-6-octenal.

Very slightly soluble in water, soluble in alcohols.

*d*₂₀²⁰: 0.848 to 0.856.

*n*_D²⁰: about 1.446.

[α]_D²⁵: about + 11.50.

Citronellal used in gas chromatography complies with the following additional test.

Assay. Examine by gas chromatography (2.2.28) as prescribed in the monograph on *Citronella oil* (1609).

The content is not less than 95.0 per cent calculated by the normalisation procedure.

Citronellol. C₁₀H₂₀O. (*M_r* 156.3). 1134900. [106-22-9]. 3,7-Dimethyloct-6-en-1-ol.

A clear, colourless liquid, practically insoluble in water, miscible with alcohol.

*d*₂₀²⁰: 0.857.

*n*_D²⁰: 1.456.

bp: 220 °C to 222 °C.

Citronellol used in gas chromatography complies with the following additional test.

Assay. Examine by gas chromatography (2.2.28) as prescribed in the monograph on *Citronella oil* (1609).

The content is not less than 95.0 per cent calculated by the normalisation procedure.

Storage: in an airtight container, protected from light.

Citronellyl acetate. C₁₂H₂₂O₂. (*M_r* 198.3). 1135000. [150-84-5]. 3,7-Dimethyl-6-octen-1-yl acetate.

*d*₂₀²⁰: 0.890.

*n*_D²⁰: 1.443.

bp: 229 °C.

Citronellyl acetate used in gas chromatography complies with the following additional test.

Assay. Examine by gas chromatography (2.2.28) as prescribed in the monograph on *Citronella oil* (1609).

The content is not less than 97.0 per cent calculated by the normalisation procedure.

Storage: in an airtight container, protected from light.

Citropten. C₁₁H₁₀O₄. (*M_r* 206.2). 1021300. [487-06-9]. Limettin. 5,7-Dimethoxy-2*H*-1-benzopyran-2-one.

Needle-shaped crystals, practically insoluble in water and in light petroleum, freely soluble in acetone and in alcohol.

mp: about 145 °C.

Chromatography. Examine by thin-layer chromatography (2.2.27), using *silica gel GF₂₅₄ R* as the coating substance. Apply to the plate 10 µl of a 1 g/l solution in *toluene R*. Develop over a path of 15 cm using a mixture of 15 volumes of *ethyl acetate R* and 85 volumes of *toluene R*. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. The chromatogram obtained shows only one principal spot.

Clobetasol propionate. C₂₅H₃₂ClFO₅. (*M_r* 467.0). 1097700. [25122-46-7]. 21-Chloro-9-fluoro-11β,17-dihydroxy-16β-methylpregna-1,4-diene-3,20-dione 17-propionate.

A white crystalline powder, insoluble in water, soluble in alcohol and in acetone.

[α]_D²⁰: about + 104 (in dioxan).

mp: about 196 °C.

Coagulation factor V solution. 1021400.

Coagulation factor V solution may be prepared by the following method or by any other method which excludes factor VIII.

Prepare the factor V reagent from fresh oxalated bovine plasma, by fractionation at 4 °C with a saturated solution of *ammonium sulphate R* prepared at 4 °C. Separate the fraction which precipitates between 38 per cent and 50 per cent of saturation, which contains factor V without significant contamination with factor VIII. Remove the ammonium sulphate by dialysis and dilute the solution with a 9 g/l solution of *sodium chloride R* to give a solution containing between 10 per cent and 20 per cent of the quantity of factor V present in fresh human normal plasma.

Determination of factor V content. Prepare two dilutions of the preparation of factor V in *imidazole buffer solution pH 7.3 R* containing 1 volume of the preparation in 10 volumes and in 20 volumes of the buffer solution respectively. Test each dilution as follows: mix 0.1 ml of *plasma substrate deficient in factor V R*, 0.1 ml of the solution to be examined, 0.1 ml of *thromboplastin R* and 0.1 ml of a 3.5 g/l solution of *calcium chloride R* and measure the coagulation times, i.e. the interval between the moment at which the calcium chloride solution is added and the first indication of the formation of fibrin, which may be observed visually or by means of a suitable apparatus.

In the same manner, determine the coagulation time (in duplicate) of four dilutions of human normal plasma in *imidazole buffer solution pH 7.3 R*, containing respectively, 1 volume in 10 (equivalent to 100 per cent of factor V), 1 volume in 50 (20 per cent), 1 volume in 100 (10 per cent), and 1 volume in 1000 (1 per cent). Using two-way logarithmic paper plot the average coagulation times for each dilution of human plasma against the equivalent percentage of factor V and read the percentage of factor V for the two dilutions of the factor V solution by interpolation. The mean of the two results gives the percentage of factor V in the solution to be examined.

Storage: in the frozen state at a temperature not higher than –20 °C.

Cobalt chloride. CoCl₂·6H₂O. (*M_r* 237.9). 1021600. [7791-13-1].

A red, crystalline powder or deep-red crystals, very soluble in water, soluble in alcohol.

Cobalt nitrate. $\text{Co}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$. (M_r 291.0). 1021700. [10026-22-9].

Small garnet-red crystals, very soluble in water.

Codeine. 1021800. [6059-47-8].

See *Codeine* (0076).

Codeine phosphate. 1021900. [52-28-8].

See *Codeine phosphate hemihydrate* (0074).

Congo red. $\text{C}_{32}\text{H}_{22}\text{N}_6\text{Na}_2\text{O}_6\text{S}_2$. (M_r 697). 1022000. [573-58-0]. Schultz No. 360.

Colour Index No. 22120.

Disodium (biphenyl-4,4'-diyl-bis-2,2'-azo)bis(1-aminonaphthalene-4-sulphonate).

A brownish-red powder, soluble in water.

Congo red paper. 1022002.

Immerse strips of filter paper for a few minutes in *congo red solution R*. Allow to dry.

Congo red solution. 1022001.

Dissolve 0.1 g of *congo red R* in a mixture of 20 ml of *alcohol R* and *water R* and dilute to 100 ml with *water R*.

Test for sensitivity. To 0.2 ml of the *congo red solution* add 100 ml of *carbon dioxide-free water R* and 0.3 ml of 0.1 M *hydrochloric acid*. The solution is blue. Not more than 0.3 ml of 0.1 M *sodium hydroxide* is required to change the colour to pink.

Colour change: pH 3.0 (blue) to pH 5.0 (pink).

Coomassie blue. 1001400. [3861-73-2].

See *acid blue 92 R*.

Coomassie blue solution. 1001401.

See *acid blue 92 solution R*.

Coomassie staining solution. 1012201.

A 1.25 g/l solution of *acid blue 83 R* in a mixture consisting of 1 volume of *glacial acetic acid R*, 4 volumes of *methanol R* and 5 volumes of *water R*. Filter.

Copper. Cu. (A_r 63.55). 1022100. [7440-50-8].

Cleaned foil, turnings, wire or powder of the pure metal of electrolytic grade.

Copper acetate. $\text{C}_4\text{H}_6\text{CuO}_4 \cdot \text{H}_2\text{O}$. (M_r 199.7). 1022200. [142-71-2].

Blue-green crystals or powder, freely soluble in boiling water, soluble in water and in alcohol, slightly soluble in glycerol (85 per cent).

Copper edetate solution. 1022300.

To 2 ml of a 20 g/l solution of *copper acetate R* add 2 ml of 0.1 M *sodium edetate* and dilute to 50 ml with *water R*.

Copper nitrate. $\text{Cu}(\text{NO}_3)_2 \cdot 3\text{H}_2\text{O}$. (M_r 241.6). 1022400. [10031-43-3]. Chloride dinitrate trihydrate.

Dark blue crystals, hygroscopic, very soluble in water giving a strongly acid reaction, freely soluble in alcohol and in dilute nitric acid.

Storage: in an airtight container.

Copper sulphate. $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$. (M_r 249.7). 1022500. [7758-99-8].

A blue powder or deep-blue crystals, slowly efflorescent, very soluble in water, slightly soluble in alcohol.

Copper sulphate solution. 1022501.

A 125 g/l solution.

Copper tetrammine, ammoniacal solution of. 1022600.

Dissolve 34.5 g of *copper sulphate R* in 100 ml of *water R* and, whilst stirring, add dropwise *concentrated ammonia R* until the precipitate which forms dissolves completely. Keeping the temperature below 20 °C, add dropwise with continuous shaking 30 ml of *strong sodium hydroxide solution R*. Filter through a sintered-glass filter (40), wash with *water R* until the filtrate is clear and take up the precipitate with 200 ml of *concentrated ammonia R*. Filter through a sintered-glass filter and repeat the filtration to reduce the residue to a minimum.

Cortisone acetate. 1097800. [50-04-4].

See *Cortisone acetate* (0321).

Coumaphos. $\text{C}_{14}\text{H}_{16}\text{ClO}_5\text{PS}$. (M_r 362.8). 1124800. [56-72-4]. mp: 91 °C to 92 °C.

A suitable certified reference solution (10 ng/μl in iso-octane) may be used.

Coumarin. $\text{C}_9\text{H}_6\text{O}_2$. (M_r 146.1). 1124900. [91-64-5]. 2H-Chromen-2-one. 2H-1-Benzopyran-2-one.

A colourless, crystalline powder or orthorhombic or rectangular crystals, very soluble in boiling water, soluble in alcohol. It dissolves in solutions of alkali hydroxides.

mp: 68 °C to 70 °C.

Coumarin used in gas chromatography complies with the following additional test.

Assay. Examine by gas chromatography (2.2.28) as prescribed in the monograph on *Cassia oil* (1496).

The content is not less than 98.0 per cent, calculated by the normalisation procedure.

Cresol. $\text{C}_7\text{H}_8\text{O}$. (M_r 108.1). 1022700. [95-48-7]. *o*-Cresol. 2-Methylphenol.

Crystals or a super-cooled liquid becoming dark on exposure to light and air, miscible with ethanol, soluble in about 50 parts of water and soluble in solutions of alkali hydroxides.

d_{20}^{20} : about 1.05.

n_D^{20} : 1.540 to 1.550.

bp: about 190 °C.

Freezing point (2.2.18): minimum 30.5 °C.

Residue on evaporation: maximum 0.1 per cent *m/m*, determined by evaporating on a water-bath and drying in an oven at 100-105 °C.

Storage: protected from light, moisture and oxygen.

Distil before use.

***p*-Cresol.** $\text{C}_7\text{H}_8\text{O}$. (M_r 108.1). 1153100. [106-44-5]. 4-Methylphenol.

Colourless or white crystals or crystalline mass.

d_{20}^{20} : about 1.02.

bp: about 202 °C.

***m*-Cresol purple.** $\text{C}_{21}\text{H}_{18}\text{O}_5\text{S}$. (M_r 382.44). 1121700. [2303-01-7]. *m*-Cresolsulphonphthalein.

An olive-green, crystalline powder, slightly soluble in water, soluble in alcohol, in glacial acetic acid and in methanol.

***m*-Cresol purple solution.** 1121701.

Dissolve 0.1 g of *m-cresol purple R* in 13 ml of 0.01 M *sodium hydroxide*, dilute to 100 ml with *water R* and mix.

Colour change: pH 1.2 (red) to pH 2.8 (yellow); pH 7.4 (yellow) to pH 9.0 (purple).

Cresol red. $C_{21}H_{18}O_5S$. (M_r 382.4). 1022800. [1733-12-6]. Cresolsulfonphthalein. 4,4'-(3*H*-2,1-Benzoxathiol-3-ylidene)bis-(2-methylphenol) *S,S*-dioxide.

A reddish-brown crystalline powder, slightly soluble in water, soluble in alcohol and in dilute solutions of alkali hydroxides.

Cresol red solution. 1022801.

Dissolve 0.1 g of *cresol red R* in a mixture of 2.65 ml of 0.1 *M* sodium hydroxide and 20 ml of *alcohol R* and dilute to 100 ml with *water R*.

Test for sensitivity. A mixture of 0.1 ml of the *cresol red* solution and 100 ml of *carbon dioxide-free water R* to which 0.15 ml of 0.02 *M* sodium hydroxide has been added is purple-red. Not more than 0.15 ml of 0.02 *M* hydrochloric acid is required to change the colour to yellow.

Colour change: pH 7.0 (yellow) to pH 8.6 (red).

Crystal violet. $C_{25}H_{30}ClN_3$. (M_r 408.0). 1022900. [548-62-9]. Schultz No. 78.

Colour Index No. 42555.

Hexamethyl-pararosaniminium chloride.

Dark-green powder or crystals, soluble in water and in alcohol.

Crystal violet solution. 1022901.

Dissolve 0.5 g of *crystal violet R* in *anhydrous acetic acid R* and dilute to 100 ml with the same solvent.

Test for sensitivity. To 50 ml of *anhydrous acetic acid R* add 0.1 ml of the *crystal violet* solution. On addition of 0.1 ml of 0.1 *M* perchloric acid the bluish-purple solution turns bluish-green.

Cupric chloride. $CuCl_2 \cdot 2H_2O$. (M_r 170.5). 1023000. [10125-13-0]. Cupric chloride dihydrate.

Greenish-blue powder or crystals, deliquescent in moist air, efflorescent in dry air, freely soluble in water, in alcohol and in methanol, sparingly soluble in acetone.

Storage: in an airtight container.

Cupri-citric solution. 1023100.

Dissolve 25 g of *copper sulphate R*, 50 g of *citric acid R* and 144 g of *anhydrous sodium carbonate R* in *water R* and dilute to 1000 ml with the same solvent.

Cupri-citric solution R1. 1023200.

Dissolve 25 g of *copper sulphate R*, 50 g of *citric acid R* and 144 g of *anhydrous sodium carbonate R* in *water R* and dilute to 1000 ml with the same solvent.

Adjust the solution so that it complies with the following requirements.

a) To 25.0 ml add 3 g of *potassium iodide R*. Add 25 ml of a 25 per cent *m/m* solution of *sulphuric acid R* with precaution and in small quantities. Titrate with 0.1 *M* sodium thiosulphate using 0.5 ml of *starch solution R*, added towards the end of the titration, as indicator.

24.5 ml to 25.5 ml of 0.1 *M* sodium thiosulphate is used in the titration.

b) Dilute 10.0 ml to 100.0 ml with *water R* and mix. To 10.0 ml of the solution, add 25.0 ml of 0.1 *M* hydrochloric acid and heat for 1 h on a water-bath. Cool, adjust with *water R* to the initial volume and titrate with 0.1 *M* sodium hydroxide, using 0.1 ml of *phenolphthalein solution R1* as indicator.

5.7 ml to 6.3 ml of 0.1 *M* sodium hydroxide is used in the titration.

c) Dilute 10.0 ml to 100.0 ml with *water R* and mix. Titrate 10.0 ml of the solution with 0.1 *M* hydrochloric acid, using 0.1 ml of *phenolphthalein solution R1* as indicator.

6.0 ml to 7.5 ml of 0.1 *M* hydrochloric acid is used in the titration.

Cupriethylenediamine hydroxide solution. 3008700. [14552-35-3].

The molar ratio of ethylenediamine to copper is 2.00 ± 0.04 .

This solution is commercially available.

Cupri-tartaric solution. 1023300.

Solution I. Dissolve 34.6 g of *copper sulphate R* in *water R* and dilute to 500 ml with the same solvent.

Solution II. Dissolve 173 g of *sodium potassium tartrate R* and 50 g of *sodium hydroxide R* in 400 ml of *water R*. Heat to boiling, allow to cool and dilute to 500 ml with *carbon dioxide-free water R*.

Mix equal volumes of the 2 solutions immediately before use.

Cupri-tartaric solution R2. 1023302.

Add 1 ml of a solution containing 5 g/l of *copper sulphate R* and 10 g/l of *potassium tartrate R* to 50 ml of *sodium carbonate solution R1*. Prepare immediately before use.

Cupri-tartaric solution R3. 1023303.

Prepare a solution containing 10 g/l of *copper sulphate R* and 20 g/l of *sodium tartrate R*. To 1.0 ml of the solution add 50 ml of *sodium carbonate solution R2*. Prepare immediately before use.

Cupri-tartaric solution R4. 1023304.

Solution I. 150 g/l *copper sulphate R*.

Solution II. Dissolve 2.5 g of *anhydrous sodium carbonate R*, 2.5 g of *potassium sodium tartrate R*, 2.0 g of *sodium hydrogen carbonate R*, and 20.0 g of *anhydrous sodium sulphate R* in *water R* and dilute to 100 ml with the same solvent.

Mix 1 part of solution I with 25 parts of solution II immediately before use.

Curcumin. $C_{21}H_{20}O_6$. (M_r 368.4). 1023500. [458-37-7]. 1,7-bis(4-Hydroxy-3-methoxyphenyl)hepta-1,6-diene-3,5-dione.

An orange-brown, crystalline powder, practically insoluble in water, soluble in glacial acetic acid.

mp: about 183 °C.

Cyanoacetic acid. $C_3H_3NO_2$. (M_r 85.1). 1097900. [372-09-8].

White to yellowish-white, hygroscopic crystals, very soluble in water.

Storage: in an airtight container.

Cyanocobalamin. 1023600. [68-19-9].

See *Cyanocobalamin* (0547).

Cyanogen bromide solution. 1023700. [506-68-3].

Add dropwise, with cooling 0.1 *M* ammonium thiocyanate to *bromine water R* until the yellow colour disappears. Prepare immediately before use.

β-Cyclodextrin for chiral chromatography, modified. 1154600.

30 per cent of 2,3-di-*O*-ethyl-6-*O*-*tert*-butyldimethylsilyl-β-cyclodextrin dissolved in *poly(dimethyl)(85)(diphenyl)(15)siloxane R*.

Cyanoguanidine. $C_2H_4N_4$. (M_r 84.1). 1023800. [461-58-5]. Dicyandiamide. 1-Cyanoguanidine.

A white, crystalline powder, sparingly soluble in water and in alcohol, practically insoluble in methylene chloride.
mp: about 210 °C.

Cyclohexane. C_6H_{12} . (M_r 84.2). 1023900. [110-82-7].

A clear, colourless, flammable liquid, practically insoluble in water, miscible with organic solvents.

d_{20}^{20} : about 0.78.

bp: about 80.5 °C.

Cyclohexane used in spectrophotometry complies with the following additional requirements.

Minimum transmittance (2.2.25), determined using *water R* as compensation liquid: 45 per cent at 220 nm, 70 per cent at 235 nm, 90 per cent at 240 nm, 98 per cent at 250 nm.

Cyclohexane R1. 1023901.

Complies with the requirements prescribed for *cyclohexane R* and with the following additional requirement.

The fluorescence, measured at 460 nm, under illumination with an excitant light beam at 365 nm, is not more intense than that of a solution containing 0.002 ppm of *quinine R* in 0.05 M sulphuric acid.

Cyclohexylamine. $C_6H_{13}N$. (M_r 99.2). 1024000. [108-91-8].

A colourless liquid, soluble in water, miscible with usual organic solvents.

n_D^{20} : about 1.460.

bp: 134 °C to 135 °C.

Cyclohexylenedinitrilotetra-acetic acid. $C_{14}H_{22}N_2O_8 \cdot H_2O$. (M_r 364.4). 1024100. *trans*-Cyclohexylene-1,2-dinitrilo-*N,N,N',N'*-tetra-acetic acid.

A white, crystalline powder.

mp: about 204 °C.

Cyclohexylmethanol. $C_7H_{14}O$. (M_r 114.2). 1135200.

[100-49-2]. Cyclohexylcarbinol.

A liquid with a slight odour of camphor, soluble in alcohol.

n_D^{25} : about 1.464.

bp: about 185 °C.

3-Cyclohexylpropionic acid. $C_9H_{16}O_2$. (M_r 156.2). 1119200. [701-97-3].

A clear liquid.

d_{20}^{20} : about 0.998.

n_D^{20} : about 1.4648.

bp: about 130 °C.

Cyhalothrin. $C_{23}H_{19}ClF_3NO_3$. (M_r 449.9). 1125000. [91465-08-6].

bp: 187 °C to 190 °C.

mp: about 49 °C.

A suitable certified reference solution (10 ng/μl in cyclohexane) may be used.

p-Cymene. $C_{10}H_{14}$. (M_r 134.2). 1113400. [99-87-6]. 1-Isopropyl-4-methylbenzene.

A colourless liquid, practically insoluble in water, soluble in alcohol.

d_{20}^{20} : about 0.858.

n_D^{20} : about 1.4895.

bp: 175 °C to 178 °C.

p-Cymene used in gas chromatography complies with the following additional test.

Assay. Examine by gas chromatography (2.2.28) as prescribed in the monograph *Peppermint oil* (0405).

Test solution. The substance to be examined.

The area of the principal peak is not less than 96.0 per cent of the area of all the peaks in the chromatogram obtained.

Cypermethrin. $C_{22}H_{19}Cl_2NO_3$. (M_r 416.3). 1125100. [52315-07-8].

bp: 170 °C to 195 °C.

mp: 60 °C to 80 °C.

A suitable certified reference solution (10 ng/μl in cyclohexane) may be used.

L-Cysteine. $C_3H_7NO_2S$. (M_r 121.1). 1024200. [52-90-4].

A powder, freely soluble in water, in alcohol and in acetic acid, practically insoluble in acetone.

Cysteine hydrochloride. 1024300. [7048-04-6].

See *Cysteine hydrochloride monohydrate* (0895).

L-Cystine. $C_6H_{12}N_2O_4S_2$. (M_r 240.3). 1024400. [56-89-3].

A white, crystalline powder, practically insoluble in water and in alcohol. It dissolves in dilute solutions of alkali hydroxides. It decomposes at 250 °C.

$[\alpha]_D^{20}$: -218 to -224, determined in 1 M hydrochloric acid.

Dantron. $C_{14}H_8O_4$. (M_r 240.2). 1024500. [117-10-2].

1,8-Dihydroxyanthraquinone. 1,8-Dihydroxyanthracene-9,10-dione.

A crystalline orange powder, practically insoluble in water, slightly soluble in alcohol, soluble in solutions of alkali hydroxides.

mp: about 195 °C.

Dantron used in the sesquiterpenic acids assay in Valerian root (0453) complies with the following additional requirements.

$A_{1\text{ cm}}^{1\%}$: 355 to 375, determined at 500 nm in 1 M potassium hydroxide.

Assay. Examine by liquid chromatography (2.2.29) as prescribed in the monograph on *Valerian Root* (0453) at the concentration of the reference solution. The content of dantron is not less than 95 per cent calculated by the normalisation procedure

o,p'-DDD. $C_{14}H_{10}Cl_4$. (M_r 320.0). 1125200. [53-19-0].

1-(2-Chlorophenyl)-1-(4-chlorophenyl)-2,2-dichloroethane.

A suitable certified reference solution (10 ng/μl in cyclohexane) may be used.

p,p'-DDD. $C_{14}H_{10}Cl_4$. (M_r 320.0). 1125300. [72-54-8].

1,1-bis(4-Chlorophenyl)-2,2-dichloroethane.

bp: about 193 °C.

mp: about 109 °C.

A suitable certified reference solution (10 ng/μl in cyclohexane) may be used.

o,p'-DDE. $C_{14}H_8Cl_4$. (M_r 318.0). 1125400. [3424-82-6].

1-(2-Chlorophenyl)-1-(4-chlorophenyl)-2,2-dichloroethylene.

A suitable certified reference solution (10 ng/μl in cyclohexane) may be used.

p,p'-DDE. $C_{14}H_8Cl_4$. (M_r 318.0). 1125500. [72-55-9].

1,1-bis(4-Chlorophenyl)-2,2-dichloroethylene.

bp: 316 °C to 317 °C.

mp: 88 °C to 89 °C.

A suitable certified reference solution (10 ng/μl in cyclohexane) may be used.

***o,p'*-DDT.** C₁₄H₉Cl₅. (*M*_r 354.5). 1125600. [789-02-6]. 1-(2-Chlorophenyl)-1-(4-chlorophenyl)-2,2,2-trichloroethane. A suitable certified reference solution (10 ng/μl in cyclohexane) may be used.

***p,p'*-DDT.** C₁₄H₉Cl₅. (*M*_r 354.5). 1125700. [50-29-3]. 1,1-bis(4-Chlorophenyl)-2,2,2-trichloroethane. bp: about 260 °C. mp: 108 °C to 109 °C.

A suitable certified reference solution (10 ng/μl in cyclohexane) may be used.

Decanal. C₁₀H₂₀O. (*M*_r 156.3). 1149200. [112-31-2]. Decyl aldehyde. Oily, colourless liquid, with a characteristic odour of orange, practically insoluble in water, soluble in chloroform. *d*₄²⁰: 0.825 to 0.829. *n*_D²⁰: 1.420 to 1.430. bp: 207 °C to 209 °C.

Decanal used in gas chromatography complies with the following additional test.

Assay. Examine by gas chromatography (2.2.28) as prescribed in the monograph on *Sweet orange oil* (1811). The content is not less than 99 per cent, calculated by the normalisation procedure.

Decane. C₁₀H₂₂. (*M*_r 142.3). 1024600. [124-18-5]. A colourless liquid, practically insoluble in water. *n*_D²⁰: about 1.411. bp: about 174 °C.

Decanol. C₁₀H₂₂O. (*M*_r 158.3). 1024700. [112-30-1]. *n*-Decyl alcohol. A viscous liquid, solidifying at about 6 °C, practically insoluble in water, soluble in alcohol. *n*_D²⁰: about 1.436. bp: about 230 °C.

Deltamethrin. C₂₂H₁₉Br₂NO₃. (*M*_r 505.2). 1125800. [52918-63-5]. bp: about 300 °C. mp: about 98 °C.

A suitable certified reference solution (10 ng/μl in cyclohexane) may be used.

Demeclocycline hydrochloride. 1145600. See *Demeclocycline hydrochloride* (0176).

Demethylflumazenil. C₁₄H₁₂FN₃O₃. (*M*_r 289.3). 1149300. [79089-72-8]. Ethyl 8-fluoro-6-oxo-5,6-dihydro-4*H*-imidazo[1,5-*a*][1,4]benzodiazepine-3-carboxylate. mp: about 288 °C. Colourless needles, soluble in dimethyl sulphoxide and in hot methanol.

2'-Deoxyuridine. C₉H₁₂N₂O₅. (*M*_r 228.2). 1024800. [951-78-0]. 1-(2-Deoxy-β-*d*-erythro-pentofuranosyl)-1*H*,3*H*-pyrimidine-2,4-dione. mp: about 165 °C.

Chromatography. Examine as prescribed in the monograph on *Idoxuridine* (0669), applying 5 μl of a 0.25 g/l solution. The chromatogram obtained shows only one principal spot.

Destaining solution. 1012202.

A mixture consisting of 1 volume of *glacial acetic acid R*, 4 volumes of *methanol R* and 5 volumes of *water R*.

Deuterated acetic acid. C₂²H₄O₂. (*M*_r 64.1). 1101100. [1186-52-3]. Tetradeuteroacetic acid. Acetic-*d*₃ acid-*d*. The degree of deuteration is not less than 99.7 per cent. *d*₂₀²⁰: about 1.12. *n*_D²⁰: about 1.368. bp: about 115 °C. mp: about 16 °C.

Deuterated acetone. C₃²H₆O. (*M*_r 64.1). 1024900. [666-52-4]. Acetone-*d*₆. (²H₆)-Acetone. The degree of deuteration is not less than 99.5 per cent. A clear, colourless liquid, miscible with water, with dimethylformamide, with ethanol and with methanol. *d*₂₀²⁰: about 0.87. *n*_D²⁰: about 1.357. bp: about 55 °C.

Water and deuterium oxide. Not more than 0.1 per cent.

Deuterated chloroform. C²HCl₃. (*M*_r 120.4). 1025000. [865-49-6]. (²H)-Chloroform. Chloroform-*d*. The degree of deuteration is not less than 99.7 per cent. A clear, colourless liquid, practically insoluble in water, miscible with acetone and with alcohol. It may be stabilised over silver foil. *d*₂₀²⁰: about 1.51. *n*_D²⁰: about 1.445. bp: about 60 °C.

Water and deuterium oxide: maximum 0.05 per cent.

Deuterated dimethyl sulphoxide. C₂²H₆OS. (*M*_r 84.2). 1025100. [2206-27-1]. (²H₆)-Dimethyl sulphoxide. Dimethyl sulphoxide-*d*₆. The degree of deuteration is not less than 99.8 per cent. A very hygroscopic liquid, practically colourless, viscous, soluble in water, in acetone and in ethanol. *d*₂₀²⁰: about 1.18. mp: about 20 °C. *Water and deuterium oxide:* maximum 0.1 per cent. *Storage:* in an airtight container.

Deuterated methanol. C²H₄O. (*M*_r 36.1). 1025200. [811-98-3]. (²H)-Methanol. Methanol-*d*. The degree of deuteration is not less than 99.8 per cent. Clear, colourless liquid miscible with water, with alcohol and with methylene chloride. *d*₂₀²⁰: about 0.888. *n*_D²⁰: about 1.326. bp: 65.4 °C.

Deuterium oxide. ²H₂O. (*M*_r 20.03). 1025300. [7789-20-0]. Deuterated water. The degree of deuteration is not less than 99.7 per cent. *d*₂₀²⁰: about 1.11. *n*_D²⁰: about 1.328. bp: about 101 °C.

Deuterium oxide R1. ²H₂O. (*M*_r 20.03). 1025301. [7789-20-0]. Deuterated water.

The degree of deuteration is not less than 99.95 per cent.

Developer solution. 1122500.

Dilute 2.5 ml of a 20 g/l solution of *citric acid R* and 0.27 ml of *formaldehyde R* to 500.0 ml with *water R*.

Dextran for chromatography, cross-linked R2. 1025500.

A bead-form dextran with a fraction range suitable for the separation of peptides and proteins with relative molecular masses of 15×10^2 to 30×10^3 . When dry, the beads have a diameter of 20 μm to 80 μm .

Dextran for chromatography, cross-linked R3. 1025600.

A bead-form dextran with a fraction range suitable for the separation of peptides and proteins with relative molecular masses of 4×10^3 to 15×10^4 . When dry, the beads have a diameter of 40 μm to 120 μm .

Dextrose. 1025700. [50-99-7].

See *glucose R*.

3,3'-Diaminobenzidine tetrahydrochloride.

$\text{C}_{12}\text{H}_{18}\text{Cl}_4\text{N}_4 \cdot 2\text{H}_2\text{O}$. (M_r 396.1). 1098000. [7411-49-6].
3,3',4,4'-Biphenyl-tetramine.

An almost white or slightly pink powder, soluble in water.
mp: about 280 °C, with decomposition.

Diatomaceous earth. 1025900. [91053-39-3].

A white or almost white, fine granular powder, made up of siliceous frustules of fossil diatoms or of debris of fossil diatoms, practically insoluble in water and in alcohol.

The substance may be identified by microscopic examination with a magnification of $\times 500$.

Diatomaceous earth for gas chromatography. 1026000.

A white or almost white, fine granular powder, made up of siliceous frustules of fossil diatoms or of debris of fossil diatoms, practically insoluble in water and in alcohol. The substance may be identified by microscopic examination with a magnification of $\times 500$. The substance is purified by treating with *hydrochloric acid R* and washing with *water R*.

Particle size. Not more than 5 per cent is retained on a sieve No. 180. Not more than 10 per cent passes a sieve No. 125.

Diatomaceous earth for gas chromatography R1. 1026100.

A white or almost white, fine granular powder, made up of siliceous frustules of fossil diatoms or of debris of fossil diatoms, practically insoluble in water and in alcohol. The substance may be identified by microscopic examination with a magnification of $\times 500$. The substance is purified by treating with *hydrochloric acid R* and washing with *water R*.

Particle size. Not more than 5 per cent is retained on a sieve No. 250. Not more than 10 per cent passes a sieve No. 180.

Diatomaceous earth for gas chromatography R2. 1026200.

A white or almost white, fine granular powder with a specific surface area of about 0.5 m^2/g , made up of siliceous frustules of fossil diatoms or of debris of fossil diatoms, practically insoluble in water and in alcohol. The substance may be identified by microscopic examination with a magnification of $\times 500$. The substance is purified by treating with *hydrochloric acid R* and washing with *water R*.

Particle size. Not more than 5 per cent is retained on a sieve No. 180. Not more than 10 per cent passes a sieve No. 125.

Diatomaceous earth for gas chromatography, silanised. 1026300.

Diatomaceous earth for gas chromatography R silanised with dimethyldichlorosilane or other suitable silanising agents.

Diatomaceous earth for gas chromatography, silanised R1. 1026400.

Prepared from crushed pink firebrick and silanised with dimethyldichlorosilane or other suitable silanising agents. The substance is purified by treating with *hydrochloric acid R* and washing with *water R*.

Diazinon. $\text{C}_{12}\text{H}_{21}\text{N}_2\text{O}_3\text{PS}$. (M_r 304.3). 1125900. [333-41-5].
bp: about 306 °C.

A suitable certified reference solution (10 $\text{ng}/\mu\text{l}$ in iso-octane) may be used.

Diazobenzenesulphonic acid solution R1. 1026500.

Dissolve 0.9 g of *sulphanilic acid R* in a mixture of 30 ml of *dilute hydrochloric acid R* and 70 ml of *water R*. To 3 ml of the solution add 3 ml of a 50 g/l solution of *sodium nitrite R*. Cool in an ice-bath for 5 min, add 12 ml of the sodium nitrite solution and cool again. Dilute to 100 ml with *water R* and keep the reagent in an ice-bath. Prepare extemporaneously but allow to stand for 15 min before use.

Dibutylamine. $\text{C}_8\text{H}_{19}\text{N}$. (M_r 129.3). 1126000. [111-92-2].
N-Butylbutan-1-amine.

Colourless liquid.

n_{D}^{20} : about 1.417.

bp: about 159 °C.

Dibutyl ether. $\text{C}_8\text{H}_{18}\text{O}$. (M_r 130.2). 1026700. [142-96-1].

A colourless, flammable liquid, practically insoluble in water, miscible with ethanol.

d_{20}^{20} : about 0.77.

n_{D}^{20} : about 1.399.

Do not distil if the dibutyl ether does not comply with the test for peroxides.

Peroxides. Place 8 ml of *potassium iodide and starch solution R* in a 12 ml ground-glass-stoppered cylinder about 1.5 cm in diameter. Fill completely with the substance to be examined, shake vigorously and allow to stand protected from light for 30 min. No colour is produced.

The name and concentration of any added stabiliser are stated on the label.

Dibutyl phthalate. $\text{C}_{16}\text{H}_{22}\text{O}_4$. (M_r 278.3). 1026800. [84-74-2].
Dibutyl benzene-1,2-dicarboxylate.

A clear, colourless or faintly coloured, oily liquid, very slightly soluble in water, miscible with acetone and with alcohol.

d_{20}^{20} : 1.043 to 1.048.

n_{D}^{20} : 1.490 to 1.495.

Dicarboxidine hydrochloride. $\text{C}_{20}\text{H}_{26}\text{Cl}_2\text{N}_2\text{O}_6$. (M_r 461.3). 1026900. [56455-90-4]. 4,4'-[(4,4'-Diaminobiphenyl-3,3'-diyl)dioxy]dibutanoic acid dihydrochloride.

Dichlofenthion. $\text{C}_{10}\text{H}_{13}\text{Cl}_2\text{O}_3\text{PS}$. (M_r 315.2). 1126100. [97-17-6].

A suitable certified reference solution (10 $\text{ng}/\mu\text{l}$ in cyclohexane) may be used.

Dichloroacetic acid. $\text{C}_2\text{H}_2\text{Cl}_2\text{O}_2$. (M_r 128.9). 1027000. [79-43-6].

Colourless liquid, miscible with water and alcohol.

d_{20}^{20} : about 1.566.

n_{D}^{20} : about 1.466.

bp: about 193 °C.

Dichloroacetic acid solution. 1027001.

Dilute 67 ml of *dichloroacetic acid R* to 300 ml with *water R* and neutralise to *blue litmus paper R* using *ammonia R*. Cool, add 33 ml of *dichloroacetic acid R* and dilute to 600 ml with *water R*.

Dichlorobenzene. C₆H₄Cl₂. (*M_r* 147.0). 1027100. [95-50-1]. 1,2-Dichlorobenzene.

A colourless, oily liquid, practically insoluble in water, soluble in ethanol.

*d*₂₀²⁰: about 1.31.

bp: about 180 °C.

2,3-Dichloro-5,6-dicyanobenzoquinone. C₈Cl₂N₂O₂. (*M_r* 227.0). 1153600. [84-58-2]. 4,5-Dichloro-3,6-dioxo-cyclohexa-1,4-diene-1,2-dicarbonitrile.

Yellow or orange crystals, soluble in dioxan and in acetic acid, slightly soluble in methylene chloride. It decomposes in water.

mp: about 214 °C.

Storage: at a temperature of 2 °C to 8 °C.

(S)-3,5-Dichloro-2,6-dihydroxy-N-[(1-ethylpyrrolidin-2-yl)methyl]benzamide hydrobromide. C₁₄H₁₉BrCl₂N₂O₃. (*M_r* 414.1). 1142600. [113310-88-6].

White, crystalline powder.

[α]_D²²: + 11.4, determined on a 15.0 g/l solution in *ethanol R*. mp: about 212 °C.

Dichlorofluorescein. C₂₀H₁₀Cl₂O₅. (*M_r* 401.2).

1027200. [76-54-0]. 2,7-Dichlorofluorescein. 2-(2,7-Dichloro-6-hydroxy-3-oxo-3H-xanthen-9-yl)benzoic acid.

A yellowish-brown to yellow-orange powder, slightly soluble in water, freely soluble in alcohol and in dilute solutions of alkali hydroxides giving a solution showing a yellowish-green fluorescence.

Dichlorophenolindophenol, sodium salt.

C₁₂H₆Cl₂NNaO₂·2H₂O. (*M_r* 326.1). 1027300. [620-45-1]. The sodium derivative of 2,6-dichloro-*N*-(4-hydroxyphenyl)-1,4-benzoquinone monoimine dihydrate.

A dark-green powder, freely soluble in water and in ethanol. The aqueous solution is dark blue; when acidified it becomes pink.

Dichlorophenolindophenol standard solution. 1027301.

Dissolve 50.0 mg of *dichlorophenolindophenol, sodium salt R* in 100.0 ml of *water R* and filter.

Standardisation. Dissolve 20.0 mg of *ascorbic acid R* in 10 ml of a freshly prepared 200 g/l solution of *metaphosphoric acid R* and dilute to 250.0 ml with *water R*. Titrate 5.0 ml rapidly with the dichloro-phenolindophenol standard solution, added from a microburette graduated in 0.01 ml, until the pink colour persists for 10 s, the titration occupying not more than 2 min. Dilute the dichlorophenolindophenol solution with *water R* to make 1 ml of the solution equivalent to 0.1 mg of ascorbic acid (C₆H₈O₆).

Storage: use within 3 days.

Standardise immediately before use.

5,7-Dichloroquinolin-8-ol. C₉H₅Cl₂NO. (*M_r* 214.1). 1157000. [773-76-2]. 5,7-Dichlorooxine.

Yellow, crystalline powder, soluble in acetone, slightly soluble in ethanol (96 per cent).

mp: about 179 °C.

Content: minimum 95.0 per cent of C₉H₅Cl₂NO.

Dichloroquinonechlorimide. C₆H₂Cl₃NO. (*M_r* 210.4). 1027400. [101-38-2]. 2,6-Dichloro-*N*-chloro-1,4-benzoquinone mono-imine.

A pale yellow or greenish-yellow crystalline powder, practically insoluble in water, soluble in alcohol and in dilute alkaline solutions.

mp: about 66 °C.

Dichlorvos. C₄H₇Cl₂O₄P. (*M_r* 221). 1101200. [62-73-7]. 2,2-Dichlorovinyl dimethyl phosphate.

Colourless or brownish-yellow liquid, soluble in water, miscible with most organic solvents.

*n*_D²⁵: about 1.452.

Dicyclohexyl. C₁₂H₂₂. (*M_r* 166.3). 1135300. [92-51-3]. Bicyclohexyl.

*d*₂₀²⁰: about 0.864.

bp: about 227 °C.

mp: about 4 °C.

Dicyclohexylamine. C₁₂H₂₃N. (*M_r* 181.3). 1027500. [101-83-7]. *N,N*-Dicyclohexylamine.

Colourless liquid, sparingly soluble in water, miscible with the usual organic solvents.

*n*_D²⁰: about 1.484.

bp: about 256 °C.

Freezing point (2.2.18): 0 °C to 1 °C.

Dicyclohexylurea. C₁₃H₂₄N₂O. (*M_r* 224.4). 1027600. [2387-23-7]. 1,3-Dicyclohexylurea.

A white, crystalline powder.

mp: about 232 °C.

Didocosahexaenoin. C₄₇H₆₈O₅. (*M_r* 713.0). 1142700.

[88315-12-2]. Diglyceride of docosahexaenoic acid (C22:6). Glycerol didocosahexaenoate. (*all-Z*)-Docosahexaenoic acid, diester with propane-1,2,3-triol.

Didodecyl 3,3'-thiodipropionate. C₃₀H₅₈O₄S. (*M_r* 514.8). 1027700. [123-28-4].

A white, crystalline powder, practically insoluble in water, freely soluble in acetone and in light petroleum, slightly soluble in alcohol.

mp: about 39 °C.

Dieldrin. C₁₂H₈Cl₆O. (*M_r* 380.9). 1126200. [60-57-1].

bp: about 385 °C.

mp: about 176 °C.

A suitable certified reference solution (10 ng/μl in cyclohexane) may be used.

Diethanolamine. C₄H₁₁NO₂. (*M_r* 105.1). 1027800. [111-42-2]. 2,2'-Iminobisethanol.

A viscous, clear, slightly yellow liquid or deliquescent crystals melting at about 28 °C, very soluble in water, in acetone and in methanol.

*d*₂₀²⁰: about 1.09.

pH (2.2.3): 10.0 to 11.5 for a 50 g/l solution.

Diethanolamine used in the test for alkaline phosphatase complies with the following additional test.

Ethanolamine: maximum 1.0 per cent. Examine by gas chromatography (2.2.28), using 3-aminopropanol *R* as the internal standard.

Internal standard solution. Dissolve 1.00 g of 3-aminopropanol *R* in *acetone R* and dilute to 10.0 ml with the same solvent.

Test solution (a). Dissolve 5.00 g of the substance to be examined in *acetone R* and dilute to 10.0 ml with the same solvent.

Test solution (b). Dissolve 5.00 g of the substance to be examined in *acetone R*, add 1.0 ml of the internal standard solution and dilute to 10.0 ml with the same solvent.

Reference solutions. Dissolve 0.50 g of *ethanolamine R* in *acetone R* and dilute to 10.0 ml with the same solvent. To 0.5 ml, 1.0 ml and 2.0 ml of this solution, add 1.0 ml of the internal standard solution and dilute to 10.0 ml with *acetone R*.

The chromatographic procedure may be carried out using:

- a column 1 m long and 4 mm in internal diameter packed with *diphenylphenylene oxide polymer R* (180 µm to 250 µm),
- *nitrogen for chromatography R* as the carrier gas at a flow rate of 40 ml/min,
- a flame-ionisation detector.

Maintain the temperature of the column at 125 °C for 3 min and then raise to 300 °C at a rate of 12 °C/min. Maintain the temperature of the injection port at 250 °C and that of the detector at 280 °C. Inject 1.0 µl of each test solution and 1.0 µl of each reference solution.

Storage: in an airtight container.

Diethoxytetrahydrofuran. C₈H₁₆O₃. (*M_r* 160.2). 1027900. [3320-90-9]. 2,5-Diethoxytetrahydrofuran. A mixture of the *cis* and *trans* isomers.

A clear, colourless or slightly yellowish liquid, practically insoluble in water, soluble in alcohol and in most other organic solvents.

*d*₂₀²⁰: about 0.98.

*n*_D²⁰: about 1.418.

Diethylamine. C₄H₁₁N. (*M_r* 73.1). 1028000. [109-89-7].

A clear, colourless, flammable liquid, strongly alkaline, miscible with water and with alcohol.

*d*₂₀²⁰: about 0.71.

bp: about 55 °C.

Diethylaminoethyl dextran. 1028200.

Anion exchange resin presented as the hydrochloride.

A powder forming gels with water.

***N,N*-Diethylaniline.** C₁₀H₁₅N. (*M_r* 149.2). 1028400. [91-66-7].

*d*₂₀²⁰: about 0.938.

bp: about 217 °C.

mp: about –38 °C.

Diethylene glycol. C₄H₁₀O₃. (*M_r* 106.1). 1028300. [111-46-6]. 2,2'-Oxydiethanol.

Content: minimum 99.5 per cent *m/m* of C₄H₁₀O₃.

A clear, colourless liquid, hygroscopic, miscible with water, with acetone and with alcohol.

*d*₂₀²⁰: about 1.118.

*n*_D²⁰: about 1.447.

bp: 244 °C to 246 °C.

Storage: in an airtight container.

***N,N*-Diethylethane-1,2-diamine.** 1028500. [100-36-7].

See *N,N*-diethylethylenediamine *R*.

***N,N*-Diethylethylenediamine.** C₆H₁₆N₂. (*M_r* 116.2).

1028500. [100-36-7].

Content: minimum 98.0 per cent of C₆H₁₆N₂.

A slightly oily liquid, colourless or slightly yellow, strong odour of ammonia, irritant to the skin, eyes and mucous membranes.

*d*₂₀²⁰: 0.827.

bp: 145 °C to 147 °C.

Water (2.5.12): maximum 1.0 per cent, determined on 0.500 g.

Di(2-ethylhexyl) phthalate. C₂₄H₃₈O₄. (*M_r* 390.5). 1028100. Di(2-ethylhexyl) benzene-1,2-dicarboxylate.

A colourless, oily liquid, practically insoluble in water, soluble in organic solvents.

*d*₂₀²⁰: about 0.98.

*n*_D²⁰: about 1.486.

Viscosity (2.2.9): about 80 mPa·s.

Diethylphenylenediamine sulphate. C₁₀H₁₈N₂O₄S. (*M_r* 262.3). 1028600. [6283-63-2]. *N,N'*-Diethyl-*p*-phenylenediamine sulphate. *N,N'*-Diethylbenzene-1,4-diamine sulphate.

A white or slightly yellow powder, soluble in water.

mp: about 185 °C, with decomposition.

Storage: protected from light.

Diethylphenylenediamine sulphate solution. 1028601.

To 250 ml of *water R* add 2 ml of *sulphuric acid R* and 25 ml of 0.02 *M* sodium edetate. Dissolve in this solution 1.1 g of *diethylphenylenediamine sulphate R* and dilute to 1000 ml with *water R*.

Do not use if the solution is not colourless.

Storage: protected from light and heat for 1 month.

Digitonin. C₅₆H₉₂O₂₉. (*M_r* 1229). 1028700. [11024-24-1]. 3β-[*O*-β-D-Glucopyranosyl-(1→3)-*O*-β-D-galactopyranosyl-(1→2)-*O*-[β-D-xylopyranosyl-(1→3)]-*O*-β-D-galactopyranosyl-(1→4)-*O*-β-D-galactopyranosyloxy]-(25*R*)-5α-spirostan-2α,15β-diol.

Crystals, practically insoluble in water, sparingly soluble in ethanol, slightly soluble in alcohol.

Digitoxin. 1028800. [71-63-6].

See *Digitoxin* (0078).

Diammonium 2,2'-azinobis(3-ethylbenzothiazoline-6-sulphonate). C₁₈H₂₄N₆O₆S₄. (*M_r* 548.7).

1153000. [30931-67-0]. ABTS. Diammonium 2,2'-(diazanediylidene)bis[3-ethyl-2,3-dihydrobenzothiazole-6-sulphonate].

Chromogenic substrate suitable for use in ELISA procedures.

Green tablets, freely soluble in water.

pH (2.2.3): 4.2 to 5.8 for a 0.1 g/l solution.

Dihydrocapsaicin. C₁₈H₂₉NO₃. (*M_r* 307.4). 1148100. [19408-84-5]. *N*-[(4-Hydroxy-3-methoxyphenyl)methyl]-8-methylnonanamide.

White, crystalline powder, practically insoluble in cold water, freely soluble in ethanol.

10,11-Dihydrocarbamazepine. C₁₅H₁₄N₂O. (*M_r* 238.3).

1028900. [3564-73-6]. 10,11-Dihydro-5*H*-dibenzo[*b*,*f*]azepine-5-carboxamide.

mp: 205 °C to 210 °C.

2,5-Dihydroxybenzoic acid. C₇H₆O₄. (*M_r* 154.1). 1148200. [490-79-9]. Gentic acid.

Light yellow crystals.

mp: about 200 °C.

5,7-Dihydroxy-4-methylcoumarin. $C_{10}H_8O_4$. (M_r 192.2). 1149400. [2107-76-8]. 5,7-Dihydroxy-4-methyl-2H-1-benzopyran-2-one.

Light yellowish powder, practically insoluble in water, sparingly soluble in alcohol.

mp: 295 °C to 303 °C.

Dihydroxynaphthalene. 1029000. [132-86-5].

See 1,3-dihydroxynaphthalene R.

1,3-Dihydroxynaphthalene. $C_{10}H_8O_2$. (M_r 160.2). 1029000. [132-86-5]. Naphthalene-1,3-diol.

A crystalline, generally brownish-violet powder, freely soluble in water and in alcohol.

mp: about 125 °C.

2,7-Dihydroxynaphthalene. $C_{10}H_8O_2$. (M_r 160.2). 1029100. [582-17-2]. Naphthalene-2,7-diol.

Needles, soluble in water and in alcohol.

mp: about 190 °C.

2,7-Dihydroxynaphthalene solution. 1029101.

Dissolve 10 mg of 2,7-dihydroxynaphthalene R in 100 ml of sulphuric acid R and allow to stand until decolorised.

Storage: use within 2 days.

5,7-Diiodoquinolin-8-ol. $C_9H_5I_2NO$. (M_r 397.0). 1157100. [83-73-8]. 5,7-Diiodooxine.

Yellowish-brown powder, sparingly soluble in acetone and in ethanol (96 per cent).

Content: minimum 95.0 per cent of $C_9H_5I_2NO$.

Di-isobutyl ketone. $C_9H_{18}O$. (M_r 142.2). 1029200. [108-83-8].

A clear, colourless liquid, slightly soluble in water, miscible with most organic solvents.

n_D^{20} : about 1.414

bp: about 168 °C.

Di-isopropyl ether. $C_6H_{14}O$. (M_r 102.2). 1029300. [108-20-3].

A clear, colourless liquid, very slightly soluble in water, miscible with alcohol.

d_{20}^{20} : 0.723 to 0.728.

bp: 67 °C to 69 °C.

Do not distil if the di-isopropyl ether does not comply with the test for peroxides.

Peroxides. Place 8 ml of potassium iodide and starch solution R in a 12 ml ground-glass-stoppered cylinder about 1.5 cm in diameter. Fill completely with the substance to be examined, shake vigorously and allow to stand protected from light for 30 min. No colour is produced.

The name and concentration of any added stabiliser are stated on the label.

Storage: protected from light.

N,N'-Diisopropylethylenediamine. $C_8H_{20}N_2$. (M_r 144.3). 1140600. [4013-94-9]. N,N'-bis(1-Methylethyl)-1,2-ethanediamine.

Colourless to yellowish, corrosive, flammable, hygroscopic liquid.

d_{20}^{20} : about 0.798.

n_D^{20} : about 1.429.

bp: about 170 °C.

4,4'-Dimethoxybenzophenone. $C_{15}H_{14}O_3$. (M_r 242.3). 1126300. [90-96-0]. bis(4-Methoxyphenyl)methanone.

A white powder, practically insoluble in water and slightly soluble in alcohol.

mp: about 142 °C.

Dimethoxypropane. $C_5H_{12}O_2$. (M_r 104.1). 1105200. [77-76-9]. 2,2-Dimethoxypropane.

A colourless liquid, decomposing on exposure to moist air or water.

d_{20}^{20} : about 0.847.

n_D^{20} : about 1.378.

bp: about 83 °C.

Dimethylacetamide. C_4H_9NO . (M_r 87.1). 1029700. [127-19-5]. N,N-Dimethylacetamide.

Content: minimum 99.5 per cent of C_4H_9NO .

A colourless liquid, miscible with water and with many organic solvents.

d_{20}^{20} : about 0.94.

n_D^{20} : about 1.437.

bp: about 165 °C.

Dimethylaminobenzaldehyde. $C_9H_{11}NO$. (M_r 149.2). 1029800. [100-10-7]. 4-Dimethylaminobenzaldehyde.

White or yellowish-white crystals, soluble in alcohol and in dilute acids.

mp: about 74 °C.

Dimethylaminobenzaldehyde solution R1. 1029801.

Dissolve 0.2 g of dimethylaminobenzaldehyde R in 20 ml of alcohol R and add 0.5 ml of hydrochloric acid R. Shake the solution with activated charcoal R and filter. The colour of the reagent is less intense than that of iodine solution R3. Prepare immediately before use.

Dimethylaminobenzaldehyde solution R2. 1029802.

Dissolve 0.2 g of dimethylaminobenzaldehyde R, without heating, in a mixture of 4.5 ml of water R and 5.5 ml of hydrochloric acid R. Prepare immediately before use.

Dimethylaminobenzaldehyde solution R6. 1029803.

Dissolve 0.125 g of dimethylaminobenzaldehyde R in a cooled mixture of 35 ml of water R and 65 ml of sulphuric acid R. Add 0.1 ml of a 50 g/l solution of ferric chloride R. Before use allow to stand for 24 h, protected from light.

Storage: when stored at room temperature it must be used within 1 week; when kept in a refrigerator, it may be stored for several months.

Dimethylaminobenzaldehyde solution R7. 1029804.

Dissolve 1.0 g of dimethylaminobenzaldehyde R in 50 ml of hydrochloric acid R and add 50 ml of alcohol R.

Storage: protected from light; use within 4 weeks.

Dimethylaminobenzaldehyde solution R8. 1029805.

Dissolve 0.25 g of dimethylaminobenzaldehyde R in a mixture of 5 g of phosphoric acid R, 45 g of water R and 50 g of anhydrous acetic acid R. Prepare immediately before use.

4-Dimethylaminocinnamaldehyde. $C_{11}H_{13}NO$. (M_r 175.2). 1029900. [6203-18-5]. 3-(4-Dimethylaminophenyl)prop-2-enal.

Orange to orange-brown crystals or powder. Sensitive to light.

mp: about 138 °C.

4-Dimethylaminocinnamaldehyde solution. 1029901.

Dissolve 2 g of 4-dimethylaminocinnamaldehyde R in a mixture of 100 ml of hydrochloric acid R1 and 100 ml of ethanol R. Dilute the solution to four times its volume with ethanol R immediately before use.

2-(Dimethylamino)ethyl methacrylate. $C_8H_{15}NO_2$. (M_r 157.2). 1147200. [2867-47-2]. 2-(Dimethylamino)ethyl 2-methylpropenoate.

d_4^{20} : about 0.930.

bp: about 187 °C.

Dimethylaminonaphthalenesulphonyl chloride.

$C_{12}H_{12}ClNO_2S$. (M_r 269.8). 1030000. [605-65-2].

5-Dimethyl-amino-1-naphthalenesulphonyl chloride.

A yellow, crystalline powder, slightly soluble in water, soluble in methanol.

mp: about 70 °C.

3-Dimethylaminophenol. $C_8H_{11}NO$. (M_r 137.2). 1156500. [99-07-0]. 3-(Dimethylamino)phenol.

Grey powder, slightly soluble in water.

mp: about 80 °C.

Dimethylaniline. $C_8H_{11}N$. (M_r 121.2). 1030100. [121-69-7]. *N,N*-Dimethylaniline.

A clear, oily liquid, almost colourless when freshly distilled, darkening on storage to reddish-brown, practically insoluble in water, freely soluble in alcohol.

n_D^{20} : about 1.558.

Distillation range (2.2.11). Not less than 95 per cent distils between 192 °C and 194 °C.

***N,N*-Dimethylaniline.** 1030100. [121-69-7].

See *Dimethylaniline R*.

2,3-Dimethylaniline. $C_8H_{11}N$. (M_r 121.2). 1105300. [87-59-2]. 2,3-Xylidine.

A yellowish liquid, sparingly soluble in water, soluble in alcohol.

d_{20}^{20} : 0.993 to 0.995.

n_D^{20} : about 1.569.

bp: about 224 °C.

2,6-Dimethylaniline. $C_8H_{11}N$. (M_r 121.2). 1030200. [87-62-7]. 2,6-Xylidine.

A colourless liquid, sparingly soluble in water, soluble in alcohol.

d_{20}^{20} : about 0.98.

2,4-Dimethyl-6-*tert*-butylphenol. $C_{12}H_{18}O$. (M_r 178.3). 1126500. [1879-09-0].

Dimethyl carbonate. $C_3H_6O_3$. (M_r 90.1). 1119300. [616-38-6]. Carbonic acid dimethyl ester.

Liquid, insoluble in water, miscible with alcohol.

d_4^{17} : 1.065.

n_D^{20} : 1.368.

bp: about 90 °C.

Dimethyldecylamine. $C_{12}H_{27}N$. (M_r 185.4). 1113500. [1120-24-7]. *N,N*-dimethyldecylamine.

Content: minimum 98.0 per cent *m/m* of $C_{12}H_{27}N$.

bp: about 234 °C.

1,1-Dimethylethylamine. $C_4H_{11}N$. (M_r 73.1). 1100900. [75-64-9]. 2-Amino-2-methylpropane. *tert*-Butylamine.

Liquid, miscible with alcohol.

d_{20}^{20} : about 0.694.

n_D^{20} : about 1.378.

bp: about 46 °C.

1,1-Dimethylethyl methyl ether. $C_5H_{12}O$. (M_r 88.1). 1013900. [1634-04-4]. 2-Methoxy-2-methylpropane. *tert*-Butyl methyl ether.

A colourless, clear, flammable liquid.

n_D^{20} : about 1.376.

Minimum transmittance (2.2.25), determined using *water R* as compensation liquid: 50 per cent at 240 nm, 80 per cent at 255 nm, 98 per cent at 280 nm.

1,1-Dimethylethyl methyl ether R1. 1126400.

Content: minimum 99.5 per cent of $C_5H_{12}O$.

d_{20}^{20} : about 0.741.

n_D^{20} : about 1.369.

bp: about 55 °C.

Dimethylformamide. C_3H_7NO . (M_r 73.1). 1030300. [68-12-2].

A clear, colourless neutral liquid, miscible with water and with alcohol.

d_{20}^{20} : 0.949 to 0.952.

bp: about 153 °C.

Water (2.5.12): maximum 0.1 per cent.

Dimethylformamide diethylacetal. $C_7H_{17}NO_2$. (M_r 147.2). 1113600. [1188-33-6]. *N,N*-Dimethylformamide diethylacetal.

n_D^{20} : about 1.40.

bp: 128 °C to 130 °C.

***N,N*-Dimethylformamide dimethylacetal.**

$C_5H_{13}NO_2$. (M_r 119.2). 1140700. [4637-24-5].

1,1-Dimethoxytrimethylamine.

Clear, colourless liquid.

d_{20}^{20} : about 0.896.

n_D^{20} : about 1.396.

bp: about 103 °C.

Dimethylglyoxime. $C_4H_8N_2O_2$. (M_r 116.1). 1030400. [95-45-4]. 2,3-Butanedione dioxime.

A white, crystalline powder or colourless crystals, practically insoluble in cold water, very slightly soluble in boiling water, soluble in alcohol.

mp: about 240 °C, with decomposition.

Sulphated ash (2.4.14): maximum 0.05 per cent.

1,3-Dimethyl-2-imidazolidinone. $C_5H_{10}N_2O$. (M_r 114.2). 1135400. [80-73-9]. *N,N'*-Dimethylethylene urea.

1,3-Dimethyl-2-imidazolidone.

n_D^{20} : 1.4720.

bp: about 224 °C.

***N,N*-Dimethyloctylamine.** $C_{10}H_{23}N$. (M_r 157.3). 1030500. [7378-99-6]. Octyldimethylamine.

Colourless liquid.

d_{20}^{20} : about 0.765.

n_D^{20} : about 1.424.

bp: about 195 °C.

2,6-Dimethylphenol. $C_8H_{10}O$. (M_r 122.2). 1030600. [576-26-1].

Colourless needles, slightly soluble in water, very soluble in alcohol.

bp: about 203 °C.

mp: 46 °C to 48 °C.

3,4-Dimethylphenol. $C_8H_{10}O$. (M_r 122.2). 1098100. [95-65-8].

White or almost white crystals, slightly soluble in water, freely soluble in alcohol.

bp: about 226 °C.

mp: 25 °C to 27 °C.

Dimethylpiperazine. $C_6H_{14}N_2$. (M_r 114.2). 1030700. [106-58-1]. 1,4-Dimethylpiperazine.

A colourless liquid, miscible with water and with alcohol.

d_{20}^{20} : about 0.85.

n_D^{20} : about 1.446.

bp: about 131 °C.

Dimethylstearamide. $C_{20}H_{41}NO$. (M_r 311.6). 1030800. *N,N*-Dimethylstearamide.

A white or almost white solid mass, soluble in many organic solvents, including acetone.

mp: about 51 °C.

Dimethylstearylamine. 1030800.

See *dimethylstearamide R*.

Dimethyl sulphone. $C_2H_6O_2S$. (M_r 94.1). 1030900. [67-71-0].

A white, crystalline powder, freely soluble in water, soluble in acetone and alcohol.

mp: 108 °C to 110 °C.

Dimethyl sulfoxide. 1029500. [67-68-5].

See *Dimethyl sulfoxide* (0763).

Dimethyl sulfoxide used in spectrophotometry complies with the following additional test.

Minimum transmittance (2.2.25), determined using *water R* as compensation liquid: 10 per cent at 262 nm, 35 per cent at 270 nm, 70 per cent at 290 nm, 98 per cent at 340 nm and at higher wavelengths.

Dimethyl sulfoxide R1. 1029501.

Content: minimum 99.7 per cent of C_2H_6OS , determined by gas chromatography.

Dimeticone. 1105400. [9016-00-6].

See *Dimeticone* (0138).

Dimidium bromide. $C_{20}H_{18}BrN_3$. (M_r 380.3). 1031100. [518-67-2]. 3,8-Diamino-5-methyl-6-phenylphenanthridinium bromide.

Dark-red crystals, slightly soluble in water at 20 °C, sparingly soluble in water at 60 °C and in alcohol.

Dimidium bromide-sulphan blue mixed solution. 1031101.

Dissolve separately 0.5 g of *dimidium bromide R* and 0.25 g of *sulphan blue R* in 30 ml of a hot mixture of 1 volume of *ethanol R* and 9 volumes of *water R*, stir, mix the two solutions, and dilute to 250 ml with the same mixture of solvents. Mix 20 ml of this solution with 20 ml of a 14.0 per cent *V/V* solution of *sulphuric acid R* previously diluted with about 250 ml of *water R* and dilute to 500 ml with *water R*.

Storage: protected from light.

Dinitrobenzene. $C_6H_4N_2O_4$. (M_r 168.1). 1031200. [528-29-0]. 1,3-Dinitrobenzene.

Yellowish crystalline powder or crystals, practically insoluble in water, slightly soluble in alcohol.

mp: about 90 °C.

Dinitrobenzene solution. 1031201.

A 10 g/l solution in *alcohol R*.

Dinitrobenzoic acid. $C_7H_4N_2O_6$. (M_r 212.1). 1031300. [99-34-3]. 3,5-Dinitrobenzoic acid.

Almost colourless crystals, slightly soluble in water, very soluble in alcohol.

mp: about 206 °C.

Dinitrobenzoic acid solution. 1031301.

A 20 g/l solution in *alcohol R*.

Dinitrobenzoyl chloride. $C_7H_3ClN_2O_5$. (M_r 230.6). 1031400. [99-33-2]. 3,5-Dinitrobenzoyl chloride.

Translucent, yellow or greenish-yellow powder or yellowish crystals, soluble in acetone and in toluene.

mp: about 68 °C.

Suitability test. To 1 ml of *ethanol R* and 0.1 g of *dinitrobenzoyl chloride R* add 0.05 ml of *dilute sulphuric acid R* and boil under a reflux condenser for 30 min. After evaporation on a water-bath add 5 ml of *heptane R* to the residue and heat to boiling. Filter the hot solution. Wash the crystals formed on cooling to room temperature with a small quantity of *heptane R* and dry in a desiccator. The crystals melt (2.2.14) at 94 °C to 95 °C.

Dinitrophenylhydrazine. $C_6H_6N_4O_4$. (M_r 198.1). 1031500. [119-26-6]. 2,4-Dinitrophenylhydrazine.

Reddish-orange crystals, very slightly soluble in water, slightly soluble in alcohol.

mp: about 203 °C (instantaneous method).

Dinitrophenylhydrazine-aceto-hydrochloric solution. 1031501.

Dissolve 0.2 g of *dinitrophenylhydrazine R* in 20 ml of *methanol R* and add 80 ml of a mixture of equal volumes of *acetic acid R* and *hydrochloric acid R1*. Prepare immediately before use.

Dinitrophenylhydrazine-hydrochloric solution. 1031502.

Dissolve by heating 0.50 g of *dinitrophenylhydrazine R* in *dilute hydrochloric acid R* and complete to 100 ml with the same solvent. Allow to cool and filter. Prepare immediately before use.

Dinitrophenylhydrazine-sulphuric acid solution. 1031503.

Dissolve 1.5 g of *dinitrophenylhydrazine R* in 50 ml of a 20 per cent *V/V* solution of *sulphuric acid R*. Prepare immediately before use.

Dinonyl phthalate. $C_{26}H_{42}O_4$. (M_r 418.6). 1031600. [28553-12-0].

A colourless to pale yellow, viscous liquid.

d_{20}^{20} : 0.97 to 0.98.

n_D^{20} : 1.482 to 1.489.

Acidity. Shake 5.0 g with 25 ml of *water R* for 1 min. Allow to stand, filter the separated aqueous layer and add 0.1 ml of *phenolphthalein solution R*. Not more than 0.3 ml of 0.1 *M sodium hydroxide* is required to change the colour of the solution (0.05 per cent, calculated as phthalic acid).

Water (2.5.12): maximum 0.1 per cent.

Diocetyl disulphide. $C_{36}H_{74}S_2$. (M_r 571.1). 1031700. [1844-09-3].

A white powder, practically insoluble in water.

mp: 53 °C to 58 °C.

2,2'-Di(octadecyloxy)-5,5'-spirobi(1,3,2-dioxaphosphorinane). $C_{41}H_{82}O_6P_2$. (M_r 733). 1031800.

White, waxy solid, practically insoluble in water, soluble in hydrocarbons.

mp: 40 °C to 70 °C.

Diocetadecyl 3,3'-thiodipropionate. $C_{42}H_{82}O_4S$. (M_r 683). 1031900. [693-36-7].

A white, crystalline powder, practically insoluble in water, freely soluble in methylene chloride, sparingly soluble in acetone, in alcohol and in light petroleum.

mp: 58 °C to 67 °C.

Dioxan. $C_4H_8O_2$. (M_r 88.1). 1032000. [123-91-1]. 1,4-Dioxan. A clear, colourless liquid, miscible with water and with most organic solvents.

d_{20}^{20} : about 1.03.

Freezing-point (2.2.18): 9 °C to 11 °C.

Water (2.5.12): maximum 0.5 per cent.

Do not distil if the dioxan does not comply with the test for peroxides.

Peroxides. Place 8 ml of *potassium iodide and starch solution R* in a 12 ml ground-glass-stoppered cylinder about 1.5 cm in diameter. Fill completely with the substance to be examined, shake vigorously and allow to stand in the dark for 30 min. No colour is produced.

Dioxan used for liquid scintillation is of a suitable analytical grade.

Dioxan solution. 1032002.

Dilute 50.0 ml of *dioxan stock solution R* to 100.0 ml with *water R*. (0.5 mg/ml of dioxan).

Dioxan solution R1. 1032003.

Dilute 10.0 ml of *dioxan solution R* to 50.0 ml with *water R*. (0.1 mg/ml of dioxan).

Dioxan stock solution. 1032001.

Dissolve 1.00 g of *dioxan R* in *water R* and dilute to 100.0 ml with the same solvent. Dilute 5.0 ml of this solution to 50.0 ml with *water R* (1.0 mg/ml).

Diphenylamine. $C_{12}H_{11}N$. (M_r 169.2). 1032100. [122-39-4].

White crystals, slightly soluble in water, soluble in alcohol.

mp: about 55 °C.

Storage: protected from light.

Diphenylamine solution. 1032101.

A 1 g/l solution in *sulphuric acid R*.

Storage: protected from light.

Diphenylamine solution R1. 1032102.

A 10 g/l solution in *sulphuric acid R*. The solution is colourless.

Diphenylamine solution R2. 1032103.

Dissolve 1 g of *diphenylamine R* in 100 ml of *glacial acetic acid R* and add 2.75 ml of *sulphuric acid R*. Use immediately.

Diphenylanthracene. $C_{26}H_{18}$. (M_r 330.4). 1032200. [1499-10-1]. 9,10-Diphenylanthracene.

Yellowish to yellow, crystalline powder, practically insoluble in water.

mp: about 248 °C.

Diphenylbenzidine. $C_{24}H_{20}N_2$. (M_r 336.4). 1032300. [531-91-9]. *N,N'*-Diphenylbenzidine. *N,N'*-Diphenylbiphenyl-4,4'-diamine.

A white or faintly grey, crystalline powder, practically insoluble in water, slightly soluble in acetone and in alcohol.

mp: about 248 °C.

Nitrates. Dissolve 8 mg in a cooled mixture of 5 ml of *water R* and 45 ml of *nitrogen-free sulphuric acid R*. The solution is colourless or very pale blue.

Sulphated ash (2.4.14): maximum 0.1 per cent.

Storage: protected from light.

Diphenylboric acid aminoethyl ester. $C_{14}H_{16}BNO$. (M_r 225.1). 1032400. [524-95-8].

A white or slightly yellow, crystalline powder, practically insoluble in water, soluble in alcohol.

mp: about 193 °C.

Diphenylcarbazine. $C_{13}H_{14}N_4O$. (M_r 242.3). 1032500. [140-22-7]. 1,5-Diphenylcarbonodihydrazide.

A white, crystalline powder which gradually becomes pink on exposure to air, very slightly soluble in water, soluble in acetone, in alcohol and in glacial acetic acid.

mp: about 170 °C.

Sulphated ash (2.4.14): maximum 0.1 per cent.

Storage: protected from light.

Diphenylcarbazine solution. 1032501.

Dissolve 0.2 g of *diphenylcarbazine R* in 10 ml of *glacial acetic acid R* and dilute to 100 ml with *ethanol R*. Prepare immediately before use.

Diphenylcarbazon. $C_{13}H_{12}N_4O$. (M_r 240.3). 1032600. [538-62-5]. 1,5-Diphenylcarbazon.

An orange-yellow, crystalline powder, practically insoluble in water, freely soluble in alcohol.

mp: about 157 °C, with decomposition.

Diphenylcarbazon mercuric reagent. 1032601.

Solution I. Dissolve 0.1 g of *diphenylcarbazon R* in *ethanol R* and dilute to 50 ml with the same solvent.

Solution II. Dissolve 1 g of *mercuric chloride R* in *ethanol R* and dilute to 50 ml with the same solvent.

Mix equal volumes of the two solutions.

1,2-Diphenylhydrazine. $C_{12}H_{12}N_2$. (M_r 184.3). 1140800. [122-66-7]. Hydrazobenzene. 1,2-Diphenyldiazane.

Orange powder.

mp: about 125 °C.

Diphenylmethanol. $C_{13}H_{12}O$. (M_r 184.2). 1145700. [91-01-0]. Benzhydrol.

A white, crystalline powder.

mp: about 66 °C.

Diphenyloxazole. $C_{15}H_{11}NO$. (M_r 221.3). 1032700. [92-71-7]. 2,5-Diphenyloxazole.

A white powder, practically insoluble in water, soluble in methanol, sparingly soluble in dioxan and in glacial acetic acid.

mp: about 70 °C.

$A_{1\text{ cm}}^{1\%}$: about 1260 determined at 305 nm in *methanol R*.

Diphenyloxazole used for liquid scintillation is of a suitable analytical grade.

Diphenylphenylene oxide polymer. 1032800.
2,6-Diphenyl-*p*-phenylene oxide polymer.

White or almost white, porous beads. The size range of the beads is specified after the name of the reagent in the tests where it is used.

Diphosphorus pentoxide. P_2O_5 . (M_r 141.9). 1032900.
[1314-56-3]. Phosphorus pentoxide. Phosphoric anhydride.
A white powder, amorphous, deliquescent. It is hydrated by water with the evolution of heat.
Storage: in an airtight container.

Dipotassium hydrogen phosphate. K_2HPO_4 . (M_r 174.2). 1033000. [7758-11-4].

A white, crystalline powder, hygroscopic, very soluble in water, slightly soluble in alcohol.
Storage: in an airtight container.

Dipotassium sulphate. K_2SO_4 . (M_r 174.3). 1033100.
[7778-80-5].

Colourless crystals, soluble in water.

Disodium arsenate. $Na_2HAsO_4 \cdot 7H_2O$. (M_r 312.0). 1102500.
[10048-95-0]. Disodium hydrogen arsenate heptahydrate.
Dibasic sodium arsenate.

Crystals, efflorescent in warm air, freely soluble in water, soluble in glycerol, slightly soluble in alcohol. The aqueous solution is alkaline to litmus.

d_{20}^{20} : about 1.87.

mp: about 57 °C when rapidly heated.

Disodium bicinchoninate. $C_{20}H_{10}N_2Na_2O_4$. (M_r 388.3). 1126600. [979-88-4]. Disodium 2,2'-biquinoline-4,4'-dicarboxylate.

Disodium hydrogen citrate. $C_6H_6Na_2O_7 \cdot 1\frac{1}{2}H_2O$. (M_r 263.1). 1033200. [144-33-2]. Sodium acid citrate.
Disodium hydrogen 2-hydroxypropane-1,2,3-tricarboxylate sesquihydrate.

A white powder, soluble in less than 2 parts of water, practically insoluble in alcohol.

Disodium hydrogen phosphate. 1033300. [10039-32-4].
See *Disodium phosphate dodecahydrate* (0118).

Disodium hydrogen phosphate solution. 1033301.

A 90 g/l solution.

Disodium hydrogen phosphate, anhydrous. Na_2HPO_4 . (M_r 142.0). 1033400. [7558-79-4].

Disodium hydrogen phosphate dihydrate. 1033500.
[10028-24-7].

See *Disodium phosphate dihydrate* (0602).

Disodium tetraborate. 1033600. [1303-96-4].

See *Borax* (0013).

Borate solution. 1033601.

Dissolve 9.55 g of *disodium tetraborate R* in *sulphuric acid R*, heating on a water-bath, and dilute to 1 litre with the same acid.

Ditalimphos. $C_{12}H_{14}NO_4PS$. (M_r 299.3). 1126700.
[5131-24-8]. *O,O*-Diethyl (1,3-dihydro-1,3-dioxo-2*H*-isoindol-2-yl)phosphonothioate.

Very slightly soluble in water, in ethyl acetate and in ethanol. A suitable certified reference solution may be used.

5,5'-Dithiobis(2-nitrobenzoic acid). $C_{14}H_8N_2O_8S_2$. (M_r 396.4). 1097300. [69-78-3]. 3-Carboxy-4-nitrophenyldisulphide. Ellman's reagent. DTNB.

Yellow powder sparingly soluble in alcohol.

mp: about 242 °C.

Dithiol. $C_7H_8S_2$. (M_r 156.3). 1033800. [496-74-2]. Toluene-3,4-dithiol. 4-Methylbenzene-1,2-dithiol.

White crystals, hygroscopic, soluble in methanol and in solutions of alkali hydroxides.

mp: about 30 °C.

Storage: in an airtight container.

Dithiol reagent. 1033801.

To 1 g of *dithiol R* add 2 ml of *thioglycollic acid R* and dilute to 250 ml with a 20 g/l solution of *sodium hydroxide R*. Prepare immediately before use.

Dithiothreitol. $C_4H_{10}O_2S_2$. (M_r 154.2). 1098200.
[27565-41-9]. *threo*-1,4-Dimercaptobutane-2,3-diol.

Slightly hygroscopic needles, freely soluble in water, in acetone and in ethanol.

Storage: in an airtight container.

Dithizone. $C_{13}H_{12}N_4S$. (M_r 256.3). 1033900. [60-10-6]. 1,5-Diphenylthiocarbazone.

A bluish-black, brownish-black or black powder, practically insoluble in water, soluble in alcohol.

Storage: protected from light.

Dithizone solution. 1033901.

A 0.5 g/l solution in *chloroform R*. Prepare immediately before use.

Dithizone solution R2. 1033903.

Dissolve 40.0 mg of *dithizone R* in *chloroform R* and dilute to 1000.0 ml with the same solvent. Dilute 30.0 ml of the solution to 100.0 ml with *chloroform R*.

Standardisation. Dissolve a quantity of *mercuric chloride R* equivalent to 0.1354 g of $HgCl_2$ in a mixture of equal volumes of *dilute sulphuric acid R* and *water R* and dilute to 100.0 ml with the same mixture of solvents. Dilute 2.0 ml of this solution to 100.0 ml with a mixture of equal volumes of *dilute sulphuric acid R* and *water R*. (This solution contains 20 ppm of Hg). Transfer 1.0 ml of the solution to a separating funnel and add 50 ml of *dilute sulphuric acid R*, 140 ml of *water R* and 10 ml of a 200 g/l solution of *hydroxylamine hydrochloride R*. Titrate with the dithizone solution; after each addition, shake the mixture twenty times and towards the end of the titration allow to separate and discard the chloroform layer. Titrate until a bluish-green colour is obtained. Calculate the equivalent in micrograms of mercury per millilitre of the dithizone solution from the expression $20/V$, where V is the volume in millilitres of the dithizone solution used in the titration.

Dithizone R1. $C_{13}H_{12}N_4S$. (M_r 256.3). 1105500. [60-10-6]. 1,5-Diphenylthiocarbazone.

Content: minimum 98.0 per cent of $C_{13}H_{12}N_4S$.

A bluish-black, brownish-black or black powder, practically insoluble in water, soluble in alcohol.

Storage: protected from light.

Divanadium pentoxide. V_2O_5 . (M_r 181.9). 1034000. [1314-62-1]. Vanadic anhydride.

Content: minimum 98.5 per cent of V_2O_5 .

A yellow-brown to rust-brown powder, slightly soluble in water, soluble in strong mineral acids and in solutions of alkali hydroxides with formation of salts.

Appearance of solution. Heat 1 g for 30 min with 10 ml of *sulphuric acid R*. Allow to cool and dilute to 10 ml with the same acid. The solution is clear (2.2.1).

Sensitivity to hydrogen peroxide. Dilute 1.0 ml of the solution prepared for the test for appearance of solution cautiously to 50.0 ml with *water R*. To 0.5 ml of the solution add 0.1 ml of a solution of *hydrogen peroxide R* (0.1 g/l of H_2O_2). The solution has a distinct orange colour compared with a blank prepared from 0.5 ml of the solution to be examined and 0.1 ml of *water R*. After the addition of 0.4 ml of hydrogen peroxide solution (0.1 g/l H_2O_2), the orange solution becomes orange-yellow.

Loss on ignition: maximum 1.0 per cent, determined on 1.00 g at 700 °C.

Assay. Dissolve 0.200 g with heating in 20 ml of a 70 per cent *m/m* solution of *sulphuric acid R*. Add 100 ml of *water R* and 0.02 *M* *potassium permanganate* until a reddish colour is obtained. Decolorise the excess of potassium permanganate by the addition of a 30 g/l solution of *sodium nitrite R*. Add 5 g of *urea R* and 80 ml of a 70 per cent *m/m* solution of *sulphuric acid R*. Cool. Using 0.1 ml of *ferroin R* as indicator, titrate the solution immediately with 0.1 *M* *ferrous sulphate* until a greenish-red colour is obtained.

1 ml of 0.1 *M* *ferrous sulphate* is equivalent to 9.095 mg of V_2O_5 .

Divanadium pentoxide solution in sulphuric acid. 1034001.

Dissolve 0.2 g of *divanadium pentoxide R* in 4 ml of *sulphuric acid R* and dilute to 100 ml with *water R*.

Docosaheptaenoic acid methyl ester. $C_{23}H_{34}O_2$. (M_r 342.5). 1142800. [301-01-9]. DHA methyl ester. Cervonic acid methyl ester. (all-*Z*)-Docosa-4,7,10,13,16,19-hexaenoic acid methyl ester.

Content: minimum 90.0 per cent of $C_{23}H_{34}O_2$, determined by gas chromatography.

Docusate sodium. 1034100. [577-11-7].

See *Docusate sodium* (1418).

Dodecyltrimethylammonium bromide. $C_{15}H_{34}BrN$. (M_r 308.4). 1135500. [1119-94-4]. *N,N,N*-Trimethyldodecan-1-aminium bromide.

White crystals.

mp: about 246 °C.

Dotriacontane. $C_{32}H_{66}$. (M_r 450.9). 1034200. [544-85-4]. *n*-Dotriacontane.

White plates, practically insoluble in water, sparingly soluble in hexane.

mp: about 69 °C.

Impurities. Not more than 0.1 per cent of impurities with the same t_R value as α -tocopherol acetate, determined by the gas chromatographic method prescribed in the monograph on α -Tocopherol acetate (0439).

Doxycycline. 1145800.

See *Doxycycline monohydrate* (0820).

Electrolyte reagent for the micro determination of water. 1113700.

Commercially available anhydrous reagent or a combination of anhydrous reagents for the coulometric titration of water, containing suitable organic bases, sulphur dioxide and iodide dissolved in a suitable solvent.

Elementary standard solution for atomic spectrometry (1.000 g/l). 5004000.

This solution is prepared, generally in acid conditions, from the element or a salt of the element whose minimum content is not less than 99.0 per cent. The quantity per litre of solution is greater than 0.995 g throughout the guaranteed period, as long as the vial has not been opened. The starting material (element or salt) and the characteristics of the final solvent (nature and acidity, etc.) are mentioned on the label.

Emetine dihydrochloride. 1034300. [316-42-7].

See *Emetine hydrochloride pentahydrate* (0081).

Emodin. $C_{15}H_{10}O_5$. (M_r 270.2). 1034400. [518-82-1]. 1,3,8-Trihydroxy-6-methylantraquinone.

Orange-red needles, practically insoluble in water, soluble in alcohol and in solutions of alkali hydroxides.

Chromatography. Examine as prescribed in the monograph on *Rhubarb* (0291); the chromatogram shows only one principal spot.

α -Endosulphan. $C_9H_6Cl_6O_3S$. (M_r 406.9). 1126800. [959-98-8].

bp: about 200 °C.

mp: about 108 °C.

A suitable certified reference solution (10 ng/ μ l in cyclohexane) may be used.

β -Endosulphan. $C_9H_6Cl_6O_3S$. (M_r 406.9). 1126900. [33213-65-9].

bp: about 390 °C.

mp: about 207 °C.

A suitable certified reference solution (10 ng/ μ l in cyclohexane) may be used.

Endrin. $C_{12}H_8Cl_6O$. (M_r 380.9). 1127000. [72-20-8].

A suitable certified reference solution (10 ng/ μ l in cyclohexane) may be used.

Erucamide. $C_{22}H_{43}NO$. (M_r 337.6). 1034500. [112-84-5]. (*Z*)-Docos-13-enoamide.

Yellowish or white powder or granules, practically insoluble in water, very soluble in methylene chloride, soluble in ethanol.

mp: about 70 °C.

Erythritol. 1113800. [149-32-6].

See *Erythritol* (1803).

Esculin. $C_{15}H_{16}O_9 \cdot 1\frac{1}{2}H_2O$. (M_r 367.3). 1119400. [531-75-9]. 6-(β -D-Glucopyranosyloxy)-7-hydroxy-2H-chromen-2-one.

A white to almost white powder or colourless crystals, sparingly soluble in water and in alcohol, freely soluble in hot water and in hot alcohol.

Chromatography (2.2.27). Examine as prescribed in the monograph on *Eleutherococcus* (1419). The chromatogram shows only one principal spot.

Estradiol. $C_{18}H_{24}O_2$. (M_r 272.4). 1135600. [50-28-2].
Estra-1,3,5(10)-triene-3,17 β -diol. β -Estradiol.

Prisms stable in air, practically insoluble in water, freely soluble in alcohol, soluble in acetone and in dioxane, sparingly soluble in vegetable oils.

mp: 173 °C to 179 °C.

17 α -Estradiol. $C_{18}H_{24}O_2$. (M_r 272.4). 1034600. [57-91-0].

A white or almost white, crystalline powder or colourless crystals.

mp: 220 °C to 223 °C.

Estragole. $C_{10}H_{12}O$. (M_r 148.2). 1034700. [140-67-0].
1-Methoxy-4-prop-2-enylbenzene.

Liquid, miscible with alcohol.

n_D^{20} : about 1.52.

bp: about 216 °C.

Estragole used in gas chromatography complies with the following test.

Assay. Examine by gas chromatography (2.2.28) under the conditions described in the monograph on *Anise oil* (0804) using the substance to be examined as the test solution.

The area of the principal peak is not less than 98.0 per cent of the total area of the peaks.

Ethanol. 1034800. [64-17-5].

See *Ethanol, anhydrous R*.

Ethanol, anhydrous. 1034800. [64-17-5].

See *Ethanol, anhydrous* (1318).

Ethanol R1. 1034801.

Complies with the requirements prescribed for the monograph *Ethanol, anhydrous* (1318) and with the following requirement.

Methanol: maximum 0.005 per cent V/V, determined by gas chromatography (2.2.28).

Test solution. Use the substance to be examined.

Reference solution. Dilute 0.50 ml of *anhydrous methanol R* to 100.0 ml with the substance to be examined. Dilute 1.0 ml of this solution to 100.0 ml with the substance to be examined.

The chromatographic procedure may be carried out using:

- a glass column 2 m long and 2 mm in internal diameter packed with *ethylvinylbenzene-divinyl-benzene copolymer R* (75 μ m to 100 μ m),
- *nitrogen for chromatography R* as the carrier gas at a flow rate of 30 ml/min,
- a flame-ionisation detector.

Maintain the temperature of the column at 130 °C, that of the injection port at 150 °C and that of the detector at 200 °C.

Inject 1 μ l of the test solution and 1 μ l of the reference solution, alternately, three times. After each chromatography, heat the column to 230 °C for 8 min. Integrate the methanol peak. Calculate the percentage methanol content from the expression:

$$\frac{a \times b}{c - b}$$

- a = percentage V/V content of methanol in the reference solution,
- b = area of the methanol peak in the chromatogram obtained with the test solution,
- c = area of the methanol peak in the chromatogram obtained with the reference solution.

Ethanol (96 per cent). 1002500. [64-17-5].

See *Ethanol (96 per cent)* (1317).

Ethanol (x per cent V/V). 1002502.

Mix appropriate volumes of *water R* and *ethanol (96 per cent) R*, allowing for the effects of warming and volume contraction inherent to the preparation of such a mixture, to obtain a solution whose final content of ethanol corresponds to the value of x .

Ethanolamine. C_2H_7NO . (M_r 61.1). 1034900. [141-43-5].
2-Aminoethanol.

A clear, colourless, viscous, hygroscopic liquid, miscible with water and with methanol.

d_{20}^{20} : about 1.04.

n_D^{20} : about 1.454.

mp: about 11 °C.

Storage: in an airtight container.

Ether. $C_4H_{10}O$. (M_r 74.1). 1035000. [60-29-7].

A clear, colourless, volatile and very mobile liquid, very flammable, hygroscopic, soluble in water, miscible with alcohol.

d_{20}^{20} : 0.713 to 0.715.

bp: 34 °C to 35 °C.

Do not distil if the ether does not comply with the test for peroxides.

Peroxides. Place 8 ml of *potassium iodide and starch solution R* in a 12 ml ground-glass-stoppered cylinder about 1.5 cm in diameter. Fill completely with the substance to be examined, shake vigorously and allow to stand in the dark for 30 min. No colour is produced.

The name and concentration of any added stabilisers are stated on the label.

Storage: in an airtight container, protected from light, at a temperature not exceeding 15 °C.

Ether, peroxide-free. 1035100.

See *Anaesthetic ether* (0367).

Ethion. $C_9H_{22}O_4P_2S_4$. (M_r 384.5). 1127100. [563-12-2].

mp: –24 °C to –25 °C.

A suitable certified reference solution (10 ng/ μ l in cyclohexane) may be used.

Ethoxychrysoidine hydrochloride. $C_{14}H_{17}ClN_4O$. (M_r 292.8). 1035200. [2313-87-3]. 4-[(4-Ethoxyphenyl)diazonyl]phenylene-1,3-diamine hydrochloride.

A reddish powder, soluble in alcohol.

Ethoxychrysoidine solution. 1035201.

A 1 g/l solution in *alcohol R*.

Test for sensitivity. To a mixture of 5 ml of *dilute hydrochloric acid R* and 0.05 ml of the ethoxy-chrysoidine solution add 0.05 ml of 0.0167 M *bromide-bromate*. The colour changes from red to light yellow within 2 min.

Ethyl acetate. $C_4H_8O_2$. (M_r 88.1). 1035300. [141-78-6].

A clear, colourless liquid, soluble in water, miscible with alcohol.

d_{20}^{20} : 0.901 to 0.904.

bp: 76 °C to 78 °C.

Ethyl acetate, treated. 1035301.

Disperse 200 g of *sulphamic acid R* in *ethyl acetate R* and make up to 1000 ml with the same solvent. Stir the suspension obtained for three days and filter through a filter paper.

Storage: use within 1 month.

Ethyl acrylate. $C_5H_8O_2$. (M_r 100.1). 1035400. [140-88-5]. Ethyl prop-2-enoate.

A colourless liquid.

d_{20}^{20} : about 0.924.

n_D^{20} : about 1.406.

bp: about 99 °C.

mp: about – 71 °C.

4-[(Ethylamino)methyl]pyridine. $C_8H_{12}N_2$. (M_r 136.2). 1101300. [33403-97-3].

A pale yellow liquid.

d_{20}^{20} : about 0.98.

n_D^{20} : about 1.516.

bp: about 98 °C.

Ethylbenzene. C_8H_{10} . (M_r 106.2). 1035800. [100-41-4].

Content: minimum 99.5 per cent *m/m* of C_8H_{10} , determined by gas chromatography. A clear, colourless liquid, practically insoluble in water, soluble in acetone, and in alcohol.

d_{20}^{20} : about 0.87.

n_D^{20} : about 1.496.

bp: about 135 °C.

Ethyl benzoate. $C_9H_{10}O_2$. (M_r 150.2). 1135700. [93-89-0].

A clear, colourless, refractive liquid, practically insoluble in water, miscible with alcohol and with light petroleum.

d_4^{25} : about 1.050.

n_D^{20} : about 1.506.

bp: 211 °C to 213 °C.

Ethyl 5-bromovalerate. $C_7H_{13}BrO_2$. (M_r 209.1). 1142900. [14660-52-7]. Ethyl 5-bromopentanoate.

Clear, colourless liquid.

d_{20}^{20} : about 1.321.

bp: 104 °C to 109 °C.

Ethyl cyanoacetate. $C_5H_7NO_2$. (M_r 113.1). 1035500. [105-56-6].

A colourless to pale yellow liquid, slightly soluble in water, miscible with alcohol.

bp: 205 °C to 209 °C, with decomposition.

Ethylene chloride. $C_2H_4Cl_2$. (M_r 99.0). 1036000. [107-06-2]. 1,2-Dichloroethane.

A clear, colourless liquid, soluble in about 120 parts of water and in 2 parts of alcohol.

d_{20}^{20} : about 1.25.

Distillation range (2.2.11). Not less than 95 per cent distils between 82 °C and 84 °C.

Ethylenediamine. $C_2H_8N_2$. (M_r 60.1). 1036500. [107-15-3]. Ethane-1,2-diamine.

A clear, colourless, fuming liquid, strongly alkaline, miscible with water and with alcohol.

bp: about 116 °C.

Ethylene bis[3,3-di(3-*tert*-butyl-4-hydroxyphenyl)butyrate]. 1035900. [32509-66-3].

See *ethylene bis[3,3-di(3-(1,1-dimethylethyl)-4-hydroxyphenyl)butyrate] R*.

Ethylene bis[3,3-di(3-(1,1-dimethylethyl)-4-hydroxyphenyl)butyrate]. $C_{50}H_{66}O_8$. (M_r 795). 1035900. [32509-66-3]. Ethylene bis[3,3-di(3-*tert*-butyl-4-hydroxyphenyl)butyrate].

A crystalline powder, practically insoluble in water and in light petroleum, very soluble in acetone and in methanol. mp: about 165 °C.

(Ethylenedinitrilo)tetra-acetic acid. $C_{10}H_{16}N_2O_8$. (M_r 292.2). 1105800. [60-00-4]. *N,N'*-1,2-Ethanediybis[*N*-(carboxymethyl)glycine]. Edetic acid.

A white crystalline powder, very slightly soluble in water. mp: about 250 °C, with decomposition.

Ethylene glycol. $C_2H_6O_2$. (M_r 62.1). 1036100. [107-21-1]. Ethane-1,2-diol.

A colourless, slightly viscous liquid, hygroscopic, miscible with water and with alcohol.

d_{20}^{20} : 1.113 to 1.115.

n_D^{20} : about 1.432.

bp: about 198 °C.

mp: about – 12 °C.

Acidity. To 10 ml add 20 ml of *water R* and 1 ml of *phenolphthalein solution R*. Not more than 0.15 ml of 0.02 *M* sodium hydroxide is required to change the colour of the indicator to pink.

Water (2.5.12): maximum 0.2 per cent

Ethylene glycol monoethyl ether. $C_4H_{10}O_2$. (M_r 90.1). 1036200. [110-80-5]. 2-Ethoxyethanol.

A clear, colourless liquid, miscible with water, with acetone and with alcohol.

d_{20}^{20} : about 0.93.

n_D^{25} : about 1.406

bp: about 135 °C.

Ethylene glycol monomethyl ether. $C_3H_8O_2$. (M_r 76.1). 1036300. [109-86-4]. 2-Methoxyethanol.

A clear, colourless liquid, miscible with water, with acetone and with alcohol.

d_{20}^{20} : about 0.97.

n_D^{20} : about 1.403.

bp: about 125 °C.

Ethylene oxide. C_2H_4O . (M_r 44.05). 1036400. [75-21-8]. Oxirane.

Colourless, flammable gas, very soluble in water and in ethanol.

Liquefaction point: about 12 °C.

Ethylene oxide solution. 1036402.

Weigh a quantity of cool *ethylene oxide stock solution R* equivalent to 2.5 mg of ethylene oxide into a cool flask and dilute to 50.0 g with *macrogol 200 R1*. Mix well and dilute 2.5 g of this solution to 25.0 ml with *macrogol 200 R1* (5 µg of ethylene oxide per gram of solution). Prepare immediately before use.

Ethylene oxide solution R1. 1036403.

Dilute 1.0 ml of cooled *ethylene oxide stock solution R* (check the exact volume by weighing) to 50.0 ml with *macrogol 200 R1*. Mix well and dilute 2.5 g of this solution to 25.0 ml with *macrogol 200 R1*. Calculate the exact amount of ethylene oxide in ppm from the volume determined by weighing and taking the density of *macrogol 200 R1* as 1.127. Prepare immediately before use.

Ethylene oxide solution R2. 1036404.

Weigh 1.00 g of cold *ethylene oxide stock solution R* (equivalent to 2.5 mg of ethylene oxide) into a cold flask containing 40.0 g of cold *macrogol 200 R1*. Mix and determine the exact mass and dilute to a calculated mass to obtain a solution containing 50 µg of ethylene oxide per gram of solution. Weigh 10.00 g into a flask containing about 30 ml of *water R*, mix and dilute to 50.0 ml with *water R* (10 µg/ml of ethylene oxide). Prepare immediately before use.

Ethylene oxide solution R3. 1036405.

Dilute 10.0 ml of *ethylene oxide solution R2* to 50.0 ml with *water R* (2 µg/ml of ethylene oxide). Prepare immediately before use.

Ethylene oxide solution R4. 1036407.

Dilute 1.0 ml of *ethylene oxide stock solution R1* to 100.0 ml with *water R*. Dilute 1.0 ml of this solution to 25.0 ml with *water R*.

Ethylene oxide solution R5. 1036408.

A 50 g/l solution of *ethylene oxide R* in *methylene chloride R*.

Either use a commercially available reagent or prepare the solution corresponding to the above-mentioned composition.

Ethylene oxide stock solution. 1036401.

All operations carried out in the preparation of these solutions must be conducted in a fume-hood. The operator must protect both hands and face by wearing polyethylene protective gloves and an appropriate face mask.

Store all solutions in an airtight container in a refrigerator at 4 °C to 8 °C. Carry out all determinations three times.

Into a dry, clean test-tube, cooled in a mixture of 1 part of *sodium chloride R* and 3 parts of crushed ice, introduce a slow current of *ethylene oxide R* gas, allowing condensation onto the inner wall of the test-tube. Using a glass syringe, previously cooled to –10 °C, inject about 300 µl (corresponding to about 0.25 g) of liquid *ethylene oxide R* into 50 ml of *macrogol 200 R1*. Determine the absorbed quantity of ethylene oxide by weighing before and after absorption (M_{e_0}). Dilute to 100.0 ml with *macrogol 200 R1*. Mix well before use.

Assay. To 10 ml of a 500 g/l suspension of *magnesium chloride R* in *ethanol R* add 20.0 ml of 0.1 M *alcoholic hydrochloric acid* in a flask. Stopper and shake to obtain a saturated solution and allow to stand overnight to equilibrate. Weigh 5.00 g of *ethylene oxide stock solution (2.5 g/l) R* into the flask and allow to stand for 30 min. Titrate with 0.1 M *alcoholic potassium hydroxide* determining the end-point potentiometrically (2.2.20).

Carry out a blank titration, replacing the substance to be examined with the same quantity of *macrogol 200 R1*.

Ethylene oxide content in milligrams per gram is given by:

$$\frac{(V_0 - V_1) \times f \times 4.404}{m}$$

Where V_0 and V_1 are the volumes of alcoholic potassium hydroxide used respectively for the blank titration and the assay,

f = factor of the alcoholic potassium hydroxide solution,

m = mass of the sample taken (g).

Ethylene oxide stock solution R1. 1036406.

A 50 mg/ml solution of *ethylene oxide R* in *methanol R*.

Ethyl formate. $C_3H_6O_2$. (M_r 74.1). 1035600. [109-94-4]. Ethyl methanoate.

A clear, colourless, flammable liquid, freely soluble in water, miscible with alcohol.

d_{20}^{20} : about 0.919.

n_D^{20} : about 1.36.

bp: about 54 °C.

2-Ethylhexane-1,3-diol. $C_8H_{18}O_2$. (M_r 146.2). 1105900. [94-96-2].

A slightly oily liquid, soluble in ethanol, 2-propanol, propylene glycol and castor oil.

d_{20}^{20} : about 0.942.

n_D^{20} : about 1.451.

bp: about 244 °C.

2-Ethylhexanoic acid. $C_8H_{16}O_2$. (M_r 144.2). 1036600. [149-57-5].

A colourless liquid.

d_{20}^{20} : about 0.91.

n_D^{20} : about 1.425.

Related substances. Examine by gas chromatography (2.2.28). Inject 1 µl of a solution prepared as follows: suspend 0.2 g of the 2-ethylhexanoic acid in 5 ml of *water R*, add 3 ml of *dilute hydrochloric acid R* and 5 ml of *hexane R*, shake for 1 min, allow the layers to separate and use the upper layer. Carry out the chromatographic procedure as prescribed in the test for 2-ethylhexanoic acid in the monograph on *Amoxicillin sodium* (0577). The sum of the area of any peaks, apart from the principal peak and the peak due to the solvent, is not greater than 2.5 per cent of the area of the principal peak.

N-Ethylmaleimide. $C_6H_7NO_2$. (M_r 125.1). 1036700. [128-53-0]. 1-Ethyl-1H-pyrrole-2,5-dione.

Colourless crystals, sparingly soluble in water, freely soluble in alcohol.

mp: 41 °C to 45 °C.

Storage: at a temperature of 2 °C to 8 °C.

Ethyl methyl ketone. 1054100. [78-93-3].

See *methyl ethyl ketone R*.

2-Ethyl-2-methylsuccinic acid. $C_7H_{12}O_4$. (M_r 160.2). 1036800. [631-31-2]. 2-Ethyl-2-methylbutanedioic acid. mp: 104 °C to 107 °C.

Ethyl parahydroxybenzoate. 1035700. [120-47-8].

See *Ethyl parahydroxybenzoate* (0900).

2-Ethylpyridine. C_7H_9N . (M_r 107.2). 1133400. [100-71-0].

Colourless or brownish liquid.

d_{20}^{20} : about 0.939.

n_D^{20} : about 1.496.

bp: about 149 °C.

Ethylvinylbenzene-divinylbenzene copolymer. 1036900.

Porous, rigid, cross-linked polymer beads. Several grades are available with different sizes of bead. The size range of the beads is specified after the name of the reagent in the tests where it is used.

Ethylvinylbenzene-divinylbenzene copolymer R1. 1036901.

Porous, rigid, cross-linked polymer beads, with a nominal specific surface area of 500 m²/g to 600 m²/g and having pores with a mean diameter of 7.5 nm. Several grades are available with different sizes of beads. The size range of the beads is specified after the name of the reagent in the tests where it is used.

Eugenol. C₁₀H₁₂O₂. (*M_r* 164.2). 1037000. [97-53-0]. 4-Allyl-2-methoxyphenol.

A colourless or pale yellow, oily liquid, darkening on exposure to air and light and becoming more viscous, practically insoluble in water, miscible with alcohol and with fatty and essential oils.

d_{20}^{20} : about 1.07.

bp: about 250 °C.

Eugenol used in gas chromatography complies with the following additional test.

Assay. Examine by gas chromatography (2.2.28) as prescribed in the monograph on *Clove oil* (1091) using the substance to be examined as the test solution.

The area of the principal peak is not less than 98.0 per cent of the total area of the peaks.

Storage: protected from light.

Euglobulins, bovine. 1037100.

Use fresh bovine blood collected into an anticoagulant solution (for example, sodium citrate solution). Discard any haemolysed blood. Centrifuge at 1500 *g* to 1800 *g* at 15 °C to 20 °C to obtain a supernatant plasma poor in platelets.

To 1 litre of bovine plasma add 75 g of *barium sulphate R* and shake for 30 min. Centrifuge at not less than 1500 *g* to 1800 *g* at 15 °C to 20 °C and draw off the clear supernatant liquid. Add 10 ml of a 0.2 mg/ml solution of *aprotinin R* and shake to ensure mixing. In a container with a minimum capacity of 30 litres in a chamber at 4 °C introduce 25 litres of *distilled water R* at 4 °C and add about 500 g of solid carbon dioxide. Immediately add, while stirring, the supernatant liquid obtained from the plasma. A white precipitate is formed. Allow to settle at 4 °C for 10 h to 15 h. Remove the clear supernatant solution by siphoning. Collect the precipitate by centrifuging at 4 °C. Suspend the precipitate by dispersing mechanically in 500 ml of *distilled water R* at 4 °C, shake for 5 min and collect the precipitate by centrifuging at 4 °C. Disperse the precipitate mechanically in 60 ml of a solution containing 9 g/l of *sodium chloride R* and 0.9 g/l *sodium citrate R* and adjust to pH 7.2 to 7.4 by adding a 10 g/l solution of *sodium hydroxide R*. Filter through a sintered glass filter; to facilitate the dissolution of the precipitate crush the particles of the precipitate with a suitable instrument. Wash the filter and the instrument with 40 ml of the chloride-citrate solution described above and dilute to 100 ml with the same solution. Freeze-dry the solution. The yields are generally 6 g to 8 g of euglobulins per litre of bovine plasma.

Test for suitability. For this test, prepare the solutions using *phosphate buffer solution pH 7.4 R* containing 30 g/l of *bovine albumin R*.

Into a test-tube 8 mm in diameter placed in a water-bath at 37 °C introduce 0.2 ml of a solution of a reference preparation of urokinase containing 100 IU/ml and 0.1 ml of a solution of *human thrombin R* containing 20 IU/ml. Add rapidly 0.5 ml of a solution containing 10 mg of bovine euglobulins per millilitre. A firm clot forms in less than 10 s. Note the time that elapses between the addition of the solution of bovine euglobulins and the lysis of the clot. The lysis time does not exceed 15 min.

Storage: protected from moisture at 4 °C; use within 1 year.

Euglobulins, human. 1037200.

For the preparation, use fresh human blood collected into an anticoagulant solution (for example sodium citrate solution) or human blood for transfusion that has been collected in plastic blood bags and which has just reached its expiry date. Discard any haemolysed blood. Centrifuge at 1500 *g* to 1800 *g* at 15 °C to obtain a supernatant plasma poor in platelets. Iso-group plasmas may be mixed.

To 1 litre of the plasma add 75 g of *barium sulphate R* and shake for 30 min. Centrifuge at not less than 15 000 *g* at 15 °C and draw off the clear supernatant liquid. Add 10 ml of a solution of *aprotinin R* containing 0.2 mg/ml and shake to ensure mixing. In a container with a minimum capacity of 30 litres in a chamber at 4 °C introduce 25 litres of *distilled water R* at 4 °C and add about 500 g of solid carbon dioxide. Immediately add while stirring the supernatant liquid obtained from the plasma. A white precipitate is formed. Allow to settle at 4 °C for 10 h to 15 h. Remove the clear supernatant solution by siphoning. Collect the precipitate by centrifuging at 4 °C. Suspend the precipitate by dispersing mechanically in 500 ml of *distilled water R* at 4 °C, shake for 5 min and collect the precipitate by centrifuging at 4 °C. Disperse the precipitate mechanically in 60 ml of a solution containing 9 g/l of *sodium chloride R* and 0.9 g/l of *sodium citrate R*, and adjust the pH to 7.2 to 7.4 by adding a 10 g/l solution of *sodium hydroxide R*. Filter through a sintered-glass filter; to facilitate the dissolution of the precipitate crush the particles of the precipitate with a suitable instrument. Wash the filter and the instrument with 40 ml of the chloride-citrate solution described above and dilute to 100 ml with the same solution. Freeze-dry the solution. The yields are generally 6 g to 8 g of euglobulins per litre of human plasma.

Test for suitability. For this test, prepare the solutions using *phosphate buffer solution pH 7.2 R* containing 30 g/l of *bovine albumin R*. Into a test-tube 8 mm in diameter placed in a water-bath at 37 °C introduce 0.1 ml of a solution of a reference preparation of streptokinase containing 10 IU of streptokinase activity per millilitre and 0.1 ml of a solution of *human thrombin R* containing 20 IU/ml. Add rapidly 1 ml of a solution containing 10 mg of human euglobulins per millilitre. A firm clot forms in less than 10 s. Note the time that elapses between the addition of the solution of human euglobulins and the lysis of the clot. The lysis time does not exceed 15 min.

Storage: in an airtight container at 4 °C; use within 1 year.

Factor Xa, bovine, coagulation. 1037300. [9002-05-5].

An enzyme which converts prothrombin to thrombin. The semi-purified preparation is obtained from liquid bovine plasma and it may be prepared by activation of the zymogen factor X with a suitable activator such as Russell's viper venom.

Store freeze-dried preparation at –20 °C and frozen solution at a temperature lower than –20 °C.

Factor Xa solution, bovine. 1037301.

Reconstitute as directed by the manufacturer and dilute with *tris(hydroxymethyl)aminomethane sodium chloride buffer solution pH 7.4 R*.

Any change in the absorbance of the solution, measured at 405 nm (2.2.25) against *tris(hydroxymethyl)aminomethane sodium chloride buffer solution pH 7.4 R* as the blank is not more than 0.15 to 0.20 per minute.

Fast blue B salt. $C_{14}H_{12}Cl_2N_4O_2$. (M_r 339.2). 1037400. [84633-94-3].

Schultz No. 490.

Colour Index No. 37235.

3,3'-Dimethoxy(biphenyl)-4,4'-bisdiazonium dichloride.

A dark green powder, soluble in water. It is stabilised by addition of zinc chloride.

Storage: in an airtight container, at a temperature between 2 °C and 8 °C.

Fast red B salt. $C_{17}H_{13}N_3O_9S_2$. (M_r 467.4). 1037500. [56315-29-8].

Schultz No. 155.

Colour Index No. 37125.

2-Methoxy-4-nitrobenzenediazonium hydrogen naphthalene-1,5-disulphonate.

An orange-yellow powder, soluble in water, slightly soluble in alcohol.

Storage: in an airtight container, protected from light, at 2 °C to 8 °C.

Fenchlorphos. $C_8H_8Cl_3O_3PS$. (M_r 321.5). 1127200. [299-84-3].

mp: about 35 °C.

A suitable certified reference solution (10 ng/μl in cyclohexane) may be used.

Fenchone. $C_{10}H_{16}O$. (M_r 152.2). 1037600. [7787-20-4]. 1,3,3-Trimethylbicyclo[2.2.1]heptan-2-one.

An oily liquid, miscible with alcohol, practically insoluble in water.

n_D^{20} : about 1.46.

bp_{15mm}: about 66 °C.

Fenchone used in gas chromatography complies with the following test.

Assay. Examine by gas chromatography (2.2.28) under the conditions described in the monograph on *Bitter fennel* (0824) using the substance to be examined as the test solution.

The area of the principal peak is not less than 98.0 per cent of the total area of the peaks.

Fenvalerate. $C_{25}H_{22}ClNO_3$. (M_r 419.9). 1127300. [51630-58-1].

bp: about 300 °C.

A suitable certified reference solution (10 ng/μl in cyclohexane) may be used.

Ferric ammonium sulphate. $FeNH_4(SO_4)_2 \cdot 12H_2O$. (M_r 482.2). 1037700. [7783-83-7]. Ammonium iron disulphate dodecahydrate.

Pale-violet crystals, efflorescent, very soluble in water, practically insoluble in alcohol.

Ferric ammonium sulphate solution R2. 1037702.

A 100 g/l solution. If necessary filter before use.

Ferric ammonium sulphate solution R5. 1037704.

Shake 30.0 g of *ferric ammonium sulphate R* with 40 ml of *nitric acid R* and dilute to 100 ml with *water R*. If the solution is turbid, centrifuge or filter it.

Storage: protected from light.

Ferric ammonium sulphate solution R6. 1037705.

Dissolve 20 g of *ferric ammonium sulphate R* in 75 ml of *water R*, add 10 ml of a 2.8 per cent V/V solution of *sulphuric acid R* and dilute to 100 ml with *water R*.

Ferric chloride. $FeCl_3 \cdot 6H_2O$. (M_r 270.3). 1037800. [10025-77-1]. Iron trichloride hexahydrate.

Yellowish-orange or brownish crystalline masses, deliquescent, very soluble in water, soluble in alcohol. On exposure to light, ferric chloride and its solutions are partly reduced.

Storage: in an airtight container.

Ferric chloride solution R1. 1037801.

A 105 g/l solution.

Ferric chloride solution R2. 1037802.

A 13 g/l solution.

Ferric sulphate pentahydrate. $Fe_2(SO_4)_3 \cdot 5H_2O$. (M_r 489.9). 1153700. [142906-29-4].

White or yellowish powder.

Ferric chloride solution R3. 1037803.

Dissolve 2.0 g of *ferric chloride R* in *ethanol R* and dilute to 100.0 ml with the same solvent.

Ferric chloride-sulphamic acid reagent. 1037804.

A solution containing 10 g/l of *ferric chloride R* and 16 g/l of *sulphamic acid R*.

Ferric nitrate. $Fe(NO_3)_3 \cdot 9H_2O$. (M_r 404). 1106100. [7782-61-8].

Content: minimum 99.0 per cent m/m of $Fe(NO_3)_3 \cdot 9H_2O$.

Light-purple crystals or crystalline mass, very soluble in water.

Free acid: not more than 0.3 per cent (as HNO_3).

Ferric sulphate. $Fe_2(SO_4)_3 \cdot xH_2O$. 1037900. [10028-22-5]. Iron(III) trisulphate hydrated.

A yellowish-white powder, very hygroscopic, decomposes in air, slightly soluble in water and in alcohol.

Storage: in an airtight container, protected from light.

Ferrocyphe. $C_{26}H_{16}FeN_6$. (M_r 468.3). 1038000. [14768-11-7]. Dicyanobis(1,10-phenanthroline)iron(II).

A violet-bronze, crystalline powder, practically insoluble in water and in alcohol.

Storage: protected from light and moisture.

Ferroin. 1038100. [14634-91-4].

Dissolve 0.7 g of *ferrous sulphate R* and 1.76 g of *phenanthroline hydrochloride R* in 70 ml of *water R* and dilute to 100 ml with the same solvent.

Test for sensitivity. To 50 ml of *dilute sulphuric acid R* add 0.15 ml of *osmium tetroxide solution R* and 0.1 ml of the ferroin. After the addition of 0.1 ml of 0.1 M ammonium and cerium nitrate the colour changes from red to light blue.

Ferrous ammonium sulphate. $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}$. (M_r 392.2). 1038200. [7783-85-9]. Diammonium iron disulphate hexahydrate.

Pale bluish-green crystals or granules, freely soluble in water, practically insoluble in alcohol.

Storage: protected from light.

Ferrous sulphate. 1038300. [7782-63-0].

See *Ferrous sulphate* (0083).

Ferrous sulphate solution R2. 1038301.

Dissolve 0.45 g of *ferrous sulphate R* in 50 ml of 0.1 M hydrochloric acid and dilute to 100 ml with carbon dioxide-free water *R*. Prepare immediately before use.

Ferulic acid. $\text{C}_{10}\text{H}_{10}\text{O}_4$. (M_r 194.2). 1149500. [1135-24-6]. 4-Hydroxy-3-methoxycinnamic acid.

3-(4-Hydroxy-3-methoxyphenyl)propenoic acid.

Faint yellow powder, freely soluble in methanol.

mp: 172.9 °C to 173.9 °C.

Ferulic acid used in the assay of eleutherosides in Eleutherococcus (1419) complies with the following additional requirement.

Assay. Examine by liquid chromatography (2.2.29) as prescribed in the monograph on *Eleutherococcus* (1419).

The content is not less than 99 per cent, calculated by the normalisation procedure.

Fibrin blue. 1101400.

Mix 1.5 g of fibrin with 30 ml of a 5 g/l solution of *indigo carmine R* in 1 per cent V/V dilute hydrochloric acid *R*. Heat the mixture to 80 °C and maintain at this temperature whilst stirring for about 30 min. Allow to cool. Filter. Wash extensively by resuspension in 1 per cent V/V dilute hydrochloric acid *R* and mixing for about 30 min; filter. Repeat the washing operation three times. Dry at 50 °C. Grind.

Fibrin congo red. 1038400.

Take 1.5 g of fibrin and leave overnight in 50 ml of a 20 g/l solution of *congo red R* in alcohol (90 per cent V/V) *R*. Filter, rinse the fibrin with water *R* and store under ether *R*.

Fibrinogen. 1038500. [9001-32-5].

See *Human fibrinogen, freeze-dried* (0024).

Fixing solution. 1122600.

To 250 ml of methanol *R*, add 0.27 ml of formaldehyde *R* and dilute to 500.0 ml with water *R*.

Fixing solution for isoelectric focusing in polyacrylamide gel. 1138700.

A solution containing 35 g of sulphosalicylic acid *R* and 100 g of trichloroacetic acid *R* per litre of water *R*.

Flufenamic acid. $\text{C}_{14}\text{H}_{10}\text{F}_3\text{NO}_2$. (M_r 281.2). 1106200. [530-78-9]. 2-[[3-(Trifluoromethyl)phenyl]amino]benzoic acid.

Pale yellow, crystalline powder or needles, practically insoluble in water, freely soluble in alcohol.

mp: 132 °C to 135 °C.

Flumazenil. 1149600. [78755-81-4].

See *Flumazenil* (1326).

Flunitrazepam. 1153800. [1622-62-4].

See *Flunitrazepam* (0717).

Fluoranthene. $\text{C}_{16}\text{H}_{10}$. (M_r 202.3). 1038600. [206-44-0]. 1,2-(1,8-Naphtylene)benzene. 1,2-Benzacenaphtene.

Yellow or yellowish-brown crystals.

bp: about 384 °C.

mp: 109 °C to 110 °C.

Fluorene. $\text{C}_{13}\text{H}_{10}$. (M_r 166.2). 1127400. [86-73-7]. Diphenylenemethane.

White crystals, freely soluble in anhydrous acetic acid, soluble in hot alcohol.

mp: 113 °C to 115 °C.

Fluorescamine. $\text{C}_{17}\text{H}_{10}\text{O}_4$. (M_r 278.3). 1135800. [38183-12-9]. 4-Phenylspiro[furan-2(3H),1'(3'H)-isobenzofuran]-3,3'-dione.

mp: 154 °C to 155 °C.

Fluorescein. $\text{C}_{20}\text{H}_{12}\text{O}_5$. (M_r 332.3). 1106300. [2321-07-5]. 3',6'-Dihydroxyspiro[isobenzofurane-1(3H),9'-[9H]xanthen]-3-one.

An orange-red powder, practically insoluble in water, soluble in warm alcohol, soluble in alkaline solutions. In solution, fluorescein displays a green fluorescence.

mp: about 315 °C.

Fluorescein-conjugated rabies antiserum. 1038700.

Immunoglobulin fraction with a high rabies antibody titre, prepared from the sera of suitable animals that have been immunised with inactivated rabies virus; the immunoglobulin is conjugated with fluorescein isothiocyanate.

2-Fluoro-2-deoxy-D-glucose. $\text{C}_6\text{H}_{11}\text{FO}_5$. (M_r 182.2). 1113900. [86783-82-6].

A white crystalline powder.

mp: 174 °C to 176 °C.

Fluorodinitrobenzene. $\text{C}_6\text{H}_3\text{FN}_2\text{O}_4$. (M_r 186.1). 1038800. [70-34-8]. 1-Fluoro-2,4-dinitrobenzene.

Pale yellow crystals, soluble in propylene glycol.

mp: about 29 °C.

1-Fluoro-2-nitro-4-(trifluoromethyl)benzene. $\text{C}_7\text{H}_3\text{F}_4\text{NO}_2$. (M_r 209.1). 1038900. [367-86-2].

mp: about 197 °C.

Folic acid. 1039000. [75708-92-8].

See *Folic acid* (0067).

Formaldehyde. 1039100. [50-00-0].

See *Formaldehyde solution R*.

Formaldehyde solution. 1039101.

See *Formaldehyde solution* (35 per cent) (0826).

Formamide. CH_3NO . (M_r 45.0). 1039200. [75-12-7].

A clear, colourless, oily liquid, hygroscopic, miscible with water and with alcohol. It is hydrolysed by water.

bp: about 103 °C, determined at a pressure of 2 kPa.

Storage: in an airtight container.

Formamide R1. 1039202.

Complies with the requirements prescribed for *formamide R* and with the following additional test.

Water (2.5.12): maximum 0.1 per cent determined with an equal volume of *anhydrous methanol R*.

Formamide, treated. 1039201.

Disperse 1.0 g of *sulphamic acid R* in 20.0 ml of *formamide R* containing 5 per cent V/V of water *R*.

Formic acid, anhydrous. CH_2O_2 . (M_r 46.03). 1039300. [64-18-6].

Content: minimum 98.0 per cent m/m of CH_2O_2 .

A colourless liquid, corrosive, miscible with water and with alcohol.

d_{20}^{20} : about 1.22.

Assay. Weigh accurately a conical flask containing 10 ml of *water R*, quickly add about 1 ml of the acid and weigh again. Add 50 ml of *water R* and titrate with 1 M sodium hydroxide, using 0.5 ml of *phenolphthalein solution R* as indicator.

1 ml of 1 M sodium hydroxide is equivalent to 46.03 mg of CH_2O_2 .

Fructose. 1106400. [57-48-7].

See *Fructose* (0188).

Fuchsin, basic. 1039400. [632-99-5].

A mixture of rosaniline hydrochloride ($\text{C}_{20}\text{H}_{20}\text{ClN}_3$; M_r 337.9; Colour Index No. 42510; Schultz No. 780) and *para*-rosaniline hydrochloride ($\text{C}_{19}\text{H}_{18}\text{ClN}_3$; M_r 323.8; Colour Index No. 42500; Schultz No. 779).

If necessary, purify in the following manner. Dissolve 1 g in 250 ml of *dilute hydrochloric acid R*. Allow to stand for 2 h at room temperature, filter and neutralise with *dilute sodium hydroxide solution R* and add 1 ml to 2 ml in excess. Filter the precipitate through a sintered-glass filter (40) and wash with *water R*. Dissolve the precipitate in 70 ml of *methanol R*, previously heated to boiling, and add 300 ml of *water R* at 80 °C. Allow to cool to room temperature, filter and dry the crystals *in vacuo*.

Crystals with a greenish-bronze sheen, soluble in water and in alcohol.

Storage: protected from light.

Fuchsin solution, decolorised. 1039401.

Dissolve 0.1 g of *basic fuchsin R* in 60 ml of *water R*. Add a solution containing 1 g of *anhydrous sodium sulphite R* or 2 g of *sodium sulphite R* in 10 ml of *water R*. Slowly and with continuous shaking add 2 ml of *hydrochloric acid R*. Dilute to 100 ml with *water R*. Allow to stand protected from light for at least 12 h, decolorise with *activated charcoal R* and filter. If the solution becomes cloudy, filter before use. If on standing the solution becomes violet, decolorise again by adding *activated charcoal R*.

Test for sensitivity. To 1.0 ml add 1.0 ml of *water R* and 0.1 ml of *aldehyde-free alcohol R*. Add 0.2 ml of a solution containing 0.1 g/l of formaldehyde (CH_2O , M_r 30.0). A pale-pink colour develops within 5 min.

Storage: protected from light.

Fuchsin solution, decolorised R1. 1039402.

To 1 g of *basic fuchsin R* add 100 ml of *water R*. Heat to 50 °C and allow to cool with occasional shaking. Allow to stand for 48 h, shake and filter. To 4 ml of the filtrate add 6 ml of *hydrochloric acid R*, mix and dilute to 100 ml with *water R*. Allow to stand for at least 1 h before use.

Fucose. $\text{C}_6\text{H}_{12}\text{O}_5$. (M_r 164.2). 1039500. [6696-41-9]. 6-Deoxy-L-galactose.

A white powder, soluble in water and in alcohol.

$[\alpha]_D^{20}$: about -76, determined on a 90 g/l solution 24 h after dissolution.

mp: about 140 °C.

Fumaric acid. $\text{C}_4\text{H}_4\text{O}_4$. (M_r 116.1). 1153200. [110-17-8]. (*E*)-Butenedioic acid.

White crystals, slightly soluble in water, soluble in alcohol, slightly soluble in acetone.

mp: about 300 °C.

Furfural. $\text{C}_5\text{H}_4\text{O}_2$. (M_r 96.1). 1039600. [98-01-1]. 2-Furaldehyde. 2-Furanecarbaldehyde.

A clear, colourless to brownish-yellow, oily liquid, miscible in 11 parts of water, miscible with alcohol.

d_{20}^{20} : 1.155 to 1.161.

Distillation range (2.2.11). Not less than 95 per cent distils between 159 °C and 163 °C.

Storage: in a dark place.

Galactose. $\text{C}_6\text{H}_{12}\text{O}_6$. (M_r 180.2). 1039700. [59-23-4]. D-(+)-Galactose.

A white, crystalline powder, freely soluble in water.

$[\alpha]_D^{20}$: +79 to +81, determined on a 100 g/l solution in *water R* containing about 0.05 per cent of NH_3 .

Gallic acid. $\text{C}_7\text{H}_6\text{O}_5 \cdot \text{H}_2\text{O}$. (M_r 188.1). 1039800. [5995-86-8]. 3,4,5-Trihydroxybenzoic acid monohydrate.

A crystalline powder or long needles, colourless or slightly yellow, soluble in water, freely soluble in hot water, in alcohol and in glycerol.

It loses its water of crystallisation at 120 °C and it melts at about 260 °C, with decomposition.

Chromatography. Examine as prescribed in the monograph on *Bearberry leaf* (1054); the chromatogram shows only one principal spot.

Gastric juice, artificial. 1039900.

Dissolve 2.0 g of *sodium chloride R* and 3.2 g of *pepsin powder R* in *water R*. Add 80 ml of 1 M *hydrochloric acid* and dilute to 1000 ml with *water R*.

GC concentric column. 1135100.

A commercially available system consisting of 2 concentrically arranged tubes. The outer tube is packed with molecular sieves and the inner tube is packed with a porous polymer mixture. The main application is the separation of gases.

Gelatin. 1040000. [9000-70-8].

See *Gelatin* (0330).

Gelatin, hydrolysed. 1040100.

Dissolve 50 g of *gelatin R* in 1000 ml of *water R*. Autoclave in saturated steam at 121 °C for 90 min and freeze dry.

Geraniol. $\text{C}_{10}\text{H}_{18}\text{O}$. (M_r 154.2). 1135900. [106-24-1]. (*E*)-3,7-Dimethylocta-2,6-dien-1-ol.

An oily liquid, slight odour of rose, practically insoluble in water, miscible with alcohol.

d_{20}^{20} : 0.890.

n_D^{20} : 1.477.

bp: 229 °C to 230 °C.

Geraniol used in gas chromatography complies with the following additional test.

Assay. Examine by gas chromatography (2.2.28) as prescribed in the monograph on *Citronella oil* (1609).

The content is not less than 98.5 per cent calculated by the normalisation procedure.

Storage: in an airtight container, protected from light

Geranyl acetate. $C_{12}H_{20}O_2$, (M_r 196.3). 1106500. [105-87-3]. (*E*)-3,7-Dimethylocta-2,6-dien-1-yl acetate.

A colourless or slightly yellow liquid, slight odour of rose and lavender.

d_{25}^{25} : 0.896 to 0.913.

n_D^{15} : about 1.463.

bp_{25} : about 138 °C.

Geranyl acetate used in gas chromatography complies with the following additional test.

Assay. Examine by gas chromatography (2.2.28) as prescribed in the monograph on *Bitter-orange-flower oil* (1175), using the substance to be examined as the test solution. The area of the principal peak is not less than 99.0 per cent of the total area of the peaks.

Ginsenoside Rb1. $C_{54}H_{92}O_{23} \cdot 3H_2O$, (M_r 1163). 1127500. [41753-43-9]. (2*S*)-3 β -di-D-Glucopyranosyl-20-di-D-glucopyranosylprotopanaxadiol. (2*S*)-3 β -[(2-*O*- β -D-Glucopyranosyl- β -D-glucopyranosyl)oxy]-20-[(6-*O*- β -D-glucopyranosyl- β -D-glucopyranosyl)oxy]-5 α -dammar-24-en-12 β -ol. (2*S*)-3 β -[(2-*O*- β -D-Glucopyranosyl- β -D-glucopyranosyl)oxy]-20-[(6-*O*- β -D-glucopyranosyl- β -D-glucopyranosyl)oxy]-4,4,8,14-tetramethyl-18-nor-5 α -cholest-24-en-12 β -ol.

A colourless solid, soluble in water, in ethanol and in methanol.

$[\alpha]_D^{20}$: + 11.3 determined on a 10 g/l solution in *methanol R*.

mp: about 199 °C.

Water (2.5.12): maximum 6.8 per cent.

Assay. Examined by liquid chromatography (2.2.29) as prescribed in the monograph on *Ginseng* (1523).

Test solution. Dissolve 3.0 mg, accurately weighted, of *ginsenoside Rb1* in 10 ml of *methanol R*.

The content is not less than 95.0 per cent calculated by the normalisation procedure.

Ginsenoside Rf. $C_{42}H_{72}O_{14} \cdot 2H_2O$, (M_r 837). 1127700. [52286-58-5]. (2*S*)-6-*O*-[β -D-Glucopyranosyl-(1 $\rightarrow$ 2)- β -D-glycopyranoside]-dammar-24-ene-3 β ,6 α ,12 β ,20-tetrol.

A colourless solid, soluble in water, in ethanol and in methanol.

$[\alpha]_D^{20}$: + 12.8 determined on a 10 g/l solution in *methanol R*.

mp: about 198 °C.

Ginsenoside Rg1. $C_{42}H_{72}O_{14} \cdot 2H_2O$, (M_r 837). 1127600. [22427-39-0]. (2*S*)-6 β -D-Glucopyranosyl-D-glucopyranosylprotopanaxatriol. (2*S*)-6 α ,20-bis(β -D-Glucopyranosyloxy)-5 α -dammar-24-ene-3 β ,12 β -diol. (2*S*)-6 α ,20-bis(β -D-Glucopyranosyloxy)-4,4,8,14-tetramethyl-18-nor-5 α -cholest-24-ene-3 β ,12 β -diol.

A colourless solid, soluble in water, in ethanol and in methanol.

$[\alpha]_D^{20}$: + 31.2 determined on a 10 g/l solution in *methanol R*.

mp: 188 °C to 191 °C.

Water (2.5.12): maximum 4.8 per cent.

Assay. Examined by liquid chromatography (2.2.29) as prescribed in the monograph on *Ginseng* (1523).

Test solution. Dissolve 3.0 mg, accurately weighted, of *ginsenoside Rg1* in 10 ml of *methanol R*.

The content is not less than 95.0 per cent calculated by the normalisation procedure.

Gitoxin. $C_{41}H_{64}O_{14}$, (M_r 781). 1040200. [4562-36-1]. Glycoside of *Digitalis purpurea* L. 3 β -(*O*-2,6-Dideoxy- β -D-ribo-hexopyranosyl-(1 $\rightarrow$ 4)-*O*-2,6-dideoxy- β -D-ribo-hexopyranosyl-(1 $\rightarrow$ 4)-2,6-dideoxy- β -D-ribo-hexopyranosyloxy)-14,16 β -dihydroxy-5 β ,14 β -card-20(22)-enolide.

A white, crystalline powder, practically insoluble in water and in most common organic solvents, soluble in pyridine.

$[\alpha]_D^{20}$: + 20 to + 24, determined on a 5 g/l solution in a mixture of equal volumes of *chloroform R* and *methanol R*. **Chromatography.** Examine as prescribed in the monograph on *Digitalis leaf* (0117); the chromatogram shows only one principal spot.

Glucosamine hydrochloride. $C_6H_{14}ClNO_5$, (M_r 215.6). 1040300. [66-84-2]. D-Glucosamine hydrochloride.

Crystals, soluble in water.

$[\alpha]_D^{20}$: + 100, decreasing to + 47.5 after 30 min, determined on a 100 g/l solution in *water R*.

Glucose. 1025700. [50-99-7].

See *Anhydrous glucose* (0177).

D-Glucuronic acid. $C_6H_{10}O_7$, (M_r 194.1). 1119700. [6556-12-3].

Content: minimum 96.0 per cent of $C_6H_{10}O_7$, calculated with reference to the substance dried *in vacuo* (2.2.32).

Soluble in water and in alcohol.

Shows mutarotation: $[\alpha]_D^{24}$: + 11.7 $\rightarrow$ + 36.3

Assay. Dissolve 0.150 g in 50 ml of *anhydrous methanol R* while stirring under nitrogen. Titrate with 0.1 *M tetrabutylammonium hydroxide*, protecting the solution from atmospheric carbon dioxide throughout solubilisation and titration. Determine the end-point potentiometrically (2.2.20).

1 ml of 0.1 *M tetrabutylammonium hydroxide* is equivalent to 19.41 mg of $C_6H_{10}O_7$.

Glutamic acid. 1040400. [56-86-0].

See *Glutamic acid* (0750).

Glutaraldehyde. $C_5H_8O_2$, (M_r 100.1). 1098300. [111-30-8].

An oily liquid, soluble in water.

n_D^{25} : about 1.434.

bp: about 188 °C.

Glutaric acid. $C_5H_8O_4$, (M_r 132.1). 1149700. [110-94-1]. Pentanedioic acid.

White, crystalline powder.

Glycerol. 1040500. [56-81-5].

See *Glycerol* (0496).

Glycerol R1. 1040501.

Glycerol complying with the monograph *Glycerol* (0496) and free from diethylene glycol when examined as described in the test for Impurity A and related substances in that monograph.

Glycerol (85 per cent). 1040600.

See *Glycerol (85 per cent)* (0497).

Glycerol (85 per cent) R1. 1040601.

Glycerol complying with the monograph *Glycerol 85 per cent* (0497) and free from diethylene glycol when examined as described in the test for Impurity A and related substances in that monograph.

Glycidol. $C_3H_6O_2$, (M_r 74.1). 1127800. [556-52-5].

A slightly viscous liquid, miscible with water.

d_4^{20} : about 1.115.

n_D^{20} : about 1.432.

Glycine. 1040700. [56-40-6].

See *Glycine* (0614).

Glycollic acid. $C_2H_4O_3$. (M_r 76.0). 1040800. [79-14-1].
2-Hydroxyacetic acid.

Crystals, soluble in water, in acetone, in alcohol and in methanol.

mp: about 80 °C.

Glycyrrhetic acid. $C_{30}H_{46}O_4$. (M_r 470.7). 1040900. [471-53-4]. Glycyrrhetinic acid. 12,13-Didehydro-3 β -hydroxy-11-oxo-olean-30-oic acid.

A mixture of α - and β -glycyrrhetic acids in which the β -isomer is predominant.

A white or yellowish-brown powder, practically insoluble in water, soluble in ethanol and in glacial acetic acid.

$[\alpha]_D^{20}$: + 145 to + 155, determined on a 10.0 g/l solution in ethanol *R*.

Chromatography. Examine by thin-layer chromatography (2.2.27) using *silica gel GF₂₅₄ R* as the coating substance; prepare the slurry using a 0.25 per cent *V/V* solution of *phosphoric acid R*. Apply to the plate 5 μ l of a 5 g/l solution of the glycyrrhetic acid in a mixture of equal volumes of *chloroform R* and *methanol R*. Develop over a path of 10 cm using a mixture of 5 volumes of *methanol R* and 95 volumes of *chloroform R*. Examine the chromatogram in ultraviolet light at 254 nm. The chromatogram shows a dark spot (R_f about 0.3) corresponding to β -glycyrrhetic acid and a smaller spot (R_f about 0.5) corresponding to α -glycyrrhetic acid. Spray with *anisaldehyde solution R* and heat at 100 °C to 105 °C for 10 min. Both spots are coloured bluish-violet. Between them a smaller bluish-violet spot may be present.

18 α -Glycyrrhetic acid. $C_{30}H_{46}O_4$. (M_r 470.7). 1127900. [1449-05-4]. (20 β)-3 β -Hydroxy-11-oxo-18 α -olean-12-en-29-oic acid.

A white or almost white powder, practically insoluble in water, soluble in ethanol, sparingly soluble in methylene chloride.

Glyoxalhydroxyanil. $C_{14}H_{12}N_2O_2$. (M_r 240.3). 1041000. [1149-16-2]. Glyoxal bis(2-hydroxyanil).

White crystals, soluble in hot alcohol.

mp: about 200 °C.

Glyoxal solution. 1098400. [107-22-2].

Contains about 40 per cent (*m/m*) glyoxal.

Assay. In a ground-glass stoppered flask place 1.000 g of glyoxal solution, 20 ml of a 70 g/l solution of *hydroxylamine hydrochloride R* and 50 ml of *water R*. Allow to stand for 30 min and add 1 ml of *methyl red mixed solution R* and titrate with 1 *M sodium hydroxide* until the colour changes from red to green. Carry out a blank titration.

1 ml of 1 *M sodium hydroxide* is equivalent to 29.02 mg of glyoxal ($C_2H_2O_2$).

Gonadotrophin, chorionic. 1041100. [9002-61-3].

See *Chorionic gonadotrophin* (0498).

Gonadotrophin, serum. 1041200.

See *Equine serum gonadotrophin for veterinary use* (0719).

Guaiacol. $C_7H_8O_2$. (M_r 124.1). 1148300. [90-05-1].
2-Methoxyphenol. 1-Hydroxy-2-methoxybenzene.

Crystalline mass or colourless or yellowish liquid, hygroscopic, slightly soluble in water, very soluble in methylene chloride, freely soluble in alcohol.

bp: about 205 °C.

mp: about 28 °C.

Guaiacum resin. 1041400.

Resin obtained from the heartwood of *Guaiacum officinale* L. and *Guaiacum sanctum* L.

Reddish-brown or greenish-brown, hard, glassy fragments; fracture shiny.

Guaiazulene. $C_{15}H_{18}$. (M_r 198.3). 1041500. [489-84-9].
1,4-Dimethyl-7-isopropylazulene.

Dark-blue crystals or blue liquid, very slightly soluble in water, miscible with fatty and essential oils and with liquid paraffin, sparingly soluble in alcohol, soluble in 500 g/l sulphuric acid and 80 per cent *m/m* phosphoric acid, giving a colourless solution.

mp: about 30 °C.

Storage: protected from light and air.

Guanidine hydrochloride. CH_5N_3HCl . (M_r 95.5). 1098500. [50-01-1].

Crystalline powder, freely soluble in water and in alcohol.

Guanine. $C_5H_5N_5O$. (M_r 151.1). 1041600. [73-40-5].
2-Amino-1,7-dihydro-6*H*-purin-6-one.

An amorphous white powder, practically insoluble in water, slightly soluble in alcohol. It dissolves in ammonia and in dilute solutions of alkali hydroxides.

Haemoglobin. 1041700. [9008-02-0].

Nitrogen: 15 per cent to 16 per cent.

Iron: 0.2 per cent to 0.3 per cent.

Loss on drying (2.2.32): maximum 2 per cent.

Sulphated ash (2.4.14): maximum 1.5 per cent.

Haemoglobin solution. 1041701.

Transfer 2 g of *haemoglobin R* to a 250 ml beaker and add 75 ml of *dilute hydrochloric acid R2*. Stir until solution is complete. Adjust the pH to 1.6 ± 0.1 (2.2.3) using 1 *M hydrochloric acid*. Transfer to a 100 ml flask with the aid of *dilute hydrochloric acid R2*. Add 25 mg of *thiomersal R*. Prepare daily, store at 5 ± 3 °C and readjust to pH 1.6 before use.

Storage: at 2 °C to 8 °C.

Harpagoside. $C_{24}H_{30}O_{11}$. (M_r 494.5). 1098600.

A white, crystalline powder, very hygroscopic, soluble in water and in alcohol.

mp: 117 °C to 121 °C.

Storage: in an airtight container.

Helium for chromatography. He. (A_r 4.003). 1041800. [7440-59-7].

Content: minimum 99.995 per cent *V/V* of He.

Heparin. 1041900. [9041-08-1].

See *Heparin sodium* (0333).

Heptachlor. $C_{10}H_5Cl_7$. (M_r 373.3). 1128000. [76-44-8].

bp: about 135 °C.

mp: about 95 °C.

A suitable certified reference solution (10 ng/ μ l in cyclohexane) may be used.

Heptachlor epoxide. $C_{10}H_5Cl_7O$. (M_r 389.3). 1128100. [1024-57-3].

bp: about 200 °C.

mp: about 160 °C.

A suitable certified reference solution (10 ng/μl in cyclohexane) may be used.

Heptafluoro-*N*-methyl-*N*-(trimethylsilyl)butanamide. $C_8H_{12}F_7NOSi$. (M_r 299.3). 1139500. [53296-64-3]. 2,2,3,3,4,4,4-Heptafluoro-*N*-methyl-*N*-(trimethylsilyl)butyramide.

Clear, colourless liquid, flammable.

n_D^{20} : about 1.351.

bp: about 148 °C.

Heptane. C_7H_{16} . (M_r 100.2). 1042000. [142-82-5].

A colourless, flammable liquid, practically insoluble in water, miscible with ethanol.

d_{20}^{20} : 0.683 to 0.686.

n_D^{20} : 1.387 to 1.388.

Distillation range (2.2.11). Not less than 95 per cent distils between 97 °C and 98 °C.

Hesperidin. $C_{28}H_{34}O_{15}$. (M_r 611). 1139000. [520-26-3]. (S)-7-[[6-*O*-(6-Deoxy- α -L-mannopyranosyl)- β -D-glucopyranosyl]oxy]-5-hydroxy-2-(3-hydroxy-4-methoxyphenyl)-2,3-dihydro-4*H*-1-benzopyran-4-one.

Hygroscopic powder, slightly soluble in water and in methanol.

mp: 258 °C to 262 °C.

Hexachlorobenzene. C_6Cl_6 . (M_r 284.8). 1128200. [118-74-1].

bp: about 332 °C.

mp: about 230 °C.

A suitable certified reference solution (10 ng/μl in cyclohexane) may be used.

α -Hexachlorocyclohexane. $C_6H_6Cl_6$. (M_r 290.8). 1128300. [319-84-6].

bp: about 288 °C.

mp: about 158 °C.

A suitable certified reference solution (10 ng/μl in cyclohexane) may be used.

β -Hexachlorocyclohexane. $C_6H_6Cl_6$. (M_r 290.8). 1128400. [319-85-7].

A suitable certified reference solution (10 ng/μl in cyclohexane) may be used.

δ -Hexachlorocyclohexane. $C_6H_6Cl_6$. (M_r 290.8). 1128500. [319-86-8].

A suitable certified reference solution (10 ng/μl in cyclohexane) may be used.

Hexacosane. $C_{26}H_{54}$. (M_r 366.7). 1042200. [630-01-3].

Colourless or white flakes.

mp: about 57 °C.

Hexadimethrine bromide. $(C_{13}H_{30}Br_2N_2)_n$. 1042300. [28728-55-4]. 1,5-Dimethyl-1,5-diazaundecamethylene polymethobromide. Poly(1,1,5,5-tetramethyl-1,5-azonia-undecamethylene dibromide).

A white, amorphous powder, hygroscopic, soluble in water.

Storage: in an airtight container.

2,2',2'',6,6',6''-Hexa(1,1-dimethylethyl)-4,4',4''-[(2,4,6-trimethyl-1,3,5-benzenetriyl)trismethylene]triphenol.

$C_{54}H_{78}O_3$. (M_r 775). 1042100. 2,2',2'',6,6',6''-Hexa-*tert*-butyl-4,4',4''-[(2,4,6-trimethyl-1,3,5-benzenetriyl)trismethylene]triphenol.

A crystalline powder, practically insoluble in water, soluble in acetone, slightly soluble in alcohol.

mp: about 244 °C.

1,1,1,3,3,3-Hexafluoropropan-2-ol. $C_3H_2F_6O$. (M_r 168.0). 1136000. [920-66-1].

Content: minimum 99.0 per cent of $C_3H_2F_6O$, determined by gas chromatography.

A clear, colourless liquid, miscible with water and with ethanol.

d_{20}^{20} : about 1.596.

bp: about 59 °C.

Hexamethyldisilazane. $C_6H_{19}NSi_2$. (M_r 161.4). 1042400. [999-97-3].

A clear, colourless liquid.

d_{20}^{20} : about 0.78.

n_D^{20} : about 1.408.

bp: about 125 °C.

Storage: in an airtight container.

Hexamethylenetetramine. $C_6H_{12}N_4$. (M_r 140.2). 1042500. [100-97-0]. Hexamine. 1,3,5,7-Tetra-azatricyclo[3.3.1.1^{3,7}]decane.

A colourless, crystalline powder, very soluble in water.

Hexane. C_6H_{14} . (M_r 86.2). 1042600. [110-54-3].

A colourless, flammable liquid, practically insoluble in water, miscible with ethanol.

d_{20}^{20} : 0.659 to 0.663.

n_D^{20} : 1.375 to 1.376.

Distillation range (2.2.11). Not less than 95 per cent distils between 67 °C and 69 °C.

Hexane used in spectrophotometry complies with the following additional test.

Minimum transmittance (2.2.25), determined using *water R* as compensation liquid: 97 per cent from 260 nm to 420 nm.

Hexylamine. $C_6H_{15}N$. (M_r 101.2). 1042700. [111-26-2]. Hexanamine.

A colourless liquid, slightly soluble in water, soluble in alcohol.

d_{20}^{20} : about 0.766.

n_D^{20} : about 1.418.

bp: 127 °C to 131 °C.

Histamine dihydrochloride. 1042800. [56-92-8].

See *Histamine dihydrochloride* (0143).

Histamine phosphate. 1042900. [23297-93-0].

See *Histamine phosphate* (0144).

Histamine solution. 1042901.

A 9 g/l solution of *sodium chloride R* containing 0.1 μg per millilitre of histamine base (as the phosphate or dihydrochloride).

Histidine monohydrochloride. $C_6H_{10}ClN_3O_2 \cdot H_2O$. (M_r 209.6). 1043000. [123333-71-1]. (RS)-2-Amino-3-(imidazol-4-yl)propionic acid hydrochloride monohydrate.

A crystalline powder or colourless crystals, soluble in water. mp: about 250 °C, with decomposition.

Chromatography. Examine as prescribed in the monograph on *Histamine dihydrochloride* (0143); the chromatogram shows only one principal spot.

Holmium oxide. Ho_2O_3 . (M_r 377.9). 1043100. [12055-62-8]. Diholmium trioxide.

A yellowish powder, practically insoluble in water.

Holmium perchlorate solution. 1043101.

A 40 g/l solution of *holmium oxide R* in a solution of *perchloric acid R* containing 141 g/l of $HClO_4$.

DL-Homocysteine. $C_4H_9NO_2S$. (M_r 135.2). 1136100. [454-29-5]. (2RS)-2-Amino-4-sulphanylbutanoic acid.

A white, crystalline powder.

mp: about 232 °C.

L-Homocysteine thiolactone hydrochloride.

C_4H_8ClNOS . (M_r 153.6). 1136200. [31828-68-9]. (3S)-3-Aminodihydrothiophen-2(3H)-one hydrochloride.

A white, crystalline powder.

mp: about 202 °C.

Hyaluronidase diluent. 1043300.

Mix 100 ml of *phosphate buffer solution pH 6.4 R* with 100 ml of *water R*. Dissolve 0.140 g of *hydrolysed gelatin R* in the solution at 37 °C.

Storage: use within 2 h.

Hydrastine hydrochloride. $C_{21}H_{22}ClNO_6$. (M_r 419.9). 1154000. [5936-28-7]. (3S)-6,7-Dimethoxy-3-[(5R)-6-methyl-5,6,7,8-tetrahydro-1,3-dioxolo[4,5-g]isoquinolin-5-yl]isobenzofuran-1(3H)-one hydrochloride.

A white powder, hygroscopic, very soluble in water and in alcohol.

$[\alpha]_D^{17}$: about + 127.

mp: about 116 °C.

Hydrastine hydrochloride used in liquid chromatography complies with the following additional test.

Assay. Examine by liquid chromatography (2.2.29) as prescribed in the monograph on *Goldenseal rhizome* (1831). The content is not less than 98 per cent, calculated by the normalisation procedure.

Hydrazine. H_4N_2 . (M_r 32.05). 1136300. [302-01-2]. Diazane.

A slightly oily liquid, colourless, with a strong odour of ammonia, miscible with water. Dilute solutions in water are commercially available.

Caution: toxic and corrosive.

n_D^{20} : about 1.470.

bp: about 113 °C.

mp: about 1.5 °C.

Hydrazine sulphate. $H_6N_2O_4S$. (M_r 130.1). 1043400. [10034-93-2].

Colourless crystals, sparingly soluble in cold water, soluble in hot water (50 °C) and freely soluble in boiling water, practically insoluble in alcohol.

Arsenic (2.4.2). 1.0 g complies with limit test A (1 ppm).

Sulphated ash (2.4.14): maximum 0.1 per cent.

Hydriodic acid. HI. (M_r 127.9). 1098900. [10034-85-2].

Prepare by distilling hydriodic acid over red phosphorus, passing *carbon dioxide R* or *nitrogen R* through the apparatus during the distillation. Use the colourless or almost colourless, constant-boiling mixture (55 per cent to 58 per cent of HI) distilling between 126 °C and 127 °C.

Place the acid in small, amber, glass-stoppered bottles previously flushed with *carbon dioxide R* or *nitrogen R*, seal with paraffin.

Storage: in a dark place.

Hydrobromic acid, 30 per cent. 1098700. [10035-10-6].

30 per cent hydrobromic acid in *glacial acetic acid R*.

Degas with caution the contents before opening.

Hydrobromic acid, dilute. 1098701.

Place 5.0 ml of 30 per cent hydrobromic acid *R* in amber vials equipped with polyethylene stoppers. Seal under *argon R* and store in the dark. Add 5.0 ml of *glacial acetic acid R* immediately before use. Shake.

Storage: in the dark.

Hydrobromic acid, 47 per cent. 1118900.

A 47 per cent *m/m* solution of hydrobromic acid in *water R*.

Hydrobromic acid, dilute R1. 1118901.

Contains 7.9 g/l of HBr.

Dissolve 16.81 g of 47 per cent hydrobromic acid *R* in *water R* and dilute to 1000 ml with the same solvent.

Hydrochloric acid. 1043500. [7647-01-0].

See *Concentrated hydrochloric acid* (0002).

Hydrochloric acid R1. 1043501.

Contains 250 g/l of HCl.

Dilute 70 g of *hydrochloric acid R* to 100 ml with *water R*.

Hydrochloric acid, brominated. 1043507.

To 1 ml of *bromine solution R* add 100 ml of *hydrochloric acid R*.

Hydrochloric acid, dilute. 1043503.

Contains 73 g/l of HCl.

Dilute 20 g of *hydrochloric acid R* to 100 ml with *water R*.

Hydrochloric acid, dilute, heavy metal-free. 1043509.

Complies with the requirements prescribed for *dilute hydrochloric acid R* and with the following maximum contents of heavy metals:

As: 0.005 ppm;

Cd: 0.003 ppm;

Cu: 0.003 ppm;

Fe: 0.05 ppm;

Hg: 0.005 ppm;

Ni: 0.004 ppm;

Pb: 0.001 ppm;

Zn: 0.005 ppm.

Hydrochloric acid, dilute R1. 1043504.

Contains 0.37 g/l of HCl.

Dilute 1.0 ml of *dilute hydrochloric acid R* to 200.0 ml with *water R*.

Hydrochloric acid, dilute R2. 1043505.

Dilute 30 ml of 1 M *hydrochloric acid* to 1000 ml with *water R*; adjust to pH 1.6 ± 0.1.

Hydrochloric acid, ethanolic. 1043506.

Dilute 5.0 ml of 1 M *hydrochloric acid* to 500.0 ml with *alcohol R*.

Hydrochloric acid, heavy metal-free. 1043510.

Complies with the requirements prescribed for *hydrochloric acid R* and with the following maximum contents of heavy metals:

As: 0.005 ppm;

Cd: 0.003 ppm;

Cu: 0.003 ppm;

Fe: 0.05 ppm;

Hg: 0.005 ppm;

Ni: 0.004 ppm;

Pb: 0.001 ppm;

Zn: 0.005 ppm.

Hydrochloric acid, lead-free. 1043508.

Complies with the requirements prescribed for *hydrochloric acid R* and with the following additional test.

Lead: maximum 20 ppb of Pb determined by atomic emission spectrometry (2.2.22, *Method I*).

Test solution. In a quartz crucible evaporate 200 g of the acid to be examined almost to dryness. Take up the residue in 5 ml of *nitric acid* prepared by sub-boiling distillation of *nitric acid R* and evaporate to dryness. Take up the residue in 5 ml of *nitric acid* prepared by sub-boiling distillation of *nitric acid R*.

Reference solutions. Prepare the reference solutions using *lead standard solution (0.1 ppm Pb) R* diluted with *nitric acid* prepared by sub-boiling distillation of *nitric acid R*. Measure the emission intensity at 220.35 nm.

Hydrocortisone acetate. 1098800. [50-03-3].

See *Hydrocortisone acetate (0334)*.

Hydrofluoric acid. HF. (M_r 20.01). 1043600. [7664-39-3].

Content: minimum 40.0 per cent *m/m* of HF.

A clear, colourless liquid.

Residue on ignition. Not more than 0.05 per cent *m/m*. Evaporate the hydrofluoric acid in a platinum crucible and gently ignite the residue to constant mass.

Assay. Weigh accurately a glass-stoppered flask containing 50.0 ml of 1 M *sodium hydroxide*. Introduce 2 g of the hydrofluoric acid and weigh again. Titrate the solution with 0.5 M *sulphuric acid*, using 0.5 ml of *phenolphthalein solution R* as indicator.

1 ml of 1 M *sodium hydroxide* is equivalent to 20.01 mg of HF.

Storage: in a polyethylene container.

Hydrogen for chromatography. H₂. (M_r 2.016). 1043700. [1333-74-0].

Content: minimum 99.95 per cent *V/V* of H₂.

Hydrogen peroxide solution, dilute. 1043800. [7722-84-1].

See *Hydrogen peroxide solution (3 per cent) (0395)*.

Hydrogen peroxide solution, strong. 1043900. [7722-84-1].

See *Hydrogen peroxide solution (30 per cent) (0396)*.

Hydrogen sulphide. H₂S. (M_r 34.08). 1044000. [7783-06-4].

A gas, slightly soluble in water.

Hydrogen sulphide solution. 1136400.

A recently prepared solution of *hydrogen sulphide R* in *water R*. The saturated solution contains about 0.4 per cent to 0.5 per cent of H₂S at 20 °C.

Hydrogen sulphide R1. H₂S. (M_r 34.08). 1106600.

Content: minimum 99.7 per cent *V/V* of H₂S.

Hydroquinone. C₆H₆O₂. (M_r 110.1). 1044100. [123-31-9]. Benzene-1,4-diol.

Fine, colourless or white needles, darkening on exposure to air and light, soluble in water and in alcohol.

mp: about 173 °C.

Storage: protected from light and air.

Hydroquinone solution. 1044101.

Dissolve 0.5 g of *hydroquinone R* in *water R*, add 20 µl of *sulphuric acid R* and dilute to 50 ml with *water R*.

4-Hydroxybenzohydrazide. C₇H₈N₂O₂. (M_r 152.2). 1145900. [5351-23-5]. *p*-Hydroxybenzohydrazide.**4-Hydroxybenzoic acid.** C₇H₆O₃. (M_r 138.1). 1106700. [99-96-7].

Crystals, slightly soluble in water, very soluble in alcohol, soluble in acetone.

mp: 214 °C to 215 °C.

2-[4-(2-Hydroxyethyl)piperazin-1-yl]ethanesulphonic acid.

C₈H₁₈N₂O₄S. (M_r 238.3). 1106800. [7365-45-9]. HEPES.

A white powder.

mp: about 236 °C, with decomposition

4-Hydroxyisophthalic acid. C₈H₆O₅. (M_r 182.1). 1106900.

[636-46-4]. 4-Hydroxybenzene-1,3-dicarboxylic acid.

Needles or platelets, very slightly soluble in water, freely soluble in alcohol.

mp: about 314 °C, with decomposition.

Hydroxylamine hydrochloride. NH₄ClO. (M_r 69.5).

1044300. [5470-11-1].

A white, crystalline powder, very soluble in water, soluble in alcohol.

Hydroxylamine hydrochloride solution R2. 1044304.

Dissolve 2.5 g of *hydroxylamine hydrochloride R* in 4.5 ml of hot *water R* and add 40 ml of *alcohol R* and 0.4 ml of *bromophenol blue solution R2*. Add 0.5 M *alcoholic potassium hydroxide* until a greenish-yellow colour is obtained. Dilute to 50.0 ml with *alcohol R*.

Hydroxylamine solution, alcoholic. 1044301.

Dissolve 3.5 g of *hydroxylamine hydrochloride R* in 95 ml of *alcohol (60 per cent V/V) R*, add 0.5 ml of a 2 g/l solution of *methyl orange R* in *alcohol (60 per cent V/V) R* and sufficient 0.5 M *potassium hydroxide in alcohol (60 per cent V/V)* to give a pure yellow colour. Dilute to 100 ml with *alcohol (60 per cent V/V) R*.

Hydroxylamine solution, alkaline. 1044302.

Immediately before use, mix equal volumes of a 139 g/l solution of *hydroxylamine hydrochloride R* and a 150 g/l solution of *sodium hydroxide R*.

Hydroxylamine solution, alkaline R1. 1044303.

Solution A. Dissolve 12.5 g of *hydroxylamine hydrochloride R* in *methanol R* and dilute to 100 ml with the same solvent.

Solution B. Dissolve 12.5 g of *sodium hydroxide R* in *methanol R* and dilute to 100 ml with the same solvent.

Mix equal volumes of solution A and solution B immediately before use.

Hydroxymethylfurfural. $C_6H_6O_3$. (M_r 126.1). 1044400. [67-47-0]. 5-Hydroxymethylfurfural.

Acicular crystals, freely soluble in water, in acetone and in alcohol.

mp: about 32 °C.

Hydroxynaphthol blue, sodium salt. $C_{20}H_{11}N_2Na_3O_{11}S_3$. (M_r 620). 1044500. [63451-35-4]. Trisodium 2,2'-dihydroxy-1,1'-azonaphthalene-3',4,6'-trisulphonate.

2-Hydroxypropylbetadex for chromatography R. 1146000. Betacyclodextrin modified by the bonding of (*R*) or (*RS*) propylene oxide groups on the hydroxyl groups.

Hydroxypropyl-β-cyclodextrin. 1128600. [94035-02-6]. See *Hydroxypropylbetadex* (1804). pH (2.2.3): 5.0-7.5 (20 g/l solution).

Hydroxyquinoline. C_9H_7NO . (M_r 145.2). 1044600. [148-24-3]. 8-Hydroxyquinoline. Quinolin-8-ol.

A white or slightly yellowish, crystalline powder, slightly soluble in water, freely soluble in acetone, in alcohol and in dilute mineral acids.

mp: about 75 °C.

Sulphated ash (2.4.14): maximum 0.05 per cent.

12-Hydroxystearic acid. $C_{18}H_{36}O_3$. (M_r 300.5). 1099000. [106-14-9]. 12-Hydroxyoctadecanoic acid.

White powder.

mp: 71 °C to 74 °C.

5-Hydroxyuracil. $C_4H_4N_2O_3$. (M_r 128.1). 1044700. [496-76-4]. Isobarbituric acid. Pyrimidine-2,4,5-triol.

A white, crystalline powder.

mp: about 310 °C, with decomposition.

Chromatography. Examined as prescribed in the monograph on *Fluorouracil* (0611), the chromatogram shows a principal spot with an R_f of about 0.3.

Storage: in an airtight container.

Hyoscine hydrobromide. 1044800. [6533-68-2]. See *Hyoscine hydrobromide* (0106).

Hyoscyamine sulphate. 1044900. [620-61-1]. See *Hyoscyamine sulphate* (0501).

Hypericin. $C_{30}H_{16}O_8$. (M_r 504.4). 1149800. [548-04-9]. 1,3,4,6,8,13-Hexahydroxy-10,11-dimethylphenanthro[1,10,9,8-*opqra*]perylene-7,14-dione.

Content: minimum 85 per cent of $C_{30}H_{16}O_8$.

Hyperoside. $C_{21}H_{20}O_{12}$. (M_r 464.4). 1045000. 2-(3,4-Dihydroxyphenyl)-3-β-D-galactopyranosyloxy-5,7-dihydroxy-chromen-4-one.

Faint yellow needles, soluble in methanol.

$[\alpha]_D^{20}$: -8.3, determined on a 2 g/l solution in *pyridine R*. mp: about 240 °C, with decomposition.

A solution in *methanol R* shows two absorption maxima (2.2.25), at 259 nm and at 364 nm.

Hypophosphorous reagent. 1045200.

Dissolve with the aid of gentle heat, 10 g of *sodium hypophosphite R* in 20 ml of *water R* and dilute to 100 ml with *hydrochloric acid R*. Allow to settle and decant or filter through glass wool.

Hypoxanthine. $C_5H_4N_4O$. (M_r 136.1). 1045300. [68-94-0]. 1*H*-Purin-6-one.

A white, crystalline powder, very slightly soluble in water, sparingly soluble in boiling water, soluble in dilute acids and in dilute alkali hydroxide solutions, decomposes without melting at about 150 °C.

Chromatography. Examine as prescribed in the monograph on *Mercaptopurine* (0096); the chromatogram shows only one principal spot.

Imidazole. $C_3H_4N_2$. (M_r 68.1). 1045400. [288-32-4].

A white, crystalline powder, soluble in water and in alcohol. mp: about 90 °C.

Iminodibenzyl. $C_{14}H_{13}N$. (M_r 195.3). 1045500. [494-19-9]. 10,11-Dihydrodibenz[*b,f*]azepine.

A pale yellow, crystalline powder, practically insoluble in water, freely soluble in acetone.

mp: about 106 °C.

Indigo carmine. $C_{16}H_8N_2Na_2O_8S_2$. (M_r 466.3). 1045600. [860-22-0].

Schultz No. 1309.

Colour Index No. 73015.

3,3'-Dioxo-2,2'-bisindolylidene-5,5'-disulphonate disodium. E 132.

It usually contains sodium chloride.

A blue or violet-blue powder or blue granules with a coppery lustre, sparingly soluble in water, practically insoluble in alcohol. It is precipitated from an aqueous solution by sodium chloride.

Indigo carmine solution. 1045601.

To a mixture of 10 ml of *hydrochloric acid R* and 990 ml of 200 g/l *nitrogen-free sulphuric acid R* add 0.2 g of *indigo carmine R*.

The solution complies with the following test.

Add 10 ml to a solution of 1.0 mg of *potassium nitrate R* in 10 ml of *water R*, rapidly add 20 ml of *nitrogen-free sulphuric acid R* and heat to boiling. The blue colour is discharged within 1 min.

Indigo carmine solution R1. 1045602.

Dissolve 4 g of *indigo carmine R* in about 900 ml of *water R* added in several portions. Add 2 ml of *sulphuric acid R* and dilute to 1000 ml with *water R*.

Standardisation. Place in a 100 ml conical flask with a wide neck 10.0 ml of *nitrate standard solution* (100 ppm NO_3^-) *R*, 10 ml of *water R*, 0.05 ml of the *indigo carmine solution R1*, and then in a single addition, but with caution, 30 ml of *sulphuric acid R*. Titrate the solution immediately, using the *indigo carmine solution R1*, until a stable blue colour is obtained.

The number of millilitres used, *n*, is equivalent to 1 mg of NO_3^- .

Indometacin. 1101500. [53-86-1].

See *Indometacin* (0092).

Iodine. 1045800. [7553-56-2].

See *Iodine* (0031).

Iodine solution R1. 1045801.

To 10.0 ml of 0.05 *M iodine* add 0.6 g of *potassium iodide R* and dilute to 100.0 ml with *water R*. Prepare immediately before use.

Iodine solution R2. 1045802.

To 10.0 ml of 0.05 M iodine add 0.6 g of potassium iodide R and dilute to 1000.0 ml with water R. Prepare immediately before use.

Iodine solution R3. 1045803.

Dilute 2.0 ml of iodine solution R1 to 100.0 ml with water R. Prepare immediately before use.

Iodine solution R4. 1045806.

Dissolve 14 g of iodine R in 100 ml of a 400 g/l solution of potassium iodide R, add 1 ml of dilute hydrochloric acid R and dilute to 1000 ml with water R.

Storage: protected from light.

Iodine solution, alcoholic. 1045804.

A 10 g/l solution in alcohol R.

Storage: protected from light.

Iodine solution, chloroformic. 1045805.

A 5 g/l solution in chloroform R.

Storage: protected from light.

Iodine bromide. IBr. (M_r 206.8). 1045900. [7789-33-5].

Bluish-black or brownish-black crystals, freely soluble in water, in alcohol and in glacial acetic acid.

bp: about 116 °C.

mp: about 40 °C.

Storage: protected from light.

Iodine bromide solution. 1045901.

Dissolve 20 g of iodine bromide R in glacial acetic acid R and dilute to 1000 ml with the same solvent.

Storage: protected from light.

Iodine chloride. ICl. (M_r 162.4). 1143000. [7790-99-0].

Black crystals, soluble in water, in acetic acid and in alcohol.
bp: about 97.4 °C.

Iodine chloride solution. 1143001.

Dissolve 1.4 g of iodine chloride R in glacial acetic acid R and dilute to 100 ml with the same acid.

Storage: protected from light.

Iodine pentoxide, recrystallised. I₂O₅. (M_r 333.8). 1046000. [12029-98-0]. Di-iodine pentoxide. Iodic anhydride.

Content: minimum 99.5 per cent of I₂O₅.

A white, crystalline powder, or white or greyish-white granules, hygroscopic, very soluble in water forming HIO₃.

Stability on heating. Dissolve 2 g, previously heated for 1 h at 200 °C, in 50 ml of water R. A colourless solution is obtained.

Assay. Dissolve 0.100 g in 50 ml of water R, add 3 g of potassium iodide R and 10 ml of dilute hydrochloric acid R. Titrate the liberated iodine with 0.1 M sodium thiosulphate, using 1 ml of starch solution R as indicator.

1 ml of 0.1 M sodium thiosulphate is equivalent to 2.782 mg of I₂O₅.

Storage: in an airtight container, protected from light.

Iodoacetic acid. C₂H₃IO₂. (M_r 185.9). 1107000. [64-69-7].

Colourless or white crystals, soluble in water and in alcohol.
mp: 82 °C to 83 °C.

2-Iodobenzoic acid. C₇H₅IO₂. (M_r 248.0). 1046100. [88-67-5].

A white or slightly yellow, crystalline powder, slightly soluble in water, soluble in alcohol.

mp: about 160 °C.

Chromatography. Examine by thin-layer chromatography (2.2.27), using cellulose for chromatography F_{254} R as the coating substance. Apply to the plate 20 µl of a solution of the 2-iodobenzoic acid, prepared by dissolving 40 mg in 4 ml of 0.1 M sodium hydroxide and diluting to 10 ml with water R. Develop over a path of about 12 cm using as the mobile phase the upper layer obtained by shaking together 20 volumes of water R, 40 volumes of glacial acetic acid R and 40 volumes of toluene R. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. The chromatogram shows only one principal spot.

Iodoethane. C₂H₅I. (M_r 155.9). 1099100. [75-03-6].

Colourless to slightly yellowish liquid, darkening on exposure to air and light, miscible with alcohol and most organic solvents.

d_{20}^{20} : about 1.95.

n_D^{20} : about 1.513.

bp: about 72 °C.

Storage: in an airtight container.

2-Iodohippuric acid. C₉H₈INO₃·2H₂O. (M_r 341.1). 1046200. [147-58-0]. 2-(2-Iodobenzamido)acetic acid.

A white or almost white, crystalline powder, sparingly soluble in water.

mp: about 170 °C.

Water (2.5.12): 9 per cent to 13 per cent, determined on 1.000 g.

Chromatography. Examine by thin-layer chromatography (2.2.27), using cellulose for chromatography F_{254} R as the coating substance. Apply to the plate 20 µl of a solution of the 2-iodohippuric acid, prepared by dissolving 40 mg in 4 ml of 0.1 M sodium hydroxide and diluting to 10 ml with water R. Develop over a path of about 12 cm using as the mobile phase the upper layer obtained by shaking together 20 volumes of water R, 40 volumes of glacial acetic acid R and 40 volumes of toluene R. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. The chromatogram shows only one principal spot.

Iodoplatinate reagent. 1046300.

To 3 ml of a 100 g/l solution of chloroplatinic acid R add 97 ml of water R and 100 ml of a 60 g/l solution of potassium iodide R.

Storage: protected from light.

Iodosulphurous reagent. 1046400.

The apparatus, which must be kept closed and dry during the preparation, consists of a 3000 ml to 4000 ml round-bottomed flask with three inlets for a stirrer and a thermometer and fitted with a drying tube. To 700 ml of anhydrous pyridine R and 700 ml of ethyleneglycol monomethyl ether R add, with constant stirring, 220 g of finely powdered iodine R, previously dried over diphosphorus pentoxide R. Continue stirring until the iodine has completely dissolved (about 30 min). Cool to –10 °C, and add quickly, still stirring, 190 g of sulphur dioxide R. Do not allow the temperature to exceed 30 °C. Cool.

Standardisation. Add about 20 ml of anhydrous methanol R to a titration vessel and titrate to the end-point with the iodosulphurous reagent (2.5.12). Introduce in an appropriate form a suitable amount of water R, accurately weighed,

and repeat the determination of water. Calculate the water equivalent in milligrams per millilitre of iodosulphurous reagent.

The minimum water equivalent is 3.5 mg of water per millilitre of reagent.

Work protected from humidity. Standardise immediately before use.

Storage: in a dry container.

5-Iodouracil. $C_4H_3IN_2O_2$. (M_r 238.0). 1046500. [696-07-1]. 5-Iodo-1*H*,3*H*-pyrimidine-2,4-dione.

mp: about 276 °C, with decomposition.

Chromatography. Examine as prescribed in the monograph on *Idoxuridine* (0669), applying 5 µl of a 0.25 g/l solution. The chromatogram obtained shows only one principal spot.

Ion-exclusion resin for chromatography. 1131000.

A resin with sulphonic acid groups attached to a polymer lattice consisting of polystyrene cross-linked with divinylbenzene.

Ion-exchange resin, strongly acidic. 1085400.

A resin in protonated form with sulphonic acid groups attached to a lattice consisting of polystyrene cross-linked with 8 per cent of divinylbenzene. It is available as spherical beads; unless otherwise prescribed, the particle size is 0.3 mm to 1.2 mm.

Capacity. 4.5 mmol to 5 mmol per gram, with a water content of 50 per cent to 60 per cent.

Preparation of a column. Unless otherwise prescribed, use a tube with a fused-in sintered glass disc having a length of 400 mm, an internal diameter of 20 mm and a filling height of about 200 mm. Introduce the resin, mixing it with *water R* and pouring the slurry into the tube, ensuring that no air bubbles are trapped between the particles. When in use, the liquid must not be allowed to fall below the surface of the resin. If the resin is in its protonated form, wash with *water R* until 50 ml requires not more than 0.05 ml of 0.1 *M* sodium hydroxide for neutralisation, using 0.1 ml of *methyl orange solution R* as indicator.

If the resin is in its sodium form or if it requires regeneration, pass about 100 ml of a mixture of equal volumes of *hydrochloric acid R1* and *water R* slowly through the column and then wash with *water R* as described above.

Iron. Fe. (A_r 55.85). 1046600. [7439-89-6].

Grey powder or wire, soluble in dilute mineral acids.

Iron salicylate solution. 1046700.

Dissolve 0.1 g of *ferric ammonium sulphate R* in a mixture of 2 ml of *dilute sulphuric acid R* and 48 ml of *water R* and dilute to 100 ml with *water R*. Add 50 ml of a 11.5 g/l solution of *sodium salicylate R*, 10 ml of *dilute acetic acid R*, 80 ml of a 136 g/l solution of *sodium acetate R* and dilute to 500 ml with *water R*. The solution should be recently prepared.

Storage: in an airtight container, protected from light.

Isatin. $C_8H_5NO_2$. (M_r 147.1). 1046800. [91-56-5]. Indoline-2,3-dione.

Small, yellowish-red crystals, slightly soluble in water, soluble in hot water and in alcohol, soluble in solutions of alkali hydroxides giving a violet colour becoming yellow on standing.

mp: about 200 °C, with partial sublimation.

Sulphated ash (2.4.14): maximum 0.2 per cent.

Isatin reagent. 1046801.

Dissolve 6 mg of *ferric sulphate R* in 8 ml of *water R* and add cautiously 50 ml of *sulphuric acid R*. Add 6 mg of *isatin R* and stir until dissolved.

The reagent should be pale yellow, but not orange or red.

Isoamyl alcohol. $C_5H_{12}O$. (M_r 88.1). 1046900. [123-51-3]. 3-Methylbutan-1-ol.

A colourless liquid, slightly soluble in water, miscible with alcohol.

bp: about 130 °C.

Isoandrosterone. $C_{19}H_{30}O_2$. (M_r 290.4). 1107100. [481-29-8]. Epiandrosterone. 3β-Hydroxy-5α-androstan-17-one.

A white powder, practically insoluble in water, soluble in organic solvents.

$[\alpha]_D^{20}$: + 88, determined on 20 g/l solution in *methanol R*.

mp: 172 °C to 174 °C.

ΔA (2.2.41): 14.24×10^3 , determined at 304 nm on a 1.25 g/l solution.

Isodrin. $C_{12}H_8Cl_6$. (M_r 364.9). 1128700. [465-73-6].

1,2,3,4,10,10-Hexachloro-1,4,4a,5,8,8a-hexahydro-endo,endo-1,4,5,8-dimethanonaphthalene.

Practically insoluble in water, soluble in common organic solvents such as acetone.

A suitable certified reference solution may be used.

Isomenthol. $C_{10}H_{20}O$. (M_r 156.3). 1047000. [23283-97-8]. (+)-*Isomenthol*: (1*S*,2*R*,5*R*)-2-isopropyl-5-methylcyclohexanol. (±)-*Isomenthol*: a mixture of equal parts of (1*S*,2*R*,5*R*)- and (1*R*,2*S*,5*S*)-2-isopropyl-5-methylcyclohexanol.

Colourless crystals, practically insoluble in water, very soluble in alcohol.

$[\alpha]_D^{20}$: (+)-*Isomenthol*: about + 24, determined on a 100 g/l solution in *alcohol R*.

bp: (+)-*Isomenthol*: about 218 °C. (±)-*Isomenthol*: about 218 °C.

mp: (+)-*Isomenthol*: about 80 °C. (±)-*Isomenthol*: about 53 °C.

(+)-**Isomenthone.** $C_{10}H_{18}O$. (M_r 154.2). 1047100. (1*R*)-*cis-p*-Menthan-3-one. (1*R*)-*cis*-2-Isopropyl-5-methylcyclohexanone.

Contains variable amounts of menthone. A colourless liquid, very slightly soluble in water, soluble in alcohol.

d_{20}^{20} : about 0.904.

n_D^{20} : about 1.453.

$[\alpha]_D^{20}$: about + 93.2.

Isomenthone used in gas chromatography complies with the following additional test.

Assay. Examine by gas chromatography (2.2.28) as prescribed in the monograph on *Peppermint oil* (0405) using the substance to be examined as the test solution.

The area of the principal peak is not less than 80.0 per cent of the total area of the peaks.

Isopropylamine. C_3H_9N . (M_r 59.1). 1119800. [75-31-0]. Propan-2-amine.

A colourless, highly volatile, flammable liquid.

n_D^{20} : about 1.374.

bp: 32 °C to 34 °C.

Isopropyl myristate. 1047200. [110-27-0].

See *Isopropyl myristate* (0725).

4-Isopropylphenol. $C_9H_{12}O$. (M_r 136.2). 1047300. [99-89-8].

Content: minimum 98 per cent of $C_9H_{12}O$.

bp: about 212 °C.

mp: 59 °C to 61 °C.

Isopulegol. $C_{10}H_{18}O$. (M_r 154.2). 1139600. [89-79-2]. (–)-Isopulegol. (1*R*,2*S*,5*R*)-2-Isopropenyl-5-methylcyclohexanol.

d_4^{20} : about 0.911.

n_D^{20} : about 1.472.

bp: about 91 °C.

Isopulegol used in gas chromatography complies with the following additional test.

Assay. Examine by gas chromatography (2.2.28) as prescribed in the monograph on *Mint oil, partly dementholised* (1838).

The content is not less than 99 per cent, calculated by the normalisation procedure.

Isoquercitroside. $C_{21}H_{20}O_{12}$. (M_r 464.4). 1136500. [21637-25-2]. Isoquercitrin. 2-(3,4-Dihydroxyphenyl)-3-(β-D-glucofuranosyloxy)-5,7-dihydroxy-4*H*-1-benzopyran-4-one. 3,3',4',5,7-Pentahydroxyflavone-3-glucoside.

Isosilibinin. $C_{25}H_{22}O_{10}$. (M_r 482.4). 1149900. [72581-71-6]. 3,5,7-Trihydroxy-2-[2-(4-hydroxy-3-methoxyphenyl)-3-hydroxymethyl-2,3-dihydro-1,4-benzodioxin-6-yl]chroman-4-one.

White to yellowish powder, practically insoluble in water, soluble in acetone and in methanol.

Kaolin, light. 1047400. [1332-58-7].

A purified native hydrated aluminium silicate. It contains a suitable dispersing agent.

A light, white powder free from gritty particles, unctuous to the touch, practically insoluble in water and in mineral acids.

Coarse particles. Place 5.0 g in a ground-glass-stoppered cylinder about 160 mm long and 35 mm in diameter and add 60 ml of a 10 g/l solution of *sodium pyrophosphate R*. Shake vigorously and allow to stand for 5 min. Using a pipette, remove 50 ml of the liquid from a point about 5 cm below the surface. To the remaining liquid add 50 ml of *water R*, shake, allow to stand for 5 min and remove 50 ml as before. Repeat the operations until a total of 400 ml has been removed. Transfer the remaining suspension to an evaporating dish. Evaporate to dryness on a water-bath and dry the residue to constant mass at 100 °C to 105 °C. The residue weighs not more than 25 mg (0.5 per cent).

Fine particles. Disperse 5.0 g in 250 ml of *water R* by shaking vigorously for 2 min. Immediately pour into a glass cylinder 50 mm in diameter and, using a pipette, transfer 20 ml to a glass dish, evaporate to dryness on a water-bath and dry to constant mass at 100 °C to 105 °C. Allow the remainder of the suspension to stand at 20 °C for 4 h and, using a pipette with its tip exactly 5 cm below the surface, withdraw a further 20 ml without disturbing the sediment, place in a glass dish, evaporate to dryness on a water-bath and dry to constant mass at 100 °C to 105 °C. The mass of the second residue is not less than 70 per cent of that of the first residue.

Kieselguhr for chromatography. 1047500.

A white or yellowish-white, light powder, practically insoluble in water, in dilute acids and in organic solvents.

Filtration rate. Use a chromatography column 0.25 m long and 10 mm in internal diameter with a sintered-glass (100) plate and two marks at 0.10 m and 0.20 m above the plate.

Place sufficient of the substance to be examined in the column to reach the first mark and fill to the second mark with *water R*. When the first drops begin to flow from the column, fill to the second mark again with *water R* and measure the time required for the first 5 ml to flow from the column. The flow rate is not less than 1 ml/min.

Appearance of the eluate. The eluate obtained in the test for filtration rate is colourless (*Method I*, 2.2.2).

Acidity or alkalinity. To 1.00 g add 10 ml of *water R*, shake vigorously and allow to stand for 5 min. Filter the suspension on a filter previously washed with hot *water R* until the washings are neutral. To 2.0 ml of the filtrate add 0.05 ml of *methyl red solution R*; the solution is yellow. To 2.0 ml of the filtrate add 0.05 ml of *phenolphthalein solution R1*; the solution is at most slightly pink.

Water-soluble substances. Place 10.0 g in a chromatography column 0.25 m long and 10 mm in internal diameter and elute with *water R*. Collect the first 20 ml of eluate, evaporate to dryness and dry the residue at 100 °C to 105 °C. The residue weighs not more than 10 mg.

Iron (2.4.9). To 0.50 g add 10 ml of a mixture of equal volumes of *hydrochloric acid R1* and *water R*, shake vigorously, allow to stand for 5 min and filter. 1.0 ml of the filtrate complies with the limit test for iron (200 ppm).

Loss on ignition: maximum 0.5 per cent. During heating to red heat (600 °C) the substance does not become brown or black.

Kieselguhr G. 1047600.

Consists of kieselguhr treated with hydrochloric acid and calcined, to which is added about 15 per cent of calcium sulphate hemihydrate.

A fine greyish-white powder; the grey colour becomes more pronounced on triturating with water. The average particle size is 10 µm to 40 µm.

Calcium sulphate content. Determine by the method prescribed for *silica gel G R*.

pH (2.2.3). Shake 1 g with 10 ml of *carbon dioxide-free water R* for 5 min. The pH of the suspension is 7 to 8.

Chromatographic separation. Examine by thin-layer chromatography (2.2.27). Prepare plates using a slurry of the kieselguhr G with a 2.7 g/l solution of *sodium acetate R*. Apply 5 µl of a solution containing 0.1 g/l of lactose, sucrose, glucose and fructose in *pyridine R*. Develop over a path of 14 cm using a mixture of 12 volumes of *water R*, 23 volumes of *2-propanol R* and 65 volumes of *ethyl acetate R*. The migration time of the solvent is about 40 min. Dry, spray onto the plate about 10 ml of *anisaldehyde solution R* and heat for 5 min to 10 min at 100 °C to 105 °C. The chromatogram shows four well-defined spots without tailing and well separated from each other.

Lactic acid. 1047800. [50-21-5].

See *Lactic acid* (0458).

Lactic reagent. 1047801.

Solution A. To 60 ml of *lactic acid R* add 45 ml of previously filtered *lactic acid R* saturated without heating with *Sudan red G R*; as lactic acid saturates slowly without heating, an excess of colorant is always necessary.

Solution B. Prepare 10 ml of a saturated solution of *aniline R*. Filter.

Solution C. Dissolve 75 mg of *potassium iodide R* in water and dilute to 70 ml with the same solvent. Add 10 ml of *alcohol R* and 0.1 g of *iodine R*. Shake.

Mix solutions A and B. Add solution C.

Lactobionic acid. $C_{12}H_{22}O_{12}$. (M_r 358.3). 1101600. [96-82-2].
A white, crystalline powder, freely soluble in water,
practically insoluble in alcohol.
mp: about 115 °C.

Lactose. 1047900. [5989-81-1].
See *Lactose* (0187).

β-Lactose. $C_{12}H_{22}O_{11}$. (M_r 342.3). 1150100. [5965-66-2].
β-D-Lactose.
White or slightly yellowish powder.
The α-D-lactose content is not greater than 35 per cent.
Assay. Gas chromatography (2.2.28): use the normalisation
procedure.

Inject an appropriate derivatised sample.

Column:

- size: $l = 30$ m, $\varnothing = 0.25$ mm,
- stationary phase: poly[(cyanopropyl)(phenyl)][dimethyl] siloxane *R* (film thickness 1 µm).

Carrier gas: helium for chromatography *R*.

Temperature:

	Time (min)	Temperature (°C)
Column	0 - 32.5	20 → 280
Injection port		250
Detector		250

Detection: flame ionisation.

The area of the peak due to β-lactose is not less than 99 per cent of the total peak area.

α-Lactose monohydrate. $C_{12}H_{22}O_{11} \cdot H_2O$. (M_r 360.3). 1150000. [5989-81-1]. α-D-Lactose monohydrate.

White powder.

The β-D-lactose content is less than 3 per cent.

Assay. Gas chromatography (2.2.28): use the normalisation
procedure.

Inject an appropriate derivatised sample.

Column:

- size: $l = 30$ m, $\varnothing = 0.25$ mm,
- stationary phase: poly(dimethyl)siloxane *R* (film thickness 1 µm).

Carrier gas: helium for chromatography *R*.

Temperature:

	Time (min)	Temperature (°C)
Column	0 - 12.5	230 → 280
Injection port		250
Detector		280

Detection: flame ionisation.

The area of the peak due to α-lactose is not less than 97 per cent of the total peak area.

Lanthanum nitrate. $La(NO_3)_3 \cdot 6H_2O$. (M_r 433.0). 1048000. [10277-43-7]. Lanthanum trinitrate hexahydrate.

Colourless crystals, deliquescent, freely soluble in water.

Storage: in an airtight container.

Lanthanum nitrate solution. 1048001.

A 50 g/l solution.

Lanthanum trioxide. La_2O_3 . (M_r 325.8). 1114000. [1312-81-8].

An almost white, amorphous powder, practically insoluble in *water R*. It dissolves in dilute solutions of mineral acids and absorbs atmospheric carbon dioxide.

Calcium: maximum 5 ppm.

Lanthanum chloride solution. 1114001.

To 58.65 g of *lanthanum trioxide R* slowly add 100 ml of *hydrochloric acid R*. Heat to boiling. Allow to cool and dilute to 1000.0 ml with *water R*.

Lauric acid. $C_{12}H_{24}O_2$. (M_r 200.3). 1143100. [143-07-7]. Dodecanoic acid.

White, crystalline powder, practically insoluble in water, freely soluble in alcohol.

mp: about 44 °C.

Lauric acid used in the assay of total fatty acids in Saw palmetto fruit (1848) complies with the following additional requirement.

Assay. Examine by gas chromatography (2.2.28) as prescribed in the monograph on *Saw palmetto fruit (1848)*.

The content of lauric acid is not less than 98 per cent, calculated by the normalisation procedure.

Lauryl alcohol. $C_{12}H_{26}O$. (M_r 186.3). 1119900. [112-53-8]. 1-Dodecanol.

d_{20}^{20} : about 0.820.

mp: 24 °C to 27 °C.

Lavandulol. $C_{10}H_{18}O$. (M_r 154.2). 1114100. [498-16-8]. (R)-5-Methyl-2-(1-methylethenyl)-4-hexen-1-ol.

An oily liquid with a characteristic odour.

d_{20}^{20} : about 0.875.

n_D^{20} : about 1.407.

$[\alpha]_D^{20}$: about – 10.2.

bp₁₃: about 94 °C.

Lavandulol used in gas chromatography complies with the following additional test.

Assay. Examine by gas chromatography (2.2.28) as prescribed in the monograph on *Lavender oil (1338)*.

Test solution. The substance to be examined.

The area of the principal peak is not less than 98.0 per cent of the area of all the peaks in the chromatogram obtained.

Lavandulyl acetate. $C_{12}H_{20}O_2$. (M_r 196.3). 1114200. [50373-59-6]. 2-Isopropenyl-5-methylhex-4-en-1-yl acetate.

A colourless liquid with a characteristic odour.

d_{20}^{20} : about 0.911.

n_D^{20} : about 1.454.

bp₁₃: 106 °C to 107 °C.

Lavandulyl acetate used in gas chromatography complies with the following additional test.

Assay. Examine by gas chromatography (2.2.28) as prescribed in the monograph on *Lavender oil (1338)*.

Test solution. The substance to be examined.

The area of the principal peak is not less than 93.0 per cent of the area of all the peaks in the chromatogram obtained.

Lead acetate. $C_4H_6O_4Pb \cdot 3H_2O$. (M_r 379.3). 1048100. [6080-56-4]. Lead di-acetate.

Colourless crystals, efflorescent, freely soluble in water, soluble in alcohol.

Lead acetate cotton. 1048101.

Immerse absorbent cotton in a mixture of 1 volume of *dilute acetic acid R* and 10 volumes of *lead acetate solution R*. Drain off the excess of liquid, without squeezing the cotton, by placing it on several layers of filter paper. Allow to dry in air.

Storage: in an airtight container.

Lead acetate paper. 1048102.

Immerse filter paper weighing about 80 g/m² in a mixture of 1 volume of *dilute acetic acid R* and 10 volumes of *lead acetate solution R*. After drying, cut the paper into strips 15 mm by 40 mm.

Lead acetate solution. 1048103.

A 95 g/l solution in *carbon dioxide-free water R*.

Lead dioxide. PbO₂. (*M_r* 239.2). 1048200. [1309-60-0].

A dark brown powder, evolving oxygen when heated, practically insoluble in water, soluble in hydrochloric acid with evolution of chlorine, soluble in dilute nitric acid in the presence of hydrogen peroxide, oxalic acid or other reducing agents, soluble in hot, concentrated alkali hydroxide solutions.

Lead nitrate. Pb(NO₃)₂. (*M_r* 331.2). 1048300. [10099-74-8].

Lead dinitrate.

A white, crystalline powder or colourless crystals, freely soluble in water.

Lead nitrate solution. 1048301.

A 33 g/l solution.

Lead subacetate solution. 1048400. [1335-32-6]. Basic lead acetate solution.

Content: 16.7 per cent *m/m* to 17.4 per cent *m/m* of Pb (*A_r* 207.2) in a form corresponding approximately to the formula C₈H₁₄O₁₀Pb₃.

Dissolve 40.0 g of *lead acetate R* in 90 ml of *carbon dioxide-free water R*. Adjust the pH to 7.5 with *strong sodium hydroxide solution R*. Centrifuge and use the clear colourless supernatant solution.

The solution remains clear when stored in a well-closed container.

Leiocarposide. C₂₇H₃₄O₁₆. (*M_r* 614.5). 1150200.

[71953-77-0]. 2-(β-D-Glucopyranosyloxy)benzyl 3-(β-D-glucopyranosyloxy)-6-hydroxy-2-methoxybenzoate. 2-[[[3-(β-D-Glucopyranosyloxy)-6-hydroxy-2-methoxybenzoyl]oxy]methyl]phenyl-β-D-glucopyranoside.

White powder, soluble in water, freely soluble in methanol, slightly soluble in alcohol.

mp: 190 °C to 193 °C.

Lemon oil. 1101700.

See *Lemon oil* (0620).

Leucine. 1048500. [61-90-5].

See *Leucine* (0771).

Levomenol. C₁₅H₂₆O. (*M_r* 222.4). 1128800. [23089-26-1]. (2*S*)-6-Methyl-2-[(1*S*)-4-methylcyclohex-3-enyl]hept-5-en-2-ol. (–)-α-Bisabolol.

Colourless, viscous liquid with a slight, characteristic odour, practically insoluble in water, freely soluble in alcohol, in methanol, in toluene, in fatty oils and in essential oils.

*d*₂₀²⁰: 0.925 to 0.935.

*n*_D²⁰: 1.492 to 1.500.

[α]_D²⁰: –54.5 to –58.0, determined on a 50 mg/ml solution in *alcohol R*.

Levomenol used for gas chromatography complies with the following additional test.

Assay. Examine by gas chromatography (2.2.28) as prescribed in the monograph on *Matricaria oil* (1836), using a 4 g/l solution in *cyclohexane R*.

The content of levomenol is not less than 95.0 per cent, calculated by the normalisation procedure.

Limonene. C₁₀H₁₆. (*M_r* 136.2). 1048600.

[5989-27-5]. D-Limonene. (+)-*p*-Mentha-1,8-diene. (*R*)-4-Isopropenyl-1-methylcyclohex-1-ene.

A colourless liquid, practically insoluble in water, soluble in alcohol.

*d*₂₀²⁰: about 0.84.

*n*_D²⁰: 1.471 to 1.474.

[α]_D²⁰: + 96 to + 106.

bp: 175 °C to 177 °C.

Limonene used in gas chromatography complies with the following additional test.

Assay. Examine by gas chromatography (2.2.28) as prescribed in the monograph on *Peppermint oil* (0405) using the substance to be examined as the test solution.

The area of the principal peak is not less than 99.0 per cent of the total area of the peaks.

Linalol. C₁₀H₁₈O. (*M_r* 154.2). 1048700. [78-70-6].

(*RS*)-3,7-Dimethylocta-1,6-dien-3-ol.

Mixture of two stereoisomers (licareol and coriandrol).

Liquid, practically insoluble in water.

*d*₂₀²⁰: about 0.860.

*n*_D²⁰: about 1.462.

bp: about 200 °C.

Linalol used in gas chromatography complies with the following test.

Assay. Examine by gas chromatography (2.2.28) under the conditions described in the monograph on *Anise oil* (0804) using the substance to be examined as the test solution.

The area of the principal peak is not less than 98.0 per cent of the total area of the peaks.

Linalyl acetate. C₁₂H₂₀O₂. (*M_r* 196.3). 1107200. [115-95-7].

(*RS*)-1,5-Dimethyl-1-vinylhex-4-enyl acetate.

A colourless or slightly yellow liquid with a strong odour of bergamot and lavender.

*d*₂₅²⁵: : 0.895 to 0.912.

*n*_D²⁰: 1.448 to 1.451.

bp: about 215 °C.

Linalyl acetate used in gas chromatography complies with the following additional test.

Assay. Examine by gas chromatography (2.2.28) as prescribed in the monograph on *Bitter-orange-flower oil* (1175), using the substance to be examined as the test solution.

The area of the principal peak is not less than 95.0 per cent of the total area of the peaks.

Lindane. C₆H₆Cl₆. (*M_r* 290.8). 1128900. [58-89-9].

γ-Hexachlorocyclohexane.

See *Lindane* (0772).

For the monograph *Wool fat* (0134), a suitable certified reference solution (10 ng/μl in cyclohexane) may be used.

Linoleic acid. $C_{18}H_{32}O_2$. (M_r 280.5). 1143200. [60-33-3]. (9Z,12Z)-Octadeca-9,12-dienoic acid.

Colourless, oily liquid.

d_4^{20} : about 0.903.

n_D^{20} : about 1.470.

Linoleic acid used in the assay of total fatty acids in Saw palmetto fruit (1848) complies with the following additional requirement.

Assay. Examine by gas chromatography (2.2.28) as prescribed in the monograph on *Saw palmetto fruit (1848)*.

The content of linoleic acid is not less than 98 per cent, calculated by the normalisation procedure.

Linolenic acid. $C_{18}H_{30}O_2$. (M_r 278.4). 1143300. [463-40-1]. (9Z,12Z,15Z)-Octadeca-9,12,15-trienoic acid.

Colourless liquid, practically insoluble in water, soluble in organic solvents.

d_4^{20} : about 0.915.

n_D^{20} : about 1.480.

Linolenic acid used in the assay of total fatty acids in Saw palmetto fruit (1848) complies with the following additional requirement.

Assay. Examine by gas chromatography (2.2.28) as prescribed in the monograph on *Saw palmetto fruit (1848)*.

The content of linolenic acid is not less than 98 per cent, calculated by the normalisation procedure.

Linolenyl alcohol. $C_{18}H_{32}O$. (M_r 264.4). 1156200. [24149-05-1]. (9Z,12Z,15Z)-octadeca-9,12,15-trien-1-ol.

Content: minimum 96 per cent of $C_{18}H_{32}O$.

Linoleyl alcohol. $C_{18}H_{34}O$. (M_r 266.5). 1155900. [506-43-4]. (9Z,12Z)-octadeca-9,12-dien-1-ol.

Relative density: 0.830.

Content: minimum 85 per cent of $C_{18}H_{34}O$.

Lithium. Li. (A_r 6.94). 1048800. [7439-93-2].

A soft metal whose freshly cut surface is silvery-grey. It rapidly tarnishes in contact with air. It reacts violently with water, yielding hydrogen and giving a solution of lithium hydroxide; soluble in methanol, yielding hydrogen and a solution of lithium methoxide; practically insoluble in light petroleum.

Storage: under light petroleum or liquid paraffin.

Lithium carbonate. Li_2CO_3 . (M_r 73.9). 1048900. [554-13-2]. Dilithium carbonate.

A white, light powder, sparingly soluble in water, very slightly soluble in alcohol. A saturated solution at 20 °C contains about 13 g/l of Li_2CO_3 .

Lithium chloride. $LiCl$. (M_r 42.39). 1049000. [7447-41-8].

Crystalline powder or granules or cubic crystals, deliquescent, freely soluble in water, soluble in acetone and in alcohol. Aqueous solutions are neutral or slightly alkaline.

Storage: in an airtight container.

Lithium hydroxide. $LiOH \cdot H_2O$. (M_r 41.96). 1049100. [1310-66-3]. Lithium hydroxide monohydrate.

A white, granular powder, strongly alkaline, it rapidly absorbs water and carbon dioxide, soluble in water, sparingly soluble in alcohol.

Storage: in an airtight container.

Lithium metaborate, anhydrous. $LiBO_2$. (M_r 49.75). 1120000. [13453-69-5].

Lithium sulphate. $Li_2SO_4 \cdot H_2O$. (M_r 128.0). 1049200. [10102-25-7]. Dilithium sulphate monohydrate.

Colourless crystals, freely soluble in water, practically insoluble in alcohol.

Litmus. 1049300. [1393-92-6].

Schultz No. 1386.

Indigo-blue fragments prepared from various species of Rocella, Lecanora or other lichens, soluble in water, practically insoluble in alcohol.

Colour change: pH 5 (red) to pH 8 (blue).

Litmus paper, blue. 1049301.

Boil 10 parts of coarsely powdered *litmus R* for 1 h with 100 parts of *alcohol R*. Decant the alcohol and add to the residue a mixture of 45 parts of *alcohol R* and 55 parts of *water R*. After 2 days decant the clear liquid. Impregnate strips of filter paper with the solution and allow to dry.

Test for sensitivity. Immerse a strip measuring 10 mm by 60 mm in a mixture of 10 ml of 0.02 M hydrochloric acid and 90 ml of *water R*. On shaking the paper turns red within 45 s.

Litmus paper, red. 1049302.

To the blue litmus extract, add *dilute hydrochloric acid R* dropwise until the blue colour becomes red. Impregnate strips of filter paper with the solution and allow to dry.

Test for sensitivity. Immerse a strip measuring 10 mm by 60 mm in a mixture of 10 ml of 0.02 M sodium hydroxide and 90 ml of *water R*. On shaking the paper turns blue within 45 s.

Loganin. $C_{17}H_{26}O_{10}$. (M_r 390.4). 1136700. [18524-94-2]. Methyl (1S,4aS,6S,7R,7aS)-1-(β-D-glucopyranosyloxy)-6-hydroxy-7-methyl-1,4a,5,6,7,7a-hexahydrocyclopenta[c]pyran-4-carboxylate.

mp: 220 °C to 221 °C.

Longifolene. $C_{15}H_{24}$. (M_r 204.4). 1150300. [475-20-7]. (1S,3aR,4S,8aS)-4,8,8-Trimethyl-9-methylenedecahydro-1,4-methanoazulene.

Oily, colourless liquid, practically insoluble in water, miscible with alcohol.

d_4^{18} : 0.9319.

n_D^{20} : 1.5050.

$[\alpha]_D^{20}$: + 42.7.

bp: 254 °C to 256 °C.

Longifolene used in gas chromatography complies with the following additional test.

Assay. Examine by gas chromatography (2.2.28) as prescribed in the monograph on *Turpentine oil, Pinus pinaster type (1627)*.

The content is not less than 98.0 per cent, calculated by the normalisation procedure.

Low-vapour-pressure hydrocarbons (type L). 1049400.

Unctuous mass, soluble in benzene and in toluene.

Lumiflavine. $C_{13}H_{12}N_4O_2$. (M_r 256.3). 1141000. [1088-56-8]. 7,8,10-Trimethylbenzo[g]pteridine-2,4(3H,10H)-dione.

Yellow powder or orange crystals, very slightly soluble in water, freely soluble in methylene chloride.

Macrogol 23 lauryl ether. 1129000.

Complies with the monograph *Macrogol lauryl ether* (1124), the nominal value for the amount of ethylene oxide reacted with lauryl alcohol being 23.

Macrogol 200. 1099200. [25322-68-3]. Polyethyleneglycol 200.

A clear, colourless or almost colourless viscous liquid, very soluble in acetone and in ethanol, practically insoluble in fatty oils.

d_{20}^{20} : about 1.127.

n_D^{20} : about 1.450.

Macrogol 200 R1. 1099201.

Introduce 500 ml of *macrogol 200 R* into a 1000 ml round bottom flask. Using a rotation evaporator remove any volatile components applying for 6 h a temperature of 60 °C and a vacuum with a pressure of 1.5 kPa to 2.5 kPa.

Macrogol 300. 1067100. [25322-68-3]. Polyethyleneglycol 300.

See *Macrogols* (1444).

Macrogol 400. 1067200. [25322-68-3]. Polyethyleneglycol 400.

See *Macrogols* (1444).

Macrogol 1000. 1067300. [25322-68-3]. Polyethyleneglycol 1000.

See *Macrogols* (1444).

Macrogol 1500. 1067400. [25322-68-3]. Polyethyleneglycol 1500.

See *Macrogols* (1444).

Macrogol 20 000. 1067600. Polyethyleneglycol 20 000.

See *Macrogols* (1444).

Macrogol 20 000 2-nitroterephthalate. 1067601.

Polyethyleneglycol 20 000 2-nitroterephthalate.

Macrogol 20 000 R modified by treating with 2-nitroterephthalate acid.

A hard, white or almost white, waxy solid, soluble in acetone.

Magnesium. Mg. (A_r 24.30). 1049500. [7439-95-4].

Silver-white ribbon, turnings or wire, or a grey powder.

Magnesium acetate. $C_4H_6MgO_4 \cdot 4H_2O$. (M_r 214.5). 1049600. [16674-78-5]. Magnesium diacetate tetrahydrate.

Colourless crystals, deliquescent, freely soluble in water and in alcohol.

Storage: in an airtight container.

Magnesium chloride. 1049700. [7791-18-6].

See *Magnesium chloride hexahydrate* (0402).

Magnesium nitrate. $Mg(NO_3)_2 \cdot 6H_2O$. (M_r 256.4). 1049800. [13446-18-9]. Magnesium nitrate hexahydrate.

Colourless, clear crystals, deliquescent, very soluble in water, freely soluble in alcohol.

Storage: in an airtight container.

Magnesium nitrate solution. 1049801.

Dissolve 17.3 g of *magnesium nitrate R* in 5 ml of *water R* warming gently and add 80 ml of *alcohol R*. Cool and dilute to 100.0 ml with the same solvent.

Magnesium nitrate solution R1. 1049802.

Dissolve 20 g of *magnesium nitrate R* ($Mg(NO_3)_2 \cdot 6H_2O$) in *deionised distilled water R* and dilute to 100 ml with the same solvent. Immediately before use, dilute 10 ml to 100 ml with *deionised distilled water R*. A volume of 5 µl will provide 0.06 mg of Mg (NO_3)₂.

Magnesium oxide. 1049900. [1309-48-4].

See *Light magnesium oxide* (0040).

Magnesium oxide R1. 1049901.

Complies with the requirements prescribed for *magnesium oxide R* with the following modifications.

Arsenic (2.4.2). Dissolve 0.5 g in a mixture of 5 ml of *water R* and 5 ml of *hydrochloric acid R1*. The solution complies with limit test A for arsenic (2 ppm).

Heavy metals (2.4.8). Dissolve 1.0 g in a mixture of 3 ml of *water R* and 7 ml of *hydrochloric acid R1*. Add 0.05 ml of *phenolphthalein solution R* and *concentrated ammonia R* until a pink colour is obtained. Neutralise the excess of ammonia by the addition of *glacial acetic acid R*. Add 0.5 ml in excess and dilute to 20 ml with *water R*. Filter, if necessary. 12 ml of the solution complies with limit test A for heavy metals (10 ppm). Prepare the standard using a mixture of 5 ml of *lead standard solution* (1 ppm Pb) *R* and 5 ml of *water R*.

Iron (2.4.9). Dissolve 0.2 g in 6 ml of *dilute hydrochloric acid R* and dilute to 10 ml with *water R*. The solution complies with the limit test for iron (50 ppm).

Magnesium oxide, heavy. 1050000. [1309-48-4].

See *Heavy magnesium oxide* (0041).

Magnesium silicate for pesticide residue analysis. 1129100. [1343-88-0].

Magnesium silicate for chromatography (60-100 mesh).

Magnesium sulphate. 1050200. [10034-99-8].

See *Magnesium sulphate heptahydrate* (0044).

Maize oil. 1050400.

See *Maize oil, refined* (1342).

Malachite green. $C_{23}H_{25}ClN_2$. (M_r 364.9). 1050500. [12333-61-9].

Schultz No. 754.

Colour Index No. 42000.

[4-[[4-(Dimethylamino)phenyl]phenylmethylene]cyclohexa-2,5-dien-1-ylidene]dimethylammonium chloride.

Green crystals with a metallic lustre, very soluble in water giving a bluish-green solution, soluble in alcohol and in methanol.

A 0.01 g/l solution in *alcohol R* shows an absorption maximum (2.2.25) at 617 nm.

Malachite green solution. 1050501.

A 5 g/l solution in *anhydrous acetic acid R*.

Malathion. $C_{10}H_{19}O_6PS_2$. (M_r 330.3). 1129200. [121-75-5].

bp: about 156 °C.

A suitable certified reference solution (10 ng/µl in iso-octane) may be used.

Maleic acid. 1050600. [110-16-7].

See *Maleic acid* (0365).

Maleic anhydride. $C_4H_2O_3$. (M_r 98.1). 1050700. [108-31-6]. Butenedioic anhydride. 2,5-Furandione.

White crystals, soluble in water forming maleic acid, very soluble in acetone and in ethyl acetate, freely soluble in toluene, soluble in alcohol with ester formation, very slightly soluble in light petroleum.

mp: about 52 °C.

Any residue insoluble in toluene does not exceed 5 per cent (maleic acid).

Maleic anhydride solution. 1050701.

Dissolve 5 g of *maleic anhydride R* in *toluene R* and dilute to 100 ml with the same solvent. Use within one month. If the solution becomes turbid, filter.

Maltitol. 1136800. [585-88-6].

See *Maltitol* (1235).

Manganese sulphate. $MnSO_4 \cdot H_2O$. (M_r 169.0). 1050900. [10034-96-5]. Manganese sulphate monohydrate.

Pale-pink, crystalline powder or crystals, freely soluble in water, practically insoluble in alcohol.

Loss on ignition: 10.0 per cent to 12.0 per cent, determined on 1.000 g at 500 °C.

Mannitol. 1051000. [69-65-8].

See *Mannitol* (0559).

Mannose. $C_6H_{12}O_6$. (M_r 180.2). 1051100. [3458-28-4]. D-(+)-Mannose.

A white, crystalline powder or small, white crystals, very soluble in water, slightly soluble in ethanol.

$[\alpha]_D^{20}$: + 13.7 + 14.7, determined on a 200 g/l solution in *water R* containing about 0.05 per cent of NH_3 .

mp: about 132 °C, with decomposition.

Meclozine hydrochloride. 1051200. [1104-22-9].

See *Meclozine hydrochloride* (0622).

Melamine. $C_3H_6N_6$. (M_r 126.1). 1051300. [108-78-1]. 1,3,5-Triazine-2,4,6-triamine.

A white, amorphous powder, very slightly soluble in water and in alcohol.

Menadione. 1051400. [58-27-5].

See *Menadione* (0507).

Menthofuran. $C_{10}H_{14}O$. (M_r 150.2). 1051500. [17957-94-7]. 3,9-Epoxy-*p*-mentha-3,8-diene. 3,6-Dimethyl-4,5,6,7-tetrahydro-benzofuran.

A slightly bluish liquid, very slightly soluble in water, soluble in alcohol.

d_{15}^{20} : about 0.965.

n_D^{20} : about 1.480.

$[\alpha]_D^{20}$: about + 93.

bp: 196 °C.

Menthofuran used in gas chromatography complies with the following additional test.

Assay. Examine by gas chromatography (2.2.28) as prescribed in the monograph on *Peppermint oil* (0405) using the substance to be examined as the test solution.

The area of the principal peak is not less than 97.0 per cent of the total area of the peaks.

Menthol. 1051600. [2216-51-5]. See *Levomenthol* (0619) and *Racemic menthol* (0623).

Menthol used in gas chromatography complies with the following additional test.

Assay. Examine by gas chromatography (2.2.28) as prescribed in the Related substances test included in the monograph on *Racemic menthol* (0623).

The area of the principal peak is not less than 98.0 per cent of the total area of the peaks, disregarding any peak due to the solvent.

Menthone. $C_{10}H_{18}O$. (M_r 154.2). 1051700. [14073-97-3]. (2*S*,5*R*)-2-Isopropyl-5-methylcyclohexanone. (-)-*trans-p*-Menthane-3-one.

Contains variable amounts of isomenthone.

A colourless liquid, very slightly soluble in water, very soluble in alcohol.

d_{20}^{20} : about 0.897.

n_D^{20} : about 1.450.

Menthone used in gas chromatography complies with the following additional test.

Assay. Examine by gas chromatography (2.2.28) as prescribed in the monograph on *Peppermint oil* (0405) using the substance to be examined as the test solution.

The area of the principal peak is not less than 90.0 per cent of the total area of the peaks.

Menthyl acetate. $C_{12}H_{22}O_2$. (M_r 198.3). 1051800. [2623-23-6]. 2-Isopropyl-5-methylcyclohexyl acetate.

A colourless liquid, slightly soluble in water, miscible with alcohol.

d_{20}^{20} : about 0.92.

n_D^{20} : about 1.447.

bp: about 228 °C.

Menthyl acetate used in gas chromatography complies with the following additional test.

Assay. Examine by gas chromatography (2.2.28) as prescribed in the monograph on *Peppermint oil* (0405) using the substance to be examined as the test solution.

The area of the principal peak is not less than 97.0 per cent of the total area of the peaks.

2-Mercaptoethanol. C_2H_6OS . (M_r 78.1). 1099300. [60-24-2].

A liquid, miscible with water.

d_{20}^{20} : about 1.116.

bp: about 157 °C.

Mercaptopurine. 1051900. [6112-76-1].

See *Mercaptopurine* (0096).

Mercuric acetate. $C_4H_6HgO_4$. (M_r 318.7). 1052000. [1600-27-7]. Mercury diacetate.

White crystals, freely soluble in water, soluble in alcohol.

Mercuric acetate solution. 1052001.

Dissolve 3.19 g of *mercuric acetate R* in *anhydrous acetic acid R* and dilute to 100 ml with the same acid. If necessary, neutralise the solution with 0.1 *M perchloric acid* using 0.05 ml of *crystal violet solution R* as indicator.

Mercuric bromide. $HgBr_2$. (M_r 360.4). 1052100. [7789-47-1]. Mercury dibromide.

White or faintly yellow crystals or a crystalline powder, slightly soluble in water, soluble in alcohol.

Mercuric bromide paper. 1052101.

In a rectangular dish place a 50 g/l solution of *mercuric bromide R* in *ethanol R* and immerse in it pieces of white filter paper weighing 80 g per square metre (speed of filtration = filtration time expressed in seconds for 100 ml of water at 20 °C with a filter surface of 10 cm² and constant pressure of 6.7 kPa: 40 s to 60 s), each measuring 1.5 cm by 20 cm and folded in two. Allow the excess liquid to drain and allow the paper to dry, protected from light, suspended over a non-metallic thread. Discard 1 cm from each end of each strip and cut the remainder into 1.5 cm squares or discs of 1.5 cm diameter.

Storage: in a glass-stoppered container wrapped with black paper.

Mercuric chloride. 1052200. [7487-94-7].

See *Mercuric chloride* (0120).

Mercuric chloride solution. 1052201.

A 54 g/l solution.

Mercuric iodide. HgI₂. (*M_r* 454.4). 1052300. [7774-29-0]. Mercury di-iodide.

A dense, scarlet, crystalline powder, slightly soluble in water, sparingly soluble in acetone and in alcohol, soluble in an excess of *potassium iodide solution R*.

Storage: protected from light.

Mercuric nitrate. Hg(NO₃)₂·H₂O. (*M_r* 342.6). 1052400. [7782-86-7]. Mercury dinitrate monohydrate.

Colourless or slightly coloured crystals, hygroscopic, soluble in water in the presence of a small quantity of nitric acid.

Storage: in an airtight container, protected from light.

Mercuric oxide. HgO. (*M_r* 216.6). 1052500. [21908-53-2]. Yellow mercuric oxide. Mercury oxide.

A yellow to orange-yellow powder, practically insoluble in water and in alcohol.

Storage: protected from light.

Mercuric sulphate solution. 1052600. [7783-35-9].

Dissolve 1 g of *mercuric oxide R* in a mixture of 20 ml of *water R* and 4 ml of *sulphuric acid R*.

Mercuric thiocyanate. Hg(SCN)₂. (*M_r* 316.7). 1052700. [592-85-8]. Mercury di(thiocyanate).

A white, crystalline powder, very slightly soluble in water, slightly soluble in alcohol, soluble in solutions of sodium chloride.

Mercuric thiocyanate solution. 1052701.

Dissolve 0.3 g of *mercuric thiocyanate R* in *ethanol R* and dilute to 100 ml with the same solvent.

Storage: use within 1 week.

Mercury. Hg. (*A_r* 200.6). 1052800. [7439-97-6].

A silver-white liquid, breaking into spherical globules which do not leave a metallic trace when rubbed on paper.

*d*₂₀²⁰: about 13.5.

bp: about 357 °C.

Mercury, nitric acid solution of. 1052801.

Carefully dissolve 3 ml of *mercury R* in 27 ml of *fuming nitric acid R*. Dilute the solution with an equal volume of *water R*.

Storage: protected from light; use within 2 months.

Mesityl oxide. C₆H₁₀O. (*M_r* 98.1). 1120100. [141-79-7]. 4-Methylpent-3-en-2-one.

Colourless, oily liquid, soluble in 30 parts of water, miscible with most organic solvents.

*d*₂₀²⁰: about 0.858.

bp: 129 °C to 130 °C.

Metanil yellow. C₁₈H₁₄N₃NaO₃S. (*M_r* 375.4). 1052900. [587-98-4].

Schultz No. 169.

Colour Index No. 13065.

Sodium 3-[4-(phenylamino)phenylazo]benzenesulphonate.

A brownish-yellow powder, soluble in water and in alcohol.

Metanil yellow solution. 1052901.

A 1 g/l solution in *methanol R*.

Test for sensitivity. To 50 ml of *anhydrous acetic acid R* add 0.1 ml of the metanil yellow solution. Add 0.05 ml of 0.1 *M perchloric acid*; the colour changes from pinkish-red to violet.

Colour change: pH 1.2 (red) to pH 2.3 (orange-yellow).

Metaphosphoric acid. (HPO₃)_x. 1053000. [37267-86-0].

Glassy lumps or sticks containing a proportion of sodium metaphosphate, hygroscopic, very soluble in water.

Nitrates. Boil 1.0 g with 10 ml of *water R*, cool, add 1 ml of *indigo carmine solution R*, 10 ml of *nitrogen-free sulphuric acid R* and heat to boiling. The blue colour is not entirely discharged.

Reducing substances: maximum 0.01 per cent, calculated as H₃PO₃. Dissolve 35.0 g in 50 ml of *water R*. Add 5 ml of a 200 g/l solution of *sulphuric acid R*, 50 mg of *potassium bromide R* and 5.0 ml of 0.02 *M potassium bromate* and heat on a water-bath for 30 min. Allow to cool and add 0.5 g of *potassium iodide R*. Titrate the liberated iodine with 0.1 *M sodium thiosulphate*, using 1 ml of *starch solution R* as indicator. Carry out a blank test.

1 ml of 0.02 *M potassium bromate* is equivalent to 4.10 mg of H₃PO₃.

Storage: in an airtight container.

Methacrylic acid. C₄H₆O₂. (*M_r* 86.1). 1101800. [79-41-4]. 2-Methylprop-2-enoic acid.

A colourless liquid.

*n*_D²⁰: about 1.431.

bp: about 160 °C.

mp: about 16 °C.

Methanesulphonic acid. CH₃O₃S. (*M_r* 96.1). 1053100. [75-75-2].

A clear, colourless liquid, solidifying at about 20 °C, miscible with water, slightly soluble in toluene, practically insoluble in hexane.

*d*₂₀²⁰: about 1.48.

*n*_D²⁰: about 1.430.

Methanol. CH₃O. (*M_r* 32.04). 1053200. [67-56-1].

A clear, colourless, flammable liquid, miscible with water and with alcohol.

*d*₂₀²⁰: 0.791 to 0.793.

bp: 64 °C to 65 °C.

Methanol R1. 1053201.

Complies with the requirements prescribed for *methanol R* and the following additional requirement.

Minimum transmittance (2.2.25), determined using *water R* as compensation liquid: 20 per cent at 210 nm, 50 per cent at 220 nm, 75 per cent at 230 nm, 95 per cent at 250 nm, 98 per cent at 260 nm and at higher wavelengths.

Methanol R2. 1053202.

Methanol R2 used in liquid chromatography complies with the following additional requirements.

Content: minimum 99.8 per cent of CH₄O (*M_r* 32.04).

Absorbance (2.2.25). The absorbance at 225 nm using *water R* as the compensation liquid is not more than 0.17.

Methanol, hydrochloric. 1053203.

Dilute 1.0 ml of *hydrochloric acid R1* to 100.0 ml with *methanol R*.

Methanol, aldehyde-free. 1053300.

Dissolve 25 g of *iodine R* in 1 litre of *methanol R* and pour the solution, with constant stirring, into 400 ml of 1 *M* *sodium hydroxide*. Add 150 ml of *water R* and allow to stand for 16h. Filter. Boil under a reflux condenser until the odour of iodoform disappears. Distil the solution by fractional distillation.

Content: maximum 0.001 per cent of aldehydes and ketones.

Methanol, anhydrous. 1053400. [67-56-1].

Treat 1000 ml of *methanol R* with 5 g of *magnesium R*. If necessary initiate the reaction by adding 0.1 ml of *mercuric chloride solution R*. When the evolution of gas has ceased, distil the liquid and collect the distillate in a dry container protected from moisture.

Water (2.5.12): maximum 0.3 g/l.

DL-Methionine. 1129400. [59-51-8].

See *DL-Methionine* (0624).

L-Methionine. 1053500. [63-68-3].

See *Methionine* (1027).

(RS)-Methotrexate. 1120200. [60388-53-6].

(RS)-2-[4-[(2,4-diaminopteridin-6-yl)methyl]methylamino]benzoylamino]pentanedioic acid.

Content: minimum 96.0 per cent of C₂₀H₂₂N₈O₅.

mp: about 195 °C.

Methoxychlor. C₁₆H₁₅Cl₃O₂. (*M_r* 345.7). 1129300. [72-43-5]. 1,1-(2,2,2-Trichloroethylidene)-bis(4-methoxybenzene).

Practically insoluble in water, freely soluble in most organic solvents.

bp: about 346 °C.

mp: 78 °C to 86 °C.

A suitable certified reference solution (10 ng/μl in iso-octane) may be used.

trans-2-Methoxycinnamaldehyde. C₁₀H₁₀O₂. (*M_r* 162.2). 1129500. [60125-24-8].

mp: 44 °C to 46 °C.

trans-2-Methoxycinnamaldehyde used in gas chromatography complies with the following additional test.

Assay. Examine by gas chromatography (2.2.28) as prescribed in the monograph on *Cassia oil* (1496).

The content is not less than 96.0 per cent, calculated by the normalisation procedure.

Methoxyphenylacetic acid. C₉H₁₀O₃. (*M_r* 166.2). 1053600. [7021-09-2]. (RS)-2-Methoxy-2-phenylacetic acid.

A white, crystalline powder or white or almost white crystals, sparingly soluble in water, freely soluble in alcohol.

mp: about 70 °C.

Methoxyphenylacetic reagent. 1053601.

Dissolve 2.7 g of *methoxyphenylacetic acid R* in 6 ml of *tetramethylammonium hydroxide solution R* and add 20 ml of *ethanol R*.

Storage: in a polyethylene container.

Methyl acetate. C₃H₆O₂. (*M_r* 74.1). 1053700. [79-20-9].

A clear, colourless liquid, soluble in water, miscible with alcohol.

*d*₂₀²⁰: about 0.933.

*n*_D²⁰: about 1.361

bp: 56 °C to 58 °C.

Methyl 4-acetylbenzoate. C₁₀H₁₀O₃. (*M_r* 178.2). 1154100. [3609-53-8].

mp: about 94 °C.

Methyl 4-acetylbenzoate reagent. 1154101.

Dissolve 0.25 g of *methyl 4-acetylbenzoate R* in a mixture of 5 ml of *sulphuric acid R* and 85 ml of cooled *methanol R*.

4-Methylaminophenol sulphate. C₁₄H₂₀N₂O₆S. (*M_r* 344.4). 1053800. [55-55-0].

Colourless crystals, very soluble in water, slightly soluble in alcohol.

mp: about 260 °C.

Methyl anthranilate. C₈H₉NO₂. (*M_r* 151.2). 1107300. [134-20-3]. Methyl 2-aminobenzoate.

Colourless crystals or a colourless or yellowish liquid, soluble in water, freely soluble in alcohol.

bp: 134 °C to 136 °C.

mp: 24 °C to 25 °C.

Methyl anthranilate used in gas chromatography complies with the following additional test.

Assay. Examine by gas chromatography (2.2.28) as prescribed in the monograph on *Bitter-orange-flower oil* (1175), using the substance to be examined as the test solution. The area of the principal peak is not less than 95.0 per cent of the total area of the peaks.

Methyl arachidate. C₂₁H₄₂O₂. (*M_r* 326.6). 1053900. [1120-28-1]. Methyl eicosanoate.

Content: minimum 98.0 per cent of C₂₁H₄₂O₂, determined by gas chromatography (2.4.22).

A white or yellow, crystalline mass, soluble in alcohol and in light petroleum.

mp: about 46 °C.

Methyl behenate. C₂₃H₄₆O₂. (*M_r* 354.6). 1107500. [929-77-1]. Methyl docosanoate.

mp: 54 °C to 55 °C.

Methylbenzothiazolone hydrazone hydrochloride.

$C_8H_{10}ClN_3S_2H_2O$. (M_r 233.7). 1055300. [38894-11-0]. 3-Methylbenzothiazol-2(3*H*)-one hydrazone hydrochloride monohydrate.

An almost white or yellowish, crystalline powder.

mp: about 270 °C.

Suitability for determination of aldehydes. To 2 ml of *aldehyde-free methanol R* add 60 µl of a 1 g/l solution of *propionaldehyde R* in *aldehyde-free methanol R* and 5 ml of a 4 g/l solution of methylbenzothiazolone hydrazone hydrochloride. Mix. Allow to stand for 30 min. Prepare a blank omitting the propionaldehyde solution. Add 25.0 ml of a 2 g/l solution of *ferric chloride R* to the test solution and to the blank, dilute to 100.0 ml with *acetone R* and mix. The absorbance (2.2.25) of the test solution, measured at 660 nm using the blank as compensation liquid, is not less than 0.62.

2-Methylbutane. C_5H_{12} . (M_r 72.2). 1099500. [78-78-4].

Isopentane.

Content: minimum 99.5 per cent of C_5H_{12} .

A very flammable colourless liquid.

d_{20}^{20} : about 0.621.

n_D^{20} : about 1.354.

bp: about 29 °C.

Water (2.5.12): maximum 0.02 per cent.

Residue on evaporation: maximum 0.0003 per cent.

Minimum transmittance (2.2.25), determined using *water R* as compensation liquid: 50 per cent at 210 nm, 85 per cent at 220 nm, 98 per cent at 240 nm and at higher wavelengths.

2-Methylbut-2-ene. C_5H_{10} . (M_r 70.1). 1055400. [513-35-9].

A very flammable liquid, practically insoluble in water, miscible with alcohol.

bp: 37.5 °C to 38.5 °C.

Methyl caprate. 1054000.

See *Methyl decanoate R*.

Methyl caproate. $C_7H_{14}O_2$. (M_r 130.2). 1120300. [106-70-7]. Methyl hexanoate.

d_{20}^{20} : about 0.885.

n_D^{20} : about 1.405.

bp: 150 °C to 151 °C.

Methyl caprylate. $C_9H_{18}O_2$. (M_r 158.2). 1120400. [111-11-5]. Methyl octanoate.

d_{20}^{20} : about 0.876.

n_D^{20} : about 1.417.

bp: 193 °C to 194 °C.

Methylcellulose 450. 1055500. [9004-67-5].

See *Methylcellulose (0345)*.

The nominal viscosity is 450 mPa·s

Methyl cinnamate. $C_{10}H_{10}O_2$. (M_r 162.2). 1099400. [103-26-4].

Colourless crystals practically insoluble in water, soluble in alcohol.

n_D^{20} : about 1.56.

bp: about 260 °C.

mp: 34 °C to 36 °C.

Methyl decanoate. $C_{11}H_{22}O_2$. (M_r 186.3). 1054000. [110-42-9]. Methyl *n*-decanoate.

Content: minimum 99.0 per cent of $C_{11}H_{22}O_2$.

A clear, colourless or yellow liquid, soluble in light petroleum.

d_{20}^{20} : 0.871 to 0.876.

n_D^{20} : 1.425 to 1.426.

Foreign substances. Examine by gas chromatography (2.2.28), injecting equal volumes of each of the following:

(I) a 0.02 g/l solution of the substance to be examined in *carbon disulphide R*, (II) a 2 g/l solution of the substance to be examined in *carbon disulphide R*, and (III) *carbon disulphide R*. Carry out the chromatographic procedure under the conditions of the test for butylated hydroxytoluene prescribed in the monograph on *Wool fat (0134)*. The total area of any peaks, apart from the solvent peak and the principal peak, in the chromatogram obtained with solution (II) is less than the area of the principal peak in the chromatogram obtained with solution (I).

3-O-Methyldopamine hydrochloride. $C_9H_{14}ClNO_2$. (M_r 203.7). 1055600. [1477-68-5]. 4-(2-Aminoethyl)-2-methoxyphenol hydrochloride.

mp: 213 °C to 215 °C.

Chromatography. Examine as prescribed in the monograph on *Dopamine hydrochloride (0664)*, applying 10 µl of a 0.075 g/l solution in *methanol R*. The chromatogram obtained shows only one principal spot.

4-O-Methyldopamine hydrochloride. $C_9H_{14}ClNO_2$. (M_r 203.7). 1055700. [645-33-0]. 5-(2-Aminoethyl)-2-methoxyphenol hydrochloride.

mp: 207 °C to 208 °C.

Chromatography. Examine as prescribed in the monograph on *Dopamine hydrochloride (0664)*, applying 10 µl of a 0.075 g/l solution in *methanol R*. The chromatogram obtained shows only one principal spot.

Methylenebisacrylamide. $C_7H_{10}N_2O_2$. (M_r 154.2). 1056000. [110-26-9]. *N,N'*-Methylenebispropenamide.

A fine, white or almost white powder, slightly soluble in water, soluble in alcohol.

mp: It melts with decomposition at a temperature above 300 °C.

Methylene blue. $C_{16}H_{18}ClN_3S_xH_2O$. (M_r 319.9 for the anhydrous substance). 1055800. [7220-79-3].

Schultz No. 1038.

Colour Index No. 52015.

3,7-Dimethylaminophenothiazin-5-ium chloride.

It occurs in different hydrated forms and may contain up to 22 per cent of water. A dark-green or bronze, crystalline powder, freely soluble in water, soluble in alcohol.

Methylene chloride. CH_2Cl_2 . (M_r 84.9). 1055900. [75-09-2]. Dichloromethane.

A colourless liquid, sparingly soluble in water, miscible with alcohol.

bp: 39 °C to 42 °C.

Methylene chloride used in fluorimetry complies with the following additional requirement.

Fluorescence. Under irradiation at 365 nm, the fluorescence (2.2.21) measured at 460 nm in a 1 cm cell is not more intense than that of a solution containing 0.002 ppm of *quinine R* in 0.5 *M* sulphuric acid measured in the same conditions.

Methylene chloride, acidified. 1055901.

To 100 ml of *methylene chloride R* add 10 ml of *hydrochloric acid R*, shake, allow to stand and separate the two layers. Use the lower layer.

Methyl eicosenoate. $C_{21}H_{40}O_2$. (M_r 324.5). 1120500. [2390-09-2]. (11*Z*)-eicos-11-enoate.

Methyl erucate. $C_{23}H_{44}O_2$. (M_r 352.6). 1146100. [1120-34-9]. Methyl *cis*-13-docosenoate.

d_{20}^{20} : about 0.871.

n_D^{20} : about 1.456.

3-O-Methylestrone. $C_{19}H_{24}O_2$. (M_r 284.4). 1137000. [1624-62-0]. 3-Methoxy-1,3,5(10)-estratrien-17-one.

White to yellowish-white powder.

$[\alpha]_D^{20}$: about + 157.

mp: about 173 °C.

Methyl ethyl ketone. C_4H_8O . (M_r 72.1). 1054100. [78-93-3]. Ethyl methyl ketone. 2-Butanone.

A clear, colourless, flammable liquid, very soluble in water, miscible with alcohol.

d_{20}^{20} : about 0.81.

bp: 79 °C to 80 °C.

Methyl green. $C_{26}H_{33}Cl_2N_3$. (M_r 458.5). 1054200. [7114-03-6].

Schultz No. 788.

Colour Index No. 42585.

4-[[4-(Dimethyl-amino)phenyl][4-(dimethyliminio)cyclohexa-2,5-dienylidene]-methylphenyl]trimethylammonium dichloride.

Green powder, soluble in water, soluble in sulphuric acid giving a yellow solution turning green on dilution with water.

Methyl green-iodomercurate paper. 1054201.

Immerse thin strips of suitable filter paper in a 40 g/l solution of *methyl green R* and allow to dry in air.

Immerse the strips for 1 h in a solution containing 140 g/l of *potassium iodide R* and 200 g/l of *mercuric iodide R*. Wash with *distilled water R* until the washings are practically colourless and allow to dry in air.

Storage: protected from light; use within 48 h.

1-Methylimidazole. $C_4H_6N_2$. (M_r 82.1). 1139700. [616-47-7]. 1-Methyl-1*H*-imidazole.

Colourless or slightly yellowish liquid.

n_D^{20} : about 1.495.

bp: 195 °C to 197 °C.

Storage: in an airtight container, protected from light.

1-Methylimidazole R1. 1139701.

Complies with the requirements described for *1-methylimidazole R* with the following additional requirement.

Content: minimum 95.0 per cent.

2-Methylimidazole. $C_4H_6N_2$. (M_r 82.1). 1143400. [693-98-1].

White, crystalline powder.

mp: about 145 °C.

Methyl isobutyl ketone. $C_6H_{12}O$. (M_r 100.2). 1054300. [108-10-1]. 4-Methyl-2-pentanone.

A clear, colourless liquid, slightly soluble in water, miscible with most organic solvents.

d_{20}^{20} : about 0.80.

bp: about 115 °C.

Distillation range (2.2.11). Distil 100 ml. The range of temperature of distillation from 1 ml to 95 ml of distillate does not exceed 4.0 °C.

Residue on evaporation: maximum 0.01 per cent, determined by evaporating on a water-bath and drying at 100-105 °C.

Methyl isobutyl ketone R1. 1054301.

Shake 50 ml of freshly distilled *methyl isobutyl ketone R* with 0.5 ml of *hydrochloric acid R1* for 1 min. Allow the phases to separate and discard the lower phase. Prepare immediately before use.

Methyl isobutyl ketone R3. 1054302.

Complies with the requirements for *methyl isobutyl ketone R* and with the following limits:

Chromium: maximum 0.02 ppm.

Copper: maximum 0.02 ppm.

Lead: maximum 0.1 ppm.

Nickel: maximum 0.02 ppm.

Tin: maximum 0.1 ppm.

Methyl laurate. $C_{13}H_{26}O_2$. (M_r 214.4). 1054400. [111-82-0]. Methyl dodecanoate.

Content: minimum 98.0 per cent of $C_{13}H_{26}O_2$, determined by gas chromatography (2.4.22).

A colourless or yellow liquid, soluble in alcohol and in light petroleum.

d_{20}^{20} : about 0.87.

n_D^{20} : about 1.431.

mp: about 5 °C.

Methyl lignocerate. $C_{25}H_{50}O_2$. (M_r 382.7). 1120600. [2442-49-1]. Methyl tetracosanoate.

Flakes.

mp: about 58 °C.

Methyl linoleate. $C_{19}H_{34}O_2$. (M_r 294.5). 1120700. [112-63-0]. Methyl (9*Z*,12*Z*)-octadeca-9,12-dienoate.

d_{20}^{20} : about 0.888.

n_D^{20} : about 1.466.

bp: 207 °C to 208 °C.

Methyl linolenate. $C_{19}H_{32}O_2$. (M_r 292.5). 1120800. [301-00-8]. Methyl (9*Z*,12*Z*,15*Z*)-octadeca-9,12,15-trienoate.

d_{20}^{20} : about 0.901.

n_D^{20} : about 1.471.

bp: about 207 °C.

Methyl margarate. $C_{18}H_{36}O_2$. (M_r 284.5). 1120900. [1731-92-6]. Methyl heptadecanoate.

White or almost white powder.

mp: 32 °C to 34 °C.

Methyl margarate used in the assay of total fatty acids in Saw palmetto fruit (1848) complies with the following additional requirement.

Assay. Examine by gas chromatography (2.2.28) as prescribed in the monograph on *Saw palmetto fruit (1848)*.

The content of methyl margarate is not less than 97 per cent, calculated by the normalisation procedure.

Methyl methacrylate. $C_5H_8O_2$. (M_r 100.1). 1054500. [80-62-6]. Methyl 2-methylprop-2-enoate.

A colourless liquid.

n_D^{20} : about 1.414.

bp: about 100 °C.

mp: about -48 °C.

It contains a suitable stabilising reagent.

Methyl myristate. $C_{15}H_{30}O_2$. (M_r 242.4). 1054600. [124-10-7]. Methyl tetradecanoate.

Content: minimum 98.0 per cent of $C_{15}H_{30}O_2$, determined by gas chromatography (2.4.22).

A colourless or slightly yellow liquid, soluble in alcohol and in light petroleum.

d_{20}^{20} : about 0.87.

n_D^{20} : about 1.437.

mp: about 20 °C.

2-Methyl-5-nitroimidazole. $C_4H_5N_3O_2$. (M_r 127.1). 1056100. [88054-22-2].

White to light yellow powder.

mp: 252 °C to 254 °C.

Content: minimum 98.0 per cent of $C_4H_5N_3O_2$.

Methyl oleate. $C_{19}H_{36}O_2$. (M_r 296.4). 1054700. [112-62-9]. Methyl (Z)-octadec-9-enoate.

Content: minimum 98.0 per cent of $C_{19}H_{36}O_2$, determined by gas chromatography (2.4.22).

A colourless or slightly yellow liquid, soluble in alcohol and in light petroleum.

d_{20}^{20} : about 0.88.

n_D^{20} : about 1.452.

Methyl orange. $C_{14}H_{14}N_3NaO_3S$. (M_r 327.3). 1054800. [547-58-0].

Schultz No. 176.

Colour Index No. 13025.

Sodium 4'-(dimethylamino)azobenzene-4-sulphonate.

An orange-yellow, crystalline powder, slightly soluble in water, practically insoluble in alcohol.

Methyl orange mixed solution. 1054801.

Dissolve 20 mg of *methyl orange R* and 0.1 g of *bromocresol green R* in 1 ml of 0.2 M sodium hydroxide and dilute to 100 ml with *water R*.

Colour change: pH 3.0 (orange) to pH 4.4 (olive-green).

Methyl orange solution. 1054802.

Dissolve 0.1 g of *methyl orange R* in 80 ml of *water R* and dilute to 100 ml with *alcohol R*.

Test for sensitivity. A mixture of 0.1 ml of the methyl orange solution and 100 ml of *carbon dioxide-free water R* is yellow. Not more than 0.1 ml of 1 M *hydrochloric acid* is required to change the colour to red.

Colour change: pH 3.0 (red) to pH 4.4 (yellow).

Methyl palmitate. $C_{17}H_{34}O_2$. (M_r 270.5). 1054900. [112-39-0]. Methyl hexadecanoate.

Content: minimum 98.0 per cent of $C_{17}H_{34}O_2$, determined by gas chromatography (2.4.22).

A white or yellow, crystalline mass, soluble in alcohol and in light petroleum.

mp: about 30 °C.

Methyl palmitoleate. $C_{17}H_{32}O_2$. (M_r 268.4). 1121000. [1120-25-8]. Methyl (9Z)-hexadec-9-enoate.

d_{20}^{20} : about 0.876.

n_D^{20} : about 1.451.

Methyl parahydroxybenzoate. 1055000. [99-76-3].

See *Methyl parahydroxybenzoate* (0409).

Methyl pelargonate. $C_{10}H_{20}O_2$. (M_r 172.3). 1143500. [1731-84-6]. Methyl nonanoate.

Clear, colourless liquid.

d_4^{20} : about 0.873.

n_D^{20} : about 1.422.

bp: 91 °C to 92 °C.

Methyl pelargonate used in the assay of total fatty acids in Saw palmetto fruit (1848) complies with the following additional requirement.

Assay. Examine by gas chromatography (2.2.28) as prescribed in the monograph on *Saw palmetto fruit (1848)*. The content of methyl pelargonate is not less than 98 per cent, calculated by the normalisation procedure.

3-Methylpentan-2-one. $C_6H_{12}O$. (M_r 100.2). 1141100. [565-61-7].

Colourless, flammable liquid.

d_{20}^{20} : about 0.815.

n_D^{20} : about 1.400.

bp: about 118 °C

4-Methylpentan-2-ol. $C_6H_{14}O$. (M_r 102.2). 1114300. [108-11-2].

A clear, colourless, volatile liquid.

d_4^{20} : about 0.802.

n_D^{20} : about 1.411.

bp: about 132 °C.

Methylphenyloxazolybenzene. $C_{26}H_{20}N_2O_2$. (M_r 392.5). 1056200. [3073-87-8]. 1,4-Bis[2-(4-methyl-5-phenyl)oxazoly]benzene.

A fine, greenish-yellow powder with a blue fluorescence or small crystals, soluble in alcohol, sparingly soluble in xylene. mp: about 233 °C.

Methylphenyloxazolybenzene used for liquid scintillation is of a suitable analytical grade.

1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine. $C_{12}H_{15}N$. (173.3). 1137100. [28289-54-5]. MPTP.

A white or almost white, crystalline powder, slightly soluble in water.

mp: about 41 °C.

Methylpiperazine. $C_5H_{12}N_2$. (M_r 100.2). 1056300. [74879-18-8]. 1-Methylpiperazine.

A colourless liquid, miscible with water and with alcohol.

d_{20}^{20} : about 0.90.

n_D^{20} : about 1.466.

bp: about 138 °C.

4-(4-Methylpiperidino)pyridine. $C_{11}H_{16}N_2$. (M_r 176.3). 1114400. [80965-30-6].

A clear liquid.

n_D^{20} : about 1.565.

2-Methylpropanol. $C_4H_{10}O$. (M_r 74.1). 1056400. [78-83-1]. Isobutyl alcohol. 2-Methylpropan-1-ol.

A clear colourless liquid, soluble in water, miscible with alcohol.

d_{20}^{20} : about 0.80.

n_D^{15} : 1.397 to 1.399.

bp: about 107 °C.

Distillation range (2.2.11). Not less than 96 per cent distils between 107 °C and 109 °C.

2-Methyl-2-propanol. $C_4H_{10}O$. (M_r 74.1). 1056500. [75-65-0]. 1,1-Dimethyl ethyl alcohol. *tert*-Butyl alcohol.

A clear, colourless liquid or crystalline mass, soluble in water, miscible with alcohol.

Freezing point (2.2.18): about 25 °C.

Distillation range (2.2.11). Not less than 95 per cent distils between 81 °C and 83 °C.

Methyl red. C₁₅H₁₅N₃O₂. (*M_r* 269.3). 1055100. [493-52-7]. Schultz No. 250.

Colour Index No. 13020.

2-(4-Dimethylamino-phenylazo)benzoic acid.

A dark-red powder or violet crystals, practically insoluble in water, soluble in alcohol.

Methyl red mixed solution. 1055101.

Dissolve 0.1 g of *methyl red R* and 50 mg of *methylene blue R* in 100 ml of *alcohol R*.

Colour change: pH 5.2 (red-violet) to pH 5.6 (green).

Methyl red solution. 1055102.

Dissolve 50 mg in a mixture of 1.86 ml of 0.1 M *sodium hydroxide* and 50 ml of *alcohol R* and dilute to 100 ml with *water R*.

Test for sensitivity. To 0.1 ml of the methyl red solution add 100 ml of *carbon dioxide-free water R* and 0.05 ml of 0.02 M *hydrochloric acid*. The solution is red. Not more than 0.1 ml of 0.02 M *sodium hydroxide* is required to change the colour to yellow.

Colour change: pH 4.4 (red) to pH 6.0 (yellow).

Methyl salicylate. 1146200. [119-36-8].

See *Methyl salicylate* (0230)

Methyl stearate. C₁₉H₃₈O₂. (*M_r* 298.5). 1055200. [112-61-8]. Methyl octadecanoate.

Content: minimum 98.0 per cent of C₁₉H₃₈O₂, determined by gas chromatography (2.4.22).

A white or yellow, crystalline mass, soluble in alcohol and in light petroleum.

mp: about 38 °C.

Methyl tricosanoate. C₂₄H₄₈O₂. (*M_r* 368.6). 1111500. [2433-97-8]. Tricosanoic acid methyl ester.

Content: minimum 99.0 per cent of C₂₄H₄₈O₂.

White crystals, practically insoluble in water, soluble in hexane.

mp: 55 °C to 56 °C.

Methyl tridecanoate. C₁₄H₂₈O₂. (*M_r* 228.4). 1121100. [1731-88-0].

A colourless or slightly yellow liquid, soluble in alcohol and in light petroleum.

*d*₂₀²⁰: about 0.86.

*n*_D²⁰: about 1.441.

mp: about 6 °C.

N-Methyltrimethylsilyl-trifluoroacetamide.

C₆H₁₂F₃NOSi. (*M_r* 199.3). 1129600. [24589-78-4]. 2,2,2-Trifluoro-N-methyl-N-(trimethylsilyl)acetamide.

*n*_D²⁰: about 1.380.

bp: 130 °C to 132 °C.

Minocycline hydrochloride. 1146300.

See *Minocycline hydrochloride* (1030).

Molecular sieve. 1056600.

Molecular sieve composed of sodium aluminosilicate. It is available as beads with a pore size of 0.4 nm and with a diameter of 2 mm.

Molecular sieve for chromatography. 1129700.

A molecular sieve composed of sodium aluminosilicate. The pore size is indicated after the name of the reagent in the tests where it is used. If necessary, the particle size is also indicated.

Molybdovanadic reagent. 1056700.

In a 150 ml beaker, mix 4 g of finely powdered *ammonium molybdate R* and 0.1 g of finely powdered *ammonium vanadate R*. Add 70 ml of *water R* and grind the particles using a glass rod. A clear solution is obtained within a few minutes. Add 20 ml of *nitric acid R* and dilute to 100 ml with *water R*.

Monodocosahexaenoin. C₂₅H₃₈O₄. (*M_r* 402.6). 1143600. [124516-13-8]. Monoglyceride of docosahexaenoic acid (C22:6). Glycerol monodocosahexaenoate. (*all-Z*)-Docosa-4,7,10,13,16,19-hexaenoic acid, monoester with propane-1,2,3-triol.

Mordant black 11. C₂₀H₁₂N₃NaO₇S. (*M_r* 461.4). 1056800. [1787-61-7].

Schultz No. 241.

Colour Index No. 14645.

Sodium 2-hydroxy-1-[(1-hydroxynaphth-2-yl)azo]-6-nitronaphthalene-4-sulphonate. Eriochrome black.

A brownish-black powder, soluble in water and in alcohol.

Storage: in an airtight container, protected from light.

Mordant black 11 triturate. 1056801.

Mix 1 g of *mordant black 11 R* with 99 g of *sodium chloride R*.

Test for sensitivity. Dissolve 50 mg in 100 ml of *water R*. The solution is brownish-violet. On addition of 0.3 ml of *dilute ammonia R1* the solution turns blue. On the subsequent addition of 0.1 ml of a 10 g/l solution of *magnesium sulphate R*, it turns violet.

Storage: in an airtight container, protected from light.

Morphine hydrochloride. 1056900.

See *Morphine hydrochloride* (0097).

Morpholine. C₄H₉NO. (*M_r* 87.1). 1057000. [110-91-8]. Tetrahydro-1,4-oxazine.

A colourless, hygroscopic liquid, flammable, soluble in water and in alcohol.

*d*₂₀²⁰: about 1.01.

Distillation range (2.2.11). Not less than 95 per cent distils between 126 °C and 130 °C.

Storage: in an airtight container.

Morpholine for chromatography. 1057001.

It complies with the requirements of *morpholine R* and with the following requirement.

Content: minimum 99.5 per cent of C₄H₉NO.

Murexide. C₈H₈N₆O₆·H₂O. (*M_r* 302.2). 1137200. 5,5'-Nitrilobis(pyrimidine-2,4,6-(1*H*,3*H*,5*H*)-trione) monoammonium salt.

Brownish-red crystalline powder, sparingly soluble in cold water, soluble in hot water, practically insoluble in alcohol, soluble in solutions of potassium hydroxide or sodium hydroxide giving a blue colour.

Myosmine. C₉H₁₀N₂. (*M_r* 146.2). 1121200. [532-12-7]. 3-(4,5-Dihydro-3*H*-pyrrol-2-yl)pyridine.

Colourless crystals.

mp: about 45 °C.

β -Myrcene. $C_{10}H_{16}$. (M_r 136.2). 1114500. [123-35-3]. 7-Methyl-3-methylenocta-1,6-diene.

An oily liquid with a pleasant odour, practically insoluble in water, miscible with alcohol, soluble in glacial acetic acid. It dissolves in solutions of alkali hydroxides.

d_4^{20} : about 0.794.

n_D^{20} : about 1.470.

β -Myrcene used in gas chromatography complies with the following additional test.

Assay. Examine by gas chromatography (2.2.28) as prescribed in the monograph on *Peppermint oil* (0405).

Test solution. The substance to be examined.

The area of the principal peak is not less than 90.0 per cent of the area of all the peaks in the chromatogram obtained.

Myristic acid. $C_{14}H_{28}O_2$. (M_r 228.4). 1143700. [544-63-8]. Tetradecanoic acid.

Colourless or white flakes.

mp: about 58.5 °C.

Myristic acid used in the assay of total fatty acids in Saw palmetto fruit (1848) complies with the following additional requirement.

Assay. Examine by gas chromatography (2.2.28) as prescribed in the monograph on *Saw palmetto fruit* (1848).

The content of myristic acid is not less than 97 per cent, calculated by the normalisation procedure.

Myristicine. $C_{11}H_{12}O_3$. (M_r 192.2). 1099600. [607-91-0]. 5-Allyl-1-methoxy-2,3-methylenedioxybenzene. 4-Methoxy-6-(prop-2-enyl)-1,3-benzodioxole.

An oily colourless liquid, practically insoluble in water, slightly soluble in ethanol, miscible with toluene and with xylene.

d_{20}^{20} : about 1.144.

n_D^{20} : about 1.540.

bp: 276 °C to 277 °C.

mp: about 173 °C.

Chromatography. Examined as prescribed in the monograph on *Star anise* (1153), the chromatogram obtained shows only one principal spot.

Myristicine used in gas chromatography complies with the following additional test.

Assay. Examine by gas chromatography (2.2.28) under the conditions prescribed in the monograph on *Nutmeg oil* (1552).

The content is not less than 95.0 per cent, calculated by the normalisation procedure.

Storage: protected from light.

Myristyl alcohol. $C_{14}H_{30}O$. (M_r 214.4). 1121300. [112-72-1]. 1-Tetradecanol.

d_{20}^{20} : about 0.823.

mp: 38 °C to 40 °C.

Naphthalene. $C_{10}H_8$. (M_r 128.2). 1057100. [91-20-3].

White crystals, practically insoluble in water, soluble in alcohol.

mp: about 80 °C.

Naphthalene used for liquid scintillation is of a suitable analytical grade.

Naphtharson. $C_{16}H_{11}AsN_2Na_2O_{10}S_2$. (M_r 576.3). 1121400. [132-33-2]. Thorin. Disodium 4-[(2-arsonophenyl)azo]-3-hydroxynaphthalene-2,7-disulphonate.

A red powder, soluble in water.

Naphtharson solution. 1121401.

A 0.58 g/l solution.

Test for sensitivity. To 50 ml of *alcohol R*, add 20 ml of *water R*, 1 ml of 0.05 M *sulphuric acid* and 1 ml of the naphtharson solution. Titrate with 0.025 M *barium perchlorate*; the colour changes from orange-yellow to orange-pink.

Storage: protected from light; use within 1 week.

α -Naphthol. $C_{10}H_8O$. (M_r 144.2). 1057300. [90-15-3]. 1-Naphthol.

A white, crystalline powder or colourless or white crystals, darkening on exposure to light, slightly soluble in water, freely soluble in alcohol.

mp: about 95 °C.

Storage: protected from light.

α -Naphthol solution. 1057301.

Dissolve 0.10 g of α -naphthol R in 3 ml of a 150 g/l solution of *sodium hydroxide R* and dilute to 100 ml with *water R*. Prepare immediately before use.

β -Naphthol. $C_{10}H_8O$. (M_r 144.2). 1057400. [135-19-3]. 2-Naphthol.

White or slightly pink plates or crystals, very slightly soluble in water, very soluble in alcohol.

mp: about 122 °C.

Storage: protected from light.

β -Naphthol solution. 1057401.

Dissolve 5 g of freshly recrystallised β -naphthol R in 40 ml of *dilute sodium hydroxide solution R* and dilute to 100 ml with *water R*. Prepare immediately before use.

β -Naphthol solution R1. 1057402.

Dissolve 3.0 mg of β -naphthol R in 50 ml of *sulphuric acid R* and dilute to 100.0 ml with the same acid. Use the recently prepared solution.

Naphtholbenzein. $C_{27}H_{20}O_3$. (M_r 392.5). 1057600. [6948-88-5]. α -Naphtholbenzein. Phenylbis(4-hydroxynaphthyl)methanol.

A brownish-red powder or shiny brownish-black crystals, practically insoluble in water, soluble in alcohol and in glacial acetic acid.

Naphtholbenzein solution. 1057601.

A 2 g/l solution in *anhydrous acetic acid R*.

Test for sensitivity. To 50 ml of *glacial acetic acid R* add 0.25 ml of the naphtholbenzein solution. The solution is brownish-yellow. Not more than 0.05 ml of 0.1 M *perchloric acid* is required to change the colour to green.

Naphthol yellow. $C_{10}H_5N_2NaO_5$. (M_r 256.2). 1136600. 2,4-Dinitro-1-naphthol, sodium salt.

Orange-yellow powder or crystals, freely soluble in water, slightly soluble in ethanol.

Naphthol yellow S. $C_{10}H_4N_2Na_2O_8S$. (M_r 358.2). 1143800. [846-70-8].

Colour Index No. 10316.

8-Hydroxy-5,7-dinitro-2-naphthalenesulphonic acid disodium salt. Disodium 5,7-dinitro-8-oxidonaphthalene-2-sulphonate.

Yellow or orange-yellow powder, freely soluble in water.

1-Naphthylacetic acid. $C_{12}H_{10}O_2$. (M_r 186.2). 1148400. [86-87-3]. (Naphthalen-1-yl)acetic acid.

White to yellow crystalline powder, very slightly soluble in water, freely soluble in acetone.

mp: about 135 °C.

Naphthylamine. $C_{10}H_9N$. (M_r 143.2). 1057700. [134-32-7]. 1-Naphthylamine.

A white, crystalline powder, turning pink on exposure to light and air, slightly soluble in water, freely soluble in alcohol.

mp: about 51 °C.

Storage: protected from light.

Naphthylethylenediamine dihydrochloride.

$C_{12}H_{16}Cl_2N_2$. (M_r 259.2). 1057800. [1465-25-4]. *N*-(1-Naphthyl)ethylene-diamine dihydrochloride.

It may contain methanol of crystallisation.

A white to yellowish-white powder, soluble in water, slightly soluble in alcohol.

Naphthylethylenediamine dihydrochloride solution. 1057801.

Dissolve 0.1 g of *naphthylethylenediamine dihydrochloride R* in *water R* and dilute to 100 ml with the same solvent. Prepare immediately before use.

Naringin. $C_{27}H_{32}O_{14}$. (M_r 580.5). 1137300. [10236-47-2]. 7-[[2-*O*-(6-Deoxy- α -L-mannopyranosyl)- β -D-glucopyranosyl]oxy]-5-hydroxy-2-(4-hydroxyphenyl)-2,3-dihydro-4*H*-chromen-4-one.

A white or almost white crystalline powder, slightly soluble in water, soluble in methanol and in dimethylformamide.

mp: about 171 °C.

Absorbance (2.2.25). Naringin dissolved in a 5 g/l solution of *dimethylformamide R* in *methanol R* shows an absorption maximum at 283 nm.

trans-Nerolidol. $C_{15}H_{26}O$. (M_r 222.4). 1107900. [40716-66-3]. 3,7,11-Trimethyldodeca-1,6,10-trien-3-ol.

A slightly yellow liquid, slight odour of lily and lily of the valley, practically insoluble in water and in glycerol, miscible with alcohol.

d_{20}^{20} : about 0.876.

n_D^{20} : about 1.479.

bp₁₂: 145 °C to 146 °C.

trans-Nerolidol used in gas chromatography complies with the following additional test.

Assay. Examine by gas chromatography (2.2.28) as prescribed in the monograph on *Bitter-orange-flower oil* (1175), using the substance to be examined as the test solution. The area of the principal peak is not less than 90.0 per cent of the total area of the peaks.

Neryl acetate. $C_{12}H_{20}O_2$. (M_r 196.3). 1108000. [141-12-8]. (*Z*)-3,7-Dimethylocta-2,6-dienyl acetate.

A colourless, oily liquid.

d_{20}^{20} : about 0.907.

n_D^{20} : about 1.460.

bp₂₅: 134 °C.

Neryl acetate used in gas chromatography complies with the following additional test.

Assay. Examine by gas chromatography (2.2.28) as prescribed in the monograph on *Bitter-orange-flower oil* (1175), using the substance to be examined as the test solution. The area of the principal peak is not less than 93.0 per cent of the total area of the peaks.

Nickel-aluminium alloy. 1058100.

Contains 48 per cent to 52 per cent of aluminium (Al; A_r 26.98) and 48 per cent to 52 per cent of nickel (Ni; A_r 58.70).

Before use, reduce to a fine powder (180).

It is practically insoluble in water and soluble in mineral acids.

Nickel-aluminium alloy (halogen-free). 1118100.

Contains 48 per cent to 52 per cent of aluminium (Al; A_r 26.98) and 48 per cent to 52 per cent of nickel (Ni; A_r 58.71).

Fine, grey powder, practically insoluble in water, soluble in mineral acids with formation of salts.

Chlorides: maximum 10 ppm.

Dissolve 0.400 g in 40 ml of a mixture of 67 volumes of *sulphuric acid R* and 33 volumes of *dilute nitric acid R*. Evaporate the solution nearly to dryness, dissolve the residue in *water R* and dilute to 20.0 ml with the same solvent. To one half-aliquot of the solution, add 1.0 ml of 0.1 *M silver nitrate*. Filter after 15 min and add 0.2 ml of sodium chloride solution (containing 10 µg of chlorides per millilitre) to the filtrate. After 5 min the solution is more opalescent than a mixture of the second half-aliquot of the solution with 1.0 ml of 0.1 *M silver nitrate*.

Nickel chloride. $NiCl_2$. (M_r 129.6). 1057900. [7718-54-9]. Nickel chloride, anhydrous.

A yellow, crystalline powder, very soluble in water, soluble in alcohol. It sublimes in the absence of air and readily absorbs ammonia. The aqueous solution is acid.

Nickel sulphate. $NiSO_4 \cdot 7H_2O$. (M_r 280.9). 1058000. [10101-98-1]. Nickel sulphate heptahydrate.

A green, crystalline powder or crystals, freely soluble in water, slightly soluble in alcohol.

Nicotinamide-adenine dinucleotide. $C_{21}H_{27}N_7O_{14}P_2$. (M_r 663). 1108100. [53-84-9]. NAD^+ .

A white powder, very hygroscopic, freely soluble in water.

Nicotinamide-adenine dinucleotide solution. 1108101.

Dissolve 40 mg of *nicotinamide-adenine dinucleotide R* in *water R* and dilute to 10 ml with the same solvent.

Prepare immediately before use.

Nile blue A. $C_{20}H_{21}N_3O_5S$. (M_r 415.5). 1058200. [3625-57-8]. Schultz No. 1029.

Colour Index No. 51180.

5-Amino-9-(diethylamino)benzo[*a*]phenoxazinylum hydrogen sulphate.

A green, crystalline powder with a bronze lustre, sparingly soluble in alcohol, in glacial acetic acid and in pyridine.

A 0.005 g/l solution in *alcohol* (50 per cent V/V) *R* shows an absorption maximum (2.2.25) at 640 nm.

Nile blue A solution. 1058201.

A 10 g/l solution in *anhydrous acetic acid R*.

Test for sensitivity. To 50 ml of *anhydrous acetic acid R* add 0.25 ml of the Nile blue A solution. The solution is blue. On the addition of 0.1 ml of 0.1 *M perchloric acid*, the colour changes to blue-green.

Colour change: pH 9.0 (blue) to pH 13.0 (red).

Ninhydrin. $C_9H_4O_3 \cdot H_2O$. (M_r 178.1). 1058300. [485-47-2]. 1,2,3-Indanetrione monohydrate.

A white or very pale yellow, crystalline powder, soluble in water and in alcohol.

Storage: protected from light.

Ninhydrin and stannous chloride reagent. 1058301.

Dissolve 0.2 g of *ninhydrin R* in 4 ml of hot *water R*, add 5 ml of a 1.6 g/l solution of *stannous chloride R*, allow to stand for 30 min, then filter and store at a temperature of 2 °C to 8 °C. Immediately before use dilute 2.5 ml of the solution with 5 ml of *water R* and 45 ml of *2-propanol R*.

Ninhydrin and stannous chloride reagent R1. 1058302.

Dissolve 4 g of *ninhydrin R* in 100 ml of *ethylene glycol monomethyl ether R*. Shake gently with 1 g of *cation exchange resin R* (300 µm to 840 µm) and filter (solution a). Dissolve 0.16 g of *stannous chloride R* in 100 ml of *buffer solution pH 5.5 R* (solution b). Immediately before use, mix equal volumes of each solution.

Ninhydrin solution. 1058303.

A 2 g/l solution of *Ninhydrin R* in a mixture of 5 volumes of *dilute acetic acid R* and 95 volumes of *butanol R*.

Ninhydrin solution R1. 1058304.

Dissolve 1.0 g of *ninhydrin R* in 50 ml of *alcohol R* and add 10 ml of *glacial acetic acid R*.

Ninhydrin solution R2. 1058305.

Dissolve 3 g of *ninhydrin R* in 100 ml of a 45.5 g/l solution of *sodium metabisulphite R*.

Ninhydrin solution R3. 1058306.

A 4 g/l solution in a mixture of 5 volumes of *anhydrous acetic acid R* and 95 volumes of *butanol R*.

Nitrazepam. 1143900. [146-22-5].

See *Nitrazepam* (0415).

Nitric acid. HNO₃. (*M_r* 63.0). 1058400. [7697-37-2].

Content: 63.0 per cent *m/m* to 70.0 per cent *m/m* of HNO₃. A clear, colourless or almost colourless liquid, miscible with water.

*d*₂₀²⁰: 1.384 to 1.416.

A 10 g/l solution is strongly acid and gives the reaction of nitrates (2.3.1).

Appearance. Nitric acid is clear (2.2.1) and not more intensely coloured than reference solution Y₆ (*Method II*, 2.2.2).

Chlorides (2.4.4). To 5 g add 10 ml of *water R* and 0.3 ml of *silver nitrate solution R2* and allow to stand for 2 min protected from light. Any opalescence is not more intense than that of a standard prepared in the same manner using 13 ml of *water R*, 0.5 ml of *nitric acid R*, 0.5 ml of *chloride standard solution* (5 ppm Cl) *R* and 0.3 ml of *silver nitrate solution R2* (0.5 ppm).

Sulphates (2.4.13). Evaporate 10 g to dryness with 0.2 g of *sodium carbonate R*. Dissolve the residue in 15 ml of *distilled water R*. The solution complies with the limit test for sulphates (2 ppm). Prepare the standard using a mixture of 2 ml of *sulphate standard solution* (10 ppm SO₄) *R* and 13 ml of *distilled water R*.

Arsenic (2.4.2). Gently heat 50 g with 0.5 ml of *sulphuric acid R* until white fumes begin to evolve. To the residue add 1 ml of a 100 g/l solution of *hydroxylamine hydrochloride R* and dilute to 2 ml with *water R*. The solution complies with limit test A for arsenic (0.02 ppm). Prepare the standard using 1.0 ml of *arsenic standard solution* (1 ppm As) *R*.

Heavy metals (2.4.8). Dilute 10 ml of the solution prepared for the limit test for iron to 20 ml with *water R*. 12 ml of the solution complies with limit test A for heavy metals (2 ppm). Prepare the standard using *lead standard solution* (2 ppm Pb) *R*.

Iron (2.4.9). Dissolve the residue from the determination of sulphated ash in 1 ml of *dilute hydrochloric acid R* and dilute to 50 ml with *water R*. 5 ml of the solution diluted to 10 ml with *water R* complies with the limit test for iron (1 ppm).

Sulphated ash. Carefully evaporate 100 g to dryness.

Moisten the residue with a few drops of *sulphuric acid R* and heat to dull red. The residue does not exceed 0.001 per cent.

Assay. To 1.50 g add about 50 ml of *water R* and titrate with 1 M *sodium hydroxide*, using 0.1 ml of *methyl red solution R* as indicator.

1 ml of 1 M *sodium hydroxide* is equivalent to 63.0 mg of HNO₃.

Storage: protected from light.

Nitric acid, cadmium- and lead-free. 1058401.

Complies with the requirements prescribed for Nitric acid *R* and with the following additional test.

Test solution. To 100 g add 0.1 g of *anhydrous sodium carbonate R* and evaporate to dryness. Dissolve the residue in *water R* heating slightly, and dilute to 50.0 ml with the same solvent.

Cadmium: maximum 0.1 ppm of cadmium (Cd) determined by atomic absorption spectrometry (*Method II*, 2.2.23) measuring the absorbance at 228.8 nm using a cadmium hollow-cathode lamp and an air-acetylene or air-propane flame.

Lead: maximum 0.1 ppm of lead (Pb) determined by atomic absorption spectrometry (*Method II*, 2.2.23) measuring the absorbance at 283.3 nm or 217.0 nm using a lead hollow-cathode lamp and an air-acetylene flame.

Nitric acid, dilute. 1058402.

Contains about 125 g/l of HNO₃ (*M_r* 63.0).

Dilute 20 g of *nitric acid R* to 100 ml with *water R*.

Nitric acid, heavy metal-free. 1058404.

Complies with the requirements prescribed for *nitric acid R* and with the following maximum contents of heavy metals:

As: 0.005 ppm;
Cd: 0.005 ppm;
Cu: 0.001 ppm;
Fe: 0.02 ppm;
Hg: 0.002 ppm;
Ni: 0.005 ppm;
Pb: 0.001 ppm;
Zn: 0.01 ppm.

Nitric acid, lead-free. 1058403.

Complies with the requirements prescribed for *Nitric acid R* and with the following additional test:

To 100 g add 0.1 g of *anhydrous sodium carbonate R* and evaporate to dryness. Dissolve the residue in *water R*, heating slightly, and dilute to 50.0 ml with the same solvent. Determine the lead content by atomic absorption spectrometry (*Method II*, 2.2.23) measuring the absorbance at 283.3 nm or 217.0 nm using a lead hollow-cathode lamp and an air-acetylene flame. It contains not more than 0.1 ppm of lead (Pb).

Nitric acid, lead-free R1. 1058405.

Nitric acid R containing not more than 1 µg/kg of lead.

Nitric acid, lead-free, dilute. 1058406.

Dilute 5 g of *lead-free nitric acid R1* to 100 ml with *deionised distilled water R*.

Nitric acid, fuming. 1058500. [52583-42-3].

A clear, slightly yellowish liquid, fuming on contact with air.
 d_{20}^{20} : about 1.5.

Nitrilotriacetic acid. $C_6H_9NO_6$. (M_r 191.1). 1137400. [139-13-9].

White crystalline powder, practically insoluble in water and in most organic solvents.
 mp: about 240 °C, with decomposition.

Nitroaniline. $C_6H_6N_2O_2$. (M_r 138.1). 1058600. [100-01-6]. 4-Nitroaniline.

A bright yellow, crystalline powder, very slightly soluble in water, sparingly soluble in boiling water, soluble in alcohol, forms water-soluble salts with strong mineral acids.
 mp: about 147 °C.

Nitrobenzaldehyde. $C_7H_5NO_3$. (M_r 151.1). 1058700. [552-89-6]. 2-Nitrobenzaldehyde.

Yellow needles, slightly soluble in water, freely soluble in alcohol, volatile in steam.
 mp: about 42 °C.

Nitrobenzaldehyde paper. 1058701.

Dissolve 0.2 g of *nitrobenzaldehyde R* in 10 ml of a 200 g/l solution of *sodium hydroxide R*. Use the solution within 1 h. Immerse the lower half of a slow filter paper strip 10 cm long and 0.8 cm to 1 cm wide. Absorb the excess reagent between two sheets of filter paper. Use within a few minutes of preparation.

Nitrobenzaldehyde solution. 1058702.

Add 0.12 g of powdered *nitrobenzaldehyde R* to 10 ml of *dilute sodium hydroxide solution R*; allow to stand for 10 min shaking frequently and filter. Prepare immediately before use.

Nitrobenzene. $C_6H_5NO_2$. (M_r 123.1). 1058800. [98-95-3].

A colourless or very slightly yellow liquid, practically insoluble in water, miscible with alcohol.
 bp: about 211 °C.

Dinitrobenzene. To 0.1 ml add 5 ml of *acetone R*, 5 ml of *water R* and 5 ml of *strong sodium hydroxide solution R*. Shake and allow to stand. The upper layer is almost colourless.

4-Nitrobenzoic acid. $C_7H_5NO_4$. (M_r 167.1). 1144000. [62-23-7].

Yellow crystals.
 mp: about 240 °C.

Nitrobenzoyl chloride. $C_7H_4ClNO_3$. (M_r 185.6). 1058900. [122-04-3]. 4-Nitrobenzoyl chloride.

Yellow crystals or a crystalline mass, decomposing in moist air, completely soluble in sodium hydroxide solution giving a yellowish-orange colour.
 mp: about 72 °C.

Nitrobenzyl chloride. $C_7H_6ClNO_2$. (M_r 171.6). 1059000. [100-14-1]. 4-Nitrobenzyl chloride.

Pale-yellow crystals, lachrymatory, practically insoluble in water, very soluble in alcohol.

4-(4-Nitrobenzyl)pyridine. $C_{12}H_{10}N_2O_2$. (M_r 214.2). 1101900. [1083-48-3].

Yellow powder.
 mp: about 70 °C.

Nitrochromic reagent. 1059100.

Dissolve 0.7 g of *potassium dichromate R* in *nitric acid R* and dilute to 100 ml with the same acid.

Nitroethane. $C_2H_5NO_2$. (M_r 75.1). 1059200. [79-24-3].

A clear, oily, colourless liquid.
 bp: about 114 °C.

Nitrofurantoin. 1099700. [67-20-9].

See *Nitrofurantoin (0101)*.

(5-Nitro-2-furyl)methylene diacetate. $C_9H_9NO_7$. (M_r 243.2). 1099800. [92-55-7]. Nitrofurfural diacetate. 5-Nitrofurfurylidene diacetate.

Yellow crystals.
 mp: about 90 °C.

Nitrogen. N_2 . (M_r 28.01). 1059300. [7727-37-9].

Nitrogen, washed and dried.

Nitrogen R1. 1059400.

Content: minimum 99.999 per cent V/V of N_2 .

Carbon monoxide: less than 5 ppm.

Oxygen: less than 5 ppm.

Nitrogen for chromatography. 1059500.

Content: minimum 99.95 per cent V/V of N_2 .

Nitrogen gas mixture. 1136900.

Nitrogen R containing 1 per cent V/V of each of the following gases: *carbon dioxide R2*, *carbon monoxide R1* and *oxygen R1*.

Nitrogen monoxide. NO . (M_r 30.01). 1108300.

Content: minimum 98.0 per cent V/V of NO .

Nitrogen, oxygen-free. 1059600.

Nitrogen R which has been freed from oxygen by passing it through *alkaline pyrogallol solution R*.

Nitromethane. CH_3NO_2 . (M_r 61.0). 1059700. [75-52-5].

A clear, colourless, oily liquid, slightly soluble in water, miscible with alcohol.

d_{20}^{20} : 1.132 to 1.134.

n_D^{20} : 1.381 to 1.383.

Distillation range (2.2.11). Not less than 95 per cent distils between 100 °C and 103 °C.

Nitro-molybdovanadic reagent. 1060100.

Solution I. Dissolve 10 g of *ammonium molybdate R* in *water R*, add 1 ml of *ammonia R* and dilute to 100 ml with *water R*.

Solution II. Dissolve 2.5 g of *ammonium vanadate R* in hot *water R*, add 14 ml of *nitric acid R* and dilute to 500 ml with *water R*.

To 96 ml of *nitric acid R* add 100 ml of solution I and 100 ml of solution II and dilute to 500 ml with *water R*.

4-Nitrophenol. $C_6H_5NO_3$. (M_r 139.1). 1146400. [100-02-7]. *p*-Nitrophenol.

Content: minimum 95 per cent of $C_6H_5NO_3$.

Colourless or slightly yellow powder, sparingly soluble in water and in methanol.

mp: about 114 °C.

N-Nitrosodiethanolamine. $C_4H_{10}N_2O_3$. (M_r 134.1). 1129800. [1116-54-7]. 2,2'-(Nitrosoimino)diethanol.

A yellow liquid, miscible with ethanol.

n_D^{20} : about 1.485.
bp: about 125 °C.

Nitrosodipropylamine. $C_6H_{14}N_2O$. (M_r 130.2). 1099900. [621-64-7]. Dipropylnitrosamine.

Liquid, soluble in ethanol and in strong acids.

d_4^{20} : about 0.915.

bp: about 78 °C.

Appropriate grade for chemiluminescence determination.

Nitrosodipropylamine solution. 1099901.

Inject 78.62 g of *ethanol R* through the septum of a vial containing *nitrosodipropylamine R*. Dilute 1/100 in *ethanol R* and place 0.5 ml aliquots in crimp-sealed vials.

Storage: in the dark at 5 °C.

Nitrotetrazolium blue. $C_{40}H_{30}Cl_2N_{10}O_6$. (M_r 818). 1060000. [298-83-9]. 3,3'-(3,3'-Dimethoxy-4,4'-diphenylene)di[2-(4-nitrophenyl)-5-phenyl-2*H*-tetrazolium] dichloride. *p*-Nitro-tetrazolium blue.

Crystals, soluble in methanol, giving a clear, yellow solution.
mp: about 189 °C, with decomposition.

Nitrous oxide. N_2O . (M_r 44.01). 1108500.

Content: minimum 99.99 per cent *V/V* of N_2O .

Nitrogen monoxide: less than 1 ppm.

Carbon monoxide: less than 1 ppm.

Nonivamide. $C_{17}H_{27}NO_3$. (M_r 293.4). 1148500. [2444-46-4]. *N*-[(4-Hydroxy-3-methoxyphenyl)methyl]nonanamide.

White, crystalline powder, practically insoluble in cold water, freely soluble in ethanol.

Nonivamide used in the test for nonivamide in Capsicum (1859) complies with the following additional requirement.

Assay. Examine by liquid chromatography (2.2.29) as prescribed in the monograph on *Capsicum (1859)*. The content of nonivamide is not less than 98.0 per cent, calculated by the normalisation procedure.

Nonylamine. $C_9H_{21}N$. (M_r 143.3). 1139800. [112-20-9]. 1-Aminononane.

Corrosive, colourless, clear liquid.

d_4^{20} : about 0.788.

n_D^{20} : about 1.433.

Nordazepam. $C_{15}H_{11}ClN_2O$. (M_r 270.7). 1060200. [340-57-8]. 7-Chloro-2,3-dihydro-5-phenyl-1*H*-1,4-benzodiazepin-2-one.

A white or pale yellow, crystalline powder, practically insoluble in water, slightly soluble in alcohol.

mp: about 216 °C.

DL-Norleucine. $C_6H_{13}NO_2$. (M_r 131.2). 1060300. [616-06-8]. (*RS*)-2-Aminohexanoic acid.

Shiny crystals, sparingly soluble in water and in alcohol, soluble in acids.

Noscapine hydrochloride. 1060500. [912-60-7].

See *Noscapine hydrochloride (0515)*.

Octadecyl [3-[3,5-bis(1,1-dimethylethyl)-4-hydroxyphenyl]-propionate]. $C_{35}H_{62}O_3$. (M_r 530.9). 1060600. [2082-79-3]. Octadecyl 3-(3,5-di-*tert*-butyl-4-hydroxyphenyl)propionate.

A white or slightly yellowish, crystalline powder, practically insoluble in water, very soluble in acetone and in hexane, slightly soluble in methanol.

mp: 49 °C to 55 °C.

Octanal. $C_8H_{16}O$. (M_r 128.2). 1150400. [124-13-0]. Octyl aldehyde.

Oily, colourless liquid. Practically insoluble in water.

d_4^{20} : 0.822.

n_D^{20} : 1.419.

bp: 171 °C.

Octanal used in gas chromatography complies with the following additional test.

Assay. Examine by gas chromatography (2.2.28) as prescribed in the monograph on *Sweet orange oil (1811)*. The content is not less than 99 per cent, calculated by the normalisation procedure.

Octanol. $C_8H_{18}O$. (M_r 130.2). 1060700. [111-87-5]. 1-Octanol. Caprylic alcohol.

A colourless liquid, practically insoluble in water, miscible with alcohol.

d_{20}^{20} : about 0.828.

bp: about 195 °C.

3-Octanone. $C_8H_{16}O$. (M_r 128.2). 1114600. [106-68-3]. Ethylpentylketone.

A colourless liquid with a characteristic odour.

d_{20}^{20} : about 0.822.

n_D^{20} : about 1.415.

bp: about 167 °C.

3-Octanone used in gas chromatography complies with the following additional test.

Assay. Examine by gas chromatography (2.2.28) as prescribed in the monograph on *Lavender oil (1338)*.

Test solution. The substance to be examined.

The area of the principal peak is not less than 98.0 per cent of the area of all the peaks in the chromatogram obtained.

Octoxinol 10. $C_{34}H_{62}O_{11}$ (average). (M_r 647). 1060800. [9002-93-1]. α -[4-(1,1,3,3-Tetramethylbutyl)phenyl]- ω -hydroxypoly-(oxyethylene).

A clear, pale-yellow, viscous liquid, miscible with water, with acetone and with alcohol, soluble in toluene.

Storage: in an airtight container.

Octylamine. $C_8H_{19}N$. (M_r 129.2). 1150500. [111-86-4]. Octan-1-amine.

Colourless liquid.

d_{20}^{20} : about 0.782.

bp: 175 °C to 179 °C.

Oleamide. $C_{18}H_{35}NO$. (M_r 281.5). 1060900. (*Z*)-Octadec-9-enoamide.

Yellowish or white powder or granules, practically insoluble in water, very soluble in methylene chloride, soluble in ethanol.

mp: about 80 °C.

Oleic acid. $C_{18}H_{34}O_2$. (M_r 282.5). 1144100. [112-80-1]. (9*Z*)-Octadec-9-enoic acid.

Clear, colourless liquid, practically insoluble in water.

d_4^{20} : about 0.891.

n_D^{20} : about 1.459.

mp: 13 °C to 14 °C.

Oleic acid used in the assay of total fatty acids in Saw palmetto fruit (1848) complies with the following additional requirement.

Assay. Examine by gas chromatography (2.2.28) as prescribed in the monograph on *Saw palmetto fruit (1848)*.

The content of oleic acid is not less than 98 per cent, calculated by the normalisation procedure.

Oleuropein. $C_{25}H_{32}O_{13}$. (M_r 540.5). 1152900. [32619-42-4]. 2-(3,4-Dihydroxyphenyl)ethyl[(2*S*,3*E*,4*S*)-3-ethylidene-2-(β -D-glucopyranosyloxy)-5-(methoxycarbonyl)-3,4-dihydro-2*H*-pyran-4-yl]acetate.

Powder, soluble in methanol.

Oleuropein used in Olive leaf (1878) complies with the following requirement.

Assay. Examine by liquid chromatography (2.2.29) as prescribed in the monograph on *Olive leaf (1878)*.

The content of oleuropein is not less than 80 per cent, calculated by the normalisation procedure.

Oleyl alcohol. $C_{18}H_{36}O$. (M_r 268.5). 1156000. [143-28-2]. (9*Z*)-octadec-9-en-1-ol.

bp: about 207 °C.

n_D^{20} : 1.460.

Content: minimum 85 per cent of $C_{18}H_{36}O$.

Olive oil. 1061000. [8001-25-0].

See *Olive oil, virgin (0518)*.

Oracet blue 2R. $C_{20}H_{14}N_2O_2$. (M_r 314.3). 1061100. [4395-65-7].

Colour Index No. 61110.

1-Amino-4-(phenylamino)anthracene-9,10-dione.

mp: about 194 °C.

Orcinol. $C_7H_8O_2 \cdot H_2O$. (M_r 142.2). 1108700. [6153-39-5]. 5-Methylbenzene-1,3-diol monohydrate.

A crystalline powder, sensitive to light.

bp: about 290 °C.

mp: 58 °C to 61 °C.

Organosilica polymer, amorphous, octadecylsilyl. 1144200.

Synthetic, spherical hybrid particles, containing both inorganic (silica) and organic (organosiloxanes) components, chemically modified at the surface by trifunctionally bonded octadecylsilyl groups.

Organosilica polymer, amorphous, polar-embedded octadecylsilyl, end-capped. 1150600.

Synthetic, spherical hybrid particles containing both inorganic (silica) and organic (organosiloxanes) components, chemically modified at the surface by the bonding of polar embedded octadecylsilyl groups.

To minimise any interaction with basic compounds, it is carefully end-capped to cover most of the remaining silanol groups. The particle size is indicated after the name of the reagent in the tests where it is used.

Osmium tetroxide. OsO_4 . (M_r 254.2). 1061200. [20816-12-0].

Light-yellow needles or a yellow, crystalline mass, hygroscopic, light sensitive, soluble in water and in alcohol.

Storage: in an airtight container.

Osmium tetroxide solution. 1061201.

A 2.5 g/l solution in 0.05 *M* sulphuric acid.

Oxalic acid. $C_2H_2O_4 \cdot 2H_2O$. (M_r 126.1). 1061400. [6153-56-6]. Ethanedioic acid dihydrate.

White crystals, soluble in water, freely soluble in alcohol.

Oxalic acid and sulphuric acid solution. 1061401.

A 50 g/l solution of *oxalic acid R* in a cooled mixture of equal volumes of *sulphuric acid R* and *water R*.

Oxazepam. 1144300. [604-75-1].

See *Oxazepam (0778)*.

Ox brain, acetone-dried. 1061300.

Cut into small pieces a fresh ox brain previously freed from vascular and connective tissue. Place in *acetone R* for preliminary dehydration. Complete the dehydration by pounding in a mortar 30 g of this material with successive quantities, each of 75 ml, of *acetone R* until a dry powder is obtained after filtration. Dry at 37 °C for 2 h or until the odour of acetone is no longer present.

2,2'-Oxybis(*N,N*-dimethylethylamine). $C_8H_{20}N_2O$. (M_r 160.3). 1141200. [3033-62-3]. bis(2-Dimethylaminoethyl) ether.

Colourless, corrosive liquid.

d_{20}^{20} : about 0.85.

n_D^{20} : about 1.430.

Oxygen. O_2 . (M_r 32.00). 1108800.

Content: minimum 99.99 per cent *V/V* of O_2 .

Nitrogen and argon: less than 100 ppm.

Carbon dioxide: less than 10 ppm.

Carbon monoxide: less than 5 ppm.

Oxygen R1. O_2 . (M_r 32.00). 1137600.

Content: minimum 99 per cent *V/V* of O_2 .

Oxytetracycline hydrochloride. 1146500.

See *Oxytetracycline hydrochloride (0198)*.

Palladium. Pd. (A_r 106.4). 1114700. [7440-05-3].

Grey white metal, soluble in hydrochloric acid.

Palladium chloride. $PdCl_2$. (M_r 177.3). 1061500. [7647-10-1].

Red crystals.

mp: 678 °C to 680 °C.

Palladium chloride solution. 1061501.

Dissolve 1 g of *palladium chloride R* in 10 ml of warm *hydrochloric acid R*. Dilute the solution to 250 ml with a mixture of equal volumes of *dilute hydrochloric acid R* and *water R*. Dilute this solution immediately before use with 2 volumes of *water R*.

Palmitic acid. $C_{16}H_{32}O_2$. (M_r 256.4). 1061600. [57-10-3]. Hexadecanoic acid.

White, crystalline scales, practically insoluble in water, freely soluble in hot alcohol.

mp: about 63 °C.

Chromatography. Examine as prescribed in the monograph on *Chloramphenicol palmitate (0473)*. The chromatogram shows only one principal spot.

Palmitic acid used in the assay of total fatty acids in Saw palmetto fruit (1848) complies with the following additional requirement.

Assay. Examine by gas chromatography (2.2.28) as prescribed in the monograph on *Saw palmetto fruit (1848)*. The content of palmitic acid is not less than 98 per cent, calculated by the normalisation procedure.

Palmitoleic acid. $C_{16}H_{30}O_2$. (M_r 254.4). 1144400. [373-49-9]. (9*Z*)-Hexadec-9-enoic acid.

Clear, colourless liquid.

bp: about 162 °C.

Palmitoleic acid used in the assay of total fatty acids in Saw palmetto fruit (1848) complies with the following additional requirement.

Assay. Examine by gas chromatography (2.2.28) as prescribed in the monograph on *Saw palmetto fruit* (1848). The content of palmitoleic acid is not less than 98 per cent, calculated by the normalisation procedure.

Palmityl alcohol. $C_{16}H_{34}O$. (M_r 242.4). 1156100. [36653-82-4]. Cetyl alcohol. 1-Hexadecanol. mp: about 48 °C.

Content: minimum 96 per cent of $C_{16}H_{34}O$.

Pancreas powder. 1061700.

See *Pancreas powder* (0350).

Papain. 1150700. [9001-73-4].

A proteolytic enzyme obtained from the latex of the green fruit and leaves of *Carica papaya* L.

Papaverine hydrochloride. 1061800. [61-25-6].

See *Papaverine hydrochloride* (0102).

Paper chromatography performance test solutions. 1150800.

Test solution (a). Sodium pertechnetate (^{99m}Tc) injection (fission) (0124) or Sodium pertechnetate (^{99m}Tc) injection (non-fission) (0283).

Test solution (b). In a closed vial mix 100 µl of a 5 g/l solution of stannous chloride *R* in 0.05 *M* hydrochloric acid and 100 MBq to 200 MBq of Sodium pertechnetate (^{99m}Tc) injection (fission) (0124) or Sodium pertechnetate (^{99m}Tc) injection (non-fission) (0283) in a volume not exceeding 2 ml.

Paper for chromatography. 1150900.

Pure cellulose grade thin paper with a smooth surface and a thickness of about 0.2 mm.

Chromatographic separation. To 2 strips of paper for chromatography *R* apply separately 2-5 µl of test solution (a) and test solution (b) of paper chromatography performance test solutions *R*. Develop over a pathlength of 3/4 of the paper height, using a mixture of equal volumes of methanol *R* and water *R*. Allow to dry and determine the distribution of radioactivity using a suitable detector. The paper is not satisfactory, unless the chromatogram obtained with test solution (a) shows a single radioactivity spot with an R_f value in the range 0.8-1.0 and the chromatogram obtained with test solution (b) shows a single radioactivity spot at the application point (R_f value in the range 0.0-0.1).

Paracetamol. 1061900. [103-90-2].

See *Paracetamol* (0049).

Paracetamol, 4-aminophenol-free. 1061901.

Recrystallise paracetamol *R* from water *R* and dry in vacuo at 70 °C; repeat the procedure until the product complies with the following test: dissolve 5 g of the dried substance in a mixture of equal volumes of methanol *R* and water *R* and dilute to 100 ml with the same mixture of solvents. Add 1 ml of a freshly prepared solution containing 10 g/l of sodium nitroprusside *R* and 10 g/l of anhydrous sodium carbonate *R*, mix and allow to stand for 30 min protected from light. No blue or green colour is produced.

Paraffin, liquid. 1062000. [8042-47-5].

See *Liquid paraffin* (0239).

Paraffin, white soft. 1062100.

A semi-liquid mixture of hydrocarbons obtained from petroleum and bleached, practically insoluble in water and in alcohol, soluble in light petroleum *RI*, the solution sometimes showing a slight opalescence.

Paraldehyde. 1151000. [123-63-7].

See *Paraldehyde* (0351).

Pararosanine hydrochloride. $C_{19}H_{18}ClN_3$. (M_r 323.8). 1062200. [569-61-9].

Schultz No. 779.

Colour Index No. 42500.

4-[bis(4-Aminophenyl)methylene]cyclohexa-2,5-dieniminium chloride.

A bluish-red, crystalline powder, slightly soluble in water, soluble in ethanol. Solutions in water and ethanol are deep-red; solutions in sulphuric acid and in hydrochloric acid are yellow.

mp: about 270 °C, with decomposition.

Decolorised pararosaniline solution. 1062201.

To 0.1 g of pararosaniline hydrochloride *R* in a ground-glass-stoppered flask add 60 ml of water *R* and a solution of 1.0 g of anhydrous sodium sulphite *R* or 2.0 g of sodium sulphite *R* or 0.75 g of sodium metabisulphite *R* in 10 ml of water *R*. Slowly and with stirring add 6 ml of dilute hydrochloric acid *R*, stopper the flask and continue stirring until dissolution is complete. Dilute to 100 ml with water *R*. Allow to stand for 12 h before use.

Storage: protected from light.

Parthenolide. $C_{15}H_{20}O_3$. (M_r 248.3). 1129900. [20554-84-1]. (4*E*)-(1*aR*,7*aS*,10*aS*,10*bS*)-1*a*,5-Dimethyl-8-methylene-2,3,6,7,7*a*,8,10*a*,10*b*-octahydro-oxireno[9,10]cyclodeca[1,2-*b*]furan-9(1*aH*)-one. (*E*)-(5*S*,6*S*)-4,5-Epoxygermacra-1(10),11(13)-dieno-12(6)-lactone.

A white, crystalline powder, very slightly soluble in water, very soluble in methylene chloride, soluble in methanol.

$[\alpha]_D^{22}$: -71.4, determined on a 2.2 g/l solution in methylene chloride *R*.

mp: 115 °C to 116 °C.

Absorbance (2.2.25). A 0.01 g/l solution in alcohol *R* shows an absorption maximum at 214 nm.

Assay. Examine by liquid chromatography (2.2.29), as prescribed in the monograph on *Feverfew* (1516), at the concentration of the reference solution. The content of parthenolide is not less than 90 per cent calculated by the normalisation procedure.

Penicillinase solution. 1062300.

Dissolve 10 g of casein hydrolysate, 2.72 g of potassium dihydrogen phosphate *R* and 5.88 g of sodium citrate *R* in 200 ml of water *R*, adjust to pH 7.2 with a 200 g/l solution of sodium hydroxide *R* and dilute to 1000 ml with water *R*. Dissolve 0.41 g of magnesium sulphate *R* in 5 ml of water *R* and add 1 ml of a 1.6 g/l solution of ferrous ammonium sulphate *R* and sufficient water *R* to produce 10 ml. Sterilise both solutions by heating in an autoclave, cool, mix, distribute in shallow layers in conical flasks and inoculate with *Bacillus cereus* (NCTC 9946). Allow the flasks to stand at 18 °C to 37 °C until growth is apparent and then maintain at 35 °C to 37 °C for 16 h, shaking constantly to ensure maximum aeration. Centrifuge and sterilise the supernatant liquid by filtration through a membrane filter. 1.0 ml of penicillinase solution contains not less than 0.4 microkatal (corresponding to the hydrolysis of not less than 500 mg of benzylpenicillin to benzylpenicilloic acid per hour) at 30 °C and pH 7, provided that the concentration of benzylpenicillin does not fall below the level necessary for enzyme saturation. The Michaelis constant for benzylpenicillin of the penicillinase in penicillinase solution is approximately 12 µg/ml.

Sterility (2.6.1). It complies with the test for sterility.

Storage: at a temperature between 0 °C and 2 °C for 2 to 3 days. When freeze-dried and kept in sealed ampoules, it may be stored for several months.

Pentaerythrityl tetrakis[3-(3,5-di(1,1-dimethylethyl)-4-hydroxyphenyl)propionate]. $C_{73}H_{108}O_{12}$. (M_r 1178). 1062400. [6683-19-8]. Pentaerythrityl tetrakis[3-(3,5-di-*tert*-butyl-4-hydroxyphenyl) propionate]. 2,2'-bis(Hydroxymethyl)propane-1,3-diol tetrakis[3-[3,5-di(1,1-dimethylethyl)-4-hydroxyphenyl]]propionate.

A white to slightly yellow, crystalline powder, practically insoluble in water, very soluble in acetone, soluble in methanol, slightly soluble in hexane.

mp: 110 °C to 125 °C.

α -form: 120 °C to 125 °C.

β -form: 110 °C to 115 °C.

Pentafluoropropanoic acid. $C_3HF_5O_2$. (M_r 164.0). 1151100. [422-64-0].

Clear, colourless liquid.

d_{20}^{20} : about 1.561.

n_D^{20} : about 1.284.

bp: about 97 °C.

Pentane. C_5H_{12} . (M_r 72.2). 1062500. [109-66-0].

A clear, colourless, flammable liquid, very slightly soluble in water, miscible with acetone and with ethanol.

d_{20}^{20} : about 0.63.

n_D^{20} : about 1.359.

bp: about 36 °C.

Pentane used in spectrophotometry complies with the following additional requirement.

Minimum transmittance (2.2.25), determined using *water R* as compensation liquid: 20 per cent at 200 nm, 50 per cent at 210 nm, 85 per cent at 220 nm, 93 per cent at 230 nm, 98 per cent at 240 nm.

1,2-Pentanediol. $C_5H_{12}O_2$. (M_r 104.2). 1155800. [5343-92-0]. (2*RS*)-Pentane-1,2-diol.

d_4^{20} : about 0.971.

n_D^{20} : about 1.439.

bp: about 201 °C.

Pentanol. $C_5H_{12}O$. (M_r 88.1). 1062600. [71-41-0]. 1-Pentanol. Colourless liquid, sparingly soluble in water, miscible with alcohol.

n_D^{20} : about 1.410.

bp: about 137 °C.

***tert*-Pentyl alcohol.** $C_5H_{12}O$. (M_r 88.1). 1062700. [75-85-4]. *tert*-Amyl alcohol. 2-Methyl-2-butanol.

A volatile, flammable liquid, freely soluble in water, miscible with alcohol and with glycerol.

d_{20}^{20} : about 0.81.

Distillation range (2.2.11). Not less than 95 per cent distils between 100 °C and 104 °C.

Storage: protected from light.

Pepsin powder. 1062800. [9001-75-6].

See *Pepsin powder (0682)*.

Perchloric acid. $HClO_4$. (M_r 100.5). 1062900. [7601-90-3]. **Content:** 70.0 per cent *m/m* to 73.0 per cent *m/m* of $HClO_4$. A clear, colourless liquid, miscible with water.

d_{20}^{20} : about 1.7.

Assay. To 2.50 g add 50 ml of *water R* and titrate with 1 *M sodium hydroxide*, using 0.1 ml of *methyl red solution R* as indicator.

1 ml of 1 *M sodium hydroxide* is equivalent to 100.5 mg of $HClO_4$.

Perchloric acid solution. 1062901.

Dilute 8.5 ml of *perchloric acid R* to 100 ml with *water R*.

Periodic acetic acid solution. 1063000.

Dissolve 0.446 g of *sodium periodate R* in 2.5 ml of a 25 per cent *V/V* solution of *sulphuric acid R*. Dilute to 100.0 ml with *glacial acetic acid R*.

Periodic acid. H_5IO_6 . (M_r 227.9). 1108900. [10450-60-9].

Crystals, freely soluble in water and soluble in alcohol.

mp: about 122 °C.

Permethrin. $C_{21}H_{20}Cl_2O_3$. (M_r 391.3). 1130000. [52645-53-1].

mp: 34 °C to 35 °C.

A suitable certified reference solution (10 ng/ μ l in cyclohexane) may be used.

Peroxide test strips. 1147800.

Use commercial test strips with a suitable scale in the range from 0 ppm to 25 ppm peroxide.

Perylene. $C_{20}H_{12}$. (M_r 252.3). 1130100. [198-55-0].

Dibenz(de,kl)anthracene.

Orange powder.

mp: about 279 °C.

Petroleum, light. 1063100. [8032-32-4].

A clear, colourless, flammable liquid without fluorescence, practically insoluble in water, miscible with alcohol.

d_{20}^{20} : 0.661 to 0.664.

Distillation range (2.2.11): 50 °C to 70 °C.

Petroleum, light R1. 1063101.

Complies with the requirements prescribed for *light petroleum R*, with the following modifications:

d_{20}^{20} : 0.630 to 0.656.

Distillation range (2.2.11): 40 °C to 60 °C.

It does not become cloudy at 0 °C.

Petroleum, light R2. 1063102.

Complies with the requirements prescribed for *light petroleum R*, with the following modifications:

d_{20}^{20} : 0.620 to 0.630.

Distillation range (2.2.11): 30 °C to 40 °C.

It does not become cloudy at 0 °C.

α -Phellandrene. $C_{10}H_{16}$. (M_r 136.2). 1130400. [4221-98-1]. (*R*)-5-Isopropyl-2-methyl-cyclohexa-1,3-diene. (–)-p-Mentha-1,5-diene.

d_{20}^{20} : about 0.839.

n_D^{20} : about 1.471.

$[\alpha]_D^{20}$: about –217.

bp: 171 °C to 174 °C.

α -Phellandrene used in gas chromatography complies with the following additional test.

Assay. Examine by gas chromatography (2.2.28) as prescribed in the monograph on *Eucalyptus oil (0390)* using the substance to be examined as the test solution.

The area of the principal peak is not less than 98.0 per cent of the total area of the peaks.

Phenanthrene. $C_{14}H_{10}$. (M_r 178.2). 1063200. [85-01-8].

White crystals, practically insoluble in water, sparingly soluble in alcohol.

mp: about 100 °C.

Phenanthroline hydrochloride. $C_{12}H_9ClN_2 \cdot H_2O$. (M_r 234.7). 1063300. [3829-86-5]. 1,10-Phenanthroline hydrochloride monohydrate.

A white or almost white, crystalline powder, freely soluble in water, soluble in alcohol.

mp: about 215 °C, with decomposition.

Phenazone. 1063400. [60-80-0].

See *Phenazone* (0421).

Phenol. 1063500. [108-95-2].

See *Phenol* (0631).

Phenolphthalein. $C_{20}H_{14}O_4$. (M_r 318.3). 1063700. [77-09-8]. 3,3-bis(4-Hydroxyphenyl)-3*H*-isobenzofuran-1-one.

A white to yellowish-white powder, practically insoluble in water, soluble in alcohol.

Phenolphthalein paper. 1063704.

Immerse strips of filter paper for a few minutes in *phenolphthalein solution R*. Allow to dry.

Phenolphthalein solution. 1063702.

Dissolve 0.1 g of *phenolphthalein R* in 80 ml of *alcohol R* and dilute to 100 ml with *water R*.

Test for sensitivity. To 0.1 ml of the phenolphthalein solution add 100 ml of *carbon dioxide-free water R*. The solution is colourless. Not more than 0.2 ml of 0.02 *M* sodium hydroxide is required to change the colour to pink.

Colour change: pH 8.2 (colourless) to pH 10.0 (red).

Phenolphthalein solution R1. 1063703.

A 10 g/l solution in *alcohol R*.

Phenol red. 1063600. [143-74-8].

Bright red or dark red, crystalline powder, very slightly soluble in water, slightly soluble in alcohol.

Phenol red solution. 1063601.

Dissolve 0.1 g of *phenol red R* in a mixture of 2.82 ml of 0.1 *M* sodium hydroxide and 20 ml of *alcohol R* and dilute to 100 ml with *water R*.

Test for sensitivity. Add 0.1 ml of the phenol red solution to 100 ml of *carbon dioxide-free water R*. The solution is yellow. Not more than 0.1 ml of 0.02 *M* sodium hydroxide is required to change the colour to reddish-violet.

Colour change: pH 6.8 (yellow) to pH 8.4 (reddish-violet).

Phenol red solution R2. 1063603.

Solution I. Dissolve 33 mg of *phenol red R* in 1.5 ml of *dilute sodium hydroxide solution R* and dilute to 100 ml with *water R*.

Solution II. Dissolve 25 mg of *ammonium sulphate R* in 235 ml of *water R*; add 105 ml of *dilute sodium hydroxide solution R* and 135 ml of *dilute acetic acid R*. Add 25 ml of solution I to solution II. If necessary, adjust the pH of the mixture to 4.7.

Phenol red solution R3. 1063604.

Solution I. Dissolve 33 mg of *phenol red R* in 1.5 ml of *dilute sodium hydroxide solution R* and dilute to 50 ml with *water R*.

Solution II. Dissolve 50 mg of *ammonium sulphate R* in 235 ml of *water R*; add 105 ml of *dilute sodium hydroxide solution R* and 135 ml of *dilute acetic acid R*.

Add 25 ml of solution I to solution II; if necessary, adjust the pH of the mixture to 4.7.

Phenoxyacetic acid. $C_8H_8O_3$. (M_r 152.1). 1063800. [122-59-8]. 2-Phenoxyethanoic acid.

Almost white crystals, sparingly soluble in water, freely soluble in alcohol, and in glacial acetic acid.

mp: about 98 °C.

Chromatography. Examine as prescribed in the monograph on *Phenoxyethylpenicillin* (0148); the chromatogram shows only one principal spot.

Phenoxybenzamine hydrochloride. $C_{18}H_{23}Cl_2NO$. (M_r 340.3). 1063900. *N*-(2-Chloroethyl)-*N*-(1-methyl-2-phenoxyethyl)-benzylamine hydrochloride.

Content: 97.0 per cent to the equivalent of 103.0 per cent of $C_{18}H_{23}Cl_2NO$, calculated with reference to the dried substance.

A white or almost white, crystalline powder, sparingly soluble in water, freely soluble in alcohol.

mp: about 138 °C.

Loss on drying (2.2.32): maximum 0.5 per cent, determined by drying over *diphosphorus pentoxide R* at a pressure not exceeding 670 Pa for 24 h.

Assay. Dissolve 0.500 g in 50.0 ml of *ethanol-free chloroform R* and extract with three quantities, each of 20 ml, of 0.01 *M* hydrochloric acid. Discard the acid extracts, filter the chloroform layer through cotton and dilute 5.0 ml of the filtrate to 500.0 ml with *ethanol-free chloroform R*. Measure the absorbance of the resulting solution in a closed cell at the maximum at 272 nm.

Calculate the content of $C_{18}H_{23}Cl_2NO$, taking the specific absorbance to be 56.3.

Storage: protected from light.

Phenoxyethanol. $C_8H_{10}O_2$. (M_r 138.2). 1064000. [122-99-6]. 2-Phenoxyethanol.

A clear, colourless, oily liquid, slightly soluble in water, freely soluble in alcohol.

d_{20}^{20} : about 1.11.

n_D^{20} : about 1.537.

Freezing point (2.2.18): minimum 12 °C.

Phenylalanine. 1064100. [63-91-2].

See *Phenylalanine* (0782).

***p*-Phenylenediamine dihydrochloride.** $C_6H_{10}Cl_2N_2$. (M_r 181.1). 1064200. [615-28-1]. 1,4-Diaminobenzene dihydrochloride.

A crystalline powder or white or slightly coloured crystals, turning reddish on exposure to air, freely soluble in water, slightly soluble in alcohol.

α -Phenylglycine. $C_8H_9NO_2$. (M_r 151.2). 1064300. [2835-06-5]. (*RS*)-2-Amino-2-phenylacetic acid.

***D*-Phenylglycine.** $C_8H_9NO_2$. (M_r 151.2). 1144500. [875-74-1]. (2*R*)-2-Amino-2-phenylacetic acid.

Content: minimum 99 per cent of $C_8H_9NO_2$.

White or almost white, crystalline powder.

Phenylhydrazine hydrochloride. $C_6H_9ClN_2$. (M_r 144.6). 1064500. [59-88-1].

A white or almost white, crystalline powder, becoming brown on exposure to air, soluble in water and in alcohol.

mp: about 245 °C, with decomposition.

Storage: protected from light.

Phenylhydrazine hydrochloride solution. 1064501.

Dissolve 0.9 g of *phenylhydrazine hydrochloride R* in 50 ml of *water R*. Decolorise with *activated charcoal R* and filter. To the filtrate add 30 ml of *hydrochloric acid R* and dilute to 250 ml with *water R*.

Phenylhydrazine-sulphuric acid solution. 1064502.

Dissolve 65 mg of *phenylhydrazine hydrochloride R*, previously recrystallised from *alcohol (85 per cent V/V) R*, in a mixture of 80 volumes of *water R* and 170 volumes of *sulphuric acid R* and dilute to 100 ml with the same mixture of solvents. Prepare immediately before use.

Phenyl isothiocyanate. C_7H_5NS . (M_r 135.2). 1121500. [103-72-0].

A liquid, insoluble in water, soluble in alcohol.

d_{20}^{20} : about 1.13.

n_D^{20} : about 1.65.

bp: about 221 °C.

mp: about –21 °C.

Use a grade suitable for protein sequencing.

1-Phenylpiperazine. $C_{10}H_{14}N_2$. (M_r 162.2). 1130500. [92-54-6].

Slightly viscous, yellow liquid, not miscible with water.

d_4^{20} : about 1.07.

n_D^{20} : about 1.588.

Phloroglucinol. $C_6H_6O_3 \cdot 2H_2O$. (M_r 162.1). 1064600. [6099-90-7]. Benzene-1,3,5-triol.

White or yellowish crystals, slightly soluble in water, soluble in alcohol.

mp: about 223 °C (instantaneous method).

Phloroglucinol solution. 1064601.

To 1 ml of a 100 g/l solution of *phloroglucinol R* in *alcohol R*, add 9 ml of *hydrochloric acid R*.

Storage: protected from light.

Phosalone. $C_{12}H_{15}ClNO_4PS_2$. (M_r 367.8). 1130200. [2310-17-0].

mp: 45 °C to 48 °C

A suitable certified reference solution (10 ng/μl in iso-octane) may be used.

Phospholipids. 1064800.

Wash a quantity of human or bovine brain, well separated from the meninges and blood vessels and liquidise in a suitable apparatus. Weigh 1000 g to 1300 g of the liquidised substance and measure the volume (*V* ml). Extract with three quantities, each of 4*V* ml, of *acetone R*, filter under reduced pressure and dry the residue at 37 °C for 18 h. Extract the residue with two quantities, each of 2*V* ml, of a mixture of 2 volumes of *light petroleum R2* and 3 volumes of *light petroleum R1*, filtering each extract on a filter paper previously moistened with the mixture of solvents. Combine the extracts and evaporate to dryness at 45 °C at a pressure not exceeding 670 Pa. Dissolve the residue in 0.2*V* ml of *ether R* and allow to stand at 4 °C until a deposit

forms. Centrifuge and evaporate the clear supernatant liquid under low pressure to a volume of 100 ml per kilogram of the liquidised substance and weigh. Allow to stand at 4 °C until a precipitate forms (12 h to 24 h) and centrifuge. Add to the clear supernatant liquid five times its volume of *acetone R*, centrifuge and reject the supernatant liquid. Dry the precipitate.

Storage: in a desiccator under vacuum, protected from light.

Phosphomolybdic acid. $12MoO_3 \cdot H_3PO_4 \cdot xH_2O$. 1064900. [51429-74-4].

Orange-yellow, fine crystals, freely soluble in water, soluble in alcohol.

Phosphomolybdic acid solution. 1064901.

Dissolve 4 g of *phosphomolybdic acid R* in *water R* and dilute to 40 ml with the same solvent. Add cautiously and with cooling 60 ml of *sulphuric acid R*. Prepare immediately before use.

Phosphomolybdotungstic reagent. 1065000.

Dissolve 100 g of *sodium tungstate R* and 25 g of *sodium molybdate R* in 700 ml of *water R*. Add 100 ml of *hydrochloric acid R* and 50 ml of *phosphoric acid R*. Heat the mixture under a reflux condenser in a glass apparatus for 10 h. Add 150 g of *lithium sulphate R*, 50 ml of *water R* and a few drops of *bromine R*. Boil to remove the excess of bromine (15 min), allow to cool, dilute to 1000 ml with *water R* and filter. The reagent should be yellow in colour. If it acquires a greenish tint, it is unsatisfactory for use but may be regenerated by boiling with a few drops of *bromine R*. Care must be taken to remove the excess of bromine by boiling.

Storage: at 2 °C to 8 °C.

Phosphomolybdotungstic reagent, dilute. 1065001.

To 1 volume of *phosphomolybdotungstic reagent R* add 2 volumes of *water R*.

Phosphoric acid. 1065100. [7664-38-2].

See *Concentrated phosphoric acid (0004)*.

Phosphoric acid, dilute. 1065101.

See *Dilute phosphoric acid (0005)*.

Phosphoric acid, dilute R1. 1065102.

Dilute 93 ml of *dilute phosphoric acid R* to 1000 ml with *water R*.

Phosphorous acid. H_3PO_3 . (M_r 82.0). 1130600. [13598-36-2].

White, very hygroscopic and deliquescent crystalline mass; slowly oxidised by oxygen (air) to H_3PO_4 .

Unstable, orthorhombic crystals, soluble in water, in alcohol and in a mixture of 3 volumes of ether and 1 volume of alcohol.

d_4^{21} : 1.651.

mp: about 73 °C.

Phosphotungstic acid solution. 1065200.

Heat under a reflux condenser for 3 h, 10 g of *sodium tungstate R* with 8 ml of *phosphoric acid R* and 75 ml of *water R*. Allow to cool and dilute to 100 ml with *water R*.

Phthalaldehyde. $C_8H_6O_2$. (M_r 134.1). 1065300. [643-79-8]. Benzene-1,2-dicarboxaldehyde.

A yellow, crystalline powder.

mp: about 55 °C.

Storage: protected from light and air.

Phthalaldehyde reagent. 1065301.

Dissolve 2.47 g of *boric acid R* in 75 ml of *water R*, adjust to pH 10.4 using a 450 g/l solution of *potassium hydroxide R* and dilute to 100 ml with *water R*. Dissolve 1.0 g of *phthalaldehyde R* in 5 ml of *methanol R*, add 95 ml of the boric acid solution and 2 ml of *thioglycollic acid R* and adjust to pH 10.4 with a 450 g/l solution of *potassium hydroxide R*.

Storage: protected from light; use within 3 days.

Phthalazine. C₈H₆N₂. (*M_r* 130.1). 1065400. [253-52-1].

Pale yellow crystals, freely soluble in water, soluble in ethanol, in ethyl acetate and in methanol.

mp: 89 °C to 92 °C.

Phthalein purple. C₃₂H₃₂N₂O₁₂·xH₂O. (*M_r* 637, anhydrous substance). 1065500. [2411-89-4]. Metalphthalein. 2,2',2'', 2'''-[*o*-Cresolphthalein-3',3''-bis(methylenenitrilo)]tetra-acetic acid. (1,3-Dihydro-3-oxo-isobenzofuran-1-ylidene)bis[(6-hydroxy-5-methyl-3,1-phenylene)bis(methyleneimino)diacetic acid].

A yellowish-white to brownish powder, practically insoluble in water, soluble in alcohol. The product may be found in commerce in the form of the sodium salt: a yellowish-white to pink powder, soluble in water, practically insoluble in alcohol.

Test for sensitivity. Dissolve 10 mg in 1 ml of *concentrated ammonia R* and dilute to 100 ml with *water R*. To 5 ml of the solution add 95 ml of *water R*, 4 ml of *concentrated ammonia R*, 50 ml of *alcohol R* and 0.1 ml of 0.1 M *barium chloride*. The solution is blue-violet. Add 0.15 ml of 0.1 M *sodium edetate*. The solution becomes colourless.

Phthalic acid. C₈H₆O₄. (*M_r* 166.1). 1065600. [88-99-3]. Benzene-1,2-dicarboxylic acid.

A white, crystalline powder, soluble in hot water and in alcohol.

Phthalic anhydride. C₈H₄O₃. (*M_r* 148.1). 1065700. [85-44-9]. Isobenzofuran-1,3-dione.

Content: minimum 99.0 per cent of C₈H₄O₃.

White flakes.

mp: 130 °C to 132 °C.

Assay. Dissolve 2.000 g in 100 ml of *water R* and boil under a reflux condenser for 30 min. Cool and titrate with 1 M *sodium hydroxide*, using *phenolphthalein solution R* as indicator.

1 ml of 1 M *sodium hydroxide* is equivalent to 74.05 mg of C₈H₄O₃.

Phthalic anhydride solution. 1065701.

Dissolve 42 g of *phthalic anhydride R* in 300 ml of *anhydrous pyridine R*. Allow to stand for 16 h.

Storage: protected from light; use within 1 week.

Picein. C₁₄H₁₈O₇. (*M_r* 298.3). 1130700. [530-14-3].

1-[4-(β-D-Glucopyranosyloxy)phenyl]ethanone.

p-(Acetylphenyl)-β-D-glucopyranoside.

mp: 194 °C to 195 °C.

Picric acid. C₆H₃N₃O₇. (*M_r* 229.1). 1065800. [88-89-1]. 2,4,6-Trinitrophenol.

Yellow prisms or plates, soluble in water and in alcohol.

Storage: moistened with *water R*.

Picric acid solution. 1065801.

A 10 g/l solution.

Picric acid solution R1. 1065802.

Prepare 100 ml of a saturated solution of *picric acid R* and add 0.25 ml of *strong sodium hydroxide solution R*.

α-Pinene. C₁₀H₁₆. (*M_r* 136.2). 1130800. [7785-70-8]. (1*R*,5*R*)-2,6,6-Trimethylbicyclo[3.1.1]hept-2-ene.

A liquid not miscible with water.

*d*₂₀²⁰: about 0.859.

*n*_D²⁰: about 1.466.

bp: 154 °C to 156 °C.

α-Pinene used in gas chromatography complies with the following additional test.

Assay. Examine by gas chromatography (2.2.28) as prescribed in the monograph on *Bitter-orange-flower oil* (1175) using the substance to be examined as the test solution.

The area of the principal peak is not less than 99.0 per cent of the total area of the peaks.

β-Pinene. C₁₀H₁₆. (*M_r* 136.2). 1109000. [18172-67-3]. 6,6-Dimethyl-2-methylenebicyclo[3.1.1]heptane.

A colourless, oily liquid, odour reminiscent of turpentine, practically insoluble in water, miscible with alcohol.

*d*₂₀²⁰: about 0.867.

*n*_D²⁰: about 1.474.

bp: 164 °C to 166 °C.

β-Pinene used in gas chromatography complies with the following additional test.

Assay. Examine by gas chromatography (2.2.28) as prescribed in the monograph on *Bitter-orange-flower oil* (1175), using the substance to be examined as the test solution. The area of the principal peak is not less than 99.0 per cent of the total area of the peaks.

Piperazine hydrate. 1065900. [142-63-2].

See *Piperazine hydrate* (0425).

Piperidine. C₅H₁₁N. (*M_r* 85.2). 1066000. [110-89-4].

Hexahydropyridine.

A colourless to slightly yellow, alkaline liquid, miscible with water, with alcohol and with light petroleum.

bp: about 106 °C.

Piperitone. C₁₀H₁₆O. (*M_r* 152.2). 1151200. [89-81-6]. 6-Isopropyl-3-methyl-cyclohex-2-en-1-one.

Pirimiphos-ethyl. C₁₃H₂₄N₃O₃PS. (*M_r* 333.4). 1130300. [23505-41-1].

mp: 15 °C to 18 °C.

A suitable certified reference solution (10 ng/μl in cyclohexane) may be used.

Plasma, platelet-poor. 1066100.

Withdraw 45 ml of human blood into a 50 ml plastic syringe containing 5 ml of a sterile 38 g/l solution of *sodium citrate R*. Without delay, centrifuge at 1500 *g* at 4 °C for 30 min. Remove the upper two-thirds of the supernatant plasma using a plastic syringe and without delay centrifuge at 3500 *g* at 4 °C for 30 min. Remove the upper two-thirds of the liquid and freeze it rapidly in suitable amounts in plastic tubes at or below −40 °C. Use plastic or silicone-treated equipment.

Plasma substrate. 1066200.

Separate the plasma from human or bovine blood collected into one-ninth its volume of a 38 g/l solution of *sodium citrate R*, or into two-sevenths its volume of a solution containing 20 g/l of *disodium hydrogen citrate R* and

25 g/l of *glucose R*. With the former, prepare the substrate on the day of collection of the blood. With the latter, prepare within two days of collection of the blood.

Storage: at $-20\text{ }^{\circ}\text{C}$.

Plasma substrate R1. 1066201.

Use water-repellent equipment (made from materials such as suitable plastics or suitably silicone-treated glass) for taking and handling blood.

Collect a suitable volume of blood from each of at least five sheep; a 285 ml volume of blood collected into 15 ml of anticoagulant solution is suitable but smaller volumes may be collected, taking the blood, either from a live animal or at the time of slaughter, using a needle attached to a suitable cannula which is long enough to reach the bottom of the collecting vessel. Discarding the first few millilitres and collecting only free-flowing blood, collect the blood in a sufficient quantity of an anticoagulant solution containing 8.7 g of *sodium citrate R* and 4 mg of *aprotinin R* per 100 ml of *water R* to give a final ratio of blood to anticoagulant solution of 19 to 1. During and immediately after collection, swirl the flask gently to ensure mixing but do not allow frothing to occur. When collection is complete, close the flask and cool to $10\text{ }^{\circ}\text{C}$ to $15\text{ }^{\circ}\text{C}$. When cold, pool the contents of all the flasks with the exception of any that show obvious haemolysis or clots and keep the pooled blood at $10\text{ }^{\circ}\text{C}$ to $15\text{ }^{\circ}\text{C}$.

As soon as possible and within 4 h of collection, centrifuge the pooled blood at 1000 *g* to 2000 *g* at $10\text{ }^{\circ}\text{C}$ to $15\text{ }^{\circ}\text{C}$ for 30 min. Separate the supernatant liquid and centrifuge it at 5000 *g* for 30 min. (Faster centrifugation, for example 20 000 *g* for 30 min, may be used if necessary to clarify the plasma, but filtration procedures should not be used.) Separate the supernatant liquid and, without delay, mix thoroughly and distribute the plasma substrate into small stoppered containers in portions sufficient for a complete heparin assay (for example 10 ml to 30 ml). Without delay, rapidly cool to a temperature below $-70\text{ }^{\circ}\text{C}$ (for example by immersing the containers into liquid nitrogen) and store at a temperature below $-30\text{ }^{\circ}\text{C}$.

The plasma is suitable for use as plasma substrate in the assay for heparin if, under the conditions of the assay, it gives a clotting time appropriate to the method of detection used and if it provides reproducible, steep log dose-response curves.

When required for use, thaw a portion of the plasma substrate in a water-bath at $37\text{ }^{\circ}\text{C}$, gently swirling until thawing is complete; once thawed it should be kept at $10\text{ }^{\circ}\text{C}$ to $20\text{ }^{\circ}\text{C}$ and used without delay. The thawed plasma substrate may be lightly centrifuged if necessary; filtration procedures should not be used.

Plasma substrate R2. 1066202.

Prepare from human blood containing less than 1 per cent of the normal amount of factor IX. Collect the blood into one-ninth its volume of a 38 g/l solution of *sodium citrate R*.

Storage: in small amounts in plastic tubes at a temperature of $-30\text{ }^{\circ}\text{C}$ or lower.

Plasma substrate R3. 1066203.

Prepare from human blood containing less than 1 per cent of the normal amount of factor XI. Collect the blood into one-ninth its volume of a 38 g/l solution of *sodium citrate R*.

Storage: in small amounts in plastic tubes at a temperature of $-30\text{ }^{\circ}\text{C}$ or lower.

Plasma substrate deficient in factor V. 1066300.

Use preferably a plasma which is congenitally deficient, or prepare it as follows: separate the plasma from human blood collected into one tenth of its volume of a 13.4 g/l solution of *sodium oxalate R*. Incubate at $37\text{ }^{\circ}\text{C}$ for 24 h to 36 h. The coagulation time determined by the method described for *coagulation factor V solution R* should be 70 s to 100 s. If the coagulation time is less than 70 s, incubate again for 12 h to 24 h.

Storage: in small quantities at a temperature of $-20\text{ }^{\circ}\text{C}$ or lower.

Plasminogen, human. 1109100. [9001-91-6].

A substance present in blood that may be activated to plasmin, an enzyme that lyses fibrin in blood clots.

Platelet substitute. 1066400.

To 0.5 g to 1 g of *phospholipid R* add 20 ml of *acetone R* and allow to stand for 2 h with frequent shaking. Centrifuge for 2 min and discard the supernatant liquid. Dry the residue using a water pump, mix with 20 ml of *chloroform R* and shake for 2 h. Filter under vacuum and suspend the residue obtained in 5 ml to 10 ml of a 9 g/l solution of *sodium chloride R*.

For use in the assay of factor IX, prepare a dilution in a 9 g/l solution of *sodium chloride R* that will give coagulation time differences between consecutive dilutions of the reference preparation of about 10 s.

Storage of the diluted suspensions: at $-30\text{ }^{\circ}\text{C}$; use within 6 weeks.

Poly[(cyanopropyl)methylphenylmethylsiloxane]. 1066500.

See *poly[(cyanopropyl)(methyl)][(phenyl)(methyl)]siloxane R*.

Poly[(cyanopropyl)(methyl)][(phenyl)(methyl)]siloxane. 1066500.

Contains 25 per cent of cyanopropyl groups, 25 per cent of phenyl groups and 50 per cent of methyl groups. (Average relative molecular mass 8000).

A very viscous liquid (viscosity about 9000 mPa.s).

d_{25}^{25} : about 1.10.

n_D^{25} : about 1.502.

Poly[(cyanopropyl)(phenyl)][dimethyl]siloxane. 1114800.

Stationary phase for gas chromatography.

Contains 6 per cent of (cyanopropyl)(phenyl) groups and 94 per cent of dimethyl groups.

Poly(cyanopropyl)(phenylmethyl)siloxane. 1066600.

Stationary phase for gas chromatography.

Contains 90 per cent of cyanopropyl groups and 10 per cent of phenylmethyl groups.

Poly(cyanopropyl)(7)(phenyl)(7)(methyl)(86)siloxane. 1109200.

Stationary phase for gas chromatography.

Polysiloxane substituted with 7 per cent of cyanopropyl groups, 7 per cent of phenyl groups and 86 per cent of dimethyl groups.

Poly(cyanopropyl)siloxane. 1066700.

Polysiloxane substituted with 100 per cent of cyanopropyl groups.

Poly(dimethyl)(diphenyl)(divinyl)siloxane. 1100000.

Stationary phase for gas chromatography.

Contains 94 per cent of methyl groups, 5 per cent of phenyl groups and 1 per cent of vinyl groups. SE54.

Poly(dimethyl)(diphenyl)siloxane. 1066900.

Stationary phase for gas chromatography.

Contains 95 per cent of methyl groups and 5 per cent of phenyl groups. DB-5, SE52.

Poly(dimethyl)(85)(diphenyl)(15)siloxane. 1154700.

Stationary phase for chromatography.

Contains 85 per cent of methyl groups and 15 per cent of phenyl groups. PS086.

Poly(dimethyl)siloxane. 1066800.

Silicone gum rubber (methyl). Organosilicon polymer with the appearance of a semi-liquid, colourless gum.

The intrinsic viscosity, determined as follows is about 115 ml·g⁻¹. Weigh 1.5 g, 1 g and 0.3 g of the substance to be examined to the nearest 0.1 mg, into 100 ml volumetric flasks. Add 40 ml to 50 ml of *toluene R*, shake until the substance is completely dissolved and dilute to 100.0 ml with the same solvent. Determine the viscosity (2.2.9) of each solution. Determine the viscosity of *toluene R* under the same conditions. Reduce the concentration of each solution by half by diluting with *toluene R*. Determine the viscosity of these solutions.

- c = concentration in grams per 100 ml,
 t_1 = flow time of the solution to be examined,
 t_2 = flow time of toluene,
 η_1 = viscosity of the solution to be examined in millipascal seconds,
 η_2 = viscosity of toluene in millipascal seconds,
 d_1 = relative density of the solution to be examined,
 d_2 = relative density of toluene.

To obtain the relative densities use the following data.

Concentration (g/100 ml)	Relative density (d_1)
0 - 0.5	1.000
0.5 - 1.25	1.001
1.25 - 2.20	1.002
2.20 - 2.75	1.003
2.75 - 3.20	1.004
3.20 - 3.75	1.005
3.75 - 4.50	1.006

The specific viscosity is obtained from the equation:

$$\eta_{sp} = \frac{\eta_1 - \eta_2}{\eta_2} = \frac{t_1 d_1}{t_2 d_2} - 1$$

and the reduced viscosity from:

$$\eta_{red} = \frac{\eta_{sp}}{c}$$

The intrinsic viscosity (η) is obtained by extrapolating the preceding equation to $c = 0$. This is done by plotting the curve η_{sp}/c or $\log \eta_{sp}/c$ as a function of c . Extrapolation to $c = 0$ gives η . The intrinsic viscosity is expressed in millilitres per gram; the value obtained must therefore be multiplied by 100.

The infrared absorption spectrum (2.2.24) obtained by applying the substance, if necessary dispersed in a few drops of *carbon tetrachloride R*, to a sodium chloride plate, does not show absorption at 3053 cm⁻¹, corresponding to vinyl groups.

Loss on drying (2.2.32): maximum 2.0 per cent, determined on 1.000 g by drying *in vacuo* at 350 °C for 15 min; maximum 0.8 per cent, determined on 2.000 g by drying at 200 °C for 2 h.

Polyether hydroxylated gel for chromatography. 1067000.

Gel with a small particle size having a hydrophilic surface with hydroxyl groups. It has an exclusion limit for dextran of relative molecular mass 2×10^5 to 2.5×10^6 .

Polyethyleneglycol adipate. (C₈H₁₂O₄)_n. (M_r (172.2)_n). 1067700.

A white, wax-like mass, practically insoluble in water.
 mp: about 43 °C.

Polyethyleneglycol succinate. (C₆H₈O₄)_n. (M_r (144.1)_n). 1067800.

A white, crystalline powder, practically insoluble in water.
 mp: about 102 °C.

Polymethacrylate gel, hydroxylated. 1151300.

Stationary phase for size-exclusion chromatography.
 Gel based on hydroxylated methacrylic acid polymer.

Polymethylphenylsiloxane. 1067900.

Stationary phase for gas chromatography.

Contains 50 per cent of methyl groups and 50 per cent of phenyl groups. (Average relative molecular mass 4000).

A very viscous liquid (viscosity about 1300 mPas).

d_{25}^{25} : about 1.09.

n_D^{25} : about 1.540.

Poly[methyl(95)phenyl(5)]siloxane. 1068000.

See *Poly(dimethyl)(diphenyl)siloxane R*.

Poly[methyl(94)phenyl(5)vinyl(1)]siloxane. 1068100.

See *Poly(dimethyl)(diphenyl)(divinyl)siloxane R*.

Polyoxyethylated castor oil. 1068200.

A light yellow liquid. It becomes clear above 26 °C.

Polysorbate 20. 1068300. [9005-64-5].

See *Polysorbate 20 (0426)*.

Polysorbate 80. 1068400. [9005-65-6].

See *Polysorbate 80 (0428)*.

Polystyrene 900-1000. 1112200. [9003-53-6].

Organic standard used for calibration in gas chromatography.

M_w : about 950.

M_w/M_n : 1.10.

Potassium bicarbonate. 1069900. [298-14-6].

See *potassium hydrogen carbonate R*.

Potassium bicarbonate solution, saturated methanolic. 1069901.

See *potassium hydrogen carbonate solution, saturated methanolic R*.

Potassium bromate. KBrO₃. (M_r 167.0). 1068700. [7758-01-2].

White granular powder or crystals, soluble in water, slightly soluble in alcohol.

Potassium bromide. 1068800. [7758-02-3]. See *Potassium bromide* (0184).

Potassium bromide used for infrared absorption spectrophotometry (2.2.24) also complies with the following requirement.

A disc 2 mm thick prepared from the substance previously dried at 250 °C for 1 h, has a substantially flat baseline over the range 4000 cm⁻¹ to 620 cm⁻¹. It exhibits no maxima with absorbance greater than 0.02 above the baseline, except maxima for water at 3440 cm⁻¹ and 1630 cm⁻¹.

Potassium carbonate. K₂CO₃. (*M_r* 138.2). 1068900. [584-08-7]. Dipotassium carbonate.

A white, granular powder, hygroscopic, very soluble in water, practically insoluble in ethanol.

Storage: in an airtight container.

Potassium chlorate. KClO₃. (*M_r* 122.6). 1069000. [3811-04-9].

A white powder, granules or crystals, soluble in water.

Potassium chloride. 1069100. [7447-40-7]. See *Potassium chloride* (0185).

Potassium chloride used for infrared absorption spectrophotometry (2.2.24) also complies with the following requirement.

A disc 2 mm thick, prepared from the substance previously dried at 250 °C for 1 h, has a substantially flat baseline over the range 4000 cm⁻¹ to 620 cm⁻¹. It exhibits no maxima with absorbance greater than 0.02 above the baseline, except maxima for water at 3440 cm⁻¹ and 1630 cm⁻¹.

Potassium chloride, 0.1 M. 1069101.

A solution of *potassium chloride R* containing the equivalent of 7.46 g of KCl in 1000.0 ml.

Potassium chromate. K₂CrO₄. (*M_r* 194.2). 1069200. [7789-00-6]. Dipotassium chromate.

Yellow crystals, freely soluble in water.

Potassium chromate solution. 1069201.

A 50 g/l solution.

Potassium citrate. 1069300. [6100-05-6].

See *Potassium citrate* (0400).

Potassium cyanide. KCN. (*M_r* 65.1). 1069400. [151-50-8].

A white, crystalline powder or white mass or granules, freely soluble in water, slightly soluble in alcohol.

Potassium cyanide solution. 1069401.

A 100 g/l solution.

Potassium cyanide solution, lead-free. 1069402.

Dissolve 10 g of *potassium cyanide R* in 90 ml of *water R*, add 2 ml of *strong hydrogen peroxide solution R* diluted 1 to 5. Allow to stand for 24 h, dilute to 100 ml with *water R* and filter.

The solution complies with the following test: take 10 ml of the solution, add 10 ml of *water R* and 10 ml of *hydrogen sulphide solution R*. No colour is evolved even after addition of 5 ml of *dilute hydrochloric acid R*.

Potassium dichromate. K₂Cr₂O₇. (*M_r* 294.2). 1069500. [7778-50-9]. Dipotassium dichromate.

Potassium dichromate used for the calibration of spectrophotometers (2.2.25) contains not less than 99.9 per cent of K₂Cr₂O₇, calculated with reference to the substance dried at 130 °C.

Orange-red crystals, soluble in water, practically insoluble in alcohol.

Assay. Dissolve 1.000 g in *water R* and dilute to 250.0 ml with the same solvent. To 50.0 ml of this solution add a freshly prepared solution of 4 g of *potassium iodide R*, 2 g of *sodium hydrogen carbonate R* and 6 ml of *hydrochloric acid R* in 100 ml of *water R* in a 500 ml flask. Stopper the flask and allow to stand protected from light for 5 min. Titrate with 0.1 M *sodium thiosulphate*, using 1 ml of *iodide-free starch solution R* as indicator.

1 ml of 0.1 M *sodium thiosulphate* is equivalent to 4.903 mg of K₂Cr₂O₇.

Potassium dichromate solution. 1069501.

A 106 g/l solution.

Potassium dichromate solution R1. 1069502.

A 5 g/l solution.

Potassium dihydrogen phosphate. 1069600. [7778-77-0]. See *Potassium dihydrogen phosphate* (0920).

Potassium dihydrogen phosphate, 0.2 M. 1069601.

A solution of *potassium dihydrogen phosphate R* containing the equivalent of 27.22 g of KH₂PO₄ in 1000.0 ml.

Potassium ferricyanide. K₃[Fe(CN)₆]. (*M_r* 329.3). 1069700. [13746-66-2]. Potassium hexacyanoferrate(III).

Red crystals, freely soluble in water.

Potassium ferricyanide solution. 1069701.

Wash 5 g of *potassium ferricyanide R* with a little *water R*, dissolve and dilute to 100 ml with *water R*. Prepare immediately before use.

Potassium ferrocyanide. K₄[Fe(CN)₆].3H₂O. (*M_r* 422.4). 1069800. [14459-95-1]. Potassium hexacyanoferrate(II).

Transparent yellow crystals, freely soluble in water, practically insoluble in alcohol.

Potassium ferrocyanide solution. 1069801.

A 53 g/l solution.

Potassium fluoride. KF. (*M_r* 58.1). 1137800. [7789-23-3].

Colourless crystals or white crystalline powder, deliquescent, soluble in water, practically insoluble in alcohol.

Potassium hydrogen carbonate. KHCO₃. (*M_r* 100.1). 1069900. [298-14-6]. Potassium bicarbonate.

Transparent, colourless crystals, freely soluble in water, practically insoluble in alcohol.

Potassium hydrogen carbonate solution, saturated methanolic. 1069901.

Dissolve 0.1 g of *potassium hydrogen carbonate R* in 0.4 ml of *water R*, heating on water-bath. Add 25 ml of *methanol R* and swirl, keeping the solution on the water-bath until dissolution is complete. Use a freshly prepared solution.

Potassium hydrogen phthalate. C₈H₅KO₄. (*M_r* 204.2). 1070000. [877-24-7]. Potassium hydrogen benzene-1,2-dicarboxylate.

White crystals, soluble in water, slightly soluble in alcohol.

Potassium hydrogen phthalate, 0.2 M. 1070001.

A solution of *potassium hydrogen phthalate R* containing the equivalent of 40.84 g of $C_8H_5KO_4$ in 1000.0 ml.

Potassium hydrogen sulphate. $KHSO_4$. (M_r 136.2). 1070100. [7646-93-7].

Colourless, transparent, hygroscopic crystals, freely soluble in water giving a strongly acid solution.

Storage: in an airtight container.

Potassium hydrogen tartrate. $C_4H_5KO_6$. (M_r 188.2). 1070200. [868-14-4]. Potassium hydrogen (2*R*,3*R*)-2,3-dihydroxybutane-1,4-dioate.

A white, crystalline powder or colourless, slightly opaque crystals, slightly soluble in water, soluble in boiling water, practically insoluble in alcohol.

Potassium hydroxide. 1070300. [1310-58-3].

See *Potassium hydroxide (0840)*.

Potassium hydroxide, alcoholic, 2 M. 1070301.

Dissolve 12 g of *potassium hydroxide R* in 10 ml of *water R* and dilute to 100 ml with *alcohol R*.

Potassium hydroxide in alcohol (10 per cent V/V), 0.5 M. 1070302.

Dissolve 28 g of *potassium hydroxide R* in 100 ml of *alcohol R* and dilute to 1000 ml with *water R*.

Potassium hydroxide solution, alcoholic. 1070303.

Dissolve 3 g of *potassium hydroxide R* in 5 ml of *water R* and dilute to 100 ml with *aldehyde-free alcohol R*.

Decant the clear solution. The solution should be almost colourless.

Potassium hydroxide solution, alcoholic R1. 1070304.

Dissolve 6.6 g of *potassium hydroxide R* in 50 ml of *water R* and dilute to 1000 ml with *ethanol R*.

Potassium iodate. KIO_3 . (M_r 214.0). 1070400. [7758-05-6].

A white, crystalline powder, soluble in water.

Potassium iodide. 1070500. [7681-11-0].

See *Potassium iodide (0186)*.

Potassium iodide and starch solution. 1070501.

Dissolve 0.75 g of *potassium iodide R* in 100 ml of *water R*. Heat to boiling and add whilst stirring a solution of 0.5 g of *soluble starch R* in 35 ml of *water R*. Boil for 2 min and allow to cool.

Test for sensitivity. A mixture of 15 ml of the potassium iodide and starch solution, 0.05 ml of *glacial acetic acid R* and 0.3 ml of *iodine solution R2* is blue.

Potassium iodide solution. 1070502.

A 166 g/l solution.

Potassium iodide solution, iodinated. 1070503.

Dissolve 2 g of *iodine R* and 4 g of *potassium iodide R* in 10 ml of *water R*. When solution is complete dilute to 100 ml with *water R*.

Potassium iodide solution, saturated. 1070504.

A saturated solution of *potassium iodide R* in *carbon dioxide-free water R*. Make sure the solution remains saturated as indicated by the presence of undissolved crystals.

Test by adding to 0.5 ml of the saturated potassium iodide solution 30 ml of a mixture of 2 volumes of *chloroform R* and 3 volumes of *glacial acetic acid R*, as well as 0.1 ml

of *starch solution R*. Any blue colour formed should be discharged by the addition of 0.05 ml of 0.1 M *sodium thiosulphate*.

Storage: protected from light.

Potassium iodobismuthate solution. 1070600.

To 0.85 g of *bismuth subnitrate R* add 40 ml of *water R*, 10 ml of *glacial acetic acid R* and 20 ml of a 400 g/l solution of *potassium iodide R*.

Potassium iodobismuthate solution R1. 1070601.

Dissolve 100 g of *tartaric acid R* in 400 ml of *water R* and add 8.5 g of *bismuth subnitrate R*. Shake for 1 h, add 200 ml of a 400 g/l solution of *potassium iodide R* and shake well. Allow to stand for 24 h and filter.

Storage: protected from light.

Potassium iodobismuthate solution R2. 1070602.

Stock solution. Suspend 1.7 g of *bismuth subnitrate R* and 20 g of *tartaric acid R* in 40 ml of *water R*. To the suspension add 40 ml of a 400 g/l solution of *potassium iodide R* and stir for 1 h. Filter. The solution may be kept for several days in brown bottles.

Spray solution. Mix immediately before use 5 ml of the stock solution with 15 ml of *water R*.

Potassium iodobismuthate solution R3. 1070604.

Dissolve 0.17 g of *bismuth subnitrate R* in a mixture of 2 ml of *glacial acetic acid R* and 18 ml of *water R*. Add 4 g of *potassium iodide R*, 1 g of *iodine R* and dilute to 100 ml with *dilute sulphuric acid R*.

Potassium iodobismuthate solution R4. 1070605.

Dissolve 1.7 g of *bismuth subnitrate R* in 20 ml of *glacial acetic acid R*. Add 80 ml of *distilled water R*, 100 ml of a 400 g/l solution of *potassium iodide R*, 200 ml of *glacial acetic acid R* and dilute to 1000 ml with *distilled water R*. Mix 2 volumes of this solution with 1 volume of a 200 g/l solution of *barium chloride R*.

Potassium iodobismuthate solution, dilute. 1070603.

Dissolve 100 g of *tartaric acid R* in 500 ml of *water R* and add 50 ml of *potassium iodobismuthate solution R1*.

Storage: protected from light.

Potassium nitrate. KNO_3 . (M_r 101.1). 1070700. [7757-79-1].

Colourless crystals, very soluble in water.

Potassium periodate. KIO_4 . (M_r 230.0). 1070800. [7790-21-8].

A white, crystalline powder or colourless crystals, soluble in water.

Potassium ferriperiodate solution. 1070801.

Dissolve 1 g of *potassium periodate R* in 5 ml of a freshly prepared 120 g/l solution of *potassium hydroxide R*. Add 20 ml of *water R* and 1.5 ml of *ferric chloride solution R1*. Dilute to 50 ml with a freshly prepared 120 g/l solution of *potassium hydroxide R*.

Potassium permanganate. 1070900. [7722-64-7].

See *Potassium permanganate (0121)*.

Potassium permanganate and phosphoric acid solution. 1070901.

Dissolve 3 g of *potassium permanganate R* in a mixture of 15 ml of *phosphoric acid R* and 70 ml of *water R*. Dilute to 100 ml with *water R*.

Potassium permanganate solution. 1070902.

A 30 g/l solution.

Potassium perrhenate. KReO_4 . (M_r 289.3). 1071000. [10466-65-6].

A white, crystalline powder, soluble in water, slightly soluble in alcohol, in methanol and in propylene glycol.

Potassium persulphate. $\text{K}_2\text{S}_2\text{O}_8$. (M_r 270.3). 1071100. [7727-21-1]. Dipotassium peroxodisulphate.

Colourless crystals or a white, crystalline powder, sparingly soluble in water, practically insoluble in alcohol. Aqueous solutions decompose at room temperature and more rapidly on warming.

Potassium plumbite solution. 1071200.

Dissolve 1.7 g of *lead acetate R*, 3.4 g of *potassium citrate R* and 50 g of *potassium hydroxide R* in *water R* and dilute to 100 ml with the same solvent.

Potassium pyroantimonate. KSb(OH)_6 . (M_r 262.9). 1071300. [12208-13-8]. Potassium hexahydroxoantimoniate.

White crystals or a white, crystalline powder, sparingly soluble in water.

Potassium pyroantimonate solution. 1071301.

Dissolve 2 g of *potassium pyroantimonate R* in 95 ml of hot *water R*. Cool quickly and add a solution containing 2.5 g of *potassium hydroxide R* in 50 ml of *water R* and 1 ml of *dilute sodium hydroxide solution R*. Allow to stand for 24 h, filter and dilute to 150 ml with *water R*.

Potassium tartrate. $\text{C}_4\text{H}_4\text{K}_2\text{O}_6 \cdot \frac{1}{2}\text{H}_2\text{O}$. (M_r 235.3). 1071400. [921-53-9]. Dipotassium (2*R*,3*R*)-2,3-dihydroxybutane-1,4-dioate hemihydrate.

White, granular powder or crystals, very soluble in water, very slightly soluble in alcohol.

Potassium tetraiodomercurate solution. 1071500.

Dissolve 1.35 g of *mercuric chloride R* in 50 ml of *water R*. Add 5 g of *potassium iodide R* and dilute to 100 ml with *water R*.

Potassium tetraiodomercurate solution, alkaline. 1071600.

Dissolve 11 g of *potassium iodide R* and 15 g of *mercuric iodide R* in *water R* and dilute to 100 ml with the same solvent. Immediately before use, mix 1 volume of this solution with an equal volume of a 250 g/l solution of *sodium hydroxide R*.

Potassium tetroxalate. $\text{C}_4\text{H}_3\text{KO}_8 \cdot 2\text{H}_2\text{O}$. (M_r 254.2). 1071700. [6100-20-5].

A white, crystalline powder, sparingly soluble in water, soluble in boiling water, slightly soluble in alcohol.

Potassium thiocyanate. KSCN . (M_r 97.2). 1071800. [333-20-0].

Colourless crystals, deliquescent, very soluble in water and in alcohol.

Storage: in an airtight container.

Potassium thiocyanate solution. 1071801.

A 97 g/l solution.

Povidone. 1068500. [9003-39-8].

See *Povidone* (0685).

Procaine hydrochloride. 1109400.

See *Procaine hydrochloride* (0050).

Proline. $\text{C}_5\text{H}_9\text{NO}_2$. (M_r 115.1). 1152200. [147-85-3]. L-Proline. (S)-Pyrrolidine-2-carboxylic acid.

White or almost white, finely crystallised powder, freely soluble in water and in mineral acids, soluble in alcohol.

Content: minimum 99.0 per cent of $\text{C}_5\text{H}_9\text{NO}_2$.

$[\alpha]_{\text{D}}^{22}$: -51 to -53, determined on a 5.0 per cent *m/V* solution in 1 *M* *hydrochloric acid*.

Propanol. $\text{C}_3\text{H}_8\text{O}$. (M_r 60.1). 1072000. [71-23-8]. 1-Propanol.

A clear colourless liquid, miscible with water and with alcohol.

d_{20}^{20} : about 0.802 to 0.806.

bp: about 97.2 °C.

Distillation range (2.2.11). Not less than 95 per cent distils between 96 °C and 99 °C.

2-Propanol. $\text{C}_3\text{H}_8\text{O}$. (M_r 60.1). 1072100. [67-63-0]. Isopropyl alcohol.

A clear, colourless, flammable liquid, miscible with water and with alcohol.

d_{20}^{20} : about 0.785.

bp: 81 °C to 83 °C.

2-Propanol R1. 1072101.

Complies with the requirements prescribed for *2-propanol R* and with the following requirements:

n_{D}^{20} : about 1.378.

Water (2.5.12): maximum 0.05 per cent, determined on 10 g.

Minimum transmittance (2.2.25), determined using *water R* as compensation liquid: 25 per cent at 210 nm, 55 per cent at 220 nm, 75 per cent at 230 nm, 95 per cent at 250 nm, 98 per cent at 260 nm.

Propetamphos. $\text{C}_{10}\text{H}_{20}\text{NO}_4\text{PS}$. (M_r 281.3). 1130900. [31218-83-4].

A suitable certified reference solution (10 ng/μl in cyclohexane) may be used.

Propidium iodide. $\text{C}_{27}\text{H}_{34}\text{I}_2\text{N}_4$. (M_r 668.4). 1154200. [25535-16-4]. 3,8-Diamino-5-[3(diethylmethylammonio)propyl]-6-phenylphenanthridinium diiodide.

Dark red solid.

Propionaldehyde. $\text{C}_3\text{H}_6\text{O}$. (M_r 58.1). 1072300. [123-38-6]. Propanal.

A liquid freely soluble in water, miscible with alcohol.

d_{20}^{20} : about 0.81.

n_{D}^{20} : about 1.365.

bp: about 49 °C.

mp: about -81 °C.

Propionic acid. $\text{C}_3\text{H}_6\text{O}_2$. (M_r 74.1). 1072400. [79-09-4].

An oily liquid, soluble in alcohol, miscible with water.

d_{20}^{20} : about 0.993.

n_{D}^{20} : about 1.387.

bp: about 141 °C.

mp: about -21 °C.

Propionic anhydride. $\text{C}_6\text{H}_{10}\text{O}_3$. (M_r 130.1). 1072500. [123-62-6].

A clear, colourless liquid, soluble in alcohol.

d_{20}^{20} : about 1.01.

bp: about 167 °C.

Propionic anhydride reagent. 1072501.

Dissolve 1 g of *toluenesulphonic acid R* in 30 ml of *glacial acetic acid R*, add 5 ml of *propionic anhydride R* and allow to stand for at least 15 min before use.

Storage: use within 24 h.

Propyl acetate. $C_5H_{10}O_2$. (M_r 102.1). 1072600. [109-60-4].
 d_{20}^{20} : about 0.888.
 bp: about 102 °C.
 mp: about –95 °C.

Propyl parahydroxybenzoate. 1072700. [94-13-3].
 See *Propyl parahydroxybenzoate* (0431).

D-Prolyl-L-phenylalanyl-L-arginine 4-nitroanilide dihydrochloride. $C_{26}H_{36}Cl_2N_8O_5$. (M_r 612). 1072800.

Propylene glycol. 1072900. [57-55-6].
 See *Propylene glycol* (0430).

Propylene oxide. C_3H_6O . (M_r 58.1). 1121800. [75-56-9].
 Colourless liquid, miscible with alcohol.

Protamine sulphate. 1073000. [53597-25-4 (salmine) 9007-31-2 (clupeine)].
 See *Protamine sulphate* (0569).

Pteric acid. $C_{14}H_{12}N_6O_3$. (M_r 312.3). 1144600. [119-24-4]. 4-[(2-Amino-4-oxo-1,4-dihydropteridin-6-yl)methyl]amino]benzoic acid.
 Crystals, soluble in solutions of alkali hydroxides.

Pulegone. $C_{10}H_{16}O$. (M_r 152.2). 1073100. [89-82-7].
 (*R*)-2-Isopropylidene-5-methylcyclohexanone.
 (+)-*p*-Menth-4-en-3-one.

An oily, colourless liquid, practically insoluble in water, miscible with alcohol.

d_{15}^{20} : about 0.936.
 n_D^{20} : 1.485 to 1.489.
 $[\alpha]_D^{20}$: +19.5 to +22.5.
 bp: 222 °C to 224 °C.

Pulegone used in gas chromatography complies with the following additional test.

Assay. Examine by gas chromatography (2.2.28) as prescribed in the monograph on *Peppermint oil* (0405) using the substance to be examined as the test solution.

The area of the principal peak is not less than 98.0 per cent of the total area of the peaks.

Putrescine. $C_4H_{12}N_2$. (M_r 88.15). 1137900. [110-60-1].
 1,4-Butanediamine. Tetramethylenediamine.

A colourless oily liquid, very soluble in water. Strong piperidine-like odour.

bp: about 159 °C.
 mp: about 23 °C.

Pyridine. C_5H_5N . (M_r 79.1). 1073200. [110-86-1].
 A clear, colourless liquid, hygroscopic, miscible with water and with alcohol.
 bp: about 115 °C.
Storage: in an airtight container.

Pyridine, anhydrous. 1073300. [110-86-1].
 Dry *pyridine R* over *anhydrous sodium carbonate R*. Filter and distil.
Water (2.5.12): maximum 0.01 per cent *m/m*.

Pyrid-2-ylamine. $C_5H_6N_2$. (M_r 94.1). 1073400. [504-29-0].
 2-Aminopyridine.
 Large crystals soluble in water and in alcohol.
 bp: about 210 °C.
 mp: about 58 °C.

Pyridylazonaphthol. $C_{15}H_{11}N_3O$. (M_r 249.3). 1073500. [85-85-8]. 1-(2-Pyridylazo)-2-naphthol.

A brick-red powder, practically insoluble in water, soluble in alcohol, in methanol and in hot dilute alkali solutions.
 mp: about 138 °C.

Pyridylazonaphthol solution. 1073501.
 A 1 g/l solution in *ethanol R*.

Test for sensitivity. To 50 ml of *water R* add 10 ml of *acetate buffer solution pH 4.4 R*, 0.10 ml of 0.02 *M* *sodium edetate* and 0.25 ml of the pyridylazonaphthol solution. After addition of 0.15 ml of a 5 g/l solution of *copper sulphate R*, the colour changes from light yellow to violet.

4-(2-Pyridylazo)resorcinol monosodium salt. $C_{11}H_8N_3NaO_2$, H_2O . (M_r 255.2). 1131500. [16593-81-0].

Orange crystalline powder.

Pyrocatechol. $C_6H_6O_2$. (M_r 110.1). 1073600. [120-80-9].
 Benzene-1,2-diol.

Colourless or slightly yellow crystals, soluble in water, in acetone and in alcohol.

mp: about 102 °C.
Storage: protected from light.

Pyrogallol. $C_6H_6O_3$. (M_r 126.1). 1073700. [87-66-1].
 Benzene-1,2,3-triol.

White crystals, becoming brownish on exposure to air and light, very soluble in water and in alcohol, slightly soluble in carbon disulphide. On exposure to air, aqueous solutions, and more rapidly alkaline solutions, become brown owing to the absorption of oxygen.

mp: about 131 °C.
Storage: protected from light.

Pyrogallol solution, alkaline. 1073701.

Dissolve 0.5 g of *pyrogallol R* in 2 ml of *carbon dioxide-free water R*. Dissolve 12 g of *potassium hydroxide R* in 8 ml of *carbon dioxide-free water R*. Mix the two solutions immediately before use.

2-Pyrrolidone. C_4H_7NO . (M_r 85.1). 1138000. [616-45-5].
 Pyrrolidin-2-one.

Liquid above 25 °C, miscible with water, with ethanol and with ethyl acetate.

d_4^{25} : 1.116.

Pyruvic acid. $C_3H_4O_3$. (M_r 88.1). 1109300. [127-17-3].
 2-Oxopropanoic acid.

A yellowish liquid, miscible with water and with ethanol.
 d_{20}^{20} : about 1.267.
 n_D^{20} : about 1.413.
 bp: about 165 °C.

Quercetin dihydrate. $C_{15}H_{10}O_7 \cdot 2H_2O$. (M_r 338.2). 1138100.
 2-(3,4-Dihydroxyphenyl)-3,5,7-trihydroxy-4*H*-1-benzopyran-4-one.

Yellow crystals or yellowish powder, practically insoluble in water, soluble in acetone and in methanol.

Water (2.5.12): maximum 12.0 per cent, determined on 0.100 g.

Assay. Examine by liquid chromatography (2.2.29) as prescribed in the monograph on *Ginkgo leaf* (1828).
 The content is not less than 90 per cent (anhydrous substance) calculated by the normalisation procedure.
Storage: protected from light.

Quercitrin. $C_{21}H_{20}O_{11}$. (M_r 448.4). 1138200. [522-12-3]. Quercetin 3-L-rhamnopyranoside. 3-[(6-Deoxy- α -L-mannopyranosyl)oxy]-2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-4H-1-benzopyran-4-one. Quercitroside.

Yellow crystals, practically insoluble in cold water, soluble in alcohol.

mp: 176 °C to 179 °C.

Chromatography. Examine as prescribed in the monograph on *Goldenrod* (1892) applying 20 μ l of the solution. After spraying, the chromatogram shows a yellowish-brown fluorescent zone with an R_f of about 0.6.

Storage: at a temperature of 2 °C to 8 °C.

Quinaldine red. $C_{21}H_{23}IN_2$. (M_r 430.3). 1073800. [117-92-0]. 2-[2-[4-(Dimethylamino)phenyl]ethenyl]-1-ethylquinolinium iodide.

Dark bluish-black powder, sparingly soluble in water, freely soluble in alcohol.

Quinaldine red solution. 1073801.

Dissolve 0.1 g of *quinaldine red R* in *methanol R* and dilute to 100 ml with the same solvent.

Colour change: pH 1.4 (colourless) to pH 3.2 (red).

Quinhydrone. $C_{12}H_{10}O_4$. (M_r 218.2). 1073900. [106-34-3]. Equimolecular compound of 1,4-benzoquinone and hydroquinone.

Dark green, lustrous crystals or a crystalline powder, slightly soluble in water, sparingly soluble in hot water, soluble in alcohol and in concentrated ammonia.

mp: about 170 °C.

Quinidine. $C_{20}H_{24}N_2O_2$. (M_r 324.4). 1074000. [56-54-2]. (S)-(6-Methoxyquinol-4-yl)[(2R,4S,5R)-5-vinylquinuclidin-2-yl]methanol.

White crystals, very slightly soluble in water, sparingly soluble in alcohol, slightly soluble in methanol.

$[\alpha]_D^{20}$: about + 260, determined on a 10 g/l solution in *ethanol R*.

mp: about 172 °C.

Storage: protected from light.

Quinidine sulphate. 1109500. [6591-63-5].

See *Quinidine sulphate* (0017).

Quinine. $C_{20}H_{24}N_2O_2$. (M_r 324.4). 1074100. [130-95-0]. (R)-(6-Methoxyquinol-4-yl)[(2S,4S,5R)-5-vinylquinuclidin-2-yl]methanol.

A white, microcrystalline powder, very slightly soluble in water, slightly soluble in boiling water, very soluble in ethanol.

$[\alpha]_D^{20}$: about – 167, determined on a 10 g/l solution in *ethanol R*.

mp: about 175 °C.

Storage: protected from light.

Quinine hydrochloride. 1074200. [6119-47-7].

See *Quinine hydrochloride* (0018).

Quinine sulphate. 1074300. [6119-70-6].

See *Quinine sulphate* (0019).

Rabbit erythrocyte suspension. 1074500.

Prepare a 1.6 per cent V/V suspension of rabbit erythrocytes as follows: defibrinate 15 ml of freshly drawn rabbit blood by shaking with glass beads, centrifuge at 2000 g for 10 min and wash the erythrocytes with three quantities, each of

30 ml, of a 9 g/l solution of *sodium chloride R*. Dilute 1.6 ml of the suspension of erythrocytes to 100 ml with a mixture of 1 volume of *phosphate buffer solution pH 7.2 R* and 9 volumes of a 9 g/l solution of *sodium chloride R*.

Raclopride tartrate. $C_{19}H_{26}Cl_2N_2O_9$. (M_r 497.3). 1144700. [98185-20-7]. Raclopride L-tartrate.

A white solid, sensitive to light, soluble in water.

$[\alpha]_D^{25}$: + 0.3, determined on a 3 g/l solution.

mp: about 141 °C.

Rapeseed oil. 1074600.

See *Rapeseed oil, refined* (1369).

Reducing mixture. 1074700.

Grind the substances added in the following order to obtain a homogeneous mixture: 20 mg of *potassium bromide R*, 0.5 g of *hydrazine sulphate R* and 5 g of *sodium chloride R*.

Resin for reversed-phase ion chromatography. 1131100.

A neutral, macroporous, high specific surface area with a non-polar character resin consisting of polymer lattice of polystyrene cross-linked with divinylbenzene.

Resin, weak cationic. 1096000.

See *weak cationic resin R*.

Resorcinol. 1074800. [108-46-3].

See *Resorcinol* (0290).

Resorcinol reagent. 1074801.

To 80 ml of *hydrochloric acid R1* add 10 ml of a 20 g/l solution of *resorcinol R* and 0.25 ml of a 25 g/l solution of *copper sulphate R* and dilute to 100.0 ml with *water R*. Prepare the solution at least 4 h before use.

Storage: at 2 °C to 8 °C for 1 week.

Rhamnose. $C_6H_{12}O_5 \cdot H_2O$. (M_r 182.2). 1074900. [6155-35-7]. L-(+)-Rhamnose. 6-Deoxy-L-mannose.

A white, crystalline powder, freely soluble in water.

$[\alpha]_D^{20}$: + 7.8 to + 8.3, determined on a 50 g/l solution in *water R* containing about 0.05 per cent of NH_3 .

Rhaponticin. $C_{21}H_{24}O_9$. (M_r 420.4). 1075000. [155-58-8]. 3-Hydroxy-5-[2-(3-hydroxy-4-methoxyphenyl)ethenyl]phenyl β -D-glucopyranoside.

A yellowish-grey, crystalline powder, soluble in alcohol and in methanol.

Chromatography. Examine as prescribed in the monograph on *Rhubarb* (0291); the chromatogram shows only one principal spot.

Rhodamine 6 G. $C_{28}H_{31}ClN_2O_3$. (M_r 479.0). 1153300. [989-38-8].

Colour Index No. 45160.

9-[2-(Ethoxycarbonyl)phenyl]-3,6-bis(ethylamino)-2,7-dimethylxanthylium chloride.

Brownish-red powder.

Rhodamine B. $C_{28}H_{31}ClN_2O_3$. (M_r 479.0). 1075100. [81-88-9]. Schultz No. 864.

Colour Index No. 45170.

[9-(2-Carboxyphenyl)-6-(diethylamino)-3H-xanthen-3-ylidene]diethylammonium chloride.

Green crystals or reddish-violet powder, very soluble in water and in alcohol.

Ribose. $C_5H_{10}O_5$. (M_r 150.1). 1109600. [50-69-1]. D-Ribose.

Soluble in water, slightly soluble in alcohol.

mp: 88 °C to 92 °C.

Ricinoleic acid. $C_{18}H_{34}O_3$. (M_r 298.5). 1100100. [141-22-0]. 12-Hydroxyoleic acid.

A yellow or yellowish-brown viscous liquid, consisting of a mixture of fatty acids obtained by the hydrolysis of castor oil, practically insoluble in water, very soluble in ethanol.

d_{20}^{20} : about 0.942.

n_D^{20} : about 1.472.

mp: about 285 °C, with decomposition.

Rosmarinic acid. $C_{18}H_{16}O_8$. (M_r 360.3). 1138300. [20283-92-5].

mp: 170 °C to 174 °C.

Ruscogenins. 1141300.

Mixture of neoruscogenin ($C_{27}H_{40}O_4$; M_r 428.6) and ruscogenin ($C_{27}H_{42}O_4$; M_r 430.6).

White powder, very slightly soluble in water, soluble in alcohol.

Ruscogenins used in liquid chromatography complies with the following additional requirement.

Assay. Examine by liquid chromatography (2.2.29) as prescribed in the monograph on *Butcher's broom (1847)*. The content is not less than 90 per cent of ruscogenins of which at least 60 per cent consists of neoruscogenin, calculated by the normalisation procedure.

Ruthenium red. $[(NH_3)_3RuORu(NH_3)_4ORu(NH_3)_5]Cl_6 \cdot 4H_2O$. (M_r 858). 1075200. [11103-72-3].

A brownish-red powder, soluble in water.

Ruthenium red solution. 1075201.

A 0.8 g/l solution in *lead acetate solution R*.

Rutin. $C_{27}H_{30}O_{16} \cdot 3H_2O$. (M_r 665). 1075300. [153-18-4]. Rutoside. 3-(*O*-6-Deoxy- α -L-mannopyranosyl-(1 $\rightarrow$ 6)- β -D-glucopyranosyloxy)-2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-4*H*-chromen-4-one.

A yellow, crystalline powder, darkening in light, very slightly soluble in water, soluble in about 400 parts of boiling water, slightly soluble in alcohol, soluble in solutions of the alkali hydroxides and in ammonia.

mp: about 210 °C, with decomposition.

A solution in *alcohol R* shows two absorption maxima (2.2.25), at 259 nm and 362 nm.

Storage: protected from light.

Sabinene. $C_{10}H_{16}$. (M_r 136.2). 1109700. [2009-00-9]. Thuj-4(10)-ene. 4-Methylene-1-isopropylbicyclo[3.1.0]hexane.

A colourless, oily liquid.

d_{25}^{25} : about 0.843.

n_D^{20} : about 1.468.

bp: 163 °C to 165 °C.

Sabinene used in gas chromatography complies with the following additional test.

Assay. Examine by gas chromatography (2.2.28) as prescribed in the monograph on *Bitter-orange-flower oil (1175)*, using the substance to be examined as the test solution.

The area of the principal peak is not less than 99.0 per cent of the total area of the peaks.

Saccharin sodium. 1131400. [128-44-9].

See *Saccharin sodium (0787)*.

Safrole. $C_{10}H_{10}O_2$. (M_r 162.2). 1131200. [94-59-7]. 5-(Prop-2-enyl)-1,3-benzodioxole. 4-Allyl-1,2-(methylenedioxy)benzene.

A colourless or slightly yellow, oily liquid, with the odour of sassafras, insoluble in water, very soluble in alcohol, miscible with hexane.

d_{20}^{20} : 1.095 to 1.096.

n_D^{20} : 1.537 to 1.538.

bp: 232 °C to 234 °C.

Freezing point: about 11 °C.

Safrole used in gas chromatography complies with the following additional test.

Assay. Examine by gas chromatography (2.2.28) as prescribed in the monograph on *Cinnamon bark oil, Ceylon (1501)*.

The content is not less than 96.0 per cent, calculated by the normalisation procedure.

Salicin. $C_{13}H_{18}O_7$. (M_r 286.3). 1131300. [138-52-3]. 2-(Hydroxymethyl)phenyl- β -D-glucopyranoside. Salicoside.

$[\alpha]_D^{20}$: -62.5 ± 2 .

mp: 199 °C to 201 °C.

Assay. Examine by liquid chromatography (2.2.29) as prescribed in the monograph on *Willow bark (1583)* at the concentration of the reference solution. The content is not less than 99.0 per cent calculated by the normalisation procedure.

Salicylaldehyde. $C_7H_6O_2$. (M_r 122.1). 1075400. [959-36-4]. 2-Hydroxybenzaldehyde.

A clear, colourless, oily liquid.

d_{20}^{20} : about 1.167.

n_D^{20} : about 1.574.

bp: about 196 °C.

mp: about -7 °C.

Salicylaldehyde azine. $C_{14}H_{12}N_2O_2$. (M_r 240.3). 1075500. [959-36-4]. 2,2'-Azinodimethyldiphenol.

Dissolve 0.30 g of *hydrazine sulphate R* in 5 ml of *water R*, add 1 ml of *glacial acetic acid R* and 2 ml of a freshly prepared 20 per cent *V/V* solution of *salicylaldehyde R* in *2-propanol R*. Mix, allow to stand until a yellow precipitate is formed. Shake with two quantities, each of 15 ml, of *methylene chloride R*. Combine the organic layers and dry over *anhydrous sodium sulphate R*. Decant or filter the solution and evaporate to dryness. Recrystallise from a mixture of 40 volumes of *methanol R* and 60 volumes of *toluene R* with cooling. Dry the crystals *in vacuo*.

mp: about 213 °C.

Chromatography. Examine as prescribed in the test for hydrazine in the monograph on *Povidone (0685)*; the chromatogram shows only one principal spot.

Salicylic acid. 1075600. [69-72-7].

See *Salicylic acid (0366)*.

Sand. 1075800.

White or slightly greyish grains of silica with a particle size between 150 μ m and 300 μ m.

Santonin. $C_{15}H_{18}O_3$. (M_r 246.3). 1122000. [481-06-1]. ($\pm$)- α -Santonin. 3,5a,9-Trimethyl-3a,5,5a,9b-tetrahydro-3*H*, 4*H*-naphtho[1,2]furan-2,8-dione.

Colourless, shiny crystals colouring yellow in light, very slightly soluble in water, freely soluble in hot ethanol, sparingly soluble in ethanol.

$[\alpha]_{\text{D}}^{18}$: –173 in ethanol.

mp: 174 °C to 176 °C.

Chromatography. Examine as prescribed in identification test C in the monograph on *Arnica flower* (1391), the chromatogram obtained with 10 µl of the solution shows a quenching zone with an R_f value of about 0.5. Spray with *anisaldehyde solution R* and examine while heating at 105 °C for 5 min to 10 min. In daylight the quenching zone is at first a yellow zone that quickly changes to a violet-red zone.

Sclareol. $\text{C}_{20}\text{H}_{36}\text{O}_2$ (M_r 308.5). 1139900. [515-03-7]. (1*R*,2*R*,4*aS*,8*aS*)-1-[(3*R*)-3-Hydroxy-3-methylpent-4-enyl]-2,5,5,8*a*-tetramethyldecahydronaphthalen-2-ol.

Odourless crystals.

$[\alpha]_{\text{D}}^{20}$: 6.7, in solution in ethanol.

bp_{19 mm}: 218 °C to 220 °C.

mp: 96 °C to 98 °C.

Sclareol used in the chromatographic profile test in the monograph on Clary sage oil (1850) complies with the following additional test.

Assay. Examine by gas chromatography (2.2.28) as prescribed in the monograph on *Clary sage oil* (1850).

The content of sclareol is not less than 97 per cent, calculated by the normalisation procedure.

SDS-PAGE running buffer. 1114900.

Dissolve 151.4 g of *tris(hydroxymethyl)aminomethane R*, 721.0 g of *glycine R* and 50.0 g of *sodium lauryl sulphate R* in *water R* and dilute to 5000 ml with the same solvent. Immediately before use, dilute to 10 times its volume with *water R* and mix. Measure the pH (2.2.3) of the diluted solution. The pH is between 8.1 and 8.8.

SDS-PAGE sample buffer (concentrated). 1115000.

Dissolve 1.89 g of *tris(hydroxymethyl)aminomethane R*, 5.0 g of *sodium lauryl sulphate R* and 50 mg of *bromophenol blue R* in *water R*. Add 25.0 ml of *glycerol R* and dilute to 100 ml with *water R*. Adjust the pH to 6.8 with *hydrochloric acid R*, and dilute to 125 ml with *water R*.

SDS-PAGE sample buffer for reducing conditions (concentrated). 1122100.

Dissolve 3.78 g of *tris(hydroxymethyl)aminomethane R*, 10.0 g of *sodium dodecyl sulphate R* and 100 mg of *bromophenol blue R* in *water R*. Add 50.0 ml of *glycerol R* and dilute to 200 ml with *water R*. Add 25.0 ml of *2-mercaptoethanol R*. Adjust to pH 6.8 (2.2.3) with *hydrochloric acid R*, and dilute to 250.0 ml with *water R*. Alternatively, dithiothreitol may be used as reducing agent instead of 2-mercaptoethanol. In this case prepare the sample buffer as follows: dissolve 3.78 g of *tris(hydroxymethyl)aminomethane R*, 10.0 g of *sodium dodecyl sulphate R* and 100 mg of *bromophenol blue R* in *water R*. Add 50.0 ml of *glycerol R* and dilute to 200 ml with *water R*. Adjust to pH 6.8 (2.2.3) with *hydrochloric acid R*, and dilute to 250.0 ml with *water R*. Immediately before use, add *dithiothreitol R* to a final concentration of 100 mM.

Selenious acid. H_2SeO_3 (M_r 129.0). 1100200. [7783-00-8].

Deliquescent crystals, freely soluble in water.

Storage: in an airtight container.

Selenium. Se. (A_r 79.0). 1075900. [7782-49-2].

A brown-red to black powder or granules, practically insoluble in water and in alcohol, soluble in nitric acid. mp: about 220 °C.

Serine. 1076000. [56-45-1].

See *Serine* (0788).

Sialic acid. 1001100. [131-48-6].

See *N-acetylneuraminic acid R*.

Silibinin. $\text{C}_{25}\text{H}_{22}\text{O}_{10}$ (M_r 482.4). 1151400. [22888-70-6]. Silybin. (2*R*,3*R*)-3,5,7-Trihydroxy-2-[(2*R*,3*R*)-3-(4-hydroxy-3-methoxyphenyl)-2-(hydroxymethyl)-2,3-dihydro-1,4-benzodioxin-6-yl]-2,3-dihydro-4*H*-1-benzopyran-4-one.

White to yellowish powder, practically insoluble in water, soluble in acetone and in methanol.

Silibinin used in the assay of Milk-thistle fruit (1860) complies with the following requirement.

Assay. Examine by liquid chromatography (2.2.29) as prescribed in the monograph on *Milk-thistle fruit* (1860).

Test solution. Dissolve 5.0 mg of silibinin, dried *in vacuo*, in *methanol R* and dilute to 50.0 ml with the same solvent.

The silibinin A and silibinin B content is not less than 95.0 per cent, calculated by the normalisation procedure.

Silica gel AGP for chiral chromatography. 1148700.

A very finely divided silica gel for chromatography consisting of spherical particles coated with α 1- acid glycoprotein. The particle size is indicated after the name of the reagent in the tests where it is used.

Silica gel, anhydrous. 1076100. [112926-00-8].

Partly dehydrated polymerised, amorphous silicic acid, absorbing at 20 °C about 30 per cent of its mass of water. It contains cobalt chloride as indicator. Practically insoluble in water, partly soluble in solutions of sodium hydroxide.

Silica gel for chromatography. 1076900.

A very finely divided (3 µm–10 µm) silica gel. The particle size is indicated after the name of the reagent in the tests where it is used.

A fine, white, homogeneous powder, practically insoluble in water and in alcohol.

Silica gel for chromatography, aminohexadecylsilyl. 1138400.

A very finely divided (3–10 µm) silica gel with a fine particle size chemically modified at the surface by the bonding of aminohexadecylsilyl groups. The particle size is indicated after the name of the reagent in the test where it is used.

A fine, white, homogeneous powder, practically insoluble in water and in alcohol.

Silica gel for chromatography, aminopropylmethylsilyl. 1102400.

Silica gel with a fine particle size (between 3 µm and 10 µm), chemically modified by bonding aminopropylmethylsilyl groups on the surface. The particle size is indicated after the name of the reagent in the tests where it is used.

A fine, white, homogeneous powder, practically insoluble in water and in alcohol.

Silica gel for chromatography, aminopropylsilyl. 1077000.

Silica gel with a fine particle size (between 3 µm and 10 µm), chemically modified by bonding aminopropylsilyl groups on the surface. The particle size is indicated after the name of the reagent in the tests where it is used.

A fine, white, homogeneous powder, practically insoluble in water and in alcohol.

Silica gel for chromatography, amylose derivative of. 1109800.

A very finely divided (10 µm) silica gel, chemically modified at the surface by the bonding of an amylose derivative. The particle size is indicated after the name of the reagent in the test where it is used.

A fine, white, homogenous powder, practically insoluble in water and in alcohol.

Silica gel for chromatography, butylsilyl. 1076200.

A very finely divided silica gel (3 µm-10 µm), chemically modified at the surface by the bonding of butylsilyl groups. The particle size is indicated after the name of the reagent in the tests where it is used.

A fine, white, homogeneous powder, practically insoluble in water and in alcohol.

Spheroidal silica: 30 nm.

Pore volume: 0.6 cm³/g.

Specific surface area: 80 m²/g.

Silica gel for chromatography, cyanosilyl. 1109900.

A very finely divided silica gel chemically modified at the surface by the bonding of cyanosilyl groups. The particle size is indicated after the name of the reagent in the tests where it is used.

A fine, white, homogeneous powder, practically insoluble in water and in alcohol.

Silica gel for chromatography, di-isobutyloctadecylsilyl. 1140000.

A very finely divided silica gel chemically modified at the surface by the bonding of di-isobutyloctadecylsilyl groups. The particle size is indicated after the name of the reagent in the tests where it is used.

Silica gel for chromatography, dimethyloctadecylsilyl. 1115100.

A very finely divided silica gel (3 µm-10 µm), chemically modified at the surface by the bonding of dimethyloctadecylsilyl groups. The particle size is indicated after the name of the reagent in the tests where it is used.

A fine, white, homogeneous powder, practically insoluble in water and in alcohol. Irregular particle size.

Specific surface area: 300 m²/g.

Silica gel for chromatography, diol. 1110000.

Spherical silica particles to which dihydroxypropyl groups are bonded. Pore size 10 nm.

Silica gel for chromatography, hexylsilyl. 1077100.

A very finely divided (3 µm-10 µm) silica gel, chemically modified at the surface by the bonding of hexylsilyl groups. The particle size is indicated after the name of the reagent in the tests where it is used.

A fine, white, homogeneous powder, practically insoluble in water and in alcohol.

Silica gel for chromatography, human albumin coated. 1138500.

A very finely divided (3 µm to 10 µm) silica gel, chemically modified at the surface by the bonding of human albumin. The particle size is indicated after the name of the reagent in the tests where it is used.

A white, fine, homogeneous powder.

Silica gel for chromatography, hydrophilic. 1077200.

A very finely divided (3 µm-10 µm) silica gel whose surface has been modified to provide hydrophilic characteristics. The particle size may be stated after the name of the reagent in the tests where it is used.

Silica gel for chromatography, nitrile. 1077300.

A very finely divided silica gel, chemically modified at the surface by the bonding of cyanopropylsilyl groups. The particle size is indicated after the name of the reagent in the test where it is used.

A fine white, homogenous powder, practically insoluble in water and in alcohol.

Silica gel for chromatography, nitrile R1. 1077400.

A very finely divided silica gel consisting of porous, spherical particles with chemically bonded nitrile groups. The particle size is indicated after the name of the reagent in the test where it is used.

A fine, white, homogeneous powder, practically insoluble in water and in alcohol.

Silica gel for chromatography, nitrile R2. 1119500.

Ultrapure silica gel, chemically modified at the surface by the introduction of cyanopropylsilyl groups. Less than 20 ppm of metals. The particle size is indicated after the name of the reagent in the tests where it is used.

A fine white, homogenous powder, practically insoluble in water and in alcohol.

Silica gel for chromatography, octadecanoylaminopropylsilyl. 1115200.

A very finely divided (3 µm-10 µm) silica gel, chemically modified at the surface by the bonding of aminopropylsilyl groups which are acylated with octadecanoyl groups. The particle size is indicated after the name of the reagent in the tests where it is used.

A fine, white, homogeneous powder, practically insoluble in water and in alcohol.

Silica gel for chromatography, octadecylsilyl. 1077500.

A very finely divided (3 µm-10 µm) silica gel, chemically modified at the surface by the bonding of octadecylsilyl groups. The particle size is indicated after the name of the reagent in the tests where it is used.

A fine, white, homogeneous powder, practically insoluble in water and in alcohol.

Silica gel for chromatography, octadecylsilyl R1. 1110100.

A very finely divided ultrapure silica gel, chemically modified at the surface by the bonding of octadecylsilyl groups.

The particle size, the pore size and the carbon loading are indicated after the name of the reagent in the tests where it is used. Less than 20 ppm of metals.

Silica gel for chromatography, octadecylsilyl R2. 1115300.

A very finely divided (15 nm pore size) ultrapure silica gel, chemically modified at the surface by the bonding of octadecylsilyl groups (20 per cent carbon load), optimised for the analysis of polycyclic aromatic hydrocarbons. The particle size is indicated after the name of the reagent in the tests where it is used.

A fine, white, homogeneous powder, practically insoluble in water and in alcohol.

Silica gel for chromatography, octadecylsilyl, base-deactivated. 1077600.

A very finely divided (3 µm-10 µm) silica gel, pretreated before the bonding of octadecylsilyl groups by careful washing and hydrolysing most of the superficial siloxane

bridges to minimise the interaction with basic components. The particle size is indicated after the name of the reagent in the tests where it is used.

A fine, white, homogeneous powder, practically insoluble in water and in alcohol.

Silica gel for chromatography, octadecylsilyl, end-capped. 1115400.

A very finely divided (3 µm-10 µm) silica gel, chemically modified at the surface by the bonding of octadecylsilyl groups. To minimise any interaction with basic compounds it is carefully end-capped to cover most of the remaining silanol groups. The particle size is indicated after the name of the reagent in the tests where it is used.

A fine, white, homogenous powder, practically insoluble in water and in alcohol.

Silica gel for chromatography, octadecylsilyl, end-capped, base-deactivated. 1108600.

A very finely divided (3 µm-10 µm) silica gel with a pore size of 10 nm and a carbon loading of 16 per cent, pre-treated before the bonding of octadecylsilyl groups by washing and hydrolysing most of the superficial siloxane bridges. To further minimise any interaction with basic compounds it is carefully end-capped to cover most of the remaining silanol groups. The particle size is indicated after the name of the reagent in the test where it is used.

A fine, white, homogeneous powder, practically insoluble in water and in alcohol.

Silica gel for chromatography, octadecylsilyl, monolithic. 1154500.

Monolithic rods of highly porous (greater than 80 per cent) metal-free silica with a bimodal pore structure, modified at the surface by the bonding of octadecylsilyl groups.

Silica gel for chromatography, octylsilyl. 1077700.

A very finely divided (3 µm-10 µm) silica gel, chemically modified at the surface by the bonding of octylsilyl groups. The particle size is indicated after the name of the reagent in the tests where it is used.

A fine, white, homogeneous powder, practically insoluble in water and in alcohol.

Silica gel for chromatography, octylsilyl R1. 1077701.

A very finely divided (3 µm-10 µm) silica gel, chemically modified at the surface by the bonding of octylsilyl and methyl groups (double bonded phase). The particle size is indicated after the name of the reagent in the tests where it is used.

A fine, white, homogeneous powder, practically insoluble in water and in alcohol.

Silica gel for chromatography, octylsilyl R2. 1077702.

Ultrapure very finely divided (10 nm pore size) silica gel, chemically modified at the surface by the bonding of octylsilyl groups (19 per cent carbon load). Less than 20 ppm of metals.

Silica gel for chromatography, octylsilyl R3. 1155200.

A very finely divided ultrapure silica gel, chemically modified at the surface by the bonding of octylsilyl groups and sterically protected with branched hydrocarbons at the silanes. The particle size is indicated after the name of the reagent in the tests where it is used.

Silica gel for chromatography, octylsilyl, base-deactivated. 1131600.

A very finely divided (3 µm-10 µm) silica gel, pretreated before the bonding of octylsilyl groups by careful washing and hydrolysing most of the superficial siloxane bridges to minimise the interaction with basic components. The particle size is indicated after the name of the reagent in the tests where it is used.

A fine, white, homogeneous powder, practically insoluble in water and in alcohol.

Silica gel for chromatography, octylsilyl, end-capped. 1119600.

A very finely divided (3 µm-10 µm) silica gel, chemically modified at the surface by the bonding of octylsilyl groups. To minimise any interaction with basic compounds, it is carefully end-capped to cover most of the remaining silanol groups. The particle size is indicated after the name of the reagent in the tests where it is used.

A fine, white, homogeneous powder, practically insoluble in water and in alcohol.

Silica gel for chromatography, octylsilyl, end-capped, base-deactivated. 1148800.

A very finely divided (3 µm-10 µm) silica gel, pre-treated before the bonding of octylsilyl groups by washing and hydrolysing most of the superficial siloxane bridges. To further minimise any interaction with basic compounds it is carefully end-capped to cover most of the remaining silanol groups. The particle size is indicated after the name of the reagent in the test where it is used.

A fine, white, homogeneous powder, practically insoluble in water and in alcohol.

Silica gel for chromatography, octylsilyl, with polar incorporated groups, end-capped. 1152600.

A very finely divided silica gel (3-10 µm). The particles are based on silica, chemically modified with a reagent providing a surface with chains having polar incorporated groups and terminating octyl groups. Furthermore, the packing material is end-capped. The particle size is indicated after the name of the reagent in the tests where it is used.

A fine, white, homogeneous powder.

Silica gel for chromatography, palmitamidopropylsilyl, end-capped. 1115200.

A very finely divided (3 µm-10 µm) silica gel, chemically modified at the surface by the bonding of palmitamidopropyl groups and end-capped with acetamidopropyl groups. The particle size is indicated after the name of the reagent in the tests where it is used.

A fine, white, homogeneous powder, practically insoluble in water and in alcohol.

Silica gel for chromatography, phenylhexylsilyl. 1153900.

A very finely divided silica gel, chemically modified at the surface by the bonding of phenylhexyl groups. The particle size is indicated after the name of the reagent in the tests where it is used.

Silica gel for chromatography, phenylsilyl. 1110200.

A very finely divided (5 µm-10 µm) silica gel, chemically modified at the surface by the bonding of phenyl groups.

Silica gel for chromatography, phenylsilyl R1. 1075700.

A very finely divided silica gel (5 µm), chemically modified at the surface by the bonding of phenyl groups. The particle size is indicated after the name of the reagent in the tests where it is used.

A fine, white, homogeneous powder, practically insoluble in water, in alcohol and in methylene chloride.

Spheroidal silica: 8 nm.

Specific surface area: 180 m²/g.

Carbon loading: 5.5 per cent.

Silica gel for chromatography, phenylsilyl, end-capped.
1154900.

A very finely divided (5-10 µm) silica gel, chemically modified at the surface by the bounding of phenyl groups. To minimise any interaction with basic compounds it is carefully end-capped to cover most of the remaining silanol groups. The particle size is indicated after the name of the reagent in the tests where it is used.

Silica gel for chromatography, strong-anion-exchange.
1077800.

A very finely divided (3 µm-10 µm) silica gel, chemically modified at the surface by the bonding of quaternary ammonium groups. The particle size is indicated after the name of the reagent in the tests where it is used.

A fine, white, homogeneous powder, practically insoluble in water and in alcohol.

pH limit of use: 2 to 8.

Silica gel for chromatography, trimethylsilyl. 1115500.

A very finely divided (3 µm-10 µm) silica gel, chemically modified at the surface by the bonding of trimethylsilyl groups. The particle size is indicated after the name of the reagent in the tests where it is used.

A fine, white, homogeneous powder, practically insoluble in water and in alcohol.

Silica gel for size-exclusion chromatography. 1077900.

A very finely divided silica gel (10 µm) with a very hydrophilic surface. The average diameter of the pores is about 30 nm. It is compatible with aqueous solutions between pH 2 and 8 and with organic solvents. It is suitable for the separation of proteins with relative molecular masses of 1×10^3 to 3×10^5 .

Silica gel G. 1076300. [112926-00-8].

Contains about 13 per cent of calcium sulphate hemihydrate.

A fine, white, homogeneous powder with a particle size of about 15 µm.

Calcium sulphate content. Place 0.25 g in a ground-glass stoppered flask, add 3 ml of *dilute hydrochloric acid R* and 100 ml of *water R* and shake vigorously for 30 min. Filter through a sintered-glass filter and wash the residue. Carry out on the combined filtrate and washings the complexometric assay of calcium (2.5.11).

1 ml of 0.1 M sodium edetate is equivalent to 14.51 mg of CaSO₄· $\frac{1}{2}$ H₂O.

pH (2.2.3). Shake 1 g for 5 min with 10 ml of *carbon dioxide-free water R*. The pH of the suspension is about 7.

Silica gel GF₂₅₄. 1076400. [112926-00-8].

Contains about 13 per cent of calcium sulphate hemihydrate and about 1.5 per cent of a fluorescent indicator having an optimal intensity at 254 nm.

A fine, white, homogeneous powder with a particle size of about 15 µm.

Calcium sulphate content. Determine by the method prescribed for *silica gel G R*.

pH (2.2.3). Complies with the test prescribed for *silica gel G R*.

Fluorescence. Examine by thin-layer chromatography (2.2.27) using *silica gel GF₂₅₄ R* as the coating substance. Apply separately to the plate at ten points increasing

volumes from 1 µl to 10 µl of a 1 g/l solution of *benzoic acid R* in a mixture of 10 volumes of *anhydrous formic acid R* and 90 volumes of *2-propanol R*. Develop over a path of 10 cm with the same mixture of solvents. After evaporating the solvents examine the chromatogram in ultraviolet light at 254 nm. The benzoic acid appears as dark spots on a fluorescent background in the upper third of the chromatogram for quantities of 2 µg and greater.

Silica gel H. 1076500. [112926-00-8].

A fine, white, homogeneous powder with a particle size of about 15 µm.

pH (2.2.3). Complies with the test prescribed for *silica gel G R*.

Silica gel H, silanised. 1076600.

Preparation of a thin layer. See *silanised silica gel HF₂₅₄ R*.

A fine, white homogeneous powder which, after being shaken with water, floats on the surface because of its water-repellent properties.

Chromatographic separation. Complies with the test prescribed for *silanised silica gel HF₂₅₄ R*.

Silica gel HF₂₅₄. 1076700.

Contains about 1.5 per cent of a fluorescent indicator having an optimal intensity at 254 nm.

A fine, white, homogeneous powder with a particle size of about 15 µm.

pH (2.3.3). Complies with the test prescribed for *silica gel G R*.

Fluorescence. Complies with the test prescribed for *silica gel GF₂₅₄ R*.

Silica gel HF₂₅₄, silanised. 1076800.

Contains about 1.5 per cent of a fluorescent indicator having an optimal intensity at 254 nm.

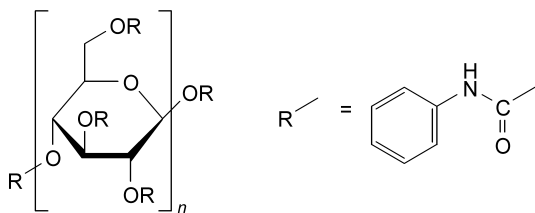
A fine, white, homogeneous powder which, after shaking with water, floats on the surface because of its water-repellent properties.

Preparation of a thin layer. Vigorously shake 30 g for 2 min with 60 ml of a mixture of 1 volume of *methanol R* and 2 volumes of *water R*. Coat carefully cleaned plates with a layer 0.25 mm thick using a spreading device. Allow the coated plates to dry in air and then heat in an oven at 100 °C to 105 °C for 30 min.

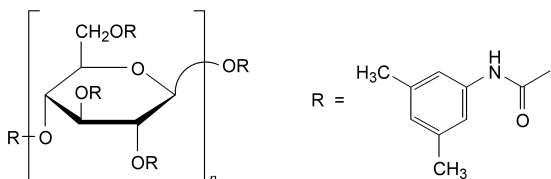
Chromatographic separation. Introduce 0.1 g each of *methyl laurate R*, *methyl myristate R*, *methyl palmitate R* and *methyl stearate R* into a 250 ml conical flask. Add 40 ml of *alcoholic potassium hydroxide solution R* and heat under a reflux condenser on a water-bath for 1 h. Allow to cool, transfer the solution to a separating funnel by means of 100 ml of *water R*, acidify (pH 2 to 3) with *dilute hydrochloric acid R* and shake with three quantities, each of 10 ml of *chloroform R*. Dry the combined chloroform extracts over *anhydrous sodium sulphate R*, filter and evaporate to dryness on a water-bath. Dissolve the residue in 50 ml of *chloroform R*. Examine by thin-layer chromatography (2.2.27), using silanised silica gel HF₂₅₄ as the coating substance. Apply to the plate at each of three separate points 10 µl of the chloroformic solution. Develop over a path of 14 cm with a mixture of 10 volumes of *glacial acetic acid R*, 25 volumes of *water R* and 65 volumes of *dioxan R*. Dry the plate at 120 °C for 30 min. Allow to cool, spray with a 35 g/l solution of *phosphomolybdic acid R* in *2-propanol R* and heat at 150 °C until the spots become visible. Treat the plate with ammonia vapour until the background is white. The chromatograms show four clearly separated, well-defined spots.

Silica gel OC for chiral separations. 1146800.

A very finely divided silica gel for chromatography (5 µm) coated with the following derivative:

**Silica gel OD for chiral separations.** 1110300.

A very finely divided silica gel for chromatography (5 µm) coated with the following derivative:

**Silicotungstic acid.** $\text{H}_4\text{SiW}_{12}\text{O}_{40} \cdot x\text{H}_2\text{O}$. 1078000. [11130-20-4].

White or yellowish-white crystals, deliquescent, very soluble in water and in alcohol.

Storage: in an airtight container.

Silicristin. $\text{C}_{25}\text{H}_{22}\text{O}_{10}$. (M_r 482.4). 1151500. [33889-69-9]. (2*R*,3*R*)-3,5,7-Trihydroxy-2-[(2*R*,3*S*)-7-hydroxy-2-(4-hydroxy-3-methoxyphenyl)-3-hydroxymethyl-2,3-dihydro-1-benzofuran-5-yl]chroman-4-one.

White to yellowish powder, practically insoluble in water, soluble in acetone and in methanol.

Silidianin. $\text{C}_{25}\text{H}_{22}\text{O}_{10}$. (M_r 482.4). 1151600. [29782-68-1]. (3*R*,3*aR*,6*R*,7*aR*,8*R*)-7*a*-Hydroxy-8-(4-hydroxy-3-methoxyphenyl)-4-[(2*R*,3*R*)-3,5,7-trihydroxy-4-oxochroman-2-yl]-2,3,3*a*,7*a*-tetrahydro-3,6-methano-1-benzofuran-7(6*aH*)-one.

White to yellowish powder, practically insoluble in water, soluble in acetone and in methanol.

Silver diethyldithiocarbamate. $\text{C}_5\text{H}_{10}\text{AgNS}_2$. (M_r 256.1). 1110400. [1470-61-7].

A pale-yellow or greyish-yellow powder, practically insoluble in water, soluble in pyridine.

It may be prepared as follows. Dissolve 1.7 g of *silver nitrate R* in 100 ml of *water R*. Separately dissolve 2.3 g of *sodium diethyldithiocarbamate R* in 100 ml of *water R*. Cool both solutions to 10 °C, then mix and while stirring collect the yellow precipitate on a sintered-glass filter and wash with 200 ml of cold *water R*. Dry the precipitate *in vacuo* for 2-3 h.

Silver diethyldithiocarbamate may be used provided it has not changed in colour or developed a strong odour.

Silver manganese paper. 1078200.

Immerse strips of slow filter paper into a solution containing 8.5 g/l of *manganese sulphate R* and 8.5 g/l of *silver nitrate R*. Maintain for a few minutes and allow to dry over *diphosphorus pentoxide R* protected from acid and alkaline vapours.

Silver nitrate. 1078300. [7761-88-8].

See *Silver nitrate* (0009).

Silver nitrate reagent. 1078305.

To a mixture of 3 ml of *concentrated ammonia R* and 40 ml of 1 *M sodium hydroxide*, add 8 ml of a 200 g/l solution of *silver nitrate R*, dropwise, with stirring. Dilute to 200 ml with *water R*.

Silver nitrate solution R1. 1078301.

A 42.5 g/l solution.

Storage: protected from light.

Silver nitrate solution R2. 1078302.

A 17 g/l solution.

Storage: protected from light.

Silver nitrate solution, ammoniacal. 1078303.

Dissolve 2.5 g of *silver nitrate R* in 80 ml of *water R* and add *dilute ammonia R1* dropwise until the precipitate has dissolved. Dilute to 100 ml with *water R*. Prepare immediately before use.

Silver nitrate solution in pyridine. 1078304.

An 85 g/l solution in *pyridine R*.

Storage: protected from light.

Silver oxide. Ag_2O . (M_r 231.7). 1078400. [20667-12-3]. Disilver oxide.

A brownish-black powder, practically insoluble in water and in alcohol, freely soluble in dilute nitric acid and in ammonia.

Storage: protected from light.

Sinensetin. $\text{C}_{20}\text{H}_{20}\text{O}_7$. (M_r 372.4). 1110500. [2306-27-6]. 3',4',5,6,7-Pentamethoxyflavone.

A white, crystalline powder, practically insoluble in water, soluble in alcohol.

mp: about 177 °C.

Absorbance (2.2.25). A solution in *methanol R* shows 3 absorption maxima, at 243 nm, 268 nm and 330 nm.

Assay. Examine by liquid chromatography (2.2.29) as prescribed in the monograph on *Java tea* (1229).

The content is not less than 95 per cent, calculated by the normalisation procedure.

Sitostanol. $\text{C}_{29}\text{H}_{52}\text{O}$. (M_r 416.7). 1140100. [19466-47-8]. Dihydro- β -sitosterol.

Content: minimum 95.0 per cent of $\text{C}_{29}\text{H}_{52}\text{O}$.

 β -Sitosterol. $\text{C}_{29}\text{H}_{50}\text{O}$. (M_r 414.7). 1140200. [83-46-5]. Stigmast-5-en-3 β -ol. 22,23-Dihydrostigmasterol.

A white powder, practically insoluble in water, sparingly soluble in tetrahydrofuran.

Content: minimum 75.0 per cent *m/m* of $\text{C}_{29}\text{H}_{50}\text{O}$, calculated with reference to the dried substance.

Assay. Gas chromatography (2.2.28), as prescribed in the monograph on *Phytosterol* (1911).

Test solution. Dissolve 0.100 g of the substance to be examined in *tetrahydrofuran R* and dilute to 10.0 ml with the same solvent. Introduce 100 µl of this solution into a suitable 3 ml flask and evaporate to dryness under *nitrogen R*. To the residue add 100 µl of a freshly prepared mixture of 50 µl of 1-methylimidazole *R* and 1.0 ml of heptafluoro-*N*-methyl-*N*-(trimethylsilyl)butanamide *R*. Close the flask tightly and heat at 100 °C for 15 min. Allow to cool. Inject 1 µl of the test solution.

Sodium. Na. (A_r 22.99). 1078500. [7440-23-5].

A metal whose freshly cut surface is bright silver-grey. It rapidly tarnishes in contact with air and is oxidised completely to sodium hydroxide and converted to sodium carbonate. It reacts violently with water, yielding hydrogen

and a solution of sodium hydroxide; soluble in anhydrous methanol, yielding hydrogen and a solution of sodium methoxide; practically insoluble in light petroleum.

Storage: under light petroleum or liquid paraffin.

Sodium acetate. 1078600. [6131-90-4].

See *Sodium acetate* (0411).

Sodium acetate, anhydrous. $C_2H_3NaO_2$. (M_r 82.0). 1078700. [127-09-3].

Colourless crystals or granules, very soluble in water, sparingly soluble in alcohol.

Loss on drying (2.2.32). Not more than 2.0 per cent, determined by drying in an oven at 100 °C to 105 °C.

Sodium ascorbate solution. 1078800. [134-03-2].

Dissolve 3.5 g of *ascorbic acid R* in 20 ml of 1 M *sodium hydroxide*. Prepare immediately before use.

Sodium azide. NaN_3 . (M_r 65.0). 1078900. [26628-22-8].

A white, crystalline powder or crystals, freely soluble in water, slightly soluble in alcohol.

Sodium bicarbonate. 1081300. [144-55-8].

See *sodium hydrogen carbonate R*.

Sodium bismuthate. $NaBiO_3$. (M_r 280.0). 1079000. [12232-99-4].

Content: minimum 85.0 per cent of $NaBiO_3$.

A yellow or yellowish-brown powder, slowly decomposing when moist or at a high temperature, practically insoluble in cold water.

Assay. Suspend 0.200 g in 10 ml of a 200 g/l solution of *potassium iodide R* and add 20 ml of *dilute sulphuric acid R*. Using 1 ml of *starch solution R* as indicator, titrate with 0.1 M *sodium thiosulphate* until an orange colour is obtained.

1 ml of 0.1 M *sodium thiosulphate* is equivalent to 14.00 mg of $NaBiO_3$.

Sodium bromide. 1154300. [7647-15-6].

See *Sodium bromide* (0190).

Sodium butanesulphonate. $C_4H_9NaO_3S$. (M_r 160.2). 1115600. [2386-54-1].

A white, crystalline powder, soluble in water.

mp: greater than 300 °C.

Sodium carbonate. 1079200. [6132-02-1].

See *Sodium carbonate decahydrate* (0191).

Sodium carbonate, anhydrous. Na_2CO_3 . (M_r 106.0). 1079300. [497-19-8]. Disodium carbonate.

A white powder, hygroscopic, freely soluble in water.

When heated to about 300 °C it loses not more than 1 per cent of its mass.

Storage: in an airtight container.

Sodium carbonate solution. 1079301.

A 106 g/l solution of *anhydrous sodium carbonate R*.

Sodium carbonate solution R1. 1079302.

A 20 g/l solution of *anhydrous sodium carbonate R* in 0.1 M *sodium hydroxide*.

Sodium carbonate solution R2. 1079303.

A 40 g/l solution of *anhydrous sodium carbonate R* in 0.2 M *sodium hydroxide*.

Sodium carbonate monohydrate. $Na_2CO_3 \cdot H_2O$. 1131700. [5968-11-6].

See *Sodium carbonate monohydrate* (0192).

Sodium cetostearyl sulphate. 1079400.

See *Sodium cetostearyl sulphate* (0847).

Sodium chloride. 1079500. [7647-14-5].

See *Sodium chloride* (0193).

Sodium chloride solution. 1079502.

A 20 per cent *m/m* solution.

Sodium chloride solution, saturated. 1079503.

Mix 1 part of *sodium chloride R* with 2 parts of *water R*, shake from time to time and allow to stand. Before use, decant the solution from any undissolved substance and filter, if necessary.

Sodium citrate. 1079600. [6132-04-3].

See *Sodium citrate* (0412).

Sodium cobaltinitrite. $Na_3[Co(NO_2)_6]$. (M_r 403.9). 1079700. [13600-98-1]. Trisodium hexanitrocobaltate(III).

Orange-yellow powder, freely soluble in water, slightly soluble in alcohol.

Sodium cobaltinitrite solution. 1079701.

A 100 g/l solution. Prepare immediately before use.

Sodium decanesulphonate. $C_{10}H_{21}NaO_3S$. (M_r 244.3). 1079800. [13419-61-9].

Crystalline powder or flakes, white or almost white, freely soluble in water, soluble in methanol.

Sodium decyl sulphate. $C_{10}H_{21}NaO_4S$. (M_r 260.3). 1138600. [142-87-0].

Content: minimum 95.0 per cent of $C_{10}H_{21}NaO_4S$.

White or almost white powder, freely soluble in water.

Sodium deoxycholate. $C_{24}H_{39}NaO_4$. (M_r 414.6). 1131800. [302-95-4]. Sodium 3 α ,12 α -dihydroxy-5 β -cholan-24-oate.

Sodium deoxyribonucleate. (About 85 per cent has a relative molecular mass of 2×10^7 or greater). 1079900. [73049-39-5].

A white, fibrous preparation obtained from calf thymus.

Test for suitability. Dissolve 10 mg in *imidazole buffer solution pH 6.5 R* and dilute to 10.0 ml with the same buffer solution (solution a). Dilute 2.0 ml of solution (a) to 50.0 ml with *imidazole buffer solution pH 6.5 R*. The absorbance (2.2.25) of the solution, measured at 260 nm, is 0.4 to 0.8.

To 0.5 ml of solution (a) add 0.5 ml of *imidazole buffer solution pH 6.5 R* and 3 ml of perchloric acid (25 g/l $HClO_4$). A precipitate is formed. Centrifuge. The absorbance of the supernatant liquid, measured at 260 nm using a mixture of 1 ml of *imidazole buffer solution pH 6.5 R* and 3 ml of perchloric acid (25 g/l $HClO_4$) as compensation liquid, is not greater than 0.3.

In each of two tubes, place 0.5 ml of solution (a) and 0.5 ml of a solution of a reference preparation of streptodornase containing 10 IU/ml in *imidazole buffer solution pH 6.5 R*. To one tube add immediately 3 ml of perchloric acid (25 g/l $HClO_4$). A precipitate is formed. Centrifuge and collect the supernatant liquid (a). Heat the other tube at 37 °C for 15 min and add 3 ml of perchloric acid (25 g/l $HClO_4$). Centrifuge and collect the supernatant liquid (b). The absorbance of supernatant liquid (b), measured at 260 nm with reference to supernatant liquid (a) is not less than 0.15.

Sodium diethyldithiocarbamate. $C_5H_{10}NNaS_2 \cdot 3H_2O$. (M_r 225.3). 1080000. [20624-25-3].

White or colourless crystals, freely soluble in water, soluble in alcohol. The aqueous solution is colourless.

Sodium dihydrogen phosphate. 1080100. [13472-35-0].

See *Sodium dihydrogen phosphate dihydrate* (0194).

Sodium dihydrogen phosphate, anhydrous. NaH_2PO_4 . (M_r 120.0). 1080200. [7558-80-7].

White powder, hygroscopic.

Storage: in an airtight container.

Sodium dihydrogen phosphate monohydrate.

$NaH_2PO_4 \cdot H_2O$. (M_r 138.0). 1080300. [10049-21-5].

White, slightly deliquescent crystals or granules, freely soluble in water, practically insoluble in alcohol.

Storage: in an airtight container.

Sodium dithionite. $Na_2S_2O_4$. (M_r 174.1). 1080400. [7775-14-6].

White or greyish-white, crystalline powder, oxidises in air, very soluble in water, slightly soluble in alcohol.

Storage: in an airtight container.

Sodium dodecyl sulphate. 1080500. [151-21-3].

See *Sodium laurilsulfate* (0098) except for the content which should be not less than 99.0 per cent.

Sodium edetate. 1080600. [6381-92-6].

See *Disodium edetate* (0232).

Sodium fluoresceinate. $C_{20}H_{10}Na_2O_5$. (M_r 376.3). 1080700. [518-47-8].

Schultz No. 880.

Colour Index No. 45350.

Fluorescein sodium. Disodium 2-(3-oxo-6-oxido-3H-xanthen-9-yl)benzoate.

An orange-red powder, freely soluble in water. Aqueous solutions display an intense yellowish-green fluorescence.

Sodium fluoride. 1080800. [7681-49-4].

See *Sodium fluoride* (0514).

Sodium formate. $CHNaO_2$. (M_r 68.0). 1122200. [141-53-7]. Sodium methanoate.

White, crystalline powder or deliquescent granules, soluble in water and in glycerol, slightly soluble in alcohol.

mp: about 253 °C.

Sodium glucuronate. $C_6H_9NaO_7 \cdot H_2O$. (M_r 234.1). 1080900. Sodium D-glucuronate monohydrate.

$[\alpha]_D^{20}$: about + 21.5, determined on a 20 g/l solution.

Sodium glycocholate. $C_{26}H_{42}NNaO_6 \cdot 2H_2O$. (M_r 523.6). 1155500. [207300-80-9]. Sodium [(3,7,12-trihydroxy-5-cholan-24-oyl)amino]acetate dihydrate. *N*-[(3,5,7,12)-3,7,12-Trihydroxy-24-oxocholan-24-yl]glycine monosodium salt dihydrate.

Content: minimum 99 per cent of $C_{26}H_{42}NNaO_6 \cdot 2H_2O$.

Sodium heptanesulphonate. $C_7H_{15}NaO_3S$. (M_r 202.3). 1081000. [22767-50-6].

A white or almost white, crystalline mass, freely soluble in water, soluble in methanol.

Sodium heptanesulphonate monohydrate. $C_7H_{15}NaO_3S \cdot H_2O$. (M_r 220.3). 1081100.

Content: minimum 96 per cent of $C_7H_{15}NaO_3S$, calculated with reference to the anhydrous substance.

A white, crystalline powder, soluble in water, very slightly soluble in ethanol.

Water (2.5.12): maximum 8 per cent, determined on 0.300 g.

Assay. Dissolve 0.150 g in 50 ml of *anhydrous acetic acid R*. Titrate with 0.1 *M perchloric acid*, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 *M perchloric acid* is equivalent to 20.22 mg of $C_7H_{15}NaO_3S$.

Sodium hexanesulphonate. $C_6H_{13}NaO_3S$. (M_r 188.2). 1081200. [2832-45-3].

A white or almost white powder, freely soluble in water.

Sodium hydrogen carbonate. 1081300. [144-55-8].

See *Sodium hydrogen carbonate* (0195).

Sodium hydrogen carbonate solution. 1081301.

A 42 g/l solution.

Sodium hydrogen sulphate. $NaHSO_4$. (M_r 120.1). 1131900. [7681-38-1]. Sodium bisulphate.

Freely soluble in water, very soluble in boiling water. It decomposes in alcohol into sodium sulphate and free sulphuric acid.

mp: about 315 °C.

Sodium hydrogensulphite. $NaHO_3S$. (M_r 104.1). 1115700. [7631-90-5].

A white, crystalline powder, freely soluble in water, sparingly soluble in alcohol.

On exposure to air, some sulphur dioxide is lost and the substance is gradually oxidated to sulphate.

Sodium hydroxide. 1081400. [1310-73-2].

See *Sodium hydroxide* (0677).

Sodium hydroxide solution. 1081401.

Dissolve 20.0 g of *sodium hydroxide R* in *water R* and dilute to 100.0 ml with the same solvent. Verify the concentration by titration with 1 *M hydrochloric acid*, using *methyl orange solution R* as indicator, and adjust if necessary to 200 g/l.

Sodium hydroxide solution, carbonate-free. 1081406.

Dissolve *sodium hydroxide R* in *carbon dioxide-free water R* to give a concentration of 500 g/l and allow to stand. Decant the clear supernatant liquid, taking precautions to avoid the introduction of carbon dioxide.

Sodium hydroxide solution, dilute. 1081402.

Dissolve 8.5 g of *sodium hydroxide R* in *water R* and dilute to 100 ml with the same solvent.

Sodium hydroxide solution, methanolic. 1081403.

Dissolve 40 mg of *sodium hydroxide R* in 50 ml of *water R*. Cool and add 50 ml of *methanol R*.

Sodium hydroxide solution, methanolic R1. 1081405.

Dissolve 200 mg of *sodium hydroxide R* in 50 ml of *water R*. Cool and add 50 ml of *methanol R*.

Sodium hydroxide solution, strong. 1081404.

Dissolve 42 g of *sodium hydroxide R* in *water R* and dilute to 100 ml with the same solvent.

Sodium hypobromite solution. 1081500.

In a bath of iced water mix 20 ml of *strong sodium hydroxide solution R* and 500 ml of *water R*, add 5 ml of *bromine solution R* and stir gently until solution is complete. Prepare immediately before use.

Sodium hypochlorite solution, strong. 1081600.

Content: 25 g/l to 30 g/l of active chlorine.

A yellowish liquid with an alkaline reaction.

Assay. Introduce into a flask, successively, 50 ml of *water R*, 1 g of *potassium iodide R* and 12.5 ml of *dilute acetic acid R*. Dilute 10.0 ml of the substance to be examined to 100.0 ml with *water R*. Introduce 10.0 ml of this solution into the flask and titrate with 0.1 M *sodium thiosulphate*, using 1 ml of *starch solution R* as indicator.

1 ml of 0.1 M *sodium thiosulphate* is equivalent to 3.546 mg of active chlorine.

Storage: protected from light.

Sodium hypophosphite. $\text{NaH}_2\text{PO}_2 \cdot \text{H}_2\text{O}$. (M_r 106.0). 1081700. [10039-56-2]. Sodium phosphinate monohydrate.

A white, crystalline powder or colourless crystals, hygroscopic, freely soluble in water, soluble in alcohol.

Storage: in an airtight container.

Sodium iodide. 1081800. [7681-82-5].

See *Sodium iodide* (0196).

Sodium laurilsulfate. 1081900. [151-21-3].

See *Sodium laurilsulfate* (0098).

Sodium lauryl sulphate. 1081900. [151-21-3].

See *Sodium laurilsulfate R*.

Sodium laurylsulphonate for chromatography.

$\text{C}_{12}\text{H}_{25}\text{NaO}_3\text{S}$. (M_r 272.4). 1132000. [2386-53-0].

White or almost white powder or crystals, freely soluble in water.

Absorbance $A_{1\text{ cm}}^{5\%}$ (2.2.25), determined in *water R*:

about 0.05 at 210 nm,

about 0.03 at 220 nm,

about 0.02 at 230 nm,

about 0.02 at 500 nm.

Sodium metabisulphite. 1082000. [7681-57-4].

See *Sodium metabisulphite* (0849).

Sodium methanesulphonate. $\text{CH}_3\text{SO}_3\text{Na}$. (M_r 118.1). 1082100. [2386-57-4].

A white, crystalline powder, hygroscopic.

Storage: in an airtight container.

Sodium molybdate. $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$. (M_r 242.0). 1082200. [10102-40-6]. Disodium molybdate dihydrate.

A white, crystalline powder or colourless crystals, freely soluble in water.

Sodium naphthoquinonesulphonate. $\text{C}_{10}\text{H}_5\text{NaO}_5\text{S}$. (M_r 260.2). 1082300. [521-24-4]. Sodium 1,2-naphthoquinone-4-sulphonate.

A yellow to orange-yellow, crystalline powder, freely soluble in water, practically insoluble in alcohol.

Sodium nitrate. NaNO_3 . (M_r 85.0). 1082400. [7631-99-4].

White powder or granules or colourless, transparent crystals, deliquescent in moist air, freely soluble in water, slightly soluble in alcohol.

Storage: in an airtight container.

Sodium nitrite. NaNO_2 . (M_r 69.0). 1082500. [7632-00-0].

Content: minimum 97.0 per cent of NaNO_2 .

A white, granular powder or a slightly yellow, crystalline powder, freely soluble in water.

Assay. Dissolve 0.100 g in 50 ml of *water R*. Add 50.0 ml of 0.02 M *potassium permanganate* and 15 ml of *dilute sulphuric acid R*. Add 3 g of *potassium iodide R*. Titrate with 0.1 M *sodium thiosulphate*, using 1.0 ml of *starch solution R* added towards the end of the titration as indicator.

1 ml of 0.02 M *potassium permanganate* is equivalent to 3.450 mg of NaNO_2 .

Sodium nitrite solution. 1082501.

A 100 g/l solution. Prepare immediately before use.

Sodium nitroprusside. $\text{Na}_2[\text{Fe}(\text{CN})_5(\text{NO})] \cdot 2\text{H}_2\text{O}$. (M_r 298.0). 1082600. [13755-38-9]. Sodium pentacyano-nitrosylferrate(III) dihydrate.

Reddish-brown powder or crystals, freely soluble in water, slightly soluble in alcohol.

Sodium octanesulphonate. $\text{C}_8\text{H}_{17}\text{NaO}_3\text{S}$. (M_r 216.3). 1082700. [5324-84-5].

Content: minimum 98.0 per cent of $\text{C}_8\text{H}_{17}\text{NaO}_3\text{S}$.

White or almost white, crystalline powder or flakes, freely soluble in water, soluble in methanol.

Absorbance. The absorbance (2.2.25) of a 54 g/l solution measured at 200 nm is not greater than 0.10 and that measured at 250 nm is not greater than 0.01.

Sodium octyl sulphate. $\text{C}_8\text{H}_{17}\text{NaO}_4\text{S}$. (M_r 232.3). 1082800. [142-31-4].

White or almost white, crystalline powder or flakes, freely soluble in water, soluble in methanol.

Sodium oxalate. $\text{C}_2\text{Na}_2\text{O}_4$. (M_r 134.0). 1082900. [62-76-0].

A white, crystalline powder, soluble in water, practically insoluble in alcohol.

Sodium pentanesulphonate. $\text{C}_5\text{H}_{11}\text{NaO}_3\text{S}$. (M_r 174.2). 1083000. [22767-49-3].

A white, crystalline solid, soluble in water.

Sodium pentanesulphonate monohydrate. $\text{C}_5\text{H}_{11}\text{NaO}_3\text{S} \cdot \text{H}_2\text{O}$. (M_r 192.2). 1132100.

A white crystalline solid, soluble in water.

Sodium perchlorate. $\text{NaClO}_4 \cdot \text{H}_2\text{O}$. (M_r 140.5). 1083100. [7791-07-3].

Content: minimum 99.0 per cent of $\text{NaClO}_4 \cdot \text{H}_2\text{O}$.

White, deliquescent crystals, very soluble in water.

Storage: in a well-closed container.

Sodium periodate. NaIO_4 . (M_r 213.9). 1083200. [7790-28-5]. Sodium metaperiodate.

Content: minimum 99.0 per cent of NaIO_4 .

A white, crystalline powder or white crystals, soluble in water and in mineral acids.

Sodium periodate solution. 1083201.

Dissolve 1.07 g of *sodium periodate R* in *water R*, add 5 ml of *dilute sulphuric acid R* and dilute to 100.0 ml with *water R*. Use a freshly prepared solution.

Sodium phosphite pentahydrate. $\text{Na}_2\text{HPO}_3 \cdot 5\text{H}_2\text{O}$. (M_r 216.0). 1132200. [13517-23-2].

A white, crystalline powder, hygroscopic, freely soluble in water.

Storage: in an airtight container.

Sodium picrate solution, alkaline. 1083300.

Mix 20 ml of *picric acid solution R* and 10 ml of a 50 g/l solution of *sodium hydroxide R* and dilute to 100 ml with *water R*.

Storage: use within 2 days.

Sodium potassium tartrate. $C_4H_4KNaO_6 \cdot 4H_2O$. (M_r 282.2). 1083500. [6381-59-5].

Colourless, prismatic crystals, very soluble in water.

Sodium pyrophosphate. $Na_4P_2O_7 \cdot 10H_2O$. (M_r 446.1). 1083600. [13472-36-1]. Tetrasodium diphosphate decahydrate.

Colourless, slightly efflorescent crystals, freely soluble in water.

Sodium rhodizonate. $C_6Na_2O_6$. (M_r 214.0). 1122300. [523-21-7]. [(3,4,5,6-Tetraoxocyclohex-1-en-1,2-ylene)dioxy]disodium.

Violet crystals, soluble in water with an orange-yellow colour.

Solutions are unstable and must be prepared on the day of use.

Sodium salicylate. 1083700. [54-21-7].

See *Sodium salicylate* (0413).

Sodium sulphate, anhydrous. 1083800. [7757-82-6].

Ignite at 600 °C to 700 °C anhydrous sodium sulphate complying with the requirements prescribed in the monograph on *Anhydrous sodium sulphate* (0099).

Loss on drying (2.2.32): maximum 0.5 per cent, determined by drying in an oven at 130 °C.

Sodium sulphate decahydrate. $Na_2SO_4 \cdot 10H_2O$. (M_r 322.2). 1132300. [7727-73-3].

See *Sodium sulphate decahydrate* (0100).

Sodium sulphide. $Na_2S \cdot 9H_2O$. (M_r 240.2). 1083900. [1313-84-4]. Disodium sulphide nonahydrate.

Colourless, rapidly yellowing crystals, deliquescent, very soluble in water.

Storage: in an airtight container.

Sodium sulphide solution. 1083901.

Dissolve 12 g of *sodium sulphide R* with heating in 45 ml of a mixture of 10 volumes of *water R* and 29 volumes of *glycerol* (85 per cent) *R*, allow to cool and dilute to 100 ml with the same mixture of solvents.

The solution should be colourless.

Sodium sulphide solution R1. 1083902.

Prepare by one of the following methods.

– Dissolve 5 g of *sodium sulphide R* in a mixture of 10 ml of *water R* and 30 ml of *glycerol R*.

– Dissolve 5 g of *sodium hydroxide R* in a mixture of 30 ml of *water R* and 90 ml of *glycerol R*. Divide the solution into 2 equal portions. Saturate 1 portion with *hydrogen sulphide R*, with cooling. Mix the 2 portions.

Storage: in a well-filled container, protected from light; use within 3 months.

Sodium sulphite. 1084000. [10102-15-5].

See *Sodium sulphite heptahydrate* (0776).

Sodium sulphite, anhydrous. 1084100. [7757-83-7].

See *Anhydrous sodium sulphite* (0775).

Sodium tartrate. $C_4H_4Na_2O_6 \cdot 2H_2O$. (M_r 230.1). 1084200. [6106-24-7]. Disodium (2*R*,3*R*)-2,3-dihydroxybutanedioate dihydrate.

White crystals or granules, very soluble in water, practically insoluble in alcohol.

Sodium taurodeoxycholate. $C_{26}H_{44}NNaO_6S \cdot H_2O$.

(M_r 539.7). 1155600. [110026-03-4]. Sodium 2-[(3,12-dihydroxy-5-cholan-24-oyl)amino]ethanesulphonate monohydrate. 2-[[[(3,5,12)-3,12-Dihydroxy-24-oxocholan-24-yl]amino]ethanesulphonic acid monosodium salt monohydrate.

Content: minimum 94 per cent of $C_{26}H_{44}NNaO_6S \cdot H_2O$.

Sodium tetradeuteriodimethylsilapentanoate.

$C_6H_9^2H_3NaO_2Si$. (M_r 172.3). 1084300. TSP. Sodium (2,2,3,3-tetradeuterio)-4,4-dimethyl-4-silapentanoate.

The degree of deuteration is not less than 99 per cent.

A white, crystalline powder, freely soluble in water, in ethanol and in methanol.

mp: about 300 °C.

Water and deuterium oxide: maximum 0.5 per cent.

Sodium tetrahydroborate. $NaBH_4$. (M_r 37.8). 1146900. [16940-66-2]. Sodium borohydride.

Colourless, hygroscopic crystals, freely soluble in water, soluble in anhydrous ethanol, decomposing at higher temperature or in the presence of acids or certain metal salts forming borax and hydrogen.

Storage: in an airtight container.

Sodium tetrahydroborate reducing solution. 1146901.

Introduce about 100 ml of *water R* into a 500 ml volumetric flask containing a stirring bar. Add 5.0 g of *sodium hydroxide R* in pellets and 2.5 g of *sodium tetrahydroborate R*. Stir until complete dissolution, dilute to 500.0 ml with *water R* and mix. Prepare immediately before use.

Sodium tetraphenylborate. $NaB(C_6H_5)_4$. (M_r 342.2). 1084400. [143-66-8].

A white or slightly yellowish, bulky powder, freely soluble in water and in acetone.

Sodium tetraphenylborate solution. 1084401.

Filter before use if necessary.

A 10 g/l solution.

Storage: use within 1 week.

Sodium thioglycollate. $C_2H_3NaO_2S$. (M_r 114.1). 1084500. [367-51-1]. Sodium mercaptoacetate.

White, granular powder or crystals, hygroscopic, freely soluble in water and in methanol, slightly soluble in alcohol.

Storage: in an airtight container.

Sodium thiosulphate. 1084600. [10102-17-7].

See *Sodium thiosulphate* (0414).

Sodium tungstate. $Na_2WO_4 \cdot 2H_2O$. (M_r 329.9). 1084700. [10213-10-2]. Disodium tungstate dihydrate.

A white, crystalline powder or colourless crystals, freely soluble in water forming a clear solution, practically insoluble in alcohol.

Sorbitol. 1084800. [50-70-4].

See *Sorbitol* (0435).

Squalane. $C_{30}H_{62}$. (M_r 422.8). 1084900. [111-01-3].
2,6,10,15,19,23-Hexamethyltetracosane.

A colourless, oily liquid, freely soluble in fatty oils, slightly soluble in acetone, in alcohol, in glacial acetic acid and in methanol.

d_{20}^{20} : 0.811 to 0.813.

n_D^{20} : 1.451 to 1.453.

Stannous chloride. $SnCl_2 \cdot 2H_2O$. (M_r 225.6). 1085000.
[10025-69-1]. Tin dichloride dihydrate.

Content: minimum 97.0 per cent of $SnCl_2 \cdot 2H_2O$.

Colourless crystals, very soluble in water, freely soluble in alcohol, in glacial acetic acid and in dilute and concentrated hydrochloric acid.

Assay. Dissolve 0.500 g in 15 ml of *hydrochloric acid R* in a ground-glass-stoppered flask. Add 10 ml of *water R* and 5 ml of *chloroform R*. Titrate rapidly with 0.05 M *potassium iodate* until the chloroform layer is colourless.

1 ml of 0.05 M *potassium iodate* is equivalent to 22.56 mg of $SnCl_2 \cdot 2H_2O$.

Stannous chloride solution. 1085001.

Heat 20 g of *tin R* with 85 ml of *hydrochloric acid R* until no more hydrogen is released. Allow to cool.

Storage: over an excess of *tin R*, protected from air.

Stannous chloride solution R1. 1085002.

Immediately before use, dilute 1 volume of *stannous chloride solution R* with 10 volumes of *dilute hydrochloric acid R*.

Stannous chloride solution R2. 1085003.

To 8 g of *stannous chloride R* add 100 ml of a 20 per cent V/V solution of *hydrochloric acid R*. Shake until dissolved, heating, if necessary, on a water-bath at 50 °C. Pass a current of *nitrogen R* for 15 min. Prepare immediately before use.

Stanolone. $C_{19}H_{30}O_2$. (M_r 290.4). 1154400. [521-18-6].
17 β -Hydroxy-5 α -androstan-3-one.

White or almost white powder.

mp: about 180 °C.

Standard solution for the micro determination of water. 1147300.

Commercially available standard solution for the coulometric titration of water, containing a certified content of water in a suitable solvent.

Staphylococcus aureus strain V8 protease. Type XVII-B. 1115800. [66676-43-5].

Microbial extracellular proteolytic enzyme. A lyophilised powder containing 500 units to 1000 units per milligram of solid.

Starch, soluble. 1085100. [9005-84-9].

A white powder.

Prepare a 20 g/l solution in hot *water R*. The solution is at most slightly opalescent and remains fluid on cooling.

Starch iodate paper. 1085101.

Immerse strips of filter paper in 100 ml of *iodide-free starch solution R* containing 0.1 g of *potassium iodate R*. Drain and allow to dry protected from light.

Starch iodide paper. 1085106.

Immerse strips of filter paper in 100 ml of *starch solution R* containing 0.5 g of *potassium iodide R*. Drain and allow to dry protected from light.

Test for sensitivity. Mix 0.05 ml of 0.1 M *sodium nitrite* with 4 ml of *hydrochloric acid R* and dilute to 100 ml with *water R*. Apply one drop of the solution to starch iodide paper; a blue spot appears.

Starch solution. 1085103.

Triturate 1.0 g of *soluble starch R* with 5 ml of *water R* and whilst stirring pour the mixture into 100 ml of boiling *water R* containing 10 mg of *mercuric iodide R*.

Carry out the test for sensitivity each time the reagent is used.

Test for sensitivity. To a mixture of 1 ml of the starch solution and 20 ml of *water R*, add about 50 mg of *potassium iodide R* and 0.05 ml of *iodine solution R1*. The solution is blue.

Starch solution, iodide-free. 1085104.

Prepare the solution as prescribed for *starch solution R* omitting the mercuric iodide. Prepare immediately before use.

Starch solution R1. 1085105.

Mix 1 g of *soluble starch R* and a small amount of cold *water R*. Add this mixture, while stirring, to 200 ml of boiling *water R*. Add 250 mg of *salicylic acid R* and boil for 3 min. Immediately remove from the heat and cool.

Storage: long storage is required, the solution shall be stored at 4 °C to 10 °C. A fresh starch solution shall be prepared when the end-point of the titration from blue to colourless fails to be sharp. If stored under refrigeration, the starch solution is stable for about 2 to 3 weeks.

Test for sensitivity. A mixture of 2 ml of *starch solution R1*, 20 ml of *water R*, about 50 mg of *potassium iodide R* and 0.05 ml of *iodine solution R1* is blue.

Starch solution R2. 1085107.

Triturate 1.0 g of *soluble starch R* with 5 ml of *water R* and whilst stirring pour the mixture into 100 ml of boiling *water R*. Use a freshly prepared solution.

Test for sensitivity. To a mixture of 1 ml of the starch solution and 20 ml of *water R*, add about 50 mg of *potassium iodide R* and 0.05 ml of *iodine solution R1*. The solution is blue.

Stearic acid. $C_{18}H_{36}O_2$. (M_r 284.5). 1085200. [57-11-4].
Octadecanoic acid.

White powder or flakes, greasy to the touch, practically insoluble in water, soluble in hot alcohol.

mp: about 70 °C.

Stearic acid used in the assay of total fatty acids in Saw palmetto fruit (1848) complies with the following additional requirement.

Assay. Examine by gas chromatography (2.2.28) as prescribed in the monograph on *Saw palmetto fruit (1848)*. The content of stearic acid is not less than 98 per cent, calculated by the normalisation procedure.

Stearyl alcohol. $C_{18}H_{38}O$. (M_r 270.5). 1156400. [112-92-5].
1-Octadecanol.

mp: about 60 °C.

Content: minimum 95 per cent of $C_{18}H_{38}O$.

Stigmasterol. $C_{29}H_{48}O$. (M_r 412.7). 1141400. [83-48-7]. (22*E*)-Stigmasta-5,22-dien-3 β -ol.
(22*E*)-24-Ethylcholesta-5,22-dien-3 β -ol.

White powder, insoluble in water.

mp: about 170 °C.

$[\alpha]_D^{22}$: about – 51 ($c = 2$ in chloroform).

Streptomycin sulphate. 1085300. [3810-74-0].

See *Streptomycin sulphate* (0053).

Strongly acidic ion-exchange resin. 1085400.

See *ion-exchange resin, strongly acidic R*.

Strontium carbonate. SrCO_3 . (M_r 147.6). 1122700. [1633-05-2].

A white, crystalline powder.

Content: minimum 99.5 per cent of SrCO_3 .

Styrene. C_8H_8 . (M_r 104.2). 1151700. [100-42-5].
Ethenylbenzene.

bp: about 145 °C.

Colourless, oily liquid, very slightly soluble in water.

Styrene-divinylbenzene copolymer. 1085500.

Porous, rigid, cross-linked polymer beads. Several grades are available with different sizes of beads. The size range of the beads is specified after the name of the reagent in the tests where it is used.

Succinic acid. $\text{C}_4\text{H}_6\text{O}_4$. (M_r 118.1). 1085600. [110-15-6].
Butanedioic acid.

A white, crystalline powder or colourless crystals, soluble in water and in alcohol.

mp: 184 °C to 187 °C.

Sucrose. 1085700. [57-50-1].

See *Sucrose* (0204).

When sucrose is used for controlling the polarimeter, it must be kept dry in a sealed ampoule.

Sudan orange. $\text{C}_{16}\text{H}_{12}\text{N}_2\text{O}$. (M_r 248.3). 1110700. [842-07-9].
Colour Index No. 12055.

1-(Phenylazo)naphthalen-2-ol. Sudan I.

An orange-red powder, practically insoluble in water, soluble in methylene chloride.

mp: about 131 °C.

Sudan red G. $\text{C}_{17}\text{H}_{14}\text{N}_2\text{O}_2$. (M_r 278.3). 1085800.

Schultz No. 149.

Colour Index No. 12150.

Solvent Red 1. 1-[(2-Methoxyphenyl)azo]naphthalen-2-ol.

A reddish-brown powder, practically insoluble in water.

Chromatography. Examine by thin-layer chromatography (2.2.27) using *silica gel G R* as the coating substance. Apply 10 µl of a 0.1 g/l solution in *methylene chloride R* and develop over a path of 10 cm with the same solvent. The chromatogram shows only one principal spot.

Sulfanilamide. $\text{C}_6\text{H}_8\text{N}_2\text{O}_2\text{S}$. (M_r 172.2). 1086100. [63-74-1].
4-Aminobenzenesulphonamide.

A white powder, slightly soluble in water, freely soluble in boiling water, in acetone, in dilute acids and in solutions of the alkali hydroxides, sparingly soluble in alcohol and in light petroleum.

mp: about 165 °C.

Sulphamic acid. $\text{H}_3\text{NO}_3\text{S}$. (M_r 97.1). 1085900. [5329-14-6].

White crystalline powder or crystals, freely soluble in water, sparingly soluble in acetone, in alcohol and in methanol.

mp: about 205 °C, with decomposition.

Sulphan blue. $\text{C}_{27}\text{H}_{31}\text{N}_2\text{NaO}_6\text{S}_2$. (M_r 566.6). 1086000. [129-17-9].

Schultz No. 769.

Colour Index No. 42045.

Acid Blue 1. Patent Blue VF. Disulphine blue.

Blue VS. Sodium [[[(4-diethylamino)phenyl](2,4-disulphonatophenyl)methylene]cyclohexa-2,5-dien-1-ylidene]diethylammonium.

A violet powder, soluble in water. Dilute solutions are blue and turn yellow on the addition of concentrated hydrochloric acid.

Sulphanilic acid. $\text{C}_6\text{H}_7\text{NO}_3\text{S}$. (M_r 173.2). 1086200. [121-57-3]. 4-Aminobenzenesulphonic acid.

Colourless crystals, sparingly soluble in water, practically insoluble in alcohol.

Sulphanilic acid solution. 1086203.

Dissolve 0.33 g of *sulphanilic acid R* in 75 ml of *water R* heating gently if necessary and dilute to 100 ml with *glacial acetic acid R*.

Sulphanilic acid solution R1. 1086201.

Dissolve 0.5 g of *sulphanilic acid R* in a mixture of 75 ml of *dilute acetic acid R* and 75 ml of *water R*.

Sulphanilic acid solution, diazotised. 1086202.

Dissolve, with warming, 0.9 g of *sulphanilic acid R* in 9 ml of *hydrochloric acid R*, and dilute to 100 ml with *water R*. Cool 10 ml of this solution in iced water and add 10 ml of an ice-cold 4.5 per cent *m/V* solution of *sodium nitrite R*. Allow to stand at 0 °C for 15 min (if stored at this temperature, the solution is stable for 3 days) and immediately before use add 20 ml of a 10 per cent *m/V* solution of *sodium carbonate R*.

Sulfathiazole. $\text{C}_9\text{H}_9\text{N}_3\text{O}_2\text{S}_2$. (M_r 255.3). 1086300. [72-14-0].
4-Amino-*N*-(thiazol-2-yl)benzenesulphonamide.

White or yellowish-white powder or crystals, very slightly soluble in water, soluble in acetone, slightly soluble in alcohol. It dissolves in dilute mineral acids and in solutions of alkali hydroxides and carbonates.

mp: about 200 °C.

Sulphomolybdic reagent R2. 1086400.

Dissolve about 50 mg of *ammonium molybdate R* in 10 ml of *sulphuric acid R*.

Sulphomolybdic reagent R3. 1086500.

Dissolve with heating 2.5 g of *ammonium molybdate R* in 20 ml of *water R*. Dilute 28 ml of *sulphuric acid R* in 50 ml of *water R*, then cool. Mix the two solutions and dilute to 100 ml with *water R*.

Storage: in a polyethylene container.

Sulphosalicylic acid. $\text{C}_7\text{H}_6\text{O}_6\text{S}_2\text{H}_2\text{O}$. (M_r 254.2). 1086600. [5965-83-3]. 2-Hydroxy-5-sulphobenzoic acid.

A white, crystalline powder or crystals, very soluble in water and in alcohol.

mp: about 109 °C.

Sulphur. 1110800. [7704-34-9].

See *Sulphur for external use* (0953).

Sulphur dioxide. SO_2 . (M_r 64.1). 1086700. [7446-09-5].
Sulphurous anhydride.

A colourless gas. When compressed it is a colourless liquid.

Sulphur dioxide R1. SO_2 . (M_r 64.1). 1110900.

Content: minimum 99.9 per cent *V/V* of SO_2 .

Sulphuric acid. H_2SO_4 . (M_r 98.1). 1086800. [7664-93-9].

Content: 95.0 per cent *m/m* to 97.0 per cent *m/m* of H_2SO_4 .

A colourless, caustic liquid with an oily consistency, highly hygroscopic, miscible with water and with alcohol producing intense heat.

d_{20}^{20} : 1.834 to 1.837.

A 10 g/l solution is strongly acid and gives the reactions of sulphates (2.3.1).

Appearance. It is clear (2.2.1) and colourless (2.2.2, Method II).

Oxidisable substances. Pour 20 g cautiously, with cooling, into 40 ml of *water R*. Add 0.5 ml of 0.002 M *potassium permanganate*. The violet colour persists for at least 5 min.

Chlorides. Pour 10 g, carefully and while cooling, into 10 ml of *water R* and after cooling dilute to 20 ml with the same solvent. Add 0.5 ml of *silver nitrate solution R2*. Allow to stand for 2 min protected from bright light. The solution is not more opalescent than a standard prepared at the same time using a mixture of 1 ml of *chloride standard solution (5 ppm Cl) R*, 19 ml of *water R* and 0.5 ml of *silver nitrate solution R2* (0.5 ppm).

Nitrates. Pour 50 g or 27.2 ml, carefully and while cooling, into 15 ml of *water R*. Add 0.2 ml of a freshly prepared 50 g/l solution of *brucine R* in *glacial acetic acid R*. After 5 min any colour is less intense than that of a reference mixture prepared in the same manner and containing 12.5 ml of *water R*, 50 g of *nitrogen-free sulphuric acid R*, 2.5 ml of *nitrate standard solution (10 ppm NO₃) R* and 0.2 ml of a 50 g/l solution of *brucine R* in *glacial acetic acid R* (0.5 ppm).

Ammonium. Pour 2.5 g, carefully and while cooling, into *water R* and dilute to 20 ml with the same solvent. Cool, and add dropwise 10 ml of a 200 g/l solution of *sodium hydroxide R*, followed by 1 ml of *alkaline potassium tetraiodomercurate solution R*. The colour of the solution is less intense than that of a mixture of 5 ml of *ammonium standard solution (1 ppm NH₄) R*, 15 ml of *water R*, 10 ml of a 200 g/l solution of *sodium hydroxide R* and 1 ml of *alkaline potassium tetraiodomercurate solution R* (2 ppm).

Arsenic (2.4.2). To 50 g add 3 ml of *nitric acid R* and evaporate carefully until the volume is reduced to about 10 ml. Cool, add to the residue 20 ml of *water R* and concentrate to 5 ml. The solution complies with limit test A for arsenic (0.02 ppm). Prepare the standard using 1.0 ml of *arsenic standard solution (1 ppm As) R*.

Heavy metals (2.4.8). Dilute 10 ml of the solution obtained in the test for iron to 20 ml with *water R*. 12 ml of the solution complies with limit test A for heavy metals (2 ppm). Prepare the standard using *lead standard solution (2 ppm Pb) R*.

Iron (2.4.9). Dissolve the residue on ignition with slight heating in 1 ml of *dilute hydrochloric acid R* and dilute to 50.0 ml with *water R*. 5 ml of the solution diluted to 10 ml with *water R* complies with the limit test for iron (1 ppm).

Residue on ignition: maximum 0.001 per cent, determined on 100 g by evaporating cautiously in a small crucible over a naked flame and igniting the residue to redness.

Assay. Weigh accurately a ground-glass-stoppered flask containing 30 ml of *water R*, introduce 0.8 ml of the sulphuric acid, cool and weigh again. Titrate with 1 M *sodium hydroxide*, using 0.1 ml of *methyl red solution R* as indicator.

1 ml of 1 M *sodium hydroxide* is equivalent to 49.04 mg of H₂SO₄.

Storage: in a ground-glass-stoppered container made of glass or other inert material.

Sulphuric acid, alcoholic, 2.5 M. 1086801.

Carefully and with constant cooling, stir 14 ml of *sulphuric acid R* into 60 ml of *ethanol R*. Allow to cool and dilute to 100 ml with *ethanol R*. Prepare immediately before use.

Sulphuric acid, alcoholic, 0.25 M. 1086802.

Dilute 10 ml of 2.5 M *alcoholic sulphuric acid R* to 100 ml with *ethanol R*. Prepare immediately before use.

Sulphuric acid, alcoholic solution of. 1086803.

Carefully and with constant cooling, stir 20 ml of *sulphuric acid R* into 60 ml of *alcohol R*. Allow to cool and dilute to 100 ml with *alcohol R*. Prepare immediately before use.

Sulphuric acid, dilute. 1086804.

Contains 98 g/l of H₂SO₄.

Add 5.5 ml of *sulphuric acid R* to 60 ml of *water R*, allow to cool and dilute to 100 ml with the same solvent.

Assay. Into a ground-glass-stoppered flask containing 30 ml of *water R*, introduce 10.0 ml of the dilute sulphuric acid. Titrate with 1 M *sodium hydroxide*, using 0.1 ml of *methyl red solution R* as indicator.

1 ml of 1 M *sodium hydroxide* is equivalent to 49.04 mg of H₂SO₄.

Sulphuric acid-formaldehyde reagent. 1086805.

Mix 2 ml of *formaldehyde solution R* with 100 ml of *sulphuric acid R*.

Sulphuric acid, heavy metal-free. 1086807.

Complies with the requirements prescribed for *sulphuric acid R* and with the following maximum contents of heavy metals:

As: 0.005 ppm;

Cd: 0.002 ppm;

Cu: 0.001 ppm;

Fe: 0.05 ppm;

Hg: 0.005 ppm;

Ni: 0.002 ppm;

Pb: 0.001 ppm;

Zn: 0.005 ppm.

Sulphuric acid, nitrogen-free. 1086806.

Complies with the requirements prescribed for *sulphuric acid R* and with the following additional test.

Nitrates. To 5 ml of *water R* add carefully 45 ml of the sulphuric acid, allow to cool to 40 °C and add 8 mg of *diphenylbenzidine R*. The solution is faint pink or very pale blue.

Sulphuric acid, nitrogen-free R1. 1086808.

Nitrogen-free sulphuric acid R containing 95.0 per cent *m/m* to 95.5 per cent *m/m* of H₂SO₄.

Sunflower oil. 1086900.

See *Sunflower oil, refined (1371)*.

Tagatose. C₆H₁₂O₆. (*M_r* 180.16). 1111000. [87-81-0]. D-lyxo-Hexulose.

White powder.

$[\alpha]_D^{20}$: -2.3 (21.9 g/l solution in *water R*).

mp: 134 °C to 135 °C.

Talc. 1087000. [14807-96-6].

See *Talc (0438)*.

Tannic acid. 1087100. [1401-55-4].

Yellowish to light-brown, glistening scales or amorphous powder, very soluble in water, freely soluble in alcohol, soluble in acetone.

Storage: protected from light.

Tartaric acid. 1087200. [87-69-4].

See *Tartaric acid* (0460).

Taxifolin. C₁₅H₁₂O₇. (*M_r* 304.3). 1151800. [480-18-2]. (2*R*,3*R*)-2-(3,4-Dihydroxyphenyl)-3,5,7-trihydroxy-2,3-dihydro-4*H*-1-benzopyran-4-one.

White or almost white powder, slightly soluble in ethanol.

A solution in *ethanol R* shows an absorption maximum (2.2.25) at 290 nm.

Tecnazene. C₆HCl₄NO₂. (*M_r* 260.9). 1132400. [117-18-0].

bp: about 304 °C.

mp: 99 °C to 100 °C.

A suitable certified reference solution (10 ng/μl in cyclohexane) may be used.

α-Terpinene. C₁₀H₁₆. (*M_r* 136.2). 1140300. [99-86-5]. 1-Isopropyl-4-methylcyclohexa-1,3-diene.

Clear, almost colourless liquid.

*d*₄²⁰: about 0.837.

*n*_D²⁰: about 1.478.

bp: about 174 °C.

α-Terpinene used in gas chromatography complies with the following additional test.

Assay. Examine by gas chromatography (2.2.28) as prescribed in the monograph on *Tea tree oil* (1837).

The content is not less than 95 per cent, calculated by the normalisation procedure.

γ-Terpinene. C₁₀H₁₆. (*M_r* 136.2). 1115900. [99-85-4]. 1-Isopropyl-4-methylcyclohexa-1,4-diene.

An oily liquid.

*d*₄¹⁵: about 0.850.

*n*_D¹⁵: 1.474 to 1.475.

bp: 183 °C to 186 °C.

γ-Terpinene used in gas chromatography complies with the following additional test.

Assay. Examine by gas chromatography (2.2.28) as prescribed in the monograph *Peppermint oil* (0405).

Test solution. The substance to be examined.

The area of the principal peak is not less than 93.0 per cent of the area of all the peaks in the chromatogram obtained.

Terpinen-4-ol. C₁₀H₁₈O. (*M_r* 154.2). 1116000. [562-74-3]. 4-Methyl-1-(1-methylethyl)cyclohex-3-en-1-ol. *p*-Menth-1-en-4-ol.

An oily, colourless liquid.

*d*₂₀²⁰: about 0.934.

*n*_D²⁰: about 1.477.

bp: 209 °C to 212 °C.

Terpinen-4-ol used in gas chromatography complies with the following additional test.

Assay. Examine by gas chromatography (2.2.28) as prescribed in the monograph on *Lavender oil* (1338).

Test solution. The substance to be examined.

The area of the principal peak is not less than 98.0 per cent of the area of all the peaks in the chromatogram obtained.

α-Terpineol. C₁₀H₁₈O. (*M_r* 154.2). 1087300. [98-55-5]. (2*S*)-2-(4-Methylcyclohex-3-enyl)-2-propanol.

Colourless crystals, practically insoluble in water, soluble in alcohol.

*d*₂₀²⁰: about 0.935.

*n*_D²⁰: about 1.483.

[α]_D²⁰: about 92.5.

mp: about 35 °C.

It may contain 1 to 3 per cent of β-terpineol.

α-Terpineol used in gas chromatography complies with the following test.

Assay. Examine by gas chromatography (2.2.28) under the conditions described in the monograph on *Anise oil* (0804).

Test solution. A 100 g/l solution in *hexane R*.

The area of the principal peak is not less than 97.0 per cent of the total area of the peaks. Disregard the peak due to hexane.

Terpinolene. C₁₀H₁₆. (*M_r* 136.2). 1140400. [586-62-9]. *p*-Mentha-1,4(8)-diene. 4-Isopropylidene-1-methylcyclohexene.

Clear, almost colourless liquid.

*d*₄²⁰: about 0.863.

*n*_D²⁰: about 1.488.

bp: about 184 °C.

Terpinolene used in gas chromatography complies with the following additional test.

Assay. Examine by gas chromatography (2.2.28) as prescribed in the monograph on *Tea tree oil* (1837).

The content is not less than 90 per cent, calculated by the normalisation procedure.

Testosterone. 1116100. [58-22-0].

See *Testosterone* (1373).

Testosterone propionate. 1087400. [57-85-2].

See *Testosterone propionate* (0297).

Tetrabutylammonium bromide. C₁₆H₃₆BrN. (*M_r* 322.4). 1087500. [1643-19-2].

White or almost white crystals.

mp: 102 °C to 104 °C.

Tetrabutylammonium dihydrogen phosphate. C₁₆H₃₈NO₄P. (*M_r* 339.5). 1087600. [5574-97-0].

White powder, hygroscopic.

pH (2.2.3): about 7.5 for a 170 g/l solution.

Absorbance (2.2.25): about 0.10 determined at 210 nm using a 170 g/l solution.

Storage: in an airtight container.

Tetrabutylammonium hydrogen sulphate. C₁₆H₃₇NO₄S. (*M_r* 339.5). 1087700. [32503-27-8].

A crystalline powder or colourless crystals, freely soluble in water and in methanol.

mp: 169 °C to 173 °C.

Absorbance (2.2.25). The absorbance of a 50 g/l solution, at wavelengths from 240 nm to 300 nm, is not greater than 0.05.

Tetrabutylammonium hydrogen sulphate R1. 1087701.

Complies with the requirements prescribed for *tetrabutylammonium hydrogen sulphate R* and with the following additional requirement:

Absorbance (2.2.25). The absorbance of a 50 g/l solution, at wavelengths from 215 nm to 300 nm, is not greater than 0.02.

Tetrabutylammonium hydroxide. $C_{16}H_{37}NO \cdot 30H_2O$. (M_r 800). 1087800. [2052-49-5].

Content: minimum 98.0 per cent of $C_{16}H_{37}NO \cdot 30H_2O$.

White or almost white crystals, soluble in water.

Assay. Dissolve 1.000 g in 100 ml of *water R*. Titrate immediately with 0.1 M *hydrochloric acid* determining the end-point potentiometrically (2.2.20). Carry out a blank titration.

1 ml of 0.1 M *hydrochloric acid* is equivalent to 80.0 mg $C_{16}H_{37}NO \cdot 30H_2O$.

Tetrabutylammonium hydroxide solution (104 g/l). 1087801. [2052-49-5].

A solution containing 104 g/l of $C_{16}H_{37}NO$ (M_r 259.5), prepared by dilution of a suitable reagent grade.

Tetrabutylammonium hydroxide solution (400 g/l). 1087802. [2052-49-5].

A solution containing 400 g/l of $C_{16}H_{37}NO$ (M_r 259.5) of a suitable grade.

Tetrabutylammonium iodide. $C_{16}H_{36}IN$. (M_r 369.4). 1087900. [311-28-4].

Content: minimum 98.0 per cent of $C_{16}H_{36}IN$.

White or slightly coloured, crystalline powder or crystals, soluble in alcohol.

Sulphated ash (2.4.14): maximum 0.02 per cent.

Assay. Dissolve 1.200 g in 30 ml of *water R*. Add 50.0 ml of 0.1 M *silver nitrate* and 5 ml of *dilute nitric acid R*. Titrate the excess of silver nitrate with 0.1 M *ammonium thiocyanate*, using 2 ml of *ferric ammonium sulphate solution R2* as indicator.

1 ml of 0.1 M *silver nitrate* is equivalent to 36.94 mg of $C_{16}H_{36}IN$.

Tetrachloroethane. $C_2H_2Cl_4$. (M_r 167.9). 1088000. [79-34-5]. 1,1,2,2-Tetrachloroethane.

A clear, colourless liquid, slightly soluble in water, miscible with alcohol.

d_{20}^{20} : about 1.59.

n_D^{20} : about 1.495.

Distillation range (2.2.11). Not less than 95 per cent distils between 145 °C and 147 °C.

Tetrachlorvinphos. $C_{10}H_9Cl_4O_4P$. (M_r 366.0). 1132500. [22248-79-9].

mp: about 95 °C.

A suitable certified reference solution (10 ng/μl in iso-octane) may be used.

Tetracos-15-enoic acid methyl ester. $C_{25}H_{48}O_2$. (M_r 380.7). 1144800. [2733-88-2]. 15-Tetracosanoic acid methyl ester. Methyl tetracos-15-enoate. Nervonic acid methyl ester.

Content: minimum 99.0 per cent of $C_{25}H_{48}O_2$, determined by gas chromatography.

Liquid.

Tetracycline hydrochloride. 1147000.

See *Tetracycline hydrochloride* (0210).

Tetradecane. $C_{14}H_{30}$. (M_r 198.4). 1088200. [629-59-4]. *n*-Tetradecane.

Content: minimum 99.5 per cent *m/m* of $C_{14}H_{30}$.

A colourless liquid.

d_{20}^{20} : about 0.76.

n_D^{20} : about 1.429.

bp: about 252 °C.

mp: about –5 °C.

Tetradecylammonium bromide. $C_{40}H_{84}BrN$. (M_r 659). 1088300. [14937-42-9]. Tetrakis(decyl)ammonium bromide.

A white or slightly coloured, crystalline powder or crystals.

mp: 88 °C to 89 °C.

Tetraethylammonium hydrogen sulphate. $C_8H_{21}NO_4S$. (M_r 227.3). 1116200. [16873-13-5].

Hygroscopic powder.

mp: about 245 °C.

Tetraethylammonium hydroxide solution. $C_8H_{21}NO$. (M_r 147.3). 1100300. [77-98-5].

A 200 g/l aqueous solution, colourless liquid, strongly alkaline.

d_{20}^{20} : about 1.01.

n_D^{20} : about 1.372.

HPLC grade.

Tetraethylene pentamine. $C_8H_{23}N_5$. (M_r 189.3). 1102000. [112-57-2]. 3,6,9-Triazaundecan-1,11-diamine.

Colourless liquid, soluble in acetone.

n_D^{20} : about 1.506.

Storage: protected from humidity and heat.

Tetraheptylammonium bromide. $C_{28}H_{60}BrN$. (M_r 490.7). 1088400. [4368-51-8].

A white or slightly coloured, crystalline powder or crystals. mp: 89 °C to 91 °C.

Tetrahexylammonium bromide. $C_{24}H_{52}BrN$. (M_r 434.6). 1152500. [4328-13-6]. *N,N,N*-Trihexylhexan-1-aminium bromide.

White, crystalline powder, hygroscopic.

mp: about 100 °C.

Tetrahexylammonium hydrogen sulphate.

$C_{24}H_{53}NO_4S$. (M_r 451.8). 1116300. [32503-34-7].

N,N,N-Trihexylhexan-1-aminium hydrogen sulphate.

White crystals.

mp: 100 °C to 102 °C.

Tetrahydrofuran. C_4H_8O . (M_r 72.1). 1088500. [109-99-9]. Tetramethylene oxide.

A clear, colourless, flammable liquid, miscible with water, with alcohol.

d_{20}^{20} : about 0.89.

Do not distil if the tetrahydrofuran does not comply with the test for peroxides.

Peroxides. Place 8 ml of *potassium iodide and starch solution R* in a 12 ml ground-glass-stoppered cylinder about 1.5 cm in diameter. Fill completely with the substance to be examined, shake vigorously and allow to stand protected from light for 30 min. No colour is produced.

Tetrahydrofuran used in spectrophotometry complies with the following additional requirement.

Minimum transmittance (2.2.25), determined using *water R* as compensation liquid: 20 per cent at 255 nm, 80 per cent at 270 nm, 98 per cent at 310 nm.

Tetrahydrofuran for chromatography R. 1147100.

It complies with the requirements of *tetrahydrofuran R* and with the following requirements:

$$d_4^{20} = 0.8892.$$

bp: about 66 °C.

Content: minimum 99.8 per cent of C₄H₈O.

Tetramethylammonium bromide. C₄H₁₂BrN. (*M_r* 154.1). 1156600. [64-20-0]. *N,N,N*-Trimethylmethanaminium bromide.

White or slightly yellow crystals, freely soluble in water.
mp: about 285 °C, with decomposition.

Tetramethylammonium chloride. C₄H₁₂ClN. (*M_r* 109.6). 1100400. [75-57-0].

Colourless crystals, soluble in water and in alcohol.
mp: about 300 °C, with decomposition.

Tetramethylammonium hydrogen sulphate. C₄H₁₃NO₄S. (*M_r* 171.2). 1116400. [80526-82-5].

Hygroscopic powder.

mp: about 295 °C.

Tetramethylammonium hydroxide. C₄H₁₃NO₅H₂O. (*M_r* 181.2). 1122800. [10424-65-4]. Tetramethylammonium hydroxide pentahydrate.

Suitable grade for HPLC.

Tetramethylammonium hydroxide solution. 1088600. [75-59-2].

Content: minimum 10.0 per cent *m/m* of C₄H₁₃NO. (*M_r* 91.2).
A clear, colourless or very pale yellow liquid, miscible with water and with alcohol.

Assay. To 1.000 g add 50 ml of *water R* and titrate with 0.05 *M* sulphuric acid, using 0.1 ml of *methyl red solution R* as indicator.

1 ml of 0.05 *M* sulphuric acid is equivalent to 9.12 mg of C₄H₁₃NO.

Tetramethylammonium hydroxide solution, dilute. 1088601.

Dilute 10 ml of *tetramethylammonium hydroxide solution R* to 100 ml with *aldehyde-free alcohol R*.
Prepare immediately before use.

Tetramethylbenzidine. C₁₆H₂₀N₂. (*M_r* 240.3). 1132600. [54827-17-7]. 3,3',5,5'-Tetramethylbiphenyl-4,4'-diamine.

A powder, practically insoluble in water, very soluble in methanol.

mp: about 169 °C.

1,1,3,3-Tetramethylbutylamine. C₈H₁₉N. (*M_r* 129.3). 1141500. [107-45-9]. 2-Amino-2,4,4-trimethylpentane.

Clear, colourless liquid.

*d*₂₀²⁰: about 0.805.

*n*_D²⁰: about 1.424.

bp: about 140 °C.

Tetramethyldiaminodiphenylmethane. C₁₇H₂₂N₂. (*M_r* 254.4). 1088700. [101-61-1]. 4,4'-Methylenebis(*N,N*-dimethylaniline).

White to bluish-white crystals or leaflets, practically insoluble in water, slightly soluble in alcohol, soluble in mineral acids.
mp: about 90 °C.

Tetramethyldiaminodiphenylmethane reagent. 1088701.

Solution A. Dissolve 2.5 g of *tetramethyldiaminodiphenylmethane R* in 10 ml of *glacial acetic acid R* and add 50 ml of *water R*.

Solution B. Dissolve 5 g of *potassium iodide R* in 100 ml of *water R*.

Solution C. Dissolve 0.30 g of *ninhydrin R* in 10 ml of *glacial acetic acid R* and add 90 ml of *water R*.

Mix solution A, solution B and 1.5 ml of solution C.

Tetramethylethylenediamine. C₆H₁₆N₂. (*M_r* 116.2). 1088800. [110-18-9]. *N,N,N',N'*-Tetramethylethylenediamine.

A colourless liquid, miscible with water and with alcohol.

*d*₂₀²⁰: about 0.78.

*n*_D²⁰: about 1.418.

bp: about 121 °C.

Tetramethylsilane. C₄H₁₂Si. (*M_r* 88.2). 1088900. [75-76-3]. TMS.

A clear, colourless liquid, very slightly soluble in water, soluble in acetone and in alcohol.

*d*₂₀²⁰: about 0.64.

*n*_D²⁰: about 1.358.

bp: about 26 °C.

Tetramethylsilane used in nuclear magnetic resonance spectrometry complies with the following additional requirement.

In the nuclear magnetic resonance spectrum of an approximately 10 per cent *V/V* solution of the tetramethylsilane in *deuterated chloroform R*, the intensity of any foreign signal, excluding those due to spinning side bands and to chloroform, is not greater than the intensity of the C-13 satellite signals located at a distance of 59.1 Hz on each side of the principal signal of tetramethylsilane.

Tetrapropylammonium chloride. C₁₂H₂₈ClN. (*M_r* 221.8). 1151900. [5810-42-4].

White, crystalline powder, sparingly soluble in water.
mp: about 241 °C.

Tetrazolium blue. C₄₀H₃₂Cl₂N₈O₂. (*M_r* 728). 1089000. [1871-22-3]. 3,3'-(3,3'-Dimethoxy[1,1'-biphenyl]-4,4'-diyl)bis[2,5-diphenyl-2*H*-tetrazolium] dichloride.

Yellow crystals, slightly soluble in water, freely soluble in alcohol and in methanol, practically insoluble in acetone.
mp: about 245 °C, with decomposition.

Tetrazolium bromide. C₁₈H₁₆BrN₅S. (*M_r* 414.3). 1152700. [298-93-1]. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide. MTT.

Thallous sulphate. Tl₂SO₄. (*M_r* 504.8). 1089100. [7446-18-6]. Dithallium sulphate.

White, rhomboid prisms, slightly soluble in water, practically insoluble in alcohol.

Thebaine. C₁₉H₂₁NO₃. (*M_r* 311.4). 1089200. [115-37-7]. (5*R*,9*R*,13*S*)-4,5-Epoxy-3,6-dimethoxy-9a-methylmorphina-6,8-diene.

A white or pale yellow, crystalline powder, very slightly soluble in water, soluble in hot ethanol and in toluene.
mp: about 193 °C.

Chromatography (2.2.27). Examine as prescribed in identification test B in the monograph on *Raw opium (0777)*, applying to the plate as a band (20 mm × 3 mm) 20 µl of a 0.5 g/l solution. The chromatogram obtained shows an orange-red or red principal band with an *R_f* of about 0.5.

Theobromine. 1138800. [83-67-0].

See *Theobromine (0298)*.

Theophylline. 1089300. [58-55-9].

See *Theophylline (0299)*.

Thiamazole. $C_4H_6N_2S$. (M_r 114.2). 1089400. [60-56-0]. Methimazole. 1-Methyl-1*H*-imidazole-2-thiol.

A white or almost white, crystalline powder, freely soluble in water, soluble in alcohol and in methylene chloride.

mp: about 145 °C.

2-(2-Thienyl)acetic acid. $C_6H_6O_2S$. (M_r 142.1). 1089500. [1918-77-0].

A brown powder.

mp: about 65 °C.

Thioacetamide. C_2H_5NS . (M_r 75.1). 1089600. [62-55-5].

A crystalline powder or colourless crystals, freely soluble in water and in alcohol.

mp: about 113 °C.

Thioacetamide reagent. 1089601.

To 0.2 ml of *thioacetamide solution R* add 1 ml of a mixture of 5 ml of *water R*, 15 ml of 1 *M* sodium hydroxide and 20 ml of glycerol (85 per cent) *R*. Heat in a water-bath for 20 s. Prepare immediately before use.

Thioacetamide solution. 1089602.

A 40 g/l solution.

Thiobarbituric acid. $C_4H_4N_2O_2S$. (M_r 144.2). 1111200. [504-17-6]. 4,6-Dihydroxy-2-sulfanylpuridine.

Thiodiethylene glycol. $C_4H_{10}O_2S$. (M_r 122.2). 1122900. [111-48-8]. Di(2-hydroxyethyl) sulphide.

A colourless or yellow, viscous liquid. It contains at least 99.0 per cent of $C_4H_{10}O_2S$.

d_{20}^{20} : about 1.18.

Thioglycollic acid. $C_2H_4O_2S$. (M_r 92.1). 1089700. [68-11-1]. 2-Mercaptoacetic acid.

A colourless liquid, miscible with water, soluble in alcohol.

Thiomersal. $C_9H_9HgNaO_2S$. (M_r 404.8). 1089800. [54-64-8]. Sodium mercuriothiolate. Sodium 2-[(ethylmercurio)thio]benzoate.

A light, yellowish-white, crystalline powder, very soluble in water, freely soluble in alcohol.

Thiourea. CH_4N_2S . (M_r 76.1). 1089900. [62-56-6].

White, crystalline powder or crystals, soluble in water and in alcohol.

mp: about 178 °C.

Threonine. 1090000. [72-19-5].

See *Threonine* (1049).

Thrombin, bovine. 1090200. [9002-04-4].

A preparation of the enzyme, obtained from bovine plasma, that converts fibrinogen into fibrin.

A yellowish-white powder.

Storage: at a temperature below 0 °C.

Thrombin, human. 1090100. [9002-04-4].

Dried human thrombin. A preparation of the enzyme which converts human fibrinogen into fibrin. It is obtained from liquid human plasma and may be prepared by precipitation with suitable salts and organic solvents under controlled conditions of pH, ionic strength and temperature.

A yellowish-white powder, freely soluble in a 9 g/l solution of sodium chloride forming a cloudy, pale yellow solution.

Storage: in a sealed, sterile container under nitrogen, protected from light, at a temperature below 25 °C.

Thrombin solution, human. 1090101.

Reconstitute *human thrombin R* as directed by the manufacturer and dilute with *tris(hydroxymethyl)aminomethane sodium chloride buffer solution pH 7.4 R* to 5 IU/ml.

Thromboplastin. 1090300.

Extract 1.5 g of *acetone-dried ox brain R* with 60 ml of *water R* at 50 °C for 10 min to 15 min, centrifuge at 1500 r/min for 2 min and decant the supernatant liquid. The extract retains its activity for several days when stored in a refrigerator. It may contain 3 g/l of *cresol R* as an antimicrobial preservative.

Thujone. $C_{10}H_{16}O$. (M_r 152.2). 1116500. [546-80-5]. 4-Methyl-1-(1-methylethyl)bicyclo[3.1.0]hexan-3-one.

A colourless or almost colourless liquid, practically insoluble in water, soluble in alcohol and in many other organic solvents.

d_{20}^{20} : about 0.925.

n_D^{20} : about 1.455.

$[\alpha]_D^{20}$: about –15.

bp: about 200 °C.

Thymine. $C_5H_6N_2O_2$. (M_r 126.1). 1090400. [65-71-4]. 5-Methylpyrimidine-2,4(1*H*,3*H*)-dione.

Short needles or plates, slightly soluble in cold water, soluble in hot water. It dissolves in dilute solution of alkali hydroxides.

Thymol. 1090500. [89-83-8]. See *Thymol* (0791).

Thymol used in gas chromatography complies with the following additional test.

Assay. Examine by gas chromatography (2.2.28) as prescribed in the monograph *Peppermint oil* (0405).

Test solution. Dissolve 0.1 g in about 10 ml of *acetone R*.

The area of the principal peak is not less than 95.0 per cent of the area of all the peaks in the chromatogram obtained. Disregard the peak due to acetone.

Thymol blue. $C_{27}H_{30}O_5S$. (M_r 466.6). 1090600. [76-61-9]. Thymolsulphonphthalein. 4,4'-(3*H*-2,1-Benzoxathiol-3-ylidene)bis(2-isopropyl-5-methylphenol) *S,S*-dioxide.

A brownish-green to greenish-blue, crystalline powder, slightly soluble in water, soluble in alcohol and in dilute solutions of alkali hydroxides.

Thymol blue solution. 1090601.

Dissolve 0.1 g of *thymol blue R* in a mixture of 2.15 ml of 0.1 *M* sodium hydroxide and 20 ml of *alcohol R* and dilute to 100 ml with *water R*.

Test for sensitivity. To 0.1 ml of the thymol blue solution add 100 ml of *carbon dioxide-free water R* and 0.2 ml of 0.02 *M* sodium hydroxide. The solution is blue. Not more than 0.15 ml of 0.02 *M* hydrochloric acid is required to change the colour to yellow.

Colour change: pH 1.2 (red) to pH 2.8 (yellow); pH 8.0 (olive-green) to pH 9.6 (blue).

Thymolphthalein. $C_{28}H_{30}O_4$. (M_r 430.5). 1090700. [125-20-2]. 3,3-bis(4-Hydroxy-5-isopropyl-2-methylphenyl)-3*H*-isobenzofuran-1-one.

A white or yellowish-white powder, practically insoluble in water, soluble in alcohol and in dilute solutions of alkali hydroxides.

Thymolphthalein solution. 1090701.

A 1 g/l solution in *alcohol R*.

Test for sensitivity. To 0.2 ml of the thymolphthalein solution add 100 ml of *carbon dioxide-free water R*. The solution is colourless. Not more than 0.05 ml of 0.1 M *sodium hydroxide* is required to change the colour to blue.

Colour change: pH 9.3 (colourless) to pH 10.5 (blue).

Tin. Sn. (*A_r* 118.7). 1090800. [7440-31-5].

Silvery-white granules, soluble in hydrochloric acid with release of hydrogen.

Arsenic (2.4.2). 0.1 g complies with limit test A (10 ppm).

Titan yellow. C₂₈H₁₉N₅Na₂O₆S₄. (*M_r* 696). 1090900. [1829-00-1].

Schultz No. 280.

Colour Index No. 19540.

Thiazol yellow. Disodium 2,2'-[(1-triazene-1,3-diyl)di-4,1-phenylene]bis-[6-methylbenzothiazole-7-sulphonate].

A yellowish-brown powder, freely soluble in water and in alcohol.

Titan yellow paper. 1090901.

Immerse strips of filter paper in *titan yellow solution R* and leave for a few minutes. Allow to dry at room temperature.

Titan yellow solution. 1090902.

A 0.5 g/l solution.

Test for sensitivity. To 0.1 ml of the titan yellow solution add 10 ml of *water R*, 0.2 ml of *magnesium standard solution* (10 ppm Mg) *R* and 1.0 ml of 1 M *sodium hydroxide*. A distinct pink colour is visible by comparison with a reference solution prepared in a similar manner omitting the magnesium.

Titanium. Ti. (*A_r* 47.88). 1091000. [7440-32-6].

Content: minimum 99 per cent of Ti.

Metal powder, fine wire (diameter not more than 0.5 mm), sponge.

mp: about 1668 °C.

Density: about 4.507 g/cm³.

Titanium dioxide. 1117900. [13463-67-7].

See *Titanium dioxide* (0150).

Titanium trichloride. TiCl₃. (*M_r* 154.3). 1091200. [7705-07-9]. Titanium(III) chloride.

Reddish-violet crystals, deliquescent, soluble in water and in alcohol.

mp: about 440 °C.

Storage: in an airtight container.

Titanium trichloride solution. 1091201.

*d*₂₀²⁰: about 1.19.

A 150 g/l solution in hydrochloric acid (100 g/l HCl).

Titanium trichloride-sulphuric acid reagent. 1091202.

Carefully mix 20 ml of *titanium trichloride solution R* with 13 ml of *sulphuric acid R*. Add sufficient *strong hydrogen peroxide solution R* to give a yellow colour. Heat until white fumes are evolved. Allow to cool. Dilute with *water R* and repeat the evaporation and addition of *water R* until a colourless solution is obtained. Dilute to 100 ml with *water R*.

TLC octadecylsilyl silica gel plate. 1148600.

Support of glass, metal or plastic coated with a layer of octadecylsilyl silica gel. The plate may contain an organic binder.

TLC octadecylsilyl silica gel F₂₅₄ plate R. 1146600.

Support of glass, metal or plastic coated with a layer of octadecylsilyl silica gel.

It contains a fluorescent indicator having a maximum absorbance in ultraviolet light at 254 nm.

TLC performance test solution. 1116600.

Prepare a mixture of 1.0 ml of each of the following solutions and dilute to 10.0 ml with *acetone R*: a 0.5 g/l solution of *Sudan red G R* in *toluene R*, a 0.5 g/l solution of *methyl orange R* in *ethanol R* prepared immediately before use, a 0.5 g/l solution of *bromocresol green R* in *acetone R* and a 0.25 g/l solution of *methyl red R* in *acetone R*.

TLC silica gel plate. 1116700.

Support of glass, metal or plastic, coated with a layer of silica gel of a suitable thickness and particle size (usually 2 µm to 10 µm for fine particle size (High Performance Thin-Layer Chromatography, HPTLC) plates and 5 µm to 40 µm for normal TLC plates). If necessary, the particle size is indicated after the name of the reagent in the tests where it is used.

The plate may contain an organic binder.

Chromatographic separation. Apply to the plate an appropriate volume (10 µl for a normal TLC plate and 1 µl to 2 µl for a fine particle size plate) of *TLC performance test solution R*. Develop over a pathlength two-thirds of the plate height, using a mixture of 20 volumes of *methanol R* and 80 volumes of *toluene R*. The plate is not satisfactory, unless the chromatogram shows four clearly separated spots, the spot of bromocresol green with an *R_f* value less than 0.15, the spot of methyl orange with an *R_f* value in the range of 0.1 to 0.25, the spot of methyl red with an *R_f* value in the range of 0.35 to 0.55 and the spot of Sudan red G with an *R_f* value in the range of 0.75 to 0.98.

TLC silica gel F₂₅₄ plate. 1116800.

It complies with the requirements prescribed for *TLC silica gel plate R* with the following modification.

It contains a fluorescent indicator having a maximum absorbance at 254 nm.

Fluorescence suppression. Apply separately to the plate at five points increasing volumes (1 µl to 10 µl for normal TLC plates and 0.2 µl to 2 µl for fine particle size plates) of a 1 g/l solution of *benzoic acid R* in a mixture of 15 volumes of *ethanol R* and 85 volumes of *cyclohexane R*. Develop over a pathlength half of the plate height with the same mixture of solvents. After evaporating the solvents examine the chromatogram in ultraviolet light at 254 nm. For normal TLC plates the benzoic acid appears as dark spots on a fluorescent background approximately in the middle of the chromatogram for quantities of 2 µg and greater. For fine particle size plates the benzoic acid appears as dark spots on a fluorescent background approximately in the middle of the chromatogram for quantities of 0.2 µg and greater.

TLC silica gel F₂₅₄, silanised plate. 1117200.

It complies with the requirements prescribed for *TLC silica gel silanised plate R* with the following modification.

It contains a fluorescent indicator having a maximum absorbance at 254 nm.

TLC silica gel G plate. 1116900.

It complies with the requirements prescribed for *TLC silica gel plate R* with the following modification.

It contains calcium sulphate hemihydrate as binder.

TLC silica gel GF₂₅₄ plate. 1117000.

It complies with the requirements prescribed for *TLC silica gel plate R* with the following modifications.

It contains calcium sulphate hemihydrate as binder and a fluorescent indicator having a maximum absorbance at 254 nm.

Fluorescence suppression. Complies with the test prescribed for *TLC silica gel F₂₅₄ plate R*.

TLC silica gel plate for chiral separations, octadecylsilyl. 1137700.

Support of glass, metal or plastic, coated with a layer of octadecylsilyl silica gel, impregnated with Cu²⁺ ions and enantiomerically pure hydroxyproline. The plate may contain an organic binder.

TLC silica gel, silanised plate. 1117100.

Support of glass, metal or plastic, coated with a layer of silanised silica gel of a suitable thickness and particle size (usually 2 µm to 10 µm for fine particle size (High Performance Thin-Layer Chromatography, HPTLC) plates and 5 µm to 40 µm for normal TLC plates). If necessary, the particle size is indicated after the name of the reagent in the tests where it is used.

The plate may contain an organic binder.

Chromatographic separation. Introduce 0.1 g each of *methyl laurate R*, *methyl myristate R*, *methyl palmitate R* and *methyl stearate R* into a 250 ml conical flask. Add 40 ml of *alcoholic potassium hydroxide solution R* and heat under a reflux condenser on a water-bath for 1 h. Allow to cool, transfer the solution to a separating funnel by means of 100 ml of *water R*, acidify (pH 2 to 3) with *dilute hydrochloric acid R* and shake with three quantities each of 10 ml of *methylene chloride R*. Dry the combined methylene chloride extracts over *anhydrous sodium sulphate R*, filter and evaporate to dryness on a water-bath. Dissolve the residue in 50 ml of *methylene chloride R*. Examine by thin-layer chromatography (2.2.27), using *silanised TLC silica gel plate R*. Apply an appropriate quantity (about 10 µl for normal TLC plates and about 1 µl to 2 µl for fine particle size plates) of the methylene chloride solution at each of three separate points. Develop over a pathlength two-thirds of the plate height with a mixture of 10 volumes of *glacial acetic acid R*, 25 volumes of *water R* and 65 volumes of *dioxan R*. Dry the plate at 120 °C for 30 min. Allow to cool, spray with a 35 g/l solution of *phosphomolybdic acid R* in *2-propanol R* and heat at 150 °C until the spots become visible. Treat the plate with ammonia vapour until the background is white. The chromatograms show four clearly separated, well-defined spots.

α-Tocopherol. 1152300. [10191-41-0].

See *all-rac-α-Tocopherol (0692)*.

α-Tocopheryl acetate. 1152400. [7695-91-2].

See *all-rac-α-Tocopheryl acetate (0439)*.

o-Tolidine. C₁₄H₁₆N₂. (M_r 212.3). 1123000. [119-93-7]. 3,3'-Dimethylbenzidine.

Content: minimum 97.0 per cent of C₁₄H₁₆N₂.

A light brownish, crystalline power.

mp: about 130 °C.

o-Tolidine solution. 1123001.

Dissolve 0.16 g of *o-tolidine R* in 30.0 ml of *glacial acetic acid R*, add 1.0 g of *potassium iodide R* and dilute to 500.0 ml with *water R*.

Toluene. C₇H₈. (M_r 92.1). 1091300. [108-88-3]. Methylbenzene.

A clear, colourless, flammable liquid, very slightly soluble in water, miscible with alcohol.

*d*₂₀²⁰: 0.865 to 0.870.

bp: about 110 °C.

Toluene, sulphur-free. 1091301.

Complies with the requirements prescribed for *toluene R* and with the following additional requirements:

Sulphur compounds. To 10 ml add 1 ml of *ethanol R* and 3 ml of *potassium plumbite solution R* and boil under a reflux condenser for 15 min. Allow to stand for 5 min. No darkening is produced in the aqueous layer.

Thiophen-related substances. Shake 2 ml with 5 ml of *isatin reagent R* for 5 min and allow to stand for 15 min. No blue colour is produced in the lower layer.

Toluenesulphonamide. C₇H₉NO₂S. (M_r 171.2). 1091500. [70-55-3]. 4-Methylbenzenesulphonamide. *p*-Toluenesulphonamide.

A white, crystalline powder, slightly soluble in water, soluble in alcohol and in solutions of alkali hydroxides.

mp: about 136 °C.

Chromatography. Examine as prescribed in the monograph on *Tolbutamide (0304)*; the chromatogram shows only one principal spot.

o-Toluenesulphonamide. C₇H₉NO₂S. (M_r 171.2). 1091400. [88-19-7]. 2-Methylbenzenesulphonamide.

A white, crystalline powder, slightly soluble in water, soluble in alcohol and in solutions of alkali hydroxides.

mp: about 156 °C.

p-Toluenesulphonamide. 1091500. [70-55-3]. See *toluenesulphonamide R*.

Toluenesulphonic acid. C₇H₉O₃S.H₂O. (M_r 190.2). 1091600. [6192-52-5]. 4-Methylbenzenesulphonic acid.

Content: minimum 87.0 per cent of C₇H₈O₃S.

A white, crystalline powder or crystals, freely soluble in water, soluble in alcohol.

o-Toluidine. C₇H₉N. (M_r 107.2). 1091700. [95-53-4]. 2-Methylaniline.

A pale-yellow liquid becoming reddish-brown on exposure to air and light, slightly soluble in water, soluble in alcohol and in dilute acids.

*d*₂₀²⁰: about 1.01.

*n*_D²⁰: about 1.569.

bp: about 200 °C.

Storage: in an airtight container, protected from light.

o-Toluidine hydrochloride. C₇H₁₀ClN. (M_r 143.6). 1117300. [636-21-5]. 2-Methylaniline hydrochloride. 2-Methylbenzenamine hydrochloride.

Content: minimum 98.0 per cent of C₇H₁₀ClN.

mp: 215 °C to 217 °C.

p-Toluidine. C₇H₉N. (M_r 107.2). 1091800. [106-49-0]. 4-Methylaniline.

Lustrous plates or flakes, slightly soluble in water, freely soluble in acetone and in alcohol.

mp: about 44 °C.

Toluidine blue. C₁₅H₁₆ClN₃S. (M_r 305.8). 1091900. [92-31-9]. Schultz No. 1041.

Colour Index No. 52040.

Toluidine Blue O. 3-Amino-7-dimethylamino-2-methylphenothiazin-5-ium chloride.

A dark-green powder, soluble in water, slightly soluble in alcohol.

Tosylarginine methyl ester hydrochloride.C₁₄H₂₃ClN₄O₄S. (*M_r* 378.9). 1092000. [1784-03-8].*N*-Tosyl-L-arginine methyl ester hydrochloride. Methyl (S)-5-guanidino-2-(4-methylbenzenesulphonamido)valerate hydrochloride.[α]_D²⁰: −12 to −16, determined on a 40 g/l solution.

mp: about 145 °C.

Tosylarginine methyl ester hydrochloride solution.
1092001.To 98.5 mg of *tosylarginine methyl ester hydrochloride R* add 5 ml of *tris(hydroxymethyl)aminomethane buffer solution pH 8.1 R* and shake to dissolve. Add 2.5 ml of *methyl red mixed solution R* and dilute to 25.0 ml with *water R*.**Tosyl-lysyl-chloromethane hydrochloride.**C₁₄H₂₂Cl₂N₂O₃S. (*M_r* 369.3). 1092100. [4238-41-9].*N*-Tosyl-L-lysyl-chloromethane hydrochloride. (3S)-7-Amino-1-chloro-3-(4-methylbenzenesulphonamido)heptan-2-one hydrochloride.[α]_D²⁰: −7 to −9, determined on a 20 g/l solution.

mp: about 155 °C, with decomposition.

A_{1 cm}^{1%}: 310 to 340, determined at 230 nm in *water R*.**Tosylphenylalanylchloromethane.** C₁₇H₁₈ClNO₃S. (*M_r* 351.9). 1092200. [402-71-1]. *N*-Tosyl-L-phenylalanylchloromethane.[α]_D²⁰: −85 to −89, determined on a 10 g/l solution in *alcohol R*.

mp: about 105 °C.

A_{1 cm}^{1%}: 290 to 320, determined at 228.5 nm in *alcohol R*.**Toxaphene.** 1132800. [8001-35-2].

A mixture of polychloro derivatives.

mp: 65 °C to 90 °C.

A suitable certified reference solution (10 ng/μl in iso-octane) may be used.

Tragacanth. 1092300. [9000-65-1].See *Tragacanth (0532)*.**Triacetin.** C₉H₁₄O₆. (*M_r* 218.2). 1092400. [102-76-1].

Propane-1,2,3-triyl triacetate.

An almost clear, colourless to yellowish liquid, soluble in water, miscible with alcohol.

*d*₂₀²⁰: about 1.16.*n*_D²⁰: about 1.43.

bp: about 260 °C.

Triamcinolone. C₂₁H₂₇FO₆. (*M_r* 394.4). 1111300. [124-94-7].

9-Fluoro-11β,16α,17,21-tetrahydroxypregna-1,4-diene-3,20-dione.

A crystalline powder.

mp: 262 °C to 263 °C.

Triamcinolone acetonide. 1133100. [76-25-5].See *Triamcinolone acetonide (0533)*.**Tributyl citrate.** C₁₈H₃₂O₇. (*M_r* 360.4). 1152800. [77-94-1].

Tributyl 2-hydroxypropane-1,2,3-tricarboxylate.

*d*₄²⁰: about 1.043.*n*_D²⁰: about 1.445.**Trichlorethylene.** 1102100.See *Trichloroethylene R*.**Trichloroacetic acid.** C₂HCl₃O₂. (*M_r* 163.4). 1092500. [76-03-9].

Colourless crystals or a crystalline mass, very deliquescent, very soluble in water and in alcohol.

Storage: in an airtight container.**Trichloroacetic acid solution.** 1092501.Dissolve 40.0 g of *trichloroacetic acid R* in *water R* and dilute to 1000.0 ml with the same solvent. Verify the concentration by titration with 0.1 M *sodium hydroxide* and adjust if necessary to 40 ± 1 g/l.**1,1,1-Trichloroethane.** C₂H₃Cl₃. (*M_r* 133.4). 1092600. [71-55-6]. Methylchloroform.

A non-flammable liquid, practically insoluble in water, soluble in acetone and in methanol.

*d*₂₀²⁰: about 1.34.*n*_D²⁰: about 1.438.

bp: about 74 °C.

Trichloroethylene. C₂HCl₃. (*M_r* 131.4). 1102100. [79-01-6].

A colourless liquid, practically insoluble in water, miscible with alcohol.

*d*₂₀²⁰: about 1.46.*n*_D²⁰: about 1.477.**Trichlorotrifluoroethane.** C₂Cl₃F₃. (*M_r* 187.4). 1092700. [76-13-1]. 1,1,2-Trichloro-1,2,2-trifluoroethane.

A colourless, volatile liquid, practically insoluble in water, miscible with acetone.

*d*₂₀²⁰: about 1.58.*Distillation range (2.2.11)*. Not less than 98 per cent distils between 47 °C and 48 °C.**Tricine.** C₆H₁₃NO₅. (*M_r* 179.2). 1138900. [5704-04-1]. *N*-[2-Hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

Use electrophoresis-grade reagent.

mp: about 183 °C.

Tricosane. C₂₃H₄₈. (*M_r* 324.6). 1092800. [638-67-5].

White crystals, practically insoluble in water, soluble in hexane.

*n*_D²⁰: about 1.447.

mp: about 48 °C.

Tridocosahexaenoin. C₆₉H₉₈O₆. (*M_r* 1023.5). 1144900. [124596-98-1]. Triglyceride of docosahexaenoic acid(C22:6). Glycerol tridocosahexaenoate. Propane-1,2,3-triyl tri-(*all-Z*)-docosa-4,7,10,13,16,19-hexaenoate.

The reagent from Nu-Chek Prep, Inc. has been found suitable.

Triethanolamine. 1092900. [102-71-6].See *Trolamine (1577)*.**Triethylamine.** C₆H₁₅N. (*M_r* 101.2). 1093000. [121-44-8]. *N,N*-Diethylethanamine.

A colourless liquid, slightly soluble in water at a temperature below 18.7 °C, miscible with alcohol.

*d*₂₀²⁰: about 0.727.*n*_D²⁰: about 1.401.

bp: about 90 °C.

Triethylenediamine. $C_6H_{12}N_2$. (M_r 112.2). 1093100. 1,4-Diazabicyclo[2.2.2]octane.

Crystals, very hygroscopic, sublimes readily at room temperature, freely soluble in water, in acetone and in ethanol.

bp: about 174 °C.

mp: about 158 °C.

Storage: in an airtight container.

Triethyl phosphonoformate. $C_7H_{15}O_5P$. (M_r 210.2). 1132900. [1474-78-8]. Ethyl (diethoxyphosphoryl)formate.

Colourless liquid.

bp_{12 mm}: about 135 °C.

Trifluoroacetic acid. $C_2HF_3O_2$. (M_r 114.0). 1093200. [76-05-1].

Content: minimum 99 per cent of $C_2HF_3O_2$.

Liquid, miscible with acetone and with alcohol.

d_{20}^{20} : about 1.53.

bp: about 72 °C.

Use a grade suitable for protein sequencing.

Storage: in an airtight container.

Trifluoroacetic anhydride. $C_4F_6O_3$. (M_r 210.0). 1093300. [407-25-0].

Colourless liquid.

d_{20}^{20} : about 1.5.

Trigonelline hydrochloride. $C_7H_8ClNO_2$. (M_r 173.6). 1117400. [6138-41-6]. 3-Carboxy-1-methylpyridinium chloride. Nicotinic acid *N*-methylbetaine hydrochloride.

A crystalline powder, very soluble in water, soluble in alcohol.

mp: about 258 °C.

Trimethylpentane. C_8H_{18} . (M_r 114.2). 1093400. [540-84-1]. Iso-octane. 2,2,4-Trimethylpentane.

A colourless, flammable liquid, practically insoluble in water, soluble in ethanol.

d_{20}^{20} : 0.691 to 0.696.

n_D^{20} : 1.391 to 1.393.

Distillation range (2.2.11). Not less than 95 per cent distils between 98 °C and 100 °C.

Trimethylpentane used in spectrophotometry complies with the following additional requirement.

Minimum transmittance (2.2.25), determined using *water R* as compensation liquid: 98 per cent from 250 nm to 420 nm.

Trimethylpentane R1. 1093401.

Complies with the requirements prescribed for *trimethylpentane R* with the following modification.

Absorbance (2.2.25). Not more than 0.07 from 220 nm to 360 nm, determined using *water R* as the compensation liquid.

***N,O*-bis(Trimethylsilyl)acetamide.** $C_8H_{21}NOSi_2$. (M_r 203.4). 1093600. [10416-59-8].

Colourless liquid.

d_{20}^{20} : about 0.83.

***N*-Trimethylsilylimidazole.** $C_6H_{12}N_2Si$. (M_r 140.3). 1100500. [18156-74-6]. 1-Trimethylsilylimidazole.

A colourless, hygroscopic liquid.

d_{20}^{20} : about 0.96.

n_D^{20} : about 1.48.

Storage: in an airtight container.

***N,O*-bis(Trimethylsilyl)trifluoroacetamide.** $C_8H_{18}F_3NOSi_2$. (M_r 257.4). 1133200. [25561-30-2]. BSTFA.

Colourless liquid.

d_{20}^{20} : about 0.97.

n_D^{20} : about 1.38.

bp_{12 mm}: about 40 °C

Trimethylsulphonium hydroxide. $C_3H_{10}OS$. (M_r 94.2). 1145000. [17287-03-5].

d_4^{20} : about 0.81.

2,4,6-Trinitrobenzene sulphonic acid. $C_6H_3N_3O_9S \cdot 3H_2O$. (M_r 347.2). 1117500. [2508-19-2].

A white, crystalline powder, soluble in water.

mp: 190 °C to 195 °C.

Triphenylmethanol. $C_{19}H_{16}O$. (M_r 260.3). 1093700. [76-84-6]. Triphenylcarbinol.

Colourless crystals, practically insoluble in water, freely soluble in alcohol.

Triphenyltetrazolium chloride. $C_{19}H_{15}ClN_4$. (M_r 334.8). 1093800. [298-96-4]. 2,3,5-Triphenyl-2*H*-tetrazolium chloride.

Content: minimum 98.0 per cent of $C_{19}H_{15}ClN_4$. A pale or dull-yellow powder, soluble in water, in acetone and in alcohol.

mp: about 240 °C, with decomposition.

Assay. Dissolve 1.000 g in a mixture of 5 ml of *dilute nitric acid R* and 45 ml of *water R*. Add 50.0 ml of 0.1 *M silver nitrate* and heat to boiling. Allow to cool, add 3 ml of *dibutyl phthalate R*, shake vigorously and titrate with 0.1 *M ammonium thiocyanate*, using 2 ml of *ferric ammonium sulphate solution R2* as indicator.

1 ml of 0.1 *M silver nitrate* is equivalent to 33.48 mg of $C_{19}H_{15}ClN_4$.

Storage: protected from light.

Triphenyltetrazolium chloride solution. 1093801.

A 5 g/l solution in *aldehyde-free alcohol R*.

Storage: protected from light.

Triscyanoethoxypropane. $C_{12}H_{17}N_3O_3$. (M_r 251.3). 1093900. 1,2,3-Tris(2-cyanoethoxy)propane.

A viscous, brown-yellow liquid, soluble in methanol. Used as a stationary phase in gas chromatography.

d_{20}^{20} : about 1.11.

Viscosity (2.2.9): about 172 mPas.

1,3,5-Tris[3,5-di(1,1-dimethylethyl)-4-hydroxybenzyl]-1,3,5-triazine-2,4,6(1*H*,3*H*,5*H*)-trione. $C_{48}H_{69}O_6N_3$. (M_r 784.1). 1094000. [27676-62-6].

A white, crystalline powder.

mp: 218 °C to 222 °C.

Tris[2,4-di(1,1-dimethylethyl)phenyl] phosphite. $C_{42}H_{63}O_3P$. (M_r 647). 1094100. [31570-04-4].

White powder.

mp: 182 °C to 186 °C.

Tris(hydroxymethyl)aminomethane. 1094200. [77-86-1]. See *Trometamol* (1053).

Tris(hydroxymethyl)aminomethane solution. 1094201.

A solution containing the equivalent of 24.22 g of $C_4H_{11}NO_3$ in 1000.0 ml.

Tris(hydroxymethyl)aminomethane solution R1. 1094202.

Dissolve 60.6 mg of *tris(hydroxymethyl)aminomethane R* and 0.234 g of *sodium chloride R* in *water R* and dilute to 100 ml with the same solvent.

Storage: at 2 °C to 8 °C; use within 3 days.

Tripotassium phosphate trihydrate. $K_3PO_4 \cdot 3H_2O$. (M_r 266.3). 1155300. [22763-03-7].

White or almost white crystalline powder, freely soluble in water.

Trisodium phosphate dodecahydrate. $Na_3PO_4 \cdot 12H_2O$. (M_r 380.1). 1094300. [10101-89-0].

Colourless or white crystals, freely soluble in water.

Trypsin. 1094500. [9002-07-7].

A proteolytic enzyme obtained by activation of trypsinogen extracted from the pancreas of beef (*Bos taurus* L.).

A white, crystalline or amorphous powder, sparingly soluble in water.

Trypsin for peptide mapping. 1094600. [9002-07-7].

Trypsin of high purity treated to eliminate chymotryptic activity.

Tryptophan. $C_{11}H_{12}N_2O_2$. (M_r 204.2). 1094700. [73-22-3].

A white or yellowish-white, crystalline powder or colourless crystals, slightly soluble in water, very slightly soluble in alcohol.

$[\alpha]_D^{20}$: about –30, determined on a 10 g/l solution.

Tyramine. $C_8H_{11}NO$. (M_r 137.2). 1117600. [51-67-2]. 4-(2-Aminoethyl)phenol.

Crystals, sparingly soluble in water, soluble in boiling ethanol.

mp: 164 °C to 165 °C.

Tyrosine. $C_9H_{11}NO_3$. (M_r 181.2). 1094800. [60-18-4]. 2-Amino-3-(4-hydroxyphenyl)propionic acid.

A white, crystalline powder or colourless or white crystals, slightly soluble in water, practically insoluble in acetone and in ethanol, soluble in dilute hydrochloric acid and in solutions of alkali hydroxides.

Chromatography. Examine as prescribed in the monograph on *Levodopa* (0038); the chromatogram shows only one principal spot.

Umbelliferone. $C_9H_6O_3$. (M_r 162.1). 1137500. [93-35-6]. 7-Hydroxycoumarin. 7-Hydroxy-2H-1-benzopyran-2-one.

Needles from water.

mp: 225 °C to 228 °C.

Urea. 1095000. [57-13-6].

See *Urea* (0743).

Uridine. $C_9H_{12}N_2O_6$. (M_r 244.2). 1095100. [58-96-8]. 1-β-D-Ribofuranosyluracil.

A white or almost white, crystalline powder, soluble in water.

mp: about 165 °C.

Ursolic acid. $C_{30}H_{48}O_3$. (M_r 456.7). 1141600. [77-52-1]. (3β)-3-Hydroxyurs-12-en-28-oic acid.

White powder, practically insoluble in water, sparingly soluble in methanol, slightly soluble in alcohol.

$[\alpha]_D^{21}$: about 67.50 (10 g/l solution in a 56.1 g/l solution of *potassium hydroxide R* in *alcohol R*).

mp: 285 °C to 288 °C.

Valencene. $C_{15}H_{24}$. (M_r 204.4). 1152100.

[4630-07-3]. 4βH,5α-Eremophila-1(10),11-diene.

(1R,7R,8aS)-1,8a-Dimethyl-7-(1-methylethenyl)-1,2,3,5,6,7,8,8a-octahydronaphthalene.

Oily, colourless to pale yellow liquid, with a characteristic odour, practically insoluble in water, soluble in alcohol.

d_4^{20} : about 0.918.

n_D^{20} : about 1.508.

bp: about 123 °C.

Valencene used in gas chromatography complies with the following additional test.

Assay. Examine by gas chromatography (2.2.28) as prescribed in the monograph on *Sweet orange oil* (1811).

The content is not less than 90 per cent, calculated by the normalisation procedure.

Valeric acid. $C_5H_{10}O_2$. (M_r 102.1). 1095200. [109-52-4]. Pentanoic acid.

A colourless liquid, soluble in water, freely soluble in alcohol.

d_{20}^{20} : about 0.94.

n_D^{20} : about 1.409.

bp: about 186 °C.

Vanillin. 1095300. [121-33-5].

See *Vanillin* (0747).

Vanillin reagent. 1095301.

Carefully add, dropwise, 2 ml of *sulphuric acid R* to 100 ml of a 10 g/l solution of *vanillin R* in *alcohol R*.

Storage: use within 48 h.

Vanillin solution, phosphoric. 1095302.

Dissolve 1.0 g of *vanillin R* in 25 ml of *alcohol R*. Add 25 ml of *water R* and 35 ml of *phosphoric acid R*.

Verbenone. $C_{10}H_{14}O$. (M_r 150.2). 1140500. [1196-01-6]. (1S,5S)-4,6,6-Trimethylbicyclo[3.1.1]hept-3-en-2-one.

Oil with a characteristic odour, practically insoluble in water, miscible with organic solvents.

d_{20}^{20} : about 0.978.

n_D^{18} : about 1.49.

$[\alpha]_D^{18}$: about +249.6.

bp: 227 °C to 228 °C.

mp: about 6.5 °C.

Verbenone used in gas chromatography complies with the following additional test.

Assay. Examine by gas chromatography (2.2.28) as prescribed in the monograph on *Rosemary oil* (1846).

The content is not less than 99 per cent, calculated by the normalisation procedure.

Vinyl acetate. $C_4H_6O_2$. (M_r 86.10). 1111800. [108-05-4]. Ethenyl acetate.

d_{20}^{20} : about 0.930.

bp: about 72 °C.

Vinyl chloride. C_2H_3Cl . (M_r 62.5). 1095400. [75-01-4].

A colourless gas, slightly soluble in organic solvents.

Vinyl polymer for chromatography, octadecyl. 1155400.

Spherical particles (5 µm) of a vinyl alcohol copolymer chemically modified by bonding of octadecyl groups on the hydroxyl groups.

Vinyl polymer for chromatography, octadecylsilyl. 1121600.

Spherical particles (5 µm) of a vinyl alcohol copolymer bonded to an octadecylsilane. Carbon content of 17 per cent.

2-Vinylpyridine. C₇H₇N. (*M_r* 105.1). 1102200. [100-69-6].

A yellow liquid, miscible in water.

*d*₂₀²⁰: about 0.97.

*n*_D²⁰: about 1.549.

1-Vinylpyrrolidin-2-one. C₆H₉NO. (*M_r* 111.1). 1111900. [88-12-0]. 1-Ethenylpyrrolidin-2-one.

Content: minimum 99.0 per cent of C₆H₉NO.

A clear colourless liquid.

Water (2.5.12): maximum 0.1 per cent, determined on 2.5 g. Use as the solvent, a mixture of 50 ml of *anhydrous methanol R* and 10 ml of *butyrolactone R*.

Assay. Examine by gas chromatography (2.2.28).

The chromatography may be carried out using

- a fused-silica column 30 m long and 0.5 mm in internal diameter the inner wall of which is coated with a 1.0 µm layer of *macrogol 20 000 R*,
- *helium for chromatography R* as the carrier gas,
- a flame-ionisation detector,

maintaining the temperature of the injection port at 190 °C and programming the temperature of the column as follows: maintain the temperature at 80 °C for 1 min and then increase it to 190 °C at a rate of 10 °C per minute. Maintain at 190 °C for 15 min. Inject 0.3 µl of the substance to be examined and adjust the flow rate of the carrier gas so that the retention time of the peak corresponding to 1-vinylpyrrolidin-2-one is about 17 min. Determine the content of C₆H₉NO by internal normalisation.

Vitexin. C₂₁H₂₀O₁₀. (*M_r* 448.4). 1133300. [3681-93-4]. Apigenin 8-glucoside.

Yellow powder.

Storage: in an airtight container, protected from light.

Water. 1095500. [7732-18-5].

See *Purified water* (0008).

Water R1. 1095508.

Prepared from *distilled water R* by multiple distillation. Remove carbon dioxide by boiling for at least 15 min before use in a boiling flask of fused silica or borosilicate glass and cool. Any other suitable method may be used. The boiling flask has been already used for the test or has been filled with *water R* and kept in an autoclave at 121 °C for at least 1 h prior to first use. When tested immediately before use, *water R1* is neutral to *methyl red solution R*, i.e. it shall produce an orange-red (not a violet-red or yellow) colour corresponding to pH 5.5 ± 0.1 when 0.05 ml of *methyl red solution R* is added to 50 ml of the water to be examined.

Conductivity: maximum 1 µS·cm⁻¹, determined at 25 °C by an in-line conductivity meter (see *Purified water* (0008)).

Water, ammonium-free. 1095501. [7732-18-5].

To 100 ml of *water R* add 0.1 ml of *sulphuric acid R*. Distil using the apparatus described for the determination of *Distillation range* (2.2.11). Reject the first 10 ml and collect the following 50 ml.

Water, carbon dioxide-free. 1095502. [7732-18-5].

Water R which has been boiled for a few minutes and protected from the atmosphere during cooling and storage.

Water for chromatography. 1095503. [7732-18-5].

Deionised *water R* with a resistivity of not less than 0.18 Mohm·m.

Water, distilled. 1095504. [7732-18-5].

Water R prepared by distillation.

Water, distilled, deionised. 1095508.

Deionised *water R* prepared by distillation with a resistivity of not less than 18 Mohm·m.

Water for injections. 1095505. [7732-18-5].

See *Water for injections* (0169).

Water, nitrate-free. 1095506. [7732-18-5].

To 100 ml of *water R* add a few milligrams of *potassium permanganate R* and of *barium hydroxide R*. Distil using the apparatus described for the determination of *Distillation range* (2.2.11). Reject the first 10 ml and collect the following 50 ml.

Water, particle-free. 1095507. [7732-18-5].

Filter *water R* through a membrane with a pore size of 0.22 µm.

Weak cationic resin. 1096000.

Polymethacrylic resin, slightly acid, with carboxyl groups present in a protonated form.

Particle size: 75 µm to 160 µm.

pH limits of use: 5 to 14.

Maximum temperature of use: 120 °C.

Xanthydrol. C₁₃H₁₀O₂. (*M_r* 198.2). 1096100. [90-46-0]. 9-Xanthanol.

Content: minimum 90.0 per cent of C₁₃H₁₀O₂.

A white to pale-yellow powder, very slightly soluble in water, soluble in alcohol and in glacial acetic acid.

It is also available as a methanolic solution containing 90 g/l to 110 g/l of xanthydrol.

mp: about 123 °C.

Assay. In a 250 ml flask dissolve 0.300 g in 3 ml of *methanol R* or use 3.0 ml of solution. Add 50 ml of *glacial acetic acid R* and, dropwise with shaking, 25 ml of a 20 g/l solution of *urea R*. Allow to stand for 12 h, collect the precipitate on a sintered-glass filter (16), wash with 20 ml of *alcohol R*, dry in an oven at 100 °C to 105 °C and weigh. 1 g of precipitate is equivalent to 0.9429 g of xanthydrol.

Storage: protected from light. If a methanolic solution is used, store in small sealed ampoules and filter before use if necessary.

Xanthydrol R1. 1096101.

Complies with the requirements prescribed for *xanthydrol R* and with the following requirement.

Content: minimum 98.0 per cent of C₁₃H₁₀O₂.

Xanthydrol solution. 1096102.

To 0.1 ml of a 100 g/l solution of *xanthydrol R* in *methanol R* add 100 ml of *anhydrous acetic acid R* and 1 ml of *hydrochloric acid R*. Allow to stand for 24 h before using.

Xylene. C₈H₁₀. (*M_r* 106.2). 1096200. [1330-20-7].

Mixture of isomers. A clear, colourless, flammable liquid, practically insoluble in water, miscible with alcohol.

d_{20}^{20} : about 0.867.

n_D^{20} : about 1.497.

bp: about 138 °C.

m-Xylene. C_8H_{10} . (M_r 106.2). 1117700. [108-38-3].
1,3-Dimethylbenzene.

A clear, colourless, flammable liquid, practically insoluble in water, miscible with alcohol.

d_{20}^{20} : about 0.884.

n_D^{20} : about 1.497.

bp: about 139 °C.

mp: about -47 °C.

o-Xylene. C_8H_{10} . (M_r 106.2). 1100600. [95-47-6].
1,2-Dimethylbenzene.

A clear, colourless, flammable liquid, practically insoluble in water, miscible with alcohol.

d_{20}^{20} : about 0.881.

n_D^{20} : about 1.505.

bp: about 144 °C.

mp: about -25 °C.

Xylenol orange. $C_{31}H_{28}N_2Na_4O_{13}S$. (M_r 761). 1096300. [3618-43-7]. Tetrasodium 3,3'-(3H-2,1-benzoxathiol-3-ylidene)bis[(6-hydroxy-5-methyl-3,1-phenylene)methyleneiminobisacetate] S,S-dioxide.

A reddish-brown crystalline powder, soluble in water.

Xylenol orange triturate. 1096301.

Triturate 1 part of *xylenol orange R* with 99 parts of *potassium nitrate R*.

Test for sensitivity. To 50 ml of *water R* add 1 ml of *dilute acetic acid R*, 50 mg of the xylenol orange triturate and 0.05 ml of *lead nitrate solution R*. Add *hexamethylenetetramine R* until the colour changes from yellow to violet-red. After addition of 0.1 ml of *0.1 M sodium edetate* the colour changes to yellow.

Xylose. 1096400. [58-86-6].

See *Xylose* (1278).

Zinc. Zn. (A_r 65.4). 1096500. [7440-66-6].

Content: minimum 99.5 per cent of Zn.

Silver-white cylinders, granules, pellets or filings with a blue sheen.

Arsenic (2.4.2). 5.0 g complies with limit test A (0.2 ppm). Dissolve in a mixture of the 15 ml of *hydrochloric acid R* and 25 ml of *water R* prescribed.

Zinc, activated. 1096501.

Place the zinc cylinders or pellets to be activated in a conical flask and add a sufficient quantity of a 50 ppm solution of *chloroplatinic acid R* to cover the metal. Allow the metal to remain in contact with the solution for 10 min, wash, drain and dry immediately.

Arsenic. To 5 g of the activated zinc add 15 ml of *hydrochloric acid R*, 25 ml of *water R*, 0.1 ml of *stannous chloride solution R* and 5 ml of *potassium iodide solution R*. Treat as described in limit test A for arsenic (2.4.2). No stain is produced on the *mercuric bromide paper R*.

Activity. Repeat the test for arsenic using the same reagents and adding a solution containing 1 µg of arsenic. An appreciable stain appears on the *mercuric bromide paper R*.

Zinc acetate. $(C_2H_3O_2)_2Zn \cdot 2H_2O$. (M_r 219.5). 1102300. [5970-45-6]. Zinc acetate dihydrate.

Bright white crystals, slightly efflorescent, freely soluble in water, soluble in alcohol. It loses its crystallisation water at 100 °C.

d_{20}^{20} : about 1.735.

mp: about 237 °C.

Zinc acetate solution. 1102301.

Mix 600 ml of *water R* with 150 ml of *glacial acetic acid R*, 54.9 g of *zinc acetate R* and stir to dissolve. Continue stirring while adding 150 ml of *concentrated ammonia R*. Cool to room temperature and adjust with *ammonia R* to pH 6.4. Dilute the mixture to 1 litre with *water R*.

Zinc chloride. 1096600. [7646-85-7].

See *Zinc chloride* (0110).

Zinc chloride-formic acid solution. 1096601.

Dissolve 20 g of *zinc chloride R* in 80 g of an 850 g/l solution of *anhydrous formic acid R*.

Zinc chloride solution, iodinated. 1096602.

Dissolve 20 g of *zinc chloride R* and 6.5 g of *potassium iodide R* in 10.5 ml of *water R*. Add 0.5 g of *iodine R* and shake for 15 min. Filter if necessary.

Storage: protected from light.

Zinc iodide and starch solution. 1096502.

To a solution of 2 g of *zinc chloride R* in 10 ml of *water R* add 0.4 g of *soluble starch R* and heat until the starch has dissolved. After cooling to room temperature add 1.0 ml of a colourless solution containing 0.10 g *zinc R* as filings and 0.2 g of *iodine R* in *water R*. Dilute the solution to 100 ml with *water R* and filter.

Storage: protected from light.

Test for sensitivity. Dilute 0.05 ml of *sodium nitrite solution R* to 50 ml with *water R*. To 5 ml of this solution add 0.1 ml of *dilute sulphuric acid R* and 0.05 ml of the zinc iodide and starch solution and mix. The solution becomes blue.

Zinc oxide. 1096700. [1314-13-2].

See *Zinc oxide* (0252).

Zinc powder. Zn. (A_r 65.4). 1096800. [7440-66-6].

Content: minimum 90.0 per cent of Zn (A_r 65.4).

A very fine, grey powder, soluble in *dilute hydrochloric acid R*.

Zinc sulphate. 1097000. [7446-20-0].

See *Zinc sulphate* (0111).

Zirconyl chloride. A basic salt corresponding approximately to the formula $ZrCl_2O \cdot 8H_2O$. 1097100. [15461-27-5].

Content: minimum 96.0 per cent of $ZrCl_2O \cdot 8H_2O$.

White or almost white, crystalline powder or crystals, freely soluble in water and in alcohol.

Assay. Dissolve 0.600 g in a mixture of 5 ml of *nitric acid R* and 50 ml of *water R*. Add 50.0 ml of *0.1 M silver nitrate* and 3 ml of *dibutyl phthalate R* and shake. Using 2 ml of *ferric ammonium sulphate solution R2* as indicator, titrate with *0.1 M ammonium thiocyanate* until a reddish-yellow colour is obtained.

1 ml of *0.1 M silver nitrate* is equivalent to 16.11 mg of $ZrCl_2O \cdot 8H_2O$.

Zirconyl nitrate. A basic salt corresponding approximately to the formula $\text{ZrO}(\text{NO}_3)_2 \cdot 2\text{H}_2\text{O}$. 1097200. [14985-18-3].

A white powder or crystals, hygroscopic, soluble in water. The aqueous solution is a clear or at most slightly opalescent liquid.

Storage: in an airtight container.

Zirconyl nitrate solution. 1097201.

A 1 g/l solution in a mixture of 40 ml of *water R* and 60 ml of *hydrochloric acid R*.

01/2005:40102

4.1.2. STANDARD SOLUTIONS FOR LIMIT TESTS

Acetaldehyde standard solution (100 ppm $\text{C}_2\text{H}_4\text{O}$). 5000100.

Dissolve 1.0 g of *acetaldehyde R* in *2-propanol R* and dilute to 100.0 ml with the same solvent. Dilute 5.0 ml of the solution to 500.0 ml with *2-propanol R*. Prepare immediately before use.

Acetaldehyde standard solution (100 ppm $\text{C}_2\text{H}_4\text{O}$) R1. 5000101.

Dissolve 1.0 g of *acetaldehyde R* in *water R* and dilute to 100.0 ml with the same solvent. Dilute 5.0 ml of the solution to 500.0 ml with *water R*. Prepare immediately before use.

Aluminium standard solution (200 ppm Al). 5000200.

Dissolve in *water R* a quantity of *aluminium potassium sulphate R* equivalent to 0.352 g of $\text{AlK}(\text{SO}_4)_2 \cdot 12\text{H}_2\text{O}$. Add 10 ml of *dilute sulphuric acid R* and dilute to 100.0 ml with *water R*.

Aluminium standard solution (100 ppm Al). 5000203.

Immediately before use, dilute with *water R* to 10 times its volume a solution containing 8.947 g of *aluminium chloride R* in 1000.0 ml of *water R*.

Aluminium standard solution (10 ppm Al). 5000201.

Immediately before use, dilute with *water R* to 100 times its volume in a solution containing *aluminium nitrate R* equivalent to 1.39 g of $\text{Al}(\text{NO}_3)_3 \cdot 9\text{H}_2\text{O}$ in 100.0 ml.

Aluminium standard solution (2 ppm Al). 5000202.

Immediately before use, dilute with *water R* to 100 times its volume a solution containing *aluminium potassium sulphate R* equivalent to 0.352 g of $\text{AlK}(\text{SO}_4)_2 \cdot 12\text{H}_2\text{O}$ and 10 ml of *dilute sulphuric acid R* in 100.0 ml.

Ammonium standard solution (100 ppm NH_4). 5000300.

Immediately before use, dilute to 25 ml with *water R* 10 ml of a solution containing *ammonium chloride R* equivalent to 0.741 g of NH_4Cl in 1000 ml.

Ammonium standard solution (2.5 ppm NH_4). 5000301.

Immediately before use, dilute with *water R* to 100 times its volume a solution containing *ammonium chloride R* equivalent to 0.741 g of NH_4Cl in 1000.0 ml.

Ammonium standard solution (1 ppm NH_4). 5000302.

Immediately before use, dilute ammonium standard solution (2.5 ppm NH_4) *R* to 2.5 times its volume with *water R*.

Antimony standard solution (100 ppm Sb). 5000401.

Dissolve *antimony potassium tartrate R* equivalent to 0.274 g of $\text{C}_4\text{H}_4\text{KO}_7\text{Sb} \cdot \frac{1}{2}\text{H}_2\text{O}$ in 500 ml of *1 M hydrochloric acid* and dilute the clear solution to 1000 ml with *water R*.

Antimony standard solution (1 ppm Sb). 5000400.

Dissolve *antimony potassium tartrate R* equivalent to 0.274 g of $\text{C}_4\text{H}_4\text{KO}_7\text{Sb} \cdot \frac{1}{2}\text{H}_2\text{O}$ in 20 ml of *hydrochloric acid R1* and dilute the clear solution to 100.0 ml with *water R*. To 10.0 ml of this solution add 200 ml of *hydrochloric acid R1* and dilute to 1000.0 ml with *water R*. To 100.0 ml of this solution add 300 ml of *hydrochloric acid R1* and dilute to 1000.0 ml with *water R*. Prepare the dilute solutions immediately before use.

Arsenic standard solution (10 ppm As). 5000500.

Immediately before use, dilute with *water R* to 100 times its volume a solution prepared by dissolving *arsenious trioxide R* equivalent to 0.330 g of As_2O_3 in 5 ml of *dilute sodium hydroxide solution R* and diluting to 250.0 ml with *water R*.

Arsenic standard solution (1 ppm As). 5000501.

Immediately before use, dilute *arsenic standard solution (10 ppm As) R* to 10 times its volume with *water R*.

Arsenic standard solution (0.1 ppm As). 5000502.

Immediately before use, dilute *arsenic standard solution (1 ppm As) R* to 10 times its volume with *water R*.

Barium standard solution (50 ppm Ba). 5000600.

Immediately before use, dilute with *distilled water R* to 20 times its volume a solution in *distilled water R* containing *barium chloride R* equivalent to 0.178 g of $\text{BaCl}_2 \cdot 2\text{H}_2\text{O}$ in 100.0 ml.

Bismuth standard solution (100 ppm Bi). 5005300.

Dissolve *bismuth R* equivalent to 0.500 g of Bi in 50 ml of *nitric acid R* and dilute to 500.0 ml with *water R*. Dilute the solution to 10 times its volume with *dilute nitric acid R* immediately before use.

Cadmium standard solution (0.1 per cent Cd). 5000700.

Dissolve *cadmium R* equivalent to 0.100 g of Cd in the smallest necessary amount of a mixture of equal volumes of *hydrochloric acid R* and *water R* and dilute to 100.0 ml with a 1 per cent *V/V* solution of *hydrochloric acid R*.

Cadmium standard solution (10 ppm Cd) . 5000701.

Immediately before use, dilute *cadmium standard solution (0.1 per cent Cd) R* to 100 times its volume with a 1 per cent *V/V* solution of *hydrochloric acid R*.

Calcium standard solution (400 ppm Ca). 5000800.

Immediately before use, dilute with *distilled water R* to 10 times its volume a solution in *distilled water R* containing *calcium carbonate R* equivalent to 1.000 g of CaCO_3 and 23 ml of *1 M hydrochloric acid* in 100.0 ml.

Calcium standard solution (100 ppm Ca). 5000801.

Immediately before use, dilute with *distilled water R* to 10 times its volume a solution in *distilled water R* containing *calcium carbonate R* equivalent to 0.624 g of CaCO_3 and 3 ml of *acetic acid R* in 250.0 ml.

Calcium standard solution (100 ppm Ca) R1. 5000804.

Immediately before use, dilute with *water R* to 10 times its volume a solution containing *anhydrous calcium chloride R* equivalent to 2.769 g of CaCl_2 in 1000.0 ml of *dilute hydrochloric acid R*.

Calcium standard solution (100 ppm Ca), alcoholic. 5000802.

Immediately before use, dilute with *alcohol R* to 10 times its volume a solution in *distilled water R* containing *calcium carbonate R* equivalent to 2.50 g of CaCO_3 and 12 ml of *acetic acid R* in 1000.0 ml.

Calcium standard solution (10 ppm Ca). 5000803.

Immediately before use, dilute with *distilled water R* to 100 times its volume a solution in *distilled water R* containing *calcium carbonate R* equivalent to 0.624 g of CaCO_3 and 3 ml of *acetic acid R* in 250.0 ml.

Chloride standard solution (50 ppm Cl). 5004100.

Immediately before use, dilute with *water R* to 10 times its volume a solution containing *sodium chloride R* equivalent to 0.824 g of NaCl in 1000.0 ml.

Chloride standard solution (8 ppm Cl). 5000900.

Immediately before use, dilute with *water R* to 100 times its volume a solution containing *sodium chloride R* equivalent to 1.32 g of NaCl in 1000.0 ml.

Chloride standard solution (5 ppm Cl). 5000901.

Immediately before use, dilute with *water R* to 100 times its volume a solution containing *sodium chloride R* equivalent to 0.824 g of NaCl in 1000.0 ml.

Chromium liposoluble standard solution (1000 ppm Cr). 5004600.

A chromium (metal) organic compound in an oil.

Chromium standard solution (0.1 per cent Cr). 5001002.

Dissolve *potassium dichromate R* equivalent to 2.83 g of $\text{K}_2\text{Cr}_2\text{O}_7$ in *water R* and dilute to 1000.0 ml with the same solvent.

Chromium standard solution (100 ppm Cr). 5001000.

Dissolve *potassium dichromate R* equivalent to 0.283 g of $\text{K}_2\text{Cr}_2\text{O}_7$ in *water R* and dilute to 1000.0 ml with the same solvent.

Chromium standard solution (0.1 ppm Cr). 5001001.

Immediately before use, dilute *chromium standard solution (100 ppm Cr) R* to 1000 times its volume with *water R*.

Cobalt standard solution (100 ppm Co). 5004300.

Dissolve *cobalt nitrate R* equivalent to 0.494 g of $\text{Co}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$ in 500 ml of *1M nitric acid* and dilute the clear solution to 1000 ml with *water R*.

Copper liposoluble standard solution (1000 ppm Cu). 5004700.

A copper (metal) organic compound in an oil.

Copper standard solution (0.1 per cent Cu). 5001100.

Dissolve *copper sulphate R* equivalent to 0.393 g of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ in *water R* and dilute to 100.0 ml with the same solvent.

Copper standard solution (10 ppm Cu). 5001101.

Immediately before use, dilute *copper standard solution (0.1 per cent Cu) R* to 100 times its volume with *water R*.

Copper standard solution (0.1 ppm Cu). 5001102.

Immediately before use, dilute *copper standard solution (10 ppm Cu) R* to 100 times its volume with *water R*.

Ferrocyanide standard solution (100 ppm $\text{Fe}(\text{CN})_6$). 5001200.

Immediately before use, dilute with *water R* to 10 times its volume a solution containing *potassium ferrocyanide R* equivalent to 0.20 g of $\text{K}_4\text{Fe}(\text{CN})_6 \cdot 3\text{H}_2\text{O}$ in 100.0 ml.

Ferricyanide standard solution (50 ppm $\text{Fe}(\text{CN})_6$). 5001300.

Immediately before use, dilute with *water R* to 100 times its volume a solution containing *potassium ferricyanide R* equivalent to 0.78 g of $\text{K}_3\text{Fe}(\text{CN})_6$ in 100.0 ml.

Fluoride standard solution (10 ppm F). 5001400.

Dissolve in *water R* *sodium fluoride R* previously dried at 300 °C for 12 h, equivalent to 0.442 g of NaF, and dilute to 1000.0 ml with the same solvent (1 ml = 0.2 mg F). Store in a polyethylene container. Immediately before use, dilute the solution to 20 times its volume with *water R*.

Fluoride standard solution (1 ppm F). 5001401.

Immediately before use, dilute *fluoride standard solution (10 ppm F) R* to 10 times its volume with *water R*.

Formaldehyde standard solution (5 ppm CH_2O). 5001500.

Immediately before use, dilute with *water R* to 200 times its volume a solution containing 1.0 g of CH_2O per litre prepared from *formaldehyde solution R*.

Germanium standard solution (100 ppm Ge). 5004400.

Dissolve *ammonium hexafluorogermanate (IV) R* equivalent to 0.307 g of $(\text{NH}_4)_2\text{GeF}_6$ in a 0.01 per cent V/V solution of *hydrofluoric acid R*. Dilute the clear solution to 1000 ml with *water R*.

Glyoxal standard solution (20 ppm $\text{C}_2\text{H}_2\text{O}_2$). 5003700.

In a 100 ml graduated flask weigh a quantity of *glyoxal solution R* corresponding to 0.200 g of $\text{C}_2\text{H}_2\text{O}_2$ and make up to volume with *ethanol R*. Immediately before use dilute the solution to 100 times its volume with the same solvent.

Glyoxal standard solution (2 ppm $\text{C}_2\text{H}_2\text{O}_2$). 5003701.

Immediately before use, dilute *glyoxal standard solution (20 ppm $\text{C}_2\text{H}_2\text{O}_2$) R* to 10 times its volume with *ethanol R*.

Hydrogen peroxide standard solution (10 ppm H_2O_2). 5005200.

Dilute 10.0 ml of *dilute hydrogen peroxide solution R* to 300.0 ml with *water R*. Dilute 10.0 ml of this solution to 1000.0 ml with *water R*. Prepare immediately before use.

Iodide standard solution (10 ppm I). 5003800.

Immediately before use, dilute with *water R* to 100 times its volume a solution containing *potassium iodide R* equivalent to 0.131 g of KI in 100.0 ml.

Iron standard solution (0.1 per cent Fe). 5001605.

Dissolve 0.100 g of Fe in the smallest amount necessary of a mixture of equal volumes of *hydrochloric acid R* and *water R* and dilute to 100.0 ml with *water R*.

Iron standard solution (250 ppm Fe). 5001606.

Immediately before use, dilute with *water R* to 40 times its volume a solution containing 4.840 g of *ferric chloride R* in a 150 g/l solution of *hydrochloric acid R* diluted to 100.0 ml.

Iron standard solution (20 ppm Fe). 5001600.

Immediately before use, dilute with *water R* to 10 times its volume a solution containing *ferric ammonium sulphate R* equivalent to 0.863 g of $\text{FeNH}_4(\text{SO}_4)_2 \cdot 12\text{H}_2\text{O}$ and 25 ml of *dilute sulphuric acid R* in 500.0 ml.

Iron standard solution (10 ppm Fe). 5001601.

Immediately before use, dilute with *water R* to 100 times its volume a solution containing *ferrous ammonium sulphate R* equivalent to 7.022 g of $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}$ and 25 ml of *dilute sulphuric acid R* in 1000.0 ml.

Iron standard solution (8 ppm Fe). 5001602.

Immediately before use, dilute with *water R* to 10 times its volume a solution containing 80 mg of *iron R* and 50 ml of *hydrochloric acid R* (220 g/l HCl) in 1000.0 ml.

Iron standard solution (2 ppm Fe). 5001603.

Immediately before use, dilute *iron standard solution (20 ppm Fe) R* to 10 times its volume with *water R*.

Iron standard solution (1 ppm Fe). 5001604.

Immediately before use, dilute *iron standard solution (20 ppm Fe) R* to 20 times its volume with *water R*.

Lead liposoluble standard solution (1000 ppm Pb). 5004800.

A lead (metal) organic compound in an oil.

Lead standard solution (0.1 per cent Pb). 5001700.

Dissolve *lead nitrate R* equivalent to 0.400 g of $\text{Pb}(\text{NO}_3)_2$ in *water R* and dilute to 250.0 ml with the same solvent.

Lead standard solution (0.1 per cent Pb) R1. 5005400.

Dissolve in *dilute lead-free nitric acid R* a quantity of *lead nitrate R* equivalent to 0.400 g of $\text{Pb}(\text{NO}_3)_2$ and dilute to 250.0 ml with the same solvent.

Lead standard solution (100 ppm Pb). 5001701.

Immediately before use, dilute *lead standard solution (0.1 per cent Pb) R* to 10 times its volume with *water R*.

Lead standard solution (10 ppm Pb). 5001702.

Immediately before use, dilute *lead standard solution (100 ppm Pb) R* to 10 times its volume with *water R*.

Lead standard solution (10 ppm Pb) R1. 5001706.

Immediately before use, dilute with *water R* to 10 times its volume a solution containing 0.160 g of *lead nitrate R* in 100 ml of *water R*, to which is added 1 ml of *lead-free nitric acid R* and dilute to 1000.0 ml.

Lead standard solution (10 ppm Pb) R2. 5005401.

Dilute *lead standard solution (0.1 per cent Pb) R1* to 100 times its volume with *dilute lead-free nitric acid R*. Use within 1 week.

Lead standard solution (2 ppm Pb). 5001703.

Immediately before use, dilute *lead standard solution (10 ppm Pb) R* to 5 times its volume with *water R*.

Lead standard solution (1 ppm Pb). 5001704.

Immediately before use, dilute *lead standard solution (10 ppm Pb) R* to 10 times its volume with *water R*.

Lead standard solution (0.5 ppm Pb). 5005402.

Dilute *lead standard solution (10 ppm Pb) R2* to 20 times its volume with *dilute lead-free nitric acid R*. Use within 1 day.

Lead standard solution (0.1 ppm Pb). 5001705.

Immediately before use, dilute *lead standard solution (1 ppm Pb) R* to 10 times its volume with *water R*.

Magnesium standard solution (100 ppm Mg). 5001800.

Immediately before use, dilute with *water R* to 10 times its volume a solution containing *magnesium sulphate R* equivalent to 1.010 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ in 100.0 ml.

Magnesium standard solution (10 ppm Mg). 5001801.

Immediately before use, dilute magnesium standard solution (100 ppm Mg) *R* to 10 times its volume with *water R*.

Magnesium standard solution (10 ppm Mg) R1. 5001802.

Immediately before use, dilute with *water R* to 100 times its volume a solution containing 8.365 g of *magnesium chloride R* in 1000.0 ml of *dilute hydrochloric acid R*.

Manganese standard solution (100 ppm Mn). 5004500.

Dissolve *manganese sulphate R* equivalent to 0.308 g of $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ in 500 ml of *1M nitric acid* and dilute the clear solution to 1000 ml with *water R*.

Mercury standard solution (1000 ppm Hg). 5001900.

Dissolve *mercuric chloride R* equivalent to 1.354 g of HgCl_2 in 50 ml of *dilute nitric acid R* and dilute to 1000.0 ml with *water R*.

Mercury standard solution (10 ppm Hg). 5001901.

Immediately before use, dilute with *water* to 100 times its volume a solution containing *mercuric chloride R* equivalent to 0.338 g of HgCl_2 in 250.0 ml.

Nickel liposoluble standard solution (1000 ppm Ni). 5004900.

A nickel (metal) organic compound in an oil.

Nickel standard solution (10 ppm Ni). 5002000.

Immediately before use, dilute with *water R* to 100 times its volume a solution containing *nickel sulphate R* equivalent to 4.78 g of $\text{NiSO}_4 \cdot 7\text{H}_2\text{O}$ in 1000.0 ml.

Nickel standard solution (0.2 ppm Ni). 5002002.

Immediately before use, dilute *nickel standard solution (10 ppm Ni) R* to 50 times its volume with *water R*.

Nickel standard solution (0.1 ppm Ni). 5002001.

Immediately before use, dilute *nickel standard solution (10 ppm Ni) R* to 100 times its volume with *water R*.

Nitrate standard solution (100 ppm NO_3). 5002100.

Immediately before use, dilute with *water R* to 10 times its volume a solution containing *potassium nitrate R* equivalent to 0.815 g of KNO_3 in 500.0 ml.

Nitrate standard solution (10 ppm NO_3). 5002101.

Immediately before use, dilute *nitrate standard solution (100 ppm NO_3) R* to 10 times its volume with *water R*.

Nitrate standard solution (2 ppm NO_3). 5002102.

Immediately before use, dilute *nitrate standard solution (10 ppm NO_3) R* to 5 times its volume with *water R*.

Palladium standard solution (500 ppm Pd). 5003600.

Dissolve 50.0 mg of *palladium R* in 9 ml of *hydrochloric acid R* and dilute to 100.0 ml with *water R*.

Palladium standard solution (20 ppm Pd). 5003602.

Dissolve 0.333 g of *palladium chloride R* in 2 ml of warm *hydrochloric acid R*. Dilute the solution to 1000.0 ml with a mixture of equal volumes of *dilute hydrochloric acid R* and *water R*. Immediately before use dilute to 10 times its volume with *water R*.

Palladium standard solution (0.5 ppm Pd). 5003601.

Dilute 1 ml of *palladium standard solution (500 ppm Pd) R* to 1000 ml with a mixture of 0.3 volumes of *nitric acid R* and 99.7 volumes of *water R*.

Phosphate standard solution (200 ppm PO₄). 5004200.

Dissolve *potassium dihydrogen phosphate R* equivalent to 0.286 g of KH₂PO₄ in *water R* and dilute to 1000.0 ml with the same solvent.

Phosphate standard solution (5 ppm PO₄). 5002200.

Immediately before use, dilute with *water R* to 100 times its volume a solution containing *potassium dihydrogen phosphate R* equivalent to 0.716 g of KH₂PO₄ in 1000.0 ml.

Platinum standard solution (30 ppm Pt). 5002300.

Immediately before use, dilute with *1 M hydrochloric acid* to 10 times its volume a solution containing 80 mg of *chloroplatinic acid R* in 100.0 ml of *1 M hydrochloric acid*.

Potassium standard solution (600 ppm K). 5005100.

Immediately before use, dilute with *water R* to 20 times its volume a solution containing *dipotassium sulphate R* equivalent to 2.676 g of K₂SO₄ in 100.0 ml.

Potassium standard solution (100 ppm K). 5002400.

Immediately before use, dilute with *water R* to 20 times its volume a solution containing *dipotassium sulphate R* equivalent to 0.446 g of K₂SO₄ in 100.0 ml.

Potassium standard solution (20 ppm K). 5002401.

Immediately before use, dilute *potassium standard solution (100 ppm K) R* to 5 times its volume with *water R*.

Selenium standard solution (100 ppm Se). 5002500.

Dissolve 0.100 g of *selenium R* in 2 ml of *nitric acid R*. Evaporate to dryness. Take up the residue in 2 ml of *water R* and evaporate to dryness; carry out three times. Dissolve the residue in 50 ml of *dilute hydrochloric acid R* and dilute to 1000.0 ml with the same acid.

Selenium standard solution (1 ppm Se). 5002501.

Immediately before use, dilute with *water R* to 40 times its volume a solution containing *selenious acid R* equivalent to 6.54 mg of H₂SeO₃ in 100.0 ml.

Silver standard solution (5 ppm Ag). 5002600.

Immediately before use, dilute with *water R* to 100 times its volume a solution containing *silver nitrate R* equivalent to 0.790 g of AgNO₃ in 1000.0 ml.

Sodium standard solution (200 ppm Na). 5002700.

Immediately before use, dilute with *water R* to 10 times its volume a solution containing *sodium chloride R* equivalent to 0.509 g of NaCl in 100.0 ml.

Sodium standard solution (50 ppm Na). 5002701.

Dilute the *sodium standard solution (200 ppm Na) R* to four times its volume with *water R*.

Strontium standard solution (1.0 per cent Sr). 5003900.

Cover with *water R*, *strontium carbonate R* equivalent to 1.6849 g of SrCO₃. Cautiously add *hydrochloric acid R* until all the solid has dissolved and there is no sign of further effervescence. Dilute to 100.0 ml with *water R*.

Sulphate standard solution (100 ppm SO₄). 5002802.

Immediately before use, dilute with *distilled water R* to 10 times its volume a solution in *distilled water R* containing *dipotassium sulphate R* equivalent to 0.181 g of K₂SO₄ in 100.0 ml.

Sulphate standard solution (10 ppm SO₄). 5002800.

Immediately before use, dilute with *distilled water R* to 100 times its volume a solution in *distilled water R* containing *dipotassium sulphate R* equivalent to 0.181 g of K₂SO₄ in 100.0 ml.

Sulphate standard solution (10 ppm SO₄) R1. 5002801.

Immediately before use, dilute with *alcohol (30 per cent V/V) R* to 100 times its volume a solution containing *dipotassium sulphate R* equivalent to 0.181 g of K₂SO₄ in 100.0 ml of *alcohol (30 per cent V/V) R*.

Sulphite standard solution (80 ppm SO₂). 5005500.

Dissolve 3.150 g of *anhydrous sodium sulphite R* in freshly prepared *distilled water R* and dilute to 100.0 ml with the same solvent. Dilute 0.5 ml to 100.0 ml with freshly prepared *distilled water R*.

Sulphite standard solution (1.5 ppm SO₂). 5002900.

Dissolve *sodium metabisulphite R* equivalent to 0.152 g of Na₂S₂O₅ in *water R* and dilute to 100.0 ml with the same solvent. Dilute 5.0 ml of this solution to 100.0 ml with *water R*. To 3.0 ml of the resulting solution, add 4.0 ml of *0.1 M sodium hydroxide* and dilute to 100.0 ml with *water R*.

Thallium standard solution (10 ppm Tl). 5003000.

Dissolve *thallous sulphate R* equivalent to 0.1235 g of Tl₂SO₄ in a 9 g/l solution of *sodium chloride R* and dilute to 1000.0 ml with the same solution. Dilute 10.0 ml of the solution to 100.0 ml with the 9 g/l solution of *sodium chloride R*.

Tin liposoluble standard solution (1000 ppm Sn). 5005000.

A tin metal organic compound in an oil.

Tin standard solution (5 ppm Sn). 5003100.

Dissolve *tin R* equivalent to 0.500 g of Sn in a mixture of 5 ml of *water R* and 25 ml of *hydrochloric acid R* and dilute to 1000.0 ml with *water R*. Dilute the solution to 100 times its volume with a 2.5 per cent V/V solution of *hydrochloric acid R* immediately before use.

Tin standard solution (0.1 ppm Sn). 5003101.

Immediately before use, dilute *tin standard solution (5 ppm Sn) R* to 50 times its volume with *water R*.

Titanium standard solution (100 ppm Ti). 5003200.

Dissolve 100.0 mg of *titanium R* in 100 ml of *hydrochloric acid R* diluted to 150 ml with *water R*, heating if necessary. Allow to cool and dilute to 1000 ml with *water R*.

Vanadium standard solution (1 g/l V). 5003300.

Dissolve in *water R* *ammonium vanadate R* equivalent to 0.230 g of NH₄VO₃ and dilute to 100.0 ml with the same solvent.

Zinc standard solution (5 mg/ml Zn). 5003400.

Dissolve 3.15 g of *zinc oxide R* in 15 ml of *hydrochloric acid R* and dilute to 500.0 ml with *water R*.

Zinc standard solution (100 ppm Zn). 5003401.

Immediately before use, dilute with *water R* to 10 times its volume a solution containing *zinc sulphate R* equivalent to 0.440 g of ZnSO₄·7H₂O and 1 ml of *acetic acid R* in 100.0 ml.

Zinc standard solution (10 ppm Zn). 5003402.

Immediately before use, dilute *zinc standard solution (100 ppm Zn) R* to 10 times its volume with *water R*.

Zinc standard solution (5 ppm Zn). 5003403.

Immediately before use, dilute *zinc standard solution (100 ppm Zn) R* to 20 times its volume with *water R*.

Zirconium standard solution (1 g/l Zr). 5003500.

Dissolve *zirconyl nitrate R* equivalent to 0.293 g of ZrO(NO₃)₂·2H₂O in a mixture of 2 volumes of *hydrochloric acid R* and 8 volumes of *water R* and dilute to 100.0 ml with the same mixture of solvents.

01/2005:40103 Phosphate buffer solution pH 3.2. 4008100.

To 900 ml of a 4 g/l solution of *sodium dihydrogen phosphate R*, add 100 ml of a 2.5 g/l solution of *phosphoric acid R*. Adjust the pH (2.2.3) if necessary.

Phosphate buffer solution pH 3.2 R1. 4008500.

Adjust a 35.8 g/l solution of *disodium hydrogen phosphate R* to pH 3.2 (2.2.3) with *dilute phosphoric acid R*. Dilute 100.0 ml of the solution to 2000.0 ml with *water R*.

Buffer solution pH 3.5. 4000600.

Dissolve 25.0 g of *ammonium acetate R* in 25 ml of *water R* and add 38.0 ml of *hydrochloric acid R1*. Adjust the pH (2.2.3) if necessary with *dilute hydrochloric acid R* or *dilute ammonia R1*. Dilute to 100.0 ml with *water R*.

Phosphate buffer solution pH 3.5. 4000700.

Dissolve 68.0 g of *potassium dihydrogen phosphate R* in *water R* and dilute to 1000.0 ml with the same solvent. Adjust the pH (2.2.3) with *phosphoric acid R*.

Buffer solution pH 3.6. 4000800.

To 250.0 ml of 0.2 M *potassium hydrogen phthalate R* add 11.94 ml of 0.2 M *hydrochloric acid*. Dilute to 1000.0 ml with *water R*.

Buffer solution pH 3.7. 4000900.

To 15.0 ml of *acetic acid R* add 60 ml of *alcohol R* and 20 ml of *water R*. Adjust to pH 3.7 (2.2.3) by the addition of *ammonia R*. Dilute to 100.0 ml with *water R*.

Buffered copper sulphate solution pH 4.0. 4001000.

Dissolve 0.25 g of *copper sulphate R* and 4.5 g of *ammonium acetate R* in *dilute acetic acid R* and dilute to 100.0 ml with the same solvent.

Acetate buffer solution pH 4.4. 4001100.

Dissolve 136 g of *sodium acetate R* and 77 g of *ammonium acetate R* in *water R* and dilute to 1000.0 ml with the same solvent; add 250.0 ml of *glacial acetic acid R* and mix.

Phthalate buffer solution pH 4.4. 4001200.

Dissolve 2.042 g of *potassium hydrogen phthalate R* in 50 ml of *water R*, add 7.5 ml of 0.2 M *sodium hydroxide* and dilute to 200.0 ml with *water R*.

Acetate buffer solution pH 4.5. 4012500.

Dissolve 77.1 g of *ammonium acetate R* in *water R*. Add 70 ml of *glacial acetic acid R* and dilute to 1000.0 ml with *water R*.

0.05 M Phosphate buffer solution pH 4.5. 4009000.

Dissolve 6.80 g of *potassium dihydrogen phosphate R* in 1000.0 ml of *water R*. The pH (2.2.3) of the solution is 4.5.

Sodium acetate buffer solution pH 4.5. 4010100.

Dissolve 63 g of *anhydrous sodium acetate R* in *water R*, add 90 ml *acetic acid R* and adjust to pH 4.5, and dilute to 1000 ml with *water R*.

Acetate buffer solution pH 4.6. 4001400.

Dissolve 5.4 g of *sodium acetate R* in 50 ml of *water R*, add 2.4 g of *glacial acetic acid R* and dilute to 100.0 ml with *water R*. Adjust the pH (2.2.3) if necessary.

Succinate buffer solution pH 4.6. 4001500.

Dissolve 11.8 g of *succinic acid R* in a mixture of 600 ml of *water R* and 82 ml of 1 M *sodium hydroxide* and dilute to 1000.0 ml with *water R*.

4.1.3. BUFFER SOLUTIONS**Buffered acetone solution. 4000100.**

Dissolve 8.15 g of *sodium acetate R* and 42 g of *sodium chloride R* in *water R*, add 68 ml of 0.1 M *hydrochloric acid* and 150 ml of *acetone R* and dilute to 500 ml with *water R*.

Buffer solution pH 2.0. 4000200.

Dissolve 6.57 g of *potassium chloride R* in *water R* and add 119.0 ml of 0.1 M *hydrochloric acid*. Dilute to 1000.0 ml with *water R*.

Phosphate buffer solution pH 2.0. 4007900.

Dissolve 8.95 g of *disodium hydrogen phosphate R* and 3.40 g of *potassium dihydrogen phosphate R* in *water R* and dilute to 1000.0 ml with the same solvent. If necessary adjust the pH (2.2.3) with *phosphoric acid R*.

Sulphate buffer solution pH 2.0. 4008900.

Dissolve 132.1 g of *ammonium sulphate R* in *water R* and dilute to 500.0 ml with the same solvent (Solution I). Carefully and with constant cooling stir 14 ml of sulphuric acid R into about 400 ml of *water R*; allow to cool and dilute to 500.0 ml with *water R* (Solution II). Mix equal volumes of solutions I and II. Adjust the pH (2.2.3) if necessary.

Buffer solution pH 2.2. 4010500.

Mix of 6.7 ml of *phosphoric acid R* with 50.0 ml of a 4 per cent solution of *dilute sodium hydroxide solution R* and dilute to 1000.0 ml with *water R*.

Buffer solution pH 2.5. 4000300.

Dissolve 100 g of *potassium dihydrogen phosphate R* in 800 ml of *water R*; adjust to pH 2.5 (2.2.3) with *hydrochloric acid R* and dilute to 1000.0 ml with *water R*.

Buffer solution pH 2.5 R1. 4000400.

To 4.9 g of *dilute phosphoric acid R* add 250 ml of *water R*. Adjust the pH (2.2.3) with *dilute sodium hydroxide solution R* and dilute to 500.0 ml with *water R*.

Phosphate buffer solution pH 2.8. 4010600.

Dissolve 7.8 g of *sodium dihydrogen phosphate R* in 900 ml of *water R*, adjust to pH 2.8 (2.2.3) with *phosphoric acid R* and dilute to 1000 ml with the same solvent.

Buffer solution pH 3.0. 4008000.

Dissolve 21.0 g of *citric acid R* in 200 ml of 1 M *sodium hydroxide* and dilute to 1000 ml with *water R*. Dilute 40.3 ml of this solution to 100.0 ml with 0.1 M *hydrochloric acid*.

Phosphate buffer solution pH 3.0. 4000500.

Mix 0.7 ml of *phosphoric acid R* with 100 ml of *water R*. Dilute to 900 ml with the same solvent. Adjust to pH 3.0 (2.2.3) with *strong sodium hydroxide solution R* and dilute to 1000 ml with *water R*.

0.1 M Phosphate buffer solution pH 3.0. 4011500.

Dissolve 12.0 g of *anhydrous sodium dihydrogen phosphate R* in *water R*, adjust the pH (2.2.3) with *dilute phosphoric acid R1* and dilute to 1000 ml with *water R*.

Phosphate buffer solution pH 3.0 R1. 4010000.

Dissolve 3.40 g of *potassium dihydrogen phosphate R* in 900 ml of *water R*. Adjust to pH 3.0 (2.2.3) with *phosphoric acid R* and dilute to 1000.0 ml with *water R*.

Acetate buffer solution pH 4.7. 4001600.

Dissolve 136.1 g of *sodium acetate R* in 500 ml of *water R*. Mix 250 ml of this solution with 250 ml of *dilute acetic acid R*. Shake twice with a freshly prepared, filtered, 0.1 g/l solution of *dithizone R* in *chloroform R*. Shake with *carbon tetrachloride R* until the extract is colourless. Filter the aqueous layer to remove traces of carbon tetrachloride.

Acetate buffer solution pH 5.0. 4009100.

To 120 ml of a 6 g/l solution of *glacial acetic acid R* add 100 ml of 0.1 M *potassium hydroxide* and about 250 ml of *water R*. Mix. Adjust the pH to 5.0 with a 6 g/l solution of *acetic acid R* or with 0.1 M *potassium hydroxide* and dilute to 1000.0 ml with *water R*.

Citrate buffer solution pH 5.0. 4010700.

Prepare a solution containing 20.1 g/l of *citric acid R* and 8.0 g/l of *sodium hydroxide R*. Adjust the pH with *dilute hydrochloric acid R*.

Phosphate buffer solution pH 5.0. 4011300.

Dissolve 2.72 g of *potassium dihydrogen phosphate R* in 800 ml of *water R*. Adjust the pH (2.2.3) with 1 M *potassium hydroxide* and dilute to 1000 ml with *water R*.

Buffer solution pH 5.2. 4001700.

Dissolve 1.02 g of *potassium hydrogen phthalate R* in 30.0 ml of 0.1 M *sodium hydroxide*. Dilute to 100.0 ml with *water R*.

0.067 M Phosphate buffer solution pH 5.4. 4012000.

Mix appropriate volumes of a 23.99 g/l solution of *disodium hydrogen phosphate R* with a 9.12 g/l solution of *sodium dihydrogen phosphate monohydrate R* to obtain pH 5.4 (2.2.3).

Acetate-edetate buffer solution pH 5.5. 4001900.

Dissolve 250 g of *ammonium acetate R* and 15 g *sodium edetate R* in 400 ml of *water R* and add 125 ml of *glacial acetic acid R*.

Buffer solution pH 5.5. 4001800.

Dissolve 54.4 g of *sodium acetate R* in 50 ml of *water R*, heating to 35 °C if necessary. After cooling, slowly add 10 ml of *anhydrous acetic acid R*. Shake and dilute to 100.0 ml with *water R*.

Phosphate buffer solution pH 5.5. 4002000.

Solution I. Dissolve 13.61 g of *potassium dihydrogen phosphate R* in *water R* and dilute to 1000.0 ml with the same solvent.

Solution II. Dissolve 35.81 g of *disodium hydrogen phosphate R* in *water R* and dilute to 1000.0 ml with the same solvent.

Mix 96.4 ml of solution I and 3.6 ml of solution II.

Phosphate-citrate buffer solution pH 5.5. 4008700.

Mix 56.85 ml of a 28.4 g/l solution of *anhydrous disodium hydrogen phosphate R* and 43.15 ml of a 21 g/l solution of *citric acid R*.

Phosphate buffer solution pH 5.6. 4011200.

Solution I. Dissolve 0.908 g of *potassium dihydrogen phosphate R* in *water R* and dilute to 100.0 ml with the same solvent.

Solution II. Dissolve 1.161 g of *dipotassium hydrogen phosphate R* in *water R* and dilute to 100.0 ml with the same solvent.

Mix 94.4 ml of solution I and 5.6 ml of solution II. If necessary, adjust to pH 5.6 (2.2.3) using solution I or solution II.

Phosphate buffer solution pH 5.8. 4002100.

Dissolve 1.19 g of *disodium hydrogen phosphate dihydrate R* and 8.25 g of *potassium dihydrogen phosphate R* in *water R* and dilute to 1000.0 ml with the same solvent.

Acetate buffer solution pH 6.0. 4002200.

Dissolve 100 g of *ammonium acetate R* in 300 ml of *water R*, add 4.1 ml of *glacial acetic acid R*, adjust the pH (2.2.3) if necessary using *ammonia R* or *acetic acid R* and dilute to 500.0 ml with *water R*.

Diethylammonium phosphate buffer solution pH 6.0. 4002300.

Dilute 68 ml of *phosphoric acid R* to 500 ml with *water R*. To 25 ml of this solution add 450 ml of *water R* and 6 ml of *diethylamine R*, adjust to pH 6 ± 0.05 (2.2.3), if necessary, using *diethylamine R* or *phosphoric acid R* and dilute to 500.0 ml with *water R*.

Phosphate buffer solution pH 6.0. 4002400.

Mix 63.2 ml of a 71.5 g/l solution of *disodium hydrogen phosphate R* and 36.8 ml of a 21 g/l solution of *citric acid R*.

Phosphate buffer solution pH 6.0 R1. 4002500.

Dissolve 6.8 g of *sodium dihydrogen phosphate R* in *water R* and dilute to 1000.0 ml with *water R*. Adjust the pH (2.2.3) with *strong sodium hydroxide solution R*.

Phosphate buffer solution pH 6.0 R2. 4002600.

To 250.0 ml of 0.2 M *potassium dihydrogen phosphate R* add 28.5 ml of 0.2 M *sodium hydroxide* and dilute to 1000.0 ml with *water R*.

Phosphate buffer solution pH 6.4. 4002800.

Dissolve 2.5 g of *disodium hydrogen phosphate R*, 2.5 g of *sodium dihydrogen phosphate R* and 8.2 g of *sodium chloride R* in 950 ml of *water R*. Adjust the pH (2.2.3) of the solution to 6.4 with 1 M *sodium hydroxide* or 1 M *hydrochloric acid*, if necessary. Dilute to 1000.0 ml with *water R*.

0.5 M Phthalate buffer solution pH 6.4. 4009200.

Dissolve 100 g of *potassium hydrogen phthalate R* in *water R* and dilute to 1000.0 ml with the same solvent. Adjust the pH (2.2.3) if necessary, using *strong sodium hydroxide solution R*.

Buffer solution pH 6.5. 4002900.

Dissolve 60.5 g of *disodium hydrogen phosphate R* and 46 g of *potassium dihydrogen phosphate R* in *water R*. Add 100 ml of 0.02 M *sodium edetate* and 20 mg of *mercuric chloride R* and dilute to 1000.0 ml with *water R*.

Imidazole buffer solution pH 6.5. 4003000.

Dissolve 6.81 g of *imidazole R*, 1.23 g of *magnesium sulphate R* and 0.73 g of *calcium sulphate R* in 752 ml of 0.1 M *hydrochloric acid*. Adjust the pH (2.2.3) if necessary and dilute to 1000.0 ml with *water R*.

0.1 M phosphate buffer solution pH 6.5. 4010800.

Dissolve 13.80 g of *sodium dihydrogen phosphate monohydrate R* in 900 ml of *distilled water R*. Adjust the pH (2.2.3) using a 400 g/l solution of *sodium hydroxide R*. Dilute to 1000 ml with *distilled water R*.

Buffer solution pH 6.6. 4003100.

To 250.0 ml of 0.2 M *potassium dihydrogen phosphate R* add 89.0 ml of 0.2 M *sodium hydroxide*. Dilute to 1000.0 ml with *water R*.

Phosphate buffered saline pH 6.8. 4003200.

Dissolve 1.0 g of *potassium dihydrogen phosphate R*, 2.0 g of *dipotassium hydrogen phosphate R* and 8.5 g of *sodium chloride R* in 900 ml of *water R*, adjust the pH (2.2.3) if necessary and dilute to 1000.0 ml with the same solvent.

Phosphate buffer solution pH 6.8. 4003300.

Mix 77.3 ml of a 71.5 g/l solution of *disodium hydrogen phosphate R* with 22.7 ml of a 21 g/l solution of *citric acid R*.

Phosphate buffer solution pH 6.8 R1. 4003400.

To 51.0 ml of a 27.2 g/l solution of *potassium dihydrogen phosphate R* add 49.0 ml of a 71.6 g/l solution of *disodium hydrogen phosphate R*. Adjust the pH (2.2.3) if necessary.

Storage: at 2 °C to 8 °C.

1 M tris-hydrochloride buffer solution pH 6.8. 4009300.

Dissolve 60.6 g of *tris(hydroxymethyl)aminomethane R* in 400 ml of *water R*. Adjust the pH (2.2.3) with *hydrochloric acid R* and dilute to 500.0 ml with *water R*.

Buffer solution pH 7.0. 4003500.

To 1000 ml of a solution containing 18 g/l of *disodium hydrogen phosphate R* and 23 g/l of *sodium chloride R* add sufficient (about 280 ml) of a solution containing 7.8 g/l of *sodium dihydrogen phosphate R* and 23 g/l of *sodium chloride R* to adjust the pH (2.2.3). Dissolve in the solution sufficient *sodium azide R* to give a 0.2 g/l solution.

Maleate buffer solution pH 7.0. 4003600.

Dissolve 10.0 g of *sodium chloride R*, 6.06 g of *tris(hydroxymethyl)aminomethane R* and 4.90 g of *maleic anhydride R* in 900 ml of *water R*. Adjust the pH (2.2.3) using a 170 g/l solution of *sodium hydroxide R*. Dilute to 1000.0 ml with *water R*.

Storage: at 2 °C to 8 °C; use within 3 days.

0.025 M Phosphate buffer solution pH 7.0. 4009400.

Mix 1 volume of 0.063 M *phosphate buffer solution pH 7.0 R* with 1.5 volumes of *water R*.

0.03 M Phosphate buffer solution pH 7.0. 4010300.

Dissolve 5.2 g of *dipotassium hydrogen phosphate R* in 900 ml of *water for chromatography R*. Adjust the solution to pH 7.0 ± 0.1 using *phosphoric acid R* and dilute to 1000 ml with *water for chromatography R*.

0.05 M Phosphate buffer solution pH 7.0. 4012400.

Mix 34 ml of *water R* and 100 ml of 0.067 M *phosphate buffer solution pH 7.0 R*.

0.063 M Phosphate buffer solution pH 7.0. 4009500.

Dissolve 5.18 g of *anhydrous disodium hydrogen phosphate R* and 3.65 g of *sodium dihydrogen phosphate monohydrate R* in 950 ml of *water R* and adjust the pH (2.2.3) with *phosphoric acid R*; dilute to 1000.0 ml with *water R*.

0.067 M Phosphate buffer solution pH 7.0. 4003800.

Solution I. Dissolve 0.908 g of *potassium dihydrogen phosphate R* in *water R* and dilute to 100.0 ml with the same solvent.

Solution II. Dissolve 2.38 g of *disodium hydrogen phosphate R* in *water R* and dilute to 100.0 ml with the same solvent.

Mix 38.9 ml of *solution I* and 61.1 ml of *solution II*. Adjust the pH (2.2.3) if necessary.

0.1 M Phosphate buffer solution pH 7.0. 4008200.

Dissolve 1.361 g of *potassium dihydrogen phosphate R* in *water R* and dilute to 100.0 ml with the same solvent. Adjust the pH (2.2.3) using a 35 g/l solution of *disodium hydrogen phosphate R*.

Phosphate buffer solution pH 7.0. 4003700.

Mix 82.4 ml of a 71.5 g/l solution of *disodium hydrogen phosphate R* with 17.6 ml of a 21 g/l solution of *citric acid R*.

Phosphate buffer solution pH 7.0 R1. 4003900.

Mix 250.0 ml of 0.2 M *potassium dihydrogen phosphate R* and 148.2 ml of a 8 g/l solution of *sodium hydroxide R*, adjust the pH (2.2.3) if necessary. Dilute to 1000.0 ml with *water R*.

Phosphate buffer solution pH 7.0 R2. 4004000.

Mix 50.0 ml of a 136 g/l solution of *potassium dihydrogen phosphate R* with 29.5 ml of 1 M *sodium hydroxide* and dilute to 100.0 ml with *water R*. Adjust the pH (2.2.3) to 7.0 ± 0.1.

Phosphate buffer solution pH 7.0 R3. 4008600.

Dissolve 5 g of *potassium dihydrogen phosphate R* and 11 g of *dipotassium hydrogen phosphate R* in 900 ml of *water R*. Adjust to pH 7.0 (2.2.3) with *dilute phosphoric acid R* or *dilute sodium hydroxide solution R*. Dilute to 1000 ml with *water R* and mix.

Phosphate buffer solution pH 7.0 R4. 4010200.

Dissolve 28.4 g of *anhydrous disodium hydrogen phosphate R* and 18.2 g of *potassium dihydrogen phosphate R* in *water R* and dilute to 500 ml with the same solvent.

Phosphate buffer solution pH 7.0 R5. 4011400.

Dissolve 28.4 g of *anhydrous disodium hydrogen phosphate R* in 800 ml of *water R*. Adjust the pH (2.2.3) using a 30 per cent *m/m* solution of *phosphoric acid R* and dilute to 1000 ml with *water R*.

Tetrabutylammonium buffer solution pH 7.0. 4010900.

Dissolve 6.16 g of *ammonium acetate R* in a mixture of 15 ml of *tetrabutylammonium hydroxide solution (400 g/l) R* and 185 ml of *water R*. Adjust the pH (2.2.3) with *nitric acid R*.

Buffered salt solution pH 7.2. 4004300.

Dissolve in *water R* 8.0 g of *sodium chloride R*, 0.2 g of *potassium chloride R*, 0.1 g of *anhydrous calcium chloride R*, 0.1 g of *magnesium chloride R*, 3.18 g of *disodium hydrogen phosphate R* and 0.2 g of *potassium dihydrogen phosphate R* and dilute to 1000.0 ml with *water R*.

Buffer solution pH 7.2. 4004100.

To 250.0 ml of 0.2 M *potassium dihydrogen phosphate R* add 175.0 ml of 0.2 M *sodium hydroxide*. Dilute to 1000.0 ml with *water R*. Adjust the pH (2.2.3) if necessary.

Phosphate-albumin buffered saline pH 7.2. 4004400.

Dissolve 10.75 g of *disodium hydrogen phosphate R*, 7.6 g of *sodium chloride R* and 10 g of *bovine albumin R* in *water R* and dilute to 1000.0 ml with the same solvent. Immediately before use adjust the pH (2.2.3) using *dilute sodium hydroxide solution R* or *dilute phosphoric acid R*.

Phosphate-albumin buffered saline pH 7.2 R1. 4009600.

Dissolve 10.75 g of *disodium hydrogen phosphate R*, 7.6 g of *sodium chloride R* and 1 g of *bovine albumin R* in *water R* and dilute to 1000.0 ml with the same solvent. Immediately before use adjust the pH (2.2.3) using *dilute sodium hydroxide solution R* or *dilute phosphoric acid R*.

Phosphate buffer solution pH 7.2. 4004200.

Mix 87.0 ml of a 71.5 g/l solution of *disodium hydrogen phosphate R* with 13.0 ml of a 21 g/l solution of *citric acid R*.

Imidazole buffer solution pH 7.3. 4004500.

Dissolve 3.4 g of *imidazole R* and 5.8 g of *sodium chloride R* in *water R*, add 18.6 ml of 1 M *hydrochloric acid* and dilute to 1000.0 ml with *water R*. Adjust the pH (2.2.3) if necessary.

Barbital buffer solution pH 7.4. 4004700.

Mix 50 ml of a solution in *water R* containing 19.44 g/l of *sodium acetate R* and 29.46 g/l of *barbital sodium R* with 50.5 ml of 0.1 M *hydrochloric acid*, add 20 ml of an 85 g/l of *sodium chloride R* and dilute to 250 ml with *water R*.

Buffer solution pH 7.4. 4004600.

Dissolve 0.6 g of *potassium dihydrogen phosphate R*, 6.4 g of *disodium hydrogen phosphate R* and 5.85 g of *sodium chloride R* in *water R*, and dilute to 1000.0 ml with the same solvent. Adjust the pH (2.2.3) if necessary.

Phosphate buffered saline pH 7.4. 4005000.

Dissolve 2.38 g of *disodium hydrogen phosphate R*, 0.19 g of *potassium dihydrogen phosphate R* and 8.0 g of *sodium chloride R* in *water*. Dilute to 1000.0 ml with the same solvent. Adjust the pH (2.2.3) if necessary.

Phosphate buffer solution pH 7.4. 4004800.

Add 250.0 ml of 0.2 M *potassium dihydrogen phosphate R* to 393.4 ml of 0.1 M *sodium hydroxide*.

Tris(hydroxymethyl)aminomethane buffer solution pH 7.4. 4012100.

Dissolve 30.3 g of *tris(hydroxymethyl)aminomethane R* in approximately 200 ml of *water R*. Add 183 ml of 1 M *hydrochloric acid*. Dilute to 500.0 ml with *water R*. Note: the pH is 7.7-7.8 at room temperature and 7.4 at 37 °C. This solution is stable for several months at 4 °C.

Tris(hydroxymethyl)aminomethane sodium chloride buffer solution pH 7.4. 4004900.

Dissolve 6.08 g of *tris(hydroxymethyl)aminomethane R*, 8.77 g of *sodium chloride R* in 500 ml of *distilled water R*. Add 10.0 g of *bovine albumin R*. Adjust the pH (2.2.3) using *hydrochloric acid R*. Dilute to 1000.0 ml with *distilled water R*.

Tris(hydroxymethyl)aminomethane sodium chloride buffer solution pH 7.4 R1. 4012200.

Dissolve 0.1 g of *bovine albumin R* in a mixture containing 2 ml of *tris(hydroxymethyl)aminomethane buffer solution pH 7.4 R* and 50 ml of a 5.84 mg/ml solution of *sodium chloride R*. Dilute to 100.0 ml with *water R*.

Borate buffer solution pH 7.5. 4005200.

Dissolve 2.5 g of *sodium chloride R*, 2.85 g of *disodium tetraborate R* and 10.5 g of *boric acid R* in *water R* and dilute to 1000.0 ml with the same solvent. Adjust the pH (2.2.3) if necessary.

Storage: at 2 °C to 8 °C.

Buffer (HEPES) solution pH 7.5. 4009700.

Dissolve 2.38 g of 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulphonic acid *R* in about 90 ml of *water R*. Adjust the pH to 7.5 with *sodium hydroxide solution R*. Dilute to 100 ml with *water R*.

0.2 M Phosphate buffer solution pH 7.5. 4005400.

Dissolve 27.22 g of *potassium dihydrogen phosphate R* in 930 ml of *water R*, adjust to pH 7.5 (2.2.3) with a 300 g/l solution of *potassium hydroxide R* and dilute to 1000.0 ml with *water R*.

0.33 M Phosphate buffer solution pH 7.5. 4005300.

Solution I. Dissolve 119.31 g of *disodium hydrogen phosphate R* in *water R* and dilute to 1000.0 ml with the same solvent.

Solution II. Dissolve 45.36 g of *potassium dihydrogen phosphate R* in *water R* and dilute to 1000.0 ml with the same solvent.

Mix 85 ml of solution I and 15 ml of solution II. Adjust the pH (2.2.3) if necessary.

0.05 M Tris-hydrochloride buffer solution pH 7.5. 4005600.

Dissolve 6.057 g of *tris(hydroxymethyl)aminomethane R* in *water R* and adjust the pH (2.2.3) with *hydrochloric acid R*. Dilute to 1000.0 ml with *water R*.

Tris(hydroxymethyl)aminomethane buffer solution pH 7.5. 4005500.

Dissolve 7.27 g of *tris(hydroxymethyl)aminomethane R* and 5.27 g of *sodium chloride R* in *water R*, and adjust the pH (2.2.3) if necessary. Dilute to 1000.0 ml with *water R*.

Sodium citrate buffer solution pH 7.8 (0.034 M sodium citrate, 0.101 M sodium chloride). 4009800.

Dissolve 10.0 g of *sodium citrate R* and 5.90 g of *sodium chloride R* in 900 ml of *water R*. Adjust the pH (2.2.3) by addition of *hydrochloric acid R* and dilute to 1000 ml with *water R*.

0.0015 M Borate buffer solution pH 8.0. 4006000.

Dissolve 0.572 g of *disodium tetraborate R* and 2.94 g of *calcium chloride R* in 800 ml of *water R*. Adjust the pH (2.2.3) with 1 M *hydrochloric acid*. Dilute to 1000.0 ml with *water R*.

Buffer solution pH 8.0. 4005900.

To 50.0 ml of 0.2 M *potassium dihydrogen phosphate R* add 46.8 ml of 0.2 M *sodium hydroxide*. Dilute to 200.0 ml with *water R*.

Buffer solution pH 8.0 R1. 4010400.

Dissolve 20 g of *dipotassium hydrogen phosphate R* in 900 ml of *water R*. Adjust the pH (2.2.3) with *phosphoric acid R*. Dilute to 1000 ml with *water R*.

0.02 M Phosphate buffer solution pH 8.0. 4006100.

To 50.0 ml of 0.2 M *potassium dihydrogen phosphate R* add 46.8 ml of 0.2 M *sodium hydroxide*. Dilute to 500.0 ml with *water R*.

0.1 M Phosphate buffer solution pH 8.0. 4008400.

Dissolve 0.523 g of *potassium dihydrogen phosphate R* and 16.73 g of *dipotassium hydrogen phosphate R* in *water R* and dilute to 1000.0 ml with the same solvent.

1 M Phosphate buffer solution pH 8.0. 4007800.

Dissolve 136.1 g of *potassium dihydrogen phosphate R* in *water R*, adjust the pH (2.2.3) with 1 M *sodium hydroxide*. Dilute to 1000.0 ml with *water R*.

Tris-hydrochloride buffer solution pH 8.0. 4012300.

Dissolve 1.21 g of *tris(hydroxymethyl)aminomethane R* and 29.4 mg of *calcium chloride R* in *water R*. Adjust the pH (2.2.3) with *1 M hydrochloric acid* and dilute to 100.0 ml with *water R*.

Tris(hydroxymethyl)aminomethane buffer solution pH 8.1. 4006200.

Dissolve 0.294 g of *calcium chloride R* in 40 ml of *tris(hydroxymethyl)aminomethane solution R* and adjust the pH (2.2.3) with *1 M hydrochloric acid*. Dilute to 100.0 ml with *water R*.

Tris-glycine buffer solution pH 8.3. 4006300.

Dissolve 6.0 g of *tris(hydroxymethyl)aminomethane R* and 28.8 g of *glycine R* in *water R* and dilute to 1000.0 ml with the same solvent. Dilute 1 volume to 10 volumes with *water R* immediately before use.

Tris-hydrochloride buffer solution pH 8.3. 4011800.

Dissolve 9.0 g of *tris(hydroxymethyl)aminomethane R* in 2.9 litres of *water R*. Adjust the pH (2.2.3) with *1 M hydrochloric acid*. Adjust the volume to 3 litres with *water R*.

Barbital buffer solution pH 8.4. 4006400.

Dissolve 8.25 g of *barbital sodium R* in *water R* and dilute to 1000.0 ml with the same solvent.

Tris-EDTA BSA buffer solution pH 8.4. 4006500.

Dissolve 6.1 g of *tris(hydroxymethyl)aminomethane R*, 2.8 g of *sodium edetate R*, 10.2 g of *sodium chloride R* and 10 g of *bovine albumin R* in *water R*, adjust to pH 8.4 (2.2.3) using *1 M hydrochloric acid* and dilute to 1000.0 ml with *water R*.

Tris(hydroxymethyl)aminomethane EDTA buffer solution pH 8.4. 4006600.

Dissolve 5.12 g of *sodium chloride R*, 3.03 g of *tris(hydroxymethyl)aminomethane R* and 1.40 g of *sodium edetate R* in 250 ml of *distilled water R*. Adjust the pH (2.2.3) to 8.4 using *hydrochloric acid R*. Dilute to 500.0 ml with *distilled water R*.

Tris acetate buffer solution pH 8.5. 4006700.

Dissolve 0.294 g of *calcium chloride R* and 12.11 g of *tris(hydroxymethyl)aminomethane R* in *water R*. Adjust the pH (2.2.3) with *acetic acid R*. Dilute to 1000.0 ml with *water R*.

Barbital buffer solution pH 8.6 R1. 4006900.

Dissolve in *water R* 1.38 g of *barbital R*, 8.76 g of *barbital sodium R* and 0.38 g of *calcium lactate R* and dilute to 1000.0 ml with the same solvent.

1.5 M tris-hydrochloride buffer solution pH 8.8. 4009900.

Dissolve 90.8 g of *tris(hydroxymethyl)aminomethane R* in 400 ml of *water R*. Adjust the pH (2.2.3) with *hydrochloric acid R* and dilute to 500.0 ml with *water R*.

Buffer (phosphate) solution pH 9.0. 4008300.

Dissolve 1.74 g of *potassium dihydrogen phosphate R* in 80 ml of *water R*, adjust the pH (2.2.3) with *1 M potassium hydroxide* and dilute to 100.0 ml with *water R*.

Buffer solution pH 9.0. 4007000.

Solution I. Dissolve 6.18 g of *boric acid R* in *0.1 M potassium chloride R* and dilute to 1000.0 ml with the same solvent.

Solution II. *0.1 M sodium hydroxide.*

Mix 1000.0 ml of solution I and 420.0 ml of solution II.

Buffer solution pH 9.0 R1. 4007100.

Dissolve 6.20 g of *boric acid R* in 500 ml of *water R* and adjust the pH (2.2.3) with *1 M sodium hydroxide* (about 41.5 ml). Dilute to 1000.0 ml with *water R*.

Ammonium chloride buffer solution pH 9.5. 4007200.

Dissolve 33.5 g of *ammonium chloride R* in 150 ml of *water R*, add 42.0 ml of *concentrated ammonia R* and dilute to 250.0 ml with *water R*.

Storage: in a polyethylene container.

Ammonium chloride buffer solution pH 10.0. 4007300.

Dissolve 5.4 g of *ammonium chloride R* in 20 ml of *water R*, add 35.0 ml of *ammonia R* and dilute to 100.0 ml with *water R*.

Diethanolamine buffer solution pH 10.0. 4007500.

Dissolve 96.4 g of *diethanolamine R* in *water R* and dilute to 400 ml with the same solvent. Add 0.5 ml of an 186 g/l solution of *magnesium chloride R* and adjust the pH (2.2.3) with *1 M hydrochloric acid*. Dilute to 500.0 ml with *water R*.

0.1 M Ammonium carbonate buffer solution pH 10.3. 4011900.

Dissolve 7.91 g of *ammonium carbonate R* in 800 ml of *water R*. Adjust the pH (2.2.3) with *dilute sodium hydroxide solution R*. Dilute to 1000.0 ml with *water R*.

Ammonium chloride buffer solution pH 10.4. 4011000.

Dissolve 70 g of *ammonium chloride R* in 200 ml of *water R*, add 330 ml of *concentrated ammonia R* and dilute to 1000.0 ml with *water R*. If necessary, adjust to pH 10.4 with *ammonia R*.

Borate buffer solution pH 10.4. 4011100.

Dissolve 24.64 g of *boric acid R* in 900 ml of *distilled water R*. Adjust the pH (2.2.3) using a 400 g/l solution of *sodium hydroxide R*. Dilute to 1000 ml with *distilled water R*.

Buffer solution pH 10.9. 4007600.

Dissolve 6.75 g of *ammonium chloride R* in *ammonia R* and dilute to 100.0 ml with the same solvent.

Total-ionic-strength-adjustment buffer. 4007700.

Dissolve 58.5 g of *sodium chloride R*, 57.0 ml of *glacial acetic acid R*, 61.5 g of *sodium acetate R* and 5.0 g of *cyclohexylene-dinitrilotetra-acetic acid R* in *water R* and dilute to 500.0 ml with the same solvent. Adjust to pH 5.0 to 5.5 with a 335 g/l solution of *sodium hydroxide R* and dilute to 1000.0 ml with *distilled water R*.

Total-ionic-strength-adjustment buffer R1. 4008800.

Solution (a). Dissolve 210 g of *citric acid R* in 400 ml of *distilled water R*. Adjust to pH 7.0 (2.2.3) with *concentrated ammonia R*. Dilute to 1000.0 ml with *distilled water R*.

Solution (b). Dissolve 132 g of *ammonium phosphate R* in *distilled water R* and dilute to 1000.0 ml with the same solvent.

Solution (c). To a suspension of 292 g of (ethylenedinitrilo)tetra-acetic acid *R* in about 500 ml of distilled water *R*, add about 200 ml of concentrated ammonia *R* to dissolve. Adjust the pH to 6 to 7 (2.2.3) with concentrated ammonia *R*. Dilute to 1000.0 ml with distilled water *R*.

Mix equal volumes of solution (a), (b), and (c) and adjust to pH 7.5 with concentrated ammonia *R*.

4.2. VOLUMETRIC ANALYSIS

01/2005:40201

4.2.1. PRIMARY STANDARDS FOR VOLUMETRIC SOLUTIONS

Primary standards for volumetric solutions are indicated by the suffix RV. Primary standards of suitable quality may be obtained from commercial sources or prepared by the following methods.

Benzoic acid. $C_7H_6O_2$. (M_r 122.1). 2000200. [65-85-0].
Sublime benzoic acid *R* in a suitable apparatus.

Potassium bromate. $KBrO_3$. (M_r 167.0). 2000300. [7758-01-2].
Crystallise potassium bromate *R* from boiling water *R*. Collect the crystals and dry to constant mass at 180 °C.

Potassium hydrogen phthalate. $C_8H_5KO_4$. (M_r 204.2). 2000400. [877-24-7].
Recrystallise potassium hydrogen phthalate *R* from boiling water *R*, collect the crystals at a temperature above 35 °C and dry to constant mass at 110 °C.

Sodium carbonate. Na_2CO_3 . (M_r 106.0). 2000500. [497-19-8].
Filter at room temperature a saturated solution of sodium carbonate *R*. Introduce slowly into the filtrate a stream of carbon dioxide *R* with constant cooling and stirring. After about 2 h, collect the precipitate on a sintered-glass filter. Wash the filter with iced water *R* containing carbon dioxide. After drying at 100 °C to 105 °C, heat to constant mass at 270 °C to 300 °C, stirring from time to time.

Sodium chloride. $NaCl$. (M_r 58.44). 2000600. [7647-14-5].
To 1 volume of a saturated solution of sodium chloride *R* add 2 volumes of hydrochloric acid *R*. Collect the crystals formed and wash with hydrochloric acid *R1*. Remove the hydrochloric acid by heating on a water-bath and dry the crystals to constant mass at 300 °C.

Sulphanilic acid. $C_6H_7NO_3S$. (M_r 173.2). 2000700. [121-57-3].
Recrystallise sulphanilic acid *R* from boiling water *R*. Filter and dry to constant mass at 100 °C to 105 °C.

Zinc. Zn . (M_r 65.4). 2000800. [7440-66-6].
Use a quality containing not less than 99.9 per cent of Zn.

01/2005:40202

4.2.2. VOLUMETRIC SOLUTIONS

Volumetric solutions are prepared according to the usual chemical analytical methods. The accuracy of the apparatus used is verified to ensure that it is appropriate for the intended use.

The concentration of volumetric solutions is indicated in terms of molarity. Molarity expresses, as the number of moles, the amount of substance dissolved in 1 litre of solution. A solution which contains x moles of substance per litre is said to be x M.

Volumetric solutions do not differ from the prescribed strength by more than 10 per cent. The molarity of the volumetric solutions is determined by an appropriate number of titrations. The repeatability does not exceed 0.2 per cent (relative standard deviation).

Volumetric solutions are standardised by the methods described below. When a volumetric solution is to be used in an assay in which the end-point is determined by an electrochemical process (for example, amperometry or potentiometry) the solution is standardised by the same method. The composition of the medium in which a volumetric solution is standardised should be the same as that in which it is to be used.

Solutions more dilute than those described are obtained by diluting the latter with carbon dioxide-free water *R*. The correction factors of these solutions are the same as those from which the dilutions were prepared.

0.1 M Acetic acid. 3008900.

Dilute 6.0 g of glacial acetic acid *R* to 1000.0 ml with water *R*.

Standardisation. To 25.0 ml of acetic acid add 0.5 ml of phenolphthalein solution *R* and titrate with 0.1 M sodium hydroxide.

0.1 M Ammonium and cerium nitrate. 3000100.

Shake for 2 min a solution containing 56 ml of sulphuric acid *R* and 54.82 g of ammonium and cerium nitrate *R*, add five successive quantities, each of 100 ml, of water *R*, shaking after each addition. Dilute the clear solution to 1000.0 ml with water *R*. Standardise the solution after 10 days.

Standardisation. To 25.0 ml of the ammonium and cerium nitrate solution add 2.0 g of potassium iodide *R* and 150 ml of water *R*. Titrate immediately with 0.1 M sodium thiosulphate, using 1 ml of starch solution *R* as indicator.
Storage: protected from light.

0.01 M Ammonium and cerium nitrate. 3000200.

To 100.0 ml of 0.1 M ammonium and cerium nitrate add, with cooling, 30 ml of sulphuric acid *R* and dilute to 1000.0 ml with water *R*.

0.1 M Ammonium and cerium sulphate. 3000300.

Dissolve 65.0 g of ammonium and cerium sulphate *R* in a mixture of 500 ml of water *R* and 30 ml of sulphuric acid *R*. Allow to cool and dilute to 1000.0 ml with water *R*.

Standardisation. To 25.0 ml of the ammonium and cerium sulphate solution add 2.0 g of potassium iodide *R* and 150 ml of water *R*. Titrate immediately with 0.1 M sodium thiosulphate, using 1 ml of starch solution *R* as indicator.

0.01 M Ammonium and cerium sulphate. 3000400.

To 100.0 ml of 0.1 M ammonium and cerium sulphate add, with cooling, 30 ml of sulphuric acid *R* and dilute to 1000.0 ml with water *R*.

0.1 M Ammonium thiocyanate. 3000500.

Dissolve 7.612 g of ammonium thiocyanate *R* in water *R* and dilute to 1000.0 ml with the same solvent.

Standardisation. To 20.0 ml of 0.1 M silver nitrate add 25 ml of water *R*, 2 ml of dilute nitric acid *R* and 2 ml of ferric ammonium sulphate solution *R2*. Titrate with the ammonium thiocyanate solution until a reddish-yellow colour is obtained.

0.1 M Barium chloride. 3000600.

Dissolve 24.4 g of *barium chloride R* in *water R* and dilute to 1000.0 ml with the same solvent.

Standardisation. To 10.0 ml of the barium chloride solution add 60 ml of *water R*, 3 ml of *concentrated ammonia R* and 0.5 mg to 1 mg of *phthalein purple R*. Titrate with *0.1 M sodium edetate*. When the solution begins to decolorise, add 50 ml of *alcohol R* and continue the titration until the blue-violet colour disappears.

0.05 M Barium perchlorate. 3000700.

Dissolve 15.8 g of *barium hydroxide R* in a mixture of 7.5 ml of *perchloric acid R* and 75 ml of *water R*, adjust the solution to pH 3 by adding *perchloric acid R* and filter if necessary. Add 150 ml of *alcohol R* and dilute to 250 ml with *water R*. Dilute to 1000.0 ml with *buffer solution pH 3.7 R*.

Standardisation. To 5.0 ml of *0.05 M sulphuric acid* add 5 ml of *water R*, 50 ml of *buffer solution pH 3.7 R* and 0.5 ml of *alizarin s solution R*. Titrate with the barium perchlorate solution until an orange-red colour appears. Standardise immediately before use.

0.025 M Barium perchlorate. 3009600.

Dilute 500.0 ml of *0.05 M barium perchlorate* to 1000.0 ml with *buffer solution pH 3.7 R*.

0.004 M Benzethonium chloride. 3000900.

Dissolve in *water R* 1.792 g of *benzethonium chloride R*, previously dried to constant mass at 100 °C to 105 °C, and dilute to 1000.0 ml with the same solvent.

Standardisation. Calculate the molarity of the solution from the content of $C_{27}H_{42}ClNO_2$ in the dried benzethonium chloride determined as follows. Dissolve 0.350 g of the dried substance in 30 ml of *anhydrous acetic acid R* and add 6 ml of *mercuric acetate solution R*. Titrate with *0.1 M perchloric acid*, using 0.05 ml of *crystal violet solution R* as indicator. Carry out a blank titration.

1 ml of *0.1 M perchloric acid* is equivalent to 44.81 mg of $C_{27}H_{42}ClNO_2$.

0.0167 M Bromide-bromate. 3001000.

Dissolve 2.7835 g of *potassium bromate RV* and 13 g of *potassium bromide R* in *water R* and dilute to 1000.0 ml with the same solvent.

0.1 M Cerium sulphate. 3001100.

Dissolve 40.4 g of *cerium sulphate R* in a mixture of 500 ml of *water R* and 50 ml of *sulphuric acid R*. Allow to cool and dilute to 1000.0 ml with *water R*.

Standardisation. To 25.0 ml of the cerium sulphate solution, add 2.0 g of *potassium iodide R* and 150 ml of *water R*. Titrate immediately with *0.1 M sodium thiosulphate* using 1 ml of *starch solution R* as indicator.

0.02 M Copper sulphate. 3001200.

Dissolve 5.0 g of *copper sulphate R* in *water R* and dilute to 1000.0 ml with the same solvent.

Standardisation. To 20.0 ml of the copper sulphate solution add 2 g of *sodium acetate R* and 0.1 ml of *pyridylazonaphthol solution R*. Titrate with *0.02 M sodium edetate* until the colour changes from violet-blue to bright green. Titrate slowly towards the end of the titration.

0.1 M Ferric ammonium sulphate. 3001300.

Dissolve 50.0 g of *ferric ammonium sulphate R* in a mixture of 6 ml of *sulphuric acid R* and 300 ml of *water R* and dilute to 1000.0 ml with *water R*.

Standardisation. To 25.0 ml of the ferric ammonium sulphate solution, add 3 ml of *hydrochloric acid R* and 2 g of *potassium iodide R*. Allow to stand for 10 min. Titrate with *0.1 M sodium thiosulphate*, using 1 ml of *starch solution R* as indicator.

1 ml of *0.1 M sodium thiosulphate* is equivalent to 48.22 mg of $FeNH_4(SO_4)_2 \cdot 12H_2O$.

0.1 M Ferrous sulphate. 3001400.

Dissolve 27.80 g of *ferrous sulphate R* in 500 ml of *dilute sulphuric acid R* and dilute to 1000.0 ml with *water R*.

Standardisation. To 25.0 ml of the ferrous sulphate solution add 3 ml of *phosphoric acid R* and titrate immediately with *0.02 M potassium permanganate*. Standardise immediately before use.

6 M Hydrochloric acid. 3001500.

Dilute 618.0 g of *hydrochloric acid R* to 1000.0 ml with *water R*.

3 M Hydrochloric acid. 3001600.

Dilute 309.0 g of *hydrochloric acid R* to 1000.0 ml with *water R*.

2 M Hydrochloric acid. 3001700.

Dilute 206.0 g of *hydrochloric acid R* to 1000.0 ml with *water R*.

1 M Hydrochloric acid. 3001800.

Dilute 103.0 g of *hydrochloric acid R* to 1000.0 ml with *water R*.

Standardisation. Dissolve 1.000 g of *sodium carbonate RV* in 50 ml of *water R*, add 0.1 ml of *methyl orange solution R* and titrate with the hydrochloric acid until the solution just becomes yellowish-red. Boil for 2 min. The solution reverts to yellow. Cool and continue the titration until a yellowish-red colour is obtained.

1 ml of *1 M hydrochloric acid* is equivalent to 53.00 mg of Na_2CO_3 .

0.1 M Hydrochloric acid. 3002100.

Dilute 100.0 ml of *1 M hydrochloric acid* to 1000.0 ml with *water R*.

Standardisation. Carry out the titration described for *1 M hydrochloric acid* using 0.100 g of *sodium carbonate RV* dissolved in 20 ml of *water R*.

1 ml of *0.1 M hydrochloric acid* is equivalent to 5.30 mg of Na_2CO_3 .

0.1 M Hydrochloric acid, alcoholic. 3008800.

Dilute 9.0 ml of *hydrochloric acid R* to 1000.0 ml with *aldehyde-free alcohol R*.

0.5 M Iodine. 3009400.

Dissolve 127 g of *iodine R* and 200 g of *potassium iodide R* in *water R* and dilute to 1000.0 ml with the same solvent.

Standardisation. To 2.0 ml of the iodine solution add 1 ml of *dilute acetic acid R* and 50 ml of *water R*. Titrate with *0.1 M sodium thiosulphate*, using *starch solution R* as indicator.

Storage: protected from light.

0.05 M Iodine. 3002700.

Dissolve 12.7 g of *iodine R* and 20 g of *potassium iodide R* in *water R* and dilute to 1000.0 ml with the same solvent.

Standardisation. To 20.0 ml of the iodine solution add 1 ml of *dilute acetic acid R* and 30 ml of *water R*. Titrate with *0.1 M sodium thiosulphate*, using *starch solution R* as indicator.

Storage: protected from light.

0.01 M Iodine. 3002900.

Add 0.3 g of *potassium iodide R* to 20.0 ml of 0.05 M iodine and dilute to 100.0 ml with *water R*.

0.1 M Lead nitrate. 3003100.

Dissolve 33 g of *lead nitrate R* in *water R* and dilute to 1000.0 ml with the same solvent.

Standardisation. Take 20.0 ml of the lead nitrate solution and carry out the determination of lead by complexometry (2.5.11).

0.05 M Lead nitrate. 3009700.

Dilute 50.0 ml of 0.1 M *Lead nitrate* to 100.0 ml with *water R*.

0.1 M Lithium methoxide. 3003300.

Dissolve 0.694 g of *lithium R* in 150 ml of *anhydrous methanol R* and dilute to 1000.0 ml with *toluene R*.

Standardisation. To 10 ml of *dimethylformamide R* add 0.05 ml of a 3 g/l solution of *thymol blue R* in *methanol R* and titrate with the lithium methoxide solution until a pure blue colour is obtained. Immediately add 0.200 g of *benzoic acid RV*. Stir to effect solution and titrate with the lithium methoxide solution until the pure blue colour is again obtained. Protect the solution from atmospheric carbon dioxide throughout the titration. From the volume of titrant used in the second titration ascertain the exact strength of the lithium methoxide solution. Standardise immediately before use.

1 ml of 0.1 M *lithium methoxide* is equivalent to 12.21 mg of $C_7H_6O_2$.

0.1 M Magnesium chloride. 3003400.

Dissolve 20.33 g of *magnesium chloride R* in *water R* and dilute to 1000.0 ml with the same solvent.

Standardisation. Carry out the determination of magnesium by complexometry (2.5.11).

1 M Nitric acid. 3003600.

Dilute 96.6 g of *nitric acid R* to 1000.0 ml with *water R*.

Standardisation. Dissolve 1.000 g of *sodium carbonate RV* in 50 ml of *water R*, add 0.1 ml of *methyl orange solution R* and titrate with the nitric acid until the solution just becomes reddish-yellow; boil for 2 min. The solution reverts to yellow. Cool and continue the titration until a reddish-yellow colour is obtained.

1 ml of 1 M *nitric acid* is equivalent to 53.00 mg of Na_2CO_3 .

0.1 M Perchloric acid. 3003900.

Place 8.5 ml of *perchloric acid R* in a volumetric flask containing about 900 ml of *glacial acetic acid R* and mix. Add 30 ml of *acetic anhydride R*, dilute to 1000.0 ml with *glacial acetic acid R*, mix and allow to stand for 24 h. Determine the water content (2.5.12) without addition of *methanol* and, if necessary, adjust the water content to between 0.1 per cent and 0.2 per cent by adding either *acetic anhydride R* or *water R*. Allow to stand for 24 h.

Standardisation. Dissolve 0.350 g of *potassium hydrogen phthalate RV* in 50 ml of *anhydrous acetic acid R*, warming gently if necessary. Allow to cool protected from the air, and titrate with the perchloric acid solution, using 0.05 ml of *crystal violet solution R* as indicator. Note the temperature of the perchloric acid solution at the time of the titration. If the temperature at which an assay is carried out is different from that at which the *perchloric acid R* has been standardised the volume used in the assay becomes:

$$V_c = V [1 + (t_1 - t_2) 0.0011]$$

where t_1 is the temperature during standardisation, and t_2 is the temperature during the assay, V_c is the corrected volume and V the observed volume.

1 ml of 0.1 M *perchloric acid* is equivalent to 20.42 mg of $C_8H_5KO_4$.

0.05 M Perchloric acid. 3004000.

Dilute 50.0 ml of 0.1 M *perchloric acid* to 100.0 ml with *anhydrous acetic acid R*.

0.033 M Potassium bromate. 3004200.

Dissolve 5.5670 g of *potassium bromate RV* in *water R* and dilute to 1000.0 ml with the same solvent.

0.02 M Potassium bromate. 3004300.

Dissolve 3.340 g of *potassium bromate RV* in *water R* and dilute to 1000.0 ml with the same solvent.

0.0167 M Potassium bromate. 3004400.

Prepare by diluting 0.033 M *Potassium bromate*.

0.0083 M Potassium bromate. 3004500.

Prepare by diluting 0.033 M *Potassium bromate*.

0.0167 M Potassium dichromate. 3004600.

Dissolve 4.90 g of *potassium dichromate R* in *water R* and dilute to 1000.0 ml with the same solvent.

Standardisation. To 20.0 ml of the potassium dichromate solution add 1 g of *potassium iodide R* and 7 ml of *dilute hydrochloric acid R*. Add 250 ml of *water R* and titrate with 0.1 M *sodium thiosulphate*, using 3 ml of *starch solution R* as indicator, until the colour changes from blue to light green.

0.1 M Potassium hydrogen phthalate. 3004700.

In a conical flask containing about 800 ml of *anhydrous acetic acid R*, dissolve 20.42 g of *potassium hydrogen phthalate RV*. Heat on a water-bath until completely dissolved, protected from humidity. Cool to 20 °C and dilute to 1000.0 ml with *anhydrous acetic acid R*.

1 M Potassium hydroxide. 3009100.

Dissolve 60 g of *potassium hydroxide R* in *carbon dioxide-free water R* and dilute to 1000.0 ml with the same solvent.

Standardisation. Titrate 20.0 ml of the potassium hydroxide solution with 1 M *hydrochloric acid*, using 0.5 ml of *phenolphthalein solution R* as indicator.

0.1 M Potassium hydroxide. 3004800.

Dissolve 6 g of *potassium hydroxide R* in *carbon dioxide-free water R* and dilute to 1000.0 ml with the same solvent.

Standardisation. Titrate 20.0 ml of the potassium hydroxide solution with 0.1 M *hydrochloric acid*, using 0.5 ml of *phenolphthalein solution R* as indicator.

0.5 M Potassium hydroxide in alcohol (60 per cent V/V). 3004900.

Dissolve 3 g of *potassium hydroxide R* in *aldehyde-free alcohol R* (60 per cent V/V) and dilute to 100.0 ml with the same solvent.

Standardisation. Titrate 20.0 ml of the alcoholic potassium hydroxide solution (60 per cent V/V) with 0.5 M *hydrochloric acid*, using 0.5 ml of *phenolphthalein solution R* as indicator.

0.5 M Potassium hydroxide, alcoholic. 3005000.

Dissolve 3 g of *potassium hydroxide R* in 5 ml of *water R* and dilute to 100.0 ml with *aldehyde-free alcohol R*.

Standardisation. Titrate 20.0 ml of the alcoholic potassium hydroxide solution with 0.5 M hydrochloric acid, using 0.5 ml of phenolphthalein solution R as indicator.

0.1 M Potassium hydroxide, alcoholic. 3005100.

Dilute 20.0 ml of 0.5 M alcoholic potassium hydroxide to 100.0 ml with aldehyde-free alcohol R.

0.01 M Potassium hydroxide, alcoholic. 3009000.

Dilute 2.0 ml of 0.5 M alcoholic potassium hydroxide to 100.0 ml with aldehyde-free alcohol R.

0.05 M Potassium iodate. 3005200.

Dissolve 10.70 g of potassium iodate R in water R and dilute to 1000.0 ml with the same solvent.

Standardisation. Dilute 25.0 ml of the potassium iodate solution to 100.0 ml with water R. To 20.0 ml of this solution add 2 g of potassium iodide R and 10 ml of dilute sulphuric acid R. Titrate with 0.1 M sodium thiosulphate, using 1 ml of starch solution R, added towards the end of the titration, as indicator.

0.001 M Potassium iodide. 3009200.

Dilute 10.0 ml of potassium iodide solution R (166 g/l) to 100.0 ml with water R. Dilute 5.0 ml of this solution to 500.0 ml with water R.

0.02 M Potassium permanganate. 3005300.

Dissolve 3.2 g of potassium permanganate R in water R and dilute to 1000.0 ml with the same solvent. Heat the solution for 1 h on a water-bath, allow to cool and filter through a sintered-glass filter.

Standardisation. To 20.0 ml of the potassium permanganate solution, add 2 g of potassium iodide R and 10 ml of dilute sulphuric acid R. Titrate with 0.1 M sodium thiosulphate, using 1 ml of starch solution R, added towards the end of the titration, as indicator. Standardise immediately before use.

Storage: protected from light.

0.1 M Silver nitrate. 3005600.

Dissolve 17.0 g of silver nitrate R in water R and dilute to 1000.0 ml with the same solvent.

Standardisation. Dissolve 0.100 g of sodium chloride RV in 30 ml of water R. Titrate with the silver nitrate solution, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M silver nitrate is equivalent to 5.844 mg of NaCl.

Storage: protected from light.

0.001 M Silver nitrate. 3009300.

Dilute 5.0 ml of silver nitrate 0.1 M to 500.0 ml with water R.

0.1 M Sodium edetate. 3005900.

Dissolve 37.5 g of sodium edetate R in 500 ml of water R, add 100 ml of 1 M sodium hydroxide and dilute to 1000.0 ml with water R.

Standardisation. Dissolve 0.120 g of zinc RV in 4 ml of hydrochloric acid R1 and add 0.1 ml of bromine water R. Drive off the excess of bromine by boiling, add dilute sodium hydroxide solution R until the solution is weakly acid or neutral and carry out the assay of zinc by complexometry (2.5.11).

1 ml of 0.1 M sodium edetate is equivalent to 6.54 mg of Zn.

Storage: in a polyethylene container.

0.02 M Sodium edetate. 3006000.

Dissolve 7.444 g of sodium edetate R in water R and dilute to 1000.0 ml with the same solvent.

Standardisation. Dissolve 0.100 g of zinc RV in 4 ml of hydrochloric acid R1 and add 0.1 ml of bromine water R. Drive off the excess of bromine by boiling. Transfer the solution to a volumetric flask and dilute to 100.0 ml with water R. Transfer 25.0 ml of the solution to a 500 ml conical flask and dilute to 200 ml with water R. Add about 50 mg of xylene orange triturate R and hexamethylenetetramine R until the solution becomes violet-pink. Add 2 g of hexamethylenetetramine R in excess. Titrate with the sodium edetate solution until the violet-pink colour changes to yellow.

1 ml of 0.02 M sodium edetate is equivalent to 1.308 mg of Zn.

1 M Sodium hydroxide. 3006300.

Dissolve 42 g of sodium hydroxide R in carbon dioxide-free water R and dilute to 1000.0 ml with the same solvent.

Standardisation. Titrate 20.0 ml of the sodium hydroxide solution with 1 M hydrochloric acid using the indicator prescribed in the assay in which 1 M sodium hydroxide is used.

If sodium hydroxide free from carbonate is prescribed, prepare it as follows. Dissolve sodium hydroxide R in water R to give a concentration of 400 g/l to 600 g/l and allow to stand. Decant the clear supernatant liquid, taking precautions to avoid the introduction of carbon dioxide, and dilute with carbon dioxide-free water R to the required molarity. The solution complies with the following test. Titrate 20.0 ml of hydrochloric acid of the same molarity with the solution of sodium hydroxide, using 0.5 ml of phenolphthalein solution R as indicator. At the end-point add just sufficient of the acid to discharge the pink colour and concentrate the solution to 20 ml by boiling. During boiling add just sufficient acid to discharge the pink colour, which should not reappear after prolonged boiling. The volume of acid used does not exceed 0.1 ml.

0.1 M Sodium hydroxide. 3006600.

Dilute 100.0 ml of 1 M sodium hydroxide to 1000.0 ml with carbon dioxide-free water R.

Standardisation. Titrate 20.0 ml of the sodium hydroxide solution with 0.1 M hydrochloric acid, using the end-point detection prescribed for the assay in which the 0.1 M sodium hydroxide is used.

Standardisation (for use in the assay of halide salts of organic bases). Dissolve 0.100 g of benzoic acid RV in a mixture of 5 ml of 0.01 M hydrochloric acid and 50 ml of alcohol R. Carry out the titration (2.2.20), using the sodium hydroxide solution. Note the volume added between the 2 points of inflexion.

1 ml of 0.1 M sodium hydroxide is equivalent to 12.21 mg of $C_7H_6O_2$.

2 M Sodium hydroxide. 3009800.

Dissolve 84 g of sodium hydroxide R in carbon dioxide-free water R and dilute to 1000.0 ml with the same solvent.

0.1 M Sodium hydroxide, ethanolic. 3007000.

To 250 ml of ethanol R add 3.3 g of strong sodium hydroxide solution R.

Standardisation. Dissolve 0.100 g of benzoic acid RV in 2 ml of water R and 10 ml of alcohol R. Titrate with the ethanolic sodium hydroxide solution, using 0.2 ml of thymolphthalein solution R as indicator. Standardise immediately before use.

1 ml of 0.1 M ethanolic sodium hydroxide is equivalent to 12.21 mg of $C_7H_6O_2$.

0.1 M Sodium methoxide. 3007100.

Cool 175 ml of *anhydrous methanol R* in iced *water R* and add, in small portions, about 2.5 g of freshly cut *sodium R*. When the metal has dissolved, dilute to 1000.0 ml with *toluene R*.

Standardisation. To 10 ml of *dimethylformamide R* add 0.05 ml of a 3 g/l solution of *thymol blue R* in *methanol R*, and titrate with the sodium methoxide solution until a pure blue colour is obtained. Immediately add 0.200 g of *benzoic acid RV*. Stir to effect solution and titrate with the sodium methoxide solution until the pure blue colour is again obtained. Protect the solution from atmospheric carbon dioxide throughout the titration. From the volume of titrant used in the second titration ascertain the exact strength of the sodium methoxide solution. Standardise immediately before use.

1 ml of 0.1 M sodium methoxide is equivalent to 12.21 mg of $C_7H_6O_2$.

0.1 M Sodium nitrite. 3007200.

Dissolve 7.5 g of *sodium nitrite R* in *water R* and dilute to 1000.0 ml with the same solvent.

Standardisation. Dissolve 0.300 g of *sulphanilic acid RV* in 50 ml of *dilute hydrochloric acid R* and carry out the determination of primary aromatic amino-nitrogen (2.5.8), using the sodium nitrite solution and determining the end-point electrometrically. Standardise immediately before use.

1 ml of 0.1 M sodium nitrite is equivalent to 17.32 mg of $C_6H_7NO_3S$.

0.1 M Sodium periodate. 3009500.

Dissolve 21.4 g of *sodium periodate R* in about 500 ml of *water R* and dilute to 1000.0 ml with the same solvent.

Standardisation. In a stoppered flask, introduce 20.0 ml of the sodium periodate solution and add 5 ml of *perchloric acid R*. Close the flask and shake. Adjust the solution to pH 6.4 (2.2.3) using a saturated solution of *sodium hydrogen carbonate R*. Add 10 ml of *potassium iodide solution R*, close, shake and allow to stand for 2 min. Titrate with 0.025 M sodium arsenite until the yellow colour almost disappears. Add 2 ml of *starch solution R* and titrate slowly until the colour is completely discharged.

0.1 M Sodium thiosulphate. 3007300.

Dissolve 25 g of *sodium thiosulphate R* and 0.2 g of *sodium carbonate R* in *carbon dioxide-free water R* and dilute to 1000.0 ml with the same solvent.

Standardisation. To 10.0 ml of 0.033 M *potassium bromate*, add 40 ml of *water R*, 10 ml of *potassium iodide solution R* and 5 ml of *hydrochloric acid R1*. Titrate with the sodium thiosulphate solution, using 1 ml of *starch solution R*, added towards the end of the titration, as indicator.

0.5 M Sulphuric acid. 3007800.

Dissolve 28 ml of sulphuric acid R in *water R* and dilute to 1000.0 ml with the same solvent.

Standardisation. Dissolve 1.000 g of *sodium carbonate RV* in 50 ml of *water R*, add 0.1 ml of *methyl orange solution R*, and titrate with the sulphuric acid until the solution begins

to turn reddish-yellow. Boil for about 2 min. The colour of the solutions reverts to yellow. Cool and titrate again until the reddish-yellow colour reappears.

1 ml of 0.5 M sulphuric acid is equivalent to 53.00 mg of Na_2CO_3 .

0.05 M Sulphuric acid. 3008000.

Dilute 100.0 ml of 0.5 M sulphuric acid to 1000.0 ml with *water R*.

Standardisation. Carry out the titration described for 0.5 M sulphuric acid, using 0.100 g of *sodium carbonate RV*, dissolved in 20 ml of *water R*.

1 ml of 0.05 M sulphuric acid is equivalent to 5.30 mg of Na_2CO_3 .

0.1 M Tetrabutylammonium hydroxide. 3008300.

Dissolve 40 g of *tetrabutylammonium iodide R* in 90 ml of *anhydrous methanol R*, add 20 g of finely powdered *silver oxide R* and shake vigorously for 1 h. Centrifuge a few millilitres of the mixture and test the supernatant liquid for iodides. If a positive reaction is obtained, add an additional 2 g of *silver oxide R* and shake for a further 30 min. Repeat this procedure until the liquid is free from iodides, filter the mixture through a fine sintered-glass filter and rinse the reaction vessel and filter with three quantities, each of 50 ml, of *toluene R*. Add the washings to the filtrate and dilute to 1000.0 ml with *toluene R*. Pass dry carbon dioxide-free nitrogen through the solution for 5 min.

Standardisation. To 10 ml of *dimethylformamide R* add 0.05 ml of a 3 g/l solution of *thymol blue R* in *methanol R* and titrate with the tetrabutylammonium hydroxide solution until a pure blue colour is obtained. Immediately add 0.200 g of *benzoic acid RV*. Stir to effect solution, and titrate with the tetrabutylammonium hydroxide solution until the pure blue colour is again obtained. Protect the solution from atmospheric carbon dioxide throughout the titration. From the volume of titrant used in the second titration ascertain the exact strength of the tetrabutylammonium hydroxide solution. Standardise immediately before use.

1 ml of 0.1 M tetrabutylammonium hydroxide is equivalent to 12.21 mg of $C_7H_6O_2$.

0.1 M Tetrabutylammonium hydroxide in 2-propanol. 3008400.

Prepare as described for 0.1 M tetrabutylammonium hydroxide using 2-propanol R instead of *toluene R* and standardise as described.

0.05 M Zinc chloride. 3008500.

Dissolve 6.82 g of *zinc chloride R*, weighed with appropriate precautions, in *water R*. If necessary, add dropwise *dilute hydrochloric acid R* until the opalescence disappears. Dilute to 1000.0 ml with *water R*.

Standardisation. To 20.0 ml of the zinc chloride solution add 5 ml of *dilute acetic acid R* and carry out the determination of zinc by complexometry (2.5.11).

0.1 M Zinc sulphate. 3008600.

Dissolve 29 g of *zinc sulphate R* in *water R* and dilute to 1000.0 ml with the same solvent.

Standardisation. To 20.0 ml of the zinc sulphate solution add 5 ml of *dilute acetic acid R* and carry out the determination of zinc by complexometry (2.5.11).

5.1. GENERAL TEXTS ON STERILITY

01/2005:50101

5.1.1. METHODS OF PREPARATION OF STERILE PRODUCTS

Sterility is the absence of viable micro-organisms. The sterility of a product cannot be guaranteed by testing; it has to be assured by the application of a suitably validated production process. It is essential that the effect of the chosen sterilisation procedure on the product (including its final container or package) is investigated to ensure effectiveness and the integrity of the product and that the procedure is validated before being applied in practice. It is recommended that the choice of the container is such as to allow the optimum sterilisation to be applied. Failure to follow meticulously a validated process involves the risk of a non-sterile product or of a deteriorated product. Revalidation is carried out whenever major changes in the sterilisation procedure, including changes in the load, take place. It is expected that the principles of good manufacturing practice (as described in, for example, the European Community Guide to GMP) will have been observed in the design of the process including, in particular, the use of:

- qualified personnel with appropriate training,
- adequate premises,
- suitable production equipment, designed for easy cleaning and sterilisation,
- adequate precautions to minimise the bioburden prior to sterilisation,
- validated procedures for all critical production steps,
- environmental monitoring and in-process testing procedures.

The precautions necessary to minimise the pre-sterilisation bioburden include the use of components with an acceptable low degree of microbial contamination. Microbiological monitoring and setting of suitable action limits may be advisable for ingredients which are liable to be contaminated because of their origin, nature or method of preparation.

The methods described here apply mainly to the inactivation or removal of bacteria, yeasts and moulds. For biological products of animal or human origin or in cases where such material has been used in the production process, it is necessary during validation to demonstrate that the process is capable of the removal or inactivation of relevant viral contamination. Guidance on this aspect is provided in, for example, the appropriate European Community Notes for Guidance.

Wherever possible, a process in which the product is sterilised in its final container (terminal sterilisation) is chosen. When a fully validated terminal sterilisation method by steam, dry heat or ionising radiation is used, parametric release, that is the release of a batch of sterilised items based on process data rather than on the basis of submitting a sample of the items to sterility testing, may be carried out, subject to the approval of the competent authority.

If terminal sterilisation is not possible, filtration through a bacteria-retentive filter or aseptic processing is used; wherever possible, appropriate additional treatment of the product (for example, heating of the product) in its final container is applied. In all cases, the container and closure are required to maintain the sterility of the product throughout its shelf-life.

Sterility Assurance Level (SAL)

Where appropriate reference is made within the methods described below, to a “sterility assurance level” or “SAL”. The achievement of sterility within any one item in a population of items submitted to a sterilisation process cannot be guaranteed nor can it be demonstrated. The inactivation of micro-organisms by physical or chemical means follows an exponential law; thus there is always a finite statistical probability that a micro-organism may survive the sterilising process. For a given process, the probability of survival is determined by the number, types and resistance of the micro-organisms present and by the environment in which the organisms exist during treatment. The SAL of a sterilising process is the degree of assurance with which the process in question renders a population of items sterile. The SAL for a given process is expressed as the probability of a non-sterile item in that population. An SAL of 10^{-6} , for example, denotes a probability of not more than one viable micro-organism in 1×10^6 sterilised items of the final product. The SAL of a process for a given product is established by appropriate validation studies.

METHODS AND CONDITIONS OF STERILISATION

Sterilisation may be carried out by one of the methods described below. Modifications to, or combinations of, these methods may be used provided that the chosen procedure is validated both with respect to its effectiveness and the integrity of the product including its container or package. For all methods of sterilisation the critical conditions of the operation are monitored in order to confirm that the previously determined required conditions are achieved throughout the batch during the whole sterilisation process. This applies in all cases including those where the reference conditions are used.

TERMINAL STERILISATION

For terminal sterilisation it is essential to take into account the non-uniformity of the physical and, where relevant, chemical conditions within the sterilising chamber. The location within the sterilising chamber that is least accessible to the sterilising agent is determined for each loading configuration of each type and size of container or package (for example, the coolest location in an autoclave). The minimum lethality delivered by the sterilising cycle and the reproducibility of the cycle are also determined in order to ensure that all loads will consistently receive the specified treatment.

Having established a terminal sterilisation process, knowledge of its performance in routine use is gained wherever possible, by monitoring and suitably recording the physical and, where relevant, chemical conditions achieved within the load in the chamber throughout each sterilising cycle.

Steam sterilisation (Heating in an autoclave). Sterilisation by saturated steam under pressure is preferred, wherever applicable, especially for aqueous preparations. For this method of terminal sterilisation the reference conditions for aqueous preparations are heating at a minimum of 121°C for 15 min. Other combinations of time and temperature may be used provided that it has been satisfactorily demonstrated that the process chosen delivers an adequate and reproducible level of lethality when operating routinely within the established tolerances. The procedures and precautions employed are such, as to give an SAL of 10^{-6} or better. Guidance concerning validation by means of the F_0 concept is provided below (5.1.5).

Knowledge of the physical conditions (temperature and pressure) within the autoclave chamber during the sterilisation procedure is obtained. The temperature is

usually measured by means of temperature-sensing elements inserted into representative containers together with additional elements at the previously established coolest part of the loaded chamber. The conditions throughout each cycle are suitably recorded, for example, as a temperature-time chart, or by any other suitable means.

Where a biological assessment is carried out, this is obtained using a suitable biological indicator (5.1.2).

Dry heat sterilisation. For this method of terminal sterilisation the reference conditions are a minimum of 160 °C for at least 2 h. Other combinations of time and temperature may be used provided that it has been satisfactorily demonstrated that the process chosen delivers an adequate and reproducible level of lethality when operated routinely within the established tolerances. The procedures and precautions employed are such as to give an SAL of 10^{-6} or better.

Dry heat sterilisation is carried out in an oven equipped with forced air circulation or other equipment specially designed for the purpose. The steriliser is loaded in such a way that a uniform temperature is achieved throughout the load. Knowledge of the temperature within the steriliser during the sterilisation procedure is usually obtained by means of temperature-sensing elements inserted into representative containers together with additional elements at the previously established coolest part of the loaded steriliser. The temperature throughout each cycle is suitably recorded.

Where a biological assessment is carried out, this is obtained using a suitable biological indicator (5.1.2).

Dry heat at temperatures greater than 220 °C is frequently used for sterilisation and depyrogenation of glassware. In this case demonstration of a 3-log reduction in heat resistant endotoxin can be used as a replacement for biological indicators (5.1.2).

Ionising radiation sterilisation. Sterilisation by this method is achieved by exposure of the product to ionising radiation in the form of gamma radiation from a suitable radioisotopic source (such as cobalt 60) or of a beam of electrons energised by a suitable electron accelerator.

In some countries there are regulations that lay down rules for the use of ionising radiation for sterilisation purposes, for example, in the appropriate European Community Notes for Guidance.

For this method of terminal sterilisation the reference absorbed dose is 25 kGy. Other doses may be used provided that it has satisfactorily been demonstrated that the dose chosen delivers an adequate and reproducible level of lethality when the process is operated routinely within the established tolerances. The procedures and precautions employed are such as to give an SAL of 10^{-6} or better.

During the sterilisation procedure the radiation absorbed by the product is monitored regularly by means of established dosimetry procedures that are independent of dose rate. Dosimeters are calibrated against a standard source at a reference radiation plant on receipt from the supplier and at suitable intervals of not longer than one year thereafter.

Where a biological assessment is carried out, this is obtained using a suitable biological indicator (5.1.2).

Gas sterilisation. This method of sterilisation is only to be used where there is no suitable alternative. It is essential that penetration by gas and moisture into the material to be sterilised is ensured and that it is followed by a process of elimination of the gas under conditions that have been previously established to ensure that any residue of gas or its transformation products in the sterilised product is below the concentration that could give rise to toxic effects during

use of the product. Guidance on this aspect with respect to the use of ethylene oxide is provided, for example, in the appropriate European Community Notes for Guidance.

Wherever possible, the gas concentration, relative humidity, temperature and duration of the process are measured and recorded. Measurements are made where sterilisation conditions are least likely to be achieved, as determined at validation.

The effectiveness of the process applied to each sterilisation load is checked using a suitable biological indicator (5.1.2).

A suitable sample of each batch is tested for sterility (2.6.1) before the batch is released.

FILTRATION

Certain active ingredients and products that cannot be terminally sterilised may be subjected to a filtration procedure using a filter of a type that has been demonstrated to be satisfactory by means of a microbial challenge test using a suitable test micro-organism. A suspension of *Pseudomonas diminuta* (ATCC 19146, NCIMB 11091 or CIP 103020) may be suitable. It is recommended that a challenge of at least 10^7 CFU per cm² of active filter surface is used and that the suspension is prepared in tryptone soya broth which, after passage through the filter, is collected aseptically and incubated aerobically at 32 °C. Such products need special precautions. The production process and environment are designed to minimise microbial contamination and are regularly subjected to appropriate monitoring procedures. The equipment, containers and closures and, wherever possible, the ingredients are subjected to an appropriate sterilisation process. It is recommended that the filtration process is carried out as close as possible to the filling point. The operations following filtration are carried out under aseptic conditions.

Solutions are passed through a bacteria-retentive membrane with a nominal pore size of 0.22 µm or less or any other type of filter known to have equivalent properties of bacteria retention. Appropriate measures are taken to avoid loss of solute by adsorption on to the filter and to avoid the release of contaminants from the filter. Attention is given to the bioburden prior to filtration, filter capacity, batch size and duration of filtration. The filter is not used for a longer period than has been approved by validation of the combination of the filter and the product in question.

The integrity of an assembled sterilising filter is verified before use and confirmed after use by carrying out tests appropriate to the type of filter used and the stage of testing, for example bubble-point, pressure hold or diffusion rate tests.

Due to the potential additional risks of the filtration method as compared with other sterilisation processes, a prefiltration through a bacteria-retentive filter may be advisable in cases where a low bioburden cannot be ensured by other means.

ASEPTIC PREPARATION

The objective of aseptic processing is to maintain the sterility of a product that is assembled from components, each of which has been sterilised by one of the above methods. This is achieved by using conditions and facilities designed to prevent microbial contamination. Aseptic processing may include aseptic filling of products into container/closure systems, aseptic blending of formulations followed by aseptic filling and aseptic packaging.

In order to maintain the sterility of the components and the product during processing, careful attention needs to be given to:

- environment,
- personnel,

- critical surfaces,
- container/closure sterilisation and transfer procedures,
- maximum holding period of the product before filling into the final container.

Process validation includes appropriate checks on all the above and checks on the process are regularly carried out by means of process simulation tests using microbial growth media which are then incubated and examined for microbial contamination (media fill tests). In addition, a suitable sample of each batch of any product that is sterilised by filtration and/or aseptically processed is tested for sterility (2.6.1) before the batch is released.

01/2005:50102

5.1.2. BIOLOGICAL INDICATORS OF STERILISATION

Biological indicators are standardised preparations of selected micro-organisms used to assess the effectiveness of a sterilisation procedure. They usually consist of a population of bacterial spores placed on an inert carrier, for example a strip of filter paper, a glass slide or a plastic tube. The inoculated carrier is covered in such a way that it is protected from any deterioration or contamination, while allowing the sterilising agent to enter into contact with the micro-organisms. Spore suspensions may be presented in sealed ampoules. Biological indicators are prepared in such a way that they can be stored under defined conditions; an expiry date is set.

Micro-organisms of the same bacterial species as the bacteria used to manufacture the biological indicators may be inoculated directly into a liquid product to be sterilised or into a liquid product similar to that to be sterilised. In this case, it must be demonstrated that the liquid product has no inhibiting effect on the spores used, especially as regards their germination.

A biological indicator is characterised by the name of the species of bacterium used as the reference micro-organism, the number of the strain in the original collection, the number of viable spores per carrier and the *D*-value. The *D*-value is the value of a parameter of sterilisation (duration or absorbed dose) required to reduce the number of viable organisms to 10 per cent of the original number. It is of significance only under precisely defined experimental conditions. Only the stated micro-organisms are present. Biological indicators consisting of more than one species of bacteria on the same carrier may be used. Information on the culture medium and the incubation conditions is supplied.

It is recommended that the indicator organisms are placed at the locations presumed, or wherever possible, found by previous physical measurement to be least accessible to the sterilising agent. After exposure to the sterilising agent, aseptic technique is used to transfer carriers of spores to the culture media, so that no contamination is present at the time of examination. Biological indicators that include an ampoule of culture medium placed directly in the packaging protecting the inoculated carrier may be used.

A choice of indicator organisms is made such that:

- a) the resistance of the test strain to the particular sterilisation method is great compared to the resistance of all pathogenic micro-organisms and to that of micro-organisms potentially contaminating the product,
- b) the test strain is non-pathogenic,
- c) the test strain is easy to culture.

After incubation, growth of the reference micro-organisms subjected to a sterilisation procedure demonstrates that the procedure has been unsatisfactory.

Steam sterilisation. The use of biological indicators intended for steam sterilisation is recommended for the validation of sterilisation cycles. Spores of *Bacillus stearothermophilus* (for example, ATCC 7953, NCTC 10007, NCIMB 8157 or CIP 52.81) are recommended. The number of viable spores exceeds 5×10^5 per carrier. The *D*-value at 121 °C exceeds 1.5 min. It is verified that exposing the biological indicators to steam at 121 ± 1 °C for 6 min leaves revivable spores, and that there is no growth of the reference micro-organisms after the biological indicators have been exposed to steam at 121 ± 1 °C for 15 min.

Dry-heat sterilisation. Spores of *Bacillus subtilis* (for example, var. *niger* ATCC 9372, NCIMB 8058 or CIP 77.18) are recommended for the preparation of biological indicators. The number of viable spores exceeds 1×10^5 per carrier and the *D*-value at 160 °C is approximately 1 min to 3 min. Dry heat at temperatures greater than 220 °C is frequently used for sterilisation and depyrogenation of glassware. In this case, demonstration of a 3 log reduction in heat resistant bacterial endotoxin can be used as a replacement for biological indicators.

Ionising radiation sterilisation. Biological indicators may be used to monitor routine operations, as an additional possibility to assess the effectiveness of the set dose of radiation energy, especially in the case of accelerated electron sterilisation. The spores of *Bacillus pumilus* (for example, ATCC 27.142, NCTC 10327, NCIMB 10692 or CIP 77.25) are recommended. The number of viable spores exceeds 1×10^7 per carrier. The *D*-value exceeds 1.9 kGy. It is verified that there is no growth of the reference micro-organisms after the biological indicators have been exposed to 25 kGy (*minimum absorbed dose*).

Gas sterilisation. The use of biological indicators is necessary for all gas sterilisation procedures, both for the validation of the cycles and for routine operations. Gas sterilisation is widely used for medical devices, isolators, chambers, etc. Use for such purposes is outside the scope of the European Pharmacopoeia. The use of spores of *Bacillus subtilis* (for example, var. *niger* ATCC 9372, NCIMB 8058 or CIP 77.18) is recommended for ethylene oxide. The number of viable spores exceeds 5×10^5 per carrier. The parameters of resistance are the following: the *D*-value exceeds 2.5 min for a test cycle involving 600 mg/l of ethylene oxide, at 54 °C and at 60 per cent relative humidity. It is verified that there is no growth of the reference micro-organisms after the biological indicators have been exposed to the test cycle described above for 60 min and that exposing the indicators to a reduced temperature cycle (600 mg/l, 30 °C and 60 per cent relative humidity) for 15 min leaves revivable spores. Exposing the indicators to 600 mg/l of ethylene oxide at 54 °C for 60 min without humidification must leave revivable spores to ensure that the biological indicator is able to reveal insufficient humidification.

01/2005:50103

5.1.3. EFFICACY OF ANTIMICROBIAL PRESERVATION

If a pharmaceutical preparation does not itself have adequate antimicrobial activity, antimicrobial preservatives may be added, particularly to aqueous preparations, to prevent proliferation or to limit microbial contamination which, during normal conditions of storage and use, particularly for multidose containers, could occur in a product and present

- critical surfaces,
- container/closure sterilisation and transfer procedures,
- maximum holding period of the product before filling into the final container.

Process validation includes appropriate checks on all the above and checks on the process are regularly carried out by means of process simulation tests using microbial growth media which are then incubated and examined for microbial contamination (media fill tests). In addition, a suitable sample of each batch of any product that is sterilised by filtration and/or aseptically processed is tested for sterility (2.6.1) before the batch is released.

01/2005:50102

5.1.2. BIOLOGICAL INDICATORS OF STERILISATION

Biological indicators are standardised preparations of selected micro-organisms used to assess the effectiveness of a sterilisation procedure. They usually consist of a population of bacterial spores placed on an inert carrier, for example a strip of filter paper, a glass slide or a plastic tube. The inoculated carrier is covered in such a way that it is protected from any deterioration or contamination, while allowing the sterilising agent to enter into contact with the micro-organisms. Spore suspensions may be presented in sealed ampoules. Biological indicators are prepared in such a way that they can be stored under defined conditions; an expiry date is set.

Micro-organisms of the same bacterial species as the bacteria used to manufacture the biological indicators may be inoculated directly into a liquid product to be sterilised or into a liquid product similar to that to be sterilised. In this case, it must be demonstrated that the liquid product has no inhibiting effect on the spores used, especially as regards their germination.

A biological indicator is characterised by the name of the species of bacterium used as the reference micro-organism, the number of the strain in the original collection, the number of viable spores per carrier and the *D*-value. The *D*-value is the value of a parameter of sterilisation (duration or absorbed dose) required to reduce the number of viable organisms to 10 per cent of the original number. It is of significance only under precisely defined experimental conditions. Only the stated micro-organisms are present. Biological indicators consisting of more than one species of bacteria on the same carrier may be used. Information on the culture medium and the incubation conditions is supplied.

It is recommended that the indicator organisms are placed at the locations presumed, or wherever possible, found by previous physical measurement to be least accessible to the sterilising agent. After exposure to the sterilising agent, aseptic technique is used to transfer carriers of spores to the culture media, so that no contamination is present at the time of examination. Biological indicators that include an ampoule of culture medium placed directly in the packaging protecting the inoculated carrier may be used.

A choice of indicator organisms is made such that:

- a) the resistance of the test strain to the particular sterilisation method is great compared to the resistance of all pathogenic micro-organisms and to that of micro-organisms potentially contaminating the product,
- b) the test strain is non-pathogenic,
- c) the test strain is easy to culture.

After incubation, growth of the reference micro-organisms subjected to a sterilisation procedure demonstrates that the procedure has been unsatisfactory.

Steam sterilisation. The use of biological indicators intended for steam sterilisation is recommended for the validation of sterilisation cycles. Spores of *Bacillus stearothermophilus* (for example, ATCC 7953, NCTC 10007, NCIMB 8157 or CIP 52.81) are recommended. The number of viable spores exceeds 5×10^5 per carrier. The *D*-value at 121 °C exceeds 1.5 min. It is verified that exposing the biological indicators to steam at 121 ± 1 °C for 6 min leaves revivable spores, and that there is no growth of the reference micro-organisms after the biological indicators have been exposed to steam at 121 ± 1 °C for 15 min.

Dry-heat sterilisation. Spores of *Bacillus subtilis* (for example, var. *niger* ATCC 9372, NCIMB 8058 or CIP 77.18) are recommended for the preparation of biological indicators. The number of viable spores exceeds 1×10^5 per carrier and the *D*-value at 160 °C is approximately 1 min to 3 min. Dry heat at temperatures greater than 220 °C is frequently used for sterilisation and depyrogenation of glassware. In this case, demonstration of a 3 log reduction in heat resistant bacterial endotoxin can be used as a replacement for biological indicators.

Ionising radiation sterilisation. Biological indicators may be used to monitor routine operations, as an additional possibility to assess the effectiveness of the set dose of radiation energy, especially in the case of accelerated electron sterilisation. The spores of *Bacillus pumilus* (for example, ATCC 27.142, NCTC 10327, NCIMB 10692 or CIP 77.25) are recommended. The number of viable spores exceeds 1×10^7 per carrier. The *D*-value exceeds 1.9 kGy. It is verified that there is no growth of the reference micro-organisms after the biological indicators have been exposed to 25 kGy (*minimum absorbed dose*).

Gas sterilisation. The use of biological indicators is necessary for all gas sterilisation procedures, both for the validation of the cycles and for routine operations. Gas sterilisation is widely used for medical devices, isolators, chambers, etc. Use for such purposes is outside the scope of the European Pharmacopoeia. The use of spores of *Bacillus subtilis* (for example, var. *niger* ATCC 9372, NCIMB 8058 or CIP 77.18) is recommended for ethylene oxide. The number of viable spores exceeds 5×10^5 per carrier. The parameters of resistance are the following: the *D*-value exceeds 2.5 min for a test cycle involving 600 mg/l of ethylene oxide, at 54 °C and at 60 per cent relative humidity. It is verified that there is no growth of the reference micro-organisms after the biological indicators have been exposed to the test cycle described above for 60 min and that exposing the indicators to a reduced temperature cycle (600 mg/l, 30 °C and 60 per cent relative humidity) for 15 min leaves revivable spores. Exposing the indicators to 600 mg/l of ethylene oxide at 54 °C for 60 min without humidification must leave revivable spores to ensure that the biological indicator is able to reveal insufficient humidification.

01/2005:50103

5.1.3. EFFICACY OF ANTIMICROBIAL PRESERVATION

If a pharmaceutical preparation does not itself have adequate antimicrobial activity, antimicrobial preservatives may be added, particularly to aqueous preparations, to prevent proliferation or to limit microbial contamination which, during normal conditions of storage and use, particularly for multidose containers, could occur in a product and present

a hazard to the patient from infection and spoilage of the preparation. Antimicrobial preservatives must not be used as a substitute for good manufacturing practice.

The efficacy of an antimicrobial preservative may be enhanced or diminished by the active constituent of the preparation or by the formulation in which it is incorporated or by the container and closure used. The antimicrobial activity of the preparation in its final container is investigated over the period of validity to ensure that such activity has not been impaired by storage. Such investigations may be carried out on samples removed from the final container immediately prior to testing.

During development of a pharmaceutical preparation, it shall be demonstrated that the antimicrobial activity of the preparation as such or, if necessary, with the addition of a suitable preservative or preservatives provides adequate protection from adverse effects that may arise from microbial contamination or proliferation during storage and use of the preparation.

The efficacy of the antimicrobial activity may be demonstrated by the test described below. The test is not intended to be used for routine control purposes.

TEST FOR EFFICACY OF ANTIMICROBIAL PRESERVATION

The test consists of challenging the preparation, wherever possible in its final container, with a prescribed inoculum of suitable micro-organisms, storing the inoculated preparation at a prescribed temperature, withdrawing samples from the container at specified intervals of time and counting the organisms in the samples so removed.

The preservative properties of the preparation are adequate if, in the conditions of the test, there is a significant fall or no increase, as appropriate, in the number of micro-organisms in the inoculated preparation after the times and at the temperatures prescribed. The criteria of acceptance, in terms of decrease in the number of micro-organisms with time, vary for different types of preparations according to the degree of protection intended (see Tables 5.1.3.-1/2/3).

Test micro-organisms

<i>Pseudomonas aeruginosa</i>	ATCC 9027; NCIMB 8626; CIP 82.118.
<i>Staphylococcus aureus</i>	ATCC 6538; NCTC 10788; NCIMB 9518; CIP 4.83.
<i>Candida albicans</i>	ATCC 10231; NCPF 3179; IP 48.72.
<i>Aspergillus niger</i>	ATCC 16404; IMI 149007; IP 1431.83.

Single-strain challenges are used and the designated micro-organisms are supplemented, where appropriate, by other strains or species that may represent likely contaminants to the preparation. It is recommended, for example, that *Escherichia coli* (ATCC 8739; NCIMB 8545; CIP 53.126) is used for all oral preparations and *Zygosaccharomyces rouxii* (NCYC 381; IP 2021.92) for oral preparations containing a high concentration of sugar.

Preparation of inoculum

Preparatory to the test, inoculate the surface of agar medium B (2.6.12) for bacteria or agar medium C without the addition of antibiotics (2.6.12) for fungi, with the recently grown stock culture of each of the specified micro-organisms. Incubate the bacterial cultures at 30-35 °C for 18-24 h, the culture of *C. albicans* at 20-25 °C for 48 h, and the culture of *A. niger* at 20-25 °C for 1 week or until good sporulation is obtained. Subcultures may be needed after revival before the

micro-organism is in its optimal state, but it is recommended that their number be kept to a minimum.

To harvest the bacterial and *C. albicans* cultures, use a sterile suspending fluid, containing 9 g/l of *sodium chloride R*, for dispersal and transfer of the surface growth into a suitable vessel. Add sufficient suspending fluid to reduce the microbial count to about 10⁸ micro-organisms per millilitre. To harvest the *A. niger* culture, use a sterile suspending fluid containing 9 g/l of *sodium chloride R* and 0.5 g/l of *polysorbate 80 R* and adjust the spore count to about 10⁸ per millilitre by adding the same solution.

Remove immediately a suitable sample from each suspension and determine the number of colony-forming units per millilitre in each suspension by plate count or membrane filtration (2.6.12). This value serves to determine the inoculum and the baseline to use in the test. The suspensions shall be used immediately.

METHOD

To count the viable micro-organisms in the inoculated products, use the agar medium used for the initial cultivation of the respective micro-organisms.

Inoculate a series of containers of the product to be examined, each with a suspension of one of the test organisms to give an inoculum of 10⁵ to 10⁶ micro-organisms per millilitre or per gram of the preparation. The volume of the suspension of inoculum does not exceed 1 per cent of the volume of the product. Mix thoroughly to ensure homogeneous distribution.

Maintain the inoculated product at 20-25 °C, protected from light. Remove a suitable sample from each container, typically 1 ml or 1 g, at zero hour and at appropriate intervals according to the type of the product and determine the number of viable micro-organisms by plate count or membrane filtration (2.6.12). Ensure that any residual antimicrobial activity of the product is eliminated by dilution, by filtration or by the use of a specific inactivator. When dilution procedures are used, due allowance is made for the reduced sensitivity in the recovery of small numbers of viable micro-organisms. When a specific inactivator is used, the ability of the system to support the growth of the test organisms is confirmed by the use of appropriate controls.

The procedure is validated to verify its ability to demonstrate the required reduction in count of viable micro-organisms.

CRITERIA OF ACCEPTANCE

The criteria for evaluation of antimicrobial activity are given in Tables 5.1.3.-1/2/3 in terms of the log reduction in the number of viable micro-organisms against the value obtained for the inoculum.

Table 5.1.3.-1. - Parenteral and ophthalmic preparations

		Log reduction				
		6 h	24 h	7 d	14 d	28 d
Bacteria	A	2	3	-	-	NR*
	B	-	1	3	-	NI**
Fungi	A	-	-	2	-	NI
	B	-	-	-	1	NI

*NR: no recover

**NI: no increase

The A criteria express the recommended efficacy to be achieved. In justified cases where the A criteria cannot be attained, for example for reasons of an increased risk of adverse reactions, the B criteria must be satisfied.

Table 5.1.3.-2. - *Topical preparations*

		Log reduction			
		2 d	7 d	14 d	28 d
Bacteria	A	2	3	-	NI
	B	-	-	3	NI
Fungi	A	-	-	2	NI
	B	-	-	1	NI

The A criteria express the recommended efficacy to be achieved. In justified cases where the A criteria cannot be attained, for example for reasons of an increased risk of adverse reactions, the B criteria must be satisfied.

Table 5.1.3.-3. - *Oral preparations*

		Log reduction	
		14 d	28 d
Bacteria		3	NI
Fungi		1	NI

The above criteria express the recommended efficacy to be achieved.

01/2005:50104

5.1.4. MICROBIOLOGICAL QUALITY OF PHARMACEUTICAL PREPARATIONS

The following chapter is published for information.

In the manufacture, packaging, storage and distribution of pharmaceutical preparations, suitable means must be taken to ensure their microbiological quality. The pharmaceutical preparations should comply with the criteria given below.

Category 1

Preparations required to be sterile by the relevant monograph on the dosage form and other preparations labelled sterile.

- Test for sterility (2.6.1).

Category 2

Preparations for topical use and for use in the respiratory tract except where required to be sterile and transdermal patches.

- Total viable aerobic count (2.6.12). Not more than 10^2 micro-organisms (aerobic bacteria plus fungi) per gram, per millilitre or per patch (including the adhesive and backing layer).
- Transdermal patches: absence of enterobacteria and certain other gram-negative bacteria, determined on 1 patch (including the adhesive and backing layer). Other preparations: not more than 10^1 enterobacteria and certain other gram-negative bacteria per gram or per millilitre (2.6.13).
- Absence of *Pseudomonas aeruginosa*, determined on 1 g, 1 ml or one patch (including the adhesive and backing layer) (2.6.13).
- Absence of *Staphylococcus aureus*, determined on 1 g, 1 ml or one patch (including the adhesive and backing layer) (2.6.13).

Category 3

A. Preparations for oral and rectal administration.

- Total viable aerobic count (2.6.12). Not more than 10^3 bacteria and not more than 10^2 fungi per gram or per millilitre.
- Absence of *Escherichia coli* (1 g or 1 ml) (2.6.13).

B. Preparations for oral administration containing raw materials of natural (animal, vegetable or mineral) origin for which antimicrobial pretreatment is not feasible and for which the competent authority accepts microbial contamination of the raw material exceeding 10^3 viable micro-organisms per gram or per millilitre. Herbal medicinal products described in category 4 are excluded.

- Total viable aerobic count (2.6.12). Not more than 10^4 bacteria and not more than 10^2 fungi per gram or per millilitre.
- Not more than 10^2 enterobacteria and certain other gram-negative bacteria per gram or per millilitre (2.6.13).
- Absence of *Salmonella* (10 g or 10 ml) (2.6.13).
- Absence of *Escherichia coli* (1 g or 1 ml) (2.6.13).
- Absence of *Staphylococcus aureus* (1 g or 1 ml) (2.6.13).

Category 4

Herbal medicinal products consisting solely of one or more herbal drugs (whole, reduced or powdered).

A. Herbal medicinal products to which boiling water is added before use.

- Total viable aerobic count (2.6.12). Not more than 10^7 bacteria and not more than 10^5 fungi per gram or per millilitre.
- Not more than 10^2 *Escherichia coli* per gram or per millilitre (2.6.13, using suitable dilutions).

B. Herbal medicinal products to which boiling water is not added before use.

- Total viable aerobic count (2.6.12). Not more than 10^5 bacteria and not more than 10^4 fungi per gram or per millilitre.
- Not more than 10^3 enterobacteria and certain other gram-negative bacteria per gram or per millilitre (2.6.13).
- Absence of *Escherichia coli* (1 g or 1 ml) (2.6.13).
- Absence of *Salmonella* (10 g or 10 ml) (2.6.13).

01/2005:50105

5.1.5. APPLICATION OF THE F_0 CONCEPT TO STEAM STERILISATION OF AQUEOUS PREPARATIONS

The following chapter is published for information.

The F_0 value of a saturated steam sterilisation process is the lethality expressed in terms of the equivalent time in minutes at a temperature of 121 °C delivered by the process to the product in its final container with reference to micro-organisms possessing a Z-value of 10.

The total F_0 of a process takes account of the heating up and cooling down phases of the cycle and can be calculated by integration of lethal rates with respect to time at discrete temperature intervals.

When a steam sterilisation cycle is chosen on the basis of the F_0 concept, great care must be taken to ensure that an adequate assurance of sterility is consistently

achieved. In addition to validating the process, it may also be necessary to perform continuous, rigorous microbiological monitoring during routine production to demonstrate that the microbiological parameters are within the established tolerances so as to give an SAL of 10^{-6} or better.

In connection with sterilisation by steam, the Z -value relates the heat resistance of a micro-organism to changes in temperature. The Z -value is the change in temperature required to alter the D -value by a factor of 10.

The D -value (or decimal reduction value) is the value of a parameter of sterilisation (duration or absorbed dose) required to reduce the number of viable organisms to 10 per cent of the original number. It is only of significance under precisely defined experimental conditions.

The following mathematical relationships apply:

$$F_0 = D_{121} (\log N_0 - \log N) = D_{121} \log IF$$

D_{121} = D -value of the reference spores (5.1.2) at 121 °C,

N_0 = initial number of viable micro-organisms,

N = final number of viable micro-organisms,

IF = inactivation factor.

$$Z = \frac{T_2 - T_1}{\log D_1 - \log D_2}$$

D_1 = D -value of the micro-organism at temperature T_1 ,

D_2 = D -value of the micro-organism at temperature T_2 .

$$IF = N_0 - N = 10^{t/D}$$

t = exposure time,

D = D -value of micro-organism in the exposure conditions.

5.2. GENERAL TEXTS ON VACCINES

01/2005:50201

5.2.1. TERMINOLOGY USED IN MONOGRAPHS ON VACCINES

For some items, alternative terms commonly used in connection with veterinary vaccines are shown in parenthesis.

Seed-lot system. A seed-lot system is a system according to which successive batches of a product are derived from the same master seed lot. For routine production, a working seed lot may be prepared from the master seed lot. The origin and the passage history of the master seed lot and the working seed lot are recorded.

Master seed lot. A culture of a micro-organism distributed from a single bulk into containers and processed together in a single operation in such a manner as to ensure uniformity and stability and to prevent contamination. A master seed lot in liquid form is usually stored at or below -70°C . A freeze-dried master seed lot is stored at a temperature known to ensure stability.

Working seed lot. A culture of a micro-organism derived from the master seed lot and intended for use in production. Working seed lots are distributed into containers and stored as described above for master seed lots.

Cell-bank system (Cell-seed system). A system whereby successive final lots (batches) of a product are manufactured by culture in cells derived from the same master cell bank (master cell seed). A number of containers from the master cell bank (master cell seed) are used to prepare a working cell bank (working cell seed). The cell-bank system (cell-seed system) is validated for the highest passage level achieved during routine production.

Master cell bank (Master cell seed). A culture of cells distributed into containers in a single operation, processed together and stored in such a manner as to ensure uniformity and stability and to prevent contamination. A master cell bank (master cell seed) is usually stored at -70°C or lower.

Working cell bank (Working cell seed). A culture of cells derived from the master cell bank (master cell seed) and intended for use in the preparation of production cell cultures. The working cell bank (working cell seed) is distributed into containers, processed and stored as described for the master cell bank (master cell seed).

Primary cell cultures. Cultures of cells obtained by trypsinisation of a suitable tissue or organ. The cells are essentially identical to those of the tissue of origin and are no more than 5 *in vitro* passages from the initial preparation from the animal tissue.

Cell lines. Cultures of cells that have a high capacity for multiplication *in vitro*. In diploid cell lines, the cells have essentially the same characteristics as those of the tissue of origin. In continuous cell lines, the cells are able to multiply indefinitely in culture and may be obtained from healthy or tumoral tissue. Some continuous cell lines have oncogenic potential under certain conditions.

Production cell culture. A culture of cells intended for use in production; it may be derived from one or more containers of the working cell bank (working cell seed) or it may be a primary cell culture.

Control cells. A quantity of cells set aside, at the time of virus inoculation, as uninfected cell cultures. The uninfected cells are incubated under similar conditions to those used for the production cell cultures.

Single harvest. Material derived on one or more occasions from a single production cell culture inoculated with the same working seed lot or a suspension derived from the working seed lot, incubated, and harvested in a single production run.

Monovalent pooled harvest. Pooled material containing a single strain or type of micro-organism or antigen and derived from a number of eggs, cell culture containers etc. that are processed at the same time.

Final bulk vaccine. Material that has undergone all the steps of production except for the final filling. It consists of one or more monovalent pooled harvests, from cultures of one or more species or types of micro-organism, after clarification, dilution or addition of any adjuvant or other auxiliary substance. It is treated to ensure its homogeneity and is used for filling the containers of one or more final lots (batches).

Final lot (Batch). A collection of closed, final containers or other final dosage units that are expected to be homogeneous and equivalent with respect to risk of contamination during filling or preparation of the final product. The dosage units are filled, or otherwise prepared, from the same final bulk vaccine, freeze-dried together (if applicable) and closed in one continuous working session. They bear a distinctive number or code identifying the final lot (batch). Where a final bulk vaccine is filled and/or freeze-dried on several separate sessions, there results a related set of final lots (batches) that are usually identified by the use of a common part in the distinctive number or code; these related final lots (batches) are sometimes referred to as sub-batches, sub-lots or filling lots.

Combined vaccine. A multicomponent preparation formulated so that different antigens are administered simultaneously. The different antigenic components are intended to protect against different strains or types of the same organism and/or different organisms. A combined vaccine may be supplied by the manufacturer either as a single liquid or freeze-dried preparation or as several constituents with directions for admixture before use.

01/2005:50202

5.2.2. CHICKEN FLOCKS FREE FROM SPECIFIED PATHOGENS FOR THE PRODUCTION AND QUALITY CONTROL OF VACCINES

INTRODUCTION

Where specified in a monograph, chickens, embryos or cell cultures used for the production or quality control of vaccines are derived from eggs produced by chicken flocks free from specified pathogens (SPF). The SPF status of a flock is ensured by means of the system described below. The list of micro-organisms given is based on current knowledge and will be updated as necessary.

GENERAL PRINCIPLES AND PROCEDURES

A flock is defined as a group of birds sharing a common environment and having their own caretakers who have no contact with non-SPF flocks. Once a flock is defined, no non-SPF birds are added to it.

For SPF flocks established on a rolling basis, all replacements are hatched and reared in the controlled environment house. Subject to the agreement of the competent authorities, SPF embryos derived from a tested SPF flock from another house on the same site may be introduced. From 8 weeks of age, these replacement birds are regarded as a flock and monitored monthly in accordance with the Subsequent Testing requirements. At point of lay, all these replacement birds are tested in accordance with the Initial Testing requirements.

The flock is housed so as to minimise the chance of contamination. It is not sited near to non-SPF flocks of birds and is housed in an isolator or on wire in a building with filtered air under positive pressure. Appropriate measures are taken to prevent access of rodents, wild birds, insects and unauthorised people.

Personnel authorised to enter must have no contact with other birds or with agents likely to infect the flock. It is advisable for personnel to shower and change clothing or to wear protective clothing before entering the chicken house.

Items taken into the flock are sterilised. The feed is suitably treated to avoid the introduction of undesirable micro-organisms and water is obtained from a chlorinated supply. No medication is given that could interfere with detection of disease in the flock.

A permanent record is kept of the general health of the flock and any abnormality is investigated. Factors to be monitored include morbidity, mortality, general physical condition, feed consumption, daily egg production and egg quality, fertility and hatchability. Dirty eggs are discarded; clean eggs may be surface-disinfected whilst warm.

The flock originates from chickens shown to be free from vertically-transmitted agents. In particular, each chicken from which the flock is derived is tested repeatedly to ensure freedom from leucosis viruses and their antibodies. In order to establish the SPF status of a flock, it is kept under SPF conditions for a test period of not less than 4 months. Each bird in the entire flock is shown to be free from evidence of infection with the agents listed below under Initial Testing after 6 weeks and at the end of the test period.

For each new generation in an established flock, all of the birds in the flock are tested at not later than 20 weeks of age, using the tests prescribed below under Initial Testing. After the initial test, monthly tests are carried out on a representative 5 per cent sample (but not less than ten and not more than two hundred birds), using the tests prescribed below under Subsequent Testing, with a final test at 4 weeks after the last collection of eggs.

For all tests, blood samples are collected from an appropriate number of birds at the specified time. The resultant serum samples are examined for antibodies against the relevant agents. Serum-neutralisation tests are done on pools of not more than five sera. All other tests are done on each individual serum. Positive and negative controls are used in all tests. The reagents used in the tests are standardised against international or European standard reagents where these are available. For avian leucosis virus, in addition to tests for antibodies carried out on serum samples, appropriate samples are taken for testing for the virus.

In addition to serological tests, clinical examination is carried out at least once per week to verify that the birds are free from fowl-pox and signs of other infections. Necropsy and, where necessary to confirm diagnosis, histopathological examination are carried out on any bird that dies to verify that there is no sign of infection. The absence of *Salmonella*

spp. is determined by cultural examination of faecal samples at least once every 4 weeks; a pool of up to ten samples may be used for the tests.

If a positive result is obtained in any test carried out to establish the SPF status of a flock, the flock may not be designated as an SPF flock. If a positive result is obtained in any test carried out on an established flock, the flock loses its SPF status. Special provisions apply to chick anaemia agent (CAA) as described below. Any chickens, embryos or cell cultures collected since the previous negative test are not suitable for use: any product made from them must be discarded and any quality control tests done with them are invalid and must be repeated.

In order to regain SPF status, the flock is maintained under SPF conditions and routine 5 per cent monthly testing shall continue except that every bird in the entire flock is tested every month for infection with the particular agent that gave the positive result. Infected birds and their progeny are removed from the flock. SPF status is regained after two such consecutive tests have yielded completely negative results.

A positive result for CAA does not necessarily exclude use of material derived from the flock, but live vaccines for use in birds less than 7 days old must be produced using material from CAA-negative flocks. Inactivated vaccines for use in birds less than 7 days old may be produced using material from flocks that have not been shown to be free from CAA, provided it has been demonstrated that the inactivation process inactivates CAA.

Permanent records of mortality and of results of flock testing are kept for a minimum of five years. Details of any deterioration in egg production or hatchability, except for accidental cases identified as being of non-infectious origin, and of any test results indicating infection with a specified agent, are immediately submitted to the user of the eggs.

INITIAL TESTING

Subject to agreement by the competent authority, other types of test may be used provided they are at least as sensitive as those indicated and of appropriate specificity.

Micro-organism	Type of test
Avian adenoviruses	Enzyme-linked immuno-sorbent assay
Avian encephalomyelitis virus	Enzyme-linked immuno-sorbent assay
Avian infectious bronchitis virus	Enzyme-linked immuno-sorbent assay
Avian infectious laryngotracheitis virus	Serum neutralisation
Avian leucosis viruses	Enzyme-linked immuno-sorbent assay for virus and serum neutralisation for antibody
Avian nephritis virus	Fluorescent antibody
Avian reoviruses	Enzyme-linked immuno-sorbent assay
Avian reticuloendotheliosis virus	Fluorescent antibody
Chick anaemia agent	Fluorescent antibody
Haemagglutinating avian adenovirus (Egg drop syndrome 76- EDS 76 virus)	Haemagglutination inhibition
Infectious bursal disease virus	Serum neutralisation against each serotype present in the country of origin
Influenza A virus	Enzyme-linked immuno-sorbent assay
Marek's disease virus	Enzyme-linked immuno-sorbent assay

Micro-organism	Type of test
Newcastle disease virus	Haemagglutination inhibition
Turkey rhinotracheitis virus	Enzyme-linked immuno-sorbent assay
<i>Mycoplasma gallisepticum</i>	Agglutination and, to confirm a positive test, haemagglutination inhibition
<i>Mycoplasma synoviae</i>	Agglutination and, to confirm a positive test, haemagglutination inhibition
<i>Salmonella pullorum</i>	Agglutination

SUBSEQUENT TESTING

Subject to agreement by the competent authority, other types of test may be used provided they are at least as sensitive as those indicated and of appropriate specificity.

Micro-organism	Type of test
Avian adenoviruses	Enzyme-linked immuno-sorbent assay
Avian encephalomyelitis virus	Enzyme-linked immuno-sorbent assay
Avian infectious bronchitis virus	Enzyme-linked immuno-sorbent assay
Avian infectious laryngotracheitis virus	Serum neutralisation
Avian leucosis viruses	Enzyme-linked immuno-sorbent assay for the antibody
Avian nephritis virus	Fluorescent antibody
Avian reoviruses	Fluorescent antibody
Avian reticuloendotheliosis virus	Fluorescent antibody
Chick anaemia agent	Fluorescent antibody
Haemagglutinating avian adenovirus	Haemagglutination inhibition
Infectious bursal disease virus	Immunodiffusion against each serotype present in the country of origin
Influenza A virus	Enzyme-linked immuno-sorbent assay
Marek's disease virus	Enzyme-linked immuno-sorbent assay
Newcastle disease virus	Haemagglutination inhibition
Turkey rhinotracheitis virus	Enzyme-linked immuno-sorbent assay
<i>Mycoplasma gallisepticum</i>	Agglutination and, to confirm a positive test, haemagglutination inhibition
<i>Mycoplasma synoviae</i>	Agglutination and, to confirm a positive test, haemagglutination inhibition
<i>Salmonella pullorum</i>	Agglutination

01/2005:50203

5.2.3. CELL SUBSTRATES FOR THE PRODUCTION OF VACCINES FOR HUMAN USE

This general chapter deals with diploid cell lines and continuous cell lines used for the production of vaccines for human use; specific issues relating to vaccines prepared by recombinant DNA technology are covered by the monograph on *Products of recombinant DNA technology* (0784). Testing to be carried out at various stages (cell seed, master cell bank, working cell bank, cells at or beyond the maximum population doubling level used for production) is indicated in

Table 5.2.3-1. General provisions for the use of cell lines and test methods are given below. Where primary cells or cells that have undergone a few passages without constitution of a cell bank are used for vaccine production, requirements are given in the individual monograph for the vaccine concerned.

Diploid cell lines. A diploid cell line has a high but finite capacity for multiplication *in vitro*.

Continuous cell lines. A continuous cell line has the capacity to multiply indefinitely *in vitro*; the cells often have differences in karyotype compared to the original cells; they may be obtained from healthy or tumoral tissue.

For injectable vaccines produced in continuous cell lines, the purification process is validated to demonstrate removal of substrate-cell DNA to a level equivalent to not more than 10 ng per single human dose, unless otherwise prescribed.

Cell-bank system. Production of vaccines in diploid and continuous cell lines is based on a cell-bank system. The *in vitro* age of the cells is counted from the master cell bank. Each working cell bank is prepared from one or more containers of the master cell bank. The use, identity and inventory control of the containers is carefully documented.

Media and substances of animal and human origin. The composition of media used for isolation and all subsequent culture is recorded in detail and if substances of animal origin are used they must be free from extraneous agents. If human albumin is used, it complies with the monograph on *Human albumin solution* (0255).

Bovine serum used for the preparation and maintenance of cell cultures is tested and shown to be sterile and free from bovine viruses, notably bovine diarrhoea virus and mycoplasmas.

Trypsin used for the preparation of cell cultures is examined by suitable methods and shown to be sterile and free from mycoplasmas and viruses, notably pestiviruses and parvoviruses.

Cell seed. The data used to assess the suitability of the cell seed comprise information, where available, on source, history and characterisation.

Source of the cell seed. For human cell lines, the following information concerning the donor is recorded: ethnic and geographical origin, age, sex, general physiological condition, tissue or organ used, results of any tests for pathogens.

For animal cell lines, the following information is recorded concerning the source of the cells: species, strain, breeding conditions, geographical origin, age, sex, general physiological condition, tissue or organ used, results of any tests for pathogens.

Cells of neural origin, such as neuroblastoma and P12 cell lines, may contain substances that concentrate agents of spongiform encephalopathies and such cells are not used for vaccine production.

History of the cell seed. The following information is recorded: the method used to isolate the cell seed, culture methods and any other procedures used to establish the master cell bank, notably any that might expose the cells to extraneous agents.

Full information may not be available on the ingredients of media used in the past for cultivation of cells, for example on the source of substances of animal origin; where justified and authorised, cell banks already established using such media may be used for vaccine production.

Characterisation of the cell seed. The following properties are investigated:

(1) the identity of the cells (for example, isoenzymes, serology, nucleic acid fingerprinting);

Table 5.2.3.-1 – Testing of cell lines

Test	Cell seed	Master cell bank (MCB)	Working cell bank (WCB)	Cells at or beyond the maximum population doubling level used for production
1. IDENTITY AND PURITY				
Morphology	+	+	+	+
Relevant selection of the following tests: biochemical (e.g. isoenzymes), immunological (e.g. histocompatibility), cytogenetic markers, nucleic acid fingerprinting	+	+	+	+
Karyotype (diploid cell lines)	+	+	+(1)	+(1)
Life span (diploid cell lines)	–	+	+	–
2. EXTRANEIOUS AGENTS				
Bacterial and fungal contamination	–	+	+	–
Mycoplasmas	–	+	+	–
Tests in cell cultures	–	–	+	–
Co-cultivation	–	–	+(2)	+(2)
Tests in animals and eggs	–	–	+(2)	+(2)
Specific tests for possible contaminants depending on the origin of the cells (see above under Infectious extraneous agents)	–	–	+(2)	+(2)
Retroviruses	–	+(3)	–	+(3)
3. TUMORIGENICITY				
Tumorigenicity	–	–	–	+(4)

(1) The diploid character is established for each working cell bank but using cells at or beyond the maximum population doubling level used for production.

(2) Testing is carried out for each working cell bank, but using cells at or beyond the maximum population doubling level used for production.

(3) Testing is carried out for the master cell bank, but using cells at or beyond the maximum population doubling level used for production.

(4) The MRC-5 cell line, the WI-38 cell line and the FRhL-2 cell line are recognised as being non-tumorigenic and they need not be tested. Tests are not carried out on cell lines that are known or assumed to be tumorigenic.

(2) the growth characteristics of the cells and their morphological properties (light and electron microscopes);

(3) for diploid cell lines, karyotype;

(4) for diploid cell lines, the *in vitro* life span in terms of population doubling level.

Cell substrate stability. Suitable viability of the cell line in the intended storage conditions must be demonstrated. For a given product to be prepared in the cell line, it is necessary to demonstrate that consistent production can be obtained with cells at passage levels at the beginning and end of the intended span of use.

Infectious extraneous agents. Cell lines for vaccine production shall be free from infectious extraneous agents. Tests for extraneous agents are carried out as shown in Table 5.2.3.-1.

Depending on the origin and culture history of the cell line, it may be necessary to carry out tests for selected, specific potential contaminants, particularly those that are known to infect latently the species of origin, for example simian virus 40 in rhesus monkeys. For cell lines of rodent origin, antibody-production tests are carried out in mice, rats and hamsters to detect species-specific viruses.

Cell lines are examined for the presence of retroviruses as described below. Cell lines that show the presence of retroviruses capable of replication are not acceptable for production of vaccines.

Tumorigenicity. For the preparation of live vaccines, the cell line must not be tumorigenic at any population doubling level used for vaccine production. Where a tumorigenic cell

line is used for the production of other types of vaccine, the purification process is validated to demonstrate that residual substrate-cell DNA is reduced to less than 10 ng per single human dose of the vaccine, unless otherwise prescribed, and that substrate-cell protein is reduced to an acceptable level.

A cell line which is known to have tumorigenic potential does not have to be tested further. If a cell line is of unknown tumorigenic potential, it is either regarded as being tumorigenic or it is tested for tumorigenicity using an *in vitro* test as described below; if the result of the *in vitro* test is negative or not clearly positive, an *in vivo* test as described below is carried out. The tests are carried out using cells at or beyond the maximum population doubling level that will be used for vaccine production.

The MRC-5, the WI-38 and the FRhL-2 cell lines are recognised as being non-tumorigenic and further testing is not necessary.

Chromosomal characterisation. Diploid cell lines shall be shown to be diploid. More extensive characterisation of a diploid cell line by karyotype analysis is required if the removal of intact cells during processing after harvest has not been validated. Samples from four passage levels evenly spaced over the life-span of the cell line are examined. A minimum of 200 cells in metaphase are examined for exact count of chromosomes and for frequency of hyperploidy, hypoploidy, polyploidy, breaks and structural abnormalities.

The MRC-5, the WI-38 and the FRhL-2 cell lines are recognised as being diploid and well characterised; where they are not genetically modified, further characterisation is not necessary.

TEST METHODS FOR CELL CULTURES

Identification. Nucleic-acid-fingerprint analysis and a relevant selection of the following are used to establish the identity of the cells:

- (1) biochemical characteristics (isoenzyme analysis),
- (2) immunological characteristics (histocompatibility antigens),
- (3) cytogenetic markers.

Contaminating cells. The nucleic-acid-fingerprint analysis carried out for identification also serves to demonstrate freedom from contaminating cells.

Bacterial and fungal contamination. The master cell bank and each working cell bank comply with the test for sterility (2.6.1), carried out using for each medium 10 ml of supernatant fluid from cell cultures. Carry out the test on 1 per cent of the containers with a minimum of two containers.

Mycoplasmas (2.6.7). The master cell bank and each working cell bank comply with the test for mycoplasmas by the culture method and the indicator cell culture method. Use one or more containers for the test.

Test for extraneous agents in cell cultures. The cells comply with the test for haemadsorbing viruses and with the tests in cell cultures for other extraneous agents given in chapter 2.6.16 under Production cell culture: control cells. If the cells are of simian origin, they are also inoculated into rabbit kidney cell cultures to test for herpesvirus B (cercopithecid herpesvirus 1).

Co-cultivation. Co-cultivate intact and disrupted cells separately with other cell systems including human cells and simian cells. Carry out examinations to detect possible morphological changes. Carry out tests on the cell culture fluids to detect haemagglutinating viruses. The cells comply with the test if no evidence of any extraneous agent is found.

Retroviruses. Examine for the presence of retroviruses using:

- (1) infectivity assays,
- (2) transmission electron microscopy,
- (3) if tests (1) and (2) give negative results, reverse transcriptase assays (in the presence of magnesium and manganese) carried out on pellets obtained by high-speed centrifugation.

Tests in animals. Inject intramuscularly (or, for suckling mice, subcutaneously) into each of the following groups of animals 10^7 viable cells divided equally between the animals in each group:

- (1) two litters of suckling mice less than 24 h old, comprising not fewer than ten animals,
- (2) ten adult mice.

Inject intracerebrally into each of ten adult mice 10^6 viable cells to detect the possible presence of lymphocytic choriomeningitis virus.

Observe the animals for at least 4 weeks. Investigate animals that become sick or show any abnormality to establish the cause of illness. The cells comply with the test if no evidence of any extraneous agent is found. The test is invalid if fewer than 80 per cent of the animals in each group remain healthy and survive to the end of the observation period.

For cells obtained from a rodent species (for example, Chinese hamster ovary cells or baby hamster kidney cells), tests for antibodies against likely viral contaminants of the species in question are carried out on animals that have received injections of the cells.

Tests in eggs. Using an inoculum of 10^6 viable cells per egg, inoculate the cells into the allantoic cavity of ten SPF embryonated hens' eggs (5.2.2) 9 to 11 days old and into the yolk sac of ten SPF embryonated hens' eggs 5 to 6 days old. Incubate for not less than 5 days. Test the allantoic fluids for the presence of haemagglutinins using mammalian and avian red blood cells; carry out the test at 5 ± 3 °C and 20-25 °C and read the results after 30 min and 60 min. The cells comply with the test if no evidence of any extraneous agent is found. The test is invalid if fewer than 80 per cent of the embryos remain healthy and survive to the end of the observation period.

Tests for tumorigenicity *in vitro*. The following test systems may be used:

- (1) colony formation in soft agar gels,
- (2) production of invasive cell growth following inoculation into organ cultures,
- (3) study of transformation activity using, for example, the 3T3 assay system for active oncogenes.

Tests for tumorigenicity *in vivo*. The test consists in establishing a comparison between the cell line and a suitable positive control (for example, HeLa or Hep2 cells).

Animal systems that have been shown to be suitable for this test include:

- (1) athymic mice (Nu/Nu genotype),
- (2) newborn mice, rats or hamsters that have been treated with antithymocyte serum or globulin,
- (3) thymectomised and irradiated mice that have been reconstituted (T⁺, B⁺) with bone marrow from healthy mice.

Whichever animal system is selected, the cell line and the reference cells are injected into separate groups of 10 animals each. In both cases, the inoculum for each animal is 10^7 cells suspended in a volume of 0.2 ml, and the injection may be by either the intramuscular or subcutaneous route. Newborn animals are treated with 0.1 ml of antithymocyte serum or globulin on days 0, 2, 7 and 14 after birth. A potent serum or globulin is one that suppresses the immune mechanisms of growing animals to the extent that the subsequent inoculum of 10^7 positive reference cells regularly produces tumours and metastases. Severely affected animals showing evident progressively growing tumours are killed before the end of the test to avoid unnecessary suffering.

At the end of the observation period all animals, including the reference group(s), are killed and examined for gross and microscopic evidence of the proliferation of inoculated cells at the site of injection and in other organs (for example lymph nodes, lungs, kidneys and liver).

In all test systems, the animals are observed and palpated at regular intervals for the formation of nodules at the sites of injection. Any nodules formed are measured in two perpendicular dimensions, the measurements being recorded regularly to determine whether there is progressive growth of the nodule. Animals showing nodules which begin to regress during the period of observation are killed before the nodules are no longer palpable, and processed for histological examination. Animals with progressively growing nodules are observed for 1-2 weeks. Among those without nodule formation, half are observed for 3 weeks and half for 12 weeks before they are killed and processed for histological examination. A necropsy is performed on each animal and includes examination for gross evidence of tumour formation at the site of injection and in other organs such as lymph nodes, lungs, brain, spleen, kidneys and liver. All tumour-like lesions and the site of injection are examined histologically. In addition, since some cell lines may give rise

to metastases without evidence of local tumour growth, any detectable regional lymph nodes and the lungs of all animals are examined histologically.

The test is invalid if fewer than nine of ten animals injected with the positive reference cells show progressively growing tumours.

01/2005:50204

5.2.4. CELL CULTURES FOR THE PRODUCTION OF VETERINARY VACCINES

Cell cultures for the production of vaccines for veterinary use comply with the requirements of this section. It may also be necessary that cell cultures used for testing of vaccines for veterinary use also comply with some or all of these requirements.

For most mammalian viruses, propagation in cell lines is possible and the use of primary cells is then not acceptable.

Permanently infected cells used for production of veterinary vaccines comply with the appropriate requirements described below. The cells shall be shown to be infected only with the agent stated.

CELL LINES

Cell lines are normally handled according to a cell-seed system. Each master cell seed is assigned a specific code for identification purposes. The master cell seed is stored in aliquots at -70°C or lower. Production of vaccine is not normally undertaken on cells more than twenty passages from the master cell seed. Where suspension cultures are used, an increase in cell numbers equivalent to approximately three population doublings is considered equivalent to one passage. If cells beyond twenty passage levels are to be used for production, it shall be demonstrated, by validation or further testing, that the production cell cultures are essentially similar to the master cell seed with regard to their biological characteristics and purity and that the use of such cells has no deleterious effect on vaccine production.

The history of the cell line shall be known and recorded in detail (for example, origin, number of passages and media used for multiplication, storage conditions).

The method of storing and using the cells, including details of how it is ensured that the maximum number of passages permitted is not exceeded during product manufacture, are recorded. A sufficient quantity of the master cell seed and each working cell seed are kept for analytical purposes.

The tests described below are carried out (as prescribed in Table 5.2.4.-1) on a culture of the master cell seed and the working cell seed or on cell cultures from the working cell seed at the highest passage level used for production and derived from a homogeneous sample demonstrated to be representative.

Characteristics of culture. The appearance of cell monolayers, before and after histological staining, is described. Information, if possible numerical data, is provided especially on the speed and rate of growth. Similarly, the presence or absence of contact inhibition, polynucleated cells and any other cellular abnormalities are specified.

Karyotype. A chromosomal examination is made of not fewer than fifty cells undergoing mitosis in the master cell seed and at a passage level at least as high as that to be used in production. Any chromosomal marker present in the master cell seed must also be found in the high passage

cells and the modal number of chromosomes in these cells must not be more than 15 per cent higher than of cells of the master cell seed. The karyotypes must be identical. If the modal number exceeds the level stated, if the chromosomal markers are not found in the working cell seed at the highest level used for production or if the karyotype differs, the cell line shall not be used for manufacture.

Table 5.2.4.-1. – *Cell culture stage at which tests are carried out*

	Master cell seed	Working cell seed	Cell from working cell seed at highest passage level
General microscopy	+	+	+
Bacteria and fungi	+	+	–
Mycoplasmas	+	+	–
Viruses	+	+	–
Identification of species	+	–	+
Karyotype	+	–	+
Tumorigenicity	+	–	–

Identification of the species. It shall be shown, by one validated method, that the master cell seed and the cells from the working cell seed at the highest passage level used for production come from the species of origin specified. When a fluorescence test is carried out and the corresponding serum to the species of origin of cells is used and shows that all the tested cells are fluorescent, it is not necessary to carry out other tests with reagents able to detect contamination by cells of other species.

Bacterial and fungal contamination. The cells comply with the test for sterility (2.6.1). The sample of cells to be examined consists of not less than the number of cells in a monolayer with an area of 70 cm^2 or, for cells grown in suspension, an approximately equivalent number of cells. The cells are maintained in culture for at least 15 days without antibiotics before carrying out the test.

Mycoplasmas (2.6.7). The cells comply with the test for mycoplasmas. The cells are maintained in culture for at least 15 days without antibiotics before carrying out the test.

Absence of contaminating viruses. The cells must not be contaminated by viruses; suitably sensitive tests, including those prescribed below, are carried out.

The monolayers tested shall have an area of at least 70 cm^2 , and shall be prepared and maintained using medium and additives, and grown under similar conditions to those used for the preparation of the vaccine. The monolayers are maintained in culture for a total of at least 28 days. Subcultures are made at 7-day intervals, unless the cells do not survive for this length of time, when the subcultures are made on the latest day possible. Sufficient cells, in suitable containers, are produced for the final subculture to carry out the tests specified below.

The monolayers are examined regularly throughout the incubation period for the possible presence of cytopathic effects and at the end of the observation period for cytopathic effects, haemadsorbent viruses and specific viruses by immuno-fluorescence and other suitable tests as indicated below.

Detection of cytopathic viruses. Two monolayers of at least 6 cm^2 each are stained with an appropriate cytological stain. The entire area of each stained monolayer is examined for any inclusion bodies, abnormal numbers of giant cells or any other lesion indicative of a cellular abnormality which might be attributable to a contaminant.

Detection of haemadsorbent viruses. Monolayers totalling at least 70 cm² are washed several times with an appropriate buffer and a sufficient volume of a suspension of suitable red blood cells added to cover the surface of the monolayer evenly. After different incubation times cells are examined for the presence of haemadsorption.

Detection of specified viruses. Tests are carried out for freedom from contaminants specific for the species of origin of the cell line and for the species for which the product is intended. Sufficient cells on suitable supports are prepared to carry out tests for the agents specified. Suitable positive controls are included in each test. The cells are subjected to suitable tests, for example using fluorescein-conjugated antibodies or similar reagents.

Tests in other cell cultures. Monolayers totalling at least 140 cm² are required. The cells are frozen and thawed at least three times and then centrifuged to remove cellular debris. Inoculate aliquots onto the following cells at any time up to 70 per cent confluency:

- primary cells of the source species;
- cells sensitive to viruses pathogenic for the species for which the vaccine is intended;
- cells sensitive to pestiviruses.

The inoculated cells are maintained in culture for at least 7 days, after which freeze-thawed extracts are prepared as above and inoculated onto sufficient fresh cultures of the same cell types to allow for the testing as described below. The cells are incubated for at least a further 7 days. The cultures are examined regularly for the presence of any cytopathic changes indicative of living organisms.

At the end of this period of 14 days, the inoculated cells are subjected to the following checks:

- freedom from cytopathic and haemadsorbent organisms, using the methods specified in the relevant paragraphs above,
- absence of pestiviruses and other specific contaminants by immunofluorescence or other validated methods as indicated in the paragraph above on Detection of Specified Viruses.

Tumorigenicity. The risk of a cell line for the target species must be evaluated and, if necessary, tests are carried out.

PRIMARY CELLS

For most mammalian vaccines, the use of primary cells is not acceptable for the manufacture of vaccines since cell lines can be used. If there is no alternative to the use of primary cells, the cells are obtained from a herd or flock free from specified pathogens, with complete protection from introduction of diseases (for example, disease barriers, filters on air inlets, suitable quarantine before introduction of animals). Chicken flocks comply with the requirements prescribed under *Chicken Flocks Free from Specified Pathogens for the Production and Quality Control of Vaccines* (5.2.2). For all other species, the herd or flock is shown to be free from relevant specified pathogens. All the breeding stock in the herd or flock intended to be used to produce primary cells for vaccine manufacture is subject to a suitable monitoring procedure including regular serological checks carried out at least twice a year and two supplementary serological examinations performed in 15 per cent of the breeding stock in the herd between the two checks mentioned above.

Wherever possible, particularly for mammalian cells, a seed-lot system is used with, for example, a master cell seed formed after less than five passages, the working cell seed being no more than five passages from the initial preparation of the cell suspension from the animal tissues.

Each master cell seed, working cell seed and cells of the highest passage of primary cells are checked in accordance with Table 5.2.4.-2 and the procedure described below. The sample tested shall cover all the sources of cells used for the manufacture of the batch. No batches of vaccine manufactured using the cells may be released if any one of the checks performed produces unsatisfactory results.

Table 5.2.4.-2. – *Cell culture stage at which tests are carried out*

	Master cell seed	Working cell seed	Highest passage level
General microscopy	+	+	+
Bacteria and fungi	+	+	–
Mycoplasmas	+	+	–
Viruses	+	+	–
Identification of species	+	–	–

Characteristics of cultures. The appearance of cell monolayers, before and after histological staining, is described. Information, if possible numerical data, is recorded, especially on the speed and rate of growth. Similarly, the presence or absence of contact inhibition, polynucleated cells and any other cellular abnormalities are specified.

Identification of species. It shall be demonstrated by one validated test that the master cell seed comes from the specified species of origin.

When a fluorescence test is carried out and the corresponding serum to the species of origin of cells is used and shows that all the tested cells are fluorescent, it is not necessary to carry out other tests with reagents able to detect contamination by cells of other species.

Bacterial and fungal sterility. The cells comply with the test for sterility (2.6.1). The sample of cells to be examined consists of not less than the number of cells in a monolayer with an area of 70 cm² or for cells grown in suspension an approximately equivalent number of cells. The cells are maintained in culture for at least 15 days without antibiotics before carrying out the test.

Mycoplasmas (2.6.7). The cells comply with the test for mycoplasmas. The cells are maintained in culture for at least 15 days without antibiotics before carrying out the test.

Absence of contaminating viruses. The cells must not be contaminated by viruses; suitably sensitive tests, including those prescribed below are carried out.

The monolayers tested shall be at least 70 cm², and shall be prepared and maintained in culture using the same medium and additives, and under similar conditions to those used for the preparation of the vaccine.

The monolayers are maintained in culture for a total of at least 28 days or for the longest period possible if culture for 28 days is impossible. Subcultures are made at 7-day intervals, unless the cells do not survive for this length of time when the subcultures are made on the latest day possible. Sufficient cells, in suitable containers are produced for the final subculture to carry out the tests specified below.

The monolayers are examined regularly throughout the incubation period for the possible presence of cytopathic effects and at the end of the observation period for cytopathic effects, haemadsorbent viruses and specific viruses by immunofluorescence and other suitable tests as indicated below.

Detection of cytopathic viruses. Two monolayers of at least 6 cm² each are stained with an appropriate cytological stain. Examine the entire area of each stained monolayer for any inclusion bodies, abnormal numbers of giant cells or any other lesion indicative of a cellular abnormality that might be attributable to a contaminant.

Detection of haemadsorbent viruses. Monolayers totalling at least 70 cm² are washed several times with a suitable buffer solution and a sufficient volume of a suspension of suitable red blood cells added to cover the surface of the monolayer evenly. After different incubation times, examine cells for the presence of haemadsorption.

Detection of specified viruses. Tests are carried out for freedom of contaminants specific for the species of origin of the cells and for the species for which the product is intended.

Sufficient cells on suitable supports are prepared to carry out tests for the agents specified. Suitable positive controls are included in each test. The cells are subjected to suitable tests using fluorescein-conjugated antibodies or similar reagents.

Tests in other cell cultures. Monolayers totalling at least 140 cm² are required. The cells are frozen and thawed at least three times and then centrifuged to remove cellular debris. Aliquots are inoculated onto the following cells at any time up to 70 per cent confluency:

- primary cells of the source species;
- cells sensitive to viruses pathogenic for the species for which the vaccine is intended;
- cells sensitive to pestiviruses.

The inoculated cells are maintained in culture for at least 7 days, after which freeze-thawed extracts are prepared as above, and inoculated onto sufficient fresh cultures of the same cell types to allow for the testing as described below. The cells are incubated for at least a further 7 days. All cultures are regularly examined for the presence of any cytopathic changes indicative of living organisms.

At the end of this period of 14 days, the inoculated cells are subjected to the following checks:

- freedom from cytopathic and haemadsorbent organisms is demonstrated using the methods specified in the relevant paragraphs above;
- relevant substrates are tested for the absence of pestiviruses and other specific contaminants by immunofluorescence or other validated methods as indicated in the paragraph above on Detection of Specified Viruses.

01/2005:50205

5.2.5. SUBSTANCES OF ANIMAL ORIGIN FOR THE PRODUCTION OF VETERINARY VACCINES

Substances of animal origin (for example, serum, trypsin and serum albumin) may be used during the manufacture of veterinary immunological products, as ingredients of culture media etc. or as added constituents of vaccines or diluents. It is recommended to reduce, wherever practicable, the use of such substances.

Certain restrictions are placed upon the use of such substances to minimise the risk associated with pathogens that may be present in them.

- The use of substances of animal origin as constituents of vaccines or diluents is not generally acceptable except where such substances are sterilised by a suitable,

validated method. Where the use of such substances has been shown to be essential and sterilisation is not possible, the criteria described under Requirements apply.

- Substances of animal origin used during production are either subjected to a suitable, validated sterilisation or inactivation procedure or the substance is tested for the absence of extraneous organisms in accordance with the Requirements below. For inactivated vaccines, the method used for inactivation of the vaccine strain may also be validated for inactivation of possible contaminants from substances of animal origin.

In addition to the restrictions described below, manufacturers must consider restrictions on the handling of substances of animal origin in the vaccine manufacturing premises.

The restrictions imposed by these sections may need to be varied in accordance with changes in the incidence of disease in the country of origin and in Europe.

REQUIREMENTS

Substances of animal origin comply with the requirements of the Pharmacopoeia (where a relevant monograph exists).

Source. The risk related to the animal diseases occurring in the country of origin of the substance and to the potential infectious diseases occurring in the source species, in relation to the proposed recipient species must be carefully evaluated. The strictest possible selection criteria must be applied, in particular for substances for use in products intended for the same species and for substances of bovine, caprine, ovine and porcine origin.

Preparation. Substances of animal origin are prepared from a homogeneous bulk designated with a batch number. A batch may contain substances derived from as many animals as desired but once defined and given a batch number, the batch is not added to or contaminated in any way.

All batches of substances shall be shown to be free from contaminants as described below and/or are subject to a validated inactivation procedure.

Inactivation. The inactivation procedure chosen shall have been shown to be capable of reducing the titre of certain potential contaminants in the substance concerned by at least 10⁶. If this reduction in titre cannot be shown experimentally, kinetic studies for the inactivation procedure must be carried out and shown to be satisfactory, taking into account the possible level of contamination.

The list of potential contaminating organisms that the procedure must be shown to be capable of inactivating must be appropriate to the particular species of origin of the substance. The evidence for the efficacy of the procedure, which must relate to the current circumstances, may take the form of references to published literature or experimental data generated by the manufacturer.

Tests. For examination of the substance for freedom from contaminants, any solids are dissolved or suspended in a suitable medium in such a way as to create a solution or suspension containing at least 300 g/l of the substance to be examined. If the substance is not soluble or where cytotoxic reactions occur, a lower concentration may be used.

Any batch of substance found to contain living organisms of any kind is unsatisfactory and is either discarded or re-processed and shown to be satisfactory.

Freedom from extraneous viruses. The solution or suspension of the solid substance or the undiluted liquid substance is tested for contaminants by suitably sensitive methods. These methods shall include tests in suitably

sensitive cell cultures, including primary cells from the same species as the substances to be examined. A proportion of the cells is passaged at least twice.

The cells are observed regularly for 21 days for cytopathic effects. At the end of each 7 day period, a proportion of the original cultures is fixed, stained and examined for cytopathic effects; a proportion is tested for haemadsorbing agents; and a proportion is tested for specific agents by appropriate serodiagnostic tests.

Bacterial and fungal contamination. Before use, substances are tested for sterility (2.6.1) and freedom from mycoplasmas (2.6.7) or sterilised to inactivate any bacterial, fungal or mycoplasmal contaminants.

01/2005:50206

5.2.6. EVALUATION OF SAFETY OF VETERINARY VACCINES

During the development of the vaccine, safety tests are carried out in the target species to show the risks from use of the vaccine. Live vaccines are prepared only from strains of organisms that have been shown to be safe.

In the tests, "dose" means that quantity of the product to be recommended for use and containing the maximum titre or potency likely to be contained in production batches. For live vaccines, a batch or batches of vaccine prepared from the least attenuated passage to be used for production shall be used in the tests.

The safety of each of the components of combined vaccines and the safety of the combined product shall be demonstrated. For inactivated vaccines, safety tests carried out on the combined vaccine may be regarded as sufficient to demonstrate the safety of the individual components.

The tests described below, modified or supplemented by tests described in the Production section of a specific monograph may be carried out as part of the tests necessary during development to demonstrate the safety of a vaccine.

A. LABORATORY TESTS

Safety of the administration of one dose. For each of the recommended routes of administration, administer one dose of vaccine to susceptible animals of each species and category for which use of the vaccine is to be recommended. This must include animals of the youngest recommended age and pregnant animals, if appropriate. The animals are observed and examined for signs of abnormal local and systemic reactions. Where appropriate, these studies shall include detailed post-mortem macroscopic and microscopic examinations of the injection site; such examinations are not usually necessary for non-food animals. Other objective criteria are recorded, such as rectal temperature (for mammals) and performance measurements. The rectal temperatures are recorded on at least the day before vaccination, at the time of vaccination, 4 h after vaccination and on the following 4 days. The animals are observed and examined until reactions may no longer be expected but, in all cases, the observation and examination period extends at least until 14 days after administration.

As part of these studies, examination of reproductive performance must also be considered when data suggest that the starting material from which the product is derived may be a risk factor. Where prescribed in a monograph, reproductive performance of males and non-pregnant and pregnant females and harmful effects on the progeny, including teratogenic and abortifacient effects, are investigated.

Safety of one administration of an overdose. An overdose of the product is administered by each recommended route of administration to animals of the most sensitive categories of the target species, including animals of the youngest age and pregnant animals, if appropriate. The overdose normally consists of 10 doses of a live vaccine or 2 doses of an inactivated product. The animals are observed and examined for signs of local and systemic reactions. Other objective criteria are recorded, such as rectal temperature (for mammals) and performance measurements. The animals are observed and examined for at least 14 days after administration.

Safety of the repeated administration of one dose. Repeated administration of one dose may be required to reveal any adverse effects induced by such administration. These tests are carried out on the most sensitive categories of the target species, using the recommended route of administration. The animals are observed and examined for at least 14 days after the last administration for signs of systemic and local reactions. Other objective criteria are recorded, such as rectal temperature (for mammals) and performance measurements.

Residues. It is not normally necessary to undertake a study of residues. However, where adjuvants and/or preservatives are used in the manufacture of veterinary vaccines, consideration shall be given to the possibility of any residue remaining in the foodstuffs. If necessary, the effects of such residues are investigated. Moreover, in the case of live vaccines for well established zoonotic diseases, the determination of residual vaccine organisms at the injection site may be required, in addition to the studies of dissemination described below.

Adverse effects on immunological functions. Where the vaccine might adversely affect the immune response of the vaccinated animal or of its progeny, suitable tests on the immunological functions are carried out.

Special requirements for live vaccines. The following laboratory tests must also be carried out with live vaccines.

a) Spread of the vaccine strain. Spread of the vaccine strain from vaccinated to unvaccinated target animals is investigated using the recommended route of administration most likely to result in spread. Moreover, it may be necessary to investigate the safety of spread to non-target species that could be highly susceptible to a live vaccine strain. An assessment must be made of how many animal-to-animal passages are likely to be sustainable under normal circumstances together with an assessment of the likely consequences.

b) Dissemination in vaccinated animal. Faeces, urine, milk, eggs, oral, nasal and other secretions shall be tested for the presence of the organism. Moreover, studies may be required of the dissemination of the vaccine strain in the body, with particular attention being paid to the predilection sites for replication of the organism. In the case of live vaccines for well-established zoonotic diseases for food-producing animals, these studies are obligatory.

c) Reversion to or increase in virulence. For attenuated vaccines, use material from the passage level that is least attenuated for the target species between the master seed lot and the final product. For other live vaccines, use material from the passage likely to have maximum virulence for the target species. The initial vaccination is carried out using the recommended route of administration most likely to lead to reversion to virulence. After this, not fewer than 5 further serial passages through animals of the target species are undertaken. Where this is not technically possible due to failure of the organism to replicate adequately, the test is repeated and as many passages as possible are carried out in

the target species. If necessary, *in vitro* propagation of the organism may be carried out between two passages *in vivo*. The passages are undertaken by the route of administration most likely to lead to reversion to virulence. Not fewer than two fully susceptible animals are used for each passage. At each passage, the presence of living vaccine-derived organisms in the material used for passage is demonstrated. The safety of material from the highest successful passage is compared with that of unpassaged material.

For particular viruses, a monograph may require more passages in more animals if there is an indication from available data that this is relevant. At least the final passage is done using animals most appropriate to the potential risk being assessed.

d) Biological properties of the vaccine strain. Other tests may be necessary to determine as precisely as possible the intrinsic biological properties of the vaccine strain (for example, neurotropism). For vector vaccines, evaluation is made of the risk of changing the tropism or virulence of the strain and where necessary specific tests are carried out. Such tests are systematically carried out where the product of a foreign gene is incorporated into the strain as a structural protein.

e) Recombination or genomic reassortment of strain. The probability of recombination or genomic reassortment with field or other strains shall be considered.

B. FIELD STUDIES

Results from laboratory studies shall normally be supplemented with supportive data from field studies.

For food-producing mammals, the studies include measurement of the rectal temperatures of a sufficient number of animals, before and after vaccination; for other mammals, such measurements are carried out if the laboratory studies indicate that there might be a problem. The size and persistence of any local reaction and the proportion of animals showing local or systemic reactions are recorded. Performance measurements are made, where appropriate.

C. ECOTOXICITY

An assessment is made of the potential harmful effects of the vaccine for the environment and any necessary precautionary measures to reduce such risks are identified. The likely degree of exposure of the environment to the vaccine is assessed taking into account: the target species and mode of administration; excretion of the product; disposal of unused vaccine. If these factors indicate that there will be significant exposure of the environment to the product, the potential ecotoxicity is evaluated taking into account the properties of the vaccine as determined, for example, in the tests described in this section.

methods of vaccination and using the recommended schedule to animals of each species and category for which use of the vaccine is to be recommended. The type of efficacy testing to be carried out varies considerably depending on the particular type of vaccine.

As part of or in addition to tests carried out during development to establish efficacy, the tests described in the Production section of a specific monograph may be carried out; the following must be taken into account.

The dose to be used is that quantity of the product to be recommended for use and containing the minimum titre or potency expected at the end of the period of validity.

For live vaccines, vaccine prepared from the most attenuated passage to be used for production is used.

The efficacy evidence must support all the claims being made. For example, claims for protection against respiratory disease must be supported by at least evidence of protection from clinical signs of respiratory disease. Where it is claimed that there is protection from infection this must be demonstrated using re-isolation techniques. If more than one claim is made, supporting evidence for each claim is required.

The influence of passively acquired and maternally derived antibodies on the efficacy of a vaccine is adequately evaluated. Any claims, stated or implied, regarding onset and duration of protection shall be supported by data from trials.

The efficacy of each of the components of multivalent and combined products shall be demonstrated using the combined vaccine.

Studies of immunological compatibility are undertaken when simultaneous administration is recommended or where it is a part of a usual vaccination schedule. Wherever a product is to be recommended as part of a vaccination scheme, the priming or booster effect or the contribution of the product to the efficacy of the scheme as a whole shall be demonstrated.

LABORATORY TESTS

In principle, demonstration of efficacy is undertaken under well-controlled laboratory conditions by challenge after administration of the product to the target animal under the recommended conditions of use. Challenge is carried out using a strain different from the one used in the production of the vaccine. In so far as possible, the conditions under which the challenge is carried out shall mimic the natural conditions for infection, for example with regard to the amount of challenge organism and the route of administration of the challenge.

If possible, the immune mechanism (cell-mediated/humoral, local/general, classes of immunoglobulin) that is initiated after the administration of the vaccine to target animals shall be determined.

FIELD TRIALS

In general, results from laboratory tests are supplemented with data from field trials, carried out with untreated control animals. Where laboratory trials cannot be supportive of efficacy, the performance of field trials alone may be acceptable.

01/2005:50207

5.2.7. EVALUATION OF EFFICACY OF VETERINARY VACCINES

During development of a vaccine, tests are carried out to demonstrate that the vaccine is efficacious when administered by each of the recommended routes and

01/2005:50208

5.2.8. MINIMISING THE RISK OF TRANSMITTING ANIMAL SPONGIFORM ENCEPHALOPATHY AGENTS VIA HUMAN AND VETERINARY MEDICINAL PRODUCTS

This chapter is identical with the Note for Guidance on Minimising the Risk of Transmitting Animal Spongiform Encephalopathy Agents via Human and Veterinary Medicinal Products - Revision 2, October 2003 [Committee for Proprietary Medicinal Products (CPMP), Committee for Veterinary Medicinal Products (CVMP), European Agency for the Evaluation of Medicinal Products].

Contents

1. INTRODUCTION
 - 1-1. Scientific background
 - 1-2. Regulatory compliance
2. SCOPE OF THE CHAPTER
3. GENERAL CONSIDERATIONS
 - 3-1. Scientific principles for minimising risk
 - 3-2. Source animals
 - 3-2-1. Geographical sourcing
 - 3-2-1-1. Bovine materials
 - 3-2-1-2. Sheep and goats (small ruminants)
 - 3-2-2. BSE negligible risk (closed) bovine herds
 - 3-3. Animal parts, body fluids and secretions as starting materials
 - 3-4. Age of animals
 - 3-5. Manufacturing Process
4. RISK ASSESSMENT OF MATERIALS OR SUBSTANCES USED IN THE MANUFACTURE AND PREPARATION OF A MEDICINAL PRODUCT IN THE CONTEXT OF REGULATORY COMPLIANCE
5. BENEFIT/RISK EVALUATION
6. SPECIFIC CONSIDERATIONS
 - 6-1. Collagen
 - 6-2. Gelatin
 - 6-3. Bovine blood derivatives
 - 6-4. Tallow derivatives
 - 6-5. Animal charcoal
 - 6-6. Milk and milk derivatives
 - 6-7. Wool derivatives
 - 6-8. Amino acids

1. INTRODUCTION

1-1. SCIENTIFIC BACKGROUND

Transmissible Spongiform Encephalopathies (TSEs) are chronic degenerative nervous diseases characterised by the accumulation of an abnormal isoform of a cellular glycoprotein known as PrP (or prion protein). The abnormal isoform of PrP (PrP^{Sc}) differs from normal PrP (PrP^C) in being highly resistant to protease and heat denaturation treatments. PrP^{Sc} is considered to be the infective agent responsible for transmitting TSE disease.

TSE diseases in animals include:

- bovine spongiform encephalopathy (BSE) in cattle,
- scrapie in sheep and goats,
- chronic wasting disease (CWD) in cervids (deer and elk),

- transmissible mink encephalopathy (TME) in farmed mink,
- feline spongiform encephalopathy (FSE) in felidae (specifically domestic cats and captive large cats), and
- spongiform encephalopathy of exotic ungulates in zoos.

In humans, spongiform encephalopathies include different forms of Creutzfeldt-Jakob Disease (CJD), Kuru, Gerstmann-Sträussler-Scheinker Syndrome (GSS), and Fatal Familial Insomnia (FFI).

Iatrogenic transmission of spongiform encephalopathies has been reported. In sheep, scrapie has been accidentally transmitted by the use of Louping Ill vaccine prepared from pooled, formaldehyde treated ovine brain and spleen in which material from scrapie-infected sheep had been inadvertently incorporated. In man, cases of transmission of CJD have been reported which have been attributed to the parenteral administration of growth hormone and gonadotropin derived from human cadaveric pituitary glands. Cases of CJD have also been attributed to the use of contaminated instruments in brain surgery and with the transplantation of human dura mater and cornea.

Interspecies TSE transmission is restricted by a number of natural barriers, transmissibility being affected by the species of origin, the prion strain, dose, route of exposure and, in some species, the host allele of the PrP gene. Species barriers can be crossed under appropriate conditions.

Bovine spongiform encephalopathy (BSE) was first recognised in the United Kingdom in 1986 and a large number of cattle and individual herds have been affected. It is clear that BSE is a food borne disease associated with feeding meat and bone meal derived from TSE affected animals. Other countries have experienced cases of BSE, either in animals imported from the United Kingdom or in indigenous animals. There is convincing evidence to show that the variant form of CJD (vCJD) is caused by the agent which is responsible for BSE in cattle. Therefore, a cautious approach continues to be warranted if biological materials from species naturally affected by TSE diseases, especially bovine species, are used for the manufacture of medicinal products.

Scrapie occurs worldwide and has been reported in most European countries. It has the highest incidence in the United Kingdom. While humans have been exposed to naturally occurring scrapie for over 200 years, there is no epidemiological evidence directly linking scrapie to spongiform encephalopathies in humans. However, there remains a theoretical and currently unquantifiable risk that some BSE-contaminated protein supplement may have been fed to sheep. If such feed causes a recurrent BSE infection in sheep, it may be diagnosed as scrapie and might as such pose a risk of human TSEs. Further, it should also be assumed that any BSE agent introduced into the small ruminant population via contaminated feed is likely to be recycled and amplified.

1-2. REGULATORY COMPLIANCE

Risk assessment. Since the use of animal-derived materials is unavoidable for the production of some medicinal products and that complete elimination of risk at source is rarely possible, the measures taken to manage the risk of transmitting animal TSEs via medicinal products represent risk minimisation rather than risk elimination. Consequently, the basis for regulatory compliance should be based on a risk assessment, taking into consideration all pertinent factors as identified in this chapter (see below).

Legal Aspects. The note for guidance has been given the force of law by virtue of Annex I to European Parliament and Council Directives 2001/82/EC and 2001/83/EC

(as amended by Commission Directive 2003/63/EC⁽¹⁾), governing the veterinary and human medicinal products, respectively. These directives require that applicants for marketing authorisation for human and veterinary medicinal products must demonstrate that medicinal products are manufactured in accordance with the latest version of this note for guidance published in the *Official Journal of the European Union*. This is a continuing obligation after the marketing authorisation has been granted.

By definition, the principle of Specified Risk Materials as defined in Regulation (EC) No 999/2001 of the European Parliament and of the Council⁽²⁾ does not apply to medicinal products. The use of substances derived from high infectivity tissues must be fully justified following an appropriate benefit/risk evaluation (see further below).

The note for guidance should be read in conjunction with the various European Community legal instruments including Commission decisions progressively implemented since 1991. Where appropriate, references to these decisions are given in the text. Position statements and explanatory notes made by the Committee for Proprietary Medicinal Products (CPMP) and Committee for Veterinary Medicinal Products (CVMP) are still applicable for the purpose of regulatory compliance unless otherwise superseded by the note for guidance.

The general monograph *Products with risk of transmitting agents of animal spongiform encephalopathies* of the European Pharmacopoeia refers to this chapter, which is identical with the note for guidance. The monograph forms the basis for issuing Certificates of Suitability as a procedure for demonstrating TSE compliance for substances and materials used in the manufacture of human and veterinary medicinal products.

Clarification of note for guidance. As the scientific understanding of TSEs, especially the pathogenesis of the diseases, is evolving, from time to time CPMP and its Biotechnology Working Party in collaboration with CVMP and its Immunologicals Working Party may be required in the future to develop supplementary guidance in the form of position statements or explanatory notes for the purpose of clarifying the note for guidance. The supplementary guidance shall be published by the Commission and on the website of the European Agency for the Evaluation of Medicinal Products (EMA) and taken into consideration accordingly in the scope of the certification of the European Directorate for the Quality of Medicines (EDQM).

Implementation of the revised note for guidance. All authorised medicinal products in the European Union have demonstrated compliance with the note for guidance on minimising the risk of transmitting animal spongiform encephalopathy agents via human and veterinary medicinal products (EMA/410/01-Rev.1) in line with the legal requirement as inscribed in Annex I to Directive 2001/82/EC (veterinary medicines) or Directive 2001/83/EC as amended by Directive 2003/63/EC (medicines for human use). The revised note for guidance is to be applied prospectively, i.e. for all medicinal products that will be authorised or whose marketing authorisation will be renewed after the time of coming into operation of the revised note for guidance.

2. SCOPE OF THE CHAPTER

TSE-RELEVANT ANIMAL SPECIES

Cattle, sheep, goats and animals that are naturally susceptible to infection with transmissible spongiform encephalopathy agents or susceptible to infection through the oral route other than humans⁽³⁾ and non-human primates are defined as “TSE-relevant animal species”⁽⁴⁾.

MATERIALS

This chapter is concerned with materials derived from “TSE-relevant animal species” that are used for the preparation of:

- active substances,
- excipients and adjuvants,
- raw and starting materials and reagents used in production (e.g. bovine serum albumin; enzymes; culture media including those used to prepare working cell banks, or new master cell banks for medicinal products which are subject to a new marketing authorisation).

This chapter is also applicable to materials that come into direct contact with the equipment used in manufacture of the medicinal product or that come in contact with the medicinal product and therefore have the potential for contamination.

Materials used in the qualification of plant and equipment, such as culture media used in media fill experiments to validate the aseptic filling process, shall be considered in compliance with this chapter provided that the constituent or constituents are derived from tissues with no detectable infectivity (category C tissues), where the risk of cross-contamination with potentially infective tissues has been considered (see section 3-3) and where the materials are sourced from a GBR I/II country (see section 3-2). Such information shall be provided in the dossier for a marketing authorisation and verified during routine inspection for compliance with Good Manufacturing Practice (GMP).

Other materials such as cleaning agents, softeners and lubricants that come into contact with the medicinal product during its routine manufacture or in the finishing stage or in the primary packaging are considered in compliance with this chapter if they are derived from tallow under the conditions described in section 6.

SEED LOTS, CELL BANKS AND ROUTINE FERMENTATION/PRODUCTION⁽⁵⁾

For the purpose of regulatory compliance, master seeds or master cell banks in marketing authorisation applications lodged after 1 July 2000 (for human medicinal products) or 1 October 2000 (for veterinary medicinal products) are covered by the note for guidance.

Master seeds and master cell banks,

- for vaccine antigens;
- for a biotechnology-derived medicinal product within the meaning of Part A of the Annex to Council Regulation (EC) No 2309/93; and
- for other medicinal products using seed lots or cell banking systems in their manufacture,

that have already been approved for the manufacture of a constituent of an authorised medicinal product shall be considered in compliance with the note for guidance even if they are incorporated in marketing authorisation

(1) O.J. L 159, 27.06.2003, p. 46

(2) O.J. L 147, 31.05.2001, p. 1

(3) Regulatory guidance and position papers have been issued by the Committee for Proprietary Medicinal Products and its Biotechnology Working Party on human tissue derived medicinal products in relation with CJD and vCJD. Such guidance can be found on <http://www.emea.eu.int>.

(4) Pigs and birds, which are animal species of particular interest for the production of medicinal products, are not naturally susceptible to infection via the oral route. Therefore they are not TSE-relevant animal species within the meaning of this chapter. Also dogs, rabbits and fish are non TSE-relevant animal species within the meaning of this chapter.

(5) See also: Position paper on the assessment of the risk of transmission of animal spongiform encephalopathy agents by master seed materials used in the production of veterinary vaccines (EMA/CVMP/019/01-February 2001 adopted by the Committee for Veterinary Medicinal products (CVMP) in July 2001, Official Journal of the European Communities C 286 of 12 October 2001, p.12.

applications lodged after 1 July 2000 (for human medicinal products) or 1 October 2000 (for veterinary medicinal products).

Master cell banks and master seeds established before 1 July 2000 (for human medicinal products) or 1 October 2000 (for veterinary medicinal products), but not yet approved as a constituent of an authorised medicinal product shall demonstrate that they fulfil the requirements of the note for guidance. If, for some raw or starting materials or reagents used for the establishment of these cell banks or seeds, full documentary evidence is not/no longer available, the applicant should present a risk assessment as described in Section 4 of the note for guidance.

Established working seeds or cell banks used in the manufacture of medicinal products authorised before 1 July 2000 (human medicines) or 1 October 2000 (veterinary medicines), which have been subjected to a properly conducted risk assessment by a competent authority of the Member States or the EMEA and declared to be acceptable, shall also be considered compliant.

However, where materials derived from the “TSE-relevant animal species” are used in fermentation/routine production processes or in the establishment of working seeds and working cell banks, the applicant must demonstrate that they fulfil the requirements of the note for guidance.

3. GENERAL CONSIDERATIONS

3-1. SCIENTIFIC PRINCIPLES FOR MINIMISING RISK

When manufacturers have a choice, the use of materials from “non TSE-relevant animal species” or non-animal origin is preferred. The rationale for using materials derived from “TSE-relevant animal species” instead of materials from “non-TSE-relevant species” or of non-animal origin should be given. If materials from “TSE-relevant animal species” have to be used, consideration should be given to all the necessary measures to minimise the risk of transmission of TSE.

Readily applicable diagnostic tests for TSE infectivity *in vivo* are not yet available. Diagnosis is based on post-mortem confirmation of characteristic brain lesions by histopathology and/or detection of PrP^{Sc} by Western blot or immunoassay. The demonstration of infectivity by the inoculation of suspect tissue into target species or laboratory animals is also used for confirmation. However, due to the long incubation periods of all TSEs, results of *in vivo* tests are available only after months or years.

Several *in vitro* diagnostic tests capable of detecting PrP^{Sc} in brain samples from infected animals have been approved for use but in the main they are less sensitive than *in vivo* infectivity assays. Nonetheless, screening of source animals by *in vitro* tests may prevent the use of animals at late stages of incubation of the disease and may provide information about the epidemiological status of a given country or region.

Minimising the risks of transmission of TSE is based upon three complementary parameters:

- the source animals and their geographical origin,
- nature of animal material used in manufacture and any procedures in place to avoid cross-contamination with higher risk materials,
- production process(es) including the quality assurance system in place to ensure product consistency and traceability.

3-2. SOURCE ANIMALS

The source materials used for the production of materials for the manufacture of medicinal products shall be derived from animals fit for human consumption following ante- and post-mortem inspection in accordance with Community or equivalent (third country) conditions, except for materials derived from live animals, which should be found healthy after clinical examination.

3-2-1. Geographical sourcing

3-2-1-1. Bovine materials

There are currently two organisations involved in the assessment of the BSE status of a specified country or zone. Firstly, the Organisation Internationale des Epizooties (OIE)⁽⁶⁾ lays down the criteria for the assessment of the status of countries in the chapter of the International Animal Health Code on bovine spongiform encephalopathy. OIE also provides a list of notified BSE cases worldwide. Secondly, the European Commission Scientific Steering Committee (SSC)⁽⁷⁾ has established a system for classifying the countries according to their geographical BSE risk (GBR).

Regulation (EC) No 999/2001 of the European Parliament and of the Council laying down rules for the prevention, control and eradication of certain transmissible spongiform encephalopathies (TSE Regulation)⁽²⁾ entered into force on 1 July 2001. While medicinal products, medical devices and cosmetics are excluded from the scope of this Regulation, the principles for the determination of BSE status should be taken into account in the categorisation of the BSE status of a given country or region.

For the purposes of this chapter, the SSC GBR classification should be used as the indicator of the status of a given country. However, when countries are categorised according to Regulation (EC) No 999/2001, this categorisation should be used.

European Commission Scientific Steering Committee Classification

The European Scientific Steering Committee classification for geographical BSE risk (GBR) gives an indication of the level of likelihood of the presence of one or more cattle clinically or pre-clinically infected with BSE in a given country or region. A definition of the four categories is provided in the following Table.

GBR level	Presence of one or more cattle clinically or pre-clinically infected with BSE in a geographical region/country
I	Highly unlikely
II	Unlikely but not excluded
III	Likely but not confirmed or confirmed at a lower level
IV	Confirmed at a higher level ⁽¹⁾

(1) ≥ 100 cases/1 Million adult cattle per year

Reports of the GBR assessment of the countries are available on the SSC website⁽⁸⁾. If the BSE status of a country has not been classified by the SSC, a risk assessment shall be submitted taking into account the SSC criteria for the GBR classification.

Where there is a choice, animals should be sourced from countries with the lowest possible GBR level unless the use of material from higher GBR countries is justified. Some of the materials identified in Section 6, “Specific Conditions” can be sourced from GBR category III and, in some cases, category IV countries, provided that the controls and

(6) <http://www.oie.int>

(7) The Scientific Steering Committee established by Commission Decision 97/404/EC shall assist the Commission to obtain the best scientific advice available on matters relating to consumer health. Since May 2003, its tasks have been taken over by the European Food Safety Agency (EFSA): <http://www.efsa.eu.int>

(8) http://europa.eu.int/comm/food/fs/sc/ssc/outcome_en.html

requirements as specified in the relevant sections below are applied. Apart from these exceptions, animals must not be sourced from category IV countries, and justifications for the use of animals from category III countries must always be provided.

3-2-1-2. Sheep and goats (small ruminants)

Naturally occurring clinical scrapie cases have been reported in a number of countries worldwide. As BSE in sheep could possibly be mistaken for scrapie, as a precautionary measure, sourcing of materials derived from small ruminants shall take into account the prevalence of both BSE and scrapie in the country and the tissues from which the materials are derived.

The principles related to “BSE Negligible risk (closed) bovine herds” (see section 3-2-2) could equally be applied in the context of small ruminants in order to develop a framework to define the TSE status of a flock of small ruminants. For sheep, because of the concern over the possibility of BSE in sheep, the use of (a) genotype(s) shown to be resistant to BSE/scrapie infection shall be considered in establishing TSE free flocks. However, goats have not been studied sufficiently with regard to a genotype specific sensitivity.

Material of small ruminant origin should preferably be sourced from countries with a long history of absence of scrapie, such as New Zealand or Australia or from proven TSE-free flocks. Justification shall be required if the material is sourced from some other origin.

3-2-2. BSE negligible risk (closed) bovine herds. The safest sourcing is from countries where the presence of BSE is highly unlikely i.e. GBR I. Other countries may have or have had cases of BSE at some point in time and the practical concept of “Negligible risk (closed) bovine herds” has been developed by the SSC and endorsed by the CPMP and CVMP. Criteria for establishing and maintaining a “BSE negligible risk (closed) bovine herd” can be found in the SSC opinion of 22-23 July 1999⁽⁹⁾.

For the time being it is not possible to quantify the reduction of the geographical BSE risk for cattle from BSE negligible risk (closed) bovine herds. However, it is expected that this risk reduction is substantial. Therefore, sourcing from such closed bovine herds shall be considered in the risk assessment in conjunction with the GBR classification of the country.

3-3. ANIMAL PARTS, BODY FLUIDS AND SECRETIONS AS STARTING MATERIALS

In a TSE infected animal, different organs and secretions have different levels of infectivity⁽¹⁰⁾. The tables in the Annex of this chapter⁽¹¹⁾ summarise current data about the distribution of infectivity and PrP^{Sc} in cattle with BSE, and in sheep and goats with scrapie.

The information in the tables is based exclusively upon observations of naturally occurring disease or primary experimental infection by the oral route (in cattle) but does not include data on models using strains of TSE that have been adapted to experimental animals, because passaged strain phenotypes can differ significantly and unpredictably from those of naturally occurring disease. Because immunohistochemical and/or Western blot detection of misfolded host protein (PrP^{Sc}) have proven to be a surrogate marker of infectivity, PrP^{Sc} testing results have

been presented in parallel with bioassay data. Tissues are grouped into three major infectivity categories, irrespective of the stage of disease:

Category A	High-infectivity tissues central nervous system (CNS) tissues that attain a high titre of infectivity in the later stages of all TSEs, and certain tissues that are anatomically associated with the CNS
Category B	Lower-infectivity tissues peripheral tissues that have tested positive for infectivity and/or PrP ^{Sc} in at least one form of TSE
Category C	Tissues with no detectable infectivity tissues that have been examined for infectivity, without any infectivity detected, and/or PrP ^{Sc} , with negative results

Category A tissues and substances derived from them shall not be used in the manufacture of medicinal products, unless justified (see Section 5).

Although the category of lower risk tissues (category B tissues) almost certainly includes some (e.g. blood) with a lower risk than others (e.g. lymphoreticular tissues), the data about infectivity levels in these tissues are too limited to subdivide the category into different levels of risk. It is also evident that the placement of a given tissue in one or another category can be disease and species specific, and subject to revision as new data emerges.

For the risk assessment (see section 4), manufacturers and/or marketing authorisation holders/applicants shall take into account the tissue classification tables in the Annex to this chapter⁽¹²⁾.

The categories in the tables are only indicative and it is important to note the following points.

- In certain situations there could be cross-contamination of tissues of different categories of infectivity. The potential risk will be influenced by the circumstances in which tissues were removed, especially by contact of tissues with lower-infectivity tissues or no detectable infectivity (categories B and C tissues) with high-infectivity tissues (category A tissues). Thus, cross-contamination of some tissues may be increased if infected animals are slaughtered by penetrative brain stunning or if the brain and/or spinal cord is sawed. The risk of cross-contamination will be decreased if body fluids are collected with minimal damage to tissue and cellular components are removed, and if foetal blood is collected without contamination from other maternal or foetal tissues including placenta, amniotic and allantoic fluids. For certain tissues, it is very difficult or impossible to prevent cross-contamination with category A tissues (e.g. skull). This has to be considered in the risk assessment.
- For certain classes of substances the stunning/slaughtering techniques used may be important in minimising the potential risk⁽¹³⁾ because of the likelihood of disseminating the brain particles into the peripheral organs, particularly to the lungs. Stunning/slaughtering techniques should be described as well as the procedures to remove high infectivity tissues. The procedures to collect the animal tissues/organs to be used and the measures in place to avoid cross-contamination with a higher risk material must also be described in detail.

(9) SSC Scientific Opinion on the conditions related to “BSE Negligible Risk (Closed) Bovine Herds” adopted at the meeting of 22-23 July 1999. http://europa.eu.int/comm/food/fs/sc/ssc/out56_en.html

(10) If materials from “TSE-relevant animal species” have to be used, consideration should be given to use of materials of the lowest category of risk.

(11) The tissue classification tables are based upon the most recent WHO guidelines on transmissible spongiform encephalopathies in relation to biological and pharmaceutical products (February 2003) WHO/BCT/QSD/03.01.

(12) The introduction of the 3-category tissue classification system does not invalidate the risk-assessments based on the previously used 4-category tissue classification, performed for authorised medicinal products.

(13) SSC opinion on stunning methods and BSE risk (the risk of dissemination of brain particles into the blood and carcass when applying certain stunning methods), adopted at the meeting of 10-11 January 2002. http://europa.eu.int/comm/food/fs/sc/ssc/out245_en.pdf

- The risk of contamination of tissues and organs with BSE-infectivity potentially harboured in central nervous material as a consequence of the stunning method used for cattle slaughtering depends on the following factors:
 - the amount of BSE-infectivity in the brain of the slaughtered animal,
 - the extent of brain damage,
 - the dissemination of brain particles in the animal body.

These factors must be considered in conjunction with the GBR classification of the source animals, the age of the animals in the case of cattle and the post-mortem testing of the cattle using a validated method.

The underlying principles indicated above would be equally applicable to sheep and goats.

The risk posed by cross-contamination will be dependent on several complementary factors including:

- measures adopted to avoid contamination during collection of tissues (see above),
- level of contamination (amount of the contaminating tissue),
- amount and type of materials collected at the same time.

Manufacturers or the marketing authorisation holders/applicants should take into account the risk with respect to cross-contamination.

3.4. AGE OF ANIMALS

As the TSE infectivity accumulates in bovine animals over an incubation period of several years, it is prudent to source from young animals.

3.5. MANUFACTURING PROCESS

The assessment of the overall TSE risk reduction of a medicinal product shall take into account the control measures instituted with respect to:

- sourcing of the raw/starting materials, and
- the manufacturing process.

Controlled sourcing is a very important criterion in achieving acceptable safety of the product, due to the documented resistance of TSE agents to most inactivation procedures.

A quality assurance system, such as ISO 9000 certification, HACCP⁽¹⁴⁾ or GMP, must be put in place for monitoring the production process and for batch delineation (i.e. definition of batch, separation of batches, cleaning between batches). Procedures shall be put in place to ensure traceability as well as self-auditing and to auditing suppliers of raw/starting materials.

Certain production procedures may contribute considerably to the reduction of the risk of TSE contamination, e.g. procedures used in the manufacture of tallow derivatives (see section 6). As such rigorous processing cannot be applied to many products, processes involving physical removal, such as precipitation and filtration to remove prion-rich material, are likely to be more appropriate than chemical treatments. A description of the manufacturing process, including in-process controls applied, shall be presented and the steps that might contribute to reduction or elimination of TSE contamination should be discussed. Whenever different manufacturing sites are involved, the steps performed at each site shall be clearly identified. The measures in place in order to ensure traceability of every production batch to the source material should be described.

Cleaning process. Cleaning of process equipment may be difficult to validate for the elimination of TSE agents. It is reported that after exposure to high titre preparations of TSE

agent, detectable infectivity can remain bound to the surface of stainless steel. The removal of all adsorbed protein by the use of sodium hydroxide or chlorine releasing disinfectants (e.g. 20 000 ppm chlorine for 1 h) have been considered acceptable approaches where equipment that cannot be replaced has been exposed to potentially contaminated material. In the case of using category A materials in the manufacture of a product, dedicated equipment shall be used, unless otherwise justified.

If risk materials are used in the manufacture of a product, cleaning procedures, including control measures, shall be put in place in order to minimise the risk of cross-contamination between production batches. This is especially important if materials from different risk categories are handled in the same plant with the same equipment.

Removal/Inactivation validation. Validation studies of removal/inactivation procedures for TSEs are difficult to interpret. It is necessary to take into consideration the nature of the spiked material and its relevance to the natural situation, the design of the study (including scaling-down of processes) and the method of detection of the agent (*in vitro* or *in vivo* assay). Further research is needed to develop an understanding of the most appropriate “spike preparation” for validation studies. Therefore, validation studies are currently not generally required. However, if claims are made for the safety of the product with respect to TSEs based on the ability of manufacturing processes to remove or inactivate TSE agents, they must be substantiated by appropriate validation studies.

In addition to appropriate sourcing, manufacturers are encouraged to continue their investigations into removal and inactivation methods to identify steps/processes that would have benefit in assuring the removal or inactivation of TSE agents. In any event, a production process wherever possible shall be designed taking account of available information on methods which are thought to inactivate or remove TSE agents.

4. RISK ASSESSMENT OF MATERIALS OR SUBSTANCES USED IN THE MANUFACTURE AND PREPARATION OF A MEDICINAL PRODUCT IN THE CONTEXT OF REGULATORY COMPLIANCE

The assessment of the risk associated with TSE needs careful consideration of all of the parameters as outlined in section 3-1 (Scientific Principles for Minimising Risk).

As indicated in the introduction to this chapter, regulatory compliance is based on a favourable outcome from a risk assessment. The risk assessments, conducted by the manufacturers and/or the marketing authorisation holders or applicants for the different materials or substances from “TSE-relevant animal species” used in the manufacture of a medicinal product shall show that all TSE risk factors have been taken into account and, where possible, risk has been minimised by application of the principles described in this chapter. TSE Certificates of suitability issued by the EDQM may be used by the marketing authorisation holders or applicants as the basis of the risk assessments.

An overall risk assessment for the medicinal product, conducted by the marketing authorisation holders or applicants, shall take into account the risk assessments for all the different materials from “TSE-relevant animal species” and, where appropriate, TSE reduction or inactivation by the manufacturing steps of the active substance and/or finished product.

The final determination of regulatory compliance rests with the competent authority.

(14) Hazard Analysis Critical Control Point.

It is incumbent upon the manufacturers and/or the marketing authorisation holders or applicants for both human and veterinary medicinal products to select and justify the control measures for a given “TSE-relevant animal species” derivative, taking into account the state of the art of science and technology.

5. BENEFIT/RISK EVALUATION

In addition to the parameters as mentioned in sections 3 and 4, the acceptability of a particular medicinal product containing materials derived from a “TSE-relevant animal species”, or which as a result of manufacture could contain these materials, shall take into account the following factors:

- route of administration of the medicinal product,
- quantity of animal material used in the medicinal product,
- maximum therapeutic dosage (daily dose and duration of treatment),
- intended use of the medicinal product and its clinical benefit.

High-infectivity tissues (category A tissues) and substances derived thereof shall not be used in manufacture of medicinal products, their starting materials and intermediate products (including active substances, excipients and reagents), unless justified. A justification why no other materials can be used shall be provided. In these exceptional and justified circumstances, the use of high-infectivity tissues could be envisaged for the manufacture of active substances, when, after performing the risk assessment as described in Section 4 of this chapter, and taking into account the intended clinical use, a positive benefit/risk assessment can be presented by the marketing authorisation applicant. Substances from category A materials, if their use is justified, must be produced from animals of GBR I countries.

6. SPECIFIC CONSIDERATIONS

The following materials prepared from “TSE-relevant animal species” are considered in compliance with this chapter provided that they meet at least the conditions specified below. The relevant information or a certificate of suitability granted by the EDQM shall be provided by the marketing authorisation applicant/holder.

6-1. COLLAGEN

Collagen is a fibrous protein component of mammalian connective tissue.

For collagen, documentation to demonstrate compliance with this chapter needs to be provided taking into account the provisions listed in sections 3 to 5. In addition, consideration should be given to the following.

- For collagen produced from bones, the conditions specified for gelatin are applicable (see below).
- Collagen produced from tissues such as hides and skins do not usually present a measurable TSE risk provided that contamination with potentially infected materials, for example spillage of blood and/or central nervous tissues, is avoided during their procurement.

6-2. GELATIN

Gelatin is a natural, soluble protein, gelling or non-gelling, obtained by the partial hydrolysis of collagen produced from bones, hides and skins, tendons and sinews of animals.

For gelatin, documentation to demonstrate compliance with this chapter needs to be provided taking into account the provisions listed in sections 3 to 5. In addition, consideration should be given to the following.

The source material used

Gelatin used in medicinal products can be manufactured from bones or hides.

Hides as the starting material. On the basis of current knowledge, hides used for gelatin production represent a much safer source material as compared to bones. However, it is highly recommended that measures should be put in place to avoid cross-contamination with potentially infected materials during procurement.

Bones as the starting material. Where bones are used to manufacture gelatin, more stringent production conditions shall be applied (see below). In any case, the removal of skulls and spinal cords from the starting material is considered as a first precautionary measure which largely affects the safety of the product. As far as practicable, bones should be sourced from countries classified as GBR I and II. Bones from category GBR III countries can be used if the gelatin is manufactured under defined conditions as indicated below and if vertebrae from cattle over 12 months of age are removed from the raw/starting materials⁽¹⁵⁾.

Manufacturing methods

No specific measures with regard to the processing conditions are required for gelatin produced from hides provided that control measures are put in place to avoid cross-contamination both during the procurement of the hides and during the manufacturing process.

However, the mode of manufacture must be taken into account where bones are used as the starting material.

- Bones (including vertebrae) for the production of gelatin using acid treatment shall be sourced only from GBR category I or II countries. An additional alkaline treatment (pH 13, 1 h) of the bones/ossein may further increase the TSE safety of acid-derived bone gelatin.

For bones sourced from a GBR category III country, the alkaline process shall be applied. However, this manufacturing method is optional for bones coming from GBR category I and II countries.

- For a typical alkaline manufacturing process, bones are finely crushed, degreased with hot water and demineralised with dilute hydrochloric acid (at a minimum of 4 per cent and pH < 1.5) over a period of at least 2 days to produce the ossein. This is followed by an alkaline treatment with saturated lime solution (pH at least 12.5) for a period of at least 20 days. The gelatin is extracted, washed, filtered and concentrated. A “flash” heat treatment (sterilisation) step using 138-140 °C for 4 s is applied. Bovine hide gelatin can also be produced by the alkaline process. Bovine bones may also be treated by an acid process. The liming step is then replaced by an acid pre-treatment where the ossein is soaked overnight at pH < 4.

6-3. BOVINE BLOOD DERIVATIVES

Foetal bovine serum is commonly used in cell cultures. Foetal bovine serum should be obtained from fetuses harvested in abattoirs from healthy dams fit for human consumption and the womb should be completely removed and the foetal blood harvested in dedicated space or area by cardiac puncture into a closed collection system using aseptic technique.

New born calf serum is obtained from calves under 20 days old and calf serum from animals under the age of 12 months. In the case of donor bovine serum, given that it may be derived from animals less than 36 months old, the TSE status

(15) Regulation (EC) No 1774/2002 of the European Parliament and of the Council laying down health rules concerning animal by-products not intended for human consumption shall apply unless justified. Regarding the manufacturing of gelatin and collagen or import of raw material for such manufacturing for use in pharmaceutical products, only material from animals fit for human consumption shall be used. The use of vertebrae from such animals from category II countries, which according to the risk assessment is safe, shall continue to be allowed.

of the donor herd shall be well defined and documented. In all cases, serum shall be collected according to specified protocols by personnel trained in these procedures to avoid cross-contamination with higher risk tissues.

For bovine blood derivatives, documentation to demonstrate compliance with this chapter needs to be provided taking into account the provisions listed in sections 3 to 5. In addition, consideration should be given to the following.

Traceability

Traceability to the slaughterhouse must be assured for each batch of serum or plasma. Slaughterhouses must have available lists of farms from which the animals are originated. If serum is produced from living animals, records must be available for each serum batch which assures the traceability to the farms.

Geographical origin

Whilst tissue infectivity of BSE in cattle is more restricted than scrapie, as a precautionary measure bovine blood must be sourced from countries classified GBR I and II, unless otherwise justified.

Stunning methods

If it is sampled from slaughtered animals, the method of slaughter is of importance to assure the safety of the material. It has been demonstrated that stunning by captive bolt stunner with or without pithing as well as by pneumatic stunner, especially if it injects air, can destroy the brain and disseminate brain material into the blood stream. Negligible risk can be expected from a non-penetrative stunner and from electro-narcosis⁽¹⁶⁾. The stunning methods must therefore be described for the bovine blood collection process.

If sourcing is allowed from countries where cases of BSE have been detected (GBR III) a non-penetrative stunner shall be used for slaughter.

6-4. TALLOW DERIVATIVES

Tallow is fat obtained from tissues including subcutaneous, abdominal and inter-muscular areas and bones. Tallow used as the starting material for the manufacture of tallow derivatives shall be category 3 material or equivalent, as defined in Regulation (EC) No 1774/2002⁽¹⁷⁾ of the European Parliament and of the Council of 3 October 2002 laying down health rules concerning animal by-products not intended for human consumption.

Tallow derivatives, such as glycerol and fatty acids, manufactured from tallow by rigorous processes are thought unlikely to be infectious and they have been the subject of specific consideration by CPMP and CVMP. For this reason, such materials manufactured under the conditions at least as rigorous as those given below shall be considered in compliance for this chapter, irrespective of the geographical origin and the nature of the tissues from which tallow derivatives are derived. Examples of rigorous processes are:

- trans-esterification or hydrolysis at not less than 200 °C for not less than 20 min under pressure (glycerol, fatty acids and fatty acid esters production),
- saponification with 12 M NaOH (glycerol and soap production):
 - batch process: at not less than 95 °C for not less than 3 h,

- continuous process: at not less than 140 °C, under pressure for not less than 8 min, or equivalent,
- distillation at 200 °C.

Tallow derivatives manufactured according to these conditions are unlikely to present any TSE risk and shall therefore be considered compliant with this chapter.

Tallow derivatives produced using other conditions must demonstrate compliance with this chapter.

6-5. ANIMAL CHARCOAL

Animal charcoal is prepared by carbonisation of animal tissues, such as bones, using high temperature at > 800 °C. Unless otherwise justified, the starting material for the manufacture of animal charcoal shall be category 3 material or equivalent, as defined in Regulation (EC) No 1774/2002 of the European Parliament and of the Council of 3 October 2002 laying down health rules concerning animal by-products not intended for human consumption. Irrespective of the geographical origin and the nature of the tissue, for the purpose of regulatory compliance, animal charcoal shall be considered in compliance with this chapter.

Charcoal manufactured according to these conditions is unlikely to present any TSE risk and shall therefore be considered compliant with this chapter. Charcoal produced using other conditions must demonstrate compliance with this chapter.

6-6. MILK AND MILK DERIVATIVES

In the light of the current scientific knowledge and irrespective of the geographical origin, milk is unlikely to present any risk of TSE contamination.

Certain materials, including lactose, are extracted from whey, the spent liquid from cheese production following coagulation. Coagulation can involve the use of calf rennet, an extract from abomasum, or rennet derived from other ruminants. The CPMP/CVMP have performed a risk assessment for lactose and other whey derivatives produced using calf rennet and concluded that the TSE risk is negligible if the calf rennet is produced in accordance with the process described in the risk assessment report⁽¹⁸⁾. The conclusion was endorsed by the SSC⁽¹⁹⁾ which has also performed an assessment of the TSE risk of rennet in general⁽²⁰⁾.

Milk derivatives manufactured according to the conditions below are unlikely to present any TSE risk and shall therefore be considered compliant with this chapter.

- The milk is sourced from healthy animals in the same conditions as milk collected for human consumption, and
- no other ruminant materials, with the exception of calf rennet, are used in the preparation of such derivatives (e.g. pancreatic enzyme digests of casein).

Milk derivatives produced using other processes or rennet derived from other ruminant species must demonstrate compliance with this chapter.

6-7. WOOL DERIVATIVES

Derivatives of wool and hair of ruminants, such as lanolin and wool alcohols derived from hair shall be considered in compliance with this chapter, provided the wool and hair are sourced from live animals.

(16) SSC Opinion on stunning methods and BSE risk (The risk of dissemination of brain particles into the blood and carcass when applying certain stunning methods) adopted at the meeting on 10-11 January 2002. http://europa.eu.int/comm/food/fs/sc/ssc/out245_en.pdf

(17) OJ L 273, 10.10.2002, p. 1

(18) Committee for Proprietary Medicinal Products and its Biotechnology Working Party conducted a risk and regulatory assessment of lactose prepared using calf rennet. The risk assessment included the source of the animals, the excision of the abomasums and the availability of well-defined quality assurance procedures. The quality of any milk replacers used as feed for the animals from which abomasums are obtained is particularly important. The report can be found on <http://www.emea.eu.int>

(19) Provisional statement on the safety of calf-derived rennet for the manufacture of lactose. Adopted by the SSC at its meeting of 4-5 April 2002. (http://europa.eu.int/comm/food/fs/sc/ssc/out255_en.pdf)

(20) The SSC issued an opinion on the safety of animal rennet in regard to risks from animal TSE and BSE in particular, adopted at its meeting of 16 May 2002. (http://europa.eu.int/comm/food/fs/sc/ssc/out265_en.pdf)

Wool derivatives produced from wool which is sourced from slaughtered animals declared “fit for human consumption” and the manufacturing process in relation to pH, temperature and duration of treatment meets at least one of the stipulated processing conditions listed below are unlikely to present any TSE risk and shall therefore be considered compliant with this chapter.

- Treatment at pH ≥ 13 (initial; corresponding to a NaOH concentration of at least 0.1 M NaOH) at ≥ 60 °C for at least 1 h. This occurs normally during the reflux stage of the organic-alkaline treatment.
- Molecular distillation at ≥ 220 °C under reduced pressure.

Wool derivatives produced using other conditions must demonstrate compliance with this chapter.

6-8. AMINO ACIDS

Amino acids can be obtained by hydrolysis of materials from various sources.

Unless otherwise justified, the starting material for the manufacture of amino acids shall be category 3 material or equivalent, as defined in Regulation (EC) No 1774/2002 of the European Parliament and of the Council of 3 October 2002 laying down health rules concerning animal by-products not intended for human consumption.

Amino acids prepared using the following processing conditions, in accordance with Commission Decision 98/256/EC⁽²¹⁾ and Commission Decision 2001/376/EC⁽²²⁾, are unlikely to present any TSE risk and shall be considered compliant with this chapter:

- amino acids produced from hides and skins by a process which involves exposure of the material to a pH of 1 to 2, followed by a pH of > 11 , followed by heat treatment at 140 °C for 30 min at 3 bar,
- the resulting amino acids or peptides must be filtered after production, and
- analysis is performed using a validated and sensitive method to control any residual intact macromolecules, with an appropriate limit set.

Amino acids prepared using other conditions must demonstrate compliance with this chapter.

Annex: major categories of infectivity

The tables below are adapted from the *WHO Guideline on Transmissible Spongiform Encephalopathies in Relation to Biological and Pharmaceutical Products* (February 2003).

Data entries are shown as follows:

- + = presence of infectivity or PrP^{TSE}⁽²³⁾,
- = absence of detectable infectivity or PrP^{TSE},
- NT = not tested,
- ? = controversial or uncertain results.

(21) OJ L 113, 15.4.1998, p. 32

(22) OJ L 132, 15.5.2001, p. 17

(23) In the main body of this chapter the abnormal isoform of the prion protein is referred to as PrP^{Sc}. However, as these tables are transcribed directly from the WHO guideline mentioned above, the WHO nomenclature for the abnormal prion protein (PrP^{TSE}) has been maintained.

Category A: High-infectivity tissues

Tissues	Cattle BSE		Sheep and goats Scrapie	
	Infectivity ¹	PrP ^{TSE}	Infectivity ¹	PrP ^{TSE}
Brain	+	+	+	+
Spinal cord	+	+	+	+
Retina, Optic nerve	+	NT	NT	+
Spinal ganglia	+	NT	NT	+
Trigeminal ganglia	+	NT	NT	+
Pituitary gland ²	–	NT	+	NT
Dura mater ²	NT	NT	NT	NT

1. Infectivity bioassays of cattle tissues have been conducted in either cattle or mice (or both); and most bioassays of sheep and/or goat tissues have been conducted only in mice. In regard to sheep and goats not all results are consistent for both species.

2. No experimental data about infectivity in human pituitary gland or dura mater have been reported, but cadaveric dura mater patches, and growth hormone derived from cadaveric pituitaries have transmitted disease to scores of people and therefore must be included in the category of high-risk tissues.

Category B: Lower-infectivity tissues

Tissues	Cattle BSE		Sheep and goats Scrapie	
	Infectivity	PrP ^{TSE}	Infectivity	PrP ^{TSE}
Peripheral Nervous system				
Peripheral nerves	–	NT	+	NT
Enteric plexuses ¹	NT	+	NT	+
Lymphoreticular tissues				
Spleen	–	–	+	+
Lymph nodes	–	–	+	+
Tonsil	+	NT	+	+
Nictitating membrane	NT	–	NT	+
Thymus	–	NT	+	NT
Alimentary tract				
Esophagus	–	NT	NT	+
Fore-stomach ² (ruminants only)	–	NT	NT	+
Stomach/ abomasum ²	–	NT	NT	+
Duodenum	–	NT	NT	+
Jejunum	–	NT	NT	+
Ileum ³	+	+	+	+
Large intestine	–	NT	+	+
Reproductive tissues				
Placenta	–	NT	+	+
Other tissues				
Lung*	–	NT	–	NT
Liver	–	NT	+	NT

Tissues	Cattle BSE		Sheep and goats Scrapie	
	Infectivity	PrP ^{TSE}	Infectivity	PrP ^{TSE}
Kidney*	–	–	–	–
Adrenal	NT	NT	+	NT
Pancreas	–	NT	+	NT
Bone marrow	+	NT	+	NT
Blood vessels	–	NT	NT	+
Olfactory mucosa	–	NT	+	NT
Gingival tissue*	NT	NT	NT	NT
Salivary gland	–	NT	+	NT
Cornea ^{4*}	NT	NT	NT	NT
Body fluids				
CSF	–	NT	+	NT
Blood ⁵	–	NT	+	–

1. In cattle, limited to the distal ileum.

2. Ruminant forestomachs (reticulum, rumen, and omasum) are widely consumed, as is the true stomach (abomasum). The abomasum of cattle (and sometimes sheep) is also a source of rennet.

3. In cattle and sheep, only the distal ileum has been bioassayed for infectivity.

4. Because only one or two cases of CJD have been plausibly attributed to corneal transplants among hundreds of thousands of recipients, cornea is categorised as a lower-risk tissue; other anterior chamber tissues (lens, aqueous humor, iris, conjunctiva) have been tested with a negative result both in vCJD and other human TSEs, and there is no epidemiological evidence that they have been associated with iatrogenic disease transmission.

5. Early reports on the transmission of disease to rodents from the blood of patients with sCJD have not been confirmed, and evaluation of the ensemble of experimental and epidemiological data relevant to TSE transmission through blood, blood components, and therapeutic plasma products fails to suggest transmission from blood of patients with any form of “classical” TSE. Not enough data has accumulated to be able to make the same statement about blood from patients with vCJD. Foetal calf blood contains no detectable infectivity, but in genotypically susceptible sheep with natural scrapie or experimentally induced BSE, transfusion of large blood volumes has transmitted disease to healthy sheep. Infectivity has also been demonstrated in studies of rodent-adapted strains of TSE.

* These tissues have been classified under category B: Lower-infectivity tissues, because infectivity and/or PrP^{TSE} have been found in human CJD (vCJD or other).

Category C: Tissues with no detected infectivity

Tissues	Cattle BSE		Sheep and goats Scrapie	
	Infectivity	PrP ^{TSE}	Infectivity	PrP ^{TSE}
Reproductive tissues				
Testis	–	NT	–	NT
Prostate/Epididymis/Seminal vesicle	–	NT	–	NT
Semen	–	NT	NT	NT
Ovary	–	NT	–	NT
Uterus (Non-gravid)	–	NT	–	NT
Placenta fluids	–	NT	NT	NT
Foetus ¹	–	NT	–	NT

Tissues	Cattle BSE		Sheep and goats Scrapie	
	Infectivity	PrP ^{TSE}	Infectivity	PrP ^{TSE}
Embryos ¹	–	NT	?	NT
Musculo-skeletal tissues				
Bone	–	NT	NT	NT
Skeletal muscle ²	–	NT	–	NT
Tongue	–	NT	NT	NT
Heart/pericardium	–	NT	–	NT
Tendon	–	NT	NT	NT
Other tissues				
Trachea	–	NT	NT	NT
Skin	–	NT	–	NT
Adipose tissue	–	NT	NT	NT
Thyroid gland	NT	NT	–	NT
Mammary gland/udder	–	NT	–	NT
Body fluids, secretions and excretions				
Milk ³	–	NT	–	NT
Colostrum ⁴	NT	NT	–	NT
Cord blood ⁴	–	NT	NT	NT
Saliva	NT	NT	–	NT
Sweat	NT	NT	NT	NT
Tears	NT	NT	NT	NT
Nasal mucus	NT	NT	NT	NT
Urine ^{4,5}	–	NT	NT	NT
Faeces	–	NT	–	NT

1. Embryos from BSE-affected cattle have not transmitted disease to mice, but no infectivity measurements have been made on foetal calf tissues other than blood (negative mouse bioassay). Calves born of dams that received embryos from BSE-affected cattle have survived for observations periods of up to seven years, and examination of the brains of both the unaffected dams and their calves revealed no spongiform encephalopathy or PrP^{TSE}.

2. Intracerebral inoculation of muscle homogenates has not transmitted disease to 1) primates from humans with sCJD; 2) mice or cattle from cattle with BSE; and 3) mice from sheep and goats with natural or experimentally-induced scrapie. However, older reports described single instances of transmission from goat and hamster muscle, and a more recent report described transmission from the muscle of wild type and transgenic mice, but as each of these studies were conducted with passaged strains of TSE, their relevance to natural disease remains undetermined. A recent human case report described a patient with CJD and inclusion body myositis with abundant PrP^{TSE} in diseased muscle. After much deliberation, the committee nevertheless elected to retain muscle in the ‘no detected infectivity’ tissue category until more information about uncomplicated natural infections becomes available.

3. Evidence that infectivity is not present in milk includes temporo-spatial epidemiologic observations failing to detect maternal transmission; clinical observations of over a hundred calves nursed by infected cows that have not developed BSE; and experimental observations that milk from infected cows has not transmitted disease when administered intracerebrally or orally to mice. Experiments are in progress in which large volumes of milk from experimentally infected cows are concentrated and tested for the presence of PrP^{TSE}.

4. Single reports of transmission of CJD infectivity from human cord blood, colostrum, and urine have never been confirmed and are considered improbable.

5. A previously unreported PrP type, termed PrPⁿ, has been identified in the urine of sporadic and familial CJD patients, but its significance for transmission risk remains to be determined.

01/2005:50300

1. INTRODUCTION

This chapter provides guidance for the design of bioassays prescribed in the European Pharmacopoeia (Ph. Eur.) and for analysis of their results. It is intended for use by those whose primary training and responsibilities are not in statistics, but who have responsibility for analysis or interpretation of the results of these assays, often without the help and advice of a statistician. The methods of calculation described in this annex are not mandatory for the bioassays which themselves constitute a mandatory part of the Ph. Eur. Alternative methods may be used, provided that they are not less reliable than those described here. A wide range of computer software is available and may be useful depending on the facilities available to, and the expertise of, the analyst.

Professional advice should be obtained in situations where: a comprehensive treatment of design and analysis suitable for research or development of new products is required; the restrictions imposed on the assay design by this chapter are not satisfied, for example particular laboratory constraints may require customized assay designs, or equal numbers of equally spaced doses may not be suitable; analysis is required for extended non-linear dose-response curves, for example as may be encountered in immunoassays. An outline of extended dose-response curve analysis for one widely used model is nevertheless included in Section 3.4 and a simple example is given in Section 5.4.

1.1. GENERAL DESIGN AND PRECISION

Biological methods are described for the assay of certain substances and preparations whose potency cannot be adequately assured by chemical or physical analysis. The principle applied wherever possible throughout these assays is that of comparison with a standard preparation so as to determine how much of the substance to be examined produces the same biological effect as a given quantity, the *Unit*, of the standard preparation. It is an essential condition of such methods of biological assay that the tests on the standard preparation and on the substance to be examined be carried out at the same time and under identical conditions.

For certain assays (determination of virus titre for example) the potency of the test sample is not expressed relative to a standard. This type of assay is dealt with in Section 4.5.

Any estimate of potency derived from a biological assay is subject to random error due to the inherent variability of biological responses and calculations of error should be made, if possible, from the results of each assay, even when the official method of assay is used. Methods for the design of assays and the calculation of their errors are, therefore, described below. In every case, before a statistical method is adopted, a preliminary test is to be carried out with an appropriate number of assays, in order to ascertain the applicability of this method.

The confidence interval for the potency gives an indication of the precision with which the potency has been estimated in the assay. It is calculated with due regard to the experimental design and the sample size. The 95 per cent confidence interval is usually chosen in biological assays. Mathematical statistical methods are used to calculate these limits so as to warrant the statement that there is a 95 per cent probability that these limits include the true potency. Whether this

precision is acceptable to the European Pharmacopoeia depends on the requirements set in the monograph for the preparation concerned.

The terms “mean” and “standard deviation” are used here as defined in most current textbooks of biometry.

The terms “stated potency” or “labelled potency”, “assigned potency”, “assumed potency”, “potency ratio” and “estimated potency” are used in this section to indicate the following concepts:

- “stated potency” or “labelled potency”: in the case of a formulated product a nominal value assigned from knowledge of the potency of the bulk material; in the case of bulk material the potency estimated by the manufacturer;
- “assigned potency”: the potency of the standard preparation;
- “assumed potency”: the provisionally assigned potency of a preparation to be examined which forms the basis of calculating the doses that would be equipotent with the doses to be used of the standard preparation;
- “potency ratio” of an unknown preparation; the ratio of equipotent doses of the standard preparation and the unknown preparation under the conditions of the assay;
- “estimated potency”: the potency calculated from assay data.

Section 9 (Glossary of symbols) is a tabulation of the more important uses of symbols throughout this annex. Where the text refers to a symbol not shown in this section or uses a symbol to denote a different concept, this is defined in that part of the text.

2. RANDOMISATION AND INDEPENDENCE OF INDIVIDUAL TREATMENTS

The allocation of the different treatments to different experimental units (animals, tubes, etc.) should be made by some strictly random process. Any other choice of experimental conditions that is not deliberately allowed for in the experimental design should also be made randomly. Examples are the choice of positions for cages in a laboratory and the order in which treatments are administered. In particular, a group of animals receiving the same dose of any preparation should not be treated together (at the same time or in the same position) unless there is strong evidence that the relevant source of variation (for example, between times, or between positions) is negligible. Random allocations may be obtained from computers by using the built-in randomisation function. The analyst must check whether a different series of numbers is produced every time the function is started.

The preparations allocated to each experimental unit should be as independent as possible. Within each experimental group, the dilutions allocated to each treatment are not normally divisions of the same dose, but should be prepared individually. Without this precaution, the variability inherent in the preparation will not be fully represented in the experimental error variance. The result will be an under-estimation of the residual error leading to:

- 1) an unjustified increase in the stringency of the test for the analysis of variance (see Sections 3.2.3 and 3.2.4),

2) an under-estimation of the true confidence limits for the test which, as shown in Section 3.2.5, are calculated from the estimate of s^2 , the residual error mean square.

3. ASSAYS DEPENDING UPON QUANTITATIVE RESPONSES

3.1. STATISTICAL MODELS

3.1.1. GENERAL PRINCIPLES

The bioassays included in the Ph. Eur. have been conceived as “dilution assays”, which means that the unknown preparation to be assayed is supposed to contain the same active principle as the standard preparation, but in a different ratio of active and inert components. In such a case the unknown preparation may in theory be derived from the standard preparation by dilution with inert components. To check whether any particular assay may be regarded as a dilution assay, it is necessary to compare the dose-response relationships of the standard and unknown preparations. If these dose-response relationships differ significantly, then the theoretical dilution assay model is not valid. Significant differences in the dose-response relationships for the standard and unknown preparations may suggest that one of the preparations contains, in addition to the active principle, other components which are not inert but which influence the measured responses.

To make the effect of dilution in the theoretical model apparent, it is useful to transform the dose-response relationship to a linear function on the widest possible range of doses. 2 statistical models are of interest as models for the bioassays prescribed: the parallel-line model and the slope-ratio model.

The application of either is dependent on the fulfilment of the following conditions:

- 1) the different treatments have been randomly assigned to the experimental units,
- 2) the responses to each treatment are normally distributed,
- 3) the standard deviations of the responses within each treatment group of both standard and unknown preparations do not differ significantly from one another.

When an assay is being developed for use, the analyst has to determine that the data collected from many assays meet these theoretical conditions.

- Condition 1 can be fulfilled by an efficient use of Section 2.
- Condition 2 is an assumption which in practice is almost always fulfilled. Minor deviations from this assumption will in general not introduce serious flaws in the analysis as long as several replicates per treatment are included. In case of doubt, a test for deviations from normality (e.g. the Shapiro-Wilk⁽¹⁾ test) may be performed.
- Condition 3 can be checked with a test for homogeneity of variances (e.g. Bartlett's⁽²⁾ test, Cochran's⁽³⁾ test). Inspection of graphical representations of the data can also be very instructive for this purpose (see examples in Section 5).

When conditions 2 and/or 3 are not met, a transformation of the responses may bring a better fulfilment of these conditions. Examples are $\ln y$, $\sqrt{y}$, y^2 .

- Logarithmic transformation of the responses y to $\ln y$ can be useful when the homogeneity of variances is not satisfactory. It can also improve the normality if the distribution is skewed to the right.

- The transformation of y to $\sqrt{y}$ is useful when the observations follow a Poisson distribution i.e. when they are obtained by counting.
- The square transformation of y to y^2 can be useful if, for example, the dose is more likely to be proportional to the area of an inhibition zone rather than the measured diameter of that zone.

For some assays depending on quantitative responses, such as immunoassays or cell-based *in vitro* assays, a large number of doses is used. These doses give responses that completely span the possible response range and produce an extended non-linear dose-response curve. Such curves are typical for all bioassays, but for many assays the use of a large number of doses is not ethical (for example, *in vivo* assays) or practical, and the aims of the assay may be achieved with a limited number of doses. It is therefore customary to restrict doses to that part of the dose-response range which is linear under suitable transformation, so that the methods of Sections 3.2 or 3.3 apply. However, in some cases analysis of extended dose-response curves may be desirable. An outline of one model which may be used for such analysis is given in Section 3.4 and a simple example is shown in Section 5.4.

There is another category of assays in which the response cannot be measured in each experimental unit, but in which only the fraction of units responding to each treatment can be counted. This category is dealt with in Section 4.

3.1.2. ROUTINE ASSAYS

When an assay is in routine use, it is seldom possible to check systematically for conditions 1 to 3, because the limited number of observations per assay is likely to influence the sensitivity of the statistical tests. Fortunately, statisticians have shown that, in symmetrical balanced assays, small deviations from homogeneity of variance and normality do not seriously affect the assay results. The applicability of the statistical model needs to be questioned only if a series of assays shows doubtful validity. It may then be necessary to perform a new series of preliminary investigations as discussed in Section 3.1.1.

2 other necessary conditions depend on the statistical model to be used:

- for the parallel-line model:
 - 4A) the relationship between the logarithm of the dose and the response can be represented by a straight line over the range of doses used,
 - 5A) for any unknown preparation in the assay the straight line is parallel to that for the standard.
- for the slope-ratio model:
 - 4B) the relationship between the dose and the response can be represented by a straight line for each preparation in the assay over the range of doses used,
 - 5B) for any unknown preparation in the assay the straight line intersects the y -axis (at zero dose) at the same point as the straight line of the standard preparation (i.e. the response functions of all preparations in the assay must have the same intercept as the response function of the standard).

Conditions 4A and 4B can be verified only in assays in which at least 3 dilutions of each preparation have been tested. The use of an assay with only 1 or 2 dilutions may be justified when experience has shown that linearity and parallelism or equal intercept are regularly fulfilled.

(1) Wilk, M.B. and Shapiro, S.S. (1968). The joint assessment of normality of several independent samples, *Technometrics* 10, 825-839.

(2) Bartlett, M.S. (1937). Properties of sufficiency and statistical tests, *Proc. Roy. Soc. London, Series A* 160, 280-282.

(3) Cochran, W.G. (1951). Testing a linear relation among variances, *Biometrics* 7, 17-32.

After having collected the results of an assay, and before calculating the relative potency of each test sample, an analysis of variance is performed, in order to check whether conditions 4A and 5A (or 4B and 5B) are fulfilled. For this, the total sum of squares is subdivided into a certain number of sum of squares corresponding to each condition which has to be fulfilled. The remaining sum of squares represents the residual experimental error to which the absence or existence of the relevant sources of variation can be compared by a series of *F*-ratios.

When validity is established, the potency of each unknown relative to the standard may be calculated and expressed as a potency ratio or converted to some unit relevant to the preparation under test e.g. an International Unit. Confidence limits may also be estimated from each set of assay data.

Assays based on the parallel-line model are discussed in Section 3.2 and those based on the slope-ratio model in Section 3.3.

If any of the 5 conditions (1, 2, 3, 4A, 5A or 1, 2, 3, 4B, 5B) are not fulfilled, the methods of calculation described here are invalid and an investigation of the assay technique should be made.

The analyst should not adopt another transformation unless it is shown that non-fulfilment of the requirements is not incidental but is due to a systematic change in the experimental conditions. In this case, testing as described in Section 3.1.1 should be repeated before a new transformation is adopted for the routine assays.

Excess numbers of invalid assays due to non-parallelism or non-linearity, in a routine assay carried out to compare similar materials, are likely to reflect assay designs with inadequate replication. This inadequacy commonly results from incomplete recognition of all sources of variability affecting the assay, which can result in underestimation of the residual error leading to large *F*-ratios.

It is not always feasible to take account of all possible sources of variation within one single assay (e.g. day-to-day variation). In such a case, the confidence intervals from repeated assays on the same sample may not satisfactorily overlap, and care should be exercised in the interpretation of the individual confidence intervals. In order to obtain a more reliable estimate of the confidence interval it may be necessary to perform several independent assays and to combine these into one single potency estimate and confidence interval (see Section 6).

For the purpose of quality control of routine assays it is recommended to keep record of the estimates of the slope of regression and of the estimate of the residual error in control charts.

- An exceptionally high residual error may indicate some technical problem. This should be investigated and, if it can be made evident that something went wrong during the assay procedure, the assay should be repeated. An unusually high residual error may also indicate the presence of an occasional outlying or aberrant observation. A response that is questionable because of failure to comply with the procedure during the course of an assay is rejected. If an aberrant value is discovered after the responses have been recorded, but can then be traced to assay irregularities, omission may be justified. The arbitrary rejection or retention of an apparently aberrant response can be a serious source of bias. In general, the rejection of observations solely because a test for outliers is significant, is discouraged.

- An exceptionally low residual error may once in a while occur and cause the *F*-ratios to exceed the critical values. In such a case it may be justified to replace the residual error estimated from the individual assay, by an average residual error based on historical data recorded in the control charts.

3.1.3. CALCULATIONS AND RESTRICTIONS

According to general principles of good design the following 3 restrictions are normally imposed on the assay design. They have advantages both for ease of computation and for precision.

- Each preparation in the assay must be tested with the same number of dilutions.
- In the parallel-line model, the ratio of adjacent doses must be constant for all treatments in the assay; in the slope-ratio model, the interval between adjacent doses must be constant for all treatments in the assay.
- There must be an equal number of experimental units to each treatment.

If a design is used which meets these restrictions, the calculations are simple. The formulae are given in Sections 3.2 and 3.3. It is recommended to use software which has been developed for this special purpose. There are several programs in existence which can easily deal with all assay-designs described in the monographs. Not all programs may use the same formulae and algorithms, but they should all lead to the same results.

Assay designs not meeting the above mentioned restrictions may be both possible and correct, but the necessary formulae are too complicated to describe in this text. A brief description of methods for calculation is given in Section 7.1. These methods can also be used for the restricted designs, in which case they are equivalent with the simple formulae.

The formulae for the restricted designs given in this text may be used, for example, to create *ad hoc* programs in a spreadsheet. The examples in Section 5 can be used to clarify the statistics and to check whether such a program gives correct results.

3.2. THE PARALLEL-LINE MODEL

3.2.1. INTRODUCTION

The parallel-line model is illustrated in Figure 3.2.1-1. The logarithm of the doses are represented on the horizontal axis with the lowest concentration on the left and the highest concentration on the right. The responses are indicated on the vertical axis. The individual responses to each treatment are indicated with black dots. The 2 lines are the calculated $\ln(\text{dose})$ -response relationship for the standard and the unknown.

Note: the natural logarithm ($\ln$ or $\log_e$) is used throughout this text. Wherever the term “antilogarithm” is used, the quantity e^x is meant. However, the Briggs or “common” logarithm ($\log$ or $\log_{10}$) can equally well be used. In this case the corresponding antilogarithm is 10^x .

For a satisfactory assay the assumed potency of the test sample must be close to the true potency. On the basis of this assumed potency and the assigned potency of the standard, equipotent dilutions (if feasible) are prepared, i.e. corresponding doses of standard and unknown are expected to give the same response. If no information on the assumed potency is available, preliminary assays are carried out over a wide range of doses to determine the range where the curve is linear.

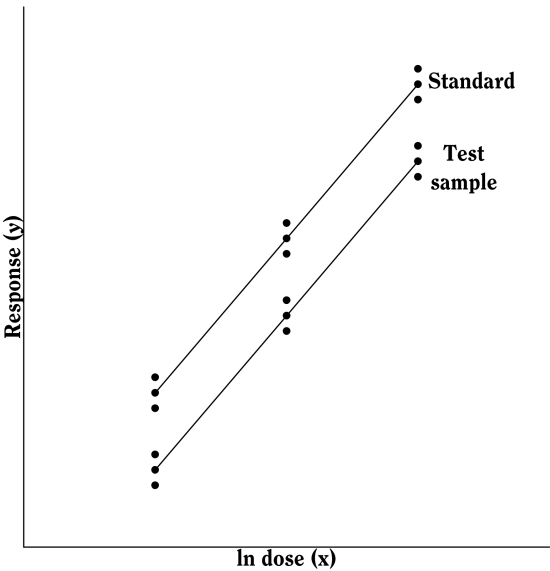


Figure 3.2.1-I. – *The parallel-line model for a 3 + 3 assay*
The more nearly correct the assumed potency of the unknown, the closer the 2 lines will be together, for they should give equal responses at equal doses. The horizontal distance between the lines represents the “true” potency of the unknown, relative to its assumed potency. The greater the distance between the 2 lines, the poorer the assumed potency of the unknown. If the line of the unknown is situated to the right of the standard, the assumed potency was overestimated, and the calculations will indicate an estimated potency lower than the assumed potency. Similarly, if the line of the unknown is situated to the left of the standard, the assumed potency was underestimated, and the calculations will indicate an estimated potency higher than the assumed potency.

3.2.2. ASSAY DESIGN

The following considerations will be useful in optimising the precision of the assay design:

- 1) the ratio between the slope and the residual error should be as large as possible,
- 2) the range of doses should be as large as possible,
- 3) the lines should be as close together as possible, i.e. the assumed potency should be a good estimate of the true potency.

The allocation of experimental units (animals, tubes, etc.) to different treatments may be made in various ways.

3.2.2.1. Completely randomised design

If the totality of experimental units appears to be reasonably homogeneous with no indication that variability in response will be smaller within certain recognisable sub-groups, the allocation of the units to the different treatments should be made randomly.

If units in sub-groups such as physical positions or experimental days are likely to be more homogeneous than the totality of the units, the precision of the assay may be increased by introducing one or more restrictions into the design. A careful distribution of the units over these restrictions permits irrelevant sources of variation to be eliminated.

3.2.2.2. Randomised block design

In this design it is possible to segregate an identifiable source of variation, such as the sensitivity variation between litters of experimental animals or the variation between Petri dishes in a diffusion microbiological assay. The design requires that every treatment be applied an equal number of times in

every block (litter or Petri dish) and is suitable only when the block is large enough to accommodate all treatments once. This is illustrated in Section 5.1.3. It is also possible to use a randomised design with repetitions. The treatments should be allocated randomly within each block. An algorithm to obtain random permutations is given in Section 8.5.

3.2.2.3. Latin square design

This design is appropriate when the response may be affected by two different sources of variation each of which can assume k different levels or positions. For example, in a plate assay of an antibiotic the treatments may be arranged in a $k \times k$ array on a large plate, each treatment occurring once in each row and each column. The design is suitable when the number of rows, the number of columns and the number of treatments are equal. Responses are recorded in a square format known as a Latin square. Variations due to differences in response among the k rows and among the k columns may be segregated, thus reducing the error. An example of a Latin square design is given in Section 5.1.2. An algorithm to obtain Latin squares is given in Section 8.6.

3.2.2.4. Cross-over design

This design is useful when the experiment can be sub-divided into blocks but it is possible to apply only 2 treatments to each block. For example, a block may be a single unit that can be tested on 2 occasions. The design is intended to increase precision by eliminating the effects of differences between units while balancing the effect of any difference between general levels of response at the 2 occasions. If 2 doses of a standard and of an unknown preparation are tested, this is known as a twin cross-over test.

The experiment is divided into 2 parts separated by a suitable time interval. Units are divided into 4 groups and each group receives 1 of the 4 treatments in the first part of the test. Units that received one preparation in the first part of the test receive the other preparation on the second occasion, and units receiving small doses in one part of the test receive large doses in the other. The arrangement of doses is shown in Table 3.2.2-I. An example can be found in Section 5.1.5.

Table 3.2.2-I. – *Arrangement of doses in cross-over design*

Group of units	Time I	Time II
1	S_1	T_2
2	S_2	T_1
3	T_1	S_2
4	T_2	S_1

3.2.3. ANALYSIS OF VARIANCE

This section gives formulae that are required to carry out the analysis of variance and will be more easily understood by reference to the worked examples in Section 5.1. Reference should also be made to the glossary of symbols (Section 9).

The formulae are appropriate for symmetrical assays where one or more preparations to be examined (T , U , etc.) are compared with a standard preparation (S). It is stressed that the formulae can only be used if the doses are equally spaced, if equal numbers of treatments per preparation are applied, and each treatment is applied an equal number of times. It should not be attempted to use the formulae in any other situation.

Apart from some adjustments to the error term, the basic analysis of data derived from an assay is the same for completely randomised, randomised block and Latin square designs. The formulae for cross-over tests do not entirely fit this scheme and these are incorporated into Example 5.1.5.

Having considered the points discussed in Section 3.1 and transformed the responses, if necessary, the values should be averaged over each treatment and each preparation, as shown in Table 3.2.3.-I. The linear contrasts, which relate to the slopes of the $\ln(\text{dose})$ -response lines, should also be formed. 3 additional formulae, which are necessary for the construction of the analysis of variance, are shown in Table 3.2.3.-II.

The total variation in response caused by the different treatments is now partitioned as shown in Table 3.2.3.-III the sums of squares being derived from the values obtained in Tables 3.2.3.-I and 3.2.3.-II. The sum of squares due to non-linearity can only be calculated if at least 3 doses per preparation are included in the assay.

The residual error of the assay is obtained by subtracting the variations allowed for in the design from the total variation in response (Table 3.2.3.-IV). In this table $\bar{y}$ represents the mean of all responses recorded in the assay. It should be noted that for a Latin square the number of replicate responses (n) is equal to the number of rows, columns or treatments (dh).

The analysis of variance is now completed as follows. Each sum of squares is divided by the corresponding number of degrees of freedom to give mean squares. The mean square for each variable to be tested is now expressed as a ratio to

the residual error (s^2) and the significance of these values (known as F -ratios) are assessed by use of Table 8.1 or a suitable sub-routine of a computer program.

3.2.4. TESTS OF VALIDITY

Assay results are said to be “statistically valid” if the outcome of the analysis of variance is as follows.

- 1) The linear regression term is significant, i.e. the calculated probability is less than 0.05. If this criterion is not met, it is not possible to calculate 95 per cent confidence limits.
- 2) The term for non-parallelism is not significant, i.e. the calculated probability is not less than 0.05. This indicates that condition 5A, Section 3.1, is satisfied;
- 3) The term for non-linearity is not significant, i.e. the calculated probability is not less than 0.05. This indicates that condition 4A, Section 3.1, is satisfied.

A significant deviation from parallelism in a multiple assay may be due to the inclusion in the assay-design of a preparation to be examined that gives an $\ln(\text{dose})$ -response line with a slope different from those for the other preparations. Instead of declaring the whole assay invalid, it may then be decided to eliminate all data relating to that preparation and to restart the analysis from the beginning.

When statistical validity is established, potencies and confidence limits may be estimated by the methods described in the next section.

Table 3.2.3.-I. – Formulae for parallel-line assays with d doses of each preparation

	Standard (S)	1 st Test sample (T)	2 nd Test sample (U, etc.)
Mean response lowest dose	S_1	T_1	U_1
Mean response 2 nd dose	S_2	T_2	U_2
...	...	...	...
Mean response highest dose	S_d	T_d	U_d
Total preparation	$P_S = S_1 + S_2 + \dots + S_d$	$P_T = T_1 + T_2 + \dots + T_d$	$P_U = \dots \text{etc.}$
Linear contrast	$L_S = 1S_1 + 2S_2 + \dots + dS_d - \frac{1}{2}(d+1)P_S$	$L_T = 1T_1 + 2T_2 + \dots + dT_d - \frac{1}{2}(d+1)P_T$	$L_U = \dots \text{etc.}$

Table 3.2.3.-II. – Additional formulae for the construction of the analysis of variance

$H_P = \frac{n}{d}$	$H_L = \frac{12n}{d^3 - d}$	$K = \frac{n(P_S + P_T + \dots)^2}{hd}$
---------------------	-----------------------------	---

Table 3.2.3.-III. – Formulae to calculate the sum of squares and degrees of freedom

Source of variation	Degrees of freedom (f)	Sum of squares
Preparations	$h - 1$	$SS_{\text{prep}} = H_P (P_S^2 + P_T^2 + \dots) - K$
Linear regression	1	$SS_{\text{reg}} = \frac{1}{h} H_L (L_S + L_T + \dots)^2$
Non-parallelism	$h - 1$	$SS_{\text{par}} = H_L (L_S^2 + L_T^2 + \dots) - SS_{\text{reg}}$
Non-linearity ^(*)	$h(d - 2)$	$SS_{\text{lin}} = SS_{\text{treat}} - SS_{\text{prep}} - SS_{\text{reg}} - SS_{\text{par}}$
Treatments	$hd - 1$	$SS_{\text{treat}} = n(S_1^2 + \dots + S_d^2 + T_1^2 + \dots + T_d^2 + \dots) - K$

(*) Not calculated for two-dose assays

Table 3.2.3-IV. – Estimation of the residual error

Source of variation	Degrees of freedom	Sum of squares
Blocks (rows)^(*)	$n - 1$	$SS_{\text{block}} = hd(R_1^2 + \dots + R_n^2) - K$
Columns^(**)	$n - 1$	$SS_{\text{col}} = hd(C_1^2 + \dots + C_n^2) - K$
Residual error^(***)	Completely randomised	$SS_{\text{res}} = SS_{\text{tot}} - SS_{\text{treat}}$
	Randomised block	$SS_{\text{res}} = SS_{\text{tot}} - SS_{\text{treat}} - SS_{\text{block}}$
	Latin square	$SS_{\text{res}} = SS_{\text{tot}} - SS_{\text{treat}} - SS_{\text{block}} - SS_{\text{col}}$
Total	$nhd - 1$	$SS_{\text{tot}} = \sum (y - \bar{y})^2$

(*) Not calculated for completely randomised designs
 (**) Only calculated for Latin square designs
 (***) Depends on the type of design

3.2.5. ESTIMATION OF POTENCY AND CONFIDENCE LIMITS

If I is the ln of the ratio between adjacent doses of any preparation, the common slope (b) for assays with d doses of each preparation is obtained from:

$$b = \frac{H_L(L_S + L_T + \dots)}{Inh} \quad (3.2.5-1)$$

and the logarithm of the potency ratio of a test preparation, for example T , is:

$$M'_T = \frac{P_T - P_S}{db} \quad (3.2.5-2)$$

The calculated potency is an estimate of the "true potency" of each unknown. Confidence limits may be calculated as the antilogarithms of:

$$CM'_T \pm \sqrt{(C-1)(CM_T'^2 + 2V)} \quad (3.2.5-3)$$

$$\text{where } C = \frac{SS_{\text{reg}}}{SS_{\text{reg}} - s^2t^2} \text{ and } V = \frac{SS_{\text{reg}}}{b^2dn}$$

The value of t may be obtained from Table 8.2 for $p = 0.05$ and degrees of freedom equal to the number of the degrees of freedom of the residual error. The estimated potency (R_T) and associated confidence limits are obtained by multiplying the values obtained by A_T after antilogarithms have been taken. If the stock solutions are not exactly equipotent on the basis of assigned and assumed potencies, a correction factor is necessary (See Examples 5.1.2 and 5.1.3).

3.2.6. MISSING VALUES

In a balanced assay, an accident totally unconnected with the applied treatments may lead to the loss of one or more responses, for example because an animal dies. If it is considered that the accident is in no way connected with the composition of the preparation administered, the exact calculations can still be performed but the formulae are necessarily more complicated and can only be given within the framework of general linear models (see Section 7.1). However, there exists an approximate method which keeps the simplicity of the balanced design by replacing the missing response by a calculated value. The loss of information is taken into account by diminishing the degrees of freedom for the total sum of squares and for the residual error by the number of missing values and using one of the formulae below for the missing values. It should be borne in mind that this is only an approximate method, and that the exact method is to be preferred.

If more than one observation is missing, the same formulae can be used. The procedure is to make a rough guess at all the missing values except one, and to use the proper formula for this one, using all the remaining values including the rough guesses. Fill in the calculated value. Continue by

similarly calculating a value for the first rough guess. After calculating all the missing values in this way the whole cycle is repeated from the beginning, each calculation using the most recent guessed or calculated value for every response to which the formula is being applied. This continues until 2 consecutive cycles give the same values; convergence is usually rapid.

Provided that the number of values replaced is small relative to the total number of observations in the full experiment (say less than 5 per cent), the approximation implied in this replacement and reduction of degrees of freedom by the number of missing values so replaced is usually fairly satisfactory. The analysis should be interpreted with great care however, especially if there is a preponderance of missing values in one treatment or block, and a biometrician should be consulted if any unusual features are encountered. Replacing missing values in a test without replication is a particularly delicate operation.

Completely randomised design

In a completely randomised assay the missing value can be replaced by the arithmetic mean of the other responses to the same treatment.

Randomised block design

The missing value is obtained using the equation:

$$y' = \frac{nB' + kT' - G'}{(n-1)(k-1)} \quad (3.2.6-1)$$

where B' is the sum of the responses in the block containing the missing value, T' the corresponding treatment total and G' is the sum of all responses recorded in the assay.

Latin square design

The missing value y' is obtained from:

$$y' = \frac{k(B' + C' + T') - 2G'}{(k-1)(k-2)} \quad (3.2.6-2)$$

where B' and C' are the sums of the responses in the row and column containing the missing value. In this case $k = n$.

Cross-over design

If an accident leading to loss of values occurs in a cross-over design, a book on statistics should be consulted (e.g. D.J. Finney, see Section 10), because the appropriate formulae depend upon the particular treatment combinations.

3.3. THE SLOPE-RATIO MODEL

3.3.1. INTRODUCTION

This model is suitable, for example, for some microbiological assays when the independent variable is the concentration of an essential growth factor below the optimal concentration of the medium. The slope-ratio model is illustrated in Figure 3.3.1-I.

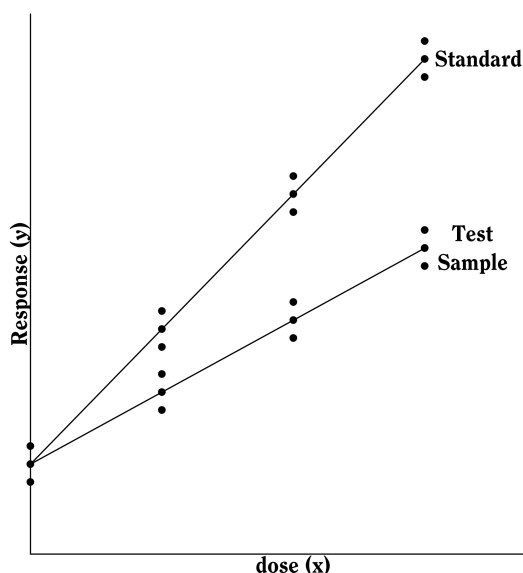


Figure 3.3.1.-I. – The slope-ratio model for a $2 \times 3 + 1$ assay

The doses are represented on the horizontal axis with zero concentration on the left and the highest concentration on the right. The responses are indicated on the vertical axis. The individual responses to each treatment are indicated with black dots. The 2 lines are the calculated dose-response relationship for the standard and the unknown under the assumption that they intersect each other at zero-dose. Unlike the parallel-line model, the doses are not transformed to logarithms.

Just as in the case of an assay based on the parallel-line model, it is important that the assumed potency is close to the true potency, and to prepare equipotent dilutions of the test preparations and the standard (if feasible). The more nearly correct the assumed potency, the closer the 2 lines will be together. The ratio of the slopes represents the “true” potency of the unknown, relative to its assumed potency. If the slope of the unknown preparation is steeper than that of the standard, the potency was underestimated and the calculations will indicate an estimated potency higher than the assumed potency. Similarly, if the slope of the unknown is less steep than that of the standard, the potency was overestimated and the calculations will result in an estimated potency lower than the assumed potency.

In setting up an experiment, all responses should be examined for the fulfilment of the conditions 1, 2 and 3 in Section 3.1. The analysis of variance to be performed in routine is described in Section 3.3.3 so that compliance with conditions 4B and 5B of Section 3.1 may be examined.

3.3.2. ASSAY DESIGN

The use of the statistical analysis presented below imposes the following restrictions on the assay:

- the standard and the test preparations must be tested with the same number of equally spaced dilutions,
- an extra group of experimental units receiving no treatment may be tested (the blanks),
- there must be an equal number of experimental units to each treatment.

As already remarked in Section 3.1.3, assay designs not meeting these restrictions may be both possible and correct, but the simple statistical analyses presented here are no longer applicable and either expert advice should be sought or suitable software should be used.

A design with 2 doses per preparation and 1 blank, the “common zero ($2h + 1$)-design”, is usually preferred, since it gives the highest precision combined with the possibility to check validity within the constraints mentioned above. However, a linear relationship cannot always be assumed to be valid down to zero-dose. With a slight loss of precision a design without blanks may be adopted. In this case 3 doses per preparation, the “common zero ($3h$)-design”, are preferred to 2 doses per preparation. The doses are thus given as follows:

- the standard is given in a high dose, near to but not exceeding the highest dose giving a mean response on the straight portion of the dose-response line,
- the other doses are uniformly spaced between the highest dose and zero dose,
- the test preparations are given in corresponding doses based on the assumed potency of the material.

A completely randomised, a randomised block or a Latin square design may be used, such as described in Section 3.2.2. The use of any of these designs necessitates an adjustment to the error sum of squares as described for assays based on the parallel-line model. The analysis of an assay of one or more test preparations against a standard is described below.

3.3.3. ANALYSIS OF VARIANCE

3.3.3.1. The ($hd + 1$)-design

The responses are verified as described in Section 3.1 and, if necessary, transformed. The responses are then averaged over each treatment and each preparation as shown in Table 3.3.3.1.-I. Additionally, the mean response for blanks (B) is calculated.

The sums of squares in the analysis of variance are calculated as shown in Tables 3.3.3.1.-I to 3.3.3.1.-III. The sum of squares due to non-linearity can only be calculated if at least 3 doses of each preparation have been included in the assay. The residual error is obtained by subtracting the variations allowed for in the design from the total variation in response (Table 3.3.3.1.-IV).

The analysis of variance is now completed as follows. Each sum of squares is divided by the corresponding number of degrees of freedom to give mean squares. The mean square for each variable to be tested is now expressed as a ratio to the residual error (s^2) and the significance of these values (known as F -ratios) are assessed by use of Table 8.1 or a suitable sub-routine of a computer program.

3.3.3.2. The (hd)-design

The formulae are basically the same as those for the ($hd + 1$)-design, but there are some slight differences.

- B is discarded from all formulae.
- $$K = \frac{n(P_S + P_T + \dots)^2}{hd}$$
- SS_{blank} is removed from the analysis of variance.
- The number of degrees of freedom for treatments becomes $hd - 1$.
- The number of degrees of freedom of the residual error and the total variance is calculated as described for the parallel-line model (see Table 3.2.3.-IV).

Validity of the assay, potency and confidence interval are found as described in Sections 3.3.4 and 3.3.5.

Table 3.3.3.1-I. – *Formulae for slope-ratio assays with d doses of each preparation and a blank*

	Standard (S)	1st Test sample (T)	2nd Test sample (U, etc.)
Mean response lowest dose	S_1	T_1	U_1
Mean response 2 nd dose	S_2	T_2	U_2
...	...	...	...
Mean response highest dose	S_d	T_d	U_d
Total preparation	$P_S = S_1 + S_2 + \dots + S_d$	$P_T = T_1 + T_2 + \dots + T_d$	$P_U = \dots$
Linear product	$L_S = 1S_1 + 2S_2 + \dots + dS_d$	$L_T = 1T_1 + 2T_2 + \dots + dT_d$	$L_U = \dots$
Intercept value	$a_S = (4d + 2)P_S - 6L_S$	$a_T = (4d + 2)P_T - 6L_T$	$a_U = \dots$
Slope value	$b_S = 2L_S - (d + 1)P_S$	$b_T = 2L_T - (d + 1)P_T$	$b_U = \dots$
Treatment value	$G_S = S_1^2 + \dots + S_d^2$	$G_T = T_1^2 + \dots + T_d^2$	$G_U = \dots$
Non-linearity(*)	$J_S = G_S - \frac{P_S^2}{d} - \frac{3b_S^2}{d^3 - d}$	$J_T = G_T - \frac{P_T^2}{d} - \frac{3b_T^2}{d^3 - d}$	$J_U = \dots$

(*) Not calculated for two-dose assays

Table 3.3.3.1-II. – *Additional formulae for the construction of the analysis of variance*

$H_B = \frac{nhd^2 - nhd}{hd^2 - hd + 4d + 2}$	$H_I = \frac{n}{4d^3 - 2d^2 - 2d}$	$a = \frac{a_S + a_T + \dots}{h(d^2 - d)}$	$K = \frac{n(B + P_S + P_T + \dots)^2}{hd + 1}$
--	------------------------------------	--	---

Table 3.3.3.1-III. – *Formulae to calculate the sum of squares and degrees of freedom*

Source of variation	Degrees of freedom (f)	Sum of squares
Regression	h	$SS_{\text{reg}} = SS_{\text{treat}} - SS_{\text{blank}} - SS_{\text{int}} - SS_{\text{lin}}$
Blanks	1	$SS_{\text{blank}} = H_B(B - a)^2$
Intersection	$h - 1$	$SS_{\text{int}} = H_I((a_S^2 + a_T^2 + \dots) - h(d^2 - d)^2 a^2)$
Non-linearity(*)	$h(d - 2)$	$SS_{\text{lin}} = n(J_S + J_T + \dots)$
Treatments	hd	$SS_{\text{treat}} = n(B^2 + G_S + G_T + \dots) - K$

(*) Not calculated for two-dose assays

3.3.4. TESTS OF VALIDITY

Assay results are said to be “statistically valid” if the outcome of the analysis of variance is as follows:

- 1) the variation due to blanks in $(hd + 1)$ -designs is not significant, i.e. the calculated probability is not smaller than 0.05. This indicates that the responses of the blanks do not significantly differ from the common intercept and the linear relationship is valid down to zero dose;
- 2) the variation due to intersection is not significant, i.e. the calculated probability is not less than 0.05. This indicates that condition 5B, Section 3.1 is satisfied;
- 3) in assays including at least 3 doses per preparation, the variation due to non-linearity is not significant, i.e. the calculated probability is not less than 0.05. This indicates that condition 4B, Section 3.1 is satisfied.

A significant variation due to blanks indicates that the hypothesis of linearity is not valid near zero dose. If this is likely to be systematic rather than incidental for the type of assay, the (hd) -design is more appropriate. Any response to blanks should then be disregarded.

When these tests indicate that the assay is valid, the potency is calculated with its confidence limits as described in Section 3.3.5.

3.3.5. ESTIMATION OF POTENCY AND CONFIDENCE LIMITS

3.3.5.1. The $(hd + 1)$ -design

The common intersection a' of the preparations can be calculated from:

$$a' = \frac{(2d + 1)B + (2d - 3)ha}{h(2d - 3) + 2d + 1} \quad (3.3.5.1-1)$$

The slope of the standard, and similarly for each of the other preparations, is calculated from:

$$b'_S = \frac{6L_S - 3d(d + 1)a'}{2d^3 + 3d^2 + d} \quad (3.3.5.1-2)$$

The potency ratio of each of the test preparations can now be calculated from:

Table 3.3.3.1-IV. – Estimation of the residual error

Source of variation	Degrees of freedom	Sum of squares	
Blocks (rows) ^(*)	$n - 1$	$SS_{block} = hd (R_1^2 + ... + R_n^2) - K$	
Columns ^(**)	$n - 1$	$SS_{col} = hd (C_1^2 + ... + C_n^2) - K$	
Residual error ^(***)	Completely randomised $(hd + 1) (n - 1)$	$SS_{res} = SS_{tot} - SS_{treat}$	
		Randomised block $hd (n - 1)$	$SS_{res} = SS_{tot} - SS_{treat} - SS_{block}$
		Latin square $(hd - 1) (n - 1)$	$SS_{res} = SS_{tot} - SS_{treat} - SS_{block} - SS_{col}$
Total	$nhd + n - 1$	$SS_{tot} = \sum (y - \bar{y})^2$	

(*) Not calculated for completely randomised designs

(**) Only calculated for Latin square designs

(***) Depends on the type of design

$$R'_T = \frac{b'_T}{b'_S} \quad (3.3.5.1.3)$$

which has to be multiplied by A_r , the assumed potency of the test preparation, in order to find the estimated potency R_r . If the step between adjacent doses was not identical for the standard and the test preparation, the potency has to be multiplied by I_s/I_r . Note that, unlike the parallel-line analysis, no antilogarithms are calculated.

The confidence interval for R'_r is calculated from:

$$CR'_T - K' \pm \sqrt{(C - 1)(CR'^2_T + 1) + K'(K' - 2CR'_T)} \quad (3.3.5.1.4)$$

$$\text{where } C = \frac{b'^2_S}{b'^2_S - s^2 t^2 V_1} \text{ and } K' = (C - 1)V_2$$

V_1 and V_2 are related to the variance and covariance of the numerator and denominator of R_r . They can be obtained from:

$$V_1 = \frac{6}{n(2d + 1)} \left(\frac{1}{d(d + 1)} + \frac{3}{2(2d + 1) + hd(d - 1)} \right) \quad (3.3.5.1.5)$$

$$V_2 = \frac{3d(d + 1)}{(3d + 1)(d + 2) + hd(d - 1)} \quad (3.3.5.1.6)$$

The confidence limits are multiplied by A_r , and if necessary by I_s/I_r .

3.3.5.2. The (hd) -design

The formulae are the same as for the $(hd + 1)$ -design, with the following modifications:

$$a' = a \quad (3.3.5.2.1)$$

$$V_1 = \frac{6}{nd(2d + 1)} \left(\frac{1}{d + 1} + \frac{3}{h(d - 1)} \right) \quad (3.3.5.2.2)$$

$$V_2 = \frac{3(d + 1)}{3(d + 1) + h(d - 1)} \quad (3.3.5.2.3)$$

3.4. EXTENDED SIGMOID DOSE-RESPONSE CURVES

This model is suitable, for example, for some immunoassays when analysis is required of extended sigmoid dose-response curves. This model is illustrated in Figure 3.4-I.

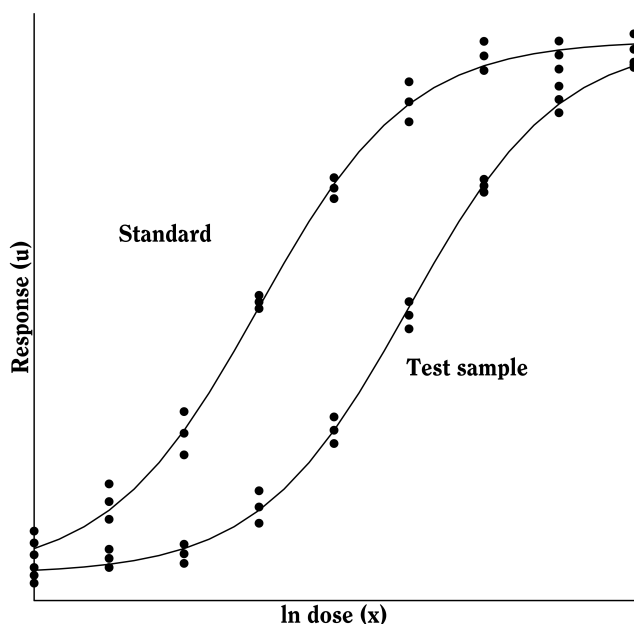


Figure 3.4-I. – The four-parameter logistic curve model
 The logarithms of the doses are represented on the horizontal axis with the lowest concentration on the left and the highest concentration on the right. The responses are indicated on the vertical axis. The individual responses to each treatment are indicated with black dots. The 2 curves are the calculated $\ln(\text{dose})$ -response relationship for the standard and the test preparation.

The general shape of the curves can usually be described by a logistic function but other shapes are also possible. Each curve can be characterised by 4 parameters: The upper asymptote (α), the lower asymptote (δ), the slope-factor (β), and the horizontal location (γ). This model is therefore often referred to as a four-parameter model. A mathematical representation of the $\ln(\text{dose})$ -response curve is:

$$u = \delta + \frac{\alpha - \delta}{1 + e^{-\beta(x - \gamma)}}$$

For a valid assay it is necessary that the curves of the standard and the test preparations have the same slope-factor, and the same maximum and minimum response level at the extreme parts. Only the horizontal location (γ) of the curves may be different. The horizontal distance between the curves is related to the “true” potency of the unknown. If the assay is used routinely, it may be sufficient to test the condition of equal upper and lower response levels when the assay is

developed, and then to retest this condition directly only at suitable intervals or when there are changes in materials or assay conditions.

The maximum-likelihood estimates of the parameters and their confidence intervals can be obtained with suitable computer programs. These computer programs may include some statistical tests reflecting validity. For example, if the maximum likelihood estimation shows significant deviations from the fitted model under the assumed conditions of equal upper and lower asymptotes and slopes, then one or all of these conditions may not be satisfied.

The logistic model raises a number of statistical problems which may require different solutions for different types of assays, and no simple summary is possible. A wide variety of possible approaches is described in the relevant literature. Professional advice is therefore recommended for this type of analysis. A simple example is nevertheless included in Section 5.4 to illustrate a “possible” way to analyse the data presented. A short discussion of alternative approaches and other statistical considerations is given in Section 7.5.

If professional advice or suitable software is not available, alternative approaches are possible: 1) if “reasonable” estimates of the upper limit (α) and lower limit (δ) are available, select for all preparations the doses with mean of the responses (u) falling between approximately 20 per cent and 80 per cent of the limits, transform responses of the selected doses to $y = \ln \left(\frac{u - \delta}{\alpha - u} \right)$ and use the parallel line model (Section 3.2) for the analysis; 2) select a range of doses for which the responses (u) or suitably transformed responses, for example $\ln(u)$, are approximately linear when plotted against $\ln(\text{dose})$; the parallel line model (Section 3.2) may then be used for analysis.

4. ASSAYS DEPENDING UPON QUANTAL RESPONSES

4.1. INTRODUCTION

In certain assays it is impossible or excessively laborious to measure the effect on each experimental unit on a quantitative scale. Instead, an effect such as death or hypoglycaemic symptoms may be observed as either occurring or not occurring in each unit, and the result depends on the number of units in which it occurs. Such assays are called quantal or all-or-none.

The situation is very similar to that described for quantitative assays in Section 3.1, but in place of n separate responses to each treatment a single value is recorded, i.e. the fraction of units in each treatment group showing a response. When these fractions are plotted against the logarithms of the doses the resulting curve will tend to be sigmoid (S-shaped) rather than linear. A mathematical function that represents this sigmoid curvature is used to estimate the dose-response curve. The most commonly used function is the cumulative normal distribution function. This function has some theoretical merit, and is perhaps the best choice if the response is a reflection of the tolerance of the units. If the response is more likely to depend upon a process of growth, the logistic distribution model is preferred, although the difference in outcome between the 2 models is usually very small.

The maximum likelihood estimators of the slope and location of the curves can be found only by applying an iterative procedure. There are many procedures which lead to the same outcome, but they differ in efficiency due to the speed of convergence. One of the most rapid methods is direct

optimisation of the maximum-likelihood function (see Section 7.1), which can easily be performed with computer programs having a built-in procedure for this purpose. Unfortunately, most of these procedures do not yield an estimate of the confidence interval, and the technique to obtain it is too complicated to describe here. The technique described below is not the most rapid, but has been chosen for its simplicity compared to the alternatives. It can be used for assays in which one or more test preparations are compared to a standard. Furthermore, the following conditions must be fulfilled:

- 1) the relationship between the logarithm of the dose and the response can be represented by a cumulative normal distribution curve,
- 2) the curves for the standard and the test preparation are parallel, i.e. they are identically shaped and may only differ in their horizontal location,
- 3) in theory, there is no natural response to extremely low doses and no natural non-response to extremely high doses.

4.2. THE PROBIT METHOD

The sigmoid curve can be made linear by replacing each response, i.e. the fraction of positive responses per group, by the corresponding value of the cumulative standard normal distribution. This value, often referred to as “normit”, ranges theoretically from $-\infty$ to $+\infty$. In the past it was proposed to add 5 to each normit to obtain “probits”. This facilitated the hand-performed calculations because negative values were avoided. With the arrival of computers the need to add 5 to the normits has disappeared. The term “normit method” would therefore be better for the method described below. However, since the term “probit analysis” is so widely spread, the term will, for historical reasons, be maintained in this text.

Once the responses have been linearised, it should be possible to apply the parallel-line analysis as described in Section 3.2. Unfortunately, the validity condition of homogeneity of variance for each dose is not fulfilled. The variance is minimal at normit = 0 and increases for positive and negative values of the normit. It is therefore necessary to give more weight to responses in the middle part of the curve, and less weight to the more extreme parts of the curve. This method, the analysis of variance, and the estimation of the potency and confidence interval are described below.

4.2.1. TABULATION OF THE RESULTS

Table 4.2.1-I is used to enter the data into the columns indicated by numbers:

- (1) the dose of the standard or the test preparation,
- (2) the number n of units submitted to that treatment,
- (3) the number of units r giving a positive response to the treatment,
- (4) the logarithm x of the dose,
- (5) the fraction $p = r/n$ of positive responses per group.

The first cycle starts here.

- (6) column Y is filled with zeros at the first iteration,
 - (7) the corresponding value $\Phi = \Phi(Y)$ of the cumulative standard normal distribution function (see also Table 8.4).
- The columns (8) to (10) are calculated with the following formulae:

$$(8) \quad Z = \frac{e^{-Y^2/2}}{\sqrt{2\pi}} \quad (4.2.1-1)$$

$$(9) \quad y = Y + \frac{p - \Phi}{Z} \quad (4.2.1-2)$$

$$(10) \quad w = \frac{nZ^2}{\Phi - \Phi^2} \quad (4.2.1-3)$$

The columns (11) to (15) can easily be calculated from columns (4), (9) and (10) as wx , wy , wx^2 , wy^2 and wxy respectively, and the sum (Σ) of each of the columns (10) to (15) is calculated separately for each of the preparations.

The sums calculated in Table 4.2.1-I are transferred to columns (1) to (6) of Table 4.2.1-II and 6 additional columns (7) to (12) are calculated as follows:

$$(7) \quad S_{xx} = \sum wx^2 - \frac{(\sum wx)^2}{\sum w} \quad (4.2.1-4)$$

$$(8) \quad S_{xy} = \sum wxy - \frac{(\sum wx)(\sum wy)}{\sum w} \quad (4.2.1-5)$$

$$(9) \quad S_{yy} = \sum wy^2 - \frac{(\sum wy)^2}{\sum w} \quad (4.2.1-6)$$

$$(10) \quad \bar{x} = \frac{\sum wx}{\sum w} \quad (4.2.1-7)$$

$$(11) \quad \bar{y} = \frac{\sum wy}{\sum w} \quad (4.2.1-8)$$

The common slope b can now be obtained as:

$$b = \frac{\sum S_{xy}}{\sum S_{xx}} \quad (4.2.1-9)$$

and the intercept a of the standard, and similarly for the test preparations is obtained as:

$$(12) \quad a = \bar{y} - b\bar{x} \quad (4.2.1-10)$$

Column (6) of the first working table can now be replaced by $Y = a + bx$ and the cycle is repeated until the difference between 2 cycles has become small (e.g. the maximum difference of Y between 2 consecutive cycles is smaller than 10^{-8}).

4.2.2. TESTS OF VALIDITY

Before calculating the potencies and confidence intervals, validity of the assay must be assessed. If at least 3 doses for each preparation have been included, the deviations from linearity can be measured as follows: add a 13th column to Table 4.2.1-II and fill it with:

$$S_{yy} - \frac{S_{xy}^2}{S_{xx}} \quad (4.2.2-1)$$

The column total is a measure of deviations from linearity and is approximately χ^2 distributed with degrees of freedom equal to $N - 2h$. Significance of this value may be assessed with the aid of Table 8.3 or a suitable sub-routine in a computer program. If the value is significant at the 0.05 probability level, the assay must probably be rejected (see Section 4.2.4).

When the above test gives no indication of significant deviations from linear regression, the deviations from parallelism are tested at the 0.05 significance level with:

$$\chi^2 = \sum \frac{S_{xy}^2}{S_{xx}} - \frac{(\sum S_{xy})^2}{\sum S_{xx}} \quad (4.2.2-2)$$

with $h - 1$ degrees of freedom.

4.2.3. ESTIMATION OF POTENCY AND CONFIDENCE LIMITS

When there are no indications for a significant departure from parallelism and linearity the $\ln(\text{potency ratio}) M'_T$ is calculated as:

$$M'_T = \frac{a_T - a_S}{b} \quad (4.2.3-1)$$

and the antilogarithm is taken. Now let $t = 1.96$ and $s = 1$. Confidence limits are calculated as the antilogarithms of:

$$CM'_T - (C-1)(\bar{x}_S - \bar{x}_T) \pm \sqrt{(C-1)\left(V \sum S_{xx} + C(M'_T - \bar{x}_S + \bar{x}_T)^2\right)} \quad (4.2.3-2)$$

$$\text{where } C = \frac{b^2 \sum S_{xx}}{b^2 \sum S_{xx} - s^2 t^2} \text{ and } V = \frac{1}{\sum w} + \frac{1}{\sum w}$$

Table 4.2.1-I. – First working table

	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)	(13)	(14)	(15)
	dose	n	r	x	p	Y	Φ	Z	y	w	wx	wy	wx^2	wy^2	wxy
S	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
										$\Sigma =$	$\Sigma =$	$\Sigma =$	$\Sigma =$	$\Sigma =$	$\Sigma =$
T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
										$\Sigma =$	$\Sigma =$	$\Sigma =$	$\Sigma =$	$\Sigma =$	$\Sigma =$
etc.															

Table 4.2.1-II. – Second working table

	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)
	Σw	Σwx	Σwy	Σwx^2	Σwy^2	Σwxy	S_{xx}	S_{xy}	S_{yy}	$\bar{x}$	$\bar{y}$	a
S	.	.	.	.	.	.	.	.	.	.	.	.
T	.	.	.	.	.	.	.	.	.	.	.	.
etc.	.	.	.	.	.	.	.	.	.	.	.	.
							$\Sigma =$	$\Sigma =$				

4.2.4. INVALID ASSAYS

If the test for deviations from linearity described in Section 4.2.2 is significant, the assay should normally be rejected. If there are reasons to retain the assay, the formulae are slightly modified. t becomes the t -value ($p = 0.05$) with the same number of degrees of freedom as used in the check for linearity and s^2 becomes the χ^2 value divided by the same number of degrees of freedom (and thus typically is greater than 1).

The test for parallelism is also slightly modified. The χ^2 value for non-parallelism is divided by its number of degrees of freedom. The resulting value is divided by s^2 calculated above to obtain an F -ratio with $h - 1$ and $N - 2h$ degrees of freedom, which is evaluated in the usual way at the 0.05 significance level.

4.3. THE LOGIT METHOD

As indicated in Section 4.1 the logit method may sometimes be more appropriate. The name of the method is derived from the logit function which is the inverse of the logistic distribution. The procedure is similar to that described for the probit method with the following modifications in the formulae for Φ and Z .

$$\Phi = \frac{1}{1 + e^{-Y}} \quad (4.3.-1)$$

$$Z = \frac{e^{-Y}}{(1 + e^{-Y})^2} \quad (4.3.-2)$$

4.4. OTHER SHAPES OF THE CURVE

The probit and logit method are almost always adequate for the analysis of quantal responses called for in the European Pharmacopoeia. However, if it can be made evident that the $\ln(\text{dose})$ -response curve has another shape than the 2 curves described above, another curve Φ may be adopted. Z is then taken to be the first derivative of Φ .

For example, if it can be shown that the curve is not symmetric, the Gompertz distribution may be appropriate (the gompit method) in which case $\Phi = 1 - e^{-e^Y}$ and $Z = e^{Y-e^Y}$.

4.5. THE MEDIAN EFFECTIVE DOSE

In some types of assay it is desirable to determine a median effective dose which is the dose that produces a response in 50 per cent of the units. The probit method can be used to determine this median effective dose (ED_{50}), but since there is no need to express this dose relative to a standard, the formulae are slightly different.

Note: a standard can optionally be included in order to validate the assay. Usually the assay is considered valid if the calculated ED_{50} of the standard is close enough to the assigned ED_{50} . What "close enough" in this context means depends on the requirements specified in the monograph.

The tabulation of the responses to the test samples, and optionally a standard, is as described in Section 4.2.1. The test for linearity is as described in Section 4.2.2. A test for parallelism is not necessary for this type of assay. The ED_{50} of test sample T , and similarly for the other samples, is obtained as described in Section 4.2.3, with the following modifications in formulae 4.2.3.-1 and 4.2.3.-2).

$$M'_T = \frac{-a_T}{b} \quad (4.5.-1)$$

$$CM'_T - (C-1)\bar{x}_T \pm \sqrt{(C-1)\left(V \sum S_{xx} + C(M'_T + \bar{x}_T)^2\right)} \quad (4.5.-2)$$

$$\text{where } V = \frac{1}{\sum w} \text{ and } C \text{ is left unchanged}$$

5. EXAMPLES

This section consists of worked examples illustrating the application of the formulae. The examples have been selected primarily to illustrate the statistical method of calculation. They are not intended to reflect the most suitable method of assay, if alternatives are permitted in the individual monographs. To increase their value as program checks, more decimal places are given than would usually be necessary. It should also be noted that other, but equivalent methods of calculation exist. These methods should lead to exactly the same final results as those given in the examples.

5.1. PARALLEL-LINE MODEL

5.1.1. TWO-DOSE MULTIPLE ASSAY WITH COMPLETELY RANDOMISED DESIGN

An assay of corticotrophin by subcutaneous injection in rats

The standard preparation is administered at 0.25 and 1.0 unit per 100 g of body mass. 2 preparations to be examined are both assumed to have a potency of 1 unit per milligram and they are administered in the same quantities as the standard. The individual responses and means per treatment are given in Table 5.1.1.-I. A graphical presentation (Figure 5.1.1.I) gives no rise to doubt the homogeneity of variance and normality of the data, but suggests problems with parallelism for preparation U .

Table 5.1.1.-I. – Response metameter y - mass of ascorbic acid (mg) per 100 g of adrenal gland

	Standard S		Preparation T		Preparation U	
	S_1	S_2	T_1	T_2	U_1	U_2
	300	289	310	230	250	236
	310	221	290	210	268	213
	330	267	360	280	273	283
	290	236	341	261	240	269
	364	250	321	241	307	251
	328	231	370	290	270	294
	390	229	303	223	317	223
	360	269	334	254	312	250
	342	233	295	216	320	216
	306	259	315	235	265	265
Mean	332.0	248.4	323.9	244.0	282.2	250.0

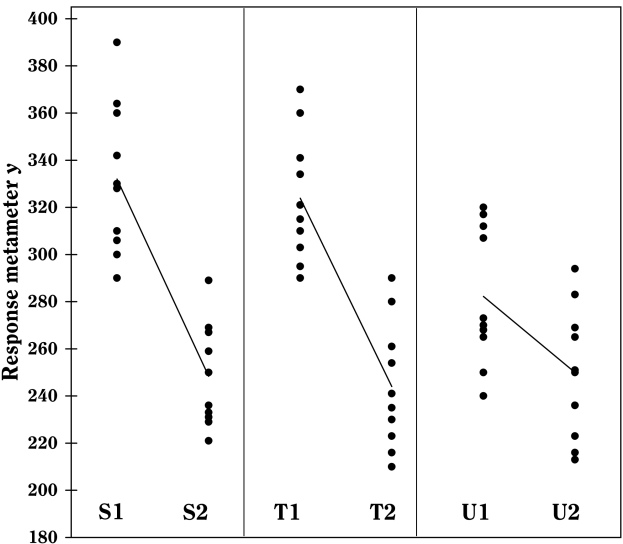


Figure 5.1.1-I.

The formulae in Tables 3.2.3-I and 3.2.3-II lead to:

$$\begin{aligned} P_s &= 580.4 & L_s &= -41.8 \\ P_T &= 567.9 & L_T &= -39.95 \\ P_U &= 532.2 & L_U &= -16.1 \\ H_P &= \frac{10}{2} = 5 & H_L &= \frac{120}{6} = 20 \end{aligned}$$

The analysis of variance can now be completed with the formulae in Tables 3.2.3-III and 3.2.3-IV. This is shown in Table 5.1.1-II.

Table 5.1.1-II. – Analysis of variance

Source of variation	Degrees of freedom	Sum of squares	Mean square	F-ratio	Probability
Preparations	2	6256.6	3128.3		
Regression	1	63 830.8	63 830.8	83.38	0.000
Non-parallelism	2	8218.2	4109.1	5.37	0.007
Treatments	5	78 305.7			
Residual error	54	41 340.9	765.57		
Total	59	119 646.6			

The analysis confirms a highly significant linear regression. Departure from parallelism, however, is also significant ($p = 0.0075$) which was to be expected from the graphical observation that preparation *U* is not parallel to the standard. This preparation is therefore rejected and the analysis repeated using only preparation *T* and the standard.

Table 5.1.1-III. – Analysis of variance without sample *U*

Source of variation	Degrees of freedom	Sum of squares	Mean square	F-ratio	Probability
Preparations	1	390.6	390.6		
Regression	1	66 830.6	66 830.6	90.5	0.000
Non-parallelism	1	34.2	34.2	0.05	0.831
Treatments	3	67 255.5			
Residual error	36	26 587.3	738.54		
Total	39	93 842.8			

The analysis without preparation *U* results in compliance with the requirements with respect to both regression and parallelism and so the potency can be calculated. The formulae in Section 3.2.5 give:

– for the common slope:

$$b = \frac{20(-41.8 - 39.95)}{\ln 4 \times 10 \times 2} = -58.970$$

– the $\ln(\text{potency ratio})$ is:

$$\begin{aligned} M'_T &= \frac{567.9 - 580.4}{2 \times (-58.970)} = 0.1060 \\ C &= \frac{66\,830.6}{66\,830.6 - 738.54 \times 2.028^2} = 1.0476 \\ V &= \frac{66\,830.6}{(-58.970)^2 \times 2 \times 10} = 0.9609 \end{aligned}$$

– and $\ln(\text{confidence limits})$ are:

$$1.0476 \times 0.1060 \pm \sqrt{0.0476 \times (1.0476 \times 0.1060^2 + 2 \times 0.9609)} = 0.1110 \pm 0.3034$$

By taking the antilogarithms we find a potency ratio of 1.11 with 95 per cent confidence limits from 0.82-1.51.

Multiplying by the assumed potency of preparation *T* yields a potency of 1.11 units/mg with 95 per cent confidence limits from 0.82 to 1.51 units/mg.

5.1.2. THREE-DOSE LATIN SQUARE DESIGN

Antibiotic agar diffusion assay using a rectangular tray

The standard has an assigned potency of 4855 IU/mg. The test preparation has an assumed potency of 5600 IU/mg. For the stock solutions 25.2 mg of the standard is dissolved in 24.5 ml of solvent and 21.4 mg of the test preparation is dissolved in 23.95 ml of solvent. The final solutions are prepared by first diluting both stock solutions to 1/20 and further using a dilution ratio of 1.5.

A Latin square is generated with the method described in Section 8.6 (see Table 5.1.2-I). The responses of this routine assay are shown in Table 5.1.2-II (inhibition zones in mm $\times$ 10). The treatment mean values are shown in Table 5.1.2-III. A graphical representation of the data (see Figure 5.1.2-I) gives no rise to doubt the normality or homogeneity of variance of the data.

The formulae in Tables 3.2.3-I and 3.2.3-II lead to:

$$\begin{aligned} P_s &= 529.667 & L_s &= 35.833 \\ P_T &= 526.333 & L_T &= 39.333 \\ H_P &= \frac{6}{3} = 2 & H_L &= \frac{72}{24} = 3 \end{aligned}$$

The analysis of variance can now be completed with the formulae in Tables 3.2.3-III and 3.2.3-IV. The result is shown in Table 5.1.2-IV.

The analysis shows significant differences between the rows. This indicates the increased precision achieved by using a Latin square design rather than a completely randomised design. A highly significant regression and no significant departure of the individual regression lines from parallelism and linearity confirms that the assay is satisfactory for potency calculations.

Table 5.1.2-I. – Distribution of treatments over the plate

	1	2	3	4	5	6
1	S_1	T_1	T_2	S_3	S_2	T_3
2	T_1	T_3	S_1	S_2	T_2	S_3
3	T_2	S_3	S_2	S_1	T_3	T_1
4	S_3	S_2	T_3	T_1	S_1	T_2
5	S_2	T_2	S_3	T_3	T_1	S_1
6	T_3	S_1	T_1	T_2	S_3	S_2

Table 5.1.2-II. – Measured inhibition zones in mm × 10

	1	2	3	4	5	6	Row mean
1	161	160	178	187	171	194	$175.2 = R_1$
2	151	192	150	172	170	192	$171.2 = R_2$
3	162	195	174	161	193	151	$172.7 = R_3$
4	194	184	199	160	163	171	$178.5 = R_4$
5	176	181	201	202	154	151	$177.5 = R_5$
6	193	166	161	186	198	182	$181.0 = R_6$
Col.	172.8	179.7	177.2	178.0	174.8	173.5	
Mean =	C_1	C_2	C_3	C_4	C_5	C_6	

Table 5.1.2-III. – Means of the treatments

	Standard S			Preparation T		
	S_1	S_2	S_3	T_1	T_2	T_3
Mean	158.67	176.50	194.50	156.17	174.67	195.50

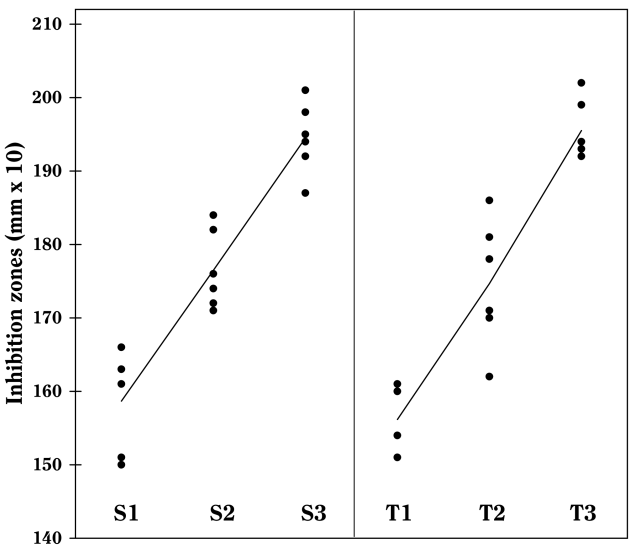


Figure 5.1.2-I.

Table 5.1.2-IV. – Analysis of variance

Source of variation	Degrees of freedom	Sum of squares	Mean square	F-ratio	Probability
Preparations	1	11.1111	11.1111		
Regression	1	8475.0417	8475.0417	408.1	0.000
Non-parallelism	1	18.3750	18.3750	0.885	0.358
Non-linearity	2	5.4722	2.7361	0.132	0.877
Treatments	5	8510			
Rows	5	412	82.40	3.968	0.012
Columns	5	218.6667	43.73	2.106	0.107
Residual error	20	415.3333	20.7667		
Total	35	9556			

The formulae in Section 3.2.5 give:

– for the common slope:

$$b = \frac{3 \times (35.833 + 39.333)}{\ln(1.5) \times 6 \times 2} = 46.346$$

– the ln(potency ratio) is:

$$M'_T = \frac{526.333 - 529.667}{3 \times 46.346} = -0.023974$$

$$C' = \frac{8475.0417}{8475.0417 - 20.7667 \times 2.086^2} = 1.0108$$

$$V = \frac{8475.0417}{46.346^2 \times 3 \times 6} = 0.2192$$

– and ln(confidence limits) are:

$$\sqrt{\frac{1.0108 \times (-0.0240) \pm \sqrt{0.0108 \times (1.0108 \times (-0.0240)^2 + 2 \times 0.2192)}}{-0.02423 \pm 0.06878}}$$

The potency ratio is found by taking the antilogarithms, resulting in 0.9763 with 95 per cent confidence limits from 0.9112-1.0456.

A correction factor of $\frac{4855 \times 25.2/24.5}{5600 \times 21.4/23.95} = 0.99799$ is necessary because the dilutions were not exactly equipotent on the basis of the assumed potency. Multiplying by this correction factor and the assumed potency of 5600 IU/mg yields a potency of 5456 IU/mg with 95 per cent confidence limits from 5092 to 5843 IU/mg.

5.1.3. FOUR-DOSE RANDOMISED BLOCK DESIGN

Antibiotic turbidimetric assay

This assay is designed to assign a potency in international units per vial. The standard has an assigned potency of 670 IU/mg. The test preparation has an assumed potency of 20 000 IU/vial. On the basis of this information the stock solutions are prepared as follows. 16.7 mg of the standard is dissolved in 25 ml solvent and the contents of one vial of the test preparation are dissolved in 40 ml solvent. The final solutions are prepared by first diluting to 1/40 and further using a dilution ratio of 1.5. The tubes are placed in a water-bath in a randomised block arrangement (see Section 8.5). The responses are listed in Table 5.1.3-I.

Inspection of Figure 5.1.3-I gives no rise to doubt the validity of the assumptions of normality and homogeneity of variance of the data. The standard deviation of S_3 is somewhat high but is no reason for concern.

The formulae in Tables 3.2.3-I and 3.2.3-II lead to:

$$\begin{aligned} P_S &= 719.4 & L_S &= -229.1 \\ P_T &= 687.6 & L_T &= -222 \\ H_P &= \frac{5}{4} = 1.25 & H_L &= \frac{60}{60} = 1 \end{aligned}$$

The analysis of variance is constructed with the formulae in Tables 3.2.3-III and 3.2.3-IV. The result is shown in Table 5.1.3-II.

Table 5.1.3-I. – Absorbances of the suspensions ($\times 1000$)

Block	Standard S				Preparation T				Mean
	S ₁	S ₂	S ₃	S ₄	T ₁	T ₂	T ₃	T ₄	
1	252	207	168	113	242	206	146	115	181.1
2	249	201	187	107	236	197	153	102	179.0
3	247	193	162	111	246	197	148	104	176.0
4	250	207	155	108	231	191	159	106	175.9
5	235	207	140	98	232	186	146	95	167.4
Mean	246.6	203.0	162.4	107.4	237.4	195.4	150.4	104.4	

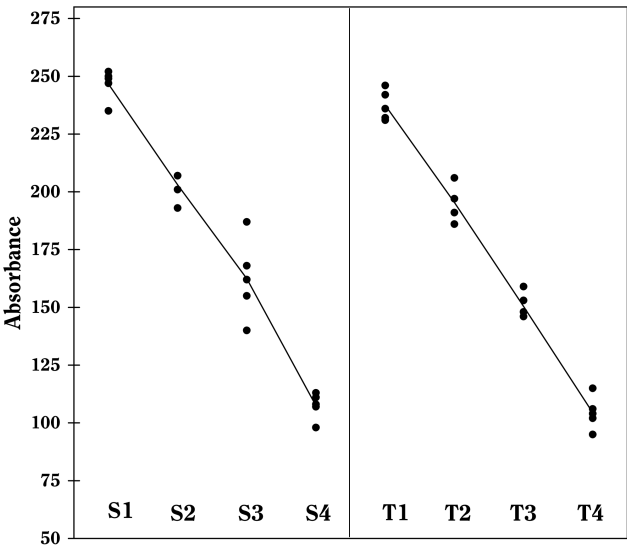


Figure 5.1.3-I.

Table 5.1.3-II. – Analysis of variance

Source of variation	Degrees of freedom	Sum of squares	Mean square	F-ratio	Probability
Preparations	1	632.025	632.025		
Regression	1	101 745.6	101 745.6	1887.1	0.000
Non-parallelism	1	25.205	25.205	0.467	0.500
Non-linearity	4	259.14	64.785	1.202	0.332
Treatments	7	102 662			
Blocks	4	876.75	219.188	4.065	0.010
Residual error	28	1509.65	53.916		
Total	39	105 048.4			

A significant difference is found between the blocks. This indicates the increased precision achieved by using a randomised block design. A highly significant regression

and no significant departure from parallelism and linearity confirms that the assay is satisfactory for potency calculations. The formulae in Section 3.2.5 give:

– for the common slope:

$$b = \frac{1 \times (-229.1 - 222)}{\ln(1.5) \times 5 \times 2} = -111.255$$

– the $\ln(\text{potency ratio})$ is:

$$\begin{aligned} M'_T &= \frac{687.6 - 719.4}{4 \times (-111.255)} = 0.071457 \\ C &= \frac{101\,745.6}{101\,745.6 - 53.916 \times 2.048^2} = 1.00223 \\ V &= \frac{101\,745.6}{(-111.255)^2 \times 4 \times 5} = 0.4110 \end{aligned}$$

– and $\ln(\text{confidence limits})$ are:

$$\begin{aligned} &1.00223 \times 0.0715 \pm \\ &\sqrt{0.00223 \times (1.00223 \times 0.0715^2 + 2 \times 0.4110)} \\ &= 0.07162 \pm 0.04293 \end{aligned}$$

The potency ratio is found by taking the antilogarithms, resulting in 1.0741 with 95 per cent confidence limits from 1.0291 to 1.1214. A correction factor of $\frac{670 \times 16.7/25}{20\,000 \times 1/40} = 0.89512$ is necessary because the dilutions were not exactly equipotent on the basis of the assumed potency. Multiplying by this correction factor and the assumed potency of 20 000 IU/vial yields a potency of 19 228 IU/vial with 95 per cent confidence limits from 18 423–20 075 IU/vial.

5.1.4. FIVE-DOSE MULTIPLE ASSAY WITH COMPLETELY RANDOMISED DESIGN

An *in-vitro* assay of three hepatitis B vaccines against a standard

3 independent two-fold dilution series of 5 dilutions were prepared from each of the vaccines. After some additional steps in the assay procedure, absorbances were measured. They are shown in Table 5.1.4-I.

Table 5.1.4-I. – Optical densities

Dilution	Standard S			Preparation T		
1:16 000	0.043	0.045	0.051	0.097	0.097	0.094
1:8000	0.093	0.099	0.082	0.167	0.157	0.178
1:4000	0.159	0.154	0.166	0.327	0.355	0.345
1:2000	0.283	0.295	0.362	0.501	0.665	0.576
1:1000	0.514	0.531	0.545	1.140	1.386	1.051

Dilution	Preparation U			Preparation V		
1:16 000	0.086	0.071	0.073	0.082	0.082	0.086
1:8000	0.127	0.146	0.133	0.145	0.144	0.173
1:4000	0.277	0.268	0.269	0.318	0.306	0.316
1:2000	0.586	0.489	0.546	0.552	0.551	0.624
1:1000	0.957	0.866	1.045	1.037	1.039	1.068

The logarithms of the optical densities are known to have a linear relationship with the logarithms of the doses. The mean responses of the $\ln$ -transformed optical densities are listed in Table 5.1.4-II. No unusual features are discovered in a graphical presentation of the data.

Table 5.1.4-II. – Means of the *ln*-transformed absorbances

S_1	-3.075	T_1	-2.344	U_1	-2.572	V_1	-2.485
S_2	-2.396	T_2	-1.789	U_2	-2.002	V_2	-1.874
S_3	-1.835	T_3	-1.073	U_3	-1.305	V_3	-1.161
S_4	-1.166	T_4	-0.550	U_4	-0.618	V_4	-0.554
S_5	-0.635	T_5	0.169	U_5	-0.048	V_5	0.047

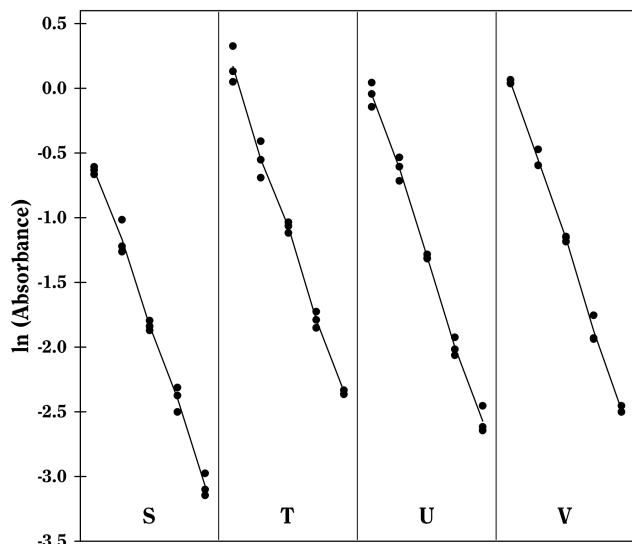


Figure 5.1.4-I.

The formulae in Tables 3.2.3-I and 3.2.3-II give:

$$\begin{aligned}
 P_S &= -9.108 & L_S &= 6.109 \\
 P_T &= -5.586 & L_T &= 6.264 \\
 P_U &= -6.544 & L_U &= 6.431 \\
 P_V &= -6.027 & L_V &= 6.384 \\
 H_P &= \frac{3}{5} = 0.6 & H_L &= \frac{36}{120} = 0.3
 \end{aligned}$$

The analysis of variance is completed with the formulae in Tables 3.2.3-III and 3.2.3-IV. This is shown in Table 5.1.4-III.

Table 5.1.4-III. – Analysis of variance

Source of variation	Degrees of freedom	Sum of squares	Mean square	F-ratio	Probability
Preparations	3	4.475	1.492		
Regression	1	47.58	47.58	7126	0.000
Non-parallelism	3	0.0187	0.006	0.933	0.434
Non-linearity	12	0.0742	0.006	0.926	0.531
Treatments	19	52.152			
Residual error	40	0.267	0.0067		
Total	59	52.42			

A highly significant regression and a non-significant departure from parallelism and linearity confirm that the potencies can be safely calculated. The formulae in Section 3.2.5 give:

– for the common slope:

$$b = \frac{0.3 \times (6.109 + 6.264 + 6.431 + 6.384)}{\ln 2 \times 3 \times 4} = 0.90848$$

– the *ln*(potency ratio) for preparation *T* is:

$$M'_T = \frac{-5.586 - (-9.108)}{5 \times 0.90848} = 0.7752$$

$$C = \frac{47.58}{47.58 - 0.0067 \times 2.021^2} = 1.00057$$

$$V = \frac{47.58}{0.9085^2 \times 5 \times 3} = 3.8436$$

– and *ln*(confidence limits) for preparation *T* are:

$$\begin{aligned}
 &1.00057 \times 0.7752 \pm \\
 &\sqrt{0.00057 \times (1.00057 \times 0.7752^2 + 2 \times 3.8436)} \\
 &= 0.7756 \pm 0.0689
 \end{aligned}$$

By taking the antilogarithms a potency ratio of 2.171 is found with 95 per cent confidence limits from 2.027 to 2.327. All samples have an assigned potency of 20 µg protein/ml and so a potency of 43.4 µg protein/ml is found for test preparation *T* with 95 per cent confidence limits from 40.5–46.5 µg protein/ml.

The same procedure is followed to estimate the potency and confidence interval of the other test preparations. The results are listed in Table 5.1.4-IV.

Table 5.1.4-IV. – Final potency estimates and 95 per cent confidence intervals of the test vaccines (in µg protein/ml)

	Lower limit	Estimate	Upper limit
Vaccine <i>T</i>	40.5	43.4	46.5
Vaccine <i>U</i>	32.9	35.2	37.6
Vaccine <i>V</i>	36.8	39.4	42.2

5.1.5. TWIN CROSS-OVER DESIGN

Assay of insulin by subcutaneous injection in rabbits

The standard preparation was administered at 1 unit and 2 units per millilitre. Equivalent doses of the unknown preparation were used based on an assumed potency of 40 units per millilitre. The rabbits received subcutaneously 0.5 ml of the appropriate solutions according to the design in Table 5.1.5-I and responses obtained are shown in Table 5.1.5-II. The large variance illustrates the variation between rabbits and the need to employ a cross-over design.

Table 5.1.5-I. – Arrangements of treatments

	Group of rabbits			
	1	2	3	4
Day 1	S_1	S_2	T_1	T_2
Day 2	T_2	T_1	S_2	S_1

Table 5.1.5-II. – Response *y*: sum of blood glucose readings (mg/100 ml) at 1 hour and 2½ hours

	Group 1		Group 2		Group 3		Group 4	
	S_1	T_2	S_2	T_1	T_1	S_2	T_2	S_1
	112	104	65	72	105	91	118	144
	126	112	116	160	83	67	119	149
	62	58	73	72	125	67	42	51
	86	63	47	93	56	45	64	107
	52	53	88	113	92	84	93	117
	110	113	63	71	101	56	73	128
	116	91	50	65	66	55	39	87
	101	68	55	100	91	68	31	71
Mean	95.6	82.8	69.6	93.3	89.9	66.6	72.4	106.8

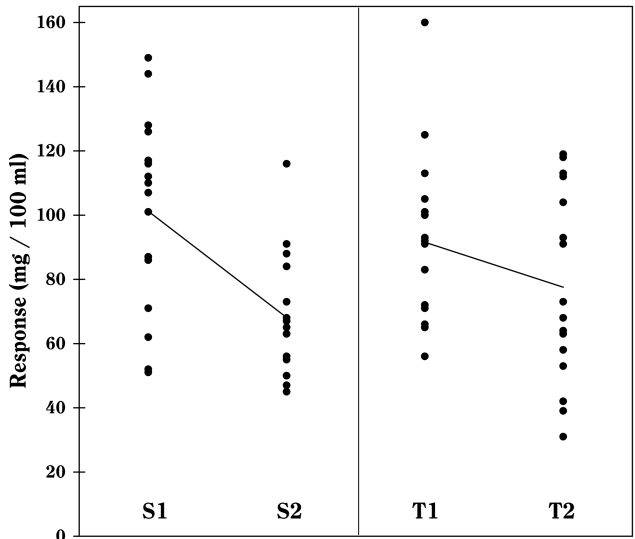


Figure 5.1.5-I.

The analysis of variance is more complicated for this assay than for the other designs given because the component of the sum of squares due to parallelism is not independent of the component due to rabbit differences. Testing of the parallelism of the regression lines involves a second error-mean-square term obtained by subtracting the parallelism component and 2 “interaction” components from the component due to rabbit differences.

3 “interaction” components are present in the analysis of variance due to replication within each group:
days × preparation; days × regression; days × parallelism.

These terms indicate the tendency for the components (preparations, regression and parallelism) to vary from day to day. The corresponding *F*-ratios thus provide checks on these aspects of assay validity. If the values of *F* obtained are significantly high, care should be exercised in interpreting the results of the assay and, if possible, the assay should be repeated.

The analysis of variance is constructed by applying the formulae given in Tables 3.2.3-I to 3.2.3-III separately for both days and for the pooled set of data. The formulae in Tables 3.2.3-I and 3.2.3-II give:

Day 1:	$P_s = 165.25$	$L_s = -13$
	$P_T = 162.25$	$L_T = -8.75$
	$H_p = \frac{8}{2} = 4$	$H_L = \frac{96}{6} = 16$
Day 2:	$P_s = 173.38$	$L_s = -20.06$
	$P_T = 176.00$	$L_T = -5.25$
	$H_p = \frac{8}{2} = 4$	$H_L = \frac{96}{6} = 16$
Pooled:	$P_s = 169.31$	$L_s = -16.53$
	$P_T = 169.13$	$L_T = -7.00$
	$H_p = \frac{16}{2} = 8$	$H_L = \frac{192}{6} = 32$

and with the formulae in Table 3.2.3-III this leads to:

Day 1	Day 2	Pooled
$SS_{\text{prep}} = 18.000$	$SS_{\text{prep}} = 13.781$	$SS_{\text{prep}} = 0.141$
$SS_{\text{reg}} = 3784.5$	$SS_{\text{reg}} = 5125.8$	$SS_{\text{reg}} = 8859.5$
$SS_{\text{par}} = 144.5$	$SS_{\text{par}} = 1755.3$	$SS_{\text{par}} = 1453.5$

The interaction terms are found as Day 1 + Day 2 - Pooled.

$$SS_{\text{days} \times \text{prep}} = 31.64$$

$$SS_{\text{days} \times \text{reg}} = 50.77$$

$$SS_{\text{days} \times \text{par}} = 446.27$$

In addition the sum of squares due to day-to-day variation is calculated as:

$$SS_{\text{days}} = \frac{1}{2}N(D_1^2 + D_2^2) - K = 478.52$$

and the sum of squares due to blocks (the variation between rabbits) as:

$$SS_{\text{block}} = 2 \sum B_i^2 - K = 39\,794.7$$

where B_i is the mean response per rabbit.
The analysis of variance can now be completed as shown in Table 5.1.5-III.

Table 5.1.5-III. – Analysis of variance

Source of variation	Degrees of freedom	Sum of squares	Mean square	<i>F</i> -ratio	Probability
Non-parallelism	1	1453.5	1453.5	1.064	0.311
Days × Prep.	1	31.6	31.6	0.023	0.880
Days × Regr.	1	50.8	50.8	0.037	0.849
Residual error between rabbits	28	38\,258.8	1366.4		
Rabbits	31	39\,794.7	1283.7		
Preparations	1	0.14	0.14	0.001	0.975
Regression	1	8859.5	8859.5	64.532	0.000
Days	1	478.5	478.5	3.485	0.072
Days × non-par.	1	446.3	446.3	3.251	0.082
Residual error within rabbits	28	3844.1	137.3		
Total	63	53\,423.2			

The analysis of variance confirms that the data fulfil the necessary conditions for a satisfactory assay: a highly significant regression, no significant departures from parallelism, and none of the three interaction components is significant.

The formulae in Section 3.2.5 give:

– for the common slope:

$$b = \frac{32 \times (-16.53 - 7)}{\ln 2 \times 16 \times 2} = -33.95$$

– the ln(potency ratio) is:

$$M'_T = \frac{169.13 - 169.31}{2 \times (-33.95)} = 0.00276$$

$$C = \frac{8859.5}{8859.5 - 137.3 \times 2.048^2} = 1.0695$$

$$V = \frac{8859.5}{(-33.95)^2 \times 2 \times 16} = 0.2402$$

– and ln(confidence limits) are:

$$1.0695 \times 0.00276 \pm \sqrt{0.0695 \times (1.0695 \times 0.00276^2 + 2 \times 0.2402)} = 0.00295 \pm 0.18279$$

By taking the antilogarithms a potency ratio of 1.003 with 95 per cent confidence limits from 0.835 to 1.204 is found. Multiplying by $A_T = 40$ yields a potency of 40.1 units per millilitre with 95 per cent confidence limits from 33.4-48.2 units per millilitre.

5.2. SLOPE-RATIO MODEL

5.2.1. A COMPLETELY RANDOMISED (0,3,3)-DESIGN

An assay of factor VIII

A laboratory carries out a chromogenic assay of factor VIII activity in concentrates. The laboratory has no experience with the type of assay but is trying to make it operational. 3 equivalent dilutions are prepared of both the standard and the test preparation. In addition a blank is prepared, although a linear dose-response relationship is not expected for low doses. 8 replicates of each dilution are prepared, which is more than would be done in a routine assay.

A graphical presentation of the data shows clearly that the dose-response relationship is indeed not linear at low doses. The responses to blanks will therefore not be used in the calculations (further assays are of course needed to justify this decision). The formulae in Tables 3.3.3.1-I and 3.3.3.1-II yield.

$$\begin{aligned} P_S &= 0.6524 & P_T &= 0.5651 \\ L_S &= 1.4693 & L_T &= 1.2656 \\ a_S &= 0.318 & a_T &= 0.318 \\ b_S &= 0.329 & b_T &= 0.271 \\ G_S &= 0.1554 & G_T &= 0.1156 \\ J_S &= 4.17 \cdot 10^{-8} & J_T &= 2.84 \cdot 10^{-6} \end{aligned}$$

and

$$H_1 = 0.09524 \quad a' = 0.05298 \quad K = 1.9764$$

and the analysis of variance is completed with the formulae in Tables 3.3.3.1-III and 3.3.3.1-IV.

A highly significant regression and no significant deviations from linearity and intersection indicate that the potency can be calculated.

Slope of standard:

$$b'_S = \frac{6 \times 1.469 - 36 \times 0.0530}{84} = 0.0822$$

Slope of test sample:

$$b'_T = \frac{6 \times 1.266 - 36 \times 0.0530}{84} = 0.0677$$

Formula 3.3.5.1-3 gives:

$$R = \frac{0.0677}{0.0822} = 0.823$$

$$C = \frac{0.0822^2}{0.0822^2 - 3.86 \cdot 10^{-6} \times 2.018^2 \times 0.0357} = 1.000083$$

$$K' = 0.000083 \times 0.75 = 0.000062$$

and the 95 per cent confidence limits are:

$$0.823 \pm \sqrt{0.000083 \times 1.678 + 0.000062 \times (-1.646)} \\ = 0.823 \pm 0.006$$

The potency ratio is thus estimated as 0.823 with 95 per cent confidence limits from 0.817 to 0.829.

Table 5.2.1-I. – Absorbances

	Blank	Standard S (in IU/ml)			Preparation T (in IU/ml)		
Conc.	B	S ₁ 0.01	S ₂ 0.02	S ₃ 0.03	T ₁ 0.01	T ₂ 0.02	T ₃ 0.03
	0.022	0.133	0.215	0.299	0.120	0.188	0.254
	0.024	0.133	0.215	0.299	0.119	0.188	0.253
	0.024	0.131	0.216	0.299	0.118	0.190	0.255
	0.026	0.136	0.218	0.297	0.120	0.190	0.258
	0.023	0.137	0.220	0.297	0.120	0.190	0.257
	0.022	0.136	0.220	0.305	0.121	0.191	0.257
	0.022	0.138	0.219	0.299	0.121	0.191	0.255
	0.023	0.137	0.218	0.302	0.121	0.190	0.254
Mean	0.0235	0.1351	0.2176	0.2996	0.1200	0.1898	0.2554

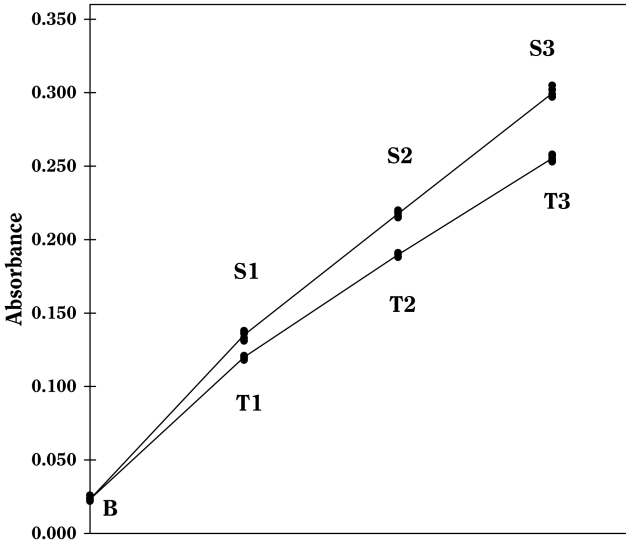


Figure 5.2.1-I.

Table 5.2.1-II. – Analysis of variance

Source of variation	Degrees of freedom	Sum of squares	Mean square	F-ratio	Probability
Regression	2	0.1917	0.0958	24 850	0.000
Intersection	1	$3 \cdot 10^{-9}$	$3 \cdot 10^{-9}$	$7 \cdot 10^{-4}$	0.978
Non-linearity	2	$2 \cdot 10^{-5}$	$1 \cdot 10^{-5}$	2.984	0.061
Treatments	5	0.1917			
Residual error	42	$1.62 \cdot 10^{-4}$	$3.86 \cdot 10^{-6}$		
Total	47	0.1919			

5.2.2. A COMPLETELY RANDOMISED (0,4,4,4)-DESIGN

An in-vitro assay of influenza vaccines

The haemagglutinin antigen (HA) content of 2 influenza vaccines is determined by single radial immunodiffusion. Both have a labelled potency of 15 µg HA per dose, which is equivalent with a content of 30 µg HA/ml. The standard has an assigned content of 39 µg HA/ml.

Standard and test vaccines are applied in 4 duplicate concentrations which are prepared on the basis of the assigned and the labelled contents. When the equilibrium

between the external and the internal reactant is established, the zone of the annulus precipitation area is measured. The results are shown in Table 5.2.2-I.

A graphical presentation of the data shows no unusual features. The formulae in Tables 3.3.3.1-I and 3.3.3.1-II yield

P_S	= 108.2	P_T	= 103.85	P_U	= 85.8
L_S	= 301.1	L_T	= 292.1	L_U	= 234.1
a_S	= 141.0	a_T	= 116.7	a_U	= 139.8
b_S	= 61.2	b_T	= 64.95	b_U	= 39.2
G_S	= 3114.3	G_T	= 2909.4	G_U	= 1917.3
J_S	= 0.223	J_T	= 2.227	J_U	= 0.083

and

H_I	= 0.0093	a'	= 11.04	K	= 14 785.8
-------	----------	------	---------	-----	------------

and the analysis of variance is completed with the formulae in Tables 3.3.3.1-III and 3.3.3.1-IV. This is shown in Table 5.2.2-II.

A highly significant regression and no significant deviations from linearity and intersection indicate that the potency can be calculated.

Slope of standard:

$$b'_S = \frac{6 \times 301.1 - 60 \times 11.04}{180} = 6.356$$

Slope of *T* is:

$$b'_T = \frac{6 \times 292.1 - 60 \times 11.04}{180} = 6.056$$

Slope of *U* is:

$$b'_U = \frac{6 \times 234.1 - 60 \times 11.04}{180} = 4.123$$

This leads to a potency ratio of $6.056/6.356 = 0.953$ for vaccine *T* and $4.123/6.356 = 0.649$ for vaccine *U*.

$$C = \frac{6.356^2}{6.356^2 - 1.068 \times 2.179^2 \times 0.0444} = 1.0056$$

$$K' = 0.0056 \times 0.625 = 0.0035$$

And the confidence limits are found with formula 3.3.5.1-4.

For vaccine *T*:

$$0.955 \pm \sqrt{0.0056 \times 1.913 + 0.0035 \times (-1.913)} = 0.955 \pm 0.063$$

For vaccine *U*:

$$0.649 \pm \sqrt{0.0056 \times 1.423 + 0.0035 \times (-1.301)} = 0.649 \pm 0.058$$

The HA content in µg/dose can be found by multiplying the potency ratios and confidence limits by the assumed content of 15 µg/dose. The results are given in Table 5.2.2-III.

Table 5.2.2-I. – Zone of precipitation area (mm²)

Conc. (µg/ml)	Standard S		Preparation T		Preparation U	
	I	II	I	II	I	II
7.5	18.0	18.0	15.1	16.8	15.4	15.7
15.0	22.8	24.5	23.1	24.2	20.2	18.6
22.5	30.4	30.4	28.9	27.4	24.2	23.1
30.0	35.7	36.6	34.4	37.8	27.4	27.0

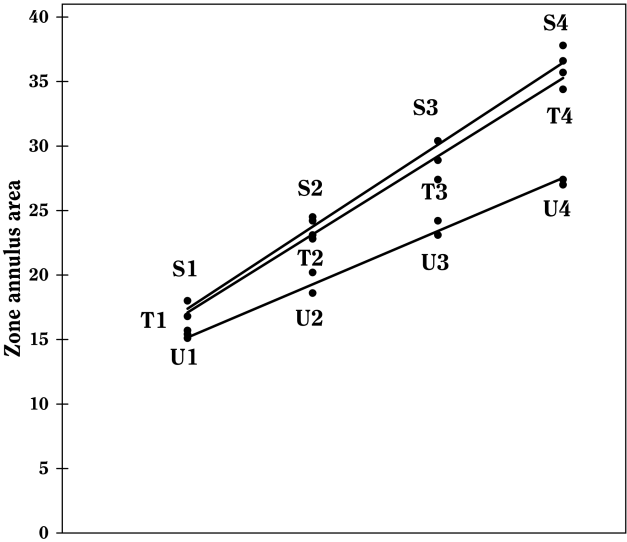


Figure 5.2.2-I.

Table 5.2.2-II. – Analysis of variance

Source of variation	Degrees of freedom	Sum of squares	Mean square	F-ratio	Probability
Regression	3	1087.7	362.6	339.5	0.000
Intersection	2	3.474	1.737	1.626	0.237
Non-linearity	6	5.066	0.844	0.791	0.594
Treatments	11	1096.2			
Residual error	12	12.815	1.068		
Total	23	1109.0			

Table 5.2.2-III. – Estimates of HA content (µg/dose)

	Lower limit	Estimate	Upper limit
Vaccin T	13.4	14.3	15.3
Vaccin U	8.9	9.7	10.6

5.3. QUANTAL RESPONSES

5.3.1. PROBIT ANALYSIS OF A TEST PREPARATION AGAINST A REFERENCE

An in-vivo assay of a diphtheria vaccine

A diphtheria vaccine (assumed potency 140 IU/vial) is assayed against a standard (assigned potency 132 IU/vial). On the basis of this information, equivalent doses are prepared and randomly administered to groups of guinea-pigs. After a given period, the animals are challenged with diphtheria toxin and the number of surviving animals recorded as shown in Table 5.3.1-I.

Table 5.3.1-I. – Raw data from a diphtheria assay in guinea-pigs

Standard (S) Assigned potency 132 IU/vial			Test preparation (T) Assumed potency 140 IU/vial		
dose (IU/ml)	chal- lenged	protect- ed	dose (I.U./ml)	chal- lenged	protect- ed
1.0	12	0	1.0	11	0
1.6	12	3	1.6	12	4
2.5	12	6	2.5	11	8
4.0	11	10	4.0	11	10

The observations are transferred to the first working table and the subsequent columns are computed as described in Section 4.2.1. Table 5.3.1-II shows the first cycle of this procedure.

The sums of the last 6 columns are then calculated per preparation and transferred to the second working table (see Table 5.3.1-III). The results in the other columns are found with formulae 4.2.1-4 to 4.2.1-10. This yields a common slope b of 1.655.

The values for Y in the first working table are now replaced by $a + bx$ and a second cycle is carried out (see Table 5.3.1-IV).

The cycle is repeated until the difference between 2 consecutive cycles has become small. The second working table should then appear as shown in Table 5.3.1-V.

Linearity is tested as described in Section 4.2.2. The χ^2 -value with 4 degrees of freedom is $0.851 + 1.070 = 1.921$ representing a p -value of 0.750 which is not significant.

Since there are no significant deviations from linearity, the test for parallelism can be carried out as described in the same section. The χ^2 -value with 1 degree of freedom is

$$(16.71 + 17.27) - \frac{14.15^2}{5.89} = 0.001 \text{ representing a } p\text{-value of } 0.974 \text{ which is not significant.}$$

The $\ln(\text{potency ratio})$ can now be estimated as described in Section 4.2.3.

$$M'_T = \frac{-1.721 - (-2.050)}{2.401} = 0.137$$

Further:

$$C = \frac{2.401^2 \times 5.893}{2.401^2 \times 5.893 - 1^2 \times 1.960^2} = 1.127$$

$$V = \frac{1}{18.37} + \frac{1}{17.96} = 0.110$$

So $\ln$ confidence limits are:

$$0.155 - 0.013 \pm \sqrt{0.127(0.649 + 1.127 \times 0.036^2)} = 0.142 \pm 0.288$$

The potency and confidence limits can now be found by taking the antilogarithms and multiplying these by the assumed potency of 140 IU/vial. This yields an estimate of 160.6 IU/vial with 95 per cent confidence limits from 121.0-215.2 IU/vial.

Table 5.3.1-II. – First working table in the first cycle

Vaccine	Dose	n	r	x	p	Y	Φ	Z	y	w	wx	wy	wx^2	wy^2	wxy
S	1.0	12	0	0.000	0.000	0	0.5	0.399	-1.253	7.64	0.00	-9.57	0.00	12.00	0.00
	1.6	12	3	0.470	0.250	0	0.5	0.399	-0.627	7.64	3.59	-4.79	1.69	3.00	-2.25
	2.5	12	6	0.916	0.500	0	0.5	0.399	0.000	7.64	7.00	0.00	6.41	0.00	0.00
	4.0	11	10	1.386	0.909	0	0.5	0.399	1.025	7.00	9.71	7.18	13.46	7.36	9.95
T	1.0	11	0	0.000	0.000	0	0.5	0.399	-1.253	7.00	0.00	-8.78	0.00	11.00	0.00
	1.6	12	4	0.470	0.333	0	0.5	0.399	-0.418	7.64	3.59	-3.19	1.69	1.33	-1.50
	2.5	11	8	0.916	0.727	0	0.5	0.399	0.570	7.00	6.42	3.99	5.88	2.27	3.66
	4.0	11	10	1.386	0.909	0	0.5	0.399	1.025	7.00	9.71	7.18	13.46	7.36	9.95

Table 5.3.1-III. – Second working table in the first cycle

Vaccine	Σw	Σwx	Σwy	Σwx^2	Σwy^2	Σwxy	S_{xx}	S_{xy}	S_{yy}	$\bar{x}$	$\bar{y}$	a
S	29.92	20.30	-7.18	21.56	22.36	7.70	7.79	12.58	20.64	0.68	-0.24	-1.36
T	28.65	19.72	-0.80	21.03	21.97	12.11	7.46	12.66	21.95	0.69	-0.03	-1.17

Table 5.3.1-IV. – First working table in the second cycle

Vaccine	Dose	n	r	x	p	Y	Φ	Z	y	w	wx	wy	wx^2	wy^2	wxy
S	1.0	12	0	0.000	0.000	-1.36	0.086	0.158	-1.911	3.77	0.00	-7.21	0.00	13.79	0.00
	1.6	12	3	0.470	0.250	-0.58	0.279	0.336	-0.672	6.74	3.17	-4.53	1.49	3.04	-2.13
	2.5	12	6	0.916	0.500	0.15	0.561	0.394	-0.001	7.57	6.94	-0.01	6.36	0.00	-0.01
	4.0	11	10	1.386	0.909	0.93	0.824	0.258	1.260	5.07	7.03	6.39	9.75	8.05	8.86
T	1.0	11	0	0.000	0.000	-1.17	0.122	0.202	-1.769	4.20	0.00	-7.43	0.00	13.14	0.00
	1.6	12	4	0.470	0.333	-0.39	0.349	0.370	-0.430	7.23	3.40	-3.11	1.60	1.34	-1.46
	2.5	11	8	0.916	0.727	0.35	0.637	0.375	0.591	6.70	6.14	3.96	5.62	2.34	3.63
	4.0	11	10	1.386	0.909	1.13	0.870	0.211	1.311	4.35	6.03	5.70	8.36	7.48	7.90

Table 5.3.1-V. – Second working table after sufficient cycles

Vaccine	Σw	Σwx	Σwy	Σwx^2	Σwy^2	Σwxy	S_{xx}	S_{xy}	S_{yy}	$\bar{x}$	$\bar{y}$	a
S	18.37	14.80	-2.14	14.85	17.81	5.28	2.93	7.00	17.56	0.81	-0.12	-2.05
T	17.96	12.64	-0.55	11.86	18.35	6.76	2.96	7.15	18.34	0.70	-0.03	-1.72

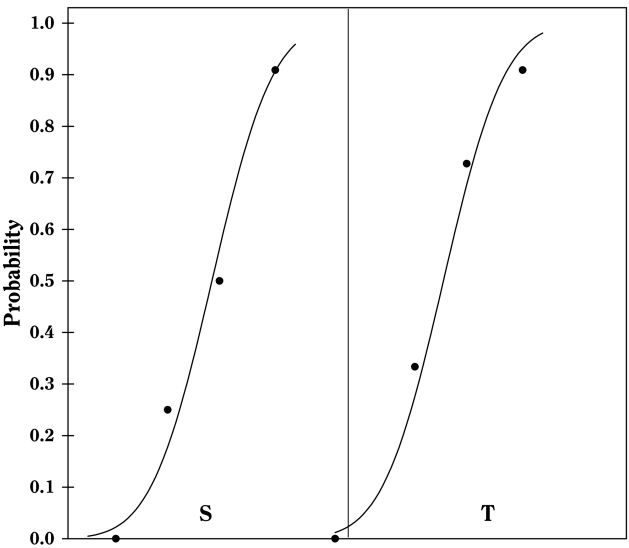


Figure 5.3.1-I.

5.3.2. LOGIT ANALYSIS AND OTHER TYPES OF ANALYSES OF A TEST PREPARATION AGAINST A REFERENCE

Results will be given for the situation where the logit method and other “classical” methods of this family are applied to the data in Section 5.3.1. This should be regarded as an exercise rather than an alternative to the probit method in this specific case. Another shape of the curve may be adopted only if this is supported by experimental or theoretical evidence.

Table 5.3.2-I. – Results by using alternative curves

	Logit	Gompit	Angle ^(*)
Φ	$\frac{1}{1 + e^{-Y}}$	$1 - e^{-e^Y}$	$\frac{1}{2} \sin Y + \frac{1}{2}$
Z	$\frac{e^{-Y}}{(1 + e^{-Y})^2}$	$e^{Y - e^Y}$	$\frac{1}{2} \cos Y$
slope b	4.101	2.590	1.717
χ^2 lin	2.15	3.56	1.50
χ^2 par	0.0066	0.168	0.0010
Potency	162.9	158.3	155.8
Lower limit	121.1	118.7	122.6
Upper limit	221.1	213.3	200.7

$$(*) \begin{cases} \text{If } Y < -\frac{1}{2}\pi \text{ then } \Phi = 0 \text{ and } Z = 0 \\ \text{If } Y > \frac{1}{2}\pi \text{ then } \Phi = 1 \text{ and } Z = 0 \end{cases}$$

5.3.3. THE ED_{50} DETERMINATION OF A SUBSTANCE USING THE PROBIT METHOD

An *in-vitro* assay of oral poliomyelitis vaccine

In an ED_{50} assay of oral poliomyelitis vaccine with 10 different dilutions in 8 replicates of 50 μ l on an ELISA-plate, results were obtained as shown in Table 5.3.3-I.

The observations are transferred to the first working table and the subsequent columns are computed as described in Section 4.2.1. Table 5.3.3-II shows the first cycle of this procedure.

Table 5.3.3-I. – Dilutions (10^x μ l of the undiluted vaccine)

-3.5	-4.0	-4.5	-5.0	-5.5	-6.0	-6.5	-7.0	-7.5	-8.0
+	+	+	+	-	-	-	-	-	-
+	+	+	+	-	-	-	-	-	-
+	+	-	-	-	-	-	-	-	-
+	+	+	+	-	-	-	-	-	-
+	+	+	-	-	-	-	-	-	-
+	+	+	+	+	-	-	-	-	-
+	+	+	+	+	-	+	-	-	-
+	+	+	+	-	+	-	-	-	-

The sums of the last 6 columns are calculated and transferred to the second working table (see Table 5.3.3-III). The results in the other columns are found with formulae 4.2.1-4 to 4.2.1-10. This yields a common slope b of -0.295 .

The values for Y in the first working table are now replaced by $a + bx$ and a second cycle is carried out. The cycle is repeated until the difference between 2 consecutive cycles has become small. The second working table should then appear as shown in Table 5.3.3-IV.

Linearity is tested as described in Section 4.2.2. The χ^2 -value with 8 degrees of freedom is 2.711 representing a p -value of 0.951 which is not significant.

The potency ratio can now be estimated as described in Section 4.5.

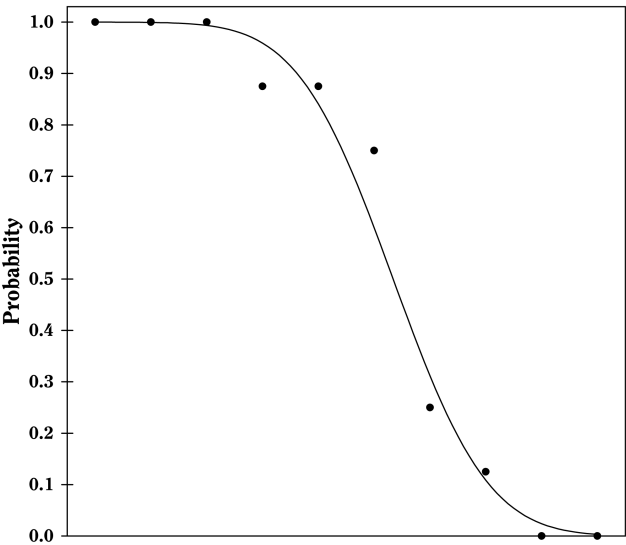


Figure 5.3.3-I.

$$M'_T = \frac{-(-7.931)}{-0.646} = -12.273$$

Further:

$$C = \frac{(-0.646)^2 \times 55.883}{(-0.646)^2 \times 55.883 - 1^2 \times 1.960^2} = 1.197$$

$$V = \frac{1}{19.39} = 0.052$$

So ln confidence limits are:

$$\begin{aligned} & -14.692 - (-2.420) \pm \sqrt{0.197 \times (2.882 + 1.197 \times 0.009^2)} \\ & = -12.272 \pm 0.754 \end{aligned}$$

This estimate is still expressed in terms of the ln(dilutions). In order to obtain estimates expressed in ln(ED_{50})/ml the values are transformed to $-M'_T + \ln\left(\frac{1000}{50}\right)$.

Since it has become common use to express the potency of this type of vaccine in terms of $\log_{10}(\text{ED}_{50})/\text{ml}$, the results have to be divided by $\ln(10)$. The potency is thus estimated as $6.63 \log_{10}(\text{ED}_{50})/\text{ml}$ with 95 per cent confidence limits from 6.30 to $6.96 \log_{10}(\text{ED}_{50})/\text{ml}$.

5.4. EXTENDED SIGMOID DOSE-RESPONSE CURVES

5.4.1. FOUR-PARAMETER LOGISTIC CURVE ANALYSIS

A serological assay of tetanus sera

As already stated in Section 3.4, this example is intended to illustrate a “possible” way to analyse the data presented, but not necessarily to reflect the “only” or the “most appropriate” way. Many other approaches can be found in the literature, but in most cases they should not yield dramatically different outcomes. A short discussion of alternative approaches and other statistical considerations is given in Section 7.5.

A guinea-pig antiserum is assayed against a standard serum (0.4 IU/ml) using an enzyme-linked immunosorbent assay technique (ELISA). 10 two-fold dilutions of each serum were applied on a 96-well ELISA plate. Each dilution was applied twice. The observed responses are listed in Table 5.4.1.-I.

For this example, it will be assumed that the laboratory has validated conditions 1 to 3 in Section 3.1.1 when the assay was being developed for routine use. In addition, the laboratory has validated that the upper limit and lower limit of the samples can be assumed to be equal.

No unusual features are discovered in a graphical representation. A least squares method of a suitable computer program is used to fit the parameters of the logistic function, assuming that the residual error terms are independent and identically distributed normal random variables. In this case, 3 parameters (α , β and δ) are needed to describe the common slope-factor and the common lower and upper asymptotes. 2 additional parameters (γ_s and γ_T) are needed to describe the horizontal location of the 2 curves.

The following estimates of the parameters are returned by the program:

$$\begin{aligned}\alpha &= 3.196 & \gamma_s &= -4.307 \\ \beta &= 1.125 & \gamma_T &= -4.684 \\ \delta &= 0.145\end{aligned}$$

In addition, the estimated residual variance (s^2) is returned as 0.001429 with 20 degrees of freedom (within-treatments variation).

In order to obtain confidence limits, and also to check for parallelism and linearity, the observed responses (u) are linearised and submitted to a weighted parallel-line analysis by the program. This procedure is very similar to that described in Section 4.2 for probit analysis with the following modifications:

$$\begin{aligned}Y &= \beta(x - \gamma) & y &= Y + \frac{\left(\frac{u - \delta}{\alpha - \delta}\right) - \Phi}{Z} \\ \Phi &= \frac{1}{1 + e^{-Y}} & w &= \frac{Z^2(\alpha - \delta)^2}{s^2} \\ Z &= \frac{e^{-Y}}{(1 + e^{-Y})^2}\end{aligned}$$

The resulting weighted analysis of variance of the transformed responses (y) using weights (w) is as follows:

There are no significant deviations from parallelism and linearity and thus the assay is satisfactory for potency calculations. If the condition of equal upper and lower asymptotes is not fulfilled, significant deviations from linearity and/or parallelism are likely to occur because the tests for linearity and parallelism reflect the goodness of fit of the complete four-parameter model. The residual error in the analysis of variance is always equal to 1 as a result of the transformation. However, a heterogeneity factor (analogous to that for the probit model) can be computed.

Table 5.3.3.-II. – First working table in the first cycle

Vaccine	Dose	<i>n</i>	<i>r</i>	<i>x</i>	<i>p</i>	<i>Y</i>	Φ	<i>Z</i>	<i>y</i>	<i>w</i>	<i>wx</i>	<i>wy</i>	<i>wx</i> ²	<i>wy</i> ²	<i>wxy</i>
<i>T</i>	10 ^{-3.5}	8	0	-8.06	0.000	0.00	0.5	0.399	-1.253	5.09	-41.04	-6.38	330.8	8.00	51.4
	10 ^{-4.0}	8	0	-9.21	0.000	0.00	0.5	0.399	-1.253	5.09	-46.91	-6.38	432.0	8.00	58.8
	10 ^{-4.5}	8	1	-10.36	0.125	0.00	0.5	0.399	-0.940	5.09	-52.77	-4.79	546.8	4.50	49.6
	10 ^{-5.0}	8	2	-11.51	0.250	0.00	0.5	0.399	-0.627	5.09	-58.63	-3.19	675.1	2.00	36.7
	10 ^{-5.5}	8	6	-12.66	0.750	0.00	0.5	0.399	0.627	5.09	-64.50	3.19	816.8	2.00	-40.4
	10 ^{-6.0}	8	7	-13.82	0.875	0.00	0.5	0.399	0.940	5.09	-70.36	4.79	972.1	4.50	-66.1
	10 ^{-6.5}	8	7	-14.97	0.875	0.00	0.5	0.399	0.940	5.09	-76.23	4.79	1140.8	4.50	-71.7
	10 ^{-7.0}	8	8	-16.12	1.000	0.00	0.5	0.399	1.253	5.09	-82.09	6.38	1323.1	8.00	-102.9
	10 ^{-7.5}	8	8	-17.27	1.000	0.00	0.5	0.399	1.253	5.09	-87.95	6.38	1518.9	8.00	-110.2
	10 ^{-8.0}	8	8	-18.42	1.000	0.00	0.5	0.399	1.253	5.09	-93.82	6.38	1728.2	8.00	-117.6

Table 5.3.3.-III. – Second working table in the first cycle

Vaccine	Σw	Σwx	Σwy	Σwx^2	Σwy^2	Σwxy	S_{xx}	S_{xy}	S_{yy}	$\bar{x}$	$\bar{y}$	<i>a</i>
<i>T</i>	50.93	-674.3	11.17	9484.6	57.50	-312.32	556.92	-164.43	55.05	-13.24	0.219	-3.690

Table 5.3.3.-IV. – Second working table after sufficient cycles

Vaccine	Σw	Σwx	Σwy	Σwx^2	Σwy^2	Σwxy	S_{xx}	S_{xy}	S_{yy}	$\bar{x}$	$\bar{y}$	<i>a</i>
<i>T</i>	19.39	-238.2	0.11	2981.1	26.05	-37.45	55.88	-36.11	26.05	-12.28	0.006	-7.931

The relative potency of the test preparation can be obtained as the antilogarithm of $\gamma_s - \gamma_r$. Multiplying by the assigned potency of the standard yields an estimate of $1.459 \times 0.4 = 0.584$ IU/ml. Formula 4.2.3.-2 gives 95 per cent confidence limits from 0.557-0.612 IU/ml.

Table 5.4.1.-I. – *Observed responses*

Standard S			Preparation to be examined T		
Dil.	Obs. 1	Obs. 2	Dil.	Obs. 1	Obs. 2
1/10	2.912	2.917	1/10	3.017	2.987
1/20	2.579	2.654	1/20	2.801	2.808
1/40	2.130	2.212	1/40	2.401	2.450
1/80	1.651	1.638	1/80	1.918	1.963
1/160	1.073	0.973	1/160	1.364	1.299
1/320	0.585	0.666	1/320	0.861	0.854
1/640	0.463	0.356	1/640	0.497	0.496
1/1280	0.266	0.234	1/1280	0.340	0.344
1/2560	0.228	0.197	1/2560	0.242	0.217
1/5120	0.176	0.215	1/5120	0.178	0.125

Table 5.4.1.-II – *Weighted analysis of variance*

Source of variation	Degrees of freedom	Chi-square	Probability
Preparations	1	0.529653	0.467
Regression	1	6599.51	0.000
Non-parallelism	1	0.0458738	0.830
Non-linearity	16	8.89337	0.918
Treatments	19	6608.98	0.000
Residual error	20	20.0000	
Total	39	6628.98	

6. COMBINATION OF ASSAY RESULTS

6.1. INTRODUCTION

Replication of independent assays and combination of their results is often needed to fulfil the requirements of the European Pharmacopoeia. The question then arises as to whether it is appropriate to combine the results of such assays and if so in what way.

2 assays may be regarded as mutually independent when the execution of either does not affect the probabilities of the possible outcomes of the other. This implies that the random errors in all essential factors influencing the result (for example, dilutions of the standard and of the preparation to be examined, the sensitivity of the biological indicator) in one assay must be independent of the corresponding random errors in the other one. Assays on successive days using the original and retained dilutions of the standard therefore are not independent assays.

There are several methods for combining the results of independent assays, the most theoretically acceptable being the most difficult to apply. 3 simple, approximate methods are described below; others may be used provided the necessary conditions are fulfilled.

Before potencies from assays based on the parallel-line or probit model are combined they must be expressed in logarithms; potencies derived from assays based on the

slope-ratio model are used as such. As the former models are more common than those based on the slope-ratio model, the symbol M denoting $\ln$ potency is used in the formulae in this section; by reading R (slope-ratio) for M , the analyst may use the same formulae for potencies derived from assays based on the slope-ratio model. All estimates of potency must be corrected for the potency assigned to each preparation to be examined before they are combined.

6.2. WEIGHTED COMBINATION OF ASSAY RESULTS

This method can be used provided the following conditions are fulfilled:

- 1) the potency estimates are derived from independent assays;
- 2) for each assay C is close to 1 (say less than 1.1);
- 3) the number of degrees of freedom of the individual residual errors is not smaller than 6, but preferably larger than 15;
- 4) the individual potency estimates form a homogeneous set (see Section 6.2.2).

When these conditions are not fulfilled this method cannot be applied. The method described in Section 6.3 may then be used to obtain the best estimate of the mean potency to be adopted in further assays as an assumed potency.

6.2.1. CALCULATION OF WEIGHTING COEFFICIENTS

It is assumed that the results of each of the n' assays have been analysed to give n' values of M with associated confidence limits. For each assay the logarithmic confidence interval L is obtained by subtracting the lower limit from the upper. A weight W for each value of M is calculated from equation 6.2.1.-1, where t has the same value as that used in the calculation of confidence limits.

$$W = \frac{4t^2}{L^2} \quad (6.2.1.-1)$$

6.2.2. HOMOGENEITY OF POTENCY ESTIMATES

By squaring the deviation of each value of M from the weighted mean, multiplying by the appropriate weight and summing over all assays, a statistic is obtained which is approximately distributed as χ^2 (see Table 8.3) and which may be used to test the homogeneity of a set of $\ln$ potency estimates:

$$\chi^2 \approx \sum_{n'} W (M - \bar{M})^2 \quad \text{where} \quad \bar{M} = \frac{\sum WM}{\sum W} \quad (6.2.2.-1)$$

If the calculated χ^2 is smaller than the tabulated value corresponding to $(n' - 1)$ degrees of freedom the potencies are homogeneous and the mean potency and limits obtained in Section 6.2.3 will be meaningful.

If the calculated value of this statistic is greater than the tabulated value, the potencies are heterogeneous. This means that the variation between individual estimates of M is greater than would have been predicted from the estimates of the confidence limits, i.e. that there is a significant variability between the assays. Under these circumstances condition 4 is not fulfilled and the equations in Section 6.2.3 are no longer applicable. Instead, the formulae in Section 6.2.4 may be used.

6.2.3. CALCULATION OF THE WEIGHTED MEAN AND CONFIDENCE LIMITS

The products WM are formed for each assay and their sum divided by the total weight for all assays to give the logarithm of the weighted mean potency.

$$\bar{M} = \frac{\sum WM}{\sum W} \quad (6.2.3.-1)$$

The standard error of the $\ln$ (mean potency) is taken to be the square root of the reciprocal of the total weight:

$$s_{\overline{M}} = \sqrt{\frac{1}{\sum W}} \quad (6.2.3-2)$$

and approximate confidence limits are obtained from the antilogarithms of the value given by

$$\overline{M} \pm t \times s_{\overline{M}} \quad (6.2.3-3)$$

where the number of degrees of freedom of t equals the sum of the number of degrees of freedom for the error mean squares in the individual assays.

6.2.4. WEIGHTED MEAN AND CONFIDENCE LIMITS BASED ON THE INTRA- AND INTER-ASSAY VARIATION

When results of several repeated assays are combined, the (χ^2 -value may be significant. The observed variation is then considered to have two components:

- the intra-assay variation $s_M^2 = 1/W$,
- the inter-assay variation $s_{\overline{M}}^2 = \frac{\sum (M - \overline{M})^2}{n'(n' - 1)}$

where $\overline{M}$ is the unweighted mean. The former varies from assay to assay whereas the latter is common to all M .

For each M a weighting coefficient is then calculated as:

$$W' = \frac{1}{s_M^2 + s_{\overline{M}}^2}$$

which replaces W in Section 6.2.3. where t is taken to be approximately 2.

6.3. UNWEIGHTED COMBINATION OF ASSAY RESULTS

To combine the n' estimates of M from n' assays in the simplest way, the mean is calculated and an estimate of its standard deviation is obtained by calculating:

$$s_{\overline{M}}^2 = \frac{\sum (M - \overline{M})^2}{n'(n' - 1)} \quad (6.3-1)$$

and the limits are:

$$\overline{M} \pm ts_{\overline{M}} \quad (6.3-2)$$

where t has $(n' - 1)$ degrees of freedom. The number n' of estimates of M is usually small, and hence the value of t is quite large.

6.4. EXAMPLE OF A WEIGHTED MEAN POTENCY WITH CONFIDENCE LIMITS

Table 6.4-I lists 6 independent potency estimates of the same preparation together with their 95 per cent confidence limits and the number of degrees of freedom of their error variances. Conditions 1, 2 and 3 in Section 6.2. are met. The $\ln$ potencies and the weights are calculated as described in Section 6.2.

Table 6.4-I. – Potency estimates and confidence intervals of 6 independent assays

Potency estimate (I.U./vial)	Lower limit (I.U./vial)	Upper limit (I.U./vial)	Degrees of freedom	$\ln$ potency M	Weight W
18 367	17 755	19 002	20	9.8183	3777.7
18 003	17 415	18 610	20	9.7983	3951.5
18 064	17 319	18 838	20	9.8017	2462.5
17 832	17 253	18 429	20	9.7887	4003.0
18 635	17 959	19 339	20	9.8328	3175.6
18 269	17 722	18 834	20	9.8130	4699.5

Homogeneity of potency estimates is assessed with formula 6.2.2-1 which gives a χ^2 of 4.42 with 5 degrees of freedom. This is not significant ($p = 0.49$) and thus all conditions are met.

A weighted mean potency is calculated with formula 6.2.3-1 which yields 9.8085.

Formula 6.2.3-2 gives a standard deviation of 0.00673 and approximate 95 per cent confidence limits of 9.7951 and 9.8218 are calculated with formula 6.2.3-3 where t has 120 degrees of freedom.

By taking the antilogarithms a potency of 18 187 IU/vial is found with 95 per cent confidence limits from 17 946-18 431 IU/vial.

7. BEYOND THIS ANNEX

It is impossible to give a comprehensive treatise of statistical methods in a pharmacopoeial text. However, the methods described in this annex should suffice for most pharmacopoeial purposes. This section tries to give a more abstract survey of alternative or more general methods that have been developed. The interested reader is encouraged to further explore the existing literature in this area. The use of more specialised statistical methods should, in any case, be left to qualified personnel.

7.1. GENERAL LINEAR MODELS

The methods given in this annex can be described in terms of general linear models (or generalised linear models to include the probit and logit methods). The principle is to define a linear structure matrix X (or design matrix) in which each row represents an observation and each column a linear effect (preparation, block, column, dose). For example: the Latin square design in example 5.1.2 would involve a matrix with 36 rows and 13 columns. 1 column for each of the preparations, 1 column for the doses, 5 columns for each block except the first, and 5 columns for each row except the first. All columns, except the one for doses, are filled with 0 or 1 depending on whether or not the observation relates to the effect. A vector Y is filled with the (transformed) observations. The effects are estimated with the formula $(X'X)^{-1}X'Y$ from which the potency estimate m can easily be derived as a ratio of relevant effects. Confidence intervals are calculated from Fieller's theorem:

$$m_l, m_u = \frac{\left[m - \frac{g v_{12}}{v_{22}} \pm \frac{t_s}{b} \sqrt{v_{11} - 2m v_{12} + m^2 v_{22} - g \left(v_{11} - \frac{v_{12}^2}{v_{22}} \right)} \right]}{(1 - g)}$$

where $g = \frac{t^2 s^2 v_{22}}{b^2}$

and v_{11} , v_{22} , v_{12} represent the variance multipliers for the numerator, the denominator and their covariance multiplier respectively. These are taken directly from $(X'X)^{-1}$ or indirectly by noting that:

$$\text{Var}(a_1 - a_2) = \text{Var}(a_1) + \text{Var}(a_2) - 2\text{Cov}(a_1, a_2)$$

$$\text{and } \text{Cov}(a_1 - a_2, b) = \text{Cov}(a_1, b) - \text{Cov}(a_2, b)$$

A full analysis of variance in which all components are partitioned is slightly more complicated as it involves a renewed definition of X with more columns to relax the assumptions of parallelism and linearity, after which the linear hypotheses can be tested. For assays depending upon quantal responses the linear effects (intercepts a_s , a_T etc. and the common slope b are found by maximising the sum over treatments of $n \ln \Phi(a_i + bx) + (n - r) \ln(1 - \Phi(a_i + bx))$ where x is the $\ln(\text{dose})$, Φ denotes the shape of the distribution and $i \in \{S, T, \dots\}$.

7.2. HETEROGENEITY OF VARIANCE

Heterogeneity of variance cannot always be solved by simply transforming the responses. A possible way to cope with this problem is to perform a weighted linear regression. In order to obtain an unbiased estimate, the weight of the observations is taken to be proportional to the reciprocal of the error variances. Since the true error variance is not always known, an iterative reweighted linear procedure may be followed. However, the calculation of the confidence interval involves new problems.

7.3. OUTLIERS AND ROBUST METHODS

The method of least squares described in this annex has the disadvantage of being very sensitive to outliers. A clear outlier may completely corrupt the calculations. This problem is often remedied by discarding the outlying result from the dataset. This policy can lead to arbitrary rejection of data and is not always without danger. It is not easy to give a general guideline on how to decide whether or not a specific observation is an outlier and it is for this reason that many robust methods have been developed. These methods are less sensitive to outliers because they give less weight to observations that are far away from the predicted value. New problems usually arise in computing confidence intervals or defining a satisfactory function to be minimised.

7.4. CORRELATED ERRORS

Absolute randomisation is not always feasible or very undesirable from a practical point of view. Thus, subsequent doses within a dilution series often exhibit correlated errors leading to confidence limits that are far too narrow. Some methods have been developed that take account of this autocorrelation effect.

7.5. EXTENDED NON-LINEAR DOSE-RESPONSE CURVES

Analysis of extended non-linear dose-response curves raises a number of statistical questions which require consideration, and for which professional advice is recommended. Some of these are indicated below.

- 1) An example using the four-parameter logistic function has been shown. However, models based on functions giving other sigmoid curves may also be used. Models incorporating additional asymmetry parameters have been suggested.
- 2) Heterogeneity of variance is common when responses cover a wide range. If the analysis ignores the heterogeneity, interpretation of results may not be correct and estimates may be biased. Use of the reciprocal of the error variances as weights is unlikely to be reliable with limited numbers of replicates. It may be appropriate to estimate a function which relates variance to mean response.
- 3) The statistical curve-fitting procedures may give different estimates depending on assumptions made about the homogeneity of the variance and on the range of responses used.
- 4) In principle, equality of upper and lower response limits for the different preparations included in an assay can be directly tested in each assay. However, interpretation of the results of these tests may not be straightforward. The tests for linearity and parallelism given by the simplified method of analysis (Example 5.4.1) indirectly incorporate tests for equality and accuracy of upper and lower limits.
- 5) Many assays include "controls" which are intended to identify the upper and/or lower response limits. However, these values may not be consistent with the statistically fitted upper and lower response limits based on the extended dose-response curve.
- 6) The simplified method of analysis given in Example 5.4.1 provides approximate confidence intervals. Other methods may also be used, for example intervals based on lack-of-fit of the completely specified model. For typical assay data, with responses covering the complete range for each preparation tested, all methods give similar results.

8. TABLES AND GENERATING PROCEDURES

The tables in this section list the critical values for the most frequently occurring numbers of degrees of freedom. If a critical value is not listed, reference should be made to more extensive tables. Many computer programs include statistical functions and their use is recommended instead of the tables in this section. Alternatively, the generating procedures given below each table can be used to compute the probability corresponding to a given statistic and number of degrees of freedom.

8.1. THE *F*-DISTRIBUTION

If an observed value is higher than the value in Table 8.1-I, it is considered to be significant (upper lines, $p = 0.05$) and highly significant (lower lines, $p = 0.01$). $df1$ is the number of degrees of freedom of the numerator and $df2$ is the number of degrees of freedom of the denominator.

Generating procedure. Let F be the F -ratio and $df1$ and $df2$ as described above. Let $\pi = 3.14159265358979...$ The procedure in Table 8.1-II will then generate the p -value.

8.2. THE *t*-DISTRIBUTION

If an observed value is higher than the value in Table 8.2-I, it is considered to be significant ($p = 0.05$) and highly significant ($p = 0.01$).

Generating procedures. The p -value for a given t with df degrees of freedom can be found with the procedures in Section 8.1 where $F = t^2$, $df1 = 1$ and $df2 = df$.

The t -value ($p = 0.05$) for a given number of degrees of freedom df can be found with the procedure in Table 8.2-II which should be accurate up to 6 decimal places.

Table 8.1-I – Critical values of the *F*-distribution

$df1 \rightarrow$	1	2	3	4	5	6	8	10	12	15	20	∞
$df2 \downarrow$												
10	4.965	4.103	3.708	3.478	3.326	3.217	3.072	2.978	2.913	2.845	2.774	2.538
	10.044	7.559	6.552	5.994	5.636	5.386	5.057	4.849	4.706	4.558	4.405	3.909
12	4.747	3.885	3.490	3.259	3.106	2.996	2.849	2.753	2.687	2.617	2.544	2.296
	9.330	6.927	5.953	5.412	5.064	4.821	4.499	4.296	4.155	4.010	3.858	3.361
15	4.543	3.682	3.287	3.056	2.901	2.790	2.641	2.544	2.475	2.403	2.328	2.066
	8.683	6.359	5.417	4.893	4.556	4.318	4.004	3.805	3.666	3.522	3.372	2.868
20	4.351	3.493	3.098	2.866	2.711	2.599	2.447	2.348	2.278	2.203	2.124	1.843
	8.096	5.849	4.938	4.431	4.103	3.871	3.564	3.368	3.231	3.088	2.938	2.421
25	4.242	3.385	2.991	2.759	2.603	2.490	2.337	2.236	2.165	2.089	2.007	1.711
	7.770	5.568	4.675	4.177	3.855	3.627	3.324	3.129	2.993	2.850	2.699	2.169
30	4.171	3.316	2.922	2.690	2.534	2.421	2.266	2.165	2.092	2.015	1.932	1.622
	7.562	5.390	4.510	4.018	3.699	3.473	3.173	2.979	2.843	2.700	2.549	2.006
50	4.034	3.183	2.790	2.557	2.400	2.286	2.130	2.026	1.952	1.871	1.784	1.438
	7.171	5.057	4.199	3.720	3.408	3.186	2.890	2.698	2.563	2.419	2.265	1.683
∞	3.841	2.996	2.605	2.372	2.214	2.099	1.938	1.831	1.752	1.666	1.571	1.000
	6.635	4.605	3.782	3.319	3.017	2.802	2.511	2.321	2.185	2.039	1.878	1.000

Table 8.1-II – Generating procedure for the *F*-distribution

If df1 is even	If df1 is odd and df2 is even	If df1 and df2 are odd
$x = df1 / (df1 + df2 / F)$	$x = df2 / (df2 + df1 * F)$	$x = \text{atn}(\text{sqr}(df1 * F / df2))$
$s = 1$	$s = 1$	$cs = \cos(x)$
$t = 1$	$t = 1$	$sn = \sin(x)$
for i=2 to (df1-2) step 2	for i=2 to (df2-2) step 2	$x = x / 2$
$t = t * x * (df2 + i - 2) / i$	$t = t * x * (df1 + i - 2) / i$	$s = 0$
$s = s + t$	$s = s + t$	$t = sn * cs / 2$
next i	next i	$v = 0$
$p = s * (1 - x) ^ (df2 / 2)$	$p = 1 - s * (1 - x) ^ (df1 / 2)$	$w = 1$
		for i=2 to (df2-1) step 2
		$s = s + t$
		$t = t * i / (i + 1) * cs * cs$
		next i
		for i=1 to (df1-2) step 2
		$v = v + w$
		$w = w * (df2 + i) / (i + 2) * sn * sn$
		next i
		$p = 1 + (t * df2 * v - x - s) / \pi * 4$

Table 8.2-I – Critical values of the *t*-distribution

df	<i>p</i> = 0.05	<i>p</i> = 0.01	df	<i>p</i> = 0.05	<i>p</i> = 0.01
1	12.706	63.656	22	2.074	2.819
2	4.303	9.925	24	2.064	2.797
3	3.182	5.841	26	2.056	2.779
4	2.776	4.604	28	2.048	2.763
5	2.571	4.032	30	2.042	2.750
6	2.447	3.707	35	2.030	2.724
7	2.365	3.499	40	2.021	2.704
8	2.306	3.355	45	2.014	2.690
9	2.262	3.250	50	2.009	2.678
10	2.228	3.169	60	2.000	2.660
12	2.179	3.055	70	1.994	2.648
14	2.145	2.977	80	1.990	2.639
16	2.120	2.921	90	1.987	2.632
18	2.101	2.878	100	1.984	2.626
20	2.086	2.845	∞	1.960	2.576

Table 8.2-II – Generating procedure for the *t*-distribution

$t = 1.959964 +$
 $2.37228 / df +$
 $2.82202 / df^2 +$
 $2.56449 / df^3 +$
 $1.51956 / df^4 +$
 $1.02579 / df^5 +$
 $0.44210 / df^7$

8.3. THE χ^2 -DISTRIBUTIONTable 8.3-I – Critical values of the χ^2 -distribution

df	<i>p</i> = 0.05	<i>p</i> = 0.01	df	<i>p</i> = 0.05	<i>p</i> = 0.01
1	3.841	6.635	11	19.675	24.725
2	5.991	9.210	12	21.026	26.217
3	7.815	11.345	13	22.362	27.688
4	9.488	13.277	14	23.685	29.141
5	11.070	15.086	15	24.996	30.578
6	12.592	16.812	16	26.296	32.000
7	14.067	18.475	20	31.410	37.566
8	15.507	20.090	25	37.652	44.314
9	16.919	21.666	30	43.773	50.892
10	18.307	23.209	40	55.758	63.691

If an observed value is higher than the value in Table 8.3-I, it is considered to be significant (*p* = 0.05) or highly significant (*p* = 0.01).

Generating procedure. Let χ^2 be the χ^2 -value and df as described above. The procedure in Table 8.3-II will then generate the p -value.

Table 8.3-II – *Generating procedure for the χ^2 -distribution*

If df is even	If df is odd
s=0	x=sqr(x2)
t=exp(-x2/2)	s=0
for i=2 to df step 2	t=x*exp(-x2/2)/sqr(pi/2)
s=s+t	for i=3 to df step 2
t=t*x2/i	s=s+t
next i	t=t*x2/i
p=1-s	next i
	p=1-s-2*phi(x)

In this procedure phi is the cumulative standard normal distribution function Φ (see Section 8.4).

8.4. THE Φ -DISTRIBUTION (THE CUMULATIVE STANDARD NORMAL DISTRIBUTION)

Table 8.4-I – *Values of the Φ -distribution*

x	Φ	x	Φ	x	Φ
0.00	0.500	1.00	0.841	2.00	0.977
0.05	0.520	1.05	0.853	2.05	0.980
0.10	0.540	1.10	0.864	2.10	0.982
0.15	0.560	1.15	0.875	2.15	0.984
0.20	0.579	1.20	0.885	2.20	0.986
0.25	0.599	1.25	0.894	2.25	0.988
0.30	0.618	1.30	0.903	2.30	0.989
0.35	0.637	1.35	0.911	2.35	0.991
0.40	0.655	1.40	0.919	2.40	0.992
0.45	0.674	1.45	0.926	2.45	0.993
0.50	0.691	1.50	0.933	2.50	0.994
0.55	0.709	1.55	0.939	2.55	0.995
0.60	0.726	1.60	0.945	2.60	0.995
0.65	0.742	1.65	0.951	2.65	0.996
0.70	0.758	1.70	0.955	2.70	0.997
0.75	0.773	1.75	0.960	2.75	0.997
0.80	0.788	1.80	0.964	2.80	0.997
0.85	0.802	1.85	0.968	2.85	0.998
0.90	0.816	1.90	0.971	2.90	0.998
0.95	0.829	1.95	0.974	2.95	0.998

The Φ -value for negative x is found from table 8.4-I as $1 - \Phi(-x)$.

Generating procedure: Let x be the x -value. The procedure in Table 8.4-II will generate the corresponding Φ -value if $0 \leq x \leq 8.15$. If x is greater than 8.15 the Φ -value can be set to 1. If x is negative, the formula given above can be used.

This procedure assumes that the computer can represent about 15 decimal places. If less digits or more digits can be represented, the procedure needs some trivial modifications.

Table 8.4-II – *Generating procedure for the Φ -distribution*

s=0
t=x
i=1
repeat
s=s+t
i=i+2
t=t*x*x/i
until t<1E-16
phi=0.5+s*exp(-x*x/2)/sqr(2*pi)

8.5. RANDOM PERMUTATIONS

Random permutations are needed in randomised block designs. The following algorithm shows how the built-in random generator of a computer can be used to create random permutations of N treatments.

Step 1. Write the N possible treatments down in a row.

Step 2. Obtain a random integer r such that $1 \leq r \leq N$.

Step 3. Exchange the r -th treatment with the N -th treatment in the row.

Step 4. Let $N = N - 1$ and repeat steps 2 to 4 until $N = 1$.

An example with 6 treatments will illustrate this algorithm.

1.	$N = 6$	S_1	S_2	S_3	T_1	T_2	T_3
2.	$r = 2$		→				←
3.		S_1	T_3	S_3	T_1	T_2	S_2
4.	$N = 5$						
2.	$r = 4$				→		←
3.		S_1	T_3	S_3	T_2	T_1	S_2
4.	$N = 4$						
2.	$r = 4$				↓		
3.		S_1	T_3	S_3	T_2	T_1	S_2
4.	$N = 3$						
2.	$r = 1$	→			←		
3.		S_3	T_3	S_1	T_2	T_1	S_2
4.	$N = 2$						
2.	$r = 1$	→		←			
3.		T_3	S_3	S_1	T_2	T_1	S_2
4.	$N = 1$						

8.6. LATIN SQUARES

The following example shows how 3 independent permutations can be used to obtain a Latin square.

1). Generate a random permutation of the N possible treatments (see Section 8.5):

T_3	S_3	S_1	T_2	T_1	S_2
-------	-------	-------	-------	-------	-------

2). A simple Latin square can now be constructed by “rotating” this permutation to the right. This can be done as follows. Write the permutation found in step 1 down on the first row. The second row consists of the same permutation, but with all treatments shifted to the right. The rightmost

treatment is put on the empty place at the left. This is repeated for all the rows until all the treatments appear once in each column:

T_3	S_3	S_1	T_2	T_1	S_2
S_2	T_3	S_3	S_1	T_2	T_1
T_1	S_2	T_3	S_3	S_1	T_2
T_2	T_1	S_2	T_3	S_3	S_1
S_1	T_2	T_1	S_2	T_3	S_3
S_3	S_1	T_2	T_1	S_2	T_3

3). Generate 2 independent random permutations of the figures 1 to N :

– one for the rows:

2	3	6	1	4	5
---	---	---	---	---	---

– and one for the columns:

3	4	6	2	5	1
---	---	---	---	---	---

4). The Latin square can now be found by sorting the rows and columns of the simple Latin square according to the 2 permutations for the rows and columns:

	3	4	6	2	5	1
2	T_3	S_3	S_1	T_2	T_1	S_2
3	S_2	T_3	S_3	S_1	T_2	T_1
6	T_1	S_2	T_3	S_3	S_1	T_2
1	T_2	T_1	S_2	T_3	S_3	S_1
4	S_1	T_2	T_1	S_2	T_3	S_3
5	S_3	S_1	T_2	T_1	S_2	T_3
	↓					
	1	2	3	4	5	6
1	S_1	T_3	T_2	T_1	S_3	S_2
2	S_2	T_2	T_3	S_3	T_1	S_1
3	T_1	S_1	S_2	T_3	T_2	S_3
4	S_3	S_2	S_1	T_2	T_3	T_1
5	T_3	T_1	S_3	S_1	S_2	T_2
6	T_2	S_3	T_1	S_2	S_1	T_3

9. GLOSSARY OF SYMBOLS

Symbol	Definition
a	Intersection of linear regression of responses on dose or $\ln(\text{dose})$
b	Slope of linear regression of responses on dose or on $\ln(\text{dose})$
d	Number of dose levels for each preparation (excluding the blank in slope-ratio assays)
e	Base of natural logarithms (= 2.71828182845905...)

Symbol	Definition
g	Statistic used in Fieller's theorem: $g = \frac{C - 1}{C}$
h	Number of preparations in an assay, including the standard preparation
m	Potency estimate obtained as a ratio of effects in general linear models
n	Number of replicates for each treatment
p	Probability of a given statistic being larger than the observed value. Also used as the ratio r/n in probit analysis
r	The number of responding units per treatment group in assays depending upon quantal responses
s	Estimate of standard deviation (= $\sqrt{s^2}$)
s^2	Estimate of residual variance given by error mean square in analysis of variance
t	Student's statistic (Table 8.2.)
u	Observed response in four-parameter analysis
v_{11}, v_{12}, v_{22}	(Co)variance multipliers for numerator and denominator of ratio m in Fieller's theorem
w	Weighting coefficient
x	The $\ln(\text{dose})$
y	Individual response or transformed response
A	Assumed potencies of test preparations when making up doses
B	Mean response to blanks in slope-ratio assays
C	Statistic used in the calculation of confidence intervals: $C = \frac{1}{1 - g}$
$C_p \dots, C_n$	Mean response to each column of a Latin square design
D_1, D_2	Mean response on time 1 or time 2 in the twin cross-over design
F	Ratio of 2 independent estimates of variance following an F -distribution (Table 8.1.)
$G_S, G_P \dots$	Treatment values used in the analysis of variance for slope-ratio assays
H_P, H_L	Multipliers used in the analysis of variance for parallel-line assays
H_P, H_I	Multipliers used in the analysis of variance for slope-ratio assays

Symbol	Definition	Symbol	Definition
I	In parallel-line assays, the ln of the ratio between adjacent doses. In slope-ratio assays, the interval between adjacent doses	Y	Vector representing the (transformed) responses in general linear models
$J_S, J_P, \dots$	Linearity values used in the analysis of variance for slope-ratio assays	Z	The first derivative of Φ
K	Correction term used in the calculation of sums of squares in the analysis of variance	α	Upper asymptote of the ln(dose)-response curve in four-parameter analysis
L	Width of confidence interval in logarithms	β	Slope-factor of the ln(dose)-response curve in four-parameter analysis
$L_S, L_P, \dots$	Linear contrasts of standard and test preparations	γ	The ln(dose) giving 50 per cent response in the four-parameter analysis
M'	ln potency ratio of a given test preparation	δ	Lower asymptote of the ln(dose)-response curve in four-parameter analysis
N	Total number of treatments in the assay (= dh)	π	3.141592653589793238...
$P_S, P_P, \dots$	Sum of standard and test preparations	Φ	Cumulative standard normal distribution function (Table 8.4.)
R	Estimated potency of a given test preparation	χ^2	Chi-square statistic (Table 8.3.)
R'	Potency ratio of a given test preparation	10. LITERATURE <i>This section lists some recommended literature for further study.</i> Finney, D.J. (1971). <i>Probit Analysis</i> , 3 rd Ed. Cambridge University Press, Cambridge. Nelder, J.A. & Wedderburn, R.W.M. (1972). Generalized linear models, <i>Journal of the Royal Statistical Society, Series A</i> 135, 370-384. DeLean, A., Munson, P.J., and Rodbard, D. (1978). <i>Simultaneous analysis of families of sigmoidal curves: Application to bioassay, radioligand assay, and physiological dose-response curves</i> , Am. J. Physiol. 235(2): E97-E102. Finney, D.J. (1978). <i>Statistical Method in Biological Assay</i> , 3 rd Ed. Griffin, London. Sokal, R.R. & Rohlf, F.R. (1981). <i>Biometry: Principles and Practice of Statistics in Biological Research</i> , 2 nd Ed. W.H. Freeman & CO, New York. Peace, K.E. (1988). <i>Biopharmaceutical Statistics for Drug Development</i> , Marcel Dekker Inc., New York/Basel. Bowerman, B.L. & O'Connell, R.T. (1990). <i>Linear Statistical Models an Applied Approach</i> , 2 nd Ed. PWS-KENT Publishing Company, Boston. Govindarajulu, Z. (2001). <i>Statistical Techniques in Bioassay</i> , 2nd revised and enlarged edition, Karger, New York.	
$R_P, \dots, R_n$	Mean response in each of rows 1 to n of a Latin square design, or in each block of a randomised block design		
S	Standard preparation		
$S_P, \dots, S_d$	Mean response to the lowest dose 1 up to the highest dose d of the standard preparation S		
SS	Sum of squares due to a given source of variation		
$T, U, V, \dots$	Test preparations		
$T_P, \dots, T_d$	Mean response to the lowest dose 1 up to the highest dose d of test preparation T		
V	Variance coefficient in the calculation of confidence limits		
W	Weighting factor in combination of assay results		
X	Linear structure or design matrix used in general linear models		

01/2005:50400

5.4. RESIDUAL SOLVENTS

LIMITING RESIDUAL SOLVENT LEVELS IN ACTIVE SUBSTANCES, EXCIPIENTS AND MEDICINAL PRODUCTS

The International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) has adopted Impurities Guidelines for Residual Solvents which prescribes limits for the content of solvents which may remain in active substances, excipients and medicinal products after processing. This guideline, the text of which is reproduced below, excludes existing marketed products. The European Pharmacopoeia is, however, applying the same principles enshrined in the guideline to existing active substances, excipients and medicinal products whether or not they are the subject of a monograph of the Pharmacopoeia. All substances and products are to be tested for the content of solvents likely to be present in a substance or product.

Where the limits to be applied comply with those given below, tests for residual solvents are not generally mentioned in specific monographs since the solvents employed may vary from one manufacturer to another and the requirements of this general chapter are applied via the general monograph on *Substances for Pharmaceutical Use (2034)*. The competent authority is to be informed of the solvents employed during the production process. This information is also given in the dossier submitted for a certificate of suitability of the monographs of the European Pharmacopoeia and is mentioned on the certificate.

Where only Class 3 solvents are used, a test for loss on drying may be applied or a specific determination of the solvent may be made. If for a Class 3 solvent a justified and authorised limit higher than 0.5 per cent is applied, a specific determination of the solvent is required.

When Class 1 residual solvents or Class 2 residual solvents (or Class 3 residual solvents which exceed the 0.5 per cent) are used, the methodology described in the general method (2.4.24) is to be applied wherever possible. Otherwise an appropriate validated method is to be employed.

When a quantitative determination of a residual solvent is carried out, the result is taken into account for the calculation of the content of the substance except where a test for drying is carried out.

IMPURITIES: GUIDELINES FOR RESIDUAL SOLVENTS (CPMP/ICH/283/95)

1. INTRODUCTION

2. SCOPE OF THE GUIDELINE

3. GENERAL PRINCIPLES

3.1. CLASSIFICATION OF RESIDUAL SOLVENTS BY RISK ASSESSMENT

3.2. METHODS FOR ESTABLISHING EXPOSURE LIMITS

3.3. OPTIONS FOR DESCRIBING LIMITS OF CLASS 2 SOLVENTS

3.4. ANALYTICAL PROCEDURES

3.5. REPORTING LEVELS OF RESIDUAL SOLVENTS

4. LIMITS OF RESIDUAL SOLVENTS

4.1. SOLVENTS TO BE AVOIDED

4.2. SOLVENTS TO BE LIMITED

4.3. SOLVENTS WITH LOW TOXIC POTENTIAL

4.4. SOLVENTS FOR WHICH NO ADEQUATE TOXICOLOGICAL DATA WAS FOUND

GLOSSARY

APPENDIX 1. LIST OF SOLVENTS INCLUDED IN THE GUIDELINE

APPENDIX 2. ADDITIONAL BACKGROUND

A2.1: ENVIRONMENTAL REGULATION OF ORGANIC VOLATILE SOLVENTS

A2.2: RESIDUAL SOLVENTS IN PHARMACEUTICALS

APPENDIX 3. METHODS FOR ESTABLISHING EXPOSURE LIMITS

1. INTRODUCTION

The objective of this guideline is to recommend acceptable amounts of residual solvents in pharmaceuticals for the safety of the patient. The guideline recommends the use of less toxic solvents and describes levels considered to be toxicologically acceptable for some residual solvents.

Residual solvents in pharmaceuticals are defined here as organic volatile chemicals that are used or produced in the manufacture of active substances or excipients, or in the preparation of medicinal products. The solvents are not completely removed by practical manufacturing techniques. Appropriate selection of the solvent for the synthesis of active substance may enhance the yield, or determine characteristics such as crystal form, purity, and solubility. Therefore, the solvent may sometimes be a critical parameter in the synthetic process. This guideline does not address solvents deliberately used as excipients nor does it address solvates. However, the content of solvents in such products should be evaluated and justified.

Since there is no therapeutic benefit from residual solvents, all residual solvents should be removed to the extent possible to meet product specifications, good manufacturing practices, or other quality-based requirements. Medicinal products should contain no higher levels of residual solvents than can be supported by safety data. Some solvents that are known to cause unacceptable toxicities (Class 1, Table 1) should be avoided in the production of active substances, excipients, or medicinal products unless their use can be strongly justified in a risk-benefit assessment. Some solvents associated with less severe toxicity (Class 2, Table 2) should be limited in order to protect patients from potential adverse effects. Ideally, less toxic solvents (Class 3, Table 3) should be used where practical. The complete list of solvents included in this guideline is given in Appendix 1.

The lists are not exhaustive and other solvents can be used and later added to the lists. Recommended limits of Class 1 and 2 solvents or classification of solvents may change as new safety data becomes available. Supporting safety data in a marketing application for a new medicinal product containing a new solvent may be based on concepts in this guideline or the concept of qualification of impurities as expressed in the guideline for active substances (Q3A,

Impurities in New Active Substances) or medicinal products (Q3B, Impurities in New Medicinal Products), or all three guidelines.

2. SCOPE OF THE GUIDELINE

Residual solvents in active substances, excipients, and in medicinal products are within the scope of this guideline. Therefore, testing should be performed for residual solvents when production or purification processes are known to result in the presence of such solvents. It is only necessary to test for solvents that are used or produced in the manufacture or purification of active substances, excipients, or medicinal product. Although manufacturers may choose to test the medicinal product, a cumulative method may be used to calculate the residual solvent levels in the medicinal product from the levels in the ingredients used to produce the medicinal product. If the calculation results in a level equal to or below that recommended in this guideline, no testing of the medicinal product for residual solvents need be considered. If however, the calculated level is above the recommended level, the medicinal product should be tested to ascertain whether the formulation process has reduced the relevant solvent level to within the acceptable amount. Medicinal product should also be tested if a solvent is used during its manufacture.

This guideline does not apply to potential new active substances, excipients, or medicinal products used during the clinical research stages of development, nor does it apply to existing marketed medicinal products.

The guideline applies to all dosage forms and routes of administration. Higher levels of residual solvents may be acceptable in certain cases such as short term (30 days or less) or topical application. Justification for these levels should be made on a case by case basis.

See Appendix 2 for additional background information related to residual solvents.

3. GENERAL PRINCIPLES

3.1. CLASSIFICATION OF RESIDUAL SOLVENTS BY RISK ASSESSMENT

The term “tolerable daily intake” (TDI) is used by the International Program on Chemical Safety (IPCS) to describe exposure limits of toxic chemicals and “acceptable daily intake” (ADI) is used by the World Health Organisation (WHO) and other national and international health authorities and institutes. The new term “permitted daily exposure” (PDE) is defined in the present guideline as a pharmaceutically acceptable intake of residual solvents to avoid confusion of differing values for ADI's of the same substance.

Residual solvents assessed in this guideline are listed in Appendix 1 by common names and structures. They were evaluated for their possible risk to human health and placed into one of three classes as follows:

Class 1 solvents: Solvents to be avoided

Known human carcinogens, strongly suspected human carcinogens, and environmental hazards.

Class 2 solvents: Solvents to be limited

Non-genotoxic animal carcinogens or possible causative agents of other irreversible toxicity such as neurotoxicity or teratogenicity.

Solvents suspected of other significant but reversible toxicities.

Class 3 solvents: Solvents with low toxic potential

Solvents with low toxic potential to man; no health-based exposure limit is needed. Class 3 solvents have PDEs of 50 mg or more per day.

3.2. METHODS FOR ESTABLISHING EXPOSURE LIMITS

The method used to establish permitted daily exposures for residual solvents is presented in Appendix 3. Summaries of the toxicity data that were used to establish limits are published in *Pharmeuropa*, Vol. 9, No. 1, Supplement April 1997.

3.3. OPTIONS FOR DESCRIBING LIMITS OF CLASS 2 SOLVENTS

Two options are available when setting limits for Class 2 solvents.

Option 1: The concentration limits in ppm stated in Table 2 can be used. They were calculated using equation (1) below by assuming a product mass of 10 g administered daily.

$$\text{Concentration (ppm)} = \frac{1000 \times \text{PDE}}{\text{dose}} \quad (1)$$

Here, PDE is given in terms of mg/day and dose is given in g/day.

These limits are considered acceptable for all substances, excipients, or products. Therefore this option may be applied if the daily dose is not known or fixed. If all excipients and active substances in a formulation meet the limits given in Option 1, then these components may be used in any proportion. No further calculation is necessary provided the daily dose does not exceed 10 g. Products that are administered in doses greater than 10 g per day should be considered under Option 2.

Option 2: It is not considered necessary for each component of the medicinal product to comply with the limits given in Option 1. The PDE in terms of mg/day as stated in Table 2 can be used with the known maximum daily dose and equation (1) above to determine the concentration of residual solvent allowed in a medicinal product. Such limits are considered acceptable provided that it has been demonstrated that the residual solvent has been reduced to the practical minimum. The limits should be realistic in relation to analytical precision, manufacturing capability, reasonable variation in the manufacturing process, and the limits should reflect contemporary manufacturing standards.

Option 2 may be applied by adding the amounts of a residual solvent present in each of the components of the medicinal product. The sum of the amounts of solvent per day should be less than that given by the PDE.

Consider an example of the use of Option 1 and Option 2 applied to acetonitrile in a medicinal product. The permitted daily exposure to acetonitrile is 4.1 mg per day; thus, the Option 1 limit is 410 ppm. The maximum administered daily mass of a medicinal product is 5.0 g, and the medicinal product contains two excipients. The composition of the medicinal product and the calculated maximum content of residual acetonitrile are given in the following table.

Component	Amount in formulation	Acetonitrile content	Daily exposure
Active substance	0.3 g	800 ppm	0.24 mg
Excipient 1	0.9 g	400 ppm	0.36 mg
Excipient 2	3.8 g	800 ppm	3.04 mg
Medicinal product	5.0 g	728 ppm	3.64 mg

Excipient 1 meets the Option 1 limit, but the drug substance, excipient 2, and medicinal product do not meet the Option 1 limit. Nevertheless, the product meets the Option 2 limit of 4.1 mg per day and thus conforms to the recommendations in this guideline.

Consider another example using acetonitrile as residual solvent. The maximum administered daily mass of a medicinal product is 5.0 g, and the medicinal product contains two excipients. The composition of the medicinal product and the calculated maximum content of residual acetonitrile is given in the following table.

Component	Amount in formulation	Acetonitrile content	Daily exposure
Active substance	0.3 g	800 ppm	0.24 mg
Excipient 1	0.9 g	2000 ppm	1.80 mg
Excipient 2	3.8 g	800 ppm	3.04 mg
Medicinal product	5.0 g	1016 ppm	5.08 mg

In this example, the product meets neither the Option 1 nor the Option 2 limit according to this summation. The manufacturer could test the medicinal product to determine if the formulation process reduced the level of acetonitrile. If the level of acetonitrile was not reduced during formulation to the allowed limit, then the manufacturer of the medicinal product should take other steps to reduce the amount of acetonitrile in the medicinal product. If all of these steps fail to reduce the level of residual solvent, in exceptional cases the manufacturer could provide a summary of efforts made to reduce the solvent level to meet the guideline value, and provide a risk-benefit analysis to support allowing the product to be utilised containing residual solvent at a higher level.

3.4. ANALYTICAL PROCEDURES

Residual solvents are typically determined using chromatographic techniques such as gas chromatography. Any harmonised procedures for determining levels of residual solvents as described in the pharmacopoeias should be used, if feasible. Otherwise, manufacturers would be free to select the most appropriate validated analytical procedure for a particular application. If only Class 3 solvents are present, a non-specific method such as loss on drying may be used.

Validation of methods for residual solvents should conform to ICH guidelines “Text on Validation of Analytical Procedures” and “Extension of the ICH Text on Validation of Analytical Procedures”.

3.5. REPORTING LEVELS OF RESIDUAL SOLVENTS

Manufacturers of pharmaceutical products need certain information about the content of residual solvents in excipients or active substances in order to meet the criteria of this guideline. The following statements are given as acceptable examples of the information that could be provided from a supplier of excipients or active substances to a pharmaceutical manufacturer. The supplier might choose one of the following as appropriate:

- Only Class 3 solvents are likely to be present. Loss on drying is less than 0.5 per cent.
- Only Class 2 solvents X, Y, ... are likely to be present. All are below the Option 1 limit
(Here the supplier would name the Class 2 solvents represented by X, Y, ...)
- Only Class 2 solvents X, Y, ... and Class 3 solvents are likely to be present. Residual Class 2 solvents are below the Option 1 limit and residual Class 3 solvents are below 0.5 per cent.

If Class 1 solvents are likely to be present, they should be identified and quantified. “Likely to be present” refers to the solvent used in the final manufacturing step and to solvents that are used in earlier manufacturing steps and not removed consistently by a validated process.

If solvents of Class 2 or Class 3 are present at greater than their Option 1 limits or 0.5 per cent, respectively, they should be identified and quantified.

4. LIMITS OF RESIDUAL SOLVENTS

4.1. SOLVENTS TO BE AVOIDED

Solvents in Class 1 should not be employed in the manufacture of active substances, excipients, and medicinal products because of their unacceptable toxicity or their deleterious environmental effect. However, if their use is unavoidable in order to produce a medicinal product with a significant therapeutic advance, then their levels should be restricted as shown in Table 1, unless otherwise justified. 1,1,1-Trichloroethane is included in Table 1 because it is an environmental hazard. The stated limit of 1500 ppm is based on a review of the safety data.

Table 1. – *Class 1 solvents in pharmaceutical products (solvents that should be avoided)*

Solvent	Concentration limit (ppm)	Concern
Benzene	2	Carcinogen
Carbon tetrachloride	4	Toxic and environmental hazard
1,2-Dichloroethane	5	Toxic
1,1-Dichloroethene	8	Toxic
1,1,1-Trichloroethane	1500	Environmental hazard

4.2. SOLVENTS TO BE LIMITED

Solvents in Table 2 should be limited in pharmaceutical products because of their inherent toxicity. PDEs are given to the nearest 0.1 mg/day, and concentrations are given to the nearest 10 ppm. The stated values do not reflect the necessary analytical precision of determination. Precision should be determined as part of the validation of the method.

Table 2. – *Class 2 solvents in pharmaceutical products*

Solvent	PDE (mg/day)	Concentration limit (ppm)
Acetonitrile	4.1	410
Chlorobenzene	3.6	360
Chloroform	0.6	60
Cyclohexane	38.8	3880
1,2-Dichloroethene	18.7	1870
Dichloromethane	6.0	600
1,2-Dimethoxyethane	1.0	100
<i>N,N</i> -Dimethylacetamide	10.9	1090
<i>N,N</i> -Dimethylformamide	8.8	880
1,4-Dioxane	3.8	380
2-Ethoxyethanol	1.6	160
Ethyleneglycol	6.2	620
Formamide	2.2	220
Hexane	2.9	290
Methanol	30.0	3000
2-Methoxyethanol	0.5	50
Methylbutylketone	0.5	50
Methylcyclohexane	11.8	1180
<i>N</i> -Methylpyrrolidone	5.3	530
Nitromethane	0.5	50
Pyridine	2.0	200

Solvent	PDE (mg/day)	Concentration limit (ppm)
Sulfolane	1.6	160
Tetrahydrofuran	7.2	720
Tetralin	1.0	100
Toluene	8.9	890
1,1,2-Trichloroethene	0.8	80
Xylene*	21.7	2170

*usually 60 per cent *m*-xylene, 14 per cent *p*-xylene, 9 per cent *o*-xylene with 17 per cent ethyl benzene

4.3. SOLVENTS WITH LOW TOXIC POTENTIAL

Solvents in Class 3 (shown in Table 3) may be regarded as less toxic and of lower risk to human health. Class 3 includes no solvent known as a human health hazard at levels normally accepted in pharmaceuticals. However, there are no long-term toxicity or carcinogenicity studies for many of the solvents in Class 3. Available data indicate that they are less toxic in acute or short-term studies and negative in genotoxicity studies. It is considered that amounts of these residual solvents of 50 mg per day or less (corresponding to 5000 ppm or 0.5 per cent under Option I) would be acceptable without justification. Higher amounts may also be acceptable provided they are realistic in relation to manufacturing capability and good manufacturing practice.

Table 3. – *Class 3 solvents which should be limited by GMP or other quality-based requirements*

Acetic acid	Heptane
Acetone	Isobutyl acetate
Anisole	Isopropyl acetate
1-Butanol	Methyl acetate
2-Butanol	3-Methyl-1-butanol
Butyl acetate	Methylethylketone
<i>tert</i> -Butylmethyl ether	Methylisobutylketone
Cumene	2-Methyl-1-propanol
Dimethyl sulphoxide	Pentane
Ethanol	1-Pentanol
Ethyl acetate	1-Propanol
Ethyl ether	2-Propanol
Ethyl formate	Propyl acetate
Formic acid	

4.4. SOLVENTS FOR WHICH NO ADEQUATE TOXICOLOGICAL DATA WAS FOUND

The following solvents (Table 4) may also be of interest to manufacturers of excipients, active substances, or medicinal products. However, no adequate toxicological data on which to base a PDE was found. Manufacturers should supply justification for residual levels of these solvents in pharmaceutical products.

Table 4. – *Solvents for which no adequate toxicological data was found*

1,1-Diethoxypropane	Methylisopropylketone
1,1-Dimethoxymethane	Methyltetrahydrofuran
2,2-Dimethoxypropane	Petroleum ether
Isooctane	Trichloroacetic acid
Isopropyl ether	Trifluoroacetic acid

GLOSSARY

Genotoxic carcinogens: Carcinogens which produce cancer by affecting genes or chromosomes.

LOEL: Abbreviation for *lowest-observed effect level*.

Lowest-observed effect level: The lowest dose of substance in a study or group of studies that produces biologically significant increases in frequency or severity of any effects in the exposed humans or animals.

Modifying factor: A factor determined by professional judgement of a toxicologist and applied to bioassay data to relate that data safely to humans.

Neurotoxicity: The ability of a substance to cause adverse effects on the nervous system.

NOEL: Abbreviation for *no-observed-effect level*.

No-observed-effect level: The highest dose of substance at which there are no biologically significant increases in frequency or severity of any effects in the exposed humans or animals.

PDE: Abbreviation for *permitted daily exposure*.

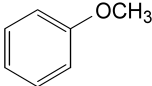
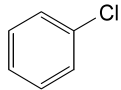
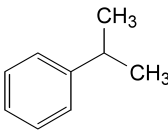
Permitted daily exposure: The maximum acceptable intake per day of residual solvent in pharmaceutical products.

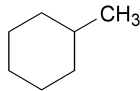
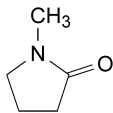
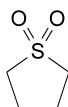
Reversible toxicity: The occurrence of harmful effects that are caused by a substance and which disappear after exposure to the substance ends.

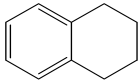
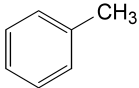
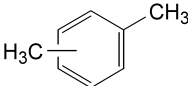
Strongly suspected human carcinogen: A substance for which there is no epidemiological evidence of carcinogenesis but there are positive genotoxicity data and clear evidence of carcinogenesis in rodents.

Teratogenicity: The occurrence of structural malformations in a developing foetus when a substance is administered during pregnancy.

APPENDIX 1. LIST OF SOLVENTS INCLUDED IN THE GUIDELINE

Solvent	Other Names	Structure	Class
Acetic acid	Ethanoic acid	CH_3COOH	Class 3
Acetone	2-Propanone Propan-2-one	CH_3COCH_3	Class 3
Acetonitrile		CH_3CN	Class 2
Anisole	Methoxybenzene		Class 3
Benzene	Benzol		Class 1
1-Butanol	<i>n</i> -Butyl alcohol Butan-1-ol	$\text{CH}_3[\text{CH}_2]_3\text{OH}$	Class 3
2-Butanol	<i>sec</i> -Butyl alcohol Butan-2-ol	$\text{CH}_3\text{CH}_2\text{CH}(\text{OH})\text{CH}_3$	Class 3
Butyl acetate	Acetic acid butyl ester	$\text{CH}_3\text{COO}[\text{CH}_2]_3\text{CH}_3$	Class 3
<i>tert</i> -Butylmethyl ether	2-Methoxy-2-methylpropane	$(\text{CH}_3)_3\text{COCH}_3$	Class 3
Carbon tetrachloride	Tetrachloromethane	CCl_4	Class 1
Chlorobenzene			Class 2
Chloroform	Trichloromethane	CHCl_3	Class 2
Cumene	Isopropylbenzene (1-Methylethyl)benzene		Class 3
Cyclohexane	Hexamethylene		Class 2
1,2-Dichloroethane	<i>sym</i> -Dichloroethane Ethylene dichloride Ethylene chloride	$\text{CH}_2\text{ClCH}_2\text{Cl}$	Class 1
1,1-Dichloroethene	1,1-Dichloroethylene Vinylidene chloride	$\text{H}_2\text{C}=\text{CCl}_2$	Class 1
1,2-Dichloroethene	1,2-Dichloroethylene Acetylene dichloride	$\text{ClHC}=\text{CHCl}$	Class 2
Dichloromethane	Methylene chloride	CH_2Cl_2	Class 2
1,2-Dimethoxyethane	Ethyleneglycol dimethyl ether Monoglyme Dimethyl cellosolve	$\text{H}_3\text{COCH}_2\text{CH}_2\text{OCH}_3$	Class 2
<i>N,N</i> -Dimethylacetamide	DMA	$\text{CH}_3\text{CON}(\text{CH}_3)_2$	Class 2
<i>N,N</i> -Dimethylformamide	DMF	$\text{HCON}(\text{CH}_3)_2$	Class 2
Dimethyl sulphoxide	Methylsulphinylmethane Methyl sulphoxide DMSO	$(\text{CH}_3)_2\text{SO}$	Class 3
1,4-Dioxane	<i>p</i> -Dioxane [1,4]Dioxane		Class 2
Ethanol	Ethyl alcohol	$\text{CH}_3\text{CH}_2\text{OH}$	Class 3
2-Ethoxyethanol	Cellosolve	$\text{CH}_3\text{CH}_2\text{OCH}_2\text{CH}_2\text{OH}$	Class 2
Ethyl acetate	Acetic acid ethyl ester	$\text{CH}_3\text{COOCH}_2\text{CH}_3$	Class 3

Solvent	Other Names	Structure	Class
Ethyleneglycol	1,2-Dihydroxyethane 1,2-Ethanediol	$\text{HOCH}_2\text{CH}_2\text{OH}$	Class 2
Ethyl ether	Diethyl ether Ethoxyethane 1,1'-Oxybisethane	$\text{CH}_3\text{CH}_2\text{OCH}_2\text{CH}_3$	Class 3
Ethyl formate	Formic acid ethyl ester	$\text{HCOOCH}_2\text{CH}_3$	Class 3
Formamide	Methanamide	HCONH_2	Class 2
Formic acid		HCOOH	Class 3
Heptane	<i>n</i> -Heptane	$\text{CH}_3[\text{CH}_2]_5\text{CH}_3$	Class 3
Hexane	<i>n</i> -Hexane	$\text{CH}_3[\text{CH}_2]_4\text{CH}_3$	Class 2
Isobutyl acetate	Acetic acid isobutyl ester	$\text{CH}_3\text{COOCH}_2\text{CH}(\text{CH}_3)_2$	Class 3
Isopropyl acetate	Acetic acid isopropyl ester	$\text{CH}_3\text{COOCH}(\text{CH}_3)_2$	Class 3
Methanol	Methyl alcohol	CH_3OH	Class 2
2-Methoxyethanol	Methyl cellosolve	$\text{CH}_3\text{OCH}_2\text{CH}_2\text{OH}$	Class 2
Methyl acetate	Acetic acid methyl ester	$\text{CH}_3\text{COOCH}_3$	Class 3
3-Methyl-1-butanol	Isoamyl alcohol Isopentyl alcohol 3-Methylbutan-1-ol	$(\text{CH}_3)_2\text{CHCH}_2\text{CH}_2\text{OH}$	Class 3
Methylbutylketone	2-Hexanone Hexan-2-one	$\text{CH}_3[\text{CH}_2]_3\text{COCH}_3$	Class 2
Methylcyclohexane	Cyclohexylmethane		Class 2
Methylethylketone	2-Butanone MEK Butan-2-one	$\text{CH}_3\text{CH}_2\text{COCH}_3$	Class 3
Methylisobutylketone	4-Methylpentan-2-one 4-Methyl-2-pentanone MIBK	$\text{CH}_3\text{COCH}_2\text{CH}(\text{CH}_3)_2$	Class 3
2-Methyl-1-propanol	Isobutyl alcohol 2-Methylpropan-1-ol	$(\text{CH}_3)_2\text{CHCH}_2\text{OH}$	Class 3
<i>N</i> -Methylpyrrolidone	1-Methylpyrrolidin-2-one 1-Methyl-2-pyrrolidinone		Class 2
Nitromethane		CH_3NO_2	Class 2
Pentane	<i>n</i> -Pentane	$\text{CH}_3[\text{CH}_2]_3\text{CH}_3$	Class 3
1-Pentanol	Amyl alcohol Pentan-1-ol Pentyl alcohol	$\text{CH}_3[\text{CH}_2]_3\text{CH}_2\text{OH}$	Class 3
1-Propanol	Propan-1-ol Propyl alcohol	$\text{CH}_3\text{CH}_2\text{CH}_2\text{OH}$	Class 3
2-Propanol	Propan-2-ol Isopropyl alcohol	$(\text{CH}_3)_2\text{CHOH}$	Class 3
Propyl acetate	Acetic acid propyl ester	$\text{CH}_3\text{COOCH}_2\text{CH}_2\text{CH}_3$	Class 3
Pyridine			Class 2
Sulfonane	Tetrahydrothiophene 1,1-dioxide		Class 2

Solvent	Other Names	Structure	Class
Tetrahydrofuran	Tetramethylene oxide Oxacyclopentane		Class 2
Tetralin	1,2,3,4-Tetrahydronaphthalene		Class 2
Toluene	Methylbenzene		Class 2
1,1,1-Trichloroethane	Methylchloroform	CH_3CCl_3	Class 1
1,1,2-Trichloroethene	Trichloroethene	$\text{HClC}=\text{CCl}_2$	Class 2
Xylene*	Dimethylbenzene Xylol		Class 2

*usually 60 per cent *m*-xylene, 14 per cent *p*-xylene, 9 per cent *o*-xylene with 17 per cent ethyl benzene.

APPENDIX 2. ADDITIONAL BACKGROUND

A2.1. ENVIRONMENTAL REGULATION OF ORGANIC VOLATILE SOLVENTS

Several of the residual solvents frequently used in the production of pharmaceuticals are listed as toxic chemicals in Environmental Health Criteria (EHC) monographs and the Integrated Risk Information System (IRIS). The objectives of such groups as the International Programme on Chemical Safety (IPCS), the United States Environmental Protection Agency (USEPA) and the United States Food and Drug Administration (USFDA) include the determination of acceptable exposure levels. The goal is protection of human health and maintenance of environmental integrity against the possible deleterious effects of chemicals resulting from long-term environmental exposure. The methods involved in the estimation of maximum safe exposure limits are usually based on long-term studies. When long-term study data are unavailable, shorter term study data can be used with modification of the approach such as use of larger safety factors. The approach described therein relates primarily to long-term or life-time exposure of the general population in the ambient environment, i.e. ambient air, food, drinking water and other media.

A2.2. RESIDUAL SOLVENTS IN PHARMACEUTICALS

Exposure limits in this guideline are established by referring to methodologies and toxicity data described in EHC and IRIS monographs. However, some specific assumptions about residual solvents to be used in the synthesis and formulation of pharmaceutical products should be taken into account in establishing exposure limits. They are:

- 1) Patients (not the general population) use pharmaceuticals to treat their diseases or for prophylaxis to prevent infection or disease.
- 2) The assumption of life-time patient exposure is not necessary for most pharmaceutical products but may be appropriate as a working hypothesis to reduce risk to human health.
- 3) Residual solvents are unavoidable components in pharmaceutical production and will often be a part of medicinal products.
- 4) Residual solvents should not exceed recommended levels except in exceptional circumstances.

5) Data from toxicological studies that are used to determine acceptable levels for residual solvents should have been generated using appropriate protocols such as those described for example, by OECD and the FDA Red Book.

APPENDIX 3. METHODS FOR ESTABLISHING EXPOSURE LIMITS

The Gaylor-Kodell method of risk assessment (Gaylor, D. W. and Kodell, R. L. Linear Interpolation algorithm for low dose assessment of toxic substance. *J. Environ. Pathology*, 4, 305, 1980) is appropriate for Class 1 carcinogenic solvents. Only in cases where reliable carcinogenicity data are available should extrapolation by the use of mathematical models be applied to setting exposure limits. Exposure limits for Class 1 solvents could be determined with the use of a large safety factor (i.e., 10 000 to 100 000) with respect to the no-observed-effect level (NOEL). Detection and quantification of these solvents should be by state-of-the-art analytical techniques.

Acceptable exposure levels in this guideline for Class 2 solvents were established by calculation of PDE values according to the procedures for setting exposure limits in pharmaceuticals (*Pharmacopeial Forum*, Nov-Dec 1989), and the method adopted by IPCS for Assessing Human Health Risk of Chemicals (*Environmental Health Criteria 170*, WHO, 1994). These methods are similar to those used by the USEPA (IRIS) and the USFDA (*Red Book*) and others. The method is outlined here to give a better understanding of the origin of the PDE values. It is not necessary to perform these calculations in order to use the PDE values tabulated in Section 4 of this document.

PDE is derived from the no-observed-effect level (NOEL), or the lowest-observed effect level (LOEL), in the most relevant animal study as follows:

$$\text{PDE} = \frac{\text{NOEL} \times \text{Weight Adjustment}}{\text{F}_1 \times \text{F}_2 \times \text{F}_3 \times \text{F}_4 \times \text{F}_5}$$

The PDE is derived preferably from a NOEL. If no NOEL is obtained, the LOEL may be used. Modifying factors proposed here, for relating the data to humans, are the same kind of “uncertainty factors” used in Environmental Health Criteria (*Environmental Health Criteria 170*, World Health Organisation, Geneva, 1994), and “modifying factors” or

“safety factors” in *Pharmacopoeial Forum*. The assumption of 100 per cent systemic exposure is used in all calculations regardless of route of administration.

The modifying factors are as follows:

F1 = A factor to account for extrapolation between species

F1 = 2 for extrapolation from dogs to humans

F1 = 2.5 for extrapolation from rabbits to humans

F1 = 3 for extrapolation from monkeys to humans

F1 = 5 for extrapolation from rats to humans

F1 = 10 for extrapolation from other animals to humans

F1 = 12 for extrapolation from mice to humans

F1 takes into account the comparative surface area: body weight ratios for the species concerned and for man. Surface area (S) is calculated as:

$$S = km^{0.67}$$

in which m = body mass, and the constant k has been taken to be 10. The body weight used in the equation are those shown below in Table A3-1.

Table A3-1. – Values used in the calculations in this document

Rat body weight	425 g
Pregnant rat body weight	330 g
Mouse body weight	28 g
Pregnant mouse body weight	30 g
Guinea-pig body weight	500 g
Rhesus monkey body weight	2.5 kg
Rabbit body weight (pregnant or not)	4 kg
Beagle dog body weight	11.5 kg
Rat respiratory volume	290 l/day
Mouse respiratory volume	43 l/day
Rabbit respiratory volume	1440 l/day
Guinea-pig respiratory volume	430 l/day
Human respiratory volume	28800 l/day
Dog respiratory volume	9000 l/day
Monkey respiratory volume	1150 l/day
Mouse water consumption	5 ml/day
Rat water consumption	30 ml/day
Rat food consumption	30 g/day

F2 = A factor of 10 to account for variability between individuals. A factor of 10 is generally given for all organic solvents, and 10 is used consistently in this guideline.

F3 = A variable factor to account for toxicity studies of short-term exposure.

F3 = 1 for studies that last at least one half-lifetime (1 year for rodents or rabbits; 7 years for cats, dogs and monkeys).

F3 = 1 for reproductive studies in which the whole period of organogenesis is covered.

F3 = 2 for a 6 month study in rodents, or a 3.5 year study in non-rodents.

F3 = 5 for a 3 month study in rodents, or a 2 year study in non-rodents.

F3 = 10 for studies of a shorter duration.

In all cases, the higher factor has been used for study durations between the time points, e.g. a factor of 2 for a 9 month rodent study.

F4 = A factor that may be applied in cases of severe toxicity, e.g. non-genotoxic carcinogenicity, neurotoxicity or teratogenicity. In studies of reproductive toxicity, the following factors are used:

F4 = 1 for foetal toxicity associated with maternal toxicity

F4 = 5 for foetal toxicity without maternal toxicity

F4 = 5 for a teratogenic effect with maternal toxicity

F4 = 10 for a teratogenic effect without maternal toxicity

F5 = A variable factor that may be applied if the no-effect level was not established.

When only a LOEL is available, a factor of up to 10 can be used depending on the severity of the toxicity.

The weight adjustment assumes an arbitrary adult human body weight for either sex of 50 kg. This relatively low weight provides an additional safety factor against the standard weights of 60 kg or 70 kg that are often used in this type of calculation. It is recognised that some adult patients weigh less than 50 kg; these patients are considered to be accommodated by the built-in safety factors used to determine a PDE. If the solvent was present in a formulation specifically intended for paediatric use, an adjustment for a lower body weight would be appropriate.

As an example of the application of this equation, consider the toxicity study of acetonitrile in mice that is summarised in *Pharmeuropa*, Vol. 9. No. 1, Supplement, April 1997, page S24. The NOEL is calculated to be 50.7 mg kg⁻¹ day⁻¹. The PDE for acetonitrile in this study is calculated as follows:

$$\text{PDE} = \frac{50.7 \text{ mg kg}^{-1} \text{ day}^{-1} \times 50 \text{ kg}}{12 \times 10 \times 5 \times 1 \times 1} = 4.22 \text{ mg day}^{-1}$$

In this example,

F1 = 12 to account for the extrapolation from mice to humans

F2 = 10 to account for differences between individual humans

F3 = 5 because the duration of the study was only 13 weeks

F4 = 1 because no severe toxicity was encountered

F5 = 1 because the no-effect level was determined

The equation for an ideal gas, $PV = nRT$, is used to convert concentrations of gases used in inhalation studies from units of ppm to units of mg/l or mg/m³. Consider as an example the rat reproductive toxicity study by inhalation of carbon tetrachloride (molecular weight 153.84) summarised in *Pharmeuropa*, Vol. 9, No. 1, Supplement, April 1997, page S9.

$$\begin{aligned}\frac{n}{V} &= \frac{P}{RT} = \frac{300 \times 10^{-6} \text{ atm} \times 153\,840 \text{ mg mol}^{-1}}{0.082 \text{ l atm K}^{-1} \text{ mol}^{-1} \times 298 \text{ K}} \\ &= \frac{46.15 \text{ mg}}{24.45 \text{ l}} = 1.89 \text{ mg/l}\end{aligned}$$

The relationship 1000 litres = 1 m³ is used to convert to mg/m³.

01/2005:50500

5.5. ALCOHOLIMETRIC TABLES

The general formula agreed by the Council of the European Communities in its Directive of 27 July 1976 on alcoholimetry served as the basis for establishing the following tables.

% V/V	% m/m	ρ_{20} (kg/m ³)
0.0	0.0	998.20
0.1	0.08	998.05
0.2	0.16	997.90
0.3	0.24	997.75
0.4	0.32	997.59
0.5	0.40	997.44
0.6	0.47	997.29
0.7	0.55	997.14
0.8	0.63	996.99
0.9	0.71	996.85
1.0	0.79	996.70
1.1	0.87	996.55
1.2	0.95	996.40
1.3	1.03	996.25
1.4	1.11	996.11
1.5	1.19	995.96
1.6	1.27	995.81
1.7	1.35	995.67
1.8	1.43	995.52
1.9	1.51	995.38
2.0	1.59	995.23
2.1	1.67	995.09
2.2	1.75	994.94
2.3	1.82	994.80
2.4	1.90	994.66
2.5	1.98	994.51
2.6	2.06	994.37
2.7	2.14	994.23
2.8	2.22	994.09
2.9	2.30	993.95
3.0	2.38	993.81
3.1	2.46	993.66
3.2	2.54	993.52
3.3	2.62	993.38
3.4	2.70	993.24
3.5	2.78	993.11
3.6	2.86	992.97
3.7	2.94	992.83
3.8	3.02	992.69

% V/V	% m/m	ρ_{20} (kg/m ³)
3.9	3.10	992.55
4.0	3.18	992.41
4.1	3.26	992.28
4.2	3.34	992.14
4.3	3.42	992.00
4.4	3.50	991.87
4.5	3.58	991.73
4.6	3.66	991.59
4.7	3.74	991.46
4.8	3.82	991.32
4.9	3.90	991.19
5.0	3.98	991.06
5.1	4.06	990.92
5.2	4.14	990.79
5.3	4.22	990.65
5.4	4.30	990.52
5.5	4.38	990.39
5.6	4.46	990.26
5.7	4.54	990.12
5.8	4.62	989.99
5.9	4.70	989.86
6.0	4.78	989.73
6.1	4.86	989.60
6.2	4.95	989.47
6.3	5.03	989.34
6.4	5.11	989.21
6.5	5.19	989.08
6.6	5.27	988.95
6.7	5.35	988.82
6.8	5.43	988.69
6.9	5.51	988.56
7.0	5.59	988.43
7.1	5.67	988.30
7.2	5.75	988.18
7.3	5.83	988.05
7.4	5.91	987.92
7.5	5.99	987.79
7.6	6.07	987.67
7.7	6.15	987.54
7.8	6.23	987.42
7.9	6.32	987.29
8.0	6.40	987.16

5. General texts

% V/V	% m/m	ρ_{20} (kg/m ³)	% V/V	% m/m	ρ_{20} (kg/m ³)
8.1	6.48	987.04	12.4	9.97	981.89
8.2	6.56	986.91	12.5	10.05	981.78
8.3	6.64	986.79	12.6	10.13	981.67
8.4	6.72	986.66	12.7	10.21	981.55
8.5	6.80	986.54	12.8	10.29	981.44
8.6	6.88	986.42	12.9	10.37	981.32
8.7	6.96	986.29			
8.8	7.04	986.17	13.0	10.46	981.21
8.9	7.12	986.05	13.1	10.54	981.10
			13.2	10.62	980.98
9.0	7.20	985.92	13.3	10.70	980.87
9.1	7.29	985.80	13.4	10.78	980.76
9.2	7.37	985.68	13.5	10.87	980.64
9.3	7.45	985.56	13.6	10.95	980.53
9.4	7.53	985.44	13.7	11.03	980.42
9.5	7.61	985.31	13.8	11.11	980.31
9.6	7.69	985.19	13.9	11.19	980.19
9.7	7.77	985.07			
9.8	7.85	984.95	14.0	11.27	980.08
9.9	7.93	984.83	14.1	11.36	979.97
			14.2	11.44	979.86
10.0	8.01	984.71	14.3	11.52	979.75
10.1	8.10	984.59	14.4	11.60	979.64
10.2	8.18	984.47	14.5	11.68	979.52
10.3	8.26	984.35	14.6	11.77	979.41
10.4	8.34	984.23	14.7	11.85	979.30
10.5	8.42	984.11	14.8	11.93	979.19
10.6	8.50	983.99	14.9	12.01	979.08
10.7	8.58	983.88			
10.8	8.66	983.76	15.0	12.09	978.97
10.9	8.75	983.64	15.1	12.17	978.86
			15.2	12.26	978.75
11.0	8.83	983.52	15.3	12.34	978.64
11.1	8.91	983.40	15.4	12.42	978.53
11.2	8.99	983.29	15.5	12.50	978.42
11.3	9.07	983.17	15.6	12.59	978.31
11.4	9.15	983.05	15.7	12.67	978.20
11.5	9.23	982.94	15.8	12.75	978.09
11.6	9.32	982.82	15.9	12.83	977.98
11.7	9.40	982.70			
11.8	9.48	982.59	16.0	12.91	977.87
11.9	9.56	982.47	16.1	13.00	977.76
			16.2	13.08	977.65
12.0	9.64	982.35	16.3	13.16	977.55
12.1	9.72	982.24	16.4	13.24	977.44
12.2	9.80	982.12	16.5	13.32	977.33
12.3	9.89	982.01	16.6	13.41	977.22

% V/V	% m/m	ρ_{20} (kg/m ³)	% V/V	% m/m	ρ_{20} (kg/m ³)
16.7	13.49	977.11			
16.8	13.57	977.00	21.0	17.04	972.48
16.9	13.65	976.89	21.1	17.13	972.37
			21.2	17.21	972.27
17.0	13.74	976.79	21.3	17.29	972.16
17.1	13.82	976.68	21.4	17.38	972.05
17.2	13.90	976.57	21.5	17.46	971.94
17.3	13.98	976.46	21.6	17.54	971.83
17.4	14.07	976.35	21.7	17.62	971.73
17.5	14.15	976.25	21.8	17.71	971.62
17.6	14.23	976.14	21.9	17.79	971.51
17.7	14.31	976.03			
17.8	14.40	975.92	22.0	17.87	971.40
17.9	14.48	975.81	22.1	17.96	971.29
			22.2	18.04	971.18
18.0	14.56	975.71	22.3	18.12	971.08
18.1	14.64	975.60	22.4	18.21	970.97
18.2	14.73	975.49	22.5	18.29	970.86
18.3	14.81	975.38	22.6	18.37	970.75
18.4	14.89	975.28	22.7	18.46	970.64
18.5	14.97	975.17	22.8	18.54	970.53
18.6	15.06	975.06	22.9	18.62	970.42
18.7	15.14	974.95			
18.8	15.22	974.85	23.0	18.71	970.31
18.9	15.30	974.74	23.1	18.79	970.20
			23.2	18.87	970.09
19.0	15.39	974.63	23.3	18.96	969.98
19.1	15.47	974.52	23.4	19.04	969.87
19.2	15.55	974.42	23.5	19.13	969.76
19.3	15.63	974.31	23.6	19.21	969.65
19.4	15.72	974.20	23.7	19.29	969.54
19.5	15.80	974.09	23.8	19.38	969.43
19.6	15.88	973.99	23.9	19.46	969.32
19.7	15.97	973.88			
19.8	16.05	973.77	24.0	19.54	969.21
19.9	16.13	973.66	24.1	19.63	969.10
			24.2	19.71	968.99
20.0	16.21	973.56	24.3	19.79	968.88
20.1	16.30	973.45	24.4	19.88	968.77
20.2	16.38	973.34	24.5	19.96	968.66
20.3	16.46	973.24	24.6	20.05	968.55
20.4	16.55	973.13	24.7	20.13	968.43
20.5	16.63	973.02	24.8	20.21	968.32
20.6	16.71	972.91	24.9	20.30	968.21
20.7	16.79	972.80			
20.8	16.88	972.70	25.0	20.38	968.10
20.9	16.96	972.59	25.1	20.47	967.99

% V/V	% m/m	ρ_{20} (kg/m ³)	% V/V	% m/m	ρ_{20} (kg/m ³)
25.2	20.55	967.87	29.5	24.18	962.83
25.3	20.63	967.76	29.6	24.27	962.71
25.4	20.72	967.65	29.7	24.35	962.58
25.5	20.80	967.53	29.8	24.44	962.46
25.6	20.88	967.42	29.9	24.52	962.33
25.7	20.97	967.31			
25.8	21.05	967.19	30.0	24.61	962.21
25.9	21.14	967.08	30.1	24.69	962.09
			30.2	24.78	961.96
26.0	21.22	966.97	30.3	24.86	961.84
26.1	21.31	966.85	30.4	24.95	961.71
26.2	21.39	966.74	30.5	25.03	961.59
26.3	21.47	966.62	30.6	25.12	961.46
26.4	21.56	966.51	30.7	25.20	961.33
26.5	21.64	966.39	30.8	25.29	961.21
26.6	21.73	966.28	30.9	25.38	961.08
26.7	21.81	966.16			
26.8	21.90	966.05	31.0	25.46	960.95
26.9	21.98	965.93	31.1	25.55	960.82
			31.2	25.63	960.70
27.0	22.06	965.81	31.3	25.72	960.57
27.1	22.15	965.70	31.4	25.80	960.44
27.2	22.23	965.58	31.5	25.89	960.31
27.3	22.32	965.46	31.6	25.97	960.18
27.4	22.40	965.35	31.7	26.06	960.05
27.5	22.49	965.23	31.8	26.15	959.92
27.6	22.57	965.11	31.9	26.23	959.79
27.7	22.65	964.99			
27.8	22.74	964.88	32.0	26.32	959.66
27.9	22.82	964.76	32.1	26.40	959.53
			32.2	26.49	959.40
28.0	22.91	964.64	32.3	26.57	959.27
28.1	22.99	964.52	32.4	26.66	959.14
28.2	23.08	964.40	32.5	26.75	959.01
28.3	23.16	964.28	32.6	26.83	958.87
28.4	23.25	964.16	32.7	26.92	958.74
28.5	23.33	964.04	32.8	27.00	958.61
28.6	23.42	963.92	32.9	27.09	958.47
28.7	23.50	963.80			
28.8	23.59	963.68	33.0	27.18	958.34
28.9	23.67	963.56	33.1	27.26	958.20
			33.2	27.35	958.07
29.0	23.76	963.44	33.3	27.44	957.94
29.1	23.84	963.32	33.4	27.52	957.80
29.2	23.93	963.20	33.5	27.61	957.66
29.3	24.01	963.07	33.6	27.69	957.53
29.4	24.10	962.95	33.7	27.78	957.39

% V/V	% m/m	ρ_{20} (kg/m ³)	% V/V	% m/m	ρ_{20} (kg/m ³)
33.8	27.87	957.26	38.0	31.53	951.18
33.9	27.95	957.12	38.1	31.62	951.02
			38.2	31.71	950.87
34.0	28.04	956.98	38.3	31.79	950.72
34.1	28.13	956.84	38.4	31.88	950.56
34.2	28.21	956.70	38.5	31.97	950.41
34.3	28.30	956.57	38.6	32.06	950.25
34.4	28.39	956.43	38.7	32.15	950.10
34.5	28.47	956.29	38.8	32.24	949.94
34.6	28.56	956.15	38.9	32.32	949.79
34.7	28.65	956.01			
34.8	28.73	955.87	39.0	32.41	949.63
34.9	28.82	955.73	39.1	32.50	949.47
			39.2	32.59	949.32
35.0	28.91	955.59	39.3	32.68	949.16
35.1	28.99	955.45	39.4	32.77	949.00
35.2	29.08	955.30	39.5	32.86	948.84
35.3	29.17	955.16	39.6	32.94	948.68
35.4	29.26	955.02	39.7	33.03	948.52
35.5	29.34	954.88	39.8	33.12	948.37
35.6	29.43	954.73	39.9	33.21	948.21
35.7	29.52	954.59			
35.8	29.60	954.44	40.0	33.30	948.05
35.9	29.69	954.30	40.1	33.39	947.88
			40.2	33.48	947.72
36.0	29.78	954.15	40.3	33.57	947.56
36.1	29.87	954.01	40.4	33.66	947.40
36.2	29.95	953.86	40.5	33.74	947.24
36.3	30.04	953.72	40.6	33.83	947.08
36.4	30.13	953.57	40.7	33.92	946.91
36.5	30.21	953.42	40.8	34.01	946.75
36.6	30.30	953.28	40.9	34.10	946.58
36.7	30.39	953.13			
36.8	30.48	952.98	41.0	34.19	946.42
36.9	30.56	952.83	41.1	34.28	946.26
			41.2	34.37	946.09
37.0	30.65	952.69	41.3	34.46	945.93
37.1	30.74	952.54	41.4	34.55	945.76
37.2	30.83	952.39	41.5	34.64	945.59
37.3	30.92	952.24	41.6	34.73	945.43
37.4	31.00	952.09	41.7	34.82	945.26
37.5	31.09	951.94	41.8	34.91	945.09
37.6	31.18	951.79	41.9	35.00	944.93
37.7	31.27	951.63			
37.8	31.35	951.48	42.0	35.09	944.76
37.9	31.44	951.33	42.1	35.18	944.59
			42.2	35.27	944.42

% V/V	% m/m	ρ_{20} (kg/m ³)	% V/V	% m/m	ρ_{20} (kg/m ³)
42.3	35.36	944.25	46.6	39.27	936.63
42.4	35.45	944.08	46.7	39.36	936.44
42.5	35.54	943.91	46.8	39.45	936.26
42.6	35.63	943.74	46.9	39.54	936.07
42.7	35.72	943.57			
42.8	35.81	943.40	47.0	39.64	935.88
42.9	35.90	943.23	47.1	39.73	935.70
			47.2	39.82	935.51
43.0	35.99	943.06	47.3	39.91	935.32
43.1	36.08	942.88	47.4	40.00	935.14
43.2	36.17	942.71	47.5	40.10	934.95
43.3	36.26	942.54	47.6	40.19	934.76
43.4	36.35	942.37	47.7	40.28	934.57
43.5	36.44	942.19	47.8	40.37	934.38
43.6	36.53	942.02	47.9	40.47	934.19
43.7	36.62	941.84			
43.8	36.71	941.67	48.0	40.56	934.00
43.9	36.80	941.49	48.1	40.65	933.81
			48.2	40.75	933.62
44.0	36.89	941.32	48.3	40.84	933.43
44.1	36.98	941.14	48.4	40.93	933.24
44.2	37.07	940.97	48.5	41.02	933.05
44.3	37.16	940.79	48.6	41.12	932.86
44.4	37.25	940.61	48.7	41.21	932.67
44.5	37.35	940.43	48.8	41.30	932.47
44.6	37.44	940.26	48.9	41.40	932.28
44.7	37.53	940.08			
44.8	37.62	939.90	49.0	41.49	932.09
44.9	37.71	939.72	49.1	41.58	931.90
			49.2	41.68	931.70
45.0	37.80	939.54	49.3	41.77	931.51
45.1	37.89	939.36	49.4	41.86	931.31
45.2	37.98	939.18	49.5	41.96	931.12
45.3	38.08	939.00	49.6	42.05	930.92
45.4	38.17	938.82	49.7	42.14	930.73
45.5	38.26	938.64	49.8	42.24	930.53
45.6	38.35	938.46	49.9	42.33	930.34
45.7	38.44	938.28			
45.8	38.53	938.10	50.0	42.43	930.14
45.9	38.62	937.91	50.1	42.52	929.95
			50.2	42.61	929.75
46.0	38.72	937.73	50.3	42.71	929.55
46.1	38.81	937.55	50.4	42.80	929.35
46.2	38.90	937.36	50.5	42.90	929.16
46.3	38.99	937.18	50.6	42.99	928.96
46.4	39.08	937.00	50.7	43.08	928.76
46.5	39.18	936.81	50.8	43.18	928.56

% V/V	% m/m	ρ_{20} (kg/m ³)	% V/V	% m/m	ρ_{20} (kg/m ³)
50.9	43.27	928.36	55.1	47.28	919.75
			55.2	47.38	919.54
51.0	43.37	928.16	55.3	47.47	919.33
51.1	43.46	927.96	55.4	47.57	919.12
51.2	43.56	927.77	55.5	47.67	918.91
51.3	43.65	927.57	55.6	47.77	918.69
51.4	43.74	927.36	55.7	47.86	918.48
51.5	43.84	927.16	55.8	47.96	918.27
51.6	43.93	926.96	55.9	48.06	918.06
51.7	44.03	926.76			
51.8	44.12	926.56	56.0	48.15	917.84
51.9	44.22	926.36	56.1	48.25	917.63
			56.2	48.35	917.42
52.0	44.31	926.16	56.3	48.45	917.20
52.1	44.41	925.95	56.4	48.54	916.99
52.2	44.50	925.75	56.5	48.64	916.77
52.3	44.60	925.55	56.6	48.74	916.56
52.4	44.69	925.35	56.7	48.84	916.35
52.5	44.79	925.14	56.8	48.93	916.13
52.6	44.88	924.94	56.9	49.03	915.91
52.7	44.98	924.73			
52.8	45.07	924.53	57.0	49.13	915.70
52.9	45.17	924.32	57.1	49.23	915.48
			57.2	49.32	915.27
53.0	45.26	924.12	57.3	49.42	915.05
53.1	45.36	923.91	57.4	49.52	914.83
53.2	45.46	923.71	57.5	49.62	914.62
53.3	45.55	923.50	57.6	49.72	914.40
53.4	45.65	923.30	57.7	49.81	914.18
53.5	45.74	923.09	57.8	49.91	913.97
53.6	45.84	922.88	57.9	50.01	913.75
53.7	45.93	922.68			
53.8	46.03	922.47	58.0	50.11	913.53
53.9	46.13	922.26	58.1	50.21	913.31
			58.2	50.31	913.09
54.0	46.22	922.06	58.3	50.40	912.87
54.1	46.32	921.85	58.4	50.50	912.65
54.2	46.41	921.64	58.5	50.60	912.43
54.3	46.51	921.43	58.6	50.70	912.22
54.4	46.61	921.22	58.7	50.80	912.00
54.5	46.70	921.01	58.8	50.90	911.78
54.6	46.80	920.80	58.9	51.00	911.55
54.7	46.90	920.59			
54.8	46.99	920.38	59.0	51.10	911.33
54.9	47.09	920.17	59.1	51.19	911.11
			59.2	51.29	910.89
55.0	47.18	919.96	59.3	51.39	910.67

5. General texts

% V/V	% m/m	ρ_{20} (kg/m ³)	% V/V	% m/m	ρ_{20} (kg/m ³)
59.4	51.49	910.45	63.7	55.82	900.69
59.5	51.59	910.23	63.8	55.92	900.46
59.6	51.69	910.01	63.9	56.02	900.23
59.7	51.79	909.78			
59.8	51.89	909.56	64.0	56.12	899.99
59.9	51.99	909.34	64.1	56.23	899.76
			64.2	56.33	899.53
60.0	52.09	909.11	64.3	56.43	899.29
60.1	52.19	908.89	64.4	56.53	899.06
60.2	52.29	908.67	64.5	56.64	898.83
60.3	52.39	908.44	64.6	56.74	898.59
60.4	52.49	908.22	64.7	56.84	898.36
60.5	52.59	908.00	64.8	56.94	898.12
60.6	52.69	907.77	64.9	57.05	897.89
60.7	52.79	907.55			
60.8	52.89	907.32	65.0	57.15	897.65
60.9	52.99	907.10	65.1	57.25	897.42
			65.2	57.36	897.18
61.0	53.09	906.87	65.3	57.46	896.94
61.1	53.19	906.64	65.4	57.56	896.71
61.2	53.29	906.42	65.5	57.67	896.47
61.3	53.39	906.19	65.6	57.77	896.23
61.4	53.49	905.97	65.7	57.87	896.00
61.5	53.59	905.74	65.8	57.98	895.76
61.6	53.69	905.51	65.9	58.08	895.52
61.7	53.79	905.29			
61.8	53.89	905.06	66.0	58.18	895.28
61.9	53.99	904.83	66.1	58.29	895.05
			66.2	58.39	894.81
62.0	54.09	904.60	66.3	58.49	894.57
62.1	54.19	904.37	66.4	58.60	894.33
62.2	54.30	904.15	66.5	58.70	894.09
62.3	54.40	903.92	66.6	58.81	893.85
62.4	54.50	903.69	66.7	58.91	893.61
62.5	54.60	903.46	66.8	59.01	893.37
62.6	54.70	903.23	66.9	59.12	893.13
62.7	54.80	903.00			
62.8	54.90	902.77	67.0	59.22	892.89
62.9	55.00	902.54	67.1	59.33	892.65
			67.2	59.43	892.41
63.0	55.11	902.31	67.3	59.54	892.17
63.1	55.21	902.08	67.4	59.64	891.93
63.2	55.31	901.85	67.5	59.74	891.69
63.3	55.41	901.62	67.6	59.85	891.45
63.4	55.51	901.39	67.7	59.95	891.20
63.5	55.61	901.15	67.8	60.06	890.96
63.6	55.72	900.92	67.9	60.16	890.72

% V/V	% m/m	ρ_{20} (kg/m ³)	% V/V	% m/m	ρ_{20} (kg/m ³)
			72.2	64.75	880.03
68.0	60.27	890.48	72.3	64.86	879.78
68.1	60.37	890.23	72.4	64.97	879.52
68.2	60.48	889.99	72.5	65.08	879.27
68.3	60.58	889.75	72.6	65.19	879.01
68.4	60.69	889.50	72.7	65.29	878.75
68.5	60.80	889.26	72.8	65.40	878.50
68.6	60.90	889.01	72.9	65.51	878.24
68.7	61.01	888.77			
68.8	61.11	888.52	73.0	65.62	877.99
68.9	61.22	888.28	73.1	65.73	877.73
			73.2	65.84	877.47
69.0	61.32	888.03	73.3	65.95	877.21
69.1	61.43	887.79	73.4	66.06	876.96
69.2	61.54	887.54	73.5	66.17	876.70
69.3	61.64	887.29	73.6	66.28	876.44
69.4	61.75	887.05	73.7	66.39	876.18
69.5	61.85	886.80	73.8	66.50	875.92
69.6	61.96	886.55	73.9	66.61	875.66
69.7	62.07	886.31			
69.8	62.17	886.06	74.0	66.72	875.40
69.9	62.28	885.81	74.1	66.83	875.14
			74.2	66.94	874.88
70.0	62.39	885.56	74.3	67.05	874.62
70.1	62.49	885.31	74.4	67.16	874.36
70.2	62.60	885.06	74.5	67.27	874.10
70.3	62.71	884.82	74.6	67.38	873.84
70.4	62.81	884.57	74.7	67.49	873.58
70.5	62.92	884.32	74.8	67.60	873.32
70.6	63.03	884.07	74.9	67.71	873.06
70.7	63.13	883.82			
70.8	63.24	883.57	75.0	67.82	872.79
70.9	63.35	883.32	75.1	67.93	872.53
			75.2	68.04	872.27
71.0	63.46	883.06	75.3	68.15	872.00
71.1	63.56	882.81	75.4	68.26	871.74
71.2	63.67	882.56	75.5	68.38	871.48
71.3	63.78	882.31	75.6	68.49	871.21
71.4	63.89	882.06	75.7	68.60	870.95
71.5	63.99	881.81	75.8	68.71	870.68
71.6	64.10	881.55	75.9	68.82	870.42
71.7	64.21	881.30			
71.8	64.32	881.05	76.0	68.93	870.15
71.9	64.43	880.79	76.1	69.04	869.89
			76.2	69.16	869.62
72.0	64.53	880.54	76.3	69.27	869.35
72.1	64.64	880.29	76.4	69.38	869.09

% V/V	% m/m	ρ_{20} (kg/m ³)	% V/V	% m/m	ρ_{20} (kg/m ³)
76.5	69.49	868.82	80.8	74.41	857.03
76.6	69.61	868.55	80.9	74.53	856.75
76.7	69.72	868.28			
76.8	69.83	868.02	81.0	74.64	856.46
76.9	69.94	867.75	81.1	74.76	856.18
			81.2	74.88	855.90
77.0	70.06	867.48	81.3	74.99	855.62
77.1	70.17	867.21	81.4	75.11	855.33
77.2	70.28	866.94	81.5	75.23	855.05
77.3	70.39	866.67	81.6	75.34	854.76
77.4	70.51	866.40	81.7	75.46	854.48
77.5	70.62	866.13	81.8	75.58	854.19
77.6	70.73	865.86	81.9	75.70	853.91
77.7	70.85	865.59			
77.8	70.96	865.32	82.0	75.82	853.62
77.9	71.07	865.05	82.1	75.93	853.34
			82.2	76.05	853.05
78.0	71.19	864.78	82.3	76.17	852.76
78.1	71.30	864.50	82.4	76.29	852.48
78.2	71.41	864.23	82.5	76.41	852.19
78.3	71.53	863.96	82.6	76.52	851.90
78.4	71.64	863.69	82.7	76.64	851.61
78.5	71.76	863.41	82.8	76.76	851.32
78.6	71.87	863.14	82.9	76.88	851.03
78.7	71.98	862.86			
78.8	72.10	862.59	83.0	77.00	850.74
78.9	72.21	862.31	83.1	77.12	850.45
			83.2	77.24	850.16
79.0	72.33	862.04	83.3	77.36	849.87
79.1	72.44	861.76	83.4	77.48	849.58
79.2	72.56	861.49	83.5	77.60	849.29
79.3	72.67	861.21	83.6	77.72	848.99
79.4	72.79	860.94	83.7	77.84	848.70
79.5	72.90	860.66	83.8	77.96	848.41
79.6	73.02	860.38	83.9	78.08	848.11
79.7	73.13	860.10			
79.8	73.25	859.83	84.0	78.20	847.82
79.9	73.36	859.55	84.1	78.32	847.53
			84.2	78.44	847.23
80.0	73.48	859.27	84.3	78.56	846.93
80.1	73.60	858.99	84.4	78.68	846.64
80.2	73.71	858.71	84.5	78.80	846.34
80.3	73.83	858.43	84.6	78.92	846.05
80.4	73.94	858.15	84.7	79.04	845.75
80.5	74.06	857.87	84.8	79.16	845.45
80.6	74.18	857.59	84.9	79.28	845.15
80.7	74.29	857.31			

% V/V	% m/m	ρ_{20} (kg/m ³)	% V/V	% m/m	ρ_{20} (kg/m ³)
85.0	79.40	844.85	89.3	84.76	831.48
85.1	79.53	844.55	89.4	84.89	831.15
85.2	79.65	844.25	89.5	85.02	830.82
85.3	79.77	843.95	89.6	85.15	830.50
85.4	79.89	843.65	89.7	85.28	830.17
85.5	80.01	843.35	89.8	85.41	829.84
85.6	80.14	843.05	89.9	85.54	829.51
85.7	80.26	842.75			
85.8	80.38	842.44	90.0	85.66	829.18
85.9	80.50	842.14	90.1	85.79	828.85
			90.2	85.92	828.52
86.0	80.63	841.84	90.3	86.05	828.19
86.1	80.75	841.53	90.4	86.18	827.85
86.2	80.87	841.23	90.5	86.31	827.52
86.3	81.00	840.92	90.6	86.44	827.18
86.4	81.12	840.62	90.7	86.57	826.85
86.5	81.24	840.31	90.8	86.71	826.51
86.6	81.37	840.00	90.9	86.84	826.17
86.7	81.49	839.70			
86.8	81.61	839.39	91.0	86.97	825.83
86.9	81.74	839.08	91.1	87.10	825.49
			91.2	87.23	825.15
87.0	81.86	838.77	91.3	87.36	824.81
87.1	81.99	838.46	91.4	87.49	824.47
87.2	82.11	838.15	91.5	87.63	824.13
87.3	82.24	837.84	91.6	87.76	823.78
87.4	82.36	837.52	91.7	87.89	823.44
87.5	82.49	837.21	91.8	88.02	823.09
87.6	82.61	836.90	91.9	88.16	822.74
87.7	82.74	836.59			
87.8	82.86	836.27	92.0	88.29	822.39
87.9	82.99	835.96	92.1	88.42	822.04
			92.2	88.56	821.69
88.0	83.11	835.64	92.3	88.69	821.34
88.1	83.24	835.32	92.4	88.83	820.99
88.2	83.37	835.01	92.5	88.96	820.63
88.3	83.49	834.69	92.6	89.10	820.28
88.4	83.62	834.37	92.7	89.23	819.92
88.5	83.74	834.05	92.8	89.37	819.57
88.6	83.87	833.73	92.9	89.50	819.21
88.7	84.00	833.41			
88.8	84.13	833.09	93.0	89.64	818.85
88.9	84.25	832.77	93.1	89.77	818.49
			93.2	89.91	818.12
89.0	84.38	832.45	93.3	90.05	817.76
89.1	84.51	832.12	93.4	90.18	817.40
89.2	84.64	831.80	93.5	90.32	817.03

5. General texts

% <i>V/V</i>	% <i>m/m</i>	ρ_{20} (kg/m ³)	% <i>V/V</i>	% <i>m/m</i>	ρ_{20} (kg/m ³)
93.6	90.46	816.66	96.9	95.16	803.70
93.7	90.59	816.30			
93.8	90.73	815.93	97.0	95.31	803.27
93.9	90.87	815.55	97.1	95.45	802.85
			97.2	95.60	802.42
94.0	91.01	815.18	97.3	95.75	801.99
94.1	91.15	814.81	97.4	95.90	801.55
94.2	91.29	814.43	97.5	96.05	801.12
94.3	91.43	814.06	97.6	96.21	800.68
94.4	91.56	813.68	97.7	96.36	800.24
94.5	91.70	813.30	97.8	96.51	799.80
94.6	91.84	812.92	97.9	96.66	799.35
94.7	91.98	812.54			
94.8	92.13	812.15	98.0	96.81	798.90
94.9	92.27	811.77	98.1	96.97	798.45
			98.2	97.12	798.00
95.0	92.41	811.38	98.3	97.28	797.54
95.1	92.55	810.99	98.4	97.43	797.08
95.2	92.69	810.60	98.5	97.59	796.62
95.3	92.83	810.21	98.6	97.74	796.15
95.4	92.98	809.82	98.7	97.90	795.68
95.5	93.12	809.42	98.8	98.06	795.21
95.6	93.26	809.02	98.9	98.22	794.73
95.7	93.41	808.63			
95.8	93.55	808.23	99.0	98.38	794.25
95.9	93.69	807.82	99.1	98.53	793.77
			99.2	98.69	793.28
96.0	93.84	807.42	99.3	98.86	792.79
96.1	93.98	807.01	99.4	99.02	792.30
96.2	94.13	806.61	99.5	99.18	791.80
96.3	94.27	806.20	99.6	99.34	791.29
96.4	94.42	805.78	99.7	99.50	790.79
96.5	94.57	805.37	99.8	99.67	790.28
96.6	94.71	804.96	99.9	99.83	789.76
96.7	94.86	804.54			
96.8	95.01	804.12	100.0	100.0	789.24

01/2005:50600

5.6. ASSAY OF INTERFERONS

The following chapter is published for information.

1. INTRODUCTION

Monographs on human interferons generally contain a bioassay based on the inhibitory activity of the interferon on the cytopathic action of a virus on a cell line in culture. In most cases, however, the virus, cell line and the assay details are not specified, in order to allow the appropriate flexibility, where the monograph covers more than one sub-class of interferon.

The present text is intended to provide outline information for the analyst on how to design, optimise and validate such an assay once an appropriate combination of cell line and cytopathic virus has been identified. A detailed procedure for a particular cytopathic antiviral assay is described as an example of a suitable method, together with information on other virus-cell line combinations and guidance on how to adapt and validate the procedure for these other combinations.

2. ANTIVIRAL (CYTOPATHIC EFFECT REDUCTION) ASSAYS

The antiviral assay of human interferons is based on the induction of a cellular response in human cells, which prevents or reduces the cytopathic effect of an infectious virus. The potency of interferon is estimated by comparing its protective effect against a viral cytopathic effect with the same effect of the appropriate reference preparation calibrated in International Units.

3. INTERFERON ASSAY USING Hep2c CELLS AND INFECTIOUS ENCEPHALOMYOCARDITIS VIRUS

The antiviral assay of human interferons described is of the cytopathic effect reduction type. It uses human Hep2c cells infected by encephalomyocarditis virus (EMCV) to measure the potencies of different human interferon test preparations. This assay has been used in three World Health Organisation (WHO) international collaborative studies of candidate International Standards for human interferon alpha, human interferon beta and human interferon gamma and has repeatedly been demonstrated to be sensitive, reliable and reproducible for potency estimations of the different types of human interferon.

For the culture of mammalian cells, all procedures are carried out using standard operating procedures for the maintenance of such cell lines in culture. Volumes of reagents are indicated for cell cultures carried out in 75 cm² flasks. Other types of containers (flasks or plates) may be used but volumes must be adapted accordingly.

3.1. MAINTENANCE AND PREPARATION OF *Her2c* CELLS

Hep2c cells are maintained and passaged in culture medium A.

Cells are stored as frozen stocks using standard operating procedures. Growing cells may be maintained in culture up to a permitted passage number of 30, after which new cultures are established from frozen stocks.

At the beginning of the assay procedure, harvest the cells from the flasks showing 90 per cent confluent monolayers using the trypsin-treatment procedure described below.

- Remove the culture medium from the flasks.
- To each flask, add 5 ml of trypsin solution heated at 37 °C (a trypsin stock solution contains 4 mg/ml of *trypsin R* and 4 mg/ml of *sodium edetate R*; immediately before use, dilute 50 times with phosphate buffered saline). Swirl the capped flask to wash the cell monolayer. Remove the excess of trypsin solution.
- Incubate the flasks for 5 min to 10 min at 37 °C. Microscopically or visually observe the cells for signs of detachment. When viewed microscopically, the cells appear rounded up or detached and free-floating. Shake the flask vigorously to detach all the cells, add approximately 5 ml of culture medium A. Shake vigorously to yield a suspension of single cells.
- To prepare the cell suspensions for the assay procedure, carefully disperse the cells by pipetting up and down to disrupt cell aggregates, count the cells and resuspend at a concentration of 6×10^5 cells/ml.

3.2. PROPAGATION OF ENCEPHALOMYOCARDITIS VIRUS

Encephalomyocarditis virus is propagated in mouse L-929 cells in order to produce a stock of progeny virus. L-929 cells are maintained by trypsin treatment and passage as described for Hep2c cells (*NOTE: it may be necessary to substitute neonatal calf serum with foetal bovine serum if the cells show poor growth*).

Take several flasks containing confluent cultures of L-929 cells. Pour off the medium from the flasks. Inoculate with 2 ml of the EMCV suspension appropriately diluted in culture medium B so that it contains approximately 2.5×10^8 plaque forming units (PFU) per millilitre. Each flask will contain $4-6 \times 10^7$ L-929 cells and therefore the multiplicity of infection (m.o.i.) will be approximately 10 PFU/cell. Carefully swirl the virus suspension over the entire cell monolayer and return the flasks to the incubator for approximately 1 h. Maintain the medium at pH 7.4 to 7.8.

After adsorption of the EMCV, add approximately 40 ml of culture medium B to each flask and return the flasks to the incubator at 37 °C for about 30 h. Maintain the medium at pH 7.4 to 7.8 to obtain a maximum virus yield. Remove the culture fluid and store at approximately 40 °C.

Place the flasks at –20 °C to freeze the cell monolayer. Then thaw to room temperature. Add approximately 5 ml of culture medium and shake the flask to disrupt the cell walls. Transfer the contents of each flask to the container of culture fluid. Transfer the culture fluid containing the EMCV to 50 ml plastic centrifuge tubes and centrifuge at approximately 500 *g* for about 10 min to remove cell debris. Dispense the clarified culture fluid into glass screw-capped bottles, in quantities of 20 ml, 10 ml, 5 ml, 1 ml, 0.5 ml or 0.2 ml, as appropriate. Store at –70 °C. Larger volumes can be thawed, dispensed into smaller quantities and re-frozen when required. The EMCV stock will retain its original titre if stored permanently at approximately –70 °C, but repeated freeze-thaw cycles or storage at higher temperatures, e.g. at approximately –20 °C, results in progressive loss of titre.

3.3. ASSAY PROCEDURE

3.3.1. Determination of the dose-response range

Preparation of the solutions

Dilute the appropriate standard for interferon (for example a specific WHO sub-type interferon standard) in culture medium A, in 10-fold dose increments, to give doses covering the range of 1000 - 0.001 IU/ml. Carry out the assay procedure in 96-well microtitre plates. To each well add 100 µl of culture medium A. Add approximately 100 µl of each dilution of the reference preparation to each well except for those intended for virus controls. Using a multichannel pipette set at 100 µl, mix the contents of the wells.

Dispensing of the cell suspension

Pour the cell suspension of Hep2c cells, which has been adjusted to contain approximately 6×10^5 cells/ml of culture medium A, into a plastic Petri-dish. Dispense the cell suspension from the Petri-dish into each well of the microtitre plates, using a multichannel pipette set at 100 µl. Incubate the plates for about 24 h in an incubator set at 37 °C and 5 per cent CO₂.

Viral infection

At this stage, using an inverted microscope, check that the monolayers of Hep2c cells are confluent, that they show a relatively even distribution of cells, that they have correct morphology and that they are healthy.

Remove most of the culture medium from the wells by inverting the plate and shaking it and blotting on a paper towel (proceed in an identical way when discarding fluids from micro-titre plates as described later). Dilute the EMCV stock with fresh culture medium A to a titre of approximately 3×10^7 PFU/ml (*NOTE: each plate requires approximately 20 ml of diluted virus, plus 5 per cent to 10 per cent of extra volume*). Dispense the diluted suspension from a 9 cm sterile Petri-dish using a multichannel pipette set at 200 µl to all wells including virus controls, but excluding cell controls. Add approximately 200 µl of culture medium A without virus to each of the cell control wells.

Return the plates to the incubator set at 37 °C and 5 per cent CO₂ for approximately 24 h.

Staining

Examine the plates microscopically to check that the EMCV has caused a cytopathic effect (c.p.e.) in the virus controls. The time interval for maximum c.p.e. may vary from one assay to the next because of inherent variation of Hep2c cells to virus challenge over a given period of continuous cultivation.

Remove most of the culture medium from the wells by discarding into an appropriate decontaminating solution (sodium hypochlorite is suitable). Dispense *phosphate buffered saline pH 7.4 R* into each well. Discard the *phosphate buffered saline pH 7.4 R* into a decontaminating solution. Dispense into each well 150 µl of staining solution. Stain the cells for approximately 30 min at room temperature. Discard the staining solution into a decontaminating solution. Dispense approximately 150 µl of fixing solution. Fix for 10 min at room temperature. Discard the fixing solution into a decontaminating solution and wash the cell monolayers by immersing the assay plates in a plastic box containing running water. Discard the water and superficially dry the plates with paper towels. Dry the assay plates at 20 °C to 37 °C until all moisture has evaporated.

Add 150 µl of 0.1 M sodium hydroxide to each well. Elute the stain by gentle agitation of the plates or by hitting them against the palm of the hand. Make sure that the stain is evenly distributed in all wells before making spectrophotometric readings.

Read the absorbance at 610 nm to 620 nm, using a microtitre plate reader, taking as a blank a well or a column of wells containing no cells and approximately 150 µl of 0.1 M sodium hydroxide.

Estimate the concentrations of interferon standard that give the maximum and minimum reduction of cytopathic effect. This is the dose response corresponding to the working range of the assay.

3.3.2. Assay procedure

Carry out the assay as described above, using:

- as test solutions, the substance to be examined, diluted in two-fold increments with culture medium A to give nominal concentrations covering the working range of the assay,
- as reference solutions, the appropriate standard for interferon (for example, a specific WHO sub-type interferon) in culture medium A, diluted in two-fold increments to give nominal concentrations covering the working range of the assay.

3.3.3. Data analysis

Results of the cytopathic effect reduction assay generally fit a sigmoidal dose-response curve, when the interferon concentration (the log of the reciprocal of the interferon dilution) is plotted versus stain absorbance.

Plot the interferon concentration (log reciprocal of dilution) versus the stain absorbance for the interferon reference preparation and for the interferon test solutions. Using the linear portion of the curve, calculate the concentration of interferon in the sample by comparing the responses for test and reference solutions, using the usual statistical methods for a parallel line assay.

4. VALIDATION OF OTHER PROCEDURES

4.1. CHOICE OF CELL LINE AND VIRUS

A number of other combinations of cell line and virus have been used in anti-viral assays for interferons. For example, EMCV has been used in combination with the A549 epithelial lung carcinoma cell line, Semliki Forest virus or Sindbis virus have been used with human fibroblasts, and vesicular stomatitis virus has been used with either human diploid fibroblasts, the human amnion WISH cell line or the Madin-Darby bovine kidney cell line. In each case the choice of the cell line/virus combination is usually based on that which gives the most sensitive response to the interferon preparation to be assayed, and gives parallel responses when comparing the test preparation and interferon standard.

4.2. CHOICE OF RESPONSE

The staining procedure described above measures remaining viable cells. A number of other responses have been used, including methyl violet or crystal violet staining, or the thiazolyl blue (MTT) conversion procedure. In each case, the method is selected on the basis of producing a suitably linear and sensitive relationship between response colour and viable cell count.

4.3. STATISTICAL VALIDATION

As with all parallel line bioassays, the assay must satisfy the usual statistical criteria of linearity of response, parallelism and variance.

4.4. VALIDATION OF ASSAY LAYOUT

As with all microtitre plate assay procedures, attention must be given to validating the assay layout. In particular, bias due to non-random pipetting order or plate edge effects must be investigated and eliminated, by randomising the assay layout, or by avoiding the use of edge wells.

REAGENTS AND CULTURE MEDIA

Culture medium A (10 per cent neonatal calf serum)

RPMI 1640 culture medium, supplemented with antibiotics if necessary (penicillin 10 000 IU/ml; streptomycin 10 ng/ml)	450 ml
L-Glutamine, 200 mM, sterile	5 ml
Neonatal calf serum	50 ml

Culture medium B (2 per cent foetal bovine serum)

RPMI 1640 culture medium, supplemented with antibiotics if necessary (penicillin 10 000 IU/ml; streptomycin 10 ng/ml)	490 ml
L-Glutamine, 200 mM, sterile	5 ml
Foetal bovine serum	10 ml

Staining solution

Naphthalene black	0.5 g
Acetic acid, glacial	90 ml
Sodium acetate, anhydrous	8.2 g
Water to produce	1000 ml

Fixing solution

Formaldehyde, 40 per cent	100 ml
Acetic acid, glacial	90 ml
Sodium acetate, anhydrous	8.2 g
Water to produce	1000 ml

01/2005:50700 ** PTB (Physikalisch-Technische Bundesanstalt, Braunschweig, Germany),

*** NPL (National Physical Laboratory, Teddington, Middlesex, UK).

5.7. TABLE OF PHYSICAL CHARACTERISTICS OF RADIONUCLIDES MENTIONED IN THE EUROPEAN PHARMACOPOEIA

The following table is given to complete the general monograph on *Radiopharmaceutical preparations (0125)*.

The values are obtained from the database of the National Nuclear Data Center (NNDC) at Brookhaven National Laboratory, Upton. N.Y., USA, directly accessible via Internet at the address: "http://www.nndc.bnl.gov/nndc/nudat/radform.html".

In case another source of information is preferred (more recent values), this source is explicitly mentioned.

Other data sources:

* DAMRI (Département des Applications et de la Métrologie des Rayonnements Ionisants, CEA Gif-sur-Yvette, France),

The uncertainty of the half-lives are given in parentheses. In principle the digits in parentheses are the standard uncertainty of the corresponding last digits of the indicated numerical value "Guide to the Expression of Uncertainty in measurement", International Organisation for Standardisation (ISO), 1993, ISBN 92-67-10188-9).

The following abbreviations are used:

e_A = Auger electrons,

ce = conversion electrons,

β^- = electrons,

β^+ = positrons,

γ = gamma rays,

X = X-rays.

Radionuclide	Half-life	Electronic emission			Photon emission		
		Type	Energy (MeV)	Emission probability (per 100 disintegrations)	Type	Energy (MeV)	Emission probability (per 100 disintegrations)
Tritium (^3H)	*12.33 (6) years	* β^-	*0.006 ^(I) (max: 0.019)	*100			
Carbon-11 (^{11}C)	20.385 (20) min	β^+	0.386 ^(I) (max: 0.960)	99.8	γ	0.511	199.5 ^(II)
Nitrogen-13 (^{13}N)	9.965 (4) min	β^+	0.492 ^(I) (max: 1.198)	99.8	γ	0.511	199.6 ^(II)
Oxygen-15 (^{15}O)	122.24 (16) s	β^+	0.735 ^(I) (max: 1.732)	99.9	γ	0.511	199.8 ^(II)
Fluorine-18 (^{18}F)	109.77 (5) min	β^+	0.250 ^(I) (max: 0.633)	96.7	γ	0.511	193.5 ^(II)
Phosphorus-32 (^{32}P)	14.26 (4) days	β^-	0.695 ^(I) (max: 1.71)	100			
Phosphorus-33 (^{33}P)	25.34 (12) days	β^-	0.076 ^(I) (max: 0.249)	100			
Sulphur-35 (^{35}S)	87.51 (12) days	β^-	0.049 ^(I) (max: 0.167)	100			
Chromium-51 (^{51}Cr)	27.7025 (24) days	e_A	0.004	67	X	0.005	22.3
					γ	0.320	9.9

(I) Mean energy of the β spectrum.

(II) Maximum emission probability corresponding to a total annihilation in the source per 100 disintegrations.

Radionuclide	Half-life	Electronic emission			Photon emission		
		Type	Energy (MeV)	Emission probability (per 100 disintegrations)	Type	Energy (MeV)	Emission probability (per 100 disintegrations)
Cobalt-56 (⁵⁶ Co)	77.27 (3) days	e _A	0.006	47	X	0.006-0.007	25
		β ⁺	0.179 ^(I) 0.631 ^(I)	0.9 18.1	γ	0.511	38.0 ^(II)
						0.847	100.0
						1.038	14.1
						1.175	2.2
						1.238	66.1
						1.360	4.3
						1.771	15.5
						2.015	3.0
						2.035	7.8
						2.598	17.0
		3.202	3.1				
3.253	7.6						
Cobalt-57 (⁵⁷ Co)	271.79 (9) days	e _A +ce	0.006-0.007	177.4	X	0.006-0.007	57
		ce	0.014 0.115 0.129	7.4 1.8 1.3	γ	0.014	9.2
						0.122	85.6
						0.136	10.7
						0.692	0.15
Cobalt-58 (⁵⁸ Co)	70.86 (7) days	e _A	0.006	49.4	X	0.006-0.007	26.3
		β ⁺	0.201 ^(I)	14.9	γ	0.511	29.9 ^(II)
						0.811	99.4
						0.864	0.7
					1.675	0.5	
Cobalt-60 (⁶⁰ Co)	5.2714 (5) years	β ⁻	0.096 ^(I) (max: 0.318)	99.9	γ	1.173	100.0
						1.333	100.0
(I) Mean energy of the β spectrum.							
(II) Maximum emission probability corresponding to a total annihilation in the source per 100 disintegrations.							

Radionuclide	Half-life	Electronic emission			Photon emission		
		Type	Energy (MeV)	Emission probability (per 100 disintegrations)	Type	Energy (MeV)	Emission probability (per 100 disintegrations)
Gallium-66 (⁶⁶ Ga)	9.49 (7) hours	e _A	0.008	21	X	0.009-0.010	19.1
		β ⁺	0.157 ^(I)	1	γ	0.511	112 ^(II)
			0.331 ^(I)	0.7		0.834	5.9
			0.397 ^(I)	3.8		1.039	37
			0.782 ^(I)	0.3		1.333	1.2
			1.90 ^(I)	50		1.919	2.1
						2.190	5.6
						2.423	1.9
						2.752	23.4
						3.229	1.5
						3.381	1.5
						3.792	1.1
						4.086	1.3
						4.295	4.1
						4.807	1.8
Gallium-67 (⁶⁷ Ga)	3.2612 (6) days	e _A	0.008	62	X	0.008-0.010	57
		ce	0.082-0.084	30.4	γ	0.091-0.093	42.4
			0.090-0.092	3.6		0.185	21.2
			0.175	0.3		0.209	2.4
						0.300	16.8
						0.394	4.7
						0.888	0.15
Germanium-68 (⁶⁸ Ge) in equilibrium with Gallium-68 (⁶⁸ Ga)	270.82 (27) days (⁶⁸ Ga: 67.629 (24) min)	e _A	0.008	42.4	X	0.009-0.010	44.1
		β ⁺	0.353 ^(I)	1.2	γ	0.511	178.3
			0.836 ^(I)	88.0		1.077	3.0
Gallium-68 (⁶⁸ Ga)	67.629 (24) min	e _A	0.008	5.1	X	0.009-0.010	4.7
		β ⁺	0.353 ^(I)	1.2	γ	0.511	178.3
			0.836 ^(I)	88.0		1.077	3.0
Krypton-81m (⁸¹ mKr)	13.10 (3) s	ce	0.176	26.4	X	0.012-0.014	17.0
			0.189	4.6			
					γ	0.190	67.6
(I) Mean energy of the β spectrum. (II) Maximum emission probability corresponding to a total annihilation in the source per 100 disintegrations.							

Radionuclide	Half-life	Electronic emission			Photon emission		
		Type	Energy (MeV)	Emission probability (per 100 disintegrations)	Type	Energy (MeV)	Emission probability (per 100 disintegrations)
Rubidium-81 (⁸¹ Rb) in equilibrium with Krypton-81m (^{81m} Kr)	4.576 (5) hours (^{81m} Kr:13.10 (3) s)	e _A	0.011	31.3	X	0.013-0.014	57.2
		ce	0.176	25.0	γ	0.190	64
			0.188	4.3		0.446	23.2
						0.457	3.0
		β ⁺	0.253 ^(I)	1.8		0.510	5.3
			0.447 ^(I)	25.0		0.511	54.2
						0.538	2.2
Strontium-89 (⁸⁹ Sr) in equilibrium with Yttrium-89m (^{89m} Y)	50.53 (7) days (^{89m} Y:16.06 (4) s)	β ⁻	0.583 ^(I) (max: 1.492)	99.99	γ	0.909	0.01
Strontium-90 (⁹⁰ Sr) in equilibrium with Yttrium-90 (⁹⁰ Y)	28.74 (4) years (⁹⁰ Y: 64.10 (8) hours)	β ⁻	0.196 ^(I) (max: 0.546)	100			
Yttrium-90 (⁹⁰ Y)	64.10 (8) hours	β ⁻	0.934 ^(I) (max: 2.280)	100			
Molybdenum-99 (⁹⁹ Mo) in equilibrium with Technetium-99m (^{99m} Tc)	65.94 (1) hours (^{99m} Tc: 6.01 (1) hours)	β ⁻	0.133 ^(I)	16.4	X	0.018-0.021	3.6
			0.290 ^(I)	1.1	γ	0.041	1.1
			0.443 ^(I)	82.4		0.141	4.5
						0.181	6
						0.366	1.2
						0.740	12.1
						0.778	4.3
Technetium-99m (^{99m} Tc)	6.01 (1) hours	ce	0.002	74	X	0.018-0.021	7.3
		e _A	0.015	2.1	γ	0.141	89.1
		ce	0.120	9.4			
			0.137-0.140	1.3			
Technetium-99 (⁹⁹ Tc)	2.11 × 10 ⁵ years	β ⁻	0.085 ^(I) (max: 0.294)	100			
(I) Mean energy of the β spectrum. (II) Maximum emission probability corresponding to a total annihilation in the source per 100 disintegrations.							

Radionuclide	Half-life	Electronic emission			Photon emission		
		Type	Energy (MeV)	Emission probability (per 100 disintegrations)	Type	Energy (MeV)	Emission probability (per 100 disintegrations)
Ruthenium-103 (¹⁰³ Ru) in equilibrium with Rhodium-103m (^{103m} Rh)	39.26 (2) days (^{103m} Rh: 56.114 (20) min)	e _A +ce	0.017	12	X	0.020-0.023	9.0
		ce	0.030-0.039	88.3	γ	0.497	91
						0.610	5.8
		β ⁻	0.031 ^(I) 0.064 ^(I)	6.6 92.2			
Indium-110 (¹¹⁰ In)	4.9 (1) hours	e _A	0.019	13.4	X	0.023-0.026	70.5
					γ	0.642	25.9
						0.658	98.3
						0.885	92.9
						0.938	68.4
Indium-110m (^{110m} In)	69.1 (5) min	e _A	0.019	5.3	X	0.023-0.026	27.8
					γ	0.511	123.4 ^(II)
						0.658	97.8
						2.129	2.1
Indium-111 (¹¹¹ In)	2.8047 (5) days	e _A	0.019	15.6	X	0.003	6.9
						0.023-0.026	82.3
					γ	0.171	90.2
						0.245	94.0
Indium-114m (^{114m} In) in equilibrium with Indium-114 (¹¹⁴ In)	49.51 (1) days (¹¹⁴ In: 71.9 (1) s)	ce	0.162 0.186-0.190 0.777 ^(I) (max: 1.985)	40 40 95	X	0.023-0.027	36.3
					γ	0.190	15.6
						0.558	3.2
						0.725	3.2
Tellurium-121m (^{121m} Te) in equilibrium with Tellure-121 (¹²¹ Te)	154.0 (7) days (¹²¹ Te: 19.16 (5) days)	e _A	0.003 0.022-0.023 0.050 0.077 0.180	88.0 7.4 33.2 40.0 6.1	X	0.026-0.031	50.5
					γ	0.212	81.4
						1.102	2.5

(I) Mean energy of the β spectrum.
(II) Maximum emission probability corresponding to a total annihilation in the source per 100 disintegrations.

Radionuclide	Half-life	Electronic emission			Photon emission					
		Type	Energy (MeV)	Emission probability (per 100 disintegrations)	Type	Energy (MeV)	Emission probability (per 100 disintegrations)			
Tellurium-121 (¹²¹ Te)	**19.16 (5) days	e _A	0.022	11.6	X	0.026-0.030	75.6			
					γ	0.470	1.4			
						0.508	17.7			
						0.573	80.3			
Iodine-123 (¹²³ I)	13.27 (8) hours	e _A	0.023	12.3	X	0.004	9.3			
					ce	0.027-0.031	86.6			
		γ	0.127	13.6						
			0.154	1.8		0.159	83.3			
			0.158	0.4		0.346	0.1			
		0.440	0.4							
		0.505	0.3							
		0.529	1.4							
		0.538	0.4							
Iodine-125 (¹²⁵ I)	59.402 (14) days	e _A +ce	0.004	80	X	0.004	15.5			
			0.023-0.035	33	γ	0.027	114			
						0.031	26			
			0.035	6.7						
Iodine-126 (¹²⁶ I)	13.11 (5) days	e _A	0.023	6	X	0.027-0.031	42.2			
					ce	γ	0.354	0.5	0.388	34
							0.634	0.1	0.491	2.9
		β ⁻	0.511	2.3 ^(II)						
			0.109 ^(I)	3.6	0.666	33				
			0.290 ^(I)	32.1	0.754	4.2				
			0.459 ^(I)	8.0	0.880	0.8				
			1.420	0.3						
		β ⁺	0.530 ^(I)	1						
(I) Mean energy of the β spectrum.										
(II) Maximum emission probability corresponding to a total annihilation in the source per 100 disintegrations.										

Radionuclide	Half-life	Electronic emission			Photon emission		
		Type	Energy (MeV)	Emission probability (per 100 disintegrations)	Type	Energy (MeV)	Emission probability (per 100 disintegrations)
Iodine-131 (¹³¹ I)	8.02070 (11) days	ce	0.46	3.5	X	0.029-0.030	3.9
			0.330	1.6	γ	0.080	2.6
		β ⁻	0.069 ^(I)	2.1		0.284	6.1
			0.097 ^(I)	7.3		0.365	81.7
			0.192 ^(I)	89.9		0.637	7.2
					0.723	1.8	
Xenon-131m (^{131m} Xe)	11.84 (7) days	e _A	0.025	6.8	X	0.004	8.3
		ce				0.030	44.0
			0.129	61		0.034	10.2
			0.159	28.5	γ	0.164	2.0
			0.163	8.3			
Iodine-133 (¹³³ I) (decays to radioactive Xenon-133)	20.8 (1) hours	β ⁻	0.140 ^(I)	3.8	γ	0.530	87
			0.162 ^(I)	3.2		0.875	4.5
			0.299 ^(I)	4.2		1.298	2.4
			0.441 ^(I)	83			
Xenon-133 (¹³³ Xe)	5.243 (1) days	e _A	0.026	5.8	X	0.004	6.3
		ce				0.031	40.3
			0.045	55.1		0.035	9.4
			0.075-0.080	9.9	γ	0.080	38.3
			0.101 ^(I)	99.0			
Xenon-133m (^{133m} Xe) (decays to radioactive Xenon-133)	2.19 (1) days	e _A	0.025	7	X	0.004	7.8
		ce				0.030	45.9
			0.199	64.0		0.034	10.6
			0.228	20.7	γ	0.233	10.0
			0.232	4.6			
(I) Mean energy of the β spectrum. (II) Maximum emission probability corresponding to a total annihilation in the source per 100 disintegrations.							

Radionuclide	Half-life	Electronic emission			Photon emission		
		Type	Energy (MeV)	Emission probability (per 100 disintegrations)	Type	Energy (MeV)	Emission probability (per 100 disintegrations)
Iodine-135 (¹³⁵ I) (decays to radioactive Xenon-135)	6.57 (2) hours	β ⁻	0.140 ^(I)	7.4	γ	0.527	13.8
			0.237 ^(I)	8		0.547	7.2
			0.307 ^(I)	8.8		0.837	6.7
			0.352 ^(I)	21.9		1.039	8.0
			0.399 ^(I)	8		1.132	22.7
			0.444 ^(I)	7.5		1.260	28.9
			0.529 ^(I)	23.8		1.458	8.7
			1.678	9.6			
1.791	7.8						
Xenon-135 (¹³⁵ Xe)	9.14 (2) hours	ce	0.214	5.5	X	0.031-0.035	5.0
		β ⁻	0.171	3.1	γ	0.250	90.2
			0.308	96.0		0.608	2.9
Caesium-137 (¹³⁷ Cs) in equilibrium with Barium-137m (^{137m} Ba)	30.04 (3) years (^{137m} Ba: 2.552 (1) min)	e _A	0.026	0.8	X	0.005 0.032-0.036	1 7
		ce	0.624	8.0		γ	0.662
			0.656	1.4			
		β ⁻	0.174 ^(I)	94.4			
			0.416 ^(I)	5.6			
Thallium-200 (²⁰⁰ Tl)	26.1 (1) hours	ce	0.285	3.4	X	0.010	32.0
			0.353	1.4		0.069-0.071	63.3
						0.08	17.5
		β ⁺	0.495 ^(I)	0.3	γ	0.368	87.2
						0.579	13.8
						0.828	10.8
						1.206	29.9
						1.226	3.4
						1.274	3.3
						1.363	3.4
						1.515	4.0
						(I) Mean energy of the β spectrum. (II) Maximum emission probability corresponding to a total annihilation in the source per 100 disintegrations.	

Radionuclide	Half-life	Electronic emission			Photon emission		
		Type	Energy (MeV)	Emission probability (per 100 disintegrations)	Type	Energy (MeV)	Emission probability (per 100 disintegrations)
Lead-201 (²⁰¹ Pb) (decays to radioactive Thallium-201)	9.33 (3) hours	e _A	0.055	3	X	0.070-0.073	69
						0.083	19
		ce	0.246	8.5	γ	0.331	79
						0.361	9.9
						0.406	2.0
						0.585	3.6
						0.692	4.3
						0.767	3.2
						0.826	2.4
						0.908	5.7
						0.946	7.9
						1.099	1.8
						1.277	1.6
Thallium-201 (²⁰¹ Tl)	72.912 (17) hours	ce	0.016-0.017	17.7	X	0.010	46.0
			0.027-0.029	4.1		0.069-0.071	73.7
			0.052	7.2		0.080	20.4
			0.084	15.4			
			0.153	2.6		0.135	2.6
					γ	0.167	10.0
Thallium-202 (²⁰² Tl)	12.23 (2) days	e _A	0.054	2.8	X	0.010	31.0
		ce	0.357	2.4		0.069-0.071	61.6
						0.080	17.1
					γ	0.440	91.4
Lead-203 (²⁰³ Pb)	51.873 (9) hours	e _A	0.055	3.0	X	0.010	37.0
		ce	0.194	13.3		0.071-0.073	69.6
						0.083	19.4
					γ	0.279	80.8
						0.401	3.4
(I) Mean energy of the β spectrum. (II) Maximum emission probability corresponding to a total annihilation in the source per 100 disintegrations.							

01/2005:50800

5.8. PHARMACOPOEIAL HARMONISATION

This general chapter is included for guidance of users. It provides information on the degree of harmonisation and consequently interchangeability of various general chapters and monographs of the European Pharmacopoeia and those of the Japanese Pharmacopoeia and United States Pharmacopoeia. The chapter does not affect in any way the status of the monographs and general chapters as the authoritative reference in any case of doubt or dispute where compliance with the European Pharmacopoeia is required.

The European Pharmacopoeia Commission recognises the utility of working with other pharmacopoeial bodies to develop harmonised monographs and general chapters. Such harmonisation is fully compatible with the declared aims of the Commission and has benefits of different kinds, notably the simplification and rationalisation of quality control methods and licensing procedures. Such harmonisation also enhances the benefits of the work of the International Conference on Harmonisation (ICH) and the Veterinary International Co-operation on Harmonisation (VICH) since some of the guidelines developed depend on pharmacopoeial general chapters for their application.

Work on harmonisation is carried out by a well-defined but informal process in the Pharmacopoeial Discussion Group (PDG), in which the European Pharmacopoeia, the Japanese

Pharmacopoeia and the United States Pharmacopoeia are associated. Information will be given in subsequent revised versions of this general chapter on items that have been dealt with by the PDG.

Where harmonisation of general chapters is carried out, the aim is to arrive at interchangeable methods or requirements so that demonstration of compliance using a general chapter from one of the three pharmacopoeias implies that the same result would be obtained using the general chapter of either of the other pharmacopoeias. If residual differences remain in harmonised general chapters, information will be given in subsequent versions of this general chapter.

Where harmonisation of monographs is carried out, the aim is to arrive at identical requirements for all attributes of a product. For some products, it can be extremely difficult to achieve complete harmonisation, for example because of differences in legal status and interpretation. It has therefore appeared to the PDG worthwhile to approve and publish monographs in which as many attributes as possible are harmonised. Information on any non-harmonised attributes will be included in subsequent versions of this general chapter.

The three Pharmacopoeias have undertaken not to make unilateral changes to harmonised monographs and general chapters but rather to apply the agreed revision procedure whereby all partners adopt a revision simultaneously.

01/2005:50900

5.9. POLYMORPHISM

Polymorphism (or crystal polymorphism) is a phenomenon related to the solid state; it is the ability of a compound in the solid state to exist in different crystalline forms having the same chemical composition. Substances that exist in a non-crystalline solid state are said to be amorphous.

When this phenomenon is observed for a chemical element (for example, sulphur), the term allotropy is used instead of polymorphism.

The term pseudopolymorphism is used to describe solvates (including hydrates), where a solvent is present in the crystal matrix in stoichiometric proportions; the term may also be extended to include compounds where the solvent is trapped in the matrix in variable proportions. However the term pseudopolymorphism is ambiguous because of its use in different circumstances. It is therefore preferable to use only the terms “solvates” and “hydrates”.

Where a monograph indicates that a substance shows polymorphism, this may be true crystal polymorphism, occurrence of solvates, allotropy or occurrence of the amorphous form.

The identity of chemical composition implies that all crystalline and amorphous forms of a given species have the same chemical behaviour in solution or as a melt; in contrast, their physico-chemical and physical characteristics (solubility, hardness, compressibility, density, melting point, etc.), and therefore their reactivity and bioavailability may be different at the solid state.

When a compound shows polymorphism, the form for which the free enthalpy is lowest at a given temperature and pressure is the most thermodynamically stable. The other forms are said to be in a metastable state. At normal temperature and pressure, a metastable form may remain unchanged or may change to a thermodynamically more stable form.

If there are several crystalline forms, one form is thermodynamically more stable at a given temperature and pressure. A given crystalline form may constitute a phase that can reach equilibrium with other solid phases and with the liquid and gas phases.

If each crystalline form is the more stable within a given temperature range, the change from one form to another is reversible and is said to be enantiotropic. The change from

one phase to another is a univariate equilibrium, so that at a given pressure this state is characterised by a transition temperature. However, if only one of the forms is stable over the entire temperature range, the change is irreversible or monotropic.

Different crystalline forms or solvates may be produced by varying the crystallisation conditions (temperature, pressure, solvent, concentration, rate of crystallisation, seeding of the crystallisation medium, presence and concentration of impurities, etc.).

The following techniques may be used to study polymorphism:

- X-ray diffraction of powders,
- X-ray diffraction of single crystals,
- thermal analysis (2.2.34) (differential scanning calorimetry, thermogravimetry, thermomicroscopy),
- microcalorimetry,
- moisture absorption analysis,
- optical and electronic microscopy,
- solid-state nuclear magnetic resonance,
- infrared absorption spectrophotometry (2.2.24),
- Raman spectrometry (2.2.48),
- measurement of solubility and intrinsic dissolution rate,
- density measurement.

These techniques are often complementary and it is indispensable to use several of them.

Pressure/temperature and energy/temperature diagrams based on analytical data are valuable tools for fully understanding the energetic relationship (enantiotropism, monotropism) and the thermodynamic stability of the individual modifications of a polymorphic compound.

For solvates, differential scanning calorimetry and thermogravimetry are preferable, combined with measurements of solubility, intrinsic dissolution rate and X-ray diffraction.

For hydrates, water sorption/desorption isotherms are determined to demonstrate the zones of relative stability.

In general, hydrates are less soluble in water than anhydrous forms, and likewise solvates are less soluble in their solvent than unsolvated forms.

01/2005:51000

5.10. CONTROL OF IMPURITIES IN SUBSTANCES FOR PHARMACEUTICAL USE

Preamble

The monographs of the European Pharmacopoeia on substances for pharmaceutical use are designed to ensure acceptable quality for users. The role of the Pharmacopoeia in public health protection requires that adequate control of impurities be provided by monographs. The quality required is based on scientific, technical and regulatory considerations.

Requirements concerning impurities are given in specific monographs and in the general monograph *Substances for pharmaceutical use (2034)*. Specific monographs and the general monograph are complementary: specific monographs prescribe acceptance criteria for impurities whereas the general monograph deals with the need for qualification, identification and reporting of any organic impurities that occur in *active substances*.

The thresholds for reporting, identification and qualification contained in the general monograph *Substances for pharmaceutical use (2034)* apply to all related substances. However, if a monograph does not contain a related substances test based on a quantitative method, any new impurities occurring above a threshold may be overlooked since the test is not capable to detect those impurities.

The provisions of the Related substances section of the general monograph *Substances for pharmaceutical use (2034)*, notably those concerning thresholds, do not apply to excipients; also excluded from the provisions of this section are: biological and biotechnological products; peptides; oligonucleotides; radiopharmaceuticals; fermentation products and semisynthetic products derived therefrom; herbal products and crude products of animal and plant origin. Although the thresholds stated in the general monograph do not apply, the general concepts of reporting, identification (wherever possible) and qualification of impurities are equally valid for these classes.

Basis for the elaboration of monographs of the European Pharmacopoeia

European Pharmacopoeia monographs are elaborated on substances that are present in medicinal products that have been authorised by the competent authorities of Parties to the *European Pharmacopoeia Convention*. Consequently, these monographs do not necessarily cover all sources of substances for pharmaceutical use on the world market.

Organic and inorganic impurities present in those substances that have been evaluated by the competent authorities are qualified with respect to safety at the maximum authorised content (at the maximum daily dose) unless new safety data that become available following evaluation justify lower limits.

European Pharmacopoeia monographs on substances for pharmaceutical use are elaborated by groups of experts and working parties collaborating with national pharmacopoeia authorities, the competent authorities for marketing authorisation, national control laboratories and the European Pharmacopoeia laboratory; they are also assisted by the producers of the substances and/or the pharmaceutical manufacturers that use these substances.

Control of impurities in substances for pharmaceutical use

The quality with respect to impurities is controlled by a set of tests within a monograph. These tests are intended to cover

organic and inorganic impurities that are relevant in view of the sources of active substances in authorised medicinal products.

Control of residual solvents is provided by the general monograph *Substances for pharmaceutical use (2034)* and general chapter 5.4. *Residual solvents*. The certificate of suitability of a monograph of the European Pharmacopoeia for a given source of a substance indicates the residual solvents that are controlled together with the specified acceptance criteria and the validated control method where this differs from those described in general chapter 2.4.24. *Identification and control of residual solvents*.

Monographs on organic chemicals usually have a test entitled "Related substances" that covers relevant organic impurities. This test may be supplemented by specific tests where the general test does not control a given impurity or where there are particular reasons (for example, safety reasons) for requiring special control.

Where a monograph has no Related substances (or equivalent) test but only specific tests, the user of a substance must nevertheless ensure that there is suitable control of organic impurities; those occurring above the identification threshold are to be identified (wherever possible) and, unless justified, those occurring above the qualification threshold are to be qualified (see also under Recommendations to users of monographs of active substances).

Where the monograph covers substances with different impurity profiles, it may have a single related substances test to cover all impurities mentioned in the Impurities section or several tests may be necessary to give control of all known profiles. Compliance may be established by carrying out only the tests relevant to the known impurity profile for the source of the substance.

Instructions for control of impurities may be included in the Production section of a monograph, for example where the only analytical method appropriate for the control of a given impurity is to be performed by the manufacturer since the method is too technically complex for general use or cannot be applied to the final drug substance and/or where validation of the production process (including the purification step) will give sufficient control.

Impurities section in monographs on active substances

The Impurities section in a monograph includes impurities (chemical structure and name wherever possible), which are usually organic, that are known to be detected by the tests prescribed in the monograph. It is based on information available at the time of elaboration or revision of the monograph and is not necessarily exhaustive. The section includes specified impurities and, where so indicated, other detectable impurities.

Specified impurities have an acceptance criterion not greater than that authorised by the competent authorities.

Other detectable impurities are potential impurities with a defined structure but not known to be normally present above the identification threshold in substances used in medicinal products that have been authorised by the competent authorities of Parties to the Convention. They are given in the Impurities section for information.

Where an impurity other than a specified impurity is found in an active substance it is the responsibility of the user of the substance to check whether it has to be identified/qualified, depending on its content, nature, maximum daily dose and relevant identification/qualification threshold, in accordance with the general monograph on *Substances for pharmaceutical use (2034)*, Related substances section.

It should be noted that specific thresholds are applied to substances exclusively for veterinary use.

Interpretation of the test for related substances in the monographs on active substances

A specific monograph on a substance for pharmaceutical use is to be read and interpreted in conjunction with the general monograph on *Substances for pharmaceutical use (2034)*.

Where a general acceptance criterion for impurities (“any other impurity”, “other impurities”, “any impurity”) equivalent to a nominal content greater than the applicable identification threshold (see the general monograph on *Substances for pharmaceutical use (2034)*) is prescribed, this is valid only for specified impurities mentioned in the Impurities section. The need for identification (wherever possible), reporting, specification and qualification of other impurities that occur must be considered according to the requirements of the general monograph. It is the responsibility of the user of the substance to determine the validity of the acceptance criteria for impurities not mentioned in the Impurities section and for those indicated as other detectable impurities.

The following examples are given as an aid in the interpretation of acceptance criteria for impurities in specific monographs. The examples correspond to different styles currently used in monographs pending harmonisation in a future edition.

Example 1

Consider a monograph on a substance for human use where the Impurities section lists 8 impurities (A-H), 2 of which are indicated to be other detectable impurities (G, H) and there are the following acceptance criteria:

- *impurity A*: not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent),
- *any other impurity*: not more than the area of the principal peak in the chromatogram obtained with reference solution (c) (0.2 per cent),
- *total*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (1.0 per cent),
- *disregard limit*: 0.25 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.05 per cent).

A substance complies with the test if:

- impurity A is present at a nominal concentration less than or equal to 0.5 per cent,
- impurities B, C, D, E, F are each present at a nominal concentration less than or equal to 0.2 per cent,
- any other impurity is present at a nominal concentration less than or equal to the identification threshold applicable for the active substance,
- the sum of nominal concentrations of impurities that are above the disregard limit is less than or equal to 1.0 per cent.

Example 2

Consider a monograph on a substance for human use where the Impurities section lists 6 impurities (A-F) with the following acceptance criteria.

In the chromatogram obtained with the test solution: the area of any peak, apart from the principal peak, is not greater than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent); the sum of the areas of all peaks, apart from the principal peak, is not greater than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (1.0 per

cent). Disregard any peak obtained with the blank and any peak with an area less than 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (b).

A substance complies with the test if:

- impurities A, B, C, D, E, F are each present at a nominal concentration less than or equal to 0.5 per cent,
- any other impurity is present at a nominal concentration less than or equal to the identification threshold applicable for the active substance,
- the sum of nominal concentrations of impurities that are above the disregard limit is less than or equal to 1.0 per cent.

Example 3

Consider a monograph on a substance for human use (for which the maximum daily dose is not more than 2 g/day) in more explicit style where the Impurities section lists 7 impurities, 6 of which (A-F) are defined as *Specified impurities* and the seventh (G) is given under *Other detectable impurities*.

Limits:

- *correction factor*: for the calculation of content, multiply the peak area of impurity A by 2.3,
- *impurity B*: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.3 per cent),
- *impurity E*: not more than 4 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.4 per cent),
- *impurities A, C, D, F*: for each impurity, not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent),
- *any other impurity*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent),
- *total*: not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (1.0 per cent),
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

A substance complies with the test if:

- impurity B is present at a nominal concentration less than or equal to 0.3 per cent,
- impurity E is present at a nominal concentration less than or equal to 0.4 per cent,
- impurities A, C, D, F are each present at a nominal concentration less than or equal to 0.2 per cent; the peak area of impurity A has been multiplied by 2.3 for the calculation of content,
- any other impurity is present at a nominal concentration less than or equal to 0.10 per cent (identification threshold),
- the sum of nominal concentrations of impurities that are above the disregard limit is less than or equal to 1.0 per cent.

Recommendations to users of monographs of active substances

Monographs give a specification for suitable quality of substances with impurity profiles corresponding to those taken into account during elaboration and/or revision of the monograph. It is the responsibility of the user of the substance to check that the monograph provides adequate control of impurities for a substance for pharmaceutical use from a given source, notably by using the procedure

for certification of suitability of the monographs of the European Pharmacopoeia.

A monograph with a related substances test based on a quantitative method (such as liquid chromatography, gas chromatography and capillary electrophoresis) provides adequate control of impurities for a substance from a given source if impurities present in amounts above the applicable identification threshold are specified impurities mentioned in the Impurities section.

If the substance contains impurities other than those mentioned in the Impurities section, it has to be verified that these impurities are detectable by the method described in the monograph, otherwise a new method must be developed and revision of the monograph must be requested. Depending on the contents found and the limits proposed, the identification and/or the qualification of these impurities must be considered.

Where a single related substances test covers different impurity profiles, only impurities for the known profile from a single source need to be reported in the certificate of analysis unless the marketing authorisation holder uses active substances with different impurity profiles.

Identification of impurities (peak assignment)

Where a monograph has an individual limit for an impurity, it is often necessary to define means of identification, for example using a reference substance, a representative chromatogram or relative retention. The user of the substance may find it necessary to identify impurities other than those for which the monograph provides a means of identification, for example to check the suitability of the specification for a given impurity profile by comparison with the Impurities section. The European Pharmacopoeia does not provide reference substances, representative chromatograms or information on relative retentions for this purpose, unless prescribed in the monograph. Users will therefore have to apply the available scientific techniques for identification.

New impurities/Specified impurities above the specified limit

Where a new manufacturing process or change in an established process leads to the occurrence of a new impurity, it is necessary to apply the provisions of the general monograph on *Substances for pharmaceutical use (2034)* regarding identification and qualification and to verify the suitability of the monograph for control of the impurity. A certificate of suitability is a means for confirming for a substance from a given source that the new impurity is adequately controlled or the certificate contains a method for control with a defined acceptance criterion. In the latter case revision of the monograph will be initiated.

Where a new manufacturing process or change in an established process leads to the occurrence of a specified impurity above the specified limit, it is necessary to apply the provisions of the general monograph on *Substances for pharmaceutical use (2034)* regarding qualification.

Chromatographic methods

General chapter 2.2.46. *Chromatographic separation techniques* deals with various aspects of impurities control.

Information is available via the EDQM web site (www.pheur.org) on commercial names for columns and other reagents and equipment found suitable during monograph development, where this is considered useful.

GLOSSARY

Disregard limit: in chromatographic tests, the nominal content at or below which peaks/signals are not taken into account for calculating a sum of impurities. The numerical values for the disregard limit and the reporting threshold are usually the same.

Identification threshold: a limit above which an impurity is to be identified.

Identified impurity: an impurity for which structural characterisation has been achieved.

Impurity: any component of a substance for pharmaceutical use that is not the chemical entity defined as the substance.

Nominal concentration: concentration calculated on the basis of the concentration of the prescribed reference and taking account of the prescribed correction factor.

Other detectable impurities: potential impurities with a defined structure that are known to be detected by the tests in a monograph but not known to be normally present above the identification threshold in substances used in medicinal products that have been authorised by the competent authorities of Parties to the Convention. They are unspecified impurities and are thus limited by a general acceptance criterion.

Potential impurity: an impurity that theoretically can arise during manufacture or storage. It may or may not actually appear in the substance. Where a potential impurity is known to be detected by the tests in a monograph but not known to be normally present in substances used in medicinal products that have been authorised by the competent authorities of Parties to the Convention, it will be included in the Impurities section under *Other detectable impurities* for information.

Qualification: the process of acquiring and evaluating data that establishes the biological safety of an individual impurity or a given impurity profile at the level(s) specified.

Qualification threshold: a limit above which an impurity is to be qualified.

Related substances: title used in monographs for general tests for organic impurities.

Reporting threshold: a limit above which an impurity is to be reported. Synonym: reporting level.

Specified impurity: an impurity that is individually listed and limited with a specific acceptance criterion in a monograph. A specified impurity can be either identified or unidentified.

Unidentified impurity: an impurity for which a structural characterisation has not been achieved and that is defined solely by qualitative analytical properties (for example, relative retention).

Unspecified impurity: an impurity that is limited by a general acceptance criterion and not individually listed with its own specific acceptance criterion.

01/2005:51100

5.11. CHARACTERS SECTION IN MONOGRAPHS

The General Notices indicate that the statements included in the Characters section are not to be interpreted in a strict sense and are not requirements. For information of users, the methods recommended to authors of monographs as the basis for statements concerning hygroscopicity, crystallinity and solubility are given below.

HYGROSCOPICITY

This method is to be carried out on a substance that complies with the test for loss on drying or water content of the monograph. The method gives an indication of the degree of hygroscopicity rather than a true determination.

Use a glass weighing vessel 50 mm in external diameter and 15 mm high. Weigh the vessel and stopper (m_1). Place the amount of substance prescribed for the test for loss on drying or water in the vessel and weigh (m_2). Place the unstoppered vessel in a desiccator at 25 °C containing a saturated solution of ammonium chloride or ammonium sulphate or place it in a climatic cabinet set at 25 ± 1 °C and 80 ± 2 per cent relative humidity. Allow to stand for 24 h. Stopper the weighing vessel and weigh (m_3).

Calculate the percentage increase in mass using the expression:

$$\frac{m_3 - m_2}{m_2 - m_1} \times 100$$

The result is interpreted as follows:

- *deliquescent*: sufficient water is absorbed to form a liquid,
- *very hygroscopic*: increase in mass is equal to or greater than 15 per cent,
- *hygroscopic*: increase in mass is less than 15 per cent and equal to or greater than 2 per cent,
- *slightly hygroscopic*: increase in mass is less than 2 per cent and equal to or greater than 0.2 per cent.

CRYSTALLINITY

This method is employed to establish the crystalline or amorphous nature of a substance.

Mount a few particles of the substance to be examined in mineral oil on a clean glass slide. Examine under a polarising microscope. Crystalline particles exhibit birefringence and extinction positions when the microscope stage is revolved.

SOLUBILITY

For this test a maximum of 111 mg of substance (for each solvent) and a maximum of 30 ml of each solvent are necessary.

Dissolving procedure

Shake vigorously for 1 min and place in a constant temperature device, maintained at a temperature of 25.0 ± 0.5 °C for 15 min. If the substance is not completely dissolved, repeat the shaking for 1 min and place the tube in the constant temperature device for 15 min.

Method

Weigh 100 mg of finely powdered substance (90) in a stoppered tube (16 mm in internal diameter and 160 mm long), add 0.1 ml of the solvent and proceed as described under Dissolving Procedure. If the substance is completely dissolved, it is *very soluble*.

If the substance is not completely dissolved, add 0.9 ml of the solvent and proceed as described under Dissolving Procedure. If the substance is completely dissolved, it is *freely soluble*.

If the substance is not completely dissolved, add 2.0 ml of the solvent and proceed as described under Dissolving Procedure. If the substance is completely dissolved, it is *soluble*.

If the substance is not completely dissolved, add 7.0 ml of the solvent and proceed as described under Dissolving Procedure. If the substance is completely dissolved, it is *sparingly soluble*.

If the substance is not completely dissolved, weigh 10 mg of finely powdered substance (90) in a stoppered tube, add 10.0 ml of the solvent and proceed as described under Dissolving Procedure. If the substance is completely dissolved, it is *slightly soluble*.

If the substance is not completely dissolved, weigh 1 mg of finely powdered substance (90) in a stoppered tube, add 10.0 ml of the solvent and proceed as described under Dissolving Procedure. If the substance is completely dissolved, it is *very slightly soluble*.

01/2005:1063

ALLERGEN PRODUCTS

Producta allergenica

DEFINITION

Allergen products are pharmaceutical preparations derived from extracts of naturally occurring source materials containing allergens, which are substances that cause or provoke allergic (hypersensitivity) disease. The allergenic components are most often of a proteinaceous nature.

Allergen products are intended for *in vivo* diagnosis or treatment of allergic (hypersensitivity) diseases attributed to these allergens.

Allergen products are available as finished products, as bulk preparations in dried form, solutions or suspensions intended to be further concentrated or diluted prior to use or as final preparations in solutions, suspensions or freeze-dried. Allergen products intended for parenteral, bronchial and conjunctival administration are sterile.

For *diagnostic use*, allergen products are usually prepared as unmodified extracts in a 50 per cent V/V solution of glycerol for skin-prick testing. For intradermal diagnosis or for provocation tests by nasal, ocular or bronchial routes, suitable dilutions of allergen products may be prepared by dilution of aqueous or glycerinated extracts, or by reconstitution immediately before use of unmodified freeze-dried extracts.

For *immunotherapy*, allergen products may be either unmodified extracts or extracts modified chemically and/or by adsorption onto different carriers (for example, aluminium hydroxide, calcium phosphate or tyrosine).

This monograph does not apply: to chemicals that are used solely for diagnosis of contact dermatitis; to chemically synthesised products; to allergens derived by rDNA technology; to finished products used on a named-patient basis. It does not necessarily apply to allergen products for veterinary use.

PRODUCTION

Allergen products are derived from a wide range of allergenic source materials. They are often prepared as bulk products intended to be further diluted or concentrated prior to use. They may be treated to modify or reduce the allergenic activity or remain unmodified.

SOURCE MATERIALS

Source materials for the preparation of allergen products are mostly pollens, moulds, mites, animal epithelia, hymenoptera venoms and certain foods.

They are described by their origin, nature, method of collection or production and pretreatment, and are stored under defined conditions which minimise deterioration.

The collection or production, as well as the handling of the source materials are such that uniform qualitative and quantitative composition is ensured as far as possible from batch to batch.

Pollens. Potential chemical contaminants, such as pesticides and heavy metals, must be minimised. Pollens contain not more than 1 per cent of foreign pollens as determined by microscopic examination. Pollens contain not more than 1 per cent of mould spores as determined by microscopic examination.

Mites and moulds. Biologically active contaminants such as mycotoxins in moulds must be minimised and any presence justified. Care must be taken to minimise any allergenic constituents of the media used for the cultivation of mites

and moulds as source materials. Culture media that contain substances of human or animal origin must be justified and, when required must be suitably treated to ensure the inactivation or elimination of possible transmissible agents of disease.

Animal epithelia. Animal epithelia must be obtained from healthy animals selected to avoid possible transmissible agents of disease.

MANUFACTURING PROCESS

Allergen products are generally obtained by extraction, and may be purified, from the source materials using appropriate methods shown to preserve the biological properties of the allergenic components. Allergen products are manufactured under conditions designed to minimise microbial growth and enzymatic degradation.

A purification procedure, if any, is designed to minimise the content of any potential irritant low-molecular-mass components or other non-allergenic components.

Allergen products may contain a suitable antimicrobial preservative. The nature and the concentration of the antimicrobial preservative have to be justified.

The manufacturing process comprises various stages.

Native allergen extracts result after separation from the extracted source materials.

Intermediate allergen products are obtained by further processing or modification of the native allergen extracts. The modification may be achieved by chemical processes (chemical conjugation) or physical processes (physical adsorption onto different carriers, for example, aluminium hydroxide, calcium phosphate or tyrosine). They may also be modified by inclusion into such vehicles as liposomes or microspheres, or by the addition of other biologically active agents to enhance efficacy or safety. Intermediate allergen products may be freeze-dried.

Bulk allergen preparations consist of products in solution or suspension which will not be further processed or modified, and are ready for dilution or filling into final containers.

IN-HOUSE REFERENCE PREPARATION

An appropriate representative preparation is selected as the In-House Reference Preparation (IHRP), characterised and used to verify batch-to-batch consistency. The IHRP is stored in suitably sized aliquots under conditions ensuring its stability, usually freeze-dried.

Characterisation of the In-House Reference Preparation.

The extent of characterisation of the IHRP depends on the nature of the allergenic source material, knowledge of the allergenic components and availability of suitable reagents, as well as the intended use. The characterised IHRP is used as reference in the batch control of native allergen extracts or intermediate allergen products and, if possible, in the batch control of final allergen preparations.

The In-House Reference Preparation (IHRP) is characterised by the protein content determination and a protein profile using relevant methods (such as isoelectric focusing, polyacrylamide gel electrophoresis, immunoelectrophoresis or molecular-mass profiling). Allergenic components may be detected by appropriate methods (for example immunoblotting or crossed radio-immunoelectrophoresis). Characterisation of the allergenic components may include identification of relevant allergens based on serological or others techniques using a pool or individual sera from allergic patients, or allergen-specific polyclonal or monoclonal antibodies. When allergen reference substances are available determination of the content of individual allergens may be performed. Individual allergens are identified according to internationally established nomenclature whenever possible.

Where possible, the biological potency of the IHRP is established by *in vivo* techniques such as skin testing, and expressed in units of biological activity. If not, for certain extracts, potency may be established by suitable immunoassays (for example, those based on the inhibition of the binding capacity of specific immunoglobulin E antibodies) or by quantitative techniques for a single major component.

IDENTIFICATION

Identity is confirmed at the intermediate or other applicable stage by comparison with the IHRP using protein profiling by appropriate methods (for example, isoelectric focusing, sodium dodecyl sulphate-polyacrylamide gel electrophoresis or immunoelectrophoresis).

TESTS

Various biochemical and immunological tests have been developed in order to characterise allergens qualitatively and quantitatively. However, some of the methods, particularly for the determination of allergenic activity and allergen profile, are not applicable to all products at present. This is because knowledge of the allergenic components or the required reagents is not available. Accordingly, allergen products have been classified in different categories with increasing test requirements, according to quality and intended use.

Where possible the following tests are applied to the final preparations. If not, they must be performed on the extracts as late as possible in the manufacturing process, for example, at the stage immediately prior to that stage (modification, dilution etc.) which renders the test not feasible on the final preparation.

Water (2.5.12). Not more than 5 per cent for freeze-dried products.

Sterility (2.6.1). Allergen products intended for parenteral, bronchial and conjunctival administration comply with the test for sterility.

Protein content: 80 per cent to 120 per cent of the stated content of a given batch. If the biological potency can be determined then the test for protein content may be omitted.

Protein profile. The protein composition determined by suitable methods corresponds to that of the IHRP.

Abnormal toxicity (2.6.9). Allergen products obtained from moulds and intended for parenteral administration (except skin-prick tests) comply with the test for abnormal toxicity for immunosera and vaccines for human use.

Various additional tests, somewith increasing selectivity, depending on the allergen product concerned can be applied, but in any case for allergen products intended for therapeutic use a validated test measuring the potency (total allergenic activity, determination of individual allergens or any other justified tests) must be applied.

Aluminium (2.5.13). Not less than 80 per cent and not more than 120 per cent of the stated amount but in any case not more than 1.25 mg per human dose unless otherwise justified and authorised, when aluminium hydroxide or aluminium phosphate is used as adsorbent.

Calcium (2.5.14). Not less than 80 per cent and not more than 120 per cent of the stated amount when calcium phosphate is used as adsorbent.

Antigen profile. The antigens are identified by means of suitable techniques using antigen-specific animal antibodies.

Allergen profile. Relevant allergenic components are identified by means of suitable techniques using allergen-specific human antibodies.

Total allergenic activity. The activity is 50 per cent to 200 per cent of the stated amount as assayed by inhibition of the binding capacity of specific immunoglobulin E antibodies or a suitable equivalent *in vitro* method.

Individual allergens: 50 per cent to 200 per cent of the stated amount, determined by a suitable method.

STORAGE

Adsorbed allergen products should not be frozen.

LABELLING

The label states:

- the biological potency and/or the protein content and/or the extraction concentration;
- the route of administration and the intended use;
- the storage conditions;
- where applicable, the name and amount of added antimicrobial preservative;
- for freeze-dried preparations:
 - the name, composition and volume of the reconstituting liquid to be added
 - the period of time within which the preparation is to be used after reconstitution;
- where applicable, that the preparation is sterile;
- where applicable the name and amount of adsorbent.

01/2005:0765

EXTRACTS

Extracta

DEFINITION

Extracts are preparations of liquid (liquid extracts and tinctures), semi-solid (soft extracts) or solid (dry extracts) consistency, obtained from herbal drugs or animal matters, which are usually in a dry state.

Different types of extract may be distinguished. Standardised extracts are adjusted within an acceptable tolerance to a given content of constituents with known therapeutic activity; standardisation is achieved by adjustment of the extract with inert material or by blending batches of extracts. Quantified extracts are adjusted to a defined range of constituents; adjustments are made by blending batches of extracts. Other extracts are essentially defined by their production process (state of the herbal drug or animal matter to be extracted, solvent, extraction conditions) and their specifications.

PRODUCTION

Extracts are prepared by suitable methods using ethanol or other suitable solvents. Different batches of the herbal drug or animal matter may be blended prior to extraction. The herbal drug or animal matter to be extracted may undergo a preliminary treatment, for example, inactivation of enzymes, grinding or defatting. In addition, unwanted matter may be removed after extraction.

Herbal drugs, animal matters and organic solvents used for the preparation of extracts comply with any relevant monograph of the Pharmacopoeia. For soft and dry extracts where the organic solvent is removed by evaporation, recovered or recycled solvent may be used, provided that the recovery procedures are controlled and monitored to ensure that solvents meet appropriate standards before re-use or admixture with other approved materials. Water used for the preparation of extracts is of suitable quality. Except for the test for bacterial endotoxins, water complying with

the section on Purified water in bulk of the monograph on *Purified water (0008)* is suitable. Potable water may be suitable if it complies with a defined specification that allows the consistent production of a suitable extract.

Where applicable, concentration to the intended consistency is carried out using suitable methods, usually under reduced pressure, and at a temperature at which deterioration of the constituents is reduced to a minimum. Essential oils that have been separated during processing may be restored to the extracts at an appropriate stage in the manufacturing process. Suitable excipients may be added at the various stages of the manufacturing process for example to improve technological qualities such as homogeneity or consistency. Suitable stabilisers and antimicrobial preservatives may also be added.

Extraction with a given solvent leads to typical proportions of characterised constituents in the extractable matter; during production of standardised and quantified extracts, purification procedures may be applied that increase these proportions with respect to the expected values; such extracts are referred to as “refined”.

IDENTIFICATION

Extracts are identified using a suitable method.

TESTS

Where applicable, as a result of analysis of the herbal drug or animal matter used for production and in view of the production process, tests for microbiological quality (5.1.4), heavy metals, aflatoxins, pesticide residues (2.8.13) in the extracts may be necessary.

ASSAY

Wherever possible, extracts are assayed by a suitable method.

LABELLING

The label states:

- the herbal drug or animal matter used,
- whether the extract is liquid, soft or dry, or whether it is a tincture,
- for standardised extracts, the content of constituents with known therapeutic activity,
- for quantified extracts, the content of constituents (markers) used for quantification,
- the ratio of the starting material to the genuine extract (DER),
- the solvent or solvents used for extraction,
- where applicable, that a fresh herbal drug or fresh animal matter has been used,
- where applicable, that the extract is “refined”,
- the name and amount of any excipient used including stabilisers and antimicrobial preservatives,
- where applicable, the percentage of dry residue.

Liquid extracts – extracta fluida

DEFINITION

Liquid extracts are liquid preparations of which, in general, 1 part by mass or volume is equivalent to 1 part by mass of the dried herbal drug or animal matter. These preparations are adjusted, if necessary, so that they satisfy the requirements for content of solvent, and, where applicable, for constituents.

PRODUCTION

Liquid extracts are prepared by using ethanol of suitable concentration or water to extract the herbal drug or animal matter, or by dissolving a soft or dry extract (which has been

produced using the same strength of extraction solvent as is used in preparing the liquid extract by direct extraction) of the herbal drug or animal matter in either ethanol of suitable concentration or water. Liquid extracts may be filtered, if necessary.

A slight sediment may form on standing, which is acceptable as long as the composition of the liquid extract is not changed significantly.

TESTS

Relative density (2.2.5). Where applicable, the liquid extract complies with the limits prescribed in the monograph.

Ethanol (2.9.10). For alcoholic liquid extracts, carry out the determination of ethanol content. The ethanol content complies with that prescribed.

Methanol and 2-propanol (2.9.11): maximum 0.05 per cent V/V of methanol and maximum 0.05 per cent V/V of 2-propanol for alcoholic liquid extracts unless otherwise prescribed.

Dry residue (2.8.16). Where applicable, the liquid extract complies with the limits prescribed in the monograph, corrected if necessary, taking into account any excipient used.

STORAGE

Protected from light.

LABELLING

The label states in addition to the requirements listed above:

- where applicable, the ethanol content in per cent V/V in the final extract.

Tinctures – tincturae

DEFINITION

Tinctures are liquid preparations which are usually obtained using either 1 part of herbal drug or animal matter and 10 parts of extraction solvent or 1 part of herbal drug or animal matter and 5 parts of extraction solvent.

PRODUCTION

Tinctures are prepared by maceration or percolation (outline methodology is given below) using only ethanol of a suitable concentration for extraction of the herbal drug or animal matter, or by dissolving a soft or dry extract (which has been produced using the same strength of extraction solvent as is used in preparing the tincture by direct extraction) of the herbal drug or animal matter in ethanol of a suitable concentration. Tinctures are filtered, if necessary.

Tinctures are usually clear. A slight sediment may form on standing which is acceptable as long as the composition of the tincture is not changed significantly.

Production by maceration. Unless otherwise prescribed, reduce the herbal drug or animal matter to be extracted to pieces of suitable size, mix thoroughly with the prescribed extraction solvent and allow to stand in a closed container for an appropriate time. The residue is separated from the extraction solvent and, if necessary, pressed out. In the latter case, the 2 liquids obtained are combined.

Production by percolation. If necessary, reduce the herbal drug or animal matter to be extracted to pieces of suitable size. Mix thoroughly with a portion of the prescribed extraction solvent and allow to stand for an appropriate time. Transfer to a percolator and allow the percolate to flow at room temperature slowly making sure that the herbal drug

or animal matter to be extracted is always covered with the remaining extraction solvent. The residue may be pressed out and the expressed liquid combined with the percolate.

01/2005:1434

TESTS

Relative density (2.2.5). Where applicable, the tincture complies with the limits prescribed in the monograph.

Ethanol (2.9.10). The ethanol content complies with that prescribed.

Methanol and 2-propanol (2.9.11): maximum 0.05 per cent V/V of methanol and maximum 0.05 per cent V/V of 2-propanol, unless otherwise prescribed.

Dry residue (2.8.16). Where applicable, the tincture complies with the limits prescribed in the monograph, corrected if necessary, taking into account any excipient used.

STORAGE

Protected from light.

LABELLING

The label states in addition to the requirements listed above:

- for tinctures other than standardised and quantified tinctures, the ratio of starting material to extraction liquid or of starting material to final tincture,
- the ethanol content in per cent V/V in the final tincture.

Soft extracts – *extracta spissa*

DEFINITION

Soft extracts are semi-solid preparations obtained by evaporation or partial evaporation of the solvent used for extraction.

TESTS

Dry residue (2.8.16). The soft extract complies with the limits prescribed in the monograph.

Solvents. Where applicable, a monograph on a soft extract prescribes a limit test for the solvent used for extraction.

STORAGE

Protected from light.

Dry extracts – *extracta sicca*

DEFINITION

Dry extracts are solid preparations obtained by evaporation of the solvent used for their production. Dry extracts usually have a loss on drying or a water content of not greater than 5 per cent *m/m*.

TESTS

Water (2.2.13). Where applicable, the dry extract complies with the limits prescribed in the monograph.

Loss on drying (2.8.17). Where applicable, the dry extract complies with the limits prescribed in the monograph.

Solvents. Where applicable, a monograph on a dry extract prescribes a limit test for the solvent used for extraction.

STORAGE

In an airtight container, protected from light.

HERBAL DRUG PREPARATIONS

Plantae medicinales praeparatore

DEFINITION

Herbal drug preparations are obtained by subjecting herbal drugs to treatments such as extraction, distillation, expression, fractionation, purification, concentration or fermentation. These include comminuted or powdered herbal drugs, tinctures, extracts, essential oils, expressed juices and processed exudates.

Herbal teas comply with the monograph on *Herbal teas* (1435).

Instant herbal teas consist of powder or granules of one or more herbal drug preparation(s) intended for the preparation of an oral solution immediately before use.

01/2005:1433

HERBAL DRUGS

Plantae medicinales

DEFINITION

Herbal drugs are mainly whole, fragmented or cut, plants, parts of plants, algae, fungi, lichen in an unprocessed state, usually in dried form but sometimes fresh. Certain exudates that have not been subjected to a specific treatment are also considered to be herbal drugs. Herbal drugs are precisely defined by the botanical scientific name according to the binominal system (genus, species, variety and author).

PRODUCTION

Herbal drugs are obtained from cultivated or wild plants. Suitable collection, cultivation, harvesting, drying, fragmentation and storage conditions are essential to guarantee the quality of herbal drugs.

Herbal drugs are, as far as possible, free from impurities such as soil, dust, dirt and other contaminants such as fungal, insect and other animal contaminations. They are not rotten. If a decontaminating treatment has been used, it is necessary to demonstrate that the constituents of the plant are not affected and that no harmful residues remain. The use of ethylene oxide is prohibited for the decontamination of herbal drugs.

IDENTIFICATION

Herbal drugs are identified using their macroscopic and microscopic descriptions and any further tests that may be required (for example, thin-layer chromatography).

TESTS

A test for foreign matter (2.8.2) is carried out, unless otherwise prescribed in the individual monographs.

A specific appropriate test may apply to herbal drugs liable to be falsified.

If appropriate, the herbal drugs comply with other tests, for example, total ash (2.4.16), ash insoluble in hydrochloric acid (2.8.1), extractable matter, swelling index (2.8.4) and bitterness value.

The test for loss on drying (2.2.32) is carried out on herbal drugs, unless otherwise prescribed in the individual monographs. A determination of water (2.2.13) is carried out for herbal drugs with a high essential oil content.

Herbal drugs comply with the requirements for pesticide residues (2.8.13). The requirements take into account the nature of the plant, where necessary the preparation in which the plant might be used, and where available the knowledge of the complete record of treatment of the batch of the plant. The content of pesticide residues may be determined by the method described in the annex to the general method.

The risk of contamination of herbal drugs by heavy metals must be considered. If an individual monograph does not prescribe limits for heavy metals or specific elements such limits may be required if justified.

Recommendations on the microbiological quality of products consisting solely of one or more herbal drugs are given in the text on *Microbiological quality of pharmaceutical preparations* (5.1.4. – Category 4).

Where necessary limits for aflatoxins may be required.

In some specific circumstances, the risk of radioactive contamination is to be considered.

ASSAY

Unless otherwise justified and authorised herbal drugs are assayed by an appropriate method.

STORAGE

Store protected from light.

01/2005:1435

HERBAL TEAS

Plantae ad ptisanam

DEFINITION

Herbal teas consist exclusively of one or more herbal drugs intended for oral aqueous preparations by means of decoction, infusion or maceration. The preparation is prepared immediately before use.

Herbal teas are usually supplied in bulk form or in sachets.

The herbal drugs used comply with the appropriate individual European Pharmacopoeia monographs or in their absence to the general monograph on *Herbal drugs* (1433).

Recommendations on the microbiological quality of herbal teas (5.1.4. – Category 4) take into account the prescribed preparation method (use of boiling or non-boiling water).

IDENTIFICATION

The identity of herbal drugs present in herbal teas is checked by botanical examinations.

TESTS

The proportion of herbal drugs present in herbal teas is checked by appropriate methods.

Herbal teas in sachets comply with the following test:

Uniformity of mass. Determine the average mass of twenty randomly chosen units as follows: weigh a single full sachet of herbal tea, open it without losing any fragments. Empty it completely using a brush. Weigh the empty sachet and calculate the mass of the contents by subtraction. Repeat the operation on the nineteen remaining sachets. Unless otherwise justified not more than two of the twenty individual masses of the contents deviate from the average

mass of the contents by more than the percentage deviation shown in the table below and none deviates by more than twice that percentage.

Average mass	Percentage deviation
less than 1.5 g	15 per cent
1.5 g to 2.0 g included	10 per cent
more than 2.0 g	7.5 per cent

STORAGE

Store protected from light.

01/2005:0084

IMMUNOSERA FOR HUMAN USE, ANIMAL

Immunosera ex animale ad usum humanum

DEFINITION

Animal immunosera for human use are liquid or freeze-dried preparations containing purified immunoglobulins or immunoglobulin fragments obtained from serum or plasma of immunised animals of different species.

The immunoglobulins or immunoglobulin fragments have the power of specifically neutralising or binding to the antigen used for immunisation. The antigens include microbial or other toxins, human antigens, suspensions of bacterial and viral antigens and venoms of snakes, scorpions and spiders. The preparation is intended for intravenous or intramuscular administration, after dilution where applicable.

PRODUCTION

GENERAL PROVISIONS

The production method shall have been shown to yield consistently immunosera of acceptable safety, potency in man and stability.

Any reagent of biological origin used in the production of immunosera shall be free of contamination with bacteria, fungi and viruses. The method of preparation includes a step or steps that have been shown to remove or inactivate known agents of infection.

Methods used for production are validated, effective, reproducible and do not impair biological activity of the product.

The production method is validated to demonstrate that the product, if tested, would comply with the test for abnormal toxicity for immunosera and vaccines for human use (2.6.9).

Reference preparation. A batch shown to be suitable in clinical trials, or a batch representative thereof, is used as the reference preparation for the tests for high molecular mass proteins and purity.

ANIMALS

The animals used are of a species approved by the competent authority, are healthy and exclusively reserved for production of immunoserum. They are tested and shown to be free from a defined list of infectious agents. The introduction of animals into a closed herd follows specified procedures, including definition of quarantine measures. Where appropriate, additional specific agents are considered depending on the geographical localisation of the establishment used for the breeding and production of the animals. The feed originates from a controlled source and no animal proteins are added. The suppliers of animals are certified by the competent authority.

If the animals are treated with antibiotics, a suitable withdrawal period is allowed before collection of blood or plasma. The animals are not treated with penicillin antibiotics. If a live vaccine is administered, a suitable waiting period is imposed between vaccination and collection of serum or plasma for immunoserum production.

IMMUNISATION

The antigens used are identified and characterised, where appropriate; where relevant, they are shown to be free from extraneous infectious agents. They are identified by their names and a batch number; information on the source and preparation are recorded.

The selected animals are isolated for at least 1 week before being immunised according to a defined schedule with booster injections at suitable intervals. Adjuvants may be used.

Animals are kept under general health surveillance and specific antibody production is controlled at each cycle of immunisation.

Animals are thoroughly examined before collection of blood or plasma. If an animal shows any pathological lesion not related to the immunisation process, it is not used, nor are any other of the animals in the group concerned, unless it is evident that their use will not impair the safety of the product.

COLLECTION OF BLOOD OR PLASMA

Collection of blood is made by venepuncture or plasmapheresis. The puncture area is shaved, cleaned and disinfected. The animals may be anaesthetised under conditions that do not influence the quality of the product. Unless otherwise prescribed, an antimicrobial preservative may be added. The blood or plasma is collected in such a manner as to maintain sterility of the product. The blood or plasma collection is conducted at a site separate from the area where the animals are kept or bred and the area where the immunoserum is purified. If the serum or plasma is stored before further processing, precautions are taken to avoid microbial contamination.

Several single plasma or serum samples may be pooled before purification. The single or pooled samples are tested before purification for the following tests.

Tests for contaminating viruses. If an antimicrobial preservative is added, it must be neutralised before carrying out the tests or the tests carried out on a sample taken before addition of the antimicrobial preservative. Each pool is tested for contaminating viruses by suitable *in vitro* tests.

Each pool is tested for viruses by inoculation to cell cultures capable of detecting a wide range of viruses relevant for the particular product.

Potency. Carry out a biological assay as indicated in the monograph and express the result in International Units per millilitre, where applicable. A validated *in vitro* method may also be used.

Protein content. Dilute the product to be examined with a 9 g/l solution of *sodium chloride R* to obtain a solution containing about 15 mg of protein in 2 ml. To 2 ml of this solution in a round-bottomed centrifuge tube add 2 ml of a 75 g/l solution of *sodium molybdate R* and 2 ml of a mixture of 1 volume of *nitrogen-free sulphuric acid R* and 30 volumes of *water R*. Shake, centrifuge for 5 min, decant the supernatant liquid and allow the inverted tube to drain on filter paper. Determine the nitrogen in the residue by the method of sulphuric acid digestion (2.5.9) and calculate the content of protein by multiplying by 6.25. The protein content is within approved limits.

PURIFICATION AND VIRAL INACTIVATION

The immunoglobulins are concentrated and purified by fractional precipitation, chromatography, immunoabsorption or by other chemical or physical methods. They may be processed further by enzyme treatment. The methods are selected and validated to avoid contamination at all steps of processing and to avoid formation of protein aggregates that effect immunobiological characteristics of the product. For products intended to consist of immunoglobulin fragments, the methods are validated to guarantee total fragmentation. The methods of purification used are such that they do not generate additional components that compromise the quality and the safety of the product.

Unless otherwise justified and authorised, validated procedures are applied for removal and/or inactivation of viruses. The procedures are selected to avoid the formation of polymers or aggregates and, unless the product is intended to consist of Fab' fragments, to minimise the splitting of F(ab')₂ into Fab' fragments.

After purification and treatment for removal and/or inactivation of viruses, a stabiliser may be added to the intermediate product, which may be stored for a period defined in the light of stability data.

Only an intermediate product that complies with the following requirements may be used in the preparation of the final bulk.

Purity. Examine by non-reducing polyacrylamide gel electrophoresis (2.2.31), by comparison with the reference preparation, the bands are compared in intensity and no additional bands are found.

FINAL BULK

The final bulk is prepared from a single intermediate product or from a pool of intermediate products obtained from animals of the same species. Intermediate products with different specificities may be pooled.

An antimicrobial preservative and a stabiliser may be added. If an antimicrobial preservative has been added to the blood or plasma, the same substance is used as antimicrobial preservative in the final bulk.

Only a final bulk that complies with the following requirements may be used in the preparation of the final lot.

Antimicrobial preservative. Where applicable, determine the amount of antimicrobial preservative by a suitable physico-chemical method. It contains not less than 85 per cent and not more than 115 per cent of the amount stated on the label.

Sterility (2.6.1). It complies with the test for sterility.

FINAL LOT

The final bulk of immunoserum is distributed aseptically into sterile, tamper-proof containers. The containers are closed so as to prevent contamination.

Only a final lot that complies with the requirements prescribed below under Identification, Tests and Assay may be released for use. Provided that the tests for osmolality, protein content, molecular-size distribution, antimicrobial preservative, stabiliser, purity, foreign proteins and albumin and the assay have been carried out with satisfactory results on the final bulk, they may be omitted on the final lot.

Reconstitute the preparation to be examined as stated on the label immediately before carrying out the identification, tests (except those for solubility and water) and assay.

IDENTIFICATION

The identity is established by immunological tests and, where necessary, by determination of biological activity. The assay may also serve for identification.

CHARACTERS

Immunosera are clear to opalescent and colourless to very faintly yellow liquids. They are free from turbidity. Freeze-dried products are white or slightly yellow powders or solid friable masses. After reconstitution they show the same characteristics as liquid preparations.

TESTS

Solubility. To a container of the preparation to be examined, add the volume of the liquid for reconstitution stated on the label. The preparation dissolves completely within the time stated on the label.

Extractable volume (2.9.17). It complies with the requirement for extractable volume.

pH (2.2.3). The pH is within the limits approved for the particular product.

Osmolality (2.2.35): minimum 240 mosmol/kg after dilution, where applicable.

Protein content: 90 per cent to 110 per cent of the amount stated on the label, and not more than 100 g/l.

Dilute the preparation to be examined with a 9 g/l solution of *sodium chloride R* to obtain a solution containing about 15 mg of protein in 2 ml. To 2 ml of this solution in a round-bottomed centrifuge tube add 2 ml of 75 g/l solution of *sodium molybdate R* and 2 ml of a mixture of 1 volume of *nitrogen-free sulphuric acid R* and 30 volumes of *water R*. Shake, centrifuge for 5 min, decant the supernatant liquid and allow the inverted tube to drain on filter paper. Determine the nitrogen in the residue by the method of sulphuric acid digestion (2.5.9) and calculate the content of protein by multiplying by 6.25.

Molecular-size distribution. Examine by liquid chromatography (2.2.29 or 2.2.30). It complies with the specification approved for the particular product.

Antimicrobial preservative. Where applicable, determine the amount of antimicrobial preservative by a suitable physicochemical method. The amount is not less than the minimum amount shown to be effective and is not greater than 115 per cent of that stated on the label.

Phenol (2.5.15): maximum 2.5 g/l for preparations containing phenol.

Stabiliser. Determine the amount of stabiliser by a suitable physico-chemical method. The preparation contains not less than 80 per cent and not more than 120 per cent of the quantity stated on the label.

Purity. Examine by non-reducing polyacrylamide gel electrophoresis (2.2.31), by comparison with the reference preparation. No additional bands are found for the preparation to be examined.

Foreign proteins. When examined by precipitation tests with specific antisera, only protein from the declared animals species is shown to be present, unless otherwise prescribed, for example where material of human origin is used during production.

Albumin. Unless otherwise prescribed in the monograph, when examined electrophoretically, the content of albumin is not greater than the limit approved for the particular product and, in any case, not greater than 3 per cent.

Water (2.5.12): maximum 3 per cent.

Sterility (2.6.1). It complies with the test for sterility.

Pyrogens (2.6.8). Unless otherwise justified and authorised, it complies with the test for pyrogens. Unless otherwise prescribed, inject 1 ml per kilogram of the rabbit's body mass.

ASSAY

Carry out a biological assay as indicated in the monograph and express the result in International Units per millilitre, where appropriate. A validated *in vitro* method may also be used.

STORAGE

Store protected from light at the temperature stated on the label. Do not allow liquid preparations to freeze.

Expiry date. The expiry date is calculated from the beginning of the assay.

LABELLING

The label states:

- the number of International Units per millilitre, where applicable,
- the amount of protein per container,
- for freeze-dried preparations,
 - the name and volume of the reconstituting liquid to be added,
 - that the immunoserum is to be used immediately after reconstitution,
 - the time required for complete dissolution,
- the route of administration,
- the storage conditions,
- the expiry date, except for containers of less than 1 ml which are individually packed. The expiry date may be omitted from the label on the container, provided it is shown on the package and the label on the package states that the container must be kept in the package until required for use,
- the animal species of origin,
- the name and amount of any antimicrobial preservative, any stabiliser and any other substance added to the immunoserum.

01/2005:0030

IMMUNOSERA FOR VETERINARY USE

Immunosera ad usum veterinarium

DEFINITION

Immunosera for veterinary use are preparations containing immunoglobulins which have the power of specifically neutralising the toxins formed by or of specifically combining with the antigen used for their preparation. They may be crude or purified.

PRODUCTION

Immunosera are obtained from the serum of healthy animals immunised by injections of toxins or toxoids, venins of snakes, viruses, suspensions of micro-organisms or other suitable antigens. If a penicillin is used during immunisation, animals must not be bled until 8 days after the last administration. One or more suitable antimicrobial preservatives may be added and are invariably added if the preparations are issued in multidose containers.

For purified immunosera, the globulins containing the immune substances may be obtained from the crude immunoserum by enzyme treatment and fractional precipitation or by other chemical or physical methods. Purified immunosera are most stable at about pH 6.

CHARACTERS

Immunosera are liquids that vary in colour according to the method of preparation. They are distributed aseptically in sterile containers which are then closed. When they are freeze-dried they consist of crusts or powders that are soluble in water.

TESTS

The following requirements refer to liquid immunosera and reconstituted freeze-dried immunosera.

pH (2.2.3). The pH of crude immunosera is 7.0 to 8.0. The pH of purified immunosera is 6.0 to 7.0.

Foreign proteins. When examined by precipitation with specific antisera, immunosera are shown to consist exclusively of proteins of the animal species used for the preparation.

Albumins. Purified immunosera comply with the test for albumins. Unless otherwise prescribed in the monograph, when examined electrophoretically, purified immunosera show not more than a trace of albumin.

Total protein Not more than 170 g/l. Carry out the determination of nitrogen by sulphuric acid digestion (2.5.9) and multiply the result by 6.25.

Phenol (2.5.15). When the immunoserum contains phenol, the concentration is not more than 5 g/l.

Sterility (2.6.1). Immunosera comply with the test for sterility. When the volume of liquid in a container is greater than 100 ml, the method of membrane filtration is used wherever possible. If this method is used, incubate the media for not less than 14 days. Where the method of membrane filtration cannot be used, the method of direct inoculation may be used.

Where the volume of liquid in each container is 20 ml or more, the minimum volume to be used for each culture medium is 10 per cent of the contents or 5 ml whichever is less.

The appropriate number of items to be tested (2.6.1) is 1 per cent of the batch with a minimum of 4 and a maximum of 10.

POTENCY

Carry out the biological assay prescribed in the monograph and express the result in International Units per millilitre when such exist.

STORAGE

Store protected from light at a temperature of 5 ± 3 °C. Liquid immunosera should not be allowed to freeze.

Expiry date. The expiry date is calculated from the beginning of the test for Potency. It applies to immunosera stored in the prescribed conditions.

LABELLING

The label states:

- the name of the preparation,
- "for veterinary use",
- the number of International Units per millilitre, where such exist,
- the batch number or other reference,
- the storage conditions,
- the expiry date,
- the animal species for which the immunoserum is intended,
- the name of the animal species of origin,
- the name and amount of any antimicrobial preservative or other substance added to the immunoserum,

- whether any substance is likely to cause an adverse reaction,
- any contra-indications to the use of the product,
- for freeze-dried immunosera:
 - the name or composition and the volume of the reconstituting liquid to be added,
 - that the immunoserum should be used immediately after reconstitution,
- the doses recommended for different species,
- the name and address of the manufacturer.

01/2005:1468

PRODUCTS OF FERMENTATION

Producta ab fermentatione

This monograph applies to indirect gene products obtained by fermentation. It is not applicable to:

- *monographs in the Pharmacopoeia concerning vaccines for human or veterinary use;*
- *products derived from continuous cell lines of human or animal origin;*
- *direct gene products that result from the transcription and translation from nucleic acid to protein, whether or not subject to post-translational modification;*
- *products obtained by semi-synthesis from a product of fermentation and those obtained by biocatalytic transformation;*
- *whole broth concentrates or raw fermentation products.*

This monograph provides general requirements for the development and manufacture of products of fermentation. These requirements are not necessarily comprehensive in a given case and requirements complementary or additional to those prescribed in this monograph may be imposed in an individual monograph or by the competent authority.

DEFINITION

For the purposes of this monograph, products of fermentation are active or inactive pharmaceutical substances produced by controlled fermentation as indirect gene products. They are primary or secondary metabolites of micro-organisms such as bacteria, yeasts, fungi and micro-algae, whether or not modified by traditional procedures or recombinant DNA (rDNA) technology. Such metabolites include vitamins, amino acids, antibiotics, alkaloids and polysaccharides. They may be obtained by batch or continuous fermentation processes followed by procedures such as extraction, concentration, purification and isolation.

PRODUCTION

Production is based on a process that has been validated and shown to be suitable. The extent of validation depends on the critical nature of the respective process step.

CHARACTERISATION OF THE PRODUCER MICRO-ORGANISM

The history of the micro-organism used for production is documented. The micro-organism is adequately characterised. This may include determination of the phenotype of the micro-organism, macroscopic and microscopic methods and biochemical tests and, if appropriate, determination of the genotype of the micro-organism and molecular genetic tests.

PROCESSES USING A SEED-LOT SYSTEM

The *master cell bank* is a homogeneous suspension or lyophilisate of the original cells distributed into individual containers for storage. The viability and productivity of the cells under the selected storage conditions and their suitability for initiating a satisfactory production process after storage must be demonstrated.

Propagation of the master cell bank may take place through a seed-lot system that uses a working cell bank.

The *working cell bank* is a homogeneous suspension or lyophilisate of the cell material derived from the master cell bank, distributed in equal volumes into individual containers for storage (for example, in liquid nitrogen).

Production may take place by batch or continuous culture and may be terminated under defined conditions.

All containers in a cell bank are stored under identical conditions. Once removed from storage, the individual ampoules, vials or culture straws are not returned to the cell bank.

PROCESSES USING STAGED GROWTH IN CULTURES

The contents of a container of the working cell bank are used, if necessary after resuspension, to prepare an inoculum in a suitable medium. After a suitable period of growth, the cultures are used to initiate the fermentation process, if necessary following preculture in a fermentor. The conditions to be used at each stage of the process are defined and must be met with each production run.

CHANGE CONTROL

If the production process is altered in a way that causes a significant change in the impurity profile of the product, the critical steps associated with this change in impurity profile are revalidated.

If a significant change has taken place in the micro-organism used for production that causes a significant change in the impurity profile of the product, the critical steps of the production process associated with this change, particularly the procedure for purification and isolation, are revalidated.

Revalidation includes demonstration that new impurities present in the product as a result of the change are adequately controlled by the test procedures. If necessary, additional or alternative tests must be introduced with appropriate limits. If the change in the process or in the micro-organism results in an increase in the level of an impurity already present, the acceptability of such an increase is addressed.

When a master cell bank is replaced, the critical steps of the production process must be revalidated to the extent necessary to demonstrate that no adverse change has occurred in the quality and safety of the product. Particular attention must be given to possible changes in the impurity profile of the product if a modified or new micro-organism is introduced into the process.

RAW MATERIALS

The raw materials employed in the fermentation and/or down-stream processing are of suitable quality for the intended purpose. They are tested to ensure that they comply with written specifications.

Levels of bioburden in media or in the inlet air for aeration are reduced to an adequately low level to ensure that if microbiological contamination occurs, it does not adversely affect the quality, purity and safety of the product. Addition of components such as nutrients, precursors, and substrates during fermentation takes place aseptically.

IN-PROCESS CONTROLS

In-process controls are in place to ensure the consistency of the conditions during fermentation and down-stream processing and of the quality of the isolated product. Particular attention must be paid to ensure that any microbial contamination that adversely affects the quality, purity and safety of the product is detected by the controls applied.

Production conditions may be monitored, as appropriate, by suitable procedures for example to control and check:

- temperature,
- pH,
- rate of aeration,
- rate of agitation,
- pressure,

and to monitor the concentration of the required product.

DOWN-STREAM PROCESSING

At the end of fermentation, the producer micro-organism is inactivated or removed. Further processing is designed to reduce residues originating from the culture medium to an acceptable level and to ensure that the desired product is recovered with consistent quality.

Various purification processes may be used, for example, charcoal treatment, ultrafiltration and solvent extraction. It must be demonstrated that the process or processes chosen reduce to a minimum or remove:

- residues from the producer micro-organism, culture media, substrates and precursors,
- unwanted transformation products of substrates and precursors.

If necessary, suitable tests are performed either as in-process controls or on the isolated product of fermentation.

IDENTIFICATION, TESTS AND ASSAY

The requirements with which the product must comply throughout its period of validity, as well as specific test methods, are stated in the individual monographs.

STORAGE

See the individual monographs.

LABELLING

See the individual monographs.

01/2005:1483

PRODUCTS WITH RISK OF TRANSMITTING AGENTS OF ANIMAL SPONGIFORM ENCEPHALOPATHIES

Producta cum possibili transmissione
vectorium encephalopathiarum
spongiformium animalium

DEFINITION

Products with risk of transmitting agents of animal spongiform encephalopathies are those derived from tissues or secretions of animals susceptible to transmissible spongiform encephalopathies other than by experimental challenge. This monograph applies to all substances or preparations obtained from such animals and to all substances or preparations where products obtained from

such animals are included as active substances or excipients or have been used during production, for example as raw or source materials, starting materials or reagents.

PRODUCTION

Production complies with chapter *Minimising the risk of transmitting animal spongiform encephalopathy agents via medicinal products* (5.2.8).

01/2005:0125

RADIOPHARMACEUTICAL PREPARATIONS

Radiopharmaceutica

DEFINITION

For the purposes of this general monograph, radiopharmaceutical preparations cover:

- radiopharmaceutical: any medicinal product which, when ready for use, contains one or more radionuclides (radioactive isotopes) included for a medicinal purpose,
- radionuclide generator: any system incorporating a fixed parent radionuclide from which is produced a daughter radionuclide which is to be removed by elution or by any other method and used in a radiopharmaceutical preparation,
- kit for radiopharmaceutical preparation: any preparation to be reconstituted and/or combined with radionuclides in the final radiopharmaceutical preparation, usually prior to its administration,
- radiopharmaceutical precursor: any other radionuclide produced for the radio-labelling of another substance prior to administration.

A nuclide is a species of atom characterised by the number of protons and neutrons in its nucleus (and hence by its atomic number Z , and mass number A) and also by its nuclear energy state. Isotopes of an element are nuclides with the same atomic number but different mass numbers. Nuclides containing an unstable arrangement of protons and neutrons will transform spontaneously to either a stable or another unstable combination of protons and neutrons with a constant statistical probability. Such nuclides are said to be radioactive and are called radionuclides. The initial unstable nuclide is referred to as the parent radionuclide and the resulting nuclide as the daughter nuclide.

The radioactive decay or transformation may involve the emission of charged particles, electron capture (EC) or isomeric transition (IT). The charged particles emitted from the nucleus may be alpha particles (helium nucleus of mass number 4) or beta particles (negatively charged, generally called electrons or positively charged, generally called positrons). The emission of charged particles from the nucleus may be accompanied by the emission of gamma rays. Gamma rays are also emitted in the process of isomeric transition. These emissions of gamma rays may be partly replaced by the ejection of electrons known as internal conversion electrons. This phenomenon, like the process of electron capture, causes a secondary emission of X-rays (due to the reorganisation of the electrons in the atom). This secondary emission may itself be partly replaced by the ejection of electrons known as Auger electrons. Radionuclides with a deficit of neutrons may decay by emitting positrons. These radionuclides are called positron emitters. Positrons are annihilated on contact with electrons,

the process being accompanied by the emission of usually two gamma photons, each with an energy of 511 keV, generally emitted at 180° to each other, termed annihilation radiation.

The decay of a radionuclide is governed by the laws of probability with a characteristic decay constant and follows an exponential law. The time in which a given quantity of a radionuclide decays to half its initial value is termed the half-life ($T_{1/2}$).

The penetrating power of each radiation varies considerably according to its nature and its energy. Alpha particles are completely absorbed in a thickness of a few micrometers to some tens of micrometers of matter. Beta particles are completely absorbed in a thickness of several millimetres to several centimetres of matter. Gamma rays are not completely absorbed but only attenuated and a tenfold reduction may require, for example, several centimetres of lead. For most absorbents, the denser the absorbent, the shorter the range of alpha and beta particles and the greater the attenuation of gamma rays.

Each radionuclide is characterised by an invariable half-life, expressed in units of time and by the nature and energy of its radiation or radiations. The energy is expressed in electronvolts (eV), kilo-electronvolts (keV) or mega-electronvolts (MeV).

Generally the term "radioactivity" is used to describe the phenomenon of radioactive decay and to express the physical quantity (activity) of this phenomenon. The radioactivity of a preparation is the number of nuclear disintegrations or transformations per unit time.

In the International System (SI), radioactivity is expressed in becquerel (Bq) which is one nuclear transformation per second. Absolute radioactivity measurements require a specialised laboratory but identification and measurement of radiation can be carried out relatively by comparing with standardised preparations provided by laboratories recognised by the competent authority.

Radionuclidic purity: the ratio, expressed as a percentage, of the radioactivity of the radionuclide concerned to the total radioactivity of the radiopharmaceutical preparation. The relevant radionuclidic impurities are listed with their limits in the individual monographs.

Radiochemical purity: the ratio, expressed as a percentage, of the radioactivity of the radionuclide concerned which is present in the radiopharmaceutical preparation in the stated chemical form, to the total radioactivity of that radionuclide present in the radiopharmaceutical preparation. The relevant radiochemical impurities are listed with their limits in the individual monographs.

Chemical purity: in monographs on radiopharmaceutical preparations chemical purity is controlled by specifying limits on chemical impurities.

Isotopic carrier: a stable isotope of the element concerned either present or added to the radioactive preparation in the same chemical form as that in which the radionuclide is present.

Specific radioactivity: the radioactivity of a radionuclide per unit mass of the element or of the chemical form concerned.

Radioactive concentration: the radioactivity of a radionuclide per unit volume.

Total radioactivity: the radioactivity of the radionuclide, expressed per unit (vial, capsule, ampoule, generator, etc).

Starting materials: all the constituents which make up the radiopharmaceutical preparations.

Period of validity: the time during which specifications described in the monograph must be fulfilled. Expiry date and, if necessary, time must be clearly stated.

PRODUCTION

A radiopharmaceutical preparation monograph describes as precisely as possible the method of production of the radionuclide. A radiopharmaceutical preparation contains its radionuclide:

- as an element in atomic or molecular form, e.g. [^{133}Xe], [^{15}O] O_2 ,
- as an ion, e.g. [^{131}I]iodide, [$^{99\text{m}}\text{Tc}$]pertechnetate,
- included in or attached to organic molecules by chelation, e.g. [^{111}In]oxine or by covalent bonding, e.g. 2- ^{18}F fluoro-2-deoxy-D-glucose.

The practical ways of producing radionuclides for use in, or as radiopharmaceutical preparations are:

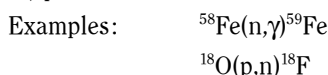
- neutron bombardment of target materials (generally in nuclear reactors),
- charged particles bombardment of target materials (in accelerators such as cyclotrons),
- nuclear fission of heavy nuclides of target materials (generally after neutron or particle bombardment),
- from a radionuclide generator.

NEUTRON OR CHARGED PARTICLE BOMBARDMENT

The nuclear reaction and the probability of its occurrence in unit time are dependent on the nature and physical properties of the target material and the nature, energy and quantity of the incident particles.

The nuclear transformation occurring through particle bombardment may be written in the form:

target nucleus (bombarding particle, emitted particle or radiation) produced nucleus.



In addition to the desired nuclear reaction adventitious transformations may occur. These will be influenced by the energy of the incident particle and the purity of the target material. Such adventitious transformations may give rise to radionuclidic impurities.

NUCLEAR FISSION

A small number of nuclides with a high atomic number are fissionable and the most frequently used reaction is the fission of uranium-235 by neutrons in a nuclear reactor. Iodine-131, molybdenum-99 and xenon-133 may be produced by nuclear fission of uranium-235. Their extraction from a mixture of more than 200 other radionuclides must be carefully controlled in order to minimise the radionuclidic impurities.

RADIONUCLIDE GENERATORS

Radionuclide generator systems use a relatively long-lived parent radionuclide which decays to a daughter radionuclide, usually with a shorter half-life.

By separating the daughter radionuclide from the parent radionuclide by a chemical or physical process, it is possible to use the daughter at a considerable distance from the production site of the generators despite its short half-life.

TARGET MATERIALS

The isotopic composition and purity of the target material will determine the relative percentages of the principal radionuclide and radionuclidic impurities. The use of isotopically enriched target material in which the abundance

of the required target nuclide has been artificially increased, can improve the production yield and the purity of the desired radionuclide.

The chemical form, the purity, the physical state and the chemical additives, as well as the bombardment conditions and the direct physical and chemical environment will determine the chemical state and chemical purity of the radionuclides which are produced.

In the production of radionuclides and particularly of short-lived radionuclides it may not be possible to determine any of these quality criteria before further processing and manufacture of radiopharmaceutical preparations. Therefore each batch of target material must be tested in test production runs before its use in routine radionuclide production and manufacture of the radiopharmaceutical preparations, to ensure that under specified conditions, the target yields the radionuclide in the desired quantity and quality specified.

The target material is contained in a holder in gaseous, liquid or solid state, in order to be irradiated by a beam of particles. For neutron bombardment, the target material is commonly contained in quartz ampoules or high purity aluminium or titanium containers. It is necessary to ascertain that no interaction can occur between the container and its contents under the irradiation conditions (temperature, pressure, time).

For charged particle bombardment, the holder for target material is usually built of aluminium or another appropriate metal, with inlet and outlet ports, a surrounding cooling system and usually a thin metal foil target window. The nature and thickness of the target window have a particular influence on the yield of the nuclear reaction and may also affect the radionuclidic purity.

The production procedure clearly describes:

- target material,
- construction of the holder for target material,
- loading of target material into the irradiation system,
- method of irradiation (bombardment),
- separation of the desired radionuclide,

and evaluates all effects on the efficiency of the production in terms of quality and quantity of the produced radionuclide.

The chemical state of the isolated radionuclide may play a major role in all further processing.

PRECURSORS FOR SYNTHESIS

Generally, these precursors are not produced on a large scale. Some precursors are synthesised by the radiopharmaceutical production laboratory, others are supplied by specialised producers or laboratories.

Tests for identity, for chemical purity and the assay must be performed by validated procedures.

When batches of precursors are accepted using data from the certificates of analysis, suitable evidence has to be established to demonstrate the consistent reliability of the supplier's analyses and at least one identity test must be conducted. It is recommended to test precursor materials in production runs before their use for the manufacture of radiopharmaceutical preparations, to ensure that under specified production conditions, the precursor yields the radiopharmaceutical preparation in the desired quantity and quality specified.

PERFORMANCE OF THE PRODUCTION SYSTEM

All operations, from the preparation of the target to the dispensing of the final radiopharmaceutical preparation, must be clearly documented including their impact on the purity of the final product and the efficiency of the procedure.

Where possible, in-process controls are performed and the results recorded at each production step to identify at which level a possible discrepancy from the normal production pathway may have occurred.

a) The production of radiopharmaceutical preparations may make use of mechanical and automated processes that are used in the pharmaceutical industry, subject to adapting these to the specificity of the radioactive starting material and to the requirements of radioprotection.

b) For radiopharmaceutical preparations containing shortlived radionuclides, such as certain positron emitters, remotely controlled production and automated radiosynthesis are generally used. For radionuclides with a very short half-life (less than 20 min) the control of the performance of the production system is an important measure to assure the quality of the radiopharmaceutical preparation before its release.

c) Any production procedure must be validated in test production runs before its use in routine manufacture of radiopharmaceutical preparations, to ensure that under specified production conditions, the production system yields the radiopharmaceutical preparation in the desired quantity and specified quality.

d) The preparation of the dosage form of the final radiopharmaceutical preparation in the practice of nuclear medicine generally involves limited radioactivity starting from ready-to-use radiopharmaceutical preparations, generators, kits and radioactive precursors. All conditions which may affect the quality of the product (e.g. radiochemical purity and sterility) must be clearly defined and must include appropriate measures for radiation protection.

IDENTIFICATION

Radioactive decay: radioactivity decays at an exponential rate with a decay constant characteristic of each radionuclide.

The curve of exponential decay (decay curve) is described by the equation:

$$A_t = A_0 e^{-\lambda t}$$

A_t = the radioactivity at time t ,

A_0 = the radioactivity at time $t = 0$,

λ = the decay constant characteristic of each radionuclide,

e = the base of Napierian logarithms.

The half-life ($T_{1/2}$) is related to the decay constant (λ) by the equation:

$$T_{1/2} = \frac{\ln 2}{\lambda} \quad (\ln 2 \approx 0.693)$$

The radionuclide is generally identified by its half-life or by the nature and energy of its radiation or radiations or by both, as prescribed in the monograph.

Measurement of half-life. The half-life is measured with a suitable detection apparatus such as an ionisation chamber, a Geiger-Müller counter, a scintillation counter (solid crystal, liquid) or a semiconductor detector. The preparation to be tested is used as such or diluted or dried in a capsule after appropriate dilution. The radioactivity chosen, having regard to experimental conditions, must be of a sufficiently high level to allow detection during several estimated half-lives, but not too high to minimise count rate losses, for example due to dead time.

The radioactive source is prepared in a manner that will avoid loss of material during handling. If it is a liquid

(solution), it is contained in bottles or sealed tubes. If it is a solid (residue from drying in a capsule), it is protected by a cover consisting of a sheet of adhesive cellulose acetate or of some other material.

The same source is measured in the same geometrical conditions and at intervals usually corresponding to half of the estimated half-life throughout a time equal to about three half-lives. The correct functioning of the apparatus is checked using a source of long half-life and, if necessary, corrections for any changes of the count rate have to be applied (see Measurement of Radioactivity).

A graph can be drawn with time as the abscissa and the logarithm of the relative instrument reading (e.g. count rate) as the ordinate. The calculated half-life differs by not more than 5 per cent from the half-life stated in the Pharmacopoeia, unless otherwise stated.

Determination of the nature and energy of the radiation.

The nature and energy of the radiation emitted may be determined by several procedures including the construction of an attenuation curve and the use of spectrometry. The attenuation curve can be used for analysis of electron radiation; spectrometry is mostly used for identification of gamma rays and detectable X-rays.

The *attenuation curve* is drawn for pure electron emitters when no spectrometer for beta rays is available or for beta/gamma emitters when no spectrometer for gamma rays is available. This method of estimating the maximum energy of beta radiation gives only an approximate value. The source, suitably mounted to give constant geometrical conditions, is placed in front of the thin window of a Geiger-Müller counter or a proportional counter. The source is protected as described above. The count rate of the source is then measured. Between the source and the counter are placed, in succession, at least six aluminium screens of increasing mass per unit area within such limits that with a pure beta emitter this count rate is not affected by the addition of further screens. The screens are inserted in such a manner that constant geometrical conditions are maintained. A graph is drawn showing, as the abscissa, the mass per unit area of the screen expressed in milligrams per square centimetre and, as the ordinate, the logarithm of the count rate for each screen examined. A graph is drawn in the same manner for a standardised preparation. The mass attenuation coefficients are calculated from the median parts of the curves, which are practically rectilinear.

The *mass attenuation coefficient* μ_m , expressed in square centimetres per milligram, depends on the energy spectrum of the beta radiation and on the nature and the physical properties of the screen. It therefore allows beta emitters to be identified. It is calculated using the equation:

$$\mu_m = \frac{\ln A_1 - \ln A_2}{m_2 - m_1}$$

m_1 = mass per unit area of the lightest screen,

m_2 = mass per unit area of the heaviest screen, m_1 and m_2 being within the rectilinear part of the curve,

A_1 = count rate for mass per unit area m_1 ,

A_2 = count rate for mass per unit area m_2 .

The mass attenuation coefficient μ_m thus calculated does not differ by more than 10 per cent from the coefficient obtained under identical conditions using a standardised preparation of the same radionuclide.

The range of beta particles is a further parameter which can be used for the determination of the beta energy. It is obtained from the graph described above as the mass per unit

area corresponding to the intersection of the extrapolations of the descending rectilinear part of the attenuation curve and the horizontal line of background radioactivity.

Liquid scintillation counting may be used to obtain spectra of α and β^- emitters (see measurement of radioactivity).

Gamma spectrometry is used to identify radionuclides by the energy and intensity of their gamma rays and X-rays.

The preferred detector for gamma and X-ray spectrometry is a germanium semiconductor detector. A thallium-activated sodium iodide scintillation detector is also used but this has a much lower energy resolution.

The gamma detector has to be calibrated using standard sources because the detection efficiency is a function of the energy of the gamma and X-rays as well as the form of the source and the source-to-detector distance. The detection efficiency may be measured using a calibrated source of the radionuclide to be measured or, for more general work, a graph of efficiency against gamma and X-ray energy may be constructed from a series of calibrated sources of various radionuclides.

The gamma and X-ray spectrum of a radionuclide which emits gamma and X-rays is unique to that nuclide and is characterised by the energies and the number of photons of particular energies emitted per transformation from one energy level to another energy level. This property contributes to the identification of radionuclides present in a source and to their quantification. It allows the estimation of the degree of radionuclidic impurity by detecting peaks other than those expected.

It is possible to establish the rate of the decay of radioactivity using gamma spectrometry since the peaks diminish in amplitude as a function of the half-life. If, in such a source, a radioactive impurity with a different half-life is present, it is possible to detect the latter by identification of the characteristic peak or peaks whose amplitudes decrease at a different rate from that expected for the particular radionuclide. A determination of the half-life of the additional peaks by repeated measurements of the sample will help to identify the impurity.

The *Table of physical characteristics of radionuclides mentioned in the European Pharmacopoeia (5.7)* summarises the most commonly accepted physical characteristics of radionuclides used in preparations which are the subject of monographs in the European Pharmacopoeia. In addition, the Table states the physical characteristics of the main potential impurities of the radionuclides mentioned in the monographs.

By “transition probability” is meant the probability of the transformation of a nucleus in a given energy state, via the transition concerned. Instead of “probability” the terms “intensity” and “abundance” are frequently used.

By “emission probability” is meant the probability of an atom of a radionuclide giving rise to the emission of the particles or radiation concerned.

Irrespective of whether the one or the other meaning is intended, probability is usually measured in terms of 100 disintegrations.

MEASUREMENT OF RADIOACTIVITY

The radioactivity of a preparation is stated at a given date and, if necessary, time.

The absolute measurement of the radioactivity of a given sample may be carried out if the decay scheme of the radionuclide is known, but in practice many corrections are required to obtain accurate results. For this reason it

is common to carry out the measurement with the aid of a primary standard source. Primary standards may not be available for short-lived radionuclides e.g. positron emitters. Measuring instruments are calibrated using suitable standards for the particular radionuclides. Standards are available from the laboratories recognised by the competent authority. Ionisation chambers and Geiger-Müller counters may be used to measure beta and beta/gamma emitters; scintillation or semiconductor counters or ionisation chambers may be used for measuring gamma emitters; low-energy beta emitters require a liquid-scintillation counter. For the detection and measurement of alpha emitters, specialised equipment and techniques are required. For an accurate comparison of radioactive sources, it is essential for samples and standards to be measured under similar conditions.

Low-energy beta emitters may be measured by liquid-scintillation counting. The sample is dissolved in a solution containing one or more often two organic fluorescent substances (primary and secondary scintillators), which convert part of the energy of disintegration into photons of light, which are detected by a photomultiplier and converted into electrical impulses. When using a liquid-scintillation counter, comparative measurements are corrected for light-quenching effects. Direct measurements are made, wherever possible, under similar conditions, (e.g. volumes and type of solutions) for the source to be examined and the standard source.

All measurements of radioactivity must be corrected by subtracting the background due to radioactivity in the environment and to spurious signals generated in the equipment itself.

With some equipment, when measurements are made at high levels of radioactivity, it may be necessary to correct for loss by coincidence due to the finite resolving time of the detector and its associated electronic equipment. For a counting system with a fixed dead time τ following each count, the correction is:

$$N = \frac{N_{obs}}{1 - N_{obs}\tau}$$

N = the true count rate per second,

N_{obs} = the observed count rate per second,

τ = the dead time, in seconds.

With some equipment this correction is made automatically. Corrections for loss by coincidence must be made before the correction for background radiation.

If the time of an individual measurement, t_m is not negligible short compared with the half-life, $T_{1/2}$, the decay during this measurement time must be taken into account. After having corrected the instrument reading (count rate, ionisation current, etc.) for background and, if necessary, for losses due to electronic effects, the decay correction during measurement time is:

$$R_{corr} = \frac{R \frac{t_m \ln 2}{T_{1/2}}}{1 - \exp\left(-\frac{t_m \ln 2}{T_{1/2}}\right)}$$

R_{corr} = instrument reading corrected to the beginning of the individual measurement,

R = instrument reading before decay correction, but already corrected for background, etc.

The results of determinations of radioactivity show variations which derive mainly from the random nature of nuclear transformation. A sufficient number of counts must be registered in order to compensate for variations in the number of transformations per unit of time. The standard deviation is the square root of the counts, so at least 10 000 counts are necessary to obtain a relative standard deviation of not more than 1 per cent (confidence interval: 1 sigma).

All statements of radioactive content are accompanied by a statement of the date and, if necessary, the time at which the measurement was made. This statement of the radioactive content must be made with reference to a time zone (GMT, CET). The radioactivity at other times may be calculated from the exponential equation or from tables.

The radioactivity of a solution is expressed per unit volume to give the radioactive concentration.

RADIONUCLIDIC PURITY

In most of the cases, to state the radionuclidic purity of a radiopharmaceutical preparation, the identity of every radionuclide present and their radioactivity must be known. The most generally useful method for examination of radionuclidic purity is that of gamma spectrometry. It is not a completely reliable method because alpha- and beta-emitting impurities are not usually easily detectable and, when sodium iodide detectors are employed, the peaks due to gamma emitting impurities are often obscured by the spectrum of the principal radionuclide.

The individual monographs prescribe the radionuclidic purity required (for example, the gamma-ray spectrum does not significantly differ from that of a standardised preparation) and may set limits for specific radionuclidic impurities (for example, cobalt-60 in cobalt-57). While these requirements are necessary, they are not in themselves sufficient to ensure that the radionuclidic purity of a preparation is sufficient for human use. The manufacturer must examine the product in detail and especially must examine preparations of radionuclides of short half-life for impurities of long half-life after a suitable period of decay. In this way, information on the suitability of the manufacturing processes and the adequacy of the testing procedures may be obtained. In cases where two or more positron emitting radionuclides need to be identified and/or differentiated, as e.g. 18F-impurities in 13N-preparations, half-life determinations need to be carried out in addition to gamma spectrometry.

Due to differences in the half-lives of the different radionuclides present in a radiopharmaceutical preparation, the radionuclidic purity changes with time. The requirement of the radionuclidic purity must be fulfilled throughout the period of validity. It is sometimes difficult to carry out these tests before authorising the release for use of the batch when the half-life of the radionuclide in the preparation is short. The test then constitutes a control of the quality of production.

RADIOCHEMICAL PURITY

The determination of radiochemical purity requires separation of the different chemical substances containing the radionuclide and estimating the percentage of radioactivity associated with the declared chemical substance. Radiochemical impurities may originate from:

- radionuclide production,
- subsequent chemical procedures,
- incomplete preparative separation,
- chemical changes during storage.

The requirement of the radiochemical purity must be fulfilled throughout the period of validity.

In principle, any method of analytical separation may be used in the determination of radiochemical purity. For example, the monographs for radiopharmaceutical products may include paper chromatography (2.2.26), thin-layer chromatography (2.2.27), electrophoresis (2.2.37), size-exclusion chromatography (2.2.30), gas chromatography (2.2.28) and liquid chromatography (2.2.29). The technical description of these analytical methods is set out in the monographs. Moreover certain precautions special to radioactivity must also be taken for radiation protection.

In a hospital environment thin-layer and paper chromatography are mostly used. In paper and thin-layer chromatography, a volume equal to that described in the monograph is deposited on the starting-line as prescribed in the general methods for chromatography. It is preferable not to dilute the preparation to be examined but it is important to avoid depositing such a quantity of radioactivity that counting losses by coincidence occur during measurement of the radioactivity. On account of the very small quantities of the radioactive material applied, a carrier may be added when specified in a particular monograph. After development, the support is dried and the positions of the radioactive areas are detected by autoradiography or by measurement of radioactivity over the length of the chromatogram, using suitable collimated counters or by cutting the strips and counting each portion. The positions of the spots or areas permit chemical identification by comparison with solutions of the same chemical substances (non-radioactive) using a suitable detection method.

Radioactivity may be measured by integration using an automatic-plotting instrument or a digital counter. The ratios of the areas under the peaks give the ratios of the radioactive concentration of the chemical substances. When the strips are cut into portions, the ratios of the quantities of radioactivity measured give the ratio of concentrations of the radioactive chemical species.

SPECIFIC RADIOACTIVITY

Specific radioactivity is usually calculated taking into account the radioactive concentration (radioactivity per unit volume) and the concentration of the chemical substance being studied, after verification that the radioactivity is attributable only to the radionuclide (radionuclidic purity) and the chemical species (radiochemical purity) concerned.

Specific radioactivity changes with time. The statement of the specific radioactivity therefore includes reference to a date and, if necessary, time. The requirement of the specific radioactivity must be fulfilled throughout the period of validity.

CHEMICAL PURITY

The determination of chemical purity requires quantification of the individual chemical impurities specified in the monograph.

ENANTIOMERIC PURITY

Where appropriate, the stereoisomeric purity has to be verified.

PHYSIOLOGICAL DISTRIBUTION

A physiological distribution test is prescribed, if necessary, for certain radiopharmaceutical preparations. The distribution pattern of radioactivity observed in specified organs, tissues or other body compartments of an appropriate animal species (usually rats or mice) can be a reliable indication of the expected distribution in humans and thus of the suitability for the intended purpose.

The individual monograph prescribes the details concerning the performance of the test and the physiological distribution requirements which must be met for the radiopharmaceutical preparation. A physiological distribution conforming to the requirements will assure appropriate distribution of the radioactive compounds to the intended biological target in humans and limits its distribution to non-target areas.

In general, the test is performed as follows.

Each of three animals is injected intravenously with the preparation to be tested. If relevant, the species, sex, strain and weight and/or age of the animals is specified in the monograph. The test injection is the radiopharmaceutical preparation as it is intended for human use. Where applicable, products are reconstituted according to the manufacturer's instructions. In some cases, dilution immediately before injection may be necessary.

The administration will normally be made via the intravenous route for which purpose the caudal vein is used. Other veins such as the saphenous, femoral, jugular or penile veins may be used in special cases. Animals showing evidence of extravasation of the injection (observed at the time of injection or revealed by subsequent assay of tissue radioactivity) are rejected from the test.

Immediately after injection each animal is placed in a separate cage which will allow collection of excreta and prevent contamination of the body surface of the animal.

At the specified time after injection, the animals are killed by an appropriate method and dissected. Selected organs and tissues are assayed for their radioactivity using a suitable instrument as described elsewhere in this monograph. The physiological distribution is then calculated and expressed in terms of the percentage of the radioactivity which is found in each of the selected organs or tissues. For this purpose the radioactivity in an organ may be related to the injected radioactivity calculated from the radioactive content of the syringe measured before and after injection. For some radiopharmaceutical preparations it may be appropriate to determine the ratio of the radioactivity in weighed samples of selected tissues (radioactivity/mass).

For a preparation to meet the requirements of the test, the distribution of radioactivity in at least two of the three animals must comply with all the specified criteria.

STERILITY

Radiopharmaceutical preparations for parenteral administration must be prepared using precautions designed to exclude microbial contamination and to ensure sterility. The test for sterility is carried out as described in the general method for sterility (2.6.1). Special difficulties arise with radiopharmaceutical preparations because of the short half-life of some radionuclides, small size of batches and the radiation hazards. It is not always possible to await the results of the test for sterility before authorisation of the release for use of the batch concerned. Parametric release (5.1.1) of the product manufactured by a fully validated process is the method of choice in such cases. When aseptic manufacturing is used, the test for sterility has to be executed as a control of the quality of production.

When the size of a batch of the radiopharmaceutical preparation is limited to one or a few samples (e.g. therapeutic or very short-lived radiopharmaceutical preparation), sampling the batch for sterility testing may not be applicable. If the radiopharmaceutical preparation is sterilised by filtration and/or aseptically processed (5.1.1) process validation is critical.

When the half-life of the radionuclide is very short (e.g. less than 20 min), the administration of the radiopharmaceutical preparation to the patient is generally on-line with a validated production system.

For safety reasons (high level of radioactivity) it is not possible to use the quantity of the radiopharmaceutical preparations as required in the test for sterility (2.6.1). The method by membrane filtration is to be preferred to limit irradiation of personnel.

Notwithstanding the requirements concerning the use of antimicrobial preservatives in *Parenteral preparations (0520)*, their addition to radiopharmaceutical preparations in multidose containers is not obligatory, unless prescribed in the monograph.

BACTERIAL ENDOTOXINS - PYROGENS

For certain radiopharmaceutical preparations a test for bacterial endotoxins is prescribed. The test is carried out as described in the general method (2.6.14), taking the necessary precautions to limit irradiation of the personnel carrying out the test.

The limit for bacterial endotoxins is indicated in the individual monograph.

When the nature of the radiopharmaceutical preparation results in an interference by inhibition or activation and it is not possible to eliminate the interfering factor(s), the test for pyrogens (2.6.8) may be specifically prescribed.

It is sometimes difficult to carry out these tests before releasing the batch for use when the half-life of the radionuclide in the preparation is short. The test then constitutes a control of the quality of production.

STORAGE

Store in an airtight container in a place that is sufficiently shielded to protect personnel from irradiation by primary or secondary emissions and that complies with national and international regulations concerning the storage of radioactive substances. During storage, containers may darken due to irradiation. Such darkening does not necessarily involve deterioration of the preparations.

Radiopharmaceutical preparations are intended for use within a short time and the end of the period of validity must be clearly stated.

LABELLING

The labelling of radiopharmaceutical preparations complies with the relevant national and European legislation.

The label on the direct container states:

- the name of the preparation and/or its reference,
- the name of the manufacturer,
- an identification number,
- for liquid and gaseous preparations: the total radioactivity in the container, or the radioactive concentration per millilitre at a stated date and, if necessary, time, and the volume of liquid in the container,
- for solid preparations (such as freeze-dried preparations): the total radioactivity at a stated date and, if necessary, time. After reconstitution with the appropriate solution, the preparation is considered as a liquid preparation,
- for capsules: the radioactivity per capsule at a stated date and, if necessary, time and the number of capsules in the container.

The labelling can be adapted in certain cases (e.g. radiopharmaceutical preparations containing short-lived radionuclides).

In addition, the label on the outer package states:

- the route of administration,
- the period of validity or the expiry date,
- the name and concentration of any added antimicrobial preservative,
- where applicable, any special storage conditions.

01/2005:0784

RECOMBINANT DNA TECHNOLOGY, PRODUCTS OF

Producta ab ADN recombinante

This monograph provides general requirements for the development and manufacture of products of recombinant DNA technology. These requirements are not necessarily comprehensive in a given case and requirements complementary or additional to those prescribed in this monograph may be imposed in an individual monograph or by the competent authority.

The monograph is not applicable to modified live organisms that are intended to be used directly in man and animals, for example as live vaccines.

DEFINITION

Products of rDNA technology are produced by genetic modification in which DNA coding for the required product is introduced, usually by means of a plasmid or a viral vector, into a suitable micro-organism or cell line, in which that DNA is expressed and translated into protein. The desired product is then recovered by extraction and purification. The cell or micro-organism before harbouring the vector is referred to as the host cell, and the stable association of the two used in the manufacturing process is referred to as the host-vector system.

PRODUCTION

Production is based on a validated seed-lot system using a host-vector combination that has been shown to be suitable to the satisfaction of the competent authority. The seed-lot system uses a master cell bank and a working cell bank derived from the master seed lot of the host-vector combination. A detailed description of cultivation, extraction and purification steps and a definition of the production batch shall be established.

The determination of the suitability of the host-vector combination and the validation of the seed-lot system include the following elements.

CLONING AND EXPRESSION

The suitability of the host-vector system, particularly as regards microbiological purity, is demonstrated by:

Characterisation of the host cell, including source, phenotype and genotype, and of the cell-culture media.

Documentation of the strategy for the cloning of the gene and characterisation of the recombinant vector including:

- i. the origin and characterisation of the gene;
- ii. nucleotide-sequence analysis of the cloned gene and the flanking control regions of the expression vector. The cloned sequences are kept to a minimum and all relevant expressed sequences are clearly identified and confirmed at the RNA level.

The DNA sequence of the cloned gene is normally confirmed at the seed-lot stage, up to and beyond the normal level of population doubling for full-scale fermentation. In certain

systems, for example, where multiple copies of the gene are inserted into the genome of a continuous cell line, it may be inappropriate to sequence the cloned gene at the production level. Under these circumstances, Southern blot analysis of total cellular DNA or sequence analysis of the messenger RNA (m RNA) may be helpful, particular attention being paid to the characterisation of the expressed protein;

- iii. the construction, genetics and structure of the complete expression vector.

Characterisation of the host-vector system including:

- i. mechanism of transfer of the vector into the host cells;
- ii. copy number, physical state and stability of the vector inside the host cell;
- iii. measures used to promote and control the expression.

CELL-BANK SYSTEM

The master cell bank is a homogeneous suspension of the original cells already transformed by the expression vector containing the desired gene, distributed in equal volumes into individual containers for storage (for example, in liquid nitrogen). In some cases it may be necessary to establish separate master cell banks for the expression vector and the host cells.

The working cell bank is a homogeneous suspension of the cell material derived from the master cell bank(s) at a finite passage level, distributed in equal volumes into individual containers for storage (for example, in liquid nitrogen).

In both cell banks, all containers are treated identically during storage and, once removed from storage, the containers are not returned to the cell stock.

The cell bank may be used for production at a finite passage level or for continuous-culture production.

Production at a finite passage level

This cultivation method is defined by a limited number of passages or population doublings which must not be exceeded during production. The maximum number of cell doublings, or passage levels, during which the manufacturing process routinely meets the criteria described below must be stated.

Continuous-culture production

By this cultivation method the number of passages or population doublings is not restricted from the beginning of production. Criteria for the harvesting as well as for the termination of production have to be defined by the manufacturer. Monitoring is necessary throughout the life of the culture; the required frequency and type of monitoring will depend on the nature of the production system and the product.

Information is required on the molecular integrity of the gene being expressed and on the phenotypic and genotypic characteristics of the host cell after long-term cultivation. The acceptance of harvests for further processing must be clearly linked to the schedule of monitoring applied and a clear definition of a "batch" of product for further processing is required.

VALIDATION OF THE CELL BANKS

Validation of the cell banks includes:

- i. stability by measuring viability and the retention of the vector;
- ii. identity of the cells by phenotypic features;
- iii. where appropriate, evidence that the cell banks are free from potentially oncogenic or infective adventitious agents (viral, bacterial, fungal or mycoplasmal). Special attention has to be given to viruses that can commonly contaminate the species from which the cell line has been derived. Certain cell lines contain endogenous viruses, for example,

retroviruses, which may not readily be eliminated. The expression of these organisms, under a variety of conditions known to cause their induction, shall be tested for;

iv. for mammalian cells, details of the tumorigenic potential of the cell bank shall be obtained.

CONTROL OF THE CELLS

The origin, form, storage, use and stability at the anticipated rate of use must be documented in full for all cell banks under conditions of storage and recovery. New cell banks must be fully validated.

VALIDATION OF THE PRODUCTION PROCESS

Extraction and purification

The capacity of each step of the extraction and purification procedure to remove and/or inactivate contaminating substances derived from the host cell or culture medium, including, in particular, virus particles, proteins, nucleic acids and added substances, must be validated.

Validation studies are carried out to demonstrate that the production process routinely meets the following criteria:

- exclusion of extraneous agents from the product. Studies including, for example, viruses with relevant physico-chemical features are undertaken, and a reduction capacity for such contaminants at each relevant stage of purification is established;
- adequate removal of vector, host-cell, culture medium and reagent-derived contaminants from the product. The reduction capacity for DNA is established by spiking. The reduction of proteins of animal origin can be determined by immunochemical methods;
- maintenance within stated limits of the yield of product from the culture;
- adequate stability of any intermediate of production and/or manufacturing when it is intended to use intermediate storage during the process.

Characterisation of the substance

The identity, purity, potency and stability of the final bulk product are established initially by carrying out a wide range of chemical, physical, immunochemical and biological tests. Prior to release, each batch of the product is tested by the manufacturer for identity and purity and an appropriate assay is carried out.

Production consistency

Suitable tests for demonstrating the consistency of the production and purification are performed. The tests include, especially characterisation tests, in-process controls and final-product tests, for example:

AMINO-ACID COMPOSITION

Partial amino-acid sequence analysis. The sequence data permit confirmation of the correct *N*-terminal processing and detection of loss of the *C*-terminal amino acids.

Peptide mapping. Peptide mapping using chemical and/or enzymatic cleavage of the protein product and analysis by a suitable method such as two-dimensional gel electrophoresis, capillary electrophoresis or liquid chromatography must show no significant difference between the test protein and the reference preparation. Peptide mapping can also be used to demonstrate correct disulphide bonding.

DETERMINATION OF MOLECULAR MASS

Cloned-gene retention. The minimum amount in percentage of the cells containing the vector or the cloned gene after cultivation is approved by the relevant authority.

Total protein. The yield of protein is determined.

Chemical purity. The purity of the protein product is analysed in comparison with a reference preparation by a suitable method such as liquid chromatography, capillary electrophoresis or sodium dodecyl sulphate polyacrylamide gel electrophoresis.

Host-cell-derived proteins. Host-cell-derived proteins are detected by immunochemical methods, using, for example, polyclonal antisera raised against protein components of the host-vector system used to manufacture the product, unless otherwise prescribed. The following types of procedure may be used: liquid-phase displacement assays (for example, radio-immunoassay), liquid-phase direct-binding assays and direct-binding assays using antigens immobilised on nitrocellulose (or similar) membranes (for example, dot-immunoblot assays, Western blots). General requirements for the validation of immunoassay procedures are given under 2.7.1. *Immunochemical Methods*. In addition, immunoassay methods for host-cell contaminants meet the following criteria:

- *Antigen preparations.* Antisera are raised against a preparation of antigens derived from the host organism, into which has been inserted the vector used in the manufacturing process that lacks the specific gene coding for the product. This host cell is cultured, and proteins are extracted, using conditions identical to those used for culture and extraction in the manufacturing process. Partly purified preparations of antigens, using some of the purification steps in the manufacturing process, may also be used for the preparation of antisera.
- *Calibration and standardisation.* Quantitative data are obtained by comparison with dose-response curves obtained using standard preparations of host-derived protein antigens. Since these preparations are mixtures of poorly defined proteins, a standard preparation is prepared and calibrated by a suitable protein determination method. This preparation is stored in a stable state suitable for use over an extended period of time.
- *Antisera.* Antisera contain high-avidity antibodies recognising as many different proteins in the antigen mixture as possible, and do not cross-react with the product.

Host-cell- and vector-derived DNA. Residual DNA is detected by hybridisation analysis, using suitably sensitive, sequence-independent analytical techniques or other suitably sensitive analytical techniques.

Hybridisation analysis

DNA in the test sample is denatured to give single-stranded DNA, immobilised on a nitrocellulose or other suitable filter and hybridised with labelled DNA prepared from the host-vector manufacturing system (DNA probes). Although a wide variety of experimental approaches is available, hybridisation methods for measurement of host-vector DNA meet the following criteria:

- *DNA probes.* Purified DNA is obtained from the host-vector system grown under the same conditions as those used in the manufacturing process. Host chromosomal DNA and vector DNA may be separately prepared and used as probes.
- *Calibration and standardisation.* Quantitative data are obtained by comparison with responses obtained using standard preparations. Chromosomal DNA probes and vector DNA probes are used with chromosomal DNA and vector DNA standards, respectively. Standard preparations are calibrated by spectroscopic measurements and stored in a state suitable for use over an extended period of time.

- *Hybridisation conditions.* The stringency of hybridisation conditions is such as to ensure specific hybridisation between probes and standard DNA preparations and the drug substances must not interfere with hybridisation at the concentrations used.

Sequence-independent techniques

Suitable procedures include: detection of sulphonated cytosine residues in single-stranded DNA (where DNA is immobilised on a filter and cytosines are derivatised *in situ*, before detection and quantitation using an antibody directed against the sulphonated group); detection of single-stranded DNA using a fragment of single-stranded DNA bound to a protein and an antibody of this protein. Neither procedure requires the use of specific host or vector DNA as an assay standard. However, the method used must be validated to ensure parallelism with the DNA standard used, linearity of response and non-interference of either the drug substance or excipients of the formulation at the dilutions used in the assay.

IDENTIFICATION, TESTS AND ASSAY

The requirements with which the final product (bulk material or dose form) must comply throughout its period of validity, as well as specific test methods, are stated in the individual monograph.

STORAGE

See the individual monographs.

LABELLING

See the individual monographs.

Polymorphism. Individual monographs do not usually specify crystalline or amorphous forms, unless bioavailability is affected. All forms of a substance for pharmaceutical use comply with the requirements of the monograph, unless otherwise indicated.

PRODUCTION

Substances for pharmaceutical use are manufactured by procedures that are designed to ensure a consistent quality and comply with the requirements of the individual monograph or approved specification.

The provisions of general chapter 5.10 apply to the control of impurities in substances for pharmaceutical use.

Whether or not it is specifically stated in the individual monograph that the substance for pharmaceutical use:

- is a recombinant protein or another substance obtained as a direct gene product based on genetic modification, where applicable, the substance also complies with the requirements of the general monograph on *Products of recombinant DNA technology (0784)*;
- is obtained from animals susceptible to transmissible spongiform encephalopathies other than by experimental challenge, where applicable, the substance also complies with the requirements of the general monograph on *Products with risk of transmitting agents of animal spongiform encephalopathies (1483)*;
- is a substance derived from a fermentation process whether or not the micro-organisms involved are modified by traditional procedures or recombinant DNA (rDNA) technology, where applicable, the substance complies with the requirements of the general monograph on *Products of fermentation (1468)*.

If solvents are used during production, they are of suitable quality. In addition, their toxicity and their residual level are taken into consideration (5.4). If water is used during production, it is of suitable quality.

If substances are produced or processed to yield a certain form or grade, that specific form or grade of the substance complies with the requirements of the monograph. Certain functionality-related tests may be described to control properties that may influence the suitability of the substance and subsequently the properties of dosage forms prepared from it.

Powdered substances may be processed to obtain a certain degree of fineness (2.9.12).

Compacted substances are processed to increase the particle size or to obtain particles of a specific form and/or to obtain a substance with a higher bulk density.

Coated active substances consist of particles of the active substance coated with one or more suitable excipients.

Granulated active substances are particles of a specified size and/or form produced from the active substance by granulation directly or with one or more suitable excipients.

If substances are processed with excipients, these excipients comply with the requirements of the relevant monograph or, where no such monograph exists, the approved specification.

CHARACTERS

The statements under the heading Characters (e.g. statements about the solubility or a decomposition point) are not to be interpreted in a strict sense and are not requirements. They are given for information.

Where a substance may show polymorphism, this may be stated under Characters in order to draw this to the attention of the user who may have to take this characteristic into consideration during formulation of a preparation.

01/2005:2034

SUBSTANCES FOR PHARMACEUTICAL USE

Corpora ad usum pharmaceuticum

The statements in this monograph are intended to be read in conjunction with individual monographs on substances in the Pharmacopoeia. Application of the monograph to other substances may be decided by the competent authority.

DEFINITION

Substances for pharmaceutical use are any organic or inorganic substances that are used as active substances or excipients for the production of medicinal products for human or veterinary use. They may be obtained from natural sources or produced by extraction from raw materials, fermentation or synthesis.

Substances for pharmaceutical use may be used as such or as starting materials for subsequent formulation to prepare medicinal products. Depending on the formulation, certain substances may be used either as active substances or excipients. Solid substances may be compacted, coated, granulated, powdered to a certain fineness or processed in other ways. Processing with addition of excipients is permitted only where this is specifically stated in the Definition of the individual monograph.

Substance for pharmaceutical use of special grade.

Unless otherwise indicated or restricted in the individual monographs, a substance for pharmaceutical use is intended for human and veterinary use, and is of appropriate quality for manufacture of all dosage forms in which it can be used.

IDENTIFICATION

Where under Identification an individual monograph contains subdivisions entitled *First identification* and *Second identification*, the test or tests that constitute the *First identification* may be used in all circumstances. The test or tests that constitute the *Second identification* may be used for identification, provided it can be demonstrated that the substance is fully traceable to a batch certified to comply with all the other requirements of the monograph.

TESTS

Polymorphism (5.9). If the nature of a crystalline or amorphous form imposes restrictions on its use in preparations, the nature of the specific crystalline or amorphous form is identified, its morphology is adequately controlled and its identity is stated on the label.

Related substances. Organic impurities in active substances are to be reported, identified wherever possible, and qualified as indicated in Table 2034.-1.

Specific thresholds may be applied for impurities known to be unusually potent or to produce toxic or unexpected pharmacological effects.

If the individual monograph does not provide suitable control for a new impurity, a suitable test for control must be developed and included in the specification for the substance.

The requirements above do not apply to biological and biotechnological products, peptides, oligonucleotides, radiopharmaceuticals, products of fermentation and semi-synthetic products derived therefrom, to crude products of animal or plant origin or herbal products.

Residual solvents are limited according to the principles defined in the chapter (5.4), using the general method (2.4.24) or other suitable methods. Where a quantitative determination of a residual solvent is carried out and a test for loss on drying is not carried out, the content of residual solvent is taken into account for calculation of the assay content of the substance.

Sterility (2.6.1). If intended for use in the manufacture of sterile dosage forms without a further appropriate sterilisation procedure, or if offered as sterile grade, the substance for pharmaceutical use complies with the test for sterility.

Bacterial endotoxins (2.6.14). If offered as bacterial endotoxin-free grade, the substance for pharmaceutical use complies with the test for bacterial endotoxins. The limit and test method (if not gelation method A) are stated in the individual monograph. The limit is calculated in accordance with *Test for bacterial endotoxins: guidelines* (2.6.14), unless a lower limit is justified from results from production batches or is required by the competent authority. Where a test for bacterial endotoxins is prescribed, a test for pyrogens is not required.

Pyrogens (2.6.8). If the test for pyrogens is justified rather than the test for bacterial endotoxins and if a pyrogen-free grade is offered, the substance for pharmaceutical use complies with the test for pyrogens. The limit and test method are stated in the individual monograph or approved by the competent authority. Based on appropriate test validation for bacterial endotoxins and pyrogens, the test for bacterial endotoxins may replace the test for pyrogens.

Additional properties. Control of additional properties (e.g. physical characteristics, functionality-related characteristics) may be necessary for individual manufacturing processes or formulations. Grades (such as sterile, endotoxin-free, pyrogen-free) may be produced with a view to manufacture of preparations for parenteral administration or other dosage forms and appropriate requirements may be specified in an individual monograph.

ASSAY

Unless justified and authorised, contents of substances for pharmaceutical use are determined. Suitable methods are used.

LABELLING

In general, labelling is subject to supranational and national regulation and to international agreements. The statements under the heading Labelling therefore are not comprehensive and, moreover, for the purposes of the Pharmacopoeia only those statements that are necessary to demonstrate compliance or non-compliance with the monograph are mandatory. Any other labelling statements are included as recommendations. When the term "label" is used in the Pharmacopoeia, the labelling statements may appear on the container, the package, a leaflet accompanying the package or a certificate of analysis accompanying the article, as decided by the competent authority.

Where appropriate, the label includes statements that the substance is:

- intended for a specific use,
- of a distinct crystalline form,
- of a specific degree of fineness,
- compacted,
- coated,
- granulated,
- sterile,
- free from bacterial endotoxins,
- free from pyrogens,
- containing gliding agents.

Where appropriate, the label indicates the degree of hydration, the nature of any added antimicrobial preservative, antioxidant or other excipient. When active substances are processed with addition of excipients, the label indicates the excipients used and the content of active substance and excipients.

Table 2034.-1. – Reporting, identification and qualification of organic impurities in active substances

Use	Maximum daily dose	Reporting threshold	Identification threshold	Qualification threshold
Human use or human and veterinary use	≤ 2 g/day	> 0.05 per cent	> 0.10 per cent or a daily intake of > 1.0 mg (whichever is the lower)	> 0.15 per cent or a daily intake of > 1.0 mg (whichever is the lower)
Human use or human and veterinary use	> 2 g/day	> 0.03 per cent	> 0.05 per cent	> 0.05 per cent
Veterinary use only	Not applicable	> 0.1 per cent	> 0.2 per cent	> 0.5 per cent

01/2005:0153

VACCINES FOR HUMAN USE

Vaccina ad usum humanum

For a combined vaccine, where there is no monograph to cover a particular combination, the vaccine complies with the monograph for each individual component, with any necessary modifications approved by the competent authority.

DEFINITION

Vaccines for human use are preparations containing antigenic substances capable of inducing a specific and active immunity in man against an infecting agent or the toxin or the antigen elaborated by it. They shall have been shown to have acceptable immunogenic activity in man with the intended vaccination schedule.

Vaccines for human use may contain: organisms inactivated by chemical or physical means that maintain adequate immunogenic properties; living organisms that are naturally avirulent or that have been treated to attenuate their virulence whilst retaining adequate immunogenic properties; antigens extracted from the organisms or secreted by them or produced by genetic engineering; the antigens may be used in their native state or may be detoxified by chemical or physical means and may be aggregated, polymerised or conjugated to a carrier to increase their immunogenicity.

Terminology used in monographs on vaccines for human use is defined in chapter 5.2.1.

Bacterial vaccines are suspensions of various degrees of opacity in colourless or almost colourless liquids, or may be freeze-dried. The concentration of living or inactivated bacteria is expressed in terms of International Units of opacity or, where appropriate, is determined by direct cell count or, for living bacteria, by viable count.

Bacterial toxoids are prepared from toxins by diminishing their toxicity to a non-detectable level or by completely eliminating it by physical or chemical procedures whilst retaining adequate immunogenic properties. The toxins are obtained from selected strains of micro-organisms. The method of production is such that the toxoid does not revert to toxin. Toxoids may be liquid or freeze-dried. They may be purified and adsorbed. Adsorbed toxoids are suspensions of white or grey particles dispersed in colourless or pale yellow liquids and may form a sediment at the bottom of the container.

Viral vaccines are prepared from viruses grown in animals, in fertilised eggs, in suitable cell cultures or in suitable tissues or by culture of genetically engineered cells. They are liquids that vary in opacity according to the type of preparation or may be freeze-dried. Liquid preparations and freeze-dried preparations after reconstitution may be coloured if a pH indicator such as phenol red has been used in the culture medium.

PRODUCTION

General provisions. Requirements for production including in-process testing are included in individual monographs. Where justified and authorised, certain tests may be omitted where it can be demonstrated, for example by validation studies, that the production process consistently ensures compliance with the test.

Unless otherwise justified and authorised, vaccines are produced using a seed-lot system. The methods of

preparation are designed to maintain adequate immunogenic properties, to render the preparation harmless and to prevent contamination with extraneous agents.

Unless otherwise justified and authorised, in the production of a final lot of vaccine, the number of passages of a virus, or the number of subcultures of a bacterium, from the master seed lot shall not exceed that used for production of the vaccine shown in clinical studies to be satisfactory with respect to safety and efficacy.

Vaccines are as far as possible free from ingredients known to cause toxic, allergic or other undesirable reactions in man. Suitable additives, including stabilisers and adjuvants may be incorporated. Penicillin and streptomycin are not used at any stage of production nor added to the final product; however, master seed lots prepared with media containing penicillin or streptomycin may, where justified and authorised, be used for production.

Substrates for propagation. Substrates for propagation comply with the relevant requirements of the Pharmacopoeia (5.2.2, 5.2.3) or in the absence of such requirements with those of the competent authority. Processing of cell banks and subsequent cell cultures is done under aseptic conditions in an area where no other cells are being handled. Serum and trypsin used in the preparation of cell suspensions shall be shown to be free from extraneous agents.

Seed lots. The strain of bacterium or virus used in a master seed lot is identified by historical records that include information on the origin of the strain and its subsequent manipulation. Suitable measures are taken to ensure that no micro-organism other than the seed strain is present in a seed lot.

Culture media. Culture media are as far as possible free from ingredients known to cause toxic, allergic or other undesirable reactions in man; if inclusion of such ingredients is necessary, it shall be demonstrated that the amount present in the final lot is reduced to such a level as to render the product safe. Approved animal (but not human) serum may be used in the growth medium for cell cultures but the medium used for maintaining cell growth during virus multiplication shall not contain serum, unless otherwise stated. Cell culture media may contain a pH indicator such as phenol red and approved antibiotics at the lowest effective concentration although it is preferable to have a medium free from antibiotics during production.

Propagation and harvest. The seed cultures are propagated and harvested under defined conditions. The purity of the harvest is verified by suitable tests as defined in the monograph.

Control cells. For vaccines produced in cell cultures, control cells are maintained and tested as prescribed. In order to provide a valid control, these cells must be maintained in conditions that are rigorously identical with those used for the production cell cultures, including use of the same batches of media and media changes.

Control eggs. For live vaccines produced in eggs, control eggs are incubated and tested as prescribed in the monograph.

Purification. Where applicable, validated purification procedures may be applied.

Inactivation. Inactivated vaccines are produced using a validated inactivation process whose effectiveness and consistency have been demonstrated. Where there are recognised potential contaminants of a harvest, for example in vaccines produced in eggs from healthy, non-SPF flocks, the inactivation process is also validated with respect to the

potential contaminants. A test for inactivation is carried out as soon as possible after the inactivation process, unless otherwise justified and authorised.

Stability of intermediates. During production of vaccines, intermediates are obtained at various stages and are stored, sometimes for long periods. Such intermediates include:

- seed lots,
- live or inactivated harvests from bacterial or viral cultures,
- purified harvests that may consist of toxins or toxoids, polysaccharides, bacterial or viral suspensions,
- purified antigens,
- adsorbed antigens,
- conjugated polysaccharides,
- final bulk vaccine,
- vaccine in the final closed container stored at a temperature lower than that used for stability studies and intended for release without re-assay.

Except where they are used within a short period of time, stability studies are carried out on the intermediates in the intended storage conditions to establish the expected extent of degradation. For final bulk vaccine, stability studies may be carried out on representative samples in conditions equivalent to those intended to be used for storage. For each intermediate (except for seed lots), a period of validity applicable for the intended storage conditions is established, where appropriate in the light of stability studies.

Final bulk. The final bulk is prepared by aseptically blending the ingredients of the vaccine.

Adsorbents. Vaccines may be adsorbed on aluminium hydroxide, aluminium phosphate, calcium phosphate or other suitable adsorbent; the adsorbents are prepared in special conditions which confer the appropriate physical form and adsorptive properties.

Antimicrobial preservatives. Antimicrobial preservatives are used to prevent spoilage or adverse effects caused by microbial contamination occurring during the use of a vaccine. Antimicrobial preservatives are not included in freeze-dried products. For single-dose liquid preparations, inclusion of antimicrobial preservatives is not normally acceptable. For multidose liquid preparations, the need for effective antimicrobial preservation is evaluated taking into account likely contamination during use and the maximum recommended period of use after breaching of the container. If an antimicrobial preservative is used, it shall be shown that it does not impair the safety or efficacy of the vaccine. Addition of antibiotics as antimicrobial preservatives is not normally acceptable.

During development studies, the effectiveness of the antimicrobial preservative throughout the period of validity shall be demonstrated to the satisfaction of the competent authority.

The efficacy of the antimicrobial preservative is evaluated as described in chapter 5.1.3. If neither the A criteria nor the B criteria can be met, then in justified cases the following criteria are applied to vaccines for human use: bacteria, no increase at 24 h and 7 days, 3 log reduction at 14 days, no increase at 28 days; fungi, no increase at 14 days and 28 days.

Final lot. For vaccines for parenteral administration, the final lot is prepared by aseptically distributing the final bulk into sterile tamper-proof containers which, after freeze-drying where applicable, are closed so as to exclude contamination.

For vaccines for administration by a non-parenteral route, the final lot is prepared by distributing the final bulk under suitable conditions into sterile, tamper-proof containers.

Degree of adsorption. During development of an adsorbed vaccine, the degree of adsorption is evaluated as part of the consistency testing. A release specification for the degree of adsorption is established in the light of results found for batches used in clinical testing. From the stability data generated for the vaccine it must be shown that at the end of the period of validity the degree of adsorption will not be less than for batches used in clinical testing.

Stability. During development studies, maintenance of potency of the final lot throughout the period of validity shall be demonstrated; the loss of potency in the recommended storage conditions is assessed and excessive loss even within the limits of acceptable potency may indicate that the vaccine is unacceptable.

Expiry date. Unless otherwise stated, the expiry date is calculated from the beginning of the assay or from the beginning of the first assay for a combined vaccine. For vaccines stored at a temperature lower than that used for stability studies and intended for release without re-assay, the expiry date is calculated from the date of removal from cold storage. If, for a given vaccine, an assay is not carried out, the expiry date is calculated from the date of an approved stability-indicating test or failing this from the date of freeze-drying or the date of filling into the final containers. For a combined vaccine where components are presented in separate containers, the expiry date is that of the component which expires first.

The expiry date applies to vaccines stored in the prescribed conditions.

Animal tests. In accordance with the provisions of the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes, tests must be carried out in such a way as to use the minimum number of animals and to cause the least pain, suffering, distress or lasting harm. The criteria for judging tests in monographs must be applied in the light of this. For example, if it is indicated that an animal is considered to show positive, infected etc. when typical clinical signs or death occur then as soon as sufficient indication of a positive result is obtained the animal in question shall be either humanely destroyed or given suitable treatment to prevent unnecessary suffering. In accordance with the General Notices, alternative test methods may be used to demonstrate compliance with the monograph and the use of such tests is particularly encouraged when this leads to replacement or reduction of animal use or reduction of suffering.

TESTS

Vaccines comply with the tests prescribed in individual monographs including, where applicable, the following:

Aluminium (2.5.13): maximum 1.25 mg of aluminium (Al) per single human dose where an aluminium adsorbent has been used in the vaccine, unless otherwise stated,

Calcium (2.5.14): maximum 1.3 mg of calcium (Ca) per single human dose where a calcium adsorbent has been used in the vaccine, unless otherwise stated,

Formaldehyde (2.4.18): maximum 0.2 g/l of free formaldehyde is present in the final product where formaldehyde has been used in the preparation of the vaccine, unless otherwise stated.

Phenol (2.5.15): maximum 2.5 g/l is present in the final product where phenol has been used in the preparation of the vaccine, unless otherwise stated.

Water (2.5.12): maximum 3.0 per cent *m/m* for freeze-dried vaccines, unless otherwise stated.

STORAGE

Store protected from light. Unless otherwise stated, the storage temperature is 5 ± 3 °C; liquid adsorbed vaccines must not be allowed to freeze.

LABELLING

The label states:

- the name of the preparation,
- a reference identifying the final lot,
- the recommended human dose and route of administration,
- the storage conditions,
- the expiry date,
- the name and amount of any antimicrobial preservative,
- the name of any antibiotic, adjuvant, flavour or stabiliser present in the vaccine,
- the name of any constituent that may cause adverse reactions and any contra-indications to the use of the vaccine,
- for freeze-dried vaccines:
 - the name or composition and the volume of the reconstituting liquid to be added,
 - the time within which the vaccine is to be used after reconstitution.

01/2005:0062
corrected

VACCINES FOR VETERINARY USE

Vaccina ad usum veterinarium

In the case of combined vaccines, for each component that is the subject of a monograph in the Pharmacopoeia, the provisions of that monograph apply to that component, modified where necessary as indicated (see Tests (Safety) below, Evaluation of safety of veterinary vaccines (5.2.6) and Evaluation of efficacy of veterinary vaccines (5.2.7)).

DEFINITION

Vaccines for veterinary use are preparations containing antigenic substances and are administered for the purpose of inducing a specific and active immunity against disease provoked by bacteria, toxins, viruses, fungi or parasites. The vaccines, live or inactivated, confer active immunity that may be transferred passively via maternal antibodies against the immunogens they contain and sometimes also against antigenically related organisms. Vaccines may contain bacteria, toxins, viruses or fungi, living or inactivated, parasites, or antigenic fractions or substances produced by these organisms and rendered harmless whilst retaining all or part of their antigenic properties; vaccines may also contain combinations of these constituents. The antigens may be produced by recombinant DNA technology. Suitable adjuvants may be included to enhance the immunising properties of the vaccines.

Terminology used in monographs on vaccines for veterinary use is defined in chapter 5.2.1.

BACTERIAL VACCINES AND BACTERIAL TOXOIDS

Bacterial vaccines and bacterial toxoids are prepared from cultures grown on suitable solid or liquid media, or by other suitable means; the requirements of this section do not apply to bacterial vaccines prepared in cell cultures or in

live animals. The strain of bacterium used may have been modified by genetic engineering. The identity, antigenic potency and purity of each bacterial culture used is carefully controlled.

Bacterial vaccines contain inactivated or live bacteria or their antigenic components; they are liquid preparations of various degrees of opacity or they may be freeze-dried.

Bacterial toxoids are prepared from toxins by diminishing their toxicity to a very low level or by completely eliminating it by physical or chemical means whilst retaining adequate immunising potency. The toxins are obtained from selected strains of specified micro-organisms grown in suitable media or are obtained by other suitable means, for example, chemical synthesis.

The toxoids may be:

- liquid,
- precipitated with alum or other suitable agent,
- purified and/or adsorbed on aluminium phosphate, aluminium hydroxide, calcium phosphate or other adsorbent prescribed in the monograph.

Bacterial toxoids are clear or slightly opalescent liquids. Adsorbed toxoids are suspensions or emulsions. Certain toxoids may be freeze-dried.

Unless otherwise indicated, statements and requirements given below for bacterial vaccines apply equally to bacterial vaccines, bacterial toxoids and products containing a combination of bacterial cells and toxoid.

VIRAL VACCINES

Viral vaccines are prepared by growth in suitable cell cultures (5.2.4), in tissues, in micro-organisms, in fertilised eggs or, where no other possibility is available, in live animals, or by other suitable means. The strain of virus used may have been modified by genetic engineering. They are liquid or freeze-dried preparations of one or more viruses or viral subunits or peptides.

Live viral vaccines are prepared from viruses of attenuated virulence or of natural low virulence for the target species.

Inactivated viral vaccines are treated by a validated procedure for inactivation of the virus and may be purified and concentrated.

VECTOR VACCINES

Vector vaccines are liquid or freeze-dried preparations of one or more types of live micro-organisms (bacteria or viruses) that are non-pathogenic or have low pathogenicity for the target species and in which have been inserted one or more genes encoding antigens that stimulate an immune response protective against other microorganisms.

PRODUCTION

The methods of preparation, which vary according to the type of vaccine, are such as to maintain the identity and immunogenicity of the antigen and to ensure freedom from contamination with extraneous agents.

Substances of animal origin used in the production of vaccines for veterinary use comply with the requirements of chapter 5.2.5. Other substances used in the preparation of vaccines for veterinary use comply with requirements of the Pharmacopoeia (where a relevant monograph exists) and are prepared in a manner that avoids contamination of the vaccine.

SUBSTRATES FOR PRODUCTION

Cell cultures used in the production of vaccines for veterinary use comply with the requirements of chapter 5.2.4.

Where a monograph refers to chicken flocks free from specified pathogens (SPF), these flocks comply with the requirements prescribed in chapter 5.2.2.

For production of inactivated vaccines, where vaccine organisms are grown in poultry embryos, such embryos are derived either from SPF flocks (5.2.2) or from healthy non-SPF flocks free from the presence of certain agents and their antibodies, as specified in the monograph. It may be necessary to demonstrate that the inactivation process is effective against specified potential contaminants. For the production of a master seed lot and for all passages of a micro-organism up to and including the working seed lot, eggs from SPF flocks (5.2.2) are used.

Where it is unavoidable to use animals or animal tissues in the production of veterinary vaccines, such animals shall be free from specified pathogens, as appropriate to the source species and the target animal for the vaccine.

MEDIA

At least the qualitative composition must be recorded of media used for seed culture preparation and for production. The grade of each named ingredient is specified. Where media or ingredients are claimed as proprietary, this is indicated and an appropriate description recorded. Ingredients that are derived from animals are specified as to the source species and country of origin, and must comply with the criteria described in chapter 5.2.5. Preparation processes for media used, including sterilisation procedures, are documented.

The addition of antibiotics during the manufacturing process is normally restricted to cell culture fluids and other media, egg inocula and material harvested from skin or other tissues.

BACTERIAL SEED LOTS

General requirements. The genus and species (and varieties where appropriate) of the bacteria used in the vaccine are stated. Bacteria used in manufacture are handled in a seed-lot system wherever possible. Each master seed lot is tested as described below. A record of the origin, date of isolation, passage history (including purification and characterisation procedures) and storage conditions is maintained for each master seed lot. Each master seed lot is assigned a specific code for identification purposes.

Propagation. The minimum and maximum number of subcultures of each master seed lot prior to the production stage are specified. The methods used for the preparation of seed cultures, preparation of suspensions for seeding, techniques for inoculation of seeds, titre and concentration of inocula and the media used, are documented. It shall be demonstrated that the characteristics of the seed material (for example, dissociation or antigenicity) are not changed by these subcultures. The conditions under which each seed lot is stored are documented.

Identity and purity. Each master seed lot is shown to contain only the species and strain of bacterium stated. A brief description of the method of identifying each strain by biochemical, serological and morphological characteristics and distinguishing it as far as possible from related strains is recorded, as is also the method of determining the purity of the strain. If the master seed lot is shown to contain living organisms of any kind other than the species and strain stated, then it is unsuitable for vaccine production.

VIRUS SEED LOTS

General requirements. Viruses used in manufacture are handled in a seed-lot system. Each master seed lot is tested as described below. A record of the origin, date of isolation, passage history (including purification and characterisation procedures) and storage conditions is maintained for each

seed lot. Each master seed lot is assigned a specific code for identification purposes. Production of vaccine is not normally undertaken using virus more than 5 passages from the master seed lot. In the tests on the master seed lot described below, the organisms used are not normally more than 5 passages from the master seed lot at the start of the tests, unless otherwise indicated.

Where the master seed lot is contained within a permanently infected master cell seed, the following tests are carried out on an appropriate volume of virus from disrupted master cell seed. Where relevant tests have been carried out on disrupted cells to validate the suitability of the master cell seed, these tests need not be repeated.

Propagation. The master seed lot and all subsequent passages are propagated on cells, on embryonated eggs or in animals that have been shown to be suitable for vaccine production (see above), and, where applicable, using substances of animal origin that meet the requirements prescribed in chapter 5.2.5.

Identification. A suitable method to identify the vaccine strain and to distinguish it as far as possible from related strains must be used.

Bacterial and fungal contamination. The master seed lot complies with the test for sterility (2.6.1).

Mycoplasmas (2.6.7). The master seed lot complies with the test for mycoplasmas (culture method and indicator cell culture method).

Absence of extraneous viruses. Monographs may contain requirements for freedom from extraneous agents, otherwise the requirements stated below apply.

Preparations of monoclonal or polyclonal antibodies containing high levels of neutralising antibody to the virus of the seed lot are made on a batch basis, using antigen that is not derived from any passage level of the virus isolate giving rise to the master seed virus. Each batch of serum is maintained at 56 °C for 30 min to inactivate complement. Each batch is shown to be free of antibodies to potential contaminants of the seed virus and is shown to be free of any non-specific inhibiting effects on the ability of viruses to infect and propagate within cells (or eggs, where applicable). If such a serum cannot be obtained, other methods are used to remove or neutralise the seed virus specifically.

If the seed lot virus would interfere with the conduct and sensitivity of a test for extraneous viruses, a sample of the master seed lot is treated with a minimum amount of the monoclonal or polyclonal antibody so that the vaccine virus is neutralised as far as possible or removed. The final virus-serum mixture shall, if possible, contain at least the virus content of 10 doses of vaccine per 0.1 ml for avian vaccines and per millilitre for other vaccines. For avian vaccines, the testing to be carried out on seed lots is given in chapters 2.6.3, 2.6.4, 2.6.5 and 2.6.6. For mammalian vaccines, the seed lot or the mixture of seed lot and antiserum is tested for freedom from extraneous agents as follows.

The mixture is inoculated onto cultures of at least 70 cm² of the required cell types. The cultures may be inoculated at any suitable stage of growth up to 70 per cent confluency. At least 1 monolayer of each type must be retained as a control. The cultures must be monitored daily for a week. At the end of this period the cultures are freeze thawed 3 times, centrifuged to remove cell debris and re-inoculated onto the same cell type as above. This is repeated twice. The final passage must produce sufficient cells in appropriate vessels to carry out the tests below.

Cytopathic and haemadsorbing agents are tested for using the methods described in the relevant sections on testing cell cultures (5.2.4) and techniques such as immuno-fluorescence are used for detection of specific contaminants for the tests in cell cultures. The master seed lot is inoculated onto:

- primary cells of the species of origin of the virus,
- cells sensitive to viruses pathogenic for the species for which the vaccine is intended,
- cells sensitive to pestiviruses.

If the master seed lot is shown to contain living organisms of any kind, other than the virus of the species and strain stated, or foreign viral antigens, then it is unsuitable for vaccine production.

INACTIVATION

Inactivated vaccines are subjected to a validated inactivation procedure. The testing of the inactivation kinetics described below is carried out once for a given production process. The rest of this section applies to each production run. When conducting tests for inactivation, it is essential to take account of the possibility that under the conditions of manufacture, organisms may be physically protected from inactivant.

Inactivation kinetics. The inactivating agent and the inactivation procedure shall be shown, under conditions of manufacture, to inactivate the vaccine micro-organism. Adequate data on inactivation kinetics shall be obtained. Normally, the time required for inactivation shall be not more than 67 per cent of the duration of the inactivation process.

Aziridine. If an aziridine compound is used as the inactivating agent then it shall be shown that no inactivating agent remains at the end of the inactivation procedure. This may be accomplished by neutralising the inactivating agent with thiosulphate and demonstrating residual thiosulphate in the inactivated harvest at the completion of the inactivation procedure.

Formaldehyde. If formaldehyde is used as the inactivating agent, then a test for free formaldehyde is carried out as prescribed under Tests.

Other inactivating agents. When other inactivation methods are used, appropriate tests are carried out to demonstrate that the inactivating agent has been removed or reduced to an acceptable residual level.

Inactivation and/or detoxification testing. A test for complete inactivation and/or detoxification is performed immediately after the inactivation and/or detoxification procedure and, if applicable, the neutralisation or removal of the inactivating or detoxifying agent.

Bacterial vaccines. The test selected shall be appropriate to the vaccine bacteria being used and shall consist of at least 2 passages in production medium or, if solid medium has been used for production, in a suitable liquid medium or in the medium prescribed in the monograph. The product complies with the test if no evidence of any live micro-organism is observed.

Bacterial toxoids. The test selected shall be appropriate to the toxin or toxins present and shall be the most sensitive available.

Viral vaccines. The test selected shall be appropriate to the vaccine virus being used and must consist of at least 2 passages in cells, embryonated eggs or, where no other suitably sensitive method is available, in animals. The quantity of cell samples, eggs or animals shall be sufficient to ensure appropriate sensitivity of the test. For tests in cell cultures, not less than 150 cm² of cell culture monolayer is

inoculated with 1.0 ml of inactivated harvest. The product complies with the test if no evidence of the presence of any live virus or other micro-organism is observed.

CHOICE OF VACCINE COMPOSITION AND CHOICE OF VACCINE STRAIN

For the choice of vaccine composition and choice of vaccine strain, important aspects to be evaluated include safety, efficacy and stability. General requirements for evaluation of safety and efficacy are given in chapter 5.2.6 and chapter 5.2.7. These requirements may be made more explicit or supplemented by the requirements of specific monographs.

For live vaccines, a maximum virus titre or bacterial count acceptable from the point of view of safety is established during development studies. This is then used as the maximum acceptable titre for each batch of vaccine at release.

Potency and immunogenicity. The tests given under the headings Potency and Immunogenicity in monographs serve 2 purposes:

- the Potency section establishes by a well-controlled test in experimental conditions, the minimum acceptable vaccinating capacity for all vaccines within the scope of the definition, which must be guaranteed throughout the period of validity;
- well-controlled experimental studies are normally a part of the overall demonstration of efficacy of a vaccine (see chapter 5.2.7); the test referred to in the section 'Immunogenicity' (which is usually a cross-reference to the Potency section) is suitable as a part of this testing.

For most vaccines, the tests cited under Potency or Immunogenicity are not suitable for the routine testing of batches.

For live vaccines, the minimum acceptable virus titre or bacterial count that gives satisfactory results in the Potency test and other efficacy studies is established during development. For routine testing it must be demonstrated for each batch that the titre or count at release is such that at the end of the period of validity, in the light of stability studies, the vaccine, stored in the recommended conditions, will contain not less than the minimum acceptable virus titre or bacterial count determined during development studies. For inactivated vaccines, if the test described under Potency is not used for routine testing, a batch potency test is established during development. The aim of the batch potency test is to ensure that each batch of vaccine would, if tested, comply with the test described under Potency or Immunogenicity. The acceptance criteria for the batch potency test are therefore established by correlation with the test described under Potency. Where a batch potency test is described in a monograph, this is given as an example of a test that is considered suitable, after establishment of correlation with the potency test; other test models can also be used.

Route of administration. During development of a vaccine, safety and immunogenicity are demonstrated for each route of administration to be recommended. The following is a non-exhaustive list of such routes of administration:

- intramuscular,
- subcutaneous,
- intravenous,
- ocular,
- oral,
- nasal,
- foot-stab,
- wing web,

- intradermal,
- intraperitoneal,
- *in ovo*.

Methods of administration. During development of a vaccine, safety and immunogenicity are demonstrated for each method of administration to be recommended. The following is a non-exhaustive list of such methods of administration:

- injection,
- drinking water,
- spray,
- eye-drop,
- scarification,
- implantation,
- immersion.

Categories of animal. Monographs may indicate that a given test is to be carried out for each category of animal of the target species for which the product is recommended or is to be recommended. The following is a non-exhaustive list of categories that are to be taken into account.

- **Mammals:**
 - pregnant animals/non-pregnant animals,
 - animals raised primarily for breeding/animals raised primarily for food production,
 - animals of the minimum age or size recommended for vaccination.
- **Avian species:**
 - birds raised primarily for egg production/birds raised primarily for production of meat,
 - birds before point of lay/birds after onset of lay.
- **Fish:**
 - broodstock fish/fish raised primarily for food production.

Stability. Evidence of stability is obtained to justify the proposed period of validity. This evidence takes the form of the results of virus titrations, bacterial counts or potency tests carried out at regular intervals until 3 months beyond the end of the shelf life on not fewer than 3 representative consecutive batches of vaccine kept under recommended storage conditions together with results from studies of moisture content (for freeze-dried products), physical tests on the adjuvant, chemical tests on substances such as the adjuvant constituents and preservatives and pH, as appropriate.

Where applicable, studies on the stability of the reconstituted vaccine are carried out, using the product reconstituted in accordance with the proposed recommendations.

FINAL BULK VACCINE

The final bulk vaccine is prepared by combining one or more batches of antigen that comply with all the relevant requirements with any auxiliary substances, such as adjuvants, stabilisers, antimicrobial preservatives and diluents.

Antimicrobial preservatives. Antimicrobial preservatives are used to prevent spoilage or adverse effects caused by microbial contamination occurring during use of a vaccine which is expected to be no longer than 10 h after first broaching. Antimicrobial preservatives are not included in freeze-dried products but, if justified, taking into account the maximum recommended period of use after reconstitution, they may be included in the diluent for multi-dose freeze-dried products. For single-dose liquid preparations, inclusion of antimicrobial preservatives is not acceptable unless justified and authorised, but may be acceptable, for

example where the same vaccine is filled in single-dose and multidose containers and is used in non-food-producing species. For multidose liquid preparations, the need for effective antimicrobial preservation is evaluated taking into account likely contamination during use and the maximum recommended period of use after broaching of the container.

During development studies the effectiveness of the antimicrobial preservative throughout the period of validity shall be demonstrated to the satisfaction of the competent authority.

The efficacy of the antimicrobial preservative is evaluated as described in chapter 5.1.3 and in addition samples are tested at suitable intervals over the proposed in use shelf-life. If neither the A criteria nor the B criteria can be met, then in justified cases the following criteria are applied to vaccines for veterinary use: bacteria, no increase from 24 h to 7 days, 3 log reduction at 14 days, no increase at 28 days; fungi, no increase at 14 days and 28 days.

Addition of antibiotics as antimicrobial preservative is generally not acceptable.

Test for inactivation and/or detoxification. For inactivated vaccines, where the auxiliary substances would interfere with a test for inactivation and/or detoxification, a test for inactivation or detoxification is carried out during preparation of the final bulk, after the different batches of antigen have been combined but before addition of auxiliary substances; the test for inactivation or detoxification may then be omitted on the final bulk and the batch.

Where there is a risk of reversion to toxicity, the test for detoxification performed at the latest stage of the production process at which the sensitivity of the test is not compromised (e.g. after the different batches of antigen have been combined but before the addition of auxiliary substances) is important to demonstrate a lack of reversion to toxicity.

In-process tests. Certain tests may be carried out on the final bulk vaccine rather than on the batch or batches prepared from it; such tests include those for antimicrobial preservatives, free formaldehyde and the potency determination for inactivated vaccines.

BATCH

Unless otherwise prescribed in the monograph, the final bulk vaccine is distributed aseptically into sterile, tamper-proof containers which are then closed so as to exclude contamination.

Only a batch that complies with each of the requirements given below under Identification, Tests and Potency or in the relevant individual monograph may be released for use. With the agreement of the competent authority, certain of the batch tests may be omitted where in-process tests give an equal or better guarantee that the batch would comply or where alternative tests validated with respect to the Pharmacopoeia method have been carried out.

The identification test can often be conveniently combined with the batch potency test to avoid unnecessary use of animals. For a given vaccine, a validated *in vitro* test can be used to avoid the unnecessary use of animals.

It is recognised that, in accordance with General Notices (1.1. General statements), for an established vaccine the routine application of the safety test will be waived by the competent authority in the interests of animal welfare when a sufficient number of consecutive production batches have been produced and found to comply with the test, thus demonstrating consistency of the manufacturing process. Significant changes to the manufacturing process may require resumption of routine testing to re-establish consistency. The number of consecutive batches to be tested

depends on a number of factors such as the type of vaccine, the frequency of production of batches and experience with the vaccine during development safety testing and during application of the batch safety test. Without prejudice to the decision of the competent authority in the light of information available for a given vaccine, testing of 10 consecutive batches is likely to be sufficient for most products. For products with an inherent safety risk, it may be necessary to continue to conduct the safety test on each batch.

Animal tests. In accordance with the provisions of the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes, tests must be carried out in such a way as to use the minimum number of animals and to cause the least pain, suffering, distress or lasting harm. The criteria for judging tests in monographs must be applied in the light of this. For example, if it is indicated that an animal is considered to be positive, infected etc. when typical clinical signs occur then as soon as it is clear that the result will not be affected the animal in question shall be either humanely killed or given suitable treatment to prevent unnecessary suffering. In accordance with the General Notices, alternative test methods may be used to demonstrate compliance with the monograph and the use of such tests is particularly encouraged when this leads to replacement or reduction of animal use or reduction of suffering.

Physical tests. A vaccine with an oily adjuvant is tested for viscosity by a suitable method and shown to be within the limits set for the product. The stability of the emulsion shall be demonstrated.

Chemical tests. Tests for the concentrations of appropriate substances such as aluminium and preservatives are carried out to show that these are within the limits set for the product.

pH. The pH of liquid products and diluents is measured and shown to be within the limits set for the product.

Water. Where applicable, the freeze-drying process is checked by a determination of water and shown to be within the limits set for the product.

IDENTIFICATION

For inactivated vaccines, the identification prescribed in monographs is usually an antibody induction test since this is applicable to all vaccines.

TESTS

The monographs also indicate tests to be carried out on each particular vaccine.

All hen eggs, chickens and chicken cell cultures for use in quality control tests shall be derived from an SPF flock (5.2.2).

Formaldehyde (2.4.18; use Method B if sodium metabisulphite has been used to neutralise excess formaldehyde). Where formaldehyde has been used in the preparation, the concentration of free formaldehyde is not greater than 0.5 g/l, unless a higher amount has been shown to be safe.

Phenol (2.5.15). When the vaccine contains phenol, the concentration is not greater than 5 g/l.

Sterility (2.6.1). Where prescribed in the monograph, vaccines comply with the test for sterility. Where the volume of liquid in a container is greater than 100 ml, the method of membrane filtration is used wherever possible. Where the method of membrane filtration cannot be used, the method

of direct inoculation may be used. Where the volume of liquid in each container is at least 20 ml, the minimum volume to be used for each culture medium is 10 per cent of the contents or 5 ml, whichever is less. The appropriate number of items to be tested (2.6.1) is 1 per cent of the batch with a minimum of 4 and a maximum of 10.

For avian live viral vaccines, for non-parenteral use only, the requirement for sterility is usually replaced by requirements for absence of pathogenic micro-organisms and for a maximum of 1 non-pathogenic micro-organism per dose.

Extraneous agents. Monographs prescribe a set of measures that taken together give an acceptable degree of assurance that the final product does not contain infectious extraneous agents. These measures include:

- 1) Production within a seed-lot system and a cell-seed system, wherever possible;
- 2) Extensive testing of seed lots and cell seed for extraneous agents;
- 3) Requirements for SPF flocks used for providing substrates for vaccine production;
- 4) Testing of substances of animal origin, which must, wherever possible, undergo an inactivation procedure;
- 5) For live vaccines, testing of the final product for infectious extraneous agents; such tests are less extensive than those carried out at earlier stages because of the guarantees given by in-process testing.

In cases of doubt, the tests intended for the seed lot of a live vaccine may also be applied to the final product. If an extraneous agent is found in such a test, the vaccine does not comply the monograph.

Avian live viral vaccines comply with the tests for extraneous agents in batches of finished products (2.6.4).

Mycoplasmas (2.6.7). Where prescribed in a monograph, the vaccine complies with the test for mycoplasmas (culture method).

Safety. In general, 2 doses of an inactivated vaccine and/or 10 doses of a live vaccine are injected by a recommended route. It may be necessary to reduce the prescribed number of doses under certain circumstances or amend the method of re-constitution and injection, for example for a combined vaccine, where it is difficult to reconstitute 10 doses of the live component in 2 doses of the inactivated component. The animals are observed for the longest period stated in the monographs. No abnormal local or systemic reaction occurs. Where several batches are prepared from the same final bulk, the safety test is carried out on the first batch and then omitted for further batches prepared from the same final bulk.

During development studies, the type and degree of reactions expected with the vaccine are defined in the light of safety testing. This definition is then used as part of the operating procedure for the batch safety test to evaluate acceptable and unacceptable reactions.

The immune status of animals to be used for the safety test is specified in the individual monograph. For most monographs, one of the 3 following categories is specified:

- 1) the animals must be free from antibodies against the virus/bacterium/toxin etc. contained in the vaccine,
- 2) the animals are preferably free from antibodies but animals with a low level of antibody may be used as long as the animals have not been vaccinated and the administration of the vaccine does not cause an anamnestic response,
- 3) the animals must not have been vaccinated against the disease the vaccine is intended to prevent.

As a general rule, category 1 is specified for live vaccines. For other vaccines, category 2 is usually specified but where most animals available for use in tests would comply with category 1, this may be specified for inactivated vaccines also. Category 3 is specified for some inactivated vaccines where determination of antibodies prior to testing is unnecessary or impractical. For poultry vaccines, as a general rule the use of SPF birds is specified.

For avian vaccines, the safety test is generally carried out using 10 SPF chickens (5.2.2), except that for vaccines not recommended for use in chickens it is carried out using 10 birds of one of the species for which the vaccine is recommended, the birds being free from antibodies against the disease agent for which the vaccine is intended to provide protection.

POTENCY

See Choice of vaccine composition and choice of vaccine strain under Production.

STORAGE

Store protected from light at a temperature of 5 ± 3 °C, unless otherwise indicated. Liquid preparations are not to be allowed to freeze, unless otherwise indicated.

LABELLING

The label states:

- that the preparation is for veterinary use,
- the volume of the preparation and the number of doses in the container,
- the route of administration,
- the type or types of bacteria or viruses used and for live vaccines the minimum and the maximum number of live bacteria or the minimum and the maximum virus titre,
- where applicable, for inactivated vaccines, the minimum potency in International Units,
- where applicable, the name and amount of antimicrobial preservative or other substance added to the vaccine,
- the name of any substance that may cause an adverse reaction,
- for freeze-dried vaccines:
 - the name or composition and the volume of the reconstituting liquid to be added,
 - the period within which the vaccine is to be used after reconstitution,
- for vaccines with an oily adjuvant, that if the vaccine is accidentally injected into man, urgent medical attention is necessary,
- the animal species for which the vaccine is intended,
- the indications for the vaccine,
- the instructions for use,
- any contra-indications to the use of the product including any required warning on the dangers of administration of an overdose,
- the doses recommended for different species.

01/2005:1579

VEGETABLE FATTY OILS

Olea herbaria

DEFINITION

Vegetable fatty oils are mainly solid or liquid triglycerides of fatty acids. They may contain small amounts of other lipids such as waxes, free fatty acids, partial glycerides or

unsaponifiable matters. Vegetable fatty oils are obtained from the seeds, the fruit or the pit/stone/kernel of various plants by expression and/or solvent extraction, then possibly refined and hydrogenated. A suitable antioxidant may be added if necessary.

Virgin oil: an oil obtained from raw materials of special quality by mechanical procedures (e.g. by cold expression or centrifugation).

Refined oil: an oil obtained by expression and/or solvent extraction, and subsequently, either alkali refining (followed by bleaching and deodorisation) or physical refining.

Hydrogenated oil: an oil obtained by expression and/or solvent extraction, and subsequently, either alkali refining or physical refining, then possible bleaching, followed by drying, hydrogenation and subsequent bleaching and deodorisation.

Only phosphoric acid and alkali refined oils are used in the preparation of parenteral dosage forms.

PRODUCTION

OBTAINING OF A CRUDE OIL

When the plant has a high oil content, the oil is generally obtained by expression under heating followed by an extraction; when the plant has a low oil content, the oil is generally obtained by direct extraction.

Mechanical procedures

A. Expression

High-pressure screw-pressing. It consists of some or all of the following steps: cleaning, drying, dehulling or decorticating, grinding, cooking and flaking.

During *cleaning* the foreign matter is eliminated. *Drying* may be necessary if the seed moisture content is higher than desirable for downstream processing. *Decorticating* is useful to obtain a high-protein meal by reduction of fibre and to reduce impurities in the oil. *Cooking* serves various purposes: completion of the breakdown of oil cells, lowering of the viscosity of the oil, coagulation of the protein in the meal, adjustment of the moisture level, sterilisation of the seed, detoxifying undesirable seed constituents (gossypol for cottonseed) and fixing certain phosphatides in the cake thus lowering subsequent refining losses. The efficacy of the expression process is such that only 3 per cent to 6 per cent of the oil is left in the cake.

Wet screw-pressing. The bunches are loaded into cages (for palm fruit) and moved into a horizontal steriliser with application of live steam and heating. The purposes of this steriliser are inactivation of enzymes, loosening of the fruit on the bunch, coagulation of proteins, etc. After heating in a digester, the pulp is fed to a screw-press. The oil is centrifugally clarified and vacuum-dried.

Pre-pressing followed by solvent extraction. The same sequence of steps is performed as above. The main function of pre-pressing is to obtain a cake of excellent permeability for the following solvent extraction stage. The extraction is performed either in a percolation type or in an immersion type apparatus. The efficacy of the solvent extraction process is such that residual oil levels in meal are generally below 1 per cent.

B. Centrifugation

Centrifugation separates the oily phase from the aqueous phase which contains water, water-soluble components and residual solid particles. This operation can be carried out using:

- self-cleaning bowl or disc centrifuges,

- super-decanter, which are horizontal turbines equipped with a cylindrical bowl that tapers slightly at one end and which contains a continuously turning screw that scrapes the sides of the bowl. The screw and the bowl rotate at different speeds. The solid particles are discarded from the tapered end of the bowl and the oil flows out from the other end.

Solvent extraction. Prior to extraction, the following steps are carried out: the seeds are tempered for about a week at a temperature below 24 °C in order to loosen the hull from the seed and allow the seed moisture to attain equilibrium. Then the seeds are cleaned, ground, dehulled and flaked. The most widely used solvent is a mixture of mainly *n*-hexane and methylpentanes (bp: 65-70 °C) commonly referred to as “hexane”. Due to the major fire and explosive risks of this mixture, liquified gases and supercritical gases may also be used.

REFINING

The objective of refining is to remove impurities and contaminants of the oil with the least possible damage to the triglycerides and with minimal loss of oil. The contents of the following substances are reduced:

- free fatty acids which may cause deterioration of the oil by oxidation, smoked taste when heated and sharp flavour (by alkali refining),
- water which favours the enzymatic hydrolysis reactions (by alkali refining, drying),
- partial glycerides which may cause foaming and bitter taste (by neutralisation, washing),
- phosphatides and phosphorous compounds which have emulsifying properties, may cause deposits, a darkening of the oil when heated, a cloudy appearance and bad organoleptic stability (by alkali refining),
- colouring matters such as chlorophyll (by alkali refining), carotenoids (by bleaching),
- glycolipids which may form colloidal solutions with water,
- free hydrocarbons, paraffin, waxes and resinous materials,
- metals (Fe, Cu, Pb, Sn, Pt, Pd, etc.) which are strong oxidation catalysts,
- pigments such as gossypol (in cottonseed oil) or mycotoxins such as aflatoxin (mainly in arachis oil),
- pesticides,
- oxidation products (aldehydes, peroxides),
- proteins having possible allergic reactions,
- unsaponifiable matters (e.g. lignins, sterols, tocopherols and other vitamins),
- polycyclic aromatic hydrocarbons.

Alkali refining. It involves the following steps: degumming, neutralisation using alkali, washing and drying.

Degumming. During this step of the refining, i.e. treatment with water and/or phosphoric acid, and/or sodium chloride, the phosphatides, phosphorous compounds and metals are eliminated. The use of this step depends on the nature of the oil.

Neutralisation with alkali. This step reduces the free fatty acid content below 0.1 per cent; the fatty acids are converted into oil-insoluble soaps, also called “soapstocks”. Other substances may be removed by adsorption on these soaps: mucilaginous substances, phosphatides, oxidation products, colouring matters, etc. All substances that become insoluble in the oil on hydration are removed. Neutralisation with alkali has the disadvantage of saponifying a portion of neutral oil if the neutralisation is not well conducted.

Washing. This operation consists in removing the excess of soaps and alkali as well as the remaining traces of metals, phosphatides and other impurities, using hot water.

Drying. The remaining water is eliminated under vacuum before any further steps, such as bleaching.

Physical refining. It involves a steam treatment of the oil under high vacuum at a temperature greater than 235 °C. This technique must be applied to oils naturally low in phosphatides and metals (palm, coconut, olive) or from which phosphatides and metals have been removed by an acid treatment using concentrated phosphoric acid followed by an adsorptive treatment with activated bleaching earth (for sunflower, rapeseed, soya-bean). Moreover it cannot be used for heat sensitive oils (cottonseed oil) which darken.

Bleaching. The common method of bleaching is by adsorption treatment of the oil, which is generally heated at 90 °C for 30 min under vacuum, with bleaching earth (natural or activated) or carbon (activated or not); synthetic silica adsorbents may also be added. Substances which have not been totally removed during refining are eliminated, for example carotenoids and chlorophyll.

Deodorisation. Deodorisation eliminates odours, volatile substances and residual extraction solvents; it involves injecting dry vapour into the oil which is kept under vacuum at a high temperature. Different temperatures are used according to the oil: 200 °C to 235 °C for 1 h 30 min to 3 h or greater than 240 °C for 30 min.

One of the main side reactions is thermic decolourisation due to the destruction of carotenoids when the temperature is greater than 150 °C. This technique provokes a loss of substances which may be distilled (free fatty acids, sterols, tocopherols, part of the refined oil) and may cause *cis-trans* isomerisation of the unsaturated fatty-acid double bonds.

WINTERISATION

Elimination of solids and waxes by filtration at low temperature (also called dewaxing). These solids and waxes could affect the appearance of the oil and cause deposits.

HYDROGENATION

The hydrogenation of the dried and/or bleached oil is performed using a catalyst (e.g. Ni, Pt, Pd), at a temperature of about 100 °C to 200 °C under hydrogen pressure. The catalyst is then removed by filtration at 90 °C. The hydrogen must be pure: free of poisons for the catalyst, water-free, low in carbon dioxide, methane and nitrogen contents. Small amounts of *trans*-fatty acids or polymers may be obtained.

CHROMATOGRAPHIC PURIFICATION

In high purity applications, mainly for parenteral uses, the oil may be further purified by passing the oil through a column containing an activated earth. A solvent may sometimes be used to improve the efficiency. High polarity molecules such as oxidised materials, acids, alcohols, partial glycerides and free sterols are preferentially removed.

When the oil is used in the preparation of parenteral dosage forms, the limits set in the monograph for the acid value, the peroxide value and the water content may be different.

LABELLING

The label states:

- where applicable, that the oil was obtained by expression or extraction,
- where applicable, that the oil is suitable for use in the manufacture of parenteral dosage forms,
- the name and concentration of any added antioxidant.

01/2005:1502 **Large-volume parenterals**

Infusions and injections supplied in containers with a nominal content of more than 100 ml.

Small-volume parenterals

Infusions and injections supplied in containers with a nominal content of 100 ml or less.

01/2005:0016

GLOSSARY

The following introductory text provides definitions and/or explanations of terms that may be found in, or used in association with, the general monographs on dosage forms, but that are not defined within them. Where relevant, reference is made to other equivalent terms that may be found in other publications or contexts.

This glossary is published for information.

Standard Term

Standard Terms for describing the pharmaceutical form of a medicinal product, the routes of administration and the containers used have been established by the European Pharmacopoeia Commission and are provided in a separate publication on Standard Terms.

Active substance

Equivalent terms: active ingredient, drug substance, medicinal substance, active pharmaceutical ingredient.

Vehicle

A vehicle is the carrier, composed of one or more excipients, for the active substance(s) in a liquid preparation.

Basis

A basis is the carrier, composed of one or more excipients, for the active substance(s) in semi-solid and solid preparations.

Conventional-release dosage forms

Conventional-release dosage forms are preparations showing a release of the active substance(s) which is not deliberately modified by a special formulation design and/or manufacturing method. In the case of a solid dosage form, the dissolution profile of the active substance depends essentially on its intrinsic properties. Equivalent term: immediate-release dosage form.

Modified-release dosage forms

Modified-release dosage forms are preparations where the rate and/or place of release of the active substance(s) is different from that of a conventional-release dosage form administered by the same route. This deliberate modification is achieved by a special formulation design and/or manufacturing method. Modified-release dosage forms include prolonged-release, delayed-release and pulsatile-release dosage forms.

Prolonged-release dosage forms

Prolonged-release dosage forms are modified-release dosage forms showing a slower release of the active substance(s) than that of a conventional-release dosage form administered by the same route. Prolonged-release is achieved by a special formulation design and/or manufacturing method. Equivalent term: extended-release dosage form.

Delayed-release dosage forms

Delayed-release dosage forms are modified-release dosage forms showing a release of the active substance(s) which is delayed. Delayed release is achieved by a special formulation design and/or manufacturing method. Delayed-release dosage forms include gastro-resistant preparations as defined in the general monographs on solid oral dosage forms.

Pulsatile-release dosage forms

Pulsatile-release dosage forms are modified-release dosage forms showing a sequential release of the active substance(s). Sequential release is achieved by a special formulation design and/or manufacturing method.

CAPSULES

Capsulae

The requirements of this monograph do not necessarily apply to preparations that are presented as capsules intended for use other than by oral administration. Requirements for such preparations may be found, where appropriate, in other general monographs, for example Rectal preparations (1145) and Vaginal preparations (1164).

DEFINITION

Capsules are solid preparations with hard or soft shells of various shapes and capacities, usually containing a single dose of active substance. They are intended for oral administration.

The capsule shells are made of gelatin or other substances, the consistency of which may be adjusted by the addition of substances such as glycerol or sorbitol. Excipients such as surface-active agents, opaque fillers, antimicrobial preservatives, sweeteners, colouring matter authorised by the competent authority and flavouring substances may be added. The capsules may bear surface markings.

The contents of capsules may be solid, liquid or of a paste-like consistency. They consist of one or more active substances with or without excipients such as solvents, diluents, lubricants and disintegrating agents. The contents do not cause deterioration of the shell. The shell, however, is attacked by the digestive fluids and the contents are released.

Where applicable, containers for capsules comply with the requirements of *Materials used for the manufacture of containers* (3.1 and subsections) and *Containers* (3.2 and subsections).

Several categories of capsules may be distinguished:

- hard capsules,
- soft capsules,
- gastro-resistant capsules,
- modified-release capsules,
- cachets.

PRODUCTION

In the manufacture, packaging, storage and distribution of capsules, suitable means are taken to ensure their microbial quality; recommendations on this aspect are provided in the text on *Microbiological quality of pharmaceutical preparations* (5.1.4).

TESTS

Uniformity of content (2.9.6). Unless otherwise prescribed or justified and authorised, capsules with a content of active substance less than 2 mg or less than 2 per cent of the total mass comply with test B for uniformity of content of single-dose preparations. If the preparation has more than one active substance, the requirement applies only to those ingredients which correspond to the above conditions.

Uniformity of mass (2.9.5). Capsules comply with the test for uniformity of mass of single-dose preparations. If the test for uniformity of content is prescribed for all the active substances, the test for uniformity of mass is not required.

Dissolution. A suitable test may be carried out to demonstrate the appropriate release of the active substance(s), for example one of the tests described in *Dissolution test for solid dosage forms (2.9.3)*.

Where a dissolution test is prescribed, a disintegration test may not be required.

STORAGE

Store at a temperature not exceeding 30 °C.

LABELLING

The label states the name of any added antimicrobial preservative.

Hard capsules

DEFINITION

Hard capsules have shells consisting of two prefabricated cylindrical sections one end of which is rounded and closed, the other being open.

PRODUCTION

The active substance(s) usually in solid form (powder or granules) are filled into one of the sections which is then closed by slipping the other section over it. The security of the closure may be strengthened by suitable means.

TESTS

Disintegration. Hard capsules comply with the test for disintegration of tablets and capsules (2.9.1). Use *water R* as the liquid medium. When justified and authorised, *0.1 M hydrochloric acid* or *artificial gastric juice R* may be used as the liquid medium. If the capsules float on the surface of the water, a disc may be added. Operate the apparatus for 30 min, unless otherwise justified and authorised and examine the state of the capsules. The capsules comply with the test if all 6 have disintegrated.

Soft capsules

DEFINITION

Soft capsules have thicker shells than those of hard capsules. The shells consist of one part and are of various shapes.

PRODUCTION

Soft capsules are usually formed, filled and sealed in one operation but for extemporaneous use, the shell may be prefabricated. The shell material may contain an active substance.

Liquids may be enclosed directly; solids are usually dissolved or dispersed in a suitable vehicle to give a solution or dispersion of a paste-like consistency.

There may be partial migration of the constituents from the capsule contents into the shell and vice versa because of the nature of the materials and the surfaces in contact.

TESTS

Disintegration. Soft capsules comply with the test for disintegration of tablets and capsules (2.9.1). Use *water R* as the liquid medium. When justified and authorised, *0.1 M hydrochloric acid* or *artificial gastric juice R* may be used as the liquid medium. Add a disc to each tube. Liquid active

substances dispensed in soft capsules may attack the disc; in such circumstances and where authorised, the disc may be omitted. Operate the apparatus for 30 min, unless otherwise justified and authorised and examine the state of the capsules. If the capsules fail to comply because of adherence to the discs, repeat the test on a further 6 capsules omitting the discs. The capsules comply with the test if all 6 have disintegrated.

Modified-release capsules

DEFINITION

Modified-release capsules are hard or soft capsules in which the contents or the shell or both contain special excipients or are prepared by a special process designed to modify the rate, the place or the time at which the active substance(s) are released.

Modified release capsules include prolonged-release capsules and delayed-release capsules.

PRODUCTION

A suitable test is carried out to demonstrate the appropriate release of the active substance(s).

Gastro-resistant capsules

DEFINITION

Gastro-resistant capsules are delayed-release capsules that are intended to resist the gastric fluid and to release their active substance or substances in the intestinal fluid. Usually they are prepared by filling capsules with granules or with particles covered with a gastro-resistant coating or in certain cases, by providing hard or soft capsules with a gastro-resistant shell (enteric capsules).

PRODUCTION

For capsules filled with granules or filled with particles covered with a gastro-resistant coating, a suitable test is carried out to demonstrate the appropriate release of the active substance(s).

TESTS

Disintegration. For capsules with a gastro-resistant shell carry out the test for disintegration (2.9.1) with the following modifications. Use *0.1 M hydrochloric acid* as the liquid medium and operate the apparatus for 2 h, or other such time as may be authorised, without the discs. Examine the state of the capsules. The time of resistance to the acid medium varies according to the formulation of the capsules to be examined. It is typically 2 h to 3 h but even with authorised deviations it must not be less than 1 h. No capsule shows signs of disintegration or rupture permitting the escape of the contents. Replace the acid by *phosphate buffer solution pH 6.8 R*. When justified and authorised, a buffer solution of pH 6.8 with added pancreas powder (for example, 0.35 g of *pancreas powder R* per 100 ml of buffer solution) may be used. Add a disc to each tube. Operate the apparatus for 60 min and examine the state of the capsules. If the capsules fail to comply because of adherence to the discs, repeat the test on a further 6 capsules omitting the discs. The capsules comply with the test if all 6 have disintegrated.

Dissolution. For capsules prepared from granules or particles already covered with a gastro-resistant coating, a suitable test is carried out to demonstrate the appropriate release of the active substance(s), for example the test described in *Dissolution test for solid dosage forms (2.9.3)*.

Cachets

DEFINITION

Cachets are solid preparations consisting of a hard shell containing a single dose of one or more active substances. The cachet shell is made of unleavened bread usually from rice flour and consists of 2 prefabricated flat cylindrical sections. Before administration, the cachets are immersed in water for a few seconds, placed on the tongue and swallowed with a draught of water.

LABELLING

The label states the method of administration of the cachets.

01/2005:1239

CHEWING GUMS, MEDICATED

Masticabilia gummis medicata

DEFINITION

Medicated chewing gums are solid, single-dose preparations with a base consisting mainly of gum that are intended to be chewed but not swallowed.

They contain one or more active substances which are released by chewing. After dissolution or dispersion of the active substances in saliva, chewing gums are intended to be used for:

- local treatment of mouth diseases,
- systemic delivery after absorption through the buccal mucosa or from the gastrointestinal tract.

PRODUCTION

Medicated chewing gums are made with a tasteless masticatory gum base that consists of natural or synthetic elastomers. They may contain other excipients such as fillers, softeners, sweetening agents, flavouring substances, stabilisers and plasticisers and authorised colouring matter.

Medicated chewing gums are manufactured by compression or by softening or melting the gum bases and adding successively the other substances. In the latter case, chewing gums are then further processed to obtain the desired gum presentation. The medicated chewing gums may be coated, for example, if necessary to protect from humidity and light.

Unless otherwise justified and authorised, a suitable test is carried out to demonstrate the appropriate release of the active ingredient(s).

In the manufacture, packaging, storage and distribution of medicated chewing gums, suitable means must be taken to ensure their microbial quality; recommendations related to this aspect are provided in the general chapter on *Microbiological quality of pharmaceutical preparations* (5.1.4).

TESTS

Uniformity of content (2.9.6). Unless otherwise prescribed or justified and authorised, medicated chewing gums with a content of active ingredient less than 2 mg or less than 2 per cent of the total mass comply with test A for uniformity of content of single-dose preparations. If the preparation contains more than one active substance, the requirement applies only to those active substances which correspond to the above conditions.

Uniformity of mass (2.9.5). Uncoated medicated chewing gums and, unless otherwise justified and authorised, coated medicated chewing gums comply with the test for uniformity

of mass of single-dose preparations. If the test for uniformity of content is prescribed for all the active substances, the test for uniformity of mass is not required.

STORAGE

Store uncoated medicated chewing gums protected from humidity and light.

01/2005:0652

EAR PREPARATIONS

Auricularia

DEFINITION

Ear preparations are liquid, semi-solid or solid preparations intended for instillation, for spraying, for insufflation, for application to the auditory meatus or as an ear wash.

Ear preparations usually contain one or more active substances in a suitable vehicle. They may contain excipients, for example, to adjust tonicity or viscosity, to adjust or stabilise the pH, to increase the solubility of the active substances, to stabilise the preparation or to provide adequate antimicrobial properties. The excipients do not adversely affect the intended medicinal action of the preparation or, at the concentrations used, cause toxicity or undue local irritation.

Preparations for application to the injured ear, particularly where the ear-drum is perforated, or prior to surgery are sterile, free from antimicrobial preservatives and supplied in single-dose containers.

Ear preparations are supplied in multi-dose or single-dose containers, provided, if necessary, with a suitable administration device which may be designed to avoid the introduction of contaminants.

Unless otherwise justified and authorised, aqueous ear preparations supplied in multidose containers contain a suitable antimicrobial preservative at a suitable concentration, except where the preparation itself has adequate antimicrobial properties.

Where applicable, containers for ear preparations comply with the requirements of *Materials used for the manufacture of containers* (3.1 and subsections) and *Containers* (3.2 and subsections).

Several categories of ear preparations may be distinguished:

- ear-drops and sprays,
- semi-solid ear preparations,
- ear powders,
- ear washes,
- ear tampons.

PRODUCTION

During the development of an ear preparation, the formulation for which contains an antimicrobial preservative, the effectiveness of the chosen preservative shall be demonstrated to the satisfaction of the competent authority. A suitable test method together with criteria for judging the preservative properties of the formulation are provided in the text on *Efficacy of antimicrobial preservation* (5.1.3).

In the manufacture, packaging, storage and distribution of ear preparations, suitable means are taken to ensure their microbial quality; recommendations on this aspect are provided in the text on *Microbiological quality of pharmaceutical preparations* (5.1.4).

Sterile ear preparations are prepared using materials and methods designed to ensure sterility and to avoid the introduction of contaminants and the growth of

micro-organisms; recommendations on this aspect are provided in the text on *Methods of preparation of sterile products* (5.1.1).

In the manufacture of ear preparations containing dispersed particles, measures are taken to ensure a suitable and controlled particle size with regard to the intended use.

TESTS

Uniformity of content (2.9.6). Unless otherwise prescribed or justified and authorised, single-dose ear preparations with a content of active substance less than 2 mg or less than 2 per cent of the total mass comply with test B for uniformity of content of single-dose preparations. If the preparation has more than one active substance, the requirement applies only to those ingredients that correspond to the above conditions.

Uniformity of mass (2.9.5). Single-dose ear preparations comply with the test for uniformity of mass of single-dose preparations. If the test for uniformity of content is prescribed for all the active substances, the test for uniformity of mass is not required.

Sterility (2.6.1). Where the label indicates that the ear preparation is sterile, it complies with the test for sterility.

STORAGE

If the preparation is sterile, store in a sterile, airtight, tamper-proof container.

LABELLING

The label states:

- the name of any added antimicrobial preservative,
- where applicable, that the preparation is sterile,
- for multidose containers, the period after opening the container after which the contents must not be used. This period does not exceed 4 weeks, unless otherwise justified and authorised.

Ear-drops and sprays

DEFINITION

Ear-drops and sprays are solutions, emulsions or suspensions of one or more active substances in liquids suitable for application to the auditory meatus without exerting harmful pressure on the ear-drum (for example, water, glycols or fatty oils). They may also be placed in the auditory meatus by means of a tampon impregnated with the liquid.

Emulsions may show evidence of phase separation but are readily redispersed on shaking. Suspensions may show a sediment which is readily dispersed on shaking to give a suspension which remains sufficiently stable to enable the correct dose to be delivered.

Ear drops are usually supplied in multidose containers of glass or suitable plastic material that are fitted with an integral dropper or with a screw cap of suitable materials incorporating a dropper and rubber or plastic teat. Alternatively, such a cap assembly is supplied separately. Sprays are usually supplied in multi-dose containers fitted with an appropriate applicator. When sprays are supplied in pressurised containers, these comply with the requirements of the monograph on *Pressurised pharmaceutical preparations* (0523).

Semi-solid ear preparations

DEFINITION

Semi-solid ear preparations are intended for application to the external auditory meatus, if necessary by means of a tampon impregnated with the preparation.

Semi-solid ear preparations comply with the requirements of the monograph on *Semi-solid preparations for cutaneous application* (0132).

They are supplied in containers fitted with a suitable applicator.

Ear powders

DEFINITION

Ear powders comply with the requirements of the monograph on *Powders for cutaneous application* (1166).

They are supplied in containers fitted with a suitable device for application or insufflation.

Ear washes

DEFINITION

Ear washes are preparations intended to cleanse the external auditory meatus. They are usually aqueous solutions with apH within physiological limits.

Ear washes intended for application to injured parts or prior to a surgical operation are sterile.

TESTS

Deliverable mass or volume (2.9.28). Ear washes supplied in single-dose containers comply with the test.

Ear tampons

DEFINITION

Ear tampons are intended to be inserted into the external auditory meatus. They comply with the requirements of the monograph on *Medicated tampons* (1155).

01/2005:1163

EYE PREPARATIONS

Ophthalmica

DEFINITION

Eye preparations are sterile liquid, semi-solid or solid preparations intended for administration upon the eyeball and/or to the conjunctiva or for insertion in the conjunctival sac.

Where applicable, containers for eye preparations comply with the requirements of *Materials used for the manufacture of containers* (3.1 and subsections) and *Containers* (3.2 and subsections).

Several categories of eye preparations may be distinguished:

- eye drops,
- eye lotions,
- powders for eye drops and eye lotions,
- semi-solid eye preparations,
- ophthalmic inserts.

PRODUCTION

During the development of an eye preparation, the formulation for which contains an antimicrobial preservative, the effectiveness of the chosen preservative shall be demonstrated to the satisfaction of the competent authority. A suitable test method together with criteria for judging the preservative properties of the formulation are provided in the text on *Efficacy of antimicrobial preservation* (5.1.3).

Eye preparations are prepared using materials and methods designed to ensure sterility and to avoid the introduction of contaminants and the growth of micro-organisms; recommendations on this aspect are provided in the text on *Methods of preparation of sterile products* (5.1.1).

In the manufacture of eye preparations containing dispersed particles, measures are taken to ensure a suitable and controlled particle size with regard to the intended use.

TESTS

Sterility (2.6.1). Eye preparations comply with the test for sterility. Applicators supplied separately also comply with the test for sterility. Remove the applicator with aseptic precautions from its package and transfer it to a tube of culture medium so that it is completely immersed. Incubate and interpret the results as described in the test for sterility.

Deliverable mass or volume (2.9.28). Liquid and semi-solid eye preparations supplied in single-dose containers comply with the test.

STORAGE

Unless otherwise prescribed, store in a sterile, airtight, tamper-proof container.

LABELLING

The label states the name of any added antimicrobial preservative.

Eye-drops

DEFINITION

Eye-drops are sterile aqueous or oily solutions or suspensions of one or more active substances intended for instillation into the eye.

Eye-drops may contain excipients, for example, to adjust the tonicity or the viscosity of the preparation, to adjust or stabilise the pH, to increase the solubility of the active substance, or to stabilise the preparation. These substances do not adversely affect the intended medicinal action or, at the concentrations used, cause undue local irritation.

Aqueous preparations supplied in multidose containers contain a suitable antimicrobial preservative in appropriate concentration except when the preparation itself has adequate antimicrobial properties. The antimicrobial preservative chosen must be compatible with the other ingredients of the preparation and must remain effective throughout the period of time during which eye-drops are in use.

If eye-drops are prescribed without antimicrobial preservatives they are supplied wherever possible in single-dose containers. Eye-drops intended for use in surgical procedures do not contain antimicrobial preservatives and are supplied in single-dose containers.

Eye-drops that are solutions, examined under suitable conditions of visibility, are practically clear and practically free from particles.

Eye-drops that are suspensions may show a sediment that is readily redispersed on shaking to give a suspension which remains sufficiently stable to enable the correct dose to be delivered.

Multidose preparations are supplied in containers that allow successive drops of the preparation to be administered. The containers contain at most 10 ml of the preparation, unless otherwise justified and authorised.

TESTS

Particle size. Unless otherwise justified and authorised, eye-drops in the form of a suspension comply with the following test: introduce a suitable quantity of the suspension into a counting cell or with a micropipette onto a slide, as appropriate, and scan under a microscope an area corresponding to 10 µg of the solid phase. For practical reasons, it is recommended that the whole sample is first scanned at low magnification (e.g. × 50) and particles greater than 25 µm are identified. These larger particles can then be measured at a larger magnification (e.g. × 200 to × 500). For each 10 µg of solid active substance, not more than 20 particles have a maximum dimension greater than 25 µm, and not more than 2 of these particles have a maximum dimension greater than 50 µm. None of the particles has a maximum dimension greater than 90 µm.

LABELLING

The label states for multidose containers, the period after opening the container after which the contents must not be used. This period does not exceed 4 weeks, unless otherwise justified and authorised.

Eye lotions

DEFINITION

Eye lotions are sterile aqueous solutions intended for use in washing or bathing the eye or for impregnating eye dressings. Eye lotions may contain excipients, for example to adjust the tonicity or the viscosity of the preparation or to adjust or stabilise the pH. These substances do not adversely affect the intended action or, at the concentrations used, cause undue local irritation.

Eye lotions supplied in multidose containers contain a suitable antimicrobial preservative in appropriate concentration except when the preparation itself has adequate antimicrobial properties. The antimicrobial preservative chosen is compatible with the other ingredients of the preparation and remains effective throughout the period of time during which the eye lotions are in use.

If eye lotions are prescribed without an antimicrobial preservative, they are supplied in single-dose containers. Eye lotions intended for use in surgical procedures or in first-aid treatment do not contain an antimicrobial preservative and are supplied in single-dose containers.

Eye lotions examined under suitable conditions of visibility, are practically clear and practically free from particles.

The containers for multidose preparations do not contain more than 200 ml of eye lotion, unless otherwise justified and authorised.

LABELLING

The label states:

- where applicable, that the contents are to be used on one occasion only,
- for multidose preparations, the period after opening the container after which the contents must not be used. This period does not exceed 4 weeks, unless otherwise justified and authorised.

Powders for eye-drops and powders for eye lotions

DEFINITION

Powders for the preparation of eye-drops and eye lotions are supplied in a dry, sterile form to be dissolved or suspended in an appropriate liquid vehicle at the time of administration.

They may contain excipients to facilitate dissolution or dispersion, to prevent caking, to adjust the tonicity, to adjust or stabilise the pH or to stabilise the preparation.

After dissolution or suspension in the prescribed liquid, they comply with the requirements for eye-drops or eye lotions, as appropriate.

TESTS

Uniformity of content (2.9.6). Unless otherwise prescribed or justified and authorised, single-dose powders for eye-drops and eye lotions with a content of active substance less than 2 mg or less than 2 per cent of the total mass comply with test B for uniformity of content of single-dose preparations. If the preparation has more than one active substance, the requirement applies only to those substances which correspond to the above condition.

Uniformity of mass (2.9.5). Single-dose powders for eye-drops and eye lotions comply with the test for uniformity of mass of single-dose preparations. If the test for uniformity of content is prescribed for all the active substances, the test for uniformity of mass is not required.

Semi-solid eye preparations

DEFINITION

Semi-solid eye preparations are sterile ointments, creams or gels intended for application to the conjunctiva. They contain one or more active substances dissolved or dispersed in a suitable basis. They have a homogeneous appearance.

Semi-solid eye preparations comply with the requirements of the monograph on *Semi-solid preparations for cutaneous application (0132)*. The basis is non-irritant to the conjunctiva.

Semi-solid eye preparations are packed in small, sterilised collapsible tubes fitted or provided with a cannula and having a content of not more than 5 g of the preparation. The tubes must be well-closed to prevent microbial contamination. Semi-solid eye preparations may also be packed in suitably designed single-dose containers. The containers, or the nozzles of tubes, are of such a shape as to facilitate administration without contamination. Tubes are tamper-proof.

TESTS

Particle size. Semi-solid eye preparations containing dispersed solid particles comply with the following test: spread gently a quantity of the preparation corresponding to at least 10 µg of solid active substance as a thin layer. Scan under a microscope the whole area of the sample. For practical reasons, it is recommended that the whole sample is first scanned at a small magnification (e.g. $\times 50$) and particles greater than 25 µm are identified. These larger particles can then be measured at a larger magnification (e.g. $\times 200$ to $\times 500$). For each 10 µg of solid active substance, not more than 20 particles have a maximum dimension greater than 25 µm, and not more than 2 of these particles have a maximum dimension greater than 50 µm. None of the particles has a maximum dimension greater than 90 µm.

Ophthalmic inserts

DEFINITION

Ophthalmic inserts are sterile, solid or semi-solid preparations of suitable size and shape, designed to be inserted in the conjunctival sac, to produce an ocular effect. They generally consist of a reservoir of active substance embedded in a matrix or bounded by a rate-controlling

membrane. The active substance, which is more or less soluble in physiological fluids, is released over a determined period of time.

Ophthalmic inserts are individually distributed into sterile containers.

PRODUCTION

In the manufacture of ophthalmic inserts, means must be taken to ensure a suitable dissolution behaviour.

TESTS

Uniformity of content (2.9.6). Ophthalmic inserts comply, where applicable, with test A for uniformity of content.

LABELLING

The label states:

- where applicable, the total quantity of active substance per insert,
- where applicable, the dose released per unit time.

01/2005:1105

FOAMS, MEDICATED

Musci medicati

Additional requirements for medicated foams may be found, where appropriate, in other general monographs, for example on Rectal preparations (1145), Vaginal preparations (1164) and Liquid preparations for cutaneous application (0927).

DEFINITION

Medicated foams are preparations consisting of large volumes of gas dispersed in a liquid generally containing one or more active substances, a surfactant ensuring their formation and various other excipients. Medicated foams are usually intended for application to the skin or mucous membranes.

Medicated foams are usually formed at the time of administration from a liquid preparation in a pressurised container. The container is equipped with a device consisting of a valve and a push button suitable for the delivery of the foam.

Medicated foams intended for use on severely injured skin and on large open wounds are sterile.

Medicated foams supplied in pressurised containers comply with the requirements of the monograph on *Pressurised pharmaceutical preparations (0523)*.

PRODUCTION

Sterile medicated foams are prepared using materials and methods designed to ensure sterility and to avoid the introduction of contaminants and the growth of micro-organisms; recommendations on this aspect are provided in the text on *Methods of preparation of sterile products (5.1.1)*.

TESTS

Relative foam density. Maintain the container at about 25 °C for at least 24 h. Taking care not to warm the container, fit a rigid tube 70 mm to 100 mm long and about 1 mm in internal diameter onto the push button. Shake the container to homogenise the liquid phase of the contents and dispense 5 ml to 10 ml of foam to waste. Tare a flat-bottomed dish with a volume of about 60 ml and about 35 mm high. Place the end of the rigid tube attached to the push button in the corner of the dish, press the push button and fill the dish uniformly, using a circular motion. After the foam has

completely expanded, level off by removing the excess foam with a slide. Weigh. Determine the mass of the same volume of *water R* by filling the same dish with *water R*.

The relative foam density is equivalent to the ratio:

$$\frac{m}{e}$$

m = mass of the test sample of foam, in grams,

e = mass of same volume of *water R*, in grams.

Carry out three measurements. None of the individual values deviate by more than 20 per cent from the mean value.

Duration of expansion. The apparatus (Figure 1105-1) consists of a 50 ml burette, 15 mm in internal diameter, with 0.1 ml graduations and fitted with a 4 mm single bore stopcock. The graduation corresponding to 30 ml is at least 210 mm from the axis of the stopcock. The lower part of the burette is connected by means of a plastic tube not longer than 50 mm and 4 mm in internal diameter to the foam-generating container equipped with a push button fitted to this connection. Maintain the container at about 25 °C for at least 24 h. Shake the container, taking care not to warm it, to homogenise the liquid phase of the contents and dispense 5 ml to 10 ml of the foam to waste. Connect the push button to the outlet of the burette. Press the button and introduce about 30 ml of foam in a single delivery. Close the stopcock and at the same time start the chronometer and read the volume of foam in the burette. Every 10 s read the growing volume until the maximum volume is reached.

Carry out three measurements. None of the times needed to obtain the maximum volume is more than 5 min.

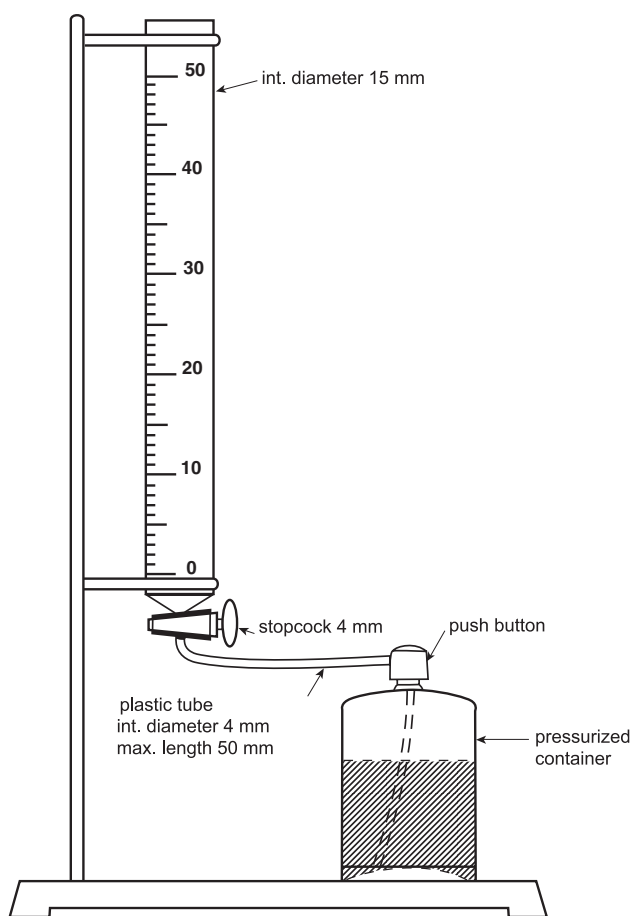


Figure 1105-1. – Apparatus for the determination of the duration of expansion

Sterility (2.6.1). When the label indicates that the preparation is sterile, it complies with the test for sterility.

LABELLING

The label states, where applicable, that the preparation is sterile.

01/2005:0499

GRANULES

Granulata

Requirements for granules to be used for the preparation of oral solutions or suspensions are given in the monograph on Liquid preparations for oral use (0672). Where justified and authorised, the requirements of this monograph do not apply to granules for veterinary use.

DEFINITION

Granules are preparations consisting of solid, dry aggregates of powder particles sufficiently resistant to withstand handling. They are intended for oral administration. Some are swallowed as such, some are chewed and some are dissolved or dispersed in water or another suitable liquid before being administered.

Granules contain one or more active substances with or without excipients and, if necessary, colouring matter authorised by the competent authority and flavouring substances.

Granules are presented as single-dose or multidose preparations. Each dose of a multidose preparation is administered by means of a device suitable for measuring the quantity prescribed. For single-dose granules, each dose is enclosed in an individual container, for example a sachet or a vial.

Where applicable, containers for granules comply with the requirements of *Materials used for the manufacture of containers* (3.1 and subsections) and *Containers* (3.2 and subsections).

Several categories of granules may be distinguished:

- effervescent granules,
- coated granules,
- gastro-resistant granules,
- modified-release granules.

PRODUCTION

In the manufacture, packaging, storage and distribution of granules, suitable means are taken to ensure their microbial quality; recommendations on this aspect are provided in the text on *Microbiological quality of pharmaceutical preparations* (5.1.4).

TESTS

Uniformity of content (2.9.6). Unless otherwise prescribed or justified and authorised, single-dose granules with a content of active substance less than 2 mg or less than 2 per cent of the total mass comply with test B for uniformity of content of single-dose preparations. If the preparation has more than one active substance, the requirement applies only to those substances which correspond to the above conditions.

Uniformity of mass (2.9.5). Single-dose granules except for coated granules comply with the test for uniformity of mass of single-dose preparations. If the test for uniformity of content is prescribed for all the active substances, the test for uniformity of mass is not required.

Uniformity of mass of delivered doses from multidose containers (2.9.27). Granules supplied in multidose containers comply with the test.

STORAGE

If the preparation contains volatile ingredients or the contents have to be protected, store in an airtight container.

Effervescent granules

DEFINITION

Effervescent granules are uncoated granules generally containing acid substances and carbonates or hydrogen carbonates which react rapidly in the presence of water to release carbon dioxide. They are intended to be dissolved or dispersed in water before administration.

TESTS

Disintegration. Place one dose of the effervescent granules in a beaker containing 200 ml of *water R* at 15-25 °C; numerous bubbles of gas are evolved. When the evolution of gas around the individual grains ceases, the granules have disintegrated, being either dissolved or dispersed in the water. Repeat the operation on 5 other doses. The preparation complies with the test if each of the 6 doses used disintegrates within 5 min.

STORAGE

In an airtight container.

Coated granules

DEFINITION

Coated granules are usually multidose preparations and consist of granules coated with one or more layers of mixtures of various excipients.

PRODUCTION

The substances used as coatings are usually applied as a solution or suspension in conditions in which evaporation of the vehicle occurs.

TESTS

Dissolution. A suitable test may be carried out to demonstrate the appropriate release of the active substance(s), for example one of the tests described in *Dissolution test for solid dosage forms (2.9.3)*.

Modified-release granules

DEFINITION

Modified-release granules are coated or uncoated granules which contain special excipients or which are prepared by special procedures, or both, designed to modify the rate, the place or the time at which the active substance or substances are released.

Modified-release granules include prolonged-release granules and delayed-release granules.

PRODUCTION

A suitable test is carried out to demonstrate the appropriate release of the active substance(s).

TESTS

Dissolution. Carry out a suitable test to demonstrate the appropriate release of the active substance(s), for example the test described in *Dissolution test for solid dosage forms (2.9.3)*.

Gastro-resistant granules

DEFINITION

Gastro-resistant granules are delayed-release granules that are intended to resist the gastric fluid and to release the active substance(s) in the intestinal fluid. These properties are achieved by covering the granules with a gastro-resistant material (enteric-coated granules) or by other suitable means.

PRODUCTION

A suitable test is carried out to demonstrate the appropriate release of the active substance(s).

TESTS

Dissolution. Carry out a suitable test to demonstrate the appropriate release of the active substance(s), for example the test described in *Dissolution test for solid dosage forms (2.9.3)*.

01/2005:0945

INTRAMAMMARY PREPARATIONS FOR VETERINARY USE

Praeparationes intramammariae ad usum veterinarium

DEFINITION

Intramammary preparations for veterinary use are sterile preparations intended for introduction into the mammary gland via the teat canal. There are two main categories: those intended for administration to lactating animals, and those intended for administration to animals at the end of lactation or to non-lactating animals for the treatment or prevention of infection.

Intramammary preparations for veterinary use are solutions, emulsions or suspensions or semi-solid preparations containing one or more active substances in a suitable vehicle. They may contain excipients such as stabilising, emulsifying, suspending and thickening agents. Suspensions may show a sediment which is readily dispersed on shaking. Emulsions may show evidence of phase separation but are readily redispersed on shaking.

Unless otherwise justified and authorised, intramammary preparations for veterinary use are supplied in containers for use on one occasion only for introduction in a single teat canal of an animal.

If supplied in multidose containers, aqueous preparations contain a suitable antimicrobial preservative at a suitable concentration, except where the preparation itself has adequate antimicrobial properties. Precautions for administration and for storage between administrations must be taken.

Where applicable, containers for intramammary preparations for veterinary use comply with the requirements of *Materials used for the manufacture of containers (3.1 and subsections)* and *Containers (3.2 and subsections)*.

PRODUCTION

During the development of a intramammary preparation for veterinary use, the formulation for which contains an antimicrobial preservative, the effectiveness of the chosen preservative shall be demonstrated to the satisfaction of the competent authority. A suitable test method together with criteria for judging the preservative properties of the formulation are provided in the text on *Efficacy of antimicrobial preservation (5.1.3)*.

Intramammary preparations for veterinary use are prepared using materials and methods designed to ensure sterility and to avoid the introduction of contaminants and the growth of micro-organisms; recommendations on this aspect are provided in the text on *Methods of preparation of sterile products* (5.1.1).

In the manufacture of intramammary preparations for veterinary use containing dispersed particles, measures are taken to ensure a suitable and controlled particle size with regard to the intended use.

TESTS

Deliverable mass or volume. Squeeze out as much as possible of the contents of ten containers according to the instructions on the label. The mean mass or volume does not differ by more than 10 per cent from the nominal mass or volume.

Sterility (2.6.1). Intramammary preparations for veterinary use comply with the test for sterility; use the technique of membrane filtration or, in justified cases, direct inoculation of the culture media. Squeeze out the contents of ten containers and mix thoroughly. For each medium, use 0.5 g to 1 g (or 0.5 ml to 1 ml as appropriate) taken from the mixed sample.

STORAGE

Store in a sterile, airtight, tamper-proof container.

LABELLING

The label states:

- the name of the active substance(s) and the mass or number of International Units of the active substance(s) that may be delivered from the container using normal technique,
- whether the preparation is intended for use in a lactating animal or a non-lactating animal,
- in the case of multidose containers, the name of any added antimicrobial preservative.

01/2005:1228

INTRARUMINAL DEVICES

Praeparationes intraruminales

The requirements of this monograph do not apply to preparations (sometimes known as boluses), such as large conventional tablets, capsules or moulded dosage forms which give immediate or prolonged release of the active substance(s). Such preparations comply with the relevant parts of the monographs on Capsules (0016) or Tablets (0478).

DEFINITION

Intraruminal devices are solid preparations each containing one or more active substances. They are intended for oral administration to ruminant animals and are designed to be retained in the rumen to deliver the active substance(s) in a continuous or pulsatile manner. The period of release of the active substance(s) may vary from days to weeks according to the nature of the formulation and/or the delivery device.

Intraruminal devices may be administered using a balling gun. Some intraruminal devices are intended to float on the surface of the ruminal fluid while others are intended to remain on the floor of the rumen or reticulum. Each device has a density appropriate for its intended purpose.

PRODUCTION

For continuous release, the intraruminal device is designed to release the active substance(s) at a defined rate over a defined period of time. This may be achieved by erosion, corrosion, diffusion, osmotic pressure or any other suitable chemical, physical or physico-chemical means.

For pulsatile-release, the intraruminal device is designed to release a specific quantity of active substance(s) at one or several defined intermediate times. This may be achieved by corrosion by ruminal fluids of the metallic elements of the intraruminal device which leads to sequential release of the constituent units which are usually in the form of tablets.

In the manufacture of intraruminal devices, means are taken to ensure an appropriate release of the active substance(s).

In the manufacture, packaging, storage and distribution of intraruminal devices, suitable means are taken to ensure their microbial quality; recommendations on this aspect are provided in the text on *Microbiological quality of pharmaceutical preparations* (5.1.4).

TESTS

Uniformity of content (2.9.6). Unless otherwise justified and authorised, constituent tablet units of intraruminal devices in which the active substances are present at levels less than 2 mg or less than 2 per cent of the total mass comply with test A for uniformity of content of single-dose preparations. If the preparation contains more than one active substance, the requirement applies only to those substances which correspond to the above conditions.

Uniformity of mass (2.9.5). Unless otherwise justified and authorised, the constituent tablet units of intraruminal devices comply with the test for uniformity of mass. If the test for uniformity of content is prescribed for all active substances, the test for uniformity of mass is not required.

LABELLING

The label states:

- for continuous-release devices, the dose released per unit time,
- for pulsatile-release devices, the dose released at specified times.

01/2005:0927

LIQUID PREPARATIONS FOR CUTANEOUS APPLICATION

Praeparationes liquidae ad usum dermicum

Where justified and authorised, the requirements of this monograph do not apply to preparations intended for systemic and veterinary use.

DEFINITION

Liquid preparations for cutaneous application are preparations of a variety of viscosities intended for local or transdermal delivery of active ingredients. They are solutions, emulsions or suspensions which may contain one or more active substances in a suitable vehicle. They may contain suitable antimicrobial preservatives, antioxidants and other excipients such as stabilisers, emulsifiers and thickeners.

Emulsions may show evidence of phase separation but are readily redispersed on shaking. Suspensions may show a sediment which is readily dispersed on shaking to give a suspension which is sufficiently stable to enable a homogeneous preparation to be delivered.

Where applicable, containers for liquid preparations for cutaneous application comply with the requirements of *Materials used for the manufacture of containers* (3.1 and subsections) and *Containers* (3.2 and subsections).

When liquid preparations for cutaneous application are dispensed in pressurised containers, the containers comply with the requirements of the monograph on *Pressurised pharmaceutical preparations* (0523).

Preparations specifically intended for use on severely injured skin are sterile.

Several categories of liquid preparations for cutaneous application may be distinguished, for example:

- shampoos,
- cutaneous foams.

PRODUCTION

During the development of a liquid preparation for cutaneous application, the formulation for which contains an antimicrobial preservative, the effectiveness of the chosen preservative shall be demonstrated to the satisfaction of the competent authority. A suitable test method together with criteria for judging the preservative properties of the formulation are provided in the text on *Efficacy of antimicrobial preservation* (5.1.3).

In the manufacture, packaging, storage and distribution of liquid preparations for cutaneous application, suitable means are taken to ensure their microbial quality; recommendations on this aspect are provided in the text on *Microbiological quality of pharmaceutical preparations* (5.1.4).

Sterile liquid preparations for cutaneous application are prepared using materials and methods designed to ensure sterility and to avoid the introduction of contaminants and the growth of micro-organisms; recommendations on this aspect are provided in the text on *Methods of preparation of sterile products* (5.1.1).

In the manufacture of liquid preparations for cutaneous application containing dispersed particles, measures are taken to ensure a suitable and controlled particle size with regard to the intended use.

TESTS

Deliverable mass or volume (2.9.28). Liquid preparations for cutaneous application supplied in single-dose containers comply with the test.

Sterility (2.6.1). Where the label indicates that the preparation is sterile, it complies with the test for sterility.

STORAGE

If the preparation is sterile, store in a sterile, airtight, tamper-proof container.

LABELLING

The label states:

- the name of any added antimicrobial preservative,
- where applicable, that the preparation is sterile.

Shampoos

DEFINITION

Shampoos are liquid or, occasionally semi-solid preparations intended for application to the scalp and subsequent washing away with water. Upon rubbing with water they usually form a foam.

They are emulsions, suspensions or solutions. Shampoos normally contain surface active agents.

Cutaneous foams

DEFINITION

Cutaneous foams comply with the requirements of the monograph on *Medicated foams* (1105).

01/2005:0672

LIQUID PREPARATIONS FOR ORAL USE

Praeparationes liquidae peroraliae

Where justified and authorised, the requirements of this monograph do not apply to liquid preparations for oral use intended for veterinary use.

DEFINITION

Liquid preparations for oral use are usually solutions, emulsions or suspensions containing one or more active substances in a suitable vehicle; they may, however, consist of liquid active substances used as such (oral liquids).

Some preparations for oral use are prepared by dilution of concentrated liquid preparations, or from powders or granules for the preparation of oral solutions or suspensions, for oral drops or for syrups, using a suitable vehicle.

The vehicle for any preparations for oral use is chosen having regard to the nature of the active substance(s) and to provide organoleptic characteristics appropriate to the intended use of the preparation.

Liquid preparations for oral use may contain suitable antimicrobial preservatives, antioxidants and other excipients such as dispersing, suspending, thickening, emulsifying, buffering, wetting, solubilising, stabilising, flavouring and sweetening agents and colouring matter, authorised by the competent authority.

Emulsions may show evidence of phase separation but are readily redispersed on shaking. Suspensions may show a sediment which is readily dispersed on shaking to give a suspension which remains sufficiently stable to enable the correct dose to be delivered.

Where applicable, containers for liquid preparations for oral use comply with the requirements of *Materials used for the manufacture of containers* (3.1 and subsections) and *Containers* (3.2 and subsections).

Several categories of preparations may be distinguished:

- oral solutions, emulsions and suspensions,
- powders and granules for oral solutions and suspensions,
- oral drops,
- powders for oral drops,
- syrups,
- powders and granules for syrups.

PRODUCTION

During the development of a preparation for oral use, the formulation for which contains an antimicrobial preservative, the effectiveness of the chosen preservative shall be demonstrated to the satisfaction of the competent authority. A suitable test method together with criteria for judging the preservative properties of the formulation are provided in the text on *Efficacy of antimicrobial preservation* (5.1.3).

In the manufacturing, packaging, storage and distribution of liquid preparations for oral use, suitable means are taken to ensure their microbial quality; recommendations on this aspect are provided in the text on *Microbiological quality of pharmaceutical preparations* (5.1.4).

In the manufacture of liquid preparations for oral use containing dispersed particles, measures are taken to ensure a suitable and controlled particle size with regard to the intended use.

TESTS

Uniformity of content (2.9.6). Unless otherwise prescribed or justified and authorised, single-dose preparations that are suspensions comply with the following test. After shaking, empty each container as completely as possible and carry out the test on the individual contents. They comply with test B for uniformity of content of single-dose preparations.

Uniformity of mass. Single-dose preparations that are solutions or emulsions comply with the following test: weigh individually the contents of 20 containers, emptied as completely as possible, and determine the average mass. Not more than 2 of the individual masses deviate by more than 10 per cent from the average mass and none deviates by more than 20 per cent.

Dose and uniformity of dose of oral drops. Into a suitable, graduated cylinder, introduce by means of the dropping device the number of drops usually prescribed for one dose or introduce by means of the measuring device, the usually prescribed quantity. The dropping speed does not exceed 2 drops per second. Weigh the liquid, repeat the addition, weigh again and carry on repeating the addition and weighing until a total of 10 masses are obtained. No single mass deviates by more than 10 per cent from the average mass. The total of 10 masses does not differ by more than 15 per cent from the nominal mass of 10 doses. If necessary, measure the total volume of 10 doses. The volume does not differ by more than 15 per cent from the nominal volume of 10 doses.

Deliverable mass or volume (2.9.28). Liquid preparations for oral use supplied in single-dose containers comply with the test.

Uniformity of mass of delivered doses from multidose containers (2.9.27). Liquid preparations for oral use supplied in multidose containers comply with the test.

LABELLING

The label states the name of any added antimicrobial preservative.

Oral solutions, emulsions and suspensions

DEFINITION

Oral solutions, emulsions and suspensions are supplied in single-dose or multi-dose containers. Each dose from a multi-dose container is administered by means of a device suitable for measuring the prescribed volume. The device is usually a spoon or a cup for volumes of 5 ml or multiples thereof or an oral syringe for other volumes.

Powders and granules for oral solutions and suspensions

DEFINITION

Powders and granules for the preparation of oral solutions or suspensions generally conform to the definitions in the monographs on *Oral powders (1165)* or *Granules (0499)* as appropriate. They may contain excipients in particular to facilitate dispersion or dissolution and to prevent caking.

After dissolution or suspension, they comply with the requirements for oral solutions or oral suspensions, as appropriate.

TESTS

Uniformity of content (2.9.6). Unless otherwise prescribed or justified and authorised, single-dose powders and single-dose granules with a content of active substance less than 2 mg or less than 2 per cent of the total mass comply with test B for uniformity of content of single-dose preparations. If the preparation has more than one active substance, the requirement applies only to those substances that correspond to the above conditions.

Uniformity of mass (2.9.5). Single-dose powders and single-dose granules comply with the test for uniformity of mass of single-dose preparations. If the test for uniformity of content is prescribed for all the active substances, the test for uniformity of mass is not required.

LABELLING

The label states:

- the method of preparation of the solution or suspension,
- the conditions and the duration of storage after constitution.

Oral drops

DEFINITION

Oral drops are solutions, emulsions or suspensions which are administered in small volumes such as drops by the means of a suitable device.

LABELLING

The label states the number of drops per millilitre of preparation or per gram of preparation if the dose is measured in drops.

Powders for oral drops

DEFINITION

Powders for the preparation of oral drops generally conform to the definition of *Oral powders (1165)*. They may contain excipients to facilitate dissolution or suspension in the prescribed liquid or to prevent caking.

After dissolution or suspension, they comply with the requirements for oral drops.

TESTS

Uniformity of content (2.9.6). Unless otherwise prescribed or justified and authorised, single-dose powders for oral drops with a content of active substance less than 2 mg or less than 2 per cent of the total mass comply with test B for uniformity of content of single-dose preparations. If the preparation has more than one active substance, the requirement applies only to those substances that correspond to the above conditions.

Uniformity of mass (2.9.5). Single-dose powders for oral drops comply with the test for uniformity of mass of single-dose preparations. If the test for uniformity of content is prescribed for all the active substances, the test for uniformity of mass is not required.

Syrups

DEFINITION

Syrups are aqueous preparations characterised by sweet taste and a viscous consistency. They may contain sucrose at a concentration of at least 45 per cent *m/m*. The sweet taste can also be obtained by using other polyols or sweetening agents. Syrups usually contain aromatic or other flavouring agents. Each dose from a multi-dose container is

administered by means of a device suitable for measuring the prescribed volume. The device is usually a spoon or a cup for volumes of 5 ml or multiples thereof.

LABELLING

The label states the name and concentration of the polyol or sweetening agent.

Powders and granules for syrups

DEFINITION

Powders and granules for syrups generally conform to the definitions in the monograph on *Oral powders (1165)* or *Granules (0499)*. They may contain excipients to facilitate dissolution.

After dissolution, they comply with the requirements for syrups.

TESTS

Uniformity of content (2.9.6). Unless otherwise prescribed or justified and authorised, single-dose powders and granules for syrups with a content of active substance less than 2 mg or less than 2 per cent of the total mass comply with test B for uniformity of content of single-dose preparations. If the preparation has more than one active substance, the requirement applies only to those substances that correspond to the above conditions.

Uniformity of mass (2.9.5). Single-dose powders and granules for syrups comply with the test for uniformity of mass of single-dose preparations. If the test for uniformity of content is prescribed for all the active substances, the test for uniformity of mass is not required.

- nasal powders,
- semi-solid nasal preparations,
- nasal washes,
- nasal sticks.

PRODUCTION

During the development of a nasal preparation, the formulation for which contains an antimicrobial preservative, the effectiveness of the chosen preservative shall be demonstrated to the satisfaction of the competent authority. A suitable test method together with criteria for judging the preservative properties of the formulation are provided in the text on *Efficacy of antimicrobial preservation (5.1.3)*.

In the manufacture, packaging, storage and distribution of nasal preparations, suitable means are taken to ensure their microbial quality; recommendations on this aspect are provided in the text on *Microbiological quality of pharmaceutical preparations (5.1.4)*.

Sterile nasal preparations are prepared using materials and methods designed to ensure sterility and to avoid the introduction of contaminants and the growth of micro-organisms; recommendations on this aspect are provided in the text on *Methods of preparation of sterile products (5.1.1)*.

In the manufacture of nasal preparations containing dispersed particles, measures are taken to ensure a suitable and controlled particle size with regard to the intended use.

TESTS

Sterility (2.6.1). Where the label states that the preparation is sterile, it complies with the test for sterility.

STORAGE

If the preparation is sterile, store in a sterile, airtight, tamper-proof container.

LABELLING

The label states:

- the name of any added antimicrobial preservative,
- where applicable, that the preparation is sterile.

Nasal drops and liquid nasal sprays

DEFINITION

Nasal drops and liquid nasal sprays are solutions, emulsions or suspensions intended for instillation or spraying into the nasal cavities.

Emulsions may show evidence of phase separation but are easily redispersed on shaking. Suspensions may show a sediment which is readily dispersed on shaking to give a suspension which remains sufficiently stable to enable the correct dose to be delivered.

Nasal drops are usually supplied in multidose containers provided with a suitable applicator.

Liquid nasal sprays are supplied in containers with atomising devices or in pressurised containers fitted with a suitable adapter and with or without a metering dose valve, which comply with the requirements of the monograph on *Pressurised pharmaceutical preparations (0523)*.

The size of droplets of the spray is such as to localise their deposition in the nasal cavity.

TESTS

Unless otherwise prescribed or justified and authorised, nasal drops supplied in single-dose containers and single doses of metered nasal sprays intended for systemic action, comply with the following tests.

01/2005:0676

NASAL PREPARATIONS

Nasalia

DEFINITION

Nasal preparations are liquid, semi-solid or solid preparations intended for administration to the nasal cavities to obtain a systemic or local effect. They contain one or more active substances. Nasal preparations are as far as possible non-irritating and do not adversely affect the functions of the nasal mucosa and its cilia. Aqueous nasal preparations are usually isotonic and may contain excipients, for example, to adjust the viscosity of the preparation, to adjust or stabilise the pH, to increase the solubility of the active substance, or to stabilise the preparation.

Nasal preparations are supplied in multidose or single-dose containers, provided, if necessary, with a suitable administration device which may be designed to avoid the introduction of contaminants.

Unless otherwise justified and authorised, aqueous nasal preparations supplied in multidose containers contain a suitable antimicrobial preservative in appropriate concentration, except where the preparation itself has adequate antimicrobial properties.

Where applicable, the containers comply with the requirements of *Materials used for the manufacture of containers (3.1 and subsections)* and *Containers (3.2 and subsections)*.

Several categories of nasal preparations may be distinguished:

- nasal drops and liquid nasal sprays,

Uniformity of mass. Nasal drops that are solutions comply with the following test: weigh individually the contents of ten containers emptied as completely as possible, and determine the average mass. Not more than two of the individual masses deviate by more than 10 per cent from the average mass and none deviates by more than 20 per cent.

Metered-dose nasal sprays that are solutions comply with the following test: discharge once to waste. Wait for not less than 5 s and discharge again to waste. Repeat this procedure for a further three actuations. Weigh the mass of the container, discharge once to waste and weigh the remaining mass of the container. Calculate the difference between the two masses. Repeat the procedure for a further nine containers. They comply with the test if not more than two of the individual values deviate by more than 25 per cent from the average value and none deviates by more than 35 per cent.

Uniformity of content (2.9.6). Nasal drops that are suspensions or emulsions comply with the following test: empty each container as completely as possible and carry out the test on the individual content. They comply with test B of uniformity of content.

Uniformity of delivered dose. Metered-dose nasal sprays that are suspensions or emulsions comply with the following test. Use an apparatus capable of quantitatively retaining the dose leaving the actuator of the atomising device.

Shake a container for 5 s and discharge once to waste. Wait for not less than 5 s, shake for 5 s and discharge again to waste. Repeat this procedure for a further three actuations. After 2 s, fire one dose of the metered-dose nasal spray into the collecting vessel by actuating the atomising device. Collect the contents of the collecting vessel by successive rinses. Determine the content of active substance in the combined rinses.

Repeat the procedure for a further nine containers.

Unless otherwise justified and authorised, the preparation complies with the test if not more than one of the individual contents is outside the limits of 75 per cent to 125 per cent and none is outside the limits of 65 per cent and 135 per cent of the average content.

If two or three individual contents are outside the limits of 75 per cent to 125 per cent but within the limits of 65 per cent to 135 per cent, repeat the test for twenty more containers. The preparation complies with the test if not more than three individual contents of the thirty individual contents are outside the limits of 75 per cent to 125 per cent and none is outside the limits of 65 per cent to 135 per cent of the average content.

Nasal powders

DEFINITION

Nasal powders are powders intended for insufflation into the nasal cavity by means of a suitable device.

They comply with the requirements of the monograph on *Powders for cutaneous application (1166)*.

The size of the particles is such as to localise their deposition in the nasal cavity and verified by adequate methods of particle-size determination.

Semi-solid nasal preparations

DEFINITION

Semi-solid nasal preparations comply with the requirements of the monograph on *Semi-solid preparations for cutaneous application (0132)*.

The containers are adapted to deliver the product to the site of application.

Nasal washes

DEFINITION

Nasal washes are generally aqueous isotonic solutions intended to cleanse the nasal cavities.

Nasal washes intended for application to injured parts or prior to a surgical operation are sterile.

TESTS

Deliverable mass or volume (2.9.28). Nasal washes supplied in single-dose containers comply with the test.

Nasal sticks

DEFINITION

Nasal sticks comply with the monograph on *Sticks (1154)*.

01/2005:1807

OROMUCOSAL PREPARATIONS

Praeparationes buccales

This monograph does not apply to dental preparations or to preparations such as chewable tablets (0478), medicated chewing gums (1239), oral lyophilisates and other solid or semi-solid preparations that are intended to be chewed or dispersed in the saliva before being swallowed. Where justified and authorised, this monograph does not apply to preparations for veterinary use.

DEFINITION

Oromucosal preparations are solid, semi-solid or liquid preparations, containing one or more active substances intended for administration to the oral cavity and/or the throat to obtain a local or systemic effect. Preparations intended for a local effect may be designed for application to a specific site within the oral cavity such as the gums (gingival preparations) or the throat (oropharyngeal preparations). Preparations intended for a systemic effect are designed to be absorbed primarily at one or more sites on the oral mucosa (e.g. sublingual preparations). Mucoadhesive preparations are intended to be retained in the oral cavity by adhesion to the mucosal epithelium and may modify systemic drug absorption at the site of application. For many oromucosal preparations, it is likely that some proportion of the active substance(s) will be swallowed and may be absorbed via the gastrointestinal tract.

Oromucosal preparations may contain suitable antimicrobial preservatives and other excipients such as dispersing, suspending, thickening, emulsifying, buffering, wetting, solubilising, stabilising, flavouring and sweetening agents. Solid preparations may in addition contain glidants, lubricants and excipients capable of modifying the release of the active substance(s).

Where applicable, containers for oromucosal preparations comply with the requirements for *Materials used for the manufacture of containers (3.1 and subsections)* and *Containers (3.2 and subsections)*.

Several categories of preparations for oromucosal use may be distinguished:

- gargles,
- mouthwashes,
- gingival solutions,
- oromucosal solutions and oromucosal suspensions,

- semi-solid oromucosal preparations (including for example gingival gel, gingival paste, oromucosal gel, oromucosal paste),
- oromucosal drops, oromucosal sprays and sublingual sprays (including oropharyngeal sprays),
- lozenges and pastilles,
- compressed lozenges,
- sublingual tablets and buccal tablets,
- oromucosal capsules,
- mucoadhesive preparations.

PRODUCTION

During the development of an oromucosal preparation containing an antimicrobial preservative, the effectiveness of the chosen preservative shall be demonstrated to the satisfaction of the competent authority. A suitable test method together with the criteria for judging the preservative properties of the formulation are provided in *5.1.3 Efficacy of antimicrobial preservation*.

In the manufacture, packaging, storage and distribution of oromucosal preparations, suitable means are taken to ensure their microbiological quality; recommendations on this aspect are provided in the text on *Microbiological quality of pharmaceutical preparations (5.1.4)*.

In the manufacture of semi-solid and liquid oromucosal preparations containing dispersed particles, measures are taken to ensure a suitable and controlled particle size with regard to the intended use.

TESTS

Uniformity of content (2.9.6). Unless otherwise prescribed or justified and authorised, single-dose preparations with a content of active substance less than 2 mg or less than 2 per cent of the total mass comply with test A (compressed and moulded dosage forms) or test B (capsules) for the uniformity of content of single-dose preparations. If the preparation contains more than one active substance, this requirement applies only to those substances that correspond to the above conditions.

Uniformity of mass (2.9.5). Solid single-dose preparations comply with the test for uniformity of mass. If the test for the uniformity of content is prescribed, or justified and authorised for all active substances, the test for uniformity of mass is not required.

LABELLING

The label states the name of any added antimicrobial preservative.

Gargles

DEFINITION

Gargles are aqueous solutions intended for gargling to obtain a local effect. They are not to be swallowed. They are supplied as ready-to-use solutions or concentrated solutions to be diluted. They may also be prepared from powders or tablets to be dissolved in water before use.

Gargles may contain excipients to adjust the pH which, as far as possible, is neutral.

Mouthwashes

DEFINITION

Mouthwashes are aqueous solutions intended for use in contact with the mucous membrane of the oral cavity, usually after dilution with water. They are not to be swallowed.

They are supplied as ready-to-use solutions or concentrated solutions to be diluted. They may also be prepared from powders or tablets to be dissolved in water before use.

Mouthwashes may contain excipients to adjust the pH which, as far as possible, is neutral.

Gingival solutions

DEFINITION

Gingival solutions are intended for administration to the gingivae by means of a suitable applicator.

Oromucosal solutions and oromucosal suspensions

DEFINITION

Oromucosal solutions and oromucosal suspensions are liquid preparations intended for administration to the oral cavity by means of a suitable applicator.

Oromucosal suspensions may show a sediment which is readily dispersible on shaking to give a suspension which remains sufficiently stable to enable the correct dose to be delivered.

Semi-solid oromucosal preparations

DEFINITION

Semi-solid oromucosal preparations are hydrophilic gels or pastes intended for administration to the oral cavity or to a specific part of the oral cavity such as the gingivae (gingival gel, gingival paste). They may be provided as single-dose preparations.

Semi-solid oromucosal preparations comply with the requirements of the monograph on *Semi-solid preparations for cutaneous use (0132)*.

Oromucosal drops, oromucosal sprays and sublingual sprays

DEFINITION

Oromucosal drops, oromucosal sprays and sublingual sprays are solutions, emulsions or suspensions intended for local or systemic effect. They are applied by instillation or spraying into the oral cavity or onto a specific part of the oral cavity such as spraying under the tongue (sublingual spray) or into the throat (oropharyngeal spray).

Emulsions may show evidence of phase separation but are readily redispersed on shaking. Suspensions may show a sediment which is readily dispersed on shaking to give a suspension which remains sufficiently stable to enable the correct dose to be delivered.

Liquid oromucosal sprays are supplied in containers with atomising devices or in pressurised containers having a suitable adaptor, with or without a metering dose valve, which comply with the requirements of the monograph on *Pressurised pharmaceutical preparations (0523)*.

The size of the droplets of the spray is such as to localise their deposition in the oral cavity or the throat as intended.

TESTS

Unless otherwise prescribed or justified and authorised, oromucosal drops supplied in single-dose containers, single-doses of metered oromucosal sprays and sublingual sprays intended for systemic action comply with the following tests.

Uniformity of mass. Oromucosal drops that are solutions comply with the following test: determine the individual masses of the contents of 10 containers emptied as completely as possible, and calculate the average mass. Not more than 2 of the individual masses deviate by more than 10 per cent from the average mass and none deviates by more than 20 per cent.

Metered-dose oromucosal sprays and sublingual sprays that are solutions comply with the following test: discharge once to waste. Wait for not less than 5 s and discharge again to waste. Repeat this procedure for a further 3 actuations. Weigh the mass of the container, discharge once to waste and weigh the remaining mass of the container. Calculate the difference between the 2 masses. Repeat the procedure for a further 9 containers. The preparation complies with the test if not more than 2 of the individual masses deviate by more than 25 per cent from the average value and none deviates by more than 35 per cent.

Uniformity of content (2.9.6). Oromucosal drops that are suspensions or emulsions comply with the following test: empty each container as completely as possible and carry out the test on the individual contents. They comply with test B of uniformity of content.

Uniformity of delivered dose. Metered-dose oromucosal sprays and sublingual sprays that are suspensions or emulsions comply with the following test: use an apparatus capable of quantitatively retaining the dose leaving the actuator of the atomising device.

Shake a container for 5 s and discharge once to waste. Wait for not less than 5 s, shake for 5 s and discharge again to waste. Repeat this procedure for a further 3 actuations. After 2 s, fire one dose of the metered-dose oromucosal spray into the collecting vessel by actuating the atomising device. Collect the contents of the collecting vessel by successive rinses. Determine the content of active substance in the combined rinses. Repeat the procedure for a further 9 containers. Unless otherwise justified and authorised, the preparation complies with the test if not more than one of the individual contents is outside the limits of 75 per cent to 125 per cent and none is outside the limits of 65 per cent to 135 per cent of the average content.

If 2 or 3 individual contents are outside the limits of 75 per cent to 125 per cent but within the limits of 65 per cent to 135 per cent, repeat the test for 20 more containers. The preparation complies with the test if not more than 3 individual contents of the 30 contents are outside the limits of 75 per cent to 125 per cent and none is outside the limits of 65 per cent to 135 per cent of the average content.

Lozenges and pastilles

DEFINITION

Lozenges and pastilles are solid, single-dose preparations intended to be sucked to obtain, usually, a local effect in the oral cavity and the throat. They contain one or more active substances, usually in a flavoured and sweetened base, and are intended to dissolve or disintegrate slowly in the mouth when sucked.

Lozenges are hard preparations prepared by moulding. Pastilles are soft, flexible preparations prepared by moulding of mixtures containing natural or synthetic polymers or gums and sweeteners.

Compressed lozenges

DEFINITION

Compressed lozenges are solid, single-dose preparations intended to be sucked to obtain a local or systemic effect. They are prepared by compression and are often rhomboid in shape.

Compressed lozenges conform with the general definition of tablets.

PRODUCTION

In the manufacture of compressed lozenges, means are taken to ensure that they possess a suitable mechanical strength to resist handling without crumbling or breaking. This may be demonstrated by examining the *Friability of uncoated tablets (2.9.7)* and the *Resistance to crushing of tablets (2.9.8)*.

TESTS

Dissolution. For compressed lozenges intended for a systemic effect, a suitable test is carried out to demonstrate the appropriate release of the active substance(s).

Sublingual tablets and buccal tablets

DEFINITION

Sublingual tablets and buccal tablets are solid, single-dose preparations to be applied under the tongue or to the buccal cavity, respectively, to obtain a systemic effect. They are prepared by compression of mixtures of powders or granulations into tablets with a shape suited for the intended use.

Sublingual tablets and buccal tablets conform to the general definition of tablets.

PRODUCTION

In the manufacture of sublingual tablets and buccal tablets, means are taken to ensure that they possess suitable mechanical strength to resist handling without crumbling or breaking. This may be demonstrated by examining the *Friability of uncoated tablets (2.9.7)* and the *Resistance to crushing of tablets (2.9.8)*.

TESTS

Dissolution. Unless otherwise justified and authorised, a suitable test is carried out to demonstrate the appropriate release of the active substance(s).

Oromucosal capsules

DEFINITION

Oromucosal capsules are soft capsules to be chewed or sucked.

Mucoadhesive preparations

DEFINITION

Mucoadhesive preparations contain one or more active substances intended for systemic absorption through the buccal mucosa over a prolonged period of time. They may be supplied as mucoadhesive buccal tablets or as other mucoadhesive solid or semi-solid preparations.

Mucoadhesive buccal tablets are prepared by compression of mono- or multi-layered tablets. They usually contain hydrophilic polymers, which on wetting with the saliva produce a flexible hydrogel that adheres to the buccal mucosa.

PRODUCTION

In the manufacture of mucoadhesive buccal tablets, means are taken to ensure that they possess suitable mechanical strength to resist handling without crumbling or breaking. This may be demonstrated by examining the *Friability of uncoated tablets* (2.9.7) and the *Resistance to crushing of tablets* (2.9.8).

TESTS

Dissolution. Unless otherwise justified and authorised, a suitable test is carried out to demonstrate the appropriate release of the active substance(s).

01/2005:0520

PARENTERAL PREPARATIONS

Parenteralia

The requirements of this monograph do not necessarily apply to products derived from human blood, to immunological preparations, or radiopharmaceutical preparations. Special requirements may apply to preparations for veterinary use depending on the species of animal for which the preparation is intended.

DEFINITION

Parenteral preparations are sterile preparations intended for administration by injection, infusion or implantation into the human or animal body.

Parenteral preparations may require the use of excipients, for example to make the preparation isotonic with blood, to adjust the pH, to increase solubility, to prevent deterioration of the active substances or to provide adequate antimicrobial properties but not to adversely affect the intended medicinal action of the preparation or, at the concentrations used, to cause toxicity or undue local irritation.

Containers for parenteral preparations are made as far as possible from materials that are sufficiently transparent to permit the visual inspection of the contents, except for implants and in other justified and authorised cases.

Where applicable, the containers for parenteral preparations comply with the requirements for *Materials used for the manufacture of containers* (3.1 and subsections) and *Containers* (3.2 and subsections).

Parenteral preparations are supplied in glass containers (3.2.1) or in other containers such as plastic containers (3.2.2, 3.2.2.1 and 3.2.9) and prefilled syringes. The tightness of the container is ensured by suitable means. Closures ensure a good seal, prevent the access of micro-organisms and other contaminants and usually permit the withdrawal of a part or the whole of the contents without removal of the closure. The plastic materials or elastomers (3.2.9) of which the closure is composed are sufficiently firm and elastic to allow the passage of a needle with the least possible shedding of particles. Closures for multidose containers are sufficiently elastic to ensure that the puncture is resealed when the needle is withdrawn.

Several categories of parenteral preparations may be distinguished:

- injections,
- infusions,
- concentrates for injections or infusions,
- powders for injections or infusions,
- implants.

PRODUCTION

During the development of a parenteral preparation, the formulation for which contains an antimicrobial preservative, the effectiveness of the chosen preservative shall be demonstrated to the satisfaction of the competent authority. A suitable test method together with criteria for judging the preservative properties of the formulation are provided under *Efficacy of antimicrobial preservation* (5.1.3).

Parenteral preparations are prepared using materials and methods designed to ensure sterility and to avoid the introduction of contaminants and the growth of micro-organisms; recommendations on this aspect are provided in the text on *Methods of preparation of sterile products* (5.1.1).

Water used in the manufacture of parenteral preparations complies with the requirements of water for injections in bulk stated in the monograph on *Water for injections* (0169).

TESTS

Particulate contamination: sub-visible particles (2.9.19).

For preparations for human use, solutions for infusion or solutions for injection supplied in containers with a nominal content of more than 100 ml comply with the test.

For preparations for veterinary use, when supplied in containers with a nominal content of more than 100 ml and when the content is equivalent to a dose of more than 1.4 ml per kilogram of body mass, solutions for infusion or solutions for injection comply with the test for particulate contamination: sub-visible particles.

Products for which the label states that the product is to be used with a final filter are exempt from these requirements.

Sterility (2.6.1). Parenteral preparations comply with the test for sterility.

STORAGE

Store in a sterile, airtight, tamper-proof container.

LABELLING

The label states:

- the name and concentration of any added antimicrobial preservative,
- where applicable, that the solution is to be used in conjunction with a final filter,
- where applicable, that the preparation is free from bacterial endotoxins or that it is apyrogenic.

Injections

DEFINITION

Injections are sterile solutions, emulsions or suspensions. They are prepared by dissolving, emulsifying or suspending the active substance(s) and any added excipients in *Water for injections* (0169), in a suitable, sterile non-aqueous liquid or in a mixture of these vehicles.

Solutions for injection, examined under suitable conditions of visibility, are clear and practically free from particles.

Emulsions for injection do not show any evidence of phase separation. Suspensions for injection may show a sediment which is readily dispersed on shaking to give a suspension which remains sufficiently stable to enable the correct dose to be withdrawn.

Multidose preparations. Multidose aqueous injections contain a suitable antimicrobial preservative at an appropriate concentration except when the preparation itself has adequate antimicrobial properties. When it is necessary to present a preparation for parenteral use in a multidose

container, the precautions to be taken for its administration and more particularly for its storage between successive withdrawals are given.

Antimicrobial preservatives. Aqueous preparations which are prepared using aseptic precautions and which cannot be terminally sterilised may contain a suitable antimicrobial preservative in an appropriate concentration.

No antimicrobial preservative is added when:

- the volume to be injected in a single dose exceeds 15 ml, unless otherwise justified,
- the preparation is intended for administration by routes where, for medical reasons, an antimicrobial preservative is not acceptable, such as intracisternally, epidurally, intrathecally or by any route giving access to the cerebrospinal fluid, or intra- or retro-ocularly.

Such preparations are presented in single-dose containers.

PRODUCTION

In the manufacture of injections containing dispersed particles, measures are taken to ensure a suitable and controlled particle size with regard to the intended use.

Single-dose preparations. The volume of the injection in a single-dose container is sufficient to permit the withdrawal and administration of the nominal dose using a normal technique.

TESTS

Uniformity of content (2.9.6). Unless otherwise prescribed or justified and authorised, single-dose suspensions for injection with a content of active substance less than 2 mg or less than 2 per cent of the total mass comply with test A for uniformity of content of single-dose preparations. If the preparation contains more than one active substance, the requirement applies only to those substances that correspond to the above conditions.

Bacterial endotoxins - pyrogens. A test for bacterial endotoxins (2.6.14) is carried out or, where justified and authorised, the test for pyrogens (2.6.8). Recommendations on the limits for bacterial endotoxins are given in chapter 2.6.14.

Preparations for human use. The preparation complies with a test for bacterial endotoxins (2.6.14) or with the test for pyrogens (2.6.8).

Preparations for veterinary use. When the volume to be injected in a single dose is 15 ml or more and is equivalent to a dose of 0.2 ml or more per kilogram of body mass, the preparation complies with a test for bacterial endotoxins (2.6.14) or with the test for pyrogens (2.6.8).

Any preparation. Where the label states that the preparation is free from bacterial endotoxins or apyrogenic, respectively, the preparation complies with a test for bacterial endotoxins (2.6.14) or with the test for pyrogens (2.6.8), respectively.

Infusions

DEFINITION

Infusions are sterile, aqueous solutions or emulsions with water as the continuous phase; they are usually made isotonic with blood. They are principally intended for administration in large volume. Infusions do not contain any added antimicrobial preservative.

Solutions for infusion, examined under suitable conditions of visibility, are clear and practically free from particles.

Emulsions for infusion do not show any evidence of phase separation.

PRODUCTION

In the manufacture of infusions containing dispersed particles, measures are taken to ensure a suitable and controlled particle size with regard to the intended use.

The volume of the infusion in the container is sufficient to permit the withdrawal and administration of the nominal dose using a normal technique (2.9.17).

TESTS

Bacterial endotoxins - pyrogens. They comply with a test for bacterial endotoxins (2.6.14) or, where justified and authorised, with the test for pyrogens (2.6.8). For the latter test, inject 10 ml per kilogram of body mass into each rabbit, unless otherwise justified and authorised.

Concentrates for injections or infusions

DEFINITION

Concentrates for injections or infusions are sterile solutions intended for injection or infusion after dilution. They are diluted to a prescribed volume with a prescribed liquid before administration. After dilution, they comply with the requirements for injections or for infusions.

TESTS

Bacterial endotoxins - pyrogens. They comply with the requirements prescribed for injections or for infusions, after dilution to a suitable volume.

Powders for injections or infusions

DEFINITION

Powders for injections or infusions are solid, sterile substances distributed in their final containers and which, when shaken with the prescribed volume of a prescribed sterile liquid, rapidly form either clear and practically particle-free solutions or uniform suspensions. After dissolution or suspension, they comply with the requirements for injections or for infusions.

Freeze-dried products for parenteral use are considered as powders for injections or infusions.

PRODUCTION

The uniformity of content and uniformity of mass of freeze-dried products for parenteral use are ensured by the in-process control of the amount of the solution prior to freeze-drying.

TESTS

Uniformity of content (2.9.6). Unless otherwise prescribed or justified and authorised, powders for injections or infusions with a content of active substance less than 2 mg or less than 2 per cent of the total mass or with a unit mass equal to or less than 40 mg comply with test A for uniformity of content of single-dose preparations. If the preparation contains more than one active substance, the requirement applies only to those substances that correspond to the above conditions.

Uniformity of mass (2.9.5). Powders for injections or infusions comply with the test for uniformity of mass of single-dose preparations. If the test for uniformity of content is prescribed for all the active substances, the test for uniformity of mass is not required.

Bacterial endotoxins - pyrogens. They comply with the requirements prescribed for injections or for infusions, after dissolution or suspension in a suitable volume of liquid.

LABELLING

The label states the instructions for the preparation of injections and infusions.

Implants

DEFINITION

Implants are sterile, solid preparations of a size and shape suitable for parenteral implantation and release the active substance(s) over an extended period of time. Each dose is provided in a sterile container.

01/2005:1011

PATCHES, TRANSDERMAL

Emplastra transcutanea

DEFINITION

Transdermal patches are flexible pharmaceutical preparations of varying sizes, containing one or more active substances. They are intended to be applied to the unbroken skin in order to deliver the active substance(s) to the systemic circulation after passing through the skin barrier.

Transdermal patches normally consist of an outer covering which supports a preparation which contains the active substance(s). The transdermal patches are covered on the site of the release surface of the preparation by a protective liner, which is removed before applying the patch to the skin.

The outer covering is a backing sheet impermeable to the active substance(s) and normally impermeable to water, designed to support and protect the preparation. The outer covering may have the same dimensions as the preparation or it may be larger. In the latter case the overlapping border of the outer covering is covered by pressure-sensitive adhesive substances which assure the adhesion of the patch to the skin.

The preparation contains the active substance(s) together with excipients such as stabilisers, solubilisers or substances intended to modify the release rate or to enhance transdermal absorption. It may be a single layer or multi-layer solid or semi-solid matrix, and in this case it is the composition and structure of the matrix which determines the diffusion pattern of the active substance(s) to the skin. The matrix may contain pressure-sensitive adhesives which assure the adhesion of the preparation to the skin. The preparation may exist as a semi-solid reservoir one side of which is a membrane which may control the release and the diffusion of the active substance(s) from the preparation. The pressure-sensitive adhesive substances may, in this case, be applied to some or all parts of the membrane, or only around the border of the membrane of the outer covering.

When applied to the dried, clean and unbroken skin, the transdermal patch adheres firmly to the skin by gentle pressure of the hand or the fingers and can be peeled off without causing appreciable injury to the skin or detachment of the preparation from the outer covering. The patch must not be irritant or sensitising to the skin, even after repeated applications.

The protective liner generally consists of a sheet of plastic or metal material. When removed, the protective liner does not detach the preparation (matrix or reservoir) or the adhesive from the patch.

Transdermal patches are normally individually enclosed in sealed sachets.

PRODUCTION

In the manufacture, packaging, storage and distribution of transdermal patches suitable means are taken to ensure their microbial quality; recommendations on this aspect are provided in the text on *Microbiological quality of pharmaceutical preparations* (5.1.4).

TESTS

Uniformity of content (2.9.6). Unless otherwise prescribed or justified and authorised, transdermal patches comply with test C for uniformity of content of single-dose preparations.

Dissolution. A suitable test may be required to demonstrate the appropriate release of the active substance(s), for example one of the tests described in *Dissolution test for transdermal patches* (2.9.4). The disc assembly method, the cell method or the rotating cylinder method may be used, as suitable, according to the composition, dimensions and shape of the patch.

A membrane may be used. It can be of various materials, such as inert porous cellulose or silicones, and must not affect the release kinetics of the active substance(s) from the patch. Furthermore, it must be free of substances that may interfere with its performance (for example grease). The membrane may be suitably treated before the tests, for example, by maintaining it in the medium to be used in the test for 24 h. Apply the membrane above the releasing surface of the patch, avoiding the formation of air bubbles. The test conditions and the requirements are to be authorised by the competent authority.

STORAGE

Store at room temperature, unless otherwise indicated.

LABELLING

The label states, where applicable, the total quantity of active substance(s) per patch, the dose released per unit time and the area of the releasing surface.

01/2005:1166

POWDERS FOR CUTANEOUS APPLICATION

Pulveres ad usum dermicum

Where justified and authorised, the requirements of this monograph do not apply to powders for cutaneous application intended for veterinary use.

DEFINITION

Powders for cutaneous application are preparations consisting of solid, loose, dry particles of varying degrees of fineness. They contain one or more active substances, with or without excipients and, if necessary, colouring matter authorised by the competent authority.

Powders for cutaneous application are presented as single-dose powders or multidose powders. They are free from grittiness. Powders specifically intended for use on large open wounds or on severely injured skin are sterile.

Multidose powders for cutaneous application may be dispensed in sifter-top containers, containers equipped with a mechanical spraying device or in pressurised containers.

Powders dispensed in pressurised containers comply with the requirements of *Pressurised pharmaceutical preparations* (0523).

Where applicable, containers for powders comply with the requirements of *Materials used for the manufacture of containers* (3.1 and subsections) and *Containers* (3.2 and subsections).

PRODUCTION

In the manufacture of powders for cutaneous application, measures are taken to ensure a suitable particle size with regard to the intended use.

In the manufacture, packaging, storage and distribution of powders for cutaneous application, suitable means are taken to ensure their microbial quality; recommendations on this aspect are provided in the text on *Microbiological quality of pharmaceutical preparations* (5.1.4).

Sterile powders for cutaneous application are prepared using materials and methods designed to ensure sterility and to avoid the introduction of contaminants and the growth of micro-organisms; recommendations on this aspect are provided in the text on *Methods of preparation of sterile products* (5.1.1).

TESTS

Fineness. If prescribed, the fineness of a powder is determined by the sieve test (2.9.12) or another appropriate method.

Uniformity of content (2.9.6). Unless otherwise prescribed or justified and authorised, single-dose powders for cutaneous application with a content of active substance less than 2 mg or less than 2 per cent of the total mass comply with test B for uniformity of content of single-dose preparations. If the preparation has more than one active substance, the requirement applies only to those substances which correspond to the above conditions.

Uniformity of mass (2.9.5). Single-dose powders for cutaneous application comply with the test for uniformity of mass of single-dose preparations. If the test for uniformity of content is prescribed for all the active substances, the test for uniformity of mass is not required.

Sterility (2.6.1). Where the label indicates that the preparation is sterile, it complies with the test for sterility.

LABELLING

The label states:

- that the preparation is for external use,
- where applicable, that the preparation is sterile.

01/2005:1165

POWDERS, ORAL

Pulveres perorales

Requirements for powders to be used for the preparation of oral solutions or suspensions are given in the monograph for Liquid preparations for oral use (0672). Where justified and authorised, the requirements of this monograph do not apply to oral powders intended for veterinary use.

DEFINITION

Oral powders are preparations consisting of solid, loose, dry particles of varying degrees of fineness. They contain one or more active substances, with or without excipients and, if necessary, colouring matter authorised by the competent authority and flavouring substances. They are generally administered in or with water or another suitable liquid. They may also be swallowed directly. They are presented as single-dose or multidose preparations.

Where applicable, containers for oral powders comply with the requirements of *Materials used for the manufacture of containers* (3.1 and subsections) and *Containers* (3.2 and subsections).

Multidose oral powders require the provision of a measuring device capable of delivering the quantity prescribed. Each dose of a single-dose powder is enclosed in an individual container, for example a sachet or a vial.

PRODUCTION

In the manufacture of oral powders, means are taken to ensure a suitable particle size with regard to the intended use.

In the manufacture, packaging, storage and distribution of oral powders, suitable means are taken to ensure their microbial quality; recommendations on this aspect are provided in the text on *Microbiological quality of pharmaceutical preparations* (5.1.4).

TESTS

Uniformity of content (2.9.6). Unless otherwise prescribed or justified and authorised, single-dose oral powders with a content of active substance less than 2 mg or less than 2 per cent of the total mass comply with test B for uniformity of content of single-dose preparations. If the preparation has more than one active substance, the requirement applies only to those substances which correspond to the above conditions.

Uniformity of mass (2.9.5). Single-dose oral powders comply with the test for uniformity of mass of single-dose preparations. If the test for uniformity of content is prescribed for all the active substances, the test for uniformity of mass is not required.

Uniformity of mass of delivered doses from multidose containers (2.9.27). Oral powders supplied in multidose containers comply with the test.

STORAGE

If the preparation contains volatile ingredients, or the contents have to be protected, store in an airtight container.

Effervescent powders

Effervescent powders are presented as single-dose or multidose preparations and generally contain acid substances and carbonates or hydrogen carbonates which react rapidly in the presence of water to release carbon dioxide. They are intended to be dissolved or dispersed in water before administration.

STORAGE

In an airtight container.

01/2005:1037

PREMIXES FOR MEDICATED FEEDING STUFFS FOR VETERINARY USE

Praeadmixta ad alimenta medicata ad usum veterinarium

DEFINITION

Mixtures of one or more active substances, usually in suitable bases, that are prepared to facilitate feeding the active substances to animals. They are used exclusively in the preparation of medicated feeding stuffs.

Premixes occur in granulated, powdered, semi-solid or liquid form. Used as powders or granules, they are free-flowing and homogeneous; any aggregates break apart during normal handling. Used in liquid form, they are homogeneous suspensions or solutions which may be obtained from thixotropic gels or structured liquids. The particle size and other properties are such as to ensure uniform distribution of the active substance(s) in the final feed. Unless otherwise justified and authorised, the instructions for use state that the concentration of a premix in granulated or powdered form is at least 0.5 per cent in the medicated feeding stuff.

PRODUCTION

Active substance. An active substance intended for incorporation into a medicated premix complies with the requirements of the relevant monograph of the European Pharmacopoeia, unless already otherwise justified and authorised for existing premixes.

TESTS

Loss on drying (2.2.32). Unless otherwise justified and authorised, for premixes occurring in granulated or powdered form, maximum 15.0 per cent, determined on 3.000 g by drying in an oven at 100-105 °C for 2 h.

LABELLING

The label states:

- the category of animal for which the premix is intended,
- the instructions for the preparation of the medicated feeding stuffs from the premix and the basic feed,
- where applicable, the time that must elapse between the cessation of feeding of the medicated feeding stuff and collection of the material intended for human consumption.

PRODUCTION

During the development of a preparation for inhalation which contains an antimicrobial preservative, the effectiveness of the chosen preservative shall be demonstrated to the satisfaction of the competent authority. A suitable test method together with the criteria for judging the preservative properties of the formulation are described in the text on *Efficacy of antimicrobial preservation (5.1.3)*.

The size of aerosol particles to be inhaled is controlled so that a significant fraction is deposited in the lung. The fine-particle characteristics of preparations for inhalation are determined by the method for *Aerodynamic assessment of fine particles (2.9.18)*.

In assessing the uniformity of delivered dose of a multidose inhaler, it is not sufficient to test a single inhaler.

Manufacturers must substitute procedures which take both inter- and intra-inhaler dose uniformity into account. A suitable procedure based on the intra-inhaler test would be to collect each of the specified doses at the beginning, middle and end of the number of doses stated on the label from separate inhalers.

Pressurised metered-dose inhalers are tested for leakage. All inhalers are tested for extraneous particulate contamination.

LABELLING

For metered-dose preparations the label states:

- the delivered dose, except for preparations for which the dose has been established as a metered-dose or as a predisposed-dose,
- where applicable, the number of deliveries from the inhaler to provide the minimum recommended dose,
- the number of deliveries per inhaler.

The label states, where applicable, the name of any added antimicrobial preservative.

Liquid preparations for inhalation

Three categories of liquid preparations for inhalation may be distinguished:

- A. preparations intended to be converted into vapour,
- B. liquid preparations for nebulisation,
- C. pressurised metered-dose preparations for inhalation.

Liquid preparations for inhalation are solutions or dispersions.

Dispersions are readily dispersible on shaking and they remain sufficiently stable to enable the correct dose to be delivered. Suitable excipients may be used.

A. PREPARATIONS INTENDED TO BE CONVERTED INTO VAPOUR

DEFINITION

Preparations intended to be converted into vapour are solutions, dispersions or solid preparations. They are usually added to hot water and the vapour generated is inhaled.

B. LIQUID PREPARATIONS FOR NEBULISATION

DEFINITION

Liquid preparations for inhalation intended to be converted into aerosols by continuously operating nebulisers or metered-dose nebulisers are solutions, suspensions or emulsions. Suitable co-solvents or solubilisers may be used to increase the solubility of the active substances.

Liquid preparations for nebulisation in concentrated form for use in continuously operating nebulisers are diluted to the prescribed volume with the prescribed liquid before use. Liquids for nebulisation may also be prepared from powders.

01/2005:0671

PREPARATIONS FOR INHALATION

Inhalanda

DEFINITION

Preparations for inhalation are liquid or solid preparations intended for administration as vapours or aerosols to the lung in order to obtain a local or systemic effect. They contain one or more active substances which may be dissolved or dispersed in a suitable vehicle.

Preparations for inhalation may, depending on the type of preparation, contain propellants, co-solvents, diluents, antimicrobial preservatives, solubilising and stabilising agents, etc. These excipients do not adversely affect the functions of the mucosa of the respiratory tract or its cilia.

Preparations for inhalation are supplied in multidose or single-dose containers. When supplied in pressurised containers, they comply with the requirements of the monograph on *Pressurised pharmaceutical preparations (0523)*.

Preparations intended to be administered as aerosols (dispersions of solid or liquid particles in a gas) are administered by one of the following devices:

- nebuliser,
- pressurised metered-dose inhaler,
- dry-powder inhaler.

The pH of the liquid preparations for use in continuously operating nebulisers is not lower than 3 and not higher than 8.5.

Suspensions and emulsions are readily dispersible on shaking and they remain sufficiently stable to enable the correct dose to be delivered.

Aqueous preparations for nebulisation supplied in multidose containers may contain a suitable antimicrobial preservative at a suitable concentration except where the preparation itself has adequate antimicrobial properties.

Continuously operating nebulisers are devices that convert liquids into aerosols by high-pressure gases, ultrasonic vibration or other methods. They allow the dose to be inhaled at an appropriate rate and particle size which ensures deposition of the preparation in the lungs.

Metered-dose nebulisers are devices that convert liquids into aerosols by high-pressure gases, ultrasonic vibration or other methods. The volume of liquid to be nebulised is metered so that the aerosol dose can be inhaled with one breath.

C. PRESSURISED METERED-DOSE PREPARATIONS FOR INHALATION

DEFINITION

Pressurised metered-dose preparations for inhalation are solutions, suspensions or emulsions supplied in special containers equipped with a metering valve and which are held under pressure with suitable propellants or suitable mixtures of liquefied propellants, which can act also as solvents. Suitable co-solvents, solubilisers and stabilisers may be added.

The delivered dose is the dose delivered from the inhaler to the patient. For some preparations, the dose has been established as a metered-dose. The metered-dose is determined by adding the amount deposited within the device to the delivered dose. It may also be determined directly.

TESTS

Uniformity of delivered dose. Containers usually operate in an inverted position. For containers that operate in an upright position, an equivalent test is applied using methods that ensure the complete collection of the delivered dose. In all cases, prepare the inhaler as directed in the instructions to the patient.

The dose collection apparatus must be capable of quantitatively capturing the delivered dose.

The following apparatus and procedure may be used.

The apparatus (Figure 0671.-1) consists of a filter-support base with an open-mesh filter-support, such as a stainless steel screen, a collection tube that is clamped or screwed to the filter-support base, and a mouthpiece adapter to ensure an airtight seal between the collection tube and the mouthpiece. Use a mouthpiece adapter which ensures that the front face of the inhaler mouthpiece is flush with the front face of the sample collection tube. The vacuum connector is connected to a system comprising a vacuum source and a flow regulator. The source should be adjusted to draw air through the complete assembly, including the filter and the inhaler to be tested, at 28.3 ± 1.5 litres/min. Air should be drawn continuously through the apparatus to avoid loss of the active substance into the atmosphere. The filter support base is designed to accommodate 25 mm diameter filter disks. The filter disk and other materials used in the construction of the apparatus must be compatible with the active substance and solvents that are used to extract the active substance from the filter. One end of the collection tube is designed to hold the filter disk tightly against the filter-support base. When assembled, the joints between the

components of the apparatus are airtight so that when a vacuum is applied to the base of the filter, all of the air drawn through the collection tube passes through the inhaler.

Unless otherwise prescribed in the instructions to the patient, shake the inhaler for 5 s and discharge one delivery to waste. Fire the inverted inhaler into the apparatus, depressing the valve for a sufficient time to ensure complete discharge. Repeat the procedure until the number of deliveries that constitute the minimum recommended dose have been sampled. Quantitatively collect the contents of the apparatus and determine the amount of active substance.

Repeat the procedure for a further 2 doses.

Discharge the device to waste, waiting not less than 5 s between actuations until $(n/2)+1$ deliveries remain, where n is the number of deliveries stated on the label. Collect 4 doses using the procedure described above.

Discharge the device to waste, waiting not less than 5 s between actuations until 3 doses remain. Collect these 3 doses using the procedure described above.

For preparations containing more than one active substance, carry out the test for uniformity of delivered dose for each active substance.

Unless otherwise justified and authorised, the preparation complies with the test if 9 out of 10 results lie between 75 per cent and 125 per cent of the average value and all lie between 65 per cent and 135 per cent. If 2 or 3 values lie outside the range of 75 per cent to 125 per cent, repeat the test for 2 more inhalers. Not more than 3 of the 30 values lie outside the range 75 per cent to 125 per cent and no value lies outside the range 65 per cent to 135 per cent.

Fine particle dose. Using an apparatus and procedure described in *Aerodynamic assessment of fine particles (2.9.18 - apparatus C or D)*, calculate the fine particle dose.

Number of deliveries per inhaler. Take one inhaler and discharge the contents to waste, actuating the valve at intervals of not less than 5 s. The total number of deliveries so discharged from the inhaler is not less than the number stated on the label (this test may be combined with the test for uniformity of delivered dose).

Powders for inhalation

DEFINITION

Powders for inhalation are presented as single-dose powders or multidose powders. To facilitate their use, active substances may be combined with a suitable carrier. They are generally administered by dry-powder inhalers. In pre-metered systems, the inhaler is loaded with powders pre-dispensed in capsules or other suitable pharmaceutical forms. For devices using a powder reservoir, the dose is created by a metering mechanism within the inhaler.

The delivered dose is the dose delivered from the inhaler. For some preparations, the dose has been established as a metered dose or as a predispensed dose. The metered dose is determined by adding the amount deposited within the device to the delivered dose. It may also be determined directly.

TESTS

Uniformity of delivered dose. In all cases, prepare the inhaler as directed in the instructions to the patient. The dose collection apparatus must be capable of quantitatively capturing the delivered dose. A dose collection apparatus similar to that described for the evaluation of pressurised metered-dose inhalers may be used provided that the dimensions of the tube and the filter can accommodate

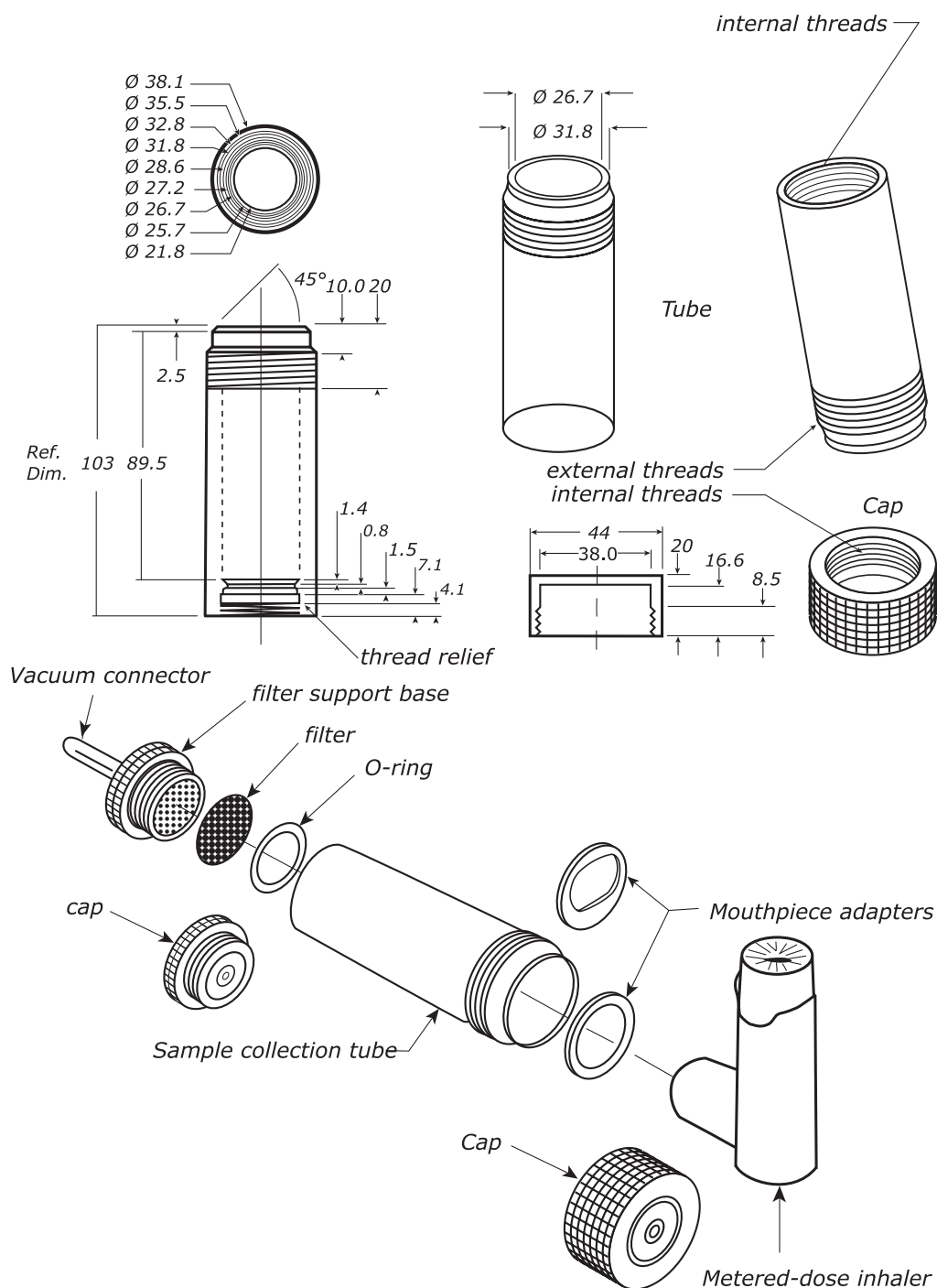


Figure 0671-1. – Dose collection apparatus for pressurised metered-dose preparations
Dimensions in millimetres

the measured flow rate. A suitable tube is defined in Figure 0671-1. Connect the tube to a flow system according to the scheme specified in Figure 0671-2 and Table 0671-1.

Unless otherwise stated, determine the test flow rate and duration using the dose collection tube, the associated flow system, a suitable differential pressure meter and a suitable volumetric flow meter, calibrated for the flow leaving the meter, according to the following procedure.

Prepare the inhaler for use and connect it to the inlet of the apparatus using a mouthpiece adapter to ensure an airtight seal. Use a mouthpiece adapter which ensures that the front face of the inhaler mouthpiece is flush with the front face of the sample collection tube. Connect one port of a differential pressure meter to the pressure reading point, P1, in Figure 0671-2 and let the other be open to the

atmosphere. Switch on the pump, open the two way valve and adjust the flow control valve until the pressure drop across the inhaler is 4.0 kPa (40.8 cm H₂O) as indicated by the differential pressure meter. Remove the inhaler from the mouthpiece adapter and without touching the flow control valve, connect a flow meter to the inlet of the sampling apparatus. If the flow rate is above 100 litres/min adjust the flow control valve to obtain a flow rate of 100 ± 5 litres/min. Note the volumetric airflow rate and define this as the test flow rate, Q, in litres per minute. Define the test flow duration, T, in seconds so that a volume of 4 litres of air is drawn through the inhaler.

Ensure that critical flow occurs in the flow control valve by the following procedure. With the inhaler in place and the test flow rate Q, measure the absolute pressure on both sides of the control valve (pressure reading points P2 and P3 in

Figure 0671.2). A ratio $P3/P2 \leq 0.5$ indicates critical flow. Switch to a more powerful pump and re-measure the test flow rate if critical flow is not indicated.

Predispensed systems. Prepare the inhaler as directed in the instructions to the patient and connect it to the apparatus using an adapter which ensures a good seal. Draw air through the inhaler using the predetermined conditions. Repeat the procedure until the number of deliveries which constitute the minimum recommended dose have been sampled. Quantitatively collect the contents of the apparatus and determine the amount of active substance.

Repeat the procedure for a further 9 doses.

Reservoir systems. Prepare the inhaler as directed in the instructions to the patient and connect it to the apparatus using an adapter which ensures a good seal. Draw air through the inhaler under the predetermined conditions. Repeat the procedure until the number of deliveries which constitute the minimum recommended dose have been sampled. Quantitatively collect the contents of the apparatus and determine the amount of active substance.

Repeat the procedure for a further 2 doses.

Discharge the device to waste until $(n/2)+1$ deliveries remain, where n is the number of deliveries stated on the label. If necessary, store the inhaler to discharge electrostatic charges. Collect 4 doses using the procedure described above.

Discharge the device to waste until 3 doses remain. If necessary, store the inhaler to discharge electrostatic charges. Collect 3 doses using the procedure described above.

For preparations containing more than 1 active substance, carry out the test for uniformity of delivered dose for each active substance.

The preparation complies with the test if 9 out of 10 results lie between 75 per cent and 125 per cent of the average value and all lie between 65 per cent and 135 per cent. If 2 or 3 values lie outside the range of 75 per cent to 125 per cent, repeat the test for 2 more inhalers. Not more than 3 of the 30 values lie outside the range 75 per cent to 125 per cent and no value lies outside the range 65 per cent to 135 per cent.

In justified and authorised cases, these ranges may be extended but no value should be greater than 150 per cent or less than 50 per cent of the average value.

Table 0671.1. – Specifications of the apparatus described in figure 0671.2

Code	Item	Description
A	Sample collection tube	Capable of quantitatively capturing the delivered dose, e.g. Dose collection tube similar to that described in Fig. 0671.1 with dimensions of 34.85 mm ID × 12 cm length (e.g. product number XX40 047 00, Millipore Corporation, Bedford, MA 01732 with modified exit tube, ID ≥ 8 mm, fitted with Gelman product number 61631), or equivalent.
B	Filter	47 mm filter, e.g. A/E glass fibre filter (Gelman Sciences, Ann Arbor, MI 48106), or equivalent.
C	Connector	ID ≥ 8 mm, e.g., short metal coupling, with low-diameter branch to P3.
D	Vacuum tubing	8 ± 0.5 mm ID × 50 ± 10 cm length, e.g., silicone tubing with an OD of 14 mm and an ID of 8 mm.
E	Two-way solenoid valve	Minimum airflow resistance orifice having an ID of ≥ 8 mm and a maximum response time of 100 ms (e.g. type 256-A08, Bürkert GmbH, D-74653 Ingelfingen), or equivalent.
F	Vacuum pump	Pump must be capable of drawing the required flow rate through the assembled apparatus with the dry powder inhaler in the mouthpiece adapter (e.g. product type 1023, 1423 or 2565, Gast Manufacturing Inc., Benton Harbor, MI 49022), or equivalent. Connect the pump to the solenoid valve using short and/or wide (≥ 10 mm ID) vacuum tubing and connectors to minimise pump capacity requirements.
G	Timer	Timer capable of driving the solenoid valve for the required time period (e.g. type G814, RS Components International, Corby, NN17 9RS, UK), or equivalent.
P1	Pressure tap	2.2 mm ID, 3.1 mm OD, flush with internal surface of the sample collection tube, centred and burr-free, 59 mm from its inlet.
P1 P2 P3	Pressure measurements	Differential pressure to atmosphere (P1) or absolute pressure (P2 and P3).
H	Flow control valve	Adjustable regulating valve with maximum $C_v \geq 1$, (e.g. type 8FV12LNSS, Parker Hannifin plc., Barnstaple, EX31 1NP, UK), or equivalent.

Fine particle dose. Using the apparatus and procedure described in *Aerodynamic assessment of fine particles (2.9.18 - apparatus C or D)*, calculate the fine particle dose.

Number of deliveries per inhaler for multidose inhalers. Discharge doses from the inhaler until empty, at the predetermined flow rate. Record the deliveries discharged.

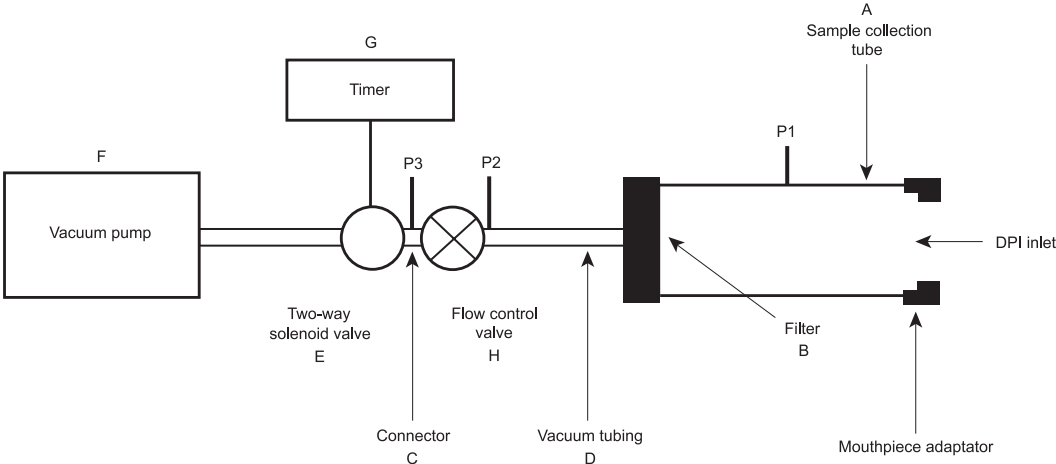


Figure 0671.2. – Apparatus suitable for measuring the uniformity of delivered dose for powder inhalers

The total number of doses delivered is not less than the number stated on the label (this test may be combined with the test for uniformity of delivered dose).

01/2005:0523

PRESSURISED PHARMACEUTICAL PREPARATIONS

01/2005:1116

Praeparationes pharmaceuticae in vasis cum pressu

PREPARATIONS FOR IRRIGATION

Praeparationes ad irrigationem

DEFINITION

Preparations for irrigation are sterile, aqueous large volume preparations intended to be used for irrigation of body cavities, wounds and surfaces, for example during surgical procedures.

Preparations for irrigation are either solutions prepared by dissolving one or more active substances, electrolytes or osmotically active substances in water complying with the requirements for *Water for injections* (0169) or they consist of such water alone. In the latter case, the preparation may be labelled as water for irrigation. Irrigation solutions are usually adjusted to be isotonic with blood.

Examined in suitable conditions of visibility, preparations for irrigation are clear and practically free from particles.

Preparations for irrigation are supplied in single-dose containers. The containers and closures comply with the requirements for containers for preparations for parenteral use (3.2.1 and 3.2.2) but the administration port of the container is incompatible with intravenous administration equipment and does not allow the preparation for irrigation to be administered with such equipment.

PRODUCTION

Preparations for irrigation are prepared using materials and methods designed to ensure sterility and to avoid the introduction of contaminants and the growth of micro-organisms; recommendations on this aspect are provided in the text on *Methods of preparation of sterile products* (5.1.1).

TESTS

Deliverable mass or volume (2.9.28). Preparations for irrigation supplied in single-dose containers comply with the test.

Sterility (2.6.1). Preparations for irrigation comply with the test for sterility.

Bacterial endotoxins (2.6.14): less than 0.5 IU/ml.

Pyrogens (2.6.8). Preparations for which a validated test for bacterial endotoxins cannot be carried out comply with the test for pyrogens. Inject per kilogram of the rabbits mass, 10 ml of the preparation, unless otherwise justified and authorised.

LABELLING

The label states:

- that the preparation is not to be used for injection,
- that the preparation is to be used for one occasion only and that any unused portion of preparation is to be discarded.

Additional requirements for preparations presented in pressurised containers may be found, where appropriate, in other general monographs, for example Preparations for inhalation (0671), Liquid preparations for cutaneous application (0927), Powders for cutaneous application (1166), Nasal preparations (0676) and Ear preparations (0652).

DEFINITION

Pressurised pharmaceutical preparations are presented in special containers under pressure of a gas and contain one or more active substances. The preparations are released from the container, upon actuation of an appropriate valve, in the form of an aerosol (dispersion of solid or liquid particles in a gas, the size of the particles being adapted to the intended use) or of a liquid or semisolid jet such as a foam. The pressure for the release is generated by suitable propellants.

The preparations consist of a solution, an emulsion or a suspension and are intended for local application to the skin or to mucous membranes of various body orifices, or for inhalation. Suitable excipients may also be used, for example solvents, solubilisers, emulsifying agents, suspending agents and lubricants for the valve to prevent clogging.

Propellants. The propellants are either gases liquefied under pressure or compressed gases or low-boiling liquids. Liquefied gases are, for example, fluorinated hydrocarbons and low-molecular-mass hydrocarbons (such as propane and butane). Compressed gases are, for example, carbon dioxide, nitrogen and nitrous oxide.

Mixtures of these propellants may be used to obtain optimal solution properties and desirable pressure, delivery and spray characteristics.

Containers. The containers are tight and resistant to the internal pressure and may be made of metal, glass, plastic or combinations of these materials. They are compatible with their contents. Glass containers are protected with a plastic coating.

Spraying device. The valve keeps the container tightly closed when not in use and regulates the delivery of the contents during use. The spray characteristics are influenced by the type of spraying device, in particular by the dimensions, number and location of orifices. Some valves provide a continuous release, others ("metering dose valves") deliver a defined quantity of product upon each valve actuation.

The various valve materials in contact with the contents are compatible with them.

Requirements for pressurised pharmaceutical preparations. Pressurised preparations are provided with a delivery device appropriate for the intended application.

Special requirements may be necessary for the selection of propellants, for particle size and the single-dose delivered by the metering valves.

LABELLING

The label states:

- the method of use,
- any precautions to be taken,

- for a container with a metering dose valve, the amount of active substance in a unit-spray.

01/2005:1145

RECTAL PREPARATIONS

Rectalia

DEFINITION

Rectal preparations are intended for rectal use in order to obtain a systemic or local effect, or they may be intended for diagnostic purposes.

Where applicable, containers for rectal preparations comply with the requirements for *Materials used for the manufacture of containers* (3.1 and subsections) and *Containers* (3.2 and subsections).

Several categories of rectal preparations may be distinguished:

- suppositories,
- rectal capsules,
- rectal solutions, emulsions and suspensions,
- powders and tablets for rectal solutions and suspensions,
- semi-solid rectal preparations,
- rectal foams,
- rectal tampons.

PRODUCTION

During the development of a rectal preparation, the formulation for which contains an antimicrobial preservative, the effectiveness of the chosen preservative shall be demonstrated to the satisfaction of the competent authority. A suitable test method together with criteria for judging the preservative properties of the formulation are provided in the text on *Efficacy of antimicrobial preservation* (5.1.3).

In the manufacture, packaging, storage and distribution of rectal preparations, suitable means are taken to ensure their microbial quality; recommendations on this aspect are provided in the text on *Microbiological quality of pharmaceutical preparations* (5.1.4).

In the manufacture of semi-solid and liquid rectal preparations containing dispersed particles measures are taken to ensure a suitable and controlled particle size with regard to the intended use.

TESTS

Uniformity of content (2.9.6). Unless otherwise prescribed or justified and authorised, solid single-dose preparations with a content of active substance less than 2 mg or less than 2 per cent of the total mass comply with test A (tablets) or test B (suppositories, rectal capsules) for uniformity of content of single-dose preparations. If the preparation contains more than one active substance, this requirement applies only to those substances that correspond to the above conditions.

Uniformity of mass (2.9.5). Solid, single-dose preparations comply with the test for uniformity of mass. If the test for uniformity of content is prescribed for all active substances, the test for uniformity of mass is not required.

Deliverable mass or volume (2.9.28). Liquid and semi-solid rectal preparations supplied in single-dose containers comply with the test.

Dissolution. A suitable test may be required to demonstrate the appropriate release of the active substance(s) from solid, single-dose preparations, for example the dissolution test for suppositories and soft capsules (2.9.3).

Where a dissolution test is prescribed, a disintegration test may not be required.

LABELLING

The label states the name of any added antimicrobial preservative.

Suppositories

DEFINITION

Suppositories are solid, single-dose preparations. The shape, volume and consistency of suppositories are suitable for rectal administration.

They contain one or more active substances dispersed or dissolved in a suitable basis which may be soluble or dispersible in water or may melt at body temperature. Excipients such as diluents, adsorbents, surface-active agents, lubricants, antimicrobial preservatives and colouring matter, authorised by the competent authority, may be added if necessary.

PRODUCTION

Suppositories are prepared by compression or moulding. If necessary, the active substance(s) are previously ground and sieved through a suitable sieve. When prepared by moulding, the medicated mass, sufficiently liquified by heating, is poured into suitable moulds. The suppository solidifies on cooling. Various excipients are available for this process, such as hard fat, macrogols, cocoa butter, and various gelatinous mixtures consisting of, for example, gelatin, water and glycerol. Where applicable, the determination of the softening time of lipophilic suppositories (2.9.22) and/or the determination of the resistance to rupture of suppositories (2.9.24) are carried out.

A suitable test is carried out to demonstrate the appropriate release of the active substance(s) from suppositories intended for modified release or for prolonged local action.

In the manufacture of suppositories containing dispersed active substances, measures are taken to ensure a suitable and controlled particle size.

TESTS

Disintegration. Unless intended for modified release or for prolonged local action, they comply with the test for disintegration of suppositories and pessaries (2.9.2). For suppositories with a fatty base, examine after 30 min and for suppositories with a water-soluble base after 60 min, unless otherwise justified and authorised.

Rectal capsules

DEFINITION

Rectal capsules (shell suppositories) are solid, single-dose preparations generally similar to soft capsules as defined in the monograph on *Capsules* (0016) except that they may have lubricating coatings. They are of elongated shape, are smooth and have a uniform external appearance.

PRODUCTION

A suitable test is carried out to demonstrate the appropriate release of the active substance(s) from rectal capsules intended for modified release or for prolonged local action.

TESTS

Disintegration. Unless intended for modified release or for prolonged local action, they comply with the test for disintegration of suppositories and pessaries (2.9.2). Examine the state of the capsules after 30 min, unless otherwise justified and authorised.

Rectal solutions, emulsions and suspensions

DEFINITION

Rectal solutions, emulsions and suspensions are liquid preparations intended for rectal use in order to obtain a systemic or local effect, or they may be intended for diagnostic purposes.

Rectal solutions, emulsions and suspensions are supplied in single-dose containers and they contain one or more active substances dissolved or dispersed in water, glycerol or macrogols or other suitable solvents. Emulsions may show evidence of phase separation but are readily redispersed on shaking. Suspensions may show a sediment which is readily dispersible on shaking to give a suspension which remains sufficiently stable to enable the correct dose to be delivered.

Rectal solutions, emulsions and suspensions may contain excipients, for example to adjust the viscosity of the preparation, to adjust or stabilise the pH, to increase the solubility of the active substance(s) or to stabilise the preparation. These substances do not adversely affect the intended medical action or, at the concentrations used, cause undue local irritation.

Rectal solutions, emulsions and suspensions are supplied in containers containing a volume in the range of 2.5 ml to 2000 ml. The container is adapted to deliver the preparation to the rectum or it is accompanied by a suitable applicator.

Powders and tablets for rectal solutions and suspensions

DEFINITION

Powders and tablets intended for the preparation of rectal solutions or suspensions are single-dose preparations which are dissolved or dispersed in water at the time of administration. They may contain excipients to facilitate dissolution or dispersion or to prevent aggregation of the particles.

After dissolution or suspension, they comply with the requirements for rectal solutions or rectal suspensions, as appropriate.

TESTS

Disintegration. Tablets for rectal solutions or suspensions disintegrate within 3 min when tested according to the test for disintegration of tablets and capsules (2.9.1) but using *water R* at 15 °C to 25 °C.

LABELLING

The label states:

- the method of preparation of the rectal solution or suspension,
- the conditions and duration of storage of the solution or suspension after constitution.

Semi-solid rectal preparations

DEFINITION

Semi-solid rectal preparations are ointments, creams or gels.

They are often supplied as single-dose preparations in containers provided with a suitable applicator.

Semi-solid rectal preparations comply with the requirements of the monograph on *Semi-solid preparations for cutaneous application* (0132).

Rectal foams

DEFINITION

Rectal foams comply with the requirements of the monograph on *Medicated foams* (1105).

Rectal tampons

DEFINITION

Rectal tampons are solid, single-dose preparations intended to be inserted into the lower part of the rectum for a limited time.

They comply with the requirements of the monograph on *Medicated tampons* (1155).

01/2005:0132

SEMI-SOLID PREPARATIONS FOR CUTANEOUS APPLICATION

Praeparationes molles ad usum dermicum

The requirements of this monograph apply to all semi-solid preparations for cutaneous application. Where appropriate, additional requirements specific to semi-solid preparations intended to be applied to particular surfaces or mucous membranes may be found in other general monographs, for example Ear preparations (0652), Nasal preparations (0676), Rectal preparations (1145), Eye preparations (1163) and Vaginal preparations (1164).

DEFINITION

Semi-solid preparations for cutaneous application are intended for local or transdermal delivery of active substances, or for their emollient or protective action. They are of homogeneous appearance.

Semi-solid preparations for cutaneous application consist of a simple or compound basis in which, usually, one or more active substances are dissolved or dispersed. According to its composition, the basis may influence the activity of the preparation.

The basis may consist of natural or synthetic substances and may be single phase or multiphase. According to the nature of the basis, the preparation may have hydrophilic or hydrophobic properties; it may contain suitable excipients such as antimicrobial preservatives, antioxidants, stabilisers, emulsifiers, thickeners and penetration enhancers.

Semi-solid preparations for cutaneous application intended for use on severely injured skin are sterile.

Where applicable, containers for semi-solid preparations for cutaneous application comply with the requirements for *Materials used for the manufacture of containers* (3.1 and subsections) and *Containers* (3.2 and subsections).

Several categories of semi-solid preparations for cutaneous application may be distinguished:

- ointments,
- creams,
- gels,
- pastes,

- poultices,
- medicated plasters.

According to their structure, ointments, creams and gels generally show viscoelastic behaviour and are non-newtonian in character e.g. plastic, pseudoplastic or thixotropic type flow at high shear rates. Pastes frequently exhibit dilatancy.

PRODUCTION

During the development of semi-solid preparations for cutaneous application whose formulation contains an antimicrobial preservative, the necessity for and the effectiveness of the chosen preservative shall be demonstrated to the satisfaction of the competent authority. A suitable test method together with criteria for judging the preservative properties of the formulation are provided in *Efficacy of antimicrobial preservation (5.1.3)*. In the manufacture, packaging, storage and distribution of semi-solid preparations for cutaneous application, suitable steps are taken to ensure their microbiological quality; recommendations on this are provided in *Microbiological Quality of Pharmaceutical Preparations (5.1.4)*. Sterile semi-solid preparations for cutaneous application are prepared using materials and methods designed to ensure sterility and to avoid the introduction of contaminants and the growth of micro-organisms; recommendations on this are provided in *Methods of Preparation of Sterile Products (5.1.1)*.

In the manufacture of semi-solid preparations for cutaneous application, suitable measures are taken to ensure that the defined rheological properties are fulfilled. Where appropriate, the following non-mandatory tests may be carried out: measurement of consistency by penetrometry (2.9.9), viscosity (apparent viscosity) (2.2.10) and a suitable test to demonstrate the appropriate release of the active substance(s).

In the manufacture of semi-solid preparations for cutaneous application containing (an) active substance(s) which is/are not dissolved in the basis (e.g. emulsions or suspensions), measures are taken to ensure appropriate homogeneity of the preparation to be delivered.

In the manufacture of semi-solid preparations for cutaneous application containing dispersed particles, measures are taken to ensure a suitable and controlled particle size with regard to the intended use.

TESTS

Deliverable mass or volume (2.9.28). Semi-solid preparations for cutaneous application supplied in single-dose containers comply with the test.

Sterility (2.6.1). Where the label indicates that the preparation is sterile, it complies with the test for sterility.

STORAGE

If the preparation contains water or other volatile ingredients, store in an airtight container. If the preparation is sterile, store in a sterile, airtight, tamper-proof container.

LABELLING

The label states:

- the name of any added antimicrobial preservative,
- where applicable, that the preparation is sterile.

Ointments

DEFINITION

An ointment consists of a single-phase basis in which solids or liquids may be dispersed.

Hydrophobic Ointments

Hydrophobic ointments can absorb only small amounts of water. Typical bases used for their formulation are hard, liquid and light liquid paraffins, vegetable oils, animal fats, synthetic glycerides, waxes and liquid polyalkylsiloxanes.

Water-emulsifying Ointments

Water-emulsifying ointments can absorb larger amounts of water and thereby produce water-in-oil or oil-in-water emulsions depending on the nature of the emulsifiers: water-in-oil emulsifying agents such as wool alcohols, sorbitan esters, monoglycerides and fatty alcohols, or oil-in-water emulsifying agents such as sulphated fatty alcohols, polysorbates, macrogol cetostearyl ether or esters of fatty acids with macrogols may be used for this purpose. Their bases are those of the hydrophobic ointments.

Hydrophilic Ointments

Hydrophilic ointments are preparations having bases that are miscible with water. The bases usually consist of mixtures of liquid and solid macrogols (polyethylene glycols). They may contain appropriate amounts of water.

Creams

DEFINITION

Creams are multiphase preparations consisting of a lipophilic phase and an aqueous phase.

Lipophilic Creams

Lipophilic creams have as the continuous phase the lipophilic phase. They contain water-in-oil emulsifying agents such as wool alcohols, sorbitan esters and monoglycerides.

Hydrophilic Creams

Hydrophilic creams have as the continuous phase the aqueous phase. They contain oil-in-water emulsifying agents such as sodium or trolamine soaps, sulphated fatty alcohols, polysorbates and polyoxyl fatty acid and fatty alcohol esters combined, if necessary, with water-in-oil emulsifying agents.

Gels

DEFINITION

Gels consist of liquids gelled by means of suitable gelling agents.

Lipophilic Gels

Lipophilic gels (oleogels) are preparations whose bases usually consist of liquid paraffin with polyethylene or fatty oils gelled with colloidal silica or aluminium or zinc soaps.

Hydrophilic Gels

Hydrophilic gels (hydrogels) are preparations whose bases usually consist of water, glycerol or propylene glycol gelled with suitable gelling agents such as starch, cellulose derivatives, carbomers and magnesium-aluminium silicates.

Pastes

DEFINITION

Pastes are semi-solid preparations for cutaneous application containing large proportions of solids finely dispersed in the basis.

Poultices

DEFINITION

Poultices consist of a hydrophilic heat-retentive basis in which solid or liquid active substances are dispersed. They are usually spread thickly on a suitable dressing and heated before application to the skin.

Medicated plasters

DEFINITION

Medicated plasters are flexible preparations containing one or more active substances. They are intended to be applied to the skin. They are designed to maintain the active substance(s) in close contact with the skin such that these may be absorbed slowly, or act as protective or keratolytic agents.

Medicated plasters consist of an adhesive basis, which may be coloured, containing one or more active substances, spread as a uniform layer on an appropriate support made of natural or synthetic material. It is not irritant or sensitising to the skin. The adhesive layer is covered by a suitable protective liner, which is removed before applying the plaster to the skin. When removed, the protective liner does not detach the preparation from the outer, supporting layer.

Medicated plasters are presented in a range of sizes directly adapted to their intended use or as larger sheets to be cut before use. Medicated plasters adhere firmly to the skin when gentle pressure is applied and can be peeled off without causing appreciable injury to the skin or detachment of the preparation from the outer, supporting layer.

TESTS

Dissolution. A suitable test may be required to demonstrate the appropriate release of the active substance(s), for example one of the tests described in *Dissolution test for transdermal patches* (2.9.4).

01/2005:1154

STICKS

Styli

Additional requirements for sticks may be found, where appropriate, in other general monographs, for example Nasal preparations (0676).

DEFINITION

Sticks are solid preparations intended for local application. They are rod-shaped or conical preparations consisting of one or more active substances alone or which are dissolved or dispersed in a suitable basis which may dissolve or melt at body temperature.

Urethral sticks and sticks for insertion into wounds are sterile.

PRODUCTION

In the manufacture, packaging, storage and distribution of sticks, suitable means are taken to ensure their microbial quality; recommendations on this aspect are provided in the text on *Microbiological quality of pharmaceutical preparations* (5.1.4).

Urethral sticks and other sterile sticks are prepared using materials and methods designed to ensure sterility and to avoid the introduction of contaminants and the growth of micro-organisms; recommendations on this aspect are provided in the text on *Methods of preparation of sterile products* (5.1.1).

In the manufacture of sticks means are taken to ensure that the preparation complies with a test for mass uniformity or, where appropriate, a test for uniformity of content.

TESTS

Sterility (2.6.1). Urethral sticks and sticks for insertion into wounds comply with the test for sterility.

LABELLING

The label states:

- the quantity of active substance(s) per stick,
- for urethral sticks and sticks to be inserted into wounds that they are sterile.

01/2005:0478

TABLETS

Compressi

The requirements of this monograph do not necessarily apply to preparations that are presented as tablets intended for use other than by oral administration. Requirements for such preparations may be found, where appropriate, in other general monographs; for example Rectal preparations (1145), Vaginal preparations (1164) and Oromucosal preparations (1807). This monograph does not apply to lozenges, oral lyophilisates, oral pastes and oral gums. Where justified and authorised, the requirements of this monograph do not apply to tablets for veterinary use.

DEFINITION

Tablets are solid preparations each containing a single dose of one or more active substances and usually obtained by compressing uniform volumes of particles. Tablets are intended for oral administration. Some are swallowed whole, some after being chewed, some are dissolved or dispersed in water before being administered and some are retained in the mouth where the active substance is liberated.

The particles consist of one or more active substances with or without excipients such as diluents, binders, disintegrating agents, glidants, lubricants, substances capable of modifying the behaviour of the preparation in the digestive tract, colouring matter authorised by the competent authority and flavouring substances.

Tablets are usually right, circular solid cylinders, the end surfaces of which are flat or convex and the edges of which may be bevelled. They may have lines or break-marks and may bear a symbol or other markings. Tablets may be coated.

Where applicable, containers for tablets comply with the requirements for *Materials used for the manufacture of containers* (3.1 and subsections) and *Containers* (3.2 and subsections).

Several categories of tablets for oral use may be distinguished:

- uncoated tablets,
- coated tablets,
- effervescent tablets,
- soluble tablets,
- dispersible tablets,
- orodispersible tablets,
- gastro-resistant tablets,
- modified-release tablets.

PRODUCTION

Tablets are usually prepared by compressing uniform volumes of particles or particle aggregates produced by granulation methods. In the manufacture of tablets, means are taken to ensure that they possess a suitable mechanical strength to avoid crumbling or breaking on handling or subsequent processing. This may be demonstrated by examining the *Friability of uncoated tablets* (2.9.7) and the *Resistance to crushing of tablets* (2.9.8). Chewable tablets are prepared to ensure that they are easily crushed by chewing.

For tablets for which subdivision is authorised, it is demonstrated to the satisfaction of the competent authority that the subdivided parts comply either with test A for *Uniformity of content of single-dose preparations* (2.9.6) or with the test for *Uniformity of mass of single-dose preparations* (2.9.5), as appropriate.

In the manufacture, packaging, storage and distribution of tablets, suitable means are taken to ensure their microbiological quality; recommendations on this aspect are provided in the text on *Microbiological quality of pharmaceutical preparations* (5.1.4).

TESTS

Uniformity of content (2.9.6). Unless otherwise prescribed or justified and authorised, tablets with a content of active substance less than 2 mg or less than 2 per cent of the total mass comply with test A for uniformity of content of single-dose preparations. If the preparation has more than one active substance, the requirement applies only to those substances which correspond to the above conditions.

Unless otherwise justified and authorised, coated tablets other than film-coated tablets comply with test A for uniformity of content of single-dose preparations irrespective of their content of active substance(s).

Uniformity of mass (2.9.5). Uncoated tablets and, unless otherwise justified and authorised, film-coated tablets comply with the test for uniformity of mass of single-dose preparations. If the test for uniformity of content is prescribed or justified and authorised for all the active substances, the test for uniformity of mass is not required.

Dissolution. A suitable test may be carried out to demonstrate the appropriate release of the active substance(s), for example one of the tests described in *Dissolution test for solid dosage forms* (2.9.3).

Where a dissolution test is prescribed, a disintegration test may not be required.

Uncoated tablets

DEFINITION

Uncoated tablets include single-layer tablets resulting from a single compression of particles and multi-layer tablets consisting of concentric or parallel layers obtained by successive compression of particles of different composition. The excipients used are not specifically intended to modify the release of the active substance in the digestive fluids.

Uncoated tablets conform to the general definition of tablets. A broken section, when examined under a lens, shows either a relatively uniform texture (single-layer tablets) or a stratified texture (multi-layer tablets) but no signs of coating.

TESTS

Disintegration. Uncoated tablets comply with the test for disintegration of tablets and capsules (2.9.1). Use *water R* as the liquid. Add a disc to each tube. Operate the apparatus for 15 min, unless otherwise justified and authorised, and examine the state of the tablets. If the tablets fail to comply because of adherence to the discs, repeat the test on a further 6 tablets omitting the discs. The tablets comply with the test if all 6 have disintegrated.

Chewable tablets are not required to comply with the test.

Coated tablets

DEFINITION

Coated tablets are tablets covered with one or more layers of mixtures of various substances such as natural or synthetic resins, gums, gelatin, inactive and insoluble fillers, sugars, plasticisers, polyols, waxes, colouring matter authorised by the competent authority and sometimes flavouring substances and active substances. The substances used as coatings are usually applied as a solution or suspension in conditions in which evaporation of the vehicle occurs. When the coating is a very thin polymeric coating, the tablets are known as film-coated tablets.

Coated tablets have a smooth surface which is often coloured and may be polished; a broken section, when examined under a lens, shows a core surrounded by one or more continuous layers with a different texture.

PRODUCTION

Where justified, uniformity of mass or uniformity of content of coated tablets other than film-coated tablets may be ensured by control of the cores.

TESTS

Disintegration. Coated tablets other than film-coated tablets comply with the test for disintegration of tablets and capsules (2.9.1). Use *water R* as the liquid. Add a disc to each tube. Operate the apparatus for 60 min, unless otherwise justified and authorised, and examine the state of the tablets. If any of the tablets has not disintegrated, repeat the test on a further 6 tablets, replacing *water R* with *0.1 M hydrochloric acid*. The tablets comply with the test if all 6 have disintegrated in the acid medium.

Film-coated tablets comply with the disintegration test prescribed above except that the apparatus is operated for 30 min, unless otherwise justified and authorised.

If coated tablets or film-coated tablets fail to comply because of adherence to the discs, repeat the test on a further 6 tablets omitting the discs. The tablets comply with the test if all 6 have disintegrated.

Chewable coated tablets are not required to comply with the test.

Effervescent tablets

DEFINITION

Effervescent tablets are uncoated tablets generally containing acid substances and carbonates or hydrogen carbonates which react rapidly in the presence of water to release carbon dioxide. They are intended to be dissolved or dispersed in water before administration.

TESTS

Disintegration. Place 1 tablet in a beaker containing 200 ml of *water R* at 15-25 °C; numerous bubbles of gas are evolved. When the evolution of gas around the tablet or its fragments ceases the tablet has disintegrated, being either dissolved or dispersed in the water so that no agglomerates of particles remain. Repeat the operation on 5 other tablets. The tablets comply with the test if each of the 6 tablets used disintegrates in the manner prescribed within 5 min, unless otherwise justified and authorised.

Soluble tablets

DEFINITION

Soluble tablets are uncoated or film-coated tablets. They are intended to be dissolved in water before administration. The solution produced may be slightly opalescent due to the added excipients used in the manufacture of the tablets.

TESTS

Disintegration. Soluble tablets disintegrate within 3 min when examined by the test for disintegration of tablets and capsules (2.9.1), but using *water R* at 15-25 °C.

Dispersible tablets

DEFINITION

Dispersible tablets are uncoated or film-coated tablets intended to be dispersed in water before administration giving a homogeneous dispersion.

TESTS

Disintegration. Dispersible tablets disintegrate within 3 min when examined by the test for disintegration of tablets and capsules (2.9.1), but using *water R* at 15-25 °C.

Fineness of dispersion. Place 2 tablets in 100 ml of *water R* and stir until completely dispersed. A smooth dispersion is produced, which passes through a sieve screen with a nominal mesh aperture of 710 µm.

Orodispersible tablets

DEFINITION

Orodispersible tablets are uncoated tablets intended to be placed in the mouth where they disperse rapidly before being swallowed.

TESTS

Disintegration. Orodispersible tablets disintegrate within 3 min when examined by the test for disintegration of tablets and capsules (2.9.1).

Modified-release tablets

DEFINITION

Modified-release tablets are coated or uncoated tablets which contain special excipients or which are prepared by special procedures, or both, designed to modify the rate, the place or the time at which the active substance(s) are released.

Modified-release tablets include prolonged-release tablets, delayed-release tablets and pulsatile-release tablets.

PRODUCTION

A suitable test is carried out to demonstrate the appropriate release of the active substance(s).

Gastro-resistant tablets

DEFINITION

Gastro-resistant tablets are delayed-release tablets that are intended to resist the gastric fluid and to release their active substance(s) in the intestinal fluid. Usually they are prepared from granules or particles already covered with a gastro-resistant coating or in certain cases by covering tablets with a gastro-resistant coating (enteric-coated tablets).

Tablets covered with a gastro-resistant coating conform to the definition of coated tablets.

PRODUCTION

For tablets prepared from granules or particles already covered with a gastro-resistant coating, a suitable test is carried out to demonstrate the appropriate release of the active substance(s).

TESTS

Disintegration. For tablets covered with a gastro-resistant coating carry out the test for disintegration (2.9.1) with the following modifications. Use *0.1 M hydrochloric acid* as the liquid. Operate the apparatus for 2 h, or other such time as may be justified and authorised, without the discs and examine the state of the tablets. The time of resistance to the acid medium varies according to the formulation of the tablets to be examined. It is typically 2 h to 3 h but even with authorised deviations is not less than 1 h. No tablet shows signs of either disintegration (apart from fragments of coating) or cracks that would allow the escape of the contents. Replace the acid by *phosphate buffer solution pH 6.8 R* and add a disc to each tube. Operate the apparatus for 60 min and examine the state of the tablets. If the tablets fail to comply because of adherence to the discs, repeat the test on a further 6 tablets omitting the discs. The tablets comply with the test if all 6 have disintegrated.

Dissolution. For tablets prepared from granules or particles already covered with a gastro-resistant coating, a suitable test is carried out to demonstrate the appropriate release of the active substance(s), for example the test described in *Dissolution test for solid dosage forms* (2.9.3).

Tablets for use in the mouth

DEFINITION

Tablets for use in the mouth are usually uncoated tablets. They are formulated to effect a slow release and local action of the active substance(s) or the release and absorption of the active substance or substances at a defined part of the mouth. They comply with the requirements of the monograph on *Oromucosal preparations* (1807).

01/2005:1155

TAMPONS, MEDICATED

Tamponae medicatae

Additional requirements for medicated tampons may be found, where appropriate, in other general monographs, for example Rectal preparations (1145), Vaginal preparations (1164) and Ear preparations (0652).

DEFINITION

Medicated tampons are solid, single-dose preparations intended to be inserted into the body cavities for a limited period of time. They consist of a suitable material such as cellulose, collagen or silicone impregnated with one or more active substances.

PRODUCTION

In the manufacture, packaging, storage and distribution of medicated tampons, suitable means are taken to ensure their microbial quality; recommendations on this aspect are provided in the text on *Microbiological quality of pharmaceutical preparations* (5.1.4).

LABELLING

The label states the quantity of active substance(s) per tampon.

01/2005:1164

VAGINAL PREPARATIONS

Vaginalia

DEFINITION

Vaginal preparations are liquid, semi-solid or solid preparations intended for administration to the vagina usually in order to obtain a local effect. They contain one or more active substances in a suitable basis.

Where appropriate, containers for vaginal preparations comply with the requirements for *Materials used for the manufacture of containers* (3.1 and subsections) and *Containers* (3.2 and subsections).

Several categories of vaginal preparations may be distinguished:

- pessaries,
- vaginal tablets,
- vaginal capsules,
- vaginal solutions, emulsions and suspensions,
- tablets for vaginal solutions and suspensions,
- semi-solid vaginal preparations,
- vaginal foams,
- medicated vaginal tampons.

PRODUCTION

In the manufacturing, packaging, storage and distribution of vaginal preparations, suitable means are taken to ensure their microbial quality; recommendations on this aspect are provided in the text on *Microbiological quality of pharmaceutical preparations* (5.1.4).

TESTS

Uniformity of content (2.9.6). Unless otherwise prescribed or justified and authorised, solid single-dose preparations with a content of active substance less than 2 mg or less than 2 per cent of the total mass comply with test A (vaginal tablets) or test B (pessaries, vaginal capsules) for uniformity of content of single-dose preparations. If the preparation has more than one active substance, the requirement applies only to those substances which correspond to the above conditions.

Uniformity of mass (2.9.5). Solid single-dose vaginal preparations comply with the test for uniformity of mass of single-dose preparations. If the test for uniformity of content is prescribed for all the active substances, the test for uniformity of mass is not required.

Deliverable mass or volume (2.9.28). Liquid and semi-solid vaginal preparations supplied in single-dose containers comply with the test.

Dissolution. A suitable test may be carried out to demonstrate the appropriate release of the active substance(s) from solid single-dose preparations, for example one of the tests described in *Dissolution test for solid dosage forms* (2.9.3).

When a dissolution test is prescribed, a disintegration test may not be required.

Pessaries

DEFINITION

Pessaries are solid, single-dose preparations. They have various shapes, usually ovoid, with a volume and consistency suitable for insertion into the vagina. They contain one or

more active substances dispersed or dissolved in a suitable basis that may be soluble or dispersible in water or may melt at body temperature. Excipients such as diluents, adsorbents, surface-active agents, lubricants, antimicrobial preservatives and colouring matter authorised by the competent authority may be added, if necessary.

PRODUCTION

Pessaries are usually prepared by moulding. Where appropriate in the manufacture of pessaries, measures are taken to ensure a suitable and controlled particle size of the active substance(s). If necessary, the active substance(s) are previously ground and sieved through a suitable sieve.

When prepared by moulding, the medicated mass, sufficiently liquified by heating, is poured into suitable moulds. The pessary solidifies on cooling. Various excipients are available for this process, such as hard fat, macrogols, cocoa butter, and various gelatinous mixtures consisting, for example, of gelatin, water and glycerol.

A suitable test is carried out to demonstrate the appropriate release of the active substance(s) from pessaries intended for prolonged local action.

Where appropriate, the determination of the resistance to rupture of pessaries (2.9.24) is carried out.

TESTS

Disintegration. Unless intended for prolonged local action, they comply with the test for disintegration of suppositories and pessaries (2.9.2). Examine the state of the pessaries after 60 min, unless otherwise justified and authorised.

Vaginal tablets

DEFINITION

Vaginal tablets are solid, single-dose preparations. They generally conform to the definitions of uncoated or film-coated tablets given in the monograph on *Tablets* (0478).

PRODUCTION

A suitable test is carried out to demonstrate the appropriate release of the active substance(s) from vaginal tablets intended for prolonged local action.

TESTS

Disintegration. Unless intended for prolonged local action, they comply with the test for disintegration of suppositories and pessaries (special method for vaginal tablets, 2.9.2). Examine the state of the tablets after 30 min, unless otherwise justified and authorised.

Vaginal capsules

DEFINITION

Vaginal capsules (shell pessaries) are solid, single-dose preparations. They are generally similar to soft capsules, differing only in their shape and size. Vaginal capsules have various shapes, usually ovoid. They are smooth and have a uniform external appearance.

PRODUCTION

A suitable test is carried out to demonstrate the appropriate release of the active substance(s) from vaginal capsules intended for prolonged local action.

TESTS

Disintegration. Unless intended for prolonged local action, they comply with the test for disintegration of suppositories and pessaries (2.9.2). Examine the state of the capsules after 30 min, unless otherwise justified and authorised.

Vaginal solutions, emulsions and suspensions

DEFINITION

Vaginal solutions, emulsions and suspensions are liquid preparations intended for a local effect, for irrigation or for diagnostic purposes. They may contain excipients, for example to adjust the viscosity of the preparation, to adjust or stabilise the pH, to increase the solubility of the active substance(s) or to stabilise the preparation. The excipients do not adversely affect the intended medical action or, at the concentrations used, cause undue local irritation.

Vaginal emulsions may show evidence of phase separation but are readily redispersed on shaking. Vaginal suspensions may show a sediment that is readily dispersed on shaking to give a suspension which remains sufficiently stable to enable a homogeneous preparation to be delivered.

They are supplied in single-dose containers. The container is adapted to deliver the preparation to the vagina or it is accompanied by a suitable applicator.

PRODUCTION

In the manufacture of vaginal suspensions measures are taken to ensure a suitable and controlled particle size with regard to the intended use.

Tablets for vaginal solutions and suspensions

DEFINITION

Tablets intended for the preparation of vaginal solutions and suspensions are single-dose preparations which are dissolved or dispersed in water at the time of administration. They may contain excipients to facilitate dissolution or dispersion or to prevent caking.

Apart from the test for disintegration, tablets for vaginal solutions or suspensions conform with the definition for *Tablets* (0478).

After dissolution or dispersion, they comply with the requirements for vaginal solutions or vaginal suspensions, as appropriate.

TESTS

Disintegration. Tablets for vaginal solutions or suspensions disintegrate within 3 min when tested according to the test for disintegration of tablets and capsules (2.9.1), but using *water R* at 15 °C to 25 °C.

LABELLING

The label states:

- the method of preparation of the vaginal solution or suspension,
- the conditions and duration of storage of the solution or suspension after constitution.

Semi-solid vaginal preparations

DEFINITION

Semi-solid vaginal preparations are ointments, creams or gels.

They are often supplied in single-dose containers. The container is provided with a suitable applicator.

Semi-solid vaginal preparations comply with the requirements of the monograph on *Semi-solid preparations for cutaneous application* (0132).

Vaginal foams

DEFINITION

Vaginal foams comply with the requirements of the monograph on *Medicated foams* (1105).

Medicated vaginal tampons

DEFINITION

Medicated vaginal tampons are solid, single-dose preparations intended to be inserted in the vagina for a limited time.

They comply with the requirements of the monograph on *Medicated tampons* (1155).

01/2005:1808

VETERINARY LIQUID PREPARATIONS FOR CUTANEOUS APPLICATION

Praeparationes liquidae veterinariae ad usum dermicum

Unless otherwise justified and authorised, veterinary liquid preparations for cutaneous application comply with the requirements of the monograph on Liquid preparations for cutaneous application (0927). In addition to these requirements, the following statements apply to veterinary liquid preparations for cutaneous application.

DEFINITION

Veterinary liquid preparations for cutaneous application are liquid preparations intended to be applied to the skin to obtain a local and/or systemic effect. They are solutions, suspensions or emulsions which may contain one or more active substances in a suitable vehicle. They may be presented as concentrates in the form of wettable powders, pastes, solutions or suspensions, which are used to prepare diluted suspensions or emulsions of active substances. They may contain suitable antimicrobial preservatives, antioxidants and other excipients such as stabilisers, emulsifiers and thickeners.

Several categories of veterinary liquid preparations for cutaneous application may be distinguished:

- cutaneous foams (see *Liquid preparations for cutaneous application* (0927)),
- dip concentrates,
- pour-on preparations,
- shampoos (see *Liquid preparations for cutaneous application* (0927)),
- spot-on preparations,
- sprays,
- teat dips,
- teat sprays,
- udder-washes.

Dip concentrates

DEFINITION

Dip concentrates are preparations containing one or more active substances, usually in the form of wettable powders, pastes, solutions or suspensions, which are used to prepare diluted solutions, suspensions or emulsions of active substances. The diluted preparations are applied by complete immersion of the animal.

Pour-on preparations

DEFINITION

Pour-on preparations contain one or more active substances for the prevention and treatment of ectoparasitic and/or endoparasitic infestations of animals. They are applied in volumes which are usually greater than 5 ml by pouring along the animal's dorsal midline.

Spot-on preparations

DEFINITION

Spot-on preparations contain one or more active substances for the prevention and treatment of ectoparasitic and/or endoparasitic infestations of animals. They are applied in volumes which are usually less than 10 ml, to a small area on the head or back, as appropriate, of the animal.

Sprays

DEFINITION

Sprays contain one or more active substances that are intended to be applied externally for therapeutic or prophylactic purposes. They are delivered in the form of an aerosol by the actuation of an appropriate valve or by means of a suitable atomising device that is either an integral part of the container or is supplied separately.

Sprays may be presented in pressurised containers (see *Pressurised pharmaceutical preparations (0523)*). When so presented, sprays usually consist of one or more active substances in a suitable vehicle held under pressure with suitable propellants or suitable mixtures of propellants. When otherwise presented, sprays are supplied in well-closed containers.

PRODUCTION

During the development and manufacture of a spray, measures are taken to ensure that the assembled product conforms to a defined spray rate and spray pattern.

Teat dips

DEFINITION

Teat dips contain one or more disinfectant active substances, usually in the form of solutions into which the teats of an animal are dipped pre- and, where necessary, post-milking to reduce the population of pathogenic micro-organisms on the surfaces. Teat dips may be supplied/presented as ready-to-use preparations or they may be prepared by dilution of teat dip concentrates. Pre- and post-milking teat dips often differ in formulation. Teat dips usually contain emollients to promote skin hydration, to soften the skin and allow healing of lesions that would otherwise harbour bacteria.

Teat sprays

DEFINITION

Teat sprays contain one or more disinfectant active substances, usually in the form of solutions which are sprayed onto the teats of an animal pre- and, where necessary, post-milking to reduce the population of pathogenic micro-organisms on the surfaces. Teat sprays may be supplied/presented as ready-to-use preparations or they may be prepared by dilution of teat spray concentrates. Pre- and post-milking sprays often differ in formulation. Teat sprays usually contain emollients to promote skin hydration, to soften the skin and allow healing of lesions that would otherwise harbour bacteria.

Udder-washes

DEFINITION

Udder-washes contain one or more disinfectant active substances, usually in the form of solutions which are sprayed onto the udder and teats of an animal to remove mud and faecal contamination before the application of teat dips or sprays. Udder-washes are usually prepared by the dilution either of concentrated preparations or of ready-to-use teat dips or teat sprays.

01/2005:1929 **Virulent mycobacteria.** Examine the working seed lot as prescribed under Tests, using 10 guinea-pigs.

BCG FOR IMMUNOTHERAPY

BCG ad immunocurationem

DEFINITION

BCG for immunotherapy is a freeze-dried preparation of live bacteria derived from a culture of the bacillus of Calmette and Guérin (*Mycobacterium bovis* BCG) whose capacity for treatment has been established.

It complies with the monograph *Vaccines for human use* (0153).

PRODUCTION

GENERAL PROVISIONS

BCG for immunotherapy shall be produced by a staff consisting of healthy persons who do not work with other infectious agents; in particular they shall not work with virulent strains of *Mycobacterium tuberculosis*, nor shall they be exposed to a known risk of tuberculosis infection. Staff are examined periodically for tuberculosis. BCG for immunotherapy is susceptible to sunlight: the procedures for production shall be so designed that all products are protected from direct sunlight and from ultraviolet light at all stages of manufacture, testing and storage.

Production is based on a seed-lot system. The production method shall have been shown to yield consistently BCG products that can be used for treatment of superficial bladder cancer and are safe. The product is prepared from cultures which are separated from the master seed lot by as few subcultures as possible and in any case not more than 8 subcultures. During the course of these subcultures the preparation is not freeze-dried more than once.

If a bioluminescence test or other biochemical method is used instead of viable count, the method is validated against the viable count for each stage of the process at which it is used.

SEED LOTS

The strain used to establish the master seed lot is chosen for and maintained to preserve its characteristics, its capacity to treat and prevent superficial bladder cancer, and its relative absence of pathogenicity for man and laboratory animals. The strain used shall be identified by historical records that include information on its origin and subsequent manipulation.

Before establishment of a working seed lot a batch is prepared and reserved for use as the comparison product. When a new working seed lot is established, a suitable test for delayed hypersensitivity in guinea-pigs is carried out on a batch of product prepared from the new working seed lot; the product is shown to be not significantly different in activity from the comparison product. Antimicrobial agent sensitivity testing is also carried out.

Only a working seed lot that complies with the following requirements may be used for propagation.

Identification. The bacteria in the working seed lot are identified as *Mycobacterium bovis* BCG using microbiological techniques, which may be supplemented by molecular biology techniques (for example, nucleic acid amplification and restriction-fragment-length polymorphism).

Bacterial and fungal contamination. Carry out the test for sterility (2.6.1), using 10 ml for each medium. The working seed lot complies with the test for sterility, except for the presence of mycobacteria.

PROPAGATION AND HARVEST

The bacteria are grown in a suitable medium for not more than 21 days by surface or submerged culture. The culture medium does not contain substances known to cause toxic or allergic reactions in human beings or to cause the bacteria to become virulent for guinea-pigs. The culture is harvested and suspended in a sterile liquid medium that protects the viability of the culture as determined by a suitable method of viable count.

FINAL BULK

The final bulk is prepared from a single harvest or by pooling a number of single harvests. A stabiliser may be added; if the stabiliser interferes with the determination of bacterial concentration on the final bulk, the determination is carried out before addition of the stabiliser.

Only final bulk that complies with the following requirements may be used in the preparation of the final lot.

Bacterial and fungal contamination. Carry out the test for sterility (2.6.1), using 10 ml of final bulk for each medium. The final bulk complies with the test for sterility, except for the presence of mycobacteria.

Count of viable units. Determine the number of viable units per millilitre by viable count on solid medium using a method suitable for the product to be examined or by a suitable biochemical method. Carry out the test in parallel on a reference preparation of the same strain.

Bacterial concentration. Determine the total bacterial concentration by a suitable method, either directly by determining the mass of the micro-organisms, or indirectly by an opacity method that has been calibrated in relation to the mass of the micro-organisms; if the bacterial concentration is determined before addition of a stabiliser, the concentration in the final bulk is established by calculation. The total bacterial concentration is within the limits approved for the particular product.

The ratio of the count of viable units to the total bacterial concentration is not less than that approved for the particular product.

FINAL LOT

The final bulk is distributed into sterile containers and freeze-dried to a moisture content favourable to the stability of the product; the containers are closed either under vacuum or under an inert gas.

Except where the filled and closed containers are stored at a temperature of -20°C or lower, the expiry date is not later than 4 years from the date of harvest.

Only a final lot that complies with the following requirement for count of viable units and with each of the requirements given below under Identification, Tests and Assay may be released for use. Provided the test for virulent mycobacteria has been carried out with satisfactory results on the final bulk, it may be omitted on the final lot.

Count of viable units. Determine the number of viable units per millilitre of the reconstituted product by viable count on solid medium using a method suitable for the product to be examined, or by a suitable biochemical method. The ratio of the count of viable units after freeze-drying to that before is not less than that approved for the particular product.

IDENTIFICATION

BCG for immunotherapy is identified by microscopic examination of the bacilli in stained smears demonstrating their acid-fast property and by the characteristic appearance

of colonies grown on solid medium. Alternatively, molecular biology techniques (for example, nucleic acid amplification) may be used.

TESTS

Virulent mycobacteria. Inject subcutaneously or intramuscularly into each of 6 guinea-pigs, each weighing 250-400 g and having received no treatment likely to interfere with the test, a quantity of the product to be examined equivalent to at least 1/25 of 1 human dose. Observe the animals for at least 42 days. At the end of this period, kill the guinea-pigs and examine by autopsy for signs of infection with tuberculosis, ignoring any minor reactions at the site of injection. Animals that die during the observation period are also examined for signs of tuberculosis. The product complies with the test if none of the guinea-pigs shows signs of tuberculosis and if not more than 1 animal dies during the observation period. If 2 animals die during this period and autopsy does not reveal signs of tuberculosis, repeat the test on 6 other guinea-pigs. The product complies with the test if not more than 1 animal dies during the 42 days following the injection and autopsy does not reveal any sign of tuberculosis.

Bacterial and fungal contamination. The reconstituted product complies with the test for sterility (2.6.1) except for the presence of mycobacteria.

Temperature stability. Maintain samples of the freeze-dried product at 37 °C for 4 weeks. Determine the number of viable units in the heated product and in unheated product as described below. The number of viable units in the heated product is within the limits approved for the particular product but in any case not less than 20 per cent of that in unheated product.

Water. Not more than the limit approved for the particular product, determined by a suitable method.

ASSAY

Determine the number of viable units in the reconstituted product by viable count on solid medium using a method suitable for the product to be examined or by a suitable validated biochemical method. The number is within the range stated on the label. Determine the number of viable units in the comparison control in parallel.

LABELLING

The label states:

- the minimum and the maximum number of viable units per dose in the reconstituted product,
- that the product must be protected from direct sunlight.

01/2005:0163

BCG VACCINE, FREEZE-DRIED

*Vaccinum tuberculosis (BCG)
cryodesiccatum*

DEFINITION

Freeze-dried BCG vaccine is a preparation of live bacteria derived from a culture of the bacillus of Calmette and Guérin (*Mycobacterium bovis* BCG) whose capacity to protect against tuberculosis has been established.

PRODUCTION

GENERAL PROVISIONS

BCG vaccine shall be produced by a staff consisting of healthy persons who do not work with other infectious agents; in particular they shall not work with virulent strains of *Mycobacterium tuberculosis*, nor shall they be exposed to a known risk of tuberculosis infection. Staff are examined periodically for tuberculosis. BCG vaccine is susceptible to sunlight: the procedures for the preparation of the vaccine shall be designed so that all cultures and vaccines are protected from direct sunlight and from ultraviolet light at all stages of manufacture, testing and storage.

Production of the vaccine is based on a seed-lot system. The production method shall have been shown to yield consistently BCG vaccines that induce adequate sensitivity to tuberculin in man, that have acceptable protective potency in animals and are safe. The vaccine is prepared from cultures which are derived from the master seed lot by as few subcultures as possible and in any case not more than 8 subcultures. During the course of these subcultures the preparation is not freeze-dried more than once.

If a bioluminescence test or other biochemical method is used instead of viable count, the method is validated against the viable count for each stage of the process at which it is used.

BACTERIAL SEED LOTS

The strain used to establish the master seed lot is chosen for and maintained to preserve its characteristics, its capacity to sensitise man to tuberculin and to protect animals against tuberculosis, and its relative absence of pathogenicity for man and laboratory animals. The strain used shall be identified by historical records that include information on its origin and subsequent manipulation.

A suitable batch of vaccine is prepared from the first working seed lot and is reserved for use as the comparison vaccine. When a new working seed lot is established, a suitable test for delayed hypersensitivity in guinea-pigs is carried out on a batch of vaccine prepared from the new working seed lot; the vaccine is shown to be not significantly different in activity from the comparison vaccine. Antimicrobial agent sensitivity testing is also carried out.

Only a working seed lot that complies with the following requirements may be used for propagation.

Identification. The bacteria in the working seed lot are identified as *Mycobacterium bovis* BCG using microbiological techniques, which may be supplemented by molecular biology techniques (for example, nucleic acid amplification and restriction-fragment-length polymorphism).

Bacterial and fungal contamination. Carry out the test for sterility (2.6.1), using 10 ml for each medium. The working seed lot complies with the test for sterility except for the presence of mycobacteria.

Virulent mycobacteria. Examine the working seed lot as prescribed under Tests, using 10 guinea-pigs.

PROPAGATION AND HARVEST

The bacteria are grown in a suitable medium for not more than 21 days by surface or submerged culture. The culture medium does not contain substances known to cause toxic or allergic reactions in humans or to cause the bacteria to become virulent for guinea-pigs. The culture is harvested and suspended in a sterile liquid medium that protects the viability of the vaccine as determined by a suitable method of viable count.

FINAL BULK VACCINE

The final bulk vaccine is prepared from a single harvest or by pooling a number of single harvests. A stabiliser may be added; if the stabiliser interferes with the determination of bacterial concentration in the final bulk vaccine, the determination is carried out before addition of the stabiliser.

Only final bulk vaccine that complies with the following requirements may be used in the preparation of the final lot.

Bacterial and fungal contamination. Carry out the test for sterility (2.6.1), using 10 ml for each medium. The final bulk vaccine complies with the test for sterility except for the presence of mycobacteria.

Count of viable units. Determine the number of viable units per millilitre by viable count on solid medium using a method suitable for the vaccine to be examined or by a suitable biochemical method. Carry out the test in parallel on a reference preparation of the same strain.

Bacterial concentration. Determine the total bacterial concentration by a suitable method, either directly by determining the mass of the micro-organisms, or indirectly by an opacity method that has been calibrated in relation to the mass of the organisms; if the bacterial concentration is determined before addition of a stabiliser, the concentration in the final bulk vaccine is established by calculation. The total bacterial concentration is within the limits approved for the particular product.

The ratio of the count of viable units to the total bacterial concentration is not less than that approved for the particular product.

FINAL LOT

The final bulk vaccine is distributed into sterile containers and freeze-dried to a moisture content favourable to the stability of the vaccine; the containers are closed either under vacuum or under an inert gas.

Except where the filled and closed containers are stored at a temperature of -20°C or lower, the expiry date is not later than 4 years from the date of harvest.

Only a final lot that complies with the following requirement for count of viable units and with each of the requirements given below under Identification, Tests and Assay may be released for use. Provided the test for virulent mycobacteria has been carried out with satisfactory results on the final bulk vaccine, it may be omitted on the final lot. Provided the test for excessive dermal reactivity has been carried out with satisfactory results on the working seed lot and on 5 consecutive final lots produced from it, the test may be omitted on the final lot.

Count of viable units. Determine the number of viable units per millilitre of the reconstituted vaccine by viable count on solid medium using a method suitable for the vaccine to be examined or by a suitable biochemical method. The ratio of the count of viable units after freeze-drying to that before is not less than that approved for the particular product.

IDENTIFICATION

BCG vaccine is identified by microscopic examination of the bacilli in stained smears demonstrating their acid-fast property and by the characteristic appearance of colonies grown on solid medium. Alternatively, molecular biology techniques (for example nucleic acid amplification) may be used.

TESTS

Virulent mycobacteria. Inject subcutaneously or intramuscularly into each of 6 guinea-pigs, each weighing 250-400 g and having received no treatment likely to interfere with the test, a quantity of vaccine equivalent to at least 50 human doses. Observe the animals for at least 42 days. At the end of this period, kill the guinea-pigs and examine by autopsy for signs of infection with tuberculosis, ignoring any minor reactions at the site of injection. Animals that die during the observation period are also examined for signs of tuberculosis. The vaccine complies with the test if none of the guinea-pigs shows signs of tuberculosis and if not more than 1 animal dies during the observation period. If 2 animals die during this period and autopsy does not reveal signs of tuberculosis repeat the test on 6 other guinea-pigs. The vaccine complies with the test if not more than 1 animal dies during the 42 days following the injection and autopsy does not reveal any sign of tuberculosis.

Bacterial and fungal contamination. The reconstituted vaccine complies with the test for sterility (2.6.1) except for the presence of mycobacteria.

Excessive dermal reactivity. Use 6 healthy, white or pale-coloured guinea-pigs, each weighing not less than 250 g and having received no treatment likely to interfere with the test. Inject intradermally into each guinea-pig, according to a randomised plan, 0.1 ml of the reconstituted vaccine and of 2 tenfold serial dilutions of the vaccine and identical doses of the comparison vaccine. Observe the lesions formed at the site of the injection for 4 weeks. The vaccine complies with the test if the reaction it produces is not markedly different from that produced by the comparison vaccine.

Temperature stability. Maintain samples of the freeze-dried vaccine at 37°C for 4 weeks. Determine the number of viable units in the heated vaccine and in unheated vaccine as described below. The number of viable units in the heated vaccine is not less than 20 per cent that in unheated vaccine.

Water. Not more than the limit approved for the particular product, determined by a suitable method.

ASSAY

Determine the number of viable units in the reconstituted vaccine by viable count on solid medium using a method suitable for the vaccine to be examined or by a suitable validated biochemical method. The number is within the range stated on the label. Determine the number of viable units in the comparison vaccine in parallel.

LABELLING

The label states:

- the minimum and maximum number of viable units per millilitre in the reconstituted vaccine,
- that the vaccine must be protected from direct sunlight.

01/2005:0154

CHOLERA VACCINE**Vaccinum cholerae****DEFINITION**

Cholera vaccine is a homogeneous suspension of a suitable strain or strains of *Vibrio cholerae* containing not less than 8×10^9 bacteria in each human dose. The human dose does not exceed 1.0 ml.

PRODUCTION

The vaccine is prepared using a seed-lot system. The vaccine consists of a mixture of equal parts of vaccines prepared from smooth strains of the 2 main serological types, Inaba and Ogawa. These may be of the classical biotype with or without the El-Tor biotype. A single strain or several strains of each type may be included. All strains must contain, in addition to their type O antigens, the heat-stable O antigen common to Inaba and Ogawa. If more than one strain each of Inaba and Ogawa are used, these may be selected so as to contain other O antigens in addition. The World Health Organisation recommends new strains which may be used if necessary, in accordance with the regulations in force in the signatory States of the Convention on the Elaboration of a European Pharmacopoeia. In order to comply with the requirements for vaccination certificates required for international travel, the vaccine must contain not less than 8×10^9 organisms of the classical biotype. Each strain is grown separately. The bacteria are inactivated either by heating the suspensions (for example, at 56 °C for 1 h) or by treatment with formaldehyde or phenol or by a combination of the physical and chemical methods.

The production method is validated to demonstrate that the product, if tested, would comply with the test for abnormal toxicity for immunosera and vaccines for human use (2.6.9) modified as follows: inject 0.5 ml of the vaccine into each mouse and 1.0 ml into each guinea pig.

IDENTIFICATION

It is identified by specific agglutination tests.

TESTS

Phenol (2.5.15). If phenol has been used in the preparation, the concentration is not more than 5 g/l.

Antibody production. Test the ability of the vaccine to induce antibodies (such as agglutinating, vibriocidal or haemagglutinating antibodies) in the guinea-pig, the rabbit or the mouse. Administer the vaccine to a group of at least 6 animals. At the end of the interval of time necessary for maximum antibody formation, determined in preliminary tests, collect sera from the animals and titrate them individually for the appropriate antibody using a suitable method. The vaccine to be examined passes the test if each serotype has elicited a significant antibody response.

Sterility (2.6.1). It complies with the test for sterility.

LABELLING

The label states:

- the method used to inactivate the bacteria,
- the number of bacteria in each human dose.

01/2005:0155

CHOLERA VACCINE, FREEZE-DRIED

Vaccinum cholerae cryodesiccatum

DEFINITION

Freeze-dried cholera vaccine is a preparation of a suitable strain or strains of *Vibrio cholerae*. The vaccine is reconstituted as stated on the label to give a uniform

suspension containing not less than 8×10^9 bacteria in each human dose. The human dose does not exceed 1.0 ml of the reconstituted vaccine.

PRODUCTION

The vaccine is prepared using a seed-lot system. The vaccine consists of a mixture of equal parts of vaccines prepared from smooth strains of the 2 main serological types, Inaba and Ogawa. These may be of the classical biotype with or without the El-Tor biotype. A single strain or several strains of each type may be included. All strains must contain, in addition to their type O antigens, the heat-stable O antigen common to Inaba and Ogawa. If more than one strain each of Inaba and Ogawa are used, these may be selected so as to contain other O antigens in addition. The World Health Organisation recommends new strains which may be used if necessary in accordance with the regulations in force in the signatory States of the Convention on the Elaboration of a European Pharmacopoeia. In order to comply with the requirements for vaccination certificates required for international travel, the vaccine must contain not less than 8×10^9 organisms of the classical biotype. Each strain is grown separately. The bacteria are inactivated either by heating the suspensions (for example, at 56 °C for 1 h) or by treatment with formaldehyde or by a combination of the physical and chemical methods. Phenol is not used in the preparation. The vaccine is distributed into sterile containers and freeze-dried to a moisture content favourable to the stability of the vaccine. The containers are then closed so as to exclude contamination.

The production method is validated to demonstrate that the product, if tested, would comply with the test for abnormal toxicity for immunosera and vaccines for human use (2.6.9) modified as follows: inject 0.5 ml of the vaccine into each mouse and 1.0 ml into each guinea pig.

IDENTIFICATION

The vaccine reconstituted as stated on the label is identified by specific agglutination tests.

TESTS

Phenol (2.5.15). If phenol has been used in the preparation, the concentration is not more than 5 g/l.

Antibody production. Test the ability of the vaccine to induce antibodies (such as agglutinating, vibriocidal or haemagglutinating antibodies) in the guinea-pig, the rabbit or the mouse. Administer the reconstituted vaccine to a group of at least 6 animals. At the end of the interval of time necessary for maximum antibody formation, determined in preliminary tests, collect sera from the animals and titrate them individually for the appropriate antibody using a suitable method. The vaccine to be examined passes the test if each serotype has elicited a significant antibody response.

Sterility (2.6.1). The reconstituted vaccine complies with the test for sterility.

LABELLING

The label states:

- the method used to inactivate the bacteria,
- the number of bacteria in each human dose.

01/2005:0444 IDENTIFICATION

DIPHTHERIA AND TETANUS VACCINE (ADSORBED)

Vaccinum diphtheriae et tetani adsorbatum

DEFINITION

Diphtheria and tetanus vaccine (adsorbed) is a preparation of diphtheria formol toxoid and tetanus formol toxoid with a mineral adsorbent. The formol toxoids are prepared from the toxins produced by the growth of *Corynebacterium diphtheriae* and *Clostridium tetani*, respectively.

PRODUCTION

GENERAL PROVISIONS

Specific toxicity of the diphtheria and tetanus components.

The production method is validated to demonstrate that the product, if tested, would comply with the following test: inject subcutaneously 5 times the single human dose stated on the label into each of 5 healthy guinea-pigs, each weighing 250-350 g, that have not previously been treated with any material that will interfere with the test. If within 42 days of the injection any of the animals shows signs of or dies from diphtheria toxæmia or tetanus, the vaccine does not comply with the test. If more than 1 animal dies from non-specific causes, repeat the test once; if more than 1 animal dies in the second test, the vaccine does not comply with the test.

BULK PURIFIED DIPHTHERIA AND TETANUS TOXOIDS

The bulk purified diphtheria and tetanus toxoids are prepared as described in the monographs on *Diphtheria vaccine (adsorbed)* (0443) and *Tetanus vaccine (adsorbed)* (0452) and comply with the requirements prescribed therein.

FINAL BULK VACCINE

The final bulk vaccine is prepared by adsorption of suitable quantities of bulk purified diphtheria toxoid and tetanus toxoid onto a mineral carrier such as hydrated aluminium phosphate or aluminium hydroxide; the resulting mixture is approximately isotonic with blood. Suitable antimicrobial preservatives may be added. Certain antimicrobial preservatives, particularly those of the phenolic type, adversely affect the antigenic activity and must not be used. Only a final bulk vaccine that complies with the following requirements may be used in the preparation of the final lot.

Antimicrobial preservative. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The amount is not less than 85 per cent and not greater than 115 per cent of the intended amount.

Sterility (2.6.1). Carry out the test for sterility using 10 ml for each medium.

FINAL LOT

The final bulk vaccine is distributed aseptically into sterile, tamper-proof containers. The containers are closed so as to prevent contamination.

Only a final lot that is satisfactory with respect to each of the requirements given below under Identification, Tests and Assay may be released for use. Provided the test for antimicrobial preservative and the assay have been carried out with satisfactory results on the final bulk vaccine, they may be omitted on the final lot.

Provided the free formaldehyde content has been determined on the bulk purified antigens or on the final bulk and it has been shown that the content in the final lot will not exceed 0.2 g/l, the test for free formaldehyde may be omitted on the final lot.

- A. Diphtheria toxoid is identified by a suitable immunochemical method (2.7.1). The following method, applicable to certain vaccines, is given as an example. Dissolve in the vaccine to be examined sufficient *sodium citrate R* to give a 100 g/l solution. Maintain at 37 °C for about 16 h and centrifuge until a clear supernatant liquid is obtained. The clear supernatant liquid reacts with a suitable diphtheria antitoxin, giving a precipitate.
- B. Tetanus toxoid is identified by a suitable immunochemical method (2.7.1). The following method, applicable to certain vaccines, is given as an example. The clear supernatant liquid obtained as described in identification test A reacts with a suitable tetanus antitoxin, giving a precipitate.

TESTS

Aluminium (2.5.13): maximum 1.25 mg per single human dose, if aluminium hydroxide or hydrated aluminium phosphate is used as the adsorbent.

Free formaldehyde (2.4.18): maximum 0.2 g/l.

Antimicrobial preservative. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The content is not less than the minimum amount shown to be effective and is not greater than 115 per cent of the quantity stated on the label.

Sterility (2.6.1). The vaccine complies with the test for sterility.

ASSAY

Diphtheria component. Carry out one of the prescribed methods for the assay of diphtheria vaccine (adsorbed) (2.7.6).

The lower confidence limit ($P = 0.95$) of the estimated potency is not less than 30 IU per single human dose.

Tetanus component. Carry out one of the prescribed methods for the assay of tetanus vaccine (adsorbed) (2.7.8).

The lower confidence limit ($P = 0.95$) of the estimated potency is not less than 40 IU per single human dose.

LABELLING

The label states:

- the minimum number of International Units of each component per single human dose,
- where applicable, that the vaccine is intended for primary vaccination of children and is not necessarily suitable for reinforcing doses or for administration to adults,
- the name and the amount of the adsorbent,
- that the vaccine must be shaken before use,
- that the vaccine is not to be frozen.

01/2005:0647

DIPHTHERIA AND TETANUS VACCINE (ADSORBED) FOR ADULTS AND ADOLESCENTS

Vaccinum diphtheriae et tetani adulti et adulescentis adsorbatum

DEFINITION

Diphtheria and tetanus vaccine (adsorbed) for adults and adolescents is a preparation of diphtheria formol toxoid and tetanus formol toxoid with a mineral adsorbent. The

formol toxoids are prepared from the toxins produced by the growth of *Corynebacterium diphtheriae* and *Clostridium tetani*, respectively. It shall have been demonstrated to the competent authority that the quantity of diphtheria toxoid used does not produce adverse reactions in subjects from the age groups for which the vaccine is intended.

PRODUCTION

GENERAL PROVISIONS

Specific toxicity of the diphtheria and tetanus components.

The production method is validated to demonstrate that the product, if tested, would comply with the following test: inject subcutaneously 5 times the single human dose stated on the label into each of 5 healthy guinea-pigs, each weighing 250-350 g, that have not previously been treated with any material that will interfere with the test. If within 42 days of the injection any of the animals shows signs of or dies from diphtheria toxæmia or tetanus, the vaccine does not comply with the test. If more than 1 animal dies from non-specific causes, repeat the test once; if more than 1 animal dies in the second test, the vaccine does not comply with the test.

BULK PURIFIED DIPHTHERIA TOXOID AND TETANUS TOXOIDS

The bulk purified diphtheria and tetanus toxoids are prepared as described in the monographs on *Diphtheria vaccine (adsorbed)* (0443) and *Tetanus vaccine (adsorbed)* (0452) and comply with the requirements prescribed therein.

FINAL BULK VACCINE

The vaccine is prepared by adsorption of suitable quantities of bulk purified diphtheria toxoid and tetanus toxoid onto a mineral carrier such as hydrated aluminium phosphate or aluminium hydroxide; the resulting mixture is approximately isotonic with blood. Suitable antimicrobial preservatives may be added. Certain antimicrobial preservatives, particularly those of the phenolic type, adversely affect the antigenic activity and must not be used.

Only a final bulk vaccine that complies with the following requirements may be used in the preparation of the final lot.

Antimicrobial preservative. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The amount is not less than 85 per cent and not greater than 115 per cent of the intended amount.

Sterility (2.6.1). Carry out the test for sterility using 10 ml for each medium.

FINAL LOT

The final bulk vaccine is distributed aseptically into sterile, tamper-proof containers. The containers are closed so as to prevent contamination.

Only a final lot that is satisfactory with respect to each of the requirements given below under Identification, Tests and Assay may be released for use. Provided the test for antimicrobial preservative and the assay have been carried out with satisfactory results on the final bulk vaccine, they may be omitted on the final lot.

Provided the free formaldehyde content has been determined on the bulk purified toxoids or on the final bulk and it has been shown that the content in the final lot will not exceed 0.2 g/l, the test for free formaldehyde may be omitted on the final lot.

IDENTIFICATION

- A. Diphtheria toxoid is identified by a suitable immunochemical method (2.7.1). The following method, applicable to certain vaccines, is given as an example. Dissolve in the vaccine to be examined sufficient *sodium citrate R* to give a 100 g/l solution. Maintain at 37 °C for about 16 h and centrifuge until a clear supernatant liquid is obtained. The clear supernatant liquid reacts with a suitable diphtheria antitoxin, giving a precipitate. If a satisfactory result is not obtained with a vaccine adsorbed on aluminium hydroxide, carry out the test as follows. Centrifuge 15 ml of the vaccine to be examined and suspend the residue in 5 ml of a freshly prepared mixture of 1 volume of a 56 g/l solution of *sodium edetate R* and 49 volumes of a 90 g/l solution of *disodium hydrogen phosphate R*. Maintain at 37 °C for not less than 6 h and centrifuge. The clear supernatant liquid reacts with a suitable diphtheria antitoxin, giving a precipitate.
- B. Tetanus toxoid is identified by a suitable immunochemical method (2.7.1). The following method, applicable to certain vaccines, is given as an example. The clear supernatant liquid obtained during identification test A reacts with a suitable tetanus antitoxin, giving a precipitate.

TESTS

Aluminium (2.5.13): maximum 1.25 mg per single human dose, if aluminium hydroxide or hydrated aluminium phosphate is used as the adsorbent.

Free formaldehyde (2.4.18): maximum 0.2 g/l.

Antimicrobial preservative. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The content is not less than the minimum amount shown to be effective and is not greater than 115 per cent of the quantity stated on the label.

Sterility (2.6.1). The vaccine complies with the test for sterility.

ASSAY

Diphtheria component. Carry out one of the prescribed methods for the assay of diphtheria vaccine (adsorbed) (2.7.6).

The lower confidence limit ($P = 0.95$) of the estimated potency is not less than 2 IU per single human dose.

Tetanus component. Carry out one of the prescribed methods for the assay of tetanus vaccine (adsorbed) (2.7.8).

The lower confidence limit ($P = 0.95$) of the estimated potency is not less than 20 IU per single human dose.

LABELLING

The label states:

- the minimum number of International Units of each component per single human dose,
- the name and the amount of the adsorbent,
- that the vaccine must be shaken before use,
- that the vaccine is not to be frozen.

01/2005:2062 FINAL BULK VACCINE

DIPHTHERIA, TETANUS AND HEPATITIS B (rDNA) VACCINE (ADSORBED)

Vaccinum diphtheriae, tetani et
hepatitidis B (ADNr) adsorbatum

DEFINITION

Diphtheria, tetanus and hepatitis B (rDNA) vaccine (adsorbed) is a combined vaccine composed of: diphtheria formol toxoid; tetanus formol toxoid; hepatitis B surface antigen (HBsAg); a mineral adsorbent such as aluminium hydroxide or hydrated aluminium phosphate.

The formol toxoids are prepared from the toxins produced by the growth of *Corynebacterium diphtheriae* and *Clostridium tetani*, respectively.

HBsAg is a component protein of hepatitis B virus; the antigen is obtained by recombinant DNA technology.

PRODUCTION

GENERAL PROVISIONS

The production method shall have been shown to yield consistently vaccines comparable with the vaccine of proven clinical efficacy and safety in man.

The content of bacterial endotoxins (2.6.14) in the bulk purified diphtheria toxoid and tetanus toxoid is determined to monitor the purification procedure and to limit the amount in the final vaccine. For each component, the content of bacterial endotoxins is less than the limit approved for the particular vaccine and in any case the contents are such that the final vaccine contains less than 100 IU per single human dose.

Reference vaccine(s). Provided valid assays can be performed, monocomponent reference vaccines may be used for the assays on the combined vaccine. If this is not possible because of interaction between the components of the combined vaccine or because of the difference in composition between monocomponent reference vaccine and the test vaccine, a batch of combined vaccine shown to be effective in clinical trials or a batch representative thereof is used as a reference vaccine. For the preparation of a representative batch, strict adherence to the production process used for the batch tested in clinical trials is necessary. The reference vaccine may be stabilised by a method that has been shown to have no effect on the assay procedure.

Specific toxicity of the diphtheria and tetanus components.

The production method is validated to demonstrate that the product, if tested, would comply with the following test: inject subcutaneously 5 times the single human dose stated on the label into each of 5 healthy guinea-pigs, each weighing 250-350 g, that have not previously been treated with any material that will interfere with the test. If within 42 days of the injection any of the animals shows signs of or dies from diphtheria toxæmia or tetanus, the vaccine does not comply with the test. If more than 1 animal dies from non-specific causes, repeat the test once; if more than 1 animal dies in the second test, the vaccine does not comply with the test.

PRODUCTION OF THE COMPONENTS

The production of the components complies with the requirements of the monographs on *Diphtheria vaccine (adsorbed)* (0443), *Tetanus vaccine (adsorbed)* (0452) and *Hepatitis B vaccine (rDNA)* (1056).

FINAL BULK VACCINE

The final bulk vaccine is prepared by adsorption, separately or together, of suitable quantities of bulk purified diphtheria toxoid, tetanus toxoid and HBsAg onto a mineral carrier such as aluminium hydroxide or hydrated aluminium phosphate. Suitable antimicrobial preservatives may be added.

Only a final bulk vaccine that complies with the following requirements may be used in the preparation of the final lot.

Antimicrobial preservative. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The amount is not less than 85 per cent and not greater than 115 per cent of the intended content.

Sterility (2.6.1). Carry out the test for sterility using 10 ml for each medium.

FINAL LOT

Only a final lot that is satisfactory with respect to the test for osmolality and with respect to each of the requirements given below under Identification, Tests and Assay may be released for use.

Provided the test for antimicrobial preservative and the assays for the diphtheria and tetanus components have been carried out with satisfactory results on the final bulk vaccine, they may be omitted on the final lot.

Provided the content of free formaldehyde has been determined on the bulk purified antigens or on the final bulk and it has been shown that the content in the final lot will not exceed 0.2 g/l, the test for free formaldehyde may be omitted on the final lot.

If an *in vivo* assay is used for the hepatitis B component, provided it has been carried out with satisfactory results on the final bulk vaccine, it may be omitted on the final lot.

Osmolality (2.2.35). The osmolality of the vaccine is within the limits approved for the particular preparation.

IDENTIFICATION

- Diphtheria toxoid is identified by a suitable immunochemical method (2.7.1). The following method, applicable to certain vaccines, is given as an example. Dissolve in the vaccine to be examined sufficient *sodium citrate R* to give a 100 g/l solution. Maintain at 37 °C for about 16 h and centrifuge until a clear supernatant liquid is obtained. The clear supernatant liquid reacts with a suitable diphtheria antitoxin, giving a precipitate.
- Tetanus toxoid is identified by a suitable immunochemical method (2.7.1). The following method, applicable to certain vaccines, is given as an example. The clear supernatant liquid obtained during identification test A reacts with a suitable tetanus antitoxin, giving a precipitate.
- The assay or, where applicable, the electrophoretic profile, serves also to identify the hepatitis B component of the vaccine.

TESTS

Aluminium (2.5.13): maximum 1.25 mg per single human dose, if aluminium hydroxide or hydrated aluminium phosphate is used as the adsorbent.

Free formaldehyde (2.4.18): maximum 0.2 g/l.

Antimicrobial preservative. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The content is not less than the minimum amount shown to be effective and is not greater than 115 per cent of the quantity stated on the label.

Sterility (2.6.1). It complies with the test for sterility.

Pyrogens (2.6.8). It complies with the test for pyrogens. Inject the equivalent of 1 human dose into each rabbit.

ASSAY

Diphtheria component. Carry out one of the prescribed methods for the assay of diphtheria vaccine (adsorbed) (2.7.6).

The lower confidence limit ($P = 0.95$) of the estimated potency is not less than 30 IU per single human dose.

Tetanus component. Carry out one of the prescribed methods for the assay of tetanus vaccine (adsorbed) (2.7.8).

The lower confidence limit ($P = 0.95$) of the estimated potency is not less than 40 IU per single human dose.

Hepatitis B component. It complies with the assay of hepatitis B vaccine (2.7.15).

LABELLING

The label states:

- the minimum number of International Units of diphtheria and tetanus toxoid per single human dose,
- the amount of HBsAg per single human dose,
- the type of cells used for production of the HBsAg component,
- where applicable, that the vaccine is intended for primary vaccination of children and is not necessarily suitable for reinforcing doses or for administration to adults,
- the name and the amount of the adsorbent,
- that the vaccine must be shaken before use,
- that the vaccine is not to be frozen.

01/2005:1931

DIPHTHERIA, TETANUS AND PERTUSSIS (ACELLULAR, COMPONENT) VACCINE (ADSORBED)

Vaccinum diphtheriae, tetani et pertussis
sine cellulis ex elementis praeparatum
adsorbatum

DEFINITION

Diphtheria, tetanus and pertussis (acellular, component) vaccine (adsorbed) is a combined vaccine composed of: diphtheria formol toxoid; tetanus formol toxoid; individually purified antigenic components of *Bordetella pertussis*; a mineral adsorbent such as aluminium hydroxide or hydrated aluminium phosphate.

The formol toxoids are prepared from the toxins produced by the growth of *Corynebacterium diphtheriae* and *Clostridium tetani*, respectively.

The vaccine contains either pertussis toxoid or a pertussis-toxin-like protein free from toxic properties, produced by expression of a genetically modified form of the corresponding gene. Pertussis toxoid is prepared from pertussis toxin by a method that renders the latter harmless while maintaining adequate immunogenic properties and avoiding reversion to toxin. The vaccine may also contain filamentous haemagglutinin, pertactin (a 69 kDa outer-membrane protein) and other defined components of *B. pertussis* such as fimbrial-2 and fimbrial-3 antigens. The latter 2 antigens may be copurified. The antigenic composition and characteristics are based on evidence of protection and freedom from unexpected reactions in the target group for which the vaccine is intended.

PRODUCTION

GENERAL PROVISIONS

The production method shall have been shown to yield consistently vaccines comparable with the vaccine of proven clinical efficacy and safety in man.

The content of bacterial endotoxins (2.6.14) in the bulk purified diphtheria toxoid, tetanus toxoid and pertussis components is determined to monitor the purification procedure and to limit the amount in the final vaccine. For each component, the content of bacterial endotoxins is less than the limit approved for the particular vaccine and, in any case, the contents are such that the final vaccine contains less than 100 IU per single human dose.

Reference vaccine(s). Provided valid assays can be performed, monocomponent reference vaccines may be used for the assays on the combined vaccine. If this is not possible because of interaction between the components of the combined vaccine or because of the difference in composition between monocomponent reference vaccine and the test vaccine, a batch of combined vaccine shown to be effective in clinical trials or a batch representative thereof is used as a reference vaccine. For the preparation of a representative batch, strict adherence to the production process used for the batch tested in clinical trials is necessary. The reference vaccine may be stabilised by a method that has been shown to have no effect on the assay procedure.

PRODUCTION OF THE COMPONENTS

The production of the components complies with the requirements of the monographs on *Diphtheria vaccine (adsorbed)* (0443), *Tetanus vaccine (adsorbed)* (0452) and *Pertussis vaccine (acellular, component, adsorbed)* (1356).

FINAL BULK VACCINE

The final bulk vaccine is prepared by adsorption of suitable quantities of bulk purified diphtheria toxoid, tetanus toxoid and pertussis components separately or together onto a mineral carrier such as aluminium hydroxide or hydrated aluminium phosphate. Suitable antimicrobial preservatives may be added.

Only a final bulk vaccine that complies with the following requirements may be used in the preparation of the final lot.

Antimicrobial preservative. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The amount is not less than 85 per cent and not greater than 115 per cent of the intended content.

Sterility (2.6.1). Carry out the test for sterility using 10 ml for each medium.

FINAL LOT

Only a final lot that is satisfactory with respect to the test for osmolality and with respect to each of the requirements given below under Identification, Tests and Assay may be released for use.

Provided the tests for absence of residual pertussis toxin, irreversibility of pertussis toxoid, free formaldehyde and antimicrobial preservative and the assay have been carried out with satisfactory results on the final bulk vaccine, they may be omitted on the final lot.

Osmolality (2.2.35). The osmolality of the vaccine is within the limits approved for the particular preparation.

IDENTIFICATION

- Diphtheria toxoid is identified by a suitable immunochemical method (2.7.1). The following method, applicable to certain vaccines, is given as an example. Dissolve in the vaccine to be examined sufficient *sodium citrate R* to give a 100 g/l solution. Maintain at 37 °C for

about 16 h and centrifuge until a clear supernatant liquid is obtained. The clear supernatant liquid reacts with a suitable diphtheria antitoxin, giving a precipitate.

- B. Tetanus toxoid is identified by a suitable immunochemical method (2.7.1). The following method, applicable to certain vaccines, is given as an example. The clear supernatant liquid obtained as described in identification test A reacts with a suitable tetanus antitoxin, giving a precipitate.
- C. The pertussis components are identified by a suitable immunochemical method (2.7.1). The following method, applicable to certain vaccines, is given as an example. The clear supernatant liquid obtained as described in identification test A reacts with specific antisera to the pertussis components of the vaccine.

TESTS

Absence of residual pertussis toxin and irreversibility of pertussis toxoid. *This test is not necessary for the product obtained by genetic modification.* Use 3 groups each of not fewer than 5 histamine-sensitive mice. Inject intraperitoneally into the first group twice the single human dose of the vaccine stored at 2-8 °C. Inject intraperitoneally into the second group twice the single human dose of the vaccine incubated at 37 °C for 4 weeks. Inject diluent into the third group of mice. After 5 days, inject into each mouse 2 mg of histamine base intraperitoneally in a volume not exceeding 0.5 ml and observe for 24 h. The test is invalid if 1 or more control mice die following histamine challenge. The vaccine complies with the test if no animal in the first or second group dies following histamine challenge. If 1 mouse dies in either or both of the first and second groups, the test may be repeated with the same number of mice or with a greater number and the results of valid tests combined; the vaccine complies with the test if, in both of the groups given the vaccine, not more than 5 per cent of the total number of mice die following histamine challenge.

The histamine sensitivity of the strain of mice used is verified at suitable intervals as follows: inject intravenously threefold dilutions of a reference pertussis toxin preparation in phosphate-buffered saline solution containing 2 g/l of gelatin and challenge with histamine as above; the strain is suitable if more than 50 per cent of the animals are sensitised by 50 ng of pertussis toxin and none of the control animals injected with only diluent and challenged similarly with histamine show symptoms of sensitisation.

Aluminium (2.5.13): maximum 1.25 mg per single human dose, if aluminium hydroxide or hydrated aluminium phosphate is used as the adsorbent.

Free formaldehyde (2.4.18): maximum 0.2 g/l.

Antimicrobial preservative. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The content is not less than the minimum amount shown to be effective and is not greater than 115 per cent of the quantity stated on the label.

Sterility (2.6.1). The vaccine complies with the test for sterility.

ASSAY

Diphtheria component. Carry out one of the prescribed methods for the assay of diphtheria vaccine (adsorbed) (2.7.6).

The lower confidence limit ($P = 0.95$) of the estimated potency is not less than the minimum potency stated on the label.

Unless otherwise justified and authorised, the minimum potency stated on the label is 30 IU per single human dose.

Tetanus component. Carry out one of the prescribed methods for the assay of tetanus vaccine (adsorbed) (2.7.8). The lower confidence limit ($P = 0.95$) of the estimated potency is not less than 40 IU per single human dose.

Pertussis component. The vaccine complies with the assay of pertussis vaccine (acellular) (2.7.16).

LABELLING

The label states:

- the minimum number of International Units of diphtheria and tetanus toxoid per single human dose,
- the names and amounts of the pertussis components per single human dose,
- where applicable, that the vaccine is intended for primary vaccination of children and is not necessarily suitable for reinforcing doses or for administration to adults,
- the name and the amount of the adsorbent,
- that the vaccine must be shaken before use,
- that the vaccine is not to be frozen,
- where applicable, that the vaccine contains a pertussis toxin-like protein produced by genetic modification.

01/2005:0445

DIPHTHERIA, TETANUS AND PERTUSSIS VACCINE (ADSORBED)

Vaccinum diphtheriae, tetani et pertussis adsorbatum

DEFINITION

Diphtheria, tetanus and pertussis vaccine (adsorbed) is a preparation of diphtheria formol toxoid and tetanus formol toxoid with a mineral adsorbent to which a suspension of inactivated *Bordetella pertussis* has been added. The formol toxoids are prepared from the toxins produced by the growth of *Corynebacterium diphtheriae* and *Clostridium tetani*, respectively.

PRODUCTION

GENERAL PROVISIONS

Specific toxicity of the diphtheria and tetanus components.

The production method is validated to demonstrate that the product, if tested, would comply with the following test: inject subcutaneously 5 times the single human dose stated on the label into each of 5 healthy guinea-pigs, each weighing 250-350 g, that have not previously been treated with any material that will interfere with the test. If within 42 days of the injection any of the animals shows signs of or dies from diphtheria toxæmia or tetanus, the vaccine does not comply with the test. If more than 1 animal dies from non-specific causes, repeat the test once; if more than 1 animal dies in the second test, the vaccine does not comply with the test.

BULK PURIFIED DIPHTHERIA AND TETANUS TOXOIDS, BULK INACTIVATED B. PERTUSSIS SUSPENSION

The bulk purified diphtheria and tetanus toxoids and the inactivated *B. pertussis* suspension are prepared as described in the monographs on *Diphtheria vaccine (adsorbed)* (0443), *Tetanus vaccine (adsorbed)* (0452) and *Pertussis vaccine (adsorbed)* (0161), respectively, and comply with the requirements prescribed therein.

FINAL BULK VACCINE

The final bulk vaccine is prepared by adsorption of suitable quantities of bulk purified diphtheria toxoid and tetanus toxoid onto a mineral carrier such as hydrated aluminium

phosphate or aluminium hydroxide and admixture of an appropriate quantity of a suspension of inactivated *B. pertussis*; the resulting mixture is approximately isotonic with blood. The *B. pertussis* concentration of the final bulk vaccine does not exceed that corresponding to an opacity of 20 IU per single human dose. If 2 or more strains of *B. pertussis* are used, the composition of consecutive lots of the final bulk vaccine shall be consistent with respect to the proportion of each strain as measured in opacity units. Suitable antimicrobial preservatives may be added to the bulk vaccine. Certain antimicrobial preservatives, particularly those of the phenolic type, adversely affect the antigenic activity and must not be used.

Only a final bulk vaccine that complies with the following requirements may be used in the preparation of the final lot.

Antimicrobial preservative. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The amount is not less than 85 per cent and not greater than 115 per cent of the intended amount.

Sterility (2.6.1). Carry out the test for sterility using 10 ml for each medium.

FINAL LOT

The final bulk vaccine is distributed aseptically into sterile, tamper-proof containers. The containers are closed so as to prevent contamination.

Only a final lot that is satisfactory with respect to each of the requirements given below under Identification, Tests and Assay may be released for use. Provided the tests for specific toxicity of the pertussis component, antimicrobial preservative and the assay have been carried out with satisfactory results on the final bulk vaccine, they may be omitted on the final lot.

Provided the free formaldehyde content has been determined on the bulk purified antigens or on the final bulk and it has been shown that the content in the final lot will not exceed 0.2 g/l, the test for free formaldehyde may be omitted on the final lot.

IDENTIFICATION

- Diphtheria toxoid is identified by a suitable immunochemical method (2.7.1). The following method, applicable to certain vaccines, is given as an example. Dissolve in the vaccine to be examined sufficient *sodium citrate R* to give a 100 g/l solution. Maintain at 37 °C for about 16 h and centrifuge until a clear supernatant liquid is obtained; reserve the precipitate for identification test C. The clear supernatant liquid reacts with a suitable diphtheria antitoxin, giving a precipitate.
- Tetanus toxoid is identified by a suitable immunochemical method (2.7.1). The following method, applicable to certain vaccines, is given as an example. The clear supernatant liquid obtained during identification test A reacts with a suitable tetanus antitoxin, giving a precipitate.
- Dissolve in the vaccine to be examined sufficient *sodium citrate R* to give a 100 g/l solution. Maintain at 37 °C for about 16 h and centrifuge to obtain a bacterial precipitate. Other suitable methods for separating the bacteria from the adsorbent may also be used. Identify pertussis vaccine by agglutination of the bacteria from the resuspended precipitate by antisera specific to *B. pertussis* or by the assay.

TESTS

Specific toxicity of the pertussis component. Use not fewer than 5 mice each weighing 14 g to 16 g for the vaccine group and for the saline control. Use mice of the same sex

or distribute males and females equally between the groups. Allow the animals access to food and water for at least 2 h before injection and during the test. Inject each mouse of the vaccine group intraperitoneally with 0.5 ml, containing a quantity of the vaccine equivalent to not less than half the single human dose. Inject each mouse of the control group with 0.5 ml of a 9 g/l sterile solution of *sodium chloride R*, preferably containing the same amount of antimicrobial preservative as that injected with the vaccine. Weigh the groups of mice immediately before the injection and 72 h and 7 days after the injection. The vaccine complies with the test if: (a) at the end of 72 h the total mass of the group of vaccinated mice is not less than that preceding the injection; (b) at the end of 7 days the average increase in mass per vaccinated mouse is not less than 60 per cent of that per control mouse; and (c) not more than 5 per cent of the vaccinated mice die during the test. The test may be repeated and the results of the tests combined.

Aluminium (2.5.13): maximum 1.25 mg per single human dose, if aluminium hydroxide or hydrated aluminium phosphate is used as the adsorbent.

Free formaldehyde (2.4.18): maximum 0.2 g/l.

Antimicrobial preservative. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The content is not less than the minimum amount shown to be effective and is not greater than 115 per cent of the quantity stated on the label.

Sterility (2.6.1). The vaccine complies with the test for sterility.

ASSAY

Diphtheria component. Carry out one of the prescribed methods for the assay of diphtheria vaccine (adsorbed) (2.7.6).

The lower confidence limit ($P = 0.95$) of the estimated potency is not less than 30 IU per single human dose.

Tetanus component. Carry out one of the prescribed methods for the assay of tetanus vaccine (adsorbed) (2.7.8).

If the test is carried out in guinea-pigs, the lower confidence limit ($P = 0.95$) of the estimated potency is not less than 40 IU per single human dose; if the test is carried out in mice, the lower confidence limit ($P = 0.95$) of the estimated potency is not less than 60 IU per single human dose.

Pertussis component. Carry out the assay of pertussis vaccine (2.7.7).

The estimated potency is not less than 4 IU per single human dose and the lower confidence limit ($P = 0.95$) of the estimated potency is not less than 2 IU per single human dose.

LABELLING

The label states:

- the minimum number of International Units of each component per single human dose,
- where applicable, that the vaccine is intended for primary vaccination of children and is not necessarily suitable for reinforcing doses or for administration to adults,
- the name and the amount of the adsorbent,
- that the vaccine must be shaken before use,
- that the vaccine is not to be frozen.

01/2005:1932

DIPHTHERIA, TETANUS, PERTUSSIS (ACELLULAR, COMPONENT) AND HAEMOPHILUS TYPE b CONJUGATE VACCINE (ADSORBED)

Vaccinum diphtheriae, tetani, pertussis
sine cellulis ex elementis praeparatum
et haemophili stirpe b coniugatum
adsorbatum

DEFINITION

Diphtheria, tetanus, pertussis (acellular, component) and haemophilus type b conjugate vaccine (adsorbed) is a combined vaccine composed of: diphtheria formol toxoid; tetanus formol toxoid; individually purified antigenic components of *Bordetella pertussis*; polyribosylribitol phosphate (PRP) covalently bound to a carrier protein; a mineral absorbent such as aluminium hydroxide or hydrated aluminium phosphate. The product may be presented with the haemophilus type b component in a separate container, the contents of which are mixed with the other components immediately before use.

The formol toxoids are prepared from the toxins produced by the growth of *Corynebacterium diphtheriae* and *Clostridium tetani* respectively.

The vaccine contains either pertussis toxoid or a pertussis-toxin-like protein free from toxic properties produced by expression of a genetically modified form of the corresponding gene. Pertussis toxoid is prepared from pertussis toxin by a method that renders the toxin harmless while maintaining adequate immunogenic properties and avoiding reversion to toxin. The acellular pertussis component may also contain filamentous haemagglutinin, pertactin (a 69 kDa outer-membrane protein) and other defined components of *B. pertussis* such as fimbrial-2 and fimbrial-3 antigens. The latter 2 antigens may be copurified. The antigenic composition and characteristics are based on evidence of protection and freedom from unexpected reactions in the target group for which the vaccine is intended.

PRP is a linear copolymer composed of repeated units of 3-β-D-ribofuranosyl-(1→1)-ribitol-5-phosphate $[(C_{10}H_{19}O_{12}P)_n]$, with a defined molecular size and derived from a suitable strain of *Haemophilus influenzae* type b. The carrier protein, when conjugated to PRP, is capable of inducing a T-cell-dependent B-cell immune response to the polysaccharide.

PRODUCTION

GENERAL PROVISIONS

The production method shall have been shown to yield consistently vaccines comparable with the vaccine of proven clinical efficacy and safety in man.

If the vaccine is presented with the haemophilus component in a separate vial, as part of consistency studies the assays of the diphtheria, tetanus and pertussis components are carried out on a suitable number of batches of vaccine reconstituted as for use. For subsequent routine control, the assays of these components may be carried out without mixing with the haemophilus component.

The content of bacterial endotoxins (2.6.14) in bulk purified diphtheria toxoid, tetanus toxoid, pertussis components and PRP conjugate is determined to monitor the purification procedure and to limit the amount in the final vaccine. For each component, the content of bacterial endotoxins is less than the limit approved for the particular vaccine; if the vaccine is presented with the haemophilus component in a separate container, the contents of the diphtheria, tetanus and pertussis antigens are in any case such that the final vial for these components contains less than 100 IU per single human dose.

The production method is validated to demonstrate that the product, if tested, would comply with the test for abnormal toxicity for immunosera and vaccines for human use (2.6.9).

During development studies and wherever revalidation is necessary, it shall be demonstrated by tests in animals that the vaccine induces a T-cell dependent B-cell immune response to PRP.

Reference vaccine(s). Provided valid assays can be performed, monocomponent reference vaccines may be used for the assays on the combined vaccine. If this is not possible because of interaction between the components of the combined vaccine or because of the difference in composition between monocomponent reference vaccine and the test vaccine, a batch of combined vaccine shown to be effective in clinical trials or a batch representative thereof is used as a reference vaccine. For the preparation of a representative batch, strict adherence to the production process used for the batch tested in clinical trials is necessary. The reference vaccine may be stabilised by a method that has been shown to have no effect on the assay procedure.

PRODUCTION OF THE COMPONENTS

The production of the components complies with the requirements of the monographs on *Diphtheria vaccine (adsorbed)* (0443), *Tetanus vaccine (adsorbed)* (0452), *Pertussis vaccine (acellular, component, adsorbed)* (1356) and *Haemophilus type b conjugate vaccine* (1219).

FINAL BULK VACCINE

Different methods of preparation may be used: a final bulk vaccine may be prepared by adsorption, separately or together, of suitable quantities of bulk purified diphtheria toxoid, tetanus toxoid, acellular pertussis components and PRP conjugate onto a mineral carrier such as aluminium hydroxide or hydrated aluminium phosphate; or 2 final bulks may be prepared and filled separately, one containing the diphtheria, tetanus and pertussis components, the other the haemophilus component, which may be freeze-dried. Suitable antimicrobial preservatives may be added.

Only a final bulk vaccine that complies with the following requirements may be used in the preparation of the final lot.

Antimicrobial preservative. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The amount is not less than 85 per cent and not greater than 115 per cent of the intended content.

Sterility (2.6.1). Carry out the test for sterility using 10 ml for each medium.

FINAL LOT

Only a final lot that is satisfactory with respect to the test for osmolality shown below and with respect to each of the requirements given below under Identification, Tests and Assay may be released for use.

Provided the tests for absence of residual pertussis toxin, irreversibility of pertussis toxoid and antimicrobial preservative and the assay have been carried out with satisfactory results on the final bulk vaccine, they may be omitted on the final lot.

Provided the free formaldehyde content has been determined on the bulk purified antigens or the final bulk and it has been shown that the content in the final lot will not exceed 0.2 g/l, the test for free formaldehyde may be omitted on the final lot.

Osmolality. (2.2.35). The osmolality of the vaccine, reconstituted where applicable, is within the limits approved for the particular preparation.

pH (2.2.3). The pH of the vaccine, reconstituted if necessary, is within the range approved for the particular product.

Free PRP. Unbound PRP is determined after removal of the conjugate, for example by anion-exchange, size-exclusion or hydrophobic chromatography, ultrafiltration or other validated methods. The amount of free PRP is not greater than that approved for the particular product.

IDENTIFICATION

If the vaccine is presented with the haemophilus component in a separate vial: identification tests A, B and C are carried out using the vial containing the diphtheria, tetanus and pertussis components; identification test D is carried out on the vial containing the haemophilus components.

- A. Diphtheria toxoid is identified by a suitable immunochemical method (2.7.1). The following method, applicable to certain vaccines, is given as an example. Dissolve in the vaccine to be examined sufficient *sodium citrate R* to give a 100 g/l solution. Maintain at 37 °C for about 16 h and centrifuge until a clear supernatant liquid is obtained. The clear supernatant liquid reacts with a suitable diphtheria antitoxin, giving a precipitate.
- B. Tetanus toxoid is identified by a suitable immunochemical method (2.7.1). The following method, applicable to certain vaccines, is given as an example. The clear supernatant liquid obtained as described in identification test A reacts with a suitable tetanus antitoxin, giving a precipitate.
- C. The pertussis components are identified by a suitable immunochemical method (2.7.1). The following method, applicable to certain vaccines, is given as an example. The clear supernatant liquid obtained as described in identification test A reacts with a specific antisera to the pertussis components of the vaccine.
- D. The haemophilus component is identified by a suitable immunochemical method (2.7.1) for PRP.

TESTS

If the product is presented with the haemophilus component in a separate container: the tests for absence of residual pertussis toxin, irreversibility of pertussis toxoid, aluminium, free formaldehyde, antimicrobial preservative and sterility are carried out on the container with the diphtheria, tetanus and pertussis components; the tests for PRP content, water (where applicable), sterility and pyrogens are carried out on the container with the haemophilus component.

If the haemophilus component is freeze-dried, some tests may be carried out on the freeze-dried product rather than on the bulk conjugate where the freeze-drying process may affect the component to be tested.

Absence of residual pertussis toxin and irreversibility of pertussis toxoid. This test is not necessary for the product obtained by genetic modification. Use 3 groups each of not fewer than 5 histamine-sensitive mice. Inject intraperitoneally into the first group twice the single human dose of the vaccine stored at 2–8 °C. Inject intraperitoneally into the second group twice the single human dose of the vaccine incubated at 37 °C for 4 weeks. Inject diluent into

the third group of mice. After 5 days, inject into each mouse 2 mg of histamine base intraperitoneally in a volume not exceeding 0.5 ml and observe for 24 h. The test is invalid if 1 or more control mice die following histamine challenge. The vaccine complies with the test if no animal in the first or second group dies following histamine challenge. If 1 mouse dies in either or both of the first and second groups, the test may be repeated with the same number of mice or with a greater number and the results of valid tests combined; the vaccine complies with the test if, in both of the groups given the vaccine, not more than 5 per cent of the total number of mice die following histamine challenge.

The histamine sensitivity of the strain of mice used is verified at suitable intervals as follows: inject intravenously threefold dilutions of a reference pertussis toxin preparation in phosphate-buffered saline solution containing 2 g/l of gelatin and challenge with histamine as above; the strain is suitable if more than 50 per cent of the animals are sensitised by 50 ng of pertussis toxin and none of the control animals injected with only diluent and challenged similarly with histamine show symptoms of sensitisation.

PRP: minimum 80 per cent of the amount of PRP stated on the label. PRP is determined either by assay of ribose (2.5.31) or phosphorus (2.5.18), by an immunochemical method (2.7.1) or by anion-exchange liquid chromatography (2.2.29) with pulsed-amperometric detection.

Aluminium (2.5.13): maximum 1.25 mg per single human dose, if aluminium hydroxide or hydrated aluminium phosphate is used as the adsorbent.

Free formaldehyde (2.4.18): maximum 0.2 g/l.

Antimicrobial preservative. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The content is not less than the minimum amount shown to be effective and is not greater than 115 per cent of the quantity stated on the label.

Water (2.5.12): maximum 3.0 per cent for the freeze-dried haemophilus component.

Sterility (2.6.1). It complies with the test for sterility.

Pyrogens (2.6.8). It complies with the test for pyrogens. Inject per kilogram of the rabbit's mass a quantity of the vaccine equivalent to: 1 µg of PRP for a vaccine with diphtheria toxoid or CRM 197 diphtheria protein as carrier; 0.1 µg of PRP for a vaccine with tetanus toxoid as carrier; 0.025 µg of PRP for a vaccine with OMP as carrier.

ASSAY

Diphtheria component. Carry out one of the prescribed methods for the assay of diphtheria vaccine (adsorbed) (2.7.6).

The lower confidence limit ($P = 0.95$) of the estimated potency is not less than the minimum potency stated on the label.

Unless otherwise justified and authorised, the minimum potency stated on the label is 30 IU per single human dose.

Tetanus component. Carry out one of the prescribed methods for the assay of tetanus vaccine (adsorbed) (2.7.8).

The lower confidence limit ($P = 0.95$) of the estimated potency is not less than 40 IU per single human dose.

Pertussis component. The vaccine complies with the assay of pertussis vaccine (acellular) (2.7.16).

LABELLING

The label states:

- the minimum number of International Units of diphtheria and tetanus toxoid per single human dose,

- the names and amounts of the pertussis components per single human dose,
- the number of micrograms of PRP per single human dose,
- the type and nominal amount of carrier protein per single human dose,
- where applicable, that the vaccine is intended for primary vaccination of children and is not necessarily suitable for reinforcing doses or for administration to adults,
- the name and the amount of the adsorbent,
- that the vaccine must be shaken before use,
- that the vaccine is not to be frozen,
- where applicable, that the vaccine contains a pertussis toxin-like protein produced by genetic modification.

01/2005:1933

DIPHTHERIA, TETANUS, PERTUSSIS (ACELLULAR, COMPONENT) AND HEPATITIS B (rDNA) VACCINE (ADSORBED)

Vaccinum diphtheriae, tetani, pertussis
sine cellulis ex elementis praeparatum et
hepatitidis B (ADNr) adsorbatum

DEFINITION

Diphtheria, tetanus, pertussis (acellular, component) and hepatitis B (rDNA) vaccine (adsorbed) is a combined vaccine composed of: diphtheria formol toxoid; tetanus formol toxoid; individually purified antigenic components of *Bordetella pertussis*; hepatitis B surface antigen; a mineral adsorbent such as aluminium hydroxide or hydrated aluminium phosphate.

The formol toxoids are prepared from the toxins produced by the growth of *Corynebacterium diphtheriae* and *Clostridium tetani*, respectively.

The vaccine contains either pertussis toxoid or a pertussis-toxin-like protein free from toxic properties, produced by expression of a genetically modified form of the corresponding gene. Pertussis toxoid is prepared from pertussis toxin by a method that renders the latter harmless while maintaining adequate immunogenic properties and avoiding reversion to toxin. The vaccine may also contain filamentous haemagglutinin, pertactin (a 69 kDa outer-membrane protein) and other defined components of *B. pertussis* such as fimbrial-2 and fimbrial-3 antigens. The latter 2 antigens may be copurified. The antigenic composition and characteristics are based on evidence of protection and freedom from unexpected reactions in the target group for which the vaccine is intended.

Hepatitis B surface antigen is a component protein of hepatitis B virus; the antigen is obtained by recombinant DNA technology.

PRODUCTION

GENERAL PROVISIONS

The production method shall have been shown to yield consistently vaccines comparable with the vaccine of proven clinical efficacy and safety in man.

The content of bacterial endotoxins (2.6.14) in the bulk purified diphtheria toxoid, tetanus toxoid and pertussis components is determined to monitor the purification procedure and to limit the amount in the final vaccine. For each component, the content of bacterial endotoxins is less than the limit approved for the particular vaccine.

Reference vaccine(s). Provided valid assays can be performed, monocomponent reference vaccines may be used for the assays on the combined vaccine. If this is not possible because of interaction between the components of the combined vaccine or because of the difference in composition between monocomponent reference vaccine and the test vaccine, a batch of combined vaccine shown to be effective in clinical trials or a batch representative thereof is used as a reference vaccine. For the preparation of a representative batch, strict adherence to the production process used for the batch tested in clinical trials is necessary. The reference vaccine may be stabilised by a method that has been shown to have no effect on the assay procedure.

PRODUCTION OF THE COMPONENTS

The production of the components complies with the requirements of the monographs on *Diphtheria vaccine (adsorbed)* (0443), *Tetanus vaccine (adsorbed)* (0452), *Pertussis vaccine (acellular, component, adsorbed)* (1356) and *Hepatitis B vaccine (rDNA)* (1056).

FINAL BULK VACCINE

The final bulk vaccine is prepared by adsorption, separately or together, of suitable quantities of bulk purified diphtheria toxoid, tetanus toxoid, acellular pertussis components and hepatitis B surface antigen onto a mineral carrier such as aluminium hydroxide or hydrated aluminium phosphate. Suitable antimicrobial preservatives may be added.

Only a final bulk vaccine that complies with the following requirements may be used in the preparation of the final lot.

Antimicrobial preservative. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The amount is not less than 85 per cent and not greater than 115 per cent of the intended content.

Sterility (2.6.1). Carry out the test for sterility using 10 ml for each medium.

FINAL LOT

Only a final lot that is satisfactory with respect to the test for osmolality and with respect to each of the requirements given below under Identification, Tests and Assay may be released for use.

Provided the tests for absence of residual pertussis toxin, irreversibility of pertussis toxoid and antimicrobial preservative and the assays for the diphtheria, tetanus and pertussis components have been carried out with satisfactory results on the final bulk vaccine, they may be omitted on the final lot.

Provided the content of free formaldehyde has been determined on the bulk purified antigens or on the final bulk and it has been shown that the content in the final lot will not exceed 0.2 g/l, the test for free formaldehyde may be omitted on the final lot.

If an *in vivo* assay is used for the hepatitis B component, provided it has been carried out with satisfactory results on the final bulk vaccine, it may be omitted on the final lot.

Osmolality (2.2.35). The osmolality of the vaccine is within the limits approved for the particular preparation.

IDENTIFICATION

- Diphtheria toxoid is identified by a suitable immunochemical method (2.7.1). The following method, applicable to certain vaccines, is given as an example. Dissolve in the vaccine to be examined sufficient *sodium citrate R* to give a 100 g/l solution. Maintain at 37 °C for about 16 h and centrifuge until a clear supernatant liquid is obtained. The clear supernatant liquid reacts with a suitable diphtheria antitoxin, giving a precipitate.

- B. Tetanus toxoid is identified by a suitable immunochemical method (2.7.1). The following method, applicable to certain vaccines, is given as an example. The clear supernatant liquid obtained as described in identification test A reacts with a suitable tetanus antitoxin, giving a precipitate.
- C. The pertussis components are identified by a suitable immunochemical method (2.7.1). The following method, applicable to certain vaccines, is given as an example. The clear supernatant liquid obtained as described in identification test A reacts with a specific antisera to the pertussis components of the vaccine.
- D. The assay or, where applicable, the electrophoretic profile, serves also to identify the hepatitis B component of the vaccine.

TESTS

Absence of residual pertussis toxin and irreversibility of pertussis toxoid. *This test is not necessary for the product obtained by genetic modification.* Use 3 groups each of not fewer than 5 histamine-sensitive mice. Inject intraperitoneally into the first group twice the single human dose of the vaccine stored at 2–8 °C. Inject intraperitoneally into the second group twice the single human dose of the vaccine incubated at 37 °C for 4 weeks. Inject diluent into the third group of mice. After 5 days, inject into each mouse 2 mg of histamine base intraperitoneally in a volume not exceeding 0.5 ml and observe for 24 h. The test is invalid if 1 or more control mice die following histamine challenge. The vaccine complies with the test if no animal in the first or second group dies following histamine challenge. If 1 mouse dies in either or both of the first and second groups, the test may be repeated with the same number of mice or with a greater number and the results of valid tests combined; the vaccine complies with the test if, in both of the groups given the vaccine, not more than 5 per cent of the total number of mice die following histamine challenge.

The histamine sensitivity of the strain of mice used is verified at suitable intervals as follows: inject intravenously threefold dilutions of a reference pertussis toxin preparation in phosphate-buffered saline solution containing 2 g/l of gelatin and challenge with histamine as above; the strain is suitable if more than 50 per cent of the animals are sensitised by 50 ng of pertussis toxin and none of the control animals injected with only diluent and challenged similarly with histamine show symptoms of sensitisation.

Aluminium (2.5.13): maximum 1.25 mg per single human dose, if aluminium hydroxide or hydrated aluminium phosphate is used as the adsorbent.

Free formaldehyde (2.4.18): maximum 0.2 g/l.

Antimicrobial preservative. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The content is not less than the minimum amount shown to be effective and is not greater than 115 per cent of the quantity stated on the label.

Sterility (2.6.1). The vaccine complies with the test for sterility.

Pyrogens (2.6.8). The vaccine complies with the test for pyrogens. Inject the equivalent of 1 human dose into each rabbit.

ASSAY

Diphtheria component. Carry out one of the prescribed methods for the assay of diphtheria vaccine (adsorbed) (2.7.6).

The lower confidence limit ($P = 0.95$) of the estimated potency is not less than the minimum potency stated on the label.

Unless otherwise justified and authorised, the minimum potency stated on the label is 30 IU per single human dose.

Tetanus component. Carry out one of the prescribed methods for the assay of tetanus vaccine (adsorbed) (2.7.8). The lower confidence limit ($P = 0.95$) of the estimated potency is not less than 40 IU per single human dose.

Pertussis component. The vaccine complies with the assay of pertussis vaccine (acellular) (2.7.16).

Hepatitis B component. The vaccine complies with the assay of hepatitis B vaccine (2.7.15).

LABELLING

The label states:

- the minimum number of International Units of diphtheria and tetanus toxoid per single human dose,
- the names and amounts of the pertussis components per single human dose,
- the amount of HBsAg per single human dose,
- the type of cells used for production of the hepatitis B component,
- where applicable, that the vaccine is intended for primary vaccination of children and is not necessarily suitable for reinforcing doses or for administration to adults,
- the name and the amount of the adsorbent,
- that the vaccine must be shaken before use,
- that the vaccine is not to be frozen,
- where applicable, that the vaccine contains a pertussis toxin-like protein produced by genetic modification.

01/2005:1934

DIPHTHERIA, TETANUS, PERTUSSIS (ACELLULAR, COMPONENT) AND POLIOMYELITIS (INACTIVATED) VACCINE (ADSORBED)

Vaccinum diphtheriae, tetani, pertussis
sine cellulis ex elementis praeparatum et
poliomyelitis inactivatum adsorbatum

DEFINITION

Diphtheria, tetanus, pertussis (acellular, component) and poliomyelitis (inactivated) vaccine (adsorbed) is a combined vaccine containing: diphtheria formol toxoid; tetanus formol toxoid; individually purified antigenic components of *Bordetella pertussis*; suitable strains of human polioviruses 1, 2 and 3 grown in suitable cell cultures and inactivated by a validated method; a mineral adsorbent such as aluminium hydroxide or hydrated aluminium phosphate.

The formol toxoids are prepared from the toxins produced by the growth of *Corynebacterium diphtheriae* and *Clostridium tetani* respectively.

The vaccine contains either pertussis toxoid or a pertussis-toxin-like protein free from toxic properties produced by expression of a genetically modified form of the corresponding gene. Pertussis toxoid is prepared from

pertussis toxin by a method that renders the toxin harmless while maintaining adequate immunogenic properties and avoiding reversion to toxin. The vaccine may also contain filamentous haemagglutinin, pertactin (a 69 kDa outer-membrane protein) and other defined components of *B. pertussis* such as fimbrial-2 and fimbrial-3 antigens. The latter 2 antigens may be copurified. The antigenic composition and characteristics are based on evidence of protection and freedom from unexpected reactions in the target group for which the vaccine is intended.

PRODUCTION

GENERAL PROVISIONS

The production method shall have been shown to yield consistently vaccines comparable with the vaccine of proven clinical efficacy and safety in man.

The production method is validated to demonstrate that the product, if tested, would comply with the test for abnormal toxicity for immunosera and vaccines for human use (2.6.9).

The content of bacterial endotoxins (2.6.14) in bulk purified diphtheria toxoid, tetanus toxoid, pertussis components and purified, inactivated monovalent poliovirus harvests is determined to monitor the purification procedure and to limit the amount in the final vaccine. For each component, the content of bacterial endotoxins is less than the limit approved for the particular vaccine and, in any case, the contents are such that the final vaccine contains less than 100 IU per single human dose.

Reference vaccine(s). Provided valid assays can be performed, monocomponent reference vaccines may be used for the assays on the combined vaccine. If this is not possible because of interaction between the components of the combined vaccine or because of the difference in composition between monocomponent reference vaccine and the test vaccine, a batch of combined vaccine shown to be effective in clinical trials or a batch representative thereof is used as a reference vaccine. For the preparation of a representative batch, strict adherence to the production process used for the batch tested in clinical trials is necessary. The reference vaccine may be stabilised by a method that has been shown to have no effect on the assay procedure.

PRODUCTION OF THE COMPONENTS

The production of the components complies with the requirements of the monographs on *Diphtheria vaccine (adsorbed)* (0443), *Tetanus vaccine (adsorbed)* (0452), *Pertussis vaccine (acellular, component, adsorbed)* (1356) and *Poliomyelitis vaccine (inactivated)* (0214).

FINAL BULK VACCINE

The final bulk vaccine is prepared by adsorption onto a mineral carrier such as aluminium hydroxide or hydrated aluminium phosphate, separately or together, of suitable quantities of bulk purified diphtheria toxoid, tetanus toxoid, acellular pertussis components and admixture of suitable quantities of purified monovalent harvests of human polioviruses 1, 2 and 3 or a suitable quantity of a trivalent pool of such purified monovalent harvests. Suitable antimicrobial preservatives may be added.

Only a final bulk vaccine that complies with the following requirements may be used in the preparation of the final lot.

Bovine serum albumin. Determined on the poliomyelitis components by a suitable immunochemical method (2.7.1) after virus harvest and before addition of the adsorbent in the preparation of the final bulk vaccine, the amount of bovine serum albumin is such that the content in the final vaccine will be not more than 50 ng per single human dose.

Antimicrobial preservative. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The amount is not less than 85 per cent and not greater than 115 per cent of the intended content.

Sterility (2.6.1). Carry out the test for sterility using 10 ml for each medium.

FINAL LOT

Only a final lot that is satisfactory with respect to the test for osmolality and with respect to each of the requirements given below under Identification, Tests and Assay may be released for use.

Provided the tests for absence of residual pertussis toxin, irreversibility of pertussis toxoid and antimicrobial preservative and the assays for the diphtheria, tetanus and pertussis components have been carried out with satisfactory results on the final bulk vaccine, they may be omitted on the final lot.

Provided the free formaldehyde content has been determined on the bulk purified antigens or on the final bulk and it has been shown that the content in the final lot will not exceed 0.2 g/l, the test for free formaldehyde may be omitted on the final lot.

Provided that the determination of D-antigen content has been carried out with satisfactory results during preparation of the final bulk before addition of the adsorbent, it may be omitted on the final lot.

Provided that the *in vivo* assay for the poliomyelitis component has been carried out with satisfactory results on the final bulk vaccine, it may be omitted on the final lot.

Osmolality (2.2.35). The osmolality of the vaccine is within the limits approved for the particular preparation.

IDENTIFICATION

- A. Diphtheria toxoid is identified by a suitable immunochemical method (2.7.1). The following method, applicable to certain vaccines, is given as an example. Dissolve in the vaccine to be examined sufficient *sodium citrate R* to give a 100 g/l solution. Maintain at 37 °C for about 16 h and centrifuge until a clear supernatant liquid is obtained. The clear supernatant liquid reacts with a suitable diphtheria antitoxin, giving a precipitate.
- B. Tetanus toxoid is identified by a suitable immunochemical method (2.7.1). The following method, applicable to certain vaccines, is given as an example. The clear supernatant liquid obtained as described in identification test A reacts with a suitable tetanus antitoxin, giving a precipitate.
- C. The pertussis components are identified by a suitable immunochemical method (2.7.1). The following method, applicable to certain vaccines, is given as an example. The clear supernatant liquid obtained as described in identification test A reacts with a specific antisera to the pertussis components of the vaccine.
- D. The vaccine is shown to contain human polioviruses 1, 2 and 3 by a suitable immunochemical method (2.7.1) such as the determination of D-antigen by enzyme-linked immunosorbent assay (ELISA).

TESTS

Absence of residual pertussis toxin and irreversibility of pertussis toxoid. *This test is not necessary for the product obtained by genetic modification.* Use 3 groups each of not fewer than 5 histamine-sensitive mice. Inject intraperitoneally into the first group twice the single human dose of the vaccine stored at 2-8 °C. Inject intraperitoneally into the second group twice the single human dose of the vaccine incubated at 37 °C for 4 weeks. Inject diluent into

the third group of mice. After 5 days, inject into each mouse 2 mg of histamine base intraperitoneally in a volume not exceeding 0.5 ml and observe for 24 h. The test is invalid if 1 or more control mice die following histamine challenge. The vaccine complies with the test if no animal in the first or second group dies following histamine challenge. If 1 mouse dies in either or both of the first and second groups, the test may be repeated with the same number of mice or with a greater number and the results of valid tests combined; the vaccine complies with the test if, in both of the groups given the vaccine, not more than 5 per cent of the total number of mice die following histamine challenge.

The histamine sensitivity of the strain of mice used is verified at suitable intervals as follows: inject intravenously threefold dilutions of a reference pertussis toxin preparation in phosphate-buffered saline solution containing 2 g/l of gelatin and challenge with histamine as above; the strain is suitable if more than 50 per cent of the animals are sensitised by 50 ng of pertussis toxin and none of the control animals injected with only diluent and challenged similarly with histamine show symptoms of sensitisation.

Aluminium (2.5.13): maximum 1.25 mg per single human dose if aluminium hydroxide or hydrated aluminium phosphate is used as the adsorbent.

Free formaldehyde (2.4.18): maximum 0.2 g/l.

Antimicrobial preservative. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The content is not less than the minimum amount shown to be effective and is not greater than 115 per cent of the quantity stated on the label.

Sterility (2.6.1). It complies with the test for sterility.

ASSAY

Diphtheria component. Carry out one of the prescribed methods for the assay of diphtheria vaccine (adsorbed) (2.7.6).

The lower confidence limit ($P = 0.95$) of the estimated potency is not less than the minimum potency stated on the label.

Unless otherwise justified and authorised, the minimum potency stated on the label is 30 IU per single human dose.

Tetanus component. Carry out one of the prescribed methods for the assay of tetanus vaccine (adsorbed) (2.7.8).

The lower confidence limit ($P = 0.95$) of the estimated potency is not less than 40 IU per single human dose.

Pertussis component. The vaccine complies with the assay of pertussis vaccine (acellular) (2.7.16).

Poliomyelitis component

D-antigen content. As a measure of consistency of production, determine the D-antigen content for human polioviruses 1, 2 and 3 by a suitable immunochemical method (2.7.1) following desorption using a reference preparation calibrated in European Pharmacopoeia D-antigen units. For each type, the content, expressed with reference to the amount of D-antigen stated on the label, is within the limits approved for the particular product.

Poliomyelitis vaccine (inactivated) BRP is calibrated in European Pharmacopoeia units and intended for use in the assay of D-antigen. The European Pharmacopoeia unit and the International Unit are equivalent.

In vivo test. The vaccine complies with the *in vivo* assay of poliomyelitis vaccine (inactivated) (2.7.20).

LABELLING

The label states:

- the minimum number of International Units of diphtheria and tetanus toxoid per single human dose,
- the names and amounts of the pertussis components per single human dose,
- the types of poliovirus contained in the vaccine,
- the nominal amount of poliovirus of each type (1, 2 and 3), expressed in European Pharmacopoeia units of D-antigen, per single human dose,
- the type of cells used for production of the poliomyelitis component,
- where applicable, that the vaccine is intended for primary vaccination of children and is not necessarily suitable for reinforcing doses or for administration to adults,
- the name and the amount of the adsorbent,
- that the vaccine must be shaken before use,
- that the vaccine is not to be frozen,
- where applicable, that the vaccine contains a pertussis toxin-like protein produced by genetic modification.

01/2005:2067

DIPHTHERIA, TETANUS, PERTUSSIS (ACELLULAR, COMPONENT), HEPATITIS B (rDNA), POLIOMYELITIS (INACTIVATED) AND HAEMOPHILUS TYPE b CONJUGATE VACCINE (ADSORBED)

Vaccinum diphtheriae, tetani, pertussis
sine cellulis ex elementis praeparatum,
hepatitidis B (ADNr), poliomyelitidis
inactivatum et haemophili stirpe b
coniugatum adsorbatum

DEFINITION

Diphtheria, tetanus, pertussis (acellular, component), hepatitis B (rDNA), poliomyelitis (inactivated) and haemophilus type b conjugate vaccine (adsorbed) is a combined vaccine composed of: diphtheria formol toxoid; tetanus formol toxoid; individually purified antigenic components of *Bordetella pertussis*; hepatitis B surface antigen (HBsAg); human polioviruses 1, 2 and 3 grown in suitable cell cultures and inactivated by a suitable method; polyribosylribitol phosphate (PRP) covalently bound to a carrier protein. The antigens in the vaccine may be adsorbed on a mineral carrier such as aluminium hydroxide or hydrated aluminium phosphate. The product may be presented with the haemophilus component in a separate container, the contents of which are mixed with the other components immediately before or during use.

The formol toxoids are prepared from the toxins produced by the growth of *Corynebacterium diphtheriae* and *Clostridium tetani* respectively.

The vaccine contains either pertussis toxoid or a pertussis-toxin-like protein free from toxic properties produced by expression of a genetically modified form of the corresponding gene. Pertussis toxoid is prepared from pertussis toxin by a method that renders the toxin harmless while maintaining adequate immunogenic properties and avoiding reversion to toxin. The acellular pertussis component may also contain filamentous haemagglutinin,

pertactin (a 69 kDa outer-membrane protein) and other defined components of *B. pertussis* such as fimbrial-2 and fimbrial-3 antigens. The latter 2 antigens may be copurified. The antigenic composition and characteristics are based on evidence of protection and freedom from unexpected reactions in the target group for which the vaccine is intended.

Hepatitis B surface antigen is a component protein of hepatitis B virus; the antigen is obtained by recombinant DNA technology.

PRP is a linear copolymer composed of repeated units of 3-β-D-ribofuranosyl-(1 → 1)-ribitol-5-phosphate [(C₁₀H₁₉O₁₂P)_n], with a defined molecular size and derived from a suitable strain of *Haemophilus influenzae* type b. The carrier protein, when conjugated to PRP, is capable of inducing a T-cell-dependent B-cell immune response to the polysaccharide.

PRODUCTION

GENERAL PROVISIONS

The production method shall have been shown to yield consistently vaccines comparable with the vaccine of proven clinical efficacy and safety in man.

If the vaccine is presented with the haemophilus component in a separate vial, as part of consistency studies the assays of the diphtheria, tetanus, pertussis, hepatitis B and poliomyelitis components are carried out on a suitable number of batches of vaccine reconstituted as for use. For subsequent routine control, the assays of these components may be carried out without mixing with the haemophilus component.

The content of bacterial endotoxins (2.6.14) in bulk purified diphtheria toxoid, bulk purified tetanus toxoid, bulk purified pertussis components, the hepatitis B surface antigen, the purified, inactivated monovalent poliovirus harvests and bulk PRP conjugate is determined to monitor the purification procedure and to limit the amount in the final vaccine. For each component, the content of bacterial endotoxins is not greater than the limit approved.

During development studies and wherever revalidation is necessary, a test for pyrogens in rabbits (2.6.8) is carried out by injection of a suitable dose of the final lot. The vaccine is shown to be acceptable with respect to absence of pyrogenic activity.

The production method is validated to demonstrate that the product, if tested, would comply with the following test for specific toxicity of the diphtheria and tetanus component: inject subcutaneously 5 times the single human dose stated on the label into each of 5 healthy guinea-pigs, each weighing 250-350 g, that have not previously been treated with any material that will interfere with the test. If within 42 days of the injection any of the animals shows signs of or dies from diphtheria, toxæmia or tetanus, the vaccine does not comply with the test. If more than 1 animal dies from non-specific causes, repeat the test once; if more than 1 animal dies in the second test, the vaccine does not comply with the test.

During development studies and wherever revalidation is necessary, it shall be demonstrated by tests in animals that the vaccine induces a T-cell-dependent B-cell immune response to PRP.

The stability of the final lot and relevant intermediates is evaluated using one or more indicator tests. For the haemophilus component, such tests may include determination of molecular size, determination of free PRP in the conjugate and kinetics of depolymerisation. Taking account of the results of the stability testing, release

requirements are set for these indicator tests to ensure that the vaccine will be satisfactory at the end of the period of validity.

Reference vaccine(s). Provided valid assays can be performed, monocomponent reference vaccines may be used for the assays on the combined vaccine. If this is not possible because of interaction between the components of the combined vaccine or because of the difference in composition between monocomponent reference vaccine and the test vaccine, a batch of combined vaccine shown to be effective in clinical trials or a batch representative thereof is used as a reference vaccine. For the preparation of a representative batch, strict adherence to the production process used for the batch tested in clinical trials is necessary. The reference vaccine may be stabilised by a method that has been shown to have no effect on the assay procedure.

PRODUCTION OF THE COMPONENTS

The production of the components complies with the requirements of the monographs on *Diphtheria vaccine (adsorbed)* (0443), *Tetanus vaccine (adsorbed)* (0452), *Pertussis vaccine (acellular, component, adsorbed)* (1356), *Hepatitis B vaccine (rDNA)* (1056), *Poliomyelitis vaccine (inactivated)* (0214) and *Haemophilus type b conjugate vaccine* (1219).

FINAL BULKS

Vaccine with all components in the same container.

The final bulk is prepared by adsorption, separately or together, of suitable quantities of bulk purified diphtheria toxoid, bulk purified tetanus toxoid, bulk purified acellular pertussis components and bulk purified hepatitis B surface antigen onto a mineral carrier such as aluminium hydroxide or hydrated aluminium phosphate and admixture of a suitable quantity of PRP conjugate and suitable quantities of purified and inactivated, monovalent harvests of human polioviruses 1, 2 and 3 or a suitable quantity of a trivalent pool of such monovalent harvests. Suitable antimicrobial preservatives may be added.

Vaccine with the haemophilus component in a separate container. The final bulk of diphtheria, tetanus, pertussis, hepatitis B and poliovirus component is prepared by adsorption, separately or together, of suitable quantities of bulk purified diphtheria toxoid, bulk purified tetanus toxoid, bulk purified acellular pertussis components and bulk purified hepatitis B surface antigen onto a mineral carrier such as aluminium hydroxide or hydrated aluminium phosphate and admixture of suitable quantities of purified and inactivated, monovalent harvests of human polioviruses 1, 2 and 3 or a suitable pool of such monovalent harvests. This final bulk is filled separately. Suitable antimicrobial preservatives may be added. The final bulk of the haemophilus component is prepared by dilution of the bulk conjugate to the final concentration with a suitable diluent. A stabiliser may be added.

Only final bulks that comply with the following requirements may be used in the preparation of the final lot.

Bovine serum albumin. Determined on the poliomyelitis components by a suitable immunochemical method (2.7.1) after purification of the harvests and before preparation of the final bulk vaccine, before addition of the adsorbent, the amount of bovine serum albumin is such that the content in the final vaccine will be not more than 50 ng per single human dose.

Antimicrobial preservative. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The amount is not less than 85 per cent and not greater than 115 per cent of the intended content.

Sterility (2.6.1). Carry out the test for sterility using 10 ml for each medium.

FINAL LOT

Where the haemophilus component is in a separate container, the final bulk of the haemophilus component is freeze-dried. Only a final lot that is satisfactory with respect to the test for osmolality shown below and with respect to each of the requirements given below under Identification, Tests and Assay may be released for use.

Provided that the tests for osmolality, for absence of residual pertussis toxin and irreversibility of pertussis toxoid and for antimicrobial preservative and the diphtheria, tetanus and pertussis component assays have been carried out with satisfactory results on the final bulk vaccine, they may be omitted on the final lot.

Provided the free formaldehyde content has been determined on the bulk purified antigens and the purified monovalent harvests or the trivalent pool of polioviruses or the final bulk and it has been shown that the content in the final lot will not exceed 0.2 g/l, the test for free formaldehyde may be omitted on the final lot.

Provided that the test for bovine serum albumin has been carried out with satisfactory results on the trivalent pool of inactivated monovalent harvests of polioviruses or on the final bulk vaccine, it may be omitted on the final lot.

If an *in vivo* assay is used for the hepatitis B component, provided it has been carried out with satisfactory results on the final bulk vaccine, it may be omitted on the final lot.

Provided the *in vivo* assay for the poliomyelitis component has been carried out with satisfactory results on the final bulk vaccine, it may be omitted on the final lot.

Free PRP. For vaccines with all components in the same container, the free PRP content is determined on the non-absorbed fraction. Unbound PRP is determined on the haemophilus component after removal of the conjugate, for example by anion-exchange, size-exclusion or hydrophobic chromatography, ultrafiltration or other validated methods. The amount of free PRP is not greater than that approved for the particular product.

Bacterial endotoxins (2.6.14): less than the limit approved for the particular product.

Osmolality (2.2.35). The osmolality of the vaccine, reconstituted where applicable, is within the limits approved for the particular preparation.

IDENTIFICATION

If the vaccine is presented with the haemophilus component in a separate vial: identification tests A, B, C, D and E are carried out using the vial containing the diphtheria, tetanus, pertussis, hepatitis B and poliomyelitis components; identification test F is carried out on the vial containing the haemophilus components.

- A. Diphtheria toxoid is identified by a suitable immunochemical method (2.7.1). The following method is given as an example. Dissolve in the vaccine to be examined sufficient sodium citrate R to give a 100 g/l solution. Maintain at 37 °C for about 16 h and centrifuge until a clear supernatant liquid is obtained. The clear supernatant liquid reacts with a suitable diphtheria antitoxin, giving a precipitate.
- B. Tetanus toxoid is identified by a suitable immunochemical method (2.7.1). The following method is given as an example. The clear supernatant liquid obtained during identification test A reacts with a suitable tetanus antitoxin, giving a precipitate.

- C. The clear supernatant liquid obtained during identification test A reacts with a specific antisera to the pertussis components of the vaccine when examined by suitable immunochemical methods (2.7.1).
- D. The hepatitis B component is identified by a suitable immunochemical method (2.7.1), for example the *in vitro* assay or by a suitable electrophoretic method (2.2.31).
- E. The vaccine is shown to contain human polioviruses 1, 2 and 3 by a suitable immunochemical method (2.7.1), such as determination of D-antigen by enzyme-linked immunosorbent assay (ELISA).
- F. The PRP and the haemophilus carrier protein are identified by a suitable immunochemical method (2.7.1).

TESTS

If the product is presented with the haemophilus component in a separate container: the tests for absence of residual pertussis toxin and irreversibility of pertussis toxoid, free formaldehyde, aluminium, antimicrobial preservative and sterility are carried out on the container with the diphtheria, tetanus, pertussis, poliomyelitis and hepatitis B components; the tests for PRP content, water, antimicrobial preservative, aluminium (where applicable) and sterility are carried out on the container with the haemophilus component.

Some tests for the haemophilus component are carried out on the freeze-dried product rather than on the bulk conjugate where the freeze-drying process may affect the component to be tested.

Absence of residual pertussis toxin and irreversibility of pertussis toxoid. This test is not necessary for the product obtained by genetic modification. Use 3 groups each of not fewer than 5 histamine-sensitive mice. Inject intraperitoneally into the first group twice the single human dose of the vaccine stored at 2-8 °C. Inject intraperitoneally into the second group twice the single human dose of the vaccine incubated at 37 °C for 4 weeks. Inject diluent into the third group of mice. After 5 days, inject into each mouse 2 mg of histamine base intraperitoneally in a volume not exceeding 0.5 ml and observe for 24 h. The test is invalid if 1 or more control mice die following histamine challenge. The vaccine complies with the test if no animal in the first or second group dies following histamine challenge. If 1 mouse dies in either or both of the first and second groups, the test may be repeated with the same number of mice or with a greater number and the results of valid tests combined; the vaccine complies with the test if, in both of the groups given the vaccine, not more than 5 per cent of the total number of mice die following histamine challenge.

The histamine sensitivity of the strain of mice used is verified at suitable intervals as follows: inject intravenously threefold dilutions of a reference pertussis toxin preparation in phosphate-buffered saline solution containing 2 g/l of gelatin and challenge with histamine as above; the strain is suitable if more than 50 per cent of the animals are sensitised by 50 ng of pertussis toxin and none of the control animals injected with only diluent and challenged similarly with histamine show symptoms of sensitisation.

PRP: minimum 80 per cent of the amount of PRP stated on the label, for a vaccine with the haemophilus component in a separate container.

For a vaccine with all components in the same container: the PRP content determined on the non-absorbed fraction is not less than that approved for the product.

PRP is determined either by assay of ribose (2.5.31) or phosphorus (2.5.18), by an immunochemical method (2.7.1) or by anion-exchange liquid chromatography (2.2.29) with pulsed-amperometric detection.

Aluminium (2.5.13): maximum 1.25 mg of aluminium (Al) per single human dose, if aluminium hydroxide or hydrated aluminium phosphate is used as the adsorbent.

Free formaldehyde (2.4.18): maximum 0.2 g/l of free formaldehyde per single human dose.

Antimicrobial preservative. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The content is not less than the minimum amount shown to be effective and is not greater than 115 per cent of the quantity stated on the label.

Water (2.5.12): maximum 3.0 per cent for the freeze-dried haemophilus component.

Sterility (2.6.1). It complies with the test for sterility.

ASSAY

Diphtheria component. Carry out one of the prescribed methods for the assay of diphtheria vaccine (adsorbed) (2.7.6).

The lower confidence limit ($P = 0.95$) of the estimated potency is not less than the minimum potency stated on the label.

Unless otherwise justified and authorised, the minimum potency stated on the label is 30 IU per single human dose.

Tetanus component. Carry out one of the prescribed methods for the assay of tetanus vaccine (adsorbed) (2.7.8).

The lower confidence limit ($P = 0.95$) of the estimated potency is not less than 40 IU per single human dose.

Pertussis component. The vaccine complies with the assay of pertussis vaccine (acellular) (2.7.16).

Hepatitis B component. The vaccine complies with the assay of hepatitis B vaccine (2.7.15).

Poliomyelitis component

D-antigen content. As a measure of consistency of production, determine the D-antigen content for human polioviruses 1, 2 and 3 by a suitable immunochemical method (2.7.1) using a reference preparation calibrated in European Pharmacopoeia D-antigen units. For each type, the content, expressed with reference to the amount of D-antigen stated on the label, is within the limits approved for the particular product. *Poliomyelitis vaccine (inactivated) BRP* is calibrated in European Pharmacopoeia units and intended for use in the assay of D antigen. The European Pharmacopoeia unit and the International Unit are equivalent.

In vivo test. The vaccine complies with the *in vivo* assay of poliomyelitis vaccine (inactivated) (2.7.20).

LABELLING

The label states:

- the minimum number of International Units of diphtheria and tetanus toxoid per single human dose,
- the names and amounts of the pertussis components per single human dose,
- the amount of HBsAg per single human dose,
- the nominal amount of poliovirus of each type (1, 2 and 3), expressed in European Pharmacopoeia Units of D-antigen, per single human dose,
- the types of cells used for production of the poliomyelitis and the hepatitis B components,
- the number of micrograms of PRP per single human dose,

- the type and nominal amount of carrier protein per single human dose,
- where applicable, that the vaccine is intended for primary vaccination of children and is not necessarily suitable for reinforcing doses or for administration to adults,
- the name and the amount of the adsorbent,
- that the vaccine must be shaken before use,
- that the vaccine is not to be frozen,
- where applicable, that the vaccine contains a pertussis toxin-like protein produced by genetic modification.

01/2005:2065

DIPHTHERIA, TETANUS, PERTUSSIS (ACELLULAR, COMPONENT), POLIOMYELITIS (INACTIVATED) AND HAEMOPHILUS TYPE b CONJUGATE VACCINE (ADSORBED)

Vaccinum diphtheriae, tetani, pertussis
sine cellulis ex elementis praeparatum
poliomyelitis inactivatum et haemophili
stirpe b coniugatum adsorbatum

DEFINITION

Diphtheria, tetanus, pertussis (acellular, component), poliomyelitis (inactivated) and haemophilus type b conjugate vaccine (adsorbed) is a combined vaccine composed of: diphtheria formol toxoid; tetanus formol toxoid; individually purified antigenic components of *Bordetella pertussis*; suitable strains of human polioviruses 1, 2 and 3 grown in suitable cell cultures and inactivated by a suitable method; polyribosylribitol phosphate (PRP) covalently bound to a carrier protein; a mineral adsorbent such as aluminium hydroxide or hydrated aluminium phosphate. The product is presented with the haemophilus component in a separate container the contents of which are mixed with the other components immediately before use.

The formol toxoids are prepared from the toxins produced by the growth of *Corynebacterium diphtheriae* and *Clostridium tetani* respectively.

The vaccine contains either pertussis toxoid or a pertussis-toxin-like protein free from toxic properties produced by expression of a genetically modified form of the corresponding gene. Pertussis toxoid is prepared from pertussis toxin by a method that renders the toxin harmless while maintaining adequate immunogenic properties and avoiding reversion to toxin. The acellular pertussis component may also contain filamentous haemagglutinin, pertactin (a 69 kDa outer-membrane protein) and other defined components of *B. pertussis* such as fimbrial-2 and fimbrial-3 antigens. The latter 2 antigens may be co-purified. The antigenic composition and characteristics are based on evidence of protection and freedom from unexpected reactions in the target group for which the vaccine is intended.

PRP is a linear copolymer composed of repeated units of 3-β-D-ribofuranosyl-(1→1)-ribitol-5-phosphate [(C₁₀H₁₉O₁₂P)_n], with a defined molecular size and derived from a suitable strain of *Haemophilus influenzae* type b. The carrier protein, when conjugated to PRP, is capable of inducing a T-cell-dependent B-cell immune response to the polysaccharide.

PRODUCTION

GENERAL PROVISIONS

The production method shall have been shown to yield consistently vaccines comparable with the vaccine of proven clinical efficacy and safety in man.

The content of bacterial endotoxins (2.6.14) in bulk purified diphtheria toxoid, tetanus toxoid, pertussis components, purified, inactivated monovalent poliovirus harvests and bulk PRP conjugate is determined to monitor the purification procedure and to limit the amount in the final vaccine. For each component, the content of bacterial endotoxins is less than the limit approved for the particular vaccine and, in any case, the contents are such that the final vaccine contains less than 100 IU per single human dose.

The production method is validated to demonstrate that the product, if tested, would comply with the test for abnormal toxicity for immunosera and vaccines for human use (2.6.9), and with the following test for specific toxicity of the diphtheria and tetanus components: inject subcutaneously 5 times the single human dose stated on the label into each of 5 healthy guinea-pigs, each weighing 250-350 g, that have not previously been treated with any material that will interfere with the test. If within 42 days of the injection any of the animals shows signs of or dies from diphtheria, toxæmia or tetanus, the vaccine does not comply with the test. If more than 1 animal dies from non-specific causes, repeat the test once; if more than 1 animal dies in the second test, the vaccine does not comply with the test.

During development studies and wherever revalidation is necessary, it shall be demonstrated by tests in animals that the vaccine induces a T-cell dependent B-cell immune response to PRP.

As part of consistency studies the assays of the diphtheria, tetanus, pertussis and poliomyelitis components are carried out on a suitable number of batches of vaccine reconstituted as for use. For subsequent routine control, the assays of these components may be carried out without mixing with the haemophilus component.

Reference vaccine(s). Provided valid assays can be performed, monocomponent reference vaccines may be used for the assays on the combined vaccine. If this is not possible because of interaction between the components of the combined vaccine or because of the difference in composition between monocomponent reference vaccine and the test vaccine, a batch of combined vaccine shown to be effective in clinical trials or a batch representative thereof is used as a reference vaccine. For the preparation of a representative batch, strict adherence to the production process used for the batch tested in clinical trials is necessary. The reference vaccine may be stabilised by a method that has been shown to have no effect on the assay procedure.

PRODUCTION OF THE COMPONENTS

The production of the components complies with the requirements of the monographs on *Diphtheria vaccine (adsorbed)* (0443), *Tetanus vaccine (adsorbed)* (0452), *Pertussis vaccine (acellular, component, adsorbed)* (1356), *Poliomyelitis vaccine (inactivated)* (0214) and *Haemophilus type b conjugate vaccine* (1219).

FINAL BULK

The final bulk of the diphtheria, tetanus, pertussis and poliomyelitis components is prepared by adsorption, separately or together, of suitable quantities of bulk purified diphtheria toxoid, bulk purified tetanus toxoid and bulk purified acellular pertussis components onto a mineral carrier such as aluminium hydroxide or hydrated aluminium phosphate and admixture of suitable quantities of purified,

monovalent harvests of human polioviruses 1, 2 and 3 or a suitable quantity of a trivalent pool of such monovalent harvests. Suitable antimicrobial preservatives may be added. The final bulk of the haemophilus component is prepared by dilution of the bulk conjugate to the final concentration with a suitable diluent. A stabiliser may be added.

Only final bulks that comply with the following requirements may be used in the preparation of the final lot.

Bovine serum albumin. Determined on the poliomyelitis components by a suitable immunochemical method (2.7.1) during preparation of the final bulk vaccine, before addition of the adsorbent, the amount of bovine serum albumin is such that the content in the final vaccine will be not more than 50 ng per single human dose.

Antimicrobial preservative. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The amount is not less than 85 per cent and not greater than 115 per cent of the intended content.

Sterility (2.6.1). Carry out the test for sterility using 10 ml for each medium.

FINAL LOT

The final bulk of the haemophilus component is freeze-dried.

Only a final lot that is satisfactory with respect to the test for osmolality shown below and with respect to each of the requirements given below under Identification, Tests and Assay may be released for use.

Provided that the test for absence of residual pertussis toxin and irreversibility of pertussis toxoid, the test for antimicrobial preservative and the assay have been carried out with satisfactory results on the final bulk vaccine, they may be omitted on the final lot.

Provided that the free formaldehyde content has been determined on the bulk purified antigens and the purified monovalent harvests or the trivalent pool of polioviruses or the final bulk and it has been shown that the content in the final lot will not exceed 0.2 g/l, the test for free formaldehyde may be omitted on the final lot.

Provided that the *in vivo* assay for the poliomyelitis component has been carried out with satisfactory results on the final bulk vaccine, it may be omitted on the final lot.

Osmolality (2.2.35). The osmolality of the vaccine, reconstituted where applicable, is within the limits approved for the particular preparation.

Free PRP. Unbound PRP is determined on the haemophilus component after removal of the conjugate, for example by anion-exchange, size-exclusion or hydrophobic chromatography, ultrafiltration or other validated methods. The amount of free PRP is not greater than that approved for the particular product.

IDENTIFICATION

Identification tests A, B, C and D are carried out using the vial containing the diphtheria, tetanus, pertussis and poliomyelitis components; identification test E is carried out on the vial containing the haemophilus component.

- A. Diphtheria toxoid is identified by a suitable immunochemical method (2.7.1). The following method, applicable to certain vaccines, is given as an example. Dissolve in the vaccine to be examined sufficient *sodium citrate R* to give a 100 g/l solution. Maintain at 37 °C for about 16 h and centrifuge until a clear supernatant liquid is obtained. The clear supernatant liquid reacts with a suitable diphtheria antitoxin, giving a precipitate.
- B. Tetanus toxoid is identified by a suitable immunochemical method (2.7.1). The following method, applicable to certain vaccines, is given as an example. The clear

supernatant liquid obtained during identification test A reacts with a suitable tetanus antitoxin, giving a precipitate.

- C. The pertussis components are identified by suitable immunochemical methods (2.7.1). The following method, applicable to certain vaccines, is given as an example. The clear supernatant liquid obtained during identification test A reacts with specific antisera to the pertussis components of the vaccine.
- D. The vaccine is shown to contain human polioviruses 1, 2 and 3 by a suitable immunochemical method (2.7.1), such as determination of D-antigen by enzyme-linked immunosorbent assay (ELISA).
- E. The haemophilus component is identified by a suitable immunochemical method (2.7.1) for PRP.

TESTS

The tests for absence of residual pertussis toxin, irreversibility of pertussis toxoid, aluminium, free formaldehyde, antimicrobial preservative and sterility are carried out on the container with the diphtheria, tetanus, pertussis and poliomyelitis components; the tests for PRP content, water, sterility and pyrogens are carried out on the container with the haemophilus component.

Some tests for the haemophilus component may be carried out on the freeze-dried product rather than on the bulk conjugate where the freeze-drying process may affect the component to be tested.

Absence of residual pertussis toxin and irreversibility of pertussis toxoid. *This test is not necessary for the product obtained by genetic modification.* Use 3 groups each of not fewer than 5 histamine-sensitive mice. Inject intraperitoneally into the first group twice the single human dose of the vaccine stored at 2–8 °C. Inject intraperitoneally into the second group twice the single human dose of the vaccine incubated at 37 °C for 4 weeks. Inject diluent into the third group of mice. After 5 days, inject into each mouse 2 mg of histamine base intraperitoneally in a volume not exceeding 0.5 ml and observe for 24 h. The test is invalid if 1 or more control mice die following histamine challenge. The vaccine complies with the test if no animal in the first or second group dies following histamine challenge. If 1 mouse dies in either or both of the first and second groups, the test may be repeated with the same number of mice or with a greater number and the results of valid tests combined; the vaccine complies with the test if, in both of the groups given the vaccine, not more than 5 per cent of the total number of mice die following histamine challenge.

The histamine sensitivity of the strain of mice used is verified at suitable intervals as follows: inject intravenously threefold dilutions of a reference pertussis toxin preparation in phosphate-buffered saline solution containing 2 g/l of gelatin and challenge with histamine as above; the strain is suitable if more than 50 per cent of the animals are sensitised by 50 ng of pertussis toxin and none of the control animals injected with only diluent and challenged similarly with histamine show symptoms of sensitisation.

PRP: minimum 80 per cent of the amount of PRP stated on the label. PRP is determined either by assay of ribose (2.5.31) or phosphorus (2.5.18), by an immunochemical method (2.7.1) or by anion-exchange liquid chromatography (2.2.29) with pulsed-ampereometric detection.

Aluminium (2.5.13): maximum 1.25 mg per single human dose, if aluminium hydroxide or hydrated aluminium phosphate is used as the adsorbent.

Free formaldehyde (2.4.18): maximum 0.2 g/l.

Antimicrobial preservative. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The content is not less than the minimum amount shown to be effective and is not greater than 115 per cent of the quantity stated on the label.

Water (2.5.12): maximum 3.0 per cent for the haemophilus component.

Sterility (2.6.1). It complies with the test for sterility.

Pyrogens (2.6.8). It complies with the test for pyrogens. Inject per kilogram of the rabbit's mass a quantity of the vaccine equivalent to: 1 µg of PRP for a vaccine with diphtheria toxoid or CRM 197 diphtheria protein as carrier; 0.1 µg of PRP for a vaccine with tetanus toxoid as carrier; 0.025 µg of PRP for a vaccine with OMP as a carrier.

ASSAY

Diphtheria component. Carry out one of the prescribed methods for the assay of diphtheria vaccine (adsorbed) (2.7.6).

Unless otherwise justified and authorised, the lower confidence limit ($P = 0.95$) of the estimated potency is not less than 30 IU per single human dose.

Tetanus component. Carry out one of the prescribed methods for the assay of tetanus vaccine (adsorbed) (2.7.8). The lower confidence limit ($P = 0.95$) of the estimated potency is not less than 40 IU per single human dose.

Pertussis component. It complies with the assay of pertussis vaccine (acellular) (2.7.16).

Poliomyelitis component

D-antigen content. As a measure of consistency of production, determine the D-antigen content for human polioviruses 1, 2 and 3 by a suitable immunochemical method (2.7.1) using a reference preparation calibrated in Ph. Eur. Units of D-antigen. For each type, the content, expressed with reference to the amount of D-antigen stated on the label, is within the limits approved for the particular product. *Poliomyelitis vaccine (inactivated) BRP* is calibrated in Ph. Eur. Units and intended for use in the assay of D-antigen. The Ph. Eur. Unit and the IU are equivalent.

In vivo test. The vaccine complies with the *in vivo* assay of poliomyelitis vaccine (inactivated) (2.7.20).

LABELLING

The label states:

- the minimum number of International Units of diphtheria and tetanus toxoid per single human dose,
- the names and amounts of the pertussis components per single human dose,
- the nominal amount of poliovirus of each type (1, 2 and 3), expressed in European Pharmacopoeia Units of D-antigen, per single human dose,
- the type of cells used for production of the poliomyelitis component,
- the number of micrograms of PRP per single human dose,
- the type and nominal amount of carrier protein per single human dose,
- where applicable, that the vaccine is intended for primary vaccination of children and is not necessarily suitable for reinforcing doses or for administration to adults,
- the name and the amount of the adsorbent,
- that the vaccine must be shaken before use,
- that the vaccine is not to be frozen,
- where applicable, that the vaccine contains a pertussis toxin-like protein produced by genetic modification.

01/2005:2061

DIPHtheria, TETANUS, PERTUSSIS AND POLIOMYELITIS (INACTIVATED) VACCINE (ADSORBED)

Vaccinum diphtheriae, tetani, pertussis et poliomyelitis inactivatum adsorbatum

DEFINITION

Diphtheria, tetanus, pertussis and poliomyelitis (inactivated) vaccine (adsorbed) is a combined vaccine containing: diphtheria formol toxoid; tetanus formol toxoid; an inactivated suspension of *Bordetella pertussis*; suitable strains of human polioviruses 1, 2 and 3 grown in suitable cell cultures and inactivated by a validated method; a mineral adsorbent such as aluminium hydroxide or hydrated aluminium phosphate.

The formol toxoids are prepared from the toxins produced by the growth of *Corynebacterium diphtheriae* and *Clostridium tetani* respectively.

PRODUCTION

GENERAL PROVISIONS

The production method shall have been shown to yield consistently vaccines comparable with the vaccine of proven clinical efficacy and safety in man.

Reference vaccine(s). Provided valid assays can be performed, monocomponent reference vaccines may be used for the assays on the combined vaccine. If this is not possible because of interaction between the components of the combined vaccine or because of the difference in composition between monocomponent reference vaccine and the test vaccine, a batch of combined vaccine shown to be effective in clinical trials or a batch representative thereof is used as a reference vaccine. For the preparation of a representative batch, strict adherence to the production process used for the batch tested in clinical trials is necessary. The reference vaccine may be stabilised by a method that has been shown to have no effect on the assay procedure.

Specific toxicity of the diphtheria and tetanus components.

The production method is validated to demonstrate that the product, if tested, would comply with the following test: inject subcutaneously 5 times the single human dose stated on the label into each of 5 healthy guinea-pigs, each weighing 250-350 g, that have not previously been treated with any material that will interfere with the test. If within 42 days of the injection any of the animals shows signs of or dies from diphtheria toxæmia or tetanus, the vaccine does not comply with the test. If more than 1 animal dies from non-specific causes, repeat the test once; if more than 1 animal dies in the second test, the vaccine does not comply with the test.

PRODUCTION OF THE COMPONENTS

The production of the components complies with the requirements of the monographs on *Diphtheria vaccine (adsorbed)* (0443), *Tetanus vaccine (adsorbed)* (0452), *Pertussis vaccine (adsorbed)* (0161) and *Poliomyelitis vaccine (inactivated)* (0214).

FINAL BULK VACCINE

The final bulk vaccine is prepared by adsorption onto a mineral carrier such as aluminium hydroxide or hydrated aluminium phosphate, separately or together, of suitable quantities of bulk purified diphtheria toxoid and bulk purified tetanus toxoid and admixture of suitable quantities of an inactivated suspension of *B. pertussis* and purified monovalent harvests of human polioviruses 1, 2 and 3

or a suitable quantity of a trivalent pool of such purified monovalent harvests. Suitable antimicrobial preservatives may be added.

Only a final bulk vaccine that complies with the following requirements may be used in the preparation of the final lot.

Bovine serum albumin. Determined on the poliomyelitis components by a suitable immunochemical method (2.7.1) during preparation of the final bulk vaccine, before addition of the adsorbent, the amount of bovine serum albumin is such that the content in the final vaccine will be not more than 50 ng per single human dose.

Antimicrobial preservative. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The amount is not less than 85 per cent and not greater than 115 per cent of the intended content.

Sterility (2.6.1). Carry out the test for sterility using 10 ml for each medium.

FINAL LOT

Only a final lot that is satisfactory with respect to the test for osmolality and with respect to each of the requirements given below under Identification, Tests and Assay may be released for use.

Provided that the tests for specific toxicity of the pertussis component and antimicrobial preservative, and the assays for the diphtheria, tetanus and pertussis components have been carried out with satisfactory results on the final bulk vaccine, they may be omitted on the final lot.

Provided that the free formaldehyde content has been determined on the bulk purified antigens, the inactivated *B. pertussis* suspension and the purified monovalent harvests or the trivalent pool of polioviruses or on the final bulk and it has been shown that the content in the final lot will not exceed 0.2 g/l, the test for free formaldehyde may be omitted on the final lot.

Provided that the *in vivo* assay for the poliomyelitis component has been carried out with satisfactory results on the final bulk vaccine, it may be omitted on the final lot.

Osmolality (2.2.35). The osmolality of the vaccine is within the limits approved for the particular preparation.

IDENTIFICATION

- Diphtheria toxoid is identified by a suitable immunochemical method (2.7.1). The following method, applicable to certain vaccines, is given as an example. Dissolve in the vaccine to be examined sufficient *sodium citrate R* to give a 100 g/l solution. Maintain at 37 °C for about 16 h and centrifuge until a clear supernatant liquid is obtained. The clear supernatant liquid reacts with a suitable diphtheria antitoxin, giving a precipitate.
- Tetanus toxoid is identified by a suitable immunochemical method (2.7.1). The following method, applicable to certain vaccines, is given as an example. The clear supernatant liquid obtained during identification test A reacts with a suitable tetanus antitoxin, giving a precipitate.
- The centrifugation residue obtained in identification A may be used. Other suitable methods for separating the bacteria from the adsorbent may also be used. Identify pertussis vaccine by agglutination of the bacteria from the resuspended precipitate by antisera specific to *B. pertussis* or by the assay of the pertussis component prescribed under Assay.
- The vaccine is shown to contain human polioviruses 1, 2 and 3 by a suitable immunochemical method (2.7.1) such as the determination of D-antigen by enzyme-linked immunosorbent assay (ELISA).

TESTS

Specific toxicity of the pertussis component. Use not fewer than 5 healthy mice each weighing 14–16 g for the vaccine group and for the saline control. Use mice of the same sex or distribute males and females equally between the groups. Allow the animals access to food and water for at least 2 h before injection and during the test. Inject each mouse of the vaccine group intraperitoneally with 0.5 ml, containing a quantity of the vaccine equivalent to not less than half the single human dose. Inject each mouse of the control group with 0.5 ml of a 9 g/l sterile solution of *sodium chloride R*, preferably containing the same amount of antimicrobial preservative as that injected with the vaccine. Weigh the groups of mice immediately before the injection and 72 h and 7 days after the injection. The vaccine complies with the test if: (a) at the end of 72 h the total mass of the group of vaccinated mice is not less than that preceding the injection; (b) at the end of 7 days the average increase in mass per vaccinated mouse is not less than 60 per cent of that per control mouse; and (c) not more than 5 per cent of the vaccinated mice die during the test. The test may be repeated and the results of the tests combined.

Aluminium (2.5.13): maximum 1.25 mg per single human dose, if aluminium hydroxide or hydrated aluminium phosphate is used as the adsorbent.

Free formaldehyde (2.4.18): maximum 0.2 g/l.

Antimicrobial preservative. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The content is not less than the minimum amount shown to be effective and is not greater than 115 per cent of the quantity stated on the label.

Sterility (2.6.1). It complies with the test for sterility.

ASSAY

Diphtheria component. Carry out one of the prescribed methods for the assay of diphtheria vaccine (adsorbed) (2.7.6).

The lower confidence limit ($P = 0.95$) of the estimated potency is not less than 30 IU per single human dose.

Tetanus component. Carry out one of the prescribed methods for the assay of tetanus vaccine (adsorbed) (2.7.8).

If the test is carried out in guinea pigs, the lower confidence limit ($P = 0.95$) of the estimated potency is not less than 40 IU per single human dose; if the test is carried out in mice, the lower confidence limit ($P = 0.95$) of the estimated potency is not less than 60 IU per single human dose.

Pertussis component. Carry out the assay of pertussis vaccine (2.7.7).

The estimated potency is not less than 4 IU per single human dose and the lower confidence limit ($P = 0.95$) of the estimated potency is not less than 2 IU per single human dose.

Poliomyelitis component

D-antigen content. As a measure of consistency of production, determine the D-antigen content for human polioviruses 1, 2 and 3 by a suitable immunochemical method (2.7.1) using a reference preparation calibrated in European Pharmacopoeia Units of D-antigen. For each type, the content, expressed with reference to the amount of D-antigen stated on the label, is within the limits approved for the particular product. *Poliomyelitis vaccine (inactivated) BRP* is calibrated in European Pharmacopoeia Units and intended for use in the assay of D-antigen. The European Pharmacopoeia Unit and the International Unit are equivalent.

In vivo test. The vaccine complies with the *in vivo* assay of poliomyelitis vaccine (inactivated) (2.7.20).

LABELLING

The label states:

- the minimum number of International Units of diphtheria and tetanus toxoid per single human dose,
- the minimum number of International Units of pertussis vaccine per single human dose,
- the nominal amount of poliovirus of each type (1, 2 and 3), expressed in European Pharmacopoeia Units of D-antigen, per single human dose,
- the type of cells used for production of the poliomyelitis component,
- where applicable, that the vaccine is intended for primary vaccination of children and is not necessarily suitable for reinforcing doses or for administration to adults,
- the name and the amount of the adsorbent,
- that the vaccine must be shaken before use,
- that the vaccine is not to be frozen.

01/2005:2066

DIPHTHERIA, TETANUS, PERTUSSIS, POLIOMYELITIS (INACTIVATED) AND HAEMOPHILUS TYPE b CONJUGATE VACCINE (ADSORBED)

Vaccinum diphtheriae, tetani, pertussis, poliomyelitis inactivatum et haemophili stirpe b coniugatum adsorbatum

DEFINITION

Diphtheria, tetanus, pertussis, poliomyelitis (inactivated) and haemophilus type b conjugate vaccine (adsorbed) is a combined vaccine composed of: diphtheria formol toxoid; tetanus formol toxoid; an inactivated suspension of *Bordetella pertussis*; suitable strains of human polioviruses 1, 2 and 3 grown in suitable cell cultures and inactivated by a suitable method; polyribosylribitol phosphate (PRP) covalently bound to a carrier protein; a mineral adsorbent such as aluminium hydroxide or hydrated aluminium phosphate. The product is presented with the haemophilus component in a separate container, the contents of which are mixed with the other components immediately before use.

The formol toxoids are prepared from the toxins produced by the growth of *Corynebacterium diphtheriae* and *Clostridium tetani* respectively.

PRP is a linear copolymer composed of repeated units of 3-β-D-ribofuranosyl-(1→1)-ribitol-5-phosphate [(C₁₀H₁₉O₁₂P)_n], with a defined molecular size and derived from a suitable strain of *Haemophilus influenzae* type b. The carrier protein, when conjugated to PRP, is capable of inducing a T-cell-dependent B-cell immune response to the polysaccharide.

PRODUCTION

GENERAL PROVISIONS

The production method shall have been shown to yield consistently vaccines comparable with the vaccine of proven clinical efficacy and safety in man.

During development studies and wherever revalidation is necessary, it shall be demonstrated by tests in animals that the vaccine induces a T-cell dependent B-cell immune response to PRP.

As part of consistency studies the assays of the diphtheria, tetanus, pertussis and poliomyelitis components are carried out on a suitable number of batches of vaccine reconstituted as for use. For subsequent routine control, the assays of these components may be carried out without mixing with the haemophilus component.

Reference vaccine(s). Provided valid assays can be performed, monocomponent reference vaccines may be used for the assays on the combined vaccine. If this is not possible because of interaction between the components of the combined vaccine or because of the difference in composition between monocomponent reference vaccine and the test vaccine, a batch of combined vaccine shown to be effective in clinical trials or a batch representative thereof is used as a reference vaccine. For the preparation of a representative batch, strict adherence to the production process used for the batch tested in clinical trials is necessary. The reference vaccine may be stabilised by a method that has been shown to have no effect on the assay procedure.

Specific toxicity of the diphtheria and tetanus components. The production method is validated to demonstrate that the product, if tested, would comply with the following test: inject subcutaneously 5 times the single human dose stated on the label into each of 5 healthy guinea-pigs, each weighing 250-350 g, that have not previously been treated with any material that will interfere with the test. If within 42 days of the injection any of the animals shows signs of or dies from diphtheria toxæmia or tetanus, the vaccine does not comply with the test. If more than 1 animal dies from non-specific causes, repeat the test once; if more than 1 animal dies in the second test, the vaccine does not comply with the test.

PRODUCTION OF THE COMPONENTS

The production of the components complies with the requirements of the monographs on *Diphtheria vaccine (adsorbed)* (0443), *Tetanus vaccine (adsorbed)* (0452), *Pertussis vaccine (adsorbed)* (0161), *Poliomyelitis vaccine (inactivated)* (0214) and *Haemophilus type b conjugate vaccine* (1219).

FINAL BULKS

The final bulk of the diphtheria, tetanus, pertussis and poliomyelitis components is prepared by adsorption, separately or together, of suitable quantities of bulk purified diphtheria toxoid, and bulk purified tetanus toxoid onto a mineral carrier such as aluminium hydroxide or hydrated aluminium phosphate and admixture of suitable quantities of an inactivated suspension of *B. pertussis* and of purified, monovalent harvests of human polioviruses 1, 2 and 3 or a suitable quantity of a trivalent pool of such monovalent harvests. Suitable antimicrobial preservatives may be added.

The final bulk of the haemophilus component is prepared by dilution of the bulk conjugate to the final concentration with a suitable diluent. A stabiliser may be added.

Only final bulks that comply with the following requirements may be used in the preparation of the final lot.

Bovine serum albumin. Determined on the poliomyelitis components by a suitable immunochemical method (2.7.1) during preparation of the final bulk vaccine, before addition of the adsorbent, the amount of bovine serum albumin is such that the content in the final vaccine will be not more than 50 ng per single human dose.

Antimicrobial preservative. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The amount is not less than 85 per cent and not greater than 115 per cent of the intended content.

Sterility (2.6.1). Carry out the test for sterility using 10 ml for each medium.

FINAL LOT

The final bulk of the haemophilus component is freeze-dried.

Only a final lot that is satisfactory with respect to the test for osmolality shown below and with respect to each of the requirements given below under Identification, Tests and Assay may be released for use.

Provided that the tests for specific toxicity of the pertussis component and antimicrobial preservative, and the assays for the diphtheria, tetanus and pertussis components have been carried out with satisfactory results on the final bulk vaccine, they may be omitted on the final lot.

Provided that the free formaldehyde content has been determined on the bulk purified antigens, the inactivated *B. pertussis* suspension and the purified monovalent harvests or the trivalent pool of polioviruses or on the final bulk and it has been shown that the content in the final lot will not exceed 0.2 g/l, the test for free formaldehyde may be omitted on the final lot.

Provided that the *in vivo* assay for the poliomyelitis component has been carried out with satisfactory results on the final bulk vaccine, it may be omitted on the final lot.

Osmolality (2.2.35). The osmolality of the vaccine, reconstituted where applicable, is within the limits approved for the particular preparation.

Free PRP. Unbound PRP is determined on the haemophilus component after removal of the conjugate, for example by anion-exchange, size-exclusion or hydrophobic chromatography, ultrafiltration or other validated methods. The amount of free PRP is not greater than that approved for the particular product.

IDENTIFICATION

Identification tests A, B, C and D are carried out using the vial containing the diphtheria, tetanus, pertussis and poliomyelitis components; identification test E is carried out on the vial containing the haemophilus component.

- Diphtheria toxoid is identified by a suitable immunochemical method (2.7.1). The following method, applicable to certain vaccines, is given as an example. Dissolve in the vaccine to be examined sufficient *sodium citrate R* to give a 100 g/l solution. Maintain at 37 °C for about 16 h and centrifuge until a clear supernatant liquid is obtained. The clear supernatant liquid reacts with a suitable diphtheria antitoxin, giving a precipitate.
- Tetanus toxoid is identified by a suitable immunochemical method (2.7.1). The following method, applicable to certain vaccines, is given as an example. The clear supernatant liquid obtained during identification test A reacts with a suitable tetanus antitoxin, giving a precipitate.
- The centrifugation residue obtained in identification A may be used. Other suitable methods for separating the bacteria from the adsorbent may also be used. Identify pertussis vaccine by agglutination of the bacteria from the resuspended precipitate by antisera specific to *B. pertussis* or by the assay of the pertussis component prescribed under Assay.

D. The vaccine is shown to contain human polioviruses 1, 2 and 3 by a suitable immunochemical method (2.7.1), such as determination of D-antigen by enzyme-linked immunosorbent assay (ELISA).

E. The haemophilus component is identified by a suitable immunochemical method (2.7.1) for PRP.

TESTS

The tests for specific toxicity of the pertussis component, aluminium, free formaldehyde, antimicrobial preservative and sterility are carried out on the container with diphtheria, tetanus, pertussis and poliomyelitis components; the tests for PRP, water, sterility and pyrogens are carried out on the container with the haemophilus component.

Some tests for the haemophilus component may be carried out on the freeze-dried product rather than on the bulk conjugate where the freeze-drying process may affect the component to be tested.

Specific toxicity of the pertussis component. Use not fewer than 5 healthy mice each weighing 14–16 g, for the vaccine group and for the saline control. Use mice of the same sex or distribute males and females equally between the groups. Allow the animals access to food and water for at least 2 h before injection and during the test. Inject each mouse of the vaccine group intraperitoneally with 0.5 ml, containing a quantity of the vaccine equivalent to not less than half the single human dose. Inject each mouse of the control group with 0.5 ml of a 9 g/l sterile solution of *sodium chloride R*, preferably containing the same amount of antimicrobial preservative as that injected with the vaccine. Weigh the groups of mice immediately before the injection and 72 h and 7 days after the injection. The vaccine complies with the test if: (a) at the end of 72 h the total mass of the group of vaccinated mice is not less than that preceding the injection; (b) at the end of 7 days the average increase in mass per vaccinated mouse is not less than 60 per cent of that per control mouse; and (c) not more than 5 per cent of the vaccinated mice die during the test. The test may be repeated and the results of the tests combined.

PRP: minimum 80 per cent of the amount of PRP stated on the label. PRP is determined either by assay of ribose (2.5.31) or phosphorus (2.5.18), by an immunochemical method (2.7.1) or by anion-exchange liquid chromatography (2.2.29) with pulsed-amprometric detection.

Aluminium (2.5.13): maximum 1.25 mg per single human dose, if aluminium hydroxide or hydrated aluminium phosphate is used as the adsorbent.

Free formaldehyde (2.4.18): maximum 0.2 g/l.

Antimicrobial preservative. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The content is not less than the minimum amount shown to be effective and is not greater than 115 per cent of the quantity stated on the label.

Water (2.5.12): maximum 3.0 per cent for the haemophilus component.

Sterility (2.6.1). It complies with the test for sterility.

Pyrogens (2.6.8). It complies with the test for pyrogens. Inject per kilogram of the rabbit's mass a quantity of the vaccine equivalent to: 1 µg of PRP for a vaccine with

diphtheria toxoid or CRM 197 diphtheria protein as carrier; 0.1 µg of PRP for a vaccine with tetanus toxoid as carrier; 0.025 µg of PRP for a vaccine with OMP as carrier.

ASSAY

Diphtheria component. Carry out one of the prescribed methods for the assay of diphtheria vaccine (adsorbed) (2.7.6).

The lower confidence limit ($P = 0.95$) of the estimated potency is not less than 30 IU per single human dose.

Tetanus component. Carry out one of the prescribed methods for the assay of tetanus vaccine (adsorbed) (2.7.8).

If the test is carried out in guinea-pigs, the lower confidence limit ($P = 0.95$) of the estimated potency is not less than 40 IU per single human dose; if the test is carried out in mice, the lower confidence limit ($P = 0.95$) of the estimated potency is not less than 60 IU per single human dose.

Pertussis component. Carry out the assay of pertussis vaccine (2.7.7).

The estimated potency is not less than 4 IU per single human dose and the lower confidence limit ($P = 0.95$) of the estimated potency is not less than 2 IU per single human dose.

Poliomyelitis component

D-antigen content. As a measure of consistency of production, determine the D-antigen content for human polioviruses 1, 2 and 3 by a suitable immunochemical method (2.7.1) using a reference preparation calibrated in European Pharmacopoeia Units of D-antigen. For each type, the content, expressed with reference to the amount of D-antigen stated on the label, is within the limits approved for the particular product. *Poliomyelitis vaccine (inactivated) BRP* is calibrated in European Pharmacopoeia Units and intended for use in the assay of D-antigen. The European Pharmacopoeia Unit and the International Unit are equivalent.

In vivo test. The vaccine complies with the *in vivo* assay of poliomyelitis vaccine (inactivated) (2.7.20).

LABELLING

The label states:

- the minimum number of International Units of diphtheria and tetanus toxoid per single human dose,
- the minimum number of International Units of pertussis vaccine per single human dose,
- the nominal amount of poliovirus of each type (1, 2 and 3), expressed in European Pharmacopoeia Units of D-antigen, per single human dose,
- the type of cells used for production of the poliomyelitis component,
- the number of micrograms of PRP per single human dose,
- the type and nominal amount of carrier protein per single human dose,
- where applicable, that the vaccine is intended for primary vaccination of children and is not necessarily suitable for reinforcing doses or for administration to adults,
- the name and the amount of the adsorbent,
- that the vaccine must be shaken before use,
- that the vaccine is not to be frozen.

01/2005:0443

DIPHTHERIA VACCINE (ADSORBED)**Vaccinum diphtheriae adsorbatum****DEFINITION**

Diphtheria vaccine (adsorbed) is a preparation of diphtheria formol toxoid with a mineral adsorbent. The formol toxoid is prepared from the toxin produced by the growth of *Corynebacterium diphtheriae*.

PRODUCTION**GENERAL PROVISIONS**

Specific toxicity. The production method is validated to demonstrate that the product, if tested, would comply with the following test: inject subcutaneously 5 times the single human dose stated on the label into each of 5 healthy guinea-pigs, each weighing 250-350 g, that have not previously been treated with any material that will interfere with the test. If within 42 days of the injection any of the animals shows signs of or dies from diphtheria toxæmia, the vaccine does not comply with the test. If more than 1 animal dies from non-specific causes, repeat the test once; if more than 1 animal dies in the second test, the vaccine does not comply with the test.

BULK PURIFIED TOXOID

For the production of diphtheria toxin, from which toxoid is prepared, seed cultures are managed in a defined seed-lot system in which toxinogenicity is conserved and, where necessary, restored by deliberate reselection. A highly toxinogenic strain of *Corynebacterium diphtheriae* with known origin and history is grown in a suitable liquid medium. At the end of cultivation, the purity of each culture is tested and contaminated cultures are discarded. Toxin-containing culture medium is separated aseptically from the bacterial mass as soon as possible. The toxin content (Lf per millilitre) is checked to monitor consistency of production. Single harvests may be pooled to prepare the bulk purified toxoid. The toxin is purified to remove components likely to cause adverse reactions in humans. The purified toxin is detoxified with formaldehyde by a method that avoids destruction of the immunogenic potency of the toxoid and reversion of the toxoid to toxin, particularly on exposure to heat. Alternatively, purification may be carried out after detoxification.

Only bulk purified toxoid that complies with the following requirements may be used in the preparation of the final bulk vaccine.

Sterility (2.6.1). Carry out the test for sterility using 10 ml for each medium.

Absence of toxin and irreversibility of toxoid. Using the same buffer solution as for the final vaccine, without adsorbent, prepare a solution of bulk purified toxoid at 100 Lf/ml. Divide the solution into 2 equal parts. Maintain 1 part at 5 ± 3 °C and the other at 37 °C for 6 weeks. Carry out a test in Vero cells for active diphtheria toxin using 50 µl/well of both samples. The sample should not contain antimicrobial preservatives and detoxifying agents should be determined to be below the concentration toxic to Vero cells. Non-specific toxicity may be eliminated by dialysis.

Use freshly trypsinised Vero cells at a suitable concentration, for example 2.5×10^5 ml⁻¹ and a reference diphtheria toxin diluted in 100 Lf/ml diphtheria toxoid. A suitable reference diphtheria toxin will contain either not less than 100 LD₅₀/ml or 67 to 133 lr/100 in 1 Lf and 25 000 to

50 000 minimal reacting doses for guinea-pig skin in 1 Lf (*diphtheria toxin BRP* is suitable for use as the reference toxin). Dilute the toxin in 100 Lf/ml diphtheria toxoid to a suitable concentration, for example 2×10^{-4} Lf/ml. Prepare serial twofold dilutions of the diluted diphtheria toxin and use undiluted test samples (50 µl/well). Distribute them in the wells of a sterile tissue culture plate containing a medium suitable for Vero cells. To ascertain that any cytotoxic effect noted is specific to diphtheria toxin, prepare in parallel dilutions where the toxin is neutralised by a suitable concentration of diphtheria antitoxin, for example 100 IU/ml. Include control wells without toxoid or toxin and with non-toxic toxoid at 100 Lf/ml on each plate to verify normal cell growth. Add cell suspension to each well, seal the plates and incubate at 37 °C for 5-6 days. Cytotoxic effect is judged to be present where there is complete metabolic inhibition of the Vero cells, indicated by the pH indicator of the medium. Confirm cytopathic effect by microscopic examination or suitable staining such as MTT dye. The test is invalid if 5×10^{-5} Lf/ml of reference diphtheria toxin in 100 Lf/ml toxoid has no cytotoxic effect on Vero cells or if the cytotoxic effect of this amount of toxin is not neutralised in the wells containing diphtheria antitoxin. The bulk purified toxoid complies with the test if no toxicity neutralisable by antitoxin is found in either sample.

Antigenic purity. Not less than 1500 Lf per milligram of protein nitrogen.

FINAL BULK VACCINE

The final bulk vaccine is prepared by adsorption of a suitable quantity of bulk purified toxoid onto a mineral carrier such as hydrated aluminium phosphate or aluminium hydroxide; the resulting mixture is approximately isotonic with blood. Suitable antimicrobial preservatives may be added. Certain antimicrobial preservatives, particularly those of the phenolic type, adversely affect the antigenic activity and must not be used.

Only a final bulk vaccine that complies with the following requirements may be used in the preparation of the final lot.

Antimicrobial preservative. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The amount is not less than 85 per cent and not greater than 115 per cent of the intended amount.

Sterility (2.6.1). Carry out the test for sterility using 10 ml for each medium.

FINAL LOT

The final bulk vaccine is distributed aseptically into sterile, tamper-proof containers. The containers are closed so as to prevent contamination.

Only a final lot that is satisfactory with respect to each of the requirements given below under Identification, Tests and Assay may be released for use. Provided the test for antimicrobial preservative and the assay have been carried out with satisfactory results on the final bulk vaccine, they may be omitted on the final lot.

Provided the free formaldehyde content has been determined on the bulk purified antigens or on the final bulk and it has been shown that the content in the final lot will not exceed 0.2 g/l, the test for free formaldehyde may be omitted on the final lot.

IDENTIFICATION

Diphtheria toxoid is identified by a suitable immunochemical method (2.7.1). The following method, applicable to certain vaccines, is given as an example. Dissolve in the vaccine to be examined sufficient *sodium citrate R* to give a 100 g/l solution. Maintain at 37 °C for about 16 h and centrifuge

until a clear supernatant liquid is obtained. The clear supernatant liquid reacts with a suitable diphtheria antitoxin, giving a precipitate.

TESTS

Aluminium (2.5.13): maximum 1.25 mg per single human dose, if aluminium hydroxide or hydrated aluminium phosphate is used as the adsorbent.

Free formaldehyde (2.4.18): maximum 0.2 g/l.

Antimicrobial preservative. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The content is not less than the minimum amount shown to be effective and is not greater than 115 per cent of the quantity stated on the label.

Sterility (2.6.1). The vaccine complies with the test for sterility.

ASSAY

Carry out one of the prescribed methods for the assay of diphtheria vaccine (adsorbed) (2.7.6).

The lower confidence limit ($P = 0.95$) of the estimated potency is not less than 30 IU per single human dose.

LABELLING

The label states:

- the minimum number of International Units per single human dose,
- where applicable, that the vaccine is intended for primary vaccination of children and is not necessarily suitable for reinforcing doses or for administration to adults,
- the name and the amount of the adsorbent,
- that the vaccine must be shaken before use,
- that the vaccine is not to be frozen.

01/2005:0646

DIPHTHERIA VACCINE (ADSORBED) FOR ADULTS AND ADOLESCENTS

Vaccinum diphtheriae adulti et adolescentis adsorbatum

DEFINITION

Diphtheria vaccine (adsorbed) for adults and adolescents is a preparation of diphtheria formol toxoid with a mineral adsorbent. The formol toxoid is prepared from the toxin produced by the growth of *Corynebacterium diphtheriae*. It shall have been demonstrated to the competent authority that the quantity of diphtheria toxoid used does not produce adverse reactions in subjects from the age groups for which the vaccine is intended.

PRODUCTION

GENERAL PROVISIONS

Specific toxicity. The production method is validated to demonstrate that the product, if tested, would comply with the following test: inject subcutaneously 5 times the single human dose stated on the label into each of 5 healthy guinea-pigs, each weighing 250-350 g, that have not previously been treated with any material that will interfere with the test. If within 42 days of the injection any of the animals shows signs of or dies from diphtheria toxæmia, the vaccine does not comply with the test. If more than 1 animal dies from non-specific causes, repeat the test once; if more than 1 animal dies in the second test, the vaccine does not comply with the test.

BULK PURIFIED TOXOID

The bulk purified toxoid is prepared as described in the monograph on *Diphtheria vaccine (adsorbed)* (0443) and complies with the requirements prescribed therein.

FINAL BULK VACCINE

The final bulk vaccine is prepared by adsorption of a suitable quantity of bulk purified toxoid onto a mineral carrier such as hydrated aluminium phosphate or aluminium hydroxide; the resulting mixture is approximately isotonic with blood. Suitable antimicrobial preservatives may be added. Certain antimicrobial preservatives, particularly those of the phenolic type, adversely affect the antigenic activity and must not be used.

Only a final bulk vaccine that complies with the following requirements may be used in the preparation of the final lot.

Antimicrobial preservative. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The amount is not less than 85 per cent and not greater than 115 per cent of the intended amount.

Sterility (2.6.1). Carry out the test for sterility using 10 ml for each medium.

FINAL LOT

The final bulk vaccine is distributed aseptically into sterile, tamper-proof containers. The containers are closed so as to prevent contamination.

Only a final lot that is satisfactory with respect to each of the requirements given below under Identification, Tests and Assay may be released for use. Provided the test for antimicrobial preservative and the assay have been carried out with satisfactory results on the final bulk vaccine, they may be omitted on the final lot.

Provided the free formaldehyde content has been determined on the bulk purified toxoid or on the final bulk and it has been shown that the content in the final lot will not exceed 0.2 g/l, the test for free formaldehyde may be omitted on the final lot.

IDENTIFICATION

Diphtheria toxoid is identified by a suitable immunochemical method (2.7.1). The following method, applicable to certain vaccines, is given as an example. Dissolve in the vaccine to be examined sufficient *sodium citrate R* to give a 100 g/l solution. Maintain at 37 °C for about 16 h and centrifuge until a clear supernatant liquid is obtained. The clear supernatant liquid reacts with a suitable diphtheria antitoxin, giving a precipitate. If a satisfactory result is not obtained with a vaccine adsorbed on aluminium hydroxide, carry out the test as follows. Centrifuge 15 ml of the vaccine to be examined and suspend the residue in 5 ml of a freshly prepared mixture of 1 volume of a 56 g/l solution of *sodium edetate R* and 49 volumes of a 90 g/l solution of *disodium hydrogen phosphate R*. Maintain at 37 °C for not less than 6 h and centrifuge. The clear supernatant liquid reacts with a suitable diphtheria antitoxin, giving a precipitate.

TESTS

Aluminium (2.5.13): maximum 1.25 mg per single human dose, if aluminium hydroxide or hydrated aluminium phosphate is used as the adsorbent.

Free formaldehyde (2.4.18): maximum 0.2 g/l.

Antimicrobial preservative. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The content is not less than the minimum amount shown to be effective and is not greater than 115 per cent of the quantity stated on the label.

Sterility (2.6.1). The vaccine complies with the test for sterility.

ASSAY

Carry out one of the prescribed methods for the assay of diphtheria vaccine (adsorbed) (2.7.6).

The lower confidence limit ($P = 0.95$) of the estimated potency is not less than 2 IU per single human dose.

LABELLING

The label states:

- the minimum number of International Units per single human dose,
- the name and the amount of the adsorbent,
- that the vaccine must be shaken before use,
- that the vaccine is not to be frozen.

01/2005:1219

HAEMOPHILUS TYPE b CONJUGATE VACCINE

Vaccinum haemophili stirpe b coniugatum

DEFINITION

Haemophilus type b conjugate vaccine is a liquid or freeze-dried preparation of a polysaccharide, derived from a suitable strain of *Haemophilus influenzae* type b, covalently bound to a carrier protein. The polysaccharide, polyribosylribitol phosphate, referred to as PRP, is a linear copolymer composed of repeated units of 3-β-D-ribofuranosyl-(1→1)-ribitol-5-phosphate $[(C_{10}H_{19}O_{12}P)_n]$, with a defined molecular size. The carrier protein, when conjugated to PRP, is capable of inducing a T-cell-dependent B-cell immune response to the polysaccharide.

PRODUCTION

GENERAL PROVISIONS

The production method shall have been shown to yield consistently haemophilus type b conjugate vaccines of adequate safety and immunogenicity in man. The production of PRP and of the carrier are based on seed-lot systems.

The production method is validated to demonstrate that the product, if tested, would comply with the test for abnormal toxicity for immunosera and vaccines for human use (2.6.9).

During development studies and wherever revalidation of the manufacturing process is necessary, it shall be demonstrated by tests in animals that the vaccine consistently induces a T-cell-dependent B-cell immune response.

The stability of the final lot and relevant intermediates is evaluated using one or more indicator tests. Such tests may include determination of molecular size, determination of free PRP in the conjugate and the immunogenicity test in mice. Taking account of the results of the stability testing, release requirements are set for these indicator tests to ensure that the vaccine will be satisfactory at the end of the period of validity.

BACTERIAL SEED LOTS

The seed lots of *H. influenzae* type b are shown to be free from contamination by methods of suitable sensitivity. These may include inoculation into suitable media, examination of colony morphology, microscopic examination of Gram-stained smears and culture agglutination with suitable specific antisera.

No complex products of animal origin are included in the menstruum used for preservation of strain viability, either for freeze-drying or for frozen storage.

It is recommended that PRP produced by the seed lot be characterised using nuclear magnetic resonance spectrometry (2.2.33).

H. INFLUENZAE TYPE b POLYSACCHARIDE (PRP)

H. influenzae type b is grown in a liquid medium that does not contain high-molecular-mass polysaccharides; if any ingredient of the medium contains blood-group substances, the process shall be validated to demonstrate that after the purification step they are no longer detectable. The bacterial purity of the culture is verified by methods of suitable sensitivity. These may include inoculation into suitable media, examination of colony morphology, microscopic examination of Gram-stained smears and culture agglutination with suitable specific antisera. The culture may be inactivated. PRP is separated from the culture medium and purified by a suitable method. Volatile matter, including water, in the purified polysaccharide is determined by a suitable method such as thermogravimetry (2.2.34); the result is used to calculate the results of certain tests with reference to the dried substance, as prescribed below.

Only PRP that complies with the following requirements may be used in the preparation of the conjugate.

Identification. PRP is identified by an immunochemical method (2.7.1) or other suitable method, for example ^1H nuclear magnetic resonance spectrometry (2.2.33).

Molecular-size distribution. The percentage of PRP eluted before a given K_0 value or within a range of K_0 values is determined by size-exclusion chromatography (2.2.30); an acceptable value is established for the particular product and each batch of PRP must be shown to comply with this limit. Limits for currently approved products, using the indicated stationary phases, are shown for information in Table 1219-1. Where applicable, the molecular-size distribution is also determined after chemical modification of the polysaccharide.

Liquid chromatography (2.2.29) with multiple-angle laser light-scattering detection may also be used for determination of molecular-size distribution.

A validated determination of the degree of polymerisation or of the weight-average molecular weight and the dispersion of molecular masses may be used instead of the determination of molecular size distribution.

Ribose (2.5.31). Not less than 32 per cent, calculated with reference to the dried substance.

Phosphorus (2.5.18): 6.8 per cent to 9.0 per cent, calculated with reference to the dried substance.

Protein (2.5.16). Not more than 1.0 per cent, calculated with reference to the dried substance. Use sufficient PRP to allow detection of proteins at concentrations of 1 per cent or greater.

Nucleic acid (2.5.17). Not more than 1.0 per cent, calculated with reference to the dried substance.

Bacterial endotoxins (2.6.14): less than 25 IU per microgram of PRP.

Residual reagents. Where applicable, tests are carried out to determine residues of reagents used during inactivation and purification. An acceptable value for each reagent is established for the particular product and each batch of PRP must be shown to comply with this limit. Where validation studies have demonstrated removal of a residual reagent, the test on PRP may be omitted.

Table 1219-1. – Product characteristics and specifications for PRP and carrier protein in currently approved products

Carrier			Haemophilus polysaccharide		Conjugation	
Type	Purity	Nominal amount per dose	Type of PRP	Nominal amount per dose	Coupling method	Procedure
Diphtheria toxoid	> 1500 Lf per milligram of nitrogen	18 µg	Size-reduced PRP K_0 : 0.6-0.7, using cross-linked agarose for chromatography R	25 µg	cyanogen bromide activation of PRP	activated diphtheria toxoid (D-AH ⁺), cyanogen bromide-activated PRP
Tetanus toxoid	> 1500 Lf per milligram of nitrogen	20 µg	PRP ≥ 50 % $\leq K_0$: 0.30, using cross-linked agarose for chromatography R	10 µg	carbodi-imide mediated	ADH-activated PRP (PRP-cov.-AH) + tetanus toxoid + EDAC
CRM 197 diphtheria protein	> 90 % of diphtheria protein	25 µg	Size-reduced PRP Dp = 15-35 or 10-35	10 µg	reductive amination (1-step method) or <i>N</i> -hydroxysuccinimide activation	direct coupling of PRP to CRM 197 (cyanoborohydride activated)
Meningococcal group B outer membrane protein (OMP)	outer membrane protein vesicles: ≤ 8 % of lipopolysaccharide	125 µg or 250 µg	Size-reduced PRP $K_0 < 0.6$, using cross-linked agarose for chromatography R or $M_w > 50 \times 10^3$	7.5 µg or 15 µg	thioether bond	PRP activation by CDI PRP-IM + BuA2 + BrAc = PRP-BuA2-BrAc + thioactivated OMP
ADH = adipic acid dihydrazide BrAc = bromoacetyl chloride BuA2 = butane-1,4-diamide CDI = carbonyldiimidazole			Dp = degree of polymerisation EDAC = 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide IM = imidazolium M_w = weight-average molecular weight			

CARRIER PROTEIN

The carrier protein is chosen so that when the PRP is conjugated it is able to induce a T-cell-dependent B-cell immune response. Currently approved carrier proteins and coupling methods are listed for information in Table 1219-1. The carrier proteins are produced by culture of suitable micro-organisms; the bacterial purity of the culture is verified; the culture may be inactivated; the carrier protein is purified by a suitable method.

Only a carrier protein that complies with the following requirements may be used in the preparation of the conjugate.

Identification. The carrier protein is identified by a suitable immunochemical method (2.7.1).

Sterility (2.6.1). Carry out the test using for each medium 10 ml or the equivalent of one-hundred doses, whichever is less.

Diphtheria toxoid. Diphtheria toxoid is produced as described in *Diphtheria vaccine (adsorbed) (0443)* and complies with the requirements prescribed therein for bulk purified toxoid.

Tetanus toxoid. Tetanus toxoid is produced as described in *Tetanus vaccine (adsorbed) (0452)* and complies with the requirements prescribed therein for bulk purified toxoid, except that the antigenic purity is not less than 1500 Lf per milligram of protein nitrogen.

Diphtheria protein CRM 197. It contains not less than 90 per cent of diphtheria CRM 197 protein, determined by a suitable method. Suitable tests are carried out, for validation or routinely, to demonstrate that the product is nontoxic.

OMP (meningococcal group B Outer Membrane Protein complex). OMP complies with the following requirements for lipopolysaccharide and pyrogens.

Lipopolysaccharide. Not more than 8 per cent of lipopolysaccharide, determined by a suitable method.

Pyrogens (2.6.8). Inject into each rabbit 0.25 µg of OMP per kilogram of body mass.

BULK CONJUGATE

PRP is chemically modified to enable conjugation; it is usually partly depolymerised either before or during this procedure. Reactive functional groups or spacers may be introduced into the carrier protein or PRP prior to conjugation. As a measure of consistency, the extent of derivatisation is monitored. The conjugate is obtained by the covalent binding of PRP and carrier protein. Where applicable, unreacted but potentially reactogenic functional groups are made unreactive by means of capping agents; the conjugate is purified to remove reagents.

Only a bulk conjugate that complies with the following requirements may be used in the preparation of the final bulk vaccine. For each test and for each particular product, limits of acceptance are established and each batch of conjugate must be shown to comply with these limits. Limits applied to currently approved products for some of these tests are listed for information in Table 1219-2. For a freeze-dried vaccine, some of the tests may be carried out on the final lot rather than on the bulk conjugate where the freeze-drying process may affect the component being tested.

PRP. The PRP content is determined by assay of phosphorus (2.5.18) or by assay of ribose (2.5.31) or by an immunochemical method (2.7.1).

Protein. The protein content is determined by a suitable chemical method (for example, 2.5.16).

PRP to protein ratio. Determine the ratio by calculation.

Molecular-size distribution. Molecular-size distribution is determined by size-exclusion chromatography (2.2.30).

Free PRP. Unbound PRP is determined after removal of the conjugate, for example by anion-exchange, size-exclusion or hydrophobic chromatography, ultrafiltration or other validated methods.

Free carrier protein. Determine the content by a suitable method, either directly or by deriving the content by calculation from the results of other tests. The amount is within the limits approved for the particular product.

Table 1219.2. – Bulk conjugate requirements for currently approved products

Test	Protein carrier			
	Diphtheria toxoid	Tetanus toxoid	CRM 197	OMP
Free PRP	< 37 %	< 20 %	< 25 %	< 15 %
Free protein	< 4 %	< 1 %, where applicable	< 1 % or < 2 %, depending on the coupling method	not applicable
PRP to protein ratio	1.25 - 1.8	0.30 - 0.55	0.3 - 0.7	0.05 - 0.1
Molecular size (K_0):				
<i>cross-linked agarose for chromatography R</i>	95 % < 0.75	60 % < 0.2	50 % 0.3 - 0.6	85 % < 0.3
<i>cross-linked agarose for chromatography R1</i>	0.6-0.7	85 % < 0.5		

Unreacted functional groups. No unreacted functional groups are detectable in the bulk conjugate unless process validation has shown that unreacted functional groups detectable at this stage are removed during the subsequent manufacturing process (for example, owing to short half-life).

Residual reagents. Removal of residual reagents such as cyanide, EDAC (ethyl dimethylaminopropyl carbodi-imide) and phenol is confirmed by suitable tests or by validation of the process.

Sterility (2.6.1). Carry out the test using for each medium 10 ml or the equivalent of 100 doses, whichever is less.

FINAL BULK VACCINE

An adjuvant, an antimicrobial preservative and a stabiliser may be added to the bulk conjugate before dilution to the final concentration with a suitable diluent.

Only a final bulk vaccine that complies with the following requirements may be used in preparation of the final lot.

Antimicrobial preservative. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical or physico-chemical method. The content is not less than 85 per cent and not greater than 115 per cent of the intended amount.

Sterility (2.6.1). It complies with the test for sterility, carried out using 10 ml for each medium.

FINAL LOT

Only a final lot that is satisfactory with respect to each of the requirements given below under Identification and Tests may be released for use. Provided the test for antimicrobial preservative has been carried out on the final bulk vaccine, it may be omitted on the final lot.

pH (2.2.3). The pH of the vaccine, reconstituted if necessary, is within the range approved for the particular product.

Free PRP. Unbound PRP is determined after removal of the conjugate, for example by anion-exchange, size-exclusion or hydrophobic chromatography, ultrafiltration or other validated methods. The amount of free PRP is not greater than that approved for the particular product.

IDENTIFICATION

The vaccine is identified by a suitable immunochemical method (2.7.1) for PRP.

TESTS

PRP. Not less than 80 per cent of the amount of PRP stated on the label. PRP is determined either by assay of ribose (2.5.31) or phosphorus (2.5.18), by an immunochemical method (2.7.1) or by anion-exchange liquid chromatography with pulsed amperometric detection (2.2.29).

Aluminium (2.5.13): maximum 1.25 mg per single human dose, if aluminium hydroxide or hydrated aluminium phosphate is used as the adsorbent.

Antimicrobial preservative. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical or physico-chemical method. The content is not less than the minimum amount shown to be effective and not greater than 115 per cent of the quantity stated on the label.

Water (2.5.12): maximum 3.0 per cent for freeze-dried vaccines.

Sterility (2.6.1). It complies with the test for sterility.

Pyrogens (2.6.8). It complies with the test for pyrogens. Inject per kilogram of the rabbit's mass a quantity of the vaccine equivalent to: 1 µg of PRP for a vaccine with diphtheria toxoid or CRM 197 diphtheria protein as carrier; 0.1 µg of PRP for a vaccine with tetanus toxoid as carrier; 0.025 µg of PRP for a vaccine with OMP as carrier.

LABELLING

The label states:

- the number of micrograms of PRP per human dose,
- the type and nominal amount of carrier protein per single human dose.

01/2005:1526

HEPATITIS A (INACTIVATED) AND HEPATITIS B (rDNA) VACCINE (ADSORBED)

Vaccinum hepatitis A inactivatum et hepatitis B (ADNr) adsorbatum

DEFINITION

Hepatitis A (inactivated) and hepatitis B (rDNA) vaccine (adsorbed) is a suspension consisting of a suitable strain of hepatitis A virus, grown in cell cultures and inactivated by a validated method, and of hepatitis B surface antigen (HBsAg), a component protein of hepatitis B virus obtained by recombinant DNA technology; the antigens are adsorbed on a mineral carrier, such as aluminium hydroxide or hydrated aluminium phosphate.

PRODUCTION

GENERAL PROVISIONS

The two components are prepared as described in the monographs on *Hepatitis A vaccine (inactivated, adsorbed) (1107)* and *Hepatitis B vaccine (rDNA) (1056)* and comply with the requirements prescribed therein.

The production method is validated to demonstrate that the product, if tested, would comply with the test for abnormal toxicity for immunosera and vaccines for human use (2.6.9).

Reference preparation. The reference preparation is part of a representative batch shown to be at least as immunogenic in animals as a batch that, in clinical studies in young healthy

adults, produced not less than 95 per cent seroconversion, corresponding to a level of neutralising antibody recognised to be protective, after a full-course primary immunisation. For hepatitis A, an antibody level not less than 20 mIU/ml determined by enzyme-linked immunosorbent assay is recognised as being protective. For hepatitis B, an antibody level not less than 10 mIU/ml against HBsAg is recognised as being protective.

FINAL BULK VACCINE

The final bulk vaccine is prepared from one or more inactivated harvests of hepatitis A virus and one or more batches of purified antigen.

Only a final bulk vaccine that complies with the following requirements may be used in the preparation of the final lot.

Antimicrobial preservative. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical or physico-chemical method. The amount is not less than 85 per cent and not greater than 115 per cent of the intended amount.

Sterility (2.6.1). The final bulk vaccine complies with the test for sterility, carried out using 10 ml for each medium.

FINAL LOT

Only a final lot that complies with each of the requirements given below under Identification, Tests and Assay may be released for use. Provided that the tests for free formaldehyde (where applicable) and antimicrobial preservative content (where applicable) have been carried out on the final bulk vaccine with satisfactory results, they may be omitted on the final lot. If the assay of the hepatitis A and/or the hepatitis B component is carried out *in vivo*, then provided it has been carried out with satisfactory results on the final bulk vaccine, it may be omitted on the final lot.

IDENTIFICATION

The vaccine is shown to contain hepatitis A virus antigen and hepatitis B surface antigen by suitable immunochemical methods (2.7.1), using specific antibodies or by the mouse immunogenicity tests described under Assay.

TESTS

Aluminium (2.5.13): maximum 1.25 mg per single human dose, if aluminium hydroxide or hydrated aluminium phosphate is used as the adsorbent.

Free formaldehyde (2.4.18): maximum 0.2 g/l.

Antimicrobial preservative. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical or physico-chemical method. The amount is not less than the minimum amount shown to be effective and is not greater than 115 per cent of that stated on the label.

Sterility (2.6.1). The vaccine complies with the test for sterility.

Bacterial endotoxins (2.6.14): less than 2 IU per human dose.

ASSAY

Hepatitis A component. The vaccine complies with the assay of hepatitis A vaccine (2.7.14).

Hepatitis B component. The vaccine complies with the assay of hepatitis B vaccine (rDNA) (2.7.15).

LABELLING

The label states:

- the amount of hepatitis A virus antigen and hepatitis B surface antigen per container,
- the type of cells used for production of the vaccine,

- the name and amount of the adsorbent used,
- that the vaccine must be shaken before use,
- that the vaccine must not be frozen.

01/2005:1107

HEPATITIS A VACCINE (INACTIVATED, ADSORBED)

Vaccinum hepatitis A inactivatum adsorbatum

DEFINITION

Hepatitis A vaccine (inactivated, adsorbed) is a suspension consisting of a suitable strain of hepatitis A virus grown in cell cultures, inactivated by a validated method and adsorbed on a mineral carrier.

PRODUCTION

Production of the vaccine is based on a virus seed-lot system and a cell-bank system. The production method shall have been shown to yield consistently vaccines that comply with the requirements for immunogenicity, safety and stability.

The production method is validated to demonstrate that the product, if tested, would comply with the test for abnormal toxicity for immunosera and vaccines for human use (2.6.9).

Unless otherwise justified and authorised, the virus in the final vaccine shall not have undergone more passages from the master seed lot than were used to prepare the vaccine shown in clinical studies to be satisfactory with respect to safety and efficacy.

Reference preparation. A part of a batch shown to be at least as immunogenic in animals as a batch that, in clinical studies in young healthy adults, produced not less than 95 per cent seroconversion, corresponding to a level of neutralising antibody accepted to be protective, after a full-course primary immunisation is used as a reference preparation. An antibody level of 20 mIU/ml determined by enzyme-linked immunosorbent assay is recognised as being protective.

SUBSTRATE FOR VIRUS PROPAGATION

The virus is propagated in a human diploid cell line (5.2.3) or in a continuous cell line approved by the competent authority.

SEED LOTS

The strain of hepatitis A virus used to prepare the master seed lot shall be identified by historical records that include information on the origin of the strain and its subsequent manipulation.

Only a seed lot that complies with the following requirements may be used for virus propagation.

Identification. Each master and working seed lot is identified as hepatitis A virus using specific antibodies.

Virus concentration. The virus concentration of each master and working seed lot is determined to monitor consistency of production.

Extraneous agents. The master and working seed lots comply with the requirements for seed lots for virus vaccines (2.6.16). In addition, if primary monkey cells have been used for isolation of the strain, measures are taken to ensure that the strain is not contaminated with simian viruses such as simian immunodeficiency virus and filoviruses.

VIRUS PROPAGATION AND HARVEST

All processing of the cell bank and subsequent cell cultures is done under aseptic conditions in an area where no other cells are being handled. Animal serum (but not human

serum) may be used in the cell culture media. Serum and trypsin used in the preparation of cell suspensions and media are shown to be free from extraneous agents. The cell culture media may contain a pH indicator, such as phenol red, and antibiotics at the lowest effective concentration. Not less than 500 ml of the cell cultures employed for vaccine production is set aside as uninfected cell cultures (control cells). Multiple harvests from the same production cell culture may be pooled and considered as a single harvest.

Only a single harvest that complies with the following requirements may be used in the preparation of the vaccine. When the determination of the ratio of virus concentration to antigen content has been carried out on a suitable number of single harvests to demonstrate production consistency, it may subsequently be omitted as a routine test.

Identification. The test for antigen content also serves to identify the single harvest.

Bacterial and fungal contamination (2.6.1). The single harvest complies with the test for sterility, carried out using 10 ml for each medium.

Mycoplasmas (2.6.7). The single harvest complies with the test for mycoplasmas, carried out using 1 ml for each medium.

Control cells. The control cells of the production cell culture comply with a test for identification and the requirements for extraneous agents (2.6.16).

Antigen content. Determine the hepatitis A antigen content by a suitable immunochemical method (2.7.1) to monitor production consistency; the content is within the limits approved for the particular product.

Ratio of virus concentration to antigen content. The consistency of the ratio of the concentration of infectious virus, determined by a suitable cell culture method, to antigen content is established by validation on a suitable number of single harvests.

PURIFICATION AND PURIFIED HARVEST

The harvest, which may be a pool of several single harvests, is purified by validated methods. If continuous cell lines are used for production, the purification process shall have been shown to reduce consistently the level of host-cell DNA.

Only a purified harvest that complies with the following requirements may be used in the preparation of the inactivated harvest.

Virus concentration. The concentration of infectious virus in the purified harvest is determined by a suitable cell culture method to monitor production consistency and as a starting point for monitoring the inactivation curve.

Antigen:total protein ratio. Determine the hepatitis A virus antigen content by a suitable immunochemical method (2.7.1). Determine the total protein by a validated method. The ratio of hepatitis A virus antigen content to total protein content is within the limits approved for the particular product.

Bovine serum albumin. Not more than 50 ng in the equivalent of a single human dose, determined by a suitable immunochemical method (2.7.1). Where appropriate in view of the manufacturing process, other suitable protein markers may be used to demonstrate effective purification.

Residual host-cell DNA. If a continuous cell line is used for virus propagation, the content of residual host-cell DNA, determined using a suitable method, is not greater than 100 pg in the equivalent of a single human dose.

Residual chemicals. If chemical substances are used during the purification process, tests for these substances are carried out on the purified harvest (or on the inactivated

harvest), unless validation of the process has demonstrated total clearance. The concentration must not exceed the limits approved for the particular product.

INACTIVATION AND INACTIVATED HARVEST

Several purified harvests may be pooled before inactivation. In order to avoid interference with the inactivation process, virus aggregation must be prevented or aggregates must be removed immediately before and/or during the inactivation process. The virus suspension is inactivated by a validated method; the method shall have been shown to be consistently capable of inactivating hepatitis A virus without destroying the antigenic and immunogenic activity; for each inactivation procedure, an inactivation curve is plotted representing residual live virus concentration measured at not fewer than three points in time (for example, on days 0, 1 and 2 of the inactivation process). If formaldehyde is used for inactivation, the presence of excess free formaldehyde is verified at the end of the inactivation process.

Only an inactivated harvest that complies with the following requirements may be used in the preparation of the final bulk vaccine.

Inactivation. Carry out an amplification test for residual infectious hepatitis A virus by inoculating a quantity of the inactivated harvest equivalent to 5 per cent of the batch or, if the harvest contains the equivalent of 30 000 doses or more, not less than 1500 doses of vaccine into cell cultures of the same type as those used for production of the vaccine; incubate for a total of not less than 70 days making not fewer than one passage of cells within that period. At the end of the incubation period, carry out a test of suitable sensitivity for residual infectious virus. No evidence of hepatitis A virus multiplication is found in the samples taken at the end of the inactivation process. Use infectious virus inocula concurrently as positive controls to demonstrate cellular susceptibility and absence of interference. Incubate for a total of not less than 70 days, making not fewer than one passage of cells within that period.

Sterility (2.6.1). The inactivated viral harvest complies with the test for sterility, carried out using 10 ml for each medium.

Bacterial endotoxins (2.6.14): less than 2 IU in the equivalent of a single human dose.

Antigen content. Determine the hepatitis A virus antigen content by a suitable immunochemical method (2.7.1).

Residual chemicals. See under Purification and purified harvest.

FINAL BULK VACCINE

The final bulk vaccine is prepared from one or more inactivated harvests. Approved adjuvants, stabilisers and antimicrobial preservatives may be added.

Only a final bulk vaccine that complies with the following requirements may be used in the preparation of the final lot.

Sterility (2.6.1). The final bulk vaccine complies with the test for sterility, carried out using 10 ml for each medium.

Antimicrobial preservative. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical or physico-chemical method. The amount is not less than 85 per cent and not greater than 115 per cent of the intended amount.

FINAL LOT

The final bulk vaccine is distributed aseptically into sterile containers. The containers are then closed so as to avoid contamination.

Only a final lot that complies with each of the requirements given below under Identification, Tests and Assay may be released for use. Provided that the tests for free formaldehyde

(where applicable) and antimicrobial preservative content (where applicable) have been carried out on the final bulk vaccine with satisfactory results, these tests may be omitted on the final lot. If the assay is carried out using mice or other animals, then provided it has been carried out with satisfactory results on the final bulk vaccine, it may be omitted on the final lot.

IDENTIFICATION

The vaccine is shown to contain hepatitis A virus antigen by a suitable immunochemical method (2.7.1) using specific antibodies or by the *in vivo* assay (2.7.14).

TESTS

Aluminium (2.5.13): maximum 1.25 mg per single human dose, if aluminium hydroxide or hydrated aluminium phosphate is used as the adsorbent.

Free formaldehyde (2.4.18): maximum 0.2 g/l.

Antimicrobial preservative. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical or physico-chemical method. The amount is not less than the minimum amount shown to be effective and is not greater than 115 per cent of that stated on the label.

Sterility (2.6.1). The vaccine complies with the test for sterility.

ASSAY

The vaccine complies with the assay of hepatitis A vaccine (2.7.14).

LABELLING

The label states the biological origin of the cells used for the preparation of the vaccine.

01/2005:1935

HEPATITIS A VACCINE (INACTIVATED, VIROSOME)

Vaccinum hepatitis A inactivatum virosomale

DEFINITION

Hepatitis A vaccine (inactivated, virosome) is a suspension of a suitable strain of hepatitis A virus grown in cell cultures and inactivated by a validated method. Virosomes composed of influenza proteins of a strain approved for the particular product and phospholipids are used as adjuvants.

PRODUCTION

GENERAL PROVISIONS

The production method shall have been shown to yield consistently vaccines comparable with the vaccine of proven clinical efficacy and safety in man.

The production method is validated to demonstrate that the product, if tested, would comply with the test for abnormal toxicity for immunosera and vaccines for human use (2.6.9).

Reference preparation. A reference preparation of inactivated hepatitis A antigen is calibrated against a batch of hepatitis A vaccine (inactivated, virosome) that, in clinical studies in young healthy adults, produced not less than 95 per cent seroconversion, corresponding to a level of neutralising antibody accepted to be protective, after a full-course primary immunisation. An antibody level not less than 20 mIU/ml determined by enzyme-linked immunosorbent assay is recognised as being protective.

PREPARATION OF HEPATITIS A ANTIGEN

Production of the hepatitis A antigen is based on a virus seed-lot system and a cell-bank system. The production method shall have been shown to yield consistently vaccines that comply with the requirements for immunogenicity, safety and stability.

Unless otherwise justified and authorised, the virus in the final vaccine shall not have undergone more passages from the master seed lot than were used to prepare the vaccine shown in clinical studies to be satisfactory with respect to safety and efficacy.

SUBSTRATE FOR PROPAGATION OF HEPATITIS A VIRUS

The virus is propagated in a human diploid cell line (5.2.3).

SEED LOTS OF HEPATITIS A VIRUS

The strain of hepatitis A virus used to prepare the master seed lot shall be identified by historical records that include information on the origin of the strain and its subsequent manipulation.

Only a seed lot that complies with the following requirements may be used for virus propagation.

Identification. Each master and working seed lot is identified as hepatitis A virus using specific antibodies.

Virus concentration. The virus concentration of each master and working seed lot is determined to monitor consistency of production.

Extraneous agents. The master and working seed lots comply with the requirements for seed lots for virus vaccines (2.6.16).

PROPAGATION AND HARVEST OF HEPATITIS A VIRUS

All processing of the cell bank and subsequent cell cultures is done under aseptic conditions in an area where no other cells are handled. Animal serum (but not human serum) may be used in the cell culture media. Serum and trypsin used in the preparation of cell suspensions and media are shown to be free from extraneous agents. The cell culture media may contain a pH indicator such as phenol red and antibiotics at the lowest effective concentration. Not less than 500 ml of the cell cultures employed for vaccine production is set aside as uninfected cell cultures (control cells). Multiple harvests from the same production cell culture may be pooled and considered as a single harvest.

Only a single harvest that complies with the following requirements may be used in the preparation of the vaccine. When the determination of the ratio of virus concentration to antigen content has been carried out on a suitable number of single harvests to demonstrate consistency, it may subsequently be omitted as a routine test.

Identification. The test for antigen content also serves to identify the single harvest.

Bacterial and fungal contamination (2.6.1). The single harvest complies with the test for sterility, carried out using 10 ml for each medium.

Mycoplasmas (2.6.7). The single harvest complies with the test for mycoplasmas.

Control cells. The control cells of the production cell culture comply with a test for identity and the requirements for extraneous agents (2.6.16).

Antigen content. Determine the hepatitis A antigen content by a suitable immunochemical method (2.7.1) to monitor production consistency; the content is within the limits approved for the particular product.

Ratio of virus concentration to antigen content. The consistency of the ratio of the concentration of infectious virus, as determined by a suitable cell culture method, to antigen content is established by validation on a suitable number of single harvests.

PURIFICATION AND PURIFIED HARVEST OF HEPATITIS A VIRUS

The harvest, which may be a pool of several single harvests, is purified by validated methods. If continuous cell lines are used for production, the purification process shall have been shown to reduce consistently the level of host-cell DNA.

Only a purified harvest that complies with the following requirements may be used in the preparation of the inactivated harvest.

Virus concentration. The concentration of infective virus in the purified harvest is determined by a suitable cell culture method to monitor production consistency and as a starting point for monitoring the inactivation curve.

Ratio of antigen to total protein. Determine the hepatitis A virus antigen content by a suitable immunochemical method (2.7.1). Determine the total protein by a validated method. The ratio of hepatitis A virus antigen content to total protein content is within the limits approved for the particular product.

Bovine serum albumin: maximum 50 ng per single human dose if foetal bovine serum is used, determined by a suitable immunochemical method (2.7.1). Where appropriate in view of the manufacturing process, other suitable protein markers may be used to demonstrate effective purification.

Residual chemicals. If chemical substances are used during the purification process, tests for these substances are carried out on the purified harvest (or on the inactivated harvest), unless validation of the process has demonstrated total clearance. The concentration must not exceed the limits approved for the particular product.

INACTIVATION AND INACTIVATED HARVEST OF HEPATITIS A VIRUS

Several purified harvests may be pooled before inactivation. In order to avoid interference with the inactivation process, virus aggregation must be prevented or aggregates must be removed immediately before and/or during the inactivation process. The virus suspension is inactivated by a validated method; the method shall have been shown to be consistently capable of inactivating hepatitis A virus without destroying the antigenic and immunogenic activity; for each inactivation procedure, an inactivation curve is plotted representing residual live virus concentration measured on at least 3 occasions (for example, on days 0, 1 and 2 of the inactivation process). If formaldehyde is used for inactivation, the presence of excess free formaldehyde is verified at the end of the inactivation process.

Only an inactivated harvest that complies with the following requirements may be used in the preparation of the final bulk vaccine.

Inactivation. Carry out an amplification test for residual infectious hepatitis A virus by inoculating a quantity of the inactivated harvest equivalent to 5 per cent of the batch or, if the harvest contains the equivalent of 30 000 doses or more, not less than 1500 doses of vaccine into cell cultures of the same type as those used for production of the vaccine; incubate for a total of not less than 70 days making not fewer than 1 passage of cells within that period. At the end of the incubation period, carry out a test of suitable sensitivity for residual infectious virus. No evidence of hepatitis A virus multiplication is found in the samples taken at the end of the

inactivation process. Use infective virus inocula concurrently as positive controls to demonstrate cellular susceptibility and absence of interference.

Sterility (2.6.1). The inactivated viral harvest complies with the test for sterility, carried out using 10 ml for each medium.

Bacterial endotoxins (2.6.14): less than 2 IU of endotoxin in the equivalent of a single human dose.

Antigen content. Determine the hepatitis A virus antigen content by a suitable immunochemical method (2.7.1).

Residual chemicals. See under Purification and purified harvest.

PREPARATION OF INACTIVATED INFLUENZA VIRUS

The production of influenza viruses is based on a seed-lot system. Working seed lots represent not more than 15 passages from the approved reassorted virus or the approved virus isolate. The final production represents 1 passage from the working seed lot. The strain of influenza virus to be used is approved by the competent authority.

SUBSTRATE FOR PROPAGATION OF INFLUENZA VIRUS

Influenza virus seed to be used in the production of vaccine is propagated in fertilised eggs from chicken flocks free from specified pathogens (5.2.2) or in suitable cell cultures (5.2.4), such as chick-embryo fibroblasts or chick kidney cells obtained from chicken flocks free from specified pathogens (5.2.2). For production, the virus is grown in the allantoic cavity of fertilised hens' eggs from healthy flocks.

SEED LOTS OF INFLUENZA VIRUS

The haemagglutinin and neuraminidase antigens of each seed lot are identified as originating from the correct strain of influenza virus by suitable methods.

Only a working virus seed lot that complies with the following requirements may be used in the preparation of the monovalent pooled harvest.

Bacterial and fungal contamination. Carry out the test for sterility (2.6.1), using 10 ml for each medium.

Mycoplasmas (2.6.7). Carry out the test for mycoplasmas, using 10 ml.

PROPAGATION AND HARVEST OF INFLUENZA VIRUS

An antimicrobial agent may be added to the inoculum. After incubation at a controlled temperature, the allantoic fluids are harvested and combined to form the monovalent pooled harvest. An antimicrobial agent may be added at the time of harvest. At no stage in the production is penicillin or streptomycin used.

POOLED HARVEST OF INFLUENZA VIRUS

To limit the possibility of contamination, inactivation is initiated as soon as possible after preparation. The virus is inactivated by a method that has been demonstrated on 3 consecutive batches to be consistently effective for the manufacturer. The inactivation process shall have been shown to be capable of inactivating the influenza virus without destroying antigenicity of haemagglutinin. The inactivation process shall also have been shown to be capable of inactivating avian leucosis viruses and mycoplasmas. If the monovalent pooled harvest is stored after inactivation, it is held at a temperature of 5 ± 3 °C. If formaldehyde solution is used, the concentration does not exceed 0.2 g/l of CH₂O at any time during inactivation; if betapropiolactone is used, the concentration does not exceed 0.1 per cent V/V at any time during inactivation.

Only a pooled harvest that complies with the following requirements may be used in the preparation of the virosomes.

Haemagglutinin antigen. Determine the content of haemagglutinin antigen by an immunodiffusion test (2.7.1), by comparison with a haemagglutinin antigen reference preparation or with an antigen preparation calibrated against it. Carry out the test at 20–25 °C.

Sterility (2.6.1). Carry out the test for sterility, using 10 ml for each medium.

Viral inactivation. Inoculate 0.2 ml of the harvest into the allantoic cavity of each of 10 fertilised eggs and incubate at 33–37 °C for 3 days. The test is not valid unless at least 8 of the 10 embryos survive. Harvest 0.5 ml of the allantoic fluid from each surviving embryo and pool the fluids. Inoculate 0.2 ml of the pooled fluid into a further 10 fertilised eggs and incubate at 33–37 °C for 3 days. The test is not valid unless at least 8 of the 10 embryos survive. Harvest about 0.1 ml of the allantoic fluid from each surviving embryo and examine each individual harvest by a haemagglutination test. If haemagglutination is found for any of the fluids, carry out for that fluid a further passage in eggs and test for haemagglutination; no haemagglutination occurs.

Ovalbumin: maximum 1 µg of ovalbumin in the equivalent of 1 human dose, determined by a suitable technique using a suitable reference preparation of ovalbumin.

Antimicrobial preservative. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The content is not less than 85 per cent and not greater than 115 per cent of the intended amount.

Residual chemicals. Tests are carried out on the monovalent pooled harvest for the chemicals used for inactivation, the limits being approved by the competent authority.

PREPARATION OF VIROSOMES

Inactivated influenza virions are solubilised using a suitable detergent and are purified by high-speed centrifugation in order to obtain supernatants containing mainly influenza antigens. After the addition of suitable phospholipids, virosomes are formed by removal of the detergent either by adsorption chromatography or another suitable technique

Only virosomes that comply with the following requirements may be used in the preparation of the final bulk vaccine.

Haemagglutinin content. Determine the content of haemagglutinin antigen by an immunodiffusion test (2.7.1), by comparison with a haemagglutinin antigen reference preparation or with an antigen preparation calibrated against it.

Phospholipids. The content and identity of the phospholipids are determined by suitable immunochemical or physico-chemical methods.

Ratio of phospholipid to haemagglutinin. The ratio of phospholipid content to haemagglutinin content is within the limits approved for the particular product.

Residual chemicals. Tests are carried out for the chemicals used during the process. The concentration of each residual chemical is within the limits approved for the particular product.

FINAL BULK VACCINE

The bulk vaccine is prepared by adding virosomes to inactivated hepatitis A viruses to yield an approved hepatitis A antigen:haemagglutinin ratio. Several bulks may be pooled, and approved stabilisers and antimicrobial preservatives may be added.

Only a final bulk vaccine that complies with the following requirements may be used in the preparation of the final lot.

Protein content. The amount of protein is determined using a suitable technique, the limits being approved by the competent authority.

Phospholipids. The content and identity of the phospholipids are determined by suitable immunochemical or physico-chemical methods. The amount of phospholipids complies with the limits approved for the particular product.

Haemagglutinin content. Determine the content of haemagglutinin antigen by an immunodiffusion test (2.7.1). The amount of haemagglutinin must not exceed the limits approved for the particular product.

Hepatitis A antigen content. Determine the hepatitis A antigen content by a suitable immunochemical method. The amount of antigen must not exceed the limits approved for the particular product.

Ratio of hepatitis A antigen to haemagglutinin. The ratio of hepatitis A antigen content to haemagglutinin content is within the limits approved for the particular product.

Ovalbumin: maximum 1 µg of ovalbumin per human dose, determined by a suitable technique using a suitable reference preparation of ovalbumin.

Virosome size. The size distribution of the virosome-hepatitis A virus mixture is within the limits approved for the particular product.

Sterility (2.6.1). The final bulk vaccine complies with the test for sterility, carried out using 10 ml for each medium.

Antimicrobial preservative. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical or physico-chemical method. The amount is not less than 85 per cent and not greater than 115 per cent of the intended amount.

Residual chemicals. If chemical substances are used during the formulation process, tests for these substances are carried out, the limits being approved by the competent authority.

FINAL LOT

The final bulk vaccine is distributed aseptically into sterile containers. The containers are then closed so as to avoid contamination.

Only a final lot that complies with each of the requirements given below under Identification, Tests and Assay may be released for use. Provided that the tests for free formaldehyde (where applicable) and antimicrobial preservative content (where applicable) have been carried out on the final bulk vaccine with satisfactory results, these tests may be omitted on the final lot. If the assay is carried out *in vivo*, provided it has been carried out with satisfactory results on the final bulk vaccine, it may be omitted on the final lot.

IDENTIFICATION

The vaccine is shown to contain hepatitis A virus antigen by a suitable immunochemical method (2.7.1) using specific antibodies.

TESTS

Free formaldehyde (2.4.18): maximum 0.2 g/l.

Antimicrobial preservative. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical or physico-chemical method. The amount is not less than the minimum amount shown to be effective and is not greater than 115 per cent of that stated on the label.

Sterility (2.6.1). The vaccine complies with the test for sterility.

Bacterial endotoxins (2.6.14): less than 2 IU of endotoxin per human dose.

ASSAY

Determine the antigen content of the vaccine using a suitable immunochemical method (2.7.1) by comparison with the reference preparation. The acceptance criteria are approved for a given reference preparation by the competent authority.

LABELLING

The label states:

- the biological origin of the cells used for the preparation of the vaccine,
- that the carrier contains influenza proteins prepared in eggs,
- that the vaccine is not to be frozen,
- that the vaccine is to be shaken before use.

01/2005:1056

HEPATITIS B VACCINE (rDNA)

Vaccinum hepatitis B (ADNr)

DEFINITION

Hepatitis B vaccine (rDNA) is a preparation of hepatitis B surface antigen (HBsAg), a component protein of hepatitis B virus; the antigen may be adsorbed on a mineral carrier such as aluminium hydroxide or hydrated aluminium phosphate. The antigen is obtained by recombinant DNA technology.

PRODUCTION

GENERAL PROVISIONS

The vaccine shall have been shown to induce specific, protective antibodies in man. The production method shall have been shown to yield consistently vaccines that comply with the requirements for immunogenicity and safety.

The production method is validated to demonstrate that the product, if tested, would comply with the test for abnormal toxicity for immunosera and vaccines for human use (2.6.9).

Hepatitis B vaccine (rDNA) is produced by the expression of the viral gene coding for HBsAg in yeast (*Saccharomyces cerevisiae*) or mammalian cells (Chinese hamster ovary (CHO) cells or other suitable cell lines), purification of the resulting HBsAg and the rendering of this antigen into an immunogenic preparation. The suitability and safety of the cells are approved by the competent authority.

The vaccine may contain the product of the S gene (major protein), a combination of the S gene and pre-S2 gene products (middle protein) or a combination of the S gene, the pre-S2 gene and pre-S1 gene products (large protein).

Reference preparation. The reference preparation is part of a representative batch shown to be at least as immunogenic in animals as a batch that, in clinical studies in young, healthy adults, produced not less than 95 per cent seroconversion, corresponding to a level of HBsAg neutralising antibody recognised to be protective, after a full-course primary immunisation. An antibody level not less than 10 mIU/ml is recognised as being protective.

CHARACTERISATION OF THE SUBSTANCE

Development studies are carried out to characterise the antigen. The complete protein, lipid and carbohydrate structure of the antigen is established. The morphological characteristics of the antigen particles are established by electron microscopy. The mean buoyant density of the antigen particles is determined by a physico-chemical

method, such as gradient centrifugation. The antigenic epitopes are characterised. The protein fraction of the antigen is characterised in terms of the primary structure (for example, by determination of the amino-acid composition, by partial amino-acid sequence analysis and by peptide mapping).

CULTURE AND HARVEST

Identity, microbial purity, plasmid retention and consistency of yield are determined at suitable production stages. If mammalian cells are used, tests for extraneous agents and mycoplasmas are performed in accordance with *Tests for extraneous agents in viral vaccines for human use (2.6.16)*, but using 200 ml of harvest in the test in cell culture for other extraneous agents.

PURIFIED ANTIGEN

Only a purified antigen that complies with the following requirements may be used in the preparation of the final bulk vaccine.

Total protein. The total protein is determined by a validated method. The content is within the limits approved for the specific product.

Antigen content and identification. The quantity and specificity of HBsAg is determined in comparison with the International Standard for HBsAg subtype *ad* or an in-house reference, by a suitable immunochemical method (2.7.1) such as radio-immunoassay (RIA), enzyme-linked immunosorbent assay (ELISA), immunoblot (preferably using a monoclonal antibody directed against a protective epitope) or single radial diffusion. The antigen/protein ratio is within the limits approved for the specific product.

The molecular weight of the major band revealed following sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) performed under reducing conditions corresponds to the value expected from the known nucleic acid and polypeptide sequences and possible glycosylation.

Antigenic purity. The purity of the antigen is determined by comparison with a reference preparation using liquid chromatography or other suitable methods such as SDS-PAGE with staining by acid blue 92 and silver. A suitable method is sensitive enough to detect a potential contaminant at a concentration of 1 per cent of total protein. Not less than 95 per cent of the total protein consists of hepatitis B surface antigen.

Composition. The content of proteins, lipids, nucleic acids and carbohydrates is determined.

Host-cell- and vector-derived DNA. If mammalian cells are used for production, not more than 10 pg of DNA in the quantity of purified antigen equivalent to a single human dose of vaccine.

Caesium. If a caesium salt is used during production, a test for residual caesium is carried out on the purified antigen. The content is within the limits approved for the specific product.

Sterility (2.6.1). The purified antigen complies with the test for sterility, carried out using 10 ml for each medium.

Additional tests on the purified antigen may be required depending on the production method used: for example, a test for residual animal serum where mammalian cells are used for production or tests for residual chemicals used during extraction and purification.

FINAL BULK VACCINE

An antimicrobial preservative and an adjuvant may be included in the vaccine.

Only a final bulk vaccine that complies with the following requirements may be used in the preparation of the final lot.

Antimicrobial preservative. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical or physico-chemical method. The amount is not less than 85 per cent and not greater than 115 per cent of the intended amount.

Sterility (2.6.1). The final bulk vaccine complies with the test for sterility, carried out using 10 ml for each medium.

FINAL LOT

Only a final lot that complies with each of the requirements given below under Identification, Tests and Assay may be released for use. Provided that the tests for free formaldehyde (where applicable) and antimicrobial preservative content (where applicable) have been carried out on the final bulk vaccine with satisfactory results, they may be omitted on the final lot. If the assay is carried out *in vivo*, then provided it has been carried out with satisfactory results on the final bulk vaccine, it may be omitted on the final lot.

IDENTIFICATION

The assay or, where applicable, the electrophoretic profile, serves also to identify the vaccine.

TESTS

Aluminium (2.5.13): maximum 1.25 mg per single human dose, if aluminium hydroxide or hydrated aluminium phosphate is used as the adsorbent.

Free formaldehyde (2.4.18): maximum 0.2 g/l.

Antimicrobial preservative. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical or physico-chemical method. The amount is not less than the minimum amount shown to be effective and is not greater than 115 per cent of that stated on the label.

Sterility (2.6.1). The vaccine complies with the test for sterility.

Pyrogens (2.6.8). The vaccine complies with the test for pyrogens. Inject the equivalent of one human dose into each rabbit.

ASSAY

The vaccine complies with the assay of hepatitis B vaccine (rDNA) (2.7.15).

LABELLING

The label states:

- the amount of HBsAg per container,
- the type of cells used for production of the vaccine,
- the name and amount of the adsorbent used,
- that the vaccine must be shaken before use,
- that the vaccine must not be frozen.

01/2005:0158

INFLUENZA VACCINE (SPLIT VIRION, INACTIVATED)

Vaccinum influenzae inactivatum
ex virorum fragmentis praeparatum

DEFINITION

Influenza vaccine (split virion, inactivated) is a sterile, aqueous suspension of a strain or strains of influenza virus, type A or B, or a mixture of strains of the two types grown individually in fertilised hens' eggs, inactivated and treated so that the integrity of the virus particles has been disrupted without diminishing the antigenic properties of

the haemagglutinin and neuraminidase antigens. The stated amount of haemagglutinin antigen for each strain present in the vaccine is 15 µg per dose, unless clinical evidence supports the use of a different amount.

The vaccine is a slightly opalescent liquid.

PRODUCTION

The production method is validated to demonstrate that the product, if tested, would comply with the test for abnormal toxicity for immunosera and vaccines for human use (2.6.9).

CHOICE OF VACCINE STRAIN

The World Health Organisation reviews the world epidemiological situation annually and if necessary recommends new strains corresponding to prevailing epidemiological evidence.

Such strains are used in accordance with the regulations in force in the signatory states of the Convention on the Elaboration of a European Pharmacopoeia. It is now common practice to use reassorted strains giving high yields of the appropriate surface antigens. The origin and passage history of virus strains shall be approved by the competent authority.

SUBSTRATE FOR VIRUS PROPAGATION

Influenza virus seed to be used in the production of vaccine is propagated in fertilised eggs from chicken flocks free from specified pathogens (5.2.2) or in suitable cell cultures (5.2.4), such as chick-embryo fibroblasts or chick kidney cells obtained from chicken flocks free from specified pathogens (5.2.2). For production, the virus of each strain is grown in the allantoic cavity of fertilised hens' eggs from healthy flocks.

VIRUS SEED LOT

The production of vaccine is based on a seed-lot system. Working seed lots represent not more than 15 passages from the approved reassorted virus or the approved virus isolate. The final vaccine represents 1 passage from the working seed lot. The haemagglutinin and neuraminidase antigens of each seed lot are identified as originating from the correct strain of influenza virus by suitable methods.

Only a working virus seed lot that complies with the following requirements may be used in the preparation of the monovalent pooled harvest.

Bacterial and fungal contamination. Carry out the test for sterility (2.6.1), using 10 ml for each medium.

Mycoplasmas (2.6.7). Carry out the test for mycoplasmas, using 10 ml.

VIRUS PROPAGATION AND HARVEST

An antimicrobial agent may be added to the inoculum. After incubation at a controlled temperature, the allantoic fluids are harvested and combined to form a monovalent pooled harvest. An antimicrobial agent may be added at the time of harvest. At no stage in the production is penicillin or streptomycin used.

MONOVALENT POOLED HARVEST

To limit the possibility of contamination, inactivation is initiated as soon as possible after preparation. The virus is inactivated by a method that has been demonstrated on three consecutive batches to be consistently effective for the manufacturer. The inactivation process shall have been shown to be capable of inactivating the influenza virus without destroying its antigenicity; the process should cause minimum alteration of the haemagglutinin and neuraminidase antigens. The inactivation process shall also have been shown to be capable of inactivating avian leucosis viruses and mycoplasmas. If the monovalent pooled harvest is stored after inactivation, it is held at a

temperature of 5 ± 3 °C. If formaldehyde solution is used, the concentration does not exceed 0.2 g/l of CH₂O at any time during inactivation; if betapropiolactone is used, the concentration does not exceed 0.1 per cent V/V at any time during inactivation.

Before or after the inactivation procedure, the monovalent pooled harvest is concentrated and purified by high-speed centrifugation or other suitable method and the virus particles are disrupted into component subunits by the use of approved procedures. For each new strain, a validation test is carried out to show that the monovalent bulk consists predominantly of disrupted virus particles.

Only a monovalent pooled harvest that complies with the following requirements may be used in the preparation of the final bulk vaccine.

Haemagglutinin antigen. Determine the content of haemagglutinin antigen by an immunodiffusion test (2.7.1), by comparison with a haemagglutinin antigen reference preparation or with an antigen preparation calibrated against it⁽¹⁾. Carry out the test at 20 °C to 25 °C.

For some vaccines, the physical form of the haemagglutinin particles prevents quantitative determination by immunodiffusion after inactivation of the virus. For these vaccines, a determination of haemagglutinin antigen is made on the monovalent pooled harvest before inactivation. The production process is validated to demonstrate suitable conservation of haemagglutinin antigen and a suitable tracer is used for formulation, for example, protein content.

Neuraminidase antigen. The presence and type of neuraminidase antigen are confirmed by suitable enzymatic or immunological methods on the first 3 monovalent pooled harvests from each working seed lot.

Sterility (2.6.1). Carry out the test for sterility, using 10 ml for each medium.

Viral inactivation. Carry out the test described below under Tests.

Chemicals used for disruption. Tests are carried out on the monovalent pooled harvest for the chemicals used for disruption, the limits being approved by the competent authority.

FINAL BULK VACCINE

Appropriate quantities of the monovalent pooled harvests are blended to make the final bulk vaccine.

Only a final bulk vaccine that complies with the following requirements may be used in the preparation of the final lot.

Antimicrobial preservative. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The content is not less than 85 per cent and not greater than 115 per cent of the intended amount.

Sterility (2.6.1). Carry out the test for sterility, using 10 ml for each medium.

FINAL LOT

The final bulk vaccine is distributed aseptically into sterile, tamper-proof containers. The containers are closed so as to prevent contamination.

Only a final lot that is satisfactory with respect to each of the requirements given below under Tests and Assay may be released for use. Provided that the test for viral inactivation has been performed with satisfactory results on each monovalent pooled harvest and that the tests for

free formaldehyde, ovalbumin and total protein have been performed with satisfactory results on the final bulk vaccine, they may be omitted on the final lot.

IDENTIFICATION

The assay serves to confirm the antigenic specificity of the vaccine.

TESTS

Viral inactivation. Inoculate 0.2 ml of the vaccine into the allantoic cavity of each of 10 fertilised eggs and incubate at 33 °C to 37 °C for 3 days. The test is not valid unless at least 8 of the 10 embryos survive. Harvest 0.5 ml of the allantoic fluid from each surviving embryo and pool the fluids. Inoculate 0.2 ml of the pooled fluid into a further 10 fertilised eggs and incubate at 33 °C to 37 °C for 3 days. The test is not valid unless at least 8 of the 10 embryos survive. Harvest about 0.1 ml of the allantoic fluid from each surviving embryo and examine each individual harvest for live virus by a haemagglutination test. If haemagglutination is found for any of the fluids, carry out for that fluid a further passage in eggs and test for haemagglutination; no haemagglutination occurs.

Total protein. Not more than 6 times the total haemagglutinin content of the vaccine as determined in the assay, but in any case, not more than 100 µg of protein per virus strain per human dose and not more than a total of 300 µg of protein per human dose.

Ovalbumin. Not more than 1 µg of ovalbumin per human dose, determined by a suitable technique using a suitable reference preparation of ovalbumin.

Free formaldehyde (2.4.18): maximum 0.2 g/l.

Antimicrobial preservative. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The content is not less than the minimum amount shown to be effective and is not greater than 115 per cent of the quantity stated on the label.

Sterility (2.6.1). It complies with the test for sterility.

Bacterial endotoxins (2.6.14): less than 100 IU per human dose.

ASSAY

Determine the content of haemagglutinin antigen by an immunodiffusion test (2.7.1), by comparison with a haemagglutinin antigen reference preparation or with an antigen preparation calibrated against it⁽¹⁾. Carry out the test at 20 °C to 25 °C. The confidence limits ($P = 0.95$) are not less than 80 per cent and not more than 125 per cent of the estimated haemagglutinin antigen content. The lower confidence limit ($P = 0.95$) is not less than 80 per cent of the amount stated on the label for each strain.

For some vaccines, quantitative determination of haemagglutinin antigen with respect to available reference preparations is not possible. An immunological identification of the haemagglutinin antigen and a semi-quantitative determination are carried out instead by suitable methods.

LABELLING

The label states:

- that the vaccine has been prepared on eggs,
- the strain or strains of influenza virus used to prepare the vaccine,
- the method of inactivation,

(1) Reference haemagglutinin antigens are available from the National Institute for Biological Standards and Control, Blanche Lane, South Mimms, Potters Bar, Hertfordshire EN6 3QG, Great Britain.

- the haemagglutinin content in micrograms per virus strain per dose,
- the season during which the vaccine is intended to protect.

01/2005:0869

INFLUENZA VACCINE (SURFACE ANTIGEN, INACTIVATED)

Vaccinum influenzae inactivatum ex corticis antigeniis praeparatum

DEFINITION

Influenza vaccine (surface antigen, inactivated) is a sterile suspension of a strain or strains of influenza virus, type A or B, or a mixture of strains of the 2 types grown individually in fertilised hens' eggs, inactivated and treated so that the preparation consists predominantly of haemagglutinin and neuraminidase antigens, without diminishing the antigenic properties of these antigens. The stated amount of haemagglutinin antigen for each strain present in the vaccine is 15 µg per dose, unless clinical evidence supports the use of a different amount. The vaccine may contain an adjuvant.

PRODUCTION

The production method is validated to demonstrate that the product, if tested, would comply with the test for abnormal toxicity for immunosera and vaccines for human use (2.6.9).

CHOICE OF VACCINE STRAIN

The World Health Organisation reviews the world epidemiological situation annually and if necessary recommends new strains corresponding to prevailing epidemiological evidence.

Such strains are used in accordance with the regulations in force in the signatory states of the Convention on the Elaboration of a European Pharmacopoeia. It is now common practice to use reassorted strains giving high yields of the appropriate surface antigens. The origin and passage history of virus strains shall be approved by the competent authority.

SUBSTRATE FOR VIRUS PROPAGATION

Influenza virus seed to be used in the production of vaccine is propagated in fertilised eggs from chicken flocks free from specified pathogens (SPF) (5.2.2) or in suitable cell cultures (5.2.4), such as chick-embryo fibroblasts or chick kidney cells obtained from SPF chicken flocks (5.2.2). For production, the virus of each strain is grown in the allantoic cavity of fertilised hens' eggs from healthy flocks.

VIRUS SEED LOT

The production of vaccine is based on a seed-lot system. Working seed lots represent not more than 15 passages from the approved reassorted virus or the approved virus isolate. The final vaccine represents one passage from the working seed lot. The haemagglutinin and neuraminidase antigens of each seed lot are identified as originating from the correct strain of influenza virus by suitable methods.

Only a working virus seed lot that complies with the following requirements may be used in the preparation of the monovalent pooled harvest.

Bacterial and fungal contamination. Carry out the test for sterility (2.6.1), using 10 ml for each medium.

Mycoplasmas (2.6.7). Carry out the test for mycoplasmas, using 10 ml.

VIRUS PROPAGATION AND HARVEST

An antimicrobial agent may be added to the inoculum. After incubation at a controlled temperature, the allantoic fluids are harvested and combined to form a monovalent pooled harvest. An antimicrobial agent may be added at the time of harvest. At no stage in the production is penicillin or streptomycin used.

MONOVALENT POOLED HARVEST

To limit the possibility of contamination, inactivation is initiated as soon as possible after preparation. The virus is inactivated by a method that has been demonstrated on 3 consecutive batches to be consistently effective for the manufacturer. The inactivation process shall have been shown to be capable of inactivating the influenza virus without destroying its antigenicity; the process should cause minimum alteration of the haemagglutinin and neuraminidase antigens. The inactivation process shall also have been shown to be capable of inactivating avian leucosis viruses and mycoplasmas. If the monovalent pooled harvest is stored after inactivation, it is held at 5 ± 3 °C. If formaldehyde solution is used, the concentration does not exceed 0.2 g/l of CH₂O at any time during inactivation; if betapropiolactone is used, the concentration does not exceed 0.1 per cent V/V at any time during inactivation.

Before or after the inactivation process, the monovalent pooled harvest is concentrated and purified by high-speed centrifugation or other suitable method. Virus particles are disrupted into component subunits by approved procedures and further purified so that the monovalent bulk consists mainly of haemagglutinin and neuraminidase antigens.

Only a monovalent pooled harvest that complies with the following requirements may be used in the preparation of the final bulk vaccine.

Haemagglutinin antigen. Determine the content of haemagglutinin antigen by an immunodiffusion test (2.7.1), by comparison with a haemagglutinin antigen reference preparation or with an antigen preparation calibrated against it⁽²⁾. Carry out the test at 20-25 °C.

Neuraminidase antigen. The presence and type of neuraminidase antigen are confirmed by suitable enzymatic or immunological methods on the first 3 monovalent pooled harvests from each working seed lot.

Sterility (2.6.1). Carry out the test for sterility, using 10 ml for each medium.

Viral inactivation. Carry out the test described below under Tests.

Purity. The purity of the monovalent pooled harvest is examined by polyacrylamide gel electrophoresis or by other approved techniques. Mainly haemagglutinin and neuraminidase antigens shall be present.

Chemicals used for disruption and purification. Tests are carried out on the monovalent pooled harvest for the chemicals used for disruption and purification, the limits being approved by the competent authority.

FINAL BULK VACCINE

Appropriate quantities of the monovalent pooled harvests are blended to make the final bulk vaccine. An adjuvant may be added.

Only a final bulk vaccine that complies with the following requirements may be used in the preparation of the final lot.

(2) Reference haemagglutinin antigens are available from the National Institute for Biological Standards and Control, Blanche Lane, South Mimms, Potters Bar, Hertfordshire EN6 3QG, Great Britain.

Antimicrobial preservative. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The content is not less than 85 per cent and not greater than 115 per cent of the intended amount.

Sterility (2.6.1). Carry out the test for sterility, using 10 ml for each medium.

FINAL LOT

The final bulk vaccine is distributed aseptically into sterile, tamper-proof containers. The containers are closed so as to prevent contamination.

Only a final lot that is satisfactory with respect to each of the requirements given below under Tests and Assay may be released for use.

Provided that the test for viral inactivation has been performed with satisfactory results on each monovalent pooled harvest and that the tests for free formaldehyde, ovalbumin and total protein have been performed with satisfactory results on the final bulk vaccine, they may be omitted on the final lot.

If the ovalbumin and formaldehyde content cannot be determined on the final lot, owing to interference from the adjuvant, they are determined on the monovalent pooled harvest, the acceptance limits being set to ensure that the limits for the final product will not be exceeded.

If the vaccine contains an adjuvant, suitable tests for identity and other relevant quality criteria are carried out on the final lot. These tests may include chemical and physical analysis, determination of particle size and determination of the number of particles per unit volume.

IDENTIFICATION

The assay serves to confirm the antigenic specificity of the vaccine.

TESTS

Viral inactivation. Inoculate 0.2 ml of the vaccine into the allantoic cavity of each of 10 fertilised eggs and incubate at 33-37 °C for 3 days. The test is not valid unless at least 8 of the 10 embryos survive. Harvest 0.5 ml of the allantoic fluid from each surviving embryo and pool the fluids. Inoculate 0.2 ml of the pooled fluid into a further 10 fertilised eggs and incubate at 33-37 °C for 3 days. The test is not valid unless at least 8 of the 10 embryos survive. Harvest about 0.1 ml of the allantoic fluid from each surviving embryo and examine each individual harvest for live virus by a haemagglutination test. If haemagglutination is found for any of the fluids, carry out for that fluid a further passage in eggs and test for haemagglutination; no haemagglutination occurs.

Total protein. Not more than 40 µg of protein other than haemagglutinin per virus strain per human dose and not more than a total of 120 µg of protein other than haemagglutinin per human dose.

Free formaldehyde (2.4.18): maximum 0.2 g/l.

Antimicrobial preservative. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The content is not less than the minimum amount shown to be effective and is not greater than 115 per cent of the quantity stated on the label.

Ovalbumin. Not more than 1 µg of ovalbumin per human dose, determined by a suitable immunochemical method (2.7.1) using a suitable reference preparation of ovalbumin.

Sterility. It complies with the test for sterility (2.6.1).

Bacterial endotoxins (2.6.14): less than 100 IU per human dose.

ASSAY

Determine the content of haemagglutinin antigen by an immunodiffusion test (2.7.1), by comparison with a haemagglutinin antigen reference preparation or with an antigen preparation calibrated against it⁽²⁾. Carry out the test at 20-25 °C. The confidence limits ($P = 0.95$) are not less than 80 per cent and not more than 125 per cent of the estimated haemagglutinin antigen content. The lower confidence limit ($P = 0.95$) is not less than 80 per cent of the amount stated on the label for each strain.

LABELLING

The label states:

- that the vaccine has been prepared on eggs,
- the strain or strains of influenza virus used to prepare the vaccine,
- the method of inactivation,
- the haemagglutinin content in micrograms per virus strain per dose,
- the season during which the vaccine is intended to protect,
- where applicable, the name and the quantity of adjuvant used.

01/2005:2053

INFLUENZA VACCINE (SURFACE ANTIGEN, INACTIVATED, VIROSOME)

Vaccinum influenzae inactivatum ex corticis antigeniis praeparatum virosomale

DEFINITION

Influenza vaccine (surface antigen, inactivated, virosome) is a sterile, aqueous suspension of a strain or strains of influenza virus, type A or B, or a mixture of strains of the 2 types grown individually in fertilised hens' eggs, inactivated and treated so that the preparation consists predominantly of haemagglutinin and neuraminidase antigens reconstituted to virosomes with phospholipids and without diminishing the antigenic properties of the antigens. The stated amount of haemagglutinin antigen for each strain present in the vaccine is 15 µg per dose, unless clinical evidence supports the use of a different amount.

CHARACTERS

The vaccine is a slightly opalescent liquid.

PRODUCTION

GENERAL PROVISIONS

The production method shall have been shown to yield consistently vaccines comparable with the vaccine of proven clinical efficacy and safety in man.

The production method is validated to demonstrate that the product, if tested, would comply with the test for abnormal toxicity for immunosera and vaccines for human use (2.6.9).

CHOICE OF VACCINE STRAIN

The World Health Organisation reviews the world epidemiological situation annually and if necessary recommends new strains corresponding to prevailing epidemiological evidence.

Such strains are used in accordance with the regulations in force in the signatory states of the Convention on the Elaboration of a European Pharmacopoeia. It is now common practice to use reassorted strains giving high yields

of the appropriate surface antigens. The origin and passage history of virus strains shall be approved by the competent authority.

SUBSTRATE FOR VIRUS PROPAGATION

Influenza virus seed to be used in the production of vaccine is propagated in fertilised eggs from chicken flocks free from specified pathogens (5.2.2) or in suitable cell cultures (5.2.4), such as chick-embryo fibroblasts or chick kidney cells obtained from chicken flocks free from specified pathogens (5.2.2). For production, the virus of each strain is grown in the allantoic cavity of fertilised hens' eggs from healthy flocks.

VIRUS SEED LOT

The production of vaccine is based on a seed lot system. Working seed lots represent not more than 15 passages from the approved reassorted virus or the approved virus isolate. The final vaccine represents 1 passage from the working seed lot. The haemagglutinin and neuraminidase antigens of each seed lot are identified as originating from the correct strain of influenza virus by suitable methods.

Only a working virus seed lot that complies with the following requirements may be used in the preparation of the monovalent pooled harvest.

Bacterial and fungal contamination. Carry out the test for sterility (2.6.1), using 10 ml for each medium.

Mycoplasmas (2.6.7). Carry out the test for mycoplasmas, using 10 ml.

VIRUS PROPAGATION AND HARVEST

An antimicrobial agent may be added to the inoculum. After incubation at a controlled temperature, the allantoic fluids are harvested and combined to form a monovalent pooled harvest. An antimicrobial agent may be added at the time of harvest.

MONOVALENT POOLED HARVEST

To limit the possibility of contamination, inactivation is initiated as soon as possible after preparation. The virus is inactivated by a method that has been demonstrated on 3 consecutive batches to be consistently effective for the manufacturer. The inactivation process shall have been shown to be capable of inactivating the influenza virus without destroying its antigenicity; the process is designed so as to cause minimum alteration of the haemagglutinin and neuraminidase antigens. The inactivation process shall also have been shown to be capable of inactivating avian leucosis viruses and mycoplasmas. If the monovalent pooled harvest is stored after inactivation, it is held at a temperature of 5 ± 3 °C. If formaldehyde solution is used, the concentration does not exceed 0.2 g/l of CH₂O at any time during inactivation; if betapropiolactone is used, the concentration does not exceed 0.1 per cent V/V at any time during inactivation.

Before or after the inactivation process, the monovalent pooled harvest is concentrated and purified by high-speed centrifugation or other suitable method.

Only a monovalent pooled harvest that complies with the following requirements may be used for the preparation of virosomes.

Haemagglutinin antigen. Determine the content of haemagglutinin antigen by an immunodiffusion test (2.7.1), by comparison with a haemagglutinin antigen reference preparation or with an antigen preparation calibrated against it⁽³⁾. Carry out the test at 20-25 °C.

Neuraminidase antigen. The presence and type of neuraminidase antigen are confirmed by suitable enzymatic or immunological methods on the first 3 monovalent pooled harvests from each working seed lot.

Viral inactivation. Carry out the test described under Tests.

PREPARATION OF MONOVALENT VIROSOMES

Virus particles are disrupted into component subunits by a suitable detergent and further purified so that the monovalent bulk consists mainly of haemagglutinin and neuraminidase antigens. After addition of suitable phospholipids, solubilisation by ultrasonication and sterile filtration, virosomal preparations are formed by removal of the detergent either by adsorption chromatography or another suitable technique. Several monovalent virosomal preparations may be pooled.

Only a monovalent virosomal preparation that complies with the following requirements may be used in the preparation of the final bulk vaccine.

Haemagglutinin antigen. Determine the content of haemagglutinin antigen by an immunodiffusion test (2.7.1), by comparison with a haemagglutinin antigen reference preparation or with an antigen preparation calibrated against it⁽³⁾. Carry out the test at 20-25 °C.

Neuraminidase antigen. The presence and type of neuraminidase antigen are confirmed by suitable enzymatic or immunological methods on the first 3 virosomal preparations from each working seed lot.

Sterility (2.6.1). Carry out the test for sterility, using 10 ml for each medium.

Purity. The purity of the virosomal preparation is examined by polyacrylamide gel electrophoresis (2.2.31) or by other approved techniques. Mainly haemagglutinin and neuraminidase antigens are present.

Residual chemicals. Tests for the chemicals used for disruption and purification are carried out on the virosomal preparation, the limits being approved by the competent authority.

Phospholipid. The content and identity of the phospholipids is determined by suitable immunochemical or physico-chemical methods.

Ratio of haemagglutinin to phospholipid. The ratio of haemagglutinin content to phospholipid content is within the limits approved for the particular product.

Virosome size distribution. The size of the virosomes, determined by a suitable method such as laser light scattering, is not less than 100 nm and not greater than 500 nm.

FINAL BULK VACCINE

Appropriate quantities of the virosomal preparations are blended to make the final bulk vaccine.

Only a final bulk vaccine that complies with the following requirements may be used in the preparation of the final lot.

Antimicrobial preservative. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The content is not less than 85 per cent and not greater than 115 per cent of the intended amount.

Sterility (2.6.1). Carry out the test for sterility, using 10 ml for each medium.

FINAL LOT

The final bulk vaccine is distributed aseptically into sterile, tamper-proof containers. The containers are closed so as to prevent contamination.

(3) Reference haemagglutinin antigens are available from the National Institute for Biological Standards and Control (NIBSC), Blanche Lane, South Mimms, Potters Bar, Hertfordshire EN6 3QC, United Kingdom.

Only a final lot that is satisfactory with respect to each of the requirements given under Tests and Assay may be released for use. Provided that the test for viral inactivation has been performed with satisfactory results on each monovalent pooled harvest and that the tests for phospholipid, ratio of haemagglutinin to phospholipid, free formaldehyde, ovalbumin and total protein have been performed with satisfactory results on the final bulk vaccine, they may be omitted on the final lot.

IDENTIFICATION

The assay serves to confirm the antigenic specificity of the vaccine.

TESTS

Viral inactivation. Inoculate 0.2 ml of the vaccine into the allantoic cavity of each of 10 fertilised eggs and incubate at 33-37 °C for 3 days. The test is not valid unless at least 8 of the 10 embryos survive. Harvest 0.5 ml of the allantoic fluid from each surviving embryo and pool the fluids. Inoculate 0.2 ml of the pooled fluid into a further 10 fertilised eggs and incubate at 33-37 °C for 3 days. The test is not valid unless at least 8 of the 10 embryos survive. Harvest about 0.1 ml of the allantoic fluid from each surviving embryo and examine each individual harvest for live virus by a haemagglutination test. If haemagglutination is found for any of the fluids, carry out for that fluid a further passage in eggs and test for haemagglutination; no haemagglutination occurs.

pH (2.2.3): 6.5 to 7.8

Total protein: maximum 40 µg of protein other than haemagglutinin per virus strain per human dose and maximum 120 µg of protein other than haemagglutinin per human dose.

Phospholipid. The content and identity of the phospholipids is determined by a suitable immunochemical or physico-chemical method.

Ratio of haemagglutinin to phospholipid. The ratio of haemagglutinin content to phospholipid content is within the limits approved for the particular product.

Free formaldehyde (2.4.18): where applicable, maximum 0.2 g/l.

Antimicrobial preservative. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The content is not less than the minimum amount shown to be effective and is not greater than 115 per cent of the quantity stated on the label.

Ovalbumin: maximum 50 ng of ovalbumin per human dose, determined by a suitable technique using a suitable reference preparation of ovalbumin.

Sterility (2.6.1). It complies with the test for sterility.

Virosome size distribution. The size of the virosomes, determined by a suitable method such as laser light scattering, is not less than 100 nm and not greater than 500 nm.

Bacterial endotoxins (2.6.14): maximum 100 IU per human dose.

ASSAY

Determine the content of haemagglutinin antigen by an immunodiffusion test (2.7.1), by comparison with a haemagglutinin antigen reference preparation or with an antigen preparation calibrated against it⁽³⁾. Carry out the test at 20-25 °C. The confidence limits ($P = 0.95$) are not less than 80 per cent and not more than 125 per cent of

the estimated haemagglutinin antigen content. The lower confidence limit ($P = 0.95$) is not less than 80 per cent of the amount stated on the label for each strain.

LABELLING

The label states:

- that the vaccine has been prepared on eggs,
- the strain or strains of influenza virus used to prepare the vaccine,
- the method of inactivation,
- the haemagglutinin content, in micrograms per virus strain per dose,
- the season during which the vaccine is intended to protect.

01/2005:0159

INFLUENZA VACCINE (WHOLE VIRION, INACTIVATED)

*Vaccinum influenzae inactivatum
ex viris integris praeparatum*

DEFINITION

Influenza vaccine (whole virion, inactivated) is a sterile, aqueous suspension of a strain or strains of influenza virus, type A or B, or a mixture of strains of the two types grown individually in fertilised hens' eggs and inactivated in such a manner that their antigenic properties are retained. The stated amount of haemagglutinin antigen for each strain present in the vaccine is 15 µg per dose, unless clinical evidence supports the use of a different amount.

The vaccine is a slightly opalescent liquid.

PRODUCTION

The production method is validated to demonstrate that the product, if tested, would comply with the test for abnormal toxicity for immunosera and vaccines for human use (2.6.9).

CHOICE OF VACCINE STRAIN

The World Health Organisation reviews the world epidemiological situation annually and if necessary recommends new strains corresponding to prevailing epidemiological evidence.

Such strains are used in accordance with the regulations in force in the signatory states of the Convention on the Elaboration of a European Pharmacopoeia. It is now common practice to use reassorted strains giving high yields of the appropriate surface antigens. The origin and passage history of virus strains shall be approved by the competent authority.

SUBSTRATE FOR VIRUS PROPAGATION

Influenza virus seed to be used in the production of vaccine is propagated in fertilised eggs from chicken flocks free from specified pathogens (5.2.2) or in suitable cell cultures (5.2.4), such as chick-embryo fibroblasts or chick kidney cells obtained from chicken flocks free from specified pathogens (5.2.2). For production, the virus of each strain is grown in the allantoic cavity of fertilised hens' eggs from healthy flocks.

VIRUS SEED LOT

The production of vaccine is based on a seed-lot system. Working seed lots represent not more than 15 passages from the approved reassorted virus or the approved virus isolate. The final vaccine represents 1 passage from the working

seed lot. The haemagglutinin and neuraminidase antigens of each seed lot are identified as originating from the correct strain of influenza virus by suitable methods.

Only a working virus seed lot that complies with the following requirements may be used in the preparation of the monovalent pooled harvest.

Bacterial and fungal contamination. Carry out the test for sterility (2.6.1), using 10 ml for each medium.

Mycoplasmas (2.6.7). Carry out the test for mycoplasmas, using 10 ml.

VIRUS PROPAGATION AND HARVEST

An antimicrobial agent may be added to the inoculum. After incubation at a controlled temperature, the allantoic fluids are harvested and combined to form a monovalent pooled harvest. An antimicrobial agent may be added at the time of harvest. At no stage in the production is penicillin or streptomycin used.

MONOVALENT POOLED HARVEST

To limit the possibility of contamination, inactivation is initiated as soon as possible after preparation. The virus is inactivated by a method that has been demonstrated on three consecutive batches to be consistently effective for the manufacturer. The inactivation process shall have been shown to be capable of inactivating the influenza virus without destroying its antigenicity; the process should cause minimum alteration of the haemagglutinin and neuraminidase antigens. The inactivation process shall also have been shown to be capable of inactivating avian leucosis viruses and mycoplasmas. If the monovalent pooled harvest is stored after inactivation, it is held at a temperature of 5 ± 3 °C. If formaldehyde solution is used, the concentration does not exceed 0.2 g/l of CH₂O at any time during inactivation; if betapropiolactone is used, the concentration does not exceed 0.1 per cent V/V at any time during inactivation.

Before or after the inactivation process, the monovalent pooled harvest is concentrated and purified by high-speed centrifugation or other suitable method.

Only a monovalent pooled harvest that complies with the following requirements may be used in the preparation of the final bulk vaccine.

Haemagglutinin antigen. Determine the content of haemagglutinin antigen by an immunodiffusion test (2.7.1), by comparison with a haemagglutinin antigen reference preparation or with an antigen preparation calibrated against it⁽⁴⁾. Carry out the test at 20 °C to 25 °C.

Neuraminidase antigen. The presence and type of neuraminidase antigen are confirmed by suitable enzymatic or immunological methods on the first 3 monovalent pooled harvests from each working seed lot.

Sterility (2.6.1). Carry out the test for sterility, using 10 ml for each medium.

Viral inactivation. Carry out the test described below under Tests.

FINAL BULK VACCINE

Appropriate quantities of the monovalent pooled harvests are blended to make the final bulk vaccine.

Only a final bulk vaccine that complies with the following requirements may be used in the preparation of the final lot.

Antimicrobial preservative. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The content is not less than 85 per cent and not greater than 115 per cent of the intended amount.

Sterility (2.6.1). Carry out the test for sterility using 10 ml for each medium.

FINAL LOT

The final bulk vaccine is distributed aseptically into sterile, tamper-proof containers. The containers are closed so as to prevent contamination.

Only a final lot that is satisfactory with respect to each of the requirements given below under Tests and Assay may be released for use. Provided that the test for viral inactivation has been performed with satisfactory results on each monovalent pooled harvest and that the tests for free formaldehyde, ovalbumin and total protein have been performed with satisfactory results on the final bulk vaccine, they may be omitted on the final lot.

IDENTIFICATION

The assay serves to confirm the antigenic specificity of the vaccine.

TESTS

Viral inactivation. Inoculate 0.2 ml of the vaccine into the allantoic cavity of each of 10 fertilised eggs and incubate at 33 °C to 37 °C for 3 days. The test is not valid unless at least 8 of the 10 embryos survive. Harvest 0.5 ml of the allantoic fluid from each surviving embryo and pool the fluids. Inoculate 0.2 ml of the pooled fluid into a further 10 fertilised eggs and incubate at 33 °C to 37 °C for 3 days. The test is not valid unless at least 8 of the 10 embryos survive. Harvest about 0.1 ml of the allantoic fluid from each surviving embryo and examine each individual harvest for live virus by a haemagglutination test. If haemagglutination is found for any of the fluids, carry out for that fluid a further passage in eggs and test for haemagglutination; no haemagglutination occurs.

Total protein. Not more than 6 times the total haemagglutinin content of the vaccine as determined in the assay, but in any case, not more than 100 µg of protein per virus strain per human dose and not more than a total of 300 µg of protein per human dose.

Ovalbumin. Not more than 1 µg of ovalbumin per human dose, determined by a suitable technique using a suitable reference preparation of ovalbumin.

Free formaldehyde (2.4.18): maximum 0.2 g/l.

Antimicrobial preservative. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The content is not less than the minimum amount shown to be effective and is not greater than 115 per cent of the quantity stated on the label.

Sterility (2.6.1). It complies with the test for sterility.

Bacterial endotoxins (2.6.14): less than 100 IU per human dose.

ASSAY

Determine the content of haemagglutinin antigen by an immunodiffusion test (2.7.1), by comparison with a haemagglutinin antigen reference preparation or with an antigen preparation calibrated against it⁽⁴⁾. Carry out the test at 20 °C to 25 °C. The confidence limits ($P = 0.95$) are not less than 80 per cent and not more than 125 per cent of

(4) Reference haemagglutinin antigens are available from the National Institute for Biological Standards and Control, Blanche Lane, South Mimms, Potters Bar, Hertfordshire EN6 3QG, Great Britain.

the estimated haemagglutinin antigen content. The lower confidence limit ($P = 0.95$) is not less than 80 per cent of the amount stated on the label for each strain.

LABELLING

The label states:

- that the vaccine has been prepared on eggs,
- the strain or strains of influenza virus used to prepare the vaccine,
- the method of inactivation,
- the haemagglutinin content in micrograms per virus strain per dose,
- the season during which the vaccine is intended to protect.

01/2005:1057
corrected

MEASLES, MUMPS AND RUBELLA VACCINE (LIVE)

Vaccinum morbillorum, parotitidis et rubellae vivum

DEFINITION

Measles, mumps and rubella vaccine (live) is a freeze-dried preparation of suitable attenuated strains of measles virus, mumps virus and rubella virus.

The vaccine is reconstituted immediately before use, as stated on the label, to give a clear liquid that may be coloured owing to the presence of a pH indicator.

PRODUCTION

The three components are prepared as described in the monographs on *Measles vaccine (live)* (0213), *Mumps vaccine (live)* (0538) and *Rubella vaccine (live)* (0162) and comply with the requirements prescribed therein.

The production method is validated to demonstrate that the product, if tested, would comply with the test for abnormal toxicity for immunosera and vaccines for human use (2.6.9).

FINAL BULK VACCINE

Virus harvests for each component are pooled and clarified to remove cells. A suitable stabiliser may be added and the pooled harvests diluted as appropriate. Suitable quantities of the pooled harvest for each component are mixed.

Only a final bulk vaccine that complies with the following requirement may be used in the preparation of the final lot.

Bacterial and fungal contamination. Carry out the test for sterility (2.6.1), using 10 ml for each medium.

FINAL LOT

For each component, a minimum virus concentration for release of the product is established such as to ensure, in the light of stability data, that the minimum concentration stated on the label will be present at the end of the period of validity.

Only a final lot that complies with the requirements for minimum virus concentration of each component for release, with the following requirement for thermal stability and with each of the requirements given below under Identification and Tests may be released for use. Provided that the tests for bovine serum albumin and, where applicable, for ovalbumin have been carried out with satisfactory results on the final bulk vaccine, they may be omitted on the final lot.

Thermal stability. Maintain samples of the final lot of freeze-dried vaccine in the dry state at 37 °C for 7 days. Determine the virus concentration as described under Assay in parallel for the heated vaccine and for unheated vaccine stored at 5 ± 3 °C. For each component, the virus concentration of the heated vaccine is not more than $1.0 \log_{10}$ lower than that of the unheated vaccine.

IDENTIFICATION

When the vaccine reconstituted as stated on the label is mixed with antibodies specific for measles virus, mumps virus and rubella virus, it is no longer able to infect cell cultures susceptible to these viruses. When the vaccine reconstituted as stated on the label is mixed with quantities of specific antibodies sufficient to neutralise any two viral components, the third viral component infects susceptible cell cultures.

TESTS

Bacterial and fungal contamination. The reconstituted vaccine complies with the test for sterility (2.6.1).

Bovine serum albumin. Not more than 50 ng per single human dose, determined by a suitable immunochemical method (2.7.1).

Ovalbumin. If the mumps component is produced in chick embryos, the vaccine contains not more than 1 µg of ovalbumin per single human dose, determined by a suitable immunochemical method (2.7.1).

Water (2.5.12). Not more than 3.0 per cent, determined by the semi-micro determination of water.

ASSAY

A. Mix the vaccine with a sufficient quantity of antibodies specific for mumps virus. Titrate the vaccine for infective measles virus at least in triplicate, using at least five cell cultures for each $0.5 \log_{10}$ dilution step or by a method of equal precision. Use an appropriate virus reference preparation to validate each assay. The estimated measles virus concentration is not less than that stated on the label; the minimum measles virus concentration stated on the label is not less than 1×10^3 CCID₅₀ per single human dose. The assay is not valid if the confidence interval ($P = 0.95$) of the logarithm of the virus concentration is greater than ± 0.3 .

Measles vaccine (live) BRP is suitable for use as a reference preparation.

B. Mix the vaccine with a sufficient quantity of antibodies specific for measles virus. Titrate the vaccine for infective mumps virus at least in triplicate, using at least five cell cultures for each $0.5 \log_{10}$ dilution step or by a method of equal precision. Use an appropriate virus reference preparation to validate each assay. The estimated mumps virus concentration is not less than that stated on the label; the minimum mumps virus concentration stated on the label is not less than 5×10^3 CCID₅₀ per single human dose. The assay is not valid if the confidence interval ($P = 0.95$) of the logarithm of the virus concentration is greater than ± 0.3 .

Mumps vaccine (live) BRP is suitable for use as a reference preparation.

C. Mix the vaccine with a sufficient quantity of antibodies specific for mumps virus. Titrate the vaccine for infective rubella virus at least in triplicate, using at least five cell cultures for each $0.5 \log_{10}$ dilution step or by a method of equal precision. Use an appropriate virus reference preparation to validate each assay. The estimated rubella virus concentration is not less than that stated on the label; the minimum rubella virus concentration stated on the label is not less than 1×10^3 CCID₅₀.

per single human dose. The assay is not valid if the confidence interval ($P = 0.95$) of the logarithm of the virus concentration is greater than ± 0.3 .

Rubella vaccine (live) BRP is suitable for use as a reference preparation.

LABELLING

The label states:

- the strains of virus used in the preparation of the vaccine,
- where applicable, that chick embryos have been used for the preparation of the vaccine,
- the type and origin of the cells used for the preparation of the vaccine,
- the minimum virus concentration for each component of the vaccine,
- that contact with disinfectants is to be avoided,
- the time within which the vaccine must be used after reconstitution,
- that the vaccine must not be given to a pregnant woman and that a woman must not become pregnant within 2 months after having the vaccine.

01/2005:0213
corrected

MEASLES VACCINE (LIVE)

Vaccinum morbillorum vivum

DEFINITION

Measles vaccine (live) is a freeze-dried preparation of a suitable attenuated strain of measles virus. The vaccine is reconstituted immediately before use, as stated on the label, to give a clear liquid that may be coloured owing to the presence of a pH indicator.

PRODUCTION

The production of vaccine is based on a virus seed-lot system and, if the virus is propagated in human diploid cells, a cell-bank system. The production method shall have been shown to yield consistently live measles vaccines of adequate immunogenicity and safety in man. Unless otherwise justified and authorised, the virus in the final vaccine shall have undergone no more passages from the master seed lot than were used to prepare the vaccine shown in clinical studies to be satisfactory with respect to safety and efficacy; even with authorised exceptions, the number of passages beyond the level used for clinical studies shall not exceed five.

The production method is validated to demonstrate that the product, if tested, would comply with the test for abnormal toxicity for immunosera and vaccines for human use (2.6.9).

SUBSTRATE FOR VIRUS PROPAGATION

The virus is propagated in human diploid cells (5.2.3) or in cultures of chick-embryo cells derived from a chicken flock free from specified pathogens (5.2.2).

SEED LOT

The strain of measles virus used shall be identified by historical records that include information on the origin of the strain and its subsequent manipulation. To avoid the unnecessary use of monkeys in the test for neurovirulence, virus seed lots are prepared in large quantities and stored at temperatures below -20°C if freeze-dried, or below -60°C if not freeze-dried.

Only a seed lot that complies with the following requirements may be used for virus propagation.

Identification. The master and working seed lots are identified as measles virus by serum neutralisation in cell culture, using specific antibodies.

Virus concentration. The virus concentration of the master and working seed lots is determined to monitor consistency of production.

Extraneous agents (2.6.16). The working seed lot complies with the requirements for seed lots.

Neurovirulence (2.6.18). The working seed lot complies with the test for neurovirulence of live virus vaccines. *Macaca* and *Cercopithecus* monkeys susceptible to measles virus are suitable for the test.

PROPAGATION AND HARVEST

All processing of the cell bank and subsequent cell cultures is done under aseptic conditions in an area where no other cells are handled. Suitable animal (but not human) serum may be used in the growth medium, but the final medium for maintaining cell growth during virus multiplication does not contain animal serum. Serum and trypsin used in the preparation of cell suspensions and culture media are shown to be free from extraneous agents. The cell culture medium may contain a pH indicator such as phenol red and suitable antibiotics at the lowest effective concentration. It is preferable to have a substrate free from antibiotics during production. Not less than 500 ml of the production cell culture is set aside as uninfected cell cultures (control cells). The viral suspensions are harvested at a time appropriate to the strain of virus being used.

Only a single harvest that complies with the following requirements may be used in the preparation of the final bulk vaccine.

Identification. The single harvest contains virus that is identified as measles virus by serum neutralisation in cell culture, using specific antibodies.

Virus concentration. The virus concentration in the single harvest is determined as prescribed under Assay to monitor consistency of production and to determine the dilution to be used for the final bulk vaccine.

Extraneous agents (2.6.16). The single harvest complies with the tests for extraneous agents.

Control cells. If human diploid cells are used for production, the control cells comply with a test for identification. They comply with the tests for extraneous agents (2.6.16).

FINAL BULK VACCINE

Virus harvests that comply with the above tests are pooled and clarified to remove cells. A suitable stabiliser may be added and the pooled harvests diluted as appropriate.

Only a final bulk vaccine that complies with the following requirement may be used in the preparation of the final lot.

Bacterial and fungal contamination. The final bulk vaccine complies with the test for sterility (2.6.1), carried out using 10 ml for each medium.

FINAL LOT

A minimum virus concentration for release of the product is established such as to ensure, in the light of stability data, that the minimum concentration stated on the label will be present at the end of the period of validity.

Only a final lot that complies with the requirements for minimum virus concentration for release, with the following requirement for thermal stability and with each of the requirements given below under Identification and Tests may be released for use. Provided that the test for bovine serum albumin has been carried out with satisfactory results on the final bulk vaccine, it may be omitted on the final lot.

Thermal stability. Maintain samples of the final lot of freeze-dried vaccine in the dry state at 37 °C for 7 days. Determine the virus concentration as described under Assay in parallel for the heated vaccine and for unheated vaccine stored at 5 ± 3 °C. The virus concentration of the heated vaccine is not more than 1.0 log₁₀ lower than that of the unheated vaccine.

IDENTIFICATION

When the vaccine reconstituted as stated on the label is mixed with specific measles antibodies, it is no longer able to infect susceptible cell cultures.

TESTS

Bacterial and fungal contamination. The reconstituted vaccine complies with the test for sterility (2.6.1).

Bovine serum albumin. Not more than 50 ng per single human dose, determined by a suitable immunochemical method (2.7.1).

Water (2.5.12). Not more than 3.0 per cent, determined by the semi-micro determination of water.

ASSAY

Titrate the vaccine for infective virus at least in triplicate, using at least five cell cultures for each 0.5 log₁₀ dilution step or by a method of equal precision. Use an appropriate virus reference preparation to validate each assay. The estimated virus concentration is not less than that stated on the label; the minimum virus concentration stated on the label is not less than 1 × 10³ CCID₅₀ per human dose. The assay is not valid if the confidence interval ($P = 0.95$) of the logarithm of the virus concentration is greater than ± 0.3.

Measles vaccine (live) BRP is suitable for use as a reference preparation.

LABELLING

The label states:

- the strain of virus used for the preparation of the vaccine,
- the type and origin of the cells used for the preparation of the vaccine,
- the minimum virus concentration,
- that contact with disinfectants is to be avoided,
- the time within which the vaccine must be used after reconstitution.

01/2005:2112

MENINGOCOCCAL GROUP C CONJUGATE VACCINE

*Vaccinum meningococcale classis C
coniugatum*

DEFINITION

Meningococcal group C conjugate vaccine is a liquid or freeze-dried preparation of purified capsular polysaccharide derived from a suitable strain of *Neisseria meningitidis* group C covalently linked to a carrier protein. Meningococcal group C polysaccharide consists of partly *O*-acetylated or *O*-deacetylated repeating units of sialic acids, linked with 2α→9 glycosidic bonds. The carrier protein, when conjugated to group C polysaccharide, is capable of inducing a T-cell-dependent B-cell immune response to the polysaccharide. The vaccine may contain an adjuvant.

PRODUCTION

GENERAL PROVISIONS

The production method shall consistently have been shown to yield meningococcal group C conjugate vaccines of satisfactory immunogenicity and safety in man. The production of meningococcal group C polysaccharide and of the carrier protein are based on seed-lot systems.

During development studies and wherever revalidation is necessary, a test for pyrogens in rabbits (2.6.8) is carried out by injection of a suitable dose of the final lot. The vaccine is shown to be acceptable with respect to absence of pyrogenic activity.

The production method is validated to demonstrate that the vaccine, if tested, would comply with the test for abnormal toxicity for immunosera and vaccines for human use (2.6.9).

During development studies and wherever revalidation of the manufacturing process is necessary, it shall be demonstrated by tests in animals that the vaccine consistently induces a T-cell-dependent B-cell immune response.

The stability of the final lot and relevant intermediates is evaluated using 1 or more indicator tests. Such tests may include determination of molecular size, determination of free saccharide in the conjugate or an immunogenicity test in animals.

BACTERIAL SEED LOTS

The bacterial strains used for master seed lots shall be identified by historical records that include information on their origin and the tests used to characterise the strain. Cultures from the working seed lot shall have the same characteristics as the strain that was used to prepare the master seed lot.

Purity of bacterial cultures is verified by methods of suitable sensitivity. These may include inoculation into suitable media, examination of colony morphology, microscopic examination of Gram-stained smears and culture agglutination with suitable specific antisera.

MENINGOCOCCAL GROUP C POLYSACCHARIDE

N. meningitidis is grown in a liquid medium that does not contain high-molecular-mass polysaccharides and is free from ingredients that will form a precipitate upon addition of cetyltrimethylammonium bromide (CTAB). The culture may be inactivated by heat and filtered before the polysaccharide is precipitated by addition of CTAB. The precipitate is further purified using suitable methods to remove nucleic acids, proteins and lipopolysaccharides and the final purification step consists of ethanol precipitation. An *O*-deacetylation step may also be included. Volatile matter, including water, in the purified polysaccharide is determined by a suitable method such as thermogravimetry (2.2.34). The value is used to calculate the results of other tests with reference to the dried substance, as prescribed below.

Only meningococcal group C polysaccharide that complies with the following requirements may be used in the preparation of the conjugate.

Protein (2.5.16): maximum 1.0 per cent, calculated with reference to the dried substance.

Nucleic acid (2.5.17): maximum 1.0 per cent, calculated with reference to the dried substance.

***O*-acetyl groups.** Examine by a suitable method (for example 2.5.19). An acceptable value is established for the particular product and each batch of meningococcal group C polysaccharide must be shown to comply with this limit.

Sialic acid (2.5.23): minimum 0.800 g of sialic acid per gram of meningococcal group C polysaccharide using *N*-acetylneuraminic acid *R* to prepare the reference solution.

Residual reagents. Where applicable, tests are carried out to determine residues of reagents used during inactivation and purification. An acceptable value for each reagent is established for the particular product and each batch of meningococcal group C polysaccharide must be shown to comply with this limit. Where validation studies have demonstrated removal of a residual reagent, the test on purified meningococcal group C polysaccharide may be omitted.

Molecular-size distribution. Examine by size-exclusion chromatography (2.2.30). An acceptable value is established for the particular product and each batch of meningococcal group C polysaccharide must be shown to comply with this limit. Where applicable, the molecular-size distribution is also determined after chemical modification of the meningococcal group C polysaccharide.

Identification and serological specificity. The identity and serological specificity are determined by a suitable immunochemical method (2.7.1) or other suitable method, for example ¹H nuclear magnetic resonance spectrometry (2.2.33).

Bacterial endotoxins (2.6.14): less than 100 IU per microgram of meningococcal group C polysaccharide.

CARRIER PROTEIN

The carrier protein is chosen so that when meningococcal group C polysaccharide is conjugated it is able to induce a T-cell-dependent immune response. Tetanus toxoid and the non-toxic mutant of diphtheria toxin-like protein, CRM197 are suitable. The carrier protein is produced by culture of a suitable microorganism, the bacterial purity of which is verified.

Only a carrier protein that complies with the following requirements may be used in preparation of the conjugate.

Identification. The carrier protein is identified by a suitable immunochemical method (2.7.1).

Diphtheria toxin-like protein. Where diphtheria toxin-like protein is used as the carrier, it contains not less than 90 per cent of CRM197, determined by a suitable method. Suitable tests are carried out, for validation or routinely, to demonstrate that the product is non-toxic.

Tetanus toxoid. Where tetanus toxoid is used as the carrier, it is produced as described in *Tetanus vaccine (adsorbed)* (0452) and complies with the requirements prescribed therein for bulk purified toxoid, except that the antigenic purity is not less than 1500 Lf per milligram of protein nitrogen.

BULK CONJUGATE

Meningococcal group C polysaccharide is chemically modified to enable conjugation; it is usually partly depolymerised either before or during this procedure. The conjugate is obtained by the covalent bonding of activated meningococcal group C oligosaccharide and carrier protein. The conjugate purification procedures are designed to remove residual reagents used for conjugation. The removal of residual reagents and reaction by-products is confirmed by suitable tests or by validation of the purification process. Only a bulk conjugate that complies with the following requirements may be used in the preparation of the final bulk vaccine. For each test and for each particular product, limits of acceptance are established and each batch of conjugate must be shown to comply with these limits.

Molecular-size distribution. Examine by size-exclusion chromatography (2.2.30). An acceptable value is established for the particular product and each batch of bulk conjugate must be shown to comply with this limit.

Saccharide. The saccharide content is determined by a suitable validated assay, for example (2.5.23). Anion-exchange liquid chromatography with pulsed amperometric detection (2.2.29) may also be used for determination of saccharide content. An acceptable value is established for the particular product and each batch of bulk conjugate must be shown to comply with this limit.

Protein. The protein content is determined by a suitable chemical method, for example (2.5.16). An acceptable value is established for the particular product and each batch of bulk conjugate must be shown to comply with this limit.

Saccharide-to-protein ratio. Determine the ratio by calculation.

Free saccharide. Unbound saccharide is determined after removal of the conjugate, for example by anion-exchange liquid chromatography, size-exclusion or hydrophobic chromatography, ultrafiltration or other validated methods. An acceptable value is established for the particular product and each batch of bulk conjugate must be shown to comply with this limit.

Free carrier protein. Determine the content, either directly by a suitable method or by deriving the content by calculation from the results of other tests. An acceptable value is established for the particular product and each batch of bulk conjugate must be shown to comply with this limit.

Residual reagents. Removal of residual reagents such as cyanide is confirmed by suitable tests or by validation of the process.

Sterility (2.6.1). It complies with the test for sterility, carried out using 10 ml for each medium or the equivalent of 100 doses, whichever is less.

FINAL BULK VACCINE

An adjuvant and a stabiliser may be added to the bulk conjugate before dilution to the final concentration with a suitable diluent.

Only a final bulk vaccine that complies with the following requirement and is within the limits approved for the particular product may be used in preparation of the final lot.

Sterility (2.6.1). It complies with the test for sterility, carried out using 10 ml for each medium.

FINAL LOT

Only a final lot that is within the limits approved for the particular product and is satisfactory with respect to each of the requirement given below under Identification, Tests and Assay may be released for use.

IDENTIFICATION

The vaccine is identified by a suitable immunochemical method (2.7.1).

TESTS

pH (2.2.3). The pH of the vaccine, reconstituted if necessary, is within ± 0.5 pH units of the limit approved for the particular product.

Aluminium (2.5.13): maximum 1.25 mg per single human dose, if aluminium hydroxide or hydrated aluminium phosphate is used as the adsorbent.

Water (2.5.12): maximum 3.0 per cent for freeze-dried vaccines.

Free saccharide. Unbound saccharide is determined after removal of the conjugate, for example by anion-exchange liquid chromatography, size-exclusion or hydrophobic chromatography, ultrafiltration or other validated methods. An acceptable value consistent with adequate

immunogenicity, as shown in clinical trials, is established for the particular product and each final lot must be shown to comply with this limit.

Sterility (2.6.1). It complies with the test for sterility.

Bacterial endotoxins (2.6.14): less than 25 IU per single human dose.

ASSAY

Saccharide: minimum 80 per cent of the amount of meningococcal group C polysaccharide stated on the label. The saccharide content is determined by a suitable validated assay, for example sialic acid assay (2.5.23) or anion-exchange liquid chromatography with pulsed amperometric detection (2.2.29).

LABELLING

The label states:

- the number of micrograms of meningococcal group C polysaccharide per human dose,
- the type and number of micrograms of carrier protein per human dose.

01/2005:0250

MENINGOCOCCAL POLYSACCHARIDE VACCINE

Vaccinum meningococcale polysaccharidicum

DEFINITION

Meningococcal polysaccharide vaccine is a freeze-dried preparation of one or more purified capsular polysaccharides obtained from one or more suitable strains of *Neisseria meningitidis* group A, group C, group Y and group W135 that are capable of consistently producing polysaccharides.

N. meningitidis group A polysaccharide consists of partly *O*-acetylated repeating units of *N*-acetylmannosamine, linked with 1 α →6 phosphodiester bonds.

N. meningitidis group C polysaccharide consists of partly *O*-acetylated repeating units of sialic acid, linked with 2 α →9 glycosidic bonds.

N. meningitidis group Y polysaccharide consists of partly *O*-acetylated alternating units of sialic acid and D-glucose, linked with 2 α →6 and 1 α →4 glycosidic bonds.

N. meningitidis group W135 polysaccharide consists of partly *O*-acetylated alternating units of sialic acid and D-galactose, linked with 2 α →6 and 1 α →4 glycosidic bonds.

The polysaccharide component or components stated on the label together with calcium ions and residual moisture account for over 90 per cent of the mass of the preparation.

PRODUCTION

Production of the meningococcal polysaccharides is based on a seed-lot system. The production method shall have been shown to yield consistently meningococcal polysaccharide vaccines of satisfactory immunogenicity and safety in man. The production method is validated to demonstrate that the product, if tested, would comply with the test for abnormal toxicity for immunosera and vaccines for human use (2.6.9).

SEED LOTS

The strains of *N. meningitidis* used for the master seed lots shall be identified by historical records that include information on their origin and by their biochemical and serological characteristics.

Cultures from each working seed lot shall have the same characteristics as the strain that was used to prepare the master seed lot. The strains have the following characteristics:

- colonies obtained from a culture are rounded, uniform in shape and smooth with a mucous, opalescent, greyish appearance,
- Gram staining reveals characteristic Gram-negative diplococci in “coffee-bean” arrangement,
- the oxidase test is positive,
- the culture utilises glucose and maltose,
- suspensions of the culture agglutinate with suitable specific antisera.

Purity of bacterial strains used for the seed lots is verified by methods of suitable sensitivity. These may include inoculation into suitable media, examination of colony morphology, microscopic examination of Gram-stained smears and culture agglutination with suitable specific antisera.

PROPAGATION AND HARVEST

The working seed lots are cultured on solid media that do not contain blood-group substances or ingredients of mammalian origin. The inoculum may undergo 1 or more subcultures in liquid medium before being used for inoculating the final medium. The liquid media used and the final medium are semisynthetic and free from substances precipitated by cetrimonium bromide (hexadecyltrimethylammonium bromide) and do not contain blood-group substances or high-molecular-mass polysaccharides.

The bacterial purity of the culture is verified by methods of suitable sensitivity. These may include inoculation into suitable media, examination of colony morphology, microscopic examination of Gram-stained smears and culture agglutination with suitable specific antisera.

The cultures are centrifuged and the polysaccharides precipitated from the supernatant by addition of cetrimonium bromide. The precipitate obtained is harvested and may be stored at –20 °C awaiting further purification.

PURIFIED POLYSACCHARIDES

The polysaccharides are purified, after dissociation of the complex of polysaccharide and cetrimonium bromide, using suitable procedures to remove successively nucleic acids, proteins and lipopolysaccharides.

The final purification step consists of ethanol precipitation of the polysaccharides which are then dried and stored at –20 °C. The loss on drying is determined by thermogravimetry (2.2.34) and the value is used to calculate the results of the other chemical tests with reference to the dried substance.

Only purified polysaccharides that comply with the following requirements may be used in the preparation of the final bulk vaccine.

Protein (2.5.16). Not more than 10 mg of protein per gram of purified polysaccharide, calculated with reference to the dried substance.

Nucleic acids (2.5.17). Not more than 10 mg of nucleic acids per gram of purified polysaccharide, calculated with reference to the dried substance.

***O*-Acetyl groups (2.5.19).** Not less than 2 mmol of *O*-acetyl groups per gram of purified polysaccharide for group A, not less than 1.5 mmol per gram of polysaccharide for group C, not less than 0.3 mmol per gram of polysaccharide for groups Y and W135, all calculated with reference to the dried substance.

Phosphorus (2.5.18). Not less than 80 mg of phosphorus per gram of group A purified polysaccharide, calculated with reference to the dried substance.

Sialic acid (2.5.23). Not less than 800 mg of sialic acid per gram of group C polysaccharide and not less than 560 mg of sialic acid per gram of purified polysaccharide for groups Y and W135, all calculated with reference to the dried substance. Use the following reference solutions.

Group C polysaccharide: a 150 mg/l solution of *N-acetylneuraminic acid R*.

Group Y polysaccharide: a solution containing 95 mg/l of *N-acetylneuraminic acid R* and 55 mg/l of *glucose R*.

Group W135 polysaccharide: a solution containing 95 mg/l of *N-acetylneuraminic acid R* and 55 mg/l of *galactose R*.

Calcium. If a calcium salt is used during purification, a determination of calcium is carried out on the purified polysaccharide; the content is within the limits approved for the particular product.

Distribution of molecular size. Examine by size-exclusion chromatography (2.2.30) using *agarose for chromatography R* or *cross-linked agarose for chromatography R*. Use a column about 0.9 m long and 16 mm in internal diameter equilibrated with a solvent having an ionic strength of 0.2 mol/kg and a pH of 7.0-7.5. Apply to the column about 2.5 mg of polysaccharide in a volume of about 1.5 ml and elute at about 20 ml/h. Collect fractions of about 2.5 ml and determine the content of polysaccharide by a suitable method. At least 65 per cent of group A polysaccharide, 75 per cent of group C polysaccharide, 80 per cent of group Y polysaccharide and 80 per cent of group W135 polysaccharide is eluted before a distribution coefficient (K_0) of 0.50 is reached. In addition, the percentages eluted before this distribution coefficient are within the limits approved for the particular product.

Identification and serological specificity. The identity and serological specificity are determined by a suitable immunochemical method (2.7.1). Identity and purity of each polysaccharide shall be confirmed; it shall be shown that there is not more than 1 per cent *m/m* of group-heterologous *N. meningitidis* polysaccharide.

Pyrogens (2.6.8). The polysaccharide complies with the test for pyrogens. Inject into each rabbit per kilogram of body mass 1 ml of a solution containing 0.025 µg of purified polysaccharide per millilitre.

FINAL BULK VACCINE

One or more purified polysaccharides of 1 or more *N. meningitidis* groups are dissolved in a suitable solvent that may contain a stabiliser. When dissolution is complete, the solution is filtered through a bacteria-retentive filter.

Only a final bulk vaccine that complies with the following requirement may be used in the preparation of the final lot.

Sterility (2.6.1). The final bulk vaccine complies with the test for sterility, carried out using 10 ml for each medium.

FINAL LOT

The final bulk vaccine is distributed aseptically into sterile containers. The containers are then closed so as to avoid contamination.

Only a final lot that is satisfactory with respect to each of the requirements prescribed below under Identification, Tests and Assay may be released for use.

CHARACTERS

A white or cream-coloured powder or pellet, freely soluble in water.

IDENTIFICATION

Carry out an identification test for each polysaccharide present in the vaccine by a suitable immunochemical method (2.7.1).

TESTS

Distribution of molecular size. Examine by size-exclusion chromatography (2.2.30). Use a column about 0.9 m long and 16 mm in internal diameter equilibrated with a solvent having an ionic strength of 0.2 mol/kg and a pH of 7.0-7.5. Apply to the column about 2.5 mg of each polysaccharide in a volume of about 1.5 ml and elute at about 20 ml/h. Collect fractions of about 2.5 ml and determine the content of polysaccharide by a suitable method.

For a divalent vaccine (group A + group C), use *cross-linked agarose for chromatography R*. The vaccine complies with the test if:

- 65 per cent of group A polysaccharide is eluted before $K_0 = 0.50$,
- 75 per cent of group C polysaccharide is eluted before $K_0 = 0.50$.

For a tetravalent vaccine (group A + group C + group Y + group W135), use *cross-linked agarose for chromatography R1* and apply a suitable immunochemical method (2.7.1) to establish the elution pattern of the different polysaccharides. The vaccine complies with the test if K_0 for the principal peak is:

- not greater than 0.70 for group A and group C polysaccharide,
- not greater than 0.57 for group Y polysaccharide,
- not greater than 0.68 for group W135 polysaccharide.

Water (2.5.12). Not more than 3.0 per cent, determined by the semi-micro determination of water.

Sterility (2.6.1). It complies with the test for sterility.

Pyrogens (2.6.8). It complies with the test for pyrogens. Inject per kilogram of the rabbit's mass 1 ml of a solution containing:

- 0.025 µg of polysaccharide for a monovalent vaccine,
- 0.050 µg of polysaccharide for a divalent vaccine,
- 0.10 µg of polysaccharide for a tetravalent vaccine.

ASSAY

Carry out an assay of each polysaccharide present in the vaccine.

For a divalent vaccine (group A + group C), use measurement of phosphorus (2.5.18) to determine the content of polysaccharide A and measurement of sialic acid (2.5.23) to determine the content of polysaccharide C. To determine sialic acid, use as reference solution a 150 mg/l solution of *N-acetylneuraminic acid R*.

For a tetravalent vaccine (group A + group C + group Y + group W135) a suitable immunochemical method (2.7.1) is used with a reference preparation of purified polysaccharide for each group.

The vaccine contains not less than 70 per cent and not more than 130 per cent of the quantity of each polysaccharide stated on the label.

LABELLING

The label states:

- the group or groups of polysaccharides (A, C, Y or W135) present in the vaccine,
- the number of micrograms of polysaccharide per human dose.

01/2005:0538
corrected**MUMPS VACCINE (LIVE)****Vaccinum parotitidis vivum****DEFINITION**

Mumps vaccine (live) is a freeze-dried preparation of a suitable attenuated strain of mumps virus. The vaccine is reconstituted immediately before use, as stated on the label, to give a clear liquid that may be coloured owing to the presence of a pH indicator.

PRODUCTION

The production of vaccine is based on a virus seed-lot system and, if the virus is propagated in human diploid cells, a cell-bank system. The production method shall have been shown to yield consistently live mumps vaccines of adequate immunogenicity and safety in man. Unless otherwise justified and authorised, the virus in the final vaccine shall have undergone no more passages from the master seed lot than were used to prepare the vaccine shown in clinical studies to be satisfactory with respect to safety and efficacy. The production method is validated to demonstrate that the product, if tested, would comply with the test for abnormal toxicity for immunosera and vaccines for human use (2.6.9).

SUBSTRATE FOR VIRUS PROPAGATION

The virus is propagated in human diploid cells (5.2.3) or in chick-embryo cells or in the amniotic cavity of chick embryos derived from a chicken flock free from specified pathogens (5.2.2).

SEED LOT

The strain of mumps virus used shall be identified by historical records that include information on the origin of the strain and its subsequent manipulation. To avoid the unnecessary use of monkeys in the test for neurovirulence, virus seed lots are prepared in large quantities and stored at temperatures below -20°C if freeze-dried, or below -60°C if not freeze-dried.

Only a seed lot that complies with the following requirements may be used for virus propagation.

Identification. The master and working seed lots are identified as mumps virus by serum neutralisation in cell culture, using specific antibodies.

Virus concentration. The virus concentration of the master and working seed lots is determined to ensure consistency of production.

Extraneous agents (2.6.16). The working seed lot complies with the requirements for seed lots.

Neurovirulence (2.6.18). The working seed lot complies with the test for neurovirulence of live virus vaccines. *Macaca* and *Cercopithecus* monkeys are suitable for the test.

PROPAGATION AND HARVEST

All processing of the cell bank and subsequent cell cultures is done under aseptic conditions in an area where no other cells are handled. Suitable animal (but not human) serum may be used in the culture media. Serum and trypsin used in the preparation of cell suspensions and culture media are shown to be free from extraneous agents. The cell culture medium may contain a pH indicator such as phenol red and suitable antibiotics at the lowest effective concentration. It is preferable to have a substrate free from antibiotics during production. Not less than 500 ml of the production cell cultures is set aside as uninfected cell cultures (control

cells). If the virus is propagated in chick embryos, 2 per cent but not less than twenty eggs are set aside as uninfected control eggs. The viral suspensions are harvested at a time appropriate to the strain of virus being used.

Only a single harvest that complies with the following requirements may be used in the preparation of the final bulk vaccine.

Identification. The single harvest contains virus that is identified as mumps virus by serum neutralisation in cell culture, using specific antibodies.

Virus concentration. The virus concentration in the single harvest is determined as prescribed under Assay to monitor consistency of production and to determine the dilution to be used for the final bulk vaccine.

Extraneous agents (2.6.16). The single harvest complies with the tests for extraneous agents.

Control cells or eggs. If human diploid cells are used for production, the control cells comply with a test for identification; the control cells and the control eggs comply with the tests for extraneous agents (2.6.16).

FINAL BULK VACCINE

Single harvests that comply with the above tests are pooled and clarified to remove cells. A suitable stabiliser may be added and the pooled harvests diluted as appropriate.

Only a final bulk vaccine that complies with the following requirement may be used in the preparation of the final lot.

Bacterial and fungal contamination. The final bulk vaccine complies with the test for sterility (2.6.1), carried out using 10 ml for each medium.

FINAL LOT

A minimum virus concentration for release of the product is established such as to ensure, in the light of stability data, that the minimum concentration stated on the label will be present at the end of the period of validity.

Only a final lot that complies with the requirements for minimum virus concentration for release, with the following requirement for thermal stability and with each of the requirements given below under Identification and Tests may be released for use. Provided that the tests for bovine serum albumin and, where applicable, for ovalbumin have been carried out with satisfactory results on the final bulk vaccine, they may be omitted on the final lot.

Thermal stability. Maintain samples of the final lot of freeze-dried vaccine in the dry state at 37°C for 7 days. Determine the virus concentration as described under Assay in parallel for the heated vaccine and for unheated vaccine stored at $5 \pm 3^{\circ}\text{C}$. The virus concentration of the heated vaccine is not more than $1.0 \log_{10}$ lower than that of the unheated vaccine.

IDENTIFICATION

When the vaccine reconstituted as stated on the label is mixed with specific mumps antibodies, it is no longer able to infect susceptible cell cultures.

TESTS

Bacterial and fungal contamination. The reconstituted vaccine complies with the test for sterility (2.6.1).

Bovine serum albumin. Not more than 50 ng per single human dose, determined by a suitable immunochemical method (2.7.1).

Ovalbumin. If the vaccine is produced in chick embryos, it contains not more than $1 \mu\text{g}$ of ovalbumin per single human dose, determined by a suitable immunochemical method (2.7.1).

Water (2.5.12). Not more than 3.0 per cent, determined by the semi-micro determination of water.

ASSAY

Titrate the vaccine for infective virus at least in triplicate, using at least five cell cultures for each 0.5 log₁₀ dilution step or by a method of equal precision. Use an appropriate virus reference preparation to validate each assay. The estimated virus concentration is not less than that stated on the label; the minimum virus concentration stated on the label is not less than 5×10^3 CCID₅₀ per human dose. The assay is not valid if the confidence interval ($P = 0.95$) of the logarithm of the virus concentration is greater than ± 0.3 .

Mumps vaccine (live) BRP is suitable for use as a reference preparation.

LABELLING

The label states:

- the strain of virus used for the preparation of the vaccine,
- that the vaccine has been prepared in chick embryos or the type and origin of cells used for the preparation of the vaccine,
- the minimum virus concentration,
- that contact with disinfectants is to be avoided,
- the time within which the vaccine must be used after reconstitution.

01/2005:0160

PERTUSSIS VACCINE

Vaccinum pertussis

DEFINITION

Pertussis vaccine is a sterile saline suspension of inactivated whole cells of one or more strains of *Bordetella pertussis*.

PRODUCTION

INACTIVATED *B. PERTUSSIS* SUSPENSION

The inactivated *B. pertussis* suspension is prepared as described in the monograph on *Pertussis vaccine (adsorbed) (0161)* and complies with the requirements prescribed therein.

FINAL BULK VACCINE

Suitable quantities of the inactivated single harvests are pooled to prepare the final bulk vaccine. Suitable antimicrobial preservatives may be added. The bacterial concentration of the final bulk vaccine does not exceed that corresponding to an opacity of 20 IU per single human dose. If 2 or more strains of *B. pertussis* are used, the composition of consecutive lots of the final bulk vaccine shall be consistent with respect to the proportion of each strain as measured in opacity units.

Only a final bulk vaccine that complies with the following requirements may be used in the preparation of the final lot.

Antimicrobial preservative. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The amount is not less than 85 per cent and not greater than 115 per cent of the intended amount.

Sterility (2.6.1). Carry out the test for sterility using 10 ml for each medium.

FINAL LOT

The final bulk vaccine is distributed aseptically into sterile, tamper-proof containers. The containers are closed so as to prevent contamination.

Only a final lot that is satisfactory with respect to each of the requirements given below under Identification, Tests and Assay may be released for use. Provided the tests for specific toxicity, free formaldehyde and antimicrobial preservative and the assay have been carried out with satisfactory results on the final bulk vaccine, they may be omitted on the final lot.

IDENTIFICATION

Identify pertussis vaccine by agglutination of the bacteria in the vaccine by antisera specific to *B. pertussis*.

TESTS

Specific toxicity. Use not fewer than 5 healthy mice each weighing 14 g to 16 g for the vaccine group and for the saline control. Use mice of the same sex or distribute males and females equally between the groups. Allow the animals access to food and water for at least 2 h before injection and during the test. Inject each mouse of the vaccine group intraperitoneally with 0.5 ml, containing a quantity of the vaccine equivalent to not less than half the single human dose. Inject each mouse of the control group with 0.5 ml of a 9 g/l sterile solution of *sodium chloride R*, preferably containing the same amount of antimicrobial preservative as that injected with the vaccine. Weigh the groups of mice immediately before the injection and 72 h and 7 days after the injection. The vaccine complies with the test if: (a) at the end of 72 h the total mass of the group of vaccinated mice is not less than that preceding the injection; (b) at the end of 7 days the average increase in mass per vaccinated mouse is not less than 60 per cent of that per control mouse; and (c) not more than 5 per cent of the vaccinated mice die during the test. The test may be repeated and the results of the tests combined.

Free formaldehyde (2.4.18): maximum 0.2 g/l.

Antimicrobial preservative. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The content is not less than the minimum amount shown to be effective and is not greater than 115 per cent of the quantity stated on the label.

Sterility (2.6.1). The vaccine complies with the test for sterility.

ASSAY

Carry out the assay of pertussis vaccine (2.7.7).

The estimated potency is not less than 4 IU per single human dose and the lower confidence limit ($P = 0.95$) of the estimated potency is not less than 2 IU per single human dose.

LABELLING

The label states:

- the minimum number of International Units per single human dose,
- that the vaccine must be shaken before use,
- that the vaccine is not to be frozen.

01/2005:1356

PERTUSSIS VACCINE (ACELLULAR, COMPONENT, ADSORBED)

Vaccinum pertussis sine cellulis ex elementis praeparatum adsorbatum

DEFINITION

Pertussis vaccine (acellular, component, adsorbed) is a preparation of individually prepared and purified antigenic components of *Bordetella pertussis* adsorbed on a mineral carrier such as aluminium hydroxide or hydrated aluminium phosphate.

The vaccine contains either pertussis toxoid or a pertussis-toxin-like protein free from toxic properties, produced by expression of a genetically modified form of the corresponding gene. Pertussis toxoid is prepared from pertussis toxin by a method that renders the latter harmless while maintaining adequate immunogenic properties and avoiding reversion to toxin. The vaccine may also contain filamentous haemagglutinin, pertactin (a 69 kDa outer-membrane protein) and other defined components of *B. pertussis* such as fimbrial-2 and fimbrial-3 antigens. The latter 2 antigens may be copurified. The antigenic composition and characteristics are based on evidence of protection and freedom from unexpected reactions in the target group for which the vaccine is intended.

PRODUCTION

The production method shall have been shown to yield consistently vaccines comparable with the vaccine of proven clinical efficacy and safety in man.

Where a genetically modified form of *B. pertussis* is used for production consistency and genetic stability shall be established in conformity with the requirements of the monograph *Products of recombinant DNA technology* (0784).

Reference vaccine. A batch of vaccine shown to be effective in clinical trials or a batch representative thereof is used as a reference vaccine. For the preparation of a representative batch, strict adherence to the production process used for the batch tested in clinical trials is necessary. The reference vaccine is preferably stabilised by a method that has been shown to have no significant effect on the assay procedure when the stabilised and non-stabilised batches are compared.

CHARACTERISATION OF COMPONENTS

During development of the vaccine, the production process shall be validated to demonstrate that it yields consistently individual components that comply with the following requirements; after demonstration of consistency, the tests need not be applied routinely to each batch.

Adenylate cyclase. Not more than 500 ng in the equivalent of 1 dose of the final vaccine, determined by immunoblot analysis or another suitable method.

Tracheal cytotoxin. Not more than 2 pmol in the equivalent of 1 dose of the final vaccine, determined by a suitable method such as a biological assay or liquid chromatography (2.2.29).

Absence of residual dermonecrotic toxin. Inject intradermally into each of 3 unweaned mice, in a volume of 0.1 ml, the amount of component or antigenic fraction equivalent to 1 dose of the final vaccine. Observe for 48 h. No dermonecrotic reaction is demonstrable.

Specific properties. The components of the vaccine are analysed by one or more of the methods shown below in order to determine their identity and specific properties (activity per unit amount of protein) in comparison with reference preparations.

Pertussis toxin. Chinese hamster ovary (CHO) cell-clustering effect and haemagglutination as *in vitro* methods; lymphocytosis-promoting activity, histamine-sensitising activity and insulin secretory activity as *in vivo* methods. The toxin shows ADP-ribosyl transferase activity using transducin as the acceptor.

Filamentous haemagglutinin. Haemagglutination and inhibition by specific antibody.

Pertactin, fimbrial-2 and fimbrial-3 antigens. Reactivity with specific antibody.

Pertussis toxoid. The toxoid induces in animals production of antibodies capable of inhibiting all the properties of pertussis toxin.

PURIFIED COMPONENTS

Production of each component is based on a seed-lot system. The seed cultures from which toxin is prepared are managed to conserve or where necessary restore toxinogenicity by deliberate selection.

None of the media used at any stage contains blood or blood products of human origin. Media used for the preparation of seed lots and inocula may contain blood or blood products of animal origin.

Pertussis toxin and, where applicable, filamentous haemagglutinin and pertactin are purified and, after appropriate characterisation, detoxified using suitable chemical reagents, by a method that avoids reversion of the toxoid to toxin, particularly on storage or exposure to heat. Other components such as fimbrial-2 and fimbrial-3 antigens are purified either separately or together, characterised and shown to be free from toxic substances. The purification procedure is validated to demonstrate appropriate clearance of substances used during culture or purification.

The content of bacterial endotoxins (2.6.14) is determined to monitor the purification procedure and to limit the amount in the final vaccine. The limits applied for the individual components are such that the final vaccine contains less than 100 IU per single human dose.

Before detoxification, the purity of the components is determined by a suitable method such as polyacrylamide gel electrophoresis (PAGE) or liquid chromatography. SDS-PAGE or immunoblot analysis with specific monoclonal or polyclonal antibodies may be used to characterise subunits. Requirements are established for each individual product.

Only purified components that comply with the following requirements may be used in the preparation of the final bulk vaccine.

Sterility (2.6.1). Carry out the test for sterility using for each medium a quantity of purified component equivalent to not less than 100 doses.

Absence of residual pertussis toxin. *This test is not necessary for the product obtained by genetic modification.* Use a group of not fewer than 5 histamine-sensitive mice each weighing 18-26 g. Inject into each mouse the equivalent of 1 human dose intravenously or twice the human dose intraperitoneally, diluted to not more than 0.5 ml with phosphate-buffered saline solution containing 2 g/l of gelatin. Inject diluent into a second group of control mice.

After 5 days, inject 2 mg of histamine base intraperitoneally in a volume not exceeding 0.5 ml and observe for 24 h. If no animal dies, the preparation complies with the test.

The histamine sensitivity of the strain of mice used is verified at suitable intervals as follows: inject threefold dilutions of a reference pertussis toxin preparation in phosphate-buffered saline solution containing 2 g/l of gelatin and challenge with histamine as above; the strain is suitable if more than 50 per cent of the animals are sensitised by 50 ng of pertussis toxin and none of the control animals injected with only diluent and challenged similarly with histamine show symptoms of sensitisation.

A validated test based on the clustering effect of the toxin for Chinese hamster ovary (CHO) cells may be used instead of the test in mice.

Residual detoxifying agents and other reagents. The content of residual detoxifying agents and other reagents is determined and shown to be below approved limits unless validation of the process has demonstrated acceptable clearance.

Antigen content. Determine the antigen content by a suitable immunochemical method (2.7.1) and protein nitrogen by sulphuric acid digestion (2.5.9) or another suitable method. The ratio of antigen content to protein nitrogen is within the limits established for the product.

FINAL BULK VACCINE

The vaccine is prepared by adsorption of suitable quantities of purified components, separately or together, onto aluminium hydroxide or hydrated aluminium phosphate. A suitable antimicrobial preservative may be added.

Only a final bulk vaccine that complies with the following requirements may be used in the preparation of the final lot.

Antimicrobial preservative. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical or physico-chemical method. The amount is not less than 85 per cent and not greater than 115 per cent of the intended content.

Sterility (2.6.1). Carry out the test for sterility using 10 ml for each medium.

FINAL LOT

Only a final lot that is satisfactory with respect to each of the requirements given below under Identification, Tests and Assay may be released for use. Provided that the tests for absence of residual pertussis toxin and irreversibility of pertussis toxoid, antimicrobial preservative, free formaldehyde and the assay have been carried out with satisfactory results on the final bulk vaccine, these tests may be omitted on the final lot.

IDENTIFICATION

Subject the vaccine to a suitable desorption procedure such as the following: dissolve in the vaccine to be examined sufficient *sodium citrate R* to give a 10 g/l solution; maintain at 37 °C for about 16 h and centrifuge until a clear supernatant liquid is obtained. Examined by a suitable immunochemical method (2.7.1), the clear supernatant liquid reacts with specific antisera to the components stated on the label.

TESTS

Absence of residual pertussis toxin and irreversibility of pertussis toxoid. *This test is not necessary for the product obtained by genetic modification.* Use 3 groups each of not fewer than 5 histamine-sensitive mice. Inject intraperitoneally into the first group twice the single human dose of the vaccine stored at 2–8 °C. Inject intraperitoneally

into the second group twice the single human dose of the vaccine incubated at 37 °C for 4 weeks. Inject diluent into the third group of mice. After 5 days, inject into each mouse 2 mg of histamine base intraperitoneally in a volume not exceeding 0.5 ml and observe for 24 h. The test is invalid if 1 or more control mice die following histamine challenge. The vaccine complies with the test if no animal in the first or second group dies following histamine challenge. If 1 mouse dies in either or both of the first and second groups, the test may be repeated with the same number of mice or with a greater number and the results of valid tests combined; the vaccine complies with the test if, in both of the groups given the vaccine, not more than 5 per cent of the total number of mice die following histamine challenge.

The histamine sensitivity of the strain of mice used is verified at suitable intervals as follows: inject intravenously threefold dilutions of a reference pertussis toxin preparation in phosphate-buffered saline solution containing 2 g/l of gelatin and challenge with histamine as above; the strain is suitable if more than 50 per cent of the animals are sensitised by 50 ng of pertussis toxin and none of the control animals injected with only diluent and challenged similarly with histamine show symptoms of sensitisation.

Aluminium (2.5.13): maximum 1.25 mg per single human dose, if aluminium hydroxide or hydrated aluminium phosphate is used as the adsorbent.

Free formaldehyde (2.4.18): maximum 0.2 g/l.

Antimicrobial preservative. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical or physico-chemical method. The amount is not less than the minimum amount shown to be effective and is not greater than 115 per cent of the quantity stated on the label.

Sterility (2.6.1). It complies with the test for sterility.

ASSAY

The capacity of the vaccine to induce the formation of specific antibodies is compared with the same capacity of a reference preparation examined in parallel; antibodies are determined using suitable immunochemical methods (2.7.1) such as enzyme-linked immunosorbent assay (ELISA). The test in mice shown below uses a three-point model but, after validation, for routine testing a single-dilution method may be used.

Requirement. The capacity to induce antibodies is not significantly ($P = 0.95$) less than that of the reference vaccine.

The following test model is given as an example of a method that has been found to be satisfactory.

Selection and distribution of test animals. Use in the test healthy mice (for example, CD1 strain) of the same stock 4 to 8 weeks old. Distribute the animals in 6 groups of a number appropriate to the requirements of the assay. Use 3 dilutions of the vaccine to be examined and 3 dilutions of a reference preparation and attribute each dilution to a group of mice. Inject intraperitoneally or subcutaneously into each mouse 0.5 ml of the dilution attributed to its group.

Collection of serum samples. 4 to 5 weeks after vaccination, bleed the mice individually under anaesthesia. Store the sera at –20 °C until tested for antibody content.

Antibody determination. Assay the individual sera for content of specific antibodies to each component using a validated method such as the ELISA test shown below.

ELISA test. Microtitre plates (poly(vinyl chloride) or polystyrene as appropriate for the specific antigen) are coated with the purified antigen at a concentration of 100 ng per well. After washing, unreacted sites are blocked by incubating with a solution of bovine serum albumin and then

washed. Two-fold dilutions of sera from mice immunised with test or reference vaccines are made on the plates. After incubation at 22-25 °C for 1 h, the plates are washed. A suitable solution of anti-mouse IgG enzyme conjugate is added to each well and incubated at 22-25 °C for 1 h. After washing, a substrate is added from which the bound enzyme conjugate liberates a chromophore which can be quantified by measurement of absorbance (2.2.25). The test conditions are designed to obtain a linear response for absorbance with respect to antibody content over the range of measurement used and absorbance values within the range 0.1 to 2.0.

A reference antiserum of assigned potency is used in the test and serves as the basis for calculation of the antibody levels in test sera. A standardised control serum is also included in the test.

The test is not valid if:

- the value found for the control serum differs by more than 2 standard deviations from the assigned value,
- the confidence limits ($P = 0.95$) are less than 50 per cent or more than 200 per cent of the estimated potency.

Calculation. The antibody titres in the sera of mice immunised with reference and test vaccines are calculated and from the values obtained the potency of the test vaccine in relation to the reference vaccine is calculated by the usual statistical methods.

LABELLING

The label states:

- the names and amounts of the components present in the vaccine,
- where applicable, that the vaccine contains a pertussis toxin-like protein produced by genetic modification,
- the name and amount of the adsorbent,
- that the vaccine must be shaken before use,
- that the vaccine is not to be frozen.

01/2005:1595
corrected

PERTUSSIS VACCINE (ACELLULAR, CO-PURIFIED, ADSORBED)

Vaccinum pertussis sine cellulis
copurificatum adsorbatum

DEFINITION

Pertussis vaccine (acellular, co-purified, adsorbed) is a preparation of antigenic components of *Bordetella pertussis* adsorbed on a mineral carrier such as aluminium hydroxide or hydrated aluminium phosphate.

The vaccine contains an antigenic fraction purified without separation of the individual components. The antigenic fraction is treated by a method that transforms pertussis toxin to toxoid, rendering it harmless while maintaining adequate immunogenic properties of all the components and avoiding reversion to toxin. The antigenic fraction is composed of pertussis toxoid, filamentous haemagglutinin, pertactin (a 69 kDa outer-membrane protein) and other defined components of *B. pertussis* such as fimbrial-2 and fimbrial-3 antigens. It may contain residual pertussis toxin up to a maximum level approved by the competent authority. The antigenic composition and characteristics are based on evidence of protection and freedom from unexpected reactions in the target group for which the vaccine is intended.

PRODUCTION

GENERAL PROVISIONS

The production method shall have been shown to yield consistently vaccines comparable with the vaccine of proven clinical efficacy and safety in man.

Reference vaccine. A batch of vaccine shown to be effective in clinical trials or a batch representative thereof is used as a reference vaccine. For the preparation of a representative batch, strict adherence to the production process used for the batch tested in clinical trials is necessary. The reference vaccine is preferably stabilised, by a method that has been shown to have no significant effect on the assay procedure when the stabilised and non-stabilised batches are compared.

CHARACTERISATION OF COMPONENTS

During development of the vaccine, the production process shall be validated to demonstrate that it yields consistently an antigenic fraction that complies with the following requirements; after demonstration of consistency, the tests need not be applied routinely to each batch.

Adenylate cyclase. Not more than 500 ng in the equivalent of 1 dose of the final vaccine, determined by immunoblot analysis or another suitable method.

Tracheal cytotoxin. Not more than 2 pmol in the equivalent of 1 dose of the final vaccine, determined by a suitable method such as a biological assay or liquid chromatography (2.2.29).

Absence of residual dermonecrotic toxin. Inject intradermally into each of 3 unweaned mice, in a volume of 0.1 ml, the amount of antigenic fraction equivalent to 1 dose of the final vaccine. Observe for 48 h. No dermonecrotic reaction is demonstrable.

Specific properties. The antigenic fraction is analysed by one or more of the methods shown below in order to determine the identity and specific properties (activity per unit amount of protein) of its components in comparison with reference preparations.

Pertussis toxin. Chinese hamster ovary (CHO) cell-clustering effect and haemagglutination as *in vitro* methods; lymphocytosis-promoting activity, histamine-sensitising activity and insulin secretory activity as *in vivo* methods. The toxin shows ADP-ribosyl transferase activity using transducin as the acceptor.

Filamentous haemagglutinin. Haemagglutination and inhibition by specific antibody.

Pertactin, fimbrial-2 and fimbrial-3 antigens. Reactivity with specific antibody.

Pertussis toxoid. The toxoid induces in animals the production of antibodies capable of inhibiting all the properties of pertussis toxin.

PURIFIED ANTIGENIC FRACTION

Production of the antigenic fraction is based on a seed-lot system. The seed cultures are managed to conserve or, where necessary, restore toxinogenicity by deliberate selection. None of the media used at any stage contains blood or blood products of human origin. Media used for the preparation of seed batches and inocula may contain blood or blood products of animal origin.

The antigenic fraction is purified and, after appropriate characterisation, detoxified using suitable reagents by a method that ensures minimal reversion of toxoid to toxin, particularly on storage or exposure to heat. The purification procedure is validated to demonstrate appropriate clearance of substances used during culture or purification.

The content of bacterial endotoxins (2.6.14) is determined to monitor the purification procedure and to limit the amount in the final vaccine. The limits applied are such that the final vaccine contains not more than 100 IU per single human dose.

Before detoxification, the purity of the antigenic fraction is determined by a suitable method such as polyacrylamide gel electrophoresis (PAGE) or liquid chromatography. SDS-PAGE or immunoblot analysis with specific monoclonal or polyclonal antibodies may be used to characterise subunits. Requirements are established for each individual product.

Only a purified antigenic fraction that complies with the following requirements may be used in the preparation of the final bulk vaccine.

Sterility (2.6.1). Carry out the test for sterility using for each medium a quantity of purified antigenic fraction equivalent to not less than 100 doses of the final vaccine.

Test for residual pertussis toxin. Use 3 groups of not fewer than 5 histamine-sensitive mice each weighing 18 g to 26 g. Using phosphate-buffered saline containing 2 g/l of gelatin, prepare a series of dilutions of the purified antigenic fraction that have been shown to yield a graded response and attribute each dilution to a separate group of mice. Inject intraperitoneally into each mouse the dilution attributed to its group. Inject diluent into a fourth group of control mice. After 5 days, inject intraperitoneally into each mouse 1 mg of histamine base in a volume not exceeding 0.5 ml. Record the number of animals that die within 24 h of histamine challenge. Calculate the weight or volume of a preparation that sensitises 50 per cent of the mice injected using a suitable statistical method such as probit analysis (see 5.3). The residual activity of pertussis toxin does not exceed that of batches shown to be safe in clinical studies.

The histamine sensitivity of the strain of mice used is verified at suitable intervals as follows: inject threefold dilutions of a reference pertussis toxin preparation in phosphate-buffered saline solution containing 2 g/l of gelatin and challenge with histamine as described above; the strain is suitable if more than 50 per cent of the animals are sensitised by 50 ng of pertussis toxin and none of the control animals injected with only diluent and challenged similarly with histamine show symptoms of sensitisation.

Pertussis toxin BRP is suitable for use as a reference pertussis toxin.

A validated test based on the clustering effect of the toxin for Chinese hamster ovary (CHO) cells may be used instead of the test in mice.

Residual detoxifying agents and other reagents. The content of residual detoxifying agents and other reagents is determined and shown to be below approved limits unless validation of the process has demonstrated acceptable clearance.

Antigen content. Determine the complete quantitative antigen composition of the antigenic fraction by suitable immunochemical methods (2.7.1) and protein nitrogen by sulphuric acid digestion (2.5.9) or another suitable method. The ratio of total antigen content to protein nitrogen is within the limits established for the product.

FINAL BULK VACCINE

The vaccine is prepared by adsorption of a suitable quantity of the antigenic fraction onto aluminium hydroxide or hydrated aluminium phosphate. A suitable antimicrobial preservative may be added.

Only a final bulk vaccine that complies with the following requirements may be used in the preparation of the final lot.

Antimicrobial preservative. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical or physico-chemical method. The amount is not less than 85 per cent and not greater than 115 per cent of the intended content.

Sterility (2.6.1). The final bulk vaccine complies with the test for sterility, carried out using 10 ml for each medium.

FINAL LOT

Only a final lot that is satisfactory with respect to each of the requirements given below under Identification, Tests and Assay may be released for use.

Provided that the tests for residual pertussis toxin, reversibility of toxoid, antimicrobial preservative, free formaldehyde and the assay have been carried out with satisfactory results on the final bulk vaccine, these tests may be omitted on the final lot.

IDENTIFICATION

Subject the vaccine to a suitable desorption procedure such as the following: dissolve in the vaccine to be examined sufficient *sodium citrate R* to give a 10 g/l solution; maintain at 37 °C for about 16 h and centrifuge until a clear supernatant liquid is obtained. Examined by a suitable immunochemical method (2.7.1), the clear supernatant liquid reacts with specific antisera to the components in the vaccine.

TESTS

Test for residual pertussis toxin. Use 3 groups of not fewer than 5 histamine-sensitive mice (see under Production) each weighing 18 g to 26 g. Using phosphate-buffered saline containing 2 g/l of gelatin, prepare a series of dilutions of the vaccine to be examined that have been shown to yield a graded response and attribute each dilution to a separate group of mice. Inject intraperitoneally into each mouse the dilution attributed to its group. Inject diluent into a fourth group of control mice. After 5 days, inject intraperitoneally into each mouse 1 mg of histamine base in a volume not exceeding 0.5 ml. Note the number of animals that die within 24 h of histamine challenge. Calculate the weight or volume of a preparation that sensitises 50 per cent of the mice injected using a suitable statistical method such as probit analysis (see 5.3). The residual activity of pertussis toxin does not exceed that of batches shown to be safe in clinical studies.

Reversibility of toxoid. Carry out the test for residual pertussis toxin described above using the vaccine incubated at 37 °C for 4 weeks in parallel with a sample stored at 2 °C to 8 °C. The degree of reversibility does not exceed that of batches shown to be safe in clinical studies.

Antimicrobial preservative. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical or physico-chemical method. The amount is not less than the minimum amount shown to be effective and is not greater than 115 per cent of the quantity stated on the label.

Aluminium (2.5.13): maximum 1.25 mg per single human dose, if aluminium hydroxide or hydrated aluminium phosphate is used as the adsorbent.

Free formaldehyde (2.4.18): maximum 0.2 g/l.

Sterility (2.6.1). It complies with the test for sterility.

ASSAY

The vaccine complies with the assay of pertussis vaccine (acellular) (2.7.16).

LABELLING

The label states:

- the names and amounts of the antigenic components present in the vaccine,
- the maximum amount of residual pertussis toxin present in the vaccine,
- the maximum degree of reversion of toxoid to toxin during the period of validity,
- the name and amount of the adsorbent,
- that the vaccine must be shaken before use,
- that the vaccine is not to be frozen.

01/2005:0161

PERTUSSIS VACCINE (ADSORBED)

Vaccinum pertussis adsorbatum

DEFINITION

Pertussis vaccine (adsorbed) is a sterile saline suspension of inactivated whole cells of one or more strains of *Bordetella pertussis* with a mineral adsorbent such as hydrated aluminium phosphate or aluminium hydroxide.

PRODUCTION

INACTIVATED *B. PERTUSSIS* SUSPENSION

Production is based on a seed-lot system. One or more strains of *B. pertussis* with known origin and history are used. Strains, culture medium and cultivation method are chosen in such a way that agglutinogens 1, 2 and 3 are present in the final vaccine. Each strain is grown for 24 h to 72 h in a liquid medium or on a solid medium; the medium used in the final cultivation stage does not contain blood or blood products. Human blood or blood products are not used in any culture media. The bacteria are harvested, washed to remove substances derived from the medium and suspended in a 9 g/l solution of sodium chloride or other suitable isotonic solution. The opacity of the suspension is determined not later than 2 weeks after harvest by comparison with the International Reference Preparation of Opacity and used as the basis of calculation for subsequent stages in vaccine preparation. The equivalence in International Units of the International Reference Preparation is stated by the World Health Organisation.

Single harvests are not used for the final bulk vaccine unless they have been shown to contain *B. pertussis* cells with the same characteristics, with regard to growth and agglutinogens, as the parent strain and to be free from contaminating bacteria and fungi. The bacteria are killed and detoxified in controlled conditions by means of a suitable chemical agent or by heating or by a combination of these methods. Freedom from live *B. pertussis* is tested using a suitable culture medium. The suspension is maintained at 5 ± 3 °C for a suitable period to diminish its toxicity.

FINAL BULK VACCINE

Suitable quantities of the inactivated single harvests are pooled to prepare the final bulk vaccine. A mineral adsorbent such as hydrated aluminium phosphate or aluminium hydroxide is added to the cell suspension. Suitable antimicrobial preservatives may be added. The bacterial concentration of the final bulk vaccine does not exceed that corresponding to an opacity of 20 IU per single human dose. If 2 or more strains of *B. pertussis* are used, the composition of consecutive lots of the final bulk vaccine shall be consistent with respect to the proportion of each strain as measured in opacity units.

Only a final bulk vaccine that complies with the following requirements may be used in the preparation of the final lot.

Antimicrobial preservative. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The amount is not less than 85 per cent and not greater than 115 per cent of the intended amount.

Sterility (2.6.1). Carry out the test for sterility using 10 ml for each medium.

FINAL LOT

The final bulk vaccine is distributed aseptically into sterile, tamper-proof containers. The containers are closed so as to prevent contamination.

Only a final lot that is satisfactory with respect to each of the requirements given below under Identification, Tests and Assay may be released for use. Provided the tests for specific toxicity, free formaldehyde and antimicrobial preservative and the assay have been carried out with satisfactory results on the final bulk vaccine, they may be omitted on the final lot.

IDENTIFICATION

Dissolve in the vaccine to be examined sufficient *sodium citrate R* to give a 100 g/l solution. Maintain at 37 °C for about 16 h and centrifuge to obtain a bacterial precipitate. Other suitable methods for separating the bacteria from the adsorbent may also be used. Identify pertussis vaccine by agglutination of the bacteria from the resuspended precipitate by antisera specific to *B. pertussis* or by the assay.

TESTS

Specific toxicity. Use not fewer than 5 healthy mice each weighing 14 g to 16 g for the vaccine group and for the saline control. Use mice of the same sex or distribute males and females equally between the groups. Allow the animals access to food and water for at least 2 h before injection and during the test. Inject each mouse of the vaccine group intraperitoneally with 0.5 ml, containing a quantity of the vaccine equivalent to not less than half the single human dose. Inject each mouse of the control group with 0.5 ml of a 9 g/l sterile solution of *sodium chloride R*, preferably containing the same amount of antimicrobial preservative as that injected with the vaccine. Weigh the groups of mice immediately before the injection and 72 h and 7 days after the injection. The vaccine complies with the test if: (a) at the end of 72 h the total mass of the group of vaccinated mice is not less than that preceding the injection; (b) at the end of 7 days the average increase in mass per vaccinated mouse is not less than 60 per cent of that per control mouse; and (c) not more than 5 per cent of the vaccinated mice die during the test. The test may be repeated and the results of the tests combined.

Aluminium (2.5.13): maximum 1.25 mg per single human dose, if aluminium hydroxide or hydrated aluminium phosphate is used as the adsorbent.

Free formaldehyde (2.4.18): maximum 0.2 g/l.

Antimicrobial preservative. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The content is not less than the minimum amount shown to be effective and is not greater than 115 per cent of the quantity stated on the label.

Sterility (2.6.1). The vaccine complies with the test for sterility.

ASSAY

Carry out the assay of pertussis vaccine (2.7.7).

The estimated potency is not less than 4 IU per single human dose and the lower confidence limit ($P = 0.95$) of the estimated potency is not less than 2 IU per single human dose.

LABELLING

The label states:

- the minimum number of International Units per single human dose,
- the name and the amount of the adsorbent,
- that the vaccine must be shaken before use,
- that the vaccine is not to be frozen.

01/2005:0966

PNEUMOCOCCAL POLYSACCHARIDE VACCINE

Vaccinum pneumococcale polysaccharidicum

DEFINITION

Pneumococcal polysaccharide vaccine consists of a mixture of equal parts of purified capsular polysaccharide antigens prepared from suitable pathogenic strains of *Streptococcus pneumoniae* whose capsules have been shown to be made up of polysaccharides that are capable of inducing satisfactory levels of specific antibodies in man. It contains the 23 immunochemically different capsular polysaccharides listed in Table 0966-1.

The vaccine is a clear, colourless liquid.

PRODUCTION

Production of the vaccine is based on a seed-lot system for each type. The production method shall have been shown to yield consistently pneumococcal polysaccharide vaccines of adequate safety and immunogenicity in man.

The production method is validated to demonstrate that the product, if tested, would comply with the test for abnormal toxicity for immunosera and vaccines for human use (2.6.9) modified as follows for the test in guinea-pigs: inject 10 human doses into each guinea-pig and observe for 12 days.

MONOVALENT BULK POLYSACCHARIDES

The bacteria are grown in a suitable liquid medium that does not contain blood-group substances or high-molecular-mass polysaccharides. The bacterial purity of the culture is verified and the culture is inactivated with phenol. Impurities are removed by such techniques as fractional precipitation, enzymatic digestion and ultrafiltration. The polysaccharide is obtained by fractional precipitation, washed, and dried in a vacuum to a residual moisture content shown to be favourable to the stability of the polysaccharide. The residual moisture content is determined by drying under reduced pressure over diphosphorus pentoxide or by thermogravimetric analysis and the value obtained is used to calculate the results of the tests shown below with reference to the dried substance. The monovalent bulk polysaccharide is stored at a suitable temperature in conditions that avoid the uptake of moisture.

Only a monovalent bulk polysaccharide that complies with the following requirements may be used in the preparation of the final bulk vaccine. Percentage contents of components, determined by the methods prescribed below, are shown in Table 0966-1.

Protein (2.5.16).

Nucleic acids (2.5.17).

Total nitrogen (2.5.9).

Phosphorus (2.5.18).

Molecular size. Determine by size-exclusion chromatography (2.2.30) using *cross-linked agarose for chromatography R* or *cross-linked agarose for chromatography R1*.

Uronic acids (2.5.22).

Hexosamines (2.5.20).

Methylpentoses (2.5.21).

O-Acetyl groups (2.5.19).

Identification (2.7.1). Confirm the identity of the monovalent bulk polysaccharide by double immunodiffusion or electroimmunodiffusion (except for polysaccharides 7F, 14 and 33F), using specific antisera.

Specificity. No reaction occurs when the antigens are tested against all the antisera specific for the other polysaccharides of the vaccine, including factor sera for distinguishing types within groups. The polysaccharides are tested at a concentration of 50 µg/ml using a method capable of detecting 0.5 µg/ml.

FINAL BULK VACCINE

The final bulk vaccine is obtained by aseptically mixing the different polysaccharide powders. The uniform mixture is aseptically dissolved in a suitable isotonic solution so that one human dose of 0.50 ml contains 25 µg of each polysaccharide. An antimicrobial preservative may be added. The solution is sterilised by filtration through a bacteria-retentive filter.

Only a final bulk vaccine that complies with the following requirements may be used in the preparation of the final lot.

Antimicrobial preservative. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The content is not less than 85 per cent and not greater than 115 per cent of the intended amount.

Sterility (2.6.1). The final bulk vaccine complies with the test for sterility, using 10 ml for each medium.

FINAL LOT

The final bulk vaccine is distributed aseptically into sterile, tamper-proof containers.

Only a final lot that is satisfactory with respect to each of the requirements given below under identification, tests and assay may be released for use. Provided that the tests for phenol and for antimicrobial preservative have been carried out with satisfactory results on the final bulk vaccine, they may be omitted on the final lot. When consistency of production has been established on a suitable number of consecutive batches, the assay may be replaced by a qualitative test that identifies each polysaccharide, provided that an assay has been performed on each monovalent bulk polysaccharide used in the preparation of the final lot.

IDENTIFICATION

The assay serves also to identify the vaccine.

TESTS

pH (2.2.3). The pH of the vaccine is 4.5 to 7.4.

Antimicrobial preservative. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The content is not less than the minimum amount shown to be effective and is not greater than 115 per cent of the quantity stated on the label.

Phenol (2.5.15). Not more than 2.5 g/l.

Sterility (2.6.1). It complies with the test for sterility.

Table 0966.-1 – Percentage contents of components of monovalent bulk polysaccharides

Molecular type*	Protein	Nucleic acids	Total nitrogen	Phosphorus	Molecular size (K_0)		Uronic acids	Hexosamines	Methyl-pentoses	O-acetyl Groups
					**	***				
1	≤ 2	≤ 2	3.5-6	0-1.5	≤ 0.15		≥ 45			≥ 1.8
2	≤ 2	≤ 2	0-1	0-1.0	≤ 0.15		≥ 15		≥ 38	
3	≤ 5	≤ 2	0-1	0-1.0	≤ 0.15		≥ 40			
4	≤ 3	≤ 2	4-6	0-1.5	≤ 0.15			≥ 40		
5	≤ 7.5	≤ 2	2.5-6.0	≤ 2		≤ 0.60	≥ 12	≥ 20		
6B	≤ 2	≤ 2	0-2	2.5-5.0		≤ 0.50			≥ 15	
7F	≤ 5	≤ 2	1.5-4.0	0-1.0	≤ 0.20				≥ 13	
8	≤ 2	≤ 2	0-1	0-1.0	≤ 0.15		≥ 25			
9N	≤ 2	≤ 1	2.2-4	0-1.0	≤ 0.20		≥ 20	≥ 28		
9V	≤ 2	≤ 2	0.5-3	0-1.0		≤ 0.45	≥ 15	≥ 13		
10A	≤ 7	≤ 2	0.5-3.5	1.5-3.5		≤ 0.65		≥ 12		
11A	≤ 3	≤ 2	0-2.5	2.0-5.0		≤ 0.40				≥ 9
12F	≤ 3	≤ 2	3-5	0-1.0	≤ 0.25			≥ 25		
14	≤ 5	≤ 2	1.5-4	0-1.0	≤ 0.30			≥ 20		
15B	≤ 3	≤ 2	1-3	2.0-4.5		≤ 0.55		≥ 15		
17A or 17F	≤ 2	≤ 2	0-1.5	0-3.5		≤ 0.45			≥ 20	
18C	≤ 3	≤ 2	0-1	2.4-4.9	≤ 0.15				≥ 14	
19A	≤ 2	≤ 2	0.6-3.5	3.0-7.0	≤ 0.45			≥ 12	≥ 20	
19F	≤ 3	≤ 2	1.4-3.5	3.0-5.5	≤ 0.20			≥ 12.5	≥ 20	
20	≤ 2	≤ 2	0.5-2.5	1.5-4.0		≤ 0.60		≥ 12		
22F	≤ 2	≤ 2	0-2	0-1.0		≤ 0.55	≥ 15		≥ 25	
23F	≤ 2	≤ 2	0-1	3.0-4.5	≤ 0.15				≥ 37	
33F	≤ 2.5	≤ 2	0-2	0-1.0		≤ 0.50				

* The different types are indicated using the Danish nomenclature.

** Cross-linked agarose for chromatography R.

*** Cross-linked agarose for chromatography R1.

Pyrogens (2.6.8). It complies with the test for pyrogens. Inject per kilogram of the rabbit's mass 1 ml of a dilution of the vaccine containing 2.5 µg/ml of each polysaccharide.

01/2005:0214

POLIOMYELITIS VACCINE (INACTIVATED)

Vaccinum poliomyelitidis inactivatum

DEFINITION

Poliomyelitis vaccine (inactivated) is a liquid preparation of suitable strains of human polioviruses 1, 2 and 3 grown in suitable cell cultures and inactivated by a validated method. It is a clear liquid that may be coloured owing to the presence of a pH indicator.

PRODUCTION

The production method shall have been shown to yield consistently vaccines of acceptable safety and immunogenicity in man.

Production of the vaccine is based on a virus seed-lot system. Cell lines are used according to a cell-bank system. If primary, secondary or tertiary monkey kidney cells are used, production complies with the requirements indicated below.

ASSAY

Determine the content of each polysaccharide by a suitable immunochemical method (2.7.1), using antisera specific for each polysaccharide contained in the vaccine, including factor sera for types within groups, and purified polysaccharides of each type as standards.

The vaccine contains not less than 70 per cent and not more than 130 per cent of the quantity stated on the label for each polysaccharide. The confidence limits ($P = 0.95$) are not less than 80 per cent and not more than 120 per cent of the estimated content.

LABELLING

The label states:

- the number of micrograms of each polysaccharide per human dose,
- the total amount of polysaccharide in the container.

Unless otherwise justified and authorised, the virus in the final vaccine shall not have undergone more passages from the master seed lot than was used to prepare the vaccine shown in clinical studies to be satisfactory with respect to safety and efficacy.

The production method is validated to demonstrate that the product, if tested, would comply with the test for abnormal toxicity for immunosera and vaccines for human use (2.6.9).

SUBSTRATE FOR VIRUS PROPAGATION

The virus is propagated in a human diploid cell line (5.2.3), in a continuous cell line (5.2.3) or in primary, secondary or tertiary monkey kidney cells.

Primary, secondary or tertiary monkey kidney cells. The following special requirements for the substrate for virus propagation apply to primary, secondary or tertiary monkey kidney cells.

Monkeys used in the preparation of kidney cell cultures for production and control of the vaccine. The animals used are of a species approved by the competent authority, in good health and, unless otherwise justified and authorised, have not been previously employed for experimental purposes. Kidney cells used for vaccine production and control are derived from monitored, closed colonies of monkeys bred in captivity, not from animals caught in the wild; a previously approved seed lot prepared using virus passaged in cells from wild monkeys may, subject to approval by the competent authority, be used for vaccine production if historical data on safety justify this.

Monitored, closed colonies of monkeys. The monkeys are kept in groups in cages. Freedom from extraneous agents is achieved by the use of animals maintained in closed colonies that are subject to continuous and systematic veterinary and laboratory monitoring for the presence of infectious agents. The supplier of animals is certified by the competent authority. Each monkey is tested serologically at regular intervals during a quarantine period of not less than 6 weeks imposed before entering the colony and then during its stay in the colony.

The monkeys used are shown to be tuberculin-negative and free from antibodies to simian virus 40 (SV40) and simian immunodeficiency virus. The blood sample used in testing for SV40 antibodies must be taken as close as possible to the time of removal of the kidneys. If *Macaca* sp. monkeys are used for production, the monkeys are also shown to be free from antibodies to herpesvirus B (cercopithecine herpesvirus 1) infection. Human herpesvirus 1 has been used as an indicator for freedom from herpesvirus B antibodies on account of the danger of handling herpesvirus B (cercopithecine herpesvirus 1).

Monkeys from which kidneys are to be removed are thoroughly examined, particularly for evidence of tuberculosis and herpesvirus B (cercopithecine herpesvirus 1) infection. If a monkey shows any pathological lesion relevant to the use of its kidneys in the preparation of a seed lot or vaccine, it is not to be used nor are any of the remaining monkeys of the group concerned unless it is evident that their use will not impair the safety of the product.

All the operations described in this section are conducted outside the area where the vaccine is produced.

Monkey cell cultures for vaccine production. Kidneys that show no pathological signs are used for preparing cell cultures. Each group of cell cultures derived from a single monkey forms a separate production cell culture giving rise to a separate single harvest.

The primary monkey kidney cell suspension complies with the test for mycobacteria (2.6.2); disrupt the cells before carrying out the test.

If secondary or tertiary cells are used, it shall be demonstrated by suitable validation tests that cell cultures beyond the passage level used for production are free from tumorigenicity.

SEED LOTS

Each of the 3 strains of poliovirus used shall be identified by historical records that include information on the origin of the strain and its subsequent manipulation.

Only a working seed lot that complies with the following requirements may be used for virus propagation.

Identification. Each working seed lot is identified as human poliovirus 1, 2 or 3 by virus neutralisation in cell culture using specific antibodies.

Virus concentration. The virus concentration of each working seed lot is determined to define the quantity of virus to be used for inoculation of production cell cultures.

Extraneous agents. The working seed lot complies with the requirements for seed lots for virus vaccines (2.6.16). In addition, if primary, secondary or tertiary monkey kidney cells have been used for isolation of the strain, measures are taken to ensure that the strain is not contaminated with simian viruses such as simian immunodeficiency virus, simian virus 40, filoviruses and herpesvirus B (cercopithecine herpesvirus 1). A working seed lot produced in primary, secondary or tertiary monkey kidney cells complies with the requirements given below under Virus propagation and harvest for single harvests produced in such cells.

PROPAGATION AND HARVEST

All processing of the cell bank and cell cultures is done under aseptic conditions in an area where no other cells or viruses are being handled. Approved animal serum (but not human serum) may be used in the cell culture media. Serum and trypsin used in the preparation of cell suspensions and media are shown to be free from extraneous agents. The cell culture media may contain a pH indicator such as phenol red and approved antibiotics at the lowest effective concentration. Not less than 500 ml of the cell cultures employed for vaccine production is set aside as uninfected cell cultures (control cells); where continuous cell lines in a fermenter are used for production, 200×10^6 cells are set aside to prepare control cells; where primary, secondary or tertiary monkey kidney cells are used for production, a cell sample equivalent to at least 500 ml of the cell suspension, at the concentration employed for vaccine production, is taken to prepare control cells.

Only a single harvest that complies with the following requirements may be used in the preparation of the vaccine. The tests for identification and bacterial and fungal contamination may be carried out instead on the purified, pooled monovalent harvest. After demonstration of consistency of production at the stage of the single harvest, the test for virus concentration may be carried out instead on the purified, pooled monovalent harvest.

Control cells. The control cells of the production cell culture comply with a test for identification (if a cell-bank system is used for production) and with the requirements for extraneous agents (2.6.16); where primary, secondary or tertiary monkey kidney cells are used, the tests in cell cultures are carried out as shown below under Test in rabbit kidney cell cultures and Test in cercopithecus kidney cell cultures).

- *Test in rabbit kidney cell cultures.* Test a sample of at least 10 ml of the pooled supernatant fluid from the control cultures for the absence of herpesvirus B (cercopithecine herpesvirus 1) and other viruses by inoculation onto rabbit kidney cell cultures. The dilution of supernatant in the nutrient medium is not

greater than 1/4 and the area of the cell layer is at least 3 cm² per millilitre of inoculum. Set aside one or more containers of each batch of cells with the same medium as non-inoculated control cells. Incubate the cultures at 37 °C and observe for at least 2 weeks. The test is not valid if more than 20 per cent of the control cells are discarded for non-specific, accidental reasons.

- **Test in cercopithecus kidney cell cultures.** Test a sample of at least 10 ml of the pooled supernatant fluid from the control cultures for the absence of SV40 virus and other extraneous agents by inoculation onto cell cultures prepared from the kidneys of cercopithecus monkeys, or other cells shown to be at least as sensitive for SV40, by the method described under Test in rabbit kidney cell cultures. The test is not valid if more than 20 per cent of the control cell cultures are discarded for non-specific, accidental reasons.

Identification. The single harvest is identified as containing human poliovirus 1, 2 or 3 by virus neutralisation in cell cultures using specific antibodies.

Virus concentration. The virus concentration of each single harvest is determined by titration of infectious virus in cell cultures.

Bacterial and fungal contamination. The single harvest complies with the test for sterility (2.6.1), carried out using 10 ml for each medium.

Mycoplasmas (2.6.7). The single harvest complies with the test for mycoplasmas, carried out using 10 ml.

Test in rabbit kidney cell cultures. Where primary, secondary or tertiary monkey kidney cells are used for production, test a sample of at least 10 ml of the single harvest for the absence of herpesvirus B (cercopithecine herpesvirus 1) and other viruses by inoculation onto rabbit kidney cell cultures as described above for the control cells.

Test in cercopithecus kidney cell cultures. Where primary, secondary or tertiary monkey kidney cells are used for production, test a sample of at least 10 ml of the single harvest for the absence of SV40 virus and other extraneous agents. Neutralise the sample by a high-titre antiserum against the specific type of poliovirus. Test the sample in primary cercopithecus kidney cell cultures or cells that have been demonstrated to be at least as susceptible for SV40. Incubate the cultures at 37 °C and observe for 14 days. At the end of this period, make at least one subculture of fluid in the same cell culture system and observe both primary cultures and subcultures for an additional 14 days.

PURIFICATION AND PURIFIED MONOVALENT HARVEST

Several single harvests of the same type may be pooled and may be concentrated. The monovalent harvest or pooled monovalent harvest is purified by validated methods. If continuous cell lines are used for production, the purification process shall have been shown to reduce consistently the content of substrate-cell DNA to not more than 100 pg per single human dose.

Only a purified monovalent harvest that complies with the following requirements may be used for the preparation of the inactivated monovalent harvest.

Identification. The virus is identified by virus neutralisation in cell cultures using specific antibodies or by determination of D-antigen.

Virus concentration. The virus concentration is determined by titration of infectious virus.

Specific activity. The ratio of the virus concentration or the D-antigen content, determined by a suitable immunochemical method (2.7.1), to the total protein content (specific activity) of the purified monovalent harvest is within the limits approved for the particular product.

INACTIVATION AND INACTIVATED MONOVALENT HARVEST

Several purified monovalent harvests of the same type may be mixed before inactivation. To avoid failures in inactivation caused by the presence of virus aggregates, filtration is carried out before and during inactivation; inactivation is started within a suitable period, preferably not more than 24 h and in any case not more than 72 h, of the prior filtration. The virus suspension is inactivated by a validated method that has been shown to inactivate poliovirus without destruction of immunogenicity; during validation studies, an inactivation curve with at least 4 points (for example, time 0 h, 24 h, 48 h and 96 h) is established showing the decrease in concentration of live virus with time. If formaldehyde is used for inactivation, the presence of an excess of formaldehyde at the end of the inactivation period is verified.

Only an inactivated monovalent harvest that complies with the following requirements may be used in the preparation of a trivalent pool of inactivated monovalent harvests or a final bulk vaccine.

Test for effective inactivation. After neutralisation of the formaldehyde with sodium bisulphite (where applicable), verify the absence of residual live poliovirus by inoculation on suitable cell cultures of 2 samples of each inactivated monovalent harvest, corresponding to at least 1500 human doses. Take one sample not later than three-quarters of the way through the inactivation period and the other at the end. Inoculate the samples in cell cultures such that the dilution of vaccine in the nutrient medium is not greater than 1/4 and the area of the cell layer is at least 3 cm² per millilitre of inoculum. Set aside one or more containers with the same medium as non-inoculated control cells. Observe the cell cultures for at least 3 weeks. Make not fewer than 2 passages from each container, one at the end of the observation period and the other 1 week before; for the passages, use cell culture supernatant and inoculate as for the initial sample. Observe the subcultures for at least 2 weeks. No sign of poliovirus multiplication is present in the cell cultures. At the end of the observation period, test the susceptibility of the cell culture used by inoculation of live poliovirus of the same type as that present in the inactivated monovalent harvest.

Sterility (2.6.1). The inactivated monovalent harvest complies with the test for sterility, carried out using 10 ml for each medium.

D-antigen content. The content of D-antigen determined by a suitable immunochemical method (2.7.1) is within the limits approved for the particular preparation.

FINAL BULK VACCINE

The final bulk vaccine is prepared directly from the inactivated monovalent harvests of human polioviruses 1, 2 and 3 or from a trivalent pool of inactivated monovalent harvests. If a trivalent pool of inactivated monovalent harvests is used, a test for effective inactivation is carried out on this pool instead of on the final bulk vaccine. A stabiliser and an antimicrobial preservative may be added.

Only a final bulk vaccine that complies with the following requirements may be used in the preparation of the final lot.

Sterility (2.6.1). The final bulk vaccine complies with the test for sterility, carried out using 10 ml for each medium.

Antimicrobial preservative. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical or physicochemical method. The amount is not less than 85 per cent and not greater than 115 per cent of the intended amount.

Inactivation. Before addition of any antimicrobial preservative, a sample of at least 1500 ml or, for a purified and concentrated vaccine, the equivalent of 1500 doses is tested for residual live poliovirus in cell cultures, as described for the inactivated monovalent harvest. If the final bulk vaccine is prepared from a trivalent pool of inactivated monovalent harvests, the test for inactivation is carried out on that pool rather than on the final bulk vaccine.

FINAL LOT

Only a final lot that complies with each of the requirements given below under Identification, Tests and Assay may be released for use. Provided that the tests for free formaldehyde and antimicrobial preservative and the *in vivo* assay have been performed with satisfactory results on the final bulk vaccine, they may be omitted on the final lot. Provided that the test for bovine serum albumin has been performed with satisfactory results on the trivalent pool of inactivated monovalent harvests or on the final bulk vaccine, it may be omitted on the final lot.

IDENTIFICATION

The vaccine is shown to contain human polioviruses 1, 2 and 3 by a suitable immunochemical method (2.7.1) such as the determination of D-antigen by enzyme-linked immunosorbent assay (ELISA).

TESTS

Free formaldehyde (2.4.18): maximum 0.2 g/l.

Antimicrobial preservative. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical or physicochemical method. The amount is not less than the minimum amount shown to be effective and is not greater than 115 per cent of that stated on the label.

Protein nitrogen content (Lowry method). Not more than 10 µg of protein nitrogen per single human dose.

Bovine serum albumin. Not more than 50 ng per single human dose, determined by a suitable immunochemical method (2.7.1).

Sterility (2.6.1). The vaccine complies with the test for sterility.

Bacterial endotoxins (2.6.14): less than 5 IU per single human dose.

ASSAY

D-antigen content. As a measure of consistency of production, determine the D-antigen content for human polioviruses 1, 2 and 3 by a suitable immunochemical method (2.7.1) using a reference preparation calibrated in European Pharmacopoeia D-antigen units. For each type, the content, expressed with reference to the amount of D-antigen stated on the label, is within the limits approved for the particular product.

Poliomyelitis vaccine (inactivated) BRP is calibrated in European Pharmacopoeia units and intended for use in the assay of D-antigen. The European Pharmacopoeia unit and the International Unit are equivalent.

In vivo test. The vaccine complies with the *in vivo* assay of poliomyelitis vaccine (inactivated) (2.7.20).

LABELLING

The label states:

- the types of poliovirus contained in the vaccine,
- the nominal amount of virus of each type (1, 2 and 3), expressed in Ph. Eur. units of D-antigen, per single human dose,
- the cell substrate used to prepare the vaccine.

01/2005:0215

POLIOMYELITIS VACCINE (ORAL)

Vaccinum poliomyelitidis perorale

DEFINITION

Oral poliomyelitis vaccine is a preparation of approved strains of live attenuated poliovirus type 1, 2 or 3 grown in *in vitro* cultures of approved cells, containing any one type or any combination of the 3 types of Sabin strains, presented in a form suitable for oral administration.

The vaccine is a clear liquid that may be coloured owing to the presence of a pH indicator.

PRODUCTION

The vaccine strains and the production method shall have been shown to yield consistently vaccines that are both immunogenic and safe in man.

The production of vaccine is based on a virus seed-lot system. Cell lines are used according to a cell-bank system. If primary monkey kidney cell cultures are used, production complies with the requirements indicated below. Unless otherwise justified and authorised, the virus in the final vaccine shall not have undergone more than 2 passages from the master seed lot.

SUBSTRATE FOR VIRUS PROPAGATION

The virus is propagated in human diploid cells (5.2.3), in continuous cell lines (5.2.3) or in primary monkey kidney cell cultures (including serially passaged cells from primary monkey kidney cells).

Primary monkey kidney cell cultures. *The following special requirements for the substrate for virus propagation apply to primary monkey kidney cell cultures.*

Monkeys used for preparation of primary monkey kidney cell cultures and for testing of virus. If the vaccine is prepared in primary monkey kidney cell cultures, animals of a species approved by the competent authority, in good health, kept in closed or intensively monitored colonies and not previously employed for experimental purposes shall be used.

The monkeys shall be kept in well-constructed and adequately ventilated animal rooms in cages spaced as far apart as possible. Adequate precautions shall be taken to prevent cross-infection between cages. Not more than 2 monkeys shall be housed per cage and cage-mates shall not be interchanged. The monkeys shall be kept in the country of manufacture of the vaccine in quarantine groups for a period of not less than 6 weeks before use. A quarantine group is a colony of selected, healthy monkeys kept in one room, with separate feeding and cleaning facilities, and having no contact with other monkeys during the quarantine period. If at any time during the quarantine period the overall death rate of a shipment consisting of one or more groups reaches 5 per cent (excluding deaths from accidents or where the cause was specifically determined not to be an infectious disease), monkeys from that entire shipment shall continue in quarantine from that time for a minimum of 6 weeks. The groups shall be kept continuously in isolation, as in

quarantine, even after completion of the quarantine period, until the monkeys are used. After the last monkey of a group has been taken, the room that housed the group shall be thoroughly cleaned and decontaminated before being used for a fresh group. If kidneys from near-term monkeys are used, the mother is quarantined for the term of pregnancy.

Monkeys from which kidneys are to be removed shall be anaesthetised and thoroughly examined, particularly for evidence of tuberculosis and cercopithecoid herpesvirus 1 (B virus) infection.

If a monkey shows any pathological lesion relevant to the use of its kidneys in the preparation of a seed lot or vaccine, it shall not be used, nor shall any of the remaining monkeys of the quarantine group concerned be used unless it is evident that their use will not impair the safety of the product.

All the operations described in this section shall be conducted outside the areas where the vaccine is produced.

The monkeys used shall be shown to be free from antibodies to simian virus 40 (SV40), simian immunodeficiency virus and spumaviruses. The blood sample used in testing for SV40 antibodies must be taken as close as possible to the time of removal of the kidneys. If *Macaca* spp. are used for production, the monkeys shall also be shown to be free from antibodies to cercopithecoid herpesvirus 1 (B virus). Human herpesvirus has been used as an indicator for freedom from B virus antibodies on account of the danger of handling cercopithecoid herpesvirus 1 (B virus).

Primary monkey kidney cell cultures for vaccine production. Kidneys that show no pathological signs are used for preparing cell cultures. If the monkeys are from a colony maintained for vaccine production, serially passaged monkey kidney cell cultures from primary monkey kidney cells may be used for virus propagation, otherwise the monkey kidney cells are not propagated in series. Virus for the preparation of vaccine is grown by aseptic methods in such cultures. If animal serum is used in the propagation of the cells, the maintenance medium after virus inoculation shall contain no added serum.

Each group of cell cultures derived from a single monkey or from foetuses from no more than 10 near-term monkeys is prepared and tested as an individual group.

VIRUS SEED LOTS

The strains of poliovirus used shall be identified by historical records that include information on the origin and subsequent manipulation of the strains.

Working seed lots are prepared by a single passage from a master seed lot and at an approved passage level from the original Sabin virus. Virus seed lots are prepared in large quantities and stored at a temperature below -60°C .

Only a virus seed lot that complies with the following requirements may be used for virus propagation.

Identification. Each working seed lot is identified as poliovirus of the given type, using specific antibodies.

Virus concentration. Determined by the method described below, the virus concentration is the basis for the quantity of virus used in the neurovirulence test.

Extraneous agents (2.6.16). If the working seed lot is produced in human diploid cells or in a continuous cell line, it complies with the requirements for seed lots for virus vaccines. If the working seed lot is produced in primary monkey kidney cell cultures, it complies with the requirements given below under Virus Propagation and Harvest and Monovalent Pooled Harvest and with the tests

in adult mice, suckling mice and guinea-pigs given under 2.6.16. **Tests for extraneous agents in viral vaccines for human use.**

Working seed lots shall be free from detectable DNA sequences from simian virus 40 (SV40).

Neurovirulence (2.6.19). Each master and working seed lot complies with the test for neurovirulence of poliomyelitis vaccine (oral). Furthermore, the seed lot shall cease to be used in vaccine production if the frequency of failure of the monovalent pooled harvests produced from it is greater than predicted statistically. This statistical prediction is calculated after each test on the basis of all the monovalent pooled harvests tested; it is equal to the probability of false rejection on the occasion of a first test (i.e. 1 per cent), the probability of false rejection on retest being negligible. If the test is carried out only by the manufacturer, the test slides are provided to the control authority for assessment. Reference preparations of the 3 types of poliovirus at the Sabin Original + 2 passage levels are available on application to Biologicals, WHO, Geneva, Switzerland.

Genetic markers. Each working seed lot is tested for its replicating properties at temperatures ranging from 36°C to 40°C as described under Monovalent Pooled Harvest.

VIRUS PROPAGATION AND HARVEST

All processing of the cell banks and subsequent cell cultures is done under aseptic conditions in an area where no other cells are handled. Approved animal (but not human) serum may be used in the media, but the final medium for maintaining cell growth during virus multiplication does not contain animal serum. Serum and trypsin used in the preparation of cell suspensions and media are shown to be free from live extraneous agents. The cell-culture medium may contain a pH indicator such as phenol red and approved antibiotics at the lowest effective concentration. It is preferable to have a substrate free from antibiotics during production. On the day of inoculation with the virus working seed lot, not less than 5 per cent or 1000 ml, whichever is less, of the cell cultures employed for vaccine production are set aside as uninfected cell cultures (control cells); special requirements, given below, apply to control cells when the vaccine is produced in primary monkey kidney cell cultures. The virus suspension is harvested not later than 4 days after virus inoculation. After inoculation of the production cell culture with the virus working seed lot, inoculated cells are maintained at a fixed temperature, shown to be suitable, within the range 33°C to 35°C ; the temperature is maintained constant to $\pm 0.5^{\circ}\text{C}$; control cell cultures are maintained at $33-35^{\circ}\text{C}$ for the relevant incubation periods.

Only a single virus harvest that complies with the following requirements may be used in the preparation of the monovalent pooled harvest.

Virus concentration. The virus concentration of virus harvests is determined as prescribed under Assay to monitor consistency of production and to determine the dilution to be used for the final bulk vaccine.

Extraneous agents (2.6.16).

Control cells. The control cells of the production cell culture from which the virus harvest is derived comply with a test for identity and with the requirements for extraneous agents (2.6.16) or, where primary monkey kidney cell cultures are used, as shown below.

Primary monkey kidney cell cultures. The following special requirements apply to virus propagation and harvest in primary monkey kidney cell cultures.

Cell cultures. On the day of inoculation with virus working seed lot, each cell culture is examined for degeneration

caused by an infective agent. If, in this examination, evidence is found of the presence in a cell culture of any extraneous agent, the entire group of cultures concerned shall be rejected.

On the day of inoculation with the virus working seed lot, a sample of at least 30 ml of the pooled fluid removed from the cell cultures of the kidneys of each single monkey or from foetuses from not more than 10 near-term monkeys is divided into 2 equal portions. 1 portion of the pooled fluid is tested in monkey kidney cell cultures prepared from the same species, but not the same animal, as that used for vaccine production. The other portion of the pooled fluid is, where necessary, tested in monkey kidney cell cultures from another species so that tests on the pooled fluids are done in cell cultures from at least 1 species known to be sensitive to SV40. The pooled fluid is inoculated into bottles of these cell cultures in such a way that the dilution of the pooled fluid in the nutrient medium does not exceed 1 in 4. The area of the cell sheet is at least 3 cm²/ml of pooled fluid. At least 1 bottle of each kind of cell culture remains uninoculated to serve as a control. If the monkey species used for vaccine production is known to be sensitive to SV40, a test in a second species is not required. Animal serum may be used in the propagation of the cells, provided that it does not contain SV40 antibody, but the maintenance medium after inoculation of test material contains no added serum except as described below.

The cultures are incubated at a temperature of 35-37 °C and are observed for a total period of at least 4 weeks. During this observation period and after not less than 2 weeks' incubation, at least 1 subculture of fluid is made from each of these cultures in the same cell culture system. The subcultures are also observed for at least 2 weeks.

Serum may be added to the original culture at the time of subculturing, provided that the serum does not contain SV40 antibody.

Fluorescent-antibody techniques may be useful for detecting SV40 virus and other viruses in the cells.

A further sample of at least 10 ml of the pooled fluid is tested for cercopithecid herpesvirus 1 (B virus) and other viruses in rabbit kidney cell cultures. Serum used in the nutrient medium of these cultures shall have been shown to be free from inhibitors of B virus. Human herpesvirus has been used as an indicator for freedom from B virus inhibitors on account of the danger of handling cercopithecid herpesvirus 1 (B virus). The sample is inoculated into bottles of these cell cultures in such a way that the dilution of the pooled fluid in the nutrient medium does not exceed 1 in 4. The area of the cell sheet is at least 3 cm²/ml of pooled fluid. At least 1 bottle of the cell cultures remains uninoculated to serve as a control.

The cultures are incubated at a temperature of 35-37 °C and observed for at least 2 weeks.

A further sample of 10 ml of the pooled fluid removed from the cell cultures on the day of inoculation with the seed lot virus is tested for the presence of extraneous agents by inoculation into human cell cultures sensitive to measles virus.

The tests are not valid if more than 20 per cent of the culture vessels have been discarded for non-specific accidental reasons by the end of the respective test periods.

If, in these tests, evidence is found of the presence of an extraneous agent, the single harvest from the whole group of cell cultures concerned is rejected.

If the presence of cercopithecid herpesvirus 1 (B virus) is demonstrated, the manufacture of oral poliomyelitis vaccine shall be discontinued and the competent authority shall

be informed. Manufacturing shall not be resumed until a thorough investigation has been completed and precautions have been taken against any reappearance of the infection, and then only with the approval of the competent authority.

If these tests are not done immediately, the samples of pooled cell-culture fluid shall be kept at a temperature of -60 °C or below, with the exception of the sample for the test for B virus, which may be held at 4 °C, provided that the test is done not more than 7 days after it has been taken.

Control cell cultures. On the day of inoculation with the virus working seed lot, 25 per cent (but not more than 2.5 litres) of the cell suspension obtained from the kidneys of each single monkey or from not more than 10 near-term monkeys is taken to prepare uninoculated control cell cultures. These control cell cultures are incubated in the same conditions as the inoculated cultures for at least 2 weeks and are examined during this period for evidence of cytopathic changes. The tests are not valid if more than 20 per cent of the control cell cultures have been discarded for non-specific, accidental reasons. At the end of the observation period, the control cell cultures are examined for degeneration caused by an infectious agent. If this examination or any of the tests required in this section shows evidence of the presence in a control culture of any extraneous agent, the poliovirus grown in the corresponding inoculated cultures from the same group shall be rejected.

Tests for haemadsorbing viruses. At the time of harvest or within 4 days of inoculation of the production cultures with the virus working seed lot, a sample of 4 per cent of the control cell cultures is taken and tested for haemadsorbing viruses. At the end of the observation period, the remaining control cell cultures are similarly tested. The tests are made as described in 2.6.16. **Tests for extraneous agents in viral vaccines for human use.**

Tests for other extraneous agents. At the time of harvest, or within 7 days of the day of inoculation of the production cultures with the working seed lot, a sample of at least 20 ml of the pooled fluid from each group of control cultures is taken and tested in 2 kinds of monkey kidney cell culture, as described above.

At the end of the observation period for the original control cell cultures, similar samples of the pooled fluid are taken and the tests referred to in this section in the 2 kinds of monkey kidney cell culture and in the rabbit cell cultures are repeated, as described above under Cell cultures.

If the presence of cercopithecid herpesvirus 1 (B virus) is demonstrated, the production cell cultures shall not be used and the measures concerning vaccine production described above must be undertaken.

The fluids collected from the control cell cultures at the time of virus harvest and at the end of the observation period may be pooled before testing for extraneous agents. A sample of 2 per cent of the pooled fluid is tested in each of the cell culture systems specified.

Single harvests.

Tests for neutralised single harvests in primary monkey kidney cell cultures. A sample of at least 10 ml of each single harvest is neutralised by a type-specific poliomyelitis antiserum prepared in animals other than monkeys. In preparing antisera for this purpose, the immunising antigens used shall be prepared in non-simian cells.

Half of the neutralised suspension (corresponding to at least 5 ml of single harvest) is tested in monkey kidney cell cultures prepared from the same species, but not the same animal, as that used for vaccine production. The other half of the neutralised suspension is tested, if necessary, in monkey

kidney cell cultures from another species so that the tests on the neutralised suspension are done in cell cultures from at least 1 species known to be sensitive to SV40.

The neutralised suspensions are inoculated into bottles of these cell cultures in such a way that the dilution of the suspension in the nutrient medium does not exceed 1 in 4. The area of the cell sheet is at least 3 cm²/ml of neutralised suspension. At least 1 bottle of each type of cell culture remains uninoculated to serve as a control and is maintained by nutrient medium containing the same concentration of the specific antiserum used for neutralisation.

Animal serum may be used in the propagation of the cells, provided that it does not contain SV40 antibody, but the maintenance medium, after the inoculation of the test material, contains no added serum other than the poliovirus neutralising antiserum, except as described below.

The cultures are incubated at a temperature of 35-37 °C and observed for a total period of at least 4 weeks. During this observation period and after not less than 2 weeks' incubation, at least 1 subculture of fluid is made from each of these cultures in the same cell-culture system. The subcultures are also observed for at least 2 weeks.

Serum may be added to the original cultures at the time of subculturing, provided that the serum does not contain SV40 antibody.

Additional tests are made for extraneous agents on a further sample of the neutralised single harvests by inoculation of 10 ml into human cell cultures sensitive to measles virus.

Fluorescent-antibody techniques may be useful for detecting SV40 virus and other viruses in the cells.

The tests are not valid if more than 20 per cent of the culture vessels have been discarded for non-specific accidental reasons by the end of the respective test periods.

If any cytopathic changes occur in any of the cultures, the causes of these changes are investigated. If the cytopathic changes are shown to be due to unneutralised poliovirus, the test is repeated. If there is evidence of the presence of SV40 or other extraneous agents attributable to the single harvest, that single harvest is rejected.

MONOVALENT POOLED HARVEST

Monovalent pooled harvests are prepared by pooling a number of satisfactory single harvests of the same virus type. Monovalent pooled harvests from continuous cell lines may be purified. Each monovalent pooled harvest is filtered through a bacteria-retentive filter.

Only a monovalent pooled harvest that complies with the following requirements may be used in the preparation of the final bulk vaccine.

Identification. Each monovalent pooled harvest is identified as poliovirus of the given type, using specific antibodies.

Virus concentration. The virus concentration is determined by the method described below and serves as the basis for calculating the dilutions for preparation of the final bulk, for the quantity of virus used in the neurovirulence test and to establish and monitor production consistency.

Genetic markers. A ratio of the replication capacities of the virus in the monovalent pooled harvest is obtained over a temperature range between 36 °C and 40 °C in comparison with the seed lot or a reference preparation for the marker tests and with appropriate rct/40- and rct/40+ strains of poliovirus of the same type. The incubation temperatures used in this test are controlled to within ± 0.1 °C. The monovalent pooled harvest passes the test if, for both the virus in the harvest and the appropriate reference material, the titre determined at 36 °C is at least 5.0 log greater than

that determined at 40 °C. If growth at 40 °C is so low that a valid comparison cannot be established, a temperature in the region of 39.0 °C to 39.5 °C is used, at which temperature the reduction in titre of the reference material must be in the range 3.0 to 5.0 log of its value at 36 °C; the acceptable minimum reduction is determined for each virus strain at a given temperature. If the titres obtained for 1 or more of the reference viruses are not concordant with the expected values, the test must be repeated.

Neurovirulence (2.6.19). Each monovalent pooled harvest complies with the test for neurovirulence of poliomyelitis vaccine (oral). If the test is carried out only by the manufacturer, the test slides are provided to the competent authority for assessment.

Primary monkey kidney cell cultures. *The following special requirements apply to monovalent pooled harvests derived from primary monkey kidney cell cultures.*

Retroviruses. The monovalent pooled harvest is examined using a reverse transcriptase assay. No indication of the presence of retroviruses is found.

Test in rabbits. A sample of the monovalent pooled harvest is tested for cercopithecid herpesvirus 1 (B virus) and other viruses by injection of not less than 100 ml into not fewer than 10 healthy rabbits each weighing 1.5-2.5 kg. Each rabbit receives not less than 10 ml and not more than 20 ml, of which 1 ml is given intradermally at multiple sites, and the remainder subcutaneously. The rabbits are observed for at least 3 weeks for death or signs of illness.

All rabbits that die after the first 24 h of the test and those showing signs of illness are examined by autopsy, and the brain and organs removed for detailed examination to establish the cause of death.

The test is not valid if more than 20 per cent of the inoculated rabbits show signs of intercurrent infection during the observation period. The monovalent pooled harvest passes the test if none of the rabbits shows evidence of infection with B virus or with other extraneous agents or lesions of any kind attributable to the bulk suspension.

If the presence of B virus is demonstrated, the measures concerning vaccine production described above under Cell cultures are taken.

Test in guinea-pigs. *If the primary monkey kidney cell cultures are not derived from monkeys kept in a closed colony, the monovalent pooled harvest shall be shown to comply with the following test.* Administer to not fewer than 5 guinea-pigs, each weighing 350-450 g, 0.1 ml of the monovalent pooled harvest by intracerebral injection and 0.5 ml by intraperitoneal injection. Measure the rectal temperature of each animal on each working day for 6 weeks. At the end of the observation period carry out autopsy on each animal.

In addition, administer to not fewer than 5 guinea-pigs 0.5 ml by intraperitoneal injection and observe as described above for 2-3 weeks. At the end of the observation period, carry out a passage from these animals to not fewer than 5 guinea-pigs using blood and a suspension of liver or spleen tissue. Measure the rectal temperature of the latter guinea-pigs for 2-3 weeks. Examine by autopsy all animals that, after the first day of the test, die or are killed because they show disease or show on 3 consecutive days a body temperature higher than 40.1 °C; carry out histological examination to detect infection with filoviruses; in addition, inject a suspension of liver or spleen tissue or of blood intraperitoneally into not fewer than 3 guinea-pigs. If any signs of infection with filoviruses are noted, confirmatory serological tests are carried out on the blood of the affected animals. The monovalent pooled harvest complies with the

01/2005:0216

test if not fewer than 80 per cent of the guinea-pigs survive to the end of the observation period and remain in good health and no animal shows signs of infection with filoviruses.

FINAL BULK VACCINE

The final bulk vaccine is prepared from one or more satisfactory monovalent pooled harvests and may contain more than one virus type. Suitable flavouring substances and stabilisers may be added.

Only a final bulk vaccine that complies with the following requirement may be used in the preparation of the final lot.

Bacterial and fungal contamination. Carry out the test for sterility (2.6.1), using 10 ml for each medium.

FINAL LOT

Only a final lot that complies with the following requirement for thermal stability and is satisfactory with respect to each of the requirements given below under Identification, Tests and Assay may be released for use.

Thermal stability. Maintain samples of the final lot at 37 °C for 48 h. Determine the total virus concentration as described under Assay in parallel for the heated vaccine and for unheated vaccine. The estimated difference between the total virus concentration of the unheated and heated vaccines is not greater than 0.5 log₁₀ infectious virus units (CCID₅₀) per single human dose.

IDENTIFICATION

The vaccine is shown to contain poliovirus of each type stated on the label, using specific antibodies.

TESTS

Bacterial and fungal contamination. The vaccine complies with the test for sterility (2.6.1).

ASSAY

Titrate for infectious virus at least in triplicate using the method described below. Use an appropriate virus reference preparation to validate each assay. If the vaccine contains more than one poliovirus type, titrate each type separately, using appropriate type-specific antiserum (or preferably a monoclonal antibody) to neutralise each of the other types present.

For a trivalent vaccine, the estimated mean virus titres must be: not less than $1 \times 10^{6.0}$ infectious virus units (CCID₅₀) per single human dose for type 1; not less than $1 \times 10^{5.0}$ infectious virus units (CCID₅₀) for type 2; and not less than $1 \times 10^{5.5}$ infectious virus units (CCID₅₀) for type 3.

For monovalent or divalent vaccine, the minimum virus titres are decided by the competent authority.

Method. Inoculate groups of 8 to 12 flat-bottomed wells in a microtitre plate with 0.1 ml of each of the selected dilutions of virus followed by a suitable cell suspension of the Hep-2 (Cincinnati) line. Incubate the plates at a suitable temperature. Examine the cultures on days 7-9. The assay is not valid if the confidence interval ($P = 0.95$) of the logarithm of the virus concentration is greater than ± 0.3 .

LABELLING

The label states:

- the types of poliovirus contained in the vaccine,
- the minimum amount of virus of each type contained in 1 single human dose,
- the cell substrate used for the preparation of the vaccine,
- that the vaccine is not to be injected.

RABIES VACCINE FOR HUMAN USE PREPARED IN CELL CULTURES

Vaccinum rabiei ex cellulis ad usum humanum

DEFINITION

Rabies vaccine for human use prepared in cell cultures is a freeze-dried preparation of a suitable strain of fixed rabies virus grown in cell cultures and inactivated by a validated method.

The vaccine is reconstituted immediately before use as stated on the label to give a clear liquid that may be coloured owing to the presence of a pH indicator.

PRODUCTION

The production of the vaccine is based on a virus seed-lot system and, if a cell line is used for virus propagation, a cell-bank system. The production method shall have been shown to yield consistently vaccines that comply with the requirements for immunogenicity, safety and stability. Unless otherwise justified and authorised, the virus in the final vaccine shall not have undergone more passages from the master seed lot than was used to prepare the vaccine shown in clinical studies to be satisfactory with respect to safety and efficacy; even with authorised exceptions, the number of passages beyond the level used for clinical studies shall not exceed five.

The production method is validated to demonstrate that the product, if tested, would comply with the test for abnormal toxicity for immunosera and vaccines for human use (2.6.9).

SUBSTRATE FOR VIRUS PROPAGATION

The virus is propagated in a human diploid cell line (5.2.3), in a continuous cell line approved by the competent authority or in cultures of chick-embryo cells derived from a flock free from specified pathogens (5.2.2).

SEED LOTS

The strain of rabies virus used shall be identified by historical records that include information on the origin of the strain and its subsequent manipulation.

Working seed lots are prepared by not more than five passages from the master seed lot.

Only a working seed lot that complies with the following tests may be used for virus propagation.

Identification. Each working seed lot is identified as rabies virus using specific antibodies.

Virus concentration. The virus concentration of each working seed lot is determined by a cell culture method using immunofluorescence, to ensure consistency of production.

Extraneous agents (2.6.16). The working seed lot complies with the requirements for virus seed lots. If the virus has been passaged in mouse brain, specific tests for murine viruses are carried out.

VIRUS PROPAGATION AND HARVEST

All processing of the cell bank and subsequent cell cultures are done under aseptic conditions in an area where no other cells are handled. Approved animal (but not human) serum may be used in the media, but the final medium for maintaining cell growth during virus multiplication does not contain animal serum; the media may contain human albumin complying with the monograph on *Human albumin solution* (0255). Serum and trypsin used in the preparation of cell suspensions and media are shown to be free from

infectious extraneous agents; trypsin complies with the monograph on *Trypsin* (0694). The cell culture media may contain a pH indicator such as phenol red and approved antibiotics at the lowest effective concentration. Not less than 500 ml of the cell cultures employed for vaccine production are set aside as uninfected cell cultures (control cells). The virus suspension is harvested on one or more occasions during incubation. Multiple harvests from the same production cell culture may be pooled and considered as a single harvest.

Only a single harvest that complies with the following requirements may be used in the preparation of the inactivated viral harvest.

Identification. The single harvest contains virus that is identified as rabies virus using specific antibodies.

Virus concentration. Titrate for infective virus in cell cultures; the titre is used to monitor consistency of production.

Control cells. The control cells of the production cell culture from which the single harvest is derived comply with a test for identification and with the requirements for extraneous agents (2.6.16).

PURIFICATION AND INACTIVATION

The virus harvest may be concentrated and/or purified by suitable methods; the virus harvest is inactivated by a validated method at a fixed, well defined stage of the process which may be before, during or after any concentration or purification. The method shall have been shown to be capable of inactivating rabies virus without destruction of the immunogenic activity. If betapropiolactone is used, the concentration shall at no time exceed 1:3500.

Only an inactivated viral suspension that complies with the following requirements may be used in the preparation of the final bulk vaccine.

Inactivation. Carry out an amplification test for residual infectious rabies virus immediately after inactivation or using a sample frozen immediately after inactivation and stored at -70°C . Inoculate a quantity of inactivated viral suspension equivalent to not less than 25 doses of vaccine into cell cultures of the same type as those used for production of the vaccine. Make a passage after 7 days. Maintain the cultures for a further 14 days and then examine the cell cultures for rabies virus using an immunofluorescence test. No rabies virus is detected.

Residual host-cell DNA. If a continuous cell line is used for virus propagation, the content of residual host-cell DNA, determined using a suitable method as described in *Products of recombinant DNA technology* (0784), is not greater than 100 pg per single human dose.

FINAL BULK VACCINE

The final bulk vaccine is prepared from one or more inactivated viral suspensions. An approved stabiliser may be added to maintain the activity of the product during and after freeze-drying.

Only a final bulk vaccine that complies with the following requirements may be used in the preparation of the final lot.

Glycoprotein content. Determine the glycoprotein content by a suitable immunochemical method (2.7.1), for example, single-radial immunodiffusion, enzyme-linked immunosorbent assay or an antibody-binding test. The content is within the limits approved for the particular product.

Sterility (2.6.1). The final bulk vaccine complies with the test for sterility, carried out using 10 ml for each medium.

FINAL LOT

The final bulk vaccine is distributed aseptically into sterile containers and freeze-dried to a moisture content shown to be favourable to the stability of the vaccine. The containers are then closed so as to avoid contamination and the introduction of moisture.

Only a final lot that complies with each of the requirements given below under Identification, Tests and Assay may be released for use. Provided that the test for inactivation has been carried out with satisfactory results on the inactivated viral suspension and the test for bovine serum albumin has been carried out with satisfactory results on the final bulk vaccine, these tests may be omitted on the final lot.

IDENTIFICATION

The vaccine is shown to contain rabies virus antigen by a suitable immunochemical method (2.7.1) using specific antibodies, preferably monoclonal; alternatively, the assay serves also to identify the vaccine.

TESTS

Inactivation. Inoculate a quantity equivalent to not less than 25 human doses of vaccine into cell cultures of the same type as those used for production of the vaccine. Make a passage after 7 days. Maintain the cultures for a further 14 days and then examine the cell cultures for rabies virus using an immunofluorescence test. No rabies virus is detected.

Bovine serum albumin. Not more than 50 ng per single human dose, determined by a suitable immunochemical method (2.7.1).

Sterility (2.6.1). The vaccine complies with the test for sterility.

Bacterial endotoxins (2.6.14): less than 25 IU per single human dose.

Pyrogens (2.6.8). The vaccine complies with the test for pyrogens. Unless otherwise justified and authorised, inject into each rabbit a single human dose of the vaccine diluted to ten times its volume.

Water (2.5.12). Not more than 3.0 per cent, determined by the semi-micro determination of water.

ASSAY

The potency of rabies vaccine is determined by comparing the dose necessary to protect mice against the effects of a lethal dose of rabies virus, administered intracerebrally, with the quantity of a reference preparation of rabies vaccine necessary to provide the same protection. For this comparison a reference preparation of rabies vaccine, calibrated in International Units, and a suitable preparation of rabies virus for use as the challenge preparation are necessary.

The International Unit is the activity contained in a stated quantity of the International Standard. The equivalence in International Units of the International Standard is stated by the World Health Organisation.

The test described below uses a parallel-line model with at least three points for the vaccine to be examined and the reference preparation. Once the analyst has experience with the method for a given vaccine, it is possible to carry out a simplified test using a single dilution of the vaccine to be examined. Such a test enables the analyst to determine that the vaccine has a potency significantly higher than the required minimum but will not give full information on the validity of each individual potency determination. The use of a single dilution allows a considerable reduction in the number of animals required for the test and must be considered by each laboratory in accordance with the

provisions of the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes.

01/2005:0162
corrected

Selection and distribution of the test animals. Use in the test healthy female mice about 4 weeks old, each weighing 11 g to 15 g, and from the same stock. Distribute the mice into six groups of a size suitable to meet the requirements for validity of the test and, for titration of the challenge suspension, four groups of five.

Preparation of the challenge suspension. Inoculate mice intracerebrally with the CVS strain of rabies virus and when the mice show signs of rabies, but before they die, sacrifice them, remove the brains and prepare a homogenate of the brain tissue in a suitable diluent. Separate gross particulate matter by centrifugation and use the supernatant liquid as the challenge suspension. Distribute the suspension in small volumes in ampoules, seal and store at a temperature below -60°C . Thaw one ampoule of the suspension and make serial dilutions in a suitable diluent. Allocate each dilution to a group of five mice and inject intracerebrally into each mouse 0.03 ml of the dilution allocated to its group. Observe the mice for 14 days. Calculate the LD_{50} of the undiluted suspension using the number in each group that, between the fifth and fourteenth days, die or develop signs of rabies.

Determination of potency of the vaccine to be examined. Prepare three fivefold serial dilutions of the vaccine to be examined and three fivefold serial dilutions of the reference preparation. Prepare the dilutions such that the most concentrated suspensions may be expected to protect more than 50 per cent of the animals to which they are administered and the least concentrated suspensions may be expected to protect less than 50 per cent of the animals to which they are administered. Allocate the six dilutions one to each of the six groups of mice and inject intraperitoneally into each mouse 0.5 ml of the dilution allocated to its group. After 7 days, prepare three identical dilutions of the vaccine to be examined and of the reference preparation and repeat the injections. Seven days after the second injection, prepare a suspension of the challenge virus such that, on the basis of the preliminary titration, 0.03 ml contains about 50 LD_{50} . Inject intracerebrally into each vaccinated mouse 0.03 ml of this suspension. Prepare three suitable serial dilutions of the challenge suspension. Allocate the challenge suspension and the three dilutions one to each of the four groups of five control mice and inject intracerebrally into each mouse 0.03 ml of the suspension or one of the dilutions allocated to its group. Observe the animals in each group for 14 days and record the number in each group that die or show signs of rabies in the period 5 days to 14 days after challenge.

The test is not valid unless: for both the vaccine to be examined and the reference preparation the 50 per cent protective dose lies between the largest and smallest doses given to the mice; the titration of the challenge suspension shows that 0.03 ml of the suspension contained not less than 10 LD_{50} ; the statistical analysis shows a significant slope and no significant deviations from linearity or parallelism of the dose-response lines; the confidence limits ($P = 0.95$) are not less than 25 per cent and not more than 400 per cent of the estimated potency.

The vaccine complies with the test if the estimated potency is not less than 2.5 IU per human dose.

LABELLING

The label states the biological origin of the cells used for the preparation of the vaccine.

RUBELLA VACCINE (LIVE)

Vaccinum rubellae vivum

DEFINITION

Rubella vaccine (live) is a freeze-dried preparation of a suitable attenuated strain of rubella virus. The vaccine is reconstituted immediately before use, as stated on the label, to give a clear liquid that may be coloured owing to the presence of a pH indicator.

PRODUCTION

The production of vaccine is based on a virus seed-lot system and a cell-bank system. The production method shall have been shown to yield consistently live rubella vaccines of adequate immunogenicity and safety in man. Unless otherwise justified and authorised, the virus in the final vaccine shall have undergone no more passages from the master seed lot than were used to prepare the vaccine shown in clinical studies to be satisfactory with respect to safety and efficacy.

The production method is validated to demonstrate that the product, if tested, would comply with the test for abnormal toxicity for immunosera and vaccines for human use (2.6.9).

SUBSTRATE FOR VIRUS PROPAGATION

The virus is propagated in human diploid cells (5.2.3).

SEED LOT

The strain of rubella virus used shall be identified by historical records that include information on the origin of the strain and its subsequent manipulation. To avoid the unnecessary use of monkeys in the test for neurovirulence, virus seed lots are prepared in large quantities and stored at temperatures below -20°C if freeze-dried, or below -60°C if not freeze-dried.

Only a seed lot that complies with the following requirements may be used for virus propagation.

Identification. The master and working seed lots are identified as rubella virus by serum neutralisation in cell culture, using specific antibodies.

Virus concentration. The virus concentration of the master and working seed lots is determined to ensure consistency of production.

Extraneous agents (2.6.16). The working seed lot complies with the requirements for seed lots.

Neurovirulence (2.6.18). The working seed lot complies with the test for neurovirulence of live virus vaccines. *Macaca* and *Cercopithecus* monkeys are suitable for the test.

PROPAGATION AND HARVEST

All processing of the cell bank and subsequent cell cultures is done under aseptic conditions in an area where no other cells are handled. Suitable animal (but not human) serum may be used in the growth medium, but the final medium for maintaining cell growth during virus multiplication does not contain animal serum. Serum and trypsin used in the preparation of cell suspensions and culture media are shown to be free from extraneous agents. The cell culture medium may contain a pH indicator such as phenol red and suitable antibiotics at the lowest effective concentration. It is preferable to have a substrate free from antibiotics during production. Not less than 500 ml of the production cell cultures is set aside as uninfected cell cultures (control cells). The temperature of incubation is controlled during the growth of the virus. The virus suspension is harvested,

on one or more occasions, within 28 days of inoculation. Multiple harvests from the same production cell culture may be pooled and considered as a single harvest.

Only a single harvest that complies with the following requirements may be used in the preparation of the final bulk vaccine.

Identification. The single harvest contains virus that is identified as rubella virus by serum neutralisation in cell culture, using specific antibodies.

Virus concentration. The virus concentration in the single harvest is determined as prescribed under Assay to monitor consistency of production and to determine the dilution to be used for the final bulk vaccine.

Extraneous agents (2.6.16). The single harvest complies with the tests for extraneous agents.

Control cells. The control cells comply with a test for identification and with the tests for extraneous agents (2.6.16).

FINAL BULK VACCINE

Single harvests that comply with the above tests are pooled and clarified to remove cells. A suitable stabiliser may be added and the pooled harvests diluted as appropriate.

Only a final bulk vaccine that complies with the following requirement may be used in the preparation of the final lot.

Bacterial and fungal contamination. The final bulk vaccine complies with the test for sterility (2.6.1), carried out using 10 ml for each medium.

FINAL LOT

A minimum virus concentration for release of the product is established such as to ensure, in the light of stability data, that the minimum concentration stated on the label will be present at the end of the period of validity.

Only a final lot that complies with the requirements for minimum virus concentration for release, with the following requirement for thermal stability and with each of the requirements given below under Identification and Tests may be released for use. Provided that the test for bovine serum albumin has been carried out with satisfactory results on the final bulk vaccine, it may be omitted on the final lot.

Thermal stability. Maintain samples of the final lot of freeze-dried vaccine in the dry state at 37 °C for 7 days. Determine the virus concentration as described under Assay in parallel for the heated vaccine and for unheated vaccine stored at 5 ± 3 °C. The virus concentration of the heated vaccine is not more than 1.0 log₁₀ lower than that of the unheated vaccine.

IDENTIFICATION

When the vaccine reconstituted as stated on the label is mixed with specific rubella antibodies, it is no longer able to infect susceptible cell cultures.

TESTS

Bacterial and fungal contamination. The reconstituted vaccine complies with the test for sterility (2.6.1).

Bovine serum albumin. Not more than 50 ng per single human dose, determined by a suitable immunochemical method (2.7.1).

Water (2.5.12). Not more than 3.0 per cent, determined by the semi-micro determination of water.

ASSAY

Titrate the vaccine for infective virus at least in triplicate, using at least 5 cell cultures for each 0.5 log₁₀ dilution step or by a method of equal precision. Use an appropriate virus

reference preparation to validate each assay. The estimated virus concentration is not less than that stated on the label; the minimum virus concentration stated on the label is not less than 1 × 10³ CCID₅₀ per human dose. The assay is not valid if the confidence interval ($P = 0.95$) of the logarithm of the virus concentration is greater than ± 0.3.

Rubella vaccine (live) BRP is suitable for use as a reference preparation.

LABELLING

The label states:

- the strain of virus used for the preparation of the vaccine,
- the type and origin of the cells used for the preparation of the vaccine,
- the minimum virus concentration,
- that contact with disinfectants is to be avoided,
- the time within which the vaccine must be used after reconstitution,
- that the vaccine must not be given to a pregnant woman and that a woman must not become pregnant within 2 months after having the vaccine.

01/2005:0452

TETANUS VACCINE (ADSORBED)

Vaccinum tetani adsorbatum

DEFINITION

Tetanus vaccine (adsorbed) is a preparation of tetanus formol toxoid with a mineral adsorbent. The formol toxoid is prepared from the toxin produced by the growth of *Clostridium tetani*.

PRODUCTION

GENERAL PROVISIONS

Specific toxicity. The production method is validated to demonstrate that the product, if tested, would comply with the following test: inject subcutaneously 5 times the single human dose stated on the label into each of 5 healthy guinea-pigs, each weighing 250-350 g, that have not previously been treated with any material that will interfere with the test. If within 21 days of the injection any of the animals shows signs of or dies from tetanus, the vaccine does not comply with the test. If more than 1 animal dies from non-specific causes, repeat the test once; if more than 1 animal dies in the second test, the vaccine does not comply with the test.

BULK PURIFIED TOXOID

For the production of tetanus toxin, from which toxoid is prepared, seed cultures are managed in a defined seed-lot system in which toxinogenicity is conserved and, where necessary, restored by deliberate reselection. A highly toxinogenic strain of *Clostridium tetani* with known origin and history is grown in a suitable liquid medium. At the end of cultivation, the purity of each culture is tested and contaminated cultures are discarded. Toxin-containing culture medium is collected aseptically. The toxin content (Lf per millilitre) is checked to monitor consistency of production. Single harvests may be pooled to prepare the bulk purified toxoid. The toxin is purified to remove components likely to cause adverse reactions in humans. The purified toxin is detoxified with formaldehyde by a method that avoids destruction of the immunogenic potency of the toxoid and reversion of toxoid to toxin, particularly on exposure to heat. Alternatively, purification may be carried out after detoxification.

Only bulk purified toxoid that complies with the following requirements may be used in the preparation of the final bulk vaccine.

Sterility (2.6.1). Carry out the test for sterility using 10 ml for each medium.

Absence of toxin and irreversibility of toxoid. Using the same buffer solution as for the final vaccine, without adsorbent, prepare a solution of bulk purified toxoid at the same concentration as in the final vaccine. Divide the dilution into 2 equal parts. Keep one of them at 5 ± 3 °C and the other at 37 °C for 6 weeks. Test both dilutions as described below. Use 15 guinea-pigs, each weighing 250-350 g and that have not previously been treated with any material that will interfere with the test. Inject subcutaneously into each of 5 guinea-pigs 5 ml of the dilution incubated at 5 ± 3 °C. Inject subcutaneously into each of 5 other guinea-pigs 5 ml of the dilution incubated at 37 °C. Inject subcutaneously into each of 5 guinea-pigs at least 500 Lf of the non-incubated bulk purified toxoid in a volume of 1 ml. The bulk purified toxoid complies with the test if during the 21 days following the injection no animal shows signs of or dies from tetanus. If more than 1 animal dies from non-specific causes, repeat the test; if more than 1 animal dies in the second test, the toxoid does not comply with the test.

Antigenic purity. Not less than 1000 Lf per milligram of protein nitrogen.

FINAL BULK VACCINE

The final bulk vaccine is prepared by adsorption of a suitable quantity of bulk purified toxoid onto a mineral carrier such as hydrated aluminium phosphate or aluminium hydroxide; the resulting mixture is approximately isotonic with blood. Suitable antimicrobial preservatives may be added. Certain antimicrobial preservatives, particularly those of the phenolic type, adversely affect the antigenic activity and must not be used.

Only final bulk vaccine that complies with the following requirements may be used in the preparation of the final lot.

Antimicrobial preservative. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The amount is not less than 85 per cent and not greater than 115 per cent of the intended amount.

Sterility (2.6.1). Carry out the test for sterility using 10 ml for each medium.

FINAL LOT

The final bulk vaccine is distributed aseptically into sterile, tamper-proof containers. The containers are closed so as to prevent contamination.

Only a final lot that is satisfactory with respect to each of the requirements given below under Identification, Tests and Assay may be released for use. Provided the test for antimicrobial preservative and the assay have been carried out with satisfactory results on the final bulk vaccine, they may be omitted on the final lot.

Provided the free formaldehyde content has been determined on the bulk purified toxoid or on the final bulk and it has been shown that the content in the final lot will not exceed 0.2 g/l, the test for free formaldehyde may be omitted on the final lot.

IDENTIFICATION

Tetanus toxoid is identified by a suitable immunochemical method (2.7.1). The following method, applicable to certain vaccines, is given as an example. Dissolve in the vaccine to be examined sufficient *sodium citrate R* to give a 100 g/l

solution. Maintain at 37 °C for about 16 h and centrifuge until a clear supernatant liquid is obtained. The clear supernatant liquid reacts with a suitable tetanus antitoxin, giving a precipitate.

TESTS

Aluminium (2.5.13): maximum 1.25 mg per single human dose, if aluminium hydroxide or hydrated aluminium phosphate is used as the adsorbent.

Free formaldehyde (2.4.18): maximum 0.2 g/l.

Antimicrobial preservative. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The content is not less than the minimum amount shown to be effective and is not greater than 115 per cent of the quantity stated on the label.

Sterility (2.6.1). The vaccine complies with the test for sterility.

ASSAY

Carry out one of the prescribed methods for the assay of tetanus vaccine (adsorbed) (2.7.8).

The lower confidence limit ($P = 0.95$) of the estimated potency is not less than 40 IU per single human dose.

LABELLING

The label states:

- the minimum number of International Units per single human dose,
- the name and the amount of the adsorbent,
- that the vaccine must be shaken before use,
- that the vaccine is not to be frozen.

01/2005:1375

TICK-BORNE ENCEPHALITIS VACCINE (INACTIVATED)

Vaccinum encephalitis ixodibus advectae inactivatum

DEFINITION

Tick-borne encephalitis vaccine (inactivated) is a liquid preparation of a suitable strain of tick-borne encephalitis virus grown in cultures of chick-embryo cells or other suitable cell cultures and inactivated by a suitable, validated method.

PRODUCTION

Production of the vaccine is based on a virus seed-lot system. The production method shall have been shown to yield consistently vaccines comparable with the vaccine of proven clinical efficacy and safety in man. Unless otherwise justified and authorised, the virus in the final vaccine shall not have undergone more passages from the master seed lot than the virus in the vaccine used in clinical trials.

The production method is validated to demonstrate that the product, if tested, would comply with the test for abnormal toxicity for immunosera and vaccines for human use (2.6.9).

SUBSTRATE FOR VIRUS PROPAGATION

The virus is propagated in chick embryo cells prepared from eggs derived from a chicken flock free from specified pathogens (5.2.2) or in other suitable cell cultures (5.2.3).

SEED LOTS

The strain of virus used is identified by historical records that include information on the origin of the strain and its subsequent manipulation. Virus seed lots are stored at or below $-60\text{ }^{\circ}\text{C}$.

Only a seed lot that complies with the following requirements may be used for virus propagation.

Identification. Each seed lot is identified as containing the vaccine strain of tick-borne encephalitis virus by a suitable immunochemical method (2.7.1), preferably using monoclonal antibodies.

Virus concentration. The virus concentration of each seed lot is determined by titration in suitable cell cultures to monitor consistency of production.

Extraneous agents (2.6.16). Each seed lot complies with the requirements for extraneous agents in viral vaccines for human use; the tests in cell cultures are carried out in human and simian cells only. For neutralisation of the vaccine virus, the use of monoclonal antibodies is preferable.

VIRUS PROPAGATION AND HARVEST

All processing of the cell cultures is performed under aseptic conditions in an area where no other cells are being handled. Serum and trypsin used in the preparation of cell suspensions and media used must be shown to be free from extraneous agents. The cell culture media may contain a pH indicator such as phenol red and approved antibiotics at the lowest effective concentration. At least 500 ml of the cell cultures employed for vaccine production is set aside as uninfected cell cultures (control cells).

Only a single harvest that complies with the following requirements may be used in the preparation of the inactivated harvest.

Identification. The single harvest is shown to contain tick-borne encephalitis virus by a suitable immunochemical method (2.7.1), preferably using monoclonal antibodies, or by virus neutralisation in cell cultures.

Bacterial and fungal contamination (2.6.1). The single harvest complies with the test for sterility, carried out using 10 ml for each medium.

Mycoplasmas (2.6.7). The single harvest complies with the test for mycoplasmas carried out using 1 ml for each medium.

Control cells. The control cells comply with the tests for extraneous agents (2.6.16). If the vaccine is produced using a cell-bank system, the control cells comply with a test for identification.

Virus concentration. Determine the virus concentration by titration in suitable cell cultures to monitor consistency of production.

INACTIVATION

To avoid interference, viral aggregates are removed by filtration immediately before the inactivation process. The virus suspension is inactivated by a validated method; the method shall have been shown to be consistently capable of inactivating tick-borne encephalitis virus without destroying the antigenic and immunogenic activity; as part of the validation studies, an inactivation curve is plotted representing residual live virus concentration measured on not fewer than three occasions. If formaldehyde is used for inactivation, the presence of an excess of free formaldehyde is verified at the end of the inactivation process.

Only an inactivated harvest that complies with the following requirements may be used in the preparation of the final bulk vaccine.

Residual infective virus. Inoculate a quantity of the inactivated harvest equivalent to not less than ten human doses of vaccine in the final lot into primary chicken fibroblast cell cultures, or other cells shown to be at least as sensitive to tick-borne encephalitis virus, with not less than 3 cm^2 of cell sheet per millilitre of inoculum. Incubate at $37 \pm 1\text{ }^{\circ}\text{C}$ for 14 days. No cytopathic effect is detected at the end of the incubation period. Collect the culture fluid and inoculate 0.03 ml intracerebrally into each of not fewer than ten mice about 4 weeks old. Observe the mice for 14 days. They show no evidence of tick-borne encephalitis virus infection.

PURIFICATION

Several inactivated single harvests may be pooled before concentration and purification by suitable methods, preferably by continuous-flow, sucrose density-gradient centrifugation.

Only a purified, inactivated harvest that complies with the following requirements may be used in the preparation of the final bulk vaccine.

Sterility (2.6.1). The purified, inactivated harvest complies with the test for sterility carried out using 10 ml for each medium.

Specific activity. Determine the antigen content of the purified, inactivated harvest by a suitable immunochemical method (2.7.1). Determine the total protein content by a suitable method. The specific activity, calculated as the antigen content per unit mass of protein, is within the limits approved for the specific product.

FINAL BULK VACCINE

The final bulk vaccine is prepared from one or more purified, inactivated harvests.

Only a final bulk vaccine that complies with the following requirement may be used in the preparation of the final lot.

Sterility (2.6.1). The final bulk vaccine complies with the test for sterility, carried out using 10 ml for each medium.

FINAL LOT

Only a final lot that is satisfactory with respect to each of the requirements given below under Identification, Tests and Assay may be released for use. Provided that the tests for free formaldehyde, bovine serum albumin (where applicable) and pyrogens and the assay have been carried out with satisfactory results on the final bulk vaccine, they may be omitted on the final lot.

IDENTIFICATION

The vaccine is shown to contain tick-borne encephalitis virus antigen by a suitable immunochemical method (2.7.1) using specific antibodies or by the mouse immunogenicity test described under Assay.

TESTS

Aluminium (2.5.13): maximum 1.25 mg per single human dose, if aluminium hydroxide or hydrated aluminium phosphate is used as the adsorbent.

Free formaldehyde (2.4.18): maximum of 0.1 g/l.

Bovine serum albumin. If bovine serum albumin has been used during production, the vaccine contains not more than 50 ng per single human dose, determined by a suitable immunochemical method (2.7.1).

Sterility (2.6.1). The vaccine complies with the test for sterility.

Pyrogens (2.6.8). The vaccine complies with the test for pyrogens. Inject into each rabbit, per kilogram of body mass, one dose of vaccine.

ASSAY

The potency is determined by comparing the dose necessary to protect a given proportion of mice against the effects of a lethal dose of tick-borne encephalitis virus, administered intraperitoneally, with the quantity of a reference preparation of tick-borne encephalitis vaccine necessary to provide the same protection. For this comparison an approved reference preparation and a suitable preparation of tick-borne encephalitis virus from an approved strain for use as the challenge preparation are necessary.

The following is cited as an example of a method that has been found suitable for a given vaccine.

Selection and distribution of test animals. Use healthy mice weighing 11 g to 17 g and derived from the same stock. Distribute the mice into not fewer than six groups of a suitable size to meet the requirements for validity of the test; for titration of the challenge suspension, use not fewer than four groups of ten mice. Use mice of the same sex or distribute males and females equally between groups.

Determination of potency of the vaccine. Prepare not fewer than three suitable dilutions of the vaccine to be examined and of the reference preparation; in order to comply with validity criteria four to five dilutions will usually be necessary. Prepare dilutions such that the most concentrated suspension is expected to protect more than 50 per cent of the animals and the least concentrated suspension less than 50 per cent. Allocate each dilution to a different group of mice and inject subcutaneously into each mouse 0.2 ml of the dilution allocated to its group. 7 days later make a second injection using the same dilution scale. 14 days after the second injection prepare a suspension of the challenge virus containing not less than 100 LD₅₀ in 0.2 ml. Inject 0.2 ml of this virus suspension intraperitoneally into each vaccinated mouse. To verify the challenge dose, prepare a series of not fewer than three dilutions of the challenge virus suspension at not greater than one-hundredfold intervals. Allocate the challenge suspension and the four dilutions, one to each of the five groups of ten mice, and inject intraperitoneally into each mouse 0.2 ml of the challenge suspension or the dilution allocated to its group. Observe the animals for 21 days after the challenge and record the number of mice that die in the period between 7 days and 21 days after the challenge.

Calculations. Calculate the results by the usual statistical methods for an assay with quantal responses (for example, 5.3.).

Validity criteria. The test is not valid unless:

- the concentration of the challenge virus is not less than 100 LD₅₀,
- for both the vaccine to be examined and the reference preparation the 50 per cent protective dose (PD₅₀) lies between the largest and smallest doses given to the mice,
- the statistical analysis shows a significant slope and no significant deviation from linearity and parallelism of the dose-response lines,
- the confidence limits ($P = 0.95$) are not less than 33 per cent and not more than 300 per cent of the estimated potency.

Potency requirement. Include all valid tests to estimate the mean potency and the confidence limits ($P = 0.95$) for the mean potency; compute weighted means with the inverse of the squared standard error as weights. The vaccine complies with the test if the estimated potency is not less than that approved by the competent authority, based on data from clinical efficacy trials.

LABELLING

The label states:

- the strain of virus used in preparation,
- the type of cells used for production of the vaccine.

01/2005:1160

TYPHOID POLYSACCHARIDE VACCINE

Vaccinum febris typhoidis polysaccharidicum

DEFINITION

Typhoid polysaccharide vaccine is a preparation of purified Vi capsular polysaccharide obtained from *Salmonella typhi* Ty 2 strain or some other suitable strain that has the capacity to produce Vi polysaccharide.

Capsular Vi polysaccharide consists of partly 3-*O*-acetylated repeated units of 2-acetyl-amino-2-deoxy-D-galactopyranuronic acid with α -(1→4) linkages.

PRODUCTION

The production of Vi polysaccharide is based on a seed-lot system. The method of production shall have been shown to yield consistently typhoid polysaccharide vaccines of adequate immunogenicity and safety in man.

The production method is validated to demonstrate that the product, if tested, would comply with the test for abnormal toxicity for immunosera and vaccines for human use (2.6.9).

BACTERIAL SEED LOTS

The strain of *S. typhi* used for the master seed lot shall be identified by historical records that include information on its origin and by its biochemical and serological characteristics. Cultures from the working seed lot shall have the same characteristics as the strain that was used to prepare the master seed lot.

Only a strain that has the following characteristics may be used in the preparation of the vaccine: (a) stained smears from a culture are typical of enterobacteria; (b) the culture utilises glucose without production of gas; (c) colonies on agar are oxidase-negative; (d) a suspension of the culture agglutinates specifically with a suitable Vi antiserum or colonies form haloes on an agar plate containing a suitable Vi antiserum.

Purity of bacterial strain used for the seed lot is verified by methods of suitable sensitivity. These may include inoculation into suitable media, examination of colony morphology, microscopic examination of Gram-stained smears and culture agglutination with suitable specific antisera.

CULTURE AND HARVEST

The working seed lot is cultured on a solid medium, which may contain blood-group substances, or a liquid medium; the inoculum obtained is transferred to a liquid medium which is used to inoculate the final medium. The liquid medium used and the final medium are semi-synthetic, free from substances that are precipitated by cetrimonium bromide and do not contain blood-group substances or high-molecular-mass polysaccharides, unless it has been demonstrated that they are removed by the purification process.

The bacterial purity of the culture is verified by methods of suitable sensitivity. These may include inoculation into suitable media, examination of colony morphology, microscopic examination of Gram-stained smears and culture agglutination with suitable specific antisera.

The culture is then inactivated at the beginning of the stationary phase by the addition of formaldehyde. Bacterial cells are eliminated by centrifugation; the polysaccharide is precipitated from the culture medium by addition of hexadecyltrimethylammonium bromide (cetrimonium bromide). The precipitate is harvested and may be stored at -20°C before purification.

PURIFIED VI POLYSACCHARIDE

The polysaccharide is purified, after dissociation of the polysaccharide/cetrimonium bromide complex, using suitable procedures to eliminate successively nucleic acids, proteins and lipopolysaccharides. The polysaccharide is precipitated as the calcium salt in the presence of ethanol and dried at $2-8^{\circ}\text{C}$; the powder obtained constitutes the purified Vi polysaccharide. The loss on drying is determined by thermogravimetry (2.2.34) and is used to calculate the results of the chemical tests shown below with reference to the dried substance.

Only a purified Vi polysaccharide that complies with the following requirements may be used in the preparation of the final bulk.

Protein (2.5.16): maximum 10 mg per gram of polysaccharide, calculated with reference to the dried substance.

Nucleic acids (2.5.17): maximum 20 mg per gram of polysaccharide, calculated with reference to the dried substance.

O-Acetyl groups (2.5.19): minimum 2 mmol per gram of polysaccharide, calculated with reference to the dried substance.

Molecular size. Examine by size-exclusion chromatography (2.2.30) using *cross-linked agarose for chromatography R*. Use a column 0.9 m long and 16 mm in internal diameter equilibrated with a solvent having an ionic strength of 0.2 mol/kg and a pH of 7.0-7.5. Apply about 5 mg of polysaccharide in a volume of 1 ml to the column and elute at about 20 ml/h. Collect fractions of about 2.5 ml. Determine the point corresponding to $K_0 = 0.25$ and make 2 pools consisting of fractions eluted before and after this point. Determine O-acetyl groups on the 2 pools (2.5.19). Not less than 50 per cent of the polysaccharide is found in the pool containing fractions eluted before $K_0 = 0.25$.

Identification. Carry out an identification test using a suitable immunochemical method (2.7.1).

Bacterial endotoxins. The content of bacterial endotoxins determined by a suitable method (2.6.14) is within the limits approved for the specific product.

FINAL BULK VACCINE

One or more batches of purified Vi polysaccharide are dissolved in a suitable solvent, which may contain an antimicrobial preservative, so that the volume corresponding to 1 dose contains 25 µg of polysaccharide and the solution is isotonic with blood (250 mosmol/kg to 350 mosmol/kg).

Only a final bulk vaccine that complies with the following tests may be used in the preparation of the final lot.

Sterility (2.6.1). The final bulk vaccine complies with the test for sterility, carried out using 10 ml for each medium.

Antimicrobial preservative. Where applicable, determine the amount of antimicrobial preservative by a suitable physicochemical method. The amount is not less than 85 per cent and not greater than 115 per cent of the intended amount.

FINAL LOT

The final bulk vaccine is distributed aseptically into sterile tamper-proof containers that are then closed so as to prevent contamination.

Only a final lot that is satisfactory with respect to each of the requirements prescribed below under Identification, Tests and Assay and with the requirement for bacterial endotoxins may be released for use. Provided the tests for free formaldehyde and antimicrobial preservative have been carried out on the final bulk vaccine, they may be omitted on the final lot.

Bacterial endotoxins. The content of bacterial endotoxins determined by a suitable method (2.6.14) is within the limit approved for the specific product.

CHARACTERS

Clear colourless liquid, free from visible particles.

IDENTIFICATION

Carry out an identification test using a suitable immunochemical method (2.7.1).

TESTS

pH (2.2.3): 6.5 to 7.5.

O-Acetyl groups: 0.085 (± 25 per cent) µmol per dose (25 µg of polysaccharide).

Test solution. Place 3 ml of the vaccine in each of 3 tubes (2 reaction solutions and 1 correction solution).

Reference solutions. Dissolve 0.150 g of *acetylcholine chloride R* in 10 ml of *water R* (stock solution containing 15 g/l of acetylcholine chloride). Immediately before use, dilute 0.5 ml of the stock solution to 50 ml with *water R* (working dilution containing 150 µg/ml of acetylcholine chloride). In 10 tubes, place in duplicate (reaction and correction solutions) 0.1 ml, 0.2 ml, 0.5 ml, 1.0 ml and 1.5 ml of the working dilution.

Prepare a blank using 3 ml of *water R*.

Make up the volume in each tube to 3 ml with *water R*. Add 0.5 ml of a mixture of 1 volume of *water R* and 2 volumes of *dilute hydrochloric acid R* to each of the correction tubes and to the blank. Add 1.0 ml of *alkaline hydroxylamine solution R* to each tube. Allow the reaction to proceed for exactly 2 min and add 0.5 ml of a mixture of 1 volume of *water R* and 2 volumes of *dilute hydrochloric acid R* to each of the reaction tubes. Add 0.5 ml of a 200 g/l solution of *ferric chloride R* in 0.2 M *hydrochloric acid* to each tube, stopper the tubes and shake vigorously to remove bubbles.

Measure the absorbance (2.2.25) of each solution at 540 nm using the blank as the compensation liquid. For each reaction solution, subtract the absorbance of the corresponding correction solution. Draw a calibration curve from the corrected absorbances for the 5 reference solutions and the corresponding content of acetylcholine chloride and read from the curve the content of acetylcholine chloride in the test solution for each volume tested. Calculate the mean of the 2 values.

1 mole of acetylcholine chloride (181.7 g) is equivalent to 1 mole of O-acetyl (43.05 g).

Free formaldehyde (2.4.18): maximum 0.2 g/l.

Antimicrobial preservative. Where applicable, determine the amount of antimicrobial preservative by a suitable physicochemical method. The content is not less than the minimum amount shown to be effective and not more than 115 per cent of the content stated on the label. If phenol has been used in the preparation, the content is not more than 2.5 g/l (2.5.15).

Sterility (2.6.1). The vaccine complies with the test for sterility.

ASSAY

Determine Vi polysaccharide by a suitable immunochemical method (2.7.1), using a reference purified polysaccharide. The estimated amount of polysaccharide per dose is 80 per cent to 120 per cent of the content stated on the label. The confidence limits ($P = 0.95$) of the estimated amount of polysaccharide are not less than 80 per cent and not more than 120 per cent.

LABELLING

The label states:

- the number of micrograms of polysaccharide per human dose (25 µg),
- the total quantity of polysaccharide in the container.

01/2005:0156

TYPHOID VACCINE

Vaccinum febris typhoidi

DEFINITION

Typhoid vaccine is a sterile suspension of inactivated *Salmonella typhi* containing not less than 5×10^8 and not more than 1×10^9 bacteria (*S. typhi*) per human dose. The human dose does not exceed 1.0 ml.

PRODUCTION

The vaccine is prepared using a seed-lot system from a suitable strain, such as Ty 2⁽⁵⁾, of *S. typhi*. The final vaccine represents not more than 3 subcultures from the strain on which were made the laboratory and clinical tests that showed it to be suitable. The bacteria are inactivated by acetone, by formaldehyde, by phenol or by heating or by a combination of the last 2 methods.

The production method is validated to demonstrate that the product, if tested, would comply with the test for abnormal toxicity for immunosera and vaccines for human use (2.6.9) modified as follows: inject 0.5 ml of the vaccine into each mouse and 1.0 ml into each guinea pig.

IDENTIFICATION

It is identified by specific agglutination.

TESTS

Phenol (2.5.15). If phenol has been used in the preparation, the concentration is not more than 5 g/l.

Antigenic power. When injected into susceptible laboratory animals, it elicits anti-O, anti-H and, to a lesser extent, anti-Vi agglutinins.

Sterility (2.6.1). It complies with the test for sterility.

LABELLING

The label states:

- the method used to inactivate the bacteria,
- the number of bacteria per human dose.

01/2005:0157

TYPHOID VACCINE, FREEZE-DRIED

Vaccinum febris typhoidi cryodesiccatum

DEFINITION

Freeze-dried typhoid vaccine is a freeze-dried preparation of inactivated *Salmonella typhi*. The vaccine is reconstituted as stated on the label to give a uniform suspension containing not less than 5×10^8 and not more than 1×10^9 bacteria (*S. typhi*) per human dose. The human dose does not exceed 1.0 ml of the reconstituted vaccine.

PRODUCTION

The vaccine is prepared using a seed-lot system from a suitable strain, such as Ty 2⁽⁶⁾, of *S. typhi*. The final vaccine represents not more than 3 subcultures from the strain on which were made the laboratory and clinical tests that showed it to be suitable. The bacteria are inactivated either by acetone or by formaldehyde or by heat. Phenol is not used in the preparation. The vaccine is distributed into sterile containers and freeze-dried to a moisture content favourable to the stability of the vaccine. The containers are then closed so as to exclude contamination.

The production method is validated to demonstrate that the product, if tested, would comply with the test for abnormal toxicity for immunosera and vaccines for human use (2.6.9) modified as follows: inject 0.5 ml of the vaccine into each mouse and 1.0 ml into each guinea pig.

IDENTIFICATION

The vaccine reconstituted as stated on the label is identified by specific agglutination.

TESTS

Phenol (2.5.15). If phenol has been used in the preparation, the concentration is not more than 5 g/l.

Antigenic power. When injected into susceptible laboratory animals, the reconstituted vaccine elicits anti-O, anti-H and, to a lesser extent, anti-Vi agglutinins.

Sterility (2.6.1). The reconstituted vaccine complies with the test for sterility.

LABELLING

The label states:

- the method used to inactivate the bacteria,
- the number of bacteria per human dose,
- that the vaccine should be used within 8 h of reconstitution.

(5) This strain is issued by the World Health Organisation Collaborating Centre for Reference and Research on Bacterial Vaccines, Human Serum and Vaccine Institute, Szallas Utea 5, H-1107, Budapest, Hungary.

(6) This strain is issued by the World Health Organisation Collaborating Centre for Reference and Research on Bacterial Vaccines, Human Serum and Vaccine Institute, Szallas Utea 5, H-1107, Budapest, Hungary.

01/2005:1055

TYPHOID VACCINE (LIVE, ORAL, STRAIN Ty 21a)

Vaccinum febris typhoidis vivum perorale (stirpe Ty 21a)

DEFINITION

Typhoid vaccine (live, oral, strain Ty 21a) is a freeze-dried preparation of live *Salmonella typhi* strain Ty 21a grown in a suitable medium. When presented in capsules, the vaccine complies with the monograph on *Capsules* (0016).

PRODUCTION

CHOICE OF VACCINE STRAIN

The main characteristic of the strain is the defect of the enzyme uridine diphosphate-galactose-4-epimerase. The activities of galactopermease, galactokinase and galactose-1-phosphate uridyl-transferase are reduced by 50 per cent to 90 per cent. Whatever the growth conditions, the strain does not contain Vi antigen. The strain agglutinates to anti-O:9 antiserum only if grown in medium containing galactose. It contains the flagellar H:d antigen and does not produce hydrogen sulphide on Kligler iron agar. The strain is nonvirulent for mice. Cells of strain Ty 21a lyse if grown in the presence of 1 per cent of galactose.

BACTERIAL SEED LOTS

The vaccine is prepared using a seed-lot system. The working seed lots represent not more than one subculture from the master seed lot. The final vaccine represents not more than four subcultures from the original vaccine on which were made the laboratory and clinical tests showing the strain to be suitable.

Only a master seed lot that complies with the following requirements may be used in the preparation of working seed lots.

Galactose metabolism. In a spectrophotometric assay, no activity of the enzyme uridine diphosphate-galactose-4-epimerase is found in the cytoplasm of strain Ty 21a compared to strain Ty 2.

Biosynthesis of lipopolysaccharide. Lipopolysaccharides are extracted by the hot-phenol method and examined by size-exclusion chromatography. Strain Ty 21a grown in medium free of galactose shows only the rough (R) type of lipopolysaccharide.

Serological characteristics. Strain Ty 21a grown in a synthetic medium without galactose does not agglutinate to specific anti-O:9 antiserum. Whatever the growth conditions, strain Ty 21a does not agglutinate to Vi antiserum. Strain Ty 21a agglutinates to H:d flagellar antiserum.

Biochemical markers. Strain Ty 21a does not produce hydrogen sulphide on Kligler iron agar. This property serves to distinguish Ty 21a from other galactose-epimerase-negative *S. typhi* strains.

Cell growth. Strain Ty 21a cells lyse when grown in the presence of 1 per cent of galactose.

BACTERIAL PROPAGATION AND HARVEST

The bacteria from the working seed lot are multiplied in a preculture, subcultured once and are then grown in a suitable medium containing 0.001 per cent of galactose at 30 °C for 13 h to 15 h. The bacteria are harvested. The harvest must be free from contaminating micro-organisms.

Only a single harvest that complies with the following requirements may be used for the preparation of the freeze-dried harvest.

pH. The pH of the culture is 6.8 to 7.5.

Optical density. The optical density of the culture, measured at 546 nm, is 6.5 to 11.0. Before carrying out the measurement, dilute the culture so that a reading in the range 0.1 to 0.5 is obtained and correct the reading to take account of the dilution.

Identification. Culture bacteria on an agar medium containing 1 per cent of galactose and bromothymol blue. Light blue, concave colonies, transparent due to lysis of cells, are formed. No yellow colonies (galactose-fermenting) are found.

FREEZE-DRIED HARVEST

The harvest is mixed with a suitable stabiliser and freeze-dried by a process that ensures the survival of at least 10 per cent of the bacteria and to a water content shown to be favourable to the stability of the vaccine. No antimicrobial preservative is added to the vaccine.

Only a freeze-dried harvest that complies with the following tests may be used for the preparation of the final bulk.

Identification. Culture bacteria are examined on an agar medium containing 1 per cent of galactose and bromothymol blue. Light blue, concave colonies, transparent due to lysis of cells, are formed. No yellow colonies (galactose-fermenting) are found.

Number of live bacteria. Not fewer than 1×10^{11} live *S. typhi* strain Ty 21a per gram.

Water (2.5.12): 1.5 per cent to 4.0 per cent, determined by the semi-micro determination of water.

FINAL BULK VACCINE

The final bulk vaccine is prepared by aseptically mixing one or more freeze-dried harvests with a suitable sterile excipient.

Only a final bulk that complies with the following requirement may be used in the preparation of the final lot.

Number of live bacteria. Not fewer than 40×10^9 live *S. typhi* strain Ty 21a per gram.

FINAL LOT

The final bulk vaccine is distributed under aseptic conditions into capsules with a gastro-resistant shell or into suitable containers.

Only a final lot that is satisfactory with respect to each of the requirements given below under Identification, Tests and Number of live bacteria may be released for use, except that in the determination of the number of live bacteria each dosage unit must contain not fewer than 4×10^9 live bacteria.

IDENTIFICATION

Culture bacteria from the vaccine to be examined on an agar medium containing 1 per cent of galactose and bromothymol blue. Light blue, concave colonies, transparent due to lysis of cells, are formed. No yellow colonies (galactose-fermenting) are found.

TESTS

Contaminating micro-organisms (2.6.12, 2.6.13). Carry out the test using suitable selective media. Determine the total viable count using the plate-count method. The number of contaminating micro-organisms per dosage unit is not greater than 10^2 bacteria and 20 fungi. No pathogenic bacterium, particularly *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and no salmonella other than strain Ty 21a are found.

Water (2.5.12): 1.5 per cent to 4.0 per cent, determined on the contents of the capsule or of the container by the semi-micro determination of water.

NUMBER OF LIVE BACTERIA

Carry out the test using not fewer than five dosage units. Homogenise the contents of the dosage units in a 9 g/l solution of *sodium chloride R* at 4 °C using a mixer in a cold room with sufficient glass beads to emerge from the liquid. Immediately after homogenisation prepare a suitable dilution of the suspension using cooled diluent and inoculate brain heart infusion agar; incubate at 36 ± 1 °C for 20 h to 36 h. The vaccine contains not fewer than 2×10^9 live *S. typhi* Ty 21a bacteria per dosage unit.

LABELLING

The label states:

- the minimum number of live bacteria per dosage unit,
- that the vaccine is for oral use only.

01/2005:0648

VARICELLA VACCINE (LIVE)

Vaccinum varicellae vivum

DEFINITION

Varicella vaccine (live) is a freeze-dried preparation of a suitable attenuated strain of *Herpesvirus varicellae*. The vaccine is reconstituted immediately before use, as stated on the label, to give a clear liquid that may be coloured owing to the presence of a pH indicator.

PRODUCTION

The production of vaccine is based on a virus seed-lot system and a cell-bank system. The production method shall have been shown to yield consistently live varicella vaccines of adequate immunogenicity and safety in man. The virus in the final vaccine shall not have been passaged in cell cultures beyond the 38th passage from the original isolated virus.

The production method is validated to demonstrate that the product, if tested, would comply with the test for abnormal toxicity for immunosera and vaccines for human use (2.6.9).

SUBSTRATE FOR VIRUS PROPAGATION

The virus is propagated in human diploid cells (5.2.3).

VIRUS SEED LOT

The strain of varicella virus shall be identified as being suitable by historical records which shall include information on the origin of the strain and its subsequent manipulation. The virus shall at no time have been passaged in continuous cell lines. Seed lots are prepared in the same kind of cells as those used for the production of the final vaccine.

To avoid the unnecessary use of monkeys in the test for neurovirulence, virus seed lots are prepared in large quantities and stored at temperatures below –20 °C, if freeze-dried, or below –60 °C, if not freeze-dried.

Only a virus seed lot that complies with the following requirements may be used for virus propagation.

Identification. The master and working seed lots are identified as varicella virus by serum neutralisation in cell culture, using specific antibodies.

Virus concentration. The virus concentration of the master and working seed lots is determined as prescribed under Assay to monitor consistency of production.

Extraneous agents (2.6.16). The working seed lot complies with the requirements for seed lots for live virus vaccines; a sample of 50 ml is taken for the test in cell cultures.

Neurovirulence (2.6.18). The working seed lot complies with the test for neurovirulence of live virus vaccines.

VIRUS PROPAGATION AND HARVEST

All processing of the cell bank and subsequent cell cultures is done under aseptic conditions in an area where no other cells are handled. Approved animal (but not human) serum may be used in the media. Serum and trypsin used in the preparation of cell suspensions and media are shown to be free from extraneous agents. The cell culture medium may contain a pH indicator such as phenol red and approved antibiotics at the lowest effective concentration. It is preferable to have a substrate free from antibiotics during production. 5 per cent, but not less than 50 ml, of the cell cultures employed for vaccine production is set aside as uninfected cell cultures (control cells). The infected cells constituting a single harvest are washed, released from the support surface and pooled. The cell suspension is disrupted by sonication.

Only a virus harvest that complies with the following requirements may be used in the preparation of the final bulk vaccine.

Identification. The virus harvest contains virus that is identified as varicella virus by serum neutralisation in cell culture, using specific antibodies.

Virus concentration. The concentration of infective virus in virus harvests is determined as prescribed under Assay to monitor consistency of production and to determine the dilution to be used for the final bulk vaccine.

Extraneous agents (2.6.16). Use 50 ml for the test in cell cultures.

Control cells. The control cells of the production cell culture from which the single harvest is derived comply with a test for identity and with the requirements for extraneous agents (2.6.16).

FINAL BULK VACCINE

Virus harvests that comply with the above tests are pooled and clarified to remove cells. A suitable stabiliser may be added and the pooled harvests diluted as appropriate.

Only a final bulk vaccine that complies with the following requirements may be used in the preparation of the final lot.

Bacterial and fungal contamination. Carry out the test for sterility (2.6.1) using 10 ml for each medium.

FINAL LOT

The final bulk vaccine is distributed aseptically into sterile, tamper-proof containers and freeze-dried to a moisture content shown to be favourable to the stability of the vaccine. The containers are then closed so as to prevent contamination and the introduction of moisture.

Only a final lot that is satisfactory with respect to each of the requirements given below under Identification, Tests and Assay may be released for use. Provided that the test for bovine serum albumin has been carried out with satisfactory results on the final bulk vaccine, it may be omitted on the final lot.

IDENTIFICATION

When the vaccine reconstituted as stated on the label is mixed with specific *Herpesvirus varicellae* antibodies, it is no longer able to infect susceptible cell cultures.

TESTS

Bacterial and fungal contamination. The reconstituted vaccine complies with the test for sterility (2.6.1).

Bovine serum albumin. Not more than 0.5 µg per human dose, determined by a suitable immunochemical method (2.7.1).

Water (2.5.12). Not more than 3.0 per cent, determined by the semi-micro determination of water.

ASSAY

Titrate for infective virus, using at least 10 cell cultures for each fourfold dilution or by a technique of equal precision. Use a suitable virus reference preparation to validate each assay. The virus concentration is not less than the minimum stated on the label.

LABELLING

The label states:

- the strain of virus used for the preparation of the vaccine,
- the type and origin of the cells used for the preparation of the vaccine,
- that contact with disinfectants is to be avoided,
- the minimum virus concentration,
- that the vaccine is not to be administered to pregnant women,
- the time within which the vaccine must be used after reconstitution.

01/2005:0537

YELLOW FEVER VACCINE (LIVE)

Vaccinum febris flavae vivum

DEFINITION

Yellow fever vaccine (live) is a freeze-dried preparation of the 17D strain of yellow fever virus grown in fertilised hen eggs. The vaccine is reconstituted immediately before use, as stated on the label, to give a clear liquid.

PRODUCTION

The production of vaccine is based on a virus seed-lot system. The production method shall have been shown to yield consistently yellow fever vaccine (live) of acceptable immunogenicity and safety for man.

The production method is validated to demonstrate that the product, if tested, would comply with the test for abnormal toxicity for immunosera and vaccines for human use (2.6.9) modified as follows for the test in guinea-pigs: inject ten human doses into each guinea-pig and observe for 21 days.

Reference preparation. In the test for neurotropism, a suitable batch of vaccine known to have satisfactory properties in man is used as the reference preparation.

SUBSTRATE FOR VIRUS PROPAGATION

Virus for the preparation of master and working seed lots and of all vaccine batches is grown in the tissues of chick embryos from a flock free from specified pathogens (5.2.2).

SEED LOTS

The 17D strain shall be identified by historical records that include information on the origin of the strain and its subsequent manipulation. Virus seed lots are prepared in large quantities and stored at a temperature below –60 °C. Master and working seed lots shall not contain any human protein or added serum.

Unless otherwise justified and authorised, the virus in the final vaccine shall be between passage levels 204 and 239 from the original isolate of strain 17D. A working seed lot shall be only one passage from a master seed lot. A working seed lot shall be used without intervening passage as the

inoculum for infecting the tissues used in the production of a vaccine lot, so that no vaccine virus is more than one passage from a seed lot that has passed all the safety tests. Only a virus seed lot that complies with the following requirements may be used for virus propagation.

Identification. The master and working seed lots are identified as containing yellow fever virus by serum neutralisation in cell culture, using specific antibodies.

Extraneous agents (2.6.16). Each working seed lot complies with the following tests:

Bacterial and fungal sterility

Mycoplasmas

Mycobacteria

Avian viruses

Test in adult mice (intraperitoneal inoculation only)

Test in guinea-pigs

Tests in monkeys. Each master and working seed lot complies with the following tests in monkeys for viraemia (viscerotropism), immunogenicity and neurotropism.

The monkeys shall be *Macaca* sp. susceptible to yellow fever virus and shall have been shown to be non-immune to yellow fever at the time of injecting the seed virus. They shall be healthy and shall not have received previously intracerebral or intraspinal inoculation. Furthermore, they shall not have been inoculated by other routes with neurotropic viruses or with antigens related to yellow fever virus. Not fewer than ten monkeys are used for each test.

Use a test dose of 0.25 ml containing the equivalent of not less than 5000 mouse LD₅₀ and not more than 50 000 mouse LD₅₀, determined by a titration for infectious virus and using the established equivalence between virus concentration and mouse LD₅₀ (see under Assay). Inject the test dose into one frontal lobe of each monkey under anaesthesia and observe the monkeys for not less than 30 days.

Viraemia (Viscerotropism). Viscerotropism is indicated by the amount of virus present in serum. Take blood from each of the test monkeys on the second, fourth and sixth days after inoculation and prepare serum from each sample.

Prepare 1:10, 1:100 and 1:1000 dilutions from each serum and inoculate each dilution into a group of at least six cell culture vessels used for the determination of the virus concentration. The seed lot complies with the test if none of the sera contains more than the equivalent of 500 mouse LD₅₀ in 0.03 ml and at most one serum contains more than the equivalent of 100 mouse LD₅₀ in 0.03 ml.

Immunogenicity. Take blood from each monkey 30 days after the injection of the test dose and prepare serum from each sample. The seed lot complies with the test if at least 90 per cent of the test monkeys are shown to be immune, as determined by examining their sera in the test for neutralisation of yellow fever virus described below.

It has been shown that a low dilution of serum (for example, 1:10) may contain non-specific inhibitors that influence this test; such serum shall be treated to remove inhibitors. Mix dilutions of at least 1:10, 1:40 and 1:160 of serum from each monkey with an equal volume of 17D vaccine virus at a dilution that will yield an optimum number of plaques with the titration method used. Incubate the serum-virus mixtures in a water-bath at 37 °C for 1 h and then cool in iced water; add 0.2 ml of each serum-virus mixture to each of four cell-culture plates and proceed as for the determination of virus concentration. Inoculate similarly ten plates with the same amount of virus plus an equal volume of a 1:10 dilution of monkey serum known to contain no neutralising antibodies to yellow fever virus. At the end of the observation period, compare the mean number of plaques in the plates

receiving virus plus non-immune serum with the mean number of plaques in the plates receiving virus plus dilutions of each monkey serum. Not more than 10 per cent of the test monkeys have serum that fails to reduce the number of plaques by 50 per cent at the 1:10 dilution.

Neurotropism. Neurotropism is assessed from clinical evidence of encephalitis, from incidence of clinical manifestations and by evaluation of histological lesions, in comparison with ten monkeys injected with the reference preparation. The seed lot is not acceptable if either the onset and duration of the febrile reaction or the clinical signs of encephalitis and pathological findings are such as to indicate a change in the properties of the virus.

Clinical evaluation. - The monkeys are examined daily for 30 days by personnel familiar with clinical signs of encephalitis in primates (if necessary, the monkeys are removed from their cage and examined for signs of motor weakness or spasticity). The seed lot is not acceptable if in the monkeys injected with it the incidence of severe signs of encephalitis, such as paralysis or inability to stand when stimulated, or mortality is greater than for the reference vaccine. These and other signs of encephalitis, such as paresis, inco-ordination, lethargy, tremors or spasticity are assigned numerical values for the severity of symptoms by a grading method. Each day each monkey in the test is given a score based on the scale:

Grade 1 - rough coat, not eating,

Grade 2 - high-pitched voice, inactive, slow moving,

Grade 3 - shaky, tremors, unco-ordinated, limb weakness,

Grade 4 - inability to stand, limb paralysis or death (a dead monkey receives a daily score of 4 from the day of death until day 30).

A clinical score for a particular monkey is the average of its daily scores; the clinical score for the seed lot is the mean of the individual monkey scores. The seed lot is not acceptable if the mean of the clinical severity scores for the group of monkeys inoculated with it is significantly greater ($P = 0.95$) than the mean for the group of monkeys injected with the reference preparation. In addition, special consideration is given to any animal showing unusually severe signs when deciding on the acceptability of the seed lot.

Histological evaluation. - Five levels of the brain are examined including:

Block I - the corpus striatum at the level of the optic chiasma,
Block II - the thalamus at the level of the mamillary bodies,
Block III - the mesencephalon at the level of the superior colliculi,

Block IV - the pons and cerebellum at the level of the superior olives,

Block V - the medulla oblongata and cerebellum at the level of the mid-inferior olivary nuclei.

Cervical and lumbar enlargements of the spinal cord are each divided equally into six blocks; 15 µm sections are cut from the tissue blocks embedded in paraffin wax and stained with galloxyanin. Numerical scores are given to each hemisection of the cord and to structures in each hemisection of the brain as listed below. Lesions are scored as follows:

Grade 1. Minimal: 1 to 3 small focal inflammatory infiltrates. Degeneration or loss of a few neurons.

Grade 2. Moderate: 4 or more focal inflammatory infiltrates. Degeneration or loss of neurons affecting not more than one third of cells.

Grade 3. Severe: moderate focal or diffuse inflammatory infiltration. Degeneration or loss of up to two thirds of the neurons.

Grade 4. Overwhelming: variable but often severe inflammatory reaction. Degeneration or loss of more than 90 per cent of neurons.

It has been found that inoculation of yellow fever vaccine into the monkey brain causes histological lesions in different anatomical formations of the central nervous system with varying frequency and severity (I. S. Levenbook *et al.*, *Journal of Biological Standardization*, 1987, 15, 305-313). Based on these two indicators, the anatomical structures can be divided into target, spared and discriminator areas. Target areas are those which show more severe specific lesions in a majority of monkeys irrespective of the degree of neurovirulence of the seed lot. Spared areas are those which show only minimal specific lesions and in a minority of monkeys. Discriminator areas are those where there is a significant increase in the frequency of more severe specific lesions with seed lots having a higher degree of neurovirulence. Discriminator and target areas for *Macaca cynomolgus* and *Macaca rhesus* monkeys are shown in Table 0537-1.

Table 0537-1

Type of monkey	Discriminator areas	Target areas
<i>Macaca cynomolgus</i>	globus pallidus	substantia nigra
	putamen	
	anterior/median thalamic nucleus	
	lateral thalamic nucleus	
<i>Macaca rhesus</i>	caudate nucleus	substantia nigra
	globus pallidus	cervical enlargement
	putamen	lumbar enlargement
	anterior/median thalamic nucleus	
	lateral thalamic nucleus	
	cervical enlargement	
	lumbar enlargement	

Scores for discriminator and target areas are used for the final evaluation of the seed lot. The individual monkey score is calculated from the sum of individual target area scores in each hemisection divided by the number of areas examined. A separate score is calculated similarly for the discriminator areas.

Mean scores for the test group are calculated in two ways: (1) by dividing the sum of the individual monkey discriminator scores by the number of monkeys and (2) by dividing the sum of the individual monkey target and discriminator scores by the number of monkeys. These two mean scores are taken into account when deciding on the acceptability of the seed lot. The seed lot is not acceptable if either of the mean lesion scores is significantly greater ($P = 0.95$) than for the reference preparation.

PROPAGATION AND HARVEST

All processing of the fertilised eggs is done under aseptic conditions in an area where no other infectious agents or cells are handled at the same time. 2 per cent but not less than twenty and not more than fifty eggs are set aside as uninfected control eggs. After inoculation and incubation at a controlled temperature, only living and typical chick embryos are harvested. The age of the embryo at the time of virus harvest is reckoned from the initial introduction of the egg into the incubator and shall be not more than 12 days. After homogenisation and clarification by centrifugation, the extract of embryonic pulp is tested as described below

and kept at -70°C or colder until further processing. Virus harvests that comply with the prescribed tests may be pooled. No human protein is added to the virus suspension at any stage during production. If stabilisers are added, they shall have been shown to have no antigenic or sensitising properties for man.

Only a single harvest that complies with the following requirements may be used in the preparation of the final bulk vaccine.

Identification. The single harvest contains virus that is identified as yellow fever virus by serum neutralisation in cell culture, using specific antibodies.

Extraneous agents (2.6.16). The single harvest complies with the tests for extraneous agents.

Control eggs (2.6.16). The control eggs comply with the tests for extraneous agents.

Virus concentration. In order to calculate the dilution for formulation of the final bulk, each single harvest is titrated as described under Assay.

FINAL BULK VACCINE

Single harvests that comply with the tests prescribed above are pooled and clarified again. A test for protein nitrogen content is carried out. A suitable stabiliser may be added and the pooled harvests diluted as appropriate.

Only a final bulk vaccine that complies with the following requirements may be used in the preparation of the final lot.

Bacterial and fungal contamination. Carry out the test for sterility (2.6.1), using 10 ml for each medium.

Protein nitrogen content. The protein nitrogen content, before the addition of any stabiliser, is not more than 0.25 mg per human dose.

FINAL LOT

The final bulk vaccine is distributed aseptically into sterile, tamper-proof containers and freeze-dried to a moisture content shown to be favourable to the stability of the vaccine. The containers are then closed so as to prevent contamination and the introduction of moisture.

Only a final lot that is satisfactory with respect to thermal stability and each of the requirements given below under Identification, Tests and Assay may be released for use. Provided that the test for ovalbumin has been performed with satisfactory results on the final bulk vaccine, it may be omitted on the final lot.

Thermal stability. Maintain samples of the final lot of freeze-dried vaccine in the dry state at 37°C for 14 days. Determine the virus concentration as described under Assay in parallel for the heated vaccine and for unheated vaccine. The difference in the virus concentration between unheated and heated vaccine does not exceed $1.0 \log_{10}$ and the virus concentration of the heated vaccine is not less than the number of plaque-forming units (PFU) equivalent to 1×10^3 mouse LD_{50} per human dose.

IDENTIFICATION

When the vaccine reconstituted as stated on the label is mixed with specific yellow fever virus antibodies, there is a significant reduction in its ability to infect susceptible cell cultures.

TESTS

Bacterial and fungal contamination. The reconstituted vaccine complies with the test for sterility (2.6.1).

Ovalbumin. Not more than $5 \mu\text{g}$ of ovalbumin per human dose, determined by a suitable immunochemical method (2.7.1).

Bacterial endotoxins (2.6.14): less than 5 IU per human dose.

Water (2.5.12). Not more than 3.0 per cent, determined by the semi-micro determination of water.

ASSAY

Titrate for infective virus in cell cultures. Use an appropriate virus reference preparation to validate each assay.

The virus concentration is not less than the equivalent in PFU of 1×10^3 mouse LD_{50} per human dose. The relationship between mouse LD_{50} and PFU is established by each laboratory and approved by the competent authority. The method shown below, or another suitable technique, may be used to determine the mouse LD_{50} .

Suggested method for determination of the mouse LD_{50}

Mouse LD_{50} . The statistically calculated quantity of virus suspension that is expected to produce fatal specific encephalitis in 50 per cent of mice of a highly susceptible strain, 4 to 6 weeks of age, after intracerebral inoculation.

Appropriate serial dilutions of the reconstituted vaccine are made in diluent for yellow fever virus (a 7.5 g/l solution of bovine albumin R in phosphate-buffered saline pH 7.4 R, or any other diluent that has been shown to be equivalent for maintaining the infectivity of the virus).

Mice of a highly susceptible strain, 4 to 6 weeks of age, are injected intracerebrally under anaesthesia with 0.03 ml of the vaccine dilution. Groups of not fewer than six mice are used for each dilution; the series of dilutions is chosen so as to cover the range 0 to 100 per cent mortality of the mice. Injection of the mice is performed immediately after the dilutions have been made. The mice are observed for 21 days and all deaths are recorded. Only survivors and deaths caused by typical yellow fever infections are counted in the computations. Mice paralysed on the twenty-first day of observation are counted as survivors.

LABELLING

The label states:

- the strain of virus used in preparation,
- that the vaccine has been prepared in chick embryos,
- the minimum virus concentration,
- that contact with disinfectants is to be avoided,
- the period of time within which the vaccine is to be used after reconstitution.

01/2005:0441

ANTHRAX SPORE VACCINE (LIVE) FOR VETERINARY USE

Vaccinum anthracis vivum
ad usum veterinarium

DEFINITION

Anthrax spore live vaccine for veterinary use consists of a suspension of live spores of an attenuated, non-capsulated strain of *Bacillus anthracis*.

PRODUCTION

The strain used is either: not lethal to the guinea-pig or the mouse; or lethal to the guinea-pig but not to the rabbit; or lethal to some rabbits. *B. anthracis* is grown in an appropriate medium. At the end of growth the spores are suspended in a stabilising solution and counted. The vaccine may contain an adjuvant.

IDENTIFICATION

B. anthracis present in the vaccine is identified by means of morphological and serological tests, culture and biochemical tests.

TESTS

Safety. Carry out the test on one of the species of animals for which the vaccine is intended. If the vaccine is intended for several species, including the goat, carry out the test on goats. Administer subcutaneously or intradermally to each of 2 animals, of the minimum age recommended for vaccination and having no antibodies against *B. anthracis*, twice the dose stated on the label for the species used and observe the animals for 14 days. No abnormal systemic reaction is produced but a local reaction may occur at the site of injection. The severity of the local reaction may vary according to the strain of the spores and the adjuvants used in the preparation, but necrosis does not occur.

Spore count. The number of live spores determined by plate count is not less than 80 per cent of that stated on the label.

Bacterial and fungal contamination. Carry out the test by microscopic examination and by inoculation of suitable media. The vaccine does not contain contaminating bacteria or fungi.

POTENCY

For a strain of *B. anthracis* which is not lethal to the guinea-pig or the mouse, the test may be carried out in guinea-pigs. For a strain which is lethal to the guinea-pig but not to the rabbit, the test may be carried out in rabbits. For a strain which is lethal to some rabbits, carry out the test in sheep.

If the test is carried out in guinea-pigs or in rabbits, use 10 healthy animals (group a). Inject subcutaneously or intradermally into each animal 1/10 of the smallest dose of the vaccine stated on the label for sheep. Observe the animals for 21 days. If more than 2 animals die from non-specific causes, repeat the test. Use as controls 3 animals of the same species and of the same origin.

If the test is carried out in sheep, use 5 healthy animals (group b). Inject subcutaneously or intradermally into each animal 1/10 of the smallest dose of the vaccine stated on the label for sheep. Observe the animals for 21 days. Use as controls 3 sheep of the same origin. Inject subcutaneously into each vaccinated animal of group (a) or group (b) at least 100 MLD and into each control animal 10 MLD of a strain of

B. anthracis pathogenic for the species of animal used in the test. Observe all the animals for 10 days. All the vaccinated animals survive and all the controls die from anthrax during the observation period. If a vaccinated animal dies after the challenge, repeat the test. If in the second test a vaccinated animal dies, the vaccine fails the test.

LABELLING

The label states:

- the strain used for preparation of the vaccine,
- the number of viable spores per millilitre.

01/2005:0744

AUJESZKY'S DISEASE VACCINE (INACTIVATED) FOR PIGS

Vaccinum morbi Aujeszkyi ad suem
inactivatum

DEFINITION

Aujeszky's disease vaccine (inactivated) for pigs consists of a suspension of an appropriate strain of Aujeszky's disease virus inactivated without affecting its immunogenic properties or a suspension of an inactivated fraction of the virus having adequate immunogenic properties.

PRODUCTION

The virus strain is grown in suitable cell cultures (5.2.4). The viral suspension is harvested and inactivated; it may be treated to fragment the virus and the viral fragments may be purified and concentrated.

The test for inactivation is carried out using two passages in the same type of cell culture as that used in the production of the vaccine or cells shown to be at least as sensitive. The quantity of inactivated virus used in the test is equivalent to not less than twenty-five doses of the vaccine. No live virus is detected.

Suitable adjuvants and antimicrobial preservatives may be added. The vaccine may be freeze-dried.

CHOICE OF VACCINE COMPOSITION

The vaccine is shown to be satisfactory with respect to safety and immunogenicity. The following tests may be used during demonstration of safety (5.2.6) and efficacy (5.2.7).

Safety

- A test is carried out in each category of animals for which the vaccine is intended (sows, fattening pigs). The animals used do not have antibodies against Aujeszky's disease virus or against a fraction of the virus. Two doses of vaccine are injected by a recommended route into each of not fewer than ten animals. After 14 days, one dose of vaccine is injected into each of the animals. The animals are observed for a further 14 days. No abnormal local or systemic reaction is produced during the 28 days of the test. If the vaccine is intended for use in pregnant sows, for the test in this category of animal the observation period is prolonged up to farrowing and any effects on gestation or the offspring are noted.
- The animals used in the test for immunogenicity are also used to evaluate safety. The rectal temperature of each vaccinated animal is measured at the time of vaccination and 6 h, 24 h and 48 h later. No animal shows a temperature rise greater than 1.5 °C and the number of animals showing a temperature greater than 41 °C does not exceed 10 per cent of the group. No other systemic reactions (for example, anorexia) are noted. At slaughter,

the injection site is examined for local reactions. No abnormal local reactions attributable to the vaccine are produced.

- C. The animals used for field trials are also used to evaluate safety. A test is carried out in each category of animals for which the vaccine is intended (sows, fattening pigs). Not fewer than three groups each of not fewer than twenty animals are used with corresponding groups of not fewer than ten controls. The rectal temperature of each vaccinated animal is measured at the time of vaccination and 6 h, 24 h and 48 h later. No animal shows a temperature rise greater than 1.5 °C and the number of animals showing a temperature greater than 41 °C does not exceed 25 per cent of the group. At slaughter, the injection site is examined for local reactions. No abnormal local reactions attributable to the vaccine are produced.

Immunogenicity. Not fewer than ten fattening pigs of the age recommended for vaccination and which do not have antibodies against Aujeszky's disease virus or against a fraction of the virus are used. The body mass of none of the pigs differs from the average body mass of the group by more than 20 per cent. Each pig is vaccinated according to the recommended schedule and by a recommended route. Five similar pigs are used as controls. At the end of the fattening period (80 kg to 90 kg), each pig is weighed and then challenged by the intranasal route with a suitable quantity of a virulent strain of Aujeszky's disease virus (challenge with at least 10^6 CCID₅₀ of a virulent strain having undergone not more than three passages and administered in not less than 4 ml of diluent has been found to be satisfactory). The titre of challenge virus is determined in swabs taken from the nasal cavity of each animal daily from the day before challenge until virus is no longer detected. Each animal is weighed 7 days after challenge or at the time of death if this occurs earlier and the average daily gain is calculated as a percentage. For each group (vaccinated and controls), the average of the average daily gains is calculated. The vaccine complies with the test if:

- all the vaccinated pigs survive and the difference between the averages of the daily gains for the two groups is not less than 1.5,
- the geometrical mean titres and the duration of excretion of the challenge virus are significantly lower in vaccinates than in controls.

The test is not valid unless all the control pigs display signs of Aujeszky's disease and the average of their daily gains is less than –0.5.

If the vaccine is intended for use in sows for the passive protection of piglets, the suitability of the strain for this purpose may be demonstrated by the following method. Eight sows which do not have antibodies against Aujeszky's disease virus or against a fraction of the virus are vaccinated according to the recommended schedule and by a recommended route; four sows are kept as controls. The piglets from the sows are challenged with a suitable quantity of a virulent strain of Aujeszky's disease virus at 6 to 10 days of age. The piglets are observed for 21 days. The vaccine is satisfactory if not less than 80 per cent protection against mortality is found in the piglets from the vaccinated sows compared to those from the control sows. The test is not valid if the average number of piglets per litter for each group is less than six.

BATCH TESTING

The test described under Potency is not necessarily carried out for routine testing of batches of vaccine. It is carried out for a given vaccine, on one or more occasions, as decided by or with the agreement of the competent authority; where

the test is not carried out a suitable, validated, alternative test is carried out, the criteria for acceptance being set with reference to a batch of vaccine that has given satisfactory results in the test described under Potency.

IDENTIFICATION

In animals having no antibodies against Aujeszky's disease virus or against a fraction of the virus, the vaccine stimulates the production of specific antibodies against Aujeszky's disease virus or the fraction of the virus used in the production of the vaccine.

TESTS

Safety. Inject two doses of the vaccine by a recommended route into each of not fewer than two pigs of the minimum age recommended for vaccination and having no antibodies against Aujeszky's disease virus or against a fraction of the virus. Observe the animals for 14 days and then inject one dose of the vaccine into each piglet. Observe the animals for a further 14 days. No abnormal local or systemic reaction occurs during the 28 days of the test.

Inactivation. Wherever possible, carry out a suitable test for residual infectious Aujeszky's disease virus using two passages in the same type of cell culture as used in the production of the vaccine or cells shown to be at least as sensitive. Otherwise, inject one dose of the vaccine subcutaneously into each of five healthy non-immunised rabbits. Observe the animals for 14 days after the injection. No abnormal reaction (in particular a local rash) occurs. If the vaccine strain is not pathogenic for the rabbit, carry out the test in two sheep.

Extraneous viruses. On the pigs used for the safety test carry out tests for antibodies. The vaccine does not stimulate the formation of antibodies, other than those against Aujeszky's disease virus, against viruses pathogenic for pigs or against viruses that could interfere with the diagnosis of infectious diseases of pigs (including the viruses of the pestivirus group).

Sterility. The vaccine complies with the test for sterility prescribed in the monograph on *Vaccines for veterinary use* (0062).

POTENCY

Use not fewer than five pigs each weighing 15 kg to 35 kg and which do not have antibodies against Aujeszky's disease virus or against a fraction of the virus. The body mass of none of the pigs differs from the average body mass of the group by more than 25 per cent. Administer to each pig by a recommended route one dose of the vaccine. Use five similar pigs as controls. Three weeks later weigh each pig and then challenge by the intranasal route with a suitable quantity of a virulent strain of Aujeszky's disease virus. Weigh each animal 7 days after challenge or at the time of death if this occurs earlier and calculate the average daily gain as a percentage. For each group (vaccinated and controls), calculate the average of the average daily gains. The vaccine passes the test if the vaccinated pigs survive and the difference between the averages of the daily gains for the two groups is not less than 1.1. The test is not valid unless all the control pigs display signs of Aujeszky's disease and the average of their daily gains is less than –0.5.

LABELLING

The label states:

- whether the vaccine strain is pathogenic for the rabbit,
- whether the vaccine is a whole-virus vaccine or a subunit vaccine.

01/2005:0745

AUJESZKY'S DISEASE VACCINE (LIVE) FOR PIGS FOR PARENTERAL ADMINISTRATION, FREEZE-DRIED

Vaccinum morbi Aujeszkyi ad suem vivum cryodesiccatum ad usum parenterale

DEFINITION

Freeze-dried Aujeszky's disease vaccine (live) for pigs for parenteral administration is a preparation of an attenuated strain of Aujeszky's disease virus. It may be administered after mixing with an adjuvant.

PRODUCTION

The virus strain is grown in suitable cell cultures (5.2.4) or in fertilised hen eggs from flocks free from specified pathogens (5.2.2). The viral suspension is harvested, mixed with a suitable stabilising liquid and freeze-dried.

CHOICE OF VACCINE STRAIN

Only a virus strain shown to be satisfactory with respect to the following characteristics may be used in the preparation of the vaccine: safety; transmissibility, including transmission across the placenta and by semen; irreversibility of attenuation; immunogenicity. The strain may have a genetic marker. The following tests may be used during demonstration of safety (5.2.6) and efficacy (5.2.7).

Safety.

- A. 10 piglets, 3 to 4 weeks old and which do not have antibodies against Aujeszky's disease virus or against a fraction of the virus, each receive by a recommended route a quantity of virus corresponding to 10 doses of vaccine. 10 piglets of the same origin and age and which do not have antibodies against Aujeszky's disease virus or against a fraction of the virus are kept as controls. The animals are observed for 21 days. The piglets remain in good health. The weight curve of the vaccinated piglets does not differ significantly from that of the controls.
- B. The animals used in the test for immunogenicity are also used to evaluate safety. The rectal temperature of each vaccinated animal is measured at the time of vaccination and 6 h, 24 h and 48 h later. No animal shows a temperature rise greater than 1.5 °C and the number of animals showing a temperature greater than 41 °C does not exceed 10 per cent of the group. No other systemic reactions (for example, anorexia) are noted. At slaughter, the injection site is examined for local reactions. No abnormal local reactions attributable to the vaccine are produced.
- C. The animals used for field trials are also used to evaluate safety. A test is carried out in each category of animals for which the vaccine is intended (sows, fattening pigs). Not fewer than 3 groups each of not fewer than 20 animals are used with corresponding groups of not fewer than 10 controls. The rectal temperature of each animal is measured at the time of vaccination and 6 h, 24 h and 48 h later. No animal shows a temperature rise greater than 1.5 °C and the number of animals showing a temperature greater than 41 °C does not exceed 25 per cent of the group. At slaughter, the injection site is examined for local reactions. No abnormal local reactions attributable to the vaccine are produced.
- D. 10 piglets, 3 to 5 days old and which do not have antibodies against Aujeszky's disease virus or against a fraction of the virus, each receive by the intranasal route

a quantity of virus corresponding to 10 doses of vaccine. The animals are observed for 21 days. None of the piglets dies or shows signs of neurological disorder attributable to the vaccine virus.

- E. This test is not necessary for gE-negative strains. 5 piglets, 3 to 5 days old, each receive $10^{4.5}$ CCID₅₀ of vaccine virus intracerebrally. None of the piglets dies or shows signs of neurological disorder.
- F. 10 piglets, 3 to 4 weeks old and which do not have antibodies against Aujeszky's disease virus or against a fraction of the virus, each receive a daily injection of 2 mg of prednisolone per kilogram of body mass for 5 consecutive days. On the third day each piglet receives a quantity of virus corresponding to 1 dose of vaccine by a recommended route. Antimicrobial agents may be administered to prevent aspecific signs. Observe the animals for 21 days following administration of the virus. The piglets remain in good health.
- G. 15 pregnant sows which do not have antibodies against Aujeszky's disease virus or against a fraction of the virus are used. Each of 5 sows receive by a recommended route a quantity of virus corresponding to 10 doses of vaccine during the fourth or fifth week of gestation. 5 other sows each receive the same dose of virus by the same route during the tenth or eleventh week of gestation. The other 5 pregnant sows are kept as controls. The number of piglets born to the vaccinated sows, any abnormalities in the piglets and the duration of gestation do not differ significantly from those of the controls. For the piglets from vaccinated sows: carry out tests for serum antibodies against Aujeszky's disease virus; carry out tests for Aujeszky's disease virus antigen in the liver and lungs of those piglets showing abnormalities and in a quarter of the remaining healthy piglets. No Aujeszky's disease virus antigen is found in piglets born to the vaccinated sows and no antibodies against Aujeszky's disease virus are found in the serum taken before ingestion of colostrum.

Virus excretion. 18 pigs, 3 to 4 weeks old and which do not have antibodies against Aujeszky's disease virus or against a fraction of the virus are used. 14 of the pigs each receive 1 dose of vaccine by the recommended route and at the recommended site and the remaining 4 pigs are kept as contact controls. Suitably sensitive tests for the virus are carried out individually on the nasal and oral secretions as follows: nasal and oral swabs are collected daily from the day before vaccination until 10 days after vaccination. The vaccine is acceptable if the virus is not isolated from the secretions collected.

Transmissibility. The test is carried out on 4 separate occasions. Each time 4 piglets, 3 to 4 weeks old and which do not have antibodies against Aujeszky's disease virus or against a fraction of the virus, each receive by a recommended route a quantity of virus corresponding to 1 dose of vaccine. 1 day after the administration, 2 other piglets of the same age which do not have antibodies against Aujeszky's disease virus or against a fraction of the virus are kept close together with them. After 5 weeks all the animals are tested for the presence of antibodies against Aujeszky's disease virus. Antibodies against Aujeszky's disease virus are not detected in any group of contact controls. All the treated piglets show an antibody response.

Reversion to virulence. 2 piglets, 3 to 5 days old and which do not have antibodies against Aujeszky's disease virus or against a fraction of the virus, each receive by the intranasal route a quantity of virus corresponding to 1 dose of vaccine. 3 to 5 days later brain, lung, tonsils and local lymph glands are taken from each piglet and the samples are pooled. 1 ml of the pooled organ suspension is administered

intranasally into each of 2 other piglets of the same age and susceptibility. This operation is then repeated not fewer than 4 times, the last time in not fewer than 5 piglets. The presence of the virus is verified at each passage by direct or indirect means. If the virus has disappeared, a second series of passages is carried out. The piglets do not die or show neurological disorders from causes attributable to the vaccine virus. There is no indication of an increase of virulence as compared with the non-passaged virus.

Immunogenicity. Not fewer than 10 fattening pigs of the age recommended for vaccination and which do not have antibodies against Aujeszky's disease virus or against a fraction of the virus are used. The body mass of none of the pigs differs from the average body mass of the group by more than 20 per cent. Each pig is vaccinated according to the recommended schedule and by a recommended route. 5 similar pigs are used as controls. At the end of the fattening period (80 kg to 90 kg), each pig is weighed and then challenged by the intranasal route with a suitable quantity of a virulent strain of Aujeszky's disease virus (challenge with at least 10^6 CCID₅₀ of a virulent strain having undergone not more than 3 passages and administered in not less than 4 ml of diluent has been found to be satisfactory). The titre of virus is determined in swabs taken from the nasal cavity of each pig daily from the day before challenge until virus is no longer detected. Each pig is weighed 7 days after challenge or at the time of death if this occurs earlier and the average daily gain is calculated as a percentage. For each group (vaccinated and controls), the average of the average daily gains is calculated. The vaccine complies with the test if:

- all the vaccinated pigs survive and the difference between the averages of the average daily gains for the 2 groups is not less than 1.5,
- the geometrical mean titres and the duration of excretion of the challenge virus are significantly lower in vaccinates than in controls.

The test is not valid unless all the control pigs display signs of Aujeszky's disease and the average of their average daily gains is less than –0.5.

If the vaccine is intended for use in sows for the passive protection of piglets, the suitability of the strain for this purpose may be demonstrated by the following method. 8 sows which do not have antibodies against Aujeszky's disease virus or against a fraction of the virus are vaccinated according to the recommended schedule and by the recommended route; 4 sows are kept as controls. The piglets from the sows are challenged with a suitable quantity of a virulent strain of Aujeszky's disease virus at 6 to 10 days of age. The piglets are observed for 21 days. The vaccine is satisfactory if not less than 80 per cent protection against mortality is found in the piglets from the vaccinated sows compared to those from the control sows. The test is not valid if the average number of piglets per litter for each group is less than 6.

BATCH TESTING

The test described under Potency is not necessarily carried out for routine testing of batches of vaccine. It is carried out for a given vaccine, on one or more occasions, as decided by or with the agreement of the competent authority; where the test is not carried out a suitable, validated alternative test is carried out, the criteria for acceptance being set with reference to a batch of vaccine that has given satisfactory results in the test described under Potency.

IDENTIFICATION

In animals having no antibodies against Aujeszky's disease virus or against a fraction of the virus, the vaccine stimulates the production of specific neutralising antibodies.

TESTS

Safety. Administer 10 doses of the vaccine in a suitable volume by a recommended route to each of not fewer than 2 pigs of the minimum age recommended for vaccination and which do not have antibodies against Aujeszky's disease virus or against a fraction of the virus. Observe the animals for 14 days. No abnormal local or systemic reaction occurs.

Extraneous viruses. Neutralise the vaccine using a monospecific antiserum or monoclonal antibodies and inoculate into cell cultures known to be sensitive to viruses pathogenic for pigs and to pestiviruses. Maintain these cultures for 14 days and make at least 1 passage during this period. No cytopathic effect develops; the cells show no evidence of the presence of haemadsorbing agents. Carry out a specific test for pestiviruses.

Sterility. The vaccine complies with the test for sterility prescribed in the monograph on *Vaccines for veterinary use* (0062).

Mycoplasmas (2.6.7). The vaccine complies with the test for mycoplasmas.

Virus titre. Titrate the reconstituted vaccine on the same substrate as used for production (cell cultures or inoculation into the allantoic cavity of fertilised hen eggs). 1 dose of the vaccine contains not less than the quantity of virus equivalent to the minimum virus titre stated on the label.

POTENCY

Use not fewer than 5 pigs weighing 15 kg to 35 kg and which do not have antibodies against Aujeszky's disease virus or against a fraction of the virus. The body mass of none of the pigs differs from the average body mass of the group by more than 25 per cent. Administer to each pig by a recommended route 1 dose of the vaccine. Use 5 similar pigs as controls. 3 weeks later weigh each pig and then challenge by the intranasal route with a suitable quantity of a virulent strain of Aujeszky's disease virus. Weigh each animal 7 days after challenge or at the time of death if this occurs earlier and calculate the average daily gain as a percentage. For each group (vaccinated and controls), calculate the average of the average daily gains. The vaccine complies with the test if all the vaccinated pigs survive and the difference between the averages of the average daily gains for the 2 groups is not less than 1.6. The test is not valid unless all the control pigs display signs of Aujeszky's disease and the average of their average daily gains is less than –0.5.

LABELLING

The label states:

- the substrate used for production of the vaccine (cell cultures or eggs),
- the minimum virus titre.

01/2005:0959

AVIAN INFECTIOUS BRONCHITIS VACCINE (INACTIVATED)

Vaccinum bronchitidis infectivae aviariae inactivatum

DEFINITION

Avian infectious bronchitis vaccine (inactivated) consists of an emulsion or a suspension of one or more serotypes of avian infectious bronchitis virus which have been inactivated in such a manner that the immunogenic activity is retained. This monograph describes vaccines intended to protect

against drop of egg production or quality; for vaccines also intended to protect against respiratory symptoms, a demonstration of efficacy additional to that described under Potency is required.

PRODUCTION

The virus is propagated in fertilised hen eggs from healthy flocks (5.2.2) or in suitable cell cultures (5.2.4).

An amplification test for residual live avian infectious bronchitis virus is carried out on each batch of antigen immediately after inactivation and on the final bulk vaccine or, if the vaccine contains an adjuvant, on the bulk antigen or the mixture of bulk antigens immediately before the addition of adjuvant; the test is carried out in fertilised hen eggs from flocks free from specified pathogens (5.2.2) or in suitable cell cultures (5.2.4) and the quantity of inactivated virus used is equivalent to not less than 10 doses of vaccine. No live virus is detected.

The vaccine may contain one or more suitable adjuvants.

CHOICE OF VACCINE COMPOSITION

The vaccine is shown to be satisfactory with respect to safety and immunogenicity for each category of chickens for which it is intended. The following test may be used during demonstration of efficacy (5.2.7).

Immunogenicity. The test described under Potency is suitable to demonstrate immunogenicity.

BATCH POTENCY TEST

The test described under Potency is not carried out for routine testing of batches of vaccine. It is carried out, for a given vaccine, on one or more occasions, as decided by or with the agreement of the competent authority; where the test is not carried out, a suitable validated test is carried out, the criteria for acceptance being set with reference to a batch of vaccine that has given satisfactory results in the test described under Potency. The following test may be used after a satisfactory correlation with the test described under Potency has been established by a statistical evaluation.

Administer 1 dose of vaccine intramuscularly to each of ten chickens, between 2 weeks of age and the minimum age stated for vaccination and from a flock free from specified pathogens and keep five hatch mates as unvaccinated controls. Collect serum samples from each chicken just before administration of the vaccine and after the period defined when testing the reference vaccine; determine the antibody titre of each serum, for each serotype in the vaccine, by a suitable serological method, for example, serum neutralisation. The antibody levels are not significantly less than those obtained with a batch that has given satisfactory results in the test described under Potency (reference vaccine). The test is not valid unless the sera collected from the unvaccinated controls and from the chickens just before the administration of the vaccine are free from detectable specific antibody.

IDENTIFICATION

In susceptible animals, the vaccine stimulates the production of specific antibodies against each of the virus serotypes in the vaccine, detectable by virus neutralisation.

TESTS

Safety. Inject a double dose of vaccine by a recommended route into each of ten 14- to 28-day-old chickens from a flock free from specified pathogens (5.2.2). Observe the chickens for 21 days. No abnormal local or systemic reaction occurs.

Inactivation.

A. For vaccine prepared with embryo-adapted strains of virus, inject two-fifths of a dose into the allantoic cavity of ten 9- to 11-day-old fertilised hen eggs from a flock free from specified pathogens (SPF hen eggs) (5.2.2) and incubate. Observe for 5 to 6 days and pool separately the allantoic liquid from eggs containing live embryos and that from eggs containing dead embryos, excluding those that die within the first 24 h after injection. Examine for abnormalities all embryos which die after 24 h of injection or which survive 5 to 6 days. No death or abnormality attributable to the vaccine virus occurs.

Inject into the allantoic cavity of each of ten 9- to 11-day-old fertilised SPF hen eggs 0.2 ml of the pooled allantoic liquid from the live embryos and into each of ten similar eggs 0.2 ml of the pooled liquid from the dead embryos and incubate for 5 to 6 days. Examine for abnormalities all embryos which die after 24 h of injection or which survive 5 to 6 days. No death or abnormality attributable to the vaccine virus occurs.

If more than 20 per cent of the embryos die at either stage repeat the test from that stage. The vaccine complies with the test if there is no death or abnormality attributable to the vaccine virus.

B. For vaccine prepared with cell-culture-adapted strains of virus, inoculate ten doses of the vaccine into suitable cell cultures. If the vaccine contains an oil adjuvant, eliminate it by suitable means. Incubate at 38 ± 1 °C for 7 days. Make a passage on another set of cell cultures and incubate at 38 ± 1 °C for 7 days. None of the cultures shows signs of infection.

Extraneous agents. Use the chickens from the test for safety. 21 days after injection of the double dose of vaccine, inject one dose by the same route into each chicken. Collect serum samples from each chicken 2 weeks later and carry out tests for antibodies to the following agents by the methods prescribed for *chicken flocks free from specified pathogens for the production and quality control of vaccines* (5.2.2): avian encephalomyelitis virus, avian leucosis viruses, haemagglutinating avian adenovirus, infectious bursal disease virus, infectious laryngotracheitis virus, influenza A virus, Marek's disease virus, Newcastle disease virus. The vaccine does not stimulate the formation of antibodies against these agents.

Sterility. The vaccine complies with the test for sterility prescribed in the monograph on *Vaccines for veterinary use* (0062).

POTENCY

Carry out a potency test for each serotype in the vaccine. Use four groups of not fewer than thirty chickens from a flock free from specified pathogens (5.2.2.), treated as follows:

Group A: unvaccinated controls.

Group B: vaccinated with inactivated avian infectious bronchitis vaccine.

Group C: vaccinated with live avian infectious bronchitis vaccine and inactivated avian infectious bronchitis vaccine according to the recommended schedule.

Group D: vaccinated with live avian infectious bronchitis vaccine.

Monitor egg production and quality in all birds from point of lay until at least 4 weeks after challenge. At the peak of lay, challenge all groups with a quantity of virulent avian infectious bronchitis virus sufficient to cause a drop in egg production or quality over three consecutive weeks during the 4 weeks following challenge. The vaccine complies with the test if egg production or quality is significantly

better in group C than in group D and significantly better in group B than in group A. The test is not valid unless there is a drop in egg production in group A compared to the normal level noted before challenge of at least 35 per cent where challenge has been made with a Massachusetts-type strain; where it is necessary to carry out a challenge with a strain of another serotype for which there is documented evidence that the strain will not cause a 35 per cent drop in egg production, the challenge must produce a drop in egg production commensurate with the documented evidence and in any case not less than 15 per cent.

LABELLING

The label states:

- the category and age of the chickens for which the vaccine is intended,
- the strains and serotypes of virus used in the production of the vaccine and the serotypes against which the vaccine is intended to protect,
- whether the strain in the vaccine is embryo-adapted or cell-culture-adapted.

01/2005:0442

AVIAN INFECTIOUS BRONCHITIS VACCINE (LIVE)

Vaccinum bronchitidis infectivae aviariae vivum

1. DEFINITION

Avian infectious bronchitis vaccine (live) is a preparation of one or more suitable strains of different types of avian infectious bronchitis virus. This monograph applies to vaccines intended for administration to chickens for active immunisation against respiratory disease caused by avian infectious bronchitis virus.

2. PRODUCTION

2-1. PREPARATION OF THE VACCINE

The vaccine virus is grown in embryonated hens' eggs or in cell cultures.

2-2. SUBSTRATE FOR VIRUS PROPAGATION

2-2-1. Embryonated hens' eggs. If the vaccine virus is grown in embryonated hens' eggs, they are obtained from flocks free from specified pathogens (SPF) (5.2.2).

2-2-2. Cell cultures. If the vaccine virus is grown in cell cultures, they comply with the requirements for cell cultures for production of veterinary vaccines (5.2.4).

2-3. SEED LOTS

2-3-1. Extraneous agents. The master seed lot complies with the tests for extraneous agents in seed lots (2.6.24). In these tests on the master seed lot, the organisms used are not more than 5 passages from the master seed lot at the start of the test.

2-4. CHOICE OF VACCINE VIRUS

The vaccine virus shall be shown to be satisfactory with respect to safety (5.2.6) and efficacy (5.2.7) for the chickens for which it is intended.

The following tests for safety (section 2-4-1), increase in virulence (section 2-4-2) and immunogenicity (section 2-4-3) may be used during the demonstration of safety and immunogenicity.

2-4-1. Safety

2-4-1-1. Safety for the respiratory tract and kidneys. Carry out the test in chickens not older than the youngest age to be recommended for vaccination. Use vaccine virus at the least attenuated passage level that will be present between the master seed lot and a batch of the vaccine. Use not fewer than 15 chickens from an SPF flock (5.2.2) and from the same origin. Administer to each chicken by the oculonasal route a quantity of the vaccine virus equivalent to not less than 10 times the maximum virus titre likely to be contained in 1 dose of the vaccine. On each of days 5, 7 and 10 after administration of the virus, kill not fewer than 5 of the chickens, take samples of trachea and kidney. Fix kidney samples for histological examination. Remove the tracheas and cut 10 rings from each trachea (3 from the top, 4 from the mid-part and 3 from the bottom); examine each ring under low magnification and score for ciliostasis on a scale from 0 (100 per cent ciliary activity) to 4 (no activity, complete ciliostasis); calculate the mean ciliostasis score (the maximum for each trachea being 40) for the 5 chickens killed on each of days 5, 7 and 10. The test is not valid if more than 10 per cent of the chickens die from causes not attributable to the vaccine virus. The vaccine virus complies with the test if:

- no chicken shows notable clinical signs of avian infectious bronchitis or dies from causes attributable to the vaccine virus,
- the average ciliostasis score is not more than 25,
- at most moderate inflammatory lesions are seen during kidney histological examination.

2-4-1-2. Safety for the reproductive tract. If the recommendations for use state or imply that the vaccine may be used in females of less than 3 weeks old that are subsequently kept to sexual maturity, it shall be demonstrated that there is no damage to development of the reproductive tract when the vaccine is given to chickens of the minimum age to be recommended for vaccination. The following test may be carried out: use not fewer than 40 female chickens not older than the minimum age recommended for vaccination and from an SPF flock (5.2.2); use the vaccine virus at the least attenuated passage level that will be present in a batch of vaccine; administer to each chicken by a recommended route a quantity of virus equivalent to not less than the maximum titre likely to be present in 1 dose of vaccine; at least 10 weeks after administration of the vaccine virus, kill the chickens and carry out macroscopic examination of the oviducts. The vaccine virus complies with the test if abnormalities are present in not more than 5 per cent of the oviducts.

2-4-2. Increase in virulence. The test for increase in virulence consists of the administration of the vaccine virus, at the least attenuated virus passage level that will be present between the master seed lot and a batch of the vaccine, to a group of five 2-week-old chickens from an SPF flock (5.2.2), sequential passages, 5 times where possible, to further similar groups and testing of the final recovered virus for increase in virulence. If the properties of the vaccine virus allow sequential passage to 5 groups via natural spreading, this method may be used, otherwise passage as described below is carried out and the maximally passaged virus that has been recovered is tested for increase in virulence. Care must be taken to avoid contamination by virus from previous passages. Administer by eye-drop a quantity of the vaccine virus that will allow recovery of virus for the passages described below. 2 to 4 days after administration of the vaccine virus, prepare a suspension from the mucosa of the trachea of each chicken and pool these samples. Administer 0.05 ml of the pooled samples by eye-drop to each of 5

other 2-week-old chickens from an SPF flock (5.2.2). Carry out this passage operation not fewer than 5 times; verify the presence of the virus at each passage. If the virus is not found at a passage level, carry out a second series of passages. Carry out the test for safety for the respiratory tract and kidney (section 2-4-1-1) and, where applicable, the test for safety for the reproductive tract (section 2-4-1-2) using the unpassaged vaccine virus and the maximally passaged virus that has been recovered. Administer the virus by the route to be recommended for vaccination likely to be the least safe. The vaccine virus complies with the test if no indication of increase in virulence of the maximally passaged virus compared with the unpassaged virus is observed. If virus is not recovered at any passage level in the first and second series of passages, the vaccine virus also complies with the test.

2-4-3. Immunogenicity. Immunogenicity is demonstrated for each strain of virus to be included in the vaccine. A test is carried out for each route and method of administration to be recommended using in each case chickens from an SPF flock (5.2.2) not older than the youngest age to be recommended for vaccination. The quantity of the vaccine virus administered to each chicken is not greater than the minimum virus titre to be stated on the label and the virus is at the most attenuated passage level that will be present in a batch of the vaccine. 1 or both of the tests below may be used during the demonstration of immunogenicity.

2-4-3-1. Ciliary activity of tracheal explants. Use not fewer than 25 chickens of the same origin and from an SPF flock (5.2.2). Vaccinate by a recommended route not fewer than 20 chickens. Maintain not fewer than 5 chickens as controls. Challenge each chicken after 21 days by eye-drop with a sufficient quantity of virulent avian infectious bronchitis virus of the same type as the vaccine virus to be tested. Kill the chickens 4 to 7 days after challenge and prepare transverse sections from the upper part (3), the middle part (4) and the lower part (3) of the trachea of each chicken. Examine all explants as soon as possible and at the latest 2 h after sampling by low-magnification microscopy for ciliary activity. For a given tracheal section, ciliary activity is considered as normal when at least 50 per cent of the internal ring shows vigorous ciliary movement. A chicken is considered not affected if not fewer than 9 out of 10 rings show normal ciliary activity.

The test is not valid if:

- fewer than 80 per cent of the control chickens show cessation or extreme loss of vigour of ciliary activity,
- and/or during the period between the vaccination and challenge more than 10 per cent of vaccinated or control chickens show abnormal clinical signs or die from causes not attributable to the vaccine.

The vaccine virus complies with the test if not fewer than 80 per cent of the vaccinated chickens show normal ciliary activity.

2-4-3-2. Virus recovery from tracheal swabs. Use not fewer than 30 chickens of the same origin and from an SPF flock (5.2.2). Vaccinate by a recommended route not fewer than 20 chickens. Maintain not fewer than 10 chickens as controls. Challenge each chicken after 21 days by eye-drop with a sufficient quantity of virulent avian infectious bronchitis virus of the same type as the vaccine virus to be tested. Kill the chickens 4 to 7 days after challenge and prepare a suspension from swabs of the tracheal mucosa of each chicken. Inoculate 0.2 ml of the suspension into the allantoic cavity of each of 5 embryonated hens' eggs, 9 to 11 days old, from an SPF flock (5.2.2). Incubate the eggs for 6-8 days after inoculation. Eggs that after 1 day of incubation do not contain a live embryo are eliminated and considered as

non-specific deaths. Record the other eggs containing a dead embryo and after 6-8 days' incubation examine each egg containing a live embryo for lesions characteristic of avian infectious bronchitis. Make successively 3 such passages. If 1 embryo of a series of eggs dies or shows characteristic lesions, the inoculum is considered to be a carrier of avian infectious bronchitis virus. The examination of a series of eggs is considered to be definitely negative if no inoculum concerned is a carrier. The test is not valid if:

- the challenge virus is re-isolated from fewer than 80 per cent of the control chickens,
- and/or during the period between vaccination and challenge more than 10 per cent of the vaccinated or control chickens show abnormal clinical signs or die from causes not attributable to the vaccine,
- and/or more than 1 egg in any group is eliminated because of non-specific embryo death.

The vaccine virus complies with the test if the challenge virus is re-isolated from not more than 20 per cent of the vaccinated chickens.

3. BATCH TESTS

3-1. Identification

3-1-1. Vaccines containing one type of virus. The vaccine, diluted if necessary and mixed with avian infectious bronchitis virus antiserum specific for the virus type, no longer infects embryonated hens' eggs from an SPF flock (5.2.2) or susceptible cell cultures (5.2.4) into which it is inoculated.

3-1-2. Vaccines containing more than one type of virus. The vaccine, diluted if necessary and mixed with type-specific antisera against each strain present in the vaccine except that to be identified, infects embryonated hens' eggs from an SPF flock (5.2.2) or susceptible cell cultures (5.2.4) into which it is inoculated whereas after further admixture with type-specific antiserum against the strain to be identified it no longer produces such infection.

3-2. Bacteria and fungi

Vaccines intended for administration by injection comply with the test for sterility prescribed in the monograph *Vaccines for veterinary use (0062)*.

Vaccines not intended for administration by injection either comply with the test for sterility prescribed in the monograph *Vaccines for veterinary use (0062)* or with the following test: carry out a quantitative test for bacterial and fungal contamination; carry out identification tests for microorganisms detected in the vaccine; the vaccine does not contain pathogenic microorganisms and contains not more than 1 non-pathogenic microorganism per dose.

Any liquid supplied with the vaccine complies with the test for sterility prescribed in the monograph *Vaccines for veterinary use (0062)*.

3-3. Mycoplasmas. The vaccine complies with the test for mycoplasmas (2.6.7).

3-4. Extraneous agents. The vaccine complies with the tests for extraneous agents in batches of finished product (2.6.25).

3-5. Safety. Use not fewer than 10 chickens from an SPF flock (5.2.2) and of the youngest age recommended for vaccination. Administer by a recommended route to each chicken, 10 doses of the vaccine. Observe the chickens at least daily for 21 days. The test is not valid if more than 20 per cent of the chickens show abnormal clinical signs or die from causes not attributable to the vaccine. The vaccine complies with the test if no chicken shows notable clinical signs of disease or dies from causes attributable to the vaccine.

3-6. **Virus titre.** Titrate the vaccine virus by inoculation into embryonated hens' eggs from an SPF flock (5.2.2) or into suitable cell cultures (5.2.4). If the vaccine contains more than 1 strain of virus, titrate each strain after having neutralised the others with type-specific avian infectious bronchitis antisera. The vaccine complies with the test if 1 dose contains for each vaccine virus not less than the minimum titre stated on the label.

3-7. **Potency.** The vaccine complies with the requirements of 1 of the tests prescribed under Immunogenicity (section 2-4-3) when administered according to the recommended schedule by a recommended route and method. It is not necessary to carry out the potency test for each batch of the vaccine if it has been carried out on a representative batch using a vaccinating dose containing not more than the minimum virus titre stated on the label.

01/2005:0960

AVIAN INFECTIOUS BURSAL DISEASE VACCINE (INACTIVATED)

Vaccinum bursitidis infectivae aviariae inactivatum

DEFINITION

Inactivated infectious avian bursal disease vaccine consists of an emulsion or a suspension of a suitable strain of infectious avian bursal disease virus type 1 which has been inactivated in such a manner that immunogenic activity is retained. The vaccine is for use in breeding domestic fowl to protect their progeny from infectious avian bursal disease.

PRODUCTION

The virus is propagated in fertilised eggs from healthy flocks, in suitable cell cultures (5.2.4) or in chickens from a flock free from specified pathogens (5.2.2).

An amplification test for residual live infectious avian bursal disease virus is carried out on each batch of antigen immediately after inactivation and on the final bulk vaccine or, if the vaccine contains an adjuvant, on the bulk antigen or the mixture of bulk antigens immediately before the addition of any adjuvant, to confirm inactivation; the test is carried out in fertilised hen eggs or in suitable cell cultures or, where chickens have been used for production of the vaccine, in chickens from a flock free from specified pathogens (5.2.2); the quantity of inactivated virus used in the test is equivalent to not less than ten doses of the vaccine. No live virus is detected.

The vaccine may contain one or more suitable adjuvants.

CHOICE OF VACCINE COMPOSITION

The vaccine is shown to be satisfactory with respect to safety (5.2.6) and immunogenicity (5.2.7). The following test may be used during demonstration of efficacy.

Immunogenicity. Each of at least twenty chickens from a flock free from specified pathogens (5.2.2), and of the recommended age for vaccination (close to the point of lay), is injected with the minimum recommended dose of vaccine by one of the recommended routes. 4 to 6 weeks later serum samples are collected from each bird and the antibody response is measured in a serum-neutralisation (SN) test. A suitable standard, calibrated in Ph. Eur. Units against *infectious avian bursal disease serum BRP*, is included in the test. The vaccine complies with the test if the mean antibody level in the sera from the vaccinated birds is at least 10 000 Ph. Eur. Units per millilitre.

Eggs are collected for hatching 5 to 7 weeks after vaccination and the test described below is carried out with 3-week-old chickens from that egg collection. Eggs are collected again towards the end of the period of lay and the test is repeated with chickens from that egg collection which are at least 15 days old.

Twenty-five chickens from vaccinated hens and ten control chickens of the same breed and age from unvaccinated hens are challenged with an eye-drop application of a quantity of a virulent strain of infectious avian bursal disease virus sufficient to produce severe signs of disease, including lesions of the bursa of Fabricius, in all unvaccinated chickens. 3 to 4 days after challenge, the bursa of Fabricius is removed from each chicken. The bursae are examined for evidence of infection by histological examination or by testing for the presence of infectious avian bursal disease antigen in an agar-gel precipitation test. The vaccine complies with the test if three or fewer of the chickens from vaccinated hens show evidence of infectious avian bursal disease infection. The test is not valid unless all the chickens from unvaccinated hens are affected.

Where there is more than one recommended route of administration, the test described under Potency is carried out in parallel with the above immunogenicity test, using different groups of birds for each recommended route. The serological response of the birds inoculated by routes other than that used in the immunogenicity test is not significantly less than that of the group vaccinated by that route.

IDENTIFICATION

In chickens with no antibodies to infectious avian bursal disease virus type 1, the vaccine stimulates the production of specific antibodies.

TESTS

Safety. Inject twice the vaccinating dose by one of the recommended routes into each of ten chickens, 14 to 28 days old, from flocks free from specified pathogens (5.2.2). Observe the birds for 21 days. No abnormal local or systemic reaction occurs. *Note: this test may be omitted if inactivation test C is carried out.*

Inactivation

A. For vaccine prepared with embryo-adapted strains of virus, inject two-fifths of a dose into the allantoic cavity or onto the chorio-allantoic membrane of ten 9- to 11-day-old fertilised hen eggs from a flock free from specified pathogens (SPF eggs) (5.2.2). Incubate the eggs and observe for 6 days. Pool separately the allantoic liquid or membranes from eggs containing live embryos, and that from eggs containing dead embryos, excluding those that die from non-specific causes within 24 h of the injection.

Inject into the allantoic cavity or onto the chorio-allantoic membrane of each of ten 9- to 11-day-old SPF eggs 0.2 ml of the pooled allantoic liquid or crushed chorio-allantoic membranes from the live embryos and, into each of ten similar eggs, 0.2 ml of the pooled liquid or membranes from the dead embryos and incubate for 6 days. Examine each embryo for lesions of infectious avian bursal disease.

If more than 20 per cent of the embryos die at either stage repeat that stage. The vaccine complies with the test if there is no evidence of lesions of infectious avian bursal disease and if, in any repeat test, not more than 20 per cent of the embryos die from non-specific causes.

Antibiotics may be used in the test to control extraneous bacterial infection.

01/2005:0587

- B. For vaccine prepared with cell-culture-adapted strains of virus, inoculate ten doses of the vaccine into suitable cell cultures. If the vaccine contains an oil adjuvant, eliminate it by suitable means. Incubate at 38 ± 1 °C for 7 days. Make a passage on another set of cell cultures and incubate at 38 ± 1 °C for 7 days. The cultures show no signs of infection.
- C. For vaccine prepared with strains of virus not adapted to embryos or cell cultures, inject two doses intramuscularly into each of twenty 14- to 28-day-old chickens from flocks free from specified pathogens (5.2.2). 4 days later, kill ten of the chickens and remove the bursa of Fabricius from each chicken. Pool the bursae and homogenise in an equal volume of a suitable liquid. Inject 1 ml of the bursal homogenate into each of a further ten chickens of the same age and from the same source. Examine microscopically the bursa of Fabricius of each remaining chicken from the first group and of each chicken from the second group 21 days after the injection; there is no evidence of infectious avian bursal disease infection. In addition there are no signs of any disease attributable to the vaccine and no abnormal local reaction develops. The chickens of the second group do not have antibodies against infectious avian bursal disease virus when examined 21 days after the injection.

Extraneous agents. Collect serum samples from each of the birds used in the safety test or inactivation test C, three weeks after vaccination. Carry out tests for antibodies to the following agents by the methods prescribed for *chicken flocks free from specified pathogens for the production and quality control of vaccines* (5.2.2): avian encephalomyelitis virus, avian leucosis viruses, haemagglutinating avian adenovirus, infectious bronchitis virus, infectious laryngotracheitis virus, influenza A virus, Marek's disease virus, Newcastle disease virus. The vaccine does not stimulate the formation of antibodies against these agents.

Sterility. The vaccine complies with the test for sterility prescribed in the monograph on *Vaccines for veterinary use* (0062).

POTENCY

Vaccinate each of not fewer than ten chickens, 4 weeks of age and from a flock free from specified pathogens (5.2.2), with one dose of vaccine by one of the recommended routes. 4 to 6 weeks later, collect serum samples from each bird and ten unvaccinated control birds of the same age and from the same source. Measure the antibody response in a serum-neutralisation test. Include in the test a suitable standard, calibrated in Ph.Eur. Units against *infectious avian bursal disease serum BRP*. The mean antibody level in the sera from the vaccinated birds is not less than 10 000 Ph. Eur. Units per millilitre. There are no antibodies in the sera of the unvaccinated birds.

LABELLING

The label states whether the strain in the vaccine is embryo-adapted or cell-culture-adapted.

AVIAN INFECTIOUS BURSAL DISEASE VACCINE (LIVE)

Vaccinum bursitidis infectivae aviariae vivum

1. DEFINITION

Avian infectious bursal disease vaccine (live) [Gumboro disease vaccine (live)] is a preparation of a suitable strain of infectious bursal disease virus type 1. This monograph applies to vaccines intended for administration to chickens for active immunisation; it applies to vaccines containing strains of low virulence but not to those containing strains of higher virulence that may be needed for disease control in certain epidemiological situations.

2. PRODUCTION

2-1. PREPARATION OF THE VACCINE

The vaccine virus is grown in embryonated hens' eggs or in cell cultures.

2-2. SUBSTRATE FOR VIRUS PROPAGATION

2-2-1. Embryonated hens' eggs. If the vaccine virus is grown in embryonated hens' eggs, they are obtained from flocks free from specified pathogens (SPF) (5.2.2).

2-2-2. Cell cultures. If the vaccine virus is grown in cell cultures, they comply with the requirements for cell cultures for production of veterinary vaccines (5.2.4).

2-3. SEED LOTS

2-3-1. Extraneous agents. The master seed lot complies with the tests for extraneous agents in seed lots (2.6.24). In these tests on the master seed lot, the organisms used are not more than 5 passages from the master seed lot at the start of the tests.

2-4. CHOICE OF VACCINE VIRUS

The vaccine virus shall be shown to be satisfactory with respect to safety (5.2.6) and efficacy (5.2.7) for the chickens for which it is intended.

The following tests for safety (section 2-4-1), damage to the bursa of Fabricius (section 2-4-2), immunosuppression (section 2-4-3), increase in virulence (section 2-4-4) and immunogenicity (section 2-4-5) may be used during the demonstration of safety and immunogenicity.

2-4-1. Safety. Carry out the test for each route and method of administration to be recommended for vaccination using in each case chickens not older than the youngest age to be recommended for vaccination. Use vaccine virus at the least attenuated passage level that will be present between the master seed lot and a batch of the vaccine. For each test, use not fewer than 20 chickens from an SPF flock (5.2.2). Administer to each chicken a quantity of the vaccine virus equivalent to not less than 10 times the maximum virus titre likely to be contained in 1 dose of the vaccine. Observe the chickens at least daily for 21 days. The test is not valid if more than 10 per cent of the chickens die from causes not attributable to the vaccine virus. The vaccine virus complies with the test if no chicken shows notable clinical signs of avian infectious bursal disease or dies from causes attributable to the vaccine virus.

2-4-2. Damage to the bursa of Fabricius. Carry out the test for the route to be recommended for vaccination likely to be the least safe using chickens not older than the youngest age to be recommended for vaccination. Use virus at the least attenuated passage level that will be present between

the master seed lot and a batch of the vaccine. Use not fewer than 20 chickens from an SPF flock (5.2.2). Administer to each chicken a quantity of the vaccine virus equivalent to 10 times the maximum titre likely to be contained in a dose of the vaccine. On each of days 7, 14, 21 and 28 after administration of the vaccine virus, kill not fewer than 5 chickens and prepare a section from the site with the greatest diameters of the bursa of Fabricius of each chicken. Carry out histological examination of the section and score the degree of bursal damage using the following scale.

- | | |
|---|---|
| 0 | No lesion, normal bursa. |
| 1 | 1 per cent to 25 per cent of the follicles show lymphoid depletion (i.e. less than 50 per cent depletion in 1 affected follicle) influx of heterophils in lesions. |
| 2 | 26 per cent to 50 per cent of the follicles show nearly complete lymphoid depletion (i.e. more than 75 per cent depletion in 1 affected follicle), affected follicles show necrosis and severe influx of heterophils may be detected. |
| 3 | 51 per cent to 75 per cent of the follicles show lymphoid depletion; affected follicles show necrosis and severe influx of heterophils is detected. |
| 4 | 76 per cent to 100 per cent of the follicles show nearly complete lymphoid depletion, hyperplasia and cyst structures are detected; affected follicles show necrosis and severe influx of heterophils is detected. |
| 5 | 100 per cent of the follicles show nearly complete lymphoid depletion; complete loss of follicular structure, thickened and folded epithelium, fibrosis of bursal tissue. |

Calculate the average score for each group of chickens. The vaccine virus complies with the test if:

- no chicken shows notable clinical signs of disease or dies from causes attributable to the vaccine virus,
- the average score for bursal damage 21 days after administration of the vaccine virus is less than or equal to 2.0 and 28 days after administration is less than or equal to 0.6,
- during the 21 days after administration a notable repopulation of the bursae by lymphocytes has taken place.

2-4-3. Immunosuppression. Carry out the tests for the route recommended for vaccination likely to be the least safe using chickens not older than the youngest age recommended for vaccination. Use vaccine virus at the least attenuated passage level that will be present between the master seed lot and a batch of the vaccine. Use not fewer than 30 chickens from an SPF flock (5.2.2). Divide them randomly into 3 groups each of not fewer than 10 and maintain the groups separately. Administer by eye-drop to each chicken of 1 group a quantity of the vaccine virus equivalent to not less than the maximum titre likely to be contained in 1 dose of the vaccine. At the time after administration when maximal bursal damage is likely to be present, as judged from the results obtained in the test for damage to the bursa of Fabricius (section 2-4-2), administer to each vaccinated chicken and to each chicken of another group 1 dose of Hitchner B1 strain Newcastle disease vaccine (live). Determine the seroresponse of each chicken of the 2 groups to the Newcastle disease virus 14 days after administration. Challenge each chicken of the 3 groups by the intramuscular route with not less than 10^5 EID₅₀ of virulent Newcastle disease virus and note the degree of protection in the 2 groups vaccinated with

Hitchner B1 strain Newcastle vaccine compared with the non-vaccinated group. The test is not valid if 1 or more of the non-vaccinated chickens does not die within 7 days of challenge. The degree of immunosuppression is estimated from the comparative seroresponses and protection rates of the 2 Hitchner B1 vaccinated groups. The vaccine complies with the test if there is no significant difference between the 2 groups.

2-4-4. Increase in virulence. The test for increase in virulence consists of the administration of the vaccine virus at the least attenuated passage level that will be present between the master seed lot and a batch of the vaccine to a group of 5 chickens from an SPF flock (5.2.2) and not older than the youngest age to be recommended for vaccination, sequential passages, 5 times where possible, to further similar groups and testing of the final recovered virus for increase in virulence. If the properties of the vaccine virus allow sequential passage to 5 groups via natural spreading, this method may be used, otherwise passage as described below is carried out and the maximally passaged virus that has been recovered is tested for increase in virulence. Care must be taken to avoid contamination by virus from previous passages. Administer by eye-drop a quantity of the vaccine virus that will allow recovery of virus for the passages described below. Prepare 3 to 4 days after administration a suspension from the bursa of Fabricius of each chicken and pool these samples. Administer 0.05 ml of the pooled samples by eye-drop to each of 5 other chickens of the same age and origin. Carry out this passage operation not fewer than 5 times; verify the presence of the virus at each passage. If the virus is not found at a passage level, carry out a second series of passages. Carry out the test for damage to the bursa of Fabricius (section 2-4-2) using the unpassaged vaccine virus and the maximally passaged virus that has been recovered. Administer the virus by the route recommended for vaccination likely to be the least safe. The vaccine virus complies with the test if no indication of increasing virulence of the maximally passaged virus compared with the unpassaged virus is observed. If virus is not recovered at any passage level in the first and second series of passages, the vaccine virus also complies with the test.

2-4-5. Immunogenicity. A test is carried out for each route and method of administration to be recommended using in each case chickens not older than the youngest age to be recommended for vaccination. The quantity of vaccine virus administered to each chicken is not greater than the minimum virus titre to be stated on the label and the virus is at the most attenuated passage level that will be present in a batch of the vaccine. Use not fewer than 30 chickens of the same origin and from an SPF flock (5.2.2). Vaccinate by a recommended route not fewer than 20 chickens. Maintain not fewer than 10 chickens as controls. Challenge each chicken after 14 days by eye-drop with a sufficient quantity of virulent avian infectious bursal disease virus. Observe the chickens at least daily for 10 days after challenge. Record the deaths due to infectious bursal disease and the surviving chickens that show clinical signs of disease. At the end of the observation period, kill all the surviving chickens and carry out histological examination for lesions of the bursa of Fabricius. The test is not valid if one or more of the following applies:

- during the observation period following challenge, fewer than 50 per cent of the control chickens show characteristic signs of avian infectious bursal disease,
- 1 or more of the surviving control chickens does not show degree 3 lesions of the bursa of Fabricius,

- during the period between the vaccination and challenge more than 10 per cent of the vaccinated or control chickens show abnormal clinical signs or die from causes not attributable to the vaccine.

The vaccine virus complies with the test if during the observation period after challenge not fewer than 90 per cent of the vaccinated chickens survive and show no notable clinical signs of disease nor degree 3 lesions of the bursa of Fabricius.

3. BATCH TESTS

3-1. Identification. The vaccine, diluted if necessary and mixed with a monospecific infectious bursal disease virus type 1 antiserum, no longer infects embryonated hens' eggs from an SPF flock (5.2.2) or susceptible cell cultures (5.2.4) into which it is inoculated.

3-2. Bacteria and fungi

Vaccines intended for administration by injection comply with the test for sterility prescribed in the monograph *Vaccines for veterinary use (0062)*.

Vaccines not intended for administration by injection either comply with the test for sterility prescribed in the monograph *Vaccines for veterinary use (0062)* or with the following test: carry out a quantitative test for bacterial and fungal contamination; carry out identification tests for microorganisms detected in the vaccine; the vaccine does not contain pathogenic microorganisms and contains not more than 1 non-pathogenic microorganism per dose.

Any liquid supplied with the vaccine complies with the test for sterility prescribed in the monograph *Vaccines for veterinary use (0062)*.

3-3. Mycoplasmas. The vaccine complies with the test for mycoplasmas (2.6.7).

3-4. Extraneous agents. The vaccine complies with the tests for extraneous agents in batches of finished product (2.6.25).

3-5. Safety. Use not fewer than 10 chickens from an SPF flock (5.2.2) and of the youngest age recommended for vaccination. Administer by a recommended route and method to each chicken 10 doses of the vaccine. Observe the chickens at least daily for 21 days. The test is not valid if more than 20 per cent of the chickens show abnormal clinical signs or die from causes not attributable to the vaccine. The vaccine complies with the test if no chicken shows notable clinical signs of disease or dies from causes attributable to the vaccine.

3-6. Virus titre. Titrate the vaccine virus by inoculation into embryonated hens' eggs from an SPF flock (5.2.2) or into suitable cell cultures (5.2.4). The vaccine complies with the test if 1 dose contains not less than the minimum virus titre stated on the label.

3-7. Potency. The vaccine complies with the requirements of the test prescribed under Immunogenicity (section 2-4-5) when administered by a recommended route and method. It is not necessary to carry out the potency test for each batch of the vaccine if it has been carried out on a representative batch using a vaccinating dose containing not more than the minimum virus titre stated on the label.

01/2005:0588

AVIAN INFECTIOUS ENCEPHALOMYELITIS VACCINE (LIVE)

Vaccinum encephalomyelitidis infectivae aviariae vivum

1. DEFINITION

Avian infectious encephalomyelitis vaccine (live) is a preparation of a suitable strain of avian encephalomyelitis virus. This monograph applies to vaccines intended for administration to non-laying breeder chickens to protect passively their future progeny and/or to prevent vertical transmission of virus via the egg.

2. PRODUCTION

2-1. PREPARATION OF THE VACCINE

The vaccine virus is grown in embryonated hens' eggs or in cell cultures.

2-2. SUBSTRATE FOR VIRUS PROPAGATION

2-2-1. Embryonated hens' eggs. If the vaccine virus is grown in embryonated hens' eggs, they are obtained from flocks free from specified pathogens (SPF) (5.2.2).

2-2-2. Cell cultures. If the vaccine virus is grown in cell cultures, they comply with the requirements for cell cultures for production of veterinary vaccines (5.2.4).

2-3. SEED LOTS

2-3-1. Extraneous agents. The master seed lot complies with the tests for extraneous agents in seed lots (2.6.24). In these tests on the master seed lot, the organisms used are not more than 5 passages from the master seed lot at the start of the tests.

2-4. CHOICE OF VACCINE VIRUS

The vaccine virus shall be shown to be satisfactory with respect to safety (5.2.6) and efficacy (5.2.7) for the chickens for which it is intended.

The following tests for safety (section 2-4-1), increase in virulence (section 2-4-2) and immunogenicity (section 2-4-3) may be used during the demonstration of safety and immunogenicity.

2-4-1. Safety. Carry out the test for each route and method of administration to be recommended for vaccination using in each case non-laying breeder chickens not older than the youngest age to be recommended for vaccination. Use vaccine virus at the least attenuated passage level that will be present between the master seed lot and a batch of the vaccine. For each test, use not fewer than 20 chickens from an SPF flock (5.2.2). Administer to each chicken a quantity of the vaccine virus equivalent to not less than 10 times the maximum virus titre likely to be contained in 1 dose of the vaccine. Observe the chickens at least daily for 21 days. The test is not valid if more than 10 per cent of the chickens show abnormal clinical signs or die from causes not attributable to the vaccine virus. The vaccine virus complies with the test if no chicken shows notable clinical signs of avian infectious encephalomyelitis or dies from causes attributable to the vaccine virus.

2-4-2. Increase in virulence. The test for increase in virulence consists of the administration of the vaccine virus at the least attenuated passage level that will be present between the master seed lot and a batch of vaccine to a group of five 1-day-old chickens from an SPF flock (5.2.2), sequential passages, 5 times where possible, to further

similar groups of 1-day-old chickens, and testing of the final recovered virus for increase in virulence. If the properties of the vaccine virus allow sequential passage to 5 groups via natural spreading, this method may be used, otherwise passage as described below is carried out and the maximally passaged virus that has been recovered is tested for increase in virulence. Care must be taken to avoid contamination by virus from previous passages. Administer by a recommended route and method, a quantity of the vaccine virus that will allow recovery of virus for the passages described below. 5 to 7 days later, prepare a suspension from the brain of each chick and pool these samples. Administer a suitable volume of the pooled samples by the oral route to each of 5 other chickens of the same age and origin. Carry out this passage operation not fewer than 5 times; verify the presence of the virus at each passage. If the virus is not found at a passage level, carry out a second series of passages. Carry out the test for safety (section 2-4-1) using the unpassaged vaccine virus and the maximally passaged vaccine virus that has been recovered. The vaccine virus complies with the test if no indication of increase in virulence of the maximally passaged virus compared with the unpassaged virus is observed. If virus is not recovered at any passage level in the first and second series of passages, the vaccine virus also complies with the test.

2-4-3. Immunogenicity. If the vaccine is recommended for passive protection of future progeny carry out test 2-4-3-1. If the vaccine is recommended for prevention of vertical transmission of virus via the egg, carry out test 2-4-3-2. A test is carried out for each route and method of administration to be recommended, using in each case chickens from an SPF flock (5.2.2) not older than the youngest age to be recommended for vaccination. The quantity of the vaccine virus administered to each chicken is not greater than the minimum titre to be stated on the label and the virus is at the most attenuated passage level that will be present in a batch of the vaccine.

2-4-3-1. Passive immunity in chickens. Vaccinate not fewer than 20 breeder chickens from an SPF flock (5.2.2). Maintain separately not fewer than 10 breeder chickens of the same age and origin as controls. At the peak of lay, hatch not fewer than 25 chickens from eggs from vaccinated breeder chickens and 10 chickens from non-vaccinated breeder chickens. At 2 weeks of age, challenge each chicken by the intracerebral route with a sufficient quantity of virulent avian encephalomyelitis virus. Observe the chickens at least daily for 21 days after challenge. Record the deaths and the number of surviving chickens that show clinical signs of disease. The test is not valid if:

- during the observation period after challenge fewer than 80 per cent of the control chickens die or show severe clinical signs of avian infectious encephalomyelitis,
- and/or during the period between the vaccination and challenge more than 15 per cent of control or vaccinated chickens show abnormal clinical signs or die from causes not attributable to the vaccine.

The vaccine virus complies with the test if during the observation period after challenge not fewer than 80 per cent of the progeny of vaccinated chickens survive and show no notable clinical signs of disease.

2-4-3-2. Passive immunity in embryos. Vaccinate not fewer than 20 breeder chickens from an SPF flock (5.2.2). Maintain separately not fewer than 10 breeder chickens of the same age and origin as controls. At the peak of lay, incubate

not fewer than 36 eggs from the 2 groups, vaccinated and controls, and carry out an embryo sensitivity test. On the sixth day of incubation inoculate 100 EID₅₀ of the Van Roekel strain of avian encephalomyelitis virus into the yolk sacs of the eggs. 12 days after inoculation examine the embryos for specific lesions of avian encephalomyelitis (muscular atrophy). Deaths during the first 24 h are considered to be non-specific. The test is not valid if fewer than 80 per cent of the control embryos show lesions of avian encephalomyelitis. The test is not valid if fewer than 80 per cent of the embryos can be given an assessment. The vaccine virus complies with the test if not fewer than 80 per cent of the embryos in the vaccinated group show no lesions of avian encephalomyelitis.

3. BATCH TESTS

3-1. Identification. The vaccine, diluted if necessary and mixed with a monospecific avian encephalomyelitis virus antiserum, no longer infects embryonated hens' eggs from an SPF flock (5.2.2) or susceptible cell cultures (5.2.4) into which it is inoculated.

3-2. Bacteria and fungi

Vaccines intended for administration by injection comply with the test for sterility prescribed in the monograph *Vaccines for veterinary use* (0062).

Vaccines not intended for administration by injection either comply with the test for sterility prescribed in the monograph *Vaccines for veterinary use* (0062) or with the following test: carry out a quantitative test for bacterial and fungal contamination; carry out identification tests for microorganisms detected in the vaccine; the vaccine does not contain pathogenic microorganisms and contains not more than 1 non-pathogenic microorganism per dose.

Any liquid supplied with the vaccine complies with the test for sterility prescribed in the monograph *Vaccines for veterinary use* (0062).

3-3. Mycoplasmas. The vaccine complies with the test for mycoplasmas (2.6.7).

3-4. Extraneous agents. The vaccine complies with the tests for extraneous agents in batches of finished product (2.6.25).

3-5. Safety. Use not fewer than 10 chickens not older than the minimum age recommended for vaccination and from an SPF flock (5.2.2). Administer by a recommended route and method to each chicken 10 doses of the vaccine. Observe the chickens at least daily for 21 days. The test is not valid if more than 20 per cent of the chickens show abnormal clinical signs or die from causes not attributable to the vaccine. The vaccine complies with the test if no chicken shows notable clinical signs of disease or dies from causes attributable to the vaccine.

3-6. Virus titre. Titrate the vaccine virus by inoculation into embryonated hens' eggs from an SPF flock (5.2.2) or into suitable cell cultures (5.2.4). The vaccine complies with the test if 1 dose contains not less than the minimum virus titre stated on the label.

3-7. Potency. Depending on the indications, the vaccine complies with the requirements of 1 or both of the tests prescribed under Immunogenicity (section 2-4-3-1, 2-4-3-2), when administered by a recommended route and method. It is not necessary to carry out the potency test for each batch of the vaccine if it has been carried out on a representative batch using a vaccinating dose containing not more than the minimum virus titre stated on the label.

01/2005:1068

AVIAN INFECTIOUS LARYNGOTRACHEITIS VACCINE (LIVE)

Vaccinum laryngotracheitidis infectivae aviariae vivum

1. DEFINITION

Avian infectious laryngotracheitis vaccine (live) is a preparation of a suitable strain of avian infectious laryngotracheitis virus (gallid herpesvirus 1). This monograph applies to vaccines intended for administration to chickens for active immunisation.

2. PRODUCTION

2-1. PREPARATION OF THE VACCINE

The vaccine virus is grown in embryonated hens' eggs or in cell cultures.

2-2. SUBSTRATE FOR VIRUS PROPAGATION

2-2-1. Embryonated hens' eggs. If the vaccine virus is grown in embryonated hens' eggs, they are obtained from flocks free from specified pathogens (SPF) (5.2.2).

2-2-2. Cell cultures. If the vaccine virus is grown in cell cultures, they comply with the requirements for cell cultures for production of veterinary vaccines (5.2.4).

2-3. SEED LOTS

2-3-1. Extraneous agents. The master seed lot complies with the tests for extraneous agents in seed lots (2.6.24). In these tests on the master seed lot, the organisms used are not more than 5 passages from the master seed lot at the start of the tests.

2-4. CHOICE OF VACCINE VIRUS

The vaccine virus shall be shown to be satisfactory with respect to safety (5.2.6) and efficacy (5.2.7) for the chickens for which it is intended.

The following tests for index of respiratory virulence (section 2-4-1), safety (section 2-4-2), increase in virulence (section 2-4-3) and immunogenicity (section 2-4-4) may be used during the demonstration of safety and immunogenicity.

2-4-1. Index of respiratory virulence. Use for the test not fewer than sixty 10-day-old chickens from an SPF flock (5.2.2). Divide them randomly into 3 groups, maintained separately. Prepare 2 tenfold serial dilutions starting from a suspension of the vaccine virus having a titre of 10^5 EID₅₀ or 10^5 CCID₅₀ per 0.2 ml or, if not possible, having the maximum attainable titre. Use vaccine virus at the least attenuated passage level that will be present in a batch of the vaccine. Allocate the undiluted virus suspension and the 2 virus dilutions each to a different group of chickens. Administer by the intratracheal route to each chicken 0.2 ml of the virus suspension attributed to its group. Observe the chickens for 10 days after administration and record the number of deaths. The index of respiratory virulence is the total number of deaths in the 3 groups divided by the total number of chickens. The vaccine virus complies with the test if its index of respiratory virulence is not greater than 0.33.

2-4-2. Safety. Carry out the test for each route and method of administration to be recommended for vaccination, using in each case chickens not older than the youngest age to be recommended for vaccination. Use vaccine virus at the least attenuated passage level that will be present between

the master seed lot and a batch of the vaccine. For each test use not fewer than 20 chickens, from an SPF flock (5.2.2). Administer to each chicken a quantity of the vaccine virus equivalent to not less than 10 times the maximum virus titre likely to be contained in 1 dose of the vaccine. Observe the chickens at least daily for 21 days. The test is not valid if more than 10 per cent of the chickens die from causes not attributable to the vaccine virus. The vaccine virus complies with the test if no chicken shows notable clinical signs of avian infectious laryngotracheitis or dies from causes attributable to the vaccine virus.

2-4-3. Increase in virulence. The test for increase in virulence consists of the administration of the vaccine virus at the least attenuated passage level that will be present between the master seed lot and a batch of the vaccine to a group of 5 chickens not more than 2 weeks old, from an SPF flock (5.2.2), sequential passages, 5 times where possible, to further similar groups and testing of the final recovered virus for increase in virulence. If the properties of the vaccine virus allow sequential passage to 5 groups via natural spreading, this method may be used, otherwise passage as described below is carried out and the maximally passaged virus that has been recovered is tested for increase in virulence. Care must be taken to avoid contamination by virus from previous passages. Administer by eye-drop a quantity of the vaccine virus that will allow recovery of virus for the passages described below. After the period shown to correspond to maximum replication of the virus, prepare a suspension from the mucosae of suitable parts of the respiratory tract of each chicken and pool these samples. Administer 0.05 ml of the pooled samples by eye-drop to each of 5 other chickens that are 2 weeks old and from an SPF flock (5.2.2). Carry out this passage operation not fewer than 5 times; verify the presence of the virus at each passage. If the virus is not found at a passage level, carry out a second series of passages. Determine the index of respiratory virulence (section 2-4-1) using the unpassaged vaccine virus and the maximally passaged virus that has been recovered; if the titre of the maximally passaged virus is less than 10^5 EID₅₀ or 10^5 CCID₅₀, prepare the tenfold, serial dilutions using the highest titre available. The vaccine virus complies with the test if no indication of increase in virulence of the maximally passaged virus compared with the unpassaged virus is observed. If virus is not recovered at any passage level in the first and second series of passages, the vaccine virus also complies with the test.

2-4-4. Immunogenicity. A test is carried out for each route and method of administration to be recommended using in each case chickens not older than the youngest age to be recommended for vaccination. The quantity of the vaccine virus administered to each chicken is not greater than the minimum virus titre to be stated on the label and the virus is at the most attenuated passage level that will be present in a batch of the vaccine. Use for the test not fewer than 30 chickens of the same origin and from an SPF flock (5.2.2). Vaccinate by a recommended route not fewer than 20 chickens. Maintain not fewer than 10 chickens as controls. Challenge each chicken after 21 days by the intratracheal route with a sufficient quantity of virulent infectious laryngotracheitis virus. Observe the chickens at least daily for 7 days after challenge. Record the deaths and the number of surviving chickens that show clinical signs of disease. At the end of the observation period kill all the surviving chickens and carry out examination for macroscopic lesions: mucoid, haemorrhagic and pseudomembranous inflammation of the trachea and orbital sinuses. The test is not valid if:

01/2005:1392

- during the observation period after challenge fewer than 90 per cent of the control chickens die or show severe clinical signs of avian infectious laryngotracheitis or notable macroscopic lesions of the trachea and orbital sinuses,
- or if during the period between the vaccination and challenge more than 10 per cent of the vaccinated or control chickens show notable clinical signs of disease or die from causes not attributable to the vaccine.

The vaccine virus complies with the test if during the observation period after challenge not fewer than 90 per cent of the vaccinated chickens survive and show no notable clinical signs of disease and/or macroscopical lesions of the trachea and orbital sinuses.

3. BATCH TESTS

3-1. Identification. The vaccine, diluted if necessary and mixed with a monospecific infectious laryngotracheitis virus antiserum, no longer infects embryonated hens' eggs from an SPF flock (5.2.2) or susceptible cell cultures (5.2.4) into which it is inoculated.

3-2. Bacteria and fungi

Vaccines intended for administration by injection comply with the test for sterility prescribed in the monograph *Vaccines for veterinary use* (0062).

Vaccines not intended for administration by injection either comply with the test for sterility prescribed in the monograph *Vaccines for veterinary use* (0062) or with the following test: carry out a quantitative test for bacterial and fungal contamination; carry out identification tests for micro-organisms detected in the vaccine; the vaccine does not contain pathogenic micro-organisms and contains not more than 1 non-pathogenic micro-organism per dose.

Any liquid supplied with the vaccine complies with test for sterility in the monograph *Vaccines for veterinary use* (0062).

3-3. Mycoplasmas. The vaccine complies with the test for mycoplasmas (2.6.7).

3-4. Extraneous agents. The vaccine complies with the tests for extraneous agents in batches of finished product (2.6.25).

3-5. Safety. Use not fewer than 10 chickens from an SPF flock (5.2.2) and of the youngest age recommended for vaccination. Administer by eye-drop to each chicken 10 doses of the vaccine. Observe the chickens at least daily for 21 days. The test is not valid if more than 20 per cent of the chickens show abnormal clinical signs or die from causes not attributable to the vaccine. The vaccine complies with the test if no chicken shows notable clinical signs of disease or dies from causes attributable to the vaccine.

3-6. Virus titre. Titrate the vaccine virus by inoculation into embryonated hens' eggs from an SPF flock (5.2.2) or into suitable cell cultures (5.2.4). The vaccine complies with the test if 1 dose contains not less than the minimum titre stated on the label.

3-7. Potency. The vaccine complies with the requirements of the test prescribed under Immunogenicity (section 2-4-4) when administered according to the recommended schedule by a recommended route and method. It is not necessary to carry out the potency test for each batch of the vaccine if it has been carried out on a representative batch using a vaccinating dose containing not more than the minimum virus titre stated on the label.

AVIAN PARAMYXOVIRUS 3 VACCINE (INACTIVATED)

Vaccinum paramyxoviris 3 aviarii inactivatum

DEFINITION

Avian paramyxovirus 3 vaccine (inactivated) consists of an emulsion or a suspension of a suitable strain of avian paramyxovirus 3 that has been inactivated in such a manner that immunogenic activity is retained. The vaccine is used for protection against loss in egg production and egg quality in turkeys.

PRODUCTION

The virus is propagated in embryonated eggs from healthy flocks or in suitable cell cultures (5.2.4).

The test for inactivation is carried out in embryonated eggs or suitable cell cultures and the quantity of inactivated virus used is equivalent to not less than ten doses of vaccine. No live virus is detected.

The vaccine may contain an adjuvant.

CHOICE OF VACCINE COMPOSITION

The vaccine is shown to be satisfactory with respect to safety (5.2.6) and immunogenicity (5.2.7) for each category of turkeys for which it is intended. The following test may be used during demonstration of immunogenicity.

Immunogenicity. The test described under Potency is suitable for demonstrating immunogenicity.

BATCH TESTING

Batch potency test. Carry out a suitable validated test for which satisfactory correlation with the test described under Potency has been established, the criteria for acceptance being set with reference to a batch that has given satisfactory results in the latter test.

IDENTIFICATION

When injected into animals free from antibodies against avian paramyxovirus 3, the vaccine stimulates the production of such antibodies.

TESTS

Safety. Inject twice the vaccinating dose by a recommended route into each of ten turkeys, 14 to 28 days old and free from antibodies against avian paramyxovirus 3. Observe the birds for 21 days. No abnormal local or systemic reaction occurs.

Inactivation. Inject two-fifths of a dose into the allantoic cavity of each of ten embryonated hen eggs, 9 to 11 days old, from flocks free from specified pathogens (5.2.2) (SPF eggs) and incubate. Observe for 6 days and pool separately the allantoic fluid from eggs containing live embryos, and that from eggs containing dead embryos, excluding those dying within 24 h of the injection. Examine embryos that die within 24 h of injection for the presence of avian paramyxovirus 3: the vaccine does not comply with the test if avian paramyxovirus 3 is found.

Inject into the allantoic cavity of each of ten SPF eggs, 9 to 11 days old, 0.2 ml of the pooled allantoic fluid from the live embryos and, into each of ten similar eggs, 0.2 ml of the pooled fluid from the dead embryos and incubate for 5 to 6 days. Test the allantoic fluid from each egg for the presence of haemagglutinins using chicken erythrocytes.

The vaccine complies with the test if there is no evidence of haemagglutinating activity and if not more than 20 per cent of the embryos die at either stage. If more than 20 per cent of the embryos die at one of the stages, repeat that stage; the vaccine complies with the test if there is no evidence of haemagglutinating activity and not more than 20 per cent of the embryos die at that stage.

Antibiotics may be used in the test to control extraneous bacterial infection.

Extraneous agents. Inject a double dose by a recommended route into each of ten chickens, 14 to 28 days old and from a flock free from specified pathogens (5.2.2). After 3 weeks, inject one dose by the same route. Collect serum samples from each chicken 2 weeks later and carry out tests for antibodies against the following agents by the methods prescribed for chicken flocks free from specified pathogens (5.2.2): avian encephalomyelitis virus, avian infectious bronchitis virus, avian leucosis viruses, egg-drop syndrome virus, avian bursal disease virus, avian infectious laryngotracheitis virus, influenza A virus, Marek's disease virus. The vaccine does not stimulate the formation of antibodies against these agents.

Sterility. The vaccine complies with the test for sterility prescribed in the monograph on *Vaccines for veterinary use* (0062).

POTENCY

Use two groups each of not fewer than twenty turkeys free from antibodies against avian paramyxovirus 3. Vaccinate one group in accordance with the recommendations for use. Keep the other group as controls. The test is invalid if serological tests carried out on serum samples obtained at the time of first vaccination show the presence of antibodies against avian paramyxovirus 3 in either vaccinates or controls or if tests carried out at the time of challenge show such antibodies in controls. At the egg-production peak, challenge the two groups by the oculo-nasal route with a sufficient quantity of a virulent strain of avian paramyxovirus 3. For not less than 6 weeks after challenge, record the number of eggs laid weekly for each group, distinguishing between normal and abnormal eggs. The vaccine complies with the test if egg production and quality are significantly better in the vaccinated group than in the control group.

01/2005:1956

AVIAN VIRAL TENOSYNOVITIS VACCINE (LIVE)

Vaccinum tenosynovitis viralis aviariae vivum

1. DEFINITION

Avian viral tenosynovitis vaccine (live) is a preparation of a suitable strain of avian tenosynovitis virus (avian orthoreovirus). This monograph applies to vaccines intended for administration to chickens for active immunisation.

2. PRODUCTION

2-1. PREPARATION OF THE VACCINE

The vaccine virus is grown in cell cultures.

2-2. SUBSTRATE FOR VIRUS PROPAGATION

2-2-1. Cell cultures. Cell cultures comply with the requirements for cell cultures for production of veterinary vaccines (5.2.4).

2-3. SEED LOTS

2-3-1. Extraneous agents. The master seed lot complies with the tests for extraneous agents in seed lots (2.6.24). In these tests on the master seed lot, the organisms used are not more than 5 passages from the master seed lot at the start of the tests.

2-4. CHOICE OF VACCINE VIRUS

The vaccine virus shall be shown to be satisfactory with respect to safety (5.2.6) and efficacy (5.2.7) for the chickens for which it is intended.

The following tests for safety (section 2-4-1), increase in virulence (section 2-4-2) and immunogenicity (section 2-4-3) may be used during the demonstration of safety and immunogenicity.

2-4-1. Safety. Carry out the test for each route and method of administration to be recommended for vaccination using in each case chickens not older than the youngest age to be recommended for vaccination. Use vaccine virus at the least attenuated passage level that will be present between the master seed lot and a batch of the vaccine. For each test use not fewer than 20 chickens, from an SPF flock (5.2.2). Administer to each chicken a quantity of the vaccine virus not less than 10 times the maximum virus titre likely to be contained in 1 dose of the vaccine. Observe the chickens at least daily for 21 days. Carry out histological examination of the joints and tendon sheaths of the legs and feet at the end of the observation period (as a basis for comparison in the test for increase in virulence). The test is not valid if more than 10 per cent of the chickens die from causes not attributable to the vaccine virus. The vaccine virus complies with the test if no chicken shows notable clinical signs of avian viral tenosynovitis or dies from causes attributable to the vaccine virus.

2-4-2. Increase in virulence. The test for increase in virulence consists of the administration of the vaccine virus at the least attenuated passage level that will be present between the master seed lot and a batch of the vaccine to a group of five 1-day-old chicks from an SPF flock (5.2.2), sequential passages, 5 times where possible, to further groups of 1-day-old chicks and testing of the final recovered virus for increase in virulence. If the properties of the vaccine virus allow sequential passage to 5 groups via natural spreading, this method may be used, otherwise passage as described below is carried out and the maximally passaged virus that has been recovered is tested for increase in virulence. Care must be taken to avoid contamination by virus from previous passages. Administer by a suitable route a quantity of the vaccine virus that will allow recovery of virus for the passages described below. Kill the chickens at the moment when the virus concentration in the most suitable material (for example, tendons, tendon sheaths and liquid exudates from the hock joints, spleen) is sufficient. Prepare a suspension from this material from each chicken and pool these samples. Administer 0.1 ml of the pooled samples by the route of administration most likely to lead to increase in virulence to each of 5 other chickens of the same age and origin. Carry out this passage operation not fewer than 5 times; verify the presence of the virus at each passage. If the virus is not found at a passage level, carry out a second series of passages. Carry out the test for safety (section 2-4-1) using the unpassaged vaccine virus and the maximally passaged vaccine virus that has been recovered. The vaccine virus complies with the test if no indication of increase in virulence of the maximally passaged virus compared with the unpassaged virus is observed. If the virus is not recovered at any passage level in the first and second series of passages, the vaccine virus also complies with the test.

2-4-3. **Immunogenicity.** A test is carried out for each route and method of administration to be recommended using in each case chickens not older than the youngest age to be recommended for vaccination. The quantity of the vaccine virus administered to each chicken is not greater than the minimum virus titre to be stated on the label and the virus is at the most attenuated passage level that will be present in a batch of the vaccine. Use not fewer than 30 chickens of the same origin and from an SPF flock (5.2.2). Administer the vaccine by a recommended route to not fewer than 20 chickens. Maintain not fewer than 10 chickens as controls. Challenge each chicken after 21 days by a suitable route with a sufficient quantity of virulent avian tenosynovitis virus. Observe the chickens at least daily for 21 days after challenge. Record the deaths and the surviving chickens that show clinical signs of disease. If the challenge is administered by the foot pad, any transient swelling of the foot pad during the first 5 days after challenge may be considered non-specific. At the end of the observation period, kill all the surviving chickens and carry out macroscopic and/or microscopic examination for lesions of the joints and tendon sheaths of the legs and feet, e.g. exudate and swelling. The test is not valid if:

- during the observation period after challenge fewer than 80 per cent of the control chickens die or show severe clinical signs of avian viral tenosynovitis or show macroscopical and/or microscopical lesions in the joints and tendon sheaths of the legs and feet,
- or if during the period between vaccination and challenge more than 10 per cent of the control or vaccinated chickens show abnormal clinical signs or die from causes not attributable to the vaccine.

The vaccine virus complies with the test if during the observation period after challenge not fewer than 90 per cent of the vaccinated chickens survive and show no notable clinical signs of disease or show macroscopical and/or microscopical lesions in the joints and tendon sheaths of the legs and feet.

3. BATCH TESTS

3-1. **Identification.** Carry out an immunostaining test in cell cultures to identify the vaccine virus.

3-2. Bacteria and fungi

Vaccines intended for administration by injection comply with the test for sterility prescribed in the monograph *Vaccines for veterinary use* (0062).

Vaccines not intended for administration by injection either comply with the test for sterility prescribed in the monograph *Vaccines for veterinary use* (0062) or with the following test: carry out a quantitative test for bacterial and fungal contamination; carry out identification tests for microorganisms detected in the vaccine; the vaccine does not contain pathogenic microorganisms and contains not more than 1 non-pathogenic microorganism per dose.

Any liquid supplied with the vaccine complies with the test for sterility prescribed in the monograph *Vaccines for veterinary use* (0062).

3-3. **Mycoplasmas.** The vaccine complies with the test for mycoplasmas (2.6.7).

3-4. **Extraneous agents.** The vaccine complies with the tests for extraneous agents in batches of finished product (2.6.25).

3-5. **Safety.** Use not fewer than 10 chickens from an SPF flock (5.2.2) and of the youngest age recommended for vaccination. Administer by a recommended route and method to each chicken 10 doses of the vaccine. Observe the chickens at least daily for 21 days. The test is not valid if more than 20 per cent of the chickens show abnormal

clinical signs or die from causes not attributable to the vaccine. The vaccine complies with the test if no chicken shows notable clinical signs of disease or dies from causes attributable to the vaccine.

3-6. **Virus titre.** Titrate the vaccine virus by inoculation into suitable cell cultures (5.2.4). The vaccine complies with the test if 1 dose contains not less than the minimum virus titre stated on the label.

3-7. **Potency.** The vaccine complies with the requirements of the test prescribed under Immunogenicity (section 2-4-3) when administered by a recommended route and method. It is not necessary to carry out the potency test for each batch of the vaccine if it has been carried out on a representative batch using a vaccinating dose containing not more than the minimum virus titre stated on the label.

01/2005:1939

BOVINE LEPTOSPIROSIS VACCINE (INACTIVATED)

Vaccinum leptospirosis bovine inactivatum

DEFINITION

Bovine leptospirosis vaccine (inactivated) is a suspension of inactivated whole organisms and/or antigenic extract(s) of one or more suitable strains of one or more of *Leptospira borgpetersenii* serovar hardjo, *Leptospira interrogans* serovar hardjo or other *L. interrogans* serovars, inactivated and prepared in such a way that adequate immunogenicity is maintained. This monograph applies to vaccines intended for active immunisation of cattle against leptospirosis.

PRODUCTION

The seed material is cultured in a suitable medium; each strain is cultivated separately. During production, various parameters such as growth rate are monitored by suitable methods; the values are within the limits approved for the particular product. Purity and identity are verified on the harvest using suitable methods. After cultivation, the bacterial harvest is inactivated by a suitable method. The antigen may be concentrated. The vaccine may contain an adjuvant.

CHOICE OF VACCINE COMPOSITION

The vaccine is shown to be satisfactory with respect to safety (5.2.6) and efficacy (5.2.7) in cattle. As part of the studies to demonstrate the suitability of the vaccine with respect to these characteristics the following tests may be carried out.

Safety

A. The test is carried out for each route of administration to be stated on the label and in animals of each category (for example, young calves, pregnant cattle) for which the vaccine is intended. For each test, use not fewer than 10 animals that do not have antibodies against *L. borgpetersenii* serovar hardjo and the principal serovars of *L. interrogans* (icterohaemorrhagiae, canicola, grippotyphosa, sejroe, hardjo, hebdomonadis, pomona, australis and autumnalis). Use a batch of vaccine containing not less than the maximum potency that may be expected in a batch of vaccine. Administer to each animal a double dose of vaccine. If the recommended schedule requires a second dose, administer 1 dose after the recommended interval. Observe the animals for at least 14 days after the last administration. Record body temperatures the day before each vaccination, at vaccination, 4 h later and daily for 4 days. If the vaccine is intended for use or may be used in pregnant cattle,

vaccinate the animals at the relevant stages of pregnancy and prolong the observation period until 1 day after calving. The vaccine complies with the test if no animal shows an abnormal local or systemic reaction or clinical signs of disease or dies from a cause attributable to the vaccine. In addition, if the vaccine is for use in pregnant animals, no adverse effects on the pregnancy and offspring are noted.

- B. The animals used for the field trials are also used to evaluate safety. Use not fewer than 3 groups of 20 animals with corresponding groups of not fewer than 10 controls in 3 different locations. Examine the injection sites for local reactions after vaccination. Record body temperatures the day before vaccination, at vaccination and on the 2 days following vaccination. The vaccine complies with the test if no animal shows an abnormal local or systemic reaction or clinical signs of disease or dies from a cause attributable to the vaccine. In addition, if the vaccine is for use in pregnant animals, no adverse effects on the pregnancy and offspring are noted.

Immunogenicity. As part of the studies to demonstrate the suitability of the vaccine with respect to immunogenicity and in support of the claims for a beneficial effect on the rates of infection and urinary excretion, the test described under Potency may be carried out for each proposed route of administration, using vaccine of minimum potency. Urine samples are collected from each animal on days 0, 14, 21, 28 and 35 post-challenge. For leptospiral species other than *L. borgpetersenii* serovar hardjo, appropriate days are determined by the characteristics of the challenge model. In the case of other serovars for which there is published evidence that the serovar has a lower tropism for the urinary tract, a lower rate of infection may be justified. Depending on their tissue tropism, for some leptospira serovars, samples from other tissues/body fluids can be used to establish whether the animals are infected or not by the challenge organism. If claims are to be made for protection against reproductive or production losses, further specific studies will be required.

BATCH POTENCY TEST

The test described under Potency is not carried out for routine testing of batches of vaccine. It is carried out, for a given vaccine, on one or more occasions, as decided by or with the agreement of the competent authority; where the test is not carried out, a suitable validated test is carried out, the criteria for acceptance being set with reference to a batch of vaccine that has given satisfactory results in the test described under Potency. The following test may be used after a suitable correlation with the test described under Potency has been established.

For each of the serovars for which protection is claimed, the antibody response from vaccinated animals is measured. Use guinea pigs weighing 250-350 g which do not have antibodies to *L. borgpetersenii* serovar hardjo and the principal serovars of *L. interrogans* (icterohaemorrhagiae, canicola, grippotyphosa, sejroe, hardjo, hebdomonadis, pomona, australis and autumnalis) and which have been obtained from a regularly tested and certified leptospira-free source. The dose to be administered to the animals is that fraction of a cattle dose which has been shown in the validation studies to provide a suitably sensitive test. Vaccinate each of 10 animals with the suitable dose. Maintain not fewer than 2 guinea-pigs as unvaccinated controls. At a given interval within the range of 19 to 23 days after the injection, collect blood from each animal and prepare serum samples. Use a suitable validated method such as a micro-agglutination test to measure the antibody responses in each sample. The

antibody levels are equal to or greater than those obtained with a batch that has given satisfactory results in the test described under Potency and there is no significant increase in antibody titre in the controls.

IDENTIFICATION

When injected into healthy seronegative animals, the vaccine stimulates the production of specific antibodies to the leptospira serovar(s) present in the vaccine.

TESTS

Safety. For vaccines recommended for use in cattle older than 6 months of age, use cattle not older than the minimum age recommended for vaccination and not younger than 6 months of age. For vaccines recommended for use in cattle less than 6 months of age, use cattle of the minimum age recommended for vaccination. Inject 2 doses of the vaccine into each of 2 cattle, free from specific antibodies to the leptospira serovar(s) present in the vaccine, by a recommended route. Observe the animals for 14 days. The animals remain in good health and no abnormal local or systemic reaction occurs.

Inactivation. Carry out a test for live leptospirae by inoculation of a specific medium. Inoculate 1 ml of the vaccine into 100 ml of the medium. Incubate at 30 °C for 14 days, subculture into a further quantity of the medium and incubate both media at 30 °C for 14 days: no growth occurs in either medium. At the same time, carry out a control test by inoculating a further quantity of the medium with the vaccine together with a quantity of a culture containing approximately 100 leptospirae and incubating at 30 °C: growth of leptospirae occurs within 14 days.

Sterility. The vaccine complies with the test for sterility prescribed in the monograph *Vaccines for veterinary use* (0062).

POTENCY

Carry out a separate test for each of the serovars for which a claim is made for a beneficial effect on the rates of infection and urinary excretion.

Use not fewer than 15 cattle of the minimum age recommended for vaccination and free from specific antibodies against *L. borgpetersenii* serovar hardjo and the principal serovars of *L. interrogans* (icterohaemorrhagiae, canicola, grippotyphosa, sejroe, hardjo, hebdomonadis, pomona, australis and autumnalis). Vaccinate not fewer than 10 animals by a recommended route and according to the recommended schedule. Keep not fewer than 5 animals as controls. 21 days after the last vaccination, infect all the animals by a suitable mucosal route with a suitable quantity of a virulent strain of the relevant serovar. Observe the animals for a further 35 days. Collect urine samples from each animal on days 0, 14, 21, 28 and 35 post-challenge. Kill surviving animals at the end of the observation period. Carry out post-mortem examination on any animal that dies and on those killed at the end of the observation period. In particular, examine the kidneys for macroscopic and microscopic signs of leptospira infection. A sample of each kidney is collected and each urine and kidney sample is tested for the presence of the challenge organisms by re-isolation or by another suitable method.

For the test conducted with *L. borgpetersenii* serovar hardjo, control animals are regarded as infected if the challenge organisms are re-isolated from at least 2 samples. The test is invalid if infection has been established in fewer than 80 per cent of the control animals.

The vaccine complies with the requirements of the test if the challenge organisms are re-isolated from any urine or kidney sample from not more than 20 per cent of the vaccinated animals.

LABELLING

The label states:

- the serovar(s) used to prepare the vaccine,
- the serovar(s) against which protection is claimed.

01/2005:1176

BOVINE PARAINFLUENZA VIRUS VACCINE (LIVE), FREEZE-DRIED

Vaccinum parainfluenzae viri bovini vivum cryodesiccatum

DEFINITION

Freeze-dried bovine parainfluenza virus vaccine (live) is a preparation of a suitable strain of bovine parainfluenza 3 virus.

PRODUCTION

The vaccine strain is grown in suitable cell cultures (5.2.4). The viral suspension is harvested, mixed with a suitable stabilising liquid and freeze-dried.

CHOICE OF VACCINE STRAIN

Only a virus strain shown to be satisfactory with respect to reversion to virulence, safety and immunogenicity may be used in the preparation of the vaccine. The following tests may be used during demonstration of safety (5.2.6) and efficacy (5.2.7).

Reversion to virulence. Administer by the intranasal route to 2 susceptible calves that do not have antibodies against bovine parainfluenza virus 3 a quantity of virus that will allow optimal re-isolation of the virus for subsequent passages. On each of days 3 to 7 after administration of the virus, take nasal swabs from each calf and collect in not more than 5 ml of a suitable medium which is then used to inoculate cell cultures to verify the presence of virus; use about 1 ml of the suspensions from swabs that contain the maximum amount of virus, as indicated by the titration in cell cultures, to inoculate 2 other calves of the same age and sensitivity; repeat these operations until 5 passages on calves have been carried out. No calf shows clinical signs attributable to the vaccinal virus. No indication of increase of virulence, compared to the original vaccinal virus is observed; account is taken of the titre of excreted virus in the nasal swabs.

Safety. The test is carried out for each recommended route of administration. Use calves of the minimum age recommended for vaccination and preferably having no antibodies against bovine parainfluenza 3 virus or, where justified, use calves with a very low level of such antibodies as long as they have not been vaccinated against bovine parainfluenza virus and administration of the vaccine does not cause an anamnestic response. Administer to 5 calves a quantity of virus corresponding to not less than 10 times the maximum virus titre that may be expected in a batch of vaccine. Observe the animals for 21 days. Measure the body temperature of each animal on the day before vaccination, at the time of vaccination and for the 4 subsequent days. No abnormal effect on body temperature and no abnormal local or systemic reactions occur.

Immunogenicity. The test described under Potency is suitable to demonstrate immunogenicity of the vaccine strain.

BATCH TESTING

If the test for potency has been carried out with satisfactory results on a representative batch of vaccine, this test may be omitted as a routine control on other batches of vaccine prepared from the same seed lot, subject to agreement by the competent authority.

IDENTIFICATION

Carry out an immunofluorescence test in suitable cell cultures, using a monospecific antiserum.

TESTS

Safety. Use calves of the minimum age recommended for vaccination and preferably having no antibodies against bovine parainfluenza 3 virus or, where justified, use calves with a very low level of such antibodies as long as they have not been vaccinated against bovine parainfluenza virus and administration of the vaccine does not cause an anamnestic response. Administer 10 doses of the vaccine by a recommended route to each of 2 calves. Observe the animals for 21 days. No abnormal local or systemic reaction occurs.

Extraneous viruses. Neutralise the vaccine using a monospecific antiserum against bovine parainfluenza 3 virus and inoculate into cell cultures known to be sensitive to viruses pathogenic for cattle. Maintain these cultures for 14 days and make at least one passage during this period. No cytopathic effect develops; the cells show no evidence of the presence of haemadsorbing agents. Carry out a specific test for pestiviruses.

Bacterial and fungal contamination. The vaccine complies with the test for sterility prescribed under *Vaccines for veterinary use (0062)*.

Mycoplasmas (2.6.7). The vaccine complies with the test for mycoplasmas.

Virus titre. Titrate the vaccine in suitable cell cultures. One dose of the vaccine contains not less than the quantity of virus equivalent to the minimum virus titre stated on the label.

POTENCY

Use not fewer than 10 calves of the minimum age recommended for vaccination and that do not have antibodies against bovine parainfluenza 3 virus; calves having low levels of such antibodies may be used if it has been demonstrated that valid results are obtained in these conditions. Collect sera from the animals before vaccination, 7 days and 14 days after the time of vaccination and just before challenge. Vaccinate not fewer than 5 of the calves according to the instructions for use. Keep 5 calves as controls. Observe the animals for 21 days and then administer to each of them by a respiratory tract route a suitable quantity of a low-passage virulent strain of bovine parainfluenza 3 virus. Monitor each animal for clinical signs, in particular respiratory symptoms, and virus shedding (by nasal swabs or tracheobronchial washing) for 14 days after challenge. The vaccine complies with the test if in vaccinated animals compared to controls there is (a) a significant reduction in mean titre and in mean duration of virus excretion and (b) a notable reduction in general and local signs (if the challenge virus used produces such signs). The test is not valid if tests for antibodies against bovine parainfluenza 3 virus on the sera indicate that there was intercurrent infection with the virus during the test or if

more than 2 of the 5 control animals show no excretion of the challenge virus, as shown by nasal swabs or samples harvested by tracheobronchial washing.

vaccine or to wild virus. The vaccine is satisfactory if it is not associated with an abnormal incidence of immediate hypersensitivity reactions.

Immunogenicity. The test described under Potency is suitable to demonstrate immunogenicity of the vaccine strain.

01/2005:1177

BOVINE RESPIRATORY SYNCYTIAL VIRUS VACCINE (LIVE), FREEZE-DRIED

*Vaccinum viri syncytialis meatus spiritus
bovini vivum cryodesiccatum*

DEFINITION

Freeze-dried bovine respiratory syncytial virus vaccine (live) is a preparation of a suitable strain of bovine respiratory syncytial virus.

PRODUCTION

The vaccine strain is grown in suitable cell cultures (5.2.4). The viral suspension is harvested, mixed with a suitable stabilising solution and freeze-dried.

CHOICE OF VACCINE STRAIN

Only a virus strain shown to be satisfactory with respect to reversion to virulence, safety and immunogenicity may be used in the preparation of the vaccine. The following tests may be used during demonstration of safety (5.2.6) and efficacy (5.2.7).

Reversion to virulence. Administer by the intranasal route to two susceptible calves that do not have antibodies against bovine respiratory syncytial virus a quantity of virus that will allow optimal re-isolation of the virus for subsequent passages. On each of days 3 to 7 after administration of the virus, take nasal swabs from each calf and collect in not more than 5 ml of a suitable medium which is then used to inoculate cell cultures to verify the presence of virus; use about 1 ml of the suspensions from swabs that contain the maximum amount of virus, as indicated by the titration in cell cultures, to inoculate two other calves of the same age and sensitivity; repeat these operations until five passages on calves have been carried out. No calf shows clinical signs attributable to the vaccinal virus. No indication of increase of virulence compared to the original vaccinal virus is observed; account is taken of the titre of excreted virus in the nasal swabs.

Safety. Safety studies are conducted in calves of the minimum age for which the vaccine is recommended and for each recommended route of administration.

- A. Administer by a recommended route to five calves, without antibodies against bovine respiratory syncytial virus, a quantity of virus corresponding to not less than ten times the maximum virus titre that may be expected in a batch of vaccine. Observe the animals for 21 days. Measure the rectal temperature of each animal on the day before vaccination, at the time of vaccination and daily for the following 7 days. No abnormal effect on body temperature and no abnormal local or systemic reaction are noted.
- B. The animals used for the field trials are also used to evaluate the incidence of hypersensitivity reactions in vaccinated animals following subsequent exposure to the

BATCH TESTING

If the test for potency has been carried out with satisfactory results on a representative batch of vaccine, this test may be omitted as a routine control on other batches of vaccine prepared from the same seed lot, subject to agreement by the competent authority.

IDENTIFICATION

Identify the vaccine by an immunofluorescence test in suitable cell cultures using a monospecific antiserum.

TESTS

Safety. Administer ten doses of the vaccine by a recommended route to each of two calves of the minimum age recommended for vaccination and that do not have antibodies against bovine respiratory syncytial virus. Observe the animals for 21 days. No abnormal local or systemic reaction occurs.

Extraneous viruses. Neutralise the vaccine using a monospecific antiserum against bovine respiratory syncytial virus and inoculate into cell cultures known to be sensitive to viruses pathogenic for cattle. Maintain the cultures for 14 days and make at least one passage during this period. No cytopathic effect develops; the cells show no evidence of the presence of haemadsorbing agents. Carry out a specific test for pestiviruses.

Bacterial and fungal contamination. The vaccine complies with the test for sterility prescribed under *Vaccines for veterinary use (0062)*.

Mycoplasmas (2.6.7). The vaccine complies with the test for mycoplasmas.

Virus titre. Titrate the vaccine in suitable cell cultures. One dose of the vaccine contains not less than the quantity of virus equivalent to the minimum titre stated on the label.

POTENCY

Use not fewer than ten calves of the minimum age recommended for vaccination and that do not have antibodies against bovine respiratory syncytial virus. Collect sera from the animals before the time of vaccination, 7 and 14 days after the time of vaccination and just before challenge. Vaccinate not fewer than five of the calves according to the instructions for use. Keep five calves as controls. Observe the animals for 21 days and then administer to each of them by a respiratory tract route a suitable quantity of a low-passage virulent strain of bovine respiratory syncytial virus. Monitor each animal for clinical signs, in particular respiratory symptoms, and virus shedding (by nasal swabs or tracheobronchial washing) for 14 days after challenge. The vaccine complies with the test if there is: (a) a significant reduction in mean titre and in mean duration of virus excretion in vaccinates compared to controls and (b) a notable reduction in general and local clinical signs in vaccinated animals (if the challenge virus used produces such signs). The test is not valid if antibodies to bovine respiratory syncytial virus are detected in any sample from control animals before challenge or if more than two of the five control animals show no excretion of the challenge virus, as shown by nasal swabs or samples harvested by tracheobronchial washing.

01/2005:1952

BOVINE VIRAL DIARRHOEA VACCINE (INACTIVATED)

Vaccinum diarrhoeae viralis bovinæ inactivatum

DEFINITION

Bovine viral diarrhoea vaccine (inactivated) is a preparation of one or more suitable strains of bovine diarrhoea virus inactivated by a suitable method. This monograph applies to vaccines intended for vaccination of heifers and cows to protect the foetus against transplacental infection.

PRODUCTION

The vaccine virus strain or strains are grown in suitable cell cultures (5.2.4).

The test for inactivation is carried out using a quantity of virus equivalent to not less than 25 doses of vaccine in cells of the same type as those used for production of the vaccine or cells shown to be at least as sensitive; the cells are passaged after 7 days and observed for a total of not less than 14 days. No infectious virus is detected.

CHOICE OF VACCINE COMPOSITION

The vaccine shall be shown to be satisfactory with respect to safety (5.2.6) and immunogenicity (5.2.7) in cattle.

The following tests may be used during the demonstration of safety and immunogenicity.

Safety. Carry out a safety test for each recommended route and for each category of cattle for which the vaccine is intended. Use cattle of the minimum age recommended for vaccination and that are free from bovine diarrhoea virus and from antibodies against the virus. Inject a double dose of vaccine into each of not fewer than 10 animals. Observe the animals for 14 days. No abnormal local or systemic reaction occurs. If the vaccine is intended for administration to pregnant cattle, carry out the test in these animals at the beginning of each trimester for which use is not contra-indicated and extend the observation period to calving. No undesirable effect on gestation or the offspring occurs. If the vaccine is intended for administration shortly before or at insemination, absence of undesirable effects on conception rate must be demonstrated.

Immunogenicity. The test for potency is suitable to demonstrate the immunogenicity of the vaccine with respect to bovine diarrhoea virus of genotype 1; if protection against bovine diarrhoea virus of genotype 2 is claimed, an additional test, similar to that described under Potency, but using bovine diarrhoea virus of genotype 2 for challenge, is carried out.

BATCH POTENCY TEST

The test described under Potency is not carried out for routine testing of batches of vaccine. It is carried out for a given vaccine on one or more occasions as decided by or with the agreement of the competent authority. Where the test is not carried out, a suitable validated test is carried out, the criteria for acceptance being set with reference to a batch of vaccine that has given satisfactory results in the test described under Potency. The following test may be used after a satisfactory correlation with the test described under Potency has been established.

Inject subcutaneously a suitable dose of the vaccine into each of 5 suitable seronegative laboratory animals or calves. Keep 2 animals as controls. A second dose of vaccine may be administered after a suitable interval if this has been shown

to provide a suitably discriminating test system. Collect blood samples before the first vaccination and at a given interval between 14 and 21 days after the last vaccination. Determine the antibody titres against bovine diarrhoea virus by seroneutralisation on suitable cell cultures. The test is invalid if the control animals show antibodies against bovine diarrhoea virus. The vaccine complies with the test if the level of antibodies is not lower than that found for a batch of vaccine that has given satisfactory results in the test described under Potency.

IDENTIFICATION

When administered to animals free from specific neutralising antibodies against bovine diarrhoea virus, the vaccine stimulates the production of such antibodies.

TESTS

Safety. Inject a double dose of the vaccine by a recommended route into each of 2 cattle not older than the minimum age recommended for vaccination and that are free from bovine diarrhoea virus and antibodies against the virus. Observe the animals for 14 days. No abnormal local or systemic reaction occurs.

Inactivation. Carry out a test for residual infectious bovine diarrhoea virus by inoculating not less than 10 doses onto cells known to be sensitive to bovine diarrhoea virus; passage the cells after 7 days and observe the second culture for not less than 7 days. No live virus is detected. If the vaccine contains an adjuvant, separate the adjuvant if possible from the liquid phase by a method that does not interfere with the detection of possible live virus.

Bacteria and fungi. The vaccine complies with the requirement for sterility prescribed in the monograph on *Vaccines for veterinary use (0062)*.

POTENCY

Use heifers free from bovine diarrhoea virus that do not have neutralising antibodies against bovine diarrhoea virus. Vaccinate not fewer than 13 animals using the recommended schedule. Keep not fewer than 7 heifers as non-vaccinated controls. Keep all the animals as one group. Inseminate the heifers. Take a blood sample from non-vaccinated heifers shortly before challenge. The test is discontinued if fewer than 10 vaccinated heifers or 5 non-vaccinated heifers are pregnant at the time of challenge. Between the 60th and 90th days of gestation, challenge the animals. For both test models described (observation until calving and harvest of foetuses at 28 days), challenge may be made by the intranasal inoculation of a suitable quantity of a non-cytopathic strain of bovine diarrhoea virus or alternatively, where the animals are observed until calving, challenge may be made by contact with a persistently viraemic animal. Observe the animals clinically from challenge either until the end of gestation or until harvest of foetuses after 28 days. If abortion occurs, examine the aborted foetus for bovine diarrhoea virus by suitable methods. If animals are observed until calving, immediately after birth and prior to ingestion of colostrum, examine all calves for viraemia and antibodies against bovine diarrhoea virus. If foetuses are harvested 28 days after challenge, examine the foetuses for bovine diarrhoea virus by suitable methods. Transplacental infection is considered to have occurred if virus is detected in foetal organs or in the blood of newborn calves or if antibodies are detected in precolostral sera of calves. The test is invalid if any of the non-vaccinated heifers have neutralising antibody before challenge or if transplacental infection fails to occur in more than 10 per cent of non-vaccinated heifers. The vaccine complies with the test if 90 per cent or more of the vaccinated animals are protected from transplacental infection.

01/2005 :0793

BRUCELLOSIS VACCINE (LIVE) (BRUCELLA MELITENSIS REV. 1 STRAIN), FREEZE-DRIED, FOR VETERINARY USE

Vaccinum brucellosis (*Brucella melitensis*
stirpe Rev. 1) vivum cryodesiccatum
ad usum veterinarium

DEFINITION

Brucellosis vaccine (live) (*Brucella melitensis* Rev. 1 strain), freeze-dried, for veterinary use is a freeze-dried suspension of live *Brucella melitensis* Rev. 1 strain. The vaccine contains not fewer than 0.5×10^9 and not more than 4×10^9 live bacteria per dose.

PRODUCTION

Brucella melitensis Rev. 1 strain is cultured in a suitable medium. The method of culture is such as to avoid bacterial dissociation and thus maintain the smooth characteristic of the culture. The bacteria are suspended in a buffer solution which may contain a suitable stabiliser. The suspension is distributed into containers and freeze-dried.

CHOICE OF VACCINE STRAIN

Only a vaccine strain shown to be satisfactory with respect to safety and immunogenicity may be used in the production of the vaccine. The following test may be used during demonstration of efficacy (5.2.7).

Immunogenicity. 40 ewe lambs, 4 to 5 months old, from a non-vaccinated flock free from brucellosis are used. For the challenge strain, a 24-hour culture of *Brucella melitensis* strain H38 in trypticase agar is used. A preliminary test using animals of the same breed and age as for the main test is carried out to determine the challenge dose, which is between 10^7 and 10^8 colony-forming units and is chosen so as to produce abortion in all non-vaccinated animals. Half the animals are vaccinated at 4 to 6 months of age, according to the recommended schedule and using the minimum recommended dose. Oestrus is synchronised in the 40 animals which are then inseminated at 10 to 12 months of age. A diagnosis of pregnancy is made on all animals 2 to 3 months after insemination and all non-gravid animals are excluded from the test. All gravid animals are challenged by conjunctival instillation of the challenge dose of *Brucella melitensis* strain H38. The occurrence of abortion is noted and aetiology confirmed by testing for the presence of the challenge strain in the aborted foetus and in the ewe (using selective media of Kuzdas and Morse or of Farrell). Tests for the presence of the challenge strain are carried out on each animal at lambing. Tests for the presence of the challenge strain in the prescapular and retromammary lymph nodes are carried out at slaughter of the animals 4 to 6 weeks after lambing. The test is not valid unless at least 70 per cent of non-vaccinated animals: show abortion caused by the challenge strain; show infection with the challenge strain at lambing; show infection of the prescapular and retromammary lymph nodes at slaughter. The vaccine complies with the test if: not more than 30 per cent of vaccinated animals show abortion caused by the challenge strain; the challenge strain is found in not more than 50 per

cent of vaccinated animals at lambing; the challenge strain is found in not more than 40 per cent of vaccinated animals at slaughter.

IDENTIFICATION

Brucella melitensis present in the vaccine is identified by suitable morphological, serological and biochemical tests and by culture: Rev. 1 strain is inhibited by addition to the suitable culture medium of 3 µg of benzylpenicillin sodium per millilitre; the strain grows on agar containing 2.5 µg of streptomycin per millilitre.

TESTS

Safety. Use 2 sheep, 4 to 6 months old and having no antibodies against *B. melitensis*. Administer 1 dose of vaccine by a recommended route to each sheep. Observe the animals for 21 days. No abnormal local or systemic reaction occurs.

Determination of dissociation phase. Examine not fewer than 200 colonies by a suitable technique. The culture of the vaccine strain is seen to be in the smooth (S) phase. Not fewer than 95 per cent of the colonies are of the smooth type.

Extraneous micro-organisms. The reconstituted vaccine does not contain extraneous micro-organisms. Verify the absence of micro-organisms other than *Brucella melitensis* Rev. 1 strain as described in the test for sterility prescribed in the monograph on *Vaccines for veterinary use (0062)*.

Live bacteria. Make a count of live bacteria on a solid medium suitable for the culture of *Brucella melitensis* Rev. 1 strain. The vaccine contains not fewer than 0.5×10^9 and not more than 4×10^9 live bacteria per dose.

Fifty per cent persistence time. Inject subcutaneously a suspension containing 10^8 live bacteria of the vaccine to be examined into each of 32 female CD1 mice, aged 5 to 6 weeks. Kill the mice in groups of eight, selected at random, 3, 6, 9 and 12 weeks later. Remove the spleens and homogenise individually and aseptically in 10 volumes of *phosphate buffered saline pH 6.8 R*. Spread the suspension on plates containing a suitable culture medium, using 0.4 ml per plate and not fewer than 3 plates per spleen (lower limit of detection: 5 bacteria per spleen). Carry out in parallel a similar test using *Brucella melitensis* Rev. 1 strain BRP (reference strain). Calculate the 50 per cent persistence time by the usual statistical methods (5.3) for probit analysis. The 50 per cent persistence time for the vaccine strain does not differ significantly from that of the reference strain. For Rev. 1 strain, the 50 per cent persistence time and confidence limits ($P = 0.05$) are usually 7.9 ± 1.2 weeks.

STORAGE

Store at a temperature of 2 °C to 8 °C.

LABELLING

The label states:

- the number of *Brucella melitensis* Rev. 1 strain per dose,
- that the vaccine is intended for sheep and goats 4 to 6 months old,
- that the vaccine may be dangerous for man,
- that the vaccine is not to be used in pregnant or lactating animals,
- that the vaccine may be dangerous for cattle and that they are not to be kept in contact with vaccinated animals.

01/2005:1953

CALF CORONAVIRUS DIARRHOEA VACCINE (INACTIVATED)

Vaccinum inactivatum diarrhoeae vituli coronaviro illatae

DEFINITION

Calf coronavirus diarrhoea vaccine (inactivated) is a preparation of one or more suitable strains of bovine coronavirus, inactivated in such a manner that immunogenic properties are maintained. The vaccine is administered to the dam to aid in the control of coronavirus diarrhoea in offspring during the first few weeks of life.

PRODUCTION

Each virus strain is grown separately in suitable cell cultures (5.2.4). The viral suspensions of each strain are harvested separately and inactivated by a method that maintains immunogenicity. The viral suspensions may be purified and concentrated.

The test for inactivation is carried out using 2 passages in cell cultures of the same type as those used for production or in cells shown to be at least as sensitive. The quantity of virus used in the test is equivalent to not less than 10 doses of vaccine. No live virus is detected.

The vaccine may contain an adjuvant.

CHOICE OF VACCINE COMPOSITION

The vaccine is shown to be satisfactory with respect to safety (5.2.6) and efficacy (5.2.7) in the pregnant cow. The following tests may be used during demonstration of safety and immunogenicity.

Safety. Carry out the test for each proposed route of administration. Administer by a proposed route and at the proposed stage or stages of pregnancy, a double dose of vaccine to each of not fewer than 10 pregnant cows that have not been vaccinated against bovine coronavirus. After the proposed interval, inject 1 dose into each cow. After each injection, measure the body temperature on the day of the injection and on the 4 following days. Observe the cows until calving. No abnormal local or systemic reaction occurs; any effects on gestation and the offspring are noted.

Immunogenicity. The test described under Potency is suitable to demonstrate immunogenicity of the strain.

BATCH TESTING

Batch potency test. The test described under Potency is not carried out for routine testing of batches of vaccine. It is carried out, for a given vaccine, on one or more occasions, as decided by or with the agreement of the competent authority; where the test is not carried out, a suitable validated test is carried out, the criteria for acceptance being set with reference to a batch of vaccine that has given satisfactory results in the test described under Potency. The following test may be used after a suitable correlation with the test described under Potency has been established.

To obtain a valid assay, it may be necessary to carry out a test using several groups of animals, each receiving a different dose. For each dose required, carry out the test as follows. Vaccinate not fewer than 5 animals of a suitable species, free from specific antibodies against bovine coronavirus, using 1 injection of a suitable dose. Maintain not fewer than 2 animals as unvaccinated controls. Where the recommended schedule requires a booster injection to be given, a booster vaccination may also be given in this test

provided it has been demonstrated that this will still provide a suitably sensitive test system. At a given interval not less than 14 days after the last injection, collect blood from each animal and prepare serum samples. Use a suitable validated test to measure the antibody response. The antibody level is not significantly less than that obtained with a batch that has given satisfactory results in the test described under Potency and there is no significant increase in antibody titre in the controls.

IDENTIFICATION

Injected into animals free from specific antibodies against bovine coronavirus, the vaccine stimulates the formation of such antibodies.

TESTS

Safety. Use cattle not less than 6 months old and preferably having no antibodies against bovine coronavirus or, where justified, use cattle with a low level of such antibodies as long as they have not been vaccinated against bovine coronavirus and administration of the vaccine does not cause an anamnestic response. Administer to each of 2 animals a double dose of vaccine by a recommended route. After 14 days, administer 1 dose to each animal. Observe the animals for 14 days. No abnormal local or systemic reaction occurs.

Inactivation. Carry out a test for residual infectious virus using 10 doses of vaccine and 2 passages in cell cultures of the same type as those used for production of the vaccine or other cell cultures of suitable sensitivity. No live virus is detected. If the vaccine contains an adjuvant which interferes with the test, separate it if possible from the liquid phase of the vaccine by a method that does not inactivate virus nor interfere in any other way with detection of live viruses.

Extraneous viruses. Carry out tests for antibodies on the cattle used for the safety test. Take a blood sample at the end of the second observation period. The vaccine does not stimulate the formation of antibodies against bovine herpes virus 1 (BHV1), bovine leukaemia virus (BLV) and bovine viral diarrhoea virus (BVDV).

Sterility. The vaccine complies with the test for sterility prescribed in the monograph on *Vaccines for veterinary use* (0062).

POTENCY

Use not fewer than 15 pregnant cows, where possible having no antibodies against bovine coronavirus. Where such cows are not available, use cows that: have not been vaccinated against bovine coronavirus; come from a farm where there is no recent history of infection with bovine coronavirus; and have a low level of antibodies against bovine coronavirus, the levels being comparable in all animals. Vaccinate not fewer than 10 pregnant cows according to the recommended schedule. Keep not fewer than 5 pregnant cows as unvaccinated controls. Starting at calving, take the colostrum and then milk from each cow and keep it in suitable conditions. Determine individually the protective activity of the colostrum and milk from each cow using calves born from healthy cows, and which may be born by Caesarean section, and maintained in an environment where they are not exposed to infection by bovine coronavirus. Feed colostrum and then milk to each calf every 6 h or according to the recommended schedule. At 5-7 days after birth, challenge each calf by the oral administration of a suitable quantity of a virulent strain of bovine coronavirus. Observe the calves for 7 days. Note the incidence, severity and duration of diarrhoea and the duration and quantity of virus excretion. The vaccine complies with the test if there

is a significant reduction in diarrhoea and virus excretion in calves given colostrum and milk from vaccinated cows compared to those given colostrum and milk from controls.

LABELLING

The label states the recommended schedule for administering colostrum and milk, *post-partum*.

01/2005:1954

CALF ROTAVIRUS DIARRHOEA VACCINE (INACTIVATED)

Vaccinum inactivatum diarrhoeae vituli rotaviro illatae

DEFINITION

Calf rotavirus diarrhoea vaccine (inactivated) is a preparation of one or more suitable strains of bovine rotavirus, inactivated in such a manner that immunogenic properties are maintained. The vaccine is administered to the dam to aid in the control of rotavirus diarrhoea in offspring during the first few weeks of life.

PRODUCTION

Each virus strain is grown separately in suitable cell cultures (5.2.4). The viral suspensions of each strain are harvested separately and inactivated by a method that maintains immunogenicity. The viral suspensions may be purified and concentrated.

The test for inactivation is carried out using 2 passages in cell cultures of the same type as those used for production or in cells shown to be at least as sensitive. The quantity of virus used in the test is equivalent to not less than 100 doses of vaccine. No live virus is detected.

The vaccine may contain an adjuvant.

CHOICE OF VACCINE COMPOSITION

The vaccine is shown to be satisfactory with respect to safety (5.2.6) and efficacy (5.2.7) in the pregnant cow. The following tests may be used during demonstration of safety and immunogenicity.

Safety. Carry out the test for each proposed route of administration. Administer by a proposed route and at the proposed stage or stages of pregnancy, a double dose of vaccine to each of not fewer than 10 pregnant cows that have not been vaccinated against bovine rotavirus. After the proposed interval, inject 1 dose into each cow. After each injection, measure the body temperature on the day of the injection and on the 4 following days. Observe the cows until calving. No abnormal local or systemic reaction occurs; any effects on gestation and the offspring are noted.

Immunogenicity. The test described under Potency is suitable to demonstrate immunogenicity of the strain.

BATCH TESTING

Batch potency test. The test described under Potency is not carried out for routine testing of batches of vaccine. It is carried out, for a given vaccine, on one or more occasions, as decided by or with the agreement of the competent authority; where the test is not carried out, a suitable validated test is carried out, the criteria for acceptance being set with reference to a batch of vaccine that has given satisfactory results in the test described under Potency. The following test may be used after a suitable correlation with the test described under Potency has been established.

To obtain a valid assay, it may be necessary to carry out a test using several groups of animals, each receiving

a different dose. For each dose required, carry out the test as follows. Vaccinate not fewer than 5 animals of a suitable species, free from specific antibodies against bovine rotavirus, using 1 injection of a suitable dose. Maintain not fewer than 2 animals as unvaccinated controls. Where the recommended schedule requires a booster injection to be given, a booster vaccination may also be given in this test provided it has been demonstrated that this will still provide a suitably sensitive test system. At a given interval not less than 14 days after the last injection, collect blood from each animal and prepare serum samples. Use a suitable validated test to measure the antibody response. The antibody level is not significantly less than that obtained with a batch that has given satisfactory results in the test described under Potency and there is no significant increase in antibody titre in the controls.

IDENTIFICATION

Injected into animals free from specific antibodies against bovine rotavirus, the vaccine stimulates the formation of such antibodies.

TESTS

Safety. Use cattle not less than 6 months old and preferably having no antibodies against bovine rotavirus or, where justified, use cattle with a low level of such antibodies as long as they have not been vaccinated against bovine rotavirus and administration of the vaccine does not cause an anamnestic response. Administer to each of 2 animals a double dose of vaccine by a recommended route. After 14 days, administer 1 dose to each animal. Observe the animals for 14 days. No abnormal local or systemic reaction occurs.

Inactivation. Carry out a test for residual infectious virus using 10 doses of vaccine and 2 passages in cell cultures of the same type as those used for production of the vaccine or other cell cultures of suitable sensitivity. No live virus is detected. If the vaccine contains an adjuvant which interferes with the test, separate it if possible from the liquid phase of the vaccine by a method that does not inactivate virus nor interfere in any other way with detection of live viruses.

Extraneous viruses. Carry out tests for antibodies on the cattle used for the safety test. Take a blood sample at the end of the second observation period. The vaccine does not stimulate the formation of antibodies against bovine herpes virus 1 (BHV 1), bovine leukaemia virus (BLV) and bovine viral diarrhoea virus (BVDV).

Sterility. The vaccine complies with the test for sterility prescribed in the monograph on *Vaccines for veterinary use* (0062).

POTENCY

Use not fewer than 15 pregnant cows, where possible having no antibodies against bovine rotavirus. Where such cows are not available, use cows that: have not been vaccinated against bovine rotavirus; come from a farm where there is no recent history of infection with bovine rotavirus; and have a low level of antibodies against bovine rotavirus, the levels being comparable in all animals. Vaccinate not fewer than 10 pregnant cows according to the recommended schedule. Keep not fewer than 5 pregnant cows as unvaccinated controls. Starting at calving, take the colostrum and then milk from each cow and keep it in suitable conditions. Determine individually the protective activity of the colostrum and milk from each cow using calves born from healthy cows, and which may be born by Caesarean section, and maintained in an environment where they are not exposed to infection by bovine rotavirus. Feed colostrum

and then milk to each calf every 6 h or according to the recommended schedule. At 5-7 days after birth, challenge each calf by the oral administration of a suitable quantity of a virulent strain of bovine rotavirus. Observe the calves for 7 days. Note the incidence, severity and duration of diarrhoea and the duration and quantity of virus excretion. The vaccine complies with the test if there is a significant reduction in diarrhoea and virus excretion in calves given colostrum and milk from vaccinated cows compared to those given colostrum and milk from controls.

LABELLING

The label states the recommended schedule for administering colostrum and milk, *post-partum*.

01/2005:1298

CANINE ADENOVIRUS VACCINE (INACTIVATED)

Vaccinum adenoviro-sis caninae inactivatum

DEFINITION

Canine adenovirus vaccine (inactivated) is a suspension of one or more suitable strains of canine adenovirus 1 (canine contagious hepatitis virus) and/or canine adenovirus 2, inactivated in such a way that adequate immunogenicity is maintained.

PRODUCTION

The test for inactivation is carried out using a quantity of virus equivalent to at least 10 doses of vaccine with 2 passages in cell cultures of the same type as those used for production or in cell cultures shown to be at least as sensitive. No live virus is detected.

The vaccine may contain an adjuvant.

CHOICE OF VACCINE COMPOSITION

The vaccine is shown to be satisfactory with respect to safety (5.2.6) and efficacy (5.2.7). The following tests may be used during demonstration of safety and immunogenicity.

Safety. Carry out the test for each recommended route of administration in animals of the minimum age recommended for vaccination. Use a batch of vaccine of the maximum potency likely to be attained.

Use for each test not fewer than 10 dogs that do not have antibodies against canine adenovirus 1 or 2. Administer to each dog a double dose of vaccine. If the recommended schedule requires a second dose, administer one dose after the recommended interval. Observe the dogs for 14 days after the last administration. No abnormal local or systemic reaction occurs.

If the vaccine is intended for use in pregnant bitches, vaccinate bitches at the stage of pregnancy or at different stages of pregnancy according to the recommended schedule. Prolong observation until 1 day after parturition. No abnormal local or systemic reaction occurs. No adverse effects on the pregnancy and offspring are noted.

Immunogenicity. For vaccines intended to protect against hepatitis, the test described under Potency is suitable for demonstration of immunogenicity. If the vaccine is indicated for protection against respiratory signs, a further test to demonstrate immunogenicity for this indication is also necessary.

BATCH TESTING

Batch potency test. The test described under Potency is not carried out for routine testing of batches of vaccine. It is carried out for a given vaccine on one or more occasions as

decided by or with the agreement of the competent authority. Where the test is not carried out, a suitable validated alternative test is carried out, the criteria for acceptance being set with reference to a batch of vaccine that has given satisfactory results in the test described under Potency.

IDENTIFICATION

When injected into susceptible animals, the vaccine stimulates the formation of specific antibodies against the type or types of canine adenovirus stated on the label.

TESTS

Safety. Use dogs of the minimum age recommended for vaccination and preferably having no canine adenovirus-neutralising antibodies or, where justified, use dogs with a low level of such antibodies as long as they have not been vaccinated against canine adenovirus and administration of the vaccine does not cause an anamnestic response. Administer a double dose of vaccine by a recommended route to each of 2 dogs. Observe the dogs for 14 days. No abnormal local or systemic reaction occurs.

Inactivation. Carry out a test for residual infectious canine adenovirus using 10 doses of vaccine by inoculation into sensitive cell cultures; make a passage after 6-8 days and maintain the cultures for 14 days. No live virus is detected. If the vaccine contains an adjuvant, separate the adjuvant from the liquid phase by a method that does not inactivate or otherwise interfere with the detection of live virus.

Sterility. The vaccine complies with the test for sterility prescribed in the monograph on *Vaccines for veterinary use* (0062).

POTENCY

Use 7 dogs of the minimum age recommended for vaccination and that do not have antibodies against canine adenovirus. Vaccinate 5 of the animals by a recommended route and according to the recommended schedule. Keep the other 2 dogs as controls. 21 days later inject intravenously into each of the 7 animals a quantity of a virulent strain of canine adenovirus sufficient to cause death or typical signs of the disease in a susceptible dog. Observe the animals for a further 21 days. Dogs displaying typical signs of serious infection with canine adenovirus are killed humanely to avoid unnecessary suffering. The test is invalid and must be repeated if one or both of the controls do not die from or display typical signs of serious infection with canine adenovirus. The vaccine complies with the test if the vaccinated animals remain in good health.

LABELLING

The label states the type or types of canine adenovirus present in the vaccine.

01/2005:1951

CANINE ADENOVIRUS VACCINE (LIVE)

Vaccinum adenoviro-sidis caninae vivum

DEFINITION

Canine adenovirus vaccine (live) is a preparation of 1 or more suitable strains of canine adenovirus 2. This monograph applies to vaccines intended for active immunisation of dogs against canine contagious hepatitis and/or respiratory disease caused by canine adenovirus.

PRODUCTION

The virus strain is propagated in suitable cell cultures (5.2.4). The viral suspension is harvested, titrated and may be mixed with a suitable stabilising solution. The vaccine may be freeze-dried.

CHOICE OF VACCINE STRAIN

The vaccine is shown to be satisfactory with respect to safety (5.2.6), absence of increase in virulence and immunogenicity (5.2.7). The following tests may be used during demonstration of safety, absence of increase in virulence and immunogenicity.

Safety. The test is carried out for each route of administration to be stated on the label. Use not fewer than 5 puppies of the minimum age recommended for vaccination and that do not have antibodies to canine adenovirus. Administer to each puppy by a recommended route a quantity of virus corresponding to not less than 10 times the maximum titre that may be expected in a dose of vaccine. Observe the dogs for 14 days. The puppies remain in good health and no abnormal local or systemic reaction occurs.

If the vaccine is intended for use or may be used in pregnant bitches, administer the virus to 5 bitches at the recommended stage or at a range of stages of pregnancy according to the recommended schedule. Prolong the observation period until 1 day after parturition. The bitches remain in good health and there is no abnormal local or systemic reaction. No adverse effects on the pregnancy or the offspring are noted.

Increase in virulence. Administer by a recommended route to each of 2 puppies, 5-7 weeks old and which do not have antibodies against canine adenovirus, a quantity of virus that will allow recovery of virus for the passages described below. Kill the puppies 4-6 days later. Remove from each puppy nasal and pharyngeal mucosa, tonsils, lung, spleen and, if they are likely to contain virus, liver and kidney. Pool the samples; administer by a suitable route, for example intranasally, 1 ml of the pooled organ suspension to each of 2 other puppies of the same age and susceptibility; carry out these operations at least 5 times; verify the presence of the virus at each passage by direct or indirect means. If the virus has disappeared, carry out a second series of passages. Inoculate virus from the highest recovered passage level to 5 puppies of the minimum age recommended for vaccination, observe for 14 days and compare the reactions that occur with those seen in the test for safety described above. There is no indication of an increase of virulence as compared with the non-passaged virus.

Immunogenicity. For vaccines intended to protect against hepatitis, test A described under Potency is suitable for demonstration of Immunogenicity. For vaccines intended to protect against respiratory signs, test B described under Potency is suitable for demonstration of immunogenicity.

BATCH TESTING

If the test for Potency has been carried out with satisfactory results on a representative batch of vaccine, this test may be omitted as a routine control on other batches of vaccine prepared from the same seed lot.

IDENTIFICATION

The vaccine mixed with monospecific antiserum against canine adenovirus 2 no longer infects susceptible cell cultures.

TESTS

Safety. Use 2 puppies not older than the minimum age recommended for vaccination and which do not have antibodies against canine adenovirus. Administer 10 doses of the vaccine to each dog by a recommended route. Observe for 14 days. The dogs remain in good health and no abnormal local or systemic reaction occurs.

Extraneous viruses. Mix the vaccine with a suitable monospecific antiserum against canine adenovirus 2 and inoculate into cell cultures known for their susceptibility to viruses pathogenic for the dog. Carry out a passage after 6-8 days and maintain the cultures for a total of 14 days. No cytopathic effect develops and the cells show no evidence of the presence of haemadsorbing agents.

Bacterial and fungal contamination. The vaccine, reconstituted if necessary, complies with the test for sterility prescribed in the monograph on *Vaccines for veterinary use* (0062).

Mycoplasmas (2.6.7). The vaccine, reconstituted if necessary, complies with the test for mycoplasmas.

Virus titre. Reconstitute the vaccine, if necessary, and titrate in suitable cell cultures. 1 dose of the vaccine contains not less than the quantity of virus equivalent to the minimum virus titre stated on the label.

POTENCY

Depending on the indications for the vaccine, it complies with test A and/or B for potency.

- A. Use 7 puppies of the minimum age recommended for vaccination and that do not have antibodies against canine adenovirus. Vaccinate 5 of the animals by a recommended route and according to the recommended schedule. Keep the other 2 dogs as controls. 21 days later, inject intravenously into each of the 7 animals a quantity of a virulent strain of canine adenovirus 1 (canine contagious hepatitis virus) sufficient to cause death or typical signs of the disease in a susceptible dog. Observe the animals for a further 21 days. Dogs displaying typical signs of serious infection with canine adenovirus are killed humanely to avoid unnecessary suffering. The test is invalid and must be repeated if 1 or both of the controls do not die from or display typical signs of serious infection with canine adenovirus. The vaccine complies with the test if the vaccinated animals remain in good health showing no clinical signs except for a possible transient elevated rectal temperature.
- B. Use 20 dogs of the minimum age recommended for vaccination and that do not have antibodies against canine adenovirus. Vaccinate 10 of the dogs by a recommended route and according to the recommended schedule. Keep the other 10 dogs as controls. 21 days later, administer intranasally to each of the 20 animals a quantity of a virulent strain of canine adenovirus 2 sufficient to cause typical signs of respiratory disease in a susceptible dog. Observe the animals daily for a further 10 days. Record the incidence of signs of respiratory and general disease in each dog (for example, sneezing, coughing, nasal and lachrymal discharge, loss of appetite). Collect nasal swabs or washings from each dog daily from days 2 to 10 after challenge and test these samples to determine the presence and titre of excreted virus. The vaccine complies with the test if there is a notable decrease in the incidence and severity of clinical signs and in virus excretion in vaccinates compared to controls.

01/2005:0448 IDENTIFICATION

**CANINE DISTEMPER VACCINE (LIVE),
FREEZE-DRIED****Vaccinum morbi Carrei vivum
cryodesiccatum ad canem****DEFINITION**

Freeze-dried canine distemper vaccine (live) is a preparation of a strain of distemper virus that is attenuated for dogs.

PRODUCTION

The virus is propagated in suitable cell cultures (5.2.4) or in fertilised hen eggs from flocks free from specified pathogens (5.2.2). The viral suspension is harvested, titrated and may be mixed with a suitable stabilising solution. The vaccine is then freeze-dried.

CHOICE OF VACCINE STRAIN

The vaccine strain is shown to be satisfactory with respect to safety (5.2.6), absence of increase in virulence and immunogenicity (5.2.7). The following tests may be used during demonstration of safety, absence of increase in virulence and immunogenicity.

Safety. The test is carried out for each recommended route of administration. Use five susceptible puppies of the minimum age recommended for vaccination and that do not have antibodies against canine distemper virus. Administer to each puppy by a recommended route a quantity of virus corresponding to not less than ten times the maximum titre that may be expected in a dose of vaccine. Observe the puppies for 42 days. The puppies remain in good health and there is no abnormal local or systemic reaction.

If the vaccine is intended for use in pregnant bitches, administer the virus to five bitches at the recommended stage of pregnancy or at a range of stages of pregnancy according to the recommended schedule. Prolong observation until 1 day after parturition. The dogs remain in good health and there is no abnormal local or systemic reaction. No adverse effects on the pregnancy or the offspring are noted.

Increase in virulence. Administer by a recommended route to each of two puppies, 5 to 7 weeks old and which do not have antibodies against canine distemper virus a quantity of virus corresponding to one dose of vaccine. Kill the puppies 5 to 10 days later, remove nasal mucosa, tonsils, thymus, spleen and the lungs and their local lymph nodes from each puppy and pool the samples; administer intranasally 1 ml of the pooled organ suspension to each of two other puppies of the same age and susceptibility; carry out these operations at least five times; verify the presence of the virus at each passage by direct or indirect means. If the virus has disappeared, carry out a second series of passages. Inoculate virus from the highest recovered passage level to puppies, observe for 42 days and compare any reactions that occur with those seen in the test for safety described above. There is no indication of an increase of virulence as compared with the non-passaged virus.

Immunogenicity. The test described under Potency may be used to demonstrate the immunogenicity of the strain.

BATCH TESTING

If the test for potency has been carried out with satisfactory results on a representative batch of vaccine, this test may be omitted as a routine control on other batches of vaccine prepared from the same seed lot.

IDENTIFICATION

The vaccine reconstituted as stated on the label and mixed with a monospecific distemper antiserum against canine distemper virus no longer provokes cytopathic effects in susceptible cell cultures.

TESTS

Safety. Use two puppies of the minimum age recommended for vaccination and which do not have antibodies against canine distemper virus. Administer ten doses of the vaccine to each dog by a recommended route. Observe for 14 days. The dogs remain in good health and no abnormal local or systemic reaction occurs.

Extraneous viruses. Mix the vaccine with a suitable monospecific antiserum against canine distemper virus and inoculate into cell cultures known for their susceptibility to viruses pathogenic for the dog. Carry out a passage after 6 to 8 days and maintain the cultures for 14 days. No cytopathic effect develops and the cells show no evidence of the presence of haemadsorbing agents.

Bacterial and fungal contamination. The reconstituted vaccine complies with the test for sterility prescribed under *Vaccines for veterinary use (0062)*.

Mycoplasmas (2.6.7). The reconstituted vaccine complies with the test for mycoplasmas.

Virus titre. Titrate the reconstituted vaccine in suitable cell cultures. One dose of the vaccine contains not less than the quantity of virus equivalent to the minimum virus titre stated on the label.

POTENCY

Use seven susceptible puppies, 8 to 16 weeks old and free from antibodies against canine distemper virus. Vaccinate five puppies according to the instructions for use. Keep the two other animals as controls. Observe all the animals for 21 days. Inject intravenously into each animal a quantity of canine distemper virus sufficient to cause in a susceptible dog death or typical signs of the disease. Observe the animals for a further 21 days. Dogs displaying typical signs of serious infection with canine distemper virus are killed humanely to avoid unnecessary suffering. The test is not valid and must be repeated if one or more of the control animals do not either die of distemper or display typical signs of serious infection. The vaccine complies with the test if the vaccinated animals remain in good health.

01/2005:0447

**CANINE LEPTOSPIROSIS VACCINE
(INACTIVATED)****Vaccinum leptospirosis caninae inactivatum****DEFINITION**

Canine leptospirosis vaccine (inactivated) is a suspension of inactivated whole organisms and/or antigenic extract(s) of one or more suitable strains of one or more of *Leptospira interrogans* serovar canicola, serovar icterohaemorrhagiae or any other epidemiologically appropriate serovar, inactivated and prepared in such a way that adequate immunogenicity is maintained. This monograph applies to vaccines intended for active immunisation of dogs against leptospirosis.

PRODUCTION

The seed material is cultured in a suitable medium; each strain is cultivated separately. During production, various parameters such as growth rate are monitored by suitable methods; the values are within the limits approved for the particular product. Purity and identity are verified on the harvest using suitable methods. After cultivation, the bacterial harvests are collected separately and inactivated by a suitable method. The antigen may be concentrated. The vaccine may contain an adjuvant.

CHOICE OF VACCINE COMPOSITION

The vaccine is shown to be satisfactory with respect to safety (5.2.6) and efficacy (5.2.7) in dogs. As part of the studies to demonstrate the suitability of the vaccine with respect to these characteristics the following tests may be carried out.

Safety. The test is carried out for each route of administration to be stated on the label and in animals of each category for which the vaccine is intended. For each test, use not fewer than 10 dogs that do not have antibodies against the principal *L. interrogans* serovars (icterohaemorrhagiae, canicola, grippotyphosa, sejroe, hardjo, hebdomonadis, pomona, australis and autumnalis). Use a batch of vaccine containing not less than the maximum antigen content and/or potency that may be expected in a batch of vaccine. Administer to each animal a double dose of vaccine. If the recommended schedule requires a second dose, administer 1 dose after the recommended interval. Observe the animals for at least 14 days after the last administration. Record body temperatures the day before each vaccination, at vaccination, 4 h later and daily for 4 days. If the vaccine is intended for use or may be used in pregnant bitches, vaccinate the animals at the recommended stage of pregnancy or at a range of stages of pregnancy and prolong the observation period until 1 day after whelping. The vaccine complies with the test if no animal shows an abnormal local or systemic reaction or clinical signs of disease or dies from a cause attributable to the vaccine. In addition, if the vaccine is for use in pregnant animals, no adverse effects on the pregnancy and offspring are noted.

Immunogenicity. As part of the studies to demonstrate the suitability of the vaccine with respect to immunogenicity and compliance with the claims to be stated on the label, the test described under Potency may be carried out for each proposed route of administration and using vaccine of minimum antigen content and/or potency.

BATCH POTENCY TEST

The test described under Potency is not carried out for routine testing of batches of vaccine. It is carried out, for a given vaccine, on one or more occasions, as decided by or with the agreement of the competent authority. Where the test is not carried out, one of the following tests may be used.

A. For vaccines with or without adjuvants

If leptospira from more than 1 serovar (for example *L. interrogans* serovar canicola and serovar icterohaemorrhagiae) has been used to prepare the vaccine, carry out a batch potency test for each serovar against which protective immunity is claimed on the label. Inject 1/40 of the dose for dogs stated on the label subcutaneously into each of 5 healthy hamsters not more than 3 months old, which do not have antibodies to the principal serovars of *L. interrogans* (icterohaemorrhagiae, canicola, grippotyphosa, sejroe, hardjo, hebdomonadis, pomona, australis and autumnalis) and which have been obtained from a regularly tested and certified leptospira-free source. After 15-20 days, inoculate intraperitoneally into each of the vaccinated animals and into an equal number of non-vaccinated controls

derived from the same certified leptospira-free source, a suitable quantity of a virulent culture of leptospirae of the serovar against which protective immunity is claimed on the label. The vaccine complies with the test if not fewer than 4 of the 5 control animals die showing typical signs of leptospira infection within 14 days of receiving the challenge suspension and if not fewer than 4 of the 5 vaccinated animals remain in good health for 14 days after the death of 4 control animals.

B. For vaccines with or without adjuvants

A suitable validated sero-response test may be carried out. Vaccinate each animal in a group of experimental animals with a suitable dose. Collect blood samples after a suitable, fixed time after vaccination. For each of the serovars present in the vaccine, an *in vitro* test is carried out on individual blood samples to determine the antibody response to one or more antigenic components which are indicators of protection and which are specific for that serovar. The criteria for acceptance are set with reference to a batch of vaccine that has given satisfactory results in the test described under Potency.

C. For vaccines without adjuvants

For each of the serovars present in the vaccine, a suitable validated *in vitro* test may be carried out to determine the content of one or more antigenic components which are indicators of protection and which are specific for that serovar. The criteria for acceptance are set with reference to a batch of vaccine that has given satisfactory results in the test described under Potency.

IDENTIFICATION

When injected into healthy seronegative animals, the vaccine stimulates the production of specific antibodies to the leptospira serovar(s) present in the vaccine. If test C is used for batch potency test, it also serves to identify the vaccine.

TESTS

Safety. Use 2 dogs of the minimum age recommended for vaccination and which do not have antibodies to the leptospira serovar(s) present in the vaccine. Administer 2 doses of the vaccine to each dog by a recommended route. Observe the animals for 14 days. The animals remain in good health and no abnormal local or systemic reaction occurs.

Inactivation. Carry out a test for live leptospirae by inoculation of a specific medium. Inoculate 1 ml of the vaccine into 100 ml of the medium. Incubate at 30 °C for 14 days, subculture into a further quantity of the medium and incubate both media at 30 °C for 14 days: no growth occurs in either medium. At the same time, carry out a control test by inoculating a further quantity of the medium with the vaccine together with a quantity of a culture containing approximately 100 leptospirae and incubating at 30 °C: growth of leptospirae occurs within 14 days.

Sterility. The vaccine complies with the test for sterility prescribed in the monograph *Vaccines for veterinary use* (0062).

POTENCY

For each type of the serovars against which protective immunity is claimed on the label, carry out a separate test with a challenge strain representative of that serovar.

Use not fewer than 12 dogs of the minimum age recommended for vaccination and free from specific antibodies against the principal serovars of *L. interrogans* (icterohaemorrhagiae, canicola, grippotyphosa, sejroe, hardjo, hebdomonadis, pomona, australis and autumnalis). Vaccinate half of the animals by a recommended route and according to the recommended schedule. Keep the

remaining animals as controls. 25-28 days after the last vaccination, infect all the animals by the conjunctival and/or intraperitoneal route with a suitable quantity of a virulent strain of the relevant *L. interrogans* serovar. Observe the animals for a further 28 days. Examine the dogs daily and record and score clinical signs observed post-challenge and any deaths that occur. If an animal shows marked signs of disease, it is killed. Monitor body temperatures each day for the first week after challenge. Collect blood samples from each animal on days 0, 2, 3, 4, 5, 8 and 11 post challenge. Collect urine samples from each animal on days 0, 3, 5, 8, 11, 14, 21 and 28 post challenge. Kill surviving animals at the end of the observation period. Carry out post-mortem examination on any animal that dies during the observation period and on the remainder when killed at the end of the observation period. In particular, examine the liver and kidneys for macroscopic and microscopic signs of leptospira infection. A sample of each kidney is collected and each blood, urine and kidney sample is tested for the presence of challenge organisms by re-isolation or by another suitable method. The blood samples are also analysed to detect biochemical and haematological changes indicative of infection and these are also scored.

The test is invalid if: samples give positive results on day 0; *L. interrogans* serovar challenge strain is re-isolated from or demonstrated by another suitable method to be present in fewer than 2 samples on fewer than 2 different days, to show infection has been established in fewer than 80 per cent of the control animals.

The vaccine complies with the test if: at least 80 per cent of the vaccinates show no more than mild signs of disease (for example, transient hyperthermia) and, depending on the *L. interrogans* serovar used for the challenge, one or more of the following is also shown:

- where the vaccine is intended to have a beneficial effect against clinical signs, the clinical scores and haematological and biochemical scores are statistically lower for the vaccinates than for the controls,
- where the vaccine is intended to have a beneficial effect against infection, the number of days that the organisms are detected in the blood is statistically lower for the vaccinates than for the controls,
- where the vaccine is intended to have a beneficial effect against urinary tract infection and excretion, the number of days that the organisms are detected in the urine and the number of kidney samples in which the organisms are detected is statistically lower for the vaccinates than for the controls.

LABELLING

The label states:

- the serovar(s) used to prepare the vaccine,
- the serovar(s) against which the protection is claimed.

01/2005:1955

CANINE PARAINFLUENZA VIRUS VACCINE (LIVE)

Vaccinum parainfluenzae viri canini vivum

DEFINITION

Canine parainfluenza virus vaccine (live) is a preparation of a suitable attenuated strain of canine parainfluenza virus for dogs. The vaccine is intended for the protection of dogs against respiratory signs of infection with parainfluenza virus of canine origin.

PRODUCTION

The virus is propagated in suitable cell cultures (5.2.4). The viral suspension is harvested, titrated and may be mixed with a suitable stabilising solution. The vaccine may be freeze-dried.

CHOICE OF VACCINE STRAIN

The vaccine is shown to be satisfactory with respect to safety, absence of increase in virulence and immunogenicity. The following tests may be used during demonstration of safety (5.2.6), absence of increase in virulence and immunogenicity (5.2.7).

Safety. The test is carried out for each route of administration stated on the label. Use not fewer than 5 susceptible puppies of the recommended minimum age for vaccination and that do not have antibodies against parainfluenza virus of canine origin. Administer to each puppy by a recommended route a quantity of virus corresponding to not less than 10 times the maximum titre that may be expected in a dose of vaccine. Observe the puppies for 21 days. The puppies remain in good health and there is no abnormal local or systemic reaction.

If the vaccine is intended for use in pregnant bitches, administer the virus to not fewer than 5 bitches at the recommended stage or stages of pregnancy and according to the recommended schedule. Prolong the observation period until 1 day after whelping. The dogs remain in good health and there is no abnormal local or systemic reaction. No adverse effects on the pregnancy or the offspring are noted.

Increase in virulence. Administer intranasally and by a recommended route to each of 2 puppies, 5 to 7 weeks old and which do not have antibodies against parainfluenza virus of canine origin, a quantity of virus that will allow recovery of virus for the passages described below. Use vaccine virus at the least attenuated passage level that will be present in a batch of the vaccine. Collect nasal swabs from each dog daily from 3 to 10 days after inoculation. Inoculate the suspension from the swabs into suitable cell cultures to verify the presence of virus. Use the suspension from the swabs that contain the maximum amount of virus and administer intranasally 1 ml of the suspension into each of 2 other puppies of the same age and susceptibility. This operation is then repeated at least 5 times. If the virus is not recovered at a given passage level, a second series of passages is carried out. Inoculate virus from the highest recovered passage level to not fewer than 5 puppies, observe for 21 days and compare any reactions that occur with those seen in the test for safety described above. There is no indication of an increase in virulence as compared with the non-passaged virus.

Immunogenicity. The test described under Potency is suitable to demonstrate the immunogenicity of the strain.

BATCH TESTING

If the test for potency has been carried out with satisfactory results on a representative batch of vaccine, this test may be omitted as a routine control on other batches of vaccine prepared from the same seed lot.

IDENTIFICATION

Carry out an immunofluorescence test in suitable cell cultures, using a monospecific antiserum.

TESTS

Safety. Use 2 puppies not older than the minimum age recommended for vaccination and which do not have antibodies against parainfluenza virus of canine origin. Administer a volume containing 10 doses of the vaccine into

each puppy by a recommended route. Observe for 14 days. The puppies remain in good health and no abnormal local or systemic reaction occurs.

Extraneous viruses. Neutralise the vaccine virus using a monospecific antiserum and inoculate into cell cultures known for their susceptibility to viruses pathogenic for the dog. Carry out a passage after 6 to 8 days and maintain the cultures for a total of 14 days. No cytopathic effect develops and the cells show no evidence of the presence of haemadsorbing agents.

Bacterial and fungal contamination. The vaccine, reconstituted if necessary, complies with the test for sterility prescribed in the monograph on *Vaccines for veterinary use* (0062).

Mycoplasmas (2.6.7). The vaccine, reconstituted if necessary, complies with the test for mycoplasmas.

Virus titre. Reconstitute the vaccine, if necessary, and titrate in suitable cell cultures. 1 dose of the vaccine contains not less than the quantity of virus equivalent to the minimum virus titre stated on the label.

POTENCY

Use not fewer than 15 susceptible puppies of the minimum age recommended for vaccination and which do not have antibodies against parainfluenza virus of canine origin. Vaccinate not fewer than 10 of the puppies according to the instructions for use. Keep not fewer than 5 other puppies as controls. Observe all the animals for not less than 21 days after the last vaccination. Administer by the intratracheal or intranasal route to each animal a quantity of a virulent strain of parainfluenza virus of canine origin sufficient to establish infection with the virus in a susceptible dog. Observe the animals for a further 14 days. Collect nasal swabs or washings from each dog daily from day 2 to 10 after challenge and test these samples for the presence of excreted virus. Use a scoring system to record the incidence of coughing in each dog. The test is not valid if more than 1 of the control animals shows neither coughing nor the excretion of the challenge virus. The vaccine complies with the test if the scores for coughing or virus excretion for the vaccinated animals are significantly lower than in the controls.

01/2005:0795

CANINE PARVOVIRUS VACCINE (INACTIVATED)

Vaccinum parvovirus caninae inactivatum

DEFINITION

Inactivated canine parvovirus vaccine is a liquid or freeze-dried preparation of canine parvovirus inactivated by a suitable method.

PRODUCTION

The virus is propagated in suitable cell cultures (5.2.4). The virus may be purified and concentrated.

A test for residual live virus is carried out on the bulk harvest of each batch to confirm inactivation of the canine parvovirus. The quantity of inactivated virus used in the test is equivalent to not less than 100 doses of the vaccine. The vaccine is inoculated into suitable non-confluent cells; after incubation for 8 days, a subculture is made using trypsinised cells. After incubation for a further 8 days, the

cultures are examined for residual live parvovirus by an immunofluorescence test. The immunofluorescence test may be supplemented by a haemagglutination test or other suitable tests on the supernatant of the cell cultures. No live virus is detected.

The vaccine may contain an adjuvant or adjuvants.

CHOICE OF VACCINE COMPOSITION

The vaccine is shown to be satisfactory with respect to safety and immunogenicity in dogs. The following test may be used in the demonstration of efficacy (5.2.7).

Immunogenicity. 7 susceptible dogs of the minimum age recommended for vaccination are used. A blood sample is drawn from each dog and tested individually for antibodies against canine parvovirus to determine susceptibility. 5 dogs are vaccinated according to the recommended schedule. 2 dogs are kept as controls. 20 to 22 days after the last vaccination each of the dogs receives by the oronasal route a suspension of pathogenic canine parvovirus. The dogs are observed for 14 days. Haemagglutination tests are carried out to detect virus in the faeces. The test is not valid unless the 2 control dogs show typical signs of the disease or leucopenia and excretion of the virus. The vaccine complies with the test if the 5 vaccinated dogs remain in excellent health and show no sign of the disease nor leucopenia and if the maximum titre of virus excreted in the faeces is less than 1/100 of the geometric mean of the maximum titres found in the controls.

IDENTIFICATION

When injected into dogs, the vaccine stimulates the production of antibodies against canine parvovirus.

TESTS

Safety. Use dogs of the minimum age recommended for vaccination and preferably having no canine parvovirus antibodies or, where justified, use dogs with a low level of such antibodies as long as they have not been vaccinated against canine parvovirus and administration of the vaccine does not cause an anamnestic response. Administer a double dose of vaccine by a recommended route to each of 2 dogs. Observe the animals for 14 days. No abnormal local or systemic reaction occurs.

Sterility. The vaccine complies with the test for sterility prescribed in the monograph on *Vaccines for veterinary use* (0062).

POTENCY

Carry out test A or test B.

A. Inject subcutaneously into each of 5 guinea-pigs, free from specific antibodies, half of the dose stated on the label. After 14 days, inject again half of the dose stated on the label. 14 days later, collect blood samples and separate the serum. Inactivate each serum by heating at 56 °C for 30 min. To 1 volume of each serum add 9 volumes of a 200 g/l suspension of *light kaolin R* in *phosphate buffered saline pH 7.4 R*. Shake each mixture for 20 min. Centrifuge, collect the supernatant liquid and mix with 1 volume of a concentrated suspension of pig erythrocytes. Allow to stand at 4 °C for 60 min and centrifuge. The dilution of the serum obtained is 1:10. Using each serum, prepare a series of twofold dilutions. To 0.025 ml of each of the latter dilutions add 0.025 ml of a suspension of canine parvovirus antigen containing 4 haemagglutinating units. Allow to stand at 37 °C for 30 min and add 0.05 ml of a suspension of pig erythrocytes containing 30×10^6 cells per millilitre. Allow to stand at 4 °C for 90 min and note the last dilution of serum that still completely inhibits haemagglutination.

The vaccine complies with the test if the median antibody titre of the sera collected after the second vaccination is not less than 1/80.

- B. Vaccinate, according to the schedule stated on the label, 2 healthy susceptible dogs, 8 to 12 weeks old and having antibody titres less than 4 ND₅₀ (50 per cent neutralising dose) per 0.1 ml of serum measured by the method described below. 14 days after vaccination, examine the serum of each animal as follows. Heat the serum at 56 °C for 30 min and prepare serial dilutions using a medium suitable for canine cells. Add to each dilution an equal volume of a virus suspension containing an amount of virus such that when the volume of serum-virus mixture appropriate for the assay system is inoculated into cell cultures, each culture receives approximately 10⁴ CCID₅₀. Incubate the mixtures at 37 °C for 1 h and inoculate 4 canine cell cultures with a suitable volume of each mixture. Incubate the cell cultures at 37 °C for 7 days, passage and incubate for a further 7 days. Examine the cultures for evidence of specific cytopathic effects and calculate the antibody titre. The vaccine complies with the test if the mean titre is not less than 32 ND₅₀ per 0.1 ml of serum. If one dog fails to respond, repeat the test using 2 more dogs and calculate the result as the mean of the titres obtained from all of the 3 dogs that have responded.

01/2005:0964

CANINE PARVOVIRUS VACCINE (LIVE)

Vaccinum parvovirus caninae vivum

DEFINITION

Canine parvovirus vaccine (live) is a preparation of a strain of canine parvovirus that is attenuated for the dog.

PRODUCTION

The attenuated virus is grown in suitable cell cultures (5.2.4).

The viral suspension is harvested, titrated and mixed with a suitable stabilising solution. The vaccine may be freeze-dried.

CHOICE OF VACCINE STRAIN

Only a virus strain shown to be satisfactory with respect to safety, irreversibility of attenuation, and immunogenicity may be used in the preparation of the vaccine. The following tests are used in the demonstration of safety (5.2.6) and efficacy (5.2.7).

Safety. Each test is carried out for each recommended route of administration.

5 susceptible puppies of the minimum age recommended for vaccination and having no haemagglutination-inhibiting antibodies against canine parvovirus are used for the test. A count of white blood cells in circulating blood is made on days 4, 2 and 0 before injection of the vaccine strain. Each puppy receives by a recommended route a quantity of virus corresponding to not less than 10 times the maximum virus titre that may be expected in a batch of vaccine and at the lowest passage level. The puppies are observed for 21 days. A count of white blood cells in circulating blood is made on days 3, 5, 7 and 10 after the injection. The puppies remain in good health and there is no abnormal local or systemic reaction. Any diminution in the number of circulating white blood cells is not greater than 50 per cent of the initial number determined as the average of the 3 values found before injection of the vaccine strain.

A quantity of virus corresponding to not less than 10 times the maximum titre that may be expected in a batch of vaccine and at the lowest passage level is administered by one of the recommended routes to each of 5 susceptible puppies. Five puppies are kept as controls. 2 puppies from each group are killed at 14 days and the 3 remaining puppies from each group at 21 days and histological examination of the thymus of each animal is carried out. Slight hypoplasia of the thymus may be evident after 14 days. The strain is not acceptable if damage is evident after 21 days.

Irreversibility of attenuation. Use 2 susceptible puppies of the minimum age recommended for vaccination and which do not have haemagglutination-inhibiting antibodies against canine parvovirus. Administer to each puppy, by a recommended route, a quantity of virus corresponding to 10 times the maximum titre that may be expected in a batch of vaccine. From the second to the tenth day after administration of the virus, the faeces are collected from each puppy and checked for the presence of the virus; faeces containing virus are pooled. 1 ml of the suspension of pooled faeces is administered by the oronasal route to each of 2 other puppies of the same age and susceptibility; this operation is carried out 4 times. The presence of virus is verified at each passage. If the virus is not found, a second identification of passages is carried out; if the virus is not found in one of the second identification of passages, the vaccine strain complies with the test. No puppy dies or shows signs attributable to the vaccine. No indication of increase of virulence compared to the original vaccinal virus is observed; account is taken, notably, of the count of white blood cells, of results of histological examination of the thymus and of the titre of excreted virus.

Immunogenicity. The test described under Potency is suitable to demonstrate immunogenicity of the strain.

BATCH TESTING

If the test for potency has been carried out with satisfactory results on a representative batch of vaccine, this test may be omitted as a routine control on other batches of vaccine prepared from the same seed lot, subject to agreement by the competent authority.

IDENTIFICATION

The vaccine is grown in a susceptible cell line in a substrate suitable for presenting for fluorescent antibody or immunoperoxidase tests. Suitable controls are included. A proportion of the cells is tested with a monoclonal antibody specific for canine parvovirus and a proportion of the cells tested with a monoclonal antibody specific for feline parvovirus. Canine parvovirus antigen is detected but no feline parvovirus is detected in the cells inoculated with the vaccine.

TESTS

Safety. Use 2 dogs of the minimum age recommended for vaccination and having no haemagglutination-inhibiting antibodies against canine parvovirus. Administer 10 doses of the vaccine to each dog by a recommended route. Observe for 14 days. No abnormal local or systemic reaction occurs.

Extraneous viruses. Mix the vaccine with a suitable antiserum against canine parvovirus and inoculate into cell cultures known for their susceptibility to viruses pathogenic for the dog. No cytopathic effect develops. There is no sign of haemagglutinating or haemadsorbing agents and no other sign of the presence of extraneous viruses.

Bacterial and fungal contamination. The vaccine, reconstituted if necessary, complies with the test for sterility prescribed in the monograph on *Vaccines for veterinary use* (0062).

Mycoplasmas (2.6.7). The vaccine, reconstituted if necessary, complies with the test for mycoplasmas.

Virus titre. Reconstitute the vaccine, if necessary, as stated on the label and titrate in suitable cell cultures. One dose of the vaccine contains not less than the quantity of virus equivalent to the minimum virus titre stated on the label.

POTENCY

Use 7 susceptible puppies of the minimum age recommended for vaccination and which do not have haemagglutination-inhibiting antibodies against canine parvovirus. Keep 2 of the puppies as controls and to each of the others inject by a recommended route the quantity of virus equivalent to the minimum titre stated on the label. Observe all the puppies for 20 to 22 days and then inoculate to each of them by the oronasal route a suspension of virulent canine parvovirus. Observe all the animals for 14 days. Carry out a haemagglutination test for the virus in the faeces. The test is not valid unless the 2 control puppies show typical signs of the disease and/or leucopenia and excretion of the virus. The vaccine complies with the test if the 5 vaccinated puppies remain in excellent health and show no sign of the disease nor leucopenia and if the maximum titre of virus excreted in the faeces is less than 1/100 of the geometric mean of the maximum titres found in the controls.

01/2005:0360

CLOSTRIDIUM BOTULINUM VACCINE FOR VETERINARY USE

*Vaccinum clostridii botulini
ad usum veterinarium*

DEFINITION

Clostridium botulinum vaccine for veterinary use is prepared from a culture in liquid medium of *Clostridium botulinum* type C or type D or a mixture of these types. The whole culture or its filtrate or a mixture of the two is inactivated in such a manner that toxicity is eliminated and immunogenic activity is retained.

The preparation may be adsorbed, precipitated or concentrated. It may be treated with a suitable adjuvant and may be freeze-dried.

The identification, the tests and the determination of potency apply to the liquid preparation and to the freeze-dried preparation reconstituted as stated on the label.

IDENTIFICATION

When injected into a healthy susceptible animal, the vaccine provokes the formation of specific antibodies against the type or types of *C. botulinum* from which the vaccine was prepared.

TESTS

Safety. Use 2 animals of one of the species for which the vaccine is intended and that have not been vaccinated against *C. botulinum*. Administer to each animal, by a recommended route, twice the maximum dose stated on the label. Observe the animals for 7 days. No abnormal local or systemic reaction occurs.

Residual toxicity. Inject 0.5 ml of the vaccine subcutaneously into each of 5 mice, each weighing 17 g to 22 g. Observe the animals for 7 days. No abnormal local or systemic reaction occurs.

Sterility. It complies with the test for sterility prescribed in the monograph on *Vaccines for veterinary use* (0062).

POTENCY

Use healthy white mice from a uniform stock, each weighing 18 g to 20 g. Use as challenge dose a quantity of a toxin of *C. botulinum* of the same type as that used in the preparation of the vaccine corresponding to 25 times the paralytic dose 50 per cent, a paralytic dose 50 per cent being the quantity of toxin which, when injected intraperitoneally into mice, causes paralysis in 50 per cent of the animals within an observation period of 7 days. If 2 types of *C. botulinum* have been used in the preparation of the vaccine, carry out the potency determination for each. Dilute the vaccine to be examined 1 in 8 using a 9 g/l solution of *sodium chloride R*. Inject 0.2 ml of the dilution subcutaneously into each of 20 mice. After 21 days, inject the challenge dose intraperitoneally into each of the vaccinated mice and into each of 10 control mice. Observe the mice for 7 days and record the number of animals which show signs of botulism. All the control mice show signs of botulism during the observation period. The vaccine passes the test if not fewer than 80 per cent of the vaccinated mice are protected.

LABELLING

The label states:

- the type or types of *C. botulinum* from which the vaccine has been prepared,
- whether the preparation is a toxoid or a vaccine prepared from a whole inactivated culture or a mixture of the two,
- that the preparation be shaken before use.

01/2005:0361

CLOSTRIDIUM CHAUVOEI VACCINE FOR VETERINARY USE

*Vaccinum clostridii chauvoei
ad usum veterinarium*

DEFINITION

Clostridium chauvoei vaccine for veterinary use is prepared from a culture in liquid medium of one or more suitable strains of *Clostridium chauvoei*. The whole culture is inactivated in such a manner that toxicity is eliminated and immunogenic activity is retained. Inactivated cultures may be treated with a suitable adjuvant.

IDENTIFICATION

The vaccine protects susceptible animals against infection with *C. chauvoei*.

TESTS

Safety. Use 2 animals of one of the species for which the vaccine is intended and that have not been vaccinated against *C. chauvoei*. Administer to each animal at a single site, by a recommended route, twice the maximum dose stated on the label. Observe the animals for 7 days. No abnormal local or systemic reaction occurs.

Sterility. It complies with the test for sterility prescribed in the monograph on *Vaccines for veterinary use* (0062).

POTENCY

Inject subcutaneously into not fewer than 10 healthy guinea-pigs, each weighing 350 g to 450 g, a quantity of the vaccine not exceeding the minimum dose stated on the label as the first dose. After 28 days, inject into the same animals a quantity of the vaccine not exceeding the minimum dose stated on the label as the second dose. 14 days after the second vaccination, inoculate intramuscularly into each

of the vaccinated guinea-pigs and into each of 5 control animals a suitable quantity of a virulent culture, or of a spore suspension, of *C. chauvoei*, activated if necessary with an activating agent such as calcium chloride. The vaccine complies with the test if not more than 10 per cent of the vaccinated guinea-pigs die from *C. chauvoei* infection within 5 days and all the control animals die from *C. chauvoei* infection within 48 h of challenge or within 72 h if a spore suspension was used for the challenge. If more than 10 per cent but not more than 20 per cent of the vaccinated animals die, repeat the test. The vaccine complies with the test if not more than 10 per cent of the second group of vaccinated animals die within 5 days and all of the second group of control animals die within 48 h of challenge or within 72 h if a spore suspension was used for the challenge. To avoid unnecessary suffering following virulent challenge, moribund animals are killed and are then considered to have died from *C. chauvoei* infection.

LABELLING

The label states that the preparation is to be shaken before use.

01/2005:0362
corrected

CLOSTRIDIUM NOVYI (TYPE B) VACCINE FOR VETERINARY USE

Vaccinum clostridii novyi B
ad usum veterinarium

DEFINITION

Clostridium novyi (type B) vaccine for veterinary use is prepared from a liquid culture of a suitable strain of *Clostridium novyi* (type B).

PRODUCTION

The whole culture or its filtrate or a mixture of the two is inactivated in such a manner that toxicity is eliminated and immunogenic activity is retained. Toxoids and/or inactivated cultures may be treated with a suitable adjuvant, after concentration if necessary.

CHOICE OF VACCINE COMPOSITION

The vaccine is shown to be satisfactory with respect to safety (5.2.6) and efficacy (5.2.7). For the latter, it shall be demonstrated that for each target species the vaccine, when administered according to the recommended schedule, stimulates an immune response (for example, induction of antibodies) consistent with the claims made for the product.

BATCH TESTING

Residual toxicity. The test for residual toxicity may be omitted by the manufacturer, since a test for detoxification is carried out immediately after the detoxification process and, when there is risk of reversion, a second test is carried out at as late a stage as possible during the production process.

Batch potency test. The test described under Potency is not necessarily carried out for routine testing of batches of vaccine. It is carried out for a given vaccine on one or more occasions as decided by or with the agreement of the competent authority. Where the test is not carried out, a suitable validated alternative test is carried out, the criteria

for acceptance being set with reference to a batch of vaccine that has given satisfactory results in the test described under Potency and that has been shown to be satisfactory with respect to immunogenicity in the target species. The following test may be used after a satisfactory correlation with the test described under Potency has been established.

Vaccinate rabbits as described under Potency and prepare sera. Determine the level of antibodies against the alpha toxin of *C. novyi* in the individual sera by a suitable method such as an immunochemical method (2.7.1) or neutralisation in cell cultures. Use a homologous reference serum calibrated in International Units of *C. novyi* alpha antitoxin. *Clostridia (multicomponent) rabbit antiserum BRP* is suitable for use as a reference serum. The vaccine complies with the test if the level of antibodies is not less than that found for a batch of vaccine that has given satisfactory results in the test described under Potency and that has been shown to be satisfactory with respect to immunogenicity in the target species.

IDENTIFICATION

The vaccine stimulates the formation of novyi alpha antitoxin when injected into animals that do not have this antitoxin.

TESTS

Safety. Administer by a recommended route, to each of 2 sheep that have not been vaccinated against *C. novyi* (type B) twice the maximum dose of the vaccine stated on the label. Observe the animals for not less than 14 days. No abnormal local or systemic reaction occurs.

Residual toxicity. Inject 0.5 ml of the vaccine subcutaneously into each of 5 mice, each weighing 17 g to 22 g. Observe the animals for 7 days. No abnormal local or systemic reaction occurs.

Sterility. It complies with the test for sterility prescribed in the monograph on *Vaccines for veterinary use* (0062).

POTENCY

Inject subcutaneously into each of not fewer than 10 healthy rabbits, 3 to 6 months old, a quantity of vaccine not exceeding the minimum dose stated on the label as the first dose. After 21 to 28 days, inject into the same animals a quantity of the vaccine not exceeding the minimum dose stated on the label as the second dose. 10 to 14 days after the second injection, bleed the rabbits and pool the sera.

The potency of the pooled sera is not less than 3.5 IU/ml.

The International Unit is the specific neutralising activity for *C. novyi* alpha toxin contained in a stated amount of the International Standard, which consists of a quantity of dried immune horse serum. The equivalence in International Units of the International Standard is stated by the World Health Organisation.

The potency of the pooled sera obtained from the rabbits is determined by comparing the quantity necessary to protect mice or other suitable animals against the toxic effects of a fixed dose of *C. novyi* alpha toxin with the quantity of a reference preparation of Clostridium novyi alpha antitoxin, calibrated in International Units, necessary to give the same protection. For this comparison, a suitable preparation of *C. novyi* alpha toxin for use as a test toxin is required. The dose of the test toxin is determined in relation to the reference preparation; the potency of the serum to be examined is determined in relation to the reference preparation using the test toxin.

Clostridia (multicomponent) rabbit antiserum BRP is suitable for use as a reference serum.

Preparation of test toxin. Prepare the test toxin from a sterile filtrate of an approximately 5-day culture in liquid medium of *C. novyi* type B and dry by a suitable method. Select the test toxin by determining for mice the L₅₀/10 dose and the LD₅₀, the observation period being 72 h.

A suitable alpha toxin contains not less than one L₅₀/10 dose in 0.05 mg and not less than 10 LD₅₀ in each L₅₀/10 dose.

Determination of test dose of toxin. Prepare a solution of the reference preparation in a suitable liquid so that it contains 1 IU/ml. Prepare a solution of the test toxin in a suitable liquid so that 1 ml contains a precisely known amount such as 1 mg. Prepare mixtures of the solution of the reference preparation and the solution of the test toxin such that each mixture contains 1.0 ml of the solution of the reference preparation (1 IU), one of a series of graded volumes of the solution of the test toxin and sufficient of a suitable liquid to bring the total volume to 2.0 ml. Allow the mixtures to stand at room temperature for 60 min. Using not fewer than 2 mice, each weighing 17 g to 22 g, for each mixture, inject a dose of 0.2 ml intramuscularly or subcutaneously into each mouse. Observe the mice for 72 h. If all the mice die, the amount of toxin present in 0.2 ml of the mixture is in excess of the test dose. If none of the mice die, the amount of toxin present in 0.2 ml of the mixture is less than the test dose. Prepare fresh mixtures such that 2.0 ml of each mixture contains 1.0 ml of the solution of the reference preparation (1 IU) and one of a series of graded volumes of the solution of the test toxin separated from each other by steps of not more than 20 per cent and covering the expected end-point. Allow the mixtures to stand at room temperature for 60 min. Using not fewer than two mice for each mixture, inject a dose of 0.2 ml intramuscularly or subcutaneously into each mouse. Observe the mice for 72 h. Repeat the determination at least once and combine the results of the separate tests that have been made with mixtures of the same composition so that a series of totals is obtained, each total representing the mortality due to a mixture of a given composition.

The test dose of toxin is the amount present in 0.2 ml of that mixture which causes the death of one half of the total number of mice injected with it.

Determination of the potency of the serum obtained from rabbits

Preliminary test. Dissolve a quantity of the test toxin in a suitable liquid so that 1 ml contains 10 times the test dose (solution of the test toxin). Prepare a series of mixtures of the solution of the test toxin and of the serum to be examined such that each mixture contains 1.0 ml of the solution of the test toxin, one of a series of graded volumes of the serum to be examined and sufficient of a suitable liquid to bring the final volume to 2.0 ml. Allow the mixtures to stand at room temperature for 60 min. Using not fewer than 2 mice for each mixture, inject a dose of 0.2 ml intramuscularly or subcutaneously into each mouse. Observe the mice for 72 h. If none of the mice dies, 0.2 ml of the mixture contains more than 0.1 IU. If all the mice die, 0.2 ml of the mixture contains less than 0.1 IU.

Final test. Prepare a series of mixtures of the solution of the test toxin and of the serum to be examined such that 2.0 ml of each mixture contains 1.0 ml of the solution of the test toxin and one of a series of graded volumes of the serum to be examined, separated from each other by steps of not more than 20 per cent and covering the expected end-point as determined by the preliminary test. Prepare further mixtures of the solution of the test toxin and of the solution of the reference preparation such that 2.0 ml of each mixture contains 1.0 ml of the solution of the test toxin and one of a series of graded volumes of the solution of the reference

preparation, in order to confirm the test dose of the toxin. Allow the mixtures to stand at room temperature for 60 min. Using not fewer than 2 mice for each mixture, proceed as described in the preliminary test. The test mixture which contains 0.1 IU in 0.2 ml is that mixture which kills the same or almost the same number of mice as the reference mixture containing 0.1 IU in 0.2 ml. Repeat the determination at least once and calculate the average of all valid estimates. The test is valid only if the reference preparation gives a result within 20 per cent of the expected value.

The confidence limits ($P = 0.95$) have been estimated to be:

- 85 per cent and 114 per cent when 2 animals per dose are used,
- 91.5 per cent and 109 per cent when 4 animals per dose are used,
- 93 per cent and 108 per cent when 6 animals per dose are used.

LABELLING

The label states:

- whether the product is a toxoid, a vaccine prepared from a whole inactivated culture or a mixture of the two,
- that the preparation is to be shaken before use,
- for each target species, the immunising effect produced (for example, antibody production, protection against signs of disease or infection).

01/2005:0363
corrected

CLOSTRIDIUM PERFRINGENS VACCINE FOR VETERINARY USE

Vaccinum clostridii perfringentis ad usum veterinarium

DEFINITION

Clostridium perfringens vaccine for veterinary use is prepared from liquid cultures of suitable strains of *Clostridium perfringens* type B, *C. perfringens* type C or *C. perfringens* type D or a mixture of these types.

PRODUCTION

The whole cultures or their filtrates or a mixture of the two are inactivated in such a manner that toxicity is eliminated and immunogenic activity is retained. Toxoids and/or inactivated cultures may be treated with a suitable adjuvant.

CHOICE OF VACCINE COMPOSITION

The vaccine is shown to be satisfactory with respect to safety (5.2.6) and efficacy (5.2.7). For the latter, it shall be demonstrated that for each target species the vaccine, when administered according to the recommended schedule, stimulates an immune response (for example, induction of antibodies) consistent with the claims made for the product.

BATCH TESTING

Residual toxicity. The test for residual toxicity may be omitted by the manufacturer, since a test for detoxification is carried out immediately after the detoxification process and, when there is risk of reversion, a second test is carried out at as late a stage as possible during the production process.

Batch potency test. The test described under Potency is not necessarily carried out for routine testing of batches of vaccine. It is carried out for a given vaccine on one or more occasions as decided by or with the agreement of the competent authority. Where the test is not carried out, a suitable validated alternative test is carried out, the criteria

for acceptance being set with reference to a batch of vaccine that has given satisfactory results in the test described under Potency and that has been shown to be satisfactory with respect to immunogenicity in the target species. The following test may be used after a satisfactory correlation with the test described under Potency has been established.

Vaccinate rabbits as described under Potency and prepare sera. Determine the level of antibodies against the beta and/or epsilon toxins of *C. perfringens* in the individual sera by a suitable method such as an immunochemical method (2.7.1) or neutralisation in cell cultures. Use a homologous reference serum calibrated in International Units of *C. perfringens* beta and/or epsilon antitoxin. *Clostridia (multicomponent) rabbit antiserum BRP* is suitable for use as a reference serum. The vaccine complies with the test if the level or levels of antibodies are not less than that found for a batch of vaccine that has given satisfactory results in the test described under Potency and that has been shown to be satisfactory with respect to immunogenicity in the target species.

IDENTIFICATION

Type B. The vaccine stimulates the formation of beta and epsilon antitoxins when injected into animals that do not have these antitoxins.

Type C. The vaccine stimulates the formation of beta antitoxin when injected into animals that do not have this antitoxin.

Type D. The vaccine stimulates the formation of epsilon antitoxin when injected into animals that do not have this antitoxin.

TESTS

Safety. Use 2 animals of one of the species for which the vaccine is intended and that have not been vaccinated against *C. perfringens*. Administer by a recommended route to each animal, twice the maximum dose stated on the label. Observe the animals for 14 days. No abnormal local or systemic reaction occurs.

Residual toxicity. Inject 0.5 ml of the vaccine subcutaneously into each of 5 mice, each weighing 17 g to 22 g. Observe the animals for 7 days. No abnormal local or systemic reaction occurs.

Sterility. It complies with the test for sterility prescribed in the monograph on *Vaccines for veterinary use (0062)*.

POTENCY

Inject subcutaneously into each of not fewer than 10 healthy rabbits, 3 to 6 months old, a quantity of vaccine not exceeding the minimum dose stated on the label as the first dose. After 21 to 28 days inject into the same animals a quantity of the vaccine not exceeding the minimum dose stated on the label as the second dose. 10 to 14 days after the second injection, bleed the rabbits and pool the sera.

Type B. The potency of the pooled sera is not less than 10 IU of beta antitoxin and not less than 5 IU of epsilon antitoxin per millilitre.

Type C. The potency of the pooled sera is not less than 10 IU of beta antitoxin per millilitre.

Type D. The potency of the pooled sera is not less than 5 IU of epsilon antitoxin per millilitre.

International standard for clostridium perfringens beta antitoxin

The International Unit is the specific neutralising activity for *C. perfringens* beta toxin contained in a stated amount of the International Standard which consists of a quantity of dried immune horse serum. The equivalence in International

Units of the International Standard is stated by the World Health Organisation.

International standard for clostridium perfringens epsilon antitoxin

The International Unit is the specific neutralising activity for *C. perfringens* epsilon toxin contained in a stated amount of the International Standard which consists of a quantity of dried immune horse serum. The equivalence in International Units of the International Standard is stated by the World Health Organisation.

The potency of the pooled sera obtained from the rabbits is determined by comparing the quantity necessary to protect mice or other suitable animals against the toxic effects of a fixed dose of *C. perfringens* beta toxin or *C. perfringens* epsilon toxin with the quantity of a reference preparation of clostridium perfringens beta antitoxin or clostridium perfringens epsilon antitoxin, as appropriate, calibrated in International Units, necessary to give the same protection. For this comparison, a suitable preparation of *C. perfringens* beta or epsilon toxin for use as a test toxin is required. The dose of the test toxin is determined in relation to the appropriate reference preparation; the potency of the serum to be examined is determined in relation to the appropriate reference preparation using the appropriate test toxin.

Clostridia (multicomponent) rabbit antiserum BRP is suitable for use as a reference serum.

Preparation of test toxin. Prepare the test toxin from a sterile filtrate of an early culture in liquid medium of *C. perfringens* type B, type C or type D as appropriate and dry by a suitable method. Use a beta or epsilon toxin as appropriate. Select the test toxin by determining for mice the L+ and the LD₅₀ for the beta toxin and the L+/10 dose and the LD₅₀ for the epsilon toxin, the observation period being 72 h.

A suitable beta toxin contains not less than one L+ in 0.2 mg and not less than 25 LD₅₀ in one L+ dose. A suitable epsilon toxin contains not less than one L+/10 dose in 0.005 mg and not less than 20 LD₅₀ in one L+/10 dose.

Determination of test dose of toxin. Prepare a solution of the reference preparation in a suitable liquid so that it contains 5 IU/ml for clostridium perfringens beta antitoxin and 0.5 IU/ml for clostridium perfringens epsilon antitoxin. Prepare a solution of the test toxin in a suitable liquid so that 1 ml contains a precisely known amount such as 10 mg for beta toxin and 1 mg for epsilon toxin. Prepare mixtures of the solution of the reference preparation and the solution of the test toxin such that each contains 2.0 ml of the solution of the reference preparation, one of a series of graded volumes of the solution of the test toxin and sufficient of a suitable liquid to bring the total volume to 5.0 ml. Allow the mixtures to stand at room temperature for 30 min. Using not fewer than 2 mice, each weighing 17 g to 22 g, for each mixture, inject a dose of 0.5 ml intravenously or intraperitoneally into each mouse. Observe the mice for 72 h. If all the mice die, the amount of toxin present in 0.5 ml of the mixture is in excess of the test dose. If none of the mice dies the amount of toxin present in 0.5 ml of the mixture is less than the test dose. Prepare fresh mixtures such that 5.0 ml of each mixture contains 2.0 ml of the solution of the reference preparation and one of a series of graded volumes of the solution of the test toxin separated from each other by steps of not more than 20 per cent and covering the expected end-point. Allow the mixtures to stand at room temperature for 30 min. Using not fewer than two mice for each mixture, inject a dose of 0.5 ml intravenously or intraperitoneally into each mouse. Observe the mice for 72 h. Repeat the determination at least once and add together the results of the separate tests that

have been made with mixtures of the same composition so that a series of totals is obtained, each total representing the mortality due to a mixture of given composition.

The test dose of toxin is the amount present in 0.5 ml of that mixture which causes the death of one half of the total number of mice injected with it.

Determination of the potency of the serum obtained from rabbits

Preliminary test. Dissolve a quantity of the test toxin in a suitable liquid so that 2.0 ml contains 10 times the test dose (solution of the test toxin). Prepare a series of mixtures of the solution of the test toxin and of the serum to be examined such that each contains 2.0 ml of the solution of the test toxin, one of a series of graded volumes of the serum to be examined and sufficient of a suitable liquid to bring the final volume to 5.0 ml. Allow the mixtures to stand at room temperature for 30 min. Using not fewer than 2 mice for each mixture, inject a dose of 0.5 ml intravenously or intraperitoneally into each mouse. Observe the mice for 72 h. If none of the mice die, 0.5 ml of the mixture contains more than 1 IU of beta antitoxin or 0.1 IU of epsilon antitoxin. If all the mice die, 0.5 ml of the mixture contains less than 1 IU of beta antitoxin or 0.1 IU of epsilon antitoxin.

Final test. Prepare a series of mixtures of the solution of the test toxin and the serum to be examined such that 5.0 ml of each mixture contains 2.0 ml of the solution of the test toxin and one of a series of graded volumes of the serum to be examined separated from each other by steps of not more than 20 per cent and covering the expected end-point as determined by the preliminary test. Prepare further mixtures of the solution of the test toxin and of the solution of the reference preparation such that 5.0 ml of each mixture contains 2.0 ml of the solution of the test toxin and one of a series of graded volumes of the solution of the reference preparation, in order to confirm the test dose of the toxin. Allow the mixtures to stand at room temperature for 30 min. Using not fewer than 2 mice for each mixture proceed as described in the preliminary test.

Beta antitoxin. The test mixture which contains 1 IU in 0.5 ml is that mixture which kills the same or almost the same number of mice as the reference mixture containing 1 IU in 0.5 ml.

Epsilon antitoxin. The test mixture which contains 0.1 IU in 0.5 ml is that mixture which kills the same or almost the same number of mice as the reference mixture containing 0.1 IU in 0.5 ml. Repeat the determination at least once and calculate the average of all valid estimates. The test is valid only if the reference preparation gives a result within 20 per cent of the expected value.

The confidence limits ($P = 0.95$) have been estimated to be:

- 85 per cent and 114 per cent when 2 animals per dose are used,
- 91.5 per cent and 109 per cent when 4 animals per dose are used,
- 93 per cent and 108 per cent when 6 animals per dose are used.

LABELLING

The label states:

- the type or types of *C. perfringens* from which the vaccine has been prepared,
- whether the preparation is a toxoid or a vaccine prepared from a whole inactivated culture or a mixture of the two,
- that the preparation is to be shaken before use,

- for each target species, the immunising effect produced (for example, antibody production, protection against signs of disease or infection).

01/2005:0364
corrected

CLOSTRIDIUM SEPTICUM VACCINE FOR VETERINARY USE

Vaccinum clostridii septic ad usum veterinarium

DEFINITION

Clostridium septicum vaccine for veterinary use is prepared from a liquid culture of a suitable strain of *Clostridium septicum*.

PRODUCTION

The whole culture or its filtrate or a mixture of the two is inactivated in such a manner that toxicity is eliminated and immunogenic activity is retained. Toxoid and/or inactivated cultures may be treated with a suitable adjuvant.

CHOICE OF VACCINE COMPOSITION

The vaccine is shown to be satisfactory with respect to safety (5.2.6) and efficacy (5.2.7). For the latter, it shall be demonstrated that for each target species the vaccine, when administered according to the recommended schedule, stimulates an immune response (for example, induction of antibodies) consistent with the claims made for the product.

BATCH TESTING

Residual toxicity. The test for residual toxicity may be omitted by the manufacturer, since a test for detoxification is carried out immediately after the detoxification process and, when there is risk of reversion, a second test is carried out at as late a stage as possible during the production process.

Batch potency test. The test described under Potency is not necessarily carried out for routine testing of batches of vaccine. It is carried out for a given vaccine on one or more occasions as decided by or with the agreement of the competent authority. Where the test is not carried out, a suitable validated alternative test is carried out, the criteria for acceptance being set with reference to a batch of vaccine that has given satisfactory results in the test described under Potency and that has been shown to be satisfactory with respect to immunogenicity in the target species. The following test may be used after a satisfactory correlation with the test described under Potency has been established. Vaccinate rabbits as described under Potency and prepare sera. Determine the level of antibodies against the toxin of *C. septicum* in the individual sera by a suitable method such as an immunochemical method (2.7.1) or neutralisation in cell cultures. Use a homologous reference serum calibrated in International Units of *C. septicum* antitoxin. *Clostridia (multicomponent) rabbit antiserum BRP* is suitable for use as a reference serum. The vaccine complies with the test if the level of antibodies is not less than that found for a batch of vaccine that has given satisfactory results in the test described under Potency and that has been shown to be satisfactory with respect to immunogenicity in the target species.

IDENTIFICATION

The vaccine stimulates the formation of *C. septicum* antitoxin when injected into animals that do not have this antitoxin.

TESTS

Safety. Use 2 animals of one of the species for which the vaccine is intended and that have not been vaccinated against *C. septicum*. Administer to each animal, by a recommended route, twice the maximum dose stated on the label. Observe the animals for 14 days. No abnormal local or systemic reaction occurs.

Residual toxicity. Inject 0.5 ml of the vaccine subcutaneously into each of 5 mice, each weighing 17 g to 22 g. Observe the animals for 7 days. No abnormal local or systemic reaction occurs.

Sterility. It complies with the test for sterility prescribed in the monograph on *Vaccines for veterinary use (0062)*.

POTENCY

Inject subcutaneously into each of not fewer than 10 healthy rabbits, 3 to 6 months old, a quantity of vaccine not exceeding the minimum dose stated on the label as the first dose. After 21 to 28 days, inject into the same animals a quantity of the vaccine not exceeding the minimum dose stated on the label as the second dose. 10 to 14 days after the second injection, bleed the rabbits and pool the sera.

The potency of the pooled sera is not less than 2.5 IU/ml.

The International Unit is the specific neutralising activity for *C. septicum* toxin contained in a stated amount of the International Standard which consists of a quantity of dried immune horse serum. The equivalence in International Units of the International Standard is stated by the World Health Organisation.

The potency of the pooled sera obtained from the rabbits is determined by comparing the quantity necessary to protect mice or other suitable animals against the toxic effects of a dose of *C. septicum* toxin with the quantity of a reference preparation of clostridium septicum antitoxin, calibrated in International Units, necessary to give the same protection. For this comparison, a suitable preparation of *C. septicum* toxin for use as a test toxin is required. The dose of the test toxin is determined in relation to the reference preparation; the potency of the serum to be examined is determined in relation to the reference preparation using the test toxin.

Clostridia (multicomponent) rabbit antiserum BRP is suitable for use as a reference serum.

Preparation of test toxin. Prepare the test toxin from a sterile filtrate of a 1- to 3-day culture of *C. septicum* in liquid medium and dry by a suitable method. Select the test toxin by determining for mice the $L_{+}/5$ dose and the LD_{50} the observation period being 72 h.

A suitable toxin contains not less than one $L_{+}/5$ dose in 1.0 mg and not less than 10 LD_{50} in each $L_{+}/5$ dose.

Determination of test dose of toxin. Prepare a solution of the reference preparation in a suitable liquid so that it contains 1.0 IU/ml. Prepare a solution of the test toxin in a suitable liquid so that 1 ml contains a precisely known amount, such as 4 mg. Prepare mixtures of the solution of the reference preparation and the solution of the test toxin such that each mixture contains 2.0 ml of the solution of the reference preparation (2 IU), one of a series of graded volumes of the solution of the test toxin and sufficient of a suitable liquid to bring the total volume to 5.0 ml. Allow the mixtures to stand at room temperature for 60 min. Using not fewer than 2 mice, each weighing 17 g to 22 g, for each mixture, inject a dose of 0.5 ml intravenously or intraperitoneally into each mouse. Observe the mice for 72 h. If all the mice die, the amount of toxin present in 0.5 ml of the mixture is in excess of the test dose. If none of the mice die, the amount of toxin present in 0.5 ml of the mixture is less

than the test dose. Prepare fresh mixtures such that 5.0 ml of each mixture contains 2.0 ml of the reference preparation (2 IU) and one of a series of graded volumes of the solution of the test toxin separated from each other by steps of not more than 20 per cent and covering the expected end-point. Allow the mixtures to stand at room temperature for 60 min. Using not fewer than 2 mice for each mixture, inject a dose of 0.5 ml intravenously or intraperitoneally into each mouse. Observe the mice for 72 h. Repeat the determination at least once and add together the results of the separate tests that have been made with mixtures of the same composition so that a series of totals is obtained, each total representing the mortality due to a mixture of a given composition.

The test dose of toxin is the amount present in 0.5 ml of that mixture which causes the death of one half of the total number of mice injected with it.

Determination of the potency of the serum obtained from rabbits

Preliminary test. Dissolve a quantity of the test toxin in a suitable liquid so that 2.0 ml contains ten times the test dose (solution of the test toxin). Prepare a series of mixtures of the solution of the test toxin and of the serum to be examined such that each contains 2.0 ml of the solution of the test toxin, one of a series of graded volumes of the serum to be examined and sufficient of a suitable liquid to bring the final volume to 5.0 ml. Allow the mixtures to stand at room temperature for 60 min. Using not fewer than 2 mice for each mixture, inject a dose of 0.5 ml intravenously or intraperitoneally into each mouse. Observe the mice for 72 h. If none of the mice dies, 0.5 ml of the mixture contains more than 0.2 IU. If all the mice die, 0.5 ml of the mixture contains less than 0.2 IU.

Final test. Prepare a series of mixtures of the solution of the test toxin and of the serum to be examined such that 5.0 ml of each mixture contains 2.0 ml of the solution of the test toxin and one of a series of graded volumes of the serum to be examined, separated from each other by steps of not more than 20 per cent and covering the expected end-point as determined by the preliminary test. Prepare further mixtures of the solution of the test toxin and of the solution of the reference preparation such that 5.0 ml of each mixture contains 2.0 ml of the solution of the test toxin and one of a series of graded volumes of the solution of the reference preparation to confirm the test dose of the toxin. Allow the mixtures to stand at room temperature for 60 min. Using not fewer than 2 mice for each mixture proceed as described in the preliminary test. The test mixture which contains 0.2 IU in 0.5 ml is that mixture which kills the same or almost the same number of mice as the reference mixture containing 0.2 IU in 0.5 ml. Repeat the determination at least once and calculate the average of all valid estimates. The test is valid only if the reference preparation gives a result within 20 per cent of the expected value.

The confidence limits ($P = 0.95$) have been estimated to be:

- 85 per cent and 114 per cent when 2 animals per dose are used,
- 91.5 per cent and 109 per cent when 4 animals per dose are used,
- 93 per cent and 108 per cent when 6 animals per dose are used.

LABELLING

The label states:

- whether the preparation is a toxoid or a vaccine prepared from a whole inactivated culture, or a mixture of the two,
- that the preparation is to be shaken before use,

- for each target species, the immunising effect produced (for example, antibody production, protection against signs of disease or infection).

01/2005:0449

DISTEMPER VACCINE (LIVE) FOR MUSTELIDS, FREEZE-DRIED

*Vaccinum morbi Carrei vivum
cryodesiccatum ad mustelidas*

DEFINITION

Freeze-dried distemper vaccine (live) for mustelids is a preparation of a strain of distemper virus that is attenuated for ferrets.

PRODUCTION

The attenuated strain is grown in suitable cell cultures (5.2.4) or in fertilised hen eggs, obtained from healthy flocks.

CHOICE OF VACCINE STRAIN

Only a virus strain shown to be satisfactory with respect to attenuation and immunogenicity may be used in the preparation of the vaccine. The following tests may be used during demonstration of safety (5.2.6) and immunogenicity (5.2.7).

Attenuation. When a volume of a viral suspension equivalent to five doses of the vaccine is injected intramuscularly into each of two susceptible ferrets, it does not provoke pathogenic effects within 21 days.

Immunogenicity. The test described under Potency is suitable to demonstrate the immunogenicity of the strain.

BATCH TESTING

If the test for potency has been carried out with satisfactory results on a representative batch of vaccine, this test may be omitted as a routine control on other batches of vaccine prepared from the same seed lot, subject to agreement by the competent authority.

IDENTIFICATION

The vaccine reconstituted as stated on the label and mixed with a specific distemper antiserum no longer provokes cytopathic effects in susceptible cell cultures or lesions on the chorio-allantoic membranes of fertilised hen eggs 9 to 11 days old.

TESTS

Safety. Inject by the route stated on the label twice the dose of the reconstituted vaccine into each of two susceptible ferrets free from distemper-virus-neutralising antibodies. Observe the animals for 21 days. They remain in normal health and no abnormal local or systemic reaction occurs.

Extraneous viruses. Mix the reconstituted vaccine with a monospecific antiserum. It no longer provokes cytopathic effects in susceptible cell cultures. It shows no evidence of haemagglutinating or haemadsorbing agents.

Bacterial and fungal contamination. The reconstituted vaccine complies with the test for sterility prescribed in the monograph on *Vaccines for veterinary use (0062)*.

Mycoplasmas (2.6.7). The reconstituted vaccine complies with the test for mycoplasmas.

Virus titre. Titrate the reconstituted vaccine in suitable cell cultures or fertilised hen eggs 9 to 11 days old. One dose of the vaccine contains not less than the quantity of virus equivalent to the minimum titre stated on the label.

POTENCY

Use seven susceptible ferrets free from distemper-virus-neutralising antibodies. Vaccinate five of the ferrets according to the instructions for use. Keep the two other animals as controls. Observe all the animals for 21 days. Inject intramuscularly into each animal a quantity of distemper virus sufficient to cause the death of a susceptible ferret. Observe the animals for a further 21 days. The test is invalid if one or both of the control ferrets do not die of distemper. The vaccine complies with the test if the vaccinated ferrets remain in normal health.

01/2005:1315

DUCK VIRAL HEPATITIS TYPE I VACCINE (LIVE)

*Vaccinum hepatitis viralis anatis
stirpe I vivum*

1. DEFINITION

Duck viral hepatitis type I vaccine (live) is a preparation of a suitable strain of duck hepatitis virus type I. This monograph applies to vaccines intended for the active immunisation of breeder ducks in order to protect passively their progeny and/or for the active immunisation of ducklings.

2. PRODUCTION

2-1. PREPARATION OF THE VACCINE

The vaccine virus is grown in embryonated hens' eggs or in cell cultures.

2-2. SUBSTRATE FOR VIRUS PROPAGATION

2-2-1. Embryonated hens' eggs. If the vaccine virus is grown in embryonated hens' eggs, they are obtained from flocks free from specified pathogens (SPF) (5.2.2).

2-2-2. Cell cultures. If the vaccine virus is grown in cell cultures, they comply with the requirements for cell cultures for production of veterinary vaccines (5.2.4).

2-3. SEED LOTS

2-3-1. Extraneous agents. The master seed lot complies with the tests for extraneous agents in seed lots (2.6.24). In these tests on the master seed lot, the organisms used are not more than 5 passages from the master seed lot at the start of the tests.

2-4. CHOICE OF VACCINE VIRUS

The vaccine virus shall be shown to be satisfactory with respect to safety (5.2.6) and efficacy (5.2.7) for the ducks for which it is intended.

The following tests for safety (section 2-4-1), increase in virulence (section 2-4-2) and immunogenicity (section 2-4-3) may be used during demonstration of safety and immunogenicity.

2-4-1. Safety. Carry out the test for each route and method of administration to be recommended for vaccination using in each case ducks not older than the youngest age to be recommended for vaccination. Use vaccine virus at the least attenuated passage level that will be present between the master seed lot and a batch of the vaccine. For each test, use not fewer than 20 susceptible domestic ducklings (*Anas platyrhynchos*) that do not have antibodies against duck hepatitis virus type I. Administer to each duckling a quantity of vaccine virus equivalent to not less than 10 times the maximum virus titre likely to be contained in 1 dose of vaccine. Observe the ducklings at least daily for 21 days. The test is not valid if more than 10 per cent of the ducklings

die from causes not attributable to the vaccine virus. The vaccine virus complies with the test if no duckling shows notable signs of duck viral hepatitis or dies from causes attributable to the vaccine virus.

2-4-2. Increase in virulence. The test for increase in virulence consists of the administration of the vaccine virus at the least attenuated passage level that will be present between the master seed lot and a batch of vaccine to a group of five 1-day-old domestic ducklings that do not have antibodies against duck hepatitis virus type I, sequential passages, 5 times where possible, to further similar groups of 1-day-old ducklings and testing of the final recovered virus for increase in virulence. If the properties of the vaccine virus allow sequential passage to 5 groups via natural spreading, this method may be used, otherwise passage as described below is carried out and the maximally passaged virus that has been recovered is tested for increase in virulence. Care must be taken to avoid contamination by virus from previous passages. Administer by the oro-nasal route a quantity of vaccine virus that will allow recovery of virus for the passages described below. 2 to 4 days later, take samples of liver from each duckling and pool the samples. Administer 1 ml of the pooled liver suspension by the oro-nasal route to each of five 1-day-old seronegative domestic ducklings. Carry out this operation 5 times. Verify the presence of the virus at each passage. If the virus is not found at a passage level, carry out a second series of passages. Observe the ducklings given the last passage at least daily for 21 days. Compare the results obtained with unpassaged virus in the safety studies and those obtained with maximally passaged virus to assess the degree of increase in virulence. If the virus is not recovered at any passage level in the first and second series of passages, it is considered not to show increase in virulence.

2-4-3. Immunogenicity. A test is carried out for each route and method of administration to be recommended, using in each case domestic ducks not older than the youngest age to be recommended for vaccination. The quantity of the vaccine virus administered to each bird is not greater than the minimum virus titre to be stated on the label and the virus is at the most attenuated passage level that will be present in a batch of the vaccine.

2-4-3-1. Vaccines for passive immunisation of ducklings. Use for the test not fewer than 15 laying ducks or ducks intended for laying, as appropriate, of the same origin and that do not have antibodies against duck hepatitis virus type I. Vaccinate by a recommended route not fewer than 10 ducks using the schedule to be recommended. Maintain not fewer than 5 ducks as controls. Starting from 4 weeks after onset of lay, collect embryonated eggs from vaccinated and control ducks and incubate them. Challenge not fewer than twenty 1-week-old ducklings representative of the vaccinated group and not fewer than 10 from the control group by the oro-nasal route with a sufficient quantity of virulent duck hepatitis virus type I. Observe the ducklings at least daily for 14 days after challenge. Record the deaths and the number of surviving ducklings that show clinical signs of disease.

The test is not valid if:

- during the observation period after challenge fewer than 70 per cent of the challenged ducklings from the control ducks die or show typical signs of the disease,
- and/or during the period between vaccination and collection of the eggs more than 10 per cent of the control or vaccinated ducks show abnormal clinical signs or die from causes not attributable to the vaccine.

The vaccine virus complies with the test if during the observation period after challenge the percentage relative protection calculated using the following expression is not less than 80 per cent:

$$\frac{V - C}{100 - C} \times 100$$

- V* = percentage of challenged ducklings from vaccinated ducks that survive to the end of the observation period without clinical signs of the disease.
- C* = percentage of challenged ducklings from unvaccinated control ducks that survive to the end of the observation period without clinical signs of the disease.

2-4-3-2. Vaccines for active immunisation of ducklings. Use for the test not fewer than 30 ducklings of the same origin and that do not have antibodies against duck hepatitis virus type I. Vaccinate by a recommended route not fewer than 20 ducklings. Maintain not fewer than 10 ducklings as controls. Challenge each duckling after at least 5 days by the oro-nasal route with a sufficient quantity of virulent duck hepatitis virus type I. Observe the ducklings at least daily for 14 days after challenge. Record the deaths and the number of surviving ducklings that show clinical signs of disease.

The test is not valid if:

- during the observation period after challenge fewer than 70 per cent of the control ducklings die or show typical signs of the disease,
- and/or during the period between vaccination and challenge more than 10 per cent of the control or vaccinated ducklings show abnormal clinical signs or die from causes not attributable to the vaccine.

The vaccine virus complies with the test if during the observation period after challenge the percentage relative protection calculated using the following expression is not less than 80 per cent:

$$\frac{V - C}{100 - C} \times 100$$

- V* = percentage of challenged vaccinated ducklings that survive to the end of the observation period without clinical signs of the disease.
- C* = percentage of challenged unvaccinated control ducklings that survive to the end of the observation period without clinical signs of the disease.

3. BATCH TESTS

3-1. Identification. The vaccine, diluted if necessary and mixed with a monospecific duck hepatitis virus type I antiserum, no longer infects embryonated hens' eggs from an SPF flock (5.2.2) or susceptible cell cultures (5.2.4) into which it is inoculated.

3-2. Bacteria and fungi

Vaccines intended for administration by injection comply with the test for sterility prescribed in the monograph *Vaccines for veterinary use (0062)*.

Vaccines not intended for administration by injection either comply with the test for sterility prescribed in the monograph *Vaccines for veterinary use (0062)* or with the following test: carry out a quantitative test for bacterial and fungal contamination; carry out identification tests for

micro-organisms detected in the vaccine; the vaccine does not contain pathogenic micro-organisms and contains not more than 1 non-pathogenic micro-organism per dose.

Any liquid supplied with the vaccine complies with the test for sterility prescribed in the monograph *Vaccines for veterinary use* (0062).

3-3. Mycoplasmas. The vaccine complies with the test for mycoplasmas (2.6.7).

3-4. Extraneous agents. The vaccine complies with the tests for extraneous agents in batches of finished product (2.6.25).

3-5. Safety. Use not fewer than 10 domestic ducks that do not have antibodies against duck hepatitis virus type I and of the youngest age recommended for vaccination. For vaccines recommended for use in ducks older than 2 weeks, ducks 2 weeks old may be used. Administer by a recommended route and method to each duck 10 doses of the vaccine. Observe the ducks at least daily for 21 days. The test is not valid if more than 20 per cent of ducks show abnormal clinical signs or die from causes not attributable to the vaccine. The vaccine complies with the test if no duck shows notable clinical signs of disease or dies from causes attributable to the vaccine.

3-6. Virus titre. Titrate the vaccine virus by inoculation into embryonated hens' eggs from an SPF flock (5.2.2) or into suitable cell cultures (5.2.4). The vaccine complies with the test if 1 dose contains not less than the minimum virus titre stated on the label.

3-7. Potency. Depending on the indications, the vaccine complies with 1 or both of the tests prescribed under Immunogenicity (section 2-4-3), when administered by a recommended route and method. It is not necessary to carry out the potency test for each batch of the vaccine if it has been carried out on a representative batch using a vaccinating dose containing not more than the minimum virus titre stated on the label.

4. LABELLING

If it has been found that the vaccine may show reversion to virulence, the label indicates the precautions necessary to avoid transmission of virulent virus to unvaccinated ducklings.

01/2005:1202

EGG DROP SYNDROME '76 VACCINE (INACTIVATED)

*Vaccinum morbi partus diminutionis
MCMLXXVI inactivatum ad pullum*

DEFINITION

Egg drop syndrome '76 vaccine (inactivated) consists of an emulsion or a suspension of a suitable strain of egg drop syndrome '76 virus (haemagglutinating avian adenovirus) which has been inactivated in such a manner that immunogenic activity is retained.

PRODUCTION

The vaccine strain is propagated in fertilised hen or duck eggs from healthy flocks or in suitable cell cultures (5.2.4).

The test for inactivation is carried out in fertilised duck eggs from a flock free from egg drop syndrome '76 virus infection or hen eggs from a flock free from specified pathogens

(5.2.2), or in suitable cell cultures, whichever is the most sensitive for the vaccine strain; the quantity of virus used in the test is equivalent to not less than ten doses of the vaccine. No live virus is detected.

The vaccine may contain adjuvants.

CHOICE OF VACCINE COMPOSITION

The vaccine is shown to be satisfactory with respect to safety and immunogenicity. The following test may be used during demonstration of efficacy (5.2.7).

Immunogenicity. The test described under Potency is suitable to demonstrate immunogenicity.

BATCH TESTING

The test described under Potency is not necessarily carried out for routine testing of batches of vaccine. It is carried out, for a given vaccine, on one or more occasions, as decided by or with the agreement of the competent authority; where the test is not carried out, an alternative validated method is used, the criteria for acceptance being set with reference to a batch of vaccine that has given satisfactory results in the test described under Potency. The following test may be used after a satisfactory correlation with the test described under Potency has been established.

Batch potency test. Vaccinate not fewer than ten 14- to 28-day-old chickens from a flock free from specified pathogens (5.2.2) with one dose of vaccine by one of the recommended routes. Four weeks later, collect serum samples from each bird and from five unvaccinated control birds of the same age and from the same source. Measure the antibody response in a haemagglutination (HA) inhibition test on each serum using four HA units of antigen and chicken erythrocytes. The test is not valid if there are specific antibodies in the sera of the unvaccinated birds. The vaccine complies with the test if the mean titre of the vaccinated group is not less than that found previously for a batch of vaccine that has given satisfactory results in the test described under Potency.

IDENTIFICATION

In chickens with no antibodies to egg drop syndrome '76 virus, the vaccine stimulates the production of specific antibodies.

TESTS

Safety. Inject a double dose by a recommended route into each of 10 chickens, 14 to 28 days old, from a flock free from specified pathogens (5.2.2). Observe the birds for 21 days. No abnormal local or systemic reaction occurs.

Inactivation

A. For a vaccine prepared in eggs, carry out the test in fertilised duck eggs from a flock free from egg drop syndrome '76 virus infection or, if it is known to provide a more sensitive test system, in hen eggs from a flock free from specified pathogens (5.2.2).

Inject two-fifths of a dose into the allantoic cavity of each of ten 10- to 14-day-old fertilised eggs that are free from parental antibodies to egg drop syndrome '76 virus. Incubate the eggs and observe for 8 days. Pool separately the allantoic fluid from eggs containing live embryos, and that from eggs containing dead embryos, excluding those that die from non-specific causes within 24 h of the injection.

Inject into the allantoic cavity of each of ten 10- to 14-day-old fertilised eggs that are free from parental antibodies to egg drop syndrome '76 virus, 0.2 ml of the pooled allantoic fluid from the live embryos and into each of ten similar eggs, 0.2 ml of the pooled allantoic fluid

from the dead embryos and incubate for 8 days. Examine the allantoic fluid from each egg for the presence of haemagglutinating activity using chicken erythrocytes.

If more than 20 per cent of the embryos die at either stage, repeat that stage. The vaccine complies with the test if there is no evidence of haemagglutinating activity and if, in any repeat test, not more than 20 per cent of the embryos die from non-specific causes.

Antibiotics may be used in the test to control extraneous bacterial infection.

- B. For a vaccine adapted to growth in cell cultures, inoculate ten doses into suitable cell cultures. If the vaccine contains an oily adjuvant, eliminate it by suitable means. Incubate the cultures at $38 \pm 1^\circ\text{C}$ for 7 days. Make a passage on another set of cell cultures and incubate at $38 \pm 1^\circ\text{C}$ for 7 days. Examine the cultures regularly and at the end of the incubation period examine the supernatant liquid for the presence of haemagglutinating activity. The vaccine complies with the test if the cell cultures show no sign of infection and if there is no haemagglutinating activity in the supernatant liquid.

Extraneous agents. Use the chickens from the test for safety. 21 days after injection of the double dose of vaccine, inject one dose by the same route into each chicken. Collect serum samples from each chicken 2 weeks later and carry out tests for antibodies to the following agents by the methods prescribed for chicken flocks free from specified pathogens (5.2.2): avian encephalomyelitis virus, avian leucosis viruses, infectious bronchitis virus, infectious bursal disease virus, infectious laryngotracheitis virus, influenza A virus, Marek's disease virus, Newcastle disease virus and, for vaccine produced in duck eggs, *Chlamydia* (by a complement-fixation test or agar gel precipitation test), duck hepatitis virus type I (by a fluorescent-antibody test or serum-neutralisation test) and Derzsy's disease virus (by a serum-neutralisation test). The vaccine does not stimulate the formation of antibodies against these agents.

Sterility. The vaccine complies with the test for sterility prescribed in the monograph on *Vaccines for veterinary use* (0062).

POTENCY

Vaccinate each of 2 groups of 30 hens from a flock free from specified pathogens (5.2.2) and of the age at which vaccination is recommended. Maintain two control groups one of 10 hens and the other of 30 hens, of the same age and from the same source as the vaccinates. Keep individual egg production records from point of lay until 4 weeks after challenge.

At 30 weeks of age, 1 group of 30 vaccinates and the group of 10 control birds are challenged with a dose of egg drop syndrome '76 virus sufficient to cause a well marked drop in egg production and/or quality. The vaccine complies with the test if the vaccinated birds show no marked drop in egg production and/or quality. The test is not valid unless there is a well marked drop in egg production and/or quality in the control birds.

When the second group of vaccinated birds and the group of 30 control birds are nearing the end of lay, challenge these birds, as before. The vaccine complies with the test if the vaccinated birds show no marked drop in egg production and/or quality. The test is not valid unless there is a well marked drop in egg production and/or quality in the control birds.

Carry out serological tests on serum samples obtained at the time of vaccination, 4 weeks later and just prior to challenge. The test is not valid if antibodies to egg drop syndrome '76 virus are detected in any sample from control birds.

LABELLING

The label states whether the strain in the vaccine is duck- or hen-embryo-adapted or cell-culture-adapted.

01/2005:1613

EQUINE HERPESVIRUS VACCINE (INACTIVATED)

Vaccinum herpesvirus equini inactivatum

DEFINITION

Equine herpesvirus vaccine (inactivated) is a preparation of one or more suitable strains of equid herpesvirus 1 and/or equid herpesvirus 4 inactivated without impairing their immunogenic activity or a suspension of an inactivated fraction of the virus.

PRODUCTION

Each strain of virus is propagated separately in suitable cell cultures (5.2.4). The viral suspensions may be purified and concentrated and are inactivated; they may be treated to fragment the virus and the viral fragments may be purified and concentrated.

The test for residual infectious equid herpesvirus is carried out using 2 passages in the same type of cell culture as that used in the production or in cell cultures shown to be at least as sensitive. The quantity of inactivated virus used in the test is equivalent to not less than 25 doses of the vaccine. No live virus is detected.

The vaccine may contain a suitable adjuvant.

CHOICE OF VACCINE COMPOSITION

The vaccine is shown to be satisfactory with respect to safety (5.2.6) and immunogenicity (5.2.7) in horses. Where a particular breed of horse is known to be especially sensitive to the vaccine, horses from that breed are included in the test for safety. The following tests may be used during demonstration of safety and immunogenicity.

Safety. A test is carried out in each category of animals for which the vaccine is intended and by each recommended route. 2 doses of vaccine are administered to each of not fewer than 10 animals which have not been previously vaccinated with an equine herpesvirus vaccine, which have at most a low antibody titre not indicative of recent infection and which do not excrete equid herpesvirus. After 14 days, 1 dose of vaccine is injected into each of the animals. The animals are observed for a further 14 days. During the 28 days of the test, no abnormal or systemic reaction occurs. If the vaccine is intended for use in pregnant horses, for the test in this category of animal, mares are vaccinated during the relevant trimester or trimesters of pregnancy and the observation period is prolonged up to foaling; any effects on gestation or the offspring are noted.

Immunogenicity. The claims of the product reflect the type of immunogenicity demonstrated (protection against the disease of the respiratory tract and/or protection against abortion). The tests described under Potency are suitable to demonstrate the immunogenicity of the strains present in the vaccine.

BATCH TESTING

The test described under Potency is not carried out for routine testing of batches of vaccine. It is carried out, for a given vaccine on one or more occasions; where the test is not carried out, an alternative validated method is used, the criteria for acceptance being set with reference to a batch of vaccine that has given satisfactory results in the tests described under Potency. The following test may be used.

Batch potency test. Vaccinate not fewer than 5 rabbits, guinea-pigs or mice with a single injection of a suitable dose. Where the schedule stated on the label requires a second injection to be given, the recommended schedule may be used in laboratory animals provided it has been demonstrated that this will still provide a suitably sensitive test system. At a given interval within the range of 14 to 21 days after the last injection, collect blood from each animal and prepare serum samples. Use a suitable validated test such as an enzyme-linked immunosorbent assay to measure the response to each of the antigens stated on the label. The antibody levels are not significantly less than those obtained with a batch that has given satisfactory results in the test described under Potency.

IDENTIFICATION

In animals having no antibodies against equid herpesvirus 1 and equid herpesvirus 4 or a fraction of the viruses, the vaccine stimulates the production of specific antibodies against the virus type or types included in the product. The method used must distinguish between antibodies against equid herpesviruses 1 and 4.

TESTS

Safety. Use for the test horses that have not been vaccinated against equid herpesviruses 1 and 4. Administer by a recommended route twice the vaccinating dose into each of not fewer than 2 horses. After 2 weeks, administer a single dose to each of the animals. Observe the animals for a further 10 days. The animals remain in good health and no abnormal local or systemic reaction occurs.

Inactivation. Carry out a test for residual infectious equid herpesvirus using not less than 25 doses of vaccine by inoculating cell cultures sensitive to equid herpesviruses 1 and 4; make a passage after 5 to 7 days and maintain the cultures for 14 days. No live virus is detected. If the vaccine contains an adjuvant, separate the adjuvant from the liquid phase, by a method that does not inactivate or otherwise interfere with the detection of live virus, or carry out a test for inactivation on the mixture of bulk antigens before addition of the adjuvant.

Sterility. The vaccine complies with the test for sterility prescribed in the monograph on *Vaccines for veterinary use* (0062).

POTENCY

The type of potency test depends on the claims for the product. For vaccines intended to protect against the disease of the respiratory tract carry out test A, using equid herpesvirus 1 and/or equid herpesvirus 4 depending on the claims for protection. For vaccines intended to protect against abortion carry out test B.

Use horses which have not been vaccinated with an equine herpesvirus vaccine, which have at most a low antibody titre not indicative of recent infection, and which do not excrete equid herpesvirus. To demonstrate that no recent infection occurs, immediately before vaccination: draw a blood sample from each animal and test individually for antibodies against equid herpesviruses 1 and 4; collect 10 ml of heparinised blood and test the washed leucocytes for equid

herpesviruses 1 and 4; collect a nasopharyngeal swab and test for equid herpesviruses 1 and 4. There is no indication of an active infection. Immediately before challenge collect a nasopharyngeal swab and test for equid herpesviruses 1 and 4. If there is an indication of virus excretion remove the animal from the test. Keep the horses in strict isolation.

- A. Use not fewer than 10 horses, not less than 6 months old. Vaccinate not fewer than 6 horses using the recommended schedule. Keep not fewer than 4 unvaccinated horses as controls. At least 2 weeks after the last vaccination, administer by nasal instillation to all horses a quantity of equid herpesvirus 1 or 4, sufficient to produce in a susceptible horse characteristic signs of the disease such as pyrexia and virus excretion (and possibly nasal discharge and coughing). Observe the horses for 14 days. Collect nasopharyngeal swabs daily from each individual animal to isolate the virus. The vaccinated horses show no more than slight signs; the signs in vaccinates are less severe than in controls. The average number of days on which virus is excreted, and the respective virus titres are significantly lower in vaccinated horses than in controls.
- B. Use not fewer than 10 pregnant mares. In addition to the testing described above, 6, 4, 3, 2 and 1 month before the first vaccination draw a blood sample from each horse and test individually for antibodies against equid herpesvirus 1 and equid herpesvirus 4. There is no evidence of recent infection or virus excretion. Vaccinate not fewer than 6 mares using the recommended schedule. Keep not fewer than 4 horses as unvaccinated controls. Between day 260 and 290 of pregnancy but not earlier than 3 weeks after the last vaccination administer by nasal instillation to all horses a quantity of equid herpesvirus 1 sufficient to produce abortion in susceptible mares. Observe the mares up to foaling or abortion. Collect samples of fetal lung and liver tissues from aborted fetuses and carry out tests for virus in cell cultures. The test is invalid if more than one control horse gives birth to a healthy foal and if the challenge virus is not isolated from the aborted fetuses. The vaccine complies with the test if not more than one vaccinated mare aborts.

LABELLING

The label states:

- the strains of virus included in the vaccine,
- the claims of protection.

01/2005:0249
corrected

EQUINE INFLUENZA VACCINE (INACTIVATED)

Vaccinum influenzae equi inactivatum

DEFINITION

Equine influenza vaccine (inactivated) is a preparation of one or more suitable strains of equine influenza virus, inactivated in such a manner that immunogenic activity is maintained. Suitable strains contain both haemagglutinin and neuraminidase.

PRODUCTION

Each strain of virus is propagated separately in fertilised hen eggs from healthy flocks or in suitable cell cultures (5.2.4). The viral suspensions may be purified and concentrated. The antigen content of the vaccine is based on the haemagglutinin content of the viral suspensions

determined as described under In-process Tests; the amount of haemagglutinin for each strain is not less than that in the vaccine shown to be satisfactory in the test for potency.

The test for residual infectious influenza virus is carried out using method A or method B whichever is the more sensitive. The quantity of inactivated virus used is equivalent to not less than 10 doses of vaccine.

- A. The vaccine is inoculated into suitable cells; after incubation for 8 days, a subculture is made. It is incubated for a further 6 to 8 days. Harvest about 0.1 ml of the supernatant and examine for live virus by a haemagglutination test. If haemagglutination is found, carry out a further passage in cell culture and test for haemagglutination; no haemagglutination occurs.
- B. Inoculate 0.2 ml into the allantoic cavity of each of 10 fertilised eggs and incubate at 33 °C to 37 °C for 3 to 4 days. The test is not valid unless not fewer than 8 of the 10 embryos survive. Harvest 0.5 ml of the allantoic fluid from each surviving embryo and pool the fluids. Inoculate 0.2 ml of the pooled fluid into a further 10 fertilised eggs and incubate at 33 °C to 37 °C for 3 to 4 days. The test is not valid unless at least 8 of the 10 embryos survive. Harvest about 0.1 ml of the allantoic fluid from each surviving embryo and examine each individual harvest for live virus by a haemagglutination test. If haemagglutination is found for any of the fluids, carry out a further passage of that fluid in eggs and test for haemagglutination; no haemagglutination occurs.

The vaccine may contain suitable adjuvants.

CHOICE OF VACCINE COMPOSITION

The choice of strains used in the vaccine is based on epidemiological data. The Office international des épizooties reviews the epidemiological data periodically and if necessary recommends new strains corresponding to prevailing epidemiological evidence. Such strains are used in accordance with the regulations in force in the signatory States of the Convention on the Elaboration of a European Pharmacopoeia.

The vaccine is shown to be satisfactory with respect to safety and immunogenicity in horses. Where a particular breed of horse is known to be especially sensitive to the vaccine, horses from that breed are included in the tests for safety. The following tests may be used during demonstration of safety (5.2.6) and efficacy (5.2.7).

Safety. A test is carried out in each category of animals for which the vaccine is intended and by each recommended route. Use animals that preferably have no equine influenza antibodies or, where justified, use animals with a low level of such antibodies as long as they have not been vaccinated against equine influenza and administration of the vaccine does not cause an anamnestic response. 2 doses of vaccine are injected by the intended route into each of not fewer than 10 animals. After 14 days, 1 dose of vaccine is injected into each of the animals. The animals are observed for a further 14 days. During the 28 days of the test, no abnormal local or systemic reaction occurs. If the vaccine is intended for use in pregnant horses, for the test in this category of animal, horses are vaccinated during the relevant trimester or trimesters of pregnancy and the observation period is prolonged up to foaling; any effects on gestation or the offspring are noted.

Immunogenicity. The test described under Potency is suitable to demonstrate the immunogenicity of the strains present in the vaccine.

A test with virulent challenge is carried out for at least one vaccine strain. For other strains in the vaccine, demonstration of immunogenicity may, where justified, be

based on the serological response induced in horses by the vaccine; justification for protection against these strains may be based on published data on the correlation of the antibody titre with protection against antigenically related strains.

Where serology is used, the test is carried out as described under Potency but instead of virulent challenge, a blood sample is drawn 2 weeks after the last vaccination and the antibody titre of each serum is determined by a suitable immunochemical method (2.7.1), such as the single radial haemolysis test or the haemagglutination-inhibition test shown below; a reference serum is used to validate the test. The acceptance criteria depend on the strain and are based on available data; for A/equine-2 virus, vaccines have usually been found satisfactory if the antibody titre of each serum is not less than 85 mm² where the single radial haemolysis test is used, or not less than 1:64 (before mixture with the suspension of antigen and erythrocytes) where the haemagglutination-inhibition test is used.

Equine influenza subtype 1 horse antiserum BRP, equine influenza subtype 2 American-like horse antiserum BRP and equine influenza subtype 2 European-like horse antiserum BRP are suitable for use as reference sera for the single radial haemolysis test.

The claims for the product reflect the type of immunogenicity demonstrated (protection against challenge or antibody production).

Single radial haemolysis. Heat each serum at 56 °C for 30 min. Perform tests on each serum using respectively the antigen or antigens prepared from the strain(s) used in the production of the vaccine. Mix 1 ml of sheep erythrocyte suspension in barbital buffer solution (1 volume of erythrocytes in 10 volumes of final suspension) with 1 ml of a suitable dilution of the influenza virus strain in barbital buffer solution and incubate the mixture at 4 °C for 30 min. To 2 ml of the virus/erythrocyte mixture, add 1 ml of a 3 g/l solution of *chromium(III) chloride hexahydrate R*, mix and allow to stand for 10 min. Heat the sensitised erythrocytes to 47 °C in a water-bath. Mix 15 ml of a 10 g/l solution of *agarose for electrophoresis R* in barbital buffer solution, 0.7 ml of sensitised erythrocyte suspension and the appropriate amount of diluted guinea-pig complement in barbital buffer solution at 47 °C. Pour the mixture into Petri dishes and allow the agar to set. Punch holes in the agar layer and place in each hole, 5 µl of the undiluted serum to be tested or control serum. Incubate the Petri dishes at 37 °C for 18 h. Measure the diameter of the haemolysis zone and calculate its area, which expresses the antibody titre, in square millimetres.

Equine influenza subtype 1 horse antiserum BRP, equine influenza subtype 2 American-like horse antiserum BRP and equine influenza subtype 2 European-like horse antiserum BRP are suitable for use as reference sera for the single radial haemolysis test.

Haemagglutination-inhibition test. Inactivate each serum by heating at 56 °C for 30 min. To one volume of each serum add three volumes of *phosphate-buffered saline pH 7.4 R* and four volumes of a 250 g/l suspension of *light kaolin R* in the same buffer solution. Shake each mixture for 10 min. Centrifuge, collect the supernatant liquid and mix with a concentrated suspension of chicken erythrocytes. Allow to stand at 37 °C for 60 min and centrifuge. The dilution of the serum obtained is 1:8. Perform tests on each serum using each antigen prepared from the strains used in the production of the vaccine. Using each diluted serum, prepare a series of twofold dilutions. To 0.025 ml of each of the latter dilutions add 0.025 ml of a suspension of antigen treated with *ether R* and containing four haemagglutinating units. Allow the mixture to stand for 30 min and add 0.05 ml of a suspension

of chicken erythrocytes containing 2×10^7 erythrocytes/ml. Allow to stand for 1 h and note the last dilution of serum that still completely inhibits haemagglutination.

IN-PROCESS TESTS

The content of haemagglutinin in the inactivated virus suspension, after purification and concentration where applicable, is determined by a suitable immunochemical method (2.7.1), such as single radial immunodiffusion, using a suitable haemagglutinin reference preparation; the content is shown to be within the limits shown to allow preparation of a satisfactory vaccine. For vaccines produced in eggs, the content of bacterial endotoxins is determined on the virus harvest to monitor production.

BATCH TESTING

The test described under Potency is not carried out for routine testing of batches of vaccine. It is carried out, for a given vaccine, on one or more occasions, as decided by or with the agreement of the competent authority; where the test is not carried out, an alternative validated method is used, the criteria for acceptance being set with reference to a batch of vaccine that has given satisfactory results in the test described under Potency. The following test may be used.

Batch potency test. Inject one dose of vaccine subcutaneously into each of 5 guinea-pigs free from specified antibodies. 21 days later, collect blood samples and separate the serum. Carry out tests on the serum for specific antibodies by a suitable immunochemical method (2.7.1) such as single radial haemolysis or haemagglutinin inhibition, using reference sera to validate the test. The antibody titres are not significantly lower than those obtained in guinea-pigs with a reference batch of vaccine shown to have satisfactory potency in horses.

IDENTIFICATION

In susceptible animals, the vaccine stimulates the production of specific antibodies.

TESTS

Safety. Use horses that preferably have no equine influenza virus antibodies or, where justified, use horses with a low level of such antibodies as long as they have not been vaccinated against equine influenza and administration of the vaccine does not cause an anamnestic response. Administer by a recommended route a double dose of vaccine to each of not fewer than 2 horses. After 2 weeks, administer a single dose to each horse. Observe the animals for a further 10 days. No abnormal local or systemic reaction occurs.

Inactivation. Inoculate 0.2 ml of the vaccine into the allantoic cavity of each of 10 fertilised eggs and incubate at 33 °C to 37 °C for 3 to 4 days. The test is not valid unless at least 8 of the 10 embryos survive. Harvest 0.5 ml of the allantoic fluid from each surviving embryo and pool the fluids. Inoculate 0.2 ml of the pooled fluid into a further 10 fertilised eggs and incubate at 33 °C to 37 °C for 3 to 4 days. The test is not valid unless not fewer than 8 of the 10 embryos survive. Harvest about 0.1 ml of the allantoic fluid from each surviving embryo and examine each individual harvest for live virus by a haemagglutination test. If haemagglutination is found for any of the fluids, carry out for that fluid a further passage in eggs and test for haemagglutination; no haemagglutination occurs.

Sterility. The vaccine complies with the test for sterility prescribed in the monograph on *Vaccines for veterinary use* (0062).

POTENCY

Carry out the potency test using a challenge strain against which the vaccine is stated to provide protection. Use where possible a recent isolate.

Use 10 horses, at least 6 months old, that do not have specific antibodies against equine influenza virus. Draw a blood sample from each animal and test individually for antibodies against equine influenza virus to determine seronegativity. Vaccinate 6 animals using the recommended schedule. Draw a second blood sample from each animal 7 days after the first vaccination and test individually for antibodies against equine influenza virus, to detect an anamnestic sero-response. Animals showing sero-conversion at this stage are excluded from the test. At least 2 weeks after the last vaccination, administer by aerosol to the 10 animals a quantity of equine influenza virus sufficient to produce characteristic signs of disease such as fever, nasal discharge and coughing in a susceptible animal. Observe the animals for 14 days. Collect nasal swabs daily from each individual animal to isolate the virus. The vaccinated animals show no more than slight signs; the controls show characteristic signs. The average number of days on which virus is excreted, and the respective virus titres are significantly lower in vaccinated animals than in control animals.

LABELLING

The label states:

- the age at which animals should be vaccinated,
- the period of time that should elapse between the first and second injections,
- any booster injections required,
- the strains of virus included in the vaccine.

01/2005:1101

FELINE CALICIVIROSI VACCINE (INACTIVATED)

Vaccinum calicivirosis felinae inactivatum

DEFINITION

Inactivated feline calicivirus vaccine is a suspension of one or more suitable strains of feline calicivirus which have been inactivated while maintaining adequate immunogenic properties or of fractions of one or more strains of feline calicivirus with adequate immunogenic properties.

PRODUCTION

The virus is grown in suitable cell lines (5.2.4). The viral suspension is harvested and inactivated.

The test for residual infectious calicivirus is carried out using 2 passages in cell cultures of the same type as those used for preparation of the vaccine or in cell cultures shown to be at least as sensitive; the quantity of inactivated virus used in the test is equivalent to not less than 25 doses of vaccine. No live virus is detected.

The vaccine may contain one or more suitable adjuvants.

CHOICE OF VACCINE COMPOSITION

The vaccine is shown to be satisfactory with respect to safety and immunogenicity in cats. The following test may be used during demonstration of efficacy (5.2.7).

Immunogenicity. The test described under Potency is suitable to demonstrate immunogenicity of the vaccine.

BATCH TESTING

Batch potency test. *The test described under Potency is not carried out for routine testing of batches of vaccine. It is carried out, for a given vaccine, on one or more occasions, as decided by or with the agreement of the competent authority; where the test is not carried out, a suitable validated alternative test is carried out, the criteria for acceptance being set with reference to a batch of vaccine that has given satisfactory results in the test described under Potency. The following test may be used after a satisfactory correlation with the test described under Potency has been established.*

Use groups of 15 seronegative mice. Administer half a dose of the vaccine to each mouse and 7 days later repeat the administration. 21 days after the first injection, take blood samples and determine the level of antibodies against feline calicivirus by an immunofluorescence technique using pools of serum from groups of 3 mice. The antibody levels are not significantly lower than those obtained with a batch of vaccine that has given satisfactory results in the test described under Potency.

IDENTIFICATION

When injected into susceptible animals, the vaccine stimulates the formation of specific antibodies against feline calicivirus.

TESTS

Safety. Use cats 8 to 12 weeks old and preferably having no feline calicivirus antibodies or, where justified, use cats with a low level of such antibodies as long as they have not been vaccinated against feline calicivirus and administration of the vaccine does not cause an anamnestic response. Administer a double dose of vaccine by a recommended route to each of 2 cats. Observe the cats for 14 days. No abnormal local or systemic reaction occurs.

Inactivation. Carry out a test for residual infectious calicivirus using 10 doses of vaccine and 2 passages in cell cultures of the same type as those used for preparation of the vaccine or in cell cultures shown to be at least as sensitive. No live virus is detected. If the vaccine contains an adjuvant that would interfere with the test, where possible separate the adjuvant from the liquid phase by a method that does not inactivate or otherwise interfere with detection of live virus.

Sterility. The reconstituted vaccine complies with the test for sterility prescribed in *Vaccines for veterinary use (0062)*.

POTENCY

Carry out a potency test for each strain of feline calicivirus in the vaccine.

Use cats 8 to 12 weeks old and that do not have antibodies against feline calicivirus. Vaccinate 10 cats by a recommended route and according to the recommended schedule. Keep 10 cats as controls. 4 weeks after the last injection, administer intranasally to each vaccinated and control cat a quantity of a virulent strain of calicivirus of the same type as the vaccine strain sufficient to produce typical signs of the disease (hyperthermia, buccal ulcers, respiratory signs) in at least 8 of the control cats. Observe the cats for 14 days after challenge; collect nasal washings daily on days 2 to 14 to test for virus excretion. Note daily the body temperature and signs of disease using the scoring system shown below. The vaccine complies with the test if the score for the vaccinated cats is significantly lower than that for the controls.

Observed signs	Score
Death	10
Depressed state	2
Temperature ≥ 39.5 °C	1
Temperature ≤ 37 °C	2
Ulcer (nasal or oral)	
– small and few in number	1
– large and numerous	3
Nasal discharge	
– slight	1
– copious	2
Ocular discharge	1
Weight loss	2
Virus excretion (total number of days):	
≤ 4 days	1
5-7 days	2
>7 days	3

01/2005:1102

FELINE CALICIVIROSI VACCINE (LIVE), FREEZE-DRIED

Vaccinum calicivirosi felinae vivum
cryodesiccatum

DEFINITION

Freeze-dried feline calicivirus vaccine (live) is a preparation of one or more suitable strains of feline calicivirus.

PRODUCTION

The virus is grown in suitable cell lines (5.2.4). The viral suspension is harvested and mixed with a suitable stabilising solution. The mixture is subsequently freeze-dried.

CHOICE OF VACCINE STRAIN

The vaccine strain shall have been shown to be satisfactory with respect to safety (including safety for pregnant queens if such use is not contra-indicated), absence of reversion to virulence, and immunogenicity. The following tests may be used during demonstration of safety (5.2.6) and efficacy (5.2.7).

Safety. To 10 cats of the minimum age stated for vaccination and which do not have antibodies against feline calicivirus, administer by a recommended route, a quantity of virus equivalent to 10 times the maximum titre that may be expected in a batch of vaccine. Observe the cats for 21 days. No abnormal local or systemic reaction occurs.

Reversion to virulence. To 2 cats which do not have antibodies against feline calicivirus administer by a recommended route a quantity of virus (for example, approximately 10 doses) that will allow maximum recovery of virus for the passages described below. 5 days later, kill the cats, remove the nasal mucus, tonsils and the trachea, mix, homogenise in 10 ml of buffered saline and decant; inoculate the supernatant intranasally into 2 other cats; carry out this operation five times. Verify the presence of virus at each passage. If the virus is not recovered, carry out a second series of passages. Observe the cats given the last passage for 21 days and compare the reactions observed with

those seen in the cats in the safety test described above. The strain complies with the test if no evidence of an increase in virulence is seen compared to the original virus.

Immunogenicity. The test described under Potency is suitable to demonstrate immunogenicity.

BATCH TESTING

If the test for potency has been carried out with satisfactory results on a representative batch of vaccine, this test may be omitted as a routine control on other batches of vaccine prepared from the same seed lot, subject to agreement by the competent authority.

IDENTIFICATION

When neutralised by one or more monospecific antisera, the reconstituted vaccine no longer infects susceptible cell cultures into which it is inoculated.

TESTS

Safety. Administer 10 doses of vaccine, by a recommended route to 2 cats, 8 to 12 weeks old and having no antibodies against feline calicivirus. Observe the cats for 14 days. No abnormal local or systemic reaction occurs.

Bacterial and fungal contamination. The reconstituted vaccine complies with the test for sterility prescribed in *Vaccines for veterinary use (0062)*.

Mycoplasmas (2.6.7). The reconstituted vaccine complies with the test for mycoplasmas.

Extraneous viruses. Neutralise the vaccine using one or more monospecific antisera and inoculate into suitable cell cultures; make at least one passage and maintain the cultures for 14 days. Examine the cultures for cytopathic effects and carry out tests for haemadsorbing agents. No signs of viral contamination occur in the cell cultures.

Virus titre. Titrate the reconstituted vaccine in susceptible cell cultures at a temperature favourable to replication of the virus. One dose of the vaccine contains not less than the quantity of virus equivalent to the minimum titre stated on the label.

POTENCY

Carry out a potency test for each strain of feline calicivirus in the vaccine.

Use cats 8 to 12 weeks old that do not have antibodies against feline calicivirus. Vaccinate 10 cats by one of the recommended routes and according to the recommended schedule. Keep 10 cats as controls. 4 weeks after the last injection, administer intranasally to each vaccinated and control cat a quantity of a virulent strain of calicivirus of the same type as the vaccine strain sufficient to produce typical signs of the disease (hyperthermia, buccal ulcers, respiratory signs) in not fewer than 8 of the control cats. Observe the cats for 14 days after challenge; collect nasal washings daily on days 2 to 14 to test for virus excretion. Note daily the body temperature and signs of disease using the scoring system shown below. The vaccine complies with the test if the score for the vaccinated cats is significantly lower than that for the controls.

Observed signs	Score
Death	10
Depressed state	2
Temperature ≥ 39.5 °C	1
Temperature ≤ 37 °C	2
Ulcer (nasal or oral)	
– small and few in number	1
– large and numerous	3
Nasal discharge	
– slight	1
– copious	2
Ocular discharge	1
Weight loss	2
Virus excretion (total number of days):	
≤ 4 days	1
5-7 days	2
>7 days	3

01/2005:0794

**FELINE INFECTIOUS ENTERITIS
(FELINE PANLEUCOPENIA) VACCINE
(INACTIVATED)**

*Vaccinum panleucopeniae felinae infectivae
inactivatum*

DEFINITION

Feline infectious enteritis (feline panleucopenia) vaccine (inactivated) is a liquid or freeze-dried preparation of feline panleucopenia virus or canine parvovirus inactivated by a suitable method.

PRODUCTION

The virus is propagated in suitable cell cultures (5.2.4). The virus is harvested and may be purified and concentrated.

The test for inactivation is carried out using a quantity of inactivated virus equivalent to not less than 100 doses of the vaccine by a validated method such as the following: inoculate into suitable non-confluent cells and after incubation for 8 days, make a subculture using trypsinised cells. After incubation for a further 8 days, examine the cultures for residual live parvovirus by an immunofluorescence test. The immunofluorescence test may be supplemented by a haemagglutination test or other suitable tests on the supernatant of the cell cultures. No live virus is detected.

The vaccine may contain an adjuvant and may be freeze-dried.

CHOICE OF VACCINE COMPOSITION

The vaccine is shown to be satisfactory with respect to safety (5.2.6) and efficacy (5.2.7) in cats. The following test may be used during demonstration of immunogenicity.

Immunogenicity. Use 10 susceptible cats, 8 to 12 weeks old. Draw a blood sample from each cat and test individually for antibodies against feline panleucopenia virus and canine parvovirus to determine susceptibility. Vaccinate 5 cats by the recommended schedule. Carry out leucocyte counts 8 days and 4 days before challenge and calculate the mean of the 2 counts to serve as the initial value. 20 to 22 days after

the last vaccination, challenge each cat by the intraperitoneal injection of a suspension of pathogenic feline panleucopenia virus. Observe the cats for 14 days. Carry out leucocyte counts on the fourth, sixth, eighth and tenth days after challenge. The test is not valid unless the 5 control cats all show on not fewer than one occasion a diminution in the number of leucocytes of at least 75 per cent of the initial value; these animals may die from panleucopenia. The vaccine complies with the test if the 5 vaccinated cats remain in excellent health and show no sign of leucopenia; that is to say, the diminution in the number of leucocytes does not exceed, in any of the four counts, 50 per cent of the initial value.

BATCH TESTING

Batch potency test. For routine testing of batches of vaccine a test based on production of haemagglutination-inhibiting antibodies in guinea-pigs may be used instead of test A or B described under Potency if a satisfactory correlation with the test for immunogenicity has been established.

IDENTIFICATION

When injected into animals, the vaccine stimulates the production of antibodies against the parvovirus present in the vaccine.

TESTS

Safety. Use cats of the minimum age recommended for vaccination and preferably having no antibodies against feline panleucopenia virus or against canine parvovirus or, where justified, use cats with a low level of such antibodies as long as they have not been vaccinated against feline panleucopenia virus or against canine parvovirus, and administration of the vaccine does not cause an anamnestic response. Administer by a recommended route a double dose of vaccine to each of 2 cats. Observe the animals for 14 days. No abnormal local or systemic reaction occurs.

Sterility. The vaccine complies with the test for sterility prescribed in the monograph on *Vaccines for veterinary use* (0062).

POTENCY

Carry out test A or test B.

- A. Use 4 cats, 8 to 12 weeks old. Draw a blood sample from each cat and test individually for antibodies against feline panleucopenia virus and canine parvovirus to determine susceptibility. Inject by a recommended route one dose of vaccine into each of 2 cats. After 21 days, draw a blood sample from each cat and separate the serum from each sample. Inactivate each serum by heating at 56 °C for 30 min. To 1 volume of each serum add 9 volumes of a 200 g/l suspension of *light kaolin R* in *phosphate buffered saline pH 7.4 R*. Shake each mixture for 20 min. Centrifuge, collect the supernatant liquid and mix with 1 volume of a concentrated suspension of pig erythrocytes. Allow to stand at 4 °C for 60 min and centrifuge. The dilution of the serum obtained is 1:10. Using each serum, prepare a series of twofold dilutions. To 0.025 ml of each of the latter dilutions add 0.025 ml of a suspension of canine parvovirus or feline panleucopenia virus antigen containing 4 haemagglutinating units. Allow to stand at 37 °C for 30 min and add 0.05 ml of a suspension of pig erythrocytes containing 30×10^6 cells per millilitre. Allow to stand at 4 °C for 90 min and note the last dilution of serum that still completely inhibits haemagglutination. The vaccine complies with the test if both vaccinated cats have developed titres of

at least 1:20. The test is not valid if either control cat develops antibodies against canine parvovirus or feline panleucopenia virus.

- B. Vaccinate according to the recommended schedule, 2 cats, 8 to 12 weeks old and having antibody titres less than 4 ND₅₀ (neutralising dose 50 per cent) per 0.1 ml of serum measured by the method described below. 14 days after vaccination, examine the serum of each animal as follows. Heat the serum at 56 °C for 30 min and prepare serial dilutions using a medium suitable for feline cells. Add to each dilution an equal volume of a virus suspension containing an amount of virus such that when the volume of serum-virus mixture appropriate for the assay system is inoculated into cell cultures, each culture receives approximately 10^4 CCID₅₀. Incubate the mixtures at 37 °C for 1 h and inoculate four feline cell cultures with a suitable volume of each mixture. Incubate the cell cultures at 37 °C for 7 days, passage and incubate for a further 7 days. Examine the cultures for evidence of specific cytopathic effects and calculate the antibody titre. The vaccine complies with the test if the mean titre is not less than 32 ND₅₀ per 0.1 ml of serum. If one cat fails to respond, repeat the test using 2 more cats and calculate the result as the mean of the titres obtained from all of the 3 cats that have responded.

01/2005:0251

FELINE INFECTIOUS ENTERITIS (FELINE PANLEUCOPENIA) VACCINE (LIVE)

Vaccinum panleucopeniae felinae infectivae vivum

DEFINITION

Feline infectious enteritis (feline panleucopenia) vaccine (live) is a preparation of a suitable strain of feline panleucopenia virus.

PRODUCTION

The virus is propagated in suitable cell cultures (5.2.4). The virus suspension is harvested and may be purified and concentrated. It is mixed with a suitable stabilising solution. The vaccine may be freeze-dried.

CHOICE OF VACCINE STRAIN

The vaccine strain shall have been shown to be satisfactory with respect to safety (including safety for pregnant queens if such use is not contra-indicated or if the virus is excreted in the faeces), absence of increase in virulence and immunogenicity.

The following tests may be used during demonstration of safety (5.2.6) and efficacy (5.2.7).

Safety. *Each test is carried out for each recommended route of administration.* Use 5 cats, of the minimum age recommended for vaccination and free from specific haemagglutination-inhibiting antibodies against feline panleucopenia virus and canine parvovirus. Make counts of leucocytes in circulating blood on days 8 and 4 before injection of the vaccine strain and calculate the mean of the 2 counts to serve as the initial value. Administer to each cat by a recommended route a quantity of virus corresponding to at least 10 times the maximum virus titre that may be expected in a batch of vaccine and at the lowest level of attenuation. Observe the animals for 21 days. Make leucocyte counts on the fourth, sixth, eighth and tenth days after inoculation. The strain complies with the test if: the

cats remain in good health and there is no abnormal local or systemic reaction; for each animal and for each blood count, the number of leucocytes is not less than 50 per cent of the initial value.

Increase in virulence. Use 2 cats of the minimum age recommended for vaccination and which do not have haemagglutination-inhibiting antibodies against feline panleucopenia virus and canine parvovirus. Administer to each cat, by a recommended route, a quantity of virus suitable to allow maximum recovery of the virus for subsequent passages. From the second to the tenth day after administration of the virus, collect the faeces from each cat and check for the presence of the virus; pool faeces containing virus. Administer 1 ml of the suspension of pooled faeces by the oronasal route to each of 2 other cats of the same age and susceptibility; repeat this operation a further 4 times. Verify the presence of virus at each passage. If the virus is not found, carry out a second series of passages; if the virus is not found in one of the second series of passages, the vaccine strain complies with the test. The vaccine strain complies with the test if: no cat dies or shows signs attributable to the vaccine; no indication of increase of virulence compared to the original vaccinal virus is observed; account is taken, notably, of the count of white blood cells, of results of histological examination of the thymus and of the titre of excreted virus.

Immunogenicity. The test described under Potency is suitable to demonstrate immunogenicity of the strain.

BATCH TESTING

If the test for potency has been carried out with satisfactory results on a representative batch of vaccine, this test may be omitted as a routine control on other batches of vaccine prepared from the same seed lot, subject to agreement by the competent authority.

IDENTIFICATION

Carry out replication of the vaccine virus in a susceptible cell line in a substrate suitable for a fluorescent antibody test or peroxidase test. Prepare suitable controls. Test a proportion of the cells with monoclonal antibodies specific for feline panleucopenia virus and a proportion with monoclonal antibodies specific for canine parvovirus. Feline panleucopenia virus is detected but no canine parvovirus is detected in the cells inoculated with the vaccine.

TESTS

Safety. Use 2 cats of the minimum age recommended for vaccination and having no antibodies against feline panleucopenia virus or against canine parvovirus. Administer 10 doses of the vaccine to each cat, by a recommended route. Observe the animals for 14 days. No abnormal local or systemic reaction occurs.

Extraneous viruses. Neutralise the vaccine using a suitable monospecific antiserum against feline panleucopenia virus and inoculate into suitable cell cultures; make at least one passage and maintain the cultures for 14 days. Examine the cultures for cytopathic effects and carry out tests for haemadsorbing agents. No signs of viral contamination occur in the cell cultures.

Bacterial and fungal contamination. The vaccine, reconstituted if necessary, complies with the test for sterility prescribed in the monograph on *Vaccines for veterinary use* (0062).

Mycoplasmas (2.6.7). The vaccine complies with the test for mycoplasmas.

Virus titre. Reconstitute the vaccine, if necessary, and titrate in suitable cell cultures. One dose of vaccine contains not less than the quantity of virus equivalent to the minimum titre stated on the label.

POTENCY

Use 10 susceptible cats, 8 to 12 weeks old. Draw a blood sample from each cat and test individually for antibodies against feline panleucopenia virus and canine parvovirus to determine susceptibility. Vaccinate 5 cats by the recommended schedule. Carry out leucocyte counts 8 days and 4 days before challenge and calculate the mean of the 2 counts to serve as the initial value. 20 to 22 days after the last vaccination, challenge each cat by the intraperitoneal injection of a suspension of pathogenic feline panleucopenia virus. Observe the cats for 14 days. Carry out leucocyte counts on the fourth, sixth, eighth and tenth days after challenge. The test is not valid unless the 5 control cats all show on not fewer than one occasion a diminution in the number of leucocytes of at least 75 per cent of the initial value; these animals may die from panleucopenia. The vaccine complies with the test if the 5 vaccinated cats remain in excellent health and show no sign of leucopenia; that is to say, the diminution in the number of leucocytes does not exceed, in any of the four counts, 50 per cent of the initial value.

LABELLING

The label states that the vaccine should not be used in pregnant queens (unless it has been shown to be safe in such conditions).

01/2005:1321

FELINE LEUKAEMIA VACCINE (INACTIVATED)

Vaccinum leucosis felinae inactivatum

DEFINITION

Feline leukaemia vaccine (inactivated) is a preparation of immunogens from a suitable strain of feline leukaemia virus.

PRODUCTION

The immunogens consist either of a suitable strain of feline leukaemia virus inactivated in such a manner that adequate immunogenicity is maintained or of a fraction of the virus with adequate immunogenic properties; the immunogenic fraction may be produced by recombinant DNA technology. Where applicable, the test for inactivation is carried out using a quantity of inactivated virus equivalent to not less than 25 doses of vaccine and two passages in the same type of cell cultures as used for the production of the vaccine or in cell cultures shown to be at least as sensitive; no live virus is detected.

The vaccine may contain an adjuvant.

CHOICE OF VACCINE COMPOSITION

The choice of the feline leukaemia virus strains and/or of the antigens included in the vaccine composition is made in such a manner as to ensure the safety (5.2.6) (including safety for pregnant queens if the vaccine may be used in such animals) and immunogenicity (5.2.7) of the vaccine. The following tests may be used during demonstration of safety and immunogenicity.

Safety. Carry out the test for each intended route of administration. Use animals that do not have antibodies against gp 70 antigen of feline leukaemia virus nor display

viraemia or antigenaemia at the time of the test; absence of antibodies and antigen is demonstrated by enzyme-linked immunosorbent assay (2.7.1).

- A. Vaccinate, according to the intended schedule, not fewer than ten cats of the minimum age recommended for vaccination. Keep five cats as controls. Record the rectal temperature of each cat on the day before each vaccination, at the time of vaccination, 4 h and 8 h later, and once per day during the four following days. Observe the animals for at least 4 weeks after the last vaccination. No abnormal local or systemic reaction occurs during the test. 1, 2 and 4 weeks after the last vaccination, submit the animals to suitable tests for evidence of an immunosuppressive effect. The vaccine complies with the test if no significant difference is observed in vaccinated animals compared with controls.
- B. Inject two doses of vaccine by one of the intended routes to not fewer than ten cats of the minimum age recommended for vaccination. At the end of the period of time stated in the instructions for use, inject one dose of vaccine to each animal. Where the instructions for use recommend it, administer a third injection after the period indicated. Observe the animals for 14 days after the last administration. The vaccine complies with the test if no abnormal local or systemic reaction occurs during the test.
- C. If the vaccine is not contra-indicated for use in pregnant queens, inject two doses of vaccine into each of not fewer than ten cats at different stages of pregnancy. Observe the cats until parturition and note any effects on gestation and the offspring. The vaccine complies with the test if no abnormal local or systemic reaction occurs during the test.

Immunogenicity. The test described under Potency is suitable to demonstrate immunogenicity of the vaccine.

IN-PROCESS CONTROL TESTS

During production, suitable immunochemical tests are carried out for the evaluation of the quality and purity of the viral antigens included in the vaccine composition. The values found are within the limits approved for the particular vaccine.

BATCH TESTING

Potency. The test described under Potency is not carried out for routine testing of batches of vaccine. It is carried out, for a given vaccine, on one or more occasions, as decided by or with the agreement of the competent authority; where the test is not carried out, a suitable validated alternative method is used, the criteria for acceptance being set with reference to a batch of vaccine that has given satisfactory results in the test described under Potency.

Bacterial endotoxins. For vaccines produced by recombinant DNA technology with a bacterial host cell such as *Escherichia coli*, a test for bacterial endotoxins (2.6.14) is carried out on each final lot or, where the nature of the adjuvant prevents performance of a satisfactory test, on the antigen immediately before addition of the adjuvant. The value found is within the limit approved for the particular vaccine and which has been shown to be safe for cats.

IDENTIFICATION

When injected into healthy, seronegative cats, the vaccine stimulates the production of specific antibodies against the antigen or antigens stated on the label.

TESTS

Safety. Use two cats of the minimum age recommended for vaccination and that do not have antibodies against feline leukaemia virus. Inject by a recommended route a double dose of vaccine into each animal. Observe the animals for 14 days. The vaccine complies with the test if no abnormal local or systemic reaction is produced.

Inactivation. If the vaccine contains inactivated virus, carry out a test for residual live feline leukaemia virus by making two passages on susceptible cell cultures. No virus is detected. If the vaccine contains an adjuvant, if possible separate the adjuvant from the liquid phase by a method that does not inactivate the virus nor interfere in any other way with the detection of virus.

Sterility. The vaccine complies with the test for sterility prescribed in *Vaccines for veterinary use* (0062).

POTENCY

Use not fewer than twenty-five susceptible cats of the minimum age recommended for vaccination, free from antibodies against the antigens of feline leukaemia virus and against the feline oncogene membrane antigen (anti-FOCMA antibodies), and showing no viraemia or antigenaemia at the time of the test. Vaccinate not fewer than fifteen cats by a recommended route in accordance with the instructions for use. Keep not fewer than ten cats as controls. Observe the animals for at least 14 days after the last administration of vaccine. Inject by the peritoneal or oronasal route, on one or several occasions, a quantity of a virulent strain of feline leukaemia virus sufficient to induce persistent viraemia or antigenaemia in not fewer than 80 per cent of susceptible animals; use for the challenge an epidemiologically relevant strain consisting predominantly of type A virus. Observe the animals for 15 weeks and, from the third week onwards, test each week for viraemia or antigenaemia (p27 protein) by suitable methods such as immunofluorescence on circulating leucocytes or enzyme-linked immunosorbent assay. A cat is considered persistently infected if it shows positive viraemia or antigenaemia for three consecutive weeks or on five occasions, consecutively or not, between the third and the fifteenth week. The test is not valid if fewer than 80 per cent of the control cats are persistently infected. The vaccine complies with the test if not fewer than 80 per cent of the vaccinated cats show no persistent infection.

LABELLING

The label states the antigen or antigens contained in the vaccine.

01/2005:1207

FELINE VIRAL RHINOTRACHEITIS VACCINE (INACTIVATED)

*Vaccinum rhinotracheitidis viralis felinae
inactivatum*

DEFINITION

Feline viral rhinotracheitis vaccine (inactivated) is a preparation of a suitable strain of feline rhinotracheitis virus (feline herpesvirus 1) that has been inactivated while maintaining adequate immunogenic properties or of an inactivated fraction of the virus having adequate immunogenic properties.

PRODUCTION

The vaccine strain is grown in suitable cell cultures (5.2.4). The viral suspension is harvested and inactivated.

The test for inactivation is carried out using 2 passages in cell cultures of the same type as those used for preparation of the vaccine or in cell cultures shown to be at least as sensitive; the quantity of virus used in the test is equivalent to not less than 25 doses of vaccine. No live virus is detected.

The virus may be fragmented and the fragments may be purified and concentrated. The vaccine may be adjuvanted; it may be freeze-dried.

CHOICE OF VACCINE COMPOSITION

The vaccine is shown to be satisfactory with respect to safety and immunogenicity in cats. The following test may be used during demonstration of efficacy (5.2.7).

Immunogenicity. The test described under Potency, carried out using vaccine prepared from the most attenuated virus that will be attained during production, is suitable to demonstrate immunogenicity of the strain.

BATCH TESTING

The test described under Potency is not necessarily carried out for routine testing of batches of vaccine. It is carried out, for a given vaccine, on one or more occasions, as decided by or with the agreement of the competent authority. Where the test is not carried out, a suitable alternative validated test is carried out, the criteria for acceptance being set with reference to a batch of vaccine that has given satisfactory results in the test described under Potency. The following test may be used after a satisfactory correlation with the test described under Potency has been established.

Batch potency test. Use a group of 15 seronegative mice. Administer half a dose of the vaccine to each mouse and 7 days later repeat the administration. 21 days after the first injection, take blood samples and determine the level of antibodies against feline rhinotracheitis virus by a suitable immunochemical method (2.7.1), such as an immunofluorescence technique using pools of serum from groups of 3 mice. The antibody levels are not significantly lower than those obtained with a batch of vaccine that has given satisfactory results in the test described under Potency.

IDENTIFICATION

When administered to susceptible animals, the vaccine stimulates the production of specific serum antibodies against feline rhinotracheitis virus or against the fraction of the virus used to produce the vaccine.

TESTS

Safety. Use cats 8 to 12 weeks old and preferably having no feline rhinotracheitis virus antibodies or antibodies to a fraction of the virus, or, where justified, use cats with a low level of such antibodies as long as they have not been vaccinated against viral rhinotracheitis and administration of the vaccine does not cause an anamnestic response. Administer a double dose of vaccine by a recommended route to each of 2 cats. Observe the cats for 14 days. No abnormal local or systemic reaction occurs.

Inactivation. Carry out a test for residual infectious feline rhinotracheitis virus using 10 doses of vaccine and 2 passages in cell cultures of the same type as those used for preparation of the vaccine or in other suitably sensitive cell cultures. No live virus is detected. If the vaccine contains an adjuvant that interferes with the test, where possible separate the adjuvant from the liquid phase by a method that does not inactivate or otherwise interfere with detection of live virus.

Sterility. The reconstituted vaccine complies with the test for sterility prescribed under *Vaccines for veterinary use* (0062).

POTENCY

Use cats 8 to 12 weeks old and which do not have antibodies against feline rhinotracheitis virus or against a fraction of the virus. Vaccinate 10 cats according to the instructions for use and keep 10 cats as controls. 4 weeks after the last administration of vaccine, administer intranasally to each of the 20 cats, a quantity of virulent feline rhinotracheitis virus sufficient to produce in susceptible cats typical signs of disease such as fever, nasal discharge and cough. Observe the cats for 14 days; collect nasal washings daily on days 2 to 14 after challenge to test for virus excretion. Note daily the body temperature and signs of disease using the scoring system shown below. If any sign of disease is observed on more than one day, record the score once only. The vaccine complies with the test if the score for the vaccinated cats is significantly lower than that for the controls.

Sign	Score
Death	10
Depressed state	2
Temperature:	
39.5 °C - 40.0 °C	1
≥ 40.0 °C	2
≤ 37.0 °C	3
Glossitis	3
Nasal discharge, slight	1
Nasal discharge, copious	2
Cough	2
Sneezing	1
Sneezing, paroxysmal	2
Ocular discharge, slight	1
Ocular discharge, serious	2
Conjunctivitis	2
Weight loss ≥ 5.0 per cent	5
Virus excretion (total number of days):	
≤ 4 days	1
5-7 days	2
> 7 days	3

01/2005:1206

FELINE VIRAL RHINOTRACHEITIS VACCINE (LIVE), FREEZE-DRIED

Vaccinum rhinotracheitidis viralis felinae vivum cryodesiccatum

DEFINITION

Freeze-dried feline viral rhinotracheitis vaccine (live) is a preparation of a suitable strain of feline rhinotracheitis virus (feline herpesvirus 1).

PRODUCTION

The vaccine strain is grown in suitable cell cultures (5.2.4). The viral suspension is harvested and mixed with a suitable stabilising solution. The mixture is subsequently freeze-dried.

CHOICE OF VACCINE STRAIN

Only a virus strain shown to be satisfactory with respect to safety, reversion to virulence and immunogenicity may be used in the preparation of the vaccine; if the vaccine is not contra-indicated in pregnant queens, the safety for such use is demonstrated. The following tests may be used during demonstration of safety (5.2.6) and efficacy (5.2.7).

Safety. Administer by a recommended route to each of ten cats, of the minimum age stated for vaccination and which do not have antibodies against feline rhinotracheitis virus, a quantity of virus equivalent to ten times the maximum titre that may be expected in a batch of vaccine. Observe the cats for 21 days. They remain in good health and show no abnormal local or systemic reaction.

Reversion to virulence. Administer by a recommended route to each of two cats that do not have antibodies against feline rhinotracheitis virus, a quantity of virus that will allow optimal re-isolation of the virus for subsequent passages (for example ten times the minimum titre to be stated on the label). Kill the cats 2 to 4 days after the inoculation and remove the nasal mucus, tonsils and local lymphatic ganglia and the trachea; mix the samples, homogenise in 10 ml of buffered saline and allow to settle; inoculate 1 ml of supernatant intranasally into two cats; repeat this operation not fewer than five times; verify the presence of virus at each passage; if the virus is not recovered, carry out a second series of passages. Observe the cats given the last passage for 21 days and compare any reactions with those seen in the safety test described above. No evidence of an increase in virulence is seen compared to the original virus.

Immunogenicity. The test described under Potency is suitable to demonstrate immunogenicity of the strain.

BATCH TESTING

If the test for potency has been carried out with satisfactory results on a representative batch of vaccine, this test may be omitted as a routine control on other batches of vaccine prepared from the same seed lot, subject to agreement by the competent authority.

IDENTIFICATION

When mixed with a monospecific antiserum, the reconstituted vaccine no longer infects susceptible cell cultures into which it is inoculated.

TESTS

Safety. Administer ten doses of the vaccine in a suitable volume by a recommended route to two cats, 8 to 12 weeks old, that do not have antibodies against feline rhinotracheitis virus. Observe the cats for 14 days. They remain in good health and show no abnormal local or systemic reaction.

Bacterial and fungal contamination. The reconstituted vaccine complies with the test for sterility prescribed in the monograph on *Vaccines for veterinary use* (0062).

Mycoplasmas (2.6.7). The reconstituted vaccine complies with the test for mycoplasmas.

Extraneous viruses. Neutralise the vaccinal virus using a monospecific antiserum and inoculate into suitable cell cultures; make at least one passage and maintain the cultures for 14 days. No cytopathic effect develops; the cells show no evidence of the presence of haemadsorbing agents or other signs of viral contamination.

Virus titre. Titrate the reconstituted vaccine in susceptible cell cultures at a temperature favourable to replication of the virus. One dose of vaccine contains not less than the quantity of virus corresponding to the minimum virus titre stated on the label.

POTENCY

Use cats 8 to 12 weeks old and which do not have antibodies against feline viral rhinotracheitis virus. Vaccinate ten cats according to the instructions for use and keep ten cats as controls. Four weeks after the last administration of vaccine, administer intranasally to each cat a quantity of virulent feline rhinotracheitis virus sufficient to produce in susceptible cats typical signs of disease such as fever, nasal discharge and cough. Observe the cats for 14 days; collect nasal washings daily on days 2 to 14 after challenge to test for virus excretion. Note daily the body temperature and signs of disease using the scoring system shown below. If any sign of disease is observed on more than one day, record the score once only. The vaccine complies with the test if the score for the vaccinated cats is significantly lower than that for the controls.

Sign	Score
Death	10
Depressed state	2
Temperature:	
39.5 °C - 40.0 °C	1
≥ 40.0 °C	2
≤ 37.0 °C	3
Glossitis	3
Nasal discharge, slight	1
Nasal discharge, copious	2
Cough	2
Sneezing	1
Sneezing, paroxysmal	2
Ocular discharge, slight	1
Ocular discharge, serious	2
Conjunctivitis	2
Weight loss ≥ 5.0 per cent	5
Virus excretion (total number of days):	
≤ 4 days	1
5-7 days	2
> 7 days	3

01/2005:0063
corrected

FOOT-AND-MOUTH DISEASE (RUMINANTS) VACCINE (INACTIVATED)

*Vaccinum aphtharum epizooticarum
inactivatum ad ruminantes*

DEFINITION

Foot-and-mouth disease (ruminants) vaccine (inactivated) is a liquid preparation containing one or more types or sub-types of foot-and-mouth disease virus inactivated without impairing their immunogenic activity.

PRODUCTION

The virus is grown either in suspensions of cattle-tongue epithelium obtained immediately after slaughter of healthy animals, or, preferably, in suitable cell cultures (5.2.4). The

virus is separated from cellular material by filtration or other suitable procedures and the virus is inactivated in suitable conditions.

The vaccine is prepared from inactivated antigen by blending with one or more adjuvants. The antigen may be concentrated and purified by a number of procedures. The concentrated antigen is used for the preparation of vaccine immediately or after storage at a temperature not exceeding -90°C , unless stability of the antigen at a higher temperature has been demonstrated.

Validation of the inactivation procedure. During inactivation, the virus titre is monitored by a sensitive and reproducible technique. The inactivation procedure is not satisfactory unless the decrease in virus titre, plotted logarithmically, is linear and extrapolation indicates that there is less than 1 infectious virus unit per 10^4 litres of liquid preparation at the end of inactivation.

BULK INACTIVATED ANTIGEN

Inactivation. A proportion of each batch of bulk inactivated antigen representing at least 200 doses is tested for freedom from infectious virus by inoculation into sensitive cell cultures. For this purpose, the sample of the inactivated antigen is concentrated to allow testing of such large samples in cell cultures. It must be shown that the selected concentration and assay systems are not detrimental to detection of infectious virus within the test sample and that the concentrated inactivated antigen does not interfere with virus replication or cause toxic changes.

Antigenicity. The antigen content of each batch of bulk inactivated antigen is determined by an *in vitro* method (for example, 146S-particle measurement by sucrose density gradient centrifugation and ultraviolet spectrophotometry at 259 nm).

Safety. A representative sample of the bulk inactivated antigen may be diluted and blended with the adjuvants for safety testing in cattle, as described below.

Sterility. The bulk inactivated antigen, the adjuvants and all dilution buffers comply with the test for sterility prescribed in the monograph on *Vaccines for veterinary use (0062)*.

Potency. A representative sample of the bulk inactivated antigen may be diluted and blended with the adjuvants for potency testing in cattle, as described below.

In situations of extreme urgency and subject to agreement by the competent authority, a batch of vaccine may be released before completion of the tests and the determination of potency if a test for sterility has been carried out on the bulk inactivated antigen and all other components of the vaccine and if the test for safety and the determination of potency have been carried out on a representative batch of vaccine prepared from the same bulk inactivated antigen. In this context, a batch is not considered to be representative unless it has been prepared using the same amount of antigen and with the same formulation as the batch to be released.

IDENTIFICATION

The serum of a susceptible animal that has been immunised with the vaccine neutralises the types or sub-types of the virus used to prepare the vaccine, when tested by a suitably sensitive method.

TESTS

Safety. Use three non-vaccinated cattle not less than 6 months old having serum free from foot-and-mouth disease antibodies and coming from regions free from foot-and-mouth disease. Inoculate each animal intradermally into the tongue at not fewer than twenty points, using 0.1 ml of the vaccine at each point. Observe the animals for not less than 4 days. No lesions or signs of foot-and-mouth disease occur. At the end of the observation period, inject into the same animals by the prescribed route three times the dose stated on the label. Observe the animals for 6 days after this inoculation. No lesions or clinical signs of foot-and-mouth disease occur on the feet or tongue and any reaction at the site of injection remains insignificant.

Sterility. It complies with the test for sterility prescribed in the monograph on *Vaccines for veterinary use (0062)*.

POTENCY

The potency of the vaccine is expressed as the number of 50 per cent cattle protective doses (PD_{50}) contained in the dose stated on the label. The PD_{50} is determined in animals given primary vaccination and challenged by the inoculation of 10 000 ID_{50} of virulent bovine virus of the same type or sub-type as that used in the preparation of the vaccine in the conditions described below.

The vaccine contains at least 3 PD_{50} per dose for cattle.

Carry out a potency test for each type and sub-type of foot-and-mouth disease virus present in the vaccine.

Use cattle not less than 6 months old, obtained from areas free from foot-and-mouth disease, which have never been vaccinated against foot-and-mouth disease and are free from antibodies neutralising the different types of foot-and-mouth disease virus. Vaccinate not fewer than three groups of not fewer than five cattle per group by the route stated on the label. Use a different dose of the vaccine for each group. Administer the different doses by injecting different volumes of the vaccine rather than by dilution of the vaccine. For example, if the label states that the injection of 2 ml corresponds to the administration of 1 dose of vaccine, a 1/4 dose of vaccine would be obtained by injecting 0.5 ml, and a 1/10 dose would be obtained by injecting 0.2 ml. Three weeks after the vaccination, challenge the vaccinated animals and a control group of two animals with a suspension of virus that has been obtained from cattle and that is fully virulent and of the same type or sub-type as that used in the preparation of the vaccine, by inoculating a total of 10 000 ID_{50} intradermally into two sites on the upper surface of the tongue (0.1 ml per site). Observe the animals for 8 days and then slaughter. Unprotected animals show lesions at sites other than the tongue. Protected animals may display lingual lesions. The test is not valid unless each control animal shows lesions on at least three feet. From the number of protected animals in each group, calculate the PD_{50} content of the vaccine.

STORAGE

The vaccine must not be frozen.

LABELLING

The label states:

- the method of preparation,
- the types and sub-types of virus used to prepare the vaccine.

01/2005:0649

FOWL-POX VACCINE (LIVE)

Vaccinum variolae gallinae vivum

1. DEFINITION

Fowl-pox vaccine (live) is a preparation of a suitable strain of avian pox virus. This monograph applies to vaccines intended for administration to chickens for active immunisation.

2. PRODUCTION

2-1. PREPARATION OF THE VACCINE

The vaccine virus is grown in embryonated hens' eggs or in cell cultures.

2-2. SUBSTRATE FOR VIRUS PROPAGATION

2-2-1. Embryonated hens' eggs. If the vaccine virus is grown in embryonated hens' eggs, they are obtained from flocks free from specified pathogens (SPF) (5.2.2).

2-2-2. Cell cultures. If the vaccine virus is grown in cell cultures, they comply with the requirements for cell cultures for production of veterinary vaccines (5.2.4).

2-3. SEED LOTS

2-3-1. Extraneous agents. The master seed lot complies with the tests for extraneous agents in seed lots (2.6.24). In these tests on the master seed lot, the organisms used are not more than 5 passages from the master seed lot at the start of the tests.

2-4. CHOICE OF VACCINE VIRUS

The vaccine virus shall be shown to be satisfactory with respect to safety (5.2.6) and efficacy (5.2.7) for the chickens for which it is intended.

The following tests for safety (section 2-4-1), increase in virulence (section 2-4-2) and immunogenicity (section 2-4-3) may be used during demonstration of safety and immunogenicity.

2-4-1. Safety. Carry out the test for each route and method of administration to be recommended for vaccination using in each case chickens not older than the youngest age to be recommended for vaccination. Use vaccine virus at the least attenuated passage level that will be present between the master seed lot and a batch of the vaccine. For each test use not fewer than 20 chickens, from an SPF flock (5.2.2). Administer to each chicken a quantity of the vaccine virus equivalent to not less than 10 times the maximum virus titre likely to be contained in a dose of the vaccine. Observe the chickens at least daily for 21 days. The test is not valid if more than 10 per cent of the chickens die from causes not attributable to the vaccine virus. The vaccine virus complies with the test if no chicken shows notable clinical signs of fowl pox or dies from causes attributable to the vaccine virus.

2-4-2. Increase in virulence. Administer by a suitable route a quantity of the vaccine virus that will allow recovery of virus for the passages described below to each of 5 chickens not older than the minimum age to be recommended for vaccination and from an SPF flock (5.2.2). Use the vaccine virus at the least attenuated passage level that will be present between the master seed lot and a batch of the vaccine. Prepare 4 to 7 days after administration a suspension from the induced skin lesions of each chicken and pool these samples. Administer 0.2 ml of the pooled samples by cutaneous scarification of the comb or other unfeathered part of the body, or by another suitable method to each of 5 other chickens not older than the minimum age

to be recommended for vaccination and from an SPF flock (5.2.2). Carry out this passage operation not fewer than 5 times; verify the presence of the virus at each passage. Care must be taken to avoid contamination by virus from previous passages. If the virus is not found at a passage level, carry out a second series of passages. Carry out the test for safety (section 2-4-1) using the unpassaged vaccine virus and the maximally passaged virus that has been recovered. Administer the virus by the route recommended for vaccination likely to be the least safe. The vaccine virus complies with the test if no indication of increase in virulence of the maximally passaged virus compared with the unpassaged virus is observed. If virus is not recovered at any passage level in the first and second series of passages, the vaccine virus also complies with the test.

2-4-3. Immunogenicity. A test is carried out for each route and method of administration to be recommended using in each case chickens not older than the youngest age to be recommended for vaccination. The quantity of the vaccine virus administered to each chicken is not greater than the minimum virus titre to be stated on the label and the virus is at the most attenuated passage level that will be present in a batch of the vaccine. Use for the test not fewer than 30 chickens of the same origin and from an SPF flock (5.2.2). Vaccinate by a recommended route not fewer than 20 chickens. Maintain not fewer than 10 chickens as controls. Challenge each chicken after 21 days by the feather-follicle route with a sufficient quantity of virulent fowl-pox virus. Observe the chickens at least daily for 21 days after challenge. Record the deaths and the number of surviving chickens that show clinical signs of disease. Examine each surviving chicken for macroscopic lesions: cutaneous lesions of the comb, wattle and other unfeathered areas of the skin and diphtherical lesions of the mucous membranes of the oro-pharyngeal area.

The test is not valid if:

- during the observation period after challenge fewer than 90 per cent of the control chickens die or show severe clinical signs of fowl pox, including notable macroscopical lesions of the skin or mucous membranes of the oro-pharyngeal area,
- and/or during the period between vaccination and challenge, more than 10 per cent of the control or vaccinated chickens show abnormal clinical signs or die from causes not attributable to the vaccine.

The vaccine virus complies with the test if during the observation period after challenge not less than 90 per cent of the vaccinated chickens survive and show no notable clinical signs of disease, including macroscopical lesions of the skin and mucous membranes of the oro-pharyngeal area.

3. BATCH TESTS

3-1. Identification. Carry out an immunostaining test in cell cultures to demonstrate the presence of the vaccine virus. For egg adapted strains, inoculate the vaccine into eggs and notice the characteristic lesions.

3-2. Bacteria and fungi

Vaccines intended for administration by injection, scarification or piercing of the wing web comply with the test for sterility prescribed in the monograph *Vaccines for veterinary use* (0062).

Vaccines not intended for administration by injection, scarification or piercing of the wing web either comply with the test for sterility prescribed in the monograph *Vaccines for veterinary use* (0062) or with the following test: carry out a quantitative test for bacterial and fungal contamination; carry out identification tests for

microorganisms detected in the vaccine; the vaccine does not contain pathogenic microorganisms and contains not more than 1 non-pathogenic microorganism per dose.

Any liquid supplied with the vaccine complies with the test for sterility prescribed in the monograph *Vaccines for veterinary use* (0062).

3-3. Mycoplasmas. The vaccine complies with the test for mycoplasmas (2.6.7).

3-4. Extraneous agents. The vaccine complies with the tests for extraneous agents in batches of finished product (2.6.25).

3-5. Safety. Use not fewer than 10 chickens from an SPF flock (5.2.2) of the youngest age recommended for vaccination. For vaccines recommended for use in chickens older than 6 weeks, chickens 6 weeks old may be used. Administer 10 doses of the vaccine to each chicken by a recommended route. Observe the chickens at least daily for 21 days. The test is not valid if more than 20 per cent of the chickens show abnormal clinical signs or die from causes not attributable to the vaccine.

The vaccine complies with the test if no chicken shows notable clinical signs of disease or dies from causes attributable to the vaccine.

3-6. Virus titre. Titrate the vaccine virus by inoculation into embryonated hens' eggs from an SPF flock (5.2.2) or into suitable cell cultures (5.2.4). The vaccine complies with the test if 1 dose contains not less than the minimum virus titre stated on the label.

3-7. Potency. The vaccine complies with the requirements of the test prescribed under Immunogenicity (section 2-4-3) when administered according to the recommended schedule by a recommended route and method. It is not necessary to carry out the potency test for each batch of the vaccine if it has been carried out on a representative batch using a vaccinating dose containing not more than the minimum virus titre stated on the label.

01/2005:1521

FURUNCULOSIS VACCINE (INACTIVATED, OIL-ADJUVANTED, INJECTABLE) FOR SALMONIDS

*Vaccinum furunculosis ad salmonidas
inactivatum cum adjuvatione oleosa
ad iniectionem*

DEFINITION

Furunculosis vaccine (inactivated, oil-adjuvanted, injectable) for salmonids is prepared from cultures of one or more suitable strains of *Aeromonas salmonicida* subsp. *salmonicida*.

PRODUCTION

The strains of *A. salmonicida* are cultured and harvested separately. The harvests are inactivated by a suitable method. They may be purified and concentrated. Whole or disrupted cells may be used and the vaccine may contain extracellular products of the bacterium released into the growth medium. The vaccine contains an oily adjuvant.

CHOICE OF VACCINE COMPOSITION

The strains included in the vaccine are shown to be suitable with respect to production of antigens of assumed immunological importance. The vaccine is shown to be

satisfactory with respect to safety (5.2.6) and efficacy (5.2.7) in the species of fish for which it is intended. The following tests may be used during demonstration of safety and immunogenicity.

Safety

A. During development of the vaccine, safety is tested on 3 different batches. A test is carried out in each species of fish for which the vaccine is intended. The fish used are from a population that does not have specific antibodies against *A. salmonicida* subsp. *salmonicida* and has not been vaccinated against or exposed to furunculosis. The test is carried out in the conditions recommended for the use of the vaccine with a water temperature not less than 10 °C. An amount of vaccine corresponding to twice the recommended dose per mass unit is administered intraperitoneally into each of not fewer than 50 fish of the minimum body mass recommended for vaccination. The fish are observed for 21 days. No abnormal local or systemic reaction occurs. The test is invalid if more than 6 per cent of the fish die from causes not attributable to the vaccine.

B. Safety is also demonstrated in field trials by administering the intended dose to a sufficient number of fish in not fewer than 2 sets of premises. Samples of 30 fish are taken on 3 occasions (after vaccination, at the middle of the rearing period and at slaughter) and examined for local reactions in the body cavity. Moderate lesions involving localised adhesions between viscera or between viscera and the abdominal wall and slight opaqueness and/or sparse pigmentation of the peritoneum are acceptable. Extensive lesions including adhesions between greater parts of the abdominal organs, massive pigmentation and/or obvious thickening and opaqueness of greater areas of the peritoneum are unacceptable if they occur in more than 10 per cent of the fish in any sample. Such lesions include adhesions that give the viscera a "one-unit" appearance and/or lead to manifest laceration of the peritoneum following evisceration.

Immunogenicity. The test described under Potency is suitable to demonstrate the immunogenicity of the vaccine.

BATCH TESTING

Batch potency test. For routine testing of batches of vaccine, the test described under Potency may be carried out using not fewer than 30 fish per group; alternatively, a suitable validated test based on antibody response may be carried out, the criteria being set with reference to a batch of vaccine that has given satisfactory results in the test described under Potency. The following test may be used after a satisfactory correlation with the test described under Potency has been established.

Use fish from a population that does not have specific antibodies against *A. salmonicida* subsp. *salmonicida* and that are within defined limits for body mass. Carry out the test at a defined temperature. Inject intraperitoneally into each of not fewer than 25 fish one dose of vaccine, according to the instructions for use. Perform mock vaccination on a control group of not fewer than 10 fish. Collect blood samples at a defined time after vaccination. Determine for each sample the level of specific antibodies against *A. salmonicida* subsp. *salmonicida* by a suitable immunochemical method (2.7.1). The vaccine complies with the test if the mean level of antibodies is not significantly lower than that found for a batch that gave satisfactory results in the test described under Potency. The test is not valid if the control group shows antibodies against *A. salmonicida* subsp. *salmonicida*.

IDENTIFICATION

01/2005:0696

When injected into fish that do not have specific antibodies against *A. salmonicida*, the vaccine stimulates the production of such antibodies.

TESTS

Safety. Use not fewer than 10 fish of one of the species for which the vaccine is intended, having, where possible, the minimum body mass recommended for vaccination; if fish of the minimum body mass are not available, use fish not greater than twice this mass. Use fish from a population that preferably does not have specific antibodies against *A. salmonicida* subsp. *salmonicida* or, where justified, use fish from a population with a low level of such antibodies as long as they have not been vaccinated against or exposed to furunculosis and administration of the vaccine does not cause an anamnestic response. Carry out the test in the conditions recommended for use of the vaccine with a water temperature not less than 10 °C. Administer intraperitoneally to each fish an amount of vaccine corresponding to twice the recommended dose per mass unit. Observe the animals for 21 days. No abnormal local or systemic reaction attributable to the vaccine occurs. The test is invalid if more than 10 per cent of the fish die from causes not attributable to the vaccine.

Sterility. The vaccine complies with the test for sterility prescribed in the monograph on *Vaccines for veterinary use* (0062).

POTENCY

Carry out the test according to a protocol defining limits of body mass for the fish, water source, water flow and temperature limits, and preparation of a standardised challenge. Vaccinate not fewer than 100 fish by a recommended route, according to the instructions for use. Perform mock vaccination on a control group of not fewer than 100 fish; mark vaccinated and control fish for identification. Keep all the fish in the same tank or mix equal numbers of controls and vaccinates in each tank if more than one tank is used. Carry out challenge by injection at a fixed time interval after vaccination, defined according to the statement regarding development of immunity. Use for challenge a culture of *A. salmonicida* subsp. *salmonicida* whose virulence has been verified. Observe the fish daily until at least 60 per cent specific mortality is reached in the control group. Plot for both vaccinates and controls a curve of specific mortality against time from challenge and determine by interpolation the time corresponding to 60 per cent specific mortality in controls. The test is invalid if the specific mortality is less than 60 per cent in the control group 21 days after the first death in the fish. Read from the curve for vaccinates the mortality (*M*) at the time corresponding to 60 per cent mortality in controls. Calculate the relative percentage survival (RPS) from the expression:

$$\left(1 - \frac{M}{60}\right) \times 100$$

The vaccine complies with the test if the RPS is not less than 80 per cent.

LABELLING

The label states information on the time needed for development of immunity after vaccination under the range of conditions corresponding to the recommended use.

INFECTIOUS BOVINE RHINOTRACHEITIS VACCINE (LIVE), FREEZE-DRIED

*Vaccinum rhinotracheitidis infectivae
bovinae vivum cryodesiccatum*

DEFINITION

Freeze-dried infectious bovine rhinotracheitis vaccine (live) is a preparation of one or more attenuated strains of infectious bovine rhinotracheitis virus (bovine herpesvirus 1).

PRODUCTION

The virus is grown in suitable cell cultures (5.2.4). The viral suspension is collected and mixed with a suitable stabilising solution. The mixture is subsequently freeze-dried.

CHOICE OF VACCINE STRAIN

Only a virus strain shown to be satisfactory with respect to the following characteristics may be used in the preparation of the vaccine: safety (including absence of abortigenicity and passage through the placenta); reversion to virulence and immunogenicity. The strain may have markers. The following tests may be used during demonstration of safety (5.2.6) and efficacy (5.2.7).

Safety. Use 5 calves 3 months old, or of the minimum age to be recommended for vaccination if this is less than 3 months, and that do not have antibodies against infectious bovine rhinotracheitis virus. Administer to each calf, by the intended route, a quantity of virus corresponding to 10 doses of vaccine. The calves are observed for 21 days. No abnormal local or systemic reaction occurs.

Abortigenicity and passage through the placenta.

24 pregnant cows that do not have antibodies against infectious bovine rhinotracheitis virus are used for the test: 8 of the cows are in the fourth month of pregnancy, 8 in the fifth month and 8 in the sixth or seventh month. A quantity of virus equivalent to ten doses of vaccine is administered by the intended route to each cow. The cows are observed until the end of pregnancy. If abortion occurs, tests for infectious bovine rhinotracheitis virus are carried out; neither the virus nor viral antigens are present in the foetus or placenta. A test for antibodies against infectious bovine rhinotracheitis virus is carried out on calves born at term before ingestion of colostrum; no such antibodies are found.

Reversion to virulence. Suitable samples are taken from the 5 calves used for the test for safety at a time when the vaccinal virus can be easily detected. The presence and titre of virus in the samples are verified. The samples are then mixed and administered intranasally to 2 other calves of the same age having no antibodies against bovine rhinotracheitis virus. 5 further serial passages are carried out. The presence of the virus is verified at each passage. No abnormal local or systemic reaction occurs.

Immunogenicity. The test described under Potency is suitable to demonstrate immunogenicity.

BATCH TESTING

If the test for potency has been carried out with satisfactory results on a representative batch of vaccine, this test may be omitted as a routine control on other batches of vaccine prepared from the same seed lot, subject to agreement by the competent authority.

IDENTIFICATION

- A. Reconstitute the vaccine as stated on the label. When mixed with a suitable quantity of a monospecific antiserum, the reconstituted vaccine is no longer able to infect susceptible cell cultures into which it is inoculated.
- B. Any markers of the strain are verified.

TESTS

Safety. Use 2 calves 3 months old or of the minimum age recommended for vaccination if this is less than 3 months, having no antibodies against bovine rhinotracheitis virus. Administer 10 doses of the reconstituted vaccine to each calf by a recommended route. Observe the calves for 21 days. No abnormal local or systemic reaction occurs.

Bacterial and fungal contamination. The reconstituted vaccine complies with the test for sterility prescribed in the monograph on *Vaccines for veterinary use (0062)*.

Mycoplasmas (2.6.7). The reconstituted vaccine complies with the test for mycoplasmas.

Extraneous viruses. Neutralise the vaccine using a monospecific antiserum and inoculate into suitable cell cultures. Maintain the cultures for 14 days and make a passage at 7 days. The cell cultures show no signs of viral contamination.

Virus titre. Titrate the reconstituted vaccine in susceptible cell cultures at a temperature favourable to replication of the virus. One dose of the vaccine contains not less than the quantity of virus equivalent to the minimum virus titre stated on the label.

POTENCY

Use susceptible calves, 2 to 3 months old and free from antibodies neutralising infectious bovine rhinotracheitis virus. Administer to five calves, by the route stated on the label a volume of the reconstituted vaccine containing a quantity of virus equivalent to the minimum virus titre stated on the label. Keep 2 calves as controls. After 21 days, administer intranasally to the seven calves a quantity of infectious bovine rhinotracheitis virus sufficient to produce typical signs of disease such as fever, ocular and nasal discharge and ulceration of the nasal mucosa in a susceptible calf. Observe the animals for 21 days. The vaccinated calves show no more than mild signs; the controls show typical signs. In not fewer than 4 of the 5 vaccinated calves, the maximum virus titre found in the nasal mucus is at least 100 times lower than the average of the maximum titres found in the control calves; the average number of days on which virus is excreted is at least 3 days less in vaccinated calves than in the control calves.

01/2005:2038

INFECTIOUS CHICKEN ANAEMIA VACCINE (LIVE)

Vaccinum anaemiae infectivae pulli vivum

1. DEFINITION

Infectious chicken anaemia vaccine (live) is a preparation of a suitable strain of chicken anaemia virus. This monograph applies to vaccines intended for administration to breeder chickens for active immunisation, to prevent excretion of the virus, to prevent or reduce egg transmission and to protect passively their future progeny.

2. PRODUCTION

2-1. PREPARATION OF THE VACCINE

The vaccine virus is grown in embryonated hens' eggs or in cell cultures.

2-2. SUBSTRATE FOR VIRUS PROPAGATION

2-2-1. Embryonated hens' eggs. If the vaccine virus is grown in embryonated hens' eggs, they are obtained from flocks free from specified pathogens (SPF) (5.2.2).

2-2-2. Cell cultures. If the vaccine virus is grown in cell cultures, they comply with the requirements for cell cultures for production of veterinary vaccines (5.2.4).

2-3. SEED LOTS

2-3-1. Extraneous agents. The master seed lot complies with the tests for extraneous agents in seed lots (2.6.24). In these tests on the master seed lot, the organisms used are not more than 5 passages from the master seed lot at the start of the tests.

2-4. CHOICE OF VACCINE VIRUS

The vaccine virus shall be shown to be satisfactory with respect to safety (5.2.6) and efficacy (5.2.7) for the chickens for which it is intended.

The following tests for safety (section 2-4-1), increase in virulence (section 2-4-2) and immunogenicity (section 2-4-3) may be used during the demonstration of safety and immunogenicity.

2-4-1. Safety

2-4-1-1. General test. Carry out the test for each route and method of administration to be recommended for vaccination in chickens not older than the youngest age to be recommended for vaccination and from an SPF flock (5.2.2). Use vaccine virus at the least attenuated passage level that will be present between the master seed lot and a batch of the vaccine. For each test use not fewer than 20 chickens. Administer to each chicken a quantity of the vaccine virus not less than 10 times the maximum virus titre likely to be contained in 1 dose of the vaccine. 14 days after vaccination, collect blood samples from half of the chickens and determine the haematocrit value. Kill these chickens and carry out post-mortem examination. Note any pathological changes attributable to chicken anaemia virus, such as thymic atrophy and specific bone-marrow lesions. Observe the remaining chickens at least daily for 21 days. The vaccine virus complies with the test if during the observation period no chicken shows notable clinical signs of chicken anaemia or dies from causes attributable to the vaccine virus.

2-4-1-2. Safety for young chicks. Use not fewer than twenty 1-day-old chicks from an SPF flock (5.2.2). Administer to each chick by the oculonasal route a quantity of the vaccine virus equivalent to not less than the maximum titre likely to be contained in 1 dose of the vaccine. Observe the chicks at least daily. Record the incidence of any clinical signs attributable to the vaccine virus, such as depression, and any deaths. 14 days after vaccination, collect blood samples from half of the chicks and determine the haematocrit value. Kill these chicks and carry out post-mortem examination. Note any pathological changes attributable to chicken anaemia virus, such as thymic atrophy and specific bone marrow lesions. Observe the remaining chicks at least daily for 21 days. Assess the extent to which the vaccine strain is pathogenic for 1-day-old susceptible chicks from the results of the clinical observations and mortality rates and the proportion of chicks examined at 14 days that show anaemia (haematocrit value less than 27 per cent) and signs of infectious chicken anaemia on post-mortem examination. The results are used to formulate the label statement on safety for young chicks.

2-4-2. Increase in virulence. The test for increase in virulence consists of the administration of the vaccine virus at the least attenuated passage level that will be present between the master seed lot and a batch of the vaccine to a group of five 1-day-old chicks from an SPF flock (5.2.2), sequential passages, 5 times where possible, to further similar groups of 1-day-old chicks and testing of the final recovered virus for increase in virulence. If the properties of the vaccine virus allow sequential passage to 5 groups via natural spreading, this method may be used, otherwise passage as described below is carried out and the maximally passaged virus that has been recovered is tested for increase in virulence. Care must be taken to avoid contamination by virus from previous passages. Administer by the intramuscular route a quantity of the vaccine virus that will allow recovery of virus for the passages described below. Prepare 7 to 9 days after administration a suspension from the liver of each chick and pool these samples. Depending on the tropism of the virus, other tissues such as spleen or bone marrow may be used. Administer 0.1 ml of the pooled samples by the intramuscular route to each of 5 other chicks of the same age and origin. Carry out this passage operation at least 5 times; verify the presence of the virus at each passage. If the virus is not found at a passage level, carry out a second series of passages. Carry out the tests for safety (section 2-4-1) using the unpassaged vaccine virus and the maximally passaged vaccine virus that has been recovered. The vaccine virus complies with the test if no indication of increase in virulence of the maximally passaged virus compared with the unpassaged virus is observed. If virus is not recovered at any passage level in the first and second series of passages, the vaccine virus also complies with the test.

2-4-3. Immunogenicity. A test is carried out for each route and method of administration to be recommended using chickens from an SPF flock (5.2.2) not older than the youngest age to be recommended for vaccination. The test for prevention of virus excretion is intended to demonstrate absence of egg transmission. The quantity of the vaccine virus administered to each chicken is not greater than the minimum titre to be stated on the label and the virus is at the most attenuated passage level that will be present in a batch of the vaccine.

2-4-3-1 Passive immunisation of chickens. Vaccinate according to the recommended schedule not fewer than 10 breeder chickens not older than the minimum age recommended for vaccination and from an SPF flock (5.2.2); keep not fewer than 10 unvaccinated breeder chickens of the same origin and from an SPF flock (5.2.2). At a suitable time after excretion of vaccine virus has ceased, collect fertilised eggs from each vaccinated and control breeder chicken and incubate them. Challenge at least 3 randomly chosen 1-day-old chickens from each vaccinated and control breeder chicken by intramuscular administration of a sufficient quantity of virulent chicken anaemia virus. Observe the chickens at least daily for 14 days after challenge. Record the deaths and the surviving chickens that show clinical signs of disease. At the end of the observation period determine the haematocrit value of each surviving chicken. Kill these chickens and carry out post-mortem examination. Note any pathological signs attributable to chicken anaemia virus, such as thymic atrophy and specific bone-marrow lesions. The test is not valid if:

- during the observation period after challenge fewer than 90 per cent of the chickens of the control breeder chickens die or show severe clinical signs of infectious

chicken anaemia, including haematocrit value under 27 per cent, and/or notable macroscopic lesions of the bone marrow and thymus,

- and/or during the period between vaccination and egg collection more than 10 per cent of vaccinated or control breeder chickens show notable clinical signs of disease or die from causes not attributable to the vaccine.

The vaccine complies with the test if during the observation period after challenge not fewer than 90 per cent of the chickens of the vaccinated breeder chickens survive and show no notable clinical signs of disease and/or macroscopic lesions of the bone marrow and thymus.

2-4-3-2. Prevention of virus excretion. Vaccinate according to the recommended schedule not fewer than 10 chickens not older than the minimum age recommended for vaccination and from an SPF flock (5.2.2). Maintain separately not fewer than 10 chickens of the same age and origin as controls. At a suitable time after excretion of vaccine virus has ceased, challenge all the chickens by intramuscular administration of a sufficient quantity of virulent chicken anaemia virus. Collect blood and faecal samples from the chickens on days 3, 5 and 7 after challenge and carry out a test for virus isolation to determine whether or not the chickens are viraemic and are excreting the virus. The test is not valid if:

- fewer than 70 per cent of the control chickens are viraemic and excrete the virus at one or more times of sampling,
- and/or during the period between vaccination and challenge more than 10 per cent of control or vaccinated chickens show abnormal clinical signs or die from causes not attributable to the vaccine.

The vaccine complies with the test if not fewer than 90 per cent of the vaccinated chickens do not develop viraemia or excrete the virus.

3 BATCH TESTING

3-1. Identification. The vaccine, diluted if necessary and mixed with a monospecific chicken anaemia virus antiserum, no longer infects susceptible cell cultures or eggs from an SPF flock (5.2.2) into which it is inoculated.

3-2. Bacteria and fungi. Vaccines intended for administration by injection comply with the test for sterility prescribed in the monograph *Vaccines for veterinary use (0062)*.

Vaccines not intended for administration by injection either comply with the test for sterility prescribed in the monograph *Vaccines for veterinary use (0062)* or with the following test: carry out a quantitative test for bacterial and fungal contamination; carry out identification tests for microorganisms detected in the vaccine; the vaccine does not contain pathogenic microorganisms and contains not more than 1 non-pathogenic microorganism per dose.

Any liquid supplied with the vaccine complies with the test for sterility prescribed in the monograph *Vaccines for veterinary use (0062)*.

3-3. Mycoplasmas. The vaccine complies with the test for mycoplasmas (2.6.7).

3-4. Extraneous agents. The vaccine complies with the tests for extraneous agents in batches of finished product (2.6.25).

3-5. Safety. Use not fewer than 10 chickens not older than the minimum age recommended for vaccination and from an SPF flock (5.2.2). Administer by a recommended route to each chicken 10 doses of the vaccine. Observe the chickens at least daily for 21 days. The test is not valid if more than 20 per cent of the chickens show abnormal clinical signs or die from causes not attributable to the vaccine. The

vaccine complies with the test if no chicken shows notable clinical signs of disease or dies from causes attributable to the vaccine.

3-6. Virus titre. Titrate the vaccine virus by inoculation into suitable cell cultures (5.2.4) or eggs from an SPF flock (5.2.2). The vaccine complies with the test if 1 dose contains not less than the minimum virus titre stated on the label.

3-7. Potency. The vaccine complies with the requirements of the tests prescribed under Immunogenicity (sections 2-4-3-1 and 2-4-3-2) when administered by a recommended route and method. It is not necessary to carry out the potency test for each batch of the vaccine if it has been carried out on a representative batch using a vaccinating dose containing not more than the minimum virus titre stated on the label.

4. LABELLING

The label states to which extent the vaccine virus causes disease if it spreads to susceptible young chicks.

01/2005:1944

MANNHEIMIA VACCINE (INACTIVATED) FOR CATTLE

Vaccinum mannheimiae inactivatum ad bovidas

DEFINITION

Mannheimia vaccine (inactivated) for cattle is a preparation from cultures of one or more suitable strains of *Mannheimia haemolytica* (formerly *Pasteurella haemolytica*). This monograph applies to vaccines intended for administration to cattle of different ages for protection against respiratory diseases caused by *M. haemolytica*.

PRODUCTION

Production of the vaccine is based on a seed-lot system. The seed material is cultured in a suitable medium; each strain is cultivated separately and identity is verified using a suitable method. During production, various parameters such as growth rate are monitored by suitable methods; the values are within the limits approved for the particular product. Purity and identity of the harvest are verified using suitable methods. After cultivation, the bacterial suspensions are collected separately and inactivated by a suitable method. The vaccine may contain an adjuvant and may be freeze-dried.

CHOICE OF VACCINE COMPOSITION

The choice of composition and the strains to be included in the vaccine is based on epidemiological data on the prevalence of the different serovars of *M. haemolytica* and on the claims being made. The vaccine is shown to be satisfactory with respect to safety (5.2.6) and efficacy (5.2.7) in cattle. As part of the studies to demonstrate the suitability of the vaccine with respect to these characteristics the following tests may be carried out.

Safety

- A. The test is carried out for each route of administration to be stated on the label and in animals of each category for which the vaccine is intended.

For each test, use not fewer than 10 animals that preferably do not have antibodies against the serovars of *M. haemolytica* or against the leucotoxin present in the vaccine. Where justified, animals with a known history

of no previous mannheimia vaccination and with low antibody titres (measured in a sensitive test system such as an ELISA) may be used.

Administer to each animal a double dose of vaccine containing not less than the maximum potency that may be expected in a batch of vaccine. Administer a single dose of vaccine to each animal after the recommended interval. Observe the animals for at least 14 days after the last administration. Record body temperature the day before vaccination, at vaccination, 2 h, 4 h and 6 h later and then daily for 4 days; note the maximum temperature increase for each animal. No abnormal local or systemic reaction occurs; the average body temperature increase for all animals does not exceed 1.5 °C and no animal shows a rise greater than 2 °C. If the vaccine is intended for use or may be used in pregnant cows, vaccinate the cows at the relevant stages of pregnancy and prolong the observation period until 1 day after parturition.

The vaccine complies with the test if no animal shows abnormal local or systemic reactions or clinical signs of disease or dies from causes attributable to the vaccine. In addition, if the vaccine is intended for use in pregnant cows, no significant effects on the pregnancy and offspring are demonstrated.

- B. The animals used for the field trials are also used to evaluate safety. Carry out a test in each category of animals for which the vaccine is intended. Use not fewer than 3 groups of 20 animals with corresponding groups of not fewer than 10 controls in 3 different locations. Examine the injection sites for local reactions after vaccination. Record body temperatures the day before vaccination, at vaccination and on the 2 days following vaccination. The vaccine complies with the test if no animal shows abnormal local or systemic reactions or clinical signs of disease or dies from causes attributable to the vaccine. The average body temperature increase for all animals does not exceed 1.5 °C and no animal shows a rise greater than 2 °C. In addition, if the vaccine is intended for use in pregnant cows, no significant effects on the pregnancy and offspring are demonstrated.

Immunogenicity. As part of the studies to demonstrate the suitability of the vaccine with respect to immunogenicity, the test described under Potency may be carried out for each proposed route of administration and using vaccine of minimum potency.

BATCH TESTING

Batch potency test. The test described under Potency is not carried out for routine testing of batches of vaccine. It is carried out, for a given vaccine, on one or more occasions, as decided by or with the agreement of the competent authority. Where the test is not carried out, a suitable validated test is carried out, the criteria for acceptance being set with reference to the results obtained with a batch of vaccine that has given satisfactory results in the test described under Potency.

Bacterial endotoxins. A test for bacterial endotoxins (2.6.14) is carried out on the final lot or, where the nature of the adjuvant prevents performance of a satisfactory test, on the bulk antigen or the mixture of bulk antigens immediately before addition of the adjuvant. The maximum acceptable amount of bacterial endotoxins is that found for a batch of vaccine that has been shown satisfactory in safety test A given under Choice of vaccine composition or in the safety test described under Tests, carried out using 10 animals. Where the latter test is used, note the maximum temperature increase for each animal; the average body temperature increase for all animals does not exceed 1.5 °C. The method

01/2005:1946

chosen for determining the amount of bacterial endotoxin present in the vaccine batch used in the safety test for determining the maximum acceptable level of endotoxin is used subsequently for testing of each batch.

IDENTIFICATION

When injected into healthy seronegative animals, the vaccine stimulates the production of specific antibodies against the serovars of *M. haemolytica* and/or against the leucotoxin present in the vaccine.

TESTS

Safety. Use 2 cattle of the minimum age recommended for vaccination that have not been vaccinated against mannheimiosis. Administer a double dose of vaccine to each animal by a recommended route. Observe the animals for 14 days. Record body temperature the day before vaccination, at vaccination, 2 h, 4 h and 6 h later and then daily for 2 days. The animals remain in good health and no abnormal local or systemic reaction occurs; a transient temperature increase not exceeding 2 °C may occur.

Sterility. It complies with the test for sterility prescribed in the monograph on *Vaccines for veterinary use (0062)*.

POTENCY

Carry out a test for each serovar for which protection is claimed on the label.

Use not fewer than 16 animals of the minimum age recommended for vaccination, free from antibodies against *M. haemolytica* and against the leucotoxin of *M. haemolytica*. Vaccinate not fewer than 8 of the animals by a recommended route and according to the recommended schedule. Keep 8 animals as controls. 21 days after the last vaccination, challenge all the animals by the intratracheal route or by another appropriate route, with a suitable quantity of a low-passage, virulent strain of a serovar of *M. haemolytica*. Observe the animals for a further 7 days; to avoid unnecessary suffering, severely ill animals are killed and are then considered to have died from the disease. During the observation period, the animals are examined for signs of disease for example, increased body temperature, dullness, abnormal breathing and the mortality is recorded. Kill surviving animals at the end of the observation period. Post-mortem examination is carried out on any animal that dies and those killed at the end of the observation period. The lungs are examined and the extent of lung lesions due to mannheimiosis is evaluated. Samples of lung tissue are collected for re-isolation of the challenge organisms. The clinical observations and lung lesions are scored and the results obtained for these parameters and the bacterial re-isolation results compared for the 2 groups.

The test is invalid if signs of *M. haemolytica* infection occur in less than 70 per cent of the control animals.

The vaccine complies with the requirements of the test if there is a significant difference between the scores obtained for the clinical and post-mortem observations in the vaccinates compared to the controls. For vaccines with a claim for a beneficial effect on the extent of infection against the serovar, the results for the infection rates are also significantly better for the vaccinates compared to the controls.

LABELLING

The label states:

- the serovar(s) of *M. haemolytica* against which protection is claimed,
- the serovar(s) of *M. haemolytica* and/or the leucotoxin present in the vaccine.

MANNHEIMIA VACCINE (INACTIVATED) FOR SHEEP

Vaccinum mannheimiae inactivatum ad ovem

DEFINITION

Mannheimia vaccine (inactivated) for sheep is a preparation of one or more suitable strains of *Mannheimia haemolytica* (formerly *Pasteurella haemolytica*). This monograph applies to vaccines intended for administration to sheep for active immunisation and to protect passively their future progeny against disease caused by *M. haemolytica*.

PRODUCTION

Production of the vaccine is based on a seed lot system. The seed material is cultured in a suitable medium; each strain is cultivated separately and identity is verified using a suitable method. During production, various parameters such as growth rate are monitored by suitable methods; the values are within the limits approved for the particular product. Purity and identity of the harvest are verified using suitable methods. After cultivation, the bacterial suspensions are collected separately and inactivated by a suitable method. The vaccine may contain an adjuvant and may be freeze-dried.

CHOICE OF VACCINE COMPOSITION

The choice of composition and the strains to be included in the vaccine is based on epidemiological data on the prevalence of the different serovars of *M. haemolytica* and on the claims being made for the product, for example active and/or passive protection. The vaccine is shown to be satisfactory with respect to safety (5.2.6) and efficacy (5.2.7) in sheep. As part of the studies to demonstrate the suitability of the vaccine with respect to these characteristics the following tests may be carried out.

Safety

- A. The test is carried out for each of the routes of administration to be stated on the label and in animals of each category (for example, young sheep, pregnant ewes) for which the vaccine is intended.

For each test, use not fewer than 10 animals that preferably do not have antibodies against the serovars of *M. haemolytica* or against the leucotoxin present in the vaccine. Where justified, animals with a known history of no previous mannheimia vaccination and with low antibody titres (measured in a sensitive test system such as an ELISA) may be used.

Administer to each animal a double dose of vaccine containing not less than the maximum potency that may be expected in a batch of vaccine. Administer a single dose of vaccine to each animal after the recommended interval. Observe the animals for at least 14 days after the last administration. Record body temperature the day before vaccination, at vaccination, 2 h, 4 h and 6 h later and then daily for 4 days; note the maximum temperature increase for each animal. No abnormal local or systemic reaction occurs; the average body temperature increase for all animals does not exceed 1.5 °C and no animal shows a rise greater than 2 °C. If the vaccine is intended for use or may be used in pregnant ewes, vaccinate the ewes at the relevant stages of pregnancy and prolong the observation period until 1 day after lambing.

The vaccine complies with the test if no animal shows abnormal local reactions or clinical signs of disease or dies from causes attributable to the vaccine. In addition, if the vaccine is intended for use in pregnant ewes, no significant effects on the pregnancy and offspring are demonstrated.

- B. The animals used for the field trials are also used to evaluate safety. Carry out a test in each category of animals for which the vaccine is intended. Use not fewer than 3 groups of 20 animals with corresponding groups of not fewer than 10 controls in 3 different locations. Examine the injection sites for local reactions after vaccination. Record body temperatures the day before vaccination, at vaccination and on the 2 days following vaccination. The vaccine complies with the test if no animal shows abnormal local or systemic reactions or clinical signs of disease or dies from causes attributable to the vaccine. The average body temperature increase for all animals does not exceed 1.5 °C and no animal shows a rise greater than 2 °C. In addition, if the vaccine is intended for use in pregnant ewes, no significant effects on the pregnancy and offspring are demonstrated.

Immunogenicity. As part of the studies to demonstrate the suitability of the vaccine with respect to immunogenicity, the tests described under Potency may be carried out for each proposed route of administration and using vaccine of minimum potency.

BATCH TESTING

Batch potency test. The relevant test or tests described under Potency are not carried out for routine testing of batches of vaccine. They are carried out, for a given vaccine, on one or more occasions, as decided by or with the agreement of the competent authority. Where the relevant test or tests are not carried out, a suitable validated batch potency test is carried out, the criteria for acceptance being set with reference to the results obtained with a batch of vaccine that has given satisfactory results in the test(s) described under Potency.

Bacterial endotoxins. A test for bacterial endotoxins (2.6.14) is carried out on the final lot or, where the nature of the adjuvant prevents performance of a satisfactory test, on the bulk antigen or the mixture of bulk antigens immediately before addition of the adjuvant. The maximum acceptable amount of bacterial endotoxins is that found for a batch of vaccine that has been shown satisfactory in safety test A given under Choice of vaccine composition or in the safety test described under Tests, carried out using 10 animals. Where the latter test is used, note the maximum temperature increase for each animal; the average body temperature increase for all animals does not exceed 1.5 °C. The method chosen for determining the amount of bacterial endotoxin present in the vaccine batch used in the safety test for determining the maximum acceptable level of endotoxin is used subsequently for testing of each batch.

IDENTIFICATION

When injected into healthy seronegative animals, the vaccine stimulates the production of specific antibodies against the serovars of *M. haemolytica* and/or against the leucotoxin present in the vaccine.

TESTS

Safety. Use 2 sheep of the minimum age recommended for vaccination or, if not available, of an age as close as possible to the minimum recommended age, and that have not been vaccinated against mannheimiosis. Administer a double dose of vaccine to each animal by a recommended route. Observe the animals for 14 days. Record body temperature the day

before vaccination, at vaccination, 2 h, 4 h and 6 h later and then daily for 2 days. The animals remain in good health and no abnormal local or systemic reaction occurs; a transient temperature increase not exceeding 2 °C may occur.

Sterility. It complies with the test for sterility prescribed in the monograph on *Vaccines for veterinary use (0062)*.

POTENCY

Active immunisation. For vaccines with claims for active immunisation due to *M. haemolytica*, carry out a test for each serovar of *M. haemolytica* for which protection is claimed on the label.

Use not fewer than 20 lambs of the minimum age recommended for vaccination, free from antibodies against *M. haemolytica* and against the leucotoxin of *M. haemolytica*. Vaccinate not fewer than 10 of the animals by a recommended route and according to the recommended schedule. Keep 10 animals as controls. 21 days after the last vaccination, challenge all the lambs by the intratracheal route or by another appropriate route, with a suitable quantity of a low-passage, virulent strain of a serovar of *M. haemolytica*. Where necessary for a given serovar, prechallenge with parainfluenza type 3 (PI3) virus or another appropriate respiratory pathogen may be used. Observe the animals for a further 7 days; to avoid unnecessary suffering, severely ill animals are killed and are then considered to have died from the disease. During the observation period, the animals are examined for signs of disease (for example, increased body temperature, dullness, abnormal respiration) and the mortality is recorded. Kill surviving animals at the end of the observation period. Post-mortem examination is carried out on any animal that dies and those killed at the end of the observation period. The lungs are examined and the extent of lung lesions due to mannheimiosis is evaluated. Samples of lung tissue are collected for re-isolation of the challenge organisms. The clinical observations and lung lesions are scored and the results obtained for these parameters and the bacterial re-isolation results compared for the 2 groups.

The test is invalid if signs of *M. haemolytica* infection occur in less than 70 per cent of the control lambs.

The vaccine complies with the requirements of the test if there is a significant difference between the scores obtained for the clinical and post-mortem observations in the vaccinates compared to the controls. For vaccines with a claim for a beneficial effect on the extent of infection against the serovar, the results for the infection rates are also significantly better for the vaccinates compared to the controls.

Passive protection. For vaccines with claims for passive protection against mannheimiosis carry out a test for each serovar of *M. haemolytica* for which protection is claimed on the label.

Use at least 6 ewes that preferably do not have antibodies against the serovars of *M. haemolytica* or against the leucotoxin present in the vaccine. Where justified, animals with a known history of no previous mannheimia vaccination, from a source with a low incidence of respiratory disease and with low antibody titres (measured in a sensitive test system such as an ELISA) may be used. Vaccinate the animals by 1 of the recommended routes, at the recommended stages of pregnancy and according to the recommended schedule. A challenge study is conducted with 20 newborn, colostrum-deprived lambs. 10 of these lambs are given colostrum from the vaccinated ewes and 10 control lambs are given colostrum or colostrum substitute without detectable antibodies to *M. haemolytica*. When the lambs are at the age claimed for the duration of the passive protection, challenge by the intratracheal route with

a suitable quantity of a low-passage, virulent strain of a serovar of *M. haemolytica*. Observe the animals for a further 7 days; to avoid unnecessary suffering, severely ill animals are killed and are then considered to have died from the disease. Observe the animals and assess the effect of the challenge on the offspring of the vaccinates and the controls as described in the test for active immunisation.

The test is invalid if clinical signs or lesions of *M. haemolytica* infection occur in less than 70 per cent of the control lambs.

The vaccine complies with the requirements of the test if there is a significant difference between the scores obtained for the clinical and post-mortem observations in the lambs from the vaccinates compared to those from the controls. For vaccines with a claim for a beneficial effect on the extent of infection against the serovar, the results for the infection rates are also significantly better for the lambs from the vaccinates compared to those from the controls.

LABELLING

The label states:

- the serovar(s) of *M. haemolytica* against which protection is claimed,
- the serovar(s) of *M. haemolytica* and/or the leucotoxin present in the vaccine,
- for vaccines for passive protection, the length of time for which the passive protection is claimed.

01/2005:0589

MAREK'S DISEASE VACCINE (LIVE)

Vaccinum morbi Marek vivum

1. DEFINITION

Marek's disease vaccine (live) is a preparation of a suitable strain or strains of Marek's disease virus (gallid herpesvirus 2 or 3) and/or turkey herpesvirus (meleagrid herpesvirus 1). This monograph applies to vaccines intended for administration to chickens for active immunisation.

2. PRODUCTION

2-1. PREPARATION OF THE VACCINE

The vaccine virus is grown in cell cultures. If the vaccine contains more than 1 type of virus, the different types are grown separately. The vaccine may be freeze-dried or stored in liquid nitrogen.

2-2. SUBSTRATE FOR VIRUS PROPAGATION

2-2-1. **Cell cultures.** The cell cultures comply with the requirements for cell cultures for production of veterinary vaccines (5.2.4).

2-3. SEED LOTS

2-3-1. **Extraneous agents.** The master seed lot complies with the tests for extraneous agents in seed lots (2.6.24). In these tests on the master seed lot, the organisms used are not more than 5 passages from the master seed lot at the start of the tests.

2-4. CHOICE OF VACCINE VIRUS

The vaccine virus shall be shown to be satisfactory with respect to safety (5.2.6) and efficacy (5.2.7) for the chickens for which it is intended.

The tests shown below for safety of the strain (section 2-4-1), increase in virulence (section 2-4-2) and immunogenicity (section 2-4-3) may be used during the demonstration of safety and immunogenicity. Additional testing may be

needed to demonstrate safety in breeds of chickens known to be particularly susceptible to Marek's disease virus, unless the vaccine is to be contra-indicated.

2-4-1. **Safety.** Carry out the test for the route to be recommended for vaccination likely to be the least safe and in the category of chickens for which the vaccine is intended likely to be the most susceptible for Marek's disease. Use vaccine virus at the least attenuated passage level that will be present between the master seed lot and a batch of the vaccine. Use not fewer than eighty 1-day-old chickens from a flock free from specified pathogens (SPF) (5.2.2). Divide them randomly into 2 groups of not fewer than 40 chickens and maintain the groups separately. Carry out examination for macroscopic lesions of each chicken that dies and of the surviving chickens at the end of the observation periods. Administer to each chicken of one group (I) a quantity of the vaccine virus equivalent to not less than 10 times the maximum virus titre likely to be contained in 1 dose of the vaccine. Administer by a suitable route to each chicken of the other group (II) a quantity of virulent Marek's disease virus that will cause mortality and/or severe macroscopic lesions of Marek's disease in not fewer than 70 per cent of the effective number of chickens within 70 days (initial number reduced by the number that die within the first 7 days of the test). Observe the chickens of group II at least daily for 70 days and those of group I at least daily for 120 days. The test is not valid if 1 or more of the following apply: more than 10 per cent of the chickens in any group die within the first 7 days of the test; fewer than 70 per cent of the effective number of chickens in group II show macroscopic lesions of Marek's disease. The vaccine virus complies with the test if:

- no chicken of group I shows notable clinical signs or macroscopic lesions of Marek's disease or dies from causes attributable to the vaccine virus,
- at 120 days the number of surviving chickens of group I is not fewer than 80 per cent of the effective number.

2-4-2. **Increase in virulence.** The test for increase in virulence is required for Marek's disease virus vaccine strains but not for turkey herpesvirus vaccine strains, which are naturally apathogenic. Administer by the intramuscular route a quantity of the vaccine virus that will allow recovery of virus for the passages described below to each of five 1-day-old chickens from an SPF flock (5.2.2). Use vaccine virus at the least attenuated passage level that will be present between the master seed lot and a batch of the vaccine. Prepare 5 to 7 days later a suspension of white blood cells of each chicken and pool these samples. Administer a suitable volume of the pooled samples by the intraperitoneal route to each of 5 other chickens that are 1-day-old and from an SPF flock (5.2.2). Carry out this passage operation not fewer than 5 times; verify the presence of the virus at each passage. Care must be taken to avoid contamination by virus from previous passages. If the virus is not found at a passage level, carry out a second series of passages. Carry out the safety test (section 2-4-1) using the unpassaged vaccine virus and the maximally passaged virus that has been recovered. Administer the virus by the route to be recommended for vaccination likely to be the least safe for use in these birds. The vaccine virus complies with the test if no indication of increase in virulence of the maximally passaged virus compared with the unpassaged virus is observed. If virus is not recovered at any passage level in the first and second series of passages, the vaccine virus also complies with the test.

2-4-3. **Immunogenicity.** A test is carried out for each route and method of administration to be recommended using in each case chickens of the youngest age to be recommended for vaccination. The quantity of the vaccine

virus administered to each chicken is not greater than the minimum virus titre to be stated on the label and the virus is at the most attenuated passage level that will be present in a batch of the vaccine. Use for the test not fewer than 60 chickens of the same origin and from an SPF flock (5.2.2). Vaccinate by a recommended route not fewer than 30 chickens. Maintain not fewer than 30 chickens as controls. Challenge each chicken after 9 days by a suitable route with a sufficient quantity of virulent Marek's disease virus. Observe the chickens at least daily for 70 days after challenge. Record the deaths and the number of surviving chickens that show clinical signs of disease. At the end of the observation period kill all the surviving chickens and carry out examination for macroscopic lesions of Marek's disease. The test is not valid if:

- during the observation period after challenge fewer than 70 per cent of the control chickens die or show severe clinical signs or macroscopic lesions of Marek's disease,
- and/or during the period between the vaccination and challenge more than 10 per cent of the control or vaccinated chickens show abnormal clinical signs or die from causes not attributable to the vaccine.

The vaccine virus complies with the test if the percentage relative protection, calculated using the following expression, is not less than 80 per cent:

$$\frac{V - C}{100 - C} \times 100$$

- V* = percentage of challenged vaccinated chickens that survive to the end of the observation period without notable clinical signs or macroscopic lesions of Marek's disease,
- C* = percentage of challenged control chickens that survive to the end of the observation period without notable clinical signs or macroscopic lesions of Marek's disease.

3. BATCH TESTS

3-1. Identification. Carry out an immunostaining test in cell cultures using monoclonal antibodies to demonstrate the presence of each type of virus stated on the label.

3-2. Bacteria and fungi. The vaccine and, where applicable, the liquid supplied with it comply with the requirement for sterility prescribed in the monograph *Vaccines for veterinary use* (0062).

3-3. Mycoplasmas. The vaccine complies with the test for mycoplasmas (2.6.7).

3-4. Extraneous agents. The vaccine complies with the tests for extraneous agents in batches of finished product (2.6.25).

3-5. Safety. Use not fewer than 10 chickens from an SPF flock (5.2.2) and not older than the youngest age recommended for vaccination. Administer by a recommended route and method to each chicken 10 doses of the vaccine. Observe the chickens at least daily for 21 days. The test is not valid if more than 20 per cent of the chickens show abnormal clinical signs or die from causes not attributable to the vaccine. The vaccine complies with the test if no chicken shows notable clinical signs of disease or dies from causes attributable to the vaccine.

3-6. Virus titre

3-6-1 Vaccines containing one type of virus. Titrate the vaccine virus by inoculation into suitable cell cultures (5.2.4). If the virus titre is determined in plaque-forming units (PFU), only primary plaques are taken into consideration. The

vaccine complies with the test if 1 dose contains not less than the minimum virus titre stated on the label.

3-6-2. Vaccines containing more than one type of virus. For vaccines containing more than 1 type of virus, titrate each virus by inoculation into suitable cell cultures (5.2.4), reading the results by immunostaining using antibodies. The vaccine complies with the test if 1 dose contains for each vaccine virus not less than the minimum virus titre stated on the label.

3-7. Potency. The vaccine complies with the requirements of the test prescribed under Immunogenicity (section 2-4-3) when administered according to the recommended schedule by a recommended route and method. It is not necessary to carry out the potency test for each batch of the vaccine if it has been carried out on a representative batch using a vaccinating dose containing not more than the minimum virus titre stated on the label.

01/2005:1943

MYXOMATOSIS VACCINE (LIVE) FOR RABBITS

*Vaccinum myxomatosis vivum
ad cuniculum*

DEFINITION

Myxomatosis vaccine (live) for rabbits is a preparation of a suitable strain of either myxoma virus that is attenuated for rabbits or Shope fibroma virus. The vaccine is intended for the active immunisation of rabbits against myxomatosis.

PRODUCTION

The virus is propagated in suitable cell cultures (5.2.4). The viral suspension is harvested, titrated and may be mixed with a suitable stabilising solution. The vaccine may be freeze-dried.

CHOICE OF VACCINE STRAIN

The vaccine is shown to be satisfactory with respect to safety, absence of increase in virulence and immunogenicity. The following tests may be used during demonstration of safety, absence of increase in virulence (5.2.6) and efficacy (5.2.7). **Safety.** The test is carried out for each route of administration to be stated on the label. Use at least 10 rabbits of the minimum age to be recommended for vaccination and that do not have antibodies against myxoma virus. Administer to each rabbit by a recommended route a quantity of virus corresponding to not less than 10 times the maximum titre that may be expected in a dose of vaccine. Observe the rabbits for 28 days. Record the body temperature the day before vaccination, at vaccination, 4 h after vaccination and then daily for 4 days; note the maximum temperature increase for each animal. No abnormal local or systemic reaction occurs; the average temperature increase does not exceed 1 °C and no animal shows a rise greater than 2 °C. A local reaction lasting less than 28 days may occur.

If the vaccine is intended for use in pregnant rabbits, administer the virus to not less than 10 pregnant rabbits according to the schedule to be recommended on the label. Prolong the observation period until 1 day after parturition. The rabbits remain in good health and there is no abnormal local or systemic reaction. No adverse effects on the pregnancy or the offspring are noted.

Increase in virulence. (This test is performed only for vaccines based on attenuated strains of myxoma virus). Administer by a recommended route to each of 2 rabbits, 5 to 7 weeks old and which do not have antibodies against

myxoma virus, a quantity of virus that will allow recovery of virus for the passages described below. Use vaccine virus at the least attenuated passage level that will be present between the master seed lot and a batch of the vaccine. Kill the rabbits 5 to 10 days after inoculation and remove from each rabbit organs, or tissues with sufficient virus to allow passage; homogenise the organs and tissues in a suitable buffer solution, centrifuge the suspension and use the supernatant for further passages. Inoculate the supernatant into suitable cell cultures to verify the presence of virus. Administer by an appropriate route, at a suitable rate, a suitable volume of the supernatant to each of 2 other rabbits of the same age and the same susceptibility. This operation is then repeated at least 5 times. If the virus has disappeared, a second series of passages is carried out. Inoculate virus from the highest recovered passage level to rabbits, observe for 28 days and compare any reactions that occur with those seen in the test for safety described above. There is no indication of an increase in virulence as compared with the non-passaged virus. If virus is not recovered in either of 2 series of passages, the vaccine virus also complies with the test.

Immunogenicity. The test described under Potency may be used to demonstrate the immunogenicity of the strain.

BATCH POTENCY TEST

If the test for potency has been carried out with satisfactory results on a representative batch of vaccine, using a vaccinating dose containing not more than the minimum virus titre stated on the label, this test may be omitted as a routine control on other batches of vaccine prepared from the same seed lot.

IDENTIFICATION

Carry out an immunofluorescence test in suitable cell cultures, using a monospecific antiserum.

TESTS

Safety. Use not fewer than 2 rabbits, not older than the minimum age recommended for vaccination, that do not have antibodies against myxoma virus and rabbit haemorrhagic disease virus and that have been reared in suitable isolation conditions to avoid contact with myxoma virus. Administer by a recommended route to each rabbit 10 doses of vaccine. Observe the rabbits at least daily for 14 days. No abnormal local or systemic reaction occurs.

Extraneous agents. At the end of the 14 day observation period of the safety test, administer by a recommended route to each rabbit, a further 10 doses of vaccine. After 14 days take a blood sample from each rabbit and carry out a test for antibodies against rabbit haemorrhagic disease virus. No antibodies are found.

Bacterial and fungal contamination. The vaccine, reconstituted if necessary, complies with the test for sterility prescribed in the monograph on *Vaccines for Veterinary Use* (0062).

Mycoplasmas (2.6.7). The vaccine, reconstituted if necessary, complies with the test for mycoplasmas.

Virus titre. Reconstitute the vaccine, if necessary, and titrate in suitable cell cultures. 1 dose of the vaccine contains not less than the quantity of virus equivalent to the minimum virus titre stated on the label.

POTENCY

Use not fewer than 15 susceptible rabbits of the minimum age to be recommended for vaccination, free from antibodies against myxoma virus and reared in suitable isolation conditions to ensure absence of contact with myxoma virus.

Administer 1 dose of vaccine to each of not fewer than 10 of the rabbits according to the instructions for use. Keep not less than 5 other rabbits as controls. Not less than 21 days after the last vaccination, administer by a suitable route to each rabbit a quantity of a virulent strain of myxoma virus sufficient to cause typical signs of myxomatosis in a susceptible rabbit. Observe the rabbits for a further 21 days. The test is not valid if fewer than 90 per cent of the control rabbits display typical signs of myxomatosis. A vaccine containing myxoma virus complies with the test if not fewer than 90 per cent of vaccinated rabbits show no signs of myxomatosis. A vaccine containing Shope fibroma virus complies with the test if not fewer than 75 per cent of vaccinated rabbits show no signs of myxomatosis.

LABELLING

The label states, where applicable, that a local reaction may occur.

01/2005:0962

NEONATAL PIGLET COLIBACILLOSIS VACCINE (INACTIVATED)

Vaccinum colibacillosis fetus a partu recentis inactivatum ad suem

DEFINITION

Neonatal piglet colibacillosis vaccine (inactivated) is prepared from cultures of one or more suitable strains of *Escherichia coli*, carrying one or more adhesins or enterotoxins. This monograph applies to vaccines administered by injection to sows and gilts for protection of newborn piglets against enteric forms of colibacillosis.

PRODUCTION

The *E. coli* strains used for production are cultured separately in a suitable medium. The cells or toxins are processed to render them safe and are blended.

The vaccine may contain one or more suitable adjuvants.

CHOICE OF VACCINE COMPOSITION

The *E. coli* strains used in the production of the vaccine are shown to be satisfactory with respect to expression of antigens and the vaccine is shown to be satisfactory with respect to safety and immunogenicity. The following tests may be used during demonstration of safety (5.2.6) and efficacy (5.2.7).

Expression of antigens. The expression of antigens that stimulate a protective immune response is verified by a suitable immunochemical method (2.7.1) carried out on the antigen obtained from each of the vaccine strains under the conditions used for the production of the vaccine.

Safety

A. Administer a double dose of vaccine by a recommended route to each of not fewer than 10 pregnant sows that have not been vaccinated against colibacillosis. Administer 1 dose of vaccine to each of the animals after the recommended interval. Observe the animals until farrowing. Record body temperature the day before vaccination, at vaccination, 2 h, 4 h and 6 h later and then daily for 4 days; note the maximum temperature increase for each animal. Note any effects on gestation or the offspring. No abnormal local or systemic reaction occurs: the average temperature increase for all animals does not exceed 1.5 °C and no animal shows a rise greater than 2 °C.

B. The animals used for field trials are also used to evaluate safety. Use not fewer than 3 groups each of not fewer than 20 animals with corresponding groups of not fewer than 10 controls. Examine the injection site for local reactions after vaccination. Record body temperature the day before vaccination, at vaccination, at the time interval after which a rise in temperature, if any, was seen in test A, and daily during the 2 days following vaccination; note the maximum temperature increase for each animal. No abnormal local or systemic reaction occurs: the average temperature increase for all animals does not exceed 1.5 °C and no animal shows a rise greater than 2 °C.

Immunogenicity. The suitability of the vaccine with respect to immunogenicity may be demonstrated by the test described under Potency.

BATCH TESTING

Batch potency test. *The test described under Potency is not carried out for routine testing of batches of vaccine. It is carried out, for a given vaccine, on one or more occasions, as decided by or with the agreement of the competent authority; where the test is not carried out, a suitable validated test is carried out, the criteria for acceptance being set with reference to a batch of vaccine that has given satisfactory results in the test described under Potency. The following test may be used after a suitable correlation with the test described under Potency has been established by a statistical evaluation.*

Use pigs not less than 3 weeks old and free from specific antibodies against the antigens stated on the label: vaccinate each of 5 pigs by the route and according to the schedule stated on the label. Maintain 2 pigs as unvaccinated controls. Alternatively, if the nature of the antigens allows reproducible results to be obtained, a test in laboratory animals (for example, guinea-pigs, mice, rabbits or rats) may be carried out. To obtain a valid assay, it may be necessary to carry out a test using several groups of animals, each receiving a different dose. For each dose, carry out the test as follows. Vaccinate not fewer than 5 animals with a single injection of a suitable dose. Maintain not fewer than 2 animals as unvaccinated controls. Where the schedule stated on the label requires a booster injection to be given, a booster vaccination may also be given in this test provided it has been demonstrated that this will still provide a suitably sensitive test system. At a given interval within the range of 14 to 21 days after the last injection, collect blood from each animal and prepare serum samples. Use a suitable validated test such as an enzyme-linked immunosorbent assay (2.7.1) to measure the antibody response to each of the antigens stated on the label. The antibody levels are not significantly less than those obtained with a batch that has given satisfactory results in the test described under Potency and there is no significant increase in antibody titre in the controls.

Where seronegative animals are not available, seropositive animals may be used in the above test. During the development of a test with seropositive animals, particular care will be required during the validation of the test system to establish that the test is suitably sensitive and to specify acceptable pass, fail and retest criteria. It will be necessary to take into account the range of possible prevaccination titres and establish the acceptable minimum titre rise after vaccination in relation to these.

Bacterial endotoxins. A test for bacterial endotoxins (2.6.14) is carried out on the final lot or, where the nature of the adjuvant prevents performance of a satisfactory test, on the bulk antigen or the mixture of bulk antigens immediately before addition of the adjuvant. The maximum acceptable

amount of bacterial endotoxins is that found for a batch of vaccine that has been shown satisfactory in safety test A given under Choice of vaccine composition or in the safety test described under Tests, carried out using 10 piglets. Where the latter test is used, note the maximum temperature increase for each animal; the average temperature increase for all animals does not exceed 1.5 °C. The method chosen for determining the amount of bacterial endotoxin present in the vaccine batch used in the safety test for determining the maximum acceptable level of endotoxin is used subsequently for testing of each batch.

IDENTIFICATION

In animals free from specific antibodies against the antigens stated on the label, the vaccine stimulates the production of antibodies against these antigens.

TESTS

Safety. Use pigs preferably having no specific antibodies against the antigens stated on the label or, where justified, pigs with a low level of such antibodies as long as they have not been vaccinated against colibacillosis and administration of the vaccine does not cause an anamnestic response. Administer to each of 2 pigs a double dose of vaccine by a recommended route. Observe the animals for 14 days. Record body temperature before vaccination, at vaccination, 2 h, 4 h and 6 h later and then daily for 2 days. No abnormal local or systemic reaction occurs; a transient temperature increase not exceeding 2 °C may occur.

Sterility. The vaccine complies with the test for sterility prescribed in the monograph on *Vaccines for veterinary use* (0062).

POTENCY

Carry out the test with a challenge strain representing each type of antigen against which the vaccine is intended to protect: if a single strain with all the necessary antigens is not available, repeat the test using different challenge strains.

Use not fewer than 8 susceptible gilts free from specific antibodies against the antigens stated on the label. Take not fewer than 4 at random and vaccinate these at the stage of pregnancy and according to the recommended vaccination scheme. Within 12 h of their giving birth, take not fewer than 15 healthy piglets from the vaccinated animals and 15 healthy piglets from the unvaccinated controls, taking at least 3 from each litter. Challenge all the piglets orally with a pathogenic strain of *E. coli* before or after colostrum feeding and using the same conditions for vaccinated animals and controls. The strain used must not be one used in the manufacture of the vaccine. Return the piglets to their dam and observe for 8 days.

On each day, note clinical signs in each piglet and score using the following scale:

- | | |
|---|----------------------------------|
| 0 | no signs |
| 1 | slight diarrhoea |
| 2 | marked diarrhoea (watery faeces) |
| 3 | dead |

Total scores for each piglet over 8 days are calculated. The test is not valid unless at least 40 per cent of the piglets from the control animals die and not more than 15 per cent of the piglets from the control animals show no signs of illness. The vaccine complies with the test if there is a significant reduction in score in the group of piglets from the vaccinated gilts compared with the group from the unvaccinated controls.

For some adhesins (for example, F5 and F41), there is published evidence that high mortality cannot be achieved under experimental conditions. If challenge has to be carried out with a strain having such adhesins: the test is not valid if fewer than 70 per cent of the control piglets show clinical signs expected with the challenge strain; the vaccine complies with the test if there is a significant reduction in score in the group of piglets from the vaccinated gilts compared with the group from the unvaccinated controls.

LABELLING

The label states the antigen or antigens contained in the vaccine that stimulate a protective immune response.

01/2005:0961

NEONATAL RUMINANT COLIBACILLOSIS VACCINE (INACTIVATED)

*Vaccinum colibacillosis fetus a partu
recentis inactivatum ad ruminantes*

DEFINITION

Neonatal ruminant colibacillosis vaccine (inactivated) is prepared from cultures of one or more suitable strains of *Escherichia coli*, carrying one or more adhesin factors or enterotoxins. This monograph applies to vaccines administered by injection to dams for protection of newborn offspring against enteric forms of colibacillosis.

PRODUCTION

The *E. coli* strains used for production are cultured separately in a suitable medium. The cells or toxins are processed to render them safe and are blended.

The vaccine may contain one or more suitable adjuvants.

CHOICE OF VACCINE COMPOSITION

The *E. coli* strains used in the production of the vaccine are shown to be satisfactory with respect to expression of antigens and the vaccine is shown to be satisfactory with respect to safety and immunogenicity. The following tests may be used during demonstration of safety (5.2.6) and efficacy (5.2.7).

Expression of antigens. The expression of antigens that stimulate a protective immune response is verified by a suitable immunochemical method (2.7.1) carried out on the antigen obtained from each of the vaccine strains under the conditions used for the production of the vaccine.

Safety

- A. A double dose of vaccine is administered to each of 10 pregnant animals of each of the species for which the vaccine is intended and that have not been vaccinated against colibacillosis. 1 dose is administered to each animal after the interval stated on the label. The animals are observed until parturition has occurred. Record body temperature the day before vaccination, at vaccination, 2 h, 4 h and 6 h later and then daily for 4 days; note the maximum temperature increase for each animal. No abnormal local or systemic reaction occurs; the average temperature increase for all animals does not exceed 1.5 °C and no animal shows a rise greater than 2 °C. Any effects on gestation or the offspring are noted.
- B. Safety is demonstrated in field trials for each species for which the vaccine is intended by administering the intended dose to at least 60 animals from 3 different

stocks, by the route and according to the schedule stated on the label. At least 30 animals from the same stocks are assigned to control groups. The animals are observed for 14 days after the last dose. No abnormal local or systemic reaction is noted and, in particular, no rise in temperature of more than 1.5 °C occurs within 2 days of administration of each dose of vaccine.

Immunogenicity. The suitability of the vaccine with respect to immunogenicity must be demonstrated for each species for which it is intended. This may be demonstrated by the test described under Potency.

BATCH TESTING

Batch potency test. *The test described under Potency is not carried out for routine testing of batches of vaccine. It is carried out, for a given vaccine, on one or more occasions, as decided by or with the agreement of the competent authority; where the test is not carried out, a suitable validated test is carried out, the criteria for acceptance being set with reference to a batch of vaccine that has given satisfactory results in the test described under Potency. The following test may be used after a suitable correlation with the test described under Potency has been established by a statistical evaluation.*

To obtain a valid assay, it may be necessary to carry out a test using several groups of animals, each receiving a different dose. For each dose required, carry out the test as follows. Vaccinate not fewer than 5 animals (for example rabbits, guinea-pigs, rats or mice), free from specific antibodies against the antigens stated on the label, using one injection of a suitable dose. Maintain 2 animals as unvaccinated controls. Where the schedule stated on the label requires a booster injection to be given, a booster vaccination may also be given in this test provided it has been demonstrated that this will still provide a suitably sensitive test system. At a given interval within the range of 14 to 21 days after the last injection, collect blood from each animal and prepare serum samples. Use a suitable validated test such as an enzyme-linked immunosorbent assay (2.7.1) to measure the antibody response to each of the protective antigens stated on the label. The antibody levels are not significantly less than those obtained with a batch that has given satisfactory results in the test described under Potency and there is no significant increase in antibody titre in the controls.

Where seronegative animals are not available, seropositive animals may be used in the above test. During the development of a test with seropositive animals, particular care will be required during the validation of the test system to establish that the test is suitably sensitive and to specify acceptable pass, fail and retest criteria. It will be necessary to take into account the range of possible prevaccination titres and establish the acceptable minimum titre rise after vaccination in relation to these.

Bacterial endotoxins. A test for bacterial endotoxins (2.6.14) is carried out on the final lot or, where the nature of the adjuvant prevents performance of a satisfactory test, on the bulk antigen or the mixture of bulk antigens immediately before addition of the adjuvant. The maximum acceptable amount of bacterial endotoxins is that found for a batch of vaccine that has been shown satisfactory in safety test A given under Choice of vaccine composition or in the safety test described under Tests, carried out using 10 animals. Where the latter test is used, note the maximum temperature increase for each animal; the average temperature increase for all animals does not exceed 1.5 °C. The method chosen for determining the amount of bacterial endotoxin present

in the vaccine batch used in the safety test for determining the maximum acceptable level of endotoxins is used subsequently for testing of each batch.

01/2005:0870

IDENTIFICATION

In animals free from specific antibodies against the antigens stated on the label, the vaccine stimulates the production of antibodies against these antigens.

TESTS

Safety. Use animals of one of the species for which the vaccine is recommended and preferably having no specific antibodies against the antigens stated on the label or, where justified, use animals with a low level of such antibodies as long as they have not been vaccinated against colibacillosis and administration of the vaccine does not cause an anamnestic response. Administer by a recommended route a double dose of vaccine to each of 2 animals. Observe the animals for 14 days. Record body temperature before vaccination, at vaccination, 2 h, 4 h and 6 h later and then daily for 2 days. No abnormal local or systemic reaction occurs; a transient temperature increase not exceeding 2 °C may occur.

Sterility. The vaccine complies with the test for sterility prescribed in the monograph on *Vaccines for veterinary use* (0062).

POTENCY

Carry out the test with a challenge strain representing each type of antigen against which the vaccine is intended to protect: if a single strain with all the necessary antigens is not available, repeat the test using different challenge strains.

Use not fewer than 15 susceptible animals of one of the species for which the vaccine is recommended and which are free from specific antibodies against the antigens stated on the label. Take not fewer than 10 at random and vaccinate these at the recommended stage of pregnancy and according to the recommended schedule. Collect colostrum from all animals after parturition and store the samples individually in conditions that maintain antibody levels. Take not fewer than 15 newborn unsuckled animals and house them in an environment ensuring absence of enteric pathogens. Allocate a colostrum sample from not fewer than 10 vaccinated dams and not fewer than 5 controls to the offspring. After birth, feed the animals with the colostrum sample allocated to it. After feeding the colostrum and within 12 h of birth, challenge the animals orally with a pathogenic strain of *E. coli* and observe for 10 days. The strain must not be one used in the manufacture of the vaccine.

On each day, note clinical signs in each animal and score using the following scale:

- 0 no signs
- 1 slight diarrhoea
- 2 marked diarrhoea (watery faeces)
- 3 dead

Total scores for each animal over 10 days are calculated. The test is not valid unless 80 per cent of the offspring from the control animals die or show severe signs of disease. The vaccine complies with the test if there is a significant reduction in score in the group of animals from vaccinated dams compared with the group from the unvaccinated controls.

LABELLING

The label states the antigen or antigens contained in the vaccine that stimulate a protective immune response.

NEWCASTLE DISEASE VACCINE (INACTIVATED)

Vaccinum pseudopestis aviariae inactivatum

DEFINITION

Newcastle disease vaccine (inactivated) [also known as avian paramyxovirus 1 vaccine (inactivated) for vaccines intended for some species] consists of an emulsion or a suspension of a suitable strain of Newcastle disease virus (avian paramyxovirus 1) that has been inactivated in such a manner that immunogenic activity is retained.

PRODUCTION

The virus is propagated in embryonated eggs from healthy flocks or in suitable cell cultures (5.2.4).

The test for inactivation is carried out in embryonated eggs or suitable cell cultures and the quantity of inactivated virus used is equivalent to not less than ten doses of vaccine. No live virus is detected.

The vaccine may contain an adjuvant.

CHOICE OF VACCINE COMPOSITION

The vaccine is shown to be satisfactory with respect to safety (5.2.6) and immunogenicity (5.2.7) for each species and category of birds for which it is intended. The following tests may be used during demonstration of immunogenicity.

Immunogenicity. For domestic fowl, the test with virulent challenge (test B) described under Potency is suitable for demonstrating immunogenicity. For other species of birds (for example, pigeons or turkeys), test C described under Potency is suitable for demonstrating immunogenicity.

BATCH TESTING

Batch potency test

For vaccines used in domestic fowl, carry out test A described under Potency but, if the nature of the product does not allow valid results to be obtained with test A or if the batch does not comply with test A, carry out test B. A test using fewer than twenty birds per group and a shorter observation period after challenge may be used if this has been shown to give a valid potency test.

For vaccines used in species other than domestic fowl, carry out a suitable validated test for which a satisfactory correlation has been established with test C described under Potency, the criteria for acceptance being set with reference to a batch that has given satisfactory results in the latter test. A test in chickens from a flock free from specified pathogens (5.2.2) and consisting of a measure of the serological response to graded amounts of vaccine (for example, 1/25, 1/50 and 1/100 dose with serum sampling 17 to 21 days later) may be used.

IDENTIFICATION

When injected into animals free from antibodies against Newcastle disease virus, the vaccine stimulates the production of such antibodies.

TESTS

Safety. If the vaccine is intended for use in domestic fowl, vaccinate ten chickens from a flock free from specified pathogens (5.2.2). If the vaccine is not for use in domestic fowl, use ten birds of one of the species for which the vaccine is intended, free from antibodies against Newcastle disease virus. Inject twice the vaccinating dose by a recommended

route into each of ten birds, 14 to 28 days old. Observe the birds for 21 days. No abnormal local or systemic reaction occurs.

Inactivation. Inject two-fifths of a dose into the allantoic cavity of each of ten embryonated hen eggs, 9 to 11 days old, from flocks free from specified pathogens (5.2.2) (SPF eggs) and incubate. Observe for 6 days and pool separately the allantoic fluid from eggs containing live embryos, and that from eggs containing dead embryos, excluding those dying within 24 h of the injection. Examine embryos that die within 24 h of injection for the presence of Newcastle disease virus: the vaccine does not comply with the test if Newcastle disease virus is found.

Inject into the allantoic cavity of each of ten SPF eggs, 9 to 11 days old, 0.2 ml of the pooled allantoic fluid from the live embryos and, into each of ten similar eggs, 0.2 ml of the pooled fluid from the dead embryos and incubate for 5 to 6 days. Test the allantoic fluid from each egg for the presence of haemagglutinins using chicken erythrocytes.

The vaccine complies with the test if there is no evidence of haemagglutinating activity and if not more than 20 per cent of the embryos die at either stage. If more than 20 per cent of the embryos die at one of the stages, repeat that stage; the vaccine complies with the test if there is no evidence of haemagglutinating activity and not more than 20 per cent of the embryos die at that stage.

Antibiotics may be used in the test to control extraneous bacterial infection.

Extraneous agents. Inject a double dose by a recommended route into each of ten chickens, 14 to 28 days old and from a flock free from specified pathogens (5.2.2). After 3 weeks, inject one dose by the same route. Collect serum samples from each chicken 2 weeks later and carry out tests for antibodies to the following agents by the methods prescribed for chicken flocks free from specified pathogens (5.2.2): avian encephalomyelitis virus, avian infectious bronchitis virus, avian leucosis viruses, egg-drop syndrome virus, avian bursal disease virus, avian infectious laryngotracheitis virus, influenza A virus, Marek's disease virus. The vaccine does not stimulate the formation of antibodies against these agents.

Sterility. The vaccine complies with the test for sterility prescribed in the monograph on *Vaccines for veterinary use* (0062).

POTENCY

For vaccines for use in domestic fowl carry out test A. If an unsatisfactory result is obtained with test A but a satisfactory result is obtained with test B, the vaccine complies with the requirements. For vaccines for use only in other species, such as pigeons, carry out test C.

A. Inject intramuscularly into each of not fewer than ten chickens, 21 to 28 days old and from a flock free from specified pathogens (5.2.2), a volume of the vaccine equivalent to 1/50 of a dose. 17 to 21 days later, collect serum samples from each vaccinated chicken and from each of not fewer than five control chickens of the same age and from the same source. Measure the antibody levels in the sera by the haemagglutination-inhibition (HI) test using the technique described below or an equivalent technique with the same numbers of

haemagglutinating units and red blood cells. The test system used must include negative and positive control sera, the latter having an HI titre of 5.0 log₂ to 6.0 log₂.

The vaccine complies with the test if the mean HI titre of the vaccinated group is equal to or greater than 4.0 log₂ and that of the unvaccinated group is 2.0 log₂ or less. If the HI titres are not satisfactory, carry out test B.

Haemagglutination-inhibition test. Inactivate the test sera by heating at 56 °C for 30 min. Add 0.025 ml to the first row of wells in a microtitre plate. Add 0.025 ml of a buffered 9 g/l solution of *sodium chloride R* at pH 7.2 to pH 7.4 to the rest of the wells. Prepare twofold dilutions of the sera across the plate. To each well add 0.025 ml of a suspension containing 4 haemagglutinating units of inactivated Newcastle disease virus. Incubate the plate at 4 °C for 1 h. Add 0.025 ml of a 1 per cent V/V suspension of red blood cells collected from chickens, 3 to 4 weeks old and free from antibodies against Newcastle disease virus. Incubate the plate at 4 °C for 1 h. The HI titre is equal to the highest dilution that produces complete inhibition.

- B. Use chickens 21 to 28 days old and from a flock free from specified pathogens (5.2.2). For vaccination, use not fewer than three groups, each of not fewer than twenty chickens; keep a group of ten chickens as controls. Choose a number of different volumes of the vaccine corresponding to the number of groups: for example, volumes equivalent to 1/25, 1/50 and 1/100 of a dose. Allocate a different volume to each vaccination group. Inject intramuscularly into each chicken the volume of vaccine allocated to its group. 17 to 21 days later, challenge all the chickens by intramuscular injection of 6 log₁₀ embryo LD₅₀ of the Herts (Weybridge 33/56) strain of Newcastle disease virus. Observe the chickens for 21 days. Calculate the PD₅₀ by standard statistical methods from the number of chickens that survive in each vaccinated group without showing any clinical evidence of Newcastle disease during the 21 days. The vaccine complies with the test if the smallest dose stated on the label corresponds to not less than 50 PD₅₀ and the lower confidence limit is not less than 35 PD₅₀ per dose. If the lower confidence limit is less than 35 PD₅₀ per dose, repeat the test; the vaccine must be shown to contain not less than 50 PD₅₀ in the repeat test. The test is not valid unless all the control birds die within 6 days of challenge.
- C. Vaccinate not fewer than twenty birds of the target species, free from antibodies against avian paramyxovirus 1, in accordance with the recommendations for use. Maintain as unvaccinated controls a group of not fewer than ten birds of the same age, from the same source and free from antibodies against avian paramyxovirus 1. The test is invalid if serum samples obtained at the time of the first vaccination show the presence of antibodies against avian paramyxovirus 1 in either vaccinates or controls or if tests carried out at the time of challenge show such antibodies in controls. 4 weeks after the last vaccination, challenge each bird intramuscularly with a sufficient quantity of virulent avian paramyxovirus 1. The test is not valid if fewer than 90 per cent of the control birds die or show serious signs of Newcastle disease virus infection. The vaccine complies with the test if not fewer than 90 per cent of the vaccinated birds survive and show no serious signs of avian paramyxovirus 1 infection.

01/2005:0450

NEWCASTLE DISEASE VACCINE (LIVE)

Vaccinum pseudopestis aviariae vivum

1. DEFINITION

Newcastle disease vaccine (live) is a preparation of a suitable strain of Newcastle disease virus (avian paramyxovirus 1). This monograph applies to vaccines intended for administration to chickens and/or other avian species for active immunisation.

2. PRODUCTION

2-1. PREPARATION OF THE VACCINE

The vaccine virus is grown in embryonated hens' eggs or in cell cultures.

2-2. SUBSTRATE FOR VIRUS PROPAGATION

2-2-1. **Embryonated hens' eggs.** If the vaccine virus is grown in embryonated hens' eggs, they are obtained from flocks free from specified pathogens (SPF) (5.2.2).

2-2-2. **Cell cultures.** If the vaccine virus is grown in cell cultures, they comply with the requirements for cell cultures for production of veterinary vaccines (5.2.4).

2-3. SEED LOTS

2-3-1. **Extraneous agents.** The master seed lot complies with the tests for extraneous agents in seed lots (2.6.24). In these tests on the master seed lot, the organisms used are not more than 5 passages from the master seed lot at the start of the tests.

2-4. CHOICE OF VACCINE VIRUS

The vaccine virus shall be shown to be satisfactory with respect to safety (5.2.6) and efficacy (5.2.7) for the birds for which it is intended.

The following tests for intracerebral pathogenicity index (section 2-4-1), amino-acid sequence (section 2-4-2), safety (section 2-4-3), increase in virulence (section 2-4-4) and immunogenicity (section 2-4-5) may be used during the demonstration of safety and immunogenicity.

2-4-1. **Intracerebral pathogenicity index.** Use vaccine virus at the least attenuated passage level that will be present in a batch of the vaccine. Inoculate the vaccine virus into the allantoic cavity of embryonated hens' eggs, 9- to 11- days-old, from an SPF flock (5.2.2). Incubate the inoculated eggs for a suitable period and harvest and pool the allantoic fluids. Use not fewer than ten 1-day-old chickens (i.e. more than 24 h but less than 40 h after hatching), from an SPF flock (5.2.2). Administer by the intracerebral route to each chick 0.05 ml of the pooled allantoic fluids containing not less than $10^{8.0}$ EID₅₀ or, if this virus quantity cannot be achieved, not less than $10^{7.0}$ EID₅₀. Observe the chickens at least daily for 8 days after administration and score them once every 24 h. A score of 0 is attributed to a chicken if it is clinically normal, 1 if it shows clinical signs of disease and 2 if it is dead. The intracerebral pathogenicity index is the mean of the scores per chicken per observation over the 8 day period.

If an inoculum of not less than $10^{8.0}$ EID₅₀ is used, the vaccine virus complies with the test if its intracerebral pathogenicity index is not greater than 0.5; if an inoculum of not less than $10^{7.0}$ EID₅₀ but less than $10^{8.0}$ EID₅₀ is used, the vaccine virus complies with the test if its intracerebral pathogenicity index is not greater than 0.4.

2-4-2. **Amino-acid sequence.** Determine the sequence of a fragment of RNA from the vaccine virus containing the region encoding for the F0 cleavage site by a suitable method. The encoded amino-acid sequence is shown to be one of the following:

	F2						Cleavage site	F1		
Site	111	112	113	114	115	116	∇	117	118	119
	Gly	Gly	Lys	Gln	Gly	Arg		Leu	Ile	Gly
or	Gly	Gly	Arg	Gln	Gly	Arg		Leu	Ile	Gly
or	Gly	Glu	Arg	Gln	Glu	Arg		Leu	Val	Gly

or equivalent with leucine at 117 and no basic amino acids at sites 111, 112, 114 and 115.

2-4-3. **Safety.** Carry out the test for each route and method of administration to be recommended for vaccination and in each avian species for which the vaccine is intended, using in each case birds not older than the youngest age to be recommended for vaccination. Use vaccine virus at the least attenuated passage level that will be present between the master seed lot and a batch of the vaccine. For tests in chickens, use not fewer than 20 chickens, from an SPF flock (5.2.2). For species other than the chicken, use not fewer than 20 birds that do not have antibodies against Newcastle disease virus. Administer to each bird a quantity of the vaccine virus equivalent to not less than 10 times the maximum virus titre likely to be contained in 1 dose of the vaccine. Observe the birds at least daily for 21 days. The test is not valid if more than 10 per cent of the birds show abnormal clinical signs or die from causes not attributable to the vaccine virus. The vaccine virus complies with the test if no bird shows notable clinical signs of Newcastle disease or dies from causes attributable to the vaccine virus.

2-4-4. **Increase in virulence.** The test for increase in virulence consists of the administration of the vaccine virus at the least attenuated passage level that will be present between the master seed lot and a batch of the vaccine to a group of 5 birds not more than 2 weeks old, sequential passages, 5 times where possible, to further similar groups and testing of the final recovered virus for increase in virulence. If the properties of the vaccine virus allow sequential passage to 5 groups via natural spreading, this method may be used, otherwise passage as described below is carried out and the maximally passaged virus that has been recovered is tested for increase in virulence. Care must be taken to avoid contamination by virus from previous passages. Carry out the test in a target species, using the chicken if it is one of the target species. For the test in chickens, use chickens from an SPF flock (5.2.2). For other species, carry out the test in birds that do not have antibodies against Newcastle disease virus. Administer by eye-drop a quantity of the vaccine virus that will allow recovery of virus for the passages described below. Observe the birds for the period shown to correspond to maximum replication of the vaccine virus, kill them and prepare a suspension from the brain of each bird and from a suitable organ depending on the tropism of the strain (for example, mucosa of the entire trachea, intestine, pancreas); pool the samples. Administer 0.05 ml of the pooled samples by eye-drop to each of 5 other birds of the same species, age and origin. Carry out this passage operation not fewer than 5 times; verify the presence of the virus at each passage. If the virus is not found at a passage level, carry out a second series of passages.

A. Carry out the test for intracerebral pathogenicity index (section 2-4-1) using unpassaged vaccine virus and the maximally passaged virus that has been recovered.

- B. Carry out the test for amino-acid sequence (section 2-4-2) using unpassaged vaccine virus and the maximally passaged virus that has been recovered.
- C. Carry out the test for safety (section 2-4-3) using unpassaged vaccine virus and the maximally passaged virus that has been recovered. Administer the virus by the route to be recommended for vaccination likely to be the least safe and to the avian species for which the vaccine is intended that is likely to be the most susceptible to Newcastle disease.

The vaccine virus complies with the test if, in the tests 2-4-4A, 2-4-4B and 2-4-4C, no indication of increase in virulence of the maximally passaged virus compared with the unpassaged virus is observed. If virus is not recovered at any passage level in the first and second series of passages, the vaccine virus also complies with the test.

2-4-5. Immunogenicity. For each avian species for which the vaccine is intended, a test is carried out for each route and method of administration to be recommended using in each case birds not older than the youngest age to be recommended for vaccination. The quantity of the vaccine virus administered to each bird is not greater than the minimum titre to be stated on the label and the virus is at the most attenuated passage level that will be present in a batch of the vaccine.

2-4-5-1. Vaccines for use in chickens. Use not fewer than 30 chickens of the same origin and from an SPF flock (5.2.2). Vaccinate by a recommended route not fewer than 20 chickens. Maintain not fewer than 10 chickens as controls. Challenge each chicken after 21 days by the intramuscular route with not less than $10^{5.0}$ embryo LD₅₀ of the Herts (Weybridge 33/56) strain of Newcastle disease virus. Observe the chickens at least daily for 14 days after challenge. Record the deaths and the number of surviving chickens that show clinical signs of disease. The test is not valid if 6 days after challenge fewer than 100 per cent of the control chickens have died or if during the period between vaccination and challenge more than 10 per cent of the vaccinated or control chickens show abnormal clinical signs or die from causes not attributable to the vaccine. The vaccine virus complies with the test if during the observation period after challenge not fewer than 90 per cent of the vaccinated chickens survive and show no notable clinical signs of Newcastle disease.

2-4-5-2. Vaccines for use in avian species other than the chicken. Use not fewer than 30 birds of the species for which the vaccine is intended for Newcastle disease, of the same origin and that do not have antibodies against avian paramyxovirus 1. Vaccinate by a recommended route not fewer than 20 birds. Maintain not fewer than 10 birds as controls. Challenge each bird after 21 days by the intramuscular route with a sufficient quantity of virulent avian paramyxovirus 1. Observe the birds at least daily for 21 days after challenge. Record the deaths and the surviving birds that show clinical signs of disease. The test is not valid if:

- during the observation period after challenge fewer than 90 per cent of the control birds die or show severe clinical signs of Newcastle disease,
- or if during the period between the vaccination and challenge more than 10 per cent of the vaccinated or control birds show abnormal clinical signs or die from causes not attributable to the vaccine.

The vaccine virus complies with the test if during the observation period after challenge not fewer than 90 per cent of the vaccinated birds survive and show no notable clinical signs of Newcastle disease. For species where there

is published evidence that it is not possible to achieve this level of protection, the vaccine complies with the test if there is a significant reduction in morbidity and mortality of the vaccinated birds compared with the control birds.

3. BATCH TESTS

3-1. Identification

3-1-1. Identification of the vaccine virus. The vaccine, diluted if necessary and mixed with a monospecific Newcastle disease virus antiserum, no longer provokes haemagglutination of chicken red blood cells or infects embryonated hens' eggs from an SPF flock (5.2.2) or susceptible cell cultures (5.2.4) into which it is inoculated.

3-1-2. Identification of the virus strain. The strain of vaccine virus is identified by a suitable method, for example using monoclonal antibodies.

3-2. Bacteria and fungi

Vaccines intended for administration by injection comply with the test for sterility prescribed in the monograph *Vaccines for veterinary use (0062)*.

Vaccines not intended for administration by injection either comply with the test for sterility prescribed in the monograph *Vaccines for veterinary use (0062)* or with the following test: carry out a quantitative test for bacterial and fungal contamination; carry out identification tests for microorganisms detected in the vaccine; the vaccine does not contain pathogenic microorganisms and contains not more than 1 non-pathogenic microorganism per dose.

Any liquid supplied with the vaccine complies with test for sterility prescribed in the monograph *Vaccines for veterinary use (0062)*.

3-3. Mycoplasmas. The vaccine complies with the test for mycoplasmas (2.6.7).

3-4. Extraneous agents. The vaccine complies with the tests for extraneous agents in batches of finished product (2.6.25).

3-5. Safety. For vaccines recommended for use in chickens, use not fewer than 10 chickens from an SPF flock (5.2.2) and of the youngest age recommended for vaccination. For vaccines recommended for use only in avian species other than the chicken, use not fewer than 10 birds of the species likely to be most sensitive to Newcastle disease, that do not have antibodies against Newcastle disease virus and of the minimum age recommended for vaccination. Administer to each bird by eye-drop, or parenterally if only parenteral administration is recommended, 10 doses of the vaccine in a volume suitable for the test. Observe the birds at least daily for 21 days. The test is not valid if more than 20 per cent of the birds show abnormal clinical signs or die from causes not attributable to the vaccine. The vaccine complies with the test if no bird shows notable clinical signs of disease or dies from causes attributable to the vaccine.

3-6. Virus titre. Titrate the vaccine virus by inoculation into embryonated hens' eggs from an SPF flock (5.2.2) or into suitable cell cultures (5.2.4). The vaccine complies with the test if 1 dose contains not less than the minimum virus titre stated on the label.

3-7. Potency. Depending on the indications, the vaccine complies with 1 or both of the tests prescribed under Immunogenicity (section 2-4-5) when administered according to the recommended schedule by a recommended route and method. If the test in section 2-4-5-2. *Vaccine for use in avian species other than the chicken* is conducted and the vaccine is recommended for use in more than 1 avian species, the test is carried out with birds of that species for which the vaccine is recommended which is likely to be the most susceptible to avian paramyxovirus 1. It is not necessary

to carry out the potency test for each batch of the vaccine if it has been carried out on a representative batch using a vaccinating dose containing not more than the minimum virus titre stated on the label.

01/2005:2072

PASTEURELLA VACCINE (INACTIVATED) FOR SHEEP

Vaccinum pasteurellae inactivatum ad ovem

DEFINITION

Pasteurella vaccine (inactivated) for sheep is a preparation of one or more suitable strains of *Pasteurella trehalosi*. This monograph applies to vaccines intended for administration to sheep to protect against disease caused by *P. trehalosi*.

PRODUCTION

Production of the vaccine is based on a seed-lot system. The seed material is cultured in a suitable medium; each strain is cultivated separately and identity is verified using a suitable method. During production, various parameters such as growth rate are monitored by suitable methods; the values are within the limits approved for the particular product. Purity and identity of the harvest are verified using suitable methods. After cultivation, the bacterial suspensions are collected separately and inactivated by a suitable method. The vaccine may contain an adjuvant and may be freeze-dried.

CHOICE OF VACCINE COMPOSITION

The choice of composition and the strains to be included in the vaccine is based on epidemiological data on the prevalence of the different serovars of *P. trehalosi*. The vaccine is shown to be satisfactory with respect to safety (5.2.6) and efficacy (5.2.7) in sheep. As part of the studies to demonstrate the suitability of the vaccine with respect to these characteristics the following tests may be carried out.

Safety

- A. The test is carried out for each of the routes of administration to be stated on the label and in animals of each category (for example, young sheep, pregnant ewes) for which the vaccine is intended.

For each test, use not fewer than 10 animals that preferably do not have antibodies against the serovars of *P. trehalosi* or against leucotoxin present in the vaccine. Where justified, animals with a known history of no previous pasteurella vaccination and with low antibody titres (measured in a sensitive test system such as an ELISA) may be used.

Administer to each animal a double dose of vaccine containing not less than the maximum potency that may be expected in a batch of vaccine. Administer a single dose of vaccine to each animal after the recommended interval. Observe the animals for at least 14 days after the last administration. Record body temperature the day before vaccination, at vaccination, 2 h, 4 h and 6 h later and then daily for 4 days; note the maximum temperature increase for each animal. No abnormal local or systemic reaction occurs; the average body temperature increase for all animals does not exceed 1.5 °C and no animal shows a rise greater than 2 °C. If the vaccine is intended for use or may be used in pregnant ewes, vaccinate the ewes at the relevant stages of pregnancy and prolong the observation period until 1 day after lambing.

The vaccine complies with the test if no animal shows abnormal local reactions or clinical signs of disease or dies from causes attributable to the vaccine. In addition, if the vaccine is intended for use in pregnant ewes, no significant effects on the pregnancy and offspring are demonstrated.

- B. The animals used for the field trials are also used to evaluate safety. Carry out a test in each category of animals for which the vaccine is intended. Use not fewer than 3 groups of 20 animals with corresponding groups of not fewer than 10 controls in 3 different locations. Examine the injection sites for local reactions after vaccination. Record body temperatures the day before vaccination, at vaccination and on the 2 days following vaccination. The vaccine complies with the test if no animal shows abnormal local or systemic reactions or clinical signs of disease or dies from causes attributable to the vaccine. The average body temperature increase for all animals does not exceed 1.5 °C and no animal shows a rise greater than 2 °C. In addition, if the vaccine is intended for use in pregnant ewes, no significant effects on the pregnancy and offspring are demonstrated.

Immunogenicity. As part of the studies to demonstrate the suitability of the vaccine with respect to immunogenicity, the test described under Potency may be carried out for each proposed route of administration and using vaccine of minimum potency.

BATCH TESTING

Batch potency test. The test described under Potency is not carried out for routine testing of batches of vaccine. It is carried out, for a given vaccine, on one or more occasions, as decided by or with the agreement of the competent authority. Where the test is not carried out, a suitable validated batch potency test is carried out, the criteria for acceptance being set with reference to the results obtained with a batch of vaccine that has given satisfactory results in the test described under Potency.

Bacterial endotoxins. A test for bacterial endotoxins (2.6.14) is carried out on the final lot or, where the nature of the adjuvant prevents performance of a satisfactory test, on the bulk antigen or the mixture of bulk antigens immediately before addition of the adjuvant. The maximum acceptable amount of bacterial endotoxins is that found for a batch of vaccine that has been shown satisfactory in safety test A given under Choice of vaccine composition or in the safety test described under Tests, carried out using 10 animals. Where the latter test is used, note the maximum temperature increase for each animal; the average body temperature increase for all animals does not exceed 1.5 °C. The method chosen for determining the amount of bacterial endotoxin present in the vaccine batch used in the safety test for determining the maximum acceptable level of endotoxin is used subsequently for testing of each batch.

IDENTIFICATION

When injected into healthy seronegative animals, the vaccine stimulates the production of specific antibodies against the serovars of *P. trehalosi* and/or against the leucotoxin present in the vaccine.

TESTS

Safety. Use 2 sheep of the minimum age recommended for vaccination or, if not available, of an age as close as possible to the minimum recommended age, and that have not been vaccinated against Pasteurella. Administer a double dose of vaccine to each animal by a recommended route. Observe the animals for 14 days. Record body temperature the day before vaccination, at vaccination, 2 h, 4 h and 6 h later and

then daily for 2 days. The animals remain in good health and no abnormal local or systemic reaction occurs; a transient temperature increase not exceeding 2 °C may occur.

Sterility. It complies with the test for sterility prescribed in the monograph on *Vaccines for veterinary use (0062)*.

POTENCY

Carry out a test for each serovar of *P. trehalosi* for which protection is claimed on the label.

Use not fewer than 20 lambs of the minimum age recommended for vaccination, free from antibodies against *P. trehalosi* and against the leucotoxin of *P. trehalosi*. Vaccinate not fewer than 10 of the animals by a recommended route and according to the recommended schedule. Keep 10 animals as controls. 21 days after the last vaccination, infect all the lambs by injection, using the subcutaneous or other suitable route, with a suitable quantity of a low-passage, virulent strain of a serovar of *P. trehalosi*. Observe the animals for a further 7 days; to avoid unnecessary suffering, severely ill animals are killed and are then considered to have died from the disease. During the observation period, the animals are examined for any signs of disease (for example, severe dullness, excess salivation) and the mortality is recorded. Kill surviving animals at the end of the observation period. Post-mortem examination is carried out on any animal that dies and those killed at the end of the observation period. The lungs, pleura, liver and spleen are examined for haemorrhages and the extent of lung consolidation due to pasteurellosis is evaluated. Samples of lung, liver and spleen tissue are collected for re-isolation of the challenge organisms. The mortality, clinical observations and the post-mortem lesions are scored and the results obtained for these parameters and the bacterial re-isolation results compared for the 2 groups.

The test is invalid if clinical signs or lesions of *P. trehalosi* infection occur in less than 70 per cent of the control lambs.

The vaccine complies with the requirements of the test if there is a significant difference between the scores obtained for the clinical and post-mortem observations in the vaccinates compared to the controls. For vaccines with a claim for a beneficial effect on the extent of infection against the serovar, the results for the infection rates are also significantly better for the vaccinates compared to the controls.

LABELLING

The label states:

- the serovar(s) of *P. trehalosi* against which protection is claimed,
- the serovar(s) of *P. trehalosi* and/or the leucotoxin present in the vaccine.

01/2005:1360

PORCINE ACTINOBACILLOSIS VACCINE (INACTIVATED)

Vaccinum actinobacillosis inactivatum
ad suem

DEFINITION

Porcine actinobacillosis vaccine (inactivated) is a liquid preparation which has one or more of the following components: inactivated *Actinobacillus pleuropneumoniae* of a suitable strain or strains; toxins, proteins or polysaccharides derived from suitable strains

of *A. pleuropneumoniae*, and treated to render them harmless; fractions of toxins derived from suitable strains of *A. pleuropneumoniae* and treated if necessary to render them harmless. This monograph applies to vaccines intended for protection of pigs against actinobacillosis.

PRODUCTION

The seed material is cultured in a suitable medium; each strain is cultivated separately. During production, various parameters such as growth rate, protein content and quantity of relevant antigens are monitored by suitable methods; the values are within the limits approved for the particular product. Purity and identity are verified on the harvest using suitable methods. After cultivation, the bacterial suspensions are collected separately and inactivated by a suitable method. They may be detoxified, purified and concentrated. The vaccine may contain an adjuvant.

CHOICE OF VACCINE COMPOSITION

The choice of strains is based on epidemiological data. The vaccine is shown to be satisfactory with respect to safety (5.2.6) and efficacy (5.2.7) in pigs. The following tests may be used during demonstration of safety and immunogenicity.

Safety

- A. Carry out a test in each category of animals for which the vaccine is intended and by each of the recommended routes of administration. Use animals that do not have antibodies against the serotypes of *A. pleuropneumoniae* or its toxins present in the vaccine. Administer a double dose of vaccine by a recommended route to each of not fewer than 10 animals. Administer a single dose of vaccine to each of the animals after the interval recommended in the instructions for use. Observe the animals for 14 days after vaccination. Record body temperature the day before vaccination, at vaccination, 2 h, 4 h and 6 h later and then daily for 4 days; note the maximum temperature increase for each animal. No abnormal local or systemic reaction occurs; the average temperature increase for all animals does not exceed 1.5 °C and no animal shows a rise greater than 2 °C. If the vaccine is intended for use in pregnant sows, for the test in this category of animals, prolong the observation period up to farrowing and note any effects on gestation or the offspring.
- B. The animals used for field trials are also used to evaluate safety. Carry out a test in each category of animals for which the vaccine is intended. Use not fewer than 3 groups each of not fewer than 20 animals with corresponding groups of not fewer than 10 controls. Examine the injection site for local reactions after vaccination. Record body temperature the day before vaccination, at vaccination, at the time interval after which a rise in temperature, if any, was seen in test A, and daily during the 2 days following vaccination; note the maximum temperature increase for each animal. No abnormal local or systemic reaction occurs; the average temperature increase for all animals does not exceed 1.5 °C and no animal shows a rise greater than 2 °C.

Immunogenicity. The test described under Potency may be used to demonstrate the immunogenicity of the vaccine.

BATCH TESTING

Batch potency test. The test described under Potency is not carried out for routine testing of batches of vaccine. It is carried out, for a given vaccine, on one or more occasions, as decided by or with the agreement of the competent authority; where the test is not carried out, a suitable validated test is carried out, the criteria for acceptance being set with reference to a batch of vaccine that has given

satisfactory results in the test described under Potency. The following test may be used after a satisfactory correlation with the test described under Potency has been established.

Inject a suitable dose subcutaneously into each of 5 seronegative mice, weighing 18-20 g. Where the schedule stated on the label requires a booster injection to be given, a booster vaccination may also be given in this test provided it has been demonstrated that this will still provide a suitably sensitive test system. Before the vaccination and at a given interval within the range of 14-21 days after the last injection, collect blood from each animal and prepare serum samples. Determine individually for each serum the titre of specific antibodies against each antigenic component stated on the label, using a suitable validated test such as enzyme-linked immunosorbent assay (2.7.1). The vaccine complies with the test if the antibody levels are not significantly lower than those obtained for a batch that has given satisfactory results in the test described under Potency.

Bacterial endotoxins. A test for bacterial endotoxins (2.6.14) is carried out on the final bulk or, where the nature of the adjuvant prevents performance of a satisfactory test, on the bulk antigen or mixture of bulk antigens immediately before addition of the adjuvant. The maximum acceptable amount of bacterial endotoxins is that found for a batch of vaccine that has been shown satisfactory in safety test A described under Choice of vaccine composition or the safety test described under Tests, carried out using 10 pigs. Where the latter test is used, note the maximum temperature increase for each animal; the average temperature increase for all animals does not exceed 1.5 °C. The method chosen for determining the amount of bacterial endotoxin present in the vaccine batch used in the safety test for determining the maximum acceptable level of endotoxin is used subsequently for batch testing.

IDENTIFICATION

When injected into healthy seronegative animals, the vaccine stimulates the production of specific antibodies against the antigenic components of *A. pleuropneumoniae* stated on the label.

TESTS

Safety. Use 2 pigs of the minimum age stated for vaccination and which do not have antibodies against the serotypes of *A. pleuropneumoniae* or its toxins present in the vaccine. Administer to each pig a double dose of vaccine by a recommended route. Observe the animals for 14 days. Record body temperature the day before vaccination, at vaccination, 2 h, 4 h and 6 h later and then daily for 2 days. No abnormal local or systemic reaction occurs; a transient temperature increase not exceeding 2 °C may occur.

Sterility. The vaccine complies with the test for sterility prescribed in the monograph on *Vaccines for veterinary use* (0062).

POTENCY

The challenge strain for the potency test is chosen to ensure challenge with each Ap toxin ⁽¹⁾ produced by the serotypes stated on the label; it may be necessary to carry out more than one test using a different challenge strain for each test.

Vaccinate according to the recommended schedule not fewer than 7 pigs, of the minimum age recommended for vaccination, which do not have antibodies against *A. pleuropneumoniae* and Ap toxins. Keep not fewer than 7 unvaccinated pigs of the same age as controls. 3 weeks after the last vaccination, challenge all the pigs intranasally

or intratracheally or by aerosol with a suitable quantity of a serotype of *A. pleuropneumoniae*. Observe the animals for 7 days; to avoid unnecessary suffering, severely ill control animals are killed and are then considered to have died from the disease. Kill all surviving animals at the end of the observation period. Carry out a post-mortem examination on all animals. Examine the lungs, the tracheobronchial lymph nodes and the tonsils for the presence of *A. pleuropneumoniae*. Evaluate the extent of lung lesions at post-mortem examination. Each of the 7 lobes of the lungs is allotted a maximum possible lesion score ⁽²⁾ of 5. The area showing pneumonia and/or pleuritis of each lobe is assessed and expressed on a scale of 0 to 5 to give the pneumonic score per lobe (the maximum total score possible for each complete lung is 35). Calculate separately for the vaccinated and the control animals the total score (the maximum score per group is 245, if 7 pigs are used per group).

The vaccine complies with the test if the vaccinated animals, when compared with controls, show lower incidence of: mortality; typical clinical signs (dyspnoea, coughing and vomiting); typical lung lesions; re-isolation of *A. pleuropneumoniae* from the lungs, the tracheobronchial lymph nodes and the tonsils. Where possible, the incidence is analysed statistically and shown to be significantly lower for vaccinates.

LABELLING

The label states:

- the antigens present in the vaccine,
- the serotypes of *A. pleuropneumoniae* for which the vaccine affords protection.

01/2005:0963

PORCINE INFLUENZA VACCINE (INACTIVATED)

Vaccinum influenzae inactivatum ad suem

DEFINITION

Porcine influenza vaccine (inactivated) is an aqueous suspension, an oily emulsion or a freeze-dried preparation of one or more inactivated strains of swine or human influenza virus. Suitable strains contain both haemagglutinin and neuraminidase.

PRODUCTION

The virus is propagated in the allantoic cavity of fertilised hen eggs from a healthy flock or in suitable cell cultures (5.2.4). Each virus strain is cultivated separately. After cultivation, the viral suspensions are collected separately and inactivated by a method that avoids destruction of the immunogenicity. If necessary, they may be purified.

An amplification test for residual infectious influenza virus is carried out on each batch of antigen immediately after inactivation by passage in the same type of substrate as that used for production (eggs or cell cultures) or a substrate shown to be at least as sensitive. The quantity of inactivated virus used in the test is equivalent to not less than 10 doses of the vaccine. No live virus is detected.

The vaccine may contain one or more suitable adjuvants; it may be freeze-dried.

(1) The nomenclature of the toxins of *A. pleuropneumoniae* is described by J. Frey *et al.*, *Journal of General Microbiology*, 1993, 139, 1723-1728.

(2) The system of lung scores is described in detail by P.C.T. Hannan, B.S. Bhogal, J.P. Fish, *Research in Veterinary Science*, 1982, 33, 76-88.

CHOICE OF VACCINE COMPOSITION

The choice of strains is based on the antigenic types and sub-types observed in Europe. The vaccine is shown to be satisfactory with respect to safety and immunogenicity for pigs. The following tests may be used during demonstration of safety (5.2.6) and efficacy (5.2.7).

Safety

- A. A test is carried out in each category of animal for which the vaccine is intended (sows, fattening pigs). The animals used do not have antibodies against swine influenza virus. 2 doses of vaccine are injected by the intended route into each of not fewer than 10 animals. After 14 days, 1 dose of vaccine is injected into each of the animals. The animals are observed for a further 14 days. During the 28 days of the test, no abnormal local or systemic reaction is produced.
- B. The animals used in the test for immunogenicity are also used to evaluate safety. The rectal temperature of each vaccinated animal is measured at the time of vaccination and 24 h and 48 h later. No abnormal effect on rectal temperature is noted nor other systemic reactions (for example, anorexia). The injection site is examined for local reactions at slaughter. No abnormal local reaction occurs.
- C. The animals used for field trials are also used to evaluate safety. A test is carried out in each category of animals for which the vaccine is intended (sows, fattening pigs). Not fewer than 3 groups each of not fewer than 20 animals in at least 2 locations are used with corresponding groups of not fewer than 10 controls. The rectal temperature of each animal is measured at the time of vaccination and 24 h and 48 h later. No abnormal effect on rectal temperature is noted. The injection site is examined for local reactions at slaughter. No abnormal local reaction occurs.

Immunogenicity. The test described under Potency carried out using an epidemiologically relevant challenge strain or strains is suitable to demonstrate the immunogenicity of the vaccine.

IN-PROCESS TESTS

For vaccines produced in eggs, the content of bacterial endotoxins is determined on the virus harvest to monitor production.

BATCH POTENCY TEST

The test described below under Potency is not carried out for routine testing of batches of vaccine. It is carried out, for a given vaccine, on one or more occasions, as decided by or with the agreement of the competent authority; where the test is not carried out, a suitable validated test is carried out, the criteria for acceptance being set with reference to a batch of vaccine that has given satisfactory results in the test described under Potency. The following test may be used after a satisfactory correlation with the test described under Potency has been established by a statistical evaluation.

Inject subcutaneously into each of 5 seronegative guinea-pigs, 5 to 7 weeks old, a quarter of the dose stated on the label. Collect blood samples before the vaccination and 21 days after vaccination. Determine for each sample the level of specific antibodies against each virus subtype in the vaccine by haemagglutination-inhibition or another suitable test. The vaccine complies with the test if the level of antibodies is not lower than that found for a batch of vaccine that gave satisfactory results in the potency test in pigs (see Potency).

IDENTIFICATION

When injected into healthy, susceptible animals, the vaccine stimulates the production of specific antibodies against the influenza virus subtypes included in the vaccine. The antibodies may be detected by a suitable immunochemical method (2.7.1).

TESTS

Safety. Use 2 pigs, free from antibodies against swine influenza virus and not older than the minimum age stated for vaccination. Inject into each pig a double dose of vaccine by the route stated on the label. Observe the animals for 14 days and then inject into each animal 1 dose of vaccine. Observe the animals for 14 days. No abnormal local or systemic reaction occurs during the 28 days of the test.

Inactivation. If the vaccine has been prepared in eggs, inoculate 0.2 ml into the allantoic cavity of each of 10 fertilised hen eggs, 9 to 11 days old. Incubate at a suitable temperature for 3 days. The death of any embryo within 24 h of inoculation is considered as non-specific mortality and the egg is discarded. The test is not valid unless at least 80 per cent of the eggs survive. Collect the allantoic fluid of each egg, pool equal quantities and carry out a second passage on fertilised eggs in the same manner. Incubate for 4 days; the allantoic fluid of these eggs shows no haemagglutinating activity.

If the vaccine has been prepared in cell cultures, carry out a suitable test for residual infectious influenza virus using 2 passages in the same type of cell culture as used in the production of vaccine. No live virus is detected. If the vaccine contains an oily adjuvant that interferes with this test, where possible separate the aqueous phase from the vaccine by means that do not diminish the capacity to detect residual infectious influenza virus.

Extraneous viruses. On the pigs used for the safety test, carry out tests for antibodies. The vaccine does not stimulate the formation of antibodies other than those against influenza virus. In particular, no antibodies against viruses pathogenic for pigs or against viruses which could interfere with the diagnosis of infectious diseases of pigs (including viruses of the pestivirus group) are detected.

Sterility. The vaccine complies with the test for sterility prescribed in the monograph on *Vaccines for veterinary use* (0062).

POTENCY

Carry out a potency test for each subtype used in the preparation of the vaccine. Use not fewer than 20 pigs of the minimum age recommended for vaccination and that do not have antibodies against swine influenza virus. Vaccinate not fewer than 10 pigs as recommended on the label and keep not fewer than 10 pigs as unvaccinated controls. Take a blood sample from all control pigs immediately before challenge. 3 weeks after the last administration of vaccine, challenge all the pigs with a suitable quantity of a virulent influenza field virus by the intratracheal route. Kill half of the vaccinated and control pigs 24 h after challenge and the other half 72 h after challenge. For each pig, measure the quantity of influenza virus in 2 lung tissue homogenates, one from the left apical, cardiac and diaphragmatic lobes, and the other from the corresponding right lung lobes. Take equivalent samples from each animal. The test is invalid if antibodies against influenza virus are found in any control pig immediately before challenge. The vaccine complies with the test if, at both times of measurement, the mean virus titre in the pooled lung tissue samples of vaccinated pigs is

significantly lower than that for control pigs, when analysed by a suitable statistical method such as the Wilcoxon Mann-Whitney test.

01/2005:0965

PORCINE PARVOVIROSIS VACCINE (INACTIVATED)

Vaccinum parvovirus inactivatum ad suem

DEFINITION

Inactivated porcine parvovirus vaccine consists of a suspension of inactivated porcine parvovirus or of a noninfectious fraction of the virus.

PRODUCTION

The virus is grown in suitable cell cultures (5.2.4). The viral suspension is harvested; the virus is inactivated by a method that avoids destruction of the immunogenicity and may be fragmented (inactivation may be by fragmentation); the virus or viral fragments may be purified and concentrated at a suitable stage of the process.

A test for residual infectious porcine parvovirus is carried out on each batch of antigen immediately after inactivation and on the final bulk or, if the vaccine contains an adjuvant, on the bulk antigen or the mixture of bulk antigens immediately before the addition of adjuvant. The quantity used in the test is equivalent to not less than 100 doses of the vaccine. The bulk harvest is inoculated into suitable non-confluent cells; after incubation for 7 days, a subculture is made using trypsinised cells. After incubation for a further 7 days, the cultures are examined for residual live parvovirus by an immunofluorescence test. No live virus is detected.

The vaccine may contain one or more suitable adjuvants.

CHOICE OF VACCINE COMPOSITION

The vaccine is shown to be satisfactory with respect to safety (including absence of adverse effects on fertility, gestation, farrowing or offspring) and immunogenicity in pigs. The following tests may be used during demonstration of safety (5.2.6) and efficacy (5.2.7).

Safety

- A. A test is carried out in each category of animals for which the vaccine is intended and by each of the recommended routes. The animals used do not have antibodies against porcine parvovirus or against a fraction of the virus. Two doses of vaccine are injected by the intended route into each of not fewer than ten animals. After 14 days, one dose of vaccine is injected into each of the animals. The animals are observed for a further 14 days. During the 28 days of the test, no abnormal local or systemic reaction occurs. If the vaccine is intended for use in pregnant sows, for the test in this category of animal, the observation period is prolonged up to farrowing and any effects on gestation or the offspring are noted.
- B. The animals used in the test for immunogenicity are also used to evaluate safety. The rectal temperature of each vaccinated animal is measured at the time of vaccination and 24 h and 48 h later. No abnormal effect on body temperature is noted nor other systemic reactions (for example, anorexia). The injection site is examined for local reactions after vaccination and at slaughter. No abnormal local reaction occurs.
- C. The animals used for field trials are also used to evaluate safety. A test is carried out in each category of animals for which the vaccine is intended (sows, gilts). Not fewer

than three groups each of not fewer than twenty animals are used with corresponding groups of not fewer than ten controls. The rectal temperature of each animal is measured at the time of vaccination and 24 h and 48 h later. No abnormal effect on body temperature is noted. The injection site is examined for local reactions after vaccination and at slaughter. No abnormal local reaction occurs.

Immunogenicity. The test described under Potency may be used to demonstrate the immunogenicity of the vaccine.

BATCH POTENCY TEST

The test described below is not carried out for routine testing of batches of vaccine. It is carried out, for a given vaccine, on one or more occasions, as decided by or with the agreement of the competent authority; where the test is not carried out, a suitable validated test is carried out, the criteria for acceptance being set with reference to a batch of vaccine that has given satisfactory results in the test described under Potency. The following test may be used after a satisfactory correlation with the test described under Potency has been established by a statistical evaluation.

Vaccinate subcutaneously not fewer than five guinea-pigs, 5 to 7 weeks old, according to the vaccination scheme stated on the label using one-fourth of the prescribed dose volume. Take blood samples after the period corresponding to maximum antibody production and carry out tests on the serum for specific antibodies by a haemagglutination-inhibition test or other suitable test. The antibody titres are not less than those obtained with a batch of vaccine shown to be satisfactory in the test in pigs (see Potency).

IDENTIFICATION

The vaccine stimulates the formation of specific antibodies against porcine parvovirus or the fraction of the virus used in the production of the vaccine when injected into susceptible animals on one or, if necessary, more than one occasion.

TESTS

Safety. Use two pigs 6 weeks to 6 months old and having no antibodies against porcine parvovirus virus or against a fraction of the virus. Inject into each animal by one of the routes stated on the label a double dose of vaccine. Observe the animals for 14 days and then inject a single dose of vaccine into each pig. Observe the animals for a further 14 days. No abnormal local or systemic reaction occurs during the 28 days of the test.

Inactivation. *This test may be omitted by the manufacturer if a test for inactivation has been carried out on the bulk vaccine, immediately before the addition of the adjuvant, where applicable.* Use a quantity of vaccine equivalent to ten doses. If the vaccine contains an oily adjuvant, break the emulsion and separate the phases. If the vaccine contains a mineral adjuvant, carry out an elution to liberate the virus. Concentrate the viral suspension 100 times by ultrafiltration or ultracentrifugation. None of the above procedures must be such as to inactivate or otherwise interfere with detection of live virus. Carry out a test for residual live virus in suitable non-confluent cells; after incubation for 7 days, make a subculture using trypsinised cells. After incubation for a further 7 days, examine the cultures for residual live parvovirus by an immunofluorescence test. No live virus is detected.

Extraneous viruses. On the pigs used for the safety test carry out tests for antibodies. The vaccine does not stimulate the formation of antibodies - other than those against porcine parvovirus - against viruses pathogenic for pigs or

against viruses which could interfere with the diagnosis of infectious diseases in pigs (including the viruses of the pestivirus group).

Sterility. The vaccine complies with the test for sterility prescribed in the monograph on *Vaccines for veterinary use* (0062).

POTENCY

Vaccinate according to the recommended schedule not less than seven gilts, 5 to 6 months old, which do not have antibodies against porcine parvovirus or against a fraction of the virus. The interval between vaccination and service is that stated on the label. Mate the gilts on two consecutive days immediately following signs of oestrus. Keep not less than five unvaccinated mated gilts of the same age as controls. At about the 40th day of gestation, challenge all gilts using a suitable strain of porcine parvovirus. Slaughter the gilts at about the 90th day of gestation and examine their foetuses for infection with porcine parvovirus as demonstrated by the presence of either virus or antibodies. The vaccine complies with the test if not fewer than 80 per cent of the total number of piglets from vaccinated gilts are protected from infection. The test is not valid unless: not fewer than seven vaccinated gilts and five control gilts are challenged; not fewer than 90 per cent of piglets from the control gilts are infected; and the average number of piglets per litter for the vaccinated gilts is not less than six.

01/2005:1361

PORCINE PROGRESSIVE ATROPHIC RHINITIS VACCINE (INACTIVATED)

*Vaccinum rhinitidis atrophicantis
ingravescentis suillae inactivatum*

DEFINITION

Porcine progressive atrophic rhinitis vaccine (inactivated) is a preparation containing either the dermonecrotic exotoxin of *Pasteurella multocida*, treated to render it harmless while maintaining adequate immunogenic activity, or a genetically modified form of the exotoxin which has adequate immunogenic activity and which is free from toxic properties; the vaccine may also contain cells and/or antigenic components of one or more suitable strains of *P. multocida* and/or *Bordetella bronchiseptica*. This monograph applies to vaccines administered to sows and gilts for protection of their progeny.

PRODUCTION

The bacterial strains used for production are cultured separately in suitable media. The toxins and/or cells are treated to render them safe.

Detoxification. A test for detoxification of the dermonecrotic exotoxin of *P. multocida* is carried out immediately after detoxification. The concentration of detoxified exotoxin used in the test is not less than that in the vaccine. The suspension complies with the test if no toxic dermonecrotic exotoxin is detected. The test for detoxification is not required where the vaccine is prepared using a toxin-like protein free from toxic properties, produced by expression of a modified form of the corresponding gene.

Antigen content. The content of the dermonecrotic exotoxin of *P. multocida* in the detoxified suspension or the toxin-like protein in the harvest is determined by a suitable immunochemical method (2.7.1), such as an enzyme-linked

immunosorbent assay and the value found is used in the formulation of the vaccine. The content of other antigens stated on the label is also determined (2.7.1).

The vaccine may contain a suitable adjuvant.

CHOICE OF VACCINE COMPOSITION

The strains used for the preparation of the vaccine are shown to be satisfactory with respect to the production of the dermonecrotic exotoxin and the other antigens claimed to be protective. The vaccine is shown to be satisfactory with respect to safety (5.2.6) and efficacy (5.2.7).

Production of antigens. The production of antigens claimed to be protective is verified by a suitable bioassay or immunochemical method (2.7.1), carried out on the antigens obtained from each of the vaccine strains under the conditions used for the production of the vaccine.

The following tests may be used during demonstration of safety and immunogenicity.

Safety

- A. Carry out the test for each route of administration stated on the label. Use pigs that do not have antibodies against the components of the vaccine, that are from a herd or herds where there are no signs of atrophic rhinitis and that have not been vaccinated against atrophic rhinitis. If the vaccine is intended for use in pregnant animals, carry out the test in pregnant sows or gilts, vaccinating them at the recommended stage of pregnancy. Administer a double dose of vaccine by a recommended route to each of not fewer than 10 sows or gilts. Administer a single dose of vaccine to each of the animals after the recommended interval. Observe the pigs until farrowing. Record body temperature the day before vaccination, at vaccination, 2 h, 4 h and 6 h later and then daily for 4 days; note the maximum temperature increase for each animal. Note any effects on gestation and the offspring. No abnormal local or systemic reaction occurs; the average temperature increase for all animals does not exceed 1.5 °C and no animal shows a rise greater than 2 °C.
- B. The animals used for field trials are also used to evaluate safety. Use not fewer than 3 groups each of not fewer than 20 animals with corresponding groups of not fewer than 10 controls. Examine the injection site for local reactions after vaccination. Record body temperature the day before vaccination, at vaccination, at the time interval after which a rise in temperature, if any, was seen in test A, and daily during the 2 days following vaccination; note the maximum temperature increase for each animal. No abnormal local or systemic reaction occurs; the average temperature increase for all animals does not exceed 1.5 °C and no animal shows a rise greater than 2 °C.

Immunogenicity. The test described under Potency is suitable for demonstration of immunogenicity.

BATCH TESTING

Batch potency test. The test described under Potency is not carried out for routine testing of batches of vaccine. It is carried out, for a given vaccine, on one or more occasions, as decided by or with the agreement of the competent authority; where the test is not carried out, a suitable validated test is carried out, the criteria for acceptance being set with reference to a batch of vaccine that has given satisfactory results in the test described under Potency. The following test may be used after a satisfactory correlation with the test described under Potency has been established. Use not fewer than 5 pigs not less than 3 weeks old and that do not have antibodies against the components of the vaccine. Vaccinate each pig by a recommended route and according to the recommended schedule. Maintain not fewer

than 2 pigs of the same origin as unvaccinated controls under the same conditions.

Alternatively, if the nature of the antigens allows reproducible results to be obtained, a test in susceptible laboratory animals may be carried out. To obtain a valid assay, it may be necessary to carry out a test using several groups of animals, each receiving a different quantity of vaccine. For each quantity of vaccine, carry out the test as follows: vaccinate not fewer than 5 animals with a suitable quantity of vaccine. Maintain not fewer than 2 animals of the same species and origin as unvaccinated controls. Where the schedule stated on the label requires a booster injection to be given, a booster vaccination may also be given in this test provided it has been demonstrated that this will still provide a suitably sensitive test system. At a given interval within the range of 14 to 21 days after the last administration, collect blood from each animal and prepare serum samples. Use a validated test such as an enzyme-linked immunosorbent assay to measure the antibody response to each of the antigens stated on the label. The test is not valid and must be repeated if there is a significant antibody titre in the controls. The vaccine complies with the test if the antibody responses of the vaccinated animals are not significantly less than those obtained with a batch of vaccine that has given satisfactory results in the test or tests (as applicable) described under Potency.

Where animals seronegative for the antigens stated on the label are not available, seropositive animals may be used in the above test. During the development of a test with seropositive animals, particular care will be required during the validation of the test system to establish that the test is suitably sensitive and to specify acceptable pass, fail and retest criteria. It will be necessary to take into account the range of prevaccination antibody titres and to establish the acceptable minimum antibody titre rise after vaccination in relation to these.

Bacterial endotoxins. A test for bacterial endotoxins (2.6.14) is carried out on the batch or, where the nature of the adjuvant prevents performance of a satisfactory test, on the bulk antigen or the mixture of bulk antigens immediately before addition of the adjuvant. The maximum acceptable amount of bacterial endotoxins is that found for a batch of vaccine shown satisfactory in safety test A given under Choice of vaccine composition or in the safety test described under Tests, carried out using 10 pigs. Where the latter test is used, note the maximum temperature increase for each animal; the average temperature increase for all animals does not exceed 1.5 °C. The method chosen for determining the amount of bacterial endotoxin present in the vaccine batch used in the safety test for determining the maximum acceptable level of endotoxin is used subsequently for testing of each batch.

IDENTIFICATION

In animals free from specific antibodies against the antigens stated on the label, the vaccine stimulates the production of antibodies against these antigens.

TESTS

Safety. Use not fewer than 2 pigs that do not have antibodies against *P. multocida* and that preferably do not have antibodies against *B. bronchiseptica*. Administer to each pig a double dose of vaccine by a recommended route. Observe the pigs for 14 days. Record body temperature the day before vaccination, at vaccination, 2 h, 4 h and 6 h later and then daily for 2 days. No abnormal local or systemic reaction occurs; a transient temperature increase not exceeding 2 °C may occur.

Sterility (2.6.1). The vaccine complies with the test for sterility prescribed in the monograph on *Vaccines for veterinary use (0062)*.

POTENCY

Use pigs that do not have antibodies against the components of the vaccine, that are from a herd or herds where there are no signs of atrophic rhinitis and that have not been vaccinated against atrophic rhinitis.

A. Vaccines containing dermonecrotic exotoxin of *P. multocida* (with or without cells of *P. multocida*).

Use not fewer than 12 breeder pigs. Vaccinate not fewer than 6 randomly chosen pigs at the stage of pregnancy or non-pregnancy and by the route and schedule stated on the label. Maintain not fewer than 6 of the remaining pigs as unvaccinated controls under the same conditions. From birth allow all the piglets from the vaccinated and unvaccinated breeder pigs to feed from their own dam.

Constitute from the progeny 2 challenge groups each of not fewer than 30 piglets chosen randomly, taking not fewer than 3 piglets from each litter. On the 2 consecutive days preceding challenge, the mucosa of the nasal cavity of the piglets may be treated by instillation of 0.5 ml of a solution of acetic acid (10 g/l C₂H₄O₂) in isotonic buffered saline pH 7.2.

Challenge each piglet at 10 days of age by the intranasal route with a sufficient quantity of a toxigenic strain of *P. multocida*.

At the age of 42 days, kill the piglets of the 2 groups and dissect the nose of each of them transversally at premolar-1. Examine the ventral and dorsal turbinates and the nasal septum for evidence of atrophy or distortion and grade the observations on the following scales:

Turbinates

- | | |
|---|---|
| 0 | no atrophy |
| 1 | slight atrophy |
| 2 | moderate atrophy |
| 3 | severe atrophy |
| 4 | very severe atrophy with almost complete disappearance of the turbinate |

The maximum score is 4 for each turbinate and 16 for the sum of the 2 dorsal and 2 ventral turbinates.

Nasal septum

- | | |
|---|-------------------------|
| 0 | no deviation |
| 1 | very slight deviation |
| 2 | deviation of the septum |

The maximum total score for the turbinates and the nasal septum is 18.

The test is not valid and must be repeated if fewer than 80 per cent of the progeny of each litter of the unvaccinated breeder pigs have a total score of at least 10. The vaccine complies with the test if a significant reduction in the total score has been demonstrated in the group from the vaccinated breeder pigs compared to that from the unvaccinated breeder pigs.

B. Vaccines containing *P. multocida* dermonecrotic exotoxin (with or without cells of *P. multocida*) and cells and/or antigenic components of *B. bronchiseptica*.

Use not fewer than 24 breeder pigs. Vaccinate not fewer than 12 randomly chosen pigs at the stage of pregnancy or non-pregnancy and by the route and schedule stated on the label. Maintain not fewer than 12 of the remaining

pigs as unvaccinated controls under the same conditions. From birth allow all the piglets from the vaccinated and unvaccinated breeder pigs to feed from their own dam.

Using groups of not fewer than 6 pigs, constitute from their progeny 2 challenge groups from vaccinated pigs and 2 groups from unvaccinated pigs each group consisting of not fewer than 30 piglets chosen randomly, taking not fewer than 3 piglets from each litter. On the 2 consecutive days preceding challenge, the mucosa of the nasal cavity of the piglets may be treated by instillation of 0.5 ml of a solution of acetic acid (10 g/l C₂H₄O₂) in isotonic buffered saline pH 7.2.

For a group of piglets from not fewer than 6 vaccinated pigs and a group from not fewer than 6 controls, challenge each piglet by the intranasal route at 10 days of age with a sufficient quantity of a toxigenic strain of *P. multocida*.

For the other group of piglets from not fewer than 6 vaccinated pigs and the other group from not fewer than 6 controls, challenge each piglet at 7 days of age by the intranasal route with a sufficient quantity of *B. bronchiseptica*. In addition, challenge each piglet at 10 days of age by the intranasal route with a sufficient quantity of a toxigenic strain of *P. multocida*.

At the age of 42 days, kill the piglets of the 4 groups and dissect the nose of each of them transversally at premolar-1. Examine the ventral and dorsal turbinates and the nasal septum for evidence of atrophy or distortion and grade the observations on the scale described above.

The test is not valid and must be repeated if fewer than 80 per cent of the progeny of each litter of the unvaccinated breeder pigs have a total score of at least 10. The vaccine complies with the test if a significant reduction in the total score has been demonstrated in the groups from the vaccinated breeder pigs compared to the corresponding group from the unvaccinated breeder pigs.

LABELLING

The label states the protective antigens present in the vaccine.

01/2005:0451

RABIES VACCINE (INACTIVATED) FOR VETERINARY USE

*Vaccinum rabiei inactivatum
ad usum veterinarium*

DEFINITION

Rabies vaccine (inactivated) for veterinary use is a liquid or freeze-dried preparation of fixed rabies virus inactivated by a suitable method.

PRODUCTION

The vaccine is prepared from virus grown either in suitable cell lines or in primary cell cultures from healthy animals (5.2.4). The virus suspension is harvested on one or more occasions within 28 days of inoculation. Multiple harvests from a single production cell culture may be pooled and considered as a single harvest. The rabies virus is inactivated by a suitable method.

Inactivation. The test for residual live rabies virus is carried out by inoculation of the inactivated virus into the same type of cell culture as that used in the production of the vaccine or a cell culture shown to be at least as sensitive; the quantity of inactivated virus used in the test is equivalent to not less than 25 doses of the vaccine. After incubation for

4 days, a subculture is made using trypsinised cells; after incubation for a further 4 days, the cultures are examined for residual live rabies virus by an immunofluorescence test. No live virus is detected.

Antigen content. The content of rabies virus glycoprotein is determined by a suitable immunochemical method (2.7.1). The content is within the limits approved for the particular preparation.

The vaccine may contain one or more adjuvants.

CHOICE OF VACCINE COMPOSITION

The vaccine is shown to be satisfactory with respect to immunogenicity for each species for which it is recommended. The suitability of the vaccine with respect to immunogenicity for carnivores (cats and dogs) is demonstrated by direct challenge. For other species, if a challenge test has been carried out for the vaccine in cats or dogs, an indirect test is carried out by determining the antibody level following vaccination of not fewer than twenty animals according to the recommended schedule; the vaccine is satisfactory if, after the period claimed for protection, the mean rabies virus antibody level in the serum of the animals is not less than 0.5 IU/ml and if not more than 10 per cent of the animals have an antibody level less than 0.1 IU/ml. The test described below may be used to demonstrate immunogenicity in cats and dogs.

Immunogenicity. Use not fewer than 35 susceptible animals of the minimum age recommended for vaccination. Take a blood sample from each animal and test individually for antibodies against rabies virus to determine susceptibility. Administer by the recommended route to each of not fewer than 25 animals one dose of vaccine. Keep not fewer than 10 animals as controls. Observe all the animals for a period equal to the claimed duration of immunity. No animal shows signs of rabies. On the last day of the claimed period for duration of immunity or later, challenge all animals by intramuscular injection of virulent rabies virus of a strain approved by the competent authority. Observe the animals for 90 days. Animals that die from causes not attributable to rabies are eliminated. The test is not valid if the number of such deaths reduces the number of vaccinated animals in the test to fewer than 25. The test is not valid unless at least eight control animals (or a statistically equivalent number if more than 10 control animals are challenged) show signs of rabies and the presence of rabies virus in their brain is demonstrated by the fluorescent-antibody test or some other suitable method. The vaccine complies with the test if not more than 2 of the 25 vaccinated animals (or a statistically equivalent number if more than 25 vaccinated animals are challenged) show signs of rabies.

BATCH TESTING

The test described under Potency is not necessarily carried out for routine testing of batches of vaccine. It is carried out, for a given vaccine, on one or more occasions, as decided by or with the agreement of the competent authority; where the test is not carried out, a suitable validated alternative method is used, the criteria for acceptance being set with reference to a batch of vaccine that has given satisfactory results in the test described above for immunogenicity or in the test described under Potency. The following test may be used after a suitable correlation with the test described above for immunogenicity or the test described under Potency has been established.

Batch potency test. Use 5 mice each weighing 18 g to 20 g. Vaccinate each mouse subcutaneously or intramuscularly using one-fifth of the recommended dose volume. Take blood samples 14 days after the injection and test the sera individually for rabies antibody using the rapid

fluorescent focus inhibition test described for *Human rabies immunoglobulin* (0723). The amount of antibody is not less than that produced by a vaccine that has been found satisfactory with respect to immunogenicity as described above or in the test described under Potency.

Antigen content. The quantity of rabies virus glycoprotein per dose, determined by a suitable immunochemical method (2.7.1), is not significantly lower than that of a batch that has been found satisfactory with respect to immunogenicity as described above or in the test described under Potency.

IDENTIFICATION

When injected into animals, the vaccine stimulates the production of specific neutralising antibodies.

TESTS

Safety. If the vaccine is intended for more than one species including one belonging to the order of Carnivora, carry out the test in dogs. Otherwise use one of the species for which the vaccine is intended. Administer, by a recommended route, a double dose of vaccine to each of 2 animals having no antibodies against rabies virus. Observe the animals for 14 days. No abnormal local or systemic reaction occurs.

Inactivation. Carry out the test using a pool of the contents of 5 containers.

For vaccines which do not contain an adjuvant, carry out a suitable amplification test for residual infectious rabies virus using the same type of cell culture as that used in the production of the vaccine or a cell culture shown to be at least as sensitive. No live virus is detected.

For vaccines that contain an adjuvant, inject intracerebrally into each of not fewer than 10 mice each weighing 11 g to 15 g 0.03 ml of a pool of at least 5 times the smallest stated dose. To avoid interference from any antimicrobial preservative or the adjuvant, the vaccine may be diluted not more than 10 times before injection. In this case or if the vaccine strain is pathogenic only for unweaned mice, carry out the test on mice 1 to 4 days old. Observe the animals for 21 days. If more than 2 animals die during the first 48 h, repeat the test. From the third to the twenty-first days following the injection, the animals show no signs of rabies and immunofluorescence tests carried out on the brains of the animals show no indication of the presence of rabies virus.

Sterility. The vaccine complies with the test for sterility prescribed in the monograph on *Vaccines for veterinary use* (0062).

POTENCY

The potency of rabies vaccine is determined by comparing the dose necessary to protect mice against the clinical effects of the dose of rabies virus defined below, administered intracerebrally, with the quantity of a reference preparation, calibrated in International Units, necessary to provide the same protection.

The International Unit is the activity of a stated quantity of the International Standard. The equivalence in International Units of the International Standard is stated by the World Health Organisation.

Rabies vaccine (inactivated) for veterinary use BRP is calibrated in International Units against the International Standard.

The test described below uses a parallel-line model with at least 3 points for the vaccine to be examined and the reference preparation. Once the analyst has experience with the method for a given vaccine, it is possible to carry

out a simplified test using one dilution of the vaccine to be examined. Such a test enables the analyst to determine that the vaccine has a potency significantly higher than the required minimum but will not give full information on the validity of each individual potency determination. It allows a considerable reduction in the number of animals required for the test and should be considered by each laboratory in accordance with the provisions of the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes.

Selection and distribution of the test animals. Use in the test healthy female mice about 4 weeks old and from the same stock. Distribute the mice into at least 10 groups of not fewer than 10 mice.

Preparation of the challenge suspension. Inoculate a group of mice intracerebrally with the CVS strain of rabies virus and when the mice show signs of rabies, but before they die, kill the mice and remove the brains and prepare a homogenate of the brain tissue in a suitable diluent. Separate gross particulate matter by centrifugation and use the supernatant liquid as challenge suspension. Distribute the suspension in small volumes in ampoules, seal and store at a temperature below -60°C . Thaw one ampoule of the suspension and make serial dilutions in a suitable diluent. Allocate each dilution to a group of mice and inject intracerebrally into each mouse 0.03 ml of the dilution allocated to its group. Observe the animals for 14 days and record the number in each group that, between the fifth and the fourteenth days, develop signs of rabies. Calculate the ID_{50} of the undiluted suspension.

Determination of potency of the vaccine to be examined. Prepare at least three serial dilutions of the vaccine to be examined and three similar dilutions of the reference preparation. Prepare the dilutions such that those containing the largest quantity of vaccine may be expected to protect more than 50 per cent of the animals into which they are injected and those containing the smallest quantities of vaccine may be expected to protect less than 50 per cent of the animals into which they are injected. Allocate each dilution to a different group of mice and inject intraperitoneally into each mouse 0.5 ml of the dilution allocated to its group. 14 days after the injection prepare a suspension of the challenge virus such that, on the basis of the preliminary titration, it contains about 50 ID_{50} in each 0.03 ml. Inject intracerebrally into each vaccinated mouse 0.03 ml of this suspension. Prepare 3 suitable serial dilutions of the challenge suspension. Allocate the challenge suspension and the 3 dilutions one to each of 4 groups of 10 unvaccinated mice and inject intracerebrally into each mouse 0.03 ml of the suspension or one of the dilutions allocated to its group. Observe the animals in each group for 14 days. The test is not valid if more than 2 mice of any group die within the first 4 days after challenge. Record the numbers in each group that show signs of rabies in the period 5 days to 14 days after challenge.

The test is not valid unless:

- for both the vaccine to be examined and the reference preparation the 50 per cent protective dose lies between the smallest and the largest dose given to the mice,
- the titration of the challenge suspension shows that 0.03 ml of the suspension contained at least 10 ID_{50} ,
- the confidence limits ($P = 0.95$) are not less than 25 per cent and not more than 400 per cent of the estimated potency,
- the statistical analysis shows a significant slope and no significant deviations from linearity or parallelism of the dose-response lines.

The vaccine complies with the test if the estimated potency is not less than 1 IU in the smallest prescribed dose.

LABELLING

The label states:

- the type of cell culture used to prepare the vaccine and the species of origin,
- the minimum number of International Units per dose,
- the minimum period for which the vaccine provides protection.

01/2005:0746

RABIES VACCINE (LIVE, ORAL) FOR FOXES

Vaccinum rabiei perorale vivum ad vulpem

DEFINITION

Rabies vaccine (live, oral) for foxes is a preparation of an immunogenic strain of an attenuated rabies virus. The vaccine is incorporated in a bait in such a manner as to enable the tests prescribed below to be performed aseptically.

PRODUCTION

The attenuated virus strain is grown in suitable cell cultures (5.2.4); if the cell cultures are of mammalian origin, they are shown to be free from rabies virus. The virus suspension is harvested on one or more occasions within 14 days of inoculation. Multiple harvests from a single cell lot may be pooled and considered as a single harvest. The viral suspension may be mixed with a suitable stabiliser. The vaccine may be freeze-dried or liquid. A freeze-dried vaccine has to be reconstituted before use.

CHOICE OF VACCINE STRAIN

Only a virus strain shown to be satisfactory with respect to immunogenicity (see Potency) and the following characteristics may be used in the preparation of the vaccine:

- when administered orally at the dose and by the method recommended for use to forty foxes, it causes no sign of rabies within 180 days of administration,
- when administered orally at ten times the recommended dose to each of ten foxes, it causes no sign of rabies within 180 days of administration,
- when administered orally at ten times the recommended dose to each of ten dogs, it causes no sign of rabies within 180 days of administration,
- when administered orally at ten times the recommended dose to each of ten cats, it causes no sign of rabies within 180 days of administration,
- in natural and experimental conditions, the virus strain does not spread from one animal to another in wild rodents,
- the virus strain has one or more stable genetic markers that may be used to discriminate the vaccine strain from other rabies virus strains.

BATCH TESTING

If the test for potency has been carried out with satisfactory results on a representative batch of vaccine, this test may be omitted as a routine control on other batches of vaccine prepared from the same seed lot.

IDENTIFICATION

- When mixed with a monospecific rabies antiserum, the vaccine is no longer able to infect susceptible cell cultures into which it is inoculated.
- A test is carried out to demonstrate the presence of the genetic marker.

TESTS

Extraneous viruses

(a) Mix the vaccine with a specific neutralising rabies virus antiserum. It no longer provokes cytopathic effects in susceptible cell cultures. It shows no evidence of haemagglutinating or haemadsorbing agents.

(b) Inoculate 1 in 10 and 1 in 1000 dilutions of the vaccine into susceptible cell cultures. Incubate at 37 °C. After 2, 4 and 6 days, stain the cells with a panel of monoclonal antibodies that do not react with the vaccine strain but that react with other strains of rabies virus (for example, street virus, Pasteur strain). The vaccine shows no evidence of contaminating rabies virus.

Bacterial and fungal contamination. The vaccine complies with the test for sterility prescribed in the monograph on *Vaccines for veterinary use (0062)*.

Mycoplasmas (2.6.7). The vaccine complies with the test for mycoplasmas.

Virus titre. Titrate the vaccine in suitable cell cultures. One dose of the vaccine contains not less than the quantity of virus equivalent to the minimum titre stated on the label.

POTENCY

Use not fewer than thirty-five foxes, at least three months old, free from rabies-neutralising antibodies. Administer orally and with the bait stated on the label to each of not fewer than twenty-five animals a volume of the vaccine containing a quantity of virus equivalent to the minimum titre stated on the label. Keep not fewer than ten animals as controls. Observe all the animals for 180 days. No animal shows signs of rabies. The test is not valid if fewer than twenty-five vaccinated animals survive after this observation period. On the 180th day after vaccination, challenge all foxes by the intramuscular injection of virulent rabies virus of a strain approved by the competent authority. Observe the animals for 90 days. Animals that die from causes not attributable to rabies are eliminated. The test is not valid if the number of such deaths reduces the number of vaccinated animals in the test to fewer than twenty-five. The vaccine complies with the test if not more than two of twenty-five vaccinated animals (or a statistically equivalent number if more than twenty-five vaccinated animals are challenged) show signs of rabies. The test is not valid unless at least nine control animals (or a statistically equivalent number if more than ten control animals are challenged) show signs of rabies and the presence of rabies virus in their brain is demonstrated by the fluorescent-antibody test or some other reliable method.

LABELLING

The label states the nature of the genetic marker of the virus strain.

01/2005:0064
corrected**SWINE ERYSIPELAS VACCINE
(INACTIVATED)****Vaccinum erysipelatis suillae inactivatum****DEFINITION**

Swine erysipelas vaccine (inactivated) is a preparation of one or more suitable strains of *Erysipelothrix rhusiopathiae* (*E. insidiosa*) inactivated by a suitable method. This monograph applies to vaccines intended to protect pigs against swine erysipelas.

PRODUCTION

The vaccine may contain an adjuvant.

CHOICE OF VACCINE COMPOSITION

The vaccine is shown to be satisfactory with respect to safety (5.2.6) and efficacy (5.2.7).

Immunogenicity. The test described under Potency is suitable to demonstrate immunogenicity of the vaccine with respect to *E. rhusiopathiae* serotypes 1 and 2. If claims are made concerning another serotype, then a further test to demonstrate immunogenicity against this serotype is necessary.

BATCH TESTING

Batch potency test. The test described under Potency is not carried out for routine testing of batches of vaccine. It is carried out, for a given vaccine, on one or more occasions, as decided by or with the agreement of the competent authority. Where the test is not carried out, a suitable validated alternative test is carried out, the criteria for acceptance being set with reference to a batch of vaccine that has given satisfactory results in the test described under Potency. The following test may be used after a satisfactory correlation with the test described under Potency has been established.

Use mice of a suitable strain (for example, NMRI) weighing 17-20 g, from a uniform stock and that do not have antibodies against swine erysipelas. Administer the vaccine to be examined to a group of 10 mice. Inject a suitable dose (usually 1/10 of the pig dose) subcutaneously into each mouse. At a given interval (for example, 21-28 days), depending on the vaccine to be examined, bleed the animals under anaesthesia. Pool the sera, using an equal volume from each mouse. Determine the level of antibodies by a suitable immunochemical method (2.7.1), for example, enzyme-linked immunosorbent assay with *erysipelas ELISA coating antigen BRP*. The antibody level is not significantly less than that obtained with a batch that has given satisfactory results in the test described under Potency.

IDENTIFICATION

Injected into animals that do not have antibodies against *E. rhusiopathiae*, it stimulates the production of such antibodies.

TESTS

Safety. Use pigs of the minimum age recommended for vaccination and preferably having no antibodies against swine erysipelas or, where justified, use pigs with a low level of such antibodies as long as they have not been vaccinated against Swine erysipelas and administration of the vaccine does not cause an anamnestic response. Administer a double dose of vaccine by a recommended route to each of 2 pigs. Observe the animals for 14 days. No abnormal local or systemic reaction occurs.

Sterility. It complies with the test for sterility prescribed in the monograph on *Vaccines for veterinary use* (0062).

POTENCY

If the vaccine contains more than 1 serotype, a test for 2 serotypes may be carried out on a single group by injecting each challenge serotype on different flanks of the animals. Validation and acceptance criteria are applied separately to the respective injection sites. If the vaccine contains more than 1 serotype, the potency test may also be carried out using a separate group for each serotype.

Use not fewer than 15 pigs not less than 12 weeks old, weighing not less than 20 kg and that do not have antibodies against swine erysipelas. Divide the animals into 2 groups. Vaccinate a group of not fewer than 10 pigs according to the recommended schedule. Maintain a group of not fewer than 5 pigs as unvaccinated controls. 3 weeks after vaccination, challenge the vaccinated animals and the control group by separate intradermal injections of 0.1 ml of a virulent strain of each of serotype 1 and serotype 2 of *E. rhusiopathiae*. Observe the animals for 7 days. The vaccine complies with the test if not fewer than 90 per cent of the vaccinated animals remain free from diamond skin lesions at the injection site. The test is invalid if fewer than 80 per cent of control animals show typical signs of disease, i.e. diamond skin lesions at the injection sites.

Swine erysipelas bacteria serotype 1 BRP and *swine erysipelas bacteria serotype 2 BRP* are suitable for use as challenge strains.

LABELLING

The label states the serotypes of *E. rhusiopathiae* included in the vaccine.

01/2005:0065

**SWINE-FEVER VACCINE (LIVE),
CLASSICAL, FREEZE-DRIED****Vaccinum pestis classicae suillae vivum
cryodesiccatum****DEFINITION**

Freeze-dried classical swine-fever vaccine (live) is a preparation obtained from a strain of classical swine-fever virus which has lost its pathogenicity for the pig by adaptation either to cell cultures or to the rabbit.

PRODUCTION

For vaccine prepared in rabbits, the seed-lot (or the vaccine) is made from the homogenised organs and/or blood of rabbits from healthy colonies, sacrificed at the peak of the temperature rise following intravenous inoculation of the virus. The vaccine is freeze-dried.

CHOICE OF VACCINE STRAIN

Only a virus strain shown to be satisfactory with respect to the following characteristics may be used in the preparation of the vaccine: safety; non-transmissibility; irreversibility of attenuation; and immunogenic properties. The following tests may be used during demonstration of safety (5.2.6) and efficacy (5.2.7).

The dose of vaccine used throughout the following tests is determined by the manufacturer on the basis of prior experiments.

Tests in pigs

Selection of animals. The piglets are 6 to 7 weeks old. The sows are primiparae. All animals are healthy and must

have had no contact with swine-fever virus and serologically must be free from swine-fever and bovine viral diarrhoea antibodies. They must have a week in which to adapt themselves to the new quarters where the tests are to be carried out.

Safety

(a) Each of five piglets receives intramuscularly as a single injection ten doses of vaccine (group a).

(b) Five piglets are immunodepressed by the daily injection of 2 mg of prednisolone per kilogram of body mass for five consecutive days and on the third day they receive one dose of vaccine (group b).

The animals of groups (a) and (b) are observed for 21 days. They must remain in good health. The temperature curve and the weight curve must not differ significantly from those of control animals.

(c) Ten non-immune pregnant sows each receive two doses of vaccine intramuscularly as a single injection between the twenty-fifth and thirty-fifth days of gestation. Ten non-immune pregnant sows of the same age and of the same origin receive instead of the two doses of vaccine an equal volume of a 9 g/l solution of *sodium chloride R*. The vaccinal virus does not cause abnormalities in the gestation or in the piglets.

Non-transmissibility. Twelve piglets of the same origin are kept together. Six are vaccinated in the normal way and the six others are kept as contact controls. After 40 days, all the pigs are challenged by intramuscular inoculation of a sufficient quantity of the challenge virus (see Potency) to kill an unvaccinated piglet in 7 days. The vaccinated piglets resist challenge whereas the contact piglets must display the typical signs of swine fever.

Irreversibility of attenuation. Each of two piglets receives one dose of vaccine intramuscularly. Seven days later, 5 ml of blood is taken from each of the piglets and the samples are pooled. 5 ml of the pooled blood is injected intramuscularly into each of two other piglets. This operation is repeated six times. The animals must not display any sign of swine fever and must show normal growth.

Immunogenic properties. The immunogenic properties may be demonstrated by the method described for the determination of potency. The quantity of the vaccinal virus corresponding to one dose of vaccine contains at least 100 PD₅₀.

IDENTIFICATION

A. For vaccines prepared in rabbits and lapinised vaccines prepared in cell cultures, inject intravenously 0.5 ml of the vaccine reconstituted as stated on the label into one or more non-immunised rabbits and one or more rabbits immunised either with an identical dose of a vaccine of the same type injected by the same route at least 10 days and at most 2 months beforehand or with a sufficient dose of antiserum administered a few hours before the injection of the vaccine. Measure the temperature of the rabbits in the morning and the evening starting 24 h after the injection and continuing until the fifth day after the injection. The vaccine is identified by its specific pyrogenic character leading to a rise in temperature of at least 1.5 °C in the non-immunised rabbits only.

B. For non-lapinised vaccines prepared in cell cultures, the serum of pigs immunised with the vaccine neutralises the virus used in the preparation of the vaccine.

TESTS

Safety. Use three piglets complying with the requirements prescribed for the selection of animals under Choice of Vaccine Strain. Inject intramuscularly into each piglet ten doses of the reconstituted vaccine as a single injection. Observe the animals for 21 days. The temperature curve remains normal and the animals remain in apparent good health and display normal growth.

Extraneous viruses. Mix the vaccine with a monospecific antiserum and inoculate into susceptible cell cultures. No cytopathic effect is produced.

Carry out a haemagglutination test using chicken red blood cells and the supernatant liquid of the cell cultures. The test is negative. Carry out a haemadsorption test on the cell cultures. The test is negative.

Use ten mice each weighing 11 g to 15 g. Inject intracerebrally into each mouse 0.03 ml of the vaccine reconstituted so that 1 ml contains 1 dose. Observe the animals for 21 days. If more than two mice die within the first 48 h, repeat the test. From the third to the twenty-first day after the injection, the mice show no abnormalities attributable to the vaccine.

Bacterial and fungal contamination. The vaccine to be examined complies with the test for sterility prescribed in the monograph on *Vaccines for veterinary use (0062)*.

Mycoplasmas (2.6.7). The vaccine complies with the test for mycoplasmas.

POTENCY

The potency is expressed as the number of 50 per cent protective doses (PD₅₀) for pigs contained in the dose indicated on the label. The vaccine contains at least 100 PD₅₀ per dose.

Use piglets complying with the requirements for selection of animals described under Choice of Vaccine Strain. To two groups of five piglets inject intramuscularly:

- 1/40 of a dose of the vaccine to be examined into each piglet of the first group,
- 1/160 of a dose of the vaccine to be examined into each piglet of the second group.

Use two piglets as controls.

Prepare the dilutions using *buffered salt solution pH 7.2 R*. On the fourteenth day after the injection, inoculate intramuscularly into each vaccinated and control animal a sufficient quantity of challenge virus to kill an unvaccinated piglet in 7 days. The challenge virus preparation consists of blood of pigs infected experimentally by virus that has not been submitted to passage in cell cultures. The control animals die within the seven days of inoculation. Observe the vaccinated animals for 14 days. From the number of animals which survive without showing any sign of swine fever, calculate the number of PD₅₀ contained in the vaccine using the usual statistical methods.

LABELLING

The label states that the vaccine has been prepared in cell cultures or in rabbits as appropriate.

01/2005:0697
corrected

TETANUS VACCINE FOR VETERINARY USE

Vaccinum tetani ad usum veterinarium

DEFINITION

Tetanus vaccine for veterinary use is a preparation of the neurotoxin of *Clostridium tetani* treated in a manner that eliminates toxicity while maintaining adequate immunogenic properties.

PRODUCTION

The *C. tetani* strain used for production is cultured in a suitable medium. The toxin is purified and then detoxified or it may be detoxified before purification. The antigenic purity is determined in Lf units of tetanus toxoid per milligram of protein and shown to be not less than the value approved for the particular product.

CHOICE OF VACCINE COMPOSITION

The *C. tetani* strain used in the preparation of the vaccine is shown to be satisfactory with respect to the production of the neurotoxin. The vaccine is shown to be satisfactory with respect to safety and immunogenicity for each species of animal for which it is intended. As part of the studies to demonstrate these characteristics, the tests described below may be used.

Production of antigens. The production of the neurotoxin of *C. tetani* is verified by a suitable immunochemical method (2.7.1) carried out on the neurotoxin obtained from the vaccine strain under the conditions used for the production of the vaccine.

Safety. Carry out the test for each recommended route of administration and species of animal for which the vaccine is intended; use animals of the minimum age recommended for vaccination and of the most sensitive category for the species.

Use not fewer than 15 animals, free from antitoxic antibodies for each test. Administer a double dose of vaccine to each animal. Administer a single dose of vaccine to each animal after the interval stated on the label. Observe the animals until 14 days after the last administration. If the vaccine is intended for use in pregnant animals, vaccinate the animals at the stage of pregnancy and according to the scheme stated on the label and prolong the observation period until 1 day after parturition. The vaccine complies with the test if no animal shows abnormal local or systemic signs of disease or dies from causes attributable to the vaccine. If the vaccine is intended for use in pregnant animals, in addition no significant effects on the gestation and the offspring are demonstrated.

Immunogenicity. The test described under Potency may be used to demonstrate immunogenicity. It shall also be demonstrated for each target species that the vaccine, administered by the recommended route, stimulates an immune response consistent with the claims for the product (for example, induction of antitoxic antibodies or induction of protective levels of antitoxic antibodies).

DETOXIFIED HARVEST

Absence of toxin and irreversibility of toxoid: carry out a test for reversion to toxicity on the detoxified harvest using 2 groups of 5 guinea-pigs, each weighing 350 g to 450 g; if the vaccine is adsorbed, carry out the test with the

shortest practical time interval before adsorption. Prepare a dilution of the detoxified harvest so that the guinea-pigs each receive 10 times the amount of toxoid (measured in Lf units) that will be present in a dose of vaccine. Divide the dilution into 2 equal parts. Keep one part at $5 \pm 3^\circ\text{C}$ and the other at 37°C for 6 weeks. Attribute each dilution to a separate group of guinea-pigs and inject into each guinea-pig the dilution attributed to its group. Observe the animals for 21 days. The toxoid complies with the test if no guinea-pig shows clinical signs of disease or dies from causes attributable to the neurotoxin of *C. tetani*.

BATCH POTENCY TEST

Where the test described under Potency is used as the batch potency test, the vaccine complies with the test if the antibody titre in International Units is not less than that found for a batch of vaccine shown to be satisfactory with respect to immunogenicity in the target species.

IDENTIFICATION

If the nature of the adjuvant allows it, carry out test A. Otherwise carry out test B.

- Dissolve in the vaccine sufficient sodium citrate R to give a 100 g/l solution. Maintain the solution at 37°C for about 16 h and centrifuge until a clear supernatant liquid is obtained. The supernatant reacts with a suitable tetanus antitoxin, giving a precipitate.
- When injected into susceptible animals, the vaccine provokes the formation of antibodies against the neurotoxin of *C. tetani*.

TESTS

Safety. Inject 5 ml of the vaccine subcutaneously as 2 equal divided doses, at separate sites into each of 5 healthy guinea-pigs, each weighing 350 g to 450 g, that have not previously been treated with any material that will interfere with the test. No abnormal local or systemic reaction occurs. If within 21 days of the injection any of the animals shows signs of or dies from tetanus, the vaccine does not comply with the test. If more than 1 animal dies from non-specific causes, repeat the test. If any animal dies in the second test, the vaccine does not comply with the test.

Sterility. The vaccine complies with the test for sterility prescribed in the monograph on *Vaccines for veterinary use* (0062).

POTENCY

Administer 1 dose of vaccine subcutaneously to each of at least 5 susceptible guinea-pigs. After 28 days, administer again 1 dose subcutaneously to each guinea-pig. 14 days after the second dose, collect blood from each guinea-pig and prepare serum samples. Determine for each serum the titre of antibodies against the neurotoxin of *C. tetani* using a suitable immunochemical method (2.7.1) such as a toxin-binding-inhibition test (ToBI test) and a homologous reference serum. Determine the average antibody titre of the serum samples.

Clostridia (multicomponent) rabbit antiserum BRP, Clostridium tetani guinea-pig antiserum for vaccines for veterinary use BRP and Clostridium tetani rabbit antiserum BRP are suitable as reference sera.

Tetanus vaccine intended for use in animals other than horses complies with the test if the average antibody titre is not less than 7.5 IU/ml.

Tetanus vaccine intended for use in horses complies with the test if the average antibody titre is not less than 30 IU/ml.

For tetanus vaccine presented as a combined vaccine for use in animals other than horses, the above test may be carried out in susceptible rabbits instead of guinea-pigs. The vaccine complies with the test if the average antibody titre of the sera of the vaccinated rabbits is not less than 2.5 IU/ml.

01/2005:1580

VIBRIOSIS (COLD-WATER) VACCINE (INACTIVATED) FOR SALMONIDS

Vaccinum vibriosidis aquae frigidae inactivatum ad salmonidas

DEFINITION

Cold-water vibriosis vaccine (inactivated) for salmonids is prepared from cultures of one or more suitable strains of *Vibrio salmonicida*.

PRODUCTION

The strains of *V. salmonicida* are cultured and harvested separately. The harvests are inactivated by a suitable method. They may be purified and concentrated. Whole or disrupted cells may be used and the vaccine may contain extracellular products of the bacterium released into the growth medium.

CHOICE OF VACCINE COMPOSITION

The strain or strains of *V. salmonicida* used are shown to be suitable with respect to production of antigens of assumed immunological importance. The vaccine is shown to be satisfactory with respect to safety (5.2.6) and immunogenicity (5.2.7) in the species of fish for which it is intended. The following tests may be used during demonstration of safety and immunogenicity.

Safety. Safety is tested in three different batches using test A, test B or both, depending on the recommendations for use.

A. Vaccines intended for administration by injection.

A test is carried out in each species of fish for which the vaccine is intended. The fish used are from a population that does not have specific antibodies against *V. salmonicida* and which has not been vaccinated against nor exposed to cold-water vibriosis. The test is carried out in the conditions recommended for the use of the vaccine with a water temperature not less than 10 °C. An amount of vaccine corresponding to twice the recommended dose per mass unit for fish of the minimum body mass recommended for vaccination is administered intraperitoneally to each of not fewer than fifty fish of the minimum recommended body mass. The fish are observed for 21 days. No abnormal local or systemic reaction occurs. The test is invalid if more than 6 per cent of the fish die from causes not attributable to the vaccine.

B. Vaccines intended for administration by immersion.

A test is carried out in each species of fish for which the vaccine is intended. The fish used are from a population that does not have specific antibodies against *V. salmonicida* and which has not been vaccinated against nor exposed to cold-water vibriosis. The test is carried out in the conditions recommended for the use of the vaccine with a water temperature not less than 10 °C. Prepare an immersion bath at twice the recommended concentration. Not fewer than fifty fish, having not less than the minimum body mass recommended for vaccination are used. The fish are bathed for twice the recommended time. The fish are observed for 21 days. No abnormal local or systemic reaction occurs. The test is invalid if more than 6 per cent of the fish die from causes not attributable to the vaccine.

C. Safety is demonstrated in addition in field trials by administering the intended dose to a sufficient number of fish distributed in not fewer than two sets of premises. No abnormal reaction occurs.

Immunogenicity. The test described under Potency, carried out for each recommended route of administration, is suitable to demonstrate immunogenicity of the vaccine.

BATCH TESTING

Batch potency test. For routine testing of batches of vaccine, the test described under Potency may be carried out using groups of not fewer than thirty fish of one of the species for which the vaccine is intended; alternatively, a suitable validated test based on antibody response may be carried out, the criteria being set with reference to a batch of vaccine that has given satisfactory results in the test described under Potency. The following test may be used after a satisfactory correlation with the test described under Potency has been established.

Use fish from a population that does not have specific antibodies against *V. salmonicida* and that are within specified limits for body mass. Carry out the test at a defined temperature. Inject into each of not fewer than twenty-five fish one dose of vaccine, according to the instructions for use. Perform mock vaccination on a control group of not fewer than ten fish. Collect blood samples at a defined time after vaccination. Determine for each sample the level of specific antibodies against *V. salmonicida* by a suitable immunochemical method (2.7.1). The vaccine complies with the test if the mean level of antibodies is not significantly lower than that found for a batch that gave satisfactory results in the test described under Potency. The test is not valid if the control group shows antibodies against *V. salmonicida*.

IDENTIFICATION

When injected into fish that do not have specific antibodies against *V. salmonicida*, the vaccine stimulates the production of such antibodies.

TESTS

Safety. Use not fewer than ten fish of one of the species for which the vaccine is intended, having, where possible, the minimum body mass recommended for vaccination; if fish of the minimum body mass are not available, use fish not greater than twice this mass. Use fish from a population that does not have specific antibodies against *V. salmonicida* and that has not been vaccinated against nor exposed to cold-water vibriosis. Carry out the test in the conditions recommended for use of the vaccine with a water temperature not less than 10 °C. For vaccines administered by injection or immersion, inject intraperitoneally into each fish an amount of vaccine corresponding to twice the recommended dose per mass unit. For vaccines administered by immersion only, use a bath with double the recommended concentration and bathe the fish for twice the recommended immersion time. Observe the animals for 21 days. No abnormal local or systemic reaction attributable to the vaccine occurs. The test is invalid if more than 10 per cent of the fish die from causes not attributable to the vaccine.

Sterility. The vaccine complies with the test for sterility prescribed in the monograph on *Vaccines for veterinary use* (0062).

POTENCY

Carry out the test according to a protocol defining limits of body mass for the fish, water source, water flow and temperature limits, and preparation of a standardised challenge. Vaccinate not fewer than one hundred fish by a

recommended route, according to the instructions for use. Perform mock vaccination on a control group of not fewer than one hundred fish; mark vaccinated and control fish for identification. Keep all the fish in the same tank or mix equal numbers of controls and vaccinates in each tank if more than one tank is used. Carry out challenge by injection at a fixed time interval after vaccination, defined according to the statement regarding development of immunity. Use for challenge a culture of *V. salmonicida* whose virulence has been verified. Observe the fish daily until at least 60 per cent specific mortality is reached in the control group. Plot for both vaccinates and controls a curve of specific mortality against time from challenge and determine by interpolation the time corresponding to 60 per cent specific mortality in controls. The test is invalid if the specific mortality is less than 60 per cent in the control group 21 days after the first death in the fish. Read from the curve for vaccinates the mortality (*M*) at the time corresponding to 60 per cent mortality in controls. Calculate the relative percentage survival (RPS) from the expression:

$$\left(1 - \frac{M}{60}\right) \times 100$$

The vaccine complies with the test if the RPS is not less than 60 per cent for vaccines administered by immersion and 90 per cent for vaccines administered by injection.

LABELLING

The label states information on the time needed for development of immunity after vaccination under the range of conditions corresponding to the recommended use.

01/2005:1581

VIBRIOSIS VACCINE (INACTIVATED) FOR SALMONIDS

Vaccinum vibriosidis ad salmonidas inactivatum

DEFINITION

Vibriosis vaccine (inactivated) for salmonids is prepared from cultures of one or more suitable strains or serovars of *Vibrio anguillarum*; the vaccine may also include *Vibrio ordalii*.

PRODUCTION

The strains of *V. anguillarum* and *V. ordalii* are cultured and harvested separately. The harvests are inactivated by a suitable method. They may be purified and concentrated. Whole or disrupted cells may be used and the vaccine may contain extracellular products of the bacterium released into the growth medium.

CHOICE OF VACCINE COMPOSITION

The strains of *V. anguillarum* and *V. ordalii* used are shown to be suitable with respect to production of antigens of assumed immunological importance. The vaccine is shown to be satisfactory with respect to safety (5.2.6) and immunogenicity (5.2.7) in the species of fish for which it is intended. The following tests may be used during demonstration of safety and immunogenicity.

Safety. Safety is tested in three different batches using test A, test B or both, depending on the recommendations for use.

A. *Vaccines intended for administration by injection.* A test is carried out in each species of fish for which the vaccine is intended. The fish used are from a population that does

not have specific antibodies against the relevant serovars of *V. anguillarum* or where applicable *V. ordalii* and which has not been vaccinated against nor exposed to vibriosis. The test is carried out in the conditions recommended for the use of the vaccine with a water temperature not less than 10 °C. An amount of vaccine corresponding to twice the recommended dose per mass unit for fish of the minimum body mass recommended for vaccination is administered intraperitoneally to each of not fewer than fifty fish of the minimum recommended body mass. The fish are observed for 21 days. No abnormal local or systemic reaction occurs. The test is invalid if more than 6 per cent of the fish die from causes not attributable to the vaccine.

B. *Vaccines intended for administration by immersion.* A test is carried out in each species of fish for which the vaccine is intended. The fish used are from a population that does not have specific antibodies against the relevant serovars of *V. anguillarum* or where applicable *V. ordalii* and which has not been vaccinated against nor exposed to vibriosis. The test is carried out in the conditions recommended for the use of the vaccine with a water temperature not less than 10 °C. Prepare an immersion bath at twice the recommended concentration. Not fewer than fifty fish having the minimum body mass recommended for vaccination are used. The fish are bathed for twice the recommended time. The fish are observed for 21 days. No abnormal local or systemic reaction occurs. The test is invalid if more than 6 per cent of the fish die from causes not attributable to the vaccine.

C. Safety is also demonstrated in field trials by administering the intended dose to a sufficient number of fish distributed in not fewer than two sets of premises. No abnormal reaction occurs.

Immunogenicity. The test described under Potency, carried out for each recommended route of administration is suitable to demonstrate immunogenicity of the vaccine.

BATCH TESTING

Batch potency test. For routine testing of batches of vaccine, the test described under Potency may be carried out using groups of not fewer than thirty fish of one of the species for which the vaccine is intended; alternatively, a suitable validated test based on antibody response may be carried out, the criteria being set with reference to a batch of vaccine that has given satisfactory results in the test described under Potency. The following test may be used after a satisfactory correlation with the test described under Potency has been established.

Use fish from a population that does not have specific antibodies against the relevant serovars of *V. anguillarum* and where applicable *V. ordalii* and that are within specified limits for body mass. Carry out the test at a defined temperature. Inject into each of not fewer than twenty-five fish one dose of vaccine, according to the instructions for use. Perform mock vaccination on a control group of not fewer than ten fish. Collect blood samples at a defined time after vaccination. Determine for each sample the level of specific antibodies against the different serovars of *V. anguillarum* and against *V. ordalii* included in the vaccine by a suitable immunochemical method (2.7.1). The vaccine complies with the test if the mean levels of antibodies are not significantly lower than those found for a batch that gave satisfactory results in the test described under Potency. The test is not valid if the control group shows antibodies against the relevant serovars of *V. anguillarum* or, where applicable, against *V. ordalii*.

IDENTIFICATION

When injected into fish that do not have specific antibodies against *V. anguillarum* and, where applicable, *V. ordalii*, the vaccine stimulates the production of such antibodies.

TESTS

Safety. Use not fewer than ten fish of one of the species for which the vaccine is intended, having, where possible, the minimum body mass recommended for vaccination; if fish of the minimum body mass are not available, use fish not greater than twice this mass. Use fish from a population that does not have specific antibodies against the relevant serovars of *V. anguillarum* and, where applicable, *V. ordalii* and which has not been vaccinated against nor exposed to vibriosis. Carry out the test in the conditions recommended for the use of the vaccine with a water temperature not less than 10 °C. For vaccines administered by injection or immersion, inject intraperitoneally into each fish an amount of vaccine corresponding to twice the recommended dose per mass unit. For vaccines administered by immersion only, use a bath with double the recommended concentration and bathe the fish for twice the recommended immersion time. Observe the animals for 21 days. No abnormal local or systemic reaction attributable to the vaccine occurs. The test is invalid if more than 10 per cent of the fish die from causes not attributable to the vaccine.

Sterility. The vaccine complies with the test for sterility prescribed in the monograph on *Vaccines for veterinary use (0062)*.

POTENCY

Carry out a separate test for each species and each serovar included in the vaccine. Carry out the test according to a protocol defining limits of body mass for the fish, water source, water flow and temperature limits, and preparation of a standardised challenge. Vaccinate not fewer than

one hundred fish by a recommended route, according to the instructions for use. Perform mock vaccination on a control group of not fewer than one hundred fish; mark vaccinated and control fish for identification. Keep all the fish in the same tank or mix equal numbers of controls and vaccinates in each tank if more than one tank is used. Carry out challenge by injection at a fixed time interval after vaccination, defined according to the statement regarding development of immunity. Use for challenge cultures of *V. anguillarum* or *V. ordalii* whose virulence has been verified. Observe the fish daily until at least 60 per cent specific mortality is reached in the control group. Plot for both vaccinates and controls a curve of specific mortality against time from challenge and determine by interpolation the time corresponding to 60 per cent specific mortality in controls. The test is invalid if the specific mortality is less than 60 per cent in the control group 21 days after the first death in the fish. Read from the curve for vaccinates the mortality (*M*) at the time corresponding to 60 per cent mortality in controls. Calculate the relative percentage survival (RPS) from the expression:

$$\left(1 - \frac{M}{60}\right) \times 100$$

The vaccine complies with the test if the RPS is not less than 60 per cent for vaccines administered by immersion and 75 per cent for vaccines administered by injection.

LABELLING

The label states:

- the serovar or serovars of *V. anguillarum* included in the vaccine,
- where applicable, that the vaccine includes *V. ordalii*,
- information on the time needed for development of immunity after vaccination under the range of conditions corresponding to the recommended use.

01/2005:0085

BOTULINUM ANTITOXIN**Immunoserum botulinicum****DEFINITION**

Botulinum antitoxin is a preparation containing antitoxic globulins that have the power of specifically neutralising the toxins formed by *Clostridium botulinum* type A, type B or type E, or any mixture of these types.

PRODUCTION

It is obtained by fractionation from the serum of horses, or other mammals, that have been immunised against *Cl. botulinum* type A, type B and type E toxins.

IDENTIFICATION

It specifically neutralises the types of *Cl. botulinum* toxins stated on the label, rendering them harmless to susceptible animals.

POTENCY

Not less than 500 IU of antitoxin per millilitre for each of types A and B and not less than 50 IU of antitoxin per millilitre for type E.

The potency of botulinum antitoxin is determined by comparing the dose necessary to protect mice against the lethal effects of a fixed dose of botulinum toxin with the quantity of the standard preparation of botulinum antitoxin necessary to give the same protection. For this comparison a reference preparation of each type of botulinum antitoxin, calibrated in International Units, and suitable preparations of botulinum toxins, for use as test toxins, are required. The potency of each test toxin is determined in relation to the specific reference preparation; the potency of the botulinum antitoxin to be examined is determined in relation to the potency of the test toxins by the same method.

International Units of the antitoxin are the specific neutralising activity for botulinum toxin type A, type B and type E contained in stated amounts of the International Standards which consist of dried immune horse sera of types A, B and E. The equivalence in International Units of the International Standard is stated from time to time by the World Health Organisation.

Selection of animals. Use mice having body masses such that the difference between the lightest and the heaviest does not exceed 5 g.

Preparation of test toxins. **CAUTION: Botulinum toxin is extremely toxic: exceptional care must be taken in any procedure in which it is employed.** Prepare type A, B and E toxins from sterile filtrates of approximately 7-day cultures in liquid medium of *Cl. botulinum* types A, B and E. To the filtrates, add 2 volumes of glycerol, concentrate, if necessary, by dialysis against glycerol and store at or slightly below 0 °C.

Selection of test toxins. Select toxins of each type for use as test toxins by determining for mice the L₊/10 dose and the LD₅₀, the observation period being 96 h. The test toxins contain at least 1000 LD₅₀ in an L₊/10 dose.

Determination of test doses of the toxins (L₊/10 dose).

Prepare solutions of the reference preparations in a suitable liquid such that each contains 0.25 IU of antitoxin per millilitre. Using each solution in turn, determine the test dose of the corresponding test toxin.

Prepare mixtures of the solution of the reference preparation and the test toxin such that each contains 2.0 ml of the solution of the reference preparation, one of a graded series

of volumes of the test toxin and sufficient of a suitable liquid to bring the total volume to 5.0 ml. Allow the mixtures to stand at room temperature, protected from light, for 60 min. Using four mice for each mixture, inject a dose of 1.0 ml intraperitoneally into each mouse. Observe the mice for 96 h. The test dose of toxin is the quantity in 1.0 ml of the mixture made with the smallest amount of toxin capable of causing, despite partial neutralisation by the reference preparation, the death of all four mice injected with the mixture within the observation period.

Determination of potency of the antitoxin. Prepare solutions of each reference preparation in a suitable liquid such that each contains 0.25 IU of antitoxin per millilitre.

Prepare solutions of each test toxin in a suitable liquid such that each contains 2.5 test doses per millilitre.

Using each toxin solution and the corresponding reference preparation in turn, determine the potency of the antitoxin. Prepare mixtures of the solution of the test toxin and the antitoxin to be examined such that each contains 2.0 ml of the solution of the test toxin, one of a graded series of volumes of the antitoxin to be examined, and sufficient of a suitable liquid to bring the total volume to 5.0 ml. Also prepare mixtures of the solution of the test toxin and the solution of the reference preparation such that each contains 2.0 ml of the solution of the test toxin, one of a graded series of volumes of the solution of the reference preparation centred on that volume (2.0 ml) that contains 0.5 IU, and sufficient of a suitable liquid to bring the total volume to 5.0 ml. Allow the mixtures to stand at room temperature, protected from light, for 60 min. Using four mice for each mixture, inject a dose of 1.0 ml intraperitoneally into each mouse. Observe the mice for 96 h.

The mixture that contains the largest volume of antitoxin that fails to protect the mice from death contains 0.5 IU. This quantity is used to calculate the potency of the antitoxin in International Units per millilitre.

The test is not valid unless all the mice injected with mixtures containing 2.0 ml or less of the solution of the reference preparation die and all those injected with mixtures containing more survive.

LABELLING

The label states the types of *Cl. botulinum* toxin neutralised by the preparation.

01/2005:0086

DIPHTHERIA ANTITOXIN**Immunoserum diphthericum****DEFINITION**

Diphtheria antitoxin is a preparation containing antitoxic globulins that have the power of specifically neutralising the toxin formed by *Corynebacterium diphtheriae*.

PRODUCTION

It is obtained by fractionation from the serum of horses, or other mammals, that have been immunised against diphtheria toxin.

IDENTIFICATION

It specifically neutralises the toxin formed by *C. diphtheriae*, rendering it harmless to susceptible animals.

ASSAY

Not less than 1000 IU of antitoxin per millilitre for antitoxin obtained from horse serum. Not less than 500 IU of antitoxin per millilitre for antitoxin obtained from the serum of other mammals.

The potency of diphtheria antitoxin is determined by comparing the dose necessary to protect guinea-pigs or rabbits against the erythrogenic effects of a fixed dose of diphtheria toxin with the quantity of the standard preparation of diphtheria antitoxin necessary to give the same protection. For this comparison a reference preparation of diphtheria antitoxin, calibrated in International Units, and a suitable preparation of diphtheria toxin, for use as a test toxin, are required. The potency of the test toxin is determined in relation to the reference preparation; the potency of the diphtheria antitoxin to be examined is determined in relation to the potency of the test toxin by the same method.

The International Unit of antitoxin is the specific neutralising activity for diphtheria toxin contained in a stated amount of the International Standard, which consists of a quantity of dried immune horse serum. The equivalence in International Units of the International Standard is stated by the World Health Organisation.

Preparation of test toxin. Prepare diphtheria toxin from cultures of *C. diphtheriae* in a liquid medium. Filter the culture to obtain a sterile toxic filtrate and store at 4 °C.

Selection of test toxin. Select a toxin for use as a test toxin by determining for guinea-pigs or rabbits the $lr/100$ dose and the minimal reacting dose, the observation period being 48 h. The test toxin has at least 200 minimal reacting doses in the $lr/100$ dose.

Minimal reacting dose. This is the smallest quantity of toxin which, when injected intracutaneously into guinea-pigs or rabbits, causes a small, characteristic reaction at the site of injection within 48 h.

The test toxin is allowed to stand for some months before being used for the assay of antitoxin. During this time its toxicity declines and the $lr/100$ dose may be increased. Determine the minimal reacting dose and the $lr/100$ dose at frequent intervals. When experiment shows that the $lr/100$ dose is constant, the test toxin is ready for use and may be used for a long period. Store the test toxin in the dark at 0 °C to 5 °C. Maintain its sterility by the addition of toluene or other antimicrobial preservative that does not cause a rapid decline in specific toxicity.

Determination of test dose of toxin ($lr/100$ dose). Prepare a solution of the reference preparation in a suitable liquid such that it contains 0.1 IU of antitoxin per millilitre.

Prepare mixtures of the solution of the reference preparation and of the test toxin such that each contains 1.0 ml of the solution of the reference preparation, one of a graded series of volumes of the test toxin and sufficient of a suitable liquid to bring the total volume to 2.0 ml. Allow the mixtures to stand at room temperature, protected from light, for 15 min to 60 min. Using two animals for each mixture, inject a dose of 0.2 ml intracutaneously into the shaven or depilated flanks of each animal. Observe the animals for 48 h.

The test dose of toxin is the quantity in 0.2 ml of the mixture made with the smallest amount of toxin capable of causing, despite partial neutralisation by the reference preparation, a small but characteristic erythematous lesion at the site of injection.

Determination of potency of the antitoxin. Prepare a solution of the reference preparation in a suitable liquid such that it contains 0.125 IU of antitoxin per millilitre.

Prepare a solution of the test toxin in a suitable liquid such that it contains 12.5 test doses per millilitre.

Prepare mixtures of the solution of the test toxin and of the antitoxin to be examined such that each contains 0.8 ml of the solution of the test toxin, one of a graded series of volumes of the antitoxin to be examined and sufficient of a suitable liquid to bring the total volume to 2.0 ml. Also prepare mixtures of the solution of the test toxin and the solution of the reference preparation such that each contains 0.8 ml of the solution of the test toxin, one of a graded series of volumes of the solution of the reference preparation centred on that volume (0.8 ml) that contains 0.1 IU and sufficient of a suitable liquid to bring the total volume to 2.0 ml. Allow the mixtures to stand at room temperature, protected from light, for 15 min to 60 min. Using two animals for each mixture, inject a dose of 0.2 ml intracutaneously into the shaven or depilated flanks of each animal. Observe the animals for 48 h.

The mixture that contains the largest volume of antitoxin that fails to protect the guinea-pigs from the erythematous effects of the toxin contains 0.1 IU. This quantity is used to calculate the potency of the antitoxin in International Units per millilitre.

The test is not valid unless all the sites injected with mixtures containing 0.8 ml or less of the solution of the reference preparation show erythematous lesions and at all those injected with mixtures containing more there are no lesions.

01/2005:0090

GAS-GANGRENE ANTITOXIN, MIXED

Immunoserum gangraenicum mixtum

DEFINITION

Mixed gas-gangrene antitoxin is prepared by mixing gas-gangrene antitoxin (novyi), gas-gangrene antitoxin (perfringens) and gas-gangrene antitoxin (septicum) in appropriate quantities.

IDENTIFICATION

It specifically neutralises the alpha toxins formed by *Clostridium novyi* (former nomenclature: *Clostridium oedematiens*), *Clostridium perfringens* and *Clostridium septicum*, rendering them harmless to susceptible animals.

ASSAY

Gas-gangrene antitoxin (novyi), not less than 1000 IU of antitoxin per millilitre; gas-gangrene antitoxin (perfringens), not less than 1000 IU of antitoxin per millilitre; gas-gangrene antitoxin (septicum) not less than 500 IU of antitoxin per millilitre.

Carry out the assay for each component, as prescribed in the monographs on *Gas-gangrene antitoxin (novyi)* (0087), *Gas-gangrene antitoxin (perfringens)* (0088) and *Gas-gangrene antitoxin (septicum)* (0089).

01/2005:0087

GAS-GANGRENE ANTITOXIN (NOVYI)

Immunoserum gangraenicum
(*Clostridium novyi*)

DEFINITION

Gas-gangrene antitoxin (novyi) is a preparation containing antitoxic globulins that have the power of neutralising the alpha toxin formed by *Clostridium novyi* (Former

nomenclature: *Clostridium oedematiens*). It is obtained by fractionation from the serum of horses, or other mammals, that have been immunised against *Cl. novyi* alpha toxin.

IDENTIFICATION

It specifically neutralises the alpha toxin formed by *Cl. novyi*, rendering it harmless to susceptible animals.

ASSAY

Not less than 3750 IU of antitoxin per millilitre.

The potency of gas-gangrene antitoxin (novyi) is determined by comparing the dose necessary to protect mice or other suitable animals against the lethal effects of a fixed dose of *Cl. novyi* toxin with the quantity of the standard preparation of gas-gangrene antitoxin (novyi) necessary to give the same protection. For this comparison a reference preparation of gas-gangrene antitoxin (novyi), calibrated in International Units, and a suitable preparation of *Cl. novyi* toxin for use as a test toxin are required. The potency of the test toxin is determined in relation to the reference preparation; the potency of the gas-gangrene antitoxin (novyi) to be examined is determined in relation to the potency of the test toxin by the same method.

The International Unit of antitoxin is the specific neutralising activity for *Cl. novyi* toxin contained in a stated amount of the International Standard, which consists of a quantity of dried immune horse serum. The equivalence in International Units of the International Standard is stated by the World Health Organisation.

Selection of animals. Use mice having body masses such that the difference between the lightest and the heaviest does not exceed 5 g.

Preparation of test toxin. Prepare the test toxin from a sterile filtrate of an approximately 5-day culture in liquid medium of *Cl. novyi*. Treat the filtrate with *ammonium sulphate R*, collect the precipitate, which contains the toxin, dry *in vacuo* over *diphosphorus pentoxide R*, powder and store dry.

Selection of test toxin. Select a toxin for use as a test toxin by determining for mice the L+ dose and the LD₅₀, the observation period being 72 h. The test toxin has an L+ dose of 0.5 mg or less and contains not less than 25 LD₅₀ in each L+ dose.

Determination of test dose of toxin (L+ dose). Prepare a solution of the reference preparation in a suitable liquid such that it contains 12.5 IU of antitoxin per millilitre.

Prepare a solution of the test toxin in a suitable liquid such that 1 ml contains a precisely known amount such as 10 mg.

Prepare mixtures of the solution of the reference preparation and the solution of the test toxin such that each contains 0.8 ml of the solution of the reference preparation, one of a graded series of volumes of the solution of the test toxin and sufficient of a suitable liquid to bring the total volume to 2.0 ml. Allow the mixtures to stand at room temperature, protected from light, for 60 min. Using six mice for each mixture, inject a dose of 0.2 ml intramuscularly into each mouse. Observe the mice for 72 h.

The test dose of toxin is the quantity in 0.2 ml of the mixture made with the smallest amount of toxin capable of causing, despite partial neutralisation by the reference preparation, the death of all six mice injected with the mixture within the observation period.

Determination of potency of the antitoxin. Prepare a solution of the reference preparation in a suitable liquid such that it contains 12.5 IU of antitoxin per millilitre.

Prepare a solution of the test toxin in a suitable liquid such that it contains 12.5 test doses per millilitre.

Prepare mixtures of the solution of the test toxin and the antitoxin to be examined such that each contains 0.8 ml of the solution of the test toxin, one of a graded series of volumes of the antitoxin to be examined and sufficient of a suitable liquid to bring the total volume to 2.0 ml. Also prepare mixtures of the solution of the test toxin and the solution of the reference preparation such that each contains 0.8 ml of the solution of the test toxin, one of a graded series of volumes of the solution of the reference preparation centred on that volume (0.8 ml) that contains 10 IU and sufficient of a suitable liquid to bring the total volume to 2.0 ml. Allow the mixtures to stand at room temperature, protected from light, for 60 min. Using six mice for each mixture, inject a dose of 0.2 ml intramuscularly into each mouse. Observe the mice for 72 h.

The mixture that contains the largest volume of antitoxin that fails to protect the mice from death contains 10 IU. This quantity is used to calculate the potency of the antitoxin in International Units per millilitre.

The test is not valid unless all the mice injected with mixtures containing 0.8 ml or less of the solution of the reference preparation die and all those injected with mixtures containing a larger volume survive.

01/2005:0088

GAS-GANGRENE ANTITOXIN (PERFRINGENS)

Immunoserum gangraenicum (*Clostridium perfringens*)

DEFINITION

Gas-gangrene antitoxin (perfringens) is a preparation containing antitoxic globulins that have the power of specifically neutralising the alpha toxin formed by *Clostridium perfringens*. It is obtained by fractionation from the serum of horses, or other mammals, that have been immunised against *Cl. perfringens* alpha toxin.

IDENTIFICATION

It specifically neutralises the alpha toxin formed by *Cl. perfringens*, rendering it harmless to susceptible animals.

ASSAY

Not less than 1500 IU of antitoxin per millilitre.

The potency of gas-gangrene antitoxin (perfringens) is determined by comparing the dose necessary to protect mice or other suitable animals against the lethal effects of a fixed dose of *Cl. perfringens* toxin with the quantity of the standard preparation of gas-gangrene antitoxin (perfringens) necessary to give the same protection. For this comparison a reference preparation of gas-gangrene antitoxin (perfringens), calibrated in International Units, and a suitable preparation of *Cl. perfringens* toxin for use as a test toxin are required. The potency of the test toxin is determined in relation to the reference preparation; the potency of the gas-gangrene antitoxin (perfringens) to be examined is determined in relation to the potency of the test toxin by the same method.

The International Unit of antitoxin is the specific neutralising activity for *Cl. perfringens* toxin contained in a stated amount of the International Standard, which consists of a quantity of dried immune horse serum. The equivalence in International Units of the International Standard is stated by the World Health Organisation.

01/2005:0089

Selection of animals. Use mice having body masses such that the difference between the lightest and the heaviest does not exceed 5 g.

Preparation of test toxin. Prepare the test toxin from a sterile filtrate of an approximately 5-day culture in liquid medium of *Cl. perfringens*. Treat the filtrate with *ammonium sulphate R*, collect the precipitate, which contains the toxin, dry *in vacuo* over *diphosphorus pentoxide R*, powder and store dry.

Selection of test toxin. Select a toxin for use as a test toxin by determining for mice the L+ dose and the LD₅₀, the observation period being 48 h. The test toxin has an L+ dose of 4 mg or less and contains not less than 20 LD₅₀ in each L+ dose.

Determination of test dose of toxin (L+ dose). Prepare a solution of the reference preparation in a suitable liquid such that it contains 5 IU of antitoxin per millilitre.

Prepare a solution of the test toxin in a suitable liquid such that 1 ml contains a precisely known amount such as 10 mg.

Prepare mixtures of the solution of the reference preparation and the solution of the test toxin such that each contains 2.0 ml of the solution of the reference preparation, one of a graded series of volumes of the solution of the test toxin and sufficient of a suitable liquid to bring the total volume to 5.0 ml. Allow the mixtures to stand at room temperature, protected from light, for 60 min. Using six mice for each mixture, inject a dose of 0.5 ml intravenously into each mouse. Observe the mice for 48 h.

The test dose of toxin is the quantity in 0.5 ml of the mixture made with the smallest amount of toxin capable of causing, despite partial neutralisation by the reference preparation, the death of all six mice injected with the mixture within the observation period.

Determination of potency of the antitoxin. Prepare a solution of the reference preparation in a suitable liquid such that it contains 5 IU of antitoxin per millilitre.

Prepare a solution of the test toxin in a suitable liquid such that it contains five test doses per millilitre.

Prepare mixtures of the solution of the test toxin and the antitoxin to be examined such that each contains 2.0 ml of the solution of the test toxin, one of a graded series of volumes of the antitoxin to be examined and sufficient of a suitable liquid to bring the total volume to 5.0 ml. Also prepare mixtures of the solution of the test toxin and the solution of the reference preparation such that each contains 2.0 ml of the solution of the test toxin, one of a graded series of volumes of the solution of the reference preparation centred on that volume (2.0 ml) that contains 10 IU and sufficient of a suitable liquid to bring the total volume to 5.0 ml. Allow the mixtures to stand at room temperature, protected from light, for 60 min. Using six mice for each mixture, inject a dose of 0.5 ml intravenously into each mouse. Observe the mice for 48 h.

The mixture that contains the largest volume of antitoxin that fails to protect the mice from death contains 10 IU. This quantity is used to calculate the potency of the antitoxin in International Units per millilitre.

The test is not valid unless all the mice injected with mixtures containing 2.0 ml or less of the solution of the reference preparation die and all those injected with mixtures containing a larger volume survive.

GAS-GANGRENE ANTITOXIN (SEPTICUM)

Immunoserum gangraenicum (*Clostridium septicum*)

DEFINITION

Gas-gangrene antitoxin (septicum) is a preparation containing antitoxic globulins that have the power of specifically neutralising the alpha toxin formed by *Clostridium septicum*. It is obtained by fractionation from the serum of horses, or other mammals, that have been immunised against *Cl. septicum* alpha toxin.

IDENTIFICATION

It specifically neutralises the alpha toxin formed by *Cl. septicum*, rendering it harmless to susceptible animals.

ASSAY

Not less than 1500 IU of antitoxin per millilitre.

The potency of gas-gangrene antitoxin (septicum) is determined by comparing the dose necessary to protect mice or other suitable animals against the lethal effects of a fixed dose of *Cl. septicum* toxin with the quantity of the standard preparation of gas-gangrene antitoxin (septicum) necessary to give the same protection. For this comparison a reference preparation of gas-gangrene antitoxin (septicum), calibrated in International Units, and a suitable preparation of *Cl. septicum* toxin for use as a test toxin are required. The potency of the test toxin is determined in relation to the reference preparation; the potency of the gas-gangrene antitoxin (septicum) to be examined is determined in relation to the potency of the test toxin by the same method.

The International Unit of antitoxin is the specific neutralising activity for *Cl. septicum* toxin contained in a stated amount of the International Standard, which consists of a quantity of dried immune horse serum. The equivalence in International Units of the International Standard is stated by the World Health Organisation.

Selection of animals. Use mice having body masses such that the difference between the lightest and the heaviest does not exceed 5 g.

Preparation of test toxin. Prepare the test toxin from a sterile filtrate of an approximately 5-day culture in liquid medium of *Cl. septicum*. Treat the filtrate with *ammonium sulphate R*, collect the precipitate, which contains the toxin, dry *in vacuo* over *diphosphorus pentoxide R*, powder and store dry.

Selection of test toxin. Select a toxin for use as a test toxin by determining for mice the L+ dose and the LD₅₀, the observation period being 72 h. The test toxin has an L+ dose of 0.5 mg or less and contains not less than 25 LD₅₀ in each L+ dose.

Determination of test dose of toxin (L+ dose). Prepare a solution of the reference preparation in a suitable liquid such that it contains 5 IU of antitoxin per millilitre.

Prepare a solution of the test toxin in a suitable liquid such that 1 ml contains a precisely known amount such as 20 mg.

Prepare mixtures of the solution of the reference preparation and the solution of the test toxin such that each contains 2.0 ml of the solution of the reference preparation, one of a graded series of volumes of the solution of the test toxin and sufficient of a suitable liquid to bring the total volume to 5.0 ml. Allow the mixtures to stand at room temperature,

protected from light, for 60 min. Using six mice for each mixture, inject a dose of 0.5 ml intravenously into each mouse. Observe the mice for 72 h.

The test dose of toxin is the quantity in 0.5 ml of the mixture made with the smallest amount of toxin capable of causing, despite partial neutralisation by the reference preparation, the death of all six mice injected with the mixture within the observation period.

Determination of potency of the antitoxin. Prepare a solution of the reference preparation in a suitable liquid such that it contains 5 IU of antitoxin per millilitre.

Prepare a solution of the test toxin in a suitable liquid such that it contains five test doses per millilitre.

Prepare mixtures of the solution of the test toxin and the antitoxin to be examined such that each contains 2.0 ml of the solution of the test toxin, one of a graded series of volumes of the antitoxin to be examined and sufficient of a suitable liquid to bring the total volume to 5.0 ml. Also prepare mixtures of the solution of the test toxin and the solution of the reference preparation such that each contains 2.0 ml of the solution of the test toxin, one of a graded series of volumes of the solution of the reference preparation centred on that volume (2.0 ml) that contains 10 IU and sufficient of a suitable liquid to bring the total volume to 5.0 ml. Allow the mixtures to stand at room temperature, protected from light, for 60 min. Using six mice for each mixture, inject a dose of 0.5 ml intravenously into each mouse. Observe the mice for 72 h.

The mixture that contains the largest volume of antitoxin that fails to protect the mice from death contains 10 IU. This quantity is used to calculate the potency of the antitoxin in International Units per millilitre.

The test is not valid unless all the mice injected with mixtures containing 2.0 ml or less of the solution of the reference preparation die and all those injected with mixtures containing more survive.

01/2005:0091

TETANUS ANTITOXIN FOR HUMAN USE

Immunoserum tetanicum ad usum humanum

DEFINITION

Tetanus antitoxin for human use is a preparation containing antitoxic globulins that have the power of specifically neutralising the toxin formed by *Clostridium tetani*.

PRODUCTION

It is obtained by fractionation from the serum of horses, or other mammals, that have been immunised against tetanus toxin.

IDENTIFICATION

It specifically neutralises the toxin formed by *Cl. tetani*, rendering it harmless to susceptible animals.

POTENCY

Not less than 1000 IU of antitoxin per millilitre when intended for prophylactic use. Not less than 3000 IU of antitoxin per millilitre when intended for therapeutic use. The potency of tetanus antitoxin is determined by comparing the dose necessary to protect guinea-pigs or mice against the paralytic effects of a fixed dose of tetanus toxin with the quantity of the standard preparation of tetanus antitoxin

necessary to give the same protection. In countries where the paralysis method is not obligatory the lethal method may be used. For this method the number of animals and the procedure are identical with those described for the paralysis method but the end-point is the death of the animal rather than the onset of paralysis and the L₅₀/10 dose is used instead of the L_p/10 dose. For this comparison a reference preparation of tetanus antitoxin, calibrated in International Units, and a suitable preparation of tetanus toxin, for use as a test toxin, are required. The potency of the test toxin is determined in relation to the reference preparation; the potency of the tetanus antitoxin to be examined is determined in relation to the potency of the test toxin by the same method.

The International Unit of antitoxin is the specific neutralising activity for tetanus toxin contained in a stated amount of the International Standard which consists of a quantity of dried immune horse serum. The equivalence in International Units of the International Standard is stated by the World Health Organisation.

Selection of animals. If mice are used, the body masses should be such that the difference between the lightest and the heaviest does not exceed 5 g.

Preparation of test toxin. Prepare the test toxin from a sterile filtrate of an approximately 9-day culture in liquid medium of *Cl. tetani*. To the filtrate add 1 to 2 volumes of glycerol and store slightly below 0 °C. Alternatively, treat the filtrate with *ammonium sulphate R*, collect the precipitate, which contains the toxin, dry *in vacuo* over *diphosphorus pentoxide R*, powder and store dry, either in sealed ampoules or *in vacuo* over *diphosphorus pentoxide R*.

Determination of test dose of toxin (L_p/10 dose). Prepare a solution of the reference preparation in a suitable liquid such that it contains 0.5 IU of antitoxin per millilitre.

If the test toxin is stored dry, reconstitute it using a suitable liquid.

Prepare mixtures of the solution of the reference preparation and the test toxin such that each contains 2.0 ml of the solution of the reference preparation, one of a graded series of volumes of the test toxin and sufficient of a suitable liquid to bring the volume to 5.0 ml. Allow the mixtures to stand at room temperature, protected from light, for 60 min. Using six mice for each mixture, inject a dose of 0.5 ml subcutaneously into each mouse. Observe the mice for 96 h. Mice that become paralysed may be killed.

The test dose of toxin is the quantity in 0.5 ml of the mixture made with the smallest amount of toxin capable of causing, despite partial neutralisation by the reference preparation, paralysis in all six mice injected with the mixture within the observation period.

Determination of potency of the antitoxin. Prepare a solution of the reference preparation in a suitable liquid such that it contains 0.5 IU of antitoxin per millilitre.

Prepare a solution of the test toxin in a suitable liquid such that it contains five test doses per millilitre.

Prepare mixtures of the solution of the test toxin and the antitoxin to be examined such that each contains 2.0 ml of the solution of the test toxin, one of a graded series of volumes of the antitoxin to be examined and sufficient of a suitable liquid to bring the total volume to 5.0 ml. Also prepare mixtures of the solution of the test toxin and the solution of the reference preparation such that each contains 2.0 ml of the solution of the test toxin, one of a graded series of volumes of the solution of the reference preparation centred on that volume (2.0 ml) that contains 1 IU and sufficient of a suitable liquid to bring the total volume to 5.0 ml. Allow the mixtures to stand at room temperature,

protected from light, for 60 min. Using six mice for each mixture, inject into each mouse subcutaneously a dose of 0.5 ml. Observe the mice for 96 h. Mice that become paralysed may be killed.

The mixture that contains the largest volume of antitoxin that fails to protect the mice from paralysis contains 1 IU. This quantity is used to calculate the potency of the antitoxin in International Units per millilitre.

The test is not valid unless all the mice injected with mixtures containing 2.0 ml or less of the solution of the reference preparation show paralysis and all those injected with mixtures containing more do not.

01/2005:0145

VIPER VENOM ANTISERUM, EUROPEAN

Immunoserum contra venena viperarum europaeorum

DEFINITION

European viper venom antiserum is a preparation containing antitoxic globulins that have the power of neutralising the venom of one or more species of viper. The globulins are obtained by fractionation of the serum of animals that have been immunised against the venom or venoms.

IDENTIFICATION

It neutralises the venom of *Vipera ammodytes*, or *Vipera aspis*, or *Vipera berus*, or *Vipera ursinii* or the mixture of these venoms stated on the label, rendering them harmless to susceptible animals.

ASSAY

Each millilitre of the preparation to be examined contains sufficient antitoxic globulins to neutralise not less than 100 mouse LD₅₀ of *Vipera ammodytes* venom or *Vipera aspis* venom and not less than 50 mouse LD₅₀ of the venoms of other species of viper.

The potency of European viper venom antiserum is determined by estimating the dose necessary to protect mice against the lethal effects of a fixed dose of venom of the relevant species of viper.

Selection of test venoms. Use venoms which have the normal physicochemical, toxicological and immunological characteristics of venoms from the particular species of vipers. They are preferably freeze-dried and stored in the dark at 5 ± 3 °C.

Select a venom for use as a test venom by determining the LD₅₀ for mice, the observation period being 48 h.

Determination of the test dose of venom. Prepare graded dilutions of the reconstituted venom in a 9 g/l solution of *sodium chloride R* or other isotonic diluent in such a manner that the middle dilution contains in 0.25 ml the dose expected to be the LD₅₀. Dilute with an equal volume of the same diluent. Using at least four mice, each weighing 18 g to 20 g, for each dilution, inject 0.5 ml intravenously into each mouse. Observe the mice for 48 h and record the number of deaths. Calculate the LD₅₀ using the usual statistical methods.

Determination of the potency of the antiserum to be examined. Dilute the reconstituted test venom so that 0.25 ml contains the test dose of 5 LD₅₀ (test venom solution). Prepare serial dilutions of the antiserum to be examined in a 9 g/l solution of *sodium chloride R* or other isotonic diluent, the dilution factor being 1.5 to 2.5. Use a sufficient number and range of dilutions to enable a mortality curve between 20 per cent and 80 per cent mortality to be established and to permit an estimation of the statistical variation.

Prepare mixtures such that 5 ml of each mixture contains 2.5 ml of one of the dilutions of the antiserum to be examined and 2.5 ml of the test venom solution. Allow the mixtures to stand in a water-bath at 37 °C for 30 min. Using not fewer than six mice, each weighing 18 g to 20 g, for each mixture, inject 0.5 ml intravenously into each mouse. Observe the mice for 48 h and record the number of deaths. Calculate the PD₅₀, using the usual statistical methods. At the same time verify the number of LD₅₀ in the test dose of venom, using the method described above. Calculate the potency of the antiserum from the expression:

$$\frac{(T_v - 1)}{PD_{50}}$$

T_v = number of LD₅₀ in the test dose of venom.

In each mouse dose of the venom-antiserum mixture at the end point there is one LD₅₀ of venom remaining unneutralised by the antiserum and it is this unneutralised venom that is responsible for the deaths of 50 per cent of the mice inoculated with the mixture. The amount of venom neutralised by the antiserum is thus one LD₅₀ less than the total amount contained in each mouse dose. Therefore, as the potency of the antiserum is defined in terms of the number of LD₅₀ of venom that are neutralised, rather than the number of LD₅₀ in each mouse dose, the expression required in the calculation of potency is $T_v - 1$ rather than T_v . Alternatively, the quantity of test venom in milligrams that is neutralised by 1 ml or some other defined volume of the antiserum to be examined may be calculated.

LABELLING

The label states the venom or venoms against which the antiserum is effective.

Warning: Because of the allergenic properties of viper venoms, inhalation of venom dust should be avoided by suitable precautions.

01/2005:0340

CLOSTRIDIUM PERFRINGENS BETA ANTITOXIN FOR VETERINARY USE

Immunoserum clostridii perfringentis beta ad usum veterinarium

DEFINITION

Clostridium perfringens beta antitoxin for veterinary use is a preparation containing principally the globulins that have the power of specifically neutralising the beta toxin formed by *Clostridium perfringens* (types B and C). It consists of the serum or a preparation obtained from the serum of animals immunised against *Cl. perfringens* beta toxin.

IDENTIFICATION

It specifically neutralises the beta toxin of *Cl. perfringens* rendering it harmless to susceptible animals.

POTENCY

The potency of the crude serum is not less than 1000 IU/ml when obtained from horses and not less than 250 IU/ml when obtained from cattle.

The potency of the concentrated serum is not less than 3000 IU/ml when obtained from horses and not less than 1000 IU/ml when obtained from cattle.

The International Unit is the specific neutralising activity for *Cl. perfringens* beta toxin contained in a stated amount of the International Standard, which consists of a quantity of dried immune horse serum. The equivalence in International Units of the International Standard is stated by the World Health Organisation.

The potency of clostridium perfringens beta antitoxin is determined by comparing the dose necessary to protect mice or other suitable animals against the toxic effects of a fixed dose of *Cl. perfringens* beta toxin with the quantity of a reference preparation of clostridium perfringens beta antitoxin, calibrated in International Units, necessary to give the same protection. For this comparison, a suitable preparation of *Cl. perfringens* beta toxin for use as a test toxin is required. The dose of the test toxin is determined in relation to the reference preparation; the potency of the clostridium perfringens beta antitoxin to be examined is determined in relation to the reference preparation using the test toxin.

Preparation of test toxin. Prepare the test toxin from a sterile filtrate of an early culture in liquid medium of *Cl. perfringens* type B or type C and dry by a suitable method.

Select the test toxin by determining for mice the L+ dose and the LD₅₀, the observation period being 72 h. A suitable beta toxin contains not less than one L+ dose in 0.2 mg and not less than 25 LD₅₀ in each L+ dose.

Determination of test dose of toxin. Prepare a solution of the reference preparation in a suitable liquid so that it contains 5 IU/ml. Prepare a solution of the test toxin in a suitable liquid so that 1 ml contains a precisely known amount such as 10 mg. Prepare mixtures of the solution of the reference preparation and the solution of the test toxin such that each mixture contains 2.0 ml of the solution of the reference preparation (10 IU), one of a series of graded volumes of the solution of the test toxin and sufficient of a suitable liquid to bring the total volume to 5.0 ml. Allow the mixtures to stand at room temperature for 30 min. Using not fewer than two mice, each weighing 17 g to 22 g, for each mixture, inject a dose of 0.5 ml intravenously or

intra-peritoneally into each mouse. Observe the mice for 72 h. If all the mice die, the amount of toxin present in 0.5 ml of the mixture is in excess of the test dose. If none of the mice dies, the amount of toxin present in 0.5 ml of the mixture is less than the test dose. Prepare fresh mixtures such that 5.0 ml of each mixture contains 2.0 ml of the solution of the reference preparation (10 IU) and one of a series of graded volumes of the solution of the test toxin separated from each other by steps of not more than 20 per cent and covering the expected end-point. Allow the mixtures to stand at room temperature for 30 min.

Using not fewer than two mice for each mixture, inject a dose of 0.5 ml intravenously or intraperitoneally into each mouse. Observe the mice for 72 h. Repeat the determination at least once and combine the results of the separate tests that have been made with mixtures of the same composition so that a series of totals is obtained, each total representing the mortality due to a mixture of a given composition. The test dose of toxin is the amount present in 0.5 ml of that mixture which causes the death of one half of the total number of mice injected with it.

Determination of the potency of the antitoxin to be examined

Preliminary test. Dissolve a quantity of the test toxin in a suitable liquid so that 2.0 ml contains 10 times the test dose. Prepare mixtures of the solution of the test toxin and the antitoxin to be examined such that each mixture contains 2.0 ml of the solution of the test toxin, one of a series of graded volumes of the antitoxin to be examined and sufficient of a suitable liquid to bring the final volume to 5.0 ml. Allow the mixtures to stand at room temperature for 30 min. Using not fewer than two mice for each mixture, inject a dose of 0.5 ml intravenously or intraperitoneally into each mouse. Observe the mice for 72 h. If none of the mice dies, 0.5 ml of the mixture contains more than 1 IU. If all the mice die, 0.5 ml of the mixture contains less than 1 IU.

Final test. Prepare mixtures of the solution of the test toxin and of the antitoxin to be examined such that 5.0 ml of each mixture contains 2.0 ml of the solution of the test toxin and one of a series of graded volumes of the antitoxin to be examined, separated from each other by steps of not more than 20 per cent and covering the expected end-point as determined by the preliminary test. Prepare further mixtures such that 5.0 ml of each mixture contains 2.0 ml of the solution of the test toxin and one of a series of graded volumes of the solution of the reference preparation, in order to confirm the test dose of the toxin. Allow the mixtures to stand at room temperature for 30 min. Using not fewer than two mice for each mixture, proceed as described in the preliminary test. The test mixture which contains, in 0.5 ml, 1 IU is that mixture which kills the same or almost the same number of mice as the reference mixture containing, in 0.5 ml, 1 IU. Repeat the determination at least once and calculate the average of all valid estimates. Estimates are valid only if the reference preparation gives a result within 20 per cent of the expected value.

The confidence limits ($P = 0.95$) have been estimated to be: 85 per cent and 114 per cent when two animals per dose are used,

91.5 per cent and 109 per cent when four animals per dose are used,

93 per cent and 108 per cent when six animals per dose are used.

LABELLING

The label states whether the product consist of crude concentrated serum.

01/2005:0341

CLOSTRIDIUM PERFRINGENS EPSILON ANTITOXIN FOR VETERINARY USE

Immunoserum clostridii perfringentis epsilon ad usum veterinarium

DEFINITION

Clostridium perfringens epsilon antitoxin for veterinary use is a preparation containing the globulins that have the power of specifically neutralising the epsilon toxin formed by *Clostridium perfringens* type D. It consists of the serum or a preparation obtained from the serum of animals immunised against *Cl. perfringens* epsilon toxin.

IDENTIFICATION

It specifically neutralises the epsilon toxin of *Cl. perfringens* type D, rendering it harmless to susceptible animals.

POTENCY

The potency of the crude serum is not less than 120 IU/ml when obtained from horses and not less than 100 IU/ml when obtained from cattle.

The potency of the concentrated serum is not less than 300 IU/ml when obtained from horses and not less than 150 IU/ml when obtained from cattle.

The International Unit is the specific neutralising activity for *Cl. perfringens* epsilon toxin contained in a stated amount of the International Standard, which consists of a quantity of dried immune horse serum. The equivalence in International Units of the International Standard is stated by the World Health Organisation.

The potency of clostridium perfringens epsilon antitoxin is determined by comparing the dose necessary to protect mice or other suitable animals against the toxic effects of a fixed dose of *Cl. perfringens* epsilon toxin with the quantity of a reference preparation of clostridium perfringens epsilon anti-toxin, calibrated in International Units, necessary to give the same protection. For this comparison, a suitable preparation of *Cl. perfringens* epsilon toxin for use as a test toxin is required. The dose of the test toxin is determined in relation to the reference preparation, the potency of the antitoxin to be examined is determined in relation to the reference preparation using the test toxin.

Preparation of test toxin. Prepare the test toxin from a sterile filtrate of an early culture in liquid medium of *Cl. perfringens* type D and dry by a suitable method.

Select the test toxin by determining for mice the $L_{+}/10$ dose and the LD_{50} , the observation period being 72 h. A suitable epsilon toxin contains not less than one $L_{+}/10$ dose in 0.005 mg and not less than 20 LD_{50} in each $L_{+}/10$ dose.

Determination of test dose of toxin. Prepare a solution of the reference preparation in a suitable liquid so that it contains 0.5 IU of antitoxin per millilitre. Prepare a solution of the test toxin in a suitable liquid so that 1 ml contains a precisely known amount such as 1 mg. Prepare mixtures of the solution of the reference preparation and the solution of the test toxin such that each mixture contains 2.0 ml of the solution of the reference preparation (1 IU), one of a series of graded volumes of the solution of the test toxin and sufficient of a suitable liquid to bring the total volume to 5.0 ml. Allow the mixtures to stand at room temperature

for 30 min. Using not fewer than two mice, each weighing 17 g to 22 g, for each mixture, inject a dose of 0.5 ml intravenously or intraperitoneally into each mouse. Observe the mice for 72 h. If all the mice die, the amount of toxin present in 0.5 ml of the mixture is in excess of the test dose. If none of the mice dies, the amount of toxin present in 0.5 ml of the mixture is less than the test dose.

Prepare similar fresh mixtures such that 5.0 ml of each mixture contains 2.0 ml of the solution of the reference preparation (1 IU) and one of a series of graded volumes of the solution of the test toxin, separated from each other by steps of not more than 20 per cent and covering the expected end-point. Allow the mixtures to stand at room temperature for 30 min. Using not fewer than two mice for each mixture, inject a dose of 0.5 ml intravenously or intraperitoneally into each mouse. Observe the mice for 72 h. Repeat the determination at least once and add together the results of the separate tests that have been made with mixtures of the same composition so that a series of totals is obtained, each total representing the mortality due to a mixture of a given composition. The test dose of the toxin is the amount present in 0.5 ml of that mixture which causes the death of one half of the total number of mice injected with it.

Determination of the potency of the antitoxin to be examined

Preliminary test. Dissolve a quantity of the test toxin in a suitable liquid so that 2.0 ml contains 10 times the test dose (solution of the test toxin). Prepare mixtures of the solution of the test toxin and of the antitoxin to be examined such that each mixture contains 2.0 ml of the solution of the test toxin, one of a series of graded volumes of the antitoxin to be examined and sufficient of a suitable liquid to bring the final volume to 5.0 ml. Allow the mixtures to stand at room temperature for 30 min. Using not fewer than two mice for each mixture, inject a dose of 0.5 ml intravenously or intra-peritoneally into each mouse. Observe the mice for 72 h. If none of the mice dies, 0.5 ml of the mixture contains more than 0.1 IU. If all the mice die, 0.5 ml of the mixture contains less than 0.1 IU.

Final test. Prepare mixtures of the solution of the test toxin and of the antitoxin to be examined such that 5.0 ml of each mixture contains 2.0 ml of the solution of the test toxin and one of a series of graded volumes of the antitoxin to be examined, separated from each other by steps of not more than 20 per cent and covering the expected end-point as determined by the preliminary test. Prepare further mixtures such that 5.0 ml of each mixture contains 2.0 ml of the solution of the test toxin and one of a series of graded volumes of the solution of the reference preparation to confirm the test dose of the toxin. Allow the mixtures to stand at room temperature for 30 min. Using not fewer than two mice for each mixture proceed as described in the preliminary test. The test mixture which contains 0.1 IU in 0.5 ml is that mixture which kills the same or almost the same number of mice as the reference mixture containing 0.1 IU in 0.5 ml. Repeat the determination at least once and calculate the average of all valid estimates. Estimates are valid only if the reference preparation gives a result within 20 per cent of the expected value.

The confidence limits ($P = 0.95$) have been estimated to be:

85 per cent and 114 per cent when 2 animals per dose are used,

91.5 per cent and 109 per cent when 4 animals per dose are used,

93 per cent and 108 per cent when 6 animals per dose are used.

LABELLING

The label states whether the product consists of crude or concentrated serum.

01/2005:0343

TETANUS ANTITOXIN FOR VETERINARY USE

Immunoserum tetanicum ad usum veterinarium

DEFINITION

Tetanus antitoxin for veterinary use is a preparation containing principally the globulins that have the power of specifically neutralising the neurotoxin formed by *Clostridium tetani*. It consists of the serum or a preparation obtained from the serum of animals immunised against tetanus toxin.

IDENTIFICATION

It specifically neutralises the neurotoxin formed by *Cl. tetani*, rendering it harmless to susceptible animals.

POTENCY

The potency of the crude serum is not less than 300 IU/ml when obtained from horses and not less than 150 IU/ml when obtained from cattle.

The potency of the concentrated serum is not less than 1000 IU/ml when obtained from horses and not less than 500 IU/ml when obtained from cattle.

The International Unit is the specific neutralising activity for tetanus toxin contained in a stated amount of the International Standard which consists of a quantity of dried immune horse serum. The equivalence in International Units of the International Standard is stated by the World Health Organisation.

The potency of tetanus antitoxin is determined by comparing the dose necessary to protect mice (or guinea-pigs) against the toxic effects of a fixed dose of tetanus toxin with the quantity of a reference preparation of tetanus antitoxin, calibrated in International Units, necessary to give the same protection. In countries where the paralysis method is not obligatory, the lethal method may be used. For this method the number of animals and the procedure are identical with those described for the paralysis method but the end-point is the death of the animal rather than the onset of paralysis and the L₅₀/10 dose and the LD₅₀ are used instead of the Lp/10 dose and the paralytic dose 50 per cent. For this comparison, a suitable preparation of tetanus toxin for use as a test toxin is required. The dose of the test toxin is determined in relation to the reference preparation; the potency of the antitoxin to be examined is determined in relation to the reference preparation using the test toxin.

Preparation of test toxin. Prepare the test toxin from a sterile filtrate of an 8 to 10 day culture in liquid medium of *Cl. tetani*. A test toxin may be prepared by adding this filtrate to *glycerol R* in the proportion of 1 volume of filtrate to 1 to 2 volumes of *glycerol R*. The solution of test toxin may be stored at or slightly below 0 °C. The toxin may also be dried by a suitable method.

Select the test toxin by determining for mice the Lp/10 dose and the paralytic dose 50 per cent. A suitable toxin contains not less than 1000 times the paralytic dose 50 per cent in one Lp/10 dose.

Lp/10 dose (Limes paralyticum). This is the smallest quantity of toxin which when mixed with 0.1 IU of antitoxin and injected subcutaneously into mice (or guinea-pigs) causes tetanic paralysis in the animals on or before the fourth day after injection.

Paralytic dose 50 per cent. This is the quantity of toxin which when injected subcutaneously into mice (or guinea-pigs) causes tetanic paralysis in one half of the animals on or before the fourth day after injection.

Determination of test dose of toxin. Reconstitute or dilute the reference preparation with a suitable liquid so that it contains 0.5 IU per millilitre. Measure or weigh a quantity of the test toxin and dilute with or dissolve in a suitable liquid. Prepare mixtures of the solution of the reference preparation and the solution of the test toxin so that each mixture will contain 0.1 IU of antitoxin in the volume chosen for injection and one of a series of graded volumes of the solution of the test toxin, separated from each other by steps of not more than 20 per cent and covering the expected end-point. Adjust each mixture with a suitable liquid to the same final volume (0.4 ml to 0.6 ml if mice are used for the test or 4.0 ml if guinea-pigs are used). Allow the mixtures to stand at room temperature for 60 min. Using not fewer than two animals for each mixture, inject the chosen volume subcutaneously into each animal. Observe the animals for 96 h and make daily records of the degree of tetanus developing in each group of animals. Repeat the test at least once and calculate the test dose as the mean of the different tests. The test dose of the toxin is the amount present in that mixture which causes tetanic paralysis in one half of the total number of animals injected with it.

When the test dose of the toxin has been determined a concentrated solution of the test toxin may be prepared in a mixture consisting of 1 volume of a 9 g/l solution of *sodium chloride R* and 1 or 2 volumes of *glycerol R*. This concentrated solution may be stored frozen and diluted as required. The specific activity of such a solution should be redetermined at frequent intervals.

Determination of the potency of the antitoxin to be examined

Preliminary test. Measure or weigh a quantity of the test toxin and dilute with or dissolve in a suitable liquid so that the solution contains five test doses per millilitre (solution of the test toxin). Prepare mixtures of the solution of the test toxin and the antitoxin to be examined so that for each mixture the volume chosen for injection contains the test dose of toxin and one of a series of graded volumes of the antitoxin to be examined. Adjust each mixture to the same final volume with a suitable liquid. Allow the mixtures to stand at room temperature for 60 min. Using not fewer than two animals for each mixture, inject the chosen volume subcutaneously into each animal. Observe the animals for 96 h and make daily records of the degree of tetanus developing in each group of animals. Using the results, select suitable mixtures for the final test.

Final test. Prepare mixtures of the solution of the test toxin and the antitoxin to be examined so that for each mixture the volume chosen for the injection contains the test dose of toxin and one of a series of graded volumes of the antitoxin to be examined, separated from each other by steps of not more than 20 per cent and covering the expected end-point as determined in the preliminary test. Prepare further mixtures with the same amount of test toxin and graded volumes of the reference preparation, centred on 0.1 IU in the volume chosen for injection, to confirm the test dose of the toxin. Adjust each mixture to the same final volume with a suitable liquid. Allow the mixtures to stand at room temperature for

60 min. Using not fewer than two animals for each mixture, inject the chosen volume subcutaneously into each animal. Observe the animals for 96 h and make daily records of the degree of tetanus developing in each group of animals. The test mixture which contains 0.1 IU in the volume injected is that mixture which causes tetanic paralysis in the same, or almost the same, number of animals as the reference mixture containing 0.1 IU in the volume injected.

Repeat the determination at least once and calculate the mean of all valid estimates. Estimates are valid only if the reference preparation gives a result within 20 per cent of the expected value.

The confidence limits ($P = 0.95$) have been estimated to be:
85 per cent and 114 per cent when 2 animals per dose are used,
91.5 per cent and 109 per cent when 3 animals per dose are used,
93 per cent and 108 per cent when 6 animals per dose are used.

LABELLING

The label states whether the product consists of crude or concentrated serum.

01/2005:1492 CHEMICAL PURITY

AMMONIA (^{13}N) INJECTION**Ammoniae (^{13}N) solutio iniectionis****DEFINITION**

Ammonia (^{13}N) injection is a sterile solution of [^{13}N]ammonia for diagnostic use. The injection contains not less than 90.0 per cent and not more than 110.0 per cent of the declared nitrogen-13 radioactivity at the date and time stated on the label. Not less than 99 per cent of the total radioactivity corresponds to nitrogen-13 in the form of [^{13}N]ammonia. Not less than 99.0 per cent of the total radioactivity corresponds to nitrogen-13.

PRODUCTION**RADIONUCLIDE PRODUCTION**

Nitrogen-13 is a radioactive isotope of nitrogen which may be produced by various nuclear reactions, such as proton irradiation of carbon-13 or oxygen-16, or deuteron irradiation of carbon-12.

RADIOCHEMICAL SYNTHESIS

[^{13}N]Ammonia may be prepared by proton irradiation of water followed by the reduction of the resulting [^{13}N]nitrates/nitrites mixture with a reducing agent. The [^{13}N]ammonia formed is distilled from the reaction mixture and trapped in a slightly acidic solution.

Other methods may produce [^{13}N]ammonia “in-target” by proton irradiation of water containing a small amount of ethanol or acetic acid, or by proton irradiation of a slurry of [^{13}C]carbon powder in water. The resulting solution can be purified, to remove radionuclidic and radiochemical impurities, using anion and cation exchange columns.

CHARACTERS

A clear, colourless solution.

Nitrogen-13 has a half-life of 9.96 min and emits positrons with a maximum energy of 1.198 MeV, followed by annihilation gamma radiation of 0.511 MeV.

IDENTIFICATION

- Record the gamma-ray spectrum using a suitable instrument. The only gamma photons have an energy of 0.511 MeV and, depending on the measurement geometry, a sum peak of 1.022 MeV may be observed.
- It complies with test (a) for radionuclidic purity (see Tests).
- Examine the chromatograms obtained in the test for radiochemical purity. The principal peak in the radiochromatogram obtained with the test solution has approximately the same retention time as the principal peak in the radiochromatogram obtained with the reference solution.

TESTS

pH (2.2.3). The pH of the injection is 5.5 to 8.5.

Sterility. It complies with the test for sterility prescribed in the monograph on *Radiopharmaceutical preparations* (0125). The injection may be released for use before completion of the test.

Bacterial endotoxins (2.6.14): less than 175/V IU/ml, V being the maximum recommended dose in millilitres. The injection may be released for use before completion of the test.

Aluminium. In a test-tube about 12 mm in internal diameter, mix 1 ml of *acetate buffer solution pH 4.6 R* and 2 ml of a 1 in 20 dilution of the preparation to be examined in *water R*. Add 0.05 ml of a 10 g/l solution of *chromazurol S R*. After 3 min, the colour of the solution is not more intense than that of a standard prepared at the same time and in the same manner using 2 ml of a 1 in 20 dilution of *aluminium standard solution (2 ppm Al) R* (2 ppm).

The injection may be released for use before completion of the test.

RADIONUCLIDIC PURITY

(a) *Half-life.* The half-life is between 9 min and 11 min.

(b) *Gamma emitting impurities.* Retain a sample of the preparation to be examined for 2 h. Examine the gamma-ray spectrum of the decayed material for the presence of radionuclidic impurities, which should, where possible, be identified and quantified. The total gamma radioactivity due to these impurities does not exceed 1.0 per cent of the total radioactivity.

The injection may be released for use before completion of tests (a) and (b).

RADIOCHEMICAL PURITY

Examine by liquid chromatography (2.2.29).

Test solution. The preparation to be examined.

Reference solution. Dilute 1.0 ml of *dilute ammonia R2* to 10.0 ml with *water R*.

The chromatographic procedure may be carried out using:

- a column 0.04 m long and 4.0 mm in internal diameter packed with *cation exchange resin R* (10 μm),
- as mobile phase at a flow rate of 2 ml/min 0.002 M *nitric acid*,
- a suitable radioactivity detector,
- a conductivity detector,
- a loop injector,

maintaining the column at a constant temperature between 20 °C and 30 °C.

Inject separately the test solution and the reference solution. The chromatogram obtained with the radioactivity detector and the test solution shows a principal peak with approximately the same retention time as the peak in the chromatogram obtained with the reference solution and the conductivity detector. Not less than 99 per cent of the total radioactivity corresponds to nitrogen-13 in the form of ammonia.

The injection may be released for use before completion of the test.

RADIOACTIVITY

Measure the radioactivity using suitable equipment by comparison with a standardised fluorine-18 solution or by using an instrument calibrated with the aid of such a solution. Standardised fluorine-18 solutions are available from laboratories recognised by the competent authority.

IMPURITIES

- [^{13}N]O₂⁻,
- [^{13}N]O₃⁻,
- [18F⁻],
- H₂[¹⁵O].

01/2005:1607 *Injection*: test sample, reference gas (b).**CARBON MONOXIDE (¹⁵O)****Carbonei monoxidum (¹⁵O)****DEFINITION**

Mixture of carbon [¹⁵O]monoxide in the gaseous phase and a suitable vehicle such as *Medicinal air (1238)*, for diagnostic use.

Purity:

- minimum 99 per cent of the total radioactivity corresponds to oxygen-15,
- minimum 97 per cent of the total radioactivity corresponds to oxygen-15 in the form of carbon monoxide (CO).

PRODUCTION**RADIONUCLIDE PRODUCTION**

Oxygen-15 is a radioactive isotope of oxygen which may be produced by various nuclear reactions such as proton irradiation of nitrogen-15 or deuteron irradiation of nitrogen-14.

RADIOCHEMICAL SYNTHESIS

In order to recover oxygen-15 as molecular oxygen from the nitrogen target gas, carrier oxygen is added at concentrations generally ranging from 0.2 per cent V/V to 1.0 per cent V/V. After irradiation, the target gas is usually reacted with activated charcoal at a temperature of about 950 °C. The activated charcoal is preconditioned before use by flushing an inert gas at the production flow rate at a temperature of about 950 °C for not less than 1 h. The carbon [¹⁵O]monoxide obtained is purified by passage through a carbon dioxide scavenger, such as soda lime, before mixing with the vehicle.

CHARACTERS

Appearance: colourless gas.

Half-life and nature of radiation of oxygen-15: see Table of physical characteristics of radionuclides (5.7).

IDENTIFICATION**A. Gamma spectrometry.**

Results: the only gamma photons have an energy of 0.511 MeV and, depending on the measurement geometry, a sum peak of 1.022 MeV may be observed.

B. It complies with the test for radionuclidic purity (see Tests).**C. Examine the chromatograms obtained in the test for radiochemical purity.**

Results: the principal peaks in the chromatogram obtained with the test gas using the radioactivity detector are similar in retention times to the principal peaks corresponding to carbon monoxide in the chromatogram obtained with reference gas (a) using the thermal conductivity detector.

TESTS

The following tests are performed on carbon [¹⁵O]monoxide as described under radiochemical synthesis before mixing with the vehicle.

Carbon monoxide. Gas chromatography (2.2.28) as described in the test for radiochemical purity.

The concentration of carbon monoxide in the test sample is determined before administration and is used to calculate the amount of carbon monoxide to be administered to the patient.

Examine the chromatogram obtained with the thermal conductivity detector and calculate the content of carbon monoxide.

RADIONUCLIDIC PURITY

Oxygen-15: minimum 99 per cent of the total radioactivity.

A. Gamma spectrometry.

Comparison: standardised fluorine-18 solution, or by using an instrument calibrated with the aid of such a solution. Standardised fluorine-18 solutions and/or standardisation services are available from the competent authority.

Results: the spectrum obtained with the solution to be examined does not differ significantly from that obtained with a standardised fluorine-18 solution.

B. Half-life: 1.9 min to 2.2 min.

The preparation may be released for use before completion of the test.

RADIOCHEMICAL PURITY

Carbon [¹⁵O]monoxide. Gas chromatography (2.2.28): use the normalisation procedure.

Test sample. Carbon [¹⁵O]monoxide as described under radiochemical synthesis.

Reference gas (a). Nitrogen gas mixture R.

Reference gas (b). Nitrogen R, containing 2.0 per cent V/V of carbon monoxide R1.

Column:

- *size*: *l* = 1.8 m, Ø1 = 6.3 mm and Ø2 = 3.2 mm,
- *stationary phase*: GC concentric column R,
- Carrier gas*: helium for chromatography R.

Flow rate: 65 ml/min.

Temperature:

- *column*: 40 °C,
- *injection port*: 40 °C,
- *thermal conductivity detector*: 70 °C.

Detection: thermal conductivity detector and radioactivity detector connected in series.

Injection: loop injector.

Run time: 10 min.

Retention times: oxygen, nitrogen and carbon monoxide eluting from the inner column = about 0.4 min; carbon dioxide eluting from the inner column = about 0.8 min; oxygen eluting from the outer column = about 2.1 min; nitrogen eluting from the outer column = about 3.1 min; carbon monoxide eluting from the outer column = about 6.2 min.

System suitability: reference gas (a):

- 5 clearly separated principal peaks are observed in the chromatogram obtained using the thermal conductivity detector,
- *resolution*: minimum of 1.5 between the peaks due to carbon dioxide eluting from the inner column and oxygen eluting from the outer column, in the chromatogram obtained using the thermal conductivity detector.

Limits: examine the chromatogram obtained with the radioactivity detector and calculate the percentage content of oxygen-15 substances from the peak areas.

- *carbon [¹⁵O]monoxide*: minimum 97 per cent of the total radioactivity.
- *disregard* the first peak corresponding to components co-eluting from the inner column.

RADIOACTIVITY

The radioactive concentration is determined before administration.

Measure the radioactivity using suitable equipment by comparison with a standardised fluorine-18 solution or by measurement in an instrument calibrated with the aid of such a solution.

01/2005:0266

CHROMIUM (^{51}Cr) EDETATE INJECTION

Chromii (^{51}Cr) edetatis solutio iniectionabilis

DEFINITION

Chromium (^{51}Cr) edetate injection is a sterile solution containing chromium-51 in the form of a complex of chromium(III) with ethylenediaminetetraacetic acid, the latter being present in excess. It may be made isotonic by the addition of sodium chloride and may contain a suitable antimicrobial preservative such as benzyl alcohol. Chromium-51 is a radioactive isotope of chromium and may be prepared by the neutron irradiation of chromium, either of natural isotopic composition or enriched in chromium-50. The injection contains not less than 90.0 per cent and not more than 110.0 per cent of the declared chromium-51 radioactivity at the date and hour stated on the label. Not less than 95 per cent of the radioactivity corresponds to chromium-51 in the form of chromium edetate. The injection contains a variable quantity of chromium (Cr) not exceeding 1 mg per millilitre.

CHARACTERS

A clear, violet solution.

Chromium-51 has a half-life of 27.7 days and emits gamma radiation.

IDENTIFICATION

- Record the gamma-ray spectrum using a suitable instrument. The spectrum does not differ significantly from that of a standardised chromium-51 solution. Standardised chromium-51 solutions are available from laboratories recognised by the competent authority. The gamma photon has an energy of 0.320 MeV.
- Examine the electropherogram obtained in the test for radiochemical purity. The distribution of radioactivity contributes to the identification of the preparation.

TESTS

pH (2.2.3). The pH of the solution is 3.5 to 6.5.

Chromium. Prepare a reference solution (1 mg per millilitre of Cr) as follows: dissolve 0.96 g of *chromic potassium sulphate R* and 2.87 g of *sodium edetate R* in 50 ml of *water R*, boil for 10 min, cool, adjust to pH 3.5 to 6.5 using *dilute sodium hydroxide solution R* and dilute to 100.0 ml with *water R*. Measure the absorbance (2.2.25) of the injection to be examined and the reference solution at the absorption maximum at 560 nm. The absorbance of the injection to be examined is not greater than that of the reference solution.

Sterility. It complies with the test for sterility prescribed in the monograph on *Radiopharmaceutical preparations (0125)*. The injection may be released for use before completion of the test.

RADIONUCLIDIC PURITY

Record the gamma-ray spectrum using a suitable instrument. The spectrum does not differ significantly from that of a standardised chromium-51 solution.

RADIOCHEMICAL PURITY

Examine by zone electrophoresis (2.2.31), using a paper strip as the support and a solution containing 0.2 g/l of *barbital sodium R* and 10 g/l of *sodium nitrate R* as the electrolyte solution. A paper with the following characteristics is suitable: mass per unit area 120 g/m²; thickness 0.22 mm; capillary rise 105 mm to 115 mm per 30 min.

Apply to the paper 10 µl of the injection as a 3 mm band at a position 10 cm from the cathode. Apply an electric field of about 30 V per centimetre for 30 min using a stabilised current. [^{51}Cr]chromium edetate moves about 5 cm towards the anode. [^{51}Cr]Chromate moves about 10 cm towards the anode and [^{51}Cr]chromic ion moves about 7 cm towards the cathode. Determine the distribution of the radioactivity using a suitable detector. Not less than 95 per cent of the total radioactivity is found in the band corresponding to [^{51}Cr]chromium edetate.

RADIOACTIVITY

Measure the radioactivity using suitable equipment by comparison with a standardised chromium-51 solution or by measurement in an instrument calibrated with the aid of such a solution.

01/2005:0710

CYANOCOBALAMIN (^{57}Co) CAPSULES

Cyanocobalamini (^{57}Co) capsulae

DEFINITION

Cyanocobalamin (^{57}Co) capsules contain [^{57}Co]- α -(5,6-dimethylbenzimidazol-1-yl)cobamide cyanide and may contain suitable auxiliary substances. Cobalt-57 is a radioactive isotope of cobalt and may be produced by proton irradiation of nickel. Cyanocobalamin (^{57}Co) may be prepared by the growth of suitable micro-organisms on a medium containing (^{57}Co) cobaltous ion. Not less than 90 per cent of the cobalt-57 is in the form of cyanocobalamin. The capsules comply with the requirements for hard capsules in the monograph on *Capsules (0016)*, unless otherwise justified and authorised.

CHARACTERS

Hard gelatin capsules.

Cobalt-57 has a half-life of 271 days and emits gamma radiation.

IDENTIFICATION

- Record the gamma-ray spectrum using a suitable instrument. The spectrum does not differ significantly from that of a standardised cobalt-57 solution. Standardised cobalt-57 and cobalt-58 solutions are available from laboratories recognised by the competent authority. The most prominent gamma photon of cobalt-57 has an energy of 0.122 MeV.
- Examine the chromatograms obtained in the test for radiochemical purity. The principal peak in the radio-chromatogram obtained with the test solution has a retention time similar to that of the peak in the chromatogram obtained with the reference solution.

TESTS

01/2005:0269

Disintegration. The capsules comply with the test for disintegration of tablets and capsules (2.9.1) except that one capsule is used in the test instead of six.

Uniformity of content. Determine by measurement in a suitable counting assembly and under identical geometrical conditions the radioactivity of each of not less than ten capsules. Calculate the average radioactivity per capsule. The radioactivity of no capsule differs by more than 10 per cent from the average. The relative standard deviation is less than 3.5 per cent.

RADIONUCLIDIC PURITY

Record the gamma-ray spectrum using a suitable instrument calibrated with the aid of standardised cobalt-57 and cobalt-58 solutions. The spectrum does not differ significantly from that of the standardised cobalt-57 solution. Determine the relative amounts of cobalt-57, cobalt-56 and cobalt-58 present. Cobalt-56 has a half-life of 78 days and its presence is shown by gamma photons of energy 0.847 MeV. Cobalt-58 has a half-life of 70.8 days and its presence is shown by gamma photons of energy 0.811 MeV. Not more than 0.1 per cent of the total radioactivity is due to cobalt-56, cobalt-58 and other radionuclidic impurities.

RADIOCHEMICAL PURITY

Examine by liquid chromatography (2.2.29).

Test solution. Dissolve the contents of a capsule in 1.0 ml of *water R* and allow to stand for 10 min. Centrifuge at 2000 r/min for 10 min. Use the supernatant.

Reference solution. Dissolve 10 mg of *cyanocobalamin CRS* in the mobile phase and dilute to 100 ml with the mobile phase. Dilute 2 ml of the solution to 100 ml with the mobile phase. Use within 1 h.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4 mm in internal diameter packed with *octylsilyl silica gel for chromatography R* (5 µm),
- as mobile phase at a flow rate of 1.0 ml/min a mixture prepared as follows: mix 26.5 volumes of *methanol R* and 73.5 volumes of a 10 g/l solution of *disodium hydrogen phosphate R* adjusted to pH 3.5 using *phosphoric acid R* and use within 2 days,
- a radioactivity detector adjusted for cobalt-57,
- as detector a spectrophotometer set at 361 nm,
- a loop injector.

Inject 100 µl of the test solution and record the chromatogram for three times the retention time of cyanocobalamin. Determine the peak areas and calculate the percentage of cobalt-57 present as cyanocobalamin. Inject 100 µl of the reference solution and record the chromatogram for 30 min.

RADIOACTIVITY

The average radioactivity determined in the test for uniformity of content is not less than 90.0 per cent and not more than 110.0 per cent of the declared cobalt-57 radioactivity, at the date stated on the label.

STORAGE

Store in an airtight container, protected from light, at a temperature of 2 °C to 8 °C.

CYANOCOBALAMIN (⁵⁷Co) SOLUTION**Cyanocobalamini (⁵⁷Co) solutio****DEFINITION**

Cyanocobalamin (⁵⁷Co) solution is a solution of [⁵⁷Co]-α-(5,6-dimethylbenzimidazol-1-yl)cobamide cyanide and may contain a stabiliser and an antimicrobial preservative. Cobalt-57 is a radioactive isotope of cobalt and may be produced by the irradiation of nickel with protons of suitable energy. Cyanocobalamin (⁵⁷Co) may be prepared by the growth of suitable micro-organisms on a medium containing (⁵⁷Co) cobaltous ion. The solution contains not less than 90.0 per cent and not more than 110.0 per cent of the declared cobalt-57 radioactivity at the date stated on the label. Not less than 90 per cent of the cobalt-57 is in the form of cyanocobalamin.

CHARACTERS

A clear, colourless or slightly pink solution. Cobalt-57 has a half-life of 271 days and emits gamma radiation.

IDENTIFICATION

- A. Record the gamma-ray spectrum using a suitable instrument. The spectrum does not differ significantly from that of a standardised cobalt-57 solution. Standardised cobalt-57 and cobalt-58 solutions are available from laboratories recognised by the competent authority. The most prominent gamma photon of cobalt-57 has an energy of 0.122 MeV.
- B. Examine the chromatograms obtained in the test for radiochemical purity. The principal peak in the radiochromatogram obtained with the solution to be examined has a retention time similar to that of the peak in the chromatogram obtained with the reference solution.

TESTS

pH (2.2.3). The pH of the solution is 4.0 to 6.0.

RADIONUCLIDIC PURITY

Record the gamma-ray spectrum using a suitable instrument calibrated with the aid of standardised cobalt-57 and cobalt-58 solutions. The spectrum does not differ significantly from that of the standardised cobalt-57 solution. Determine the relative amounts of cobalt-57, cobalt-56 and cobalt-58 present. Cobalt-56 has a half-life of 78 days and its presence is shown by gamma photons of energy 0.847 MeV. Cobalt-58 has a half-life of 70.8 days and its presence is shown by gamma photons of energy 0.811 MeV. Not more than 0.1 per cent of the total radioactivity is due to cobalt-56, cobalt-58 and other radionuclidic impurities.

RADIOCHEMICAL PURITY

Examine by liquid chromatography (2.2.29).

Reference solution. Dissolve 10 mg of *cyanocobalamin CRS* in the mobile phase and dilute to 100 ml with the mobile phase. Dilute 2 ml of the solution to 100 ml with the mobile phase. Use within 1 h.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4 mm in internal diameter packed with *octylsilyl silica gel for chromatography R* (5 µm),

- as mobile phase at a flow rate of 1.0 ml/min a mixture prepared as follows: mix 26.5 volumes of *methanol R* and 73.5 volumes of a 10 g/l solution of *disodium hydrogen phosphate R* adjusted to pH 3.5 using *phosphoric acid R* and use within 2 days,
- a radioactivity detector adjusted for cobalt-57,
- as detector a spectrophotometer set at 361 nm,
- a loop injector.

Inject 100 µl of the solution to be examined and record the chromatogram for three times the retention time of cyanocobalamin. Determine the peak areas and calculate the percentage of cobalt-57 present as cyanocobalamin. Inject 100 µl of the reference solution and record the chromatogram for 30 min.

RADIOACTIVITY

Measure the radioactivity using suitable counting equipment by comparison with a standardised cobalt-57 solution.

STORAGE

Store protected from light at a temperature of 2 °C to 8 °C.

01/2005:1505

CYANOCOBALAMIN (^{58}Co) CAPSULES

Cyanocobalamini (^{58}Co) capsulae

DEFINITION

Cyanocobalamin (^{58}Co) capsules contain [^{58}Co]- α -(5,6-dimethylbenzimidazol-1-yl)cobamide cyanide and may contain suitable auxiliary substances. Cobalt-58 is a radioactive isotope of cobalt and may be produced by neutron irradiation of nickel. Cyanocobalamin (^{58}Co) may be prepared by the growth of suitable micro-organisms on a medium containing (^{58}Co) cobaltous ion. Not less than 84 per cent of the cobalt-58 is in the form of cyanocobalamin. The capsules comply with the requirements for hard capsules in the monograph on *Capsules (0016)*, unless otherwise justified and authorised. The average radioactivity is not less than 90.0 per cent and not more than 110.0 per cent of the declared cobalt-58 radioactivity at the date stated on the label.

CHARACTERS

Hard gelatin capsules.

Cobalt-58 has a half-life of 70.9 days and emits beta (β^+) radiation and gamma radiation.

IDENTIFICATION

- Record the gamma-ray spectrum using a suitable instrument. The spectrum does not differ significantly from that of a standardised cobalt-58 solution. Standardised cobalt-58 solutions are available from laboratories recognised by the competent authority. The most prominent gamma photons of cobalt-58 have energies of 0.511 MeV (annihilation radiation) and 0.811 MeV.
- Examine the chromatograms obtained in the test for radiochemical purity. The principal peak in the radiochromatogram obtained with the test solution has a retention time similar to that of the peak in the chromatogram obtained with the reference solution.

TESTS

Disintegration. The capsules comply with the test for disintegration of tablets and capsules (2.9.1) except that one capsule is used in the test instead of six.

Uniformity of content. Determine by measurement in a suitable counting assembly and under identical geometrical conditions the radioactivity of each of not less than ten capsules. Calculate the average radioactivity per capsule. The radioactivity of no capsule differs by more than 10 per cent from the average. The relative standard deviation is less than 3.5 per cent.

RADIONUCLIDIC PURITY

Record the gamma-ray spectrum using a suitable instrument calibrated with the aid of standardised cobalt-58, cobalt-57 and cobalt-60 solutions. The spectrum does not differ significantly from that of the standardised cobalt-58 solution. Standardised cobalt-58, cobalt-57 and cobalt-60 solutions are available from laboratories recognised by the competent authority. Determine the relative amounts of cobalt-58, cobalt-57 and cobalt-60 present. Cobalt-57 has a half-life of 272 days and its presence is shown by gamma photons of energy 0.122 MeV. Cobalt-60 has a half-life of 5.27 years and its presence is shown by gamma photons of energies 1.173 MeV and 1.333 MeV. Not more than 1 per cent of the total radioactivity is due to cobalt-60 and not more than 2 per cent of the total radioactivity is due to cobalt-57, cobalt-60 and other radionuclidic impurities.

RADIOCHEMICAL PURITY

Examine by liquid chromatography (2.2.29).

Test solution. Dissolve the contents of a capsule in 1.0 ml of *water R* and allow to stand for 10 min. Centrifuge at 2000 r/min for 10 min. Use the supernatant.

Reference solution. Dissolve 10 mg of *cyanocobalamin CRS* in the mobile phase and dilute to 100 ml with the mobile phase. Dilute 2 ml of the solution to 100 ml with the mobile phase. Use within 1 h of preparation.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4 mm in internal diameter packed with *octylsilyl silica gel for chromatography R* (5 µm),
- as mobile phase at a flow rate of 1.0 ml/min a mixture prepared as follows: mix 26.5 volumes of *methanol R* and 73.5 volumes of a 10 g/l solution of *disodium hydrogen phosphate R*, adjusted to pH 3.5 with *phosphoric acid R* and use within 2 days,
- a radioactivity detector adjusted for cobalt-58,
- as detector a spectrophotometer set at 361 nm,
- a loop injector.

Inject 100 µl of the test solution and record the chromatogram for three times the retention time of cyanocobalamin. Determine the peak areas and calculate the percentage of cobalt-58 present as cyanocobalamin. Inject 100 µl of the reference solution and record the chromatogram for 30 min.

RADIOACTIVITY

The average radioactivity determined in the test for uniformity of content is not less than 90.0 per cent and not more than 110.0 per cent of the declared cobalt-58 radioactivity, at the date stated on the label.

STORAGE

Store in an airtight container, protected from light, at a temperature of 2 °C to 8 °C.

01/2005:0270

CYANOCOBALAMIN (^{58}Co) SOLUTION**Cyanocobalamini (^{58}Co) solutio****DEFINITION**

Cyanocobalamin (^{58}Co) solution is a solution of [^{58}Co]- α -(5,6-dimethylbenzimidazol-1-yl)cobamide cyanide and may contain a stabiliser and an antimicrobial preservative. Cobalt-58 is a radioactive isotope of cobalt and may be produced by neutron irradiation of nickel. Cyanocobalamin (^{58}Co) may be prepared by the growth of suitable micro-organisms on a medium containing (^{58}Co) cobaltous ion. The solution contains not less than 90.0 per cent and not more than 110.0 per cent of the declared cobalt-58 radioactivity at the date stated on the label. Not less than 90 per cent of the cobalt-58 is in the form of cyanocobalamin.

CHARACTERS

A clear, colourless or slightly pink solution.

Cobalt-58 has a half-life of 70.8 days and emits beta (β^+) radiation and gamma radiation.

IDENTIFICATION

- A. Record the gamma-ray spectrum using a suitable instrument. The spectrum does not differ significantly from that of a standardised cobalt-58 solution. Standardised cobalt-58, cobalt-57 and cobalt-60 solutions are available from laboratories recognised by the competent authority. The most prominent gamma photons of cobalt-58 have energies of 0.511 MeV (annihilation radiation) and 0.811 MeV.
- B. Examine the chromatograms obtained in the test for radiochemical purity. The principal peak in the radiochromatogram obtained with the solution to be examined has a retention time similar to that of the peak in the chromatogram obtained with the reference solution.

TESTS

pH (2.2.3). The pH of the solution is 4.0 to 6.0.

RADIONUCLIDIC PURITY

Record the gamma-ray spectrum using a suitable instrument having adequate resolution and calibrated with the aid of standardised cobalt-58, cobalt-57 and cobalt-60 solutions. The spectrum does not differ significantly from that of the standardised cobalt-58 solution. Determine the relative amounts of cobalt-58, cobalt-57 and cobalt-60 present. Cobalt-57 has a half-life of 271 days and its presence is shown by gamma photons of energy 0.122 MeV. Cobalt-60 has a half-life of 5.27 years and its presence is shown by gamma photons of energies 1.173 MeV and 1.332 MeV. Not more than 1 per cent of the total radioactivity is due to cobalt-60 and not more than 2 per cent of the total radioactivity is due to cobalt-57, cobalt-60 and other radionuclidic impurities.

RADIOCHEMICAL PURITY

Examine by liquid chromatography (2.2.29).

Reference solution. Dissolve 10 mg of *cyanocobalamin CRS* in the mobile phase and dilute to 100 ml with the mobile phase. Dilute 2 ml of the solution to 100 ml with the mobile phase. Use within 1 h.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4 mm in internal diameter packed with *octylsilyl silica gel for chromatography R* (5 μm),

- as mobile phase at a flow rate of 1.0 ml/min a mixture prepared as follows: mix 26.5 volumes of *methanol R* and 73.5 volumes of a 10 g/l solution of *disodium hydrogen phosphate R* adjusted to pH 3.5 using *phosphoric acid R* and use within 2 days,
- a radioactivity detector adjusted for cobalt-58,
- as detector a spectrophotometer set at 361 nm,
- a loop injector.

Inject 100 μl of the solution to be examined and record the chromatogram for three times the retention time of cyanocobalamin. Determine the peak areas and calculate the percentage of cobalt-58 present as cyanocobalamin. Inject 100 μl of the reference solution and record the chromatogram for 30 min.

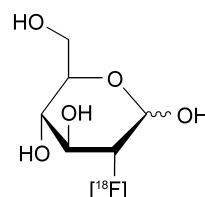
RADIOACTIVITY

Measure the radioactivity using suitable counting equipment by comparison with a standardised cobalt-58 solution or by measurement in an instrument calibrated with the aid of such a solution.

STORAGE

Store protected from light at a temperature of 2 °C to 8 °C.

01/2005:1325

FLUDEOXYGLUCOSE (^{18}F) INJECTION**Fludeoxyglucosi (^{18}F) solutio iniectionis****DEFINITION**

Fludeoxyglucose (^{18}F) injection is a sterile solution of 2- ^{18}F]fluoro-2-deoxy-D-glucopyranose (2- ^{18}F]fluoro-2-deoxy-D-glucose) for diagnostic use. The injection contains not less than 90.0 per cent and not more than 110.0 per cent of the declared fluorine-18 radioactivity at the date and time stated on the label. Not less than 95 per cent of the radioactivity corresponds to fluorine-18 in the form of 2- ^{18}F]fluoro-2-deoxy-D-glucose and 2- ^{18}F]fluoro-2-deoxy-D-mannose, with the 2- ^{18}F]fluoro-2-deoxy-D-mannose fraction not exceeding 10 per cent of the total radioactivity. Not less than 99.0 per cent of the radioactivity corresponds to fluorine-18. The content of 2-fluoro-2-deoxy-D-glucose is not more than 10 mg per maximum recommended dose of injection.

PRODUCTION**RADIONUCLIDE PRODUCTION**

Fluorine-18 is a radioactive isotope of fluorine which may be produced by various nuclear reactions induced by proton irradiation of oxygen-18, deuteron irradiation of neon-20, helium-3 or helium-4 irradiation of oxygen-16.

RADIOCHEMICAL SYNTHESIS

2- ^{18}F]Fluoro-2-deoxy-D-glucose may be prepared by various chemical synthetic pathways, which lead to different products in terms of specific radioactivity, by-products and possible impurities.

Most widely used is the method of phase transfer catalysed nucleophilic substitution of 1,3,4,6-tetra-*O*-acetyl-2-*O*-trifluoromethanesulphonyl- β -D-mannopyranose with [^{18}F]fluoride. Generally, [^{18}F]fluoride is adsorbed on an anion-exchange resin and eluted with a solution of potassium carbonate which is then evaporated to dryness. Addition of a phase transfer catalyst such as an aminopolyether in dry acetonitrile may be used to enhance the nucleophilicity of the [^{18}F]fluoride so that it reacts easily with the tetra-acetylated mannosyltriflate at elevated temperature. Hydrolysis under either alkaline or acidic conditions yields 2-[^{18}F]fluoro-2-deoxy-D-glucose. Hydrolysis using hydrochloric acid may lead to the formation of 2-chloro-2-deoxy-D-glucose. Hydrolysis under alkaline conditions may lead to the formation of 2-[^{18}F]fluoro-2-deoxy-D-mannose as a by-product.

Variations of the method substitute the aminopolyether by a tetra-alkyl ammonium salt, or use solid phase catalysed nucleophilic substitution on derivatised anion-exchange resin, e.g. derivatised with 4-(4-methylpiperidino)pyridine.

Electrophilic pathways for production of 2-[^{18}F]fluoro-2-deoxy-D-glucose proceed by the reaction of molecular [^{18}F]fluorine or [^{18}F]acetylhypofluorite with 3,4,6-tri-*O*-acetyl-D-glucal. [^{18}F]Acetylhypofluorite is obtained by conversion of molecular [^{18}F]fluorine on a solid complex of acetic acid and potassium acetate. The production of molecular [^{18}F]fluorine requires the addition of small amounts of fluorine to the neon target gas, usually from 0.1 per cent to 1 per cent, resulting in the reduction of the specific radioactivity of the end-product. Hydrolysis of the *O*-acetyl protected [^{18}F]fluorinated sugar yields 2-[^{18}F]fluoro-2-deoxy-D-glucose and usually small amounts of 2-[^{18}F]fluoro-2-deoxy-D-mannose.

The preparation can be purified by serial chromatography on combinations of ion-retardation resin, ion-exchange resin, alumina and octadecyl derivatised silica gel. Removal of the phase transfer catalyst can be achieved by different methods, all using combinations of separation cartridges.

Production systems and their performance comply with the requirements set by the competent authority.

STARTING MATERIALS

1. Target materials

Each batch of target material must be tested in special production runs before its use in routine fluorine-18 production and manufacture of the preparation, to ensure that under specified conditions, the target yields fluorine-18 in the desired quantity and quality.

2. Precursors for organic synthesis

It is recommended to test the precursors in production runs before their use for the manufacture of the preparation, to ensure that under specified production conditions, the precursors yield the preparation in the desired quantity and quality.

1,3,4,6-Tetra-*O*-acetyl-2-*O*-trifluoromethanesulphonyl- β -D-mannopyranose. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the *Ph. Eur.* reference spectrum of 1,3,4,6-tetra-*O*-acetyl-2-*O*-trifluoromethanesulphonyl- β -D-mannopyranose.

Melting point (2.2.14): 119 °C to 122 °C.

3,4,6-Tri-*O*-acetyl-D-glucal. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the *Ph. Eur.* reference spectrum of 3,4,6-tri-*O*-acetyl-D-glucal.

Melting point (2.2.14): 53 °C to 55 °C.

CHARACTERS

A clear, colourless or slightly yellow solution.

Fluorine-18 has a half-life of 109.8 min and emits positrons with a maximum energy of 0.633 MeV, followed by annihilation gamma radiation of 0.511 MeV.

IDENTIFICATION

- Record the gamma-ray spectrum using a suitable instrument. The only gamma photons have an energy of 0.511 MeV; and depending on the measurement geometry, a sum peak of 1.022 MeV may be observed.
- It complies with the test for radionuclidic purity (see Tests).
- Examine the chromatograms obtained in test (a) for radiochemical purity. The principal peak in the radiochromatogram obtained with the test solution has approximately the same retention time as the principal peak in the chromatogram obtained with reference solution (b).

TESTS

pH (2.2.3). The pH of the injection is 4.5 to 8.5.

Sterility. It complies with the test for sterility prescribed in the monograph on *Radiopharmaceutical preparations (0125)*. The injection may be released for use before completion of the test.

Bacterial endotoxins (2.6.14): less than 175/V IU/ml, V being the maximum recommended dose in millilitres. The injection may be released for use before completion of the test.

CHEMICAL PURITY

Particular tests for chemical purity may be omitted if the substances mentioned are not used or cannot be formed in the production process

(a) 2-Fluoro-2-deoxy-D-glucose and 2-chloro-2-deoxy-D-glucose. Examine by liquid chromatography (2.2.29).

Test solution. The preparation to be examined.

Reference solution (a). Dissolve 10 mg of glucose R in water R and dilute to 100 ml with the same solvent.

Reference solution (b). Dissolve 10 mg of 2-fluoro-2-deoxy-D-glucose R in water R and dilute to V with the same solvent, V being the maximum recommended dose in millilitres.

Reference solution (c). Dissolve 1.0 mg of 2-chloro-2-deoxy-D-glucose R in water R and dilute to 2.0 ml with the same solvent. Dilute 1 ml of this solution to V with the same solvent, V being the maximum recommended dose in millilitres.

The chromatographic procedure may be carried out using:

- a column 0.25 m long and 4.0 mm in internal diameter packed with *strongly basic anion-exchange resin for chromatography R* (10 μm),
- as mobile phase at a flow rate of 1 ml/min 0.1 M sodium hydroxide protected against contamination by carbon dioxide,
- a suitable radioactivity detector for radiochemical purity testing,
- a detector suitable for carbohydrates in the required concentration range,
- a loop injector,

maintaining the column at a constant temperature between 20 °C and 30 °C.

Equilibrate the column with the mobile phase until a stable baseline is achieved.

Inject separately reference solutions (a), (b) and (c).

If the validation studies exclude the formation of 2-chloro-2-deoxy-D-glucose inject separately reference

solutions (a) and (b). Continue the chromatography for twice the retention time of D-glucose, 2-fluoro-2-deoxy-D-glucose and when required, 2-chloro-2-deoxy-D-glucose respectively.

Inject the test solution. The chromatogram obtained with the detector for carbohydrates shows a principal peak corresponding to D-glucose (test solutions from nucleophilic pathways) or 2-fluoro-2-deoxy-D-glucose (test solutions from electrophilic pathways). When the chromatograms are recorded in the prescribed conditions, 2-chloro-2-deoxy-D-glucose elutes after 2-fluoro-2-deoxy-D-glucose, but their corresponding peaks may not be completely resolved. In the chromatogram obtained with the test solution, the areas of the peaks corresponding to 2-fluoro-2-deoxy-D-glucose and 2-chloro-2-deoxy-D-glucose are not greater than the areas of the peaks in the chromatograms obtained with reference solution (b) and/or reference solution (c) (10 mg of 2-fluoro-2-deoxy-D-glucose per V and 0.5 mg of 2-chloro-2-deoxy-D-glucose per V respectively).

(b) Aminopolyether. This test is performed only on the bulk solution before addition of sodium chloride by the producer and it is not intended for the final preparation to be injected. Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel plate R*.

Test solution. The preparation to be examined.

Reference solution. Dissolve 0.110 g of *aminopolyether R* in *water R* and dilute to 10.0 ml with the same solvent. Dilute 0.2 ml of this solution to V with the same solvent, V being the maximum recommended dose in millilitres.

Apply separately to the plate 2 μl of the test solution and 2 μl of the reference solution. Develop over a path of about 8 cm using a mixture of 1 volume of *ammonia R* and 9 volumes of *methanol R*. Allow the plate to dry in air for 15 min. Expose the plate to iodine vapour for at least 10 min. In the chromatogram obtained with the test solution the spot corresponding to aminopolyether is not more intense than the spot in the chromatogram obtained with the reference solution (2.2 mg per V).

(c) Tetra-alkyl ammonium salts. Examine by liquid chromatography (2.2.29).

Test solution. The preparation to be examined.

Reference solution. Dilute 2.1 ml of 0.1 *M* *tetrabutylammonium hydroxide* to 20 ml with *water R*. Dilute 1 ml of this solution to V with the same solvent, V being the maximum recommended dose in millilitres.

The chromatographic procedure may be carried out using:

- a column 0.125 m long and 4.0 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (5 μm),
 - as mobile phase at a flow rate of 0.6 ml/min a mixture of 25 volumes of a 0.95 g/l solution of *toluenesulphonic acid R* and 75 volumes of *acetonitrile R*,
 - as detector a spectrophotometer set at 254 nm,
 - a loop injector,
- maintaining the column at a constant temperature between 20 °C and 30 °C.

Equilibrate the column with the mobile phase until a stable baseline is obtained.

Inject the reference solution. Continue the chromatography for twice the retention time of tetrabutylammonium ions.

Inject the test solution. In the chromatogram obtained with the test solution, the area of the peak corresponding to tetrabutylammonium ions is not greater than the area of the peak in the chromatogram obtained with the reference solution (2.75 mg per V).

(d) Solid phase derivatisation agent 4-(4-methylpiperidino)pyridine. Examine by ultraviolet spectrophotometry (2.2.25).

Test solution. The preparation to be examined.

Reference solution. Dissolve 20 mg of 4-(4-methylpiperidino)pyridine *R* in *water R* and dilute to 100.0 ml with the same solvent. Dilute 0.1 ml of this solution to V with the same solvent, V being the maximum recommended dose in millilitres.

Measure the absorbance of the test solution and the reference solution at the maximum of 263 nm. The absorbance of the test solution is not greater than that of the reference solution (0.02 mg per V).

(e) Residual solvents (2.4.24). The concentration of acetonitrile does not exceed 4.1 mg per V , V being the maximum recommended dose in millilitres. The injection may be released for use before completion of the test.

RADIONUCLIDIC PURITY

Record the gamma-ray spectrum using a suitable instrument. The half-life is between 105 min and 115 min. The injection may be released for use before completion of the test.

RADIOCHEMICAL PURITY

A. Examine by liquid chromatography (2.2.29) as described in test (a) for chemical purity.

When the chromatograms obtained with the radioactivity detector are recorded in the prescribed conditions, the principal peak in the chromatogram obtained with the test solution has the same retention time as the peak obtained with reference solution (b) using the carbohydrate detector. The retention times of 2- ^{18}F fluoro-2-deoxy-D-mannose and ^{18}F fluoride are approximately 90 per cent and approximately 50 per cent of that of 2- ^{18}F fluoro-2-deoxy-D-glucose respectively. Other peaks in the chromatogram may be due to partially acetylated 2- ^{18}F fluoro-2-deoxy-D-glucose derivatives.

Calculate the percentage content of ^{18}F fluorinated substances from the areas of the peaks in the chromatogram obtained with the test solution. The sum of the percentages of radioactivity corresponding to 2- ^{18}F fluoro-2-deoxy-D-glucose and 2- ^{18}F fluoro-2-deoxy-D-mannose is not less than 95 per cent of the total radioactivity with the 2- ^{18}F fluoro-2-deoxy-D-mannose fraction not exceeding 10 per cent of the total radioactivity.

The method may underestimate or miss unhydrolysed or partially hydrolysed 2- ^{18}F fluoro-2-deoxytetra-acetyl-D-glucose, since these intermediate reaction products may further hydrolyse to the desired end-product under the chromatographic conditions.

B. Examine by thin-layer chromatography (2.2.27) using a *TLC silica gel plate R*.

Test solution. The preparation to be examined.

Apply 2 μl to 10 μl to the plate. Develop over a path of 8 cm using a mixture of 5 volumes of *water R* and 95 volumes of *acetonitrile R*. Allow the plate to dry in air for 15 min. Determine the distribution of radioactivity using a suitable detector. Not less than 95 per cent of the total radioactivity is found in the spot corresponding to 2-fluoro-2-deoxy-D-glucose (R_f about 0.45).

Possible contaminants are ^{18}F fluoride (R_f 0.0); partially acetylated 2- ^{18}F fluoro-2-deoxy-D-glucose derivatives (R_f about 0.8-0.95).

RADIOACTIVITY

Measure the radioactivity using suitable counting equipment by comparison with a standardised fluorine-18 solution or using an instrument calibrated with the aid of such a solution. Standardised fluorine-18 solutions are available from laboratories recognised by the competent authority.

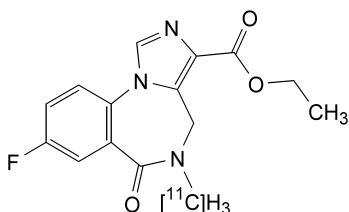
LABELLING

The accompanying information specifies the particular synthetic pathway of production. The label on the actual container states the maximum recommended dose in millilitres.

01/2005:1917

FLUMAZENIL (*N*-[¹¹C]METHYL) INJECTION

Flumazenil (*N*-[¹¹C]methyl)
solutio iniectionis



DEFINITION

Sterile solution of ethyl 8-fluoro-5-[¹¹C]methyl-6-oxo-5,6-dihydro-4*H*-imidazo[1,5-*a*][1,4]benzodiazepine-3-carboxylate which may contain a stabiliser such as ascorbic acid.

Content: 90 per cent to 110 per cent of the declared carbon-11 radioactivity at the date and time stated on the label.

Content of flumazenil: maximum 50 µg in the maximum recommended dose in millilitres.

PRODUCTION

RADIONUCLIDE PRODUCTION

Carbon-11 is a radioactive isotope of carbon which is most commonly produced by proton irradiation of nitrogen. Depending on the addition of either trace amounts of oxygen or small amounts of hydrogen, the radioactivity is obtained as [¹¹C]carbon dioxide or [¹¹C]methane, respectively.

RADIOCHEMICAL SYNTHESIS

[5-Methyl-¹¹C]flumazenil may be prepared by *N*-alkylation of ethyl 8-fluoro-6-oxo-5,6-dihydro-4*H*-imidazo[1,5-*a*][1,4]benzodiazepine-3-carboxylate (demethylflumazenil) with iodo[¹¹C]methane or [¹¹C]methyl trifluoromethanesulphonate.

Synthesis of iodo[¹¹C]methane

Iodo[¹¹C]methane may be produced from [¹¹C]carbon dioxide or from [¹¹C]methane. The most frequently used method is reduction of [¹¹C]carbon dioxide with lithium aluminium hydride. The [¹¹C]methanolate formed is reacted with hydriodic acid. Alternatively [¹¹C]methane, either obtained directly in the target or by on-line processes from [¹¹C]carbon dioxide, is reacted with iodine.

Synthesis of [¹¹C]methyl trifluoromethanesulphonate

[¹¹C]methyl trifluoromethanesulphonate may be prepared from iodo[¹¹C]methane using a solid support such as graphitised carbon, impregnated with silver trifluoromethanesulphonate.

Synthesis of [5-methyl-¹¹C]flumazenil

The most widely used method to obtain [5-methyl-¹¹C]flumazenil is the *N*-alkylation of demethylflumazenil with iodo[¹¹C]methane in alkaline conditions in a solvent such as dimethylformamide or acetone. The resulting [5-methyl-¹¹C]flumazenil can be purified by semi-preparative liquid chromatography. For example, a column packed with octadecylsilyl silica gel for chromatography eluted with a mixture of ethanol and water is suitable.

PRECURSOR FOR SYNTHESIS

Demethylflumazenil

Melting point (2.2.14): 286 °C to 289 °C.

Infrared absorption spectrophotometry (2.2.24).

Comparison: Ph. Eur. reference spectrum of demethylflumazenil.

CHARACTERS

Appearance: clear, colourless solution.

Half-life and nature of radiation of carbon-11: see Table of physical characteristics of radionuclides (5.7).

IDENTIFICATION

A. Gamma-ray spectrometry.

Results: the only gamma photons have an energy of 0.511 MeV and, depending on the measurement geometry, a sum peak of 1.022 MeV may be observed.

B. It complies with test B for radionuclidic purity (see Tests).

C. Examine the chromatograms obtained in the test for radiochemical purity.

Results: the principal peak in the radiochromatogram obtained with the test solution is similar in retention time to the principal peak in the chromatogram obtained with reference solution (a).

TESTS

pH (2.2.3): 6.0 to 8.0.

Sterility. It complies with the test for sterility prescribed in the monograph on *Radiopharmaceutical preparations* (0125). The injection may be released for use before completion of the test.

Bacterial endotoxins (2.6.14): less than 175/V IU/ml, *V* being the maximum recommended dose in millilitres. The injection may be released for use before completion of the test.

Flumazenil and impurity A. Liquid chromatography (2.2.29).

Test solution. The preparation to be examined.

Reference solution (a). Dissolve 2.5 mg of flumazenil *R* in 5 ml of methanol *R*.

Reference solution (b). Dissolve 2.5 mg of demethylflumazenil *R* in 50 ml of methanol *R*.

Reference solution (c). To 0.1 ml of reference solution (a) add 0.1 ml of reference solution (b) and dilute to *V* with a 0.9 g/l solution of sodium chloride *R*, *V* being the maximum recommended dose in millilitres.

Reference solution (d). Dilute 0.1 ml of reference solution (a) to 50 ml with methanol *R*. Dilute 1.0 ml of this solution to *V* with a 0.9 g/l solution of sodium chloride *R*, *V* being the maximum recommended dose in millilitres.

Column:

- *size*: *l* = 0.15 m, Ø = 3.9 mm,
- *stationary phase*: spherical octadecylsilyl silica gel for chromatography *R* (5 µm) with a specific surface area of 440 m²/g, a pore size of 100 nm and a carbon loading of 19 per cent,

- *temperature*: maintain at a constant temperature between 20–30 °C.

Mobile phase: methanol R, water R (45:55 V/V).

Flow rate: 1 ml/min.

Detection: spectrophotometer at 260 nm and radioactivity detector connected in series.

Injection: 100 µl.

Run time: 10 min.

Relative retention with reference to flumazenil: impurity A = about 0.74.

System suitability: reference solution (c):

- *resolution*: minimum 2.5 between the peaks due to flumazenil and impurity A.

Limits: examine the chromatogram obtained with the spectrophotometer:

- *flumazenil*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (c) (50 µg/V),
- *impurity A*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (c) (5 µg/V),
- *any other impurity*: not more than the area of the principal peak in the chromatogram obtained with reference solution (d) (1 µg/V).

Residual solvents are limited according to the principles defined in the general chapter (5.4), using the general method (2.4.24). The preparation may be released for use before completion of the test.

RADIONUCLIDIC PURITY

Carbon-11: minimum 99 per cent of the total radioactivity.

The preparation may be released for use before completion of the test.

A. Gamma-ray spectrometry.

Results: the spectrum obtained with the solution to be examined does not differ significantly from that obtained with a standardised fluorine-18 solution.

B. Half-life: 19.9 min to 20.9 min.

RADIOCHEMICAL PURITY

Liquid chromatography (2.2.29) as described in the test for flumazenil and impurity A, with the following modifications.

Injection: test solution and reference solution (a); if necessary, dilute the test solution to a radioactivity concentration suitable for the detector.

Limit: examine the chromatogram obtained with the radioactivity detector:

- [*5-methyl-¹¹C*]flumazenil: minimum 95 per cent of the total radioactivity.

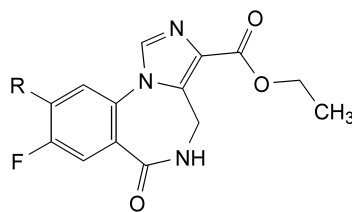
RADIOACTIVITY

Determine the radioactivity using a calibrated instrument.

LABELLING

The label states the maximum recommended dose in millilitres.

IMPURITIES



- A. R = H: ethyl 8-fluoro-6-oxo-5,6-dihydro-4H-imidazo[1,5-a][1,4]benzodiazepine-3-carboxylate (demethylflumazenil),
- B. R = CH₂-CO-CH₃: ethyl 8-fluoro-6-oxo-9-(2-oxopropyl)-5,6-dihydro-4H-imidazo[1,5-a][1,4]benzodiazepine-3-carboxylate (acetone addition compound of demethylflumazenil).

01/2005:0555

GALLIUM (⁶⁷Ga) CITRATE INJECTION

Gallii (⁶⁷Ga) citratis solutio iniectionis

DEFINITION

Gallium (⁶⁷Ga) citrate injection is a sterile solution of gallium-67 in the form of gallium citrate. It may be made isotonic by the addition of sodium chloride and sodium citrate and may contain a suitable antimicrobial preservative such as benzyl alcohol. Gallium-67 is a radioactive isotope of gallium and may be obtained by the irradiation, with protons of suitable energy, of zinc which may be enriched in zinc-68. Gallium-67 may be separated from zinc by solvent extraction or column chromatography. The injection contains not less than 90.0 per cent and not more than 110.0 per cent of the declared gallium-67 radioactivity at the date and hour stated on the label. Not more than 0.2 per cent of the total radioactivity is due to gallium-66.

CHARACTERS

A clear, colourless solution.

Gallium-67 has a half-life of 3.26 days and emits gamma radiation.

IDENTIFICATION

- A. Record the gamma-ray spectrum using a suitable instrument. The spectrum does not differ significantly from that of a standardised gallium-67 solution when measured either by direct comparison or by use of an instrument calibrated with the aid of such a solution. Standardised gallium-67 solutions are available from laboratories recognised by the competent authority. The most prominent gamma photons have energies of 0.093 MeV, 0.185 MeV and 0.300 MeV.
- B. To 0.2 ml of the injection to be examined add 0.2 ml of a solution containing 1 g/l of *ferric chloride R* and 0.1 per cent V/V of *hydrochloric acid R* and mix. Compare the colour with that of a solution containing 9 g/l of *benzyl alcohol R* and 7 g/l of *sodium chloride R* treated in the same manner. A yellow colour develops in the test solution only.

TESTS

pH (2.2.3). The pH of the injection is 5.0 to 8.0.

Zinc. To 0.1 ml of the injection to be examined add 0.9 ml of *water R*, 5 ml of *acetate buffer solution pH 4.7 R*, 1 ml of a 250 g/l solution of *sodium thiosulphate R* and 5.0 ml of a dithizone solution prepared as follows: dissolve 10 mg

of *dithizone R* in 100 ml of *methyl ethyl ketone R* allow to stand for 5 min, filter and immediately before use dilute the solution to ten times its volume with *methyl ethyl ketone R*. Shake vigorously for 2 min and separate the organic layer. Measure the absorbance (2.2.25) of the organic layer at 530 nm, using the organic layer of a blank solution as the compensation liquid. The absorbance is not greater than that of the organic layer obtained with 0.1 ml of *zinc standard solution (5 ppm Zn) R* treated in the same manner.

Sterility. It complies with the test for sterility prescribed in the monograph on *Radiopharmaceutical preparations (0125)*. The injection may be released for use before completion of the test.

RADIONUCLIDIC PURITY

Record the gamma-ray spectrum using a suitable instrument. The spectrum does not differ significantly from that of a standardised gallium-67 solution, apart from any differences attributable to the presence of gallium-66. Gallium-66 has a half-life of 9.4 h and its most prominent gamma photon has an energy of 1.039 MeV. Not more than 0.2 per cent of the total radioactivity is due to gallium-66.

RADIOACTIVITY

Measure the radioactivity using suitable counting equipment by comparison with a standardised gallium-67 solution or by measurement in an instrument calibrated with the aid of such a solution.

01/2005:1922

HUMAN ALBUMIN INJECTION, IODINATED (¹²⁵I)

Iodinati (¹²⁵I) humani albumini solutio iniectabilis

DEFINITION

Sterile, endotoxin-free solution of human albumin labelled with iodine-125. It may contain a suitable buffer and an antimicrobial preservative. The human albumin used complies with the requirements of the monograph on *Human albumin solution (0255)*.

Content: 90 per cent to 110 per cent of the declared iodine-125 radioactivity at the date stated on the label.

Purity:

- minimum of 99.0 per cent of the total radioactivity corresponds to iodine-125,
- minimum of 80 per cent of the total radioactivity is associated with the albumin fractions II to V,
- maximum of 5 per cent of the total radioactivity corresponds to unbound iodide.

Content of albumin: 95 per cent to 105 per cent of the declared albumin content stated on the label.

CHARACTERS

Appearance: clear, colourless to yellowish solution.

Half-life and nature of radiation of iodine-125: see Table of physical characteristics of radionuclides (5.7).

IDENTIFICATION

A. Gamma-ray and X-ray spectrometry.

Comparison: standardised iodine-125 solution, or by using a calibrated instrument. Standardised iodine-125 solutions and/or standardisation services are available from the competent authority.

Results: the spectrum obtained with the preparation to be examined does not differ significantly from that obtained with a standardised iodine-125 solution, apart from any differences attributable to the presence of iodine-126. The most prominent photon has an energy of 0.027 MeV, corresponding to the characteristic X-ray of tellurium, gamma photons of an energy of 0.035 MeV are also present. Iodine-126 has a half-life of 13.11 days and its most prominent gamma photons have energies of 0.388 MeV and 0.666 MeV.

B. Examine by a suitable immunoelectrophoresis technique (2.7.1). Using antiserum to normal human serum, compare normal human serum and the preparation to be examined, both diluted if necessary. The main component of the preparation to be examined corresponds to the main component of the normal human serum. The diluted solution may show the presence of small quantities of other plasma proteins.

TESTS

pH (2.2.3): 5.0 to 9.0.

Albumin

Reference solution. Dilute *human albumin solution R* with a 9 g/l solution of *sodium chloride R* to a concentration of 5 mg of albumin per millilitre.

To 1.0 ml of the preparation to be examined and to 1.0 ml of the reference solution add 4.0 ml of *biuret reagent R* and mix. After exactly 30 min, measure the absorbance (2.2.25) of each solution at 540 nm, using as the compensation liquid a 9 g/l solution of *sodium chloride R* treated in the same manner. From the absorbances measured, calculate the content of albumin in the injection to be examined in milligrams per millilitre.

Sterility. It complies with the test for sterility prescribed in the monograph on *Radiopharmaceutical preparations (0125)*.

Bacterial endotoxins (2.6.14): less than 175/V IU/ml, V being the maximum recommended dose in millilitres.

RADIONUCLIDIC PURITY

Iodine-125: minimum 99.0 per cent of the total radioactivity. Gamma-ray and X-ray spectroscopy.

Comparison: standardised solution of iodine-125.

Determine the relative amounts of iodine-125 and iodine-126 present.

RADIOCHEMICAL PURITY

Iodine-125 in albumin fractions II to V, iodine-125 corresponding to unbound iodide. Size-exclusion chromatography (2.2.30).

Test solution. Mix 0.25 ml of the preparation to be examined with 0.25 ml of the mobile phase. Use immediately after mixing.

Reference solution. *Human albumin solution R* or another appropriate human albumin standard diluted with the mobile phase to a suitable albumin concentration.

Column:

- size: $l = 0.6$ m, $\varnothing = 7.5$ mm,
- stationary phase: silica gel for size-exclusion chromatography R,
- temperature: 25 °C.

Mobile phase: dissolve 11.24 g of *potassium dihydrogen phosphate R*, 42.0 g of *disodium hydrogen phosphate R*, 11.70 g of *sodium chloride R* in 2000 ml of *water R*.

Flow rate: 0.6 ml/min.

Detection: spectrophotometer at 280 nm and radioactivity detector set for iodine-125 connected in series.

Injection: loop injector.

Run time: 85 min.

Retention times:

Peak No.	Fraction	Description of the compound	Retention time (min)
1	I	High molecular mass compound	18 - 20
2	II	Poly III albumin	23 - 24
3	III	Poly II albumin	25 - 26
4	IV	Poly I albumin	28
5	V	Human serum albumin	29 - 31
6	VI	Iodide	43 - 45

The main peak in the chromatogram obtained with the reference solution corresponds to fraction V.

Limits:

- *radioactivity in fractions II to V:* minimum 80 per cent of the total radioactivity applied to the column,
- *iodine-125 in fraction VI:* maximum 5 per cent of the total radioactivity.

RADIOACTIVITY

Measure the radioactivity using suitable equipment by comparison with a standardised iodine-125 solution or by measurement with a calibrated instrument.

LABELLING

The label states:

- the amount of albumin,
- the maximum volume to be injected.

01/2005:1227

INDIUM (¹¹¹In) CHLORIDE SOLUTION

Indii (¹¹¹In) chloridi solutio

DEFINITION

Indium (¹¹¹In) chloride solution is a sterile solution of indium-111 as the chloride in aqueous hydrochloric acid containing no additives. Indium-111 is a radioactive isotope of indium and may be produced by the irradiation of cadmium with protons of suitable energy. The solution contains not less than 90.0 per cent and not more than 110.0 per cent of the declared indium-111 radioactivity at the date and hour stated on the label. Not more than 0.25 per cent of the total radioactivity is due to radionuclides other than indium-111. Not less than 95 per cent of the radioactivity corresponds to indium-111 in the form of ionic indium(III). The method of preparation is such that no carrier is added and the specific radioactivity is not less than 1.85 GBq of indium-111 per microgram of indium.

CHARACTERS

A clear, colourless solution.

Indium-111 has a half-life of 2.8 days and emits gamma radiation and X-rays.

IDENTIFICATION

- Carry out the test after allowing sufficient time for short-lived impurities such as indium-110m to decay. Record the gamma-ray and X-ray spectrum using a suitable instrument. The spectrum does not differ significantly from that of a standardised indium-111 solution apart from any differences due to the presence of indium-114m, when measured either by direct comparison or by using an instrument calibrated with the aid of such a solution. Standardised indium-111 and indium-114m solutions are available from laboratories recognised by the competent authority. The most prominent gamma photons of indium-111 have energies of 0.171 MeV and 0.245 MeV.
- To 100 µl of *silver nitrate solution R2* add 50 µl of the solution. A white precipitate is formed.
- It complies with the test for pH (see Tests).
- Examine the chromatogram obtained in the test for radiochemical purity. The principal peak has an *R_f* value of 0.5 to 0.8.

TESTS

pH (2.2.3). The pH of the solution is 1.0 to 2.0.

Cadmium. Not more than 0.40 µg/ml, determined by electrothermal atomic absorption spectrometry (2.2.23, *Method I*).

Test solution. Dilute 0.05 ml of the solution to be examined to a suitable volume with a suitable concentration of *hydrochloric acid R*.

Reference solutions. Prepare the reference solutions using *cadmium standard solution (0.1 per cent Cd) R*, diluted as necessary with the same concentration of *hydrochloric acid R* as in the solution to be examined.

Measure the absorbance at 228.8 nm using a cadmium hollow-cathode lamp as source of radiation.

Copper. Not more than 0.15 µg/ml, determined by electrothermal atomic absorption spectrometry (2.2.23, *Method I*).

Test solution. Dilute 0.1 ml the solution to be examined to a suitable volume with a suitable concentration of *hydrochloric acid R*.

Reference solutions. Prepare the reference solutions using *copper standard solution (0.1 per cent) R* diluted as necessary with the same concentration of *hydrochloric acid R* as the solution to be examined.

Measure the absorbance at 324.8 nm using a copper hollow-cathode lamp as source of radiation.

Iron. Not more than 0.60 µg/ml, determined by electrothermal atomic absorption spectrometry (2.2.23, *Method I*).

Test solution. Dilute 0.1 ml of the solution to be examined to a suitable volume with a suitable concentration of *hydrochloric acid R*.

Reference solutions. Prepare the reference solutions using *iron standard solution (0.1 per cent Fe) R* diluted as necessary with the same concentration of *hydrochloric acid R* as the solution to be examined.

Measure the absorbance at 248.3 nm using an iron hollow-cathode lamp as source of radiation.

Sterility. It complies with the test for sterility prescribed in the monograph on *Radiopharmaceutical preparations (0125)*. The solution may be released for use before completion of the test.

RADIONUCLIDIC PURITY

Record the gamma-ray and X-ray spectrum using a suitable instrument. The spectrum does not differ significantly from that of a standardised solution of indium-111 apart from any differences due to the presence of indium-114m.

Indium-114m. Carry out the test after allowing sufficient time for short-lived impurities such as indium-110m to decay. Take a volume equivalent to 30 MBq and record the gamma-ray spectrum using a suitable detector with a shield of lead, 6 mm thick, placed between the sample and the detector. The response in the region corresponding to the 0.558 MeV photon and the 0.725 MeV photon of indium-114m does not exceed that obtained using 75 kBq of a standardised solution of indium-114m (0.25 per cent) measured under the same conditions, when all measurements are calculated with reference to the date and hour of administration. Standardised indium-111 and indium-114m solutions are available from laboratories recognised by the competent authority.

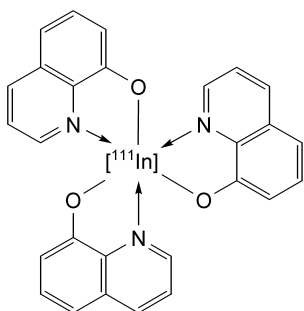
RADIOCHEMICAL PURITY

Examine by thin-layer chromatography (2.2.27) using silica gel as the coating substance on a glass-fibre sheet. Apply to the plate 5 μl of the solution to be examined. Develop immediately over a path of 15 cm using a 9.0 g/l solution of *sodium chloride R* adjusted to $\text{pH } 2.3 \pm 0.05$ with *dilute hydrochloric acid R*. Allow the plate to dry in a current of cold air. Determine the distribution of radioactivity using a suitable detector. Indium-111 chloride migrates with an R_f value of 0.5 to 0.8. Not less than 95 per cent of the total radioactivity of the chromatogram corresponds to indium-111 chloride.

RADIOACTIVITY

Measure the radioactivity using suitable counting equipment by comparison with a standardised indium-111 solution or by measurement in an instrument calibrated with the aid of such a solution.

01/2005:1109

INDIUM (^{111}In) OXINE SOLUTIONIndii (^{111}In) oxini solutio $\text{C}_{27}\text{H}_{18}[^{111}\text{In}]\text{N}_3\text{O}_3$ M_r 547.2**DEFINITION**

Indium (^{111}In) oxine solution is a sterile solution of indium-111 in the form of a complex with 8-hydroxyquinoline. It may contain suitable surface active agents and may be made iso-tonic by the addition of sodium chloride and a suitable buffer. Indium-111 is a radioactive isotope of indium and may be produced by the irradiation of cadmium with protons of suitable energy. The solution contains not less than 90.0 per cent and not more than 110.0 per cent of the declared indium-111 radioactivity at the date and hour stated on the

label. Not more than 0.25 per cent of the total radioactivity is due to radionuclides other than indium-111. Not less than 90 per cent of the radioactivity corresponds to indium-111 complexed with oxine. The method of preparation is such that no carrier is added and the specific radioactivity is not less than 1.85 GBq of indium-111 per microgram of indium.

CHARACTERS

A clear, colourless solution.

Indium-111 has a half-life of 2.8 days and emits gamma radiation and X-rays.

IDENTIFICATION

- Carry out the test after allowing sufficient time for short-lived impurities such as indium-110m to decay. Record the gamma-ray and X-ray spectrum using a suitable instrument. The spectrum does not differ significantly from that of a standardised indium-111 solution apart from any differences due to the presence of indium-114m, when measured either by direct comparison or by using an instrument calibrated with the aid of such a solution. Standardised indium-111 and indium-114m solutions are available from laboratories recognised by the competent authority. The most prominent gamma photons of indium-111 have energies of 0.171 MeV and 0.245 MeV.
- Place 5 mg to 10 mg of *magnesium oxide R* in a glass container of approximately 20 mm in internal diameter. Add 20 μl of the solution to be examined. Examine in ultraviolet light at 365 nm. Bright yellow fluorescence is produced.
- The distribution of radioactivity between the organic and aqueous phases in the test for radiochemical purity contributes to the identification of the preparation.

TESTS

pH (2.2.3). The pH of the solution is 6.0 to 7.5.

Sterility. It complies with the test for sterility prescribed in the monograph on *Radiopharmaceutical preparations* (0125). The solution may be released for use before completion of the test.

RADIONUCLIDIC PURITY

Record the gamma-ray and X-ray spectrum using a suitable instrument. The spectrum does not differ significantly from that of a standardised solution of indium-111, apart from any differences due to the presence of indium-114m.

Indium-114m. Carry out the test after allowing sufficient time for short-lived impurities such as indium-110m to decay. Take a volume equivalent to 30 MBq and record the gamma-ray spectrum using a suitable detector with a shield of lead, 6 mm thick, placed between the sample and the detector. The response in the region corresponding to the 0.558 MeV photon and the 0.725 MeV photon of indium-114m does not exceed that obtained using 75 kBq of a standardised solution of indium-114m (0.25 per cent) measured under the same conditions, when all measurements are calculated with reference to the date and hour of administration. (It should be noted that indium (^{111}In) oxine solution is a precursor used in the *in vitro* labelling of white blood cells or platelets prior to their re-injection into the patient. It is not intended for direct administration). Standardised indium-111 and indium-114m solutions are available from laboratories recognised by the competent authority.

RADIOCHEMICAL PURITY

To a silanised separating funnel containing 3 ml of a 9 g/l solution of *sodium chloride R* add 100 μl of the solution to be examined and mix. Add 6 ml of *octanol R* and shake

vigorously. Allow the phases to separate and then run the lower layer into a suitable vial for counting. Allow the upper layer to drain completely into a similar vial. Add 1 ml of *octanol R* to the funnel, shake vigorously and drain into the vial containing the organic fraction. Add 5 ml of *dilute hydrochloric acid R* to the funnel, shake vigorously and drain these rinsings into a third vial. Seal each vial and, using a suitable instrument, measure the radioactivity in each. Calculate the radiochemical purity by expressing the radioactivity of the indium-111 oxine complex, found in the organic phase, as a percentage of the radioactivity measured in the three solutions. Not less than 90 per cent of the radioactivity corresponds to indium-111 complexed with oxine.

RADIOACTIVITY

Measure the radioactivity using suitable counting equipment by comparison with a standardised indium-111 solution or by measurement in an instrument calibrated with the aid of such a solution.

01/2005:0670

INDIUM (¹¹¹In) PENTETATE INJECTION

Indii (¹¹¹In) pentetatis solutio iniectionabilis

DEFINITION

Indium (¹¹¹In) pentetate injection is a sterile and apyrogenic solution containing indium-111 in the form of indium diethylenetriaminepenta-acetate. It may contain calcium and may be made isotonic by the addition of sodium chloride and a suitable buffer. Indium-111 is a radioactive isotope of indium which may be obtained by proton irradiation, of appropriate energy, of cadmium which may be enriched with cadmium-111 or cadmium-112. The injection contains not less than 90 per cent and not more than 110 per cent of the declared indium-111 radioactivity at the date and hour stated on the label. The radioactivity due to indium-114m is not greater than 0.2 per cent of the total radioactivity at the date and hour of administration. Not less than 95 per cent of the radioactivity corresponds to indium-111 complexed with pentetate.

CHARACTERS

A clear, colourless solution.

Indium-111 has a half-life of 2.8 days and emits gamma radiation and X-rays.

IDENTIFICATION

- Record the gamma-ray and X-ray spectrum using a suitable instrument. The spectrum does not differ significantly from that of a standardised indium-111 solution apart from any differences due to the presence of indium-114m, when measured either by direct comparison or by using an instrument calibrated with the aid of such a solution. Standardised indium-111 and indium-114m solutions are available from laboratories recognised by the competent authority. The most prominent gamma photons of indium-111 have energies of 0.171 MeV and 0.245 MeV.
- Examine the chromatogram obtained in the test for radiochemical purity. The distribution of radioactivity contributes to the identification of the preparation.

TESTS

pH (2.2.3). The pH of the injection is 7.0 to 8.0.

Cadmium. Not more than 5 µg of Cd per millilitre, determined by atomic absorption spectrometry (2.2.23, *Method II*).

Test solution. Mix 0.1 ml of the injection to be examined with 0.9 ml of a mixture of 1 volume of *hydrochloric acid R* and 99 volumes of *water R*.

Reference solutions. Prepare the reference solutions using *cadmium standard solution (0.1 per cent Cd) R* and diluting with a mixture of 1 volume of *hydrochloric acid R* and 99 volumes of *water R*.

Measure the absorbance at 228.8 nm using a cadmium hollow-cathode lamp as source of radiation and an air-acetylene flame.

Uncomplexed diethylenetriaminepenta-acetic acid.

In a micro test-tube, mix 100 µl of the injection to be examined with 100 µl of a freshly prepared 1 g/l solution of *hydroxynaphthol blue, sodium salt R* in 1 M *sodium hydroxide*. Add 50 µl of a 0.15 g/l solution of *calcium chloride R*. The solution remains pinkish-violet or changes from blue to pinkish-violet (0.4 mg/ml).

Sterility. It complies with the test for sterility prescribed in the monograph on *Radiopharmaceutical preparations (0125)*. The injection may be released for use before completion of the test.

Bacterial endotoxins (2.6.14): less than 14/V IU/ml, V being the maximum recommended dose in millilitres.

RADIONUCLIDIC PURITY

Record the gamma-ray and X-ray spectrum using a suitable instrument. The spectrum does not differ significantly from that of a standardised solution of indium-111 apart from any differences due to the presence of indium-114m.

Indium-114m. Retain a sample of the injection to be examined for a sufficient time to allow the indium-111 radioactivity to decay to a sufficiently low level to permit the detection of radionuclidic impurities. Record the gamma-ray spectrum of the decayed material in a suitable instrument calibrated with the aid of a standardised indium-114m solution. Indium-114m has a half-life of 49.5 days and its most prominent gamma photon has an energy of 0.190 MeV. The radioactivity due to indium-114m is not greater than 0.2 per cent of the total radioactivity at the date and hour of administration.

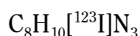
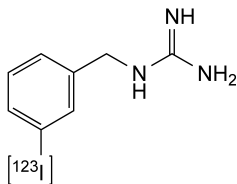
RADIOCHEMICAL PURITY

Examine by thin-layer chromatography (2.2.27) using silica gel as the coating substance on a glass-fibre sheet. Heat the plate at 110 °C for 10 min. Use a plate such that during development the mobile phase migrates over a distance of 10 cm to 15 cm in about 10 min.

Apply to the plate 5 µl to 10 µl of the injection to be examined and allow to dry. Develop over a path of 10 cm to 15 cm using a 9 g/l solution of *sodium chloride R*. Allow the plate to dry in air. Determine the distribution of radioactivity using a suitable detector. Indium pentetate complex migrates near to the solvent front. The radioactivity of the spot corresponding to indium pentetate complex represents not less than 95 per cent of the total radioactivity of the chromatogram.

RADIOACTIVITY

Measure the radioactivity using suitable counting equipment by comparison with a standardised indium-111 solution or by measurement in an instrument calibrated with the aid of such a solution.

01/2005:1113
corrected**IOBENGUANE (^{123}I) INJECTION****Iobenguani (^{123}I) solutio iniectionis****DEFINITION**

Iobenguane (^{123}I) injection is a sterile, bacterial-endotoxin free solution of 1-(3- ^{123}I iodobenzyl)guanidine or its salts. It may contain a suitable buffer, a suitable labelling catalyst such as ionic copper, a suitable labelling stabiliser such as ascorbic acid and antimicrobial preservatives. Iodine-123 is a radioactive isotope of iodine and may be obtained by proton irradiation of xenon enriched in xenon-124 (not less than 98 per cent) followed by the decay of caesium-123 formed via xenon-123. The injection contains not less than 90.0 per cent and not more than 110.0 per cent of the declared iodine-123 radioactivity at the date and hour stated on the label. Not less than 95 per cent of the radioactivity corresponds to iodine-123 in the form of iobenguane. The specific radioactivity is not less than 10 GBq of iodine-123 per gram of iobenguane base. Not more than 0.35 per cent of the total radioactivity is due to radionuclides other than iodine-123.

CHARACTERS

A clear, colourless or slightly yellow solution.

Iodine-123 has a half-life of 13.2 h and emits gamma radiation and X-rays.

IDENTIFICATION

- A. Record the gamma-ray and X-ray spectrum using a suitable instrument. The spectrum does not differ significantly from that of a standardised iodine-123 solution apart from any differences attributable to the presence of iodine-125, tellurium-121 and other radionuclidic impurities. The most prominent gamma photon of iodine-123 has an energy of 0.159 MeV. Iodine-125 has a half-life of 59.4 days and emits an X-ray of 0.027 MeV and a photon of 0.035 MeV. Tellurium-121 has a half-life of 19.2 days and the most prominent photons have energies of 0.507 MeV and 0.573 MeV. Standardised iodine-123, iodine-125 and tellurium-121 solutions are available from laboratories recognised by the competent authority.
- B. Examine the chromatogram obtained in the test for radiochemical purity. The distribution of the radioactivity contributes to the identification of the preparation.

TESTS

pH (2.2.3). The pH of the solution is 3.5 to 8.0.

Specific radioactivity. The specific radioactivity is calculated from the results obtained in the test for radiochemical purity. Determine the content of iobenguane sulphate from the areas of the peaks corresponding to iobenguane in the

chromatograms obtained with the test solution and reference solution (b). Calculate the concentration as iobenguane base by multiplying the result obtained in the assay by 0.85.

Sterility. It complies with the test for sterility prescribed in the monograph on *Radiopharmaceutical preparations* (0125). The injection may be released for use before completion of the test.

Bacterial endotoxins (2.6.14): less than 175/ V IU/ml, V being the maximum recommended dose in millilitres.

RADIONUCLIDIC PURITY

Record the gamma-ray spectrum using a suitable instrument. Determine the relative amounts of iodine-125, tellurium-121 and other radionuclidic impurities present. No radionuclides with longer half-lives than iodine-125 are detected. For the determination of iodine-125, tellurium-121 and other radionuclidic impurities, retain the solution to be examined for a sufficient time to allow the radioactivity of iodine-123 to decrease to a level which permits the detection of radionuclidic impurities. Record the gamma-ray spectrum and the X-ray spectrum of the decayed material using a suitable instrument. Not more than 0.35 per cent of the total radioactivity is due to radionuclides other than iodine-123. The injection may be released for use before completion of the test.

RADIOCHEMICAL PURITY

Examine by liquid chromatography (2.2.29).

Test solution. The injection to be examined.

Reference solution (a). Dissolve 0.100 g of *sodium iodide R* in the mobile phase and dilute to 100 ml with the mobile phase.

Reference solution (b). Dissolve 20.0 mg of *iobenguane sulphate CRS* in 50 ml of the mobile phase and dilute to 100.0 ml with the mobile phase.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4.0 mm in internal diameter packed with *silica gel for chromatography R* (5 μm),
- as mobile phase at a flow rate of 1.0 ml/min a mixture of 1 volume of an 80 g/l solution of *ammonium nitrate R*, 2 volumes of *dilute ammonia R2* and 27 volumes of *methanol R*,
- a suitable radioactivity detector,
- a spectrophotometer set at 254 nm and provided with a flow-cell,
- a 10 μl loop injector.

Inject the test solution and the reference solutions. Not less than 95 per cent of the radioactivity of the chromatogram is found in the peak corresponding to iobenguane. Not more than 4 per cent of the radioactivity is found in the peak corresponding to iodide and not more than 1 per cent of the radioactivity is found in other peaks.

RADIOACTIVITY

Measure the radioactivity using a suitable counting apparatus by comparison with a standardised iodine-123 solution or by measurement in an instrument calibrated with the aid of such a solution.

STORAGE

Store protected from light.

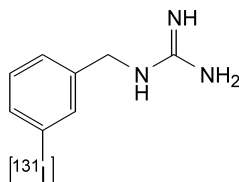
LABELLING

The label states the specific radioactivity expressed in GBq of iodine-123 per gram of iobenguane base.

01/2005:1111

IOBENGUANE (^{131}I) INJECTION FOR DIAGNOSTIC USE

Iobenguani (^{131}I) solutio iniectionis
ad usum diagnosticum


 $\text{C}_8\text{H}_{10}[\text{}^{131}\text{I}]\text{N}_3$

DEFINITION

Iobenguane (^{131}I) injection for diagnostic use is a sterile, bacterial endotoxin-free solution of 1-(3- $[\text{}^{131}\text{I}]$ iodobenzyl)guanidine or its salts. It may contain a suitable buffer. It may also contain a suitable labelling catalyst such as ionic copper and a suitable labelling stabiliser such as ascorbic acid. It may contain antimicrobial preservatives. Iodine-131 is a radioactive isotope of iodine and may be obtained by neutron irradiation of tellurium or by extraction of uranium fission products. The injection contains not less than 90.0 per cent and not more than 110.0 per cent of the declared iodine-131 radioactivity at the date and hour stated on the label. Not less than 94 per cent of the radioactivity corresponds to iodine-131 in the form of iobenguane. The specific radioactivity is not less than 20 GBq of iodine-131 per gram of iobenguane base.

CHARACTERS

A clear, colourless or slightly yellow solution.

Iodine-131 has a half-life of 8.04 days and emits beta and gamma radiation.

IDENTIFICATION

- Record the gamma-ray spectrum using a suitable instrument. The spectrum does not differ significantly from that of a standardised iodine-131 solution by direct comparison with such a solution. Standardised iodine-131 solutions are available from laboratories recognised by the competent authority. The most prominent gamma photon of iodine-131 has an energy of 0.365 MeV.
- Examine the chromatogram obtained in the test for radiochemical purity. The distribution of the radioactivity contributes to the identification of the preparation.

TESTS

pH (2.2.3). The pH of the solution is 3.5 to 8.0.

Specific radioactivity. The specific radioactivity is calculated from the results obtained in the test for radiochemical purity. Determine the content of iobenguane sulphate from the areas of the peaks corresponding to iobenguane in the chromatograms obtained with the test solution and reference solution (b). Calculate the concentration as iobenguane base by multiplying the result obtained in the assay by 0.85.

Sterility. It complies with the test for sterility prescribed in the monograph on *Radiopharmaceutical preparations* (0125). The injection may be released for use before completion of the test.

Bacterial endotoxins (2.6.14): less than 175/V IU/ml, V being the maximum recommended dose in millilitres.

RADIONUCLIDIC PURITY

Record the gamma-ray spectrum using a suitable instrument. The spectrum does not differ significantly from that of a standardised iodine-131 solution. Determine the relative amounts of iodine-131, iodine-133, iodine-135 and other radionuclidic impurities present. Iodine-133 has a half-life of 20.8 h and its most prominent gamma photons have energies of 0.530 MeV and 0.875 MeV. Iodine-135 has a half-life of 6.55 h and its most prominent gamma photons have energies of 0.527 MeV, 1.132 MeV and 1.260 MeV. Not less than 99.9 per cent of the total radioactivity is due to iodine-131.

RADIOCHEMICAL PURITY

Examine by liquid chromatography (2.2.29).

Test solution. The injection to be examined.

Reference solution (a). Dissolve 0.100 g of sodium iodide R in the mobile phase and dilute to 100 ml with the mobile phase.

Reference solution (b). Dissolve 20.0 mg of iobenguane sulphate CRS in 50 ml of the mobile phase and dilute to 100.0 ml with the mobile phase.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4.0 mm in internal diameter packed with silica gel for chromatography R (5 μm),
- as mobile phase at a flow rate of 1.0 ml/min a mixture of 1 volume of an 80 g/l solution of ammonium nitrate R, 2 volumes of dilute ammonia R2 and 27 volumes of methanol R,
- a suitable radioactivity detector,
- a spectrophotometer set at 254 nm and provided with a flow-cell,
- a 10 μl loop injector.

Inject the test solution and the reference solutions. Not less than 94 per cent of the radioactivity of the chromatogram is found in the peak corresponding to iobenguane. Not more than 5 per cent of the radioactivity is found in the peak corresponding to iodide and not more than 1 per cent of the radioactivity is found in other peaks.

RADIOACTIVITY

Measure the radioactivity using a suitable counting apparatus by comparison with a standardised iodine-131 solution or by measurement in an instrument calibrated with the aid of such a solution.

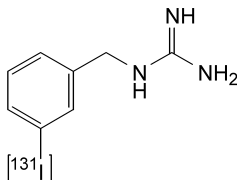
STORAGE

Store protected from light.

LABELLING

The label states the specific radioactivity expressed in gigabecquerels of iodine-131 per gram of iobenguane base.

01/2005:1112 RADIONUCLIDIC PURITY

IOBENGUANE (¹³¹I) INJECTION FOR THERAPEUTIC USEIobenguani (¹³¹I) solutio iniectionis ad usum therapeuticumC₈H₁₀ [¹³¹I] N₃**DEFINITION**

Iobenguane (¹³¹I) injection for therapeutic use is a sterile, bacterial endotoxin-free solution of 1-(3-[¹³¹I]iodobenzyl)guanidine or its salts. It may contain a suitable buffer, a suitable labelling catalyst such as ionic copper, a suitable labelling stabiliser such as ascorbic acid and antimicrobial preservatives. Iodine-131 is a radioactive isotope of iodine and may be obtained by neutron irradiation of tellurium or by extraction of uranium fission products. The injection contains not less than 90.0 per cent and not more than 110.0 per cent of the declared iodine-131 radioactivity at the date and hour stated on the label. Not less than 92 per cent of the radioactivity corresponds to iodine-131 in the form of iobenguane. The specific radioactivity is not less than 400 GBq of iodine-131 per gram of iobenguane base.

CHARACTERS

A clear, colourless or slightly yellow solution.

Iodine-131 has a half-life of 8.04 days and emits beta and gamma radiation.

IDENTIFICATION

- A. Record the gamma-ray spectrum using a suitable instrument. The spectrum does not differ significantly from that of a standardised iodine-131 solution by direct comparison with such a solution. Standardised iodine-131 solutions are available from laboratories recognised by the competent authority. The most prominent gamma photon of iodine-131 has an energy of 0.365 MeV.
- B. Examine the chromatogram obtained in the test for radio-chemical purity. The distribution of the radioactivity contributes to the identification of the preparation.

TESTS

pH (2.2.3). The pH of the solution is 3.5 to 8.0.

Specific radioactivity. The specific radioactivity is calculated from the results obtained in the test for radiochemical purity. Determine the content of iobenguane sulphate from the areas of the peaks corresponding to iobenguane in the chromatograms obtained with the test solution and reference solution (b). Calculate the concentration as iobenguane base by multiplying the result obtained in the assay by 0.85.

Sterility. It complies with the test for sterility prescribed in the monograph on *Radiopharmaceutical preparations* (0125). The injection may be released for use before completion of the test.

Bacterial endotoxins (2.6.14): less than 175/V IU/ml, V being the maximum recommended dose in millilitres.

Record the gamma-ray spectrum using a suitable instrument. The spectrum does not differ significantly from that of a standardised iodine-131 solution. Determine the relative amounts of iodine-131, iodine-133, iodine-135 and other radionuclidic impurities present. Iodine-133 has a half-life of 20.8 h and its most prominent gamma photons have energies of 0.530 MeV and 0.875 MeV. Iodine-135 has a half-life of 6.55 h and its most prominent gamma photons have energies of 0.527 MeV, 1.132 MeV and 1.260 MeV. Not less than 99.9 per cent of the total radioactivity is due to iodine-131.

RADIOCHEMICAL PURITY

Examine by liquid chromatography (2.2.29).

Test solution. The injection to be examined.

Reference solution (a). Dissolve 0.100 g of *sodium iodide R* in the mobile phase and dilute to 100 ml with the mobile phase.

Reference solution (b). Dissolve 20.0 mg of *iobenguane sulphate CRS* in 50 ml of the mobile phase and dilute to 100.0 ml with the mobile phase.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4.0 mm in internal diameter packed with *silica gel for chromatography R* (5 µm),
- as mobile phase at a flow rate of 1.0 ml/min a mixture of 1 volume of an 80 g/l solution of *ammonium nitrate R*, 2 volumes of *dilute ammonia R2* and 27 volumes of *methanol R*,
- a suitable radioactivity detector,
- a spectrophotometer set at 254 nm and provided with a flow-cell,
- a 10 µl loop injector.

Inject the test solution and the reference solutions. Not less than 92 per cent of the radioactivity of the chromatogram is found in the peak corresponding to iobenguane. Not more than 7 per cent of the radioactivity is found in the peak corresponding to iodide and not more than 1 per cent of the radioactivity is found in other peaks.

RADIOACTIVITY

Measure the radioactivity using a suitable counting apparatus by comparison with a standardised iodine-131 solution or by measurement in an instrument calibrated with the aid of such a solution.

STORAGE

Store protected from light.

LABELLING

The label states the specific radioactivity expressed in gigabecquerels of iodine-131 per gram of iobenguane base.

01/2005:1533

KRYPTON (^{81m}Kr) INHALATION GASKryptonum (^{81m}Kr) ad inhalationem**DEFINITION**

Krypton (^{81m}Kr) inhalation gas is a mixture of krypton-81m and a suitable vehicle such as air.

Krypton-81m is formed by decay of its parent radionuclide rubidium-81. Rubidium-81 has a half-life of 4.58 h.

The krypton-81m formed is separated from the rubidium-81 with a flow of a suitable gas in a rubidium/krypton generator. Rubidium-81 is produced by proton irradiation of

krypton isotopes or by helium-3 or helium-4 irradiation of bromine. After separation of rubidium-81 from the target, it is retained by a suitable support.

Krypton-81m is eluted at a suitable flow rate with a vehicle such as air. The level of moisture required in the eluent depends on the type of generator used. The transport tube for administration has a defined length and inner diameter. The radioactivity concentration is determined before administration.

The radioactivity due to radionuclides other than krypton-81m is not greater than 0.1 per cent, expressed as a percentage of the total radioactivity in the preparation and calculated with reference to the date and time of administration.

CHARACTERS

A clear, colourless gas.

Krypton-81m has a half-life of 13.1 s and emits gamma radiation.

IDENTIFICATION

- A. Record the gamma-ray and X-ray spectrum using a suitable instrument. The gamma photon of krypton-81m has an energy of 0.190 MeV.
- B. The half-life is 11.8 s to 14.4 s.

TESTS

RADIONUCLIDIC PURITY

Elute the generator as prescribed. Pass a sufficient amount (2 litres to 10 litres) of eluate at a suitable flow rate through a suitable absorber such as water. Determine the amount of radioactivity eluted. Allow the krypton-81m to decay for 5 min and record the gamma and X-ray spectrum of the residual radioactivity on the absorber using a suitable instrument. Examine the gamma-ray and X-ray spectrum of the absorber for the presence of radioactive impurities, which must be identified and quantified. The absorbed radioactivity is not more than 0.1 per cent of the radioactivity passed through the absorber, calculated with reference to the date and time of administration.

RADIOACTIVITY

Determine the radioactive concentration of the preparation using suitable equipment such as an ionisation chamber or a gamma ray spectrometer. The measurement equipment may be calibrated by reference to a primary calibrated instrument at a laboratory recognised by the competent authority. The radioactivity is measured under defined operating conditions, such as gas flow rate and measurement geometry, that are identical to those used for the calibration of the instrument.

STORAGE

The storage conditions apply to the generator.

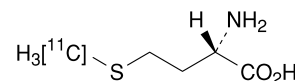
LABELLING

The labelling conditions apply to the generator.

01/2005:1617
corrected

L-METHIONINE ([¹¹C]METHYL) INJECTION

L-Methionini ([¹¹C]methyl)
solutio iniectionis



DEFINITION

Sterile solution of (2S)-2-amino-4-([¹¹C]methylsulphanyl)butanoic acid for diagnostic use.

Content: 90 per cent to 110 per cent of the declared carbon-11 radioactivity at the date and time stated on the label.

Purity:

- minimum of 99 per cent of the total radioactivity corresponds to carbon-11,
- minimum of 95 per cent of the total radioactivity corresponds to carbon-11 in the form of L-[methyl-¹¹C]methionine and D-[methyl-¹¹C]methionine,
- maximum of 10 per cent of the total radioactivity corresponds to carbon-11 in the form of D-[methyl-¹¹C]methionine.

Content of methionine: maximum of 2 mg per maximum recommended dose in millilitres.

PRODUCTION

RADIONUCLIDE PRODUCTION

Carbon-11 is a radioactive isotope of carbon which is most commonly produced by proton irradiation of nitrogen. Depending on the addition of either trace amounts of oxygen or small amounts of hydrogen, the radioactivity is obtained as [¹¹C]carbon dioxide or [¹¹C]methane.

RADIOCHEMICAL SYNTHESIS

L-[Methyl-¹¹C]methionine can be prepared by various chemical synthetic pathways. All methods rely on the alkylation of the sulphide anion of L-homocysteine with [¹¹C]methyl iodide or [¹¹C]methyl triflate. Variations in the procedures used to generate the sulphide anion of L-homocysteine and methods to obtain [¹¹C]methyl iodide lead to negligible differences with respect to quality in terms of specific radioactivity, enantiomeric purity and possible chemical and radiochemical impurities.

Synthesis of [¹¹C]methyl iodide

[¹¹C]Methyl iodide can be obtained either starting from [¹¹C]carbon dioxide or from [¹¹C]methane. The most frequently used method is the reduction of [¹¹C]carbon dioxide with lithium aluminium hydride. The formed [¹¹C]methanol is reacted with hydroiodic acid. Alternatively [¹¹C]methane, either obtained directly in the target or by on-line processes from [¹¹C]carbon dioxide, is reacted with iodine.

Synthesis of [¹¹C]methyl triflate

[¹¹C]methyl triflate can be prepared from [¹¹C]methyl iodide using a silver triflate-impregnated solid support such as graphitised carbon.

Synthesis of L-[methyl-¹¹C]methionine

The most widely used method to obtain L-[methyl-¹¹C]methionine is the alkylation of the sulphide anion, generated from L-homocysteine thiolactone, with

[¹¹C]methyl iodide or [¹¹C]methyl triflate in alkaline conditions in a solvent such as acetone. The L-[methyl-¹¹C]methionine obtained can be purified by semi-preparative liquid chromatography. For example, a column packed with octadecylsilyl silica gel for chromatography eluted with a 9 g/l solution of sodium chloride is suitable.

L-Homocysteine thiolactone hydrochloride

Specific optical rotation (2.2.7): + 20.5 to + 21.5, determined on a 10 g/l solution at 25 °C.

Infrared absorption spectrophotometry (2.2.24).

Comparison: Ph. Eur. reference spectrum of L-homocysteine thiolactone hydrochloride.

CHARACTERS

Appearance: clear, colourless solution.

Half-life and nature of radiation of carbon-11: see Table of physical characteristics of radionuclides (5.7).

IDENTIFICATION

A. Gamma-ray spectrometry.

Results: the only gamma photons have an energy of 0.511 MeV and, depending on the measurement geometry, a sum peak of 1.022 MeV may be observed.

B. It complies with the test for radionuclidic purity (see Tests).

C. Examine the chromatograms obtained in the test for radiochemical purity.

Results: the principal peak in the radiochromatogram obtained with the test solution is similar in retention time to the principal peak in the chromatogram obtained with reference solution (b).

TESTS

pH (2.2.3): 4.5 to 8.5.

Sterility. It complies with the test for sterility prescribed in the monograph on *Radiopharmaceutical preparations* (0125). The injection may be released for use before completion of the test.

Bacterial endotoxins (2.6.14): less than 175/V IU/ml, V being the maximum recommended dose in millilitres. The injection may be released for use before completion of the test.

CHEMICAL PURITY

Impurity A, impurity B and methionine. Liquid chromatography (2.2.29).

Test solution. The preparation to be examined.

Reference solution (a). Dissolve 0.6 mg of L-homocysteine thiolactone hydrochloride R, 2 mg of DL-homocysteine R and 2 mg of DL-methionine R in water R and dilute to V, V being the maximum recommended dose in millilitres.

Reference solution (b). Dissolve 2 mg of L-methionine R in the same solvent as used in the test solution and dilute to 10 ml with the same solvent.

Column:

- size: $l = 0.25$ m, $\varnothing = 4.6$ mm,
- stationary phase: spherical octadecylsilyl silica gel for chromatography R (5 μ m) with a specific surface of 220 m²/g, a pore size of 8 nm and a carbon loading of 6.2 per cent,
- temperature: 25 °C.

Mobile phase: 1.4 g/l solution of potassium dihydrogen phosphate R.

Flow rate: 1 ml/min.

Detection: spectrophotometer at 225 nm and radioactivity detector connected in series.

Injection: loop injector.

Run time: 10 min.

Relative retention with reference to methionine (retention time = about 2.6 min): impurity B = about 0.8, impurity A = about 2.7.

System suitability: reference solution (a):

- resolution: minimum of 2.5 between the peaks due to methionine and impurity B.

Limits: examine the chromatogram obtained with the spectrophotometer:

- impurity A: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (a) (0.6 mg/V),
- impurity B: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (a) (2 mg/V),
- methionine: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (a) (2 mg/V).

Residual solvents (2.4.24): maximum 50 mg/V for the concentration of acetone, V being the maximum recommended dose in millilitres. The preparation may be released for use before completion of the test.

RADIONUCLIDIC PURITY

Carbon-11: minimum 99 per cent of the total radioactivity.

A. Gamma-ray spectroscopy.

Comparison: standardised fluorine-18 solution, or by using an instrument calibrated with the aid of such a solution. Standardised fluorine-18 solutions and/or standardisation services are available from the competent authority.

Results: the spectrum obtained with the solution to be examined does not differ significantly from that obtained with a standardised fluorine-18 solution.

B. Half-life: 19.9 min to 20.9 min.

The preparation may be released for use before completion of the test.

RADIOCHEMICAL PURITY

L-[Methyl-¹¹C]methionine and impurity E. Liquid chromatography (2.2.29) as described in the test for impurity A, impurity B and methionine.

Injection: test solution and reference solution (b).

Limits: examine the chromatogram obtained with the radioactivity detector:

- total of L-[methyl-¹¹C]methionine and impurity E: minimum of 95 per cent of the total radioactivity,
- other peaks in the chromatogram may be due to impurity C, impurity D and impurity F.

ENANTIOMERIC PURITY

Impurity E. Thin-layer chromatography (2.2.27).

Test solution. The preparation to be examined.

Reference solution (a). Dissolve 2 mg of L-methionine R in water R and dilute to 10 ml with the same solvent.

Reference solution (b). Dissolve 4 mg of DL-methionine R in water R and dilute to 10 ml with the same solvent.

Plate: TLC octadecylsilyl silica gel plate for chiral separations R.

Mobile phase: methanol R, water R (50:50 V/V).

Application: 2–10 μ l.

Development: over a path of 8 cm.

Drying: in air for 5 min.

Detection: spray with a 2 g/l solution of *ninhydrin R* in *ethanol R* and heat at 60 °C for 10 min. Determine the distribution of radioactivity using a suitable detector.

Retention factors: L-[methyl- ^{11}C]methionine = about 0.58; impurity E = about 0.51.

System suitability: the chromatogram obtained with reference solution (b) shows 2 clearly separated spots.

Limits:

- total of L-[methyl- ^{11}C]methionine and impurity E: minimum 95 per cent of the total radioactivity,
- impurity E: maximum 10 per cent of the total radioactivity.

The preparation may be released for use before completion of the test.

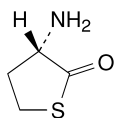
RADIOACTIVITY

Measure the radioactivity using suitable equipment by comparison with a standardised fluorine-18 solution or by measurement in an instrument calibrated with the aid of such a solution.

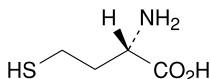
LABELLING

The accompanying information specifies the maximum recommended dose in millilitres.

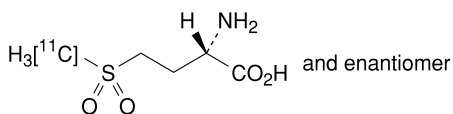
IMPURITIES



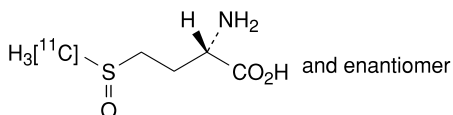
- A. (3*S*)-3-aminodihydrothiophen-2(3*H*)-one (L-homocysteine thiolactone),



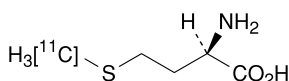
- B. (2*S*)-2-amino-4-sulphanylbutanoic acid (L-homocysteine),



- C. (2*RS*)-2-amino-4-([^{11}C]methylsulphonyl)butanoic acid (DL-[methyl- ^{11}C]methionine *S,S*-dioxide),



- D. (2*RS*)-2-amino-4-([^{11}C]methylsulphonyl)butanoic acid (DL-[methyl- ^{11}C]methionine *S*-oxide),

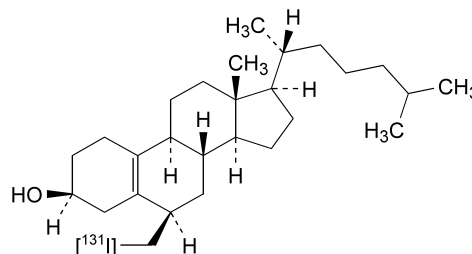


- E. (2*R*)-2-amino-4-([^{11}C]methylsulphanyl)butanoic acid (D-[methyl- ^{11}C]methionine),

- F. [^{11}C]methanol

NORCHOLESTEROL INJECTION, IODINATED (^{131}I)

Norcholesteroli iodinati (^{131}I)
solutio iniectionis



DEFINITION

Iodinated (^{131}I) norcholesterol injection is a sterile, bacterial endotoxin-free solution of 6β-[^{131}I]iodomethyl-19-norcholest-5(10)-en-3β-ol. It may contain a suitable emulsifier such as polysorbate 80 and a suitable antimicrobial preservative such as benzyl alcohol. Iodine-131 is a radioactive isotope of iodine and may be obtained by neutron irradiation of tellurium or by extraction from uranium fission products. The injection contains not less than 90.0 per cent and not more than 110.0 per cent of the declared iodine-131 radioactivity at the date and hour stated on the label. Not less than 85 per cent of the radioactivity corresponds to iodine-131 in the form of 6β-[^{131}I]iodomethyl-19-norcholest-5(10)-en-3β-ol. Not more than 5 per cent of the radioactivity corresponds to iodine-131 in the form of iodide. The specific radioactivity is 3.7 GBq to 37 GBq per gram of 6β-iodomethylnorcholesterol.

CHARACTERS

A clear or slightly turbid, colourless or pale yellow solution. Iodine-131 has a half-life of 8.04 days and emits beta and gamma radiation.

IDENTIFICATION

- Record the gamma-ray spectrum using a suitable instrument. The spectrum does not differ significantly from that of a standardised iodine-131 solution by direct comparison with such a solution. The most prominent photon of iodine-131 has an energy of 0.365 MeV. Standardised iodine-131 solutions are available from laboratories recognised by the competent authority.
- Examine the chromatogram obtained in test (a) for radiochemical purity. The distribution of radioactivity contributes to the identification of the preparation.

TESTS

pH (2.2.3). The pH of the solution is between 3.5 and 8.5.

Sterility. It complies with the test for sterility prescribed in the monograph on *Radiopharmaceutical preparations* (0125). The injection may be released for use before completion of the test.

Bacterial endotoxins (2.6.14): less than 175/*V* IU/ml, *V* being the maximum recommended dose in millilitres.

RADIONUCLIDIC PURITY

Record the gamma-ray spectrum using a suitable instrument. The spectrum does not differ significantly from that of a standardised iodine-131 solution. Determine the relative amounts of iodine-131, iodine-133, iodine-135 and other

radionuclidic impurities present. Iodine-133 has a half-life of 20.8 h and its most prominent gamma photons have energies of 0.530 MeV and 0.875 MeV. Iodine-135 has a half-life of 6.55 h and its most prominent gamma photons have energies of 0.527 MeV, 1.132 MeV and 1.260 MeV. Not less than 99.9 per cent of the total radioactivity is due to iodine-131.

RADIOCHEMICAL PURITY

a) Examine by thin-layer chromatography (2.2.27) using silica gel GF₂₅₄ R as the coating substance.

Test solution. The injection to be examined.

Carrier solution. Dissolve 10 mg of potassium iodide R, 20 mg of potassium iodate R and 0.1 g of sodium hydrogen carbonate R in distilled water R and dilute to 10 ml with the same solvent.

Apply to the plate up to 5 µl of the test solution and 10 µl of the carrier solution on the same spot. Develop over a path of 15 cm (about 60 min) using chloroform R. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. Determine the distribution of radioactivity using a suitable detector. In the chromatogram obtained, not less than 85 per cent of the total radioactivity is found in the spot corresponding to 6β-iodomethyl-19-norcholest-5(10)-en-3β-ol at an *R_f* value of about 0.5. Iodide ion remains near the starting-line.

b) Examine by thin-layer chromatography (2.2.27) using silica gel GF₂₅₄ R as the coating substance.

Test solution. The injection to be examined.

Carrier solution. Dissolve 10 mg potassium iodide R, 20 mg of potassium iodate R and 0.1 g of sodium hydrogen carbonate R in distilled water R and dilute to 10 ml with the same solvent.

Apply to the plate 10 µl of the carrier solution and then up to 5 µl of the test solution on the same spot. Develop over a path of 15 cm (about 90 min) using a mixture of equal volumes of chloroform R and ethanol R. Allow the plate to dry in air. Expose the plate to ultraviolet light at 254 nm for 5 min. A yellow spot corresponding to iodide develops at an *R_f* value of about 0.5. Determine the distribution of radioactivity using a suitable detector. The main peak of radioactivity is near to the solvent front. Other iodocholesterols migrate near the solvent front. In the chromatogram obtained, not more than 5 per cent of the total radioactivity is found in the spot corresponding to iodide.

RADIOACTIVITY

Measure the radioactivity using suitable counting equipment by comparison with a standardised iodine-131 solution or by measurement in an instrument calibrated with the aid of such a solution.

STORAGE

Store protected from light at a temperature not exceeding – 18 °C.

01/2005:1620

OXYGEN (¹⁵O)

Oxygenium (¹⁵O)

DEFINITION

Mixture of [¹⁵O]oxygen in the gaseous phase and a suitable vehicle such as Medicinal air (1238), for diagnostic use.

Purity:

- minimum 99 per cent of the total radioactivity corresponds to oxygen-15,

- minimum 97 per cent of the total radioactivity corresponds to oxygen-15 in the form of oxygen (O₂).

PRODUCTION

RADIONUCLIDIC PRODUCTION

Oxygen-15 is a radioactive isotope of oxygen which may be produced by various nuclear reactions such as proton irradiation of nitrogen-15 or deuteron irradiation of nitrogen-14.

RADIOCHEMICAL SYNTHESIS

In order to recover oxygen-15 as molecular oxygen from the nitrogen target gas, carrier oxygen is added at concentrations generally ranging from 0.2 per cent V/V to 1.0 per cent V/V. After irradiation, the target gas is usually passed through activated charcoal and a carbon dioxide scavenger, such as soda lime, before mixing with the vehicle.

CHARACTERS

Appearance: colourless gas.

Half-life and nature of radiation of oxygen-15: see Table of physical characteristics of radionuclides (5.7).

IDENTIFICATION

A. Gamma spectrometry.

Results: the only gamma photons have an energy of 0.511 MeV and, depending on the measurement geometry, a sum peak of 1.022 MeV may be observed.

B. It complies with the test for radionuclidic purity (see Tests).

C. Examine the chromatograms obtained in the test for radiochemical purity.

Results: the retention times of the principal peaks in the chromatogram obtained with the test gas using the radioactivity detector are similar to those of the principal peaks corresponding to oxygen in the chromatogram obtained with the reference gas using the thermal conductivity detector.

TESTS

The following tests are performed on [¹⁵O]oxygen as described under radiochemical synthesis before mixing with the vehicle.

RADIONUCLIDIC PURITY

Oxygen-15: minimum 99 per cent of the total radioactivity.

A. Gamma spectrometry.

Comparison: standardised fluorine-18 solution, or by using an instrument calibrated with the aid of such a solution. Standardised fluorine-18 solutions and/or standardisation services are available from the competent authority.

Results: the spectrum obtained with the solution to be examined does not differ significantly from that obtained with a standardised fluorine-18 solution.

B. Half-life: 1.9 min to 2.2 min.

The preparation may be released for use before completion of the test.

RADIOCHEMICAL PURITY

Oxygen-15 in the form of O₂. Gas chromatography (2.2.28): use the normalisation procedure.

Test sample. [¹⁵O]oxygen as described under radiochemical synthesis.

Reference gas. Nitrogen gas mixture R.

Column:

- size: l = 1.8 m, Ø1 = 6.3 mm and Ø2 = 3.2 mm,

– *stationary phase*: GC concentric column R.

Carrier gas: helium for chromatography R.

Flow rate: 65 ml/min.

Temperature:

– *column*: 40 °C,

– *injection port*: 40 °C,

– *thermal conductivity detector*: 70 °C.

Detection: thermal conductivity detector and radioactivity detector connected in series.

Injection: loop injector.

Run time: 10 min.

Retention times: oxygen, nitrogen and carbon monoxide eluting from the inner column = about 0.4 min; carbon dioxide eluting from the inner column = about 0.8 min; oxygen eluting from the outer column = about 2.1 min; nitrogen eluting from the outer column = about 3.1 min; carbon monoxide eluting from the outer column = about 6.2 min.

System suitability: reference gas:

- 5 clearly separated principal peaks are observed in the chromatogram obtained using the thermal conductivity detector,
- *resolution*: minimum of 1.5 between the peaks due to carbon dioxide eluting from the inner column and oxygen eluting from the outer column, in the chromatogram obtained using the thermal conductivity detector.

Limits: examine the chromatogram obtained with the radioactivity detector and calculate the percentage content of oxygen-15 substances from the peak areas.

- *oxygen-15 gas in the form of O₂*: minimum 97 per cent of the total radioactivity,
- disregard the first peak corresponding to components co-eluting from the inner column.

RADIOACTIVITY

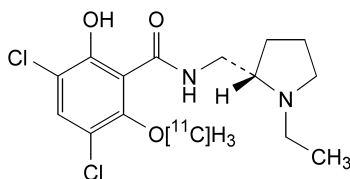
The radioactive concentration is determined before administration.

Measure the radioactivity using suitable equipment by comparison with a standardised fluorine-18 solution or by measurement in an instrument calibrated with the aid of such a solution.

01/2005:1924

RACLOPRIDE ([¹¹C]METHOXY) INJECTION

Raclopridi ([¹¹C]methoxy)
solutio iniectionis



DEFINITION

Sterile solution of 3,5-dichloro-N-[(2S)-1-ethylpyrrolidin-2-yl]methyl]-2-hydroxy-6-([¹¹C]methoxy)benzamide.

Content: 90 per cent to 110 per cent of the declared carbon-11 radioactivity at the date and time stated on the label.

Purity:

- minimum of 99 per cent of the total radioactivity corresponds to carbon-11,
- minimum of 95 per cent of the total radioactivity corresponds to carbon-11 in the form of [¹¹C]methoxy-raclopride.

Content of raclopride: maximum of 10 µg per maximum recommended dose in millilitres.

PRODUCTION

RADIONUCLIDE PRODUCTION

Carbon-11 is a radioactive isotope of carbon most commonly produced by proton irradiation of nitrogen. Depending on the addition of either trace amounts of oxygen or small amounts of hydrogen, the radioactivity is obtained as [¹¹C]carbon dioxide or [¹¹C]methane, respectively.

RADIOCHEMICAL SYNTHESIS

[¹¹C]methoxy-raclopride may be prepared by O-alkylation of the corresponding phenolate anion (S)-3,5-dichloro-2,6-dihydroxy-N-[(1-ethylpyrrolidin-2-yl)methyl]benzamide with iodo[¹¹C]methane or [¹¹C]methyl trifluoromethanesulphonate.

Synthesis of iodo[¹¹C]methane

Iodo[¹¹C]methane may be produced from [¹¹C]carbon dioxide or from [¹¹C]methane. The most frequently used method is reduction of [¹¹C]carbon dioxide with lithium aluminium hydride. The lithium aluminium [¹¹C]methanolate formed is reacted with hydroiodic acid to iodo[¹¹C]methane via [¹¹C]methanol. Alternatively [¹¹C]methane, either obtained directly in the target or by on-line processes from [¹¹C]carbon dioxide, is reacted with iodine.

Synthesis of [¹¹C]methyl trifluoromethanesulphonate

[¹¹C]Methyl trifluoromethanesulphonate may be prepared from iodo[¹¹C]methane using a solid support such as graphitised carbon impregnated with silver trifluoromethanesulphonate.

Synthesis of [¹¹C]methoxy-raclopride

Methylation with iodo[¹¹C]methane is performed under alkaline conditions in a solvent such as dimethyl sulphoxide. The methylation with [¹¹C]methyl trifluoromethanesulphonate is performed in a solvent such as dimethylformamide or acetone. The resulting [¹¹C]methoxy-raclopride may be purified by semi-preparative liquid chromatography using, for example, a column packed with octadecylsilyl silica gel for chromatography eluted with a mixture of 25 volumes of acetonitrile and 75 volumes of 0.01 M phosphoric acid.

PRECURSOR FOR SYNTHESIS

(S)-3,5-Dichloro-2,6-dihydroxy-N-[(1-ethylpyrrolidin-2-yl)methyl]benzamide hydrobromide

Melting point (2.2.14): 211 °C to 213 °C.

Specific optical rotation (2.2.7): + 11.3 to + 11.5, determined on a 15.0 g/l solution in *ethanol R* at 22 °C.

CHARACTERS

Appearance: clear, colourless solution.

Half-life and nature of radiation of carbon-11: see Table of physical characteristics of radionuclides (5.7).

IDENTIFICATION

A. Gamma-ray spectrometry.

Results: the only gamma photons have an energy of 0.511 MeV and, depending on the measurement geometry, a sum peak of 1.022 MeV may be observed.

B. It complies with test B for radionuclidic purity (see Tests).

- C. Examine the chromatograms obtained in the test for radiochemical purity.

Results: the principal peak in the radiochromatogram obtained with the test solution is similar in retention time to the principal peak in the chromatogram obtained with reference solution (d).

TESTS

pH (2.2.3): 4.5 to 8.5.

Sterility. It complies with the test for sterility prescribed in the monograph on *Radiopharmaceutical preparations* (0125). The injection may be released for use before completion of the test.

Bacterial endotoxins (2.6.14): less than 175/V IU/ml, V being the maximum recommended dose in millilitres. The injection may be released for use before completion of the test.

CHEMICAL PURITY

Raclopride and impurity A. Liquid chromatography (2.2.29).

Test solution. The preparation to be examined.

Reference solution (a). Dissolve 7.2 mg of *raclopride tartrate R* in *water R* and dilute to 50 ml with the same solvent.

Reference solution (b). Dissolve 1.2 mg of (*S*)-3,5-dichloro-2,6-dihydroxy-*N*-[(1-ethylpyrrolidin-2-yl)methyl]benzamide hydrobromide *R* in *methanol R* and dilute to 100 ml with the same solvent.

Reference solution (c). To 0.1 ml of reference solution (a) add 0.1 ml of reference solution (b) and dilute to V with *water R*, V being the maximum recommended dose in millilitres.

Reference solution (d). Dilute 1.0 ml of reference solution (a) to 10.0 ml with *water R*.

Column:

- **size:** *l* = 0.05 m, Ø = 4.6 mm,
- **stationary phase:** spherical *end-capped octadecylsilyl silica gel for chromatography R* (3.5 µm) with a specific surface area of 175 m²/g, a pore size of 12.5 nm, a pore volume of 0.7 cm³/g and a carbon loading of 15 per cent,
- **temperature:** 30 °C.

Mobile phase: dissolve 2 g of *sodium heptanesulphonate R* in 700 ml of *water R*, adjust to pH 3.9 with *phosphoric acid R* and dilute to 1000 ml with *acetonitrile R*.

Flow rate: 1 ml/min.

Detection: spectrophotometer at 220 nm and radioactivity detector connected in series.

Injection: loop injector; inject the test solution and reference solutions (b) and (c).

Run time: 10 min.

Relative retention with reference to raclopride: impurity A = about 0.46.

System suitability: reference solution (c):

- **resolution:** minimum of 5 between the peaks due to raclopride and to impurity A.

Limits: examine the chromatogram obtained with the spectrophotometer:

- **raclopride:** not more than the area of the corresponding peak in the chromatogram obtained with reference solution (c) (10 µg/V),
- **impurity A:** not more than the area of the corresponding peak in the chromatogram obtained with reference solution (c) (1 µg/V).

Residual solvents are limited according to the principles defined in the general chapter (5.4), using the general method (2.4.24). The preparation may be released for use before completion of the test.

RADIONUCLIDIC PURITY

Carbon-11: minimum 99 per cent of the total radioactivity.

The preparation may be released for use before completion of the test.

A. Gamma-ray spectrometry.

Comparison: standardised fluorine-18 solution, or by using a calibrated instrument. Standardised fluorine-18 solutions and/or standardisation services are available from the competent authority.

Results: the spectrum obtained with the solution to be examined does not differ significantly from that obtained with a standardised fluorine-18 solution.

B. Half-life. 19.9 min to 20.9 min.

RADIOCHEMICAL PURITY

Liquid chromatography (2.2.29) as described in the test for raclopride and impurity A with the following modifications.

Injection: test solution and reference solution (d).

Limits: examine the chromatogram obtained with the radioactivity detector:

- [*Methoxy-¹¹C*] raclopride: minimum of 95 per cent of the total radioactivity.

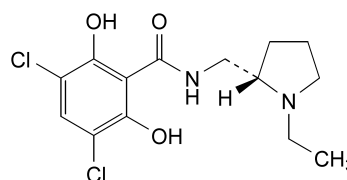
RADIOACTIVITY

Mesure the radioactivity using suitable equipment by comparison with a standardised fluorine-18 solution or by using a calibrated instrument.

LABELLING

The accompanying information specifies the maximum recommended dose in millilitres.

IMPURITIES

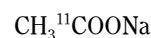


- A. 3,5-dichloro-*N*-[(2*S*)-1-ethylpyrrolidin-2-yl]methyl]-2,6-dihydroxybenzamide.

01/2005:1920

SODIUM ACETATE ([1-¹¹C]) INJECTION

Natrii acetatis ([1-¹¹C]) solutio iniectabilis



DEFINITION

Sterile solution of sodium [1-¹¹C]acetate, in equilibrium with [1-¹¹C]acetic acid.

Content: 90 per cent to 110 per cent of the declared carbon-11 radioactivity at the date and time stated on the label.

PRODUCTION

RADIONUCLIDE PRODUCTION

Carbon-11 is a radioactive isotope of carbon which is most commonly produced by proton irradiation of nitrogen. By the addition of trace amounts of oxygen, the radioactivity is obtained as [^{11}C]carbon dioxide.

RADIOCHEMICAL SYNTHESIS

[^{11}C]Carbon dioxide may be separated from the target gas mixture by cryogenic trapping or by trapping on a molecular sieve at room temperature. [^{11}C]Carbon dioxide is then released from the trap using an inert gas such as nitrogen at a temperature higher than the trapping temperature.

[1- ^{11}C]Acetate is usually prepared by reaction of [^{11}C]carbon dioxide with methylmagnesium bromide in organic solvents such as ether or tetrahydrofuran.

Hydrolysis of the product yields [1- ^{11}C]acetic acid. It is purified by chromatographic procedures. The eluate is diluted with sodium chloride solution.

PRECURSOR FOR SYNTHESIS

Methylmagnesium bromide. The reactivity of methylmagnesium bromide is tested by decomposition of a defined amount with water. The amount of hydrogen released during this reaction is not less than 90 per cent of the theoretical value.

CHARACTERS

Appearance: clear, colourless solution.

Half-life and nature of radiation of carbon-11: see Table of physical characteristics of radionuclides (5.7).

IDENTIFICATION

A. Gamma-ray spectrometry.

Results: the only gamma photons have an energy of 0.511 MeV and, depending on the measurement geometry, a sum peak of 1.022 MeV may be observed.

B. It complies with test B for radionuclidic purity (see Tests).

C. Examine the chromatograms obtained in the test for radiochemical purity.

Results: the principal peak in the radiochromatogram obtained with the test solution is similar in retention time to the principal peak in the chromatogram obtained with the reference solution.

TESTS

pH (2.2.3): 4.5 to 8.5.

Sterility. It complies with the test for sterility prescribed in the monograph on *Radiopharmaceutical preparations (0125)*. The injection may be released for use before completion of the test.

Bacterial endotoxins (2.6.14): less than 175/V IU/ml, V being the maximum recommended dose in millilitres. The injection may be released for use before completion of the test.

CHEMICAL PURITY

Acetate. Liquid chromatography (2.2.29).

Test solution. The preparation to be examined.

Reference solution. Dissolve 28 mg of sodium acetate R in water R and dilute to V, V being the maximum recommended dose in millilitres.

Column:

- size: $l = 0.25\text{ m}$, $\varnothing = 4.0\text{ mm}$,
- stationary phase: strongly basic anion exchange resin for chromatography R (10 μm),
- temperature: 25 °C.

Mobile phase: 0.1 M sodium hydroxide protected from atmospheric carbon dioxide.

Flow rate: 1 ml/min.

Detection: spectrophotometer at 220 nm and radioactivity detector connected in series.

Injection: loop injector.

Run time: 10 min.

System suitability: reference solution:

- resolution: minimum 4.0 between the peaks due to hold-up volume and acetate.

Limit: examine the chromatograms obtained with the spectrophotometer:

- acetate: not more than the area of the corresponding peak in the chromatogram obtained with the reference solution (20 mg per V).

Residual solvents are limited according to the principles defined in the general chapter (5.4), using the general method (2.4.24). The preparation may be released for use before completion of the test.

RADIONUCLIDIC PURITY

Carbon-11: minimum 99 per cent of the total radioactivity.

The preparation may be released for use before completion of the tests.

A. Gamma-ray spectrometry.

Comparison: standardised fluorine-18 solution, or by using a calibrated instrument. Standardised fluorine-18 solutions and/or standardisation services are available from laboratories recognised by the competent authority.

Results: the spectrum obtained with the solution to be examined does not differ significantly from that obtained with a standardised fluorine-18 solution.

B. Half-life: 19.9 min to 20.9 min.

RADIOCHEMICAL PURITY

[1- ^{11}C]Acetate. Liquid chromatography (2.2.29) as described in the test for acetate.

Limit: examine the chromatograms obtained with the spectrophotometer and the radioactivity detector:

- total of [1- ^{11}C]acetate: minimum 95 per cent of the total radioactivity.

RADIOACTIVITY

Measure the radioactivity using suitable equipment by comparison with a standardised fluorine-18 solution or by measurement with a calibrated instrument.

LABELLING

The accompanying information specifies the maximum recommended dose in millilitres.

01/2005:0279

SODIUM CHROMATE (^{51}Cr) STERILE SOLUTIONNatrii chromatis (^{51}Cr) solutio sterilis

DEFINITION

Sodium chromate (^{51}Cr) sterile solution is a sterile solution of sodium [^{51}Cr]chromate made isotonic by the addition of sodium chloride. Chromium-51 is a radioactive isotope of chromium and may be prepared by neutron irradiation of chromium, either of natural isotopic composition or enriched in chromium-50. The solution contains not less than 90.0 per cent and not more than 110.0 per cent of

the declared chromium-51 radioactivity at the date and hour stated on the label. Not less than 90 per cent of the radioactivity corresponds to chromium-51 in the form of chromate. The specific radioactivity is not less than 370 MBq of chromium-51 per milligram of chromate ion.

CHARACTERS

A clear, colourless or slightly yellow solution.

Chromium-51 has a half-life of 27.7 days and emits gamma radiation.

IDENTIFICATION

- A. Record the gamma-ray spectrum using a suitable instrument. The spectrum does not differ significantly from that of a standardised chromium-51 solution. Standardised chromium-51 solution are available from laboratories recognised by the competent authority. The gamma photon has an energy of 0.320 MeV.
- B. Examine the chromatogram obtained in the test for radiochemical purity. The distribution of radioactivity contributes to the identification of the preparation.

TESTS

pH (2.2.3). The pH of the solution is 6.0 to 8.5.

Total chromate. Not more than 2.7 µg of chromate ion (CrO₄²⁻) per megabecquerel. Measure the absorbance of the solution (2.2.25) at the absorption maximum at 370 nm. Calculate the content of chromate using the absorbance of a standard consisting of a 1.7 mg/l solution of *potassium chromate R*. If necessary, adjust the solution to be examined and the standard to pH 8.0 by adding *sodium hydrogen carbonate solution R*.

Sterility. It complies with the test for sterility prescribed in the monograph on *Radiopharmaceutical preparations (0125)*. The solution may be released for use before completion of the test.

RADIONUCLIDIC PURITY

Record the gamma-ray spectrum using a suitable instrument. The spectrum does not differ significantly from that of a standardised chromium-51 solution.

RADIOCHEMICAL PURITY

Examine by ascending paper chromatography (2.2.26). Apply to the paper a quantity of the solution sufficient for the detection method. Begin the development immediately and develop for 2.5 h using a mixture of 25 volumes of *ammonia R*, 50 volumes of *alcohol R* and 125 volumes of *water R*. Chromic ions remain on the starting line. Determine the distribution of the radioactivity using a suitable detector. Not less than 90 per cent of the total radioactivity of the chromatogram is found in the spot with an *R_f* value of about 0.9, corresponding to sodium chromate.

RADIOACTIVITY

Measure the radioactivity using suitable counting equipment by comparison with a standardised chromium-51 solution or by measurement in an instrument calibrated with the aid of such a solution.

01/2005:2100

SODIUM FLUORIDE (¹⁸F) INJECTION

Natrii fluoridi (¹⁸F) solutio iniectabilis

DEFINITION

Sterile solution containing fluorine-18 in the form of sodium fluoride. It may contain carrier fluoride and a suitable buffer.

Content:

- *fluorine-18*: 90 per cent to 110 per cent of the declared fluorine-18 radioactivity at the date and hour stated on the label,
- *fluoride*: maximum 4.52 mg per maximum recommended dose in millilitres.

PRODUCTION

The radionuclide fluorine-18 is most commonly produced by proton irradiation of water enriched in oxygen-18. Fluorine-18 in the form of fluoride is recovered from the target water, generally by adsorption and desorption from anion-exchange resins or electrochemical deposition and redissolution.

CHARACTERS

Appearance: clear, colourless solution.

Half-life and nature of radiation of fluorine-18: see *Table of physical characteristics of radionuclides (5.7)*.

IDENTIFICATION

- A. Gamma-ray spectrometry.

Results: the only gamma photons have an energy of 0.511 MeV and, depending on the measurement geometry, a sum peak of 1.022 MeV may be observed.

- B. It complies with test B for radionuclidic purity (see Tests).
- C. Examine the chromatograms obtained in the test for radiochemical purity (see Tests).

Results: the principal peak in the radiochromatogram obtained with the test solution is similar in retention time to the principal peak in the chromatogram obtained with the reference solution. In the chromatogram obtained with the reference solution, the peak due to fluoride is negative.

TESTS

pH (2.2.3): 5.0 to 8.5.

Fluoride. Liquid chromatography (2.2.29).

Test solution. The preparation to be examined.

Reference solution. Dissolve 10 mg of *sodium fluoride R* in *water R* and dilute to *V* with the same solvent, *V* being the maximum recommended dose in millilitres.

Column:

- *size*: *l* = 0.25 m, Ø = 4 mm,
- *stationary phase*: *strongly basic anion-exchange resin for chromatography R* (10 µm),
- *temperature*: constant, between 20 °C and 30 °C.

Mobile phase: 4 g/l solution of *sodium hydroxide R*, protected from atmospheric carbon dioxide.

Flow rate: 1 ml/min.

Detection: spectrophotometer at 220 nm and a radioactivity detector connected in series.

Injection: 20 µl.

Run time: 15 min.

System suitability: examine the chromatogram obtained with the reference solution using the spectrophotometer:

- *signal-to-noise ratio*: minimum 10 for the principal peak,
- *retention time of fluoride*: minimum 3 times the hold-up time.

Limit: examine the chromatogram obtained with the spectrophotometer:

- *fluoride*: not more than the area of the corresponding peak in the chromatogram obtained with the reference solution (4.52 mg/*V*).

Sterility. It complies with the test for sterility prescribed in the monograph on *Radiopharmaceutical preparations (0125)*. The injection may be released for use before completion of the test.

Bacterial endotoxins (2.6.14): less than $175/V \text{ IU/ml}$, V being the maximum recommended dose in millilitres. The injection may be released for use before completion of the test.

RADIONUCLIDIC PURITY

Fluorine-18: minimum 99.9 per cent of the total radioactivity.

The preparation may be released for use before completion of the tests.

A. Gamma-ray spectrometry.

Determine the amount of fluorine-18 and radionuclidic impurities with a half-life longer than 2 h. For the detection and quantification of impurities, retain the preparation to be examined for a sufficient time to allow the fluorine-18 to decay to a level which permits the detection of impurities.

Results: the spectrum obtained with the preparation to be examined does not differ significantly from that of a background spectrum.

B. Half-life: 105 min to 115 min.

RADIOCHEMICAL PURITY

[^{18}F]fluoride. Liquid chromatography (2.2.29) as described in the test for fluoride. If necessary, dilute the test solution with *water R* to obtain a radioactivity concentration suitable for the radioactivity detector.

Limit: examine the chromatogram obtained with the radioactivity detector:

- [^{18}F]fluoride: minimum 98.5 per cent of the total radioactivity.

RADIOACTIVITY

Determine the radioactivity using a calibrated instrument.

LABELLING

The label states the maximum recommended dose in millilitres.

01/2005:0563

SODIUM IODIDE (^{123}I) INJECTION

Natrii iodidi (^{123}I) solutio iniectabilis

DEFINITION

Sterile solution containing iodine-123 in the form of sodium iodide; it may contain sodium thiosulphate or some other suitable reducing agent and a suitable buffer.

Content: 90 per cent to 110 per cent of the declared iodine-123 radioactivity at the date and hour stated on the label.

PRODUCTION

Iodine-123 is obtained by proton irradiation of xenon enriched in xenon-124 (minimum 98 per cent) followed by the decay of xenon-123 which is formed directly and by the decay of caesium-123. No carrier iodide is added.

CHARACTERS

Appearance: clear, colourless solution.

Half-life and nature of radiation of iodine-123: see *Table of physical characteristics of radionuclides (5.7)*.

IDENTIFICATION

A. Gamma-ray spectrometry.

Results: the spectrum obtained with the preparation to be examined does not differ significantly from that of a standardised iodine-123 solution. The most prominent gamma photon has an energy of 0.159 MeV and is accompanied by the principal X-ray of 0.027 MeV.

B. Examine the chromatograms obtained in the test for radiochemical purity.

Results: the principal peak in the radiochromatogram obtained with the test solution is similar in retention time to the principal peak in the chromatogram obtained with reference solution (a).

TESTS

pH (2.2.3): 7.0 to 10.0.

Sterility. It complies with the test for sterility prescribed in the monograph on *Radiopharmaceutical preparations (0125)*. The preparation may be released for use before completion of the test.

RADIONUCLIDIC PURITY

Iodine-123: minimum 99.65 per cent of the total radioactivity.

Gamma-ray spectrometry.

Determine the relative amounts of iodine-123, iodine-125, tellurium-121 and other radionuclidic impurities present. For the detection of tellurium-121 and iodine-125, retain the preparation to be examined for a sufficient time to allow iodine-123 to decay to a level which permits the detection of radionuclidic impurities. No radionuclides with a half-life longer than that of iodine-125 are detected.

The preparation may be released for use before completion of the test.

RADIOCHEMICAL PURITY

[^{123}I]iodide. Liquid chromatography (2.2.29).

Test solution. Dilute the preparation to be examined with a 2 g/l solution of *sodium hydroxide R* to a radioactive concentration suitable for the detector. Add an equal volume of a solution containing 1 g/l of *potassium iodide R*, 2 g/l of *potassium iodate R* and 10 g/l of *sodium hydrogen carbonate R* and mix.

Reference solution (a). Dilute 1 ml of a 26.2 mg/l solution of *potassium iodide R* to 10 ml with *water R*.

Reference solution (b). Dilute 1 ml of a 24.5 mg/l solution of *potassium iodate R* to 10 ml with *water R*. Mix equal volumes of this solution and reference solution (a).

Column:

- **size:** $l = 0.25 \text{ m}$, $\varnothing = 4.0 \text{ mm}$,
- **stationary phase:** octadecylsilyl silica gel for chromatography *R* (5 μm),
- **temperature:** constant between 20 °C and 30 °C.

Mobile phase: dissolve 5.85 g of *sodium chloride R* in 1000 ml of *water R*, add 0.65 ml of *octylamine R* and adjust to pH 7.0 with *dilute phosphoric acid R*; add 50 ml of *acetonitrile R* and mix.

Flow rate: 1.5 ml/min.

Detection: spectrophotometer at 220 nm and a radioactivity detector connected in series.

Injection: 20 μl .

Run time: 12 min.

Relative retention with reference to iodide (retention time = about 5 min): iodate = 0.2 to 0.3.

System suitability: reference solution (b):

- **resolution:** minimum 2 between the peaks due to iodide and iodate in the chromatogram recorded with the spectrophotometer.

Limit: examine the chromatogram obtained with the test solution using the radioactivity detector and locate the peak due to iodide by comparison with the chromatogram obtained with reference solution (a) using the spectrophotometer:

- [^{123}I]iodide: minimum 95 per cent of the total radioactivity.

RADIOACTIVITY

Determine the radioactivity using a calibrated instrument.

LABELLING

The label states any substance added.

IMPURITIES

A. [^{123}I]iodate ion.

01/2005:0938

SODIUM IODIDE (^{131}I) CAPSULES FOR DIAGNOSTIC USE

Natrii iodidi (^{131}I) capsulae ad usum diagnosticum

DEFINITION

Capsules for diagnostic use containing iodine-131 in the form of sodium iodide on a solid support; they may contain sodium thiosulphate or some other suitable reducing agents and a suitable buffering substance. A package contains 1 or more capsules.

Content:

- iodine-131: maximum 37 MBq per capsule; the average radioactivity determined in the test for uniformity of content is 90 per cent to 110 per cent of the declared iodine-131 radioactivity at the date and hour stated on the label;
- iodide: maximum 20 µg per capsule.

PRODUCTION

Iodine-131 is obtained by neutron irradiation of tellurium or by extraction from uranium fission products. No carrier iodide is added.

CHARACTERS

Half-life and nature of radiation of iodine-131: see *Table of physical characteristics of radionuclides* (5.7).

IDENTIFICATION

A. Gamma-ray spectrometry.

Results: the spectrum obtained with the preparation to be examined does not differ significantly from that of a standardised iodine-131 solution. The most prominent gamma photon has an energy of 0.365 MeV.

B. Examine the chromatograms obtained in the test for radiochemical purity.

Results: the principal peak in the radiochromatogram obtained with test solution (b) is similar in retention time to the principal peak in the chromatogram obtained with reference solution (a).

TESTS

Disintegration: the contents of the capsule dissolve completely within 15 min.

In a water-bath at 37 °C, warm in a small beaker about 20 ml of a 2.0 g/l solution of *potassium iodide R*. Add a capsule to be examined. Stir magnetically at 20 r/min.

Uniformity of content. Determine the radioactivity of each of not fewer than 10 capsules. Calculate the average radioactivity per capsule. The radioactivity of no capsule differs by more than 10 per cent from the average, the relative standard deviation is not greater than 3.5 per cent.

Iodide. Liquid chromatography (2.2.29).

Test solution (a). Dissolve a capsule to be examined in 10 ml of *water R*. Filter through a 0.2 µm filter.

Test solution (b). Dissolve a capsule to be examined in *water R*. Filter through a 0.2 µm filter and dilute the filtrate with a 2 g/l solution of *sodium hydroxide R* to a radioactive concentration suitable for the detector. Add an equal volume of a solution containing 1 g/l of *potassium iodide R*, 2 g/l of *potassium iodate R* and 10 g/l of *sodium hydrogen carbonate R* and mix.

Reference solution (a). Dilute 1 ml of a 26.2 mg/l solution of *potassium iodide R* to 10 ml with *water R*.

Reference solution (b). Dilute 1 ml of a 24.5 mg/l solution of *potassium iodate R* to 10 ml with *water R*. Mix equal volumes of this solution and reference solution (a).

Blank solution. Prepare a solution containing 2 mg/ml of each constituent stated on the label, apart from iodide.

Column:

- **size:** $l = 0.25\text{ m}$, $\varnothing = 4.0\text{ mm}$,
- **stationary phase:** *octadecylsilyl silica gel for chromatography R* (5 µm),
- **temperature:** constant between 20 °C and 30 °C.

Mobile phase: dissolve 5.85 g of *sodium chloride R* in 1000 ml of *water R*, add 0.65 ml of *octylamine R* and adjust to pH 7.0 with *dilute phosphoric acid R*; add 50 ml of *acetonitrile R* and mix.

Flow rate: 1.5 ml/min.

Detection: spectrophotometer at 220 nm and radioactivity detector connected in series.

Injection: 20 µl of test solution (a), reference solutions (a) and (b) and the blank solution.

Run time: 12 min.

Relative retention with reference to iodide (retention time = about 5 min): iodate = 0.2 to 0.3.

System suitability:

- in the chromatogram obtained with the blank solution, none of the peaks has a retention time similar to that of the peak due to iodide,
- **resolution:** minimum 2 between the peaks due to iodide and iodate in the chromatogram obtained with reference solution (b) recorded with the spectrophotometer.

Limit: examine the chromatograms obtained with the spectrophotometer:

- **iodide:** not more than the area of the corresponding peak in the chromatogram obtained with reference solution (a) (20 µg/capsule).

RADIONUCLIDIC PURITY

Iodine-131: minimum 99.9 per cent of the total radioactivity.

Gamma-ray spectrometry.

Determine the relative amounts of iodine-131, iodine-133, iodine-135 and other radionuclidic impurities present.

RADIOCHEMICAL PURITY

[^{131}I]iodide. Liquid chromatography (2.2.29) as described in the test for iodide with the following modifications.

Injection: 20 μl of test solution (b) and reference solution (a).

Limit: examine the chromatogram obtained with the test solution using the radioactivity detector and locate the peak due to iodide by comparison with the chromatogram obtained with reference solution (a) using the spectrophotometer:

- [^{131}I]iodide: minimum 95 per cent of the total radioactivity.

RADIOACTIVITY

Determine the radioactivity of the package using a calibrated instrument.

LABELLING

The label states any substance added and the number of capsules in the package.

IMPURITIES

A. [^{131}I]iodate ion.

01/2005:0281

SODIUM IODIDE (^{131}I) SOLUTION**Natrii iodidi (^{131}I) solutio****DEFINITION**

Solution containing iodine-131 in the form of sodium iodide and also sodium thiosulphate or some other suitable reducing agent. It may contain a suitable buffer.

Content:

- *iodine-131*: 90 per cent to 110 per cent of the declared radioactivity at the date and hour stated on the label,
- *iodide*: maximum 20 μg in the maximum recommended dose in millilitres.

PRODUCTION

Iodine-131 is a radioactive isotope of iodine and may be obtained by neutron irradiation of tellurium or by extraction from uranium fission products. No carrier iodide is added.

CHARACTERS

Appearance: clear, colourless solution.

Half-life and nature of radiation of iodine-131: see Table of physical characteristics of radionuclides (5.7).

IDENTIFICATION

A. Gamma-ray spectrometry.

Results: the spectrum obtained with the preparation to be examined does not differ significantly from that of a standardised iodine-131 solution. The most prominent gamma photon has an energy of 0.365 MeV.

B. Examine the chromatograms obtained in the test for iodide.

Results: the principal peak in the radiochromatogram obtained with test solution (a) is similar in retention time to the principal peak in the chromatogram obtained with reference solution (a).

TESTS

pH (2.2.3): 7.0 to 10.0.

Sterility. If intended for parenteral use, it complies with the test for sterility prescribed in the monograph on *Radiopharmaceutical preparations* (0125). The solution may be released for use before completion of the test.

Iodide. Liquid chromatography (2.2.29).

Test solution (a). The preparation to be examined.

Test solution (b). Dilute the preparation to be examined with 0.05 M sodium hydroxide until the radioactivity is equivalent to about 74 MBq/ml. Add an equal volume of a solution containing 1 g/l of *potassium iodide R*, 2 g/l of *potassium iodate R* and 10 g/l of *sodium hydrogen carbonate R* and mix.

Reference solution (a). Dilute 1 ml of a 26.2 mg/l solution of *potassium iodide R* to *V* with *water R*, *V* being the maximum recommended dose in millilitres.

Reference solution (b). Dilute 1 ml of a 24.5 mg/l solution of *potassium iodate R* to *V* with *water R*, *V* being the maximum recommended dose in millilitres. Mix equal volumes of this solution and of reference solution (a).

Blank solution. Prepare a solution containing 2 mg/ml of each of the components stated on the label, apart from iodide.

Column:

- **size:** $l = 0.25\text{ m}$, $\varnothing = 4.0\text{ mm}$,
- **stationary phase:** octadecylsilyl silica gel for chromatography *R* (5 μm),
- **temperature:** maintain at a constant temperature between 20 °C and 30 °C.

Use stainless steel tubing.

Mobile phase: dissolve 5.844 g of *sodium chloride R* in 1000 ml of *water R*, add 650 μl of *octylamine R* and adjust to pH 7.0 with *phosphoric acid R*; add 50 ml of *acetonitrile R* and mix.

Flow rate: 1.5 ml/min.

Detection: spectrophotometer at 220 nm and radioactivity detector connected in series.

Injection: 25 μl ; inject test solution (a), the blank solution and reference solutions (a) and (b).

Run time: 12 min.

Relative retention with reference to iodide (retention time = about 5 min): iodate = 0.2 to 0.3.

System suitability:

- in the chromatogram obtained with the blank solution, none of the peaks shows a retention time similar to that of the peak due to iodide,
- **resolution:** minimum 2 between the peaks due to iodide and iodate in the chromatogram obtained with reference solution (b) recorded with the spectrophotometer.

Limit: examine the chromatogram obtained with the spectrophotometer; locate the peak due to iodide by comparison with the chromatogram obtained with reference solution (a):

- *iodide*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (a).

RADIONUCLIDIC PURITY

Iodine-131: minimum 99.9 per cent of the total radioactivity. Gamma-ray spectrometry.

Determine the relative amounts of iodine-131, iodine-133, iodine-135 and other radionuclidic impurities present.

RADIOCHEMICAL PURITY

[^{131}I]iodide. Liquid chromatography (2.2.29) as described in the test for iodide with the following modification.

Injection: test solution (b).

Limit: examine the chromatogram obtained with the radioactivity detector:

- [^{131}I]iodide: minimum 95 per cent of the total radioactivity.

RADIOACTIVITY

Measure the radioactivity using suitable equipment by comparison with a standardised iodine-131 solution or by using a calibrated instrument.

LABELLING

The label states:

- any substance added,
- the maximum recommended dose, in millilitres,
- where applicable, that the preparation is suitable for use in the manufacture of parenteral dosage forms.

IMPURITIES

A. [^{131}I]iodate ion.

01/2005:2121

SODIUM IODIDE (^{131}I) SOLUTION FOR RADIOLABELLING

Natrii iodidi (^{131}I) solutio ad radio-signandum

DEFINITION

Strongly alkaline solution containing iodine-131 in the form of sodium iodide. It does not contain a reducing agent.

Content: 90 per cent to 110 per cent of the declared iodine-131 radioactivity at the date and hour stated on the label.

PRODUCTION

Iodine-131 may be obtained by neutron irradiation of tellurium or by extraction from uranium fission products. No carrier iodide is added.

CHARACTERS

Appearance: clear, colourless solution.

Half-life and nature of radiation of iodine-131: see Table of physical characteristics of radionuclides (5.7).

IDENTIFICATION

A. Gamma-ray spectrometry.

Results: the spectrum obtained with the preparation to be examined does not differ significantly from that of a standardised iodine-131 solution. The most prominent gamma photon of iodine-131 has an energy of 0.365 MeV.

B. Examine the chromatograms obtained in the test for radiochemical purity (see Tests).

Results: the principal peak in the radiochromatogram obtained with the test solution is similar in retention time to the principal peak in the chromatogram obtained with reference solution (a).

TESTS

Alkalinity (2.2.4). The preparation is strongly alkaline.

RADIONUCLIDIC PURITY

Iodine-131: minimum 99.9 per cent of the total radioactivity.

Gamma-ray spectrometry.

Determine the relative amounts of iodine-130, iodine-131, iodine-133, iodine-135 and other radionuclidic impurities present.

RADIOCHEMICAL PURITY

[^{131}I]iodide. Liquid chromatography (2.2.29).

Test solution. Dilute the preparation to be examined with an equal volume of a solution containing 1 g/l of *potassium iodide R*, 2 g/l of *potassium iodate R* and 10 g/l of *sodium hydrogen carbonate R* and mix. If necessary, first dilute the preparation to be examined with a 2 g/l solution of *sodium hydroxide R* to ensure that the final mixture has a radioactivity concentration suitable for the radioactivity detector.

Reference solution (a). Dissolve 10 mg of *potassium iodide R* in *water R* and dilute to 10 ml with the same solvent.

Reference solution (b). Dissolve 20 mg of *potassium iodate R* in *water R* and dilute to 10 ml with the same solvent. Mix equal volumes of this solution and reference solution (a).

Column:

- **size:** $l = 0.25$ m, $\varnothing = 4.0$ mm,
- **stationary phase:** octadecylsilyl silica gel for chromatography *R* (5 μm),
- **temperature:** constant, between 20 °C and 30 °C.

Use stainless steel tubing.

Mobile phase: dissolve 5.85 g of *sodium chloride R* in 1000 ml of *water R*, add 0.65 ml of *octylamine R* and adjust to pH 7.0 with *dilute phosphoric acid R*; add 50 ml of *acetonitrile R* and mix.

Flow rate: 1.5 ml/min.

Detection: spectrophotometer at 220 nm and a radioactivity detector connected in series.

Injection: 20 μl .

Run time: 12 min.

Relative retention with reference to iodide (retention time = about 5 min): iodate = 0.2 to 0.3.

System suitability: reference solution (b):

- **resolution:** minimum 2 between the peaks due to iodide and iodate in the chromatogram recorded with the spectrophotometer.

Limit: examine the chromatogram obtained with the radioactivity detector:

- [^{131}I]iodide: minimum 95 per cent of the total radioactivity.

RADIOACTIVITY

Determine the radioactivity using a calibrated instrument.

LABELLING

The label states:

- the method of production of iodine-131,
- the vehicle and any substance added,
- that the preparation is not for direct human use.

IMPURITIES

A. [^{131}I]iodate ion.

01/2005:0564

SODIUM IODOHIPPURATE (^{123}I) INJECTION

Natrii iodohippurati (^{123}I) solutio iniectionis

DEFINITION

Sodium iodohippurate (^{123}I) injection is a sterile solution of sodium (2-[^{123}I]iodobenzamido)acetate. It may contain a suitable buffer and a suitable antimicrobial preservative

such as benzyl alcohol. Iodine-123 is a radioactive isotope of iodine and may be obtained by proton irradiation of xenon enriched in xenon-124 (not less than 98 per cent) followed by the decay of caesium-123 formed via xenon-123. The injection contains not less than 90.0 per cent and not more than 110.0 per cent of the declared iodine-123 radioactivity at the date and hour stated on the label. Not less than 96 per cent of the radioactivity corresponds to iodine-123 in the form of sodium 2-iodohippurate. The specific radioactivity is 0.74 GBq to 10.0 GBq of iodine-123 per gram of sodium 2-iodohippurate. Not more than 0.35 per cent of the total radioactivity is due to radionuclides other than iodine-123.

CHARACTERS

A clear, colourless liquid.

Iodine-123 has a half-life of 13.2 h and emits gamma radiation and X-rays.

IDENTIFICATION

- A. Record the gamma-ray and X-ray spectrum using a suitable instrument. The spectrum does not differ significantly from that of a standardised iodine-123 solution apart from any differences attributable to the presence of iodine-125, tellurium-121 and other radionuclidic impurities. Standardised iodine-123, iodine-125 and tellurium-121 solutions are available from laboratories recognised by the national authorities. The most prominent gamma photon of iodine-123 has an energy of 0.159 MeV and is accompanied by an X-ray of 0.027 MeV. Iodine-125 has a half-life of 59.4 days and emits an X-ray of 0.027 MeV and a photon of 0.035 MeV. Tellurium-121 has a half-life of 19.2 days and the most prominent photons have energies of 0.507 MeV and 0.573 MeV.
- B. Examine the chromatograms obtained in the test for radiochemical purity. The spot corresponding to the main peak of radioactivity in the chromatogram obtained with the test solution is similar in position to the spot corresponding to 2-iodohippuric acid in the chromatogram obtained with the reference solution.

TESTS

pH (2.2.3). The pH of the solution is 3.5 to 8.5.

Sterility. It complies with the test for sterility prescribed in the monograph on *Radiopharmaceutical preparations* (0125). The injection may be released for use before completion of the test.

RADIONUCLIDIC PURITY

Record the gamma-ray spectrum using a suitable instrument. Determine the relative amounts of iodine-125, tellurium-121 and other radionuclidic impurities present. No radionuclides with longer half lives than iodine-125 are detected. For the determination of iodine-125, tellurium-121 and other radionuclidic impurities, retain the solution to be examined for a sufficient time to allow the radioactivity of iodine-123 to decrease to a level which permits the detection of radionuclidic impurities. Record the gamma-ray spectrum and X-ray spectrum of the decayed material using a suitable instrument. Not more than 0.35 per cent of the total radioactivity is due to radionuclides other than iodine-123. The injection may be released for use before completion of the test.

RADIOCHEMICAL PURITY

Examine by thin-layer chromatography (2.2.27) using *silica gel GF₂₅₄* R as the coating substance.

Test solution. Dissolve 1 g of *potassium iodide* R in 10 ml of *water* R, add 1 volume of this solution to 10 volumes of the injection to be examined and use within 10 min of mixing.

If necessary, dilute with the reference solution (carrier) to give a radioactive concentration sufficient for the detection method, for example 3.7 MBq per millilitre.

Reference solution (carrier). Dissolve 40 mg of *2-iodohippuric acid* R and 40 mg of *2-iodobenzoic acid* R in 4 ml of 0.1 M *sodium hydroxide*, add 10 mg of *potassium iodide* R and dilute to 10 ml with *water* R.

Apply separately to the plate 10 µl of each solution. Develop over a path of 12 cm (about 75 min) using a mixture of 1 volume of *water* R, 4 volumes of *glacial acetic acid* R, 20 volumes of *butanol* R and 80 volumes of *toluene* R. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. The chromatogram obtained with the reference solution shows a spot corresponding to 2-iodohippuric acid and nearer to the solvent front a spot corresponding to 2-iodobenzoic acid. Iodide ion remains near the starting-line. Determine the distribution of radioactivity using a suitable detector. In the chromatogram obtained with the test solution, not less than 96 per cent of the total radioactivity is found in the spot corresponding to 2-iodohippuric acid and not more than 2 per cent of the total radioactivity is found in either of the spots corresponding to 2-iodobenzoic acid and to iodide ion.

RADIOACTIVITY

Measure the radioactivity using suitable counting equipment by comparison with a standardised iodine-123 solution or by measurement in an instrument calibrated with the aid of such a solution.

STORAGE

Store protected from light.

LABELLING

The label states whether or not the preparation is suitable for renal plasma-flow studies.

01/2005:0282

SODIUM IODOHIPPURATE (¹³¹I) INJECTION

Natrii iodohippurati (¹³¹I) solutio iniectionis

DEFINITION

Sodium iodohippurate (¹³¹I) injection is a sterile solution of sodium 2-(2-[¹³¹I]iodobenzamido)acetate. It may contain a suitable buffer and a suitable antimicrobial preservative such as benzyl alcohol. Iodine-131 is a radioactive isotope of iodine and may be obtained by neutron irradiation of tellurium or by extraction from uranium fission products. The injection contains not less than 90.0 per cent and not more than 110.0 per cent of the declared iodine-131 radioactivity at the date and hour stated on the label. Not less than 96 per cent of the iodine-131 is in the form of sodium 2-iodohippurate. The specific radioactivity is 0.74 GBq to 7.4 GBq of iodine-131 per gram of sodium 2-iodohippurate.

CHARACTERS

A clear, colourless liquid.

Iodine-131 has a half-life of 8.04 days and emits beta and gamma radiation.

IDENTIFICATION

- A. Record the gamma-ray spectrum using a suitable instrument. The spectrum does not differ significantly from that of a standardised iodine-131 solution. Standardised iodine-131 solutions are available from

laboratories recognised by the competent authority. The most prominent gamma photon of iodine-131 has an energy of 0.365 MeV.

- B. Examine the chromatograms obtained in the test for radiochemical purity. The main peak of radioactivity in the chromatogram obtained with the test solution is similar in position to the spot corresponding to 2-iodohippuric acid in the chromatogram obtained with the reference solution.

TESTS

pH (2.2.3). The pH of the injection is 6.0 to 8.5.

Sterility. It complies with the test for sterility prescribed in the monograph on *Radiopharmaceutical preparations* (0125). The injection may be released for use before completion of the test.

RADIONUCLIDIC PURITY

Record the gamma-ray spectrum using a suitable instrument. The spectrum does not differ significantly from that of a standardised iodine-131 solution. Determine the relative amounts of iodine-131, iodine-133, iodine-135 and other radionuclidic impurities present. Iodine-133 has a half-life of 20.8 h and its most prominent gamma photons have energies of 0.530 MeV and 0.875 MeV. Iodine-135 has a half-life of 6.55 h and its most prominent gamma photons have energies of 0.527 MeV, 1.132 MeV and 1.260 MeV. Not less than 99.9 per cent of the total radioactivity is due to iodine-131.

RADIOCHEMICAL PURITY

Examine by thin-layer chromatography (2.2.27) using *silica gel GF₂₅₄* R as the coating substance.

Test solution. Dissolve 1 g of *potassium iodide R* in 10 ml of *water R*, add 1 volume of this solution to 10 volumes of the injection to be examined and use within 10 min of mixing. If necessary dilute with the reference solution (carrier) to give a radioactive concentration sufficient for the detection method, for example 3.7 MBq per millilitre.

Reference solution (carrier). Dissolve 40 mg of *2-iodohippuric acid R* and 40 mg of *2-iodobenzoic acid R* in 4 ml of *0.1 M sodium hydroxide*, add 10 mg of *potassium iodide R* and dilute to 10 ml with *water R*.

Apply separately to the plate 10 µl of each solution. Develop over a path of 12 cm (about 75 min) using a mixture of 1 volume of *water R*, 4 volumes of *glacial acetic acid R*, 20 volumes of *butanol R* and 80 volumes of *toluene R*. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. The chromatogram obtained with the reference solution shows a spot corresponding to 2-iodohippuric acid and nearer to the solvent front a spot corresponding to 2-iodobenzoic acid. Iodide ion remains near the starting-line. Determine the distribution of radioactivity using a suitable detector. In the chromatogram obtained with the test solution, not less than 96 per cent of the total radioactivity is found in the spot corresponding to 2-iodohippuric acid and not more than 2 per cent of the total radioactivity is found in either of the spots corresponding to 2-iodobenzoic acid and to iodide ion.

RADIOACTIVITY

Measure the radioactivity using suitable counting equipment by comparison with a standardised iodine-131 solution or by measurement in an instrument calibrated with the aid of such a solution.

STORAGE

Store protected from light.

LABELLING

The label states that the preparation is not necessarily suitable for renal plasma-flow studies.

01/2005:0124

SODIUM PERTECHNETATE (^{99m}Tc) INJECTION (FISSION)

Natrii pertechnetatis (^{99m}Tc) fissionis formati solutio iniectionis

This monograph applies to sodium pertechnetate (^{99m}Tc) injection obtained from molybdenum-99 extracted from fission products of uranium. Sodium pertechnetate (^{99m}Tc) injection obtained from molybdenum-99 produced by the neutron irradiation of molybdenum is described in the monograph on Sodium pertechnetate (^{99m}Tc) injection (non-fission) (0283).

DEFINITION

Sodium pertechnetate (^{99m}Tc) injection (fission) is a sterile solution containing technetium-99m in the form of pertechnetate ion and made isotonic by the addition of sodium chloride. Technetium-99m is a radionuclide formed by the decay of molybdenum-99. Molybdenum-99 is a radioactive isotope of molybdenum extracted from uranium fission products. The injection contains not less than 90.0 per cent and not more than 110.0 per cent of the declared technetium-99m radioactivity at the date and hour stated on the label. Not less than 95 per cent of the radioactivity corresponds to technetium-99m in the form of pertechnetate ion.

The radioactivity due to radionuclides other than technetium-99m, apart from that due to technetium-99 resulting from the decay of technetium-99m, is not greater than that shown below, expressed as a percentage of the total radioactivity and calculated with reference to the date and hour of administration.

molybdenum-99	0.1 per cent
iodine-131	5×10^{-3} per cent
ruthenium-103	5×10^{-3} per cent
strontium-89	6×10^{-5} per cent
strontium-90	6×10^{-6} per cent
alpha-emitting impurities	1×10^{-7} per cent
other gamma-emitting impurities	0.01 per cent

The injection may be prepared from a sterile preparation of molybdenum-99 under aseptic conditions.

CHARACTERS

A clear, colourless solution.

Technetium-99m has a half-life of 6.02 h and emits gamma radiation.

IDENTIFICATION

Record the gamma-ray spectrum using a suitable instrument. The spectrum does not differ significantly from that of a standardised technetium-99m solution either by direct comparison or by measurement in an instrument calibrated with the aid of such a solution. Standardised technetium-99m, molybdenum-99, iodine-131, ruthenium-103, strontium-89 and strontium/yttrium-90

solutions are available from laboratories recognised by the competent authority. The most prominent gamma photon of technetium-99m has an energy of 0.140 MeV.

TESTS

pH (2.2.3). The pH of the injection is 4.0 to 8.0.

Aluminium. In a test tube about 12 mm in internal diameter, mix 1 ml of *acetate buffer solution pH 4.6 R* and 2 ml of a 1 in 2.5 dilution of the injection in *water R*. Add 0.05 ml of a 10 g/l solution of *chromazurol S R*. After 3 min, the colour of the solution is not more intense than that of a standard prepared at the same time and in the same manner using 2 ml of *aluminium standard solution (2 ppm Al) R* (5 ppm).

Sterility. It complies with the test for sterility prescribed in the monograph on *Radiopharmaceutical preparations (0125)*. The injection may be released for use before completion of the test.

RADIONUCLIDIC PURITY

Preliminary test. To obtain an approximate estimate before use of the injection, take a volume equivalent to 37 MBq and determine the gamma-ray spectrum using a sodium iodide detector with a shield of lead, of thickness 6 mm, interposed between the sample and the detector. The response in the region corresponding to the 0.740 MeV photon of molybdenum-99 does not exceed that obtained using 37 kBq of a standardised molybdenum-99 solution measured under the same conditions, when all measurements are expressed with reference to the date and hour of administration.

Definitive test. Retain a sample of the injection for a sufficient time to allow the technetium-99m radioactivity to decay to a sufficiently low level to permit the detection of radionuclidic impurities. All measurements of radioactivity are expressed with reference to the date and hour of administration.

- **Molybdenum-99.** Record the gamma-ray spectrum of the decayed material in a suitable instrument calibrated with the aid of a standardised molybdenum-99 solution. The most prominent photons have energies of 0.181 MeV, 0.740 MeV and 0.778 MeV. Molybdenum-99 has a half-life of 66.0 h. Not more than 0.1 per cent of the total radioactivity is due to molybdenum-99.
- **Iodine-131.** Record the gamma-ray spectrum of the decayed material in a suitable instrument calibrated with the aid of a standardised iodine-131 solution. The most prominent photon has an energy of 0.365 MeV. Iodine-131 has a half-life of 8.04 days. Not more than 5×10^{-3} per cent of the total radioactivity is due to iodine-131.
- **Ruthenium-103.** Record the gamma-ray spectrum of the decayed material in a suitable instrument calibrated using a standardised ruthenium-103 solution. The most prominent photon has an energy of 0.497 MeV. Ruthenium-103 has a half-life of 39.3 days. Not more than 5×10^{-3} per cent of the total radioactivity is due to ruthenium-103.
- **Strontium-89.** Determine the presence of strontium-89 in the decayed material with an instrument suitable for the detection of beta rays, by comparison with a standardised strontium-89 solution. It is usually necessary first to carry out chemical separation of the strontium so that the standard and the sample may be compared in the same physical and chemical form. Strontium-89 decays with a beta emission of 1.492 MeV maximum energy and has a half-life of 50.5 days. Not more than 6×10^{-5} per cent of the total radioactivity is due to strontium-89.
- **Strontium-90.** Determine the presence of strontium-90 in the decayed material with an instrument suitable for the detection of beta rays. To distinguish strontium-90 from

strontium-89, compare the radioactivity of yttrium-90, the daughter nuclide of strontium-90, with an yttrium-90 standard after the chemical separation of the yttrium. If prior chemical separation of the strontium is necessary, the conditions of radioactive equilibrium must be ensured. The yttrium-90 standard and the sample must be compared in the same physical and chemical form. Strontium-90 and yttrium-90 decay with respective beta emissions of 0.546 MeV and 2.284 MeV maximum energy and half-lives of 29.1 years and 64.0 h. Not more than 6×10^{-6} per cent of the total radioactivity is due to strontium-90.

- **Other gamma-emitting impurities.** Examine the gamma-ray spectrum of the decayed material for the presence of other radionuclidic impurities, which should, where possible, be identified and quantified. The total gamma radioactivity due to these impurities does not exceed 0.01 per cent of the total radioactivity.
- **Alpha-emitting impurities.** Measure the alpha radioactivity of the decayed material to detect any alpha-emitting radionuclidic impurities, which should, where possible, be identified and quantified. The total alpha radioactivity due to these impurities does not exceed 1×10^{-7} per cent of the total radioactivity.

RADIOCHEMICAL PURITY

Examine by descending paper chromatography (2.2.26).

Test solution. Dilute the preparation to be examined with *water R* to a suitable radioactive concentration.

Apply 5 μl of the test solution. Develop for 2 h using a mixture of 20 volumes of *water R* and 80 volumes of *methanol R*. Allow the paper to dry. Determine the distribution of radioactivity using a suitable detector. Not less than 95 per cent of the total radioactivity is in the spot corresponding to pertechnetate ion, which has an R_f value of about 0.6.

RADIOACTIVITY

Measure the radioactivity using suitable counting equipment by comparison with a standardised technetium-99m solution or by measurement in an instrument calibrated with the aid of such a solution.

01/2005:0283

SODIUM PERTECHNETATE (^{99m}Tc) INJECTION (NON-FISSION)

Natrii pertechnetatis (^{99m}Tc) sine fissione formati solutio iniectionabilis

This monograph applies to sodium pertechnetate (^{99m}Tc) injection obtained from molybdenum-99 produced by neutron irradiation of molybdenum. Sodium pertechnetate (^{99m}Tc) injection obtained from molybdenum-99 extracted from fission products of uranium is described in the monograph on Sodium pertechnetate (^{99m}Tc) injection (fission) (0124).

DEFINITION

Sodium pertechnetate (^{99m}Tc) injection (non-fission) is a sterile solution containing technetium-99m in the form of pertechnetate ion and made isotonic by the addition of sodium chloride. Technetium-99m is a radionuclide formed by the decay of molybdenum-99. Molybdenum-99 is a radioactive isotope of molybdenum produced by neutron irradiation of molybdenum. The injection contains not less than 90.0 per cent and not more than 110.0 per cent of the declared technetium-99m radioactivity at the date and

hour stated on the label. Not less than 95 per cent of the radioactivity corresponds to technetium-99m in the form of pertechnetate ion.

The radioactivity due to radionuclides other than technetium-99m, apart from that due to technetium-99 resulting from the decay of technetium-99m is not greater than that shown below, expressed as a percentage of the total radioactivity and calculated with reference to the date and hour of administration.

Molybdenum-99	0.1 per cent
Other radionuclidic impurities	0.01 per cent

The injection may be prepared from a sterile preparation of molybdenum-99 under aseptic conditions.

CHARACTERS

A clear, colourless solution.

Technetium-99m has a half-life of 6.02 h and emits gamma radiation.

IDENTIFICATION

- A. Record the gamma-ray spectrum using a suitable instrument. The spectrum does not differ significantly from that of a standardised technetium-99m solution either by direct comparison or by using an instrument calibrated with the aid of such a solution. Standardised technetium-99m and molybdenum-99 solutions are available from laboratories recognised by the competent authority. The most prominent gamma photon of technetium-99m has an energy of 0.140 MeV.
- B. Examine the chromatogram obtained in the test for radiochemical purity. The distribution of radioactivity contributes to the identification of the preparation.

TESTS

pH (2.2.3). The pH of the injection is 4.0 to 8.0.

Aluminium. In a test tube about 12 mm in internal diameter, mix 1 ml of *acetate buffer solution pH 4.6 R* and 2 ml of a 1 in 2.5 dilution of the injection in *water R*. Add 0.05 ml of a 10 g/l solution of *chromazurol S R*. After 3 min, the colour of the solution is not more intense than that of a standard prepared at the same time in the same manner using 2 ml of *aluminium standard solution (2 ppm Al) R* (5 ppm).

Sterility. It complies with the test for sterility prescribed in the monograph on *Radiopharmaceutical preparations (0125)*. The injection may be released for use before completion of the test.

RADIONUCLIDIC PURITY

Preliminary test. To obtain an approximate estimate before use of the injection, take a volume equivalent to 37 MBq and record the gamma-ray spectrum using a sodium iodide detector with a shield of lead, of thickness 6 mm, interposed between the sample and the detector. The response in the region corresponding to the 0.740 MeV photon of molybdenum-99 does not exceed that obtained using 37 kBq of a standardised solution of molybdenum-99 measured under the same conditions, when all measurements are expressed with reference to the date and hour of administration.

Definitive test. Retain a sample of the injection for a sufficient time to allow the technetium-99m radioactivity to decay to a sufficiently low level to permit the detection of radionuclidic impurities. All measurements of radioactivity are expressed with reference to the date and hour of administration.

- *Molybdenum-99.* Record the gamma-ray spectrum of the decayed material in a suitable instrument calibrated using a standardised molybdenum-99 solution. The most prominent gamma photons have energies of 0.181 MeV, 0.740 MeV and 0.778 MeV. Molybdenum-99 has a half-life of 66.0 h. Not more than 0.1 per cent of the total radioactivity is due to molybdenum-99.
- *Other gamma-emitting impurities.* Examine the gamma-ray spectrum of the decayed material for the presence of other radionuclidic impurities, which should, where possible, be identified and quantified. The total radioactivity due to other radionuclidic impurities does not exceed 0.01 per cent of the total radioactivity.

RADIOCHEMICAL PURITY

Examine by descending paper chromatography (2.2.26).

Test solution. Dilute the injection with *water R* to a suitable radioactive concentration.

Apply 5 µl of the test solution. Develop for 2 h using a mixture of 20 volumes of *water R* and 80 volumes of *methanol R*. Allow the paper to dry in air. Determine the distribution of radioactivity using a suitable detector. Not less than 95 per cent of the total radioactivity is found in the spot corresponding to pertechnetate ion, which has an *R_f* value of about 0.6.

RADIOACTIVITY

Measure the radioactivity using suitable counting equipment by comparison with a standardised technetium-99m solution or by measurement in an instrument calibrated with the aid of such a solution.

01/2005:0284

SODIUM PHOSPHATE (³²P) INJECTION

Natrii phosphatis (³²P) solutio iniectabilis

DEFINITION

Sodium phosphate (³²P) injection is a sterile solution of disodium and monosodium (³²P) orthophosphates made isotonic by the addition of sodium chloride. Phosphorus-32 is a radioactive isotope of phosphorus and may be produced by neutron irradiation of sulphur. The injection contains not less than 90.0 per cent and not more than 110.0 per cent of the declared phosphorus-32 radioactivity at the date and hour stated on the label. Not less than 95 per cent of the radioactivity corresponds to phosphorus-32 in the form of orthophosphate ion. The specific radioactivity is not less than 11.1 MBq of phosphorus-32 per milligram of orthophosphate ion.

CHARACTERS

A clear, colourless solution.

Phosphorus-32 has a half-life of 14.3 days and emits beta radiation.

IDENTIFICATION

- A. Record the beta-ray spectrum or the beta-ray absorption curve using a suitable method. The spectrum or curve does not differ significantly from that of a standardised phosphorus-32 solution obtained under the same conditions. Standardised phosphorus-32 solutions are available from laboratories recognised by the competent authority. The maximum energy of the beta radiation is 1.71 MeV.
- B. Examine the chromatogram obtained in the test for radiochemical purity. The distribution of radioactivity contributes to the identification of the preparation.

TESTS

pH (2.2.3). The pH of the injection is 6.0 to 8.0.

Phosphates. Dilute the injection with *water R* to give a radioactive concentration of 370 kBq of phosphorus-32 per millilitre. Mix in a volumetric flask, with shaking, 1.0 ml of the solution with a mixture of 0.5 ml of a 2.5 g/l solution of *ammonium vanadate R*, 0.5 ml of *ammonium molybdate solution R* and 1 ml of *perchloric acid R* and dilute to 5.0 ml with *water R*. After 30 min, the solution is not more intensely coloured than a standard prepared at the same time in the same manner using 1.0 ml of a solution containing 33 mg of orthophosphate ion per litre.

Sterility. It complies with the test for sterility prescribed in the monograph on *Radiopharmaceutical preparations (0125)*. The injection may be released for use before completion of the test.

RADIONUCLIDIC PURITY

Record the beta-ray spectrum or the beta-ray absorption curve using a suitable method. The spectrum or curve does not differ significantly from that of a standardised phosphorus-32 solution obtained under the same conditions.

RADIOCHEMICAL PURITY

Examine by ascending paper chromatography (2.2.26). **Test solution.** Dilute the injection with *water R* until the radioactivity is equivalent to 10 000 to 20 000 counts per minute per 10 µl

Reference solution. Prepare a solution of *phosphoric acid R* containing 2 mg of phosphorus per millilitre.

Using a strip of paper 25 mm wide and about 300 mm long, apply 10 µl of the reference solution. Apply to the same starting-point 10 µl of the test solution. Develop for 16 h using a mixture of 0.3 ml of *ammonia R*, 5 g of *trichloroacetic acid R*, 25 ml of *water R* and 75 ml of *2-propanol R*. Allow the paper to dry in air. Determine the position of the inactive phosphoric acid by spraying with a 50 g/l solution of *perchloric acid R* and then with a 10 g/l solution of *ammonium molybdate R*. Expose the paper to *hydrogen sulphide R*. A blue colour develops. Determine the position of the radioactive spot by autoradiography or by measuring the radioactivity over the whole length of the chromatogram. Not less than 95 per cent of the total radioactivity of the chromatogram is found in the spot corresponding to phosphoric acid.

RADIOACTIVITY

Measure the radioactivity using suitable counting equipment by comparison with a standardised phosphorus-32 solution or by measurement in an instrument calibrated with the aid of such a solution.

01/2005:1475

STRONTIUM (⁸⁹Sr) CHLORIDE INJECTION

Strontii (⁸⁹Sr) chloridi solutio iniectionabilis

DEFINITION

Strontium (⁸⁹Sr) chloride injection is a sterile solution of [⁸⁹Sr]strontium chloride. Strontium-89 is a radioactive isotope of strontium and is produced by neutron irradiation of strontium enriched in strontium-88. The injection contains not less than 90.0 per cent and not more than 110.0 per cent of the declared strontium-89 radioactivity at the date stated on the label. Not more than 0.6 per cent of

the total radioactivity is due to radionuclides other than strontium-89. The specific radioactivity is not less than 1.8 MBq of strontium-89 per milligram of strontium. The injection contains 6.0 mg/ml to 12.5 mg/ml of strontium.

CHARACTERS

A clear, colourless solution.

Strontium-89 has a half-life of 50.5 days and emits beta radiation with a maximum energy of 1.492 MeV.

IDENTIFICATION

- Record the gamma-ray and X-ray spectrum using a suitable instrument. The spectrum does not differ significantly from that of a standardised strontium-89 solution, when measured either by direct comparison or by using an instrument calibrated with the aid of such a solution. Standardised strontium-89 solutions are available from laboratories recognised by the competent authority. The gamma photon detected has an energy of 0.909 MeV and is due to the short-lived daughter product, yttrium-89m (formed in 0.01 per cent of the disintegrations), in equilibrium with the strontium-89.
- To 0.1 ml of the injection to be examined, add 1 ml of a freshly prepared 1 g/l solution of *sodium rhodizonate R*. Mix and allow to stand for 1 min. A reddish-brown precipitate is formed.
- To 0.1 ml of *silver nitrate solution R2* add 50 µl of the injection to be examined. A white precipitate is formed.

TESTS

pH (2.2.3). The pH of the solution is 4.0 to 7.5.

Note: the following tests for aluminium, iron and lead may be carried out simultaneously with the test for strontium. If this is not the case, the reference solutions are prepared such that they contain strontium at approximately the same concentration as in the test solution.

Aluminium. Not more than 2 µg/ml, determined by atomic emission spectrometry (plasma or arc method) (2.2.22, *Method I*).

Test solution. Dilute 0.2 ml of the injection to be examined to a suitable volume with *dilute nitric acid R*.

Reference solutions. Prepare the reference solutions using *aluminium standard solution (10 ppm Al) R* diluted as necessary with *dilute nitric acid R*.

Iron. Not more than 5 µg/ml, determined by atomic emission spectrometry (plasma or arc method) (2.2.22, *Method I*).

Test solution. Dilute 0.2 ml of the injection to be examined to a suitable volume with *dilute nitric acid R*.

Reference solutions. Prepare the reference solutions using *iron standard solution (20 ppm Fe) R* diluted as necessary with *dilute nitric acid R*.

Lead. Not more than 5 µg/ml, determined by atomic emission spectrometry (plasma or arc method) (2.2.22, *Method I*).

Test solution. Dilute 0.2 ml of the injection to be examined to a suitable volume with *dilute nitric acid R*.

Reference solutions. Prepare the reference solutions using *lead standard solution (10 ppm Pb) R* diluted as necessary with *dilute nitric acid R*.

Strontium. 6.0 mg/ml to 12.5 mg/ml. Examine by atomic emission spectrometry (2.2.22, *Method I*).

Test solution. Dilute 0.2 ml of the injection to be examined to a suitable volume with *dilute nitric acid R*.

Reference solutions. Prepare the reference solutions using *strontium standard solution (1.0 per cent Sr) R* diluted as necessary with *dilute nitric acid R*.

Sterility. It complies with the test for sterility prescribed in the monograph on *Radiopharmaceutical preparations* (0125).

RADIONUCLIDIC PURITY

Gamma emitters. Record the gamma-ray and X-ray spectrum of the injection to be examined using a suitable instrument. Not more than 0.4 per cent of the total radioactivity in the preparation to be examined is due to gamma emitting radionuclides other than yttrium-89m.

Beta emitters. Evaporate to dryness 100 µl of the injection to be examined under a radiant heat source. Dissolve the residue in 2 ml of *47 per cent hydrobromic acid R*, evaporate to dryness under the radiant heat source and dissolve the residue in 2 ml of *dilute hydrobromic acid R1*. Transfer the solution to the top of a column, 5 mm to 6 mm in diameter, packed with approximately 2 ml of *cationic exchange resin R1* (100 µm to 250 µm), previously conditioned with *dilute hydrobromic acid R1* and elute the column with the same solvent until 10 ml of eluate has been collected into a container containing 50 µl of a 15 g/l solution of *anhydrous sodium sulphate R* in *1 M hydrochloric acid*.

To a liquid scintillation vial add an appropriate volume of scintillation liquid followed by 1 ml of *water R*, 0.1 ml of a 15 g/l solution of *anhydrous sodium sulphate R* in *1 M hydrochloric acid* and 100 µl of eluate. Shake to obtain a clear solution. Using suitable counting equipment determine the radioactivity due to sulphur-35 and phosphorus-32 in the sample.

Taking into account the recovery efficiency of the separation, counting efficiency and radioactive decay, determine the radioactive concentration of sulphur-35 and phosphorus-32 in the sample and hence the percentage of total beta emitting impurities in the injection to be examined. Not more than 0.2 per cent of the total radioactivity in the injection to be examined is due to the sum of sulphur-35 and phosphorus-32 radioactivities.

RADIOACTIVITY

Measure the radioactivity using suitable equipment by comparison with a standardised strontium-89 solution or by measurement in an instrument calibrated with the aid of such a solution.

01/2005:0126

TECHNETIUM (^{99m}Tc) COLLOIDAL RHENIUM SULPHIDE INJECTION

Rhenii sulfidi colloidalis et technetii (^{99m}Tc) solutio iniectionabilis

DEFINITION

Technetium (^{99m}Tc) colloidal rhenium sulphide injection is a sterile, apyrogenic colloidal dispersion of rhenium sulphide the micelles of which are labelled with technetium-99m. It is stabilised with gelatin. The injection contains not less than 90.0 per cent and not more than 110.0 per cent of the declared technetium-99m radioactivity at the date and hour stated on the label. Not less than 92 per cent of the radioactivity corresponds to technetium-99m in colloidal form. The pH of the injection may be adjusted by the addition of a suitable buffer such as a citrate buffer solution. The injection contains a variable amount of colloidal rhenium sulphide, not exceeding 0.22 mg of rhenium (Re) per millilitre, according to the method of preparation.

It is prepared from sodium pertechnetate (^{99m}Tc) injection (fission or non-fission) using suitable sterile, apyrogenic ingredients and calculating the ratio of radionuclidic impurities with reference to the date and hour of administration.

CHARACTERS

A light-brown liquid.

Technetium-99m has a half-life of 6.02 h and emits gamma radiation.

IDENTIFICATION

- Record the gamma-ray spectrum using a suitable instrument. The spectrum does not differ significantly from that of a standardised technetium-99m solution either by direct comparison or by using an instrument calibrated with the aid of such a solution. Standardised technetium-99m and molybdenum-99 solutions are available from laboratories recognised by the competent authority. The most prominent gamma photon of technetium-99m has an energy of 0.140 MeV.
- Examine the chromatogram obtained in the test for radiochemical purity. The distribution of radioactivity contributes to the identification of the injection.
- To 1 ml add 5 ml of *hydrochloric acid R*, 5 ml of a 50 g/l solution of *thiourea R* and 1 ml of a 200 g/l solution of *stannous chloride R* in *hydrochloric acid R*. A yellow colour is produced.

TESTS

pH (2.2.3). The pH of the injection is 4.0 to 7.0.

Rhenium

Test solution. Use 1 ml of the injection to be examined.

Reference solutions. Using a solution containing 100 µg of *potassium perrhenate R* (equivalent to 60 ppm of Re) and 240 µg of *sodium thiosulphate R* per millilitre, prepare a suitable range of solutions and dilute to the same final volume with *water R*.

To the test solution and to 1 ml of each of the reference solutions add 5 ml of *hydrochloric acid R*, 5 ml of a 50 g/l solution of *thiourea R* and 1 ml of a 200 g/l solution of *stannous chloride R* in *hydrochloric acid R* and dilute to 25.0 ml with *water R*. Allow to stand for 40 min and measure the absorbance (2.2.25) of each solution at 400 nm, using a reagent blank as the compensation liquid. Using the absorbances obtained with the reference solutions, draw a calibration curve and calculate the concentration of rhenium in the injection to be examined.

Physiological distribution. Inject a volume not greater than 0.2 ml into the caudal vein of each of three mice each weighing 20 g to 25 g. Sacrifice the mice 20 min after the injection, remove the liver, spleen and lungs and measure the radioactivity in the organs using a suitable instrument. Measure the radioactivity in the rest of the body after having removed the tail. Determine the percentage of radioactivity in the liver, the spleen and the lungs from the expression:

$$\frac{A}{B} \times 100$$

- A* = radioactivity of the organ concerned,
B = total radioactivity in the liver, the spleen, the lungs and the rest of the body.

In each of the three mice at least 80 per cent of the radioactivity is found in the liver and spleen and not more than 5 per cent in the lungs. If the distribution of radioactivity in one of the three mice does not correspond

to the prescribed proportions, repeat the test on a further three mice. The preparation complies with the test if the prescribed distribution of radioactivity is found in five of the six mice used. The injection may be released for use before completion of the test.

Sterility. It complies with the test for sterility prescribed in the monograph on *Radiopharmaceutical preparations (0125)*. The injection may be released for use before completion of the test.

Pyrogens. It complies with the test for pyrogens prescribed in the monograph on *Radiopharmaceutical preparations (0125)*. Inject not less than 0.1 ml per kilogram of the rabbit's mass. The injection may be released for use before completion of the test.

RADIOCHEMICAL PURITY

Examine by ascending paper chromatography (2.2.26). Apply to the paper 10 µl of the injection. Develop immediately over a path of 10 cm to 15 cm using a 9 g/l solution of *sodium chloride R*. Allow the paper to dry. Determine the distribution of radioactivity using a suitable detector. Technetium-99m in colloidal form remains at the starting-point and pertechnetate ion migrates with an *R_f* of about 0.6. There may be other impurities with an *R_f* of 0.8 to 0.9. The radioactivity corresponding to technetium-99m in colloidal form represents not less than 92 per cent of the total radioactivity of the chromatogram.

RADIOACTIVITY

Measure the radioactivity using suitable counting equipment by comparison with a standardised technetium-99m solution or by measurement in an instrument calibrated with the aid of such a solution.

LABELLING

The label states, in particular, the quantity of rhenium per millilitre.

01/2005:0131

TECHNETIUM (^{99m}Tc) COLLOIDAL SULPHUR INJECTION

Sulfuris colloidalis et technetii (^{99m}Tc) solutio iniectabilis

DEFINITION

Technetium (^{99m}Tc) colloidal sulphur injection is a sterile, apyrogenic colloidal dispersion of sulphur, the micelles of which are labelled with technetium-99m. It may be stabilised with a colloid-protecting substance based on gelatin. The injection contains not less than 90.0 per cent and not more than 110.0 per cent of the declared technetium-99m radioactivity at the date and hour stated on the label. Not less than 92 per cent of the radioactivity corresponds to technetium-99m in colloidal form. The pH of the injection may be adjusted by the addition of a suitable buffer, such as an acetate, citrate or phosphate buffer solution. The injection contains a variable amount of colloidal sulphur, according to the method of preparation.

It is prepared from sodium pertechnetate (^{99m}Tc) injection (fission or non-fission) using suitable sterile, apyrogenic ingredients and calculating the ratio of radionuclidic impurities with reference to the date and hour of administration.

CHARACTERS

A clear to opalescent, colourless to yellowish liquid.

Technetium-99m has a half-life of 6.02 h and emits gamma radiation.

IDENTIFICATION

- Record the gamma-ray spectrum using a suitable instrument. The spectrum does not differ significantly from that of a standardised technetium-99m solution either by direct comparison or by using an instrument calibrated with the aid of such a solution. Standardised technetium-99m and molybdenum-99 solutions are available from laboratories recognised by the competent authority. The most prominent gamma photon of technetium-99m has an energy of 0.140 MeV.
- Examine the chromatogram obtained in the test for radiochemical purity. The distribution of radioactivity contributes to the identification of the injection.
- In a test-tube 100 mm long and 16 mm in internal diameter, evaporate 0.2 ml of the injection to dryness. Dissolve the sulphur by shaking the residue with 0.2 ml of *pyridine R* and add about 20 mg of *benzoïn R*. Cover the open end of the tube with a filter paper moistened with *lead acetate solution R*. Heat the test-tube in a bath containing glycerol at 150 °C. The paper slowly becomes brown.

TESTS

pH (2.2.3). The pH of the injection is 4.0 to 7.0.

Physiological distribution. Inject a volume not greater than 0.2 ml into the caudal vein of each of 3 mice, each weighing 20 g to 25 g. Sacrifice the mice 20 min after the injection, remove the liver, spleen and lungs and measure the radioactivity in the organs using a suitable instrument. Measure the radioactivity in the rest of the body after having removed the tail. Determine the percentage of radioactivity in the liver, the spleen and the lungs from the expression:

$$\frac{A}{B} \times 100$$

- A* = radioactivity of the organ concerned,
B = total radioactivity in the liver, the spleen, the lungs and the rest of the body.

In each of the 3 mice at least 80 per cent of the radioactivity is found in the liver and spleen and not more than 5 per cent in the lungs. If the distribution of radioactivity in 1 of the 3 mice does not correspond to the prescribed proportions, repeat the test on a further 3 mice. The preparation complies with the test if the prescribed distribution of radioactivity is found in 5 of the 6 mice used. The injection may be released for use before completion of the test.

Sterility. It complies with the test for sterility prescribed in the monograph on *Radiopharmaceutical preparations (0125)*. The injection may be released for use before completion of the test.

Pyrogens. It complies with the test for pyrogens prescribed in the monograph on *Radiopharmaceutical preparations (0125)*. Inject not less than 0.1 ml per kilogram of the rabbit's mass. The injection may be released for use before completion of the test.

RADIOCHEMICAL PURITY

Examine by ascending paper chromatography (2.2.26). Apply to the paper 10 µl of the injection. Develop immediately over a path of 10 cm to 15 cm with a 9 g/l solution of *sodium chloride R*. Allow the paper to dry. Determine the distribution of radioactivity using a suitable detector. Technetium-99m in colloidal form remains at the starting-point and pertechnetate ion migrates with an *R_f*

of 0.6. There may be other impurities of R_f 0.8 to 0.9. The radioactivity corresponding to technetium-99m in colloidal form represents not less than 92 per cent of the total radioactivity of the chromatogram.

RADIOACTIVITY

Measure the radioactivity using suitable counting equipment by comparison with a standardised technetium-99m solution or by measurement in an instrument calibrated with the aid of such a solution.

01/2005:0689

TECHNETIUM (^{99m}Tc) COLLOIDAL TIN INJECTION

Stanni colloidalis et technetii (^{99m}Tc) solutio iniectionabilis

DEFINITION

Technetium (^{99m}Tc) colloidal tin injection is a sterile, colloidal dispersion of tin labelled with technetium-99m. The injection contains a variable quantity of tin not exceeding 1 mg of Sn per millilitre; it contains fluoride ions, it may be stabilised with a suitable, apyrogenic colloid-protecting substance and it may contain a suitable buffer. The injection contains not less than 90.0 per cent and not more than 110.0 per cent of the declared technetium-99m radioactivity at the date and hour stated on the label. Not less than 95 per cent of the radioactivity corresponds to technetium-99m in colloidal form.

It is prepared from sodium pertechnetate (^{99m}Tc) injection (fission or non-fission) using suitable sterile ingredients and calculating the ratio of radionuclidic impurities with reference to the date and hour of administration. Syringes for handling the eluate intended for labelling of the final product, or the final product, should not contain rubber parts.

CHARACTERS

A clear or opalescent, colourless liquid.

Technetium-99m has a half life of 6.02 h and emits gamma radiation.

IDENTIFICATION

- Record the gamma-ray spectrum using a suitable instrument. The spectrum does not differ significantly from that of a standardised technetium-99m solution either by direct comparison or by using an instrument calibrated with the aid of such a solution. Standardised technetium-99m and molybdenum-99 solutions are available from laboratories recognised by the competent authority. The most prominent gamma photon of technetium-99m has an energy of 0.140 MeV.
- Mix 0.05 ml of *zirconyl nitrate solution R* with 0.05 ml of *alizarin S solution R*. Add 0.05 ml of the injection to be examined. A yellow colour is produced.

TESTS

pH (2.2.3). The pH of the injection to be examined is 4.0 to 7.0.

Tin

Test solution. Dilute 3.0 ml of the injection to be examined to 50.0 ml with 1 M hydrochloric acid.

Reference solution. Dissolve 0.115 g of *stannous chloride R* in 1 M hydrochloric acid and dilute to 1000.0 ml with the same acid.

To 1.0 ml of each solution add 0.4 ml of a 20 g/l solution of *sodium laurilsulfate R*, 0.05 ml of *thioglycollic acid R*, 0.1 ml of *dithiol reagent R* and 3.0 ml of 0.2 M hydrochloric acid. Mix. Measure the absorbance (2.2.25) of each solution at 540 nm, using 0.2 M hydrochloric acid as the compensation liquid. The absorbance of the test solution is not greater than that of the reference solution (1 mg of Sn per millilitre).

Physiological distribution. Inject not more than 0.2 ml into a caudal vein of each of three mice, each weighing 20 g to 25 g. Kill the mice 20 min after the injection and remove the liver, spleen and lungs. Measure the radioactivity in the organs using a suitable instrument. Measure the radioactivity in the rest of the body, after having removed the tail. Determine the percentage of radioactivity in the liver, the spleen and the lungs with respect to the total radioactivity of all organs and the rest of the body excluding the tail.

In each of the three mice at least 80 per cent of the radioactivity is found in the liver and spleen and not more than 5 per cent in the lungs. If the distribution of radioactivity in one of the three mice does not correspond to the prescribed proportions, repeat the test on a further three mice. The preparation complies with the test if the prescribed distribution of radioactivity is found in five of the six mice used.

Sterility. It complies with the test for sterility prescribed in the monograph on *Radiopharmaceutical preparations (0125)*. The injection may be released for use before completion of the test.

RADIOCHEMICAL PURITY

Examine by thin-layer chromatography (2.2.27) using silica gel as the coating substance on a glass-fibre sheet. Heat the plate at 110 °C for 10 min. Use a plate such that during development the mobile phase migrates over a distance of 10 cm to 15 cm in about 10 min.

Apply to the plate 5 µl to 10 µl of the injection to be examined. Develop immediately over a path of 10 cm to 15 cm using a 9 g/l solution of *sodium chloride R* purged with *nitrogen R*. Allow the plate to dry. Determine the distribution of radioactivity using a suitable detector. Technetium-99m in colloidal form remains at the starting point and pertechnetate ion migrates near to the solvent front. Not less than 95 per cent of the technetium-99m radioactivity corresponds to technetium in colloidal form.

RADIOACTIVITY

Measure the radioactivity using suitable counting equipment by comparison with a standardised technetium-99m solution or by measurement in an instrument calibrated with the aid of such a solution.

01/2005:0585

TECHNETIUM (^{99m}Tc) ETIFENIN INJECTION

Technetii (^{99m}Tc) et etifenini solutio iniectionabilis

DEFINITION

Technetium (^{99m}Tc) etifenin injection is a sterile solution which may be prepared by mixing sodium pertechnetate (^{99m}Tc) injection (fission or non-fission) with solutions of etifenin [(2,6-diethylphenyl)carbamoylmethylimino]di-acetic acid; $\text{C}_{16}\text{H}_{22}\text{N}_2\text{O}_5$] and stannous chloride. The injection contains a variable quantity of tin (Sn) not exceeding 0.2 mg/ml. The injection contains not less than 90.0 per cent and not more than 110.0 per cent of the declared

technetium-99m radioactivity at the date and hour stated on the label. Not less than 95.0 per cent of the radioactivity corresponds to technetium-99m complexed with etifenin.

It is prepared from sodium pertechnetate (^{99m}Tc) injection (fission or non-fission) using suitable, sterile ingredients and calculating the ratio of radionuclidic impurities with reference to the date and hour of administration.

CHARACTERS

A clear, colourless solution.

Technetium-99m has a half-life of 6.02 h and emits gamma radiation.

IDENTIFICATION

A. Record the gamma-ray spectrum using a suitable instrument. The spectrum does not differ significantly from that of a standardised technetium-99m solution either by direct comparison or by using an instrument calibrated with the aid of such a solution. Standardised technetium-99m and molybdenum-99 solutions are available from laboratories recognised by the competent authority. The most prominent gamma photon of technetium-99m has an energy of 0.140 MeV.

B. Examine by liquid chromatography (2.2.29).

Test solution. Dilute the injection to be examined with *methanol R* to obtain a solution containing about 1 mg of etifenin per millilitre.

Reference solution. Dissolve 5.0 mg of *etifenin CRS* in *methanol R* and dilute to 5.0 ml with the same solvent.

The chromatographic procedure may be carried out using:

- a column 0.25 m long and 4.6 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (5 µm to 10 µm),
- as mobile phase at a flow rate of 1 ml/min a mixture of 20 volumes of *methanol R* and 80 volumes of a 14 g/l solution of *potassium dihydrogen phosphate R* adjusted to pH 2.5 by the addition of *phosphoric acid R*,
- a spectrophotometer set at 230 nm.

Inject 20 µl of each solution. The principal peak in the chromatogram obtained with the test solution has a similar retention time to the principal peak in the chromatogram obtained with the reference solution.

TESTS

pH (2.2.3). The pH of the injection is 4.0 to 6.0.

Physiological distribution. Inject 0.1 ml (equivalent to about 3.7 MBq) into a caudal vein of each of three mice, each weighing 20 g to 25 g. Kill the mice 1 h after the injection. Remove the liver, gall-bladder, small intestine, large intestine and kidneys, collecting excreted urine. Measure the radioactivity in the organs using a suitable instrument. Measure the radioactivity of the rest of the body, after having removed the tail. Determine the percentage of radioactivity in each organ from the expression:

$$\frac{A}{B} \times 100$$

A = radioactivity of the organ concerned,

B = radioactivity of all organs and the rest of the body, excluding the tail.

In not fewer than two mice the sum of the percentages of radioactivity in the gall-bladder and small and large intestine is not less than 80 per cent. Not more than 3 per cent of the radioactivity is present in the liver, and not more than 2 per cent in the kidneys

Tin

Test solution. Dilute 1.0 ml of the injection to be examined to 5.0 ml with 1 M *hydrochloric acid*.

Reference solution. Prepare a reference solution containing 0.075 mg of *stannous chloride R* per millilitre in 1 M *hydrochloric acid*.

To 1.0 ml of each solution add 0.4 ml of a 20 g/l solution of *sodium laurilsulfate R*, 0.05 ml of *thioglycolic acid R*, 0.1 ml of *dithiol reagent R* and 3.0 ml of 0.2 M *hydrochloric acid*. Mix. Measure the absorbance (2.2.25) of each solution at 540 nm, using 0.2 M *hydrochloric acid* as the compensation liquid. The absorbance of the test solution is not greater than that of the reference solution (0.2 mg of Sn per millilitre).

Sterility. It complies with the test for sterility prescribed in the monograph on *Radiopharmaceutical preparations* (0125). The injection may be released for use before completion of the test.

RADIOCHEMICAL PURITY

Examine by thin-layer chromatography (2.2.27) using silicic acid as the coating substance on a glass-fibre sheet. Heat the plate at 110 °C for 10 min. The plate used should be such that during development the mobile phase moves over a distance of 10 cm to 15 cm in about 15 min.

Apply to the plate 5 µl to 10 µl of the injection to be examined. Develop immediately over a path of 10 cm to 15 cm using a 9 g/l solution of *sodium chloride R*. Allow the plate to dry. Determine the distribution of radioactivity using a suitable detector. Technetium-99m complexed with etifenin migrates almost to the middle of the chromatogram and pertechnetate ion migrates with the solvent front. Impurities in colloidal form remain at the starting point. The radioactivity corresponding to technetium-99m complexed with etifenin represents not less than 95.0 per cent of the total radioactivity of the chromatogram.

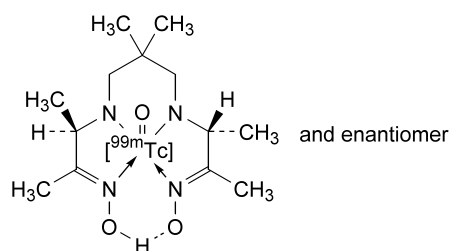
RADIOACTIVITY

Measure the radioactivity using suitable counting equipment by comparison with a standardised technetium-99m solution or by measurement in an instrument calibrated with the aid of such a solution.

01/2005:1925

TECHNETIUM (^{99m}Tc) EXAMETAZIME INJECTION

Technetii (^{99m}Tc) exametazimi solutio iniectionis



DEFINITION

Sterile solution of lipophilic technetium-99m exametazime which may be prepared by dissolving a racemic mixture of (3*RS*,9*RS*)-4,8-diaza-3,6,6,9-tetramethylundecane-2,10-dione bisoxime in the presence of a stannous salt in *Sodium pertechnetate (^{99m}Tc) injection (fission) (0124)* or *Sodium pertechnetate (^{99m}Tc) injection (non-fission) (0283)*. It may contain stabilisers and inert additives.

Content: 90 per cent to 110 per cent of the declared technetium-99m radioactivity at the date and time stated on the label.

Purity: minimum of 80 per cent of the total radioactivity corresponds to lipophilic technetium-99m exametazime and its *meso* isomer.

CHARACTERS

Appearance: clear solution.

Half-life and nature of radiation of technetium-99m: see *Table of physical characteristics of radionuclides (5.7)*.

IDENTIFICATION

A. Gamma-ray spectrometry.

Comparison: standardised technetium-99m solution, or by using a calibrated instrument. Standardised technetium-99m solutions and/or standardisation services are available from the competent authority.

Results: the spectrum obtained with the solution to be examined does not differ significantly from that obtained with a standardised technetium-99m solution. The most prominent gamma photon has an energy of 0.141 MeV.

B. Examine the chromatograms obtained in the test Impurity A under Radiochemical purity.

Results: the principal peak in the chromatogram obtained with the test solution is similar in retention time to the peak due to lipophilic technetium-99m exametazime in the chromatogram obtained with the reference solution.

TESTS

pH (2.2.3): 5.0 to 10.0.

Sterility. It complies with the test for sterility prescribed in the monograph on *Radiopharmaceutical preparations (0125)*. The injection may be released for use before completion of the test.

RADIOCHEMICAL PURITY

Impurity C. Thin-layer chromatography (2.2.27).

Test solution. The preparation to be examined.

Plate: TLC silica gel plate R; use a glass-fibre plate.

Mobile phase: 9 g/l solution of sodium chloride R.

Application: about 5 μl .

Development: immediate, over 2/3 of the plate.

Drying: in air.

Detection: determine the distribution of radioactivity using a suitable detector.

Retention factors: impurity C = 0.8 to 1.0; lipophilic technetium-99m exametazime and impurities A, B, D and E do not migrate.

Limits:

- **impurity C:** maximum 10 per cent of the total radioactivity.

Total of lipophilic technetium-99m exametazime and impurity A. Thin-layer chromatography (2.2.27).

Test solution. The preparation to be examined.

Plate: TLC silica gel plate R; use a glass-fibre plate.

Mobile phase: methyl ethyl ketone R.

Application: about 5 μl .

Development: immediate, over 2/3 of the plate.

Drying: in air.

Detection: determine the distribution of radioactivity using a suitable detector.

Retention factors: lipophilic technetium-99m exametazime = 0.8 to 1.0, impurity A = 0.8 to 1.0, impurity C = 0.8 to 1.0; impurities B, D and E do not migrate.

Limits: calculate the percentage of radioactivity due to impurities B, D and E from test B (B) and the percentage of the radioactivity due to impurity C from test A (A). Calculate the total percentage of lipophilic technetium-99m exametazime and impurity A from the expression:

$$100 - A - B$$

- **total of lipophilic technetium-99m exametazime and impurity A:** minimum 80 per cent of the total radioactivity.

Impurity A. Liquid chromatography (2.2.29).

Test solution. The preparation to be examined.

Reference solution. Dissolve the contents of a vial of *meso-rich exametazime CRS* in 0.5 ml of a 9 g/l solution of sodium chloride R and transfer to a lead-shielded, nitrogen-filled vial. Add 6 μl of a freshly prepared 1 g/l solution of stannous chloride R in 0.05 M hydrochloric acid and 2.5 ml of sodium pertechnetate (^{99m}Tc) injection (fission or non-fission) containing 370-740 MBq. Mix carefully and use within 30 min of preparation.

Column:

- **size:** $l = 0.25 \text{ m}$, $\varnothing = 4.6 \text{ mm}$,
- **stationary phase:** spherical base-deactivated end-capped octadecylsilyl silica gel for chromatography R (5 μm) with a pore size of 13 nm and a carbon loading of 11 per cent.

Mobile phase: mix 33 volumes of acetonitrile R and 67 volumes of 0.1 M phosphate buffer solution pH 3.0 R.

Flow rate: 1.5 ml/min.

Detection: radioactivity detector.

Injection: loop injector.

Run time: 20 min.

Relative retention with reference to lipophilic technetium-99m exametazime: impurity A = about 1.2.

System suitability: reference solution:

- chromatogram similar to the chromatogram provided with *meso-rich exametazime CRS*,
- **resolution:** minimum of 2 between the peaks due to lipophilic technetium-99m exametazime and to impurity A.

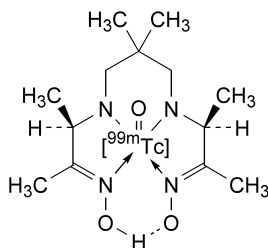
Limits:

- **impurity A:** maximum 5 per cent of the radioactivity due to lipophilic technetium-99m exametazime and impurity A.

RADIOACTIVITY

Measure the radioactivity using suitable equipment by comparison with a standardised technetium-99m solution or by using a calibrated instrument.

IMPURITIES



- A. *meso* isomer of lipophilic technetium-99m exametazime,
- B. technetium-99m in colloidal form,
- C. [^{99m}Tc]pertechnetate ion,
- D. non lipophilic technetium-99m exametazime complex,
- E. *meso* isomer of non lipophilic technetium-99m exametazime complex.

01/2005:1047

TECHNETIUM (^{99m}Tc) GLUCONATE INJECTION

Technetii (^{99m}Tc) gluconatis solutio iniectionis

DEFINITION

Technetium (^{99m}Tc) gluconate injection is a sterile solution, which may be prepared by mixing solutions of calcium gluconate and a stannous salt or other suitable reducing agent with sodium pertechnetate (^{99m}Tc) injection (fission or non-fission). The injection contains not less than 90.0 per cent and not more than 110.0 per cent of the declared technetium-99m radioactivity at the date and hour stated on the label. Not less than 90 per cent of the radioactivity corresponds to technetium-99m gluconate complex.

It is prepared from sodium pertechnetate (^{99m}Tc) injection (fission or non-fission) using suitable sterile ingredients and calculating the ratio of radionuclidic impurities with reference to the date and hour of administration.

CHARACTERS

A slightly opalescent solution.

Technetium-99m has a half-life of 6.02 h and emits gamma radiation.

IDENTIFICATION

- A. Record the gamma-ray spectrum using a suitable instrument. The spectrum does not differ significantly from that of a standardised technetium-99m solution either by direct comparison or by using an instrument calibrated with the aid of such a solution. Standardised technetium-99m and molybdenum-99 solutions are available from laboratories recognised by the competent authority. The most prominent gamma photon of technetium-99m has an energy of 0.140 MeV.
- B. 5 μl of the solution complies with identification A prescribed in the monograph on *Calcium gluconate* (0172).
- C. Examine the chromatograms obtained in the test for radiochemical purity. The distribution of the radioactivity contributes to the identification of the preparation.

TESTS

pH (2.2.3). The pH of the solution is 6.0 to 8.5.

Physiological distribution. Inject a volume not greater than 0.2 ml into the caudal vein of each of three rats weighing 150 g to 250 g. Measure the radioactivity of the syringe before and after injection. Sacrifice the rats 30 min after the injection. Remove at least 1 g of blood by a suitable method and remove the kidneys, the liver, the bladder plus voided urine and the tail. Weigh the sample of blood.

Determine the radioactivity in the organs, the blood sample and the tail using a suitable instrument. Calculate the percentage of radioactivity in each organ and in 1 g of blood with respect to the total radioactivity calculated as the difference between the two measurements made on the syringe minus the activity in the tail. Correct the blood concentration by multiplying by a factor of $m/200$ where m is the body mass of the rat in grams.

In not fewer than two of the three rats used, the radioactivity in the kidneys is not less than 15 per cent, that in the bladder plus voided urine is not less than 20 per cent and that in the liver is not more than 5 per cent. The radioactivity in the blood, after correction, is not more than 0.50 per cent.

Sterility. It complies with the test for sterility prescribed in the monograph on *Radiopharmaceutical preparations* (0125). The injection may be released for use before completion of the test.

RADIOCHEMICAL PURITY

Examine by thin-layer chromatography (2.2.27) using silica gel as the coating substance on a glass-fibre sheet. Heat the plate at 110 °C for 10 min. Use a plate such that during development the mobile phase migrates over a distance of 10 cm to 15 cm in about 10 min.

a) Apply to the plate 5 μl to 10 μl of the solution to be examined. Develop immediately over a path of 10 cm to 15 cm using a 9 g/l solution of *sodium chloride R*. Allow the plate to dry. Determine the distribution of radioactivity using a suitable detector. Impurities in colloidal form remain at the starting point. Technetium gluconate complex and pertechnetate ion migrate near to the solvent front.

b) Apply to the plate 5 μl to 10 μl of the solution to be examined and allow to dry. Develop over a path of 10 cm to 15 cm using *methyl ethyl ketone R*. Dry in a current of warm air. Determine the distribution of radioactivity using a suitable detector. Pertechnetate ion impurity migrates near to the solvent front. Technetium gluconate complex and technetium in colloidal form remain at the starting point.

The sum of the percentages of radioactivity corresponding to impurities in the chromatograms obtained in test (a) and (b) does not exceed 10 per cent.

RADIOACTIVITY

Measure the radioactivity using suitable counting equipment by comparison with a standardised technetium-99m solution or by measurement in an instrument calibrated with the aid of such a solution.

01/2005:0640

TECHNETIUM (^{99m}Tc) HUMAN ALBUMIN INJECTION

Technetii (^{99m}Tc) humani albumini solutio iniectionis

DEFINITION

Technetium (^{99m}Tc) human albumin injection is a sterile, apyrogenic solution of human albumin labelled with technetium-99m. It contains a reducing substance, such as a tin salt in an amount not exceeding 1 mg of Sn per millilitre;

it may contain a suitable buffer and an antimicrobial preservative. Although, at present, no definite value for a maximum limit of tin can be fixed, available evidence tends to suggest the importance of keeping the ratio of tin to albumin as low as possible. The human albumin used complies with the requirements of the monograph on *Human albumin solution* (0255). The injection contains not less than 90.0 per cent and not more than 110.0 per cent of the declared technetium-99m radioactivity at the date and hour stated on the label. The injection contains not less than 90.0 per cent and not more than 110.0 per cent of the quantity of albumin stated on the label. Not less than 80 per cent of the radioactivity is associated with the albumin fractions II to V. Not more than 5.0 per cent of the radioactivity due to technetium-99m corresponds to free pertechnetate, as determined by the method described in the test for radiochemical purity.

It is prepared from sodium pertechnetate (^{99m}Tc) injection (fission or non-fission) using suitable sterile and apyrogenic ingredients and calculating the ratio of radionuclidic impurities with reference to the date and hour of administration.

CHARACTERS

A clear, colourless or pale-yellow solution.

Technetium-99m has a half-life of 6.02 h and emits gamma radiation.

IDENTIFICATION

- A. Record the gamma-ray spectrum using a suitable instrument. The spectrum does not differ significantly from that of a standardised technetium-99m solution either by direct comparison or by using an instrument calibrated with the aid of such a solution. Standardised technetium-99m and molybdenum-99 solutions are available from laboratories recognised by the competent authority. The most prominent gamma photon of technetium-99m has an energy of 0.140 MeV.
- B. Using a suitable range of species-specific antisera, carry out precipitation tests on the preparation to be examined. The test is to be carried out using antisera specific to the plasma proteins of each species of domestic animal currently used in the preparation of materials of biological origin in the country concerned. The injection is shown to contain proteins of human origin and gives negative results with antisera specific to plasma proteins of other species.
- C. Examine by a suitable immunoelectrophoresis technique. Using antiserum to normal human serum, compare normal human serum and the injection to be examined, both diluted if necessary. The main component of the injection to be examined corresponds to the main component of the normal human serum. The diluted solution may show the presence of small quantities of other plasma proteins.

TESTS

pH (2.2.3). The pH of the injection is 2.0 to 6.5.

Albumin

Reference solution. Dilute *human albumin solution R* with a 9 g/l solution of *sodium chloride R* to a concentration of 5 mg of albumin per millilitre.

To 1.0 ml of the injection to be examined and to 1.0 ml of the reference solution add 4.0 ml of *biuret reagent R* and mix. After exactly 30 min, measure the absorbance (2.2.25) of each solution at 540 nm, using as the compensation liquid a 9 g/l solution of *sodium chloride R* treated in the

same manner. From the absorbances measured, calculate the content of albumin in the injection to be examined in milligrams per millilitre.

Tin

Test solution. To 1.0 ml of the injection to be examined add 1.0 ml of 2 M *hydrochloric acid*. Heat in a water-bath at 100 °C for 30 min. Cool and centrifuge at 300 g for 10 min. Dilute 1.0 ml of the supernatant liquid to 10 ml with 1 M *hydrochloric acid*.

Reference solution. Dissolve 95 mg of *stannous chloride R* in 1 M *hydrochloric acid* and dilute to 1000.0 ml with the same acid.

To 1.0 ml of each solution add 0.4 ml of a 20 g/l solution of *sodium laurilsulfate R*, 0.05 ml of *thioglycollic acid R*, 0.1 ml of *dithiol reagent R* and 3.0 ml of 0.2 M *hydrochloric acid*. Mix. Measure the absorbance (2.2.25) of each solution at 540 nm, using 0.2 M *hydrochloric acid* as the compensation liquid. The absorbance of the test solution is not greater than that of the reference solution (1 mg of Sn per millilitre).

Physiological distribution. Inject a volume not greater than 0.5 ml and containing not more than 1.0 mg of albumin into a suitable vein such as a caudal vein or a saphenous vein of each of three male rats, each weighing 150 g to 250 g. Measure the radioactivity in the syringe before and after the injection. Kill the rats 30 min after the injection. Take one millilitre of blood by a suitable method and remove the liver and, if a caudal vein has been used for the injection, the tail. Using a suitable instrument determine the radioactivity in 1 ml of blood, in the liver and, if a caudal vein has been used for the injection, in the tail. Determine the percentage of radioactivity in the liver and in 1 ml of blood with respect to the total radioactivity calculated as the difference between the measurements made on the syringe minus the activity in the tail (if a caudal vein has been used for the injection). Correct the blood concentration by multiplying by a factor of $m/200$ where m is the body mass of the rat in grams. In not fewer than two of the three rats used, the radioactivity in the liver is not more than 15 per cent and that in blood, after correction, is not less than 3.5 per cent.

Sterility. It complies with the test for sterility prescribed in the monograph on *Radiopharmaceutical preparations* (0125). The injection may be released for use before completion of the test.

Bacterial endotoxins (2.6.14): less than 175 V IU/ml, V being the maximum recommended dose in millilitres.

RADIOCHEMICAL PURITY

- A. Examine by thin-layer chromatography (2.2.27) using silica gel as the coating substance on a glass-fibre sheet. Heat the plate at 110 °C for 10 min. Use a plate such that during development the mobile phase migrates over a distance of 10 cm to 15 cm in about 10 min. Apply to the plate 5 µl to 10 µl of the injection to be examined and allow to dry. Develop over a path of 10 cm to 15 cm using *methyl ethyl ketone R*. Allow the plate to dry. Determine the distribution of radioactivity using a suitable detector. The technetium-99m human albumin complex remains at the starting-point and pertechnetate ion migrates near to the solvent front. Not more than 5.0 per cent of the technetium-99m radioactivity corresponds to technetium in the form of pertechnetate ion.
- B. Examine by size-exclusion chromatography (2.2.30). **Mobile phase (concentrated).** Dissolve 1.124 g of *potassium dihydrogen phosphate R*, 4.210 g of *disodium hydrogen phosphate R*, 1.17 g of *sodium chloride R* and 0.10 g of *sodium azide R* in 100 ml of *water R*.

Test solution. Mix 0.25 ml of the injection to be examined with 0.25 ml of the mobile phase (concentrated). Use immediately after dilution.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.6 m long and 7.5 mm in internal diameter, packed with *silica gel for size-exclusion chromatography R*,
- as the mobile phase at a flow rate of 0.6 ml/min a mixture of equal volumes of mobile phase (concentrated) and *water R*,
- a radioactivity detector set for technetium-99m,
- a loop injector.

Inject 200 µl of the test solution. Continue the chromatography for at least 10 min after background level is reached.

Peaks are eluted with the following retention times:

I	High molecular mass compound	19-20 min
II	Poly III-albumin	23-24 min
III	Poly II-albumin	25-27 min
IV	Poly I-albumin	28-29 min
V	Human serum albumin	32-33 min
VI	Tin colloid	40-47 min
VII	Pertechnetate	48 min

At least 80 per cent of the radioactivity applied to the column is associated with the albumin fractions II to V.

RADIOACTIVITY

Measure the radioactivity using suitable counting equipment by comparison with a standardised technetium-99m solution or by measurement in an instrument calibrated with the aid of such a solution.

LABELLING

The label states:

- the amount of albumin,
- the amount of tin, if any.

01/2005:0296

TECHNETIUM (^{99m}Tc) MACROSALB INJECTION

Technetii (^{99m}Tc) macrosalbi suspensio iniectionabilis

DEFINITION

Technetium (^{99m}Tc) macrosalb injection is a sterile, apyrogenic suspension of human albumin in the form of irregular insoluble aggregates obtained by denaturing human albumin in aqueous solution; the particles are labelled with technetium-99m. The injection contains reducing substances, such as tin salts in an amount not exceeding 3 mg of Sn per millilitre; it may contain a suitable buffer such as acetate, citrate or phosphate buffer and also non-denatured human albumin and an antimicrobial preservative such as benzyl alcohol. The human albumin employed complies with the requirements prescribed in the monograph on *Human albumin solution (0255)*. The injection contains not less than 90.0 per cent and not more than 110.0 per cent of the declared technetium-99m radioactivity at the date and hour stated on the label. Not less than 90 per cent of the technetium-99m is bound

to the particles of the suspension as determined by the test for non-filterable radioactivity. The particles have a typical diameter between 10 µm and 100 µm. The specific radioactivity is not less than 37 MBq of technetium-99m per milligram of aggregated albumin at the date and hour of administration.

It is prepared from sodium pertechnetate (^{99m}Tc) injection (fission or non-fission) using suitable sterile and apyrogenic ingredients and calculating the ratio of radionuclidic impurities with reference to the date and hour of administration.

CHARACTERS

A white suspension which may separate on standing.

Technetium-99m has a half-life of 6.02 h and emits gamma radiation.

IDENTIFICATION

- A. Record the gamma-ray spectrum using a suitable instrument. The spectrum does not differ significantly from that of a standardised technetium-99m solution either by direct comparison or by using an instrument calibrated with the aid of such a solution. Standardised technetium-99m and molybdenum-99 solutions are available from laboratories recognised by the competent authority. The most prominent gamma photon of technetium-99m has an energy of 0.140 MeV.
- B. The tests for non-filterable radioactivity and particle size contribute to the identification of the preparation.
- C. Transfer 1 ml of the injection to a centrifuge tube and centrifuge at 2500 *g* for 5 min to 10 min. Decant the supernatant liquid. To the residue add 5 ml of *cupri-tartaric solution R2*, mix and allow to stand for 10 min. If necessary, heat to dissolve the particles and allow to cool. Add rapidly 0.5 ml of *dilute phosphomolybdotungstic reagent R*, mixing immediately. A blue colour develops.

TESTS

pH (2.2.3). The pH of the injection is 3.8 to 7.5.

Non-filterable radioactivity. Use a polycarbonate membrane filter 13 mm to 25 mm in diameter, 10 µm thick and with circular pores 3 µm in diameter. Fit the membrane into a suitable holder. Place 0.2 ml of the injection on the membrane and filter, adding 20 ml of a 9 g/l solution of *sodium chloride R* during the filtration. The radioactivity remaining on the membrane represents not less than 90 per cent of the total radioactivity of the injection.

Particle size. Examine using a microscope. Dilute the injection if necessary so that the number of particles is just low enough for individual particles to be distinguished. Using a syringe fitted with a needle having a calibre not less than 0.35 mm, place a suitable volume in a suitable counting chamber such as a haemocytometer cell, taking care not to overfill the chamber. Allow the suspension to settle for 1 min and carefully add a cover slide without squeezing the sample. Scan an area corresponding to at least 5000 particles. Not more than 10 particles have a maximum dimension greater than 100 µm. No particle having a maximum dimension greater than 150 µm is present.

Aggregated albumin

Test solution. Transfer a volume of the injection expected to contain about 1 mg of aggregated albumin to a centrifuge tube and centrifuge at about 2500 *g* for 5 min to 10 min. Decant the supernatant liquid. Resuspend the sediment in 2.0 ml of a 9 g/l solution of *sodium chloride R*. Centrifuge at 2500 *g* for 5 min to 10 min. Decant the supernatant liquid.

Resuspend the sediment in 5.0 ml of *sodium carbonate solution R1*. Heat in a water-bath at 80 °C to 90 °C to dissolve the aggregated albumin. Allow to cool, transfer to a volumetric flask and dilute to 10.0 ml with *sodium carbonate solution R1*.

Reference solutions. Prepare a range of reference solutions containing 0.05 mg to 0.2 mg of human albumin per millilitre in *sodium carbonate solution R1*.

Introduce 3.0 ml of each solution separately into 25 ml flasks. To each flask add 15.0 ml of *cupri-tartaric solution R2*, mix and allow to stand for 10 min. Add rapidly 1.5 ml of *dilute phosphomolybdotungstic reagent R* and mix immediately. Allow to stand for 30 min and measure the absorbance (2.2.25) at 750 nm using *sodium carbonate solution R1* as the compensation liquid. Using the absorbances obtained with the reference solutions, draw a calibration curve and calculate the content of aggregated albumin in the injection.

Tin

Test solution. To 1.0 ml of the injection add 1.0 ml of 2 M *hydrochloric acid*. Heat in a water-bath for 30 min. Cool and centrifuge for 10 min at 300 g. Dilute 1.0 ml of the supernatant liquid to 25.0 ml with 1 M *hydrochloric acid*.

Reference solution. Dissolve 0.115 g of *stannous chloride R* in 1 M *hydrochloric acid* and dilute to 1000.0 ml with the same acid.

To 1.0 ml of each solution add 0.4 ml of a 20 g/l solution of *sodium laurilsulfate R*, 0.05 ml of *thioglycollic acid R*, 0.1 ml of *dithiol reagent R* and 3.0 ml of 0.2 M *hydrochloric acid*. Mix. Measure the absorbance (2.2.25) of each solution at 540 nm, using 0.2 M *hydrochloric acid* as the compensation liquid. The absorbance of the test solution is not greater than that of the reference solution (3 mg of Sn per millilitre).

Physiological distribution. Inject a volume not greater than 0.2 ml into the caudal vein of each of three rats weighing 150 g to 250 g. Kill the rats 15 min after the injection, remove the liver, the spleen and the lungs and measure the radioactivity in the organs using a suitable instrument. Measure the radioactivity in the rest of the body, including the blood, after having removed the tail. Determine the percentage of radioactivity in the lungs, the liver and the spleen from the expression:

$$\frac{A}{B} \times 100$$

- A = radioactivity of the organ concerned,
B = total radioactivity in the liver, the spleen, the lungs and the rest of the body.

In not fewer than two of the three rats used, at least 80 per cent of the radioactivity is found in the lungs and not more than a total of 5 per cent in the liver and spleen. The injection may be released for use before completion of the test.

Sterility. It complies with the test for sterility prescribed in the monograph on *Radiopharmaceutical preparations (0125)*. The injection may be released for use before completion of the test.

Pyrogens. It complies with the test for pyrogens prescribed in the monograph on *Radiopharmaceutical preparations (0125)*. Inject into the animals not less than 0.1 ml per kilogram of the rabbit's mass. The injection may be released for use before completion of the test.

RADIOACTIVITY

Measure the radioactivity using suitable counting equipment by comparison with a standardised technetium-99m solution or by measurement in an instrument calibrated with the aid of such a solution.

LABELLING

The label states:

- that the preparation should be shaken before use,
- the quantity of tin per millilitre, if any,
- that the preparation is not to be used if after shaking, the suspension does not appear homogeneous.

01/2005:0641

TECHNETIUM (^{99m}Tc) MEDRONATE INJECTION

Technetii (^{99m}Tc) medronati solutio iniectionis

DEFINITION

Technetium (^{99m}Tc) medronate injection is a sterile solution which may be prepared by mixing solutions of sodium methylenediphosphonate and a stannous salt with sodium pertechnetate (^{99m}Tc) injection (fission or non-fission). The injection contains a variable quantity of tin (Sn) not exceeding 3 mg/ml; it may contain antimicrobial preservatives, antioxidants, stabilisers and buffers. The injection contains not less than 90.0 per cent and not more than 110.0 per cent of the declared technetium-99m radioactivity at the date and hour stated on the label. Radioactivity present as chemical forms other than technetium-99m medronate complex is not greater than 5.0 per cent of the total radioactivity.

It is prepared from sodium pertechnetate (^{99m}Tc) injection (fission or non-fission) using suitable sterile ingredients and calculating the ratio of radionuclidic impurities with reference to the date and hour of administration.

CHARACTERS

A clear, colourless solution.

Technetium-99m has a half-life of 6.02 h and emits gamma radiation.

IDENTIFICATION

- A. Record the gamma-ray spectrum using a suitable instrument. The spectrum does not differ significantly from that of a standardised technetium-99m solution either by direct comparison or by using an instrument calibrated with the aid of such a solution. Standardised technetium-99m and molybdenum-99 solutions are available from laboratories recognised by the competent authority. The most prominent gamma photon of technetium-99m has an energy of 0.140 MeV.
- B. Examine the chromatograms obtained in the test for radiochemical purity. The distribution of the radioactivity contributes to the identification of the preparation.
- C. Examine by thin-layer chromatography (2.2.27) using cellulose as the coating substance.

Test solution. Dilute the injection to be examined with *water R* to obtain a solution containing about 0.1 mg to 0.5 mg of sodium medronate per millilitre.

Reference solution. Dissolve a suitable quantity (1 mg to 5 mg) of *medronic acid CRS* in a mixture of a 9.0 g/l solution of *sodium chloride R* and *water R* and dilute to

10 ml with the same solvent so as to obtain a solution similar to the test solution with regard to medronate and sodium chloride concentrations.

Apply separately to the plate 10 µl of each solution. Develop over a path of 12 cm to 14 cm (development time about 4 h) using a mixture of 20 volumes of 2-propanol R, 30 volumes of 1 M hydrochloric acid and 60 volumes of methyl ethyl ketone R. Allow the plate to dry in air and spray with ammonium molybdate solution R4. Expose the plate to ultraviolet light at 254 nm for about 10 min. The principal spot in the chromatogram obtained with the test solution is similar in position and colour to the spot in the chromatogram obtained with the reference solution.

TESTS

pH (2.2.3). The pH of the solution is 3.5 to 7.5.

Tin

Test solution. Dilute 1.0 ml of the solution to 50.0 ml with 1 M hydrochloric acid.

Reference solution. Dissolve 0.115 g of stannous chloride R in 1 M hydrochloric acid and dilute to 1000.0 ml with the same acid.

To 1.0 ml of each solution add 0.4 ml of a 20 g/l solution of sodium laurilsulfate R, 0.05 ml of thioglycollic acid R, 0.1 ml of dithiol reagent R and 3.0 ml of 0.2 M hydrochloric acid. Mix. Measure the absorbance (2.2.25) of each solution at 540 nm, using 0.2 M hydrochloric acid as compensation liquid. The absorbance of the test solution is not greater than that of the reference solution (3 mg of Sn per millilitre).

Physiological distribution. Inject a volume not greater than 0.2 ml, equivalent to not more than 0.05 mg of sodium medronate into a suitable vein such as a caudal vein or the saphenous vein of each of three rats, each weighing 150 g to 250 g. Measure the radioactivity in the syringe before and after injection. Kill the rats 2 h after the injection. Remove one femur, the liver, and some blood. Weigh the blood. Remove the tail if a caudal vein has been used for the injection. Using a suitable instrument measure the radioactivity in the femur, liver, and blood, and in the tail if a caudal vein has been used for the injection. Determine the percentage of radioactivity in each sample from the expression:

$$\frac{A}{B} \times 100$$

- A* = radioactivity of the sample concerned,
B = total radioactivity, which is equal to the difference between the two measurements made on the syringe minus the radioactivity in the tail if a caudal vein has been used for the injection.

Calculate the radioactivity per unit mass in the blood. Correct the blood concentration by multiplying by a factor *m*/200 where *m* is the body mass of the rat in grams.

In not fewer than two of the three rats: not less than 1.5 per cent of the radioactivity is found in the femur; not more than 1.0 per cent is found in the liver and not more than 0.05 per cent per gram is found in the blood.

Sterility. It complies with the test for sterility prescribed in the monograph on *Radiopharmaceutical preparations* (0125). The injection may be released for use before completion of the test.

RADIOCHEMICAL PURITY

Examine by thin-layer chromatography (2.2.27) using silica gel as the coating substance on a glass-fibre sheet. Use plates such that during development, the mobile phase migrates

10 cm to 15 cm in about 10 min. Determine hydrolysed technetium and technetium in colloidal form by test (a) and pertechnetate ion by test (b).

(a) Apply to the plate 5 µl to 10 µl of the injection. Develop immediately over a path of 10 cm to 15 cm using a 136 g/l solution of sodium acetate R. Allow the plate to dry in air. Determine the distribution of radioactivity using a suitable detector. Hydrolysed technetium and technetium in colloidal form remain at the starting point. Technetium medronate complex and pertechnetate ion migrate near to the solvent front.

(b) Apply to the plate 5 µl to 10 µl of the injection and dry quickly. Develop over a path of 10 cm to 15 cm using methyl ethyl ketone R. Allow the plate to dry. Determine the distribution of radioactivity using a suitable detector. Pertechnetate ion migrates near to the solvent front. Technetium medronate complex and technetium in colloidal form remain at the starting-point.

The percentage of radioactivity corresponding to pertechnetate ion in the chromatogram obtained in test (b) is not greater than 2.0 per cent and the sum of the percentages of radioactivity corresponding to impurities in the chromatograms obtained in test (a) and test (b) (including pertechnetate ion) is not greater than 5.0 per cent.

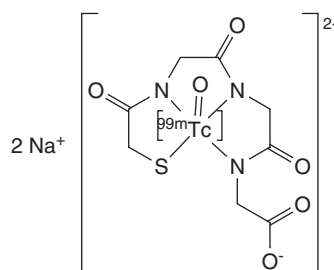
RADIOACTIVITY

Measure the radioactivity using suitable counting equipment by comparison with a standardised technetium-99m solution or by measurement in an instrument calibrated with the aid of such a solution.

01/2005:1372

TECHNETIUM (^{99m}Tc) MERTIATIDE INJECTION

Technetii (^{99m}Tc) mertiatidi solutio iniectionis



DEFINITION

Technetium (^{99m}Tc) mertiatide injection is a sterile solution which may be prepared by either heating a mixture containing S-benzoylmercaptoacetyltriglycine (betiatide), a weak chelating agent such as tartrate, a stannous salt and sodium pertechnetate (^{99m}Tc) injection (fission or non-fission), or by mixing solutions of mercaptoacetyltriglycine (mertiatide), a stannous salt and sodium pertechnetate (^{99m}Tc) injection (fission or non-fission) at alkaline pH. It may contain stabilisers and a buffer. The injection contains not less than 90.0 per cent and not more than 110.0 per cent of the declared technetium-99m radioactivity at the date and time stated on the label. Not less than 94 per cent of the radioactivity corresponds to technetium-99m in the form of [^{99m}Tc]technetium mertiatide.

CHARACTERS

A clear, colourless solution.

Technetium-99m has a half-life of 6.02 h and emits gamma radiation.

IDENTIFICATION

- A. Record the gamma-ray spectrum using a suitable instrument. The spectrum does not differ significantly from that of a standardised technetium-99m solution either by direct comparison or by using an instrument calibrated with the aid of such a solution. Standardised technetium-99m solutions are available from laboratories recognised by the competent authority. The most prominent gamma photon of technetium-99m has an energy of 0.140 MeV.
- B. Examine the chromatogram obtained in test (b) for radiochemical purity. The principal peak in the chromatogram obtained with the test solution has approximately the same retention time as the principal peak in the chromatogram obtained with the reference solution.

TESTS

pH (2.2.3). The pH of the injection is 5.0 to 7.5.

Sterility. It complies with the test for sterility prescribed in the monograph on *Radiopharmaceutical preparations* (0125). The injection may be released for use before completion of the test.

RADIOCHEMICAL PURITY

(a) Examine by ascending paper chromatography (2.2.26) using a suitable paper as the stationary phase.

Test solution. The solution to be examined.

Apply 2 μl of the test solution to the paper. Develop over a path of 15 cm using a mixture of 40 volumes of *water R* and 60 volumes of *acetonitrile R*. Allow the paper to dry and determine the distribution of the radioactivity using a suitable detector. Not more than 2.0 per cent of the total radioactivity is retained at the origin (R_f value 0.0 to 0.1).

(b) Examine by liquid chromatography (2.2.29).

Test solution. The solution to be examined.

Reference solution. Dissolve with heating on a water-bath 5 mg of *S-benzylmercaptoacetyltryglycine CRS* in 5 ml of *water R*. To 1 ml of this solution in a closed vial filled with *nitrogen R*, add 0.5 ml of a 40 g/l solution of *sodium potassium tartrate R*, 25 μl of a 4 g/l solution of *stannous chloride R* in 0.05 M *hydrochloric acid R* and 370 MBq to 740 MBq of sodium pertechnetate (^{99m}Tc) injection (fission or non-fission) in a volume not exceeding 3 ml. Heat the mixture on a water-bath for 10 min and allow to cool to room temperature.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4.0 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (5 μm),
- as mobile phase at a flow rate of 1.0 ml/min:
 - Mobile phase A.** A mixture of 7 volumes of *ethanol R* with 93 volumes of a solution of a 1.36 g/l solution of *potassium dihydrogen phosphate R*, adjusted to pH 6.0 with 0.1 M *sodium hydroxide*,
 - Mobile phase B.** A mixture of 10 volumes of *water R* with 90 volumes of *methanol R*,
- a suitable radioactivity detector,
- a 20 μl loop injector.

Equilibrate the column with mobile phase A for 20 min. Inject the test solution and the reference solution. Switch 10 min after each injection to mobile phase B and continue the chromatographic procedure for 15 min.

The test is not valid unless in the chromatogram obtained with the test solution the principal peak has approximately the same retention time as the principal peak in the chromatogram obtained with the reference solution. In the chromatogram obtained with the test solution the sum of the areas preceding the principal peak (corresponding to hydrophilic impurities, including [^{99m}Tc]pertechnetate) is not greater than 3.0 per cent of the sum of the areas of all peaks. The sum of the peaks following the principal peak (corresponding to lipophilic impurities) is not greater than 4.0 percent of the sum of the area of all peaks.

Not less than 94 per cent of the radioactivity corresponds to [^{99m}Tc]technetium mertiatide.

RADIOACTIVITY

Measure the radioactivity using suitable counting equipment by comparison with a standardised technetium-99m solution or by measurement in an instrument calibrated with the aid of such a solution.

01/2005:0570

TECHNETIUM (^{99m}Tc) MICROSPHERES INJECTION

Technetii (^{99m}Tc) microsphaerarum suspensio iniectabilis

DEFINITION

Technetium (^{99m}Tc) microspheres injection is a sterile, apyrogenic suspension of human albumin which has been denatured to form spherical insoluble particles; the particles are labelled with technetium-99m. The injection contains reducing substances, such as tin salts in an amount not exceeding 3 mg of Sn per millilitre; it may contain a suitable buffer such as acetate, citrate or phosphate and additives such as wetting agents. The human albumin used complies with the requirements of the monograph on *Human albumin solution* (0255). The injection contains not less than 90.0 per cent and not more than 110.0 per cent of the declared technetium-99m radioactivity at the date and hour stated on the label. Not less than 95 per cent of the technetium-99m is bound to the particles of the suspension as determined by the test for non-filterable radioactivity. The particles have a typical diameter between 10 μm and 50 μm . The radioactivity is not less than 185 MBq of technetium-99m per million particles at the date and hour of administration.

Technetium (^{99m}Tc) microspheres injection is prepared from sodium pertechnetate (^{99m}Tc) injection (fission or non-fission) using suitable sterile and apyrogenic ingredients and calculating the ratio of radionuclidic impurities with reference to the date and hour of administration.

CHARACTERS

A suspension of white, yellow or artificially coloured particles which may separate on standing.

Technetium-99m has a half-life of 6.02 h and emits gamma radiation.

IDENTIFICATION

- A. Record the gamma-ray spectrum using a suitable instrument. The spectrum does not differ significantly from that of a standardised technetium-99m solution either by direct comparison or by using an instrument calibrated with the aid of such a solution. Standardised technetium-99m and molybdenum-99 solutions are

available from laboratories recognised by the competent authority. The most prominent gamma photon of technetium-99m has an energy of 0.140 MeV.

- B. The tests for non-filterable radioactivity and particle size contribute to the identification of the preparation.
- C. Transfer 1 ml of the injection to a centrifuge tube and centrifuge at 2500 *g* for 5 min to 10 min. Decant the supernatant liquid. To the residue add 5 ml of *cupri-tartaric solution R2*, mix and allow to stand for 10 min. If necessary, heat to dissolve the particles and allow to cool. Add rapidly 0.5 ml of *dilute phosphomolybdotungstic reagent R*, mix immediately and allow to stand. A blue colour develops.

TESTS

pH (2.2.3). The pH of the injection is 4.0 to 9.0.

Non-filterable radioactivity. Use a polycarbonate membrane filter 13 mm to 25 mm in diameter, 10 µm thick and with circular pores 3 µm in diameter. Fit the membrane into a suitable holder. Place 0.2 ml of the injection on the membrane and filter, adding 20 ml of a 9 g/l solution of *sodium chloride R* during the filtration. The radioactivity remaining on the membrane represents not less than 95 per cent of the total radioactivity of the injection.

Particle size. Examine using a microscope. Dilute the injection if necessary so that the number of particles is just low enough for individual particles to be distinguished. Using a syringe fitted with a needle having a calibre not less than 0.35 mm, place a suitable volume in a suitable counting chamber such as a haemocytometer cell, taking care not to overfill the chamber. Allow the suspension to settle for 1 min and carefully add a cover slide without squeezing the sample. Scan an area corresponding to at least 5000 particles. The particles have a uniform spherical appearance. Not more than 10 particles have a maximum dimension greater than 75 µm. No particle having a maximum dimension greater than 100 µm is present.

Number of particles. Examine using a microscope. Fill a suitable counting chamber such as a haemocytometer cell with a suitable dilution of the injection taking care that particles do not separate during the transfer. Count the number of particles in the chamber. Repeat this procedure twice and calculate the number of particles per millilitre of the injection.

Tin

Test solution. To 1.0 ml of the injection add 0.5 ml of *sulphuric acid R* and 1.5 ml of *nitric acid R*. Heat and evaporate to approximately 1 ml. Add 2 ml of *water R* and evaporate again to approximately 1 ml. Repeat this procedure twice, cool and dilute to 25.0 ml with 1 *M hydrochloric acid*.

Reference solution. Dissolve 0.115 g of *stannous chloride R* in 1 *M hydrochloric acid* and dilute to 1000.0 ml with the same acid.

To 1.0 ml of each solution add 0.4 ml of a 20 g/l solution of *sodium laurilsulfate R*, 0.05 ml of *thioglycollic acid R*, 0.1 ml of *dithiol reagent R* and 3.0 ml of 0.2 *M hydrochloric acid*. Mix. Measure the absorbance (2.2.25) of each solution at 540 nm, using 0.2 *M hydrochloric acid* as the compensation liquid. The absorbance of the test solution is not greater than that of the reference solution (3 mg of Sn per millilitre).

Physiological distribution. Inject a volume not greater than 0.2 ml into a caudal vein of each of three rats weighing 150 g to 250 g. Sacrifice the rats 15 min after the injection, remove the liver, the spleen and the lungs and measure the radioactivity in the organs using a suitable instrument. Measure the radioactivity in the rest of the body, including

the blood and voided urine, after having discarded the tail. Determine the percentage of radioactivity in the liver, the spleen and the lungs from the expression:

$$\frac{A}{B} \times 100$$

- A* = radioactivity of the organ concerned,
B = total radioactivity in the liver, the spleen, the lungs and the rest of the body, including voided urine.

In not fewer than two of the three rats used, not less than 80 per cent of the radioactivity is found in the lungs and not more than a total of 5 per cent in the liver and spleen. The injection may be released for use before completion of the test.

Sterility. It complies with the test for sterility prescribed in the monograph on *Radiopharmaceutical preparations (0125)*. The injection may be released for use before completion of the test.

Pyrogens. It complies with the test for pyrogens prescribed in the monograph on *Radiopharmaceutical preparations (0125)*. Inject not less than 0.1 ml per kilogram of the rabbit's mass. The injection may be released for use before completion of the test.

RADIOACTIVITY

Measure the radioactivity using suitable counting equipment by comparison with a standardised technetium-99m solution or by measurement in an instrument calibrated with the aid of such a solution.

LABELLING

The label states:

- the quantity of tin per millilitre, if any,
- that the preparation should be shaken before use.

01/2005:0642

TECHNETIUM (^{99m}Tc) PENTETATE INJECTION

Technetii (^{99m}Tc) pentetatis
 solutio iniectionis

DEFINITION

Technetium (^{99m}Tc) pentetate injection is a sterile solution which may be prepared by mixing solutions of sodium diethylenetriaminepenta-acetate or calcium trisodium diethylenetriaminepenta-acetate and a stannous salt with a solution of sodium pertechnetate (^{99m}Tc). It contains a variable quantity of tin (Sn) not exceeding 1 mg/ml; it may contain suitable antimicrobial preservatives, antioxidants, stabilisers and buffers. The injection contains not less than 90.0 per cent and not more than 110.0 per cent of the declared technetium-99m radioactivity at the date and hour stated on the label. Not less than 95.0 per cent of the radioactivity corresponds to technetium-99m complexed with sodium pentetate or calcium trisodium pentetate.

It is prepared from sodium pertechnetate (^{99m}Tc) injection (fission or non-fission) using suitable, sterile ingredients and calculating the ratio of radionuclidic impurities with reference to the date and hour of administration.

CHARACTERS

A clear, colourless or slightly yellow solution.

Technetium-99m has a half life of 6.02 h and emits gamma radiation.

IDENTIFICATION

- Record the gamma-ray spectrum using a suitable instrument. The spectrum does not differ significantly from that of a standardised technetium-99m solution either by direct comparison or by using an instrument calibrated with the aid of such a solution. Standardised technetium-99m and molybdenum-99 solutions are available from laboratories recognised by the competent authority. The most prominent gamma photon of technetium-99m has an energy of 0.140 MeV.
- Examine the chromatograms obtained in the test for radiochemical purity. The distribution of radioactivity contributes to the identification of the preparation.
- Place in a clean, dry 10 ml glass tube a volume of the injection to be examined containing 2 mg of pentetate. Dilute, if necessary, to 1 ml with *water R*. Place in a second tube 1 ml of *water R* (blank). To each tube add 0.1 ml of a 1 g/l solution of *nickel sulphate R*, 0.5 ml of a 50 per cent *V/V* solution of *glacial acetic acid R* and 0.75 ml of a 50 g/l solution of *sodium hydroxide R*. Mix and verify that the pH is not above 5. To each tube add 0.1 ml of a 10 g/l solution of *dimethylglyoxime R* in *alcohol R*. Mix and allow to stand for 2 min. Adjust the pH in each tube to not less than 12 by adding a 100 g/l solution of *sodium hydroxide R*. Mix and check that the pH is not below 12. Allow to stand for 2 min. Heat the tubes gently on a water-bath for 2 min. The solution in the tube containing the injection to be examined remains clear and colourless throughout. The solution in the blank tube becomes red on addition of dimethylglyoxime solution and a red precipitate is formed when the tube is heated on a water-bath.

TESTS

pH (2.2.3). The pH of the injection is 4.0 to 7.5.

Tin

Test solution. Dilute 1.5 ml of the injection to 25.0 ml with 1 M hydrochloric acid.

Reference solution. Dissolve 0.115 g of *stannous chloride R* in 1 M hydrochloric acid and dilute to 1000.0 ml with the same acid.

To 1.0 ml of each solution add 0.4 ml of a 20 g/l solution of *sodium laurilsulfate R*, 0.05 ml of *thioglycolic acid R*, 0.1 ml of *dithiol reagent R* and 3.0 ml of 0.2 M hydrochloric acid. Mix. Measure the absorbance (2.2.25) of each solution at 540 nm, using 0.2 M hydrochloric acid as the compensation liquid. The absorbance of the test solution is not greater than that of the reference solution (1 mg of Sn per millilitre).

Sterility. It complies with the test for sterility prescribed in the monograph on *Radiopharmaceutical preparations* (0125). The injection may be released for use before completion of the test.

RADIOCHEMICAL PURITY

Examine by thin-layer chromatography (2.2.27) using silica gel as the coating substance on a glass-fibre sheet. Heat the plate at 110 °C for 10 min. Use a plate such that during development the mobile phase migrates over a distance of 10 cm to 15 cm in about 10 min.

(a) Apply to the plate 5 µl to 10 µl of the injection to be examined. Develop immediately over a path of 10 cm to 15 cm using a 9 g/l solution of *sodium chloride R*. Allow the plate to dry in air. Determine the distribution of radioactivity

using a suitable detector. Impurities in colloidal form remain at the starting point. Technetium pentetate complex and pertechnetate ion migrate near to the solvent front.

(b) Apply to the plate 5 µl to 10 µl of the injection to be examined and allow to dry. Develop over a path of 10 cm to 15 cm using *methyl ethyl ketone R*. Allow the plate to dry. Determine the distribution of radioactivity using a suitable detector. Pertechnetate ion migrates near to the solvent front. Technetium pentetate complex and impurities in colloidal form remain at the starting point.

The sum of the percentages of radioactivity corresponding to impurities in the chromatograms obtained in test (a) and (b) does not exceed 5.0 per cent.

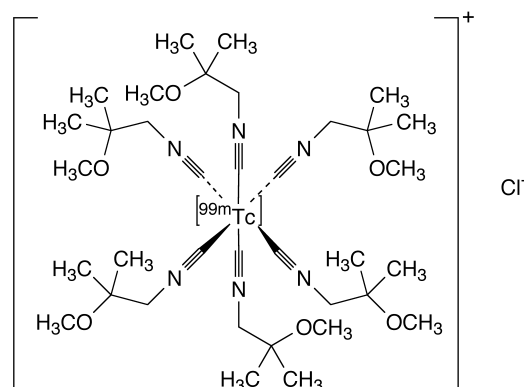
RADIOACTIVITY

Measure the radioactivity using suitable counting equipment by comparison with a standardised technetium-99m solution or by measurement in an instrument calibrated with the aid of such a solution.

01/2005:1926

TECHNETIUM (^{99m}Tc) SESTAMIBI INJECTION

Technetii (^{99m}Tc) sestamibi solutio iniectionis



DEFINITION

Sterile solution of (OC-6-11)-hexakis[1-(isocyanato-κC)-2-methoxy-2-methylpropane][^{99m}Tc]technetium(I) chloride, which may be prepared by heating a mixture containing [tetraakis(2-methoxy-2-methylpropyl-1-isocyanide)copper (1+)] tetrafluoroborate, a weak chelating agent, a stannous salt and *Sodium pertechnetate (^{99m}Tc) injection (fission)* (0124) or *Sodium pertechnetate (^{99m}Tc) injection (non-fission)* (0283).

Content: 90 per cent to 110 per cent of the declared technetium-99m radioactivity at the date and hour stated on the label.

CHARACTERS

Appearance: clear, colourless solution.

Half-life and nature of radiation of technetium-99m: see *Table of physical characteristics of radionuclides* (5.7).

IDENTIFICATION

A. Gamma-ray spectrometry.

Results: the spectrum obtained with the solution to be examined does not differ significantly from that of a standardised technetium-99m solution. The most prominent gamma photon has an energy of 0.141 MeV.

- B. Examine the chromatograms obtained in the test for impurity C under Radiochemical purity.

Results: the principal peak in the radiochromatogram obtained with the test solution is similar in retention time to the principal peak in the radiochromatogram obtained with the reference solution.

TESTS

pH (2.2.3): 5.0 to 6.0.

Sterility. It complies with the test for sterility prescribed in the monograph on *Radiopharmaceutical preparations* (0125). The injection may be released for use before completion of the test.

RADIOCHEMICAL PURITY

Impurity A and other polar impurities. Thin-layer chromatography (2.2.27).

Test solution. The preparation to be examined.

Plate: TLC octadecylsilyl silica gel plate R.

Mobile phase: mix 10 volumes of tetrahydrofuran R, 20 volumes of a 38.5 g/l solution of ammonium acetate R, 30 volumes of methanol R and 40 volumes of acetonitrile R.

Application: about 5 µl.

Development: immediately over a path of 6 cm.

Drying: in air.

Detection: determine the distribution of radioactivity using a radioactivity detector.

Retention factors: impurity B and apolar impurities = 0 to 0.1; impurity C and technetium-99m sestamibi = 0.3 to 0.6; impurity A and other polar impurities = 0.9 to 1.0.

Limit: see test for impurity B.

Impurity B. Paper chromatography (2.2.26). *If no activity is found at retention factor 0 to 0.1 in the test for impurity A and other polar impurities, impurity B is absent and the test for impurity B may be omitted.*

Test solution. The preparation to be examined.

Paper: paper for chromatography R.

Mobile phase: mix equal volumes of acetonitrile R, 0.5 M acetic acid and a 20 g/l solution of sodium chloride R.

Application: about 5 µl.

Development: over a path of 10 cm.

Drying: in air.

Detection: determine the distribution of radioactivity using a radioactivity detector.

Retention factors: impurity B = 0 to 0.1; impurity A, impurity C and technetium-99m sestamibi = 0.8 to 1.0.

Limit:

- *sum of impurity A and other polar impurities, and impurity B:* maximum 5 per cent of the total radioactivity.

Impurity C. Liquid chromatography (2.2.29).

Test solution. The preparation to be examined.

Reference solution. To a vial of sestamibi labelling kit CRS add 3 ml of a 9 g/l solution of sodium chloride R containing 700 MBq to 900 MBq of sodium pertechnetate (^{99m}Tc) injection (fission or non-fission). Heat the mixture in a water-bath for 10 min and allow to cool to room temperature.

Column:

- *size:* $l = 0.25$ m, $\varnothing = 4.6$ mm,

- *stationary phase:* spherical base-deactivated end-capped octadecylsilyl silica gel for chromatography R (5 µm).

Mobile phase: mix 20 volumes of acetonitrile R, 35 volumes of a 6.6 g/l solution of ammonium sulphate R and 45 volumes of methanol R.

Flow rate: 1.5 ml/min.

Detection: radioactivity detector.

Injection: 25 µl.

Run time: 25 min.

Relative retention with reference to technetium-99m sestamibi: impurity C = about 1.3.

System suitability: reference solution:

- the chromatogram is similar to the chromatogram provided with sestamibi labelling kit CRS,
- *relative retention* with reference to technetium-99m sestamibi: impurity C = minimum 1.2.

Limits:

- *impurity C:* not more than 3 per cent of the total radioactivity,
- *technetium-99m sestamibi:* minimum 94 per cent of the total radioactivity.

Calculate the percentage of radioactivity due to technetium-99m sestamibi from the expression:

$$\frac{(100 - B) \times T}{100}$$

B = percentage of radioactivity due to impurity B determined in the test for impurity B under Radiochemical purity,

T = area of the peak due to technetium-99m sestamibi in the chromatogram obtained with the test solution.

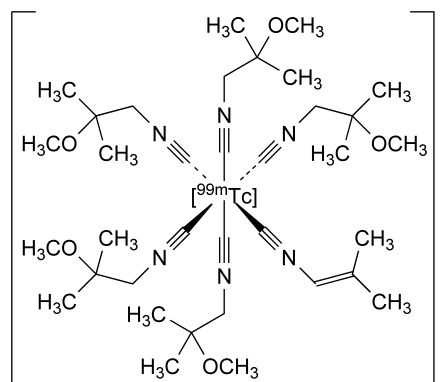
RADIOACTIVITY

Determine the radioactivity using a calibrated instrument.

IMPURITIES

A. [^{99m}Tc]O₄[−]: (^{99m}Tc)pertechnetate ion,

B. technetium-99m in colloidal form,



C. (OC-6-22)-pentakis[1-(isocyanido-κC)-2-methoxy-2-methylpropane][1-(isocyanido-κC)-2-methylprop-1-ene][^{99m}Tc]technetium (1+).

01/2005:0643

TECHNETIUM (^{99m}Tc) SUCCIMER INJECTION

Technetii (^{99m}Tc) succimeri solutio iniectionabilis

DEFINITION

Technetium (^{99m}Tc) succimer injection is a sterile solution of *meso*-2,3-dimercaptosuccinic acid labelled with technetium-99m. It contains a reducing substance, such as a tin salt in an amount not exceeding 1 mg of Sn per millilitre, and may contain stabilisers, antioxidants such as ascorbic acid, and inert additives. The injection contains not less than 90.0 per cent and not more than 110.0 per cent of the declared technetium-99m radioactivity at the date and hour stated on the label. Not less than 95.0 per cent of the radioactivity corresponds to technetium-99m succimer complex.

It is prepared from sodium pertechnetate (^{99m}Tc) injection (fission or non-fission) using suitable sterile ingredients and calculating the ratio of radionuclidic impurities with reference to the date and hour of administration. Syringes for handling the eluate intended for labelling of the final product, or for handling the final product should not contain rubber parts.

CHARACTERS

A clear, colourless solution.

Technetium-99m has a half life of 6.02 h and emits gamma radiation.

IDENTIFICATION

- Record the gamma-ray spectrum using a suitable instrument. The spectrum does not differ significantly from that of a standardised technetium-99m solution either by direct comparison or by using an instrument calibrated with the aid of such a solution. Standardised technetium-99m and molybdenum-99 solutions are available from laboratories recognised by the competent authority. The most prominent gamma photon of technetium-99m has an energy of 0.140 MeV.
- Examine the chromatogram obtained in the test for radiochemical purity. The distribution of the radioactivity contributes to the identification of the preparation.
- Place 1 ml of the injection to be examined in a test-tube and add 1 ml of a 20 g/l solution of *sodium nitroprusside R* and 0.1 ml of *glacial acetic acid R*. Mix. Place carefully at the top of the solution a layer of *concentrated ammonia R*. A violet ring develops between the layers.

TESTS

pH (2.2.3). The pH of the injection is 2.3 to 3.5.

Tin

Test solution. Dilute 1.5 ml of the injection to be examined to 25.0 ml with 1 M hydrochloric acid.

Reference solution. Dissolve 0.115 g of *stannous chloride R* in 1 M hydrochloric acid and dilute to 1000.0 ml with the same acid.

To 1.0 ml of each solution add 0.4 ml of a 20 g/l solution of *sodium laurilsulfate R*, 0.05 ml of *thioglycollic acid R*, 0.1 ml of *dithiol reagent R* and 3.0 ml of 0.2 M hydrochloric acid. Mix. Allow to stand for 60 min. Measure the absorbance (2.2.25) of each solution at 540 nm, using 0.2 M hydrochloric

acid as the compensation liquid. The absorbance of the test solution is not greater than that of the reference solution (1 mg of Sn per millilitre).

Physiological distribution. Inject a volume not greater than 0.2 ml and containing not more than 0.1 mg of dimercaptosuccinic acid into a suitable vein, such as a caudal vein or a saphenous vein, of each of three rats each weighing 150 g to 250 g. Measure the radioactivity in the syringe before and after the injection. Kill the rats 1 h after the injection. Remove the kidneys, the liver, the stomach, the lungs and, if a caudal vein has been used for the injection, the tail. Using a suitable instrument determine the radioactivity in the organs and, if a caudal vein has been used for injection, in the tail. Determine the percentage of radioactivity in each organ with respect to the total radioactivity calculated as the difference between the two measurements made on the syringe minus the activity in the tail (if a caudal vein has been used for the injection).

In not fewer than two of the three rats used, the radioactivity in the kidneys is not less than 40 per cent, that in the liver is not more than 10.0 per cent, that in the stomach is not more than 2.0 per cent and that in the lungs is not more than 5.0 per cent.

Sterility. It complies with the test for sterility prescribed in the monograph on *Radiopharmaceutical preparations* (0125). The injection may be released for use before completion of the test.

RADIOCHEMICAL PURITY

Examine by thin-layer chromatography (2.2.27) using silica gel as the coating substance on a glass-fibre sheet. Heat the plate at 110 °C for 10 min. Use a plate such that during development the mobile phase migrates over a distance of 10 cm to 15 cm in about 10 min.

Apply to the plate 5 µl to 10 µl of the injection to be examined. Develop immediately over a path of 10 cm to 15 cm using *methyl ethyl ketone R*. Allow the plate to dry. Determine the distribution of radioactivity using a suitable detector. Technetium succimer complex remains at the starting point. Pertechnetate ion migrates near to the solvent front. Not less than 95.0 per cent of the total radioactivity is found in the spot corresponding to technetium succimer complex. The radioactivity corresponding to pertechnetate ion represents not more than 2.0 per cent of the total radioactivity.

RADIOACTIVITY

Measure the radioactivity using suitable counting equipment by comparison with a standardised technetium-99m solution or by measurement in an instrument calibrated with the aid of such a solution.

STORAGE

Store protected from light.

01/2005:0129

TECHNETIUM (^{99m}Tc) TIN PYROPHOSPHATE INJECTION

Stanni pyrophosphatis et technetii (^{99m}Tc) solutio iniectionabilis

DEFINITION

Technetium (^{99m}Tc) tin pyrophosphate injection is a sterile, apyrogenic solution which may be prepared by mixing solutions of sodium pyrophosphate and stannous chloride with sodium pertechnetate (^{99m}Tc) injection (fission or non-fission). The injection contains not less than 90.0 per

cent and not more than 110.0 per cent of the declared technetium-99m radioactivity at the date and hour stated on the label. Not less than 90 per cent of the radioactivity corresponds to technetium-99m complexed with tin pyrophosphate. The injection contains a quantity of sodium pyrophosphate ($\text{Na}_4\text{P}_2\text{O}_7 \cdot 10\text{H}_2\text{O}$) that may vary from 1 mg to 50 mg per millilitre and a variable quantity of tin (Sn) not exceeding 3.0 mg per millilitre.

It is prepared from sodium pertechnetate (^{99m}Tc) injection (fission or non-fission) using suitable sterile, apyrogenic ingredients and calculating the ratio of radionuclidic impurities with reference to the date and hour of administration.

CHARACTERS

A clear, colourless solution.

Technetium-99m has a half-life of 6.02 h and emits gamma radiation.

IDENTIFICATION

- Record the gamma-ray spectrum using a suitable instrument. The spectrum does not differ significantly from that of a standardised technetium-99m solution either by direct comparison or by using an instrument calibrated with the aid of such a solution. Standardised technetium-99m and molybdenum-99 solutions are available from laboratories recognised by the competent authority. The most prominent gamma photon of technetium-99m has an energy of 0.140 MeV.
- Examine the chromatograms obtained in the test for radiochemical purity. The distribution of radioactivity contributes to the identification of the injection.
- To 1 ml add 1 ml of *acetic acid R*. Heat on a water-bath for 1 h. After cooling, add 10 ml of *nitro-vanadomolybdic reagent R* and allow to stand for 30 min. A yellow colour develops.
- To 1 ml add 2 ml of a 30 per cent *V/V* solution of *sulphuric acid R*, 1 ml of *hydrochloric acid R*, 0.05 ml of *thioglycollic acid R*, 0.4 ml of a 20 g/l solution of *sodium laurilsulfate R* and 0.1 ml of *dithiol reagent R* and allow to stand for 30 min. A pink colour develops.

TESTS

pH (2.2.3). The pH of the injection is 6.0 to 7.0.

Sodium pyrophosphate

Test solution. Use 1 ml of the injection to be examined or a suitable dilution of it.

Reference solutions. Using a solution containing *sodium pyrophosphate R* and *stannous chloride R* in the same proportions as in the injection to be examined, prepare a range of solutions and dilute to the same final volume with *water R*.

To the test solution and to 1 ml of each of the reference solutions add successively 10 ml of a 1 g/l solution of *disodium hydrogen phosphate R*, 10 ml of *iron standard solution* (8 ppm Fe) *R*, 5 ml of *glacial acetic acid R* and 5 ml of a 1 g/l solution of *hydroxylamine hydrochloride R*. Dilute each solution to 40 ml with *water R* and heat in a water-bath at 40 °C for 1 h. To each solution add 4 ml of a 1 g/l solution of *phenanthroline hydrochloride R* and dilute to 50.0 ml with *water R*. Measure the absorbance (2.2.25) of each solution at 515 nm using as the compensation liquid a reagent blank containing hydrochloric acid (1.1 g/l HCl) instead of the *iron standard solution* (8 ppm Fe) *R*. Using

the absorbances obtained with the reference solutions, draw a calibration curve and calculate the concentration of sodium pyrophosphate in the injection to be examined.

Tin

Test solution. Use 1 ml of the injection to be examined or a suitable dilution of it.

Reference solutions. Using a solution in hydrochloric acid (6.2 g/l HCl) containing *sodium pyrophosphate R* and *stannous chloride R* in the same proportions as in the injection to be examined, prepare a range of solutions and dilute to the same volume with hydrochloric acid (6.2 g/l HCl).

To the test solution and to 1 ml of each of the reference solutions add 2 ml of a 300 g/l solution of *sulphuric acid R*, 1 ml of *hydrochloric acid R*, 0.05 ml of *thioglycollic acid R*, 0.4 ml of a 20 g/l solution of *sodium laurilsulfate R* and 0.1 ml of *dithiol reagent R* and dilute to 15 ml with hydrochloric acid (6.2 g/l HCl). Allow the solutions to stand for 30 min and measure the absorbance (2.2.25) of each solution at 530 nm, using as the compensation liquid a reagent blank containing the same quantity of *sodium pyrophosphate R* as the injection to be examined. Using the absorbances obtained with the reference solutions, draw a calibration curve and calculate the concentration of tin in the injection to be examined.

Sterility. It complies with the test for sterility prescribed in the monograph on *Radiopharmaceutical preparations* (0125). The injection may be released for use before completion of the test.

Pyrogens. It complies with the test for pyrogens prescribed in the monograph on *Radiopharmaceutical preparations* (0125). Inject not less than 0.1 ml per kilogram of the rabbit's mass. The injection may be released for use before completion of the test.

RADIOCHEMICAL PURITY

(a) Examine by thin-layer chromatography (2.2.27) using silica gel as the coating substance on a glass-fibre sheet. Heat the plate at 110 °C for 10 min. The plate used should be such that during development the mobile phase migrates over a distance of 10 cm to 15 cm in about 10 min.

Apply to the plate 5 µl to 10 µl of the injection and dry in a stream of nitrogen. Develop over a path of 10 cm to 15 cm using *methyl ethyl ketone R* through which nitrogen has been bubbled in the chromatography tank for 10 min immediately before the chromatography. Allow the plate to dry. Determine the distribution of radioactivity using a suitable detector. The technetium-99m tin pyrophosphate complex remains at the starting-point and pertechnetate ion migrates with an *R_f* of 0.95 to 1.0.

(b) Examine by thin-layer chromatography (2.2.27) using silica gel as the coating substance on a glass-fibre sheet. Heat the plate at 110 °C for 10 min. The plate used should be such that during development the mobile phase migrates over a distance of 10 cm to 15 cm in about 10 min.

Apply to the plate 5 µl to 10 µl of the injection. Develop immediately over a path of 10 cm to 15 cm using a 136 g/l solution of *sodium acetate R*. Allow the plate to dry and measure the distribution of radioactivity using a suitable detector. Impurities in colloidal form remain at the starting-point and technetium-99m tin pyrophosphate complex and pertechnetate ion migrate with an *R_f* of 0.9 to 1.0.

Add together the percentages of radioactivity corresponding to impurities in the chromatograms obtained in test (a) and test (b). The sum does not exceed 10 per cent.

RADIOACTIVITY

Measure the radioactivity using suitable counting equipment by comparison with a standardised technetium-99m solution or by measurement in an instrument calibrated with the aid of such a solution.

LABELLING

The label states, in particular, the quantity of sodium pyrophosphate per millilitre and the quantity of tin per millilitre.

01/2005:0571

THALLOUS (^{201}Tl) CHLORIDE INJECTION

Thallosi (^{201}Tl) chloridi solutio iniectionabilis

DEFINITION

Thallous (^{201}Tl) chloride injection is a sterile solution of thallium-201 in the form of thallous chloride. It may be made isotonic by the addition of *Sodium chloride* (0193) and may contain a suitable antimicrobial preservative such as *Benzyl alcohol* (0256). Thallium-201 is a radioactive isotope of thallium formed by the decay of lead-201. Lead-201 is a radioactive isotope of lead and may be obtained by irradiation, with protons of suitable energy, of thallium which may be enriched in thallium-203. Thallium-201 may be separated from lead-201 by passing through a column of an ion-exchange resin. The injection contains not less than 90.0 per cent and not more than 110.0 per cent of the declared thallium-201 radioactivity at the date and hour stated on the label. Not more than 2.0 per cent of the total radioactivity is due to thallium-202 and not less than 97.0 per cent is due to thallium-201. Not less than 95.0 per cent of the radioactivity is due to thallium in the form of thallous ions. The specific radioactivity is not less than 3.7 GBq per milligram of thallium.

CHARACTERS

A clear, colourless solution.

Thallium-201 has a half-life of 3.05 days and emits gamma radiation and X-rays.

IDENTIFICATION

- A. Record the gamma-ray and X-ray spectrum using a suitable instrument. The spectrum does not differ significantly from that of a standardised thallium-201 solution when measured either by direct comparison or by use of an instrument calibrated with the aid of such a solution. Standardised thallium-201 and thallium-202 solutions are available from laboratories recognised by the competent authority. The most prominent gamma photons have energies of 0.135 MeV, 0.166 MeV and 0.167 MeV. The X-rays have energies of 0.069 MeV to 0.083 MeV.
- B. Examine the electropherogram obtained in the test for radiochemical purity. The distribution of radioactivity contributes to the identification of the preparation.

TESTS

pH (2.2.3). The pH of the injection is 4.0 to 7.0.

Thallium. To 0.5 ml of the injection add 0.5 ml of hydrochloric acid (220 g/l HCl) and 0.05 ml of *bromine water R* and mix. Add 0.1 ml of a 30 g/l solution of *sulphosalicylic acid R*. After decolorisation add 1.0 ml of a 1 g/l solution of *rhodamine B R*. Add 4 ml of *toluene R* and shake for 60 s. Separate the toluene layer. The toluene

layer is not more intensely coloured than the toluene layer of a standard prepared at the same time in the same manner using 0.5 ml of *thallium standard solution* (10 ppm Tl) R.

Sterility. It complies with the test for sterility prescribed in the monograph on *Radiopharmaceutical preparations* (0125). The injection may be released for use before completion of the test.

RADIONUCLIDIC PURITY

Record the gamma-ray and X-ray spectrum using a suitable instrument calibrated with the aid of standardised thallium-201 and thallium-202 solutions. The spectrum does not differ significantly from that of the standardised thallium-201 solution. Determine the relative amounts of thallium-201 and thallium-202 and other radionuclidic impurities present. Thallium-202 has a half-life of 12.2 days and its most prominent gamma photon has an energy of 0.440 MeV. Thallium-200 has a half-life of 1.09 days and its most prominent gamma photons have energies of 0.368 MeV, 0.579 MeV, 0.828 MeV and 1.206 MeV. Lead-201 has a half-life of 9.4 h and its most prominent gamma photon has an energy of 0.331 MeV. Lead-203 has a half-life of 2.17 days and its most prominent gamma photon has an energy of 0.279 MeV. Not more than 2.0 per cent of the total radioactivity is due to thallium-202 and not less than 97.0 per cent is due to thallium-201.

RADIOCHEMICAL PURITY

Examine by zone electrophoresis (2.2.31), using a suitable strip of cellulose acetate, as the support and a 18.6 g/l solution of *sodium edetate R* as the electrolyte solution. Soak the strip in the electrolyte solution for 45-60 min. Remove the strip with forceps taking care to handle the outer edges only. Place the strip between 2 absorbent pads and blot to remove excess solution.

Test solution. Mix equal volumes of the injection to be examined and the electrolyte solution.

Apply not less than 5 µl of the test solution to the centre of the strip and mark the point of application. Apply an electric field of 17 V/cm for at least 10 min. Allow the strip to dry in air. Determine the distribution of radioactivity using suitable equipment. Not less than 95.0 per cent of the radioactivity migrates towards the cathode.

RADIOACTIVITY

Measure the radioactivity using suitable counting equipment by comparison with a standardised thallium-201 solution or by measurement in an instrument calibrated with the aid of such a solution.

01/2005:0112

TRITIATED (^3H) WATER INJECTION

Aquae tritiatae (^3H) solutio iniectionabilis

DEFINITION

Tritiated (^3H) water injection is water for injections in which some of the water molecules contain tritium atoms in place of protium atoms. It may be made isotonic by the addition of sodium chloride. Tritium (^3H) may be obtained by the neutron irradiation of lithium. The injection contains not less than 90.0 per cent and not more than 110.0 per cent of the declared tritium activity at the date stated on the label.

CHARACTERS

A clear, colourless liquid.

Tritium has a half-life of 12.3 years and emits beta radiation.

IDENTIFICATION

Record the beta-ray spectrum by the method prescribed in the test for radionuclidic purity. The spectrum does not differ significantly from that of a standardised tritiated (³H) water. Standardised tritiated (³H) water is available from laboratories recognised by the competent authority. The maximum energy of the beta radiation is 0.019 MeV.

TESTS

pH (2.2.3). The pH of the injection is 4.5 to 7.0.

Sterility. It complies with the test for sterility prescribed in the monograph on *Radiopharmaceutical preparations* (0125).

RADIONUCLIDIC PURITY

(a) Mix 100 µl of a suitable dilution of the injection with 10 ml of a scintillation liquid consisting of 1000 ml of *dioxan R*, 100 g of *naphthalene R*, 7 g of *diphenyloxazole R* and 0.3 g of *methylphenyloxazolylbenzene R*, the reagents being of an analytical grade suitable for liquid scintillation. Measure the radioactivity of the mixture in a liquid scintillation counter fitted with a discriminator. The count should be about 5000 impulses per second at the lowest setting of the discriminator. Record the count at different discriminator settings. For each measurement count at least 10 000 impulses over a period of at least 1 min. Immediately determine in the same conditions the count for a standardised tritiated (³H) water having approximately the same activity.

Plot the counts at each discriminator setting, correcting for background activity, on semi-logarithmic paper, the discriminator settings being in arbitrary units as the abscissae. The vertical distance between the two curves obtained is constant. They obey the mathematical relationship:

$$\frac{\frac{A_1}{B_1} - \frac{A_2}{B_2}}{\frac{A_1}{B_1}} \times 100 < 20$$

- A_1 = radioactivity recorded for the standardised preparation at the lowest discriminator setting,
 B_1 = radioactivity recorded for the preparation to be examined at the lowest discriminator setting,
 A_2 = radioactivity recorded for the standard at the discriminator setting such that $A_2 \approx A_1 \times 10^{-3}$,
 B_2 = radioactivity recorded for the preparation to be examined at the latter discriminator setting.

(b) Record the gamma-ray spectrum. The instrument registers only background activity.

RADIOCHEMICAL PURITY

Place a quantity of the injection equivalent to about 2 µCi (74 kBq), diluted to 50 ml with *water R*, in an all-glass distillation apparatus of the type used for the determination of distillation range (2.2.11). Determine the radioactive concentration. Distil until about 25 ml of distillate has been collected. Precautions must be taken to avoid contamination of the air. If the test is carried out in a fume cupboard, the equipment must be protected from draughts. Determine the radioactive concentration of the distillate and of the liquid remaining in the distillation flask. Neither of the radioactive concentrations determined after distillation differs by more than 5 per cent from the value determined before distillation.

RADIOACTIVITY

Determine the radioactivity using a liquid scintillation counter.

01/2005:1582

WATER (¹⁵O) INJECTIONAquae (¹⁵O) solutio iniectionabilis

DEFINITION

Water (¹⁵O) injection is a sterile solution of [¹⁵O]water for diagnostic use. The injection contains not less than 90.0 per cent and not more than 110.0 per cent of the declared oxygen-15 radioactivity at the date and time stated on the label. Not less than 99 per cent of the total radioactivity corresponds to oxygen-15 in the form of water.

PRODUCTION

RADIONUCLIDE PRODUCTION

Oxygen-15 is a radioactive isotope of oxygen which may be produced by various nuclear reactions such as proton irradiation of nitrogen-15 or deuteron irradiation of nitrogen-14.

RADIOCHEMICAL SYNTHESIS

In order to recover oxygen-15 as molecular oxygen from the nitrogen target gas, carrier oxygen is added at concentrations generally ranging from 0.2 per cent V/V to 1.0 per cent V/V. [¹⁵O]Water can be prepared from [¹⁵O]oxygen by reaction with hydrogen using a suitable catalyst.

An alternative method is to produce [¹⁵O]water “in-target” by adding hydrogen to the irradiated target gas at a concentration generally ranging from 2 per cent V/V to 5 per cent V/V.

The [¹⁵O]water vapour contained in the gas-stream is either bubbled through a reservoir of a sterile 9 g/l solution of sodium chloride, or is exchanged by diffusion into such a solution through a membrane filter for dialysis.

Ammonia is a possible chemical impurity in [¹⁵O]water. This may arise either from catalytic conversion of hydrogen and nitrogen on the catalyst or by radiolysis if “in-target” production is used. In addition, there is the possibility of contamination by oxygen-15-labelled oxides of nitrogen. Although these contaminants can be effectively removed from the gas phase by soda lime and charcoal adsorbers, they may break through and be present in the final preparation.

CHARACTERS

A clear, colourless solution.

Oxygen-15 has a half-life of 2.04 min and emits positrons with a maximum energy of 1.732 MeV, followed by annihilation gamma radiation of 0.511 MeV.

IDENTIFICATION

- Record the gamma-ray spectrum using a suitable instrument. The only gamma photons have an energy of 0.511 MeV and, depending on the measurement geometry, a sum peak of 1.022 MeV may be observed.
- It complies with the test for radionuclidic purity (see Tests).
- Examine the chromatogram obtained in the test for radiochemical purity. The retention time of the second peak is due to the radioactivity eluting in the void volume.

TESTS

pH (2.2.3). The pH of the injection is 5.5 to 8.5.

Sterility. It complies with the test for sterility prescribed in the monograph on *Radiopharmaceutical preparations* (0125). The injection may be released for use before completion of the test.

01/2005:0133

Bacterial endotoxins (2.6.14): less than 175/V IU/ml, V being the maximum administered volume in millilitres. The injection may be released for use before completion of the test.

CHEMICAL PURITY

(a) *Ammonium* (2.4.1). 1 ml complies with the limit test for ammonium (10 ppm).

(b) *Nitrates*. To 1 ml add 49 ml of *nitrate-free water R*. Place 5 ml of this solution in a test-tube immersed in iced water, add 0.4 ml of a 100 g/l solution of *potassium chloride R*, 0.1 ml of *diphenylamine solution R* and, dropwise with shaking, 5 ml of *sulphuric acid R*. Transfer the tube to a water-bath at 50 °C. After 15 min, any blue colour in the solution is not more intense than that in a standard prepared at the same time in the same manner using a mixture of 4.5 ml of *nitrate-free water R* and 0.5 ml of *nitrate standard solution* (2 ppm NO_3) *R* (10 ppm).

The injection may be released for use before completion of tests (a) and (b).

RADIONUCLIDIC PURITY

Record the gamma-ray spectrum using a suitable instrument. The spectrum does not differ significantly from that of a standardised fluorine-18 solution. Standardised fluorine-18 solutions are available from the laboratories recognised by the competent authority.

The half-life is between 1.9 min and 2.2 min. Not less than 99 per cent of total radioactivity corresponds to oxygen-15.

The injection may be released for use before completion of the test.

RADIOCHEMICAL PURITY

Examine by liquid chromatography (2.2.29).

Test solution. The preparation to be examined.

The chromatographic procedure may be carried out using:

- a column 0.25 m long and 4.0 mm in internal diameter packed with *aminopropylsilyl silica gel for chromatography R* (10 μm),
- as mobile phase at a flow rate of 1 ml/min a 10 g/l solution of *potassium dihydrogen phosphate R* adjusted to pH 3 with *phosphoric acid R*,
- a suitable radioactivity detector,
- a loop injector,
- an internal recovery detection system, consisting of a loop of the chromatographic tubing between the injector and the column through the radioactivity detector, which has been calibrated for count recovery,

maintaining the column at a constant temperature between 20 °C and 30 °C.

Inject the test solution. Continue the chromatography for 10 min. In the chromatogram obtained, the first peak corresponds to the injected radioactivity of the test solution, the second peak corresponds to the amount of radioactivity as ^{15}O water. Calculate the percentage content of ^{15}O water from the areas of the peaks in the chromatogram obtained with the test solution. Not less than 99 per cent of the total radioactivity injected corresponds to oxygen-15 in the form of water.

The injection may be released for use before completion of the test.

RADIOACTIVITY

Measure the radioactivity using suitable equipment by comparison with a standardised fluorine-18 solution or by using an instrument calibrated with the aid of such a solution.

XENON (^{133}Xe) INJECTION

Xenoni (^{133}Xe) solutio iniectabilis

DEFINITION

Xenon (^{133}Xe) injection is a sterile solution of xenon-133 that may be made isotonic by the addition of sodium chloride. Xenon-133 is a radioactive isotope of xenon and is obtained by separation from the other products of uranium fission. The injection contains not less than 80 per cent and not more than 130 per cent of the declared xenon-133 radioactivity at the date and hour stated on the label.

The injection is presented in a container that allows the contents to be removed without introducing air bubbles. The container is filled as completely as possible and any gas bubble present does not occupy more than 1 per cent of the volume of the injection as judged by visual comparison with a suitable standard.

CHARACTERS

A clear, colourless solution.

Xenon-133 has a half-life of 5.29 days and emits beta and gamma radiation and X-rays.

IDENTIFICATION

Record the gamma-ray and X-ray spectrum using a suitable instrument. The spectrum does not differ significantly from that of a standardised xenon-133 solution in a 9 g/l solution of *sodium chloride R*, apart from any differences attributable to the presence of xenon-131m and xenon-133m. If standardised xenon-133 solutions are not readily available, suitable standardised ionisation chambers are obtainable from laboratories recognised by the relevant competent authority. The most prominent gamma photon of xenon-133 has an energy of 0.081 MeV and there is an X-ray (resulting from internal conversion) of 0.030 MeV to 0.035 MeV. Xenon-131m has a half-life of 11.9 days and emits a gamma photon of 0.164 MeV. Xenon-133m has a half-life of 2.19 days and emits a gamma photon of 0.233 MeV.

TESTS

pH (2.2.3). The pH of the injection is 5.0 to 8.0.

Sterility. It complies with the test for sterility prescribed in the monograph on *Radiopharmaceutical preparations* (0125). The injection may be released for use before completion of the test.

RADIONUCLIDIC PURITY

(a) Record the gamma-ray and X-ray spectrum using a suitable instrument. The spectrum does not differ significantly from that of a standardised xenon-133 solution in a 9 g/l solution of *sodium chloride R*, apart from any differences attributable to the presence of xenon-131m and xenon-133m.

(b) Transfer 2 ml of the injection to an open flask and pass a current of air through the solution for 30 min, taking suitable precautions concerning the dispersion of radioactivity. Measure the residual beta and gamma activity of the solution. The activity does not differ significantly from the background activity detected by the instrument.

RADIOACTIVITY

Weigh the container with its contents. Determine its total radioactivity using suitable counting equipment by comparison with a standardised xenon-133 solution or by measurement in an instrument calibrated with the aid of such a solution, operating in strictly identical conditions. If an ionisation chamber is used its inner wall should be such

that the radiation is not seriously attenuated. Remove at least half the contents and re-weigh the container. Measure the radioactivity of the container and the remaining contents as described above. From the measurements, calculate the radioactive concentration of xenon-133 in the injection.

CAUTION

Significant amounts of xenon-133 may be present in the closures and on the walls of the container. This must be taken into account in applying the rules concerning the transport and storage of radioactive substances and in disposing of used containers

01/2005:90004 TESTS

INTRODUCTION

The following monographs apply to sutures for human use: Catgut, sterile (0317), Sutures, sterile non-absorbable (0324), Sutures, sterile synthetic absorbable braided (0667) and Sutures, sterile synthetic absorbable monofilament (0666). They cover performance characteristics of sutures and may include methods of identification. Sutures are medical devices as defined in Directive 93/42/EEC.

These monographs can be applied to show compliance with essential requirements as defined in Article 3 of Directive 93/42/EEC covering the following:

Physical performance characteristics: diameter, breaking load, needle attachment, packaging, sterility, information supplied by the manufacturer (see Section 13 of Annex 1 of Directive 93/42/EEC), labelling.

To show compliance with other essential requirements, the application of appropriate harmonised standards as defined in Article 5 of Directive 93/42/EEC may be considered.

01/2005:0317

CATGUT, STERILE

Chorda resorbilis sterilis

DEFINITION

Sterile catgut consists of sutures prepared from collagen taken from the intestinal membranes of mammals. After cleaning, the membranes are split longitudinally into strips of varying width, which, when assembled in small numbers, according to the diameter required, are twisted under tension, dried, polished, selected and sterilised. The sutures may be treated with chemical substances such as chromium salts to prolong absorption and glycerol to make them supple, provided such substances do not reduce tissue acceptability.

Appropriate harmonised standards may be considered when assessing compliance with respect to origin and processing of raw materials and with respect to biocompatibility.

Sterile catgut is a surgical wound-closure device. Being an absorbable suture it serves to approximate tissue during the healing period and is subsequently metabolised by proteolytic activity.

PRODUCTION

Production complies with relevant regulations on the use of animal tissues in medical devices notably concerning the risk of transmission of animal spongiform encephalopathy agents.

Appropriate harmonised standards may apply with respect to appropriate validated methods of sterilisation, environmental control during manufacturing, labelling and packaging.

It is essential for the effectiveness and the performance characteristics during use and during the functional lifetime of catgut that the following physical properties are specified: consistent diameter, sufficient initial strength and firm needle attachment.

The requirements outlined below have been established, taking into account stresses which occur during normal conditions of use. These requirements can be used to demonstrate that individual production batches of sterile catgut are suitable for wound closure according to usual surgical techniques.

If stored in a preserving liquid, remove the sutures from the sachet and measure promptly and in succession the length, diameter and breaking load. If stored in the dry state, immerse the sutures in alcohol R or a 90 per cent V/V solution of 2-propanol R for 24 h and proceed with the measurements as indicated below.

Length. Measure the length without applying to the suture more tension than is necessary to keep it straight. The length of each suture is not less than 90 per cent of the length stated on the label and does not exceed 350 cm.

Diameter. Carry out the test on 5 sutures. Use a suitable instrument capable of measuring with an accuracy of at least 0.002 mm and having a circular pressor foot 10 mm to 15 mm in diameter. The pressor foot and the moving parts attached to it are weighted so as to apply a total load of 100 ± 10 g to the suture being tested. When making the measurement, lower the pressor foot slowly to avoid crushing the suture. Measure the diameter at intervals of 30 cm over the whole length of the suture. For a suture less than 90 cm in length, measure at 3 points approximately evenly spaced along the suture. The suture is not subjected to more tension than is necessary to keep it straight during measurement. The average of the measurements carried out on the sutures being tested and not less than two-thirds of the measurements taken on each suture are within the limits given in the columns under A in Table 0317-1 for the gauge number concerned. None of the measurements is outside the limits given in the columns under B in Table 0317-1 for the gauge number concerned.

Table 0317-1.— *Diameters and Breaking Loads*

Gauge number	Diameter (millimetres)				Breaking load (newtons)	
	A		B		C	D
	min.	max.	min.	max.		
0.1	0.010	0.019	0.005	0.025	-	-
0.2	0.020	0.029	0.015	0.035	-	-
0.3	0.030	0.039	0.025	0.045	0.20	0.05
0.4	0.040	0.049	0.035	0.060	0.30	0.10
0.5	0.050	0.069	0.045	0.085	0.40	0.20
0.7	0.070	0.099	0.060	0.125	0.70	0.30
1	0.100	0.149	0.085	0.175	1.8	0.40
1.5	0.150	0.199	0.125	0.225	3.8	0.70
2	0.200	0.249	0.175	0.275	7.5	1.8
2.5	0.250	0.299	0.225	0.325	10	3.8
3	0.300	0.349	0.275	0.375	12.5	7.5
3.5	0.350	0.399	0.325	0.450	20	10
4	0.400	0.499	0.375	0.550	27.5	12.5
5	0.500	0.599	0.450	0.650	38.0	20.0
6	0.600	0.699	0.550	0.750	45.0	27.5
7	0.700	0.799	0.650	0.850	60.0	38.0
8	0.800	0.899	0.750	0.950	70.0	45.0

Minimum breaking load. The minimum breaking load is determined over a simple knot formed by placing one end of a suture held in the right hand over the other end held in the left hand, passing one end over the suture and through the loop so formed (see Figure 0317-1) and pulling the knot tight. Carry out the test on 5 sutures. Submit sutures of length greater than 75 cm to 2 measurements and shorter

sutures to one measurement. Determine the breaking load using a suitable tensiometer. The apparatus has 2 clamps for holding the suture, one of which is mobile and is driven at a constant rate of 30 cm/min. The clamps are designed so that the suture being tested can be attached without any possibility of slipping. At the beginning of the test the length of suture between the clamps is 12.5 cm to 20 cm and the knot is midway between the clamps. Set the mobile clamp in motion and note the force required to break the suture. If the suture breaks in a clamp or within 1 cm of it, the result is discarded and the test repeated on another suture. The average of all the results, excluding those legitimately discarded, is equal to or greater than the value given in column C in Table 0317-1 and no individual result is less than that given in column D for the gauge number concerned.

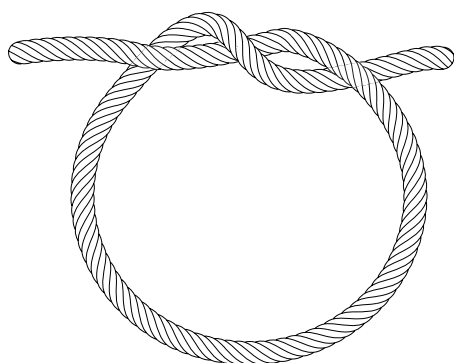


Figure 0317-1. – Simple knot

Soluble chromium compounds. Place 0.25 g in a conical flask containing 1 ml of *water R* per 10 mg of catgut. Stopper the flask, allow to stand at 37 ± 0.5 °C for 24 h, cool and decant the liquid. Transfer 5 ml to a small test tube and add 2 ml of a 10 g/l solution of *diphenylcarbazide R* in *alcohol R* and 2 ml of *dilute sulphuric acid R*. The solution is not more intensely coloured than a standard prepared at the same time using 5 ml of a solution containing 2.83 µg of *potassium dichromate R* per millilitre, 2 ml of *dilute sulphuric acid R* and 2 ml of a 10 g/l solution of *diphenylcarbazide R* in *alcohol R* (1 ppm of Cr).

Needle attachment. If the catgut is supplied with an eyeless needle attached that is not stated to be detachable, it complies with the test for needle attachment. Carry out the test on 5 sutures. Use a suitable tensiometer, such as that described for the determination of the minimum breaking load. Fix the needle and suture (without knot) in the clamps of the apparatus in such a way that the swaged part of the needle is completely free of the clamp and in line with the direction of pull on the suture. Set the mobile clamp in motion and note the force required to break the suture or to detach it from the needle. The average of the 5 determinations and all individual values are not less than the respective values given in Table 0317-2 for the gauge number concerned. If not more than one individual value fails to meet the individual requirement, repeat the test on an additional 10 sutures. The catgut complies with the test if none of these 10 values is less than the individual value in Table 0317-2 for the gauge number concerned.

STORAGE (PACKAGING)

Sterile catgut sutures are presented in individual sachets that maintain sterility and allow the withdrawal and use of the sutures in aseptic conditions. Sterile catgut may be stored dry or in a preserving liquid to which an antimicrobial agent but not an antibiotic may be added.

Sutures in their individual sachets (primary packaging) are kept in a protective cover (box) which maintains the physical and mechanical properties until the time of use.

The application of appropriate harmonised standards for packaging of medical devices shall be considered.

Table 0317-2. – Minimum Strengths of Needle Attachment

Gauge number	Mean value (newtons)	Individual values (newtons)
0.5	0.50	0.25
0.7	0.80	0.40
1	1.7	0.80
1.5	2.3	1.1
2	4.5	2.3
2.5	5.6	2.8
3	6.8	3.4
3.5	11.0	4.5
4	15.0	4.5
5	18.0	6.0

LABELLING

Reference may be made to the appropriate harmonised standards for labelling of medical devices.

The details strictly necessary for the user to identify the product properly are indicated on or in each sachet (primary packaging) and on the protective cover (box) and include at least:

- gauge number,
- length in centimetres or metres,
- if appropriate, that the needle is detachable,
- name of the product,
- intended use (surgical suture, absorbable).

01/2005:0324

SUTURES, STERILE NON-ABSORBABLE

Fila non resorbilia sterilia

DEFINITION

Sterile non-absorbable sutures are sutures which, when introduced into a living organism, are not metabolised by that organism. Sterile non-absorbable sutures vary in origin, which may be animal, vegetable, metallic or synthetic. They occur as cylindrical monofilaments or as multifilament sutures consisting of elementary fibres which are assembled by twisting, cabling or braiding; they may be sheathed; they may be treated to render them non-capillary, and they may be coloured.

Appropriate harmonised standards may be considered when assessing compliance with respect to origin and processing of raw materials and with respect to biocompatibility.

Sterile non-absorbable surgical sutures serve to approximate tissue during the healing period and provide continuing wound support.

Commonly used materials include the following:

Silk (*Filum bombycis*)

Sterile braided silk suture is obtained by braiding a number of threads, according to the diameter required, of degummed silk obtained from the cocoons of the silkworm *Bombyx mori* L.

Linen (Filum lini)

Sterile linen thread consists of the pericyclic fibres of the stem of *Linum usitatissimum* L. The elementary fibres, 2.5 cm to 5 cm long, are assembled in bundles 30 cm to 80 cm long and spun into continuous lengths of suitable diameter.

Poly(ethylene terephthalate) (Filum ethyleni polyterephthalici)

Sterile poly(ethylene terephthalate) suture is obtained by drawing poly(ethylene terephthalate) through a suitable die. The suture is prepared by braiding very fine filaments in suitable numbers, depending on the gauge required.

Polyamide-6 (Filum polyamidicum-6)

Sterile polyamide-6 suture is obtained by drawing through a suitable die a synthetic plastic material formed by the polymerisation of ϵ -caprolactam. It consists of smooth, cylindrical monofilaments or braided filaments, or lightly twisted sutures sheathed with the same material.

Polyamide-6/6 (Filum polyamidicum-6/6)

Sterile polyamide-6/6 suture is obtained by drawing through a suitable die a synthetic plastic material formed by the polycondensation of hexamethylenediamine and adipic acid. It consists of smooth, cylindrical monofilaments or braided filaments, or lightly twisted sutures sheathed with the same material.

Polypropylene (Filum polypropylenicum)

Polypropylene suture is obtained by drawing polypropylene through a suitable die. It consists of smooth cylindrical mono-filaments.

Monofilament and multifilament stainless steel (Filum aciei irrubiginibilis monofilamentum/multifilamentum)

Sterile stainless steel sutures have a chemical composition as specified in ISO 5832-1 - Metallic Materials for surgical implants - Part 1: Specification for wrought stainless steel and comply with ISO 10334 - Implants for surgery - Malleable wires for use as sutures and other surgical applications.

Stainless steel sutures consist of smooth, cylindrical monofilaments or twisted filaments or braided filaments.

Poly(vinylidene difluoride) (PVDF) (Filum poly(vinylideni difluoridum))

Sterile PVDF suture is obtained by drawing through a suitable die a synthetic plastic material which is formed by polymerisation of 1,1-difluoroethylene. It consists of smooth cylindrical monofilaments.

IDENTIFICATION

Non-absorbable sutures may be identified by chemical tests. Materials from natural origin may also be identified by microscopic examination of the morphology of these fibres. For synthetic materials, identification by infrared spectrophotometry (2.2.24) or by differential scanning calorimetry may be applied.

Identification of silk

- A. Dissect the end of a suture, using a needle or fine tweezers, to isolate a few individual fibres. The fibres are sometimes marked with very fine longitudinal striations parallel to the axis of the suture. Examined under a microscope, a cross-section is more or less triangular to semi-circular, with rounded edges and without a lumen.
- B. Impregnate isolated fibres with *iodinated potassium iodide solution R*. The fibres are coloured pale yellow.

Identification of linen

- A. Dissect the end of a suture, using a needle or fine tweezers, to isolate a few individual fibres. Examined under a microscope, the fibres are seen to be 12 μ m

to 31 μ m wide and, along the greater part of their length, have thick walls, sometimes marked with fine longitudinal striations, and a narrow lumen. The fibres gradually narrow to a long, fine point. Sometimes there are unilateral swellings with transverse lines.

- B. Impregnate isolated fibres with *iodinated zinc chloride solution R*. The fibres are coloured violet-blue.

Identification of poly(ethyleneterephthalate)

It is practically insoluble in most of the usual organic solvents, but is attacked by strong alkaline solutions. It is incompatible with phenols.

- A. 50 mg dissolves with difficulty when heated in 50 ml of *dimethylformamide R*.
- B. To about 50 mg add 10 ml of *hydrochloric acid R1*. The material remains intact even after immersion for 6 h.

Identification of polyamide-6

It is practically insoluble in the usual organic solvents; it is not attacked by dilute alkaline solutions (for example a 100 g/l solution of *sodium hydroxide R*) but is attacked by dilute mineral acids (for example a 20 g/l solution of *sulphuric acid R*), by hot *glacial acetic acid R* and by a 70 per cent *m/m* solution of *anhydrous formic acid R*.

- A. Heat about 50 mg with 0.5 ml of *hydrochloric acid R1* in a sealed glass tube at 110 °C for 18 h and allow to stand for 6 h. No crystals appear.
- B. 50 mg dissolves in 20 ml of a 70 per cent *m/m* solution of *anhydrous formic acid R*.

Identification of polyamide-6/6

It is practically insoluble in the usual organic solvents; it is not attacked by dilute alkaline solutions (for example a 100 g/l solution of *sodium hydroxide R*) but is attacked by dilute mineral acids (for example a 20 g/l solution of *sulphuric acid R*), by hot *glacial acetic acid R* and by an 80 per cent *m/m* solution of *anhydrous formic acid R*.

- A. In contact with a flame it melts and burns, forming a hard globule of residue and gives off a characteristic odour resembling that of celery.
- B. Place about 50 mg in an ignition tube held vertically and heat gently until thick fumes are evolved. When the fumes fill the tube, withdraw it from the flame and insert a strip of *nitrobenzaldehyde paper R*. A violet-brown colour slowly appears on the paper and fades slowly in air; it disappears almost immediately on washing with *dilute sulphuric acid R*.
- C. To about 50 mg add 10 ml of *hydrochloric acid R1*. The material disintegrates in the cold and dissolves within a few minutes.
- D. 50 mg does not dissolve in 20 ml of a 70 per cent *m/m* solution of *anhydrous formic acid R* but dissolves in 20 ml of an 80 per cent *m/m* solution of *anhydrous formic acid R*.

Identification of polypropylene

Polypropylene is soluble in decahydronaphthalene, 1-chloronaphthalene and trichloroethylene. It is not soluble in alcohol, in ether and in cyclohexanone.

- A. It softens at temperatures between 160 °C and 170 °C. It burns with a blue flame giving off an odour of burning paraffin wax and of octyl alcohol.
- B. To 0.25 g add 10 ml of *toluene R* and boil under a reflux condenser for about 15 min. Place a few drops of the solution on a disc of *sodium chloride R* slide and evaporate the solvent in an oven at 80 °C. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *polypropylene CRS*.

- C. To 2 g add 100 ml of *water R* and boil under a reflux condenser for 2 h. Allow to cool. The relative density (2.2.5) of the material is 0.89 g/ml to 0.91 g/ml, determined using a hydrostatic balance.

Identification of stainless steel

Stainless steel sutures are identified by confirming that the composition is in accordance with ISO 5832 Part 1.

Identification of poly(vinylidene difluoride)

It is soluble in warm dimethylformamide. It is insoluble in ethanol, hot and cold isopropyl alcohol, ethyl acetate, tetrachlorethylene.

- A. The strand melts between 170 °C and 180 °C. It melts in a flame and does not burn after removal of the flame. Place a small piece of suture on an annealed copper wire or sheet. Heat in an oxidising flame. No green colour is produced.
- B. Dissolve 0.25 g of the suture in 10 ml of *dimethylformamide R* and boil under a reflux condenser for about 15 min. Place a few drops of the solution on a *sodium chloride R* slide and evaporate the solvent in an oven at 80 °C (1 h). Examine by infrared absorption spectrophotometry (2.2.24), comparing with the *Ph. Eur. reference spectrum of poly(vinylidene difluoride)*.
- C. To 2 g of suture add 100 ml of *water R* and boil under a reflux condenser for 2 h. Allow to cool. The relative density (2.2.5) of the material is 1.71 to 1.78.

PRODUCTION

The appropriate harmonised standards may apply with respect to appropriate validated methods of sterilisation, environmental control during manufacturing, labelling and packaging.

It is essential for the effectiveness and the performance characteristics during use and during the functional lifetime of these sutures that the following physical properties are specified: consistent diameter, sufficient initial strength and firm needle attachment.

The requirements below have been established, taking into account stresses which occur during normal conditions of use. These requirements can be used to demonstrate that individual production batches of these sutures are suitable for wound closure in accordance with usual surgical techniques.

TESTS

Remove the sutures from the sachet and measure promptly and in succession the length, diameter and minimum load.

If linen is tested the sutures are conditioned as follows: if stored in the dry state, expose to an atmosphere with a relative humidity of 65 ± 5 per cent at 20 ± 2 °C for 4 h immediately before measuring the diameter and for the determination of minimum breaking load immerse in *water R* at room temperature for 30 min immediately before carrying out the test.

Length. Measure the length without applying more tension than is necessary to keep them straight. The length of the suture is not less than 95 per cent of the length stated on the label and does not exceed 400 cm.

Diameter. Unless otherwise prescribed, measure the diameter by the following method using 5 sutures. Use a suitable mechanical instrument capable of measuring with an accuracy of at least 0.002 mm and having a circular pressor foot 10-15 mm in diameter. The pressor foot and the moving parts attached to it are weighted so as to apply a total load of 100 ± 10 g to the suture being tested. When

making the measurements, lower the pressor foot slowly to avoid crushing the suture. Measure the diameter at intervals of 30 cm over the whole length of the suture. For a suture less than 90 cm in length, measure at 3 points approximately evenly spaced along the suture. During the measurement submit monofilament sutures to a tension not greater than that required to keep them straight. Submit multifilament sutures to a tension not greater than one-fifth of the minimum breaking load shown in column C of Table 0324.-1 appropriate to the gauge number and type of material concerned or 10 N whichever is less. Stainless steel sutures do not require tension to be applied during the measurement of diameter. For multifilament sutures of gauge number above 1.5 make 2 measurements at each point, the second measurement being made after rotating the suture through 90°. The diameter of that point is the average of the 2 measurements. The average of the measurements carried out on the sutures being tested and not less than two-thirds of the measurements taken on each suture are within the limits given in the column under A in Table 0324.-1 for the gauge number concerned. None of the measurements are outside the limits given in the columns under B in Table 0324.-1 for the gauge number concerned.

Minimum breaking load. Unless otherwise prescribed, determine the minimum breaking load by the following method using sutures in the condition in which they are presented. The minimum breaking load is determined over a simple knot formed by placing one end of a suture held in the right hand over the other end held in the left hand, passing one end over the suture and through the loop so formed (see Figure 0324.-1) and pulling the knot tight. For stainless steel sutures gauges 3.5 and above, the minimum breaking load is determined on a straight pull. Carry out the test on 5 sutures. Submit sutures of length greater than 75 cm to 2 measurements and shorter sutures to 1 measurement. Determine the breaking load using a suitable tensiometer. The apparatus has 2 clamps for holding the suture, 1 of which is mobile and is driven at a constant rate of 30 cm/min. The clamps are designed so that the suture being tested can be attached without any possibility of slipping. At the beginning of the test the length of suture between the clamps is 12.5 cm to 20 cm and the knot is midway between the clamps. Set the mobile clamp in motion and note the force required to break the suture. If the suture breaks in a clamp or within 1 cm of it, the result is discarded and the test repeated on another suture. The average of all the results, excluding those legitimately discarded, is equal to or greater than the value given in column C in Table 0324.-1 and no value is less than that given in column D for the gauge number and type of material concerned.

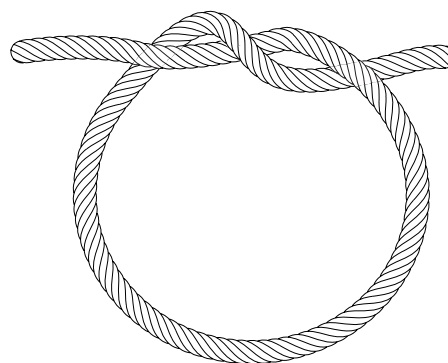


Figure 0324.-1. – Simple knot

Needle attachment. If the sutures are supplied with an eyeless needle attached that is not stated to be detachable, they comply with the test for needle attachment. Carry out

Table 0324.-1. – *Diameters and minimum breaking loads*

Gauge number	Diameter (millimetres)				Minimum breaking load (newtons)					
	A		B		Linen thread		All other non-absorbable strands		Stainless steel	
	min.	max.	min.	max.	C	D	C	D	C	D
0.05	0.005	0.009	0.003	0.012	-	-	0.01	-		
0.1	0.010	0.019	0.005	0.025	-	-	0.03	-		
0.15	0.015	0.019	0.012	0.025	-	-	0.06	0.01		
0.2	0.020	0.029	0.015	0.035	-	-	0.1	-		
0.3	0.030	0.039	0.025	0.045	-	-	0.35	0.06		
0.4	0.040	0.049	0.035	0.060	-	-	0.60	0.15	1.1	
0.5	0.050	0.069	0.045	0.085	-	-	1.0	0.35	1.6	
0.7	0.070	0.099	0.060	0.125	1.0	0.3	1.5	0.60	2.7	
1	0.100	0.149	0.085	0.175	2.5	0.6	3.0	1.0	5.3	4.0
1.5	0.150	0.199	0.125	0.225	5.0	1.0	5.0	1.5	8.0	6.0
2	0.200	0.249	0.175	0.275	8.0	2.5	9.0	3.0	13.3	10.0
2.5	0.250	0.299	0.225	0.325	9.0	5.0	13.0	5.0	15.5	11.6
3	0.300	0.349	0.275	0.375	11.0	8.0	15.0	9.0	17.7	13.3
3.5	0.350	0.399	0.325	0.450	15.0	9.0	22.0	13.0	33.4	25.0
4	0.400	0.499	0.375	0.550	18.0	11.0	27.0	15.0	46.7	35.0
5	0.500	0.599	0.450	0.650	26.0	15.0	35.0	22.0	57.9	43.4
6	0.600	0.699	0.550	0.750	37.0	18.0	50.0	27.0	89.4	67.0
7	0.700	0.799	0.650	0.850	50.0	26.0	62.0	35.0	111.8	83.9
8	0.800	0.899	0.750	0.950	65.0	37.0	73.0	50.0	133.4	100.1
9	0.900	0.999	0.850	1.050					156.0	117.0
10	1.000	1.099	0.950	1.150					178.5	133.9

the test on 5 sutures. Use a suitable tensiometer, such as that described for the determination of the minimum breaking load. Fix the needle and suture (without knot) in the clamps of the apparatus in such a way that the swaged part of the needle is completely free of the clamp and in line with the direction of pull on the suture. Set the mobile clamp in motion and note the force required to break the suture or to detach it from the needle. The average of the 5 determinations and all individual values are not less than the respective values given in Table 0324.-2 for the gauge number concerned. If not more than 1 individual value fails to meet the individual requirement, repeat the test on an additional 10 sutures. The attachment complies with the test if none of these 10 values is less than the individual value in Table 0324.-2 for the gauge number concerned.

Extractable colour. Sutures that are dyed and intended to remain so during use comply with the test for extractable colour. Place 0.25 g of the suture to be examined in a conical flask, add 25.0 ml of *water R* and cover the mouth of the flask with a short-stemmed funnel. Boil for 15 min, cool and adjust to the original volume with *water R*. Depending on the colour of the suture, prepare the appropriate reference solution as described in Table 0324.-3 using the primary colour solutions (2.2.2).

The test solution is not more intensely coloured than the appropriate reference solution.

Table 0324.-2. – *Minimum strengths of needle attachment*

Gauge number	Mean value (newtons)	Individual value (newtons)
0.4	0.50	0.25
0.5	0.80	0.40
0.7	1.7	0.80
1	2.3	1.1
1.5	4.5	2.3
2	6.8	3.4
2.5	9.0	4.5
3	11.0	4.5
3.5	15.0	4.5
4	18.0	6.0
5	18.0	7.0
6	25.0	12.5
7	25.0	12.5
8	50.0	25
9	50.0	25
10	75.0	37.5

Table 0324.-3. – *Colour reference solutions*

Colour of strand	Composition of reference solution (parts by volume)			<i>Water R</i>
	Red primary solution	Yellow primary solution	Blue primary solution	
Yellow-brown	0.2	1.2	-	8.6
Pink-red	1.0	-	-	9.0
Green-blue	-	-	2.0	8.0
Violet	1.6	-	8.4	-

Monomer and oligomers. Polyamide-6 suture additionally complies with the following test for monomer and oligomers. In a continuous-extraction apparatus, treat 1.00 g with 30 ml of *methanol R* at a rate of at least 3 extractions per hour for 7 h. Evaporate the extract to dryness, dry the residue at 110 °C for 10 min, allow to cool in a desiccator and weigh. The residue weighs not more than 20 mg (2 per cent).

STORAGE (PACKAGING)

Sterile non-absorbable sutures are presented in a suitable sachet that maintains sterility and allows the withdrawal and use of a suture in aseptic conditions. They may be stored dry or in a preserving liquid to which an antimicrobial agent but no antibiotic may be added.

Sterile non-absorbable sutures are intended to be used only on the occasion when the sachet is first opened.

Sutures in their individual sachets (primary packaging) are kept in a protective cover (box) which maintains the physical and mechanical properties until the time of use.

The application of appropriate harmonised standards for packaging of medical devices shall be considered in addition.

LABELLING

Reference may be made to the appropriate harmonised standards for the labelling of medical devices.

The details strictly necessary for the user to identify the product properly are indicated on or in each sachet (primary packaging) and on the protective cover (box) and include at least:

- gauge number,
- length, in centimetres or metres,
- if appropriate, that the needle is detachable,
- name of the product,
- intended use (surgical suture, non-absorbable),
- if appropriate, that the suture is coloured,
- if appropriate, the structure (braided, monofilament, sheathed).

01/2005:0667

SUTURES, STERILE SYNTHETIC ABSORBABLE BRAIDED

Fila resorbilia synthetica torta sterilia

DEFINITION

Sterile synthetic absorbable braided sutures consist of sutures prepared from a synthetic polymer, polymers or copolymers which, when introduced into a living organism,

are absorbed by that organism and cause no undue tissue irritation. They consist of completely polymerised material. They occur as multifilament sutures consisting of elementary fibres which are assembled by braiding. The sutures may be treated to facilitate handling and they may be coloured.

Appropriate harmonised standards may be considered when assessing compliance with respect to origin and processing of raw materials and with respect to biocompatibility.

Sterile synthetic absorbable braided sutures are wound-closure devices. Being absorbable they serve to approximate tissue during the healing period and subsequently lose tensile strength by hydrolysis.

PRODUCTION

Appropriate harmonised standards may apply with respect to appropriate validated methods of sterilisation, environmental control during manufacturing, labelling and packaging.

It is essential for the effectiveness and the performance characteristics during use and during the functional lifetime of these sutures that the following physical properties are specified: consistent diameter, sufficient initial strength and firm needle attachment.

The requirements below have been established, taking into account stresses which occur during normal conditions of use. These requirements can be used to demonstrate that individual production batches of these sutures are suitable for wound closure according to usual surgical techniques.

TESTS

Carry out the following tests on the sutures in the state in which they are removed from the sachet.

Length. Measure the length of the suture without applying more tension than is necessary to keep it straight. The length of each suture is not less than 95 per cent of the length stated on the label and does not exceed 400 cm.

Diameter. Unless otherwise prescribed, measure the diameter by the following method, using five sutures in the condition in which they are presented. Use a suitable instrument capable of measuring with an accuracy of at least 0.002 mm and having a circular pressor foot 10 mm to 15 mm in diameter. The pressor foot and the moving parts attached to it are weighted so as to apply a total load of 100 ± 10 g to the suture being tested. When making the measurements, lower the pressor foot slowly to avoid crushing the suture. Measure the diameter at intervals of 30 cm over the whole length of the suture. For a suture less than 90 cm in length, measure at three points approximately evenly spaced along the suture. During the measurement, submit the sutures to a tension not greater than one-fifth of the minimum breaking load shown in column C of Table 0667.-1 appropriate to the gauge number and type of material or 10 N whichever is less. For sutures of gauge number above 1.5 make two measurements at each point, the second measurement being made after rotating the suture through 90°. The diameter of that point is the average of the two measurements. The average of the measurements carried out on the sutures being tested and not less than two-thirds of the measurements taken on each suture are within the limits given in the columns under A in Table 0667.-1 for the gauge

number concerned. None of the measurements is outside the limits given in the columns under B in Table 0667-1 for the gauge number concerned.

Table 0667-1. – *Diameters and breaking loads*

Gauge number	Diameter (millimetres)				Breaking load (newtons)	
	A		B		C	D
	min.	max.	min.	max.		
0.01	0.001	0.004	0.0008	0.005	-	-
0.05	0.005	0.009	0.003	0.012	-	-
0.1	0.010	0.019	0.005	0.025	-	-
0.2	0.020	0.029	0.015	0.035	-	-
0.3	0.030	0.039	0.025	0.045	0.45	0.23
0.4	0.040	0.049	0.035	0.060	0.70	0.35
0.5	0.050	0.069	0.045	0.085	1.4	0.7
0.7	0.070	0.099	0.060	0.125	2.5	1.3
1	0.100	0.149	0.085	0.175	6.8	3.4
1.5	0.150	0.199	0.125	0.225	9.5	4.8
2	0.200	0.249	0.175	0.275	17.7	8.9
2.5	0.250	0.299	0.225	0.325	21.0	10.5
3	0.300	0.349	0.275	0.375	26.8	13.4
3.5	0.350	0.399	0.325	0.450	39.0	18.5
4	0.400	0.499	0.375	0.550	50.8	25.4
5	0.500	0.599	0.450	0.650	63.5	31.8
6	0.600	0.699	0.550	0.750	-	-
7	0.700	0.799	0.650	0.850	-	-

Minimum breaking load. The minimum breaking load is determined over a simple knot formed by placing one end of a suture held in the right hand over the other end held in the left hand, passing one end over the suture and through the loop so formed (see Figure 0667-1) and pulling the knot tight.

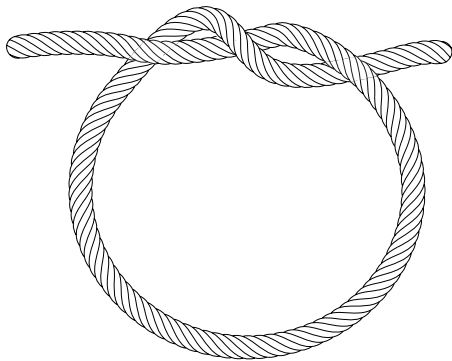


Figure 0667-1. – *Simple knot*

Carry out the test on five sutures. Submit sutures of length greater than 75 cm to two measurements and shorter sutures to one measurement. Determine the breaking load using a suitable tensiometer. The apparatus has two clamps for holding the suture, one of which is mobile and is driven at a constant rate of 25 cm to 30 cm per minute. The clamps are designed so that the suture being tested can be attached without any possibility of slipping. At the beginning of the test the length of suture between the clamps is 12.5 cm to 20 cm and the knot is midway between the clamps. Set the mobile clamp in motion and note the force required to break the suture. If the suture breaks in a clamp or within

1 cm of it, the result is discarded and the test repeated on another suture. The average of all the results excluding those legitimately discarded is equal to or greater than the value given in column C in Table 0667-1 and no individual result is less than that given in column D for the gauge number concerned.

Needle attachment. If the suture is supplied with an eyeless needle attached that is not stated to be detachable the attachment, it complies with the test for needle attachment. Carry out the test on five sutures. Use a suitable tensiometer, such as that described for the determination of the minimum breaking load. Fix the needle and suture (without knot) in the clamps of the apparatus in such a way that the swaged part of the needle is completely free of the clamp and in line with the direction of pull on the suture. Set the mobile clamp in motion and note the force required to break the suture or to detach it from the needle. The average of the five determinations and all individual values are not less than the respective values given in Table 0667-2 for the gauge number concerned. If not more than one individual value fails to meet the individual requirement, repeat the test on an additional ten sutures. The attachment complies with the test if none of the ten values is less than the individual value in Table 0667-2 for the gauge number concerned.

Table 0667-2. – *Minimum strengths of needle attachment*

Gauge number	Mean value (newtons)	Individual value (newtons)
0.4	0.50	0.25
0.5	0.80	0.40
0.7	1.7	0.80
1	2.3	1.1
1.5	4.5	2.3
2	6.8	3.4
2.5	9.0	4.5
3	11.0	4.5
3.5	15.0	4.5
4	18.0	6.0
5	18.0	7.0

STORAGE (PACKAGING)

Sterile synthetic absorbable braided sutures are presented in a suitable sachet that maintains sterility and allows the withdrawal and use of the sutures in aseptic conditions. The sutures must be stored dry.

They are intended to be used only on the occasion when the sachet is first opened.

Sutures in their individual sachets (primary packaging) are kept in a protective cover (box) which maintains the physical and mechanical properties until the time of use.

The application of appropriate harmonised standards for packaging of medical devices may be considered in addition.

LABELLING

Reference may be made to the appropriate harmonised standards for the labelling of medical devices.

The details strictly necessary for the user to identify the product properly are indicated on or in each sachet (primary packaging) and on the protective cover (box) and include at least:

- gauge number,
- length in centimetres or metres,
- if appropriate, that the needle is detachable,

- name of the product,
- intended use (surgical absorbable suture),
- if appropriate, that the suture is coloured,
- the structure (braided).

01/2005:0666

SUTURES, STERILE SYNTHETIC ABSORBABLE MONOFILAMENT

Fila resorbilia synthetica monofilamenta sterilia

DEFINITION

Sterile synthetic absorbable monofilament sutures consist of sutures prepared from a synthetic polymer, polymers or copolymers which, when introduced into a living organism, are absorbed by that organism and cause no undue tissue irritation. They consist of completely polymerised material. They occur as monofilament sutures. The sutures may be treated to facilitate handling and they may be coloured.

Appropriate harmonised standards may be considered when assessing compliance with respect to origin and processing of raw materials and with respect to biocompatibility.

Sterile synthetic absorbable monofilament sutures are wound-closure devices. Being absorbable they serve to approximate tissue during the healing period and subsequently lose tensile strength by hydrolysis.

PRODUCTION

The appropriate harmonised standards may apply with respect to appropriate validated methods of sterilisation, environmental control during manufacturing, labelling and packaging.

It is essential for the effectiveness and the performance characteristics during use and during the functional lifetime of these sutures that the following physical properties are specified: consistent diameter, sufficient initial strength and firm needle attachment.

The requirements below have been established, taking into account stresses which occur during normal conditions of use. These requirements can be used to demonstrate that individual production batches of these sutures are suitable for wound closure according to usual surgical techniques.

TESTS

Carry out the following tests on the sutures in the state in which they are removed from the sachet.

Length. Measure the length of the suture without applying more tension than is necessary to keep it straight. The length of each suture is not less than 95 per cent of the length stated on the label and does not exceed 400 cm.

Diameter. Unless otherwise prescribed, measure the diameter by the following method, using five sutures in the condition in which they are presented. Use a suitable instrument capable of measuring with an accuracy of at least 0.002 mm and having a circular pressor foot 10 mm to 15 mm in diameter. The pressor foot and the moving parts attached to it are weighted so as to apply a total load of 100 ± 10 g to the suture being tested. When making the measurements, lower the pressor foot slowly to avoid crushing the suture. Measure the diameter at intervals of 30 cm over the whole length of the suture. For a suture less than 90 cm in length, measure at three points approximately evenly spaced along

the suture. During the measurement, submit the sutures to a tension not greater than that required to keep them straight. The average of the measurements carried out on the sutures being tested and not less than two-thirds of the measurements taken on each suture are within the limits given in the columns under A in Table 0666.-1 for the gauge number concerned. None of the measurements is outside the limits given in the columns under B in Table 0666.-1 for the gauge number concerned.

Table 0666.-1. – *Diameters and breaking loads*

Gauge number	Diameter (millimetres)				Breaking load (newtons)	
	A		B		C	D
	min.	max.	min.	max.		
0.5	0.050	0.094	0.045	0.125	1.4	0.7
0.7	0.095	0.149	0.075	0.175	2.5	1.3
1	0.150	0.199	0.125	0.225	6.8	3.4
1.5	0.200	0.249	0.175	0.275	9.5	4.7
2	0.250	0.339	0.225	0.375	17.5	8.9
3	0.340	0.399	0.325	0.450	26.8	13.4
3.5	0.400	0.499	0.375	0.550	39.0	18.5
4	0.500	0.570	0.450	0.600	50.8	25.4
5	0.571	0.610	0.500	0.700	63.5	31.8

Minimum breaking load. The minimum breaking load is determined over a simple knot formed by placing one end of a suture held in the right hand over the other end held in the left hand, passing one end over the suture and through the loop so formed (see Figure 0666.-1) and pulling the knot tight.

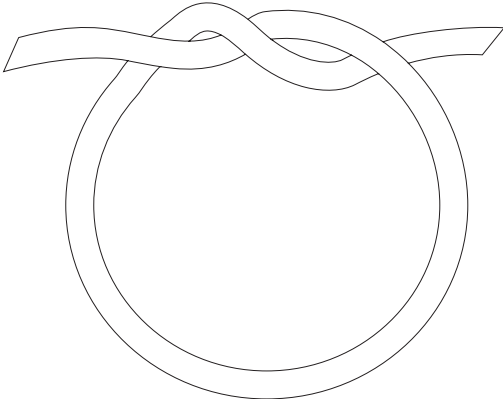


Figure 0666.-1. – *Simple knot*

Carry out the test on five sutures. Submit sutures of length greater than 75 cm to two measurements and shorter sutures to one measurement. Determine the breaking load using a suitable tensiometer. The apparatus has two clamps for holding the suture, one of which is mobile and is driven at a constant rate of 25 cm to 30 cm per minute. The clamps are designed so that the suture being tested can be attached without any possibility of slipping. At the beginning of the test the length of suture between the clamps is 12.5 cm to 20 cm and the knot is midway between the clamps. Set the mobile clamp in motion and note the force required to break the suture. If the suture breaks in a clamp or within 1 cm of it, the result is discarded and the test repeated on another suture. The average of all the results excluding those legitimately discarded is equal to or greater than the value given in column C in Table 0666.-1 and no individual result is less than that given in column D for the gauge number concerned.

Needle attachment. If the suture is supplied with an eyeless needle attached that is not stated to be detachable, the attachment complies with the test for needle attachment. Carry out the test on five sutures. Use a suitable tensiometer, such as that described for the determination of the minimum breaking load. Fix the needle and suture (without knot) in the clamps of the apparatus in such a way that the swaged part of the needle is completely free of the clamp and in line with the direction of pull on the suture. Set the mobile clamp in motion and note the force required to break the suture or to detach it from the needle. The average of the five determinations and all individual values are not less than the respective values given in Table 0666-2 for the gauge number concerned. If not more than one individual value fails to meet the individual requirement, repeat the test on an additional ten sutures. The attachment complies with the test if none of the ten values is less than the individual value in Table 0666-2 for the gauge number concerned.

Table 0666-2. – *Minimum strengths of needle attachment*

Gauge number	Mean value (newtons)	Individual value (newtons)
0.5	0.80	0.40
0.7	1.7	0.80
1	2.3	1.1
1.5	4.5	2.3
2	6.8	3.4
2.5	9.0	4.5
3	11.0	4.5
3.5	15.0	4.5
4	18.0	6.0
5	18.0	7.0

STORAGE (PACKAGING)

Sterile synthetic absorbable monofilament sutures are presented in a suitable sachet that maintains sterility and allows the withdrawal and use of the sutures in aseptic conditions. The sutures must be stored dry.

They are intended to be used only on the occasion when the sachet is first opened.

Sutures in their individual sachets (primary packaging) are kept in a protective cover (box) which maintains the physical and mechanical properties until the time of use.

The application of appropriate harmonised standards for packaging of medical devices may be considered in addition.

LABELLING

Reference may be made to appropriate harmonised standards for the labelling of medical devices.

The details strictly necessary for the user to identify the product properly are indicated on or in each sachet (primary packaging) and on the protective cover (box) and include at least:

- gauge number,
- length in centimetres or metres,
- if appropriate, that the needle is detachable,
- name of the product,
- intended use (surgical absorbable suture),
- if appropriate, that the suture is coloured,
- the structure (monofilament).

01/2005:0660 concerned. None of the measurements is outside the limits given in the columns under B in Table 0660.-1 for the gauge number concerned.

CATGUT, STERILE, IN DISTRIBUTOR
FOR VETERINARY USE

Chorda resorbilis sterilis in fuso
ad usum veterinarium

DEFINITION

Sterile catgut in distributor for veterinary use consists of strands prepared from collagen taken from the intestinal membranes of mammals. After cleaning, the membranes are split longitudinally into strips of varying width, which, when assembled in small numbers, according to the diameter required, are twisted under tension, dried, polished, selected and sterilised. The strands may be treated with chemical substances such as chromium salts to prolong absorption and glycerol to make them supple, provided such substances do not reduce tissue acceptability.

The strand is presented in a distributor that allows the withdrawal and use of all or part of it in aseptic conditions. The design of the distributor is such that with suitable handling the sterility of the content is maintained even when part of the strand has been withdrawn. It may be stored dry or in a preserving liquid to which an antimicrobial preservative but not an antibiotic may be added.

TESTS

If stored in a preserving liquid, remove the strand from the distributor and measure promptly and in succession the length, diameter and breaking load. If stored in the dry state, immerse the strand in alcohol R or a 90 per cent V/V solution of 2-propanol R for 24 h and proceed with the measurements as indicated above.

Length. Measure the length without applying to the strand more tension than is necessary to keep it straight. The length is not less than 95 per cent of the length stated on the label. If the strand consists of several sections joined by knots, the length of each section is not less than 2.5 m.

Diameter. Carry out the test using a suitable instrument capable of measuring with an accuracy of at least 0.002 mm and having a circular pressor foot 10 mm to 15 mm in diameter. The pressor foot and the moving parts attached to it are weighted so as to apply a total load of 100 ± 10 g to the strand being tested. When making the measurements, lower the pressor foot slowly to avoid crushing the strand. Make not fewer than one measurement per 2 m of length. If the strand consists of several sections joined by knots, make not fewer than three measurements per section. In any case make not fewer than twelve measurements. Make the measurements at points evenly spaced along the strand or along each section. The strand is not subjected to more tension than is necessary to keep it straight during measurement. The average of the measurements carried out on the strand being tested and not less than two-thirds of the individual measurements are within the limits given in the column under A in Table 0660.-1 for the gauge number

Table 0660.-1. – *Diameters and breaking loads*

Gauge number	Diameter (millimetres)				Breaking load (newtons)	
	A		B		C	D
	min.	max.	min.	max.		
1	0.100	0.149	0.085	0.175	1.8	0.4
1.5	0.150	0.199	0.125	0.225	3.8	0.7
2	0.200	0.249	0.175	0.275	7.5	1.8
2.5	0.250	0.299	0.225	0.325	10	3.8
3	0.300	0.349	0.275	0.375	12.5	7.5
3.5	0.350	0.399	0.325	0.450	20	10
4	0.400	0.499	0.375	0.550	27.5	12.5
5	0.500	0.599	0.450	0.650	38.4	20.0
6	0.600	0.699	0.550	0.750	45.0	27.5
7	0.700	0.799	0.650	0.850	60.0	38.0
8	0.800	0.899	0.750	0.950	70.0	45.0

Minimum breaking load. The minimum breaking load is determined over a simple knot formed by placing one end of a strand held in the right hand over the other end held in the left hand, passing one end over the strand and through the loop so formed (see Figure 0660.-1) and pulling the knot tight.

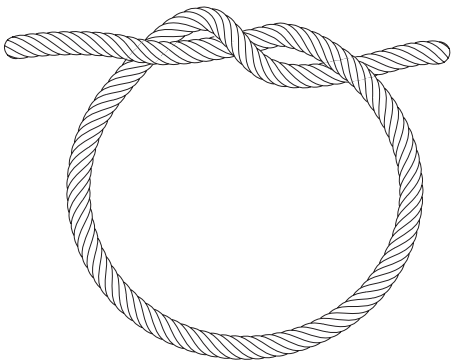


Figure 0660.-1. – *Simple knot*

Make not fewer than one measurement per 2 m of length. If the strand consists of several sections joined by knots, make not fewer than three measurements per section and, in any case, not fewer than one measurement per 2 m of length at points evenly spaced along the strand or along each section. Determine the breaking load using a suitable tensiometer. The apparatus has two clamps for holding the strand, one of which is mobile and is driven at a constant rate of 30 cm per minute. The clamps are designed so that the strand being tested can be attached without any possibility of slipping. At the beginning of the test the length of strand between the clamps is 12.5 cm to 20 cm and the knot is midway between the clamps. Set the mobile clamp in motion and note the force required to break the strand. If the strand breaks in a clamp or within 1 cm of it, the result is discarded and the test repeated on another part of the strand. The average of all the results, excluding those legitimately discarded, is equal to or greater than the value in column C and no value is less than that given in column D in Table 0660.-1 for the gauge number concerned.

Soluble chromium compounds. Place 0.25 g in a conical flask containing 1 ml of *water R* per 10 mg of catgut. Stopper the flask, allow to stand at 37 ± 0.5 °C for 24 h, cool and decant the liquid. Transfer 5 ml to a small test tube and add 2 ml of a 10 g/l solution of *diphenylcarbazide R* in *alcohol R* and 2 ml of *dilute sulphuric acid R*. The solution is not more intensely coloured than a standard prepared at the same time using 5 ml of a solution containing 2.83 µg of *potassium dichromate R* per millilitre, 2 ml of *dilute sulphuric acid R* and 2 ml of a 10 g/l solution of *diphenylcarbazide R* in *alcohol R* (1 ppm of Cr).

Sterility (2.6.1). It complies with the test for sterility as applied to catgut and other surgical sutures. Carry out the test on three sections, each 30 cm long, cut off respectively from the beginning, the centre and the end of the strand.

STORAGE

Store protected from light and heat.

LABELLING

The label states:

- the gauge number,
- the length in centimetres or in metres.

01/2005:0608

LINEN THREAD, STERILE, IN DISTRIBUTOR FOR VETERINARY USE

Filum lini sterile in fuso
ad usum veterinarium

DEFINITION

Sterile linen thread in distributor for veterinary use consists of the pericyclic fibres of the stem of *Linum usitatissimum* L. The elementary fibres, 2.5 cm to 5 cm long, are assembled in bundles 30 cm to 80 cm long and spun into continuous lengths of suitable diameter. The thread may be creamy-white or may be coloured with colouring matter authorised by the competent authority. The thread is sterilised.

IDENTIFICATION

- A. Dissect the end of a thread, using a needle or fine tweezers, to isolate a few individual fibres. Examined under a microscope, the fibres are seen to be 12 µm to 31 µm wide and, along the greater part of their length, have thick walls, sometimes marked with fine longitudinal striations, and a narrow lumen. The fibres gradually narrow to a long, fine point. Sometimes there are unilateral swellings with transverse lines.
- B. Impregnate isolated fibres with *iodinated zinc chloride solution R*. The fibres are coloured violet-blue.

TESTS

It complies with the tests prescribed in the monograph on *Strands, sterile non-absorbable, in distributor for veterinary use (0605)*.

If stored in a dry state, expose to an atmosphere with a relative humidity of 65 ± 5 per cent at 20 ± 2 °C for 4 h immediately before measuring the diameter and for the determination of minimum breaking load immerse in water R at room temperature for 30 min immediately before carrying out the test.

STORAGE

See the monograph on *Strands, sterile non-absorbable, in distributor for veterinary use (0605)*.

LABELLING

See the monograph on *Strands, sterile non-absorbable, in distributor for veterinary use (0605)*.

01/2005:0609

POLYAMIDE 6 SUTURE, STERILE, IN DISTRIBUTOR FOR VETERINARY USE

Filum polyamidicum-6 sterile in fuso
ad usum veterinarium

DEFINITION

Sterile polyamide 6 suture in distributor for veterinary use is obtained by drawing through a suitable die a synthetic plastic material formed by the polymerisation of ε-caprolactam. It consists of smooth, cylindrical monofilaments or braided filaments, or lightly twisted strands sheathed with the same material. It may be coloured with colouring matter authorised by the competent authority. The suture is sterilised.

CHARACTERS

It is practically insoluble in the usual organic solvents; it is not attacked by dilute alkaline solutions (for example a 100 g/l solution of sodium hydroxide) but is attacked by dilute mineral acids (for example 20 g/l sulphuric acid), by hot glacial acetic acid and by 70 per cent *m/m* formic acid.

IDENTIFICATION

- A. Heat about 50 mg with 0.5 ml of *hydrochloric acid R1* in a sealed glass tube at 110 °C for 18 h and allow to stand for 6 h. No crystals appear.
- B. To about 50 mg add 10 ml of *hydrochloric acid R1*. The material disintegrates in the cold and dissolves completely within a few minutes.
- C. It dissolves in a 70 per cent *m/m* solution of *anhydrous formic acid R*.

TESTS

It complies with the tests prescribed in the monograph on *Strands, sterile non-absorbable, in distributor for veterinary use (0605)* and with the following test:

Monomer and oligomers. In a continuous-extraction apparatus, treat 1.00 g with 30 ml of *methanol R* at a rate of at least three extractions per hour for 7 h. Evaporate the extract to dryness, dry the residue at 110 °C for 10 min, allow to cool in a desiccator and weigh. The residue weighs not more than 20 mg (2 per cent).

STORAGE

See the monograph on *Strands, sterile non-absorbable, in distributor for veterinary use (0605)*.

LABELLING

See the monograph on *Strands, sterile non-absorbable, in distributor for veterinary use (0605)*.

The label states whether the suture is braided, monofilament or sheathed.

01/2005:0610

01/2005:0607

POLYAMIDE 6/6 SUTURE, STERILE, IN DISTRIBUTOR FOR VETERINARY USE

Filum polyamidicum-6/6 sterile in fuso ad usum veterinarium

DEFINITION

Sterile polyamide 6/6 suture in distributor for veterinary use is obtained by drawing through a suitable die a synthetic plastic material formed by the polycondensation of hexamethylene-diamine and adipic acid. It consists of smooth, cylindrical monofilaments or braided filaments, or lightly twisted strands sheathed with the same material. It may be coloured with authorised colouring matter or pigments authorised by the competent authority. The suture is sterilised.

CHARACTERS

It is practically insoluble in the usual organic solvents; it is not attacked by dilute alkaline solutions (for example a 100 g/l solution of sodium hydroxide) but is attacked by dilute mineral acids (for example 20 g/l sulphuric acid), by hot glacial acetic acid and by 80 per cent *m/m* formic acid.

IDENTIFICATION

- A. In contact with a flame it melts and burns, forming a hard globule of residue and gives off a characteristic odour resembling that of celery.
- B. Place about 50 mg in an ignition tube held vertically and heat gently until thick fumes are evolved. When the fumes fill the tube, withdraw it from the flame and insert a strip of *nitrobenzaldehyde paper R*. A violet-brown colour slowly appears on the paper and fades slowly in air; it disappears immediately on washing with *dilute sulphuric acid R*.
- C. To about 50 mg add 10 ml of *hydrochloric acid R1*. The material disintegrates in the cold and dissolves within a few minutes.
- D. It does not dissolve in a 70 per cent *m/m* solution of *anhydrous formic acid R* but dissolves in an 80 per cent *m/m* solution of *anhydrous formic acid R*.

TESTS

It complies with the tests prescribed in the monograph on *Strands, sterile non-absorbable, in distributor for veterinary use (0605)*.

STORAGE

See the monograph on *Strands, sterile non-absorbable, in distributor for veterinary use (0605)*.

LABELLING

See the monograph on *Strands, sterile non-absorbable, in distributor for veterinary use (0605)*.

The label states whether the suture is braided, monofilament or sheathed.

POLY(ETHYLENE TEREPHTHALATE) SUTURE, STERILE, IN DISTRIBUTOR FOR VETERINARY USE

Filum ethyleni polyterephthalici sterile in fuso ad usum veterinarium

DEFINITION

Sterile poly(ethylene terephthalate) suture in distributor for veterinary use is obtained by drawing poly(ethylene terephthalate) through a suitable die. The suture is prepared by braiding very fine filaments in suitable numbers, depending on the gauge required. It may be whitish in colour, or may be coloured with authorised colouring matter or pigments authorised by the competent authority. The suture is sterilised.

CHARACTERS

It is practically insoluble in most of the usual organic solvents, but is attacked by strong alkaline solutions. It is incompatible with phenols.

IDENTIFICATION

- A. It dissolves with difficulty when heated in *dimethylformamide R* and in *dichlorobenzene R*.
- B. To about 50 mg add 10 ml of *hydrochloric acid R1*. The material remains intact even after immersion for 6 h.

TESTS

It complies with the tests prescribed in the monograph on *Strands, sterile non-absorbable, in distributor for veterinary use (0605)*.

STORAGE

See the monograph on *Strands, sterile non-absorbable, in distributor for veterinary use (0605)*.

LABELLING

See the monograph on *Strands, sterile non-absorbable, in distributor for veterinary use (0605)*.

01/2005:0606

SILK SUTURE, STERILE, BRAIDED, IN DISTRIBUTOR FOR VETERINARY USE

Filum bombycis tortum sterile in fuso ad usum veterinarium

DEFINITION

Sterile braided silk suture in distributor for veterinary use is obtained by braiding a variable number of threads, according to the diameter required, of degummed silk obtained from the cocoons of the silkworm *Bombyx mori* L. It may be coloured with colouring matter authorised by the competent authority. The suture is sterilised.

IDENTIFICATION

- A. Dissect the end of a strand, using a needle or fine tweezers, to isolate a few individual fibres. The fibres are sometimes marked with very fine longitudinal striations parallel to the axis of the strand. Examined under a microscope, a cross-section is more or less triangular or semi-circular, with rounded edges and without a lumen.

B. Impregnate isolated fibres with *iodinated potassium iodide solution R*. The fibres are coloured pale yellow.

TESTS

It complies with the tests prescribed in the monograph on *Strands, sterile non-absorbable, in distributor for veterinary use (0605)*.

STORAGE

See the monograph on *Strands, sterile non-absorbable, in distributor for veterinary use (0605)*.

LABELLING

See the monograph on *Strands, sterile non-absorbable, in distributor for veterinary use (0605)*.

01/2005:0605

STRANDS, STERILE NON-ABSORBABLE, IN DISTRIBUTOR FOR VETERINARY USE

Fila non resorbilia sterilia in fuso ad usum veterinarium

DEFINITION

The statements in this monograph are intended to be read in conjunction with the individual monographs on sterile non-absorbable strands in distributor for veterinary use in the Pharmacopoeia. The requirements do not necessarily apply to sterile non-absorbable strands which are not the subject of such monographs.

Sterile non-absorbable strands in distributor for veterinary use are strands which, when introduced into a living organism, are not metabolised by that organism. Sterile non-absorbable strands vary in origin, which may be animal, vegetable or synthetic. They occur as cylindrical monofilaments or as multifilament strands. Multifilament strands consist of elementary fibres which are assembled by twisting, cabling or braiding. Such strands may be sheathed. Sterile non-absorbable strands may be treated to render them non-capillary, and they may be coloured with colouring matter or pigments authorised by the competent authority. The strands are sterilised.

They are presented in a suitable distributor that allows the withdrawal and use of all or part of the strand in aseptic conditions. The design of the distributor is such that with suitable handling the sterility of the content is maintained even when part of the strand has been removed. They may be stored dry or in a preserving liquid to which an antimicrobial preservative but not an antibiotic may be added.

TESTS

Remove the strand from the distributor and measure promptly and in succession the length, diameter and minimum breaking load.

Length. Measure the length in the condition in which the strand is presented and without applying more tension than is necessary to keep it straight. The length of the strand is not less than 95 per cent of the length stated on the label.

Diameter. Unless otherwise prescribed, measure the diameter by the following method using the strand in the condition in which it is presented. Use a suitable instrument capable of measuring with an accuracy of at least 0.002 mm and having a circular pressor foot 10 mm to 15 mm in diameter. The pressor foot and the moving parts attached to it are weighted so as to apply a total load of 100 ± 10 g to

the strand being tested. When making the measurements, lower the pressor foot slowly to avoid crushing the strand. Make not fewer than one measurement per 2 m of length and in any case not fewer than 12 measurements at points evenly spaced along the strand. During the measurement submit monofilament strands to a tension not greater than that required to keep them straight. Submit multifilament strands to a tension not greater than one-fifth of the minimum breaking load shown in column C of Table 0605.-1 appropriate to the gauge number and type of material concerned or 10 N whichever is less. For multifilament strands of gauge number above 1.5 make two measurements at each point, the second measurement being made after rotating the strand through 90°. The diameter of that point is the average of the two measurements. The average of the measurements carried out on the strand being tested and not less than two-thirds of the individual measurements are within the limits given in the columns under A in Table 0605.-1 for the gauge number concerned. None of the measurements is outside the limits given in the columns under B in Table 0605.-1 for the gauge number concerned.

Table 0605.-1. – *Diameters and minimum breaking loads*

Gauge number	Diameter (millimetres)				Minimum breaking load (newtons)			
	A		B		Linen thread		All other non-absorbable strands	
	min.	max.	min.	max.	C	D	C	D
0.5	0.050	0.069	0.045	0.085	-	-	1.0	0.35
0.7	0.070	0.099	0.060	0.125	1.0	0.3	1.5	0.60
1	0.100	0.149	0.085	0.175	2.5	0.6	3.0	1.0
1.5	0.150	0.199	0.125	0.225	5.0	1.0	5.0	1.5
2	0.200	0.249	0.175	0.275	8.0	2.5	9.0	3.0
2.5	0.250	0.299	0.225	0.325	9.0	5.0	13.0	5.0
3	0.300	0.349	0.275	0.375	11.0	8.0	15.0	9.0
3.5	0.350	0.399	0.325	0.450	15.0	9.0	22.0	13.0
4	0.400	0.499	0.375	0.550	18.0	11.0	27.0	15.0
5	0.500	0.599	0.450	0.650	26.0	15.0	35.0	22.0
6	0.600	0.699	0.550	0.750	37.0	18.0	50.0	27.0
7	0.700	0.799	0.650	0.850	50.0	26.0	62.0	35.0
8	0.800	0.899	0.750	0.950	65.0	37.0	73.0	50.0

Minimum breaking load. Unless otherwise prescribed, determine the minimum breaking load by the following method using the strand in the condition in which it is presented. The minimum breaking load is determined over a simple knot formed by placing one end of a strand held in the right hand over the other end held in the left hand, passing one end over the strand and through the loop so formed (see Figure 0605.-1) and pulling the knot tight.

Make not fewer than one measurement per 2 m of length at points evenly spaced along the strand. Determine the breaking load using a suitable tensiometer. The apparatus has two clamps for holding the strand, one of which is mobile and is driven at a constant rate of 30 cm per minute. The clamps are designed so that the strand being tested can be attached with-out any possibility of slipping. At the beginning of the test the length of strand between the clamps is 12.5 cm to 20 cm and the knot is midway between the clamps. Set the mobile clamp in motion and note the force required to break the strand. If the strand breaks in a clamp or within 1 cm of it, the result is discarded and the test repeated on another part of the strand. The average of all

the results, excluding those legitimately dis-carded, is equal to or greater than the value in column C and no value is less than that given in column D in Table 0605.-1 for the gauge number and type of material concerned.

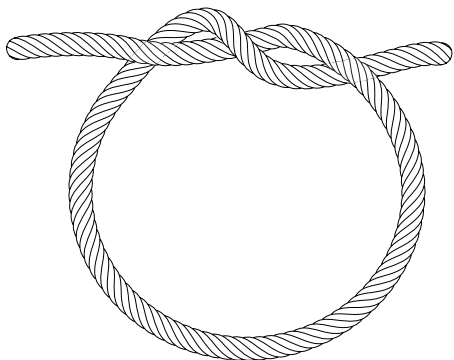


Figure 0605.-1. – Simple knot

Sterility (2.6.1). They comply with the test for sterility as applied to catgut and other surgical sutures. Carry out the test on three sections each 30 cm long, cut off respectively from the beginning, the centre and the end of the strand.

Extractable colour. Strands that are dyed and intended to remain so during use comply with the test for extractable colour. Place 0.25 g of the strand to be examined in a conical flask, add 25.0 ml of *water R* and cover the mouth of the flask with a short-stemmed funnel. Boil for 15 min, cool and

adjust to the original volume with *water R*. Depending on the colour of the strand, prepare the appropriate reference solution as described in Table 0605.-2 using the primary colour solutions (2.2.2).

Table 0605.-2. – Colour reference solutions

Colour of strand	Composition of reference solution (parts by volume)			
	Red primary solution	Yellow primary solution	Blue primary solution	Water
Yellow - brown	0.2	1.2	–	8.6
Pink - red	1.0	–	–	9.0
Green - blue	–	–	2.0	8.0
Violet	1.6	–	8.4	–

The test solution is not more intensely coloured than the appropriate reference solution.

STORAGE

Store protected from light and heat.

LABELLING

The label states:

- the gauge number,
- the length in centimetres or in metres,
- where appropriate, that the strand is coloured and intended to remain so during use.

01/2005:90006

INTRODUCTION

All general texts and other monographs of the European Pharmacopoeia that are relevant to homoeopathy are applicable.

The "Homoeopathy" chapter of the European Pharmacopoeia contains general monographs and individual monographs describing starting materials and preparations used virtually exclusively for homoeopathic medicines. Reference to these monographs for other purposes may be authorised by licensing authorities.

01/2005:2045

HERBAL DRUGS FOR HOMOEOPATHIC PREPARATIONS

Plantae medicinales ad praeparationes homoeopathicas

DEFINITION

Herbal drugs for homoeopathic preparations are mainly whole, fragmented or cut, plants, parts of plants including algae, fungi or lichens in an unprocessed state, usually in fresh form but sometimes dried. Certain exudates that have not been subjected to a specific treatment are also considered to be herbal drugs for homoeopathic preparations. Herbal drugs for homoeopathic preparations are precisely defined by the botanical scientific name of the source species according to the binomial system (genus, species, variety and author).

PRODUCTION

Herbal drugs for homoeopathic preparations are obtained from cultivated or wild plants. Suitable cultivation, harvesting, collection, sorting, drying, fragmentation and storage conditions are essential to guarantee the quality of herbal drugs for homoeopathic preparations.

Herbal drugs for homoeopathic preparations are, as far as possible, free from impurities such as soil, dust, dirt and other contaminants such as fungal, insect and other animal contaminants. They do not present signs of decay.

If a decontaminating treatment has been used, it is necessary to demonstrate that the constituents of the plant are not affected and that no harmful residues remain. The use of ethylene oxide is prohibited for the decontamination of herbal drugs for homoeopathic preparations.

Adequate measures have to be taken in order to ensure that the microbiological quality of homoeopathic preparations containing one or more herbal drugs comply with the recommendations given in the text on *Microbiological quality of pharmaceutical preparations* (5.1.4).

IDENTIFICATION

Herbal drugs for homoeopathic preparations are identified using their macroscopic and, where necessary, microscopic descriptions and any further tests that may be required (for example, thin-layer chromatography).

TESTS

When a fresh plant is used as a starting material for the manufacture of homoeopathic preparations, the content of foreign matter is as low as possible; if necessary, the maximum content of foreign matter is indicated in the individual monographs. When a dried plant is used as a

starting material for the manufacture of homoeopathic preparations, a test for foreign matter (2.8.2) is carried out, unless otherwise prescribed in the individual monographs.

A specific appropriate test may apply to herbal drugs for homoeopathic preparations liable to be falsified.

If appropriate, the herbal drugs for homoeopathic preparations comply with other tests, for example, total ash (2.4.16) and bitterness value (2.8.15).

The test for loss on drying (2.2.32) is carried out on dried herbal drugs for homoeopathic preparations. A determination of water (2.2.13) is carried out on herbal drugs for homoeopathic preparations with a high essential oil content. The water content of fresh herbal drugs for homoeopathic preparations is determined by an appropriate method.

Herbal drugs for homoeopathic preparations comply with the requirements for pesticide residues (2.8.13). The requirements take into account the nature of the plant, where necessary the preparation in which the plant might be used, and where available the knowledge of the complete record of treatment of the batch of the plant. The content of pesticide residues may be determined by the method described in the annex to the general method.

The risk of contamination of herbal drugs for homoeopathic preparations by heavy metals must be considered. If an individual monograph does not prescribe limits for heavy metals or specific elements, such limits may be required if justified.

Limits for aflatoxins may be required.

In some specific circumstances, the risk of radioactive contamination is to be considered.

ASSAY

Where applicable, herbal drugs for homoeopathic preparations are assayed by an appropriate method.

STORAGE

Fresh herbal drugs are processed as rapidly as possible after harvesting; they may also be stored deep-frozen or in ethanol (96 per cent V/V) or in alcohol of a given concentration. Store dried herbal drugs protected from light.

01/2005:1038

HOMOEOPATHIC PREPARATIONS

Praeparationes homoeopathicas

DEFINITION

Homoeopathic preparations are prepared from substances, products or preparations called stocks, in accordance with a homoeopathic manufacturing procedure. A homoeopathic preparation is usually designated by the Latin name of the stock, followed by an indication of the degree of dilution.

Raw materials

Raw materials for the production of homoeopathic preparations may be of natural or synthetic origin.

For raw materials of zoological or human origin, adequate measures are taken to minimise the risk of agents of infection in the homoeopathic preparations. For this purpose, it is demonstrated that:

- the method of production includes a step or steps that have been shown to remove or inactivate agents of infection,

- where applicable, raw materials of zoological origin comply with the monograph on *Products with risk of transmitting agents of animal spongiform encephalopathies (1483)*,
- where applicable, the animals and the tissues used to obtain the raw materials comply with the health requirements of the competent authorities for animals for human consumption,
- for materials of human origin, the donor follows the recommendations applicable to human blood donors and to donated blood (see *Human plasma for fractionation (0853)*), unless otherwise justified and authorised.

A raw material of botanical, zoological or human origin may be used either in the fresh state or in the dried state. Where appropriate, fresh material may be kept deep-frozen. Raw materials of botanical origin comply with the requirements of the monograph on *Herbal drugs for homoeopathic preparations (2045)*.

Where justified and authorised for transportation or storage purposes, fresh plant material may be kept in ethanol (96 per cent V/V) or in alcohol of a suitable concentration, provided the whole material including the storage medium is used for processing.

Raw materials comply with any requirements of the relevant monographs of the European Pharmacopoeia.

Vehicles

Vehicles are excipients used for the preparation of certain stocks or for the potentisation process. They may include for example: purified water, alcohol of a suitable concentration, glycerol and lactose.

Vehicles comply with any requirements of the relevant monographs of the European Pharmacopoeia.

Stocks

Stocks are substances, products or preparations used as starting materials for the production of homoeopathic preparations. A stock is usually one of the following: a mother tincture or a glycerol macerate, for raw materials of botanical, zoological or human origin, or the substance itself, for raw materials of chemical or mineral origin.

Mother tinctures comply with the requirements of the monograph on *Mother tinctures for homoeopathic preparations (2029)*.

Glycerol macerates are liquid preparations obtained from raw materials of botanical, zoological or human origin by using glycerol or a mixture of glycerol and either alcohol of a suitable concentration or a solution of sodium chloride of a suitable concentration.

Potentisation

Dilutions and triturations are obtained from stocks by a process of potentisation in accordance with a homoeopathic manufacturing procedure: this means successive dilutions and succussions, or successive appropriate triturations, or a combination of the 2 processes.

The potentisation steps are usually one of the following:

- 1 part of the stock plus 9 parts of the vehicle; they may be designated as “D”, “DH” or “X” (decimal),
- 1 part of the stock plus 99 parts of the vehicle; they may be designated as “C” or “CH” (centesimal).

The number of potentisation steps defines the degree of dilution; for example, “D3”, “3 DH” or “3X” means 3 decimal potentisation steps, and “C3”, “3 CH” or “3C” means 3 centesimal potentisation steps.

“LM-” (or “Q-”) potencies are manufactured according to a specific procedure.

Dosage forms

A dosage form of a homoeopathic preparation complies with any relevant dosage form monograph in the European Pharmacopoeia and with the following:

- for the purpose of dosage forms for homoeopathic use “active substances” are considered to be “dilutions or triturations of homoeopathic stocks”,
- these dosage forms are prepared using appropriate excipients,
- the test for uniformity of content is normally not appropriate. However, in certain circumstances, it is required.

Homoeopathic dosage form “pillule”

Pillules for homoeopathic use are solid preparations obtained from sucrose, lactose or other suitable excipients. They may be prepared by impregnation of preformed pillules with a dilution or dilutions of homoeopathic stocks or by progressive addition of these excipients and the addition of a dilution or dilutions of homoeopathic stocks. They are intended for oral or sublingual use.

Homoeopathic dosage form “tablet”

Tablets for homoeopathic use are solid preparations obtained from sucrose, lactose or other suitable excipients according to the monograph on *Tablets (0478)*. They may either be prepared by compressing one or more solid active substances with the excipients or by impregnating preformed tablets with a dilution or dilutions of homoeopathic stocks. The preformed tablets for impregnation are obtained from sucrose, lactose or other suitable excipients according to the monograph on *Tablets (0478)*. They are intended for oral or sublingual use.

01/2005:2029

MOTHER TINCTURES FOR HOMOEOPATHIC PREPARATIONS

Tincturae maternae ad praeparationes homoeopathicas

DEFINITION

Mother tinctures for homoeopathic preparations are liquid preparations obtained by the solvent action of a suitable vehicle upon raw materials. The raw materials are usually in the fresh form but may be dried. Mother tinctures for homoeopathic preparations may also be obtained from plant juices, with, or without the addition of a vehicle. For some preparations, the matter to be extracted may undergo a preliminary treatment.

PRODUCTION

Mother tinctures for homoeopathic preparations are prepared by maceration, digestion, infusion, decoction, fermentation or as described in the individual monographs, usually using alcohol of suitable concentration.

Mother tinctures for homoeopathic preparations are obtained using a fixed proportion of raw material to solvent, taking the moisture content of the raw material into account, unless otherwise justified and authorised.

If fresh plants are used, suitable procedures are used to ensure freshness. The competent authorities may require that the freshness is demonstrated by means of a suitable test.

Mother tinctures for homoeopathic preparations are usually clear. A slight sediment may form on standing and that is acceptable as long as the composition of the tincture is not changed significantly.

The manufacturing process is defined so that it is reproducible.

Production by maceration. Unless otherwise prescribed, reduce the matter to be extracted to pieces of suitable size, mix thoroughly and extract according to the prescribed extraction method with the prescribed extraction solvent. Allow to stand in a closed vessel for the prescribed time. The residue is separated from the extraction solvent and, if necessary, pressed out. In the latter case, the 2 liquids obtained are combined.

Adjustment of the contents. Adjustment of the content of constituents may be carried out if necessary, either by adding the extraction solvent of suitable concentration, or by adding another mother tincture for homoeopathic preparations of the vegetable or animal matter used for the preparation.

IDENTIFICATION

Where applicable, at least 1 chromatographic identification test is carried out.

TESTS

The limits in an individual monograph are set to include official methods of production. Specific limits will apply to each defined method of production.

If the test for relative density is carried out, the test for ethanol need not be carried out, and vice versa.

Relative density (2.2.5). The mother tincture for homoeopathic preparations complies with the limits prescribed in the monograph.

Ethanol (2.9.10). The ethanol content complies with that prescribed in the monograph.

Methanol and 2-propanol (2.9.11): maximum 0.05 per cent V/V of methanol and maximum 0.05 per cent V/V of 2-propanol, unless otherwise prescribed.

Dry residue (2.8.16). Where applicable, the mother tincture for homoeopathic preparations complies with the limits prescribed in the monograph.

Pesticides (2.8.13). Where applicable, the mother tincture for homoeopathic preparations complies with the test. This requirement is met if the herbal drug has been shown to comply with the test.

ASSAY

Where applicable, an assay with quantitative limits is performed.

STORAGE

Protected from light. A maximum storage temperature may be specified.

LABELLING

The label states:

- that the product is a mother tincture for homoeopathic preparations (designated as "TM" or "Ø"),
- the name of the raw material using the Latin title of the European Pharmacopoeia monograph where one exists,
- the method of preparation,
- the ethanol content or other solvent content, in per cent V/V, in the mother tincture,
- the ratio of raw material to mother tincture,
- where applicable, the storage conditions.

01/2005:1599

ARSENIOUS TRIOXIDE FOR HOMOEOPATHIC PREPARATIONS

Arsenii trioxidum ad praeparationes homoeopathicas

As₂O₃M_r 197.8

DEFINITION

Content: 99.5 per cent to 100.5 per cent of As₂O₃.

CHARACTERS

Appearance: white or almost white powder.

Solubility: practically insoluble to sparingly soluble in water. It dissolves in solutions of alkali hydroxides and carbonates.

IDENTIFICATION

- Dissolve 20 mg in 1 ml of *dilute hydrochloric acid R*, add 4 ml of *water R* and 0.1 ml of *sodium sulphide solution R*. The resulting yellow precipitate is soluble in *dilute ammonia R1*.
- Dissolve 20 mg in 1 ml of *hydrochloric acid R1*, add 5 ml of *hypophosphorous reagent R* and heat for 15 min on a water-bath. A black precipitate develops.

TESTS

Appearance of solution. A 100 g/l solution in *dilute ammonia R1* is clear (2.2.1) and colourless (2.2.2, *Method II*).

Sulphides. Dissolve 1.0 g in 10.0 ml of *dilute sodium hydroxide solution R*. Add 0.05 ml of *lead acetate solution R*. Any colour in the test solution is not more intense than that in a standard prepared at the same time and in the same manner using a mixture of 10.0 ml of a 0.015 g/l solution of *sodium sulphide R* in *dilute sodium hydroxide solution R* and 0.05 ml of *lead acetate solution R* (20 ppm).

ASSAY

Dissolve 40.0 mg in a mixture of 10 ml of *water R* and 10 ml of *dilute sodium hydroxide solution R*. Add 10 ml of *dilute hydrochloric acid R* and 3 g of *sodium hydrogen carbonate R* and mix. Add 1 ml of *starch solution R* and titrate with 0.05 M iodine.

1 ml of 0.05 M iodine is equivalent to 4.946 mg of As₂O₃.

01/2005:2030

COMMON STINGING NETTLE FOR HOMOEOPATHIC PREPARATIONS

Urtica dioica ad praeparationes homoeopathicas

DEFINITION

Whole, fresh, flowering plant of *Urtica dioica* L.

CHARACTERS

Macroscopic characters described under Identification A. The plant causes an itching, burning sensation on the skin.

IDENTIFICATION

- Common stinging nettle is perennial. The taproot sends out creeping subterranean rhizomes, more or less 4-angled in transverse section, from which extend

adventitious secondary roots and very numerous brownish hairy rootlets. The stipes are erect, generally unbranched, 3 mm to 5 mm in diameter and 0.3 m to 1.5 m high, rarely up to 2.5 m high, 4-angled, greyish-green and covered in short hairs and stinging hairs.

The decussate leaves are 30 mm to 150 mm long and 20 mm to 80 mm wide. The petiole is hispid and usually slightly less than one-third the length of the lamina. The leaf blade is ovate, acuminate, cordate or rounded at the base, and coarsely dentate; the apical tooth is distinctly larger than the lateral teeth. The upper side of the leaves is dark green and usually matt, both sides bear short serried hairs intermingled with long stinging hairs. The 2 stipules are linear-subulate and free. The inflorescences growing from the leaf axils are complex, the flowers unisexual, and, particularly in male plants, generally distinctly longer than the petiole. After shedding their pollen, male inflorescences are erect at an oblique angle or horizontal; female inflorescences are pendent when the fruit is ripe. All flowers have long stalks. The perianth of the male flowers is divided half-way down into equal green lobes, widest at their base, with short bristles and stinging hairs at the margins. The stamens are equal and opposite to the perianth segments, each with a long, whitish filament that curves inwards before pollen is shed and spreads out afterwards. The ovary is rudimentary, button or cup-shaped. The perianth of the female flowers is downy or bristly on the outside and consists of outer, and 2 inner segments; the inner segments are about twice the length of the outer ones. The hypogynous, ovate, unilocular ovary bears a large capitate stigma with a brush-like shock of hair. As the one-seeded fruit grows ripe, the 2 inner segments of the perianth fold around it like wings.

B. It complies with the test for *Urtica urens* (see Tests).

TESTS

Urtica urens. The margin of the lamina is not serrate with teeth twice as long as wide. The clusters of flowers in the axils are longer than the petiole of the leaf. Unisexual, apetalous flowers are not together on the same plant and in the same cluster.

Foreign matter (2.8.2): maximum 5 per cent.

Loss on drying (2.2.32): minimum 65.0 per cent, determined on 5.0 g of finely cut drug by drying in an oven at 100-105 °C for 2 h, if performed to demonstrate the freshness of the drug.

Mother tincture

The mother tincture complies with the requirements of the general monograph on *Mother tinctures for homoeopathic preparations* (2029).

PRODUCTION

The mother tincture of *Urtica dioica* L. is prepared by maceration using alcohol of a suitable concentration.

CHARACTERS

Appearance: greenish-brown or orange-brown liquid.

IDENTIFICATION

Thin-layer chromatography (2.2.27).

Test solution. The mother tincture to be examined.

Reference solution. Dissolve 10 mg of *phenylalanine R* and 10 mg of *serine R* in a mixture of equal volumes of *methanol R* and *water R* and dilute to 10 ml with the same mixture of solvents.

Plate: *TLC silica gel plate R.*

Mobile phase: *glacial acetic acid R, water R, acetone R, butanol R* (10:20:35:35 V/V/V/V).

Application: 20 µl, as bands.

Development: over a path of 10 cm.

Drying: in air.

Detection: spray with a 1 g/l solution of *ninhydrin R* in *alcohol R*. Heat the plate at 105-110 °C for 5-10 min then examine in daylight within 10 min.

Results: see below the sequence of the zones present in the chromatograms obtained with the reference solution and the test solution.

Top of the plate	
Phenylalanine: a violet to reddish-brown zone	4 red to violet zones
Serine: a reddish-violet zone	A violet zone A violet zone
Reference solution	Test solution

TESTS

Relative density (2.2.5): 0.930 to 0.950.

Ethanol (2.9.10): 40 per cent V/V to 56 per cent V/V.

Dry residue (2.8.16): minimum 1.1 per cent.

01/2005:1610

COPPER FOR HOMOEOPATHIC PREPARATIONS

Cuprum ad praeparationes homoeopathicas

Cu

A_r 63.5

DEFINITION

Content: 99.0 per cent to 101.0 per cent of Cu.

CHARACTERS

Appearance: reddish-brown powder.

Solubility: practically insoluble in water, soluble in hydrochloric acid and in nitric acid, practically insoluble in alcohol.

IDENTIFICATION

A. To 2 ml of solution S (see Tests) add 0.5 ml of *potassium ferrocyanide solution R*. A reddish-brown precipitate is formed.

B. To 5 ml of solution S add 0.6 ml of *ammonia R*. A blue precipitate is formed. Add 2 ml of *ammonia R*. The precipitate disappears; the solution has an intense blue colour.

TESTS

Solution S. Dissolve 2.0 g in 10 ml of *nitric acid R*. After nitrous fumes are no longer evolved, dilute to 60 ml with *distilled water R*.

Acidity or alkalinity. To 5.0 g add 20 ml of *carbon dioxide-free water R*. Boil for 1 min. Cool. Filter and dilute to 25.0 ml with *carbon dioxide free water R*. To 10 ml of the solution add 0.1 ml of *bromothymol blue solution R1*.

Not more than 0.5 ml of 0.01 M hydrochloric acid or 0.01 M sodium hydroxide is required to change the colour of the indicator.

Chlorides (2.4.4): maximum 100 ppm.

15 ml of solution S complies with the limit test for chlorides.

Sulphates (2.4.13): maximum 300 ppm.

15 ml of solution S complies with the limit test for sulphates.

Iron: maximum 50 ppm.

Atomic absorption spectrometry (2.2.23, Method I).

Test solution. Dissolve 1.00 g in 5 ml of nitric acid R and dilute to 50.0 ml with water R.

Reference solutions. Prepare the reference solutions using iron standard solution (20 ppm Fe) R, diluted as necessary with a 1 per cent V/V solution of nitric acid R.

Source: iron hollow-cathode lamp.

Wavelength: 248.3 nm.

Flame: air-acetylene.

Lead: maximum 100 ppm.

Atomic absorption spectrometry (2.2.23, Method I).

Test solution. Use the test solution prepared for the test for iron.

Reference solutions. Prepare the reference solutions using lead standard solution (0.1 per cent Pb) R, diluted as necessary with a 1 per cent V/V solution of nitric acid R.

Source: lead hollow-cathode lamp.

Wavelength: 283.3 nm.

Flame: air-acetylene.

Zinc: maximum 50 ppm.

Atomic absorption spectrometry (2.2.23, Method I).

Test solution. Use the test solution prepared for the test for iron.

Reference solutions. Prepare the reference solutions using zinc standard solution (100 ppm Zn) R, diluted as necessary with a 1 per cent V/V solution of nitric acid R.

Source: zinc hollow-cathode lamp.

Wavelength: 213.9 nm.

Flame: air-acetylene.

ASSAY

Dissolve 0.100 g in 5 ml of nitric acid R. Heat to expel the nitrous fumes. Add 200 ml of water R and neutralise (2.2.3) with dilute ammonia R1. Add 1 g of ammonium chloride R and 3 mg of murexide R. Titrate with 0.1 M sodium edetate until the colour changes from green to violet.

1 ml of 0.1 M sodium edetate is equivalent to 6.354 mg of Cu.

01/2005:2023

GARLIC FOR HOMOEOPATHIC PREPARATIONS

Allium sativum ad praeparationes homoeopathicas

DEFINITION

Fresh bulb of *Allium sativum* L.

CHARACTERS

Macroscopic characters described under identification.

It has a characteristic odour after cutting.

IDENTIFICATION

The bulb is generally 3 cm to 5 cm broad and almost spherical; the flat base bears the remnants of numerous short greyish-brown adventitious roots. The bulb consists of about 10 daughter bulbs (cloves) arranged roughly in a circle around a central axis. Individual daughter bulbs are 1 cm to 3 cm long, laterally compressed and convex on the dorsal side. Each daughter bulb has a tough, white or reddish skin around a fleshy tubular leaf, investing a more or less rounded elongated cone of leaf primordia and vegetative apex.

TESTS

Foreign matter (2.8.2). It complies with the test for foreign matter.

Water (2.2.13): minimum 55.0 per cent, determined on 10.0 g of the finely cut drug, if performed to demonstrate the freshness of the drug.

Mother tincture

The mother tincture complies with the requirements of the general monograph on *Mother tinctures for homoeopathic preparations* (2029).

PRODUCTION

The mother tincture of *Allium sativum* L. is prepared by maceration of the cut drug using alcohol of a suitable concentration.

CHARACTERS

Appearance: brownish-yellow liquid.

It has a peculiar and unpleasant aromatic odour.

IDENTIFICATION

A. To 2 ml of the mother tincture to be examined, add 0.2 ml of dilute sodium hydroxide solution R. A yellowish-white precipitate develops.

B. Thin-layer chromatography (2.2.27).

Test solution. Extract 5 ml of the mother tincture to be examined with 2 quantities, each of 10 ml, of ether R. Combine the ether layers and dry over anhydrous sodium sulphate R. Filter and evaporate the filtrate in a water-bath at low temperature. Dissolve the residue in 0.4 ml of methanol R.

Reference solution. Dissolve 10 mg of resorcinol R, 10 mg of thymol R and 30 mg of gallic acid R in 10 ml of methanol R.

Plate: TLC silica gel F₂₅₄ plate R.

Mobile phase: anhydrous formic acid R, toluene R, di-isopropyl ether R (10:40:50 V/V/V).

Application: 40 µl of the test solution and 10 µl of the reference solution.

Development: over a path of 10 cm.

Drying: in air.

Detection: examine in ultraviolet light at 254 nm and identify gallic acid; spray with anisaldehyde solution R, heat to 105–110 °C for 5–10 min. Examine in daylight within 10 min.

Results: see below the sequence of the zones present in the chromatograms obtained with the reference solution and the test solution. Other zones may also be visible in the chromatogram obtained with the test solution.

Top of the plate	
Thymol: an orange-red zone	An intense reddish-violet zone
	An intense reddish-violet zone
	A violet zone
	A yellowish or greenish zone
Resorcinol: an intense orange-red zone	
Gallic acid: a yellow zone	A violet zone
(UV at 254 nm: a fluorescent quenching zone)	A greenish-yellow zone
	A violet zone may be present
Reference solution	Test solution

TESTS

Relative density (2.2.5): 0.885 to 0.960.

Ethanol (2.9.10): 50 per cent V/V to 70 per cent V/V.

Dry residue (2.8.16): minimum 4.0 per cent.

STORAGE

In an airtight container, protected from light.

01/2005:2024

HONEY BEE FOR HOMOEOPATHIC PREPARATIONS

Apis mellifera
ad praeparationes homoeopathicas

DEFINITION

Live worker honey bee (*Apis mellifera* L.).

CHARACTERS

Characters described under Identification.

PRODUCTION

If the bee has been exposed to treatment to prevent or cure diseases, appropriate steps are taken to ensure that the levels of residues are as low as possible.

IDENTIFICATION

The body of a honey bee is about 15 mm long, black, with a silky sheen, and covered with red hairs with a touch of grey. The broad tibiae are without spines. The posterior margins of the segments and legs are brown, with gradual transition to orange-red. The claws are two-membered, the maxillary palps single-membered. On the hind legs are baskets or scoops invested with bristles. The wings have 3 complete cubital cells, with the radial cell twice as long as it is wide; the 3 cells on the lower margin and the 3 middle cells are closed. A duct connects the barbed sting with the poison sac.

Mother tincture

The mother tincture complies with the requirements of the general monograph on *Mother tinctures for homoeopathic preparations* (2029).

PRODUCTION

The mother tincture of *Apis mellifera* L. is prepared by maceration using alcohol of a suitable concentration.

CHARACTERS

Pale yellow liquid that may darken on storage.

IDENTIFICATION

Thin-layer chromatography (2.2.27).

Test solution. The mother tincture to be examined.

Reference solution. Dissolve 12 mg of 4-aminobutanoic acid R, 12 mg of leucine R and 12 mg of proline R in 5 ml of water R and dilute to 50 ml with alcohol R.

Plate: TLC silica gel plate R.

Mobile phase: water R, ethanol R (17:63 V/V).

Application: 20 µl, as bands.

Development: over a path of 10 cm.

Drying: in air.

Detection: spray with ninhydrin solution R and heat at 100-105 °C for 10 min; examine in daylight.

Results: see below the sequence of the zones present in the chromatograms obtained with the reference and test solutions. Other zones may also be visible.

Top of the plate	
Leucine: a pink zone	A pink zone
	A pink zone
	A pink zone
	A pink zone
Proline: an orange-yellow zone	An orange-yellow zone
4-Aminobutanoic acid: a pink zone	A pink zone
Reference solution	Test solution

TESTS

Relative density (2.2.5): 0.890 to 0.910.

Ethanol (2.9.10): 60 per cent V/V to 70 per cent V/V.

Dry residue (2.8.16): minimum 0.30 per cent.

01/2005:2028

HYPERICUM FOR HOMOEOPATHIC PREPARATIONS

Hypericum perforatum
ad praeparationes homoeopathicas

DEFINITION

Whole, fresh plant of *Hypericum perforatum* L., at the beginning of the flowering period.

CHARACTERS

Macroscopic characters described under Identification.

IDENTIFICATION

The perennial plant consists of a spindle-shaped root and a branched rhizome, giving rise to long, decumbent runners. The cylindrical, erect stem is woody at the base, 0.2 m to 1 m long, branched in the upper part, with 2 raised longitudinal lines.

The leaves are opposite, sessile, exstipulate, oblong-oval and 15 mm to 30 mm long. The leaf margins show black glandular dots, and many small translucent oil glands are present on the entire surface and are visible by transmitted light.

The flowers are regular and form corymbose clusters at the apex of the stem. They have 5 green, lanceolate sepals with acuminate apices, and black oil glands near the entire margins; 5 orange-yellow petals, much longer than the sepals, with black oil glands near the terminal margins only; 3 staminal blades, each divided into many orange-yellow stamens and 3 carpels surmounted by red styles. Each petal is asymmetrically linear-ovate in shape, with one of the margin entire and the other dentate.

TESTS

Foreign matter (2.8.2): maximum 4 per cent of fruits and maximum 1 per cent of other foreign matter.

Loss on drying (2.2.32): if performed to demonstrate the freshness of the drug, minimum 55 per cent, determined on 5.0 g of finely cut drug by drying in an oven at 100-105 °C.

Mother tincture

The mother tincture complies with the requirements of the general monograph on *Mother tinctures for homoeopathic preparations* (2029).

PRODUCTION

The mother tincture of *Hypericum perforatum* L. is prepared by maceration using alcohol of a suitable concentration.

CHARACTERS

Dark red to brownish red liquid.

IDENTIFICATION

Thin-layer chromatography (2.2.27).

Test solution. The mother tincture to be examined.

Reference solution. Dissolve 5 mg of *rutin R*, 1 mg of *hypericin R* and 5 mg of *hyperoside R* in *methanol R* and dilute to 5 ml with the same solvent.

Plate: TLC silica gel plate *R*.

Mobile phase: *anhydrous formic acid R*, *water R*, *ethyl acetate R* (6:9:90 V/V/V).

Application: 10 µl of the test solution and 5 µl of the reference solution, as 10 mm bands.

Development: over a path of 10 cm.

Drying: at 100-105 °C for 10 min.

Detection: spray with a 10 g/l solution of *diphenylboric acid aminoethyl ester R* in *methanol R* and then a 50 g/l solution of *macrogol 400 R* in *methanol R*. Examine the plates after 30 min in ultraviolet light at 365 nm.

Results: see below the sequence of the zones present in the chromatograms obtained with the reference solution and the test solution. In the chromatogram obtained with the test solution, the zone due to rutin may be weak or even absent. The chromatogram obtained with the test solution shows a group of zones that may be blue or yellow, with a *R_f* similar to that of the zone due to hyperoside in the chromatogram obtained with the reference solution. Other weak zones may also be visible.

Top of the plate	
Hypericin: a red zone	A yellow to blue zone 2 red zones
Hyperoside: a yellow to orange zone	Several zones Blue or yellow zones
Rutin: a yellow to orange zone	A yellow to orange zone
Reference solution	Test solution

TESTS

Relative density (2.2.5): 0.900 to 0.920.

Ethanol (2.9.10): 60 per cent V/V to 75 per cent V/V.

Dry residue (2.8.16): minimum 1.3 per cent.

01/2005:2026
corrected

IRON FOR HOMOEOPATHIC PREPARATIONS

Ferrum ad praeparationes homoeopathicas

Fe

A_r 55.85

DEFINITION

Obtained by reduction or sublimation as a fine blackish-grey powder.

Content: 97.5 per cent to 101.0 per cent.

CHARACTERS

Appearance: fine, blackish-grey powder, without metallic lustre.

Solubility: practically insoluble in water and in alcohol. It dissolves with heating in dilute mineral acids.

IDENTIFICATION

Dissolve 50 mg in 2 ml of *dilute sulphuric acid R* and dilute to 10 ml with *water R*. The solution gives reaction (a) of iron (2.3.1).

TESTS

Solution S. To 10.0 g add 40 ml of *water R*. Boil for 1 min. Cool, filter and dilute to 50.0 ml with *water R*.

Alkalinity. To 10 ml of solution S add 0.1 ml of *bromothymol blue solution R1*. Not more than 0.1 ml of 0.01 M *hydrochloric acid* is required to change the colour of the indicator to yellow.

Substances insoluble in hydrochloric acid. Dissolve 2.00 g in 40 ml of *hydrochloric acid R*. Heat on a water-bath. As soon as fumes are no longer evolved, filter through a sintered-glass filter No. 16 (2.1.2). Rinse with *water R*. Dry the residue in an oven at 100-105 °C for 1 h. The residue weighs a maximum of 20 mg (1.0 per cent).

Substances soluble in water. Evaporate 10.0 ml of solution S on a water-bath and dry at 100-105 °C for 1 h. The residue weighs a maximum of 2 mg (0.1 per cent).

Chlorides (2.4.4): maximum 50 ppm.

Dilute 5 ml of solution S to 15 ml with *water R*. The solution complies with the limit test for chlorides.

Sulphides and phosphides. In a 100 ml conical flask carefully mix 1.0 g with 10 ml of *dilute hydrochloric acid R*. Within 30 s *lead acetate paper R* moistened with *water R* and placed over the mouth of the flask is not coloured more intensely than light brown by the resulting fumes.

Arsenic (2.4.2): maximum 5 ppm.

Boil 0.2 g in 25 ml of *dilute hydrochloric acid R* until completely dissolved. The solution complies with limit test A.

Copper: maximum 50 ppm.

Atomic absorption spectrometry (2.2.23, *Method I*).

Test solution. Dissolve 1.00 g in a mixture of 60 ml of *dilute hydrochloric acid R* and 10 ml of *dilute hydrogen peroxide solution R*. Reduce to a volume of 5 ml and dilute to 50.0 ml with *water R*.

Reference solutions. Prepare the reference solutions using *copper standard solution (0.1 per cent Cu) R*, diluted as necessary with a 1 per cent V/V solution of *hydrochloric acid R*.

Source: copper hollow-cathode lamp.

Wavelength: 324.8 nm.

Flame: air-acetylene.

Lead: maximum 50 ppm.

Atomic absorption spectrometry (2.2.23, *Method I*).

Test solution. In a separating funnel, place 20 ml of the test solution prepared for the test for copper. Add 25 ml of *lead-free hydrochloric acid R*. Stir with 3 quantities, each of 25 ml, of *di-isopropyl ether R*. Collect the aqueous layer.

Add 0.10 g of *sodium sulphate decahydrate R*. Evaporate to dryness. Take up the residue with 1 ml of *lead-free nitric acid R* and dilute to 20 ml with *water R*.

Reference solutions. Prepare the reference solutions using *lead standard solution (0.1 per cent Pb) R*, diluted as necessary with a 10 per cent V/V solution of *nitric acid R* containing 5 g/l of *sodium sulphate decahydrate R*.

Source: lead hollow-cathode lamp.

Wavelength: 217 nm.

Flame: air-acetylene.

ASSAY

Stir for 10 min 0.100 g in a hot solution of 1.25 g of *copper sulphate R* in 20 ml of *water R* in a 100 ml conical flask with a ground-glass stopper. Filter rapidly and wash the filter. Combine the filtrate and the washings, acidify with *dilute sulphuric acid R* and titrate with 0.02 M *potassium permanganate* until a pink colour is obtained.

1 ml of 0.02 M *potassium permanganate* is equivalent to 5.585 mg of Fe.

LABELLING

The label indicates whether the iron for homoeopathic preparations is obtained by reduction or sublimation.

01/2005:1624

SAFFRON FOR HOMOEOPATHIC PREPARATIONS

Croci stigma ad praeparationes homoeopathicas

DEFINITION

Dried stigmas of *Crocus sativus* L. usually joined by the base to a short style.

CHARACTERS

Saffron has a characteristic, aromatic odour.

It has the macroscopic and microscopic characters described under identification tests A and B.

IDENTIFICATION

- A. The dark brick-red stigmas, when dry, are 20 mm to 40 mm long and after soaking with water, about 35 mm to 50 mm long. The tubes, gradually widening at the top, are incised on one side, the upper margin is open and finely crenated. The style connecting the 3 stigmas is pale yellow and not more than 5 mm long.
- B. Examine under a microscope using *chloral hydrate solution R*. It shows the following diagnostic characters: elongated epidermal cells, frequently with a short, central papilla; in water they release a yellow colouring matter; the upper border of the stigma has finger-shaped papillae, up to 150 µm long; between them are single, globular pollen grains, about 100 µm wide, with a finely pitted exine, vascular bundles with small spirally thickened vessels and no fibres.
- C. Carefully crush pieces of the drug to coarse particles and moisten with 0.2 ml of *phosphomolybdic acid solution R*. The particles turn blue within 1-2 min or they have a blue areole around them.
- D. Examine by thin-layer chromatography (2.2.27).

Test solution. Carefully crush 0.1 g of the drug with a glass rod and moisten with 0.2 ml of *water R*. After 3 min add 5 ml of *methanol R*, allow to stand for 20 min, protected from light, and filter through a plug of glass wool.

Reference solution. Dissolve 5 mg of *naphthol yellow R* in 5 ml of *methanol R* and add a solution of 5 mg of *Sudan red G R* in 5 ml of *methylethylene chloride R*.

Plate: TLC silica gel F₂₅₄ plate R.

Mobile phase: *water R*, 2-propanol R, *ethyl acetate R* (10:25:65 V/V/V).

Application: 10 µl of the test solution and 5 µl of the reference solution as bands.

Development: over a path of 10 cm.

Drying: in air.

Detection: examine in daylight.

Results: see below the sequence of the zones present in the chromatograms obtained with the reference and test solutions.

Top of the plate	
A red zone	2 yellow zones An intense yellow zone (crocin)
A yellow zone	
Reference solution	Test solution

Detection: in ultraviolet light at 254 nm.

Results: see below the sequence of the zones present in the chromatograms obtained with the reference and test solutions.

Top of the plate	
A red zone	1 or 2 quenching zones
A yellow zone	A quenching zone
Reference solution	Test solution

Detection: spray with *anisaldehyde solution R* and examine in daylight while heating at 100-105 °C for 5-10 min.

Results: see below the sequence of the zones present in the chromatograms obtained with the reference and test solutions.

Top of the plate	
A red zone	1 or 2 red to reddish-violet zones
A blue to bluish-green zone	A red to reddish-violet zone
	2 blue to bluish-green zones
	An intense blue to bluish-green zone (crocin)
Reference solution	Test solution

- E. Dilute 0.1 ml of the test solution (see Identification test D) with 1 ml of *methanol R*. Deposit 0.1 ml of this solution on a filter paper, allow to dry and spray with a 10 g/l

solution of *diphenylboric acid aminoethyl ester R* in *methanol R*. Examine in ultraviolet light at 365 nm. The spot shows an intense orange-yellow fluorescence.

TESTS

Colouring power. Introduce 0.10 g into a 5 ml volumetric flask and add to 5.0 ml with *distilled water R*. Close the flask and shake every 30 min for 8 h. Then allow to stand for 16 h. Dilute 1.0 ml to 500.0 ml with *distilled water R*. The absorbance (2.2.25) measured at 440 nm using *distilled water R* as the compensation liquid, is not less than 0.44.

Foreign matter. Examine the drug microscopically. No parts with rough walls, no crystals and no pollen grains containing 3 germinal pores are present.

Loss on drying (2.2.32): maximum 10.0 per cent, determined on 0.200 g by drying in an oven at 100-105 °C.

Total ash (2.4.16): maximum 7.0 per cent, determined on the residue obtained in the test for loss on drying.

01/2005:0307

ACACIA**Acaciae gummi****DEFINITION**

Acacia is the air-hardened, gummy exudate flowing naturally from or obtained by incision of the trunk and branches of *Acacia senegal* L. Willdenow, other species of *Acacia* of African origin and *Acacia seyal* Del.

CHARACTERS

Acacia is almost completely but very slowly soluble, after about 2 h, in twice its mass of water leaving only a very small residue of vegetable particles; the liquid obtained is colourless or yellowish, dense, viscous, adhesive, translucent and weakly acid to blue litmus paper. Acacia is practically insoluble in alcohol.

It has the macroscopic and microscopic characters described under identification tests A and B.

IDENTIFICATION

- A. Acacia occurs as yellowish-white, yellow or pale amber, sometimes with a pinkish tint, friable, opaque, spheroidal, oval or reniform pieces (tears) of a diameter from about 1 cm to 3 cm, frequently with a cracked surface, easily broken into irregular, whitish or slightly yellowish angular fragments with conchoidal fracture and a glassy and transparent appearance. In the centre of an unbroken tear there is sometimes a small cavity.
- B. Reduce to a powder (355). The powder is white or yellowish-white. Examine under a microscope using *glycerol R* (50 per cent *V/V*). The powder presents angular, irregular, colourless, transparent fragments. Only traces of starch or vegetable tissues are visible. No stratified membrane is apparent.
- C. Examine the chromatograms obtained in the test for glucose and fructose. The chromatogram obtained with the test solution shows three zones due to galactose, arabinose and rhamnose. No other important zones are visible, particularly in the upper part of the chromatogram.
- D. Dissolve 1 g of the powdered drug (355) in 2 ml of *water R* by stirring frequently for 2 h. Add 2 ml of *alcohol R*. After shaking, a white, gelatinous mucilage is formed which becomes fluid on adding 10 ml of *water R*.

TESTS

Solution S. Dissolve 3.0 g of the powdered drug (355) in 25 ml of *water R* by stirring for 30 min. Allow to stand for 30 min and dilute to 30 ml with *water R*.

Insoluble matter. To 5.0 g of the powdered drug (355) add 100 ml of *water R* and 14 ml of *dilute hydrochloric acid R*, boil gently for 15 min, shaking frequently, and filter while hot through a tared sintered-glass filter. Wash with hot *water R* and dry at 100-105 °C. The residue weighs not more than 25 mg (0.5 per cent).

Glucose and fructose. Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel plate R*.

Test solution. To 0.100 g of the powdered drug (355) in a thick-walled centrifuge tube add 2 ml of a 100 g/l solution of *trifluoroacetic acid R*, shake vigorously to dissolve the forming gel, stopper the tube and heat the mixture at 120 °C for 1 h. Centrifuge the hydrolysate, transfer the clear supernatant carefully into a 50 ml flask, add 10 ml

of *water R* and evaporate the solution to dryness under reduced pressure. To the resulting clear film add 0.1 ml of *water R* and 0.9 ml of *methanol R*. Centrifuge to separate the amorphous precipitate. Dilute the supernatant, if necessary, to 1 ml with *methanol R*.

Reference solution. Dissolve 10 mg of *arabinose R*, 10 mg of *galactose R*, 10 mg of *glucose R*, 10 mg of *rhamnose R* and 10 mg of *xylose R* in 1 ml of *water R* and dilute to 10 ml with *methanol R*.

Apply to the plate as bands 10 µl of each solution. Develop over a path of 10 cm using a mixture of 10 volumes of a 16 g/l solution of *sodium dihydrogen phosphate R*, 40 volumes of *butanol R* and 50 volumes of *acetone R*. Dry the plate in a current of warm air for a few minutes and develop again over a path of 15 cm using the same mobile phase. Dry the plate at 110 °C for 10 min, spray with *anisaldehyde solution R* and heat again at 110 °C for 10 min. The chromatogram obtained with the reference solution shows five clearly separated coloured zones due to galactose (greyish-green to green), glucose (grey), arabinose (yellowish-green), xylose (greenish-grey to yellowish-grey) and rhamnose (yellowish-green), in order of increasing *R_f* value. The chromatogram obtained with the test solution shows no grey zone and no greyish-green zone between the zones corresponding to galactose and arabinose in the chromatogram obtained with the reference solution.

Starch, dextrin and agar. To 10 ml of solution S previously boiled and cooled add 0.1 ml of 0.05 *M iodine*. No blue or reddish-brown colour develops.

Sterculia gum

- A. Place 0.2 g of the powdered drug (355) in a 10 ml ground-glass-stoppered cylinder graduated in 0.1 ml. Add 10 ml of *alcohol (60 per cent V/V) R* and shake. Any gel formed occupies not more than 1.5 ml.
- B. To 1.0 g of the powdered drug (355) add 100 ml of *water R* and shake. Add 0.1 ml of *methyl red solution R*. Not more than 5.0 ml of 0.01 *M sodium hydroxide* is required to change the colour of the indicator.

Tannins. To 10 ml of solution S add 0.1 ml of *ferric chloride solution R1*. A gelatinous precipitate is formed, but neither the precipitate nor the liquid shows a dark blue colour.

Tragacantha. Examine the chromatograms obtained in the test for glucose and fructose. The chromatogram obtained with the test solution shows no greenish-grey to yellowish-grey zone corresponding to the zone of xylose in the chromatogram obtained with the reference solution.

Loss on drying (2.2.32). Not more than 15.0 per cent, determined on 1.000 g of the powdered drug (355) by drying in an oven at 100-105 °C.

Total ash (2.4.16). Not more than 4.0 per cent.

Microbial contamination. Total viable aerobic count (2.6.12) not more than 10⁴ micro-organisms per gram, determined by plate count. It complies with the test for *Escherichia coli* (2.6.13).

01/2005:0308

ACACIA, SPRAY-DRIED**Acaciae gummi dispersione desiccatum****DEFINITION**

Spray-dried acacia is obtained from a solution of acacia.

CHARACTERS

It dissolves completely and rapidly, after about 20 min, in twice its mass of water. The liquid obtained is colourless or yellowish, dense, viscous, adhesive, translucent and weakly acid to blue litmus paper. Spray-dried acacia is practically insoluble in alcohol.

IDENTIFICATION

- A. Examined under a microscope, in *alcohol R*, the powder is seen to consist predominantly of spheroidal particles about 4 µm to 40 µm in diameter, with a central cavity containing one or several air-bubbles; a few minute flat fragments are present. Only traces of starch granules are visible. No vegetable tissue is seen.
- B. Examine the chromatograms obtained in the test for glucose and fructose. The chromatogram obtained with the test solution shows 3 zones due to galactose, arabinose and rhamnose. No other important zones are visible, particularly in the upper part of the chromatogram.
- C. Dissolve 1 g of the drug to be examined in 2 ml of *water R* by stirring frequently for 20 min. Add 2 ml of *alcohol R*. After shaking a white gelatinous mucilage is formed which becomes fluid on adding 10 ml of *water R*.

TESTS

Solution S. Dissolve 3.0 g of the drug to be examined in 25 ml of *water R* by stirring for 10 min. Allow to stand for 20 min and dilute to 30 ml with *water R*.

Glucose and fructose. Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel plate R*.

Test solution. To 0.100 g in a thick-walled centrifuge tube add 2 ml of a 100 g/l solution of *trifluoroacetic acid R*, shake vigorously to dissolve the forming gel, stopper the tube and heat the mixture at 120 °C for 1 h. Centrifuge the hydrolysate, transfer the clear supernatant carefully into a 50 ml flask, add 10 ml of *water R* and evaporate the solution to dryness under reduced pressure. To the resulting clear film add 0.1 ml of *water R* and 0.9 ml of *methanol R*. Centrifuge to separate the amorphous precipitate. Dilute the supernatant, if necessary, to 1 ml with *methanol R*.

Reference solution. Dissolve 10 mg of *arabinose R*, 10 mg of *galactose R*, 10 mg of *glucose R*, 10 mg of *rhamnose R* and 10 mg of *xylose R* in 1 ml of *water R* and dilute to 10 ml with *methanol R*.

Apply to the plate as bands 10 µl of each solution. Develop over a path of 10 cm using a mixture of 10 volumes of a 16 g/l solution of *sodium dihydrogen phosphate R*, 40 volumes of *butanol R* and 50 volumes of *acetone R*. Dry the plate in a current of warm air for a few minutes and develop again over a path of 15 cm using the same mobile phase. Dry the plate at 110 °C for 10 min, spray with *anisaldehyde solution R* and heat again at 110 °C for 10 min. The chromatogram obtained with the reference solution shows 5 clearly separated coloured zones due to galactose (greyish-green to green), glucose (grey), arabinose (yellowish-green) xylose (greenish-grey to yellowish-grey) and rhamnose (yellowish-green), in order of increasing R_f value. The chromatogram obtained with the test solution shows no grey zone and no greyish-green zone between the zones corresponding to galactose and arabinose in the chromatogram obtained with the reference solution.

Starch, dextrin and agar. To 10 ml of solution S previously boiled and cooled add 0.1 ml of 0.05 M *iodine*. No blue or reddish-brown colour develops.

Sterculia gum

- A. Place 0.2 g in a 10 ml ground-glass-stoppered cylinder graduated in 0.1 ml. Add 10 ml of *alcohol (60 per cent V/V) R* and shake. Any gel formed occupies not more than 1.5 ml.
- B. To 1.0 g add 100 ml of *water R* and shake. Add 0.1 ml of *methyl red solution R*. Not more than 5.0 ml of 0.01 M *sodium hydroxide* is required to change the colour of the indicator.

Tannins. To 10 ml of solution S add 0.1 ml of *ferric chloride solution R1*. A gelatinous precipitate is formed, but neither the precipitate nor the liquid shows a dark blue colour.

Tragacantha. Examine the chromatograms obtained in the test for Glucose and fructose. The chromatogram obtained with the test solution shows no greenish-grey to yellowish-grey zone corresponding to the zone of xylose in the chromatogram obtained with the reference solution.

Loss on drying (2.2.32). Not more than 10.0 per cent, determined on 1.000 g by drying in an oven at 100-105 °C.

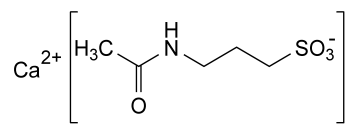
Total ash (2.4.16). Not more than 4.0 per cent.

Microbial contamination. Total viable aerobic count (2.6.12) not more than 10⁴ micro-organisms per gram, determined by plate count. It complies with the test for *Escherichia coli* (2.6.13).

01/2005:1585

ACAMPROSATE CALCIUM

Acamprosatum calcicum

C₁₀H₂₀CaN₂O₈S₂M_r 400.5

DEFINITION

Calcium bis[3-(acetylamino)propane-1-sulphonate].

Content: 98.0 per cent to 102.0 per cent (dried substance).

CHARACTERS

Appearance: white powder.

Solubility: freely soluble in water, practically insoluble in alcohol and in methylene chloride.

IDENTIFICATION

- A. Infrared absorption spectrophotometry (2.2.24).

Comparison: *Ph. Eur. reference spectrum of acamprosate calcium*.

- B. It gives reaction (a) of calcium (2.3.1).

TESTS

Solution S. Dissolve 5.0 g in *carbon dioxide-free water R* and dilute to 100 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and colourless (2.2.2, *Method II*).

pH (2.2.3): 5.5 to 7.0 for solution S.

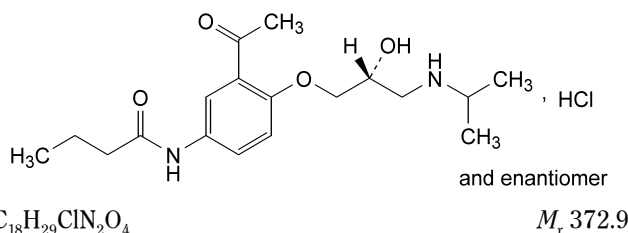
Impurity A. Liquid chromatography (2.2.29).

Test solution. Dissolve 0.40 g of the substance to be examined in *distilled water R* and dilute to 20.0 ml with the same solvent. Dilute 10.0 ml of this solution to 100.0 ml with *borate buffer solution pH 10.4 R*. Place 3.0 ml of the solution obtained in a 25 ml ground-glass-stoppered

01/2005:0871

ACEBUTOLOL HYDROCHLORIDE

Acebutololi hydrochloridum

 $C_{18}H_{29}ClN_2O_4$

DEFINITION

N-[3-Acetyl-4-[(2*RS*)-2-hydroxy-3-[(1-methylethyl)amino]propoxy]phenyl]butanamide hydrochloride.

Content: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

Appearance: white or almost white, crystalline powder.

Solubility: freely soluble in water and in alcohol, very slightly soluble in acetone and in methylene chloride.

mp: about 143 °C.

IDENTIFICATION

First identification: B, D.

Second identification: A, C, D.

- A. Dissolve 20.0 mg in a 0.1 per cent *V/V* solution of *hydrochloric acid R* and dilute to 100.0 ml with the same acid solution. Dilute 5.0 ml to 100.0 ml with a 0.1 per cent *V/V* solution of *hydrochloric acid R*. Examined between 220 nm and 350 nm (2.2.25), the solution shows 2 absorption maxima, at 233 nm and 322 nm. The specific absorbance at the maximum at 233 nm is 555 to 605.

- B. Infrared absorption spectrophotometry (2.2.24).

Preparation: discs.

Comparison: *acebutolol hydrochloride CRS*.

- C. Thin-layer chromatography (2.2.27).

Test solution. Dissolve 20 mg of the substance to be examined in *methanol R* and dilute to 20 ml with the same solvent.

Reference solution (a). Dissolve 20 mg of *acebutolol hydrochloride CRS* in *methanol R* and dilute to 20 ml with the same solvent.

Reference solution (b). Dissolve 20 mg of *pindolol CRS* in *methanol R* and dilute to 20 ml with the same solvent. To 1 ml add 1 ml of reference solution (a).

Plate: *TLC silica gel F₂₅₄ plate R*.

Mobile phase: *perchloric acid R*, *methanol R*, *water R* (5:395:600 *V/V/V*).

Application: 10 µl.

Development: over 3/4 of the plate.

Drying: in air.

Detection: examine in ultraviolet light at 254 nm.

System suitability: the chromatogram obtained with reference solution (b) shows 2 clearly separated principal spots.

Results: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a).

- D. It gives reaction (a) of chlorides (2.3.1).

tube. Add 0.15 ml of a freshly prepared 5 g/l solution of *fluorescamine R* in *acetonitrile R*. Shake immediately and vigorously for 30 s. Place in a water-bath at 50 °C for 30 min. Cool under a stream of cold water. Centrifuge and filter the supernatant through a suitable membrane filter (0.45 µm), 25 mm in diameter.

Reference solution. Dissolve 50 mg of *acamprosate impurity A CRS* in *distilled water R* and dilute to 200.0 ml with the same solvent. Dilute 0.4 ml of the solution to 100.0 ml with *borate buffer solution pH 10.4 R*. Treat 3.0 ml of this solution in the same way as the test solution

Column:

- *size*: *l* = 0.15 m, Ø = 4.6 mm,
- *stationary phase*: *spherical octadecylsilyl silica gel for chromatography R* (5 µm) with a specific surface area of 170 m²/g and a pore size of 12 nm.

Mobile phase: *acetonitrile R*, *methanol R*, *0.1 M phosphate buffer solution pH 6.5 R* (10:10:80 *V/V/V*).

Flow rate: 1 ml/min.

Detection: spectrophotometer at 261 nm.

Injection: 20 µl.

Run time: 6 times the retention time of impurity A

Retention times: *fluorescamine* = about 4 min; *impurity A* = about 8 min; *acamprosate* is not detected by this system.

Limits:

- *impurity A*: not more than the area of the corresponding peak in the chromatogram obtained with the reference solution (0.05 per cent).

Heavy metals (2.4.8): maximum 10 ppm.

Dissolve 2.0 g in *distilled water R* and dilute to 20 ml with the same solvent. 12 ml of the solution complies with limit test A. Prepare the standard using 10 ml of *lead standard solution (1 ppm Pb) R*.

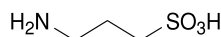
Loss on drying (2.2.32): maximum 0.4 per cent, determined on 1.000 g by drying in an oven at 100–105 °C.

ASSAY

To 4 g of *cation exchange resin R* (75–150 µm) add 20 ml of *distilled water R* and stir magnetically for 10 min. Introduce this suspension into a glass column, 45 cm long and 2.2 cm in internal diameter, equipped with a polytetrafluoroethylene flow cap covered by a glass-wool plug. Allow a few millilitres of this solution to flow, then place a plug of glass wool over the resin. Pass 50 ml of *1 M hydrochloric acid* through the column. The pH of the eluate is close to 1. Wash with 3 quantities, each of 200 ml, of *distilled water R* to obtain an eluate at pH 6. Dissolve 0.100 g of the substance to be examined in 15 ml of *distilled water R*. Pass through the column and wash with 3 quantities, each of 25 ml, of *distilled water R*, collecting the eluate. Allow to elute until an eluate at pH 6 is obtained. Titrate the solution obtained with *0.1 M sodium hydroxide*, determining the end-point potentiometrically (2.2.20).

1 ml of *0.1 M sodium hydroxide* corresponds to 20.02 mg of $C_{10}H_{20}CaN_2O_8S_2$.

IMPURITIES



- A. 3-aminopropane-1-sulphonic acid (homotaurine).

TESTS

Appearance of solution. The solution is not more opalescent than reference suspension II (2.2.1) and not more intensely coloured than reference solution BY₅ (2.2.2, Method II).

Dissolve 0.5 g in *water R* and dilute to 10 ml with the same solvent.

pH (2.2.3): 5.0 to 7.0.

Dissolve 0.20 g in *carbon dioxide-free water R* and dilute to 20 ml with the same solvent.

Related substances. Liquid chromatography (2.2.29).

Test solution. Dissolve 0.100 g of the substance to be examined in mobile phase A and dilute to 50.0 ml with mobile phase A.

Reference solution (a). Dissolve 20.0 mg of the substance to be examined in mobile phase A and dilute to 100.0 ml with mobile phase A. Dilute 0.5 ml to 50.0 ml with mobile phase A.

Reference solution (b). Dissolve 5.0 mg of *acebutolol impurity I CRS* in 10.0 ml of *acetonitrile R* and dilute to 25.0 ml with mobile phase A. Dilute 1.0 ml to 50.0 ml with mobile phase A.

Reference solution (c). Mix 2.0 ml of reference solution (a) and 1.0 ml of reference solution (b) and dilute to 10.0 ml with mobile phase A.

Reference solution (d). Dissolve 5.0 mg of *acebutolol impurity C CRS* in 10 ml of *acetonitrile R* and dilute to 25.0 ml with mobile phase A. Dilute 0.5 ml to 50.0 ml with mobile phase A.

Reference solution (e). Dissolve 5.0 mg of *acebutolol impurity B CRS* in 10.0 ml of *acetonitrile R* and dilute to 25.0 ml with mobile phase A. Dilute 1.0 ml to 50.0 ml with mobile phase A.

Column:

- **size:** $l = 0.125$ m, $\varnothing = 4$ mm,
- **stationary phase:** *end-capped octadecylsilyl silica gel for chromatography R* (5 μ m),
- **temperature:** 40 °C.

Mobile phase:

- **mobile phase A:** mix 2.0 ml of *phosphoric acid R*, and 3.0 ml of *triethylamine R* and dilute to 1000 ml with *water R*,
- **mobile phase B:** mix equal volumes of *acetonitrile R* and mobile phase A,

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)
0 - 2	98	2
2 - 30.5	98 → 10	2 → 90
30.5 - 41	10	90
41 - 42	10 → 98	90 → 2
42 - 50	98	2

Flow rate: 1.2 ml/min.

Detection: spectrophotometer at 240 nm.

Injection: 25 μ l.

System suitability: reference solution (c):

- **resolution:** minimum 7.0 between the peaks due to impurity I and acebutolol.

Limits:

- **impurity B:** not more than the area of the principal peak in the chromatogram obtained with reference solution (e) (0.2 per cent),
- **impurity C:** not more than the area of the principal peak in the chromatogram obtained with reference solution (d) (0.1 per cent),
- **impurity I:** not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent),
- **any other impurity:** not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent),
- **total of impurities:** not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent),
- **disregard limit:** half the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

Heavy metals (2.4.8): maximum 20 ppm.

Dissolve 0.50 g of the substance to be examined in 20.0 ml of *water R*. The solution complies with limit test E. Prepare the standard by diluting 10.0 ml of *lead standard solution* (1 ppm Pb) *R* to 20.0 ml with *water R*.

Loss on drying (2.2.32): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 100-105 °C for 3 h.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.300 g in 50 ml of *alcohol R* and add 1 ml of 0.1 M *hydrochloric acid*. Carry out a potentiometric titration (2.2.20), using 0.1 M *sodium hydroxide*. Read the volume added between the 2 points of inflexion.

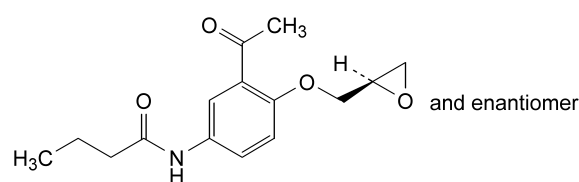
1 ml of 0.1 M *sodium hydroxide* is equivalent to 37.29 mg of C₁₈H₂₉ClN₂O₄.

STORAGE

Protected from light.

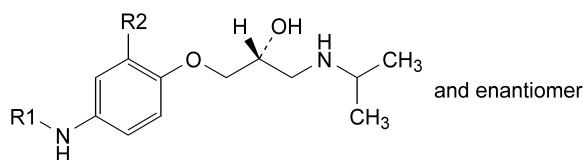
IMPURITIES

Specified impurities: A, B, C, D, E, F, G, H, I, J, K.

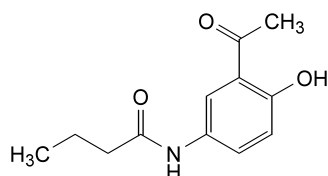


A. *N*-[3-acetyl-4-[(2*RS*)-oxiran-2-ylmethoxy]phenyl]butanamide,

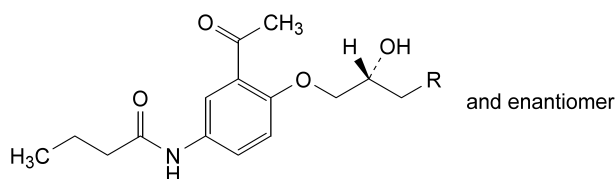
01/2005:1281



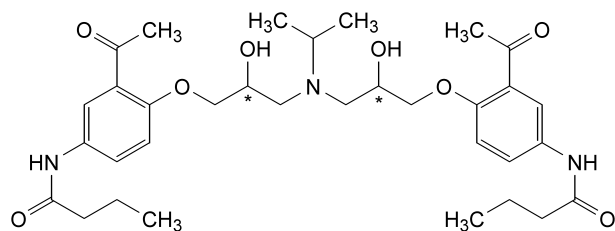
- B. R1 = R2 = CO-CH₃: *N*-[3-acetyl-4-[(2*RS*)-2-hydroxy-3-[(1-methylethyl)amino]propoxy]phenyl]acetamide (diacetolol),
- D. R1 = H, R2 = CO-CH₃: 1-[5-amino-2-[(2*RS*)-2-hydroxy-3-[(1-methylethyl)amino]propoxy]phenyl]ethanone,
- E. R1 = CO-CH₂-CH₂-CH₃, R2 = H: *N*-[4-[(2*RS*)-2-hydroxy-3-[(1-methylethyl)amino]propoxy]phenyl]butanamide,
- J. R1 = CO-CH₂-CH₃, R2 = CO-CH₃: *N*-[3-acetyl-4-[(2*RS*)-2-hydroxy-3-[(1-methylethyl)amino]propoxy]phenyl]propanamide,
- K. R1 = R2 = CO-CH₂-CH₂-CH₃: *N*-[3-butanoyl-4-[(2*RS*)-2-hydroxy-3-[(1-methylethyl)amino]propoxy]phenyl]butanamide,



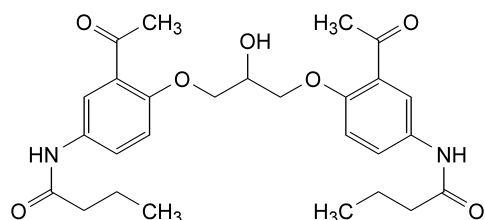
- C. *N*-(3-acetyl-4-hydroxyphenyl)butanamide,



- F. R = OH: *N*-[3-acetyl-4-[(2*RS*)-2,3-dihydroxypropoxy]phenyl]butanamide,
- I. R = NH-CH₂-CH₃: *N*-[3-acetyl-4-[(2*RS*)-3-(ethylamino)-2-hydroxypropoxy]phenyl]butanamide,



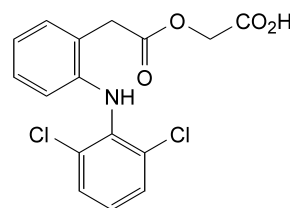
- G. *N,N'*-[[1-(1-methylethyl)imino]bis[(2-hydroxypropane-1,3-diyl)oxy(3-acetyl-1,4-phenylene)]]dibutanamide (biamine),



- H. *N,N'*-[(2-hydroxypropane-1,3-diyl)bis[oxy(3-acetyl-1,4-phenylene)]]dibutanamide,

ACECLOFENAC

Aceclofenacum

C₁₆H₁₃Cl₂NO₄M_r 354.2

DEFINITION

[[[2-[(2,6-Dichlorophenyl)amino]phenyl]acetyl]oxy]acetic acid.

Content: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

Appearance: white or almost white, crystalline powder.

Solubility: practically insoluble in water, freely soluble in acetone, soluble in alcohol.

IDENTIFICATION

First identification: B.

Second identification: A, C.

- A. Dissolve 50.0 mg in *methanol R* and dilute to 100.0 ml with the same solvent. Dilute 2.0 ml of the solution to 50.0 ml with *methanol R*. Examined between 220 nm and 370 nm (2.2.25), the solution shows an absorption maximum at 275 nm. The specific absorbance at the absorption maximum is 320 to 350.
- B. Infrared absorption spectrophotometry (2.2.24).
Comparison: Ph. Eur. reference spectrum of aceclofenac.
- C. Dissolve about 10 mg in 10 ml of *alcohol R*. To 1 ml of the solution, add 0.2 ml of a mixture, prepared immediately before use, of equal volumes of a 6 g/l solution of *potassium ferricyanide R* and a 9 g/l solution of *ferric chloride R*. Allow to stand protected from light for 5 min. Add 3 ml of a 10.0 g/l solution of *hydrochloric acid R*. Allow to stand protected from light for 15 min. A blue colour develops and a precipitate is formed.

TESTS

Related substances. Liquid chromatography (2.2.29).
Prepare the solutions immediately before use.

Test solution. Dissolve 50.0 mg of the substance to be examined in a mixture of 30 volumes of mobile phase A and 70 volumes of mobile phase B and dilute to 25.0 ml with the same mixture of solvents.

Reference solution (a). Dissolve 21.6 mg of *diclofenac sodium CRS* in a mixture of 30 volumes of mobile phase A and 70 volumes of mobile phase B and dilute to 50.0 ml with the same mixture of solvents.

Reference solution (b). Dilute 2.0 ml of the test solution to 10.0 ml with a mixture of 30 volumes of mobile phase A and 70 volumes of mobile phase B.

Reference solution (c). Mix 1.0 ml of reference solution (a) and 1.0 ml of reference solution (b) and dilute to 100.0 ml with a mixture of 30 volumes of mobile phase A and 70 volumes of mobile phase B.

Reference solution (d). Dissolve 4.0 mg of *aceclofenac impurity F CRS*, 2.0 mg of *aceclofenac impurity H CRS* and 2.0 mg of *diclofenac impurity A CRS* (aceclofenac impurity I) in a mixture of 30 volumes of mobile phase A and 70 volumes of mobile phase B and dilute to 10.0 ml with the same mixture of solvents.

Reference solution (e). Mix 1.0 ml of reference solution (b) and 1.0 ml of reference solution (d) and dilute to 100.0 ml with a mixture of 30 volumes of mobile phase A and 70 volumes of mobile phase B.

Column:

- **size:** $l = 0.25$ m, $\varnothing = 4.6$ mm,
- **stationary phase:** spherical *end-capped octadecylsilyl silica gel for chromatography R* (5 μ m) with a pore size of 10 nm and a carbon loading of 19 per cent,
- **temperature:** 40 °C.

Mobile phase:

- **mobile phase A:** 1.12 g/l solution of *phosphoric acid R* adjusted to pH 7.0 using a 42 g/l solution of *sodium hydroxide R*,
- **mobile phase B:** *water R*, *acetonitrile R* (1:9 V/V),

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)
0 - 25	70 → 50	30 → 50
25 - 30	50 → 20	50 → 80
30 - 50	20	80
50 - 52	20 → 70	80 → 30
52 - 65	70	30

Flow rate: 1.0 ml/min.

Detection: spectrophotometer at 275 nm.

Injection: 10 μ l; inject the test solution and reference solutions (c) and (e).

Relative retention with reference to aceclofenac (retention time = about 14 min): impurity A = about 0.8; impurity G = about 1.3; impurity H = about 1.5; impurity I = about 2.3; impurity D = about 2.6; impurity B = about 2.7; impurity E = about 2.8; impurity C = about 3.0; impurity F = about 3.2.

System suitability: reference solution (c):

- **resolution:** minimum 5.0 between the peaks due to aceclofenac and to impurity A.

Limits:

- **impurity A:** not more than the area of the corresponding peak in the chromatogram obtained with reference solution (c) (0.2 per cent),
- **impurities B, C, D, E, G:** for each impurity, not more than the area of the peak due to aceclofenac in the chromatogram obtained with reference solution (e) (0.2 per cent),
- **impurity F:** not more than the area of the corresponding peak in the chromatogram obtained with reference solution (e) (0.2 per cent),
- **impurity H:** not more than the area of the corresponding peak in the chromatogram obtained with reference solution (e) (0.1 per cent),

- **impurity I:** not more than the area of the corresponding peak in the chromatogram obtained with reference solution (e) (0.1 per cent),
- **any other impurity:** not more than half the area of the peak due to aceclofenac in the chromatogram obtained with reference solution (e) (0.1 per cent),
- **total:** not more than 0.7 per cent,
- **disregard limit:** 0.1 times the area of the peak due to aceclofenac in the chromatogram obtained with reference solution (e) (0.02 per cent).

Heavy metals (2.4.8): maximum 10 ppm.

To 2.0 g in a silica crucible, add 2 ml of *sulphuric acid R* to wet the substance. Heat progressively to ignition and continue heating until an almost white or at most a greyish residue is obtained. Carry out the ignition at a temperature not exceeding 800 °C. Allow to cool. Add 3 ml of *hydrochloric acid R* and 1 ml of *nitric acid R*. Heat and evaporate slowly to dryness. Cool and add 1 ml of a 100 g/l solution of *hydrochloric acid R* and 10.0 ml of *distilled water R*. Neutralise with a 1.0 g/l solution of *ammonia R* using 0.1 ml of *phenolphthalein solution R* as indicator. Add 2.0 ml of a 60 g/l solution of *anhydrous acetic acid R* and dilute to 20 ml with *distilled water R*. 12 ml of the solution complies with limit test A. Prepare the standard using *lead standard solution (1 ppm Pb) R*.

Loss on drying (2.2.32): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 100-105 °C.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

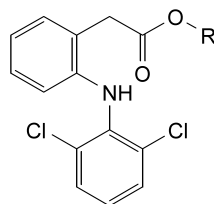
Dissolve 0.300 g in 40 ml of *methanol R*. Titrate with 0.1 M *sodium hydroxide*, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *sodium hydroxide* is equivalent to 35.42 mg of $C_{16}H_{13}Cl_2NO_4$.

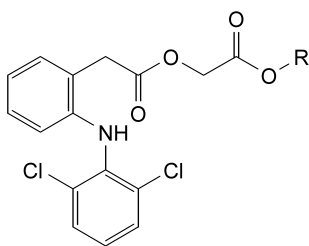
STORAGE

In an airtight container, protected from light.

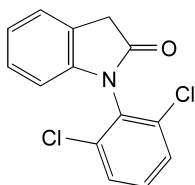
IMPURITIES



- A. R = H: [2-[(2,6-dichlorophenyl)amino]phenyl]acetic acid (diclofenac),
- B. R = CH₃: methyl [2-[(2,6-dichlorophenyl)amino]phenyl]acetate (methyl ester of diclofenac),
- C. R = C₂H₅: ethyl [2-[(2,6-dichlorophenyl)amino]phenyl]acetate (ethyl ester of diclofenac),



- D. $R = \text{CH}_3$: methyl [[2-[(2,6-dichlorophenyl)amino]phenyl]acetyl]oxy]acetate (methyl ester of aceclofenac),
- E. $R = \text{C}_2\text{H}_5$: ethyl [[2-[(2,6-dichlorophenyl)amino]phenyl]acetyl]oxy]acetate (ethyl ester of aceclofenac),
- F. $R = \text{CH}_2\text{-C}_6\text{H}_5$: benzyl [[2-[(2,6-dichlorophenyl)amino]phenyl]acetyl]oxy]acetate (benzyl ester of aceclofenac),
- G. $R = \text{CH}_2\text{-CO}_2\text{H}$: [[[[2-[(2,6-dichlorophenyl)amino]phenyl]acetyl]oxy]acetyl]oxy]acetic acid (acetic aceclofenac),
- H. $R = \text{CH}_2\text{-CO-O-CH}_2\text{-CO}_2\text{H}$: [[[[[2-[(2,6-dichlorophenyl)amino]phenyl]acetyl]oxy]acetyl]oxy]acetic acid (diacetic aceclofenac),

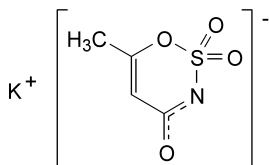


- I. 1-(2,6-dichlorophenyl)-1,3-dihydro-2H-indol-2-one.

01/2005:1282

ACESULFAME POTASSIUM

Acesulfamum kalicum

 $\text{C}_4\text{H}_4\text{KNO}_4\text{S}$ M_r 201.2

DEFINITION

Acesulfame potassium contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of potassium 6-methyl-1,2,3-oxathiazin-4-olate 2,2-dioxide, calculated with reference to the dried substance.

CHARACTERS

A white, crystalline powder or colourless crystals, soluble in water, very slightly soluble in acetone and in alcohol.

IDENTIFICATION

First identification: A, C.

Second identification: B, C.

- A. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *acesulfame potassium CRS*. Examine the substances prepared as discs.
- B. Examine by thin-layer chromatography (2.2.27), using *cellulose for chromatography R* as the coating substance.

Test solution. Dissolve 5 mg of the substance to be examined in *water R* and dilute to 5 ml with the same solvent.

Reference solution (a). Dissolve 5 mg of *acesulfame potassium CRS* in *water R* and dilute to 5 ml with the same solvent.

Reference solution (b). Dissolve 5 mg of *acesulfame potassium CRS* and 5 mg of *saccharin sodium R* in *water R* and dilute to 5 ml with the same solvent.

Apply to the plate as bands 5 μl of each solution. Develop twice over a path of 15 cm using a mixture of 10 volumes of *concentrated ammonia R*, 60 volumes of *acetone R* and 60 volumes of *ethyl acetate R*. Dry the plate in a current of warm air and examine in ultraviolet light at 254 nm. The principal band in the chromatogram obtained with the test solution is similar in position and size to the principal band in the chromatogram obtained with reference solution (a). The test is not valid unless the chromatogram obtained with reference solution (b) shows two clearly separated bands.

- C. 0.5 ml of solution S (see Tests) gives reaction (b) of potassium (2.3.1).

TESTS

Solution S. Dissolve 10.0 g in *carbon dioxide-free water R* and dilute to 50 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and colourless (2.2.2, *Method II*).

Acidity or alkalinity. To 20 ml of solution S add 0.1 ml of *bromothymol blue solution R1*. Not more than 0.2 ml of 0.01 M *hydrochloric acid* or 0.01 M *sodium hydroxide* is required to change the colour of the indicator.

Acetylacetamide. Examine by thin-layer chromatography (2.2.27), using a suitable silica gel as the coating substance.

Test solution. Dissolve 0.80 g of the substance to be examined in *water R* and dilute to 10 ml with the same solvent.

Reference solution (a). Dissolve 50 mg of *acetylacetamide R* in *water R* and dilute to 25 ml with the same solvent. To 5 ml of the solution add 45 ml of *water R* and dilute to 100 ml with *methanol R*.

Reference solution (b). To 10 ml of reference solution (a) add 1 ml of the test solution and dilute to 20 ml with *methanol R*.

Apply separately to the plate 5 μl of each solution. Develop over a path of 15 cm using a mixture of 2 volumes of *water R*, 15 volumes of *alcohol R* and 74 volumes of *ethyl acetate R*. Allow the plate to dry in air until the solvents are completely removed. Spray the plate with *vanillin phosphoric solution R* and heat at 120 °C for about 10 min. Examine in daylight. Any spot corresponding to acetylacetamide in the chromatogram obtained with the test solution, is not more intense than the spot in the chromatogram obtained with reference solution (a) (0.125 per cent). The test is not valid unless the chromatogram obtained with reference solution (a) shows a clearly visible spot and the chromatogram obtained with reference solution (b) shows two clearly separated spots.

Impurity B and related substances. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 0.100 g of the substance to be examined in *water R* and dilute to 10.0 ml with the same solvent.

Reference solution (a). Dissolve 20.0 mg of *acesulfame potassium impurity B CRS* in *water R* and dilute to 500.0 ml with the same solvent. Dilute 0.5 ml of this solution to 100.0 ml with *water R*.

Reference solution (b). Dissolve 10.0 mg of *acesulfame potassium impurity B CRS* and 10.0 mg of *acesulfame potassium CRS* in *water R* and dilute to 500.0 ml with the same solvent. Dilute 5.0 ml of this solution to 100.0 ml with *water R*.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4.6 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (3 µm),
- as mobile phase at a flow rate of 1 ml/min a mixture of 40 volumes of *acetonitrile R* and 60 volumes of a 3.3 g/l solution of *tetrabutylammonium hydrogen sulphate R*,
- as detector a spectrophotometer set at 234 nm.

Inject 20 µl of reference solution (b). Adjust the sensitivity of the system so that the height of the two principal peaks is at least 50 per cent of the full scale of the recorder. The test is not valid unless the resolution between the two peaks due to acesulfame and impurity B is at least 3.0.

Inject 20 µl of the test solution and 20 µl of reference solution (a). Continue the chromatography of the test solution for at least three times the retention time of the principal peak. In the chromatogram obtained with the test solution the area of any peak apart from the principal peak is not greater than the area of the principal peak in the chromatogram obtained with reference solution (a) (20 ppm).

Fluorides. Not more than 3 ppm of F, determined potentiometrically (2.2.36, *Method I*) using as indicator electrode a fluoride selective-membrane electrode and as reference electrode a silver-silver chloride electrode.

Test solution. Dissolve 3.000 g of the substance to be examined in *distilled water R*, add 15.0 ml of *total ionic strength adjustment buffer R1* and dilute to 50.0 ml with *distilled water R*.

Reference solutions. To 0.5 ml, 1.0 ml, 1.5 ml and 3.0 ml of *fluoride standard solution (10 ppm F) R* add 15.0 ml of *total ionic strength adjustment buffer R1* and dilute to 50.0 ml with *distilled water R*.

Carry out the measurements of each solution.

Heavy metals (2.4.8). 12 ml of solution S complies with the limit test A for heavy metals (5 ppm). Prepare the standard using *lead standard solution (1 ppm Pb) R*.

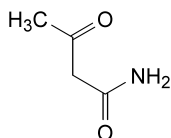
Loss on drying (2.2.32). Not more than 1.0 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C for 3 h.

ASSAY

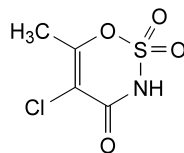
Dissolve 0.150 g in 50 ml of *anhydrous acetic acid R*. Titrate with 0.1 M *perchloric acid*, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *perchloric acid* is equivalent to 20.12 mg of C₄H₆N₄O₃S₂.

IMPURITIES



A. 3-oxobutanamide (acetylacetamide),

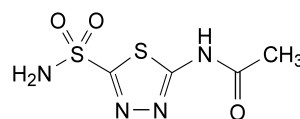


B. 5-chloro-6-methyl-1,2,3-oxathiazin-4(3*H*)-one 2,2-dioxide.

01/2005:0454

ACETAZOLAMIDE

Acetazolamidum



C₄H₆N₄O₃S₂

*M*_r 222.2

DEFINITION

Acetazolamide contains not less than 98.5 per cent and not more than the equivalent of 101.0 per cent of *N*-(5-sulphamoyl-1,3,4-thiadiazol-2-yl)acetamide, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, crystalline powder, very slightly soluble in water, slightly soluble in alcohol. It dissolves in dilute solutions of alkali hydroxides.

IDENTIFICATION

First identification: A, B.

Second identification: A, C, D.

- Dissolve 30.0 mg in 0.01 M *sodium hydroxide* and dilute to 100.0 ml with the same solvent. Dilute 10.0 ml of the solution to 100.0 ml with 0.01 M *sodium hydroxide* (solution A). Examined between 230 nm and 260 nm (2.2.25), solution A shows an absorption maximum at 240 nm. The specific absorbance at the maximum is 162 to 176. Dilute 25.0 ml of solution A to 100.0 ml with 0.01 M *sodium hydroxide*. Examined between 260 nm and 350 nm, the solution shows an absorption maximum at 292 nm. The specific absorbance at the maximum is 570 to 620.
- Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *acetazolamide CRS*. If the spectra obtained in the solid state with the substance to be examined and the reference substance show differences, dissolve separately the substance to be examined and the reference substance in *alcohol R*, evaporate to dryness, prepare new discs and record the spectra.
- Introduce about 20 mg into a test-tube and add 4 ml of *dilute hydrochloric acid R* and 0.2 g of *zinc powder R*. Immediately place a piece of *lead acetate paper R* over the mouth of the tube. The paper shows a brownish-black colour.
- Dissolve about 25 mg in a mixture of 0.1 ml of *dilute sodium hydroxide solution R* and 5 ml of *water R*. Add 0.1 ml of *copper sulphate solution R*. A greenish-blue precipitate is formed.

TESTS

Appearance of solution. Dissolve 1.0 g in 10 ml of 1 M sodium hydroxide. The solution is not more opalescent than reference suspension II (2.2.1) and not more intensely coloured than reference solution Y₅ or BY₅ (2.2.2, Method II).

Related substances. Examine by thin-layer chromatography (2.2.27), using silica gel GF₂₅₄ R as the coating substance.

Test solution. Dissolve 50 mg of the substance to be examined in a mixture of equal volumes of alcohol R and ethyl acetate R and dilute to 10 ml with the same mixture of solvents.

Reference solution. Dilute 1 ml of the test solution to 100 ml with a mixture of equal volumes of alcohol R and ethyl acetate R.

Apply separately to the plate 20 µl of each solution. Use the tank without lining the walls and allow to saturate for 1 h before development. Develop over a path of 15 cm using a freshly prepared mixture of 20 volumes of concentrated ammonia R, 30 volumes of ethyl acetate R and 50 volumes of 2-propanol R. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. Any spot in the chromatogram obtained with the test solution, apart from the principal spot, is not more intense than the spot in the chromatogram obtained with the reference solution (1 per cent).

Sulphates (2.4.13). To 0.4 g add 20 ml of distilled water R and dissolve by heating to boiling. Allow to cool with frequent shaking and filter. 15 ml of the filtrate complies with the limit test for sulphates (500 ppm).

Heavy metals (2.4.8). 1.0 g complies with limit test C for heavy metals (20 ppm). Prepare the standard using 2 ml of lead standard solution (10 ppm Pb) R.

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.200 g in 25 ml of dimethylformamide R. Titrate with 0.1 M ethanolic sodium hydroxide, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M ethanolic sodium hydroxide is equivalent to 22.22 mg of C₄H₆N₄O₃S₂.

IDENTIFICATION

A. A 100 g/l solution is strongly acid (2.2.4).

B. To 0.03 ml add 3 ml of water R and neutralise with dilute sodium hydroxide solution R. The solution gives reaction (b) of acetates (2.3.1).

TESTS

Solution S. Dilute 20 ml to 100 ml with distilled water R.

Appearance. It is clear (2.2.1) and colourless (2.2.2, Method II).

Freezing point (2.2.18). Not less than 14.8 °C.

Reducing substances. To 5.0 ml add 10.0 ml of water R and mix. To 5.0 ml of the solution add 6 ml of sulphuric acid R, cool and add 2.0 ml of 0.0167 M potassium dichromate. Allow to stand for 1 min and add 25 ml of water R and 1 ml of a freshly prepared 100 g/l solution of potassium iodide R. Titrate with 0.1 M sodium thiosulphate, using 1.0 ml of starch solution R as indicator. Not less than 1.0 ml of 0.1 M sodium thiosulphate solution is required.

Chlorides (2.4.4). 10 ml of solution S diluted to 15 ml with water R complies with the limit test for chlorides (25 mg/l).

Sulphates (2.4.13). 15 ml of solution S complies with the limit test for sulphates (50 mg/l).

Iron (2.4.9). 5.0 ml of solution (a) obtained in the test for heavy metals diluted to 10.0 ml with water R complies with the limit test for iron (5 ppm).

Heavy metals (2.4.8). Dissolve the residue obtained in the test for residue on evaporation by heating with two quantities, each of 15 ml, of water R and dilute to 50.0 ml (solution (a)). 12 ml of solution (a) complies with limit test A for heavy metals (5 ppm). Prepare the standard using lead standard solution (2 ppm Pb) R.

Residue on evaporation. Evaporate 20 g to dryness on a water-bath and dry at 100 °C to 105 °C. The residue weighs not more than 2.0 mg (0.01 per cent).

ASSAY

Weigh accurately a conical flask with a ground-glass stopper containing 25 ml of water R. Add 1.0 ml of the substance to be examined and weigh again accurately. Add 0.5 ml of phenolphthalein solution R and titrate with 1 M sodium hydroxide.

1 ml of 1 M sodium hydroxide is equivalent to 60.1 mg of C₂H₄O₂.

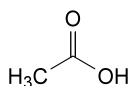
STORAGE

Store in an airtight container.

01/2005:0590

ACETIC ACID, GLACIAL

Acidum aceticum glaciale

C₂H₄O₂M_r 60.1

DEFINITION

Glacial acetic acid contains not less than 99.0 per cent *m/m* and not more than the equivalent of 100.5 per cent *m/m* of C₂H₄O₂.

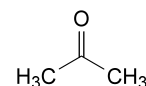
CHARACTERS

A crystalline mass or a clear, colourless, volatile liquid, miscible with water, with alcohol and with methylene chloride.

01/2005:0872

ACETONE

Acetonum

C₃H₆OM_r 58.08

DEFINITION

Acetone is propan-2-one.

CHARACTERS

A volatile clear, colourless liquid, miscible with water and with alcohol. The vapour is flammable.

IDENTIFICATION

- A. To 1 ml, add 3 ml of *dilute sodium hydroxide solution R* and 0.3 ml of a 25 g/l solution of *sodium nitroprusside R*. An intense red colour is produced which becomes violet with the addition of 3.5 ml of *acetic acid R*.
- B. To 10 ml of a 0.1 per cent V/V solution in *alcohol (50 per cent V/V) R*, add 1 ml of a 10 g/l solution of *nitrobenzaldehyde R* in the same solvent and 0.5 ml of *strong sodium hydroxide solution R*. Allow to stand for about 2 min and acidify with *acetic acid R*. A greenish-blue colour is produced.

TESTS

Appearance of solution. To 10 ml add 10 ml of *water R*. The solution is clear (2.2.1) and colourless (2.2.2, *Method II*).

Acidity or alkalinity. To 5 ml add 5 ml of *carbon dioxide-free water R*, 0.15 ml of *phenolphthalein solution R* and 0.5 ml of 0.01 M *sodium hydroxide*. The solution is pink. Add 0.7 ml of 0.01 M *hydrochloric acid* and 0.05 ml of *methyl red solution R*. The solution is red or orange.

Relative density (2.2.5). 0.790 to 0.793.

Related substances. Examine by gas chromatography (2.2.28).

Test solution. The substance to be examined.

Reference solution. To 0.5 ml of *methanol R* add 0.5 ml of *2-propanol R* and dilute to 100.0 ml with the test solution. Dilute 1.0 ml to 10.0 ml with the test solution.

The chromatographic procedure may be carried out using:

- a fused-silica column 50 m long and 0.3 mm in internal diameter coated with a film (0.5 µm) of *macrogol 20 000 R*,
- *helium for chromatography R* as the carrier gas with a split ratio of about 50:1 and at a linear flow of 21 cm/s,
- a flame-ionisation detector,

maintaining the temperature of the column at 45 °C until injection, then raising the temperature at a rate of 5 °C per minute to 100 °C and maintaining the temperature of the injection port at 150 °C and that of the detector at 250 °C.

Inject 1 µl of the test solution and 1 µl of the reference solution.

When the chromatograms are recorded in the conditions described above, the substances are eluted in the following order: acetone, methanol, 2-propanol.

Continue the chromatography for three times the retention time of acetone (which is about 5.3 min).

The test is not valid unless, in the chromatogram obtained with the reference solution, the resolution between the peaks corresponding to methanol and 2-propanol is at least 1.0.

In the chromatogram obtained with the test solution: the area of any peak corresponding to methanol or 2-propanol is not greater than the difference between the areas of the corresponding peaks in the chromatogram obtained with the reference solution and the areas of the corresponding peaks in the chromatogram obtained with the test solution (0.05 per cent V/V for each impurity); the area of any peak, apart from the principal peak and any peaks corresponding to methanol and 2-propanol, is not greater than the difference between the area of the methanol peak in the chromatogram obtained with the reference solution and the area of the corresponding peak in the chromatogram obtained with the test solution (0.05 per cent V/V for each of the additional impurities).

Matter insoluble in water. To 1 ml add 19 ml of *water R*. The solution is clear (2.2.1).

Reducing substances. To 30 ml add 0.1 ml of 0.02 M *potassium permanganate* and allow to stand in the dark for 2 h. The mixture is not completely decolourised.

Residue on evaporation. Evaporate 20.0 g to dryness on a water-bath and dry at 100 °C to 105 °C. The residue weighs not more than 1 mg (50 ppm).

Water (2.5.12). Not more than 3 g/l, determined on 10.0 ml by the semi-micro determination of water. Use 20 ml of *anhydrous pyridine R* as solvent.

STORAGE

Store protected from light.

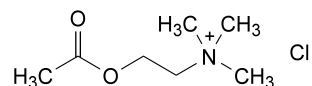
IMPURITIES

- A. CH₃-OH: methanol,
B. propan-2-ol.

01/2005:1485
corrected

ACETYLCHOLINE CHLORIDE

Acetylcholini chloridum



C₇H₁₆ClNO₂

M_r 181.7

DEFINITION

2-(Acetyloxy)-N,N,N-trimethylethanaminium chloride.

Content: 98.5 per cent to 101.5 per cent (dried substance).

CHARACTERS

Appearance: white or almost white crystalline powder or colourless crystals, very hygroscopic.

Solubility: very soluble in water, freely soluble in alcohol, slightly soluble in methylene chloride.

IDENTIFICATION

First identification: B, E.

Second identification: A, C, D, E.

- A. Melting point (2.2.14): 149 °C to 152 °C.

Introduce the substance to be examined into a capillary tube. Dry in an oven at 100-105 °C for 3 h. Seal the tube and determine the melting point.

- B. Infrared absorption spectrophotometry (2.2.24).

Comparison: *acetylcholine chloride CRS*.

- C. Examine the chromatograms obtained in the test for related substances.

Results: the principal band in the chromatogram obtained with test solution (b) is similar in position, colour and size to the principal band in the chromatogram obtained with reference solution (b).

- D. To 15 mg add 10 ml of *dilute sodium hydroxide solution R*, 2 ml of 0.02 M *potassium permanganate* and heat. The vapours formed change the colour of *red litmus paper R* to blue.

- E. 0.5 ml of solution S (see Tests) gives reaction (a) of chlorides (2.3.1).

TESTS

Solution S. Dissolve 5.0 g in *carbon dioxide-free water R* and dilute to 50 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and not more intensely coloured than reference solution Y₆ or BY₆ (2.2.2, Method II).

Acidity. Dilute 1 ml of solution S to 10 ml with *carbon dioxide-free water R*. Add 0.05 ml of *phenolphthalein solution R*. Not more than 0.4 ml of 0.01 M *sodium hydroxide* is required to change the colour of the indicator to pink.

Related substances. Thin-layer chromatography (2.2.27). Prepare the solutions immediately before use.

Test solution (a). Dissolve 0.30 g of the substance to be examined in *methanol R* and dilute to 3.0 ml with the same solvent.

Test solution (b). Dilute 1 ml of test solution (a) to 10 ml with *methanol R*.

Reference solution (a). Dilute 1 ml of test solution (a) to 100 ml with *methanol R*.

Reference solution (b). Dissolve 20.0 mg of *acetylcholine chloride CRS* in *methanol R* and dilute to 2.0 ml with the same solvent.

Reference solution (c). Dissolve 20 mg of *choline chloride R* in *methanol R*, add 0.4 ml of test solution (a) and dilute to 2.0 ml with *methanol R*.

Plate: TLC silica gel plate R.

Mobile phase: mix 20 volumes of a 40 g/l solution of *ammonium nitrate R*, 20 volumes of *methanol R* and 60 volumes of *acetonitrile R*.

Application: 5 µl as bands of 10 mm by 2 mm.

Development: over 2/3 of the plate.

Detection: spray with *potassium iodobismuthate solution R3*.

System suitability: the chromatogram obtained with reference solution (c) shows 2 clearly separated bands.

Limits:

- **any impurity:** any bands in the chromatogram obtained with test solution (a), apart from the principal band, are not more intense than the principal band in the chromatogram obtained with reference solution (a) (1 per cent).

Trimethylamine. Dissolve 0.1 g in 10 ml of *sodium carbonate solution R* and heat to boiling. No vapours appear which turn *red litmus paper R* blue.

Heavy metals (2.4.8): maximum 10 ppm.

12 ml of solution S complies with limit test A. Prepare the standard using *lead standard solution (1 ppm Pb) R*.

Loss on drying (2.2.32): maximum 1.0 per cent, determined on 1.000 g by drying in an oven at 100–105 °C for 3 h.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on the residue obtained in the test for loss on drying.

ASSAY

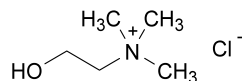
Dissolve 0.200 g in 20 ml of *carbon dioxide-free water R*. Neutralise with 0.01 M *sodium hydroxide* using 0.15 ml of *phenolphthalein solution R* as indicator. Add 20.0 ml of 0.1 M *sodium hydroxide* and allow to stand for 30 min. Titrate with 0.1 M *hydrochloric acid*.

1 ml of 0.1 M *sodium hydroxide* is equivalent to 18.17 mg of C₇H₁₆ClNO₂.

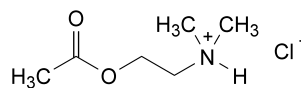
STORAGE

In ampoules, protected from light.

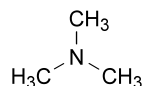
IMPURITIES



A. 2-hydroxy-*N,N,N*-trimethylethanaminium chloride (choline chloride),



B. 2-(acetoxy)-*N,N*-dimethylethanaminium chloride,

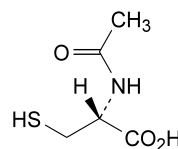


C. *N,N*-dimethylmethanamine.

01/2005:0967
corrected

ACETYLCYSTEINE

Acetylcysteinum



C₅H₉NO₃S

M_r 163.2

DEFINITION

Acetylcysteine contains not less than 98.0 per cent and not more than the equivalent of 101.0 per cent of (2*R*)-2-(acetylamino)-3-sulfanylpropanoic acid, calculated with reference to the dried substance.

CHARACTERS

A white, crystalline powder or colourless crystals, freely soluble in water and in alcohol, practically insoluble in methylene chloride.

IDENTIFICATION

First identification: A, C.

Second identification: A, B, D, E.

- It complies with the test for specific optical rotation (see Tests).
- Melting point (2.2.14): 104 °C to 110 °C.
- Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *acetylcysteine CRS*. Examine the substances prepared as discs using *potassium bromide R*.
- Examine the chromatograms obtained in the test for related substances. The retention time and size of the principal peak in the chromatogram obtained with test solution (b) are approximately the same as those of the principal peak in the chromatogram obtained with reference solution (b).
- To 0.5 ml of solution S (see Tests) add 0.05 ml of a 50 g/l solution of *sodium nitroprusside R* and 0.05 ml of *concentrated ammonia R*. A dark violet colour develops.

TESTS

Solution S. Dissolve 1.0 g in *carbon dioxide-free water R* and dilute to 20 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and colourless (2.2.2, Method II).

pH (2.2.3). To 2 ml of solution S add 8 ml of *carbon dioxide-free water R* and mix. The pH of the solution is 2.0 to 2.8.

Specific optical rotation (2.2.7). In a 25 ml volumetric flask, mix 1.25 g with 1 ml of a 10 g/l solution of *sodium edetate R*. Add 7.5 ml of a 40 g/l solution of *sodium hydroxide R*, mix and dissolve. Dilute to 25.0 ml with *phosphate buffer solution pH 7.0 R2*. The specific optical rotation is + 21.0 to + 27.0, calculated with reference to the dried substance.

Related substances. Examine by liquid chromatography (2.2.29). *Except where otherwise prescribed, prepare the solutions immediately before use.*

Test solution (a). Suspend 0.80 g of the substance to be examined in 1 ml of 1 M *hydrochloric acid* and dilute to 100.0 ml with *water R*.

Test solution (b). Dilute 5.0 ml of test solution (a) to 100.0 ml with *water R*. Dilute 5.0 ml of the solution to 50.0 ml with *water R*.

Test solution (c). Use test solution (a) after storage for at least 1 h.

Reference solution (a). Suspend 4.0 mg of *acetylcysteine CRS*, 4.0 mg of *L-cystine R*, 4.0 mg of *L-cysteine R*, 4.0 mg of *acetylcysteine impurity C CRS* and 4.0 mg of *acetylcysteine impurity D CRS* in 1 ml of 1 M *hydrochloric acid* and dilute to 100.0 ml with *water R*.

Reference solution (b). Suspend 4.0 mg of *acetylcysteine CRS* in 1 ml of 1 M *hydrochloric acid* and dilute to 100.0 ml with *water R*.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (5 µm),
- as mobile phase at a flow rate at 1.0 ml/min a mixture prepared as follows: stir 3 volumes of *acetonitrile R* and 97 volumes of *water R* in a beaker; adjust to pH 3.0 with *phosphoric acid R*,
- as detector a spectrophotometer set at 220 nm.

When the chromatograms are recorded in the prescribed conditions, the retention times are: *L-cystine*, about 2.2 min; *L-cysteine*, about 2.4 min; 2-methyl-2-thiazoline-4-carboxylic acid, originating in test solution (c), about 3.3 min; *acetylcysteine*, about 6.4 min; *acetylcysteine impurity C*, about 12 min; *acetylcysteine impurity D*, about 14 min. Inject 20 µl of reference solution (a). The test is not valid unless:

- in the chromatogram obtained with reference solution (a), the resolution between the peaks due to *L-cystine* and *L-cysteine* is at least 1.5,
- in the chromatogram obtained with reference solution (a), the resolution between the peaks due to *acetylcysteine impurity C* and *acetylcysteine impurity D* is at least 2.0.

Inject 20 µl of 0.01 M *hydrochloric acid* as a blank. Inject three times 20 µl of reference solution (a), 20 µl of reference solution (b) and 20 µl of each test solution. Continue the chromatography for five times the retention time of *acetylcysteine* which is about 30 min.

From the chromatogram obtained with test solution (a), calculate the percentage content of the known impurities and the unknown impurities using the following expressions:

$$\text{known impurity} = \frac{A_1 \times m_2 \times 100}{A_2 \times m_1}$$

$$\text{unknown impurity} = \frac{A_3 \times m_3 \times 100}{A_4 \times m_1}$$

A_1 = peak area of individual impurity (*L-cystine*, *L-cysteine*, *acetylcysteine impurity C* and *acetylcysteine impurity D*) in the chromatogram obtained with test solution (a),

A_2 = peak area of the corresponding individual impurity (*L-cystine*, *L-cysteine*, *acetylcysteine impurity C* and *acetylcysteine impurity D*) in the chromatogram obtained with reference solution (a),

A_3 = peak area of unknown impurity in the chromatogram obtained with test solution (a),

A_4 = peak area of *acetylcysteine* in the chromatogram obtained with reference solution (b),

m_1 = mass of the substance to be examined in test solution (a),

m_2 = mass of the individual impurity in reference solution (a),

m_3 = mass of *acetylcysteine* in reference solution (b).

The percentage content of each known impurity and of each unknown impurity is not greater than 0.5 per cent. The sum of the calculated percentage contents of known and unknown impurities is not greater than 0.5 per cent. Disregard any peak due to the solvent, any peak appearing at retention time of about 3.3 min corresponding to 2-methyl-2-thiazoline-4-carboxylic acid and any peak with an area less than 0.1 times that of the principal peak in the chromatogram obtained with reference solution (b).

Heavy metals (2.4.8). 2.0 g complies with limit test C for heavy metals (10 ppm). Prepare the standard using 2 ml of *lead standard (10 ppm Pb) R*.

Zinc. Not more than 10 ppm of Zn, determined by atomic absorption spectrometry (2.2.23, Method II).

Test solution. Dissolve 1.00 g in 0.001 M *hydrochloric acid* and dilute to 50.0 ml with the same acid.

Reference solutions. Prepare the reference solutions using *zinc standard solution (5 mg/ml Zn) R*, diluted with 0.001 M *hydrochloric acid*.

Measure the absorbance at 213.8 nm using a zinc hollow-cathode lamp as the source of radiation, an air-acetylene flame and a correction procedure for non-specific absorption.

Loss on drying (2.2.32). Not more than 1.0 per cent, determined on 1.000 g by drying in an oven *in vacuo* at 70 °C for 3 h.

Sulphated ash (2.4.14). Not more than 0.2 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.140 g in 60 ml of *water R* and add 10 ml of *dilute hydrochloric acid R*. After cooling in iced water, add 10 ml of *potassium iodide solution R* and titrate with 0.05 M *iodine*, using 1 ml of *starch solution R* as indicator.

1 ml of 0.05 M *iodine* is equivalent to 16.32 mg of $\text{C}_5\text{H}_9\text{NO}_3\text{S}$.

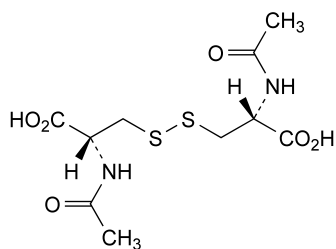
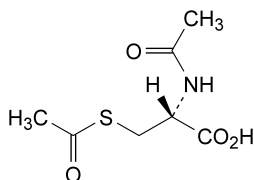
STORAGE

Store protected from light.

IMPURITIES

A. *L-cystine*,

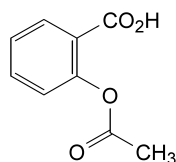
B. *L-cysteine*,

C. *N,N'*-diacetyl-L-cystine,D. *N,S*-diacetyl-L-cysteine.

01/2005:0309

ACETYLSALICYLIC ACID

Acidum acetylsalicylicum

 $C_9H_8O_4$ M_r 180.2**DEFINITION**

Acetylsalicylic acid contains not less than 99.5 per cent and not more than the equivalent of 101.0 per cent of 2-(acetoxy)benzoic acid, calculated with reference to the dried substance.

CHARACTERS

A white, crystalline powder or colourless crystals, slightly soluble in water, freely soluble in alcohol.

It melts at about 143 °C (instantaneous method).

IDENTIFICATION

First identification: A, B.

Second identification: B, C, D.

- Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *acetylsalicylic acid CRS*.
- To 0.2 g add 4 ml of *dilute sodium hydroxide solution R* and boil for 3 min. Cool and add 5 ml of *dilute sulphuric acid R*. A crystalline precipitate is formed. Filter, wash the precipitate and dry at 100 °C to 105 °C. The melting point (2.2.14) is 156 °C to 161 °C.
- In a test tube mix 0.1 g with 0.5 g of *calcium hydroxide R*. Heat the mixture and expose to the fumes produced a piece of filter paper impregnated with 0.05 ml of *nitrobenzaldehyde solution R*. A greenish-blue or greenish-yellow colour develops on the paper. Moisten the paper with *dilute hydrochloric acid R*. The colour becomes blue.
- Dissolve with heating about 20 mg of the precipitate obtained in identification test B in 10 ml of *water R* and cool. The solution gives reaction (a) of salicylates (2.3.1).

TESTS

Appearance of solution. Dissolve 1.0 g in 9 ml of *alcohol R*. The solution is clear (2.2.1) and colourless (2.2.2, *Method II*).

Related substances. Examine by liquid chromatography (2.2.29). Prepare the solutions immediately before use.

Test solution. Dissolve 0.10 g of the substance to be examined in *acetonitrile for chromatography R* and dilute to 10.0 ml with the same solvent.

Reference solution (a). Dissolve 50.0 mg of *salicylic acid R* in the mobile phase and dilute to 50.0 ml with the mobile phase. Dilute 1.0 ml of this solution to 100.0 ml with the mobile phase.

Reference solution (b). Dissolve 10.0 mg of *salicylic acid R* in the mobile phase and dilute to 10.0 ml with the mobile phase. To 1.0 ml of this solution add 0.2 ml of the test solution and dilute to 100.0 ml with the mobile phase.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4.6 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (5 µm),
- as mobile phase at a flow rate of 1 ml/min a mixture of 2 volumes of *phosphoric acid R*, 400 volumes of *acetonitrile for chromatography R* and 600 volumes of *water R*,
- as detector a spectrophotometer set at 237 nm.

Inject 10 µl of each solution. Continue the chromatography of the test solution for seven times the retention time of acetylsalicylic acid. The test is not valid unless in the chromatogram obtained with reference solution (b), the resolution between the two principal peaks is at least 6.0.

In the chromatogram obtained with the test solution the area of any peak, apart from the principal peak, is not greater than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent); the sum of the areas of all the peaks is not greater than 2.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.25 per cent). Disregard any peak with an area less than 0.25 times the area of the principal peak in the chromatogram obtained with reference solution (a).

Heavy metals (2.4.8). Dissolve 1.0 g in 12 ml of *acetone R* and dilute to 20 ml with *water R*. 12 ml of this solution complies with limit test B for heavy metals (20 ppm). Prepare the standard using lead standard solution (1 ppm Pb) obtained by diluting *lead standard solution (100 ppm Pb) R* with a mixture of 6 volumes of *water R* and 9 volumes of *acetone R*.

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying *in vacuo*.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

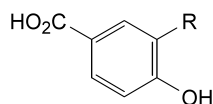
In a flask with a ground-glass stopper, dissolve 1.000 g in 10 ml of *alcohol R*. Add 50.0 ml of 0.5 M *sodium hydroxide*. Close the flask and allow to stand for 1 h. Using 0.2 ml of *phenolphthalein solution R* as indicator, titrate with 0.5 M *hydrochloric acid*. Carry out a blank titration.

1 ml of 0.5 M *sodium hydroxide* is equivalent to 45.04 mg of $C_9H_8O_4$.

STORAGE

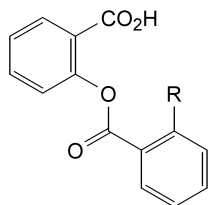
Store in an airtight container.

IMPURITIES



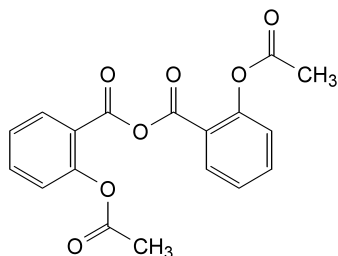
- A. R = H: 4-hydroxybenzoic acid,
 B. R = CO₂H: 4-hydroxybenzene-1,3-dicarboxylic acid
 (4-hydroxyisophthalic acid),

- C. salicylic acid,



- D. R = O-CO-CH₃: 2-[[2-(acetyloxy)benzoyl]oxy]benzoic acid
 (acetylsalicylsalicylic acid),

- E. R = OH: 2-[(2-hydroxybenzoyl)oxy]benzoic acid
 (salicylsalicylic acid),



- F. 2-(acetyloxy)benzoic anhydride (acetylsalicylic anhydride).

Second identification: A, C, D, E.

- A. It complies with the test for optical rotation (see Tests).
 B. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *N*-acetyltryptophan CRS.

- C. Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel F₂₅₄ plate R*.

Test solution. Dissolve 50 mg of the substance to be examined in 0.2 ml of *concentrated ammonia R* and dilute to 10 ml with *water R*.

Reference solution (a). Dissolve 50 mg of *N*-acetyltryptophan CRS in 0.2 ml of *concentrated ammonia R* and dilute to 10 ml with *water R*.

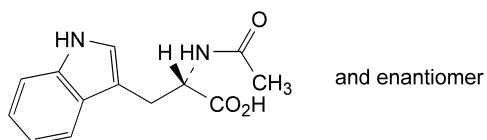
Reference solution (b). Dissolve 10 mg of *tryptophan R* in the test solution and dilute to 2 ml with the same solution.

Apply to the plate 2 µl of each solution. Develop over a path of 10 cm using a mixture of 25 volumes of *glacial acetic acid R*, 25 volumes of *water R* and 50 volumes of *butanol R*. Dry the plate in an oven at 100-105 °C for 15 min and examine in ultraviolet light at 254 nm. The principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a). The test is not valid unless the chromatogram obtained with reference solution (b) shows two clearly separated spots.

- D. Dissolve about 2 mg in 2 ml of *water R*. Add 2 ml of *dimethylaminobenzaldehyde solution R6*. Heat on a water-bath. A blue or greenish-blue colour develops.
 E. It gives the reaction of acetyl (2.3.1). Proceed as described for substances hydrolysable only with difficulty.

TESTS

01/2005:1383

N-ACETYLTRYPTOPHAN*N*-AcetyltryptophanumC₁₃H₁₄N₂O₃M_r 246.3

DEFINITION

N-Acetyltryptophan contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of (RS)-2-acetylamino-3-(1*H*-indol-3-yl)propanoic acid, calculated with reference to the dried substance.

PRODUCTION

Tryptophan used for the production of *N*-acetyltryptophan complies with the test for 1,1'-ethylidenebistryptophan and other related substances in the monograph on *Tryptophan* (1272).

CHARACTERS

A white or almost white, crystalline powder, or colourless crystals, slightly soluble in water, very soluble in alcohol. It dissolves in dilute solutions of alkali hydroxides.

It melts at about 205 °C.

IDENTIFICATION

First identification: A, B.

Appearance of solution. Dissolve 1.0 g in a 40 g/l solution of *sodium hydroxide R* and dilute to 100 ml with the same alkaline solution. The solution is clear (2.2.1) and not more intensely coloured than reference solution Y₇ or GY₇ (2.2.2, *Method II*).

Optical rotation (2.2.7). Dissolve 2.50 g in a 40 g/l solution of *sodium hydroxide R* and dilute to 25.0 ml with the same alkaline solution. The angle of optical rotation is -0.1° to + 0.1°.

Related substances. Examine by liquid chromatography (2.2.29).

Buffer solution pH 2.3. Dissolve 3.90 g of *sodium dihydrogen phosphate R* in 1000 ml of *water R*. Add about 700 ml of a 2.9 g/l solution of *phosphoric acid R* and adjust the pH to 2.3 with the same acidic solution.

Prepare the solutions immediately before use.

Test solution. Dissolve 0.10 g of the substance to be examined in a mixture of 50 volumes of *acetonitrile R* and 50 volumes of *water R* and dilute to 20.0 ml with the same mixture of solvents.

Reference solution (a). Dilute 1.0 ml of the test solution to 100.0 ml with a mixture of 10 volumes of *acetonitrile R* and 90 volumes of *water R*.

Reference solution (b). Dissolve 1.0 mg of 1,1'-ethylidenebis(*tryptophan*) CRS in a mixture of 10 volumes of *acetonitrile R* and 90 volumes of *water R* and dilute to 100.0 ml with the same mixture of solvents.

Reference solution (c). To 4.0 ml of reference solution (a), add 20.0 ml of reference solution (b) and dilute to 100.0 ml with a mixture of 10 volumes of *acetonitrile R* and 90 volumes of *water R*.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4.6 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (5 µm),
- as mobile phase at a flow rate of 0.7 ml/min:

Mobile phase A. A mixture of 115 volumes of *acetonitrile R* and 885 volumes of buffer solution pH 2.3,

Mobile phase B. A mixture of 350 volumes of *acetonitrile R* and 650 volumes of buffer solution pH 2.3,

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)	Comment
	100	0	equilibration
0 - 10	100	0	isocratic
10 - 45	100 → 0	0 → 100	linear gradient
45 - 65	0	100	isocratic
65 - 66	0 → 100	100 → 0	linear gradient
66 - 80	100	0	re-equilibration

- as detector a spectrophotometer set at 220 nm, maintaining the temperature of the column at 40 °C.

When the chromatograms are recorded in the prescribed conditions, the retention times are about 29 min for *N*-acetyltryptophan and about 34 min for 1,1'-ethyldienebis(tryptophan). Adjust the sensitivity of the system so that the height of the peak due to *N*-acetyltryptophan in the chromatogram obtained with reference solution (a) is at least 50 per cent of the full scale of the recorder.

Inject 20 µl of reference solution (c). The test is not valid unless in the chromatogram obtained, the resolution between the peaks corresponding to *N*-acetyltryptophan and 1,1'-ethyldienebis(tryptophan) is at least 8.0. If necessary, adjust the time programme for the elution gradient. An increase in the duration of elution with mobile phase A produces longer retention times and a better resolution.

Inject 20 µl of the test solution and 20 µl of reference solution (a). Continue the chromatography of the test solution for 1.8 times the retention time of *N*-acetyltryptophan. In the chromatogram obtained with the test solution: the area of any peak, apart from the principal peak, is not greater than 0.25 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.25 per cent); the sum of the areas of all the peaks, apart from the principal peak, is not greater than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent). Disregard any peak due to the solvent and any peak with an area less than 0.01 times that of the peak due to *N*-acetyltryptophan in the chromatogram obtained with reference solution (a).

Ammonium (2.4.1). 0.10 g complies with limit test B for ammonium (200 ppm). Prepare the standard using 0.2 ml of *ammonium standard solution* (100 ppm NH₄) *R*.

Iron (2.4.9). Dissolve 1.0 g in 50 ml of *hydrochloric acid R1*, with heating at 50 °C. Allow to cool. In a separating funnel, shake with three quantities, each of 10 ml, of *methyl isobutyl ketone R1*, shaking for 3 min each time. To the combined organic layers add 10 ml of *water R* and shake for 3 min. The aqueous layer complies with the limit test for iron (10 ppm).

Heavy metals (2.4.8). 2.0 g complies with limit test C for heavy metals (10 ppm). Prepare the standard using 2 ml of *lead standard solution* (10 ppm Pb) *R*.

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 100-105 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.200 g in 5 ml of *methanol R*. Add 50 ml of *ethanol R*. Titrate with 0.1 *M* sodium hydroxide, determining the end-point potentiometrically (2.2.20).

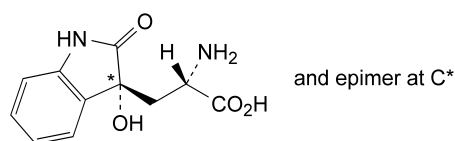
1 ml of 0.1 *M* sodium hydroxide is equivalent to 24.63 mg of C₁₃H₁₄N₂O₃.

STORAGE

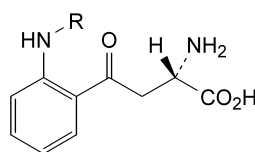
Store protected from light.

IMPURITIES

A. tryptophan,

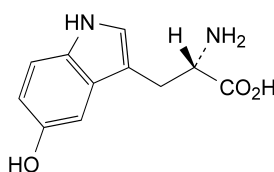


B. (S)-2-amino-3-[(3*RS*)-3-hydroxy-2-oxo-2,3-dihydro-1*H*-indol-3-yl]propanoic acid (dioxindolylalanine),

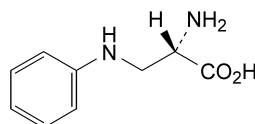


C. R = H: (S)-2-amino-4-(2-aminophenyl)-4-oxobutanoic acid (kynurenine),

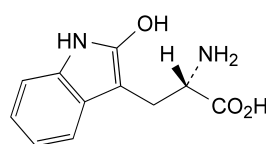
E. R = CHO: (S)-2-amino-4-[2-(formylamino)phenyl]-4-oxobutanoic acid (*N*-formylkynurenine),



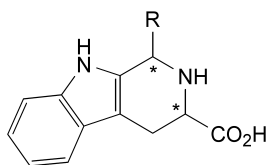
D. (S)-2-amino-3-(5-hydroxy-1*H*-indol-3-yl)propanoic acid (5-hydroxytryptophan),



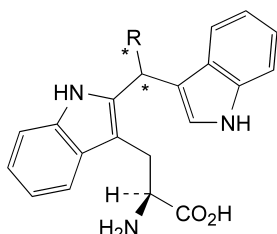
F. (S)-2-amino-3-(phenylamino)propanoic acid (3-phenylaminoalanine),



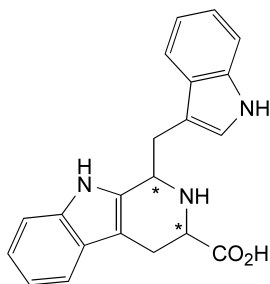
G. (S)-2-amino-3-(2-hydroxy-1*H*-indol-3-yl)propanoic acid (2-hydroxytryptophan),



- H. R = H: (3*RS*)-1,2,3,4-tetrahydro-9*H*-β-carboline-3-carboxylic acid,
 I. R = CH₃: 1-methyl-1,2,3,4-tetrahydro-9*H*-β-carboline-3-carboxylic acid,



- J. R = CHOH-CH₂-OH: (S)-2-amino-3-[2-[2,3-dihydroxy-1-(1*H*-indol-3-yl)propyl]-1*H*-indol-3-yl]propanoic acid,
 K. R = H: (S)-2-amino-3-[2-(1*H*-indol-3-ylmethyl)-1*H*-indol-3-yl]propanoic acid,

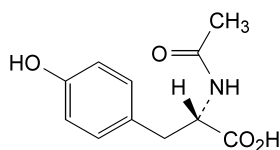


- L. 1-(1*H*-indol-3-ylmethyl)-1,2,3,4-tetrahydro-9*H*-β-carboline-3-carboxylic acid.

01/2005:1384

N-ACETYLTYROSINE

N-Acetyltyrosinum

C₁₁H₁₃NO₄M_r 223.2

DEFINITION

N-Acetyltyrosine contains not less than 98.5 per cent and not more than the equivalent of 101.0 per cent of (2*S*)-2-(acetylamino)-3-(4-hydroxyphenyl)propanoic acid, calculated with reference to the dried substance.

CHARACTERS

A white, crystalline powder or colourless crystals, freely soluble in water, practically insoluble in cyclohexane.

IDENTIFICATION

First identification: A, B.

Second identification: A, C, D.

- A. It complies with the test for specific optical rotation (see Tests).

- B. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *N*-acetyltyrosine CRS. Examine the substances prepared as discs.
 C. Examine the chromatograms obtained in the test for related substances in ultraviolet light at 254 nm. The principal spot obtained with test solution (b) is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a).
 D. Solution S (see Tests) is strongly acid (2.2.4).

TESTS

Solution S. Dissolve 2.50 g in *water R* and dilute to 100.0 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and colourless (2.2.2, *Method II*).

Specific optical rotation (2.2.7). Dilute 10.0 ml of solution S to 25.0 ml with *water R*. The specific optical rotation is + 46 to + 49, calculated with reference to the dried substance.

Related substances. Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel F₂₅₄* plate *R*.

Test solution (a). Dissolve 0.80 g of the substance to be examined in 6 ml of a mixture of equal volumes of *glacial acetic acid R* and *water R* and dilute to 10 ml with *ethanol R*.

Test solution (b). Dilute 1 ml of test solution (a) to 10 ml with *ethanol R*.

Reference solution (a). Dissolve 80 mg of *N*-acetyltyrosine CRS in a mixture of 3 volumes of *water R*, 3 volumes of *glacial acetic acid R* and 94 volumes of *ethanol R* and dilute to 10 ml with the same mixture of solvents.

Reference solution (b). Dilute 0.5 ml of test solution (b) to 10 ml with *ethanol R*.

Reference solution (c). Dissolve 40 mg of *tyrosine CRS* in 20 ml of a mixture of equal volumes of *water R* and *glacial acetic acid R* and dilute to 50 ml with *ethanol R*.

Apply separately to the plate 5 µl of each solution. Develop over a path of 10 cm using a mixture of 10 volumes of *water R*, 15 volumes of *glacial acetic acid R* and 75 volumes of *ethyl acetate R*. Allow the plate to dry in air. Examine in ultraviolet light at 254 nm. Any spot in the chromatogram obtained with test solution (a), apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.5 per cent). Spray with *ninhydrin solution R* and heat at 100 °C to 105 °C for 10 min. Examine in daylight. Any spot corresponding to tyrosine is not more intense than the spot in the chromatogram obtained with reference solution (c) (1 per cent).

Chlorides (2.4.4). Dilute 10 ml of solution S to 15 ml with *water R*. The solution complies with the limit test for chlorides (200 ppm).

Sulphates (2.4.13). Dissolve 1.0 g in *distilled water R* and dilute to 20 ml with the same solvent. The solution complies with the limit test for sulphates (200 ppm).

Ammonium. Prepare a cell consisting of two watch-glasses 60 mm in diameter placed edge to edge. To the inner wall of the upper watch-glass stick a piece of *red litmus paper R* 5 mm square and wetted with a few drops of *water R*. Finely powder the substance to be examined, place 50 mg in the lower watch-glass and dissolve in 0.5 ml of *water R*. To the solution add 0.30 g of *heavy magnesium oxide R*. Briefly triturate with a glass rod. Immediately close the cell by putting the two watch-glasses together. Heat at 40 °C for 15 min. The litmus paper is not more intensely blue

coloured than a standard prepared at the same time and in the same manner using 0.1 ml of *ammonium standard solution* (100 ppm NH_4) *R*, 0.5 ml of *water R* and 0.30 g of *heavy magnesium oxide R* (200 ppm).

Iron (2.4.9). In a separating funnel, dissolve 0.5 g in 10 ml of *dilute hydrochloric acid R*. Shake with three quantities, each of 10 ml, of *methyl isobutyl ketone R1*, shaking for 3 min each time. To the combined organic layers add 10 ml of *water R* and shake for 3 min. The aqueous layer complies with the limit test for iron (20 ppm).

Heavy metals (2.4.8). Dissolve 2.0 g in *water R* and dilute to 20 ml with the same solvent. 12 ml of the solution complies with limit test A for heavy metals (10 ppm). Prepare the standard using *lead standard solution* (1 ppm Pb) *R*.

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 100–105 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

Pyrogens (2.6.8). If intended for use in the manufacture of parenteral dosage forms without a further appropriate procedure for the removal of pyrogens, it complies with the test for pyrogens. Inject per kilogram of the rabbit's mass 1.0 ml of a freshly prepared solution in *water for injections R* containing per millilitre 10.0 mg of the substance to be examined and 9.0 mg of pyrogen-free *sodium chloride R*.

ASSAY

Dissolve 0.180 g in 50 ml of *carbon dioxide-free water R*. Titrate with 0.1 *M sodium hydroxide*, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 *M sodium hydroxide* is equivalent to 22.32 mg of $\text{C}_{11}\text{H}_{13}\text{NO}_4$.

STORAGE

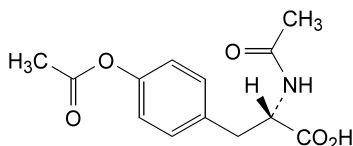
Store protected from light. If the substance is sterile, store in a sterile, airtight, tamper-proof container.

LABELLING

The label states, where applicable, that the substance is apyrogenic.

IMPURITIES

A. tyrosine,

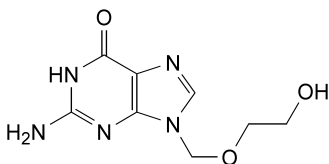


B. (2*S*)-2-(acetylamino)-3-[4-(acetoxyphe-nyl)]propanoic acid (diacetyltyrosine).

01/2005:0968

ACICLOVIR

Aciclovirum



$\text{C}_8\text{H}_{11}\text{N}_5\text{O}_3$

M_r 225.2

DEFINITION

Aciclovir contains not less than 98.5 per cent and not more than the equivalent of 101.0 per cent of 2-amino-9-[(2-hydroxyethoxy)methyl]-1,9-dihydro-6*H*-purin-6-one, calculated with reference to the anhydrous substance.

CHARACTERS

A white or almost white, crystalline powder, slightly soluble in water, freely soluble in dimethyl sulphoxide, very slightly soluble in alcohol. It dissolves in dilute solutions of mineral acids and alkali hydroxides.

IDENTIFICATION

Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *aciclovir CRS*.

TESTS

Appearance of solution. Dissolve 0.25 g in 0.1 *M sodium hydroxide* and dilute to 25 ml with the same solvent. The solution is clear (2.2.1) and not more intensely coloured than reference solution Y_7 (2.2.2, *Method II*).

Related substances.

A. Examine by thin-layer chromatography (2.2.27), using *silica gel GF₂₅₄ R* as the coating substance.

Prepare the solutions immediately before use.

Test solution. Dissolve 0.1 g of the substance to be examined in *dimethyl sulphoxide R* and dilute to 10 ml with the same solvent.

Reference solution. Dissolve 10 mg of *aciclovir impurity A CRS* in *dimethyl sulphoxide R* and dilute to 20 ml with the same solvent. Dilute 1 ml of the solution to 10 ml with *dimethyl sulphoxide R*.

Apply to the plate 10 µl of each solution. Keep the spots compact by drying in a current of warm air. Allow the plate to cool and develop over a path of 10 cm with a mixture of 2 volumes of *concentrated ammonia R*, 20 volumes of *methanol R* and 80 volumes of *methylene chloride R*. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. In the chromatogram obtained with the test solution, any spot with an R_f value greater than that of the principal spot is not more intense than the spot in the chromatogram obtained with the reference solution (0.5 per cent).

B. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 50.0 mg of the substance to be examined in 10 ml of a mixture of 20 volumes of *glacial acetic acid R* and 80 volumes of *water R* and dilute to 100.0 ml with the mobile phase.

Reference solution (a). Dilute 1.0 ml of the test solution to 200.0 ml with the mobile phase.

Reference solution (b). Dissolve 20 mg of *aciclovir CRS* and 20 mg of *aciclovir impurity A CRS* in a mixture of 20 volumes of *glacial acetic acid R* and 80 volumes of *water R* and dilute to 100.0 ml with the same mixture of solvents. Dilute 1.0 ml of the solution to 10.0 ml with the mobile phase.

Reference solution (c). Dissolve 7 mg of *guanine R* in 0.1 *M sodium hydroxide* and dilute to 100.0 ml with the same solution. Dilute 1.0 ml to 20.0 ml with the mobile phase.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.10 m long and 4.6 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (3 µm),
- as mobile phase at a flow rate of 2 ml/min a mixture prepared as follows: dissolve 6.0 g of *sodium dihydrogen phosphate R* and 1.0 g of *sodium decanesulphonate R* in 900 ml of *water R* and adjust to pH 3 ± 0.1 with *phosphoric acid R*; add 40 ml of *acetonitrile R* and dilute to 1 litre with *water R*,
- as detector a spectrophotometer set at 254 nm,
- a loop injector.

Inject 20 µl of each solution. Record the chromatograms for seven times the retention time of aciclovir. The test is not valid unless in the chromatogram obtained with reference solution (b), the number of theoretical plates calculated for the peak due to aciclovir impurity A is at least 1500 and its mass distribution ratio is at least 7 (V_0 can be calculated using *dimethyl sulfoxide R*). In the chromatogram obtained with the test solution: the area of any peak corresponding to guanine is not greater than that of the peak in the chromatogram obtained with reference solution (c) (0.7 per cent); the area of any peak apart from the principal peak and any peak corresponding to guanine is not greater than the area of the peak in the chromatogram obtained with reference solution (a) (0.5 per cent) and the sum of the areas of such peaks is not greater than twice the area of the peak in the chromatogram obtained with reference solution (a) (1 per cent). Disregard any peak with an area less than 0.05 times that of the principal peak in the chromatogram obtained with reference solution (a).

Water (2.5.12). Not more than 6.0 per cent, determined on 0.500 g by the semi-micro determination of water.

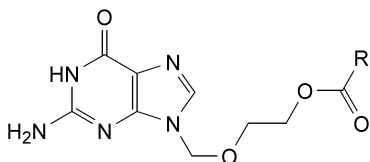
Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

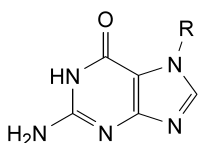
Dissolve 0.150 g in 60 ml of *anhydrous acetic acid R*. Titrate with 0.1 M *perchloric acid*, determining the end-point potentiometrically (2.2.20). Carry out a blank titration.

1 ml of 0.1 M *perchloric acid* is equivalent to 22.52 mg of $C_8H_{11}N_5O_3$.

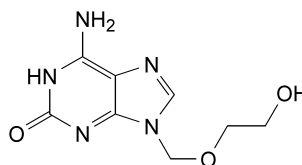
IMPURITIES



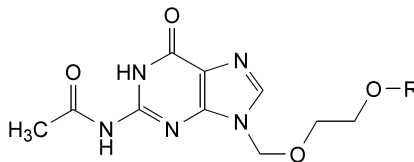
- A. R = CH_3 : 2-[(2-amino-6-oxo-1,6-dihydro-9H-purin-9-yl)methoxy]ethyl acetate,
- D. R = C_6H_5 : 2-[(2-amino-6-oxo-1,6-dihydro-9H-purin-9-yl)methoxy]ethyl benzoate,



- B. R = H: 2-amino-1,7-dihydro-6H-purin-6-one (guanine),
- C. R = $CH_2OCH_2CH_2OH$: 2-amino-7-[(2-hydroxyethoxy)methyl]-1,7-dihydro-6H-purin-6-one,



- E. 6-amino-9-[(2-hydroxyethoxy)methyl]-1,9-dihydro-2H-purin-2-one,

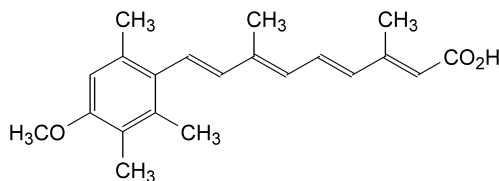


- F. R = H: *N*-[9-[(2-hydroxyethoxy)methyl]-6-oxo-6,9-dihydro-1H-purin-2-yl]acetamide,
- G. R = $CO-CH_3$: 2-[[2-(acetylamino)-6-oxo-1,6-dihydro-9H-purin-9-yl]methoxy]ethyl acetate,
- H. R = $CO-C_6H_5$: 2-[[2-(acetylamino)-6-oxo-1,6-dihydro-9H-purin-9-yl]methoxy]ethyl benzoate.

01/2005:1385
corrected

ACITRETIN

Acitretinum



$C_{21}H_{26}O_3$

M_r 326.4

DEFINITION

Acitretin contains not less than 98.0 per cent and not more than the equivalent of 102.0 per cent of (all-*E*)-9-(4-methoxy-2,3,6-trimethylphenyl)-3,7-dimethylnona-2,4,6,8-tetraenoic acid, calculated with reference to the dried substance.

CHARACTERS

A yellow or greenish-yellow, crystalline powder, practically insoluble in water, sparingly soluble in tetrahydrofuran, slightly soluble in acetone and in alcohol, very slightly soluble in cyclohexane. It is sensitive to air, heat and light, especially in solution.

Carry out all operations as rapidly as possible and avoid exposure to actinic light; use freshly prepared solutions.

IDENTIFICATION

First identification: B.

Second identification: A, C.

- A. Dissolve 15.0 mg in 10 ml of *tetrahydrofuran R* and dilute immediately to 100.0 ml with the same solvent. Dilute 2.5 ml of this solution to 100.0 ml with *tetrahydrofuran R*. Examined between 300 nm and 400 nm (2.2.25), the solution shows an absorption maximum at 358 nm. The specific absorbance at the maximum is 1350 to 1475.
- B. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *acitretin CRS*. Examine the substances prepared as discs.

- C. Examine the chromatograms obtained in the assay.
The principal peak in the chromatogram obtained with the test solution has a retention time similar to that of the principal peak in the chromatogram obtained with reference solution (a).

TESTS

Related substances. Examine by liquid chromatography (2.2.29) as described under Assay.

Inject 10 µl each of reference solutions (b) and (c) and of the test solution (a). Adjust the sensitivity of the system so that the height of the principal peak in the chromatogram obtained with reference solution (b) is not less than 40 per cent of the full scale of the recorder. The test is not valid unless the resolution between the peaks due to acitretin and tretinoin in the chromatogram obtained with reference solution (b) is at least 2.0. If necessary, adjust the concentration of *ethanol R*. Continue the chromatography of the test solution (a) for 2.5 times the retention time of the principal peak. In the chromatogram obtained with the test solution (a), the area of any peak, apart from the principal peak, is not greater than the area of the peak due to acitretin in the chromatogram obtained with reference solution (c) (0.3 per cent); the sum of the areas of any peaks, apart from the principal peak, is not greater than the area of the peak due to acitretin in the chromatogram obtained with reference solution (b) (1.0 per cent). Disregard any peak with an area less than 0.1 times that of the principal peak in the chromatogram obtained with reference solution (c).

Heavy metals (2.4.8). 2.0 g complies with limit test C (20 ppm). Prepare the standard using 2 ml of *lead standard solution* (10 ppm Pb) *R*.

Palladium. Not more than 10 ppm of Pd, determined by atomic absorption spectrometry (2.2.23, *Method I*).

Test solution. Introduce 2.0 g of the substance to be examined into a quartz beaker and add 3 ml of *magnesium nitrate solution R*. Heat in a muffle furnace to 350 °C at a rate of 40 °C/min to incinerate the content. Ignite at about 450 °C for 8 h and then at 550 °C for a further hour. Dissolve the residue in a mixture of 0.75 ml of *hydrochloric acid R* and 0.25 ml of *nitric acid R* warming gently. Cool, transfer the solution into a volumetric flask with *water R* and dilute to 50.0 ml with the same solvent.

Reference solution. Dissolve 0.163 g of *heavy magnesium oxide R* in a mixture of 0.5 ml of *nitric acid R*, 1.5 ml of *hydrochloric acid R* and 50 ml of *water R*, add 2.0 ml of *palladium standard solution* (20 ppm Pd *R*) and dilute to 100.0 ml with *water R*.

Measure the absorbance at 247.6 nm using a palladium hollow-cathode lamp as source of radiation and an air-acetylene flame.

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying *in vacuo* at 100 °C for 4 h.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Carry out the assay protected from light, use amber volumetric flasks and prepare the solutions immediately before use.

Examine by liquid chromatography (2.2.29).

Test solution (a). Dissolve 25.0 mg of the substance to be examined in 5 ml of *tetrahydrofuran R* and dilute immediately to 100.0 ml with *ethanol R*.

Test solution (b). Dilute 10.0 ml of test solution (a) to 25.0 ml with *ethanol R*.

Reference solution (a). Dissolve 25.0 mg of *acitretin CRS* in 5 ml of *tetrahydrofuran R* and dilute immediately to 100.0 ml with *ethanol R*. Dilute 10.0 ml to 25.0 ml with *ethanol R*.

Reference solution (b). Dissolve 1.0 mg of *tretinoin CRS* in *ethanol R* and dilute to 20.0 ml with the same solvent. Mix 5.0 ml of the solution with 2.5 ml of reference solution (a) and dilute to 100.0 ml with *ethanol R*.

Reference solution (c). Dilute 2.5 ml of reference solution (a) to 50.0 ml with *ethanol R*. Dilute 3.0 ml of the solution to 20.0 ml with *ethanol R*.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4 mm in internal diameter packed with microparticulate *octadecylsilyl silica gel for chromatography R* (5 µm), with a carbon loading of 20 per cent, a specific surface area of 200 m²/g and a pore size of 15 nm,
- as mobile phase at a flow rate of 0.6 ml/min a 0.3 per cent V/V solution of *glacial acetic acid R* in a mixture of 8 volumes of *water R* and 92 volumes of *ethanol R*,
- as detector a spectrophotometer set at 360 nm,
- a sampler kept at 4 °C,

maintaining the temperature of the column at 25 °C.

When the chromatograms are recorded in the prescribed conditions, the retention times are: impurity A about 4.8 min, tretinoin about 5.2 min, acitretin about 6.2 min and impurity B about 10.2 min.

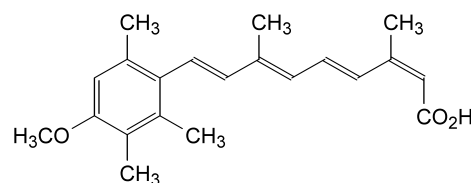
Inject 10 µl of reference solution (a) 6 times. The test is not valid unless the relative standard deviation of the peak area for acitretin is at most 1.0 per cent. If necessary, adjust the integration parameters. Inject alternately the test solution (b) and reference solution (a).

STORAGE

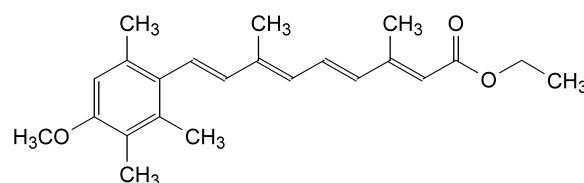
In an airtight container, protected from light, at a temperature of 2 °C to 8 °C.

It is recommended that the contents of an opened container be used as soon as possible and any unused part be protected by an atmosphere of inert gas.

IMPURITIES



A. (2Z,4E,6E,8E)-9-(4-methoxy-2,3,6-trimethylphenyl)-3,7-dimethylnona-2,4,6,8-tetraenoic acid,

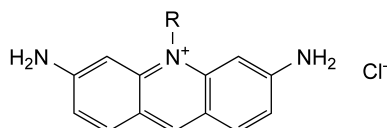


B. ethyl (all-*E*)-9-(4-methoxy-2,3,6-trimethylphenyl)-3,7-dimethylnona-2,4,6,8-tetraenoate.

01/2005:2043

ACRIFLAVINIUM MONOCHLORIDE

Acriflavinii monochloridum



R	Mol. Formula	M_r
H	$C_{13}H_{12}ClN_3$	245.7
CH ₃	$C_{14}H_{14}ClN_3$	259.7

DEFINITION

Mixture of 3,6-diamino-10-methylacridinium chloride and 3,6-diaminoacridine hydrochloride.

Content: 95.0 per cent to 105.0 per cent (anhydrous substance).

CHARACTERS

Appearance: reddish-brown powder, hygroscopic.

Solubility: freely soluble in water, sparingly soluble in alcohol, very slightly soluble in methylene chloride.

IDENTIFICATION

A. Thin-layer chromatography (2.2.27).

Test solution. Dilute 0.5 ml of solution S (see Tests) to 10 ml with *alcohol R*.

Reference solution. Dissolve 10 mg of *acriflavinium monochloride CRS* in *alcohol R* and dilute to 10 ml with the same solvent.

Plate: TLC silica gel plate *R*.

Mobile phase: glacial acetic acid *R*, water *R*, butanol *R* (1:1:4 V/V/V).

Application: 5 µl.

Development: over 2/3 of the plate.

Drying: at 100-105 °C.

Detection: examine in ultraviolet light at 365 nm.

Results: the 2 principal spots in the chromatogram obtained with the test solution are similar in position, fluorescence and size to the principal spots in the chromatogram obtained with the reference solution.

B. To 0.1 mg add 20 ml of *water R*. The solution obtained shows a yellowish-green fluorescence. Add 1 ml of *dilute sodium hydroxide solution R*. The fluorescence disappears.

C. It complies with the test for pH (see Tests).

TESTS

Solution S. Dissolve with heating 0.500 g in 15 ml of *carbon dioxide-free water R*; allow to cool and dilute to 25.0 ml with the same solvent.

pH (2.2.3): 4.5 to 7.5 for solution S.

Composition. Liquid chromatography (2.2.29): use the normalisation procedure.

Test solution. Dissolve 10.0 mg of the substance to be examined in the mobile phase and dilute to 25.0 ml with the mobile phase.

Reference solution (a). Dilute 1.0 ml of the test solution to 100.0 ml with the mobile phase.

Reference solution (b). Dilute 1.0 ml of reference solution (a) to 10.0 ml with the mobile phase.

Column:

- **size:** $l = 0.25$ m, $\varnothing = 4.6$ mm,
- **stationary phase:** octadecylsilyl silica gel for chromatography *R* (5 µm).

Mobile phase: dissolve 1.0 g of *sodium octanesulphonate R* and 5 ml of *triethylamine R* in a mixture of 400 ml of *acetonitrile R* and 600 ml of *phosphate buffer solution pH 2.8 R*.

Flow rate: 1 ml/min.

Detection: spectrophotometer at 262 nm.

Injection: 10 µl.

Run time: 30 min.

System suitability: reference solution (a):

- **resolution:** minimum 3.5 between the 2 principal peaks.

Limits:

- **first principal peak:** 30.0 per cent to 40.0 per cent,
- **second principal peak:** 50.0 per cent to 60.0 per cent,
- **any other peak:** maximum 6.0 per cent and not more than 2 such peaks have a peak area of more than 2.0 per cent,
- **disregard limit:** 0.5 times the area of the peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

Heavy metals (2.4.8): maximum 40 ppm.

0.5 g complies with limit test D. Prepare the standard using 2 ml of *lead standard solution (10 ppm Pb) R*.

Water (2.5.12): maximum 10.0 per cent, determined on 0.250 g.

Sulphated ash (2.4.14): maximum 3.5 per cent, determined on 1.0 g.

ASSAY

Prepare the solutions immediately before use and protected from light.

Determine the water content immediately before the assay.

Dissolve 0.100 g in 500.0 ml of *water R*. Dilute 5.0 ml of the solution to 250.0 ml with 0.1 M *hydrochloric acid*. Measure the absorbance (2.2.25) at the maximum at 262 nm.

Calculate the content of the sum of $C_{14}H_{14}ClN_3$ and $C_{13}H_{12}ClN_3$ taking the specific absorbance to be 1820.

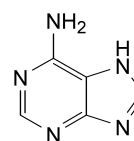
STORAGE

In an airtight container, protected from light.

01/2005:0800

ADENINE

Adeninum

 $C_5H_5N_5$ M_r 135.1

DEFINITION

Adenine contains not less than 98.5 per cent and not more than the equivalent of 101.0 per cent of 7H-purin-6-amine, calculated with reference to the dried substance.

CHARACTERS

A white powder, very slightly soluble in water and in alcohol. It dissolves in dilute mineral acids and in dilute solutions of alkali hydroxides.

IDENTIFICATION

First identification: A.

Second identification: B, C.

- Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *adenine CRS*. Examine the substances prepared as discs.
- Examine the chromatograms obtained in the test for related substances. The principal spot in the chromatogram obtained with test solution (b) is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a).
- To 1 g add 3.5 ml of *propionic anhydride R* and boil for 15 min with stirring. Cool. To the resulting crystalline mass add 15 ml of *light petroleum R* and heat to boiling with vigorous stirring. Cool and filter. Wash the precipitate with two quantities, each of 5 ml, of *light petroleum R*. Dissolve the precipitate in 10 ml of *water R* and boil for 1 min. Filter the mixture at 30 °C to 40 °C. Allow to cool. Filter, and dry the precipitate at 100 °C to 105 °C for 1 h. The melting point (2.2.14) of the precipitate is 237 °C to 241 °C.

TESTS

Solution S. Suspend 2.5 g in 50 ml of *distilled water R* and boil for 3 min. Cool and dilute to 50 ml with *distilled water R*. Filter. Use the filtrate as solution S.

Appearance of solution. Dissolve 0.5 g in *dilute hydrochloric acid R* and dilute to 50 ml with the same acid. The solution is clear (2.2.1) and colourless (2.2.2, *Method II*).

Acidity or alkalinity. To 10 ml of solution S add 0.1 ml of *bromothymol blue solution R1* and 0.2 ml of 0.01 M *sodium hydroxide*. The solution is blue. Add 0.4 ml of 0.01 M *hydrochloric acid*. The solution is yellow.

Related substances. Examine by thin-layer chromatography (2.2.27), using *silica gel GF₂₅₄ R* as the coating substance.

Test solution (a). Dissolve 0.10 g of the substance to be examined in *dilute acetic acid R*, with heating if necessary, and dilute to 10 ml with the same acid.

Test solution (b). Dilute 1 ml of test solution (a) to 10 ml with *dilute acetic acid R*.

Reference solution (a). Dissolve 10 mg of *adenine CRS* in *dilute acetic acid R*, with heating if necessary, and dilute to 10 ml with the same acid.

Reference solution (b). Dilute 1 ml of test solution (b) to 20 ml with *dilute acetic acid R*.

Reference solution (c). Dissolve 10 mg of *adenine CRS* and 10 mg of *adenosine R* in *dilute acetic acid R*, with heating if necessary, and dilute to 10 ml with the same acid.

Apply to the plate 5 µl of each solution. Develop over a path of 12 cm using a mixture of 20 volumes of *concentrated ammonia R*, 40 volumes of *ethyl acetate R* and 40 volumes of *propanol R*. Dry the plate in a current of warm air and examine in ultraviolet light at 254 nm. Any spot in the chromatogram obtained with test solution (a), apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.5 per cent). The test is not valid unless the chromatogram obtained with reference solution (c) shows two clearly separated spots.

Chlorides (2.4.4). To 10 ml of solution S add 1 ml of *concentrated ammonia R* and 3 ml of *silver nitrate solution R2*. Filter. Wash the precipitate with a little *water R*

and dilute the filtrate to 15 ml with *water R*. The solution complies with the limit test for chlorides (100 ppm). When carrying out the test, add 2 ml of *dilute nitric acid R* instead of 1 ml of *dilute nitric acid R*.

Sulphates (2.4.13). Dilute 10 ml of solution S to 15 ml with *distilled water R*. The solution complies with the limit test for sulphates (300 ppm).

Ammonium. Prepare a cell consisting of two watch-glasses 60 mm in diameter placed edge to edge. To the inner wall of the upper watch-glass stick a piece of *red litmus paper R* 5 mm square and wetted with a few drops of *water R*. Finely powder the substance to be examined, place 0.5 g in the lower watch-glass and suspend in 0.5 ml of *water R*. To the suspension add 0.30 g of *heavy magnesium oxide R*. Briefly triturate with a glass rod. Immediately close the cell by putting the two watch-glasses together. Heat at 40 °C for 15 min. The litmus paper is not more intensely blue coloured than a standard prepared at the same time and in the same manner using 0.05 ml of *ammonium standard solution* (100 ppm NH_4) *R*, 0.5 ml of *water R* and 0.30 g of *heavy magnesium oxide R* (10 ppm).

Heavy metals (2.4.8). 1.0 g complies with limit test C for heavy metals (20 ppm). Prepare the standard using 2 ml of *lead standard solution* (10 ppm Pb) *R*.

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

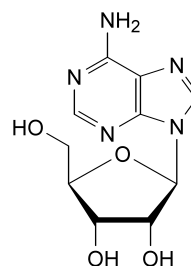
Dissolve 0.100 g in a mixture of 20 ml of *acetic anhydride R* and 30 ml of *anhydrous acetic acid R*. Titrate with 0.1 M *perchloric acid*, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *perchloric acid* is equivalent to 13.51 mg of $\text{C}_5\text{H}_5\text{N}_5$.

01/2005:1486

ADENOSINE

Adenosinum

 $\text{C}_{10}\text{H}_{13}\text{N}_5\text{O}_4$ M_r 267.2

DEFINITION

Adenosine contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of 9-β-D-ribofuranosyl-9H-purin-6-amine, calculated with reference to the dried substance.

CHARACTERS

A white, crystalline powder slightly soluble in water, soluble in hot water, practically insoluble in alcohol and in methylene chloride. It dissolves in dilute mineral acids.

It melts at about 234 °C.

IDENTIFICATION

Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *adenosine CRS*.

TESTS

Solution S. Suspend 5.0 g in 100 ml of *distilled water R* and heat to boiling. Allow to cool, filter with the aid of vacuum and dilute to 100 ml with *distilled water R*.

Appearance of solution. Solution S is colourless (2.2.2, *Method II*).

Acidity or alkalinity. To 10 ml of solution S, add 0.1 ml of *bromocresol purple solution R* and 0.1 ml of 0.01 M *hydrochloric acid*. The solution is yellow. Add 0.4 ml of 0.01 M *sodium hydroxide*, the solution is violet-blue.

Specific optical rotation (2.2.7). Dissolve 1.25 g in 1 M *hydrochloric acid* and dilute to 50.0 ml with the same acid. Determined within 10 min and calculated with reference to the dried substance, the specific optical rotation is -45 to -49 .

Related substances. Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel F₂₅₄ plate R*.

Test solution. Dissolve 0.20 g of the substance to be examined in *dilute acetic acid R* with slight heating and dilute to 5 ml with the same acid.

Reference solution (a). Dilute 1 ml of the test solution to 100 ml with *water R*.

Reference solution (b). Dissolve 10 mg of *adenosine CRS* and 10 mg of *adenine CRS* in *dilute acetic acid R*, with heating if necessary, and dilute to 10 ml with the same acid.

Apply to the plate 5 µl of each solution. Develop over a path of 12 cm using a mixture of 10 volumes of *water R*, 30 volumes of *concentrated ammonia R* and 60 volumes of *propanol R*. Allow the plate to dry in a current of warm air and examine in ultraviolet light at 254 nm. Any spot in the chromatogram obtained with the test solution, apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (a) (1 per cent). Spray with a 5 g/l solution of *potassium permanganate R* in 1 M *sodium hydroxide*. Allow the plate to dry in a current of warm air and examine in daylight. Any spot in the chromatogram obtained with the test solution, apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (a) (1 per cent). The test is not valid unless the chromatogram obtained with reference solution (b) shows two clearly separated spots.

Chlorides (2.4.4). Dilute 10 ml of solution S to 15 ml with *water R*. The solution complies with the limit test for chlorides (100 ppm).

Sulphates (2.4.13). 15 ml of solution S complies with the limit test for sulphates (200 ppm).

Ammonium (2.4.1). 0.5 g complies with limit test B for ammonium (10 ppm). Prepare the standard using 5 ml of *ammonium standard solution* (1 ppm NH_4) *R*.

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

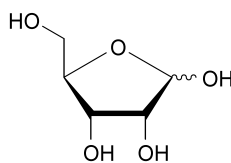
ASSAY

Dissolve 0.200 g, warming slightly if necessary, in a mixture of 20 ml of *acetic anhydride R* and 30 ml of *anhydrous acetic acid R*. Titrate with 0.1 M *perchloric acid*, determining the end-point potentiometrically (2.2.20).

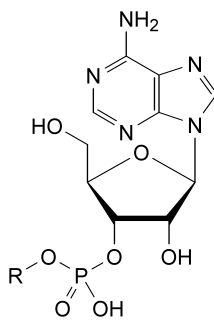
1 ml of 0.1 M *perchloric acid* is equivalent to 26.72 mg of $\text{C}_{10}\text{H}_{18}\text{N}_5\text{O}_4$.

IMPURITIES

A. adenine,



B. D-ribose,



C. R = H: adenosine 3'-(dihydrogen phosphate),

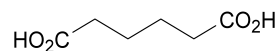
D. R = PO_3H_2 : adenosine 3'-(trihydrogen diphosphate),

E. R = $\text{PO}_2\text{H-O-PO}_3\text{H}_2$: adenosine 3'-(tetrahydrogen triphosphate).

01/2005:1586

ADIPIC ACID

Acidum adipicum



$\text{C}_6\text{H}_{10}\text{O}_4$

M_r 146.1

DEFINITION

Hexanedioic acid.

Content: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

Appearance: white, crystalline powder.

Solubility: sparingly soluble in water, soluble in boiling water, freely soluble in alcohol and in methanol, soluble in acetone.

IDENTIFICATION

A. Melting point (2.2.14): 151 °C to 154 °C.

B. Infrared absorption spectrophotometry (2.2.24).

Comparison: *adipic acid CRS*.

TESTS

Solution S. Dissolve 5.0 g with heating in *distilled water R* and dilute to 50 ml with the same solvent. Allow to cool and to crystallise. Filter through a sintered-glass filter (40). Wash the filter with *distilled water R*. Collect the filtrate and the washings until a volume of 50 ml is obtained.

Appearance of solution. The solution is clear (2.2.1) and colourless (2.2.2, *Method II*).

Dissolve 1.0 g in *methanol R* and dilute to 20 ml with the same solvent.

Related substances. Liquid chromatography (2.2.29).

Test solution. Dissolve 0.20 g of the substance to be examined in the mobile phase and dilute to 10.0 ml with the mobile phase.

Reference solution (a). Dissolve 20 mg of *glutaric acid R* in 1.0 ml of the test solution and dilute to 10.0 ml with the mobile phase.

Reference solution (b). Dilute 1.0 ml of the test solution to 100.0 ml with the mobile phase, dilute 1.0 ml of the solution to 10.0 ml with the mobile phase.

Column:

- **size:** $l = 0.125$ m, $\varnothing = 4.0$ mm,
- **stationary phase:** spherical *octadecylsilyl silica gel for chromatography R* (5 μ m) with a specific surface area of 350 m²/g and a pore size of 10 nm,
- **temperature:** 30 °C.

Mobile phase: mix 3 volumes of *acetonitrile R* and 97 volumes of a 24.5 g/l solution of *dilute phosphoric acid R*.

Flow rate: 1 ml/min.

Detection: spectrophotometer at 209 nm.

Injection: 20 μ l.

Run time: 3 times the retention time of adipic acid.

System suitability: reference solution (a):

- **resolution:** minimum 9.0 between the peaks due to glutaric acid and adipic acid.

Limits:

- **any impurity:** not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent),
- **total:** not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent),
- **disregard limit:** 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

Chlorides (2.4.4): maximum 200 ppm.

2.5 ml of solution S diluted to 15 ml with *water R* complies with the limit test for chlorides.

Nitrates: maximum 30 ppm.

To 1 ml of solution S add 2 ml of *concentrated ammonia R*, 0.5 ml of a 10 g/l solution of *manganese sulphate R*, 1 ml of a 10 g/l solution of *sulfanilamide R* and dilute to 20 ml with *water R*. Add 0.10 g of *zinc powder R* and cool in iced water for 30 min; shake from time to time. Filter and cool 10 ml of the filtrate in iced water. Add 2.5 ml of *hydrochloric acid R1* and 1 ml of a 10 g/l solution of *naphthylethylenediamine dihydrochloride R*. Allow to stand at room temperature. After 15 min the mixture is not more intensely coloured than a standard prepared at the same time and in the same manner, using 1.5 ml of *nitrate standard solution* (2 ppm NO₃) *R* instead of 1 ml of solution S. The test is invalid if a blank solution prepared at the same time and in the same manner, using 1 ml of *water R* instead of 1 ml of solution S, is more intensely coloured than a 2 mg/l solution of *potassium permanganate R*.

Sulphates (2.4.13): maximum 500 ppm.

3 ml of solution S diluted to 15 ml with *distilled water R* complies with the limit test for sulphates.

Iron (2.4.9): maximum 10 ppm.

10 ml of solution S complies with the limit test for iron.

Heavy metals (2.4.8): maximum 10 ppm.

12 ml of solution S complies with limit test A. Prepare the standard using *lead standard solution* (1 ppm Pb) *R*.

Loss on drying (2.2.32): maximum 0.2 per cent, determined on 1.000 g by drying in an oven at 100–105 °C.

Sulphated ash (2.4.14): maximum 0.1 per cent.

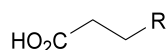
Melt 1.0 g completely over a gas burner, then ignite the melted substance with the burner. After ignition, lower or remove the flame in order to prevent the substance from boiling and keep it burning until completely carbonised. Carry out the test for sulphated ash using the residue.

ASSAY

Dissolve 60.0 mg in 50 ml of *water R*. Add 0.2 ml of *phenolphthalein solution R* and titrate with 0.1 M *sodium hydroxide*.

1 ml of 0.1 M *sodium hydroxide* is equivalent to 7.31 mg of C₆H₁₀O₄.

IMPURITIES



A. R = CH₂-CO₂H: pentanedioic acid (glutaric acid),

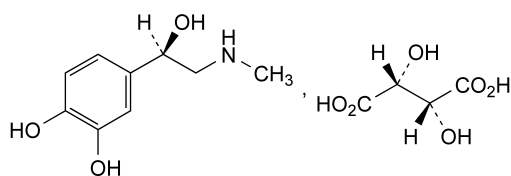
B. R = CO₂H: butanedioic acid (succinic acid),

C. R = [CH₂]₃-CO₂H: heptanedioic acid (pimelic acid).

01/2005:0254

ADRENALINE TARTRATE

Adrenalini tartras



C₁₃H₁₉NO₉

*M*_r 333.3

DEFINITION

Adrenaline tartrate contains not less than 98.5 per cent and not more than the equivalent of 101.0 per cent of (1*R*)-1-(3,4-dihydroxyphenyl)-2-(methylamino)ethanol hydrogen (2*R*,3*R*)-2,3-dihydroxybutanedioate, calculated with reference to the dried substance.

CHARACTERS

A white or greyish-white, crystalline powder, freely soluble in water, slightly soluble in ethanol (96 per cent).

IDENTIFICATION

- A. Dissolve 2 g in 20 ml of a 5 g/l solution of *sodium metabisulphite R* and make alkaline by addition of *ammonia R*. Keep the mixture in iced water for 1 h and filter. Reserve the filtrate for identification test C. Wash the precipitate with 3 quantities, each of 2 ml, of *water R*, with 5 ml of *ethanol* (96 per cent) *R* and finally with 5 ml of *ether R*. Dry *in vacuo* for 3 h. The specific optical rotation (2.2.7) of the precipitate (adrenaline base) is –50 to –54, determined using a 20.0 g/l solution in 0.5 M *hydrochloric acid*.

B. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with adrenaline base prepared by the same method from a suitable amount of *adrenaline tartrate CRS*. Use adrenaline base prepared as described under identification test A. Examine the substances prepared as discs.

C. 0.2 ml of the filtrate obtained in identification test A gives reaction (b) of tartrates (2.3.1).

TESTS

Appearance of solution. Dissolve 0.5 g in *water R* and dilute to 10 ml with the same solvent. Examine the solution immediately. The solution is not more opalescent than reference suspension II (2.2.1) and is not more intensely coloured than reference solution BY₅ (2.2.2, *Method II*).

Adrenalone. Dissolve 50.0 mg in 0.01 M *hydrochloric acid* and dilute to 25.0 ml with the same acid. The absorbance (2.2.25) of the solution measured at 310 nm is not greater than 0.10.

Noradrenaline. Examine by thin-layer chromatography (2.2.27), using *silica gel G R* as the coating substance.

Test solution. Dissolve 0.25 g of the substance to be examined in *water R* and dilute to 10 ml with the same solvent. Prepare immediately before use.

Reference solution (a). Dissolve 12.5 mg of *noradrenaline tartrate CRS* in *water R* and dilute to 10 ml with the same solvent. Prepare immediately before use.

Reference solution (b). Dilute 2 ml of reference solution (a) to 10 ml with *water R*.

Reference solution (c). Mix 2 ml of the test solution with 2 ml of reference solution (b).

Apply separately to the plate as bands 20 mm by 2 mm 6 µl of the test solution, 6 µl of reference solution (a), 6 µl of reference solution (b) and 12 µl of reference solution (c). Allow to dry and spray the bands with a saturated solution of *sodium bicarbonate R*. Allow the plate to dry in air and spray the bands twice with *acetic anhydride R*, drying between the 2 sprayings. Heat the plate at 50 °C for 90 min. Develop over a path of 15 cm using a mixture of 0.5 volumes of *anhydrous formic acid R*, 50 volumes of *acetone R* and 50 volumes of *methylethyl chloride R*. Allow the plate to dry in air and spray with a solution freshly prepared by mixing 2 volumes of *ethylenediamine R* and 8 volumes of *methanol R* and adding 2 volumes of a 5 g/l solution of *potassium ferricyanide R*. Dry the plate at 60 °C for 10 min and examine in ultraviolet light at 254 nm and 365 nm. In the chromatogram obtained with the test solution, any zone situated between the 2 most intense zones is not more intense than the corresponding zone in the chromatogram obtained with reference solution (b) (1.0 per cent). The test is not valid unless the chromatogram obtained with reference solution (c) shows between the 2 most intense zones a clearly separated zone corresponding to the most intense zone in the chromatogram obtained with reference solution (a).

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.00 g by drying *in vacuo* for 18 h.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.300 g in 50 ml of *anhydrous acetic acid R*, heating gently if necessary. Titrate with 0.1 M *perchloric acid* until a bluish-green colour is obtained using 0.1 ml of *crystal violet solution R* as indicator.

1 ml of 0.1 M *perchloric acid* is equivalent to 33.33 mg of C₁₃H₁₉NO₉.

STORAGE

In an airtight container, or preferably in a sealed tube under vacuum or under an inert gas, protected from light.

01/2005:0310

AGAR

Agar

DEFINITION

Agar consists of the polysaccharides from various species of Rhodophyceae mainly belonging to the genus *Gelidium*. It is prepared by treating the algae with boiling water; the extract is filtered whilst hot, concentrated and dried.

CHARACTERS

Agar has a mucilaginous taste.

It occurs in the form of a powder or in crumpled strips 2 mm to 5 mm wide or sometimes in flakes, colourless to pale yellow, translucent, somewhat tough and difficult to break, becoming more brittle on drying.

It has the microscopic characters described under identification test A.

IDENTIFICATION

- Examine under a microscope. When mounted in 0.005 M *iodine*, the strips or flakes are partly stained brownish-violet. Magnified 100 times, they show numerous minute, colourless, ovoid or rounded grains on an amorphous background; occasional brown, round to ovoid spores with a reticulated surface, measuring up to 60 µm, may be present. Reduce to a powder, if necessary. The powder is yellowish-white. Examine under a microscope using 0.005 M *iodine*. The powder presents angular fragments with numerous grains similar to those seen in the strips and flakes; some of the fragments are stained brownish-violet.
- Dissolve 0.1 g with heating in 50 ml of *water R*. Cool. To 1 ml of the mucilage carefully add 3 ml of *water R* so as to form two separate layers. Add 0.1 ml of 0.05 M *iodine*. A dark brownish-violet colour appears at the interface. Mix. The liquid becomes pale yellow.
- Heat 5 ml of the mucilage prepared for identification test B on a water-bath with 0.5 ml of *hydrochloric acid R* for 30 min. Add 1 ml of *barium chloride solution R1*. A white turbidity develops within 30 min.
- Heat 0.5 g with 50 ml of *water R* on a water-bath until dissolved. Only a few fragments remain insoluble. During cooling, the solution gels between 35 °C and 30 °C. Heat the gel thus obtained on a water-bath; it does not liquefy below 80 °C.

TESTS

Swelling index (2.8.4). The swelling index, determined on the powdered drug (355), is not less than 10 and is within 10 per cent of the value stated on the label.

Insoluble matter. To 5.00 g of the powdered drug (355) add 100 ml of *water R* and 14 ml of *dilute hydrochloric acid R*. Boil gently for 15 min with frequent stirring. Filter the hot liquid through a tared, sintered-glass filter (160), rinse the filter with hot *water R* and dry at 100 °C to 105 °C. The residue weighs not more than 50 mg (1.0 per cent).

Gelatin. To 1.00 g add 100 ml of *water R* and heat on a water-bath until dissolved. Allow to cool to 50 °C. To 5 ml of the solution add 5 ml of *picric acid solution R*. No turbidity appears within 10 min.

Loss on drying (2.2.32). Not more than 20.0 per cent, determined on 1.000 g of the powdered drug (355) by drying in an oven at 100 °C to 105 °C.

Total ash (2.4.16). Not more than 5.0 per cent.

Microbial contamination. Total viable aerobic count (2.6.12) not more than 10³ micro-organisms per gram, determined by plate-count. It complies with the tests for *Escherichia coli* and *Salmonella* (2.6.13).

LABELLING

The label states the swelling index.

01/2005:1587

AGRIMONY

Agrimoniae herba

DEFINITION

Dried flowering tops of *Agrimonia eupatoria* L.

Content: minimum 2.0 per cent of tannins, expressed as pyrogallol (C₆H₆O₃; *M_r* 126.1) (dried drug).

CHARACTERS

Macroscopic and microscopic characters described under identification tests A and B.

IDENTIFICATION

- A. The stem is green or, more usually, reddish, cylindrical and infrequently branched. It is covered with long, erect or tangled hairs. The leaves are compound imparipennate with 3 or 6 opposite pairs of leaflets, with 2 or 3 smaller leaflets between. The leaflets are deeply dentate to serrate, dark green on the upper surface, greyish and densely tomentose on the lower face. The flowers are small and form a terminal spike. They are pentamerous and borne in the axils of hairy bracts, the calyces closely surrounded by numerous terminal hooked spires, which occur on the rim of the hairy receptacle. The petals are free, yellow and deciduous. Fruit-bearing obconical receptacles, with deep furrows and hooked bristles, are usually present at the base of the inflorescence.
- B. Reduce to a powder (355). The powder is yellowish-green to grey. Examine under a microscope using *chloral hydrate solution R*. The powder shows numerous straight of bent, unicellular, long thick-walled (about 500 µm) trichomes finely warty and sometimes spirally marked; fragments of parenchyma with prisms and cluster crystals of calcium oxalate; fragments of leaf epidermis with sinuous walls, those of the lower epidermis with abundant stomata, mostly anomocytic but occasionally anisocytic; ovoid to subspherical-pollen grains, with 3 pores and a smooth exine; fragments of glandular trichomes with a multicellular uniseriate stalk and a spherical unicellular or quadricellular head; groups of fibres and spiral and banded-fitted vessels from the stem.
- C. Thin-layer chromatography (2.2.27).
Test solution. To 2.0 g of the powdered drug (355) add 20 ml of *methanol R*. Heat with shaking at 40 °C for 10 min. Filter.
Reference solution. Dissolve 1.0 mg of *rutin R* and 1.0 mg of *isoquercitroside R* in 2 ml of *methanol R*.

Plate: TLC silica gel plate *R*.

Mobile phase: anhydrous formic acid *R*, water *R*, ethyl acetate *R* (10:10:80 V/V/V).

Application: 10 µl, as bands.

Development: over a path of 12 cm.

Drying: at 100-105 °C.

Detection: spray the still warm plate with a 10 g/l solution of *diphenylboric acid aminoethyl ester R* in *methanol R* and then with a 50 g/l solution of *macrogol 400 R* in *methanol R*. Allow the plate to dry in air for 30 min. Examine in ultraviolet light at 365 nm

Results: see below the sequence of the zones present in the chromatograms obtained with the reference and test solutions.

Top of the plate	
Isoquercitroside: an orange fluorescent zone	An orange fluorescent zone may be present (quercitroside)
	An orange fluorescent zone (isoquercitroside)
Rutin: an orange fluorescent zone	An orange fluorescent zone (hyperoside)
	An orange fluorescent zone (rutin)
Reference solution	Test solution

TESTS

Foreign matter (2.8.2): it complies with the test for foreign matter.

Loss on drying (2.2.32): maximum 10.0 per cent, determined on 1.000 g of the powdered drug (355) by drying in an oven at 100-105 °C for 2 h.

Total ash (2.4.16): maximum 10.0 per cent.

ASSAY

Carry out the determination of tannins in herbal drugs (2.8.14). Use a 1.000 g of powdered drug (180).

01/2005:1238
corrected

AIR, MEDICINAL

Aer medicinalis

DEFINITION

Compressed ambient air.

Content: 20.4 per cent V/V to 21.4 per cent V/V of oxygen (O₂).

CHARACTERS

A colourless, odourless gas.

Solubility: at a temperature of 20 °C and a pressure of 101 kPa, 1 volume dissolves in about 50 volumes of water.

PRODUCTION

Carbon dioxide: maximum 500 ppm V/V, determined using an infrared analyser (2.5.24).

Gas to be examined. Use the substance to be examined. It must be filtered to avoid stray light phenomena.

Reference gas (a). Use a mixture of 79 per cent V/V of *nitrogen R1* and 21 per cent V/V of *oxygen R* containing less than 1 ppm V/V of *carbon dioxide R1*.

Reference gas (b). Use a mixture of 79 per cent V/V of *nitrogen R1* and 21 per cent V/V of *oxygen R* containing 500 ppm V/V of *carbon dioxide R1*.

Calibrate the apparatus and set the sensitivity using reference gases (a) and (b). Measure the content of carbon dioxide in the gas to be examined.

Carbon monoxide: maximum 5 ppm V/V, determined using an infrared analyser (2.5.25).

Gas to be examined. Use the substance to be examined. It must be filtered to avoid stray light phenomena.

Reference gas (a). Use a mixture of 79 per cent V/V of nitrogen R1 and 21 per cent V/V of oxygen R containing less than 1 ppm V/V of carbon monoxide R.

Reference gas (b). Use a mixture of 79 per cent V/V of nitrogen R1 and 21 per cent V/V of oxygen R containing 5 ppm V/V of carbon monoxide R.

Calibrate the apparatus and set the sensitivity using reference gases (a) and (b). Measure the content of carbon monoxide in the gas to be examined.

Sulphur dioxide: maximum 1 ppm V/V, determined using an ultraviolet fluorescence analyser (Figure 1238-1).

The apparatus consists of the following:

- a system generating ultraviolet radiation with a wavelength of 210 nm, made up of an ultraviolet lamp, a collimator, and a selective filter; the beam is blocked periodically by a chopper rotating at high speeds;
- a reaction chamber, through which flows the gas to be examined;
- a system that detects radiation emitted at a wavelength of 350 nm, made up of a selective filter, a photomultiplier tube and an amplifier.

Gas to be examined. Use the substance to be examined. It must be filtered.

Reference gas (a). Use a mixture of 79 per cent V/V of nitrogen R1 and 21 per cent V/V of oxygen R.

Reference gas (b). Use a mixture of 79 per cent V/V of nitrogen R1 and 21 per cent V/V of oxygen R containing 0.5 ppm V/V to 2 ppm V/V of sulphur dioxide R1.

Calibrate the apparatus and set the sensitivity using reference gases (a) and (b). Measure the content of sulphur dioxide in the gas to be examined.

Oil: maximum 0.1 mg/m³ calculated for atmospheric pressure and at 0 °C, determined using a measuring system such as described in Figure 1238-2.

The apparatus consists of the following:

- an on-off valve (1),
- a three-way valve (2),
- an oil cone (3),
- a bypass line (4),
- a pressure-regulator (5),
- a flow-metering device (6).

All of the apparatus is cleaned beforehand, using trichlorotrifluoroethane R free from oil and grease.

Place a micro fibre-glass filter in the oil cone (3); this filter has the following characteristics: 100 per cent borosilicate glass without binder; resistant to heat treatment at 500 °C (to eliminate organic traces); 99.999 per cent retention efficiency for NaCl particles with a diameter of 0.6 µm. Close the on-off valve (1); the substance to be examined enters the bypass line (4) and purges the three-way valve (2), the pressure-regulator (5) and the flow-metering device (6). Close the inlet valve of the compression and filtration system: open the on-off valve (1) and set the three-way valve (2) to the position allowing passage between the oil

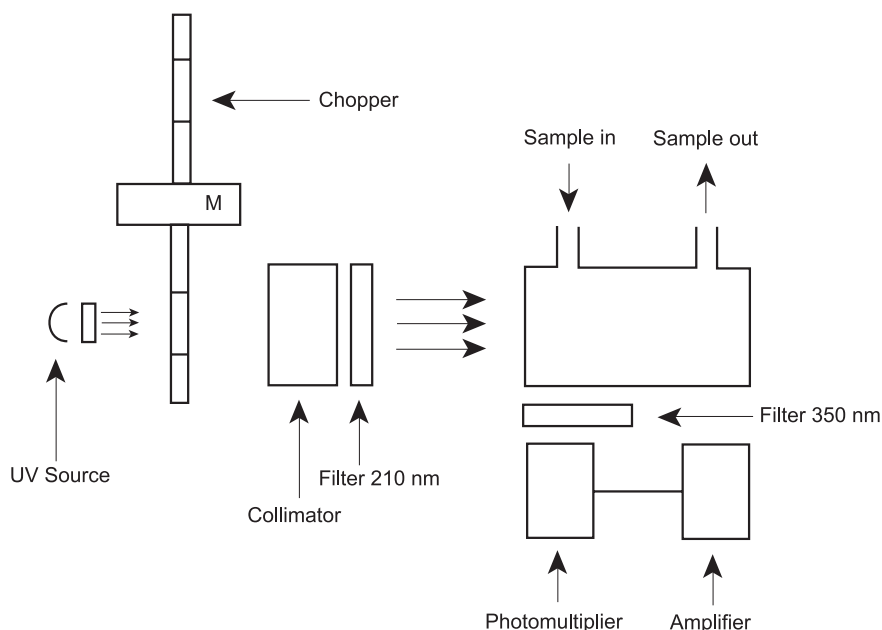


Figure 1238-1. – UV fluorescence analyser

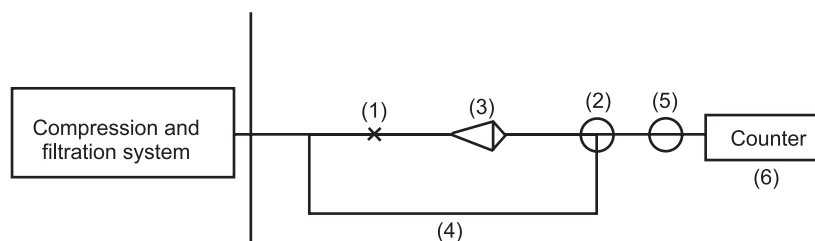


Figure 1238-2. – Measuring system for oil

cone and the pressure-regulator. Open the inlet valve and set the pressure-regulator (5) so that the flow indicated by the flow-metering device (6) is 20 litres/min. Pass 100.0 litres of the substance to be examined through the system.

Test solution. Remove the micro fibre-glass filter and place in an airtight container. Carefully cut up the micro fibre-glass filter and place the pieces in 25.0 ml of *trichlorotrifluoroethane R*.

Reference solutions. Prepare the reference solutions with quantities of oil (used for the lubrication of the compression system) ranging from 0.05 µg/ml to 0.5 µg/ml in *trichlorotrifluoroethane R*.

Measure the absorbance of the test solution and the reference solutions, using an appropriate infrared spectrophotometer, at 2960.3 cm⁻¹, 2927.7 cm⁻¹ and 2855.0 cm⁻¹. The sum of the 3 absorbances gives the absorbance of the oil. Use potassium bromide cells with a pathlength of several centimetres.

Plot the calibration curve from the absorbances obtained with the reference solutions and determine the quantity of oil from this curve.

Nitrogen monoxide and nitrogen dioxide: maximum 2 ppm V/V in total, determined using a chemiluminescence analyser (2.5.26).

Gas to be examined. Use the substance to be examined.

Reference gas (a). Use a mixture of 79 per cent V/V of *nitrogen R1* and 21 per cent V/V of *oxygen R* containing less than 0.05 ppm V/V of nitrogen monoxide and nitrogen dioxide.

Reference gas (b). Use a mixture of 2 ppm V/V of *nitrogen monoxide R* in *nitrogen R1*.

Calibrate the apparatus and set the sensitivity using reference gases (a) and (b). Measure the content of nitrogen monoxide and nitrogen dioxide in the gas to be examined.

Water: maximum 67 ppm V/V, determined using an electrolytic hygrometer (2.5.28), except where the competent authority decides that the following limit applies to medicinal air generated on-site and distributed in pipe-line systems operating at a pressure not greater than 10 bars and a temperature not less than 5 °C: maximum 870 ppm V/V, determined using an electrolytic hygrometer (2.5.28).

Assay. Determine the concentration of oxygen in air using a paramagnetic analyser (2.5.27).

IDENTIFICATION

First identification: C.

Second identification: A, B.

- A. In a conical flask containing the substance to be examined, place a glowing wood splinter. The splinter remains glowing.
- B. Use a gas burette (Figure 1238-3) of 25 ml capacity in the form of a chamber in the middle of which is a tube graduated in 0.2 per cent between 19.0 per cent and 23.0 per cent, and isolated at each end by a tap with a conical barrel. The lower tap is joined to a tube with an olive-shaped nozzle and is used to introduce the gas into the apparatus. A cylindrical funnel above the upper tap is used to introduce the absorbent solution. Wash the burette with *water R* and dry. Open the 2 taps. Connect the nozzle to the source of the substance to be examined and set the flow rate to 1 litre/min. Flush the burette by passing the substance to be examined through it for 1 min. Close the lower tap of the burette and immediately afterwards the upper tap. Rapidly disconnect the burette from the source of the substance to be examined. Rapidly give a half turn to the upper tap to eliminate any excess

pressure in the burette. Keeping the burette vertical, fill the funnel with a freshly prepared mixture of 21 ml of a 560 g/l solution of *potassium hydroxide R* and 130 ml of a 200 g/l solution of *sodium dithionite R*. Open the upper tap slowly. The solution absorbs the oxygen and enters the burette. Allow to stand for 10 min without shaking. Read the level of the liquid meniscus on the graduated part of the burette. This figure represents the percentage V/V of oxygen. The value read is 20.4 to 21.4.

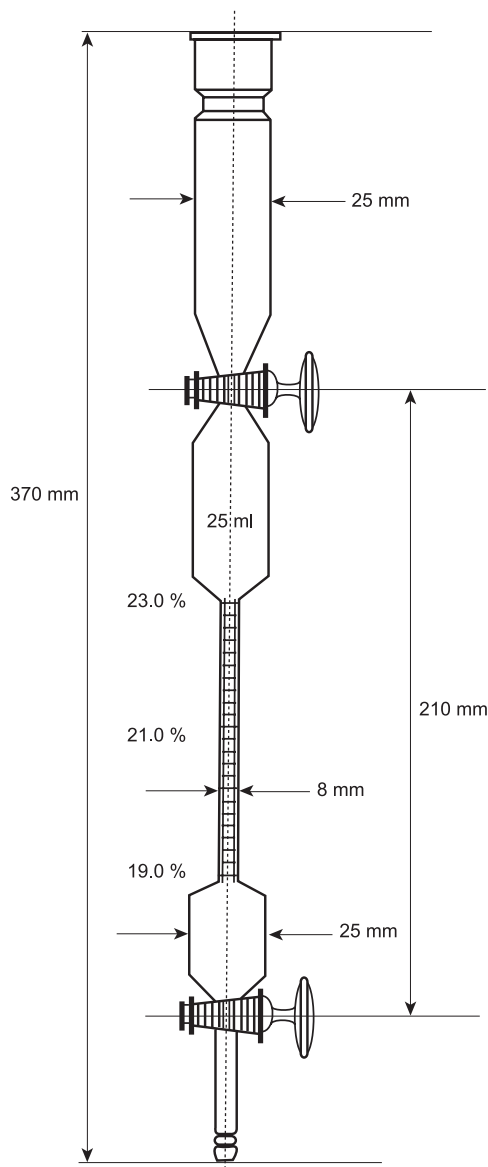


Figure 1238-3. – Gas burette

C. It complies with the limits of the assay.

TESTS

Carbon dioxide: maximum 500 ppm V/V, determined using a carbon dioxide detector tube (2.1.6).

Sulphur dioxide: maximum 1 ppm V/V, determined using a sulphur dioxide detector tube (2.1.6).

Oil: maximum 0.1 mg/m³, determined using an oil detector tube (2.1.6).

Nitrogen monoxide and nitrogen dioxide: maximum 2 ppm V/V, determined using a nitrogen monoxide and nitrogen dioxide detector tube (2.1.6).

Carbon monoxide: maximum 5 ppm V/V, determined using a carbon monoxide detector tube (2.1.6).

Water vapour: maximum 67 ppm V/V, determined using a water vapour detector tube (2.1.6), except where the competent authority decides that the following limit applies to medicinal air generated on-site and distributed in pipe-line systems operating at a pressure not greater than 10 bars and a temperature not less than 5 °C: maximum 870 ppm V/V, determined using a water vapour detector tube (2.1.6).

STORAGE

As a gas, in suitable containers complying with the legal regulations or as a gas supplied by a pipe network.

IMPURITIES

- A. carbon dioxide,
- B. sulphur dioxide,
- C. nitrogen monoxide,
- D. nitrogen dioxide,
- E. oil,
- F. carbon monoxide,
- G. water.

01/2005:1684

AIR, SYNTHETIC MEDICINAL

Aer medicinalis artificiosus

DEFINITION

Mixture of *Nitrogen* (1247) and *Oxygen* (0417).

Content: 95.0 per cent to 105.0 per cent of the nominal value which is between 21.0 per cent V/V to 22.5 per cent V/V of oxygen (O₂).

CHARACTERS

Colourless and odourless gas.

Solubility: at a temperature of 20 °C and a pressure of 101 kPa, 1 volume dissolves in about 50 volumes of water.

PRODUCTION

Water (2.5.28): maximum 67 ppm V/V.

Assay (2.5.27). Carry out the determination of oxygen in gases.

IDENTIFICATION

First identification: C.

Second identification: A, B.

- A. In a conical flask containing the substance to be examined, place a glowing splinter of wood. The splinter remains glowing.

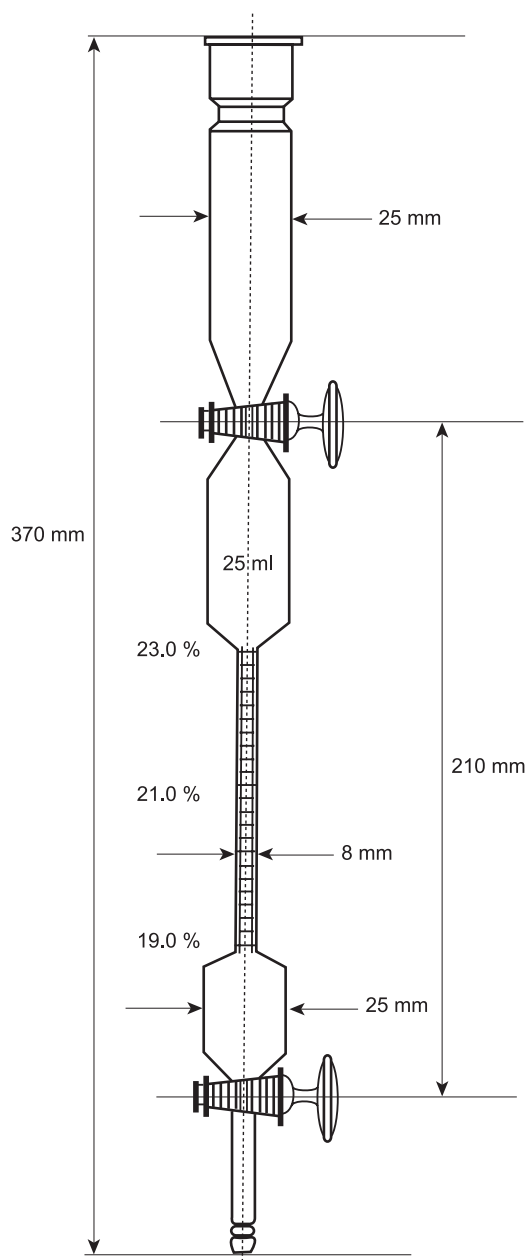


Figure 1684.-1. – Gas burette

- B. Use a gas burette (Figure 1684.-1) of 25 ml capacity in the form of a chamber, in the middle of which is a tube graduated in 0.2 per cent between 19.0 per cent and 23.0 per cent, and isolated at each end by a tap with a conical barrel. The lower tap is joined to a tube with an olive-shaped nozzle and is used to introduce the gas into the apparatus. A cylindrical funnel above the upper tap is used to introduce the absorbent solution. Wash the burette with *water R* and dry. Open both taps. Connect the nozzle to the source of the substance to be examined and set the flow rate to 1 litre/min. Flush the burette by passing the substance to be examined through it for 1 min. Close the lower tap of the burette and immediately afterwards the upper tap. Rapidly disconnect the burette from the source of the substance to be examined. Rapidly give a half turn of the upper tap to eliminate any excess pressure in the burette. Keeping the burette vertical, fill the funnel with a freshly prepared mixture of 21 ml of a 560 g/l solution of *potassium hydroxide R* and 130 ml of a 200 g/l solution of *sodium dithionite R*. Open the upper tap slowly. The solution absorbs the oxygen and enters the burette. Allow to stand for 10 min without

shaking. Read the level of the liquid meniscus on the graduated part of the burette. This figure represents the percentage *V/V* of oxygen. The value read is 95.0 per cent to 105.0 per cent of the nominal value.

C. It complies with the limits of the assay.

TESTS

Water vapour: maximum 67 ppm *V/V*, determined using a water vapour detector tube (2.1.6).

STORAGE

As a compressed gas in suitable containers complying with the legal regulations or as a compressed gas supplied by a pipe network, after mixing of the components.

LABELLING

The label states the nominal content of O₂ in per cent *V/V*.

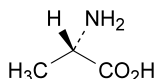
IMPURITIES

A. water.

01/2005:0752

ALANINE

Alaninum



C₃H₇NO₂

*M*_r 89.1

DEFINITION

Alanine contains not less than 98.5 per cent and not more than the equivalent of 101.0 per cent of (*S*)-2-aminopropanoic acid, calculated with reference to the dried substance.

CHARACTERS

White or almost white, crystalline powder or colourless crystals, freely soluble in water, very slightly soluble in alcohol.

IDENTIFICATION

First identification: A, B.

Second identification: A, C, D.

- It complies with the test for specific optical rotation (see Tests).
- Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *alanine CRS*. Examine the substances prepared as discs.
- Examine the chromatograms obtained in the test for ninhydrin-positive substances. The principal spot in the chromatogram obtained with test solution (b) is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).
- Dissolve 0.5 g in a mixture of 1 ml of *water R*, 0.5 ml of a 100 g/l solution of *sodium nitrite R* and 0.25 ml of *hydrochloric acid R1*. Shake. Gas is given off. Add 2 ml of *dilute sodium hydroxide solution R*, followed by 0.25 ml of *iodinated potassium iodide solution R*. After about 30 min, a yellow precipitate with a characteristic odour is formed.

TESTS

Solution S. Dissolve 2.5 g in *distilled water R* and dilute to 50 ml with the same solvent.

Appearance of solution. Dilute 10 ml of solution S to 20 ml with *water R*. The solution is clear (2.2.1) and not more intensely coloured than reference solution BY₆ (2.2.2, *Method II*).

Specific optical rotation (2.2.7). Dissolve 2.50 g in *hydrochloric acid R1* and dilute to 25.0 ml with the same acid. The specific optical rotation is + 13.5 to + 15.5, calculated with reference to the dried substance.

Ninhydrin-positive substances. Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel plate R*.

Test solution (a). Dissolve 0.10 g in *water R* and dilute to 10 ml with the same solvent.

Test solution (b). Dilute 1 ml of test solution (a) to 50 ml with *water R*.

Reference solution (a). Dissolve 10 mg of *alanine CRS* in *water R* and dilute to 50 ml with the same solvent.

Reference solution (b). Dilute 5 ml of test solution (b) to 20 ml with *water R*.

Reference solution (c). Dissolve 10 mg of *alanine CRS* and 10 mg of *glycine CRS* in *water R* and dilute to 25 ml with the same solvent.

Apply separately to the plate 5 µl of each solution. Allow the plate to dry in air. Develop over a path of 15 cm with a mixture of 20 volumes of *glacial acetic acid R*, 20 volumes of *water R* and 60 volumes of *butanol R*. Allow the plate to dry in air. Spray with *ninhydrin solution R*. Heat the plate at 100 °C to 105 °C for 15 min. Any spot in the chromatogram obtained with test solution (a), apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.5 per cent). The test is not valid unless the chromatogram obtained with reference solution (c) shows two clearly separated spots.

Chlorides (2.4.4). Dilute 5 ml of solution S to 15 ml with *water R*. The solution complies with the limit test for chlorides (200 ppm).

Sulphates (2.4.13). Dilute 10 ml of solution S to 15 ml with *distilled water R*. The solution complies with the limit test for sulphates (300 ppm).

Ammonium (2.4.1). 50 mg complies with limit test B for ammonium (200 ppm). Prepare the standard using 0.1 ml of *ammonium standard solution (100 ppm NH₄) R*.

Iron (2.4.9). In a separating funnel, dissolve 1.0 g in 10 ml of *dilute hydrochloric acid R*. Shake with three quantities, each of 10 ml, of *methyl isobutyl ketone R1*, shaking for 3 min each time. To the combined organic layers add 10 ml of *water R* and shake for 3 min. The aqueous layer complies with the limit test for iron (10 ppm).

Heavy metals (2.4.8). Dissolve 2.0 g in *water R* and dilute to 20 ml with the same solvent. 12 ml of the solution complies with limit test A for heavy metals (10 ppm). Prepare the standard using *lead standard solution (1 ppm Pb) R*.

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 80.0 mg in 3 ml of *anhydrous formic acid R*. Add 30 ml of *anhydrous acetic acid R*. Using 0.1 ml of *naphtholbenzein solution R* as indicator, titrate with 0.1 *M perchloric acid*, until the colour changes from brownish-yellow to green.

1 ml of 0.1 *M perchloric acid* is equivalent to 8.91 mg of C₃H₇NO₂.

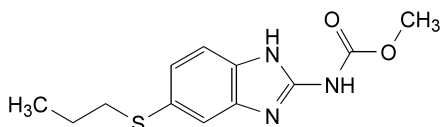
STORAGE

Store protected from light.

01/2005:1386

ALBENDAZOLE

Albendazolum


 $C_{12}H_{15}N_3O_2S$
 M_r 265.3

DEFINITION

Albendazole contains not less than 98.0 per cent and not more than the equivalent of 102.0 per cent of methyl [5-(propylsulphonyl)-1*H*-benzimidazol-2-yl]carbamate, calculated with reference to the dried substance.

CHARACTERS

A white or faintly yellowish powder, practically insoluble in water, freely soluble in anhydrous formic acid, very slightly soluble in methylene chloride, practically insoluble in alcohol.

IDENTIFICATION

Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *albendazole CRS*. Examine the substances prepared as discs.

TESTS

Appearance of solution. Dissolve 0.10 g in a mixture of 1 volume of *anhydrous formic acid R* and 9 volumes of *methylene chloride R* and dilute to 10 ml with the same mixture of solvents. The solution is clear (2.2.1) and not more intensely coloured than reference solution BY₆ (2.2.2, Method II).

Related substances. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 25.0 mg of the substance to be examined in 5 ml of *methanol R* containing 1 per cent V/V of *sulphuric acid R* and dilute to 50.0 ml with the mobile phase.

Reference solution (a). Dissolve 10.0 mg of the substance to be examined in 10 ml of *methanol R* containing 1 per cent V/V of *sulphuric acid R* and dilute to 100.0 ml with the mobile phase. Dilute 0.5 ml of this solution to 20.0 ml with the mobile phase.

Reference solution (b). Dissolve 50.0 mg of the substance to be examined and 50 mg of *oxybendazole CRS* in 5 ml of *methanol R* containing 1 per cent V/V of *sulphuric acid R* and dilute to 100.0 ml with the mobile phase.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4.6 mm in internal diameter packed with spherical *end-capped octadecylsilyl silica gel for chromatography R* (5 µm) with a pore size of 10 nm and a carbon loading of 19 per cent,
- as the mobile phase at a flow rate of 0.7 ml/min a mixture of 300 volumes of a 1.67 g/l solution of *ammonium dihydrogen phosphate R* and 700 volumes of *methanol R*,
- as detector a spectrophotometer set at 254 nm.

Inject 20 µl of reference solution (a). Adjust the sensitivity of the system so that the height of the principal peak in the chromatogram obtained is at least 50 per cent of the full scale of the recorder. Inject 20 µl of reference solution (b). The test is not valid unless the resolution between the peaks corresponding to albendazole and oxybendazole is at least 3.0.

Inject 20 µl of the test solution. Continue the chromatography for 1.5 times the retention time of albendazole. When the chromatograms are recorded in the prescribed conditions, the approximate relative retention times are: 0.80 for impurity A, 0.43 for impurities B and C, 0.40 for impurity D, 0.47 for impurity E and 0.57 for impurity F. In the chromatogram obtained with the test solution the area of any peak, apart from the principal peak, is not greater than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.75 per cent) and the sum of the areas of any such peaks is not greater than 3 times the area of the principal peak in the chromatogram obtained with reference solution (a) (1.5 per cent). Disregard any peak with an area less than 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (a).

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 100–105 °C for 4 h.

Sulphated ash (2.4.14). Not more than 0.2 per cent, determined on 1.0 g.

ASSAY

In order to avoid overheating during the titration, mix thoroughly throughout and stop the titration immediately after the end-point has been reached.

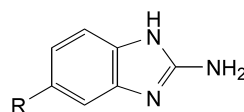
Dissolve 0.250 g in 3 ml of *anhydrous formic acid R* and add 40 ml of *anhydrous acetic acid R*. Titrate with 0.1 *M perchloric acid*, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 *M perchloric acid* is equivalent to 26.53 mg of $C_{12}H_{15}N_3O_2S$.

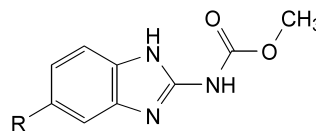
STORAGE

Store protected from light.

IMPURITIES



- A. R = S-CH₂-CH₂-CH₃: 5-(propylsulphonyl)-1*H*-benzimidazol-2-amine,
 D. R = SO₂-CH₂-CH₂-CH₃: 5-(propylsulphonyl)-1*H*-benzimidazol-2-amine,



- B. R = SO-CH₂-CH₂-CH₃: methyl [5-(propylsulphonyl)-1*H*-benzimidazol-2-yl]carbamate,
 C. R = SO₂-CH₂-CH₂-CH₃: methyl [5-(propylsulphonyl)-1*H*-benzimidazol-2-yl]carbamate,
 E. R = H: methyl (1*H*-benzimidazol-2-yl)carbamate,
 F. R = S-CH₃: methyl [5-(methylsulphonyl)-1*H*-benzimidazol-2-yl]carbamate.

01/2005:1387

ALCHEMILLA**Alchemillae herba****DEFINITION**

Alchemilla consists of the whole or cut, dried, flowering, aerial parts of *Alchemilla vulgaris* L. *sensu latiore*. It contains not less than 6.0 per cent of tannins, expressed as pyrogallol (C₆H₆O₃, *M_r* 126.1), calculated with reference to the dried drug.

CHARACTERS

It has the macroscopic and microscopic characters described under identification tests A and B.

IDENTIFICATION

A. The greyish-green, partly brownish-green, radical leaves which are the main part of the drug are reniform to slightly semicircular with a diameter generally up to 8 cm, seldom up to 11 cm and have 7 to 9, or 11 lobes and a long petiole. The smaller, cauline leaves, which have a pair of large stipules at the base, have 5 to 9 lobes and a shorter petiole or they are sessile. The leaves are densely pubescent especially on the lower surface and have a coarsely serrated margin. Young leaves are folded with a whitish-silvery pubescence; older leaves are slightly pubescent and have a finely meshed venation, prominent on the lower surface. The greyish-green to yellowish-green petiole is pubescent, about 1 mm in diameter, with an adaxial groove. The apetalous flowers are yellowish-green to light green and about 3 mm in diameter. The calyx is double with 4 small segments of the epicalyx alternating with 4 larger sepals, subacute to triangular. They are 4 short stamens and a single carpel with a capitate stigma. The greyish-green to yellowish-green stem is pubescent, more or less longitudinally wrinkled and hollow.

B. Reduce to a powder (355). The powder is greyish-green. Examine under a microscope, using *chloral hydrate solution R*. The powder shows unicellular, narrow trichomes up to 1 mm long partly tortuous, acuminate, and bluntly pointed at the apex, with thick lignified walls, somewhat enlarged and pitted at the base; fragments of leaves with 2 layers of palisade parenchyma, the upper layer of which is 2 to 3 times longer than the lower layer and with spongy parenchyma, containing scattered cluster crystals of calcium oxalate, up to 25 µm in diameter; leaf fragments in surface view with sinuous to wavy epidermal cells, the anticlinal walls unevenly thickened and beaded, anomocytic stomata (2.8.3); groups of vascular tissue and lignified fibres from the petioles and stems, the vessels spirally thickened or with bordered pits; occasional thin-walled conical trichomes, about 300 µm long; thin-walled parenchyma containing cluster crystals of calcium oxalate; spherical pollen grains, about 15 µm in diameter, with 3 distinct pores and a granular exine; occasional fragments of the ovary wall with cells containing a single crystal of calcium oxalate.

C. Thin-layer chromatography (2.2.27).

Test solution. To 0.5 g of the powdered drug (355) add 5 ml of *methanol R* and heat in a water-bath at 70 °C under a reflux condenser for 5 min. Cool and filter.

Reference solution. Dissolve 1.0 mg of *caffeic acid R* and 1.0 mg of *chlorogenic acid R* in 10 ml of *methanol R*.

Plate: TLC silica gel plate *R*.

Mobile phase: *anhydrous formic acid R*, *water R*, *ethyl acetate R* (8:8:84 V/V/V).

Application: 20 µl as bands for the test solution, 10 µl as bands for the reference solution.

Development: over a path of 10 cm.

Drying: at 100-105 °C for 5 min.

Detection: spray with a 10 g/l solution of *diphenylboric acid aminoethyl ester R* in *methanol R*. Subsequently spray with a 50 g/l solution of *macrogol 400 R* in *methanol R*. Allow the plate to dry in air for about 30 min. Examine in ultraviolet light at 365 nm.

Results: see below the sequence of the zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other fluorescent zones may be present in the chromatogram obtained with the test solution.

Top of the plate	
Caffeic acid: a light blue fluorescent zone Chlorogenic acid: a light blue fluorescent zone	Two red fluorescent zones (chlorophyll)
	One or two intense light blue fluorescent zones
	One or several intense green to greenish yellow fluorescent zone
	An intense yellow to orange fluorescent zone
Reference solution	Test solution

TESTS

Foreign matter (2.8.2): maximum 2 per cent.

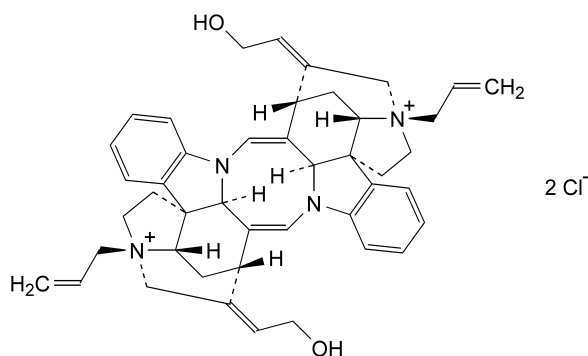
Loss on drying (2.2.32): maximum 10.0 per cent, determined on 1.000 g of powdered drug (355) by drying in an oven at 100-105 °C for 2 h.

Total ash (2.4.16): maximum 12.0 per cent.

ASSAY

Carry out the determination of tannins in herbal drugs (2.8.14). Use 0.50 g of the powdered drug (355).

01/2005:1285

ALCURONIUM CHLORIDE**Alcuronii chloridum**C₄₄H₅₀Cl₂N₄O₂*M_r* 738**DEFINITION**

Alcuronium chloride contains not less than 98.0 per cent and not more than the equivalent of 102.0 per cent of (1*R*,3*aS*,10*S*,11*aS*,12*R*,14*aS*,19*aS*,20*bS*,21*S*,22*aS*,23*E*,26*E*)-23,26-bis(2-hydroxyethylidene)-1,12-bis(prop-2-enyl)-2,3,11,11*a*,13,14,22,22*a*-octahydro-10*H*,21*H*-1,21:10,12-diethano-19*aH*,20*bH*-[1,5]diazocino[1,2,3-*lm*:5,6,7-

l'm [dipyrrolo[2',3'-d:2'',3''':d]dicarbazoledium dichloride (4,4'-didesmethyl-4,4'-bis(prop-2-enyl)toxi-ferin I dichloride), calculated with reference to the anhydrous, propan-2-ol-free substance.

CHARACTERS

A white or slightly greyish-white, crystalline powder, freely soluble in water and in methanol, soluble in alcohol, practically insoluble in cyclohexane.

Carry out the identification, tests and assay as rapidly as possible avoiding exposure to actinic light.

IDENTIFICATION

First identification: A, C.

Second identification: B, C.

- A. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *alcuronium chloride CRS*.
- B. Examine by thin-layer chromatography (2.2.27) using a *TLC silica gel plate R*.

Test solution. Dissolve 10 mg of the substance to be examined in *methanol R* and dilute to 10 ml with the same solvent.

Reference solution. Dissolve 10 mg of *alcuronium chloride CRS* in *methanol R* and dilute to 10 ml with the same solvent.

Apply to the plate 10 µl of each solution. Develop over a path of 15 cm using a mixture of 15 volumes of a 58.4 g/l solution of *sodium chloride R*, 35 volumes of *dilute ammonia R2* and 50 volumes of *methanol R*. Allow the plate to dry in air for 10 min and then spray with 0.1 M *ammonium and cerium nitrate*. The principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

- C. It gives reaction (a) of chlorides (2.3.1).

TESTS

Solution S. Dissolve 0.250 g in *carbon dioxide-free water R* and dilute to 25.0 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and not more intensely coloured than reference solution Y₆, BY₆ or B₆ (2.2.2, *Method I*).

Acidity or alkalinity. To 10 ml of solution S add 0.1 ml of *methyl red solution R* and 0.2 ml of 0.01 M *hydrochloric acid*. The solution is red. Add 0.4 ml of 0.01 M *sodium hydroxide*. The solution is yellow.

Specific optical rotation (2.2.7): –430 to –451, determined on solution S and calculated with reference to the anhydrous, propan-2-ol-free substance.

Propan-2-ol. Not more than 1.0 per cent (2.4.24, *System A*).

Related substances. Examine by liquid chromatography (2.2.29).

Solvent mixture. Mix 100 ml of *methanol R*, 200 ml of *acetonitrile R* and 200 ml of a 6.82 g/l solution of *potassium dihydrogen phosphate R*. Dissolve 1.09 g of *sodium laurylsulphonate for chromatography R* in the mixture and adjust the apparent pH to 8.0 with a 100 g/l solution of *sodium hydroxide R*.

Test solution. Dissolve 0.20 g of the substance to be examined in the solvent mixture and dilute to 100.0 ml with the solvent mixture.

Reference solution (a). Dilute 0.5 ml of the test solution to 100.0 ml with the solvent mixture.

Reference solution (b). Dilute 4.0 ml of reference solution (a) to 10.0 ml with the solvent mixture.

Reference solution (c). Dilute 1.0 ml of reference solution (a) to 10.0 ml with the solvent mixture.

Reference solution (d). To 5.0 ml of the test solution add 5.0 mg of *allylstrychnine bromide CRS*, dissolve in the solvent mixture and dilute to 100.0 ml with the solvent mixture.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4 mm in internal diameter packed with *octylsilyl silica gel for chromatography R* (5 µm),
- as mobile phase at a flow rate of 1.2 ml/min a solution prepared as follows: mix 200 ml of *methanol R*, 400 ml of *acetonitrile R* and 400 ml of a 6.82 g/l solution of *potassium dihydrogen phosphate R*. Dissolve 2.18 g of *sodium laurylsulphonate for chromatography R* in the mixture and adjust the apparent pH to 5.4 with a 100 g/l solution of *phosphoric acid R*,
- as detector a spectrophotometer set at 254 nm.

Inject 10 µl of reference solution (b). Adjust the sensitivity of the system so that the height of the peak due to alcuronium is at least 10 per cent of the full scale of the recorder.

Inject 10 µl of reference solution (d). The test is not valid unless the resolution between the peak corresponding to *N*-allylstrychnine and alcuronium is at least 4.0. Inject 10 µl of the test solution, 10 µl of reference solution (a) and 10 µl of reference solution (c). Continue the chromatography of the test solution for twice the retention time of the peak corresponding to alcuronium. In the chromatogram obtained with the test solution the area of any peak, apart from the principal peak, is not greater than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent) and not more than one such peak has an area greater than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent); the sum of the areas of all the peaks, apart from the principal peak, is not greater than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (1 per cent). Disregard any peak with an area less than the area of the principal peak in the chromatogram obtained with reference solution (c) (0.05 per cent).

Water (2.5.12). Not more than 5.0 per cent, determined on 0.500 g by the semi-micro determination of water.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

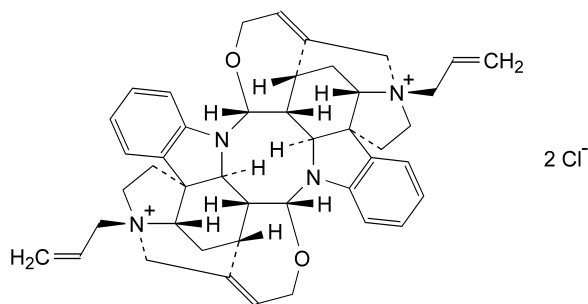
Dissolve 0.300 g by stirring in 70 ml of *acetic anhydride R* for 1 min. Titrate with 0.1 M *perchloric acid* until the colour changes from violet-blue to greenish-blue, using 0.1 ml of *crystal violet solution R* as indicator.

1 ml of 0.1 M *perchloric acid* is equivalent to 36.9 mg of C₄₄H₅₀Cl₂N₄O₂.

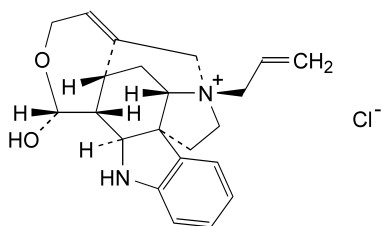
STORAGE

Store in an airtight container under nitrogen, protected from light, at a temperature of 2 °C to 8 °C.

IMPURITIES



- A. (1R,3aS,9R,9aR,10R,11aS,12R,14aS,19aS,20R,20aR,20bS,21R,22aS)-1,12-bis(prop-2-enyl)-2,3,9a,11,11a,13,14,19a,20a,21,22,22a-dodecahydro-10H,20bH-1,23:12,27-dimethano-9,10:20,21-bis(epoxyprop[2]eno)-9H,20H-[1,5]diazocino[1,2,3-lm:5,6,7-l'm']dipyrrolo[2',3'-d:2'',3''-d']dicarbazole-11,12-dichloride (4,4'-diallylcarcurin V dichloride),

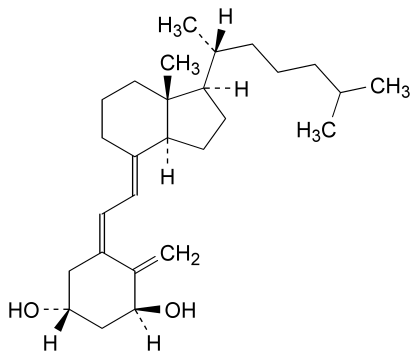


- B. (4bS,7R,7aS,8aR,13R,13aR,13bS)-13-hydroxy-7-(prop-2-enyl)-5,6,7a,8,8a,11,13,13a,13b,14-decahydro-7,9-methano-7H-oxepino[3,4-a]pyrrolo[2,3-d]carbazolium chloride ((4R,17R)-4-allyl-17,18-epoxy-17-hydroxy-19,20-didehydrocuranium chloride).

01/2005:1286

ALFACALCIDOL

Alfalcaldidolum

 $C_{27}H_{44}O_2$ M_r 400.6

DEFINITION

Alfalcaldidol contains not less than 97.0 per cent and not more than the equivalent of 102.0 per cent of (5Z,7E)-9,10-secocholesta-5,7,10(19)-triene-1 α ,3 β -diol.

CHARACTERS

White or almost white crystals, practically insoluble in water, freely soluble in alcohol, soluble in fatty oils. It is sensitive to air, heat and light.

A reversible isomerisation to pre-alfalcaldidol takes place in solution, depending on temperature and time. The activity is due to both compounds.

IDENTIFICATION

- A. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the *Ph. Eur. reference spectrum of alfalcaldidol*.
- B. Examine the chromatograms obtained in the assay. The principal peak in the chromatogram obtained with the test solution is similar in retention time and size to the principal peak in the chromatogram obtained with reference solution (a).

TESTS

Related substances. Examine by liquid chromatography (2.2.29) as described under Assay. Calculate the percentage content of related substances, apart from pre-alfalcaldidol, that are eluted within twice the retention time of alfalcaldidol from the areas of the peaks in the chromatogram obtained with the test solution by the normalisation procedure. The content of any individual related substance is not greater than 0.5 per cent and the sum of the related substances is not greater than 1.0 per cent. Disregard any peak below 0.1 per cent.

ASSAY

Carry out the assay as rapidly as possible, avoiding exposure to actinic light and air.

Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 1.0 mg of the substance to be examined without heating in 10.0 ml of the mobile phase.

Reference solution (a). Dissolve 1.0 mg of *alfalcaldidol CRS* without heating in 10.0 ml of the mobile phase.

Reference solution (b). Dilute reference solution (a) 100 times with the mobile phase.

Reference solution (c). Heat 2 ml of reference solution (a) in a water-bath at 80 °C under a reflux condenser for 2 h and cool.

The chromatographic procedure may be carried out using:

- a column 0.25 m long and 4.0 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R2* (5 μ m),
- as mobile phase at a flow rate of 2.0 ml/min a mixture of 1 volume of *ammonia R*, 200 volumes of *water R* and 800 volumes of *acetonitrile R*,
- as detector a spectrophotometer set at 265 nm,
- a loop injector.

Inject 100 μ l of reference solution (c) and record the chromatogram. Make a total of six injections. When the chromatograms are recorded in the prescribed conditions, the retention time for pre-alfalcaldidol, relative to alfalcaldidol, is about 1.3. The assay is not valid unless the relative standard deviation of the response for alfalcaldidol is at most 1 per cent and the resolution between the peaks due to pre-alfalcaldidol and alfalcaldidol is at least 4.0; adjust the proportions of the constituents of the mobile phase, if necessary, to obtain this resolution.

Inject 100 μ l of reference solution (a) and 100 μ l of reference solution (b) and record the chromatograms. Inject 100 μ l of the test solution and record the chromatogram in the same manner, continuing the chromatography for twice the retention time of the principal peak.

STORAGE

Store under nitrogen, in an airtight container, protected from light, at a temperature of 2 °C to 8 °C.

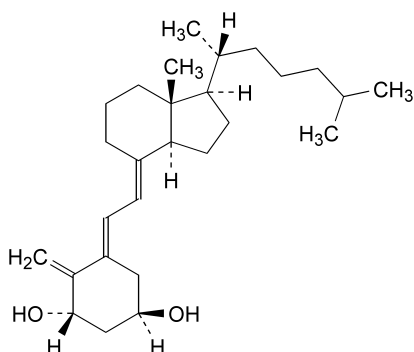
The contents of an opened container are to be used immediately.

IMPURITIES

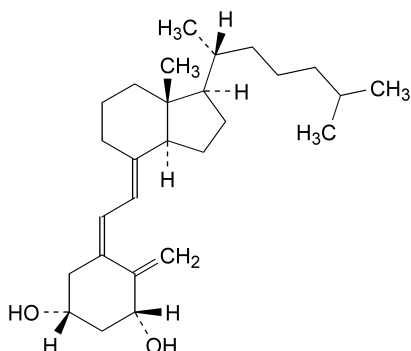
01/2005:1487

ALFADEX

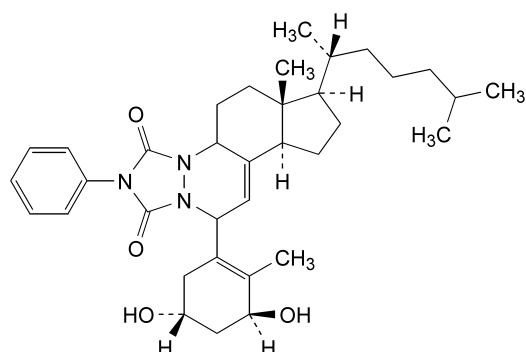
Alfadexum



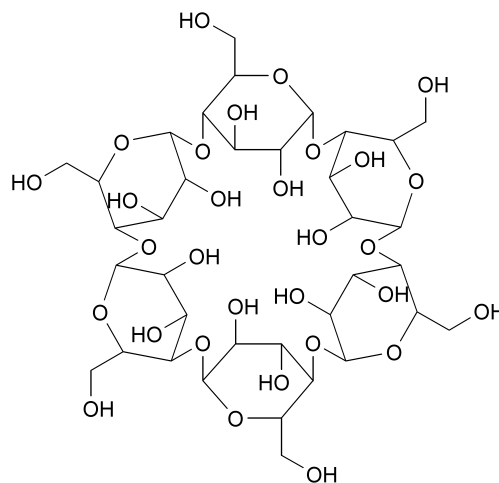
A. (5*E*,7*E*)-9,10-secocholesta-5,7,10(19)-triene-1 α ,3 β -diol (*trans*-alfacalcidol),



B. (5*Z*,7*E*)-9,10-secocholesta-5,7,10(19)-triene-1 β ,3 β -diol (1 β -calcidol),



C. triazoline adduct of pre-alfacalcidol.

[C₆H₁₀O₅]₆*M*_r 973

DEFINITION

Alfadex contains not less than 98.0 per cent and not more than the equivalent of 101.0 per cent of cyclohexakis-(1→4)-(α-D-glucopyranosyl) (cyclomaltohexaose or α-cyclodextrin), calculated with reference to the dried substance.

CHARACTERS

A white or almost white, amorphous or crystalline powder, freely soluble in water and in propylene glycol, practically insoluble in ethanol and in methylene chloride.

IDENTIFICATION

- It complies with the test for specific optical rotation (see Tests).
- Examine the chromatograms obtained in the assay. The retention time and size of the principal peak in the chromatogram obtained with test solution (b) are approximately the same as those of the principal peak in the chromatogram obtained with reference solution (c).
- Dissolve 0.2 g in 2 ml of *iodine solution R4* by warming on a water-bath, and allow to stand at room temperature; a yellowish-brown precipitate is formed.

TESTS

Solution S. Dissolve 1.000 g in *carbon dioxide-free water R* and dilute to 100.0 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1).

pH (2.2.3). The pH of a mixture of 30 ml of solution S and 1 ml of a 223.6 g/l solution of *potassium chloride R* is 5.0 to 8.0.

Specific optical rotation (2.2.7): + 147 to + 152, determined on solution S and calculated with reference to the dried substance.

Reducing sugars

Test solution. To 1 ml of solution S add 1 ml of *cupri-tartaric solution R4*. Heat on a water-bath for 10 min, cool to room temperature. Add 10 ml of *ammonium molybdate reagent R1* and allow to stand for 15 min.

Reference solution. Prepare a reference solution at the same time and in the same manner as the test solution, using 1 ml of a 0.02 g/l solution of *glucose R*.

Measure the absorbance of the test solution and the reference solution (2.2.25) at the maximum at 740 nm using *water R* as the compensation liquid. The absorbance of the test solution is not greater than that of the reference solution (0.2 per cent).

Light-absorbing impurities. Examine solution S between 230 nm and 750 nm (2.2.25). Between 230 nm and 350 nm, the absorbance is not greater than 0.10. Between 350 nm and 750 nm, the absorbance is not greater than 0.05.

Related substances. Examine by liquid chromatography (2.2.29) as described under Assay. Inject test solution (a) and reference solution (b). In the chromatogram obtained with test solution (a): the areas of any peaks corresponding to betadex or gammacyclodextrin are not greater than half of the area of the corresponding peaks in the chromatogram obtained with reference solution (b) (0.25 per cent); the sum of the areas of all the peaks, apart from the principal peak and any peaks corresponding to betadex or gammacyclodextrin, is not greater than half of the area of the peak corresponding to alfadex in the chromatogram obtained with reference solution (b) (0.5 per cent).

Heavy metals (2.4.8). 2.0 g complies with limit test C for heavy metals (10 ppm). Prepare the standard using 2 ml of *lead standard solution* (10 ppm Pb) *R*.

Loss on drying (2.2.32). Not more than 11 per cent, determined on 1.000 g by drying in an oven at 120 °C for 2 h.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Examine by liquid chromatography (2.2.29).

Test solution (a). Dissolve 0.25 g of the substance to be examined in *water R* with heating, cool and dilute to 25.0 ml with the same solvent.

Test solution (b). Dilute 5.0 ml of test solution (a) to 50.0 ml with *water R*.

Reference solution (a). Dissolve 25.0 mg of *betadex CRS*, 25.0 mg of *gammacyclodextrin CRS* and 50.0 mg of *alfadex CRS* in *water R* and dilute to 50.0 ml with the same solvent.

Reference solution (b). Dilute 5.0 ml of reference solution (a) to 50.0 ml with *water R*.

Reference solution (c). Dissolve 25.0 mg of *alfadex CRS* in *water R* and dilute to 25.0 ml with the same solvent.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4.6 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (10 µm),
- as mobile phase at a flow rate of 1.5 ml/min a mixture of 10 volumes of *methanol R* and 90 volumes of *water R*,
- as detector a differential refractometer,
- a 50 µl loop injector.

Equilibrate the column with the mobile phase at a flow rate of 1.5 ml/min for about 3 h. Inject reference solution (a) 5 times and record the chromatograms for 3.5 times the retention time of alfadex. Adjust the sensitivity of the system so that the height of the peak corresponding to gammacyclodextrin is 55 per cent to 75 per cent of the full scale of the recorder. The retention time of alfadex is about 10 min, the relative retention of gammacyclodextrin is about 0.7 and that of betadex is about 2.2. The test is not valid unless the resolution between the peaks corresponding to

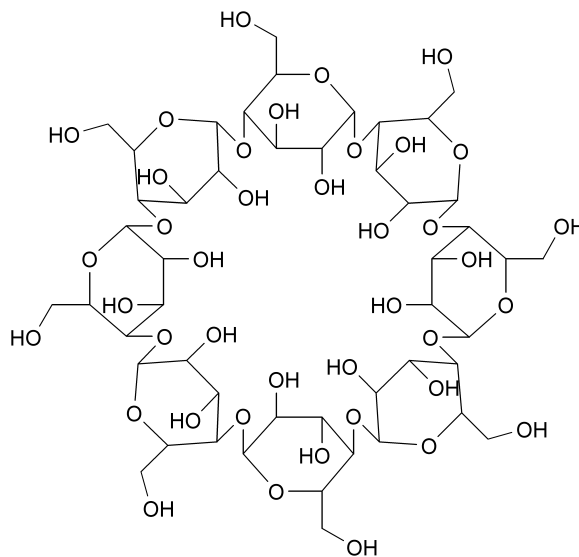
gammacyclodextrin and alfadex is at least 1.5 and the relative standard deviation for the area of the peak corresponding to alfadex is less than 2.0 per cent. If necessary, adjust the concentration of methanol in the mobile phase to achieve the required resolution. Inject alternately test solution (b) and reference solution (c). Calculate the percentage content of $[C_6H_{10}O_5]_6$ from the area of the principal peak in each of the chromatograms obtained and from the declared content of *alfadex CRS*.

STORAGE

Store in an airtight container.

IMPURITIES

A. betadex,

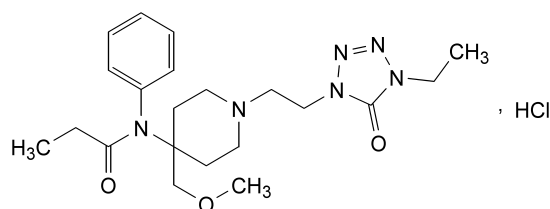


B. cyclooctakis-(1→4)-(α-D-glucopyranosyl) (cyclomaltooctaose or γ-cyclodextrin).

01/2005:1062

ALFENTANIL HYDROCHLORIDE

Alfentanili hydrochloridum



$C_{21}H_{33}ClN_6O_3$

M_r 453.0

DEFINITION

Alfentanil hydrochloride contains not less than 98.5 per cent and not more than the equivalent of 101.5 per cent of *N*-[1-[2-(4-ethyl-4,5-dihydro-5-oxo-1*H*-tetrazol-1-yl)ethyl]-4-(methoxymethyl)piperidin-4-yl]-*N*-phenylpropanamide hydrochloride, calculated with reference to the anhydrous substance.

CHARACTERS

A white or almost white powder, freely soluble in water, in alcohol and in methanol.

It melts at about 140 °C, with decomposition.

IDENTIFICATION

- A. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the *Ph. Eur. reference spectrum of alfentanil hydrochloride*.
- B. Dissolve 50 mg in a mixture of 0.4 ml of *ammonia R* and 2 ml of *water R*. Mix, allow to stand for 5 min and filter. Acidify the filtrate with *dilute nitric acid R*. It gives reaction (a) of chlorides (2.3.1).

TESTS

Appearance of solution. Dissolve 0.2 g in *water R* and dilute to 20 ml with the same solvent. The solution is clear (2.2.1) and colourless (2.2.2, *Method II*).

Related substances. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 0.100 g of the substance to be examined in *methanol R* and dilute to 10.0 ml with the same solvent.

Reference solution (a). In order to prepare the *in situ* degradation compound (alfentanil impurity E), dissolve 10 mg of the substance to be examined in 10.0 ml of *dilute hydrochloric acid R*. Heat on a water-bath under a reflux condenser for 4 h. Neutralise with 10.0 ml of *dilute sodium hydroxide solution R*. Evaporate to dryness on a water-bath. Cool and take up the residue in 10 ml of *methanol R*. Filter.

Reference solution (b). Dilute 1.0 ml of the test solution to 100.0 ml with *methanol R*. Dilute 5.0 ml of this solution to 20.0 ml with *methanol R*.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.1 m long and 4.6 mm in internal diameter packed with *octadecylsilyl silica for chromatography R* (3 µm),
- as mobile phase at a flow rate of 1.5 ml/min, a gradient programme using the following conditions:
Mobile phase A. A 5 g/l solution of *ammonium carbonate R* in a mixture of 10 volumes of *tetrahydrofuran R* and 90 volumes of *water R*,
Mobile phase B. *Acetonitrile R*,

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)	Comment
0 - 15	90 → 40	10 → 60	linear gradient
15 - 20	40	60	isocratic elution
20 - 25	90	10	switch to initial composition
25 = 0	90	10	restart gradient

- as detector a spectrophotometer set at 220 nm.

Equilibrate the column for at least 30 min with *acetonitrile R* and then equilibrate at the initial eluent composition for at least 5 min. Adjust the sensitivity of the system so that the height of the principal peak in the chromatogram obtained with 10 µl of reference solution (b) is at least 50 per cent of the full scale of the recorder.

Inject 10 µl of reference solution (a). When the chromatograms are recorded in the prescribed conditions the retention times are: alfentanil impurity E about 6 min and alfentanil hydrochloride about 7 min. Disregard any other peak. The test is not valid unless the resolution between the peaks corresponding to alfentanil hydrochloride and alfentanil impurity E is at least 4.0. If necessary, adjust the concentration of acetonitrile in the mobile phase or adjust the time programme for the linear-gradient elution.

Inject 10 µl of *methanol R* as a blank, 10 µl of the test solution and 10 µl of reference solution (b). In the chromatogram obtained with the test solution: the area of any peak,

apart from the principal peak, is not greater than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.25 per cent); the sum of the areas of all such peaks is not greater than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent). Disregard any peak obtained with the blank-run, and any peak with an area less than 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

Water (2.5.12): 3.0 per cent to 4.0 per cent, determined on 0.500 g by the semi-micro determination of water.

ASSAY

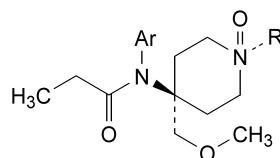
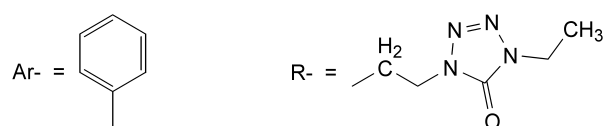
Dissolve 0.350 g in 50 ml of a mixture of 1 volume of *alcohol R* and 4 volumes of *water R* and add 5.0 ml of 0.01 *M hydrochloric acid*. Titrate with 0.1 *M sodium hydroxide*, determining the end-point potentiometrically (2.2.20). Read the volume added between the two points of inflexion.

1 ml of 0.1 *M sodium hydroxide* is equivalent to 45.30 mg of C₂₁H₃₃ClN₆O₃.

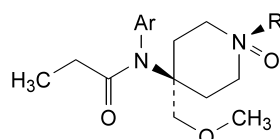
STORAGE

Store protected from light.

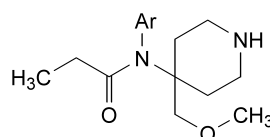
IMPURITIES



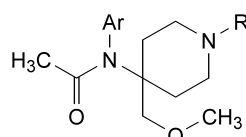
A. *cis*-N-[1-[2-(4-ethyl-4,5-dihydro-5-oxo-1H-tetrazol-1-yl)ethyl]-4-(methoxymethyl)piperidin-4-yl]-N-phenylpropanamide *N*-oxide,



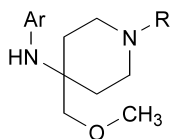
B. *trans*-N-[1-[2-(4-ethyl-4,5-dihydro-5-oxo-1H-tetrazol-1-yl)ethyl]-4-(methoxymethyl)piperidin-4-yl]-N-phenylpropanamide *N*-oxide,



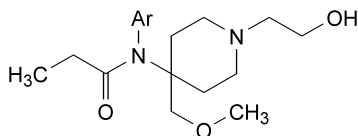
C. *N*-[4-(methoxymethyl)piperidin-4-yl]-*N*-phenylpropanamide,



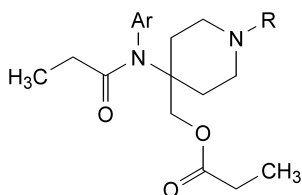
D. *N*-[1-[2-(4-ethyl-4,5-dihydro-5-oxo-1H-tetrazol-1-yl)ethyl]-4-(methoxymethyl)piperidin-4-yl]-*N*-phenylacetamide,



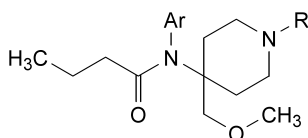
- E. 1-ethyl-1,4-dihydro-4-[2-[[4-(methoxymethyl)-4-phenylamino]piperidin-1-yl]ethyl]-5H-tetrazol-5-one,



- F. N-[1-(2-hydroxyethyl)-4-(methoxymethyl)piperidin-4-yl]-N-phenylpropanamide,



- G. N-[1-[2-(4-ethyl-4,5-dihydro-5-oxo-1H-tetrazol-1-yl)ethyl]-4-(propanoyloxymethyl)piperidin-4-yl]-N-phenylpropanamide,

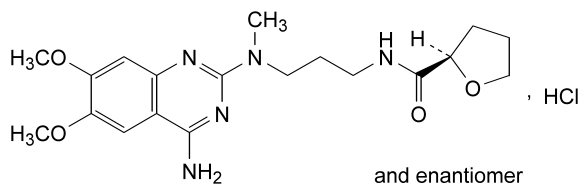


- H. N-[1-[2-(4-ethyl-4,5-dihydro-5-oxo-1H-tetrazol-1-yl)ethyl]-4-(methoxymethyl)piperidin-4-yl]-N-phenylbutanamide.

01/2005:1287

ALFUZOSIN HYDROCHLORIDE

Alfuzosini hydrochloridum

C₁₉H₂₈ClN₅O₄M_r 425.9

DEFINITION

Alfuzosin hydrochloride contains not less than 98.5 per cent and not more than the equivalent of 101.0 per cent of (*RS*)-N-[3-[(4-amino-6,7-dimethoxyquinazolin-2-yl)(methyl)amino]propyl]tetrahydrofuran-2-carboxamide hydrochloride, calculated with reference to the anhydrous substance.

CHARACTERS

A white or almost white, crystalline powder, slightly hygroscopic, freely soluble in water, sparingly soluble in alcohol, practically insoluble in methylene chloride.

IDENTIFICATION

- A. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *alfuzosin hydrochloride CRS*. Examine the substances prepared as discs.

- B. To 1 ml of solution S (see Tests) add 1 ml of *water R*. The solution gives reaction (a) of chlorides (2.3.1).

TESTS

Solution S. Dissolve 0.500 g in *carbon dioxide-free water R* and dilute to 25.0 ml with the same solvent.

pH (2.2.3). The pH of freshly prepared solution S is 4.0 to 6.0.

Optical rotation (2.2.7). The angle of optical rotation, determined on solution S, is -0.10° to $+0.10^\circ$.

Related substances. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 20.0 mg of the substance to be examined in the mobile phase and dilute to 100.0 ml with the mobile phase.

Reference solution (a). Dilute 1.0 ml of the test solution to 50.0 ml with the mobile phase. Dilute 5.0 ml of the solution to 20.0 ml with the mobile phase.

Reference solution (b). Dissolve 5 mg of *alfuzosin impurity A CRS* in the mobile phase and dilute to 25 ml with the mobile phase. To 1 ml of the solution, add 1 ml of the test solution and dilute to 100 ml with the mobile phase.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.15 m long and 4.6 mm in internal diameter packed with microparticulate *octadecylsilyl silica gel for chromatography R* (5 µm), with a carbon loading of 18.5 per cent, a specific surface area of 320 m²/g, a pore size of 15 nm, and end-capped with hexamethyldisilane,
- as mobile phase at a flow rate of 1.5 ml/min a mixture of 1 volume of *tetrahydrofuran R*, 20 volumes of *acetonitrile R* and 80 volumes of a solution of sodium perchlorate prepared as follows: dilute 5.0 ml of *perchloric acid R* in 900 ml of *water R*, adjust to pH 3.5 with *dilute sodium hydroxide solution R* and dilute to 1000 ml with *water R*,
- as detector a spectrophotometer set at 254 nm.

Inject 20 µl of reference solution (b). Adjust the sensitivity of the system so that the height of the two peaks in the chromatogram obtained is at least 50 per cent of the full scale of the recorder. The test is not valid unless the resolution between the peaks corresponding to alfuzosin and alfuzosin impurity A is at least 3.0. Inject 20 µl of the test solution and 20 µl of reference solution (a). In the chromatogram obtained with the test solution: the area of any peak, apart from the principal peak, is not greater than 0.6 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.3 per cent); the sum of the areas of all the peaks, apart from the principal peak, is not greater than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent). Disregard any peak with an area less than 0.025 times that of the principal peak in the chromatogram obtained with reference solution (a).

Water (2.5.12). Not more than 2.0 per cent, determined on 0.500 g by the semi-micro determination of water.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

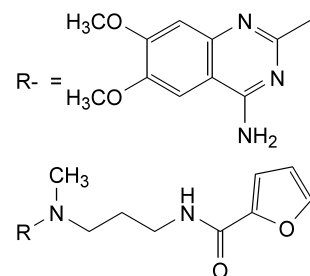
Dissolve 0.300 g in a mixture of 40 ml of *anhydrous acetic acid R* and 40 ml of *acetic anhydride R*. Titrate with 0.1 M *perchloric acid*, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *perchloric acid* is equivalent to 42.59 mg of C₁₉H₂₈ClN₅O₄.

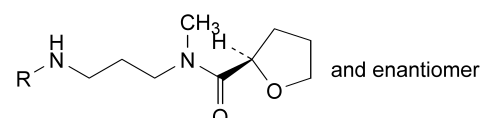
STORAGE

Store in an airtight container, protected from light.

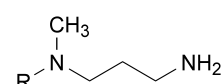
IMPURITIES



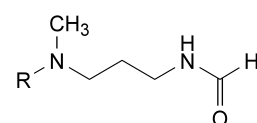
- A. *N*-[3-[(4-amino-6,7-dimethoxyquinazolin-2-yl)(methyl)amino]propyl]furan-2-carboxamide,
 B. R-Cl: 2-chloro-6,7-dimethoxyquinazolin-4-amine,



- C. (*RS*)-*N*-[3-[(4-amino-6,7-dimethoxyquinazolin-2-yl)amino]propyl]-*N*-methyltetrahydrofuran-2-carboxamide,



- D. *N*-(4-amino-6,7-dimethoxyquinazolin-2-yl)-*N*-methylpropane-1,3-diamine,



- E. *N*-[3-[(4-amino-6,7-dimethoxyquinazolin-2-yl)(methyl)amino]propyl]formamide.

01/2005:0591

ALGINIC ACID

Acidum alginicum

DEFINITION

Alginic acid is a mixture of polyuronic acids [(C₆H₈O₆)_{*n*}] composed of residues of D-mannuronic and L-guluronic acid and is obtained mainly from algae belonging to the Phaeophyceae. A small proportion of the carboxyl groups may be neutralised. It contains not less than 19.0 per cent and not more than 25.0 per cent of carboxyl groups (COOH), calculated with reference to the dried substance.

CHARACTERS

A white or pale yellowish-brown, crystalline or amorphous powder, very slightly soluble or practically insoluble in alcohol, practically insoluble in organic solvents. It swells in water but does not dissolve; it dissolves in solutions of alkali hydroxides.

IDENTIFICATION

- A. To 0.2 g add 20 ml of *water R* and 0.5 ml of *sodium carbonate solution R*. Shake and filter. To 5 ml of the filtrate add 1 ml of *calcium chloride solution R*. A voluminous gelatinous mass is formed.

- B. To 5 ml of the filtrate obtained in identification test A add 0.5 ml of a 123 g/l solution of *magnesium sulphate R*. No voluminous gelatinous mass is formed.
 C. To 5 mg add 5 ml of *water R*, 1 ml of a freshly prepared 10 g/l solution of *1,3-dihydroxynaphthalene R* in *alcohol R* and 5 ml of *hydrochloric acid R*. Boil gently for 3 min, cool, add 5 ml of *water R*, and shake with 15 ml of *di-isopropyl ether R*. Carry out a blank test. The upper layer obtained with the substance to be examined exhibits a deeper bluish-red colour than that obtained with the blank.

TESTS

Chlorides. Not more than 1.0 per cent. To 2.50 g add 50 ml of *dilute nitric acid R*, shake for 1 h and dilute to 100.0 ml with *dilute nitric acid R*. Filter. To 50.0 ml of the filtrate add 10.0 ml of 0.1 *M silver nitrate* and 5 ml of *toluene R*. Titrate with 0.1 *M ammonium thiocyanate*, using 2 ml of *ferric ammonium sulphate solution R2* as indicator and shaking vigorously towards the end point.

1 ml of 0.1 *M silver nitrate* is equivalent to 3.545 mg of Cl.

Heavy metals (2.4.8). 1.0 g complies with the limit test F for heavy metals (20 ppm). Prepare the standard using 2 ml of *lead standard solution (10 ppm Pb) R*.

Loss on drying (2.2.32). Not more than 15.0 per cent, determined on 0.1000 g by drying in an oven at 100 °C to 105 °C for 4 h.

Sulphated ash (2.4.14). Not more than 8.0 per cent, determined on 0.100 g and calculated with reference to the dried substance.

Microbial contamination. Total viable aerobic count (2.6.12) not more than 10² micro-organisms per gram, determined by plate-count. It complies with the tests for *Escherichia coli* and *Salmonella* (2.6.13).

ASSAY

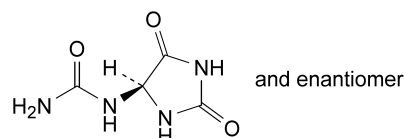
To 0.2500 g add 25 ml of *water R*, 25.0 ml of 0.1 *M sodium hydroxide* and 0.2 ml of *phenolphthalein solution R*. Titrate with 0.1 *M hydrochloric acid*.

1 ml of 0.1 *M sodium hydroxide* is equivalent to 4.502 mg of carboxyl groups (COOH).

01/2005:1288

ALLANTOIN

Allantoinum

C₄H₆N₄O₃M_r 158.1

DEFINITION

Allantoin contains not less than 98.5 per cent and not more than the equivalent of 101.0 per cent of (*RS*)-(2,5-dioxoimidazolidin-4-yl)urea.

CHARACTERS

A white, crystalline powder, slightly soluble in water, very slightly soluble in alcohol.

It melts at about 225 °C, with decomposition.

IDENTIFICATION

First identification: A.

Second identification: B, C, D.

- A. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *allantoin CRS*.
- B. Examine the chromatograms obtained in the test for related substances. The principal spot in the chromatogram obtained with test solution (b) is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).
- C. Boil 20 mg with a mixture of 1 ml of *dilute sodium hydroxide solution R* and 1 ml of *water R*. Allow to cool. Add 1 ml of *dilute hydrochloric acid R*. To 0.1 ml of the solution add 0.1 ml of a 100 g/l solution of *potassium bromide R*, 0.1 ml of a 20 g/l solution of *resorcinol R* and 3 ml of *sulphuric acid R*. Heat for 5 min to 10 min on a water-bath. A dark blue colour develops, which becomes red after cooling and pouring into about 10 ml of *water R*.
- D. Heat about 0.5 g. Ammonia vapour is evolved, which turns *red litmus paper R* blue.

TESTS

Solution S. Dissolve 0.5 g in *carbon dioxide-free water R*, with heating if necessary, and dilute to 100 ml with the same solvent.

Acidity or alkalinity. To 5 ml of solution S add 5 ml of *carbon dioxide-free water R*, 0.1 ml of *methyl red solution R* and 0.2 ml of 0.01 M *sodium hydroxide*. The solution is yellow. Add 0.4 ml of 0.01 M *hydrochloric acid*. The solution is red.

Optical rotation (2.2.7). The angle of optical rotation, determined on solution S, is -0.10° to $+0.10^\circ$.

Reducing substances. Shake 1.0 g with 10 ml of *water R* for 2 min. Filter. Add 1.5 ml of 0.02 M *potassium permanganate*. The solution must remain violet for at least 10 min.

Related substances. Examine by thin-layer chromatography (2.2.27), using a suitable *cellulose for chromatography R* as the coating substance.

Test solution (a). Dissolve 0.10 g of the substance to be examined in 5.0 ml of *water R* with heating. Allow to cool. Dilute to 10 ml with *methanol R*. Use the solution immediately after preparation.

Test solution (b). Dilute 1 ml of test solution (a) to 10 ml with a mixture of 1 volume of *methanol R* and 1 volume of *water R*.

Reference solution (a). Dissolve 10 mg of *allantoin CRS* in a mixture of 1 volume of *methanol R* and 1 volume of *water R* and dilute to 10 ml with the same mixture of solvents.

Reference solution (b). Dissolve 10 mg of *urea R* in 10 ml of *water R*. Dilute 1 ml of this solution to 10 ml with *methanol R*.

Reference solution (c). Mix 1 ml of reference solution (a) and 1 ml of reference solution (b).

Apply to the plate 10 µl of test solution (a) and 5 µl each of test solution (b), reference solution (a), reference solution (b) and reference solution (c). Develop over a path of 10 cm using a mixture of 15 volumes of *glacial acetic acid R*, 25 volumes of *water R* and 60 volumes of *butanol R*. Allow the plate to dry in air. Spray the plate with a 5 g/l solution of *dimethylaminobenzaldehyde R* in a mixture of 1 volume of *hydrochloric acid R* and 3 volumes of *methanol R*. Dry the plate in a current of hot air. Examine in daylight after 30 min. Any spot in the chromatogram obtained with test

solution (a), apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.5 per cent). The test is not valid unless the chromatogram obtained with reference solution (c) shows two clearly separated principal spots.

Loss on drying (2.2.32). Not more than 0.1 per cent, determined on 1.000 g by drying in an oven at 100–105 °C.

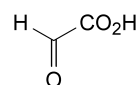
Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 120.0 mg in 40 ml of *water R*. Titrate with 0.1 M *sodium hydroxide*, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *sodium hydroxide* is equivalent to 15.81 mg of $C_5H_4N_4O$.

IMPURITIES



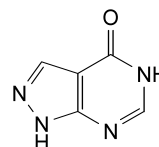
A. glyoxylic acid,

B. urea.

01/2005:0576

ALLOPURINOL

Allopurinolum


 $C_5H_4N_4O$
 M_r 136.1

DEFINITION

1,5-Dihydro-4H-pyrazolo[3,4-d]pyrimidin-4-one.

Content: 98.0 per cent to 102.0 per cent (dried substance).

CHARACTERS

Appearance: white or almost white powder.

Solubility: very slightly soluble in water and in alcohol. It dissolves in dilute solutions of alkali hydroxides.

IDENTIFICATION

First identification: B.

Second identification: A, C, D.

- A. Dissolve 10 mg in 1 ml of a 4 g/l solution of *sodium hydroxide R* and dilute to 100.0 ml with a 10.3 g/l solution of *hydrochloric acid R*. Dilute 10.0 ml of this solution to 100.0 ml with a 10.3 g/l solution of *hydrochloric acid R*. Examined between 220 nm and 350 nm (2.2.25), the solution shows an absorption maximum at 250 nm and an absorption minimum at 231 nm. The ratio of the absorbance measured at the absorption minimum at 231 nm to that measured at the absorption maximum at 250 nm is 0.52 to 0.62.
- B. Infrared absorption spectrophotometry (2.2.24).

Preparation: discs.

Comparison: *allopurinol CRS*.

C. Dissolve 0.3 g in 2.5 ml of *dilute sodium hydroxide solution R* and add 50 ml of *water R*. Add slowly and with shaking 5 ml of *silver nitrate solution R1*. A white precipitate is formed which does not dissolve on the addition of 5 ml of *ammonia R*.

D. Thin-layer chromatography (2.2.27).

Test solution. Dissolve 20 mg of the substance to be examined in *concentrated ammonia R* and dilute to 10 ml with the same solvent.

Reference solution. Dissolve 20 mg of *allopurinol CRS* in *concentrated ammonia R* and dilute to 10 ml with the same solvent.

Plate: TLC silica gel F_{254} plate *R*.

Mobile phase: *ethanol R*, *methylene chloride R* (40:60 V/V).

Application: 10 µl.

Development: over 2/3 of the plate.

Drying: in air.

Detection: examine in ultraviolet light at 254 nm.

Results: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with the reference solution.

TESTS

Related substances. Liquid chromatography (2.2.29).

Prepare the solutions immediately before use. Store them and inject them at 8 °C using a cooled autosampler.

Test solution (a). Dissolve 25.0 mg of the substance to be examined in 2.5 ml of a 4 g/l solution of *sodium hydroxide R* and dilute immediately to 50.0 ml with the mobile phase.

Test solution (b). Dissolve 20.0 mg of the substance to be examined in 5.0 ml of a 4 g/l solution of *sodium hydroxide R* and dilute immediately to 250.0 ml with the mobile phase.

Reference solution (a). Dilute 2.0 ml of test solution (a) to 100.0 ml with the mobile phase. Dilute 5.0 ml of this solution to 100.0 ml with the mobile phase.

Reference solution (b). Dissolve 5.0 mg of *allopurinol impurity A CRS*, 5.0 mg of *allopurinol impurity B CRS* and 5.0 mg of *allopurinol impurity C CRS* in 5.0 ml of a 4 g/l solution of *sodium hydroxide R* and dilute immediately to 100.0 ml with the mobile phase. Dilute 1.0 ml of this solution to 100.0 ml with the mobile phase.

Reference solution (c). Dissolve 20.0 mg of *allopurinol CRS* in 5.0 ml of a 4 g/l solution of *sodium hydroxide R* and dilute immediately to 250.0 ml with the mobile phase.

Column:

- *size:* $l = 0.25$ m, $\varnothing = 4.6$ mm,
- *stationary phase:* octadecylsilyl silica gel for chromatography *R* (5 µm).

Mobile phase: 1.25 g/l solution of *potassium dihydrogen phosphate R*.

Flow rate: 1.4 ml/min.

Detection: spectrophotometer at 230 nm.

Injection: 20 µl of test solution (a) and reference solutions (a) and (b).

Run time: twice the retention time of allopurinol.

Elution order: impurity A, impurity B, impurity C, allopurinol.

Retention time: allopurinol = about 10 min.

System suitability: reference solution (b):

- *resolution:* minimum 1.1 between the peaks due to impurity B and impurity C.

Limits:

- *impurity A:* not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent),
- *impurity B:* not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent),
- *impurity C:* not more than the area of the corresponding peak in the chromatogram obtained with reference solution (b) (0.1 per cent),
- *any other impurity:* for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent),
- *sum of impurities other than A, B and C:* not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.3 per cent),
- *disregard limit:* 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

Impurities D and E. Liquid chromatography (2.2.29).

Prepare the solutions immediately before use. Store them and inject them at 8 °C using a cooled autosampler.

Solution A: 1.25 g/l solution of *potassium dihydrogen phosphate R*.

Test solution. Dissolve 50.0 mg of the substance to be examined in 5.0 ml of a 4 g/l solution of *sodium hydroxide R* and dilute immediately to 100.0 ml with solution A.

Reference solution. Dissolve 5.0 mg of *allopurinol impurity D CRS* and 5.0 mg of *allopurinol impurity E CRS* in 5.0 ml of a 4 g/l solution of *sodium hydroxide R* and dilute immediately to 100.0 ml with solution A. Dilute 1.0 ml of this solution to 100.0 ml with solution A.

Column:

- *size:* $l = 0.05$ m, $\varnothing = 4.6$ mm,
- *stationary phase:* base-deactivated octadecylsilyl silica gel for chromatography *R* (3 µm).

Mobile phase: *methanol R*, 1.25 g/l solution of *potassium dihydrogen phosphate R* (10:90 V/V).

Flow rate: 2 ml/min.

Detection: spectrophotometer at 230 nm.

Injection: 20 µl.

Run time: 1.5 times the retention time of impurity E.

Retention times: impurity D = about 3.6 min; impurity E = about 4.5 min.

System suitability: reference solution:

- *resolution:* minimum 2.0 between the peaks due to impurity D and impurity E.

Limits:

- *impurity D:* not more than the area of the corresponding peak in the chromatogram obtained with the reference solution (0.1 per cent),
- *impurity E:* not more than the area of the corresponding peak in the chromatogram obtained with the reference solution (0.1 per cent).

Heavy metals (2.4.8): maximum 20 ppm.

1.0 g complies with limit test C. Prepare the standard using 2 ml of *lead standard solution (10 ppm Pb) R*.

Loss on drying (2.2.32): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 100–105 °C.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

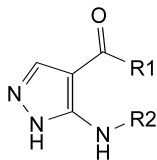
Liquid chromatography (2.2.29) as described in the test for related substances with the following modification.

Injection: test solution (b) and reference solution (c).

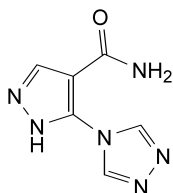
Calculate the percentage content of $C_5H_4N_4O$ from the areas of the peaks and the declared content of *allopurinol CRS*.

IMPURITIES

Specified impurities: A, B, C, D, E.



- A. R1 = NH₂, R2 = H: 5-amino-1*H*-pyrazole-4-carboxamide,
 B. R1 = NH₂, R2 = CHO: 5-(formylamino)-1*H*-pyrazole-4-carboxamide,
 D. R1 = O-CH₂-CH₃, R2 = H: ethyl 5-amino-1*H*-pyrazole-4-carboxylate,
 E. R1 = O-CH₂-CH₃, R2 = CHO: ethyl 5-(formylamino)-1*H*-pyrazole-4-carboxylate,



- C. 5-(4*H*-1,2,4-triazol-4-yl)-1*H*-pyrazole-4-carboxamide.

01/2005:2010
corrected

ALMAGATE

Almagatum

Al₂Mg₆C₂O₂₀H₁₄·4H₂O

M_r 630

DEFINITION

Hydrated aluminium magnesium hydroxycarbonate.

Content:

- aluminium: 15.0 per cent to 17.0 per cent (calculated as Al₂O₃),
- magnesium: 36.0 per cent to 40.0 per cent (calculated as MgO),
- carbonic acid: 12.5 per cent to 14.5 per cent (calculated as CO₂).

CHARACTERS

Appearance: white or almost white, fine crystalline powder.

Solubility: practically insoluble in water, in alcohol and in methylene chloride. It dissolves with effervescence and heating in dilute mineral acids.

IDENTIFICATION

- A. Infrared absorption spectrophotometry (2.2.24).

Comparison: *Ph. Eur. reference spectrum of almagate*.

- B. Dissolve 150 mg in *dilute hydrochloric acid R* and dilute to 20 ml with the same acid. 2 ml of the solution gives the reaction of aluminium (2.3.1).

- C. 2 ml of the solution prepared under identification test B gives the reaction of magnesium (2.3.1).

TESTS

pH (2.2.3): 9.1 to 9.7.

Disperse 4.0 g in 100 ml of *carbon dioxide-free water R*, stir for 2 min and filter.

Neutralising capacity. Carry out the test at 37 °C. Disperse 0.5 g in 100 ml of *water R*, heat, add 100.0 ml of 0.1 *M* *hydrochloric acid*, previously heated and stir continuously; the pH (2.2.3) of the solution between 5 min and 20 min is not less than 3.0 and not greater than 4.5. Add 10.0 ml of 0.5 *M* *hydrochloric acid*, previously heated, stir continuously for 1 h and titrate with 0.1 *M* *sodium hydroxide* to pH 3.5; not more than 20.0 ml of 0.1 *M* *sodium hydroxide* is required.

Chlorides (2.4.4): maximum 0.1 per cent.

Dissolve 0.33 g in 5 ml of *dilute nitric acid R* and dilute to 100 ml with *water R*. 15 ml of the solution complies with the limit test for chlorides. Prepare simultaneously the standard by diluting 0.7 ml of *dilute nitric acid R* to 5 ml with *water R* and adding 10 ml of *chloride standard solution* (5 ppm Cl) *R*.

Sulphates (2.4.13): maximum 0.4 per cent.

Dissolve 0.25 g in 5 ml of *dilute hydrochloric acid R* and dilute to 100 ml with *distilled water R*. 15 ml of the solution complies with the limit test for sulphates. Prepare simultaneously the standard by adding 0.8 ml of *dilute hydrochloric acid R* to 15 ml of *sulphate standard solution* (10 ppm SO₄) *R*.

Sodium: maximum 150 ppm.

Atomic absorption spectrometry (2.2.23, *Method I*).

Test solution. Dissolve 0.25 g in 50 ml of a 103 g/l solution of *hydrochloric acid R*.

Reference solutions. Prepare the reference solutions using *sodium standard solution* (200 ppm Na) *R*, diluted as necessary with a 103 g/l solution of *hydrochloric acid R*.

Heavy metals (2.4.8): maximum 20 ppm.

Dissolve 1.0 g in *dilute hydrochloric acid R* and dilute to 20.0 ml with the same acid. 12 ml of the solution complies with limit test A. Prepare the standard using *lead standard solution* (1 ppm Pb) *R*.

Loss on ignition: 43.0 per cent to 49.0 per cent, determined on 1.000 g by ignition at 900 °C.

Microbial contamination. Total viable aerobic count (2.6.12) not more than 10³ micro-organisms per gram determined by plate count. It complies with the tests for *Escherichia coli* and *Pseudomonas aeruginosa* (2.6.13).

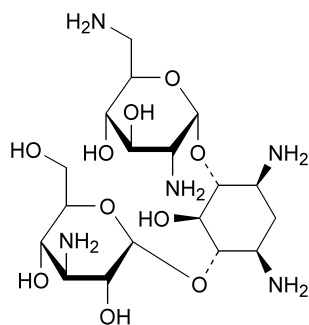
ASSAY

Aluminium. Dissolve 1.000 g in 5 ml of *hydrochloric acid R*, heating if necessary. Allow to cool to room temperature and dilute to 100.0 ml with *water R* (solution A). Introduce 10.0 ml of solution A into a 250 ml conical flask, add 25.0 ml of 0.05 *M* *sodium edetate*, 20 ml of *buffer solution pH 3.5 R*, 40 ml of *ethanol R* and 2 ml of a freshly prepared 0.25 g/l solution of *dithizone R* in *ethanol R*. Titrate the excess of sodium edetate with 0.05 *M* *zinc sulphate* until the colour changes from greenish-violet to pink.

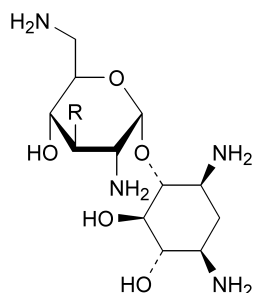
1 ml of 0.05 *M* *sodium edetate* is equivalent to 2.549 mg of Al₂O₃.

Magnesium. Introduce 10.0 ml of solution A prepared in the assay of aluminium into a 500 ml conical flask, add 200 ml of *water R*, 20 ml of *triethanolamine R* with shaking, 10 ml of

IMPURITIES

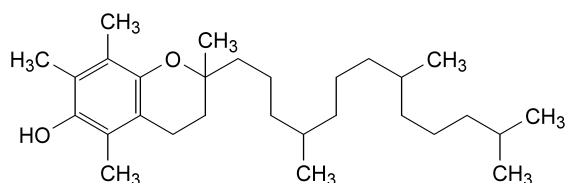


- A. 4-O-(3-amino-3-deoxy- α -D-glucopyranosyl)-2-deoxy-6-O-(2,6-diamino-2,6-dideoxy- α -D-glucopyranosyl)-L-streptamine (kanamycin B),



- B. R = H: 2-deoxy-4-O-(2,6-diamino-2,3,6-trideoxy- α -D-ribohexopyranosyl)-D-streptamine (nebramine),
C. R = OH: 2-deoxy-4-O-(2,6-diamino-2,6-dideoxy- α -D-glucopyranosyl)-D-streptamine (neamine).

01/2005:0692

all-*rac*- α -TOCOPHEROLint-*rac*- α -Tocopherolum $C_{29}H_{50}O_2$ M_r 430.7

DEFINITION

all-*rac*-2,5,7,8-Tetramethyl-2-(4,8,12-trimethyltridecyl)-3,4-dihydro-2*H*-1-benzopyran-6-ol.

Content: 96.0 per cent to 101.5 per cent.

CHARACTERS

Appearance: clear, colourless or yellowish-brown, viscous, oily liquid.

Solubility: practically insoluble in water, freely soluble in acetone, in ethanol, in methylene chloride and in fatty oils.

IDENTIFICATION

First identification: A, B.

Second identification: A, C.

- A. Optical rotation (2.2.7): -0.01° to $+0.01^\circ$.

Dissolve 2.50 g in *ethanol R* and dilute to 25.0 ml with the same solvent.

- B. Infrared absorption spectrophotometry (2.2.24).

Comparison: α -tocopherol CRS.

- C. Thin-layer chromatography (2.2.27).

Test solution. Dissolve 10 mg of the substance to be examined in 2 ml of *cyclohexane R*.

Reference solution. Dissolve 10 mg of α -tocopherol CRS in 2 ml of *cyclohexane R*.

Plate: TLC silica gel F_{254} plate *R*.

Mobile phase: *ether R*, *cyclohexane R* (20:80 V/V).

Application: 10 μ l.

Development: over 2/3 of the plate.

Drying: in a current of air.

Detection: examine in ultraviolet light at 254 nm.

Results: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with the reference solution.

TESTS

Related substances. Gas chromatography (2.2.28): use the normalisation procedure.

Internal standard solution. Dissolve 1.0 g of *squalane R* in *cyclohexane R* and dilute to 100.0 ml with the same solvent.

Test solution (a). Dissolve 0.100 g of the substance to be examined in 10.0 ml of the internal standard solution.

Test solution (b). Dissolve 0.100 g of the substance to be examined in 10 ml of *cyclohexane R*.

Reference solution (a). Dissolve 0.100 g of α -tocopherol CRS in 10.0 ml of the internal standard solution.

Reference solution (b). Dissolve 10 mg of the substance to be examined and 10 mg of α -tocopheryl acetate *R* in *cyclohexane R* and dilute to 100.0 ml with the same solvent.

Reference solution (c). Dissolve 10 mg of all-*rac*- α -tocopherol for peak identification CRS in *cyclohexane R* and dilute to 1 ml with the same solvent.

Reference solution (d). Dilute 1.0 ml of test solution (b) to 100.0 ml with *cyclohexane R*. Dilute 1.0 ml of this solution to 10.0 ml with *cyclohexane R*.

Column:

- **material:** fused silica,
- **size:** $l = 30$ m, $\varnothing = 0.25$ mm,
- **stationary phase:** poly(dimethyl)siloxane *R* (film thickness 0.25 μ m).

Carrier gas: helium for chromatography *R*.

Flow rate: 1 ml/min.

Split ratio: 1:100.

Temperature:

- **column:** 280 $^\circ$ C,
- **injection port and detector:** 290 $^\circ$ C.

Detection: flame ionisation.

Injection: 1 μ l of test solution (b) and reference solutions (b), (c) and (d).

Run time: twice the retention time of all-*rac*- α -tocopherol.

Relative retention with reference to all-*rac*- α -tocopherol (retention time = about 13 min): *squalane* = about 0.5; impurity A = about 0.7; impurity B = about 0.8. Use the chromatogram supplied with the CRS and the chromatogram obtained with reference solution (c) to identify the peaks due to impurities A and B.

System suitability: reference solution (b):

- **resolution:** minimum 3.5 between the peaks due to all-*rac*- α -tocopherol and α -tocopheryl acetate.

Limits:

- **impurity A:** maximum 0.5 per cent,

- *impurity B*: maximum 1.5 per cent,
- *any other impurity*: for each impurity, maximum 0.25 per cent,
- *total*: maximum 2.5 per cent,
- *disregard limit*: the area of the principal peak in the chromatogram obtained with reference solution (d) (0.1 per cent).

ASSAY

Gas chromatography (2.2.28) as described in the test for related substances with the following modification.

Injection: test solution (a) and reference solution (a).

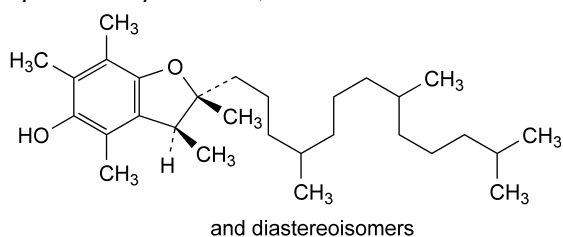
Calculate the percentage content of $C_{29}H_{50}O_2$ taking into account the declared content of α -tocopherol CRS.

STORAGE

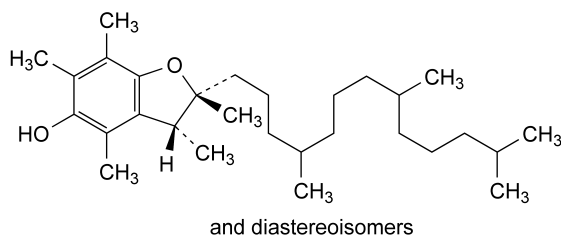
Under an inert gas, protected from light.

IMPURITIES

Specified impurities: A, B.

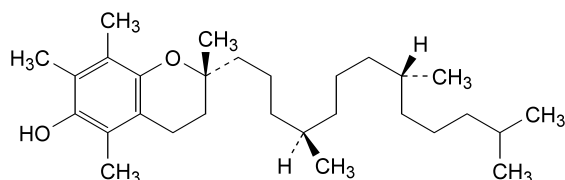


- A. all-*rac-trans*-2,3,4,6,7-pentamethyl-2-(4,8,12-trimethyltridecyl)-2,3-dihydrobenzofuran-5-ol,



- B. all-*rac-cis*-2,3,4,6,7-pentamethyl-2-(4,8,12-trimethyltridecyl)-2,3-dihydrobenzofuran-5-ol.

01/2005:1256

RRR- α -TOCOPHEROL**RRR- α -Tocopherolum** $C_{29}H_{50}O_2$ M_r 430.7**DEFINITION**

RRR- α -Tocopherol contains not less than 96.0 per cent and not more than the equivalent of 102.0 per cent of (2*R*)-2,5,7,8-tetramethyl-2-[(4*R*,8*R*)-4,8,12-trimethyltridecyl]-3,4-dihydro-2*H*-1-benzopyran-6-ol.

CHARACTERS

A clear, colourless or yellowish-brown, viscous, oily liquid, practically insoluble in water, freely soluble in acetone, in ethanol, in methylene chloride and in fatty oils.

IDENTIFICATION

First identification: B, D.

Second identification: A, C, D.

A. It complies with the test for absorbance (see Tests).

B. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with α -tocopherol CRS.

C. Examine by thin-layer chromatography (2.2.27), using a TLC silica gel F_{254} plate R.

Test solution. Dissolve 10 mg of the substance to be examined in 2 ml of cyclohexane R.

Reference solution. Dissolve 10 mg of α -tocopherol CRS in 2 ml of cyclohexane R.

Apply separately to the plate 10 μ l of each solution.

Develop over a path of 15 cm using a mixture of 20 volumes of ether R and 80 volumes of cyclohexane R. Dry the plate in a current of air and examine in ultraviolet light at 254 nm. The principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with the reference solution. Spray the plate with a mixture of 10 volumes of hydrochloric acid R, 40 volumes of a 2.5 g/l solution of ferric chloride R in alcohol R and 40 volumes of a 10 g/l solution of phenanthroline hydrochloride R in alcohol R. After 1 h to 2 h, the principal spots are orange.

D. RRR- α -tocopherol is dextrorotatory (2.2.7). The specific optical rotation after oxidation to the quinone form is not less than + 24.

Dissolve 1.0 g of the substance to be examined in 50 ml of ether R. To the solution add 20 ml of a 100 g/l solution of potassium ferricyanide R in an 8 g/l solution of sodium hydroxide R and shake for 3 min. Wash the ether solution with four quantities, each of 50 ml, of water R, discard the washings and dry the ether layer over anhydrous sodium sulphate R. Evaporate the ether on a water-bath under reduced pressure or in an atmosphere of nitrogen until a few millilitres remain, then complete the evaporation removing the last traces of ether without the application of heat. Immediately dissolve the residue in 5.0 ml of trimethylpentane R and determine the optical rotation.

Calculate the specific optical rotation of the substance in the test solution using as *c* the number of grams of RRR- α -tocopherol in 1000 ml.

TESTS

Absorbance (2.2.25). Dissolve 0.100 g in ethanol R and dilute to 100 ml with the same solvent (solution A). Dilute 10.0 ml of solution A to 100.0 ml with ethanol R (solution B). Measure the absorbance of solution B at the maximum at 292 nm and of solution A at the minimum at 255 nm. The specific absorbance at the maximum is 72.0 to 76.0 and that at the minimum is 5.5 to 8.0.

Acid value (2.5.1). Not more than 2.0, determined on 2.00 g.

Heavy metals (2.4.8). 0.50 g complies with limit test D for heavy metals (20 ppm). Prepare the standard using 1 ml of lead standard solution (10 ppm Pb) R.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g. Use sulphuric acid R instead of dilute sulphuric acid R.

ASSAY

Examine by gas chromatography (2.2.28), using dotriacontane R as the internal standard.

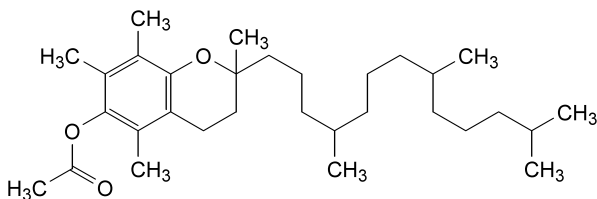
STORAGE

Store in an airtight, well-filled container, protected from light.

LABELLING

The label states the content of α -tocopherol acetate, expressed in grams per 100 g of concentrate.

01/2005:0439

all-*rac*- α -TOCOPHERYL ACETATEint-*rac*- α -Tocopherylis acetas $C_{31}H_{52}O_3$ M_r 472.7

DEFINITION

all-*rac*-2,5,7,8-Tetramethyl-2-(4,8,12-trimethyltridecyl)-3,4-dihydro-2*H*-1-benzopyran-6-yl acetate.

Content: 96.5 per cent to 101.0 per cent.

CHARACTERS

Appearance: clear, colourless or slightly greenish-yellow, viscous, oily liquid.

Solubility: practically insoluble in water, freely soluble in acetone, in ethanol and in fatty oils.

IDENTIFICATION

First identification: A, B.

Second identification: A, C.

A. Optical rotation (2.2.7): -0.01° to $+0.01^\circ$.

Dissolve 2.50 g in *ethanol R* and dilute to 25.0 ml with the same solvent.

B. Infrared absorption spectrophotometry (2.2.24).

Comparison: α -tocopheryl acetate CRS.

C. Thin-layer chromatography (2.2.27).

Test solution. Dissolve about 10 mg of the substance to be examined in 2 ml of *cyclohexane R*.

Reference solution. Dissolve about 10 mg of α -tocopheryl acetate CRS in 2 ml of *cyclohexane R*.

Plate: TLC silica gel F_{254} plate R.

Mobile phase: *ether R*, *cyclohexane R* (20:80 V/V).

Application: 10 μ l.

Development: over 2/3 of the plate.

Drying: in a current of air.

Detection: examine in ultraviolet light at 254 nm.

Results: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with the reference solution.

TESTS

Related substances. Gas chromatography (2.2.28): use the normalisation procedure.

Internal standard solution. Dissolve 1.0 g of *squalane R* in *cyclohexane R* and dilute to 100.0 ml with the same solvent.

Test solution (a). Dissolve 0.100 g of the substance to be examined in 10.0 ml of the internal standard solution.

Test solution (b). Dissolve 0.100 g of the substance to be examined in 10 ml of *cyclohexane R*.

Reference solution (a). Dissolve 0.100 g of α -tocopheryl acetate CRS in 10.0 ml of the internal standard solution.

Reference solution (b). Dissolve 10 mg of the substance to be examined and 10 mg of α -tocopherol R in *cyclohexane R* and dilute to 100.0 ml with the same solvent.

Reference solution (c). Dissolve 10 mg of all-*rac*- α -tocopheryl acetate for peak identification CRS in *cyclohexane R* and dilute to 1 ml with the same solvent.

Reference solution (d). Dilute 1.0 ml of test solution (b) to 100.0 ml with *cyclohexane R*. Dilute 1.0 ml of this solution to 10.0 ml with *cyclohexane R*.

Column:

- *material*: fused silica,
- *size*: $l = 30$ m, $\varnothing = 0.25$ mm,
- *stationary phase*: poly(dimethyl)siloxane R (film thickness 0.25 μ m).

Carrier gas: helium for chromatography R.

Flow rate: 1 ml/min.

Split ratio: 1:100.

Temperature:

- *column*: 280 $^\circ$ C,
- *injection port and detector*: 290 $^\circ$ C.

Detection: flame ionisation.

Injection: 1 μ l of test solution (b) and reference solutions (a), (b), (c) and (d).

Run time: twice the retention time of all-*rac*- α -tocopheryl acetate.

Relative retention with reference to all-*rac*- α -tocopheryl acetate (retention time = about 15 min): squalane = about 0.4; impurity A = about 0.7; impurity B = about 0.8; impurity C = about 0.9; use the chromatogram supplied with the CRS to identify the peaks due to impurities A and B in the chromatogram obtained with reference solution (c).

System suitability:

- *resolution*: minimum 3.5 between the peaks due to impurity C and all-*rac*- α -tocopheryl acetate in the chromatogram obtained with reference solution (b),
- in the chromatogram obtained with reference solution (a), the area of the peak due to impurity C is not greater than 0.2 per cent of the area of the peak due to all-*rac*- α -tocopheryl acetate.

Limits:

- *impurities A, C*: for each impurity, maximum 0.5 per cent,
- *impurity B*: maximum 1.5 per cent,
- *any other impurity*: for each impurity, maximum 0.25 per cent,
- *total*: maximum 2.5 per cent,
- *disregard limit*: the area of the principal peak in the chromatogram obtained with reference solution (d) (0.1 per cent).

ASSAY

Gas chromatography (2.2.28) as described in the test for related substances with the following modification.

Injection: test solution (a) and reference solution (a).

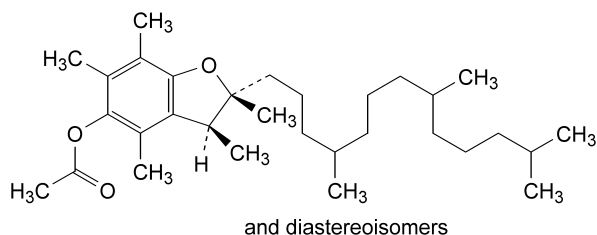
Calculate the percentage content of $C_{31}H_{52}O_3$ taking into account the declared content of α -tocopheryl acetate CRS.

STORAGE

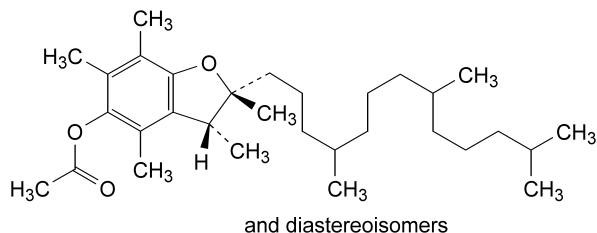
Protected from light.

IMPURITIES

Specified impurities: A, B, C.

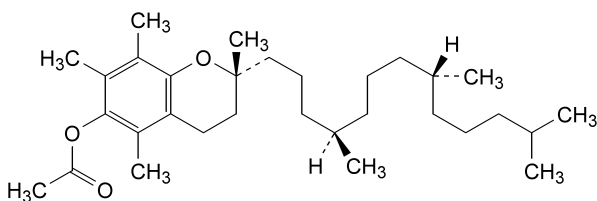


- A. all-*rac-trans*-2,3,4,6,7-pentamethyl-2-(4,8,12-trimethyltridecyl)-2,3-dihydrobenzofuran-5-yl acetate,



- B. all-*rac-cis*-2,3,4,6,7-pentamethyl-2-(4,8,12-trimethyltridecyl)-2,3-dihydrobenzofuran-5-yl acetate,
C. all-*rac*- α -tocopherol.

01/2005:1257

RRR- α -TOCOPHERYL ACETATERRR- α -Tocopherylis acetasC₃₁H₅₂O₃M_r 472.7

DEFINITION

RRR- α -Tocopheryl acetate contains not less than 96.0 per cent and not more than the equivalent of 102.0 per cent of (2*R*)-2,5,7,8-tetramethyl-2-[(4*R*,8*R*)-4,8,12-trimethyltridecyl]-3,4-dihydro-2*H*-1-benzopyran-6-yl acetate.

CHARACTERS

A clear, pale greenish-yellow, viscous, oily liquid, practically insoluble in water, freely soluble in acetone, in ethanol and in fatty oils, soluble in alcohol.

IDENTIFICATION

First identification: B, D.

Second identification: A, C, D.

- A. It complies with the test for absorbance (see Tests).
B. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with α -tocopheryl acetate CRS.
C. Examine by thin-layer chromatography (2.2.27), using a TLC silica gel F₂₅₄ plate *R*.

Test solution (a). Dissolve 10 mg of the substance to be examined in 2 ml of cyclohexane *R*.

Test solution (b). In a ground glass-stoppered tube, dissolve 10 mg of the substance to be examined in 2 ml of 2.5 *M* alcoholic sulphuric acid *R*. Heat on a water-bath for 5 min. Cool and add 2 ml of water *R* and 2 ml of cyclohexane *R*. Shake for 1 min. Use the upper layer.

Reference solution (a). Dissolve 10 mg of α -tocopheryl acetate CRS in 2 ml of cyclohexane *R*.

Reference solution (b). Prepare as described for test solution (b), using α -tocopheryl acetate CRS instead of the substance to be examined.

Apply separately to the plate 10 μ l of each solution. Develop over a path of 15 cm using a mixture of 20 volumes of ether *R* and 80 volumes of cyclohexane *R*. Dry the plate in a current of air and examine in ultraviolet light at 254 nm. The principal spot in the chromatogram obtained with test solution (a) is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a). In the chromatograms obtained with test solution (b) and reference solution (b), there are two spots: the spot with the higher *R_f* value is due to α -tocopheryl acetate and corresponds to the spot in the chromatogram obtained with reference solution (a); the spot with the lower *R_f* value is due to α -tocopherol. Spray the plate with a mixture of 10 volumes of hydrochloric acid *R*, 40 volumes of a 2.5 g/l solution of ferric chloride *R* in alcohol *R* and 40 volumes of a 10 g/l solution of phenanthroline hydrochloride *R* in alcohol *R*. In the chromatograms obtained with test solution (b) and reference solution (b), the spot due to α -tocopherol is orange.

- D. After saponification, the resulting RRR- α -tocopherol is dextrorotatory (2.2.7). The specific optical rotation after oxidation to the quinone form is at least + 24.

Carry out the test avoiding exposure to actinic light. Transfer 1.0 g of the substance to be examined to a 250 ml round-bottomed flask with a ground-glass stopper; dissolve in 30 ml of ethanol *R* and heat under reflux for 3 min. While the solution is boiling, add, through the condenser, 20 ml of 2 *M* alcoholic potassium hydroxide solution *R*. Continue heating under reflux for 20 min and, without cooling, add 4.0 ml of hydrochloric acid *R* dropwise through the condenser. Cool, rinse the condenser with 10 ml of ethanol *R*, transfer the contents of the flask to a 500 ml separating funnel. Rinse the flask with four quantities, each of 25 ml, of water *R* and four quantities, each of 25 ml, of ether *R*. Add the rinsings to the separating funnel. Shake vigorously for 2 min, allow the layers to separate and collect each of the two layers in individual separating funnels. Shake the aqueous layer with two quantities, each of 50 ml, of ether *R* and add these extracts to the main ether extract. Wash the combined ether extracts with four quantities, each of 100 ml, of water *R* and discard the washings.

To the ether solution add 40 ml of a 100 g/l solution of potassium ferricyanide *R* in an 8 g/l solution of sodium hydroxide *R* and shake for 3 min. Wash the ether solution with four quantities, each of 50 ml, of water *R*, discard the washings and dry the ether layer over anhydrous sodium sulphate *R*. Evaporate the ether on a water-bath under reduced pressure or in an atmosphere of nitrogen until a few millilitres remain, then complete the evaporation removing the last traces of ether without the application of heat. Immediately dissolve the residue in 25.0 ml of trimethylpentane *R* and determine the optical rotation.

ASSAY

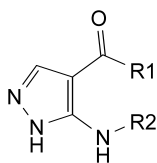
Liquid chromatography (2.2.29) as described in the test for related substances with the following modification.

Injection: test solution (b) and reference solution (c).

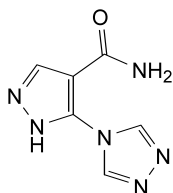
Calculate the percentage content of $C_5H_4N_4O$ from the areas of the peaks and the declared content of *allopurinol CRS*.

IMPURITIES

Specified impurities: A, B, C, D, E.



- A. R1 = NH₂, R2 = H: 5-amino-1*H*-pyrazole-4-carboxamide,
 B. R1 = NH₂, R2 = CHO: 5-(formylamino)-1*H*-pyrazole-4-carboxamide,
 D. R1 = O-CH₂-CH₃, R2 = H: ethyl 5-amino-1*H*-pyrazole-4-carboxylate,
 E. R1 = O-CH₂-CH₃, R2 = CHO: ethyl 5-(formylamino)-1*H*-pyrazole-4-carboxylate,



- C. 5-(4*H*-1,2,4-triazol-4-yl)-1*H*-pyrazole-4-carboxamide.

01/2005:2010
corrected

ALMAGATE

Almagatum

Al₂Mg₆C₂O₂₀H₁₄·4H₂O

M_r 630

DEFINITION

Hydrated aluminium magnesium hydroxycarbonate.

Content:

- aluminium: 15.0 per cent to 17.0 per cent (calculated as Al₂O₃),
- magnesium: 36.0 per cent to 40.0 per cent (calculated as MgO),
- carbonic acid: 12.5 per cent to 14.5 per cent (calculated as CO₂).

CHARACTERS

Appearance: white or almost white, fine crystalline powder.

Solubility: practically insoluble in water, in alcohol and in methylene chloride. It dissolves with effervescence and heating in dilute mineral acids.

IDENTIFICATION

- A. Infrared absorption spectrophotometry (2.2.24).

Comparison: *Ph. Eur. reference spectrum of almagate*.

- B. Dissolve 150 mg in *dilute hydrochloric acid R* and dilute to 20 ml with the same acid. 2 ml of the solution gives the reaction of aluminium (2.3.1).

- C. 2 ml of the solution prepared under identification test B gives the reaction of magnesium (2.3.1).

TESTS

pH (2.2.3): 9.1 to 9.7.

Disperse 4.0 g in 100 ml of *carbon dioxide-free water R*, stir for 2 min and filter.

Neutralising capacity. Carry out the test at 37 °C. Disperse 0.5 g in 100 ml of *water R*, heat, add 100.0 ml of 0.1 *M* *hydrochloric acid*, previously heated and stir continuously; the pH (2.2.3) of the solution between 5 min and 20 min is not less than 3.0 and not greater than 4.5. Add 10.0 ml of 0.5 *M* *hydrochloric acid*, previously heated, stir continuously for 1 h and titrate with 0.1 *M* *sodium hydroxide* to pH 3.5; not more than 20.0 ml of 0.1 *M* *sodium hydroxide* is required.

Chlorides (2.4.4): maximum 0.1 per cent.

Dissolve 0.33 g in 5 ml of *dilute nitric acid R* and dilute to 100 ml with *water R*. 15 ml of the solution complies with the limit test for chlorides. Prepare simultaneously the standard by diluting 0.7 ml of *dilute nitric acid R* to 5 ml with *water R* and adding 10 ml of *chloride standard solution* (5 ppm Cl) *R*.

Sulphates (2.4.13): maximum 0.4 per cent.

Dissolve 0.25 g in 5 ml of *dilute hydrochloric acid R* and dilute to 100 ml with *distilled water R*. 15 ml of the solution complies with the limit test for sulphates. Prepare simultaneously the standard by adding 0.8 ml of *dilute hydrochloric acid R* to 15 ml of *sulphate standard solution* (10 ppm SO₄) *R*.

Sodium: maximum 150 ppm.

Atomic absorption spectrometry (2.2.23, *Method I*).

Test solution. Dissolve 0.25 g in 50 ml of a 103 g/l solution of *hydrochloric acid R*.

Reference solutions. Prepare the reference solutions using *sodium standard solution* (200 ppm Na) *R*, diluted as necessary with a 103 g/l solution of *hydrochloric acid R*.

Heavy metals (2.4.8): maximum 20 ppm.

Dissolve 1.0 g in *dilute hydrochloric acid R* and dilute to 20.0 ml with the same acid. 12 ml of the solution complies with limit test A. Prepare the standard using *lead standard solution* (1 ppm Pb) *R*.

Loss on ignition: 43.0 per cent to 49.0 per cent, determined on 1.000 g by ignition at 900 °C.

Microbial contamination. Total viable aerobic count (2.6.12) not more than 10³ micro-organisms per gram determined by plate count. It complies with the tests for *Escherichia coli* and *Pseudomonas aeruginosa* (2.6.13).

ASSAY

Aluminium. Dissolve 1.000 g in 5 ml of *hydrochloric acid R*, heating if necessary. Allow to cool to room temperature and dilute to 100.0 ml with *water R* (solution A). Introduce 10.0 ml of solution A into a 250 ml conical flask, add 25.0 ml of 0.05 *M* *sodium edetate*, 20 ml of *buffer solution pH 3.5 R*, 40 ml of *ethanol R* and 2 ml of a freshly prepared 0.25 g/l solution of *dithizone R* in *ethanol R*. Titrate the excess of sodium edetate with 0.05 *M* *zinc sulphate* until the colour changes from greenish-violet to pink.

1 ml of 0.05 *M* *sodium edetate* is equivalent to 2.549 mg of Al₂O₃.

Magnesium. Introduce 10.0 ml of solution A prepared in the assay of aluminium into a 500 ml conical flask, add 200 ml of *water R*, 20 ml of *triethanolamine R* with shaking, 10 ml of

01/2005:1064

ammonium chloride buffer solution pH 10.0 R and 50 mg of *mordant black 11 triturate R*. Titrate with *0.05 M sodium edetate* until the colour changes from violet to pure blue.

1 ml of *0.05 M sodium edetate* is equivalent to 2.015 mg of MgO.

Carbonic acid: 12.5 per cent to 14.5 per cent.

Test sample. Place 7.00 mg of the substance to be examined in a tin capsule. Seal the capsule.

Reference sample. Place 7.00 mg of *almagate CRS* in a tin capsule. Seal the capsule.

Introduce separately the test sample and the reference sample into a combustion chamber of a CHN analyser purged with *helium for chromatography R* and maintained at a temperature of 1020 °C. Simultaneously, introduce *oxygen R* at a pressure of 276 kPa and a flow rate of 20 ml/min and allow complete combustion of the sample. Sweep the combustion gases through a reduction reactor and separate the gases formed by gas chromatography (2.2.28).

Column:

– *size:* $l = 2$ m, $\varnothing = 4$ mm,

– *stationary phase:* *ethylvinylbenzene-divinylbenzene copolymer R1*.

Carrier gas: *helium for chromatography R*.

Flow rate: 100 ml/min.

Temperature:

– *column:* 65 °C,

– *detector:* 190 °C.

Detection: thermal conductivity.

Run time: 16 min.

System suitability:

- average percentage of carbon in 5 reference samples must be within ± 0.2 per cent of the value assigned to the CRS. The difference between the upper and the lower values of the percentage of carbon in these samples must be below 0.2 per cent.

Calculate the percentage content of carbonic acid in the test sample according to the following formula:

$$C \times K \times \frac{A}{m}$$

- C = percentage content of carbonic acid in the reference sample,
- K = mean value for the 5 reference samples of the ratio of the mass in milligrams to the area of the peak due to carbonic acid,
- A = area of the peak due to carbonic acid in the chromatogram obtained with the test sample,
- m = sample mass, in milligrams.

STORAGE

In an airtight container.

ALMOND OIL, REFINED

Amygdalae oleum raffinatum

DEFINITION

Refined almond oil is the fatty oil from the ripe seeds of *Prunus dulcis* (Miller) D.A. Webb var. *dulcis* or *Prunus dulcis* (Miller) D.A. Webb var. *amara* (D.C.) Buchheim or a mixture of both varieties by cold expression. It is then refined. A suitable antioxidant may be added.

CHARACTERS

A pale yellow, clear liquid, slightly soluble in alcohol, miscible with light petroleum.

It solidifies at about -18 °C and has a relative density of about 0.916.

IDENTIFICATION

- A. Carry out the identification of fatty oils by thin-layer chromatography (2.3.2). The chromatogram obtained is comparable with the type chromatogram for almond oil.
- B. It complies with the test for composition of fatty acids (see Tests).

TESTS

Absorbance (2.2.25). Dissolve 0.100 g in *cyclohexane R* and dilute to 10.0 ml with the same solvent. The absorbance measured at the maximum between 264 nm and 276 nm is 0.2 to 6.0.

Acid value (2.5.1). Not more than 0.5, determined on 5.0 g.

Peroxide value (2.5.5). Not more than 5.0.

Unsaponifiable matter (2.5.7). Not more than 0.7 per cent, determined on 5.0 g.

Composition of fatty acids (2.4.22, Method A). The fatty acid fraction of the oil has the following composition:

- *saturated fatty acids of chain length less than C_{16}* : not more than 0.1 per cent,
- *palmitic acid*: 4.0 per cent to 9.0 per cent,
- *palmitoleic acid* (equivalent chain length on polyethyleneglycol adipate 16.3): not more than 0.6 per cent,
- *margaric acid*: not more than 0.2 per cent,
- *stearic acid*: not more than 3.0 per cent,
- *oleic acid* (equivalent chain length on polyethyleneglycol adipate 18.3): 62.0 per cent to 86.0 per cent,
- *linoleic acid* (equivalent chain length on polyethyleneglycol adipate 18.9): 20.0 per cent to 30.0 per cent,
- *linolenic acid* (equivalent chain length on polyethyleneglycol adipate 19.7): not more than 0.4 per cent,
- *arachidic acid*: not more than 0.2 per cent,
- *eicosenoic acid* (equivalent chain length on polyethyleneglycol adipate 20.3): not more than 0.3 per cent,
- *behenic acid*: not more than 0.2 per cent,
- *erucic acid* (equivalent chain length on polyethyleneglycol adipate 22.3): not more than 0.1 per cent.

Sterols. Carry out the test for sterols in fatty oils (2.4.23).

The sterol fraction of the oil has the following composition:

- *cholesterol*: not more than 0.7 per cent,
- *campesterol*: not more than 5.0 per cent,

- *stigmasterol*: not more than 4.0 per cent,
- *β-sitosterol*: 73.0 per cent to 87.0 per cent,
- *Δ⁵-avenasterol*: at least 5.0 per cent,
- *Δ⁷-avenasterol*: not more than 3.0 per cent,
- *Δ⁷-stigmastenol*: not more than 3.0 per cent,
- *brassicasterol*: not more than 0.3 per cent.

Water (2.5.32). If intended for use in the manufacture of parenteral dosage forms, not more than 0.1 per cent, determined on 5.00 g by the micro-determination of water.

STORAGE

Store in a well-filled container, protected from light.

LABELLING

The label states:

- where applicable, that the substance is suitable for use in the manufacture of parenteral dosage forms,
- the name and concentration of any added antioxidant.

01/2005:0261

ALMOND OIL, VIRGIN

Amygdalae oleum virginale

DEFINITION

Virgin almond oil is the fatty oil obtained by cold expression from the ripe seeds of *Prunus dulcis* (Miller) D.A. Webb var. *dulcis* or *Prunus dulcis* (Miller) D.A. Webb var. *amara* (D.C.) Buchheim or a mixture of both varieties.

CHARACTERS

A yellow, clear, liquid, slightly soluble in alcohol, miscible with light petroleum.

It solidifies at about –18 °C and has a relative density of about 0.916.

IDENTIFICATION

First identification: A, C.

Second identification: A, B.

- It complies with the test for absorbance (see Tests).
- Carry out the identification of fatty oils by thin-layer chromatography (2.3.2). The chromatogram obtained is comparable with the type chromatogram for almond oil.
- It complies with the test for composition of fatty acids (see Tests).

TESTS

Absorbance (2.2.25). Dissolve 0.100 g in *cyclohexane R* and dilute to 10.0 ml with the same solvent. The absorbance measured at the maximum between 264 nm and 276 nm is not greater than 0.2. The ratio of the absorbance at 232 nm to that at 270 nm is greater than 7.

Acid value (2.5.1). Not more than 2.0, determined on 5.0 g.

Peroxide value (2.5.5). Not more than 15.0.

Unsaponifiable matter (2.5.7). Not more than 0.7 per cent, determined on 5.0 g.

Composition of fatty acids (2.4.22, *Method A*). The fatty acid fraction of the oil has the following composition:

- *saturated fatty acids of chain length less than C₁₆*: not more than 0.1 per cent,
- *palmitic acid*: 4.0 per cent to 9.0 per cent,
- *palmitoleic acid* (equivalent chain length on polyethyleneglycol adipate 16.3): not more than 0.6 per cent,

- *margaric acid*: not more than 0.2 per cent,
- *stearic acid*: not more than 3.0 per cent,
- *oleic acid* (equivalent chain length on polyethyleneglycol adipate 18.3): 62.0 per cent to 86.0 per cent,
- *linoleic acid* (equivalent chain length on polyethyleneglycol adipate 18.9): 20.0 per cent to 30.0 per cent,
- *linolenic acid* (equivalent chain length on polyethyleneglycol adipate 19.7): not more than 0.4 per cent,
- *arachidic acid*: not more than 0.2 per cent,
- *eicosenoic acid* (equivalent chain length on polyethyleneglycol adipate 20.3): not more than 0.3 per cent,
- *behenic acid*: not more than 0.2 per cent,
- *erucic acid* (equivalent chain length on polyethyleneglycol adipate 22.3): not more than 0.1 per cent.

Sterols. Carry out the test for sterols in fatty oils (2.4.23).

The sterol fraction of the oil has the following composition:

- *cholesterol*: not more than 0.7 per cent,
- *campesterol*: not more than 4.0 per cent,
- *stigmasterol*: not more than 3.0 per cent,
- *β-sitosterol*: 73.0 per cent to 87.0 per cent,
- *Δ⁵-avenasterol*: at least 10.0 per cent,
- *Δ⁷-avenasterol*: not more than 3.0 per cent,
- *Δ⁷-stigmastenol*: not more than 3.0 per cent,
- *brassicasterol*: not more than 0.3 per cent.

STORAGE

Store in a well-filled container, protected from light.

01/2005:0257

ALOES, BARBADOS

Aloe barbadensis

DEFINITION

Barbados aloes consists of the concentrated and dried juice of the leaves of *Aloe barbadensis* Miller. It contains not less than 28.0 per cent of hydroxyanthracene derivatives, expressed as barbaloin (C₂₁H₂₂O₉; *M_r* 418.4) and calculated with reference to the dried drug.

CHARACTERS

Dark brown masses, slightly shiny or opaque with a conchoidal fracture, or a brown powder, soluble in hot alcohol, partly soluble in boiling water.

IDENTIFICATION

- Examine by thin-layer chromatography (2.2.27), using *silica gel G R* as the coating substance.

Test solution. To 0.25 g of the powdered drug add 20 ml of *methanol R* and heat to boiling in a water-bath. Shake for a few minutes and decant the solution. Store at about 4 °C and use within 24 h.

Reference solution. Dissolve 25 mg of *barbaloin R* in *methanol R* and dilute to 10 ml with the same solvent. Apply separately to the plate as bands 20 mm by not more than 3 mm 10 µl of each solution. Develop over a path of 10 cm using a mixture of 13 volumes of *water R*, 17 volumes of *methanol R* and 100 volumes of *ethyl acetate R*. Allow the plate to dry in air, spray with a 100 g/l solution of *potassium hydroxide R* in *methanol R* and examine in ultraviolet light at 365 nm.

The chromatogram obtained with the test solution shows in the central part a zone of yellow fluorescence (barbaloin) similar in position to the zone corresponding to barbaloin in the chromatogram obtained with the reference solution and in the lower part a zone of light-blue fluorescence (aloesine). Heat the plate at 110 °C for 5 min. In the chromatogram obtained with the test solution, a zone of violet fluorescence appears just below the zone corresponding to barbaloin.

- B. Shake 1 g of the powdered drug with 100 ml of boiling *water R*. Cool, add 1 g of *talc R* and filter. To 10 ml of the filtrate add 0.25 g of *disodium tetraborate R* and heat to dissolve. Pour 2 ml of the solution into 20 ml of *water R*. Yellowish-green fluorescence appears which is particularly marked in ultraviolet light at 365 nm.
- C. To 5 ml of the filtrate obtained in identification test B add 1 ml of freshly prepared *bromine water R*. A brownish-yellow precipitate is formed and the supernatant liquid is violet.

TESTS

Loss on drying (2.2.32). Not more than 12.0 per cent, determined on 1.000 g of the powdered drug by drying in an oven at 100 °C to 105 °C.

Total ash (2.4.16). Not more than 2.0 per cent.

ASSAY

Carry out the assay protected from bright light.

Introduce 0.300 g of powdered drug (180) into a 250 ml conical flask. Moisten with 2 ml of *methanol R*, add 5 ml of *water R* warmed to about 60 °C, mix, add a further 75 ml of *water R* at about 60 °C and shake for 30 min. Cool, filter into a volumetric flask, rinse the conical flask and filter with 20 ml of *water R*, add the rinsings to the volumetric flask and dilute to 1000.0 ml with *water R*. Transfer 10.0 ml of this solution to a 100 ml round-bottomed flask containing 1 ml of a 600 g/l solution of *ferric chloride R* and 6 ml of *hydrochloric acid R*. Heat in a water-bath under a reflux condenser for 4 h, with the water level above that of the liquid in the flask. Allow to cool, transfer the solution to a separating funnel, rinse the flask successively with 4 ml of *water R*, 4 ml of 1 M *sodium hydroxide* and 4 ml of *water R* and add the rinsings to the separating funnel. Shake the contents of the separating funnel with three quantities, each of 20 ml, of *ether R*. Wash the combined ether layers with two quantities, each of 10 ml, of *water R*. Discard the washings and dilute the organic phase to 100.0 ml with *ether R*. Evaporate 20.0 ml carefully to dryness on a water-bath and dissolve the residue in 10.0 ml of a 5 g/l solution of *magnesium acetate R* in *methanol R*. Measure the absorbance (2.2.25) at 512 nm using *methanol R* as the compensation liquid.

Calculate the percentage content of hydroxyanthracene derivatives, as barbaloin, from the expression:

$$\frac{A \times 19.6}{m}$$

i.e. taking the specific absorbance of barbaloin to be 255.

A = absorbance at 512 nm,

m = mass of the substance to be examined in grams.

STORAGE

Store in an airtight container, protected from light.

01/2005:0258

ALOES, CAPE

Aloe capensis

DEFINITION

Cape aloes consists of the concentrated and dried juice of the leaves of various species of *Aloe*, mainly *Aloe ferox* Miller and its hybrids. It contains not less than 18.0 per cent of hydroxyanthracene derivatives, expressed as barbaloin ($C_{21}H_{22}O_9$; M_r 418.4) and calculated with reference to the dried drug.

CHARACTERS

Dark brown masses tinged with green and having a shiny conchoidal fracture, or a greenish-brown powder, soluble in hot alcohol, partly soluble in boiling water.

IDENTIFICATION

- A. Examine the chromatograms obtained in the test for Barbados aloes. The chromatogram obtained with the test solution shows in the central part a zone of yellow fluorescence (barbaloin) similar in position to the zone corresponding to barbaloin in the chromatogram obtained with the reference solution and in the lower part two zones of yellow fluorescence (aloinosides A and B) and a zone of blue fluorescence (aloesine).
- B. Shake 1 g of the powdered drug with 100 ml of boiling *water R*. Cool, add 1 g of *talc R* and filter. To 10 ml of the filtrate add 0.25 g of *disodium tetraborate R* and heat to dissolve. Pour 2 ml of the solution into 20 ml of *water R*. A yellowish-green fluorescence appears which is particularly marked in ultraviolet light at 365 nm.
- C. To 5 ml of the filtrate obtained in identification test B add 1 ml of freshly prepared *bromine water R*. A yellow precipitate is formed. The supernatant liquid is not coloured violet.

TESTS

Barbados aloes. Examine by thin-layer chromatography (2.2.27), using *silica gel G R* as the coating substance.

Test solution. To 0.25 g of the powdered drug add 20 ml of *methanol R* and heat to boiling in a water-bath. Shake for a few minutes and decant the solution. Store at about 4 °C and use within 24 h.

Reference solution. Dissolve 25 mg of *barbaloin R* in *methanol R* and dilute to 10 ml with the same solvent.

Apply separately to the plate as bands 20 mm by not more than 3 mm 10 µl of each solution. Develop over a path of 10 cm using a mixture of 13 volumes of *water R*, 17 volumes of *methanol R* and 100 volumes of *ethyl acetate R*. Allow the plate to dry in air, spray with a 100 g/l solution of *potassium hydroxide R* in *methanol R*. Heat the plate at 110 °C for 5 min and examine in ultraviolet light at 365 nm. The chromatogram obtained with the test solution shows no zone of violet fluorescence just below the zone corresponding to barbaloin.

Loss on drying (2.2.32). Not more than 10.0 per cent, determined on 1.000 g of the powdered drug by drying in an oven at 100 °C to 105 °C.

Total ash (2.4.16). Not more than 2.0 per cent.

ASSAY

Carry out the assay protected from bright light.

Introduce 0.400 g of powdered drug (180) into a 250 ml conical flask. Moisten with 2 ml of *methanol R*, add 5 ml of *water R* warmed to about 60 °C, mix, add a further 75 ml of *water R* at about 60 °C and shake for 30 min. Cool, filter into a volumetric flask, rinse the conical flask and filter with 20 ml of *water R*, add the rinsings to the volumetric flask and dilute to 1000.0 ml with *water R*. Transfer 10.0 ml of this solution to a 100 ml round-bottomed flask containing 1 ml of a 600 g/l solution of *ferric chloride R* and 6 ml of *hydrochloric acid R*. Heat in a water-bath under a reflux condenser for 4 h, with the water level above that of the liquid in the flask. Allow to cool, transfer the solution to a separating funnel, rinse the flask successively with 4 ml of *water R*, 4 ml of 1 M *sodium hydroxide* and 4 ml of *water R* and add the rinsings to the separating funnel. Shake the contents of the separating funnel with three quantities, each of 20 ml, of *ether R*. Wash the combined ether layers with two quantities, each of 10 ml, of *water R*. Discard the washings and dilute the organic phase to 100.0 ml with *ether R*. Evaporate 20.0 ml carefully to dryness on a water-bath and dissolve the residue in 10.0 ml of a 5 g/l solution of *magnesium acetate R* in *methanol R*. Measure the absorbance (2.2.25) at 512 nm using *methanol R* as the compensation liquid.

Calculate the percentage content of barbaloin from the expression:

$$\frac{A \times 19.6}{m}$$

i.e. taking the specific absorbance of hydroxyanthracene derivatives, as barbaloin, to be 255.

A = absorbance at 512 nm,

m = mass of the substance to be examined in grams.

STORAGE

Store in an airtight container, protected from light.

01/2005:0259

ALOE DRY EXTRACT, STANDARDISED

Aloes extractum siccum normatum

DEFINITION

Standardised aloes dry extract is prepared from Barbados aloes or Cape aloes, or a mixture of the two, by treatment with boiling water. It is adjusted, if necessary, to contain not less than 19.0 per cent and not more than 21.0 per cent of hydroxyanthracene derivatives, expressed as barbaloin ($C_{21}H_{22}O_9$; M_r 418.4) and calculated with reference to the dried extract.

CHARACTERS

A brown or yellowish-brown powder, sparingly soluble in boiling water.

IDENTIFICATION

A. Examine by thin-layer chromatography (2.2.27), using *silica gel G R* as the coating substance.

Test solution. To 0.25 g of the preparation to be examined add 20 ml of *methanol R* and heat to boiling in a water-bath. Shake for a few minutes and decant the solution. Store at about 4 °C and use within 24 h.

Reference solution. Dissolve 25 mg of *barbaloin R* in *methanol R* and dilute to 10 ml with the same solvent.

Apply separately to the plate as bands 20 mm by not more than 3 mm 10 µl of each solution. Develop over a path of 10 cm using a mixture of 13 volumes of *water R*, 17 volumes of *methanol R* and 100 volumes of *ethyl acetate R*. Allow the plate to dry in air, spray with a 100 g/l solution of *potassium hydroxide R* in *methanol R* and examine in ultraviolet light at 365 nm. The chromatogram obtained with the test solution shows in the central part a zone of yellow fluorescence (barbaloin) similar in position to the zone corresponding to barbaloin in the chromatogram obtained with the reference solution and in the lower part there is a zone of light blue fluorescence (aloesine). In the lower part of the chromatogram obtained with the test solution two zones of yellow fluorescence (aloinosides A and B) (Cape aloes) and one zone of violet fluorescence just below the zone corresponding to barbaloin (Barbados aloes) may be present.

B. Shake 1 g of the preparation to be examined with 100 ml of boiling *water R*. Cool, add 1 g of *talc R* and filter. To 10 ml of the filtrate add 0.25 g of *disodium tetraborate R* and heat to dissolve. Pour 2 ml of the solution into 20 ml of *water R*. A yellowish-green fluorescence appears which is particularly marked in ultraviolet light at 365 nm.

TESTS

Loss on drying (2.8.17): maximum 4.0 per cent m/m .

Total ash (2.4.16). Not more than 2.0 per cent.

ASSAY

Carry out the assay protected from bright light.

Introduce 0.400 g into a 250 ml conical flask. Moisten with 2 ml of *methanol R*, add 5 ml of *water R* warmed to about 60 °C, mix, add a further 75 ml of *water R* at about 60 °C and shake for 30 min. Cool, filter into a volumetric flask, rinse the conical flask and filter with 20 ml of *water R*, add the rinsings to the volumetric flask and dilute to 1000.0 ml with *water R*. Transfer 10.0 ml of this solution to a 100 ml round-bottomed flask containing 1 ml of a 600 g/l solution of *ferric chloride R* and 6 ml of *hydrochloric acid R*. Heat in a water-bath under a reflux condenser for 4 h, with the water level above that of the liquid in the flask. Allow to cool, transfer the solution to a separating funnel, rinse the flask successively with 4 ml of *water R*, 4 ml of 1 M *sodium hydroxide* and 4 ml of *water R*, and add the rinsings to the separating funnel. Shake the contents of the separating funnel with three quantities, each of 20 ml, of *ether R*. Wash the combined ether layers with two quantities, each of 10 ml, of *water R*. Discard the washings and dilute the organic phase to 100.0 ml with *ether R*. Evaporate 20.0 ml carefully to dryness on a water-bath and dissolve the residue in 10.0 ml of a 5 g/l solution of *magnesium acetate R* in *methanol R*. Measure the absorbance (2.2.25) at 512 nm using *methanol R* as the compensation liquid.

Calculate the percentage content of hydroxyanthracene derivatives, as barbaloin, from the expression:

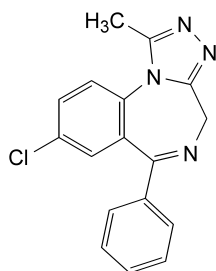
$$\frac{A \times 19.6}{m}$$

i.e. taking the specific absorbance of barbaloin to be 255.

A = absorbance at 512 nm,

m = mass of the substance to be examined in grams.

01/2005:1065

ALPRAZOLAM**Alprazolamum** $C_{17}H_{13}ClN_4$ M_r 308.8**DEFINITION**

Alprazolam contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of 8-chloro-1-methyl-6-phenyl-4*H*-[1,2,4]triazolo[4,3-*a*][1,4]benzodiazepine, calculated with reference to the dried substance.

CHARACTERS

A white, crystalline powder, practically insoluble in water, freely soluble in methylene chloride sparingly soluble in acetone and in alcohol.

It shows polymorphism.

IDENTIFICATION

First identification: B.

Second identification: A, C.

A. Dissolve the substance to be examined in the smallest necessary quantity of *ethyl acetate R* and evaporate to dryness on a water-bath. Thoroughly mix 5.0 mg of the substance to be examined with 5.0 mg of *alprazolam CRS*. The melting point (2.2.14) of the mixture does not deviate from the melting point of the substance to be examined by more than 2 °C.

B. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *alprazolam CRS*. If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in the minimum volume of *ethyl acetate R*, evaporate to dryness on a water-bath and record new spectra using the residues. Examine the substances prepared as discs.

C. Examine by thin-layer chromatography (2.2.27), using *silica gel GF₂₅₄ R* as the coating substance.

Test solution. Dissolve 10 mg of the substance to be examined in *methanol R* and dilute to 10 ml with the same solvent.

Reference solution (a). Dissolve 10 mg of *alprazolam CRS* in *methanol R* and dilute to 10 ml with the same solvent.

Reference solution (b). Dissolve 10 mg of *alprazolam CRS* and 10 mg of *midazolam CRS* in *methanol R* and dilute to 10 ml with the same solvent.

Apply separately to the plate 5 µl of each solution. Develop over a path or 12 cm using a mixture of 2 volumes of *glacial acetic acid R*, 15 volumes of *water R*, 20 volumes of *methanol R* and 80 volumes of *ethyl acetate R*. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. The principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the

chromatogram obtained with reference solution (a). The test is not valid unless the chromatogram obtained with reference solution (b) shows two clearly separately spots.

TESTS

Related substances. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 0.100 g of the substance to be examined in *dimethylformamide R* and dilute to 10.0 ml with the same solvent.

Reference solution (a). Dissolve 2 mg of *alprazolam CRS* and 2 mg of *triazolam CRS* in *dimethylformamide R* and dilute to 100.0 ml with the same solvent.

Reference solution (b). Dilute 5.0 ml of the test solution to 100.0 ml with *dimethylformamide R*. Dilute 0.5 ml of this solution to 10.0 ml with *dimethylformamide R*.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4.6 mm in internal diameter packed with *phenylsilyl silica gel for chromatography R1* (5 µm),
- as mobile phase at a flow rate of 2 ml/min and at a temperature of 40 °C a gradient programme using the following conditions:

Mobile phase A. A mixture of 44 volumes of buffer solution and 56 volumes of *methanol R*.

Mobile phase B. A mixture of 5 volumes of buffer solution and 95 volumes of *methanol R*.

Buffer solution. Dissolve 7.7 g of *ammonium acetate R* in 1000 ml of *water R* and adjust to pH 4.2 with *glacial acetic acid R*,

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)	Comment
0	98	2	isocratic
15	98	2	begin linear gradient
35	1	99	begin isocratic
40	1	99	end chromatogram and switch to initial equilibration
41	98	2	begin equilibration
50 = 0	98	2	end equilibration

- as detector a spectrophotometer set at 254 nm.

Equilibrate the column for at least 30 min with the initial eluent composition. For subsequent chromatograms, use the conditions described from 40 min to 50 min. Adjust the sensitivity of the system so that the height of the principal peak in the chromatogram obtained with reference solution (b) is at least 50 per cent of the full scale of the recorder.

Inject 10 µl of reference solution (a). When the chromatogram is recorded in the prescribed conditions the retention times are: triazolam, about 9 min and alprazolam, about 10 min. The test is not valid unless the resolution between the peaks corresponding to alprazolam and triazolam is at least 1.5.

Inject separately 10 µl of *dimethylformamide R* as a blank, 10 µl of the test solution and 10 µl of reference solution (b). In the chromatogram obtained with the test solution, the sum of the areas of the peaks, apart from the principal peak, is not greater than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.25 per cent). Disregard any peak in the chromatogram obtained with the blank and any peak with an area less than 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (b).

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

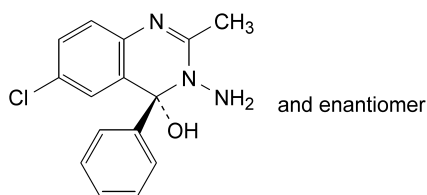
Dissolve 0.140 g in 50 ml of a mixture of 3 volumes of *anhydrous acetic acid R* and 2 volumes of *acetic anhydride R*. Titrate with 0.1 M perchloric acid, determining the end-point potentiometrically (2.2.20). Titrate to the second point of inflexion.

1 ml of 0.1 M perchloric acid is equivalent to 15.44 mg of $C_{17}H_{13}ClN_4$.

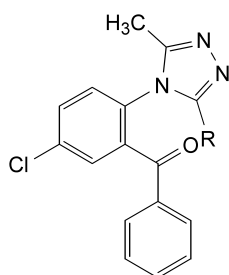
STORAGE

Store protected from light.

IMPURITIES



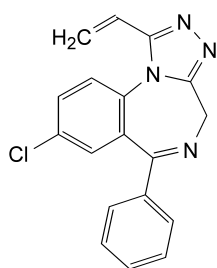
- A. (4*RS*)-3-amino-6-chloro-2-methyl-4-phenyl-3,4-dihydroquinazolin-4-ol,



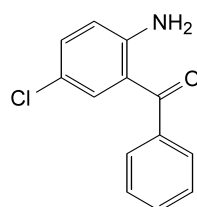
- B. R = CH₂OH: [5-chloro-2-[3-(hydroxymethyl)-5-methyl-4*H*-1,2,4-triazol-4-yl]phenyl]phenylmethanone,

- C. R = H: [5-chloro-2-[3-methyl-4*H*-1,2,4-triazol-4-yl]phenyl]phenylmethanone,

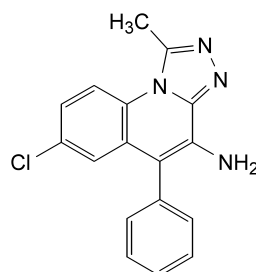
- F. R = CH₂Cl: [5-chloro-2-[3-(chloromethyl)-5-methyl-4*H*-1,2,4-triazol-4-yl]phenyl]phenylmethanone,



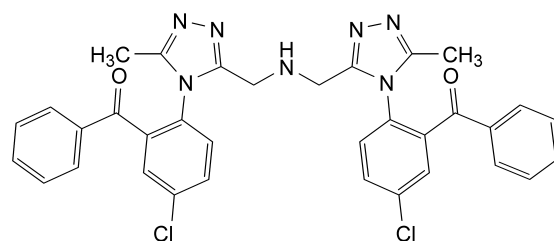
- D. 8-chloro-1-ethenyl-6-phenyl-4*H*-[1,2,4]triazolo[4,3-*a*][1,4]benzodiazepine,



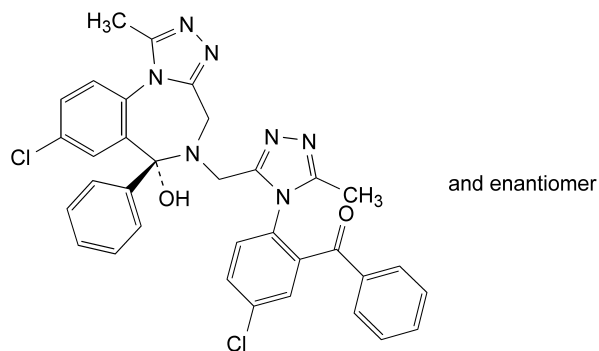
- E. (2-amino-5-chlorophenyl)phenylmethanone,



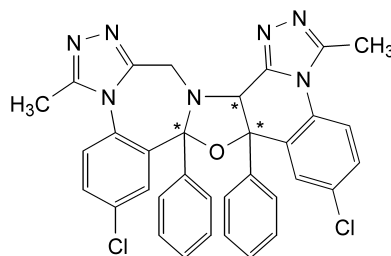
- G. 7-chloro-1-methyl-5-phenyl[1,2,4]triazolo[4,3-*a*]quinolin-4-amine,



- H. bis[[4-(2-benzoyl-4-chlorophenyl)-5-methyl-4*H*-1,2,4-triazol-3-yl]methyl]amine,



- I. [5-chloro-2-[3-[(6*RS*)-8-chloro-6-hydroxy-1-methyl-6-phenyl-4*H*-[1,2,4]triazolo[4,3-*a*][1,4]benzodiazepin-5(6*H*)-yl]methyl]-5-methyl-4*H*-1,2,4-triazol-4-yl]phenyl]phenylmethanone,

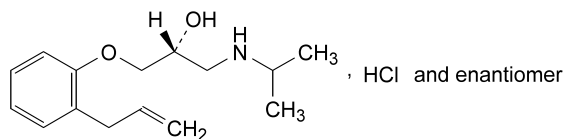


- J. 2,17-dichloro-6,13-dimethyl-18*b*,19*a*-diphenyl-8*b*,19*a*-dihydro-10*H*,18*bH*-[1,2,4]triazolo[4''',3''':1'',2'']quinolo[3'',4'':4',5']oxazolo[3',2'-*d*']-1,2,4-triazolo[4,3-*a*][1,4]benzodiazepine.

01/2005:0876

ALPRENOLOL HYDROCHLORIDE

Alprenololi hydrochloridum

 $C_{15}H_{24}ClNO_2$ M_r 285.8

DEFINITION

Alprenolol hydrochloride contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of (2*RS*)-1-[(1-methylethylamino)-3-[2-(prop-2-enyl)phenoxy]propan-2-ol hydrochloride, calculated with reference to the dried substance.

CHARACTERS

A white, crystalline powder or colourless crystals, very soluble in water, freely soluble in alcohol and in methylene chloride.

IDENTIFICATION

First identification: B, D.

Second identification: A, C, D.

A. Melting point (2.2.14): 108 °C to 112 °C.

B. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *alprenolol hydrochloride CRS*.

C. Examine the chromatograms obtained in test A for related substances in daylight, after exposure to iodine vapour for 30 min. The principal spot in the chromatogram obtained with test solution (b) is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).

D. It gives reaction (a) of chlorides (2.3.1).

TESTS

Solution S. Dissolve 1.0 g in *carbon dioxide-free water R* and dilute to 50 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and not more intensely coloured than reference solution B₉ (2.2.2, *Method II*).

Acidity or alkalinity. To 10 ml of solution S add 0.2 ml of *methyl red solution R* and 0.2 ml of 0.01 *M hydrochloric acid*. The solution is red. Add 0.4 ml of 0.01 *M sodium hydroxide*. The solution is yellow.

1-Isopropylamino-3-(2-prop-1-enylphenoxy)propan-2-ol.

Dissolve 0.25 g in *alcohol R* and dilute to 25 ml with the same solvent. The absorbance (2.2.25) measured at 297 nm is not greater than 0.20 (0.1 per cent).

Related substances

A. Examine by thin-layer chromatography (2.2.27), using *silica gel G R* as the coating substance.

Test solution (a). Dissolve 0.50 g of the substance to be examined in *methanol R* and dilute to 10 ml with the same solvent.

Test solution (b). Dilute 1 ml of test solution (a) to 50 ml with *methanol R*.

Reference solution (a). Dissolve 10 mg of *alprenolol hydrochloride CRS* in *methanol R* and dilute to 10 ml with the same solvent.

Reference solution (b). Dissolve 10 mg of *alprenolol hydrochloride CRS* and 10 mg of *oxprenolol hydrochloride CRS* in *methanol R* and dilute to 10 ml with the same solvent.

Reference solution (c). Dilute 5 ml of test solution (b) to 50 ml with *methanol R*.

Place two beakers each containing 30 ml of *ammonia R* at the bottom of the chamber which is saturated with the mobile phase and *ammonia R* for at least 1 h before use. Apply separately to the plate 5 µl of each solution. Develop over a path of 15 cm using a mixture of 5 volumes of *methanol R* and 95 volumes of *ethyl acetate R*. Dry the plate at 100 °C for 15 min. Expose the plate to iodine vapour for up to 6 h. In the chromatogram obtained with test solution (a) any spot with an R_f value higher than that of the principal spot is not more intense than the spot in the chromatogram obtained with reference solution (c). The test is not valid unless the chromatogram obtained with reference solution (b) shows two clearly separated spots.

B. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 20.0 mg of the substance to be examined in the mobile phase and dilute to 10.0 ml with the mobile phase.

Reference solution (a). Dissolve 4.0 mg of *alprenolol hydrochloride CRS* and 0.8 mg of 4-*isopropylphenol R* in the mobile phase and dilute to 100.0 ml with the mobile phase.

Reference solution (b). Dilute 4.0 ml of the test solution to 100.0 ml with the mobile phase. Dilute 1.0 ml of the solution to 10.0 ml with the mobile phase.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.15 m long and 4 mm in internal diameter packed with *octylsilyl silica gel for chromatography R* (5 µm),
- as mobile phase at a flow rate of 1 ml/min a mixture prepared as follows: mix 0.656 g of *sodium octanesulphonate R* with 150 ml of *acetonitrile R* and dilute to 500 ml with phosphate buffer pH 2.8 (prepared from 1.78 g of *phosphoric acid R* and 15.6 g of *sodium dihydrogen phosphate R* diluted to 2000 ml with *water R*),
- as detector a spectrophotometer set at 280 nm.

Equilibrate the column with the mobile phase at a flow rate of 1 ml/min for about 1 h.

Adjust the sensitivity of the system so that the height of the principal peak in the chromatogram obtained with 20 µl of reference solution (b) is at least 50 per cent of the full scale of the recorder.

Inject 20 µl of reference solution (a). When the chromatograms are recorded under the prescribed conditions, the retention times are: *alprenolol hydrochloride* about 11 min; 4-*isopropylphenol* about 18 min. The test is not valid unless, in the chromatogram obtained with reference solution (a), the resolution between the peaks corresponding to *alprenolol hydrochloride* and 4-*isopropylphenol* is at least 5; if necessary, adjust the concentration of sodium octanesulphonate and/or acetonitrile in the mobile phase (increase the concentration of sodium octanesulphonate to increase the retention time of *alprenolol hydrochloride* and increase the concentration of acetonitrile to decrease the retention times of both compounds).

01/2005:1488

Inject separately 20 µl of the test solution and 20 µl of reference solution (b). Continue the chromatography for twice the retention time of the principal peak. In the chromatogram obtained with the test solution, the sum of the areas of any peaks apart from the principal peak is not greater than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.4 per cent). Disregard any peak with an area less than 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (b).

Heavy metals (2.4.8). Dissolve 2.0 g in 20 ml of *water R*. 12 ml of the solution complies with limit test A for heavy metals (10 ppm). Prepare the standard using *lead standard solution* (1 ppm Pb) *R*.

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying over *diphosphorus pentoxide R* at a pressure not exceeding 2.7 kPa.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

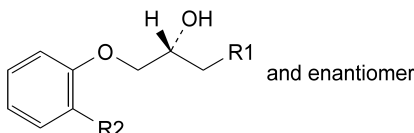
Dissolve 0.400 g in 25 ml of a mixture of equal volumes of *ethanol R* and *water R*. Add 10 ml of 0.01 M *hydrochloric acid*. Carry out a potentiometric titration (2.2.20), using 0.1 M *sodium hydroxide*. Read the volume added between the two points of inflexion.

1 ml of 0.1 M *sodium hydroxide* is equivalent to 28.58 mg of $C_{15}H_{24}ClNO_2$.

STORAGE

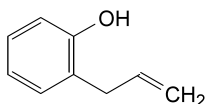
Store protected from light.

IMPURITIES

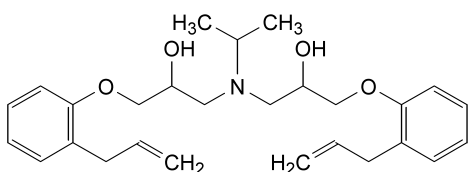


A. R1 = OH, R2 = $CH_2-CH=CH_2$: (2*RS*)-3-[2-(prop-2-enyl)phenoxy]propan-1,2-diol,

C. R1 = $NH-CH(CH_3)_2$, R2 = $CH=CH-CH_3$: (2*RS*)-1-[(1-methylethyl)amino]-3-[2-(prop-1-enyl)phenoxy]propan-2-ol,



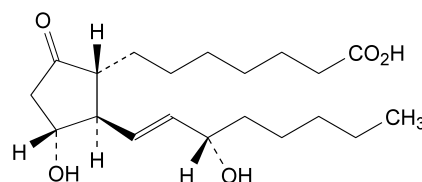
B. 2-(prop-2-enyl)phenol,



D. 1,1'-(1-methylethyl)imino]bis[3-[2-(prop-2-enyl)phenoxy]propan-2-ol].

ALPROSTADIL

Alprostadilum


 $C_{20}H_{34}O_5$
 M_r 354.5

DEFINITION

7-[(1*R*,2*R*,3*R*)-3-Hydroxy-2-[(1*E*,3*S*)-3-hydroxyoct-1-enyl]-5-oxocyclopentyl]heptanoic acid.

Content: 95.0 per cent to 102.5 per cent (anhydrous substance).

CHARACTERS

Appearance: white or slightly yellowish, crystalline powder.

Solubility: practically insoluble in water, freely soluble in alcohol, soluble in acetone, slightly soluble in ethyl acetate.

IDENTIFICATION

A. Specific optical rotation (2.2.7): -60 to -70 (anhydrous substance).

Immediately before use, dissolve 50 mg in *alcohol R* and dilute to 10.0 ml with the same solvent.

B. Infrared absorption spectrophotometry (2.2.24).

Preparation: discs.

Comparison: *alprostadil CRS*.

C. Examine the chromatograms obtained in the assay.

Results: the principal peak in the chromatogram obtained with the test solution is similar in retention time and size to the principal peak in the chromatogram obtained with the reference solution.

TESTS

Related substances. Liquid chromatography (2.2.29).

Prepare the solutions protected from light.

Test solution. Dissolve 10.0 mg of the substance to be examined in a mixture of equal volumes of *acetonitrile R1* and *water R* and dilute to 10.0 ml with the same mixture of solvents.

Reference solution (a). Dilute 100 µl of the test solution to 20.0 ml with a mixture of equal volumes of *acetonitrile R1* and *water R*.

Reference solution (b). Dissolve 1.0 mg of *dinoprostone impurity C CRS* (alprostadil impurity H) and 1.0 mg of *alprostadil CRS* in a mixture of equal volumes of *acetonitrile R1* and *water R* and dilute to 20.0 ml with the same mixture of solvents.

Reference solution (c). In order to prepare *in situ* the degradation compounds (impurity A and impurity B), dissolve 1 mg of the substance to be examined in 100 µl of 1 M *sodium hydroxide* (the solution becomes brownish-red), wait for 3 min and add 100 µl of 1 M *phosphoric acid* (yellowish-white opalescent solution); dilute to 5.0 ml with a mixture of equal volumes of *acetonitrile R1* and *water R*.

System A

Column:

— *size*: $l = 0.25$ m, $\varnothing = 4.0$ mm,

- *stationary phase*: octylsilyl base-deactivated silica gel for chromatography R (4 µm) with a pore size of 6 nm,
- *temperature*: 35 °C.

Mobile phase:

- *mobile phase A*. Dissolve 3.9 g of *sodium dihydrogen phosphate R* in *water R* and dilute to 1000.0 ml with the same solvent; adjust to pH 2.5 with a 2.9 g/l solution of *phosphoric acid R* (approximately 600 ml is required); to 740 ml of the buffer solution add 260 ml of *acetonitrile R1*;
- *mobile phase B*. Dissolve 3.9 g of *sodium dihydrogen phosphate R* in *water R* and dilute to 1000.0 ml with the same solvent; adjust to pH 2.5 with a 2.9 g/l solution of *phosphoric acid R* (approximately 600 ml is required); to 200 ml of the buffer solution add 800 ml of *acetonitrile R1*;

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)
0 - 75	100	0
75 - 76	100 → 0	0 → 100
76 - 86	0	100
86 - 87	0 → 100	100 → 0
87 - 102	100	0

Flow rate: 1 ml/min.

Detection: spectrophotometer at 200 nm.

Injection: 20 µl loop injector.

System suitability:

- *retention time*: alprostadil = about 63 min,
- *resolution*: minimum of 1.5 between the peaks due to impurity H and alprostadil in the chromatogram obtained with reference solution (b).

System B

Use the same conditions as for system A with the following mobile phase and elution programme:

- *mobile phase A*. Dissolve 3.9 g of *sodium dihydrogen phosphate R* in *water R* and dilute to 1000.0 ml with the same solvent; adjust to pH 2.5 with a 2.9 g/l solution of *phosphoric acid R* (approximately 600 ml is required); to 600 ml of the buffer solution add 400 ml of *acetonitrile R1*;
- *mobile phase B*. Use mobile phase B as described under system A;

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)
0 - 50	100	0
50 - 51	100 → 0	0 → 100
51 - 61	0	100
61 - 62	0 → 100	100 → 0
62 - 72	100	0

System suitability:

- *relative retentions* with reference to alprostadil (retention time = about 7 min): impurity A = about 2.4; impurity B = about 2.6,
- *resolution*: minimum of 1.5 between the peaks due to impurity A and impurity B in the chromatogram obtained with reference solution (c).

Carry out the test according to system A and B.

Limits:

- *correction factors*: multiply the areas of the corresponding peaks using the correction factors in Table 1488-1 to obtain the corrected areas,

Table 1488-1

Impurity	Relative retention (system A)	Relative retention (system B)	Correction factor
impurity G	0.80	-	0.7
impurity F	0.88	-	0.8
impurity D	0.90	-	1.0
impurity H	0.96	-	0.7
impurity E	1.10	-	0.7
impurity C	-	1.36	1.9
impurity K	-	1.85	0.06
impurity A	-	2.32	0.7
impurity B	-	2.45	1.5
impurity I	-	4.00	1.0
impurity J	-	5.89	1.0

- *impurity A (corrected area)*: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (a) (1.5 per cent),
- *impurity B (corrected area)*: not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent),
- *any other impurity (corrected area)*: not more than 1.8 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.9 per cent), and not more than 1 such peak has an area greater than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent). Evaluate impurities appearing at relative retentions less than 1.2 by system A and impurities appearing at relative retentions greater than 1.2 by system B,
- *total (corrected area)*: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (a) (1.5 per cent),
- *disregard limit*: 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

Water (2.5.32): maximum 0.5 per cent, determined on 50 mg.

ASSAY

Liquid chromatography (2.2.29) as described in the test for related substances, system A. *Prepare the solutions protected from light.*

Test solution. Dissolve 10.0 mg of the substance to be examined in a mixture of equal volumes of *acetonitrile R1* and *water R* and dilute to 25.0 ml with the same mixture of solvents. Dilute 3.0 ml of the solution to 20.0 ml with a mixture of equal volumes of *acetonitrile R1* and *water R*.

Reference solution. Dissolve 10.0 mg of *alprostadil CRS* in a mixture of equal volumes of *acetonitrile R1* and *water R* and dilute to 25.0 ml with the same mixture of solvents. Dilute 3.0 ml of the solution to 20.0 ml with a mixture of equal volumes of *acetonitrile R1* and *water R*.

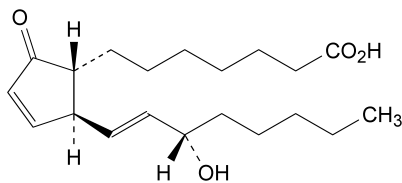
Injection: 20 µl.

Calculate the percentage content of C₂₀H₃₄O₅.

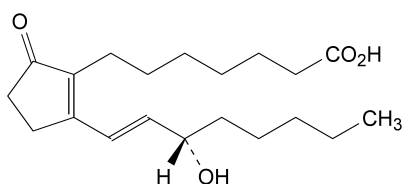
STORAGE

At a temperature of 2 °C to 8 °C.

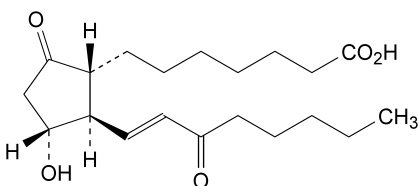
IMPURITIES



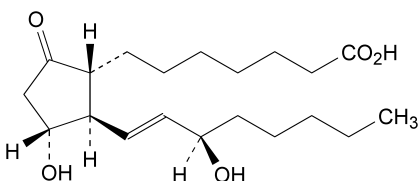
A. 7-[(1*R*,2*S*)-2-[(1*E*,3*S*)-3-hydroxyoct-1-enyl]-5-oxocyclopent-3-enyl]heptanoic acid (prostaglandin A₁),



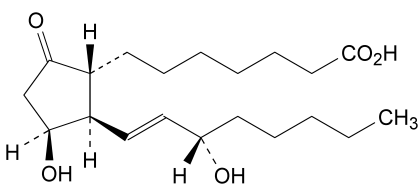
B. 7-[2-[(1*E*,3*S*)-3-hydroxyoct-1-enyl]-5-oxocyclopent-1-enyl]heptanoic acid (prostaglandin B₁),



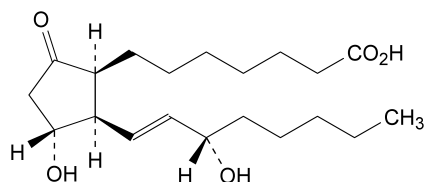
C. 7-[(1*R*,2*R*,3*R*)-3-hydroxy-2-[(1*E*)-3-oxooct-1-enyl]-5-oxocyclopentyl]heptanoic acid (15-oxoprostaglandin E₁),



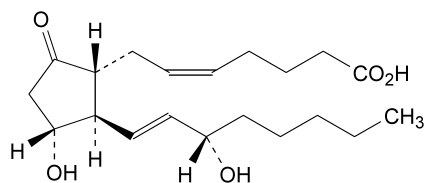
D. 7-[(1*R*,2*R*,3*R*)-3-hydroxy-2-[(1*E*,3*R*)-3-hydroxyoct-1-enyl]-5-oxocyclopentyl]heptanoic acid (15-epiprostaglandin E₁),



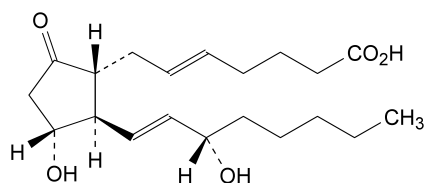
E. 7-[(1*R*,2*R*,3*S*)-3-hydroxy-2-[(1*E*,3*S*)-3-hydroxyoct-1-enyl]-5-oxocyclopentyl]heptanoic acid (11-epiprostaglandin E₁),



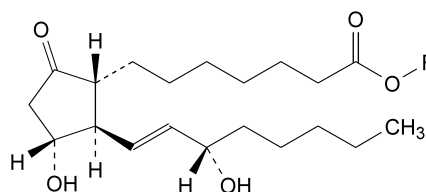
F. 7-[(1*S*,2*R*,3*R*)-3-hydroxy-2-[(1*E*,3*S*)-3-hydroxyoct-1-enyl]-5-oxocyclopentyl]heptanoic acid (8-epiprostaglandin E₁),



G. (5*Z*)-7-[(1*R*,2*R*,3*R*)-3-hydroxy-2-[(1*E*,3*S*)-3-hydroxyoct-1-enyl]-5-oxocyclopentyl]hept-5-enoic acid (dinoprostone),

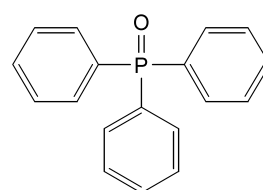


H. (5*E*)-7-[(1*R*,2*R*,3*R*)-3-hydroxy-2-[(1*E*,3*S*)-3-hydroxyoct-1-enyl]-5-oxocyclopentyl]hept-5-enoic acid ((5*E*)-prostaglandin E₂),



I. R = CH₂-CH₃: ethyl 7-[(1*R*,2*R*,3*R*)-3-hydroxy-2-[(1*E*,3*S*)-3-hydroxyoct-1-enyl]-5-oxocyclopentyl]heptanoate (prostaglandin E₁, ethyl ester),

J. R = CH(CH₃)₂: 1-methylethyl 7-[(1*R*,2*R*,3*R*)-3-hydroxy-2-[(1*E*,3*S*)-3-hydroxyoct-1-enyl]-5-oxocyclopentyl]heptanoate (prostaglandin E₁, isopropyl ester),



K. triphenylphosphine oxide.

01/2005:1170
corrected

ALTEPLASE FOR INJECTION

Alteplasmum ad iniectionabile

```

SYQVICRDEK  TQMIYQQHQS  WLRPVLRSNR  VEYCWCNSGR
AQCHSVPVKs  CSEPRCFNGG  TCQQALYFSD  FVCQCPEGFA
GKCCEIDTRA  TCYEDQGISI  RGTWSTAESG  AECTNWNSSA
LAQKPYSGR  PDAIRLGLGN  HNYCRNPDRD  SKPWCYVFKA
GKYSSEFCST  PACSEGNSDC  YFGNGSAYRG  THSLTESGAS
CLPWNMILI  GKVYTAQNPS  AQALGLGKHN  YCNRPDGDAK
PWCHVLKNRR  LTWEYCDVPS  CSTCGLRQYS  QPQFR
                                     IKGGL
FADIASHPWQ  AAIFAKHRRS  PGERFLCGGI  LISSCWILSA
AHCQERFPP  HHLTIVILGRT  YRVVPGEESQ  KFEVEKYIVH
KEFDDDTYDN  DIALQLKSD  SSRCAQESSV  VRTVCLPPAD
LQLPDWTECE  LSGYGKHEAL  SPFYSERLKE  AHVRLYPSSR
CTSQHLLNRT  VTDNMLCAGD  TRSGGPQANL  HDACQDSSGG
PLVCLNDGRM  TLVGIIISWGL  GCGQKDVPGV  YTKVTNYLDW
IRDNMRP

```

DEFINITION

Alteplase for injection is a sterile, freeze-dried preparation of alteplase, a tissue plasminogen activator produced by recombinant DNA technology. It has a potency of not less than 500 000 IU per milligram of protein.

Tissue plasminogen activator binds to fibrin clots and activates plasminogen, leading to the generation of plasmin and to the degradation of fibrin clots or blood coagulates.

Alteplase consists of 527 amino acids with a calculated relative molecular mass of 59 050 without consideration of the carbohydrate moieties attached at positions Asn 117, Asn 184 and Asn 448. The total relative molecular mass is approximately 65 000. Alteplase is cleaved by plasmin between amino-acids 275 and 276 into a two-chain form (A chain and B chain) that are connected by a disulphide bridge between Cys 264 and Cys 395. The single-chain form and the two-chain form show comparable fibrinolytic activity *in vitro*.

PRODUCTION

Alteplase is produced by recombinant DNA synthesis in cell culture; the fermentation takes place in serum-free medium.

The purification process is designed to remove efficiently potential impurities, such as antibiotics, DNA and protein contaminants derived both from the host cell and from the production medium, and potential viral contaminants.

If alteplase is stored in bulk form, stability (maintenance of potency) in the intended storage conditions must be demonstrated.

The production, purification and product consistency are checked by a number of analytical methods described below, carried out routinely as in-process controls.

Protein content. The protein concentration of alteplase solutions is determined by measuring the absorbance (2.2.25) of the protein solution at 280 nm and at 320 nm, using formulation buffer as the compensation liquid. If

dilution of alteplase samples is necessary, the samples are diluted in formulation buffer. For the calculation of the alteplase concentration the absorbance value ($A_{280} - A_{320}$) is divided by the specific absorption coefficient for alteplase of 1.9.

Potency. The potency of alteplase is determined in an *in-vitro* clot-lysis assay as described under Assay. The specific activity of bulk alteplase is approximately 580 000 IU per milligram of alteplase.

N-terminal sequence. N-terminal sequencing is applied to determine the correct N-terminal sequence and to determine semiquantitatively additional cleavage sites in the alteplase molecule, for example at position AA 275-276 or at position AA 27-28. The N-terminal sequence must conform with the sequence of human tissue plasminogen activator.

Isoelectric focusing. The consistency in the microheterogeneity of glycosylation of the alteplase molecule can be demonstrated by isoelectric focusing (IEF). A complex banding pattern with ten major and several minor bands in the pH range 6.5-8.5 is observed. Denaturing conditions are applied to achieve a good separation of differently charged variants of alteplase. The broad charge distribution observed is due to a population of molecules, which differ in the fine structure of biantennary and triantennary complex-type carbohydrate residues, with different degrees of substitution with sialic acids. The banding pattern of alteplase test samples must be consistent with the pattern of alteplase reference standard.

Single-chain alteplase content. The alteplase produced by CHO (Chinese hamster ovary) cells in serum-free medium is predominantly single-chain alteplase. The single-chain form can be separated from the two-chain form by gel-permeation liquid chromatography under reducing conditions as described under Single-chain content (see Tests). The single-chain alteplase content in bulk samples must be higher than 60 per cent.

Tryptic-peptide mapping. The primary structure of the alteplase molecule is verified by tryptic-peptide mapping as described under Identification B. The reduced and carboxymethylated molecule is cleaved by trypsin into about fifty peptides, which are separated by reverse-phase liquid chromatography. A characteristic chromatogram (fingerprint) is obtained. The identity of the tryptic-peptide map of a given alteplase sample with the profile of a well-characterised reference standard is an indirect confirmation of the amino-acid sequence, because even single amino-acid exchanges in individual peptides can be detected by this sensitive technique. In addition, complex peaks of the glycopeptides can be isolated from the tryptic-peptide map and separated in a second dimension, either by reverse-phase liquid chromatography under modified conditions or by capillary electrophoresis. By this two-dimensional separation of glycopeptide variants, lot-to-lot consistency of the microheterogeneity of glycosylation can be demonstrated.

The tryptic-peptide map of alteplase samples must be consistent with the tryptic-peptide map of alteplase reference standard.

Monomer content. The monomer content of alteplase is measured by gel-permeation liquid chromatography under non-reduced conditions as described under Monomer content (see Tests). The monomer content of alteplase bulk samples must be higher than 95 per cent.

Type I/Type II alteplase content. CHO cells produce two glycosylation variants of alteplase. Type I alteplase contains one polymannose-type glycosylation at position Asn 117 and

two complex-type glycosylation sites at positions Asn 184 and Asn 448. Type II alteplase is only glycosylated at positions Asn 117 and Asn 448.

The ratio of Type I/Type II alteplase is constant in the range of 45 to 65 per cent of Type I and 35 to 55 per cent of Type II. The content of alteplase Type I and Type II can be determined by a densitometric scan of SDS-PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis) gel. Plasmin-treated samples of alteplase, which are reduced and carboxymethylated before loading on the gel, are separated into three bands: Type I alteplase A-chain (AA 1-275), Type II alteplase A-chain (AA 1-275) and alteplase B-chain (AA 276-527). The ratio of Type I/Type II alteplase is determined from a calibration curve, which is obtained by a densitometric scan of defined mixtures of purified Type I alteplase and Type II alteplase standards.

SDS-PAGE. SDS-PAGE (silver staining) is used to demonstrate purity of the alteplase bulk material and the integrity of the alteplase molecule. For alteplase bulk samples, no additional protein bands compared to reference standard or degradation products must occur in SDS-PAGE gels at a loading amount of 2.5 µg alteplase protein per lane and a limit of detection of 5 ng per protein (BSA) band.

Bacterial endotoxins (2.6.14): less than 1 IU per milligram of alteplase.

Sialic acids. Dialyse samples and alteplase reference standard against enzyme buffer (8.9 g/l *sodium chloride R*, 4.1 g/l *sodium acetate R*, pH 5.5) using a membrane with a cut-off point corresponding to a relative molecular mass of 10 000 for globular proteins. After dialysis, determine the protein concentration. Add 5 µl of calcium chloride solution (19.98 per cent *m/m calcium chloride R*) to 1 ml of protein solution. Add 10 milliunits of neuraminidase per milligram of protein. Incubate this solution at 37 °C for about 17 h.

Prepare standard dilutions between 1.56 mg/ml and 25.0 mg/ml from an *N-acetylneuraminic acid R* reference stock solution with a concentration of 50 mg/ml. Pipette 0.2 ml of the sample and of the protein reference standard in duplicate into reagent tubes. Pipette also 0.2 ml of the standard dilutions into reagent tubes. Add 0.25 ml of periodate reagent (5.4 g/l solution of *sodium periodate R* in a 1.25 per cent *V/V* solution of *sulphuric acid R*), mix and incubate for 30 min at 37 °C. Add 0.2 ml of arsenite reagent (20 g/l solution of *sodium arsenite R* in a 1.55 per cent *V/V* solution of *hydrochloric acid R*) and mix. A yellowish-brown colour develops and disappears. Add 2.0 ml of a 28.9 g/l solution of *thiobarbituric acid R* and mix. Heat the closed tubes in boiling water for 7.5 min and then cool them in an ice-bath for 5 min. Add 2.0 ml of a mixture of *butanol R* and *hydrochloric acid R* (95:5) and mix. Centrifuge the tubes at 3000 r/min for 3 min. Measure the absorbance of the butanol-HCl layer at 552 nm within 30 min using the butanol-HCl mixture as the compensation solution. Perform a linear-regression analysis for the *N-acetylneuraminic acid* standard. The molar content of *N-acetylneuraminic acid* for the samples and for alteplase reference standard is calculated from the calibration curve. The sialic acids content for the test samples must be within the range 70 to 130 per cent of alteplase reference standard, which contains about 3 moles of sialic acids per mole of alteplase.

Neutral sugars. Dilute alteplase samples and the reference standard in the assay buffer, containing 34.8 g/l of *arginine R*, 0.1 g/l of *polysorbate 80 R* and adjusted to pH 7.4 with *phosphoric acid R*, to a protein concentration of 50 µg/ml. Prepare the following concentrations of mannose in the same assay buffer for a calibration curve: 20, 30, 40, 50 and 60 µg/ml. Pipette 2 ml of alteplase samples

and reference standard, as well as 2 ml of each mannose concentration in duplicate in reagent tubes. Add 50 µl of *phenol R*, followed by 5 ml of *sulphuric acid R* in each reagent tube. Incubate the mixture for 30 min at room temperature. Measure the absorbance at 492 nm for each tube. Read the content of neutral sugars from the mannose calibration curve. The neutral sugar content is expressed in moles of neutral sugar per mole of alteplase, taking into account the dilution factor for alteplase samples and reference standard and using a relative molecular mass of 180.2 for mannose and a relative molecular mass of 59 050 for the alteplase protein moiety. The neutral sugar content of the alteplase samples must be in the range of 70 to 130 per cent compared to alteplase reference standard, which contains about 12 moles of neutral sugar per mole of alteplase.

CHARACTERS

A white or slightly yellow powder or solid friable mass.

Reconstitute the preparation as stated on the label immediately before carrying out the Identification, Tests (except those for solubility and water) and Assay.

IDENTIFICATION

A. The assay serves also to identify the preparation.

B. Tryptic-peptide mapping. Examine by liquid chromatography (2.2.29).

Test solution. Dilute the preparation to be examined with *water R* to obtain a solution containing about 1 mg of alteplase per millilitre. Dialyse about 2.5 ml of the solution for at least 12 h into a solution containing 480 g/l of *urea R*, 44 g/l of *tris(hydroxymethyl)aminomethane R* and 1.5 g/l of *disodium edetate R* and adjusted to pH 8.6, using a membrane with a cut-off point corresponding to a relative molecular mass of 10 000 for globular proteins. Measure the volume of the solution, transfer it to a clean test-tube and add per millilitre 10 µl of a 156 g/l solution of *dithiothreitol R*. Allow to stand for 4 h, cool in iced water and add per millilitre of solution 25 µl of a freshly prepared 190 g/l solution of *iodoacetic acid R*. Allow to stand in the dark for 30 min. Add per millilitre 50 µl of *dithiothreitol* solution to stop the reaction. Dialyse for 24 h against an 8 g/l solution of *ammonium hydrogen carbonate R*. Add 1 part of *trypsin for peptide mapping R* to 100 parts of the protein and allow to stand for 6 h to 8 h. Repeat the addition of trypsin and allow to stand for a total of 24 h.

Reference solution. Prepare as for the test solution using *alteplase CRS* instead of the preparation to be examined.

The chromatographic procedure may be carried out using:

- a column 0.1 m long and 4.6 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (5 µm to 10 µm),
Mobile phase A. A 8 g/l solution of *sodium dihydrogen phosphate R*, adjusted to pH 2.85 with *phosphoric acid R*, filtered and degassed,
Mobile phase B. *Acetonitrile R* 75 per cent *V/V* in mobile phase A,
- as detector a spectrophotometer set at 210 nm.

Equilibrate the system with mobile phase A at a flow rate of 1 ml/min. After injection of the solution, increase the proportion of mobile phase B at a rate of 0.44 per cent per minute until the ratio of mobile phase A to mobile phase B is 60:40 and then increase the proportion of mobile phase B at a rate of 1.33 per cent per minute until the ratio of mobile phase A to mobile phase B is 20:80

and then continue elution with this mixture for a further 10 min. Record the chromatogram for the reference solution: the test is not valid unless the resolution of peaks 6 (peptides 268-275) and 7 (peptides 1-7) is at least 1.5; $b_{0.5a}$ and $b_{0.5b}$ are not more than 0.4 min. Inject about 100 µl of the test solution and record the chromatogram. Verify the identity of the peaks by comparison with the chromatograms of the reference solution. There should not be any additional significant peaks or shoulders, a significant peak or shoulder being defined as one with an area response equal to or greater than 5 per cent of peak 19 (peptides 278-296); no significant peak is missing. A type chromatogram for identification of the peaks cited is given at the end of the monograph (see Figure 1170.-1).

TESTS

Appearance of solution. The reconstituted preparation is clear (2.2.1) and not more intensely coloured than reference solution Y₇ (2.2.2, Method II).

pH (2.2.3): 7.1 to 7.5.

Solubility. Add the volume of the liquid stated on the label. The preparation dissolves completely within 2 min at 20 °C to 25 °C.

Protein content. Prepare a solution of the substance to be examined with an accurately known concentration of about 1 g/l. Using a 34.8 g/l solution of *arginine R* adjusted to pH 7.3 with *phosphoric acid R*, dilute an accurately measured volume of the solution of the substance to be examined so that the absorbance measured at the maximum at about 280 nm is 0.5 to 1.0 (*test solution*). Measure the absorbance (2.2.25) of the solution at the maximum at about 280 nm and at 320 nm using the arginine solution as the compensation liquid. Calculate the protein content in the portion of alteplase taken from the expression:

$$\frac{V(A_{280} - A_{320})}{1.9}$$

in which V is the volume of arginine solution required to prepare the test solution, A_{280} is the absorbance at the maximum at about 280 nm and A_{320} is the absorbance at 320 nm.

Single-chain content. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve the preparation to be examined in *water R* to obtain a solution containing about 1 mg of alteplase per millilitre. Place about 1 ml of the solution in

a tube, add 3 ml of a 3 g/l solution of *dithiothreitol R* in the mobile phase, place a cap on the tube and heat at about 80 °C for 3 min to 5 min.

The chromatographic procedure may be carried out using:

- a column 0.6 m long and 7.5 mm in internal diameter packed with silica-based rigid, hydrophilic gel with spherical particles 10 µm to 13 µm in diameter, suitable for size-exclusion chromatography,
- as mobile phase at a flow rate of 0.5 ml/min a solution containing 30 g/l of *sodium dihydrogen phosphate R* and 1 g/l of *sodium dodecyl sulphate R*, adjusted to pH 6.8 with *dilute sodium hydroxide solution R*,
- as detector a spectrophotometer set at 214 nm.

Inject about 50 µl of the test solution and record the chromatogram. The chromatogram shows two major peaks corresponding to single-chain and two-chain alteplase. Calculate the relative amount of single-chain alteplase from the peak area values.

The test is not valid unless: the number of theoretical plates calculated on the basis of the single-chain alteplase peak is at least 1000. The content of single-chain alteplase is not less than 60 per cent of the total amount of alteplase-related substances found.

Monomer content. Examine by liquid chromatography (2.2.29).

Test solution. Reconstitute the preparation to be examined to obtain a solution containing about 1 mg per millilitre.

The chromatographic procedure may be carried out using:

- a column 0.6 m long and 7.5 mm in internal diameter packed with silica-based rigid, hydrophilic gel with spherical particles 10 µm to 13 µm in diameter, suitable for size-exclusion chromatography,
- as mobile phase at a flow rate of 0.5 ml/min a solution containing 30 g/l of *sodium dihydrogen phosphate R* and 1 g/l of *sodium dodecyl sulphate R*, adjusted to pH 6.8 with *dilute sodium hydroxide solution R*,
- as detector a spectrophotometer set at 214 nm.

Inject the test solution and record the chromatogram. The test is not valid unless the number of theoretical plates calculated for the alteplase monomer peak is at least 1000. Measure the response for all peaks, i.e. peaks corresponding to alteplase species of different molecular masses. Calculate the relative content of monomer from the area values of these peaks. The monomer content for alteplase must be at least 95 per cent.

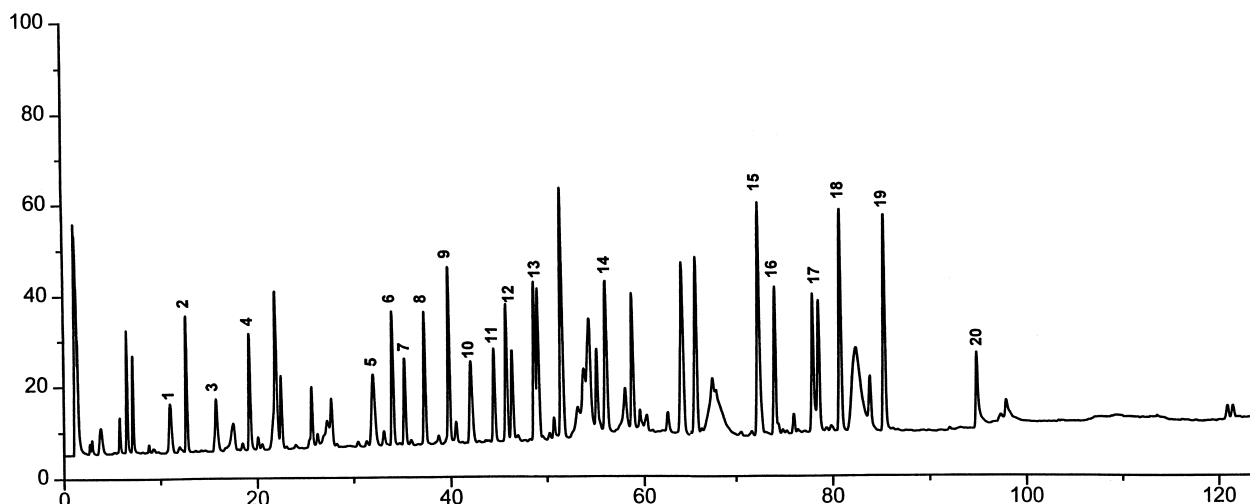


Figure 1170.-1. – Chromatogram for tryptic-peptide mapping of alteplase

Water (2.5.12). Not more than 4.0 per cent, determined by the semi-micro determination of water.

Bacterial endotoxins (2.6.14): less than 1 IU per milligram of protein.

Sterility (2.6.1). It complies with the test for sterility.

ASSAY

The potency of alteplase is determined by comparing its ability to activate plasminogen to form plasmin with the same capacity of a reference preparation calibrated in International Units. The formation of plasmin is measured by the determination of the lysis time of a fibrin clot in given conditions.

The International Unit is the activity of a stated quantity of the International Standard of alteplase. The equivalence in International Units of the International Standard is stated by the World Health Organisation.

Solvent buffer. A solution containing 1.38 g/l of *sodium dihydrogen phosphate monohydrate R*, 7.10 g/l of *anhydrous disodium hydrogen phosphate R*, 0.20 g/l of *sodium azide R* and 0.10 g/l of *polysorbate 80 R*.

Human thrombin solution. A solution of *human thrombin R* containing 33 IU/ml in solvent buffer.

Human fibrinogen solution. A 2 g/l solution of *fibrinogen R* in solvent buffer.

Human plasminogen solution. A 1 g/l solution of *human plasminogen R* in solvent buffer.

Test solutions. Using a solution of the substance to be examined containing 1 g/l, prepare serial dilutions using solvent buffer, for example 1:5000, 1:10 000, 1:20 000.

Reference solutions. Using a solution of *alteplase CRS* having an accurately known concentration of about 1 g/l (580 000 IU of alteplase per millilitre), prepare five serial dilutions using *water R* to obtain reference solutions having known concentrations in the range 9.0 IU/ml to 145 IU/ml.

To each of a set of labelled glass test-tubes, add 0.5 ml of human thrombin solution. Allocate each test and reference solution to a separate tube and add to each tube 0.5 ml of the solution allocated to it. To each of a second set of labelled glass tubes, add 20 µl of human plasminogen solution, and 1 ml of human fibrinogen solution, mix and store on ice. Beginning with the reference/thrombin mixture containing the lowest number of International Units per millilitre, record the time and separately add 200 µl of each of the thrombin mixtures to the test tubes containing the plasminogen-fibrinogen mixture. Using a vortex mixture, intermittently mix the contents of each tube for a total of 15 s and carefully place in a rack in a circulating water-bath at 37 °C. A visibly turbid clot forms within 30 s and bubbles subsequently form within the clot. Record the clot-lysis time as the time between the first addition of alteplase solution and the moment when the last bubble rises to the surface. Using a least-squares fit, determine the equation of the line using the logarithms of the concentrations of the reference preparation in International Units per millilitre versus the logarithms of the values of their clot-lysis times in seconds, according to the equation:

$$\log t = a + b(\log U_s)$$

in which t is the clot-lysis time, U_s the activity in International Units per millilitre of the reference preparation, b is the slope and a the y -intercept of the line. The test is not valid unless the correlation coefficient is -0.9900 to -1.0000 . From the line equation and the clot-lysis time for the test solution, calculate the logarithm of the activity U_A from the equation:

$$\log U_A = \frac{[(\log t) - a]}{b}$$

Calculate the alteplase activity in International Units per millilitre from the expression:

$$D \times U_A$$

in which D is the dilution factor for the test solution. Calculate the specific activity in the portion of the substance to be examined from the expression:

$$\frac{U_A}{P}$$

in which P is the concentration of protein obtained in the test for protein content.

The estimated potency is not less than 90 per cent and not more than 110 per cent of the stated potency.

STORAGE

Store in a colourless, glass container, under vacuum or under an inert gas, protected from light, at a temperature between 2 °C and 30 °C.

LABELLING

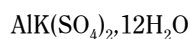
The label states:

- the number of International Units per container,
- the amount of protein per container.

01/2005:0006

ALUM

Alumen



M_r 474.4

DEFINITION

Alum contains not less than 99.0 per cent and not more than the equivalent of 100.5 per cent of $\text{AlK}(\text{SO}_4)_2 \cdot 12\text{H}_2\text{O}$.

CHARACTERS

A granular powder or colourless, transparent, crystalline masses, freely soluble in water, very soluble in boiling water, soluble in glycerol, practically insoluble in alcohol.

IDENTIFICATION

- A. Solution S (see Tests) gives the reactions of sulphates (2.3.1).
- B. Solution S gives the reaction of aluminium (2.3.1).
- C. Shake 10 ml of solution S with 0.5 g of *sodium bicarbonate R* and filter. The filtrate gives reaction (a) of potassium (2.3.1).

TESTS

Solution S. Dissolve 2.5 g in *water R* and dilute to 50 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and colourless (2.2.2, *Method II*).

pH (2.2.3). Dissolve 1.0 g in *carbon dioxide-free water R* and dilute to 10 ml with the same solvent. The pH of the solution is 3.0 to 3.5.

Ammonium (2.4.1). To 1 ml of solution S add 4 ml of *water R*. Dilute 0.5 ml of the solution to 14 ml with the same solvent. The solution complies with the limit test for ammonium (0.2 per cent).

Iron (2.4.9). 2 ml of solution S diluted to 10 ml with *water R* complies with the limit test for iron (100 ppm). Use in this test 0.3 ml of *thioglycollic acid R*.

Heavy metals (2.4.8). 12 ml of solution S complies with limit test A for heavy metals (20 ppm). Prepare the standard using *lead standard solution (1 ppm Pb) R*.

ASSAY

Dissolve 0.900 g in 20 ml of *water R* and carry out the complexometric titration of aluminium (2.5.11).

1 ml of 0.1 M *sodium edetate* is equivalent to 47.44 mg of $\text{AlK}(\text{SO}_4)_2 \cdot 12\text{H}_2\text{O}$.

01/2005:0971

ALUMINIUM CHLORIDE HEXAHYDRATE

Aluminii chloridum hexahydricum

$\text{AlCl}_3 \cdot 6\text{H}_2\text{O}$

M_r 241.4

DEFINITION

Aluminium chloride hexahydrate contains not less than 95.0 per cent and not more than the equivalent of 101.0 per cent of $\text{AlCl}_3 \cdot 6\text{H}_2\text{O}$.

CHARACTERS

A white or slightly yellow, crystalline powder or colourless crystals, deliquescent, very soluble in water, freely soluble in alcohol, soluble in glycerol.

IDENTIFICATION

- Dilute 0.1 ml of solution S2 (see Tests) to 2 ml with *water R*. The solution gives reaction (a) of chlorides (2.3.1).
- Dilute 0.3 ml of solution S2 to 2 ml with *water R*. The solution gives the reaction of aluminium (2.3.1).

TESTS

Solution S1. Dissolve 10.0 g in *distilled water R* and dilute to 100 ml with the same solvent.

Solution S2. Dilute 50 ml of solution S1 to 100 ml with *water R*.

Appearance of solution. Solution S2 is clear (2.2.1) and not more intensely coloured than reference solution B₇ (2.2.2, *Method II*).

Sulphates (2.4.13). 15 ml of solution S1 complies with the limit test for sulphates (100 ppm).

Iron (2.4.9). 10 ml of solution S1 complies with the limit test for iron (10 ppm).

Alkali and alkaline-earth metals. To 20 ml of solution S2 add 100 ml of *water R* and heat to boiling. To the hot solution add 0.2 ml of *methyl red solution R*. Add *dilute ammonia RI* until the colour of the indicator changes to yellow and dilute to 150 ml with *water R*. Heat to boiling and filter. Evaporate 75 ml of the filtrate to dryness on a water-bath and ignite to constant mass. The residue weighs not more than 2.5 mg (0.5 per cent).

Heavy metals (2.4.8). 12 ml of solution S1 complies with limit test A for heavy metals (20 ppm). Prepare the standard using *lead standard solution (2 ppm Pb) R*.

Water (2.5.12): 42.0 per cent to 48.0 per cent, determined on 50.0 mg by the semi-micro determination of water.

ASSAY

Dissolve 0.500 g in 25.0 ml of *water R*. Carry out the complexometric titration of aluminium (2.5.11). Titrate with 0.1 M *zinc sulphate* until the colour of the indicator changes from greyish-green to pink. Carry out a blank titration.

1 ml of 0.1 M *sodium edetate* is equivalent to 24.14 mg of $\text{AlCl}_3 \cdot 6\text{H}_2\text{O}$.

STORAGE

Store in an airtight container.

01/2005:1664

ALUMINIUM HYDROXIDE, HYDRATED, FOR ADSORPTION

Aluminii hydroxidum hydricum
ad adsorptionem

$[\text{AlO}(\text{OH})]_n \cdot n\text{H}_2\text{O}$

DEFINITION

Content: 90.0 per cent to 110.0 per cent of the content of aluminium stated on the label.

NOTE: shake the gel vigorously for at least 30 s immediately before examining.

CHARACTERS

Appearance: white or almost white, translucent, viscous, colloidal gel. A supernatant may be formed upon standing.

Solubility: a clear or almost clear solution is obtained with alkali hydroxide solutions and mineral acids.

IDENTIFICATION

Solution S (see Tests) gives the reaction of aluminium.

To 10 ml of solution S add about 0.5 ml of *dilute hydrochloric acid R* and about 0.5 ml of *thioacetamide reagent R*. No precipitate is formed. Add dropwise 5 ml of *dilute sodium hydroxide solution R*. Allow to stand for 1 h. A gelatinous white precipitate is formed which dissolves upon addition of 5 ml of *dilute sodium hydroxide solution R*. Gradually add 5 ml of *ammonium chloride solution R* and allow to stand for 30 min. The gelatinous white precipitate is re-formed.

TESTS

Solution S. Add 1 g to 4 ml of *hydrochloric acid R*. Heat at 60 °C for 1 h, cool, dilute to 50 ml with *distilled water R* and filter if necessary.

pH (2.2.3): 5.5 to 8.5.

Adsorption power. Dilute the substance to be examined with *distilled water R* to obtain an aluminium concentration of 5 mg/ml. Prepare *bovine albumin R* solutions with the following concentrations of bovine albumin: 0.5 mg/ml, 1 mg/ml, 2 mg/ml, 3 mg/ml, 5 mg/ml and 10 mg/ml. If necessary, adjust the gel and the *bovine albumin R* solutions to pH 6.0 with *dilute hydrochloric acid R* or *dilute sodium hydroxide solution R*.

For adsorption, mix 1 part of the diluted gel with 4 parts of each of the solutions of *bovine albumin R* and allow to stand at room temperature for 1 h. During this time shake the mixture vigorously at least 5 times. Centrifuge or filter through a non-protein-retaining filter. Immediately determine the protein content (2.5.33, *Method 2*) of either the supernatant or the filtrate.

Iron (2.4.9). 2 ml of solution S diluted to 10 ml with *water R* complies with the limit test for iron (100 ppm). Use in this test 0.3 ml of *thioglycollic acid R*.

Heavy metals (2.4.8). 12 ml of solution S complies with limit test A for heavy metals (20 ppm). Prepare the standard using *lead standard solution (1 ppm Pb) R*.

ASSAY

Dissolve 0.900 g in 20 ml of *water R* and carry out the complexometric titration of aluminium (2.5.11).

1 ml of 0.1 M *sodium edetate* is equivalent to 47.44 mg of $\text{AlK}(\text{SO}_4)_2 \cdot 12\text{H}_2\text{O}$.

01/2005:0971

ALUMINIUM CHLORIDE HEXAHYDRATE

Aluminii chloridum hexahydricum

$\text{AlCl}_3 \cdot 6\text{H}_2\text{O}$

M_r 241.4

DEFINITION

Aluminium chloride hexahydrate contains not less than 95.0 per cent and not more than the equivalent of 101.0 per cent of $\text{AlCl}_3 \cdot 6\text{H}_2\text{O}$.

CHARACTERS

A white or slightly yellow, crystalline powder or colourless crystals, deliquescent, very soluble in water, freely soluble in alcohol, soluble in glycerol.

IDENTIFICATION

- A. Dilute 0.1 ml of solution S2 (see Tests) to 2 ml with *water R*. The solution gives reaction (a) of chlorides (2.3.1).
- B. Dilute 0.3 ml of solution S2 to 2 ml with *water R*. The solution gives the reaction of aluminium (2.3.1).

TESTS

Solution S1. Dissolve 10.0 g in *distilled water R* and dilute to 100 ml with the same solvent.

Solution S2. Dilute 50 ml of solution S1 to 100 ml with *water R*.

Appearance of solution. Solution S2 is clear (2.2.1) and not more intensely coloured than reference solution B₇ (2.2.2, *Method II*).

Sulphates (2.4.13). 15 ml of solution S1 complies with the limit test for sulphates (100 ppm).

Iron (2.4.9). 10 ml of solution S1 complies with the limit test for iron (10 ppm).

Alkali and alkaline-earth metals. To 20 ml of solution S2 add 100 ml of *water R* and heat to boiling. To the hot solution add 0.2 ml of *methyl red solution R*. Add *dilute ammonia RI* until the colour of the indicator changes to yellow and dilute to 150 ml with *water R*. Heat to boiling and filter. Evaporate 75 ml of the filtrate to dryness on a water-bath and ignite to constant mass. The residue weighs not more than 2.5 mg (0.5 per cent).

Heavy metals (2.4.8). 12 ml of solution S1 complies with limit test A for heavy metals (20 ppm). Prepare the standard using *lead standard solution (2 ppm Pb) R*.

Water (2.5.12): 42.0 per cent to 48.0 per cent, determined on 50.0 mg by the semi-micro determination of water.

ASSAY

Dissolve 0.500 g in 25.0 ml of *water R*. Carry out the complexometric titration of aluminium (2.5.11). Titrate with 0.1 M *zinc sulphate* until the colour of the indicator changes from greyish-green to pink. Carry out a blank titration.

1 ml of 0.1 M *sodium edetate* is equivalent to 24.14 mg of $\text{AlCl}_3 \cdot 6\text{H}_2\text{O}$.

STORAGE

Store in an airtight container.

01/2005:1664

ALUMINIUM HYDROXIDE, HYDRATED, FOR ADSORPTION

Aluminii hydroxidum hydricum
ad adsorptionem

$[\text{AlO}(\text{OH})]_n \cdot n\text{H}_2\text{O}$

DEFINITION

Content: 90.0 per cent to 110.0 per cent of the content of aluminium stated on the label.

NOTE: shake the gel vigorously for at least 30 s immediately before examining.

CHARACTERS

Appearance: white or almost white, translucent, viscous, colloidal gel. A supernatant may be formed upon standing.

Solubility: a clear or almost clear solution is obtained with alkali hydroxide solutions and mineral acids.

IDENTIFICATION

Solution S (see Tests) gives the reaction of aluminium.

To 10 ml of solution S add about 0.5 ml of *dilute hydrochloric acid R* and about 0.5 ml of *thioacetamide reagent R*. No precipitate is formed. Add dropwise 5 ml of *dilute sodium hydroxide solution R*. Allow to stand for 1 h. A gelatinous white precipitate is formed which dissolves upon addition of 5 ml of *dilute sodium hydroxide solution R*. Gradually add 5 ml of *ammonium chloride solution R* and allow to stand for 30 min. The gelatinous white precipitate is re-formed.

TESTS

Solution S. Add 1 g to 4 ml of *hydrochloric acid R*. Heat at 60 °C for 1 h, cool, dilute to 50 ml with *distilled water R* and filter if necessary.

pH (2.2.3): 5.5 to 8.5.

Adsorption power. Dilute the substance to be examined with *distilled water R* to obtain an aluminium concentration of 5 mg/ml. Prepare *bovine albumin R* solutions with the following concentrations of bovine albumin: 0.5 mg/ml, 1 mg/ml, 2 mg/ml, 3 mg/ml, 5 mg/ml and 10 mg/ml. If necessary, adjust the gel and the *bovine albumin R* solutions to pH 6.0 with *dilute hydrochloric acid R* or *dilute sodium hydroxide solution R*.

For adsorption, mix 1 part of the diluted gel with 4 parts of each of the solutions of *bovine albumin R* and allow to stand at room temperature for 1 h. During this time shake the mixture vigorously at least 5 times. Centrifuge or filter through a non-protein-retaining filter. Immediately determine the protein content (2.5.33, *Method 2*) of either the supernatant or the filtrate.

It complies with the test if no bovine albumin is detectable in the supernatant or filtrate of the 2 mg/ml *bovine albumin R* solution (maximum level of adsorption) and in the supernatant or filtrate of *bovine albumin R* solutions of lower concentrations. Those containing 3 mg/ml, 5 mg/ml and 10 mg/ml *bovine albumin R* solutions may show bovine albumin in the supernatant or filtrate, proportional to the amount of bovine albumin in the solutions.

Sedimentation. If necessary, adjust the substance to be examined to pH 6.0 using *dilute hydrochloric acid R* or *dilute sodium hydroxide solution R*. Dilute with *distilled water R* to obtain an aluminium concentration of approximately 5 mg/ml. If the aluminium content of the substance to be examined is lower than 5 mg/ml, adjust to pH 6.0 and dilute with a 9 g/l solution of *sodium chloride R* to obtain an aluminium concentration of about 1 mg/ml. After shaking for at least 30 s, place 25 ml of the preparation in a 25 ml graduated cylinder and allow to stand for 24 h.

It complies with the test if the volume of the clear supernatant is less than 5 ml for the gel with an aluminium content of about 5 mg/ml.

It complies with the test if the volume of the clear supernatant is less than 20 ml for the gel with an aluminium content of about 1 mg/ml.

Chlorides (2.4.4): maximum 0.33 per cent.

Dissolve 0.5 g in 10 ml of *dilute nitric acid R* and dilute to 500 ml with *water R*.

Nitrates: maximum 100 ppm.

Place 5 g in a test-tube immersed in ice-water, add 0.4 ml of a 100 g/l solution of *potassium chloride R*, 0.1 ml of *diphenylamine solution R* and, dropwise with shaking, 5 ml of *sulphuric acid R*. Transfer the tube to a water-bath at 50 °C. After 15 min, any blue colour in the solution is not more intense than that in a standard prepared at the same time and in the same manner using 5 ml of *nitrate standard solution (100 ppm NO₃) R*.

Sulphates (2.4.13): maximum 0.5 per cent.

Dilute 2 ml of solution S to 20 ml with *water R*.

Ammonium (2.4.1): maximum 50 ppm.

1.0 g complies with limit test B. Prepare the standard using 0.5 ml of *ammonium standard solution (100 ppm NH₄) R*.

Arsenic (2.4.2, Method A): maximum 1 ppm, determined on 1 g.

Iron (2.4.9): maximum 10 ppm, determined on 1 g.

Heavy metals (2.4.8): maximum 20 ppm.

Dissolve 2.0 g in 10 ml of *dilute nitric acid R* and dilute to 20 ml with *water R*. The solution complies with limit test A. Prepare the standard using *lead standard solution (2 ppm Pb) R*.

Bacterial endotoxins (2.6.14): less than 5 IU of endotoxin per milligram of aluminium, if intended for use in the manufacture of an adsorbed product without a further appropriate procedure for the removal of bacterial endotoxins.

ASSAY

Dissolve 10.00 g in 10 ml of *hydrochloric acid R1*, heating on a water-bath. Cool and dilute to 20 ml with *water R*. To 10 ml of the solution, add *dilute ammonia R1* until a precipitate is obtained. Add the smallest quantity of *dilute hydrochloric acid R* needed to dissolve the precipitate and dilute to 20 ml with *water R*. Carry out the complexometric titration of aluminium (2.5.11). Carry out a blank titration.

STORAGE

At a temperature not exceeding 30 °C. Do not allow to freeze. If the substance is sterile, store in a sterile, airtight, tamper-proof container.

LABELLING

The label states:

- where applicable, that the substance is free from bacterial endotoxins,
- the declared content of aluminium.

01/2005:1388

ALUMINIUM MAGNESIUM SILICATE

Aluminii magnesii silicas

DEFINITION

Aluminium magnesium silicate is a mixture of particles with colloidal particle size of montmorillonite and saponite, free from grit and nonswellable ore. It contains not less than 95.0 per cent and not more than 105.0 per cent of the amount of aluminium and magnesium stated on the label.

CHARACTERS

Almost white powder, granules or plates, practically insoluble in water and in organic solvents.

It swells in water to produce a colloidal dispersion.

IDENTIFICATION

- Fuse 1 g with 2 g of *anhydrous sodium carbonate R*. Warm the residue with *water R* and filter. Acidify the filtrate with *hydrochloric acid R* and evaporate to dryness on a water-bath. 0.25 g of the residue gives the reaction of silicates (2.3.1).
- Dissolve the remainder of the residue obtained in identification test A in a mixture of 5 ml of *dilute hydrochloric acid R* and 10 ml of *water R*. Filter and add *ammonium chloride buffer solution pH 10.0 R*. A white, gelatinous precipitate is formed. Centrifuge, keep the supernatant for identification C. Dissolve the remaining precipitate in *dilute hydrochloric acid R*. The solution gives the reaction of aluminium (2.3.1).
- The supernatant liquid obtained after centrifugation in identification test B gives the reaction of magnesium (2.3.1).

TESTS

pH (2.2.3). Disperse 5.0 g in 100 ml of *carbon dioxide-free water R*. The pH of the dispersion is 9.0 to 10.0.

Arsenic (2.4.2). Transfer 16.6 g to a 250 ml beaker containing 100 ml of *dilute hydrochloric acid R*. Mix, cover with a watch glass and boil gently, with occasional stirring, for 15 min. Allow the insoluble matter to settle and decant the supernatant liquid through a rapid-flow filter paper into a 250 ml volumetric flask, retaining as much sediment as possible in the beaker. To the residue in the beaker add 25 ml of hot *dilute hydrochloric acid R*, stir, heat to boiling, allow the insoluble matter to settle and decant the supernatant liquid through the filter into the volumetric flask. Repeat the extraction with four additional quantities, each of 25 ml, of hot *dilute hydrochloric acid R*, decanting each supernatant liquid through the filter into the volumetric flask. At the last extraction, transfer as much of the insoluble matter as possible onto the filter. Allow to cool the combined filtrates to room temperature and dilute to 250.0 ml with *dilute*

hydrochloric acid R. Dilute 5.0 ml of the solution to 25.0 ml with *dilute hydrochloric acid R*. The solution complies with limit test A for arsenic (3 ppm).

Lead. Not more than 15 ppm of Pb, determined by atomic absorption spectrometry (2.2.23, *Method I*).

Test solution. Transfer 10.0 g to a 250 ml beaker containing 100 ml of *dilute hydrochloric acid R*. Mix, cover with a watch glass and boil for 15 min. Allow to cool to room temperature and allow the insoluble matter to settle. Decant the supernatant liquid through a rapid-flow filter paper into a 400 ml beaker. To the insoluble matter in the 250 ml beaker add 25 ml of hot *water R*. Stir, allow the insoluble matter to settle and decant the supernatant liquid through the filter into the 400 ml beaker. Repeat the extraction with two additional quantities, each of 25 ml, of *water R*, decanting each supernatant liquid through the filter into the 400 ml beaker. Wash the filter with 25 ml of hot *water R*, collecting this filtrate in the 400 ml beaker. Concentrate the combined filtrates by gently boiling to about 20 ml. If a precipitate appears, add about 0.1 ml of *nitric acid R*, heat to boiling and allow to cool to room temperature. Filter the concentrated extracts through a rapid-flow filter paper into a 50 ml volumetric flask. Transfer the remaining contents of the 400 ml beaker through the filter paper and into the flask with *water R*. Dilute to 50.0 ml with *water R*.

Reference solutions. Prepare the reference solutions using *lead standard solution (10 ppm Pb) R*, diluted if necessary with *water R*.

Measure the absorbance at 217 nm, using a lead hollow-cathode lamp as source of radiation and an oxidising air-acetylene flame.

Loss on drying (2.2.32). Not more than 8.0 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C.

Microbial contamination. Total viable aerobic count (2.6.12) not more than 10^3 micro-organisms per gram determined by plate count. It complies with the test for *Escherichia coli* (2.6.13).

ASSAY

Aluminium. Determine by atomic absorption spectrometry (2.2.23, *Method I*).

Test solution. In a platinum crucible mix 0.200 g with 1.0 g of *lithium metaborate R*. Heat slowly at first and ignite at 1000 °C to 1200 °C for 15 min. Cool, place the crucible in a 100 ml beaker containing 25 ml of *dilute nitric acid R* and add an additional 50 ml of *dilute nitric acid R*, filling and submerging the crucible. Place a polytetrafluoroethylene coated magnetic stirring bar in the crucible and stir gently with a magnetic stirrer until dissolution is complete. Pour the contents into a 250 ml beaker and remove the crucible. Warm the solution and transfer through a rapid-flow filter paper into a 250 ml volumetric flask, wash the filter and beaker with *water R* and dilute to 250.0 ml with *water R* (solution A). To 20.0 ml of this solution add 20 ml of a 10 g/l solution of *sodium chloride R* and dilute to 100.0 ml with *water R*.

Reference solutions. Dissolve 1.000 g of *aluminium R* in a mixture of 10 ml of *hydrochloric acid R* and 10 ml of *water R* by gentle heating. Cool and dilute to 1000.0 ml with *water R* (1 mg of aluminium per millilitre). Into three identical volumetric flasks, each containing 0.20 g of *sodium chloride R*, introduce 2.0 ml, 5.0 ml and 10.0 ml of the solution, respectively and dilute to 100.0 ml with *water R*. Measure the absorbance at 309 nm using an aluminium hollow-cathode lamp as the source of radiation and an oxidising acetylene-nitrous oxide flame.

Calculate the content of aluminium in the substance to be examined.

Magnesium. Determine by atomic absorption spectrometry (2.2.23, *Method I*).

Test solution. Dilute 25.0 ml of solution A, prepared in the assay for aluminium, to 50.0 ml with *water R*. To 5.0 ml of this solution add 20.0 ml of *lanthanum nitrate solution R* and dilute to 100.0 ml with *water R*.

Reference solutions. Place 1.000 g of *magnesium R* in a 250 ml beaker containing 20 ml of *water R* and carefully add 20 ml of *hydrochloric acid R*, warming if necessary to dissolve. Transfer the solution to a volumetric flask and dilute to 1000.0 ml with *water R* (1 mg of magnesium per millilitre). Dilute 5.0 ml of the solution to 250.0 ml with *water R*. Into four identical volumetric flasks, introduce 5.0 ml, 10.0 ml, 15.0 ml and 20.0 ml of the solution, respectively. To each flask add 20.0 ml of *lanthanum nitrate solution R* and dilute to 100.0 ml with *water R*.

Measure the absorbance at 285 nm using a magnesium hollow-cathode lamp as source of radiation and a reducing air-acetylene flame.

Calculate the content of magnesium in the substance to be examined.

LABELLING

The label states the content of aluminium and magnesium.

01/2005:0311

ALUMINIUM OXIDE, HYDRATED

Aluminii oxidum hydricum

DEFINITION

Hydrated aluminium oxide contains the equivalent of not less than 47.0 per cent and not more than 60.0 per cent of Al_2O_3 (M_r 102.0).

CHARACTERS

A white, amorphous powder, practically insoluble in water. It dissolves in dilute mineral acids and in solutions of alkali hydroxides.

IDENTIFICATION

Solution S (see Tests) gives the reaction of aluminium (2.3.1).

TESTS

Solution S. Dissolve 2.5 g in 15 ml of *hydrochloric acid R*, heating on a water-bath. Dilute to 100 ml with *distilled water R*.

Appearance of solution. Solution S is not more opalescent than reference suspension II (2.2.1) and not more intensely coloured than reference solution GY₆ (2.2.2, *Method II*).

Alkaline impurities. Shake 1.0 g with 20 ml of *carbon dioxide-free water R* for 1 min and filter. To 10 ml of the filtrate add 0.1 ml of *phenolphthalein solution R*. Any pink colour disappears on the addition of 0.3 ml of 0.1 M *hydrochloric acid*.

Neutralising capacity. Carry out the test at 37 °C. Disperse 0.5 g in 100 ml of *water R*, heat, add 100.0 ml of 0.1 M *hydrochloric acid*, previously heated, and stir continuously; the pH (2.2.3) of the solution after 10 min, 15 min and 20 min is not less than 1.8, 2.3 and 3.0 respectively and is at no time greater than 4.5. Add 10.0 ml of 0.5 M *hydrochloric acid*, previously heated, stir continuously for 1 h and titrate with 0.1 M *sodium hydroxide* to pH 3.5; not more than 35.0 ml of 0.1 M *sodium hydroxide* is required.

Chlorides (2.4.4). Dissolve 0.1 g with heating in 10 ml of *dilute nitric acid R* and dilute to 100 ml with *water R*. 5 ml of the solution diluted to 15 ml with *water R* complies with the limit test for chlorides (1 per cent).

Sulphates (2.4.13). Dilute 4 ml of solution S to 100 ml with *distilled water R*. 15 ml of the solution complies with the limit test for sulphates (1 per cent).

Arsenic (2.4.2). 10 ml of solution S complies with limit test A for arsenic (4 ppm).

Heavy metals (2.4.8). Neutralise 20 ml of solution S with *concentrated ammonia R*, using *metanil yellow solution R* as an external indicator. Filter, if necessary, and dilute to 30 ml with *water R*. 12 ml of the solution complies with limit test A for heavy metals (60 ppm). Prepare the standard using 10 ml of *lead standard solution (1 ppm Pb) R*.

Microbial contamination. Total viable aerobic count (2.6.12) not more than 10^3 micro-organisms per gram, determined by plate count. It complies with the tests for enterobacteria and certain other gram-negative bacteria and with the test for *Escherichia coli* (2.6.13).

ASSAY

Dissolve 0.800 g in 10 ml of *hydrochloric acid R1*, heating on a water-bath. Cool and dilute to 50.0 ml with *water R*. To 10.0 ml of the solution, add *dilute ammonia R1* until a precipitate begins to appear. Add the smallest quantity of *dilute hydrochloric acid R* needed to dissolve the precipitate and dilute to 20 ml with *water R*. Carry out the complexometric titration of aluminium (2.5.11).

1 ml of 0.1 M sodium edetate is equivalent to 5.098 mg of Al_2O_3 .

STORAGE

Store in an airtight container, at a temperature below 30 °C.

01/2005:1598

ALUMINIUM PHOSPHATE, HYDRATED

Aluminii phosphas hydricus

$\text{AlPO}_4 \cdot x \text{H}_2\text{O}$ M_r 122.0 (anhydrous substance)

DEFINITION

Content: 94.0 per cent to 102.0 per cent of AlPO_4 (M_r 122.0) (ignited substance).

CHARACTERS

Appearance: white or almost white powder.

Solubility: very slightly soluble in water, practically insoluble in alcohol. It dissolves in dilute solutions of mineral acids and alkali hydroxides.

IDENTIFICATION

- Solution S (see Tests) gives reaction (b) of phosphates (2.3.1).
- Solution S gives the reaction of aluminium (2.3.1).

TESTS

Solution S. Dissolve 2.00 g in *dilute hydrochloric acid R* and dilute to 100 ml with the same acid.

Appearance of solution. Solution S is clear (2.2.1) and colourless (2.2.2, Method II).

pH (2.2.3): 5.5 to 7.2

Shake 4.0 g with *carbon dioxide-free water R* and dilute to 100 ml with the same solvent.

Chlorides (2.4.4): maximum 1.3 per cent.

Dissolve 50.0 mg in 10 ml of *dilute nitric acid R* and dilute to 200 ml with *water R*. 15 ml of the solution complies with the limit test for chlorides.

Soluble phosphates: maximum 1.0 per cent, calculated as PO_4^{3-} .

Test solution. Stir 5.0 g with 150 ml of *water R* for 2 h. Filter and wash the filter with 50 ml of *water R*. Combine the filtrate and the washings and dilute to 250.0 ml with *water R*. Dilute 10.0 ml of this solution to 100.0 ml with *water R*.

Reference solution (a). Dissolve 2.86 g of *potassium dihydrogen phosphate R* in *water R* and dilute to 100 ml with the same solvent.

Reference solution (b). Dilute 1 ml of reference solution (a) to 5 ml with *water R*.

Reference solution (c). Dilute 3 ml of reference solution (a) to 5 ml with *water R*.

Treat each solution as follows. To 5.0 ml add 4 ml of *dilute sulphuric acid R*, 1 ml of *ammonium molybdate solution R*, 5 ml of *water R* and 2 ml of a solution containing 0.10 g of 4-methylaminophenol sulphate R, 0.5 g of anhydrous sodium sulphite R and 20.0 g of sodium metabisulphite R in 100 ml of *water R*. Shake and allow to stand for 15 min. Dilute to 25.0 ml with *water R* and allow to stand for a further 15 min. Measure the absorbance (2.2.25) at 730 nm. Calculate the content of soluble phosphates from a calibration curve prepared using reference solutions (a), (b) and (c) after treatment.

Sulphates (2.4.13): maximum 0.6 per cent.

Dilute 8 ml of solution S to 100 ml with *distilled water R*. 15 ml of the solution complies with the limit test for sulphates.

Arsenic (2.4.2): maximum 1 ppm.

1.0 g complies with limit test A.

Heavy metals (2.4.8): maximum 20 ppm.

Dissolve 1.0 g in *dilute hydrochloric acid R* and dilute to 20 ml with the same acid. 12 ml of the solution complies with limit test A. Prepare the standard using *lead standard solution (1 ppm Pb) R*.

Loss on ignition. 10.0 per cent to 20.0 per cent, determined on 1.000 g at 800 °C.

Neutralising capacity. Add 0.50 g to 30 ml of 0.1 M *hydrochloric acid* previously heated to 37 °C and maintain at this temperature for 15 min while stirring. The pH (2.2.3) of the mixture after 15 min at 37 °C is 2.0 to 2.5.

ASSAY

Dissolve 0.400 g in 10 ml of *dilute hydrochloric acid R* and dilute to 100.0 ml with *water R*. To 10.0 ml of the solution, add 10.0 ml of 0.1 M sodium edetate and 30 ml of a mixture of equal volumes of *ammonium acetate solution R* and *dilute acetic acid R*. Boil for 3 min, then cool. Add 25 ml of *alcohol R* and 1 ml of a freshly prepared 0.25 g/l solution of *dithizone R* in *alcohol R*. Titrate the excess of sodium edetate with 0.1 M *zinc sulphate* until the colour changes to pink.

1 ml of 0.1 M sodium edetate is equivalent to 12.20 mg of AlPO_4 .

STORAGE

In an airtight container.

01/2005:0165

01/2005:0463
corrected

ALUMINIUM SULPHATE

Aluminii sulfas

DEFINITION

Aluminium sulphate contains not less than 51.0 per cent and not more than the equivalent of 59.0 per cent of $\text{Al}_2(\text{SO}_4)_3$ (M_r 342.1). It contains a variable quantity of water of crystallisation.

CHARACTERS

Colourless, lustrous crystals or crystalline masses, soluble in cold water, freely soluble in hot water, practically insoluble in alcohol.

IDENTIFICATION

- A. Solution S (see Tests) gives reaction (a) of sulphates (2.3.1).
B. Solution S gives the reaction of aluminium (2.3.1).

TESTS

Solution S. Dissolve 2.5 g in *water R* and dilute to 50 ml with the same solvent.

Appearance of solution. Solution S is not more opalescent than reference suspension III (2.2.1) and is colourless (2.2.2, *Method II*).

pH (2.2.3). Dissolve 0.5 g in *carbon dioxide-free water R* and dilute to 25 ml with the same solvent. The pH of the solution is 2.5 to 4.0.

Alkali and alkaline-earth metals. To 20 ml of solution S add 100 ml of *water R*, heat and add 0.1 ml of *methyl red solution R*. Add *dilute ammonia R1* until the colour of the indicator changes to yellow. Dilute to 150 ml with *water R*, heat to boiling and filter. Evaporate 75 ml of the filtrate to dryness on a water-bath and ignite. The residue weighs not more than 2 mg (0.4 per cent).

Ammonium (2.4.1). 0.4 ml of solution S diluted to 14 ml with *water R* complies with the limit test for ammonium (500 ppm).

Iron (2.4.9). 2 ml of solution S diluted to 10 ml with *water R* complies with the limit test for iron (100 ppm). Use 0.3 ml of *thioglycollic acid R* in this test.

Heavy metals (2.4.8). Dilute 8 ml of solution S to 20 ml with *water R*. 12 ml of the solution complies with limit test A for heavy metals (50 ppm). Prepare the standard using *lead standard solution (1 ppm Pb) R*.

ASSAY

Dissolve 0.500 g in 20 ml of *water R*. Carry out the complexometric titration of aluminium (2.5.11).

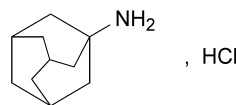
1 ml of 0.1 M *sodium edetate* is equivalent to 17.11 mg of $\text{Al}_2(\text{SO}_4)_3$.

STORAGE

Store in an airtight container.

AMANTADINE HYDROCHLORIDE

Amantadini hydrochloridum

 $\text{C}_{10}\text{H}_{18}\text{ClN}$ M_r 187.7

DEFINITION

Amantadine hydrochloride contains not less than 98.5 per cent and not more than the equivalent of 101.0 per cent of tricyclo[3.3.1.1^{3,7}]decan-1-amine hydrochloride, calculated with reference to the anhydrous substance.

CHARACTERS

A white or almost white, crystalline powder, freely soluble in water and in alcohol. It sublimes on being heated.

IDENTIFICATION

First identification: A, D.

Second identification: B, C, D.

- A. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *amantadine hydrochloride CRS*. Examine the substances prepared as discs.
B. To 0.1 g add 1 ml of *pyridine R*, mix and add 0.1 ml of *acetic anhydride R*. Heat to boiling for about 10 s. Pour the hot solution into 10 ml of *dilute hydrochloric acid R*, cool to 5 °C and filter. The precipitate, washed with *water R* and dried *in vacuo* at 60 °C for 1 h, melts (2.2.14) at 147 °C to 151 °C.
C. Dissolve 0.2 g in 1 ml of 0.1 M *hydrochloric acid*. Add 1 ml of a 500 g/l solution of *sodium nitrite R*. A white precipitate is formed.
D. 1 ml of solution S (see Tests) gives reaction (a) of chlorides (2.3.1).

TESTS

Solution S. Dissolve 2.5 g in *carbon dioxide-free water R* and dilute to 25 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and not more intensely coloured than reference solution Y₇ (2.2.2, *Method II*).

Acidity or alkalinity. Dilute 2 ml of solution S to 10 ml with *carbon dioxide-free water R*. Add 0.1 ml of *methyl red solution R* and 0.2 ml of 0.01 M *sodium hydroxide*. The solution is yellow. Add 0.4 ml of 0.01 M *hydrochloric acid*. The solution is red.

Related substances. Examine by gas chromatography (2.2.28).

Test solution. Dissolve 0.10 g of the substance to be examined in 2 ml of *water R*. Add 2 ml of a 200 g/l solution of *sodium hydroxide R* and 2 ml of *chloroform R*. Shake for 10 min. Separate the chloroform layer, dry over *anhydrous sodium sulphate R* and filter.

The chromatographic procedure may be carried out using:

- a glass column 1.8 m long and 2 mm in internal diameter with a packing prepared as follows: mix 19.5 g of *silanised diatomaceous earth for gas chromatography R* with 60 ml of a 3.3 g/l solution of *potassium hydroxide R* in *methanol R* and evaporate the solvent under reduced

pressure while rotating the mixture slowly (support); dissolve 0.4 g of *low-vapour-pressure hydrocarbons (type L) R* in 60 ml of *toluene R* (dissolution requires up to 5 h), add this solution to the support and evaporate the solvent under reduced pressure while rotating the mixture slowly,

- *nitrogen for chromatography R* as the carrier gas at a flow rate of 30 ml/min,
- a flame-ionisation detector.

Programme the temperature of the column linearly at a rate of 6 °C per minute from 100 °C to 200 °C. Maintain the temperature of the injection port at 220 °C and that of the detector at 300 °C. Inject 1 µl or the chosen volume of the test solution. Continue the chromatography for a period at least 2.5 times the retention time of the principal peak. In the chromatogram the sum of the area of any peaks apart from that corresponding to amantadine does not exceed 1 per cent of the total area of the peaks; no peak, apart from that corresponding to amantadine, has an area exceeding 0.3 per cent of the total area. Disregard the peak corresponding to the solvent during the evaluation.

Heavy metals (2.4.8). 12 ml of solution S complies with limit test A for heavy metals (20 ppm). Prepare the standard using *lead standard solution (2 ppm Pb) R*.

Water (2.5.12). Not more than 0.5 per cent, determined on 2.000 g by the semi-micro determination of water.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

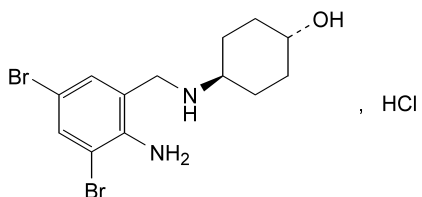
Dissolve 0.150 g in a mixture of 5.0 ml of 0.01 M *hydrochloric acid* and 50 ml of *alcohol R*. Carry out a potentiometric titration (2.2.20), using 0.1 M *sodium hydroxide*. Read the volume added between the two points of inflexion.

1 ml of 0.1 M *sodium hydroxide* is equivalent to 18.77 mg of C₁₃H₁₈ClN.

01/2005:1489

AMBROXOL HYDROCHLORIDE

Ambroxoli hydrochloridum

C₁₃H₁₉Br₂ClN₂O*M*_r 414.6

DEFINITION

trans-4-[(2-Amino-3,5-dibromobenzyl)amino]cyclohexanol hydrochloride.

Content: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

Appearance: white or yellowish crystalline powder.

Solubility: sparingly soluble in water, soluble in methanol, practically insoluble in methylene chloride.

IDENTIFICATION

First identification: B, D.

Second identification: A, C, D.

A. Dissolve 20.0 mg in 0.05 M *sulphuric acid* and dilute to 100.0 ml with the same acid. Dilute 2.0 ml of the solution to 10.0 ml with 0.05 M *sulphuric acid*. Examined between 200 nm and 350 nm (2.2.25), the solution shows two absorption maxima at 245 nm and 310 nm. The ratio of the absorbance measured at 245 nm to that measured at 310 nm is 3.2 to 3.4.

B. Infrared absorption spectrophotometry (2.2.24).

Comparison: *ambroxol hydrochloride CRS*.

C. Examine by thin-layer chromatography (2.2.27).

Test solution. Dissolve 50 mg of the substance to be examined in *methanol R* and dilute to 5 ml with the same solvent.

Reference solution. Dissolve 50 mg of *ambroxol hydrochloride CRS* in *methanol R* and dilute to 5 ml with the same solvent.

Plate: *TLC silica gel F₂₅₄ plate R*.

Mobile phase: concentrated *ammonia R*, 1-propanol *R*, *ethyl acetate R*, *hexane R* (1:10:20:70 V/V/V/V).

Application: 10 µl.

Development: over 2/3 of the plate.

Drying: in air.

Detection: examine in ultraviolet light at 254 nm.

Results: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with the reference solution.

D. Dissolve 25 mg in 2.5 ml of *water R*, mix with 1.0 ml of dilute *ammonia R1* and allow to stand for 5 min. Filter and acidify the filtrate with dilute *nitric acid R*. The filtrate gives reaction (a) of chlorides (2.3.1).

TESTS

Solution S. Dissolve 0.75 g in *methanol R* and dilute to 15 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and not more intensely coloured than reference solution Y₆ (2.2.2, *Method II*).

pH (2.2.3): 4.5 to 6.0.

Dissolve 0.2 g in *carbon dioxide-free water R* and dilute to 20 ml with the same solvent.

Related substances. Liquid chromatography (2.2.29).

Prepare the solutions immediately before use.

Test solution. Dissolve 50.0 mg of the substance to be examined in *water R* and dilute to 50.0 ml with the same solvent.

Reference solution (a). Dilute 5.0 ml of the test solution to 250.0 ml with *water R*. Dilute 1.0 ml of this solution to 20.0 ml with the mobile phase.

Reference solution (b). Dissolve 5 mg of the substance to be examined in 0.2 ml of *methanol R* and add 0.04 ml of a mixture of 1 volume of *formaldehyde solution R* and 99 volumes of *water R*. Heat at 60 °C for 5 min. Evaporate to dryness under a current of nitrogen. Dissolve the residue in 5 ml of *water R* and dilute to 20 ml with the mobile phase.

Column:

- **size:** *l* = 0.25 m, Ø = 4.0 mm,
- **stationary phase:** *octadecylsilyl silica gel for chromatography R* (5 µm).

Mobile phase: a mixture of equal volumes of *acetonitrile R* and a solution prepared as follows: dissolve 1.32 g of *ammonium phosphate R* in 900 ml of *water R*, adjust to pH 7.0 with *phosphoric acid R* and dilute to 1000 ml with *water R*.

Flow rate: 1 ml/min.

Detection: spectrophotometer at 248 nm.

Injection: 20 µl.

Sensitivity: reference solution (a).

Run time: 3 times the retention time of the principal peak in the chromatogram obtained with the test solution.

System suitability:

- *resolution:* minimum of 4.0 between the peaks due to impurity B and amroxol in the chromatogram obtained with reference solution (b).

Limits:

- *any impurity:* not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent),
- *total:* not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.3 per cent),
- *disregard limit:* 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (a).

Heavy metals (2.4.8): maximum 20 ppm.

1.0 g complies with limit test C. Prepare the standard using 2 ml of *lead standard solution* (10 ppm Pb) R.

Loss on drying (2.2.32): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 100–105 °C.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

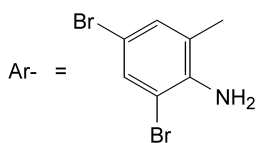
Dissolve 0.300 g in 70 ml of *alcohol R* and add 5 ml of 0.01 M *hydrochloric acid*. Carry out a potentiometric titration (2.2.20), using 0.1 M *sodium hydroxide*. Read the volume added between the two points of inflexion.

1 ml of 0.1 M *sodium hydroxide* is equivalent to 41.46 mg of C₁₃H₁₉Br₂ClN₂O.

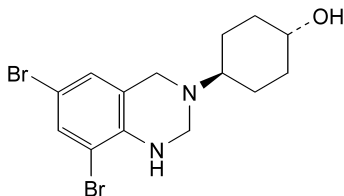
STORAGE

Store protected from light.

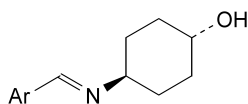
IMPURITIES



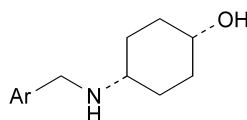
A. Ar-CH₂OH: (2-amino-3,5-dibromophenyl)methanol,



B. *trans*-4-(6,8-dibromo-1,4-dihydroquinazolin-3(2H)-yl)cyclohexanol,



C. *trans*-4-[(*E*)-2-amino-3,5-dibromobenzyliden]amino]cyclohexanol,



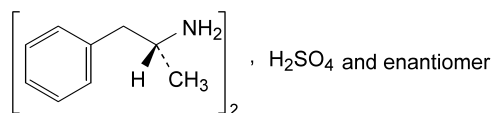
D. *cis*-4-[(2-amino-3,5-dibromobenzyl)amino]cyclohexanol,

E. Ar-CH=O: 2-amino-3,5-dibromobenzaldehyde.

01/2005:0368

AMFETAMINE SULPHATE

Amfetamini sulfas



C₁₈H₂₈N₂O₄S

M_r 368.5

DEFINITION

Amfetamine sulphate contains not less than 99.0 per cent and not more than the equivalent of 100.5 per cent of bis[(2*RS*)-1-phenylpropan-2-amine] sulphate, calculated with reference to the dried substance.

CHARACTERS

A white powder, freely soluble in water, slightly soluble in alcohol.

IDENTIFICATION

First identification: A, B, E.

Second identification: A, C, D, E.

- The angle of optical rotation (2.2.7) of solution S (see Tests), measured in a 2 dm tube, is –0.04 to +0.04.
- Examine by infrared absorption spectrophotometry (2.2.24), comparing with the *Ph. Eur. reference spectrum of amfetamine sulphate*. Examine as a mull in *liquid paraffin R*.
- To 50 ml of solution S add 5 ml of *strong sodium hydroxide solution R* and 0.5 ml of *benzoyl chloride R* and shake. Continue to add *benzoyl chloride R* in portions of 0.5 ml until no further precipitate is formed. Filter, wash the precipitate with *water R* and recrystallise twice from a mixture of equal volumes of *alcohol R* and *water R* and dry at 100 °C to 105 °C. The crystals melt (2.2.14) at 131 °C to 135 °C.
- To about 2 mg add 1 ml of *sulphuric acid-formaldehyde reagent R*. An orange colour develops and quickly becomes dark-brown.
- Solution S gives reaction (a) of sulphates (2.3.1).

TESTS

Solution S. Dissolve 2.0 g in *carbon dioxide-free water R* and dilute to 100 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and colourless (2.2.2, *Method II*).

Acidity or alkalinity. To 25 ml of solution S add 0.1 ml of *methyl red solution R*. Not more than 0.1 ml of 0.01 M *hydrochloric acid* or 0.01 M *sodium hydroxide* is required to change the colour of the indicator.

Loss on drying (2.2.32). Not more than 1.0 per cent, determined on 1.00 g by drying in an oven at 100 °C to 105 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.300 g in 30 ml of *anhydrous acetic acid R*. Titrate with 0.1 M *perchloric acid*, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *perchloric acid* is equivalent to 36.85 mg of $C_{18}H_{28}N_2O_4S$.

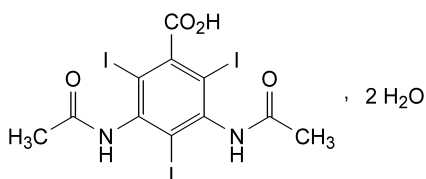
STORAGE

Store protected from light.

01/2005:0873

AMIDOTRIZOIC ACID DIHYDRATE

Acidum amidotrizoicum dihydricum


 $C_{11}H_9I_3N_2O_4 \cdot 2H_2O$
 M_r 650

DEFINITION

Amidotrizoic acid dihydrate contains not less than 98.5 per cent and not more than the equivalent of 101.0 per cent of 3,5-bis(acetylamino)-2,4,6-triodobenzoic acid, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, crystalline powder, very slightly soluble in water and in alcohol. It dissolves in dilute solutions of alkali hydroxides.

IDENTIFICATION

First identification: A.

Second identification: B, C.

- Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *amidotrizoic acid dihydrate CRS*.
- Examine the chromatograms obtained in the test for related substances (see Tests). The principal spot in the chromatogram obtained with test solution (b) is similar in position and size to the principal spot in the chromatogram obtained with reference solution (b).
- Heat 50 mg gently in a small porcelain dish over a naked flame. Violet vapour is evolved.

TESTS

Appearance of solution. Dissolve 1.0 g in *dilute sodium hydroxide solution R* and dilute to 20 ml with the same solvent. The solution is clear (2.2.1) and colourless (2.2.2, *Method II*).

Related substances. Examine by thin-layer chromatography (2.2.27), using *silica gel GF₂₅₄ R* as the coating substance.

Test solution (a). Dissolve 0.50 g of the substance to be examined in a 3 per cent V/V solution of *ammonia R* in *methanol R* and dilute to 10 ml with the same solvent.

Test solution (b). Dilute 1 ml of test solution (a) to 10 ml with a 3 per cent V/V solution of *ammonia R* in *methanol R*.

Reference solution (a). Dilute 1 ml of test solution (b) to 50 ml with a 3 per cent V/V solution of *ammonia R* in *methanol R*.

Reference solution (b). Dissolve 50 mg of *amidotrizoic acid dihydrate CRS* in a 3 per cent V/V solution of *ammonia R* in *methanol R* and dilute to 10 ml with the same solvent.

Apply separately to the plate 2 µl of each solution. Develop over a path of 15 cm using a mixture of 20 volumes of *anhydrous formic acid R*, 25 volumes of *methyl ethyl ketone R* and 60 volumes of *toluene R*. Allow the plate to dry until the solvents have evaporated and examine in ultraviolet light at 254 nm. Any spot in the chromatogram obtained with test solution (a), apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (a) (0.2 per cent).

Halides. Dissolve 0.55 g in a mixture of 4 ml of *dilute sodium hydroxide solution R* and 15 ml of *water R*. Add 6 ml of *dilute nitric acid R* and filter. 15 ml of the filtrate complies with the limit test for chlorides (2.4.4) (150 ppm expressed as chloride).

Free aromatic amines. *Maintain the solutions and reagents in iced water protected from bright light.* To 0.50 g in a 50 ml volumetric flask add 15 ml of *water R*. Shake and add 1 ml of *dilute sodium hydroxide solution R*. Cool in iced water, add 5 ml of a freshly prepared 5 g/l solution of *sodium nitrite R* and 12 ml of *dilute hydrochloric acid R*. Shake gently and allow to stand for exactly 2 min after adding the hydrochloric acid. Add 10 ml of a 20 g/l solution of *ammonium sulphamate R*. Allow to stand for 5 min, shaking frequently, and add 0.15 ml of a 100 g/l solution of *α-naphthol R* in *alcohol R*. Shake and allow to stand for 5 min. Add 3.5 ml of *buffer solution pH 10.9 R*, mix and dilute to 50.0 ml with *water R*. The absorbance (2.2.25), measured within 20 min at 485 nm using as the compensation liquid a solution prepared at the same time and in the same manner but omitting the substance to be examined, is not greater than 0.30.

Heavy metals (2.4.8). Dissolve 2.0 g in 4 ml of *dilute sodium hydroxide solution R* and dilute to 20 ml with *water R*. 12 ml of this solution complies with limit test A for heavy metals (20 ppm). Prepare the standard using *lead standard solution* (2 ppm Pb) *R*.

Loss on drying (2.2.32): 4.5 per cent to 7.0 per cent, determined on 0.500 g by drying in an oven at 100 °C to 105 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

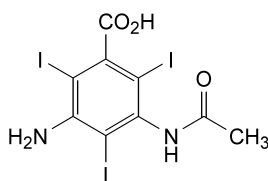
To 0.150 g in a 250 ml round-bottomed flask add 5 ml of *strong sodium hydroxide solution R*, 20 ml of *water R*, 1 g of *zinc powder R* and a few glass beads. Boil under a reflux condenser for 30 min. Allow to cool and rinse the condenser with 20 ml of *water R*, adding the rinsings to the flask. Filter through a sintered-glass filter and wash the filter with several quantities of *water R*. Collect the filtrate and washings. Add 40 ml of *dilute sulphuric acid R* and titrate immediately with 0.1 M *silver nitrate*. Determine the end-point potentiometrically (2.2.20), using a suitable electrode system such as silver-mercurous sulphate.

1 ml of 0.1 M *silver nitrate* is equivalent to 20.47 mg of $C_{11}H_9I_3N_2O_4$.

STORAGE

Store protected from light.

IMPURITIES

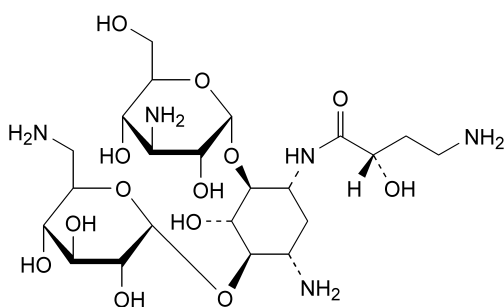


A. 3-(acetylamino)-5-amino-2,4,6-triiodobenzoic acid.

01/2005:1289

AMIKACIN

Amikacinum

 $C_{22}H_{43}N_5O_{13}$ M_r 585.6

DEFINITION

Amikacin is 6-*O*-(3-amino-3-deoxy- α -D-glucopyranosyl)-4-*O*-(6-amino-6-deoxy- α -D-glucopyranosyl)-1-*N*-[(2*S*)-4-amino-2-hydroxybutanoyl]-2-deoxy-D-streptamine, an antimicrobial substance obtained from kanamycin A. It contains not less than 96.5 per cent and not more than the equivalent of 102.5 per cent of $C_{22}H_{43}N_5O_{13}$, calculated with reference to the anhydrous substance.

CHARACTERS

A white or almost white powder, sparingly soluble in water, slightly soluble in methanol, practically insoluble in acetone and in alcohol.

IDENTIFICATION

- Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum of *amikacin CRS*.
- Examine by thin-layer chromatography (2.2.27) using a *TLC silica gel plate R*.

Test solution. Dissolve 25 mg of the substance to be examined in *water R* and dilute to 10 ml with the same solvent.

Reference solution (a). Dissolve 25 mg of *amikacin CRS* in *water R* and dilute to 10 ml with the same solvent.

Reference solution (b). Dissolve 5 mg of *kanamycin monosulphate CRS* in 1 ml of the test solution and dilute to 10 ml with *water R*.

Apply to the plate 5 μ l of each solution. Develop over a path of 15 cm using the lower layer of a mixture of equal volumes of *concentrated ammonia R*, *methanol R* and *methylene chloride R*. Allow the plate to dry in air and spray with *ninhydrin solution R1*. Heat at 110 °C for 5 min. The principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution (a). The identification test is not valid unless the chromatogram obtained with reference solution (b) shows two clearly separated spots.

TESTS

pH (2.2.3). Dissolve 0.1 g in *carbon dioxide-free water R* and dilute to 10 ml with the same solvent. The pH of the solution is 9.5 to 11.5.

Specific optical rotation (2.2.7). Dissolve 0.50 g in *water R* and dilute to 25.0 ml with the same solvent. The specific optical rotation is + 97 to + 105, calculated with reference to the anhydrous substance.

Related substances. Examine by liquid chromatography (2.2.29), as prescribed under Assay.

Inject 20 μ l of reference solution (a). Adjust the sensitivity of the system so that the height of the principal peak in the chromatogram obtained is at least 50 per cent of the full scale of the recorder. Inject 20 μ l of reference solution (c). The test is not valid unless, in the chromatogram obtained, the resolution between the peaks corresponding to amikacin and impurity A is at least 3.5 (see Figure 1289-1). Inject 20 μ l of test solution (a). Continue the chromatography for four times the retention time of amikacin. In the chromatogram obtained with test solution (a): the area of any peak corresponding to impurity A is not greater than the area of the principal peak in the chromatogram obtained with reference solution (a) (1 per cent); the area of any peak, apart from the principal peak and any peak corresponding to impurity A, is not greater than half the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent); the sum of the areas of any such peaks is not greater than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (1.5 per cent). Disregard any peak due to the blank and any peak with an area less than 0.1 times that of the principal peak in the chromatogram obtained with reference solution (a).

Water (2.5.12). Not more than 8.5 per cent, determined on 0.200 g by the semi-micro determination of water.

Sulphated ash (2.4.14). Not more than 0.5 per cent, determined on 1.0 g.

ASSAY

Examine by liquid chromatography (2.2.29).

Test solution (a). Dissolve 0.100 g of the substance to be examined in *water R* and dilute to 10.0 ml with the same solvent. In a round-glass-stoppered vial, add 0.2 ml of this solution to 2.0 ml of a 10 g/l solution of *2,4,6-trinitrobenzene sulphonic acid R*. Then add 3.0 ml of *pyridine R* and close the vial tightly. Shake vigorously for 30 s and heat in a water-bath at 75 °C for 45 min. Cool in cold water for 2 min and add 2 ml of *glacial acetic acid R*. Shake vigorously for 30 s.

Test solution (b). Dissolve 50.0 mg of the substance to be examined in *water R* and dilute to 50.0 ml with the same solvent. Then prepare as prescribed for test solution (a).

Reference solution (a). Dissolve 10.0 mg of *amikacin impurity A CRS* in *water R* and dilute to 100.0 ml with the same solvent. Then prepare as prescribed for test solution (a).

Reference solution (b). Dissolve 50.0 mg of *amikacin CRS* in *water R* and dilute to 50.0 ml with the same solvent. Then prepare as prescribed for test solution (a).

Reference solution (c). Dissolve 5 mg of *amikacin CRS* and 5 mg of *amikacin impurity A CRS* in *water R* and dilute to 50 ml with the same solvent. Then prepare as prescribed for test solution (a).

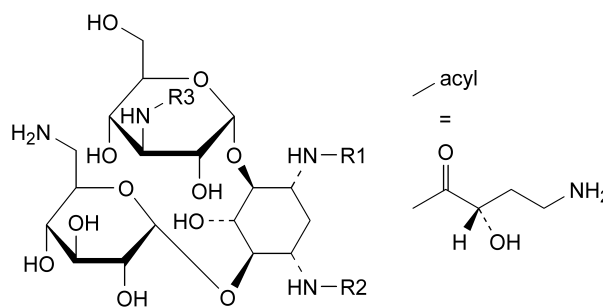
Blank solution. Prepare as described for test solution (a) using 0.2 ml of *water R*.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4.6 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (5 µm),
- as mobile phase at a flow rate of 1 ml/min a mixture of 30 volumes of a 2.7 g/l solution of *potassium dihydrogen phosphate R* adjusted to pH 6.5 with a 22 g/l solution of *potassium hydroxide R* and 70 volumes of *methanol R*,
- as detector a spectrophotometer set at 340 nm, maintaining the temperature of the column at 30 °C and that of the solutions to be examined at 10 °C.

Inject 20 µl of reference solution (b). Adjust the sensitivity of the system so that the height of the principal peak is at least 50 per cent of the full scale of the recorder. Inject reference solution (b) six times. The assay is not valid unless the relative standard deviation of the peak area for amikacin is 2.0 per cent. Inject test solution (b) and reference solution (b).

IMPURITIES



- A. R1 = R3 = H, R2 = acyl: 4-*O*-(3-amino-3-deoxy- α -D-glucopyranosyl)-6-*O*-(6-amino-6-deoxy- α -D-glucopyranosyl)-1-*N*-[(2*S*)-4-amino-2-hydroxybutanoyl]-2-deoxy-L-streptamine,
- B. R1 = R2 = acyl, R3 = H: 4-*O*-(3-amino-3-deoxy- α -D-glucopyranosyl)-6-*O*-(6-amino-6-deoxy- α -D-glucopyranosyl)-1,3-*N*-bis[(2*S*)-4-amino-2-hydroxybutanoyl]-2-deoxy-L-streptamine,
- C. R1 = R2 = H, R3 = acyl: 4-*O*-(6-amino-6-deoxy- α -D-glucopyranosyl)-6-*O*-[3-[[[(2*S*)-4-amino-2-hydroxybutanoyl]amino]-3-deoxy- α -D-glucopyranosyl]-2-deoxy-D-streptamine,
- D. R1 = R2 = R3 = H: kanamycin.

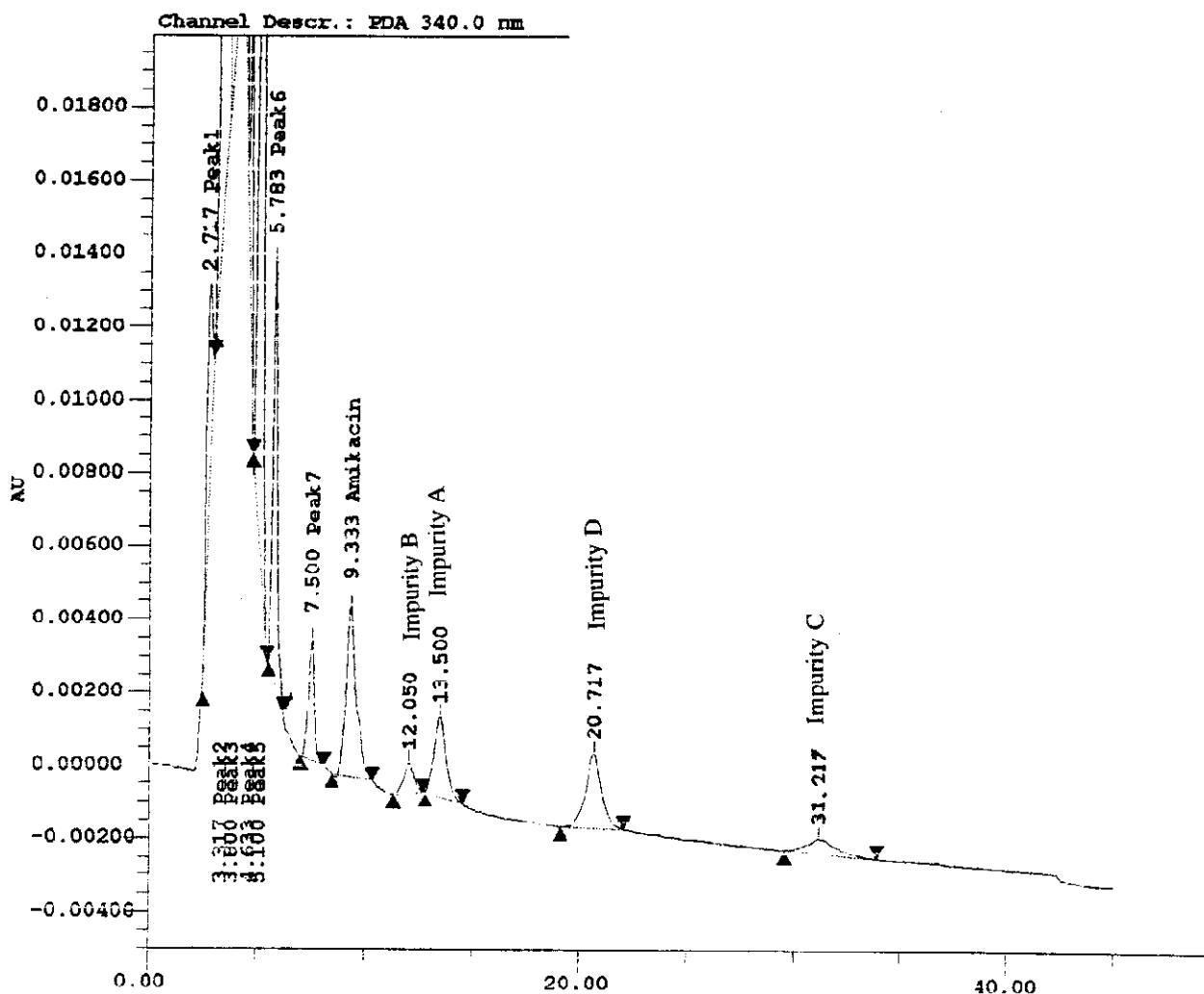
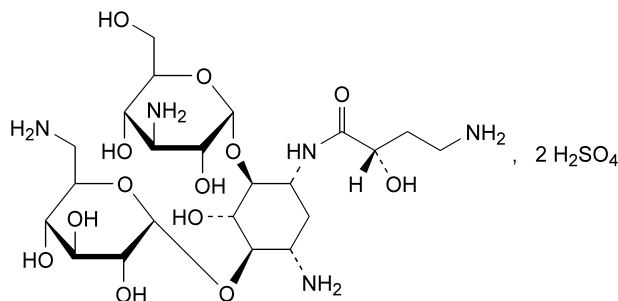


Figure 1289-1. – Chromatogram for the test for related substances of amikacin

01/2005:1290
corrected

AMIKACIN SULPHATE

Amikacini sulfas

 $C_{22}H_{47}N_5O_{21}S_2$ M_r 782

DEFINITION

Amikacin sulphate is 6-*O*-(3-amino-3-deoxy- α -D-glucopyranosyl)-4-*O*-(6-amino-6-deoxy- α -D-glucopyranosyl)-1-*N*-[(2*S*)-4-amino-2-hydroxybutanoyl]-2-deoxy-D-streptamine sulphate, an antimicrobial substance obtained from kanamycin A. It contains not less than 72.3 per cent and not more than the equivalent of 76.8 per cent of $C_{22}H_{43}N_5O_{13}$, calculated with reference to the dried substance.

CHARACTERS

A white or almost white powder, freely soluble in water, practically insoluble in acetone and in alcohol.

IDENTIFICATION

- Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *amikacin sulphate CRS*.
- Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel plate R*.

Test solution. Dissolve 25 mg of the substance to be examined in *water R* and dilute to 10 ml with the same solvent.

Reference solution (a). Dissolve 25 mg of *amikacin sulphate CRS* in *water R* and dilute to 10 ml with the same solvent.

Reference solution (b). Dissolve 5 mg of *kanamycin monosulphate CRS* in 1 ml of the test solution and dilute to 10 ml with *water R*.

Apply to the plate 5 μ l of each solution. Develop over a path of 15 cm using the lower layer of a mixture of equal volumes of *concentrated ammonia R*, *methanol R* and *methylene chloride R*. Allow the plate to dry in air and spray with *ninhydrin solution RI*. Heat at 110 °C for 5 min. The principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution (a). The identification test is not valid unless the chromatogram obtained with reference solution (b) shows two clearly separated spots.

- It gives reaction (a) of sulphates (2.3.1).

TESTS

pH (2.2.3). Dissolve 0.1 g in *carbon dioxide-free water R* and dilute to 10 ml with the same solvent. The pH of the solution is 2.0 to 4.0.

Specific optical rotation (2.2.7). Dissolve 0.50 g in *water R* and dilute to 25.0 ml with the same solvent. The specific optical rotation is + 76 to + 84, calculated with reference to the dried substance.

Related substances. Examine by liquid chromatography (2.2.29), as prescribed under Assay.

Inject 20 μ l of reference solution (a). Adjust the sensitivity of the system so that the height of the principal peak in the chromatogram obtained is at least 50 per cent of the full scale of the recorder. Inject 20 μ l of reference solution (c). The test is not valid unless in the chromatogram obtained, the resolution between the peaks corresponding to amikacin and impurity A is at least 3.5 (see Figure 1290.-1). Inject 20 μ l of test solution (a). Continue the chromatography for four times the retention time of amikacin. In the chromatogram obtained with test solution (a): the area of any peak corresponding to impurity A is not greater than the area of the principal peak in the chromatogram obtained with reference solution (a) (1 per cent); the area of any peak, apart from the principal peak and any peak corresponding to impurity A, is not greater than half the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent); the sum of the areas of any such peaks is not greater than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (1.5 per cent). Disregard any peak due to the blank, any peak eluting before the principal peak and any peak with an area less than 0.1 times that of the principal peak in the chromatogram obtained with reference solution (a).

Sulphate: 23.3 per cent to 25.8 per cent of sulphate (SO_4), calculated with reference to the dried substance. Dissolve 0.250 g in 100 ml of *water R* and adjust the solution to pH 11 using *concentrated ammonia R*. Add 10.0 ml of 0.1 *M barium chloride* and about 0.5 mg of *phthalein purple R*. Titrate with 0.1 *M sodium edetate* adding 50 ml of *alcohol R* when the colour of the solution begins to change and continue the titration until the violet-blue colour disappears. 1 ml of 0.1 *M barium chloride* is equivalent to 9.606 mg of sulphate (SO_4).

Loss on drying (2.2.32). Not more than 13.0 per cent, determined on 0.500 g by drying in an oven at 100-105 °C at a pressure not exceeding 0.7 kPa for 3 h.

Pyrogens (2.6.8). If intended for use in the manufacture of parenteral dosage forms without a further appropriate procedure for the removal of pyrogens, it complies with the test for pyrogens. Inject per kilogram of the rabbit's mass 5 ml of a solution containing 25 mg of the substance to be examined in *water for injections R*.

ASSAY

Examine by liquid chromatography (2.2.29).

Test solution (a). Dissolve 0.100 g of the substance to be examined in *water R* and dilute to 10.0 ml with the same solvent. In a round-glass-stoppered vial, add 0.2 ml of this solution to 2.0 ml of a 10 g/l solution of 2,4,6-trinitrobenzene sulphonic acid *R*. Then add 3.0 ml of *pyridine R* and close the vial tightly. Shake vigorously for 30 s and heat on a water-bath at 75 °C for 2 h. Cool in cold water for 2 min and add 2 ml of *glacial acetic acid R*. Shake vigorously for 30 s.

Test solution (b). Dissolve 50.0 mg of the substance to be examined in *water R* and dilute to 50.0 ml with the same solvent. Then prepare as prescribed for test solution (a).

Reference solution (a). Dissolve 10.0 mg of *amikacin impurity A CRS* in *water R* and dilute to 100.0 ml with the same solvent. Then prepare as prescribed for test solution (a).

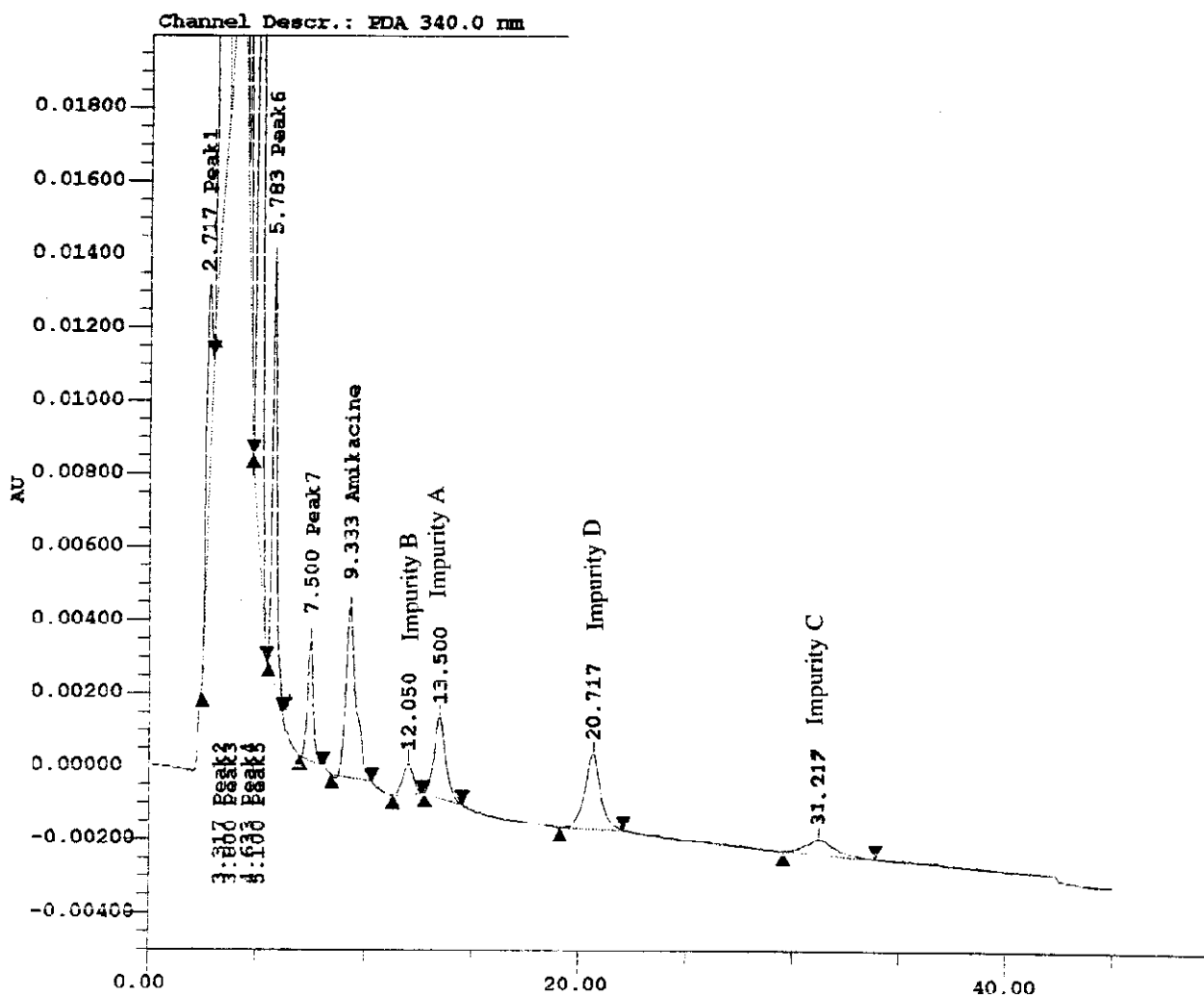


Figure 1290-1. – Chromatogram for the test for related substances of amikacin sulphate

Reference solution (b). Dissolve 50.0 mg of amikacin sulphate CRS in water R and dilute to 50.0 ml with the same solvent. Then prepare as prescribed for test solution (a).

Reference solution (c). Dissolve 5 mg of amikacin sulphate CRS and 5 mg of amikacin impurity A CRS in water R and dilute to 50 ml with the same solvent. Then prepare as prescribed for test solution (a).

Blank solution. Prepare as described for test solution (a) using 0.2 ml of water R.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4.6 mm in internal diameter packed with octadecylsilyl silica gel for chromatography R (5 µm),
- as mobile phase at a flow rate of 1 ml/min a mixture of 30 volumes of a 2.7 g/l solution of potassium dihydrogen phosphate R adjusted to pH 6.5 with a 22 g/l solution of potassium hydroxide R and 70 volumes of methanol R,

– as detector a spectrophotometer set at 340 nm,

maintaining the temperature of the column at 30 °C and that of the solutions to be examined at 10 °C.

Inject 20 µl of reference solution (b). Adjust the sensitivity of the system so that the height of the principal peak is at least 50 per cent of the full scale of the recorder. Inject reference solution (b) six times. The assay is not valid unless the relative standard deviation of the peak area for amikacin is 2.0 per cent. Inject test solution (b) and reference solution (b).

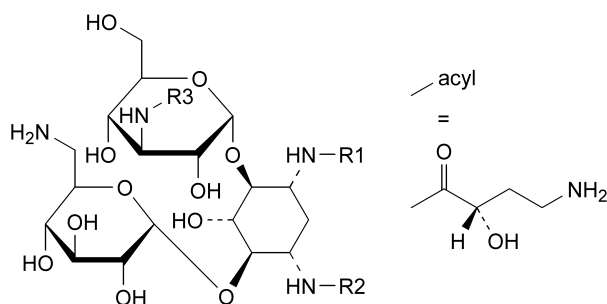
STORAGE

If the substance is sterile, store in a sterile, airtight, tamper-proof container.

LABELLING

The label states, where applicable, that the substance is apyrogenic.

IMPURITIES

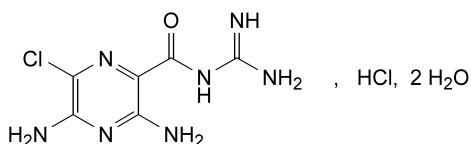


- A. R1 = R3 = H, R2 = acyl: 4-*O*-(3-amino-3-deoxy- α -D-glucopyranosyl)-6-*O*-(6-amino-6-deoxy- α -D-glucopyranosyl)-1-*N*-[(2*S*)-4-amino-2-hydroxybutanoyl]-2-deoxy-L-streptamine,
- B. R1 = R2 = acyl, R3 = H: 4-*O*-(3-amino-3-deoxy- α -D-glucopyranosyl)-6-*O*-(6-amino-6-deoxy- α -D-glucopyranosyl)-1,3-*N*-bis[(2*S*)-4-amino-2-hydroxybutanoyl]-2-deoxy-L-streptamine,
- C. R1 = R2 = H, R3 = acyl: 4-*O*-(6-amino-6-deoxy- α -D-glucopyranosyl)-6-*O*-[3-[[[(2*S*)-4-amino-2-hydroxybutanoyl]amino]-3-deoxy- α -D-glucopyranosyl]-2-deoxy-D-streptamine,
- D. R1 = R2 = R3 = H: kanamycin.

01/2005:0651

AMILORIDE HYDROCHLORIDE

Amiloridi hydrochloridum

 $C_6H_9Cl_2N_7O \cdot 2H_2O$ M_r 302.1

DEFINITION

Amiloride hydrochloride contains not less than 98.0 per cent and not more than the equivalent of 101.0 per cent of 3,5-diamino-*N*-carbamimidoyl-6-chloropyrazine-2-carboxamide, calculated with reference to the anhydrous substance.

CHARACTERS

A pale-yellow to greenish-yellow powder, slightly soluble in water and in ethanol.

IDENTIFICATION

First identification: A, D.

Second identification: B, C, D.

- A. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *amiloride hydrochloride CRS*.
- B. Examine by thin-layer chromatography (2.2.27), using a suitable silica gel as the coating substance.
- Test solution.* Dissolve 40 mg of the substance to be examined in *methanol R* and dilute to 10 ml with the same solvent.
- Reference solution.* Dissolve 40 mg of *amiloride hydrochloride CRS* in *methanol R* and dilute to 10 ml with the same solvent.

Apply to the plate 5 μ l of each solution. Develop over a path of 12 cm using a freshly prepared mixture of 6 volumes of *dilute ammonia R1*, 6 volumes of *water R* and 88 volumes of *dioxan R*. Allow the plate to dry in air and examine in ultraviolet light at 365 nm. The principal spot in the chromatogram obtained with the test solution is similar in position, fluorescence and size to the principal spot in the chromatogram obtained with the reference solution.

- C. Dissolve about 10 mg in 10 ml of *water R*. Add 10 ml of a 200 g/l solution of *cetrimide R*, 0.25 ml of *dilute sodium hydroxide solution R* and 1 ml of *bromine water R*. A greenish-yellow colour is produced. Add 2 ml of *dilute hydrochloric acid R*. The solution becomes deep yellow and shows blue fluorescence in ultraviolet light at 365 nm.
- D. It gives reaction (b) of chlorides (2.3.1).

TESTS

Free acid. Dissolve 1.0 g in a mixture of 50 ml of *methanol R* and 50 ml of *water R* and titrate with 0.1 *M* sodium hydroxide, determining the end-point potentiometrically (2.2.20). Not more than 0.3 ml of 0.1 *M* sodium hydroxide is required to reach the end-point.

Related substances. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 20.0 mg of the substance to be examined in a mixture of 1 volume of *acetonitrile R* and 3 volumes of *water R* and dilute to 10.0 ml with the same mixture of solvents.

Reference solution (a). Dilute 1.0 ml of the test solution to 100.0 ml with a mixture of 1 volume of *acetonitrile R* and 3 volumes of *water R*.

Reference solution (b). Dilute 1.0 ml of reference solution (a) to 10.0 ml with a mixture of 1 volume of *acetonitrile R* and 3 volumes of *water R*.

Reference solution (c). Dissolve 20.0 mg of *amiloride impurity A CRS* in a mixture of 1 volume of *acetonitrile R* and 3 volumes of *water R* and dilute to 20.0 ml with the same mixture of solvents. Dilute 1.0 ml of the solution to 100.0 ml with a mixture of 1 volume of *acetonitrile R* and 3 volumes of *water R*.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4.6 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (5 μ m),
- as mobile phase at a flow rate of 1 ml/min a mixture of 5 volumes of *tetramethylammonium hydroxide solution R*, 250 volumes of *acetonitrile R* and 745 volumes of *water R*; adjust to pH 7.0 using a mixture of 1 volume of *phosphoric acid R* and 9 volumes of *water R*,
- as detector a spectrophotometer set at 254 nm.

Inject 20 μ l of reference solution (c). Adjust the concentration of acetonitrile in the mobile phase so that the retention time of impurity A is 5 min to 6 min (an increase in the concentration of acetonitrile results in a shorter retention time). Inject 20 μ l of reference solution (a). Adjust the concentration of tetramethylammonium hydroxide and of phosphoric acid keeping the pH at 7.0 so that the retention time of amiloride is 9 min to 12 min (an increase in the concentration results in a shorter retention time for amiloride). Inject 20 μ l of reference solution (b). The test is not valid unless the signal-to-noise ratio for the peak due to amiloride is at least 5.0.

Inject 20 μ l of the test solution and 20 μ l of reference solution (c). Record the chromatograms for five times the retention time of amiloride. In the chromatogram obtained

with the test solution, the sum of the areas of the peaks, apart from the peak corresponding to amiloride, is not greater than the area of the peak corresponding to impurity A in the chromatogram obtained with reference solution (c) (0.5 per cent). Disregard any peak with an area less than 10 per cent of the area of the peak corresponding to impurity A in the chromatogram obtained with reference solution (c).

Water (2.5.12): 11.0 per cent to 13.0 per cent, determined on 0.200 g by the semi-micro determination of water.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

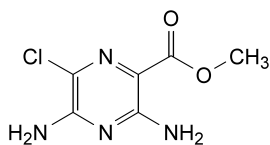
Dissolve 0.200 g in a mixture of 5.0 ml of 0.01 M hydrochloric acid and 50 ml of alcohol R. Carry out a potentiometric titration (2.2.20), using 0.1 M sodium hydroxide. Read the volume added between the two points of inflexion.

1 ml of 0.1 M sodium hydroxide is equivalent to 26.61 mg of C₇H₇Cl₂N₂O.

STORAGE

Store protected from light.

IMPURITIES

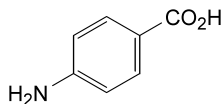


A. methyl 3,5-diamino-6-chloropyrazine-2-carboxylate.

01/2005:1687

4-AMINO BENZOIC ACID

Acidum 4-aminobenzoicum



C₇H₇NO₂

M_r 137.1

DEFINITION

4-Aminobenzoic acid.

Content: 99.0 per cent to 101.0 per cent (anhydrous substance).

CHARACTERS

Appearance: white or slightly yellow, crystalline powder.

Solubility: slightly soluble in water, freely soluble in alcohol. It dissolves in dilute solutions of alkali hydroxides.

IDENTIFICATION

First identification: B.

Second identification: A, C.

A. Melting point (2.2.14): 186 °C to 189 °C.

B. Infrared absorption spectrophotometry (2.2.24).

Comparison: 4-aminobenzoic acid CRS.

C. Thin-layer chromatography (2.2.27).

Test solution. Dissolve 20 mg of the substance to be examined in methanol R and dilute to 20 ml with the same solvent.

Reference solution (a). Dissolve 20 mg of 4-aminobenzoic acid CRS in methanol R and dilute to 20 ml with the same solvent.

Reference solution (b). Dissolve 10 mg of 4-nitrobenzoic acid R in 10 ml of reference solution (a).

Plate: suitable silica gel with a fluorescent indicator having an optimal intensity at 254 nm as the coating substance.

Mobile phase: glacial acetic acid R, hexane R, methylene chloride R (5:20:75 V/V/V).

Application: 1 µl.

Development: over a path of 10 cm.

Drying: in air.

Detection: examine in ultraviolet light at 254 nm.

System suitability: the chromatogram obtained with reference solution (b) shows 2 clearly separated spots.

Results: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a).

TESTS

Appearance of solution. The solution is clear (2.2.1) and not more intensely coloured than reference solution B₅ (2.2.2, Method II).

Dissolve 1.0 g in alcohol R and dilute to 20 ml with the same solvent.

Related substances. Liquid chromatography (2.2.29).

Test solution. Dissolve 25.0 mg of the substance to be examined in the mobile phase and dilute to 100.0 ml with the mobile phase.

Reference solution. Dissolve 25.0 mg of 4-nitrobenzoic acid R and 25.0 mg of benzocaine R in methanol R and dilute to 100.0 ml with the same solvent. Dilute 1.0 ml to 50.0 ml with the mobile phase. Dilute 1.0 ml of this solution to 10.0 ml with the mobile phase.

Column:

- size: $l = 0.12$ m, $\varnothing = 4.0$ mm,
- stationary phase: octylsilyl silica gel for chromatography R (5 µm).

Mobile phase: mix 20 volumes of a mixture of 70 volumes of acetonitrile R and 80 volumes of methanol R, and 80 volumes of a solution containing 1.5 g/l of potassium dihydrogen phosphate R and 2.5 g/l of sodium octanesulphonate R adjusted to pH 2.2 with phosphoric acid R.

Flow rate: 1.0 ml/min.

Detection: spectrophotometer at 270 nm.

Injection: 20 µl.

Run time: 11 times the retention time of 4-aminobenzoic acid.

Relative retention with reference to 4-aminobenzoic acid (retention time = about 3 min): impurity A = about 4; impurity B = about 9.

Limits:

- **impurity A:** not more than the area of the corresponding peak in the chromatogram obtained with the reference solution (0.2 per cent),
- **impurity B:** not more than the area of the corresponding peak in the chromatogram obtained with the reference solution (0.2 per cent),
- **any other impurity:** not more than 0.5 times the area of the peak due to impurity A in the chromatogram obtained with the reference solution (0.1 per cent),
- **total:** not more than 2.5 times the area of the peak due to impurity A in the chromatogram obtained with the reference solution (0.5 per cent),

- *disregard limit*: 0.1 times the area of the peak due to impurity A in the chromatogram obtained with the reference solution (0.02 per cent).

Impurity C and impurity D. Gas chromatography (2.2.28).

Internal standard solution. Dissolve 20.0 mg of *lauric acid R* in *methylene chloride R* and dilute to 100.0 ml with the same solvent.

Test solution. Dissolve 1.000 g of the substance to be examined in 10.0 ml of an 84 g/l solution of *sodium hydroxide R* and extract with 2 quantities, each of 10 ml, of *methylene chloride R*. Combine and wash with 5 ml of *water R*; filter through *anhydrous sodium sulphate R*. Wash the filter with *methylene chloride R*. Evaporate in a water-bath at 50–60 °C to obtain a volume of about 1–5 ml. Add 1.0 ml of the internal standard solution and dilute to 10.0 ml with *methylene chloride R*.

Reference solution (a). Dissolve 20.0 mg of *aniline R* in *methylene chloride R* and dilute to 100.0 ml with the same solvent.

Reference solution (b). Dissolve 20.0 mg of *p-toluidine R* in *methylene chloride R* and dilute to 100.0 ml with the same solvent.

Reference solution (c). Dilute 0.50 ml of reference solution (a), 0.50 ml of reference solution (b) and 10.0 ml of the internal standard solution to 100.0 ml with *methylene chloride R*.

Column:

- *material*: fused silica,
- *size*: $l = 30$ m, $\varnothing = 0.32$ mm,
- *stationary phase*: poly[methyl(95)phenyl(5)] siloxane *R* (film thickness 0.5 µm).

Carrier gas: helium for chromatography *R*.

Flow rate: 1.0 ml/min.

Split ratio: 1:10.

Temperature:

	Time (min)	Temperature (°C)
Column	0 - 4	130
	4 - 6.5	130 → 180
	6.5 - 11.5	180
Injection port		280
Detector		300

Detection: flame ionisation.

Injection: 2 µl; inject the test solution and reference solution (c).

Retention time: internal standard = about 9.5 min.

Limits:

- *impurity C*: calculate the ratio (*R*) of the area of the peak due to impurity C to the area of the peak due to the internal standard from the chromatogram obtained with reference solution (c); calculate the ratio of the area of the peak due to impurity C to the area of the peak due to the internal standard from the chromatogram obtained with the test solution: this ratio is not greater than *R* (10 ppm),
- *impurity D*: calculate the ratio (*R*) of the area of the peak due to impurity D to the area of the peak due to the internal standard from the chromatogram obtained with reference solution (c); calculate the ratio of the area of the peak due to impurity D to the area of the peak due to the internal standard from the chromatogram obtained with the test solution: this ratio is not greater than *R* (10 ppm).

Iron (2.4.9): maximum 40 ppm.

Dissolve 0.250 g in 3 ml of *alcohol R* and dilute to 10.0 ml with *water R*.

Heavy metals (2.4.8): maximum 20 ppm.

1.0 g complies with limit test C. Prepare the standard using 2 ml of *lead standard solution (10 ppm Pb) R*.

Water (2.5.12): maximum 0.2 per cent, determined on 1.00 g.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

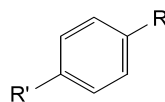
Dissolve 0.100 g with heating in 50 ml of *carbon dioxide-free water R*. Titrate with 0.1 *M sodium hydroxide* determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 *M sodium hydroxide* is equivalent to 13.71 mg of $C_6H_{13}NO_2$.

STORAGE

Protected from light.

IMPURITIES

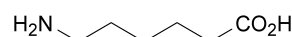


- A. $R = CO_2H$, $R' = NO_2$: 4-nitrobenzoic acid,
 B. $R = CO-O-C_2H_5$, $R' = NH_2$: benzocaine,
 C. $R = H$, $R' = NH_2$: aniline,
 D. $R = CH_3$, $R' = NH_2$: 4-methylaniline (*p*-toluidine).

01/2005:0874

AMINOCAPROIC ACID

Acidum aminocaproicum



$C_6H_{13}NO_2$

M_r 131.2

DEFINITION

Aminocaproic acid contains not less than 98.5 per cent and not more than the equivalent of 101.0 per cent of 6-aminohexanoic acid, calculated with reference to the dried substance.

CHARACTERS

A white, crystalline powder or colourless crystals, freely soluble in water, slightly soluble in alcohol.

It melts at about 205 °C with decomposition.

IDENTIFICATION

First identification: A.

Second identification: B, C, D.

- A. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *aminocaproic acid CRS*. Examine the substances prepared as discs.
- B. Examine the chromatograms obtained in the test for ninhydrin-positive substances. The principal spot in the chromatogram obtained with the test solution (b) is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).

- C. Dissolve 0.5 g in 4 ml of a mixture of equal volumes of *dilute hydrochloric acid R* and *water R*. Evaporate to dryness by heating on a water-bath. Dry the residue in a desiccator. Dissolve the residue in about 2 ml of boiling *ethanol R*. Allow to cool and maintain at 4 °C to 8 °C for 3 h. Filter under reduced pressure. The residue washed with about 10 ml of *acetone R* and dried at 60 °C for 30 min, melts (2.2.14) at 131 °C to 133 °C.
- D. Dissolve about 5 mg in 0.5 ml of *distilled water R*. Add 3 ml of *dimethylformamide R* and 2 ml of *ascorbic acid solution R*. Heat on a water-bath. An orange colour develops.

TESTS

Solution S. dissolve 10.0 g in *carbon dioxide-free water R* and dilute to 50.0 ml with the same solvent.

Appearance of solution. Solution S is colourless (2.2.2, *Method II*) and remains clear (2.2.1) on standing for 24 h.

pH (2.2.3). The pH of solution S is 7.5 to 8.0.

Absorbance (2.2.25).

- A. The absorbance of solution S at 287 nm is not more than 0.10 and at 450 nm is not more than 0.03.
- B. Place 2.0 g in an even layer in a shallow dish 9 cm in diameter, cover and allow to stand at 98 °C to 102 °C for 72 h. Dissolve in *water R* and dilute to 10.0 ml with the same solvent. The absorbance of the solution at 287 nm is not more than 0.15 and at 450 nm is not more than 0.03.

Ninhydrin-positive substances. Examine by thin-layer chromatography (2.2.27), using a suitable silica gel as the coating substance.

Test solution (a). Dissolve 0.10 g of the substance to be examined in *water R* and dilute to 10 ml with the same solvent.

Test solution (b). Dilute 1 ml of test solution (a) to 50 ml with *water R*.

Reference solution (a). Dissolve 10 mg of *aminocaproic acid CRS* in *water R* and dilute to 50 ml with the same solvent.

Reference solution (b). Dilute 5 ml of test solution (b) to 20 ml with *water R*.

Reference solution (c). Dissolve 10 mg of *aminocaproic acid CRS* and 10 mg of *leucine CRS* in *water R* and dilute to 25 ml with the same solvent.

Apply separately to the plate 5 µl of each solution. Allow the plate to dry in air. Develop over a path of 15 cm using a mixture of 20 volumes of *glacial acetic acid R*, 20 volumes of *water R* and 60 volumes of *butanol R*. Dry the plate in a current of warm air. Spray with *ninhydrin solution R* and heat at 100 °C to 105 °C for 15 min. Any spot in the chromatogram obtained with the test solution (a), apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.5 per cent). The test is not valid unless the chromatogram obtained with reference solution (c) shows two clearly separated principal spots.

Heavy metals (2.4.8). 12 ml of solution S complies with limit test A for heavy metals (10 ppm). Prepare the standard using *lead standard solution* (2 ppm Pb) *R*.

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

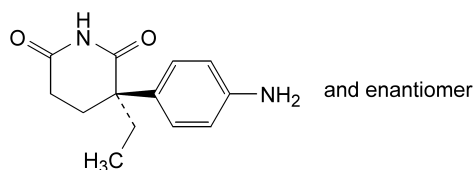
Dissolve 0.100 g in 20 ml of *anhydrous acetic acid R*. Using 0.1 ml of *crystal violet solution R* as indicator, titrate with 0.1 M *perchloric acid* until the colour changes from bluish-violet to bluish-green.

1 ml of 0.1 M *perchloric acid* is equivalent to 13.12 mg of C₆H₁₃N₂O₂.

01/2005:1291

AMINOGLUTETHIMIDE

Aminoglutethimidum

C₁₃H₁₆N₂O₂M_r 232.3

DEFINITION

Aminoglutethimide contains not less than 98.0 per cent and not more than the equivalent of 101.5 per cent of (3*RS*)-3-(4-aminophenyl)-3-ethylpiperidine-2,6-dione, calculated with reference to the dried substance.

CHARACTERS

A white or slightly yellow, crystalline powder, practically insoluble in water, freely soluble in acetone, soluble in methanol.

IDENTIFICATION

First identification: B.

Second identification: A, C.

A. Melting point (2.2.14). 150 °C to 154 °C.

B. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *aminoglutethimide CRS*. Examine the substances prepared as discs.

C. Examine by thin-layer chromatography (2.2.27), using as the coating substance a suitable silica gel with a fluorescent indicator having an optimal intensity at 254 nm.

Test solution. Dissolve 25 mg of the substance to be examined in *acetone R* and dilute to 5 ml with the same solvent.

Reference solution (a). Dissolve 25 mg of *aminoglutethimide CRS* in *acetone R* and dilute to 5 ml with the same solvent.

Reference solution (b). Dissolve 25 mg of *aminoglutethimide CRS* and 25 mg of *glutethimide CRS* in *acetone R* and dilute to 5 ml with the same solvent.

Apply to the plate 5 µl of each solution. Develop over a path of 15 cm using a mixture of 0.5 volumes of *glacial acetic acid R*, 15 volumes of *methanol R* and 85 volumes of *ethyl acetate R*. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. The principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a). The identification is not valid unless the chromatogram obtained with reference solution (b) shows two clearly separated spots.

TESTS

Solution S. Dissolve 1.0 g in *methanol R* and dilute to 20.0 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and not more intensely coloured than reference solution Y₇ (2.2.2, Method II).

Optical rotation (2.2.7). The angle of optical rotation, determined on solution S, is -0.10° to $+0.10^{\circ}$.

3-Aminoglutethimide and other related substances. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 0.10 g of the substance to be examined in a mixture of equal volumes of *methanol R* and *acetate buffer solution pH 5.0 R* and dilute to 50.0 ml with the same mixture of solvents.

Reference solution (a). Dissolve 5.0 mg of *aminoglutethimide impurity A CRS* in a mixture of equal volumes of *methanol R* and *acetate buffer solution pH 5.0 R* and dilute to 25.0 ml with the same mixture of solvents.

Reference solution (b). Dilute 1.0 ml of reference solution (a) to 10.0 ml with a mixture of equal volumes of *methanol R* and *acetate buffer solution pH 5.0 R*.

Reference solution (c). Dilute 1.0 ml of the test solution to 100.0 ml with a mixture of equal volumes of *methanol R* and *acetate buffer solution pH 5.0 R*.

Reference solution (d). Dilute 1.0 ml of the test solution to 10.0 ml with reference solution (a).

The chromatographic procedure may be carried out using:

- a stainless steel column 0.15 m long and 3.9 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (4 μ m),
- as mobile phase at a flow rate of 1.3 ml/min a mixture of 27 volumes of *methanol R* and 73 volumes of *acetate buffer solution pH 5.0 R*,
- as detector a spectrophotometer set at 240 nm,

maintaining the temperature of the column at 40 °C.

Inject 10 μ l of reference solution (d). When the chromatograms are recorded in the prescribed conditions, the retention times are: aminoglutethimide about 9 min and impurity A about 12 min. Adjust the sensitivity of the system so that the height of the principal peak in the chromatogram obtained with reference solution (d) is at least 60 per cent of the full scale of the recorder. The test is not valid unless in the chromatogram obtained with reference solution (d), the resolution between the peaks corresponding to aminoglutethimide and impurity A is at least 2.0.

Inject 10 μ l of the test solution, 10 μ l of reference solution (b) and 10 μ l of reference solution (c). Continue the chromatography of the test solution for four times the retention time of the principal peak. In the chromatogram obtained with the test solution: the area of any peak due to impurity A is not greater than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (2 per cent); the sum of the areas of any peaks apart from the principal peak and the peak due to impurity A is not greater than the area of the principal peak in the

chromatogram obtained with reference solution (c) (1 per cent); the sum of the contents of all the impurities is not greater than 2.0 per cent. Disregard any peak with an area less than 0.05 times that of the principal peak in the chromatogram obtained with reference solution (c).

Azoglutethimide. Not more than 300 ppm, determined by liquid chromatography (2.2.29). *Carry out the test protected from light. Use shaking, not sonication or heat, to dissolve the reference substance and the substance to be examined.*

Test solution. Dissolve 0.100 g of the substance to be examined in *dimethyl sulphoxide R* and dilute to 100.0 ml with the same solvent.

Reference solution. Dissolve 3.0 mg of *aminoglutethimide impurity D CRS* in *dimethyl sulphoxide R* and dilute to 100.0 ml with the same solvent. Dilute 1.0 ml of this solution to 100.0 ml with *dimethyl sulphoxide R*.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.12 m long and 4 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (5 μ m),
- as mobile phase at a flow of 1.0 ml/min a mixture prepared as follows: dissolve 0.285 g of *sodium edetate R* in *water R*, add 7.5 ml of *dilute acetic acid R* and 50 ml of 0.1 M *potassium hydroxide* and dilute to 1000 ml with *water R*; adjust to pH 5.0 with *glacial acetic acid R*; mix 350 ml of this solution with 650 ml of *methanol R*,
- as detector a spectrophotometer set at 328 nm.

Inject 10 μ l of each solution. The test is not valid unless: in the chromatogram obtained with the test solution the number of theoretical plates calculated for the principal peak is at least 3300; the mass distribution ratio of the principal peak is 2.0 to 5.0 and the symmetry factor of the principal peak is less than 1.2. The area of the peak due to impurity D in the chromatogram obtained with the test solution is not greater than that of the principal peak in the chromatogram obtained with the reference solution.

Sulphates (2.4.13). Dilute 6 ml of solution S to 15 ml with *distilled water R*. The solution complies with the limit test for sulphates (500 ppm).

Heavy metals (2.4.8). Dissolve 2.0 g in 15 ml of *acetone R* and dilute to 20 ml with *water R*. 12 ml of the solution complies with limit test B for heavy metals (10 ppm). Prepare the standard using lead standard solution (1 ppm Pb) obtained by diluting *lead standard solution (100 ppm Pb) R* with a mixture of 15 ml of *acetone R* and 5 ml of *water R*.

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C.

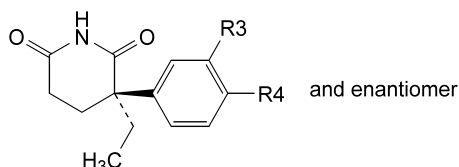
Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

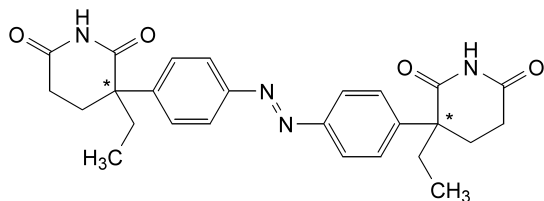
Dissolve 0.180 g in 50 ml of *anhydrous acetic acid R* and titrate with 0.1 M *perchloric acid*, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *perchloric acid* is equivalent to 23.23 mg of C₁₃H₁₆N₂O₂.

IMPURITIES



- A. R3 = NH₂, R4 = H: (3*RS*)-3-(3-aminophenyl)-3-ethylpiperidine-2,6-dione (3-aminoglutethimide),
 B. R3 = NO₂, R4 = H: (3*RS*)-3-ethyl-3-(3-nitrophenyl)piperidine-2,6-dione,
 C. R3 = H, R4 = NO₂: (3*RS*)-3-ethyl-3-(4-nitrophenyl)piperidine-2,6-dione,

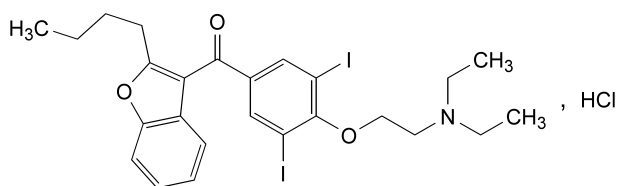


- D. 3,3'-[diazenediylbis(4,1-phenylene)]bis(3-ethylpiperidine-2,6-dione) (azoglutethimide).

01/2005:0803

AMIODARONE HYDROCHLORIDE

Amiodaroni hydrochloridum

C₂₅H₃₀ClN₂O₃M_r 682

DEFINITION

(2-Butylbenzofuran-3-yl)[4-[2-(diethylamino)ethoxy]-3,5-diiodophenyl]methanone hydrochloride.

Content: 98.5 per cent to 101.0 per cent (dried substance).

CHARACTERS

Appearance: white or almost white, fine crystalline powder.

Solubility: very slightly soluble in water, freely soluble in methylene chloride, soluble in methanol, sparingly soluble in alcohol.

IDENTIFICATION

- A. Infrared absorption spectrophotometry (2.2.24).

Comparison: amiodarone hydrochloride CRS.

- B. It gives reaction (b) of chlorides (2.3.1).

TESTS

Appearance of solution. The solution is clear (2.2.1) and not more intensely coloured than reference solution GY₅ or BY₅ (2.2.2, Method II).

Dissolve 1.0 g in *methanol R* and dilute to 20 ml with the same solvent.

pH (2.2.3): 3.2 to 3.8.

Dissolve 1.0 g in *carbon dioxide-free water R*, heating at 80 °C, cool and dilute to 20 ml with the same solvent.

Impurity H. Thin-layer chromatography (2.2.27). Prepare the solutions immediately before use and keep protected from bright light.

Test solution. Dissolve 0.5 g of the substance to be examined in *methylene chloride R* and dilute to 5.0 ml with the same solvent.

Reference solution. Dissolve 10 mg of (2-chloroethyl)diethylamine hydrochloride *R* in *methylene chloride R* and dilute to 50.0 ml with the same solvent.

Plate: TLC silica gel F₂₅₄ plate *R*.

Mobile phase: anhydrous formic acid *R*, methanol *R*, methylene chloride *R* (5:10:85 V/V/V).

Application: 5 µl.

Development: over a path of 15 cm.

Drying: in a current of cold air until the odour of solvents is no longer perceptible.

Detection: spray with *potassium iodobismuthate solution R1* and then with *dilute hydrogen peroxide solution R*. Examine immediately in daylight.

Limit:

- *impurity H*: any spot corresponding to impurity H is not more intense than the spot in the chromatogram obtained with the reference solution (0.2 per cent).

Related substances. Liquid chromatography (2.2.29).

Buffer solution pH 4.9. To 800 ml of *water R*, add 3.0 ml of *glacial acetic acid R*, adjust to pH 4.9 with *dilute ammonia R1* and dilute to 1000 ml with *water R*.

Test solution. Dissolve 0.125 g of the substance to be examined in a mixture of equal volumes of *acetonitrile R* and *water R* and dilute to 25.0 ml with the same mixture of solvents.

Reference solution. Dissolve 10 mg of *amiodarone impurity D CRS*, 10 mg of *amiodarone impurity E CRS* and 10.0 mg of *amiodarone hydrochloride CRS* in *methanol R* and dilute to 50.0 ml with the same solvent. Dilute 1.0 ml of the solution to 20.0 ml with a mixture of equal volumes of *acetonitrile R* and *water R*.

Column:

- *size*: *l* = 0.15 m, Ø = 4.6 mm,
- *stationary phase*: octadecylsilyl silica gel for chromatography *R* (5 µm),
- *temperature*: 30 °C.

Mobile phase: buffer solution pH 4.9, *methanol R*, *acetonitrile R* (30:30:40 V/V/V).

Flow rate: 1 ml/min.

Detection: spectrophotometer at 240 nm.

Injection: 10 µl.

Run time: twice the retention time of amiodarone.

Relative retention with reference to amiodarone (retention time = about 24 min): *impurity A* = about 0.26; *impurity D* = about 0.29; *impurity E* = about 0.37; *impurity B* = about 0.49; *impurity C* = about 0.55; *impurity G* = about 0.62; *impurity F* = about 0.69.

System suitability: reference solution:

- *resolution*: minimum 3.5 between the peaks due to *impurity D* and *impurity E*.

Limits:

- *any impurity*: not more than the area of the peak due to amiodarone in the chromatogram obtained with the reference solution (0.2 per cent),
- *total*: not more than 2.5 times the area of the peak due to amiodarone in the chromatogram obtained with the reference solution (0.5 per cent),

- *disregard limit*: 0.1 times the area of the peak due to amiodarone in the chromatogram obtained with the reference solution (0.02 per cent).

Iodides: maximum 150 ppm.

Prepare the test and reference solutions simultaneously.

Solution A. Add 1.50 g of the substance to be examined to 40 ml of *water R* at 80 °C and shake until completely dissolved. Cool and dilute to 50.0 ml with *water R*.

Test solution. To 15.0 ml of solution A add 1.0 ml of 0.1 M hydrochloric acid and 1.0 ml of 0.05 M potassium iodate. Dilute to 20.0 ml with *water R*. Allow to stand protected from light for 4 h.

Reference solution. To 15.0 ml of solution A add 1.0 ml of 0.1 M hydrochloric acid, 1.0 ml of a 88.2 mg/l solution of potassium iodide *R* and 1.0 ml of 0.05 M potassium iodate. Dilute to 20.0 ml with *water R*. Allow to stand protected from light for 4 h.

Measure the absorbances (2.2.25) of the solutions at 420 nm, using as the compensation liquid a mixture of 15.0 ml of solution A and 1.0 ml of 0.1 M hydrochloric acid diluted to 20.0 ml with *water R*. The absorbance of the test solution is not greater than half the absorbance of the reference solution.

Heavy metals (2.4.8): maximum 20 ppm.

1.0 g complies with limit test C. Prepare the standard using 2 ml of *lead standard solution* (10 ppm Pb) *R*.

Loss on drying (2.2.32): maximum 0.5 per cent, determined on 1.000 g by drying at 50 °C at a pressure not exceeding 0.3 kPa for 4 h.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

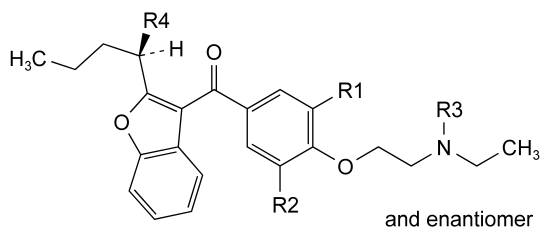
Dissolve 0.600 g in a mixture of 5.0 ml of 0.01 M hydrochloric acid and 75 ml of *alcohol R*. Carry out a potentiometric titration (2.2.20), using 0.1 M sodium hydroxide. Read the volume added between the 2 points of inflexion.

1 ml of 0.1 M sodium hydroxide is equivalent to 68.18 mg of $C_{25}H_{30}ClN_3O_4S$.

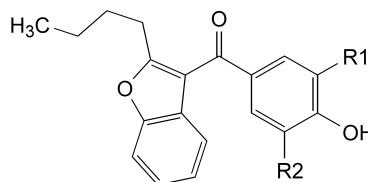
STORAGE

Protected from light, at a temperature not exceeding 30 °C.

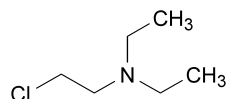
IMPURITIES



- A. R1 = R2 = R4 = H, R3 = C₂H₅: (2-butylbenzofuran-3-yl)[4-[2-(diethylamino)ethoxy]phenyl]methanone,
- B. R1 = R2 = I, R3 = R4 = H: (2-butylbenzofuran-3-yl)[4-[2-(ethylamino)ethoxy]-3,5-diiodophenyl]methanone,
- C. R1 = I, R2 = R4 = H, R3 = C₂H₅: (2-butylbenzofuran-3-yl)[4-[2-(diethylamino)ethoxy]-3-iodophenyl]methanone,
- G. R1 = R2 = I, R3 = C₂H₅, R4 = OCH₃: [2-[(1*RS*)-1-methoxybutyl]benzofuran-3-yl][4-[2-(diethylamino)ethoxy]-3,5-diiodophenyl]methanone,



- D. R1 = R2 = I: (2-butylbenzofuran-3-yl)(4-hydroxy-3,5-diiodophenyl)methanone,
- E. R1 = R2 = H: (2-butylbenzofuran-3-yl)(4-hydroxyphenyl)methanone,
- F. R1 = I, R2 = H: (2-butylbenzofuran-3-yl)(4-hydroxy-3-iodophenyl)methanone,

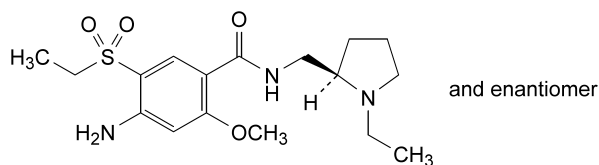


- H. 2-chloro-*N,N*-diethylethanamine (2-chlorotriethylamine, (2-chloroethyl)diethylamine).

01/2005:1490

AMISULPRIDE

Amisulpridum



C₁₇H₂₇N₃O₄S

*M*_r 369.5

DEFINITION

4-Amino-*N*-[[[(2*RS*)-1-ethylpyrrolidin-2-yl]methyl]-5-(ethylsulphonyl)-2-methoxybenzamide.

Content: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

Appearance: white or almost white, crystalline powder.

Solubility: practically insoluble in water, freely soluble in methylene chloride, sparingly soluble in ethanol.

mp: about 126 °C.

IDENTIFICATION

Infrared absorption spectrophotometry (2.2.24).

Comparison: amisulpride CRS.

TESTS

Appearance of solution. The solution is not more opalescent than reference suspension II (2.2.1) and not more intensely coloured than reference solution Y₆ (2.2.2, *Method II*).

Dissolve 1.0 g in 3 ml of a mixture of 1 volume of *acetic acid R* and 4 volumes of *water R* and dilute to 20 ml with *water R*.

Optical rotation (2.2.7): −0.10° to +0.10°.

Dissolve 5.0 g in *dimethylformamide R* and dilute to 50.0 ml with the same solvent.

Impurity A. Thin-layer chromatography (2.2.27).

Test solution. Dissolve 0.20 g in *methanol R* and dilute to 10 ml with the same solvent.

Reference solution (a). Dissolve 20 mg of *amisulpride impurity A CRS* in *methanol R* and dilute to 100 ml with the same solvent. Dilute 2 ml of the solution to 20 ml with *methanol R*.

Reference solution (b). Dilute 1 ml of the test solution to 10 ml with reference solution (a).

Plate: TLC silica gel G plate R.

Mobile phase: the upper layer obtained after shaking a mixture of a 50 per cent V/V solution of *concentrated ammonia R*, *ethanol R* and *di-isopropyl ether R* (10:25:65 V/V/V).

Application: 10 µl.

Development: over a path of 12 cm.

Drying: in air.

Detection: spray with *ninhydrin solution R* and heat at 100-105 °C for 15 min.

System suitability: the chromatogram obtained with reference solution (b) shows 2 clearly separated spots.

Limit:

- **impurity A:** any spot corresponding to impurity A is not more intense than the spot in the chromatogram obtained with reference solution (a) (0.1 per cent).

Related substances. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 0.10 g in 30 ml of *methanol R* and dilute to 100.0 ml with mobile phase B.

Reference solution (a). Dilute 5.0 ml of the test solution to 100.0 ml with a mixture of 30 volumes of mobile phase A and 70 volumes of mobile phase B. Dilute 1.0 ml of the solution to 25.0 ml with a mixture of 30 volumes of mobile phase A and 70 volumes of mobile phase B.

Reference solution (b). Dissolve 5 mg of *amisulpride impurity B CRS* in 5 ml of the test solution and dilute to 50 ml with a mixture of 30 volumes of mobile phase A and 70 volumes of mobile phase B. Dilute 1 ml of the solution to 10 ml with a mixture of 30 volumes of mobile phase A and 70 volumes of mobile phase B.

Column:

- **size:** $l = 0.25$ m, $\varnothing = 4.6$ mm,
- **stationary phase:** *octylsilyl silica gel for chromatography R* (5 µm) with a carbon loading of 16 per cent, a specific surface area of 330 m²/g and a pore size of 7.5 nm.

Mobile phase:

- **mobile phase A:** *methanol R*,
- **mobile phase B:** a 0.7 g/l solution of *sodium octanesulphonate R* in a 0.25 per cent V/V solution of *dilute sulphuric acid R*,

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)
0 - 18	30 → 36	70 → 64
18 - 35	36 → 52	64 → 48
35 - 45	52	48
45 - 46	52 → 30	48 → 70
46 - 56	30	70

Flow rate: 1.5 ml/min.

Detection: spectrophotometer at 225 nm.

Injection: 10 µl.

System suitability: reference solution (b):

- **resolution:** minimum 2.0 between the peaks due to amisulpride and impurity B.

Limits:

- **any impurity:** not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent),
- **total:** not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.3 per cent),
- **disregard limit:** 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.02 per cent).

Chlorides (2.4.4): maximum 200 ppm.

Shake 0.5 g with 30 ml of *water R* for 10 min. Filter. 15 ml of the filtrate complies with the limit test for chlorides.

Heavy metals (2.4.8): maximum 10 ppm.

Dissolve 4.0 g by gently heating in 5 ml of *dilute acetic acid R*. Allow to cool and dilute to 20 ml with *water R*. 12 ml of the solution complies with limit test A. Prepare the standard using *lead standard solution (2 ppm Pb) R*.

Loss on drying (2.2.32): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 100-105 °C for 3 h.

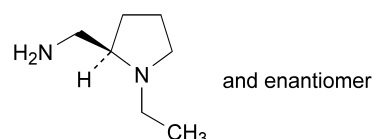
Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

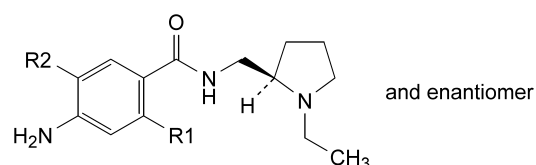
Dissolve 0.300 g with shaking in a mixture of 5 ml of *acetic anhydride R* and 50 ml of *anhydrous acetic acid R*. Titrate with 0.1 M *perchloric acid*, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *perchloric acid* is equivalent to 36.95 mg of C₁₇H₂₇N₃O₄S.

IMPURITIES



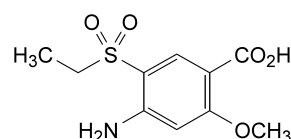
A. [(2*RS*)-1-ethylpyrrolidin-2-yl]methanamine,



B. R1 = OH, R2 = SO₂-CH₂-CH₃: 4-amino-*N*-[[(2*RS*)-1-ethylpyrrolidin-2-yl]methyl]-5-(ethylsulphonyl)-2-hydroxybenzamide,

C. R1 = OCH₃, R2 = I: 4-amino-*N*-[[(2*RS*)-1-ethylpyrrolidin-2-yl]methyl]-5-iodo-2-methoxybenzamide,

D. R1 = OCH₃, R2 = SO₂-CH₃: 4-amino-*N*-[[(2*RS*)-1-ethylpyrrolidin-2-yl]methyl]-2-methoxy-5-(methylsulphonyl)benzamide,

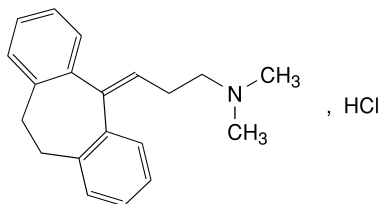


E. 4-amino-5-(ethylsulphonyl)-2-methoxybenzoic acid.

01/2005:0464

AMITRIPTYLINE HYDROCHLORIDE

Amitriptylini hydrochloridum

 $C_{20}H_{24}ClN$ M_r 313.9

DEFINITION

Amitriptyline hydrochloride contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of 3-(10,11-dihydro-5*H*-dibenzo[*a,d*]annulen-5-ylidene)-*N*,*N*-dimethylpropan-1-amine hydrochloride, calculated with reference to the dried substance.

CHARACTERS

A white or almost white powder or colourless crystals, freely soluble in water, in alcohol and in methylene chloride.

IDENTIFICATION

First identification: C, E.

Second identification: A, B, D, E.

- A. Melting point (2.2.14): 195 °C to 199 °C.
- B. Dissolve 25.0 mg in *methanol R* and dilute to 100.0 ml with the same solvent. Dilute 5.0 ml of the solution to 100.0 ml with *methanol R*. Examined between 230 nm and 350 nm (2.2.25), the solution shows an absorption maximum at 239 nm. The specific absorbance at the maximum is 435 to 475.
- C. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the *Ph. Eur. reference spectrum of amitriptyline hydrochloride*.
- D. Dissolve 0.1 g in 10 ml of *dilute sulphuric acid R*. Add 2 ml of a saturated solution of *potassium permanganate R*. The violet colour of the solution disappears quickly. Heat on a water-bath until the brown precipitate is almost completely dissolved. Cool, shake with 15 ml of *ether R* to remove the white turbidity and discard the ether layer. To the aqueous layer add 5 ml of *concentrated ammonia R* and shake for 2 min. Add 3 ml of *methylene chloride R* and shake again. A violet-red colour is produced in the lower layer.
- E. 50 mg gives reaction (b) of chlorides (2.3.1).

TESTS

Appearance of solution. Dissolve 1.25 g in *water R* and dilute to 25 ml with the same solvent. The solution is clear (2.2.1) and not more intensely coloured than reference solution B₇ (2.2.2, *Method II*).

Acidity or alkalinity. Dissolve 0.20 g in *carbon dioxide-free water R* and dilute to 10 ml with the same solvent. Add 0.1 ml of *methyl red solution R* and 0.2 ml of 0.01 *M sodium hydroxide*. The solution is yellow. Add 0.4 ml of 0.01 *M hydrochloric acid*. The solution is red.

Related substances. Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel plate R*. Prepare the solutions in subdued light and develop the chromatograms protected from light.

Test solution. Dissolve 0.20 g of the substance to be examined in *alcohol R* and dilute to 10 ml with the same solvent.

Reference solution (a). Dissolve 10 mg of *dibenzosuberone CRS* in *alcohol R* and dilute to 10 ml with the same solvent. Dilute 1 ml of the solution to 100 ml with *alcohol R*.

Reference solution (b). Dissolve 10 mg of *cyclobenzaprine hydrochloride CRS* in *alcohol R* and dilute to 10 ml with the same solvent. Dilute 2 ml of the solution to 50 ml with *alcohol R*.

Apply to the plate 5 µl of each solution. Develop in an unsaturated tank over a path of 14 cm using a mixture of 3 volumes of *diethylamine R*, 15 volumes of *ethyl acetate R* and 85 volumes of *cyclohexane R*. Heat the plate at 100-105 °C for 10 min and spray with a freshly prepared mixture of 4 volumes of *formaldehyde solution R* and 96 volumes of *sulphuric acid R*. Heat at 100-105 °C for 10 min and examine immediately in ultraviolet light at 365 nm and at 254 nm. In the chromatogram obtained with the test solution: any spots corresponding to dibenzosuberone and cyclobenzaprine hydrochloride are not more intense than the spots in the chromatograms obtained with reference solutions (a) (0.05 per cent) and (b) (0.2 per cent), respectively; any spot, apart from the principal spot and any spots corresponding to dibenzosuberone and cyclobenzaprine hydrochloride, is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.2 per cent).

Heavy metals (2.4.8). 1.0 g complies with limit test F (20 ppm). Prepare the standard using 2 ml of *lead standard solution* (10 ppm Pb) *R*.

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 100-105 °C for 2 h.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

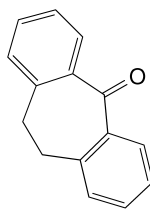
Dissolve 0.250 g in 30 ml of *alcohol R*. Titrate with 0.1 *M sodium hydroxide*, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 *M sodium hydroxide* is equivalent to 31.39 mg of $C_{20}H_{24}ClN$.

STORAGE

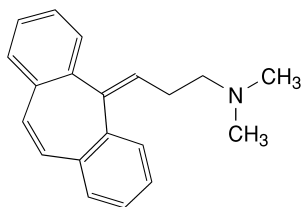
Store protected from light.

IMPURITIES

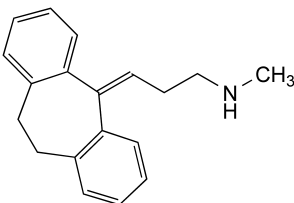


- A. 10,11-dihydro-5*H*-dibenzo[*a,d*]annulen-5-one (dibenzosuberone),

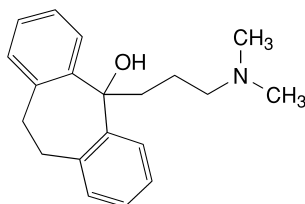
01/2005:1491



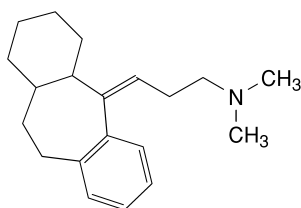
B. 3-(5*H*-dibenzo[*a,d*][7]annulen-5-ylidene)-*N,N*-dimethylpropan-1-amine (cyclobenzaprine),



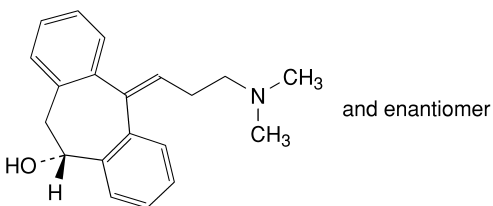
C. 3-(10,11-dihydro-5*H*-dibenzo[*a,d*][7]annulen-5-ylidene)-*N*-methylpropan-1-amine,



D. 5-[3-(dimethylamino)propyl]-10,11-dihydro-5*H*-dibenzo[*a,d*][7]annulen-5-ol,



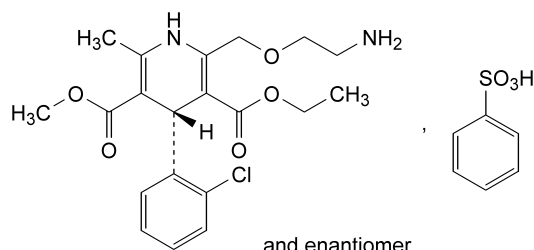
E. 3-(1,2,3,4,4a,10,11,11a-octahydro-5*H*-dibenzo[*a,d*][7]annulen-5-ylidene)-*N,N*-dimethylpropan-1-amine,



F. (10*RS*)-5-[3-(dimethylamino)propylidene]-10,11-dihydro-5*H*-dibenzo[*a,d*][7]annulen-10-ol.

AMLODIPINE BESILATE

Amlodipini besilas



and enantiomer

$C_{26}H_{31}ClN_2O_8S$

M_r 567.1

DEFINITION

3-Ethyl 5-methyl (4*RS*)-2-[(2-aminoethoxy)methyl]-4-(2-chlorophenyl)-6-methyl-1,4-dihydropyridine-3,5-dicarboxylate benzenesulphonate.

Content: 97.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

Appearance: white or almost white powder.

Solubility: slightly soluble in water, freely soluble in methanol, sparingly soluble in ethanol, slightly soluble in 2-propanol.

IDENTIFICATION

First identification: A.

Second identification: B, C.

A. Infrared absorption spectrophotometry (2.2.24).

Comparison: amlodipine besilate CRS.

Preparation: mulls.

B. Examine the chromatograms obtained in test A for related substances in ultraviolet light at 366 nm.

Results: the principal spot in the chromatogram obtained with test solution (b) is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (b).

C. Dissolve 5.0 mg in a 1 per cent *V/V* solution of 0.1 *M* hydrochloric acid in methanol *R* and dilute to 100.0 ml with the same acid solution. Examined between 300 nm and 400 nm (2.2.25), the solution shows an absorption maximum at 360 nm. The specific absorbance at the maximum is 113 to 121.

TESTS

Optical rotation (2.2.7): -0.10° to $+0.10^\circ$.

Dissolve 0.250 g in methanol *R* and dilute to 25.0 ml with the same solvent.

Related substances

A. Thin-layer chromatography (2.2.27).

Test solution (a). Dissolve 0.140 g of the substance to be examined in methanol *R* and dilute to 2.0 ml with the same solvent.

Test solution (b). Dilute 1.0 ml of test solution (a) to 10.0 ml with methanol *R*.

Reference solution (a). Dissolve 70.0 mg of amlodipine besilate CRS in 1.0 ml of methanol *R*.

Reference solution (b). Dilute 1 ml of reference solution (a) to 10 ml with methanol *R*.

Reference solution (c). Dilute 3.0 ml of test solution (b) to 100.0 ml with *methanol R*.

Reference solution (d). Dilute 1.0 ml of test solution (b) to 100.0 ml with *methanol R*.

Plate: TLC silica gel F_{254} plate *R*.

Mobile phase: the upper layer of a mixture of *glacial acetic acid R*, *water R* and *methyl isobutyl ketone R* (25:25:50 V/V/V).

Application: 10 μ l.

Development: over a path of 15 cm.

Drying: for 15 min at 80 °C.

Detection: examine in ultraviolet light at 254 nm and 366 nm.

System suitability: the chromatogram obtained with reference solution (a) shows 2 clearly separated minor spots with R_f values of about 0.18 and 0.22.

Limits: in the chromatogram obtained with test solution (a):

- **any impurity:** any spot, apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (c) (0.3 per cent) and at most 2 spots are more intense than the spot in the chromatogram obtained with reference solution (d) (0.1 per cent).

B. Liquid chromatography (2.2.29).

Test solution (a). Dissolve 50.0 mg of the substance to be examined in the mobile phase and dilute to 50.0 ml with the mobile phase.

Test solution (b). Dilute 5.0 ml of test solution (a) to 100.0 ml with the mobile phase.

Reference solution (a). Dissolve 50.0 mg of *amlodipine besilate CRS* in the mobile phase and dilute to 50.0 ml with the mobile phase. Dilute 5.0 ml of the solution to 100.0 ml with the mobile phase.

Reference solution (b). Dilute 3.0 ml of test solution (a) to 100.0 ml with the mobile phase and dilute 5.0 ml of the solution to 50.0 ml with the mobile phase.

Reference solution (c). Dissolve 5 mg of the substance to be examined in 5 ml of *strong hydrogen peroxide solution R*. Heat at 70 °C for 45 min.

Column:

- **size:** $l = 0.15$ m, $\varnothing = 3.9$ mm,
- **stationary phase:** *octadecylsilyl silica gel for chromatography R* (5 μ m).

Mobile phase: mix 15 volumes of *acetonitrile R*, 35 volumes of *methanol R* and 50 volumes of a solution prepared as follows: dissolve 7.0 ml of *triethylamine R* in 1 litre of *water R* and adjust to pH 3.0 ± 0.1 with *phosphoric acid R*.

Flow rate: 1.0 ml/min.

Detection: spectrophotometer at 237 nm.

Injection: 10 μ l; inject test solution (a) and reference solutions (b) and (c).

Run time: 3 times the retention time of amlodipine.

Relative retention with reference to amlodipine (retention time = about 7 min): impurity D = about 0.5.

System suitability: reference solution (c):

- **resolution:** minimum 4.5 between the peaks corresponding to amlodipine and impurity D.

Limits:

- **correction factor:** for the calculation of content, multiply the peak area of impurity D by 2,

- **impurity D:** not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.3 per cent),
- **total of other impurities:** not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.3 per cent); disregard any peak due to benzene sulphonate (relative retention = about 0.2),
- **disregard limit:** 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.03 per cent).

Water (2.5.12): maximum 0.5 per cent, determined on 3.000 g.

Sulphated ash (2.4.14): maximum 0.2 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (2.2.29) as described in the test for related substances with the following modification.

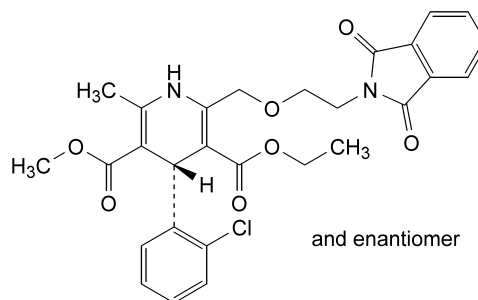
Injection: test solution (b), reference solution (a).

Calculate the percentage content of amlodipine besilate from the areas of the peaks and the declared content of $C_{26}H_{31}ClN_2O_8S$ in *amlodipine besilate CRS*.

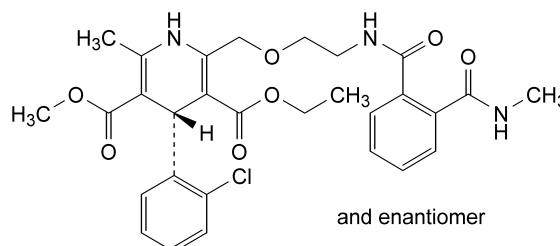
STORAGE

In an airtight container, protected from light.

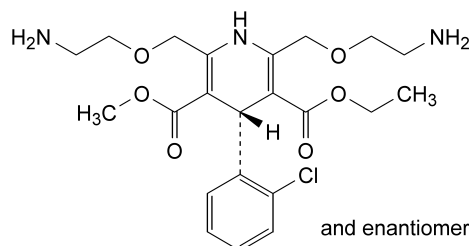
IMPURITIES



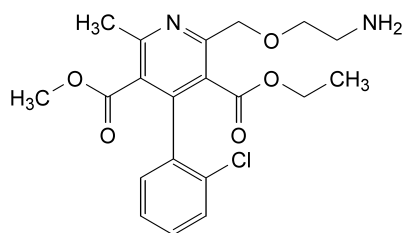
A. 3-ethyl 5-methyl (4RS)-4-(2-chlorophenyl)-2-[[2-(1,3-dioxo-1,3-dihydro-2H-isindol-2-yl)ethoxy]methyl]-6-methyl-1,4-dihydropyridine-3,5-dicarboxylate,



B. 3-ethyl 5-methyl (4RS)-4-(2-chlorophenyl)-6-methyl-2-[[2-[(methylcarbamoyl)benzoyl]amino]ethoxy]methyl]-1,4-dihydropyridine-3,5-dicarboxylate,



C. ethyl methyl (4RS)-2,6-bis[(2-aminoethoxy)methyl]-4-(2-chlorophenyl)-1,4-dihydropyridine-3,5-dicarboxylate,



D. 3-ethyl 5-methyl 2-[(2-aminoethoxy)methyl]-4-(2-chlorophenyl)-6-methylpyridine-3,5-dicarboxylate.

01/2005:0877

AMMONIA SOLUTION, CONCENTRATED

Ammoniae solutio concentrata

DEFINITION

Concentrated ammonia solution contains not less than 25.0 per cent *m/m* and not more than 30.0 per cent *m/m* of ammonia (NH_3 ; M_r 17.03).

CHARACTERS

A clear, colourless liquid, very caustic, miscible with water and with alcohol.

IDENTIFICATION

- Relative density (2.2.5): 0.892 to 0.910.
- It is strongly alkaline (2.2.4).
- To 0.5 ml add 5 ml of *water R*. Bubble air through the solution and lead the gaseous mixture obtained over the surface of a solution containing 1 ml of 0.1 *M hydrochloric acid* and 0.05 ml of *methyl red solution R*. The colour changes from red to yellow. Add 1 ml of *sodium cobaltinitrite solution R*. A yellow precipitate is formed.

TESTS

Solution S. Evaporate 220 ml almost to dryness on a water-bath. Cool, add 1 ml of *dilute acetic acid R* and dilute to 20 ml with *distilled water R*.

Appearance of solution. To 2 ml add 8 ml of *water R*. The solution is clear (2.2.1) and colourless (2.2.2, *Method II*).

Oxidisable substances. Cautiously add, whilst cooling, 8.8 ml to 100 ml of *dilute sulphuric acid R*. Add 0.75 ml of 0.002 *M potassium permanganate*. Allow to stand for 5 min. The solution remains faintly pink.

Pyridine and related substances. Measure the absorbance (2.2.25) at 252 nm using *water R* as the compensation liquid. The absorbance is not greater than 0.06 (2 ppm calculated as pyridine).

Carbonates. To 10 ml in a test-tube with a ground-glass neck add 10 ml of *calcium hydroxide solution R*. Stopper immediately and mix. Any opalescence in the solution is not more intense than that in a standard prepared at the same time and in the same manner using 10 ml of a 0.1 g/l solution of *anhydrous sodium carbonate R* (60 ppm).

Chlorides (2.4.4). Dilute 5 ml of solution S to 15 ml with *water R*. The solution complies with the limit test for chlorides (1 ppm).

Sulphates (2.4.13). Dilute 3 ml of solution S to 15 ml with *distilled water R*. The solution complies with the limit test for sulphates (5 ppm).

Iron (2.4.9). Dilute 4 ml of solution S to 10 ml with *water R*. The solution complies with the limit test for iron (0.25 ppm).

Heavy metals (2.4.8). Dilute 4 ml of solution S to 20 ml with *water R*. 12 ml of the solution complies with limit test A for heavy metals (1 ppm). Prepare the standard using *lead standard solution* (2 ppm Pb) *R*.

Residue on evaporation. Evaporate 50 ml to dryness on a water-bath and dry at 100 °C to 105 °C for 1 h. The residue weighs not more than 1 mg (0.02 g/l).

ASSAY

Weigh accurately a flask with a ground-glass neck containing 50.0 ml of 1 *M hydrochloric acid*. Add 2 ml of the substance to be examined and re-weigh. Add 0.1 ml of *methyl red solution R* as indicator. Titrate with 1 *M sodium hydroxide* until the colour changes from red to yellow.

1 ml of 1 *M hydrochloric acid* is equivalent to 17.03 mg of NH_3 .

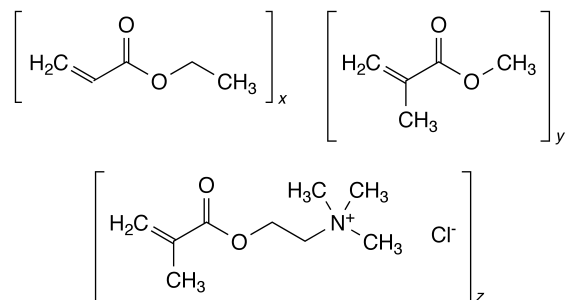
STORAGE

Store protected from air, at a temperature not exceeding 20 °C.

01/2005:2081

AMMONIO METHACRYLATE COPOLYMER (TYPE A)

Ammonio methacrylatis copolymerum A



DEFINITION

Poly(ethyl propenoate-co-methyl 2-methylpropenoate-co-2-(trimethylammonio)ethyl 2-methylpropenoate) chloride having a mean relative molecular mass of about 150 000.

The ratio of ethyl propenoate groups to methyl 2-methylpropenoate groups to 2-(trimethylammonio)ethyl 2-methylpropenoate groups is about 1:2:0.2.

Content of ammonio methacrylate groups: 8.9 per cent to 12.3 per cent (dried substance).

CHARACTERS

Appearance: colourless to white or almost white granules or powder.

Solubility: practically insoluble in water, freely soluble in ethanol and in methylene chloride giving clear to cloudy solutions. Due to the polymeric nature of the substance, a stirring time of up to 5 h may be necessary.

IDENTIFICATION

A. Infrared absorption spectrophotometry (2.2.24).

Preparation: drop solution S (see Tests) on a disc of *potassium bromide R* and dry *in vacuo* at 70 °C for 2 h.

Comparison: *Ph. Eur. reference spectrum of ammonio methacrylate copolymer (type A)*.

B. It complies with the test for viscosity (see Tests).

C. It complies with the limits of the assay.

TESTS

Solution S. Dissolve a quantity of the substance to be examined corresponding to 12.5 g of the dried substance in a mixture of 35.0 g of *acetone R* and 52.5 g of *2-propanol R*.

Viscosity (2.2.10): maximum 15 mPa·s, determined on solution S.

Apparatus: rotating viscosimeter.

Dimensions:

- *spindle*: diameter = 25.15 mm; height = 90.74 mm; shaft diameter = 4.0 mm;
- *cylinder*: diameter = 27.62 mm; height = 0.135 m.

Stirring speed: 30 r/min.

Volume of solution: 16 ml of solution S.

Temperature: 20 °C.

Appearance of a film. Spread 2 ml of solution S evenly on a glass plate. Upon drying a clear film is formed.

Monomers. Liquid chromatography (2.2.29).

Solution A. Dissolve 3.5 g of *sodium perchlorate R* in *water for chromatography R* and dilute to 100 ml with the same solvent.

Test solution. Dissolve 5.00 g of the substance to be examined in *methanol R* and dilute to 50.0 ml with the same solvent. To 10.0 ml of this solution add 5.0 ml of solution A, dropwise while continuously stirring. Remove the precipitated polymer by centrifugation. Use the clear supernatant solution.

Reference solution. Dissolve 50.0 mg of *ethyl acrylate R* and 10.0 mg of *methyl methacrylate R* in *methanol R* and dilute to 50.0 ml with the same solvent. Dilute 1.0 ml of the solution to 100.0 ml with *methanol R*. Add 10 ml of this solution to 5 ml of solution A.

Column:

- *size*: $l = 0.12$ m, $\varnothing = 4.6$ mm,
- *stationary phase*: octadecylsilyl silica gel for chromatography R (7 µm).

Mobile phase: dilute *phosphoric acid R* with *water for chromatography R* to obtain a solution at pH 2.0; mix 800 ml of this solution and 200 ml of *methanol R*, filter and degas.

Flow rate: 2.0 ml/min.

Detection: spectrophotometer at 202 nm.

Injection: 50 µl.

System suitability: reference solution:

- *resolution*: minimum 1.5 between the peaks due to impurity A and impurity B.

Limits:

- *impurity A*: not more than the area of the corresponding peak in the chromatogram obtained with the reference solution (100 ppm),
- *impurity B*: not more than 2.5 times the area of the corresponding peak in the chromatogram obtained with the reference solution (50 ppm).

Methanol (2.4.24, System A): maximum 1.5 per cent.

Heavy metals (2.4.8): maximum 20 ppm.

1.0 g complies with limit test C. Prepare the standard using 2.0 ml of *lead standard solution (10 ppm Pb R)*.

Loss on drying (2.2.32): maximum 3.0 per cent, determined on 1.000 g by drying *in vacuo* at 80 °C for 5 h.

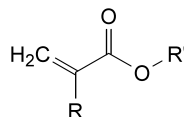
ASSAY

Dissolve 1.000 g in a mixture of 3 ml of *anhydrous formic acid R* and 30 ml of *anhydrous acetic acid R* and heat to dissolve. Add 20 ml of *acetic anhydride R*. Titrate with 0.1 M *perchloric acid*, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *perchloric acid* is equivalent to 20.77 mg of $C_9H_{18}O_2NCl$ (ammonio methacrylate groups).

IMPURITIES

Specified impurities: A, B.



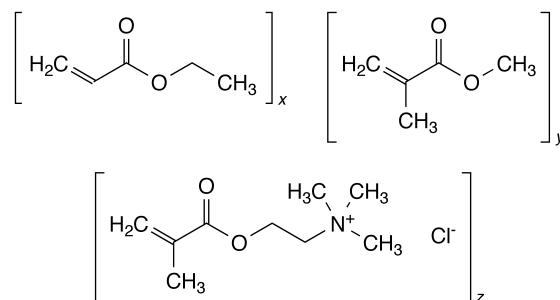
A. R = H, R' = C_2H_5 : ethyl propenoate (ethyl acrylate),

B. R = R' = CH_3 : methyl 2-methylpropenoate (methyl methacrylate).

01/2005:2082

AMMONIO METHACRYLATE COPOLYMER (TYPE B)

Ammonio methacrylatis copolymerum B



DEFINITION

Poly(ethyl propenoate-co-methyl 2-methylpropenoate-co-2-(trimethylammonio)ethyl 2-methylpropenoate) chloride having a mean relative molecular mass of about 150 000.

The ratio of ethyl propenoate groups to methyl 2-methylpropenoate groups to 2-(trimethylammonio)ethyl 2-methylpropenoate groups is about 1:2:0.1.

Content of ammonio methacrylate groups: 4.5 per cent to 7.0 per cent (dried substance).

CHARACTERS

Appearance: colourless to white or almost white granules or powder.

Solubility: practically insoluble in water, freely soluble in ethanol and in methylene chloride giving clear to cloudy solutions. Due to the polymeric nature of the substance, a stirring time of up to 5 h may be necessary.

IDENTIFICATION

A. Infrared absorption spectrophotometry (2.2.24).

Preparation: drop solution S (see Tests) on a disc of *potassium bromide R* and dry *in vacuo* at 70 °C for 2 h.

Comparison: *Ph. Eur. reference spectrum of ammonio methacrylate copolymer (type B)*.

B. It complies with the test for viscosity (see Tests).

C. It complies with the limits of the assay.

TESTS

Solution S. Dissolve a quantity of the substance to be examined corresponding to 12.5 g of the dried substance in a mixture of 35.0 g of *acetone R* and 52.5 g of *2-propanol R*.

Viscosity (2.2.10): maximum 15 mPa·s, determined on solution S.

Apparatus: rotating viscosimeter.

Dimensions:

- *spindle*: diameter = 25.15 mm; height = 90.74 mm; shaft diameter = 4.0 mm;
- *cylinder*: diameter = 27.62 mm; height = 0.135 m.

Stirring speed: 30 r/min.

Volume of solution: 16 ml of solution S.

Temperature: 20 °C.

Appearance of a film. Spread 2 ml of solution S evenly on a glass plate. Upon drying a clear film is formed.

Monomers. Liquid chromatography (2.2.29).

Solution A. Dissolve 3.5 g of *sodium perchlorate R* in *water for chromatography R* and dilute to 100 ml with the same solvent.

Test solution. Dissolve 5.00 g of the substance to be examined in *methanol R* and dilute to 50.0 ml with the same solvent. To 10.0 ml of this solution add 5.0 ml of solution A, dropwise while continuously stirring. Remove the precipitated polymer by centrifugation. Use the clear supernatant solution.

Reference solution. Dissolve 50.0 mg of *ethyl acrylate R* and 10.0 mg of *methyl methacrylate R* in *methanol R* and dilute to 50.0 ml with the same solvent. Dilute 1.0 ml of the solution to 100.0 ml with *methanol R*. Add 10 ml of this solution to 5 ml of solution A.

Column:

- *size*: $l = 0.12$ m, $\varnothing = 4.6$ mm,
- *stationary phase*: octadecylsilyl silica gel for chromatography R (7 μ m).

Mobile phase: dilute *phosphoric acid R* with *water for chromatography R* to obtain a solution at pH 2.0; mix 800 ml of this solution and 200 ml of *methanol R*, filter and degas.

Flow rate: 2.0 ml/min.

Detection: spectrophotometer at 202 nm.

Injection: 50 μ l.

System suitability: reference solution:

- *resolution*: minimum 1.5 between the peaks due to impurity A and impurity B.

Limits:

- *impurity A*: not more than the area of the corresponding peak in the chromatogram obtained with the reference solution (100 ppm),
- *impurity B*: not more than 2.5 times the area of the corresponding peak in the chromatogram obtained with the reference solution (50 ppm).

Methanol (2.4.24, System A): maximum 1.5 per cent.

Heavy metals (2.4.8): maximum 20 ppm.

1.0 g complies with limit test C. Prepare the standard using 2.0 ml of *lead standard solution (10 ppm Pb) R*.

Loss on drying (2.2.32): maximum 3.0 per cent, determined on 1.000 g by drying *in vacuo* at 80 °C for 5 h.

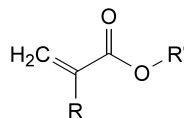
ASSAY

Dissolve 2.000 g in a mixture of 3 ml of *anhydrous formic acid R* and 30 ml of *anhydrous acetic acid R* and heat to dissolve. Add 20 ml of *acetic anhydride R*. Titrate with 0.1 M *perchloric acid*, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *perchloric acid* is equivalent to 20.77 mg of $\text{C}_9\text{H}_{18}\text{O}_2\text{NCl}$ (ammonio methacrylate groups).

IMPURITIES

Specified impurities: A, B.



A. R = H, R' = C_2H_5 : ethyl propenoate (ethyl acrylate),

B. R = R' = CH_3 : methyl 2-methylpropenoate (methyl methacrylate).

01/2005:1389

AMMONIUM BROMIDE

Ammonii bromidum

NH_4Br

M_r 97.9

DEFINITION

Content: 98.5 per cent to 100.5 per cent (dried substance).

CHARACTERS

Appearance: white or almost white, crystalline powder or colourless crystals, hygroscopic.

Solubility: freely soluble in water, sparingly soluble in alcohol.

It becomes yellow when exposed to light or air.

IDENTIFICATION

A. It gives reaction (a) of bromides (2.3.1).

B. 10 ml of solution S (see Tests) gives the reaction of ammonium salts (2.3.1).

TESTS

Solution S. Dissolve 10.0 g in *carbon dioxide-free water R* prepared from *distilled water R* and dilute to 100 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and colourless (2.2.2, *Method II*).

Acidity or alkalinity. To 10 ml of solution S add 0.05 ml of *methyl red solution R*. Not more than 0.5 ml of 0.01 M *hydrochloric acid* or 0.01 M *sodium hydroxide* is required to change the colour of the indicator.

Bromates. To 10 ml of solution S add 1 ml of *starch solution R*, 0.1 ml of a 100 g/l solution of *potassium iodide R* and 0.25 ml of 0.5 M *sulphuric acid* and allow to stand protected from light for 5 min. No blue or violet colour develops.

Chlorides: maximum 0.6 per cent.

In a conical flask, dissolve 1.000 g in 20 ml of *dilute nitric acid R*. Add 5 ml of *strong hydrogen peroxide solution R* and heat on a water-bath until the solution is completely decolorised. Wash down the sides of the flask with a little *water R* and heat on a water-bath for 15 min. Allow to cool, dilute to 50 ml with *water R* and add 5.0 ml of 0.1 M *silver nitrate* and 1 ml of *dibutyl phthalate R*. Shake and titrate

with 0.1 M ammonium thiocyanate using 5 ml of ferric ammonium sulphate solution R2 as indicator. Not more than 1.7 ml of 0.1 M silver nitrate is used. Note the volume of 0.1 M silver nitrate used (see Assay). Carry out a blank test.

Iodides. To 5 ml of solution S add 0.15 ml of ferric chloride solution R1 and 2 ml of methylene chloride R. Shake and allow to separate. The lower layer is colourless (2.2.2, Method I).

Sulphates (2.4.13): maximum 100 ppm.

15 ml of solution S complies with the limit test for sulphates.

Iron (2.4.9): maximum 20 ppm.

5 ml of solution S diluted to 10 ml with water R complies with the limit test for iron.

Magnesium and alkaline-earth metals (2.4.7): maximum 200 ppm, calculated as Ca.

10.0 g complies with the limit test for magnesium and alkaline-earth metals. The volume of 0.01 M sodium edetate used does not exceed 5.0 ml.

Heavy metals (2.4.8): maximum 10 ppm.

12 ml of solution S complies with limit test A. Prepare the standard using lead standard solution (1 ppm Pb) R.

Loss on drying (2.2.32): maximum 1.0 per cent, determined on 1.000 g by drying in an oven at 100-105 °C.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 1.500 g in water R and dilute to 100.0 ml with the same solvent. To 10.0 ml of the solution add 50 ml of water R, 5 ml of dilute nitric acid R, 25.0 ml of 0.1 M silver nitrate and 2 ml of dibutyl phthalate R. Shake. Titrate with 0.1 M ammonium thiocyanate using 2 ml of ferric ammonium sulphate solution R2 as indicator and shaking vigorously towards the end-point.

1 ml of 0.1 M silver nitrate is equivalent to 9.794 mg of NH₄Br.

Calculate the percentage content of NH₄Br from the expression:

$$a - 2.763 b$$

a = percentage content of NH₄Br and NH₄Cl obtained in the assay and calculated as NH₄Br,

b = percentage content of Cl obtained in the test for chlorides.

STORAGE

In an airtight container, protected from light.

DEFINITION

Ammonium chloride contains not less than 99.0 per cent and not more than the equivalent of 100.5 per cent of NH₄Cl, calculated with reference to the dried substance.

CHARACTERS

A white, crystalline powder or colourless crystals, freely soluble in water.

IDENTIFICATION

A. It gives the reactions of chlorides (2.3.1).

B. 10 ml of solution S (see Tests) gives the reaction of ammonium salts (2.3.1).

TESTS

Solution S. Dissolve 10.0 g in carbon dioxide-free water R prepared from distilled water R and dilute to 100 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and colourless (2.2.2, Method II).

Acidity or alkalinity. To 10 ml of solution S add 0.05 ml of methyl red solution R. Not more than 0.5 ml of 0.01 M hydrochloric acid or 0.01 M sodium hydroxide is required to change the colour of the indicator.

Bromides and iodides. To 10 ml of solution S add 0.1 ml of dilute hydrochloric acid R and 0.05 ml of chloramine solution R. After 1 min, add 2 ml of chloroform R and shake vigorously. The chloroform layer remains colourless (2.2.2, Method I).

Sulphates (2.4.13). 10 ml of solution S diluted to 15 ml with distilled water R complies with the limit test for sulphates (150 ppm).

Calcium (2.4.3). 5 ml of solution S diluted to 15 ml with distilled water R complies with the limit test for calcium (200 ppm).

Iron (2.4.9). 5 ml of solution S diluted to 10 ml with water R complies with the limit test for iron (20 ppm).

Heavy metals (2.4.8). 12 ml of solution S complies with limit test A (10 ppm). Prepare the standard using lead standard solution (1 ppm Pb) R.

Loss on drying (2.2.32). Not more than 1.0 per cent, determined on 1.00 g by drying in an oven at 100-105 °C for 2 h.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 2.0 g.

01/2005:0007 ASSAY

Dissolve 1.000 g in 20 ml of water R and add a mixture of 5 ml of formaldehyde solution R, previously neutralised to phenolphthalein solution R, and 20 ml of water R. After 1 min to 2 min, titrate slowly with 1 M sodium hydroxide, using a further 0.2 ml of the same indicator.

1 ml of 1 M sodium hydroxide is equivalent to 53.49 mg of NH₄Cl.

AMMONIUM CHLORIDE

Ammonii chloridum

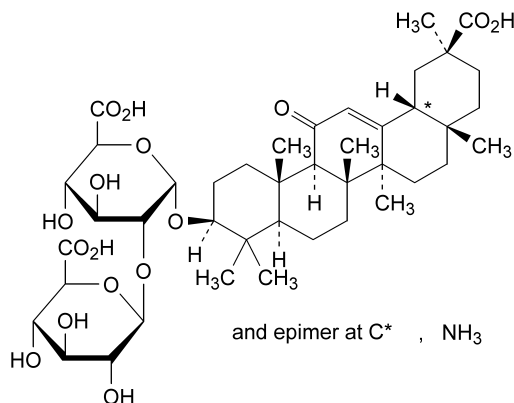
NH₄Cl

M_r 53.49

01/2005:1772 *Reference solution (a).* Dilute 1.0 ml of the test solution to 20.0 ml with the mobile phase.

AMMONIUM GLYCYRRHIZATE

Ammonii glycyrrhizas



$C_{42}H_{65}NO_{16}$

M_r 840

DEFINITION

Mixture of ammonium 18 α - and 18 β -glycyrrhizate (ammonium salt of (20 β)-3 β -[[2-*O*-(β -D-glucopyranosyluronic acid)- α -D-glucopyranosyluronic acid]oxy]-11-oxoolean-12-en-29-oic acid), the 18 β -isomer being the main component.

Content: 98.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

Appearance: white or yellowish-white, hygroscopic powder.

Solubility: slightly soluble in water, very slightly soluble in anhydrous ethanol, practically insoluble in acetone. It dissolves in dilute solutions of acids and of alkali hydroxides.

IDENTIFICATION

A. Infrared absorption spectrophotometry (2.2.24).

Comparison: ammonium glycyrrhizate CRS.

B. Dissolve 0.1 g in 20 ml of *water R*, add 2 ml of *dilute sodium hydroxide solution R* and heat cautiously. On heating, the solution gives off vapours that may be identified by the alkaline reaction of wet litmus paper (2.3.1).

TESTS

Appearance of solution. The solution is clear (2.2.1) and not more intensely coloured than reference solution BY₇ (2.2.2, *Method I*).

Dissolve 1.0 g in *ethanol (20 per cent V/V) R* and dilute to 100.0 ml with the same solvent.

Specific optical rotation (2.2.7): + 49.0 to + 54.0 (anhydrous substance).

Dissolve 0.5 g in *ethanol (50 per cent V/V) R* and dilute to 50.0 ml with the same solvent.

Related substances. Liquid chromatography (2.2.29).

Test solution. Dissolve 0.100 g of the substance to be examined in the mobile phase and dilute to 100.0 ml with the mobile phase.

Reference solution (b). Dissolve 50 mg of *ammonium glycyrrhizate CRS* in the mobile phase and dilute to 50.0 ml with the mobile phase. Dilute 1.0 ml of the solution to 20.0 ml with the mobile phase.

Column:

- *size:* $l = 0.25$ m, $\varnothing = 4.0$ mm,
- *stationary phase:* octadecylsilyl silica gel for chromatography R (5-10 μ m).

Mobile phase: glacial acetic acid R, acetonitrile R, water R (6:380:614 V/V/V).

Flow rate: 1.2 ml/min.

Detection: spectrophotometer at 254 nm.

Injection: 10 μ l.

Run time: 3 times the retention time of 18 β -glycyrrhizic acid.

Relative retention with reference to 18 β -glycyrrhizic acid (retention time = about 8 min): impurity A = about 0.8; 18 α -glycyrrhizic acid = about 1.2.

System suitability: reference solution (b):

- *resolution:* minimum 2.0 between the peaks due to 18 β -glycyrrhizic acid and 18 α -glycyrrhizic acid.

Limits:

- *18 α -glycyrrhizic acid:* not more than twice the sum of the areas of the peaks in the chromatogram obtained with reference solution (a) (10.0 per cent),
- *impurity A:* not more than the sum of the areas of the peaks in the chromatogram obtained with reference solution (a) (5.0 per cent),
- *any other impurity:* for each impurity, not more than 0.4 times the sum of the areas of the peaks in the chromatogram obtained with reference solution (a) (2.0 per cent),
- *total of other impurities:* not more than 1.4 times the sum of the areas of the peaks in the chromatogram obtained with reference solution (a) (7.0 per cent),
- *disregard limit:* 0.04 times the sum of the areas of the peaks in the chromatogram obtained with reference solution (a) (0.2 per cent).

Heavy metals (2.4.8): maximum 20 ppm.

1.0 g complies with limit test C. Prepare the reference solution using 2 ml of *lead standard solution (10 ppm Pb) R*.

Water (2.5.12): maximum 6.0 per cent, determined on 0.250 g.

Sulphated ash (2.4.14): maximum 0.2 per cent, determined on 1.0 g.

ASSAY

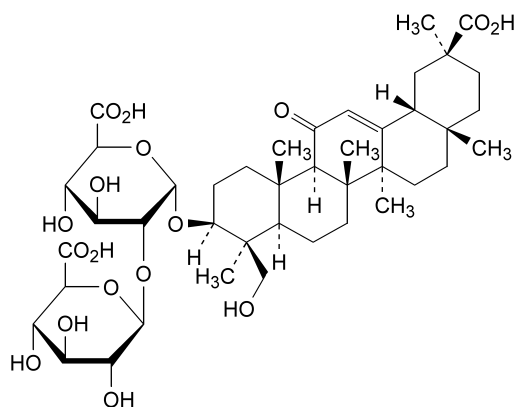
Dissolve 0.600 g in 60 ml of *acetic acid R* heating at 80 °C if necessary. Cool. Titrate with 0.1 M *perchloric acid*, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *perchloric acid* is equivalent to 84.0 mg of $C_{42}H_{65}NO_{16}$.

STORAGE

In an airtight container.

IMPURITIES



- A. (4β,20β)-3β-[[2-O-(β-D-glucopyranosyluronic acid)-α-D-glucopyranosyluronic acid]oxy]-23-hydroxy-11-oxoolean-12-en-29-oic acid (24-hydroxyglycyrrhizinic acid).

01/2005:1390

AMMONIUM HYDROGEN CARBONATE

Ammonii hydrogenocarbonas

 NH_4HCO_3 M_r 79.1

DEFINITION

Ammonium hydrogen carbonate contains not less than 98.0 per cent and not more than 101.0 per cent of the equivalent of ammonium hydrogen carbonate.

CHARACTERS

A fine, white, crystalline powder or white crystals, slightly hygroscopic, freely soluble in water, practically insoluble in alcohol.

It volatilises rapidly at 60 °C. The volatilisation takes place slowly at ambient temperatures if the substance is slightly moist. It is in a state of equilibrium with ammonium carbamate.

IDENTIFICATION

- It gives the reaction of carbonates and bicarbonates (2.3.1).
- Dissolve 50 mg in 2 ml of *water R*. The solution gives the reaction of ammonium salts (2.3.1).

TESTS

Solution S. Dissolve 14.0 g in 100 ml of *distilled water R*. Boil to remove the ammonia, allow to cool and dilute to 100.0 ml with *distilled water R*.

Chlorides (2.4.4). Dilute 5 ml of solution S to 15 ml with *water R*. The solution complies with the limit test for chlorides (70 ppm).

Sulphates (2.4.13). 15 ml of solution S complies with the limit test for sulphates (70 ppm).

Iron (2.4.9). Dilute 1.8 ml of solution S to 10 ml with *water R*. The solution complies with the limit test for iron (40 ppm).

Heavy metals (2.4.8). Dissolve cautiously 2.5 g in 25 ml of 1 M *hydrochloric acid*. 12 ml of the solution complies with limit test A for heavy metals (10 ppm). Prepare the standard using *lead standard solution* (1 ppm Pb) *R*.

ASSAY

Dissolve cautiously 1.0 g in 20.0 ml of 0.5 M *sulphuric acid* and dilute to 50 ml with *water R*. Boil, cool and titrate the excess of acid with 1 M *sodium hydroxide*, using 0.1 ml of *methyl red solution R* as indicator.

1 ml of 0.5 M *sulphuric acid* is equivalent to 79.1 mg of NH_4HCO_3 .

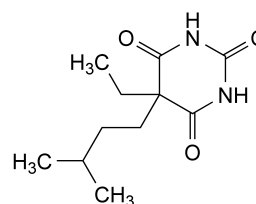
STORAGE

Store in an airtight container.

01/2005:0594

AMOBARBITAL

Amobarbitalum

 $\text{C}_{11}\text{H}_{18}\text{N}_2\text{O}_3$ M_r 226.3

DEFINITION

Amobarbital contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of 5-ethyl-5-(3-methylbutyl)pyrimidin-2,4,6(1*H*,3*H*,5*H*)-trione, calculated with reference to the dried substance.

CHARACTERS

A white, crystalline powder, very slightly soluble in water, freely soluble in alcohol, soluble in methylene chloride. It forms water-soluble compounds with alkali hydroxides and carbonates and with ammonia.

IDENTIFICATION

First identification: A, B.

Second identification: A, C, D.

- Determine the melting point (2.2.14) of the substance to be examined. Mix equal parts of the substance to be examined and *amobarbital CRS* and determine the melting point of the mixture. The difference between the melting points (which are about 157 °C) is not greater than 2 °C.
- Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *amobarbital CRS*.
- Examine by thin-layer chromatography (2.2.27), using *silica gel GF₂₅₄ R* as the coating substance.

Test solution. Dissolve 0.1 g of the substance to be examined in *alcohol R* and dilute to 100 ml with the same solvent.

Reference solution. Dissolve 0.1 g of *amobarbital CRS* in *alcohol R* and dilute to 100 ml with the same solvent.

Apply separately to the plate 10 µl of each solution. Develop over a path of 18 cm using the lower layer from a mixture of 5 volumes of *concentrated ammonia R*, 15 volumes of *alcohol R* and 80 volumes of *chloroform R*. Examine immediately in ultraviolet light at 254 nm. The principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with the reference solution.

D. It gives the reaction of non-nitrogen substituted barbiturates (2.3.1).

TESTS

Appearance of solution. Dissolve 1.0 g in a mixture of 4 ml of *dilute sodium hydroxide solution R* and 6 ml of *water R*. The solution is clear (2.2.1) and not more intensely coloured than reference solution Y₆ (2.2.2, *Method II*).

Acidity or alkalinity. To 1.0 g add 50 ml of *water R* and boil for 2 min. Allow to cool and filter. To 10 ml of the filtrate add 0.15 ml of *methyl red solution R* and 0.1 ml of 0.01 M *sodium hydroxide*. The solution is yellow. Add 0.2 ml of 0.01 M *hydrochloric acid*. The solution is red.

Related substances. Examine by thin-layer chromatography (2.2.27), using *silica gel GF₂₅₄ R* as the coating substance.

Test solution. Dissolve 1.0 g of the substance to be examined in *alcohol R* and dilute to 100 ml with the same solvent.

Reference solution. Dilute 0.5 ml of the test solution to 100 ml with *alcohol R*.

Apply separately to the plate 20 µl of each solution. Develop over a path of 15 cm using the lower layer from a mixture of 5 volumes of *concentrated ammonia R*, 15 volumes of *alcohol R* and 80 volumes of *chloroform R*. Examine the plate immediately in ultraviolet light at 254 nm. Any spot in the chromatogram obtained with the test solution, apart from the principal spot, is not more intense than the spot in the chromatogram obtained with the reference solution. Spray with *diphenylcarbazone mercuric reagent R*. Allow the plate to dry in air and spray with freshly prepared *alcoholic potassium hydroxide solution R* diluted 1 in 5 with *aldehyde-free alcohol R*. Heat at 100 °C to 105 °C for 5 min and examine immediately. Any spot in the chromatogram obtained with the test solution, apart from the principal spot, is not more intense than the spot in the chromatogram obtained with the reference solution (0.5 per cent).

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

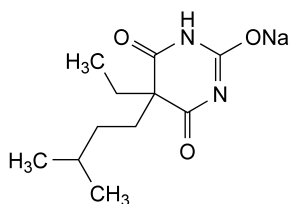
Dissolve 0.100 g in 5 ml of *pyridine R*. Add 0.5 ml of *thymolphthalein solution R* and 10 ml of *silver nitrate solution in pyridine R*. Titrate with 0.1 M *ethanolic sodium hydroxide* until a pure blue colour is obtained. Carry out a blank titration.

1 ml of 0.1 M *ethanolic sodium hydroxide* is equivalent to 11.31 mg of C₁₁H₁₈N₂O₃.

01/2005:0166

AMOBARBITAL SODIUM

Amobarbitalum natricum

C₁₁H₁₇N₂NaO₃M_r 248.3

DEFINITION

Amobarbital sodium contains not less than 98.5 per cent and not more than the equivalent of 102.0 per cent of sodium derivative of 5-ethyl-5-(3-methylbutyl)pyrimidin-2,4,6(1*H*,3*H*,5*H*)-trione, calculated with reference to the dried substance.

CHARACTERS

A white, granular powder, hygroscopic, very soluble in carbon dioxide-free water (a small fraction may be insoluble), freely soluble in alcohol.

IDENTIFICATION

First identification: A, B, E.

Second identification: A, C, D, E.

A. Acidify 10 ml of solution S (see Tests) with *dilute hydrochloric acid R* and shake with 20 ml of *ether R*. Separate the ether layer, wash with 10 ml of *water R*, dry over *anhydrous sodium sulphate R* and filter. Evaporate the filtrate to dryness and dry the residue at 100 °C to 105 °C (test residue). Repeat the operations using 0.1 g of *amobarbital sodium CRS* (reference residue). Determine the melting point (2.2.14) of the test residue. Mix equal parts of the test residue and the reference residue and determine the melting point of the mixture. The difference between the melting points (which are about 157 °C) is not greater than 2 °C.

B. Examine by infrared absorption spectrophotometry (2.2.24), comparing the spectrum obtained with the reference residue prepared from *amobarbital sodium CRS* with that obtained with the test residue (see identification test A).

C. Examine by thin-layer chromatography (2.2.27), using *silica gel GF₂₅₄ R* as the coating substance.

Test solution. Dissolve 0.1 g of the substance to be examined in *alcohol R* and dilute to 100 ml with the same solvent.

Reference solution. Dissolve 0.1 g of *amobarbital sodium CRS* in *alcohol R* and dilute to 100 ml with the same solvent.

Apply separately to the plate 10 µl of each solution. Develop over a path of 18 cm using the lower layer of a mixture of 5 volumes of *concentrated ammonia R*, 15 volumes of *alcohol R* and 80 volumes of *chloroform R*. Examine immediately in ultraviolet light at 254 nm. The principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with the reference solution.

D. It gives the reaction of non-nitrogen substituted barbiturates (2.3.1).

E. It gives reaction (a) of sodium (2.3.1).

TESTS

Solution S. Dissolve 5.0 g in *alcohol (50 per cent V/V) R* and dilute to 50 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and not more intensely coloured than reference solution Y₇ (2.2.2, *Method II*).

pH (2.2.3). Dissolve 5.0 g in *carbon dioxide-free water R* and dilute to 50 ml with the same solvent. Disregard any slight residue. The pH of the solution is not more than 11.0.

Related substances. Examine by thin-layer chromatography (2.2.27), using *silica gel GF₂₅₄ R* as the coating substance.

Test solution. Dissolve 1.0 g of the substance to be examined in *alcohol R* and dilute to 100 ml with the same solvent.

Reference solution. Dilute 0.5 ml of the test solution to 100 ml with *alcohol R*.

Apply separately to the plate 20 µl of each solution. Develop over a path of 15 cm using the lower layer of a mixture of 5 volumes of *concentrated ammonia R*, 15 volumes of *alcohol R* and 80 volumes of *chloroform R*. Examine the plate immediately in ultraviolet light at 254 nm. Spray with *diphenylcarbazone mercuric reagent R*. Allow the plate to dry in air and spray with freshly prepared *alcoholic potassium hydroxide solution R* diluted 1 in 5 with *aldehyde-free alcohol R*. Heat at 100 °C to 105 °C for 5 min and examine immediately. When examined in ultraviolet light and after spraying, any spot in the chromatogram obtained with the test solution, apart from the principal spot, is not more intense than the spot in the chromatogram obtained with the reference solution (0.5 per cent). Disregard any spot at the starting-point.

Loss on drying (2.2.32). Not more than 3.0 per cent, determined on 0.50 g by drying in an oven at 130 °C.

ASSAY

Dissolve 0.200 g in 5 ml of *ethanol R*. Add 0.5 ml of *thymolphthalein solution R* and 10 ml of *silver nitrate solution in pyridine R*. Titrate with 0.1 M *ethanolic sodium hydroxide* until a pure blue colour is obtained. Carry out a blank titration.

1 ml of 0.1 M *ethanolic sodium hydroxide* is equivalent to 24.83 mg of $C_{16}H_{18}N_3NaO_5S$.

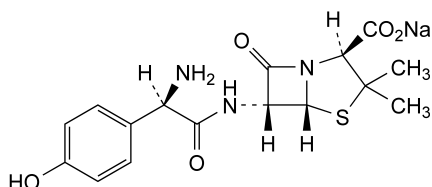
STORAGE

Store in an airtight container.

01/2005:0577
corrected

AMOXICILLIN SODIUM

Amoxicillinum natricum



$C_{16}H_{18}N_3NaO_5S$

M_r 387.4

DEFINITION

Amoxicillin sodium contains not less than 89.0 per cent and not more than the equivalent of 102.0 per cent of sodium (2*S*,5*R*,6*R*)-6-[(2*R*)-2-amino-2-(4-hydroxyphenyl)acetyl]amino]-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylate, calculated with reference to the anhydrous substance.

CHARACTERS

A white or almost white powder, very hygroscopic, very soluble in water, sparingly soluble in ethanol, very slightly soluble in acetone.

IDENTIFICATION

First identification: A, D.

Second identification: B, C, D.

A. Dissolve 0.250 g in 5 ml of *water R*, add 0.5 ml of *dilute acetic acid R*, swirl and allow to stand for 10 min in iced water. Filter the crystals and wash with 2 ml to 3 ml

of a mixture of 1 volume of *water R* and 9 volumes of *acetone R*, then dry in an oven at 60 °C for 30 min. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *amoxicillin trihydrate CRS*.

B. Examine by thin-layer chromatography (2.2.27), using a *TLC silanised silica gel plate R*.

Test solution. Dissolve 25 mg of the substance to be examined in 10 ml of *sodium hydrogen carbonate solution R*.

Reference solution (a). Dissolve 25 mg of *amoxicillin trihydrate CRS* in 10 ml of *sodium hydrogen carbonate solution R*.

Reference solution (b). Dissolve 25 mg of *amoxicillin trihydrate CRS* and 25 mg of *ampicillin trihydrate CRS* in 10 ml of *sodium hydrogen carbonate solution R*.

Apply to the plate 1 µl of each solution. Develop over a path of 15 cm using a mixture of 10 volumes of *acetone R* and 90 volumes of a 154 g/l solution of *ammonium acetate R*, the pH of which has been adjusted to 5.0 with *glacial acetic acid R*. Allow the plate to dry in air and expose it to iodine vapour until the spots appear. Examine in daylight. The principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a). The test is not valid unless the chromatogram obtained with reference solution (b) shows 2 clearly separated spots.

C. Place about 2 mg in a test-tube about 150 mm long and 15 mm in diameter. Moisten with 0.05 ml of *water R* and add 2 ml of *sulphuric acid-formaldehyde reagent R*. Mix the contents of the tube by swirling; the solution is practically colourless. Place the test-tube in a water-bath for 1 min; a dark yellow colour develops.

D. It gives reaction (a) of sodium (2.3.1).

TESTS

Appearance of solution. Dissolve 1.0 g in *water R* and dilute to 10.0 ml with the same solvent. Immediately after dissolution, the solution is not more opalescent than reference suspension II (2.2.1). The solution may show an initial, but transient, pink colour. After 5 min, the absorbance measured at 430 nm (2.2.25) is not greater than 0.20.

pH (2.2.3). Dissolve 2.0 g in *carbon dioxide-free water R* and dilute to 20 ml with the same solvent. The pH of the solution is 8.0 to 10.0.

Specific optical rotation (2.2.7). Dissolve 62.5 mg in a 4 g/l solution of *potassium hydrogen phthalate R* and dilute to 25.0 ml with the same solution. The specific optical rotation is + 240 to + 290, calculated with reference to the anhydrous substance.

Related substances. Examine by liquid chromatography (2.2.29) as described under Assay. Inject 50 µl of reference solution (d) and elute isocratically until elution of the amoxicillin peak. Inject 50 µl of test solution (b) and start the elution isocratically. Immediately after elution of the amoxicillin peak start the following linear gradient. If the mobile phase composition has been adjusted to achieve the required resolution, the adjusted composition will apply at time zero in the gradient.

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)	Comment
0 - 25	92 → 0	8 → 100	linear gradient
25 - 40	0	100	isocratic
40 - 55	92	8	re-equilibration

Inject mobile phase A and use the same elution pattern to obtain a blank. Inject reference solution (e). The 3 principal peaks eluted after the main peak correspond to amoxicillin diketopiperazine, amoxicillin dimer (impurity J; $n = 1$) and amoxicillin trimer (impurity J; $n = 2$) and, compared to the principal peak they have a relative retention time of about 3.4, 4.1 and 4.5, respectively. In the chromatogram obtained with test solution (b): the area of any peak corresponding to amoxicillin dimer is not greater than 3 times the area of the principal peak in the chromatogram obtained with reference solution (d) (3 per cent); the area of any peak, apart from the principal peak and any peak corresponding to amoxicillin dimer, is not greater than twice the area of the principal peak in the chromatogram obtained with reference solution (d) (2 per cent); the sum of the areas of all the peaks, apart from the principal peak, is not greater than 9 times the area of the principal peak in the chromatogram obtained with reference solution (d) (9 per cent). Disregard any peak with an area less than 0.1 times that of the principal peak in the chromatogram obtained with reference solution (d).

N,N-Dimethylaniline (2.4.26, *Method A or B*). Not more than 20 ppm.

2-Ethylhexanoic acid (2.4.28). Not more than 0.8 per cent *m/m*.

Heavy metals (2.4.8). 1.0 g complies with limit test C for heavy metals (20 ppm). Prepare the standard using 2 ml of *lead standard solution* (10 ppm Pb) R.

Water (2.5.12). Not more than 3.0 per cent, determined on 0.400 g by the semi-micro determination of water.

Bacterial endotoxins (2.6.14): less than 0.25 IU/mg, if intended for use in the manufacture of parenteral dosage forms without a further appropriate procedure for the removal of bacterial endotoxins.

ASSAY

Examine by liquid chromatography (2.2.29).

Test solution (a). Dissolve 30.0 mg of the substance to be examined in mobile phase A and dilute to 50.0 ml with the same mobile phase.

Test solution (b). Prepare immediately before use. Dissolve 30.0 mg of the substance to be examined in mobile phase A and dilute to 20.0 ml with the same mobile phase.

Reference solution (a). Dissolve 30.0 mg of *amoxicillin trihydrate CRS* in mobile phase A and dilute to 50.0 ml with the same mobile phase.

Reference solution (b). Dissolve 4.0 mg of *cefadroxil CRS* in mobile phase A and dilute to 50 ml with the same mobile phase. To 5.0 ml of this solution add 5.0 ml of reference solution (a) and dilute to 100 ml with mobile phase A.

Reference solution (c). Dilute 1.0 ml of reference solution (a) to 20.0 ml with mobile phase A. Dilute 1.0 ml of the solution to 50.0 ml with mobile phase A.

Reference solution (d). Dilute 2.0 ml of reference solution (a) to 20.0 ml with mobile phase A. Dilute 5.0 ml of the solution to 20.0 ml with mobile phase A.

Reference solution (e). To 0.20 g of *amoxicillin trihydrate R* add 1.0 ml of *water R*. Shake and add dropwise *dilute sodium hydroxide solution R* to obtain a solution. The pH of the solution is about 8.5. Store the solution at room temperature for 4 h. Dilute 0.5 ml of the solution to 50.0 ml with mobile phase A.

The chromatographic procedure may be carried out using:

- a column 0.25 m long and 4.6 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (5 μ m),

- as mobile phase at a flow rate of 1.0 ml/min:

Mobile phase A. Mix 1 volume of *acetonitrile R* and 99 volumes of a 25 per cent V/V solution of 0.2 M *potassium dihydrogen phosphate R* adjusted to pH 5.0 with *dilute sodium hydroxide solution R*,

Mobile phase B. Mix 20 volumes of *acetonitrile R* and 80 volumes of a 25 per cent V/V solution of 0.2 M *potassium dihydrogen phosphate R* adjusted to pH 5.0 with *dilute sodium hydroxide solution R*,

- as detector a spectrophotometer set at 254 nm.

Equilibrate the column with a mobile phase ratio A:B of 92:8. Inject 50 μ l of reference solution (b). The test is not valid unless in the chromatogram obtained, the resolution between the peaks corresponding to amoxicillin and cefadroxil is at least 2.0 (if necessary, adjust the ratio A:B of the mobile phase) and the mass distribution ratio for the first peak (amoxicillin) is 1.3 to 2.5. Inject reference solution (c). Adjust the system to obtain a peak with a signal-to-noise ratio of at least 3. Inject reference solution (a) 6 times. The test is not valid unless the relative standard deviation for the area of the principal peak is at most 1.0 per cent. Inject test solution (a) and reference solution (a).

Calculate the percentage content of amoxicillin sodium by multiplying the percentage content of amoxicillin by 1.060.

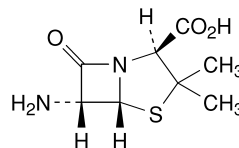
STORAGE

Store in an airtight container. If the substance is sterile, store in a sterile, airtight, tamper-proof container.

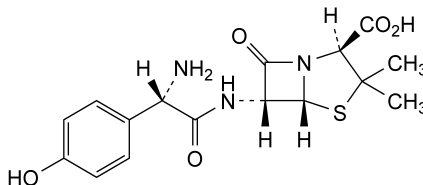
LABELLING

The label states, where applicable, that the substance is free from bacterial endotoxins.

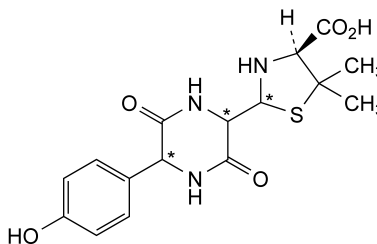
IMPURITIES



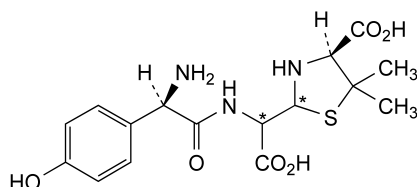
- A. (2*S*,5*R*,6*R*)-6-amino-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid (6-aminopenicillanic acid),



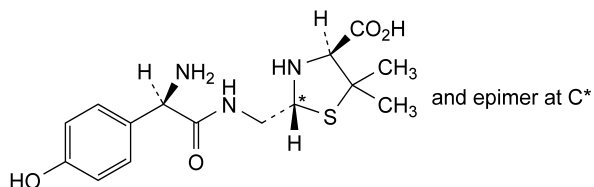
- B. (2*S*,5*R*,6*R*)-6-[(2*S*)-2-amino-2-(4-hydroxyphenyl)acetyl]amino-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid (L-amoxicillin),



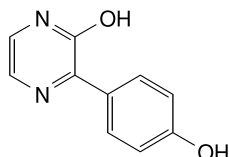
- C. (4*S*)-2-[5-(4-hydroxyphenyl)-3,6-dioxopiperazin-2-yl]-5,5-dimethylthiazolidine-4-carboxylic acid (amoxicillin diketopiperazines),



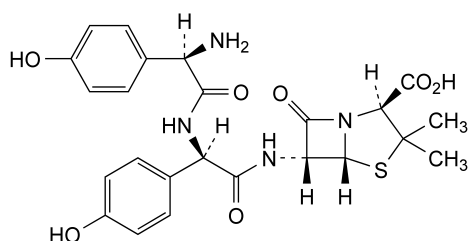
D. (4*S*)-2-[[[(2*R*)-2-amino-2-(4-hydroxyphenyl)acetyl]amino]carboxymethyl]-5,5-dimethylthiazolidine-4-carboxylic acid (penicilloic acids of amoxicillin),



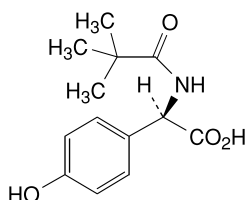
E. (2*RS*,4*S*)-2-[[[(2*R*)-2-amino-2-(4-hydroxyphenyl)acetyl]amino]methyl]-5,5-dimethylthiazolidine-4-carboxylic acid (penilloic acids of amoxicillin),



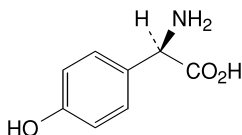
F. 3-(4-hydroxyphenyl)pyrazin-2-ol,



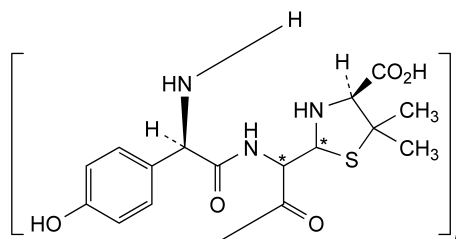
G. (2*S*,5*R*,6*R*)-6-[[[(2*R*)-2-[[[(2*R*)-2-amino-2-(4-hydroxyphenyl)acetyl]amino]-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid (D-(4-hydroxyphenyl)glycyl]amox-icillin),



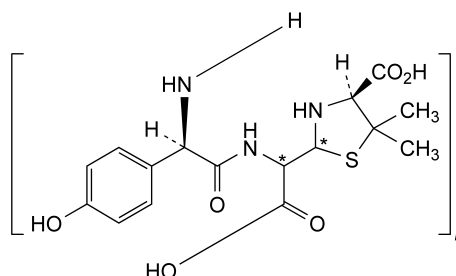
H. (2*R*)-2-[(2,2-dimethylpropanoyl)amino]-2-(4-hydroxyphenyl)acetic acid,



I. (2*R*)-2-amino-2-(4-hydroxyphenyl)acetic acid,



J. co-oligomers of amoxicillin and penicilloic acids of amoxicillin,

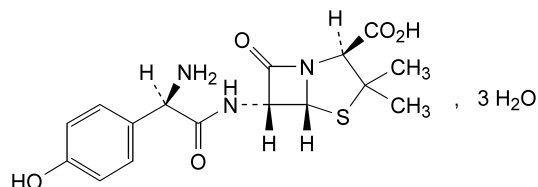


K. oligomers of penicilloic acids of amoxicillin.

01/2005:0260

AMOXICILLIN TRIHYDRATE

Amoxicillinum trihydricum


 $C_{16}H_{19}N_3O_5S \cdot 3H_2O$
 M_r 419.4

DEFINITION

Amoxicillin trihydrate contains not less than 95.0 per cent and not more than the equivalent of 102.0 per cent of (2*S*,5*R*,6*R*)-6-[[[(2*R*)-2-[[[(2*R*)-2-amino-2-(4-hydroxyphenyl)acetyl]amino]-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid, calculated with reference to the anhydrous substance.

CHARACTERS

A white or almost white, crystalline powder, slightly soluble in water, very slightly soluble in alcohol, practically insoluble in fatty oils. It dissolves in dilute acids and dilute solutions of alkali hydroxides.

IDENTIFICATION

First identification: A.

Second identification: B, C.

A. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *amoxicillin trihydrate CRS*.

B. Examine by thin-layer chromatography (2.2.27), using *silanised silica gel H R* as the coating substance.

Test solution. Dissolve 25 mg of the substance to be examined in 10 ml of *sodium hydrogen carbonate solution R*.

Reference solution (a). Dissolve 25 mg of *amoxicillin trihydrate CRS* in 10 ml of *sodium hydrogen carbonate solution R*.

Reference solution (b). Dissolve 25 mg of *amoxicillin trihydrate CRS* and 25 mg of *ampicillin trihydrate CRS* in 10 ml of *sodium hydrogen carbonate solution R*.

Apply to the plate 1 µl of each solution. Develop over a path of 15 cm using a mixture of 10 volumes of *acetone R* and 90 volumes of a 154 g/l solution of *ammonium acetate R*, the pH of which has been adjusted to 5.0 with *glacial acetic acid R*. Allow the plate to dry in air and expose it to iodine vapour until the spots appear. Examine in daylight. The principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a). The test is not valid unless the chromatogram obtained with reference solution (b) shows 2 clearly separated spots.

- C. Place about 2 mg in a test-tube about 150 mm long and 15 mm in diameter. Moisten with 0.05 ml of *water R* and add 2 ml of *sulphuric acid-formaldehyde reagent R*. Mix the contents of the tube by swirling; the solution is practically colourless. Place the test-tube in a water-bath for 1 min; a dark yellow colour develops.

TESTS

Solution S. With the aid of ultrasound or gentle heating, dissolve 0.100 g in *carbon dioxide-free water R* and dilute to 50.0 ml with the same solvent.

Appearance of solution. Dissolve 1.0 g in 10 ml of 0.5 M *hydrochloric acid*. Dissolve separately 1.0 g in 10 ml of *dilute ammonia R2*. Immediately after dissolution, the solutions are not more opalescent than reference suspension II (2.2.1).

pH (2.2.3). The pH of solution S is 3.5 to 5.5.

Specific optical rotation (2.2.7): + 290 to + 315, determined on solution S and calculated with reference to the anhydrous substance.

Related substances. Examine by liquid chromatography (2.2.29) adjusting the ratio A:B of the mobile phase and the attenuation as described under Assay. Inject reference solution (d). Inject a freshly prepared test solution (b) and start the elution isocratically with the chosen mobile phase. Immediately after elution of the amoxicillin peak start a linear gradient elution to reach a mobile phase A:B of 0:100 over a period of 25 min. Continue the chromatography with mobile phase B for 15 min, then equilibrate the column for 15 min with the mobile phase chosen originally. Inject mobile phase A and use the same elution gradient to obtain a blank. In the chromatogram obtained with test solution (b), the area of any peak, apart from the principal peak and any peak observed in the blank chromatogram, is not greater than the area of the principal peak in the chromatogram obtained with reference solution (d) (1 per cent).

***N,N*-Dimethylaniline (2.4.26, Method A or B).** Not more than 20 ppm.

Water (2.5.12): 11.5 per cent to 14.5 per cent, determined on 0.100 g by the semi-micro determination of water.

Sulphated ash (2.4.14). Not more than 1.0 per cent, determined on 1.0 g.

ASSAY

Examine by liquid chromatography (2.2.29).

Test solution (a). Dissolve 30.0 mg of the substance to be examined in mobile phase A and dilute to 50.0 ml with the same mobile phase.

Test solution (b). Dissolve 30.0 mg of the substance to be examined in mobile phase A and dilute to 20.0 ml with the same mobile phase.

Reference solution (a). Dissolve 30.0 mg of *amoxicillin trihydrate CRS* in mobile phase A and dilute to 50.0 ml with the same mobile phase.

Reference solution (b). Dissolve 4.0 mg of *cefadroxil CRS* in mobile phase A and dilute to 50 ml with the same mobile phase. To 5.0 ml of this solution add 5.0 ml of reference solution (a) and dilute to 100 ml with mobile phase A.

Reference solution (c). Dilute 1.0 ml of reference solution (a) to 20.0 ml with mobile phase A. Dilute 1.0 ml of this solution to 50.0 ml with mobile phase A.

Reference solution (d). Dilute 2.0 ml of reference solution (a) to 20.0 ml with mobile phase A. Dilute 5.0 ml of this solution to 20.0 ml with mobile phase A.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4.6 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (5 µm),
- as mobile phase at a flow rate of 1.0 ml/min:

Mobile phase A. A mixture of 1 volume of *acetonitrile R* and 99 volumes of buffer solution pH 5.0,

Mobile phase B. A mixture of 20 volumes of *acetonitrile R* and 80 volumes of buffer solution pH 5.0,

Prepare the buffer solution as follows: to 250 ml of 0.2 M *potassium dihydrogen phosphate R* add *dilute sodium hydroxide solution R* to pH 5.0 and dilute to 1000.0 ml with *water R*,

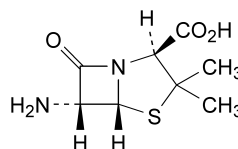
- as detector a spectrophotometer set at 254 nm,
- a 50 µl loop injector.

Equilibrate the column with a mobile phase with ratio A:B of 92:8. Inject reference solution (b). The assay is not valid unless the resolution between the 2 principal peaks is at least 2.0. If necessary, adjust the ratio A:B of the mobile phase. The mass distribution ratio for the first peak (amoxicillin) is 1.3 to 2.5. Inject reference solution (c). Adjust the system to obtain a peak with a signal-to-noise ratio of at least 3. Inject reference solution (a) 6 times. The test is not valid unless the relative standard deviation for the area of the principal peak is at most 1.0 per cent. Inject alternately test solution (a) and reference solution (a).

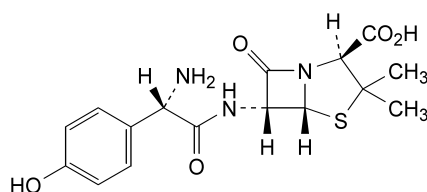
STORAGE

Store in an airtight container.

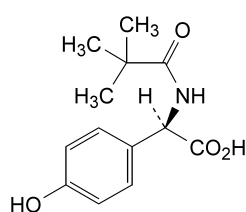
IMPURITIES



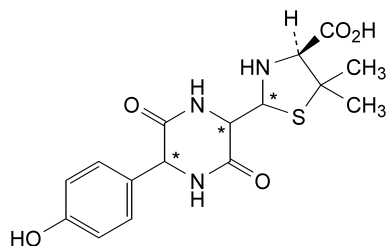
- A. (2*S*,5*R*,6*R*)-6-amino-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid (6-aminopenicillanic acid),



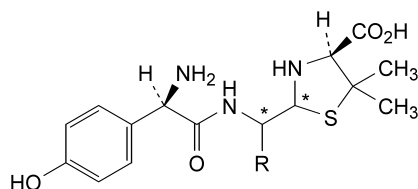
- B. (2*S*,5*R*,6*R*)-6-[[[(2*S*)-2-amino-2-(4-hydroxyphenyl)acetyl]amino]-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid (L-amoxicillin),



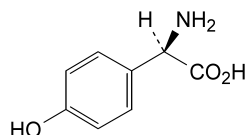
- H. (2*R*)-2-[(2,2-dimethylpropanoyl)amino]-2-(4-hydroxyphenyl)acetic acid,



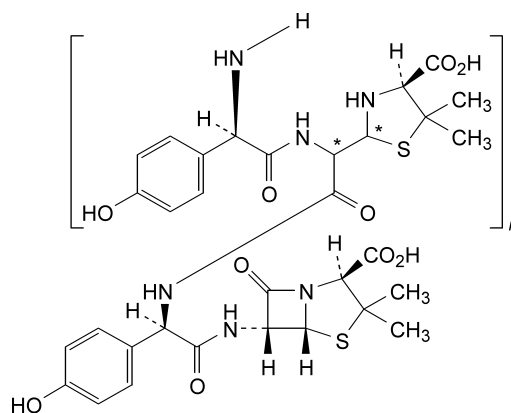
- C. (4*S*)-2-[5-(4-hydroxyphenyl)-3,6-dioxopiperazin-2-yl]-5,5-dimethylthiazolidine-4-carboxylic acid (amoxicillin diketopiperazines),



- D. R = CO₂H: (4*S*)-2-[[[(2*R*)-2-amino-2-(4-hydroxyphenyl)acetyl]amino]carboxymethyl]-5,5-dimethylthiazolidine-4-carboxylic acid (penicilloic acids of amoxicillin),

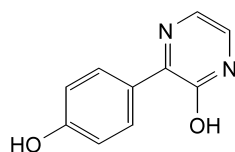


- I. (2*R*)-2-amino-2-(4-hydroxyphenyl)acetic acid,

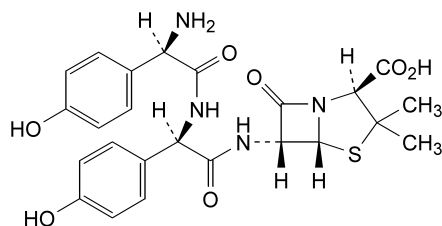


- J. co-oligomers of amoxicillin and of penicilloic acids of amoxicillin,

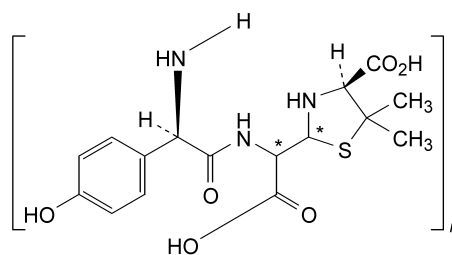
- E. R = H: (2*RS*,4*S*)-2-[[[(2*R*)-2-amino-2-(4-hydroxyphenyl)acetyl]amino]methyl]-5,5-dimethylthiazolidine-4-carboxylic acid (penicilloic acids of amoxicillin),



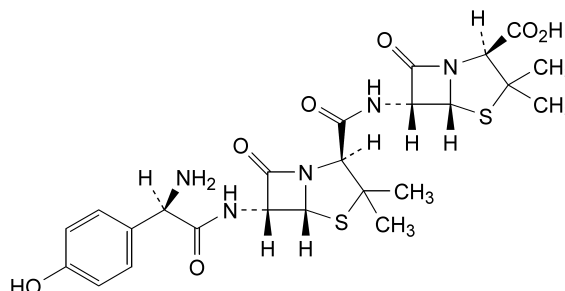
- F. 3-(4-hydroxyphenyl)pyrazin-2-ol,



- G. (2*S*,5*R*,6*R*)-6-[[[(2*R*)-2-[[[(2*R*)-2-amino-2-(4-hydroxyphenyl)acetyl]amino]-2-(4-hydroxyphenyl)acetyl]amino]-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid (D-(4-hydroxyphenyl)glycylamoxicillin),



- K. oligomers of penicilloic acids of amoxicillin,

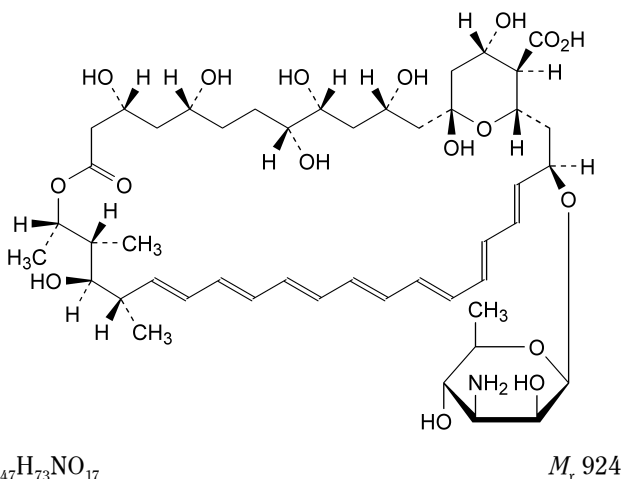


- L. (2*S*,5*R*,6*R*)-6-[[[(2*S*,5*R*,6*R*)-6-[[[(2*R*)-2-amino-2-(4-hydroxyphenyl)acetyl]amino]-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxyl]amino]-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid (6-APA amoxicillin amide).

01/2005:1292

AMPHOTERICIN B

Amphotericinum B



DEFINITION

Amphotericin B is a mixture of antifungal polyenes produced by the growth of certain strains of *Streptomyces nodosus* or by any other means. It consists mainly of amphotericin B which is (1*R*,3*S*,5*R*,6*R*,9*R*,11*R*,15*S*,16*R*,17*R*,18*S*,19*E*,21*E*,23*E*, 25*E*, 27*E*,29*E*,31*E*,33*R*,35*S*,36*R*,37*S*)-33-[(3-amino-3,6-dideoxy-β-D-mannopyranosyl)oxy]-1,3,5,6,9,11,17,37-octahydroxy-15,16,18-trimethyl-13-oxo-14,39-dioxabicyclo[33.3.1]nonatriaconta-19,21,23,25,27,29,31-heptaene-36-carboxylic acid. The potency is not less than 750 IU/mg, calculated with reference to the dried substance.

CHARACTERS

A yellow or orange powder, practically insoluble in water, soluble in dimethyl sulphoxide and in propylene glycol, slightly soluble in dimethylformamide, very slightly soluble in methanol, practically insoluble in alcohol.

It is sensitive to light in dilute solutions and is inactivated at low pH values.

IDENTIFICATION

- A. Dissolve 25 mg in 5 ml of *dimethyl sulphoxide R* and dilute to 50 ml with *methanol R*. Dilute 2 ml of the solution to 200 ml with *methanol R*. Examined between 300 nm and 450 nm (2.2.25), the solution shows 3 absorption maxima at 362 nm, 381 nm and 405 nm. The ratio of the absorbance measured at 362 nm to that measured at 381 nm is 0.57 to 0.61. The ratio of the absorbance measured at 381 nm to that measured at 405 nm is 0.87 to 0.93.
- B. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *amphotericin B CRS*. If the spectra obtained show differences dry the substance to be examined at 60 °C at a pressure not exceeding 0.7 kPa for 1 h and prepare a new spectrum.
- C. To 1 ml of a 0.5 g/l solution in *dimethyl sulphoxide R*, add 5 ml of *phosphoric acid R* to form a lower layer, avoiding mixing the 2 liquids. A blue ring is immediately

produced at the junction of the liquids. Mix, an intense blue colour is produced. Add 15 ml of *water R* and mix; the solution becomes pale yellow.

TESTS

Content of tetraenes. Not more than 10.0 per cent and not more than 5.0 per cent if intended for use in the manufacture of parenteral dosage forms, determined by the following method.

Test solution. Dissolve 50.0 mg in 5 ml of *dimethyl sulphoxide R* and dilute to 50.0 ml with *methanol R*. Dilute 4.0 ml of the solution to 50.0 ml with *methanol R*.

Reference solution (a). Dissolve 50.0 mg of *amphotericin B CRS* in 5 ml of *dimethyl sulphoxide R* and dilute to 50.0 ml with *methanol R*. Dilute 4.0 ml of the solution to 50.0 ml with *methanol R*.

Reference solution (b). Dissolve 25.0 mg of *nystatin CRS* in 25 ml of *dimethyl sulphoxide R* and dilute to 250.0 ml with *methanol R*. Dilute 4.0 ml of the solution to 50.0 ml with *methanol R*.

Measure the absorbances (2.2.25) of the test solution and of reference solutions (a) and (b) at the maxima at 282 nm and 304 nm respectively, using a 0.8 per cent V/V solution of *dimethyl sulphoxide R* in *methanol R* as the compensation liquid. Calculate the specific absorbance of the substance being examined, of *nystatin CRS* and of *amphotericin B CRS* at both wavelengths, each with reference to the dried substance, and calculate the percentage content of tetraenes using the following expression:

$$F + \frac{100 (B_1 S_2 - B_2 S_1)}{(N_2 B_1 - N_1 B_2)}$$

S_1 and S_2 = specific absorbances of the substance to be examined at 282 nm and 304 nm respectively,

N_1 and N_2 = specific absorbance of *nystatin CRS* at 282 nm and 304 nm respectively,

B_1 and B_2 = specific absorbance of *amphotericin B CRS* at 282 nm and 304 nm respectively,

F = declared content of tetraenes in *amphotericin B CRS*.

Loss on drying (2.2.32). Not more than 5.0 per cent, determined on 1.000 g by drying in an oven at 60 °C at a pressure not exceeding 0.7 kPa.

Sulphated ash (2.4.14). Not more than 3.0 per cent and not more than 0.5 per cent if intended for use in the manufacture of parenteral dosage forms, determined on 1.0 g.

Bacterial endotoxins (2.6.14): less than 1.0 IU/mg, if intended for use in the manufacture of parenteral dosage forms without a further appropriate procedure for the removal of bacterial endotoxins.

ASSAY

Protect all solutions from light throughout the assay. Dissolve 25.0 mg in *dimethyl sulphoxide R* and dilute, with shaking, to 25.0 ml with the same solvent. Under constant stirring of this stock solution, dilute with *dimethyl sulphoxide R* to obtain solutions of appropriate concentrations (the following concentrations have been found suitable: 44.4, 66.7 and 100 IU/ml). Final solutions are prepared by diluting 1 to 20 with 0.2 M phosphate buffer solution pH 10.5 so that they all contain 5 per cent V/V of dimethyl sulphoxide. Prepare the reference and the test solutions simultaneously. Carry out the microbiological assay of antibiotics (2.7.2).

STORAGE

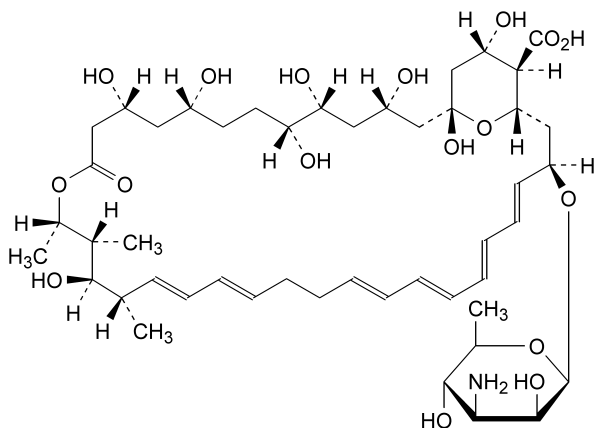
Store protected from light, at a temperature of 2 °C to 8 °C. If the substance is sterile, store in a sterile, airtight, tamper-proof container.

LABELLING

The label states:

- where applicable, that the substance is free from bacterial endotoxins,
- where applicable, that the substance is suitable for use in the manufacture of parenteral dosage forms.

IMPURITIES

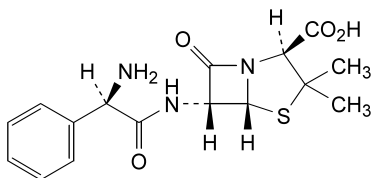


A. amphotericin A (tetraene).

01/2005:0167

AMPICILLIN, ANHYDROUS

Ampicillinum anhydricum



$C_{16}H_{19}N_3O_4S$

M_r 349.4

DEFINITION

Anhydrous ampicillin contains not less than 96.0 per cent and not more than the equivalent of 100.5 per cent of (2*S*,5*R*,6*R*)-6-[[[(2*R*)-2-amino-2-phenylacetyl]amino]-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid, calculated with reference to the anhydrous substance.

CHARACTERS

A white, crystalline powder, sparingly soluble in water, practically insoluble in acetone, in alcohol and in fatty oils. It dissolves in dilute solutions of acids and of alkali hydroxides. It shows polymorphism.

IDENTIFICATION

First identification: A, D.

Second identification: B, C, D.

- Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *anhydrous ampicillin CRS*. Examine the substances prepared as discs using *potassium bromide R*.
- Examine by thin-layer chromatography (2.2.27), using *silanised silica gel H R* as the coating substance.

Test solution. Dissolve 25 mg of the substance to be examined in 10 ml of *sodium hydrogen carbonate solution R*.

Reference solution (a). Dissolve 25 mg of *anhydrous ampicillin CRS* in 10 ml of *sodium hydrogen carbonate solution R*.

Reference solution (b). Dissolve 25 mg of *amoxicillin trihydrate CRS* and 25 mg of *anhydrous ampicillin CRS* in 10 ml of *sodium hydrogen carbonate solution R*.

Apply separately to the plate 1 µl of each solution. Develop over a path of 15 cm using a mixture of 10 volumes of *acetone R* and 90 volumes of a 154 g/l solution of *ammonium acetate R*, the pH of which has been adjusted to 5.0 with *glacial acetic acid R*. Allow the plate to dry in air and expose it to iodine vapour until the spots appear. Examine in daylight. The principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a). The test is not valid unless the chromatogram obtained with reference solution (b) shows 2 clearly separated spots.

- Place about 2 mg in a test-tube about 150 mm long and 15 mm in diameter. Moisten with 0.05 ml of *water R* and add 2 ml of *sulphuric acid-formaldehyde reagent R*. Mix the contents of the tube by swirling; the solution is practically colourless. Place the test-tube in a water-bath for 1 min; a dark yellow colour develops.

- It complies with the test for water (see Tests).

TESTS

Appearance of solution. Dissolve 1.0 g in 10 ml of 1 *M hydrochloric acid*. Separately dissolve 1.0 g in 10 ml of *dilute ammonia R2*. Immediately after dissolution, the solutions are not more opalescent than reference suspension II (2.2.1).

pH (2.2.3). Dissolve 0.1 g in *carbon dioxide-free water R* and dilute to 40 ml with the same solvent. The pH of the solution is 3.5 to 5.5.

Specific optical rotation (2.2.7). Dissolve 62.5 mg in *water R* and dilute to 25.0 ml with the same solvent. The specific optical rotation is + 280 to + 305, calculated with reference to the anhydrous substance.

Related substances. Examine by liquid chromatography (2.2.29) as described under Assay. Inject reference solution (c) and elute isocratically. Inject freshly prepared test solution (b) and start the elution isocratically. Immediately after elution of the ampicillin peak start the following linear gradient. If the mobile phase composition has been adjusted to achieve the required resolution, the adjusted composition will apply at time zero in the gradient.

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)
0	85	15
30	0	100
45	0	100

Equilibrate the column with the originally chosen mobile phase for 15 min. Inject mobile phase A and use the same elution gradient to obtain a blank. In the chromatogram obtained with test solution (b), the area of any peak, apart from the principal peak and any peak observed in the blank chromatogram, is not greater than the area of the principal peak in the chromatogram obtained with reference solution (c) (1.0 per cent).

***N,N*-Dimethylaniline** (2.4.26, *Method B*). Not more than 20 ppm.

Water (2.5.12). Not more than 2.0 per cent, determined on 0.300 g by the semi-micro determination of water.

Sulphated ash (2.4.14). Not more than 0.5 per cent, determined on 1.0 g.

ASSAY

Examine by liquid chromatography (2.2.29).

Test solution (a). Dissolve 27.0 mg of the substance to be examined in mobile phase A and dilute to 50.0 ml with the same solvent.

Test solution (b). Dissolve 27.0 mg of the substance to be examined in mobile phase A and dilute to 10.0 ml with the same solvent.

Reference solution (a). Dissolve 27.0 mg of *anhydrous ampicillin CRS* in mobile phase A and dilute to 50.0 ml with the same solvent.

Reference solution (b). Dissolve 2.0 mg of *cefradine CRS* in mobile phase A and dilute to 50 ml with the same solvent. To 5.0 ml of this solution add 5.0 ml of reference solution (a).

Reference solution (c). Dilute 1.0 ml of reference solution (a) to 20.0 ml with mobile phase A.

Reference solution (d). Dilute 1.0 ml of reference solution (c) to 25.0 ml with mobile phase A.

The chromatographic procedure may be carried out using:

- a column 0.25 m long and 4.6 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (5 µm),

- as mobile phase at a flow rate of 1.0 ml/min:

Mobile phase A. A mixture of 0.5 ml of *dilute acetic acid R*, 50 ml of *0.2 M potassium dihydrogen phosphate R* and 50 ml of *acetonitrile R*, diluted to 1000 ml with *water R*,

Mobile phase B. A mixture of 0.5 ml of *dilute acetic acid R*, 50 ml of *0.2 M potassium dihydrogen phosphate R* and 400 ml of *acetonitrile R*, diluted to 1000 ml with *water R*,

- as detector a spectrophotometer set at 254 nm,
- a 50 µl loop injector.

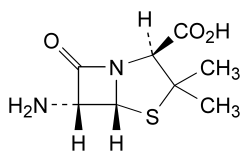
Equilibrate the column with a mobile phase with ratio A:B of 85:15. Inject reference solution (b). The test is not valid unless the resolution between the two principal peaks is at least 3.0. If necessary, adjust the ratio A:B of the mobile phase. The mass distribution ratio for the first peak (ampicillin) is 2.0 to 2.5. Inject reference solution (d). Adjust the system to obtain a peak with a signal-to-noise ratio of at least 3. Inject reference solution (a) 6 times. The test is not valid unless the relative standard deviation for the area of the principal peak is at most 1.0 per cent. Inject alternately test solution (a) and reference solution (a).

Calculate the percentage content of ampicillin.

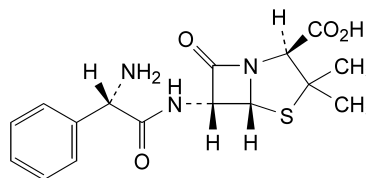
STORAGE

Store in an airtight container, at a temperature not exceeding 30 °C.

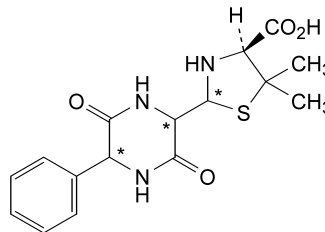
IMPURITIES



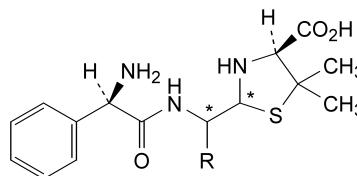
- A. (2*S*,5*R*,6*R*)-6-amino-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid (6-aminopenicillanic acid),



- B. (2*S*,5*R*,6*R*)-6-[(2*S*)-2-amino-2-phenylacetyl]amino]-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid (L-ampicillin),

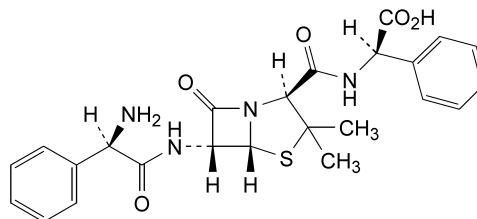


- C. (4*S*)-2-(3,6-dioxo-5-phenylpiperazin-2-yl)-5,5-dimethylthiazolidine-4-carboxylic acid (diketopiperazines of ampicillin),

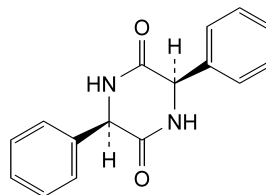


- D. R = CO₂H: (4*S*)-2-[[[(2*R*)-2-amino-2-phenylacetyl]amino]carboxymethyl]-5,5-dimethylthiazolidine-4-carboxylic acid (penicilloic acids of ampicillin),

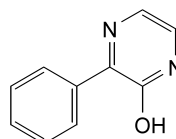
- F. R = H: (2*RS*,4*S*)-2-[[[(2*R*)-2-amino-2-phenylacetyl]amino]methyl]-5,5-dimethylthiazolidine-4-carboxylic acid (penilloic acids of ampicillin),



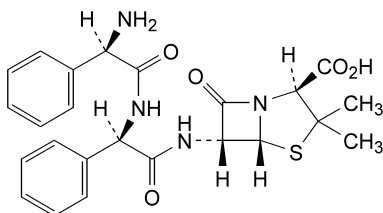
- E. (2*R*)-2-[[[(2*S*,5*R*,6*R*)-6-[(2*R*)-2-amino-2-phenylacetyl]amino]-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]hept-2-yl]carbonyl]amino]-2-phenylacetic acid (ampicillinyl-D-phenylglycine),



- G. (3*R*,6*R*)-3,6-diphenylpiperazine-2,5-dione,

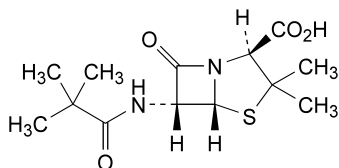


- H. 3-phenylpyrazin-2-ol,

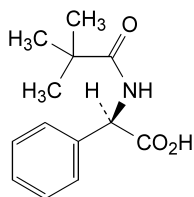
01/2005:0578
corrected**AMPICILLIN SODIUM**

Ampicillinum natricum

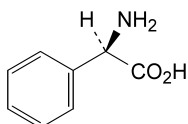
- I. (2*S*,5*R*,6*R*)-6-[[[(2*R*)-2-[(2*R*)-2-amino-2-phenylacetyl]amino]-2-phenylacetyl]amino]-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid (D-phenylglycylampicillin),



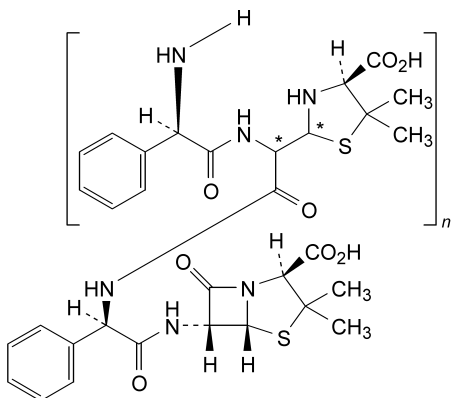
- J. (2*S*,5*R*,6*R*)-6-[(2,2-dimethylpropanoyl)amino]-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid,



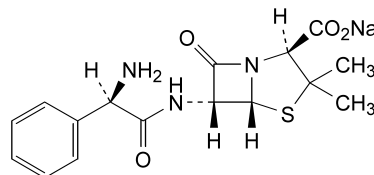
- K. (2*R*)-2-[(2,2-dimethylpropanoyl)amino]-2-phenylacetic acid,



- L. (2*R*)-2-amino-2-phenylacetic acid (D-phenylglycine),



- M. co-oligomers of ampicillin and of penicilloic acids of ampicillin.

 $C_{16}H_{18}N_3NaO_4S$ M_r 371.4**DEFINITION**

Ampicillin sodium contains not less than 91.0 per cent and not more than the equivalent of 100.5 per cent of sodium (2*S*,5*R*,6*R*)-6-[[[(2*R*)-2-amino-2-phenylacetyl]amino]-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylate, calculated with reference to the anhydrous substance.

CHARACTERS

A white or almost white powder, hygroscopic, freely soluble in water, sparingly soluble in acetone, practically insoluble in fatty oils and in liquid paraffin.

IDENTIFICATION

First identification: A, D.

Second identification: B, C, D.

- A. Dissolve 0.250 g in 5 ml of *water R*, add 0.5 ml of *dilute acetic acid R*, swirl and allow to stand for 10 min in iced water. Filter the crystals through a small sintered-glass filter (40), applying suction, wash with 2 ml to 3 ml of a mixture of 1 volume of *water R* and 9 volumes of *acetone R* and dry in an oven at 60 °C for 30 min. Examine the crystals by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *ampicillin trihydrate CRS*.

- B. Examine by thin-layer chromatography (2.2.27), using *silanised silica gel H R* as the coating substance.

Test solution. Dissolve 25 mg of the substance to be examined in 10 ml of *sodium hydrogen carbonate solution R*.

Reference solution (a). Dissolve 25 mg of *ampicillin trihydrate CRS* in 10 ml of *sodium hydrogen carbonate solution R*.

Reference solution (b). Dissolve 25 mg of *amoxicillin trihydrate CRS* and 25 mg of *ampicillin trihydrate CRS* in 10 ml of *sodium hydrogen carbonate solution R*.

Apply separately to the plate 1 µl of each solution. Develop over a path of 15 cm using a mixture of 10 volumes of *acetone R* and 90 volumes of a 154 g/l solution of *ammonium acetate R*, the pH of which has been adjusted to 5.0 with *glacial acetic acid R*. Allow the plate to dry in air and expose it to iodine vapour until the spots appear. Examine in daylight. The principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a). The test is not valid unless the chromatogram obtained with reference solution (b) shows two clearly separated spots.

- C. Place about 2 mg in a test-tube about 150 mm long and 15 mm in diameter. Moisten with 0.05 ml of *water R* and add 2 ml of *sulphuric acid-formaldehyde reagent R*.

Mix the contents of the tube by swirling; the solution is practically colourless. Place the test-tube in a water-bath for 1 min; a dark yellow colour develops.

D. It gives reaction (a) of sodium (2.3.1).

TESTS

Appearance of solution. Place 1.0 g in a conical flask and add slowly and with continuous swirling 10 ml of 1 M hydrochloric acid. Separately dissolve 1.0 g in water R and dilute to 10.0 ml with the same solvent. Immediately after dissolution, the solutions are not more opalescent than reference suspension II (2.2.1). The absorbance (2.2.25) of the solution in water measured at 430 nm is not greater than 0.15.

pH (2.2.3). Dissolve 2.0 g in carbon dioxide-free water R and dilute to 20 ml with the same solvent. The pH of the solution, measured 10 min after dissolution, is 8.0 to 10.0.

Specific optical rotation (2.2.7). Dissolve 62.5 mg in a 4 g/l solution of potassium hydrogen phthalate R and dilute to 25.0 ml with the same solvent. The specific optical rotation is + 258 to + 287, calculated with reference to the anhydrous substance.

Related substances. Examine by liquid chromatography (2.2.29) as described under Assay. Inject 50 µl of reference solution (d) and elute isocratically until elution of the ampicillin peak. Inject 50 µl of test solution (b) and start the elution isocratically. Immediately after elution of the ampicillin peak start the following linear gradient. If the mobile phase composition has been adjusted to achieve the required resolution, the adjusted composition will apply at time zero in the gradient.

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)	Comment
0 - 30	85 → 0	15 → 100	linear gradient
30 - 45	0	100	isocratic
45 - 60	85	15	re-equilibration

Inject mobile phase A and use the same elution pattern to obtain a blank. Inject reference solution (e) and use the same elution pattern. The chromatogram obtained with reference solution (e), shows an ampicillin peak and a dimer peak, with a retention time of 2.8 relative to ampicillin. In the chromatogram obtained with test solution (b), the area of any peak corresponding to ampicillin dimer is not greater than 4.5 times the area of the principal peak in the chromatogram obtained with reference solution (d) (4.5 per cent); the area of any peak, apart from the principal peak and any peak corresponding to ampicillin dimer, is not greater than twice the area of the principal peak in the chromatogram obtained with reference solution (d) (2 per cent). Disregard any peak due to the blank.

N,N-Dimethylaniline (2.4.26, Method B). Not more than 20 ppm.

2-Ethylhexanoic acid (2.4.28). Not more than 0.8 per cent m/m.

Methylene chloride. Not more than 0.2 per cent m/m, determined by gas chromatography (2.2.28), using ethylene chloride R as the internal standard.

Internal standard solution. Dissolve 1.0 ml of ethylene chloride R in water R and dilute to 500.0 ml with the same solvent.

Test solution (a). Dissolve 1.0 g of the substance to be examined in water R and dilute to 10.0 ml with the same solvent.

Test solution (b). Dissolve 1.0 g of the substance to be examined in water R, add 1.0 ml of the internal standard solution and dilute to 10.0 ml with water R.

Reference solution. Dissolve 1.0 ml of methylene chloride R in water R and dilute to 500.0 ml with the same solvent. To 1.0 ml of the solution add 1.0 ml of the internal standard solution and dilute to 10.0 ml with water R.

The chromatographic procedure may be carried out using:

- a glass column 1.5 m long and 4 mm in internal diameter packed with diatomaceous earth for gas chromatography R impregnated with 10 per cent m/m of macrogol 1000 R,
- nitrogen for chromatography R as the carrier gas at a flow rate of 40 ml/min,
- a flame-ionisation detector,

maintaining the temperature of the column at 60 °C, that of the injection port at 100 °C and that of the detector at 150 °C. Calculate the content of methylene chloride taking its density at 20 °C to be 1.325 g/ml.

Heavy metals (2.4.8). 1.0 g complies with limit test C for heavy metals (20 ppm). Prepare the standard using 2 ml of lead standard solution (10 ppm Pb) R.

Water (2.5.12). Not more than 2.0 per cent, determined on 0.300 g by the semi-micro determination of water.

Bacterial endotoxins (2.6.14): less than 0.15 IU/mg, if intended for use in the manufacture of parenteral dosage forms without a further appropriate procedure for the removal of bacterial endotoxins.

ASSAY

Examine by liquid chromatography (2.2.29).

Test solution (a). Dissolve 31.0 mg of the substance to be examined in mobile phase A and dilute to 50.0 ml with mobile phase A.

Test solution (b). Prepare immediately before use. Dissolve 31.0 mg of the substance to be examined in mobile phase A and dilute to 10.0 ml with mobile phase A.

Reference solution (a). Dissolve 27.0 mg of anhydrous ampicillin CRS in mobile phase A and dilute to 50.0 ml with mobile phase A.

Reference solution (b). Dissolve 2.0 mg of cefradine CRS in mobile phase A and dilute to 50 ml with mobile phase A. To 5.0 ml of the solution add 5.0 ml of reference solution (a).

Reference solution (c). Dilute 1.0 ml of reference solution (a) to 20.0 ml with mobile phase A. Dilute 1.0 ml of the solution to 25.0 ml with mobile phase A.

Reference solution (d). Dilute 1.0 ml of reference solution (a) to 20.0 ml with mobile phase A.

Reference solution (e). To 0.20 g of the substance to be examined add 1.0 ml of water R. Heat the solution at 60 °C for 1 h. Dilute 0.5 ml of the solution to 50.0 ml with mobile phase A.

The chromatographic procedure may be carried out using:

- a column 0.25 m long and 4.6 mm in internal diameter packed with octadecylsilyl silica gel for chromatography R (5 µm),
- as mobile phase at a flow rate of 1.0 ml/min:

Mobile phase A. A mixture of 0.5 ml of dilute acetic acid R, 50 ml of 0.2 M potassium dihydrogen phosphate R and 50 ml of acetonitrile R, diluted to 1000 ml with water R,

Mobile phase B. A mixture of 0.5 ml of dilute acetic acid R, 50 ml of 0.2 M potassium dihydrogen phosphate R and 400 ml of acetonitrile R, diluted to 1000 ml with water R,

- as detector a spectrophotometer set at 254 nm.

Equilibrate the column with a mobile phase with A:B ratio of 85:15. Inject 50 µl of reference solution (b). The assay is not valid unless in the chromatogram obtained, the resolution between the two principal peaks is at least 3.0. If necessary, adjust the A:B ratio of the mobile phase. The mass distribution ratio for the first peak (ampicillin) is 2.0 to 2.5. Inject 50 µl of reference solution (c). Adjust the system to obtain a peak with a signal-to-noise ratio of at least 3. Inject reference solution (a) six times. The test is not valid unless the relative standard deviation for the area of the principal peak is at most 1.0 per cent. Inject alternately test solution (a) and reference solution (a).

Calculate the percentage content of ampicillin sodium by multiplying the percentage content of ampicillin by 1.063.

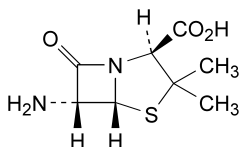
STORAGE

Store in an airtight container. If the substance is sterile, store in a sterile, airtight, tamper-proof container.

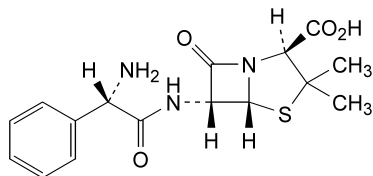
LABELLING

The label states, where applicable, that the substance is free from bacterial endotoxins.

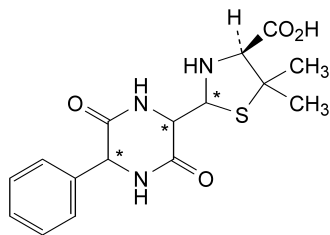
IMPURITIES



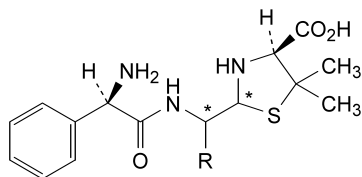
- A. (2*S*,5*R*,6*R*)-6-amino-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid (6-aminopenicillanic acid),



- B. (2*S*,5*R*,6*R*)-6-[(2*S*)-2-amino-2-phenylacetyl]amino-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid (L-ampicillin),

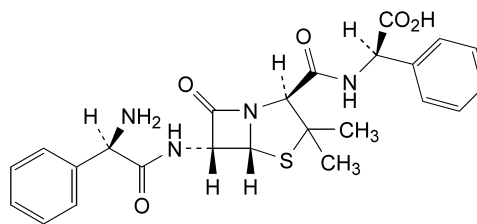


- C. (4*S*)-2-(3,6-dioxo-5-phenylpiperazin-2-yl)-5,5-dimethylthiazolidine-4-carboxylic acid (diketopiperazines of ampicillin),

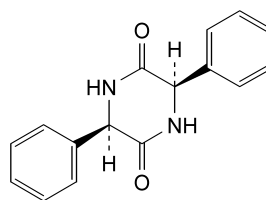


- D. R = CO₂H: (4*S*)-2-[[[(2*R*)-2-amino-2-phenylacetyl]amino]carboxymethyl]-5,5-dimethylthiazolidine-4-carboxylic acid (penicilloic acids of ampicillin),

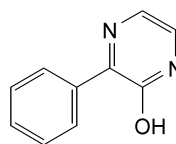
- F. R = H: (2*R*,4*S*)-2-[[[(2*R*)-2-amino-2-phenylacetyl]amino]methyl]-5,5-dimethylthiazolidine-4-carboxylic acid (penicilloic acids of ampicillin),



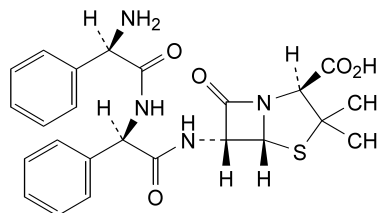
- E. (2*R*)-2-[[[(2*S*,5*R*,6*R*)-6-[(2*R*)-2-amino-2-phenylacetyl]amino]-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]hept-2-yl]carbonyl]amino]-2-phenylacetic acid (ampicillinyl-D-phenylglycine),



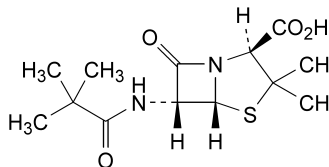
- G. (3*R*,6*R*)-3,6-diphenylpiperazine-2,5-dione,



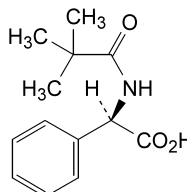
- H. 3-phenylpyrazin-2-ol,



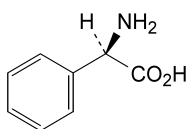
- I. (2*S*,5*R*,6*R*)-6-[[[(2*R*)-2-[(2*R*)-2-amino-2-phenylacetyl]amino]-2-phenylacetyl]amino]-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid (D-phenylglycylampicillin),



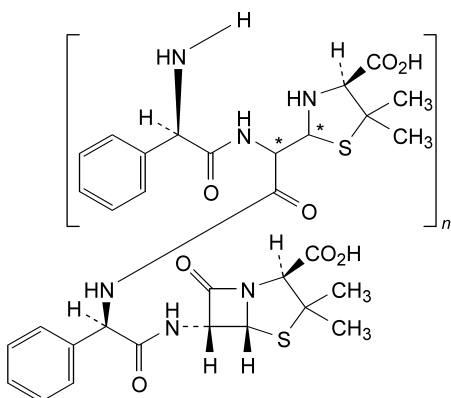
- J. (2*S*,5*R*,6*R*)-6-[(2,2-dimethylpropanoyl)amino]-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid,



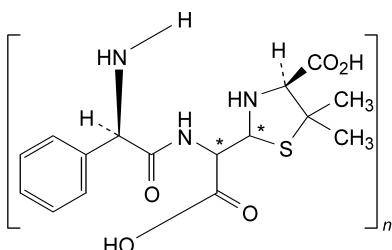
- K. (2*R*)-2-[(2,2-dimethylpropanoyl)amino]-2-phenylacetic acid,



L. (2*R*)-2-amino-2-phenylacetic acid (D-phenylglycine),



M. co-oligomers of ampicillin and of penicilloic acids of ampicillin,

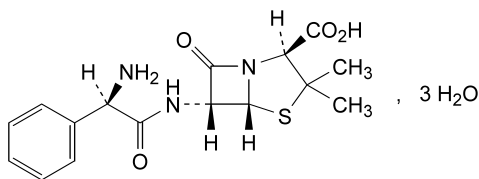


N. oligomers of penicilloic acids of ampicillin.

01/2005:0168

AMPICILLIN TRIHYDRATE

Ampicillinum trihydricum



$C_{16}H_{19}N_3O_4S \cdot 3H_2O$

M_r 403.5

DEFINITION

Ampicillin trihydrate contains not less than 96.0 per cent and not more than the equivalent of 100.5 per cent of (2*S*,5*R*,6*R*)-6-[(2*R*)-2-amino-2-phenylacetyl]amino-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid, calculated with reference to the anhydrous substance.

CHARACTERS

A white, crystalline powder, slightly soluble in water, practically insoluble in alcohol and in fatty oils. It dissolves in dilute solutions of acids and of alkali hydroxides.

IDENTIFICATION

First identification: A, D.

Second identification: B, C, D.

A. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *ampicillin trihydrate CRS*.

B. Examine by thin-layer chromatography (2.2.27), using *silanised silica gel H R* as the coating substance.

Test solution. Dissolve 25 mg of the substance to be examined in 10 ml of *sodium hydrogen carbonate solution R*.

Reference solution (a). Dissolve 25 mg of *ampicillin trihydrate CRS* in 10 ml of *sodium hydrogen carbonate solution R*.

Reference solution (b). Dissolve 25 mg of *amoxicillin trihydrate CRS* and 25 mg of *ampicillin trihydrate CRS* in 10 ml of *sodium hydrogen carbonate solution R*.

Apply separately to the plate 1 µl of each solution. Develop over a path of 15 cm using a mixture of 10 volumes of *acetone R* and 90 volumes of a 154 g/l solution of *ammonium acetate R*, the pH of which has been adjusted to 5.0 with *glacial acetic acid R*. Allow the plate to dry in air and expose it to iodine vapour until the spots appear. Examine in daylight. The principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a). The test is not valid unless the chromatogram obtained with reference solution (b) shows 2 clearly separated spots.

C. Place about 2 mg in a test-tube about 150 mm long and 15 mm in diameter. Moisten with 0.05 ml of *water R* and add 2 ml of *sulphuric acid-formaldehyde reagent R*. Mix the contents of the tube by swirling; the solution is practically colourless. Place the test-tube in a water-bath for 1 min; a dark yellow colour develops.

D. It complies with the test for water (see Tests).

TESTS

Appearance of solution. Dissolve 1.0 g in 10 ml of 1 *M hydrochloric acid*. Separately dissolve 1.0 g in 10 ml of *dilute ammonia R2*. Immediately after dissolution, the solutions are not more opalescent than reference suspension II (2.2.1).

pH (2.2.3). Dissolve 0.1 g in *carbon dioxide-free water R* and dilute to 40 ml with the same solvent. The pH of the solution is 3.5 to 5.5.

Specific optical rotation (2.2.7). Dissolve 62.5 mg in *water R* and dilute to 25.0 ml with the same solvent. The specific optical rotation is + 280 to + 305, calculated with reference to the anhydrous substance.

Related substances. Examine by liquid chromatography (2.2.29) as described under Assay. Inject reference solution (c) and elute isocratically. Inject freshly prepared test solution (b) and start the elution isocratically. Immediately after elution of the ampicillin peak start the following linear gradient. If the mobile phase composition has been adjusted to achieve the required resolution, the adjusted composition will apply at time zero in the gradient.

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)
0	85	15
30	0	100
45	0	100

Equilibrate the column with the originally chosen mobile phase for 15 min. Inject mobile phase A and use the same elution gradient to obtain a blank. In the chromatogram obtained with test solution (b), the area of any peak, apart from the principal peak and any peak observed in the blank chromatogram, is not greater than the area of the principal peak in the chromatogram obtained with reference solution (c) (1.0 per cent).

***N,N*-Dimethylaniline** (2.4.26, *Method B*). Not more than 20 ppm.

Water (2.5.12). 12.0 per cent to 15.0 per cent, determined on 0.100 g by the semi-micro determination of water.

Sulphated ash (2.4.14). Not more than 0.5 per cent, determined on 1.0 g.

ASSAY

Examine by liquid chromatography (2.2.29).

Test solution (a). Dissolve 31.0 mg of the substance to be examined in mobile phase A and dilute to 50.0 ml with the same solvent.

Test solution (b). Dissolve 31.0 mg of the substance to be examined in mobile phase A and dilute to 10.0 ml with the same solvent.

Reference solution (a). Dissolve 27.0 mg of *anhydrous ampicillin CRS* in mobile phase A and dilute to 50.0 ml with the same solvent.

Reference solution (b). Dissolve 2.0 mg of *cefradine CRS* in mobile phase A and dilute to 50 ml with the same solvent. To 5.0 ml of this solution add 5.0 ml of reference solution (a).

Reference solution (c). Dilute 1.0 ml of reference solution (a) to 20.0 ml with mobile phase A.

Reference solution (d). Dilute 1.0 ml of reference solution (c) to 25.0 ml with mobile phase A.

The chromatographic procedure may be carried out using:

- a column 0.25 m long and 4.6 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (5 µm),
- as mobile phase at a flow rate of 1.0 ml/min:
Mobile phase A. A mixture of 0.5 ml of *dilute acetic acid R*, 50 ml of 0.2 M *potassium dihydrogen phosphate R*, 50 ml of *acetonitrile R* diluted to 1000 ml with *water R*,
Mobile phase B. A mixture of 0.5 ml of *dilute acetic acid R*, 50 ml of 0.2 M *potassium dihydrogen phosphate R* and 400 ml of *acetonitrile R* diluted to 1000 ml with *water R*,
- as detector a spectrophotometer set at 254 nm,
- a 50 µl loop injector.

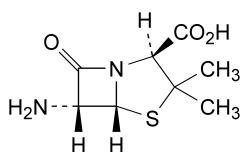
Equilibrate the column with a mobile phase with ratio A:B of 85:15. Inject reference solution (b). The test is not valid unless the resolution between the two principal peaks is at least 3.0. If necessary, adjust the ratio A:B of the mobile phase. The mass distribution ratio for the first peak (ampicillin) is 2.0 to 2.5. Inject reference solution (d). Adjust the system to obtain a peak with a signal-to-noise ratio of at least 3. Inject reference solution (a) 6 times. The test is not valid unless the relative standard deviation for the area of the principal peak is at most 1.0 per cent. Inject alternately test solution (a) and reference solution (a).

Calculate the percentage content of ampicillin.

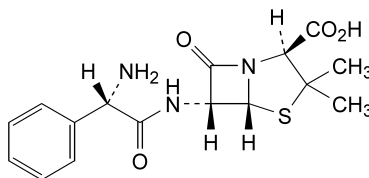
STORAGE

Store in an airtight container, at a temperature not exceeding 30 °C.

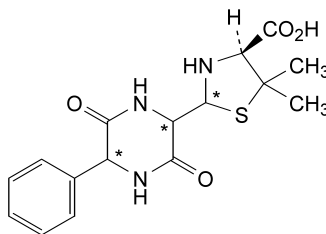
IMPURITIES



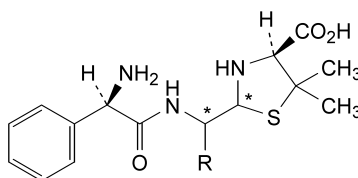
- A. (2*S*,5*R*,6*R*)-6-amino-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid (6-aminopenicillanic acid),



- B. (2*S*,5*R*,6*R*)-6-[(2*S*)-2-amino-2-phenylacetyl]amino]-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid (L-ampicillin),

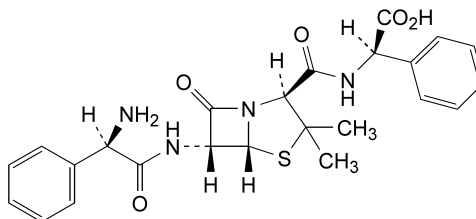


- C. (4*S*)-2-(3,6-dioxo-5-phenylpiperazin-2-yl)-5,5-dimethylthiazolidine-4-carboxylic acid (diketopiperazines of ampicillin),

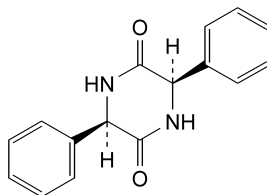


- D. R = CO₂H: (4*S*)-2-[[[(2*R*)-2-amino-2-phenylacetyl]amino]carboxymethyl]-5,5-dimethylthiazolidine-4-carboxylic acid (penicilloic acids of ampicillin),

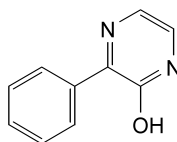
- F. R = H: (2*RS*,4*S*)-2-[[[(2*R*)-2-amino-2-phenylacetyl]amino]methyl]-5,5-dimethylthiazolidine-4-carboxylic acid (penilloic acids of ampicillin),



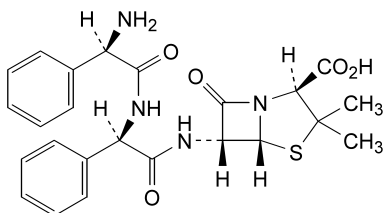
- E. (2*R*)-2-[[[(2*S*,5*R*,6*R*)-6-[(2*R*)-2-amino-2-phenylacetyl]amino]-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]hept-2-yl]carbonyl]amino]-2-phenylacetic acid (ampicillinyl-D-phenylglycine),



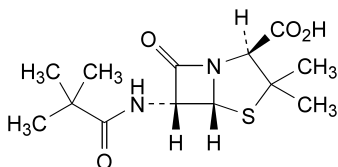
- G. (3*R*,6*R*)-3,6-diphenylpiperazine-2,5-dione,



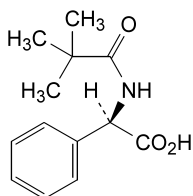
- H. 3-phenylpyrazin-2-ol,



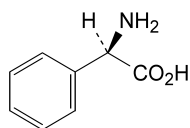
- I. (2*S*,5*R*,6*R*)-6-[[[(2*R*)-2-[(2*R*)-2-amino-2-phenylacetyl]amino]-2-phenylacetyl]amino]-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid (D-phenylglycylampicillin),



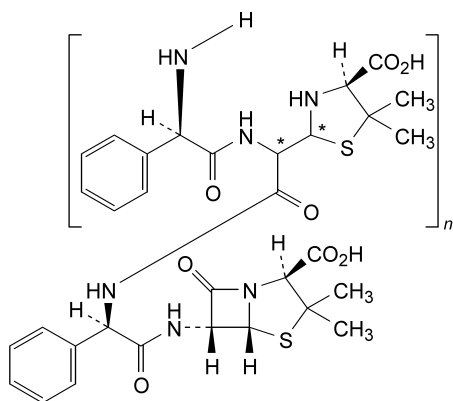
- J. (2*S*,5*R*,6*R*)-6-[(2,2-dimethylpropanoyl)amino]-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid,



- K. (2*R*)-2-[(2,2-dimethylpropanoyl)amino]-2-phenylacetic acid,



- L. (2*R*)-2-amino-2-phenylacetic acid (D-phenylglycine),



- M. co-oligomers of ampicillin and of penicilloic acids of ampicillin.

Content: minimum 2.0 ml/kg of essential oil (dried drug).

CHARACTERS

Bitter taste.

Macroscopic and microscopic characters described under identification tests A and B.

IDENTIFICATION

- A. The rhizome is greyish-brown or reddish-brown, transversely annulated. The base bears greyish-brown or reddish-brown, cylindrical, longitudinally furrowed, occasionally branched roots often with incompletely encircling, transverse ridges. The apex sometimes shows remnants of stem and leaf bases. The fracture is uneven. The transversely cut surface shows a greyish-white, spongy, distinctly radiate bark, in which the secretory channels are visible as brown spots, and a bright yellow to greyish-yellow wood which, in the rhizome, surrounds the greyish or brownish-white pith.
- B. Reduce to a powder (355). The powder is brownish-white. Examine under a microscope using *chloral hydrate solution R*. The powder shows: fragments of cork consisting of several layers of thin-walled greyish-brown or reddish-brown cells; fragments of large, yellowish-brown secretory channels; fragments of medullary rays 2 to 4 cells wide; fragments of xylem with medullary rays and radially arranged, lignified vessels with reticulate thickening. Examine under a microscope using a 50 per cent *V/V* solution of *glycerol R*. The powder shows numerous, simple starch granules 2 µm to 4 µm in diameter.
- C. Examine the chromatograms obtained in the test for lovage root.

Results: see below the sequence of the zones present in the chromatograms obtained with the reference solution and the test solution.

Top of the plate	
_____	_____
Eugenol: (marked at 254 nm)	
Coumarin: (marked at 254 nm)	
_____	_____
	An intense blue fluorescent zone
	Yellow fluorescent zones
	A blue fluorescent zone
	A yellow fluorescent zone
	An intense blue fluorescent zone
Reference solution	Test solution

TESTS

Lovage root. Thin-layer chromatography (2.2.27).

Test solution. To 1.0 g of the freshly powdered drug (355) add 5 ml of *methanol R* and boil for 30 s. Cool and filter.

Reference solution. Dissolve 5 mg of *coumarin R* and 25 µl of *eugenol R* in 10 ml of *methanol R*.

Plate: *TLC silica gel F₂₅₄ plate R*.

Mobile phase: *methylene chloride R*, *toluene R* (50:50 *V/V*).

Application: 20 µl, as bands.

Development: twice over a path of 10 cm.

Drying: in air.

Detection: examine in ultraviolet light at 254 nm. Mark the quenching zones due to coumarin and eugenol on the chromatogram obtained with the reference solution. Examine in ultraviolet light at 365 nm.

01/2005:1857

ANGELICA ROOT

Angelicae radix

DEFINITION

Whole or cut, carefully dried rhizome and root of *Angelica archangelica* L. (*Archangelica officinalis* Hoffm.).

Results: the chromatogram obtained with the test solution shows no pale blue to white fluorescent zone between the zones of coumarin and eugenol in the chromatogram obtained with the reference solution.

Foreign matter (2.8.2): maximum 5 per cent of leaf bases and stem bases, maximum 5 per cent of discoloured pieces and maximum 1 per cent of other foreign matter.

Loss on drying (2.2.32): maximum 10.0 per cent, determined on 1.000 g of the powdered drug (355) by drying in an oven at 100-105 °C for 2 h.

Total ash (2.4.16): maximum 10.0 per cent.

Ash insoluble in hydrochloric acid (2.8.1): maximum 2.0 per cent.

ASSAY

Carry out the determination of essential oils in vegetable drugs (2.8.12). Reduce the drug to a powder (500) and immediately use 40.0 g for the determination. Use a 2 litre round-bottomed flask, 10 drops of *liquid paraffin R*, 500 ml of *water R* as distillation liquid and 0.50 ml of *xylene R* in the graduated tube. Distil at a rate of 2-3 ml/min for 4 h.

01/2005:0804
corrected

ANISE OIL

Anisi aetheroleum

DEFINITION

Essential oil obtained by steam distillation from the dry ripe fruits of *Pimpinella anisum L.*

CHARACTERS

Appearance: clear, colourless or pale yellow liquid.

IDENTIFICATION

First identification: B.

Second identification: A.

A. Thin-layer chromatography (2.2.27).

Test solution. Dissolve 1 g of the substance to be examined in *toluene R* and dilute to 10 ml with the same solvent.

Reference solution. Dissolve 10 µl of *linalol R*, 30 µl of *anisaldehyde R* and 200 µl of *anethole R* in *toluene R* and dilute to 15 ml with the same solvent. Dilute 1 ml of this solution to 5 ml with *toluene R*.

Plate: TLC silica gel F_{254} plate *R*.

Mobile phase: *ethyl acetate R*, *toluene R* (7:93 V/V).

Application: 5 µl as bands of 10 mm (for normal TLC plates) or 2 µl as bands of 10 mm (for fine particle size plates).

Development: over a path of 15 cm (for normal TLC plates) or over a path of 6 cm (for fine particle size plates).

Drying: in air.

Detection A: examine in ultraviolet light at 254 nm.

Results A: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other zones may be present in the chromatogram obtained with the test solution.

Top of the plate	
Anethole: a quenching zone _____	A very strong quenching zone (anethole) _____
Anisaldehyde: a quenching zone _____	A quenching zone A quenching zone (anisaldehyde) _____
Reference solution	Test solution

Detection B: spray with *methyl 4-acetylbenzoate reagent R* and heat at 100-105 °C for 10 min; examine the still hot plate in daylight within 5 min.

Results B: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other zones may be present in the chromatogram obtained with the test solution.

Top of the plate	
Anethole: a brown zone _____	A violet-brown zone (monoterpene hydrocarbons) (solvent front) A very strong brown zone (anethole), distinctly separated _____
Anisaldehyde: a yellow zone _____	A grey zone A yellow zone (anisaldehyde) _____
Linalol: a grey zone	A grey zone (linalol) A grey zone
Reference solution	Test solution

B. Examine the chromatograms obtained in the test for chromatographic profile.

Results: the characteristic peaks in the chromatogram obtained with the test solution are similar in retention time to those in the chromatogram obtained with the reference solution.

TESTS

Relative density (2.2.5): 0.980 to 0.990.

Refractive index (2.2.6): 1.552 to 1.561.

Freezing point (2.2.18): 15 °C to 19 °C.

Fenchone. Gas chromatography (2.2.28) as described in the test for chromatographic profile with the following modifications.

Test solution. Dissolve 400 µl of the substance to be examined in 2.0 ml of *hexane R*.

Reference solution (a). Dilute 10 µl of *fenchone R* to 1.2 g with *hexane R*.

Reference solution (b). Dilute 100 µl of reference solution (a) to 100 ml with *hexane R*.

System suitability: reference solution (b):

— *signal-to-noise ratio*: minimum 10 for the principal peak.

Limit:

— *fenchone*: maximum 0.01 per cent.

Foeniculin. Gas chromatography (2.2.28) as described in the test for chromatographic profile with the following modifications.

Test solution. The substance to be examined.

Reference solution (a). Dilute 10 mg of the test solution to 1.000 g with *hexane R*. Dilute 0.5 ml of this solution to 100 ml with *hexane R*.

Reference solution (b). *Foeniculin* for peak identification CRS.

System suitability:

- the chromatogram obtained with reference solution (b) is similar to the chromatogram provided with *foeniculin* for peak identification CRS,
- *signal-to-noise ratio*: minimum 10 for the principal peak in the chromatogram obtained with reference solution (a).

Limit: locate the peak due to foeniculin by comparison with the chromatogram provided with *foeniculin* for peak identification CRS.

- *foeniculin*: maximum 0.01 per cent.

Fatty oils and resinified essential oils (2.8.7). It complies with the test for fatty oils and resinified essential oils.

Chromatographic profile. Gas chromatography (2.2.28): use the normalisation procedure.

Test solution. Dissolve 200 µl of the substance to be examined in 1.0 ml of *hexane R*.

Reference solution. To 1.0 ml of *hexane R*, add 20 µl of *linalol R*, 20 µl of *estragole R*, 20 µl of *α-terpineol R*, 60 µl of *anethole R* and 30 µl of *anisaldehyde R*.

Column:

- *material*: fused silica,
- *size*: $l = 30$ m, $\varnothing = 0.25$ mm,
- *stationary phase*: *macrogol 20 000 R* (film thickness 0.25 µm).

Carrier gas: *helium for chromatography R*.

Flow rate: 1.0 ml/min.

Split ratio: 1:100.

Temperature:

	Time (min)	Temperature (°C)
Column	0 – 5	60
	5 – 80	60 → 210
	80 – 95	210
Injection port		200
Detector		220

Detection: flame ionisation.

Injection: 0.2 µl.

Elution order: order indicated in the composition of the reference solution. Record the retention times of these substances.

System suitability: reference solution:

- *resolution*: minimum 1.5 between the peaks due to *estragole* and *α-terpineol*.

Using the retention times determined from the chromatogram obtained with the reference solution, locate the components of the reference solution in the chromatogram obtained with the test solution and locate *cis*-anethole and *pseudoisoeugenyl 2-methylbutyrate* using the chromatogram shown in Figure 0804.-1 (disregard any peak due to *hexane*). Determine the percentage content of these components. The percentages are within the following ranges:

- *linalol*: less than 1.5 per cent,
- *estragole*: 0.5 per cent to 5.0 per cent,
- *α-terpineol*: less than 1.2 per cent,
- *cis*-anethole: 0.1 per cent to 0.4 per cent,
- *trans*-anethole: 87 per cent to 94 per cent,
- *anisaldehyde*: 0.1 per cent to 1.4 per cent,
- *pseudoisoeugenyl 2-methylbutyrate*: 0.3 per cent to 2.0 per cent.

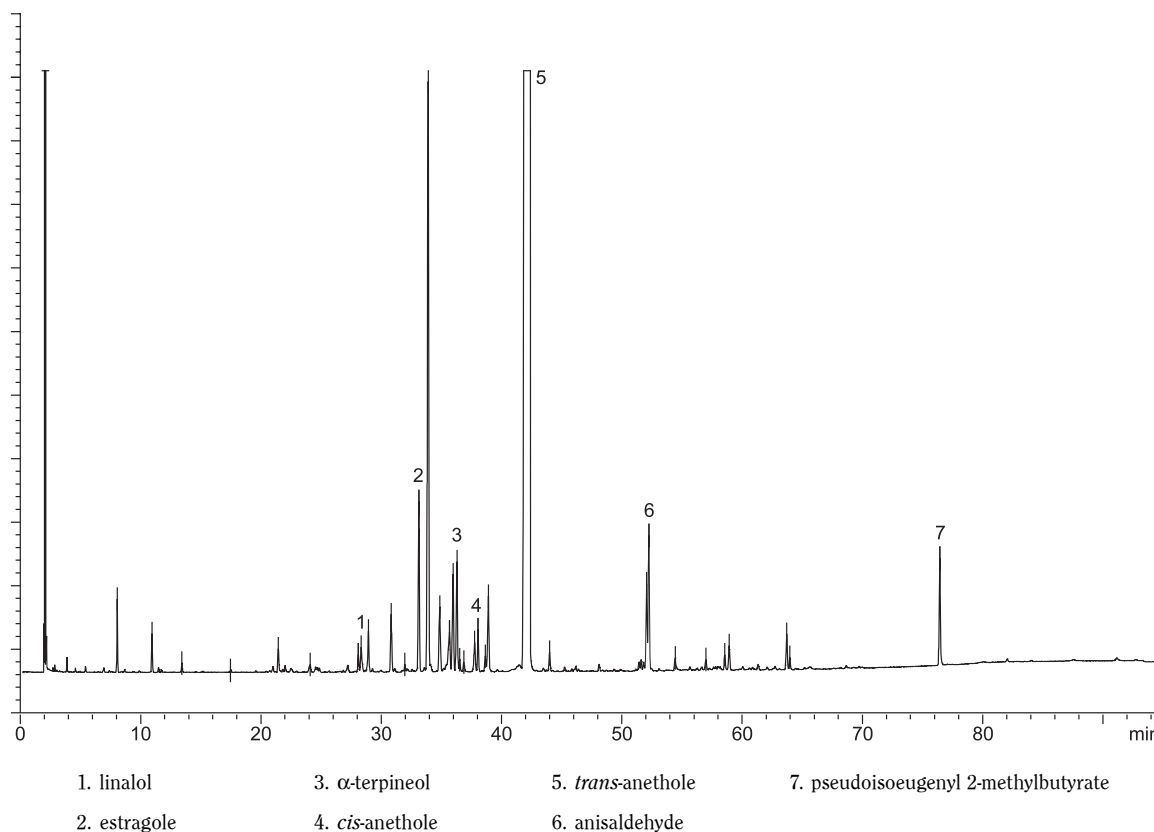


Figure 0804.-1. – Chromatogram for the test for chromatographic profile of anise oil

STORAGE

In a well-filled, airtight container, protected from light and at a temperature not exceeding 25 °C.

01/2005:0262

ANISEED

Anisi fructus

DEFINITION

Aniseed consists of the whole dry cremocarp of *Pimpinella anisum* L. It contains not less than 20 ml/kg of essential oil.

CHARACTERS

Aniseed has an odour reminiscent of anethole.

The fruit is a cremocarp and generally entire; a small fragment of the thin, rigid, slightly curved pedicel is frequently attached.

It has the macroscopic and microscopic characters described under identification tests A and B.

IDENTIFICATION

- A. The cremocarp is ovoid or pyriform and slightly compressed laterally, yellowish-green or greenish-grey, 3 mm to 5 mm long and up to 3 mm wide, surmounted by a stylopod with 2 short, reflexed stylar points. The mericarps are attached by their tops to the carpophore with a plane commissural surface and a convex dorsal surface, the latter being covered with short, warty trichomes visible using a lens; the fruit shows 5 primary ridges, running longitudinally, comprising 3 dorsal ridges and 2 lateral ridges, non-prominent, and lighter in colour.
- B. Reduce to a powder (355). The powder is greenish-yellow to brownish-green. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters: whole or broken trichomes, mostly unicellular, sometimes curved, with blunt apex and warty cuticle; fragments of epidermis with striated cuticle, occasional anomocytic stomata; fragments of numerous narrow, branched vittae, fragments of endosperm containing aleurone grains and micro-rosettes of calcium oxalate; oblong sclereids from the commissural zone and bundles of sclerenchymatous fibres from the carpophore and the pedicel. Starch is absent.
- C. Examine by thin-layer chromatography (2.2.27), using *silica gel GF₂₅₄ R* as the coating substance.
Test solution. Shake 0.10 g of the powdered drug (1500) with 2 ml of *methylene chloride R* for 15 min. Filter and carefully evaporate the filtrate to dryness on a water-bath at 60 °C. Dissolve the residue in 0.5 ml of *toluene R*.
Reference solution. Dissolve 3 µl of *anethole R* and 40 µl of *olive oil R* in 1 ml of *toluene R*.
 Apply to the plate at 2 cm intervals 2 µl and 3 µl of the test solution and 1 µl, 2 µl and 3 µl of the reference solution. Develop over a path of 10 cm using *toluene R*. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. The chromatograms show a quenching zone (anethole) in the central part against a light background. Spray the plate with a freshly prepared 200 g/l solution of *phosphomolybdic acid R* in *alcohol R*, using 10 ml for a plate 200 mm square, and heat at 120 °C for 5 min. The spots corresponding to anethole appear blue against a yellow background. In the chromatogram obtained with 2 µl of the test solution, the spot corresponding to anethole is intermediate in size between the spots

corresponding to anethole in the chromatograms obtained with 1 µl and 3 µl of the reference solution. The chromatograms obtained with the test solution show in the lower third a blue spot (triglycerides) similar in position to the spot in the lower third of the chromatograms obtained with the reference solution (triglycerides of olive oil).

TESTS

Foreign matter (2.8.2). It complies with the test for foreign matter.

Water (2.2.13). Not more than 70 ml/kg, determined by distillation on 20.0 g of the powdered drug.

Total ash (2.4.16). Not more than 12.0 per cent.

Ash insoluble in hydrochloric acid (2.8.1). Not more than 2.5 per cent.

ASSAY

Carry out the determination of essential oils in vegetable drugs (2.8.12). Use a 250 ml round-bottomed flask, 100 ml of *water R* as the distillation liquid and 0.50 ml of *xylene R* in the graduated tube. Reduce the drug to a coarse powder and immediately use 10.0 g for the determination. Distil at a rate of 2.5-3.5 ml/min for 2 h.

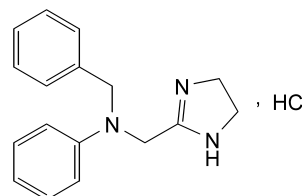
STORAGE

Store protected from light.

01/2005:0972

ANTAZOLINE HYDROCHLORIDE

Antazolini hydrochloridum

C₁₇H₂₀ClN₃M_r 301.8

DEFINITION

Antazoline hydrochloride contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of *N*-benzyl-*N*[(4,5-dihydro-1*H*-imidazol-2-yl)methyl]aniline hydrochloride, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, crystalline powder, sparingly soluble in water, soluble in alcohol, slightly soluble in methylene chloride.

It melts at about 240 °C, with decomposition.

IDENTIFICATION

First identification: A, D.

Second identification: B, C, D.

- A. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *antazoline hydrochloride CRS*. Examine the substances as discs prepared using *potassium chloride R*.
- B. Examine the chromatograms obtained in the test for related substances in daylight after spraying. The principal spot in the chromatogram obtained with test

solution (b) is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (b).

- C. To 5 ml of solution S (see Tests) add, drop by drop, *dilute sodium hydroxide solution R* until an alkaline reaction is produced. Filter. The precipitate, washed with two quantities, each of 10 ml, of *water R* and dried in a desiccator under reduced pressure, melts (2.2.14) at 119 °C to 123 °C.

- D. It gives reaction (a) of chlorides (2.3.1).

TESTS

Solution S. Dissolve 2.0 g in *carbon dioxide-free water R* prepared from *distilled water R*, heating at 60 °C if necessary. Allow to cool and dilute to 100 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and not more intensely coloured than reference solution Y₇ (2.2.2, *Method II*).

Acidity or alkalinity. To 10 ml of solution S add 0.2 ml of *methyl red solution R*. Not more than 0.1 ml of 0.01 M *hydrochloric acid* or 0.01 M *sodium hydroxide* is required to change the colour of the indicator.

Related substances. Examine by thin-layer chromatography (2.2.27), using *silica gel GF₂₅₄ R* as the coating substance. Heat the plate at 110 °C for 15 min before using.

Test solution (a). Dissolve 0.10 g of the substance to be examined in *methanol R* and dilute to 5 ml with the same solvent.

Test solution (b). Dilute 1 ml of test solution (a) to 5 ml with *methanol R*.

Reference solution (a). Dilute 0.5 ml of test solution (a) to 100 ml with *methanol R*.

Reference solution (b). Dissolve 20 mg of *antazoline hydrochloride CRS* in *methanol R* and dilute to 5 ml with the same solvent.

Reference solution (c). Dissolve 20 mg of *xylometazoline hydrochloride CRS* in 1 ml of test solution (a) and dilute to 5 ml with *methanol R*.

Apply to the plate 5 µl of each solution. Develop over a path of 15 cm using a mixture of 5 volumes of *diethylamine R*, 10 volumes of *methanol R* and 85 volumes of *ethyl acetate R*. Dry the plate in a current of warm air for 15 min. Examine in ultraviolet light at 254 nm. The test is not valid unless the chromatogram obtained with reference solution (c) shows two clearly separated principal spots. Spray with a mixture of equal volumes of a 200 g/l solution of *ferric chloride R* and a 5 g/l solution of *potassium ferricyanide R*. Examine immediately in daylight. Any spot in the chromatogram obtained with test solution (a), apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (a) (0.5 per cent).

Heavy metals (2.4.8). 1.0 g complies with limit test C for heavy metals (20 ppm). Prepare the standard using 2 ml of *lead standard solution (10 ppm Pb) R*.

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C for 3 h.

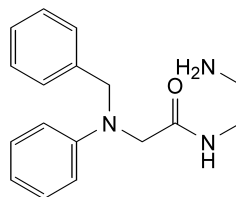
Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on the residue obtained in the test for loss on drying.

ASSAY

Dissolve 0.250 g in 100 ml of *alcohol R*. Add 0.1 ml of *phenolphthalein solution R1*. Titrate with 0.1 M *alcoholic potassium hydroxide*.

1 ml of 0.1 M *alcoholic potassium hydroxide* is equivalent to 30.18 mg of C₁₇H₂₀ClN₃.

IMPURITIES



- A. *N*-(2-aminoethyl)-2-(benzylphenylamino)acetamide.

01/2005:0209

ANTICOAGULANT AND PRESERVATIVE SOLUTIONS FOR HUMAN BLOOD

Solutiones anticoagulantes et sanguinem humanum conservantes

DEFINITION

Anticoagulant and preservative solutions for human blood are sterile and pyrogen-free solutions prepared with water for injections, filtered, distributed in the final containers and sterilised. The content of sodium citrate (C₆H₅Na₃O₇·2H₂O), glucose monohydrate (C₆H₁₂O₆·H₂O) or anhydrous glucose (C₆H₁₂O₆) and sodium dihydrogen phosphate dihydrate (NaH₂PO₄·2H₂O) is not less than 95.0 per cent and not more than 105.0 per cent of that stated in the formulae below. The content of citric acid monohydrate (C₆H₈O₇·H₂O) or anhydrous citric acid (C₆H₈O₇) is not less than 90.0 per cent and not more than 110.0 per cent of that stated in the formulae below. Subject to agreement by the competent authority, other substances, such as red-cell preservatives, may be included in the formula provided that their name and concentration are stated on the label.

Anticoagulant and preservative solutions for human blood are presented in airtight, tamper-proof containers of glass (3.2.1) or plastic (3.2.3).

Anticoagulant acid-citrate-glucose solutions (ACD)

	A	B
<i>Sodium citrate (0412)</i>	22.0 g	13.2 g
<i>Citric acid monohydrate (0456)</i>	8.0 g	4.8 g
or <i>Citric acid, anhydrous (0455)</i>	7.3 g	4.4 g
<i>Glucose monohydrate (0178)*</i>	24.5 g	14.7 g
or <i>Glucose, anhydrous (0177)*</i>	22.3 g	13.4 g
<i>Water for injections (0169) to</i>	1000.0 ml	1000.0 ml
Volume to be used per 100 ml of blood	15.0 ml	25.0 ml

*The competent authority may require that the substances comply with the test for pyrogens given in the monographs on *Glucose monohydrate (0178)* and *Glucose, anhydrous (0177)*, respectively.

CHARACTERS

A colourless or faintly yellow, clear liquid, practically free from particles.

IDENTIFICATION

- A. Examine by thin-layer chromatography (2.2.27), using *silica gel G R* as the coating substance.

Test solution. Dilute 2 ml of the solution to be examined (for formula A) or 3 ml (for formula B) to 100 ml with a mixture of 2 volumes of *water R* and 3 volumes of *methanol R*.

Reference solution (a). Dissolve 10 mg of *glucose CRS* in a mixture of 2 volumes of *water R* and 3 volumes of *methanol R* and dilute to 20 ml with the same mixture of solvents.

Reference solution (b). Dissolve 10 mg each of *glucose CRS*, *lactose CRS*, *fructose CRS* and *sucrose CRS* in a mixture of 2 volumes of *water R* and 3 volumes of *methanol R* and dilute to 20 ml with the same mixture of solvents.

Apply separately to the plate 2 µl of each solution and thoroughly dry the starting points. Develop over a path of 15 cm using a mixture of 10 volumes of *water R*, 15 volumes of *methanol R*, 25 volumes of *anhydrous acetic acid R* and 50 volumes of *ethylene chloride R*. The volumes of solvents have to be measured accurately since a slight excess of water produces cloudiness. Dry the plate in a current of warm air. Repeat the development immediately, after renewing the mobile phase. Dry the plate in a current of warm air and spray evenly with a solution of 0.5 g of *thymol R* in a mixture of 5 ml of *sulphuric acid R* and 95 ml of *alcohol R*. Heat at 130 °C for 10 min. The principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a). The test is not valid unless the chromatogram obtained with reference solution (b) shows 4 clearly separated spots.

- B. To 2 ml add 5 ml of *cupri-citric solution R*. Heat to boiling. An orange precipitate is formed and the solution becomes yellow.
- C. To 2 ml (for formula A) add 3 ml of *water R* or to 4 ml (for formula B) add 1 ml of *water R*. The solution gives the reaction of citrates (2.3.1).
- D. 0.5 ml gives reaction (b) of sodium (2.3.1).

TESTS

pH (2.2.3). The pH of the solution to be examined is 4.7 to 5.3.

Hydroxymethylfurfural. To 2.0 ml add 5.0 ml of a 100 g/l solution of *p-toluidine R* in *2-propanol R* containing 10 per cent V/V of *glacial acetic acid R* and 1.0 ml of a 5 g/l solution of *barbituric acid R*. The absorbance (2.2.25), determined at 550 nm after allowing the mixture to stand for 2 min to 3 min, is not greater than that of a standard prepared at the same time in the same manner using 2.0 ml of a solution containing 5 ppm of *hydroxymethylfurfural R* for formula A or 3 ppm of *hydroxymethylfurfural R* for formula B.

Sterility (2.6.1). They comply with the test for sterility.

Pyrogens (2.6.8). They comply with the test for pyrogens. Dilute with a pyrogen-free, 9 g/l solution of *sodium chloride R* to obtain a solution containing approximately 5 g/l of sodium citrate. Inject 10 ml of the diluted solution per kilogram of the rabbit's mass.

ASSAY

Citric acid. To 10.0 ml (for formula A) or to 20.0 ml (for formula B) add 0.1 ml of *phenolphthalein solution R1*. Titrate with 0.2 M *sodium hydroxide* until a pink colour is obtained.

1 ml of 0.2 M *sodium hydroxide* is equivalent to 14.01 mg of $C_6H_8O_7 \cdot H_2O$ or to 12.81 mg of $C_6H_8O_7$.

Sodium citrate. Prepare a chromatography column 0.10 m long and 10 mm in internal diameter and filled with *strongly acidic ion-exchange resin R* (300 µm to 840 µm). Maintain a 1 cm layer of liquid above the resin at all times. Wash the column with 50 ml of de-ionised *water R* at a flow rate of 12-14 ml/min.

Dilute 10.0 ml of the solution to be examined (for formula A) or 15.0 ml (for formula B) to about 40 ml with de-ionised *water R* in a beaker and transfer to the column reservoir, washing the beaker 3 times with a few millilitres of de-ionised *water R*. Allow the solution to run through the column at a flow rate of 12-14 ml/min and collect the eluate. Wash the column with 2 quantities, each of 30 ml, and with one quantity of 50 ml, of de-ionised *water R*. The column can be used for 3 successive determinations before regeneration with 3 times its volume of *dilute hydrochloric acid R*. Titrate the combined eluate and washings (about 150 ml) with 0.2 M *sodium hydroxide*, using 0.1 ml of *phenolphthalein solution R1* as indicator.

Calculate the content of sodium citrate in grams per litre from the following expressions:

For formula A: $1.961n - 1.40C$

or $1.961n - 1.53C'$

For formula B: $1.307n - 1.40C$

or $1.307n - 1.53C'$

n = number of millilitres of 0.2 M *sodium hydroxide* used in the titration,

C = content of citric acid monohydrate in grams per litre determined as prescribed above,

C' = content of anhydrous citric acid in grams per litre determined as prescribed above.

Reducing sugars. Dilute 5.0 ml (for formula A) or 10.0 ml (for formula B) to 100.0 ml with *water R*. Introduce 25.0 ml of the solution into a 250 ml conical flask with ground-glass neck and add 25.0 ml of *cupri-citric solution R1*. Add a few pieces of porous material, attach a reflux condenser, heat so that boiling begins within 2 min and boil for exactly 10 min. Cool and add 3 g of *potassium iodide R* dissolved in 3 ml of *water R*. Add 25 ml of a 25 per cent m/m solution of *sulphuric acid R* with caution and in small quantities. Titrate with 0.1 M *sodium thiosulphate* using 0.5 ml of *starch solution R*, added towards the end of the titration, as indicator (n_1 ml). Carry out a blank titration using 25.0 ml of *water R* (n_2 ml).

Calculate the content of reducing sugars as anhydrous glucose or as glucose monohydrate, as appropriate, from the Table 0209-1.

Table 0209.1

Volume of 0.1 M sodium thiosulphate ($n_2 - n_1$ ml)	Anhydrous glucose in milligrams	Glucose mono- hydrate in milligrams
8	19.8	21.6
9	22.4	24.5
10	25.0	27.2
11	27.6	30.2
12	30.3	33.1
13	33.0	36.1
14	35.7	39.0
15	38.3	42.1
16	41.3	45.2

STORAGE

Store in an airtight, tamper-proof container, protected from light.

LABELLING

The label states:

- the composition and volume of the solution,
- the maximum amount of blood to be collected in the container.

Anticoagulant citrate-phosphate-glucose solution (CPD)

<i>Sodium citrate</i> (0412)	26.3 g
<i>Citric acid monohydrate</i> (0456)	3.27 g
or <i>Citric acid, anhydrous</i> (0455)	2.99 g
<i>Glucose monohydrate</i> (0178)*	25.5 g
or <i>Glucose, anhydrous</i> (0177)*	23.2 g
<i>Sodium dihydrogen phosphate dihydrate</i> (0194)	2.51 g
<i>Water for injections</i> (0169) to	1000.0 ml
Volume to be used per 100 ml of blood	14.0 ml

*The competent authority may require that the substances comply with the test for pyrogens given in the monographs on *Glucose monohydrate* (0178) and *Glucose, anhydrous* (0177), respectively.

CHARACTERS

A colourless or faintly yellow, clear liquid, practically free from particles.

IDENTIFICATION

A. Examine by thin-layer chromatography (2.2.27), using *silica gel G R* as the coating substance.

Test solution. Dilute 2 ml of the solution to be examined to 100 ml with a mixture of 2 volumes of *water R* and 3 volumes of *methanol R*.

Reference solution (a). Dissolve 10 mg of *glucose CRS* in a mixture of 2 volumes of *water R* and 3 volumes of *methanol R* and dilute to 20 ml with the same mixture of solvents.

Reference solution (b). Dissolve 10 mg each of *glucose CRS*, *lactose CRS*, *fructose CRS* and *sucrose CRS* in a mixture of 2 volumes of *water R* and 3 volumes of *methanol R* and dilute to 20 ml with the same mixture of solvents.

Apply separately to the plate 2 µl of each solution and thoroughly dry the starting points. Develop over a path of 15 cm using a mixture of 10 volumes of *water R*, 15 volumes of *methanol R*, 25 volumes of *anhydrous acetic acid R* and 50 volumes of *ethylene chloride R*. The volumes of solvents have to be measured accurately since a slight excess of water produces cloudiness. Dry the plate in a current of warm air. Repeat the development immediately, after renewing the mobile phase. Dry the plate in a current of warm air and spray evenly with a solution of 0.5 g of *thymol R* in a mixture of 5 ml of *sulphuric acid R* and 95 ml of *alcohol R*. Heat at 130 °C for 10 min. The principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a). The test is not valid unless the chromatogram obtained with reference solution (b) shows 4 clearly separated spots.

B. To 2 ml add 5 ml of *cupri-citric solution R*. Heat to boiling. An orange precipitate is formed and the solution becomes yellow.

C. To 2 ml add 3 ml of *water R*. The solution gives the reaction of citrates (2.3.1).

D. 1 ml gives reaction (b) of phosphates (2.3.1).

E. 0.5 ml gives reaction (b) of sodium (2.3.1).

TESTS

pH (2.2.3). The pH of the solution is 5.3 to 5.9.

Hydroxymethylfurfural. To 2.0 ml add 5.0 ml of a 100 g/l solution of *p-toluidine R* in *2-propanol R* containing 10 per cent V/V of *glacial acetic acid R* and 1.0 ml of a 5 g/l solution of *barbituric acid R*. The absorbance (2.2.25), determined at 550 nm after allowing the mixture to stand for 2 min to 3 min, is not greater than that of a standard prepared at the same time in the same manner using 2.0 ml of a solution containing 5 ppm of *hydroxymethylfurfural R*.

Sterility (2.6.1). They comply with the test for sterility.

Pyrogens (2.6.8). They comply with the test for pyrogens. Dilute with a pyrogen-free, 9 g/l solution of *sodium chloride R* to obtain a solution containing approximately 5 g/l of sodium citrate. Inject 10 ml of the diluted solution per kilogram of the rabbit's mass.

ASSAY

Sodium dihydrogen phosphate. Dilute 10.0 ml to 100.0 ml with *water R*. To 10.0 ml of this solution add 10.0 ml of *nitro-vanado-molybdic reagent R*. Mix and allow to stand at 20 °C to 25 °C for 30 min. At the same time and in the same manner, prepare a reference solution using 10.0 ml of a standard solution containing 0.219 g of *potassium dihydrogen phosphate R* per litre. Measure the absorbance (2.2.25) of the 2 solutions at 450 nm using as the compensation liquid a solution prepared in the same manner using 10 ml of *water R*. Calculate the content of sodium dihydrogen phosphate dihydrate (P) in grams per litre from the expression:

$$\frac{11.46 \times C \times A_1}{A_2}$$

C = concentration of *potassium dihydrogen phosphate R* in the standard solution in grams per litre,

A_1 = absorbance of the test solution,

A_2 = absorbance of the reference solution.

Citric acid. To 20.0 ml add 0.1 ml of *phenolphthalein solution R1* and titrate with 0.2 M sodium hydroxide.

Calculate the content of citric acid monohydrate (C), or anhydrous citric acid (C'), in grams per litre from the equations:

$$C = 0.7005n - 0.4490P$$

$$C' = 0.6404n - 0.4105P$$

n = number of millilitres of 0.2 M sodium hydroxide used in the titration,

P = content of sodium dihydrogen phosphate dihydrate in grams per litre determined as prescribed above.

Sodium citrate. Prepare a chromatography column 0.10 m long and 10 mm in internal diameter and filled with *strongly acidic ion-exchange resin R* (300 µm to 840 µm). Maintain a 1 cm layer of liquid above the resin at all times. Wash the column with 50 ml of de-ionised *water R* at a flow rate of 12-14 ml/min.

Dilute 10.0 ml of the solution to be examined to about 40 ml with de-ionised *water R* in a beaker and transfer to the column reservoir, washing the beaker 3 times with a few millilitres of de-ionised *water R*. Allow the solution to run through the column at a flow rate of 12-14 ml/min and collect the eluate. Wash the column with 2 quantities, each of 30 ml, and with one quantity of 50 ml, of de-ionised *water R*. The column can be used for 3 successive determinations before regeneration with 3 times its volume of *dilute hydrochloric acid R*. Titrate the combined eluate and washings (about 150 ml) with 0.2 M sodium hydroxide, using 0.1 ml of *phenolphthalein solution R1* as indicator.

Calculate the content of sodium citrate in grams per litre from the following expressions:

$$1.961n - 1.257P - 1.40C$$

$$1.961n - 1.257P - 1.53C'$$

n = number of millilitres of 0.2 M sodium hydroxide used in the titration,

P = content of sodium dihydrogen phosphate dihydrate in grams per litre determined as prescribed above,

C = content of citric acid monohydrate in grams per litre determined as prescribed above,

C' = content of anhydrous citric acid in grams per litre determined as prescribed above.

Reducing sugars. Dilute 5.0 ml to 100.0 ml with *water R*. Introduce 25.0 ml of the solution into a 250 ml conical flask with ground-glass neck and add 25.0 ml of *cupri-citric solution R1*. Add a few pieces of porous material, attach a reflux condenser, heat so that boiling begins within 2 min and boil for exactly 10 min. Cool and add 3 g of *potassium iodide R* dissolved in 3 ml of *water R*. Add 25 ml of a 25 per cent *m/m* solution of *sulphuric acid R* with caution and in small quantities. Titrate with 0.1 M sodium thiosulphate using 0.5 ml of *starch solution R*, added towards the end of the titration, as indicator (*n*₁ ml). Carry out a blank titration using 25.0 ml of *water R* (*n*₂ ml).

Calculate the content of reducing sugars as anhydrous glucose or as glucose monohydrate, as appropriate, from the Table 0209-1.

STORAGE

Store in an airtight, tamper-proof container, protected from light.

LABELLING

The label states:

- the composition and volume of the solution,
- the maximum amount of blood to be collected in the container.

01/2005:1928

ANTI-T LYMPHOCYTE IMMUNOGLOBULIN FOR HUMAN USE, ANIMAL

Immunoglobulinum anti-T lymphocytorum ex animale ad usum humanum

DEFINITION

Anti-T lymphocyte animal immunoglobulin for human use is a liquid or freeze-dried preparation containing immunoglobulins, obtained from serum or plasma of animals, mainly rabbits or horses, immunised with human lymphocytic antigens.

The immunoglobulin has the property of diminishing the number and function of immunocompetent cells, in particular T-lymphocytes. The preparation contains principally immunoglobulin G. It may contain antibodies against other lymphocyte subpopulations and against other cells. The preparation is intended for intravenous administration, after dilution with a suitable diluent where applicable.

Applicable provisions of the monograph on *Immunosera for human use, animal* (0084) are stated below.

PRODUCTION

GENERAL PROVISIONS

The production method has been shown to yield consistently immunoglobulins of acceptable safety, potency in man and stability.

Any reagent of biological origin used in production shall be free of contamination with bacteria, fungi and viruses. The method of preparation includes a step or steps that have been shown to remove or inactivate known agents of infection.

During development studies, it shall be demonstrated that the production method yields a product that:

- does not transmit infectious agents,
- is characterised by a defined pattern of immunological activity, notably: antigen binding, complement-dependent and independent cytotoxicity, cytokine release, induction of T-cell activation, cell death,
- does not contain antibodies that cross-react with human tissues to a degree that would impair clinical safety,
- has a defined maximum content of anti-thrombocyte antibody activity,
- has a defined maximum content of haemoglobin.

The product has been shown, by suitable tests in animals and evaluation during clinical trials, to be well tolerated.

Reference preparation. A batch shown to be suitable for checking the validity of the assay and whose efficacy has been demonstrated in clinical trials, or a batch representative thereof.

ANIMALS

The animals used are of a species approved by the competent authority, are healthy and exclusively reserved for production of anti-T lymphocyte immunoglobulin. They are tested and shown to be free from a defined list of infectious agents. The introduction of animals into a closed herd follows specified procedures, including definition of quarantine measures. Where appropriate, tests for additional specific agents are considered depending on the geographical localisation of the establishment used for the breeding and production of the animals. The feed originates from a controlled source and no animal proteins are added. The suppliers of animals are certified by the competent authority.

If the animals are treated with antibiotics, a suitable withdrawal period is allowed before collection of blood or plasma. The animals are not treated with penicillin antibiotics. If a live vaccine is administered, a suitable waiting period is imposed between vaccination and collection of serum or plasma for immunoglobulin production.

The species, origin and identification number of the animals are specified.

IMMUNISATION

The antigens used are identified and characterised, where appropriate. They are identified by their names and a batch number; information on the source and preparation are recorded.

The selected animals are isolated for at least 1 week before being immunised according to a defined schedule with booster injections at suitable intervals. Adjuvants may be used.

Animals are kept under general health surveillance and specific antibody production is controlled at each cycle of immunisation.

Animals are thoroughly examined before collection of blood or plasma. If an animal shows any pathological lesion not related to the immunisation process, it is not used, nor are any other of the animals in the group concerned, unless it is evident that their use will not impair the safety of the product.

Human antigens such as continuously growing T-lymphocyte cell lines or thymocytes are used to immunise the animals. Cells may be subjected to a sorting procedure. The immunising antigens are shown to be free from infectious agents by validated methods for relevant blood-borne pathogens, notably hepatitis B virus (HBV), hepatitis C virus (HCV) and human immunodeficiency virus (HIV) and other relevant adventitious agents originating from the preparation of the antigen. The cells used comply with defined requirements for purity of the cell population and freedom from adventitious agents.

COLLECTION OF BLOOD OR PLASMA

Collection of blood is made by venepuncture or plasmapheresis. The puncture area is shaved, cleaned and disinfected. The animals may be anaesthetised under conditions that do not influence the quality of the product.

No antimicrobial preservative is added to the plasma and serum samples. The blood or plasma is collected in such a manner as to maintain sterility of the product. The blood or plasma collection is conducted at a site separate from the area where the animals are kept or bred and the area where the immunoglobulin is purified. If the serum or plasma is stored before further processing, precautions are taken to avoid microbial contamination.

Several single plasma or serum samples may be pooled before purification. The single or pooled samples are tested before purification for the following tests.

Tests for contaminating viruses. Each pool is tested for contaminating viruses by suitable *in vitro* tests including inoculation to cell cultures capable of detecting a wide range of viruses relevant for the particular product. Where applicable, *in vitro* tests for contaminating viruses are carried out on the adsorbed pool, after the last production stage that may introduce viral contaminants.

PURIFICATION AND VIRAL INACTIVATION

The immunoglobulins are concentrated and purified by fractional precipitation, chromatography, immuno-adsorption or by other suitable chemical or physical methods. The methods are selected and validated to avoid contamination at all steps of processing and to avoid formation of protein aggregates that effect immunobiological characteristics of the product.

Unless otherwise justified and authorised, validated procedures are applied for removal and/or inactivation of viruses.

After purification and treatment for removal and/or inactivation of viruses, a stabiliser may be added to the intermediate product, which may be stored for a period defined in the light of stability data.

Only an intermediate product that complies with the following requirements may be used in the preparation of the final bulk.

If the method of preparation includes a step for adsorption of cross-reacting anti-human antibodies using material from human tissues and/or red blood cells, the human materials are submitted to a validated procedure for inactivation of infectious agents, unless otherwise justified and authorised. If erythrocytes are used for adsorption, the donors for such materials comply with the requirements for donors of blood and plasma of the monograph on *Human plasma for fractionation (0853)*. If other human material is used, it is shown by validated methods to be free from relevant blood-borne pathogens, notably HBV, HCV and HIV. If substances are used for inactivation or removal of viruses, it shall have been shown that any residues present in the final product have no adverse effects on the patients treated with the anti-T lymphocyte immunoglobulin.

FINAL BULK

The final bulk is prepared from a single intermediate product or from a pool of intermediate products obtained from animals of the same species. A stabiliser may be added. No antimicrobial preservative is added either during the manufacturing procedure or for preparation of the final bulk solution. During manufacturing, the solution is passed through a bacteria-retentive filter.

FINAL LOT

The final bulk of anti-T-lymphocyte immunoglobulin is distributed aseptically into sterile, tamper-proof containers. The containers are closed as to prevent contamination. Only a final lot that complies with the requirements prescribed below under Identification, Tests and Assay may be released for use.

CHARACTERS

The liquid preparation is clear or slightly opalescent and colourless or pale yellow. The freeze-dried preparation is a white or slightly yellow powder or solid friable mass, which after reconstitution gives a liquid preparation corresponding to the description above.

IDENTIFICATION

A. Using a suitable range of species-specific antisera, carry out precipitation tests on the preparation to be examined. It is recommended that the test be carried

out using antisera specific to the plasma proteins of each species of domestic animal commonly used in the preparation of materials of biological origin in the country concerned and antisera specific to human plasma proteins. The preparation is shown to contain proteins originating from the animal used for the anti-T lymphocyte immunoglobulin production.

B. Examine by a suitable immunoelectrophoresis technique. Using antiserum to normal serum of the animal used for production, compare this serum and the preparation to be examined, both diluted to a concentration that will allow a clear gammaglobulin precipitation arc to be obtained on the gel. The main component of the preparation to be examined corresponds to the IgG component of normal serum of the animal used for production.

C. The preparation complies with the assay.

TESTS

Solubility. To a container of the preparation to be examined, add the volume of the liquid for reconstitution stated on the label. The preparation dissolves completely within the time stated on the label.

Extractable volume (2.9.17). It complies with the requirement for extractable volume.

pH (2.2.3). The pH is within the limits approved for the particular product.

Osmolality (2.2.35): minimum 240 mosmol/kg after dilution, where applicable.

Proteins (2.5.33): 90 per cent to 110 per cent of the amount stated on the label.

Stabiliser. Determine the amount of stabiliser by a suitable physico-chemical method. The preparation contains not less than 80 per cent and not more than 120 per cent of the quantity stated on the label.

Distribution of molecular size. Size exclusion chromatography (2.2.30).

Test solution. Dilute the preparation to be examined with a 9 g/l solution of *sodium chloride R* to a concentration suitable for the chromatographic system used. A concentration in the range 2-20 g/l is usually suitable.

Reference solution. Dilute *human immunoglobulin BRP* with a 9 g/l solution of *sodium chloride R* to the same protein concentration as the test solution.

Column:

- **size:** $l = 0.6$ m, $\varnothing = 7.5$ mm,
- **stationary phase:** *silica gel for size-exclusion chromatography R*, a grade suitable for fractionation of globular proteins in the molecular mass range of 20 000 to 200 000.

Mobile phase: dissolve 4.873 g of *disodium hydrogen phosphate dihydrate R*, 1.741 g of *sodium dihydrogen phosphate monohydrate R* and 11.688 g of *sodium chloride R* in 1 litre of *water R*.

Flow rate: 0.5 ml/min.

Detection: spectrophotometer at 280 nm.

Injection: 50-600 µg of protein.

Retention time: identify the peaks in the chromatogram obtained with the test solution by comparison with the chromatogram obtained with the reference solution; any peak with a retention time shorter than that of dimer corresponds to polymers and aggregates.

System suitability:

- **reference solution:** the principal peak corresponds to IgG monomer and there is a peak corresponding to dimer with a retention time relative to monomer of 0.85 ± 0.05 ,
- **test solution:** the relative retentions of monomer and dimer are 1 ± 0.05 with reference to the corresponding peaks in the chromatogram obtained with the reference solution.

Limits:

- **total monomer and dimer:** at least 95 per cent of the total area of the peaks,
- **total polymers and aggregates:** maximum 5 per cent of the total area of the peaks.

Purity. Polyacrylamide gel electrophoresis (2.2.31), under non-reducing and reducing conditions.

Resolving gel. Non-reducing conditions: 8 per cent acrylamide; reducing conditions: 12 per cent acrylamide.

Test solution. Dilute the preparation to be examined to a protein concentration of 0.5-2 mg/ml.

Reference solution. Dilute the reference preparation to the same protein concentration as the test solution.

Application: 10 µl.

Detection: Coomassie staining.

Results: compared with the electropherogram of the reference solution, no additional bands are found in the electropherogram of the test solution.

Anti-A and anti-B haemagglutinins (2.6.20). The 1 to 64 dilution does not show agglutination.

Where applicable, dilute the preparation to be examined as prescribed for use before preparing the dilutions for the test.

Haemolysins. Prepare a 1 to 64 dilution of the preparation to be examined, diluted if necessary as stated on the label. Take 6 aliquots of the 1 to 64 dilution. To 1 volume of 3 of the aliquots, add 1 volume of a 10 per cent V/V suspension of group A1, group B and group O erythrocytes in a 9 g/l solution of *sodium chloride R*, respectively. To 1 volume of the remaining 3 aliquots, add 1 volume of a 10 per cent V/V suspension of group A1, group B and group O erythrocytes in a 9 g/l solution of *sodium chloride R*, respectively, and to each aliquot 1 volume of fresh group AB serum (as a source of complement). Mix and incubate at 37 °C for 1 h. Examine the supernatant liquids for haemolysis. No signs of haemolysis are present.

Thrombocyte antibodies. Examined by a suitable method, the level of thrombocyte antibodies is shown to be below that approved for the specific product.

Water (2.5.12): maximum 3 per cent.

Sterility (2.6.1). It complies with the test for sterility.

Pyrogens (2.6.8). Unless otherwise justified and authorised, it complies with the test for pyrogens. Unless otherwise prescribed, inject 1 ml per kilogram of the rabbit's body mass.

ASSAY

The biological activity is determined by measuring the complement-dependent cytotoxicity on target cells. Flow cytometry is performed with read-out of dead cells stained using propidium iodide. The activity is expressed as the concentration of anti-T lymphocyte immunoglobulin in milligrams per millilitre which mediates 50 per cent cytotoxicity.

Lymphocyte separation medium. Commercial separation media with low viscosity and a density of 1.077 g/ml.

Complement. Commercial complement is suitable.

Buffered salt solution pH 7.2. Dissolve 8.0 g of *sodium chloride R*, 0.2 g of *potassium chloride R*, 3.18 g of *disodium hydrogen phosphate R* and 0.2 g of *potassium dihydrogen phosphate R* in *water R* and dilute to 1000.0 ml with the same solvent.

Buffer solution for flow cytometry. Add 40 ml of 0.1 per cent V/V *sodium azide R* and 10 ml of foetal calf serum to 440 ml of buffered salt solution pH 7.2. The foetal calf serum is inactivated at 56 °C for 30 min prior to use. Store at 4 °C.

Propidium iodide solution. Dissolve *propidium iodide R* in buffered salt solution pH 7.2, to a concentration of 1 mg/ml. Store this stock solution at 2-8 °C and use within 1 month. For the assay, dilute this solution with buffer solution for flow cytometry, to obtain a concentration of 5 µg/ml. Store at 2-8 °C and use within 3 h.

Microtitre plates. Plates used to prepare immunoglobulin dilutions are U- or V-bottomed polystyrene or poly(vinyl chloride) plates without surface treatment.

Micronic tubes. Suitable for flow cytometry measurement.

Cell suspension. Collect blood in anticoagulant from at least one healthy donor. Immediately isolate the peripheral blood mononuclear cells (PBMC) by gradient centrifugation in lymphocyte separation medium so that the PBMC form a visible clean interface between the plasma and the separation medium. Collect the layer containing the cells and dispense into centrifuge tubes containing buffered salt solution pH 7.2. Centrifuge at 400 *g* at 2-8 °C for 10 min. Discard the supernatant. Suspend the cell pellet in buffer solution for flow cytometry. Repeat the centrifugation and resuspension procedure of the cells twice. After the third centrifugation, resuspend the cell pellet in 1 ml of buffer solution for flow cytometry. Determine the number and vitality of the cells using a haemocytometer. Cell viability of at least 90 per cent is required. Adjust the cell number to 7×10^6 /ml by adding buffer solution for flow cytometry. Store the cell suspension at 4 °C and use within 12 h.

If necessary, the first PBMC pellet may be resuspended in buffered salt solution pH 7.2 containing 20 per cent foetal calf serum and stored overnight at 2 °C. Centrifuge at 400 *g* at 2-8 °C for 10 min. Discard the supernatant. Suspend the cell pellet in buffer solution for flow cytometry. Determine the number and vitality of the cells using a haemocytometer. Cell viability of at least 90 per cent is required. Adjust the cell number to 7×10^6 /ml by adding buffer solution for flow cytometry.

It is also possible for cells to be immediately frozen and stored in nitrogen using the following method.

Buffer solution for freezing. To 20 ml of cell culture medium, add 25 ml of foetal calf serum and 5 ml of dimethyl sulphoxide (DMSO). Store this solution at 2-8 °C and use within 3 h.

20×10^6 cells per ampoule are frozen. These ampoules are stored in liquid nitrogen.

Buffer solution for thawing. To 450 ml of cell culture medium, add 50 ml of foetal calf serum. Store this solution at 2-8 °C and use within 3 h.

Each ampoule is thawed in a water-bath at 37 °C with shaking. Cell suspension is repeated in a buffer solution for thawing. Centrifuge at 200 *g* at 2-8 °C for 10 min. Discard the supernatant. Suspend the cell pellet in buffer solution for flow cytometry. Repeat the procedure for centrifugation and resuspension of cells once. After the second centrifugation, resuspend the cells pellet in 1 ml of buffer solution for flow cytometry. Determine the number and vitality of the cells using a haemocytometer. Cell viability of at least 90 per cent

is required. Adjust the cell number to 7×10^6 /ml by adding buffer solution for flow cytometry. Store the cell suspension at 4 °C and use within 3 h.

Test solutions. For freeze-dried preparations, reconstitute as stated on the label. Prepare 3 independent series of not fewer than 7 dilutions using buffer solution for flow cytometry as diluent.

Reference solutions. For freeze-dried preparations, reconstitute according to the instructions for use. Prepare 3 independent dilution series of not fewer than 7 dilutions using buffer solution for flow cytometry as diluent.

Distribute 75 µl of each of the dilutions of the test solution or reference solution to each of a series of wells of a microtitre plate. Add 25 µl of the cell suspension of PBMC into each well. Add 25 µl of rabbit complement to each of the wells. Incubate at 37 °C for 30 min.

Centrifuge the plates at 200 *g* at 4 °C for 8 min, discard the supernatant and keep the plate on ice. Preparation for flow cytometry measurement is done step-wise by using a certain number of wells in order to allow labelling with *propidium iodide R* solution and measurement within a defined time period. Resuspend carefully the cell pellet of a certain number of wells with 200 µl of propidium iodide solution. Transfer the suspension into tubes. Incubate at 25 °C for 10 min then place immediately on ice.

Proceed with fluorescence measurement in a flow cytometer. Define a region including all propidium iodide-positive cells on the basis of Forward-Scattered, light (FSC) and fluorescence (FL2 or FL3 for propidium iodide). Measure the percentage of propidium iodide-positive cells, without gating but excluding debris. Analyse at least 3000 cells for each of the test and reference solutions.

Use the percentages of dead cells to estimate the potency as the concentration in milligrams per millilitre of the preparation to be examined necessary to induce 50 per cent of cytotoxicity by fitting a sigmoidal dose response curve to the data obtained with the test and the reference preparations and by using a 4-parameter logistic model (see, for example, chapter 5.3) and suitable software. The test is not valid unless the percentage of propidium iodide-positive cells at the lower asymptote of the curve is less than 15 per cent and the percentage of propidium iodide-positive cells at the upper asymptote of the curve is at least 80 per cent.

The estimated activity is 70 per cent to 130 per cent of the activity approved for the particular product.

The confidence limits ($P = 0.95$) are not less than 80 per cent and not more than 125 per cent of the estimated potency.

STORAGE

Protected from light at the temperature stated on the label.

Expiry date. The expiry date is calculated from the beginning of the assay.

LABELLING

The label states:

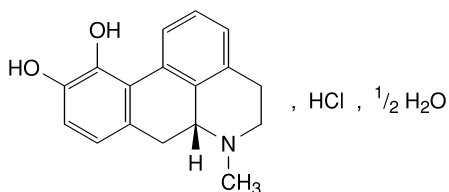
- for liquid preparations, the volume of the preparation in the container and the protein content,
- for freeze-dried preparations:
 - the name and the volume of the reconstitution liquid to be added,
 - the quantity of protein in the container,
 - that the immunosera are to be used immediately after reconstitution,
 - the time required for complete dissolution,
- the animal species of origin,

- the name and amount of stabiliser, where applicable,
- the dilution to be made before use of the product.

01/2005:0136

APOMORPHINE HYDROCHLORIDE

Apomorphini hydrochloridum

 $C_{17}H_{18}ClNO_2 \cdot 1/2 H_2O$ M_r 312.8

DEFINITION

(6a*R*)-6-Methyl-5,6,6a,7-tetrahydro-4*H*-dibenzo[*de*, *g*]quinoline-10,11-diol hydrochloride hemihydrate.

Content: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

Appearance: white or slightly yellowish-brown or green-tinged greyish, crystalline powder or crystals; on exposure to air and light, the green tinge becomes more pronounced.

Solubility: sparingly soluble in water and in alcohol, practically insoluble in toluene.

IDENTIFICATION

First identification: B, D.

Second identification: A, C, D.

- A. Dissolve 10.0 mg in 0.1 *M* hydrochloric acid and dilute to 100.0 ml with the same acid. Dilute 10.0 ml of the solution to 100.0 ml with 0.1 *M* hydrochloric acid. Examined between 230 nm and 350 nm (2.2.25), the solution shows an absorption maximum at 273 nm and a shoulder at 300 nm to 310 nm. The specific absorbance at the maximum is 530 to 570.
- B. Infrared absorption spectrophotometry (2.2.24).
Comparison: Ph. Eur. reference spectrum of apomorphine hydrochloride.
- C. To 5 ml of solution S (see Tests) add a few millilitres of sodium hydrogen carbonate solution *R* until a permanent, white precipitate is formed. The precipitate slowly becomes greenish. Add 0.25 ml of 0.05 *M* iodine and shake. The precipitate becomes greyish-green. Collect the precipitate. The precipitate dissolves in ether *R* giving a purple solution, in methylene chloride *R* giving a violet-blue solution and in alcohol *R* giving a blue solution.
- D. To 2 ml of solution S add 0.1 ml of nitric acid *R*. Mix and filter. The filtrate gives reaction (a) of chlorides (2.3.1).

TESTS

Solution S. Dissolve 0.25 g without heating in carbon dioxide-free water *R* and dilute to 25 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and not more intensely coloured than reference solution BY₅ or GY₅ (2.2.2, Method II).

pH (2.2.3): 4.0 to 5.0 for solution S.

Specific optical rotation (2.2.7): –48 to –52 (dried substance).

Dissolve 0.25 g in 0.02 *M* hydrochloric acid and dilute to 25.0 ml with the same acid.

Related substances. Liquid chromatography (2.2.29).

Test solution. Dissolve 0.25 g of the substance to be examined in a 1 per cent V/V solution of glacial acetic acid *R* and dilute to 100.0 ml with the same solution.

Reference solution (a). Dilute 1.0 ml of the test solution to 10.0 ml with a 1 per cent V/V solution of glacial acetic acid *R*. Dilute 1.0 ml to 100.0 ml with a 1 per cent V/V solution of glacial acetic acid *R*.

Reference solution (b). Dissolve 25 mg of boldine *R* in a 1 per cent V/V solution of glacial acetic acid *R* and dilute to 10.0 ml with the same solvent. To 1 ml of this solution, add 1 ml of the test solution and dilute to 10.0 ml with a 1 per cent V/V solution of glacial acetic acid *R*.

Column:

- *size*: $l = 0.15$ m, $\varnothing = 4.6$ mm,
- *stationary phase*: octadecylsilyl silica gel for chromatography *R* (5 μ m),
- *temperature*: 35 °C.

Mobile phase:

- *mobile phase A*: 1.1 g/l solution of sodium octanesulphonate *R*, adjusted to pH 2.2 using a 50 per cent *m/m* solution of phosphoric acid *R*,
- *mobile phase B*: acetonitrile *R*,

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)
0 - 30	85 → 68	15 → 32
30 - 35	68	32
35 - 45	68 → 85	32 → 15

Flow rate: 1.5 ml/min.

Detection: spectrophotometer at 280 nm.

Injection: 10 μ l.

System suitability: reference solution (b):

- *resolution*: minimum 2.5 between the peaks due to boldine and apomorphine.

Limits:

- *any impurity*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent),
- *total*: not more than 8 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.8 per cent),
- *disregard limit*: 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.02 per cent).

Heavy metals (2.4.8): maximum 20 ppm.

1.0 g complies with limit test C. Prepare the standard using 2 ml of lead standard solution (10 ppm Pb) *R*.

Loss on drying (2.2.32): 2.5 per cent to 4.2 per cent, determined on 1.000 g by drying in an oven at 100–105 °C.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

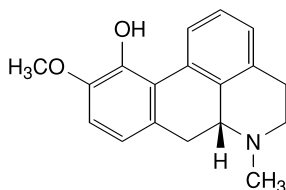
Dissolve 0.250 g in a mixture of 5.0 ml of 0.01 *M* hydrochloric acid and 50 ml of alcohol *R*. Carry out a potentiometric titration (2.2.20), using 0.1 *M* sodium hydroxide. Read the volume added between the first 2 points of inflexion.

1 ml of 0.1 M sodium hydroxide is equivalent to 30.38 mg of $C_{17}H_{18}ClNO_2$.

STORAGE

In an airtight container, protected from light.

IMPURITIES



A. (6aR)-10-methoxy-6-methyl-5,6,6a,7-tetrahydro-4H-dibenzo[de,g]quinolin-11-ol (apocodeine),

B. morphine.

01/2005:0580

APROTININ

Aprotininum

DEFINITION

Aprotinin is a polypeptide consisting of a chain of 58 amino acids. It inhibits stoichiometrically the activity of several proteolytic enzymes such as chymotrypsin, kallikrein, plasmin and trypsin. It contains not less than 3.0 Ph. Eur. U. of aprotinin activity per milligram, calculated with reference to the dried substance.

PRODUCTION

The animals from which aprotinin is derived must fulfil the requirements for the health of animals suitable for human consumption to the satisfaction of the competent authority.

The manufacturing process is validated to demonstrate suitable inactivation or removal of any contamination by viruses or other infectious agents.

The method of manufacture is validated to demonstrate that the product, if tested, would comply with the following tests.

Abnormal toxicity (2.6.9). Inject into each mouse a quantity of the substance to be examined containing 2 Ph. Eur. U. dissolved in a sufficient quantity of *water for injections R* to give a volume of 0.5 ml.

Histamine (2.6.10): maximum 0.2 µg of histamine base per 3 Ph. Eur. U.

CHARACTERS

Appearance: almost white powder, hygroscopic.

Solubility: soluble in water and in isotonic solutions, practically insoluble in organic solvents.

IDENTIFICATION

A. Thin-layer chromatography (2.2.27).

Test solution. Solution S (see Tests).

Reference solution. Aprotinin solution BRP.

Plate: TLC silica gel G plate R.

Mobile phase: *water R*, *glacial acetic acid R* (80:100 V/V) containing 100 g/l of *sodium acetate R*.

Application: 10 µl.

Development: over a path of 12 cm.

Drying: in air.

Detection: spray with a solution of 0.1 g of *ninhydrin R* in a mixture of 6 ml of a 10 g/l solution of *cupric chloride R*, 21 ml of *glacial acetic acid R* and 70 ml of *ethanol R*. Dry the plate at 60 °C.

Results: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

B. Determine the ability of the substance to be examined to inhibit trypsin activity using the method described below.

Test solution. Dilute 1 ml of solution S to 50 ml with *buffer solution pH 7.2 R*.

Trypsin solution. Dissolve 10 mg of *trypsin BRP* in 0.002 M *hydrochloric acid* and dilute to 100 ml with the same acid.

Casein solution. Dissolve 0.2 g of *casein R* in *buffer solution pH 7.2 R* and dilute to 100 ml with the same buffer solution.

Precipitating solution. Mix 1 volume of *glacial acetic acid R*, 49 volumes of *water R* and 50 volumes of *ethanol R*.

Mix 1 ml of the test solution with 1 ml of the trypsin solution. Allow to stand for 10 min and add 1 ml of the casein solution. Incubate at 35 °C for 30 min. Cool in iced water and add 0.5 ml of the precipitating solution. Shake and allow to stand at room temperature for 15 min. The solution is cloudy. Carry out a blank test under the same conditions using *buffer solution pH 7.2 R* instead of the test solution. The solution is not cloudy.

TESTS

Solution S. Prepare a solution of the substance to be examined containing 15 Ph. Eur. U./ml, calculated from the activity stated on the label.

Appearance of solution. Solution S is clear (2.2.1).

Absorbance (2.2.25): maximum 0.80 by measuring at the absorption maximum at 277 nm.

Prepare a solution of the substance to be examined containing 3.0 Ph. Eur. U./ml.

Protein impurities of higher molecular mass. Size-exclusion chromatography (2.2.30).

Use *cross-linked dextran for chromatography R2*. Use a 180 g/l solution of *anhydrous acetic acid R* to swell the gel and as the eluent. Prepare a column of gel 0.8 m to 1.0 m long and 25 mm in diameter, taking care to avoid the introduction of air bubbles. Place at the top of the column a quantity of the substance to be examined containing 300 Ph. Eur. U. dissolved in 1 ml of a 180 g/l solution of *anhydrous acetic acid R* and allow to elute. Collect the eluate in fractions of 2 ml. Measure the absorbance (2.2.25) of each fraction at the absorption maximum at 277 nm and plot the values on a graph. The chromatogram obtained does not present an absorption maximum before the elution of the aprotinin.

Loss on drying (2.2.32): maximum 6.0 per cent, determined on 0.100 g by drying *in vacuo*.

Bacterial endotoxins (2.6.14): less than 0.14 IU per European Pharmacopoeia Unit of aprotinin, if intended for use in the manufacture of parenteral dosage forms without a further appropriate procedure for the removal of bacterial endotoxins.

ASSAY

The activity of aprotinin is determined by measuring its inhibitory action on a solution of trypsin of known activity. The inhibiting activity of the aprotinin is calculated from the difference between the initial activity and the residual activity of the trypsin.

The inhibiting activity of aprotinin is expressed in European Pharmacopoeia Units. 1 Ph. Eur. U. inhibits 50 per cent of the enzymatic activity of 2 microkatal of trypsin.

Use a reaction vessel with a capacity of about 30 ml and provided with:

- a device that will maintain a temperature of 25 ± 0.1 °C;
- a stirring device, such as a magnetic stirrer;
- a lid with 5 holes for accommodating the electrodes, the tip of a burette, a tube for the admission of nitrogen and the introduction of the reagents.

An automatic or manual titration apparatus may be used. In the latter case the burette is graduated in 0.05 ml and the pH-meter is provided with a wide reading scale and glass and calomel electrodes.

Test solution. Prepare a solution of the substance to be examined in 0.0015 M borate buffer solution pH 8.0 R expected to contain 1.67 Ph. Eur. U./ml (about 0.6 mg (*m* mg) per millilitre).

Trypsin solution. Prepare a solution of *trypsin BRP* containing about 0.8 microkatal per millilitre (about 1 mg/ml), using 0.001 M hydrochloric acid as the solvent. Use a freshly prepared solution and keep in iced water.

Trypsin and aprotinin solution. To 4.0 ml of the trypsin solution add 1.0 ml of the test solution. Dilute immediately to 40.0 ml with 0.0015 M borate buffer solution pH 8.0 R. Allow to stand at room temperature for 10 min and then keep in iced water. Use within 6 h of preparation.

Dilute trypsin solution. Dilute 0.5 ml of the trypsin solution to 10.0 ml with 0.0015 M borate buffer solution pH 8.0 R. Allow to stand at room temperature for 10 min and then keep in iced water.

Maintain an atmosphere of nitrogen in the reaction flask and stir continuously; introduce 9.0 ml of 0.0015 M borate buffer solution pH 8.0 R and 1.0 ml of a freshly prepared 6.9 g/l solution of *benzoylarginine ethyl ester hydrochloride R*. Adjust to pH 8.0 with 0.1 M sodium hydroxide. When the temperature has reached equilibrium at 25 ± 0.1 °C, add 1.0 ml of the trypsin and aprotinin solution and start a timer. Maintain at pH 8.0 by the addition of 0.1 M sodium hydroxide and note the volume added every 30 s. Continue the reaction for 6 min. Determine the number of millilitres of 0.1 M sodium hydroxide used per second (n_1 ml). Carry out, under the same conditions, a titration using 1.0 ml of the dilute trypsin solution. Determine the number of millilitres of 0.1 M sodium hydroxide used per second (n_2 ml).

Calculate the aprotinin activity in European Pharmacopoeia Units per milligram from the expression:

$$\frac{4000 (2n_2 - n_1)}{m}$$

The estimated activity is not less than 90 per cent and not more than 110 per cent of the activity stated on the label.

STORAGE

In an airtight, tamper-proof container, protected from light.

LABELLING

The label states:

- the number of European Pharmacopoeia Units of aprotinin activity per milligram,

- where applicable, that the substance is free from bacterial endotoxins.

01/2005:0579

APROTININ CONCENTRATED SOLUTION

Aprotinini solutio concentrata

DEFINITION

Aprotinin concentrated solution is a solution of aprotinin, a polypeptide consisting of a chain of 58 amino acids, which inhibits stoichiometrically the activity of several proteolytic enzymes such as chymotrypsin, kallikrein, plasmin and trypsin. It contains not less than 15.0 Ph. Eur. U. of aprotinin activity per millilitre.

PRODUCTION

The animals from which aprotinin is derived must fulfil the requirements for the health of animals suitable for human consumption to the satisfaction of the competent authority. The manufacturing process is validated to demonstrate suitable inactivation or removal of any contamination by viruses or other infectious agents.

The method of manufacture is validated to demonstrate that the product, if tested, would comply with the following tests.

Abnormal toxicity (2.6.9). Inject into each mouse a quantity of the preparation to be examined containing 2 Ph. Eur. U. diluted with a sufficient quantity of *water for injections R* to give a volume of 0.5 ml.

Histamine (2.6.10): maximum 0.2 µg of histamine base per 3 Ph. Eur. U.

CHARACTERS

Appearance: clear and colourless liquid.

IDENTIFICATION

A. Thin-layer chromatography (2.2.27).

Test solution. Solution S (see Tests).

Reference solution. Aprotinin solution BRP.

Plate: TLC silica gel G plate R.

Mobile phase: *water R*, *glacial acetic acid R* (80:100 V/V) containing 100 g/l of *sodium acetate R*.

Application: 10 µl.

Development: over a path of 12 cm.

Drying: in air.

Detection: spray with a solution of 0.1 g of *ninhydrin R* in a mixture of 6 ml of a 10 g/l solution of *cupric chloride R*, 21 ml of *glacial acetic acid R* and 70 ml of *ethanol R*. Dry the plate at 60 °C.

Results: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

B. Determine the ability of the preparation to be examined to inhibit trypsin activity using the method described below.

Test solution. Dilute 1 ml of solution S to 50 ml with *buffer solution pH 7.2 R*.

Trypsin solution. Dissolve 10 mg of *trypsin BRP* in 0.002 M hydrochloric acid and dilute to 100 ml with the same acid.

Casein solution. Dissolve 0.2 g of *casein R* in *buffer solution pH 7.2 R* and dilute to 100 ml with the same buffer solution.

Precipitating solution. Mix 1 volume of *glacial acetic acid R*, 49 volumes of *water R* and 50 volumes of *ethanol R*.

Mix 1 ml of the test solution with 1 ml of the trypsin solution. Allow to stand for 10 min and add 1 ml of the casein solution. Incubate at 35 °C for 30 min. Cool in iced water and add 0.5 ml of the precipitating solution. Shake and allow to stand at room temperature for 15 min. The solution is cloudy. Carry out a blank test under the same conditions using *buffer solution pH 7.2 R* instead of the test solution. The solution is not cloudy.

TESTS

Solution S. Prepare a solution containing 15 Ph. Eur. U./ml, if necessary by dilution on the basis of the activity stated on the label.

Appearance of solution. Solution S is clear (2.2.1).

Absorbance (2.2.25): maximum 0.80 by measuring at the absorption maximum at 277 nm.

Prepare from the concentrated solution a dilution containing 3.0 Ph. Eur. U./ml.

Protein impurities of higher molecular mass. Size-exclusion chromatography (2.2.30).

Freeze-dry the preparation to be examined using a pressure of 2.7 Pa and a temperature of –30 °C; the operation, including freeze-drying and a period of drying at 15–25 °C, takes 6–12 h.

Use *cross-linked dextran for chromatography R2*. Use a 180 g/l solution of *anhydrous acetic acid R* to swell the gel and as the eluent. Prepare a column of gel 0.8 m to 1.0 m long and 25 mm in diameter, taking care to avoid the introduction of air bubbles. Place at the top of the column a quantity of the preparation to be examined containing 300 Ph. Eur. U. dissolved in 1 ml of a 180 g/l solution of *anhydrous acetic acid R* and allow to elute. Collect the eluate in fractions of 2 ml. Measure the absorbance (2.2.25) of each fraction at the absorption maximum at 277 nm and plot the values on a graph. The chromatogram obtained does not present an absorption maximum before the elution of the aprotinin.

Specific activity of the dry residue: minimum 3.0 Ph. Eur. U. of aprotinin activity per milligram of dry residue.

Evaporate 25.0 ml to dryness in a water-bath, dry the residue at 110 °C for 15 h and weigh. From the mass of the residue and the activity determined as described below, calculate the number of European Pharmacopoeia Units per milligram of dry residue.

Bacterial endotoxins (2.6.14): less than 0.14 IU per European Pharmacopoeia Unit of aprotinin, if intended for use in the manufacture of parenteral dosage forms without a further appropriate procedure for the removal of bacterial endotoxins.

ASSAY

The activity of aprotinin is determined by measuring its inhibitory action on a solution of trypsin of known activity. The inhibiting activity of the aprotinin is calculated from the difference between the initial activity and the residual activity of the trypsin.

The inhibiting activity of aprotinin is expressed in European Pharmacopoeia Units. 1 Ph. Eur. U. inhibits 50 per cent of the enzymatic activity of 2 microkatal of trypsin.

Use a reaction vessel with a capacity of about 30 ml and provided with:

- a device that will maintain a temperature of 25 ± 0.1 °C;
- a stirring device, such as a magnetic stirrer;
- a lid with 5 holes for accommodating the electrodes, the tip of a burette, a tube for the admission of nitrogen and the introduction of the reagents.

An automatic or manual titration apparatus may be used. In the latter case the burette is graduated in 0.05 ml and the pH-meter is provided with a wide reading scale and glass and calomel electrodes.

Test solution. With 0.0015 M borate buffer solution pH 8.0 R prepare an appropriate dilution (D) of the concentrated solution expected on the basis of the stated potency to contain 1.67 Ph. Eur. U./ml.

Trypsin solution. Prepare a solution of *trypsin BRP* containing about 0.8 microkatal per millilitre (about 1 mg/ml), using 0.001 M hydrochloric acid as the solvent. Use a freshly prepared solution and keep in iced water.

Trypsin and aprotinin solution. To 4.0 ml of the trypsin solution add 1.0 ml of the test solution. Dilute immediately to 40.0 ml with 0.0015 M borate buffer solution pH 8.0 R. Allow to stand at room temperature for 10 min and then keep in iced water. Use within 6 h of preparation.

Dilute trypsin solution. Dilute 0.5 ml of the trypsin solution to 10.0 ml with 0.0015 M borate buffer solution pH 8.0 R. Allow to stand at room temperature for 10 min and then keep in iced water.

Maintain an atmosphere of nitrogen in the reaction flask and stir continuously; introduce 9.0 ml of 0.0015 M borate buffer solution pH 8.0 R and 1.0 ml of a freshly prepared 6.9 g/l solution of *benzoylarginine ethyl ester hydrochloride R*. Adjust to pH 8.0 with 0.1 M sodium hydroxide. When the temperature has reached equilibrium at 25 ± 0.1 °C, add 1.0 ml of the trypsin and aprotinin solution and start a timer. Maintain at pH 8.0 by the addition of 0.1 M sodium hydroxide and note the volume added every 30 s. Continue the reaction for 6 min. Determine the number of millilitres of 0.1 M sodium hydroxide used per second (n_1 ml). Carry out, under the same conditions, a titration using 1.0 ml of the dilute trypsin solution. Determine the number of millilitres of 0.1 M sodium hydroxide used per second (n_2 ml).

Calculate the aprotinin activity in European Pharmacopoeia Units per millilitre from the expression:

$$4000 (2n_2 - n_1) \times D$$

D = dilution factor of the aprotinin concentrated solution to be examined in order to obtain a solution containing 1.67 Ph. Eur. U./ml.

The estimated activity is not less than 90 per cent and not more than 110 per cent of the activity stated on the label.

STORAGE

In an airtight, tamper-proof container, protected from light.

LABELLING

The label states:

- the number of European Pharmacopoeia Units of aprotinin activity per millilitre.
- where applicable, that the solution is free from bacterial endotoxins.

01/2005:1171

ARACHIS OIL, HYDROGENATED**Arachidis oleum hydrogenatum****DEFINITION**

Hydrogenated arachis oil is the product obtained by refining, bleaching, hydrogenating and deodorising oil obtained from the shelled seeds of *Arachis hypogaea* L. Each type of hydrogenated arachis oil is characterised by its nominal drop point.

CHARACTERS

A white or faintly yellowish, soft mass which melts to a clear, pale yellow liquid when heated, practically insoluble in water, freely soluble in methylene chloride and in light petroleum (bp: 65 °C to 70 °C), very slightly soluble in alcohol.

IDENTIFICATION

First identification: A, B.

Second identification: A, C.

- A. It complies with the test for drop point (see Tests).
- B. Carry out the identification of fatty oils by thin-layer chromatography (2.3.2). The chromatogram obtained is similar to the typical chromatogram for arachis oil.
- C. It complies with the test for composition of fatty acids.

TESTS

Drop point (2.2.17): 32 °C to 43 °C. Within this range the drop point does not differ by more than 3 °C from the nominal value.

Acid value (2.5.1). Not more than 0.5. Dissolve 10.0 g in 50 ml of the prescribed solvent by heating on a water-bath.

Peroxide value (2.5.5). Not more than 5.0. Dissolve 5.0 g in 30 ml of the prescribed solvent by heating on a water-bath.

Unsaponifiable matter (2.5.7). Not more than 1.0 per cent.

Alkaline impurities (2.4.19). It complies with the test for alkaline impurities in fatty oils.

Composition of fatty acids (2.4.22, Method A).

The chromatographic procedure may be carried out using:

- a capillary fused-silica column 25 m long and 0.25 mm in internal diameter coated with *poly(cyanopropyl)siloxane R* (film thickness 0.2 µm),
- *helium for chromatography R* as the carrier gas at a flow rate of 0.7 ml/min,
- a flame-ionisation detector,
- a split injector (1:100),

maintaining the temperature of the column at 180 °C for 20 min and that of the injection port and of the detector at 250 °C.

The fatty acid fraction of the oil has the following composition:

- *saturated fatty acids of chain length less than C₁₄*: not more than 0.5 per cent,
- *myristic acid*: not more than 0.5 per cent,
- *palmitic acid*: 7.0 per cent to 16.0 per cent,
- *stearic acid*: 3.0 per cent to 19.0 per cent,
- *oleic acid and isomers* (C_{18:1}, equivalent chain length on *poly(cyanopropyl)siloxane 18.5 to 18.8*): 54.0 per cent to 78.0 per cent,

- *linoleic acid and isomers* (C_{18:2}, equivalent chain length on *poly(cyanopropyl)siloxane 19.4 to 19.8*): not more than 10.0 per cent,
- *arachidic acid*: 1.0 per cent to 3.0 per cent,
- *eicosenoic acids* (C_{20:1}, equivalent chain length on *poly(cyanopropyl)siloxane 20.4 to 20.7*): not more than 2.1 per cent,
- *behenic acid*: 1.0 per cent to 5.0 per cent,
- *erucic acid and isomers* (C_{22:1}, equivalent chain length on *poly(cyanopropyl)siloxane 22.4 to 22.6*): not more than 0.5 per cent,
- *lignoceric acid*: 0.5 per cent to 3.0 per cent.

Nickel. Not more than 1 ppm of Ni, determined by atomic absorption spectrometry (2.2.23, Method II).

Test solution. Into a platinum or silica crucible previously tared after ignition introduce 5.0 g. Cautiously heat and introduce into the substance a wick formed from twisted ashless filter paper. Ignite the wick. When the substance has ignited stop heating. After combustion, ignite in a muffle furnace at about 600 °C. Continue ignition until white ash is obtained. After cooling, take up the residue with two quantities, each of 2 ml, of *dilute hydrochloric acid R* and transfer into a 25 ml graduated flask. Add 0.3 ml of *nitric acid R* and dilute to 25.0 ml with *water R*.

Reference solutions. Prepare three reference solutions by adding 1.0 ml, 2.0 ml and 4.0 ml of *nickel standard solution* (0.2 ppm Ni) *R* to 2.0 ml of the test solution and diluting to 10.0 ml with *water R*.

Measure the absorbance at 232 nm using a nickel hollow-cathode lamp as a source of radiation, a graphite furnace as an atomic generator and *argon R* as the carrier gas.

Water (2.5.12). Not more than 0.3 per cent, determined on 1.000 g by the semi-micro determination of water.

STORAGE

Store protected from light.

LABELLING

The label states the nominal drop point.

01/2005:0263

ARACHIS OIL, REFINED**Arachidis oleum raffinatum****DEFINITION**

The refined fatty oil obtained from the shelled seeds of *Arachis hypogaea* L. A suitable antioxidant may be added.

CHARACTERS

Appearance: clear, yellowish, viscous liquid.

Solubility: very slightly soluble in alcohol, miscible with light petroleum.

Relative density: about 0.915.

It solidifies at about 2 °C.

IDENTIFICATION

It complies with the identification test for fatty oils by thin-layer chromatography (2.3.2). The chromatogram obtained is similar to the typical chromatogram for arachis oil.

TESTS

Acid value (2.5.1): maximum 0.5, determined on 10.0 g.

Peroxide value (2.5.5): maximum 5.0.

01/2005:0806

Unsaponifiable matter (2.5.7): maximum 1.0 per cent, determined on 5.0 g.

Alkaline impurities (2.4.19). It complies with the test for alkaline impurities in fatty oils.

Composition of fatty acids. Gas chromatography (2.4.22, Method A).

Reference solution (a). Prepare 0.50 g of a mixture of the calibrating substances as indicated in Table 0263-1. Dissolve in *heptane R* and dilute to 50 ml with the same solvent.

Table 0263-1

Calibrating substances	Composition (per cent m/m)
<i>Methyl palmitate R</i>	10
<i>Methyl stearate R</i>	5
<i>Methyl oleate R</i>	40
<i>Methyl linoleate R</i>	25
<i>Methyl linolenate R</i>	2
<i>Methyl arachidate R</i>	5
<i>Methyl eicosenoate R</i>	3
<i>Methyl behenate R</i>	5
<i>Methyl erucate R</i>	2
<i>Methyl lignocerate R</i>	3

Composition of the fatty acid fraction of the oil:

- **saturated fatty acids of chain length less than C_{16} :** maximum 0.4 per cent,
- **palmitic acid:** 7.0 per cent to 16.0 per cent,
- **stearic acid:** 1.3 per cent to 6.5 per cent,
- **oleic acid** (equivalent chain length on polyethyleneglycol adipate 18.3): 35.0 per cent to 72.0 per cent,
- **linoleic acid** (equivalent chain length on polyethyleneglycol adipate 18.9): 13.0 per cent to 43.0 per cent,
- **linolenic acid** (equivalent chain length on polyethyleneglycol adipate 19.7): maximum 0.6 per cent,
- **arachidic acid:** 0.5 per cent to 3.0 per cent,
- **eicosenoic acid** (equivalent chain length on polyethyleneglycol adipate 20.3): 0.5 per cent to 2.1 per cent,
- **behenic acid:** 1.0 per cent to 5.0 per cent,
- **erucic acid** (equivalent chain length on polyethyleneglycol adipate 22.3): maximum 0.5 per cent,
- **lignoceric acid:** 0.5 per cent to 3.0 per cent.

Water (2.5.12): maximum 0.3 per cent, determined on 3.00 g, if intended for use in the manufacture of parenteral dosage forms.

STORAGE

In a well-filled container, protected from light.

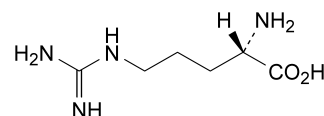
LABELLING

The label states:

- where applicable, that the substance is suitable for use in the manufacture of parenteral dosage forms,
- the name and concentration of any added antioxidant.

ARGININE

Argininum


 $C_6H_{14}N_4O_2$
 M_r 174.2

DEFINITION

Arginine contains not less than 98.5 per cent and not more than the equivalent of 101.0 per cent of (S)-2-amino-5-guanidinopentanoic acid, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, crystalline powder or colourless crystals, freely soluble in water, very slightly soluble in alcohol.

IDENTIFICATION

First identification: A, C.

Second identification: A, B, D, E.

- It complies with the test for specific optical rotation (see Tests).
- Solution S (see Tests) is strongly alkaline (2.2.4).
- Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *arginine CRS*. Examine the substances prepared as discs.
- Examine the chromatograms obtained in the test for ninhydrin-positive substances. The principal spot in the chromatogram obtained with test solution (b) is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).
- Dissolve about 25 mg in 2 ml of *water R*. Add 1 ml of α -naphthol solution R and 2 ml of a mixture of equal volumes of *strong sodium hypochlorite solution R* and water. A red colour develops.

TESTS

Solution S. Dissolve 2.5 g in *distilled water R* and dilute to 50 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and not more intensely coloured than reference solution BY₆ (2.2.2, Method II).

Specific optical rotation (2.2.7). Dissolve 2.00 g in *hydrochloric acid R1* and dilute to 25.0 ml with the same acid. The specific optical rotation is + 25.5 to + 28.5, calculated with reference to the dried substance.

Ninhydrin-positive substances. Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel plate R*.

Test solution (a). Dissolve 0.10 g of the substance to be examined in *dilute hydrochloric acid R* and dilute to 10 ml with the same acid.

Test solution (b). Dilute 1 ml of test solution (a) to 50 ml with *water R*.

Reference solution (a). Dissolve 10 mg of *arginine CRS* in 0.1 M *hydrochloric acid* and dilute to 50 ml with the same acid.

Reference solution (b). Dilute 5 ml of test solution (b) to 20 ml with *water R*.

Reference solution (c). Dissolve 10 mg of *arginine CRS* and 10 mg of *lysine hydrochloride CRS* in 0.1 M hydrochloric acid and dilute to 25 ml with the same acid.

Apply to the plate 5 µl of each solution. Allow the plate to dry in air. Develop over a path of 15 cm using a mixture of 30 volumes of *concentrated ammonia R* and 70 volumes of *2-propanol R*. Dry the plate at 100 °C to 105 °C until the ammonia disappears completely. Spray with *ninhydrin solution R* and heat at 100 °C to 105 °C for 15 min. Any spot in the chromatogram obtained with test solution (a), apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.5 per cent). The test is not valid unless the chromatogram obtained with reference solution (c) shows two clearly separated spots.

Chlorides (2.4.4). To 5 ml of solution S add 0.5 ml of *dilute nitric acid R* and dilute to 15 ml with *water R*. The solution complies with the limit test for chlorides (200 ppm).

Sulphates (2.4.13). To 10 ml of solution S, add 1.7 ml of *dilute hydrochloric acid R* and dilute to 15 ml with *distilled water R*. The solution complies with the limit test for sulphates (300 ppm).

Ammonium (2.4.1). 50 mg complies with limit test B for ammonium (200 ppm). Prepare the standard using 0.1 ml of *ammonium standard solution (100 ppm NH₄) R*.

Iron (2.4.9). In a separating funnel, dissolve 1.0 g in 10 ml of *dilute hydrochloric acid R*. Shake with three quantities, each of 10 ml, of *methyl isobutyl ketone RI*, shaking for 3 min each time. To the combined organic layers add 10 ml of *water R* and shake for 3 min. The aqueous layer complies with the limit test for iron (10 ppm).

Heavy metals (2.4.8). Dissolve 2.0 g in *water R* and dilute to 20 ml with the same solvent. 12 ml of the solution complies with limit test A for heavy metals (10 ppm). Prepare the standard using *lead standard solution (1 ppm Pb) R*.

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.150 g in 50 ml of *water R*. Using 0.2 ml of *methyl red mixed solution R* as indicator, titrate with 0.1 M *hydrochloric acid* until the colour changes from green to violet-red.

1 ml of 0.1 M *hydrochloric acid* is equivalent to 17.42 mg of C₁₀H₂₁N₅O₆.

STORAGE

Store protected from light.

DEFINITION

(2S)-2-Amino-5-guanidinopentanoic acid (2S)-2-aminobutanedioate.

Content: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

Appearance: white granules or powder.

Solubility: very soluble in water, practically insoluble in alcohol and in methylene chloride.

IDENTIFICATION

A. It complies with the test for specific optical rotation (see Tests).

B. Infrared absorption spectrophotometry (2.2.24).

Comparison: *arginine aspartate CRS*.

C. Examine the chromatograms obtained in the test for ninhydrin-positive substances.

Results: the 2 principal spots in the chromatogram obtained with test solution (b) are similar in position, colour and size to the 2 principal spots in the chromatogram obtained with reference solution (a).

TESTS

Solution S. Dissolve 5.0 g in *carbon dioxide-free water R* and dilute to 50 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and not more intensely coloured than reference solution Y₇ (2.2.2, *Method II*).

pH (2.2.3): 6.0 to 7.0 for solution S.

Specific optical rotation (2.2.7): + 25 to + 27 (dried substance).

Dissolve 2.50 g in *dilute hydrochloric acid R* and dilute to 25.0 ml with the same acid.

Ninhydrin-positive substances. Thin-layer chromatography (2.2.27).

Test solution (a). Dissolve 0.20 g of the substance to be examined in *water R* and dilute to 10 ml with the same solvent.

Test solution (b). Dilute 1 ml of test solution (a) to 10 ml with *water R*.

Reference solution (a). Dissolve 25 mg of *arginine R* and 25 mg of *aspartic acid R* in *water R* and dilute to 25 ml with the same solvent.

Reference solution (b). Dilute 2 ml of reference solution (a) to 50 ml with *water R*.

Plate: *TLC silica gel G plate R*.

Mobile phase: *ammonia R, propanol R* (36:64 V/V).

Application: 5 µl.

Development: over 2/3 of the plate.

Drying: at 100-105 °C for 10 min.

Detection: spray with *ninhydrin solution R* and heat at 100-105 °C for 10 min.

System suitability: reference solution (b):

— the chromatogram shows 2 clearly separated principal spots.

Limit: test solution (a):

— *any impurity:* any spots, apart from the 2 principal spots, are not more intense than each of the 2 principal spots in the chromatogram obtained with reference solution (b) (0.2 per cent).

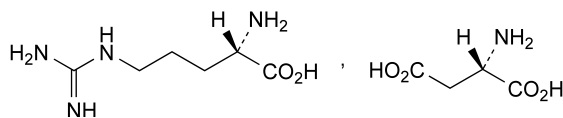
Chlorides (2.4.4): maximum 200 ppm.

Dilute 2.5 ml of solution S to 15 ml with *water R*.

01/2005:2096

ARGININE ASPARTATE

Arginini aspartas



C₁₀H₂₁N₅O₆

M_r 307.3

Sulphates (2.4.13): maximum 300 ppm.

To 0.5 g add 2.5 ml of *dilute hydrochloric acid R* and dilute to 15 ml with *distilled water R*. Examine after 30 min.

Ammonium (2.4.1): maximum 100 ppm, determined on 100 mg.

Heavy metals (2.4.8): maximum 20 ppm.

12 ml of solution S complies with limit test A. Prepare the standard using *lead standard solution (2 ppm Pb) R*.

Loss on drying (2.2.32): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 60 °C for 24 h.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

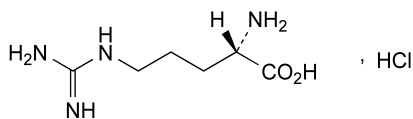
Dissolve 80.0 mg in 2 ml of *anhydrous formic acid R*. Add 50 ml of *anhydrous acetic acid R*. Titrate with 0.1 M *perchloric acid*, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *perchloric acid* is equivalent to 10.24 mg of $C_{10}H_{21}N_5O_6$.

01/2005:0805

ARGININE HYDROCHLORIDE

Arginini hydrochloridum


 $C_6H_{15}ClN_4O_2$
 M_r 210.7

DEFINITION

Arginine hydrochloride contains not less than 98.5 per cent and not more than the equivalent of 101.0 per cent of the hydrochloride of (S)-2-amino-5-guanidinopentanoic acid, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, crystalline powder or colourless crystals, freely soluble in water, very slightly soluble in alcohol.

IDENTIFICATION

First identification: A, B, E.

Second identification: A, C, D, E.

- It complies with the test for specific optical rotation (see Tests).
- Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *arginine hydrochloride CRS*. Examine the substances prepared as discs.
- Examine the chromatograms obtained in the test for ninhydrin-positive substances. The principal spot in the chromatogram obtained with test solution (b) is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).
- Dissolve about 25 mg in 2 ml of *water R*. Add 1 ml of α -naphthol solution *R* and 2 ml of a mixture of equal volumes of *strong sodium hypochlorite solution R* and *water R*. A red colour develops.
- About 20 mg gives reaction (a) of chlorides (2.3.1).

TESTS

Solution S. Dissolve 2.5 g in *distilled water R* and dilute to 50 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and not more intensely coloured than reference solution BY₆ (2.2.2, *Method II*).

Specific optical rotation (2.2.7). Dissolve 2.00 g in *hydrochloric acid R1* and dilute to 25.0 ml with the same acid. The specific optical rotation is + 21.0 to + 23.5, calculated with reference to the dried substance.

Ninhydrin-positive substances. Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel plate R*.

Test solution (a). Dissolve 0.10 g of the substance to be examined in *water R* and dilute to 10 ml with the same solvent.

Test solution (b). Dilute 1 ml of test solution (a) to 50 ml with *water R*.

Reference solution (a). Dissolve 10 mg of *arginine hydrochloride CRS* in *water R* and dilute to 50 ml with the same solvent.

Reference solution (b). Dilute 5 ml of test solution (b) to 20 ml with *water R*.

Reference solution (c). Dissolve 10 mg of *arginine hydrochloride CRS* and 10 mg of *lysine hydrochloride CRS* in *water R* and dilute to 25 ml with the same solvent.

Apply to the plate 5 µl of each solution. Allow the plate to dry in air. Develop over a path of 15 cm using a mixture of 30 volumes of *concentrated ammonia R* and 70 volumes of *2-propanol R*. Dry the plate at 100 °C to 105 °C until the ammonia disappears completely. Spray with *ninhydrin solution R* and heat at 100 °C to 105 °C for 15 min. Any spot in the chromatogram obtained with test solution (a), apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.5 per cent). The test is not valid unless the chromatogram obtained with reference solution (c) shows two clearly separated spots.

Sulphates (2.4.13). Dilute 10 ml of solution S to 15 ml with *distilled water R*. The solution complies with the limit test for sulphates (300 ppm).

Ammonium (2.4.1). 50 mg complies with limit test B for ammonium (200 ppm). Prepare the standard using 0.1 ml of *ammonium standard solution (100 ppm NH₄) R*.

Iron (2.4.9). In a separating funnel, dissolve 1.0 g in 10 ml of *dilute hydrochloric acid R*. Shake with three quantities, each of 10 ml, of *methyl isobutyl ketone R1*, shaking for 3 min each time. To the combined organic layers add 10 ml of *water R* and shake for 3 min. The aqueous layer complies with the limit test for iron (10 ppm).

Heavy metals (2.4.8). Dissolve 2.0 g in *water R* and dilute to 20 ml with the same solvent. 12 ml of the solution complies with limit test A for heavy metals (10 ppm). Prepare the standard using *lead standard solution (1 ppm Pb) R*.

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.180 g in 3 ml of *anhydrous formic acid R*. Add 30 ml of *anhydrous acetic acid R*. Using 0.1 ml of *naphtholbenzein solution R* as indicator, titrate with 0.1 M *perchloric acid* until the colour changes from brownish-yellow to green.

1 ml of 0.1 M perchloric acid is equivalent to 21.07 mg of $C_6H_{15}ClN_4O_2$.

STORAGE

Store protected from light.

01/2005:1391

ARNICA FLOWER

Arnicae flos

DEFINITION

Arnica flower consists of the whole or partially broken, dried flower-heads of *Arnica montana* L. It contains not less than 0.40 per cent *m/m* of total sesquiterpene lactones expressed as dihydrohelenalin tiglate, calculated with reference to the dried drug.

CHARACTERS

It has an aromatic odour.

The capitulum when spread out, is about 20 mm in diameter and about 15 mm deep, and has a peduncle of 2 cm to 3 cm in length. The involucre consists of eighteen to twenty-four elongated lanceolate bracts, with acute apices, arranged in one or two rows: the bracts, 8 mm to about 10 mm long, are green with yellowish-green external hairs visible under a lens. The receptacle, about 6 mm in diameter, is convex, alveolate and covered with hairs. Its periphery bears about twenty ligulate florets 20 mm to 30 mm long; the disc bears a greater number of tubular florets about 15 mm long. The ovary, 4 mm to 8 mm long, is crowned by a pappus of whitish bristles 4 mm to 8 mm long. Some brown achenes, crowned or not by a pappus, may be present.

It has the macroscopic and microscopic characters described under identification tests A and B.

IDENTIFICATION

- A. The involucre consists of elongated oval bracts with acute apices; the margin is ciliated. The ligulate floret has a reduced calyx crowned by fine, shiny, whitish bristles, bearing small coarse trichomes. The orange-yellow corolla bears seven to ten parallel veins and ends in three small lobes. The stamens, with free anthers, are incompletely developed. The narrow, brown ovary bears a stigma divided into two branches curving outwards. The tubular floret is actinomorphic. The ovary and the calyx are similar to those of the ligulate floret. The short corolla has five reflexed triangular lobes; the five fertile stamens are fused at the anthers.
- B. Separate the capitulum into its different parts. Reduce to a powder (355). Examine under a microscope using chloral hydrate solution R. The powder shows the following characteristics. The epidermises of the bracts of the involucre have stomata and trichomes, which are more abundant on the outer (abaxial) surface. There are several different types of trichomes: uniseriate multicellular covering trichomes, varying in length from 50 µm to 500 µm, particularly abundant on the margins of the bract; secretory trichomes with uni- or biseriate multicellular stalks and with multicellular, globular heads, about 300 µm long, abundant on the outer surface of the bract; secretory trichomes with uniseriate multicellular stalks and with multicellular, globular heads, about 80 µm long, abundant on the inner surface of the bract. The epidermis of the ligulate corolla consists

of lobed or elongated cells, a few stomata and trichomes of different types: covering trichomes, with very sharp ends, whose length may exceed 500 µm, consisting of one to three proximal cells with thickened walls and two to four distal cells with thin walls; secretory trichomes with biseriate multicellular heads; secretory trichomes with multicellular stalks and multicellular globular heads. The ligule ends in rounded papillose cells. The epidermis of the ovary is covered with trichomes: secretory trichomes with short stalks and multicellular globular heads; twinned covering trichomes usually consisting of two longitudinally united cells, with common punctuated walls; their ends are sharp and sometimes bifid. The epidermises of the calyx consist of elongated cells bearing short, unicellular, covering trichomes pointing towards the upper end of the bristle. The pollen grains have a diameter of about 30 µm and are rounded, with a spiny exine; they have three germinal pores.

- C. Examine the chromatograms obtained in the test for *Calendula officinalis* L. - *Heterotheca inuloides*. The chromatogram obtained with the test solution shows, in the middle, a fluorescent blue zone corresponding to the zone due to chlorogenic acid in the chromatogram obtained with the reference solution; it shows, above this zone, three fluorescent yellowish-brown to orange-yellow zones, and above these three zones a fluorescent greenish-yellow zone corresponding to astragalin. The zone located below the astragalin zone corresponds to isoquercitroside; the zone located just below this zone corresponds to luteolin-7-glucoside. It also shows a fluorescent greenish-blue zone below the zone due to caffeic acid in the chromatogram obtained with the reference solution.

TESTS

Foreign matter (2.8.2). Not more than 5.0 per cent.

***Calendula officinalis* L. - *Heterotheca inuloides*.** Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel plate R*.

Test solution. To 2.00 g of the powdered drug (710) add 10 ml of *methanol R*. Heat in a water-bath at 60 °C for 5 min with shaking. Cool and filter.

Reference solution. Dissolve 2.0 mg of *caffeic acid R*, 2.0 mg of *chlorogenic acid R* and 5.0 mg of *rutin R* in *methanol R* and dilute to 30 ml with the same solvent.

Apply separately to the plate, as bands, 15 µl of each solution. Develop over a path of 15 cm using a mixture of 10 volumes of *anhydrous formic acid R*, 10 volumes of *water R*, 30 volumes of *methyl ethyl ketone R* and 50 volumes of *ethyl acetate R*. Allow the plate to dry in air for a few minutes. Spray with a 10 g/l solution of *diphenylboric acid aminoethyl ester R* in *methanol R* and then with a 50 g/l solution of *macrogol 400 R* in *methanol R*. Heat for 5 min at 100 °C to 105 °C. Allow the plate to dry in air and examine in ultraviolet light at 365 nm. The chromatogram obtained with the reference solution shows in the lower part an orange-yellow fluorescent zone (rutin), in the middle part a fluorescent zone due to chlorogenic acid and in the upper part a light bluish fluorescent zone (caffeic acid). The chromatogram obtained with the test solution does not show a fluorescent orange-yellow zone corresponding to rutin in the chromatogram obtained with the reference solution, nor does it show a zone below the zone corresponding to rutin.

Loss on drying (2.2.32). Not more than 10.0 per cent, determined on 1.000 g of the powdered drug (355) by drying in an oven at 100 °C to 105 °C for 2 h.

Total ash (2.4.16). Not more than 10.0 per cent.

ASSAY

Examine by liquid chromatography (2.2.29), using *santonin R* as the internal standard.

Internal standard solution. Dissolve immediately before use 0.010 g of *santonin R* accurately weighed in 10.0 ml of *methanol R*.

Test solution. In a 250 ml round-bottomed flask introduce 1.00 g of the powdered drug (355), add 50 ml of a mixture of equal volumes of *methanol R* and *water R* and heat under a reflux condenser in a water-bath at 50 °C to 60 °C for 30 min, shaking frequently. Allow to cool and filter through a paper filter. Add the paper filter, cut in pieces, to the residue in the round-bottomed flask, add 50 ml of a mixture of equal volumes of *methanol R* and *water R* and heat under a reflux condenser in a water-bath at 50 °C to 60 °C for 30 min, shaking frequently. Repeat this procedure twice. To the combined filtrate add 3.00 ml of the internal standard solution and evaporate to 18 ml under reduced pressure. Rinse the round-bottomed flask with *water R* and dilute, with the washings, to 20.0 ml. Transfer the solution to a chromatography column about 0.15 m long and about 30 mm in internal diameter containing 15 g of *kieselguhr for chromatography R*. Allow to stand for 20 min. Elute with 200 ml of a mixture of equal volumes of *ethyl acetate R* and *methylene chloride R*. Evaporate the eluate to dryness in a 250 ml round-bottomed flask. Dissolve the residue in 10.0 ml of *methanol R* and add 10.0 ml of *water R*. Add 7.0 g of *neutral aluminium oxide R*, shake for 120 s, centrifuge (at 5000 *g* for 10 min) and filter through a paper filter. Evaporate 10.0 ml of the filtrate to dryness. Dissolve the residue in 3.0 ml of a mixture of equal volumes of *methanol R* and *water R* and filter.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.12 m long and 4 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (4 µm),
- as mobile phase at a flow rate of 1.2 ml/min:
Mobile phase A. Water R,
Mobile phase B. Methanol R,

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)	Comment
0 - 3	62	38	isocratic
3 - 20	62 → 55	38 → 45	linear gradient
20 - 30	55	45	isocratic
30 - 55	55 → 45	45 → 55	linear gradient
55 - 57	45 → 0	55 → 100	linear gradient
57 - 70	0	100	isocratic
70 - 90	62	38	isocratic

- as detector a spectrophotometer set at 225 nm,
- a 20 µl loop injector.

Calculate the percentage of total sesquiterpene lactones, expressed as dihydrohelenalin tiglate, from the expression:

$$\frac{S_{LS} \times C \times V \times 1.187 \times 100}{S_s \times m \times 1000}$$

- S_{LS} = area of all peaks corresponding to sesquiterpene lactones appearing after the santonin peak in the chromatogram obtained with the test solution,
- S_s = area of the peak corresponding to santonin in the chromatogram obtained with the test solution,

- m = mass of the drug to be examined, in grams,
- C = concentration of santonin in the internal standard solution used for the test solution, in milligrams per millilitre,
- V = volume of the internal standard solution used for the test solution, in millilitres,
- 1.187 = peak correlation factor between dihydrohelenalin tiglate and santonin.

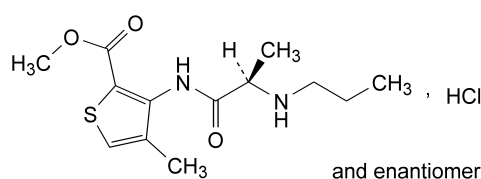
STORAGE

Store protected from light.

01/2005:1688

ARTICAINE HYDROCHLORIDE

Articaini hydrochloridum



$C_{13}H_{21}ClN_2O_3S$

M_r 320.8

DEFINITION

Methyl 4-methyl-3-[[[(2*RS*)-2-(propylamino)propanoyl]amino]thiophene-2-carboxylate hydrochloride.

Content: 98.5 per cent to 101.0 per cent (dried substance).

CHARACTERS

Appearance: white or almost white, crystalline powder.

Solubility: freely soluble in water and in alcohol.

IDENTIFICATION

First identification: B, D.

Second identification: A, C, D.

A. Dissolve 50.0 mg in a 1 g/l solution of *hydrochloric acid R* and dilute to 100.0 ml with the same acid. Dilute 5.0 ml of the solution to 100.0 ml with a 1 g/l solution of *hydrochloric acid R*. Examined between 200 nm and 350 nm (2.2.25), the solution shows an absorption maximum at 272 nm. The specific absorbance at the maximum is 290 to 320.

B. Infrared absorption spectrophotometry (2.2.24).

Preparation: place dropwise 20 µl of the test solution on 300 mg discs.

Test solution. Dissolve 0.1 g in 5 ml of *water R*, add 3 ml of a saturated solution of *sodium hydrogen carbonate R* and shake twice with 2 ml of *methylene chloride R*. Combine the methylene chloride layers, dilute to 5.0 ml with *methylene chloride R* and dry over *anhydrous sodium sulphate R*.

Comparison: *articaine hydrochloride CRS*.

C. Thin-layer chromatography (2.2.27).

Test solution. Dissolve 20 mg of the substance to be examined in 5 ml of *alcohol R*.

Reference solution. Dissolve 20 mg of *articaine hydrochloride CRS* in 5 ml of *alcohol R*.

Plate: TLC silica gel F_{254} plate *R*.

Mobile phase: *triethylamine R*, *ethyl acetate R*, *heptane R* (10:35:65 V/V/V).

Application: 5 µl.

Development: over a path of 15 cm.

Drying: in air.

Detection: examine in ultraviolet light at 254 nm.

Results: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with the reference solution.

D. It gives reaction (a) of chlorides (2.3.1).

TESTS

Solution S. Dissolve 0.50 g in *water R* and dilute to 10 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and not more intensely coloured than reference solution BY₆ (2.2.2, Method I).

pH (2.2.3): 4.2 to 5.2.

Dissolve 0.20 g in *carbon dioxide-free water R* and dilute to 20.0 ml with the same solvent.

Related substances. Liquid chromatography (2.2.29).

Test solution. Dissolve 10.0 mg of the substance to be examined in the mobile phase and dilute to 10.0 ml with the mobile phase.

Reference solution (a). Dilute 1.0 ml of the test solution to 100.0 ml with the mobile phase. Dilute 1.0 ml of this solution to 10.0 ml with the mobile phase.

Reference solution (b). Dissolve 10.0 mg of *articaine impurity A CRS* and 5.0 mg of *articaine impurity E CRS* in the mobile phase and dilute to 100.0 ml with the mobile phase.

Reference solution (c). Add 1.0 ml of reference solution (b) to 50.0 mg of *articaine hydrochloride CRS* and dilute to 50 ml with the mobile phase.

Reference solution (d). Dilute 1.0 ml of reference solution (b) to 50.0 ml with the mobile phase.

Column:

- *size:* $l = 0.25$ m, $\varnothing = 4.6$ mm,
- *stationary phase:* spherical *end-capped octadecylsilyl silica gel for chromatography R* (5 μ m) with a specific surface area of 335 m²/g and a carbon loading of 19 per cent,
- *temperature:* 45 °C.

Mobile phase: mix 25 volumes of *acetonitrile R* and 75 volumes of a solution prepared as follows: dissolve 2.02 g of *sodium heptanesulphonate R* and 4.08 g of *potassium dihydrogen phosphate R* in *water R* and dilute to 1000 ml with the same solvent. Adjust to pH 2.0 with *phosphoric acid R*.

Flow rate: 1 ml/min.

Detection: spectrophotometer at 276 nm.

Injection: 10 μ l; inject the test solution and reference solutions (a), (c) and (d).

Run time: 5 times the retention time of articaine.

Relative retentions with reference to articaine (retention time = about 9.3 min): impurity B = about 0.6; impurity D = about 0.7; impurity A = about 0.8; impurity E = about 0.86; impurity F = about 0.9;

impurity G = about 1.7; impurity H = about 2.1; impurity I = about 2.6; impurity C = about 3.6; impurity J = about 4.0.

System suitability: reference solution (c):

- *resolution:* minimum 1.2 between the peaks due to impurity A and impurity E.

Limits:

- *impurity A:* not more than the area of the corresponding peak in the chromatogram obtained with reference solution (d) (0.2 per cent),
- *any other impurity:* not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent),
- *total of other impurities:* not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent),
- *disregard limit:* half the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

Heavy metals (2.4.8): maximum 5 ppm.

Dissolve 4.0 g in 20.0 ml of *water R*. 12 ml of the solution complies with limit test A. Prepare the standard using *lead standard solution (1 ppm Pb) R*.

Loss on drying (2.2.32): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 100–105 °C for 5 h.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.250 g in a mixture of 5.0 ml of 0.01 M *hydrochloric acid* and 50 ml of *alcohol R*. Carry out a potentiometric titration (2.2.20) using 0.1 M *sodium hydroxide*. Read the volume added between the 2 points of inflexion.

1 ml of 0.1 M *sodium hydroxide* is equivalent to 32.08 mg of C₁₃H₂₁ClN₂O₃S.

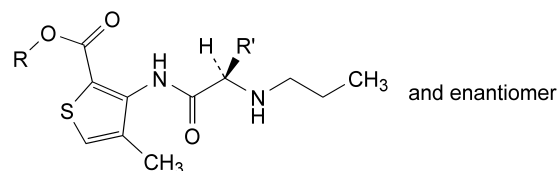
STORAGE

Protected from light.

IMPURITIES

Specified impurities: A, B, C.

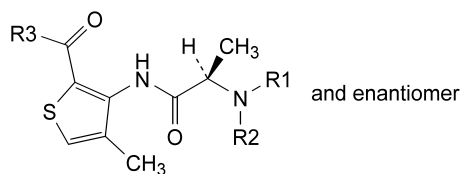
Other detectable impurities: D, E, F, G, H, I, J.



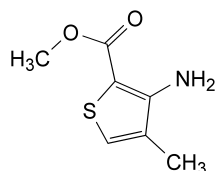
A. R = CH₃, R' = H: methyl 3-[[2-(propylamino)acetyl]amino]-4-methylthiophene-2-carboxylate (acetamidoarticaine),

B. R = H, R' = CH₃: 4-methyl-3-[[[(2RS)-2-(propylamino)propanoyl]amino]thiophene-2-carboxylic acid (articaine acid),

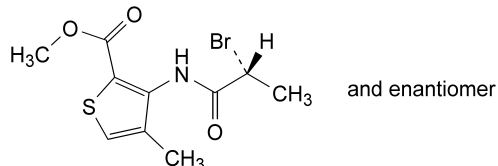
C. R = CH(CH₃)₂, R' = CH₃: 1-methylethyl 4-methyl-3-[[[(2RS)-2-(propylamino)propanoyl]amino]thiophene-2-carboxylate (articaine isopropyl ester),



- D. R1 = CH₂-CH₃, R2 = H, R3 = OCH₃: methyl 3-[[[(2*RS*)-2-(ethylamino)propanoyl]amino]-4-methylthiophene-2-carboxylate (ethylarticaïne),
- E. R1 = CH(CH₃)₂, R2 = H, R3 = OCH₃: methyl 4-methyl-3-[[[(2*RS*)-2-[(1-methylethyl)amino]propanoyl]amino]thiophene-2-carboxylate (isopropylarticaïne),
- F. R1 = CH₂-CH₂-CH₃, R2 = H, R3 = NH-CH₂-CH₂-CH₃: 4-methyl-*N*-propyl-3-[[[(2*RS*)-2-(propylamino)propanoyl]amino]thiophene-2-carboxamide (articaïne acid propionamide),
- G. R1 = (CH₂)₃-CH₃, R2 = H, R3 = OCH₃: methyl 3-[[[(2*RS*)-2-(butylamino)propanoyl]amino]-4-methylthiophene-2-carboxylate (butylarticaïne),
- H. R1 = R2 = CH₂-CH₂-CH₃, R3 = OCH₃: methyl 3-[[[(2*RS*)-2-(dipropylamino)propanoyl]amino]-4-methylthiophene-2-carboxylate (dipropylarticaïne),



- I. methyl 3-amino-4-methylthiophene-2-carboxylate (3-aminoarticaïne),

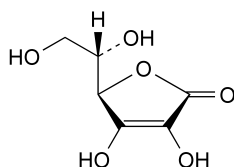


- J. methyl 3-[[[(2*RS*)-2-bromopropanoyl]amino]-4-methylthiophene-2-carboxylate (bromo compound).

01/2005:0253

ASCORBIC ACID

Acidum ascorbicum

C₆H₈O₆M_r 176.1

DEFINITION

Ascorbic acid contains not less than 99.0 per cent and not more than the equivalent of 100.5 per cent of (5*R*)-5-[(1*S*)-1,2-dihydroxyethyl]-3,4-dihydroxyfuran-2(5*H*)-one.

CHARACTERS

A white or almost white, crystalline powder or colourless crystals, becoming discoloured on exposure to air and moisture, freely soluble in water, soluble in alcohol.

It melts at about 190 °C, with decomposition.

IDENTIFICATION

First identification: B, C.

Second identification: A, C, D.

- A. Dissolve 0.10 g in *water R* and dilute immediately to 100.0 ml with the same solvent. To 10 ml of 0.1 *M hydrochloric acid*, add 1.0 ml of the solution and dilute to 100.0 ml with *water R*. Measure the absorbance (2.2.25) at the maximum at 243 nm immediately after dissolution. The specific absorbance at the maximum is 545 to 585.
- B. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *ascorbic acid CRS*. Examine the substance prepared as discs containing 1 mg.
- C. The pH (2.2.3) of solution S (see Tests) is 2.1 to 2.6.
- D. To 1 ml of solution S add 0.2 ml of *dilute nitric acid R* and 0.2 ml of *silver nitrate solution R2*. A grey precipitate is formed.

TESTS

Solution S. Dissolve 1.0 g in *carbon dioxide-free water R* and dilute to 20 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and not more intensely coloured than reference solution BY₇ (2.2.2, *Method II*).

Specific optical rotation (2.2.7). Dissolve 2.50 g in *water R* and dilute to 25.0 ml with the same solvent. The specific optical rotation is + 20.5 to + 21.5.

Oxalic acid. Dissolve 0.25 g in 5 ml of *water R*. Neutralise to *red litmus paper R* using *dilute sodium hydroxide solution R* and add 1 ml of *dilute acetic acid R* and 0.5 ml of *calcium chloride solution R* (test solution). Prepare a reference solution as follows: dissolve 70 mg of *oxalic acid R* in *water R* and dilute to 500 ml with the same solvent; to 5 ml of this solution add 1 ml of *dilute acetic acid R* and 0.5 ml of *calcium chloride solution R* (reference solution). Allow the solutions to stand for 1 h. Any opalescence in the test solution is not more intense than that in the reference solution (0.2 per cent).

Copper. Not more than 5 ppm of Cu, determined by atomic absorption spectrometry (2.2.23, *Method I*).

Test solution. Dissolve 2.0 g of the substance to be examined in 0.1 *M nitric acid* and dilute to 25.0 ml with the same acid.

Reference solutions. Prepare reference solutions containing 0.2 ppm, 0.4 ppm and 0.6 ppm of Cu by diluting *copper standard solution* (10 ppm Cu) *R* with 0.1 *M nitric acid*.

Measure the absorbance at 324.8 nm using a copper hollow-cathode lamp as a source of radiation and an air-acetylene flame. Adjust the zero of the apparatus using 0.1 *M nitric acid*.

Iron. Not more than 2 ppm of Fe, determined by atomic absorption spectrometry (2.2.23, *Method I*).

Test solution. Dissolve 5.0 g of the substance to be examined in 0.1 *M nitric acid* and dilute to 25.0 ml with the same acid.

Reference solutions. Prepare reference solutions containing 0.2 ppm, 0.4 ppm and 0.6 ppm of Fe by diluting *iron standard solution* (20 ppm Fe) *R* with 0.1 *M nitric acid*.

Measure the absorbance at 248.3 nm using an iron hollow-cathode lamp as a source of radiation and an air-acetylene flame. Adjust the zero of the apparatus using 0.1 *M nitric acid*.

Heavy metals (2.4.8). Dissolve 2.0 g in *water R* and dilute to 20 ml with the same solvent. 12 ml of the solution complies with limit test A for heavy metals (10 ppm). Prepare the standard using *lead standard solution* (1 ppm Pb) *R*.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.150 g in a mixture of 10 ml of *dilute sulphuric acid R* and 80 ml of *carbon dioxide-free water R*. Add 1 ml of *starch solution R*. Titrate with 0.05 M *iodine* until a persistent violet-blue colour is obtained.

1 ml of 0.05 M *iodine* is equivalent to 8.81 mg of $C_{6}H_8O_6$.

STORAGE

Store in a non-metallic container, protected from light.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.160 g in 50 ml of *methanol R*. Add 30 ml of *water R* and 1 ml of *starch solution R*. Titrate with 0.05 M *iodine* until a persistent violet-blue colour is obtained.

1 ml of 0.05 M *iodine* is equivalent to 20.73 mg of $C_{22}H_{38}O_7$.

STORAGE

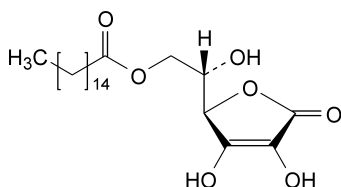
Store in an airtight container, protected from light, at a temperature of 8 °C to 15 °C.

01/2005:1600

01/2005:0807

ASCORBYL PALMITATE

Ascorbylis palmitas



$C_{22}H_{38}O_7$

M_r 414.5

DEFINITION

Ascorbyl palmitate contains not less than 98.0 per cent and not more than the equivalent of 100.5 per cent of (2S)-2-[(5R)-3,4-dihydroxy-5-oxo-2,5-dihydrofuran-2-yl]-2-hydroxyethyl hexadecanoate, calculated with reference to the dried substance.

CHARACTERS

A white or yellowish-white powder, practically insoluble in water, freely soluble in alcohol and in methanol, practically insoluble in methylene chloride and in fatty oils.

IDENTIFICATION

- It complies with the test for specific optical rotation (see Tests).
- Examine by infrared absorption spectrophotometry (2.2.24), comparing with the *Ph. Eur. reference spectrum of ascorbyl palmitate*.
- Dissolve about 10 mg in 5 ml of *methanol R*. The solution decolourises *dichlorophenolindophenol standard solution R*.

TESTS

Solution S. Dissolve 2.50 g in *methanol R* and dilute to 25.0 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and not more intensely coloured than reference solution BY₄ (2.2.2, *Method I*).

Specific optical rotation (2.2.7): + 21 to + 24, determined on solution S and calculated with reference to the dried substance.

Heavy metals (2.4.8). 2.0 g complies with limit test C for heavy metals (10 ppm). Prepare the standard using 2 ml of *lead standard solution (10 ppm Pb) R*.

Loss on drying (2.2.32). Not more than 1.0 per cent, determined on 1.000 g by drying *in vacuo* at 60 °C for 5 h.

ASH LEAF

Fraxini folium

DEFINITION

Dried leaf of *Fraxinus excelsior* L. or *Fraxinus oxyphylla* M. Bieb.

Content: minimum 2.5 per cent of total hydroxycinnamic acid derivatives expressed as chlorogenic acid ($C_{16}H_{18}O_9$; M_r 354.3) (dried drug).

CHARACTERS

Macroscopic and microscopic characters described under identification tests A and B.

IDENTIFICATION

- The leaf consists of leaflets which are sometimes detached and separated from the rachis. The leaflet is about 6 cm long and 3 cm wide. Each leaflet is subsessile or shortly petiolate, oblong, lanceolate, somewhat unequal at the base, acuminate at the apex, with fine acute teeth on the margins, the upper surface is dark green and the lower surface is greyish-green. The midrib and secondary veins are whitish and prominent on the lower surface.
- Reduce to a powder (355). The powder is greyish-green. Examined under a microscope, using *chloral hydrate solution R*. The powder shows fragments of the lamina in surface view, with the lower epidermis showing numerous anomocytic stomata (2.8.3.) and some of the cells of the upper epidermis with cuticular striations; occasional uniseriate, conical covering trichomes composed of 1 or 2 cells with thick walls and a striated cuticle; rare peltate glands with a unicellular stalk and a shield-like glandular head composed of 8 radiating cells; groups of fibres and fragments of vascular tissue from the veins.
- Examine the chromatograms obtained in the test for *Fraxinus ornus* L.

Results: see below the sequence of the zones present in the chromatograms obtained with the reference and test solutions. Furthermore, other fluorescent zones are present in the chromatogram obtained with the test solution.

Top of the plate	
	A light blue fluorescence zone
	An intense blue fluorescent zone (acteoside)
Chlorogenic acid: a blue fluorescent zone	A blue fluorescent zone (chlorogenic acid)
Rutin: an orange fluorescent zone	An orange fluorescent zone (rutin)
Reference solution	Test solution

TESTS

01/2005:2086

Foreign matter (2.8.2): maximum 3.0 per cent of stems and 2.0 per cent of other foreign matter.

Fraxinus ornus L. Thin-layer chromatography (2.2.27).

Test solution. To 1 g of the powdered drug (355) add 20 ml of *methanol R*. Heat with shaking at 40 °C for 10 min. Filter.

Reference solution. Dissolve 5.0 mg of *rutin R* and 5.0 mg of *chlorogenic acid R* in 10 ml of *methanol R*.

Plate: TLC silica gel plate *R*.

Mobile phase: *anhydrous formic acid R*, *water R*, *ethyl acetate R* (10:10:80 V/V/V).

Application: 10 µl as bands.

Development: over a path of 10 cm.

Drying: in air.

Detection: spray with a solution containing 10 g/l of *diphenylboric acid aminoethyl ester R* and 50 g/l of *macrogol 400 R* in *methanol R*. Examine in ultraviolet light at 365 nm.

Results: in the case of a substitution by *F. ornus*, the chromatogram obtained with the test solution does not show the zones of acteoside, chlorogenic acid and rutin.

Loss on drying (2.2.32): maximum 10.0 per cent, determined on 1.000 g of the powdered drug (355) by drying in an oven at 100–105 °C for 2 h.

Total ash (2.4.16): maximum 12.0 per cent.

ASSAY

Test solution (a). To 0.300 g of the powdered drug (355) add 95 ml of *alcohol (50 per cent V/V) R*. Boil in a water-bath under a reflux condenser for 30 min. Allow to cool and filter. Rinse the filter with 5 ml of *alcohol (50 per cent V/V) R*. Combine the filtrate and the rinsings in a volumetric flask and dilute to 100.0 ml with *alcohol (50 per cent V/V) R*.

Test solution (b). To 1.0 ml of test solution (a) in a test tube, add 2 ml of 0.5 M *hydrochloric acid*, 2 ml of a solution prepared by dissolving 10 g of *sodium nitrite R* and 10 g of *sodium molybdate R* in 100 ml of *water R*, then add 2 ml of *dilute sodium hydroxide solution R* and dilute to 10.0 ml with *water R*; mix.

Immediately measure the absorbance (2.2.25) of test solution (b) at 525 nm, using as compensation liquid a solution prepared as follows: mix 1.0 ml of test solution (a), 2 ml of 0.5 M *hydrochloric acid* and 2 ml of *dilute sodium hydroxide solution R* and dilute to 10.0 ml with *water R*.

Calculate the percentage content of total hydroxycinnamic acid derivatives expressed as chlorogenic acid, from the expression:

$$\frac{A \times 5.3}{m}$$

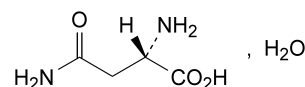
taking the specific absorbance of chlorogenic acid at 525 nm to be 188.

A = absorbance at 525 nm,

m = mass of the test sample in grams.

ASPARAGINE MONOHYDRATE

Asparaginum monohydricum

C₄H₈N₂O₃·H₂O*M_r* 150.1

DEFINITION

(2*S*)-2,4-Diamino-4-oxobutanoic acid monohydrate.

Content: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

Appearance: white, crystalline powder or colourless crystals.

Solubility: slightly soluble in water, practically insoluble in alcohol and in methylene chloride.

IDENTIFICATION

First identification: A, B.

Second identification: A, C.

A. It complies with the test for specific optical rotation (see Tests).

B. Infrared absorption spectrophotometry (2.2.24).

Comparison: *asparagine monohydrate CRS*.

C. Examine the chromatograms obtained in the test for ninhydrin-positive substances.

Results: the principal spot in the chromatogram obtained with test solution (b) is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (c).

TESTS

Solution S. Dissolve with heating 2.0 g in *carbon dioxide-free water R* and dilute to 100 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and colourless (2.2.2, *Method II*).

pH (2.2.3): 4.0 to 6.0 for solution S.

Specific optical rotation (2.2.7): + 33.7 to + 36.0 (dried substance).

Dissolve 2.50 g in a 309.0 g/l solution of *hydrochloric acid R* and dilute to 25.0 ml with the same acid.

Ninhydrin-positive substances. Thin-layer chromatography (2.2.27).

Test solution (a). Dissolve 0.25 g of the substance to be examined in *water R*, heating to not more than 40 °C, and dilute to 10 ml with the same solvent.

Test solution (b). Dilute 1 ml of test solution (a) to 10 ml with *water R*.

Reference solution (a). Dilute 1.0 ml of test solution (a) to 200 ml with *water R*.

Reference solution (b). Dissolve 25 mg of *glutamic acid R* in *water R*, add 1 ml of test solution (a) and dilute to 10 ml with *water R*.

Reference solution (c). Dissolve 25 mg of *asparagine monohydrate CRS* in *water R* and dilute to 10 ml with the same solvent.

Plate: TLC silica gel *G* plate *R*.

Mobile phase: *glacial acetic acid R*, *water R*, *butanol R* (25:25:50 V/V/V).

Application: 5 µl.

Development: over half of the plate.

Drying: at 110 °C for 15 min.

Detection: spray with *ninhydrin solution R* and heat at 110 °C for 10 min.

System suitability: reference solution (b):

- the chromatogram shows 2 clearly separated principal spots.

Limit: test solution (a):

- *any impurity:* any spot, apart from the principal spot, is not more intense than the principal spot in the chromatogram obtained with reference solution (a) (0.5 per cent).

Chlorides (2.4.4): maximum 200 ppm.

Dilute 12.5 ml of solution S to 15 ml with *water R*.

Sulphates (2.4.13): maximum 200 ppm.

To 0.75 g add 2.5 ml of *dilute hydrochloric acid R* and dilute to 15 ml with *distilled water R*. Examine after 30 min.

Ammonium (2.4.1, *Method B*): maximum 0.1 per cent, determined on 10 mg.

Iron (2.4.9): maximum 10 ppm.

Dissolve 1.0 g in *dilute hydrochloric acid R* and dilute to 10 ml with the same acid. Shake 3 times with 10 ml of *methyl isobutyl ketone R1* for 3 min. Wash the combined organic phases with 10 ml of *water R* for 3 min. The aqueous phase complies with the limit test for iron.

Heavy metals (2.4.8): maximum 10 ppm.

Dissolve 2.0 g in a mixture of 3 ml of *dilute hydrochloric acid R* and 15 ml of *water R* with gentle warming if necessary. Dilute to 20 ml with *water R*. 12 ml of the solution complies with limit test A. Prepare the standard using *lead standard solution (1 ppm Pb) R*.

Loss on drying (2.2.32): 10.5 per cent to 12.5 per cent, determined on 1.000 g by drying in an oven at 60 °C for 24 h.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.110 g in 5 ml of *anhydrous formic acid R*. Add 50 ml of *anhydrous acetic acid R*. Titrate with 0.1 M *perchloric acid*, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *perchloric acid* is equivalent to 13.21 mg of C₁₄H₁₈N₂O₅.

IMPURITIES

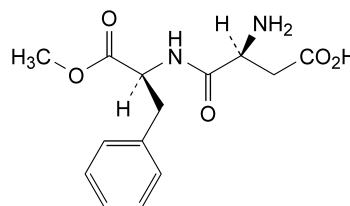
Specified impurities: A, B.

A. aspartic acid,

B. glutamic acid.

ASPARTAME

Aspartamum



C₁₄H₁₈N₂O₅

M_r 294.3

DEFINITION

Aspartame contains not less than 98.0 per cent and not more than the equivalent of 102.0 per cent of (3*S*)-3-amino-4-[[[(1*S*)-1-benzyl-2-methoxy-2-oxoethyl]amino]-4-oxobutanoic acid, calculated with reference to the dried substance.

CHARACTERS

A white, crystalline powder, slightly hygroscopic, sparingly soluble or slightly soluble in water and in alcohol, practically insoluble in hexane and in methylene chloride.

IDENTIFICATION

First identification: B.

Second identification: A, C, D.

A. Dissolve 0.1 g in *alcohol R* and dilute to 100 ml with the same solvent. Examined between 230 nm and 300 nm (2.2.25), the solution shows absorption maxima at 247 nm, 252 nm, 258 nm and 264 nm.

B. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *aspartame CRS*. Examine the substances prepared as discs.

C. Examine by thin-layer chromatography (2.2.27), using *silica gel G R* as the coating substance.

Test solution. Dissolve 15 mg of the substance to be examined in 2.5 ml of *water R* and dilute to 10 ml with *acetic acid R*.

Reference solution. Dissolve 15 mg of *aspartame CRS* in 2.5 ml of *water R* and dilute to 10 ml with *acetic acid R*.

Apply to the plate 20 µl of each solution. Develop over a path of 15 cm using a mixture of 2 volumes of *water R*, 4 volumes of *anhydrous formic acid R*, 30 volumes of *methanol R* and 64 volumes of *methylene chloride R*. Allow the plate to dry in air. Spray with *ninhydrin solution R* and heat at 100 °C to 105 °C for 15 min. The spot in the chromatogram obtained with the test solution is similar in position, colour and size to the spot in the chromatogram obtained with the reference solution.

D. Dissolve about 20 mg in 5 ml of *methanol R* and add 1 ml of *alkaline hydroxylamine solution R1*. Heat on a water-bath for 15 min. Allow to cool and adjust to about pH 2 with *dilute hydrochloric acid R*. Add 0.1 ml of *ferric chloride solution R1*. A brownish-red colour is produced.

TESTS

Solution S. Dissolve 0.8 g in *carbon dioxide-free water R* and dilute to 100 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and not more intensely coloured than reference solution GY₆ (2.2.2, *Method II*).

Conductivity (2.2.38). Not more than $30 \mu\text{S cm}^{-1}$. Dissolve 0.80 g in *carbon dioxide-free water R* prepared from *distilled water R* and dilute to 100.0 ml with the same solvent. Measure the conductivity of the solution (C_1) and that of the water used for preparing the solution (C_2). The readings must be stable within 1 per cent over a period of 30 s. Calculate the conductivity of the solution of the substance to be examined from the expression:

$$C_1 - 0.992 C_2$$

Specific optical rotation (2.2.7). Dissolve 2.00 g in a 690 g/l solution of *anhydrous formic acid R* and dilute to 50.0 ml with the same solution. The specific optical rotation is + 14.5 to + 16.5, measured within 30 min of preparation of the solution and calculated with reference to the dried substance.

Related substances. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 0.60 g of the substance to be examined in a mixture of 1.5 volumes of *glacial acetic acid R* and 98.5 volumes of *water R* and dilute to 100.0 ml with the same mixture of solvents.

Reference solution (a). Dissolve 4.5 mg of *aspartame impurity A CRS* in a mixture of 1.5 volumes of *glacial acetic acid R* and 98.5 volumes of *water R* and dilute to 50.0 ml with the same mixture of solvents.

Reference solution (b). Dissolve 30.0 mg of *phenylalanine R* in a mixture of 15 volumes of *glacial acetic acid R* and 85 volumes of *water R* and dilute to 100.0 ml with the same mixture of solvents. Dilute 1.0 ml of the solution to 10.0 ml with *water R*.

Reference solution (c). Dilute 5.0 ml of the test solution to 10.0 ml with *water R*. Dilute 3.0 ml of the solution to 100.0 ml with *water R*.

Reference solution (d). Dissolve 30.0 mg of *L-aspartyl-L-phenylalanine R* in a mixture of 15 volumes of *glacial acetic acid R* and 85 volumes of *water R* and dilute to 100.0 ml with the same mixture of solvents. Dilute 1.0 ml of the solution to 10.0 ml with *water R*. Mix 1.0 ml of this solution with 1.0 ml of reference solution (b).

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4.0 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* ($5 \mu\text{m}$ to $10 \mu\text{m}$),
- as mobile phase at a flow rate of 1 ml/min a mixture of 10 volumes of *acetonitrile R* and 90 volumes of a 6.8 g/l solution of *potassium dihydrogen phosphate R* previously adjusted to pH 3.7 with *phosphoric acid R*,
- as detector a spectrophotometer set at 220 nm.

Inject 20 μl of reference solution (c). Adjust the sensitivity of the system so that the height of the principal peak in the chromatogram obtained is not less than 50 per cent of the full scale of the recorder. Inject 20 μl of reference solution (d). The test is not valid unless in the chromatogram obtained, the resolution between the peaks due to phenylalanine and *L-aspartyl-L-phenylalanine* is at least 3.5. Inject separately 20 μl of each solution. Continue the chromatography for twice the retention time of aspartame. In the chromatogram obtained with the test solution: the area of any peak corresponding to aspartame impurity A is not greater than the area of the principal peak in the chromatogram obtained with reference solution (a) (1.5 per cent); the area of any peak corresponding to phenylalanine is not greater than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent); the sum of the areas of any other peaks, apart from the principal peak,

is not greater than the area of the principal peak in the chromatogram obtained with reference solution (c) (1.5 per cent). Disregard any peak due to the solvent.

Heavy metals (2.4.8). 1.0 g complies with limit test C for heavy metals (10 ppm). Prepare the standard using 1 ml of *lead standard solution (10 ppm Pb) R*.

Loss on drying (2.2.32). Not more than 4.5 per cent, determined on 1.000 g by drying in an oven at 100°C to 105°C .

Sulphated ash (2.4.14). Not more than 0.2 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.250 g in 1.5 ml of *anhydrous formic acid R* and 60 ml of *anhydrous acetic acid R*. Titrate immediately with 0.1 M *perchloric acid*, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *perchloric acid* is equivalent to 29.43 mg of $\text{C}_{14}\text{H}_{18}\text{N}_2\text{O}_5$.

STORAGE

Store in an airtight container.

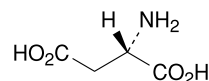
IMPURITIES

- 2-(5-benzyl-3,6-dioxopiperazin-2-yl)acetic acid (diketopiperazine),
- L*-aspartyl-*L*-phenylalanine,
- phenylalanine.

01/2005:0797

ASPARTIC ACID

Acidum asparticum



$\text{C}_4\text{H}_7\text{NO}_4$

M_r 133.1

DEFINITION

Aspartic acid contains not less than 98.5 per cent and not more than the equivalent of 101.5 per cent of (2*S*)-2-aminobutanedioic acid, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, crystalline powder or colourless crystals, slightly soluble in water, practically insoluble in alcohol. It dissolves in dilute mineral acids and in dilute solutions of alkali hydroxides.

IDENTIFICATION

First identification: A, C.

Second identification: A, B, D.

- It complies with the test for specific optical rotation (see Tests).
- A suspension of 1 g in 10 ml of *water R* is strongly acid (2.2.4).
- Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *aspartic acid CRS*. Examine the substances prepared as discs.

D. Examine the chromatograms obtained in the test for ninhydrin-positive substances. The principal spot in the chromatogram obtained with test solution (b) is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).

TESTS

Appearance of solution. Dissolve 0.5 g in 1 M hydrochloric acid and dilute to 10 ml with the same acid. The solution is clear (2.2.1) and not more intensely coloured than reference solution BY₆ (2.2.2, Method II).

Specific optical rotation (2.2.7). Dissolve 2.000 g in hydrochloric acid R1 and dilute to 25.0 ml with the same acid. The specific optical rotation is + 24.0 to + 26.0, calculated with reference to the dried substance.

Ninhydrin-positive substances. Examine by thin-layer chromatography (2.2.27), using a TLC silica gel plate R.

Test solution (a). Dissolve 0.10 g of the substance to be examined in 2 ml of ammonia R and dilute to 10 ml with water R.

Test solution (b). Dilute 1 ml of test solution (a) to 50 ml with water R.

Reference solution (a). Dissolve 10 mg of aspartic acid CRS in 2 ml of dilute ammonia R1 and dilute to 50 ml with water R.

Reference solution (b). Dilute 5 ml of test solution (b) to 20 ml with water R.

Reference solution (c). Dissolve 10 mg of aspartic acid CRS and 10 mg of glutamic acid CRS in 2 ml of dilute ammonia R1 and dilute to 25 ml with water R.

Apply separately to the plate 5 µl of each solution. Allow the plate to dry in air. Develop over a path of 15 cm using a mixture of 20 volumes of glacial acetic acid R, 20 volumes of water R and 60 volumes of butanol R. Allow the plate to dry in air, spray with ninhydrin solution R. Heat at 100-105 °C for 15 min. Any spot in the chromatogram obtained with test solution (a), apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.5 per cent). The test is not valid unless the chromatogram obtained with reference solution (c) shows 2 clearly separated principal spots.

Chlorides (2.4.4). Dissolve 0.25 g in 3 ml of dilute nitric acid R and dilute to 15 ml with water R. The solution, to which 1 ml of water R is added instead of dilute nitric acid R, complies with the limit test for chlorides (200 ppm).

Sulphates (2.4.13). Dissolve 0.5 g in 4 ml of hydrochloric acid R and dilute to 15 ml with distilled water R. The solution complies with the limit test for sulphates (300 ppm). Carry out the evaluation of the test after 30 min.

Ammonium. (2.4.1) 50 mg complies with limit test B (200 ppm). Prepare the standard using 0.1 ml of ammonium standard solution (100 ppm NH₄) R.

Iron (2.4.9). In a separating funnel, dissolve 1.0 g in 10 ml of dilute hydrochloric acid R. Shake with 3 quantities, each of 10 ml, of methyl isobutyl ketone R1, shaking for 3 min each time. To the combined organic layers add 10 ml of water R and shake for 3 min. The aqueous layer complies with the limit test for iron (10 ppm).

Heavy metals (2.4.8). 2.0 g complies with limit test D (10 ppm). Prepare the standard using 2 ml of lead standard solution (10 ppm Pb) R.

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 100-105 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.100 g in 50 ml of carbon dioxide-free water R, with slight heating if necessary. Cool and add 0.1 ml of bromothymol blue solution R1. Titrate with 0.1 M sodium hydroxide until the colour changes from yellow to blue.

1 ml of 0.1 M sodium hydroxide is equivalent to 13.31 mg of C₂₈H₃₁FN₄O.

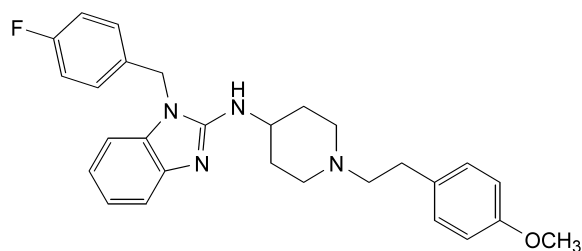
STORAGE

Protected from light.

01/2005:1067

ASTEMIZOLE

Astemizolum



C₂₈H₃₁FN₄O

M_r 458.6

DEFINITION

Astemizole contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of 1-(4-fluorobenzyl)-N-[1-[2-(4-methoxyphenyl)ethyl]piperidin-4-yl]-1H-benzimidazol-2-amine, calculated with reference to the dried substance.

CHARACTERS

A white or almost white powder, practically insoluble in water, freely soluble in methylene chloride and in methanol, soluble in alcohol.

IDENTIFICATION

First identification: A, B.

Second identification: A, C, D.

A. Melting point (2.2.14): 175 °C to 178 °C.

B. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with astemizole CRS. Examine the substances prepared as discs.

C. Examine by thin layer chromatography (2.2.27), using a suitable octadecylsilyl silica gel as the coating substance. **Test solution.** Dissolve 30 mg of the substance to be examined in methanol R and dilute to 5 ml with the same solvent.

Reference solution (a). Dissolve 30 mg of astemizole CRS in methanol R and dilute to 5 ml with the same solvent.

Reference solution (b). Dissolve 30 mg of astemizole CRS and 30 mg of ketoconazole CRS in methanol R and dilute to 5 ml with the same solvent.

Apply separately to the plate 5 µl of each solution. Develop over a path of 15 cm using a mixture of 20 volumes of ammonium acetate solution R, 40 volumes of dioxan R and 40 volumes of methanol R. Dry the plate in a current of warm air for 15 min and expose it to iodine vapour until the spots appear. Examine in daylight. The principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the

chromatogram obtained with reference solution (a). The test is not valid unless the chromatogram obtained with reference solution (b) shows two clearly separated spots.

- D. Mix about 5 mg with 45 mg of *heavy magnesium oxide R* and ignite in a crucible until an almost white residue is obtained (usually less than 5 min). Allow to cool, add 1 ml of *water R*, 0.05 ml of *phenolphthalein solution R1* and about 1 ml of *dilute hydrochloric acid R* to render the solution colourless. Filter. To a freshly prepared mixture of 0.1 ml of *alizarin S solution R* and 0.1 ml of *zirconyl nitrate solution R*, add 1 ml of the filtrate. Mix, allow to stand for 5 min and compare the colour of the solution with that of a blank prepared in the same manner. The test solution is yellow and the blank is red.

TESTS

Appearance of solution. Dissolve 0.2 g in *methanol R* and dilute to 20 ml with the same solvent. The solution is clear (2.2.1) and is not more intensely coloured than reference solution Y₇ (2.2.2, *Method II*).

Related substances. Examine by liquid chromatography (2.2.29). Prepare the solutions immediately before use.

Test solution. Dissolve 0.100 g of the substance to be examined in *methanol R* and dilute to 10.0 ml with the same solvent.

Reference solution (a). Dissolve 2.5 mg of *astemizole CRS* and 25 mg of *ketoconazole CRS* in *methanol R* and dilute to 100.0 ml with the same solvent.

Reference solution (b). Dilute 1.0 ml of the test solution to 100.0 ml with *methanol R*. Dilute 5.0 ml of this solution to 20.0 ml with *methanol R*.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.1 m long and 4.6 mm in internal diameter packed with *base-deactivated octadecylsilyl silica gel for chromatography R* (3 µm),
- as mobile phase at a flow rate of 1.5 ml/min, a linear gradient programme as follows:

Mobile phase A. A 17 g/l solution of *tetrabutylammonium hydrogen sulphate R*,

Mobile phase B. *Acetonitrile R*,

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)	Comment
0	95	5	start gradient
15	80	20	end gradient
18	0	100	purge time
23	95	5	switch to initial equilibration
28 = 0	95	5	start of next chromatogram

- as detector a spectrophotometer set at 278 nm.

Equilibrate the column for at least 30 min with *acetonitrile R* and then equilibrate the initial elution composition for at least 5 min.

Adjust the sensitivity of the system so that the height of the principal peak in the chromatogram obtained with 10 µl of reference solution (b) is at least 50 per cent of the full scale of the recorder.

Inject 10 µl of reference solution (a). When the chromatograms are recorded in the prescribed conditions the retention times are: *ketoconazole*, about 8 min and *astemizole*, about 9 min. The test is not valid unless the resolution between the peaks corresponding to *ketoconazole* and *astemizole* is at least 1.5. If necessary, adjust the

final concentration of acetonitrile or the percentage of tetrabutylammonium hydrogen sulphate in the mobile phase or adjust the time programme for the linear gradient elution.

Inject separately 10 µl of *methanol R* as a blank, 10 µl of the test solution and 10 µl of reference solution (b). In the chromatogram obtained with the test solution: the area of any peak, apart from the principal peak, is not greater than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.25 per cent); the sum of the areas of all the peaks, apart from the principal peak, is not greater than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent). Disregard any peak obtained with the blank and any peak with an area less than 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (b).

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

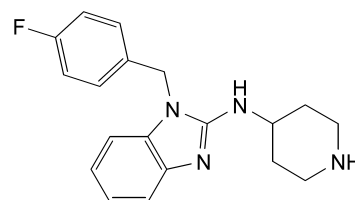
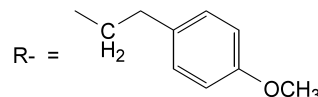
Dissolve 0.200 g in 50 ml of a mixture of 1 volume of *anhydrous acetic acid R* and 7 volumes of *methyl ethyl ketone R*. Titrate with 0.1 M *perchloric acid*, using 0.2 ml of *naphtholbenzein solution R* as indicator.

1 ml of 0.1 M *perchloric acid* is equivalent to 22.93 mg of C₂₈H₃₁N₄O.

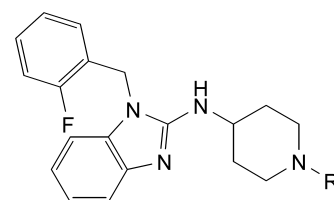
STORAGE

Store protected from light.

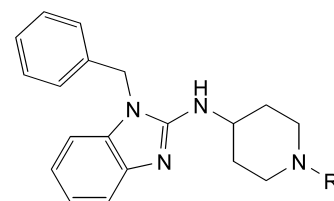
IMPURITIES



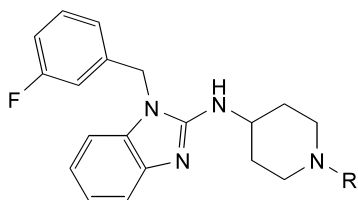
A. 1-(4-fluorobenzyl)-N-(piperidin-4-yl)-1H-benzimidazol-2-amine,



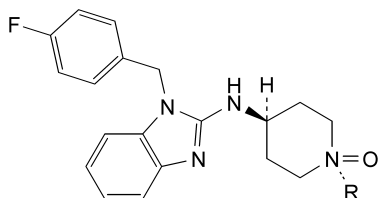
B. 1-(2-fluorobenzyl)-N-[1-[2-(4-methoxyphenyl)ethyl]piperidin-4-yl]-1H-benzimidazol-2-amine,



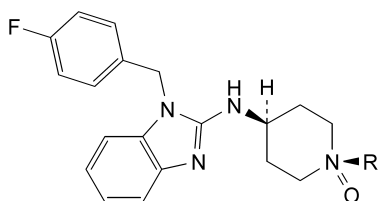
C. 1-benzyl-N-[1-[2-(4-methoxyphenyl)ethyl]piperidin-4-yl]-1H-benzimidazol-2-amine,



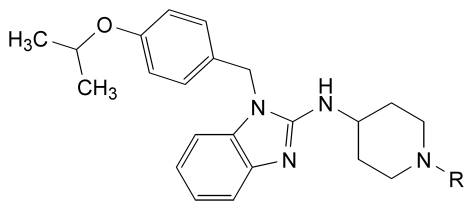
- D. 1-(3-fluorobenzyl)-N-[1-[2-(4-methoxyphenyl)ethyl]piperidin-4-yl]-1H-benzimidazol-2-amine,



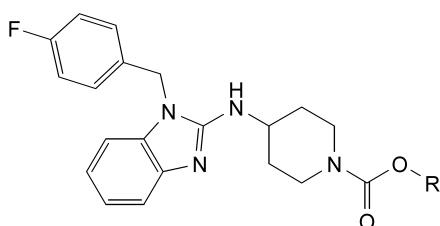
- E. 1-(4-fluorobenzyl)-N-[cis-1-[2-(4-methoxyphenyl)ethyl]piperidin-4-yl 1-oxide]-1H-benzimidazol-2-amine,



- F. 1-(4-fluorobenzyl)-N-[trans-1-[2-(4-methoxyphenyl)ethyl]piperidin-4-yl 1-oxide]-1H-benzimidazol-2-amine,



- G. N-[1-[2-(4-methoxyphenyl)ethyl]piperidin-4-yl]-1-[4-(1-methylethoxy)benzyl]-1H-benzimidazol-2-amine,

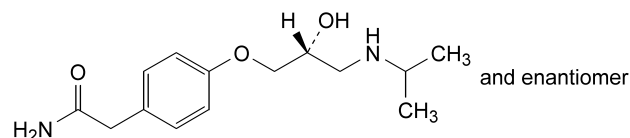


- H. 2-(4-methoxyphenyl)ethyl 4-[[1-(4-fluorobenzyl)-1H-benzimidazol-2-yl]amino]piperidin-1-carboxylate.

01/2005:0703

ATENOLOL

Atenololum



C₁₄H₂₂N₂O₃

M_r 266.3

DEFINITION

Atenolol contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of 2-[4-[(2*RS*)-2-hydroxy-3-[(1-methylethyl)amino]propoxy]phenyl]acetamide, calculated with reference to the dried substance.

CHARACTERS

A white or almost white powder, sparingly soluble in water, soluble in ethanol, slightly soluble in methylene chloride.

IDENTIFICATION

First identification: C.

Second identification: A, B, D.

A. Melting point (2.2.14): 152 °C to 155 °C.

B. Dissolve 0.100 g in *methanol R* and dilute to 100 ml with the same solvent. Dilute 10.0 ml of this solution to 100 ml with *methanol R*. Examined between 230 nm and 350 nm (2.2.25), the solution shows two absorption maxima, at 275 nm and 282 nm. The ratio of the absorbance measured at the maximum at 275 nm to that measured at the maximum at 282 nm is 1.15 to 1.20.

C. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *atenolol CRS*.

D. Examine by thin-layer chromatography (2.2.27), using *silica gel GF₂₅₄ R* as the coating substance.

Test solution. Dissolve 10 mg of the substance to be examined in 1 ml of *methanol R*.

Reference solution. Dissolve 10 mg of *atenolol CRS* in 1 ml of *methanol R*.

Apply separately to the plate 10 µl of each solution. Develop over a path of 15 cm using a mixture of 1 volume of *concentrated ammonia R1* and 99 volumes of *methanol R*. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. The principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with the reference solution.

TESTS

Solution S. Dissolve 0.10 g in *water R* and dilute to 10 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and not more intensely coloured than degree 6 of the range of reference solutions of the most appropriate colour (2.2.2, *Method II*).

Optical rotation (2.2.7). Determined on solution S, the angle of optical rotation is + 0.10° to − 0.10°.

Related substances. Examine by liquid chromatography (2.2.29).

Test solution (a). Dissolve 50.0 mg of the substance to be examined in 20 ml of the mobile phase and dilute to 25.0 ml with the mobile phase.

Test solution (b). Dissolve 50.0 mg of the substance to be examined in 0.1 ml of *dimethyl sulfoxide R*, if necessary applying gentle heat by placing the containing vessel in a water-bath for a few seconds, and dilute to 25.0 ml with the mobile phase.

Reference solution (a). Dilute 0.5 ml of test solution (a) to 100.0 ml with the mobile phase.

Reference solution (b). Dissolve 50.0 mg of *atenolol for column validation CRS* in 0.1 ml of *dimethyl sulfoxide R*, if necessary applying gentle heat by placing the containing vessel in a water-bath for a few seconds, and dilute to 25.0 ml with the mobile phase.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.15 m long and 4.6 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (5 µm),
- as mobile phase at a flow rate of 1.0 ml/min a mixture prepared as follows: dissolve 1.0 g of *sodium octanesulphonate R* and 0.4 g of *tetrabutylammonium hydrogen sulphate R* in 1 litre of a mixture of 20 volumes of *tetrahydrofuran R*, 180 volumes of *methanol R* and 800 volumes of a 3.4 g/l solution of *potassium dihydrogen phosphate R*; adjust to pH 3.0 with *phosphoric acid R*,
- as detector a spectrophotometer set at 226 nm.

Equilibrate the column with the mobile phase at a flow rate of 1.0 ml/min for about 30 min.

Adjust the sensitivity of the system so that the height of the principal peak in the chromatogram obtained with 10 µl of reference solution (a) is at least 50 per cent of the full scale of the recorder.

Inject 10 µl of reference solution (b). The resulting chromatogram is similar to that of the specimen chromatogram provided with *atenolol for column validation CRS* in that the peak due to bis-ether precedes and is separated from that due to tertiary amine which normally appears as a doublet. If necessary, adjust the concentration of sodium octanesulphonate in the mobile phase. Increasing the concentration of sodium octanesulphonate increases the retention time of the tertiary amine.

Inject separately 10 µl of test solution (a) and reference solution (a). Continue the chromatography for four times the retention time of the principal peak. In the chromatogram obtained with test solution (a): the area of any peak apart from the principal peak is not greater than half the area of the principal peak in the chromatogram obtained with reference solution (a) (0.25 per cent); the sum of the areas of all the peaks, apart from the principal peak, is not greater than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent). Disregard any peak with an area less than 0.1 times of that of the principal peak in the chromatogram obtained with reference solution (a).

If the substance to be examined is found to contain more than 0.15 per cent of bis-ether, its compliance is confirmed by repeating the chromatography using 10 µl of test solution (b).

Chlorides (2.4.4). Dissolve 50 mg in a mixture of 1 ml of *dilute nitric acid R* and 15 ml of *water R*. The solution, without further addition of *dilute nitric acid R*, complies with the limit test for chlorides (0.1 per cent).

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C.

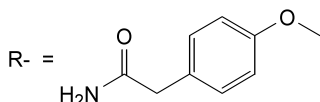
Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

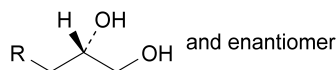
Dissolve 0.200 g in 80 ml of *anhydrous acetic acid R*. Titrate with 0.1 M *perchloric acid*, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *perchloric acid* is equivalent to 26.63 mg of C₁₄H₂₂N₂O₃.

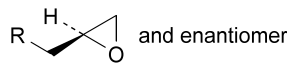
IMPURITIES



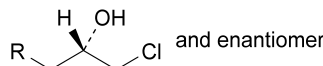
A. R-H: 2-(4-hydroxyphenyl)acetamide,



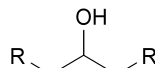
B. 2-[4-[(2*RS*)-2,3-dihydroxypropoxy]phenyl]acetamide,



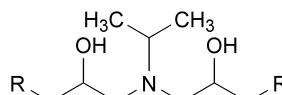
C. 2-[4-[(2*RS*)-oxiran-2-yl]methoxy]phenyl]acetamide,



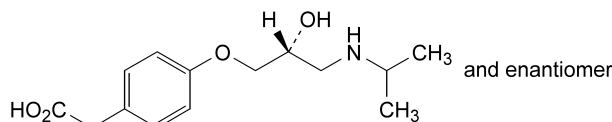
D. 2-[4-[(2*RS*)-3-chloro-2-hydroxypropoxy]phenyl]acetamide,



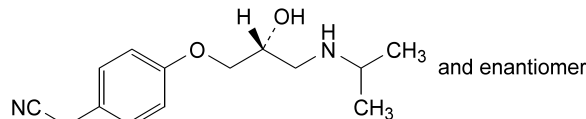
E. 2,2'-[2-hydroxypropan-1,3-diylbis(oxy-4,1-phenylene)]diacetamide,



F. 2,2'-[(1-methylethyl)iminobis(2-hydroxypropan-3,1-diyoxy-4,1-phenylene)]diacetamide,



G. 2-[4-[(2*RS*)-2-hydroxy-3-[(1-methylethyl)amino]propoxy]phenyl]acetic acid,

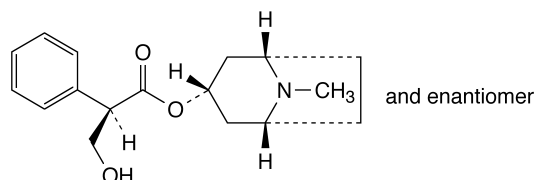


H. 2-[4-[(2*RS*)-2-hydroxy-3-[(1-methylethyl)amino]propoxy]phenyl]acetonitrile.

01/2005:2056

ATROPINE

Atropinum



C₁₇H₂₃NO₃

M_r 289.4

DEFINITION

(1*R*,3*r*,5*S*)-8-Methyl-8-azabicyclo[3.2.1]oct-3-yl (2*RS*)-3-hydroxy-2-phenylpropanoate.

Content: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

Appearance: white, crystalline powder or colourless crystals.

Solubility: very slightly soluble in water, freely soluble in alcohol and in methylene chloride.

IDENTIFICATION

First identification: A, B, E.

Second identification: A, C, D, E.

A. Melting point (2.2.14): 115 °C to 119 °C.

B. Infrared absorption spectrophotometry (2.2.24).

Comparison: Ph. Eur. reference spectrum of atropine.

C. Thin-layer chromatography (2.2.27).

Test solution. Dissolve 10 mg of the substance to be examined in *methanol R* and dilute to 10 ml with the same solvent.

Reference solution. Dissolve 10 mg of *atropine sulphate CRS* in *methanol R* and dilute to 10 ml with the same solvent.

Plate: TLC silica gel plate *R*.

Mobile phase: concentrated ammonia *R*, water *R*, acetone *R* (3:7:90 V/V/V).

Application: 10 µl.

Development: over half of the plate.

Drying: at 100-105 °C for 15 min.

Detection: after cooling, spray with dilute potassium iodobismuthate solution *R*.

Results: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

D. Place about 3 mg in a porcelain crucible and add 0.2 ml of *fuming nitric acid R*. Evaporate to dryness on a water-bath. Dissolve the residue in 0.5 ml of a 30 g/l solution of *potassium hydroxide R* in *methanol R*. A violet colour develops.

E. It complies with the test for optical rotation (see Tests).

TESTS

Optical rotation (2.2.7): -0.70° to $+0.05^{\circ}$.

Dissolve 1.25 g in *alcohol R* and dilute to 25.0 ml with the same solvent. Measure in a 2 dm tube.

Related substances. Liquid chromatography (2.2.29).

Test solution. Dissolve 50 mg of the substance to be examined in mobile phase A and dilute to 50 ml with mobile phase A. Dilute 10 ml of the solution to 50 ml with mobile phase A.

Reference solution (a). Dilute 1.0 ml of the test solution to 100.0 ml with mobile phase A. Dilute 1.0 ml of this solution to 10.0 ml with mobile phase A.

Reference solution (b). Dissolve 5 mg of *atropine for system suitability CRS* in mobile phase A and dilute to 25 ml with mobile phase A.

Column:

- size: $l = 0.125$ m, $\varnothing = 4$ mm,
- stationary phase: octylsilyl silica gel for chromatography *R* (4 µm).

Mobile phase:

- mobile phase A: to 606 ml of a 7.0 g/l solution of *potassium dihydrogen phosphate R* adjusted to pH 3.3 with 0.05 M *phosphoric acid*, add 3.5 g of *sodium dodecyl sulphate R* and 320 ml of *acetonitrile R*,
- mobile phase B: *acetonitrile R*,

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)
0 - 12	100	0
12 - 25	100 → 70	0 → 30
25 - 26	70 → 100	30 → 0
26 - 30	100	0

Flow rate: 1 ml/min.

Detection: spectrophotometer at 210 nm.

Injection: 10 µl.

System suitability: reference solution (b):

- the chromatogram obtained is similar to the chromatogram supplied with *atropine for system suitability CRS*,
- peak-to-valley ratio: minimum 20, where H_p = height above the baseline of the peak due to impurity B and H_v = height above the baseline of the lowest point of the curve separating this peak from the peak due to atropine.

Limits: locate the impurities by comparing with the chromatogram obtained with reference solution (b) and with the chromatogram supplied with *atropine for system suitability CRS*:

- impurity B: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.3 per cent),
- impurity A: not more than 4 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.4 per cent),
- any other impurity: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent),
- total: not more than 15 times the area of the principal peak in the chromatogram obtained with reference solution (a) (1.5 per cent),
- disregard limit: the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent); disregard peaks due to the blank.

Loss on drying (2.2.32): maximum 0.2 per cent, determined on 1.000 g by drying in an oven at 100-105 °C for 2 h.

ASSAY

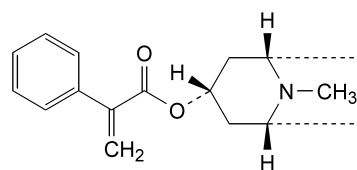
Dissolve 0.250 g in 40 ml of *anhydrous acetic acid R* and heat if necessary. Allow the solution to cool. Titrate with 0.1 M *perchloric acid*, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *perchloric acid* is equivalent to 28.94 mg of $C_{17}H_{23}NO_3$.

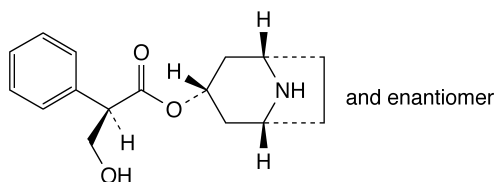
STORAGE

Protected from light.

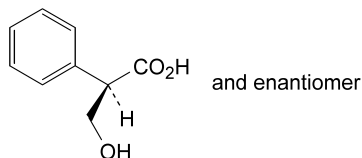
IMPURITIES



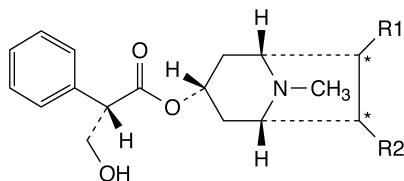
A. (1*R*,3*r*,5*S*)-8-methyl-8-azabicyclo[3.2.1]oct-3-yl 2-phenylpropenoate (apoa tropine),



- B. (1*R*,3*r*,5*S*)-8-azabicyclo[3.2.1]oct-3-yl (2*RS*)-3-hydroxy-2-phenylpropanoate (noratropine),



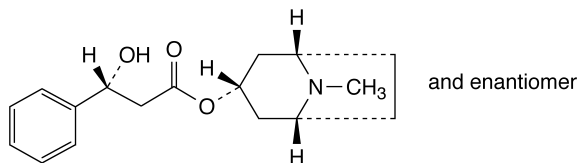
- C. (2*RS*)-3-hydroxy-2-phenylpropanoic acid (tropic acid),



- D. R1 = OH, R2 = H: (1*R*,3*S*,5*R*,6*RS*)-6-hydroxy-8-methyl-8-azabicyclo[3.2.1]oct-3-yl (2*S*)-3-hydroxy-2-phenylpropanoate (6-hydroxyhyoscyamine),

- E. R1 = H, R2 = OH: (1*S*,3*R*,5*S*,6*RS*)-6-hydroxy-8-methyl-8-azabicyclo[3.2.1]oct-3-yl (2*S*)-3-hydroxy-2-phenylpropanoate (7-hydroxyhyoscyamine),

- F. hyoscine,

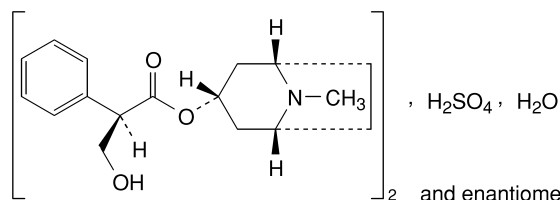


- G. (1*R*,3*r*,5*S*)-8-methyl-8-azabicyclo[3.2.1]oct-3-yl (3*RS*)-3-hydroxy-3-phenylpropanoate (isolittorine).

01/2005:0068

ATROPINE SULPHATE

Atropini sulfas

C₃₄H₄₈N₂O₁₀S₂H₂OM_r 695

DEFINITION

Atropine sulphate contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of bis[(1*R*,3*r*,5*S*)-8-methyl-8-azabicyclo[3.2.1]oct-3-yl (2*RS*)-3-hydroxy-2-phenylpropanoate] sulphate, calculated with reference to the anhydrous substance.

CHARACTERS

A white, crystalline powder or colourless crystals, very soluble in water, freely soluble in alcohol.

It melts at about 190 °C with decomposition, determined on the substance dried at 135 °C for 15 min.

IDENTIFICATION

First identification: A, B, E.

Second identification: C, D, E, F.

- An aqueous solution shows almost no optical rotation (see Tests).
- Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *atropine sulphate CRS*.
- Dissolve about 50 mg in 5 ml of *water R* and add 5 ml of *picric acid solution R*. The precipitate, washed with *water R* and dried at 100 °C to 105 °C for 2 h, melts (2.2.14) at 174 °C to 179 °C.
- To about 1 mg add 0.2 ml of *fuming nitric acid R* and evaporate to dryness in a water-bath. Dissolve the residue in 2 ml of *acetone R* and add 0.1 ml of a 30 g/l solution of *potassium hydroxide R* in *methanol R*. A violet colour develops.
- It gives the reactions of sulphates (2.3.1).
- It gives the reaction of alkaloids (2.3.1).

TESTS

pH (2.2.3). Dissolve 0.6 g in *carbon dioxide-free water R* and dilute to 30 ml with the same solvent. The pH of the solution is 4.5 to 6.2.

Optical rotation (2.2.7). Dissolve 2.50 g in *water R* and dilute to 25.0 ml with the same solvent. The angle of optical rotation measured in a 2 dm tube is −0.50° to +0.05°.

Foreign alkaloids and decomposition products. Examine by thin-layer chromatography (2.2.27), using *silica gel G R* as the coating substance.

Test solution. Dissolve 0.2 g of the substance to be examined in *methanol R* and dilute to 10 ml with the same solvent.

Reference solution (a). Dilute 1 ml of the test solution to 100 ml with *methanol R*.

Reference solution (b). Dilute 5 ml of reference solution (a) to 10 ml with *methanol R*.

Apply separately to the plate 10 µl of each solution. Develop over a path of 10 cm using a mixture of 90 volumes of *acetone R*, 7 volumes of *water R* and 3 volumes of *concentrated ammonia R*. Dry the plate at 100 °C to 105 °C for 15 min. Allow to cool and spray with *dilute potassium iodobismuthate solution R* until the spots appear. Any spot in the chromatogram obtained with the test solution, apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (a) (1.0 per cent) and not more than one such spot is more intense than the spot in the chromatogram obtained with reference solution (b) (0.5 per cent).

Apoatropine. Dissolve 0.10 g in 0.01 *M hydrochloric acid* and dilute to 100.0 ml with the same acid. Determine the absorbance (2.2.25) at 245 nm. The specific absorbance is not greater than 4.0, calculated with reference to the anhydrous substance (about 0.5 per cent).

Water (2.5.12). 2.0 per cent to 4.0 per cent, determined on 0.50 g by the semi-micro determination of water.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.500 g in 30 ml of *anhydrous acetic acid R*, warming if necessary. Cool the solution. Titrate with 0.1 *M perchloric acid* and determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M perchloric acid is equivalent to 67.68 mg of $C_{34}H_{48}N_2O_{10}S$.

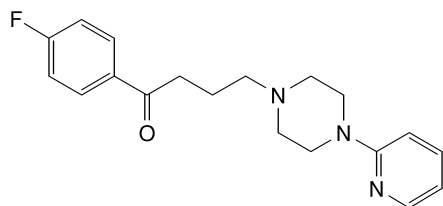
STORAGE

Store protected from light.

01/2005:1708

AZAPERONE FOR VETERINARY USE

Azaperonum ad usum veterinarium



$C_{34}H_{48}FN_3O$

M_r 327.4

DEFINITION

1-(4-Fluorophenyl)-4-[4-(pyridin-2-yl)piperazin-1-yl]butan-1-one.

Content: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

Appearance: white or almost white powder.

Solubility: practically insoluble in water, freely soluble in acetone and in methylene chloride, soluble in alcohol.

It shows polymorphism.

IDENTIFICATION

Infrared absorption spectrophotometry (2.2.24).

Preparation: discs.

Comparison: azaperone CRS.

If the spectra obtained show differences, dissolve the substance to be examined and the reference substance separately in *acetone R*, evaporate to dryness and record new spectra using the residues.

TESTS

Appearance of solution. The solution is clear (2.2.1) and not more intensely coloured than reference solution Y_6 (2.2.2, *Method II*).

Dissolve 1.0 g in 25 ml of a 14 g/l solution of *tartaric acid R*.

Related substances. Liquid chromatography (2.2.29).

Test solution. Dissolve 0.100 g of the substance to be examined in *methanol R* and dilute to 10.0 ml with the same solvent.

Reference solution (a). Dissolve 5.0 mg of *azaperone CRS* and 6.0 mg of *benperidol CRS* in *methanol R* and dilute to 200.0 ml with the same solvent.

Reference solution (b). Dilute 1.0 ml of the test solution to 100.0 ml with *methanol R*. Dilute 5.0 ml of the solution to 20.0 ml with *methanol R*.

Column:

- *size*: $l = 0.10$ m, $\varnothing = 4.6$ mm,
- *stationary phase*: base-deactivated octadecylsilyl silica gel for chromatography *R* (3 μ m),
- *temperature*: 25 °C.

Mobile phase:

- *mobile phase A*: dissolve 1.4 g of *anhydrous sodium sulphate R* in 900 ml of *water R*, add 16.0 ml of 0.01 M *sulphuric acid* and dilute to 1000 ml with *water R*,
- *mobile phase B*: *methanol R*.

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)
0 - 15	95 → 20	5 → 80
15 - 20	20	80
20 - 21	20 → 95	80 → 5

Flow rate: 1.5 ml/min.

Detection: spectrophotometer at 230 nm.

Equilibration: at least 4 min with the mobile phase at the initial composition.

Injection: 10 μ l.

Relative retention with reference to azaperone (retention time = about 9 min): impurity A = about 0.9; impurity B = about 1.1; impurity C = about 1.15.

System suitability: reference solution (a):

- *resolution*: minimum 8.0 between the peaks due to azaperone and to benperidol.

Limits:

- *impurity A*: not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.25 per cent),
- *total of impurities B and C*: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.75 per cent),
- *total*: not more than 4 times the area of the principal peak in the chromatogram obtained with reference solution (b) (1.0 per cent),
- *disregard limit*: 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

Loss on drying (2.2.32): maximum 0.5 per cent, determined on 1.000 g by drying *in vacuo* at 60 °C for 4 h.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

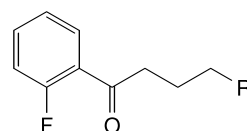
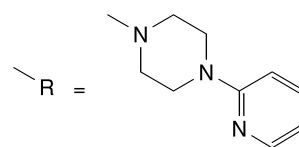
Dissolve 0.130 g in 70 ml of a mixture of 1 volume of *anhydrous acetic acid R* and 7 volumes of *methyl ethyl ketone R*. Titrate with 0.1 M *perchloric acid*, using 0.2 ml of *naphtholbenzein solution R* as indicator.

1 ml of 0.1 M *perchloric acid* is equivalent to 16.37 mg of $C_{34}H_{48}FN_3O$.

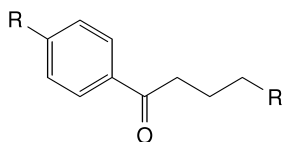
STORAGE

Protected from light.

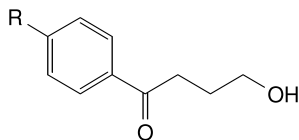
IMPURITIES



A. 1-(2-fluorophenyl)-4-[4-(pyridin-2-yl)piperazin-1-yl]butan-1-one,



- B. 4-[4-(pyridin-2-yl)piperazin-1-yl]-1-[4-[4-(pyridin-2-yl)piperazin-1-yl]phenyl]butan-1-one,

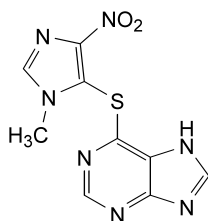


- C. 4-hydroxy-1-[4-[4-(pyridin-2-yl)piperazin-1-yl]phenyl]butan-1-one.

01/2005:0369

AZATHIOPRINE

Azathioprinum

C₉H₇N₇O₂SM_r 277.3

DEFINITION

Azathioprine contains not less than 98.5 per cent and not more than the equivalent of 101.0 per cent of 6-[(1-methyl-4-nitro-1H-imidazol-5-yl)sulfanyl]-7H-purine, calculated with reference to the dried substance.

CHARACTERS

A pale-yellow powder, practically insoluble in water and in alcohol. It is soluble in dilute solutions of alkali hydroxides and sparingly soluble in dilute mineral acids.

IDENTIFICATION

- Dissolve 0.150 g in 30 ml of *dimethyl sulphoxide R* and dilute to 500.0 ml with 0.1 M *hydrochloric acid*. Dilute 25.0 ml of the solution to 1000.0 ml with 0.1 M *hydrochloric acid*. Examined between 230 nm and 350 nm (2.2.25), the solution shows an absorption maximum at 280 nm. The specific absorbance at the maximum is 600 to 660.
- Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *azathioprine CRS*.
- To about 20 mg add 100 ml of *water R*, heat and filter. To 5 ml of the filtrate add 1 ml of *hydrochloric acid R* and about 10 mg of *zinc powder R*. Allow to stand for 5 min. The solution becomes yellow. Filter, cool in iced water, add 0.1 ml of *sodium nitrite solution R* and 0.1 g of *sulphamic acid R* and shake until the bubbles disappear. Add 1 ml of *β-naphthol solution R*. A pale-pink precipitate is formed.

TESTS

Acidity or alkalinity. To 0.5 g add 25 ml of *carbon dioxide-free water R*, shake for 15 min and filter. To 20 ml of the filtrate add 0.1 ml of *methyl red solution R*. Not more than 0.2 ml of 0.01 M *hydrochloric acid* or 0.01 M *sodium hydroxide* is required to change the colour of the indicator.

Chloromethylnitroimidazole and mercaptopurine. Examine by thin-layer chromatography (2.2.27), using *cellulose for chromatography F₂₅₄ R* as the coating substance.

Test solution. Dissolve 0.2 g of the substance to be examined in *dilute ammonia R1* and dilute to 10 ml with the same solvent. Prepare immediately before use.

Reference solution (a). Dissolve 10 mg of *chloromethylnitroimidazole CRS* in *dilute ammonia R1* and dilute to 50 ml with the same solvent. Prepare immediately before use.

Reference solution (b). Dissolve 10 mg of *mercaptopurine R* in *dilute ammonia R1* and dilute to 50 ml with the same solvent. Prepare immediately before use.

Apply separately to the plate 5 µl of each solution. Develop over a path of 15 cm using *butanol R* saturated with *dilute ammonia R1*. Dry the plate at 50 °C and examine in ultraviolet light at 254 nm. In the chromatogram obtained with the test solution, any spots corresponding to chloromethyl-nitroimidazole and mercaptopurine are not more intense than the spots in the chromatograms obtained with reference solution (a) (1.0 per cent) and reference solution (b) (1.0 per cent), respectively.

Loss on drying (2.2.32). Not more than 1.0 per cent, determined on 0.50 g by drying in an oven at 100 °C to 105 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.250 g in 25 ml of *dimethylformamide R*. Titrate with 0.1 M *tetrabutylammonium hydroxide*, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *tetrabutylammonium hydroxide* is equivalent to 27.73 mg of C₉H₇N₇O₂S.

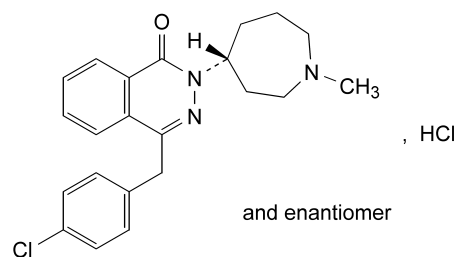
STORAGE

Store protected from light.

01/2005:1633
corrected

AZELASTINE HYDROCHLORIDE

Azelastini hydrochloridum

C₂₂H₂₅Cl₂N₃OM_r 418.4

DEFINITION

4-(4-Chlorobenzyl)-2-[(4*RS*)-1-methylhexahydro-1*H*-azepin-4-yl]phthalazin-1(2*H*)-one hydrochloride.

Content: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

Appearance: white or almost white, crystalline powder.

Solubility: sparingly soluble in water, soluble in ethanol and in methylene chloride.

IDENTIFICATION

A. Infrared absorption spectrophotometry (2.2.24).

Comparison: azelastine hydrochloride CRS.

B. Solution S (see Tests) gives reaction (a) of chlorides (2.3.1).

TESTS

Solution S. Dissolve 1.0 g in carbon dioxide-free water R and dilute to 100 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and colourless (2.2.2, Method II).

Acidity or alkalinity. To 10 ml of solution S add 0.2 ml of bromothymol blue solution R1. Not more than 0.1 ml of 0.01 M hydrochloric acid or 0.01 M sodium hydroxide is required to change the colour of the solution.

Related substances. Liquid chromatography (2.2.29).

Solvent mixture: acetonitrile for chromatography R, water R (45:55 V/V).

Test solution. Dissolve 0.125 g of the substance to be examined in the solvent mixture and dilute to 50.0 ml with the solvent mixture.

Reference solution (a). Dilute 1.0 ml of the test solution to 100.0 ml with the solvent mixture. Dilute 1.0 ml of this solution to 10.0 ml with the solvent mixture.

Reference solution (b). Dissolve 1 mg of azelastine impurity B CRS, 1 mg of azelastine impurity D CRS and 1 mg of azelastine impurity E CRS in the test solution and dilute to 20 ml with the test solution.

Column:

- size: $l = 0.25$ m, $\varnothing = 4.6$ mm,
- stationary phase: nitrile silica gel for chromatography R (10 μ m),
- temperature: 30 °C.

Mobile phase: dissolve 2.16 g of sodium octanesulphonate R and 0.68 g of potassium dihydrogen phosphate R in 740 ml of water for chromatography R, adjust to pH 3.0-3.1 with dilute phosphoric acid R, add 260 ml of acetonitrile for chromatography R and mix.

Flow rate: 2.0 ml/min.

Detection: spectrophotometer at 210 nm.

Injection: 10 μ l.

Run time: twice the retention time of azelastine.

Relative retention with reference to azelastine (retention time = about 8-9 min): impurity A = about 0.2; impurity B = about 0.3; impurity C = about 0.4; impurity D = about 0.6; impurity E = about 1.4.

System suitability: reference solution (b):

- resolution: minimum 4.0 between the peaks due to impurity B and impurity D,
- the peaks due to impurity D and impurity E are baseline separated from the principal peak.

Limits:

- correction factors: for the calculation of contents, multiply the peak areas of the following impurities by the corresponding correction factor: impurity B = 3.6; impurity D = 0.7; impurity E = 2.1;

- impurities A, B, C, D, E: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent);
- any other impurity: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent);
- total: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent);
- disregard limit: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

Loss on drying (2.2.32): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 100-105 °C.

ASSAY

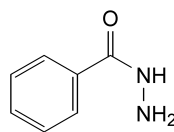
In order to avoid overheating in the reaction medium, mix thoroughly throughout and stop the titration immediately after the end-point has been reached.

Dissolve 0.300 g in 5 ml of anhydrous formic acid R. Add 30 ml of acetic anhydride R. Titrate quickly with 0.1 M perchloric acid, determining the end-point potentiometrically (2.2.20).

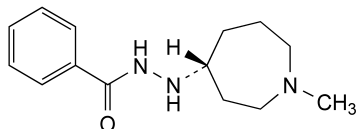
1.0 ml of 0.1 M perchloric acid is equivalent to 41.84 mg of $C_{22}H_{25}Cl_2N_3O$.

IMPURITIES

Specified impurities: A, B, C, D, E.

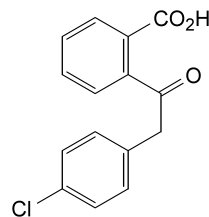


A. benzoyldiazane (benzohydrazide),

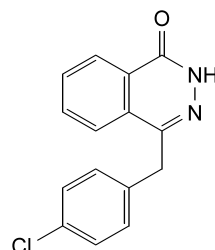


and enantiomer

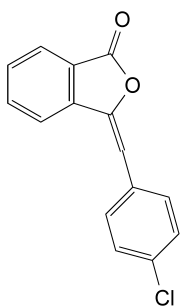
B. 1-benzoyl-2-[(4RS)-1-methylhexahydro-1H-azepin-4-yl]diazane,



C. 2-[(4-chlorophenyl)acetyl]benzoic acid,



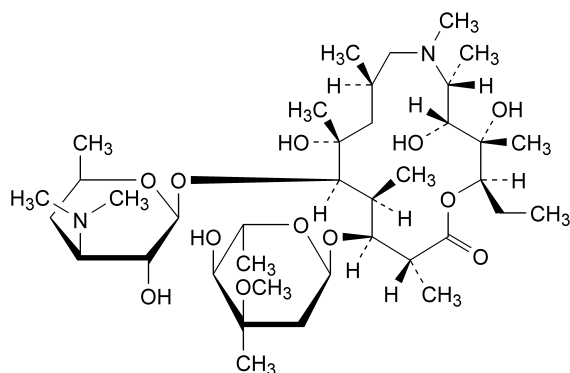
D. 4-(4-chlorobenzyl)phthalazin-1(2H)-one,



E. 3-(4-chlorobenzylidene)isobenzofuran-1(3H)-one.

01/2005:1649
corrected**AZITHROMYCIN**

Azithromycinum

 $C_{38}H_{72}N_2O_{12}$ M_r 749**DEFINITION**

(2*R*,3*S*,4*R*,5*R*,8*R*,10*R*,11*R*,12*S*,13*S*,14*R*)-13-[(2,6-Dideoxy-3-*C*-methyl-3-*O*-methyl- α -L-*ribo*-hexopyranosyl)oxy]-2-ethyl-3,4,10-trihydroxy-3,5,6,8,10,12,14-heptamethyl-11-[[3,4,6-trideoxy-3-(dimethylamino)- β -D-*xylo*-hexopyranosyl]oxy]-1-oxa-6-azacyclopentadecan-15-one.

Content: 94.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

Appearance: white or almost white powder.

Solubility: practically insoluble in water, freely soluble in ethanol and in methylene chloride.

IDENTIFICATION

A. Infrared absorption spectrophotometry (2.2.24).

Comparison: azithromycin CRS.

If the spectra obtained show differences, prepare further spectra using 90 g/l solutions in *methylene chloride R*.

B. Examine the chromatograms obtained in the assay.

Results: the principal peak in the chromatogram obtained with test solution (b) is similar in retention time and size to the principal peak in the chromatogram obtained with reference solution (a).

TESTS

Solution S. Dissolve 0.500 g in *ethanol R* and dilute to 50.0 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and colourless (2.2.2, *Method II*).

pH (2.2.3): 9.0 to 11.0.

Dissolve 0.100 g in 25.0 ml of *methanol R* and dilute to 50.0 ml with *carbon dioxide-free water R*.

Specific optical rotation (2.2.7): -45 to -49 (anhydrous substance), determined on solution S.

Related substances. Liquid chromatography (2.2.29).

Prepare the solutions immediately before use.

Solvent mixture: acetonitrile *R*, water *R* (40:60 V/V).

Test solution (a). Dissolve 0.100 g of the substance to be examined in the solvent mixture and dilute to 25.0 ml with the solvent mixture.

Test solution (b). Dilute 5.0 ml of test solution (a) to 20.0 ml with the solvent mixture.

Reference solution (a). Dissolve 50.0 mg of *azithromycin CRS* in the solvent mixture and dilute to 50.0 ml with the solvent mixture.

Reference solution (b). Dilute 1.0 ml of test solution (a) to 100.0 ml with the solvent mixture.

Reference solution (c). Dissolve 5.0 mg of *azithromycin CRS* and 5.0 mg of *azithromycin impurity A CRS* in the solvent mixture and dilute to 50 ml with the solvent mixture.

Reference solution (d). Dissolve 2 mg of *azithromycin impurity B CRS* in the solvent mixture and dilute to 50 ml with the solvent mixture. Use this solution only for identification of the peak due to impurity B.

Column:

- **size:** $l = 0.25$ m, $\varnothing = 4.6$ mm,
- **stationary phase:** end-capped polar-embedded octadecylsilyl amorphous organosilica polymer *R* (5 μ m),
- **temperature:** 70 °C.

Mobile phase: mix 10 volumes of a 34.84 g/l solution of *dipotassium hydrogen phosphate R* adjusted to pH 6.5 with *phosphoric acid R*, 35 volumes of *acetonitrile R* and 55 volumes of *water R*.

Flow rate: 1.0 ml/min.

Detection: spectrophotometer at 215 nm.

Injection: 100 μ l; inject test solution (a) and reference solutions (b), (c) and (d).

Run time: 4.5 times the retention time of azithromycin.

Relative retention with reference to azithromycin (retention time = about 26 min): impurity D = about 0.37; impurity J = about 0.39; impurity A = about 0.42; impurity I = about 0.5; impurity C = about 0.65; impurity K = about 0.9; impurity F = about 1.6; impurity B = about 1.7; impurity G = about 2.8.

System suitability: reference solution (c):

- **resolution:** minimum 7.0 between the peaks due to impurity A and azithromycin.

Limits:

- **impurity B:** not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (2.0 per cent),
- **any other impurity:** for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (1.0 per cent),
- **total:** not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (5.0 per cent),
- **disregard limit:** 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent).

Heavy metals (2.4.8): maximum 25 ppm.

Dissolve 2.0 g in a mixture of 15 volumes of *water R* and 85 volumes of *ethanol R* and dilute to 20 ml with the same mixture of solvents. 12 ml of the solution complies with limit test B. Prepare the standard using lead standard solution (2.5 ppm Pb) obtained by diluting *lead standard solution (100 ppm Pb) R* with a mixture of 15 volumes of *water R* and 85 volumes of *ethanol R*.

Water (2.5.12): 1.8 per cent to 6.5 per cent, determined on 0.20 g.

Sulphated ash (2.4.14): maximum 0.2 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (2.2.29) as described in the test for related substances, with the following modifications.

Injection: 25 µl; inject test solution (b) and reference solution (a).

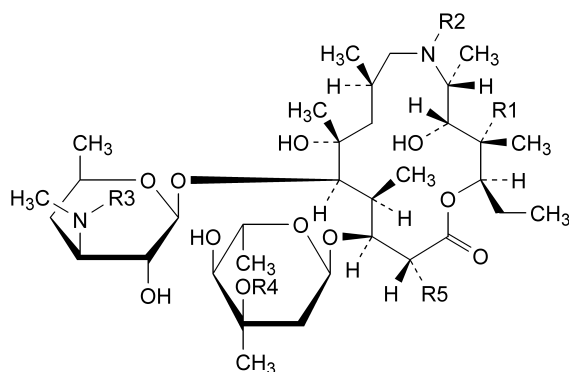
Calculate the percentage content of $C_{38}H_{72}N_2O_{12}$ using the declared content of *azithromycin CRS*.

STORAGE

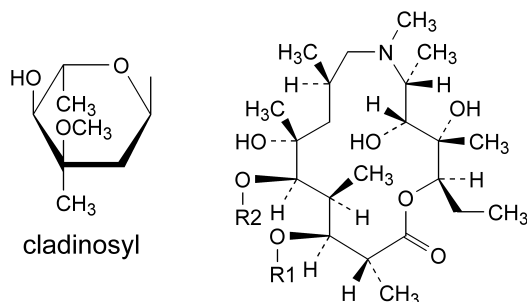
In an airtight container.

IMPURITIES

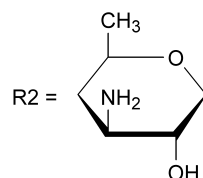
Specified impurities: A, B, C, D, E, F, G, H, I, J, K.



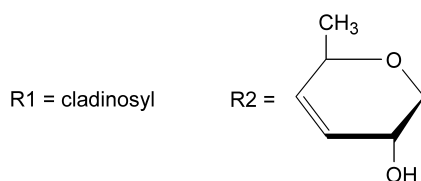
- A. R1 = OH, R2 = H, R3 = R4 = R5 = CH₃:
6-demethylazithromycin,
- B. R1 = H, R2 = R3 = R4 = R5 = CH₃: 3-deoxyazithromycin
(azithromycin B),
- C. R1 = OH, R2 = R3 = R5 = CH₃, R4 = H:
3'-O-demethylazithromycin (azithromycin C),
- D. R1 = OH, R2 = R3 = R4 = CH₃, R5 = CH₂OH:
14-demethyl-14-(hydroxymethyl)azithromycin
(azithromycin F),
- F. R1 = OH, R2 = R4 = R5 = CH₃, R3 = CHO:
3'-N-demethyl-3'-N-formylazithromycin,
- G. R1 = OH, R2 = R4 = R5 = CH₃, R3 = SO₂-C₆H₄-CH₃: 3'-N-demethyl-3'-N-[(4-methylphenyl)sulphonyl]azithromycin,
- I. R1 = OH, R2 = R4 = R5 = CH₃, R3 = H:
3'-N-demethylazithromycin,



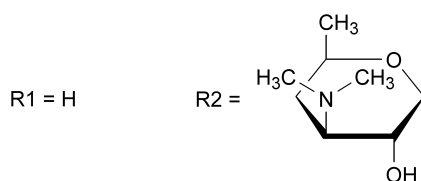
R1 = cladinosyl



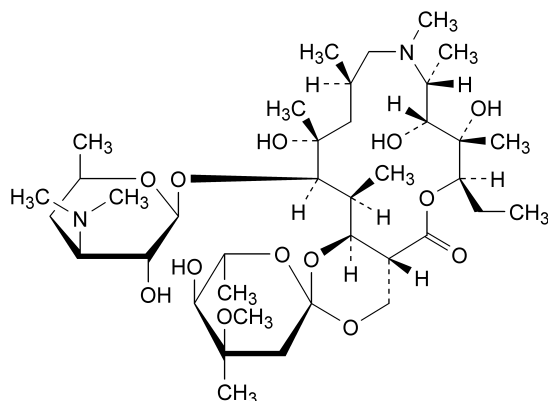
E. 3'-(*N,N*-didemethyl)azithromycin (aminoazithromycin),



H. 3'-de(dimethylamino)-3',4'-didehydroazithromycin,



J. decladinosylazithromycin,

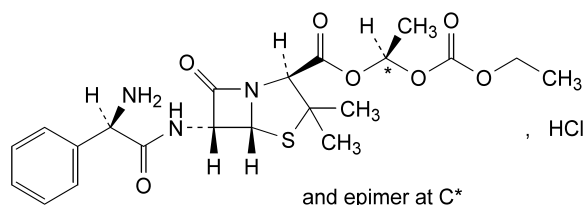


K. (2*S*,4'*R*,4*aR*,5'*S*,6'*S*,7*R*,8*S*,9*R*,10*R*,13*R*,15*R*,16*R*,17*S*,17*aS*)-7-ethyl-5',8,9,15-tetrahydroxy-4'-methoxy-4',6',8,10,11,13,15,17-octamethyl-16-[[3,4,6-trideoxy-3-(dimethylamino)-β-D-xylohexopyranosyl]oxy]octadecahydro-5*H*-spiro[1,3-dioxino[4,5-*m*][1,6]oxazacyclopentadecine-2,2'-[2*H*]pyran]-5-one (azithromycin E).

01/2005:0808

BACAMPICILLIN HYDROCHLORIDE

Bacampicillini hydrochloridum

 $C_{21}H_{28}ClN_3O_7S$ M_r 502.0

DEFINITION

Bacampicillin hydrochloride contains not less than 95.0 per cent and not more than the equivalent of 102.0 per cent of (1*RS*)-1-[(ethoxycarbonyl)oxy]ethyl (2*S*,5*R*,6*R*)-6-[(2*R*)-2-amino-2-phenylacetyl]amino]-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylate hydrochloride, calculated with reference to the anhydrous, solvent-free substance.

PRODUCTION

If manufactured by a process that may leave residues of dimethylaniline in the substance and/or by a process using starting materials or intermediates which contain residues of dimethylaniline, it complies with the following test:

N,N-Dimethylaniline (2.4.26, *Method A*). Not more than 20 ppm.

CHARACTERS

A white or almost white powder or granules, hygroscopic, soluble in water, freely soluble in alcohol, soluble in methylene chloride.

IDENTIFICATION

First identification: A, D.

Second identification: B, C, D.

- A. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *bacampicillin hydrochloride CRS*.
- B. Examine by thin-layer chromatography (2.2.27), using a *TLC silanised silica gel plate R*.

Test solution. Dissolve 10 mg of the substance to be examined in 2 ml of *methanol R*.

Reference solution (a). Dissolve 10 mg of *bacampicillin hydrochloride CRS* in 2 ml of *methanol R*.

Reference solution (b). Dissolve 10 mg each of *bacampicillin hydrochloride CRS*, *talampicillin hydrochloride CRS* and *pivampicillin CRS* in 2 ml of *methanol R*.

Apply to the plate 1 µl of each solution. Develop over a path of 15 cm using a mixture of 10 volumes of a 272 g/l solution of *sodium acetate R*, the pH of which has been adjusted to 5.0 with *glacial acetic acid R*, 40 volumes of *water R* and 50 volumes of *alcohol R*. Dry the plate in a current of warm air, then spray with *ninhydrin solution R1* and heat at 60 °C for 10 min. The principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).

The test is not valid unless the chromatogram obtained with reference solution (b) shows three clearly separated spots.

- C. Place about 2 mg in a test-tube about 150 mm long and 15 mm in diameter. Moisten with 0.05 ml of *water R* and add 2 ml of *sulphuric acid-formaldehyde reagent R*. Mix the contents of the tube by swirling; the solution is practically colourless. Place the test-tube on a water-bath for 1 min; a dark yellow colour develops.
- D. Dissolve about 25 mg in 2 ml of *water R*. Add 2 ml of *dilute sodium hydroxide solution R* and shake. Wait a few minutes and add 3 ml of *dilute nitric acid R* and 0.5 ml of *silver nitrate solution R1*. A white precipitate is formed. Add 0.5 ml of *concentrated ammonia R*. The precipitate dissolves.

TESTS

Appearance of solution. Dissolve 0.200 g in 20 ml of *water R*; the solution is not more opalescent than reference suspension II (2.2.1). Dissolve 0.500 g in 10 ml of *water R*; the absorbance (2.2.25) of the solution measured at 430 nm is not greater than 0.10.

pH (2.2.3). Dissolve 1.0 g in *carbon dioxide-free water R* and dilute to 50 ml with the same solvent. The pH of the solution is 3.0 to 4.5.

Specific optical rotation (2.2.7). Dissolve 0.250 g in *water R* and dilute to 25.0 ml with the same solvent. The specific optical rotation is + 175 to + 195, calculated with reference to the anhydrous, solvent-free substance.

Butyl acetate and ethyl acetate. Not more than 2.0 per cent *m/m* of butyl acetate, not more than 4.0 per cent *m/m* of ethyl acetate and not more than 5.0 per cent *m/m* for the sum determined by head-space gas chromatography (2.2.28), using the method of standard additions.

Sample solution. Dissolve 50.0 mg of the substance to be examined in *water R* and dilute to 10.0 ml with the same solvent.

The chromatographic procedure may be carried out using System A of the test for residual solvents (2.4.24) and the following static head-space injection conditions:

- equilibration temperature: 60 °C,
- equilibration time: 20 min.

Related substances. Examine by liquid chromatography (2.2.29) as prescribed in the Assay. Inject 20 µl of reference solution (b). Adjust the sensitivity of the system so that the height of the principal peak in the chromatogram obtained is at least 50 per cent of the full scale of the recorder. Inject 20 µl of reference solution (d). The test is not valid unless in the chromatogram obtained, the peak corresponding to ampicillin is separated from the peaks due to the solvent. Inject 20 µl of the test solution and continue the chromatography for 3.5 times the retention time of the principal peak. In the chromatogram obtained with the test solution: the area of any peak, apart from the principal peak, is not greater than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (1.5 per cent); the sum of the areas of all the peaks, apart from the principal peak, is not greater than three times the area of the principal peak in the chromatogram obtained with reference solution (b) (3 per cent). Disregard any peak with an area less than 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (b).

Water (2.5.12). Not more than 0.8 per cent, determined on 0.300 g by the semi-micro determination of water.

Sulphated ash (2.4.14). Not more than 1.5 per cent, determined on 1.0 g.

ASSAY

Examine by liquid chromatography (2.2.29). Prepare the test solution and reference solutions (a), (b) and (d) immediately before use.

Phosphate buffer A. Dissolve 1.4 g of sodium dihydrogen phosphate monohydrate *R* in water *R* and dilute to about 800 ml with the same solvent. Adjust to pH 3.0 with dilute phosphoric acid *R* and dilute to 1000.0 ml with water *R*.

Phosphate buffer B. Dissolve 2.75 g of sodium dihydrogen phosphate monohydrate *R* and 2.3 g of disodium hydrogen phosphate dihydrate *R* in water *R* and dilute to about 1800 ml with the same solvent. Adjust to pH 6.8, if necessary, using dilute phosphoric acid *R* or dilute sodium hydroxide solution *R* and dilute to 2000.0 ml with water *R*.

Test solution. Dissolve 30.0 mg of the substance to be examined in phosphate buffer A and dilute to 100.0 ml with the same solution.

Reference solution (a). Dissolve 30.0 mg of bacampicillin hydrochloride CRS in phosphate buffer A and dilute to 100.0 ml with the same solution.

Reference solution (b). Dilute 1.0 ml of reference solution (a) to 100.0 ml with phosphate buffer A.

Reference solution (c). Dissolve 30 mg of the substance to be examined in phosphate buffer B and dilute to 100 ml with the same solution. Heat the solution at 80 °C for about 30 min.

Reference solution (d). Dissolve 20 mg of ampicillin trihydrate CRS in phosphate buffer A and dilute to 250 ml with the same solution. Dilute 5 ml of the solution to 100 ml with phosphate buffer A.

The chromatographic procedure may be carried out using:

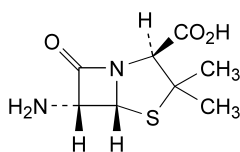
- a column 0.05 m long and 3.9 mm in internal diameter packed with octadecylsilyl silica gel for chromatography *R* (5 µm),
- as mobile phase at a flow rate of 1.0 ml/min a mixture of 30 volumes of acetonitrile *R* and 70 volumes of a 0.06 per cent *m/m* solution of tetrahexylammonium hydrogen sulphate *R* in phosphate buffer B,
- as detector a spectrophotometer set at 220 nm.

Inject 20 µl of reference solution (a). Adjust the sensitivity of the system so that the height of the principal peak in the chromatogram obtained is at least 50 per cent of the full scale of the recorder. Inject 20 µl of reference solution (c). The test is not valid unless, in the chromatogram obtained, the retention time of a degradation product eluting just after bacampicillin is 1.12 to 1.38 relative to bacampicillin. If necessary adjust the concentration of tetrahexylammonium hydrogen sulphate in the mobile phase. Inject reference solution (a) six times. The test is not valid unless the relative standard deviation of the peak area for bacampicillin is at most 1.0 per cent. Inject alternately the test solution and reference solution (a). Calculate the percentage content of bacampicillin hydrochloride.

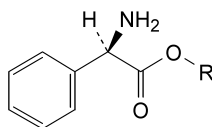
STORAGE

Store in an airtight container.

IMPURITIES

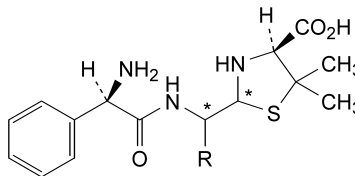


- A. (2*S*,5*R*,6*R*)-6-amino-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid (6-aminopenicillanic acid),



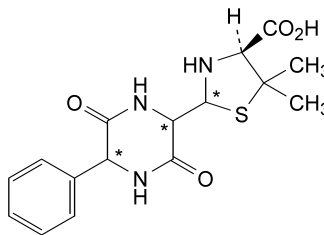
- B. *R* = H: (2*R*)-2-amino-2-phenylacetic acid (D-phenylglycine),

- G. *R* = CH₃: methyl (2*R*)-2-amino-2-phenylacetate (methyl D-phenylglycinate),

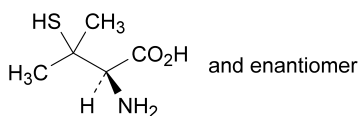


- C. *R* = H: (2*RS*,4*S*)-2-[[[(2*R*)-2-amino-2-phenylacetyl]amino]methyl]-5,5-dimethylthiazolidine-4-carboxylic acid (penilloic acids of ampicillin),

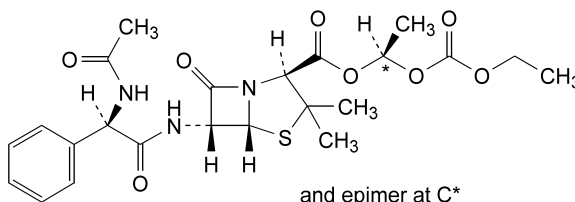
- D. *R* = CO₂H: (4*S*)-2-[[[(2*R*)-2-amino-2-phenylacetyl]amino]carboxymethyl]-5,5-dimethylthiazolidine-4-carboxylic acid (penicilloic acids of ampicillin),



- E. (4*S*)-2-(3,6-dioxo-5-phenylpiperazin-2-yl)-5,5-dimethylthiazolidine-4-carboxylic acid (diketopiperazines of ampicillin),



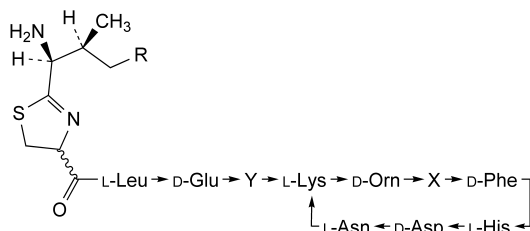
- F. (2*RS*)-2-amino-3-methyl-3-sulphanylbutanoic acid (DL-penicillamine),



- H. (1*RS*)-1-[(ethoxycarbonyl)oxy]ethyl (2*S*,5*R*,6*R*)-6-[[[(2*R*)-2-(acetyl)amino]-2-phenylacetyl]amino]-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylate (*N*-acetyl bacampicillin),

- I. ampicillin.

01/2005:0465 pH (2.2.3): 6.0 to 7.0 for solution S.

BACITRACIN**Bacitracinum**

Name	Mol. Formula	X	Y	R
Bacitracin A	C ₆₆ H ₁₀₃ N ₁₇ O ₁₆ S	L-Ile	L-Ile	CH ₃
Bacitracin B1	C ₆₅ H ₁₀₁ N ₁₇ O ₁₆ S	L-Ile	L-Ile	H
Bacitracin B2	C ₆₅ H ₁₀₁ N ₁₇ O ₁₆ S	L-Val	L-Ile	CH ₃
Bacitracin B3	C ₆₅ H ₁₀₁ N ₁₇ O ₁₆ S	L-Ile	L-Val	CH ₃

DEFINITION

Mixture of antimicrobial polypeptides produced by certain strains of *Bacillus licheniformis* or *Bacillus subtilis*, the main components being bacitracins A, B1, B2 and B3.

Content: minimum 60 IU/mg (dried substance).

CHARACTERS

Appearance: white or almost white powder, hygroscopic.

Solubility: freely soluble in water and in alcohol.

IDENTIFICATION

First identification: B, C.

Second identification: A, C.

A. Thin-layer chromatography (2.2.27).

Test solution. Dissolve 10 mg of the substance to be examined in a 3.4 g/l solution of *hydrochloric acid R* and dilute to 1.0 ml with the same solution.

Reference solution. Dissolve 10 mg of *bacitracin zinc CRS* in a 3.4 g/l solution of *hydrochloric acid R* and dilute to 1.0 ml with the same solution.

Plate: TLC silica gel plate R.

Mobile phase: glacial acetic acid R, water R, butanol R (1:2:4 V/V/V).

Application: 10 µl.

Development: over half of the plate.

Drying: at 100-105 °C.

Detection: spray with *ninhydrin solution R1* and heat at 110 °C for 5 min.

Results: the spots in the chromatogram obtained with the test solution are similar in position, size and colour to the spots in the chromatogram obtained with the reference solution.

B. It complies with the test for composition (see Tests).

C. Ignite 0.2 g. An insignificant residue remains which is not yellow at high temperature. Allow to cool. Dissolve the residue in 0.1 ml of *dilute hydrochloric acid R*. Add 5 ml of *water R* and 0.2 ml of *strong sodium hydroxide solution R*. No white precipitate is formed.

TESTS

Solution S. Dissolve 0.25 g in *carbon dioxide-free water R* and dilute to 25 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1).

Composition. Liquid chromatography (2.2.29): use the normalisation procedure. *Prepare the solutions immediately before use.*

Test solution. Dissolve 0.100 g of the substance to be examined in 50.0 ml of the mobile phase.

Reference solution (a). Suspend 20.0 mg of *bacitracin zinc CRS* in *water R*, add 0.2 ml of *dilute hydrochloric acid R* and dilute to 10.0 ml with *water R*.

Reference solution (b). Dilute 5.0 ml of the test solution to 100.0 ml with the mobile phase.

Reference solution (c). Dilute 1.0 ml of reference solution (b) to 10.0 ml with the mobile phase.

Reference solution (d). Dissolve 50.0 mg of the substance to be examined in 25.0 ml of a 40 g/l solution of *sodium edetate R* adjusted to pH 7.0 with *dilute sodium hydroxide R*. Heat in a boiling water-bath for 30 min. Cool to room temperature.

Blank solution. A 40 g/l solution of *sodium edetate R* adjusted to pH 7.0 with *dilute sodium hydroxide R*.

Column:

— **size:** $l = 0.25$ m, $\varnothing = 4.6$ mm,

— **stationary phase:** end-capped octadecylsilyl silica gel for chromatography R (5 µm).

Mobile phase: add 520 volumes of *methanol R1*, 40 volumes of *acetonitrile R* and 300 volumes of *water R* to 100 volumes of a 34.8 g/l solution of *dipotassium hydrogen phosphate R* adjusted to pH 6.0 with a 27.2 g/l solution of *potassium dihydrogen phosphate R*.

Flow rate: 1.0 ml/min.

Detection: spectrophotometer at 254 nm.

Injection: 100 µl; inject the blank, the test solution and reference solutions (a) and (c).

Run time: 3 times the retention time of bacitracin A.

Relative retention with reference to bacitracin A (retention time = 15 min to 25 min): bacitracin B1 = about 0.6; bacitracin B3 = about 0.8; impurity E = about 2.5.

If necessary, adjust the composition of the mobile phase by changing the amount of organic modifier whilst keeping the ratio constant between methanol and acetonitrile.

System suitability: reference solution (a):

— **peak-to-valley ratio:** minimum of 1.2, where H_p = height above the baseline of the peak due to bacitracin B1 and H_v = height above the baseline of the lowest point of the curve separating this peak from the peak due to bacitracin B2.

Limits:

— **bacitracin A:** minimum 40.0 per cent,

— **sum of bacitracins A, B1, B2 and B3:** minimum 70.0 per cent,

— **disregard limit:** the area of the peak due to bacitracin A in the chromatogram obtained with reference solution (c) (0.5 per cent); disregard any peak observed in the blank run.

Related peptides. Liquid chromatography (2.2.29) as described in the test for composition.

See Figure 0465-1.

Limit:

— **sum of the areas of all peaks eluting before the peak due to bacitracin B1:** maximum 20.0 per cent.

Impurity E. Liquid chromatography (2.2.29) as described in the test for composition.

See Figure 0465-2.

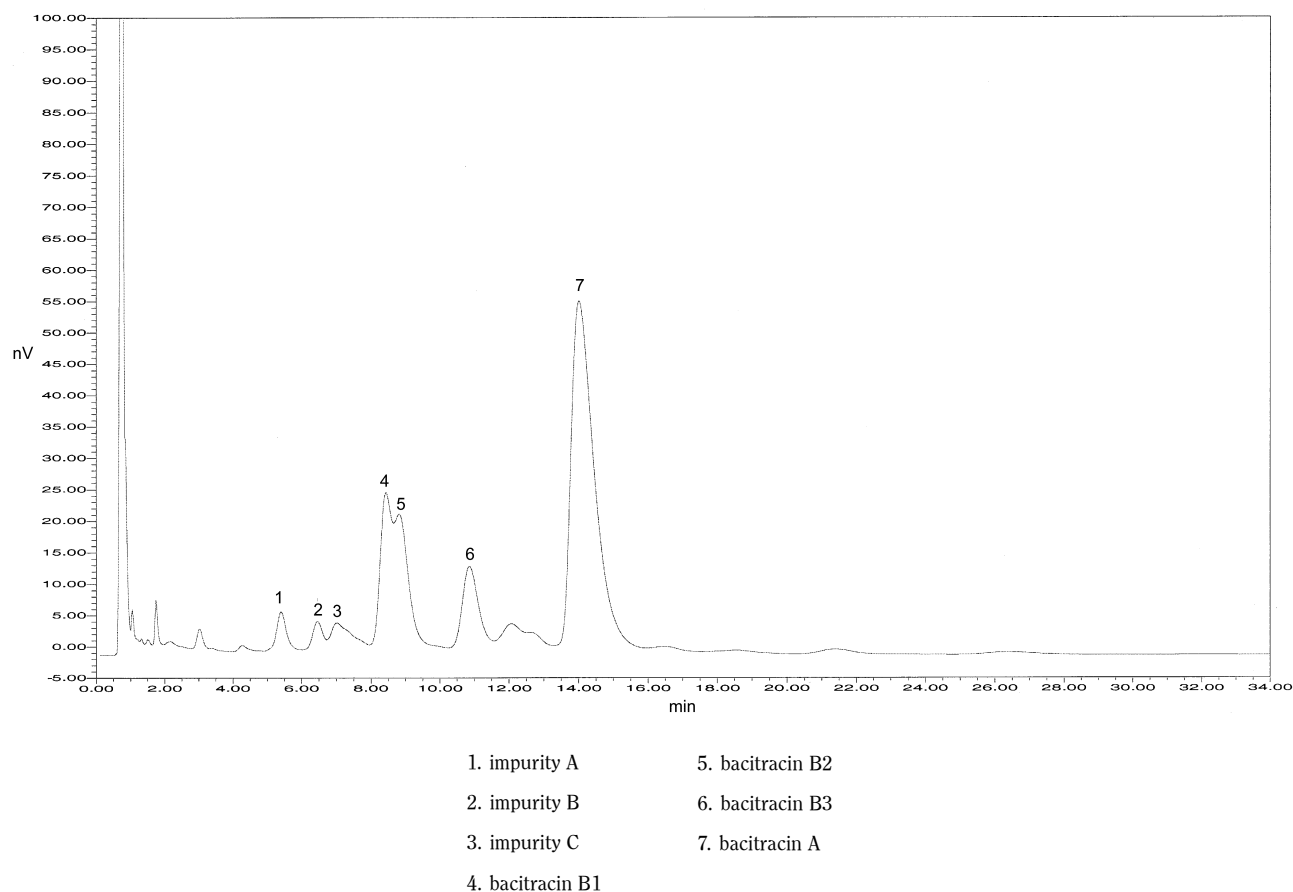


Figure 0465.-1. – Chromatogram of the test for composition in bacitracin obtained with the test solution at 254 nm

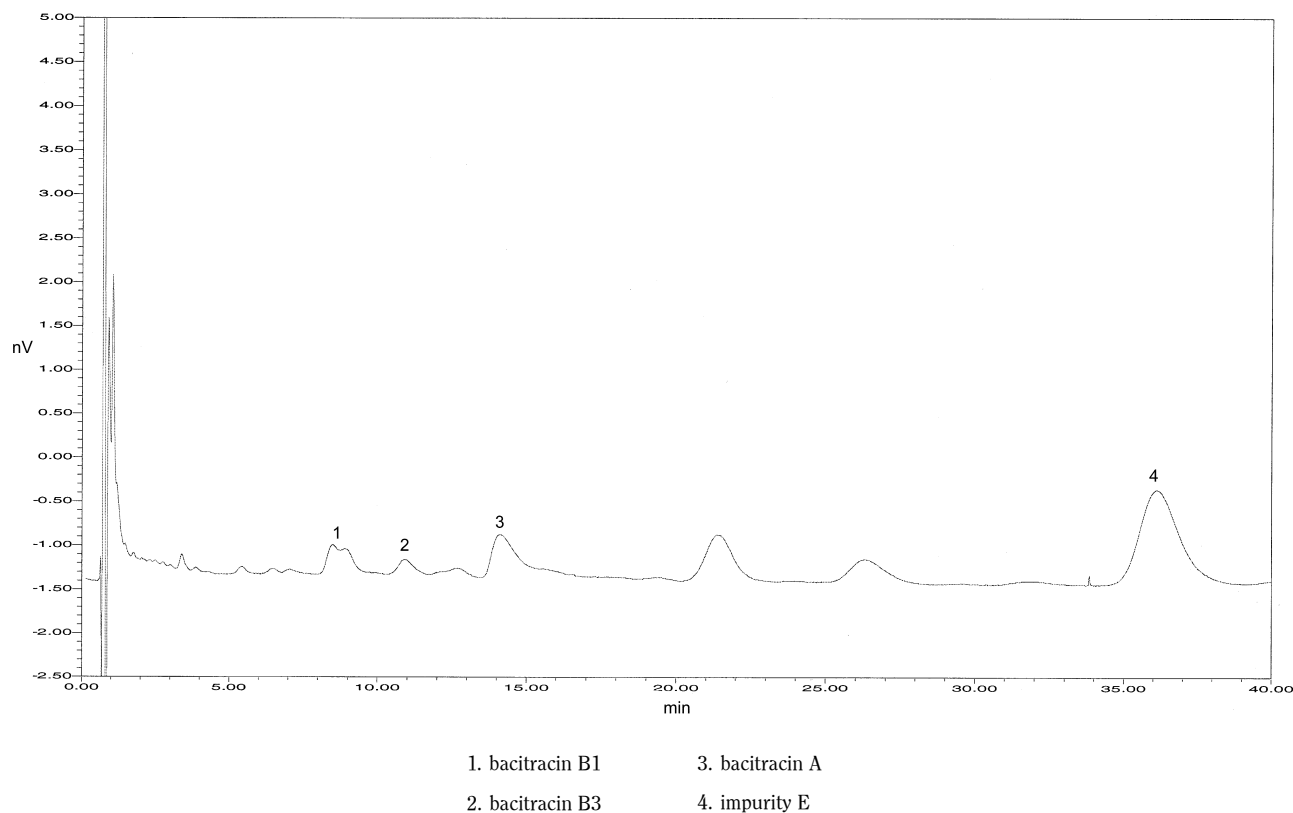


Figure 0465.-2. – Chromatogram of the test for impurity E in bacitracin obtained with reference solution (d) at 300 nm

Detection: spectrophotometer at 254 nm; spectrophotometer at 300 nm for reference solution (d).

Injection: test solution and reference solutions (b) and (d).

Limit:

- **impurity E:** not more than 1.2 times the area of the principal peak in the chromatogram obtained with reference solution (b) (6.0 per cent).

Loss on drying (2.2.32): maximum 5.0 per cent, determined on 1.000 g by drying at 60 °C over *diphosphorus pentoxide R* at a pressure not exceeding 0.1 kPa for 3 h.

Sulphated ash (2.4.14): maximum 1.0 per cent, determined on 1.0 g.

Sterility (2.6.1). If intended for the preparation of ophthalmic dosage forms without a further appropriate sterilisation procedure, it complies with the test for sterility.

Bacterial endotoxins (2.6.14): less than 0.8 IU/mg, if intended for use in the manufacture of ophthalmic dosage forms without a further appropriate procedure for the removal of bacterial endotoxins.

ASSAY

Carry out the microbiological assay of antibiotics (2.7.2). Use *bacitracin zinc CRS* as the reference substance.

STORAGE

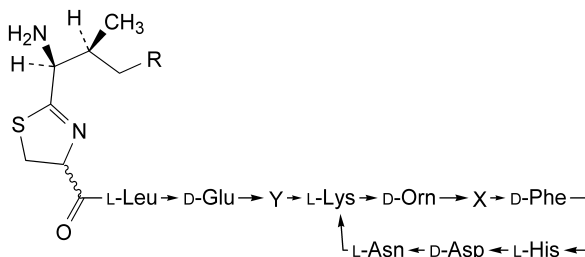
In an airtight container at 2 °C to 8 °C. If the substance is sterile, store in a sterile, airtight, tamper-proof container.

LABELLING

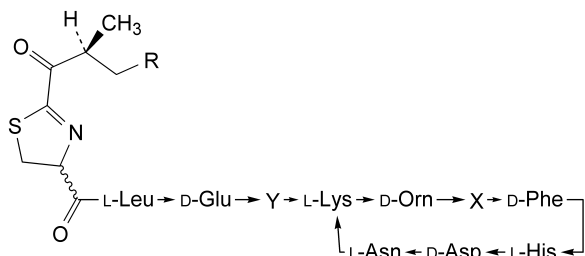
The label states, where applicable:

- that the substance is sterile,
- that the substance is free from bacterial endotoxins.

IMPURITIES



- A. X = L-Val, Y = L-Ile, R = H: bacitracin C1,
 B. X = L-Ile, Y = L-Val, R = H: bacitracin C2,
 C. X = Y = L-Val, R = CH₃: bacitracin C3,
 D. X = Y = L-Val, R = H: bacitracin E,



- E. X = Y = L-Ile, R = CH₃: bacitracin F,
 F. X = Y = L-Ile, R = H: bacitracin H1,
 G. X = L-Val, Y = L-Ile, R = CH₃: bacitracin H2,
 H. X = L-Ile, Y = L-Val, R = CH₃: bacitracin H3,
 I. X = L-Val, Y = L-Ile, R = H: bacitracin I1,
 J. X = L-Ile, Y = L-Val, R = H: bacitracin I2,

K. X = Y = L-Val, R = CH₃: bacitracin I3.

01/2005:0466

BACITRACIN ZINC

Bacitracinum zincum

DEFINITION

Zinc complex of bacitracin, which consists of a mixture of antimicrobial polypeptides produced by certain strains of *Bacillus licheniformis* or *Bacillus subtilis*, the main components being bacitracins A, B1, B2 and B3.

Content: minimum 60 IU/mg (dried substance).

CHARACTERS

Appearance: white or light yellowish-grey powder, hygroscopic.

Solubility: slightly soluble in water and in alcohol.

IDENTIFICATION

First identification: B, C.

Second identification: A, C.

A. Thin-layer chromatography (2.2.27).

Test solution. Dissolve 10 mg of the substance to be examined in 0.5 ml of *dilute hydrochloric acid R* and dilute to 1.0 ml with *water R*.

Reference solution. Dissolve 10 mg of *bacitracin zinc CRS* in 0.5 ml of *dilute hydrochloric acid R* and dilute to 1.0 ml with *water R*.

Plate: *TLC silica gel plate R*.

Mobile phase: *glacial acetic acid R, water R, butanol R* (1:2:4 V/V/V).

Application: 10 µl.

Development: over half of the plate.

Drying: at 100-105 °C.

Detection: spray with *ninhydrin solution R1* and heat at 110 °C for 5 min.

Results: the spots in the chromatogram obtained with the test solution are similar in position, size and colour to the spots in the chromatogram obtained with the reference solution.

B. It complies with the test for composition (see Tests).

C. Ignite about 0.15 g, allow to cool and dissolve the residue in 1 ml of *dilute hydrochloric acid R*. Add 4 ml of *water R*. The solution gives the reaction of zinc (2.3.1).

TESTS

pH (2.2.3): 6.0 to 7.5.

Shake 1.0 g for about 1 min with 10 ml of *carbon dioxide-free water R* and filter.

Composition. Liquid chromatography (2.2.29): use the normalisation procedure. *Prepare the solutions immediately before use.*

Test solution. Dissolve 0.100 g of the substance to be examined in 50.0 ml of a 40 g/l solution of *sodium edetate R* adjusted to pH 7.0 with *dilute sodium hydroxide R*.

Reference solution (a). Dissolve 20.0 mg of *bacitracin zinc CRS* in 10.0 ml of a 40 g/l solution of *sodium edetate R* adjusted to pH 7.0 with *dilute sodium hydroxide R*.

Reference solution (b). Dilute 5.0 ml of the test solution to 100.0 ml with *water R*.

Reference solution (c). Dilute 1.0 ml of reference solution (b) to 10.0 ml with *water R*.

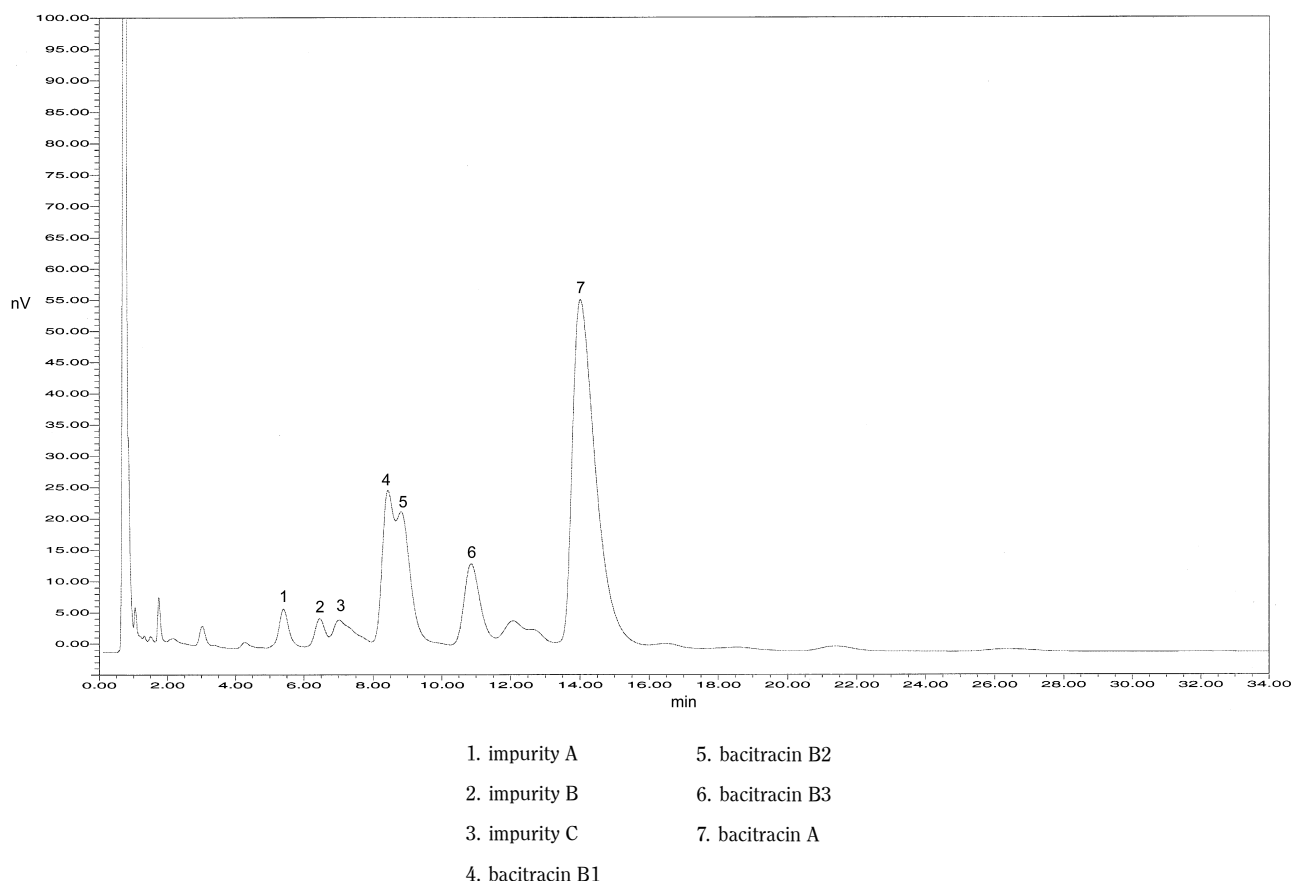


Figure 0466.-1. – Chromatogram of the test for composition in bacitracin zinc obtained with the test solution at 254 nm

Reference solution (d). Dissolve 50.0 mg of the substance to be examined in 25.0 ml of a 40 g/l solution of *sodium edetate R* adjusted to pH 7.0 with *dilute sodium hydroxide R*. Heat in a boiling water-bath for 30 min. Cool to room temperature.

Blank solution. A 40 g/l solution of *sodium edetate R* adjusted to pH 7.0 with *dilute sodium hydroxide R*.

Column:

- size: $l = 0.25$ m, $\varnothing = 4.6$ mm,
- stationary phase: end-capped octadecylsilyl silica gel for chromatography *R* (5 μ m).

Mobile phase: add 520 volumes of *methanol R1*, 40 volumes of *acetonitrile R* and 300 volumes of *water R* to 100 volumes of a 34.8 g/l solution of *dipotassium hydrogen phosphate R*, adjusted to pH 6.0 with a 27.2 g/l solution of *potassium dihydrogen phosphate R*.

Flow rate: 1.0 ml/min.

Detection: spectrophotometer at 254 nm.

Injection: 100 μ l; inject the blank, the test solution and reference solutions (a) and (c).

Run time: 3 times the retention time of bacitracin A.

Relative retention with reference to bacitracin A (retention time = 15 min to 25 min): bacitracin B1 = about 0.6; bacitracin B3 = about 0.8; impurity E = about 2.5.

If necessary, adjust the composition of the mobile phase by changing the amount of organic modifier whilst keeping the ratio constant between methanol and acetonitrile.

System suitability: reference solution (a):

- **peak-to-valley ratio:** minimum of 1.2, where H_p = height above the baseline of the peak due to bacitracin B1 and H_v = height above the baseline of the lowest point of the curve separating this peak from the peak due to bacitracin B2.

Limits:

- **bacitracin A:** minimum 40.0 per cent,
- **sum of bacitracins A, B1, B2 and B3:** minimum 70.0 per cent.
- **disregard limit:** the area of the peak due to bacitracin A in the chromatogram obtained with reference solution (c) (0.5 per cent); disregard any peak observed in the blank run.

Related peptides. Liquid chromatography (2.2.29) as described in the test for composition.

See Figure 0466.-1.

Limit:

- **sum of the areas of all peaks eluting before the peak due to bacitracin B1:** maximum 20.0 per cent.

Impurity E. Liquid chromatography (2.2.29) as described in the test for composition.

See Figure 0466.-2.

Detection: spectrophotometer at 254 nm; spectrophotometer at 300 nm for reference solution (d).

Injection: test solution and reference solutions (b) and (d).

Limit:

- **impurity E:** not more than 1.2 times the area of the principal peak in the chromatogram obtained with reference solution (b) (6.0 per cent).

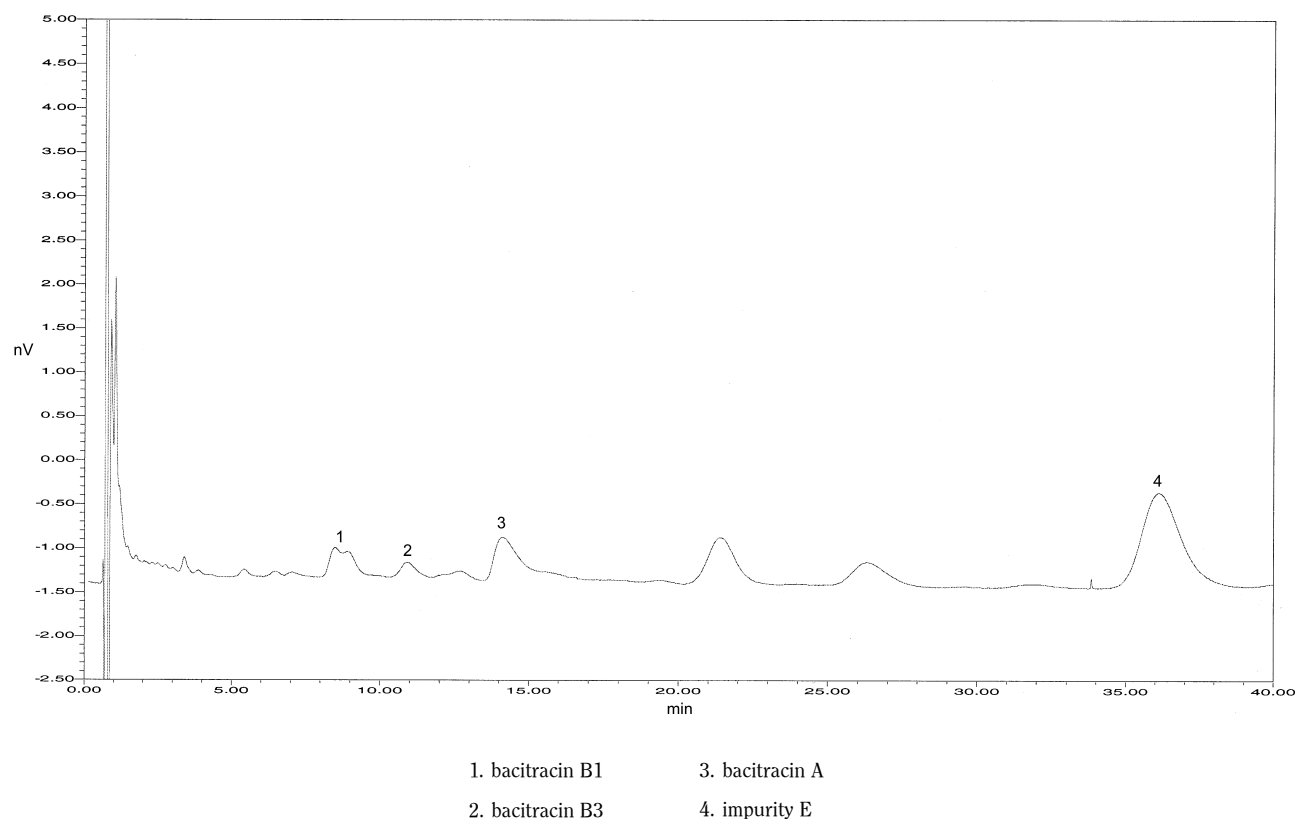


Figure 0466.-2. — Chromatogram of the test for impurity E in bacitracin zinc obtained with reference solution (d) at 300 nm

Zinc: 4.0 per cent to 6.0 per cent (dried substance).

Dissolve 0.200 g in a mixture of 2.5 ml of *dilute acetic acid R* and 2.5 ml of water. Add 50 ml of *water R*, 50 mg of *xylene orange triturate R* and sufficient *hexamethylenetetramine R* to produce a red colour. Add 2 g of *hexamethylenetetramine R* in excess. Titrate with 0.01 M *sodium edetate* until a yellow colour is obtained.

1 ml of 0.01 M *sodium edetate* is equivalent to 0.654 mg of Zn.

Loss on drying (2.2.32): maximum 5.0 per cent, determined on 1.000 g by drying at 60 °C over *diphosphorus pentoxide R* at a pressure not exceeding 0.1 kPa for 3 h.

Sterility (2.6.1). If intended for administration by spraying into internal body cavities without a further appropriate sterilisation procedure, it complies with the test for sterility.

Pyrogens (2.6.8). If intended for administration by spraying into internal body cavities without a further appropriate procedure for the removal of pyrogens, it complies with the test for pyrogens. Inject per kilogram of the rabbit's mass 1 ml of the supernatant liquid obtained by centrifuging a suspension containing 11 mg per millilitre in a 9 g/l solution of *sodium chloride R*.

ASSAY

Suspend 50.0 mg in 5 ml of *water R*, add 0.5 ml of *dilute hydrochloric acid R* and dilute to 100.0 ml with *water R*. Allow the solution to stand for 30 min. Carry out the microbiological assay of antibiotics (2.7.2).

STORAGE

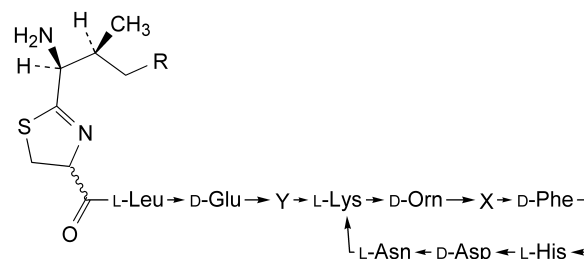
In an airtight container. If the substance is sterile, store in a sterile, airtight, tamper-proof container.

LABELLING

The label states, where applicable:

- that the substance is sterile,
- that the substance is apyrogenic.

IMPURITIES

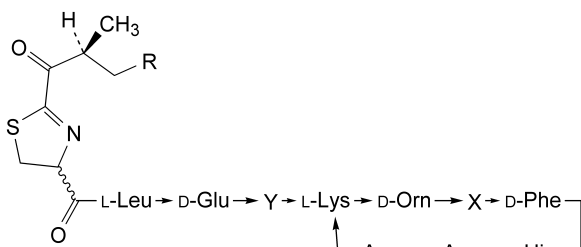


A. X = L-Val, Y = L-Ile, R = H: bacitracin C1,

B. X = L-Ile, Y = L-Val, R = H: bacitracin C2,

C. X = Y = L-Val, R = CH₃: bacitracin C3,

D. X = Y = L-Val, R = H: bacitracin E,



E. X = Y = L-Ile, R = CH₃: bacitracin F,

F. X = Y = L-Ile, R = H: bacitracin H1,

G. X = L-Val, Y = L-Ile, R = CH₃: bacitracin H2,

H. X = L-Ile, Y = L-Val, R = CH₃: bacitracin H3,

I. X = L-Val, Y = L-Ile, R = H: bacitracin I1,

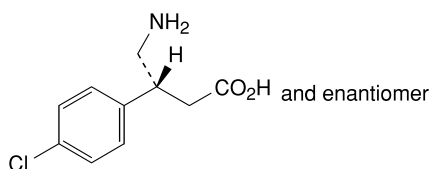
J. X = L-Ile, Y = L-Val, R = H: bacitracin I2,

K. X = Y = L-Val, R = CH₃: bacitracin I3.

01/2005:0653

BACLOFEN

Baclofenum



C₁₀H₁₂ClNO₂

M_r 213.7

DEFINITION

Baclofen contains not less than 98.0 per cent and not more than the equivalent of 101.0 per cent of (3*RS*)-4-amino-3-(4-chlorophenyl)butanoic acid, calculated with reference to the anhydrous substance.

CHARACTERS

A white or almost white powder, slightly soluble in water, very slightly soluble in alcohol, practically insoluble in acetone. It dissolves in dilute mineral acids and in dilute solutions of alkali hydroxides.

It shows polymorphism.

IDENTIFICATION

First identification: B.

Second identification: A, C.

- Dissolve 70 mg in *water R* and dilute to 100.0 ml with the same solvent. Examined between 220 nm and 320 nm (2.2.25), the solution shows three absorption maxima, at 259 nm, 266 nm and 275 nm. The specific absorbances at these maxima are 9.8 to 10.8, 11.5 to 12.7 and 8.4 to 9.3, respectively. The test is not valid unless in the test for resolution (2.2.25) the ratio of absorbances is at least 1.5.
- Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *baclofen CRS*. Examine as discs prepared using 3 mg of substance and 300 mg of *potassium bromide R*. If the spectra obtained with the substance to be examined and the reference substance show differences, dissolve 0.1 g of each of the substances separately in 1 ml of *dilute sodium hydroxide solution R* and add 10 ml of *alcohol R* and 1 ml of *dilute acetic acid R*. Allow to stand for 1 h. Filter, wash the precipitate with *alcohol R* and dry *in vacuo*. Prepare new discs and record the spectra.

- Examine by thin-layer chromatography (2.2.27), using *silica gel G R* as the coating substance.

Test solution. Dissolve 10 mg of the substance to be examined in the mobile phase (see below) and dilute to 10 ml with the mobile phase.

Reference solution. Dissolve 10 mg of *baclofen CRS* in the mobile phase and dilute to 10 ml with the mobile phase.

Apply separately to the plate 5 µl of each solution.

Develop over a path of 12 cm using a mixture of 5 volumes of *anhydrous formic acid R*, 5 volumes of *water R*, 20 volumes of *methanol R*, 30 volumes of *chloroform R* and 40 volumes of *ethyl acetate R*. Allow the solvents to evaporate. Spray with *ninhydrin solution R3* until the plate is slightly wet. Place the plate in an oven maintained at 100 °C for 10 min. Examine in daylight. The principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

TESTS

Appearance of solution. Dissolve 0.50 g in 1 M *sodium hydroxide* and dilute to 25 ml with the same solvent. The solution is not more opalescent than reference suspension II (2.2.1) and not more intensely coloured than reference solution BY₅ (2.2.2, *Method II*).

Related substances. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 25.0 mg of the substance to be examined in the mobile phase and dilute to 10.0 ml with the mobile phase.

Reference solution (a). Dissolve 25.0 mg of *baclofen impurity A CRS* in the mobile phase and dilute to 10.0 ml with the mobile phase.

Reference solution (b). Dilute 1.0 ml of reference solution (a) to 100.0 ml with the mobile phase.

Reference solution (c). Dilute 2.0 ml of the test solution to 100.0 ml with the mobile phase.

Reference solution (d). Dilute 2.0 ml of the test solution and 2.0 ml of reference solution (a) to 100.0 ml with the mobile phase.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4.0 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (10 µm),
- as mobile phase at a flow-rate of 2.0 ml/min a mixture prepared as follows: dissolve 1.822 g of *sodium hexanesulphonate R* in 1 litre of a mixture of 560 volumes of *water R*, 440 volumes of *methanol R* and 5 volumes of *glacial acetic acid R*,
- as detector a spectrophotometer set at 266 nm.

Adjust the sensitivity of the system so that the height of the principal peak in the chromatogram obtained with 20 µl of reference solution (c) is at least 50 per cent of the full scale of the recorder. Inject 20 µl of reference solution (d). The test is not valid unless the resolution between the peaks corresponding to baclofen and baclofen impurity A is at least 2.0. Inject separately 20 µl of the test solution, reference solution (b) and reference solution (c). Continue the chromatography for five times the retention time of the principal peak. In the chromatogram obtained with the test solution: the area of the peak corresponding to baclofen impurity A is not greater than the area of the principal peak in the chromatogram obtained with reference solution (b) (1.0 per cent); the sum of the areas of all the peaks, apart

from the principal peak, is not greater than the area of the principal peak in the chromatogram obtained with reference solution (c) (2.0 per cent).

Water (2.5.12). Not more than 1.0 per cent, determined on 1.000 g by the semi-micro determination of water.

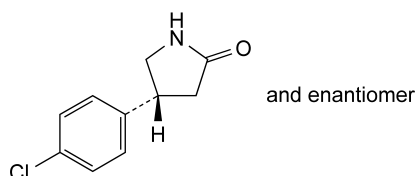
Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

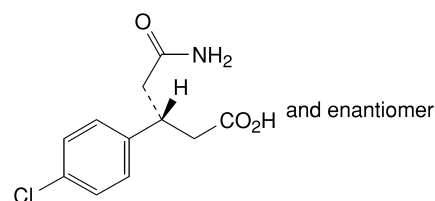
Dissolve 0.1500 g in 50 ml of *anhydrous acetic acid R*. Titrate with 0.1 M *perchloric acid*, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *perchloric acid* is equivalent to 21.37 mg of $C_{10}H_{12}ClNO_2$.

IMPURITIES



A. (4*RS*)-4-(4-chlorophenyl)pyrrolidin-2-one,

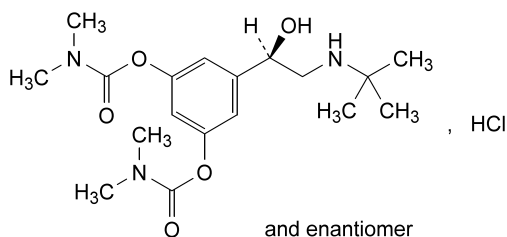


B. (3*RS*)-5-amino-3-(4-chlorophenyl)-5-oxopentanoic acid.

01/2005:1293

BAMBUTEROL HYDROCHLORIDE

Bambuteroli hydrochloridum



$C_{18}H_{30}ClN_3O_5$

M_r 403.9

DEFINITION

Bambuterol hydrochloride contains not less than 98.5 per cent and not more than the equivalent of 101.5 per cent of 5-[(1*RS*)-2-[(1,1-dimethylethyl)amino]-1-hydroxyethyl]-1,3-phenylene bis(dimethylcarbamate) hydrochloride, calculated with reference to the anhydrous substance.

CHARACTERS

A white or almost white, crystalline powder, freely soluble in water, soluble in alcohol.

It shows polymorphism.

IDENTIFICATION

A. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *bambuterol hydrochloride CRS*. Examine the substances prepared as discs. If the spectra obtained

show differences, dissolve the substance to be examined and the reference substance in a mixture of 1 volume of *water R* and 6 volumes of *acetone R*, cool in ice to precipitate and dry both precipitates *in vacuo* at 50 °C to constant weight. Record new spectra using the residues.

B. It gives reaction (a) of chlorides (2.3.1).

TESTS

Solution S. Dissolve 4.0 g in *carbon dioxide-free water R* and dilute to 20.0 ml with the same solvent.

Acidity or alkalinity. To 10 ml of solution S add 0.2 ml of *methyl red solution R* and 0.2 ml of 0.01 M *hydrochloric acid*. The solution is red. Add 0.4 ml of 0.01 M *sodium hydroxide*. The solution is yellow.

Optical rotation (2.2.7). Dilute 1 ml of solution S to 10 ml with *carbon dioxide-free water R*. The angle of optical rotation is -0.10° to $+0.10^\circ$.

Related substances. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 5.0 mg of the substance to be examined in the mobile phase and dilute to 10.0 ml with the mobile phase.

Reference solution (a). Dissolve 1.0 mg of *formoterol fumarate dihydrate CRS* in the mobile phase and dilute to 10.0 ml with the mobile phase. Mix 0.8 ml of the solution with 0.4 ml of the test solution and dilute to 100.0 ml with the mobile phase.

Reference solution (b). Dilute 1.0 ml of the test solution to 50.0 ml with the mobile phase. Dilute 2.0 ml of the solution to 20.0 ml with the mobile phase.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.15 m long and 4.6 mm in internal diameter packed with *base-deactivated octadecylsilyl silica gel for chromatography R* (5 μ m),
- as mobile phase at a flow rate of 1.5 ml/min a mixture prepared as follows: dissolve 1.3 g of *sodium octanesulphonate R* in 430 ml of a mixture of 25 volumes of *acetonitrile R1* and 75 volumes of *methanol R*; then mix this solution with 570 ml of 0.050 M phosphate buffer pH 3.0 (dissolve 6.90 g of *sodium dihydrogen phosphate monohydrate R* in *water R* and dilute to 1000 ml with *water R*, adjust the pH to 3.0 with a 50 g/l solution of *dilute phosphoric acid R*),
- as detector a spectrophotometer set at 214 nm.

Adjust the sensitivity of the system so that the height of the principal peak in the chromatogram obtained with 20 μ l of reference solution (b) is about 50 per cent of the full scale of the recorder.

Inject 20 μ l of reference solution (a). When the chromatograms are recorded under the prescribed conditions, the retention times are: *formoterol* about 7 min and *bambuterol* about 9 min. The test is not valid unless the resolution between the peaks corresponding to *bambuterol* and *formoterol* is at least 5.0. Continue the chromatography of the test solution for 1.5 times the retention time of *bambuterol*. If necessary adjust the composition of the mobile phase. Increase the content of phosphate buffer to increase the retention time.

Inject separately 20 μ l of the mobile phase, 20 μ l of the test solution and 20 μ l of reference solution (b). In the chromatogram obtained with the test solution the area of any peak, apart from the principal peak, is not greater than the area of the principal peak obtained with reference solution (b) (0.2 per cent); the sum of the areas of all the peaks, apart from the principal peak, is not greater than three times the area of the principal peak in the

chromatogram obtained with reference solution (b) (0.6 per cent). Disregard any peak due to the mobile phase and any peak with an area less than 0.25 times the area of the principal peak obtained with reference solution (b).

Water (2.5.12). Not more than 0.5 per cent, determined on 0.500 g by the semi-micro determination of water.

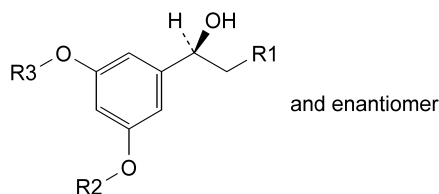
Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

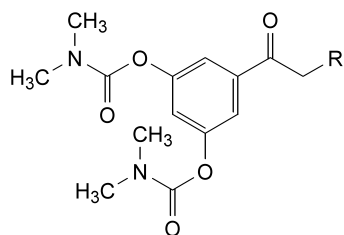
Dissolve 0.320 g in 50 ml of *alcohol R* and add 5 ml of 0.01 *M* hydrochloric acid. Carry out a potentiometric titration (2.2.20), using 0.1 *M* sodium hydroxide. Read the volume added between the two points of inflexion.

1 ml of 0.1 *M* sodium hydroxide is equivalent to 40.39 mg of $C_{18}H_{30}ClN_3O_5$.

IMPURITIES



- A. $R_1 = NH-C(CH_3)_3$, $R_2 = R_3 = H$: (1*RS*)-1-(3,5-dihydroxyphenyl)-2-[(1,1-dimethylethyl)amino]ethanol (terbutaline),
- B. $R_1 = OH$, $R_2 = R_3 = CO-N(CH_3)_2$: 5-[(1*RS*)-1,2-dihydroxyethyl]-1,3-phenylene bis(dimethylcarbamate),
- C. $R_1 = NH-C(CH_3)_3$, $R_2 = H$, $R_3 = CO-N(CH_3)_2$: 3-[(1*RS*)-2-[(1,1-dimethylethyl)amino]-1-hydroxyethyl]-5-hydroxyphenyl dimethylcarbamate,
- D. $R_1 = H$, $R_2 = R_3 = CO-N(CH_3)_2$: 5-[(1*RS*)-1-hydroxyethyl]-1,3-phenylene bis(dimethylcarbamate),



- E. $R = H$: 5-acetyl-1,3-phenylene bis(dimethylcarbamate),
- F. $R = NH-C(CH_3)_3$: 5-[[[(1,1-dimethylethyl)amino]acetyl]-1,3-phenylene bis(dimethylcarbamate).

DEFINITION

Barbital contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of 5,5-diethylpyrimidine-2,4,6-(1*H*,3*H*,5*H*)-trione, calculated with reference to the dried substance.

CHARACTERS

A white, crystalline powder or colourless crystals, slightly soluble in water, soluble in boiling water and in alcohol. It forms water-soluble compounds with alkali hydroxides and carbonates and with ammonia.

IDENTIFICATION

First identification: A, B.

Second identification: A, C, D.

- A. Determine the melting point (2.2.14) of the substance to be examined. Mix equal parts of the substance to be examined and *barbital CRS* and determine the melting point of the mixture. The difference between the melting points (which are about 190 °C) is not greater than 2 °C.
- B. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *barbital CRS*.
- C. Examine by thin-layer chromatography (2.2.27), using *silica gel GF₂₅₄ R* as the coating substance.

Test solution. Dissolve 75 mg of the substance to be examined in *alcohol R* and dilute to 25 ml with the same solvent.

Reference solution. Dissolve 75 mg of *barbital CRS* in *alcohol R* and dilute to 25 ml with the same solvent.

Apply separately to the plate 10 µl of each solution. Develop over a path of 18 cm using the lower layer of a mixture of 5 volumes of *concentrated ammonia R*, 15 volumes of *alcohol R* and 80 volumes of *chloroform R*. Examine immediately in ultraviolet light at 254 nm. The principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with the reference solution.

- D. It gives the reaction of non-nitrogen substituted barbiturates (2.3.1).

TESTS

Appearance of solution. Dissolve 1.0 g in a mixture of 4 ml of *dilute sodium hydroxide solution R* and 6 ml of *water R*. The solution is clear (2.2.1) and not more intensely coloured than reference solution Y_6 (2.2.2, *Method II*).

Acidity. Boil 1.0 g with 50 ml of *water R* for 2 min, allow to cool and filter. To 10 ml of the filtrate add 0.15 ml of *methyl red solution R*. The solution is orange-yellow. Not more than 0.1 ml of 0.1 *M* sodium hydroxide is required to produce a pure yellow colour.

Related substances. Examine by thin-layer chromatography (2.2.27), using *silica gel GF₂₅₄ R* as the coating substance.

Test solution. Dissolve 1.0 g of the substance to be examined in *alcohol R* and dilute to 100 ml with the same solvent.

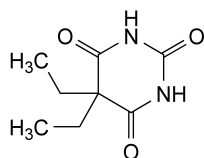
Reference solution. Dilute 0.5 ml of the test solution to 100 ml with *alcohol R*.

Apply separately to the plate 20 µl of each solution. Develop over a path of 15 cm using the lower layer of a mixture of 5 volumes of *concentrated ammonia R*, 15 volumes of *alcohol R* and 80 volumes of *chloroform R*. Examine immediately in ultraviolet light at 254 nm. Spray with *diphenylcarbazone mercuric reagent R*. Allow the plate to dry in air and spray with freshly prepared *alcoholic potassium hydroxide solution R* diluted 1 in 5 with

01/2005:0170

BARBITAL

Barbitalum



$C_8H_{12}N_2O_3$

M_r 184.2

aldehyde-free alcohol R. Heat at 100 °C to 105 °C for 5 min and examine immediately. When examined in ultraviolet light and after spraying, any spot in the chromatogram obtained with the test solution, apart from the principal spot, is not more intense than the spot in the chromatogram obtained with the reference solution (0.5 per cent).

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.00 g by drying in an oven at 100 °C to 105 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 85.0 mg in 5 ml of *pyridine R*. Add 0.5 ml of *thymolphthalein solution R* and 10 ml of *silver nitrate solution in pyridine R*. Titrate with 0.1 M *ethanolic sodium hydroxide* until a pure blue colour is obtained. Carry out a blank titration.

1 ml of 0.1 M *ethanolic sodium hydroxide* is equivalent to 9.21 mg of $C_8H_{12}N_2O_3$.

01/2005:0010

BARIUM SULPHATE

Barii sulfas

BaSO₄M_r 233.4

CHARACTERS

A fine, heavy, white powder, free from gritty particles, practically insoluble in water and in organic solvents. It is very slightly soluble in acids and in solutions of alkali hydroxides.

IDENTIFICATION

- A. Boil 0.2 g with 5 ml of a 500 g/l solution of *sodium carbonate R* for 5 min, add 10 ml of *water R*, filter and acidify a part of the filtrate with *dilute hydrochloric acid R*. The solution gives the reactions of sulphates (2.3.1).
- B. Wash the residue collected in the preceding test with three successive small quantities of *water R*. To the residue add 5 ml of *dilute hydrochloric acid R*, filter and add to the filtrate 0.3 ml of *dilute sulphuric acid R*. A white precipitate is formed that is insoluble in *dilute sodium hydroxide solution R*.

TESTS

Solution S. To 20.0 g add 40 ml of *distilled water R* and 60 ml of *dilute acetic acid R*. Boil for 5 min, filter and dilute the cooled filtrate to 100 ml with *distilled water R*.

Acidity or alkalinity. Heat 5.0 g with 20 ml of *carbon dioxide-free water R* on a water-bath for 5 min and filter. To 10 ml of the filtrate add 0.05 ml of *bromothymol blue solution R1*. Not more than 0.5 ml of 0.01 M *hydrochloric acid* or 0.01 M *sodium hydroxide* is required to change the colour of the indicator.

Acid-soluble substances. Evaporate 25 ml of solution S to dryness on a water-bath and dry to constant mass at 100–105 °C. The residue weighs not more than 15 mg (0.3 per cent).

Oxidisable sulphur compounds. Shake 1.0 g with 5 ml of *water R* for 30 s and filter. To the filtrate add 0.1 ml of *starch solution R*, dissolve 0.1 g of *potassium iodide R* in the mixture, add 1.0 ml of a freshly prepared 3.6 mg/l solution of *potassium iodate R* and 1 ml of 1 M *hydrochloric acid*

and shake well. The colour of the solution is more intense than that of a standard prepared at the same time and in the same manner omitting the potassium iodate.

Soluble barium salts. To 10 ml of solution S add 1 ml of *dilute sulphuric acid R*. After 1 h, any opalescence in the solution is not more intense than that in a mixture of 10 ml of solution S and 1 ml of *distilled water R*.

Phosphates. To 1.0 g add a mixture of 3 ml of *dilute nitric acid R* and 7 ml of *water R*. Heat on a water-bath for 5 min, filter and dilute the filtrate to 10 ml with *water R*. Add 5 ml of *molybdovanadic reagent R*. After 5 min, any yellow colour in the test solution is not more intense than that in a standard prepared at the same time and in the same manner using 10 ml of *phosphate standard solution* (5 ppm PO₄) R (50 ppm).

Arsenic (2.4.2). In a small long-necked combustion flask, shake 0.5 g with 2 ml of *nitric acid R* and 30 ml of *water R*, insert a small funnel in the neck of the flask, heat in an inclined position on a water-bath for 2 h, allow to cool, adjust to the original volume with *water R* and filter. Wash the residue by decantation with three quantities, each of 5 ml, of *water R*. Combine the filtrate and washings, add 1 ml of *sulphuric acid R*, evaporate to dryness on a water-bath, and heat until copious white fumes are evolved. Dissolve the residue in 10 ml of *dilute sulphuric acid R* and add 10 ml of *water R*. The solution complies with limit test A for arsenic (2 ppm).

Heavy metals (2.4.8). Dilute 10 ml of solution S to 20 ml with *water R*, 12 ml of this solution complies with limit test A for heavy metals (10 ppm). Prepare the standard using *lead standard solution* (1 ppm Pb) R.

Loss on ignition. Not more than 2.0 per cent, determined on 1.0 g at 600 °C.

Sedimentation. Place 5.0 g in a glass-stoppered, 50 ml graduated cylinder having the 50 ml graduation mark 14 cm from the base. Add *water R* to 50 ml, shake the mixture for 5 min and allow to stand for 15 min. The barium sulphate does not settle below the 15 ml mark.

01/2005:1975

BASIC BUTYLATED METHACRYLATE COPOLYMER

Copolymerum methacrylatis butylati basicum

DEFINITION

Copolymer of 2-(dimethylamino)ethyl methacrylate, butyl methacrylate and methyl methacrylate having a mean relative molecular mass of about 150 000. The ratio of 2-(dimethylamino)ethyl methacrylate groups to butyl methacrylate and methyl methacrylate groups is about 2:1:1. *Content of dimethylaminoethyl groups*: 20.8 per cent to 25.5 per cent (dried substance).

CHARACTERS

Appearance: colourless or yellowish granules or white powder, slightly hygroscopic.

Solubility: practically insoluble in water, freely soluble in methylene chloride. It dissolves slowly in alcohol.

IDENTIFICATION

- A. Infrared absorption spectrophotometry (2.2.24).

Comparison: Ph. Eur. reference spectrum of basic butylated methacrylate copolymer.

B. It complies with the limits of the assay.

TESTS

Solution S. Dissolve 12.5 g in a mixture of 35.0 g of *acetone R* and 52.5 g of *2-propanol R*.

Viscosity (2.2.10): 3 mPas to 6 mPas, determined on solution S.

Apparatus: rotating viscosimeter.

Dimensions:

- *spindle*: diameter = 25.15 mm, height = 90.74 mm, shaft diameter = 4 mm,
- *cylinder*: diameter = 27.62 mm, height = 0.135 m.

Stirring speed: 30 r/min.

Volume of solution: 16 ml of solution S.

Temperature: 20 °C.

Absorbance (2.2.25): maximum 0.30 at 420 nm, determined on solution S.

Appearance of film. Spread evenly 1.0 ml of solution S on a glass plate. Upon drying a clear film is formed.

Monomers: maximum 0.3 per cent, for the sum of contents of butyl methacrylate, methyl methacrylate and 2-dimethylaminoethyl methacrylate calculated by procedures A and B.

A. Butyl methacrylate and methyl methacrylate. Liquid chromatography (2.2.29).

Test solution. Dissolve 1.00 g of the substance to be examined in *phosphate buffer solution pH 2.0 R* and dilute to 50.0 ml with the same buffer solution.

Reference solution. Dissolve 10.0 mg of *butyl methacrylate R* and 10.0 mg of *methyl methacrylate R* in 10.0 ml of *acetonitrile R* and dilute to 50.0 ml with *water R*. Dilute 1.0 ml of the solution to 50.0 ml with *water R*.

Column:

- *size*: $l = 0.125$ m, $\varnothing = 4.6$ mm,
- *stationary phase*: octadecylsilyl silica gel for chromatography *R* (7 μ m).

Mobile phase: *phosphate buffer solution pH 2.0 R*, *methanol R* (45:55 *V/V*).

Flow rate: 2.0 ml/min.

Detection: spectrophotometer at 205 nm.

Injection: 50 μ l.

B. 2-Dimethylaminoethyl methacrylate. Liquid chromatography (2.2.29).

Test solution. Dissolve 1.00 g of the substance to be examined in *tetrahydrofuran R* and dilute to 50.0 ml with the same solvent.

Reference solution. Dissolve 10.0 mg of *2-(dimethylamino)ethyl methacrylate R* in *tetrahydrofuran R* and dilute to 50.0 ml with the same solvent. Dilute 2.0 ml of the solution to 50.0 ml with *tetrahydrofuran R*.

Column:

- *size*: $l = 0.125$ m, $\varnothing = 4.6$ mm,
- *stationary phase*: aminopropylsilyl silica gel for chromatography *R* (10 μ m).

Mobile phase: mix 25 volumes of a 3.404 g/l solution of *potassium dihydrogen phosphate R* and 75 volumes of *tetrahydrofuran R*.

Flow rate: 2.0 ml/min.

Detection: spectrophotometer at 215 nm.

Injection: 50 μ l.

Heavy metals (2.4.8): maximum 20 ppm.

2.0 g complies with limit test C. Prepare the standard using 4.0 ml of *lead standard solution (10 ppm Pb) R*.

Loss on drying (2.2.32): maximum 2.0 per cent, determined on 1.000 g by drying in an oven at 110 °C for 3 h.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

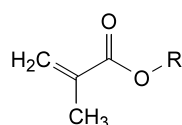
Dissolve 0.200 g in a mixture of 4 ml of *water R* and 96 ml of *anhydrous acetic acid R*. Titrate with 0.1 *M perchloric acid*, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 *M perchloric acid* is equivalent to 7.21 mg of C_4H_9NO .

STORAGE

In an airtight container.

IMPURITIES



A. $\text{R} = [\text{CH}_2]_3\text{-CH}_3$: butyl methacrylate,

B. $\text{R} = \text{CH}_3$: methyl methacrylate,

C. $\text{R} = \text{CH}_2\text{-CH}_2\text{-N}(\text{CH}_3)_2$: 2-(dimethylamino)ethyl methacrylate.

01/2005:1054

BEARBERRY LEAF

Uvae ursi folium

DEFINITION

Whole or cut, dried leaf of *Arctostaphylos uva-ursi* (L.) Spreng.

Content: minimum 7.0 per cent of anhydrous arbutin ($C_{12}H_{16}O_7$; M_r 272.3) (dried drug).

CHARACTERS

Macroscopic and microscopic characters described under identification tests A and B.

IDENTIFICATION

- A. The leaf, shiny and dark green on the adaxial surface, lighter on the abaxial surface, is normally 7 mm to 30 mm long and 5 mm to 12 mm wide. The entire leaf is obovate with smooth margins, somewhat reflexed downwards, narrowing at the base into a short petiole. The leaf is obtuse or retuse at its apex. The lamina is thick and coriaceous. The venation, pinnate and finely reticulate, is clearly visible on both surfaces. The adaxial surface is marked with sunken veinlets, giving it a characteristic grainy appearance. Only the young leaf has ciliated margins. Old leaves are glabrous.
- B. Reduce to a powder (355). The powder is green to greenish-grey or yellowish-green. Examine under a microscope using *chloral hydrate solution R*. The powder consists of fragments of epidermises which, seen in surface view, show polygonal cells covered by a thick smooth cuticle, and with straight, thick and irregularly pitted walls; anomocytic stomata (2.8.3), surrounded by 5 to 11 subsidiary cells and scars of hair bases only on the abaxial epidermis; fragments of palisade parenchyma, with 3 or 4 layers of cells of unequal lengths, and spongy

parenchyma; groups of lignified fibres from the pericycle, with rows of cells containing prisms of calcium oxalate; occasional conical, unicellular covering trichomes.

C. Thin-layer chromatography (2.2.27).

Test solution. To 0.5 g of the powdered drug (355) add 5 ml of a mixture of equal volumes of *methanol R* and *water R*, and heat under a reflux condenser for 10 min. Filter whilst hot. Wash the flask and the filter with a mixture of equal volumes of *methanol R* and *water R* and dilute to 5 ml with the same mixture of solvents.

Reference solution. Dissolve 25 mg of *arbutin R*, 25 mg of *gallic acid R* and 25 mg of *hydroquinone R* in *methanol R* and dilute to 10.0 ml with the same solvent.

Plate: TLC silica gel G plate *R*.

Mobile phase: *anhydrous formic acid R*, *water R*, *ethyl acetate R* (6:6:88 V/V/V).

Application: 10 µl of the reference solution and 20 µl of the test solution, as bands.

Development: over a path of 15 cm.

Drying: at 105–110 °C until the mobile phase has evaporated.

Detection: spray with a 10 g/l solution of *dichloroquinonechlorimide R* in *methanol R*. Next spray with a 20 g/l solution of *anhydrous sodium carbonate R*.

Results: see below the sequence of the zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, 2 or 3 blue bands and several brown or brownish-grey bands may be present in the chromatogram obtained with the test solution.

Top of the plate	
Hydroquinone: a blue zone	A blue zone
Gallic acid: a brownish zone	A brownish zone
_____	_____
_____	_____
Arbutin: a light blue zone	A light blue zone (arbutin)
Reference solution	Test solution

TESTS

Foreign matter (2.8.2): maximum 5 per cent of stems and maximum 3 per cent of other foreign matter.

Leaves of different colour: maximum 10 per cent, determined in the same manner as foreign matter (2.8.2).

Loss on drying (2.2.32): maximum 10.0 per cent, determined on 1.000 g of the powdered drug (355) by drying in an oven at 100–105 °C for 2 h.

Total ash (2.4.16): maximum 5.0 per cent.

ASSAY

Liquid chromatography (2.2.29).

Test solution. In a 100 ml flask with a ground-glass neck, place 0.800 g of the powdered drug (250). Add 20 ml of *water R* and heat under a reflux condenser on a water-bath for 30 min. Allow to cool and filter the liquid through a plug of absorbent cotton. Add the absorbent cotton to the residue in the 100 ml flask and extract with 20 ml of *water R* under a reflux condenser on a water-bath for 30 min. Allow to cool and filter through a paper filter. Combine the filtrates and dilute to 50.0 ml with *water R*. Filter the liquid through a paper filter. Discard the first 10 ml of the filtrate.

Reference solution. Dissolve 50.0 mg of *arbutin R* in the mobile phase and dilute to 50.0 ml with the mobile phase.

Column:

– **size:** $l = 0.25$ m, $\varnothing = 4$ mm,

– **stationary phase:** *base-deactivated octadecylsilyl silica gel for chromatography R* (5 µm).

Mobile phase: *methanol R*, *water R* (10:90 V/V).

Flow rate: 1.2 ml/min.

Detection: spectrophotometer at 280 nm.

Calculate the percentage content of arbutin using the following expression:

$$\frac{F_1 \times m_2 \times 100}{F_2 \times m_1}$$

F_1 = area of the peak due to arbutin in the chromatogram obtained with the test solution,

F_2 = area of the peak due to arbutin in the chromatogram obtained with the reference solution,

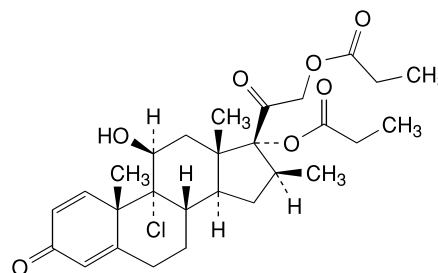
m_1 = mass of the drug to be examined, in grams,

m_2 = mass of *arbutin R* in the reference solution, in grams.

01/2005:0654

BECLOMETASONE DIPROPIONATE

Beclometasoni dipropionas



$C_{28}H_{37}ClO_7$

M_r 521.1

DEFINITION

Beclometasone dipropionate contains not less than 96.0 per cent and not more than the equivalent of 103.0 per cent of 9-chloro-11β,17,21-trihydroxy-16β-methylpregna-1,4-diene-3,20-dione 17,21-dipropionate, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, crystalline powder, practically insoluble in water, freely soluble in acetone, sparingly soluble in alcohol.

It melts at about 210 °C, with decomposition.

IDENTIFICATION

First identification: A, D.

Second identification: B, C, D.

A. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *beclometasone dipropionate CRS*. If the spectra obtained in the solid state with the substance to be examined and the reference substance show differences, record further spectra using 50 g/l solutions in *chloroform R*.

B. Examine by thin-layer chromatography (2.2.27), using as the coating substance a suitable silica gel with a fluorescent indicator having an optimal intensity at 254 nm.

Test solution (a). Dissolve 25 mg of the substance to be examined in *methanol R* with gentle heating and dilute to 5 ml with the same solvent. (This solution is also used to prepare test solution (b)). Dilute 2 ml of the solution to 10 ml with *chloroform R*.

Test solution (b). Transfer 2 ml of the solution obtained during preparation of test solution (a) to a 15 ml glass tube with a ground-glass stopper or a polytetrafluoroethylene cap. Add 10 ml of *saturated methanolic potassium hydrogen carbonate solution R* and immediately pass a stream of *nitrogen R* briskly through the solution for 5 min. Stopper the tube. Heat in a water-bath at 45 °C, protected from light, for 3 h. Allow to cool.

Reference solution (a). Dissolve 25 mg of *beclometasone dipropionate CRS* in *methanol R* with gentle heating and dilute to 5 ml with the same solvent. (This solution is also used to prepare reference solution (b)). Dilute 2 ml of the solution to 10 ml with *chloroform R*.

Reference solution (b). Transfer 2 ml of the solution obtained during preparation of reference solution (a) to a 15 ml glass tube with a ground-glass stopper or a polytetrafluoroethylene cap. Add 10 ml of *saturated methanolic potassium hydrogen carbonate solution R* and immediately pass a stream of *nitrogen R* briskly through the solution for 5 min. Stopper the tube. Heat in a water-bath at 45 °C, protected from light, for 3 h. Allow to cool.

Apply separately to the plate 5 µl of each solution. Prepare the mobile phase by mixing 1.2 volumes of *water R* and 8 volumes of *methanol R* and adding the mixture to a mixture of 15 volumes of *ether R* and 77 volumes of *methylene chloride R*. Develop over a path of 15 cm. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. The chromatogram obtained with test solution (b) shows not fewer than two distinct spots which are similar in position and size to the spots in the chromatogram obtained with reference solution (b). The principal spot in the chromatogram obtained with test solution (a) is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a). Spray with *alcoholic solution of sulphuric acid R*. Heat at 120 °C for 10 min or until the spots appear. Allow to cool. Examine the plate in daylight and in ultraviolet light at 365 nm. The chromatogram obtained with test solution (b) shows not fewer than two distinct spots, similar in position, colour in daylight, fluorescence in ultraviolet light at 365 nm and size to the spots in the chromatogram obtained with reference solution (b). The principal spot in the chromatogram obtained with test solution (a) is similar in position, colour in daylight, fluorescence in ultraviolet light at 365 nm and size to the principal spot in the chromatogram obtained with reference solution (a). The principal spots in the chromatograms obtained with test solution (b) and reference solution (b) have R_f values distinctly lower than the principal spots in the chromatograms obtained with test solution (a) and reference solution (a).

- C. Add about 2 mg to 2 ml of *sulphuric acid R* and shake to dissolve. Within 5 min, a deep reddish-brown colour develops. Add the solution to 10 ml of *water R* and mix. The colour is discharged and a clear solution remains.
- D. Treat 25 mg by the oxygen-flask method (2.5.10). Use a mixture of 1 ml of 1 M *sodium hydroxide* and 20 ml of *water R* to absorb the combustion products. The solution gives reaction (a) of chlorides (2.3.1).

TESTS

Specific optical rotation (2.2.7). Dissolve 0.250 g in *dioxan R* and dilute to 25.0 ml with the same solvent. The specific optical rotation is + 88 to + 94, calculated with reference to the dried substance.

Absorbance (2.2.25). Dissolve 50.0 mg in *alcohol R* and dilute to 100.0 ml with the same solvent. Dilute 2.0 ml of the solution to 50.0 ml with *alcohol R*. The specific absorbance at the maximum at 238 nm is 284 to 302, calculated with reference to the dried substance.

Related substances. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 62.5 mg of the substance to be examined in the mobile phase and dilute to 25.0 ml with the mobile phase.

Reference solution (a). Dissolve 2 mg of *beclometasone 17-propionate CRS* and 2 mg of *beclometasone 21-propionate CRS* in the mobile phase and dilute to 50.0 ml with the mobile phase.

Reference solution (b). Dilute 1.0 ml of the test solution to 50.0 ml with the mobile phase.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4.6 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (5 µm),
- as mobile phase at a flow rate of 1 ml/min a mixture prepared as follows: mix 600 ml of *acetonitrile R* and 350 ml of *water R*, allow to cool, dilute to 1000 ml with *water R* and mix again,
- as detector a spectrophotometer set at 254 nm and with the sensitivity adjusted so that the height of the principal peak in the chromatogram obtained with reference solution (b) is 70 per cent to 90 per cent of the full scale.

Equilibrate the column with the mobile phase at a flow rate of 1 ml/min for about 45 min. When the chromatograms are recorded in the conditions described above, the retention times are: *beclometasone 17-propionate*, about 6.2 min; *beclometasone 21-propionate*, about 6.7 min; *beclometasone dipropionate*, 13 min to 14 min. Inject 20 µl of reference solution (a). The system is not suitable unless the resolution between the peaks corresponding to *beclometasone 17-propionate* and *beclometasone 21-propionate* is at least 1.4; if this resolution is not achieved, adjust the concentration of *acetonitrile* in the mobile phase. Verify the repeatability by making five separate injections of 20 µl of reference solution (b); the system is not suitable unless the relative standard deviation for the area of the principal peak in the chromatograms obtained with reference solution (b) is less than 2.0 per cent.

Inject separately 20 µl of the test solution and 20 µl of reference solution (b). Continue the chromatography for twice the retention time of the principal peak. In the chromatogram obtained with the test solution: the area of any peak, apart from the principal peak, is not greater than the area of the principal peak in the chromatogram obtained with reference solution (b) (2.0 per cent) and not more than one such peak has an area greater than half the area of the principal peak in the chromatogram obtained with reference solution (b) (1.0 per cent); the sum of the areas of all the peaks, apart from the principal peak, is not greater than 1.25 times the area of the principal peak in the chromatogram obtained with reference solution (b) (2.5 per cent). Disregard any peak due to the solvent and any peak with an area less than 0.025 times the area of the principal peak in the chromatogram obtained with reference solution (b).

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C for 3 h.

ASSAY

Protect the solutions from light throughout the assay. The coloured reaction products tend to adsorb to the surface of glassware. To avoid low results, it is recommended that the glassware be treated with the coloured reaction products before use and that the treated glassware be reserved for this assay and washed only with water R between assays.

Dissolve an accurately weighed quantity in sufficient *aldehyde-free alcohol R* to produce a solution containing 340 µg to 360 µg in 10.0 ml. Prepare a reference solution at the same time and in the same manner, using *beclometasone dipropionate CRS*. Into two 25 ml volumetric flasks, introduce respectively 10.0 ml of each solution. Into a third identical flask, introduce 10 ml of *aldehyde-free alcohol R*. To each flask add 2.0 ml of *triphenyltetrazolium chloride solution R*. Displace the air from the flasks using *oxygen-free nitrogen R*, immediately add 2.0 ml of *dilute tetramethylammonium hydroxide solution R* and again displace the air with *oxygen-free nitrogen R*. Stopper the flasks, mix the contents by gentle swirling and allow to stand in a water-bath at 35 °C for 2 h. Cool rapidly and dilute to 25.0 ml with *aldehyde-free alcohol R*. Immediately measure the absorbance (2.2.25) of the test solution and of the reference solution at the maximum at 485 nm, using a closed 1 cm cell and as the compensation liquid the solution prepared from 10 ml of *aldehyde-free alcohol R*. Treat the test solution and the reference solution in such a manner that the period of time that elapses between the addition of the *dilute tetramethylammonium hydroxide solution R* and measurement of the absorbance is exactly the same for each solution.

Calculate the content of $C_{28}H_{37}ClO_7$ from the absorbances measured and the concentrations of the solutions.

STORAGE

Store protected from light.

01/2005:0069

BEESWAX, WHITE

Cera alba

DEFINITION

Wax obtained by bleaching yellow beeswax.

CHARACTERS

Appearance: white or yellowish-white pieces or plates, translucent when thin, with a fine-grained, matt and non-crystalline fracture; when warmed in the hand they become soft and malleable.

It has an odour similar to that of yellow beeswax, though fainter and never rancid. It is tasteless and does not stick to the teeth.

Solubility: practically insoluble in water, partially soluble in hot alcohol (90 per cent V/V) and completely soluble in fatty and essential oils.

Relative density: about 0.960.

TESTS

Drop point (2.2.17): 61 °C to 66 °C.

Melt the beeswax by heating on a water-bath, pour onto a glass plate and allow to cool to a semi-solid mass. Fill the metal cup by inserting the wider end into the beeswax and repeating the procedure until beeswax extrudes from the narrow opening. Remove the excess with a spatula and insert the thermometer immediately. Remove the beeswax displaced. Allow to stand at room temperature for at least 12 h before determining the drop point.

Acid value: 17.0 to 24.0.

To 2.00 g (*m* g), in a 250 ml conical flask fitted with a reflux condenser, add 40 ml of *xylene R* and a few glass beads. Heat until the substance is dissolved. Add 20 ml of *alcohol R* and 0.5 ml of *phenolphthalein solution R1* and titrate the hot solution with 0.5 M *alcoholic potassium hydroxide* until a red colour persists for at least 10 s (n_1 ml). Carry out a blank test (n_2 ml).

$$\text{Acid value} = \frac{28.05 (n_1 - n_2)}{m}$$

Ester value (2.5.2): 70 to 80.

Saponification value: 87 to 104.

To 2.00 g (*m* g), in a 250 ml conical flask fitted with a reflux condenser, add 30 ml of a mixture of equal volumes of *alcohol R* and *xylene R* and a few glass beads. Heat until the substance is dissolved. Add 25.0 ml of 0.5 M *alcoholic potassium hydroxide* and heat under a reflux condenser for 3 h. Titrate the hot solution immediately with 0.5 M *hydrochloric acid*, using 1 ml of *phenolphthalein solution R1* as indicator (n_1 ml). Reheat the solution to boiling several times during the course of the titration. Carry out a blank test (n_2 ml).

$$\text{Saponification value} = \frac{28.05 (n_2 - n_1)}{m}$$

Ceresin, paraffins and certain other waxes. To 3.0 g, in a 100 ml round-bottomed flask, add 30 ml of a 40 g/l solution of *potassium hydroxide R* in *aldehyde-free alcohol R* and boil gently under a reflux condenser for 2 h. Remove the condenser and immediately insert a thermometer. Place the flask in a water-bath at 80 °C and allow to cool, swirling the solution continuously. No precipitate is formed until 65 °C, although the solution may be slightly opalescent. Above 65 °C, the solution may become cloudy and precipitates may be formed. At 59 °C, the solution is cloudy.

Glycerol and other polyols: maximum 0.5 per cent *m/m*, calculated as glycerol.

To 0.20 g add 10 ml of *alcoholic potassium hydroxide solution R* and heat on a water-bath under a reflux condenser for 30 min. Add 50 ml of *dilute sulphuric acid R*, cool and filter. Rinse the flask and the filter with *dilute sulphuric acid R*. Combine the filtrate and washings and dilute to 100.0 ml with *dilute sulphuric acid R*. Place 1.0 ml of the solution in a test-tube, add 0.5 ml of a 10.7 g/l solution of *sodium periodate R*, mix and allow to stand for 5 min. Add 1.0 ml of *decolorised fuchsin solution R* and mix. Any precipitate disappears. Place the tube in a beaker containing water at 40 °C. During cooling observe for 10-15 min. Any violet-blue colour in the solution is not more intense than that in a standard prepared at the same time and in the same manner using 1.0 ml of a 10 mg/l solution of *glycerol R* in *dilute sulphuric acid R*.

01/2005:0070

BEESWAX, YELLOW**Cera flava****DEFINITION**

Wax obtained by melting the walls of the honeycomb made by the honey-bee, *Apis mellifera* L., with hot water and removing foreign matter.

CHARACTERS

Appearance: yellow or light brown pieces or plates with a fine-grained, matt and non-crystalline fracture; when warmed in the hand they become soft and malleable.

It has a faint odour, characteristic of honey. It is tasteless and does not stick to the teeth.

Solubility: practically insoluble in water, partially soluble in hot alcohol (90 per cent V/V) and completely soluble in fatty and essential oils.

Relative density: about 0.960.

TESTS

Drop point (2.2.17): 61 °C to 66 °C.

Melt the beeswax by heating on a water-bath, pour onto a glass plate and allow to cool to a semi-solid mass. Fill the metal cup by inserting the wider end into the beeswax and repeating the procedure until beeswax extrudes from the narrow opening. Remove the excess with a spatula and insert the thermometer immediately. Remove the beeswax displaced. Allow to stand at room temperature for at least 12 h before determining the drop point.

Acid value: 17.0 to 22.0.

To 2.00 g (*m* g), in a 250 ml conical flask fitted with a reflux condenser, add 40 ml of *xylene R* and a few glass beads. Heat until the substance is dissolved. Add 20 ml of *alcohol R* and 0.5 ml of *phenolphthalein solution R1* and titrate the hot solution with 0.5 M *alcoholic potassium hydroxide* until a red colour persists for at least 10 s (*n*₁ ml). Carry out a blank test (*n*₂ ml).

$$\text{Acid value} = \frac{28.05 (n_1 - n_2)}{m}$$

Ester value (2.5.2): 70 to 80.

Saponification value: 87 to 102.

To 2.00 g (*m* g), in a 250 ml conical flask fitted with a reflux condenser, add 30 ml of a mixture of equal volumes of *alcohol R* and *xylene R* and a few glass beads. Heat until the substance is dissolved. Add 25.0 ml of 0.5 M *alcoholic potassium hydroxide* and heat under a reflux condenser for 3 h. Titrate the hot solution immediately with 0.5 M *hydrochloric acid*, using 1 ml of *phenolphthalein solution R1* as indicator (*n*₁ ml). Reheat the solution to boiling several times during the course of the titration. Carry out a blank test (*n*₂ ml).

$$\text{Saponification value} = \frac{28.05 (n_2 - n_1)}{m}$$

Ceresin, paraffins and certain other waxes. To 3.0 g, in a 100 ml round-bottomed flask, add 30 ml of a 40 g/l solution of *potassium hydroxide R* in *aldehyde-free alcohol R* and boil gently under a reflux condenser for 2 h. Remove the condenser and immediately insert a thermometer. Place the flask in a water-bath at 80 °C and allow to cool, swirling the solution continuously. No precipitate is formed until 65 °C,

although the solution may be slightly opalescent. Above 65 °C, the solution may become cloudy and precipitates may be formed. At 59 °C, the solution is cloudy.

Glycerol and other polyols: maximum 0.5 per cent *m/m*, calculated as glycerol.

To 0.20 g add 10 ml of *alcoholic potassium hydroxide solution R* and heat on a water-bath under a reflux condenser for 30 min. Add 50 ml of *dilute sulphuric acid R*, cool and filter. Rinse the flask and the filter with *dilute sulphuric acid R*. Combine the filtrate and washings and dilute to 100.0 ml with *dilute sulphuric acid R*. Place 1.0 ml of the solution in a test-tube, add 0.5 ml of a 10.7 g/l solution of *sodium periodate R*, mix and allow to stand for 5 min. Add 1.0 ml of *decolorised fuchsin solution R* and mix. Any precipitate disappears. Place the tube in a beaker containing water at 40 °C. During cooling observe for 10-15 min. Any violet-blue colour in the solution is not more intense than that in a standard prepared at the same time and in the same manner using 1.0 ml of a 10 mg/l solution of *glycerol R* in *dilute sulphuric acid R*.

01/2005:0221

BELLADONNA LEAF**Belladonnae folium****DEFINITION**

Belladonna leaf consists of the dried leaf or of the dried leaf and flowering, and occasionally fruit-bearing, tops of *Atropa belladonna* L. It contains not less than 0.30 per cent of total alkaloids, calculated as hyoscyamine (*M_r* 289.4) with reference to the drug dried at 100 °C to 105 °C. The alkaloids consist mainly of hyoscyamine together with small quantities of hyoscyne (scopolamine).

CHARACTERS

Belladonna leaf has a slightly nauseous odour.

It has the macroscopic and microscopic characters described under identification tests A and B.

IDENTIFICATION

- The leaves are green to brownish-green, slightly darker on the upper surface, often crumpled and rolled and partly matted together in the drug. The leaf is petiolate and the base of the lamina is acute and decurrent and the margin entire. The flowering stems are flattened and bear at each node a pair of leaves unequal in size, in the axils of which occur singly the flowers or occasionally fruits. The flowers have a gamosepalous calyx and campanulate corolla. The fruits are globular berries, green to brownish-black and surrounded by the persistent calyx with widely spread lobes.
- Reduce to a powder (355). The powder is dark green. Examine under a microscope, using *chloral hydrate solution R*. The powder shows the following diagnostic characters: fragments of leaf lamina showing sinuous-walled epidermal cells, a striated cuticle and numerous stomata predominantly present on the lower epidermis (anisocytic and also some anomocytic); multicellular uniseriate covering trichomes with smooth cuticle, glandular trichomes with unicellular heads and multicellular, uniseriate stalks or with multicellular heads and unicellular stalks; parenchyma cells including rounded cells containing microspheoidal crystals of calcium oxalate; annular and spirally thickened vessels. The powdered drug may also show the following: fibres and reticulately thickened vessels from the

stems; subspherical pollen grains, 40 µm to 50 µm in diameter, with three germinal pores, three furrows and an extensively pitted exine; fragments of the corolla, with a papillose epidermis or bearing numerous covering or glandular trichomes of the types previously described; brownish-yellow seed fragments containing irregularly sclerified and pitted cells of the testa.

- C. Shake 1 g of powdered drug with 10 ml of 0.05 M sulphuric acid for 2 min. Filter and add to the filtrate 1 ml of concentrated ammonia R and 5 ml of water R. Shake cautiously with 15 ml of ether R, avoiding formation of an emulsion. Separate the ether layer and dry over anhydrous sodium sulphate R. Filter and evaporate the ether in a porcelain dish. Add 0.5 ml of fuming nitric acid R and evaporate to dryness on a water-bath. Add 10 ml of acetone R and, dropwise, a 30 g/l solution of potassium hydroxide R in alcohol R. A deep violet colour develops.
- D. Examine the chromatogram obtained in the chromatography test. The principal zones in the chromatogram obtained with the test solution are similar in position, colour and size to the principal zones in the chromatogram obtained with the same volume of the reference solution.

TESTS

Chromatography. Examine by thin-layer chromatography (2.2.27), using silica gel G R as the coating substance.

Test solution. To 0.6 g of powdered drug (180) add 15 ml of 0.05 M sulphuric acid, shake for 15 min and filter. Wash the filter with 0.05 M sulphuric acid until 20 ml of filtrate is obtained. To the filtrate add 1 ml of concentrated ammonia R and shake with two quantities, each of 10 ml, of peroxide-free ether R. If necessary, separate by centrifugation. Dry the combined ether layers over anhydrous sodium sulphate R, filter and evaporate to dryness on a water-bath. Dissolve the residue in 0.5 ml of methanol R.

Reference solution. Dissolve 50 mg of hyoscyamine sulphate R in 9 ml of methanol R. Dissolve 15 mg of hyoscine hydrobromide R in 10 ml of methanol R. Mix 1.8 ml of the hyoscine hydrobromide solution and 8 ml of the hyoscyamine sulphate solution.

Apply separately to the plate as bands 20 mm by 3 mm 10 µl and 20 µl of each solution, leaving 1 cm between the bands. Develop over a path of 10 cm using a mixture of 3 volumes of concentrated ammonia R, 7 volumes of water R and 90 volumes of acetone R. Dry the plate at 100 °C to 105 °C for 15 min, allow to cool and spray with potassium iodobismuthate solution R2, using about 10 ml for a plate 200 mm square, until the orange or brown zones become visible against a yellow background. The zones in the chromatograms obtained with the test solution are similar to those in the chromatograms obtained with the reference solution with respect to their position (hyoscyamine in the lower third, hyoscine in the upper third of the chromatogram) and their colour. The zones in the chromatograms obtained with the test solution are at least equal in size to the corresponding zones in the chromatogram obtained with the same volume of the reference solution. Faint secondary zones may appear, particularly in the middle of the chromatogram obtained with 20 µl of the test solution or near the starting-point in the chromatogram obtained with 10 µl of the test solution.

Spray the plate with sodium nitrite solution R until the coating is transparent. Examine after 15 min. The zones corresponding to hyoscyamine in the chromatograms obtained with the test solution and the reference solution change from brown to reddish-brown but not to greyish-blue (atropine) and any secondary zones disappear.

Foreign matter (2.8.2). Not more than 3 per cent of stem having a diameter exceeding 5 mm.

Total ash (2.4.16). Not more than 16.0 per cent.

Ash insoluble in hydrochloric acid (2.8.1). Not more than 4.0 per cent

ASSAY

Take about 50 g of drug and completely reduce it to a powder (180). Using the powder, determine the loss on drying and the total alkaloids.

a) Determine the loss on drying (2.2.32) on 2.000 g, by drying in an oven at 100 °C to 105 °C.

b) Moisten 10.00 g of powder with a mixture of 5 ml of ammonia R, 10 ml of alcohol R and 30 ml of peroxide-free ether R and mix thoroughly. Transfer the mixture to a suitable percolator, if necessary with the aid of the extracting mixture. Allow to macerate for 4 h and percolate with a mixture of 1 volume of chloroform R and 3 volumes of peroxide-free ether R until the alkaloids are completely extracted. Evaporate to dryness a few millilitres of the liquid flowing from the percolator, dissolve the residue in 0.25 M sulphuric acid and verify the absence of alkaloids using potassium tetraiodomercurate solution R. Concentrate the percolate to about 50 ml by distilling on a water-bath and transfer it to a separating funnel, rinsing with peroxide-free ether R. Add a quantity of peroxide-free ether R equal to at least 2.1 times the volume of the percolate to produce a liquid of a density well below that of water. Shake the solution with no fewer than three quantities, each of 20 ml, of 0.25 M sulphuric acid, separate the two layers by centrifugation if necessary and transfer the acid layers to a second separating funnel. Make the acid layer alkaline with ammonia R and shake with three quantities, each of 30 ml, of chloroform R. Combine the chloroform layers, add 4 g of anhydrous sodium sulphate R and allow to stand for 30 min with occasional shaking. Decant the chloroform and wash the sodium sulphate with three quantities, each of 10 ml, of chloroform R. Add the washings to the chloroform extract, evaporate to dryness on a water-bath and heat in an oven at 100 °C to 105 °C for 15 min. Dissolve the residue in a few millilitres of chloroform R, add 20.0 ml of 0.01 M sulphuric acid and remove the chloroform by evaporation on a water-bath. Titrate the excess of acid with 0.02 M sodium hydroxide using methyl red mixed solution R as indicator.

Calculate the percentage content of total alkaloids, expressed as hyoscyamine, from the expression:

$$\frac{57.88 \times (20 - n)}{(100 - d) \times m}$$

d = loss on drying as a percentage,

n = volume of 0.02 M sodium hydroxide used in millilitres,

m = mass of drug used in grams.

STORAGE

Store protected from light.

01/2005:1294

BELLADONNA LEAF DRY EXTRACT, STANDARDISED

Belladonnae folii extractum siccum normatum

DEFINITION

Standardised belladonna leaf dry extract is produced from *Belladonna leaf* (0221). It contains not less than 0.95 per cent and not more than 1.05 per cent of total alkaloids, calculated as hyoscyamine ($C_{17}H_{23}NO_3$, M_r 289.4), with reference to the dried extract.

PRODUCTION

The extract is produced from the drug and ethanol (70 per cent V/V) using an appropriate procedure.

CHARACTERS

A hygroscopic brown or greenish powder.

IDENTIFICATION

A. Examine by thin-layer chromatography (2.2.27), using a suitable silica gel as the coating substance.

Test solution. To 1 g of the extract to be examined add 5.0 ml of *methanol R*. Shake for 2 min and filter.

Reference solution. Dissolve 1.0 mg of *chlorogenic acid R* and 2.5 mg of *rutin R* in 10 ml of *methanol R*.

Apply to the plate, as bands, 20 µl of each solution. Develop over a path of 15 cm using a mixture of 10 volumes of *anhydrous formic acid R*, 10 volumes of *water R*, 30 volumes of *methyl ethyl ketone R* and 50 volumes of *ethyl acetate R*. Dry the plate at 100 °C to 105 °C and spray the warm plate with a 10 g/l solution of *diphenylboric acid aminoethyl ester R* in *methanol R*. Subsequently spray the plate with a 50 g/l solution of *macrogol 400 R* in *methanol R*. Allow the plate to dry in air for 30 min and examine in ultraviolet light at 365 nm. The chromatograms obtained with the reference solution and the test solution show in the central part a light blue fluorescent zone (chlorogenic acid) and in the lower part a yellowish-brown fluorescent zone (rutin). Furthermore, the chromatogram obtained with the test solution shows a little above the start a yellowish-brown fluorescent zone and directly above a yellow fluorescent zone, a yellow or yellowish-brown fluorescent zone between the zone due to rutin and the zone due to chlorogenic acid. Further zones may be present.

B. Examine the chromatogram obtained in the test for atropine. The principal zones in the chromatogram obtained with the test solution are similar in position and colour to the principal zones in the chromatogram obtained with the reference solution.

TESTS

Atropine. Examine by thin-layer chromatography (2.2.27), using a suitable silica gel as the coating substance.

Test solution. To 0.20 g of the extract to be examined add 10.0 ml of 0.05 M *sulphuric acid*, shake for 2 min and filter. Add 1.0 ml of *concentrated ammonia R* and shake with two quantities, each of 10 ml, of *peroxide-free ether R*. If necessary, separate by centrifugation. Dry the combined ether layers over about 2 g of *anhydrous sodium sulphate R*, filter and evaporate to dryness on a water-bath. Dissolve the residue in 0.5 ml of *methanol R*.

Reference solution. Dissolve 50 mg of *hyoscyamine sulphate R* in 9 ml of *methanol R*. Dissolve 15 mg of *hyoscyne hydrobromide R* in 10 ml of *methanol R*. Mix 1.8 ml of the hyoscyne hydrobromide solution and 8 ml of the hyoscyamine sulphate solution.

Apply to the plate, as bands, 20 µl of each solution. Develop over a path of 10 cm using a mixture of 3 volumes of *concentrated ammonia R*, 7 volumes of *water R* and 90 volumes of *acetone R*. Dry the plate at 100 °C to 105 °C for 15 min, allow to cool and spray with *potassium iodobismuthate solution R2*, until the orange or brown zones become visible against a yellow background. The zones in the chromatogram obtained with the test solution are similar to those in the chromatogram obtained with the reference solution with respect to their position (hyoscyamine in the lower third, hyoscyne in the upper third of the chromatogram) and their colour. Other faint zones may be present in the chromatogram obtained with the test solution. Spray the plate with *sodium nitrite solution R* until the coating is transparent. Examine after 15 min. The zones corresponding to hyoscyamine in the chromatograms obtained with the test solution and the reference solution change from orange or brown to reddish-brown but not to greyish-blue (atropine).

Loss on drying (2.8.17): maximum 5.0 per cent.

Microbial contamination. Total viable aerobic count (2.6.12) not more than 10^4 micro-organisms per gram of which not more than 10^2 fungi per gram, determined by plate count. It complies with the tests for *Escherichia coli* and for *Salmonella* (2.6.13).

ASSAY

At each extraction stage it is necessary to check that the alkaloids have been completely extracted. If the extraction is into the organic phase this is done by evaporating to dryness a few millilitres of the last organic layer, dissolving the residue in 0.25 M *sulphuric acid* and verifying the absence of alkaloids using *potassium tetraiodomercurate solution R*. If the extraction is into the acid aqueous phase, this is done by taking a few millilitres of the last acid aqueous phase and verifying the absence of alkaloids using *potassium tetraiodomercurate solution R*.

Disperse 3.00 g in a mixture of 5 ml of *ammonia R* and 15 ml of *water R*. Shake with no fewer than three quantities, each of 40 ml of a mixture of 1 volume of *methylene chloride R* and 3 volumes of *peroxide-free ether R* until the alkaloids are completely extracted. Concentrate the combined organic layers to about 50 ml by distilling on a water-bath and transfer the resulting liquid to a separating funnel, rinsing with *peroxide-free ether R*. Add a quantity of *peroxide-free ether R* equal to at least 2.1 times the volume of the liquid to produce a layer having a density well below that of water. Shake the resulting solution with no fewer than three quantities, each of 20 ml, of 0.25 M *sulphuric acid* until the alkaloids are completely extracted. Separate the layers by centrifugation, if necessary and transfer the acid layers to a second separating funnel. Make the combined acid layers alkaline with *ammonia R* and shake with no fewer than three quantities, each of 30 ml, of *methylene chloride R* until the alkaloids are completely extracted. Combine the organic layers, add 4 g of *anhydrous sodium sulphate R* and allow to stand for 30 min with occasional shaking. Decant the methylene chloride and wash the sodium sulphate with three quantities, each of 10 ml, of *methylene chloride R*. Combine the organic extracts, evaporate to dryness on a water-bath. Heat the residue in an oven at 100 °C to 105 °C for 15 min. Dissolve the residue in a few millilitres of *methylene chloride R*, evaporate to dryness on a water-bath

and again heat the residue in an oven at 100 °C to 105 °C for 15 min. Dissolve the residue in a few millilitres of *methylene chloride R*, add 20.0 ml of 0.01 M sulphuric acid and remove the methylene chloride by evaporation on a water-bath. Titrate the excess of acid with 0.02 M sodium hydroxide using *methyl red mixed solution R* as indicator.

Calculate the percentage content of total alkaloids, expressed as hyoscyamine, from the expression:

$$\frac{57.88 \times (20 - n)}{100 \times m}$$

- n = volume of 0.02 M sodium hydroxide used, in millilitres,
 m = mass of the substance to be examined, in grams.

STORAGE

Store in an airtight container, protected from light.

01/2005:1812

BELLADONNA LEAF TINCTURE, STANDARDISED

Belladonnae folii tinctura normata

DEFINITION

Tincture produced from *Belladonna leaf* (0221).

Content: 0.027 per cent to 0.033 per cent of total alkaloids, calculated as hyoscyamine ($C_{17}H_{23}NO_3$; M_r 289.4). The alkaloids consist mainly of hyoscyamine together with small quantities of hyoscyne.

PRODUCTION

The tincture is produced from 1 part of the powdered drug (355) and 10 parts of ethanol (70 per cent V/V) by a suitable procedure.

IDENTIFICATION

A. Thin-layer chromatography (2.2.27).

Test solution. Evaporate to dryness 10.0 ml of the tincture to be examined in a water-bath at 40 °C under reduced pressure. Dissolve the residue in 1.0 ml of *methanol R*.

Reference solution. Dissolve 1.0 mg of *chlorogenic acid R* and 2.5 mg of *rutin R* in 10 ml of *methanol R*.

Plate: TLC silica gel plate R.

Mobile phase: *anhydrous formic acid R*, *water R*, *methyl ethyl ketone R*, *ethyl acetate R* (10:10:30:50 V/V/V/V).

Application: 40 µl, as bands.

Development: over a path of 15 cm.

Drying: at 100-105 °C.

Detection: spray the warm plate with a 10 g/l solution of *diphenylboric acid aminoethyl ester R* in *methanol R*; subsequently spray the plate with a 50 g/l solution of *macrogol 400 R* in *methanol R*; allow the plate to dry in air for 30 min and examine in ultraviolet light at 365 nm.

Results: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other fluorescent zones may be present in the chromatogram obtained with the test solution.

Top of the plate	
Chlorogenic acid: a light blue fluorescent zone	A light blue fluorescent zone (chlorogenic acid) A yellow or yellowish-brown fluorescent zone
Rutin: a yellowish-brown fluorescent zone	A bluish-grey fluorescent zone A yellow fluorescent zone A yellowish-brown fluorescent zone
Reference solution	Test solution

B. Examine the chromatograms obtained in the test for atropine, detection A.

Results A: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Faint secondary zones may appear, particularly in the middle of the chromatogram obtained with 40 µl of the test solution or near the starting point in the chromatogram obtained with 20 µl of the test solution.

Top of the plate	
Hyoscyne: a brownish-orange zone	A brownish-orange zone (hyoscyne) Faint secondary zones
Hyoscyamine: a brownish-orange zone	A brownish-orange zone (hyoscyamine) Faint secondary zones
Reference solution	Test solution

TESTS

Atropine. Thin-layer chromatography (2.2.27).

Test solution. To 15.0 ml of the tincture to be examined add 15 ml of 0.05 M sulphuric acid. Filter. Add 1 ml of concentrated ammonia R to the filtrate and shake with 2 quantities, each of 10 ml, of peroxide-free ether R. Separate by centrifugation if necessary. Dry the combined ether layers over anhydrous sodium sulphate R. Filter and evaporate to dryness on a water-bath. Dissolve the residue in 0.5 ml of *methanol R*.

Reference solution. Dissolve 50 mg of *hyoscyamine sulphate R* in 9 ml of *methanol R*. Dissolve 15 mg of *hyoscyne hydrobromide R* in 10 ml of *methanol R*. Mix 1.8 ml of the hyoscyne hydrobromide solution and 8 ml of the hyoscyamine sulphate solution.

Plate: TLC silica gel plate R.

Mobile phase: concentrated ammonia R, *water R*, *acetone R* (3:7:90 V/V/V).

Application: 20 µl and 40 µl of each solution, as bands.

Development: over a path of 10 cm.

Drying: at 100-105 °C for 15 min.

Detection A: spray with *potassium iodobismuthate solution R2*.

Detection B: spray with *sodium nitrite solution R* until the plate is transparent. Examine after 15 min.

Results B: the zones due to hyoscyamine in the chromatograms obtained with the test solution and the reference solution change from brownish-orange to reddish-brown but not to greyish-blue (atropine) and any secondary zones disappear.

Ethanol (2.9.10): 64 per cent V/V to 69 per cent V/V.

ASSAY

Evaporate 50.0 g of the tincture to be examined to a volume of about 10 ml. Transfer quantitatively to a separating funnel, with the minimum volume of *alcohol (70 per cent V/V) R*. Add 5 ml of *ammonia R* and 15 ml of *water R*. Shake with not fewer than 3 quantities each of 40 ml of a mixture of 1 volume of *methylene chloride R* and 3 volumes of *peroxide-free ether R*, carefully to avoid emulsion, until the alkaloids are completely extracted. Combine the organic layers and concentrate the solution to a volume of about 50 ml by distilling on a water-bath. Transfer the resulting solution quantitatively to a separating funnel, rinsing with *peroxide-free ether R*. Add a quantity of *peroxide-free ether R* equal to at least 2.1 times the volume of the solution to produce a layer having a density well below that of water. Shake the resulting solution with not fewer than 3 quantities each of 20 ml of *0.25 M sulphuric acid* until the alkaloids are completely extracted. Separate the layers by centrifugation if necessary and transfer the layers to a separating funnel. Make the combined layers alkaline with *ammonia R* and shake with not fewer than 3 quantities each of 30 ml of *methylene chloride R* until the alkaloids are completely extracted. Combine the organic layers, add 4 g of *anhydrous sodium sulphate R* and allow to stand for 30 min with occasional shaking. Decant the methylene chloride and filter. Wash the sodium sulphate with 3 quantities each of 10 ml of *methylene chloride R*. Combine the organic extracts, evaporate to dryness on a water-bath. Heat the residue in an oven at 100-105 °C for 15 min. Dissolve the residue in a few millilitres of *methylene chloride R*, evaporate to dryness on a water-bath and heat the residue in an oven at 100-105 °C for 15 min again. Dissolve the residue in a few millilitres of *methylene chloride R*. Add 20.0 ml of *0.01 M sulphuric acid* and remove the methylene chloride by evaporation on a water-bath. Titrate the excess of acid with *0.02 M sodium hydroxide* using *methyl red mixed solution R* as indicator. Calculate the percentage content of total alkaloids, expressed as hyoscyamine, from the expression:

$$\frac{57.88 \times (20 - n)}{100 \times m}$$

n = volume of *0.02 M sodium hydroxide* used, in millilitres,

m = mass of drug used, in grams.

01/2005:0222

BELLADONNA, PREPARED

Belladonnae pulvis normatus

DEFINITION

Prepared belladonna is belladonna leaf powder (180) adjusted if necessary by adding powdered lactose or belladonna leaf powder with a lower alkaloidal content to contain 0.28 per cent to 0.32 per cent of total alkaloids, calculated as hyoscyamine (M_r 289.4) with reference to the dried drug.

CHARACTERS

Slightly nauseous odour.

IDENTIFICATION

- The powder is dark green. Examine under a microscope, using *chloral hydrate solution R*. The powder shows the following diagnostic characters: fragments of leaf lamina showing sinuous-walled epidermal cells, a striated cuticle and numerous stomata predominantly present on the lower epidermis (anisocytic and also some anomocytic); multicellular uniseriate covering trichomes with smooth cuticle, glandular trichomes with unicellular heads and multicellular, uniseriate stalks or with multicellular heads and unicellular stalks; parenchyma cells including rounded cells containing microsphenoidal crystals of calcium oxalate; annular and spirally thickened vessels. The powdered drug may also show the following: fibres and reticulately thickened vessels from the stems; subspherical pollen grains, 40 µm to 50 µm in diameter, with three germinal pores, three furrows and an extensively pitted exine; fragments of the corolla, with a papillose epidermis or bearing numerous covering or glandular trichomes of the types previously described; brownish-yellow seed fragments containing irregularly sclerified and pitted cells of the testa. Examined in *glycerol (85 per cent) R*, it may be seen to contain lactose crystals.
- Shake 1 g with 10 ml of *0.05 M sulphuric acid* for 2 min. Filter and add to the filtrate 1 ml of *concentrated ammonia R* and 5 ml of *water R*. Shake cautiously with 15 ml of *ether R*, avoiding formation of an emulsion. Separate the ether layer and dry over *anhydrous sodium sulphate R*. Filter and evaporate the ether in a porcelain dish. Add 0.5 ml of *fuming nitric acid R* and evaporate to dryness on a water-bath. Add 10 ml of *acetone R* and, dropwise, a 30 g/l solution of *potassium hydroxide R* in *alcohol R*. A deep violet colour develops.
- Examine the chromatogram obtained in the chromatography test. The principal zones in the chromatogram obtained with the test solution are similar in position, colour and size to the principal zones in the chromatogram obtained with the same volume of the reference solution.

TESTS

Chromatography. Examine by thin-layer chromatography (2.2.27), using *silica gel G R* as the coating substance.

Test solution. To 0.6 g add 15 ml of *0.05 M sulphuric acid*, shake for 15 min and filter. Wash the filter with *0.05 M sulphuric acid* until 20 ml of filtrate is obtained. To the filtrate add 1 ml of *concentrated ammonia R* and shake with two quantities, each of 10 ml, of *peroxide-free ether R*. If necessary, separate by centrifugation. Dry the combined ether layers over *anhydrous sodium sulphate R*, filter and evaporate to dryness on a water-bath. Dissolve the residue in 0.5 ml of *methanol R*.

Reference solution. Dissolve 50 mg of *hyoscyamine sulphate R* in 9 ml of *methanol R*. Dissolve 15 mg of *hyoscine hydrobromide R* in 10 ml of *methanol R*. Mix 1.8 ml of the hyoscine hydrobromide solution and 8 ml of the hyoscyamine sulphate solution.

Apply separately to the plate as bands 20 mm by 3 mm 10 µl and 20 µl of each solution, leaving 1 cm between the bands. Develop over a path of 10 cm using a mixture of 3 volumes of *concentrated ammonia R*, 7 volumes of *water R* and 90 volumes of *acetone R*. Dry the plate at 100 °C to 105 °C for 15 min, allow to cool and spray with *potassium iodobismuthate solution R2*, using about

10 ml for a plate 200 mm square, until the orange or brown zones become visible against a yellow background. The zones in the chromatograms obtained with the test solution are similar to those in the chromatograms obtained with the reference solution with respect to their position (hyoscyamine in the lower third, hyoscine in the upper third of the chromatogram) and their colour. The zones in the chromatograms obtained with the test solution are at least equal in size to the corresponding zones in the chromatogram obtained with the same volume of the reference solution. Faint secondary zones may appear, particularly in the middle of the chromatogram obtained with 20 µl of the test solution or near the starting-point in the chromatogram obtained with 10 µl of the test solution. Spray the plate with *sodium nitrite solution R* until the coating is transparent. Examine after 15 min. The zones corresponding to hyoscyamine in the chromatograms obtained with the test solution and the reference solution change from brown to reddish-brown but not to greyish-blue (atropine) and any secondary zones disappear.

Loss on drying (2.2.32). Not more than 5.0 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C.

Total ash (2.4.16). Not more than 16.0 per cent.

Ash insoluble in hydrochloric acid (2.8.1). Not more than 4.0 per cent.

ASSAY

a) Determine the loss on drying (2.2.32) on 2.000 g, by drying in an oven at 100 °C to 105 °C.

b) Moisten 10.00 g with a mixture of 5 ml of *ammonia R*, 10 ml of *alcohol R* and 30 ml of *peroxide-free ether R* and mix thoroughly. Transfer the mixture to a suitable percolator, if necessary with the aid of the extracting mixture. Allow to macerate for 4 h and percolate with a mixture of 1 volume of *chloroform R* and 3 volumes of *peroxide-free ether R* until the alkaloids are completely extracted. Evaporate to dryness a few millilitres of the liquid flowing from the percolator, dissolve the residue in 0.25 M sulphuric acid and verify the absence of alkaloids using *potassium tetraiodomercurate solution R*. Concentrate the percolate to about 50 ml by distilling on a water-bath and transfer it to a separating funnel, rinsing with *peroxide-free ether R*. Add a quantity of *peroxide-free ether R* equal to at least 2.1 times the volume of the percolate to produce a liquid of a density well below that of water. Shake the solution with no fewer than three quantities, each of 20 ml, of 0.25 M sulphuric acid, separate the two layers by centrifugation if necessary and transfer the acid layers to a second separating funnel. Make the acid layer alkaline with *ammonia R* and shake with three quantities, each of 30 ml, of *chloroform R*. Combine the chloroform layers, add 4 g of *anhydrous sodium sulphate R* and allow to stand for 30 min with occasional shaking. Decant the chloroform and wash the sodium sulphate with three quantities, each of 10 ml, of *chloroform R*. Add the washings to the chloroform extract, evaporate to dryness on a water-bath and heat in an oven at 100 °C to 105 °C for 15 min. Dissolve the residue in a few millilitres of *chloroform R*, add 20.0 ml of 0.01 M sulphuric acid and remove the chloroform by evaporation on a water-bath. Titrate the excess of acid with 0.02 M sodium hydroxide using *methyl red mixed solution R* as indicator.

Calculate the percentage content of total alkaloids, expressed as hyoscyamine, from the expression:

$$\frac{57.88 \times (20 - n)}{(100 - d) \times m}$$

d = loss on drying as a percentage,

n = volume of 0.02 M sodium hydroxide used in millilitres,

m = mass of drug used in grams.

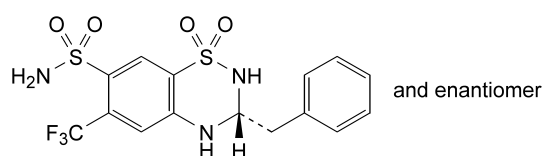
STORAGE

Store in an airtight container, protected from light.

01/2005:0370

BENDROFLUMETHIAZIDE

Bendroflumethiazidum



C₁₅H₁₄F₃N₃O₄S₂

M_r 421.4

DEFINITION

Bendroflumethiazide contains not less than 98.0 per cent and not more than the equivalent of 102.0 per cent of 3-benzyl-3,4-dihydro-6-(trifluoromethyl)-2*H*-1,2,4-benzothiadiazine-7-sulphonamide 1,1-dioxide, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, crystalline powder, practically insoluble in water, freely soluble in acetone, soluble in alcohol.

IDENTIFICATION

First identification: A.

Second identification: B, C, D.

- Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *bendroflumethiazide CRS*. Examine the substances prepared in the form of discs.
- Examine the chromatograms obtained in the test for related substances. The principal spot in the chromatogram obtained with 4 µl of the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).
- In a test-tube, heat 0.5 ml of a saturated solution of *chromic acid cleansing mixture R* in a naked flame until white fumes appear in the upper part of the tube. The solution wets the side of the tube and there is no appearance of greasiness. Add about 2 mg of the substance to be examined and heat again in a naked flame until white fumes appear. The solution does not wet the side of the tube and does not pour easily from the tube.
- Heat about 5 mg with a mixture of 5 ml of *potassium permanganate solution R* and 1 ml of *sulphuric acid R*. An odour of benzaldehyde is produced.

TESTS

Related substances. Examine by thin-layer chromatography (2.2.27), using *silica gel G R* as the coating substance.

Test solution. Dissolve 25 mg of the substance to be examined in *acetone R* and dilute to 5 ml with the same solvent.

Reference solution (a). Dissolve 25 mg of *bendroflumethiazide CRS* in *acetone R* and dilute to 5 ml with the same solvent.

Reference solution (b). Dilute 1 ml of reference solution (a) to 100 ml with *acetone R*.

Apply separately to the plate 4 µl and 20 µl of the test solution, 4 µl of reference solution (a) and 20 µl of reference solution (b). Develop over a path of 15 cm using *ethyl acetate R*. Dry the plate in a current of air for 10 min and spray with a mixture of equal volumes of *alcoholic solution of sulphuric acid R* and *alcohol R*; use about 10 ml for a plate 200 mm square and spray in small portions, allowing the solvent to evaporate each time to avoid excessive wetting. Heat at 100 °C to 105 °C for 30 min and immediately place the plate above, but not in contact with, 10 ml of a saturated solution of *sodium nitrite R* in a glass tank. Carefully add 0.5 ml of *sulphuric acid R* to the sodium nitrite solution, close the tank, and allow to stand for 15 min. Remove the plate, heat in a ventilated oven at 40 °C for 15 min and spray with three quantities, each of 5 ml, of a freshly prepared 5 g/l solution of *naphthylethylenediamine dihydrochloride R* in *alcohol R*. Examine the plate by transmitted light. Any spot in the chromatogram obtained with 20 µl of the test solution, apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (b) (1.0 per cent).

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.00 g by drying in an oven at 100 °C to 105 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g

ASSAY

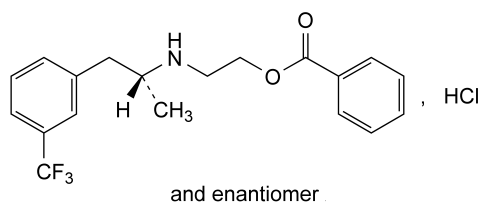
Dissolve 0.200 g in 50 ml of *anhydrous pyridine R*. Titrate with 0.1 M *tetrabutylammonium hydroxide*, determining the end-point potentiometrically at the second point of inflexion (2.2.20). Carry out a blank titration.

1 ml of 0.1 M *tetrabutylammonium hydroxide* is equivalent to 21.07 mg of $C_{15}H_{14}F_3N_3O_4S_2$.

01/2005:1601
corrected

BENFLUOREX HYDROCHLORIDE

Benfluorexi hydrochloridum



$C_{19}H_{21}ClF_3NO_2$

M_r 387.8

DEFINITION

2-[[[(1*S*)-1-Methyl-2-[3-(trifluoromethyl)phenyl]ethyl]amino]ethyl benzoate hydrochloride.

Content: 98.5 per cent to 101.0 per cent (dried substance).

CHARACTERS

Appearance: white or almost white powder.

Solubility: slightly soluble in water, freely soluble in methanol, soluble in methylene chloride, sparingly soluble or soluble in alcohol.

It shows polymorphism.

IDENTIFICATION

A. Infrared absorption spectrophotometry (2.2.24).

Preparation: mulls in *liquid paraffin R*.

Comparison: *benfluorex hydrochloride CRS*.

If the spectra obtained show differences, heat the substance to be examined and the reference substance separately in an oven at 150 °C for 3 h and record new spectra.

B. It gives reaction (a) of chlorides (2.3.1).

TESTS

Optical rotation (2.2.7): -0.10° to $+0.10^\circ$.

Dissolve 0.2 g in *ethanol R* and dilute to 20.0 ml with the same solvent.

Impurity B. Gas chromatography (2.2.28): use the normalisation procedure.

Test solution. Dissolve 0.30 g of the substance to be examined in *methylene chloride R* and dilute to 20 ml with the same solvent. Transfer to a separating funnel, add 10 ml of a 40 g/l solution of *sodium hydroxide R*. Shake the flask vigorously and allow the phases to separate. Collect the organic layer.

Reference solution. Dissolve 0.30 g of *benfluorex hydrochloride for system suitability CRS* in *methylene chloride R* and dilute to 20 ml with the same solvent. Transfer to a separating funnel and add 10 ml of a 40 g/l solution of *sodium hydroxide R*. Shake the flask vigorously and allow the phases to separate. Collect the organic layer.

Column:

- **material:** fused silica,
- **size:** $l = 25$ m, $\varnothing = 0.32$ mm,
- **stationary phase:** macrogol 20 000 R (film thickness 0.2 µm).

Carrier gas: *hydrogen for chromatography R*.

Linear velocity: 75 cm/s.

Split ratio: 1:35.

Temperature:

- **column:** 220 °C,
- **injection port and detector:** 250 °C.

Detection: flame ionisation.

Injection: 1 µl.

Run time: 1.5 times the retention time of benfluorex.

Relative retention with reference to benfluorex (retention time = about 4.5 min): **impurity B** = about 1.1.

System suitability:

- **peak-to-valley ratio:** minimum 2.5, where H_p = height above the baseline of the peak due to impurity B, and H_v = height above the baseline of the lowest point of the curve separating this peak from the peak due to benfluorex.

Limit:

- **impurity B:** maximum 0.1 per cent.

Related substances, other than impurity B. Liquid chromatography (2.2.29).

Test solution. Dissolve 60.0 mg of the substance to be examined in 50 ml of *acetonitrile R* and dilute to 100.0 ml with *water R*.

Reference solution (a). Dissolve 60.0 mg of *benfluorex hydrochloride for system suitability CRS* in 50 ml of *acetonitrile R* and dilute to 100.0 ml with *water R*.

Reference solution (b). Dilute 1.0 ml of the test solution to 100.0 ml with a mixture of equal volumes of *acetonitrile R* and *water R*. Dilute 5.0 ml of this solution to 50.0 ml with the same mixture of solvents.

Column:

- **size:** $l = 0.15$ m, $\varnothing = 4.6$ mm,
- **stationary phase:** silica gel bonded with alkylamide groups (5 μ m),
- **temperature:** 60 °C.

Mobile phase: a mixture of equal volumes of *acetonitrile R* and a solution containing 2.18 g/l of *potassium dihydrogen phosphate R* adjusted to pH 2.5 with *phosphoric acid R* and 6.5 g/l of *sodium decyl sulphate R*.

Flow rate: 1.4 ml/min.

Detection: spectrophotometer at 210 nm.

Injection: 10 μ l.

Run time: 3 times the retention time of *benfluorex*.

Relative retention with reference to *benfluorex* (retention time = about 5 min): impurity A = about 0.9.

System suitability:

- **signal-to-noise ratio:** minimum 20 for the principal peak in the chromatogram obtained with reference solution (b),
- **peak-to-valley ratio:** minimum 2.5, where H_p = height above the baseline of the peak due to impurity A, and H_v = height above the baseline of the lowest point of the curve separating this peak from the peak due to *benfluorex* in the chromatogram obtained with reference solution (a).

Limits:

- **any impurity:** not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent),
- **total:** not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent),
- **disregard limit:** 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

Heavy metals (2.4.8): maximum 20 ppm.

1.0 g complies with limit test C. Prepare the standard using 2 ml of *lead standard solution (10 ppm Pb) R*.

Loss on drying (2.2.32): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 100–105 °C.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

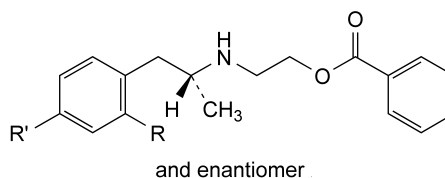
ASSAY

In order to avoid overheating in the reaction medium, mix thoroughly throughout and stop the titration immediately after the end-point has been reached.

Dissolve 0.250 g rapidly in 2.0 ml of *anhydrous formic acid R* and add 50.0 ml of *acetic anhydride R*. Titrate immediately with 0.1 M *perchloric acid*, determining the end-point potentiometrically (2.2.20).

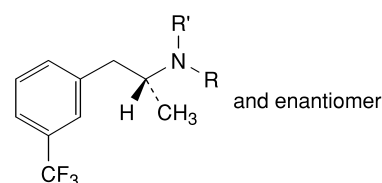
1 ml of 0.1 M *perchloric acid* is equivalent to 38.78 mg of $C_{19}H_{21}ClF_3NO_2$.

IMPURITIES



- A. R = CF_3 , R' = H: 2-[[[(1*RS*)-1-methyl-2-[2-(trifluoromethyl)phenyl]ethyl]amino]ethyl benzoate,
- B. R = H, R' = CF_3 : 2-[[[(1*RS*)-1-methyl-2-[4-(trifluoromethyl)phenyl]ethyl]amino]ethyl benzoate,

C. benzoic acid,

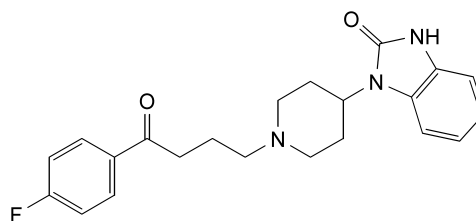


- D. R = CH_2CH_2OH , R' = H: 2-[[[(1*RS*)-1-methyl-2-[3-(trifluoromethyl)phenyl]ethyl]amino]ethanol,
- E. R = CH_2CH_2OH , R' = $CO-C_6H_5$: *N*-(2-hydroxyethyl)-*N*[[[(1*RS*)-1-methyl-2-[3-(trifluoromethyl)phenyl]ethyl]amino]ethyl]benzamide,
- F. R = $CH_2CH_2O-CO-C_6H_5$, R' = $CO-C_6H_5$: 2-[benzoyl[(1*RS*)-1-methyl-2-[3-(trifluoromethyl)phenyl]ethyl]amino]ethyl benzoate.

01/2005:1172

BENPERIDOL

Benperidolum



$C_{22}H_{24}FN_3O_2$

M_r 381.4

DEFINITION

Benperidol contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of 1-[1-[4-(4-fluorophenyl)-4-oxobutyl]piperidin-4-yl]-1,3-dihydro-2*H*-benzimidazol-2-one, calculated with reference to the dried substance.

CHARACTERS

A white or almost white powder, practically insoluble in water, freely soluble in dimethylformamide, soluble in methylene chloride, slightly soluble in alcohol.

It shows polymorphism.

IDENTIFICATION

First identification: A.

Second identification: B, C, D.

- A. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *benperidol CRS*. Examine the substances prepared as discs. If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the

reference substance separately in the minimum volume of *methyl isobutyl ketone R*, evaporate to dryness and record new spectra using the residues.

- B. Examine by thin-layer chromatography (2.2.27), using as the coating substance a suitable silica gel with a fluorescent indicator having an optimal intensity at 254 nm.

Test solution. Dissolve 30 mg of the substance to be examined in a mixture of 1 volume of *acetone R* and 9 volumes of *methanol R* and dilute to 10 ml with the same mixture of solvents.

Reference solution (a). Dissolve 30 mg of *benperidol CRS* in a mixture of 1 volume of *acetone R* and 9 volumes of *methanol R* and dilute to 10 ml with the same mixture of solvents.

Reference solution (b). Dissolve 30 mg of *benperidol CRS* and 30 mg of *droperidol CRS* in a mixture of 1 volume of *acetone R* and 9 volumes of *methanol R* and dilute to 10 ml with the same mixture of solvents.

Apply to the plate 10 µl of each solution. Develop over a path of 15 cm using a mixture of 1 volume of *acetone R* and 9 volumes of *methanol R*. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. The principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a). The test is not valid unless the chromatogram obtained with reference solution (b) shows two clearly separated spots.

- C. Dissolve about 10 mg in 5 ml of *ethanol R*. Add 0.5 ml of *dinitrobenzene solution R* and 0.5 ml of *2 M alcoholic potassium hydroxide R*. A violet colour is produced which becomes brownish-red after 20 min.
- D. Mix about 5 mg with 45 mg of *heavy magnesium oxide R* and ignite in a crucible until an almost white residue is obtained (usually less than 5 min). Allow to cool, add 1 ml of *water R*, 0.05 ml of *phenolphthalein solution R1* and about 1 ml of *dilute hydrochloric acid R* to render the solution colourless. Filter. To a freshly prepared mixture of 0.1 ml of *alizarin S solution R* and 0.1 ml of *zirconyl nitrate solution R*, add 1.0 ml of the filtrate. Mix, allow to stand for 5 min and compare the colour of the solution with that of a blank prepared in the same manner. The test solution is yellow and the blank is red.

TESTS

Related substances. Examine by liquid chromatography (2.2.29). Prepare the solutions immediately before use.

Test solution. Dissolve 0.10 g of the substance to be examined in *dimethylformamide R* and dilute to 10.0 ml with the same solvent.

Reference solution (a). Dissolve 2.5 mg of *benperidol CRS* and 2.5 mg of *droperidol CRS* in *dimethylformamide R* and dilute to 100.0 ml with the same solvent.

Reference solution (b). Dilute 1.0 ml of the test solution to 100.0 ml with *dimethylformamide R*. Dilute 5.0 ml of this solution to 20.0 ml with *dimethylformamide R*.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.1 m long and 4.6 mm in internal diameter packed with *base-deactivated octadecylsilyl silica gel for chromatography R* (3 µm),
- as mobile phase at a flow rate of 1.5 ml/min:

Mobile phase A. A 10 g/l solution of *tetrabutylammonium hydrogen sulphate R*,

Mobile phase B. *Acetonitrile R*,

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)	Comment
0 - 15	100 → 60	0 → 40	linear gradient
15 - 20	60	40	isocratic elution
20 - 25	100	0	switch to initial eluent composition
25 = 0	100	0	restart gradient

– as detector a spectrophotometer set at 275 nm.

Equilibrate the column for at least 30 min with *acetonitrile R* and then equilibrate with the initial eluent composition for at least 5 min.

Adjust the sensitivity of the system so that the height of the principal peak in the chromatogram obtained with 10 µl of reference solution (b) is at least 50 per cent of the full scale of the recorder.

Inject 10 µl of reference solution (a). When the chromatogram is recorded in the prescribed conditions, the retention times are: *benperidol* about 6.5 min and *droperidol* about 7 min. The test is not valid unless the resolution between the peaks corresponding to *benperidol* and *droperidol* is at least 2.0. If necessary, adjust the concentration of *acetonitrile* in the mobile phase or adjust the time programme for the linear gradient.

Inject 10 µl of *dimethylformamide R* as a blank, 10 µl of the test solution and 10 µl of reference solution (b). In the chromatogram obtained with the test solution: the area of any peak, apart from the principal peak, is not greater than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.25 per cent); the sum of the areas of all the peaks, apart from the principal peak, is not greater than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent). Disregard any peak obtained with the blank and any peak with an area less than 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (b).

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g in a platinum crucible.

ASSAY

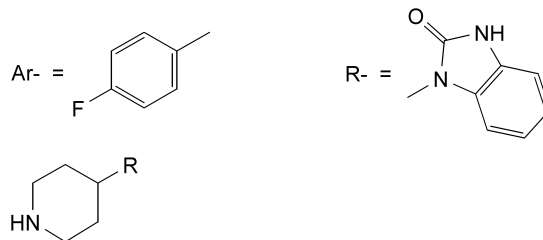
Dissolve 0.300 g in 50 ml of a mixture of 1 volume of *anhydrous acetic acid R* and 7 volumes of *methyl ethyl ketone R* and titrate with 0.1 M *perchloric acid*, using 0.2 ml of *naphtholbenzein solution R* as indicator.

1 ml of 0.1 M *perchloric acid* is equivalent to 38.14 mg of $C_{22}H_{24}FN_3O_2$.

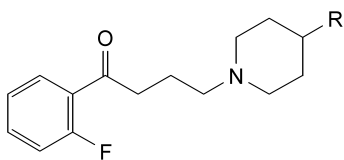
STORAGE

Store protected from light.

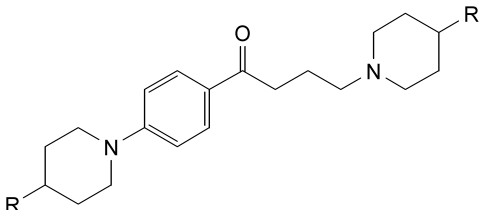
IMPURITIES



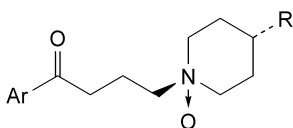
A. 1-(piperidin-4-yl)-1,3-dihydro-2H-benzimidazol-2-one,



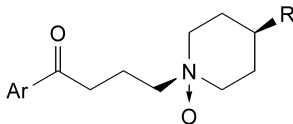
- B. 1-[1-[4-(2-fluorophenyl)-4-oxobutyl]piperidin-4-yl]-1,3-dihydro-2H-benzimidazol-2-one,



- C. 1-[1-[4-oxo-4-[4-[4-(2-oxo-2,3-dihydro-1H-benzimidazol-1-yl)piperidin-1-yl]phenyl]butyl]piperidin-4-yl]-1,3-dihydro-2H-benzimidazol-2-one,



- D. *cis*-1-[1-[4-(4-fluorophenyl)-4-oxobutyl]piperidin-4-yl]-1-oxide]-1,3-dihydro-2H-benzimidazol-2-one,

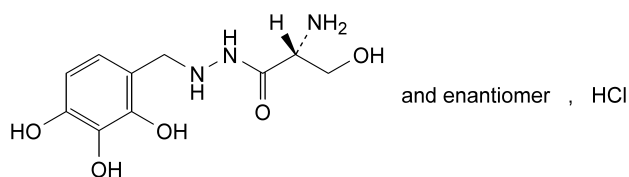


- E. *trans*-1-[1-[4-(4-fluorophenyl)-4-oxobutyl]piperidin-4-yl]-1-oxide]-1,3-dihydro-2H-benzimidazol-2-one.

01/2005:1173

BENSERAZIDE HYDROCHLORIDE

Benserazidi hydrochloridum

C₁₀H₁₆ClN₃O₅M_r 293.7

DEFINITION

Benserazide hydrochloride contains not less than 98.5 per cent and not more than the equivalent of 101.0 per cent of (*RS*)-2-amino-3-hydroxy-2'-(2,3,4-trihydroxybenzyl)propanohydrazide hydrochloride, calculated with reference to the anhydrous substance.

CHARACTERS

A white or yellowish-white or orange-white, crystalline powder, freely soluble in water, very slightly soluble in ethanol, practically insoluble in acetone.

It shows polymorphism.

IDENTIFICATION

- A. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *benserazide hydrochloride CRS*. Examine the substances prepared as discs. If the spectra obtained show

differences, dissolve the substance to be examined and the reference substance separately in hot *methanol R*, evaporate to dryness and record new spectra using the residues.

- B. Solution S (see Tests) gives reaction (b) of chlorides (2.3.1).

TESTS

Solution S. Dissolve 1.0 g in *carbon dioxide-free water R* and dilute to 100 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and not more intensely coloured than reference solution BY₆ (2.2.2, Method II).

pH (2.2.3). The pH of solution S is 4.0 to 5.0.

Optical rotation (2.2.7). The angle of optical rotation, determined on solution S, is −0.05 to +0.05.

Related substances. Examine by liquid chromatography (2.2.29).

Prepare the solutions using the mobile phase cooled to 4 °C and inject immediately.

Test solution. Dissolve 0.10 g of the substance to be examined in the mobile phase and dilute to 100.0 ml with the mobile phase.

Reference solution. Dissolve 5.0 mg of *benserazide impurity A CRS* and 5.0 mg of *benserazide hydrochloride CRS* in the mobile phase and dilute to 50.0 ml with the mobile phase. Dilute 5.0 ml of the solution to 100.0 ml with the mobile phase.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.125 m long and 4 mm in internal diameter packed with *octylsilyl silica gel for chromatography R* (5 µm),
- as mobile phase at a flow rate of 1.2 ml/min a mixture prepared as follows: dissolve 4.76 g of *potassium dihydrogen phosphate R* in 800 ml of *water R*; add 200 ml of *acetonitrile R* and 1.22 g of *sodium decanesulphonate R*; adjust to pH 3.5 with *phosphoric acid R*,
- as detector a spectrophotometer set at 220 nm.

Inject 20 µl of the reference solution. The test is not valid unless the resolution between the peaks corresponding to impurity A (first peak) and benserazide (second peak) is at least 2.0.

Inject 20 µl of the test solution. Continue the chromatography for nine times the retention time of benserazide. In the chromatogram obtained with the test solution: the area of any peak due to impurity A is not greater than the area of the corresponding peak in the chromatogram obtained with the reference solution (0.5 per cent); the area of any peak, apart from the principal peak and any peak due to impurity A, is not greater than the area of the peak due to benserazide in the chromatogram obtained with the reference solution (0.5 per cent); the sum of the areas of any such peaks is not greater than twice the area of the peak due to benserazide in the chromatogram obtained with the reference solution (1 per cent). Disregard any peak with an area less than 0.1 times that of the peak due to benserazide in the chromatogram obtained with the reference solution.

Heavy metals (2.4.8). 1.0 g complies with limit test C for heavy metals (20 ppm). Prepare the standard using 2 ml of *lead standard solution (10 ppm Pb) R*.

Water (2.5.12). Not more than 1.0 per cent, determined on 0.500 g by the semi-micro determination of water.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

In order to avoid overheating during the titration, mix thoroughly throughout and stop the titration immediately after the end-point has been reached.

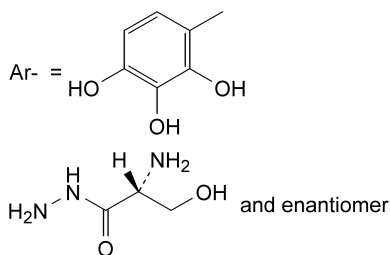
Dissolve 0.250 g in 5 ml of *anhydrous formic acid R*. Add 70 ml of *anhydrous acetic acid R*. Titrate immediately with 0.1 M *perchloric acid*, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *perchloric acid* is equivalent to 29.37 mg of $C_{10}H_{16}ClN_3O_5$.

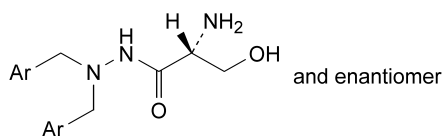
STORAGE

Store protected from light.

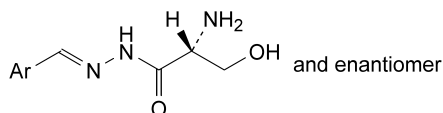
IMPURITIES



A. (RS)-2-amino-3-hydroxypropanohydrazide,



B. (RS)-2-amino-3-hydroxy-2',2'-bis(2,3,4-trihydroxybenzyl)propanohydrazide,



C. (RS)-2-amino-3-hydroxy-2'-(2,3,4-trihydroxybenzylidene)propanohydrazide.

01/2005:0467

BENTONITE

Bentonitum

DEFINITION

Bentonite is a natural clay containing a high proportion of montmorillonite, a native hydrated aluminium silicate in which some aluminium and silicon atoms may be replaced by other atoms such as magnesium and iron.

CHARACTERS

A very fine, homogeneous, greyish-white powder with a more or less yellowish or pinkish tint, practically insoluble in water and in aqueous solutions. It swells with a little water forming a malleable mass.

IDENTIFICATION

A. To 0.5 g in a metal crucible add 1 g of *potassium nitrate R* and 3 g of *sodium carbonate R* and heat until the mixture melts. Allow to cool. To the residue add 20 ml of boiling *water R*, mix and filter. Wash the residue with 50 ml of *water R*. To the residue add 1 ml of *hydrochloric acid R*

and 5 ml of *water R*. Filter. To the filtrate add 1 ml of *strong sodium hydroxide solution R* and filter. To the filtrate add 3 ml of *ammonium chloride solution R*. A gelatinous white precipitate is formed.

B. It complies with the test for swelling power with water (see Tests).

C. 0.25 g gives the reaction of silicates (2.3.1).

TESTS

Alkalinity. To 2 g add 100 ml of *carbon dioxide-free water R* and shake for 5 min. To 5 ml of the suspension add 0.1 ml of *thymolphthalein solution R*. The liquid becomes bluish. Add 0.1 ml of 0.1 M *hydrochloric acid*. The liquid is decolourised within 5 min.

Coarse particles. To 20 g add 1000 ml of *water R* and mix for 15 min using a high-speed mixer capable of operating at not less than 5000 r/min. Transfer to a wet sieve (75), tared after drying at 100 °C to 105 °C. Wash with three quantities, each of 500 ml, of *water R*, ensuring that any agglomerates have been dispersed. Dry at 100 °C to 105 °C and weigh. The particles on the sieve weigh not more than 0.1 g (0.5 per cent).

Sedimentation volume. To 6.0 g add 200 ml of *water R* and mix for 20 min using a high-speed mixer capable of operating at 10 000 r/min. Transfer 100 ml of the suspension to a graduated cylinder. Allow to stand for 24 h. The volume of the clear supernatant liquid is not greater than 2 ml.

Swelling power with water. Add 2.0 g in twenty portions to 100 ml of a 10 g/l solution of *sodium laurilsulfate R* in a 100 ml graduated cylinder about 30 mm in diameter. Allow 2 min between additions for each portion to settle. Allow to stand for 2 h. The apparent volume of the sediment is not less than 22 ml.

Heavy metals (2.4.8). To 5.0 g add 7.5 ml of *dilute hydrochloric acid R* and 27.5 ml of *water R*. Boil for 5 min. Centrifuge and filter the supernatant liquid. Wash the centrifugation residue with *water R* and filter. Dilute the combined filtrates to 50.0 ml with *water R*. To 5 ml of the solution add 5 ml of *water R*, 10 ml of *hydrochloric acid R* and 25 ml of *methyl isobutyl ketone R* and shake for 2 min. Separate the layers. Evaporate the aqueous layer to dryness on a water-bath. Dissolve the residue in 1 ml of *acetic acid R*, dilute to 25 ml with *water R* and filter. 12 ml of the filtrate complies with limit test A for heavy metals (50 ppm). Prepare the standard using *lead standard solution (1 ppm Pb) R*.

Loss on drying (2.2.32). Not more than 15 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C.

Microbial contamination. Total viable aerobic count (2.6.12) not more than 10^3 micro-organisms per gram, determined by plate-count.

01/2005:0372

BENZALKONIUM CHLORIDE

Benzalkonii chloridum

DEFINITION

Benzalkonium chloride is a mixture of alkylbenzyltrimethylammonium chlorides, the alkyl groups having chain lengths of C_8 to C_{18} . It contains not less than 95.0 per cent and not more than the equivalent of 104.0 per cent of alkylbenzyltrimethylammonium chlorides, calculated as $C_{22}H_{40}ClN$ (M_r 354.0) with reference to the anhydrous substance.

CHARACTERS

A white or yellowish-white powder or gelatinous, yellowish-white fragments, hygroscopic, soapy to the touch, very soluble in water and in alcohol. On heating it forms a clear molten mass. An aqueous solution froths copiously when shaken.

IDENTIFICATION

- A. Dissolve 80 mg in *water R* and dilute to 100 ml with the same solvent. Examined between 220 nm and 350 nm (2.2.25), the solution shows three absorption maxima, at 257 nm, 263 nm and 269 nm, and a shoulder at about 250 nm.
- B. To 2 ml of solution S (see Tests) add 0.1 ml of *glacial acetic acid R* and, dropwise, 1 ml of *sodium tetraphenylborate solution R*. A white precipitate is formed. Filter. Dissolve the precipitate in a mixture of 1 ml of *acetone R* and 5 ml of *alcohol R*, heating to not more than 70 °C. Add *water R* dropwise to the warm solution until a slight opalescence forms. Heat gently until the solution is clear and allow to cool. White crystals separate. Filter, wash with three quantities, each of 10 ml, of *water R* and dry *in vacuo* over *diphosphorus pentoxide R* or *anhydrous silica gel R* at a temperature not exceeding 50 °C. The crystals melt (2.2.14) at 127 °C to 133 °C.
- C. To 5 ml of *dilute sodium hydroxide solution R* add 0.1 ml of *bromophenol blue solution R1* and 5 ml of *chloroform R* and shake. The chloroform layer is colourless. Add 0.1 ml of solution S and shake. The chloroform layer becomes blue.
- D. To 2 ml of solution S add 1 ml of *dilute nitric acid R*. A white precipitate is formed which dissolves on the addition of 5 ml of *alcohol R*. The solution gives reaction (a) of chlorides (2.3.1).

TESTS

Solution S. Dissolve 1.0 g in *carbon dioxide-free water R* and dilute to 100 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and not more intensely coloured than reference solution Y₆ (2.2.2, Method II).

Acidity or alkalinity. To 50 ml of solution S add 0.1 ml of *bromocresol purple solution R*. Not more than 0.1 ml of 0.1 M *hydrochloric acid* or 0.1 M *sodium hydroxide* is required to change the colour of the indicator.

Amines and amine salts. Dissolve 5.0 g with heating in 20 ml of a mixture of 3 volumes of 1 M *hydrochloric acid* and 97 volumes of *methanol R* and add 100 ml of 2-propanol *R*. Pass a stream of *nitrogen R* slowly through the solution. Gradually add 12.0 ml of 0.1 M *tetrabutylammonium hydroxide* and record the potentiometric titration curve (2.2.20). If the curve shows two points of inflexion, the volume of titrant added between the two points is not greater than 5.0 ml. If the curve shows no point of inflexion, the substance to be examined does not comply with the test. If the curve shows one point of inflexion, repeat the test but add 3.0 ml of a 25.0 g/l solution of *dimethyldecylamine R* in 2-propanol *R* before the titration. If the titration curve after addition of 12.0 ml of the titrant shows only one point of inflexion, the substance to be examined does not comply with the test.

Water (2.5.12). Not more than 10 per cent, determined on 0.300 g by the semi-micro determination of water.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 2.00 g in *water R* and dilute to 100.0 ml with the same solvent. Transfer 25.0 ml of the solution to a separating funnel, add 25 ml of *chloroform R*, 10 ml of 0.1 M *sodium hydroxide* and 10.0 ml of a freshly prepared 50 g/l solution of *potassium iodide R*. Shake well, allow to separate and discard the chloroform layer. Shake the aqueous layer with three quantities, each of 10 ml, of *chloroform R* and discard the chloroform layers. To the aqueous layer add 40 ml of *hydrochloric acid R*, allow to cool and titrate with 0.05 M *potassium iodate* until the deep-brown colour is almost discharged. Add 2 ml of *chloroform R* and continue the titration, shaking vigorously, until the chloroform layer no longer changes colour. Carry out a blank titration on a mixture of 10.0 ml of the freshly prepared 50 g/l solution of *potassium iodide R*, 20 ml of *water R* and 40 ml of *hydrochloric acid R*.

1 ml of 0.05 M *potassium iodate* is equivalent to 35.4 mg of C₂₂H₄₀ClN.

01/2005:0371

BENZALKONIUM CHLORIDE
SOLUTION

Benzalkonii chloridi solutio

DEFINITION

Benzalkonium chloride solution is an aqueous solution of a mixture of alkylbenzyltrimethylammonium chlorides, the alkyl groups having chain lengths of C₈ to C₁₈. Benzalkonium chloride solution contains not less than 475 g/l and not more than 525 g/l of alkylbenzyltrimethylammonium chlorides, calculated as C₂₂H₄₀ClN (*M_r* 354.0). The solution may contain alcohol.

CHARACTERS

A clear, colourless or slightly yellowish liquid, miscible with water and with alcohol. It froths copiously when shaken.

IDENTIFICATION

- A. Dilute 0.3 ml to 100 ml with *water R*. Examined between 220 nm and 350 nm (2.2.25), the solution shows three absorption maxima, at 257 nm, 263 nm and 269 nm, and a shoulder at about 250 nm.
- B. To 0.05 ml add 2 ml of *water R*, 0.1 ml of *glacial acetic acid R* and, dropwise, 1 ml of *sodium tetraphenylborate solution R*. A white precipitate is formed. Filter. Dissolve the precipitate in a mixture of 1 ml of *acetone R* and 5 ml of *alcohol R*, heating to not more than 70 °C. Add *water R* dropwise to the warm solution until a slight opalescence forms. Heat gently until the solution is clear and allow to cool. White crystals separate. Filter, wash with three quantities, each of 10 ml, of *water R* and dry *in vacuo* over *diphosphorus pentoxide R* or *anhydrous silica gel R* at a temperature not exceeding 50 °C. The crystals melt (2.2.14) at 127 °C to 133 °C.
- C. To 5 ml of *dilute sodium hydroxide solution R* add 0.1 ml of *bromophenol blue solution R1* and 5 ml of *chloroform R* and shake. The chloroform layer is colourless. Add 0.05 ml of the solution to be examined and shake. The chloroform layer becomes blue.
- D. To 0.05 ml add 1 ml of *dilute nitric acid R*. A white precipitate is formed which dissolves on the addition of 5 ml of *alcohol R*. The solution gives reaction (a) of chlorides (2.3.1).

TESTS

Solution S. Dilute 2.0 g to 100 ml with *carbon dioxide-free water R*.

Appearance of solution. Solution S is clear (2.2.1) and not more intensely coloured than reference solution Y₆ (2.2.2, Method II).

Acidity or alkalinity. To 50 ml of solution S add 0.1 ml of *bromocresol purple solution R*. Not more than 0.1 ml of 0.1 M *hydrochloric acid* or 0.1 M *sodium hydroxide* is required to change the colour of the indicator.

Amines and amine salts. Mix 10.0 g, while heating, with 20 ml of a mixture of 3 volumes of 1 M *hydrochloric acid* and 97 volumes of *methanol R* and add 100 ml of 2-propanol *R*. Pass a stream of *nitrogen R* slowly through the solution. Gradually add 12.0 ml of 0.1 M *tetrabutylammonium hydroxide* and record the potentiometric titration curve (2.2.20). If the curve shows two points of inflexion, the volume of titrant added between the two points is not greater than 5.0 ml. If the curve shows no point of inflexion, the solution to be examined does not comply with the test. If the curve shows one point of inflexion, repeat the test but add 3.0 ml of a 25.0 g/l solution of *dimethyldecylamine R* in 2-propanol *R* before the titration. If the titration curve after the addition of 12.0 ml of the titrant shows only one point of inflexion, the solution to be examined does not comply with the test.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Determine the density (2.2.5) of the solution to be examined. Dilute 4.00 g to 100.0 ml with *water R*. Transfer 25.0 ml of the solution to a separating funnel, add 25 ml of *chloroform R*, 10 ml of 0.1 M *sodium hydroxide* and 10.0 ml of a freshly prepared 50 g/l solution of *potassium iodide R*. Shake well, allow to separate and discard the chloroform layer. Shake the aqueous layer with three quantities, each of 10 ml, of *chloroform R* and discard the chloroform layers. To the aqueous layer add 40 ml of *hydrochloric acid R*, allow to cool and titrate with 0.05 M *potassium iodate* until the deep-brown colour is almost discharged. Add 2 ml of *chloroform R* and continue the titration, shaking vigorously, until the chloroform layer no longer changes colour. Carry out a blank titration on a mixture of 10.0 ml of the freshly prepared 50 g/l solution of *potassium iodide R*, 20 ml of *water R* and 40 ml of *hydrochloric acid R*.

1 ml of 0.05 M *potassium iodate* is equivalent to 35.4 mg of C₂₂H₄₀ClN.

LABELLING

The label states the content of alcohol, if any.

DEFINITION

Benzbromarone contains not less than 98.0 and not more than the equivalent of 101.0 per cent of (3,5-dibromo-4-hydroxyphenyl)(2-ethylbenzofuran-3-yl)methanone, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, crystalline powder, practically insoluble in water, freely soluble in acetone and in methylene chloride, sparingly soluble in alcohol.

It melts at about 152 °C.

IDENTIFICATION

- Examine by infrared absorption spectrophotometry (2.2.24), comparing with the *Ph. Eur. reference spectrum of benzbromarone*.
- By means of a copper wire, previously ignited, introduce a small amount of the substance into the non-illuminated part of a flame. The colour of the flame becomes green.

TESTS

Appearance of solution. Dissolve 1.25 g in *dimethylformamide R* and dilute to 25 ml with the same solvent. The solution is clear (2.2.1) and not more intensely coloured than reference solution Y₅ (2.2.2, Method II).

Acidity or alkalinity. Shake 0.5 g with 10 ml of *carbon dioxide-free water R* for 1 min and filter. To 2.0 ml of the filtrate add 0.1 ml of *methyl red solution R* and 0.1 ml of 0.01 M *hydrochloric acid*. The solution is red. Add 0.3 ml of 0.01 M *sodium hydroxide*. The solution is yellow.

Related substances. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 0.125 g of the substance in 30 ml of *methanol R* and dilute to 50.0 ml with the mobile phase.

Reference solution (a). Dilute 1.0 ml of the test solution to 100 ml with the mobile phase. Dilute 1 ml of this solution to 10 ml with the mobile phase.

Reference solution (b). Dissolve 10 mg of *benzarone CRS* in the mobile phase and dilute to 20 ml with the mobile phase.

Reference solution (c). To 5 ml of reference solution (b) add 1 ml of the test solution and dilute to 100 ml with the mobile phase.

The chromatographic procedure may be carried out using:

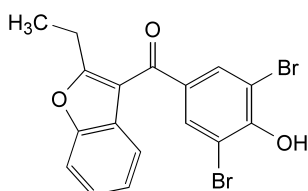
- a stainless steel column 0.25 m long and 4.6 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (5 µm),
- as mobile phase at a flow rate of 1.5 ml/min a mixture of 5 volumes of *glacial acetic acid R*, 25 volumes of *acetonitrile R*, 300 volumes of *water R* and 990 volumes of *methanol R*,
- as detector a spectrophotometer set at 231 nm.

Inject 20 µl of reference solution (c). Adjust the sensitivity of the system so that the heights of the principal peaks in the chromatogram obtained are at least 50 per cent of the full scale of the recorder. The test is not valid unless the resolution between the first peak (impurity C) and the second peak (benzbromarone) is at least 10.0.

Inject 20 µl of the test solution and 20 µl of reference solution (a). Continue the chromatography of the test solution for 2.5 times the retention time of benzbromarone. If peaks other than the principal peak are observed in the chromatogram obtained with the test solution, these may be due to impurity A or to impurity B. When the

BENZBROMARONE

Benzbromaronum



C₁₇H₁₂Br₂O₃

M_r 424.1

chromatograms are recorded in the prescribed conditions, the relative retention times are: impurity A about 0.6 and impurity B about 2.

In the chromatogram obtained with the test solution: the area of the peak corresponding to impurity A is not greater than four times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.4 per cent); the area of the peak corresponding to impurity B is not greater than ten times the area of the principal peak in the chromatogram obtained with reference solution (a) (1 per cent); the area of any peak, apart from the principal peak and the peaks corresponding to impurity A and impurity B, is not greater than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent); the sum of the areas of any such peaks is not greater than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent). Disregard any peak with an area less than 0.2 times that of the principal peak in the chromatogram obtained with reference solution (a).

Halides expressed as chlorides (2.4.4). Shake 1.25 g of the substance to be examined with a mixture of 5 ml of *dilute nitric acid R* and 15 ml of *water R*. Filter. Rinse the filter with *water R* and dilute the filtrate to 25 ml with the same solvent. Dilute 2.5 ml to 15 ml with *water R*. The solution obtained complies with the limit test for chlorides (400 ppm).

Iron (2.4.9). Moisten the residue obtained in the test for sulphated ash with 2 ml of *hydrochloric acid R* and evaporate to dryness on a water-bath. Add 0.05 ml of *hydrochloric acid R* and 10 ml of *water R* and heat until boiling for 1 min. Allow to cool. Rinse the crucible with *water R*, collect the rinsings and dilute to 25 ml with *water R*. Dilute 2 ml of this solution to 10 ml with *water R*. The solution complies with the limit test for iron (125 ppm).

Heavy metals (2.4.8). 0.5 g complies with limit test C for heavy metals (20 ppm). Prepare the standard using 1 ml of *lead standard solution (10 ppm Pb) R*.

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven *in vacuo* at 50 °C for 4 h.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

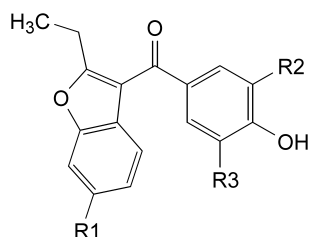
Dissolve 0.300 g in 60 ml of *methanol R*. Stir until completely dissolved and add 10 ml of *water R*. Titrate with 0.1 M *sodium hydroxide*, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *sodium hydroxide* is equivalent to 42.41 mg of $C_{17}H_{12}Br_2O_3$.

STORAGE

Store protected from light.

IMPURITIES



A. R1 = R2 = H, R3 = Br: (3-bromo-4-hydroxyphenyl)(2-ethylbenzofuran-3-yl)methanone,

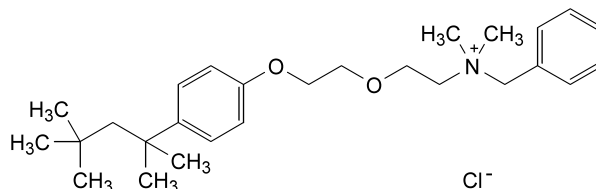
B. R1 = R2 = R3 = Br: (6-bromo-2-ethylbenzofuran-3-yl)(3,5-dibromo-4-hydroxyphenyl)methanone,

C. R1 = R2 = R3 = H: (2-ethylbenzofuran-3-yl)(4-hydroxyphenyl)methanone (benzarone).

01/2005:0974

BENZETHONIUM CHLORIDE

Benzethonii chloridum



$C_{27}H_{42}ClNO_2$

M_r 448.1

DEFINITION

Benzethonium chloride contains not less than 97.0 per cent and not more than the equivalent of 103.0 per cent of *N*-benzyl-*N,N*-dimethyl-2-[2-[4-(1,1,3,3-tetramethylbutyl)phenoxy]ethoxy]ethanaminium chloride, calculated with reference to the dried substance.

CHARACTERS

A white or yellowish-white powder, very soluble in water and in alcohol, freely soluble in methylene chloride.

An aqueous solution froths copiously when shaken.

IDENTIFICATION

A. Melting point (2.2.14): 158 °C to 164 °C, after drying at 105 °C for 4 h.

B. Examine by thin-layer chromatography (2.2.27), using as the coating substance a suitable silica gel with a fluorescent indicator having an optimal intensity at 254 nm.

Test solution. Dissolve 25 mg of the substance to be examined in *water R* and dilute to 5 ml with the same solvent.

Reference solution. Dissolve 25 mg of *benzethonium chloride CRS* in *water R* and dilute to 5 ml with the same solvent.

Apply to the plate 20 µl of each solution. Develop over a path of 12 cm using a mixture of 5 volumes of *water R*, 5 volumes of *glacial acetic acid R* and 100 volumes of *methanol R*. Dry the plate in a current of warm air and examine in ultraviolet light at 254 nm. The principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with the reference solution.

C. To 5 ml of *dilute sodium hydroxide solution R* add 0.1 ml of *bromophenol blue solution R1* and 5 ml of *methylene chloride R* and shake. The lower layer is colourless. Add 0.1 ml of solution S (see Tests) and shake. A blue colour develops in the lower layer.

D. To 2 ml of solution S add 1 ml of *dilute nitric acid R*. A white precipitate is formed which dissolves upon addition of 5 ml of *alcohol R*. The solution gives reaction (a) of chlorides (2.3.1).

TESTS

Solution S. Dissolve 5.0 g in *carbon dioxide-free water R* and dilute to 50 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and not more intensely coloured than reference solution Y₆ (2.2.2, Method II).

Acidity or alkalinity. To 25 ml of solution S add 0.1 ml of *phenolphthalein solution R*. The solution is colourless. Add 0.3 ml of 0.01 M *sodium hydroxide*. The solution is pink. Add 0.1 ml of *methyl red solution R* and 0.5 ml of 0.01 M *hydrochloric acid*. The solution is orange-red.

Volatile bases and salts of volatile bases (2.4.1). 0.20 g complies with limit test B for ammonium (50 ppm). Prepare the standard using 0.1 ml of *ammonium standard solution (100 ppm NH₄) R*. Replace heavy magnesium oxide by 2.0 ml of *strong sodium hydroxide solution R*.

Loss on drying (2.2.32). Not more than 5.0 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C for 4 h.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 2.000 g in *water R* and dilute to 100.0 ml with the same solvent. Transfer 25.0 ml of the solution to a separating funnel, add 10 ml of a 4 g/l solution of *sodium hydroxide R*, 10.0 ml of a freshly prepared 50 g/l solution of *potassium iodide R* and 25 ml of *methylene chloride R*. Shake vigorously, allow to separate and discard the lower layer. Shake the upper layer with three quantities, each of 10 ml, of *methylene chloride R* and discard the lower layers. To the upper layer add 40 ml of *hydrochloric acid R*, allow to cool and titrate with 0.05 M *potassium iodate* until the deep brown colour is almost discharged. Add 4 ml of *methylene chloride R* and continue the titration, shaking vigorously, until the lower layer is no longer brown. Carry out a blank titration using a mixture of 10.0 ml of a freshly prepared 50 g/l solution of *potassium iodide R*, 20 ml of *water R* and 40 ml of *hydrochloric acid R*.

1 ml of 0.05 M *potassium iodate* is equivalent to 44.81 mg of C₉H₁₁NO₂.

STORAGE

Store protected from light.

Second identification: A, C, D.

A. Melting point (2.2.14): 89 °C to 92 °C.

B. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *benzocaine CRS*.

C. To about 50 mg in a test-tube add 0.2 ml of a 500 g/l solution of *chromium trioxide R*. Cover the mouth of the tube with a piece of filter paper moistened with a freshly prepared mixture of equal volumes of a 50 g/l solution of *sodium nitroprusside R* and a 200 g/l solution of *piperazine hydrate R*. Boil gently for at least 30 s. A blue colour develops on the filter paper.

D. Dissolve about 50 mg in *alcohol R* and dilute to 100 ml with the same solvent. 2 ml of the solution gives the reaction of primary aromatic amines (2.3.1).

TESTS

Appearance of solution. Dissolve 1.0 g in *alcohol R* and dilute to 20 ml with the same solvent. The solution is clear (2.2.1) and colourless (2.2.2, Method II).

Acidity or alkalinity. Dissolve 0.5 g in 10 ml of *alcohol R* previously neutralised to 0.05 ml of *phenolphthalein solution R*. Add 10 ml of *carbon dioxide-free water R*. The solution remains colourless and not more than 0.5 ml of 0.01 M *sodium hydroxide* is required to change the colour of the indicator.

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.00 g by drying *in vacuo*.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.400 g in a mixture of 25 ml of *hydrochloric acid R* and 50 ml of *water R*. Carry out the determination of primary aromatic amino-nitrogen (2.5.8).

1 ml of 0.1 M *sodium nitrite* is equivalent to 16.52 mg of C₉H₁₁NO₂.

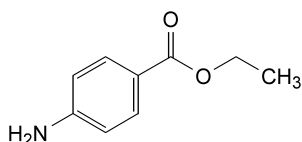
STORAGE

Store protected from light.

01/2005:0011

BENZOCAINE

Benzocainum



C₉H₁₁NO₂

M_r 165.2

DEFINITION

Benzocaine contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of ethyl 4-aminobenzoate, calculated with reference to the dried substance.

CHARACTERS

A white, crystalline powder or colourless crystals, very slightly soluble in water, freely soluble in alcohol.

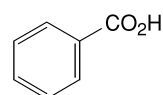
IDENTIFICATION

First identification: A, B.

01/2005:0066

BENZOIC ACID

Acidum benzoicum



C₇H₆O₂

M_r 122.1

DEFINITION

Benzoic acid contains not less than 99.0 per cent and not more than the equivalent of 100.5 per cent of benzenecarboxylic acid.

CHARACTERS

A white, crystalline powder or colourless crystals, odourless or with a very slight characteristic odour, slightly soluble in water, soluble in boiling water, freely soluble in alcohol and in fatty oils.

IDENTIFICATION

A. Melting point (2.2.14): 121 °C to 124 °C.

B. Solution S (see Tests) gives reaction (a) of benzoates (2.3.1).

01/2005:0704

TESTS

Solution S. Dissolve 5.0 g in *alcohol R* and dilute to 100 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and colourless (2.2.2, *Method II*).

Carbonisable substances. Dissolve 0.5 g with shaking in 5 ml of *sulphuric acid R*. After 5 min, the solution is not more intensely coloured than reference solution Y₅ (2.2.2, *Method I*).

Oxidisable substances. Dissolve 0.2 g in 10 ml of boiling *water R*. Cool, shake and filter. To the filtrate add 1 ml of *dilute sulphuric acid R* and 0.2 ml of 0.02 M *potassium permanganate*. After 5 min, the solution is still coloured pink.

Halogenated compounds and halides.

All glassware used must be chloride-free and may be prepared by soaking overnight in a 500 g/l solution of nitric acid R, rinsed with water R and stored full of water R. It is recommended that glassware be reserved for this test.

Solution (a). Dissolve 6.7 g of the substance to be examined in a mixture of 40 ml of 1 M *sodium hydroxide* and 50 ml of *alcohol R* and dilute to 100.0 ml with *water R*. To 10.0 ml of this solution add 7.5 ml of *dilute sodium hydroxide solution R* and 0.125 g of *nickel-aluminium alloy R* and heat on a water-bath for 10 min. Allow to cool to room temperature, filter into a 25 ml volumetric flask and wash with three quantities, each of 2 ml, of *alcohol R*. Dilute the filtrate and washings to 25.0 ml with *water R*. This solution is used to prepare solution A.

Solution (b). In the same manner, prepare a similar solution without the substance to be examined. This solution is used to prepare solution B.

In four 25 ml volumetric flasks, place separately 10 ml of solution (a), 10 ml of solution (b), 10 ml of *chloride standard solution (8 ppm Cl) R* (used to prepare solution C) and 10 ml of *water R*. To each flask add 5 ml of *ferric ammonium sulphate solution R5*, mix and add dropwise and with swirling 2 ml of *nitric acid R* and 5 ml of *mercuric thiocyanate solution R*. Shake. Dilute the contents of each flask to 25.0 ml with *water R* and allow the solutions to stand in a water-bath at 20 °C for 15 min. Measure at 460 nm the absorbance (2.2.25) of solution A using solution B as the compensation liquid, and the absorbance of solution C using the solution obtained with 10 ml of *water R* as the compensation liquid. The absorbance of solution A is not greater than that of solution C (300 ppm).

Heavy metals (2.4.8). 12 ml of solution S complies with limit test B for heavy metals (10 ppm). Prepare the standard using a mixture of 5 ml of *lead standard solution (1 ppm Pb) R* and 5 ml of *alcohol R*.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

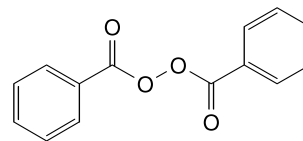
ASSAY

Dissolve 0.200 g in 20 ml of *alcohol R* and titrate with 0.1 M *sodium hydroxide*, using 0.1 ml of *phenol red solution R* as indicator, until the colour changes from yellow to violet-red.

1 ml of 0.1 M *sodium hydroxide* is equivalent to 12.21 mg of C₇H₆O₂.

BENZOYL PEROXIDE, HYDROUS

Benzoylis peroxidum cum aqua

C₁₄H₁₀O₄M_r 242.2

DEFINITION

Content:

- dibenzoyl peroxide: 70.0 per cent to 77.0 per cent,
- water: minimum 20.0 per cent.

CHARACTERS

Appearance: white, amorphous or granular powder.

Solubility: practically insoluble in water, soluble in acetone, soluble in methylene chloride with the separation of water, slightly soluble in alcohol.

It loses water rapidly on exposure to air with a risk of explosion.

Mix the entire sample thoroughly before carrying out the following tests.

IDENTIFICATION

First identification: B

Second identification: A, C, D.

- Dissolve 80.0 mg in *alcohol R* and dilute to 100.0 ml with the same solvent. Dilute 10.0 ml of the solution to 100.0 ml with *alcohol R* (solution A). Dilute 10.0 ml of solution A to 100.0 ml with *alcohol R* (solution B). Examined between 250 nm and 300 nm (2.2.25), solution A shows an absorption maximum at 274 nm and a shoulder at about 282 nm. Examined between 220 nm and 250 nm, solution B shows an absorption maximum at 235 nm. The ratio of the absorbance at the maximum at 235 nm (solution B) to that at the maximum at 274 nm (solution A) is 1.17 to 1.21.
- Infrared absorption spectrophotometry (2.2.24).
Comparison: Ph. Eur. reference spectrum of hydrous benzoyl peroxide.
- Dissolve about 25 mg in 2 ml of *acetone R*. Add 1 ml of a 10 g/l solution of *diethylphenylenediamine sulphate R* and mix. A red colour develops which quickly darkens and becomes dark violet within 5 min.
- To 1 g add 5 ml of *alcohol R*, 5 ml of *dilute sodium hydroxide solution R* and 10 ml of *water R*. Boil the mixture under reflux for 20 min. Cool. The solution gives reaction (c) of benzoates (2.3.1).

TESTS

Acidity. Dissolve a quantity of the substance to be examined containing the equivalent of 1.0 g of dibenzoyl peroxide in 25 ml of *acetone R*, add 75 ml of *water R* and filter. Wash the residue with two quantities, each of 10 ml, of *water R*. Combine the filtrate and the washings and add 0.25 ml of *phenolphthalein solution R1*. Not more than 1.25 ml of 0.1 M *sodium hydroxide* is required to change the colour of the indicator. Carry out a blank test.

Related substances. Liquid chromatography (2.2.29).
Prepare the solutions immediately before use.

Test solution. Dissolve a quantity of the substance to be examined containing the equivalent of 0.10 g of dibenzoyl peroxide in *acetonitrile R* and dilute to 50 ml with the same solvent.

Reference solution (a). Dilute 1.0 ml of the test solution to 100.0 ml with *acetonitrile R*. Dilute 1.0 ml of this solution to 10.0 ml with *acetonitrile R*.

Reference solution (b). Dissolve 30.0 mg of *benzoic acid R* in the mobile phase and dilute to 100.0 ml with the mobile phase. Dilute 1.0 ml of the solution to 10.0 ml with the mobile phase.

Reference solution (c). Dissolve 50.0 mg of *ethyl benzoate R* in the mobile phase and dilute to 100.0 ml with the mobile phase. Dilute 1.0 ml of the solution to 100.0 ml with the mobile phase.

Reference solution (d). Dissolve 50.0 mg of *benzaldehyde R* in the mobile phase and dilute to 100.0 ml with the mobile phase. Dilute 1.0 ml of the solution to 100.0 ml with the mobile phase.

Reference solution (e). Dissolve 30.0 mg of *benzoic acid R* and 30.0 mg of *benzaldehyde R* in the mobile phase and dilute to 100.0 ml with the mobile phase. Dilute 1.0 ml of the solution to 10.0 ml with the mobile phase.

Column:

- size: $l = 0.25$ m, $\varnothing = 4.6$ mm,
- stationary phase: octadecylsilyl silica gel for chromatography *R* (10 μ m),

Mobile phase: glacial acetic acid *R*, acetonitrile *R*, water *R* (1:500:500 V/V/V).

Flow rate: 1 ml/min.

Detection: spectrophotometer at 235 nm.

Injection: 20 μ l loop injector.

Run time: 2 times the retention time of dibenzoyl peroxide.

Relative retention with reference to dibenzoyl peroxide (retention time = about 28.4 min): impurity B = about 0.15; impurity A = about 0.2; impurity C = about 0.4.

System suitability: reference solution (e):

- resolution: minimum 6 between the peaks corresponding to benzoic acid and benzaldehyde.

Limits:

- impurity A: not more than the area of the principal peak in the chromatogram obtained with reference solution (d) (0.25 per cent),
- impurity B: not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (1.5 per cent),
- impurity C: not more than the area of the principal peak in the chromatogram obtained with reference solution (c) (0.25 per cent),
- any other impurity: not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent),
- disregard limit: 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.02 per cent).

Chlorides (2.4.4): maximum 0.4 per cent.

Dissolve a quantity of the substance to be examined containing the equivalent of 0.5 g of dibenzoyl peroxide in 15 ml of *acetone R*. Add, while stirring, 50 ml of 0.05 *M* nitric acid. Allow to stand for 10 min and filter. Wash the residue with 2 quantities, each of 10 ml, of 0.05 *M* nitric acid. Combine the filtrate and the washings and dilute to 100 ml with 0.05 *M* nitric acid. 2.5 ml of the solution diluted to 15.0 ml with water *R* complies with the limit test for chlorides.

ASSAY

Solution (a). Dissolve 2.500 g immediately before use in 75 ml of *dimethylformamide R* and dilute to 100.0 ml with the same solvent.

Dibenzoyl peroxide. To 5.0 ml of solution (a) add 20 ml of *acetone R* and 3 ml of a 500 g/l solution of *potassium iodide R* and mix. Allow to stand for 1 min. Titrate with 0.1 *M* sodium thiosulphate using 1 ml of *starch solution R*, added towards the end of the titration, as indicator. Carry out a blank titration.

1 ml of 0.1 *M* sodium thiosulphate is equivalent to 12.11 mg of $C_{14}H_{10}O_4$.

Water (2.5.12). Carry out the semi-micro determination of water, using 5.0 ml of solution (a). Use as the solvent a mixture of 20.0 ml of *anhydrous methanol R* and 3.0 ml of a 100 g/l solution of *potassium iodide R* in *dimethylformamide R*. After adding solution (a), stir for 5 min before starting the titration. Carry out a blank determination.

Calculate the percentage content of water using the expression:

$$\frac{(n_1 - n_2) \times w \times 2}{m} + (p \times 0.0744)$$

n_1 = number of millilitres of *iodosulphurous reagent R* used in the sample determination,

n_2 = number of millilitres of *iodosulphurous reagent R* used in the blank determination,

w = water equivalent of *iodosulphurous reagent R* in milligrams of water per millilitre of reagent,

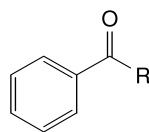
m = mass of the substance to be examined used for the preparation of solution (a) in grams,

p = percentage content of dibenzoyl peroxide.

STORAGE

In a container that has been treated to reduce static discharge and that has a device for release of excess pressure, at a temperature of 2 °C to 8 °C, protected from light.

IMPURITIES



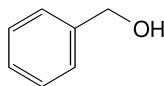
A. R = H: benzaldehyde,

B. R = OH: benzoic acid,

C. R = O-CH₂-CH₃: ethyl benzoate.

01/2005:0256
corrected**BENZYL ALCOHOL**

Alcohol benzylicus

 C_7H_8O M_r 108.1**DEFINITION**

Phenylmethanol.

Content: 98.0 per cent to 100.5 per cent.**CHARACTERS***Appearance:* clear, colourless, oily liquid.*Solubility:* soluble in water, miscible with alcohol and with fatty and essential oils.*Relative density:* 1.043 to 1.049.**IDENTIFICATION**

Infrared absorption spectrophotometry (2.2.24).

Comparison: Ph. Eur. reference spectrum of benzyl alcohol.**TESTS****Appearance of solution.** Shake 2.0 ml with 60 ml of *water R*. It dissolves completely. The solution is clear (2.2.1) and colourless (2.2.2, *Method II*).**Acidity.** To 10 ml add 10 ml of *alcohol R* and 1 ml of *phenolphthalein solution R*. Not more than 1 ml of 0.1 M *sodium hydroxide* is required to change the colour of the indicator to pink.**Refractive index** (2.2.6): 1.538 to 1.541.**Peroxide value** (2.5.5): maximum 5.**Benzaldehyde and other related substances.** Gas chromatography (2.2.28).*Test solution.* The substance to be examined.*Standard solution (a).* Dissolve 0.100 g of *ethylbenzene R* in the test solution and dilute to 10.0 ml with the same solution. Dilute 2.0 ml of this solution to 20.0 ml with the test solution.*Standard solution (b).* Dissolve 2.000 g of *dicyclohexyl R* in the test solution and dilute to 10.0 ml with the same solution. Dilute 2.0 ml of this solution to 20.0 ml with the test solution.*Reference solution (a).* Dissolve 0.750 g of *benzaldehyde R* and 0.500 g of *cyclohexylmethanol R* in the test solution and dilute to 25.0 ml with the test solution. Add 1.0 ml of this solution to a mixture of 2.0 ml of standard solution (a) and 3.0 ml of standard solution (b) and dilute to 20.0 ml with the test solution.*Reference solution (b).* Dissolve 0.250 g of *benzaldehyde R* and 0.500 g of *cyclohexylmethanol R* in the test solution and dilute to 25.0 ml with the test solution. Add 1.0 ml of this solution to a mixture of 2.0 ml of standard solution (a) and 2.0 ml of standard solution (b) and dilute to 20.0 ml with the test solution.*Column:*

- *material:* fused silica,
- *size:* $l = 30$ m, $\varnothing = 0.32$ mm,

– *stationary phase:* macrogol 20 000 R (film thickness 0.5 μ m).*Carrier gas:* helium for chromatography R.*Linear velocity:* 25 cm/s.*Temperature:*

	Time (min)	Temperature (°C)
Column	0 - 34	50 → 220
	34 - 69	220
Injection port		200
Detector		310

Detection: flame ionisation.*Benzyl alcohol not intended for parenteral use.**Injection:* without air-plug, 0.1 μ l of the test solution and 0.1 μ l of reference solution (a).*Relative retention* with reference to benzyl alcohol (retention time = about 26 min): ethylbenzene = about 0.28; dicyclohexyl = about 0.59; benzaldehyde = about 0.68; cyclohexylmethanol = about 0.71.*System suitability:* reference solution (a):

- *resolution:* minimum 3.0 between the peaks corresponding to benzaldehyde and to cyclohexylmethanol.

In the chromatogram obtained with the test solution, verify that there are no peaks with the same retention time as the standards.

Limits:

- *benzaldehyde:* not more than the difference between the area of the peak due to benzaldehyde in the chromatogram obtained with reference solution (a) and the area of the peak due to benzaldehyde in the chromatogram obtained with the test solution (0.15 per cent).
- *cyclohexylmethanol:* not more than the difference between the area of the peak due to cyclohexylmethanol in the chromatogram obtained with reference solution (a) and the area of the peak due to cyclohexylmethanol in the chromatogram obtained with the test solution (0.10 per cent).
- *total of other peaks with a relative retention less than that of benzyl alcohol:* not more than 4 times the area of the peak due to ethylbenzene in the chromatogram obtained with reference solution (a) (0.04 per cent).
- *total of peaks with a relative retention greater than that of benzyl alcohol:* not more than the area of the peak due to dicyclohexyl in the chromatogram obtained with reference solution (a) (0.3 per cent).
- *disregard limit:* 0.01 times the area of the peak due to ethylbenzene in the chromatogram obtained with reference solution (a) (0.0001 per cent).

*Benzyl alcohol intended for parenteral use.**Injection:* without air-plug, 0.1 μ l of the test solution and 0.1 μ l of reference solution (b).*Relative retention* with reference to benzyl alcohol (retention time = about 26 min): ethylbenzene = about 0.28; dicyclohexyl = about 0.59; benzaldehyde = about 0.68; cyclohexylmethanol = about 0.71.*System suitability:* reference solution (b):

- *resolution:* minimum 3.0 between the peaks corresponding to benzaldehyde and to cyclohexylmethanol.

In the chromatogram obtained with the test solution, verify that there are no peaks with the same retention time as the standards.

01/2005:0705

Limits:

- *benzaldehyde*: not more than the difference between the area of the peak due to benzaldehyde in the chromatogram obtained with reference solution (b) and the area of the peak due to benzaldehyde in the chromatogram obtained with the test solution (0.05 per cent).
- *cyclohexylmethanol*: not more than the difference between the area of the peak due to cyclohexylmethanol in the chromatogram obtained with reference solution (b) and the area of the peak due to cyclohexylmethanol in the chromatogram obtained with the test solution (0.10 per cent).
- *total of other peaks with a relative retention less than that of benzyl alcohol*: not more than twice the area of the peak due to ethylbenzene in the chromatogram obtained with reference solution (b) (0.02 per cent).
- *total of peaks with a relative retention greater than that of benzyl alcohol*: not more than the area of the peak due to dicyclohexyl in the chromatogram obtained with reference solution (b) (0.2 per cent).
- *disregard limit*: 0.01 times the area of the peak due to ethylbenzene in the chromatogram obtained with reference solution (b) (0.0001 per cent).

Residue on evaporation: maximum 0.05 per cent.

After ensuring that the substance to be examined complies with the test for peroxide value, evaporate 10.0 g to dryness on a water-bath, dry at 100–105 °C for 1 h and allow to cool in a desiccator. The residue weighs a maximum of 5 mg.

ASSAY

To 0.900 g (m g) add 15.0 ml of a freshly prepared mixture of 1 volume of *acetic anhydride R* and 7 volumes of *pyridine R* and boil under a reflux condenser for 30 min. Cool and add 25 ml of *water R*. Using 0.25 ml of *phenolphthalein solution R* as indicator, titrate with 1 M *sodium hydroxide* (n_1 ml). Carry out a blank titration (n_2 ml).

Calculate the percentage content of C_7H_8O from the expression:

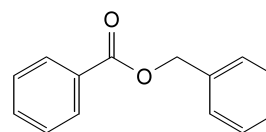
$$\frac{10.81 (n_2 - n_1)}{m}$$

STORAGE

In an airtight container, under nitrogen, protected from light at a temperature between 2 °C and 8 °C.

LABELLING

The label states, where applicable, that the substance is suitable for use in the manufacture of parenteral dosage forms.

BENZYL BENZOATE**Benzylis benzoas** $C_{14}H_{12}O_2$ M_r 212.2**DEFINITION**

Benzyl benzoate contains not less than 99.0 per cent and not more than the equivalent of 100.5 per cent of phenylmethyl benzoate.

CHARACTERS

Colourless or almost colourless crystals or a colourless or almost colourless, oily liquid, practically insoluble in water, miscible with alcohol, with methylene chloride and with fatty and essential oils.

It boils at about 320 °C.

IDENTIFICATION

First identification: A.

Second identification: B, C.

- Examine by infrared absorption spectrophotometry (2.2.24), comparing with the *Ph. Eur. reference spectrum of benzyl benzoate*.
- To 2 g add 25 ml of *alcoholic potassium hydroxide solution R* and boil under a reflux condenser for 2 h. Remove the ethanol on a water-bath, add 50 ml of *water R* and distil; collect about 25 ml of distillate and use it for identification test C. Acidify the liquid remaining in the distillation flask with *dilute hydrochloric acid R*; a white precipitate is formed, which, when washed with *water R* and dried *in vacuo*, melts (2.2.14) at 121 °C to 124 °C.
- To the distillate obtained in identification test B add 2.5 g of *potassium permanganate R* and 5 ml of *dilute sodium hydroxide solution R*. Boil under a reflux condenser for 15 min, cool and filter. Acidify the filtrate with *dilute hydrochloric acid R*; a white precipitate is formed which, when washed with *water R* and dried *in vacuo*, melts (2.2.14) at 121 °C to 124 °C.

TESTS

Acidity. Dissolve 2.0 g in *alcohol R* and dilute to 10 ml with the same solvent. Titrate with 0.1 M *sodium hydroxide* using *phenolphthalein solution R* as indicator. Not more than 0.2 ml is required to change the colour of the indicator to pink.

Relative density (2.2.5): 1.118 to 1.122.

Refractive index (2.2.6): 1.568 to 1.570.

Freezing point (2.2.18). Not less than 17.0 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

To 2.000 g add 50.0 ml of 0.5 M *alcoholic potassium hydroxide* and boil gently under a reflux condenser for 1 h. Titrate the hot solution with 0.5 M *hydrochloric acid* using 1 ml of *phenolphthalein solution R* as indicator. Carry out a blank determination.

1 ml of 0.5 M alcoholic potassium hydroxide is equivalent to 106.1 mg of $C_{14}H_{12}O_2$.

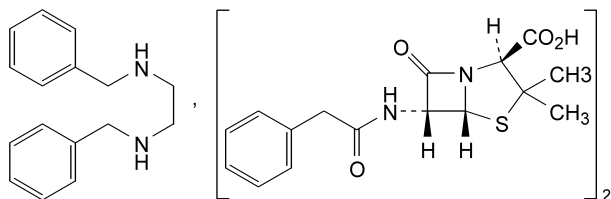
STORAGE

Store in an airtight, well-filled container, protected from light.

01/2005:0373

BENZYLPENICILLIN, BENZATHINE

Benzylpenicillinum benzathinum



$C_{48}H_{56}N_6O_8S_2$

M_r 909

DEFINITION

Benzathine benzylpenicillin is *N,N'*-dibenzylethane-1,2-diamine compound (1:2) with (2*S*,5*R*,6*R*)-3,3-dimethyl-7-oxo-6-[(phenylacetyl)amino]-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid. It contains not less than 96.0 per cent and not more than the equivalent of 102.0 per cent of benzathine benzylpenicillin and not less than 24.0 per cent and not more than 27.0 per cent of *N,N'*-dibenzylethylenediamine (benzathine $C_{16}H_{20}N_2$; M_r 240.3), both calculated with reference to the anhydrous substance. It contains a variable quantity of water. Dispersing or suspending agents may be added.

CHARACTERS

A white powder, very slightly soluble in water, freely soluble in dimethylformamide and in formamide, slightly soluble in alcohol.

IDENTIFICATION

First identification: A.

Second identification: B, C, D.

- A. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with benzathine benzylpenicillin CRS.
- B. Examine by thin-layer chromatography (2.2.27), using a TLC silanised silica gel plate R.

Test solution. Dissolve 25 mg of the substance to be examined in 5 ml of methanol R.

Reference solution. Dissolve 25 mg of benzathine benzylpenicillin CRS in 5 ml of methanol R.

Apply to the plate 1 µl of each solution. Develop over a path of 15 cm using a mixture of 30 volumes of acetone R and 70 volumes of a 154 g/l solution of ammonium acetate R, the pH of which has been adjusted to 7.0 with ammonia R. Allow the plate to dry in air and expose it to iodine vapour until the spots appear. Examine in daylight. The two principal spots in the chromatogram obtained with the test solution are similar in position, colour and size to the two principal spots in the chromatogram obtained with the reference solution. The test is not valid unless the chromatogram obtained with the reference solution shows two clearly separated spots.

- C. Place about 2 mg in a test-tube about 150 mm long and 15 mm in diameter. Moisten with 0.05 ml of water R and add 2 ml of sulphuric acid-formaldehyde reagent R. Mix the contents of the tube by swirling; the solution is practically colourless. Place the test-tube on a water-bath for 1 min; a reddish-brown colour develops.
- D. To 0.1 g add 2 ml of 1 M sodium hydroxide and shake for 2 min. Shake the mixture with two quantities, each of 3 ml, of ether R. Evaporate the combined ether layers to dryness and dissolve the residue in 1 ml of alcohol (50 per cent V/V) R. Add 5 ml of picric acid solution R, heat at 90 °C for 5 min and allow to cool slowly. Separate the crystals and recrystallise from alcohol (25 per cent V/V) R containing 10 g/l of picric acid R. The crystals melt (2.2.14) at about 214 °C.

TESTS

Acidity or alkalinity. To 0.50 g add 100 ml of carbon dioxide-free water R and shake for 5 min. Filter through a sintered-glass filter. To 20 ml of the filtrate add 0.1 ml of bromothymol blue solution R1. The solution is green or yellow. Not more than 0.2 ml of 0.02 M sodium hydroxide is required to change the colour of the indicator to blue.

Related substances. Liquid chromatography (2.2.29). Prepare the solutions immediately before use, using sonication (for about 2 min) to dissolve the samples. Avoid any over heating during the sample preparation.

Test solution. Dissolve 70.0 mg of the substance to be examined in 25 ml of methanol R and dilute to 50.0 ml with a solution containing 6.8 g/l of potassium dihydrogen phosphate R and 1.02 g/l of disodium hydrogen phosphate R.

Reference solution (a). Dissolve 70.0 mg of benzathine benzylpenicillin CRS in 25 ml of methanol R and dilute to 50.0 ml with a solution containing 6.8 g/l of potassium dihydrogen phosphate R and 1.02 g/l of disodium hydrogen phosphate R.

Reference solution (b). Dilute 1.0 ml of reference solution (a) to 100.0 ml with mobile phase A.

Column:

- size: $l = 0.25$ m, $\varnothing = 4.0$ mm,
- stationary phase: end-capped octadecylsilyl silica gel for chromatography R (5 µm),
- temperature: 40 °C.

Mobile phase:

- mobile phase A: mix 10 volumes of a 34 g/l solution of potassium dihydrogen phosphate R adjusted to pH 3.5 with phosphoric acid R, 30 volumes of methanol R and 60 volumes of water R,
- mobile phase B: mix 10 volumes of a 34 g/l solution of potassium dihydrogen phosphate R adjusted to pH 3.5 with phosphoric acid R, 30 volumes of water R and 60 volumes of methanol R,

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)
0 - 10	75	25
10 - 20	75 → 0	25 → 100
20 - 55	0	100
55 - 70	75	25

Flow rate: 1 ml/min.

Detection: spectrophotometer at 220 nm.

Injection: 20 µl; inject the test solution and the reference solutions.

System suitability: reference solution (a)

- *relative retention* with reference to benzylpenicillin: benzathine = 0.3 to 0.4; impurity C = about 2.4. If necessary, adjust the concentration of methanol in the mobile phase.

Limits:

- *impurity C*: not more than twice the sum of the areas of the 2 principal peaks in the chromatogram obtained with reference solution (b) (2 per cent),
- *any other impurity*: not more than the sum of the areas of the 2 principal peaks in the chromatogram obtained with reference solution (b) (1 per cent),
- *disregard limit*: 0.05 times the sum of the areas of the 2 principal peaks in the chromatogram obtained with reference solution (b) (0.05 per cent).

Water (2.5.12): 5.0 per cent to 8.0 per cent, determined on 0.300 g by the semi-micro determination of water.

Bacterial endotoxins (2.6.14, Method E). Suspend 20 mg of the substance to be examined in 20 ml of a solution of 0.1 M sodium hydroxide diluted 1 to 100, shake thoroughly and centrifuge. The supernatant contains less than 0.13 IU/ml, if intended for use in the manufacture of parenteral dosage forms without a further appropriate procedure for the removal of bacterial endotoxins.

ASSAY

Liquid chromatography (2.2.29) as described in the test for related substances.

Mobile phase: phosphate buffer solution pH 3.5 R, methanol R, water R (10:35:55 V/V/V).

Injection: 20 μ l; inject the test solution and reference solution (a).

Calculate the percentage contents of benzathine and of benzathine benzylpenicillin. Calculate the latter by multiplying the percentage content of benzylpenicillin by 1.36.

STORAGE

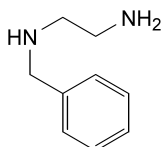
Store in an airtight container. If the substance is sterile, store in a sterile, airtight, tamper-proof container.

LABELLING

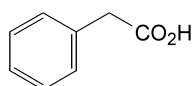
The label states:

- where applicable, the name and quantity of any added dispersing or suspending agent,
- where applicable, that the substance is free from bacterial endotoxins.

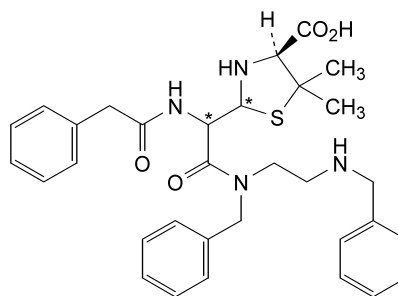
IMPURITIES



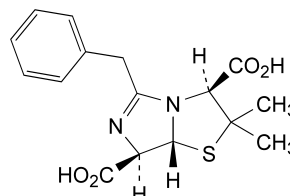
A. monobenzylethylenediamine,



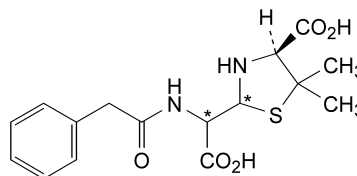
B. phenylacetic acid,



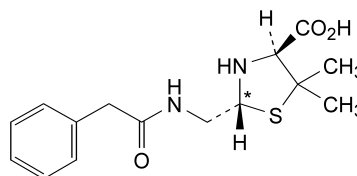
C. benzylpenicilloic acids benzathide,



D. (3*S*,7*R*,7*aR*)-5-benzyl-2,2-dimethyl-2,3,7,7*a*-tetrahydroimidazo[5,1-*b*]thiazole-3,7-dicarboxylic acid (penillic acid of benzylpenicillin).



E. (4S)-2-[carboxy[(phenylacetyl)amino]methyl]-5,5-dimethylthiazolidine-4-carboxylic acid (penicilloic acids of benzylpenicillin).



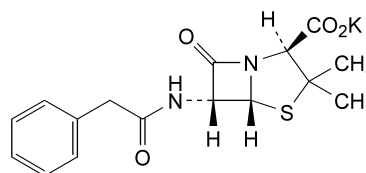
and epimer at C*

F. (2*R*,4*S*)-2-[[[(phenylacetyl)amino]methyl]-5,5-dimethylthiazolidine-4-carboxylic acid (penilloic acids of benzylpenicillin).

01/2005:0113

BENZYL PENICILLIN POTASSIUM

Benzylpenicillinum kalicum


$$\text{C}_{16}\text{H}_{17}\text{KN}_2\text{O}_4\text{S}$$
 $M_r 372.5$

DEFINITION

Benzylpenicillin potassium is potassium (2*S*,5*R*,6*R*)-3,3-dimethyl-7-oxo-6-[(phenylacetyl)amino]-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylate, a substance produced by the growth of certain strains of *Penicillium notatum* or related organisms, or obtained by any other

means. It contains not less than 96.0 per cent and not more than the equivalent of 102.0 per cent of benzylpenicillin potassium, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, crystalline powder, very soluble in water, practically insoluble in fatty oils and in liquid paraffin.

IDENTIFICATION

First identification: A, D.

Second identification: B, C, D.

- A. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *benzylpenicillin potassium CRS*.
- B. Examine by thin-layer chromatography (2.2.27), using a *TLC silanised silica gel plate R*.

Test solution. Dissolve 25 mg of the substance to be examined in 5 ml of *water R*.

Reference solution (a). Dissolve 25 mg of *benzylpenicillin potassium CRS* in 5 ml of *water R*.

Reference solution (b). Dissolve 25 mg of *benzylpenicillin potassium CRS* and 25 mg of *phenoxymethylpenicillin potassium CRS* in 5 ml of *water R*.

Apply to the plate 1 µl of each solution. Develop over a path of 15 cm using a mixture of 30 volumes of *acetone R* and 70 volumes of a 154 g/l solution of *ammonium acetate R*, the pH of which has been adjusted to 5.0 with *glacial acetic acid R*. Allow the plate to dry in air and expose it to iodine vapour until the spots appear. Examine in daylight. The principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a). The test is not valid unless the chromatogram obtained with reference solution (b) shows 2 clearly separated spots.

- C. Place about 2 mg in a test-tube about 150 mm long and 15 mm in diameter. Moisten with 0.05 ml of *water R* and add 2 ml of *sulphuric acid-formaldehyde reagent R*. Mix the contents of the tube by swirling; the solution is practically colourless. Place the test-tube on a water-bath for 1 min; a reddish-brown colour develops.
- D. It gives reaction (a) of potassium (2.3.1).

TESTS

pH (2.2.3). Dissolve 2.0 g in *carbon dioxide-free water R* and dilute to 20 ml with the same solvent. The pH of the solution is 5.5 to 7.5.

Specific optical rotation (2.2.7). Dissolve 0.500 g in *carbon dioxide-free water R* and dilute to 25.0 ml with the same solvent. The specific optical rotation is + 270 to + 300, calculated with reference to the dried substance.

Absorbance (2.2.25). Dissolve 94.0 mg in *water R* and dilute to 50.0 ml with the same solvent. Measure the absorbance of the solution at 325 nm, 280 nm and at the maximum at 264 nm, diluting the solution, if necessary, for the measurement at 264 nm. The absorbances at 325 nm and 280 nm do not exceed 0.10 and that at the maximum at 264 nm is 0.80 to 0.88, calculated on the basis of the undiluted (1.88 g/l) solution. Verify the resolution of the apparatus (2.2.25); the ratio of the absorbances is at least 1.7.

Related substances. Examine by liquid chromatography (2.2.29) as described under Assay. Inject 20 µl of reference solution (d) and elute isocratically with the chosen mobile

phase. Inject 20 µl of test solution (b) and start the elution isocratically. Immediately after elution of the benzylpenicillin peak start the following linear gradient:

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)	Comment
0 - 20	70 → 0	30 → 100	linear gradient
20 - 35	0	100	isocratic
35 - 50	70	30	re-equilibration

Inject *water R* and use the same elution pattern to obtain a blank. In the chromatogram obtained with test solution (b), the area of any peak, apart from the principal peak, is not greater than the area of the principal peak in the chromatogram obtained with reference solution (d) (1 per cent).

Loss on drying (2.2.32). Not more than 1.0 per cent, determined on 1.000 g by drying in an oven at 100-105 °C.

Bacterial endotoxins (2.6.14, *Method E*): less than 0.16 IU/mg, if intended for use in the manufacture of parenteral dosage forms without a further appropriate procedure for the removal of bacterial endotoxins.

ASSAY

Examine by liquid chromatography (2.2.29). *Prepare the solutions immediately before use.*

Test solution (a). Dissolve 50.0 mg of the substance to be examined in *water R* and dilute to 50.0 ml with the same solvent.

Test solution (b). Dissolve 80.0 mg of the substance to be examined in *water R* and dilute to 20.0 ml with the same solvent.

Reference solution (a). Dissolve 50.0 mg of *benzylpenicillin sodium CRS* in *water R* and dilute to 50.0 ml with the same solvent.

Reference solution (b). Dissolve 10 mg of *benzylpenicillin sodium CRS* and 10 mg of *phenylacetic acid CRS* in *water R* and dilute to 50 ml with the same solvent.

Reference solution (c). Dilute 1.0 ml of reference solution (a) to 20.0 ml with *water R*. Dilute 1.0 ml of the solution to 50.0 ml with *water R*.

Reference solution (d). Dilute 4.0 ml of reference solution (a) to 100.0 ml with *water R*.

The chromatographic procedure may be carried out using:

- a column 0.25 m long and 4.6 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (5 µm),

- as mobile phase at a flow rate of 1.0 ml/min:

Mobile phase A. Mix 10 volumes of a 68 g/l solution of *potassium dihydrogen phosphate R* adjusted to pH 3.5 with a 500 g/l solution of *dilute phosphoric acid R*, 30 volumes of *methanol R* and 60 volumes of *water R*,

Mobile phase B. Mix 10 volumes of a 68 g/l solution of *potassium dihydrogen phosphate R* adjusted to pH 3.5 with a 500 g/l solution of *dilute phosphoric acid R*, 40 volumes of *water R* and 50 volumes of *methanol R*,

- as detector a spectrophotometer set at 225 nm.

Equilibrate the column with a mobile phase ratio A:B of 70:30. Inject 20 µl of reference solution (b). The test is not valid unless the resolution between the 2 principal peaks is at least 6.0 (if necessary, adjust the ratio A:B of the mobile phase) and the mass distribution ratio for the second peak (benzylpenicillin) is 4.0 to 6.0. Inject 20 µl of reference solution (c). Adjust the system to obtain a peak with a signal-to-noise ratio of at least 3.

Calculate the percentage content of benzylpenicillin potassium by multiplying the percentage content of benzylpenicillin sodium by 1.045.

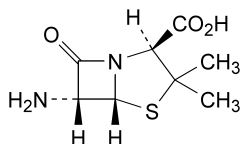
STORAGE

Store in an airtight container. If the substance is sterile, store in a sterile, airtight, tamper-proof container.

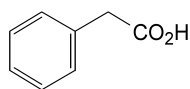
LABELLING

The label states, where applicable, that the substance is free from bacterial endotoxins.

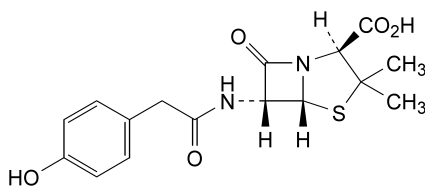
IMPURITIES



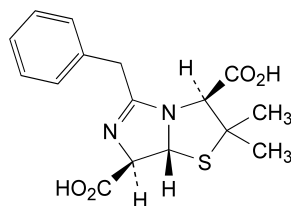
- A. (2*S*,5*R*,6*R*)-6-amino-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid (6-aminopenicillanic acid),



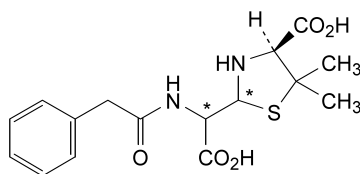
- B. phenylacetic acid,



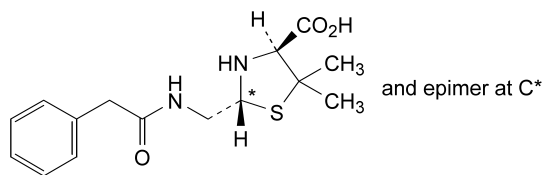
- C. (2*S*,5*R*,6*R*)-6-[(4-hydroxyphenyl)acetyl]amino-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid,



- D. (3*S*,7*R*,7*aR*)-5-benzyl-2,2-dimethyl-2,3,7,7a-tetrahydroimidazo[5,1-*b*]thiazole-3,7-dicarboxylic acid (penillic acid of benzylpenicillin),



- E. (4*S*)-2-[carboxy[(phenylacetyl)amino]methyl]-5,5-dimethylthiazolidine-4-carboxylic acid (penicilloic acids of benzylpenicillin),

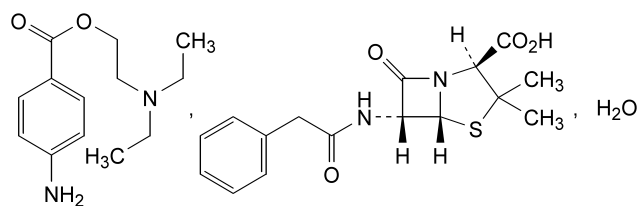


- F. (2*RS*,4*S*)-2-[(phenylacetyl)amino]methyl-5,5-dimethylthiazolidine-4-carboxylic acid (penilloic acids of benzylpenicillin).

01/2005:0115

BENZYLPENICILLIN, PROCAINE

Benzylpenicillinum procainum



$C_{29}H_{38}N_4O_6S \cdot H_2O$

M_r 588.7

DEFINITION

Procaine benzylpenicillin is the monohydrate of the (2*S*,5*R*,6*R*)-3,3-dimethyl-7-oxo-6-[(phenylacetyl)amino]-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid compound with 2-(diethylamino)ethyl 4-aminobenzoate. It contains not less than 96.0 per cent and not more than the equivalent of 102.0 per cent of procaine benzylpenicillin and not less than 39.0 per cent and not more than 42.0 per cent of procaine ($C_{13}H_{20}N_2O_2$; M_r 236.3), both calculated with reference to the anhydrous substance. Dispersing or suspending agents (for example, lecithin and polysorbate 80) may be added.

CHARACTERS

A white, crystalline powder, slightly soluble in water, sparingly soluble in alcohol.

IDENTIFICATION

First identification: A.

Second identification: B, C, D.

- A. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *procaine benzylpenicillin CRS*.
B. Examine by thin-layer chromatography (2.2.27), using a *TLC silanised silica gel plate R*.

Test solution. Dissolve 25 mg of the substance to be examined in 5 ml of *acetone R*.

Reference solution. Dissolve 25 mg of *procaine benzylpenicillin CRS* in 5 ml of *acetone R*.

Apply to the plate 1 µl of each solution. Develop over a path of 15 cm using a mixture of 30 volumes of *acetone R* and 70 volumes of a 154 g/l solution of *ammonium acetate R*, the pH of which has been adjusted to 7.0 with *ammonia R*. Allow the plate to dry in air and expose it to iodine vapour until the spots appear. Examine in daylight. The 2 principal spots in the chromatogram obtained with the test solution are similar in position, colour and size to the 2 principal spots in the chromatogram obtained with the reference solution. The test is not valid unless the chromatogram obtained with the reference solution shows 2 clearly separated spots.

- C. Place about 2 mg in a test-tube about 150 mm long and 15 mm in diameter. Moisten with 0.05 ml of *water R* and add 2 ml of *sulphuric acid-formaldehyde reagent R*. Mix the contents of the tube by swirling; the solution is practically colourless. Place the test-tube on a water-bath for 1 min; a reddish-brown colour develops.
- D. Dissolve 0.1 g in 2 ml of *dilute hydrochloric acid R* and use the solution which may be turbid. The solution gives the reaction of primary aromatic amines (2.3.1).

TESTS

pH (2.2.3). Dissolve 50 mg in *carbon dioxide-free water R* and dilute to 15 ml with the same solvent. Shake until dissolution is complete. The pH of the solution is 5.0 to 7.5.

Specific optical rotation (2.2.7). Dissolve 0.250 g in a mixture of 2 volumes of *water R* and 3 volumes of *acetone R* and dilute to 25.0 ml with the same mixture of solvents. The specific optical rotation is + 165 to + 180, calculated with reference to the anhydrous substance.

Related substances. Examine by liquid chromatography (2.2.29) as prescribed under Assay. Inject 10 µl of reference solution (c). Adjust the sensitivity of the system so that the height of the peak due to benzylpenicillin is at least 50 per cent of the full scale of the recorder. Inject 10 µl of test solution (a) and continue the chromatography for 1.5 times the retention time of the benzylpenicillin peak. In the chromatogram obtained with test solution (a): the area of any peak corresponding to 4-aminobenzoic acid is not greater than the area of the corresponding peak in the chromatogram obtained with reference solution (c) (0.024 per cent); the area of any peak, apart from the 2 principal peaks and any peak corresponding to 4-aminobenzoic acid, is not greater than the area of the peak corresponding to benzylpenicillin in the chromatogram obtained with reference solution (c) (1 per cent).

Water (2.5.12). 2.8 per cent to 4.2 per cent, determined on 0.500 g by the semi-micro determination of water.

Bacterial endotoxins (2.6.14, Method E): less than 0.10 IU/mg, if intended for use in the manufacture of parenteral dosage forms without a further appropriate procedure for the removal of bacterial endotoxins.

ASSAY

Examine by liquid chromatography (2.2.29). *Prepare the solutions immediately before use.*

Test solution (a). Dissolve 70.0 mg of the substance to be examined in the mobile phase and dilute to 50.0 ml with the mobile phase.

Test solution (b). Dissolve 70.0 mg of the substance to be examined in the mobile phase and dilute to 100.0 ml with the mobile phase.

Reference solution (a). Dissolve 70.0 mg of *procaine benzylpenicillin CRS* in the mobile phase and dilute to 100.0 ml with the mobile phase.

Reference solution (b). Dissolve 4 mg of *4-aminobenzoic acid R* in reference solution (a) and dilute to 25 ml with the same solution.

Reference solution (c). Dissolve 16.8 mg of *4-aminobenzoic acid R* in *water R* and dilute to 50.0 ml with the same solvent. Dilute 1.0 ml of the solution to 10.0 ml with *water R*. To 1.0 ml of this solution, add 1.0 ml of test solution (a) and dilute to 100.0 ml with the mobile phase.

The chromatographic procedure may be carried out using:

- as mobile phase at a flow rate of 1.75 ml/min a mixture prepared as follows: mix 250 ml of *acetonitrile R*, 250 ml of *water R* and 500 ml of a solution containing 14 g/l of *potassium dihydrogen phosphate R* and 6.5 g/l of *tetrabutylammonium hydroxide solution (400 g/l) R* adjusted to pH 7.0 with *1 M potassium hydroxide*; adjust the mixture to pH 7.2 with *dilute phosphoric acid R*, if necessary,
 - as detector a spectrophotometer set at 225 nm.
- Inject 10 µl of reference solution (b). When the chromatogram is recorded in the prescribed conditions, the substances elute in the following order: 4-aminobenzoic acid, procaine, benzylpenicillin. Adjust the sensitivity of the system so that the height of the peak corresponding to 4-aminobenzoic acid is at least 50 per cent of the full scale of the recorder. The test is not valid unless, the resolution between the first peak (4-aminobenzoic acid) and the second peak (procaine) is at least 2.0. If necessary, adjust the concentration of acetonitrile in the mobile phase. Inject reference solution (a) 6 times. The test is not valid unless the relative standard deviation for the areas of the 2 peaks is at most 1.0 per cent. Inject alternately test solution (b) and reference solution (a). Calculate the percentage contents of procaine and procaine benzylpenicillin.

STORAGE

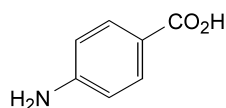
Store in an airtight container. If the substance is sterile, store in a sterile, airtight, tamper-proof container.

LABELLING

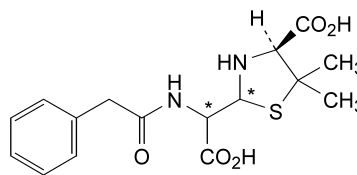
The label states:

- where applicable, the name and quantity of any added dispersing or suspending agents,
- where applicable, that the substance is free from bacterial endotoxins.

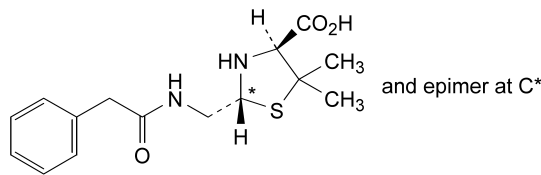
IMPURITIES



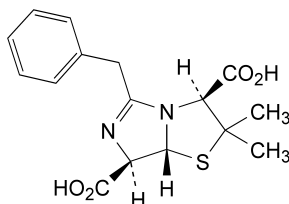
A. 4-aminobenzoic acid,



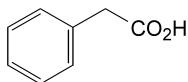
B. (4*S*)-2-[[carboxy[(phenylacetyl)amino]methyl]-5,5-dimethylthiazolidine-4-carboxylic acid (penicilloic acids of benzylpenicillin),



C. (2*RS*,4*S*)-2-[[[(phenylacetyl)amino]methyl]-5,5-dimethylthiazolidine-4-carboxylic acid (penicilloic acids of benzylpenicillin),



- D. (3*S*,7*R*,7*aR*)-5-benzyl-2,2-dimethyl-2,3,7,7*a*-tetrahydroimidazo[5,1-*b*]thiazole-3,7-dicarboxylic acid (penillic acid of benzylpenicillin),

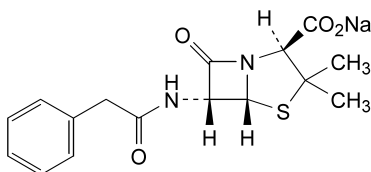


- E. phenylacetic acid.

01/2005:0114

BENZYLPENICILLIN SODIUM

Benzylpenicillinum natrium

 $C_{16}H_{17}N_2NaO_4S$ M_r 356.4

DEFINITION

Benzylpenicillin sodium is sodium (2*S*,5*R*,6*R*)-3,3-dimethyl-7-oxo-6-[(phenylacetyl)amino]-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylate, a substance produced by the growth of certain strains of *Penicillium notatum* or related organisms, or obtained by any other means. It contains not less than 96.0 per cent and not more than the equivalent of 102.0 per cent of benzylpenicillin sodium, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, crystalline powder, very soluble in water, practically insoluble in fatty oils and in liquid paraffin.

IDENTIFICATION

First identification: A, D.

Second identification: B, C, D.

- A. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *benzylpenicillin sodium CRS*.
- B. Examine by thin-layer chromatography (2.2.27), using a *TLC silanised silica gel plate R*.

Test solution. Dissolve 25 mg of the substance to be examined in 5 ml of *water R*.

Reference solution (a). Dissolve 25 mg of *benzylpenicillin sodium CRS* in 5 ml of *water R*.

Reference solution (b). Dissolve 25 mg of *benzylpenicillin sodium CRS* and 25 mg of *phenoxymethylpenicillin potassium CRS* in 5 ml of *water R*.

Apply to the plate 1 μ l of each solution. Develop over a path of 15 cm using a mixture of 30 volumes of *acetone R* and 70 volumes of a 154 g/l solution of *ammonium acetate R*, the pH of which has been adjusted to 5.0 with *glacial acetic acid R*. Allow the plate to dry in air and expose it to iodine vapour until the spots appear. Examine

in daylight. The principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a). The test is not valid unless the chromatogram obtained with reference solution (b) shows 2 clearly separated spots.

- C. Place about 2 mg in a test-tube about 150 mm long and 15 mm in diameter. Moisten with 0.05 ml of *water R* and add 2 ml of *sulphuric acid-formaldehyde reagent R*. Mix the contents of the tube by swirling; the solution is practically colourless. Place the test-tube on a water-bath for 1 min; a reddish-brown colour develops.

- D. It gives reaction (a) of sodium (2.3.1).

TESTS

pH (2.2.3). Dissolve 2.0 g in *carbon dioxide-free water R* and dilute to 20 ml with the same solvent. The pH of the solution is 5.5 to 7.5.

Specific optical rotation (2.2.7). Dissolve 0.500 g in *carbon dioxide-free water R* and dilute to 25.0 ml with the same solvent. The specific optical rotation is + 285 to + 310, calculated with reference to the dried substance.

Absorbance (2.2.25). Dissolve 90.0 mg in *water R* and dilute to 50.0 ml with the same solvent. Measure the absorbance of the solution at 325 nm, at 280 nm and at the maximum at 264 nm, diluting the solution, if necessary, for the measurement at 264 nm. The absorbances at 325 nm and 280 nm are not greater than 0.10 and the absorbance at the maximum at 264 nm is 0.80 to 0.88, calculated on the basis of the undiluted (1.80 g/l) solution. Verify the resolution of the apparatus (2.2.25); the ratio of the absorbances is at least 1.7.

Related substances. Liquid chromatography (2.2.29) as described under Assay. Inject 20 μ l of reference solution (d) and elute isocratically with the chosen mobile phase. Inject 20 μ l of test solution (b) and start the elution isocratically. Immediately after elution of the benzylpenicillin peak start the following linear gradient:

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)	Comment
0 - 20	70 $\rightarrow$ 0	30 $\rightarrow$ 100	linear gradient
20 - 35	0	100	isocratic
35 - 50	70	30	re-equilibration

Inject *water R* and use the same elution pattern to obtain a blank. In the chromatogram obtained with test solution (b), the area of any peak, apart from the principal peak, is not greater than the area of the principal peak in the chromatogram obtained with reference solution (d) (1 per cent).

2-Ethylhexanoic acid (2.4.28). Not more than 0.5 per cent *m/m*.

Loss on drying (2.2.32). Not more than 1.0 per cent, determined on 1.000 g by drying in an oven at 100-105 °C.

Bacterial endotoxins (2.6.14, *Method E*): less than 0.16 IU/mg, if intended for use in the manufacture of parenteral dosage forms without a further appropriate procedure for the removal of bacterial endotoxins.

ASSAY

Liquid chromatography (2.2.29). *Prepare the solutions immediately before use.*

Test solution (a). Dissolve 50.0 mg of the substance to be examined in *water R* and dilute to 50.0 ml with the same solvent.

Test solution (b). Dissolve 80.0 mg of the substance to be examined in *water R* and dilute to 20.0 ml with the same solvent.

Reference solution (a). Dissolve 50.0 mg of *benzylpenicillin sodium CRS* in *water R* and dilute to 50.0 ml with the same solvent.

Reference solution (b). Dissolve 10 mg of *benzylpenicillin sodium CRS* and 10 mg of *phenylacetic acid CRS* in *water R* and dilute to 50 ml with the same solvent.

Reference solution (c). Dilute 1.0 ml of reference solution (a) to 20.0 ml with *water R*. Dilute 1.0 ml of the solution to 50.0 ml with *water R*.

Reference solution (d). Dilute 4.0 ml of reference solution (a) to 100.0 ml with *water R*.

The chromatographic procedure may be carried out using:

- a column 0.25 m long and 4.6 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (5 µm),
- as mobile phase at a flow rate of 1.0 ml/min:

Mobile phase A. Mix 10 volumes of a 68 g/l solution of *potassium dihydrogen phosphate R* adjusted to pH 3.5 with a 500 g/l solution of *dilute phosphoric acid R*, 30 volumes of *methanol R* and 60 volumes of *water R*.

Mobile phase B. Mix 10 volumes of a 68 g/l solution of *potassium dihydrogen phosphate R* adjusted to pH 3.5 with a 500 g/l solution of *dilute phosphoric acid R*, 40 volumes of *water R* and 50 volumes of *methanol R*.

- as detector a spectrophotometer set at 225 nm.

Equilibrate the column with a mobile phase ratio A:B of 70:30. Inject 20 µl of reference solution (b). The test is not valid unless the resolution between the 2 principal peaks is at least 6.0 (if necessary, adjust the ratio A:B of the mobile phase) and the mass distribution ratio for the second peak (benzylpenicillin) is 4.0 to 6.0. Inject 20 µl of reference solution (c). Adjust the system to obtain a peak with a signal-to-noise ratio of at least 3.

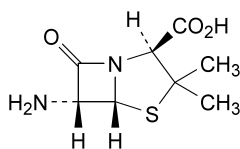
STORAGE

Store in an airtight container. If the substance is sterile, store in a sterile, airtight, tamper-proof container.

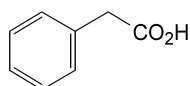
LABELLING

The label states, where applicable, that the substance is free from bacterial endotoxins.

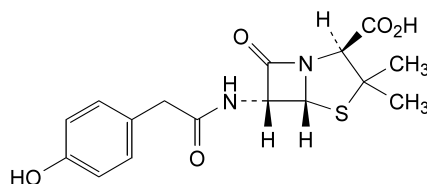
IMPURITIES



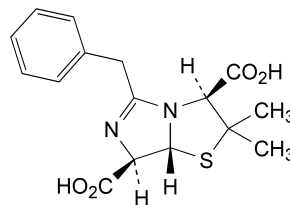
- A. (2*S*,5*R*,6*R*)-6-amino-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid (6-aminopenicillanic acid),



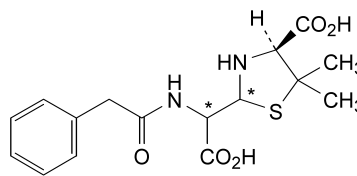
- B. phenylacetic acid,



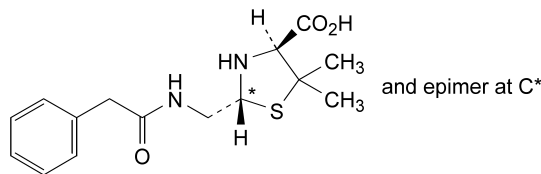
- C. (2*S*,5*R*,6*R*)-6-[(4-hydroxyphenyl)acetyl]amino-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid,



- D. (3*S*,7*R*,7*aR*)-5-benzyl-2,2-dimethyl-2,3,7,7*a*-tetrahydroimidazo[5,1-*b*]thiazole-3,7-dicarboxylic acid (penillic acid of benzylpenicillin),



- E. (4*S*)-2-[carboxy[(phenylacetyl)amino]methyl]-5,5-dimethylthiazolidine-4-carboxylic acid (penicilloic acids of benzylpenicillin),

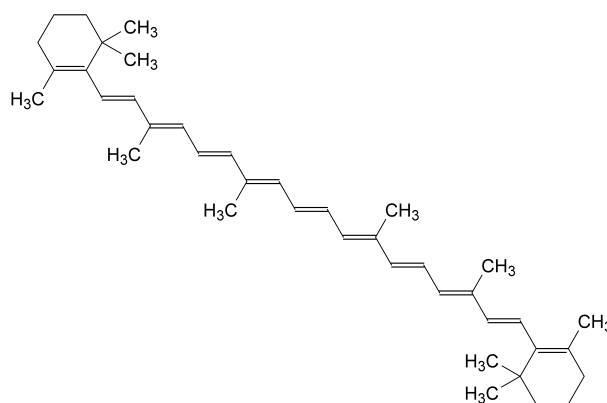


- F. (2*RS*,4*S*)-2-[(phenylacetyl)amino]methyl]-5,5-dimethylthiazolidine-4-carboxylic acid (penilloic acids of benzylpenicillin).

01/2005:1069

BETACAROTENE

Betacarotenum



C₄₀H₅₆

M_r 536.9

DEFINITION

01/2005:1070

Betacarotene contains not less than 96.0 per cent and not more than the equivalent of 101.0 per cent of (all-*E*)-3,7,12,16-Tetramethyl-1,18-bis(2,6,6-trimethylcyclohex-1-enyl)octadeca-1,3,5,7,9,11,13,15,17-nonaene, calculated with reference to the dried substance.

CHARACTERS

A brown-red or brownish-red, crystalline powder, practically insoluble in water, slightly soluble in cyclohexane, practically insoluble in ethanol. It is sensitive to air, heat and light, especially in solution.

Carry out all operations as rapidly as possible avoiding exposure to actinic light; use freshly prepared solutions.

IDENTIFICATION

Dissolve 50.0 mg in 10 ml of *chloroform R* and dilute immediately to 100.0 ml with *cyclohexane R*. Dilute 5.0 ml of this solution to 100.0 ml with *cyclohexane R* (solution A; use solution A also for the test for related substances). Dilute 5.0 ml of solution A to 50.0 ml with *cyclohexane R*. (Solution B; use solution B also for the test for related substances and for the assay). Determine the absorbance (2.2.25) of solution B at 455 nm and at 483 nm using *cyclohexane R* as the compensation liquid. The ratio of the absorbance at 455 nm to that at 483 nm is between 1.14 and 1.18.

TESTS

Related substances. Determine the absorbance (2.2.25) of solution B at 455 nm and that of solution A at 340 nm, used in Identification. The ratio of the absorbance at 455 nm to that at 340 nm is not less than 1.5.

Heavy metals (2.4.8). 2.0 g complies with limit test D for heavy metals (10 ppm). Prepare the standard using 2 ml of *lead standard solution (10 ppm Pb) R*.

Loss on drying (2.2.32). Not more than 0.2 per cent, determined on 1.000 g by drying *in vacuo* over *diphosphorus pentoxide R* at 40 °C for 4 h.

Sulphated ash (2.4.14). Not more than 0.2 per cent, determined on 1.0 g, moistened with a mixture of 2 ml of *dilute sulphuric acid R* and 5 ml of *alcohol R*.

ASSAY

Measure the absorbance (2.2.25) of solution B used in Identification at the maximum at 455 nm, using *cyclohexane R* as the compensation liquid.

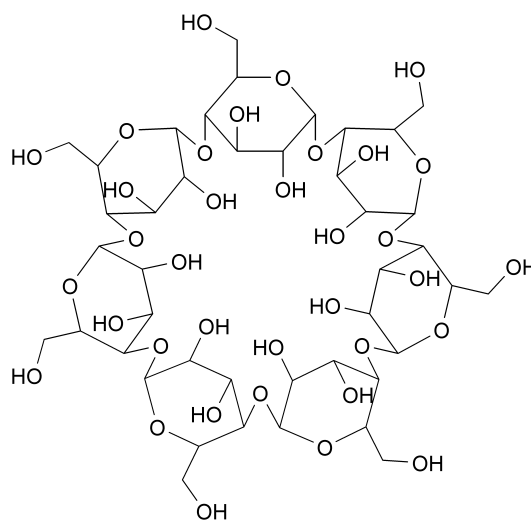
Calculate the content of C₄₀H₅₆ taking the specific absorbance to be 2500.

STORAGE

Store in an airtight container, protected from light, at a temperature not exceeding 25 °C.

BETADEX

Betadexum

[C₆H₁₀O₅]₇M_r 1135

DEFINITION

Betadex (betacyclodextrin) contains not less than 98.0 per cent and not more than the equivalent of 101.0 per cent of cyclo-α-(1→4)-D-heptagluco-pyranoside, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, amorphous or crystalline powder, sparingly soluble in water, freely soluble in propylene glycol, practically insoluble in ethanol and in methylene chloride.

IDENTIFICATION

- It complies with the test for specific optical rotation (see Tests).
- Examine the chromatograms obtained in the assay. The retention time and size of the principal peak in the chromatogram obtained with test solution (b) are approximately the same as those of the principal peak in the chromatogram obtained with reference solution (c).
- Dissolve 0.2 g in 2 ml of *iodine solution R4* by warming on a water-bath, and allow to stand at room temperature. A yellowish-brown precipitate is formed.

TESTS

Solution S. Dissolve 1.000 g in *carbon dioxide-free water R* with heating, allow to cool and dilute to 100.0 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1).

pH (2.2.3). To 10 ml of solution S add 0.1 ml of a saturated solution of *potassium chloride R*. The pH of the solution is 5.0 to 8.0.

Specific optical rotation (2.2.7): + 160 to + 164, determined on solution S and calculated with reference to the dried substance.

Reducing sugars

Test solution. To 1 ml of solution S add 1 ml of *cupri-tartaric solution R4*. Heat on a water-bath for 10 min, cool to room temperature. Add 10 ml of *ammonium molybdate reagent R1* and allow to stand for 15 min.

Reference solution. Prepare a reference solution at the same time and in the same manner as the test solution, using 1 ml of a 0.02 g/l solution of *glucose R*.

Measure the absorbance of the test solution and the reference solution (2.2.25) at the maximum at 740 nm using *water R* as the compensation liquid. The absorbance of the test solution is not greater than that of the reference solution (0.2 per cent).

Light-absorbing impurities. Examine solution S between 230 nm and 750 nm (2.2.25). Between 230 nm and 350 nm, the absorbance is not greater than 0.10. Between 350 nm and 750 nm, the absorbance is not greater than 0.05.

Related substances. Examine by liquid chromatography (2.2.29), as described under Assay. Inject separately test solution (a) and reference solution (b). In the chromatogram obtained with test solution (a): the areas of any peaks corresponding to gammacyclodextrin and alphacyclodextrin are not greater than half of the area of the corresponding peaks in the chromatogram obtained with reference solution (b) (0.25 per cent); the sum of the areas of all the peaks, apart from the principal peak and any peaks corresponding to alphacyclodextrin and gammacyclodextrin, is not greater than half of the area of the peak corresponding to betadex in the chromatogram obtained with reference solution (b) (0.5 per cent).

Residual solvents. Not more than 10 ppm of trichloroethylene and not more than 10 ppm of toluene. Examine by head-space gas chromatography (2.2.28), using the standard additions method and *ethylene chloride R* as the internal standard.

Test solutions. In each of four identical 20 ml flasks, dissolve 500 mg of the substance to be examined in *water R* and add 0.10 g of *calcium chloride R* and 30 µl of *α-amylase solution R*. Add 1 ml of reference solutions (a), (b), (c) and (d), adding a different solution to each flask. Dilute to 10 ml with *water R*.

Reference solutions. Prepare reference solution (a) containing 10 µl of *ethylene chloride R* per litre. From reference solution (a), prepare reference solutions (b), (c) and (d) containing per litre: 5 µl, 10 µl and 15 µl each of *trichloroethylene R* and of *toluene R*.

The chromatographic procedure may be carried out using:

- a fused-silica column 25 m long and 0.32 mm in internal diameter coated with a layer about 1 µm thick of *macrogol 20 000 R*,
- *helium for chromatography R* as the carrier gas,
- a flame-ionisation detector,

maintaining the temperature of the column at 50 °C, that of the injection port at 140 °C and that of the detector at 280 °C. Place the samples in a thermostated chamber at 45 °C for 2 h. Inject 200 µl of the head-space of each flask and repeat each test at least three times. The retention time of toluene is about 10 min. The test is not valid unless: the resolutions between the peaks corresponding to trichloroethylene and toluene and between the peaks corresponding to toluene and ethylene chloride are greater than 1.1 and the relative standard deviations of the ratios of the areas of the peaks corresponding to trichloroethylene and toluene to that of the peak corresponding to ethylene chloride are less than 5 per cent.

Calculate the content of trichloroethylene and of toluene taking their relative densities to be 1.46 and 0.87, respectively.

Heavy metals (2.4.8). 1.0 g complies with limit test C for heavy metals (10 ppm). Prepare the standard using 1 ml of *lead standard solution (10 ppm Pb) R*.

Loss on drying (2.2.32). Not more than 16.0 per cent, determined on 1.000 g by drying in an oven at 120 °C for 2 h.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Examine by liquid chromatography (2.2.29).

Test solution (a). Dissolve 0.25 g of the substance to be examined in *water R* with heating, cool and dilute to 25.0 ml with the same solvent.

Test solution (b). Dilute 5.0 ml of test solution (a) to 50.0 ml with *water R*.

Reference solution (a). Dissolve 25.0 mg of *alfadex CRS*, 25.0 mg of *gammacyclodextrin CRS* and 50.0 mg of *betadex CRS* in *water R* and dilute to 50.0 ml with the same solvent.

Reference solution (b). Dilute 5.0 ml of reference solution (a) to 50.0 ml with *water R*.

Reference solution (c). Dissolve 25.0 mg of *betadex CRS* in *water R* and dilute to 25.0 ml with the same solvent.

The chromatographic procedure may be carried out using:

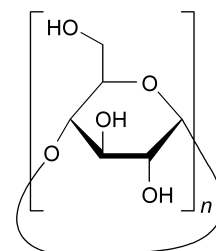
- a stainless steel column 0.25 m long and 4.6 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (10 µm),
- as mobile phase at a flow rate of 1.5 ml/min a mixture of 10 volumes of *methanol R* and 90 volumes of *water R*,
- as detector a differential refractometer,
- a 50 µl loop injector.

Equilibrate the column with the mobile phase at a flow rate of 1.5 ml/min for about 3 h. Inject each solution. Record the chromatograms for 1.5 times the retention time of betadex. Adjust the sensitivity of the detector so that the height of the peak corresponding to gammacyclodextrin, in the chromatogram obtained with reference solution (a), is 55 per cent to 75 per cent of the full scale of the recorder. The retention time of betadex is about 10 min, the relative retention time of gammacyclodextrin is about 0.3 and that of alfadex is about 0.45. The test is not valid unless the resolution between the peaks corresponding to gammacyclodextrin and alfadex is not less than 1.5, and the relative standard deviation of the area of the peak corresponding to betadex is less than 2.0 per cent. If necessary, adjust the concentration of methanol in the mobile phase to achieve the required resolution. Calculate the percentage content of $[C_6H_{10}O_5]_n$ from the area of the principal peak in each of the chromatograms obtained with test solution (b) and reference solution (c) and the declared content of *betadex CRS*.

STORAGE

Store in an airtight container.

IMPURITIES



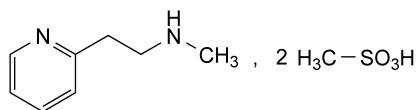
A. $n = 6$: alfadex,

B. $n = 8$: gammacyclodextrin.

01/2005:1071 TESTS

BETAHISTINE MESILATE

Betahistini mesilas

 $C_{10}H_{20}N_2O_6S_2$ M_r 328.4

DEFINITION

Betahistine mesilate contains not less than 98.0 per cent and not more than the equivalent of 101.0 per cent of *N*-methyl-2-(pyridin-2-yl)ethanamine bis(methanesulphonate), calculated with reference to the anhydrous, 2-propanol-free substance.

PRODUCTION

The production method must be evaluated to determine the potential for formation of alkyl mesilates, which is particularly likely to occur if the reaction medium contains lower alcohols. Where necessary, the production method is validated to demonstrate that alkyl mesilates are not detectable in the final product.

CHARACTERS

A white, crystalline powder, very hygroscopic, very soluble in water, freely soluble in alcohol, very slightly soluble in 2-propanol.

IDENTIFICATION

First identification: B.

Second identification: A, C, D.

A. Melting point (2.2.14): 108 °C to 112 °C.

B. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *betahistine mesilate* CRS. Examine the substances prepared as discs.

C. Examine by thin-layer chromatography (2.2.27), using a suitable silica gel with a fluorescent indicator having an optimal intensity at 254 nm as the coating substance.

Test solution. Dissolve 10 mg of the substance to be examined in *alcohol* R and dilute to 2 ml with the same solvent.

Reference solution. Dissolve 10 mg of *betahistine mesilate* CRS, in *alcohol* R and dilute to 2 ml with the same solvent.

Apply to the plate 2 µl of each solution. Develop over a path of 15 cm using a mixture of 0.75 volumes of *concentrated ammonia* R, 15 volumes of *ethyl acetate* R and 30 volumes of *methanol* R. Dry the plate at 110 °C for 10 min and examine in ultraviolet light at 254 nm. The principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with the reference solution.

D. To 0.1 g add 5 ml of *dilute hydrochloric acid* R and shake for about 5 min. Add 1 ml of *barium chloride solution* R1. The solution remains clear. To a further 0.1 g add 0.5 g of *anhydrous sodium carbonate* R, mix and ignite until a white residue is obtained. Allow to cool and dissolve the residue in 7 ml of *water* R. The solution gives reaction (a) of sulphates (2.3.1).

Solution S. Dissolve 5.0 g in *carbon dioxide-free water* R prepared from *distilled water* R, and dilute to 50 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and colourless (2.2.2, *Method II*).

pH (2.2.3). The pH of solution S is 2.0 to 3.0.

Related substances. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 50 mg of the substance to be examined in the mobile phase and dilute to 10.0 ml with the mobile phase.

Reference solution (a). Dissolve 10 mg of *betahistine mesilate* CRS and 10 mg of *2-vinylpyridine* R in the mobile phase and dilute to 50.0 ml with the mobile phase. Dilute 2.0 ml of the solution to 50.0 ml with the mobile phase.

Reference solution (b). Dilute 1.0 ml of the test solution to 100.0 ml with the mobile phase.

Reference solution (c). Dilute 2.0 ml of reference solution (b) to 10.0 ml with the mobile phase.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4.6 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography* R (5 µm),
- as mobile phase at a flow rate of 1 ml/min a mixture prepared as follows: dissolve 2.0 g of *sodium dodecyl sulphate* R in a mixture of 15 volumes of a 10 per cent V/V solution of *sulphuric acid* R, 35 volumes of a 17 g/l solution of *tetrabutylammoniumhydrogen sulphate* R and 650 volumes of *water* R; adjust to pH 3.3 using *dilute sodium hydroxide solution* R and mix with 300 volumes of *acetonitrile* R,
- as detector a spectrophotometer set at 260 nm.

Inject 20 µl of reference solution (a). When using a recorder, adjust the sensitivity of the system so that the height of the first peak in the chromatogram obtained with reference solution (a) is not less than 70 per cent of the full scale of the recorder. The test is not valid unless: in the chromatogram obtained with reference solution (a), the resolution between the peaks corresponding to 2-vinylpyridine and betahistine mesilate is at least 3.5.

Inject 20 µl of the test solution and of reference solutions (b) and (c). Continue the chromatography for 3 times the retention time of betahistine mesilate (which is about 8 min). In the chromatogram obtained with the test solution the area of any peak, apart from the principal peak, is not greater than the area of the principal peak in the chromatogram obtained with reference solution (c) (0.2 per cent); the sum of the areas of any peaks, apart from the principal peak, is not greater than half of the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent).

Disregard any peak with an area less than 0.025 times that of the principal peak in the chromatogram obtained with reference solution (b).

2-Propanol. Not more than 0.5 per cent, determined by the test for residual solvents (2.4.24).

Chlorides (2.4.4). To 14 ml of solution S add 1 ml of *water* R. The solution complies with the limit test for chlorides (35 ppm).

Sulphates (2.4.13). Dilute 6 ml of solution S to 15 ml with *distilled water* R. The solution complies with the limit test for sulphates (250 ppm).

Heavy metals (2.4.8). 12 ml of solution S complies with limit test A for heavy metals (20 ppm). Prepare the standard using *lead standard solution* (2 ppm Pb) R.

Water (2.5.12). Not more than 2.0 per cent, determined on 0.50 g by the semi-micro determination of water.

ASSAY

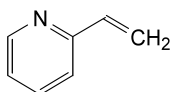
Dissolve 0.140 g in 50 ml of a mixture of 1 volume of *anhydrous acetic acid* R and 7 volumes of *acetic anhydride* R. Titrate with 0.1 M *perchloric acid*, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *perchloric acid* is equivalent to 16.42 mg of $C_{22}H_{29}FO_5$.

STORAGE

Store in an airtight container.

IMPURITIES

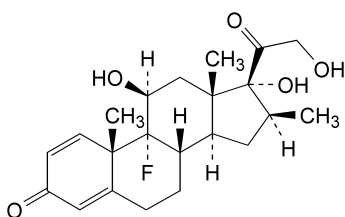


A. 2-ethenylpyridine.

01/2005:0312

BETAMETHASONE

Betamethasonum



$C_{22}H_{29}FO_5$

M_r 392.5

DEFINITION

Betamethasone contains not less than 97.0 per cent and not more than the equivalent of 103.0 per cent of 9-fluoro-11β,17,21-trihydroxy-16β-methylpregna-1,4-diene-3,20-dione, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, crystalline powder, practically insoluble in water, sparingly soluble in ethanol, very slightly soluble in methylene chloride.

IDENTIFICATION

First identification: B, C.

Second identification: A, C, D, E.

- A. Dissolve 10.0 mg in *ethanol* R and dilute to 100.0 ml with the same solvent. Place 2.0 ml of the solution in a stoppered tube, add 10.0 ml of *phenylhydrazine-sulphuric acid solution* R, mix and heat in a water-bath at 60 °C for 20 min. Cool immediately. The absorbance (2.2.25) of the solution measured at 419 nm is not greater than 0.10.
- B. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *betamethasone CRS*. If the spectra obtained in the solid state with the substance to be examined and the reference substance show differences, dissolve the substance to be

examined and the reference substance separately in the smallest necessary quantity of *methylene chloride* R and evaporate to dryness on a water-bath. Using the residues, record the spectra again.

- C. Examine by thin-layer chromatography (2.2.27), using as the coating substance a suitable silica gel with a fluorescent indicator having an optimal intensity at 254 nm.

Test solution. Dissolve 10 mg of the substance to be examined in a mixture of 1 volume of *methanol* R and 9 volumes of *methylene chloride* R and dilute to 10 ml with the same mixture of solvents.

Reference solution (a). Dissolve 20 mg of *betamethasone CRS* in a mixture of 1 volume of *methanol* R and 9 volumes of *methylene chloride* R and dilute to 20 ml with the same mixture of solvents.

Reference solution (b). Dissolve 10 mg of *dexamethasone CRS* in reference solution (a) and dilute to 10 ml with the same solution.

Apply separately to the plate 5 µl of each solution.

Develop over a path of 15 cm using a mixture of 5 volumes of *butanol* R saturated with *water* R, 10 volumes of *toluene* R and 85 volumes of *ether* R. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. The principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a). Spray with *alcoholic solution of sulphuric acid* R. Heat at 120 °C for 10 min or until the spots appear. Allow to cool. Examine the chromatograms in daylight and in ultraviolet light at 365 nm. The principal spot in the chromatogram obtained with the test solution is similar in position, colour in daylight, fluorescence in ultraviolet light at 365 nm and size to the principal spot in the chromatogram obtained with reference solution (a). The test is not valid unless the chromatogram obtained with reference solution (b) shows two spots which may however not be completely separated.

- D. Mix about 5 mg with 45 mg of *heavy magnesium oxide* R and ignite in a crucible until an almost white residue is obtained (usually less than 5 min). Allow to cool, add 1 ml of *water* R, 0.05 ml of *phenolphthalein solution* R1 and about 1 ml of *dilute hydrochloric acid* R to render the solution colourless. Filter. Add 1.0 ml of the filtrate to a freshly prepared mixture of 0.1 ml of *alizarin S solution* R and 0.1 ml of *zirconyl nitrate solution* R. Mix, allow to stand for 5 min and compare the colour of the solution with that of a blank prepared in the same manner. The test solution is yellow and the blank is red.
- E. Add about 2 mg to 2 ml of *sulphuric acid* R and shake to dissolve. Within 5 min, a deep reddish-brown colour develops. Add the solution to 10 ml of *water* R and mix. The colour is discharged and a clear solution remains.

TESTS

Specific optical rotation (2.2.7). Dissolve 0.125 g in *methanol* R and dilute to 25.0 ml with the same solvent. The specific optical rotation is + 118 to + 126, calculated with reference to the dried substance.

Related substances. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 25.0 mg of the substance to be examined in a mixture of equal volumes of *acetonitrile* R and *methanol* R and dilute to 10.0 ml with the same solvent.

Reference solution (a). Dissolve 2 mg of *betamethasone CRS* and 2 mg of *methylprednisolone CRS* in mobile phase A and dilute to 100.0 ml with the same mobile phase.

Reference solution (b). Dilute 1.0 ml of the test solution to 100.0 ml with mobile phase A.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4.6 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (5 µm),
- as mobile phase at a flow rate of 2.5 ml/min, a linear-gradient programme using the following conditions:

Mobile phase A. In a 1000 ml volumetric flask mix 250 ml of *acetonitrile R* with 700 ml of *water R* and allow to equilibrate; adjust the volume to 1000 ml with *water R* and mix again,

Mobile phase B. *Acetonitrile R*,

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)	Comment
0	100	0	isocratic
15	100	0	begin linear gradient
40	0	100	end chromatogram, return to 100A
41	100	0	begin equilibration with A
46 = 0	100	0	end equilibration, begin next chromatogram

- as detector a spectrophotometer set at 254 nm, maintaining the temperature of the column at 45 °C.

Equilibrate the column with mobile phase B at a flow rate of 2.5 ml/min for at least 30 min and then with mobile phase A for 5 min. For subsequent chromatograms, use the conditions described from 40 min to 46 min.

Adjust the sensitivity of the system so that the height of the principal peak in the chromatogram obtained with 20 µl of reference solution (b) is not less than 50 per cent of the full scale of the recorder.

Inject 20 µl of reference solution (a). When the chromatograms are recorded in the conditions described above, the retention times are: methylprednisolone, about 11.5 minutes and betamethasone, about 12.5 minutes. The test is not valid unless the resolution between the peaks corresponding to methylprednisolone and betamethasone is at least 1.5; if necessary, adjust the concentration of acetonitrile in mobile phase A.

Inject separately 20 µl of the mixture of equal volumes of *acetonitrile R* and *methanol R* as a blank, 20 µl of the test solution and 20 µl of reference solution (b). In the chromatogram obtained with the test solution: the area of any peak, apart from the principal peak, is not greater than the area of the principal peak in the chromatogram obtained with reference solution (b) (1.0 per cent) and not more than one such peak has an area greater than half the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent); the sum of the areas of all the peaks, apart from the principal peak, is not greater than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (2.0 per cent). Disregard any peak due to the blank and any peak with an area less than 0.05 times the area of the principal peak in the chromatogram obtained with reference solution (b).

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 0.500 g by drying in an oven at 100 °C to 105 °C.

ASSAY

Dissolve 0.100 g in *alcohol R* and dilute to 100.0 ml with the same solvent. Dilute 2.0 ml of the solution to 100.0 ml with *alcohol R*. Measure the absorbance (2.2.25) at the maximum at 238.5 nm.

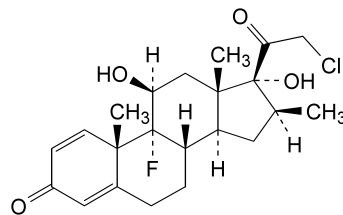
Calculate the content of C₂₂H₂₉FO₅ taking the specific absorbance to be 395.

STORAGE

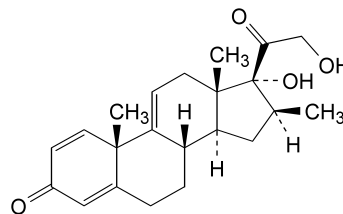
Store protected from light.

IMPURITIES

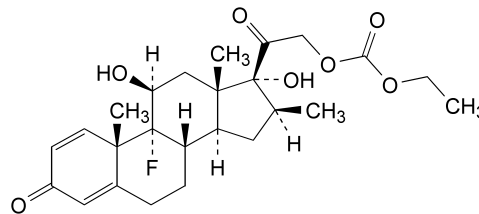
A. dexamethasone,



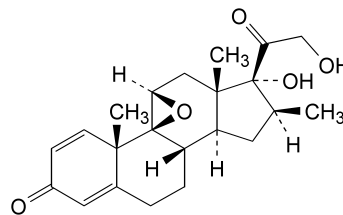
B. 21-chloro-9-fluoro-11β,17-dihydroxy-16β-methylpregna-1,4-diene-3,20-dione,



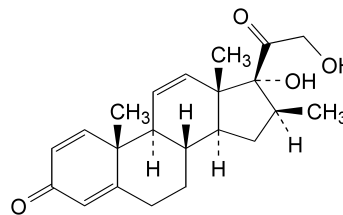
C. 17,21-dihydroxy-16β-methylpregna-1,4,9(11)-triene-3,20-dione,



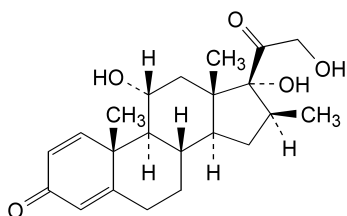
D. 9-fluoro-11β,17-dihydroxy-16β-methyl-3,20-dioxopregna-1,4-dien-21-yl ethoxycarboxylate,



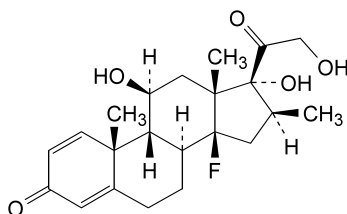
E. 9,11β-epoxy-17,21-dihydroxy-16β-methyl-9β-pregna-1,4-diene-3,20-dione,



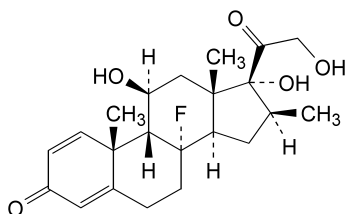
F. 17,21-dihydroxy-16β-methylpregna-1,4,11-triene-3,20-dione,



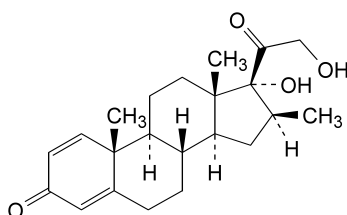
G. 11 α ,17,21-trihydroxy-16 β -methylpregna-1,4-diene-3,20-dione,



H. 14-fluoro-11 β ,17,21-trihydroxy-16 β -methyl-8 α ,9 β ,14 β -pregna-1,4-diene-3,20-dione,



I. 8-fluoro-11 β ,17,21-trihydroxy-16 β -methyl-8 α ,9 β -pregna-1,4-diene-3,20-dione,

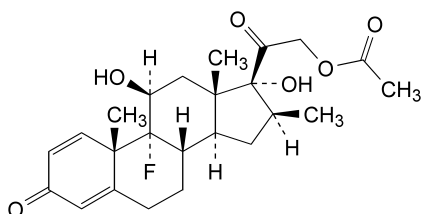


J. 17,21-dihydroxy-16 β -methylpregna-1,4-diene-3,20-dione.

01/2005:0975

BETAMETHASONE ACETATE

Betamethasoni acetat



$C_{24}H_{31}FO_6$

M_r 434.5

DEFINITION

Betamethasone acetate contains not less than 97.0 per cent and not more than the equivalent of 103.0 per cent of 9-fluoro-11 β ,17-dihydroxy-16 β -methyl-3,20-dioxopregna-1,4-diene-21-yl acetate, calculated with reference to the anhydrous substance.

CHARACTERS

A white or almost white, crystalline powder, practically insoluble in water, freely soluble in acetone, soluble in alcohol and in methylene chloride.

It shows polymorphism.

IDENTIFICATION

First identification: B, C.

Second identification: A, C, D, E, F.

- Dissolve 10.0 mg in *ethanol R* and dilute to 100.0 ml with the same solvent. Place 2.0 ml of this solution in a ground-glass-stoppered tube, add 10.0 ml of *phenylhydrazine-sulphuric acid solution R*, mix and heat in a water-bath at 60 °C for 20 min. Cool immediately. The absorbance (2.2.25) of the solution measured at 419 nm is not more than 0.10.
- Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *betamethasone acetate CRS*. If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in the minimum volume of *methanol R*, evaporate to dryness on a water-bath and record new spectra using the residues.
- Examine by thin-layer chromatography (2.2.27), using as the coating substance a suitable silica gel with a fluorescent indicator having an optimal intensity at 254 nm.

Test solution. Dissolve 10 mg of the substance to be examined in a mixture of 1 volume of *methanol R* and 9 volumes of *methylene chloride R* and dilute to 10 ml with the same mixture of solvents.

Reference solution (a). Dissolve 20 mg of *betamethasone acetate CRS* in a mixture of 1 volume of *methanol R* and 9 volumes of *methylene chloride R* and dilute to 20 ml with the same mixture of solvents.

Reference solution (b). Dissolve 10 mg of *prednisolone acetate CRS* in reference solution (a) and dilute to 10 ml with the same solution.

Apply to the plate 5 μ l of each solution. Prepare the mobile phase by adding a mixture of 1.2 volumes of *water R* and 8 volumes of *methanol R* to a mixture of 15 volumes of *ether R* and 77 volumes of *methylene chloride R*. Develop over a path of 15 cm. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. The principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a). Spray with *alcoholic solution of sulphuric acid R*. Heat at 120 °C for 10 min or until the spots appear. Allow to cool. Examine the plate in daylight and in ultraviolet light at 365 nm. The principal spot in the chromatogram obtained with the test solution is similar in position, colour in daylight, fluorescence in ultraviolet light at 365 nm and size to the principal spot in the chromatogram obtained with reference solution (a). The test is not valid unless the chromatogram obtained with reference solution (b) shows two clearly separated spots.

- Add about 2 mg to 2 ml of *sulphuric acid R* and shake to dissolve. Within 5 min, a deep reddish-brown colour develops. Add the solution to 10 ml of *water R* and mix. The colour is discharged and a clear solution remains.
- Mix about 5 mg with 45 mg of *heavy magnesium oxide R* and ignite in a crucible until an almost white residue is obtained (usually less than 5 min). Allow to cool, add 1 ml of *water R*, 0.05 ml of *phenolphthalein solution R1* and about 1 ml of *dilute hydrochloric acid R* to render the solution colourless. Filter. To a freshly prepared mixture

of 0.1 ml of *alizarin S solution R* and 0.1 ml of *zirconyl nitrate solution R*, add 1.0 ml of the filtrate. Mix, allow to stand for 5 min and compare the colour of the solution with that of a blank prepared in the same manner. The test solution is yellow and the blank is red.

F. About 10 mg gives the reaction of acetyl (2.3.1).

TESTS

Specific optical rotation (2.2.7). Dissolve 0.250 g in *dioxan R* and dilute to 25.0 ml with the same solvent. The specific optical rotation is + 120 to + 128, calculated with reference to the anhydrous substance.

Related substances. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 25.0 mg of the substance to be examined in 4 ml of *acetonitrile R* and dilute to 10.0 ml with the same solvent.

Reference solution (a). Dissolve 2 mg of *betamethasone acetate CRS* and 2 mg of *dexamethasone acetate CRS* in the mobile phase and dilute to 100.0 ml with the mobile phase.

Reference solution (b). Dilute 1.0 ml of the test solution to 100.0 ml with the mobile phase.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4.6 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (5 µm),
- as mobile phase at a flow rate of 1 ml/min a mixture prepared as follows: in a 1000 ml volumetric flask mix 380 ml of *acetonitrile R* with 550 ml of *water R* and allow to equilibrate; dilute to 1000 ml with *water R* and mix again,
- as detector a spectrophotometer set at 254 nm.

Equilibrate the column with the mobile phase at a flow rate of 1 ml/min for about 30 min.

Adjust the sensitivity of the system so that the height of the principal peak in the chromatogram obtained with 20 µl of reference solution (b) is at least 50 per cent of the full scale of the recorder.

Inject 20 µl of reference solution (a). When the chromatograms are recorded in the prescribed conditions, the retention times are: betamethasone acetate about 19 min and dexamethasone acetate about 22 min. The test is not valid unless the resolution between the peaks due to betamethasone acetate and dexamethasone acetate is at least 3.3; if necessary, adjust slightly the concentration of acetonitrile in the mobile phase.

Inject 20 µl of the test solution and 20 µl of reference solution (b). Continue the chromatography for 2.5 times the retention time of the principal peak in the chromatogram obtained with the test solution. In the chromatogram obtained with the test solution: the area of any peak, apart from the principal peak, is not greater than half the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent); the sum of the areas of all the peaks, apart from the principal peak, is not greater than 1.25 times the area of the principal peak in the chromatogram obtained with reference solution (b) (1.25 per cent). Disregard any peak with an area less than 0.05 times the area of the principal peak in the chromatogram obtained with reference solution (b).

Water (2.5.12). Not more than 4.0 per cent, determined on 0.100 g by the semi-micro determination of water.

ASSAY

Dissolve 0.100 g in *alcohol R* and dilute to 100.0 ml with the same solvent. Dilute 2.0 ml of the solution to 100.0 ml with *alcohol R*. Measure the absorbance (2.2.25) at the maximum at 240 nm.

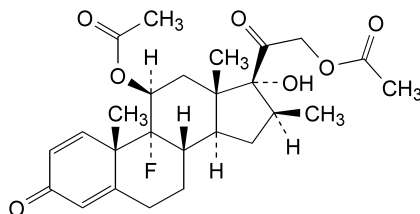
Calculate the content of $C_{24}H_{31}FO_6$ taking the specific absorbance to be 350.

STORAGE

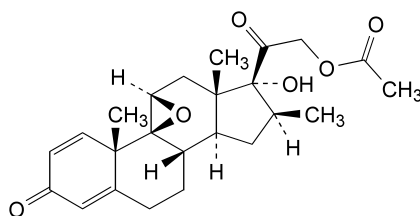
Store protected from light.

IMPURITIES

- A. betamethasone,
B. dexamethasone acetate,



- C. betamethasone 11,21-diacetate,

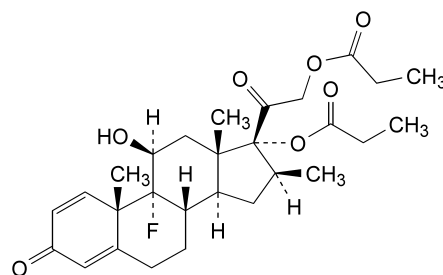


- D. 9,11β-epoxy-17-hydroxy-16β-methyl-3,20-dioxo-9β-pregna-1,4-diene-21-yl acetate.

01/2005:0809

BETAMETHASONE DIPROPIONATE

Betamethasoni dipropionas



$C_{28}H_{37}FO_7$

M_r 504.6

DEFINITION

Betamethasone dipropionate contains not less than 97.0 per cent and not more than the equivalent of 103.0 per cent of 9-fluoro-11β-hydroxy-16β-methyl-3,20-dioxopregna-1,4-diene-17,21-diyl dipropanoate, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, crystalline powder, practically insoluble in water, freely soluble in acetone and in methylene chloride, sparingly soluble in alcohol.

IDENTIFICATION

First identification: B, C.

Second identification: A, D, E, F.

- A. Dissolve 10.0 mg in *ethanol R* and dilute to 100.0 ml with the same solvent. Place 2.0 ml of this solution in a ground-glass-stoppered tube, add 10.0 ml of *phenylhydrazine-sulphuric acid solution R*, mix and heat in a water-bath at 60 °C for 20 min. Cool immediately. The absorbance (2.2.25) of the solution measured at 419 nm is not more than 0.10.
- B. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *betamethasone dipropionate CRS*.
- C. Examine by thin-layer chromatography (2.2.27), using as the coating substance a suitable silica gel with a fluorescent indicator having an optimal intensity at 254 nm.

Test solution. Dissolve 10 mg of the substance to be examined in a mixture of 1 volume of *methanol R* and 9 volumes of *methylene chloride R* and dilute to 10 ml with the same mixture of solvents.

Reference solution (a). Dissolve 10 mg of *betamethasone dipropionate CRS* in a mixture of 1 volume of *methanol R* and 9 volumes of *methylene chloride R* and dilute to 10 ml with the same mixture of solvents.

Reference solution (b). Dissolve 10 mg of *desoxycortone acetate CRS* in a mixture of 1 volume of *methanol R* and 9 volumes of *methylene chloride R* and dilute to 10 ml with the same mixture of solvents. Dilute 5 ml of this solution to 10 ml with reference solution (a).

Apply to the plate 5 µl of each solution. Prepare the mobile phase by adding a mixture of 1.2 volumes of *water R* and 8 volumes of *methanol R* to a mixture of 15 volumes of *ether R* and 77 volumes of *methylene chloride R*. Develop over a path of 15 cm. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. The principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a). Spray the plate with *alcoholic solution of sulphuric acid R*. Heat at 120 °C for 10 min or until the spots appear. Allow to cool. Examine in daylight and in ultraviolet light at 365 nm. The principal spot in the chromatogram obtained with the test solution is similar in position, colour in daylight, fluorescence in ultraviolet light at 365 nm and size to the principal spot in the chromatogram obtained with reference solution (a). The test is not valid unless the chromatogram obtained with reference solution (b) shows two clearly separated spots.

- D. Examine by thin-layer chromatography (2.2.27), using as the coating substance a suitable silica gel with a fluorescent indicator having an optimal intensity at 254 nm.

Test solution (a). Dissolve 25 mg of the substance to be examined in *methanol R* with gentle heating and dilute to 5 ml with the same solvent. This solution is also used to prepare test solution (b). Dilute 2 ml of the solution to 10 ml with *methylene chloride R*.

Test solution (b). Transfer 2 ml of the solution obtained during preparation of test solution (a) to a 15 ml glass tube with a ground-glass stopper or a polytetrafluoroethylene cap. Add 10 ml of *saturated methanolic potassium hydrogen carbonate solution R* and immediately pass a current of *nitrogen R* briskly through the solution for 5 min. Stopper the tube. Heat in a water-bath at 45 °C, protected from light, for 2 h. Allow to cool.

Reference solution (a). Dissolve 25 mg of *betamethasone dipropionate CRS* in *methanol R* with gentle heating and dilute to 5 ml with the same solvent. This solution is also used to prepare reference solution (b). Dilute 2 ml of the solution to 10 ml with *methylene chloride R*.

Reference solution (b). Transfer 2 ml of the solution obtained during preparation of reference solution (a) to a 15 ml glass tube with a ground-glass stopper or a polytetrafluoroethylene cap. Add 10 ml of *saturated methanolic potassium hydrogen carbonate solution R* and immediately pass a current of *nitrogen R* briskly through the solution for 5 min. Stopper the tube. Heat in a water-bath at 45 °C, protected from light, for 2 h. Allow to cool.

Apply to the plate 5 µl of each solution. Prepare the mobile phase by adding a mixture of 1.2 volumes of *water R* and 8 volumes of *methanol R* to a mixture of 15 volumes of *ether R* and 77 volumes of *methylene chloride R*. Develop over a path of 15 cm. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. The principal spot in each of the chromatograms obtained with the test solutions is similar in position and size to the principal spot in the chromatogram obtained with the corresponding reference solution. Spray the plate with *alcoholic solution of sulphuric acid R*. Heat at 120 °C for 10 min or until the spots appear. Allow to cool. Examine in daylight and in ultraviolet light at 365 nm. The principal spot in each of the chromatograms obtained with the test solutions is similar in position, colour in daylight, fluorescence in ultraviolet light at 365 nm and size to the principal spot in the chromatogram obtained with the corresponding reference solution. The principal spot in each of the chromatograms obtained with test solution (b) and reference solution (b) has an R_f value distinctly lower than that of the principal spots in each of the chromatograms obtained with test solution (a) and reference solution (a).

- E. Add about 2 mg to 2 ml of *sulphuric acid R* and shake to dissolve. Within 5 min, a deep reddish-brown colour develops. Add the solution to 10 ml of *water R* and mix. The colour is discharged and a clear solution remains.
- F. Mix about 5 mg with 45 mg of *heavy magnesium oxide R* and ignite in a crucible until an almost white residue is obtained (usually less than 5 min). Allow to cool, add 1 ml of *water R*, 0.05 ml of *phenolphthalein solution R1* and about 1 ml of *dilute hydrochloric acid R* to render the solution colourless. Filter. Add 1.0 ml of the filtrate to a freshly prepared mixture of 0.1 ml of *alizarin S solution R* and 0.1 ml of *zirconyl nitrate solution R*. Mix, allow to stand for 5 min and compare the colour of the solution with that of a blank prepared in the same manner. The test solution is yellow and the blank is red.

TESTS

Specific optical rotation (2.2.7). Dissolve 0.250 g in *dioxan R* and dilute to 25.0 ml with the same solvent. The specific optical rotation is + 63 to + 70, calculated with reference to the dried substance.

Related substances. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 62.5 mg of the substance to be examined in the mobile phase and dilute to 25.0 ml with the mobile phase.

Reference solution (a). Dissolve 2.5 mg of *betamethasone dipropionate CRS* and 2.5 mg of *beclometasone dipropionate CRS* in the mobile phase and dilute to 50.0 ml with the same solvent.

Reference solution (b). Dilute 1.0 ml of the test solution to 50.0 ml with the mobile phase.

01/2005:0810

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4.6 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (5 µm),
- as mobile phase at a flow rate of 1 ml/min a mixture prepared as follows: mix carefully 350 ml of *water R* with 600 ml of *acetonitrile R* and allow to equilibrate; adjust the volume to 1000 ml with *water R* and mix again,
- as detector a spectrophotometer set at 254 nm.

Adjust the sensitivity so that the height of the principal peak in the chromatogram obtained with reference solution (b) is 70 per cent to 90 per cent of the full scale of the recorder.

Equilibrate the column with the mobile phase at a flow rate of 1 ml/min for about 45 min. Inject 20 µl of reference solution (a). When the chromatograms are recorded in the prescribed conditions, the retention times are: betamethasone dipropionate, about 9 min; beclomethasone dipropionate, about 10.7 min. The test is not valid unless the resolution between the peaks corresponding to betamethasone dipropionate and beclomethasone dipropionate is at least 2.5; if necessary, adjust the concentration of acetonitrile in the mobile phase.

Inject separately 20 µl of the test solution and 20 µl of reference solution (b). Continue the chromatography for 2.5 times the retention time of the principal peak. In the chromatogram obtained with the test solution: the area of any peak apart from the principal peak is not greater than 0.75 times the area of the principal peak in the chromatogram obtained with reference solution (b) (1.5 per cent) and not more than one such peak has an area greater than half the area of the principal peak in the chromatogram obtained with reference solution (b) (1 per cent); the sum of the areas of all the peaks, apart from the principal peak, is not greater than 1.25 times the area of the principal peak in the chromatogram obtained with reference solution (b) (2.5 per cent). Disregard any peak with an area less than 0.025 times the area of the principal peak in the chromatogram obtained with reference solution (b).

Loss on drying (2.2.32). Not more than 1.0 per cent, determined on 0.500 g by drying in an oven at 100–105 °C.

ASSAY

Dissolve 50.0 mg in *alcohol R* and dilute to 100.0 ml with the same solvent. Dilute 2.0 ml of the solution to 50.0 ml with *alcohol R*. Measure the absorbance (2.2.25) at the maximum at 240 nm.

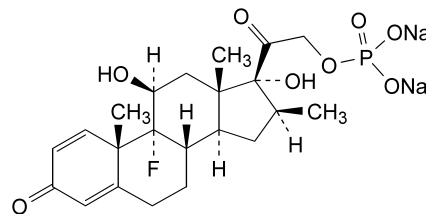
Calculate the content of $C_{22}H_{28}FO_7$ taking the specific absorbance to be 305.

STORAGE

Store protected from light.

BETAMETHASONE SODIUM PHOSPHATE

Betamethasoni natrii phosphas


 $C_{22}H_{28}FNa_2O_8P$
 M_r 516.4

DEFINITION

Betamethasone sodium phosphate contains not less than 96.0 per cent and not more than the equivalent of 103.0 per cent of 9-fluoro-11β,17-dihydroxy-16β-methyl-3,20-dioxopregna-1,4-diene-21-yl disodium phosphate, calculated with reference to the anhydrous substance.

CHARACTERS

A white or almost white powder, very hygroscopic, freely soluble in water, slightly soluble in alcohol, practically insoluble in methylene chloride.

IDENTIFICATION

First identification: B, C.

Second identification: A, C, D, E, F.

- Dissolve 10.0 mg in 5 ml of *water R* and dilute to 100.0 ml with *ethanol R*. Place 2.0 ml of this solution in a ground-glass-stoppered tube, add 10.0 ml of *phenylhydrazine-sulphuric acid solution R*, mix and heat in a water-bath at 60 °C for 20 min. Cool immediately. The absorbance (2.2.25) of the solution measured at the maximum at 450 nm is not more than 0.10.
- Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *betamethasone sodium phosphate CRS*. If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in the minimum volume of *alcohol R*, evaporate to dryness on a water-bath and record new spectra using the residues.
- Examine by thin-layer chromatography (2.2.27), using as the coating substance a suitable silica gel with a fluorescent indicator having an optimal intensity at 254 nm.

Test solution. Dissolve 10 mg of the substance to be examined in *methanol R* and dilute to 10 ml with the same solvent.

Reference solution (a). Dissolve 10 mg of *betamethasone sodium phosphate CRS* in *methanol R* and dilute to 10 ml with the same solvent.

Reference solution (b). Dissolve 10 mg of *prednisolone sodium phosphate CRS* in *methanol R* and dilute to 10 ml with the same solvent. Dilute 5 ml of this solution to 10 ml with reference solution (a).

Apply to the plate 5 µl of each solution. Develop over a path of 15 cm using a mixture of 20 volumes of *glacial acetic acid R*, 20 volumes of *water R* and 60 volumes of *butanol R*. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. The principal spot in the chromatogram obtained with the test solution is

similar in position and size to the principal spot in the chromatogram obtained with reference solution (a). Spray the plate with *alcoholic solution of sulphuric acid R*. Heat at 120 °C for 10 min or until the spots appear. Allow to cool. Examine in daylight and in ultraviolet light at 365 nm. The principal spot in the chromatogram obtained with the test solution is similar in position, colour in daylight, fluorescence in ultraviolet light at 365 nm and size to the principal spot in the chromatogram obtained with reference solution (a). The test is not valid unless the chromatogram obtained with reference solution (b) shows two spots which may however not be completely separated.

- D. Add about 2 mg to 2 ml of *sulphuric acid R* and shake to dissolve. Within 5 min, an intense reddish-brown colour develops. Add the solution to 10 ml of *water R* and mix. The colour is discharged and a clear solution remains.
- E. Mix about 5 mg with 45 mg of *heavy magnesium oxide R* and ignite in a crucible until an almost white residue is obtained (usually less than 5 min). Allow to cool, add 1 ml of *water R*, 0.05 ml of *phenolphthalein solution R1* and about 1 ml of *dilute hydrochloric acid R* to render the solution colourless. Filter. Add 1.0 ml of the filtrate to a freshly prepared mixture of 0.1 ml of *alizarin S solution R* and 0.1 ml of *zirconyl nitrate solution R*. Mix, allow to stand for 5 min and compare the colour of the solution with that of a blank prepared in the same manner. The test solution is yellow and the blank is red.
- F. To about 40 mg add 2 ml of *sulphuric acid R* and heat gently until white fumes are evolved. Add *nitric acid R* dropwise, continue the heating until the solution is almost colourless and cool. Add 2 ml of *water R*, heat until white fumes are again evolved, cool, add 10 ml of *water R* and neutralise to *red litmus paper R* with *dilute ammonia R1*. The solution gives reaction (a) of sodium (2.3.1) and reaction (b) of phosphates (2.3.1).

TESTS

Solution S. Dissolve 1.0 g in *carbon dioxide-free water R* and dilute to 20 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and not more intensely coloured than reference solution B₇ (2.2.2, Method II).

pH (2.2.3). Dilute 1 ml of solution S to 5 ml with *carbon dioxide-free water R*. The pH of the solution is 7.5 to 9.0.

Specific optical rotation (2.2.7). Dissolve 0.250 g in *water R* and dilute to 25.0 ml with the same solvent. The specific optical rotation is + 98 to + 104, calculated with reference to the anhydrous substance.

Related substances. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 62.5 mg of the substance to be examined in the mobile phase and dilute to 25.0 ml with the mobile phase.

Reference solution (a). Dissolve 25 mg of *betamethasone sodium phosphate CRS* and 25 mg of *dexamethasone sodium phosphate CRS* in the mobile phase and dilute to 25.0 ml with the mobile phase. Dilute 1.0 ml of this solution to 25.0 ml with the mobile phase.

Reference solution (b). Dilute 1.0 ml of the test solution to 50.0 ml with the mobile phase.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4.6 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (5 µm),
- as mobile phase at a flow rate of 1 ml/min a mixture prepared as follows: in a 250 ml conical flask, weigh 1.360 g of *potassium dihydrogen phosphate R* and 0.600 g of *hexylamine R*, mix and allow to stand for 10 min and then dissolve in 185 ml of *water R*; add 65 ml of *acetonitrile R*, mix and filter (0.45 µm),
- as detector a spectrophotometer set at 254 nm.

Equilibrate the column with the mobile phase at a flow rate of 1 ml/min for about 45 min.

Adjust the sensitivity of the system so that the height of the principal peak in the chromatogram obtained with reference solution (b) is 70 per cent to 90 per cent of the full scale of the recorder.

Inject 20 µl of reference solution (a). When the chromatograms are recorded in the conditions described above, the retention times are: betamethasone sodium phosphate about 14 min; dexamethasone sodium phosphate about 15.5 min. The test is not valid unless the resolution between the peaks corresponding to betamethasone sodium phosphate and dexamethasone sodium phosphate is at least 2.0; if necessary, increase the concentration of acetonitrile or increase the concentration of water in the mobile phase.

Inject 20 µl of the test solution and 20 µl of reference solution (b). Continue the chromatography for twice the retention time of the principal peak. In the chromatogram obtained with the test solution: the area of any peak, apart from the principal peak, is not greater than the area of the principal peak in the chromatogram obtained with reference solution (b) (2 per cent) and not more than one such peak has an area greater than half the area of the principal peak in the chromatogram obtained with reference solution (b) (1 per cent); the sum of the areas of all the peaks, apart from the principal peak, is not greater than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (3 per cent). Disregard any peak with an area less than 0.025 times the area of the principal peak in the chromatogram obtained with reference solution (b).

Inorganic phosphate. Dissolve 50 mg in *water R* and dilute to 100 ml with the same solvent. To 10 ml of this solution add 5 ml of *molybdovanadic reagent R*, mix and allow to stand for 5 min. Any yellow colour in the solution is not more intense than that in a standard prepared at the same time and in the same manner using 10 ml of *phosphate standard solution (5 ppm PO₄) R* (1 per cent).

Water (2.5.12). Not more than 8.0 per cent, determined on 0.200 g by the semi-micro determination of water.

ASSAY

Dissolve 0.100 g in *water R* and dilute to 100.0 ml with the same solvent. Dilute 5.0 ml of the solution to 250.0 ml with *water R*. Measure the absorbance (2.2.25) at the maximum at 241 nm.

Calculate the content of C₂₂H₂₈FNa₂O₈P taking the specific absorbance to be 297.

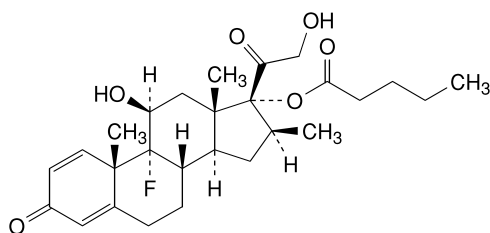
STORAGE

Store in an airtight container, protected from light.

01/2005:0811

BETAMETHASONE VALERATE

Betamethasoni valeras

 $C_{27}H_{37}FO_6$ M_r 476.6**DEFINITION**

Betamethasone valerate contains not less than 97.0 per cent and not more than the equivalent of 103.0 per cent of 9-fluoro-11 β ,21-dihydroxy-16 β -methyl-3,20-dioxopregna-1,4-dien-17-yl pentanoate, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, crystalline powder, practically insoluble in water, freely soluble in acetone and in methylene chloride, soluble in alcohol.

It melts at about 192 °C, with decomposition.

IDENTIFICATION

First identification: C, D.

Second identification: A, B, E, F, G.

- A. It complies with the test for specific optical rotation (see Tests).
- B. Dissolve 10.0 mg in *ethanol R* and dilute to 100.0 ml with the same solvent. Place 2.0 ml of this solution in a ground-glass-stoppered tube, add 10.0 ml of *phenylhydrazine-sulphuric acid solution R*, mix and heat in a water-bath at 60 °C for 20 min. Cool immediately. The absorbance (2.2.25) of the solution measured at 419 nm is not more than 0.10.
- C. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *betamethasone 17-valerate CRS*. If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in the minimum volume of *chloroform R*, evaporate to dryness on a water-bath and record new spectra using the residues.
- D. Examine by thin-layer chromatography (2.2.27), using as the coating substance a suitable silica gel with a fluorescent indicator having an optimal intensity at 254 nm.

Test solution. Dissolve 10 mg of the substance to be examined in a mixture of 1 volume of *methanol R* and 9 volumes of *methylene chloride R* and dilute to 10 ml with the same mixture of solvents.

Reference solution (a). Dissolve 10 mg of *betamethasone 17-valerate CRS* in a mixture of 1 volume of *methanol R* and 9 volumes of *methylene chloride R* and dilute to 10 ml with the same mixture of solvents.

Reference solution (b). Dissolve 10 mg of *betamethasone 21-valerate CRS* in a mixture of 1 volume of *methanol R* and 9 volumes of *methylene chloride R* and dilute to 10 ml with the same mixture of solvents. Dilute 5 ml of this solution to 10 ml with reference solution (a).

Apply to the plate 5 μ l of each solution. Prepare the mobile phase by adding a mixture of 1.2 volumes of *water R* and 8 volumes of *methanol R* to a mixture of 15 volumes of *ether R* and 77 volumes of *methylene chloride R*. Develop over a path of 15 cm. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. The principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a). Spray the plate with *alcoholic solution of sulphuric acid R*. Heat at 120 °C for 10 min or until the spots appear. Allow to cool. Examine in daylight and in ultraviolet light at 365 nm. The principal spot in the chromatogram obtained with the test solution is similar in position, colour in daylight, fluorescence in ultraviolet light at 365 nm and size to the principal spot in the chromatogram obtained with reference solution (a). The test is not valid unless the chromatogram obtained with reference solution (b) shows two clearly separated spots.

- E. Examine by thin-layer chromatography (2.2.27), using as the coating substance a suitable silica gel with a fluorescent indicator having an optimal intensity at 254 nm.

Test solution (a). Dissolve 25 mg of the substance to be examined in *methanol R* with gentle heating and dilute to 5 ml with the same solvent. This solution is also used to prepare test solution (b). Dilute 2 ml of the solution to 10 ml with *methylene chloride R*.

Test solution (b). Transfer 2 ml of the solution obtained during preparation of test solution (a) to a 15 ml glass tube with a ground-glass stopper or a polytetrafluoroethylene cap. Add 10 ml of *saturated methanolic potassium hydrogen carbonate solution R* and immediately pass a current of *nitrogen R* briskly through the solution for 5 min. Stopper the tube. Heat in a water-bath at 45 °C, protected from light, for 3 h. Allow to cool.

Reference solution (a). Dissolve 25 mg of *betamethasone 17-valerate CRS* in *methanol R* with gentle heating and dilute to 5 ml with the same solvent. This solution is also used to prepare reference solution (b). Dilute 2 ml of the solution to 10 ml with *methylene chloride R*.

Reference solution (b). Transfer 2 ml of the solution obtained during preparation of reference solution (a) to a 15 ml glass tube with a ground glass-stopper or a polytetrafluoroethylene cap. Add 10 ml of *saturated methanolic potassium hydrogen carbonate solution R* and immediately pass a current of *nitrogen R* briskly through the solution for 5 min. Stopper the tube. Heat in a water-bath at 45 °C, protected from light, for 3 h. Allow to cool.

Apply to the plate 5 μ l of each solution. Prepare the mobile phase by adding a mixture of 1.2 volumes of *water R* and 8 volumes of *methanol R* to a mixture of 15 volumes of *ether R* and 77 volumes of *methylene chloride R*. Develop over a path of 15 cm. Allow the plate to dry in air and examine under ultraviolet light at 254 nm. The principal spot in each of the chromatograms obtained with the test solutions is similar in position and size to the principal spot in the chromatogram obtained with the corresponding reference solution. Spray with *alcoholic solution of sulphuric acid R*. Heat at 120 °C for 10 min or until the spots appear. Allow to cool. Examine in daylight and in ultraviolet light at 365 nm. The principal spot in each of the chromatograms obtained with the test solutions is similar in position, colour in daylight, fluorescence in ultraviolet light at 365 nm and size to the principal spot in the chromatograms obtained with the corresponding reference solution. The principal

spot in each of the chromatograms obtained with test solution (b) and reference solution (b) has an R_f value distinctly lower than that of the principal spots in each of the chromatograms obtained with test solution (a) and reference solution (a).

- F. Add about 2 mg to 2 ml of *sulphuric acid R* and shake to dissolve. Within 5 min, a deep reddish-brown colour develops. Add the solution to 10 ml of *water R* and mix. The colour is discharged and a clear solution remains.
- G. Mix about 5 mg with 45 mg of *heavy magnesium oxide R* and ignite in a crucible until an almost white residue is obtained (usually less than 5 min). Allow to cool, add 1 ml of *water R*, 0.05 ml of *phenolphthalein solution R1* and about 1 ml of *dilute hydrochloric acid R* to render the solution colourless. Filter. Add 1.0 ml of the filtrate to a freshly prepared mixture of 0.1 ml of *alizarin S solution R* and 0.1 ml of *zirconyl nitrate solution R*. Mix, allow to stand for 5 min and compare the colour of the solution with that of a blank prepared in the same manner. The test solution is yellow and the blank is red.

TESTS

Specific optical rotation (2.2.7). Dissolve 0.250 g in *dioxan R* and dilute to 25.0 ml with the same solvent. The specific optical rotation is + 75 to + 82, calculated with reference to the dried substance.

Related substances. Examine by liquid chromatography (2.2.29).

Solution A. To 1000 ml of the mobile phase add 1 ml of *glacial acetic acid R* and mix carefully.

Test solution. Dissolve 62.5 mg of the substance to be examined in solution A and dilute to 25.0 ml with solution A.

Reference solution (a). Dissolve 2 mg of *betamethasone 17-valerate CRS* and 2 mg of *betamethasone 21-valerate CRS* in solution A and dilute to 50.0 ml with solution A.

Reference solution (b). Dilute 1.0 ml of the test solution to 50.0 ml with solution A.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4.6 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (5 µm),
- as mobile phase at a flow rate of 1 ml/min a mixture prepared as follows: mix 350 ml of *water R* with 600 ml of *acetonitrile R* and allow to equilibrate; adjust the volume to 1000 ml with *water R* and mix again,
- as detector a spectrophotometer set at 254 nm.

Equilibrate the column with the mobile phase for about 45 min.

Adjust the sensitivity so that the height of the principal peak in the chromatogram obtained with reference solution (b) is 70 per cent to 90 per cent of the full scale of the recorder.

Inject 20 µl of reference solution (a). When the chromatograms are recorded in the prescribed conditions, the retention times are: betamethasone 17-valerate, about 7 min; betamethasone 21-valerate, about 9 min. The test is not valid unless the resolution between the peaks corresponding to betamethasone 17-valerate and betamethasone 21-valerate is at least 5.0; if necessary, adjust the concentration of acetonitrile in the mobile phase.

Inject 20 µl of the test solution and 20 µl of reference solution (b). Continue the chromatography for 2.5 times the retention time of the principal peak. In the chromatogram obtained with the test solution: the area of any peak apart from the principal peak is not greater than 0.75 times the area of the principal peak in the chromatogram obtained with reference solution (b) (1.5 per cent) and not more than

one such peak has an area greater than half the area of the principal peak in the chromatogram obtained with reference solution (b) (1.0 per cent); the sum of the areas of all the peaks, apart from the principal peak, is not greater than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (3.0 per cent). Disregard any peak with an area less than 0.025 times the area of the principal peak in the chromatogram obtained with reference solution (b).

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 100–105 °C.

ASSAY

Dissolve 50.0 mg in *alcohol R* and dilute to 100.0 ml with the same solvent. Dilute 2.0 ml of the solution to 50.0 ml with *alcohol R*. Measure the absorbance (2.2.25) at the maximum at 240 nm.

Calculate the content of $C_{27}H_{37}FO_6$ taking the specific absorbance to be 325.

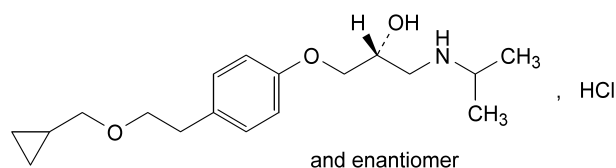
STORAGE

Store protected from light.

01/2005:1072

BETAXOLOL HYDROCHLORIDE

Betaxololi hydrochloridum



$C_{18}H_{30}ClNO_3$

M_r 343.9

DEFINITION

Betaxolol hydrochloride contains not less than 98.5 per cent and not more than the equivalent of 101.5 per cent of (2*RS*)-1-[4-[2-(cyclopropylmethoxy)ethyl]phenoxy]-3-[(1-methylethyl)amino]propan-2-ol hydrochloride, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, crystalline powder, very soluble in water, freely soluble in alcohol, soluble in methylene chloride.

IDENTIFICATION

First identification: B, D.

Second identification: A, C, D.

A. Melting point (2.2.14): 113 °C to 117 °C.

B. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *betaxolol hydrochloride CRS*.

C. Examine by thin-layer chromatography (2.2.27), using as the coating substance *octadecylsilyl silica gel for chromatography R* with a fluorescent indicator having an optimal intensity at 254 nm.

Test solution. Dissolve 10 mg of the substance to be examined in 1 ml of *methanol R*.

Reference solution (a). Dissolve 20 mg of *betaxolol hydrochloride CRS* in 2 ml of *methanol R*.

Reference solution (b). Dissolve 10 mg of *oxprenolol hydrochloride CRS* in 1 ml of reference solution (a).

Apply separately to the plate 2 µl of each solution. Develop over a path of 10 cm using a mixture of 0.5 volumes of *perchloric acid R*, 50 volumes of *methanol R* and 50 volumes of *water R*. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. The principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a). Spray the plate with a 50 g/l solution of *vanillin R* in a mixture of 5 volumes of *sulphuric acid R*, 10 volumes of *glacial acetic acid R* and 85 volumes of *methanol R*. Heat the plate at 100 °C to 105 °C until the colour of the spots reaches maximum intensity (10 min to 15 min). Examine in daylight. The principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a). The test is not valid unless the chromatogram obtained with reference solution (b) shows two clearly separated spots.

D. It gives reaction (a) of chlorides (2.3.1).

TESTS

Appearance of solution. Dissolve 0.5 g in *water R* and dilute to 25 ml with the same solvent. The solution is clear (2.2.1) and colourless (2.2.2, *Method II*).

Acidity or alkalinity. Dissolve 0.20 g in *carbon dioxide-free water R* and dilute to 20 ml with the same solvent. Add 0.2 ml of *methyl red solution R* and 0.2 ml of 0.01 M *hydrochloric acid*. The solution is red. Add 0.4 ml of 0.01 M *sodium hydroxide*. The solution is yellow.

Related substances. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 10.0 mg of the substance to be examined in the mobile phase and dilute to 5.0 ml with the mobile phase.

Reference solution (a). Dissolve 8 mg of the substance to be examined and 4 mg of *betaxolol impurity A CRS* in 20.0 ml of the mobile phase.

Reference solution (b). Dilute 1.0 ml of the test solution to 100.0 ml with the mobile phase.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4 mm in internal diameter packed with *octylsilyl silica gel for chromatography R* (5 µm),
- as the mobile phase at a flow rate of 1.5 ml/min a mixture prepared as follows: mix 175 ml of *acetonitrile R* with 175 ml of *methanol R* and dilute the mixture to 1 litre with a 3.4 g/l solution of *potassium dihydrogen phosphate R*, previously adjusted to pH 3.0 with *phosphoric acid R*,
- as the detector a spectrophotometer set at 273 nm,
- a loop injector.

Inject 20 µl of each solution. Continue the chromatography for at least four times the retention time of the principal peak in the chromatogram obtained with the test solution. In the chromatogram obtained with the test solution: the area of any peak apart from the principal peak is not greater than 0.3 times the area of the peak in the chromatogram obtained with reference solution (b) (0.3 per cent) and the sum of the areas of such peaks is not greater than the area of the peak in the chromatogram obtained with reference solution (b) (1 per cent). The test is not valid unless the resolution between the peaks due to *betaxolol impurity A* and *betaxolol* in the chromatogram obtained with reference solution (a) is at least 2.0. Disregard any peak with an area less than 0.025 times that of the peak in the chromatogram obtained with reference solution (b).

Heavy metals (2.4.8). Dissolve 2.0 g in 20 ml of *water R*. 12 ml of the solution complies with limit test A for heavy metals (10 ppm). Prepare the standard using 10 ml of *lead standard solution* (1 ppm Pb) *R*.

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

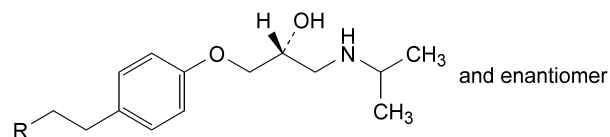
Dissolve 0.300 g in a mixture of 10.0 ml of 0.01 M *hydrochloric acid* and 50 ml of *alcohol R*. Carry out a potentiometric titration (2.2.20), using 0.1 M *sodium hydroxide*. Read the volume added between the two points of inflexion.

1 ml of 0.1 M *sodium hydroxide* is equivalent to 34.39 mg of C₁₈H₃₀ClNO₃.

STORAGE

Store protected from light.

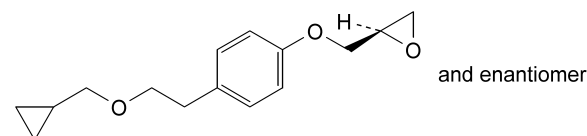
IMPURITIES



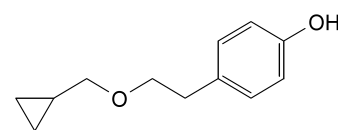
A. R = H: (2RS)-1-[4-(2-ethoxyethyl)phenoxy]-3-[(1-methylethyl)amino]propan-2-ol,

B. R = OH: (2RS)-1-[4-(2-hydroxyethyl)phenoxy]-3-[(1-methylethyl)amino]propan-2-ol,

E. R = O-CH₂-CH₂-CH₂-CH₃: (2RS)-1-[4-(2-butoxyethyl)phenoxy]-3-[(1-methylethyl)amino]propan-2-ol,



C. 2-[4-[2-(cyclopropylmethoxy)ethyl]phenoxy]methyl]oxirane,

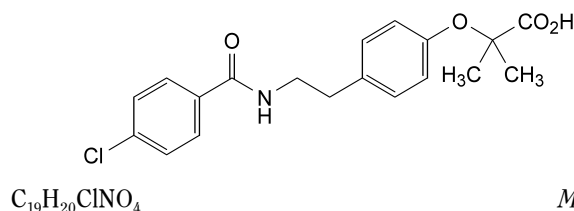


D. 4-[2-(cyclopropylmethoxy)ethyl]phenol.

01/2005:1394

BEZAFIBRATE

Bezafibratum



M_r 361.8

DEFINITION

Bezafibrate contains not less than 98.0 per cent and not more than the equivalent of 102.0 per cent of 2-[4-[2-[(4-chlorobenzoyl)amino]ethyl]phenoxy]-2-methylpropanoic acid, calculated with reference to the dried substance.

CHARACTERS

A white or almost white crystalline powder, practically insoluble in water, freely soluble in dimethylformamide, sparingly soluble in acetone and in alcohol. It dissolves in dilute solutions of alkali hydroxides.

It shows polymorphism.

IDENTIFICATION

First identification: A, B.

Second identification: A, C.

A. Melting point (2.2.14): 181 °C to 185 °C.

B. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *bezafibrate* CRS. Examine the substances prepared as discs. If the spectra obtained show differences, dissolve the substance to be examined and the reference substance separately in *methanol R* and evaporate to dryness. Dry the residues *in vacuo* at 80 °C for 1 h and record new spectra using the residues.

C. Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel F₂₅₄ plate R*.

Test solution. Dissolve 10 mg of the substance to be examined in *methanol R* and dilute to 5 ml with the same solvent.

Reference solution. Dissolve 10 mg of *bezafibrate* CRS in *methanol R* and dilute to 5 ml with the same solvent.

Apply to the plate 5 µl of each solution. Develop over a path of 10 cm using a mixture of 2.7 volumes of *glacial acetic acid R*, 30 volumes of *methyl ethyl ketone R* and 60 volumes of *xylene R*. Dry the plate at 120 °C for at least 15 min and examine in ultraviolet light at 254 nm. The principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with the reference solution.

TESTS

Solution S. Dissolve 1.0 g in *dimethylformamide R* and dilute to 20 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and not more intensely coloured than reference solution BY₅ (2.2.2, *Method II*).

Related substances. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 50.0 mg of the substance to be examined in the mobile phase and dilute to 100.0 ml with the mobile phase.

Reference solution (a). Dilute 10.0 ml of the test solution to 100.0 ml with the mobile phase. Dilute 5.0 ml of this solution to 100.0 ml with the mobile phase.

Reference solution (b). Dilute 5.0 ml of reference solution (a) to 50.0 ml with the mobile phase.

Reference solution (c). To 1 ml of the test solution, add 1 ml of 0.1 M *hydrochloric acid* and evaporate to dryness on a hot plate. Dissolve the residue in 20 ml of the mobile phase. The chromatographic procedure may be carried out using:

- a stainless steel column 0.125 m long and 4 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (5 µm),

- as mobile phase at a flow rate of 1 ml/min a mixture of 40 volumes of a 2.72 g/l solution of *potassium dihydrogen phosphate R* adjusted to pH 2.3 with *phosphoric acid R* and 60 volumes of *methanol R*,
- as detector a spectrophotometer set at 228 nm.

Inject separately 20 µl of the test solution and 20 µl of reference solutions (a), (b) and (c). When the chromatogram is recorded in the prescribed conditions, the retention times are: impurity A about 3 min, impurity B about 3.5 min, bezafibrate about 6.0 min, impurity C about 9 min, impurity D about 14 min and impurity E about 37 min. Continue the chromatography for the time necessary to detect the ester, which, depending on the route of synthesis, may be impurity C, D or E. The test is not valid unless: in the chromatogram obtained with reference solution (c) the resolution between the two principal peaks is at least 5.0 and the principal peak in the chromatogram obtained with reference solution (b) has a signal-to-noise ratio of at least 5. In the chromatogram obtained with the test solution: the area of any peak, apart from the principal peak, is not greater than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent); the sum of the areas of all the peaks, apart from the principal peak, is not greater than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.75 per cent). Disregard any peak with an area less than 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (a).

Chlorides (2.4.4). Dilute 10 ml of solution S to 50 ml with *water R*. Filter the resultant suspension through a wet filter previously washed with *water R* until free from chlorides. 15 ml of the filtrate complies with the limit test for chlorides (300 ppm). Prepare the standard using 9 ml of *chloride standard solution (5 ppm Cl) R* and 6 ml of *water R*.

Heavy metals (2.4.8). 2.0 g complies with limit test C for heavy metals (10 ppm). Prepare the standard using 2 ml of *lead standard solution (10 ppm Pb) R*.

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 100-105 °C.

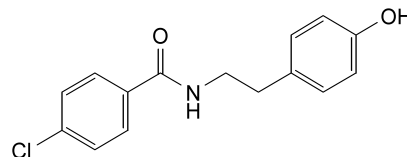
Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

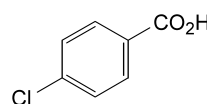
Dissolve 0.300 g in 50 ml of a mixture of 25 volumes of *water R* and 75 volumes of *alcohol R*. Using 0.1 ml of *phenolphthalein solution R* as indicator, titrate with 0.1 M *sodium hydroxide* until a pink colour is obtained. Carry out a blank titration.

1 ml of 0.1 M *sodium hydroxide* is equivalent to 36.18 mg of C₁₉H₂₀ClNO₄.

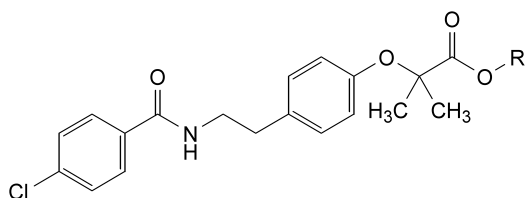
IMPURITIES



A. 4-chloro-*N*-[2-(4-hydroxyphenyl)ethyl]benzamide (chlorobenzoyltyramine),



B. 4-chlorobenzoic acid,

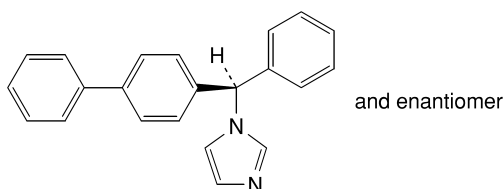


- C. R = CH₃: methyl 2-[4-[2-[(4-chlorobenzoyl)amino]ethyl]phenoxy]-2-methylpropanoate,
- D. R = CH₂-CH₃: ethyl 2-[4-[2-[(4-chlorobenzoyl)amino]ethyl]phenoxy]-2-methylpropanoate,
- E. R = CH₂-CH₂-CH₂-CH₃: butyl 2-[4-[2-[(4-chlorobenzoyl)amino]ethyl]phenoxy]-2-methylpropanoate.

01/2005:1395

BIFONAZOLE

Bifonazolum

C₂₂H₁₈N₂M_r 310.4**DEFINITION**

Bifonazole contains not less than 98.0 per cent and not more than the equivalent of 100.5 per cent of 1-[(*RS*)-(biphenyl-4-yl)phenylmethyl]-1*H*-imidazole, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, crystalline powder, practically insoluble in water, sparingly soluble in ethanol.

It shows polymorphism.

IDENTIFICATION

Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *bifonazole CRS*. If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in the minimum volume of 2-propanol *R*, evaporate to dryness and record new spectra using the residues.

TESTS

Optical rotation (2.2.7). Dissolve 0.20 g in 20.0 ml of *methanol R*. The angle of optical rotation is -0.10° to $+0.10^\circ$.

Related substances. Examine by liquid chromatography (2.2.29).

Buffer solution pH 3.2. Mix 2.0 ml of *phosphoric acid R* with *water R* and dilute to 1000.0 ml with the same solvent. Adjust to pH 3.2 (2.2.3) with *triethylamine R*.

Test solution. Dissolve 50.0 mg of the substance to be examined in 25 ml of *acetonitrile R* and dilute to 50.0 ml with buffer solution pH 3.2.

Reference solution (a). Dilute 0.25 ml of the test solution to 50.0 ml with buffer solution pH 3.2.

Reference solution (b). Dissolve 25.0 mg of *imidazole R* (impurity C) in *acetonitrile R* and dilute to 25.0 ml with the same solvent. Dilute 0.25 ml of the solution to 100.0 ml with buffer solution pH 3.2.

Reference solution (c). Dissolve 34.2 mg of 4-[(*RS*)-(biphenyl-4-yl)phenylmethyl]-1*H*-imidazole trifluoroacetate *CRS* (corresponding to 25.0 mg of impurity B base) in *acetonitrile R* and dilute to 25.0 ml with the same solvent.

Reference solution (d). Dilute 0.25 ml of reference solution (c) to 50.0 ml with buffer solution pH 3.2.

Reference solution (e). Mix 0.25 ml of the test solution and 0.25 ml of reference solution (c) and dilute to 50.0 ml with buffer solution pH 3.2.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.125 m long and 4.6 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (5 µm),
- as mobile phase at a flow rate of 1 ml/min a gradient programme using the following conditions:

Mobile phase A. A mixture of 20 volumes of *acetonitrile R* and 80 volumes of buffer solution pH 3.2,

Mobile phase B. A mixture of 20 volumes of buffer solution pH 3.2 and 80 volumes of *acetonitrile R*,

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)	Comment
0 - 8	60	40	isocratic elution
8 - 12	60 → 10	40 → 90	linear gradient
12 - 30	10	90	isocratic elution
30 - 32	10 → 60	90 → 40	switch to initial eluent composition
32 - 40	60	40	equilibration
40 = 0	60	40	restart isocratic elution

- as detector a spectrophotometer set at 210 nm, maintaining the column temperature at 40 °C.

Adjust the sensitivity of the system so that the height of the bifonazole peak in the chromatogram obtained with 50 µl of reference solution (e) is at least 50 per cent of the full scale of the recorder.

Inject 50 µl of reference solution (e). When the chromatogram is recorded in the prescribed conditions the retention times are: impurity B about 4 min and bifonazole about 4.5 min. The test is not valid unless the resolution between the peaks corresponding to impurity B and bifonazole is at least 2.5.

Inject 50 µl of the test solution and 50 µl each of reference solutions (a), (b) and (d). In the chromatogram obtained with the test solution: the area of any peak corresponding to impurity C is not greater than the corresponding peak in the chromatogram obtained with reference solution (b) (0.25 per cent); the area of any peak corresponding to impurity B is not greater than 3 times the area of the corresponding peak in the chromatogram obtained with reference solution (d) (1.5 per cent); none of the peaks, apart from the principal peak and the peaks corresponding to impurities B and C, has an area greater than the area of the peak in the chromatogram obtained with reference solution (a) (0.5 per cent); the sum of the areas of all the peaks, apart from the principal peak, is not greater than 4 times the area of the principal peak in the chromatogram obtained with reference solution (a) (2 per cent). Disregard any peak whose area is less than 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (a).

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 100-105 °C.

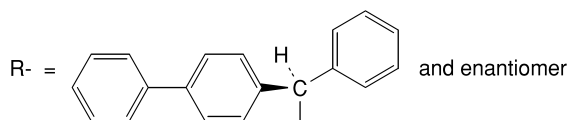
Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

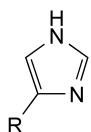
Dissolve 0.250 g in 80 ml of *anhydrous acetic acid R*. Titrate with 0.1 M *perchloric acid*, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *perchloric acid* is equivalent to 31.04 mg of $C_{22}H_{18}N_2$.

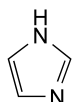
IMPURITIES



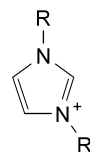
A. R-OH: (RS)-(biphenyl-4-yl)phenylmethanol,



B. 4-[(RS)-(biphenyl-4-yl)phenylmethyl]-1H-imidazole,



C. 1H-imidazole,



D. 1,3-bis[(biphenyl-4-yl)phenylmethyl]-1H-imidazolium ion.

01/2005:1588

BILBERRY FRUIT, DRIED

Myrtilli fructus siccus

DEFINITION

Dried ripe fruit of *Vaccinium myrtillus* L.

Content: minimum 1.0 per cent of tannins, expressed as pyrogallol ($C_6H_6O_3$; M_r 126.1) (dried drug).

CHARACTERS

Dried bilberry has a sweet and slightly astringent taste. Macroscopic and microscopic characters described under identification tests A and B.

IDENTIFICATION

- Dried bilberry is a dark blue, subglobular, shrunken berry about 5 mm in diameter, with a scar at the lower end and surmounted by the persistent calyx, which appears as a circular fold and the remains of the style. The deep violet, fleshy mesocarp contains numerous small, brown, ovoid seeds.
- Reduce to a powder (355). The powder is violet-brown. Examine under a microscope using *chloral hydrate solution R*. The powder shows: violet-pink sclereids from the endocarp and the mesocarp, usually aggregated, with thick, channelled walls; reddish-brown fragments of the

epicarp consisting of polygonal cells with moderately thickened walls; brownish-yellow fragments of the outer seed testa made up of elongated cells with U-shaped thickened walls; clusters and prisms crystals of various size of calcium oxalate.

C. Thin-layer chromatography (2.2.27)

Test solution. To 2 g of the powdered drug (355) add 20 ml of *methanol R*. Shake for 15 min and filter.

Reference solution. Dissolve 5 mg of *chrysanthemin R* in 10 ml of *methanol R*.

Plate: TLC silica gel plate *R*.

Mobile phase: *anhydrous formic acid R*, *water R*, *butanol R* (16:19:65 V/V/V).

Application: 10 µl, as bands.

Development: over a path of 10 cm.

Drying: in air.

Detection: examine in daylight.

Results: see below the sequence of the zones present in the chromatograms obtained with the reference and test solutions.

Top of the plate	
Chrysanthemin: a violet-red zone	A violet-red zone of low intensity
	A principal violet-red zone
	A compact set of other principal zones:
	– a violet-red zone
	– several violet-blue zones
Reference solution	Test solution

TESTS

Foreign matter (2.8.2): it complies with the test for foreign matter.

Loss on drying (2.2.32): maximum 12.0 per cent, determined on 1.000 g of the powdered drug by drying in an oven at 100-105 °C for 2 h.

Total ash (2.4.16): maximum 5.0 per cent.

ASSAY

Carry out the determination of tannins in herbal drugs (2.8.14). Use 1.500 g of the powdered drug (355).

01/2005:1602

BILBERRY FRUIT, FRESH

Myrtilli fructus recens

DEFINITION

Fresh or frozen ripe fruit of *Vaccinium myrtillus* L.

Content: minimum 0.30 per cent of anthocyanins, expressed as cyanidin-3-glucoside chloride (chrysanthemin, $C_{21}H_{21}ClO_{11}$; M_r 485.5) (dried drug).

CHARACTERS

Sweet and slightly astringent taste.

Macroscopic and microscopic characters described under identification tests A and B.

IDENTIFICATION

- The fresh fruit is a blackish-blue globular berry about 5 mm in diameter. Its lower end shows a scar or, rarely, a fragment of the pedicel. The upper end is flattened and surmounted by the remains of the persistent style

and of the calyx, which appears as a circular fold. The violet, fleshy mesocarp includes 4 to 5 locules containing numerous small, brown, ovoid seeds.

- B. The crushed fresh fruit is violet-red. Examine under a microscope using *chloral hydrate solution R*. It shows violet-pink sclereids from the endocarp and the mesocarp, usually aggregated, with thick, channelled walls; reddish-brown fragments of the epicarp consisting of polygonal cells with moderately thickened walls; brownish-yellow fragments of the outer layer of the testa composed of elongated cells with U-shaped thickened walls; cluster crystals of calcium oxalate.

- C. Thin-layer chromatography (2.2.27).

Test solution. To 5 g of the freshly crushed drug, add 20 ml of *methanol R*. Stir for 15 min and filter.

Reference solution. Dissolve 5 mg of *chrysanthemin R* in 10 ml of *methanol R*.

Plate: TLC silica gel plate *R*.

Mobile phase: *anhydrous formic acid R*, *water R*, *butanol R* (16:19:65 V/V/V).

Application: 10 µl, as bands.

Development: over a path of 10 cm.

Drying: in air.

Detection: examine in daylight.

Results: see below the sequence of the zones present in the chromatograms obtained with the reference solution and the test solution.

Top of the plate	
Chrysanthemin: a violet-red zone	A violet-red zone
	A principal violet-red zone
	A compact set of other principal zones:
	– a violet-red zone
	– several violet-blue zones
Reference solution	Test solution

TESTS

Total ash (2.4.16): maximum 0.6 per cent.

Foreign matter (2.8.2): it complies with the test for foreign matter.

Loss on drying (2.2.32): 80.0 per cent to 90.0 per cent, determined on 5.000 g of the freshly crushed drug by drying in an oven at 100-105 °C.

ASSAY

Crush 50 g extemporaneously. To about 5.00 g of the crushed, accurately weighed drug, add 95 ml of *methanol R*. Stir mechanically for 30 min. Filter into a 100.0 ml volumetric flask. Rinse the filter and dilute to 100.0 ml with *methanol R*. Prepare a 50-fold dilution of this solution in a 0.1 per cent V/V solution of *hydrochloric acid R* in *methanol R*.

Measure the absorbance (2.2.25) of the solution at 528 nm, using a 0.1 per cent V/V solution of *hydrochloric acid R* in *methanol R* as the compensation liquid.

Calculate the percentage content of anthocyanins, expressed as cyanidin-3-glucoside chloride, from the expression:

$$\frac{A \times 5000}{718 \times m}$$

718 = specific absorbance of cyanidin-3-glucoside chloride at 528 nm,

A = absorbance at 528 nm,

m = mass of the substance to be examined in grams.

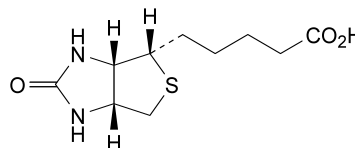
STORAGE

When frozen, store at or below –18 °C.

01/2005:1073

BIOTIN

Biotinum



C₁₀H₁₆N₂O₃S

M_r 244.3

DEFINITION

Biotin contains not less than 98.5 per cent and not more than the equivalent of 101.0 per cent of 5-[(3a*S*,4*S*,6a*R*)-2-oxohexahydrothieno[3,4-*d*]imidazol-4-yl]pentanoic acid, calculated with reference to the dried substance.

CHARACTERS

A white, crystalline powder or colourless crystals, very slightly soluble in water and in alcohol, practically insoluble in acetone. It dissolves in dilute solutions of alkali hydroxides.

IDENTIFICATION

First identification: A.

Second identification: B, C.

- A. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *biotin CRS*.
- B. Examine the chromatograms obtained in the test for related substances (see Tests). The principal spot in the chromatogram obtained with test solution (b) is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a).
- C. Dissolve about 10 mg in 20 ml of *water R* with heating. Allow to cool. Add 0.1 ml of *bromine water R*. The bromine water is decolourised.

TESTS

Solution S. Dissolve 0.250 g in a 4 g/l solution of *sodium hydroxide R* and dilute to 25.0 ml with the same alkaline solution.

Appearance of solution. Solution S is clear (2.2.1) and colourless (2.2.2, *Method II*).

Specific optical rotation (2.2.7). The specific optical rotation is + 89 to + 93, determined on solution S and calculated with reference to the dried substance.

Related substances. Examine by thin-layer chromatography (2.2.27), using as the coating substance a suitable silica gel (5 µm). Prepare the solutions immediately before use and keep protected from bright light.

Test solution (a). Dissolve 50 mg of the substance to be examined in *glacial acetic acid R* and dilute to 10 ml with the same solvent.

Test solution (b). Dilute 1 ml of test solution (a) to 10 ml with *glacial acetic acid R*.

Reference solution (a). Dissolve 5 mg of *biotin CRS* in *glacial acetic acid R* and dilute to 10 ml with the same solvent.

Reference solution (b). Dilute 1 ml of test solution (b) to 20 ml with *glacial acetic acid R*.

Reference solution (c). Dilute 1 ml of test solution (b) to 40 ml with *glacial acetic acid R*.

Apply to the plate 10 µl of each solution. Develop over a path of 15 cm using a mixture of 5 volumes of *methanol R*, 25 volumes of *glacial acetic acid R* and 75 volumes of *toluene R*. Dry the plate in a current of warm air. Allow to cool and spray with *4-dimethylaminocinnamaldehyde solution R*. Examine immediately in daylight. Any spot in the chromatogram obtained with test solution (a), apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.5 per cent) and at most one such spot is more intense than the spot in the chromatogram obtained with reference solution (c) (0.25 per cent).

Heavy metals (2.4.8). 1.0 g complies with limit test C for heavy metals (10 ppm). Prepare the standard using 10 ml of *lead standard solution (1 ppm Pb) R*.

Loss on drying (2.2.32). Not more than 1.0 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

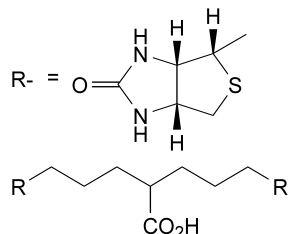
Suspend 0.200 g in 5 ml of *dimethylformamide R*. Heat until the substance has dissolved completely. Add 50 ml of *ethanol R* and titrate with 0.1 M *tetrabutylammonium hydroxide*, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *tetrabutylammonium hydroxide* is equivalent to 24.43 mg of C₁₀H₁₆N₂O₃S.

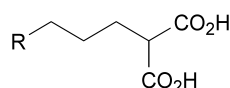
STORAGE

Store protected from light.

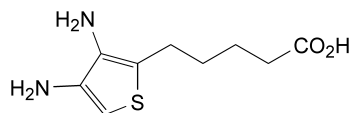
IMPURITIES



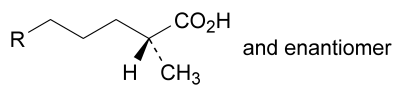
A. di[3-[(3aS,4S,6aR)-2-oxohexahydrothieno[3,4-d]imidazol-4-yl]propyl]acetic acid,



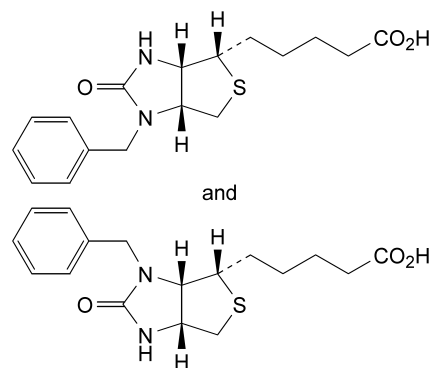
B. 4-[(3aS,4S,6aR)-2-oxohexahydrothieno[3,4-d]imidazol-4-yl]butane-1,1-dicarboxylic acid,



C. 5-(3,4-diamino-2-thienyl)pentanoic acid,



D. 2-methyl-5-[(3aS,4S,6aR)-2-oxohexahydrothieno[3,4-d]imidazol-4-yl]pentanoic acid,

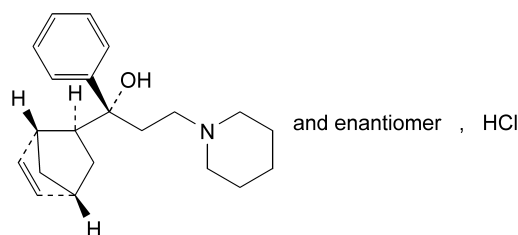


E. 5-[(3aS,4S,6aR)-3-benzyl-2-oxohexahydrothieno[3,4-d]imidazol-4-yl]pentanoic acid and 5-[(3aS,4S,6aR)-1-benzyl-2-oxohexahydrothieno[3,4-d]imidazol-4-yl]pentanoic acid.

01/2005:1074

BIPERIDEN HYDROCHLORIDE

Biperideni hydrochloridum



C₂₁H₃₀ClNO

M_r 347.9

DEFINITION

(1*RS*)-1-[(1*RS*,2*SR*,4*RS*)-Bicyclo[2.2.1]hept-5-en-2-yl]-1-phenyl-3-(piperidin-1-yl)propan-1-ol hydrochloride.

Content: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

Appearance: white, crystalline powder.

Solubility: slightly soluble in water and in alcohol, very slightly soluble in methylene chloride.

mp: about 280 °C, with decomposition.

IDENTIFICATION

First identification: A, D.

Second identification: B, C, D.

A. Infrared absorption spectrophotometry (2.2.24).

Comparison: *biperiden hydrochloride CRS*.

B. Thin-layer chromatography (2.2.27).

Test solution. Dissolve 25 mg of the substance to be examined in *methanol R* and dilute to 5 ml with the same solvent.

Reference solution (a). Dissolve 25 mg of *biperiden hydrochloride CRS* in *methanol R* and dilute to 5 ml with the same solvent.

Reference solution (b). Dissolve 5 mg of *biperiden impurity A CRS* in reference solution (a) and dilute to 2 ml with the same solution.

Plate: TLC silica gel F_{254} plate *R*.

Mobile phase: *diethylamine R*, *methanol R*, *toluene R* (1:1:20 *V/V/V*).

Application: 5 µl.

Development: over a path of 15 cm.

Drying: in air.

Detection A: examine in ultraviolet light at 254 nm.

Results A: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a).

Detection B: spray with *dilute potassium iodobismuthate solution R* and then with *sodium nitrite solution R* and examine in daylight.

Results B: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).

System suitability: reference solution (b):

- the chromatogram shows 2 clearly separated spots.

C. To about 20 mg add 5 ml of *phosphoric acid R*. A green colour develops.

D. It gives reaction (a) of chlorides (2.3.1).

TESTS

Solution S. Dissolve 0.10 g in *carbon dioxide-free water R*, heating gently if necessary, and dilute to 50 ml with the same solvent.

Appearance of solution. Solution S is not more opalescent than reference suspension II (2.2.1) and is colourless (2.2.2, *Method II*).

pH (2.2.3): 5.0 to 6.5 for solution S.

Related substances. Gas chromatography (2.2.28).

Test solution. Dissolve 0.10 g of the substance to be examined in *methanol R* and dilute to 10 ml with the same solvent.

Reference solution (a). Dilute 0.5 ml of the test solution to 100 ml with *methanol R*. Dilute 10 ml of this solution to 50 ml with *methanol R*.

Reference solution (b). Dissolve 5 mg of the substance to be examined and 5 mg of *biperiden impurity A CRS* in *methanol R* and dilute to 5 ml with the same solvent. Dilute 1 ml of the solution to 10 ml with *methanol R*.

Column:

- **material:** fused silica,
- **size:** $l = 50$ m, $\varnothing = 0.25$ mm,
- **stationary phase:** *poly(dimethyl)(diphenyl)(divinyl)siloxane R* (film thickness 0.25 µm).

Carrier gas: *nitrogen for chromatography R*.

Flow rate: 0.4 ml/min.

Split ratio: 1:250.

Temperature:

	Time (min)	Temperature (°C)
Column	0 - 5	200
	5 - 40	200 → 270
Injection port		250
Detector		300

Detection: flame ionisation.

Injection: 2 µl.

Run time: twice the retention time of biperiden.

Relative retention with reference to biperiden: impurities A, B and C = between 0.95 and 1.05.

System suitability:

- **resolution:** minimum 2.5 between the peak due to biperiden (1st peak) and the peak due to impurity A (2nd peak) in the chromatogram obtained with reference solution (b),
- **signal-to-noise ratio:** minimum 6 for the principal peak in the chromatogram obtained with reference solution (a).

Limits:

- **impurities A, B, C:** for each impurity, maximum 0.50 per cent of the area of the principal peak,
- **any other impurity:** for each impurity, maximum 0.10 per cent of the area of the principal peak,
- **total of impurities A, B and C:** maximum 1.0 per cent of the area of the principal peak,
- **total of impurities other than A, B and C:** maximum 0.50 per cent of the area of the principal peak,
- **disregard limit:** 0.05 per cent of the area of the principal peak.

Impurity F (2.4.24): maximum 2 ppm.

Heavy metals (2.4.8): maximum 20 ppm.

1.0 g complies with limit test D. Prepare the standard using 2 ml of *lead standard solution (10 ppm Pb) R*.

Loss on drying (2.2.32): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 100-105 °C for 2 h.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.200 g in 60 ml of *alcohol R*. In a closed vessel, titrate with 0.1 M *alcoholic potassium hydroxide*, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *alcoholic potassium hydroxide* is equivalent to 34.79 mg of $C_{21}H_{30}ClNO$.

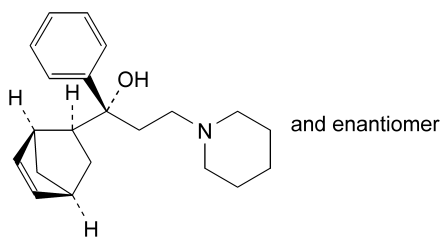
STORAGE

In an airtight container, protected from light.

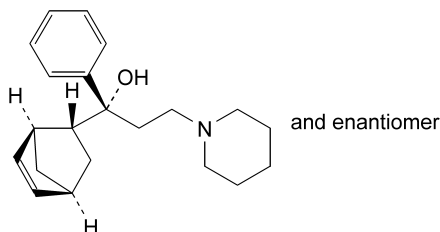
IMPURITIES

Specified impurities: A, B, C, F.

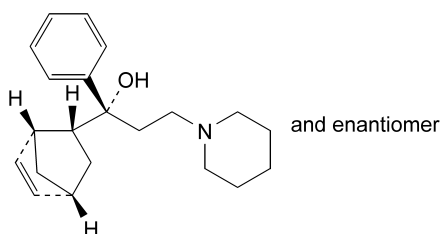
Other detectable impurities: D, E.



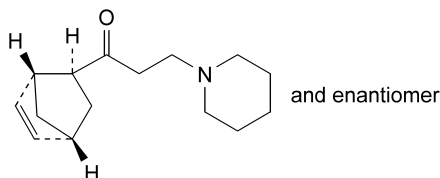
- A. (1*RS*)-1-[(1*SR*,2*SR*,4*SR*)-bicyclo[2.2.1]hept-5-en-2-yl]-1-phenyl-3-(piperidin-1-yl)propan-1-ol (*endo* form),



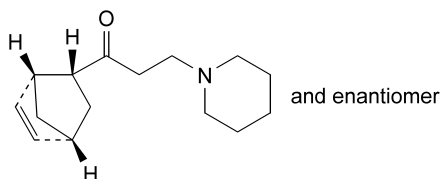
- B. (1*RS*)-1-[(1*SR*,2*RS*,4*SR*)-bicyclo[2.2.1]hept-5-en-2-yl]-1-phenyl-3-(piperidin-1-yl)propan-1-ol,



- C. (1*RS*)-1-[(1*RS*,2*RS*,4*RS*)-bicyclo[2.2.1]hept-5-en-2-yl]-1-phenyl-3-(piperidin-1-yl)propan-1-ol,



- D. 1-[(1*RS*,2*SR*,4*RS*)-bicyclo[2.2.1]hept-5-en-2-yl]-3-(piperidin-1-yl)propan-1-one,



- E. 1-[(1*RS*,2*RS*,4*RS*)-bicyclo[2.2.1]hept-5-en-2-yl]-3-(piperidin-1-yl)propan-1-one,

- F. benzene.

01/2005:1174

BIRCH LEAF

Betulae folium

DEFINITION

Birch leaf consists of the whole or fragmented dried leaves of *Betula pendula* Roth and/or *Betula pubescens* Ehrh. as well as hybrids of both species. It contains not less than 1.5 per cent of flavonoids, calculated as hyperoside ($C_{21}H_{20}O_{12}$; M_r 464.4) with reference to the dried drug.

CHARACTERS

It has the macroscopic and microscopic characters described under identification tests A and B.

IDENTIFICATION

- A. The leaves of both species are dark green on the adaxial surface and a lighter greenish-grey colour on the abaxial surface; they show a characteristic dense reticulate venation. The veins are light brown to almost white.

The leaves of *Betula pendula* are glabrous and show closely spaced glandular pits on both surfaces. The leaves of *Betula pendula* are 3 cm to 7 cm long and 2 cm to 5 cm wide; the petiole is long and the doubly dentate lamina is triangular to rhomboid and broadly cuneate or truncate at the base. The angle on each side is unrounded or slightly rounded, and the apex is long and acuminate.

The leaves of *Betula pubescens* show few glandular trichomes and are slightly pubescent on both surfaces. The abaxial surface shows small bundles of yellowish-grey trichomes at the branch points of the veins. The leaves of *Betula pubescens* are slightly smaller, oval to rhomboid and more rounded. They are more roughly and more regularly dentate. The apex is neither long nor acuminate.

- B. Reduce to a powder (355). The powder is greenish-grey. Examine under a microscope using *chloral hydrate solution R*. The powder shows numerous fragments of lamina with straight-walled epidermal cells and cells of the lower epidermis surrounding anomocytic stomata (2.8.3). Peltate large glands usually measuring 100 µm to 120 µm are found on the upper and lower epidermises. The mesophyll fragments contain calcium oxalate crystals. Fragments of radial vascular bundles and sclerenchyma fibres are accompanied by crystal sheaths. If *Betula pubescens* is present, the powder also contains unicellular covering trichomes with very thick walls, about 80 µm to 600 µm long, usually between 100 µm and 200 µm.
- C. Examine by thin-layer chromatography (2.2.27), using as the coating substance a suitable silica gel.

Test solution. To 1 g of the powdered drug (355) add 10 ml of *methanol R*. Heat in a water-bath at 60 °C for 5 min. Cool and filter the solution.

Reference solution. Dissolve 1 mg of *caffeic acid R* and 1 mg of *chlorogenic acid R*, 2.5 mg of *hyperoside R* and 2.5 mg of *rutin R* in 10 ml of *methanol R*.

Apply separately to the plates as bands, 10 µl of each solution. Develop over a path of 10 cm using a mixture of 10 volumes of *anhydrous formic acid R*, 10 volumes of *water R*, 30 volumes of *methyl ethyl ketone R* and 50 volumes of *ethyl acetate R*. Dry the plate in a current of warm air. Spray with a 10 g/l solution of *diphenylboric acid aminoethyl ester R* in *methanol R*. Subsequently spray the plate with a 50 g/l solution of *macrogol 400 R* in *methanol R*. Allow the plate to dry in air for 30 min and examine in ultraviolet light at 365 nm. The chromatogram obtained with the reference solution shows three zones in its lower half: in increasing order of R_f a yellowish-brown fluorescent zone (rutin), a light blue fluorescent zone (chlorogenic acid) and a yellowish-brown fluorescent zone (hyperoside), and in its upper third, a light blue fluorescent zone (caffeic acid).

The chromatogram obtained with the test solution shows three zones similar in position and fluorescence to the zones due to rutin, chlorogenic acid and hyperoside in the chromatogram obtained with the reference solution. The zone corresponding to rutin is very faint and the zone corresponding to hyperoside is intense. It also shows other yellowish-brown faint fluorescence zones

01/2005:0595

between the zones corresponding to caffeic acid and chlorogenic acid in the chromatogram obtained with the reference solution. Near the solvent front, the red fluorescent zone due to chlorophylls is visible. In the chromatogram obtained with the test solution, between this zone and the zone corresponding to caffeic acid in the chromatogram obtained with the reference solution, there is a brownish-yellow zone corresponding to quercetin.

TESTS

Foreign matter (2.8.2). Not more than 3 per cent of fragments of female catkins and not more than 3 per cent of other foreign matter.

Loss on drying (2.2.32). Not more than 10.0 per cent, determined on 1.000 g of powdered drug (355) by drying in an oven at 100 °C to 105 °C for 2 h.

Total ash (2.4.16). Not more than 5.0 per cent.

ASSAY

Stock solution. In a 100 ml round-bottomed flask introduce 0.200 g of the powdered drug (355), 1 ml of a 5 g/l solution of *hexamethylenetetramine R*, 20 ml of *acetone R* and 2 ml of *hydrochloric acid R1*. Boil the mixture under a reflux condenser for 30 min. Filter the liquid through a plug of absorbent cotton in a 100 ml flask. Add the absorbent cotton to the residue in the round-bottomed flask and extract with two quantities, each of 20 ml, of *acetone R*, each time boiling under a reflux condenser for 10 min. Allow to cool to room temperature, filter the liquid through a plug of absorbent cotton then filter the solution through a filter-paper in the volumetric flask, and dilute to 100.0 ml with *acetone R* by rinsing of the flask and filter. Introduce 20.0 ml of the solution into a separating funnel, add 20 ml of *water R* and extract the mixture with one quantity of 15 ml and then three quantities, each of 10 ml, of *ethyl acetate R*. Combine the ethyl acetate extracts in a separating funnel, rinse with two quantities, each of 50 ml, of *water R*, and filter the extract over 10 g of *anhydrous sodium sulphate R* into a 50 ml volumetric flask and dilute to 50.0 ml with *ethyl acetate R*.

Test solution. To 10.0 ml of the stock solution add 1 ml of *aluminium chloride reagent R* and dilute to 25.0 ml with a 5 per cent V/V solution of *glacial acetic acid R* in *methanol R*.

Compensation solution. Dilute 10.0 ml of the stock solution to 25.0 ml with a 5 per cent V/V solution of *glacial acetic acid R* in *methanol R*.

Measure the absorbance (2.2.25) of the test solution after 30 min, by comparison with the compensation solution at 425 nm.

Calculate the percentage content of flavonoids, calculated as hyperoside, from the expression:

$$\frac{A \times 1.25}{m}$$

i.e. taking the specific absorbance of hyperoside to be 500.

A = absorbance at 425 nm,

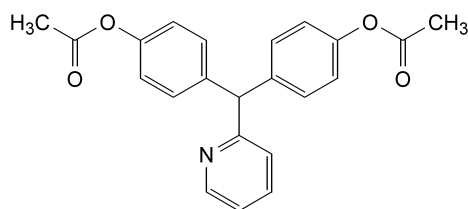
m = mass of the substance to be examined, in grams.

STORAGE

Store protected from light.

BISACODYL

Bisacodylum


 $C_{22}H_{19}NO_4$
 M_r 361.4

DEFINITION

Bisacodyl contains not less than 98.0 per cent and not more than the equivalent of 101.0 per cent of 4,4'-(pyridin-2-ylmethylenediphenyl diacetate, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, crystalline powder, practically insoluble in water, soluble in acetone, sparingly soluble in alcohol. It dissolves in dilute mineral acids.

IDENTIFICATION

First identification: C.

Second identification: A, B, D.

- Melting point (2.2.14): 131 °C to 135 °C.
- Dissolve 10.0 mg in a 6 g/l solution of *potassium hydroxide R* in *methanol R* and dilute to 100.0 ml with the same alkaline solvent. Dilute 10.0 ml of this solution to 100.0 ml with a 6 g/l solution of *potassium hydroxide R* in *methanol R*. Examined between 220 nm and 350 nm (2.2.25), the solution shows an absorption maximum at 248 nm and a shoulder at about 290 nm. The specific absorbance at the maximum is 632 to 672.
- Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *bisacodyl CRS*. If the spectra obtained with the substance to be examined and the reference substance show differences, dissolve separately the substance to be examined and the reference substance in *chloroform R*, evaporate to dryness and record the spectra again.
- Spray the chromatograms obtained in the test for related substances with a mixture of equal volumes of 0.05 M *iodine* and *dilute sulphuric acid R*. Examine in daylight. The principal spot in the chromatogram obtained with test solution (b) is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a).

TESTS

Acidity or alkalinity. To 1.0 g add 20 ml of *carbon dioxide-free water R*, shake, heat to boiling, cool and filter. Add 0.2 ml of 0.01 M *sodium hydroxide* and 0.1 ml of *methyl red solution R*. The solution is yellow. Not more than 0.4 ml of 0.01 M *hydrochloric acid* is required to change the colour of the indicator to red.

Related substances. Examine by thin-layer chromatography (2.2.27), using *silica gel GF₂₅₄ R* as the coating substance.

Test solution (a). Dissolve 0.20 g of the substance to be examined in *acetone R* and dilute to 10 ml with the same solvent.

Test solution (b). Dilute 1 ml of test solution (a) to 10 ml with *acetone R*.

Reference solution (a). Dissolve 20 mg of *bisacodyl CRS* in *acetone R* and dilute to 10 ml with the same solvent.

Reference solution (b). Dilute 1 ml of test solution (a) to 100 ml with *acetone R*.

Reference solution (c). Dilute 5 ml of reference solution (b) to 10 ml with *acetone R*.

Apply separately to the plate 10 µl of each solution. Develop over a path of 10 cm using a mixture of 50 volumes of *xylene R* and 50 volumes of *methyl ethyl ketone R*. Allow the plate to dry in air, if necessary heating at 100 °C to 105 °C, and examine in ultraviolet light at 254 nm. Any spot in the chromatogram obtained with test solution (a), apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (b) (1.0 per cent) and not more than one such spot is more intense than the spot in the chromatogram obtained with reference solution (c) (0.5 per cent).

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 0.500 g by drying in an oven at 100 °C to 105 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.300 g in 60 ml of *anhydrous acetic acid R*. Titrate with 0.1 M *perchloric acid* determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *perchloric acid* is equivalent to 36.14 mg of $C_{22}H_{19}NO_4$.

STORAGE

Store protected from light.

01/2005:0012

BISMUTH SUBCARBONATE

Bismuthi subcarbonas

DEFINITION

Bismuth subcarbonate contains not less than 80.0 per cent and not more than 82.5 per cent of Bi (A, 209.0), calculated with reference to the dried substance.

CHARACTERS

A white or almost white powder, practically insoluble in water and in alcohol. It dissolves with effervescence in mineral acids.

IDENTIFICATION

- It gives the reaction of carbonates (2.3.1).
- It gives the reactions of bismuth (2.3.1).

TESTS

Solution S. Shake 5.0 g with 10 ml of *water R* and add 20 ml of *nitric acid R*. Heat to dissolve, cool and dilute to 100 ml with *water R*.

Appearance of solution. Solution S is not more opalescent than reference suspension II (2.2.1) and is colourless (2.2.2, *Method II*).

Chlorides (2.4.4). To 6.6 ml of solution S add 4 ml of *nitric acid R* and dilute to 50 ml with *water R*. 15 ml of the solution complies with the limit test for chlorides (500 ppm).

Nitrates. To 0.25 g in a 125 ml conical flask, add 20 ml of *water R*, 0.05 ml of *indigo carmine solution R1* and then, as a single addition but with caution, 30 ml of *sulphuric acid R*. Titrate immediately with *indigo carmine solution R1* until a stable blue colour is obtained. Not more than *n* ml of the titrant is required, *n* ml being the volume corresponding to 1 mg of NO_3 (0.4 per cent).

Alkali and alkaline-earth metals. To 1.0 g add 10 ml of *water R* and 10 ml of *acetic acid R*. Boil for 2 min, cool and filter. Wash the residue with 20 ml of *water R*. To the combined filtrate and washings add 2 ml of *dilute hydrochloric acid R* and 20 ml of *water R*. Boil and pass *hydrogen sulphide R* through the boiling solution until no further precipitate is formed. Filter, wash the residue with *water R*, evaporate the combined filtrate and washings to dryness on a water-bath and add 0.5 ml of *sulphuric acid R*. Ignite gently and allow to cool. The residue weighs not more than 10 mg (1.0 per cent).

Arsenic (2.4.2). To 0.5 g in a distillation flask add 5 ml of *water R* and 7 ml of *sulphuric acid R*, allow to cool and add 5 g of *reducing mixture R* and 10 ml of *hydrochloric acid R*. Heat the contents of the flask to boiling gradually over 15 min to 30 min and continue heating at such a rate that the distillation proceeds steadily until the volume in the flask is reduced by half or until 5 min after the air-condenser has become full of steam. It is important that distillation be discontinued before fumes of sulphur trioxide appear. Collect the distillate in a tube containing 15 ml of *water R* cooled in ice-water. Wash down the condenser with *water R* and dilute the distillate to 25 ml with the same solvent. The solution complies with limit test A for arsenic (5 ppm). Prepare the standard using a mixture of 2.5 ml of *arsenic standard solution (1 ppm As) R* and 22.5 ml of *water R*.

Copper. To 5 ml of solution S, add 2 ml of *ammonia R* and dilute to 50 ml with *water R*. Filter. To 10 ml of the filtrate add 1 ml of a 1 g/l solution of *sodium diethyldithiocarbamate R*. The solution is not more intensely coloured than a standard prepared at the same time in the same manner using a mixture of 0.25 ml of *copper standard solution (10 ppm Cu) R* and 9.75 ml of *water R* instead of 10 ml of the filtrate (50 ppm).

Lead. Not more than 20 ppm of Pb, determined by atomic absorption spectrometry (2.2.23, *Method II*).

Test solution. Dissolve 12.5 g of the substance to be examined in 75 ml of a mixture of equal volumes of *lead-free nitric acid R* and *water R*. Boil for 1 min, cool and dilute to 100.0 ml with *water R*.

Reference solutions. Prepare the reference solutions using appropriate quantities of lead standard solution and a 37 per cent V/V solution of *lead-free nitric acid R*.

Measure the absorbance at 283.3 nm, using a hollow-cathode lamp as source of radiation and an air-acetylene flame. Depending on the apparatus, the line at 217.0 nm may be used.

Silver. To 2.0 g add 1 ml of *water R* and 4 ml of *nitric acid R*. Heat gently until dissolved and dilute to 11 ml with *water R*. Cool and add 2 ml of 1 M *hydrochloric acid*. Allow to stand for 5 min, protected from light. Any opalescence in the solution is not more intense than that in a standard prepared at the same time in the same manner using a mixture of 10 ml of *silver standard solution (5 ppm Ag) R*, 1 ml of *nitric acid R* and 2 ml of 1 M *hydrochloric acid* (25 ppm).

Loss on drying (2.2.32). Not more than 1.0 per cent, determined on 1.000 g by drying in an oven at 100-105 °C.

ASSAY

Dissolve 0.500 g in 3 ml of *nitric acid R* and dilute to 250 ml with *water R*. Carry out the complexometric titration of bismuth (2.5.11).

1 ml of 0.1 M *sodium edetate* is equivalent to 20.90 mg of Bi.

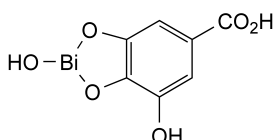
STORAGE

Store protected from light.

01/2005:1493

BISMUTH SUBGALLATE

Bismuthi subgallas


 $C_7H_5BiO_6$
 M_r 394.1

DEFINITION

Complex of bismuth and gallic acid.

Content: 48.0 per cent to 51.0 per cent of Bi (A_r 209.0) (dried substance).

CHARACTERS

Appearance: yellow powder.

Solubility: practically insoluble in water and in alcohol. It dissolves in mineral acids with decomposition and in solutions of alkali hydroxides, producing a reddish-brown liquid.

IDENTIFICATION

- A. Mix 0.1 g with 5 ml of *water R* and 0.1 ml of *phosphoric acid R*. Heat to boiling and maintain boiling for 2 min. Cool and filter. To the filtrate, add 1.5 ml of *ferric chloride solution R1*, a blackish-blue colour develops.
- B. It gives reaction (b) of bismuth (2.3.1).

TESTS

Solution S. In a porcelain or quartz dish, ignite 1.0 g, increasing the temperature very gradually. Heat in a muffle furnace at 600 ± 25 °C for 2 h. Cool and dissolve the residue with warming in 4 ml of a mixture of equal volumes of *lead-free nitric acid R* and *water R* and dilute to 20 ml with *water R*.

Acidity. Shake 1.0 g with 20 ml of *water R* for 1 min and filter. To the filtrate add 0.1 ml of *methyl red solution R*. Not more than 0.15 ml of 0.1 M *sodium hydroxide* is required to change the colour of the indicator to yellow.

Chlorides (2.4.4): maximum 200 ppm.

To 0.5 g add 10 ml of *dilute nitric acid R*. Heat on a water-bath for 5 min and filter. Dilute 5 ml of the filtrate to 15 ml with *water R*.

Nitrates: maximum 0.2 per cent.

To 1.0 g add 25 ml of *water R* then 25 ml of a mixture of 2 volumes of *sulphuric acid R* and 9 volumes of *water R*. Heat at about 50 °C for 1 min with stirring and filter. To 10 ml of the filtrate, carefully add 30 ml of *sulphuric acid R*. The solution is not more intensely brownish-yellow coloured than a reference solution prepared at the same

time as follows: to 0.4 g of *gallic acid R*, add 20 ml of *nitrate standard solution* (100 ppm NO_3) *R* and 30 ml of a mixture of 2 volumes of *sulphuric acid R* and 9 volumes of *water R*, then filter; to 10 ml of the filtrate, carefully add 30 ml of *sulphuric acid R*.

Copper: maximum 50 ppm.

Atomic absorption spectrometry (2.2.23, *Method I*).

Test solution. Solution S.

Reference solutions. Prepare the reference solutions using *copper standard solution* (10 ppm Cu) *R* and diluting with a 6.5 per cent V/V solution of *lead-free nitric acid R*.

Source: copper hollow-cathode lamp.

Wavelength: 324.7 nm.

Atomisation device: air-acetylene flame.

Lead: maximum 20 ppm.

Atomic absorption spectrometry (2.2.23, *Method II*).

Test solution. Solution S.

Reference solutions. Prepare the reference solutions using *lead standard solution* (10 ppm Pb) *R* and diluting with a 6.5 per cent V/V solution of *lead-free nitric acid R*.

Source: lead hollow-cathode lamp.

Wavelength: 283.3 nm (depending on the apparatus, the line at 217.0 nm may be used).

Atomisation device: air-acetylene flame.

Silver: maximum 25 ppm.

Atomic absorption spectrometry (2.2.23, *Method I*).

Test solution. Solution S.

Reference solutions. Prepare the reference solutions using *silver standard solution* (5 ppm Ag) *R* and diluting with a 6.5 per cent V/V solution of *lead-free nitric acid R*.

Source: silver hollow-cathode lamp.

Wavelength: 328.1 nm.

Atomisation device: air-acetylene flame.

Substances not precipitated by ammonia: maximum 1.0 per cent.

In a porcelain or quartz dish, ignite 2.0 g, increasing the temperature very gradually to 600 °C; allow to cool. Moisten the residue with 2 ml of *nitric acid R*, evaporate to dryness on a water-bath and carefully heat and ignite once more at 600 °C. After cooling, dissolve the residue in 5 ml of *nitric acid R* and dilute to 20 ml with *water R*. To 10 ml of this solution, add *concentrated ammonia R* until alkaline and filter. Wash the residue with *water R*, evaporate the combined filtrate and washings to dryness on a water-bath and add 0.3 ml of *dilute sulphuric acid R*. Ignite, the residue weighs a maximum of 10 mg.

Loss on drying (2.2.32): maximum 7.0 per cent, determined on 1.000 g by drying in an oven at 100-105 °C for 3 h.

ASSAY

To 0.300 g add 10 ml of a mixture of equal volumes of *nitric acid R* and *water R*, heat to boiling and maintain boiling for 2 min. Add 0.1 g of *potassium chlorate R*, heat to boiling and maintain boiling for 1 min. Add 10 ml of *water R* and heat until the solution becomes colourless. To the hot solution, add 200 ml of *water R* and 50 mg of *xylene orange triturate R*. Titrate with 0.1 M *sodium edetate* until a yellow colour is obtained.

1 ml of 0.1 M *sodium edetate* is equivalent to 20.90 mg of Bi.

STORAGE

Protected from light.

01/2005:1494

BISMUTH SUBNITRATE, HEAVY**Bismuthi subnitras ponderosum** $4[\text{BiNO}_3(\text{OH})_2] \cdot \text{BiO}(\text{OH})$ M_r 1462**DEFINITION**

Heavy bismuth subnitrate contains not less than 71.0 per cent and not more than 74.0 per cent of Bi (A_r 209.0), calculated with reference to the dried substance.

CHARACTERS

A white powder, practically insoluble in water and in alcohol. It dissolves in mineral acids with decomposition.

IDENTIFICATION

- A. Dilute 1 ml of solution S1 (see Tests) to 5 ml with *water R*, add 0.3 ml of *potassium iodide solution R*. A black precipitate is formed which dissolves into an orange solution with the addition of 2 ml of *potassium iodide solution R*.
- B. It gives reaction (b) of bismuth (2.3.1).
- C. It gives the reaction of nitrates (2.3.1).
- D. The pH (2.2.3) of solution S2 (see Tests) is not more than 2.0.

TESTS

Solution S1. Shake 5.0 g by gently heating in 10 ml of *water R*, add 20 ml of *nitric acid R*. Heat until dissolution, cool and dilute to 100 ml with *water R*.

Solution S2. Place 1.00 g in a 20 ml volumetric flask and add 2.0 ml of *lead-free nitric acid R*. Allow acid attack to take place without heating and if necessary warm slightly at the end to dissolve the test sample completely. Add 10 ml of *water R*, shake and add, in small fractions, 4.5 ml of *lead-free ammonia R*; shake, allow to cool, dilute to 20.0 ml with *water R*, shake again and allow the solids to settle. The clear supernatant solution is solution S2.

Acidity. Suspend 1.0 g in 15 ml of *water R* and shake several times. Allow to stand for 5 min and filter. To 10 ml of the filtrate, add 0.5 ml of *phenolphthalein solution R1*. Not more than 0.5 ml of 0.1 M *sodium hydroxide* is required to change the colour of the indicator to pink.

Chlorides (2.4.4). To 5.0 ml of solution S1, add 3 ml of *nitric acid R* and dilute to 15 ml with *water R*. The solution complies with the limit test for chlorides (200 ppm).

Copper. Not more than 50 ppm of Cu, determined by atomic absorption spectrometry (2.2.23, *Method I*).

Test solution. Solution S2.

Reference solutions. Prepare the reference solutions using *copper standard solution* (10 ppm Cu) *R* and diluting with a 37 per cent V/V solution of *lead-free nitric acid R*.

Measure the absorbance at 324.7 nm using a copper hollow-cathode lamp as source of radiation and an air-acetylene flame.

Lead. Not more than 20 ppm of Pb, determined by atomic absorption spectrometry (2.2.23, *Method II*).

Test solution. Solution S2.

Reference solutions. Prepare the reference solutions using appropriate quantities of *lead standard solution* (10 ppm Pb) *R* and diluting with a 37 per cent V/V solution of *lead-free nitric acid R*.

Measure the absorbance at 283.3 nm, using a lead hollow-cathode lamp as source of radiation and an air-acetylene flame. Depending on the apparatus, the line at 217.0 nm may be used.

Silver. Not more than 25 ppm of Ag, determined by atomic absorption spectrometry (2.2.23, *Method I*).

Test solution. Solution S2.

Reference solutions. Prepare the reference solutions using *silver standard solution* (5 ppm Ag) *R* and diluting with a 37 per cent V/V solution of *lead-free nitric acid R*.

Measure the absorbance at 328.1 nm using a silver hollow-cathode lamp as source of radiation and an air-acetylene flame.

Substances not precipitated by ammonia. To 20 ml of solution S1, add *concentrated ammonia R* until alkaline and filter. Wash the residue with *water R*, and evaporate the combined filtrate and washings to dryness on a water-bath. Add 0.3 ml of *dilute sulphuric acid R* and ignite. The residue weighs not more than 10 mg (1.0 per cent).

Loss on drying (2.2.32). Not more than 3.0 per cent, determined on 1.000 g by drying in an oven at 100-105 °C.

ASSAY

Dissolve with heating 0.250 g in 10 ml of a mixture of 2 volumes of *perchloric acid R* and 5 volumes of *water R*. To the hot solution, add 200 ml of *water R* and 50 mg of *xylenol orange triturate R*. Titrate with 0.1 M *sodium edetate* until a yellow colour is obtained.

1 ml of 0.1 M *sodium edetate* is equivalent to 20.90 mg of Bi.

01/2005:1495

BISMUTH SUBSALICYLATE**Bismuthi subsalicylas** $\text{C}_7\text{H}_5\text{BiO}_4$ M_r 362.1**DEFINITION**

Complex of bismuth and salicylic acid.

Content: 56.0 per cent to 59.4 per cent of Bi (A_r 209.0) (dried substance).

CHARACTERS

Appearance: white powder.

Solubility: practically insoluble in water and in alcohol. It dissolves in mineral acids with decomposition.

IDENTIFICATION

- A. To 0.5 g add 10 ml of *hydrochloric acid R1*. Heat on a boiling water-bath for 5 min. Cool and filter. Retain the filtrate for identification test B. Wash the residue with *dilute hydrochloric acid R* and then with *water R*. Dissolve the residue in 0.5-1 ml of *dilute sodium hydroxide solution R*. Add 15 ml of *water R*. Neutralise with *dilute hydrochloric acid R*. The solution gives reaction (a) of salicylates (2.3.1).
- B. The filtrate obtained in identification test A gives reaction (b) of bismuth (2.3.1).

TESTS

Solution S. In a porcelain or quartz dish, ignite 1.0 g, increasing the temperature very gradually. Heat in a muffle furnace at 600 ± 25 °C for 2 h. Cool and dissolve the residue with warming in 4 ml of a mixture of equal volumes of *lead-free nitric acid R* and *water R* and dilute to 20 ml with *water R*.

Acidity. Shake 2.0 g with 30 ml of *ether R* for 1 min and filter. To the filtrate add 30 ml of *alcohol R* and 0.1 ml of *thymol blue solution R*. Not more than 0.35 ml of 0.1 M *sodium hydroxide* is required to change the colour of the indicator to blue.

Chlorides (2.4.4): maximum 200 ppm.

Dissolve 0.250 g in a mixture of 2 ml of *nitric acid R*, 5 ml of *water R* and 8 ml of *methanol R*.

Nitrates: maximum 0.4 per cent.

To 0.1 g add 10 ml of *water R* and, with caution, 20 ml of *sulphuric acid R* and stir. The solution is not more intensely yellow coloured than a reference solution prepared at the same time using 0.1 g of *salicylic acid R*, 6 ml of *water R*, 4 ml of *nitrate standard solution* (100 ppm NO_3^-) *R* and 20 ml of *sulphuric acid R*.

Copper: maximum 50 ppm.

Atomic absorption spectrometry (2.2.23, *Method I*).

Test solution. Solution S.

Reference solutions. Prepare the reference solutions using *copper standard solution* (10 ppm Cu) *R* and diluting with a 6.5 per cent V/V solution of *lead-free nitric acid R*.

Source: copper hollow-cathode lamp.

Wavelength: 324.7 nm.

Atomisation device: air-acetylene flame.

Lead: maximum 20 ppm.

Atomic absorption spectrometry (2.2.23, *Method II*).

Test solution. Solution S.

Reference solutions. Prepare the reference solutions using *lead standard solution* (10 ppm Pb) *R* and diluting with a 6.5 per cent V/V solution of *lead-free nitric acid R*.

Source: lead hollow-cathode lamp.

Wavelength: 283.3 nm (depending on the apparatus, the line at 217.0 nm may be used).

Atomisation device: air-acetylene flame.

Silver: maximum 25 ppm.

Atomic absorption spectrometry (2.2.23, *Method I*).

Test solution. Solution S.

Reference solutions. Prepare the reference solutions using *silver standard solution* (5 ppm Ag) *R* and diluting with a 6.5 per cent V/V solution of *lead-free nitric acid R*.

Source: silver hollow-cathode lamp.

Wavelength: 328.1 nm.

Atomisation device: air-acetylene flame.

Soluble bismuth: maximum 40 ppm.

Atomic absorption spectrometry (2.2.23, *Method I*).

Test solution. Suspend 5.0 g in 100 ml of *water R*. Stir constantly for 2 h at 20–23 °C. Filter through filter paper (slow filtration) then through a cellulose micropore membrane filter (0.1 µm). To 10.0 ml of clear filtrate, add 0.1 ml of *nitric acid R*.

Reference solutions. Prepare the reference solutions using *bismuth standard solution* (100 ppm Bi) *R* and diluting with a mixture of equal volumes of *dilute nitric acid R* and *water R*.

Source: bismuth hollow-cathode lamp.

Wavelength: 223.06 nm.

Atomisation device: air-acetylene flame.

Loss on drying (2.2.32): maximum 1.0 per cent, determined on 1.000 g by drying in an oven at 100–105 °C.

ASSAY

Dissolve with heating 0.300 g in 10 ml of a mixture of 2 volumes of *perchloric acid R* and 5 volumes of *water R*. To the hot solution, add 200 ml of *water R* and 50 mg of *xylene orange triturate R*. Titrate with 0.1 M *sodium edetate* until a yellow colour is obtained.

1 ml of 0.1 M *sodium edetate* is equivalent to 20.90 mg of Bi.

STORAGE

Protected from light.

01/2005:1826

BITTER-FENNEL FRUIT OIL

Foeniculi amari fructus aetheroleum

DEFINITION

Essential oil obtained by steam distillation from the ripe fruits of *Foeniculum vulgare* Miller, ssp. *vulgare* var. *vulgare*.

Content:

- fenchone: 12.0 per cent to 25.0 per cent,
- *trans*-anethole: 55.0 per cent to 75.0 per cent.

CHARACTERS

Appearance: clear, colourless or pale yellow liquid.

It has a characteristic odour.

IDENTIFICATION

First identification: B.

Second identification: A.

A. Thin-layer chromatography (2.2.27).

Test solution. Dissolve 0.1 ml of the oil to be examined in 5 ml of *toluene R*.

Reference solution. Dissolve 10 µl of *fenchone R* and 80 µl of *anethole R* in 5 ml of *toluene R*.

Plate: TLC silica gel plate *R*.

Mobile phase: *ethyl acetate R*, *toluene R* (5:95 V/V).

Application: 10 µl as bands.

Development: over a path of 15 cm.

Drying: in air.

Detection: spray with a freshly prepared 200 g/l solution of *phosphomolybdic acid R* in *ethanol* (96 per cent) *R* and heat at 150 °C for 15 min; examine in daylight.

Results: see below the sequence of the zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other zones may be present in the chromatogram obtained with the test solution.

Top of the plate	
Anethole: a dark blue to dark violet zone	A dark blue to dark violet zone (anethole)
_____	_____
Fenchone: a blue or bluish-grey zone	A blue or bluish-grey zone (fenchone)
_____	_____
Reference solution	Test solution

B. Examine the chromatograms obtained in the test for chromatographic profile.

Results: the characteristic peaks in the chromatogram obtained with the test solution are similar in retention time to those in the chromatogram obtained with the reference solution.

TESTS

Relative density (2.2.5): 0.961 to 0.975.

Refractive index (2.2.6): 1.528 to 1.539.

Optical rotation (2.2.7): + 10.0° to + 24.0°.

Chromatographic profile. Gas chromatography (2.2.28): use the normalisation procedure.

Test solution. Dissolve 0.20 ml of the oil to be examined in *heptane R* and dilute to 10.0 ml with the same solvent.

Reference solution. Dissolve 20 µl of *α-pinene R*, 20 µl of *limonene R*, 50 µl of *fenchone R*, 20 µl of *estragole R*, 100 µl of *anethole R* and 20 µl of *anisaldehyde R* in *heptane R* and dilute to 10.0 ml with the same solvent.

Column:

- **material:** fused silica,
- **size:** *l* = 60 m, *Ø* = 0.25 mm,
- **stationary phase:** *macrogol 20 000 R* (film thickness 0.25 µm).

Carrier gas: *helium for chromatography R*.

Flow rate: 1 ml/min.

Split ratio: 1:200.

Temperature:

	Time (min)	Temperature (°C)
Column	0 – 4	60
	4 – 26	60 → 170
	26 – 41	170
Injection port		220
Detector		270

Detection: flame ionisation.

Injection: 1.0 µl.

Elution order: order indicated in the composition of the reference solution. Record the retention times of these substances.

System suitability: reference solution:

- **resolution:** minimum 5.0 between the peaks due to *estragole* and *trans-anethole*.

Using the retention times determined from the chromatogram obtained with the reference solution, locate the components of the reference solution on the chromatogram obtained with the test solution and locate *cis-anethole* using Figure 1826-1. (Disregard the peak due to *heptane*).

Determine the percentage content of each of these components. The percentages are within the following ranges:

- *α-pinene*: 1.0 per cent to 10.0 per cent,
- *limonene*: 0.9 per cent to 5.0 per cent,
- *fenchone*: 12.0 per cent to 25.0 per cent,
- *estragole*: maximum 6.0 per cent,

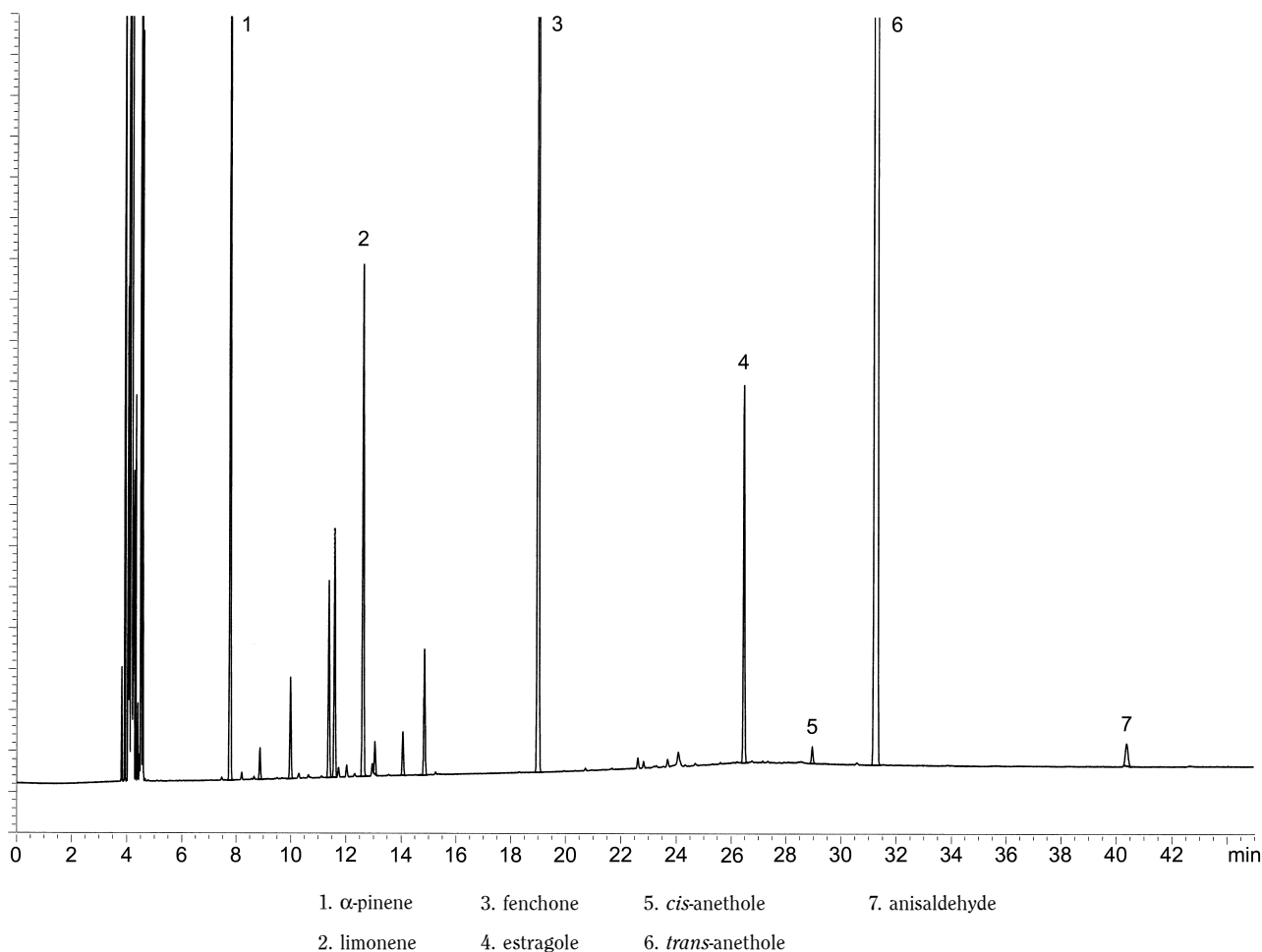


Figure 1826-1. – Chromatogram for the test for chromatographic profile of bitter-fennel fruit oil

- *cis-anethole*: maximum 0.5 per cent,
- *trans-anethole*: 55.0 per cent to 75.0 per cent,
- *anisaldehyde*: maximum 2.0 per cent.

The ratio of α -pinene content to limonene content is greater than 1.0.

STORAGE

In a well-filled, airtight container, protected from light and at a temperature not exceeding 25 °C.

01/2005:1603

BITTER-ORANGE EPICARP AND MESOCARP

Aurantii amari epicarpium et mesocarpium

DEFINITION

Dried epicarp and mesocarp of the ripe fruit of *Citrus aurantium* L. ssp. *aurantium* (*C. aurantium* L. ssp. *amara* Engl.) partly freed from the white spongy tissue of the mesocarp and endocarp.

Content: minimum 20 ml/kg of essential oil (anhydrous drug).

CHARACTERS

Aromatic odour and spicy bitter taste.

Macroscopic and microscopic characters described under identification A and B.

IDENTIFICATION

- The drug consists of elliptical to irregular pieces 5 cm to 8 cm long, 3 cm to 5 cm broad and about 3 mm thick. The outer surface is yellowish to reddish-brown and distinctly punctate, the inner surface is yellowish to brownish-white.
- Reduce to a powder (355). The powder is light brown. Examine under a microscope using *chloral hydrate solution R*. The powder shows small polygonal cells with slightly thickened anticlinal walls, filled with orange-red chromatophores, and very occasional anomocytic stomata (2.8.3); fragments of the hypodermic showing collenchymatous thickening; groups of parenchyma with each cell containing a prism crystal of calcium oxalate; fragments of lysigenous oil glands; parenchyma containing crystals of hesperidin which dissolve in a 20 g/l *potassium hydroxide R* solution giving a yellow colour.

- Thin-layer chromatography (2.2.27).

Test solution. To 1.0 g of the powdered drug (710) add 10 ml of *methanol R* and heat in a water-bath at 65 °C for 5 min shaking frequently. Allow to cool and filter.

Reference solution. Dissolve 1.0 mg of *naringin R* and 1.0 mg of *caffeic acid R* in 1 ml of *methanol R*.

Plate: TLC silica gel plate *R*.

Mobile phase: *water R*, *anhydrous formic acid R*, *ethyl acetate R* (10:15:75 V/V/V).

Application: 20 μ l, as bands.

Development: over a path of 10 cm.

Drying: in air, and heat in an oven at 110-120 °C for 5 min.

Detection: spray the warm plate with a 10 g/l solution of *diphenylboric acid aminoethyl ester R* in *methanol R* and then with a 50 g/l solution of *macrogol 400 R* in *methanol R*. After at least 1 h, examine in ultraviolet light at 365 nm.

Results: see below the sequence of the zones present in the chromatograms obtained with the reference and test solutions. Furthermore, other fluorescent zones are present in the chromatogram obtained with the test solution.

Top of the plate	
Caffeic acid: a light blue fluorescent zone	A light blue fluorescent zone
	A light blue fluorescent zone
Naringin: a dark green fluorescent zone	A light blue fluorescent zone
	A light blue fluorescent zone
	A dark green fluorescent zone (naringin)
	A red fluorescent zone (neoeriocitrin)
	An orange fluorescent zone
Reference solution	Test solution

TESTS

Foreign matter (2.8.2): it complies with the test for foreign matter.

Water (2.2.13): maximum 10.0 per cent, determined on 20.0 g of powdered drug (355) by distillation.

Total ash (2.4.16): maximum 7.0 per cent

Extractable matter: minimum 6.0 per cent.

To 2.000 g of the powdered drug (250) add a mixture of 3 ml of *water R* and 7 ml of *alcohol R* and extract for 2 h, shaking frequently. Filter, evaporate 2.000 g of the filtrate to dryness on a water-bath and dry in an oven at 100-105 °C for 3 h. Allow to cool in a desiccator over *diphosphorus pentoxide R* and weigh. The residue weighs a minimum of 120 mg.

ASSAY

Carry out the determination of essential oil in vegetable drugs (2.8.12). Use a 500 ml round-bottomed flask, 200 ml of *water R* as the distillation liquid and 0.5 ml of *xylene R* in the graduated tube. Reduce the drug to a powder (710) and immediately use 15.0 g for the determination. Distil at a rate of 2-3 ml/min for 90 min.

01/2005:1604

BITTER-ORANGE-EPICARP AND MESOCARP TINCTURE

Aurantii amari epicarpium et mesocarpium tinctura

DEFINITION

Tincture produced from *Bitter-orange epicarp and mesocarp* (1603).

PRODUCTION

The tincture is produced from 1 part of the freshly powdered drug (2000) and 5 parts of *alcohol* (70 per cent V/V/V) by an appropriate procedure.

CHARACTERS

Liquid with a bitter taste.

IDENTIFICATION

Examine by thin-layer chromatography (2.2.27).

Test solution. The tincture to be examined.

Reference solution. Dissolve 1.0 mg of *naringin R* and 1.0 mg of *caffeic acid R* in 1 ml of *methanol R*.

Plate: *TLC silica gel plate R*.

Mobile phase: *water R, anhydrous formic acid R, ethyl acetate R* (10:15:75 *V/V/V*).

Application: 20 µl, as bands.

Development: over a path of 10 cm.

Drying: in air, and heat in an oven at 110-120 °C for 5 min.

Detection: spray the warm plate with a 10 g/l solution of *diphenylboric acid aminoethyl ester R* in *methanol R* and then with a 50 g/l solution of *macrogol 400 R* in *methanol R*. After 1 h, examine in ultraviolet light at 365 nm.

Results: see below the sequence of the zones present in the chromatograms obtained with the reference and test solutions. Furthermore, other zones are present in the chromatogram obtained with the test solution.

Top of the plate	
Caffeic acid: a light blue fluorescent zone	A light blue fluorescent zone
	A light blue fluorescent zone
	A light blue fluorescent zone
	A light blue fluorescent zone
Naringin: a dark green fluorescent zone	A dark green fluorescent zone (naringin)
	A red fluorescent zone (neoneriocitrin)
	An orange fluorescent zone
Reference solution	Test solution

TESTS

Ethanol content (2.9.10): 63 per cent to 67 per cent *V/V*.

Methanol and 2-propanol (2.9.11): maximum 0.05 per cent *V/V* of methanol and maximum 0.05 per cent *V/V* of 2-propanol.

Dry residue: minimum 6.0 per cent *m/m*, determined on 2.00 g of tincture to be examined.

01/2005:1810

BITTER-ORANGE FLOWER

Aurantii amari flos

DEFINITION

Whole, dried, unopened flower of *Citrus aurantium* L. ssp. *aurantium* (*C. aurantium* L. ssp. *amara* Engl.).

Content: minimum 8.0 per cent of total flavonoids, expressed as naringin ($C_{27}H_{32}O_{14}$; M_r 580.5) (dried drug).

CHARACTERS

Macroscopic and microscopic characters described under identification tests A and B.

IDENTIFICATION

A. The flower buds are white or yellowish-white and may reach up to 25 mm in length. The dialypetalous corolla is composed of 5 thick, oblong and concave petals dotted with oil glands visible under a hand lens; the short, yellowish-green persistent gamosepalous calyx has 5 spreading sepals, connate at the base and forming a star-shaped structure attached to the yellowish-green

peduncle which is about 5 mm to 10 mm long. The flower buds contain at least 20 stamens with yellow anthers and with filaments fused at the base into groups of 4 or 5; the ovary is superior, brownish-black and spherical, consists of 8 to 10 multi-ovular loculi and is surrounded at the base by an annular granular hypogynous disc; the thick, cylindrical style ends in a capitate stigma.

B. Reduce to a powder (355). The powder is brownish-yellow. Examine under a microscope using *chloral hydrate solution R*. The powder shows numerous spherical pollen grains, with a finely pitted exine and 3 to 5 germinal pores; fragments of the epidermis of the sepals with unicellular trichomes and with large prism crystals of calcium oxalate in the underlying mesophyll; fragments of the epidermis of the petals with a distinctly striated cuticle; fragments of large schizolysigenous oil glands which measure up to 100 µm in diameter, numerous anomocytic stomata (2.8.3). Examine under a microscope using a 300 g/l solution of *potassium hydroxide R*. The powder shows yellow crystals of hesperidin.

C. Examine the chromatograms obtained in the test for sweet-orange flower.

Results: see below the sequence of the zones present in the chromatograms obtained with the reference solution and the test solution.

Top of the plate	
Hesperidin: a greenish-yellow fluorescent zone Naringin: a yellow fluorescent zone	A weak yellow fluorescent zone
	A weak yellow fluorescent zone
	A greenish-yellow fluorescent zone (hesperidin)
	A yellow fluorescent zone (naringin)
	A red fluorescent zone (neoneriocitrin)
	A yellow fluorescent zone (diosmin and neodiosmin)
Reference solution	Test solution

TESTS

Sweet-orange flower. Thin-layer chromatography (2.2.27).

Test solution. To 0.5 g of the powdered drug (355), add 5 ml of *methanol R*. Heat with stirring at 40 °C for 10 min. Filter.

Reference solution. Dissolve 3.0 mg of *naringin R* and 3.0 mg of *hesperidin R* in 10 ml of *methanol R*.

Plate: *TLC silica gel plate R*.

Mobile phase: *water R, anhydrous formic acid R, ethyl acetate R* (10:15:75 *V/V/V*).

Application: 10 µl, as bands.

Development: over a path of 10 cm.

Drying: in air, then heat in an oven at 110-120 °C for 5 min.

Detection: spray the hot plate with a 10 g/l solution of *diphenylboric acid aminoethyl ester R* in *methanol R* and then with a 50 g/l solution of *macrogol 400 R* in *methanol R*. After at least 1 h, examine in ultraviolet light at 365 nm.

Results: the chromatogram obtained with the test solution shows a yellow zone similar in position to the zone of naringin in the chromatogram obtained with the reference solution and immediately below it a red zone (neoneriocitrin).

Foreign matter (2.8.2): maximum 2 per cent.

Loss on drying (2.2.32): maximum 11.0 per cent, determined on 1.000 g of the powdered drug (355) by drying in an oven at 100-105 °C.

Total ash (2.4.16): maximum 10.0 per cent.

ASSAY

Stock solution. To 0.175 g of the powdered drug (355) add 95 ml of *alcohol (50 per cent V/V) R*. Heat on a water-bath under a reflux condenser for 30 min. Allow to cool and filter through a sintered-glass filter. Rinse the filter with 5 ml of *alcohol (50 per cent V/V) R*. Combine the filtrate and the rinsings in a volumetric flask and dilute to 100.0 ml with *alcohol (50 per cent V/V) R*.

Test solution. Into a test tube (10 mm × 180 mm) introduce 0.150 g of powdered (250) *magnesium R*, a magnetic stirring bar 25 mm long and 2.00 ml of the stock solution. Maintain the test tube upright, centrifuge at 125 *g* and carefully add dropwise, especially at the beginning, 2.0 ml of *hydrochloric acid R*, and then 6.0 ml of *alcohol (50 per cent V/V) R*. Stopper the tube and mix by inverting.

Compensation solution. Into a second tube, introduce 2.00 ml of the stock solution and carefully add dropwise, especially at the beginning, 2.0 ml of *hydrochloric acid R* and then 6.0 ml of *alcohol (50 per cent V/V) R*.

After 10 min, measure the absorbance (2.2.25) of the test solution at 530 nm.

Calculate the percentage content of total flavonoids, expressed as naringin, from the expression:

$$\frac{A \times 9.62}{m}$$

i.e. taking the value of the specific absorbance of the reaction product of naringin to be 52.

A = absorbance at 530 nm,

m = mass of the substance to be examined, in grams.

01/2005:1175

BITTER-ORANGE-FLOWER OIL

Aurantii amari floris aetheroleum

DEFINITION

Bitter-orange-flower oil is obtained by steam distillation from the fresh flowers of *Citrus aurantium* L. subsp. *aurantium* (*C. aurantium* L. subsp. *amara* Engl.).

CHARACTERS

A clear, pale-yellow or dark-yellow liquid, with a characteristic odour reminiscent of bitter-orange flowers, miscible with alcohol, with light petroleum, with fatty oils and with liquid paraffin.

IDENTIFICATION

First identification: *B*.

Second identification: *A*.

A. Examine in ultraviolet light at 365 nm the chromatograms obtained in the test for bergapten. Before spraying with the reagent, the chromatogram obtained with the test solution shows a band similar in position and fluorescence to that corresponding to methyl anthranilate in the chromatogram obtained with the reference solution. Other bands may be visible. Examine in ultraviolet light at 365 nm after spraying with the reagent. The chromatogram obtained with the reference solution shows in the upper half a band of brownish-orange fluorescence corresponding to linalyl acetate, in the lower half a band of brownish-orange fluorescence corresponding to linalol and immediately below, a band of yellow-greenish fluorescence corresponding to bergapten.

The chromatogram obtained with the test solution shows two bands similar in position and fluorescence to the bands corresponding to linalyl acetate and to linalol in the chromatogram obtained with the reference solution. Other bands may also be present.

B. Examine the chromatograms obtained in the test for chromatographic profile. The retention times of the principal peaks in the chromatogram obtained with the test solution are approximately the same as those of the peaks in the chromatogram obtained with the reference solution.

TESTS

Relative density (2.2.5): 0.866 to 0.880.

Refractive index (2.2.6): 1.468 to 1.474.

Optical rotation (2.2.7): + 1.5° to + 11.5°.

Acid value (2.5.1). Not more than 2.0.

Bergapten. Examine by thin-layer chromatography (2.2.27), using a suitable silica gel as the coating substance.

Test solution. Dissolve 0.1 g of the substance to be examined in *alcohol R* and dilute to 5.0 ml with the same solvent.

Reference solution. Dissolve 5 µl of *methyl anthranilate R*, 10 µl of *linalol R*, 20 µl of *linalyl acetate R* and 10 mg of *bergapten R* in *alcohol R* and dilute to 10.0 ml with the same solvent.

Apply separately to the plate, as bands, 10 µl of each solution. Develop over a path of 15 cm using a mixture of 15 volumes of *ethyl acetate R* and 85 volumes of *toluene R*. Allow the plate to dry in air and examine in ultraviolet light at 365 nm. The chromatogram obtained with the reference solution shows in the middle a band of blue fluorescence (methyl anthranilate) and below a band of greenish-yellow fluorescence (bergapten). Spray with *anisaldehyde solution R*. Heat the plate at 100 °C to 105 °C for 10 min. Examine in ultraviolet light at 365 nm. The chromatogram obtained with the test solution does not show a band corresponding to that due to bergapten (essential oil of bitter-orange peel) in the chromatogram obtained with the reference solution.

Chromatographic profile. Examine by gas chromatography (2.2.28).

Test solution. The substance to be examined.

Reference solution. Dissolve 20 µl of *β-pinene R*, 5 µl of *sabinene R*, 40 µl of *limonene R*, 40 µl of *linalol R*, 20 µl of *linalyl acetate R*, 5 µl of *α-terpineol R*, 5 µl of *neryl acetate R*, 5 µl of *geranyl acetate R*, 5 µl of *trans-nerolidol R* and 5 µl of *methyl anthranilate R* in 1 ml of *hexane R*.

The chromatographic procedure may be carried out using:

- a fused-silica capillary column 25 m to 60 m long and about 0.25 mm in internal diameter, impregnated with *macrogol 20 000 R* as the bonded phase,
- *helium for chromatography R* as the carrier gas at a flow rate of 1.5 ml/min,
- a flame-ionisation detector,
- a split ratio of 1:100,

maintaining the temperature of the column at 75 °C for 4 min, then raising the temperature at a rate of 4 °C/min to 230 °C and maintaining at 230 °C for 20 min, maintaining the temperature of the injection port and of the detector at 270 °C.

Inject about 0.1 µl of the reference solution. When the chromatograms are recorded in the prescribed conditions, the components elute in the order indicated in the composition of the reference solution. Record the retention times of these substances.

The test is not valid unless: the number of theoretical plates is not less than 30 000, calculated from the limonene peak at 110 °C; the resolution between the peaks due to β -pinene and to sabinene is not less than 1.5.

Inject about 0.2 μ l of the substance to be examined. Using the retention times determined from the chromatogram obtained with the reference solution, locate the components of the reference solution on the chromatogram obtained with the test solution (disregard the peak due to hexane).

Determine the percentage content of each of these components by the normalisation procedure.

The percentages are within the following ranges:

- *β -pinene*: 7.0 per cent to 17.0 per cent,
- *limonene*: 9.0 per cent to 18.0 per cent,
- *linalol*: 18.0 per cent to 42.0 per cent,
- *linalyl acetate*: 3.0 per cent to 16.0 per cent,
- *α -terpineol*: 2.0 per cent to 7.0 per cent,
- *neryl acetate*: 1.0 per cent to 3.0 per cent,
- *geranyl acetate*: 1.5 per cent to 4.0 per cent,
- *trans-nerolidol*: 1.0 per cent to 9.0 per cent,
- *methhyl anthranilate*: 0.1 per cent to 1.0 per cent.

STORAGE

Store in a well-filled, airtight container, protected from light and heat.

01/2005:1858

BLACK HOREHOUND

Ballotae nigrae herba

DEFINITION

Dried flowering tops of *Ballota nigra* L.

Content: minimum 1.5 per cent of total *ortho*-dihydroxycinnamic acid derivatives, expressed as acteoside ($C_{29}H_{36}O_{15}$; M_r 624.6) (dried drug).

CHARACTERS

Macroscopic and microscopic characters described under identification tests A and B.

IDENTIFICATION

- A. Stems conspicuously four-angled, longitudinally striated, dark green or reddish-brown and more or less pubescent. Leaves greyish-green, petiolate, lamina ovate to orbicular, 2 cm to 4 cm wide, margin irregularly crenate, cuneate to cordate at the base; both surfaces covered with abundant whitish hairs; venation pinnate, prominent on the lower surface, slightly depressed on the upper. Flowers sessile or very shortly pedicellate, calyx infundibuliform, densely pubescent, with 10 prominent ribs and 5 subequal, broadly ovate teeth; corolla purple, tube slightly shorter than the calyx tube, bilabiate, the upper lip pubescent on the outer surface, the lower lip with 3 lobes, the middle lobe notched.
- B. Reduce to a powder (355). The powder is greyish-green and slightly flocculent. Examine under a microscope using *chloral hydrate solution R*. The powder shows numerous long, uniseriate, multicellular covering trichomes consisting of 4 or more cells, thickened and swollen at the junctions, with slightly lignified and pitted walls; fewer glandular trichomes, some with a unicellular or multicellular stalk and a globose, uni- or bicellular head, others with a unicellular stalk and a multicellular

head; fragments of the leaf epidermis with sinuous walls, those from the lower epidermis with numerous stomata, some diacytic (2.8.3) but the majority anomocytic; epidermis of the corolla composed of polygonal cells, those of the inner epidermis papillose; pollen grains subspherical with 3 pores and a smooth exine; groups of collenchyma and lignified, spirally thickened and bordered pitted vessels, from the stem.

C. Thin-layer chromatography (2.2.27).

Test solution. To 2 g of the powdered drug (355) add 100 ml of *methanol R*. Heat on a water-bath under a reflux condenser for 30 min. Allow to cool. Filter. Evaporate the filtrate under reduced pressure until a volume of about 10 ml is obtained.

Reference solution. Dissolve 2.5 mg of *rutin R* and 1 mg of *chlorogenic acid R* in 10 ml of *methanol R*.

Plate: TLC silica gel plate *R*.

Mobile phase: *anhydrous formic acid R*, *glacial acetic acid R*, *water R*, *ethyl acetate R* (7.5:7.5:18:67 V/V/V/V).

Application: 20 μ l, as bands.

Development: over a path of 15 cm.

Drying: in air.

Detection: spray with a solution containing 10 g/l of *diphenylboric acid aminoethyl ester R* and 50 g/l of *macrogol 400 R* in *methanol R*. Allow to dry in a current of warm air. Examine in ultraviolet light at 365 nm after 30 min.

Results: see below the sequence of the zones present in the chromatogram obtained with the reference solution and the test solution. Furthermore, other fluorescent zones may be present in the chromatogram obtained with the test solution.

Top of the plate	
Chlorogenic acid: a light blue fluorescent zone Rutin: an orange-yellow fluorescent zone	A reddish fluorescent zone
	A faint yellow fluorescent zone
	A light blue fluorescent zone (caffeoylmalic acid)
	A greenish-blue fluorescent zone (acteoside)
	A yellow-brown fluorescent zone (luteolin 7-lactate)
	A greenish-blue fluorescent zone (forsythoside B)
	2 greenish-blue fluorescent zones (arenarioside)
	A yellow fluorescent zone (luteolin 7-lactate glucoside).
	A faint greenish-blue fluorescent zone (ballotetroside).
Reference solution	Test solution

TESTS

Foreign matter (2.8.2): maximum 2 per cent *m/m*.

Loss on drying (2.2.32): maximum 12.0 per cent, determined on 1.000 g of the powdered drug (355) in an oven at 100-105 °C for 2 h.

Total ash (2.4.16): maximum 13.0 per cent.

ASSAY

Stock solution. Place 1.000 g of the powdered drug (355) in a flask. Add 90 ml of *alcohol (50 per cent V/V) R*. Heat under a reflux condenser on a water-bath for 30 min. Allow to cool and filter collecting the filtrate into a 100 ml

volumetric flask. Rinse the flask and the filter with 10 ml of *alcohol* (50 per cent V/V) *R*. Add the rinsings to the filtrate and dilute to 100.0 ml with *alcohol* (50 per cent V/V) *R*.

Test solution. In a 10 ml volumetric flask add successively, with shaking after each addition, 1.0 ml of the stock solution, 2 ml of 0.5 *M* hydrochloric acid, 2 ml of a solution containing 100 g/l of sodium nitrite *R* and 100 g/l of sodium molybdate *R*, 2 ml of dilute sodium hydroxide solution *R* and dilute to 10.0 ml with water *R*.

Compensation liquid. In a 10 ml volumetric flask, add 1.0 ml of the stock solution, 2 ml of 0.5 *M* hydrochloric acid, 2 ml of dilute sodium hydroxide solution *R* and dilute to 10.0 ml with water *R*.

Measure immediately the absorbance (2.2.25) of the test solution, by comparison with the compensation liquid at 525 nm.

Calculate the percentage content of total *ortho*-dihydroxycinnamic acid derivatives, calculated as acteoside, from the expression:

$$\frac{A \times 1000}{185 \times m}$$

i.e. taking the specific absorbance of acteoside to be 185 at 525 nm.

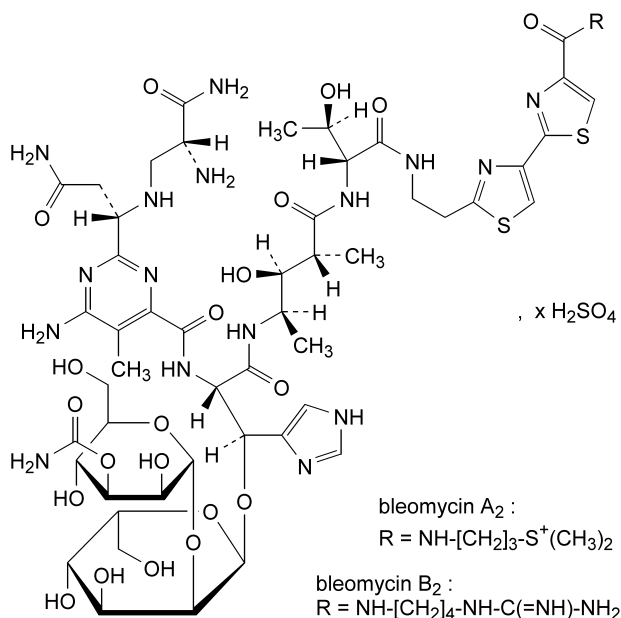
A = absorbance of the test solution at 525 nm,

m = mass of the substance to be examined, in grams.

01/2005:0976

BLEOMYCIN SULPHATE

Bleomycini sulfas



DEFINITION

Bleomycin sulphate is the sulphate of a mixture of glycopeptides produced by *Streptomyces verticillus* or by any other means; the two principal components of the mixture are *N*1-[3-(dimethylsulphonio)propyl]bleomycinamide (bleomycin A₂) and *N*1-4-(guanidobutyl)bleomycinamide (bleomycin B₂). The potency is not less than 1500 IU/mg, calculated with reference to the dried substance.

CHARACTERS

A white or yellowish-white powder, very hygroscopic, very soluble in water, slightly soluble in ethanol, practically insoluble in acetone.

IDENTIFICATION

A. Examine the chromatograms obtained in the test for composition. The retention times and sizes of the two principal peaks in the chromatogram obtained with the test solution are approximately the same as those of the two principal peaks in the chromatogram obtained with reference solution (a).

B. It gives the reactions of sulphates (2.3.1).

TESTS

Appearance of solution. Dissolve 0.200 g in water *R* and dilute to 10.0 ml with the same solvent. The solution is clear (2.2.1). The absorbance (2.2.25) measured at 430 nm is not greater than 0.10.

pH (2.2.3). Dissolve 50 mg in carbon dioxide-free water *R* and dilute to 10 ml with the same solvent. The pH of the solution is 4.5 to 6.0.

Composition. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 25.0 mg of the substance to be examined in water *R* and dilute to 50.0 ml with the same solvent.

Reference solution (a). Dissolve 25.0 mg of bleomycin sulphate CRS in water *R* and dilute to 50.0 ml with the same solvent.

Reference solution (b). Dilute 1.5 ml of reference solution (a) to 100.0 ml with water *R*.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4.6 mm in internal diameter packed with octadecylsilyl silica gel for chromatography *R* (7 µm),
- gradient elution at a flow rate of 1.2 ml/min with a mobile phase initially composed of 10 per cent V/V of methanol *R* and 90 per cent V/V of a mixture prepared as follows: dissolve 0.960 g of sodium pentanesulphonate *R* in 900 ml of acetic acid (4.8 g/l C₂H₄O₂), add 1.86 g of sodium edetate *R*, dilute to 1000 ml with the same solvent and adjust to pH 4.3 using ammonia *R*; increasing the proportion of methanol *R* to 40 per cent V/V over 60 min and continuing with the final mixture for about 20 min, until demethylbleomycin A₂ is eluted (retention time 1.5 to 2.5, relative to bleomycin A₂),
- as detector a spectrophotometer set at 254 nm,
- a 20 µl loop injector.

Inject reference solution (a). The test is not valid unless the resolution between the two principal peaks is at least 5. Inject reference solution (b). The test is not valid unless the signal-to-noise ratio calculated for the principal peak is at least 20.

Inject reference solution (a) six times. The test is not valid unless the relative standard deviation of the area of the principal peak is at most 2 per cent.

Inject the test solution. The composition, calculated by the normalisation procedure and disregarding any peak with an area less than 0.1 per cent of the total, is: bleomycin A₂ (first principal peak) 55 per cent to 70 per cent; bleomycin B₂ (second principal peak) 25 per cent to 32 per cent; sum of bleomycin A₂ and bleomycin B₂ not less than 85 per cent; demethylbleomycin A₂ (retention time relative to bleomycin A₂ 1.5 to 2.5) not more than 5.5 per cent; other related substances not more than 9.5 per cent.

Copper. Not more than 200 ppm of Cu, determined by atomic absorption spectrometry (2.2.23, *Method I*).

Test solution. Dissolve 50 mg in *water R* and dilute to 10.0 ml with the same solvent.

Reference solution. Dilute 1.0 ml of *copper standard solution (10 ppm Cu) R* to 10.0 ml with *water R*.

Measure the absorbance at 324.7 nm using a copper hollow-cathode lamp as source of radiation and an air-acetylene flame.

Loss on drying (2.2.32). Not more than 3.0 per cent, determined on 50 mg by drying at 60 °C at a pressure not exceeding 670 Pa for 3 h.

Bacterial endotoxins (2.6.14): less than 5 IU/mg, if intended for use in the manufacture of parenteral dosage forms without a further appropriate procedure for the removal of bacterial endotoxins.

ASSAY

Carry out the microbiological assay of antibiotics (2.7.2), using the diffusion method. Use *bleomycin sulphate CRS* as the reference substance.

STORAGE

Store in an airtight container, at a temperature of 2 °C to 8 °C. If the substance is sterile, store in a sterile, airtight, tamper-proof container.

LABELLING

The label states, where applicable, that the substance is free from bacterial endotoxins.

IMPURITIES

- A. R = OH: bleomycinic acid,
 B. R = NH-[CH₂]₃-NH-[CH₂]₄-NH₂: bleomycin A₅,
 C. R = NH-[CH₂]₄-NH-C(=NH)-NH-[CH₂]₄-NH-C(=NH)-NH₂: bleomycin B₄,
 D. R = NH-[CH₂]₃-S-CH₃: demethylbleomycin A₂.

01/2005:1605

BOGBEAN LEAF

Menyanthidis trifoliatae folium

DEFINITION

Dried, entire or fragmented leaf of *Menyanthes trifoliata* L.

CHARACTERS

Very bitter and persistent taste.

Macroscopic and microscopic characters described under identification tests A and B.

IDENTIFICATION

- A. The leaf is long-petiolated, trifoliate, with long sheaths from the base; the petiole is up to 5 mm in diameter and strongly striated longitudinally. The lamina is divided into equal leaflets, sessile, obovate up to 10 cm long and up to 5 cm wide, with an entire, occasionally sinuous margin with brownish or reddish hydathodes and a spatulate base; it is glabrous, dark green on the upper surface and paler green on the lower surface, with a wide, whitish, finely striated prominent midrib.
- B. Reduce to a powder (355). The powder is yellowish-green. Examine under a microscope using *chloral hydrate solution R*. The powder shows fragments of upper epidermis with polyhedral cells and thin wavy walls; fragments of lower epidermis with sinuous walls;

anomocytic stomata (2.8.3), on both surfaces, with the subsidiary cells showing radiating striations; epidermal cells from the veins straight walled and papillose; fragments of mesophyll parenchyma with large intercellular spaces (aerenchyma); irregular cells with rare sclereids; fragments of spiral or annular vessels.

C. Thin-layer chromatography (2.2.27).

Test solution. To 1.0 g of the powdered drug (355) add 10 ml of *methanol R*. Heat, with stirring, in a water-bath at 60 °C for 5 min. Allow to cool and filter. Evaporate to dryness under reduced pressure in a water-bath at 60 °C. Dissolve the residue in 2.0 ml of *methanol R*.

Reference solution. Dissolve 5 mg of *loganin R* in 15 ml of *methanol R*.

Plate: *TLC silica gel plate R*.

Mobile phase: *water R, methanol R, ethyl acetate R* (8:15:77 V/V/V).

Application: 30 µl, as bands.

Development: over a path of 15 cm.

Drying: in air.

Detection: spray with *vanillin reagent R*. Heat in an oven at 100-105 °C for 10 min. Examine in daylight.

Results: see below the sequence of the zones present in the chromatograms obtained with the reference and test solutions. Furthermore, other zones are present in the chromatogram obtained with the test solution.

Top of the plate	
Loganin: a greyish-violet zone	A violet zone
	An intense blue zone
	A violet to greyish-violet zone
	A grey to greyish-blue zone
	A brownish zone
Reference solution	Test solution

TESTS

Foreign matter (2.8.2): it complies with the test for foreign matter.

Loss on drying (2.2.32): maximum 10.0 per cent, determined on 1.000 g of the powdered drug (355) by drying in an oven at 100-105 °C for 2 h.

Total ash (2.4.16): maximum 10.0 per cent.

Bitterness value (2.8.15): minimum 3000.

01/2005:1396

BOLDO LEAF

Boldi folium

DEFINITION

Boldo leaf consists of the whole or fragmented dried leaf of *Peumus boldus* Molina. The whole drug contains not less than 20.0 ml/kg and not more than 40.0 ml/kg and the fragmented drug not less than 15.0 ml/kg of essential oil. It contains not less than 0.1 per cent of total alkaloids, expressed as boldine, (C₁₉H₂₁NO₄; *M_r* 327.4), calculated with reference to the anhydrous drug.

CHARACTERS

Boldo leaf has an aromatic odour especially when rubbed.

It has the microscopic and macroscopic characters described under Identification tests A and B.

IDENTIFICATION

A. The leaf is oval or elliptical usually 5 cm long with a short petiole, an obtuse or slightly emarginate or mucronate apex and an equal and rounded base; the margin is entire and slightly undulate and the thickened edges are more or less revolute. The lamina is greyish-green, thick, tough and brittle. The upper surface is rough with numerous prominent small protuberances and a depressed venation. The lower surface is finely pubescent, with the protuberances less well-marked, and a prominent, pinnate venation.

B. Reduce to a powder (355). The powder is greyish-green. Examine under a microscope, using *chloral hydrate solution R*. The powder shows fragments of the upper epidermis and underlying hypodermis with straight or slightly sinuous thickened and beaded walls, those of the lower epidermis with numerous stomata surrounded by four to seven subsidiary cells; solitary, bifurcated or stellate clustered unicellular covering trichomes with more or less thickened and lignified wall; fragments of the lamina showing a two-layered palisade; debris of the spongy mesophyll including numerous, large rounded oil cells and parenchyma containing fine needle-shaped crystals; thick walled fibres and lignified, pitted parenchymatous cells associated with vascular tissue from the veins.

C. Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel plate R*.

Test solution. Add to 0.5 g of the powdered drug (355) a mixture of 1 ml of *dilute hydrochloric acid R* and 20 ml of *water R* and heat on a water-bath under reflux for 10 min. Cool and filter. Add to the filtrate 2 ml of *dilute ammonia R1* and extract with two quantities, each of 20 ml of *ether R* avoiding emulsifying. Combine the organic layers and evaporate the solvent on a water-bath. Dissolve the residue in 1.0 ml of *methanol R*.

Reference solution. Dissolve 2 mg of *boldine R* in 5 ml of *methanol R*.

Apply to the plate as bands 20 µl of the test solution and 10 µl of the reference solution. Develop over a path of 15 cm using a mixture of 10 volumes of *methanol R*, 10 volumes of *diethylamine R* and 80 volumes of *toluene R*. Allow the plate to dry in air. Spray the plate with *potassium iodobismuthate solution R2*. Allow the plate to dry in air for 5 min and then spray the plate with *sodium nitrite solution R*. Examine in daylight. The chromatograms show in the lower third the brown to reddish-brown zone of boldine. The chromatogram obtained with the test solution shows several brownish zones above and below the zone corresponding to boldine.

TESTS

Foreign matter (2.8.2). Not more than 4 per cent of twigs and 2 per cent of other foreign matter.

Water (2.2.13). Not more than 100 ml/kg determined by distillation of 20.0 g of the powdered drug (355).

Total ash (2.4.16). Not more than 13.0 per cent.

ASSAY

Essential oil. Carry out the determination of essential oils in vegetable drugs (2.8.12). Use 10.0 g of the freshly crushed drug, a 1000 ml flask and 300 ml of *water R* as the distillation liquid. Distil at a rate of 2 ml/min to 3 ml/min for 3 h.

Alkaloids. Examine by liquid chromatography (2.2.29).

Test solution. To 1.000 g (m_1) of the powdered drug (355) add 50 ml of *dilute hydrochloric acid R*. Shake in a

water-bath at 80 °C for 30 min. Filter, take up the residue with 50 ml of *dilute hydrochloric acid R* and shake in a water-bath at 80 °C for 30 min. Filter and repeat the operation once on the residue obtained. Filter. Combine the cooled filtrates and shake with 100 ml of a mixture of equal volumes of *ethyl acetate R* and *hexane R*. Adjust the aqueous layer to pH 9.5 with *dilute ammonia R1*. Shake successively with 100 ml, 50 ml and 50 ml of *methylene chloride R* and combine the lower layers and evaporate to dryness under reduced pressure. In a 10.0 ml volumetric flask dilute the residue to 10.0 ml with the mobile phase.

Reference solution. In a 100.0 ml volumetric flask dissolve 12 mg (m_2) of *boldine R* in 100.0 ml of the mobile phase. Dilute 1.0 ml of the solution to 10.0 ml with the mobile phase.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4.6 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (5 µm),
- as mobile phase at a flow rate of 1.5 ml/min a mixture of 16 volumes of solution A and 84 volumes of solution B,

Solution A. Mix 99.8 ml of *acetonitrile R* and 0.2 ml of *diethylamine R*,

Solution B. Mix 99.8 ml of *water R* and 0.2 ml of *diethylamine R*, adjusted to pH 3 with *formic acid R*,

– as detector a spectrophotometer set at 304 nm.

Inject 20 µl of each solution. When the chromatograms are recorded in the prescribed conditions, the retention times relative to boldine are: isoboldine about 0.9; isocorydine *N*-oxide about 1.8; laurotetanine about 2.2; isocorydine about 2.8 and *N*-methyllaurotetanine about 3.2. Additional peaks may be present.

Calculate the percentage content of total alkaloids expressed as boldine from the expression:

$$\frac{\sum A_1 \times m_2}{A_2 \times m_1}$$

m_1 = mass of the substance to be examined, in grams,

m_2 = mass of *boldine R*, in grams,

ΣA_1 = sum of the areas of the peaks due to the six alkaloids identified in the chromatogram obtained with the test solution,

A_2 = area of the peak due to boldine in the chromatogram obtained with the reference solution.

STORAGE

Store protected from light.

01/2005:0013

BORAX

Borax

$\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$

M_r 381.4

DEFINITION

Borax contains not less than 99.0 per cent and not more than the equivalent of 103.0 per cent of disodium tetraborate decahydrate.

CHARACTERS

A white, crystalline powder, colourless crystals or crystalline masses, efflorescent, soluble in water, very soluble in boiling water, freely soluble in glycerol.

IDENTIFICATION

- A. To 1 ml of solution S (see Tests) add 0.1 ml of *sulphuric acid R* and 5 ml of *methanol R* and ignite. The flame has a green border.
- B. To 5 ml of solution S add 0.1 ml of *phenolphthalein solution R*. The solution is red. On the addition of 5 ml of *glycerol (85 per cent) R* the colour disappears.
- C. Solution S gives the reactions of sodium (2.3.1).

TESTS

Solution S. Dissolve 4.0 g in *carbon dioxide-free water R* prepared from *distilled water R* and dilute to 100 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and colourless (2.2.2, *Method II*).

pH (2.2.3). The pH of solution S is 9.0 to 9.6.

Sulphates (2.4.13). 15 ml of solution S complies with the limit test for sulphates (50 ppm). Use 1.0 ml of *acetic acid R* instead of the 0.5 ml prescribed. Prepare the standard using a mixture of 3 ml of *sulphate standard solution (10 ppm SO₄) R* and 12 ml of *distilled water R*.

Ammonium (2.4.1). 6 ml of solution S diluted to 14 ml with *water R* complies with the limit test for ammonium (10 ppm). Prepare the standard using a mixture of 2.5 ml of *ammonium standard solution (1 ppm NH₄) R* and 7.5 ml of *water R*.

Arsenic (2.4.2). 5 ml of solution S complies with limit test A for arsenic (5 ppm).

Calcium (2.4.3). 15 ml of solution S complies with the limit test for calcium (100 ppm). Prepare the standard using a mixture of 6 ml of *calcium standard solution (10 ppm Ca) R* and 9 ml of *distilled water R*.

Heavy metals (2.4.8). 12 ml of solution S complies with limit test A for heavy metals (25 ppm). Prepare the standard using *lead standard solution (1 ppm Pb) R*.

ASSAY

Dissolve 20 g of *mannitol R* in 100 ml of *water R*, heating if necessary, cool, add 0.5 ml of *phenolphthalein solution R* and neutralise with 0.1 M *sodium hydroxide* until a pink colour is obtained. To this solution add 3.00 g of the substance to be examined, heat until dissolution is complete, cool, and titrate with 1 M *sodium hydroxide* until the pink colour reappears.

1 ml of 1 M *sodium hydroxide* is equivalent to 0.1907 g of Na₂B₄O₇·10H₂O.

01/2005:0001

BORIC ACID

Acidum boricum

H₃BO₃M_r 61.8

DEFINITION

Boric acid contains not less than 99.0 per cent and not more than the equivalent of 100.5 per cent of H₃BO₃.

CHARACTERS

A white, crystalline powder, colourless, shiny plates greasy to the touch, or white crystals, soluble in water and in alcohol, freely soluble in boiling water and in glycerol (85 per cent).

IDENTIFICATION

- A. Dissolve 0.1 g by gently heating in 5 ml of *methanol R*, add 0.1 ml of *sulphuric acid R* and ignite the solution. The flame has a green border.
- B. Solution S (see Tests) is acid (2.2.4).

TESTS

Solution S. Dissolve 3.3 g in 80 ml of boiling *distilled water R*, cool and dilute to 100 ml with *carbon dioxide-free water R* prepared from *distilled water R*.

Appearance of solution. Solution S is clear (2.2.1) and colourless (2.2.2, *Method II*).

pH (2.2.3). The pH of solution S is 3.8 to 4.8.

Solubility in alcohol. Dissolve 1.0 g in 10 ml of boiling *alcohol R*. The solution is not more opalescent than reference suspension II (2.2.1) and is colourless (2.2.2, *Method II*).

Organic matter. It does not darken on progressive heating to dull redness.

Sulphates (2.4.13). 10 ml of solution S diluted to 15 ml with *distilled water R* complies with the limit test for sulphates (450 ppm).

Heavy metals (2.4.8). 12 ml of solution S complies with limit test A for heavy metals (15 ppm). Prepare the standard using a mixture of 2.5 ml of *lead standard solution (2 ppm Pb) R* and 7.5 ml of *water R*.

ASSAY

Dissolve 1.000 g with heating in 100 ml of *water R* containing 15 g of *mannitol R*. Titrate with 1 M *sodium hydroxide*, using 0.5 ml of *phenolphthalein solution R* as indicator, until a pink colour is obtained.

1 ml of 1 M *sodium hydroxide* is equivalent to 61.8 mg of H₃BO₃.

01/2005:2113

BOTULINUM TOXIN TYPE A
FOR INJECTION

Toxinum botulinicum typum A ad iniectabile

DEFINITION

Botulinum toxin type A for injection is a dried preparation containing purified botulinum neurotoxin type A which may be present in the form of a complex with haemagglutinins and non-toxic proteins. Botulinum neurotoxin type A or its haemagglutinin complex is prepared by a suitable purification process of the liquid supernatant from a broth-culture of a suitable strain of *Clostridium botulinum* type A.

The purified complexes consist of several proteins and can be of various sizes. The largest complex (relative molecular mass of about 900 000) consists of a 150 000 relative molecular mass neurotoxin, a 130 000 relative molecular mass non-toxic protein and various haemagglutinins ranging between relative molecular mass 14 000 and 43 000. The purified toxin moiety is composed of only the same 150 000 relative molecular mass neurotoxin as is found in the 900 000 relative molecular mass neurotoxin complex, which is initially produced as a single chain and further cleaved (nicked) by endogenous proteases into a fully active, disulphide-linked, 54 000 relative molecular mass light chain and a 97 000 relative molecular mass heavy chain.

The preparation is reconstituted before use, as stated on the label.

PRODUCTION

GENERAL PROVISIONS

Production of the toxin is based on seed cultures, managed in a defined seed-lot system in which the ability to produce toxin is conserved. The production method must be shown to yield consistently product of activity and profile comparable to that of lots shown in clinical studies to be of adequate safety and efficacy.

The production method is validated to demonstrate that the product, if tested, would comply with the general test of abnormal toxicity (2.6.9) using not less than the maximum human clinical dose, in the presence of a suitable amount of specific botulinum type A antitoxin used for neutralisation.

The production method and stability of the finished product and relevant intermediates are evaluated using the tests below. Such tests include the specific toxin activity per milligram of protein of purified toxin in an appropriate functional model of toxin activity and may be supported by tests confirming the presence of botulinum toxin type A, and, if appropriate, associated non-toxic proteins.

BACTERIAL SEED LOTS

A highly toxigenic strain of *C. botulinum* of known toxin type A and confirmed absence of genes encoding other botulinum toxins (particularly botulinum toxin type B), with known origin and history, is grown using suitable media. The bacterial strain, used for the master seed lot, shall be identified by historical records that include information on its origin and the tests used to characterise the strain. These will include morphological, cultural, biochemical, genetic and serological properties of the strain. The master seed lot and the working seed lot, where applicable, must be demonstrated to have identical profiles. Only a seed lot that complies with the following requirements may be used.

Identification. Each seed lot is identified as containing pure cultures of *C. botulinum* type A bacteria with no extraneous bacterial or fungal contamination.

Microbial purity. Each seed lot complies with the requirements for absence of contaminating micro-organisms. The purity of bacterial cultures is verified by methods of suitable sensitivity. These may include inoculation into suitable media and examination of colony morphology.

Phenotypic parameters. Each seed lot must have a known fatty acid profile, sugar fermentation profile (glucose, lactose, mannose, etc.) and proteolytic activity and must demonstrate relevant lipase, lecithinase and gelatinase activity.

Genetic purity. Each seed lot must have information on the toxin gene sequence and comply with requirements for the absence of other genes encoding other toxin serotypes.

Production of active toxin. A bacterial strain producing a high yield of active toxin, as determined by an acute toxicity assay, is suitable. Seed lots should demonstrate a capability of producing at least a minimum toxicity level appropriate for the manufacturing process and scale.

MANUFACTURER'S REFERENCE PREPARATIONS

During development, reference preparations are established for subsequent verification of batch consistency during production and for control of the bulk purified toxin and finished product. They are derived from representative batches of botulinum toxin type A that are characterised as described under Bulk Purified Toxin.

The reference preparations are suitably characterised for their intended purpose and are stored in suitably sized aliquots under conditions ensuring their suitability.

BULK PURIFIED TOXIN

C. botulinum type A strain is grown anaerobically, in suitable media, from which cultures are selected for step-up incubations under a suitably controlled anaerobic atmosphere through the seed culture and bulk fermentation stages to allow maximum production of toxin. The toxin is purified by suitable methods to remove nucleic acids and components likely to cause adverse reactions.

Only a purified toxin that complies with the following requirements may be used in the preparation of the final bulk. For each test and for each product, limits of acceptance are established and each new purified toxin must comply with these limits.

Residual reagents. Removal of residual reagents used in purification steps is confirmed by suitable limit tests or by validation of the process.

Nucleic acids. Removal of nucleic acids is confirmed by suitable limit tests or by validation of the process.

Immunological identity. The presence of specific type A toxin is confirmed by a suitable immunochemical method (2.7.1).

Specific activity. The specific activity is confirmed in a mouse model of toxicity or by *in vivo/ex vivo* methods validated with respect to the LD₅₀ assay and expressed in mouse LD₅₀ units per milligram of protein. Specific activity must not be less than 1×10^8 mouse LD₅₀ units per milligram of protein for the 150 000 relative molecular mass neurotoxin and must not be less than 1×10^7 mouse LD₅₀ units per milligram of protein for the 900 000 relative molecular mass neurotoxin complex.

Protein. The total protein concentration is determined by a suitable method. An acceptable value is established for the product and each batch must be shown to comply with the limits.

Protein profile. Identity and protein composition are determined by polyacrylamide gel electrophoresis (2.2.31) under reducing or non-reducing conditions or by other suitable physicochemical methods such as size-exclusion chromatography (2.2.30), comparing with suitable reference standards.

Total viable count. It complies with the limits approved for the particular product.

FINAL BULK

The final bulk is prepared by adding approved excipients to the bulk purified toxin. The solution is filtered through a bacteria-retentive filter. If human albumin is added, it complies with the monograph on *Human albumin solution* (0255).

FINAL LOT

The final bulk is distributed aseptically into sterile, tamper-proof containers. Uniformity of fill is verified during filling and the test for uniformity of content (2.9.6) is not required. The containers are closed so as to prevent contamination.

Only a final lot that is within the limits approved for the particular product and is satisfactory with respect to each of the requirements given below under Identification, Tests and Assay may be released for use.

pH (2.2.3). The pH of the reconstituted product is within ± 0.5 pH units of the limit approved for the particular product.

Water: not more than the limit approved for the particular product.

IDENTIFICATION

The presence of botulinum toxin type A is confirmed by a suitable immunochemical method (2.7.1).

TESTS

Sterility (2.6.1). It complies with the test for sterility.

Bacterial endotoxins (2.6.14): less than 10 IU per vial.

ASSAY

The potency of the reconstituted product is determined by an LD50 assay in mice or by a method validated with respect to the LD50 assay. The potency is expressed in terms of the LD50 for mice or relative to the reference preparation.

For determination of the LD50, graded doses of the product are injected intraperitoneally into groups of mice and the LD50 is calculated by the usual statistical methods (5.3) from the mouse lethality in each group. A suitable reference preparation is assayed in parallel; the potency of the toxin is expressed relative to the reference or the value found for the reference is within suitable limits defined in terms of the assigned potency.

After validation with respect to the LD50 assay (reference method), the product may also be assayed by other methods that are preferable in terms of animal welfare, including 1 of the following:

1. endopeptidase assay *in vitro*;
2. *ex vivo* assay using the mouse phrenic nerve diaphragm;
3. mouse bioassay using paralysis as the end-point.

For these other methods, the potency is calculated with respect to a suitable reference preparation calibrated in mouse LD50 units.

The estimated potency is not less than 80 per cent and not more than 125 per cent of the stated potency. The confidence limits ($P = 0.95$) are not less than 80 per cent and not more than 125 per cent of the estimated potency.

The test may be repeated but when more than 1 test is performed, the results of all valid tests must be combined in the estimate of potency.

LABELLING

The label states:

- the number of units of toxin per vial with a statement that units are product specific and not applicable to other preparations containing botulinum toxin type A,
- the name and the volume of the diluent to be added for reconstitution of a dried product.

DEFINITION

Bromazepam contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of 7-bromo-5-(pyridin-2-yl)-1,3-dihydro-2*H*-1,4-benzodiazepin-2-one, calculated with reference to the dried substance.

CHARACTERS

A white or yellowish, crystalline powder, practically insoluble in water, sparingly soluble in alcohol and in methylene chloride.

IDENTIFICATION

First identification: B.

Second identification: A, C, D, E.

- A. Dissolve 50.0 mg in *methanol R* and dilute to 100.0 ml with the same solvent. Dilute 1.0 ml of the solution to 100.0 ml with *methanol R*. Examined between 220 nm and 350 nm (2.2.25), the solution shows an absorption maximum at 233 nm and a shoulder at about 260 nm and may show a broad absorption maximum at about 325 nm. The specific absorbance at the maximum at 233 nm is 980 to 1080.
- B. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *bromazepam CRS*. Examine the substances prepared as discs.
- C. Examine by thin-layer chromatography (2.2.27), using as the coating substance a suitable silica gel with a fluorescent indicator having an optimal intensity at 254 nm.

Test solution. Dissolve 10 mg of the substance to be examined in a mixture of 1 volume of *methanol R* and 9 volumes of *methylene chloride R* and dilute to 10 ml with the same mixture of solvents.

Reference solution (a). Dissolve 10 mg of *bromazepam CRS* in a mixture of 1 volume of *methanol R* and 9 volumes of *methylene chloride R* and dilute to 10 ml with the same mixture of solvents.

Reference solution (b). Dissolve 10 mg of *bromazepam CRS* and 10 mg of *temazepam CRS* in a mixture of 1 volume of *methanol R* and 9 volumes of *methylene chloride R* and dilute to 10 ml with the same mixture of solvents.

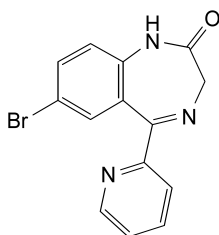
Apply separately to the plate 5 µl of each solution. Develop over a path of 10 cm using a mixture of 30 volumes of *diethylamine R* and 70 volumes of *ether R*. Dry the plate in a current of air and examine in ultraviolet light at 254 nm. The principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a). The test is not valid unless the chromatogram obtained with reference solution (b) shows two clearly separated principal spots.

- D. Dissolve about 20 mg in 5 ml of *methanol R*. Add 5 ml of *water R* and 1 ml of a 10 g/l solution of *ferrous ammonium sulphate R*. A violet colour develops.
- E. To 0.15 g in a porcelain crucible add 0.5 g of *anhydrous sodium carbonate R*. Heat over an open flame for 10 min. Allow to cool. Take up the residue in 10 ml of *dilute nitric acid R* and filter. To 1 ml of the filtrate add 1 ml of *water R*. The solution gives reaction (a) of bromides (2.3.1).

01/2005:0879

BROMAZEPAM

Bromazepamum

C₁₄H₁₀BrN₃OM_r 316.2

TESTS

Appearance of solution. Dissolve 0.5 g in a mixture of 1 volume of *methanol R* and 4 volumes of *tetrahydrofuran R* and dilute to 20 ml with the same mixture of solvents. The solution is clear (2.2.1).

Related substances. Examine by thin-layer chromatography (2.2.27), using *silica gel GF₂₅₄ R* as the coating substance. Prepare the solutions immediately before use and carry out the test protected from light.

Test solution. Dissolve 50 mg of the substance to be examined in a mixture of 1 volume of *methanol R* and 9 volumes of *methylene chloride R* and dilute to 5 ml with the same mixture of solvents.

Reference solution. Dilute 1 ml of the test solution to 20 ml with a mixture of 1 volume of *methanol R* and 9 volumes of *methylene chloride R*. Dilute 2 ml of the solution to 50 ml with a mixture of 1 volume of *methanol R* and 9 volumes of *methylene chloride R*.

Apply separately to the plate 5 µl of each solution. Develop over a path of 7.5 cm using a mixture of 5 volumes of *alcohol R*, 5 volumes of *triethylamine R*, 20 volumes of *methylene chloride R* and 70 volumes of *light petroleum R1*. Dry the plate in a current of air for 20 min and examine in ultraviolet light at 254 nm. Any spot in the chromatogram obtained with the test solution, apart from the principal spot, is not more intense than the spot in the chromatogram obtained with the reference solution (0.2 per cent).

Loss on drying (2.2.32). Not more than 0.2 per cent, determined on 1.000 g by drying at 80 °C at a pressure not exceeding 2.7 kPa for 4 h.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

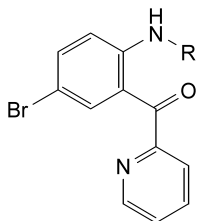
ASSAY

Dissolve 0.250 g in 20 ml of *anhydrous acetic acid R*. Add 50 ml of *acetic anhydride R*. Titrate with 0.1 M *perchloric acid*, determining the end-point potentiometrically (2.2.20). 1 ml of 0.1 M *perchloric acid* is equivalent to 31.62 mg of C₁₄H₁₀BrN₃O.

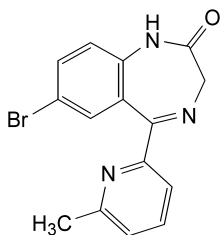
STORAGE

Store protected from light.

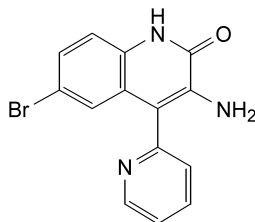
IMPURITIES



- A. R = H: (2-amino-5-bromophenyl)(pyridin-2-yl)methanone,
 B. R = CO-CH₂-Cl: *N*-[4-bromo-2-(pyridin-2-yl)carbonyl]phenyl]-2-chloroacetamide,



- C. 7-bromo-5-(6-methylpyridin-2-yl)-1,3-dihydro-2H-1,4-benzodiazepin-2-one,

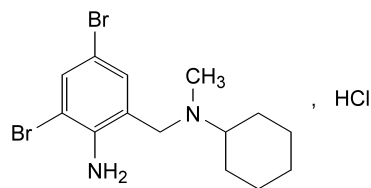


- D. 3-amino-6-bromo-4-(pyridin-2-yl)quinolin-2(1H)-one.

01/2005:0706

BROMHEXINE HYDROCHLORIDE

Bromhexini hydrochloridum



C₁₄H₂₁Br₂ClN₂

M_r 412.6

DEFINITION

N-(2-Amino-3,5-dibromobenzyl)-*N*-methylcyclohexanamine hydrochloride.

Content: 98.5 per cent to 101.5 per cent (dried substance).

CHARACTERS

Appearance: white or almost white, crystalline powder.

Solubility: very slightly soluble in water, slightly soluble in alcohol and in methylene chloride.

It shows polymorphism.

IDENTIFICATION

First identification: A, E.

Second identification: B, C, D, E.

A. Infrared absorption spectrophotometry (2.2.24).

Comparison: *bromhexine hydrochloride CRS*.

If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in *methanol R*, evaporate to dryness and record new spectra using the residues.

B. Thin-layer chromatography (2.2.27).

Test solution. Dissolve 20 mg of the substance to be examined in *methanol R* and dilute to 10 ml with the same solvent.

Reference solution. Dissolve 20 mg of *bromhexine hydrochloride CRS* in *methanol R* and dilute to 10 ml with the same solvent.

Plate: TLC silica gel *F₂₅₄* plate *R*.

Mobile phase: glacial acetic acid *R*, water *R*, butanol *R* (17:17:66 V/V/V).

Application: 20 µl.

Development: over 3/4 of the plate.

Drying: in air.

Detection: examine in ultraviolet light at 254 nm.

Results: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with the reference solution.

- C. Dissolve about 25 mg in a mixture of 1 ml of *dilute sulphuric acid R* and 50 ml of *water R*. Add 2 ml of *methylene chloride R* and 5 ml of *chloramine solution R* and shake. A brownish-yellow colour develops in the lower layer.
- D. Dissolve about 1 mg in 3 ml of *0.1 M hydrochloric acid*. The solution gives the reaction of primary aromatic amines (2.3.1).
- E. Dissolve about 20 mg in 1 ml of *methanol R* and add 1 ml of *water R*. The solution gives reaction (a) of chlorides (2.3.1).

TESTS

Appearance of solution. The solution is clear (2.2.1) and not more intensely coloured than reference solution Y₆ (2.2.2, Method II).

Dissolve 0.6 g in *methanol R* and dilute to 20 ml with the same solvent.

Related substances. Liquid chromatography (2.2.29).

Test solution. Dissolve 50 mg of the substance to be examined in *methanol R* and dilute to 10.0 ml with the same solvent.

Reference solution (a). Dissolve 5 mg of *bromhexine impurity C CRS* in *methanol R*, add 1.0 ml of the test solution and dilute to 10.0 ml with the same solvent.

Reference solution (b). Dilute 1.0 ml of the test solution to 100.0 ml with *methanol R*. Dilute 1.0 ml of this solution to 10.0 ml with *methanol R*.

Column:

- size: $l = 0.12$ m, $\varnothing = 4.6$ mm,
- stationary phase: end-capped octadecylsilyl silica gel for chromatography R (3 μ m).

Mobile phase: mix 0.50 ml of *phosphoric acid R* in 950 ml of *water R*, adjust to pH 7.0 with *triethylamine R* (about 1.5 ml) and dilute to 1000 ml with *water R*; mix 20 volumes of this solution with 80 volumes of *acetonitrile R*.

Flow rate: 1.0 ml/min.

Detection: spectrophotometer at 248 nm.

Injection: 10 μ l.

Run time: 2.5 times the retention time of bromhexine.

Relative retention with reference to bromhexine (retention time = about 11 min): impurity A = about 0.1; impurity B = about 0.2; impurity C = about 0.4; impurity D = about 0.5.

System suitability: reference solution (a):

- resolution: minimum 12.0 between the peaks due to impurity C and bromhexine.

Limits:

- any impurity: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent), and not more than 1 such peak has an area greater than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent),
- total: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.3 per cent),
- disregard limit: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

Loss on drying (2.2.32): maximum 1.0 per cent, determined on 1.000 g by drying in an oven at 100–105 °C.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.300 g in 70 ml of *alcohol R* and add 1 ml of *0.1 M hydrochloric acid*. Carry out a potentiometric titration (2.2.20), using *0.1 M sodium hydroxide*. Read the volume between the 2 points of inflexion.

1 ml of *0.1 M sodium hydroxide* is equivalent to 41.26 mg of C₁₄H₂₁Br₂ClN₂.

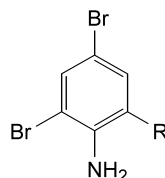
STORAGE

Protected from light.

IMPURITIES

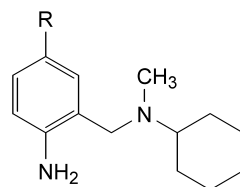
Specified impurities: A, B, C, D.

Other detectable impurities: E.



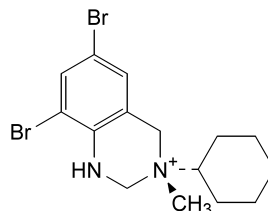
A. R = CH₂OH: (2-amino-3,5-dibromophenyl)methanol,

B. R = CHO: 2-amino-3,5-dibromobenzaldehyde,



C. R = H: *N*-(2-aminobenzyl)-*N*-methylcyclohexanamine,

D. R = Br: *N*-(2-amino-5-bromobenzyl)-*N*-methylcyclohexanamine,



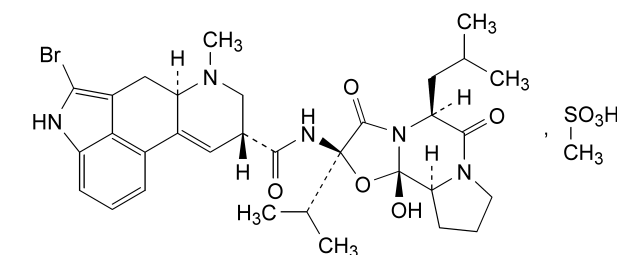
and enantiomer

E. (3*RS*)-6,8-dibromo-3-cyclohexyl-3-methyl-1,2,3,4-tetrahydroquinazolin-3-ium.

01/2005:0596

BROMOCRIPTINE MESILATE

Bromocriptini mesilas



C₃₃H₄₄BrN₅O₈S

*M*_r 751

DEFINITION

Bromocriptine mesilate contains not less than 98.0 per cent and not more than the equivalent of 101.0 per cent of (6*aR*,9*R*)-5-bromo-*N*[(2*R*,5*S*,10*aS*,10*bS*)-10*b*-hydroxy-

2-(1-methylethyl)-5-(2-methylpropyl)-3,6-dioxooctahydro-8*H*-oxazolo[3,2-*a*]pyrrolo[2,1-*c*]pyrazin-2-yl]-7-methyl-4,6,6a,7,8,9-hexahydroindolo[4,3-*fg*]quinoline-9-carboxamide monomethanesulphonate, calculated with reference to the dried substance.

PRODUCTION

The production method must be evaluated to determine the potential for formation of alkyl mesilates, which is particularly likely to occur if the reaction medium contains lower alcohols. Where necessary, the production method is validated to demonstrate that alkyl mesilates are not detectable in the final product.

CHARACTERS

A white or slightly coloured, fine crystalline powder, very sensitive to light, practically insoluble in water, freely soluble in methanol, soluble in alcohol, sparingly soluble in methylene chloride.

The identification, tests and assay are to be carried out as rapidly as possible, protected from light.

IDENTIFICATION

First identification: B.

Second identification: A, C, D, E.

A. Dissolve 10.0 mg in 10 ml of *methanol R* and dilute to 200.0 ml with 0.01 *M* hydrochloric acid. Examined between 250 nm and 380 nm (2.2.25), the solution shows an absorption maximum at 305 nm and a minimum at 270 nm. The specific absorbance at the maximum is 120 to 135, calculated with reference to the dried substance.

B. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *bromocriptine mesilate CRS*.

C. Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel G plate R*. Prepare the solutions immediately before use.

Test solution. Dissolve 10 mg of the substance to be examined in a mixture of 3 volumes of *alcohol R*, 3 volumes of *methanol R* and 4 volumes of *methylene chloride R* and dilute to 10 ml with the same mixture of solvents.

Reference solution. Dissolve 10 mg of *bromocriptine mesilate CRS* in a mixture of 3 volumes of *alcohol R*, 3 volumes of *methanol R* and 4 volumes of *methylene chloride R* and dilute to 10 ml with the same mixture of solvents.

Apply to the plate 10 µl of each solution. Develop immediately in an unsaturated tank over a path of 15 cm using a mixture of 0.1 volumes of *concentrated ammonia R*, 1.5 volumes of *water R*, 3 volumes of *2-propanol R*, 88 volumes of *methylene chloride R* and 100 volumes of *ether R*. Dry the plate in a current of cold air for 2 min. Spray with *ammonium molybdate solution R3*. Dry the plate at 100 °C until the spots appear (about 10 min). The principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

D. To 0.1 g add 5 ml of *dilute hydrochloric acid R* and shake for about 5 min. Filter and add 1 ml of *barium chloride solution R1*. The filtrate remains clear. To a further 0.1 g add 0.5 g of *anhydrous sodium carbonate R*, mix and ignite until a white residue is obtained. Allow to cool and dissolve the residue in 7 ml of *water R* (solution A). Solution A gives reaction (a) of sulphates (2.3.1).

E. Solution A obtained in identification test D gives reaction (a) of bromides (2.3.1).

TESTS

Appearance of solution. Dissolve 0.25 g in *methanol R* and dilute to 25 ml with the same solvent. The solution is clear (2.2.1) and not more intensely coloured than reference solution B₅, BY₅ or Y₅ (2.2.2, *Method II*).

pH (2.2.3). Dissolve 0.2 g in a mixture of 2 volumes of *methanol R* and 8 volumes of *carbon dioxide-free water R* and dilute to 20 ml with the same mixture of solvents. The pH of the solution is 3.1 to 3.8.

Specific optical rotation (2.2.7). Dissolve 0.100 g in a mixture of equal volumes of *methanol R* and *methylene chloride R* and dilute to 10.0 ml with the same mixture of solvents. The specific optical rotation is + 95 to + 105, calculated with reference to the dried substance.

Related substances. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 0.500 g of the substance to be examined in 5.0 ml of *methanol R* and dilute to 10.0 ml with *buffer solution pH 2.0 R*.

Reference solution (a). Dilute 1.0 ml of the test solution to 100.0 ml with a mixture of equal volumes of *buffer solution pH 2.0 R* and *methanol R*.

Reference solution (b). Dilute 1.0 ml of reference solution (a) to 10.0 ml with a mixture of equal volumes of *buffer solution pH 2.0 R* and *methanol R*.

Reference solution (c). Dissolve 5.0 mg of *bromocriptine impurity A CRS* in a mixture of equal volumes of *buffer solution pH 2.0 R* and *methanol R* and dilute to 5.0 ml with the same mixture of solvents.

Reference solution (d). Dissolve 5.0 mg of *bromocriptine impurity B CRS* in a mixture of equal volumes of *buffer solution pH 2.0 R* and *methanol R* and dilute to 5.0 ml with the same mixture of solvents.

Reference solution (e). Mix 0.5 ml of reference solution (c) and 0.5 ml of reference solution (d) and dilute to 10.0 ml with a mixture of equal volumes of *buffer solution pH 2.0 R* and *methanol R*.

Reference solution (f). Dilute 1.0 ml of reference solution (c) to 100.0 ml with a mixture of equal volumes of *buffer solution pH 2.0 R* and *methanol R*.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.12 m long and 4 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (5 µm),

- as mobile phase at a flow rate of 2 ml/min:

Mobile phase A. A 0.791 g/l solution of *ammonium carbonate R*,

Mobile phase B. *Acetonitrile R*,

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)	Comment
0 - 30	90 → 40	10 → 60	linear gradient
30 - 45	40	60	isocratic

- as detector a spectrophotometer set at 300 nm.

Inject 20 µl of reference solution (e) and adjust the sensitivity of the system so that the heights of the 2 peaks are about 20 per cent of the full scale of the recorder. The test is not valid unless the resolution between the peaks corresponding to impurity A and impurity B is at least 1.1.

Inject 20 µl of all other solutions. In the chromatogram obtained with the test solution: the area of the peak corresponding to impurity A is not greater than the area of the principal peak in the chromatogram obtained with reference solution (f) (0.02 per cent) and the area of the

peak corresponding to impurity C, with a relative retention of about 1.2, is not greater than 4 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.4 per cent); the area of any peak, apart from the principal peak and the peak corresponding to impurity C, is not greater than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent) and not more than one such peak has an area greater than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent); the sum of the areas of all the peaks, apart from the principal peak, is not greater than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (1.5 per cent). Disregard any peak, apart from the peak due to impurity A, with an area less than half the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

Loss on drying (2.2.32). Not more than 3.0 per cent, determined on 0.500 g by drying *in vacuo* at 80 °C for 5 h.

ASSAY

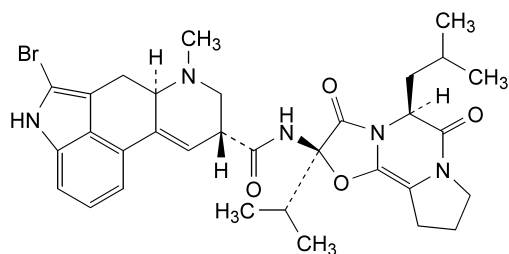
Dissolve 0.500 g in 80 ml of a mixture of 10 volumes of *anhydrous acetic acid R* and 70 volumes of *acetic anhydride R*. Titrate with 0.1 M *perchloric acid*, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *perchloric acid* is equivalent to 75.1 mg of $C_{33}H_{44}BrN_5O_8S$.

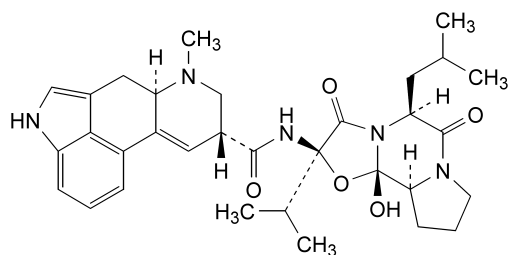
STORAGE

Store in an airtight container, protected from light, at a temperature not exceeding –15 °C.

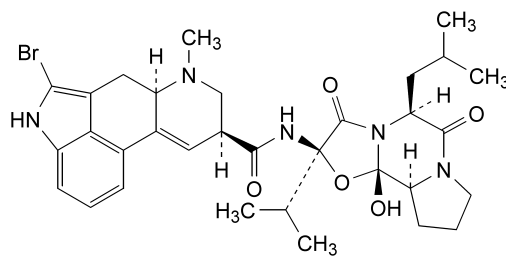
IMPURITIES



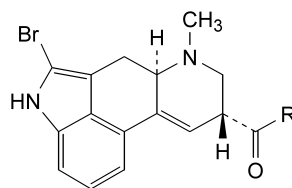
- A. (6a*R*,9*R*)-5-bromo-*N*-[(2*R*,5*S*)-2-(1-methylethyl)-5-(2-methylpropyl)-3,6-dioxo-2,3,5,6,9,10-hexahydro-8*H*-oxazolo[3,2-*a*]pyrrolo[2,1-*c*]pyrazin-2-yl]-7-methyl-4,6,6a,7,8,9-hexahydroindolo[4,3-*fg*]quinoline-9-carboxamide (2-bromodehydro- α -ergocriptine),



- B. (6a*R*,9*R*)-*N*-[(2*R*,5*S*,10a*S*,10b*S*)-10b-hydroxy-2-(1-methylethyl)-5-(2-methylpropyl)-3,6-dioxooctahydro-8*H*-oxazolo[3,2-*a*]pyrrolo[2,1-*c*]pyrazin-2-yl]-7-methyl-4,6,6a,7,8,9-hexahydroindolo[4,3-*fg*]quinoline-9-carboxamide (α -ergocriptine),

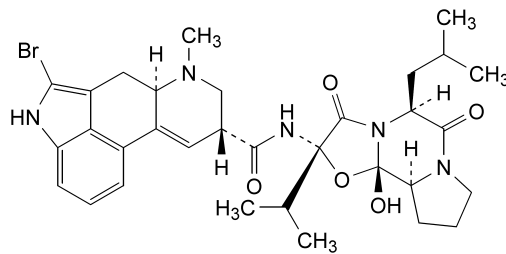


- C. (6a*R*,9*S*)-5-bromo-*N*-[(2*R*,5*S*,10a*S*,10b*S*)-10b-hydroxy-2-(1-methylethyl)-5-(2-methylpropyl)-3,6-dioxooctahydro-8*H*-oxazolo[3,2-*a*]pyrrolo[2,1-*c*]pyrazin-2-yl]-7-methyl-4,6,6a,7,8,9-hexahydroindolo[4,3-*fg*]quinoline-9-carboxamide ((9*S*)-2-bromo- α -ergocriptine),

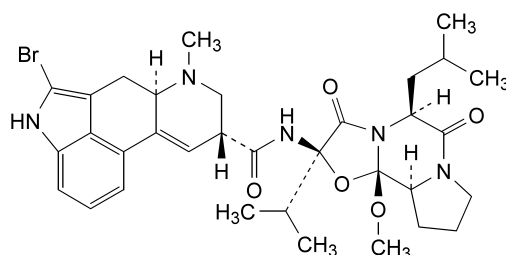


- D. R = OH: (6a*R*,9*R*)-5-bromo-7-methyl-4,6,6a,7,8,9-hexahydroindolo[4,3-*fg*]quinoline-9-carboxylic acid,

- E. R = NH₂: (6a*R*,9*R*)-5-bromo-7-methyl-4,6,6a,7,8,9-hexahydroindolo[4,3-*fg*]quinoline-9-carboxamide,

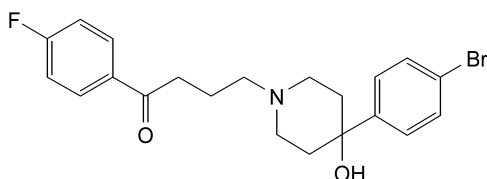


- F. (6a*R*,9*R*)-5-bromo-*N*-[(2*S*,5*S*,10a*S*,10b*S*)-10b-hydroxy-2-(1-methylethyl)-5-(2-methylpropyl)-3,6-dioxooctahydro-8*H*-oxazolo[3,2-*a*]pyrrolo[2,1-*c*]pyrazin-2-yl]-7-methyl-4,6,6a,7,8,9-hexahydroindolo[4,3-*fg*]quinoline-9-carboxamide ((2'*S*)-2-bromo- α -ergocriptine),



- G. (6a*R*,9*R*)-5-bromo-*N*-[(2*R*,5*S*,10a*S*,10b*S*)-10b-methoxy-2-(1-methylethyl)-5-(2-methylpropyl)-3,6-dioxooctahydro-8*H*-oxazolo[3,2-*a*]pyrrolo[2,1-*c*]pyrazin-2-yl]-7-methyl-4,6,6a,7,8,9-hexahydroindolo[4,3-*fg*]quinoline-9-carboxamide (2-bromo-10'*b*-*O*-methyl- α -ergocriptine).

01/2005:1178

BROMPERIDOL**Bromperidolum** $C_{21}H_{23}BrFNO_2$ M_r 420.3**DEFINITION**

Bromperidol contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of 4-[4-(4-bromophenyl)-4-hydroxypiperidin-1-yl]-1-(4-fluorophenyl)butan-1-one, calculated with reference to the dried substance.

CHARACTERS

A white or almost white powder, practically insoluble in water, sparingly soluble in methanol and in methylene chloride, slightly soluble in alcohol.

IDENTIFICATION

First identification: B, E.

Second identification: A, C, D, E.

- A. Melting point (2.2.14): 156 °C to 159 °C.
- B. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *bromperidol CRS*. Examine the substances prepared as discs.
- C. Examine by thin layer chromatography (2.2.27), using as the coating substance a suitable octadecylsilyl silica gel.
- Test solution.* Dissolve 10 mg of the substance to be examined in *methanol R* and dilute to 10 ml with the same solvent.
- Reference solution (a).* Dissolve 10 mg of *bromperidol CRS* in *methanol R* and dilute to 10 ml with the same solvent.
- Reference solution (b).* Dissolve 10 mg of *bromperidol CRS* and 10 mg of *haloperidol CRS* in *methanol R* and dilute to 10 ml with the same solvent.
- Apply separately to the plate 1 µl of each solution. Develop in an unsaturated tank over a path of 15 cm using a mixture of 10 volumes of *tetrahydrofuran R*, 45 volumes of *methanol R* and 45 volumes of a 58 g/l solution of *sodium chloride R*. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. The principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a). The test is not valid unless the chromatogram obtained with reference solution (b) shows two spots which may, however, not be completely separated.
- D. Dissolve about 10 mg in 5 ml of *ethanol R*. Add 0.5 ml of *dinitrobenzene solution R* and 0.5 ml of 2 M *alcoholic potassium hydroxide solution R*. A violet colour is produced that becomes brownish-red after 20 min.
- E. To 0.1 g in a porcelain crucible add 0.5 g of *anhydrous sodium carbonate R*. Heat over an open flame for 10 min. Allow to cool. Take up the residue with 5 ml of *dilute*

nitric acid R and filter. To 1 ml of the filtrate add 1 ml of *water R*. The solution gives reaction (a) of bromides (2.3.1).

TESTS

Appearance of solution. Dissolve 0.2 g in 20 ml of a 1 per cent V/V solution of *lactic acid R*. The solution is clear (2.2.1) and not more intensely coloured than reference solution Y₇ (2.2.2, *Method II*).

Related substances. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 0.100 g of the substance to be examined in *methanol R* and dilute to 10.0 ml with the same solvent.

Reference solution (a). Dissolve 2.5 mg of *bromperidol CRS* and 5.0 mg of *haloperidol CRS* in *methanol R* and dilute to 50.0 ml with the same solvent.

Reference solution (b). Dilute 5.0 ml of the test solution to 100.0 ml with *methanol R*. Dilute 1.0 ml of this solution to 10.0 ml with *methanol R*.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.1 m long and 4.0 mm in internal diameter packed with *base-deactivated octadecylsilyl silica gel for chromatography R* (3 µm),
- as mobile phase at a flow rate of 1.5 ml/min:
Mobile phase A. A 17 g/l solution of *tetrabutylammonium hydrogen sulphate R*,
Mobile phase B. *Acetonitrile R*,

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)	Comment
0 - 15	90 → 50	10 → 50	linear gradient
15 - 20	50	50	isocratic elution
20 - 25	90	10	switch to initial eluent composition
25 = 0	90	10	restart gradient

- as detector a spectrophotometer set at 230 nm.

Equilibrate the column for at least 30 min with *acetonitrile R* and then equilibrate with the initial eluent composition for at least 5 min.

Adjust the sensitivity of the system so that the height of the principal peak in the chromatogram obtained with 10 µl of reference solution (b) is at least 50 per cent of the full scale of the recorder.

Inject 10 µl of reference solution (a). When the chromatogram is recorded in the prescribed conditions, the retention times are: *haloperidol*, about 5.5 min and *bromperidol* about 6 min. The test is not valid unless the resolution between the peaks due to *haloperidol* and *bromperidol* is at least 3.0. If necessary, adjust the concentration of *acetonitrile* in the mobile phase or adjust the time programme for the linear gradient elution.

Inject separately 10 µl of *methanol R* as a blank, 10 µl of the test solution and 10 µl of reference solution (b). In the chromatogram obtained with the test solution: the area of any peak, apart from the principal peak, is not greater than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent); the sum of the areas of all peaks, apart from the principal peak, is not greater than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (1 per cent). Disregard any peak obtained with the blank and any peak with an area less than 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (b).

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 100-105 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g in a platinum crucible.

ASSAY

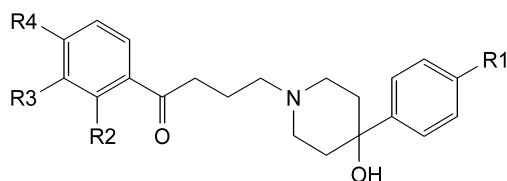
Dissolve 0.300 g in 50 ml of a mixture of 1 volume of *anhydrous acetic acid R* and 7 volumes of *methyl ethyl ketone R*. Titrate with 0.1 M *perchloric acid*, using 0.2 ml of *naphtholbenzein solution R* as indicator.

1 ml of 0.1 M *perchloric acid* is equivalent to 42.03 mg of $C_{31}H_{41}BrFNO_3$.

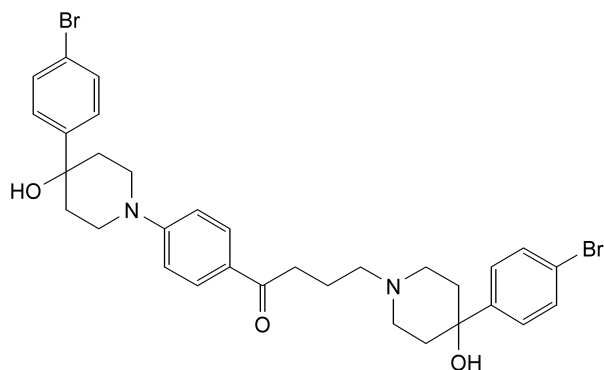
STORAGE

Store protected from light.

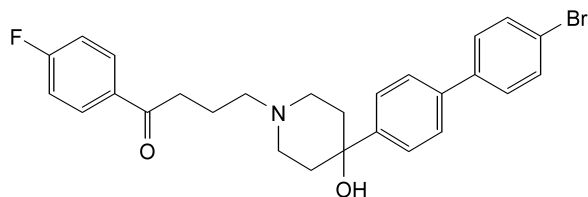
IMPURITIES



- A. R1 = R2 = R3 = H, R4 = F: 1-(4-(4-fluorophenyl)-4-(4-hydroxy-4-phenylpiperidin-1-yl)butan-1-one,
- B. R1 = Br, R2 = F, R3 = R4 = H: 4-[4-(4-bromophenyl)-4-hydroxypiperidin-1-yl]-1-(2-fluorophenyl)butan-1-one,
- C. R1 = C₆H₅, R2 = R3 = H, R4 = F: 4-[4-(biphenyl-4-yl)-4-hydroxypiperidin-1-yl]-1-(4-fluorophenyl)butan-1-one,
- D. R1 = Br, R2 = H, R3 = C₂H₅, R4 = F: 4-[4-(4-bromophenyl)-4-hydroxypiperidin-1-yl]-1-(3-ethyl-4-fluorophenyl)butan-1-one,



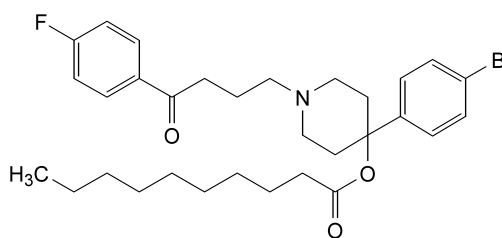
- E. 4-[4-(4-bromophenyl)-4-hydroxypiperidin-1-yl]-1-[4-(4-bromophenyl)-4-hydroxypiperidin-1-yl]phenyl]butan-1-one,



- F. 4-[4-(4'-bromobiphenyl-4-yl)-4-hydroxypiperidin-1-yl]-1-(4-fluorophenyl)butan-1-one.

BROMPERIDOL DECANOATE

Bromperidoli decanoas



$C_{31}H_{41}BrFNO_3$

M_r 574.6

DEFINITION

Bromperidol decanoate contains not less than 98.5 per cent and not more than the equivalent of 101.0 per cent of 4-(4-bromophenyl)-1-[4-(4-fluorophenyl)-4-oxobutyl]piperidin-4-yl decanoate, calculated with reference to the dried substance.

CHARACTERS

A white or almost white powder, practically insoluble in water, very soluble in methylene chloride, soluble in alcohol. It melts at about 60 °C.

IDENTIFICATION

- A. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *bromperidol decanoate CRS*. Examine the substances prepared as mulls in *liquid paraffin R*.
- B. To 0.1 g in a porcelain crucible add 0.5 g of *anhydrous sodium carbonate R*. Heat over an open flame for 10 min. Allow to cool. Take up the residue with 5 ml of *dilute nitric acid R* and filter. To 1 ml of the filtrate add 1 ml of *water R*. The solution gives reaction (a) of bromides (2.3.1).

TESTS

Appearance of solution. Dissolve 2.0 g in *methylene chloride R* and dilute to 20 ml with the same solvent. The solution is clear (2.2.1) and not more intensely coloured than reference solution B₅ (2.2.2, *Method II*).

Related substances. Examine by liquid chromatography (2.2.29). Prepare the solutions immediately before use and protect from light.

Test solution. Dissolve 0.100 g of the substance to be examined in *methanol R* and dilute to 10.0 ml with the same solvent.

Reference solution (a). Dissolve 2.5 mg of *bromperidol decanoate CRS* and 2.5 mg of *haloperidol decanoate CRS* in *methanol R* and dilute to 50.0 ml with the same solvent.

Reference solution (b). Dilute 5.0 ml of the test solution to 100.0 ml with *methanol R*. Dilute 1.0 ml of this solution to 10.0 ml with *methanol R*.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.1 m long and 4.0 mm in internal diameter packed with *base-deactivated octadecylsilyl silica gel for chromatography R* (3 µm),
- as mobile phase at a flow rate of 1.5 ml/min:

Mobile phase A. A 27 g/l solution of *tetrabutylammonium hydrogen sulphate R*,

Mobile phase B. *Acetonitrile R*,

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)	Comment
0 - 30	80 → 40	20 → 60	linear gradient
30 - 35	40	60	isocratic elution
35 - 40	40 → 80	60 → 20	switch to initial eluent composition
40 = 0	80	20	restart gradient

— as detector a spectrophotometer set at 230 nm.

Equilibrate the column for at least 30 min with *acetonitrile R* and then equilibrate at the initial eluent composition for at least 5 min.

Adjust the sensitivity of the system so that the height of the principal peak in the chromatogram obtained with 10 µl of reference solution (b) is at least 50 per cent of the full scale of the recorder.

Inject 10 µl of reference solution (a). When the chromatogram is recorded in the prescribed conditions, the retention times are: haloperidol decanoate about 24 min; bromperidol decanoate about 24.5 min. The test is not valid unless the resolution between the peaks due to haloperidol decanoate and bromperidol decanoate is at least 1.5. If necessary, adjust the gradient or the time programme for the linear gradient elution.

Inject separately 10 µl of *methanol R* as a blank, 10 µl of the test solution and 10 µl of reference solution (b). In the chromatogram obtained with the test solution: the area of any peak apart from the principal peak, is not greater than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent); the sum of the areas of all the peaks apart from the principal peak is not greater than three times the area of the principal peak in the chromatogram obtained with reference solution (b) (1.5 per cent). Disregard any peak due to the blank and any peak with an area less than 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (b).

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying *in vacuo* at 30 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g in a platinum crucible.

ASSAY

Dissolve 0.450 g in 50 ml of a mixture of 1 volume of *anhydrous acetic acid R* and 7 volumes of *methyl ethyl ketone R*. Titrate with 0.1 M *perchloric acid* using 0.2 ml of *naphtholbenzein solution R* as indicator.

1 ml of 0.1 M *perchloric acid* is equivalent to 57.46 mg of C₃₁H₄₁BrFNO₃.

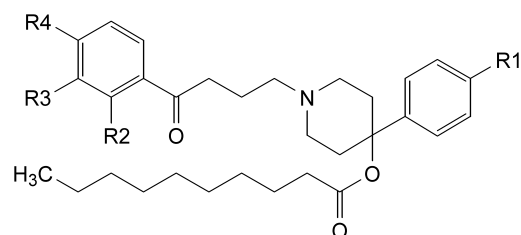
STORAGE

Store at a temperature below 25 °C, protected from light.

IMPURITIES

Specified impurities: A, B, C, D, E, F, G, H, I, J, K.

Other detectable impurities: L.

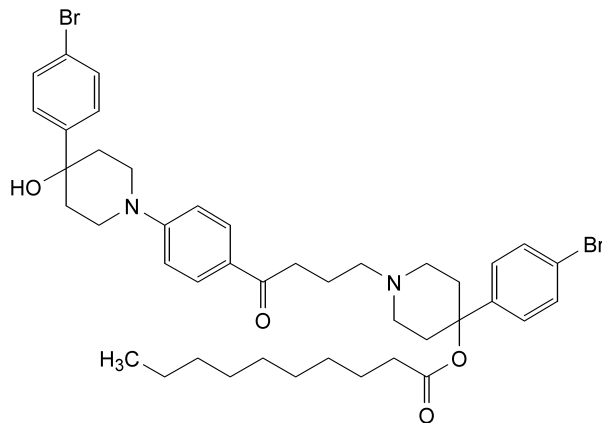


A. R1 = R2 = R3 = H, R4 = F: 1-[4-(4-fluorophenyl)-4-oxobutyl]-4-phenylpiperidin-4-yl decanoate,

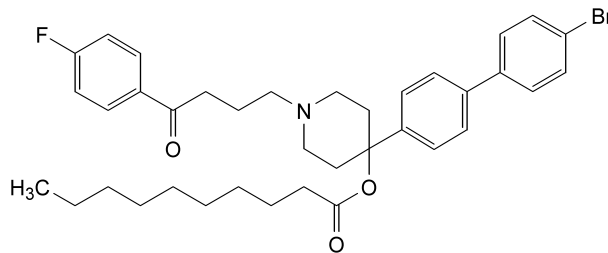
B. R1 = Br, R2 = F, R3 = R4 = H: 4-(4-bromophenyl)-1-[4-(2-fluorophenyl)-4-oxobutyl]-piperidin-4-yl decanoate,

C. R1 = Br, R2 = H, R3 = C₂H₅, R4 = F: 4-(4-bromophenyl)-1-[4-(3-ethyl-4-fluorophenyl)-4-oxobutyl]-piperidin-4-yl decanoate,

F. R1 = C₆H₅, R2 = R3 = H, R4 = F: 4-(biphenyl-4-yl)-1-[4-(4-fluorophenyl)-4-oxobutyl]piperidin-4-yl decanoate,

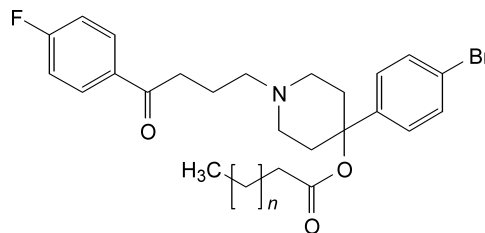


D. 4-(4-bromophenyl)-1-[4-[4-[4-(4-bromophenyl)-4-hydroxypiperidin-1-yl]phenyl]-4-oxobutyl]piperidin-4-yl decanoate,



E. 4-(4'-bromobiphenyl-4-yl)-1-[4-(4-fluorophenyl)-4-oxobutyl]piperidin-4-yl decanoate,

G. bromperidol,

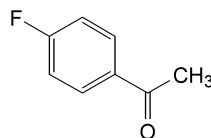


H. n = 5: 4-(4-bromophenyl)-1-[4-(4-fluorophenyl)-4-oxobutyl]piperidin-4-yl octanoate,

I. n = 6: 4-(4-bromophenyl)-1-[4-(4-fluorophenyl)-4-oxobutyl]piperidin-4-yl nonanoate,

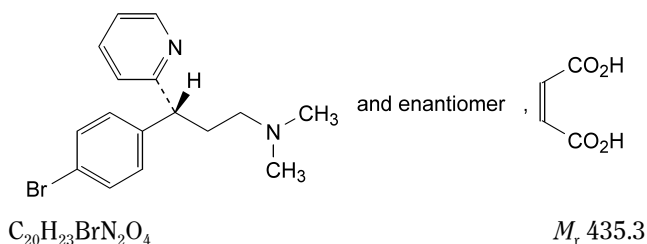
J. n = 8: 4-(4-bromophenyl)-1-[4-(4-fluorophenyl)-4-oxobutyl]piperidin-4-yl undecanoate,

K. n = 9: 4-(4-bromophenyl)-1-[4-(4-fluorophenyl)-4-oxobutyl]piperidin-4-yl dodecanoate,



L. 1-(4-fluorophenyl)ethanone.

01/2005:0977

BROMPHENIRAMINE MALEATE**Brompheniramine maleate****DEFINITION**

Brompheniramine maleate contains not less than 98.0 per cent and not more than the equivalent of 101.0 per cent of (3*RS*)-3-(4-bromophenyl)-*N,N*-dimethyl-3-(pyridin-2-yl)propan-1-amine (*Z*)-butenedioate, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, crystalline powder, soluble in water, freely soluble in alcohol, in methanol and in methylene chloride.

IDENTIFICATION

First identification: A, B, C, D, E.

Second identification: A, B, E, F.

- A. Melting point (2.2.14): 130 °C to 135 °C.
- B. Dissolve 65 mg in 0.1 *M* hydrochloric acid and dilute to 100.0 ml with the same acid. Dilute 5.0 ml of this solution to 100.0 ml with 0.1 *M* hydrochloric acid. Examined between 220 nm and 320 nm (2.2.25), the solution shows an absorption maximum at 265 nm. The specific absorbance at the maximum is 190 to 210.
- C. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with brompheniramine maleate CRS. Examine the substances prepared as discs using potassium bromide *R*.
- D. Examine the chromatograms obtained in the test for related substances. The retention time and size of the principal peak in the chromatogram obtained with the test solution are approximately the same as those of the principal peak in the chromatogram obtained with reference solution (a). The chromatogram obtained with reference solution (c) shows two principal peaks with retention times corresponding to the retention times of the peaks obtained with reference solutions (a) and (b).
- E. Examine by thin-layer chromatography (2.2.27), using as the coating substance a suitable silica gel with a fluorescent indicator having an optimal intensity at 254 nm.

Test solution. Dissolve 0.10 g of the substance to be examined in methanol *R* and dilute to 5.0 ml with the same solvent.

Reference solution. Dissolve 56 mg of maleic acid *R* in methanol *R* and dilute to 10 ml with the same solvent. Apply to the plate 5 µl of each solution. Develop over a path of 12 cm using a mixture of 3 volumes of water *R*, 7 volumes of anhydrous formic acid *R*, 20 volumes of methanol *R* and 70 volumes of di-isopropyl ether *R*. Allow the plate to dry in a current of air for a few minutes and examine in ultraviolet light at 254 nm. The chromatogram obtained with the test solution shows

two clearly separated spots. The upper spot is similar in position and size to the spot in the chromatogram obtained with the reference solution.

- F. To 0.15 g in a porcelain crucible add 0.5 g of anhydrous sodium carbonate *R*. Heat over an open flame for 10 min. Allow to cool. Take up the residue in 10 ml of dilute nitric acid *R* and filter. To 1 ml of the filtrate add 1 ml of water *R*. The solution gives reaction (a) of bromides (2.3.1).

TESTS

Appearance of solution. Dissolve 2.0 g in methanol *R* and dilute to 20 ml with the same solvent. The solution is clear (2.2.1) and not more intensely coloured than reference solution BY₆ (2.2.2, Method II).

pH (2.2.3). Dissolve 0.20 g in 20 ml of carbon dioxide-free water *R*. The pH of the solution is 4.0 to 5.0.

Optical rotation (2.2.7). Dissolve 2.5 g in water *R* and dilute to 25.0 ml with the same solvent. The angle of optical rotation measured in a 2 dm tube is −0.2° to +0.2°.

Related substances. Examine by gas chromatography (2.2.28).

Test solution. Dissolve 0.10 g of the substance to be examined in 10 ml of methylene chloride *R*.

Reference solution (a). Dissolve 10 mg of brompheniramine maleate CRS in methylene chloride *R* and dilute to 1 ml with the same solvent.

Reference solution (b). Dissolve 5 mg of chlorphenamine maleate CRS in methylene chloride *R* and dilute to 1 ml with the same solvent.

Reference solution (c). To 0.5 ml of the test solution add 0.5 ml of reference solution (b).

The chromatographic procedure may be carried out using:

- a glass column 2.3 m long and 2 mm in internal diameter packed with acid- and base-washed silanised diatomaceous earth for gas chromatography *R* (135–175 µm) impregnated with 3 per cent *m/m* of polymethylphenylsiloxane *R*,
- nitrogen for chromatography *R* as the carrier gas at a flow rate of 20 ml/min,
- a flame-ionisation detector,

maintaining the temperature of the column at 205 °C and that of the injection port and of the detector at 250 °C.

Inject 1 µl of each solution. The test is not valid unless, in the chromatogram obtained with reference solution (c), the resolution between the peaks corresponding to brompheniramine and chlorphenamine is at least 1.5. After injecting the test solution, continue the chromatography for at least 2.5 times the retention time of the principal peak. In the chromatogram obtained with the test solution: the sum of the areas of the peaks, apart from the principal peak, is not greater than 1 per cent of the area of the principal peak; none of the peaks, apart from the principal peak, has an area greater than 0.4 per cent of the area of the principal peak. Disregard any peak with an area less than 0.1 per cent of that of the peak corresponding to brompheniramine in the chromatogram obtained with the test solution.

Heavy metals (2.4.8). 1.0 g complies with limit test C for heavy metals (20 ppm). Prepare the standard using 2 ml of lead standard solution (10 ppm Pb) *R*.

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 100–105 °C for 3 h.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.260 g in 50 ml of *anhydrous acetic acid R*. Titrate with 0.1 M *perchloric acid*, determining the end-point potentiometrically (2.2.20).

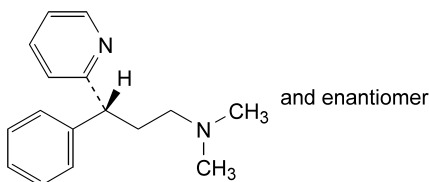
1 ml of 0.1 M *perchloric acid* is equivalent to 21.77 mg of $C_{20}H_{23}BrN_2O_4$.

STORAGE

Store protected from light.

IMPURITIES

- A. chlorphenamine,
B. dexchlorpheniramine,

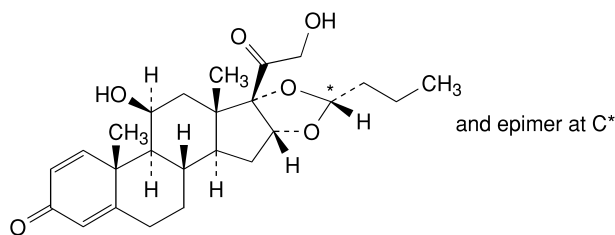


- C. (3*R*)-*N,N*-dimethyl-3-phenyl-3-(pyridin-2-yl)propan-1-amine (pheniramine).

01/2005:1075

BUDESONIDE

Budesonidum

 $C_{25}H_{34}O_6$ M_r 430.5

DEFINITION

Budesonide contains not less than 98.0 per cent and not more than the equivalent of 102.0 per cent of a mixture of the C-22*S* (epimer A) and the C-22*R* (epimer B) epimers of 16 α ,17-[(1*R**S*)-butylidenebis(oxy)]-11 β ,21-dihydroxypregna-1,4-diene-3,20-dione, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, crystalline powder, practically insoluble in water, freely soluble in methylene chloride, sparingly soluble in alcohol.

IDENTIFICATION

First identification: A.

Second identification: B, C, D.

- A. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *budesonide CRS*. Examine the substances prepared as discs.
- B. Examine by thin-layer chromatography (2.2.27), using as the coating substance a suitable silica gel with a fluorescent indicator having an optimal intensity at 254 nm.

Test solution. Dissolve 25 mg of the substance to be examined in a mixture of 1 volume of *methanol R* and 9 volumes of *methylene chloride R* and dilute to 10 ml with the same mixture of solvents.

Reference solution (a). Dissolve 25 mg *budesonide CRS* in a mixture of 1 volume of *methanol R* and 9 volumes of *methylene chloride R* and dilute to 10 ml with the same mixture of solvents.

Reference solution (b). Dissolve 12.5 mg *triamcinolone acetone CRS* in reference solution (a) and dilute to 5 ml with the same solution.

Apply separately to the plate 5 μ l of each solution. Prepare the mobile phase by adding a mixture of 1.2 volumes of *water R* and 8 volumes of *methanol R* to a mixture of 15 volumes of *ether R* and 77 volumes of *methylene chloride R*. Develop over a path of 15 cm. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. The principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a). Spray with *alcoholic solution of sulphuric acid R*. Heat at 120 °C for 10 min or until spots appear. Allow to cool. Examine in daylight and in ultraviolet light at 365 nm. The principal spot in the chromatogram obtained with the test solution is similar in position, colour in daylight, fluorescence in ultraviolet light at 365 nm and size to the principal spot in the chromatogram obtained with the reference solution (a). The test is not valid unless the chromatogram obtained with reference solution (b) shows two clearly separated spots.

- C. Dissolve about 2 mg in 2 ml of *sulphuric acid R*. Within 5 min, a yellow colour develops. Within 30 min, the colour changes to brown or reddish-brown. Add cautiously the solution to 10 ml of *water R* and mix. The colour fades and a clear solution remains.

- D. Dissolve about 1 mg in 2 ml of a solution containing 2 g of *phosphomolybdic acid R* dissolved in a mixture of 10 ml of *dilute sodium hydroxide solution R*, 15 ml of *water R* and 25 ml of *glacial acetic acid R*. Heat for 5 min on a water-bath. Cool in iced water for 10 min and add 3 ml of *dilute sodium hydroxide solution R*. The solution is blue.

TESTS

Related substances. Examine by liquid chromatography (2.2.29) as described under Assay.

Inject 20 μ l of reference solution (a). Adjust the sensitivity of the system so that the height of the peak corresponding to epimer B (the first of the two principal peaks) in the chromatogram obtained is not less than 50 per cent of the full scale of the recorder. Inject 20 μ l of the test solution, 20 μ l of reference solution (a) and 20 μ l of reference solution (b). Continue the chromatography for 1.5 times the retention time of epimer B. In the chromatogram obtained with the test solution: the area of any peak, apart from the peaks corresponding to epimer A and epimer B, is not greater than the sum of the areas of the epimer peaks in the chromatogram obtained with reference solution (b) (0.5 per cent); the sum of the areas of any such peaks is not greater than the sum of the areas of the epimer peaks in the chromatogram obtained with reference solution (a) (1.5 per cent). Disregard any peak with an area less than 0.1 times the sum of the areas of the epimer peaks in the chromatogram obtained with reference solution (b).

Epimer A. Examine by liquid chromatography (2.2.29) as described under Assay.

Inject 20 µl of the test solution. The content of epimer A (second peak) is 40.0 per cent to 51.0 per cent of the sum of the areas of the two epimer peaks of budesonide.

Methanol. Not more than 0.1 per cent, determined by head-space gas chromatography using the standard addition method (2.2.28).

Test solution. Dissolve 2.000 g of the substance to be examined in *dimethylacetamide R* and dilute to 20.0 ml with the same solvent.

Reference solution. Dilute 0.500 g of *methanol R* in *dimethylacetamide R* and dilute to 100.0 ml with the same solvent.

The following head-space conditions may be used:

- equilibration temperature: 80 °C,
- equilibration time: 30 min,
- transfer-line temperature: 85 °C,
- pressurisation time: 10 s,
- injection time: 10 s.

The chromatographic procedure may be carried out using:

- a fused-silica capillary column 30 m long and 0.32 mm in internal diameter coated with *macrogol 20 000 R* (film thickness 1 µm),
- *nitrogen for chromatography R* as the carrier gas at a pressure of 55 kPa,
- a flame-ionisation detector,

maintaining the temperature of the column at 50 °C for 5 min, then raising the temperature at a rate of 30 °C per minute to 220 °C and maintaining at 220 °C for 2 min, and maintaining the temperature of the injection port at 250 °C and that of the detector at 300 °C.

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C.

ASSAY

Examine by liquid chromatography (2.2.29). *Protect the solutions from light throughout the assay.*

Test solution. Dissolve 25.0 mg of the substance to be examined in 15 ml of *acetonitrile R* and dilute to 50.0 ml with *phosphate buffer solution pH 3.2 R*. Allow to stand for at least 15 min before use.

Reference solution (a). Dilute 15.0 ml of the test solution to 100.0 ml with the mobile phase. Dilute 1.0 ml of the solution to 10.0 ml with the mobile phase.

Reference solution (b). Dilute 5.0 ml of the test solution to 100.0 ml with the mobile phase. Dilute 1.0 ml of the solution to 10.0 ml with the mobile phase.

Reference solution (c). Dissolve 25.0 mg of *budesonide CRS* in 15 ml of *acetonitrile R* and dilute to 50.0 ml with *phosphate buffer solution pH 3.2 R*.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.12 m long and 4.6 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (5 µm),
- as mobile phase at a flow rate of 1.5 ml/min a mixture of 32 volumes of *acetonitrile R* and 68 volumes of *phosphate buffer solution pH 3.2 R*,
- as detector a spectrophotometer set at 240 nm.

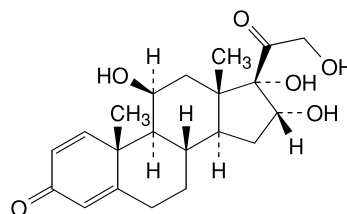
Inject 20 µl of reference solution (c). Adjust the sensitivity of the system so that the height of the peak corresponding to epimer B (the first of the two principal peaks) in the

chromatogram obtained is not less than 50 per cent of the full scale of the recorder. When the chromatograms are recorded in the prescribed conditions, the retention time for epimer B is about 16 min. If necessary, adjust the concentration of acetonitrile in the mobile phase (increasing the concentration to decrease the retention time). The test is not valid unless: in the chromatogram obtained with reference solution (c), the resolution between the peaks corresponding to epimer A and epimer B is not less than 1.5; the number of theoretical plates determined from the epimer B peak is at least 4000; the symmetry factor for the same peak is less than 1.5.

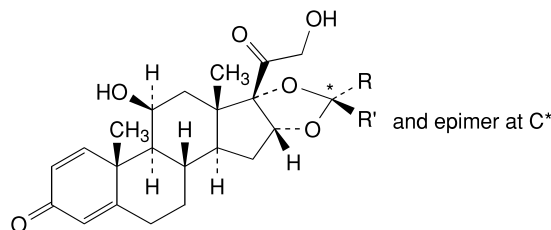
Inject 20 µl of reference solution (c) six times. The assay is not valid unless the relative standard deviation of the sum of the peak areas of the two epimers is at most 1.0 per cent. Inject alternatively the test solution and reference solution (c).

Calculate the percentage content of $C_{25}H_{34}O_6$ from the sum of the areas of the two epimer peaks.

IMPURITIES

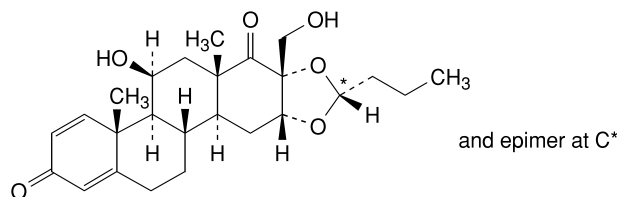


A. 11β,16α,17,21-tetrahydroxypregna-1,4-diene-3,20-dione,

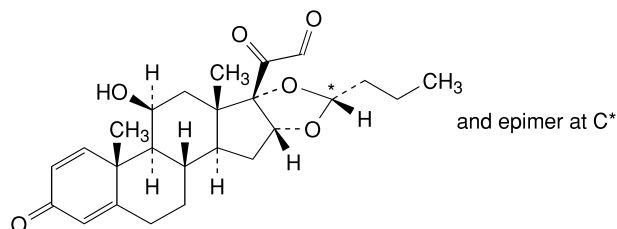


B. R = CH₃, R' = H: 16α,17-[(1RS)-ethylidenebis(oxy)]-11β,21-dihydroxypregna-1,4-diene-3,20-dione,

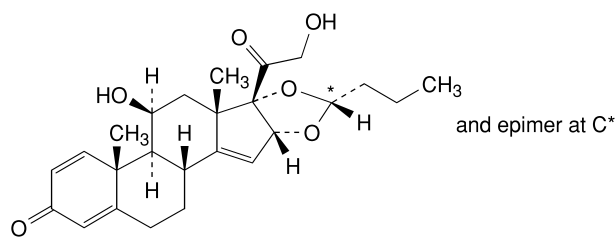
F. R = R' = CH₃: 16α,17-[1-methylethylidenebis(oxy)]-11β,21-dihydroxypregna-1,4-diene-3,20-dione,



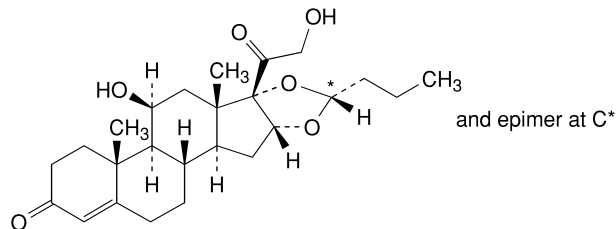
C. 16α,17-[(1RS)-butylidenebis(oxy)]-11β-hydroxy-17-(hydroxymethyl)-D-homoandrosta-1,4-diene-3,17a-dione,



D. 16α,17-[(1RS)-butylidenebis(oxy)]-11β-hydroxy-3,20-dioxopregna-1,4-dien-21-al,



E. 16α,17-[(1*RS*)-butylidenebis(oxy)]-11β,21-dihydroxypregna-1,4,14-triene-3,20-dione,

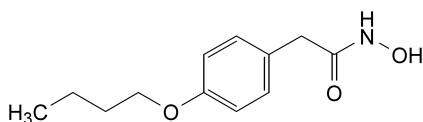


G. 16α,17-[(1*RS*)-butylidenebis(oxy)]-11β,21-dihydroxypregna-4-ene-3,20-dione.

01/2005:1179

BUFEXAMAC

Bufexamacum



C₁₂H₁₇NO₃

*M*_r 223.3

DEFINITION

Bufexamac contains not less than 98.5 per cent and not more than the equivalent of 101.5 per cent of 2-(4-butoxyphenyl)-*N*-hydroxyacetamide, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, crystalline powder, practically insoluble in water, soluble in dimethylformamide, slightly soluble in ethyl acetate and in methanol.

IDENTIFICATION

First identification: B.

Second identification: A, C.

- Dissolve 20 mg in *methanol R* and dilute to 20 ml with the same solvent. Dilute 1 ml of the solution to 50 ml with *methanol R*. Examined between 210 nm and 360 nm (2.2.25), the solution shows three absorption maxima, at 228 nm, 277 nm and 284 nm.
- Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *bufexamac CRS*. Examine the substances prepared as discs.
- Examine by thin-layer chromatography (2.2.27), using as the coating substance a suitable silica gel with a fluorescent indicator having an optimal intensity at 254 nm.

Test solution. Dissolve 10 mg of the substance to be examined in *methanol R* and dilute to 5 ml with the same solvent.

Reference solution (a). Dissolve 20 mg of *bufexamac CRS* in *methanol R* and dilute to 10 ml with the same solvent.

Reference solution (b). Dissolve 10 mg of *salicylic acid R* in reference solution (a) and dilute to 5 ml with the same solution.

Apply to the plate 10 µl of each solution. Develop over a path of 15 cm using a mixture of 4 volumes of *glacial acetic acid R*, 20 volumes of *dioxan R* and 90 volumes of *toluene R*. Dry the plate in a current of warm air and examine in ultraviolet light at 254 nm. The principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a). The test is not valid unless the chromatogram obtained with reference solution (b) shows two clearly separated spots.

TESTS

Related substances. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 50.0 mg of the substance to be examined in the mobile phase and dilute to 20.0 ml with the same solvent.

Reference solution (a). Dilute 5.0 ml of the test solution to 25.0 ml with the mobile phase. Dilute 1.0 ml of the solution to 100.0 ml with the mobile phase.

Reference solution (b). Dissolve 5 mg of *bufexamac CRS* and 5 mg of *salicylic acid R* in the mobile phase and dilute to 10 ml with the mobile phase. Dilute 1 ml of the solution to 10 ml with the mobile phase.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4.6 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (5 µm) having a specific surface area of 350 m²/g and a pore size of 10 nm,
- as mobile phase at a flow rate of 1 ml/min a mixture of 30 volumes of a 1.4 g/l solution of *dipotassium hydrogen phosphate R* and 70 volumes of *methanol R* adjusted to pH 3.6 with *dilute phosphoric acid R*,
- as detector a spectrophotometer set at 275 nm.

Inject 20 µl of reference solution (a) and 20 µl of reference solution (b). Adjust the sensitivity of the system so that the height of the principal peak in the chromatogram obtained with reference solution (a) is at least 50 per cent of the full scale of the recorder. The test is not valid unless in the chromatogram obtained with reference solution (b) the resolution between the peaks corresponding to salicylic acid and to bufexamac is at least 2.0.

Inject 20 µl of the test solution. Continue the chromatography for four times the retention time of bufexamac. In the chromatogram obtained with the test solution: the area of any peak, apart from the principal peak is not greater than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent); the sum of the areas of all the peaks, apart from the principal peak, is not greater than 2.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent). Disregard any peak due to the solvent and any peak with an area less than 0.05 times that of the principal peak in the chromatogram obtained with reference solution (a).

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying *in vacuo* at 80 °C for 3 h.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

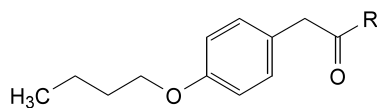
Dissolve 0.200 g in 50 ml of *dimethylformamide R*. Titrate with 0.1 *M lithium methoxide*, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M lithium methoxide is equivalent to 22.33 mg of $C_{12}H_{17}NO_3$.

STORAGE

Store protected from light.

IMPURITIES

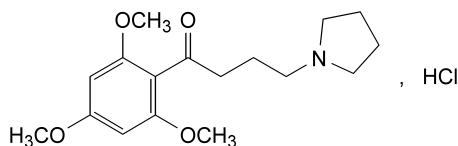


- A. R = OH: 2-(4-butoxyphenyl)acetic acid,
 B. R = OCH₃: methyl 2-(4-butoxyphenyl)acetate,
 C. R = OC₄H₉: butyl 2-(4-butoxyphenyl)acetate,
 D. R = NH₂: 2-(4-butoxyphenyl)acetamide.

01/2005:1398

BUFLOMEDIL HYDROCHLORIDE

Buflomedili hydrochloridum



$C_{17}H_{26}ClNO_4$

M_r 343.9

DEFINITION

4-(Pyrrolidin-1-yl)-1-(2,4,6-trimethoxyphenyl)butan-1-one hydrochloride.

Content: 98.5 per cent to 101.5 per cent (dried substance).

CHARACTERS

Appearance: white or almost white, microcrystalline powder.

Solubility: freely soluble in water, soluble in alcohol, very slightly soluble in acetone.

mp: about 195 °C, with decomposition.

IDENTIFICATION

First identification: B, D.

Second identification: A, C, D.

- A. Dissolve 25.0 mg in *alcohol R* and dilute to 50.0 ml with the same solvent. Dilute 2.0 ml to 20.0 ml with *alcohol R*. Examined between 220 nm and 350 nm (2.2.25), the solution shows an absorption maximum at 275 nm. The specific absorbance at the maximum is 143 to 149.

- B. Infrared absorption spectrophotometry (2.2.24).

Preparation: discs.

Comparison: buflomedil hydrochloride CRS.

- C. Thin-layer chromatography (2.2.27).

Test solution. Dissolve 40 mg of the substance to be examined in *methanol R* and dilute to 2 ml with the same solvent.

Reference solution. Dissolve 40 mg of buflomedil hydrochloride CRS in *methanol R* and dilute to 2 ml with the same solvent.

Plate: TLC silica gel F₂₅₄ plate R.

Mobile phase: triethylamine R, 2-propanol R, toluene R (5:50:50 V/V/V).

Application: 10 µl.

Development: over a path of 15 cm.

Drying: in air.

Detection: examine in ultraviolet light at 254 nm.

Results: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with the reference solution.

- D. It gives reaction (a) of chlorides (2.3.1).

TESTS

Solution S. Dissolve 2.5 g in carbon dioxide-free water R and dilute to 50 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and colourless (2.2.2, Method II).

pH (2.2.3): 5.0 to 6.5 for solution S.

Related substances. Liquid chromatography (2.2.29).

Test solution. Dissolve 0.10 g of the substance to be examined in the mobile phase and dilute to 10.0 ml with the mobile phase.

Reference solution (a). Dilute 0.5 ml of the test solution to 100.0 ml with the mobile phase. Dilute 5.0 ml of this solution to 10.0 ml with the mobile phase.

Reference solution (b). Dissolve 2 mg of buflomedil impurity B CRS in the mobile phase. Add 0.5 ml of the test solution and dilute to 100 ml with the mobile phase.

Column:

- size: $l = 0.25$ m, $\varnothing = 4.6$ mm,
- stationary phase: end-capped octadecylsilyl silica gel for chromatography R (5 µm),
- temperature: 40 °C.

Mobile phase: mix 45 volumes of acetonitrile R and 55 volumes of a 9.25 g/l solution of potassium dihydrogen phosphate R adjusted to pH 2.5 with phosphoric acid R.

Flow rate: 1 ml/min.

Detection: spectrophotometer at 210 nm.

Injection: 10 µl.

Run time: twice the retention time of buflomedil.

Retention time: buflomedil = about 5 min.

System suitability: reference solution (b):

- resolution: minimum 5.0 between the peaks due to buflomedil and impurity B.

Limits:

- any impurity: not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.25 per cent),
- total: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent),
- disregard limit: 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

Heavy metals (2.4.8): maximum 10 ppm.

2.0 g complies with limit test C. Prepare the standard using 2 ml of lead standard solution (10 ppm Pb) R.

Loss on drying (2.2.32): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 100–105 °C for 2 h.

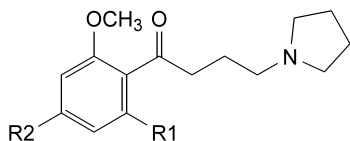
Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.300 g in 15 ml of anhydrous acetic acid R and add 35 ml of acetic anhydride R. Titrate with 0.1 M perchloric acid, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M perchloric acid is equivalent to 34.39 mg of $C_{17}H_{26}ClNO_4$.

IMPURITIES

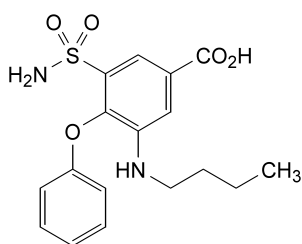


- A. $R_1 = OH$, $R_2 = OCH_3$: 4-(pyrrolidin-1-yl)-1-(2-hydroxy-4,6-dimethoxyphenyl)butan-1-one,
 B. $R_1 = OCH_3$, $R_2 = OH$: 4-(pyrrolidin-1-yl)-1-(4-hydroxy-2,6-dimethoxyphenyl)butan-1-one,
 C. $R_1 = R_2 = OH$: 4-(pyrrolidin-1-yl)-1-(2,4-dihydroxy-6-methoxyphenyl)butan-1-one.

01/2005:1076

BUMETANIDE

Bumetanidum



$C_{17}H_{20}N_2O_5S$

M_r 364.4

DEFINITION

3-(Butylamino)-4-phenoxy-5-sulphamoylbenzoic acid.

Content: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

Appearance: white, crystalline powder.

Solubility: practically insoluble in water, soluble in acetone and in alcohol, slightly soluble in methylene chloride. It dissolves in dilute solutions of alkali hydroxides.

It shows polymorphism.

mp: about 233 °C.

IDENTIFICATION

Infrared absorption spectrophotometry (2.2.24).

Comparison: bumetanide CRS.

If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in acetone R, evaporate to dryness and record new spectra using the residues.

TESTS

Appearance of solution. The solution is clear (2.2.1) and colourless (2.2.2, Method II).

Dissolve 0.1 g in a 6 g/l solution of potassium hydroxide R and dilute to 20 ml with the same solution.

Related substances. Liquid chromatography (2.2.29).

Test solution. Dissolve 50 mg of the substance to be examined in the mobile phase and dilute to 25.0 ml with the mobile phase.

Reference solution (a). Dilute 1.0 ml of the test solution to 100.0 ml with the mobile phase. Dilute 1.0 ml of this solution to 10.0 ml with the mobile phase.

Reference solution (b). Dissolve 2 mg of bumetanide impurity A CRS and 2 mg of bumetanide impurity B CRS in the mobile phase and dilute to 10.0 ml with the mobile phase. Dilute 1.0 ml of this solution to 100.0 ml with the mobile phase.

Column:

- size: $l = 0.15$ m, $\varnothing = 4.6$ mm,
- stationary phase: end-capped octylsilyl silica gel for chromatography R (3.5 μ m).

Mobile phase: mix 70 volumes of methanol R, 25 volumes of water for chromatography R and 5 volumes of a 27.2 g/l solution of potassium dihydrogen phosphate R previously adjusted to pH 7.0 with a 280 g/l solution of potassium hydroxide R; add tetrahexylammonium bromide R to this mixture to obtain a concentration of 2.17 g/l.

Flow rate: 1.0 ml/min.

Detection: spectrophotometer at 254 nm.

Injection: 10 μ l.

Run time: 5 times the retention time of bumetanide.

Relative retention with reference to bumetanide (retention time = about 6 min): impurity B = about 0.4; impurity A = about 0.6; impurity D = about 2.5; impurity C = about 4.4.

System suitability: reference solution (b):

- resolution: minimum 2.0 between the peaks due to impurity A and impurity B.

Limits:

- impurities A, B, C, D: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent),
- other impurities: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent),
- total: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent),
- disregard limit: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

Loss on drying (2.2.32): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 100-105 °C for 4 h.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.300 g in 50 ml of alcohol R. Add 0.1 ml of phenol red solution R. Titrate with 0.1 M sodium hydroxide until a violet-red colour is obtained. Carry out a blank titration.

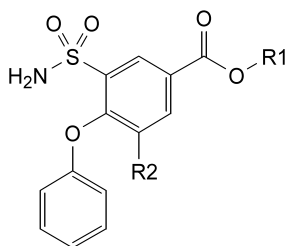
1 ml of 0.1 M sodium hydroxide is equivalent to 36.44 mg of $C_{17}H_{20}N_2O_5S$.

STORAGE

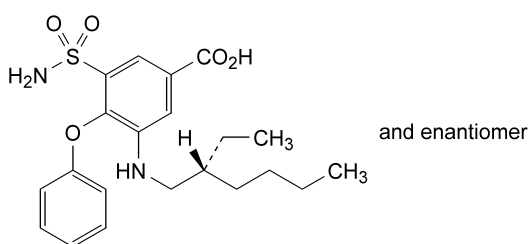
Protected from light.

IMPURITIES

Specified impurities: A, B, C, D.



- A. R1 = H, R2 = NO₂: 3-nitro-4-phenoxy-5-sulphamoylbenzoic acid,
 B. R1 = H, R2 = NH₂: 3-amino-4-phenoxy-5-sulphamoylbenzoic acid,
 C. R1 = C₄H₉, R2 = NH-C₄H₉: butyl 3-(butylamino)-4-phenoxy-5-sulphamoylbenzoate,

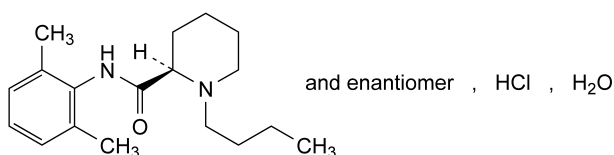


- D. 3-[[[(2RS)-2-ethylhexyl]amino]-4-phenoxy-5-sulphamoylbenzoic acid.

01/2005:0541

BUPIVACAINE HYDROCHLORIDE

Bupivacaini hydrochloridum

C₁₈H₂₉ClN₂O₂M_r 342.9

DEFINITION

(2RS)-1-Butyl-N-(2,6-dimethylphenyl)piperidine-2-carboxamide hydrochloride monohydrate.

Content: 98.5 per cent to 101.0 per cent (dried substance).

CHARACTERS

Appearance: white, crystalline powder or colourless crystals.

Solubility: soluble in water, freely soluble in alcohol.

mp: about 254 °C, with decomposition.

IDENTIFICATION

First identification: A, D.

Second identification: B, C, D.

- A. Infrared absorption spectrophotometry (2.2.24).

Preparation: discs of potassium bromide R.

Comparison: bupivacaine hydrochloride CRS.

- B. Thin-layer chromatography (2.2.27).

Test solution. Dissolve 25 mg of the substance to be examined in methanol R and dilute to 5 ml with the same solvent.

Reference solution. Dissolve 25 mg of bupivacaine hydrochloride CRS in methanol R and dilute to 5 ml with the same solvent.

Plate: TLC silica gel G plate R.

Mobile phase: concentrated ammonia R, methanol R (0.1:100 V/V).

Application: 5 µl.

Development: over a path of 10 cm.

Drying: in air.

Detection: spray with dilute potassium iodobismuthate solution R.

Results: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

- C. Dissolve 0.1 g in 10 ml of water R, add 2 ml of dilute sodium hydroxide solution R and shake with 2 quantities, each of 15 ml, of ether R. Dry the combined ether layers over anhydrous sodium sulphate R and filter. Evaporate the ether, recrystallise the residue from alcohol (90 per cent V/V) R and dry under reduced pressure. The crystals melt (2.2.14) at 105 °C to 108 °C.

- D. It gives reaction (a) of chlorides (2.3.1).

TESTS

Solution S. Dissolve 1.0 g in carbon dioxide-free water R and dilute to 50 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and colourless (2.2.2, Method II).

Acidity or alkalinity. To 10 ml of solution S add 0.2 ml of 0.01 M sodium hydroxide; the pH (2.2.3) is not less than 4.7. Add 0.4 ml of 0.01 M hydrochloric acid; the pH is not greater than 4.7.

Related substances. Gas chromatography (2.2.28).

Internal standard solution. Dissolve 25 mg of methyl behenate R in methylene chloride R and dilute to 500 ml with the same solvent.

Test solution. Dissolve 50.0 mg of the substance to be examined in 2.5 ml of water R, add 2.5 ml of dilute sodium hydroxide solution R and extract with 2 quantities, each of 5 ml, of the internal standard solution. Filter the lower layer.

Reference solution (a). Dissolve 10 mg of the substance to be examined, 10 mg of bupivacaine impurity B CRS and 10 mg of bupivacaine impurity E CRS in 2.5 ml of water R, add 2.5 ml of dilute sodium hydroxide solution R and extract with 2 quantities, each of 5 ml, of the internal standard solution. Filter the lower layer and dilute to 20 ml with the internal standard solution.

Reference solution (b). Dilute 1.0 ml of the test solution to 100.0 ml with the internal standard solution.

Reference solution (c). Dilute 5.0 ml of reference solution (b) to 10.0 ml with the internal standard solution.

Reference solution (d). Dilute 1.0 ml of reference solution (b) to 10.0 ml with the internal standard solution.

Column:

- material: fused silica,
- size: l = 30 m, Ø = 0.32 mm,
- stationary phase: poly(dimethyl)(diphenyl)siloxane R (film thickness 0.25 µm).

Carrier gas: helium for chromatography R.

Flow rate: 2.5 ml/min.

Split ratio: 1:12.

Temperature:

	Time (min)	Temperature (°C)
	0	180
Column	0 - 10	180 → 230
	10 - 15	230
Injection port		250
Detector		250

Detection: flame ionisation.

Injection: 1 µl.

Relative retention with reference to bupivacaine (retention time = about 10 min): impurity C = about 0.5; impurity A = about 0.6; impurity B = about 0.7; impurity D = about 0.8; impurity E = about 1.1; internal standard = about 1.4.

System suitability: reference solution (a):

- resolution: minimum 3.0 between the peaks due to bupivacaine and impurity E.

Limits:

- impurity B: calculate the ratio (*R*) of the area of the principal peak to the area of the peak due to the internal standard from the chromatogram obtained with reference solution (c); from the chromatogram obtained with the test solution, calculate the ratio of the area of the peak due to impurity B to the area of the peak due to the internal standard: this ratio is not greater than *R* (0.5 per cent),
- any other impurity: calculate the ratio (*R*) of the area of the principal peak to the area of the peak due to the internal standard from the chromatogram obtained with reference solution (d); from the chromatogram obtained with the test solution, calculate the ratio of the area of any peak, apart from the principal peak, the peak due to impurity B and the peak due to the internal standard, to the area of the peak due to the internal standard: this ratio is not greater than *R* (0.1 per cent),
- total: calculate the ratio (*R*) of the area of the principal peak to the area of the peak due to the internal standard from the chromatogram obtained with reference solution (b); from the chromatogram obtained with the test solution, calculate the ratio of the sum of the areas of any peaks, apart from the principal peak and the peak due to the internal standard, to the area of the peak due to the internal standard: this ratio is not greater than *R* (1.0 per cent),
- disregard limit: ratio less than 0.01 times *R* (0.01 per cent).

2,6-Dimethylaniline: maximum 100 ppm.

Dissolve 0.50 g in *methanol R* and dilute to 10 ml with the same solvent. To 2 ml of the solution add 1 ml of a freshly prepared 10 g/l solution of *dimethylaminobenzaldehyde R* in *methanol R* and 2 ml of *glacial acetic acid R* and allow to stand for 10 min. Any yellow colour in the solution is not more intense than that in a standard prepared at the same time and in the same manner using 2 ml of a 5 mg/l solution of *2,6-dimethylaniline R* in *methanol R*.

Heavy metals (2.4.8): maximum 10 ppm.

Dissolve 2.0 g in a mixture of 15 volumes of *water R* and 85 volumes of *methanol R* and dilute to 20 ml with the same mixture of solvents. 12 ml of the solution complies with limit

test B. Prepare the standard using lead standard solution (1 ppm Pb) obtained by diluting *lead standard solution (100 ppm Pb) R* with a mixture of 15 volumes of *water R* and 85 volumes of *methanol R*.

Loss on drying (2.2.32): 4.5 per cent to 6.0 per cent, determined on 1.000 g by drying in an oven at 100-105 °C.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

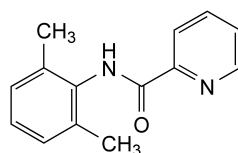
Dissolve 0.250 g in a mixture of 20 ml of *water R* and 25 ml of *alcohol R*. Add 5.0 ml of 0.01 M *hydrochloric acid*. Carry out a potentiometric titration (2.2.20), using 0.1 M *ethanolic sodium hydroxide*. Read the volume added between the 2 points of inflexion.

1 ml of 0.1 M *ethanolic sodium hydroxide* is equivalent to 32.49 mg of C₁₈H₂₉ClN₂O.

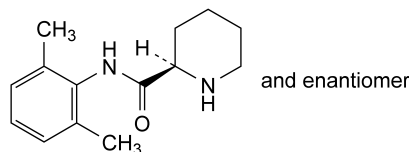
STORAGE

Protected from light.

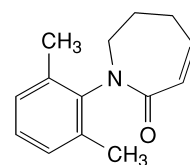
IMPURITIES



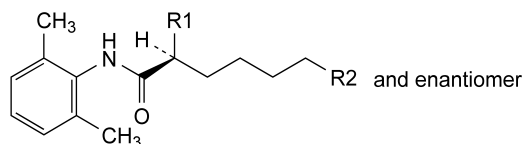
A. *N*-(2,6-dimethylphenyl)pyridine-2-carboxamide,



B. (2*RS*)-*N*-(2,6-dimethylphenyl)piperidine-2-carboxamide,

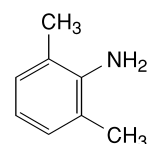


C. 1-(2,6-dimethylphenyl)-1,5,6,7-tetrahydro-2*H*-azepin-2-one,



D. R1 = R2 = Cl: (2*RS*)-2,6-dichloro-*N*-(2,6-dimethylphenyl)hexanamide,

E. R1 = H, R2 = NH-(CH₂)₃-CH₃: 6-(butylamino)-*N*-(2,6-dimethylphenyl)hexanamide,

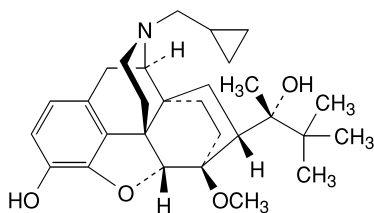


F. 2,6-dimethylaniline.

01/2005:1180 — as detector a spectrophotometer set at 288 nm, maintaining the temperature of the column at 40 °C.

BUPRENORPHINE

Buprenorphinum



$C_{29}H_{41}NO_4$

M_r 467.6

DEFINITION

Buprenorphine contains not less than 98.5 per cent and not more than the equivalent of 101.0 per cent of (2S)-2-[17-(cyclopropylmethyl)-4,5 α -epoxy-3-hydroxy-6-methoxy-6 α ,14-ethano-14 α -morphinan-7 α -yl]-3,3-dimethylbutan-2-ol, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, crystalline powder, very slightly soluble in water, freely soluble in acetone, soluble in methanol, slightly soluble in cyclohexane. It dissolves in dilute solutions of acids.

It melts at about 217 °C.

IDENTIFICATION

Examine by infrared absorption spectrophotometry (2.2.24), comparing with the *Ph. Eur. reference spectrum of buprenorphine*.

TESTS

Solution S. Dissolve 0.250 g in *ethanol R* and dilute to 25.0 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and colourless (2.2.2, *Method II*).

Specific optical rotation (2.2.7): – 103 to – 107, determined on solution S and calculated with reference to the dried substance.

Related substances. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 25.0 mg of the substance to be examined in the mobile phase and dilute to 10.0 ml with the mobile phase.

Reference solution (a). Dissolve 5.0 mg of the substance to be examined in 2.0 ml of *methanol R*. Add 0.25 ml of 2 M *hydrochloric acid*.

Reference solution (b). Dilute 0.5 ml of the test solution to 200.0 ml with the mobile phase.

Reference solution (c). Dilute 0.65 ml of the test solution to 100.0 ml with the mobile phase.

Reference solution (d). Dilute 4.0 ml of reference solution (b) to 10.0 ml with the mobile phase.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4.6 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (5 μ m),
- as mobile phase at a flow rate of about 1 ml/min, a mixture of 10 volumes of a 10 g/l solution of *ammonium acetate R* and 60 volumes of *methanol R*,

as detector a spectrophotometer set at 288 nm, maintaining the temperature of the column at 40 °C. Inject 20 μ l of reference solution (a). Adjust the flow rate so that the retention time of the peak corresponding to buprenorphine is about 15 min. The test is not valid unless the chromatogram obtained with reference solution (a) presents 2 peaks, the first having a retention time of 0.93 relative to the second peak (buprenorphine). Inject 20 μ l of each solution. Record the chromatogram of the test solution for 2.5 times the retention time of the principal peak. In the chromatogram obtained with the test solution: the area of any peak, apart from the principal peak, is not greater than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.25 per cent); the sum of the areas of such peaks is not greater than the area of the peak in the chromatogram obtained with reference solution (c) (0.65 per cent). Disregard any peak with an area less than that of the principal peak in the chromatogram obtained with reference solution (d).

Loss on drying (2.2.32). Not more than 1.0 per cent, determined on 1.000 g by drying in an oven at 100–105 °C.

ASSAY

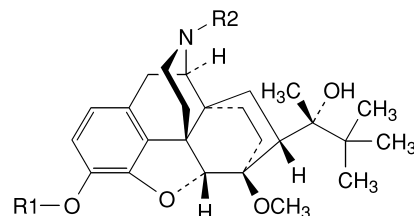
Dissolve 0.400 g in 40 ml of *anhydrous acetic acid R*. Titrate with 0.1 M *perchloric acid* until the colour changes from violet-blue to green, using 0.1 ml of *crystal violet solution R* as indicator.

1 ml of 0.1 M *perchloric acid* is equivalent to 46.76 mg of $C_{29}H_{41}NO_4$.

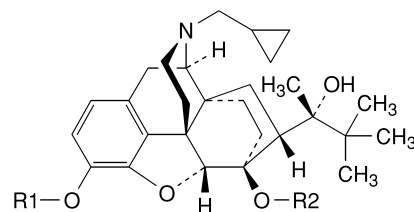
STORAGE

Store protected from light.

IMPURITIES



- R1 = H, R2 = $CH_2CH_2CH=CH_2$: (2S)-2-[17-(but-3-enyl)-4,5 α -epoxy-3-hydroxy-6-methoxy-6 α ,14-ethano-14 α -morphinan-7 α -yl]-3,3-dimethylbutan-2-ol,
- R1 = R2 = H: (2S)-2-(4,5 α -epoxy-3-hydroxy-6-methoxy-6 α ,14-ethano-14 α -morphinan-7 α -yl)-3,3-dimethylbutan-2-ol,
- R1 = CH_3 , R2 = CN: 4,5 α -epoxy-7 α -(1S)-1-hydroxy-1,2,2-trimethylpropyl]-3,6-dimethoxy-6 α ,14-ethano-14 α -morphinan-17-carbonitrile,

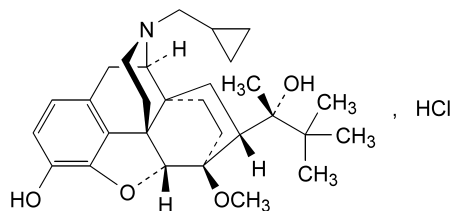


- R1 = R2 = CH_3 : (2S)-2-[17-(cyclopropylmethyl)-4,5 α -epoxy-3,6-dimethoxy-6 α ,14-ethano-14 α -morphinan-7 α -yl]-3,3-dimethylbutan-2-ol (3-O-methylbuprenorphine),
- R1 = R2 = H: (2S)-2-[17-(cyclopropylmethyl)-4,5 α -epoxy-3,6-dihydroxy-6 α ,14-ethano-14 α -morphinan-7 α -yl]-3,3-dimethylbutan-2-ol (6-O-desmethylbuprenorphine).

01/2005:1181

BUPRENORPHINE HYDROCHLORIDE

Buprenorphini hydrochloridum

 $C_{29}H_{42}ClNO_4$ M_r 504.1

DEFINITION

Buprenorphine hydrochloride contains not less than 98.5 per cent and not more than the equivalent of 101.0 per cent of (2S)-2-[17-(cyclopropylmethyl)-4,5 α -epoxy-3-hydroxy-6-methoxy-6 α ,14-ethano-14 α -morphinan-7 α -yl]-3,3-dimethylbutan-2-ol hydrochloride, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, crystalline powder, sparingly soluble in water, freely soluble in methanol, soluble in alcohol, practically insoluble in cyclohexane.

IDENTIFICATION

- Examine by infrared absorption spectrophotometry (2.2.24), comparing with the *Ph. Eur. reference spectrum of buprenorphine hydrochloride*.
- 3 ml of solution S (see Tests) gives reaction (a) of chlorides (2.3.1).

TESTS

Solution S. Dissolve 0.250 g in 5.0 ml of *methanol R* and while stirring dilute to 25.0 ml with *carbon dioxide-free water R*.

Appearance of solution. Solution S is clear (2.2.1) and colourless (2.2.2, *Method II*).

Acidity or alkalinity. To 10.0 ml of solution S add 0.05 ml of *methyl red solution R*. Not more than 0.2 ml of 0.02 M *sodium hydroxide* or 0.02 M *hydrochloric acid* is required to change the colour of the indicator.

Specific optical rotation (2.2.7). Dissolve 0.100 g in *methanol R* and dilute to 10.0 ml with the same solvent. The specific optical rotation is -92 to -98 , calculated with reference to the dried substance.

Related substances. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 25.0 mg of the substance to be examined in the mobile phase and dilute to 10.0 ml with the mobile phase.

Reference solution (a). Dissolve 5 mg of the substance to be examined in 2.0 ml of *methanol R*. Add 0.25 ml of 2 M *hydrochloric acid*.

Reference solution (b). Dilute 0.5 ml of the test solution to 200.0 ml with the mobile phase.

Reference solution (c). Dilute 0.65 ml of the test solution to 100.0 ml with the mobile phase.

Reference solution (d). Dilute 4.0 ml of reference solution (b) to 10.0 ml with the mobile phase.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4.6 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (5 μ m),
- as the mobile phase at a flow rate of about 1 ml/min, a mixture of 10 volumes of a 10 g/l solution of *ammonium acetate R* and 60 volumes of *methanol R*,
- as detector a spectrophotometer set at 288 nm, maintaining the temperature of the column at 40 $^{\circ}$ C.

Inject 20 μ l of reference solution (a). Adjust the flow rate so that the retention time of the peak corresponding to buprenorphine is about 15 min. The test is not valid unless the chromatogram obtained with reference solution (a) presents two peaks, the first having a retention time of 0.93 relative to the second peak (buprenorphine). Inject separately 20 μ l of each solution. Record the chromatogram of the test solution for 2.5 times the retention time of the main peak. In the chromatogram obtained with the test solution: the area of any peak, apart from the principal peak, is not greater than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.25 per cent) and the sum of the areas of such peaks is not greater than the area of the peak in the chromatogram obtained with reference solution (c) (0.65 per cent). Disregard any peak with an area less than that of the principal peak in the chromatogram obtained with reference solution (d).

Loss on drying (2.2.32). Not more than 1.0 per cent, determined on 1.000 g by heating in an oven at 115-120 $^{\circ}$ C.

ASSAY

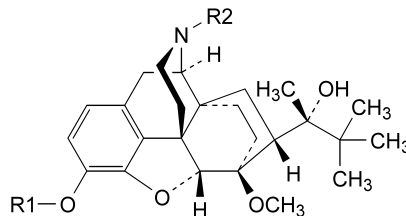
Dissolve 0.400 g in 40 ml of *anhydrous acetic acid R* and add 10 ml of *acetic anhydride R*. Titrate with 0.1 M *perchloric acid*, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *perchloric acid* is equivalent to 50.41 mg of $C_{29}H_{42}ClNO_4$.

STORAGE

Store protected from light.

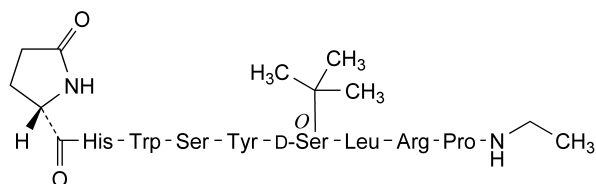
IMPURITIES



- $R_1 = H$, $R_2 = CH_2-CH_2-CH=CH_2$: (2S)-2-[17-(but-3-enyl)-4,5 α -epoxy-3-hydroxy-6-methoxy-6 α ,14-ethano-14 α -morphinan-7 α -yl]-3,3-dimethylbutan-2-ol,
- $R_1 = H$, $R_2 = H$: (2S)-2-(4,5 α -epoxy-3-hydroxy-6-methoxy-6 α ,14-ethano-14 α -morphinan-7 α -yl)-3,3-dimethylbutan-2-ol,
- $R_1 = CH_3$, $R_2 = CN$: 4,5 α -epoxy-7 α -(1S)-1-hydroxy-1,2,2-trimethylpropyl]-3,6-dimethoxy-6 α ,14-ethano-14 α -morphinan-17-carbonitrile.

01/2005:1077 **Related substances.** Liquid chromatography (2.2.29).**BUSERELIN**

Buserelinum

 $C_{60}H_{86}N_{16}O_{13}$ M_r 1239**DEFINITION**

5-Oxo-L-prolyl-L-histidyl-L-tryptophyl-L-seryl-L-tyrosyl-O-(1,1-dimethylethyl)-D-seryl-L-leucyl-L-arginyl-L-prolylethylamide.

Synthetic nonapeptide analogue of human gonadotrophin-releasing hormone GnRH with agonistic activity to gonadorelin. It is obtained by chemical synthesis and is available as an acetate.

Content: 95.0 per cent to 102.0 per cent (anhydrous, acetic acid-free substance).

CHARACTERS

Appearance: white or slightly yellowish powder, hygroscopic.

Solubility: sparingly soluble in water and in dilute acids.

IDENTIFICATION

A. Examine the chromatograms obtained in the assay.

Results: the principal peak in the chromatogram obtained with the test solution is similar in retention time and size to the principal peak in the chromatogram obtained with reference solution (b).

B. Nuclear magnetic resonance spectrometry (2.2.33).

Preparation: 4 mg/ml solution in a mixture of 20 volumes of *deuterated acetic acid R* and 80 volumes of *deuterium oxide R*.

Results: the 1H NMR spectrum obtained is qualitatively similar to the *Ph. Eur. reference spectrum of buserelin*.

C. Amino acid analysis (2.2.56). For protein hydrolysis use Method 1 and for analysis use Method 1.

Express the content of each amino acid in moles. Calculate the relative proportions of the amino acids, taking one-sixth of the sum of the number of moles of glutamic acid, histidine, tyrosine, leucine, arginine, proline as equal to one. The values fall within the following limits: serine 1.4 to 2.0; proline 0.8 to 1.2; glutamic acid 0.9 to 1.1; leucine 0.9 to 1.1; tyrosine 0.9 to 1.1; histidine 0.9 to 1.1 and arginine 0.9 to 1.1. Not more than traces of other amino acids are present, with the exception of tryptophan.

TESTS

Appearance of solution. A 10 g/l solution is clear (2.2.1) and not more intensely coloured than reference solution Y₇ (2.2.2, *Method II*).

Specific optical rotation (2.2.7): –49 to –58 (anhydrous, acetic acid-free substance), determined on a 10 g/l solution.

Specific absorbance (2.2.25): 49 to 56 by measuring at the absorption maximum at 278 nm (anhydrous, acetic acid-free substance).

Dissolve 10.0 mg in 100.0 ml of 0.01 M hydrochloric acid.

Test solution. Dissolve 5.0 mg of the substance to be examined in 5.0 ml of the mobile phase.

Reference solution (a). Dissolve the contents of a vial of *D-His-buserelin CRS* in the mobile phase. Dilute an appropriate volume of this solution in the mobile phase to obtain a final concentration of 1 mg/ml. Add 1.0 ml of the test solution to 1.0 ml of this solution.

Reference solution (b). Dissolve the contents of a vial of *buserelin CRS* in the mobile phase. Dilute an appropriate volume of this solution in the mobile phase to obtain a final concentration of 1.0 mg/ml.

Reference solution (c). Dilute 1.0 ml of the test solution to 100.0 ml with the mobile phase.

Column:

- size: $l = 0.25$ m, $\varnothing = 4$ mm,
- stationary phase: octadecylsilyl silica gel for chromatography R (5 μ m).

Mobile phase: mix 200 ml of *acetonitrile R* and 700 ml of an 11.2 g/l solution of *phosphoric acid R* and adjust to pH 2.5 with *triethylamine R*.

Flow rate: 0.8 ml/min.

Detection: spectrophotometer at 220 nm.

Injection: 10 μ l of the test solution, reference solution (a) and reference solution (c).

Relative retention with reference to buserelin (retention time = about 36 min): impurity B = about 0.76; impurity C = about 0.83; impurity A = about 0.90; impurity D = about 0.94; impurity E = about 0.94.

System suitability: reference solution (a):

- resolution: minimum 1.5 between peaks due to impurity A and buserelin.

Limits:

- sum of impurities D and E: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (c) (3 per cent),
- any other impurity: for each impurity, not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (c) (3 per cent),
- total: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (c) (5 per cent),
- disregard limit: 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.1 per cent).

Acetic acid (2.5.34): 3.0 per cent to 7.0 per cent.

Test solution. Dissolve 20.0 mg of the substance to be examined in a mixture of 5 volumes of mobile phase B and 95 volumes of mobile phase A and dilute to 10.0 ml with the same mixture of solvents.

Water (2.5.12): maximum 4.0 per cent, determined on 80.0 mg.

Bacterial endotoxins (2.6.14): less than 55.5 IU/mg, if intended for use in the manufacture of parenteral dosage forms without a further appropriate procedure for the removal of bacterial endotoxins.

ASSAY

Liquid chromatography (2.2.29) as described in the test for related substances with the following modification.

Injection: 10 μ l of the test solution and reference solution (b). Calculate the content of buserelin ($C_{60}H_{86}N_{16}O_{13}$) using the areas of the peaks of the chromatograms obtained and the stated content of $C_{60}H_{86}N_{16}O_{13}$ in *buserelin CRS*.

STORAGE

In an airtight container, protected from light, at a temperature of 2 °C to 8 °C.

If the substance is sterile, store in an airtight, sterile, tamper-proof container.

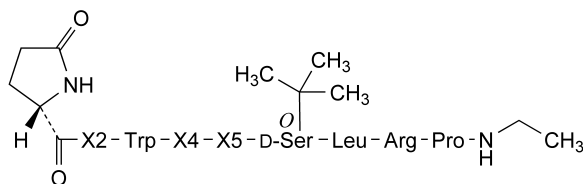
LABELLING

The label states:

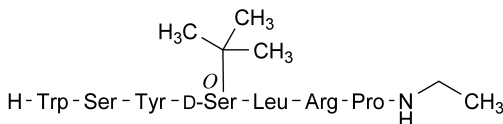
- the mass of peptide in the container,
- where applicable, that the substance is free from bacterial endotoxins.

IMPURITIES

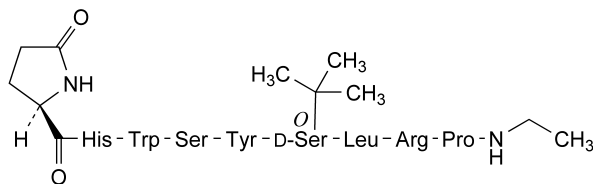
Specified impurities: A, B, C, D, E.



- A. X2 = D-His, X4 = L-Ser, X5 = L-Tyr: [2-D-histidine]buserelin,
 B. X2 = L-His, X4 = D-Ser, X5 = L-Tyr: [4-D-serine]buserelin,
 D. X2 = L-His, X4 = L-Ser, X5 = D-Tyr: [5-D-tyrosine]buserelin,



- C. buserelin-(3-9)-peptide,

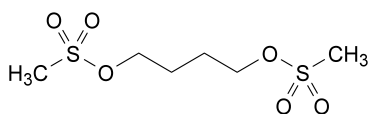


- E. [1-(5-oxo-D-proline)]buserelin.

01/2005:0542

BUSULFAN

Busulfanum



$C_6H_{14}O_6S_2$

M_r 246.3

DEFINITION

Busulfan contains not less than 99.0 per cent and not more than the equivalent of 100.5 per cent of butane-1,4-diyl di(methanesulphonate), calculated with reference to the dried substance.

CHARACTERS

A white or almost white, crystalline powder, very slightly soluble in water, freely soluble in acetone and in acetonitrile, very slightly soluble in alcohol.

It melts at about 116 °C.

IDENTIFICATION

First identification: A.

Second identification: B, C, D.

A. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with busulfan CRS.

B. Examine by thin-layer chromatography (2.2.27), using silica gel G R as the coating substance.

Test solution. Dissolve 20 mg of the substance to be examined in 2 ml of acetone R.

Reference solution. Dissolve 20 mg of busulfan CRS in 2 ml of acetone R.

Apply separately to the plate 5 µl of each solution.

Develop over a path of 15 cm using a mixture of equal volumes of acetone R and toluene R. Dry the plate in a stream of hot air and spray with anisaldehyde solution R. Heat the plate at 120 °C. The principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

C. To 0.1 g add 5 ml of 1 M sodium hydroxide. Heat until a clear solution is obtained. Allow to cool. To 2 ml of the solution add 0.1 ml of potassium permanganate solution R. The colour changes from purple through violet to blue and finally to green. Filter and add 1 ml of ammoniacal silver nitrate solution R. A precipitate is formed.

D. To 0.1 g add 0.1 g of potassium nitrate R and 0.25 g of sodium hydroxide R, mix and heat to fusion. Allow to cool and dissolve the residue in 5 ml of water R. Adjust to pH 1 to 2 using dilute hydrochloric acid R. The solution gives reaction (a) of sulphates (2.3.1).

TESTS

Appearance of solution. Dissolve 0.25 g in 20 ml of acetonitrile R, dilute to 25 ml with water R and examine immediately. The solution is clear (2.2.1) and not more intensely coloured than reference solution B₇ (2.2.2, Method II).

Acidity. Dissolve 0.20 g with heating in 50 ml of ethanol R. Add 0.1 ml of methyl red solution R. Not more than 0.05 ml of 0.1 M sodium hydroxide is required to change the colour of the indicator.

Loss on drying (2.2.32). Not more than 2.0 per cent, determined on 1.000 g by drying *in vacuo* at 60 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

To 0.250 g add 50 ml of water R. Shake. Boil under a reflux condenser for 30 min and, if necessary, make up to the initial volume with water R. Allow to cool. Using 0.3 ml of phenolphthalein solution R as indicator, titrate with 0.1 M sodium hydroxide until a pink colour is obtained.

1 ml of 0.1 M sodium hydroxide is equivalent to 12.32 mg of $C_6H_{14}O_6S_2$.

STORAGE

Store in an airtight container, protected from light.

01/2005:1847

BUTCHER'S BROOM

Rusci rhizoma

DEFINITION

Dried, whole or fragmented underground parts of *Ruscus aculeatus* L.

Content: minimum 1.0 per cent of total sapogenins, expressed as ruscogenins [mixture of neoruscogenin ($C_{27}H_{40}O_4$; M_r 428.6) and ruscogenin ($C_{27}H_{42}O_4$; M_r 430.6)] (dried drug).

CHARACTERS

Macroscopic and microscopic characters described under identification tests A and B.

IDENTIFICATION

- A. The rhizome consists of yellowish, branched, articulated, somewhat knotty pieces, cylindrical or subconical, about 5 cm to 10 cm long and about 5 mm thick. The surface is marked with thin annulations about 1 mm to 3 mm wide, separated from one another; rounded scars of the aerial stems are present on the upper surface. On the lower surface numerous roots, or their scars, occur; the roots are about 2 mm in diameter and similar in colour to the rhizome. The outer layer is easily detached, revealing a yellowish-white, very hard central cylinder.
- B. Reduce to a powder (355). The powder is yellowish. Examine under a microscope using *chloral hydrate solution R*. The powder shows groups of sclereids from the ground tissue of the rhizome, variously-shaped cells, ranging from rounded to elongated or rectangular; the walls are moderately thickened and distinctly beaded, with large, rounded to oval pits. Fragments of the endodermis composed of a single layer of irregularly-thickened cells. Groups of rounded parenchymatous cells, thickened at the corners, with small, triangular intercellular spaces; thin-walled parenchyma containing raphides of calcium oxalate. Groups of thick-walled fibres and small vessels, up to about 50 µm in diameter, the walls showing numerous small, slit-shaped pits.
- C. Thin-layer chromatography (2.2.27)

Test solution. Introduce 1.0 g of the powdered drug (355) and 50 ml of *dilute hydrochloric acid R* into a 100 ml flask with a ground-glass neck. Heat on a water-bath under a reflux condenser for 40 min. Allow to cool and extract the unfiltered mixture with 3 quantities, each of 25 ml, of *methylene chloride R*. Combine the organic solutions and dry over *anhydrous sodium sulphate R*. Filter and evaporate to dryness. Dissolve the residue in 5 ml of *methanol R*.

Reference solution. Dissolve 1 mg of *ruscogenins R* and 1 mg of *stigmaterol R* in *methanol R* and dilute to 5 ml with the same solvent.

Plate: TLC silica gel plate *R*.

Mobile phase: *methanol R*, *methylene chloride R* (7:93 V/V).

Application: 10 µl, as bands.

Development: over a path of 15 cm.

Drying: in air.

Detection: spray with *vanillin reagent R*, dry the plate in an oven at 100-105 °C for 1 min and examine in daylight.

Results: see below the sequence of the zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other weak zones may be present in the chromatogram obtained with the test solution.

Top of the plate	
Stigmaterol: a violet zone	Several zones of various colours A violet zone
Ruscogenins: a yellow zone	A violet zone A yellow zone (ruscogenins) Several zones of various colours
Reference solution	Test solution

TESTS

Foreign matter (2.8.2): maximum 5 per cent.

Loss on drying (2.2.32): maximum 12.0 per cent, determined on 1.000 g of the powdered drug (355) by drying in an oven at 100-105 °C for 2 h.

Total ash (2.4.16): maximum 12.0 per cent.

Ash insoluble in hydrochloric acid (2.8.1): maximum 5.0 per cent.

ASSAY

Liquid chromatography (2.2.29).

Test solution. To 2.000 g of the powdered drug (355), add 60 ml of *ethanol R*, 15 ml of *water R* and 0.2 g of *potassium hydroxide R*. Extract under reflux on a water-bath for 4 h. Allow to cool and filter into a 100 ml volumetric flask. Rinse the extraction flask and the residue in the filter with 3 quantities, each of 10 ml, of *ethanol R* and add the rinsings to the volumetric flask. Dilute to 100.0 ml with *ethanol R*. Introduce 25.0 ml of the solution into a round-bottomed flask fitted to a rotary evaporator and evaporate to dryness. Dissolve the residue in 10 ml of *butanol R*, add 3 ml of *hydrochloric acid R1* and 8 ml of *water R*. Heat under reflux on a water-bath for 1 h. Allow to cool and transfer the liquid into a separating funnel, rinse the round-bottomed flask with 2 quantities, each of 10 ml, of *butanol R*. Add the rinsings to the separating funnel. Extract with 3 quantities, each of 20 ml, of *butanol R* saturated with *water R*. Combine the butanolic extracts and evaporate to dryness using a rotary evaporator. Dissolve the residue in 20 ml of *methanol R* and transfer to a 100 ml volumetric flask. Rinse the extraction flask with 20 ml, 20 ml and 10 ml of *methanol R* and add the rinsings to the volumetric flask. Dilute to 100 ml with *methanol R*.

Reference solution. Dissolve 5.0 mg of *ruscogenins R* in 100 ml of *methanol R*.

Column:

- size: $l = 0.25$ m, $\varnothing = 4.6$ mm,
- stationary phase: octadecylsilyl silica gel for chromatography *R* (5 µm).

Mobile phase:

- mobile phase A: *water R*,
- mobile phase B: *acetonitrile for chromatography R*,

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)
0 - 25	40	60
25 - 27	40 → 0	60 → 100
27 - 37	0	100
37 - 39	0 → 40	100 → 60
39 - 42	40	60

Flow rate: 1.2 ml/min.

Detection: spectrophotometer at 203 nm.

Injection: 20 µl.

Retention time with reference to neoruscogenin (retention time = about 16 min): ruscogenin = about 1.2.

System suitability: reference solution:

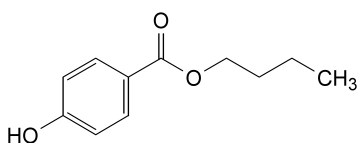
- **resolution:** minimum 1.5 between the peaks due to neoruscogenin and ruscogenin.

Calculate the percentage content of sapogenins expressed as ruscogenins (neoruscogenin and ruscogenin) in the test solution by comparing the areas of the peaks in the chromatograms obtained with the test solution and the reference solution.

01/2005:0881

BUTYL PARAHYDROXYBENZOATE

Butylis parahydroxybenzoas



$C_{11}H_{14}O_3$

M_r 194.2

DEFINITION

Butyl 4-hydroxybenzoate.

Content: 98.0 per cent to 102.0 per cent.

CHARACTERS

Appearance: white or almost white, crystalline powder or colourless crystals.

Solubility: very slightly soluble in water, freely soluble in alcohol and in methanol.

IDENTIFICATION

First identification: A, B.

Second identification: A, C, D.

A. Melting point (2.2.14): 68 °C to 71 °C.

B. Infrared absorption spectrophotometry (2.2.24).

Comparison: butyl parahydroxybenzoate CRS.

C. Examine the chromatograms obtained in the test for related substances.

Results: the principal spot in the chromatogram obtained with test solution (b) is similar in position and size to the principal spot in the chromatogram obtained with reference solution (b).

D. To about 10 mg in a test-tube add 1 ml of *sodium carbonate solution R*, boil for 30 s and cool (solution A). To a further 10 mg in a similar test-tube add 1 ml of *sodium carbonate solution R*; the substance partly dissolves (solution B). Add at the same time to solution A and solution B 5 ml of *aminopyrazolone solution R* and 1 ml of *potassium ferricyanide solution R* and mix. Solution B is yellow to orange-brown. Solution A is orange to red, the colour being clearly more intense than any similar colour which may be obtained with solution B.

TESTS

Solution S. Dissolve 1.0 g in *alcohol R* and dilute to 10 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and not more intensely coloured than reference solution BY₆ (2.2.2, Method II).

Acidity. To 2 ml of solution S add 3 ml of *alcohol R*, 5 ml of *carbon dioxide-free water R* and 0.1 ml of *bromocresol green solution R*. Not more than 0.1 ml of 0.1 M *sodium hydroxide* is required to change the colour of the indicator to blue.

Related substances. Thin-layer chromatography (2.2.27).

Test solution (a). Dissolve 0.10 g of the substance to be examined in *acetone R* and dilute to 10 ml with the same solvent.

Test solution (b). Dilute 1 ml of test solution (a) to 10 ml with *acetone R*.

Reference solution (a). Dilute 0.5 ml of test solution (a) to 100 ml with *acetone R*.

Reference solution (b). Dissolve 10 mg of *butyl parahydroxybenzoate CRS* in *acetone R* and dilute to 10 ml with the same solvent.

Reference solution (c). Dissolve 10 mg of *propyl parahydroxybenzoate R* in 1 ml of test solution (a) and dilute to 10 ml with *acetone R*.

Plate: suitable octadecylsilyl silica gel with a fluorescent indicator having an optimal intensity at 254 nm as the coating substance.

Mobile phase: *glacial acetic acid R*, *water R*, *methanol R* (1:30:70 V/V/V).

Application: 2 µl.

Development: over a path of 15 cm.

Drying: in air.

Detection: examine in ultraviolet light at 254 nm.

System suitability: the chromatogram obtained with reference solution (c) shows 2 clearly separated principal spots.

Limits:

- **any impurity:** any spot in the chromatogram obtained with test solution (a), apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (a) (0.5 per cent).

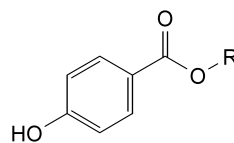
Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

To 1.000 g add 20.0 ml of 1 M *sodium hydroxide*. Heat at about 70 °C for 1 h. Cool rapidly in an ice bath. Prepare a blank in the same manner. Carry out the titration on the solutions at room temperature. Titrate the excess sodium hydroxide with 0.5 M *sulphuric acid*, continuing the titration until the second point of inflexion and determining the end-point potentiometrically (2.2.20).

1 ml of 1 M *sodium hydroxide* is equivalent to 194.2 mg of $C_{11}H_{14}O_3$.

IMPURITIES

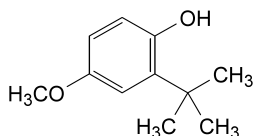


- A. R = H: 4-hydroxybenzoic acid,
- B. R = CH₃: methyl 4-hydroxybenzoate,
- C. R = CH₂-CH₃: ethyl 4-hydroxybenzoate,
- D. R = CH₂-CH₂-CH₃: propyl 4-hydroxybenzoate.

01/2005:0880

BUTYLHYDROXYANISOLE

Butylhydroxyanisolum

 $C_{11}H_{16}O_2$ M_r 180.3**DEFINITION**

Butylhydroxyanisole is 2-(1,1-dimethylethyl)-4-methoxyphenol containing not more than 10 per cent of 3-(1,1-dimethylethyl)-4-methoxyphenol.

CHARACTERS

A white, yellowish or slightly pinkish, crystalline powder, practically insoluble in water, very soluble in methylene chloride, freely soluble in alcohol and in fatty oils. It dissolves in dilute solutions of alkali hydroxides.

IDENTIFICATION

- A. Examine the chromatograms obtained in the test for related substances. The principal spot in the chromatogram obtained with test solution (b) is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).
- B. To 0.5 ml of solution S (see Tests) add 10 ml of *aminopyrazolone solution R* and 1 ml of *potassium ferricyanide solution R*. Mix and add 10 ml of *methylene chloride R*. Shake vigorously. After separation, the organic layer is red.
- C. Dissolve about 10 mg in 2 ml of *alcohol R*. Add 1 ml of a 1 g/l solution of *testosterone propionate R* in *alcohol R* and 2 ml of *dilute sodium hydroxide solution R*. Heat in a water-bath at 80 °C for 10 min and allow to cool. A red colour develops.

TESTS

Solution S. Dissolve 2.5 g in *alcohol R* and dilute to 25 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and not more intensely coloured than intensity 5 of the range of reference solutions of the most appropriate colour (2.2.2, *Method II*).

Related substances. Examine by thin-layer chromatography (2.2.27), using *silica gel G R* as the coating substance.

Test solution (a). Dissolve 0.25 g of the substance to be examined in *methylene chloride R* and dilute to 10 ml with the same solvent.

Test solution (b). Dilute 1 ml of test solution (a) to 10 ml with *methylene chloride R*.

Reference solution (a). Dissolve 25 mg of *butylhydroxyanisole CRS* in *methylene chloride R* and dilute to 10 ml with the same solvent.

Reference solution (b). Dilute 1 ml of reference solution (a) to 20 ml with *methylene chloride R*.

Reference solution (c). Dissolve 50 mg of *hydroquinone R* in 5 ml of *alcohol R* and dilute to 100 ml with *methylene chloride R*. Dilute 1 ml of this solution to 10 ml with *methylene chloride R*.

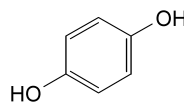
Apply separately to the plate 5 µl of each solution. Develop over a path of 10 cm using *methylene chloride R*. Allow the plate to dry in air and spray with a freshly prepared mixture of 10 volumes of *potassium ferricyanide solution R*, 20 volumes of *ferric chloride solution R1* and 70 volumes of *water R*. In the chromatogram obtained with test solution (a): any violet-blue spot with an R_f value of about 0.35 (corresponding to 3-(1,1-dimethylethyl)-4-methoxyphenol) is not more intense than the principal spot in the chromatogram obtained with reference solution (a) (10 per cent); any spot corresponding to hydroquinone is not more intense than the principal spot in the chromatogram obtained with reference solution (c) (0.2 per cent); any spot, apart from the principal spot and any spots corresponding to 3-(1,1-dimethylethyl)-4-methoxyphenol and hydroquinone, is not more intense than the principal spot in the chromatogram obtained with reference solution (b) (0.5 per cent).

Heavy metals (2.4.8). 1.0 g complies with limit test C for heavy metals (10 ppm). Prepare the standard using 1 ml of *lead standard solution (10 ppm Pb) R*.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

STORAGE

Store protected from light.

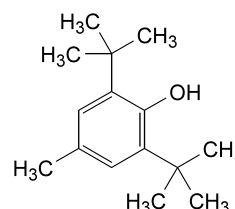
IMPURITIES

- A. benzene-1,4-diol (hydroquinone).

01/2005:0581

BUTYLHYDROXYTOLUENE

Butylhydroxytoluenum

 $C_{15}H_{24}O$ M_r 220.4**DEFINITION**

Butylhydroxytoluene is 2,6-bis(1,1-dimethylethyl)-4-methylphenol.

CHARACTERS

A white or yellowish-white, crystalline powder, practically insoluble in water, very soluble in acetone, freely soluble in alcohol and in vegetable oils.

IDENTIFICATION

First identification: A, C.

Second identification: A, B, D.

- A. It complies with the test for the freezing-point (see Tests).
- B. Dissolve 0.500 g in *ethanol R* and dilute to 100.0 ml with the same solvent. Dilute 1.0 ml of this solution to 100.0 ml with *ethanol R*. Examined between 230 nm and 300 nm (2.2.25), the solution shows an absorption maximum at 278 nm. The specific absorbance at the maximum is 80 to 90.

- C. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *butylhydroxytoluene CRS*.
- D. Dissolve about 10 mg in 2 ml of *alcohol R*. Add 1 ml of a 1 g/l solution of *testosterone propionate R* in *alcohol R* and 2 ml of *dilute sodium hydroxide solution R*. Heat in a water-bath at 80 °C for 10 min and allow to cool. A blue colour develops.

TESTS

Appearance of solution. Dissolve 1.0 g in *methanol R* and dilute to 10 ml with the same solvent. The solution is clear (2.2.1) and not more intensely coloured than reference solution Y₅ or BY₅ (2.2.2, *Method II*).

Freezing-point (2.2.18): 69 °C to 70 °C.

Related substances. Examine by thin-layer chromatography (2.2.27), using *silica gel G R* as the coating substance.

Test solution. Dissolve 0.2 g of the substance to be examined in *methanol R* and dilute to 10.0 ml with the same solvent.

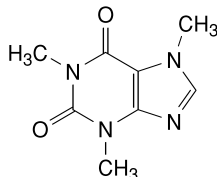
Reference solution. Dilute 1 ml of the test solution to 200 ml with *methanol R*.

Apply separately to the plate 10 µl of each solution. Develop over a path of 15 cm using *methylene chloride R*. Dry the plate in air and spray with a freshly prepared mixture of 10 volumes of *potassium ferricyanide solution R*, 20 volumes of *ferric chloride solution R1* and 70 volumes of *water R*. Any spot in the chromatogram obtained with the test solution, apart from the principal spot, is not more intense than the spot in the chromatogram obtained with the reference solution (0.5 per cent).

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

CAFFEINE

Coffeinum

 $C_8H_{10}N_4O_2$ M_r 194.2

DEFINITION

Caffeine contains not less than 98.5 per cent and not more than the equivalent of 101.5 per cent of 1,3,7-trimethyl-3,7-dihydro-1*H*-purine-2,6-dione, calculated with reference to the dried substance.

CHARACTERS

A white, crystalline powder or silky, white crystals, sublimes readily, sparingly soluble in water, freely soluble in boiling water, slightly soluble in ethanol. It dissolves in concentrated solutions of alkali benzoates or salicylates.

IDENTIFICATION

First identification: A, B, E.

Second identification: A, C, D, E, F.

- A. Melting point (2.2.14): 234 °C to 239 °C.
- B. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *caffeine CRS*.
- C. To 2 ml of a saturated solution add 0.05 ml of *iodinated potassium iodide solution R*. The solution remains clear. Add 0.1 ml of *dilute hydrochloric acid R*. A brown precipitate is formed. Neutralise with *dilute sodium hydroxide solution R*. The precipitate dissolves.
- D. In a glass-stoppered tube, dissolve about 10 mg in 0.25 ml of a mixture of 0.5 ml of *acetylacetone R* and 5 ml of *dilute sodium hydroxide solution R*. Heat in a water-bath at 80 °C for 7 min. Cool and add 0.5 ml of *dimethylaminobenzaldehyde solution R2*. Heat again in a water-bath at 80 °C for 7 min. Allow to cool and add 10 ml of *water R*. An intense blue colour develops.
- E. It complies with the test for loss on drying (see Tests).
- F. It gives the reaction of xanthines (2.3.1).

TESTS

Solution S. Dissolve 0.5 g with heating in 50 ml of *carbon dioxide-free water R* prepared from *distilled water R*, cool and dilute to 50 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and colourless (2.2.2, *Method II*).

Acidity. To 10 ml of solution S add 0.05 ml of *bromothymol blue solution R1*. The solution is green or yellow. Not more than 0.2 ml of 0.01 *M sodium hydroxide* is required to change the colour of the indicator to blue.

01/2005:0267 Related substances. Examine by thin-layer chromatography (2.2.27) using *silica gel GF₂₅₄ R* as the coating substance.

Test solution. Dissolve 0.2 g of the substance to be examined in a mixture of 4 volumes of *methanol R* and 6 volumes of *methylene chloride R* and dilute to 10 ml with the same mixture of solvents.

Reference solution. Dilute 0.5 ml of the test solution to 100 ml with a mixture of 4 volumes of *methanol R* and 6 volumes of *methylene chloride R*.

Apply to the plate 10 µl of each solution. Develop over a path of 15 cm using a mixture of 10 volumes of *concentrated ammonia R*, 30 volumes of *acetone R*, 30 volumes of *methylene chloride R* and 40 volumes of *butanol R*. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. Any spot in the chromatogram obtained with the test solution, apart from the principal spot, is not more intense than the spot in the chromatogram obtained with the reference solution (0.5 per cent).

Sulphates (2.4.13). 15 ml of solution S complies with the limit test for sulphates (500 ppm). Prepare the standard using a mixture of 7.5 ml of *sulphate standard solution (10 ppm SO₄) R* and 7.5 ml of *distilled water R*.

Heavy metals (2.4.8). 1.0 g complies with limit test C for heavy metals (20 ppm). Prepare the standard using 2 ml of *lead standard solution (10 ppm Pb) R*.

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 100-105 °C for 1 h.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.170 g with heating in 5 ml of *anhydrous acetic acid R*. Allow to cool, add 10 ml of *acetic anhydride R* and 20 ml of *toluene R*. Titrate with 0.1 *M perchloric acid*, determining the end-point potentiometrically (2.2.20).

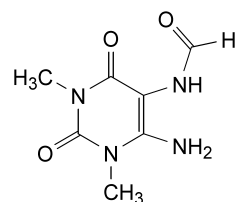
1 ml of 0.1 *M perchloric acid* is equivalent to 19.42 mg of $C_8H_{10}N_4O_2$.

IMPURITIES

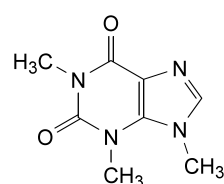
Specified impurities: A.

Other detectable impurities: B, C.

A. theophylline,



B. *N*-[6-amino-1,3-dimethyl-2,4(1*H*,3*H*)-dioxypyrimidin-5-yl]formamide,

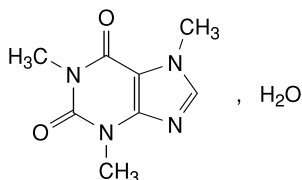


C. 1,3,9-trimethyl-3,9-dihydro-1*H*-purine-2,6-dione (isocaffeine).

01/2005:0268

CAFFEINE MONOHYDRATE

Coffeinum monohydricum

 $C_8H_{10}N_4O_2 \cdot H_2O$ M_r 212.2

DEFINITION

Caffeine monohydrate contains not less than 98.5 per cent and not more than the equivalent of 101.5 per cent of 1,3,7-trimethyl-3,7-dihydro-1*H*-purine-2,6-dione, calculated with reference to the dried substance.

CHARACTERS

A white, crystalline powder or silky, white crystals, sublimes readily, sparingly soluble in water, freely soluble in boiling water, slightly soluble in ethanol. It dissolves in concentrated solutions of alkali benzoates or salicylates.

IDENTIFICATION

First identification: A, B, E.

Second identification: A, C, D, E, F.

- Melting point (2.2.14): 234 °C to 239 °C, determined after drying at 100-105 °C.
- Dry the substance to be examined at 100-105 °C. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *caffeine CRS*.
- To 2 ml of a saturated solution add 0.05 ml of *iodinated potassium iodide solution R*. The solution remains clear. Add 0.1 ml of *dilute hydrochloric acid R*. A brown precipitate is formed. Neutralise with *dilute sodium hydroxide solution R*. The precipitate dissolves.
- In a glass-stoppered tube, dissolve about 10 mg in 0.25 ml of a mixture of 0.5 ml of *acetylacetone R* and 5 ml of *dilute sodium hydroxide solution R*. Heat in a water-bath at 80 °C for 7 min. Cool and add 0.5 ml of *dimethylaminobenzaldehyde solution R2*. Heat again in a water-bath at 80 °C for 7 min. Allow to cool and add 10 ml of *water R*. An intense blue colour develops.
- It complies with the test for loss on drying (see Tests).
- It gives the reaction of xanthines (2.3.1).

TESTS

Solution S. Dissolve 0.5 g with heating in 50 ml of *carbon dioxide-free water R* prepared from *distilled water R*, cool and dilute to 50 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and colourless (2.2.2, *Method II*).

Acidity. To 10 ml of solution S add 0.05 ml of *bromothymol blue solution R1*. The solution is green or yellow. Not more than 0.2 ml of 0.01 *M sodium hydroxide* is required to change the colour of the indicator to blue.

Related substances. Examine by thin-layer chromatography (2.2.27) using *silica gel GF₂₅₄ R* as the coating substance.

Test solution. Dissolve 0.2 g of the substance to be examined in a mixture of 4 volumes of *methanol R* and 6 volumes of *methylene chloride R* and dilute to 10 ml with the same mixture of solvents.

Reference solution. Dilute 0.5 ml of the test solution to 100 ml with a mixture of 4 volumes of *methanol R* and 6 volumes of *methylene chloride R*.

Apply to the plate 10 µl of each solution. Develop over a path of 15 cm using a mixture of 10 volumes of *concentrated ammonia R*, 30 volumes of *acetone R*, 30 volumes of *methylene chloride R* and 40 volumes of *butanol R*. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. Any spot in the chromatogram obtained with the test solution, apart from the principal spot, is not more intense than the spot in the chromatogram obtained with the reference solution (0.5 per cent).

Sulphates (2.4.13). 15 ml of solution S complies with the limit test for sulphates (500 ppm). Prepare the standard using a mixture of 7.5 ml of *sulphate standard solution (10 ppm SO₄) R* and 7.5 ml of *distilled water R*.

Heavy metals (2.4.8). 1.0 g complies with limit test C for heavy metals (20 ppm). Prepare the standard using 2 ml of *lead standard solution (10 ppm Pb) R*.

Loss on drying (2.2.32). 5.0 per cent to 9.0 per cent, determined on 1.000 g by drying in an oven at 100-105 °C for 1 h.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.170 g, previously dried at 100-105 °C, with heating in 5 ml of *anhydrous acetic acid R*. Allow to cool, add 10 ml of *acetic anhydride R* and 20 ml of *toluene R*. Titrate with 0.1 *M perchloric acid*, determining the end-point potentiometrically (2.2.20).

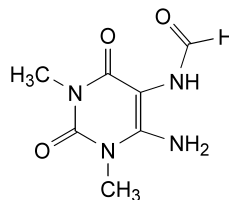
1 ml of 0.1 *M perchloric acid* is equivalent to 19.42 mg of $C_8H_{10}N_4O_2$.

IMPURITIES

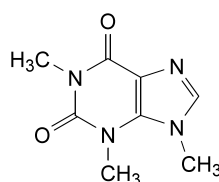
Specified impurities: A.

Other detectable impurities: B, C.

A. theophylline,

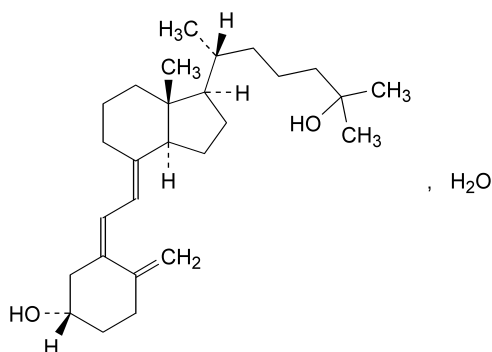


B. *N*-[6-amino-1,3-dimethyl-2,4(1*H*, 3*H*)-dioxypyrimidin-5-yl]formamide,



C. 1,3,9-trimethyl-3,9-dihydro-1*H*-purine-2,6-dione (isocaffeine).

01/2005:1295 Examine by liquid chromatography (2.2.29).

CALCIFEDIOL**Calcifediolum** $C_{27}H_{44}O_2 \cdot H_2O$ M_r 418.7**DEFINITION**

Calcifediol is the monohydrate of (5*Z*,7*E*)-9,10-seccholesta-5,7,10(19)-triene-3 β ,25-diol. It contains not less than 97.0 per cent and not more than the equivalent of 102.0 per cent of $C_{27}H_{44}O_2$, calculated with reference to the anhydrous substance.

CHARACTERS

White or almost white crystals, practically insoluble in water, freely soluble in alcohol, soluble in fatty oils. It is sensitive to air, heat and light.

A reversible isomerisation to pre-calcifediol takes place in solution, depending on temperature and time. The activity is due to both compounds.

IDENTIFICATION

- A. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the *Ph. Eur. reference spectrum of calcifediol*, prepared using 2 mg of the substance to be examined and 225 mg of *potassium bromide R*.
- B. Examine the chromatograms obtained in the assay. The principal peak in the chromatogram obtained with the test solution is similar in retention time and approximate size to the principal peak in the chromatogram obtained with reference solution (a).

TESTS

Related substances. Examine by liquid chromatography (2.2.29) as described under Assay. Calculate the percentage content of related substances, apart from pre-calcifediol, that are eluted within twice the retention time of calcifediol from the areas of the peaks in the chromatogram obtained with the test solution by the normalisation procedure. The content of any individual related substance is not greater than 0.5 per cent and the sum of the related substances is not greater than 1.0 per cent. Disregard any peak below 0.1 per cent.

Water (2.5.32): 3.8 per cent to 5.0 per cent, determined on 10.0 mg by the micro determination of water.

ASSAY

Carry out the assay as rapidly as possible, avoiding exposure to actinic light and air.

Test solution. Dissolve 1.0 mg of the substance to be examined without heating in 10.0 ml of the mobile phase.

Reference solution (a). Dissolve 1.0 mg of *calcifediol CRS* without heating in 10.0 ml of the mobile phase.

Reference solution (b). Dilute reference solution (a) 100 times with the mobile phase.

Reference solution (c). Heat 2 ml of reference solution (a) in a water-bath at 80 °C under a reflux condenser for 2 h and cool.

The chromatographic procedure may be carried out using:

- a column 0.15 m long and 4.6 mm in internal diameter packed with *octylsilyl silica gel for chromatography R1* (5 μ m),
- as mobile phase at a flow rate of 1.5 ml/min a mixture of 200 volumes of *water R* and 800 volumes of *methanol R*,
- as detector a spectrophotometer set at 265 nm,
- a loop injector.

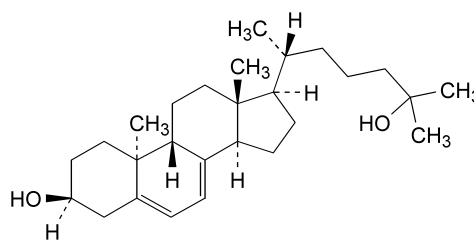
Inject 50 μ l of reference solution (c) and record the chromatogram. Make a total of six injections. When the chromatograms are recorded in the prescribed conditions, the retention time for pre-calcifediol, relative to calcifediol, is about 1.3. The assay is not valid unless the relative standard deviation of the response for calcifediol is at most 1 per cent and the resolution between the peaks due to pre-calcifediol and calcifediol is at least 5.0; adjust the proportions of the constituents of the mobile phase, if necessary, to obtain this resolution.

Inject 50 μ l of reference solution (a) and 50 μ l of reference solution (b) and record the chromatograms. Inject 50 μ l of the test solution and record the chromatogram in the same manner, continuing the chromatography for twice the retention time of the principal peak.

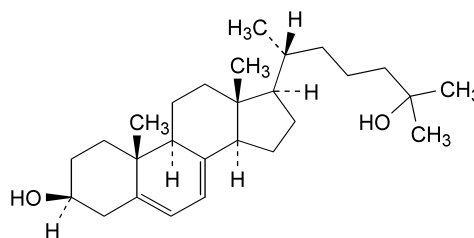
STORAGE

Store under nitrogen, in an airtight container, protected from light, at a temperature of 2 °C to 8 °C.

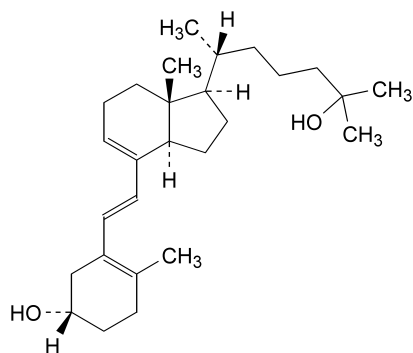
The contents of an opened container are to be used immediately.

IMPURITIES

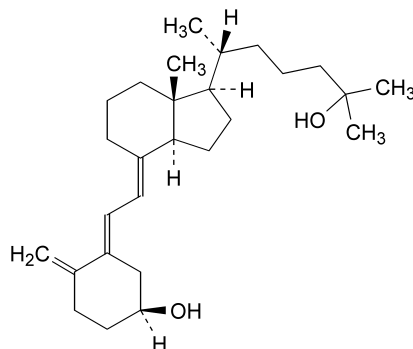
- A. 9 β ,10 α -cholesta-5,7-diene-3 β ,25-diol,



- B. cholesta-5,7-diene-3 β ,25-diol,

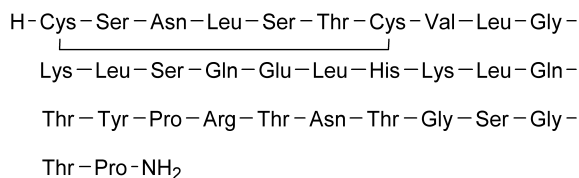


C. (6E)-9,10-secocholesta-5(10),6,8-triene-3β,25-diol,



D. (5E,7E)-9,10-secocholesta-5,7,10(19)-triene-3β,25-diol.

01/2005:0471

CALCITONIN (SALMON)**Calcitoninum salmonis** $\text{C}_{145}\text{H}_{240}\text{N}_{44}\text{O}_{48}\text{S}_2$ M_r 3432**DEFINITION**

Calcitonin (salmon) is a synthetic polypeptide having the structure determined for salmon calcitonin I. It lowers the calcium concentration in plasma of mammals by diminishing the rate of bone resorption. It is obtained by chemical synthesis and is available as an acetate. Calcitonin (salmon) contains not less than 90.0 per cent and not more than the equivalent of 105.0 per cent of the peptide $\text{C}_{145}\text{H}_{240}\text{N}_{44}\text{O}_{48}\text{S}_2$, calculated with reference to the anhydrous, acetic acid-free substance.

By convention, for the purpose of labelling calcitonin (salmon) preparations, 1 mg of calcitonin (salmon) ($\text{C}_{145}\text{H}_{240}\text{N}_{44}\text{O}_{48}\text{S}_2$) is equivalent to 6000 I.U. of biological activity.

CHARACTERS

A white or almost white powder, freely soluble in water.

IDENTIFICATION

A. Examine by thin-layer chromatography (2.2.27), using *cellulose for chromatography R1* as the coating substance.

Test solution. Dissolve 4 mg of the substance to be examined in 2 ml of a mixture of 2 volumes of *acetic acid R* and 98 volumes of *water R*. Prepare immediately before use.

Reference solution. Dissolve the contents of a vial of *calcitonin (salmon) CRS* in a mixture of 2 volumes of *acetic acid R* and 98 volumes of *water R* to obtain a concentration of 2.0 mg/ml. Prepare immediately before use.

Apply to the plate 1 µl of each solution. Develop over a path of 15 cm using a mixture of 6 volumes of *glacial acetic acid R*, 20 volumes of *pyridine R*, 24 volumes of *water R* and 30 volumes of *butanol R*. Allow the plate to dry in air for 1 h, heat at 110 °C for 10 min and spray the hot plate with *strong sodium hypochlorite solution R* diluted with *water R* immediately before use to contain 5 g/l of available chlorine. Dry in a current of cold air until a sprayed area of the plate below the line of application gives at most a very faint blue colour with a drop of *potassium iodide and starch solution R*; avoid prolonged exposure to cold air. Spray with *potassium iodide and starch solution R* until the spots are clearly visible. The principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

B. Examine the chromatograms obtained in the Assay. The retention time of the principal peak in the chromatogram obtained with the test solution is similar to that of the principal peak in the chromatogram obtained with the reference solution.

TESTS

Acetic acid (2.5.34): 4.0 per cent to 15.0 per cent.

Test solution. Dissolve 10.0 mg of the substance to be examined in a mixture of 5 volumes of mobile phase B and 95 volumes of mobile phase A and dilute to 10.0 ml with the same mixture of solvents.

Amino acids. Examine by means of an amino-acid analyser. Standardise the apparatus with a mixture containing equimolar amounts of ammonia, glycine and the L-form of the following amino acids:

lysine	histidine	arginine
serine	methionine	glutamic acid
isoleucine	proline	leucine
aspartic acid	alanine	tyrosine
threonine	valine	phenylalanine

together with half the equimolar amount of L-cystine. For the validation of the method, an appropriate internal standard, such as *DL-norleucine R*, is used.

Test solution. Place 1.0 mg of the substance to be examined in a rigorously cleaned hard-glass tube 100 mm long and 6 mm in internal diameter. Add a suitable amount of a 50 per cent V/V solution of *hydrochloric acid R*. Immerse the tube in a freezing mixture at -5 °C, reduce the pressure to below 133 Pa and seal. Heat at 110 °C to 115 °C for 16 h. Cool, open the tube, transfer the contents to a 10 ml flask with the aid of five quantities, each of 0.2 ml, of *water R* and evaporate to dryness over *potassium hydroxide R* under reduced pressure. Take up the residue in *water R* and evaporate to dryness over *potassium hydroxide R* under reduced pressure; repeat these operations once. Take up the residue in a buffer solution suitable for the amino-acid

analyser used and dilute to a suitable volume with the same buffer solution. Apply a suitable volume to the amino-acid analyser.

Express the content of each amino acid in moles. Calculate the relative proportions of the amino acids taking as equivalent to 1 the sum, divided by 20, of the number of moles of aspartic acid, glutamic acid, proline, glycine, valine, leucine, histidine, arginine and lysine. The values fall within the following limits: aspartic acid 1.8 to 2.2; glutamic acid 2.7 to 3.3; proline 1.7 to 2.3; glycine 2.7 to 3.3; valine 0.9 to 1.1; leucine 4.5 to 5.3; histidine 0.9 to 1.1; arginine 0.9 to 1.1; lysine 1.8 to 2.2; serine 3.2 to 4.2; threonine 4.2 to 5.2; tyrosine 0.7 to 1.1; half-cystine 1.4 to 2.1.

Related peptides. Examine by liquid chromatography (2.2.29) as described under Assay.

Inject 20 µl of the test solution. In the chromatogram obtained, the area of any peak apart from the principal peak is not greater than 3.0 per cent of the total area of the peaks. The sum of the areas of all the peaks, apart from the principal peak, is not greater than 5.0 per cent of the total area of the peaks. Disregard any peak due to the solvent and any peak with an area less than 0.1 per cent of that of the principal peak.

Water (2.5.32). Not more than 10.0 per cent, determined by the micro determination of water.

Acetic acid and water. Not more than 20 per cent, calculated by adding together the percentage contents of acetic acid and water determined by the methods described above.

Bacterial endotoxins (2.6.14): less than 1000 IU/mg, if intended for use in the manufacture of parenteral dosage forms without a further appropriate procedure for the removal of bacterial endotoxins.

ASSAY

Examine by liquid chromatography (2.2.29).

Test solution. Prepare a 1.0 mg/ml solution of the substance to be examined in mobile phase A.

Reference solution. Dissolve the contents of a vial of *calcitonin (salmon) CRS* in mobile phase A to obtain a concentration of 1.0 mg/ml.

Resolution solution. Dissolve the contents of a vial of *N-acetyl-cys¹calcitonin CRS* in 400 µl of mobile phase A and add 100 µl of the test solution.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4.6 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (5 µm),
- as mobile phase at a flow rate of 1.0 ml/min:

Mobile phase A. Dissolve 3.26 g of *tetramethylammonium hydroxide R* in 900 ml of *water R*, adjust the pH to 2.5 with *phosphoric acid R* and mix with 100 ml of *acetonitrile for chromatography R*; filter and degas,

Mobile phase B. Dissolve 1.45 g of *tetramethylammonium hydroxide R* in 400 ml of *water R*, adjust the pH to 2.5 with *phosphoric acid R* and mix with 600 ml of *acetonitrile for chromatography R*; filter and degas,

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)	Comment
0 - 30	72 → 48	28 → 52	linear gradient
30 - 32	48 → 72	52 → 28	switch to initial eluent composition
32 - 55	72	28	re-equilibration

- as detector a spectrophotometer set at 220 nm, maintaining the temperature of the column at 65 °C.

Equilibrate the column with a mixture of 72 volumes of mobile phase A and 28 volumes of mobile phase B.

Inject 20 µl of the resolution solution. When the chromatogram is recorded in the prescribed conditions, the relative retention of *N-acetyl-cys¹calcitonin* is about 1.15 relative to the principal peak. The test is not valid unless the resolution between the peaks corresponding to calcitonin and *N-acetyl-cys¹calcitonin* is at least 5.0 and the symmetry factor for the *N-acetyl-cys¹calcitonin* peak is not greater than 2.5. If necessary, adjust the initial A:B ratio of the mobile phase.

Inject 20 µl of the test solution and 20 µl of the reference solution.

Calculate the content of calcitonin (salmon) (C₁₄₅H₂₄₀N₄₄O₄₈S₂) from the peak areas in the chromatograms obtained with the test solution and the reference solution and the declared content of C₁₄₅H₂₄₀N₄₄O₄₈S₂ in *calcitonin (salmon) CRS*. Proceed with tangential integration of the peak areas.

STORAGE

Store protected from light at a temperature between 2 °C and 8 °C. If the substance is sterile, store in a sterile, airtight, tamper-proof container.

LABELLING

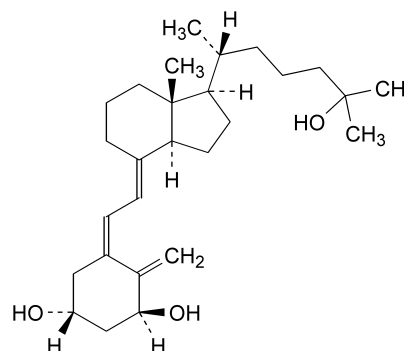
The label states:

- the calcitonin peptide content (C₁₄₅H₂₄₀N₄₄O₄₈S₂),
- where applicable, that the substance is free from bacterial endotoxins.

01/2005:0883

CALCITRIOL

Calcitriolum



C₂₇H₄₄O₃

M_r 416.6

DEFINITION

Calcitriol contains not less than 97.0 per cent and not more than the equivalent of 103.0 per cent of (5Z,7E)-9,10-secocholesta-5,7,10(19)-triene-1α,3β,25-triol.

CHARACTERS

White or almost white crystals, practically insoluble in water, freely soluble in alcohol, soluble in fatty oils. It is sensitive to air, heat and light.

A reversible isomerisation to pre-calcitriol takes place in solution, depending on temperature and time. The activity is due to both compounds.

IDENTIFICATION

- A. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the *Ph. Eur. reference spectrum of calcitriol*.

B. Examine the chromatograms obtained in the assay.

The retention time and size of the principal peak in the chromatogram obtained with the test solution are approximately the same as those of the principal peak in the chromatogram obtained with reference solution (a).

TESTS

Related substances. Examine by liquid chromatography (2.2.29) as described under Assay. Calculate the percentage content of related substances, apart from pre-calcitriol, that are eluted within twice the retention time of calcitriol from the areas of the peaks in the chromatogram obtained with the test solution by the normalisation procedure. The content of any individual related substance is not greater than 0.5 per cent and the sum of the related substances is not greater than 1.0 per cent. Disregard any peak with an area less than 0.1 times that of the peak in the chromatogram obtained with reference solution (b) (0.1 per cent).

ASSAY

Carry out the assay as rapidly as possible, avoiding exposure to actinic light and air.

Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 1.000 mg of the substance to be examined without heating in 10.0 ml of the mobile phase.

Reference solution (a). Dissolve 1.000 mg of calcitriol CRS without heating in 10.0 ml of the mobile phase.

Reference solution (b). Dilute 1.0 ml of reference solution (a) to 100.0 ml with the mobile phase.

Reference solution (c). Keep 2 ml of reference solution (a) for 30 min at 80 °C.

The chromatographic procedure may be carried out using:

- a column 0.25 m long and 4.6 mm in internal diameter packed with *octylsilyl silica gel for chromatography R1* (5 µm),
- as mobile phase at a flow rate of 1.0 ml/min, a mixture of 450 volumes of a solution containing 1.0 g/l of *tris(hydroxymethyl)aminomethane R* adjusted to pH 7.0 to 7.5 with *phosphoric acid R*, and of 550 volumes of *acetonitrile R*,
- as detector a spectrophotometer set at 230 nm,
- a loop injector,

maintaining the temperature of the column at 40 °C.

Inject 50 µl of reference solution (c) and six times 50 µl of reference solution (a). When the chromatograms are recorded in the prescribed conditions, the retention time for pre-calcitriol, relative to calcitriol, is about 0.9. The assay is not valid unless the number of theoretical plates calculated for the peak due to calcitriol in the chromatogram obtained with reference solution (a) is at least 10 000, the relative standard deviation of the peak area due to calcitriol is at most 1 per cent and the resolution between the peaks due to calcitriol and pre-calcitriol is at least 3.5.

Inject 50 µl of reference solution (b) and 50 µl of the test solution. Continue the chromatography for twice the retention time of calcitriol.

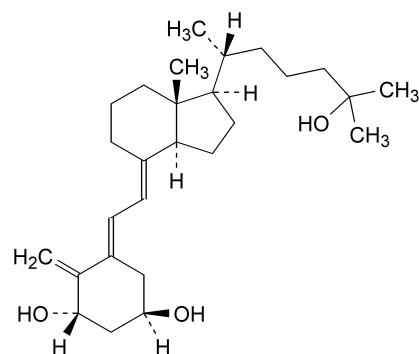
Calculate the percentage content of calcitriol.

STORAGE

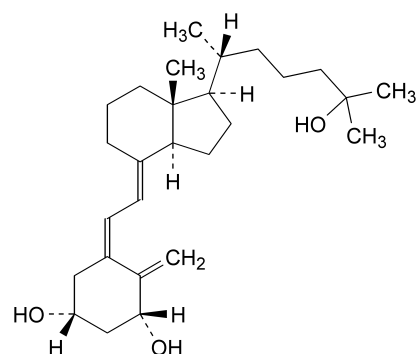
Store under nitrogen, in an airtight container, protected from light, at a temperature of 2 °C to 8 °C.

The contents of an opened container are to be used immediately.

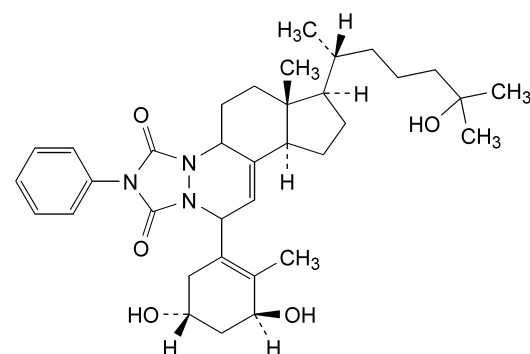
IMPURITIES



A. (5*E*,7*E*)-9,10-secocholesta-5,7,10(19)-triene-1 α ,3 β ,25-triol (*trans*-calcitriol),



B. (5*Z*,7*E*)-9,10-secocholesta-5,7,10(19)-triene-1 β ,3 β ,25-triol (1 β -calcitriol),

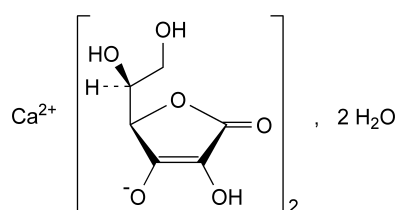


C. (6*aR*,7*R*,9*aR*)-11-[(3*S*,5*R*)-3,5-dihydroxy-2-methylcyclohex-1-enyl]-7-[(1*R*)-5-hydroxy-1,5-dimethylhexyl]-6*a*-methyl-2-phenyl-5,6,6*a*,7,8,9,9*a*,11-octahydro-1*H*,4*aH*-cyclopenta[*f*]1,2,4-triazolo[1,2-*a*]cinnoline-1,3(2*H*)-dione (triazoline adduct of pre-calcitriol).

01/2005:1182

CALCIUM ASCORBATE

Calcii ascorbas



$C_{12}H_{14}CaO_{12} \cdot 2H_2O$

M_r 426.3

DEFINITION

Calcium ascorbate contains not less than 99.0 per cent and not more than the equivalent of 100.5 per cent of calcium di[(*R*)-2-[(*S*)-1,2-dihydroxyethyl]-4-hydroxy-5-oxo-2*H*-furan-3-olate] dihydrate.

CHARACTERS

A white or slightly yellowish, crystalline powder, freely soluble in water, practically insoluble in alcohol.

IDENTIFICATION

First identification: A, B, E.

Second identification: A, C, D, E.

- A. It complies with the test for specific optical rotation (see Tests).
- B. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the *Ph. Eur. reference spectrum of calcium ascorbate*.
- C. Dilute 1 ml of solution S (see Tests) to 10 ml with *water R*. To 2 ml of the solution add 0.2 ml of a 100 g/l solution of *ferrous sulphate R*. A deep violet colour develops.
- D. To 1 ml of solution S add 0.2 ml of *dilute nitric acid R* and 0.2 ml of *silver nitrate solution R2*. A grey precipitate is formed.
- E. The substance gives reaction (b) of calcium (2.3.1).

TESTS

Solution S. Dissolve 5.00 g in *carbon dioxide-free water R* and dilute to 50.0 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and not more intensely coloured than reference solution Y₆ (2.2.2, *Method II*). Examine the colour of the solution immediately after preparation of the solution.

pH (2.2.3). The pH of solution S is 6.8 to 7.4.

Specific optical rotation (2.2.7): + 95 to + 97, determined using freshly prepared solution S and calculated with reference to the dried substance.

Fluorides. Not more than 10 ppm of F, determined potentiometrically (2.2.36, *Method I*) using as indicator electrode a fluoride-selective electrode and as reference electrode a silver-silver chloride electrode.

Test solution. In a 50 ml volumetric flask, dissolve 1.000 g in a 10.3 g/l solution of *hydrochloric acid R*, add 5.0 ml of *fluoride standard solution (1 ppm F) R* and dilute to 50.0 ml with a 10.3 g/l solution of *hydrochloric acid R*. To 20.0 ml of the solution add 20.0 ml of *total-ionic-strength-adjustment buffer R* and 3 ml of an 82 g/l solution of *anhydrous sodium acetate R*. Adjust to pH 5.2 with *ammonia R* and dilute to 50.0 ml with *distilled water R*.

Reference solutions. To 0.25 ml, 0.5 ml, 1.0 ml, 2.0 ml and 5.0 ml of *fluoride standard solution (10 ppm F) R* add 20.0 ml of *total-ionic-strength-adjustment buffer R* and dilute to 50.0 ml with *distilled water R*.

Carry out the measurements for each solution. Calculate the concentration of fluorides using the calibration curve, taking into account the addition of fluoride to the test solution.

Copper. Not more than 5 ppm of Cu, determined by atomic absorption spectrometry (2.2.23, *Method I*).

Test solution. Dissolve 2.0 g of the substance to be examined in a 9.7 g/l solution of *nitric acid R* and dilute to 25.0 ml with the same acid solution.

Reference solutions. Prepare the reference solutions using *copper standard solution (10 ppm Cu) R*, diluting with a 9.7 g/l solution of *nitric acid R*.

Measure the absorbance at 324.8 nm using a copper hollow-cathode lamp as source of radiation and an air-acetylene flame.

Iron. Not more than 2 ppm of Fe, determined by atomic absorption spectrometry (2.2.23, *Method I*).

Test solution. Dissolve 5.0 g of the substance to be examined in a 9.7 g/l solution of *nitric acid R* and dilute to 25.0 ml with the same acid solution.

Reference solutions. Prepare the reference solutions using *iron standard solution (10 ppm Fe) R*, diluting with a 9.7 g/l solution of *nitric acid R*.

Measure the absorbance at 248.3 nm using an iron hollow-cathode lamp as the radiation source and an air-acetylene flame.

Heavy metals (2.4.8). 2.0 g complies with limit test D for heavy metals (10 ppm). Prepare the standard using 2.0 ml of *lead standard solution (10 ppm Pb) R*.

Loss on drying (2.2.32). Not more than 0.1 per cent, determined on 1.000 g by drying in an oven at 100-105 °C for 2 h.

ASSAY

Dissolve 80.0 mg in a mixture of 10 ml of *dilute sulphuric acid R* and 80 ml of *carbon dioxide-free water R*. Add 1 ml of *starch solution R*. Titrate with 0.05 *M* iodine until a persistent violet-blue colour is obtained.

1 ml of 0.05 *M* iodine is equivalent to 10.66 mg of C₁₂H₁₄CaO₁₂·2H₂O.

STORAGE

Store in a non-metallic container, protected from light.

01/2005:0014

CALCIUM CARBONATE

Calcii carbonas

CaCO₃M_r 100.1

DEFINITION

Calcium carbonate contains not less than 98.5 per cent and not more than the equivalent of 100.5 per cent of CaCO₃, calculated with reference to the dried substance.

CHARACTERS

A white powder, practically insoluble in water.

IDENTIFICATION

- A. It gives the reaction of carbonates (2.3.1).
- B. 0.2 ml of solution S (see Tests) gives the reactions of calcium (2.3.1).

TESTS

Solution S. Dissolve 5.0 g in 80 ml of *dilute acetic acid R*. When the effervescence ceases, boil the solution for 2 min, allow to cool, dilute to 100 ml with *dilute acetic acid R* and filter, if necessary, through a sintered-glass filter.

Substances insoluble in acetic acid. Wash any residue obtained during the preparation of solution S with four quantities, each of 5 ml, of hot *water R* and dry at 100-105 °C for 1 h. The residue weighs not more than 10 mg (0.2 per cent).

Chlorides (2.4.4). 3 ml of solution S diluted to 15 ml with *water R* complies with the limit test for chlorides (330 ppm).

Sulphates (2.4.13). 1.2 ml of solution S diluted to 15 ml with *distilled water R* complies with the limit test for sulphates (0.25 per cent).

Arsenic (2.4.2). 5 ml of solution S complies with limit test A for arsenic (4 ppm).

Barium. To 10 ml of solution S add 10 ml of *calcium sulphate solution R*. After at least 15 min, any opalescence in the solution is not more intense than that in a mixture of 10 ml of solution S and 10 ml of *distilled water R*.

Iron (2.4.9). Dissolve 50 mg in 5 ml of *dilute hydrochloric acid R* and dilute to 10 ml with *water R*. The solution complies with the limit test for iron (200 ppm).

Magnesium and alkali metals. Dissolve 1.0 g in 12 ml of *dilute hydrochloric acid R*. Boil the solution for about 2 min and add 20 ml of *water R*, 1 g of *ammonium chloride R* and 0.1 ml of *methyl red solution R*. Add *dilute ammonia RI* until the colour of the indicator changes and then 2 ml in excess. Heat to boiling and add 50 ml of hot *ammonium oxalate solution R*. Allow to stand for 4 h, dilute to 100 ml with *water R* and filter through a suitable filter. To 50 ml of the filtrate add 0.25 ml of *sulphuric acid R*. Evaporate to dryness on a water-bath and ignite to constant mass at 600 °C. The residue weighs not more than 7.5 mg (1.5 per cent).

Heavy metals (2.4.8). 12 ml of solution S complies with limit test A for heavy metals (20 ppm). Prepare the standard using *lead standard solution (1 ppm Pb) R*.

Loss on drying (2.2.32). Not more than 2.0 per cent, determined on 1.000 g by drying in an oven at 200 °C.

ASSAY

Dissolve 0.150 g in a mixture of 3 ml of *dilute hydrochloric acid R* and 20 ml of *water R*. Boil for 2 min, allow to cool and dilute to 50 ml with *water R*. Carry out the complexometric titration of calcium (2.5.11).

1 ml of 0.1 M *sodium edetate* is equivalent to 10.01 mg of CaCO_3 .

TESTS

Solution S. Dissolve 10.0 g in *carbon dioxide-free water R* prepared from *distilled water R* and dilute to 100 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and not more intensely coloured than reference solution Y_6 (2.2.2, *Method II*).

Acidity or alkalinity. To 10 ml of freshly prepared solution S add 0.1 ml of *phenolphthalein solution R*. If the solution is red, not more than 0.2 ml of 0.01 M *hydrochloric acid* is required to discharge the colour and if the solution is colourless, not more than 0.2 ml of 0.01 M *sodium hydroxide* is required to turn it red.

Sulphates (2.4.13): maximum 300 ppm.

5 ml of solution S diluted to 15 ml with *distilled water R* complies with the limit test for sulphates.

Aluminium. To 10 ml of solution S add 2 ml of *ammonium chloride solution R* and 1 ml of *dilute ammonia RI* and boil the solution. No turbidity or precipitate is formed.

If intended for use in the manufacture of dialysis solutions, it complies with the following test for aluminium (2.4.17) which replaces the test prescribed above: maximum 1 ppm.

Prescribed solution. Dissolve 4 g in 100 ml of *water R* and add 10 ml of *acetate buffer solution pH 6.0 R*.

Reference solution. Mix 2 ml of *aluminium standard solution (2 ppm Al) R*, 10 ml of *acetate buffer solution pH 6.0 R* and 98 ml of *water R*.

Blank solution. Mix 10 ml of *acetate buffer solution pH 6.0 R* and 100 ml of *water R*.

Barium. To 10 ml of solution S add 1 ml of *calcium sulphate solution R*. After at least 15 min, any opalescence in the solution is not more intense than that in a mixture of 10 ml of solution S and 1 ml of *distilled water R*.

Iron (2.4.9): maximum 10 ppm.

10 ml of solution S complies with the limit test for iron.

Magnesium and alkali metals: maximum 0.5 per cent.

To a mixture of 20 ml of solution S and 80 ml of *water R* add 2 g of *ammonium chloride R* and 2 ml of *dilute ammonia RI*, heat to boiling and pour into the boiling solution a hot solution of 5 g of *ammonium oxalate R* in 75 ml of *water R*. Allow to stand for 4 h, dilute to 200 ml with *water R* and filter through a suitable filter. To 100 ml of the filtrate add 0.5 ml of *sulphuric acid R*. Evaporate to dryness on a water-bath and ignite to constant mass at 600 °C. The residue weighs a maximum of 5 mg.

Heavy metals (2.4.8): maximum 20 ppm.

12 ml of solution S complies with limit test A. Prepare the standard using *lead standard solution (2 ppm Pb) R*.

ASSAY

Dissolve 0.280 g in 100 ml of *water R* and carry out the complexometric titration of calcium (2.5.11).

1 ml of 0.1 M *sodium edetate* is equivalent to 14.70 mg of $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$.

LABELLING

The label states, where applicable, that the substance is suitable for use in the manufacture of dialysis solutions.

STORAGE

In an airtight container.

01/2005:0015

CALCIUM CHLORIDE DIHYDRATE

Calcii chloridum dihydricum

 $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$
 M_r 147.0

DEFINITION

Content: 97.0 per cent to 103.0 per cent of $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$.

CHARACTERS

Appearance: white, crystalline powder, hygroscopic.

Solubility: freely soluble in water, soluble in alcohol.

IDENTIFICATION

A. Solution S (see Tests) gives reaction (a) of chlorides (2.3.1).

B. It gives the reactions of calcium (2.3.1).

C. It complies with the limits of the assay.

01/2005:0707

CALCIUM CHLORIDE HEXAHYDRATE**Calcii chloridum hexahydricum** $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$ M_r 219.1**DEFINITION**

Calcium chloride hexahydrate contains not less than 97.0 per cent and not more than the equivalent of 103.0 per cent of $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$.

CHARACTERS

A white, crystalline mass or colourless crystals, very soluble in water, freely soluble in alcohol.

It freezes at about 29 °C.

IDENTIFICATION

A. Solution S (see Tests) gives reaction (a) of chlorides (2.3.1).

B. It gives the reactions of calcium (2.3.1).

C. It complies with the limits of the assay.

TESTS

Solution S. Dissolve 15.0 g in *carbon dioxide-free water R* prepared from *distilled water R* and dilute to 100 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and not more intensely coloured than reference solution Y_6 (2.2.2, *Method II*).

Acidity or alkalinity. To 10 ml of freshly prepared solution S add 0.1 ml of *phenolphthalein solution R*. If the solution is red, not more than 0.2 ml of 0.01 M *hydrochloric acid* is required to discharge the colour and if the solution is colourless, not more than 0.2 ml of 0.01 M *sodium hydroxide* is required to turn it red.

Sulphates (2.4.13). 5 ml of solution S diluted to 15 ml with *distilled water R* complies with the limit test for sulphates (200 ppm).

Aluminium. To 10 ml of solution S add 2 ml of *ammonium chloride solution R* and 1 ml of *dilute ammonia R1*. Heat to boiling. No turbidity or precipitate is formed.

If intended for use in the manufacture of dialysis solutions, it complies with the following test which replaces the test for aluminium prescribed above.

Dissolve 6 g in 100 ml of *water R* and add 10 ml of *acetate buffer solution pH 6.0 R*. The solution complies with the limit test for aluminium (1 ppm). Use as the reference solution a mixture of 2 ml of *aluminium standard solution* (2 ppm Al) *R*, 10 ml of *acetate buffer solution pH 6.0 R* and 98 ml of *water R*. To prepare the blank use a mixture of 10 ml of *acetate buffer solution pH 6.0 R* and 100 ml of *water R*.

Barium. To 10 ml of solution S add 1 ml of *calcium sulphate solution R*. After at least 15 min, any opalescence in the solution is not more intense than that in a mixture of 10 ml of solution S and 1 ml of *distilled water R*.

Iron (2.4.9). 10 ml of solution S complies with the limit test for iron (7 ppm).

Magnesium and alkali metals. To a mixture of 20 ml of solution S and 80 ml of *water R* add 2 g of *ammonium chloride R* and 2 ml of *dilute ammonia R1*, heat to boiling and pour into the boiling solution a hot solution of 5 g of *ammonium oxalate R* in 75 ml of *water R*. Allow to stand for 4 h, dilute to 200 ml with *water R* and filter through

a suitable filter. To 100 ml of the filtrate add 0.5 ml of *sulphuric acid R*. Evaporate to dryness on a water-bath and ignite to constant mass at 600 °C. The residue weighs not more than 5 mg (0.3 per cent).

Heavy metals (2.4.8). 12 ml of solution S complies with limit test A for heavy metals (15 ppm). Prepare the standard using *lead standard solution* (2 ppm Pb) *R*.

ASSAY

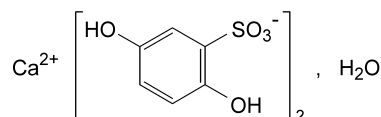
Dissolve 0.200 g in 100 ml of *water R*. Carry out the complexometric titration of calcium (2.5.11).

1 ml of 0.1 M *sodium edetate* is equivalent to 21.91 mg of $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$.

LABELLING

The label states, where applicable, that the substance is suitable for use in the manufacture of dialysis solutions.

01/2005:1183

CALCIUM DOBESILATE MONOHYDRATE**Calcii dobesilas monohydricum** $\text{C}_{12}\text{H}_{10}\text{CaO}_{10}\text{S}_2\text{H}_2\text{O}$ M_r 436.4**DEFINITION**

Calcium dobesilate monohydrate contains not less than 99.0 per cent and not more than the equivalent of 102.0 per cent of calcium di(2,5-dihydroxybenzenesulphonate), calculated with reference to the anhydrous substance.

CHARACTERS

A white or almost white, hygroscopic powder, very soluble in water, freely soluble in ethanol, very slightly soluble in 2-propanol, practically insoluble in methylene chloride.

IDENTIFICATION

A. Dissolve 0.100 g in *water R* and dilute to 200.0 ml with the same solvent. Dilute 5.0 ml of the solution to 100.0 ml with *water R*. Examined between 210 nm and 350 nm (2.2.25), the solution shows two absorption maxima, at 221 nm and 301 nm. The specific absorbance at the maximum at 301 nm is 174 to 181.

B. Mix 1 ml of *ferric chloride solution R2*, 1 ml of a freshly prepared 10 g/l solution of *potassium ferricyanide R* and 0.1 ml of *nitric acid R*. To this mixture add 5 ml of freshly prepared solution S (see Tests): a blue colour and a precipitate are immediately produced.

C. 2 ml of freshly prepared solution S (see Tests) gives reaction (b) of calcium (2.3.1).

TESTS

Solution S. Dissolve 10.0 g in *carbon dioxide-free water R* and dilute to 100 ml with the same solvent.

Appearance of solution. Solution S, when freshly prepared, is clear (2.2.1) and colourless (2.2.2, *Method II*).

pH (2.2.3). The pH of solution S is 4.5 to 6.0.

Hydroquinone. Examine by thin-layer chromatography (2.2.27), using as the coating substance a suitable silica gel with a fluorescent indicator having an optimal intensity at 254 nm.

Test solution. Dissolve 2.0 g of the substance to be examined in *water R* and dilute to 10 ml with the same solvent.

Reference solution. Dissolve 10 mg of *hydroquinone R* in *water R* and dilute to 50 ml with the same solvent.

Apply to the plate 10 µl of each solution and dry the starting points in a current of cool air. Develop over a path of 15 cm using a mixture of 20 volumes of *methylene chloride R*, 30 volumes of *methyl acetate R* and 50 volumes of *ethyl acetate R*. Dry the plate in a current of hot air and examine in ultraviolet light at 254 nm. Any spot corresponding to hydroquinone in the chromatogram obtained with the test solution is not more intense than the principal spot in the chromatogram obtained with the reference solution (0.1 per cent).

Heavy metals (2.4.8). 1.0 g complies with limit test C for heavy metals (15 ppm). Prepare the standard using 1.5 ml of *lead standard solution (10 ppm Pb) R*.

Iron (2.4.9). 10 ml of solution S complies with the limit test for iron (10 ppm).

Water (2.5.12): 4.0 per cent to 6.0 per cent, determined on 0.500 g by the semi-micro determination of water.

ASSAY

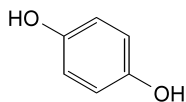
Dissolve 0.200 g in a mixture of 10 ml of *water R* and 40 ml of *dilute sulphuric acid R*. Titrate with 0.1 M *cerium sulphate*, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *cerium sulphate* is equivalent to 10.45 mg of $C_{12}H_{10}CaO_{10}S_2$.

STORAGE

Store in an airtight container, protected from light.

IMPURITIES

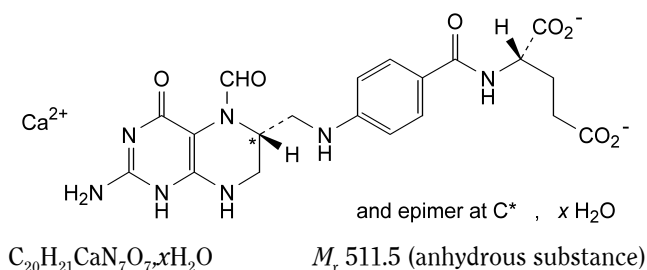


A. benzene-1,4-diol (hydroquinone).

01/2005:0978

CALCIUM FOLINATE

Calcii folinas



DEFINITION

Calcium folinate contains not less than 97.0 per cent and not more than the equivalent of 102.0 per cent of calcium (2S)-2-[[4-[[[(6RS)-2-amino-5-formyl-4-oxo-1,4,5,6,7,8-hexahydropteridin-6-yl]methyl]amino]benzoyl]amino]pentanedioate and not less than 7.54 per cent and not more than the equivalent of 8.14 per cent of Ca, both calculated with reference to the anhydrous and solvent-free substance.

CHARACTERS

A white or light yellow, amorphous or crystalline powder, sparingly soluble in water, practically insoluble in acetone and in alcohol. The amorphous form may produce supersaturated solutions in water.

IDENTIFICATION

First identification: A, B, D.

Second identification: A, C, D.

- It complies with the test for specific optical rotation (see Tests).
- Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *calcium folinate CRS*. Examine the substances prepared as discs. If the spectra obtained show differences, dissolve the substance to be examined and the reference substance separately in the minimum quantity of *water R* and add dropwise sufficient *acetone R* to produce sufficient precipitate. Allow to stand for 15 min, collect the precipitate by centrifugation, wash the precipitate with two small quantities of *acetone R* and dry. Record new spectra using the residues.
- Examine by thin-layer chromatography (2.2.27), using *cellulose for chromatography F_{254 R}* as the coating substance.

Test solution. Dissolve 15 mg of the substance to be examined in a 3 per cent V/V solution of *ammonia R* and dilute to 5 ml with the same solvent.

Reference solution. Dissolve 15 mg of *calcium folinate CRS* in a 3 per cent V/V solution of *ammonia R* and dilute to 5 ml with the same solvent.

Apply to the plate 5 µl of each solution. Develop over a path of 15 cm using the lower layer of a mixture of 1 volume of *isoamyl alcohol R* and 10 volumes of a 50 g/l solution of *citric acid R* previously adjusted to pH 8 with *ammonia R*. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. The principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with the reference solution.

D. It gives reaction (b) of calcium (2.3.1).

Carry out the tests and the assay as rapidly as possible, protected from actinic light.

TESTS

Solution S. Dissolve 1.25 g in *carbon dioxide-free water R*, heating at 40 °C if necessary, and dilute to 50.0 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1). The absorbance (2.2.25) of solution S, measured at 420 nm using *water R* as the compensation liquid, is not greater than 0.60.

pH (2.2.3). The pH of solution S is 6.8 to 8.0.

Specific optical rotation (2.2.7). The specific optical rotation is + 14.4 to + 18.0, determined on solution S and calculated with reference to the anhydrous and solvent-free substance.

Acetone, ethanol and methanol. Not more than 0.5 per cent of acetone, not more than 3.0 per cent of ethanol and not more than 0.5 per cent of methanol. Examine by head-space gas chromatography (2.2.28) using the standard additions method.

Test solution. Dissolve 0.25 g of the substance to be examined in *water R* and dilute to 10.0 ml with the same solvent.

Reference solution. Dilute 0.125 g of *acetone R*, 0.750 g of *ethanol R* and 0.125 g of *methanol R* in *water R* and dilute to 1000.0 ml with *water R*.

The chromatographic procedure may be carried out using:

- a fused-silica column 10 m long and 0.32 mm in internal diameter coated with *styrene-divinylbenzene copolymer R*,
- *nitrogen for chromatography R* as the carrier gas at a flow rate of 4 ml/min,
- a flame-ionisation detector,

raising the temperature of the column from 125 °C to 185 °C at a rate of 10 °C/min and maintaining at 185 °C until the total run time is 15 min. Maintain the temperature of the injection port at 250 °C and that of the detector at 250 °C. Place the samples in a thermostatically controlled chamber at 80 °C for 20 min and pressurise them for 30 s. Repeat the injections at least three times.

Related substances. Examine the chromatograms obtained in the Assay. In the chromatogram obtained with the test solution: the area of any peak corresponding to formylfolic acid is not greater than the area of the principal peak in the chromatogram obtained with reference solution (c) (1 per cent); the area of any peak, apart from the principal peak and any peak corresponding to formylfolic acid, is not greater than the area of the principal peak in the chromatogram obtained with reference solution (b) (1 per cent); the sum of the areas of all the peaks, apart from the principal peak, is not greater than 2.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (2.5 per cent). Disregard any peak with an area less than that of the principal peak in the chromatogram obtained with reference solution (d).

Chlorides (2.4.4). Dissolve 67 mg in 10 ml of *water R* and add 3 ml of *acetic acid R*. Filter and wash the precipitate with five quantities, each of 5 ml, of *water R*. Collect the filtrate and washings and dilute to 100 ml with *water R*. 15 ml of this solution complies with the limit test for chlorides (0.5 per cent).

Heavy metals (2.4.8). 1.0 g complies with limit test F for heavy metals (50 ppm). Prepare the standard using 5 ml of *lead standard solution (10 ppm Pb) R*.

Platinum. Not more than 20 ppm of Pt, determined by atomic absorption spectrometry (2.2.23, *Method II*).

Test solution. Dissolve 1.00 g in *water R* and dilute to 100.0 ml with the same solvent.

Reference solutions. Prepare the reference solutions using *platinum standard solution (30 ppm Pt) R*, diluted as necessary with a mixture of 1 volume of *nitric acid R* and 99 volumes of *water R*.

Measure the absorbance at 265.9 nm using a platinum hollow-cathode lamp as source of radiation.

Water (2.5.12). Not more than 17.0 per cent, determined on 0.200 g (ground to a very fine powder) by the semi-micro determination of water. Stir the substance to be examined in the titration solvent for about 6 min before titrating and use a suitable titrant that does not contain pyridine.

Bacterial endotoxins (2.6.14): less than 0.5 IU/mg, if intended for use in the manufacture of parenteral dosage forms without a further appropriate procedure for the removal of bacterial endotoxins.

ASSAY

Calcium. Dissolve 0.400 g in 150 ml of *water R* and dilute to 300 ml with the same solvent. Carry out the complexometric titration of calcium (2.5.11).

1 ml of 0.1 M *sodium edetate* is equivalent to 4.008 mg of Ca.

Calcium folinate. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 10.0 mg of the substance to be examined in *water R* and dilute to 10.0 ml with the same solvent.

Reference solution (a). Dissolve 10.0 mg of *calcium folinate CRS* in *water R* and dilute to 10.0 ml with the same solvent.

Reference solution (b). Dilute 1.0 ml of reference solution (a) to 100.0 ml with *water R*.

Reference solution (c). Dissolve 10.0 mg of *formylfolic acid CRS* in the mobile phase and dilute to 100.0 ml with the mobile phase. Dilute 1.0 ml of this solution to 10.0 ml with *water R*.

Reference solution (d). Dilute 1.0 ml of reference solution (b) to 10.0 ml with *water R*.

Reference solution (e). Dilute 5.0 ml of reference solution (c) to 10.0 ml with reference solution (b).

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (5 µm),
- as mobile phase at a flow rate of 1 ml/min a mixture prepared as follows: mix 220 ml of *methanol R* and 780 ml of a solution containing 2.0 ml of *tetrabutylammonium hydroxide solution (400 g/l) R* and 2.2 g of *disodium hydrogen phosphate R* previously adjusted to pH 7.8 with *phosphoric acid R*,
- as detector a spectrophotometer set at 280 nm, maintaining the temperature of the column at 40 °C.

Inject 10 µl of each solution. Continue the chromatography for 2.5 times the retention time of the principal peak in the chromatogram obtained with the test solution. The assay is not valid unless, in the chromatogram obtained with reference solution (e), the resolution between the peaks corresponding to calcium folinate and formylfolic acid is at least 2.2 and the relative standard deviation of the area of the principal peak for six replicate injections of reference solution (a) is at most 2.0 per cent.

Calculate the percentage content of $C_{20}H_{21}CaN_7O_7$ from the peak areas and the declared content of *calcium folinate CRS*.

STORAGE

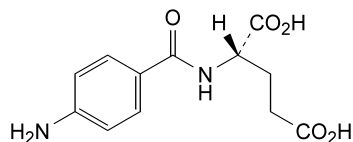
Store in an airtight container, protected from light. If the substance is sterile, store in a sterile, airtight, tamper-proof container.

LABELLING

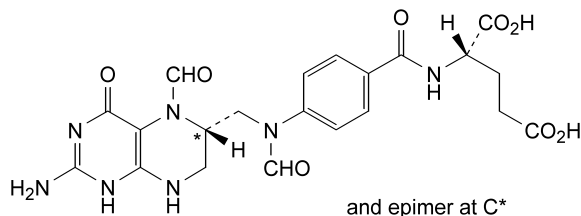
The label states, where applicable, that the substance is free from bacterial endotoxins.

IMPURITIES

01/2005:1399

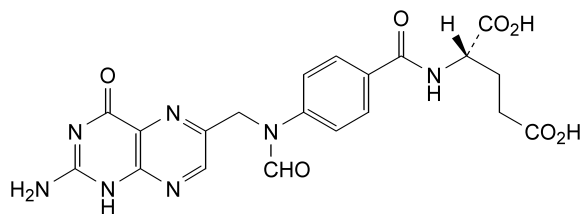


A. (2S)-2-[(4-aminobenzoyl)amino]pentanedioic acid,

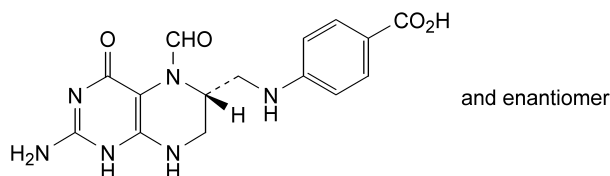


B. (2S)-2-[[4-[[[(6RS)-2-amino-5-formyl-4-oxo-1,4,5,6,7,8-hexahydropteridin-6-yl]methyl]formylamino]benzoyl]amino]pentanedioic acid (5,10-diformyltetrahydrofolic acid),

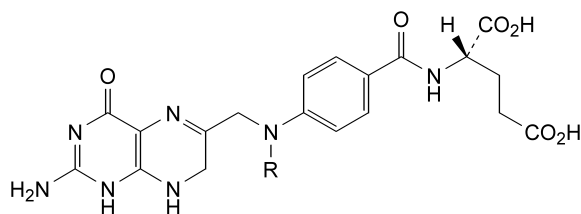
C. folic acid,



D. (2S)-2-[[4-[[[(2-amino-4-oxo-1,4-dihydropteridin-6-yl)methyl]formylamino]benzoyl]amino]pentanedioic acid (10-formylfolic acid),



E. 4-[[[(6RS)-2-amino-5-formyl-4-oxo-1,4,5,6,7,8-hexahydropteridin-6-yl]methyl]amino]benzoic acid (5-formyltetrahydroptericoic acid),

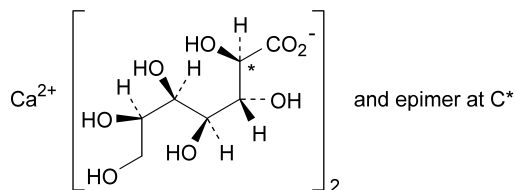


F. R = CHO: (2S)-2-[[4-[[[(2-amino-4-oxo-1,4,7,8-tetrahydropteridin-6-yl)methyl]formylamino]benzoyl]amino]pentanedioic acid (10-formyldihydrofolic acid),

G. R = H: (2S)-2-[[4-[[[(2-amino-4-oxo-1,4,7,8-tetrahydropteridin-6-yl)methyl]amino]benzoyl]amino]pentanedioic acid (dihydrofolic acid).

CALCIUM GLUCOHEPTONATE

Calcii glucoheptonas

 $C_{14}H_{26}CaO_{16}$ M_r 490.4

DEFINITION

Calcium glucoheptonate is a mixture, in variable proportions, of calcium di(D-glycero-D-gulo-heptonate) and calcium di(D-glycero-D-ido-heptonate). It contains not less than 98.0 per cent and not more than the equivalent of 102.0 per cent of calcium 2,3,4,5,6,7-hexahydroxyheptanoate, calculated with reference to the dried substance.

CHARACTERS

A white or very slightly yellow, amorphous powder, hygroscopic, very soluble in water, practically insoluble in acetone and in alcohol.

IDENTIFICATION

A. Examine by thin-layer chromatography (2.2.27), using *cellulose for chromatography R1* as the coating substance.

Test solution. Dissolve 20 mg of the substance to be examined in 1 ml of *water R*.

Reference solution (a). Dissolve 20 mg of *calcium glucoheptonate CRS* in 1 ml of *water R*.

Reference solution (b). Dissolve 10 mg of *calcium gluconate CRS* in 0.5 ml of the test solution and dilute to 1 ml with *water R*.

Apply separately to the plate, as bands 20 mm by 2 mm, 10 µl of each solution. Allow the tank to saturate for 10 min. Develop over a path of 12 cm using a freshly prepared mixture of 20 volumes of *anhydrous formic acid R*, 20 volumes of *water R*, 30 volumes of *acetone R* and 30 volumes of *butanol R*. Allow the plate to dry in air and spray with 0.02 M *potassium permanganate*. The principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a). The test is not valid unless the chromatogram obtained with reference solution (b) shows two clearly separated spots.

B. 0.2 ml of solution S (see Tests) gives reaction (b) of calcium (2.3.1).

TESTS

Solution S. Dissolve 10.0 g in *carbon dioxide-free water R* prepared from *distilled water R* and dilute to 100 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and not more intensely coloured than reference solution Y₆ (2.2.2, *Method II*).

pH (2.2.3). The pH of solution S is 6.0 to 8.0.

Reducing sugars. Dissolve 1.0 g in 5 ml of *water R* with the aid of gentle heat. Cool and add 20 ml of *cupri-citric solution R* and a few glass beads. Heat so that boiling begins after 4 min and maintain boiling for 3 min. Cool

01/2005:0172

rapidly and add 100 ml of a 2.4 per cent *V/V* solution of *glacial acetic acid R* and 20.0 ml of 0.025 *M* *iodine*. With continuous shaking, add 25 ml of a mixture of 6 volumes of *hydrochloric acid R* and 94 volumes of *water R* until the precipitate dissolves, titrate the excess of iodine with 0.05 *M* *sodium thiosulphate* using 1 ml of *starch solution R* added towards the end of the titration, as indicator. Not less than 12.6 ml of 0.05 *M* *sodium thiosulphate* is required (1 per cent expressed as glucose).

Cyanide. Dissolve 5.0 g in 50 ml of *water R* and add 2.0 g of *tartaric acid R*. Place this solution in a distillation apparatus (2.2.11). The plain bend adapter attached to the end of the condenser has a vertical part that is long enough to extend to 1 cm from the bottom of a 50 ml test-tube used as a receiver. Place 10 ml of *water R* and 2 ml of 0.1 *M* *sodium hydroxide* into the receiver. Distil, collect 25 ml of distillate and dilute to 50 ml with *water R*. To 25 ml of this solution add 25 mg of *ferrous sulphate R* and boil for a short time. After cooling to about 70 °C add 10 ml of *hydrochloric acid R1*. After 30 min, filter the solution and wash the filter. A yellow spot appears on the filter; there is no blue or green spot.

Chlorides (2.4.4). To 5 ml of solution S, add 10 ml of *water R*. The solution complies with the limit test for chlorides (100 ppm).

Sulphates (2.4.13). 15 ml of solution S complies with the limit test for sulphates (100 ppm).

Iron (2.4.9). Dilute 2.5 ml of solution S to 10 ml with *water R*. The solution complies with the limit test for iron (40 ppm).

Heavy metals (2.4.8). Dissolve 2.0 g in 10 ml of *buffer solution pH 3.5 R* and dilute to 20 ml with *water R*. 12 ml of the solution complies with limit test A for heavy metals (10 ppm). Prepare the standard using *lead standard solution (1 ppm Pb) R*.

Loss on drying (2.2.32). Not more than 5.0 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C for 3 h.

Bacterial endotoxins (2.6.14): less than 167 IU/g, if intended for use in the manufacture of parenteral dosage forms without a further appropriate procedure for the removal of bacterial endotoxins.

ASSAY

Dissolve 0.800 g in a mixture of 150 ml of *water R* and 2 ml of 3 *M* *hydrochloric acid*. While stirring, add 12.5 ml of 0.1 *M* *sodium edetate*, 15 ml of 1 *M* *sodium hydroxide* and 0.3 g of *hydroxynaphthol blue, sodium salt R*. Titrate with 0.1 *M* *sodium edetate* until the colour changes from violet to pure blue.

1 ml of 0.1 *M* *sodium edetate* is equivalent to 49.04 mg of $\text{C}_{14}\text{H}_{26}\text{CaO}_{16}$.

STORAGE

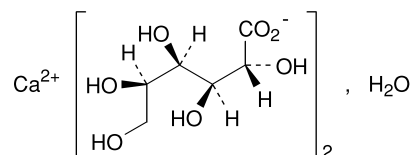
In an airtight container. If the substance is sterile, store in a sterile, airtight, tamper-proof container.

LABELLING

The label states, where applicable, that the substance is free from bacterial endotoxins.

CALCIUM GLUCONATE

Calcii gluconas


 $\text{C}_{12}\text{H}_{22}\text{CaO}_{14}\cdot\text{H}_2\text{O}$
 M_r 448.4

DEFINITION

Calcium gluconate contains not less than 98.5 per cent and not more than the equivalent of 102.0 per cent of calcium D-gluconate monohydrate.

CHARACTERS

A white, crystalline or granular powder, sparingly soluble in water, freely soluble in boiling water.

IDENTIFICATION

A. Examine by thin-layer chromatography (2.2.27), using *silica gel G R* as the coating substance.

Test solution. Dissolve 20 mg of the substance to be examined in 1 ml of *water R*, heating if necessary in a water-bath at 60 °C.

Reference solution. Dissolve 20 mg of *calcium gluconate CRS* in 1 ml of *water R*, heating if necessary in a water-bath at 60 °C.

Apply separately to the plate 5 µl of each solution. Develop over a path of 10 cm using a mixture of 10 volumes of *ethyl acetate R*, 10 volumes of *concentrated ammonia R*, 30 volumes of *water R* and 50 volumes of *alcohol R*. Dry the plate at 100 °C for 20 min. Allow to cool and spray with a 50 g/l solution of *potassium dichromate R* in a 40 per cent *m/m* solution of *sulphuric acid R*. After 5 min, the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

B. Solution S (see Tests) gives the reactions of calcium (2.3.1).

TESTS

Solution S. Dissolve 1.0 g in *water R* heated to 60 °C and dilute to 50 ml with the same solvent.

Appearance of solution. At 60 °C, solution S is not more intensely coloured than reference solution Y_6 (2.2.2, *Method II*). After cooling, it is not more opalescent than reference suspension II (2.2.1).

Organic impurities and boric acid. Introduce 0.5 g into a porcelain dish previously rinsed with *sulphuric acid R* and placed in a bath of iced water. Add 2 ml of cooled *sulphuric acid R* and mix. No yellow or brown colour develops. Add 1 ml of *chromotrope II B solution R*. A violet colour develops and does not become dark blue. Compare the colour obtained with that of a mixture of 1 ml of *chromotrope II B solution R* and 2 ml of cooled *sulphuric acid R*.

Sucrose and reducing sugars. Dissolve 0.5 g in a mixture of 2 ml of *hydrochloric acid R1* and 10 ml of *water R*. Boil for 5 min, allow to cool, add 10 ml of *sodium carbonate solution R* and allow to stand. Dilute to 25 ml with *water R*.

and filter. To 5 ml of the filtrate add 2 ml of *cupri-tartaric solution R* and boil for 1 min. Allow to stand for 2 min. No red precipitate is formed.

Chlorides (2.4.4). 12.5 ml of solution S diluted to 15 ml with *water R* complies with the limit test for chlorides (200 ppm).

Sulphates (2.4.13). Dissolve 10.0 g with heating in a mixture of 10 ml of *acetic acid R* and 90 ml of *distilled water R*. 15 ml of the solution complies with the limit test for sulphates (100 ppm).

Heavy metals (2.4.8). 2.0 g complies with limit test D for heavy metals (10 ppm). Heat the substance to be examined gradually and with care until it is almost completely transformed into a white mass and then ignite. Prepare the standard using 2 ml of *lead standard solution (10 ppm Pb) R*.

Magnesium and alkali metals. Dissolve 1.00 g in 100 ml of boiling *water R*, add 10 ml of *ammonium chloride solution R*, 1 ml of *ammonia R* and, dropwise, 50 ml of hot *ammonium oxalate solution R*. Allow to stand for 4 h, dilute to 200 ml with *water R* and filter. Evaporate 100 ml of the filtrate to dryness and ignite. The residue weighs not more than 2 mg (0.4 per cent).

Microbial contamination. Total viable aerobic count (2.6.12) not more than 10^3 micro-organisms per gram, determined by plate count.

ASSAY

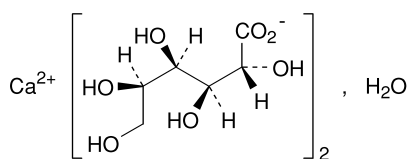
Dissolve 0.8000 g in 20 ml of hot *water R*, allow to cool and dilute to 300 ml with *water R*. Carry out the complexometric titration of calcium (2.5.11).

1 ml of 0.1 M *sodium edetate* is equivalent to 44.84 mg of $C_{12}H_{22}CaO_{14} \cdot H_2O$.

01/2005:0979

CALCIUM GLUCONATE FOR INJECTION

Calcii gluconas ad iniectionabile



$C_{12}H_{22}CaO_{14} \cdot H_2O$

M_r 448.4

DEFINITION

Calcium gluconate for injection contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of calcium D-gluconate monohydrate.

CHARACTERS

A white, crystalline or granular powder, sparingly soluble in water, freely soluble in boiling water.

IDENTIFICATION

A. Examine by thin-layer chromatography (2.2.27), using *silica gel G R* as the coating substance.

Test solution. Dissolve 20 mg of the substance to be examined in 1 ml of *water R*, heating if necessary in a water-bath at 60 °C.

Reference solution. Dissolve 20 mg of *calcium gluconate CRS* in 1 ml of *water R*, heating if necessary in a water-bath at 60 °C.

Apply to the plate 5 µl of each solution. Develop over a path of 10 cm using a mixture of 10 volumes of *concentrated ammonia R*, 10 volumes of *ethyl acetate R*, 30 volumes of *water R* and 50 volumes of *alcohol R*. Dry the plate at 100 °C for 20 min. Allow to cool and spray with a 50 g/l solution of *potassium dichromate R* in a 40 per cent *m/m* solution of *sulphuric acid R*. After 5 min, the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

B. About 20 mg gives reaction (b) of calcium (2.3.1).

TESTS

Solution S. To 10.0 g add 90 ml of boiling *distilled water R* and boil with stirring, for not more than 10 s, until completely dissolved. Dilute to 100.0 ml with the same solvent.

Appearance of solution. Solution S at 60 °C is not more intensely coloured than reference solution B₇ (2.2.2, *Method II*). After cooling to 20 °C, it is not more opalescent than reference suspension II (2.2.1).

pH (2.2.3). Dissolve 1.0 g in 20 ml of *carbon dioxide-free water R*, heating on a water-bath. The pH of the solution is 6.4 to 8.3.

Organic impurities and boric acid. Introduce 0.5 g into a porcelain dish previously rinsed with *sulphuric acid R* and placed in a bath of iced water. Add 2 ml of cooled *sulphuric acid R* and mix. No yellow or brown colour develops. Add 1 ml of *chromotrope II B solution R*. A violet colour develops and does not become dark blue. The solution is not more intensely coloured than that of a mixture of 1 ml of *chromotrope II B solution R* and 2 ml of cooled *sulphuric acid R*.

Oxalate. Not more than 100 ppm, determined by liquid chromatography (2.2.29).

Test solution. Dissolve 1.00 g of the substance to be examined in *water for chromatography R* and dilute to 100.0 ml with the same solvent.

Reference solution. Dissolve 1.00 g of the substance to be examined in *water for chromatography R*, add 0.5 ml of a 0.152 g/l solution of *sodium oxalate R* in *water for chromatography R* and dilute to 100.0 ml with the same solvent.

The chromatographic procedure may be carried out using:

- a guard column 30 mm long and 4 mm in internal diameter packed with a suitable strong anion exchange resin (30 µm to 50 µm),
- two columns each 0.25 m long and 4 mm in internal diameter packed with a suitable strong anion exchange resin (30 µm to 50 µm),
- a micromembrane anion-suppressor column, connected in series with the guard and analytical columns; the anion-suppressor column is equipped with a micromembrane that separates the mobile phase from the suppressor regeneration solution, flowing countercurrent to the mobile phase at a rate of 4 ml/min,
- as mobile phase at a flow rate of 2 ml/min a solution prepared as follows: dissolve 0.212 g of *anhydrous sodium carbonate R* and 63 mg of *sodium hydrogen carbonate R* in *water for chromatography R* and dilute to 1000.0 ml with the same solvent,
- as suppressor regeneration solution a 1.23 g/l solution of *sulphuric acid R* in *water for chromatography R*,
- a conductance detector,
- a loop injector.

Inject 50 µl of the reference solution five times. The test is not valid unless the relative standard deviation of the peak area for oxalate in the chromatogram obtained with the reference solution is at most 2.0 per cent.

Inject 50 µl of each solution three times. Calculate the content of oxalate in parts per million using the following expression:

$$\frac{S_T \times 50}{S_R - S_T}$$

S_T = area of the oxalate peak in the chromatogram obtained with the test solution,

S_R = area of the oxalate peak in the chromatogram obtained with the reference solution.

Sucrose and reducing sugars. Dissolve 0.5 g in a mixture of 2 ml of *hydrochloric acid R1* and 10 ml of *water R*. Boil for 5 min, allow to cool, add 10 ml of *sodium carbonate solution R* and allow to stand for 10 min. Dilute to 25 ml with *water R* and filter. To 5 ml of the filtrate add 2 ml of *cupri-tartaric solution R* and boil for 1 min. Allow to stand for 2 min. No red precipitate is formed.

Chlorides (2.4.4). To 10 ml of previously filtered solution S add 5 ml of *water R*. The solution complies with the limit test for chlorides (50 ppm).

Phosphates (2.4.11). Dilute 1 ml of solution S to 100 ml with *water R*. The solution complies with the limit test for phosphates (100 ppm).

Sulphates (2.4.13). 15 ml of previously filtered solution S complies with the limit test for sulphates (50 ppm). Prepare the standard using a mixture of 7.5 ml of *sulphate standard solution (10 ppm SO₄) R* and 7.5 ml of *distilled water R*.

Iron. Not more than 5 ppm of Fe, determined by atomic absorption spectrometry (2.2.23, *Method I*).

Test solution. Introduce 2.0 g into a 100 ml polytetrafluoroethylene beaker and add 5 ml of *nitric acid R*. Boil, evaporating almost to dryness. Add 1 ml of *strong hydrogen peroxide solution R* and evaporate again almost to dryness. Repeat the hydrogen peroxide treatment until a clear solution is obtained. Using 2 ml of *nitric acid R*, transfer the solution into a 25 ml volumetric flask. Dilute to 25.0 ml with *dilute hydrochloric acid R*. In the same manner, prepare a compensation solution using 0.65 g of *calcium chloride R1* instead of the substance to be examined.

Reference solutions. Prepare the reference solutions from *iron solution (20 ppm Fe) R* diluted with *dilute hydrochloric acid R*.

Measure the absorbance at 248.3 nm, using an iron hollow-cathode lamp as source of radiation and an air-acetylene flame. Carry out a basic correction using a deuterium lamp.

Magnesium and alkali metals. To 0.50 g add a mixture of 1.0 ml of *dilute acetic acid R* and 10.0 ml of *water R* and rapidly boil, whilst shaking, until completely dissolved. To the boiling solution add 5.0 ml of *ammonium oxalate solution R* and allow to stand for at least 6 h. Filter through a sintered-glass filter (1.6) into a porcelain crucible. Carefully evaporate the filtrate to dryness and ignite. The residue weighs not more than 2 mg (0.4 per cent).

Heavy metals (2.4.8). 12 ml of solution S complies with limit test A for heavy metals (10 ppm). Prepare the standard using *lead standard solution (1 ppm Pb) R*.

Bacterial endotoxins (2.6.14): less than 167 IU/g.

Microbial contamination. Total viable aerobic count (2.6.12) not more than 10² micro-organisms per gram, determined by plate count. It complies with the tests for *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* (2.6.13).

ASSAY

Dissolve 0.350 g in 20 ml of hot *water R*, allow to cool and dilute to 300 ml with *water R*. Carry out the complexometric titration of calcium (2.5.11). Use 50 mg of *calconecarboxylic acid triturate R*.

1 ml of 0.1 M *sodium edetate* is equivalent to 44.84 mg of C₁₂H₂₂CaO₁₄·H₂O.

01/2005:0980

CALCIUM GLYCEROPHOSPHATE

Calcii glycerophosphas

C₃H₇CaO₆P

M_r 210.1

DEFINITION

Calcium glycerophosphate is a mixture in variable proportions of calcium (RS)-2,3-dihydroxypropyl phosphate, and of calcium 2-hydroxy-1-(hydroxymethyl)ethyl phosphate, which may be hydrated. Calcium glycerophosphate contains not less than 18.6 per cent and not more than 19.4 per cent of Ca, calculated with reference to the dried substance.

CHARACTERS

A white powder, hygroscopic, sparingly soluble in water, practically insoluble in alcohol.

IDENTIFICATION

- Mix 1 g with 1 g of *potassium hydrogen sulphate R* in a test tube fitted with a glass tube. Heat strongly and direct the white vapour towards a piece of filter paper impregnated with a freshly prepared 10 g/l solution of *sodium nitroprusside R*. The filter paper develops a blue colour in contact with *piperidine R*.
- Ignite 0.1 g in a crucible. Take up the residue with 5 ml of *nitric acid R* and heat on a water-bath for 1 min. Filter. The filtrate gives reaction (b) of phosphates (2.3.1).
- It gives reaction (b) of calcium (2.3.1).

TESTS

Solution S. Dissolve 1.5 g at room temperature in *carbon dioxide-free water R* prepared from *distilled water R* and dilute to 150 ml with the same solvent.

Appearance of solution. Solution S is not more opalescent than reference suspension III (2.2.1).

Acidity or alkalinity. To 100 ml of solution S add 0.1 ml of *phenolphthalein solution R*. Not more than 1.5 ml of 0.1 M *hydrochloric acid* or 0.5 ml of 0.1 M *sodium hydroxide* is required to change the colour of the indicator.

Citric acid. Shake 5.0 g with 20 ml of *carbon dioxide-free water R* and filter. To the filtrate add 0.15 ml of *sulphuric acid R* and filter again. To the filtrate add 5 ml of *mercuric sulphate solution R* and heat to boiling. Add 0.5 ml of a 3.2 g/l solution of *potassium permanganate R* and again heat to boiling. No precipitate is formed.

Glycerol and alcohol-soluble substances. Shake 1.000 g with 25 ml of *alcohol R* for 1 min. Filter. Evaporate the filtrate to dryness on a water-bath and dry the residue at 70 °C for 1 h. The residue weighs not more than 5 mg (0.5 per cent).

Chlorides (2.4.4). Dissolve 0.1 g in a mixture of 2 ml of *acetic acid R* and 8 ml of *water R* and dilute to 15 ml with *water R*. The solution complies with the limit test for chlorides (500 ppm).

Phosphates (2.4.11). Dilute 2.5 ml of solution S to 100 ml with *water R*. The solution complies with the limit test for phosphates (400 ppm).

Sulphates (2.4.13). 15 ml of solution S complies with the limit test for sulphates (0.1 per cent).

Arsenic (2.4.2). Dissolve 0.33 g in *water R* and dilute to 25 ml with the same solvent. The solution complies with limit test A for arsenic (3 ppm).

Iron (2.4.9). 0.20 g complies with the limit test for iron (50 ppm).

Heavy metals (2.4.8). Dissolve 2.0 g in 10 ml of *buffer solution pH 3.5 R* and dilute to 20 ml with *water R*. 12 ml of the solution complies with limit test A for heavy metals (20 ppm). Prepare the standard using *lead standard solution (2 ppm Pb) R*.

Loss on drying (2.2.32). Not more than 12.0 per cent, determined on 1.000 g by drying in an oven at 150 °C for 4 h.

ASSAY

Dissolve 0.200 g in *water R*. Carry out the complexometric titration of calcium (2.5.11).

1 ml of 0.1 M *sodium edetate* is equivalent to 4.008 mg of Ca.

01/2005:0981
corrected

CALCIUM HYDROGEN PHOSPHATE, ANHYDROUS

Calcii hydrogenophosphas anhydricus

CaHPO_4

M_r 136.1

DEFINITION

Content: 98.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

Appearance: white, crystalline powder, or colourless crystals.

Solubility: practically insoluble in water and in alcohol. It dissolves in dilute hydrochloric acid and in dilute nitric acid.

IDENTIFICATION

- It complies with the limits of the assay.
- Dissolve 0.1 g in a mixture of 5 ml of *dilute nitric acid R* and 5 ml of *water R*. The solution gives reaction (b) of phosphates (2.3.1).
- Dissolve 5 mg in 5 ml of *acetic acid R*. The solution gives reaction (b) of calcium (2.3.1).

TESTS

Solution S. Dissolve 2.5 g in 20 ml of *dilute hydrochloric acid R*. Filter if necessary. Add *dilute ammonia R1* until a precipitate is formed. Dissolve by adding the minimum quantity needed of *dilute hydrochloric acid R* and dilute to 50 ml with *distilled water R*.

Carbonates. Shake 0.5 g with 5 ml of *carbon dioxide-free water R* and add 1 ml of *hydrochloric acid R*. No effervescence is produced.

Chlorides (2.4.4): maximum 330 ppm.

Dissolve 0.5 g in a mixture of 1 ml of *nitric acid R* and 10 ml of *water R* and dilute to 50 ml with *water R*. 15 ml of the solution complies with the limit test for chlorides.

Fluorides (2.4.5): maximum 100 ppm.

0.5 g complies with the limit test for fluorides.

Sulphates (2.4.13): maximum 0.5 per cent.

To 1 ml of solution S add 2 ml of *dilute hydrochloric acid R* and dilute to 25 ml with *distilled water R*. 15 ml of the solution complies with the limit test for sulphates.

Arsenic (2.4.2): maximum 10 ppm.

2 ml of solution S complies with limit test A.

Barium. To 10 ml of filtered solution S add 0.5 ml of *dilute sulphuric acid R*. Allow to stand for 15 min. Any opalescence in the solution is not more intense than that in a mixture of 10 ml of solution S and 0.5 ml of *distilled water R*.

Iron (2.4.9): maximum 400 ppm.

Dilute 0.5 ml of solution S to 10 ml with *water R*. The solution complies with the limit test for iron.

Heavy metals (2.4.8): maximum 40 ppm.

Dilute 10 ml of solution S to 20 ml with *water R*. 12 ml of the solution complies with limit test A. Prepare the standard using *lead standard solution (1 ppm Pb) R*.

Loss on drying (2.2.32): maximum 2.0 per cent, determined on 1.000 g by drying in an oven at 150 °C for 2 h.

ASSAY

Dissolve 0.250 g in a mixture of 1 ml of *hydrochloric acid R1* and 5 ml of *water R*. Add 25.0 ml of 0.1 M *sodium edetate* and dilute to 200 ml with *water R*. Neutralise with *concentrated ammonia R*, add 10 ml of *ammonium chloride buffer solution pH 10.0 R* and about 50 mg of *mordant black 11 triturate R*. Titrate the excess of sodium edetate with 0.1 M *zinc sulphate* until the colour changes from blue to violet. Carry out a blank titration.

1 ml of 0.1 M *sodium edetate* is equivalent to 13.61 mg of CaHPO_4 .

01/2005:0116

CALCIUM HYDROGEN PHOSPHATE DIHYDRATE

Calcii hydrogenophosphas dihydricus

$\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$

M_r 172.1

DEFINITION

Content: 98.0 per cent to 105.0 per cent.

CHARACTERS

Appearance: white, crystalline powder.

Solubility: practically insoluble in cold water and in alcohol. It dissolves in dilute hydrochloric acid and in dilute nitric acid.

IDENTIFICATION

- Dissolve 0.1 g in a mixture of 5 ml of *dilute nitric acid R* and 5 ml of *water R*. The solution gives reaction (b) of phosphates (2.3.1).
- 5 mg gives reaction (b) of calcium (2.3.1).
- It complies with the limits of the assay.

TESTS

Solution S. Dissolve 2.5 g in 20 ml of *dilute hydrochloric acid R*, filter if necessary and add *dilute ammonia R1* until a precipitate is formed. Add just sufficient *dilute hydrochloric acid R* to dissolve the precipitate and dilute to 50 ml with *distilled water R*.

Carbonates. Shake 0.5 g with 5 ml of *carbon dioxide-free water R* and add 1 ml of *hydrochloric acid R*. No effervescence is produced.

Chlorides (2.4.4): maximum 330 ppm.

Dissolve 0.5 g in a mixture of 1 ml of *nitric acid R* and 10 ml of *water R* and dilute to 50 ml with *water R*. 15 ml of the solution complies with the limit test for chlorides.

Fluorides (2.4.5): maximum 100 ppm.

0.5 g complies with the limit test for fluorides.

Sulphates (2.4.13): maximum 0.5 per cent.

To 1 ml of solution S add 2 ml of *dilute hydrochloric acid R* and dilute to 25 ml with *distilled water R*. 15 ml of the solution complies with the limit test for sulphates.

Arsenic (2.4.2): maximum 10 ppm.

2 ml of solution S complies with limit test A.

Barium. To 10 ml of solution S add 0.5 ml of *dilute sulphuric acid R*. After 15 min, any opalescence in the solution is not more intense than that in a mixture of 10 ml of solution S and 0.5 ml of *distilled water R*.

Iron (2.4.9): maximum 400 ppm.

0.5 ml of solution S diluted to 10 ml with *water R* complies with the limit test for iron.

Heavy metals (2.4.8): maximum 40 ppm.

Dilute 10 ml of solution S to 20 ml with *water R*. 12 ml of the solution complies with limit test A. Prepare the standard using *lead standard solution (1 ppm Pb) R*.

ASSAY

Dissolve 0.300 g in a mixture of 1 ml of *hydrochloric acid R1* and 5 ml of *water R*. Add 25.0 ml of *0.1 M sodium edetate* and dilute to 200 ml with *water R*. Neutralise with *concentrated ammonia R*, add 10 ml of *ammonium chloride buffer solution pH 10.0 R* and about 50 mg of *mordant black 11 triturate R*. Titrate the excess of sodium edetate with *0.1 M zinc sulphate* until the colour changes from blue to violet. Carry out a blank titration.

1 ml of *0.1 M sodium edetate* is equivalent to 17.21 mg of $\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$.

01/2005:1078
corrected

CALCIUM HYDROXIDE

Calcii hydroxidum

Ca(OH)_2

M_r 74.1

DEFINITION

Calcium hydroxide contains not less than 95.0 per cent and not more than the equivalent of 100.5 per cent of Ca(OH)_2 .

CHARACTERS

A white or almost white, fine powder, practically insoluble in water.

IDENTIFICATION

A. To 0.80 g in a mortar, add 10 ml of *water R* and 0.5 ml of *phenolphthalein solution R* and mix. The suspension turns red. On addition of 17.5 ml of *1 M hydrochloric acid*, the suspension becomes colourless without effervescing. The red colour occurs again when the mixture is triturated for 1 min. On addition of a further 6 ml of *1 M hydrochloric acid* and triturating, the solution becomes colourless.

B. Dissolve about 0.1 g in *dilute hydrochloric acid R* and dilute to 10 ml with *water R*. 5 ml of the solution give reaction (b) of calcium (2.3.1).

TESTS

Matter insoluble in hydrochloric acid. Dissolve 2.0 g in 30 ml of *hydrochloric acid R*. Boil the solution and filter. Wash the residue with hot *water R*. The residue weighs not more than 10 mg (0.5 per cent).

Carbonates. Not more than 5.0 per cent of CaCO_3 .

Add 5.0 ml of *1 M hydrochloric acid* to the titrated solution obtained under Assay and titrate with *1 M sodium hydroxide* using 0.5 ml of *methyl orange solution R* as indicator.

1 ml of *1 M hydrochloric acid* is equivalent to 50.05 mg of CaCO_3 .

Chlorides (2.4.4). Dissolve 0.30 g in a mixture of 2 ml of *nitric acid R* and 10 ml of *water R* and dilute to 30 ml with *water R*. 15 ml of the solution complies with the limit test for chlorides (330 ppm).

Sulphates (2.4.13). Dissolve 0.15 g in a mixture of 5 ml of *dilute hydrochloric acid R* and 10 ml of *distilled water R* and dilute to 60 ml with *distilled water R*. 15 ml of the solution complies with the limit test for sulphates (0.4 per cent).

Arsenic (2.4.2). Dissolve 0.50 g in 5 ml of *brominated hydrochloric acid R* and dilute to 50 ml with *water R*. 25 ml of the solution complies with the limit test A for arsenic (4 ppm).

Magnesium and alkali metals. Dissolve 1.0 g in a mixture of 10 ml of *hydrochloric acid R* and 40 ml of *water R*. Boil and add 50 ml of a 63 g/l solution of *oxalic acid R*. Neutralise with *ammonia R* and dilute to 200 ml with *water R*. Allow to stand for 1 h and filter through a suitable filter. To 100 ml of the filtrate, add 0.5 ml of *sulphuric acid R*. Cautiously evaporate to dryness and ignite. The residue weighs not more than 20 mg (4.0 per cent calculated as sulphates).

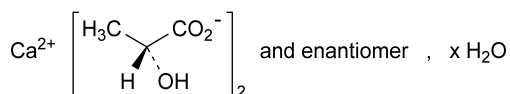
Heavy metals (2.4.8). Dissolve 1.0 g in 10 ml of *hydrochloric acid R1* and evaporate to dryness on a water-bath. Dissolve the residue in 20 ml of *water R* and filter. 12 ml of the filtrate complies with limit test A for heavy metals (20 ppm). Prepare the standard using *lead standard solution (1 ppm Pb) R*.

ASSAY

To 1.500 g in a mortar, add 20 ml to 30 ml of *water R* and 0.5 ml of *phenolphthalein solution R*. Titrate with *1 M hydrochloric acid* by triturating the substance until the red colour disappears. The final solution is used in the tests for carbonates.

1 ml of *1 M hydrochloric acid* is equivalent to 37.05 mg of Ca(OH)_2 .

01/2005:0468

CALCIUM LACTATE PENTAHYDRATE**Calcii lactas pentahydricus**
 $\text{C}_6\text{H}_{10}\text{CaO}_6 \cdot x\text{H}_2\text{O}$ with $x \simeq 5$ M_r 218.2 (anhydrous substance)
DEFINITION

Calcium lactate pentahydrate contains not less than 98.0 per cent and not more than the equivalent of 102.0 per cent of calcium bis(2-hydroxypropanoate) or of a mixture of calcium (*R*)-, (*S*)- and (*RS*)-2-hydroxypropionates, calculated with reference to the dried substance. It contains not less than 22.0 per cent and not more than 27.0 per cent of water, determined by the loss on drying.

CHARACTERS

A white or almost white, crystalline or granular powder, slightly efflorescent, soluble in water, freely soluble in boiling water, very slightly soluble in alcohol.

IDENTIFICATION

- A. It complies with the test for loss on drying (see Tests).
- B. It gives the reaction of lactates (2.3.1).
- C. It gives reaction (b) of calcium (2.3.1).

TESTS

Solution S. Dissolve 5.0 g with heating in *carbon dioxide-free water R* prepared from *distilled water R*, allow to cool and dilute to 100 ml with the same solvent.

Appearance of solution. Solution S is not more opalescent than reference suspension II (2.2.1) and not more intensely coloured than reference solution BY₆ (2.2.2, *Method II*).

Acidity or alkalinity. To 10 ml of solution S add 0.1 ml of *phenolphthalein solution R* and 0.5 ml of 0.01 *M hydrochloric acid*. The solution is colourless. Not more than 2.0 ml of 0.01 *M sodium hydroxide* is required to change the colour of the indicator to pink.

Volatile fatty acids. In a 100 ml ground-glass-stoppered flask stir 0.5 g with 1 ml of *phosphoric acid R*. Close the flask. Heat cautiously at 50 °C for 10 min. No unpleasant odour resembling that of the lower fatty acids is recognisable immediately after opening the flask.

Chlorides (2.4.4). 5 ml of solution S diluted to 15 ml with *water R* complies with the limit test for chlorides (200 ppm).

Sulphates (2.4.13). 7.5 ml of solution S diluted to 15 ml with *distilled water R* complies with the limit test for sulphates (400 ppm).

Barium. To 10 ml of solution S add 1 ml of *calcium sulphate solution R*. Allow to stand for 15 min. Any opalescence in the solution is not more intense than that in a mixture of 1 ml of *distilled water R* and 10 ml of solution S.

Heavy metals (2.4.8). 12 ml of solution S complies with limit test A for heavy metals (20 ppm). Prepare the standard using *lead standard solution* (1 ppm Pb) *R*.

Iron (2.4.9). 4 ml of solution S diluted to 10 ml with *water R* complies with the limit test for iron (50 ppm).

Magnesium and alkali salts. To 20 ml of solution S add 20 ml of *water R*, 2 g of *ammonium chloride R* and 2 ml of *dilute ammonia RI*. Heat to boiling and rapidly add 40 ml of hot *ammonium oxalate solution R*. Allow to stand for

4 h, dilute to 100.0 ml with *water R* and filter. To 50.0 ml of the filtrate add 0.5 ml of *sulphuric acid R*, evaporate to dryness and ignite the residue to constant mass at 600 °C. The residue weighs not more than 5 mg (1 per cent).

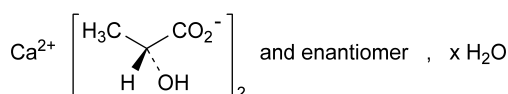
Loss on drying (2.2.32): 22.0 per cent to 27.0 per cent, determined on 0.500 g by drying in an oven at 125 °C.

ASSAY

Dissolve 0.200 g in *water R* and dilute to 300 ml with the same solvent. Carry out the complexometric titration of calcium (2.5.11).

1 ml of 0.1 *M sodium edetate* is equivalent to 21.82 mg of $\text{C}_6\text{H}_{10}\text{CaO}_6$.

01/2005:0469

CALCIUM LACTATE TRIHYDRATE**Calcii lactas trihydricus**
 $\text{C}_6\text{H}_{10}\text{CaO}_6 \cdot x\text{H}_2\text{O}$ with $x \simeq 3$ M_r 218.2 (anhydrous substance)
DEFINITION

Calcium lactate trihydrate contains not less than 98.0 per cent and not more than the equivalent of 102.0 per cent of calcium bis(2-hydroxypropanoate) or of a mixture of calcium (*R*)-, (*S*)- and (*RS*)-2-hydroxypropionates, calculated with reference to the dried substance. It contains not less than 15.0 per cent and not more than 20.0 per cent of water, determined by the loss on drying.

CHARACTERS

A white or almost white, crystalline or granular powder, soluble in water, freely soluble in boiling water, very slightly soluble in alcohol.

IDENTIFICATION

- A. It complies with the test for loss on drying (see Tests).
- B. It gives the reaction of lactates (2.3.1).
- C. It gives reaction (b) of calcium (2.3.1).

TESTS

Solution S. Dissolve 5.0 g with heating in *carbon dioxide-free water R* prepared from *distilled water R*, allow to cool and dilute to 100 ml with the same solvent.

Appearance of solution. Solution S is not more opalescent than reference suspension II (2.2.1) and not more intensely coloured than reference solution BY₆ (2.2.2, *Method II*).

Acidity or alkalinity. To 10 ml of solution S add 0.1 ml of *phenolphthalein solution R* and 0.5 ml of 0.01 *M hydrochloric acid*. The solution is colourless. Not more than 2.0 ml of 0.01 *M sodium hydroxide* is required to change the colour of the indicator to pink.

Volatile fatty acids. In a 100 ml ground-glass-stoppered flask stir 0.5 g with 1 ml of *phosphoric acid R*. Close the flask. Heat cautiously at 50 °C for 10 min. No unpleasant odour resembling that of the lower fatty acids is recognisable immediately after opening the flask.

Chlorides (2.4.4). 5 ml of solution S diluted to 15 ml with *water R* complies with the limit test for chlorides (200 ppm).

Sulphates (2.4.13). 7.5 ml of solution S diluted to 15 ml with *distilled water R* complies with the limit test for sulphates (400 ppm).

Barium. To 10 ml of solution S add 1 ml of *calcium sulphate solution R*. Allow to stand for 15 min. Any opalescence in the solution is not more intense than that in a mixture of 1 ml of *distilled water R* and 10 ml of solution S.

Heavy metals (2.4.8). 12 ml of solution S complies with limit test A for heavy metals (20 ppm). Prepare the standard using *lead standard solution* (1 ppm Pb) *R*.

Iron (2.4.9). 4 ml of solution S diluted to 10 ml with *water R* complies with the limit test for iron (50 ppm).

Magnesium and alkali salts. To 20 ml of solution S add 20 ml of *water R*, 2 g of *ammonium chloride R* and 2 ml of *dilute ammonia R1*. Heat to boiling and rapidly add 40 ml of hot *ammonium oxalate solution R*. Allow to stand for 4 h, dilute to 100.0 ml with *water R* and filter. To 50.0 ml of the filtrate add 0.5 ml of *sulphuric acid R*, evaporate to dryness and ignite the residue to constant mass at 600 °C. The residue weighs not more than 5 mg (1 per cent).

Loss on drying (2.2.32): 15.0 per cent to 20.0 per cent, determined on 0.500 g by drying in an oven at 125 °C.

ASSAY

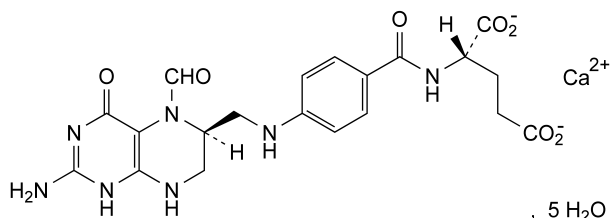
Dissolve 0.200 g in *water R* and dilute to 300 ml with the same solvent. Carry out the complexometric titration of calcium (2.5.11).

1 ml of 0.1 M *sodium edetate* is equivalent to 21.82 mg of $C_{20}H_{21}CaN_7O_7$.

01/2005:1606

CALCIUM LEVOFOLINATE PENTAHYDRATE

Calcii levofolinas pentahydricus



$C_{20}H_{21}CaN_7O_7 \cdot 5H_2O$

M_r 511.5 (anhydrous substance)

DEFINITION

Calcium (2S)-2-[[4-[[[(6S)-2-amino-5-formyl-4-oxo-1,4,5,6,7,8-hexahydropteridin-6-yl]methyl]amino]benzoyl]amino]pentanedioate pentahydrate.

Content:

- calcium levofolinate ($C_{20}H_{21}CaN_7O_7$; M_r 511.5): 97.0 per cent to 102.0 per cent (anhydrous and solvent-free substance).
- calcium (Ca; A_r 40.08): 7.54 per cent to 8.14 per cent (anhydrous and solvent-free substance).

CHARACTERS

Appearance: white or light yellow, amorphous or crystalline powder, hygroscopic.

Solubility: slightly soluble in water, practically insoluble in acetone and in alcohol.

IDENTIFICATION

First identification: A, B, D.

Second identification: A, C, D.

A. It complies with the test for specific optical rotation (see Tests).

B. Infrared absorption spectrophotometry (2.2.24).

Preparation: discs.

Comparison: *calcium folinate CRS*.

If the spectra obtained show differences, dissolve the substance to be examined and the reference substance separately in the minimum quantity of *water R* and add dropwise sufficient *acetone R* to produce a precipitate. Allow to stand for 15 min, collect the precipitate by centrifugation, wash the precipitate twice with a minimum quantity of *acetone R* and dry. Record new spectra using the residues.

C. Thin-layer chromatography (2.2.27).

Test solution. Dissolve 15 mg of the substance to be examined in a 3 per cent V/V solution of *ammonia R* and dilute to 5 ml with the same solvent.

Reference solution. Dissolve 15 mg of *calcium folinate CRS* in a 3 per cent V/V solution of *ammonia R* and dilute to 5 ml with the same solvent.

Plate: *cellulose for chromatography F₂₅₄ R*.

Mobile phase: the lower layer of a mixture of 1 volume of *isoamyl alcohol R* and 10 volumes of a 50 g/l solution of *citric acid R* previously adjusted to pH 8 with *ammonia R*.

Application: 5 µl.

Development: over a path of 15 cm.

Drying: in air.

Detection: examine in ultraviolet light at 254 nm.

Results: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with the reference solution.

D. It gives reaction (b) of calcium (2.3.1).

Carry out the tests and the assay as rapidly as possible, protected from bright light.

TESTS

Solution S. Dissolve 0.40 g in *carbon dioxide-free water R*, heating at 40 °C if necessary, and dilute to 50.0 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and its absorbance (2.2.25) at 420 nm has a maximum of 0.25.

pH (2.2.3): 7.5 to 8.5 for solution S.

Specific optical rotation (2.2.7): –10 to –15 (anhydrous and solvent-free substance).

Dissolve 0.200 g in *tris(hydroxymethyl)aminomethane solution R* previously adjusted to pH 8.1 with *sodium hydroxide solution R* or *hydrochloric acid R1* and dilute to 20.0 ml with the same solvent.

Acetone and ethanol. Head-space gas chromatography (2.2.28): use the standard additions method.

Test solution. Dissolve 0.25 g of the substance to be examined in *water R* and dilute to 10.0 ml with the same solvent.

Reference solution. Dissolve 0.125 g of *acetone R* and 0.750 g of *ethanol R* in *water R* and dilute to 1000.0 ml with *water R*.

Column:

- **material:** fused silica,
 - **size:** $l = 10$ m, $\varnothing = 0.32$ mm,
 - **stationary phase:** *styrene-divinylbenzene copolymer R*.
- Carrier gas:** *nitrogen for chromatography R*.
- Flow rate:** 4 ml/min.

Static head-space conditions which may be used:

- **equilibration temperature:** 80 °C,

- *equilibration time*: 20 min,
- *pressurisation time*: 30 s.

Temperature:

	Time (min)	Temperature (°C)
Column	0 - 14	80 → 220
Injection port		110
Detector		270

Detection: flame ionisation.

Injection: at least 3 times.

Limits:

- *acetone*: maximum 0.5 per cent,
- *ethanol*: maximum 3.0 per cent.

Related substances. Liquid chromatography (2.2.29).

Test solution. Dissolve 10.0 mg of the substance to be examined in *water R* and dilute to 10.0 ml with the same solvent.

Reference solution (a). Dissolve 10.0 mg of *calcium folinate CRS* in *water R* and dilute to 10.0 ml with the same solvent.

Reference solution (b). Dilute 1.0 ml of reference solution (a) to 100.0 ml with *water R*.

Reference solution (c). Dissolve 10.0 mg of *formylfolic acid CRS* in the mobile phase and dilute to 100.0 ml with the mobile phase. Dilute 1.0 ml of this solution to 10.0 ml with *water R*.

Reference solution (d). Dilute 1.0 ml of reference solution (b) to 20.0 ml with *water R*.

Reference solution (e). Dilute 5.0 ml of reference solution (c) to 10.0 ml with reference solution (b).

Column:

- *size*: $l = 0.25$ m, $\varnothing = 4$ mm,
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 μ m),
- *temperature*: 40 °C.

Mobile phase: mix 220 ml of *methanol R* and 780 ml of a solution containing 2.0 ml of *tetrabutylammonium hydroxide solution* (400 g/l) *R* and 2.2 g of *disodium hydrogen phosphate R* previously adjusted to pH 7.8 with *phosphoric acid R*. If necessary adjust the concentration of *methanol R* to achieve the prescribed resolution.

Flow rate: 1 ml/min.

Detection: spectrophotometer at 280 nm.

Injection: 10 μ l.

Run time: 2.5 times the retention time of the principal peak in the chromatogram obtained with the test solution.

System suitability: reference solution (e):

- *resolution*: minimum of 2.2 between the peaks due to folinate and to impurity D.

Limits:

- *impurity D*: not more than 0.8 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.8 per cent),
- *any other impurity*: not more than 0.8 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.8 per cent),
- *total of other impurities*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (2.0 per cent),

- *disregard limit*: area of the principal peak in the chromatogram obtained with reference solution (d) (0.05 per cent).

Impurity H. Liquid chromatography (2.2.29): use the normalisation procedure.

Test solution. Dissolve 50.0 mg of the substance to be examined in *water R* and dilute to 100.0 ml with the same solvent.

Reference solution (a). Dissolve 10.0 mg of *calcium folinate CRS* in *water R* and dilute to 20.0 ml with the same solvent.

Reference solution (b). Dilute 1.0 ml of reference solution (a) to 100.0 ml with *water R*.

Column:

- *size*: $l = 0.15$ m, $\varnothing = 4$ mm,
- *stationary phase*: *human albumin coated silica gel for chromatography R* (5 μ m),
- *temperature*: 40 °C.

Mobile phase: dissolve 9.72 g of *sodium dihydrogen phosphate R* in 890 ml of *water R* and adjust to pH 5.0 with *sodium hydroxide solution R*. Add 100 ml of *2-propanol R* and 10 ml of *acetonitrile R*.

Flow rate: 1 ml/min.

Detection: spectrophotometer at 286 nm.

Injection: 10 μ l.

Retention times: levofolate = about 9 min;
impurity H = about 19 min.

System suitability:

- *resolution*: minimum of 5.0 between the peaks due to levofolate and to impurity H in the chromatogram obtained with reference solution (a). The sum of the areas of the 2 peaks is 100 per cent. The peak area of impurity H is 48 per cent to 52 per cent. In the chromatogram obtained with reference solution (b) 2 clearly visible peaks are obtained.

Limits:

- *impurity H*: maximum 0.5 per cent.

Chlorides: maximum 0.5 per cent.

Dissolve 0.300 g in 50 ml of *water R* heating at 40 °C if necessary. Add 10 ml of *2 M nitric acid* and titrate with *0.005 M silver nitrate* determining the end-point potentiometrically (2.2.20).

1 ml of *0.005 M silver nitrate* is equivalent to 0.177 mg of Cl.

Platinum: maximum 10 ppm.

Atomic absorption spectrometry (2.2.23, *Method II*).

Test solution. Dissolve 1.0 g in *water R* and dilute to 100.0 ml with the same solvent.

Reference solutions. Prepare the reference solutions using *platinum standard solution* (30 ppm Pt) *R*, diluted as necessary with a mixture of 1 volume of *nitric acid R* and 99 volumes of *water R*.

Source: platinum hollow-cathode lamp.

Wavelength: 265.9 nm.

Heavy metals (2.4.8): maximum 50 ppm.

1.0 g complies with limit test F. Prepare the standard using 5 ml of *lead standard solution* (10 ppm Pb) *R*.

Water (2.5.12): 10.0 per cent to 17.0 per cent, determined on 0.200 g (ground to a very fine powder). Stir the substance to be examined in the titration solvent for about 15 min before titrating and use *iodosulphurous reagent R* as titrant.

Bacterial endotoxins (2.6.14): less than 0.5 IU/mg, if intended for use in the manufacture of parenteral dosage forms without a further appropriate procedure for the removal of bacterial endotoxins.

ASSAY

Calcium. Dissolve 0.400 g in 150 ml of *water R* and dilute to 300 ml with the same solvent. Carry out the complexometric titration of calcium (2.5.11).

1 ml of 0.1 M sodium edetate is equivalent to 4.008 mg of Ca.

Calcium folinate. Liquid chromatography (2.2.29) as described in the test for related substances.

Calculate the percentage content of $C_{20}H_{21}CaN_7O_7$ from the areas of the peaks in the chromatograms obtained with the test solution and reference solution (a) and the declared content of *calcium folinate CRS*.

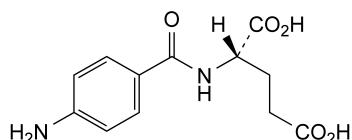
STORAGE

In an airtight container, protected from light. If the substance is sterile, store in a sterile, airtight, tamper-proof container.

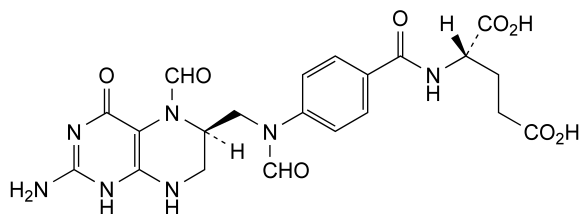
LABELLING

The label states, where applicable, that the substance is free from bacterial endotoxins.

IMPURITIES

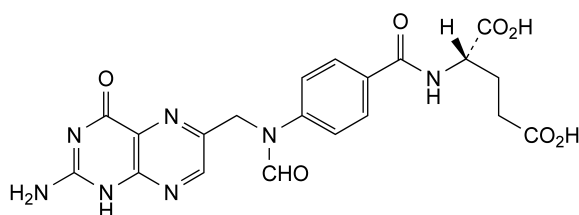


A. (2S)-2-[(4-aminobenzoyl)amino]pentanedioic acid,

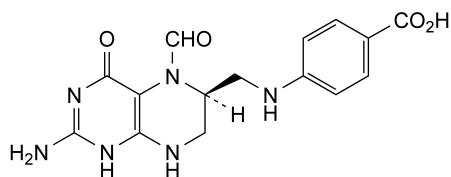


B. (2S)-2-[[4-[[[(6R)-2-amino-5-formyl-4-oxo-1,4,5,6,7,8-hexahydropteridin-6-yl]methyl]formylamino]benzoyl]amino]pentanedioic acid (5,10-diformyltetrahydrofolic acid),

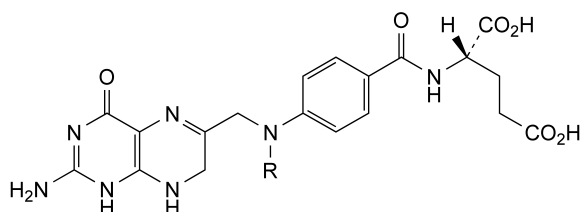
C. folic acid,



D. (2S)-2-[[4-[(2-amino-4-oxo-1,4-dihydropteridin-6-yl)methyl]formylamino]benzoyl]amino]pentanedioic acid (10-formylfolic acid),

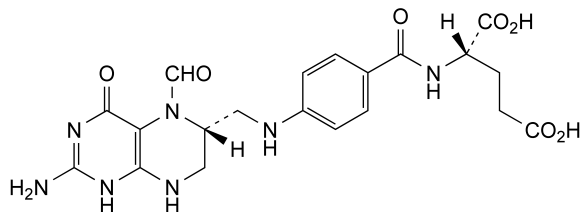


E. 4-[[[(6S)-2-amino-5-formyl-4-oxo-1,4,5,6,7,8-hexahydropteridin-6-yl]methyl]amino]benzoic acid (5-formyltetrahydropterioic acid),



F. R = CHO: (2S)-2-[[4-[[[(2-amino-4-oxo-1,4,7,8-tetrahydropteridin-6-yl)methyl]formylamino]benzoyl]amino]pentanedioic acid (10-formyldihydrofolic acid),

G. R = H: (2S)-2-[[4-[[[(2-amino-4-oxo-1,4,7,8-tetrahydropteridin-6-yl)methyl]amino]benzoyl]amino]pentanedioic acid (dihydrofolic acid),

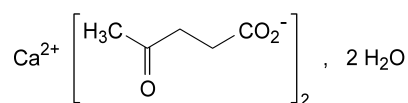


H. (2S)-2-[[4-[[[(6R)-2-amino-5-formyl-4-oxo-1,4,5,6,7,8-hexahydropteridin-6-yl]methyl]amino]benzoyl]amino]pentanedioic acid.

01/2005:1296

CALCIUM LEVULINATE DIHYDRATE

Calcii laevulinas dihydricum



$C_{10}H_{14}CaO_6 \cdot 2H_2O$

M_r 306.3

DEFINITION

Calcium levulinate dihydrate contains not less than 98.0 per cent and not more than the equivalent of 101.0 per cent of calcium di(4-oxopentanoate), calculated with reference to the dried substance.

CHARACTERS

A white or almost white, crystalline powder, freely soluble in water, very slightly soluble in alcohol, practically insoluble in methylene chloride.

IDENTIFICATION

First identification: A, D, E.

Second identification: B, C, D, E.

A. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *calcium levulinate dihydrate CRS*.

B. Examine by thin-layer chromatography (2.2.27), using a suitable silica gel as the coating substance.

Test solution. Dissolve 60 mg of the substance to be examined in *water R* and dilute to 1 ml with the same solvent.

Reference solution. Dissolve 60 mg of *calcium levulinate dihydrate CRS* in *water R* and dilute to 1 ml with the same solvent.

Apply separately to the plate 10 µl of each solution.

Develop over a path of 10 cm using a mixture of 10 volumes of *concentrated ammonia R*, 10 volumes of *ethyl acetate R*, 30 volumes of *water R* and 50 volumes

of *alcohol R*. Dry the plate at 100 °C to 105 °C for 20 min and allow to cool. Spray with a 30 g/l solution of *potassium permanganate R*. Dry the plate in a current of warm air for about 5 min or until the spots become yellow. Examine in daylight. The principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

C. To 1 ml of solution S (see Tests), add 20 ml of a 2.5 g/l solution of *dinitrophenylhydrazine R* in *dilute hydrochloric acid R*. Allow to stand for 15 min. Filter, wash the precipitate with *water R*. Dry the precipitate in an oven at 100 °C to 105 °C. The melting point (2.2.14) is 203 °C to 210 °C.

D. It gives reaction (b) of calcium (2.3.1).

E. It complies with the test for loss on drying (see Tests).

TESTS

Solution S. Dissolve 10.0 g in *carbon dioxide-free water R* prepared from *distilled water R* and dilute to 100.0 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and not more intensely coloured than reference solution Y₆ (2.2.2, *Method II*).

pH (2.2.3). The pH of solution S is 6.8 to 7.8.

Oxidisable substances. To 1 ml of solution S, add 10 ml of *water R*, 1 ml of *dilute sulphuric acid R* and 0.25 ml of a 3.0 g/l solution of *potassium permanganate R*. Mix. After 5 min, the violet colour of the mixture is still visible.

Sucrose and reducing sugars. To 5 ml of solution S, add 2 ml of *hydrochloric acid R1* and dilute to 10 ml with *water R*. Heat to boiling for 5 min and allow to cool. Add 10 ml of *sodium carbonate solution R*. Allow to stand for 5 min, dilute to 25 ml with *water R* and filter. To 5 ml of the filtrate, add 2 ml of *cupri-tartaric solution R* and heat to boiling for 1 min. No red precipitate is formed.

Chlorides (2.4.4). Dilute 10 ml of solution S to 15 ml with *water R*. The solution complies with the limit test for chlorides (50 ppm).

Sulphates (2.4.13). Dilute 7.5 ml of solution S to 15 ml with *distilled water R*. The solution complies with the limit test for sulphates (200 ppm).

Magnesium and alkali metals. To 10 ml of solution S, add 80 ml of *water R*, 10 ml of *ammonium chloride solution R* and 1 ml of *ammonia R*. Heat to boiling. To the boiling solution, add dropwise 50 ml of warm *ammonium oxalate solution R*. Allow to stand for 4 h, then dilute to 200 ml with *water R* and filter. To 100 ml of the filtrate, add 0.5 ml of *sulphuric acid R*. Evaporate to dryness on a water-bath and ignite to constant mass at 600 °C. The residue weighs not more than 5.0 mg (1.0 per cent).

Heavy metals (2.4.8). 12 ml of solution S complies with limit test A for heavy metals (10 ppm). Prepare the standard using *lead standard solution (1 ppm Pb) R*.

Loss on drying (2.2.32): 11.0 per cent to 12.5 per cent, determined on 0.200 g by drying at 100 °C to 105 °C.

Pyrogens (2.6.8). If intended for use in the manufacture of parenteral dosage forms without a further appropriate procedure for the removal of pyrogens, it complies with the test for pyrogens. Inject per kilogram of the rabbit's mass 4 ml of a solution containing per millilitre 50 mg of the substance to be examined.

ASSAY

Dissolve 0.240 g in 50 ml of *water R*. Carry out the complexometric titration of calcium (2.5.11).

1 ml of 0.1 M *sodium edetate* is equivalent to 27.03 mg of C₁₀H₁₄CaO₆.

STORAGE

Store protected from light.

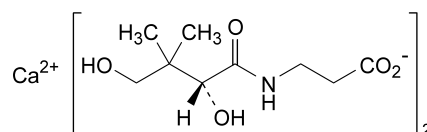
LABELLING

The label states, where applicable, that the substance is apyrogenic.

01/2005:0470

CALCIUM PANTOTHENATE

Calcii pantothenas



C₁₈H₃₂CaN₂O₁₀

M_r 476.5

DEFINITION

Calcium pantothenate contains not less than 98.0 per cent and not more than the equivalent of 101.0 per cent of calcium bis[3-[(2*R*)-2,4-dihydroxy-3,3-dimethylbutanoyl]amino]propanoate], calculated with reference to the dried substance.

CHARACTERS

A white powder, slightly hygroscopic, freely soluble in water, slightly soluble in alcohol.

IDENTIFICATION

- It complies with the test for specific optical rotation (see Tests).
- Examine the chromatograms obtained in the test for 3-aminopropionic acid. The principal spot in the chromatogram obtained with test solution (b) is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).
- To 1 ml of solution S (see Tests) add 1 ml of *dilute sodium hydroxide solution R* and 0.1 ml of *copper sulphate solution R*. A blue colour develops.
- It gives reaction (a) of calcium (2.3.1).

TESTS

Solution S. Dissolve 2.50 g in *carbon dioxide-free water R* and dilute to 50.0 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and colourless (2.2.2, *Method II*).

pH (2.2.3). The pH of solution S is 6.8 to 8.0.

Specific optical rotation (2.2.7): + 25.5 to + 27.5, determined on solution S and calculated with reference to the dried substance.

3-Aminopropionic acid. Examine by thin-layer chromatography (2.2.27), using *silica gel G R* as the coating substance.

Test solution (a). Dissolve 0.2 g of the substance to be examined in *water R* and dilute to 5 ml with the same solvent.

Test solution (b). Dilute 1 ml of test solution (a) to 10 ml with *water R*.

Reference solution (a). Dissolve 20 mg of *calcium pantothenate CRS* in *water R* and dilute to 5 ml with the same solvent.

Reference solution (b). Dissolve 10 mg of *3-aminopropionic acid R* in *water R* and dilute to 50 ml with the same solvent. Apply separately to the plate 5 µl of each solution. Develop over a path of 12 cm using a mixture of 35 volumes of *water R* and 65 volumes of *ethanol R*. Dry the plate in a current of air and spray with *ninhydrin solution R1*. Heat at 110 °C for 10 min. Any spot corresponding to 3-aminopropionic acid in the chromatogram obtained with test solution (a) is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.5 per cent).

Chlorides (2.4.4). 5 ml of solution S diluted to 15 ml with *water R* complies with the limit test for chlorides (200 ppm).

Heavy metals (2.4.8). 12 ml of solution S complies with limit test A for heavy metals (20 ppm). Prepare the standard using *lead standard solution (1 ppm Pb) R*.

Loss on drying (2.2.32). Not more than 3.0 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C.

ASSAY

Dissolve 0.180 g in 50 ml of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid* determining the end-point potentiometrically (2.2.20).

1 ml of *0.1 M perchloric acid* is equivalent to 23.83 mg of $C_{18}H_{32}CaN_2O_{10}$.

STORAGE

Store in an airtight container.

01/2005:1052

CALCIUM PHOSPHATE

Tricalcii phosphas

DEFINITION

Calcium phosphate consists of a mixture of calcium phosphates. It contains not less than 35.0 per cent and not more than the equivalent of 40.0 per cent of Ca (A_r 40.08).

CHARACTERS

A white or almost white powder, practically insoluble in water. It dissolves in dilute hydrochloric acid and in dilute nitric acid.

IDENTIFICATION

- Dissolve 0.1 g in 5 ml of a 25 per cent *V/V* solution of *nitric acid R*. The solution gives reaction (b) of phosphates (2.3.1).
- It gives reaction (b) of calcium (2.3.1). Filter before adding *potassium ferrocyanide solution R*.
- It complies with the limits of the assay.

TESTS

Solution S. Dissolve 2.50 g in 20 ml of *dilute hydrochloric acid R*. If the solution is not clear, filter it. Add *dilute ammonia R1* dropwise until a precipitate is formed. Dissolve the precipitate by adding *dilute hydrochloric acid R* and dilute to 50 ml with *distilled water R*.

Chlorides (2.4.4). Dissolve 0.22 g in a mixture of 1 ml of *nitric acid R* and 10 ml of *water R* and dilute to 100 ml with *water R*. 15 ml of the solution complies with the limit test for chlorides (0.15 per cent).

Fluorides. Not more than 75 ppm of F, determined potentiometrically (2.2.36, *Method I*) using a fluoride-selective indicator electrode and a silver-silver chloride reference electrode.

Test solution. In a 50 ml volumetric flask, dissolve 0.250 g in *0.1 M hydrochloric acid*, add 5.0 ml of *fluoride standard solution (1 ppm F) R* and dilute to 50.0 ml with *0.1 M hydrochloric acid*. To 20.0 ml of the solution add 20.0 ml of *total-ionic-strength-adjustment buffer R* and 3 ml of an 82 g/l solution of *anhydrous sodium acetate R*. Adjust to pH 5.2 with *ammonia R* and dilute to 50.0 ml with *distilled water R*.

Reference solutions. To 5.0 ml, 2.0 ml, 1.0 ml, 0.5 ml and 0.25 ml of *fluoride standard solution (10 ppm F) R* add 20.0 ml of *total-ionic-strength-adjustment buffer R* and dilute to 50.0 ml with *distilled water R*.

Carry out the measurements on 20.0 ml of each solution. Calculate the concentration of fluorides using the calibration curve, taking into account the addition of fluoride to the test solution.

Sulphates (2.4.13). Dilute 1 ml of solution S to 25 ml with *distilled water R*. 15 ml of the solution complies with the limit test for sulphates (0.5 per cent).

Arsenic (2.4.2). 5 ml of solution S complies with limit test A for arsenic (4 ppm).

Iron (2.4.9). Dilute 0.5 ml of solution S to 10 ml with *water R*. The solution complies with the limit test for iron (400 ppm).

Heavy metals (2.4.8). Dilute 13 ml of solution S to 20 ml with *water R*. 12 ml of the solution complies with limit test A for heavy metals (30 ppm). Prepare the standard using *lead standard solution (1 ppm Pb) R*.

Acid-insoluble matter. Dissolve 5.0 g in a mixture of 10 ml of *hydrochloric acid R* and 30 ml of *water R*. Filter, wash the residue with *water R* and dry to constant mass at 100 °C to 105 °C. The residue weighs not more than 10 mg (0.2 per cent).

Loss on ignition. Not more than 8.0 per cent, determined on 1.000 g by ignition at 800 °C for 30 min.

ASSAY

Dissolve 0.200 g in a mixture of 1 ml of *hydrochloric acid R1* and 5 ml of *water R*. Add 25.0 ml of *0.1 M sodium edetate* and dilute to 200 ml with *water R*. Adjust to about pH 10 with *concentrated ammonia R*. Add 10 ml of *ammonium chloride buffer solution pH 10.0 R* and a few milligrams of *mordant black 11 triturate R*. Titrate the excess sodium edetate with *0.1 M zinc sulphate* until the colour changes from blue to violet.

1 ml of *0.1 M sodium edetate* is equivalent to 4.008 mg of Ca.

01/2005:0882

CALCIUM STEARATE

Calcii stearas

DEFINITION

Calcium stearate is a mixture of calcium salts of different fatty acids consisting mainly of stearic acid [$(C_{17}H_{35}COO)_2Ca$; M_r 607] and palmitic acid [$(C_{15}H_{31}COO)_2Ca$; M_r 550.9] with minor proportions of other fatty acids. It contains not less than 6.4 per cent and not more than 7.4 per cent of Ca (A_r 40.08), calculated with reference to the dried substance. The

fatty acid fraction contains not less than 40.0 per cent of stearic acid and the sum of stearic acid and palmitic acid is not less than 90.0 per cent.

CHARACTERS

A fine, white or almost white, crystalline powder, practically insoluble in water and in alcohol.

IDENTIFICATION

First identification: C, D.

Second identification: A, B, D.

- The residue obtained in the preparation of solution S (see Tests) has a freezing point (2.2.18) not lower than 53 °C.
- The acid value of the fatty acids (2.5.1) is 195 to 210, determined on 0.200 g of the residue obtained in the preparation of solution S dissolved in 25 ml of the prescribed mixture of solvents.
- Examine the chromatograms obtained in the test for fatty acid composition. The retention times of the principal peaks in the chromatogram obtained with the test solution are approximately the same as those of the principal peaks in the chromatogram obtained with the reference solution.
- Neutralise 5 ml of solution S to *red litmus paper R* using *strong sodium hydroxide solution R*. The solution gives reaction (b) of calcium (2.3.1).

TESTS

Solution S. To 5.0 g add 50 ml of *peroxide-free ether R*, 20 ml of *dilute nitric acid R* and 20 ml of *distilled water R*. Boil under a reflux condenser until dissolution is complete. Allow to cool. In a separating funnel, separate the aqueous layer and shake the ether layer with 2 quantities, each of 5 ml, of *distilled water R*. Combine the aqueous layers, wash with 15 ml of *peroxide-free ether R* and dilute the aqueous layer to 50 ml with *distilled water R* (solution S). Evaporate the ether layer to dryness and dry the residue at 100-105 °C. Keep the residue for identification tests A and B.

Acidity or alkalinity. To 1.0 g add 20 ml of *carbon dioxide-free water R* and boil for 1 min with continuous shaking. Cool and filter. To 10 ml of the filtrate add 0.05 ml of *bromothymol blue solution R1*. Not more than 0.5 ml of 0.01 M *hydrochloric acid* or 0.01 M *sodium hydroxide* is required to change the colour of the indicator.

Chlorides (2.4.4). Dilute 0.5 ml of solution S to 15 ml with *water R*. The solution complies with the limit test for chlorides (0.1 per cent).

Sulphates (2.4.13). Dilute 0.5 ml of solution S to 15 ml with *distilled water R*. The solution complies with the limit test for sulphates (0.3 per cent).

Cadmium. Not more than 3 ppm of Cd, determined by atomic absorption spectrometry (2.2.23, *Method II*).

Test solution. Place 50.0 mg of the substance to be examined in a polytetrafluoroethylene digestion bomb and add 0.5 ml of a mixture of 1 volume of *hydrochloric acid R* and 5 volumes of *cadmium- and lead-free nitric acid R*. Allow to digest at 170 °C for 5 h. Allow to cool. Dissolve the residue in *water R* and dilute to 5.0 ml with the same solvent.

Reference solutions. Prepare the reference solutions using *cadmium standard solution (10 ppm Cd) R*, diluted if necessary with a 1 per cent V/V solution of *hydrochloric acid R*.

Measure the absorbance at 228.8 nm using a cadmium hollow-cathode lamp as source of radiation and a graphite furnace as atomic generator.

Lead. Not more than 10 ppm of Pb, determined by atomic absorption spectrometry (2.2.23, *Method II*).

Test solution. Use the solution described in the test for cadmium.

Reference solutions. Prepare the reference solutions using *lead standard solution (10 ppm Pb) R*, diluted if necessary with *water R*.

Measure the absorbance at 283.3 nm using a lead hollow-cathode lamp as source of radiation and a graphite furnace as atomic generator. Depending on the apparatus, the line at 217.0 nm may be used.

Nickel. Not more than 5 ppm of Ni, determined by atomic absorption spectrometry (2.2.23, *Method II*).

Test solution. Use the solution described in the test for cadmium.

Reference solutions. Prepare the reference solutions using *nickel standard solution (10 ppm Ni) R*, diluted if necessary with *water R*.

Measure the absorbance at 232.0 nm using a nickel hollow-cathode lamp as source of radiation and a graphite furnace as atomic generator.

Loss on drying (2.2.32). Not more than 6.0 per cent, determined on 1.000 g by drying in an oven at 100-105 °C.

Microbial contamination. Total viable aerobic count (2.6.12) not more than 10³ micro-organisms per gram, determined by plate count. It complies with the test for *Escherichia coli* (2.6.13).

ASSAY

Calcium. To 0.500 g in a 250 ml conical flask add 50 ml of a mixture of equal volumes of *butanol R* and *ethanol R*, 5 ml of *concentrated ammonia R*, 3 ml of *ammonium chloride buffer solution pH 10.0 R*, 30.0 ml of 0.1 M *sodium edetate* and 15 mg of *mordant black 11 triturate R*. Heat to 45-50 °C until the solution is clear. Cool and titrate with 0.1 M *zinc sulphate* until the colour changes from blue to violet. Carry out a blank titration.

1 ml of 0.1 M *sodium edetate* is equivalent to 4.008 mg of Ca.

Fatty acid composition. Examine by gas chromatography (2.2.28).

Test solution. In a conical flask fitted with a reflux condenser, dissolve 0.10 g of the substance to be examined in 5 ml of *boron trifluoride-methanol solution R*. Boil under a reflux condenser for 10 min. Add 4 ml of *heptane R* through the condenser and boil again under a reflux condenser for 10 min. Allow to cool. Add 20 ml of a *saturated sodium chloride solution R*. Shake and allow the layers to separate. Remove about 2 ml of the organic layer and dry over 0.2 g of *anhydrous sodium sulphate R*. Dilute 1.0 ml of the solution to 10.0 ml with *heptane R*.

Reference solution. Prepare the reference solution in the same manner as the test solution using 50.0 mg of *palmitic acid CRS* and 50.0 mg of *stearic acid CRS* instead of calcium stearate.

The chromatographic procedure may be carried out using:

- a fused-silica column 30 m long and 0.32 mm in internal diameter coated with *macrogol 20 000 R* (film thickness 0.5 µm),
- helium for chromatography R* as the carrier gas at a flow rate of 2.4 ml/min,

— a flame-ionisation detector,
with the following temperature programme:

	Time (min)	Temperature (°C)	Rate (°C/min)	Comment
Column	0 - 2	70	-	isothermal
	2 - 36	70 → 240	5	linear gradient
	36 - 41	240	-	isothermal
Injection port		220		
Detector		260		

Inject 1 µl of the reference solution. When the chromatogram is recorded in the prescribed conditions, the retention time of methyl palmitate relative to that of methyl stearate is about 0.88. The test is not valid unless, in the chromatogram obtained with the reference solution, the resolution between the peaks corresponding to methyl stearate and methyl palmitate is at least 5.0.

Inject 1 µl of the test solution. Calculate the percentage content of stearic acid and palmitic acid from the areas of the peaks in the chromatogram obtained with the test solution by the normalisation procedure, disregarding the peak due to the solvent.

Iron (2.4.9). To 0.25 g add a mixture of 5 ml of *hydrochloric acid R* and 20 ml of *water R*. Heat to boiling, cool and filter. 10 ml of the filtrate complies with the limit test for iron (100 ppm).

Heavy metals (2.4.8). To 2.5 g add a mixture of 2 ml of *hydrochloric acid R* and 15 ml of *water R*. Heat to boiling. Cool and then add 0.5 ml of *phenolphthalein solution R*. Cautiously add *concentrated ammonia R* until the colour changes to pink. Add 0.5 ml of *glacial acetic acid R* and dilute to 25 ml with *water R*. Filter. 12 ml of the filtrate complies with limit test A for heavy metals (20 ppm). Prepare the standard using *lead standard solution (2 ppm Pb) R*.

Loss on ignition: 18.0 per cent to 22.0 per cent, determined on 1.000 g by ignition to constant mass at 800 °C.

ASSAY

Dissolve 0.150 g in 120 ml of *water R*. Carry out the complexometric titration of calcium (2.5.11).

1 ml of 0.1 M *sodium edetate* is equivalent to 17.22 mg of $\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$.

01/2005:1297

CALENDULA FLOWER

Calendulae flos

DEFINITION

Calendula flower consists of the whole or cut, dried, and fully opened flowers which have been detached from the receptacle of the cultivated, double-flowered varieties of *Calendula officinalis* L. It contains not less than 0.4 per cent of flavonoids, calculated as hyperoside ($\text{C}_{21}\text{H}_{20}\text{O}_{12}$, M_r 464.4) with reference to the dried drug.

CHARACTERS

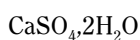
It has the macroscopic and microscopic characters described under identification tests A and B.

IDENTIFICATION

- The ligulate florets consist of a yellow or orange-yellow ligule, about 3 mm to 5 mm wide and about 7 mm in the middle part, with a three toothed apex and a hairy, partly sickle-shaped yellowish-brown to orange-brown tube with a projecting style and a bifid stigma occasionally with a partly bent yellowish-brown to orange-brown ovary. The tubular florets, about 5 mm long, are present and consist of the yellow, orange-red or red-violet five lobed corolla and the yellowish-brown or orange-brown tube, hairy in its lower part, mostly with a partly bent yellowish-brown to orange-brown ovary.
- Reduce to a powder (355). The powder is yellowish-brown. Examine under a microscope using *chloral hydrate solution R*. The powder shows fragments of the corollas containing light yellow oil droplets, some with fairly large anomocytic stomata (2.8.3), others containing prisms and very small cluster crystals of calcium oxalate; covering trichomes biseriate, multicellular and conical, glandular trichomes with a uniseriate or biseriate, multicellular biseriate stalk and a large, ovoid, biseriate and multicellular head; spherical pollen grains up to about 40 µm in diameter with a sharply spiny exine and three germinal pores; occasional fragments of the stigmas with short, bulbous papillae.
- Examine by thin-layer chromatography (2.2.27), using a suitable silica gel as the coating substance.

CALCIUM SULPHATE DIHYDRATE

Calcii sulfas dihydricus


 M_r 172.2

DEFINITION

Calcium sulphate dihydrate contains not less than 98.0 per cent and not more than the equivalent of 102.0 per cent of $\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$.

CHARACTERS

A white, fine powder, very slightly soluble in water, practically insoluble in alcohol.

IDENTIFICATION

- It complies with the test for loss on ignition (see Tests).
- Solution S (see Tests) gives reaction (a) of sulphates (2.3.1).
- Solution S gives reaction (a) of calcium (2.3.1).

TESTS

Solution S. Dissolve 1.0 g in 50 ml of a 10 per cent V/V solution of *hydrochloric acid R* by heating at 50 °C for 5 min. Allow the solution to cool.

Acidity or alkalinity. Shake 1.5 g with 15 ml of *carbon dioxide-free water R* for 5 min. Allow to stand for 5 min and filter. To 10 ml of the filtrate, add 0.1 ml of *phenolphthalein solution R* and 0.25 ml of 0.01 M *sodium hydroxide*. The solution is red. Add 0.30 ml of 0.01 M *hydrochloric acid*. The solution is colourless. Add 0.2 ml of *methyl red solution R*. The solution is reddish-orange.

Chlorides (2.4.4). Shake 0.5 g with 15 ml of *water R* for 5 min. Allow to stand for 15 min and filter. Dilute 5 ml of the filtrate to 15 ml with *water R*. The solution complies with the limit test for chlorides (300 ppm).

Arsenic (2.4.2). 5 ml of solution S complies with limit test A for arsenic (10 ppm).

Test solution. To 1.0 g of the powdered drug (500) add 10 ml of *methanol R* and heat on a water-bath under a reflux condenser for 10 min. Cool and filter.

Reference solution. Dissolve 1.0 mg of *caffeic acid R*, 1.0 mg of *chlorogenic acid R* and 2.5 mg of *rutin R* in 10 ml of *methanol R*.

Apply to the plate, as bands, 20 µl of the test solution and 10 µl of the reference solution. Develop over a path of 10 cm using a mixture of 10 volumes of *anhydrous formic acid R*, 10 volumes of *water R* and 80 volumes of *ethyl acetate R*. Allow the plate to dry at 100 °C to 105 °C and spray the still warm plate with a 10 g/l solution of *diphenylboric acid aminoethyl ester R* in *methanol R* and then spray with a 50 g/l solution of *macrogol 400 R* in *methanol R*. Allow the plate to dry in air for 30 min and examine in ultraviolet light at 365 nm. The chromatogram obtained with the reference solution shows in the lower part a yellowish-brown fluorescent zone (rutin), in the middle part a light bluish fluorescent zone (chlorogenic acid) and in the upper part a light bluish fluorescent zone (caffeic acid). The chromatogram obtained with the test solution shows a yellowish-brown fluorescent zone corresponding in position to the zone due to rutin in the chromatogram obtained with the reference solution, below and directly above it, it shows a yellowish-green fluorescent zone and a light bluish fluorescent zone corresponding to the zone due to chlorogenic acid in the chromatogram obtained with the reference solution, a yellowish-green fluorescent zone above it and a light bluish fluorescent zone shortly below the zone due to caffeic acid in the chromatogram obtained with the reference solution. Further zones are present.

TESTS

Foreign matter (2.8.2). Not more than 5 per cent of bracts and not more than 2 per cent of other foreign matter.

Loss on drying (2.2.32). Not more than 12.0 per cent, determined on 1.000 g of the powdered drug (500) by drying in an oven at 100 °C to 105 °C for 2 h.

Total ash (2.4.16). Not more than 10.0 per cent.

ASSAY

Stock solution. In a 100 ml round-bottomed flask introduce 0.800 g of the powdered drug (500), 1 ml of a 5 g/l solution of *hexamethylenetetramine R*, 20 ml of *acetone R* and 7 ml of *hydrochloric acid R1*. Boil the mixture under a reflux condenser for 30 min. Filter the liquid through a plug of absorbent cotton in a 100 ml flask. Add the absorbent cotton to the residue in the round-bottomed flask and extract with two quantities, each of 20 ml, of *acetone R*, each time boiling under a reflux condenser for 10 min. Allow to cool to room temperature, filter the liquid through a plug of absorbent cotton then filter the combined acetone solution through a filter-paper in the volumetric flask and dilute to 100.0 ml with *acetone R* by rinsing of the flask and the filter. Introduce 20.0 ml of the solution into a separating funnel, add 20 ml of *water R* and extract the mixture with one quantity of 15 ml and then three quantities, each of 10 ml, of *ethyl acetate R*. Combine the ethyl acetate extracts in a separating funnel, rinse with two quantities, each of 50 ml, of *water R*, filter the extract over 10 g of *anhydrous sodium sulphate R* in to a 50 ml volumetric flask and dilute to 50.0 ml with *ethyl acetate R*.

Test solution. To 10.0 ml of the stock solution add 1 ml of *aluminium chloride reagent R* and dilute to 25.0 ml with a 5 per cent V/V solution of *glacial acetic acid R* in *methanol R*.

Compensation solution. Dilute 10.0 ml of the stock solution to 25.0 ml with a 5 per cent V/V solution of *glacial acetic acid R* in *methanol R*.

Measure the absorbance (2.2.25) of the test solution after 30 min, by comparison with the compensation solution at 425 nm.

Calculate the percentage content of flavonoids, calculated as hyperoside, from the expression:

$$\frac{A \times 1.25}{m}$$

i.e. taking the specific absorbance of hyperoside to be 500.

A = absorbance at 425 nm,

m = mass of the substance to be examined in grams.

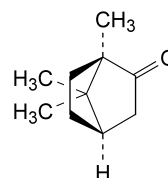
STORAGE

Store protected from light.

01/2005:1400

D-CAMPHOR

D-Camphora



$C_{10}H_{16}O$

M_r 152.2

DEFINITION

(1*R*,4*R*)-1,7,7-Trimethylbicyclo[2.2.1]heptan-2-one.

CHARACTERS

Appearance: white, crystalline powder or friable, crystalline masses.

Highly volatile even at room temperature.

Solubility: slightly soluble in water, very soluble in alcohol and in light petroleum, freely soluble in fatty oils, very slightly soluble in glycerol.

IDENTIFICATION

First identification: A, C.

Second identification: A, B, D.

A. Specific optical rotation (see Tests).

B. Melting point (2.2.14): 175 °C to 179 °C.

C. Infrared absorption spectrophotometry (2.2.24).

Comparison: *racemic camphor CRS*.

D. Dissolve 1.0 g in 30 ml of *methanol R*. Add 1.0 g of *hydroxylamine hydrochloride R* and 1.0 g of *anhydrous sodium acetate R*. Boil under a reflux condenser for 2 h. Allow to cool and add 100 ml of *water R*. Filter, wash the precipitate obtained with 10 ml of *water R* and recrystallise from 10 ml of a mixture of 4 volumes of *alcohol R* and 6 volumes of *water R*. The crystals, dried *in vacuo*, melt (2.2.14) at 118 °C to 121 °C.

TESTS

Carry out the weighings and dissolution rapidly.

Solution S. Dissolve 2.50 g in 10 ml of *alcohol R* and dilute to 25.0 ml with the same solvent.

Test solution. To 1.0 g of the powdered drug (500) add 10 ml of *methanol R* and heat on a water-bath under a reflux condenser for 10 min. Cool and filter.

Reference solution. Dissolve 1.0 mg of *caffeic acid R*, 1.0 mg of *chlorogenic acid R* and 2.5 mg of *rutin R* in 10 ml of *methanol R*.

Apply to the plate, as bands, 20 µl of the test solution and 10 µl of the reference solution. Develop over a path of 10 cm using a mixture of 10 volumes of *anhydrous formic acid R*, 10 volumes of *water R* and 80 volumes of *ethyl acetate R*. Allow the plate to dry at 100 °C to 105 °C and spray the still warm plate with a 10 g/l solution of *diphenylboric acid aminoethyl ester R* in *methanol R* and then spray with a 50 g/l solution of *macrogol 400 R* in *methanol R*. Allow the plate to dry in air for 30 min and examine in ultraviolet light at 365 nm. The chromatogram obtained with the reference solution shows in the lower part a yellowish-brown fluorescent zone (rutin), in the middle part a light bluish fluorescent zone (chlorogenic acid) and in the upper part a light bluish fluorescent zone (caffeic acid). The chromatogram obtained with the test solution shows a yellowish-brown fluorescent zone corresponding in position to the zone due to rutin in the chromatogram obtained with the reference solution, below and directly above it, it shows a yellowish-green fluorescent zone and a light bluish fluorescent zone corresponding to the zone due to chlorogenic acid in the chromatogram obtained with the reference solution, a yellowish-green fluorescent zone above it and a light bluish fluorescent zone shortly below the zone due to caffeic acid in the chromatogram obtained with the reference solution. Further zones are present.

TESTS

Foreign matter (2.8.2). Not more than 5 per cent of bracts and not more than 2 per cent of other foreign matter.

Loss on drying (2.2.32). Not more than 12.0 per cent, determined on 1.000 g of the powdered drug (500) by drying in an oven at 100 °C to 105 °C for 2 h.

Total ash (2.4.16). Not more than 10.0 per cent.

ASSAY

Stock solution. In a 100 ml round-bottomed flask introduce 0.800 g of the powdered drug (500), 1 ml of a 5 g/l solution of *hexamethylenetetramine R*, 20 ml of *acetone R* and 7 ml of *hydrochloric acid R1*. Boil the mixture under a reflux condenser for 30 min. Filter the liquid through a plug of absorbent cotton in a 100 ml flask. Add the absorbent cotton to the residue in the round-bottomed flask and extract with two quantities, each of 20 ml, of *acetone R*, each time boiling under a reflux condenser for 10 min. Allow to cool to room temperature, filter the liquid through a plug of absorbent cotton then filter the combined acetone solution through a filter-paper in the volumetric flask and dilute to 100.0 ml with *acetone R* by rinsing of the flask and the filter. Introduce 20.0 ml of the solution into a separating funnel, add 20 ml of *water R* and extract the mixture with one quantity of 15 ml and then three quantities, each of 10 ml, of *ethyl acetate R*. Combine the ethyl acetate extracts in a separating funnel, rinse with two quantities, each of 50 ml, of *water R*, filter the extract over 10 g of *anhydrous sodium sulphate R* in to a 50 ml volumetric flask and dilute to 50.0 ml with *ethyl acetate R*.

Test solution. To 10.0 ml of the stock solution add 1 ml of *aluminium chloride reagent R* and dilute to 25.0 ml with a 5 per cent V/V solution of *glacial acetic acid R* in *methanol R*.

Compensation solution. Dilute 10.0 ml of the stock solution to 25.0 ml with a 5 per cent V/V solution of *glacial acetic acid R* in *methanol R*.

Measure the absorbance (2.2.25) of the test solution after 30 min, by comparison with the compensation solution at 425 nm.

Calculate the percentage content of flavonoids, calculated as hyperoside, from the expression:

$$\frac{A \times 1.25}{m}$$

i.e. taking the specific absorbance of hyperoside to be 500.

A = absorbance at 425 nm,

m = mass of the substance to be examined in grams.

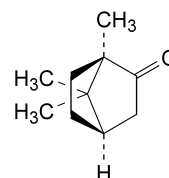
STORAGE

Store protected from light.

01/2005:1400

D-CAMPHOR

D-Camphora



$C_{10}H_{16}O$

M_r 152.2

DEFINITION

(1*R*,4*R*)-1,7,7-Trimethylbicyclo[2.2.1]heptan-2-one.

CHARACTERS

Appearance: white, crystalline powder or friable, crystalline masses.

Highly volatile even at room temperature.

Solubility: slightly soluble in water, very soluble in alcohol and in light petroleum, freely soluble in fatty oils, very slightly soluble in glycerol.

IDENTIFICATION

First identification: A, C.

Second identification: A, B, D.

A. Specific optical rotation (see Tests).

B. Melting point (2.2.14): 175 °C to 179 °C.

C. Infrared absorption spectrophotometry (2.2.24).

Comparison: *racemic camphor CRS*.

D. Dissolve 1.0 g in 30 ml of *methanol R*. Add 1.0 g of *hydroxylamine hydrochloride R* and 1.0 g of *anhydrous sodium acetate R*. Boil under a reflux condenser for 2 h. Allow to cool and add 100 ml of *water R*. Filter, wash the precipitate obtained with 10 ml of *water R* and recrystallise from 10 ml of a mixture of 4 volumes of *alcohol R* and 6 volumes of *water R*. The crystals, dried *in vacuo*, melt (2.2.14) at 118 °C to 121 °C.

TESTS

Carry out the weighings and dissolution rapidly.

Solution S. Dissolve 2.50 g in 10 ml of *alcohol R* and dilute to 25.0 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and colourless (2.2.2, Method II).

Acidity or alkalinity. To 10 ml of solution S add 0.1 ml of *phenolphthalein solution R1*. The solution is colourless. Not more than 0.2 ml of 0.1 M sodium hydroxide is required to change the colour of the indicator.

Specific optical rotation (2.2.7): + 40.0 to + 43.0, determined on solution S.

Related substances. Gas chromatography (2.2.28).

Test solution. Dissolve 2.50 g of the substance to be examined in *heptane R* and dilute to 25.0 ml with the same solvent.

Reference solution (a). Dilute 1.0 ml of the test solution to 100.0 ml with *heptane R*.

Reference solution (b). Dilute 10.0 ml of reference solution (a) to 20.0 ml with *heptane R*.

Reference solution (c). Dissolve 0.50 g of *borneol R* in *heptane R* and dilute to 25.0 ml with the same solvent. Dilute 5.0 ml of the solution to 50.0 ml with *heptane R*.

Reference solution (d). Dissolve 50 mg of *linalol R* and 50 mg of *bornyl acetate R* in *heptane R* and dilute to 100.0 ml with the same solvent.

Column:

- size: $l = 30$ m, $\varnothing = 0.25$ mm,
- stationary phase: *macrogol 20 000 R* (0.25 μ m).

Carrier gas: *helium for chromatography R*.

Split ratio: 1:70.

Flow rate: 45 cm/s.

Temperature:

	Time (min)	Temperature (°C)
Column	0 - 10	50
	10 - 35	50 → 100
	35 - 45	100 → 200
	45 - 55	200
Injection port		220
Detector		250

Detection: flame ionisation.

Injection: 1 μ l.

System suitability: reference solution (d).

- resolution: minimum 3.0 between the peaks due to *bornyl acetate* and to *linalol*.

Limits:

- *borneol*: not more than the area of the principal peak in the chromatogram obtained with reference solution (c) (2.0 per cent),
- any other impurity: not more than half of the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent),
- total of other impurities: not more than 4 times the area of the principal peak in the chromatogram obtained with reference solution (a) (4.0 per cent),
- disregard limit: 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

Halogens: maximum 100 ppm.

Dissolve 1.0 g in 10 ml of *2-propanol R* in a distillation flask. Add 1.5 ml of *dilute sodium hydroxide solution R* and 50 mg of *nickel-aluminium alloy R*. Heat on a water-bath until the

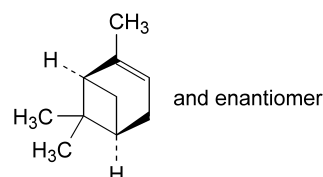
2-propanol R has evaporated. Allow to cool and add 5 ml of *water R*. Mix and filter through a wet filter previously washed with *water R* until free from chlorides. Dilute the filtrate to 10.0 ml with *water R*. To 5.0 ml of the solution, add *nitric acid R* dropwise until the precipitate which forms is redissolved and dilute to 15 ml with *water R*. The solution complies with the limit test for chlorides (2.4.4).

Residue on evaporation (2.8.9): maximum 0.05 per cent.

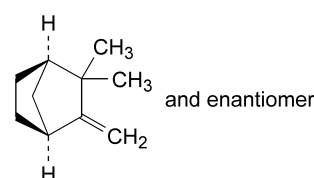
Evaporate 2.0 g on a water-bath and dry in an oven at 100-105 °C for 1 h. The residue weighs a maximum of 1 mg.

Water. Dissolve 1 g in 10 ml of *light petroleum R*. The solution is clear (2.2.1).

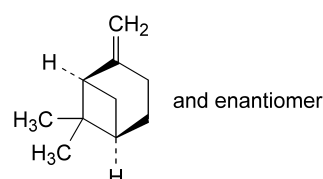
IMPURITIES



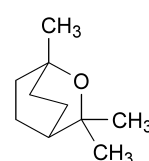
A. 2,6,6-trimethylbicyclo[3.1.1]hept-2-ene (α -pinene),



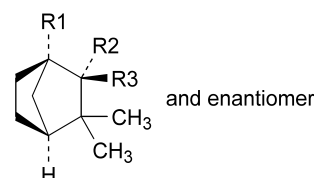
B. 2,2-dimethyl-3-methylenebicyclo[2.2.1]heptane (camphene),



C. 6,6-dimethyl-2-methylenebicyclo[3.1.1]heptane (β -pinene),



D. 3,3-dimethyl-2-oxabicyclo[2.2.2]octane (cineole),

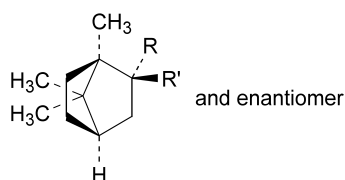


E. R1 = CH₃, R2 + R3 = O: 1,3,3-trimethylbicyclo[2.2.1]heptan-2-one (fenchone),

F. R1 = CH₃, R2 = OH, R3 = H: *exo*-1,3,3-trimethylbicyclo[2.2.1]heptan-2-ol (fenchol),

G. R1 = H, R2 = OH, R3 = CH₃: *exo*-2,3,3-trimethylbicyclo[2.2.1]heptan-2-ol (camphene hydrate),

H. R1 = H, R2 = CH₃, R3 = OH: *endo*-2,3,3-trimethylbicyclo[2.2.1]heptan-2-ol (methylcamphenilol),

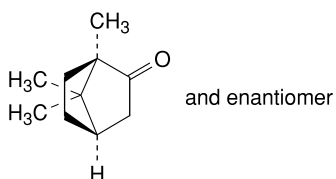


- I. R = OH, R' = H: *exo*-1,7,7-trimethylbicyclo[2.2.1]heptan-2-ol (*exo*-borneol),
 J. R = H, R' = OH: *endo*-1,7,7-trimethylbicyclo[2.2.1]heptan-2-ol (*endo*-borneol).

01/2005:0655

CAMPHOR, RACEMIC

Camphora racemica

C₁₀H₁₆OM_r 152.2

DEFINITION

Racemic camphor is (1*RS*,4*RS*)-1,7,7-trimethylbicyclo[2.2.1]heptan-2-one.

CHARACTERS

A white, crystalline powder or friable, crystalline masses, highly volatile even at room temperature, slightly soluble in water, very soluble in alcohol and in light petroleum, freely soluble in fatty oils, very slightly soluble in glycerol.

IDENTIFICATION

First identification: A, C.

Second identification: A, B, D.

- A. It complies with the test for optical rotation (see Tests).
 B. Melting point (2.2.14): 172 °C to 180 °C.
 C. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *racemic camphor CRS*. Examine the substances as mulls in *liquid paraffin R*.
 D. Dissolve 1.0 g in 30 ml of *methanol R*. Add 1.0 g of *hydroxylamine hydrochloride R* and 1.0 g of *anhydrous sodium acetate R*. Boil under a reflux condenser for 2 h. Allow to cool and add 100 ml of *water R*. A precipitate is formed. Filter, wash with 10 ml of *water R* and recrystallize from 10 ml of a mixture of 4 volumes of *alcohol R* and 6 volumes of *water R*. The crystals, dried *in vacuo*, melt (2.2.14) at 118 °C to 121 °C.

TESTS

Carry out the weighings rapidly.

Solution S. Dissolve 2.50 g in 10 ml of *alcohol R* and dilute to 25.0 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and colourless (2.2.2, *Method II*).

Acidity or alkalinity. Dissolve 1.0 g in 10 ml of *alcohol R* and add 0.1 ml of *phenolphthalein solution R1*. The solution is colourless. Not more than 0.2 ml of 0.1 M *sodium hydroxide* is required to change the colour of the indicator.

Optical rotation (2.2.7): +0.15° to −0.15°, determined on solution S.

Related substances. Examine by gas chromatography (2.2.28).

Test solution. Dissolve 50 mg of the substance to be examined in *hexane R* and dilute to 50.0 ml with the same solvent.

Reference solution (a). Dissolve 50 mg of the substance to be examined and 50 mg of *bornyl acetate R* in *hexane R* and dilute to 50.0 ml with the same solvent.

Reference solution (b). Dilute 1.0 ml of the test solution to 200.0 ml with *hexane R*.

The chromatographic procedure may be carried out using:

- a column 2 m long and 2 mm in internal diameter packed with *diatomaceous earth for gas chromatography R* impregnated with 10 per cent *m/m* of *macrogol 20 000 R*,
- *nitrogen for chromatography R* as the carrier gas at a flow rate of 30 ml/min,
- a flame-ionisation detector,

maintaining the temperature of the column at 130 °C and that of the injection port and the detector at 200 °C.

Inject 1 µl of each solution and adjust the sensitivity of the system so that the height of the principal peak in the chromatogram obtained with the test solution is about 80 per cent of the full scale of the recorder. Record the chromatograms for three times the retention time of camphor.

The test is not valid unless in the chromatogram obtained with reference solution (a), the resolution between the peaks corresponding to camphor and bornyl acetate is not less than 1.5; in the chromatogram obtained with reference solution (b), the principal peak has a signal-to-noise ratio of at least 5. In the chromatogram obtained with the test solution: the sum of the areas of the peaks, apart from the principal peak, is not greater than 4 per cent of the area of the principal peak; none of the peaks, apart from the principal peak, has an area greater than 2 per cent of the area of the principal peak. Disregard any peak with an area less than that of the peak in the chromatogram obtained with reference solution (b).

Halogens. Dissolve 1.0 g in 10 ml of *2-propanol R* in a distillation flask. Add 1.5 ml of *dilute sodium hydroxide solution R* and 50 mg of *nickel-aluminium alloy R*. Heat on a water-bath until the *2-propanol R* has evaporated. Allow to cool and add 5 ml of *water R*. Mix and filter through a wet filter previously washed with *water R* until free from chlorides. Dilute the filtrate to 10.0 ml with *water R*. To 5.0 ml of the solution, add *nitric acid R* dropwise until the precipitate which forms is redissolved and dilute to 15 ml with *water R*. The solution complies with the limit test for chlorides (2.4.4) (100 ppm).

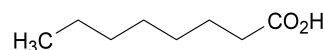
Water. Dissolve 1 g in 10 ml of *light petroleum R*. The solution is clear (2.2.1).

Residue on evaporation. Evaporate 2.0 g on a water-bath and dry at 100 °C to 105 °C for 1 h. The residue weighs not more than 1 mg (0.05 per cent).

01/2005:1401

CAPRYLIC ACID

Acidum caprylicum

C₈H₁₆O₂M_r 144.2

DEFINITION

Caprylic acid contains not less than 99.0 per cent and not more than the equivalent of 100.5 per cent of octanoic acid, calculated with reference to the anhydrous substance.

CHARACTERS

A clear, colourless or slightly yellowish, oily liquid, very slightly soluble in water, very soluble in acetone and in alcohol. It dissolves in dilute solutions of alkali hydroxides.

IDENTIFICATION

- A. It complies with the test for relative density (see Tests).
 B. Examine the chromatograms obtained in the test for related substances. The retention time and size of the principal peak in the chromatogram obtained with the test solution are approximately the same as those of the principal peak in the chromatogram obtained with reference solution (a).

TESTS

Appearance. The substance to be examined is clear (2.2.1) and not more intensely coloured than reference solution Y₅ (2.2.2, Method II).

Relative density (2.2.5): 0.909 to 0.912.

Related substances. Examine by gas chromatography (2.2.28).

Test solution. Dissolve 0.10 g of the substance to be examined in *ethyl acetate R* and dilute to 10.0 ml with the same solvent.

Reference solution (a). Dissolve 0.10 g of *caprylic acid CRS* in *ethyl acetate R* and dilute to 10.0 ml with the same solvent.

Reference solution (b). Dilute 1.0 ml of the test solution to 100.0 ml with *ethyl acetate R*. Dilute 5.0 ml of the solution to 50.0 ml with *ethyl acetate R*.

The chromatographic procedure may be carried out using:

- a fused-silica column 30 m long and 0.25 mm in internal diameter coated with *macrogol 20 000 2-nitroterephthalate R* (film thickness 0.25 µm),
- *helium for chromatography R* as the carrier gas at a flow rate of 1.5 ml/min,
- a flame-ionisation detector,
- a split ratio of 1:100,

with the following temperature programme:

	Time (min)	Temperature (°C)	Rate (°C/min)	Comment
Column	0 - 1	100	–	isothermal
	1 - 25	100 → 220	5	linear gradient
	25 - 35	220	–	isothermal
Injection port		250		
Detector		250		

Inject 1 µl of reference solution (b). The test is not valid unless in the chromatogram obtained the principal peak has a signal-to-noise ratio of at least 5.

Inject 1 µl of the test solution and 1 µl of reference solution (a). Calculate the percentage content of related substances from the areas of the peaks in the chromatogram obtained with the test solution by the normalisation procedure, disregarding any peaks with an area less than 0.5 times the area of the peak in the chromatogram obtained with reference solution (b). The content of any related substance is not greater than 0.3 per cent and the sum of the contents is not greater than 0.5 per cent.

Heavy metals (2.4.8). Dissolve 2.0 g in *alcohol R* and dilute to 20 ml with the same solvent. 12 ml of the solution complies with limit test B for heavy metals (10 ppm). Prepare the standard using 1 ml of *lead standard solution (10 ppm Pb) R* and 9 ml of *alcohol R*.

Water (2.5.12). Not more than 0.7 per cent, determined on 1.000 g by the semi-micro determination of water.

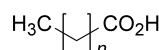
Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

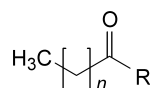
Dissolve 0.125 g in 25 ml of *alcohol R*. Titrate with 0.1 M *sodium hydroxide*, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *sodium hydroxide* is equivalent to 14.42 mg of C₈H₁₆O₂.

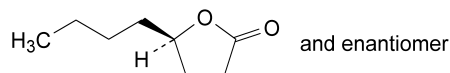
IMPURITIES



- A. $n = 4$: hexanoic acid,
 B. $n = 5$: heptanoic acid,
 C. $n = 7$: nonanoic acid,
 D. $n = 8$: decanoic acid,
 E. valproic acid,



- F. R = OCH₃, $n = 6$: methyl octanoate,
 G. R = OC₂H₅, $n = 6$: ethyl octanoate,
 H. R = OCH₃, $n = 8$: methyl decanoate,
 I. R = CH₃, $n = 8$: undecan-2-one,



- J. 5-butyltetrahydrofuran-2-one (γ-hydroxyoctanoic acid lactone).

01/2005:1184

CAPRYLOCAPROYL
MACROGOLGLYCERIDES

Macrogolglyceridorum caprylocaprates

DEFINITION

Caprylocaproyl macrogolglycerides are mixtures of monoesters, diesters and triesters of glycerol and monoesters and diesters of macrogols with a mean relative molecular mass between 200 and 400. They are obtained by partial alcoholysis of medium-chain triglycerides using macrogol or by esterification of glycerol and macrogol with caprylic acid and capric acid or a mixture of glycerol esters and condensates of ethylene oxide with caprylic acid (octanoic acid) and capric acid (decanoic acid). They may contain free macrogols.

CHARACTERS

Pale-yellow oily liquids, dispersible in hot water, freely soluble in methylene chloride.

The relative density at 20 °C is about 1.0, the refractive index at 20 °C is about 1.4.

IDENTIFICATION

- A. Examine by thin-layer chromatography (2.2.27), using a suitable silica gel as the coating substance.

Test solution. Dissolve 1.0 g of the substance to be examined in *methylene chloride R* and dilute to 20 ml with the same solvent.

Apply to the plate 50 µl of the test solution. Develop over a path of 15 cm using a mixture of 30 volumes of *hexane R* and 70 volumes of *ether R*. Allow the plate to dry in air. Spray with a 0.1 g/l solution of *rhodamine B R* in *alcohol R* and examine in ultraviolet light at 365 nm. The chromatogram shows a spot corresponding to triglycerides with an R_f value of about 0.9 (R_{st} 1) and spots corresponding to 1,3-diglycerides (R_{st} 0.7), to 1,2-diglycerides (R_{st} 0.6), to monoglycerides (R_{st} 0.1) and to esters of macrogol (R_{st} 0).

- B. They comply with the test for hydroxyl value (see Tests).
C. They comply with the test for saponification value (see Tests).
D. They comply with the test for fatty acid composition (see Tests).

TESTS

Viscosity (2.2.9). The ranges are presented in Table 1184.-1, determined at 20 ± 0.5 °C.

Table 1184.-1

Ethylene oxide units per molecule (nominal value)	Type of macrogol	Viscosity (mPa.s)
4	200	30 to 50
6	300	60 to 80
8	400	80 to 110

Acid value (2.5.1). Not more than 2.0, determined on 2.0 g.

Hydroxyl value (2.5.3, *Method A*). The ranges are presented in Table 1184.-2, determined on 1.0 g.

Table 1184.-2

Ethylene oxide units per molecule (nominal value)	Type of macrogol	Hydroxyl value
4	200	80 to 120
6	300	140 to 180
8	400	170 to 205

Peroxide value (2.5.5). Not more than 6.0, determined on 2.0 g.

Saponification value (2.5.6). The ranges are presented in Table 1184.-3, determined on 2.0 g.

Table 1184.-3

Ethylene oxide units per molecule (nominal value)	Type of macrogol	Saponification value
4	200	265 to 285
6	300	170 to 190
8	400	85 to 105

Alkaline impurities. Introduce into a test-tube 5.0 g and carefully add a mixture, neutralised if necessary with 0.01 M *hydrochloric acid* or with 0.01 M *sodium hydroxide*, of 0.05 ml of a 0.4 g/l solution of *bromophenol blue R* in *alcohol R*, 0.3 ml of *water R* and 10 ml of *alcohol R*. Shake and allow to stand. Not more than 1.0 ml of 0.01 M *hydrochloric acid* is required to change the colour of the upper layer to yellow.

Free glycerol. Not more than 5.0 per cent. Dissolve 1.20 g in 25.0 ml of *methylene chloride R*. Heat if necessary. After cooling, add 100 ml of *water R*. Shake and add 25.0 ml of a 6 g/l solution of *periodic acid R*. Shake and allow to stand for 30 min. Add 40 ml of a 75 g/l solution of *potassium iodide R*. Allow to stand for 1 min. Add 1 ml of *starch solution R*. Titrate the iodine with 0.1 M *sodium thiosulphate*. Carry out a blank titration.

1 ml of 0.1 M *sodium thiosulphate* is equivalent to 2.3 mg of glycerol.

Fatty acid composition (2.4.22, *Method A*). The fatty acid fraction has the following composition:

- *caproic acid*: not more than 2.0 per cent,
- *caprylic acid*: 50.0 per cent to 80.0 per cent,
- *capric acid*: 20.0 per cent to 50.0 per cent,
- *lauric acid*: not more than 3.0 per cent,
- *myristic acid*: not more than 1.0 per cent.

Ethylene oxide and dioxan (2.4.25). Not more than 1 ppm of ethylene oxide and not more than 10 ppm of dioxan.

Heavy metals (2.4.8). 2.0 g complies with limit test C for heavy metals (10 ppm). Prepare the standard using 2 ml of *lead standard solution* (10 ppm Pb) *R*.

Water (2.5.12). Not more than 1.0 per cent, determined on 1.0 g by the semi-micro determination of water. Use a mixture of 30 volumes of *anhydrous methanol R* and 70 volumes of *methylene chloride R* as solvent.

Total ash (2.4.16). Not more than 0.1 per cent, determined on 1.0 g.

LABELLING

The label states the type of macrogol used (mean relative molecular mass) or the number of ethylene oxide units per molecule (nominal value).

01/2005:1859

CAPSICUM

Capsici fructus

DEFINITION

Dried ripe fruits of *Capsicum annum* L. var. *minimum* (Miller) Heiser and small-fruited varieties of *Capsicum frutescens* L.

Content: minimum 0.4 per cent of total capsaicinoids expressed as capsaicin ($C_{18}H_{27}NO_3$; M_r 305.4) (dried drug).

CHARACTERS

Extremely pungent taste.

Macroscopic and microscopic characters described under identification tests A and B.

IDENTIFICATION

- A. The fruit is yellowish-orange to reddish-brown, oblong conical with an obtuse apex, about 1 cm to 3 cm long and up to 1 cm in diameter at the widest part, occasionally attached to a 5-toothed inferior calyx and a straight pedicel. Pericarp somewhat shrivelled, glabrous, enclosing about 10 to 20 flat, reniform seeds 3 mm to 4 mm long, either loose or attached to a reddish dissepiment.
- B. Reduce to a powder (355). The powder is orange. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters: fragments of the pericarp having an outer epicarp with cells often arranged in rows of 5 to

7, cuticle uniformly striated; parenchymatous cells frequently containing droplets of red oil, occasionally containing microspenoidal crystals of calcium oxalate; endocarp with characteristic island groups of sclerenchymatous cells, the groups being separated by thin-walled parenchymatous cells. Fragments of the seeds having an epispem composed of large, greenish-yellow, sinuous-walled sclereids with thin outer walls and strongly and unevenly thickened radial and inner walls which are conspicuously pitted; endosperm parenchymatous cells with drops of fixed oil and aleurone grains 3 µm to 6 µm in diameter. Occasional fragments from the calyx having an outer epidermis with anisocytic stomata (2.8.3), inner epidermis with many trichomes but no stomata; trichomes glandular, with uniseriate stalks and multicellular heads; mesophyll with many idioblasts containing microspenoidal crystals of calcium oxalate.

C. Thin-layer chromatography (2.2.27).

Test solution. To 0.50 g of the powdered drug (500) add 5.0 ml of *ether R*, shake for 5 min and filter.

Reference solution. Dissolve 2 mg of *capsaicin R* and 2 mg of *dihydrocapsaicin R* in 5.0 ml of *ether R*.

Plate: TLC octadecylsilyl silica gel plate *R*.

Mobile phase: water *R*, methanol *R* (20:80 V/V).

Application: 20 µl, as bands.

Development: over a path of 12 cm.

Drying: in air.

Detection: spray with a 5 g/l solution of *dichloroquinonechlorimide R* in *methanol R*.

Expose the plate to ammonia vapour until blue zones appear. Examine in daylight.

Results: see below the sequence of the zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other zones may be present in the chromatogram obtained with the test solution.

Top of the plate	
_____	_____
Capsaicin: a blue zone	A blue zone (capsaicin)
Dihydrocapsaicin: a blue zone	A blue zone (dihydrocapsaicin)
_____	_____
Reference solution	Test solution

TESTS

Nonivamide. Liquid chromatography (2.2.29).

Test solution. To 2.5 g of the powdered drug (500) add 100 ml of *methanol R*. Allow to macerate for 30 min.

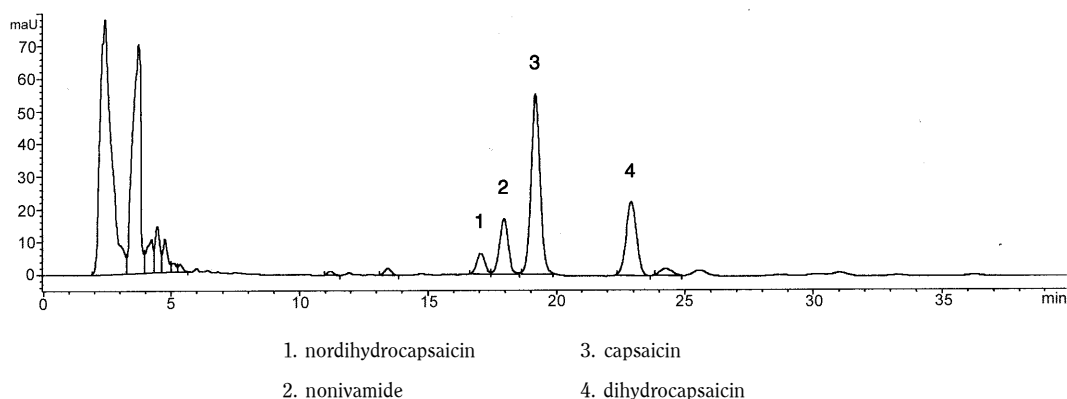


Figure 1859-1. – Chromatogram for the test for nonivamide and the assay of capsaicin

Place in an ultrasonic bath for 15 min. Filter into a 100 ml volumetric flask, rinse the flask and filter with *methanol R*. Dilute to 100.0 ml with *methanol R*.

Reference solution. Dissolve 20.0 mg of *capsaicin R* and 4.0 mg of *nonivamide R* in 100.0 ml of *methanol R*.

Column:

- size: $l = 0.25$ m, $\varnothing = 4.6$ mm,
- stationary phase: phenylsilyl silica gel for chromatography *R* (5 µm),
- temperature: 30 °C.

Mobile phase: mixture of 40 volumes of *acetonitrile R* and 60 volumes of a 1 g/l solution of *phosphoric acid R*.

Flow rate: 1.0 ml/min.

Detection: spectrophotometer at 225 nm.

Injection: 10 µl.

Elution order: elution order similar to that obtained in Figure 1859-1.

System suitability: reference solution:

- resolution: minimum 3.0 between the peaks due to capsaicin and nonivamide.

Limit: calculate the percentage content of nonivamide from the expression:

$$\frac{F_1 \times m_2 \times p_1}{F_2 \times m_1}$$

F_1 = area of the peak corresponding to nonivamide in the chromatogram obtained with the test solution,

F_2 = area of the peak corresponding to nonivamide in the chromatogram obtained with the reference solution,

m_1 = mass of the drug to be examined in grams,

m_2 = mass of nonivamide used to prepare the reference solution in grams,

p_1 = percentage content of nonivamide in the reagent.

- *nonivamide*: maximum 5.0 per cent of the total capsaicinoid content.

Foreign matter (2.8.2): maximum 2 per cent *m/m*. Fruits of *C. annuum* L. var. *longum* (Sendtn.) are absent.

Loss on drying (2.2.32): maximum 11.0 per cent, determined on 1.000 g of the powdered drug (500) by drying in an oven at 100-105 °C for 2 h.

Total ash (2.4.16): maximum 10.0 per cent.

ASSAY

Liquid chromatography (2.2.29) as described in the test for nonivamide.

Calculate the percentage content of capsaicinoids from the expression:

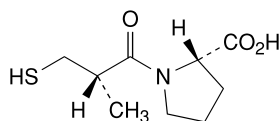
$$\frac{(F_3 + F_5 + F_6) \times m_4 \times p_2}{F_4 \times m_3}$$

- F_3 = area of the peak corresponding to capsaicin in the chromatogram obtained with the test solution,
 F_4 = area of the peak corresponding to capsaicin in the chromatogram obtained with the reference solution,
 F_5 = area of the peak corresponding to dihydrocapsaicin in the chromatogram obtained with the test solution,
 F_6 = area of the peak corresponding to nordihydrocapsaicin in the chromatogram obtained with the test solution,
 m_3 = mass of the drug to be examined in grams,
 m_4 = mass of capsaicin used to prepare the reference solution in grams,
 p_2 = percentage content of capsaicin in the reagent.

01/2005:1079

CAPTOPRIL

Captoprilum

 $C_9H_{15}NO_3S$ M_r 217.3

DEFINITION

(2S)-1-[(2S)-2-Methyl-3-sulphanylpropanoyl]pyrrolidine-2-carboxylic acid.

Content: 98.0 per cent to 101.5 per cent (dried substance).

CHARACTERS

Appearance: white or almost white crystalline powder.

Solubility: freely soluble in water, in methylene chloride and in methanol. It dissolves in dilute solutions of alkali hydroxides.

IDENTIFICATION

Infrared absorption spectrophotometry (2.2.24).

Comparison: captopril CRS.

TESTS

Solution S. Dissolve 0.5 g in carbon dioxide-free water R and dilute to 25.0 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and colourless (2.2.2, Method II).

pH (2.2.3): 2.0 to 2.6 for solution S.

Specific optical rotation (2.2.7): -127 to -132 (dried substance).

Dissolve 0.250 g in ethanol R and dilute to 25.0 ml with the same solvent.

Related substances. Liquid chromatography (2.2.29).

Test solution. Dissolve 50 mg of the substance to be examined in the mobile phase and dilute to 100.0 ml with the mobile phase.

Reference solution (a). Dilute 2.0 ml of the test solution to 100.0 ml with the mobile phase.

Reference solution (b). Dissolve 10 mg of the substance to be examined in the mobile phase, add 1 ml of 0.05 M iodine and dilute to 100.0 ml with the mobile phase. Dilute 10.0 ml of the solution to 100.0 ml with the mobile phase.

Column:

- size: $l = 0.125$ m, $\varnothing = 4$ mm,
- stationary phase: octylsilyl silica gel for chromatography R (5 μ m).

Mobile phase: phosphoric acid R, methanol R, water R (0.05:50:50 V/V/V).

Flow rate: 1 ml/min.

Detection: spectrophotometer at 220 nm.

Injection: 20 μ l.

Run time: 3 times the retention time of captopril.

System suitability: reference solution (b):

- the chromatogram shows 3 peaks,
- resolution: minimum of 2.0 between the last 2 eluting principal peaks,

Limits:

- any impurity: not more than half the area of the principal peak in the chromatogram obtained with reference solution (a) (1.0 per cent),
- total: not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (2.0 per cent),
- disregard limit: 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent). Disregard any peak with a retention time less than 1.4 min.

Heavy metals (2.4.8): maximum 20 ppm.

1.0 g complies with limit test C. Prepare the standard using 2 ml of lead standard solution (10 ppm Pb) R.

Loss on drying (2.2.32): maximum 1.0 per cent, determined on 1.000 g by drying under high vacuum at 60 °C for 3 h.

Sulphated ash (2.4.14): maximum 0.2 per cent, determined on 1.0 g.

ASSAY

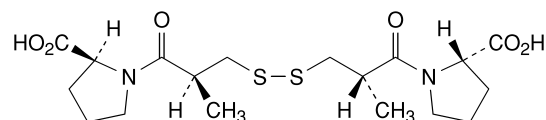
Dissolve 0.150 g in 30 ml of water R. Titrate with 0.05 M iodine, determining the end-point potentiometrically (2.2.20). Use a combined platinum electrode.

1 ml of 0.05 M iodine is equivalent to 21.73 mg of $C_9H_{15}NO_3S$.

STORAGE

In an airtight container.

IMPURITIES



A. (2S,2'S)-1,1'-[disulphanediylbis[(2S)-2-methyl-1-oxopropane-3,1-diyl]-bis[pyrrolidine-2-carboxylic] acid (captopril-disulphide).

01/2005:1080

CARAWAY FRUIT

Carvi fructus

DEFINITION

Caraway fruit consists of the whole, dry mericarp of *Carum carvi* L. It contains not less than 30 ml/kg of essential oil, calculated with reference to the anhydrous drug.

CHARACTERS

Caraway fruit has an odour reminiscent of carvone.

It has the macroscopic and microscopic characters described under identification tests A and B.

IDENTIFICATION

A. The fruit is a cremocarp of almost cylindrical shape. It is generally 3 mm to 6.5 mm long and 1 mm to 1.5 mm wide. The mericarps, usually free, are greyish-brown to brown, glabrous, mostly sickle-shaped, with both ends sharply terminated. Each bears five prominent narrow ridges. When cut transversely the profile shows an almost regular pentagon and four vittae on the dorsal surface and two on the commissural surface may be seen with a lens.

B. Reduce to a powder (355). The powder is yellowish-brown. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters: fragments of the secretory cells composed of yellowish-brown to brown, thin-walled, polygonal secretory cells, frequently associated with a layer of thin-walled transversely elongated cells, 8-12 µm wide; fragments of the epicarp with thick-walled cells and occasional anomocytic stomata (2.8.3); numerous endosperm fragments containing aleurone grains, droplets of fatty oil and microcrystals of calcium oxalate in rosette formation; spiral vessels accompanied by sclerenchymatous fibres; rarely some fibre bundles from the carpophore; groups of rectangular to sub-rectangular sclereids from the mesocarp with moderately thickened and pitted walls may be present.

C. Examine by thin-layer chromatography (2.2.27) using a suitable silica gel as the coating substance.

Test solution. Shake 0.5 g of the powdered drug (710) with 5.0 ml of *ethyl acetate R* for 2 min to 3 min. Filter over 2 g of *anhydrous sodium sulphate R*. Use the filtrate as the test solution.

Reference solution. Dissolve 2 µl of *carvone R* and 5 µl of *olive oil R* in 1.0 ml of *ethyl acetate R*.

Apply to the plate as bands 20 µl of the test solution and 10 µl of the reference solution. Develop over a path of 10 cm using a mixture of 5 volumes of *ethyl acetate R* and 95 volumes of *toluene R*. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. The chromatograms of the test solution and of the reference solution show a quenching zone (carvone) in the central part against a light background. Spray with *anisaldehyde solution R* and heat under observation at 100 °C to 105 °C for 2 to 4 min. Examine in daylight. The zones corresponding to carvone appear strong orange-brown. The chromatogram obtained with the test solution shows above the zone corresponding to carvone a violet zone corresponding to triglycerides similar in position and colour to the zone in the chromatogram obtained with the reference solution (triglycerides of olive oil). The chromatogram obtained with the test solution shows close

to the solvent front a weak violet zone corresponding to terpene hydrocarbons and in the lower part some weak, mostly violet-greyish and brownish zones.

TESTS

Foreign matter (2.8.2). It complies with the test for foreign matter.

Water (2.2.13). Not more than 100 ml/kg, determined on 10.0 g of powdered drug by distillation.

Total ash (2.4.16). Not more than 7.0 per cent.

ASSAY

Carry out the determination of essential oils in vegetable drugs (2.8.12). Use a 500 ml round-bottomed flask, 200 ml of *water R* as the distillation liquid and 0.50 ml of *xylene R* in the graduated tube. Reduce the drug to a powder (710) and immediately use 10.0 g for the determination. Distil at a rate of 2-3 ml/min for 90 min.

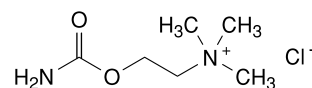
STORAGE

Store protected from light.

01/2005:1971

CARBACHOL

Carbacholum

C₆H₁₅ClN₂O₂M_r 182.7

DEFINITION

2-(Carbamoyloxy)-N,N,N-trimethylethanaminium chloride.

Content: 99.0 per cent to 101.5 per cent (dried substance).

CHARACTERS

Appearance: white, crystalline, hygroscopic powder.

Solubility: very soluble in water, sparingly soluble in alcohol, practically insoluble in acetone.

IDENTIFICATION

First identification: A, C.

Second identification: B, C.

A. Infrared absorption spectrophotometry (2.2.24).

Comparison: *carbachol CRS*.

B. Examine the chromatograms obtained in the test for related substances.

Results: the principal spot in the chromatogram obtained with test solution (b) is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).

C. 0.5 ml of solution S (see Tests) gives reaction (a) of chlorides (2.3.1).

TESTS

Solution S. Dissolve 2.5 g in *carbon dioxide-free water R* and dilute to 25 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and colourless (2.2.2, *Method II*).

Acidity or alkalinity. To 2.0 ml of solution S, add 0.05 ml of *methyl red mixed solution R*. Not more than 0.2 ml of 0.01 M *hydrochloric acid* or 0.01 M *sodium hydroxide* is required to change the colour of the indicator.

Related substances. Thin-layer chromatography (2.2.27).

01/2005:0543

Prepare the solutions immediately before use.

Test solution (a). Dissolve 0.20 g of the substance to be examined in *methanol R* and dilute to 5.0 ml with the same solvent.

Test solution (b). Dilute 2.0 ml of test solution (a) to 20.0 ml with *methanol R*.

Reference solution (a). Dissolve 20 mg of *carbachol CRS* in *methanol R* and dilute to 5.0 ml with the same solvent.

Reference solution (b). Dissolve 8 mg of *choline chloride R* and 8 mg of *acetylcholine chloride CRS* in *methanol R* and dilute to 10.0 ml with the same solvent. Dilute 5.0 ml to 10.0 ml with *methanol R*.

Plate: *cellulose for chromatography R* as the coating substance.

Mobile phase: *water R*, *methanol R* (10:90 V/V).

Application: 10 µl.

Development: over 2/3 of the plate.

Detection: spray with *potassium iodobismuthate solution R3*.

System suitability: the chromatogram obtained with reference solution (b) shows 2 clearly separated spots.

Limits: in the chromatogram obtained with test solution (a):

- **any impurity:** any spot, apart from the principal spot, is not more intense than one or other of the 2 principal spots in the chromatogram obtained with reference solution (b) (1 per cent). Compare the spots with the spot of the most appropriate colour in the chromatogram obtained with reference solution (b).

Heavy metals (2.4.8): maximum 20 ppm.

12 ml of solution S complies with limit test A. Prepare the standard using *lead standard solution (2 ppm Pb) R*.

Loss on drying (2.2.32): maximum 1.0 per cent, determined on 1.000 g by drying in an oven at 100–105 °C for 2 h.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g of the residue obtained in the test for loss on drying.

ASSAY

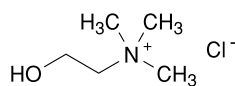
Dissolve 0.150 g in a mixture of 10 ml of *anhydrous acetic acid R* and 40 ml of *acetic anhydride R*. Titrate with 0.1 M *perchloric acid*. Determine the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *perchloric acid* is equivalent to 18.27 mg of $C_{15}H_{12}N_2O_2$.

STORAGE

In an airtight container, protected from light.

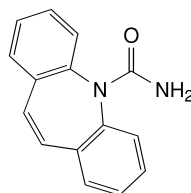
IMPURITIES



A. 2-hydroxy-*N,N,N*-trimethylethanaminium chloride (*choline chloride*).

CARBAMAZEPINE

Carbamazepinum


 $C_{15}H_{12}N_2O$
 M_r 236.3

DEFINITION

5*H*-dibenzo[*b,f*]azepine-5-carboxamide.

Content: 98.0 per cent to 102.0 per cent (dried substance).

CHARACTERS

Appearance: white or almost white crystalline powder.

Solubility: very slightly soluble in water, freely soluble in methylene chloride, sparingly soluble in acetone and in alcohol.

It shows polymorphism; the acceptable crystalline form corresponds to *carbamazepine CRS*.

IDENTIFICATION

A. Melting point (2.2.14): 189 °C to 193 °C.

B. Infrared absorption spectrophotometry (2.2.24).

Comparison: *carbamazepine CRS*.

Preparation: examine the substances as discs without prior treatment.

TESTS

Acidity or alkalinity. To 1.0 g add 20 ml of *carbon dioxide-free water R*, shake for 15 min and filter. To 10 ml of the filtrate add 0.05 ml of *phenolphthalein solution R1* and 0.5 ml of 0.01 M *sodium hydroxide*; the solution is red. Add 1.0 ml of 0.01 M *hydrochloric acid*; the solution is colourless. Add 0.15 ml of *methyl red solution R*; the solution is red.

Related substances. Liquid chromatography (2.2.29).

Test solution (a). Dissolve 0.150 g of the substance to be examined in *methanol R2* and dilute to 50.0 ml with the same solvent. Sonicate. Dilute 10.0 ml of this solution to 20.0 ml with *water R*.

Test solution (b). Dilute 10.0 ml of test solution (a) to 50.0 ml with a mixture of equal volumes of *methanol R2* and *water R*.

Reference solution (a). Dissolve 7.5 mg of *carbamazepine CRS*, 7.5 mg of *carbamazepine impurity A CRS* and 7.5 mg of *iminodibenzyl R* (impurity E) in *methanol R2* and dilute to 100.0 ml with the same solvent. Dilute 1.0 ml of this solution to 50.0 ml with a mixture of equal volumes of *methanol R2* and *water R*.

Reference solution (b). Dissolve 0.150 g of *carbamazepine CRS* in *methanol R2* and dilute to 50.0 ml with the same solvent. Dilute 5.0 ml of this solution to 50.0 ml with a mixture of equal volumes of *methanol R2* and *water R*.

Column:

– size: $l = 0.25$ m, $\varnothing = 4.6$ mm,

– stationary phase: *nitrile silica gel for chromatography R1* (10 µm).

Mobile phase: tetrahydrofuran *R*, methanol *R2*, water *R* (3:12:85 *V/V/V*). To 1000 ml of this solution add 0.2 ml of anhydrous formic acid *R* and 0.5 ml of triethylamine *R*.

Flow rate: 2.0 ml/min.

Detection: a spectrophotometer at 230 nm.

Injection: 20 µl; inject test solution (a) and reference solution (a).

Run time: 6 times the retention time of carbamazepine which is about 10 min.

Relative retention with reference to carbamazepine: impurity B = about 0.7; impurity A = about 0.9; impurity C = about 1.6; impurity D = about 3.5; impurity E = about 5.1.

System suitability:

- **resolution:** minimum of 1.7 between the peaks due to carbamazepine and impurity A in the chromatogram obtained with reference solution (a).

Limits:

- **impurity A:** not more than the area of the corresponding peak in the chromatogram obtained with reference solution (a) (0.1 per cent),
- **impurity E:** not more than the area of the corresponding peak in the chromatogram obtained with reference solution (a) (0.1 per cent),
- **any other impurity:** not more than the area of the peak due to carbamazepine in the chromatogram obtained with reference solution (a) (0.1 per cent),
- **total:** not more than 5 times the area of the peak due to carbamazepine in the chromatogram obtained with reference solution (a) (0.5 per cent),
- **disregard limit:** 0.5 times the area of the peak due to carbamazepine in the chromatogram obtained with reference solution (a) (0.05 per cent).

Chlorides (2.4.4): maximum 140 ppm.

Suspend 0.715 g in 20 ml of water *R* and boil for 10 min. Cool and dilute to 20 ml with water *R*. Filter through a membrane filter (nominal pore size: 0.8 µm). Dilute 10 ml of the filtrate to 15 ml with water *R*. This solution complies with the limit test for chlorides.

Heavy metals (2.4.8): maximum 20 ppm.

1.0 g complies with limit test C. Prepare the standard using 2 ml of lead standard solution (10 ppm Pb) *R*.

Loss on drying (2.2.32): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 100–105 °C for 2 h.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (2.2.29) as described in the test for related substances.

Injection: test solution (b) and reference solution (b).

System suitability:

- **repeatability:** reference solution (b).

Calculate the percentage content *m/m* of dried substance.

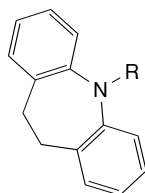
STORAGE

In an airtight container.

IMPURITIES

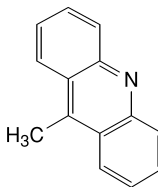
Specified impurities: A, B, C, D, E.

Other detectable impurities: F

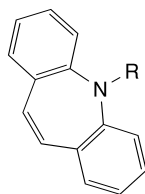


A. R = CO-NH₂: 10,11-dihydro-5*H*-dibenzo[*b,f*]azepine-5-carboxamide (10,11-dihydrocarbamazepine),

E. R = H: 10,11-dihydro-5*H*-dibenzo[*b,f*]azepine (iminodibenzyl),



B. 9-methylacridine,



C. R = CO-NH-CO-NH₂: (5*H*-dibenzo[*b,f*]azepin-5-ylcarbonyl)urea (*N*-carbamoylcarbamazepine),

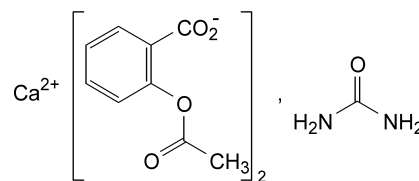
D. R = H: 5*H*-dibenzo[*b,f*]azepine (iminostilbene),

F. R = CO-Cl: 5*H*-dibenzo[*b,f*]azepine-5-carbonyl chloride (5-chlorocarbonyliminostilbene).

01/2005:1185

CARBASALATE CALCIUM

Carbasalatium calcicum



C₁₉H₁₈CaN₂O₉

M_r 458.4

DEFINITION

Carbasalate calcium contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of an equimolecular compound of calcium di[2-(acetyloxy)benzoate] and urea, calculated with reference to the anhydrous substance.

CHARACTERS

A white, crystalline powder, freely soluble in water and dimethylformamide, practically insoluble in acetone and in anhydrous methanol.

Protect the substance from moisture during handling. Examination in aqueous solutions has to be performed immediately after preparation.

IDENTIFICATION

First identification: B, E.

Second identification: A, C, D, E.

- A. Dissolve 0.250 g in *water R* and dilute to 100.0 ml with the same solvent. To 1.0 ml of the solution add 75 ml of *water R* and 5 ml of *dilute hydrochloric acid R*, mix and dilute with *water R* to 100.0 ml. Examined immediately after preparation between 220 nm and 350 nm (2.2.25), the solution shows two absorption maxima, at 228 nm and 276 nm. The specific absorbances at the maxima are 363 to 379 and 49 to 53, respectively.
- B. Examine by infrared spectrophotometry (2.2.24), comparing with the *Ph. Eur. reference spectrum of carbasalate calcium*.
- C. Dissolve 0.1 g in 10 ml of *water R*, boil for 2 min and cool. The solution gives reaction (a) of salicylates (2.3.1).
- D. Heat 0.2 g with 0.2 g of *sodium hydroxide R*; a yellow to yellowish-brown colour is produced and the vapour turns *red litmus paper R* blue.
- E. It gives reaction (a) of calcium (2.3.1).

TESTS

Appearance of solution. Dissolve 2.5 g in 50 ml of *water R*. The solution is not more opalescent than reference suspension II (2.2.1) and is colourless (2.2.2, *Method II*).

Related substances. In a 100 ml volumetric flask, dissolve 0.150 g in 10 ml of 0.1 M *tetrabutylammonium hydroxide* in 2-propanol. Allow to stand for 10 min shaking occasionally. Add 8.0 ml of 0.1 M *hydrochloric acid* and 20.0 ml of a 19 g/l solution of *disodium tetraborate R* and mix. While swirling continuously, add 2.0 ml of a 10 g/l solution of *aminopyrazolone R* and 2.0 ml of a 10 g/l solution of *potassium ferricyanide R*. Allow to stand for 2 min, dilute to 100.0 ml with *water R*, mix and allow to stand for 20 min. Measure the absorbance (2.2.25) of the solution at the maximum at 505 nm using *water R* as the compensation liquid. The absorbance is not greater than 0.125 (0.1 per cent, expressed as acetylsalicylsalicylic acid).

Salicylic acid. In a 100 ml volumetric flask, dissolve 0.200 g in 80 ml of *water R* and add 10 ml of a 10 g/l solution of *ferric nitrate R* in a 80 g/l solution of *dilute nitric acid R*. Dilute to 100.0 ml with *water R*. Immediately after preparation, measure the absorbance (2.2.25) of the solution at the maximum of 525 nm using *water R* as the compensation liquid. The absorbance is not greater than 0.115 (0.5 per cent, expressed as salicylic acid).

Sodium. Not more than 0.1 per cent, determined by atomic emission spectrometry (2.2.22, *Method I*) using 1.0 g dissolved in 500.0 ml of *water R*.

Heavy metals (2.4.8). Dissolve 2.0 g in 8 ml of *water R*, with heating, cool and add 12 ml of *acetone R*. 12 ml of the solution complies with limit test B for heavy metals (10 ppm). Prepare the standard using 10 ml of *lead standard solution* (1 ppm Pb) *R*.

Water (2.5.12). Not more than 0.1 per cent, determined on 1.000 g by the semi-micro determination of water. Use a mixture of 15 ml of *anhydrous methanol R* and 15 ml of *dimethylformamide R* as the solvent.

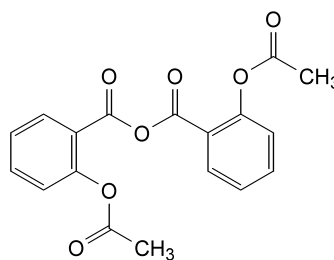
ASSAY

In a flask with a ground-glass stopper, dissolve 0.400 g in 25 ml of *water R*. Add 25.0 ml of 0.1 M *sodium hydroxide*. Close the flask and allow to stand for 2 h. Titrate the excess of alkali with 0.1 M *hydrochloric acid*, using 0.2 ml of *phenolphthalein solution R*. Carry out a blank titration. 1 ml of 0.1 M *sodium hydroxide* is equivalent to 22.92 mg of $C_{10}H_{14}N_2O_4 \cdot H_2O$.

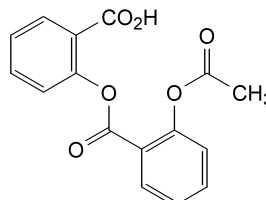
STORAGE

Store in an airtight container.

IMPURITIES



- A. 2-(acetyloxy)benzoic anhydride,



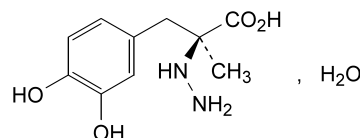
- B. 2-[[2-(acetyloxy)benzoyl]oxy]benzoic acid (acetylsalicylsalicylic acid),

- C. 2-hydroxybenzoic acid (salicylic acid).

01/2005:0755
corrected

CARBIDOPA

Carbidopum



$C_{10}H_{14}N_2O_4 \cdot H_2O$

M_r 244.2

DEFINITION

Carbidopa contains not less than 98.5 per cent and not more than the equivalent of 101.0 per cent of (2S)-3-(3,4-dihydroxyphenyl)-2-hydrazino-2-methylpropanoic acid, calculated with reference to the dried substance.

CHARACTERS

A white or yellowish-white powder, slightly soluble in water, very slightly soluble in alcohol, practically insoluble in methylene chloride. It dissolves in dilute solutions of mineral acids.

IDENTIFICATION

First identification: A, C.

Second identification: A, B, D, E.

- A. It complies with the test for specific optical rotation (see Tests).
- B. Dissolve 50.0 mg in a 8.5 g/l solution of *hydrochloric acid R* in *methanol R* and dilute to 100.0 ml with the same acid. Dilute 10.0 ml of this solution to 100.0 ml with a 8.5 g/l solution of *hydrochloric acid R* in *methanol R*. Examined between 230 nm and 350 nm (2.2.25), the solution shows an absorption maximum at 283 nm. The specific absorbance at the maximum is 135 to 150, calculated with reference to the dried substance.
- C. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *carbidopa CRS*. Examine the substances prepared as discs.

- D. Shake vigorously about 5 mg with 10 ml of *water R* for 1 min and add 0.3 ml of *ferric chloride solution R2*. An intense green colour is produced, which quickly turns to reddish-brown.
- E. Suspend about 20 mg in 5 ml of *water R* and add 5 ml of *cupri-tartaric solution R*. On heating, the colour of the solution changes to dark brown and a red precipitate is formed.

TESTS

Appearance of solution. Dissolve 0.25 g in 25 ml of 1 M *hydrochloric acid*. The solution is clear (2.2.1) and not more intensely coloured than reference solution BY₆ or B₆ (2.2.2, *Method II*).

Specific optical rotation (2.2.7). With the aid of an ultrasonic bath, dissolve completely 0.250 g in *aluminium chloride solution R* and dilute to 25.0 ml with the same solution. The specific optical rotation is -22.5 to -26.5 , calculated with reference to the dried substance.

Hydrazine. Examine by thin-layer chromatography (2.2.27), using *silanised silica gel H R* as the coating substance.

Test solution (a). Dissolve 0.50 g in *dilute hydrochloric acid R* and dilute to 2.0 ml with the same acid.

Test solution (b). Place 25 g of *strongly basic anion exchange resin R* into each of two conical flasks with ground-glass stoppers. To each, add 150 ml of *carbon dioxide-free water R* and shake from time to time during 30 min. Decant the liquid from both flasks and repeat the process with further quantities, each of 150 ml, of *carbon dioxide-free water R*.

Take two 100 ml measuring cylinders 3.5 cm to 4.5 cm in internal diameter and label these A and B. Into cylinder A, transfer as completely as possible the resin from one conical flask using 60 ml of *carbon dioxide-free water R*; into cylinder B, transfer the second quantity of resin, this time using 20 ml of *carbon dioxide-free water R*.

Into each cylinder, insert a gas-inlet tube, the end of which has an internal diameter of 2 mm to 3 mm and which reaches almost to the bottom of the cylinder. Pass a rapid stream of *nitrogen for chromatography R* through each mixture so that homogeneous suspensions are formed. After 30 min, without interrupting the gas flow, add 1.0 ml of test solution (a) to cylinder A; after 1 min stop the gas flow into cylinder A and transfer the contents, through a moistened filter paper, into cylinder B. After 1 min, stop the gas flow to cylinder B and pour the solution immediately through a moistened filter paper into a freshly prepared mixture of 1 ml of a 200 g/l solution of *salicylaldehyde R* in *methanol R* and 20 ml of *phosphate buffer solution pH 5.5 R* in a conical flask, shake thoroughly for 1 min and heat in a water-bath at 60 °C for 15 min. The liquid becomes clear. Allow to cool, add 2.0 ml of *toluene R* and shake vigorously for 2 min. Transfer the mixture into a centrifuge tube and centrifuge.

Separate the toluene layer in a 100 ml separating funnel and shake vigorously with two quantities, each of 20 ml, of a 200 g/l solution of *sodium metabisulphite R* and finally with two quantities, each of 50 ml, of *water R*. Separate the toluene layer.

Reference solution (a). Dissolve 10 mg of *hydrazine sulphate R* in *dilute hydrochloric acid R* and dilute to 50 ml with the same acid. Dilute 1.0 ml of this solution to 10.0 ml with *dilute hydrochloric acid R*.

Reference solution (b). Prepare the solution at the same time and in the same manner as described for test solution (b) using 1.0 ml of reference solution (a) in place of 1.0 ml of test solution (a).

Apply separately to the plate 10 µl of test solution (b) and 10 µl of reference solution (b). Develop over a path of 10 cm using a mixture of 1 volume of *water R* and 2 volumes of *methanol R*. Allow the plate to dry in air. Examine in ultraviolet light at 365 nm. Any spot in the chromatogram obtained with test solution (b) showing a yellow fluorescence is not more intense than the corresponding spot in the chromatogram obtained with reference solution (b) (20 ppm of hydrazine).

Methyldopa and methylcarbidopa. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 0.100 g of the substance to be examined in 0.1 M *hydrochloric acid* and dilute to 10.0 ml with the same acid.

Reference solution (a). Dissolve the contents of a vial of *methylcarbidopa CRS* in 0.1 M *hydrochloric acid*, add 1 mg of *methyldopa CRS* and dilute to 20.0 ml with the same acid.

Reference solution (b). Dissolve 5 mg of *carbidopa CRS* and 5 mg of *methyldopa CRS* in 0.1 M *hydrochloric acid* and dilute to 10.0 ml with the same acid.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4.6 mm in internal diameter packed with *octylsilyl silica gel for chromatography R* (5 µm),
- as mobile phase at a flow rate of 1 ml/min a mixture of 2 volumes of *methanol R* and 98 volumes of a 14 g/l solution of *potassium dihydrogen phosphate R*,
- as detector a spectrophotometer set at 282 nm,
- a loop injector.

Inject 20 µl of each solution. The test is not valid unless the resolution between the peaks corresponding to methyldopa and carbidopa in the chromatogram obtained with reference solution (b) is greater than 4.0. In the chromatogram obtained with the test solution, the areas of any peaks corresponding to methyldopa and methylcarbidopa are not greater than the areas of the corresponding peaks in the chromatogram obtained with reference solution (a) (0.5 per cent).

Heavy metals (2.4.8). 1.0 g complies with limit test C for heavy metals (20 ppm). Prepare the standard using 2 ml of *lead standard solution (10 ppm Pb) R*.

Loss on drying (2.2.32): 6.9 per cent to 7.9 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

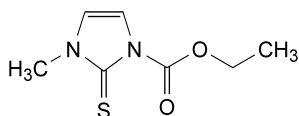
Dissolve 0.150 g with gentle heating in 75 ml of *anhydrous acetic acid R*. Titrate with 0.1 M *perchloric acid*, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *perchloric acid* is equivalent to 22.62 mg of C₁₀H₁₄N₂O₄.

STORAGE

Store protected from light.

01/2005:0884

CARBIMAZOLE**Carbimazolum** $C_7H_{10}N_2O_2S$ M_r 186.2**DEFINITION**

Ethyl 3-methyl-2-thioxo-2,3-dihydro-1H-imidazole-1-carboxylate.

Content: 98.0 per cent to 102.0 per cent (dried substance).

CHARACTERS

Appearance: white or yellowish-white, crystalline powder.

Solubility: slightly soluble in water, soluble in acetone and in alcohol.

IDENTIFICATION

First identification: B.

Second identification: A, C, D.

A. Melting point (2.2.14): 122 °C to 125 °C.

B. Infrared absorption spectrophotometry (2.2.24).

Preparation: discs.

Comparison: carbimazole CRS.

C. Thin-layer chromatography (2.2.27).

Test solution. Dissolve 10 mg of the substance to be examined in *methylene chloride R* and dilute to 10 ml with the same solvent.

Reference solution. Dissolve 10 mg of carbimazole CRS in *methylene chloride R* and dilute to 10 ml with the same solvent.

Plate: TLC silica gel GF₂₅₄ plate R.

Mobile phase: acetone R, *methylene chloride R* (20:80 V/V).

Application: 10 µl.

Development: over a path of 15 cm.

Drying: in air for 30 min.

Detection: examine in ultraviolet light at 254 nm.

Results: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with the reference solution.

D. Dissolve about 10 mg in a mixture of 50 ml of *water R* and 0.05 ml of *dilute hydrochloric acid R*. Add 1 ml of *potassium iodobismuthate solution R*. A red precipitate is formed.

TESTS

Impurity A and other related substances. Liquid chromatography (2.2.29).

Test solution. Dissolve 5.0 mg of the substance to be examined in 10.0 ml of a mixture of 20 volumes of *acetonitrile R* and 80 volumes of *water R*. Use this solution within 5 min of preparation.

Reference solution (a). Dissolve 5 mg of *thiamazole R* and 0.10 g of carbimazole CRS in a mixture of 20 volumes of *acetonitrile R* and 80 volumes of *water R* and dilute to 100.0 ml with the same mixture of solvents. Dilute 1.0 ml of this solution to 10.0 ml with a mixture of 20 volumes of *acetonitrile R* and 80 volumes of *water R*.

Reference solution (b). Dissolve 5.0 mg of *thiamazole R* in a mixture of 20 volumes of *acetonitrile R* and 80 volumes of *water R* and dilute to 10.0 ml with the same mixture of solvents. Dilute 1.0 ml of this solution to 100.0 ml with a mixture of 20 volumes of *acetonitrile R* and 80 volumes of *water R*.

Column:

- *size:* $l = 0.15$ m, $\varnothing = 3.9$ mm,
- *stationary phase:* octadecylsilyl silica gel for chromatography R (5 µm).

Mobile phase: *acetonitrile R*, *water R* (10:90 V/V).

Flow rate: 1 ml/min.

Detection: spectrophotometer at 254 nm.

Injection: 10 µl.

Run time: 1.5 times the retention time of carbimazole.

Retention time: carbimazole = about 6 min.

System suitability: reference solution (a):

- *resolution:* minimum 5.0 between the peaks due to impurity A and carbimazole.

Limits:

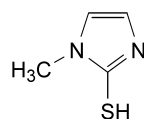
- *impurity A:* not more than half the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent),
- *any other impurity:* not more than 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent).

Loss on drying (2.2.32): maximum 0.5 per cent, determined on 1.000 g by drying in a desiccator over *diphosphorus pentoxide R* at a pressure not exceeding 0.7 kPa for 24 h.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

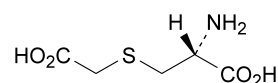
ASSAY

Dissolve 50.0 mg in *water R* and dilute to 500.0 ml with the same solvent. To 10.0 ml add 10 ml of *dilute hydrochloric acid R* and dilute to 100.0 ml with *water R*. Measure the absorbance (2.2.25) at the maximum at 291 nm. Calculate the content of $C_7H_{10}N_2O_2S$ taking the specific absorbance to be 557.

IMPURITIES

A. 1-methyl-1H-imidazole-2-thiol (thiamazole).

01/2005:0885

CARBOCISTEINE**Carbocisteinum** $C_5H_9NO_4S$ M_r 179.2**DEFINITION**

Carbocisteine contains not less than 98.5 per cent and not more than the equivalent of 101.0 per cent of (2R)-2-amino-3-[(carboxymethyl)sulphonyl]propanoic acid, calculated with reference to the dried substance.

CHARACTERS

A white, crystalline powder, practically insoluble in water and in alcohol. It dissolves in dilute mineral acids and in dilute solutions of alkali hydroxides.

IDENTIFICATION

First identification: A, B.

Second identification: A, C, D.

- A. It complies with the test for specific optical rotation (see Tests).
- B. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *carbocisteine CRS*. Examine the substances prepared as discs.
- C. Examine the chromatograms obtained in the test for ninhydrin-positive substances. The principal spot in the chromatogram obtained with test solution (b) is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).
- D. Dissolve 0.1 g in 4.5 ml of *dilute sodium hydroxide solution R*. Heat on a water-bath for 10 min. Cool and add 1 ml of a 25 g/l solution of *sodium nitroprusside R*. A dark red colour is produced, which changes to brown and then to yellow within a few minutes.

TESTS

Solution S. Disperse 5.00 g in 20 ml of *water R* and add dropwise with shaking 2.5 ml of *strong sodium hydroxide solution R*. Adjust to pH 6.3 with *1 M sodium hydroxide* and dilute to 50.0 ml with *water R*.

Appearance of solution. Solution S is clear (2.2.1) and colourless (2.2.2, *Method II*).

pH (2.2.3). Shake 0.2 g with 20 ml of *carbon dioxide-free water R*. The pH of the suspension is 2.8 to 3.0.

Specific optical rotation (2.2.7): –32.5 to –35.5, determined on solution S and calculated with reference to the dried substance.

Ninhydrin-positive substances. Examine by thin-layer chromatography (2.2.27), using a suitable silica gel as the coating substance.

Test solution (a). Dissolve 0.10 g of the substance to be examined in *dilute ammonia R2* and dilute to 10 ml with the same solvent.

Test solution (b). Dilute 1 ml of test solution (a) to 50 ml with *water R*.

Reference solution (a). Dissolve 10 mg of *carbocisteine CRS* in *dilute ammonia R2* and dilute to 50 ml with the same solvent.

Reference solution (b). Dilute 5 ml of test solution (b) to 20 ml with *water R*.

Reference solution (c). Dissolve 10 mg of *carbocisteine CRS* and 10 mg of *arginine hydrochloride CRS* in 5 ml of *dilute ammonia R2* and dilute to 25 ml with *water R*.

Apply separately to the plate 5 µl of each solution. Allow the plate to dry in air. Develop over a path of 15 cm using a mixture of 20 volumes of *glacial acetic acid R*, 20 volumes of *water R* and 60 volumes of *butanol R*. Dry the plate in a current of warm air. Spray with *ninhydrin solution R* and heat at 100 °C to 105 °C for 15 min. Any spot in the chromatogram obtained with test solution (a), apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.5 per cent). The test is not valid unless the chromatogram obtained with reference solution (c) shows two clearly separated principal spots.

Chlorides (2.4.4). Dissolve 33 mg in 5 ml of *dilute nitric acid R* and dilute to 15 ml with *water R*. The solution, without further addition of nitric acid, complies with the limit test for chlorides (0.15 per cent).

Sulphates (2.4.13). Dissolve 0.5 g in 5 ml of *dilute hydrochloric acid R* and dilute to 15 ml with *distilled water R*. The solution complies with the limit test for sulphates (300 ppm).

Heavy metals (2.4.8). 2.0 g complies with limit test D for heavy metals (10 ppm). Prepare the standard using 2 ml of *lead standard solution (10 ppm Pb) R*.

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C for 2 h.

Sulphated ash (2.4.14). Not more than 0.3 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.150 g in 10 ml of *anhydrous formic acid R* with slight heating and shake until dissolution is complete. Add 50 ml of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (2.2.20).

1 ml of *0.1 M perchloric acid* is equivalent to 17.92 mg of $C_5H_9NO_4S$.

STORAGE

Store protected from light.

01/2005:1299

CARBOMERS

Carbomera

DEFINITION

High molecular mass polymers of acrylic acid cross-linked with polyalkenyl ethers of sugars or polyalcohols.

Content: 56.0 per cent to 68.0 per cent of carboxylic acid (–COOH) groups (dried substance).

CHARACTERS

Appearance: white, fluffy powder, hygroscopic.

Solubility: swells in water and in other polar solvents after dispersion and neutralisation with sodium hydroxide solution.

IDENTIFICATION

First identification: A, E.

Second identification: B, C, D, E.

A. Infrared absorption spectrophotometry (2.2.24).

Main bands: at 2960 cm^{-1} , 1720 cm^{-1} , 1455 cm^{-1} , 1415 cm^{-1} , 1250 cm^{-1} , 1175 cm^{-1} and 800 cm^{-1} , with the strongest band at 1720 cm^{-1} .

B. Adjust a 10 g/l dispersion to about pH 7.5 with *1 M sodium hydroxide*. A highly viscous gel is formed.

C. Add 2 ml of a 100 g/l solution of *calcium chloride R* with continuous stirring to 10 ml of the gel from test B. A white precipitate is immediately produced.

D. Add 0.5 ml of *thymol blue solution R* to 10 ml of a 10 g/l dispersion. An orange colour is produced. Add 0.5 ml of *cresol red solution R* to 10 ml of a 10 g/l dispersion. A yellow colour is produced.

E. It complies with the apparent nominal viscosity indicated on the label.

TESTS

Apparent viscosity: the nominal apparent viscosity is in the range 300 mPa.s to 115 000 mPa.s. For a product with a nominal apparent viscosity of 20 000 mPa.s or greater, the apparent viscosity is 70.0 per cent to 130.0 per cent of the value stated on the label; for a product with a nominal apparent viscosity less than 20 000 mPa.s, the apparent viscosity is 50.0 per cent to 150.0 per cent of the value stated on the label.

Dry the substance to be examined *in vacuo* at 80 °C for 1 h. Carefully add 2.50 g of the previously dried substance to be examined to 500 ml of *water R* in a 1000 ml beaker while stirring continuously at 1000 ± 50 r/min, with the stirrer shaft set at an angle of 60° to one side of the beaker. Add the previously dried substance over a period of 45-90 s, at a uniform rate, ensuring that loose aggregates of powder are broken up and continue stirring at 1000 ± 50 r/min for 15 min. Remove the stirrer, and place the beaker containing the dispersion in a water-bath at 25 ± 0.2 °C for 30 min. Insert the stirrer to a depth necessary to ensure that air is not drawn into the dispersion, and while stirring at 300 ± 25 r/min, titrate with a glass-calomel electrode system to pH 7.3-7.8 by adding a 180 g/l solution of *sodium hydroxide R* below the surface, determining the end-point potentiometrically (2.2.20). The total volume of the 180 g/l solution of *sodium hydroxide R* used is about 6.2 ml. Allow 2-3 min before the final pH determination. If the final pH exceeds 7.8, discard the preparation, and prepare another using a smaller amount of sodium hydroxide for titration. Return the neutralised preparation to the water-bath at 25 °C for 1 h, then perform the viscosity determination without delay to avoid slight viscosity changes that occur 75 min after neutralisation. Determine the viscosity (2.2.10) with a rotating viscometer with a spindle rotating at 20 r/min, using a spindle suitable for the expected apparent viscosity.

Free acrylic acid. Liquid chromatography (2.2.29).

Test solution. Mix 0.125 g of the substance to be examined with a 25 g/l solution of *aluminium potassium sulphate R* and dilute to 25.0 ml with the same solution. Heat the suspension at 50 °C for 20 min with shaking. Then shake the suspension at room temperature for 60 min. Centrifuge and use the clear supernatant solution as the test solution.

Reference solution. Dissolve 62.5 mg of *acrylic acid R* in a 25 g/l solution of *aluminium potassium sulphate R* and dilute to 100.0 ml with the same solution. Dilute 1.0 ml of this solution to 50.0 ml with a 25 g/l solution of *aluminium potassium sulphate R*.

Column:

- **size:** $l = 0.12$ m, $\varnothing = 4.6$ mm,
- **stationary phase:** octadecylsilyl silica gel for chromatography *R* (5 µm).

Mobile phase:

- **mobile phase A:** a 1.361 g/l solution of *potassium dihydrogen phosphate R*, adjusted to pH 2.5 using *dilute phosphoric acid R*,
- **mobile phase B:** equal volumes of a 1.361 g/l solution of *potassium dihydrogen phosphate R* and *acetonitrile for chromatography R*.

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)
0 - 8	100	0
8 - 9	100 → 0	0 → 100
9 - 20	0	100
20 - 21	0 → 100	100 → 0
21 - 30	100	0

Flow rate: 1 ml/min.

Detection: spectrophotometer at 205 nm.

Injection: 20 µl.

Retention time: acrylic acid = about 6.0 min.

Limit:

- **acrylic acid:** not more than the area of the corresponding peak in the chromatogram obtained with the reference solution (0.25 per cent).

Benzene. Gas chromatography (2.4.24, *System A*).

Solvent solution. Dissolve 0.100 g of *benzene R* in *dimethyl sulphoxide R* and dilute to 100.0 ml with the same solvent. Dilute 1.0 ml of the solution to 100.0 ml with *water R*. Dilute 1.0 ml of this solution to 100.0 ml with *water R*.

Test solution. Weigh 50.0 mg of the substance to be examined into an injection vial and add 5.0 ml of *water R* and 1.0 ml of *dimethyl sulphoxide R*.

Reference solution. Weigh 50.0 mg of the substance to be examined into an injection vial and add 4.0 ml of *water R*, 1.0 ml of *dimethyl sulphoxide R* and 1.0 ml of the solvent solution.

Close the vials with a tight rubber membrane stopper coated with polytetrafluoroethylene and secure with an aluminium crimped cap. Shake to obtain a homogeneous dispersion.

Stratic head-space conditions which may be used:

- **equilibration temperature:** 80 °C,
- **equilibration time:** 60 min,
- **transfer line temperature:** 90 °C.

Injection: 1 ml of the gaseous phase of the test solution and 1 ml of the gaseous phase of the reference solution; repeat these injections twice more.

System suitability:

- **repeatability:** maximum relative standard deviation of the differences in area between the analyte peaks obtained from the 3 replicate pair injections of the reference solution and the test solution is 15 per cent.

Limit:

- **benzene:** the mean area of the peak corresponding to benzene in the chromatograms obtained with the test solution is not greater than half the mean area of the peak corresponding to benzene in the chromatograms obtained with the reference solution (2 ppm).

Heavy metals (2.4.8): maximum 20 ppm.

1.0 g complies with limit test C. Prepare the standard using 2 ml of *lead standard solution (10 ppm Pb) R*.

Loss on drying (2.2.32): maximum 3.0 per cent, determined on 1.000 g by drying *in vacuo* at 80 °C for 60 min.

Sulphated ash (2.4.14): maximum 4.0 per cent, determined on 1.0 g.

ASSAY

Slowly add 50 ml of *water R* to 0.120 g whilst stirring and heating at 60 °C for 15 min. Stop heating, add 150 ml of *water R* and continue stirring for 30 min. Add 2 g

of *potassium chloride R* and titrate with 0.2 M *sodium hydroxide*, determining the end-point potentiometrically (2.2.20).

1 ml of 0.2 M *sodium hydroxide* is equivalent to 9.0 mg of carboxylic acid (-COOH) groups.

STORAGE

In an airtight container.

LABELLING

The label states the nominal apparent viscosity.

01/2005:0375

CARBON DIOXIDE

Carbonei dioxidum

CO₂

M_r 44.01

DEFINITION

Content: minimum 99.5 per cent V/V of CO₂ in the gaseous phase.

This monograph applies to carbon dioxide for medicinal use.

CHARACTERS

Appearance: colourless gas.

Solubility: at 20 °C and at a pressure of 101 kPa, 1 volume dissolves in about 1 volume of water.

PRODUCTION

Examine the gaseous phase.

If the test is performed on a cylinder of gas, keep the cylinder of the substance to be examined at room temperature for not less than 6 h before carrying out the tests. Keep the cylinder in the vertical position with the outlet valve uppermost.

Carbon monoxide. Gas chromatography (2.2.28).

Gas to be examined. The substance to be examined.

Reference gas. A mixture containing 5 ppm V/V of *carbon monoxide R* in *nitrogen R1*.

Column:

- *material*: stainless steel,

- *size*: *l* = 2 m, Ø = 4 mm,

- *stationary phase*: an appropriate molecular sieve for chromatography (0.5 nm).

Carrier gas: *helium for chromatography R*.

Flow rate: 60 ml/min.

Temperature:

- *column*: 50 °C,

- *injection port and detector*: 130 °C.

Detection: flame ionisation with methaniser.

Injection: loop injector.

Adjust the injected volumes and the operating conditions so that the height of the peak due to carbon monoxide in the chromatogram obtained with the reference gas is at least 35 per cent of the full scale of the recorder.

Limit:

- *carbon monoxide*: not more than the area of the corresponding peak in the chromatogram obtained with the reference gas (5 ppm V/V).

Nitrogen monoxide and nitrogen dioxide: maximum 2 ppm V/V in total, determined using a chemiluminescence analyser (2.5.26).

Gas to be examined. The substance to be examined.

Reference gas (a). *Carbon dioxide R1*.

Reference gas (b). A mixture containing 2 ppm V/V of *nitrogen monoxide R* in *carbon dioxide R1* or in *nitrogen R1*.

Calibrate the apparatus and set the sensitivity using reference gases (a) and (b). Measure the content of nitrogen monoxide and nitrogen dioxide in the gas to be examined.

If nitrogen is used instead of carbon dioxide in reference gas (b), multiply the result obtained by the quenching correction factor in order to correct the quenching effect on the analyser response caused by the carbon dioxide matrix effect.

The quenching correction factor is determined by applying a known reference mixture of nitrogen monoxide in carbon dioxide and comparing the actual content with the content indicated by the analyser which has been calibrated with a NO/N₂ reference mixture.

$$\text{Quenching correction factor} = \frac{\text{actual nitrogen monoxide content}}{\text{indicated nitrogen monoxide content}}$$

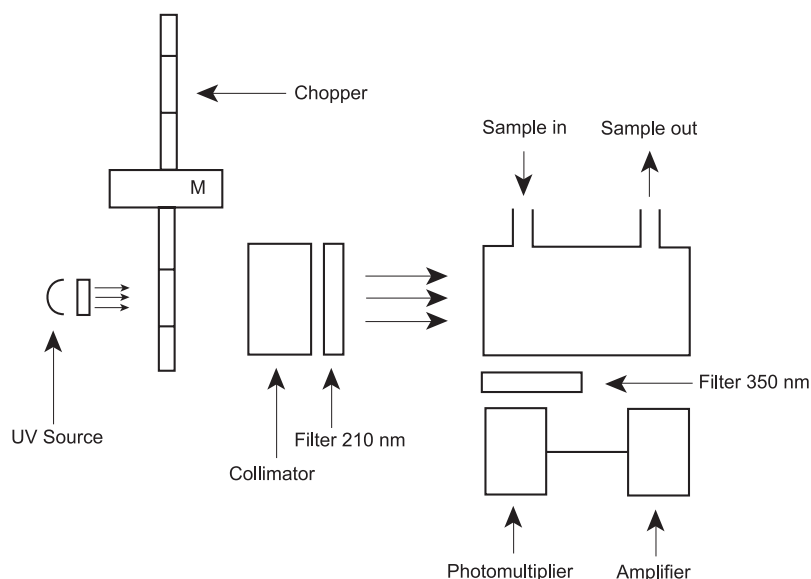


Figure 0375.-1.– UV Fluorescence Analyser

Total sulphur: maximum 1 ppm V/V, determined using an ultraviolet fluorescence analyser after oxidation of the sulphur compounds by heating at 1000 °C (Figure 0375.-1).

The apparatus consists of the following:

- a system generating ultraviolet radiation with a wavelength of 210 nm, made up of an ultraviolet lamp, a collimator, and a selective filter; the beam is blocked periodically by a chopper rotating at high speed,
- a reaction chamber through which flows the previously filtered gas to be examined,
- a system that detects radiation emitted at a wavelength of 350 nm, made up of a selective filter, a photomultiplier tube and an amplifier.

Gas to be examined. The substance to be examined.

Reference gas (a). Carbon dioxide R1.

Reference gas (b). A mixture containing between 0.5 ppm V/V and 2 ppm V/V of hydrogen sulphide R1 in carbon dioxide R1.

Calibrate the apparatus and set the sensitivity using reference gases (a) and (b). Pass the gas to be examined through a quartz oven heated to 1000 °C. Oxygen R is circulated in the oven at a tenth of the flow rate of the gas to be examined. Measure the sulphur dioxide content in the gaseous mixture leaving the oven.

Water: maximum 67 ppm V/V, determined using an electrolytic hygrometer (2.5.28).

Assay. Infrared analyser (2.5.24).

Gas to be examined. The substance to be examined. It must be filtered to avoid stray light phenomena.

Reference gas (a). Carbon dioxide R1.

Reference gas (b). A mixture containing 95.0 per cent V/V of carbon dioxide R1 and 5.0 per cent V/V of nitrogen R1.

Calibrate the apparatus and set the sensitivity using reference gases (a) and (b). Measure the content of carbon dioxide in the gas to be examined.

IDENTIFICATION

First identification: A.

Second identification: B, C.

A. Infrared absorption spectrophotometry (2.2.24).

Comparison: Ph. Eur. reference spectrum of carbon dioxide.

B. Place a glowing splinter of wood in an atmosphere of the substance to be examined. It is extinguished.

C. Pass a stream of the substance to be examined through barium hydroxide solution R. A white precipitate is formed which dissolves with effervescence in dilute acetic acid R.

TESTS

Examine the gaseous phase.

If the test is performed on a cylinder of gas, keep the cylinder of the substance to be examined at room temperature for not less than 6 h before carrying out the tests. Keep the cylinder in the vertical position with the outlet valve uppermost.

Carbon monoxide: maximum 5 ppm V/V, determined using a carbon monoxide detector tube (2.1.6).

Hydrogen sulphide: maximum 1 ppm V/V, determined using a hydrogen sulphide detector tube (2.1.6).

Nitrogen monoxide and nitrogen dioxide: maximum 2 ppm V/V in total, determined using a nitrogen monoxide and nitrogen dioxide detector tube (2.1.6).

Sulphur dioxide: maximum 2 ppm V/V, determined using a sulphur dioxide detector tube (2.1.6).

Water vapour: maximum 67 ppm V/V, determined using a water vapour detector tube (2.1.6).

STORAGE

Store liquefied under pressure in suitable containers complying with the legal regulations.

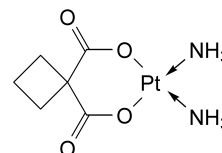
IMPURITIES

- nitrogen monoxide,
- nitrogen dioxide,
- carbon monoxide,
- total sulphur,
- water.

01/2005:1081

CARBOPLATIN

Carboplatinum



$C_6H_{12}N_2O_4Pt$

M_r 371.3

DEFINITION

Carboplatin contains not less than 98.0 per cent and not more than the equivalent of 102.0 per cent of (SP-4-2)-diammine[cyclobutan-1,1-dicarboxylato(2-)-O,O']platin, calculated with reference to the dried substance.

CHARACTERS

A colourless, crystalline powder, sparingly soluble in water, very slightly soluble in acetone and in alcohol.

It melts at about 200 °C, with decomposition.

IDENTIFICATION

Examine by infrared absorption spectrophotometry (2.2.24), comparing with the Ph. Eur. reference spectrum of carboplatin.

TESTS

Solution S1. Dissolve 0.25 g in carbon dioxide-free water R and dilute to 25 ml with the same solvent.

Solution S2. Dissolve 1.0 g in water R, heating slightly if necessary, and dilute to 40 ml with the same solvent. Filter if necessary.

Appearance of solution. Solution S1 is clear (2.2.1) and colourless (2.2.2, Method II).

Impurity B and acidity. To 10 ml of solution S1 add 0.1 ml of phenolphthalein solution R1. The solution is colourless. Not more than 0.7 ml of 0.01 M sodium hydroxide is required to change the colour of the indicator to pink (0.5 per cent calculated as impurity B).

Related substances. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 20.0 mg of the substance to be examined in a mixture of equal volumes of acetonitrile R and water R and dilute to 20.0 ml with the same mixture.

Reference solution. Dilute 0.5 ml of the test solution to 200.0 ml with the mobile phase.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4.6 mm in internal diameter packed with *aminopropylsilyl silica gel for chromatography R* (5 µm),
- as mobile phase at a flow rate of 2 ml/min a mixture of 130 volumes of *water R* and 870 volumes of *acetonitrile R*,
- a 10 µl loop injector,
- as detector a spectrophotometer set at 230 nm.

Inject the test solution. The test is not valid unless:

- the mass distribution ratio, D_m , is not less than 4.0,
- the number of theoretical plates, n , is not less than 5000,
- the symmetry factor is not more than 2.0.

If necessary adjust the concentration of acetonitrile in the mobile phase.

Inject the test solution and the reference solution. Continue the chromatography for 2.5 times the retention time of the principal peak. In the chromatogram obtained with the test solution; the area of any peak apart from the principal peak is not greater than the area of the principal peak in the chromatogram obtained with the reference solution (0.25 per cent); the sum of the areas of all the peaks apart from the principal peak is not greater than twice the area of the principal peak in the chromatogram obtained with the reference solution (0.5 per cent). Disregard any peak with an area less than 0.2 times the area of the principal peak in the chromatogram obtained with the reference solution.

Chlorides (2.4.4). Dilute 10 ml of solution S2 to 15 ml with *water R*. The solution complies with the limit test for chlorides (100 ppm). Prepare the standard using 5 ml of *chloride standard solution (5 ppm Cl) R*.

Ammonium (2.4.1). 0.20 g complies with the limit test B for ammonium (100 ppm). Prepare the standard using 0.2 ml of *ammonium standard solution (100 ppm NH₄) R*.

Silver. Not more than 10 ppm of Ag, determined by atomic emission spectrometry (2.2.22, *Method I*).

Test solution. Dissolve 0.50 g in a 1 per cent V/V solution of *nitric acid R* and dilute to 50.0 ml with the same acid.

Reference solutions. Prepare the reference solutions using *silver standard solution (5 ppm Ag) R*, diluted with a 1 per cent V/V solution of *nitric acid R*.

Measure the intensity emitted at 328.1 nm.

Soluble barium. Not more than 10 ppm of Ba, determined by atomic emission spectrometry (2.2.22, *Method I*).

Test solution. Use the solution described in the test for silver.

Reference solutions. Prepare the reference solutions using *barium standard solution (50 ppm Ba) R*, diluted with a 1 per cent V/V solution of *nitric acid R*.

Measure the intensity emitted at 455.4 nm.

Heavy metals (2.4.8). 12 ml of solution S2 complies with limit test A for heavy metals (20 ppm). Prepare the standard using 5 ml of *lead standard solution (1 ppm Pb R)*.

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C.

ASSAY

Use the residue obtained in the test for loss on drying.

Ignite 0.200 g of the residue to constant mass at 800 °C.

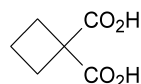
1 mg of the residue is equivalent to 1.903 mg of C₁₂H₂₄N₂O₄Pt.

STORAGE

Store protected from light.

IMPURITIES

A. cisplatin,

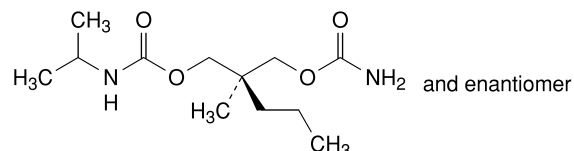


B. cyclobutane-1,1-dicarboxylic acid.

01/2005:1689

CARISOPRODOL

Carisoprodolum



C₁₂H₂₄N₂O₄

M_r 260.3

DEFINITION

(2*RS*)-2-[(Carbamoyloxy)methyl]-2-methylpentyl (1-methylethyl)carbamate.

Content: 98.0 per cent to 102.0 per cent (dried substance).

CHARACTERS

Appearance: white or almost white, fine powder.

Solubility: very slightly soluble in water, freely soluble in acetone, in alcohol and in methylene chloride.

IDENTIFICATION

First identification: A, B.

Second identification: A, C, D.

A. Melting point (2.2.14): 92 °C to 95 °C.

B. Infrared absorption spectrophotometry (2.2.24).

Comparison: *carisoprodol CRS*.

C. Examine the chromatograms obtained in the test for related substances.

Results: the principal spot in the chromatogram obtained with test solution (b) is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (d).

D. Dissolve 0.2 g in 15 ml of a 28 g/l solution of *potassium hydroxide R* in *alcohol R* and boil under a reflux condenser for 15 min. Add 0.5 ml of *glacial acetic acid R* and 1 ml of a 50 g/l solution of *cobalt nitrate R* in *ethanol R*. An intense blue colour develops.

TESTS

Optical rotation (2.2.7): −0.10° to +0.10°.

Dissolve 2.5 g in *alcohol R* and dilute to 25.0 ml with the same solvent.

Related substances. Thin-layer chromatography (2.2.27).

Test solution (a). Dissolve 0.20 g of the substance to be examined in *methylene chloride R* and dilute to 10 ml with the same solvent.

Test solution (b). Dilute 1 ml of test solution (a) to 10 ml with *methylene chloride R*.

Reference solution (a). Dissolve 5.0 mg of *meprobamate CRS* in *methylene chloride R* and dilute to 50 ml with the same solvent.

Reference solution (b). Dilute 1 ml of test solution (b) to 50 ml with *methylene chloride R*.

Reference solution (c). Dilute 5 ml of reference solution (b) to 10 ml with *methylene chloride R*.

Reference solution (d). Dissolve 20 mg of *carisoprodol CRS* in *methylene chloride R* and dilute to 10 ml with the same solvent.

Reference solution (e). Dissolve 10 mg of *carisoprodol impurity A CRS* in 5 ml of reference solution (d) and dilute to 50 ml with *methylene chloride R*.

Plate: TLC silica gel plate *R*.

Mobile phase: acetone *R*, *methylene chloride R* (20:80 V/V).

Application: 5 µl.

Development: over a path of 15 cm.

Drying: in air for 15 min.

Detection: spray with a solution prepared as follows: dissolve 5 g of *phosphomolybdic acid R* in a mixture of 50 ml of *glacial acetic acid R* and 10 ml of *sulphuric acid R*, and dilute to 100 ml with *glacial acetic acid R*. Heat the plate at 100-105 °C for 30 min.

System suitability:

- the chromatogram obtained with reference solution (c) shows 1 clearly visible spot,
- the chromatogram obtained with reference solution (e) shows 2 clearly separated spots.

Limits: in the chromatogram obtained with test solution (a):

- **impurity D:** any spot due to impurity D is not more intense than the spot in the chromatogram obtained with reference solution (a) (0.5 per cent),
- **any other impurity:** any spot, apart from the principal spot and any spot due to impurity D, is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.2 per cent).

Heavy metals (2.4.8): maximum 10 ppm.

2.0 g complies with limit test C. Prepare the standard using 2 ml of *lead standard solution (10 ppm Pb) R*.

Loss on drying (2.2.32): maximum 0.5 per cent, determined on 1.000 g *in vacuo* at 60 °C for 3 h.

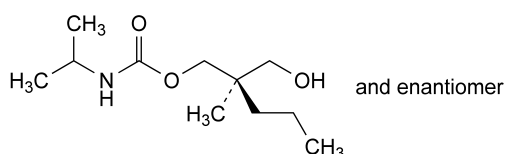
Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

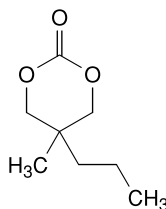
Dissolve 0.100 g in 15 ml of a 25 per cent V/V solution of *sulphuric acid R* and boil under a reflux condenser for 3 h. Cool, dissolve by cautiously adding 30 ml of *water R*, cool again and place in a steam-distillation apparatus. Add 40 ml of *strong sodium hydroxide solution R* and distil immediately by passing steam through the mixture. Collect the distillate into 40 ml of a 40 g/l solution of *boric acid R* until the total volume in the receiver reaches about 200 ml. Add 0.25 ml of *methyl red mixed solution R*. Titrate with 0.1 M *hydrochloric acid*, until the colour changes from green to violet. Carry out a blank titration.

1 ml of 0.1 M *hydrochloric acid* is equivalent to 13.02 mg of $C_{12}H_{24}N_2O_4$.

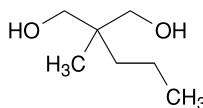
IMPURITIES



- A. (2*RS*)-2-(hydroxymethyl)-2-methylpentyl (1-methylethyl)carbamate,



- B. 5-methyl-5-propyl-1,3-dioxan-2-one,



- C. 2-methyl-2-propylpropane-1,3-diol,

- D. meprobamate.

01/2005:0886

CARMELLOSE CALCIUM

Carmellosum calcicum

DEFINITION

Calcium salt of a partly *O*-carboxymethylated cellulose.

CHARACTERS

Appearance: white or yellowish-white powder, hygroscopic after drying.

Solubility: practically insoluble in acetone, in alcohol and in toluene. It swells with water to form a suspension.

IDENTIFICATION

- Shake 0.1 g thoroughly with 10 ml of *water R*. Add 2 ml of *dilute sodium hydroxide solution R* and allow to stand for 10 min (solution A). Dilute 1 ml of solution A to 5 ml with *water R*. To 0.05 ml add 0.5 ml of a 0.5 g/l solution of *chromotropic acid, sodium salt R* in a 75 per cent *m/m* solution of *sulphuric acid R* and heat on a water-bath for 10 min. A reddish-violet colour develops.
- Shake 5 ml of solution A obtained in identification test A with 10 ml of *acetone R*. A white, flocculent precipitate is produced.
- Shake 5 ml of solution A obtained in identification test A with 1 ml of *ferric chloride solution R1*. A brown, flocculent precipitate is formed.
- Ignite 1 g and dissolve the residue in a mixture of 5 ml of *acetic acid R* and 10 ml of *water R*. Filter if necessary and boil the filtrate for a few minutes. Cool and neutralise with *dilute ammonia R1*. The solution gives reaction (a) of calcium (2.3.1).

TESTS

Solution S. Shake 1.0 g with 50 ml of *distilled water R*, add 5 ml of *dilute sodium hydroxide solution R* and dilute to 100 ml with *distilled water R*.

Alkalinity. Shake 1.0 g thoroughly with 50 ml of *carbon dioxide-free water R* and add 0.05 ml of *phenolphthalein solution R*. No red colour develops.

Chlorides (2.4.4): maximum 0.36 per cent.

Heat 28 ml of solution S with 10 ml of *dilute nitric acid R* on a water-bath until a flocculent precipitate is produced. Cool, centrifuge and separate the supernatant liquid. Wash the precipitate with 3 quantities, each of 10 ml, of *water R*, centrifuging each time. Combine the supernatant liquid and the washings and dilute to 100 ml with *water R*. To 25 ml

add 6 ml of *dilute nitric acid R* and dilute to 50 ml with *water R*. Dilute 10 ml of the solution to 15 ml with *water R*.

Sulphates (2.4.13): maximum 1 per cent.

Heat 20 ml of solution S with 1 ml of *hydrochloric acid R* on a water-bath until a flocculent precipitate is produced. Cool, centrifuge and separate the supernatant liquid. Wash the precipitate with 3 quantities, each of 10 ml, of *distilled water R*, centrifuging each time. Combine the supernatant liquid and the washings and dilute to 100 ml with *distilled water R*. To 25 ml add 1 ml of *dilute hydrochloric acid R* and dilute to 50 ml with *distilled water R*.

Heavy metals (2.4.8): maximum 20 ppm.

1.0 g complies with limit test D. Prepare the standard using 2 ml of *lead standard solution (10 ppm Pb) R*.

Loss on drying (2.2.32): maximum 10.0 per cent, determined on 1.000 g by drying in an oven at 100–105 °C for 4 h.

Sulphated ash (2.4.14): 10.0 per cent to 20.0 per cent, determined on 1.0 g in a platinum crucible.

STORAGE

In an airtight container.

01/2005:0472

CARMELLOSE SODIUM

Carmellosum natricum

DEFINITION

Carmellose sodium (carboxymethylcellulose sodium) is the sodium salt of a partly *O*-carboxymethylated cellulose. It contains not less than 6.5 per cent and not more than 10.8 per cent of sodium (Na), calculated with reference to the dried substance.

CHARACTERS

A white or almost white, granular powder, hygroscopic after drying, practically insoluble in acetone, in ethanol and in toluene. It is easily dispersed in water giving colloidal solutions.

IDENTIFICATION

- To 10 ml of solution S (see Tests) add 1 ml of *copper sulphate solution R*. A blue, cotton-like precipitate is formed.
- Boil 5 ml of solution S for a few minutes. No precipitate is formed.
- The solution prepared from the sulphated ash in the test for heavy metals gives the reactions of sodium (2.3.1).

TESTS

Solution S. Sprinkle a quantity of the substance to be examined equivalent to 1.0 g of the dried substance onto 90 ml of *carbon dioxide-free water R* at 40 °C to 50 °C stirring vigorously. Continue stirring until a colloidal solution is obtained, cool and dilute to 100 ml with *carbon dioxide-free water R*.

Appearance of solution. Solution S is not more opalescent than reference suspension III (2.2.1) and not more intensely coloured than reference solution Y₆ (2.2.2, *Method II*).

pH (2.2.3). The pH of solution S is 6.0 to 8.0.

Apparent viscosity. While stirring, introduce a quantity of the substance to be examined equivalent to 2.00 g of the dried substance into 50 ml of *water R* heated to 90 °C. For a product of low viscosity, use if necessary, the quantity required to give the concentration indicated on the label. Allow to cool, dilute to 100.0 ml with *water R* and stir until dissolution is complete. Determine the viscosity (2.2.10) using a rotating viscometer at 20 °C and a shear rate of 10 s⁻¹. If it is impossible to obtain a shear rate of exactly 10 s⁻¹, use a shear rate slightly higher and a rate slightly lower and interpolate. The apparent viscosity is not less than 75 per cent and not more than 140 per cent of the value stated on the label.

Sodium glycollate. Place a quantity of the substance to be examined equivalent to 0.500 g of dried substance in a beaker. Add 5 ml of *acetic acid R* and 5 ml of *water R*. Stir until dissolution is complete (about 30 min). Add 80 ml of *acetone R* and 2 g of *sodium chloride R*. Filter through a fast filter paper impregnated with *acetone R* into a volumetric flask, rinse the beaker and filter with *acetone R* and dilute the filtrate to 100.0 ml with the same solvent. Allow to stand for 24 h without shaking. Use the clear supernatant liquid to prepare the test solution.

In a volumetric flask, dissolve 0.310 g of *glycollic acid R*, previously dried *in vacuo* over *diphosphorus pentoxide R*, in *water R* and dilute to 1000.0 ml with the same solvent. Place 5.0 ml of this solution in a volumetric flask, add 5 ml of *acetic acid R* and allow to stand for about 30 min. Add 80 ml of *acetone R* and 2 g of *sodium chloride R* and dilute to 100.0 ml with *acetone R*. Use this solution to prepare the reference solution.

Place 2.0 ml of each solution in a separate 25 ml volumetric flask. Heat on a water-bath to eliminate acetone. Cool to room temperature and add 5.0 ml of *2,7-dihydroxynaphthalene solution R* to each flask. Shake and add 15.0 ml of *2,7-dihydroxynaphthalene solution R*. Close the flasks with aluminium foil and heat on a water-bath for 20 min. Cool under running water and dilute to 25.0 ml with *sulphuric acid R*. Within 10 min, transfer 10.0 ml of each solution to a flat-bottomed tube. Examine the solutions viewing vertically. The test solution is not more intensely coloured than the reference solution (0.4 per cent).

Chlorides (2.4.4). Dilute 2 ml of solution S to 15 ml with *water R*. The solution complies with the limit test for chlorides (0.25 per cent).

Heavy metals (2.4.8). To the residue obtained in the determination of the sulphated ash, add 1 ml of *hydrochloric acid R* and evaporate on a water-bath. Take up the residue in 20 ml of *water R*. 12 ml of the solution complies with limit test A for heavy metals (20 ppm). Prepare the standard using *lead standard solution (1 ppm Pb) R*.

Loss on drying (2.2.32). Not more than 10.0 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C.

Sulphated ash (2.4.14): 20.0 per cent to 33.3 per cent, determined on 1.0 g using a mixture of equal volumes of *sulphuric acid R* and *water R* and calculated with reference to the dried substance. These limits correspond to a content of 6.5 per cent to 10.8 per cent of sodium (Na).

LABELLING

The label states the apparent viscosity in millipascal seconds for a 20 g/l solution; for a product of low viscosity, the label states the concentration of the solution to be used and the apparent viscosity in millipascal seconds.

01/2005:1186

CARMELLOSE SODIUM, LOW-SUBSTITUTED

Carmellosum natricum, substitutum humile

DEFINITION

Low-substituted carmellose sodium (sodium carboxymethylcellulose) is the sodium salt of a partly *O*-(carboxymethylated) cellulose. It contains not less than 2.0 per cent and not more than 4.5 per cent of sodium (Na), calculated with reference to the dried substance.

CHARACTERS

A white or almost white powder or short fibres, practically insoluble in acetone, in ethanol and in toluene. It swells in water to form a gel.

IDENTIFICATION

- Shake 1 g with 100 ml of a 100 g/l solution of *sodium hydroxide R*. A suspension is produced.
- Shake 1 g with 50 ml of *water R*. Transfer 1 ml of the mixture to a test tube, add 1 ml of *water R* and 0.05 ml of a freshly prepared 40 g/l solution of α -*naphthol R* in *methanol R*. Incline the test tube and add carefully 2 ml of *sulphuric acid R* down the side so that it forms a lower layer. A reddish-purple colour develops at the interface.
- It complies with the test for sulphated ash (2.4.14) (see Tests).
- The solution prepared from the sulphated ash for the test for heavy metals gives reaction (a) of sodium (2.3.1).

TESTS

pH (2.2.3). Shake 1 g with 100 ml of *carbon dioxide-free water R* for 5 min. Centrifuge. The pH of the suspension is 6.0 to 8.5.

Sodium chloride and sodium glycollate. The sum of the percentages of sodium chloride and sodium glycollate is not more than 0.5 per cent, calculated with reference to the dried substance.

Sodium chloride. Place 5.00 g in a 250 ml conical flask, add 50 ml of *water R* and 5 ml of *strong hydrogen peroxide solution R* and heat on a water bath for 20 min, stirring occasionally to ensure total hydration. Cool, add 100 ml of *water R* and 10 ml of *nitric acid R*. Titrate with 0.05 *M silver nitrate* determining the end-point potentiometrically (2.2.20) using a silver-based indicator electrode and a double-junction reference electrode containing a 100 g/l solution of *potassium nitrate R* in the outer jacket and a standard filling solution in the inner jacket.

1 ml of 0.05 *M silver nitrate* is equivalent to 2.922 mg of NaCl.

Sodium glycollate. Place a quantity of the substance to be examined equivalent to 0.500 g of the dried substance in a beaker. Add 5 ml of *glacial acetic acid R* and 5 ml of *water R* and stir to ensure total hydration (about 30 min). Add 80 ml of *acetone R* and 2 g of *sodium chloride R*. Stir for several minutes to ensure complete precipitation of the carboxymethylcellulose. Filter through a fast filter paper impregnated with *acetone R* into a volumetric flask, rinse the beaker and filter with *acetone R* and dilute the filtrate to 100.0 ml with the same solvent. Allow to stand for 24 h without shaking. Use the clear supernatant as the test solution.

Prepare the reference solutions as follows: in a 100 ml volumetric flask, dissolve 0.100 g of *glycollic acid R*, previously dried *in vacuo* over *diphosphorus pentoxide R*, in *water R* and dilute to 100.0 ml with the same solvent. Transfer 0.5 ml, 1.0 ml, 1.5 ml and 2.0 ml of the solution to separate volumetric flasks; dilute the contents of each flask to 5.0 ml with *water R*, add 5 ml of *glacial acetic acid R*, dilute to 100.0 ml with *acetone R* and mix.

Transfer 2.0 ml of the test solution and 2.0 ml of each of the reference solutions to separate 25 ml volumetric flasks. Heat the uncovered flasks in a water-bath to eliminate the acetone. Allow to cool and add 5.0 ml of *2,7-dihydroxynaphthalene solution R* to each flask. Mix, add a further 15.0 ml of *2,7-dihydroxynaphthalene solution R* and mix again. Close the flasks with aluminium foil and heat in a water-bath for 20 min. Cool and dilute to 25.0 ml with *sulphuric acid R*.

Measure the absorbance (2.2.25) of each solution at 540 nm. Prepare a blank using 2.0 ml of a solution containing 5 per cent *V/V* each of *glacial acetic acid R* and *water R* in *acetone R*. Prepare a standard curve using the absorbances obtained with the reference solutions. From the standard curve and the absorbance of the test solution, determine the mass *a*, in milligrams, of glycollic acid in the substance to be examined and calculate the content of sodium glycollate from the formula:

$$\frac{10 \times 1.29 \times a}{(100 - b) m}$$

1.29 = the factor converting glycollic acid to sodium glycollate,

b = the loss on drying as a percentage,

m = the mass of the substance to be examined, in grams.

Water-soluble substances. Not more than 70.0 per cent. Disperse 5.00 g in 400.0 ml of *water R* and stir for 1 min every 10 min during the first 30 min. Allow to stand for 1 h and centrifuge, if necessary. Decant 100.0 ml of the supernatant liquid onto a fast filter paper in a vacuum filtration funnel, apply vacuum and collect 75.0 ml of the filtrate. Evaporate to dryness and dry the residue at 100-105 °C for 4 h.

Heavy metals (2.4.8). To the residue obtained in the determination of the sulphated ash add 1 ml of *hydrochloric acid R* and evaporate on a water-bath. Take up the residue in 20 ml of *water R*. 12 ml of the solution complies with limit test A for heavy metals (20 ppm). Prepare the standard using *lead standard solution (1 ppm Pb) R*.

Loss on drying (2.2.32). Not more than 10.0 per cent, determined on 1.000 g by drying in an oven at 100-105 °C.

Sulphated ash (2.4.14): 6.5 per cent to 13.5 per cent, determined on 1.0 g using a mixture of equal volumes of *sulphuric acid R* and *water R* and calculated with reference to the dried substance. These limits correspond to a content of 2.0 per cent to 4.5 per cent of Na.

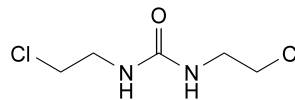
The following test concerning the pharmaco-technological properties may be carried out depending on the intended formulation. It is not a mandatory requirement.

Settling volume. In a 100 ml graduated cylinder, place 20 ml of *2-propanol R*, add 5.0 g of the substance to be examined and shake vigorously. Dilute to 30 ml with *2-propanol R* then to 50 ml with *water R* and shake vigorously. Within 15 min, repeat the shaking three times. Allow to stand for 4 h and determine the volume of the settled mass (15.0 ml to 35.0 ml).

01/2005:1187 STORAGE

Store in an airtight container, protected from light, at a temperature of 2 °C to 8 °C.

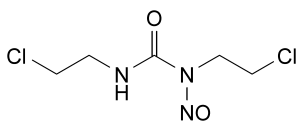
IMPURITIES



A. 1,3-bis(2-chloroethyl)urea.

CARMUSTINE

Carmustinum



$C_5H_9Cl_2N_3O_2$

M_r 214.1

DEFINITION

Carmustine contains not less than 98.0 per cent and not more than the equivalent of 102.0 per cent of 1,3-bis(2-chloroethyl)-1-nitrosourea, calculated with reference to the anhydrous substance.

CHARACTERS

A yellowish, granular powder, very slightly soluble in water, very soluble in methylene chloride, freely soluble in ethanol. It melts at about 31 °C with decomposition.

IDENTIFICATION

Examine by infrared absorption spectrophotometry(2.2.24), comparing with the *Ph. Eur. reference spectrum of carmustine*. Examine the melted substances prepared as films.

TESTS

1,3-bis(2-chloroethyl)urea (impurity A). Examine by thin-layer chromatography (2.2.27), using a suitable silica gel as the coating substance.

Test solution. Dissolve 0.10 g of the substance to be examined in *methylene chloride R* and dilute to 5 ml with the same solvent.

Reference solution (a). Dissolve 2 mg of *carmustine impurity A CRS* in *methylene chloride R* and dilute to 10 ml with the same solvent.

Reference solution (b). Dilute 1 ml of the test solution to 10 ml with *methylene chloride R*. To 5 ml of this solution, add 5 ml of reference solution (a).

Apply separately to the plate 2 µl of each solution. Develop over a path of 10 cm using a mixture of 10 volumes of *methanol R* and 90 volumes of *methylene chloride R*. Allow the plate to dry in air. Spray with *diethylamine R* and heat at 125 °C for 10 min. Allow to cool and spray with *silver nitrate solution R2*. Expose to ultraviolet light at 365 nm until brown to black spots appear. Any spot corresponding to carmustine impurity A in the chromatogram obtained with the test solution is not more intense than the spot in the chromatogram obtained with reference solution (a) (1 per cent). The test is not valid unless the chromatogram obtained with reference solution (b) shows two clearly separated spots.

Water (2.5.12). Not more than 1.0 per cent, determined on 0.50 g by the semi-micro determination of water.

ASSAY

Dissolve 0.100 g in 30 ml of *ethanol R* and dilute to 100.0 ml with *water R*. Dilute 3.0 ml of the solution to 100.0 ml with *water R*. Measure the absorbance (2.2.25) at the maximum at 230 nm.

Calculate the content of $C_5H_9Cl_2N_3O_2$ taking the specific absorbance to be 270.

01/2005:0597

CARNAUBA WAX

Cera carnauba

DEFINITION

Purified wax obtained from the leaves of *Copernicia cerifera* Mart.

CHARACTERS

Appearance: pale yellow or yellow powder, flakes or hard masses.

Solubility: practically insoluble in water, soluble on heating in ethyl acetate and in xylene, practically insoluble in alcohol.

Relative density: about 0.97.

IDENTIFICATION

Thin-layer chromatography (2.2.27).

Test solution. Dissolve 0.10 g of the substance to be examined with heating in 5 ml of *chloroform R*. Use the warm solution.

Reference solution. Dissolve 5 mg of *menthol R*, 5 µl of *menthyl acetate R* and 5 mg of *thymol R* in 10 ml of *toluene R*.

Plate: TLC silica gel plate *R*.

Mobile phase: *ethyl acetate R*, *chloroform R* (2:98 V/V).

Application: 30 µl of the test solution and 10 µl of the reference solution as bands 20 mm by 3 mm.

Development: over half of the plate.

Drying: in air.

Detection: spray with a freshly prepared 200 g/l solution of *phosphomolybdic acid R* in *alcohol R* (about 10 ml for a 20 cm plate). Heat at 100-105 °C for 10-15 min.

Results: the chromatogram obtained with the reference solution shows in the lower part a dark blue zone (menthol), above this zone a reddish zone (thymol) and in the upper part a dark blue zone (menthyl acetate). The chromatogram obtained with the test solution shows a large blue zone (triacontanol = melissyl alcohol) at a level between the thymol and menthol zones in the chromatogram obtained with the reference solution. Further blue zones are visible in the upper part of the chromatogram obtained with the test solution, at levels between those of the menthyl acetate and thymol zones in the chromatogram obtained with the reference solution; above these zones further zones are visible in the chromatogram obtained with the test solution; the zone with the highest R_f value is very pronounced. A number of faint zones are visible below the triacontanol zone and the starting point is coloured blue.

TESTS

Appearance of solution. The solution is clear (2.2.1) and not more intensely coloured than a 50 mg/l solution of *potassium dichromate R* (2.2.2, Method II).

Dissolve 0.10 g with heating in *chloroform R* and dilute to 10 ml with the same solvent.

Melting point (2.2.15): 80 °C to 88 °C.

Melt the substance to be examined carefully on a water-bath before introduction into the capillary tubes. Allow the tubes to stand in the refrigerator for 24 h or at 0 °C for 2 h.

Acid value: 2 to 7.

To 2.000 g (*m* g) in a 250 ml conical flask fitted with a reflux condenser add 40 ml of *xylene R* and a few glass beads. Heat with stirring until the substance is completely dissolved. Add 20 ml of *alcohol R* and 1 ml of *bromothymol blue solution R3* and titrate the hot solution with 0.5 M *alcoholic potassium hydroxide* until a green colour persisting for at least 10 s is obtained (*n*₁ ml). Carry out a blank test (*n*₂ ml). Calculate the acid value from the expression:

$$\frac{28.05 (n_1 - n_2)}{m}$$

Saponification value: 78 to 95.

To 2.000 g (*m* g) in a 250 ml conical flask fitted with a reflux condenser add 40 ml of *xylene R* and a few glass beads. Heat with stirring until the substance is completely dissolved. Add 20 ml of *alcohol R* and 20.0 ml of 0.5 M *alcoholic potassium hydroxide*. Boil under a reflux condenser for 3 h. Add 1 ml of *phenolphthalein solution R1* and titrate the hot solution immediately with 0.5 M *hydrochloric acid* until the red colour disappears. Repeat the heating and titration until the colour no longer reappears on heating (*n*₃ ml). Carry out a blank test (*n*₄ ml). Calculate the saponification value from the expression:

$$\frac{28.05 (n_4 - n_3)}{m}$$

Total ash (2.4.16): maximum 0.25 per cent, determined on 2.0 g.

STORAGE

Protected from light.

Solubility: soluble in water, sparingly soluble in methanol, slightly soluble in alcohol, practically insoluble in methylene chloride.

IDENTIFICATION

A. Infrared absorption spectrophotometry (2.2.24).

Comparison: Ph. Eur. reference spectrum of *carteolol hydrochloride*.

B. It gives reaction (a) of chlorides (2.3.1).

TESTS

Appearance of solution. The solution is clear (2.2.1) and colourless (2.2.2, Method II).

Dissolve 0.300 g in *water R* and dilute to 10 ml with the same solvent.

pH (2.2.3): 5.0 to 6.0.

Dissolve 0.250 g in *carbon dioxide-free water R* and dilute to 25 ml with the same solvent.

Related substances. Liquid chromatography (2.2.29).

Test solution. Dissolve 20.0 mg of the substance to be examined in the mobile phase and dilute to 10.0 ml with the mobile phase.

Reference solution (a). Dilute 1.0 ml of the test solution to 100.0 ml with the mobile phase.

Reference solution (b). Dilute 1.0 ml of reference solution (a) to 10.0 ml with the mobile phase.

Reference solution (c). Dissolve 10 mg of *carteolol for system suitability CRS* in the mobile phase and dilute to 5 ml with the mobile phase.

Reference solution (d). Dilute 5.0 ml of reference solution (b) to 10.0 ml with the mobile phase.

Column:

- size: *l* = 0.25 m, Ø = 4.6 mm,
- stationary phase: octadecylsilyl silica gel for chromatography R (5 µm).

Mobile phase: mix 1 volume of *methanol R2*, 20 volumes of *acetonitrile R* and 79 volumes of a 2.82 g/l solution of *sodium hexanesulphonate R*.

Flow rate: 1 ml/min.

Detection: spectrophotometer at 252 nm.

Injection: 20 µl.

System suitability:

- the chromatogram obtained with reference solution (c) is similar to the chromatogram provided with *carteolol for system suitability CRS*; the peaks due to impurity H and carteolol show base-line separation,
- *signal-to-noise ratio*: minimum 10 for the principal peak in the chromatogram obtained with reference solution (d),
- *number of theoretical plates*: minimum 6000, calculated for the principal peak in the chromatogram obtained with reference solution (a).

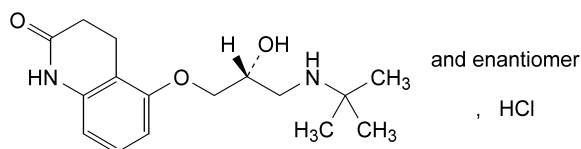
Limits: locate impurity H by comparison with the chromatogram provided with *carteolol for system suitability CRS*,

- *impurity H*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent),
- *any other impurity*: not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent),
- *total*: not more than half the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent),

01/2005:1972

CARTEOLOL HYDROCHLORIDE

Carteololi hydrochloridum



C₁₆H₂₅N₂O₃Cl

*M*_r 328.8

DEFINITION

5-[(2*RS*)-3-[(1,1-Dimethylethyl)amino]-2-hydroxypropoxy]-3,4-dihydroquinolin-2(1*H*)-one hydrochloride.

Content: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

Appearance: white crystals or crystalline powder.

- *disregard limit*: 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.02 per cent).

Loss on drying (2.2.32): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 100–105 °C for 3 h.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

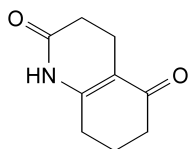
Dissolve 0.250 g in 60 ml of *alcohol R*. Add 5.0 ml of 0.01 M *hydrochloric acid*. Carry out a potentiometric titration (2.2.20), using 0.1 M *sodium hydroxide*. Read the volume added between the 2 points of inflexion.

1 ml of 0.1 M *sodium hydroxide* is equivalent to 32.88 mg of $C_{16}H_{25}N_2O_3Cl$.

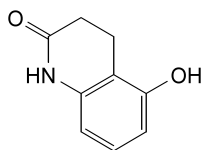
STORAGE

In an airtight container.

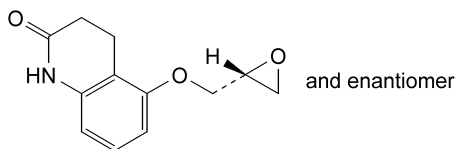
IMPURITIES



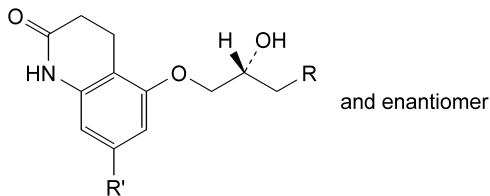
- A. 4,6,7,8-tetrahydroquinoline-2,5(1*H*,3*H*)-dione,



- B. 5-hydroxy-3,4-dihydroquinolin-2(1*H*)-one,



- C. 5-[(2*RS*)-oxiran-2-yl]methoxy-3,4-dihydroquinolin-2(1*H*)-one,

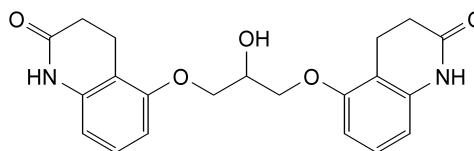


- D. R = Cl, R' = H: 5-[(2*RS*)-3-chloro-2-hydroxypropoxy]-3,4-dihydroquinolin-2(1*H*)-one,

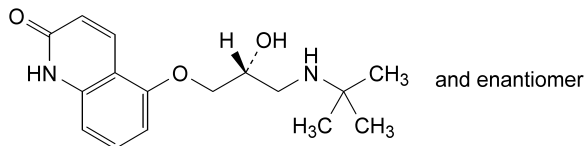
- F. R = OCH₃, R' = H: 5-[(2*RS*)-2-hydroxy-3-methoxypropoxy]-3,4-dihydroquinolin-2(1*H*)-one,

- G. R = OH, R' = H: 5-[(2*RS*)-2,3-dihydroxypropoxy]-3,4-dihydroquinolin-2(1*H*)-one,

- I. R = NH-C(CH₃)₃, R' = Br: 7-bromo-5-[(2*RS*)-3-[(1,1-dimethylethyl)amino]-2-hydroxypropoxy]-3,4-dihydroquinolin-2(1*H*)-one,



- E. 5,5'-[(2-hydroxypropan-1,3-diyl)bis(oxy)]bis(3,4-dihydroquinolin-2(1*H*)-one),

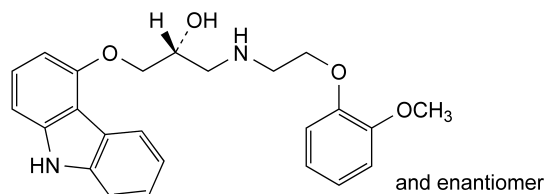


- H. 5-[(2*RS*)-3-[(1,1-dimethylethyl)amino]-2-hydroxypropoxy]quinolin-2(1*H*)-one.

01/2005:1745

CARVEDILOL

Carvedilolum



$C_{24}H_{26}N_2O_4$

M_r 406.5

DEFINITION

(2*RS*)-1-(9*H*-Carbazol-4-yloxy)-3-[[2-(2-methoxyphenoxy)ethyl]amino]propan-2-ol.

Content: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

Appearance: white or almost white, crystalline powder.

Solubility: practically insoluble in water, slightly soluble in alcohol, practically insoluble in dilute acids.

It shows polymorphism.

IDENTIFICATION

Infrared absorption spectrophotometry (2.2.24).

Comparison: *Ph. Eur. reference spectrum of carvedilol*.

If the spectrum obtained shows differences, dissolve the substance to be examined in 2-propanol *R*, evaporate to dryness and record a new spectrum using the residue.

TESTS

Related substances. Liquid chromatography (2.2.29).

Test solution. Dissolve 25.0 mg of the substance to be examined in the mobile phase and dilute to 25.0 ml with the mobile phase.

Reference solution (a). Dilute 1.0 ml of the test solution to 100.0 ml with the mobile phase. Dilute 1.0 ml of this solution to 10.0 ml with the mobile phase.

Reference solution (b). Dissolve 5.0 mg of *carvedilol impurity C CRS* in 5.0 ml of the test solution and dilute to 100.0 ml with the mobile phase.

Reference solution (c). Dilute 1.0 ml of reference solution (b) to 100.0 ml with the mobile phase. Dilute 2.0 ml of this solution to 10.0 ml with the mobile phase.

Column:

– *size*: $l = 0.125$ m, $\varnothing = 4.6$ mm,

- *stationary phase*: octylsilyl silica gel for chromatography R (5 µm),
- *temperature*: 55 °C.

Mobile phase: dissolve 1.77 g of *potassium dihydrogen phosphate* R in *water* R and dilute to 650 ml with the same solvent; adjust to pH 2.0 with *phosphoric acid* R and add 350 ml of *acetonitrile* R.

Flow rate: 1.0 ml/min.

Detection: spectrophotometer at 240 nm.

Injection: 20 µl.

Run time: 8 times the retention time of carvedilol.

Relative retention with reference to carvedilol (retention time = about 4 min): impurity A = about 0.6; impurity C = about 3.5; impurity B = about 6.7.

System suitability: reference solution (b):

- *resolution*: minimum 17 between the peaks due to carvedilol and to impurity C.

Limits:

- *correction factor*: for the calculation of content, multiply the peak area of impurity A by 2,
- *impurity A*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent),
- *impurity C*: not more than twice the area of the corresponding peak in the chromatogram obtained with reference solution (c) (0.02 per cent),
- *any other impurity*: not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent),
- *total*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent),
- *disregard limit*: the area of the principal peak in the chromatogram obtained with reference solution (c) (0.01 per cent).

Heavy metals (2.4.8): maximum 10 ppm.

2.0 g complies with limit test C. Prepare the standard using 2.0 ml of *lead standard solution* (10 ppm Pb) R.

Loss on drying (2.2.32): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 100–105 °C.

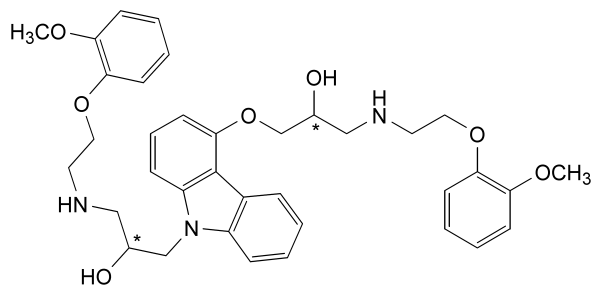
Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

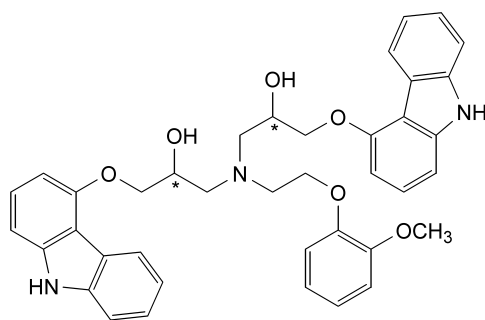
Dissolve 0.350 g in 60 ml of *anhydrous acetic acid* R. Titrate with 0.1 M *perchloric acid*, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *perchloric acid* is equivalent to 40.65 mg of C₂₄H₂₆N₂O₄.

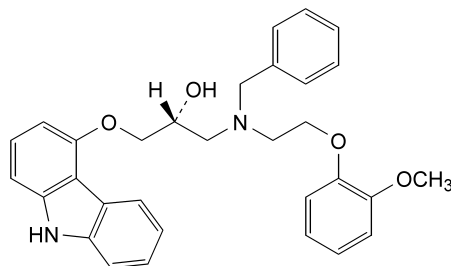
IMPURITIES



- A. 1-[[9-[2-hydroxy-3-[[2-(2-methoxyphenoxy)ethyl]amino]propyl]-9H-carbazol-4-yl]oxy]-3-[[2-(2-methoxyphenoxy)ethyl]amino]propan-2-ol,



- B. 1,1'-[[2-(2-methoxyphenoxy)ethyl]nitrilo]bis[3-(9H-carbazol-4-yloxy)propan-2-ol],



and enantiomer

- C. (2*RS*)-1-[benzyl[2-(2-methoxyphenoxy)ethyl]amino]-3-(9H-carbazol-4-yloxy)propan-2-ol.

01/2005:0105

CASCARA

Rhamni purshianae cortex

DEFINITION

Cascara consists of the dried, whole or fragmented bark of *Rhamnus purshianus* D.C. (*Frangula purshiana* (D.C.) A. Gray ex J. C. Cooper). It contains not less than 8.0 per cent of hydroxyanthracene glycosides of which not less than 60 per cent consists of cascariosides, both expressed as cascarioside A (C₂₇H₃₂O₁₄; *M_r* 580.5) and calculated with reference to the dried drug.

CHARACTERS

It has the macroscopic and microscopic characters described under identification tests A and B.

IDENTIFICATION

- A. The bark occurs in slightly channelled or nearly flat pieces, usually 1 mm to 5 mm in thickness, usually varying greatly in length and width. The outer surface is grey or dark greyish-brown and shows occasional lenticels that are orientated transversally. It is usually more or less completely covered by a whitish coat of lichens, epiphytic moss and foliaceous liverwort. The inner surface is yellow to reddish-brown or almost black with fine longitudinal striations; it turns red when treated with alkali. The yellow fracture is short and granular in the outer part and somewhat fibrous at the inner part.
- B. Reduce to a powder (355). The powder is yellowish-brown. Examine under a microscope using *chloral hydrate solution* R. The powder shows: bundles of partly lignified phloem fibres accompanied by crystal sheaths containing prisms of calcium oxalate; groups of sclereids accompanied by crystal sheaths; cluster crystals of calcium oxalate; some parenchymatous cells contain a yellow substance which becomes deep red when treated with alkali; cork cells and, frequently, epiphytes; the latter may be liverworts, entire or in fragments, having a

lamina one cell thick without a midrib and composed of isodiametric cells, or leaves of mosses, having a lamina one cell thick composed of elongated cells and possessing a midrib several cells thick.

- C. Examine the chromatograms obtained in the test for “Other species of *Rhamnus*; anthrones” after spraying with a 50 g/l solution of *potassium hydroxide R* in *alcohol (50 per cent V/V) R* and heating. The chromatogram obtained with the test solution shows several reddish-brown zones with different intensities: there are four faint zones, three being situated at about the mid-point of the chromatogram and one in the lower third and there is a strong zone in the upper third of the chromatogram. Examine in ultraviolet light at 365 nm. The chromatogram obtained with the test solution shows several zones with the same fluorescence, situated above and particularly below (cascarosides) that corresponding to barbaloin in the chromatogram obtained with the reference solution.
- D. Heat 0.2 g of the powdered drug (180) with 50 ml of *water R* on a water-bath for 15 min. Allow to cool and filter. To 10 ml of the filtrate add 20 ml of *hydrochloric acid R1* and heat on a water-bath for 15 min. Allow to cool, transfer to a separating funnel and shake with three quantities, each of 20 ml, of *ether R*. Reserve the aqueous layer (solution A).
- (a) Combine the three ether extracts and shake with 10 ml of *dilute ammonia R2*. The aqueous layer becomes reddish-violet.
- (b) Transfer solution A to a small flask, add 5 g of *ferric chloride R* and heat on a water-bath for 30 min. Allow to cool, transfer to a separating funnel and shake with 15 ml of *ether R*. Wash the ether layer with 10 ml of *water R*, discard the water and shake the ether layer with 5 ml of *dilute ammonia R2*. A red colour develops in the aqueous layer.

TESTS

Other species of *Rhamnus*; anthrones. Examine by thin-layer chromatography (2.2.27), using a suitable silica gel as the coating substance.

Test solution. To 0.5 g of the powdered drug (180) add 5 ml of *alcohol (70 per cent V/V) R* and heat to boiling. Cool and centrifuge. Decant the supernatant solution immediately and use within 30 min.

Reference solution. Dissolve 20 mg of *barbaloin R* in *alcohol (70 per cent V/V) R* and dilute to 10 ml with the same solvent.

Apply separately to the plate as bands 10 µl of each solution. Develop over a path of 10 cm using a mixture of 13 volumes of *water R*, 17 volumes of *methanol R* and 100 volumes of *ethyl acetate R*. Allow the plate to dry for 5 min, spray with about 10 ml of a 50 g/l solution of *potassium hydroxide R* in *alcohol (50 per cent V/V) R* and heat at 100 °C to 105 °C for 15 min. Examine the chromatograms immediately after heating. The chromatogram obtained with the reference solution shows, in the central part, a reddish-brown zone corresponding to barbaloin. Examine in ultraviolet light at 365 nm. The zone corresponding to barbaloin shows intense yellowish-brown fluorescence. In the chromatogram obtained with the test solution, no zone with orange-brown fluorescence is seen between the zone of barbaloin and the zones of cascarosides.

Apply to another plate as a band 10 µl of the test solution and develop as described above. Allow the plate to dry for not longer than 5 min and spray immediately with a 5 g/l

solution of *nitrotetrazolium blue R* in *methanol R*. Examine the chromatogram immediately. No violet or greyish-blue zones appear.

Foreign matter (2.8.2). Not more than 1 per cent.

Loss on drying (2.2.32). Not more than 10.0 per cent, determined on 1.000 g of the powdered drug (180) by drying in an oven at 100 °C to 105 °C for 2 h.

Total ash (2.4.16). Not more than 7.0 per cent.

ASSAY

Carry out the assay protected from bright light in one day.

Stir 1.00 g of the powdered drug (180) into 100 ml of boiling *water R* and continue boiling and stirring for 5 min. Allow to cool, dilute to 100.0 ml with *water R*, shake, filter and discard the first 20 ml of filtrate. Transfer 10.0 ml of the filtrate to a separating funnel, add 0.1 ml of 1 M *hydrochloric acid* and shake with two quantities, each of 20 ml, of a mixture of 1 volume of *ether R* and 3 volumes of *hexane R*. Wash the combined organic extracts with 5 ml of *water R*, discard the organic layer and return the rinsings to the aqueous layer. Shake the combined aqueous layers with four quantities, each of 30 ml, of *ethyl acetate R* freshly saturated with *water R*, (to 150 ml of *ethyl acetate R* add 15 ml of *water R*, shake for 3 min and allow to stand) on each occasion allowing separation to take place until the organic layer is clear. Combine the ethyl acetate extracts. Use the aqueous layer for the assay for cascarosides and the organic layer for the assay for hydroxyanthracene glycosides other than cascarosides.

Hydroxyanthracene glycosides other than cascarosides.

Transfer the organic layer to a suitable flask and remove the solvent by distillation, evaporating almost to dryness. Dissolve the residue in 0.3 ml to 0.5 ml of *methanol R* and transfer to a volumetric flask, rinsing the first flask with warm *water R* and adding the rinsings to the methanolic solution. Allow to cool and dilute to 50.0 ml with *water R*. Transfer 20.0 ml of the solution to a 100 ml round-bottomed flask with a ground-glass neck and containing 2 g of *ferric chloride R* and 12 ml of *hydrochloric acid R*. Attach a reflux condenser and place the flask in a water-bath so that the level of the water is above that of the liquid in the flask and heat for 4 h. Allow to cool, transfer the solution to a separating funnel and rinse the flask successively with 3 ml to 4 ml of 1 M *sodium hydroxide* and 3 ml to 4 ml of *water R*, adding the rinsings to the separating funnel. Shake the contents of the separating funnel with three quantities, each of 30 ml, of a mixture of 1 volume of *ether R* and 3 volumes of *hexane R*. Wash the combined organic layers with two quantities, each of 10 ml, of *water R* and discard the rinsings. Dilute the organic phase to 100.0 ml with the mixture of ether and hexane. Take 20.0 ml, evaporate carefully to dryness on a water-bath and dissolve the residue in 10.0 ml of a 5 g/l solution of *magnesium acetate R* in *methanol R*. Measure the absorbance (2.2.25) at 515 nm using *methanol R* as the compensation liquid.

Calculate the percentage content of hydroxyanthracene glycosides, expressed as cascaroside A, from the expression:

$$\frac{A \times 6.95}{m}$$

i.e. taking the specific absorbance to be 180.

A = absorbance at 515 nm,

m = mass of the sample in grams.

Measure the absorbance of the test solution at 440 nm. If the ratio of the absorbance at 515 nm to that at 440 nm is less than 2.4, the assay is not valid and must be repeated.

Cascarosides. Dilute the aqueous layer reserved for this assay to 50.0 ml with *water R*. Treat 20.0 ml of the solution as described above in the assay of hydroxyanthracene glycosides other than cascarosides.

Measure the absorbance of the test solution at 440 nm. If the ratio of the absorbance at 515 nm to that at 440 nm is less than 2.4, the assay is not valid and must be repeated.

Calculate the percentage content of cascarosides, expressed as cascaroside A from the expression:

$$\frac{A \times 6.95}{m}$$

i.e. taking the specific absorbance to be 180.

A = absorbance at 515 nm,

m = mass of the sample in grams.

Measure the absorbance of the test solution at 440 nm. If the ratio of the absorbance at 515 nm to that at 440 nm is less than 2.7, the assay is not valid and must be repeated.

STORAGE

Store protected from light.

01/2005:1496

CASSIA OIL

Cinnamomi cassiae aetheroleum

DEFINITION

Cassia oil is obtained by steam distillation of the leaves and young branches of *Cinnamomum cassia* Blume (*C. aromaticum* Nees).

CHARACTERS

A clear, mobile, yellow to reddish-brown liquid, with a characteristic odour reminiscent of cinnamic aldehyde.

IDENTIFICATION

First identification: B.

Second identification: A.

A. Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel plate R*.

Test solution. Dissolve 0.5 ml of the substance to be examined in *acetone R* and dilute to 10 ml with the same solvent.

Reference solution. Dissolve 50 µl of *trans-cinnamic aldehyde R*, 10 µl of *eugenol R* and 50 mg of *coumarin R* in *acetone R* and dilute to 10 ml with the same solvent.

Apply to the plate, as bands, 10 µl of each solution. Develop over a path of 15 cm using a mixture of 10 volumes of *methanol R* and 90 volumes of *toluene R*. Allow the plate to dry in air and examine in ultraviolet light at 365 nm. The zone of blue fluorescence in the chromatogram obtained with the test solution is similar in position and colour to the zone in the chromatogram obtained with the reference solution (coumarin). Spray the plate with *anisaldehyde solution R*. Examine in daylight while heating at 100-105 °C for 5-10 min. The chromatogram obtained with the reference solution shows in its upper part a violet zone (eugenol) and above this zone a greenish-blue zone (*trans-cinnamic aldehyde*). The chromatogram obtained with the test solution shows a zone similar in position and colour to the zone due to *trans-cinnamic aldehyde* in the chromatogram obtained

with the reference solution and may show a very faint zone corresponding to eugenol. Other faint zones are present.

B. Examine the chromatograms obtained in the test for chromatographic profile. The retention times of the principal peaks in the chromatogram obtained with the test solution are similar to those in the chromatogram obtained with the reference solution. Eugenol may be absent from the chromatogram obtained with the test solution.

TESTS

Relative density (2.2.5): 1.052 to 1.070.

Refractive index (2.2.6): 1.600 to 1.614.

Optical rotation (2.2.7): -1° to $+1^{\circ}$.

Chromatographic profile. Examine by gas chromatography (2.2.28).

Test solution. The substance to be examined.

Reference solution. Dissolve 100 µl of *trans-cinnamic aldehyde R*, 10 µl of *cinnamyl acetate R*, 10 µl of *eugenol R*, 20 mg of *coumarin R* and 10 µl of *trans-2-methoxycinnamaldehyde R* in 1 ml of *acetone R*.

The chromatographic procedure may be carried out using:

- a fused-silica column 60 m long and about 0.25 mm in internal diameter coated with *macrogol 20 000 R* as the bonded phase,
- *helium for chromatography R* as the carrier gas at a flow rate of 1.5 ml/min,
- a flame-ionisation detector,
- a split ratio of 1:100,

with the following temperature programme:

	Time (min)	Temperature (°C)	Rate (°C/min)	Comment
Column	0 - 10	60	–	isothermal
	10 - 75	60 → 190	2	linear gradient
	75 - 160	190		isothermal
Injection port		200		
Detector		240		

Inject 0.2 µl of the reference solution. When the chromatogram is recorded in the prescribed conditions, the components elute in the order indicated in the composition of the reference solution. Depending on the operating conditions and the state of the column, coumarin may elute before or after *trans-2-methoxycinnamaldehyde*. Record the retention times of these substances.

The test is not valid unless the resolution between the peaks corresponding to coumarin and *trans-2-methoxycinnamaldehyde* is at least 1.5.

Inject 0.2 µl of the test solution. Using the retention times determined from the chromatogram obtained with the reference solution, locate the components of the reference solution in the chromatogram obtained with the test solution. Determine the percentage content of each of these components by the normalisation procedure.

The percentages are within the following ranges:

- *trans-cinnamic aldehyde*: 70 per cent to 90 per cent,
- *cinnamyl acetate*: 1.0 per cent to 6.0 per cent,
- *eugenol*: less than 0.5 per cent,
- *coumarin*: 1.5 per cent to 4.0 per cent,
- *trans-2-methoxycinnamaldehyde*: 3.0 per cent to 15 per cent.

STORAGE

Store in a well-filled, airtight container, protected from light and heat.

01/2005:1497
corrected

CASTOR OIL, HYDROGENATED

Ricini oleum hydrogenatum

DEFINITION

Hydrogenated castor oil is the oil obtained by hydrogenation of *Virgin Castor oil* (0051). It consists mainly of the triglyceride of 12-hydroxystearic acid.

CHARACTERS

A fine, almost white or pale yellow powder or almost white or pale yellow masses or flakes, practically insoluble in water, freely soluble in methylene chloride, slightly soluble in light petroleum, very slightly soluble in ethanol.

IDENTIFICATION

- Melting point (2.2.14): 83 °C to 88 °C.
- It complies with the test for hydroxyl value (see Tests).
- It complies with the test for composition of fatty acids (see Tests).

TESTS

Acid value (2.5.1). Not more than 4.0, determined on 10.0 g dissolved in 75 ml of hot *alcohol R*.

Hydroxyl value (2.5.3, *Method A*): 145 to 165, determined on a warm solution.

Iodine value (2.5.4). Not more than 5.0.

Alkaline impurities. Dissolve 1.0 g by gentle heating in a mixture of 1.5 ml of *alcohol R* and 3 ml of *toluene R*. Add 0.05 ml of a 0.4 g/l solution of *bromophenol blue R* in *alcohol R*. Not more than 0.2 ml of 0.01 M *hydrochloric acid* is required to change the colour of the indicator to yellow.

Composition of fatty acids. Gas chromatography (2.4.22) with the following modifications.

Use the mixture of calibrating substances in Table 2.4.22.-3.

Test solution. Introduce 75 mg of the substance to be examined in a 10 ml centrifuge tube with a screw cap. Dissolve in 2 ml of 1,1-dimethylethyl methyl ether R1 by shaking and heat gently (50-60 °C). Add, when still warm, 1 ml of a 12 g/l solution of *sodium R* in *anhydrous methanol R*, prepared with the necessary precautions, and mix vigorously for at least 5 min. Add 5 ml of *distilled water R* and mix vigorously for about 30 s. Centrifuge for 15 min at 1500 g. Use the upper layer.

Reference solution. Dissolve 50 mg of *methyl 12-hydroxystearate CRS* and 50 mg of *methyl stearate CRS* in 10.0 ml of 1,1-dimethylethyl methyl ether R1.

The chromatographic procedure may be carried out using:

- a fused-silica column 30 m long and 0.25 mm in internal diameter coated with *macrogol 20 000 R* (film thickness 0.25 µm),
- helium for chromatography R* as the carrier gas at a flow rate of 0.9 ml/min,
- a flame-ionisation detector,
- a split injector (1:100),

maintaining the temperature of the column at 215 °C for 55 min and maintaining the temperature of the injection port and that of the detector at 250 °C.

Inject 1 µl of each solution.

Calculate the fraction of each fatty acid using the following expression:

$$A_{x,s,c} / \sum A_{x,s,c} \times 100 \text{ per cent } m/m$$

$A_{x,s,c}$ = corrected peak area of the fatty acid in the test solution:

$$A_{x,s,c} = A_{x,s} \times R_c$$

R_c = relative correction factor for the peak corresponding to methyl 12-hydroxystearate:

$$R_c = \frac{m_{1,r} \times A_{2,r}}{A_{1,r} \times m_{2,r}}$$

R_c = 1 for peaks corresponding to each of the other specified fatty acids or any unspecified fatty acid,

$m_{1,r}$ = mass of methyl 12-hydroxystearate in the reference solution,

$m_{2,r}$ = mass of methyl stearate in the reference solution,

$A_{1,r}$ = area of any peak corresponding to methyl 12-hydroxystearate in the chromatogram obtained with the reference solution,

$A_{2,r}$ = area of any peak corresponding to methyl stearate in the chromatogram obtained with the reference solution,

$A_{x,s}$ = area of the peaks corresponding to any specified or unspecified fatty acid methyl esters.

The fatty acid fraction of the oil has the following composition:

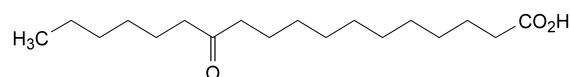
- palmitic acid*: not more than 2.0 per cent,
- stearic acid*: 7.0 per cent to 14.0 per cent,
- arachidic acid*: not more than 1.0 per cent,
- 12-oxostearic acid* (equivalent chain length on macrogol 20 000: 22.7): not more than 5.0 per cent,
- 12-hydroxystearic acid* (equivalent chain length on macrogol 20 000: 23.9): 78.0 per cent to 91.0 per cent,
- any other fatty acid*: not more than 3.0 per cent.

Nickel (2.4.27). Not more than 1 ppm of Ni.

STORAGE

Store in a well-filled container.

IMPURITIES



A. 12-oxostearic acid.

01/2005:0051
corrected

CASTOR OIL, VIRGIN

Ricini oleum virginale

DEFINITION

Fatty oil obtained by cold expression from the seeds of *Ricinus communis* L. A suitable antioxidant may be added.

CHARACTERS

Appearance: clear, almost colourless or slightly yellow, viscous, hygroscopic liquid.

Solubility: slightly soluble in light petroleum, miscible with alcohol and with glacial acetic acid.

Relative density: about 0.958.

Refractive index: about 1.479.

IDENTIFICATION

First identification: D.

Second identification: A, B, C.

A. It complies with the test for optical rotation (see Tests).

B. It complies with the test for hydroxyl value (see Tests).

C. Iodine value (2.5.4): 82 to 90.

D. It complies with the test for composition of fatty acids (see Tests).

TESTS

Optical rotation (2.2.7): + 3.5° to + 6.0°.

Specific absorbance (2.2.25): maximum 1.0, determined at the absorption maximum at 269 nm ± 1 nm.

To 1.0 g add *alcohol R* and dilute to 100.0 ml with the same solvent.

Acid value (2.5.1): maximum 2.0.

Dissolve 5.0 g in 25 ml of the prescribed mixture of solvents.

Hydroxyl value (2.5.3, *Method A*): minimum 150.

Peroxide value (2.5.5): maximum 10.0.

Unsaponifiable matter (2.5.7): maximum 0.8 per cent, determined on 5.0 g.

Composition of fatty acids. Gas chromatography (2.4.22) with the following modifications.

Use the mixture of calibrating substances in Table 2.4.22.-3.

Test solution. Introduce 75 mg of the substance to be examined into a 10 ml centrifuge tube with a screw cap. Dissolve in 2 ml of *1,1-dimethylethyl methyl ether R1* with shaking and heat gently (50-60 °C). Add, when still warm, 1 ml of a 12 g/l solution of *sodium R* in *anhydrous methanol R*, prepared with the necessary precautions, and mix vigorously for at least 5 min. Add 5 ml of *distilled water R* and mix vigorously for about 30 s. Centrifuge for 15 min at 1500 g. Use the upper layer.

Reference solution. Dissolve 50 mg of *methyl ricinoleate CRS* and 50 mg of *methyl stearate CRS* in 10.0 ml of *1,1-dimethylethyl methyl ether R1*.

Column:

- **material:** fused silica,
- **size:** $l = 30$ m, $\varnothing = 0.25$ mm,
- **stationary phase:** *macrogol 20 000 R* (film thickness 0.25 µm).

Carrier gas: *helium for chromatography R*.

Flow rate: 0.9 ml/min.

Split ratio: 1:100.

Temperature:

	Time (min)	Temperature (°C)
Column	0 - 55	215
Injection port		250
Detector		250

Detection: flame ionisation.

Injection: 1 µl.

Calculate the percentage content of each fatty acid by normalisation.

Correct the area of the peak due to methyl ricinoleate, multiplying by a factor R calculated using the following expression:

$$R = \frac{m_1 \times A_2}{A_1 \times m_2}$$

m_1 = mass of methyl ricinoleate in the reference solution,

m_2 = mass of methyl stearate in the reference solution,

A_1 = area of the peak due to methyl ricinoleate in the chromatogram obtained with the reference solution,

A_2 = area of the peak due to methyl stearate in the chromatogram obtained with the reference solution.

Composition of the fatty-acid fraction of the oil:

- *palmitic acid*: maximum 2.0 per cent,
- *stearic acid*: maximum 2.5 per cent,
- *oleic acid and isomers* (C18:1 equivalent chain length on *macrogol 20 000*: 18.3): 2.5 per cent to 6.0 per cent,
- *linoleic acid* (C18:2 equivalent chain length on *macrogol 20 000*: 18.8): 2.5 per cent to 7.0 per cent,
- *linolenic acid* (C18:3 equivalent chain length on *macrogol 20 000*: 19.2): maximum 1.0 per cent,
- *eicosenoic acid* (C20:1 equivalent chain length on *macrogol 20 000*: 20.2): maximum 1.0 per cent,
- *ricinoleic acid* (equivalent chain length on *macrogol 20 000*: 23.9): 85.0 per cent to 92.0 per cent,
- *any other fatty acid*: maximum 1.0 per cent.

Water (2.5.12): maximum 0.3 per cent, determined on 5.0 g.

STORAGE

In an airtight, well-filled container, protected from light.

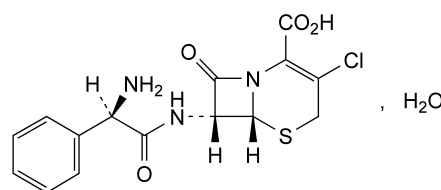
LABELLING

The label states the name and concentration of any added antioxidant.

01/2005:0986
corrected

CEFACLOR

Cefaclorum



$C_{15}H_{14}ClN_3O_4S \cdot H_2O$

M_r 385.8

DEFINITION

Cefaclor is the monohydrate of (6*R*,7*R*)-7-[[*(2R*)-2-amino-2-phenylacetyl]amino]-3-chloro-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid. It contains not

less than 96.0 per cent and not more than the equivalent of 102.0 per cent of $C_{15}H_{14}ClN_3O_4S$, calculated with reference to the anhydrous substance.

CHARACTERS

A white or slightly yellow powder, slightly soluble in water, practically insoluble in methanol and in methylene chloride.

IDENTIFICATION

Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *cefaclor CRS*.

TESTS

pH (2.2.3). Suspend 0.250 g in *carbon dioxide-free water R* and dilute to 10 ml with the same solvent. The pH of the suspension is 3.0 to 4.5.

Specific optical rotation (2.2.7). Dissolve 0.250 g in a 10 g/l solution of *hydrochloric acid R* and dilute to 25.0 ml with the same solution. The specific optical rotation is + 101 to + 111, calculated with reference to the anhydrous substance.

Related substances. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 50.0 mg of the substance to be examined in 10.0 ml of a 2.7 g/l solution of *sodium dihydrogen phosphate R* adjusted to pH 2.5 with *phosphoric acid R*.

Reference solution (a). Dissolve 2.5 mg of *cefaclor CRS* and 5.0 mg of *delta-3-cefaclor CRS* in 100.0 ml of a 2.7 g/l solution of *sodium dihydrogen phosphate R* adjusted to pH 2.5 with *phosphoric acid R*.

Reference solution (b). Dilute 1.0 ml of the test solution to 100.0 ml with a 2.7 g/l solution of *sodium dihydrogen phosphate R* adjusted to pH 2.5 with *phosphoric acid R*.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4.6 mm in internal diameter packed with *end-capped octadecylsilyl silica gel for chromatography R* (5 µm),
- as mobile phase at a flow rate of 1.0 ml/min:

Mobile phase A. Adjust a 7.8 g/l solution of *sodium dihydrogen phosphate R* to pH 4.0 with *phosphoric acid R*,

Mobile phase B. Mix 450 ml of *acetonitrile R* with 550 ml of mobile phase A,

- as detector a spectrophotometer set at 220 nm,
- a 20 µl loop injector.

Equilibrate the column with a mixture of 5 volumes of mobile phase B and 95 volumes of mobile phase A for at least 15 min between each analysis. Inject the solutions. Operate by gradient elution increasing the concentration of mobile phase B continuously and linearly by 0.67 per cent V/V per minute for 30 min (25 per cent V/V). Then increase the concentration of mobile phase B continuously and linearly by 5 per cent V/V per minute for 15 min (100 per cent V/V). Finally elute with mobile phase B for 10 min. Change the composition to a mixture of 5 volumes of mobile phase B and 95 volumes of mobile phase A to re-equilibrate the column.

Inject reference solution (a). The test is not valid unless the resolution between the peaks due to cefaclor and delta-3-cefaclor is at least 2 and the symmetry factor of the cefaclor peak is at most 1.2. If necessary, adjust the acetonitrile content of the mobile phase.

Inject the test solution and reference solution (b). In the chromatogram obtained with the test solution: the area of any peak, apart from the principal peak and any peaks due to the mobile phase, is not greater than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent); and the sum of all such peaks is not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (2 per cent). Disregard any peak with an area less than 0.1 times that of the principal peak in the chromatogram obtained with reference solution (b).

Heavy metals (2.4.8). 1.0 g complies with limit test C (30 ppm). Prepare the standard using 3 ml of *lead standard solution* (10 ppm Pb) R.

Water (2.5.12). 3.0 per cent to 6.5 per cent, determined on 0.200 g by the semi-micro determination of water.

ASSAY

Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 15.0 mg of the substance to be examined in the mobile phase and dilute to 50.0 ml with the mobile phase.

Reference solution (a). Dissolve 15.0 mg of *cefaclor CRS* in the mobile phase and dilute to 50.0 ml with the mobile phase.

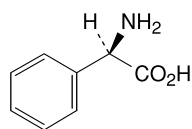
Reference solution (b). Dissolve 15.0 mg of *cefaclor CRS* and 15.0 mg of *delta-3-cefaclor CRS* in the mobile phase and dilute to 50.0 ml with the mobile phase.

The chromatographic procedure may be carried out using:

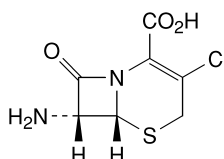
- a stainless steel column 0.25 m long and 4.6 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (5 µm),
- as mobile phase at a flow rate of 1.5 ml/min a mixture prepared by adding 220 ml of *methanol R* to a mixture of 780 ml of *water R*, 10 ml of *triethylamine R* and 1 g of *sodium pentanesulphonate R*, adjusted to pH 2.5 with *phosphoric acid R*,
- as detector a spectrophotometer set at 265 nm,
- a 20 µl loop injector.

Inject reference solution (b). The assay is not valid unless the resolution between the peaks due to cefaclor and delta-3-cefaclor is at least 2.5. Adjust the concentration of methanol in the mobile phase, if necessary. The assay is not valid unless the symmetry factor of the cefaclor peak is at most 1.5. Inject reference solution (a) six times. The assay is not valid unless the relative standard deviation of the peak area of cefaclor is at most 1.0 per cent. Inject alternately the test solution and reference solution (a).

IMPURITIES



A. (2*R*)-2-amino-2-phenylacetic acid (phenylglycine),

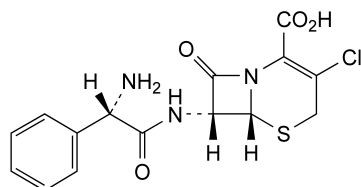


B. (6*R*,7*R*)-7-amino-3-chloro-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid,

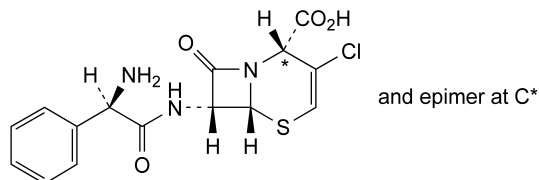
01/2005:0813
corrected

CEFADROXIL MONOHYDRATE

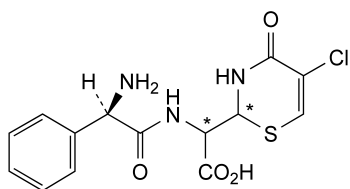
Cefadroxilum monohydricum



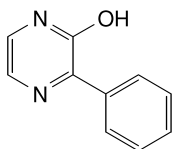
C. (6*R*,7*R*)-7-[[*(2S)*-2-amino-2-phenylacetyl]amino]-3-chloro-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid,



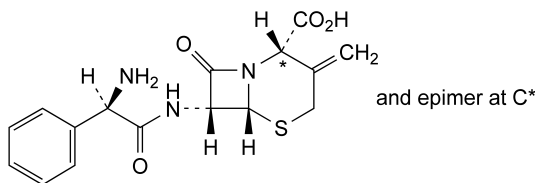
D. (2*R*,6*R*,7*R*)- and (2*S*,6*R*,7*R*)-7-[[*(2R)*-2-amino-2-phenylacetyl]amino]-3-chloro-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-3-ene-2-carboxylic acid (delta-3-cefaclor),



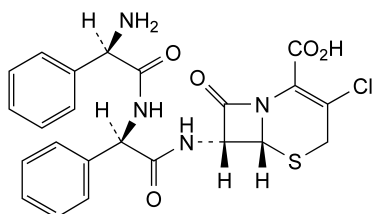
E. 2-[[*(2R)*-2-amino-2-phenylacetyl]amino]-2-(5-chloro-4-oxo-3,4-dihydro-2*H*-1,3-thiazin-2-yl)acetic acid,



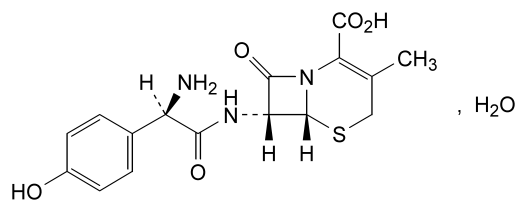
F. 3-phenylpyrazin-2-ol.



G. (2*R*,6*R*,7*R*)- and (2*S*,6*R*,7*R*)-7-[[*(2R)*-2-amino-2-phenylacetyl]amino]-3-methylene-8-oxo-5-thia-1-azabicyclo[4.2.0]octane-2-carboxylic acid (isocefalexine),



H. (6*R*,7*R*)-7-[[*(2R)*-2-[[*(2R)*-2-amino-2-phenylacetyl]amino]-2-phenylacetyl]amino]-3-chloro-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid (*N*-phenylglycyl cefaclor).


 $C_{16}H_{17}N_3O_5S \cdot H_2O$
 M_r 381.4

DEFINITION

(6*R*,7*R*)-7-[[*(2R)*-2-Amino-2-(4-hydroxyphenyl)acetyl]amino]-3-methyl-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid monohydrate.

Content: 95.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

Appearance: white or almost white powder.

Solubility: slightly soluble in water, very slightly soluble in alcohol.

IDENTIFICATION

Infrared absorption spectrophotometry (2.2.24).

TESTS

pH (2.2.3): 4.0 to 6.0.

Suspend 1.0 g in *carbon dioxide-free water R* and dilute to 20 ml with the same solvent.

Specific optical rotation (2.2.7): + 165 to + 178 (anhydrous substance).

Dissolve 0.500 g in *water R* and dilute to 50.0 ml with the same solvent.

Absorbance (2.2.25). Dissolve 20.0 mg in *phosphate buffer solution pH 6.0 R* and dilute to 100.0 ml with the same solvent. The absorbance of the solution determined at 330 nm is not greater than 0.05. Dilute 10.0 ml of the solution to 100.0 ml with *phosphate buffer solution pH 6.0 R*. Examined between 235 nm and 340 nm, the diluted solution shows an absorption maximum at 264 nm. The specific absorbance at this maximum is 225 to 250 (anhydrous substance).

Related substances. Liquid chromatography (2.2.29).

Test solution. Dissolve 50.0 mg of the substance to be examined in mobile phase A and dilute to 50.0 ml with mobile phase A.

Reference solution (a). Dissolve 10.0 mg of *D-α-(4-hydroxyphenyl)glycine CRS* (impurity A) in mobile phase A and dilute to 10.0 ml with mobile phase A.

Reference solution (b). Dissolve 10.0 mg of *7-aminodesacetoxycephalosporanic acid CRS* (impurity B) in *phosphate buffer solution pH 7.0 R5* and dilute to 10.0 ml with the same buffer solution.

Reference solution (c). Dilute 1.0 ml of reference solution (a) and 1.0 ml of reference solution (b) to 100.0 ml with mobile phase A.

Reference solution (d). Dissolve 10 mg of *dimethylformamide R* and 10 mg of *dimethylacetamide R* in mobile phase A and dilute to 10.0 ml with mobile phase A. Dilute 1.0 ml to 100.0 ml with mobile phase A.

Reference solution (e). Dilute 1.0 ml of reference solution (c) to 25.0 ml with mobile phase A.

Column:

- **size:** $l = 0.10$ m, $\varnothing = 4.6$ mm,
- **stationary phase:** spherical *octadecylsilyl silica gel for chromatography R* (5 μ m).

Mobile phase:

- **mobile phase A:** *phosphate buffer solution pH 5.0 R*,
- **mobile phase B:** *methanol R2*,

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)
0 - 1	98	2
1 - 20	98 $\rightarrow$ 70	2 $\rightarrow$ 30
20 - 23	70 $\rightarrow$ 98	30 $\rightarrow$ 2
23 - 30	98	2

Flow rate: 1.5 ml/min.

Detection: spectrophotometer at 220 nm.

Injection: 20 μ l; inject the test solution and reference solutions (c), (d) and (e).

Relative retention with reference to cefadroxil (retention time = about 6 min): *dimethylformamide* = about 0.4; *dimethylacetamide* = about 0.75.

System suitability:

- **resolution:** minimum 5.0 between the peaks due to impurity A and to impurity B in the chromatogram obtained with reference solution (c),
- **signal-to-noise ratio:** minimum 10 for the second peak in the chromatogram obtained with reference solution (e).

Limits:

- **impurity A:** not more than the area of the first peak in the chromatogram obtained with reference solution (c) (1.0 per cent),
- **any other impurity:** not more than the area of the second peak in the chromatogram obtained with reference solution (c) (1.0 per cent),
- **total:** not more than 3 times the area of the second peak in the chromatogram obtained with reference solution (c) (3.0 per cent),
- **disregard limit:** 0.05 times the area of the second peak in the chromatogram obtained with reference solution (c) (0.05 per cent); disregard the peaks due to *dimethylformamide* and *dimethylacetamide*.

***N,N*-Dimethylaniline (2.4.26, Method B):** maximum 20 ppm.

Water (2.5.12): 4.0 per cent to 6.0 per cent, determined on 0.200 g.

Sulphated ash (2.4.14): maximum 0.5 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (2.2.29).

Test solution. Dissolve 50.0 mg of the substance to be examined in the mobile phase and dilute to 100.0 ml with the mobile phase.

Reference solution (a). Dissolve 50.0 mg of *cefadroxil CRS* in the mobile phase and dilute to 100.0 ml with the mobile phase.

Reference solution (b). Dissolve 5 mg of *cefadroxil CRS* and 50 mg of *amoxicillin trihydrate CRS* in the mobile phase and dilute to 100 ml with the mobile phase.

Column:

- **size:** $l = 0.25$ m, $\varnothing = 4.6$ mm,
- **stationary phase:** *octadecylsilyl silica gel for chromatography R* (5 μ m).

Mobile phase: *acetonitrile R*, a 2.72 g/l solution of *potassium dihydrogen phosphate R* (4:96 V/V).

Flow rate: 1 ml/min.

Detection: spectrophotometer at 254 nm.

Injection: 20 μ l.

System suitability: reference solution (b):

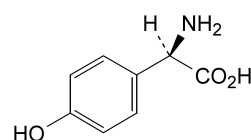
- **resolution:** minimum 5.0 between the peaks due to cefadroxil and to amoxicillin.

Calculate the percentage content of cefadroxil.

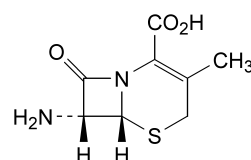
STORAGE

Protected from light.

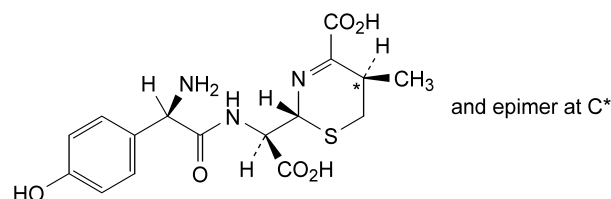
IMPURITIES



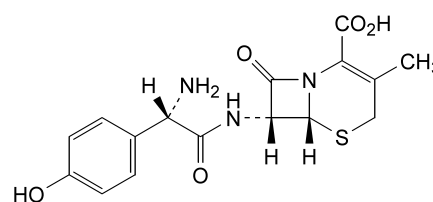
A. (2*R*)-2-amino-2-(4-hydroxyphenyl)acetic acid,



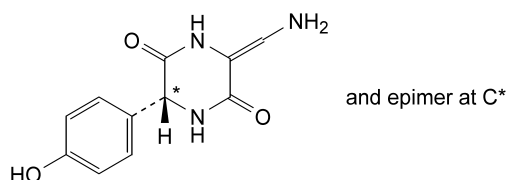
B. (6*R*,7*R*)-7-amino-3-methyl-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid (7-ADCA),



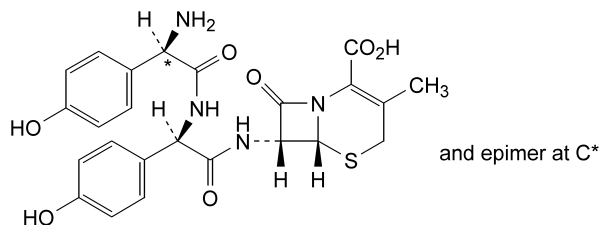
C. (2*R*,5*RS*)-2-[(*R*)-[(2*R*)-2-amino-2-(4-hydroxyphenyl)acetyl]amino]carboxymethyl]-5-methyl-5,6-dihydro-2*H*-1,3-thiazine-4-carboxylic acid,



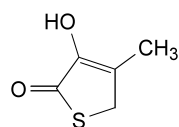
D. (6*R*,7*R*)-7-[(2*S*)-2-amino-2-(4-hydroxyphenyl)acetyl]amino]-3-methyl-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid (L-cefadroxil),



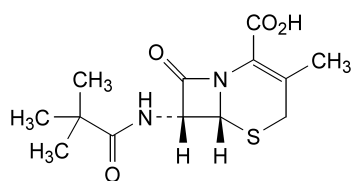
- E. (6*RS*)-3-(aminomethylene)-6-(4-hydroxyphenyl)piperazine-2,5-dione,



- F. (6*R*,7*R*)-7-[[[(2*R*)-2-[(2*RS*)-2-amino-2-(4-hydroxyphenyl)acetyl]amino]-3-methyl-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid,



- G. 3-hydroxy-4-methylthiophen-2(5*H*)-one,

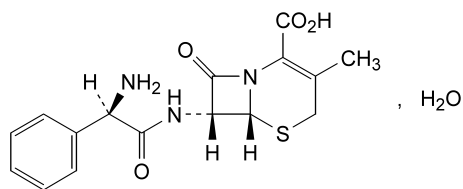


- H. (6*R*,7*R*)-7-[(2,2-dimethylpropanoyl)amino]-3-methyl-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid (7-ADCA pivalamide).

01/2005:0708

CEFALEXIN MONOHYDRATE

Cefalexinum monohydricum



$C_{16}H_{17}N_3O_4S \cdot H_2O$

M_r 365.4

DEFINITION

(6*R*,7*R*)-7-[[[(2*R*)-2-Amino-2-phenylacetyl]amino]-3-methyl-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid monohydrate.

Content: 95.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

Appearance: white or almost white, crystalline powder.

Solubility: sparingly soluble in water, practically insoluble in alcohol.

IDENTIFICATION

Infrared absorption spectrophotometry (2.2.24).

Comparison: cefalexin monohydrate CRS.

TESTS

pH (2.2.3): 4.0 to 5.5.

Dissolve 50 mg in carbon dioxide-free water *R* and dilute to 10 ml with the same solvent.

Specific optical rotation (2.2.7): + 149 to + 158 (anhydrous substance).

Dissolve 0.125 g in phthalate buffer solution pH 4.4 *R* and dilute to 25.0 ml with the same solvent.

Absorbance (2.2.25). Dissolve 50 mg in water *R* and dilute to 100.0 ml with the same solvent. The absorbance of the solution determined at 330 nm is not greater than 0.05. Dilute 2.0 ml of the solution to 50.0 ml with water *R*. Examined between 220 nm and 300 nm, the diluted solution shows an absorption maximum at 262 nm. The specific absorbance at this maximum is 220 to 245, calculated with reference to the anhydrous substance.

Related substances. Liquid chromatography (2.2.29).

Test solution. Dissolve 50.0 mg of the substance to be examined in mobile phase A and dilute to 50.0 ml with mobile phase A.

Reference solution (a). Dissolve 10.0 mg of *D*-phenylglycine *R* in mobile phase A and dilute to 10.0 ml with mobile phase A.

Reference solution (b). Dissolve 10.0 mg of 7-aminodesacetoxycephalosporanic acid CRS in phosphate buffer solution pH 7.0 *R5* and dilute to 10.0 ml with mobile phase A.

Reference solution (c). Dilute 1.0 ml of reference solution (a) and 1.0 ml of reference solution (b) to 100.0 ml with mobile phase A.

Reference solution (d). Dissolve 10 mg of dimethylformamide *R* and 10 mg of dimethylacetamide *R* in mobile phase A and dilute to 10.0 ml with mobile phase A. Dilute 1.0 ml to 100.0 ml with mobile phase A.

Reference solution (e). Dilute 1.0 ml of reference solution (c) to 20.0 ml with mobile phase A.

Reference solution (f). Dissolve 10 mg of cefotaxime sodium CRS in mobile phase A and dilute to 10.0 ml with mobile phase A. To 1.0 ml of the solution add 1.0 ml of the test solution and dilute to 100 ml with mobile phase A.

Column:

- size: $l = 0.10$ m, $\varnothing = 4.6$ mm,
- stationary phase: spherical octadecylsilyl silica gel for chromatography *R* (5 μ m).

Mobile phase:

- mobile phase A: phosphate buffer solution pH 5.0 *R*,
- mobile phase B: methanol *R2*,

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)
0 - 1	98	2
1 - 20	98 $\rightarrow$ 70	2 $\rightarrow$ 30
20 - 23	70 $\rightarrow$ 98	30 $\rightarrow$ 2
23 - 30	98	2

Flow rate: 1.5 ml/min.

Detection: spectrophotometer at 220 nm.

Injection: 20 μ l; inject the test solution and reference solutions (c), (d), (e) and (f).

System suitability:

- **resolution:** minimum of 2.0 between the peaks due to impurity A and to impurity B in the chromatogram obtained with reference solution (c) and minimum of 1.5 between the peaks due to cefalexin and to cefotaxime in the chromatogram obtained with reference solution (f).

Limits:

- **impurity B:** not more than the area of the second peak in the chromatogram obtained with reference solution (c) (1.0 per cent),
- **any other impurity** (disregard the peaks due to dimethylformamide and dimethylacetamide): not more than the area of the first peak in the chromatogram obtained with reference solution (c) (1.0 per cent),
- **total:** not more than 3 times the area of the first peak in the chromatogram obtained with reference solution (c) (3.0 per cent),
- **disregard limit:** the area of the second peak in the chromatogram obtained with reference solution (e) (0.05 per cent).

N,N-Dimethylaniline (2.4.26, *Method B*): maximum 20 ppm.

Water (2.5.12): 4.0 per cent to 8.0 per cent, determined on 0.300 g.

Sulphated ash (2.4.14): maximum 0.2 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (2.2.29).

Test solution. Dissolve 50.0 mg of the substance to be examined in *water R* and dilute to 100.0 ml with the same solvent.

Reference solution (a). Dissolve 50.0 mg of *cefalexin monohydrate CRS* in *water R* and dilute to 100.0 ml with the same solvent.

Reference solution (b). Dissolve 10 mg of *cefradine CRS* in 20 ml of reference solution (a) and dilute to 100 ml with *water R*.

Column:

- **size:** $l = 0.25$ m, $\varnothing = 4.6$ mm,
- **stationary phase:** octadecylsilyl silica gel for chromatography *R* (5 μ m).

Mobile phase: *methanol R*, *acetonitrile R*, a 13.6 g/l solution of *potassium dihydrogen phosphate R*, *water R* (2:5:10:83 *V/V/V/V*).

Flow rate: 1.5 ml/min.

Detection: spectrophotometer at 254 nm.

Injection: 20 μ l.

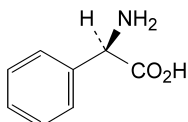
System suitability: reference solution (b):

- **resolution:** minimum 4.0 between the peaks due to cefalexin and to cefradine.

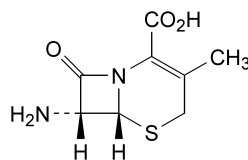
Calculate the percentage content of cefalexin monohydrate.

STORAGE

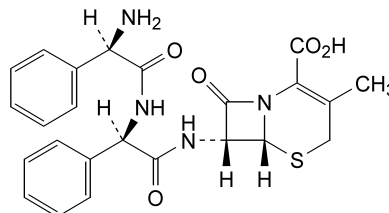
Protected from light.

IMPURITIES

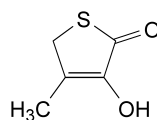
A. (2*R*)-2-amino-2-phenylacetic acid (D-phenylglycine),



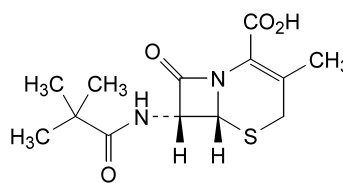
B. (6*R*,7*R*)-7-amino-3-methyl-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid (7-aminodesacetoxycephalosporanic acid, 7-ADCA),



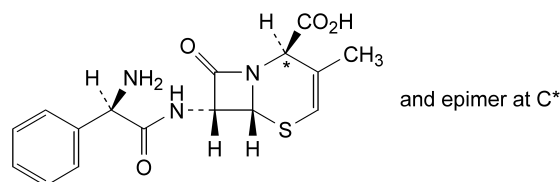
C. (6*R*,7*R*)-7-[(2*R*)-2-[(2*R*)-2-amino-2-phenylacetyl]amino]-2-phenylacetyl]amino]-3-methyl-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid,



D. 3-hydroxy-4-methylthiophen-2(5*H*)-one,

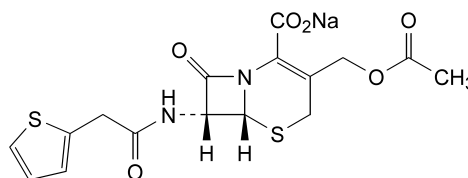


E. (6*R*,7*R*)-7-[(2,2-dimethylpropanoyl)amino]-3-methyl-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid (7-ADCA pivalamide),



F. (2*RS*,6*R*,7*R*)-7-[(2*R*)-2-amino-2-phenylacetyl]amino]-3-methyl-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-3-ene-2-carboxylic acid (delta-2-cefalexin).

01/2005:0987

CEFALOTIN SODIUM**Cefalotinum natricum**

$C_{16}H_{15}N_2NaO_6S_2$

M_r 418.4

DEFINITION

Cefalotin sodium contains not less than 96.0 per cent and not more than the equivalent of 101.0 per cent of sodium (6*R*, 7*R*)-3-[(acetyloxy)methyl]-8-oxo-7-[[2-(thiophen-2-yl)acetyl]-amino]-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylate, calculated with reference to the anhydrous substance.

CHARACTERS

A white or almost white powder, freely soluble in water, slightly soluble in ethanol.

IDENTIFICATION

- A. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *cefalotin sodium CRS*.
- B. It gives reaction (a) of sodium (2.3.1).

TESTS

Solution S. Dissolve 2.50 g in *carbon dioxide-free water R* and dilute to 25.0 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1). The absorbance (2.2.25) of solution S measured at 450 nm is not greater than 0.20.

pH (2.2.3). The pH of solution S is 4.5 to 7.0.

Specific optical rotation (2.2.7). Dissolve 1.25 g in *water R* and dilute to 25.0 ml with the same solvent. The specific optical rotation is + 124 to + 134, calculated with reference to the anhydrous substance.

Related substances. Examine by liquid chromatography (2.2.29) as described under Assay. Inject the test solution and reference solution (b). Continue the chromatography for at least four times the retention time of the principal peak. In the chromatogram obtained with the test solution: the area of any peak, apart from the principal peak, is not greater than the area of the principal peak in the chromatogram obtained with reference solution (b) (1 per cent) and the sum of the areas of any such peaks is not greater than three times the area of the principal peak in the chromatogram obtained with reference solution (b) (3 per cent). Disregard any peak with an area less than 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent).

***N,N*-Dimethylaniline (2.4.26, Method B).** Not more than 20 ppm.

2-Ethylhexanoic acid (2.4.28). Not more than 0.5 per cent *m/m*.

Water (2.5.12). Not more than 1.5 per cent, determined on 0.500 g by the semi-micro determination of water.

Bacterial endotoxins (2.6.14): less than 0.13 IU/mg, if intended for use in the manufacture of parenteral dosage forms without a further appropriate procedure for the removal of bacterial endotoxins.

ASSAY

Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 25.0 mg of the substance to be examined in the mobile phase and dilute to 25.0 ml with the mobile phase.

Reference solution (a). Dissolve 25.0 mg of *cefalotin sodium CRS* in the mobile phase and dilute to 25.0 ml with the mobile phase.

Reference solution (b). Dilute 1.0 ml of reference solution (a) to 100.0 ml with the mobile phase.

Reference solution (c). Heat 5 ml of reference solution (a) in a water-bath at 90 °C for 10 min. Cool and inject immediately.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4.6 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (5 µm),
 - as mobile phase at a flow rate of 1.0 ml/min a solution prepared as follows: dissolve 17 g of *sodium acetate R* in 790 ml of *water R*, add 0.6 ml of *glacial acetic acid R* and adjust if necessary to pH 5.8 to 6.0 with *dilute sodium hydroxide solution R* or *glacial acetic acid R*, add 150 ml of *acetonitrile R* and 70 ml of *ethanol R* and mix,
 - as detector a spectrophotometer set at 254 nm,
 - a 10 µl loop injector,
- maintaining the temperature of the column at 40 °C.

Inject reference solution (c). Adjust the sensitivity of the system to obtain peaks with a height corresponding to at least half the full scale of the recorder. The chromatogram obtained shows two principal peaks corresponding to cefalotin and desacetylcefalotin. The assay is not valid unless the resolution between these two peaks is at least 9.0. Adjust the concentration of acetonitrile in the mobile phase if necessary. The assay is not valid unless the symmetry factor of the cefalotin peak is at most 1.8. Inject reference solution (a) six times. The assay is not valid unless the relative standard deviation of the peak area for cefalotin is at most 1.0 per cent. Inject alternately the test solution and reference solution (a).

Calculate the percentage content of cefalotin sodium.

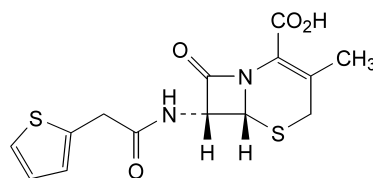
STORAGE

Store in an airtight container, protected from light. If the substance is sterile, store in a sterile, airtight, tamper-proof container.

LABELLING

The label states, where applicable, that the substance is free from bacterial endotoxins.

IMPURITIES

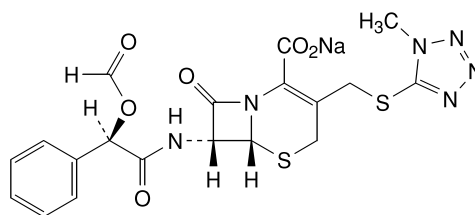


- A. (6*R*,7*R*)-3-methyl-8-oxo-7-[[2-(thiophen-2-yl)acetyl]amino]-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid (desacetoxycefalotin).

01/2005:1402
corrected

CEFAMANDOLE NAFATE

Cefamandoli nafas



C₁₉H₁₇N₆NaO₆S₂

*M*_r 512.5

DEFINITION

Sodium (6*R*,7*R*)-7-[[*(2R)*-2-(formyloxy)-2-phenylacetyl]amino]-3-[[*(1-methyl-1*H*-tetrazol-5-yl)sulphonyl* methyl]-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylate with sodium carbonate.

Content:

- cefamandole (C₁₈H₁₈N₆O₅S₂): 84.0 per cent to 93.0 per cent (anhydrous and sodium carbonate-free substance),
- sodium carbonate (Na₂CO₃): 4.8 per cent to 6.4 per cent.

CHARACTERS

Appearance: white or almost white powder.

Solubility: freely soluble in water, sparingly soluble in methanol.

IDENTIFICATION

A. Infrared absorption spectrophotometry (2.2.24).

Preparation: discs.

Comparison: cefamandole nafate CRS.

B. It gives reaction (a) of sodium (2.3.1).

TESTS

Solution S. Dissolve 2.5 g in carbon dioxide-free water *R* and dilute to 25 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and its absorbance (2.2.25) at 475 nm has a maximum of 0.03.

pH: 6.0 to 8.0 for solution S, measured after 30 min.

Specific optical rotation (2.2.7): –25.0 to –33.0 (anhydrous and sodium carbonate-free substance).

Dissolve 1.00 g in acetate buffer solution pH 4.7 *R* and dilute to 10.0 ml with the same solvent.

Related substances. Liquid chromatography (2.2.29). Prepare the solutions immediately before use.

Solvent mixture. Mix 18 volumes of acetonitrile *R* and 75 volumes of a 10 per cent V/V solution of triethylamine *R* previously adjusted to pH 2.5 with phosphoric acid *R*.

Test solution. Dissolve 0.100 g of the substance to be examined in the solvent mixture and dilute to 10.0 ml with the solvent mixture.

Reference solution (a). Dilute 1 ml of the test solution to 10 ml with the solvent mixture, then heat the solution at 60 °C for 30 min.

Reference solution (b). Dilute 1.0 ml of the test solution to 100.0 ml with the solvent mixture.

Column:

- size: *l* = 0.25 m, Ø = 4.6 mm,
- stationary phase: octadecylsilyl silica gel for chromatography *R* (5 µm).

Mobile phase:

- triethylamine phosphate solution: dissolve 2.0 g of sodium pentanesulphonate *R* in 350 ml of water *R*, add 40 ml of triethylamine *R*, adjust to pH 2.5 with phosphoric acid *R* and dilute to 700 ml with water *R*,
- mobile phase A: mix 1 volume of triethylamine phosphate solution and 2 volumes of water *R*,
- mobile phase B: mix equal volumes of triethylamine phosphate solution, methanol *R* and acetonitrile *R*.

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)
0 - 1	100	0
1 - 35	100 → 0	0 → 100
35 - 45	0	100
45 - 50	0 → 100	100 → 0

Flow rate: 1.5 ml/min.

Detection: spectrophotometer at 254 nm.

Injection: 20 µl loop injector.

System suitability: reference solution (a):

- resolution: minimum 5.0 between the peaks due to cefamandole and to cefamandole nafate.

Limits:

- any impurity: not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (1 per cent),
- total: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (5 per cent),
- disregard limit: 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent).

Cefamandole free acid: maximum 9.5 per cent (anhydrous and sodium carbonate-free substance), determined by liquid chromatography as described under Assay.

2-Ethylhexanoic acid (2.4.28): maximum 0.3 per cent *m/m*.

Heavy metals (2.4.8): maximum 20 ppm.

1.0 g complies with limit test C. Prepare the standard using 2 ml of lead standard solution (10 ppm Pb) *R*.

Water (2.5.12): maximum 2.0 per cent, determined on 0.500 g.

Bacterial endotoxins (2.6.14): less than 0.15 IU/mg, if intended for use in the manufacture of parenteral dosage forms without a further appropriate procedure for the removal of bacterial endotoxins.

ASSAY

Cefamandole. Liquid chromatography (2.2.29). Prepare the solutions immediately before use.

Test solution. Dissolve 50.0 mg of the substance to be examined in the mobile phase and dilute to 100.0 ml with the mobile phase.

Reference solution (a). Dissolve 50.0 mg of cefamandole nafate CRS in the mobile phase and dilute to 100.0 ml with the mobile phase.

Reference solution (b). Dilute 1 ml of the test solution to 10 ml with the mobile phase, then heat the solution at 60 °C for 30 min.

Column:

- size: *l* = 0.25 m, Ø = 4.6 mm,
- stationary phase: octadecylsilyl silica gel for chromatography *R* (5 µm).

Mobile phase: mix 25 volumes of acetonitrile *R* and 75 volumes of a 10 per cent V/V solution of triethylamine *R* previously adjusted to pH 2.5 with phosphoric acid *R*.

Flow rate: 1.0 ml/min.

Detection: spectrophotometer at 254 nm.

Injection: 20 µl loop injector.

System suitability:

- resolution: minimum of 7.0 between the 2 principal peaks in the chromatogram obtained with reference solution (b),

- *repeatability*: maximum relative standard deviation of 0.8 per cent after a series of single injections of a minimum 3 freshly prepared reference solutions (a).

Calculate the percentage content of cefamandole ($C_{18}H_{18}N_6O_5S_2$) from the sum of the contents of cefamandole nafate and cefamandole free acid using the declared contents of *cefamandole nafate CRS*. 1 mg of cefamandole nafate is equivalent to 0.9025 mg of cefamandole.

Sodium carbonate. Dissolve 0.500 g in 50 ml of *water R*. Titrate with 0.1 M *hydrochloric acid*, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *hydrochloric acid* is equivalent to 10.6 mg of Na_2CO_3 .

STORAGE

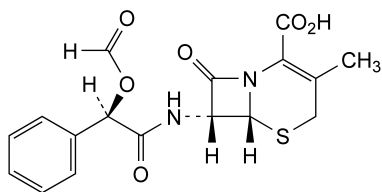
In an airtight container, protected from light. If the substance is sterile, store in a sterile, airtight, tamper-proof container.

LABELLING

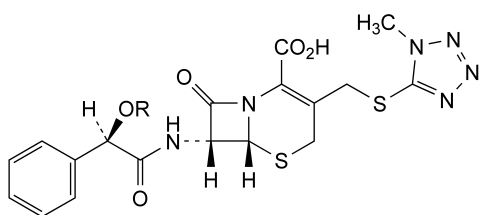
The label states:

- that the substance contains sodium carbonate,
- where applicable, that the substance is free from bacterial endotoxins.

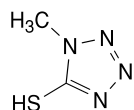
IMPURITIES



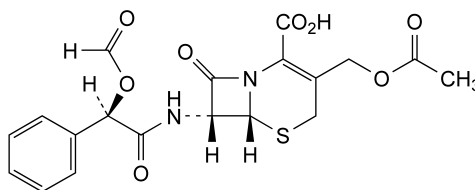
- A. (6*R*,7*R*)-7-[[[(2*R*)-2-(formyloxy)-2-phenylacetyl]amino]-3-methyl-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid (formylmandeloyl-7-amino-desacetoxy-cephalosporanic acid),



- B. R = H: (6*R*,7*R*)-7-[[[(2*R*)-2-hydroxy-2-phenylacetyl]amino]-3-[[[(1-methyl-1*H*-tetrazol-5-yl)sulphonyl]methyl]-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid (cefamandole),
- C. R = CO-H₃C: (6*R*,7*R*)-7-[[[(2*R*)-2-(acetyloxy)-2-phenylacetyl]amino]-3-[[[(1-methyl-1*H*-tetrazol-5-yl)sulphonyl]methyl]-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid (*O*-acetylcefamandole),



- D. 1-methyl-1*H*-tetrazole-5-thiol,

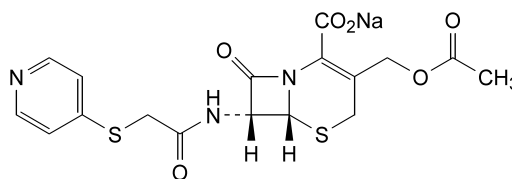


- E. (6*R*,7*R*)-7-[[[(2*R*)-2-(formyloxy)-2-phenylacetyl]amino]-3-[(acetyloxy)methyl]-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid (formylmandeloyl-7-ACA).

01/2005:1650

CEFAPIRIN SODIUM

Cefapirinum natriicum



$C_{17}H_{16}N_3NaO_6S_2$

M_r 445.5

DEFINITION

Sodium (6*R*,7*R*)-3-[(acetyloxy)methyl]-8-oxo-7-[[[(pyridin-4-yl)sulphonyl]acetyl]amino]-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylate.

Content: 96.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

Appearance: white or pale yellow powder.

Solubility: soluble in water, practically insoluble in methylene chloride.

IDENTIFICATION

- A. Infrared absorption spectrophotometry (2.2.24).

Comparison: *cefapirin sodium CRS*.

- B. It gives reaction (a) of sodium (2.3.1).

TESTS

Appearance of solution. Dissolve 2.0 g in *water R* and dilute to 10.0 ml with the same solvent. The solution is clear (2.2.1). Dilute 5.0 ml to 10.0 ml with *water R*. The absorbance (2.2.25) of this solution at 450 nm is maximum 0.25.

pH (2.2.3): 6.5 to 8.5.

Dissolve 0.100 g in *carbon dioxide-free water R* and dilute to 10.0 ml with the same solvent.

Specific optical rotation (2.2.7): + 150 to + 165 (anhydrous substance).

Dissolve 0.500 g in *water R* and dilute to 25.0 ml with the same solvent.

Related substances. Liquid chromatography (2.2.29).

Prepare the solutions immediately before use.

Test solution. Dissolve 42 mg of the substance to be examined in the mobile phase and dilute to 200.0 ml with the mobile phase.

Reference solution (a). Dissolve 42 mg of *cefapirin sodium CRS* in the mobile phase and dilute to 200.0 ml with the mobile phase.

Reference solution (b). Dilute 1.0 ml of the test solution to 100.0 ml with the mobile phase.

Reference solution (c). Dilute 1.0 ml of reference solution (b) to 20.0 ml with the mobile phase.

Reference solution (d). Mix 1 ml of the test solution, 8 ml of the mobile phase and 1 ml of *hydrochloric acid R1*. Heat at 60 °C for 10 min.

Column:

- **size:** $l = 0.30$ m, $\varnothing = 4$ mm,
- **stationary phase:** octadecylsilyl silica gel for chromatography *R* (10 μ m).

Mobile phase: mix 80 ml of *dimethylformamide R*, 4.0 ml of *glacial acetic acid R* and 20 ml of a 4.5 per cent (*m/m*) solution of *potassium hydroxide R*. Dilute to 2 litres with *water R*.

Flow rate: 2.0 ml/min.

Detection: spectrophotometer at 254 nm.

Injection: 20 μ l of the test solution and reference solutions (b), (c) and (d).

Run time: twice the retention time of cefapirin.

Relative retention with reference to cefapirin (retention time = about 13 min): impurity B = about 0.3; impurity C = about 0.5; impurity A = about 0.75.

System suitability: reference solution (d):

- **resolution:** minimum 2.0 between the peaks due to cefapirin and impurity A.

Limits:

- **any impurity:** for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (1.0 per cent), and not more than 1 such peak has an area greater than 0.3 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.3 per cent),
- **total:** not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (2.0 per cent),
- **disregard limit:** area of the principal peak in the chromatogram obtained with reference solution (c) (0.05 per cent).

***N,N*-Dimethylaniline** (2.4.26, *Method B*): maximum 20 ppm.

2-Ethylhexanoic acid (2.4.28): maximum 0.5 per cent.

Water (2.5.12): maximum 2.0 per cent, determined on 0.300 g.

Bacterial endotoxins (2.6.14): less than 0.17 IU/mg, if intended for use in the manufacture of parenteral dosage forms without a further appropriate procedure for the removal of bacterial endotoxins.

ASSAY

Liquid chromatography (2.2.29) as described in the test for related substances with the following modification.

Injection: test solution and reference solution (a).

Calculate the percentage content of $C_{17}H_{16}N_3NaO_6S_2$.

STORAGE

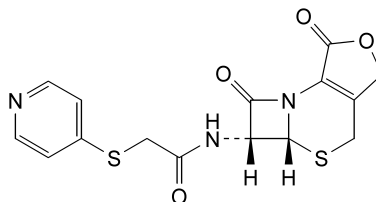
Protected from light. If the substance is sterile, store in a sterile, tamper-proof container.

LABELLING

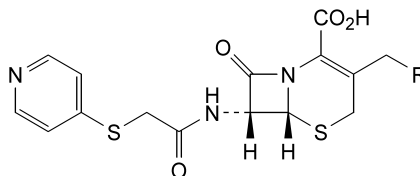
The label states, where applicable, that the substance is free from bacterial endotoxins.

IMPURITIES

Specified impurities: A, B, C.



A. (5a*R*,6*R*)-6-[[[(pyridin-4-yl)sulphonyl]acetyl]amino]-5a,6-dihydro-3*H*,7*H*-azeto[2,1-*b*]furo[3,4-*d*][1,3]thiazine-1,7(4*H*)-dione (deacetylcefapirin lactone),



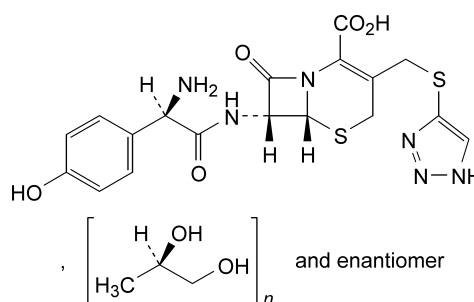
B. R = OH: (6*R*,7*R*)-3-(hydroxymethyl)-8-oxo-7-[[[(pyridin-4-yl)sulphonyl]acetyl]amino]-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid (deacetylcefapirin),

C. R = H: (6*R*,7*R*)-3-methyl-8-oxo-7-[[[(pyridin-4-yl)sulphonyl]acetyl]amino]-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid (deacetoxycefapirin).

01/2005:1403

CEFATRIZINE PROPYLENE GLYCOL

Cefatrizinum propylen glycolum



$C_{18}H_{18}N_6O_5S_2 \cdot (C_3H_8O_2)_n$

M_r 462.5 (base)

DEFINITION

Cefatrizine propylene glycol contains not less than 95.0 per cent and not more than the equivalent of 102.0 per cent of (6*R*,7*R*)-7-[[[(2*R*)-2-amino-2-(4-hydroxyphenyl)acetyl]amino]-8-oxo-3-[[[(1*H*-1,2,3-triazol-4-yl)sulphonyl]methyl]-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid, calculated with reference to the anhydrous and propylene glycol free substance. It consists of cefatrizine and propane-1,2-diol (molecular proportions about 1:1). It contains not less than 13.0 per cent and not more than 18.0 per cent of propylene glycol.

CHARACTERS

A white or almost white powder, slightly soluble in water, practically insoluble in alcohol and in methylene chloride.

IDENTIFICATION

- Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *cefatrizine propylene glycol CRS*.
- Examine the chromatograms obtained in the test for propylene glycol. The retention time and size of the principal peak in the chromatogram obtained with the

test solution are approximately the same as those of the principal peak in the chromatogram obtained with reference solution (b).

TESTS

Specific optical rotation (2.2.7). Dissolve 0.400 g of the substance to be examined in 1 M hydrochloric acid and dilute to 20.0 ml with the same solvent. The specific optical rotation is + 63 to + 69, calculated with reference to the anhydrous, propylene glycol free substance.

Propylene glycol. 13.0 per cent to 18.0 per cent, determined by gas chromatography (2.2.28), using dimethylacetamide R as the internal standard.

Internal standard solution. Dissolve 1.0 g of dimethylacetamide R in a mixture of 20 volumes of acetone R and 80 volumes of water R, and dilute to 50.0 ml with the same mixture of solvents.

Test solution. Introduce 0.40 g of the substance to be examined into a ground-glass-stoppered test tube. Add 3.0 ml of the internal standard solution, 1.0 ml of a mixture of 20 volumes of acetone R and 80 volumes of water R and 2.0 ml of hydrochloric acid R. Seal the test tube and stir the solution.

Reference solution (a). Dissolve 2.0 g of propylene glycol R in a mixture of 20 volumes of acetone R and 80 volumes of water R and dilute to 100.0 ml with the same solvent.

Reference solution (b). Introduce into a ground-glass-stoppered test tube 1.0 ml of reference solution (a) and 1.0 ml of the internal standard solution.

The chromatographic procedure may be carried out using:

- a stainless steel column 2 m long and 2 mm in internal diameter packed with ethylvinylbenzene-divinylbenzene copolymer R (150-180 µm),
- nitrogen for chromatography R as the carrier gas at a flow rate of about 30 ml/min,
- a flame-ionisation detector,

maintaining the temperature of the column at 200 °C and that of the injection port and of the detector at 250 °C.

Inject separately 1 µl of the test solution and 1 µl of reference solution (b).

7-ACA triazole and other related substances. Examine by liquid chromatography (2.2.29) as described under Assay. Adjust the sensitivity of the system so that the height of the principal peak obtained with 20 µl of reference solution (c) is at least 50 per cent of the full scale of the recorder. Inject 20 µl of the test solution and continue the chromatography for at least twice the retention time of the principal peak. In the chromatogram obtained with the test solution: the area of any peak corresponding to cefatrizine impurity A is not greater than that of the peak due to cefatrizine impurity A in the chromatogram obtained with reference solution (d) (0.5 per cent); the area of any peak apart from the principal peak and any peak corresponding to cefatrizine impurity A is not greater than the area of the principal peak in the chromatogram obtained with reference solution (c) (0.6 per cent); the sum of the areas of any such peaks is not greater than 3.5 times the area of the principal peak in the chromatogram obtained with reference solution (c) (2.1 per cent). Disregard any peak with an area less than 0.05 times that of the principal peak in the chromatogram obtained with reference solution (c).

Water (2.5.12). Not more than 1.5 per cent, determined on 0.500 g by the semi-micro determination of water.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 60.0 mg of the substance to be examined in the mobile phase and dilute to 100.0 ml with the mobile phase.

Reference solution (a). Dissolve 60.0 mg of cefatrizine propylene glycol CRS in the mobile phase and dilute to 100.0 ml with the mobile phase.

Reference solution (b). Dissolve 30.0 mg of cefatrizine impurity A CRS in buffer solution pH 7.0 R and dilute to 100.0 ml with the same buffer solution.

Reference solution (c). Dilute 0.6 ml of reference solution (a) to 100.0 ml with the mobile phase.

Reference solution (d). Dilute 1.0 ml of reference solution (b) to 100.0 ml with buffer solution pH 7.0 R.

Reference solution (e). To 1.0 ml of reference solution (a) add 1.0 ml of reference solution (b) and dilute to 10.0 ml with the mobile phase.

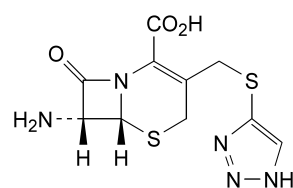
The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4 mm in internal diameter packed with octadecylsilyl silica gel for chromatography R (5 µm),
- as mobile phase at a flow rate of 2 ml/min a mixture of 5 volumes of acetonitrile R and 95 volumes of a 2.72 g/l solution of potassium dihydrogen phosphate R in water R,
- as detector a spectrophotometer set at 272 nm.

Adjust the sensitivity of the system so that the height of the principal peak obtained with 20 µl of reference solution (a) is at least 50 per cent of the full scale of the recorder. Inject 20 µl of reference solution (a) six times. The test is not valid unless the relative standard deviation is at most 1.0 per cent. Inject reference solution (e). The test is not valid unless the resolution between the peaks corresponding to cefatrizine and cefatrizine impurity A is at least 5.0.

Inject alternately 20 µl of the test solution and 20 µl of reference solution (a) and calculate the percentage content of cefatrizine using the chromatogram obtained with reference solution (a).

IMPURITIES

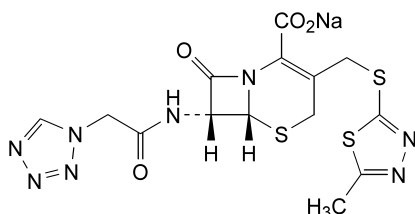


- A. (6R,7R)-7-amino-8-oxo-3-[(1H-1,2,3-triazol-4-yl)sulphanyl]methyl-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid (7-ACA triazole).

01/2005:0988

CEFAZOLIN SODIUM

Cefazolinum natricum

 $C_{14}H_{13}N_8NaO_4S_3$ M_r 476.5

DEFINITION

Sodium (6*R*,7*R*)-3-[[[(5-methyl-1,3,4-thiadiazol-2-yl)sulphanyl]methyl]-8-oxo-7-[(1*H*-tetrazol-1-ylacetyl)amino]-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylate.

Content: 95.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

Appearance: white or almost white powder, very hygroscopic.

Solubility: freely soluble in water, very slightly soluble in alcohol.

It shows polymorphism.

IDENTIFICATION

A. Infrared absorption spectrophotometry (2.2.24).

Preparation: dissolve 0.150 g in 5 ml of *water R*, add 0.5 ml of *dilute acetic acid R*, swirl and allow to stand for 10 min in iced water. Filter the precipitate and rinse with 1-2 ml of *water R*. Dissolve in a mixture of 1 volume of *water R* and 9 volumes of *acetone R*. Evaporate the solvent almost to dryness, then dry in an oven at 60 °C for 30 min.

Comparison: *cefazolin CRS*.

B. It gives reaction (a) of sodium (2.3.1).

TESTS

Solution S. Dissolve 2.50 g in *carbon dioxide-free water R* and dilute to 25.0 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and its absorbance (2.2.25) at 430 nm has a maximum of 0.15.

pH (2.2.3): 4.0 to 6.0 for solution S.

Specific optical rotation (2.2.7): -15 to -24 (anhydrous substance).

Dissolve 1.25 g in *water R* and dilute to 25.0 ml with the same solvent.

Absorbance (2.2.25). Dissolve 0.100 g in *water R* and dilute to 100.0 ml with the same solvent. Dilute 2.0 ml of the solution to 100.0 ml with *sodium hydrogen carbonate solution R*. Examined between 220 nm and 350 nm, the solution shows an absorption maximum at 272 nm. The specific absorbance at the maximum is 260 to 300 (anhydrous substance).

Related substances. Liquid chromatography (2.2.29).

Test solution. Dissolve 50.0 mg of the substance to be examined in mobile phase A and dilute to 20.0 ml with the same mobile phase.

Reference solution (a). Dilute 1.0 ml of the test solution to 100.0 ml with mobile phase A.

Reference solution (b). Dissolve 20 mg of the substance to be examined in 10 ml of a 2 g/l solution of *sodium hydroxide R*. Allow to stand for 15-30 min. Dilute 1.0 ml of the solution to 20 ml with mobile phase A.

Column:

- *size*: $l = 0.125$ m, $\varnothing = 4.0$ mm,
- *stationary phase*: octadecylsilyl silica gel for chromatography *R* (3 μ m),
- *temperature*: 45 °C.

Mobile phase:

- *mobile phase A*: solution containing 14.54 g/l of *disodium hydrogen phosphate R* and 3.53 g/l of *potassium dihydrogen phosphate R*,
- *mobile phase B*: *acetonitrile for chromatography R*,

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)	λ (nm)
0 - 1	98	2	210
1 - 2	98	2	254
2 - 4	98 → 85	2 → 15	254
4 - 10	85 → 60	15 → 40	254
10 - 11.5	60 → 35	40 → 65	254
11.5 - 12	35	65	254
12 - 15	35 → 98	65 → 2	254
15 - 16	98	2	254
16 - 21	98	2	210

Flow rate: 1.2 ml/min.

Detection: spectrophotometer at 210 nm and at 254 nm (see table above).

Injection: 5 μ l.

System suitability: reference solution (b):

- *resolution*: minimum 2.0 between the peaks due to cefazolin and to impurity I (see Figure 0988-1).

Limits:

- *any impurity* (seen at 210 nm or 254 nm): not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (1.0 per cent),
- *total*: not more than 3.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (3.5 per cent),
- *disregard limit*: 0.05 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

***N,N*-Dimethylaniline** (2.4.26, *Method B*): maximum 20 ppm.

Water (2.5.12): maximum 6.0 per cent, determined on 0.300 g.

Bacterial endotoxins (2.6.14): less than 0.15 IU/mg, if intended for use in the manufacture of parenteral dosage forms without a further appropriate procedure for the removal of bacterial endotoxins.

ASSAY

Liquid chromatography (2.2.29).

Test solution. Dissolve 50.0 mg of the substance to be examined in the mobile phase and dilute to 50.0 ml with the mobile phase.

Reference solution (a). Dissolve 50.0 mg of *cefazolin CRS* in the mobile phase and dilute to 50.0 ml with the mobile phase.

Reference solution (b). Dissolve 5.0 mg of *cefuroxime sodium CRS* in 10.0 ml of reference solution (a) and dilute to 100.0 ml with the mobile phase.

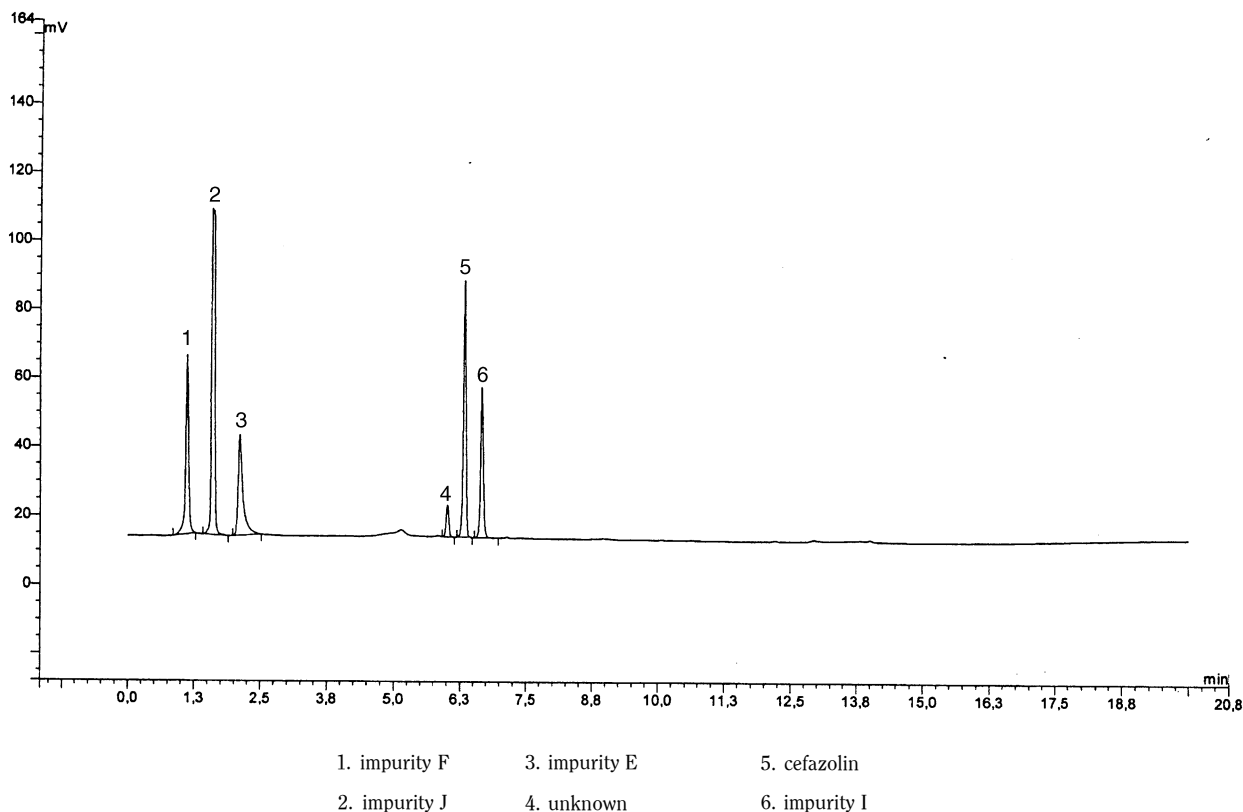


Figure 0988.-1. – Chromatogram of reference solution (b) (in situ degradation) for the test for related substances of cefazolin sodium

Column:

- size: $l = 0.25$ m, $\varnothing = 4.6$ mm,
- stationary phase: octadecylsilyl silica gel for chromatography R (5 μ m).

Mobile phase: mix 10 volumes of acetonitrile R and 90 volumes of a solution containing 2.77 g/l of disodium hydrogen phosphate R and 1.86 g/l of citric acid R .

Flow rate: 1.0 ml/min.

Detection: spectrophotometer at 270 nm.

Injection: 20 μ l.

System suitability: reference solution (b):

- resolution: minimum 2.0 between the peaks due to cefazolin and cefuroxime.

Calculate the percentage content of cefazolin sodium by multiplying the percentage content of cefazolin by 1.048.

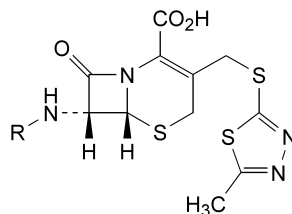
STORAGE

In an airtight container, protected from light. If the substance is sterile, store in a sterile, airtight, tamper-proof container.

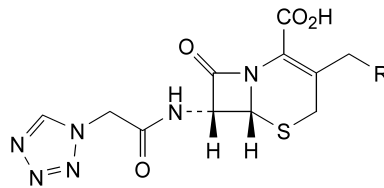
LABELLING

The label states, where applicable, that the substance is free from bacterial endotoxins.

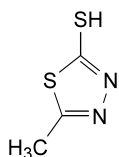
IMPURITIES



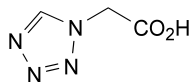
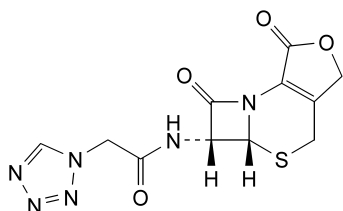
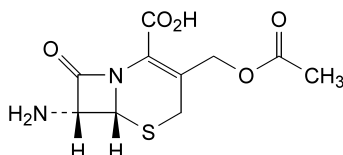
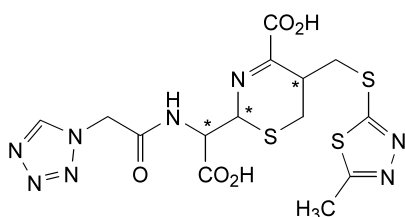
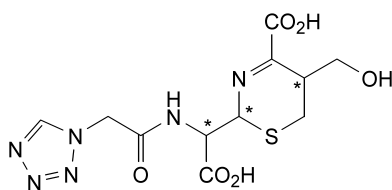
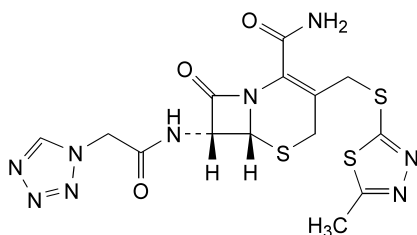
- A. R = H: (6*R*,7*R*)-7-amino-3-[[[(5-methyl-1,3,4-thiadiazol-2-yl)sulphonyl]methyl]-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid,
- B. R = CO-C(CH₃)₃: (6*R*,7*R*)-7-[(2,2-dimethylpropanoyl)amino]-3-[[[(5-methyl-1,3,4-thiadiazol-2-yl)sulphonyl]methyl]-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid,



- C. R = H: (6*R*,7*R*)-3-methyl-8-oxo-7-[(1*H*-tetrazol-1-ylacetyl)amino]-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid,
- D. R = O-CO-CH₃: (6*R*,7*R*)-3-(acetyloxy)methyl-8-oxo-7-[(1*H*-tetrazol-1-ylacetyl)amino]-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid,

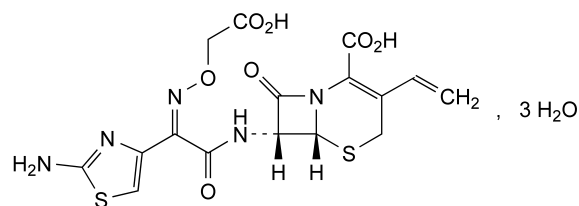
01/2005:1188
corrected

E. 5-methyl-1,3,4-thiadiazol-2-thiol (MMTD),

F. (1*H*-tetrazol-1-yl)acetic acid,G. (5a*R*,6*R*)-6-[(1*H*-tetrazol-1-ylacetyl)amino]-5a,6-dihydro-3*H*,7*H*-azeto[2,1-*b*]furo[3,4-*d*][1,3]thiazine-1,7(4*H*)-dione,H. (6*R*,7*R*)-3-[(acetyloxy)methyl]-7-amino-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid (7-ACA),I. 2-[carboxy[(1*H*-tetrazol-1-ylacetyl)amino]methyl]-5-[(5-methyl-1,3,4-thiadiazol-2-yl)sulphonyl]methyl]-5,6-dihydro-2*H*-1,3-thiazine-4-carboxylic acid (cefazoloic acid),J. 2-[carboxy[(1*H*-tetrazol-1-ylacetyl)amino]methyl]-5-(hydroxymethyl)-5,6-dihydro-2*H*-1,3-thiazine-4-carboxylic acid (hydrolysed cefazoloic acid),K. (6*R*,7*R*)-3-[(5-methyl-1,3,4-thiadiazol-2-yl)sulphonyl]methyl]-8-oxo-7-[(1*H*-tetrazol-1-ylacetyl)amino]-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxamide (cefazolinamide).

CEFIXIME

Cefiximum

 $C_{16}H_{15}N_5O_7S_2 \cdot 3H_2O$ M_r 507.5

DEFINITION

Cefixime is (6*R*,7*R*)-7-[(*Z*)-2-(2-aminothiazol-4-yl)-2-[(carboxymethoxy)imino]acetyl]amino]-3-ethenyl-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid trihydrate. It contains not less than 95.0 per cent and not more than the equivalent of 101.0 per cent of $C_{16}H_{15}N_5O_7S_2$, calculated with reference to the anhydrous and ethanol-free substance.

CHARACTERS

A white or almost white powder, slightly hygroscopic, slightly soluble in water, soluble in methanol, sparingly soluble in ethanol, practically insoluble in ethyl acetate.

IDENTIFICATION

Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *cefixime CRS*. If the spectra obtained show differences, dissolve the substance to be examined and the reference substance separately in *methanol R*, evaporate to dryness and record new spectra using the residues.

TESTS

pH (2.2.3). Suspend 0.5 g in *carbon dioxide-free water R* and dilute to 10 ml with the same solvent. The pH of the suspension is 2.6 to 4.1.

Related substances. Examine by liquid chromatography (2.2.29) as described under Assay. Inject reference solution (b). Adjust the sensitivity of the system so that the height of the principal peak in the chromatogram obtained is at least 50 per cent of the full scale of the recorder. Inject the test solution and continue the chromatography for 3 times the retention time of the principal peak. In the chromatogram obtained with the test solution: the area of any peak, apart from the principal peak, is not greater than half the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent); the sum of the areas of all the peaks, apart from the principal peak, is not greater than 3 times the area of the principal peak in the chromatogram obtained with reference solution (b) (3 per cent). Disregard any peak with an area less than 0.1 times that of the principal peak in the chromatogram obtained with reference solution (b).

Ethanol (2.4.24). Not more than 1.0 per cent *m/m*, determined by head-space gas chromatography (2.2.28), using the standard additions method.

Sample solution. Dissolve 0.250 g of the substance to be examined in a mixture of 1 volume of *dimethylacetamide R* and 4 volumes of *water R* and dilute to 25.0 ml with the same mixture of solvents.

Water (2.5.12): 9.0 per cent to 12.0 per cent, determined on 0.200 g by the semi-micro determination of water.

Sulphated ash (2.4.14). Not more than 0.2 per cent, determined on 1.0 g.

ASSAY

Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 25.0 mg of the substance to be examined in the mobile phase and dilute to 25.0 ml with the mobile phase.

Reference solution (a). Dissolve 25.0 mg of *cefixime CRS* in the mobile phase and dilute to 25.0 ml with the mobile phase.

Reference solution (b). Dilute 1.0 ml of reference solution (a) to 100.0 ml with the mobile phase.

Reference solution (c). Dissolve 10 mg of *cefixime CRS* in 10 ml of *water R*. Heat on a water-bath for 45 min. Cool and inject immediately.

The chromatographic procedure may be carried out using:

- a column 0.125 m long and 4 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (5 µm),
- as mobile phase at a flow rate of 1.0 ml/min a mixture of 250 volumes of *acetonitrile R* and 750 volumes of a tetrabutylammonium hydroxide solution prepared as follows: dissolve 8.2 g of *tetrabutylammonium hydroxide R* in *water R* and dilute to 800 ml with the same solvent; adjust to pH 6.5 with *dilute phosphoric acid R* and dilute to 1000 ml with *water R*,
- as detector a spectrophotometer set at 254 nm,
- a 10 µl loop injector,

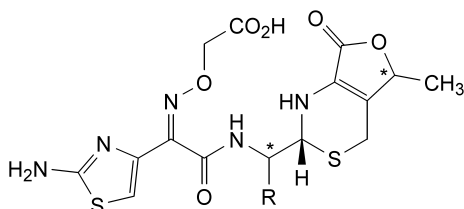
maintaining the temperature of the column at 40 °C.

Inject reference solution (c). Adjust the sensitivity of the system so that the heights of the principal peaks are at least 20 per cent of the full scale of the recorder. The test is not valid unless the resolution between the 2 principal peaks (*cefixime* and *E*-isomer) is at least 2.0. If necessary, adjust the concentration of acetonitrile in the mobile phase. Inject reference solution (a) 6 times. The test is not valid unless the relative standard deviation of the peak area for *cefixime* is at most 1.0 per cent. Inject alternately the test solution and reference solution (a).

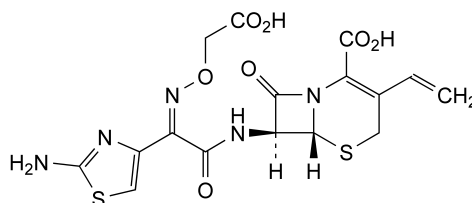
STORAGE

Store in an airtight container, protected from light.

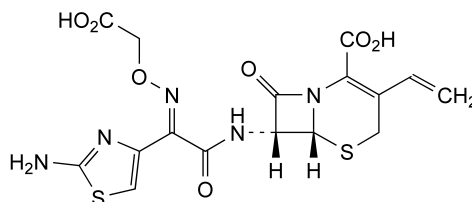
IMPURITIES



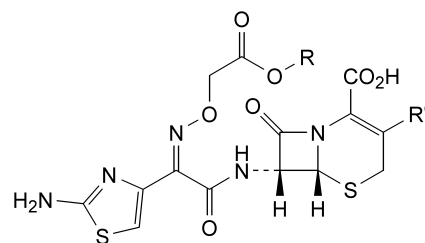
- A. R = CO₂H: 2-[[[(Z)-2-(2-aminothiazol-4-yl)-2-[(carboxymethoxy)imino]acetyl]amino]-2-[(2R)-5-methyl-7-oxo-1,2,5,7-tetrahydro-4H-furo[3,4-d][1,3]thiazin-2-yl]acetic acid,
- B. R = H: 2-[[[(Z)-1-(2-aminothiazol-4-yl)-2-[[[(2R,5RS)-5-methyl-7-oxo-1,2,5,7-tetrahydro-4H-furo[3,4-d][1,3]thiazin-2-yl]methyl]amino]-2-oxoethylidene]amino]oxy]acetic acid,



- C. (6*R*,7*S*)-7-[[[(*Z*)-2-(2-aminothiazol-4-yl)-2-[(carboxymethoxy)imino]acetyl]amino]-3-ethenyl-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid (*cefixime* 7-epimer),



- D. (6*R*,7*R*)-7-[[[(*E*)-2-(2-aminothiazol-4-yl)-2-[(carboxymethoxy)imino]acetyl]amino]-3-ethenyl-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid (*cefixime E*-isomer),



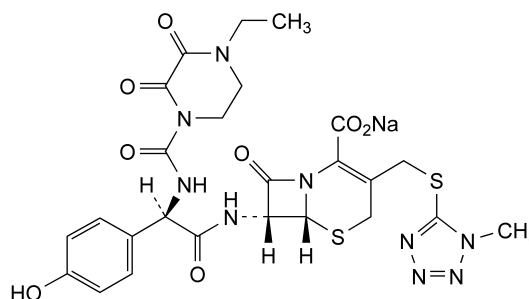
- E. R = H, R' = CH₃: (6*R*,7*R*)-7-[[[(*Z*)-2-(2-aminothiazol-4-yl)-2-[(carboxymethoxy)imino]acetyl]amino]-3-methyl-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid,

- F. R = C₂H₅, R' = CH=CH₂: (6*R*,7*R*)-7-[[[(*Z*)-2-(2-aminothiazol-4-yl)-2-[(2-ethoxy-2-oxoethoxy)imino]acetyl]amino]-3-ethenyl-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid.

01/2005:1404

CEFOPERAZONE SODIUM

Cefoperazonum natricum



C₂₅H₂₆N₉NaO₈S₂

*M*_r 668

DEFINITION

Cefoperazone sodium contains not less than 95.0 per cent and not more than the equivalent of 102.0 per cent of sodium (6*R*,7*R*)-7-[[[(2*R*)-2-[[[(4-ethyl-2,3-dioxopiperazin-1-yl)carbonyl]amino]-2-(4-hydroxyphenyl)acetyl]amino]-3-[[[(1-methyl-1*H*-tetrazol-5-yl)sulphonyl]methyl]-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylate, calculated with reference to the anhydrous and acetone-free substance.

CHARACTERS

A white or slightly yellow powder, hygroscopic, freely soluble in water, soluble in methanol, slightly soluble in alcohol.

If crystalline, it shows polymorphism.

IDENTIFICATION

- A. Dissolve the substance to be examined in *methanol R* and evaporate to dryness. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the *Ph. Eur. reference spectrum of cefoperazone sodium*.
- B. Examine the chromatograms obtained in the assay. The retention time and size of the principal peak in the chromatogram obtained with test solution (a) are approximately the same as those of the principal peak in the chromatogram obtained with reference solution (a).
- C. It gives reaction (a) of sodium (2.3.1).

TESTS

Appearance of solution. Dissolve 2.5 g in *water R* and dilute to 25.0 ml with the same solvent. The solution is clear (2.2.1). The absorbance of the solution measured at 430 nm (2.2.25) is not greater than 0.15.

pH (2.2.3). Dissolve 2.5 g in *carbon dioxide-free water R* and dilute to 10 ml with the same solvent. The pH of the solution is 4.5 to 6.5.

Related substances. Examine by liquid chromatography (2.2.29) as prescribed under Assay. Inject 20 µl of reference solution (b) and adjust the sensitivity of the system so that the height of the principal peak in the chromatogram obtained is at least 50 per cent of the full scale of the recorder. Inject 20 µl of test solution (b). Continue the chromatography for at least 2.5 times the retention time of the principal peak. In the chromatogram obtained with test solution (b): the area of any peak, apart from the principal peak, is not greater than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (1.5 per cent); the sum of the areas of any such peaks is not greater than 4.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (4.5 per cent). Disregard any peak with an area less than 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (b).

Acetone. Not more than 2.0 per cent, determined by head-space gas chromatography (2.2.28), using the method of standard additions.

Sample solution. Dissolve 0.500 g of the substance to be examined in *water R* and dilute to 10.0 ml with the same solvent.

Solvent solution. Dissolve 0.350 g of *acetone R* in *water R* and dilute to 100.0 ml with the same solvent. Dilute 10.0 ml of the solution to 100.0 ml with *water R*.

Prepare each of 4 injection vials as shown in the table below:

Vial No.	Sample solution (ml)	Solvent solution (ml)	Water R (ml)
1	1.0	0	4.0
2	1.0	1.0	3.0
3	1.0	2.0	2.0
4	1.0	3.0	1.0

The chromatographic procedure may be carried out using system B of the test for residual solvents (2.4.24) and the following static head-space injection conditions:

- equilibration time: 15 min,

- transfer-line temperature: 110 °C,

maintaining the temperature of the column at 40 °C for 10 min.

Heavy metals (2.4.8). 2.0 g complies with limit test C (5 ppm). Prepare the standard using 1 ml of *lead standard solution (10 ppm Pb) R*.

Water (2.5.12). Not more than 5.0 per cent, determined on 0.200 g.

Bacterial endotoxins (2.6.14): less than 0.20 IU/mg, if intended for use in the manufacture of parenteral dosage forms without a further appropriate procedure for the removal of bacterial endotoxins.

ASSAY

Examine by liquid chromatography (2.2.29). *Prepare the solutions immediately before use.*

Test solution (a). Dissolve 25.0 mg of the substance to be examined in the mobile phase and dilute to 250.0 ml with the mobile phase.

Test solution (b). Dissolve 25.0 mg of the substance to be examined in the mobile phase and dilute to 50.0 ml with the mobile phase.

Reference solution (a). Dissolve 25.0 mg of *cefoperazone dihydrate CRS* in the mobile phase and dilute to 250.0 ml with the mobile phase.

Reference solution (b). Dilute 5.0 ml of reference solution (a) to 100.0 ml with the mobile phase.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.15 m long and 4.6 mm in internal diameter packed with *end-capped octadecylsilyl silica gel for chromatography R* (5 µm),
- as mobile phase at a flow rate of 1 ml/min a mixture of 884 volumes of *water R*; 110 volumes of *acetonitrile R*; 3.5 volumes of a 60 g/l solution of *acetic acid R*; 2.5 volumes of a triethylammonium acetate solution prepared by diluting 14 ml of *triethylamine R* and 5.7 ml of *glacial acetic acid R* to 100 ml with *water R*,
- as detector a spectrophotometer set at 254 nm.

Inject 20 µl of reference solution (a). When the chromatogram is recorded in the prescribed conditions, the retention time is about 15 min for cefoperazone. Adjust the sensitivity of the system so that the height of the principal peak in the chromatogram obtained is at least 50 per cent of the full scale of the recorder. The test is not valid unless in the chromatogram obtained with reference solution (a), the number of theoretical plates calculated for the principal peak is at least 5000 and its symmetry factor is at most 1.6. If necessary adjust the content of *acetonitrile R* in the mobile phase. Inject reference solution (a) 6 times. The test is not valid unless the relative standard deviation of the peak area is at most 1.0 per cent. Inject alternately test solution (a) and reference solution (a).

Calculate the percentage content of cefoperazone sodium by multiplying the percentage content of cefoperazone by 1.034.

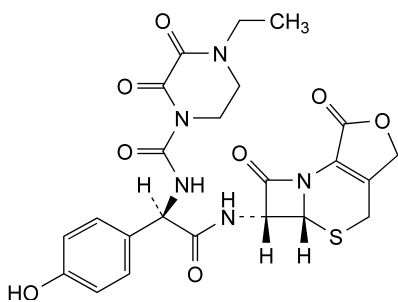
STORAGE

In an airtight container, protected from light, at a temperature of 2 °C to 8 °C. If the substance is sterile, store in a sterile, airtight, tamper-proof container.

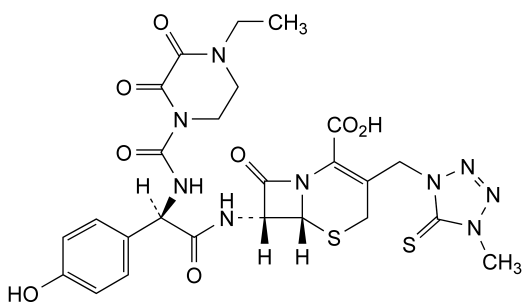
LABELLING

The label states, where applicable, that the substance is free from bacterial endotoxins.

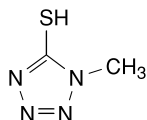
IMPURITIES



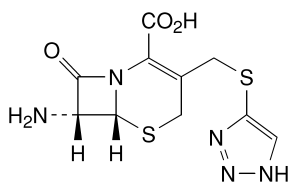
- A. (5a*R*,6*R*)-6-[[[(2*R*)-2-[[[(4-ethyl-2,3-dioxopiperazin-1-yl)carbonyl]amino]-2-(4-hydroxyphenyl)acetyl]amino]-5a,6-dihydro-3*H*,7*H*-azeto[2,1-*b*]furo[3,4-*d*][1,3]thiazine-1,7(4*H*)-dione,



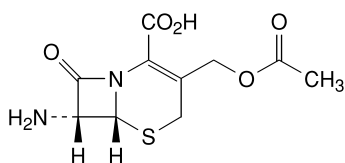
- B. (6*R*,7*R*)-7-[[[(2*R*)-2-[[[(4-ethyl-2,3-dioxopiperazin-1-yl)carbonyl]amino]-2-(4-hydroxyphenyl)acetyl]amino]-3-[(4-methyl-5-thioxo-4,5-dihydro-1*H*-tetrazol-1-yl)methyl]-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid,



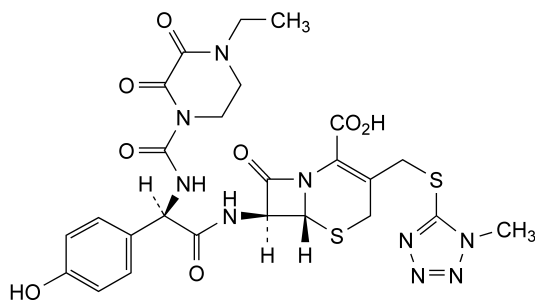
- C. 1-methyl-1*H*-tetrazole-5-thiol,



- D. (6*R*,7*R*)-7-amino-8-oxo-3-[(1*H*-1,2,3-triazol-4-ylsulphanyl)methyl]-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid (7-TACA),



- E. (6*R*,7*R*)-3-[(acetyloxy)methyl]-7-amino-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid (7-ACA),

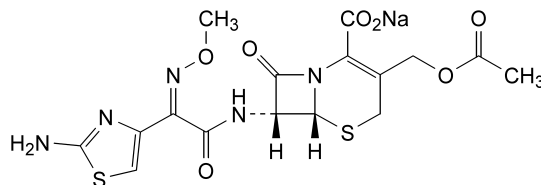


- F. (6*R*,7*S*)-7-[[[(2*R*)-2-[[[(4-ethyl-2,3-dioxopiperazine-1-yl)carbonyl]amino]-2-(4-hydroxyphenyl)acetyl]amino]-3-[[[(1-methyl-1*H*-tetrazol-5-yl)sulphanyl)methyl]-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid.

01/2005:0989
corrected

CEFOTAXIME SODIUM

Cefotaximum natricum



$C_{16}H_{16}N_5NaO_7S_2$

M_r 477.4

DEFINITION

Cefotaxime sodium contains not less than 96.0 per cent and not more than the equivalent of 101.0 per cent of sodium (6*R*,7*R*)-3-[(acetyloxy)methyl]-7-[[[(*Z*)-2-(2-aminothiazol-4-yl)-2-(methoxyimino)acetyl]amino]-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylate, calculated with reference to the dried substance.

CHARACTERS

A white or slightly yellow powder, hygroscopic, freely soluble in water, sparingly soluble in methanol.

IDENTIFICATION

- A. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *cefotaxime sodium CRS*.

- B. It gives reaction (a) of sodium (2.3.1).

TESTS

Solution S. Dissolve 2.5 g in *carbon dioxide-free water R* and dilute to 25.0 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1). Add 1 ml of *glacial acetic acid R* to 10 ml of solution S. The solution, examined immediately, is clear. The absorbance (2.2.25) of solution S measured at 430 nm is not greater than 0.20.

pH (2.2.3). The pH of solution S is 4.5 to 6.5.

Specific optical rotation (2.2.7). Dissolve 0.100 g in *water R* and dilute to 10.0 ml with the same solvent. The specific optical rotation is + 58 to + 64, calculated with reference to the dried substance.

Absorbance (2.2.25). Dissolve 20.0 mg in *water R* and dilute to 100.0 ml with the same solvent. Dilute 10.0 ml of this solution to 100.0 ml with *water R*. The specific absorbance determined at the maximum at 235 nm is 360 to 390, calculated with reference to the dried substance.

Related substances. Examine by liquid chromatography (2.2.29) as described under Assay.

Inject the test solution and reference solution (b). Continue the chromatography for at least eight times the retention time of the main peak. In the chromatogram obtained with the test solution: the area of any peak, apart from the principal peak, is not greater than the area of the principal peak in the chromatogram obtained with reference solution (b) (1 per cent); the sum of the areas of such peaks is at most three times the area of the principal peak in the chromatogram obtained with reference solution (b) (3 per cent).

***N,N*-Dimethylaniline** (2.4.26, *Method B*). Not more than 20 ppm.

2-Ethylhexanoic acid (2.4.28). Not more than 0.5 per cent *m/m*.

Loss on drying (2.2.32). Not more than 3.0 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C.

Bacterial endotoxins (2.6.14): less than 0.05 IU/mg, if intended for use in the manufacture of parenteral dosage forms without a further appropriate procedure for removal of bacterial endotoxins.

ASSAY

Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 25.0 mg of the substance to be examined in the mobile phase and dilute to 25.0 ml with the same solvent.

Reference solution (a). Dissolve 25.0 mg of *cefotaxime sodium CRS* in the mobile phase and dilute to 25.0 ml with the same solvent.

Reference solution (b). Dilute 1.0 ml of reference solution (a) to 100.0 ml with the mobile phase.

Reference solution (c). Add 1.0 ml of *dilute hydrochloric acid R* to 4.0 ml of the test solution. Heat the solution at 40 °C for 2 h. Add 5.0 ml of *buffer solution pH 6.6 R* and 1.0 ml of *dilute sodium hydroxide solution R*.

The chromatographic procedure may be carried out using:

- a column 0.25 m long and 4.6 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (5 µm),
- as mobile phase at a flow rate of 1.0 ml/min a mixture prepared as follows: dissolve 3.5 g of *potassium dihydrogen phosphate R* and 11.6 g of *disodium hydrogen phosphate R* in 1000 ml of *water R* at pH 7.0 and add 180 ml of *methanol R*,
- as detector a spectrophotometer set at 235 nm,
- an injector with a fixed loop of 10 µl.

Inject reference solution (a) and reference solution (c). Adjust the attenuation to obtain principal peaks from reference solution (c) with a height of at least 50 per cent of the full-scale deflection of the recorder. The test is not valid unless cefotaxime is eluted as the second of the principal peaks and the resolution between the two principal peaks is at least 3.5. If necessary, use another stationary phase or

adjust the methanol content of the mobile phase. The test is not valid if the symmetry factor of the cefotaxime peak is greater than 2.0. Inject reference solution (a) six times. The test is not valid unless the relative standard deviation of the area of the cefotaxime peak is at most 1.0 per cent. Inject alternately the test solution and reference solution (a).

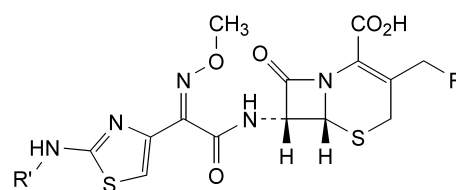
STORAGE

Store in an airtight container, protected from light at a temperature not exceeding 30 °C. If the substance is sterile, store in an sterile, airtight, tamper-proof container.

LABELLING

The label states, where applicable, that the substance is free from bacterial endotoxins.

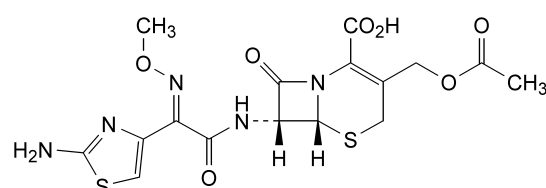
IMPURITIES



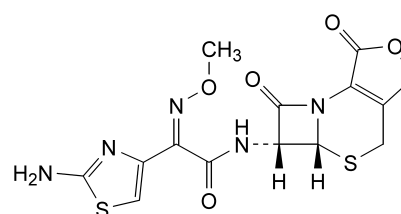
A. R = R' = H: (6*R*,7*R*)-7-[[*(Z)*-2-(2-aminothiazol-4-yl)-2-methoxyiminoacetyl]amino]-3-methyl-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid (desacetocefotaxime),

B. R = OH, R' = H: (6*R*,7*R*)-7-[[*(Z)*-2-(2-aminothiazol-4-yl)-2-methoxyiminoacetyl]amino]-3-(hydroxymethyl)-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid (desacetylcefotaxime),

C. R = O-CO-CH₃, R' = CHO: (6*R*,7*R*)-3-[(acetyloxy)methyl]-7-[[*(Z)*-2-[2-(formylamino)thiazol-4-yl]-2-(methoxyimino)acetyl]amino]-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid (*N*-formylcefotaxime),



D. (6*R*,7*R*)-3-[(acetyloxy)methyl]-7-[[*(E)*-2-(2-aminothiazol-4-yl)-2-(methoxyimino)acetyl]amino]-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid (*E*-cefotaxime),

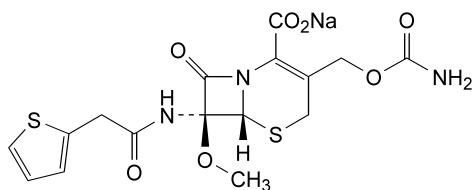


E. (5*aR*,6*R*)-6-[[*(Z)*-2-(2-aminothiazol-4-yl)-2-(methoxyimino)acetyl]amino]-5*a*,6-dihydro-3*H*,7*H*-azeto[2,1-*b*]furo[3,4-*d*][1,3]thiazine-1,7(4*H*)-dione (deacetylcefotaxime lactone).

01/2005:0990

CEFOXITIN SODIUM

Cefoxitinum natricum

 $C_{16}H_{16}N_3NaO_7S_2$ M_r 449.4**DEFINITION**

Sodium (6*R*,7*S*)-3-[(carbamoyloxy)methyl]-7-methoxy-8-oxo-7-[[thiophen-2-yl)acetyl]amino]-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylate.

Content: 95.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

Appearance: white or almost white powder, very hygroscopic.

Solubility: very soluble in water, sparingly soluble in alcohol.

IDENTIFICATION

A. Infrared absorption spectrophotometry (2.2.24).

Comparison: cefoxitin sodium CRS.

B. It gives reaction (a) of sodium (2.3.1).

TESTS

Solution S. Dissolve 2.50 g in carbon dioxide-free water *R* and dilute to 25 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and not more intensely coloured than intensity 5 of the range of reference solutions of the most appropriate colour (2.2.2, *Method II*).

pH (2.2.3): 4.2 to 7.0.

Dilute 2 ml of solution S to 20 ml with carbon dioxide-free water *R*.

Specific optical rotation (2.2.7): + 206 to + 214 (anhydrous substance).

Dissolve 0.250 g in methanol *R* and dilute to 25.0 ml with the same solvent.

Absorbance (2.2.25). Dissolve 0.100 g in water *R* and dilute to 100.0 ml with the same solvent. Dilute 2.0 ml of the solution to 100.0 ml with sodium hydrogen carbonate solution *R*. Examined between 220 nm and 350 nm, the solution shows an absorption maximum at 236 nm and a broad absorption maximum at about 262 nm. The specific absorbance at this broad maximum is 190 to 210 (anhydrous substance).

Related substances. Liquid chromatography (2.2.29).

Prepare the solutions immediately before use.

Solution A. Dilute 20 ml of a 34.8 g/l solution of dipotassium hydrogen phosphate *R* adjusted to pH 6.8 with phosphoric acid *R* to 1000 ml with water *R*.

Test solution. Dissolve 50.0 mg of the substance to be examined in solution A and dilute to 10.0 ml with the same solution.

Reference solution (a). Dilute 1.0 ml of the test solution to 100.0 ml with solution A.

Reference solution (b). To 1.0 ml of the test solution add 7.0 ml of water *R* and 2.0 ml of methanol *R*. Add 25 mg of sodium carbonate *R*, stir for 10 min at room temperature, then heat in a water-bath at 70 °C for 30 min. Allow to cool. Add 3 drops of glacial acetic acid *R* and 1 ml of the test solution and mix.

Column:

– size: $l = 0.25$ m, $\varnothing = 4.6$ mm,

– stationary phase: phenylsilyl silica gel for chromatography *R* (5 μ m) with a specific surface area of 300 m²/g and a pore size of 7 nm.

Mobile phase:

– mobile phase A: water *R* adjusted to pH 2.7 with anhydrous formic acid *R*,

– mobile phase B: acetonitrile *R*,

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)
0 - 12	90	10
12 - 37	90 → 80	10 → 20
37 - 50	80 → 60	20 → 40
50 - 55	60 → 20	40 → 80
55 - 60	20	80
60 - 62	20 → 90	80 → 10
62 - 70	90	10

Flow rate: 1 ml/min.

Detection: spectrophotometer at 235 nm.

Injection: 50 μ l.

Relative retentions with reference to cefoxitin (retention time = about 34 min): impurity A = about 0.82; impurity B = about 1.16; impurity C = about 1.27; impurity D = about 1.31.

System suitability: reference solution (b):

– resolution: minimum 5.0 between the 2 principal peaks.

Limits:

– any impurity: not more than half the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent),

– total: not more than 4 times the area of the principal peak in the chromatogram obtained with reference solution (a) (4.0 per cent),

– disregard limit: 0.05 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

Water (2.5.12): maximum 1.0 per cent, determined on 0.500 g.

Bacterial endotoxins (2.6.14): less than 0.13 IU/mg, if intended for use in the manufacture of parenteral dosage forms without a further appropriate procedure for the removal of bacterial endotoxins.

ASSAY

Liquid chromatography (2.2.29).

Test solution. Dissolve 25.0 mg of the substance to be examined in water *R* and dilute to 25.0 ml with the same solvent.

Reference solution (a). Dissolve 25.0 mg of cefoxitin sodium CRS in water *R* and dilute to 25.0 ml with the same solvent.

Reference solution (b). Dissolve 20.0 mg of 2-(2-thienyl)acetic acid *R* in water *R* and dilute to 25.0 ml with the same solvent.

Reference solution (c). Mix 1.0 ml of reference solution (a) and 5.0 ml of reference solution (b).

Column:

- **size:** $l = 0.25$ m, $\varnothing = 4.6$ mm,
- **stationary phase:** octadecylsilyl silica gel for chromatography *R* (5 μ m).

Mobile phase: acetic acid *R*, acetonitrile *R*, water *R* (1:19:81 *V/V/V*).

Flow rate: 1 ml/min.

Detection: spectrophotometer at 254 nm.

Injection: 20 μ l; inject the test solution and reference solutions (a) and (c).

System suitability: reference solution (c):

- **resolution:** minimum 3.5 between the 2 principal peaks. Calculate the percentage content of cefoxitin sodium.

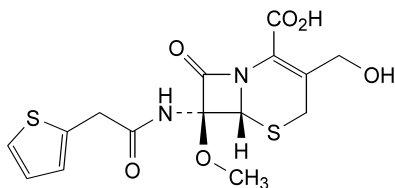
STORAGE

In an airtight container. If the substance is sterile, store in a sterile, airtight, tamper-proof container.

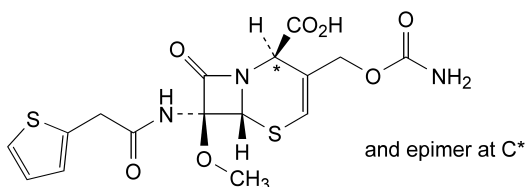
LABELLING

The label states, where applicable, that the substance is free from bacterial endotoxins.

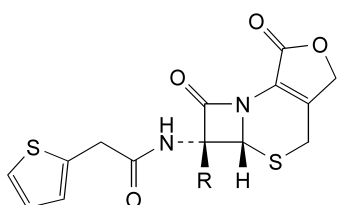
IMPURITIES



- A. (6*R*,7*S*)-3-(hydroxymethyl)-7-methoxy-8-oxo-7-[(thiophen-2-yl)acetyl]amino]-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid (decarbamoylcefexitin),



- B. (2*RS*,6*R*,7*S*)-3-[(carbamoyloxy)methyl]-7-methoxy-8-oxo-7-[(thiophen-2-yl)acetyl]amino]-5-thia-1-azabicyclo[4.2.0]oct-3-ene-2-carboxylic acid (delta-3-cefoxitin),

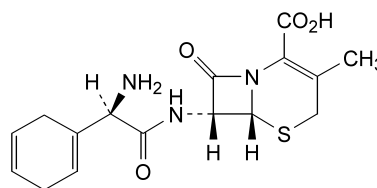


- C. $R = H$: (5*aR*,6*R*)-6-[(thiophen-2-yl)acetyl]amino]-5*a*,6-dihydro-3*H*,7*H*-azeto[2,1-*b*]furo[3,4-*d*][1,3]thiazine-1,7(4*H*)-dione (cefalotin lactone),

- D. $R = OCH_3$: (5*aR*,6*S*)-6-methoxy-6-[(thiophen-2-yl)acetyl]amino]-5*a*,6-dihydro-3*H*,7*H*-azeto[2,1-*b*]furo[3,4-*d*][1,3]thiazine-1,7(4*H*)-dione (cefoxitin lactone).

CEFRADINE

Cefradinum



$C_{16}H_{19}N_3O_4S$

M_r 349.4

DEFINITION

Cefradine contains not less than 90.0 per cent of (6*R*,7*R*)-7-[[[(2*R*)-2-amino-2-(cyclohexa-1,4-dienyl)acetyl]amino]-3-methyl-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid, calculated with reference to the anhydrous substance. The sum of the percentage contents of $C_{16}H_{19}N_3O_4S$ and of cefalexin ($C_{16}H_{17}N_3O_4S$; M_r 347.4) is not less than 95.0 per cent and not more than the equivalent of 102.0 per cent, calculated with reference to the anhydrous substance.

CHARACTERS

A white or slightly yellow, hygroscopic powder, sparingly soluble in water, practically insoluble in alcohol.

IDENTIFICATION

Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *cefradine CRS*. If the spectra obtained in the solid state show differences, dissolve 30 mg of the substance to be examined and of the reference substance separately in 10 ml of *methanol R*, evaporate to dryness at 40 °C at a pressure less than 2 kPa and record new spectra using the residues.

TESTS

Solution S. Dissolve 2.50 g in *sodium carbonate solution R* and dilute to 25.0 ml with the same solvent.

Appearance of solution. Solution S is not more opalescent than reference suspension II (2.2.1). Allow solution S to stand for 5 min. The absorbance of solution S measured at 450 nm (2.2.25) is not greater than 0.60.

pH (2.2.3). Dissolve 0.100 g in *carbon dioxide-free water R* and dilute to 10 ml with the same solvent. The pH of the solution is 3.5 to 6.0.

Specific optical rotation (2.2.7). Dissolve 0.250 g in *acetate buffer solution pH 4.6 R* and dilute to 25.0 ml with the same solvent. The specific optical rotation is + 80 to + 90, calculated with reference to the anhydrous substance.

Absorbance (2.2.25). Dissolve 50.0 mg in *water R* and dilute to 100.0 ml with the same solvent. The absorbance measured at 330 nm is not greater than 0.05. Dilute 2.0 ml of the solution to 50.0 ml with *water R*. Examined between 220 nm and 300 nm, the diluted solution shows an absorption maximum at 262 nm. The specific absorbance at this maximum is 215 to 240, calculated with reference to the anhydrous substance.

Related substances. Examine by thin-layer chromatography (2.2.27), using *silica gel G R* as the coating substance. Impregnate the plate by development with a 5 per cent *V/V*

solution of *tetradecane R* in *hexane R*. Allow the solvent to evaporate and carry out the chromatography in the same direction as the impregnation.

Test solution. Dissolve 0.25 g of the substance to be examined in *dilute hydrochloric acid R* and dilute to 10 ml with the same acid.

Reference solution (a). Dilute 1 ml of the test solution to 100 ml with *dilute hydrochloric acid R*.

Reference solution (b). Dissolve 25 mg of *7-aminodesacetoxycephalosporanic acid CRS* in *dilute hydrochloric acid R* and dilute to 10 ml with the same acid (*reference solution (b')*). Dilute 1 ml of this solution to 10 ml with *dilute hydrochloric acid R*.

Reference solution (c). Dissolve 25 mg of *cyclohexa-1,4-dienylglycine CRS* in *dilute hydrochloric acid R* and dilute to 10 ml with the same acid (*reference solution (c')*). Dilute 1 ml of this solution to 10 ml with *dilute hydrochloric acid R*.

Reference solution (d). Dissolve 0.25 g of the substance to be examined in a mixture of 1 ml of reference solution (b') and 1 ml of reference solution (c') and dilute to 10 ml with *dilute hydrochloric acid R*.

Apply to the plate 5 µl of each solution. Develop over a path of 15 cm using a mixture of 3 volumes of *acetone R*, 80 volumes of a 72 g/l solution of *disodium hydrogen phosphate R* and 120 volumes of a 21 g/l solution of *citric acid R*. Dry the plate by heating at 90 °C for 3 min. Spray the hot plate with a 1 g/l solution of *ninhydrin R* in the mobile phase. Heat the plate at 90 °C for 15 min and allow to cool. In the chromatogram obtained with the test solution: any spot corresponding to 7-aminodesacetoxycephalosporanic acid is not more intense than the spot in the chromatogram obtained with reference solution (b) (1.0 per cent); any spot corresponding to cyclohexa-1,4-dienylglycine (the position of which is defined by comparison with the chromatogram obtained with reference solution (d)), is not more intense than the spot in the chromatogram obtained with reference solution (c) (1.0 per cent); any spot, apart from the principal spot and the spots corresponding to 7-aminodesacetoxycephalosporanic acid and to cyclohexa-1,4-dienylglycine, is not more intense than the principal spot in the chromatogram obtained with reference solution (a) (1.0 per cent). The test is not valid unless the chromatogram obtained with reference solution (d) shows 3 clearly separated spots.

Cefalexin. Not more than 5.0 per cent, calculated with reference to the anhydrous substance and determined by liquid chromatography (2.2.29), as prescribed under Assay. Inject separately the test solution and reference solution (b).

***N,N*-Dimethylaniline** (2.4.26, *Method B*). Not more than 20 ppm.

Water (2.5.12). Not more than 6.0 per cent, determined on 0.300 g by the semi-micro determination of water.

Sulphated ash (2.4.14). Not more than 0.2 per cent, determined on 1.0 g.

ASSAY

Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 50.0 mg of the substance to be examined in the mobile phase and dilute to 100.0 ml with the mobile phase.

Reference solution (a). Dissolve 50.0 mg of *cefradine CRS* in the mobile phase and dilute to 100.0 ml with the mobile phase.

Reference solution (b). Dissolve 10.0 mg of *cefradine CRS* and 10.0 mg of *cefalexin CRS* in the mobile phase and dilute to 100.0 ml with the mobile phase.

The chromatographic procedure may be carried out using:

- a column 0.25 m long and 4.6 mm in internal diameter, packed with *octadecylsilyl silica gel for chromatography R* (5 µm or 10 µm),
- as mobile phase at a flow rate of 1.0 ml/min a mixture of 1 volume of *dilute acetic acid R*, 17 volumes of a 36.2 g/l solution of *sodium acetate R*, 200 volumes of *methanol R* and 782 volumes of *water R*,
- as detector a spectrophotometer set at 254 nm,
- a 20 µl loop injector.

Inject reference solution (b). Adjust the sensitivity of the detector so that the height of the peaks is at least half the full scale of the recorder. The test is not valid unless the resolution between the peaks corresponding to cefalexin and cefradine is at least 4. If necessary, adjust the methanol content of the mobile phase. Inject reference solution (a) 6 times. The test is not valid unless the relative standard deviation of the peak area for cefradine is at most 1.0 per cent. Inject separately the test solution and reference solution (a).

Calculate the percentage content of cefradine and cefalexin.

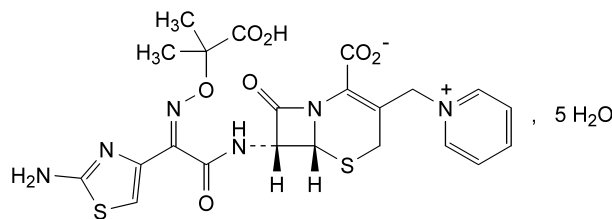
STORAGE

Store in an airtight container, protected from light, at a temperature of 2 °C to 8 °C.

01/2005:1405

CEFTAZIDIME

Ceftazidimum



$C_{22}H_{22}N_6O_7S_2 \cdot 5H_2O$

M_r 637

DEFINITION

Ceftazidime is (6*R*,7*R*)-7-[[*Z*]-2-[(2-aminothiazol-4-yl)-2-[(1-carboxy-1-methylethoxy)imino]acetyl]amino]-8-oxo-3-[(1-pyridinio)methyl]-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylate pentahydrate. It contains not less than 95.0 per cent and not more than 102.0 per cent of $C_{22}H_{22}N_6O_7S_2$, calculated with reference to the anhydrous substance.

CHARACTERS

A white or almost white, crystalline powder, slightly soluble in water and in methanol, practically insoluble in acetone and in alcohol. It dissolves in acid and alkali solutions.

IDENTIFICATION

Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *ceftazidime CRS*.

TESTS

Solution S. Dissolve 0.25 g in *carbon dioxide-free water R* and dilute to 50 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and colourless (2.2.2, *Method II*).

pH (2.2.3). The pH of solution S is 3.0 to 4.0.

Related substances

A. Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel F₂₅₄ plate R*.

Test solution. Dissolve 0.100 g of the substance to be examined in a 36 g/l solution of *disodium hydrogen phosphate R* and dilute to 2.0 ml with the same solution.

Reference solution. Dilute 1 ml of the test solution to 200 ml with a 36 g/l solution of *disodium hydrogen phosphate R*.

Apply to the plate 2 µl of each solution. Develop over a path of 15 cm using a mixture of 6 volumes of *butanol R*, 26 volumes of *sodium acetate buffer solution pH 4.5 R*, 32 volumes of *butyl acetate R* and 32 volumes of *glacial acetic acid R*. Dry the plate in a current of warm air and examine in ultraviolet light at 254 nm. Any spot with an *R_f* value greater than that of the principal spot in the chromatogram obtained with the test solution is not more intense than the spot in the chromatogram obtained with the reference solution (0.5 per cent).

B. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 0.100 g of the substance to be examined in the mobile phase and dilute to 20.0 ml with the mobile phase. Dilute 5.0 ml of the solution to 20.0 ml with the mobile phase.

Reference solution (a). Dissolve 5.0 mg of *ceftazidime impurity A CRS* in the mobile phase and dilute to 20.0 ml with the mobile phase. Dilute 1.0 ml of the solution to 20.0 ml with the mobile phase.

Reference solution (b). Dissolve 5 mg of *ceftazidime impurity A CRS* and 5 mg of *ceftazidime CRS* in the mobile phase and dilute to 20.0 ml with the mobile phase. Dilute 1.0 ml of the solution to 20.0 ml with the mobile phase.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4.6 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (5 µm),
- as mobile phase at a flow rate of 1.3 ml/min a mixture of 7 volumes of *acetonitrile R* and 93 volumes of a 22.6 g/l solution of *ammonium dihydrogen phosphate R*, adjusted to pH 3.9 with a 10 per cent *V/V* solution of *phosphoric acid R*,
- as detector a spectrophotometer set at 255 nm, maintaining the temperature of the column at 35 °C.

Inject 20 µl of reference solution (b). Adjust the sensitivity of the system so that the heights of the 2 peaks in the chromatogram obtained are at least 50 per cent of the full scale of the recorder. The test is not valid unless in the chromatogram obtained, the resolution between the peaks corresponding to ceftazidime and impurity A is at least 5.9. Inject 20 µl of the test solution and 20 µl of reference solution (a). Continue the chromatography of the test solution for 3 times the retention time of ceftazidime. In the chromatogram obtained with the test solution: the area of any peak, apart from the principal peak, is not greater than half the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent); the sum of the areas of all the peaks, apart from the principal peak, is not greater than twice the area of the principal peak in the chromatogram

obtained with reference solution (a) (2 per cent).

Disregard any peak with an area less than 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (a).

Impurity F. Not more than 500 ppm, determined by liquid chromatography (2.2.29). *Prepare the solutions immediately before use.*

Test solution. Dissolve 0.500 g of the substance to be examined in a 10 per cent *V/V* solution of *phosphate buffer solution pH 7.0 R4* and dilute to 100.0 ml with the same solvent.

Reference solution (a). Dissolve 1.00 g of *pyridine R* in *water R* and dilute to 100.0 ml with the same solvent. Dilute 5.0 ml of the solution to 200.0 ml with *water R*. To 1.0 ml of the solution, add 10 ml of *phosphate buffer solution pH 7.0 R4* and dilute to 100.0 ml with *water R*.

Reference solution (b). Dilute 1.0 ml of the test solution to 200.0 ml with a 10 per cent *V/V* solution of *phosphate buffer solution pH 7.0 R4*. To 1.0 ml of the solution add 20 ml of reference solution (a) and dilute to 200 ml with a 10 per cent *V/V* solution of *phosphate buffer solution pH 7.0 R4*.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4.6 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (5 µm),
- as mobile phase at a flow rate of 1.0 ml/min a mixture of 8 volumes of a 28.8 g/l solution of *ammonium dihydrogen phosphate R* previously adjusted to pH 7.0 with *ammonia R*, 24 volumes of *acetonitrile R* and 68 volumes of *water R*,
- as detector a spectrophotometer set at 255 nm,

Inject 20 µl of reference solution (b). The test is not valid unless in the chromatogram obtained, the resolution between the peaks due to ceftazidime and to impurity F is at least 7.0.

Inject 20 µl of reference solution (a). Adjust the sensitivity of the system so that the height of the principal peak in the chromatogram obtained is at least 50 per cent of the full scale of the recorder. Inject reference solution (a) 6 times. The test is not valid unless the relative standard deviation of the area of the principal peak is at most 1.0 per cent. Inject alternately 20 µl of the test solution and 20 µl of reference solution (a).

Water (2.5.12): 13.0 per cent to 15.0 per cent, determined on 0.200 g by the semi-micro determination of water.

Bacterial endotoxins (2.6.14): less than 0.10 IU/mg, if intended for use in the manufacture of parenteral dosage forms without a further appropriate procedure for the removal of bacterial endotoxins.

ASSAY

Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 25.0 mg of the substance to be examined in the mobile phase and dilute to 25.0 ml with the mobile phase.

Reference solution (a). Dissolve 25.0 mg of *ceftazidime CRS* in the mobile phase and dilute to 25.0 ml with the mobile phase.

Reference solution (b). Dissolve 5 mg of *ceftazidime impurity A CRS* in 5.0 ml of reference solution (a).

The chromatographic procedure may be carried out using:

- a column 0.15 m long and 4.6 mm in internal diameter packed with *hexylsilyl silica gel for chromatography R* (5 µm),

- as mobile phase at a flow rate of 2 ml/min a mixture prepared as follows: dissolve 4.26 g of *disodium hydrogen phosphate R* and 2.73 g of *potassium dihydrogen phosphate R* in 980 ml of *water R*, then add 20 ml of *acetonitrile R*,
- as detector a spectrophotometer set at 245 nm.

Inject 20 µl of reference solution (b). Adjust the sensitivity of the system so that the heights of the 2 principal peaks in the chromatogram obtained are at least 50 per cent of the full scale of the recorder. The test is not valid unless, in the chromatogram obtained, the resolution between the peaks corresponding to ceftriaxone and impurity A is at least 1.0. Inject alternately the test solution and reference solution (a). Calculate the percentage content of ceftriaxone.

STORAGE

Store in an airtight container. If the substance is sterile, store in a sterile, airtight, tamper-proof container.

LABELLING

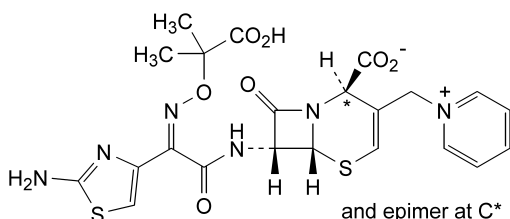
The label states, where applicable, that the substance is free from bacterial endotoxins.

IMPURITIES

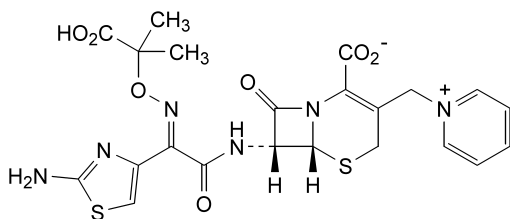
By liquid chromatography (related substances test): A, B, C.

By thin-layer chromatography (related substances test): D, E.

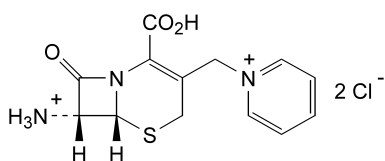
By liquid chromatography (impurity F test): F.



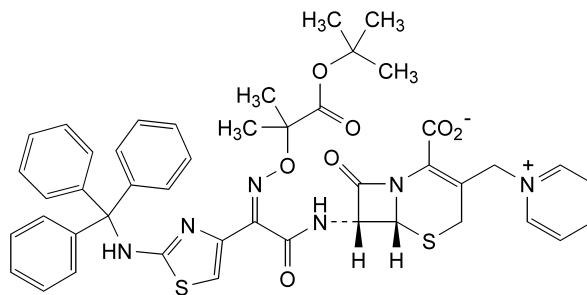
- A. (2*RS*,6*R*,7*R*)-7-[[[(*Z*)-2-(2-aminothiazol-4-yl)-2-[(1-carboxy-1-methylethoxy)imino]acetyl]amino]-8-oxo-3-[(1-pyridinio)methyl]-5-thia-1-azabicyclo[4.2.0]oct-3-ene-2-carboxylate (Δ-2-ceftazidime),



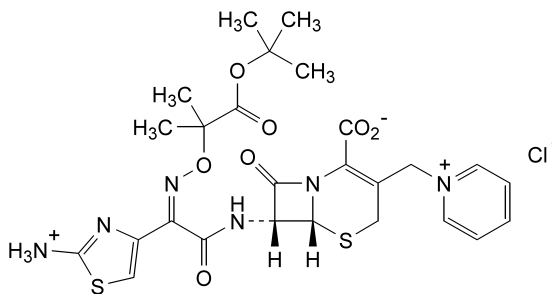
- B. (6*R*,7*R*)-7-[[[(*E*)-2-(2-aminothiazol-4-yl)-2-[(1-carboxy-1-methylethoxy)imino]acetyl]amino]-8-oxo-3-[(1-pyridinio)methyl]-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylate,



- C. (6*R*,7*R*)-2-carboxy-8-oxo-3-(pyridinimethyl)-5-thia-1-azabicyclo[4.2.0]oct-2-en-7-aminium dichloride,



- D. (6*R*,7*R*)-7-[[[(*Z*)-2-[[2-(1,1-dimethylethoxy)-1,1-dimethyl-2-oxoethoxy]imino]-2-[[2-[(triphenylmethyl)amino]thiazol-4-yl]acetyl]amino]-8-oxo-3-(pyridinimethyl)-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylate,



- E. (6*R*,7*R*)-7-[[[(*Z*)-2-(2-ammoniothiazol-4-yl)-2-[[2-(1,1-dimethylethoxy)-1,1-dimethyl-2-oxoethoxy]imino]acetyl]amino]-8-oxo-3-(pyridinimethyl)-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylate chloride,

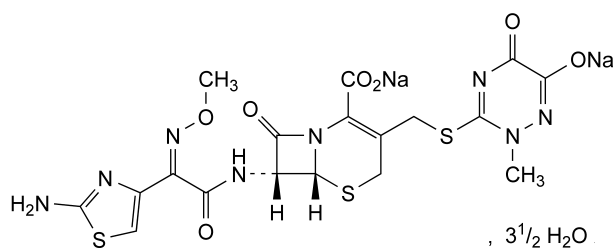


- F. pyridine.

01/2005:0991

CEFTRIAZONE SODIUM

Ceftriaxonum natricum



C₁₈H₁₆N₈Na₂O₇S₃, 3½ H₂O

M_r 662

DEFINITION

Disodium (6*R*,7*R*)-7-[[[(*Z*)-2-(2-aminothiazol-4-yl)(methoxyimino)acetyl]amino]-3-[[[(2-methyl-6-oxido-5-oxo-2,5-dihydro-1,2,4-triazin-3-yl)sulphonyl]methyl]-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylate.

Content: 96.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

Appearance: almost white or yellowish, crystalline powder, slightly hygroscopic.

Solubility: freely soluble in water, sparingly soluble in methanol, very slightly soluble in ethanol.

IDENTIFICATION

A. Infrared absorption spectrophotometry (2.2.24).

Comparison: ceftriaxone sodium CRS.

B. It gives reaction (a) of sodium (2.3.1).

TESTS

Solution S. Dissolve 2.40 g in *carbon dioxide-free water R* and dilute to 20.0 ml with the same solvent.

Appearance of solution. The solution is clear (2.2.1) and not more intensely coloured than reference solution Y₅ or BY₅ (2.2.2).

Dilute 2 ml of solution S to 20 ml with *water R*.

pH (2.2.3): 6.0 to 8.0 for solution S.

Specific optical rotation (2.2.7): –155 to –170 (anhydrous substance).

Dissolve 0.250 g in *water R* and dilute to 25.0 ml with the same solvent.

Related substances. Liquid chromatography (2.2.29).

Test solution. Dissolve 30.0 mg of the substance to be examined in the mobile phase and dilute to 100.0 ml with the mobile phase.

Reference solution (a). Dissolve 30.0 mg of *ceftriaxone sodium CRS* in the mobile phase and dilute to 100.0 ml with the mobile phase.

Reference solution (b). Dissolve 5.0 mg of *ceftriaxone sodium CRS* and 5.0 mg of *ceftriaxone impurity A CRS* in the mobile phase and dilute to 100.0 ml with the mobile phase.

Reference solution (c). Dilute 1.0 ml of the test solution to 100.0 ml with the mobile phase.

Column:

- *size:* $l = 0.25$ m, $\varnothing = 4.6$ mm,
- *stationary phase:* octadecylsilyl silica gel for chromatography R (5 μ m).

Mobile phase: dissolve 2.0 g of *tetradecylammonium bromide R* and 2.0 g of *tetraheptylammonium bromide R* in a mixture of 440 ml of *water R*, 55 ml of 0.067 M phosphate buffer solution pH 7.0 R, 5.0 ml of citrate buffer solution pH 5.0 prepared by dissolving 20.17 g of *citric acid R* in 800 ml of *water R*, adjusting topH 5.0 with *strong sodium hydroxide solution R* and diluting to 1000.0 ml with *water R*, and 500 ml of *acetonitrile R*.

Flow rate: 1.5 ml/min.

Detection: spectrophotometer at 254 nm.

Injection: 20 μ l; inject the test solution and reference solutions (b) and (c).

Run time: twice the retention time of ceftriaxone.

System suitability: reference solution (b):

- *resolution:* minimum of 3.0 between the peaks due to ceftriaxone and impurity A.

Limits:

- *any impurity:* not more than the area of the principal peak in the chromatogram obtained with reference solution (c) (1 per cent),
- *total:* not more than 4 times the area of the principal peak in the chromatogram obtained with reference solution (c) (4 per cent),
- *disregard limit:* 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.1 per cent).

N,N-Dimethylaniline (2.4.26, *Method B*): maximum 20 ppm.

2-Ethylhexanoic acid (2.4.28): maximum 0.8 per cent *m/m*.

Water (2.5.12): 8.0 per cent to 11.0 per cent, determined on 0.100 g.

Bacterial endotoxins (2.6.14): less than 0.20 IU/mg, if intended for use in the manufacture of parenteral dosage forms without a further appropriate procedure for the removal of bacterial endotoxins.

ASSAY

Liquid chromatography (2.2.29) as described in the test for related substances.

Injection: test solution and reference solution (a).

Calculate the percentage content of ceftriaxone sodium.

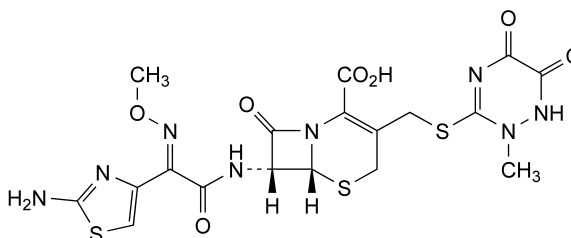
STORAGE

In an airtight container protected from light. If the substance is sterile, store in a sterile, airtight, tamper-proof container.

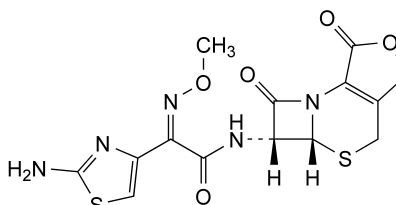
LABELLING

The label states, where applicable, that the substance is free from bacterial endotoxins.

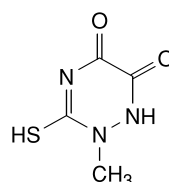
IMPURITIES



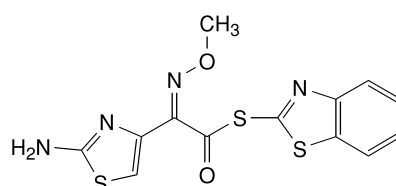
A. (6*R*,7*R*)-7-[[[(*E*)-(2-aminothiazol-4-yl)(methoxyimino)acetyl]amino]-3-[(2-methyl-5,6-dioxo-1,2,5,6-tetrahydro-1,2,4-triazin-3-yl)sulphonyl]methyl]-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid (*E*-isomer),



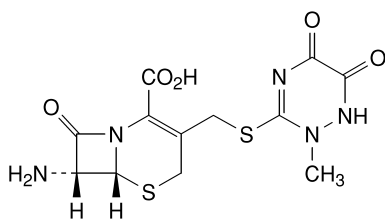
B. (5*aR*,6*R*)-6-[[[(*Z*)-(2-aminothiazol-4-yl)(methoxyimino)acetyl]amino]-5*a*,6-dihydro-3*H*,7*H*-azeto[2,1-*b*]furo[3,4-*d*][1,3]thiazine-1,7(4*H*)-dione,



C. 2-methyl-3-sulphonyl-1,2-dihydro-1,2,4-triazin-5,6-dione,



D. *S*-benzothiazol-2-yl (*Z*)-(2-aminothiazol-4-yl)(methoxyimino)thioacetate,

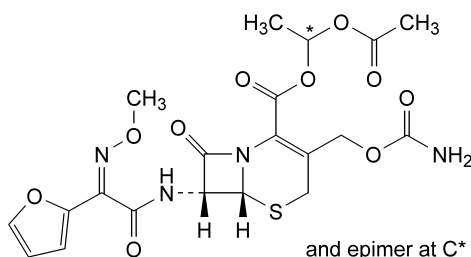


E. (6*R*,7*R*)-7-amino-3-[[2-methyl-5,6-dioxo-1,2,5,6-tetrahydro-1,2,4-triazin-3-yl]sulphonyl]methyl]-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid.

01/2005:1300

CEFUROXIME AXETIL

Cefuroximum axetili



$C_{20}H_{22}N_4O_{10}S$

M_r 510.5

DEFINITION

Cefuroxime axetil contains not less than 96.0 per cent and not more than the equivalent of 102.0 per cent of a mixture of the 2 diastereoisomers of (1*R*,5*S*)-1-(acetyloxy)ethyl (6*R*,7*R*)-3-[(carbamoyloxy)methyl]-7-[[2-(furan-2-yl)-2-(methoxyimino)acetyl]amino]-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylate, calculated with reference to the anhydrous and acetone-free substance.

CHARACTERS

A white or almost white powder, slightly soluble in water, soluble in acetone, in ethyl acetate and in methanol, slightly soluble in alcohol.

IDENTIFICATION

- Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *cefuroxime axetil CRS*.
- Examine the chromatograms obtained in the Assay. The retention time and size of the principal peaks in the chromatogram obtained with the test solution are the same as those of the peaks due to diastereoisomers A and B of cefuroxime axetil in the chromatogram obtained with reference solution (d).

TESTS

Diastereoisomer ratio. Examine by liquid chromatography (2.2.29) as described under Assay. In the chromatogram obtained with the test solution, the ratio of the peak due to cefuroxime axetil diastereoisomer A to the sum of the peaks due to cefuroxime axetil diastereoisomers A and B is between 0.48 and 0.55 by the normalisation procedure.

Related substances. Examine by liquid chromatography (2.2.29) as described under Assay. Calculate the percentage content of related substances from the areas of the peaks in the chromatogram obtained with the test solution by the normalisation procedure, disregarding any peak with an area less than 0.05 times that of the 2 principal peaks in the chromatogram obtained with reference solution (a). The percentage sum of the pair of peaks corresponding to the *E*-isomers located by comparison with the chromatogram obtained with reference solution (c) is not greater than 1.0 per cent, the percentage sum of the pair of peaks corresponding to the Δ^3 -isomers located by comparison with the chromatogram obtained with reference solution (b) is not greater than 1.5 per cent and the area of any other secondary peak is not greater than 0.5 per cent. The sum of related substances is not greater than 3.0 per cent.

Acetone (2.4.24). Not more than 1.1 per cent.

Water (2.5.12). Not more than 1.5 per cent, determined on 0.400 g by the semi-micro determination of water.

ASSAY

Examine by liquid chromatography (2.2.29).

Test solution. Prepare the solution immediately before use. Dissolve 10.0 mg of the substance to be examined in the mobile phase and dilute to 50.0 ml with the mobile phase.

Reference solution (a). Dilute 1.0 ml of the test solution to 100.0 ml with the mobile phase.

Reference solution (b). Heat 5 ml of the test solution at 60 °C for 1 h to generate the Δ^3 -isomers.

Reference solution (c). Expose 5 ml of the test solution to ultraviolet light at 254 nm for 24 h to generate *E*-isomers.

Reference solution (d). Prepare the solution immediately before use. Dissolve 10.0 mg of *cefuroxime axetil CRS* in the mobile phase and dilute to 50.0 ml with the mobile phase.

The chromatographic procedure may be carried out using:

- a column 0.25 m long and 4.6 mm in internal diameter packed with *trimethylsilyl silica gel for chromatography R* (5 μ m),
- as mobile phase at a flow rate of 1.0 ml/min a mixture of 38 volumes of *methanol R* and 62 volumes of a 23 g/l solution of *ammonium dihydrogen phosphate R*,
- as detector a spectrophotometer set at 278 nm.

Inject 20 μ l each of reference solutions (a), (b), (c) and (d). When the chromatograms are recorded in the prescribed conditions the retention times relative to cefuroxime axetil diastereoisomer A (second peak) are approximately 0.9 for cefuroxime axetil diastereoisomer B, 1.2 for the cefuroxime axetil Δ^3 -isomers and 1.7 and 2.1 for the *E*-isomers. The test is not valid unless in the chromatogram obtained with reference solution (d), the resolution between the peaks corresponding to cefuroxime axetil diastereoisomers A and B is at least 1.5. In the chromatogram obtained with reference solution (b), the resolution between the peaks corresponding to cefuroxime axetil diastereoisomer A and cefuroxime axetil Δ^3 -isomer is at least 1.5.

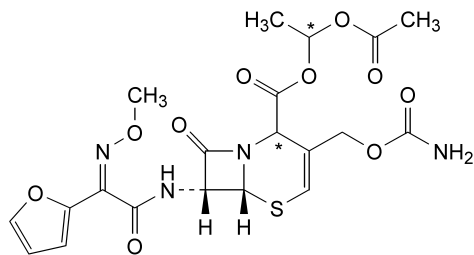
Inject reference solution (d) solution 6 times. The assay is not valid unless the relative standard deviation of the sum of the peaks corresponding to cefuroxime axetil diastereoisomers A and B is at most 2.0 per cent.

Calculate the percentage content of $C_{20}H_{22}N_4O_{10}S$ from the sum of areas of the two diastereoisomer peaks and the declared content of $C_{20}H_{22}N_4O_{10}S$ in *cefuroxime axetil CRS*.

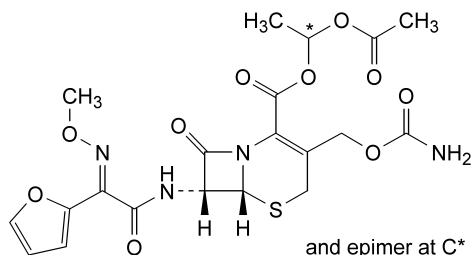
STORAGE

Store in an airtight container, protected from light.

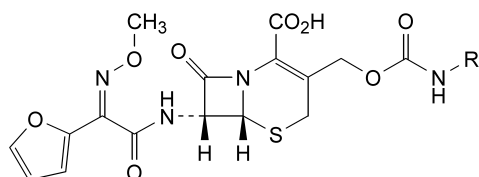
IMPURITIES



- A. 1-(acetyloxy)ethyl (6R,7R)-3-[(carbamoyloxy)methyl]-7-[(Z)-2-(furan-2-yl)-2-(methoxyimino)acetyl]amino]-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-3-ene-2-carboxylate (Δ^3 -isomers),



- B. (1RS)-1-(acetyloxy)ethyl (6R,7R)-3-[(carbamoyloxy)methyl]-7-[(E)-2-(furan-2-yl)-2-(methoxyimino)acetyl]amino]-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylate (E-isomers),



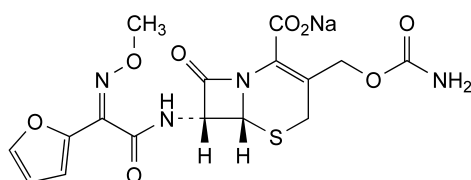
- C. R = CO-CCl₃: (6R,7R)-7-[(Z)-2-(furan-2-yl)-2-(methoxyimino)acetyl]amino]-8-oxo-3-[[[(trichloroacetyl)carbamoyl]oxy]methyl]-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid,

- D. R = H: cefuroxime.

01/2005:0992

CEFUROXIME SODIUM

Cefuroximum natrium

C₁₆H₁₅N₄NaO₈SM_r 446.4

DEFINITION

Sodium (6R,7R)-3-[(carbamoyloxy)methyl]-7-[(Z)-2-(furan-2-yl)-2-(methoxyimino)acetyl]amino]-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylate.

Content: 96.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

Appearance: white or almost white powder, slightly hygroscopic.

Solubility: freely soluble in water, very slightly soluble in alcohol.

IDENTIFICATION

A. Infrared absorption spectrophotometry (2.2.24).

Comparison: cefuroxime sodium CRS.

B. It gives reaction (a) of sodium (2.3.1).

TESTS

Solution S. Dissolve 2.0 g in carbon dioxide-free water R and dilute to 20.0 ml with the same solvent.

Appearance of solution. Solution S is not more opalescent than reference suspension II (2.2.1). The absorbance (2.2.25) of solution S measured at 450 nm is not greater than 0.25.

pH (2.2.3): 5.5 to 8.5.

Dilute 2 ml of solution S to 20 ml with carbon dioxide-free water R.

Specific optical rotation (2.2.7): + 59 to + 66 (anhydrous substance).

Dissolve 0.500 g in acetate buffer solution pH 4.6 R and dilute to 25.0 ml with the same buffer solution.

Related substances. Liquid chromatography (2.2.29).

Test solution (a). Dissolve 25.0 mg of the substance to be examined in water R and dilute to 25.0 ml with the same solvent.

Test solution (b). Dilute 5.0 ml of test solution (a) to 50.0 ml with water R.

Reference solution (a). Dissolve 25.0 mg of cefuroxime sodium CRS in water R and dilute to 25.0 ml with the same solvent. Dilute 5.0 ml to 50.0 ml with water R.

Reference solution (b). Place 20 ml of reference solution (a) in a water-bath at 80 °C for 15 min. Cool and inject immediately.

Reference solution (c). Dilute 1.0 ml of test solution (a) to 100.0 ml with water R.

Column:

- size: $l = 0.125$ m, $\varnothing = 4.6$ mm,
- stationary phase: hexylsilyl silica gel for chromatography R (5 μ m).

Mobile phase: mix 1 volume of acetonitrile R and 99 volumes of an acetate buffer solution pH 3.4, prepared by dissolving 6.01 g of glacial acetic acid R and 0.68 g of sodium acetate R in water R and diluting to 1000 ml with the same solvent.

Flow rate: 1.5 ml/min.

Detection: spectrophotometer at 273 nm.

Injection: 20 μ l loop injector; inject test solution (a) and reference solutions (b) and (c).

Run time: 4 times the retention time of cefuroxime.

System suitability: reference solution (b):

- resolution: minimum of 2.0 between the peaks due to cefuroxime and to impurity A.

Limits:

- impurity A: not more than the area of the principal peak in the chromatogram obtained with reference solution (c) (1.0 per cent),
- any other impurity: not more than the area of the principal peak in the chromatogram obtained with reference solution (c) (1.0 per cent),
- total: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (c) (3.0 per cent),

- *disregard limit*: 0.05 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.05 per cent).

***N,N*-Dimethylaniline** (2.4.26, *Method B*): maximum 20 ppm.

2-Ethylhexanoic acid (2.4.28): maximum 0.5 per cent *m/m*.

Water (2.5.12): maximum 3.5 per cent, determined on 0.400 g.

Bacterial endotoxins (2.6.14): less than 0.10 IU/mg, if intended for use in the manufacture of parenteral dosage forms without a further appropriate procedure for the removal of bacterial endotoxins.

ASSAY

Liquid chromatography (2.2.29) as described in the test for related substances with the following modification.

Injection: test solution (b) and reference solution (a).

Calculate the percentage content of cefuroxime sodium.

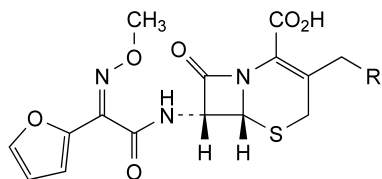
STORAGE

In an airtight container. If the substance is sterile, store in a sterile, airtight, tamper-proof container.

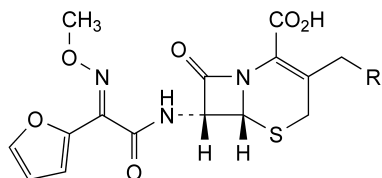
LABELLING

The label states, where applicable, that the substance is free from bacterial endotoxins.

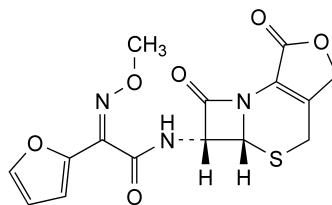
IMPURITIES



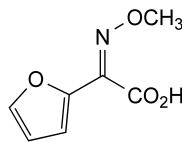
- A. R = OH: (6*R*,7*R*)-7-[[*(Z)*-(furan-2-yl)(methoxyimino)acetyl]amino]-3-(hydroxymethyl)-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid (descarbamoylcefuroxime),
- B. R = O-CO-CH₃: (6*R*,7*R*)-3-[(acetyloxy)methyl]-7-[[*(Z)*-(furan-2-yl)(methoxyimino)acetyl]amino]-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid,
- C. R = H: (6*R*,7*R*)-7-[[*(Z)*-(furan-2-yl)(methoxyimino)acetyl]amino]-3-methyl-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid,
- D. R = O-CO-NH-CO-CCl₃: (6*R*,7*R*)-7-[[*(Z)*-(furan-2-yl)(methoxyimino)acetyl]amino]-8-oxo-3-[[[trichloroacetyl]carbamoyl]oxy]methyl]-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid,



- E. R = O-CO-NH₂: (6*R*,7*R*)-3-[(carbamoyloxy)methyl]-7-[[*(E)*-(furan-2-yl)(methoxyimino)acetyl]amino]-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid (*trans*-cefuroxime),
- F. R = OH: (6*R*,7*R*)-7-[[*(E)*-(furan-2-yl)(methoxyimino)acetyl]amino]-3-(hydroxymethyl)-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid,
- G. R = O-CO-CH₃: (6*R*,7*R*)-3-[(acetyloxy)methyl]-7-[[*(E)*-(furan-2-yl)(methoxyimino)acetyl]amino]-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid,



- H. (5*aR*,6*R*)-6-[[*(Z)*-(furan-2-yl)(methoxyimino)acetyl]amino]-5*a*,6-dihydro-3*H*,7*H*-azeto[2,1-*b*]furo[3,4-*d*][1,3]thiazine-1,7(4*H*)-dione,

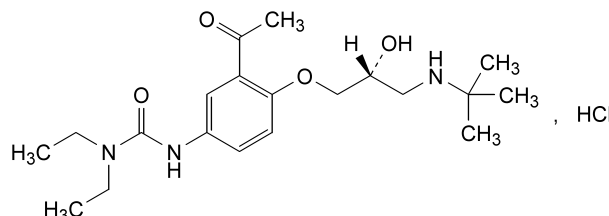


- I. (*Z*)-(furan-2-yl)(methoxyimino)acetic acid.

01/2005:1632

CELIPROLOL HYDROCHLORIDE

Celiprololi hydrochloridum



and enantiomer

C₂₀H₃₄ClN₃O₄

M_r 416.0

DEFINITION

3-[3-Acetyl-4-[(2*RS*)-3-[(1,1-dimethylethyl)amino]-2-hydroxypropoxy]phenyl]-1,1-diethylurea hydrochloride.

Content: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

Appearance: white or very slightly yellow, crystalline powder.

Solubility: freely soluble in water and in methanol, soluble in alcohol, very slightly soluble in methylene chloride.

It shows polymorphism.

IDENTIFICATION

- A. Infrared absorption spectrophotometry (2.2.24).

Comparison: celiprolol hydrochloride CRS.

If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in *methanol R*, evaporate to dryness and record new spectra using the residues.

- B. It gives reaction (a) of chlorides (2.3.1).

TESTS

Optical rotation (2.2.7): −0.10° to +0.10°.

Dissolve 1.0 g in *water R* and dilute to 10.0 ml with the same solvent.

Related substances. Liquid chromatography (2.2.29). Prepare the solutions immediately before use.

Test solution. Dissolve 0.100 g of the substance to be examined in mobile phase A and dilute to 20.0 ml with mobile phase A.

Reference solution (a). Dissolve 2 mg of the substance to be examined and 2 mg of *acebutolol hydrochloride R* in mobile phase A and dilute to 50.0 ml with mobile phase A.

Reference solution (b). Dissolve 10 mg of the substance to be examined in 2 ml of mobile phase A and allow to stand for 24 h (for identification of impurity A).

Reference solution (c). Dilute 1.0 ml of the test solution to 100.0 ml with mobile phase A. Dilute 1.0 ml of this solution to 10.0 ml with mobile phase A.

Reference solution (d). Dissolve 10 mg of *celiprolol for peak identification CRS* in mobile phase A and dilute to 2 ml with mobile phase A.

Reference solution (e). This solution is only prepared if required (see below) and is used to determine the identity of impurity I which co-elutes with impurity H (the 2 impurities originate from different synthesis routes). Dissolve 2 mg of *celiprolol impurity I CRS* in mobile phase A and dilute to 20 ml with mobile phase A. Dilute 1.0 ml of the solution to 10.0 ml with mobile phase A.

Column:

- size: $l = 0.15$ m, $\varnothing = 4.6$ mm,
- stationary phase: octylsilyl silica gel for chromatography R (5 μ m),
- temperature: 30 °C.

Mobile phase:

- mobile phase A: mix 91 ml of *tetrahydrofuran R*, 63 ml of *acetonitrile R1*, 0.6 ml of *pentafluoropropanoic acid R* and 0.2 ml of *trifluoroacetic acid R*; dilute to 1000 ml with *water R*;
- mobile phase B: *acetonitrile R1*;

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)
0 - 50	100 → 80	0 → 20
50 - 51	80 → 100	20 → 0
51 - 65	100	0

Flow rate: 1.4 ml/min.

Detection: spectrophotometer at 232 nm.

Injection: 10 μ l.

Relative retention with reference to celiprolol (retention time = about 10 min): impurity A = about 0.3; impurity D = about 0.7; impurity G = about 1.2; impurity B = about 1.4; impurity F = about 1.6; impurity C = about 2.2; impurity H or I = about 2.5; impurity E = about 3.9.

System suitability: reference solution (a):

- resolution: minimum 4.0 between the peaks due to celiprolol and acebutolol.

Limits: use the chromatogram supplied with *celiprolol for peak identification CRS* and the chromatogram obtained with reference solution (d) to identify the peaks due to impurities B, E and F:

- correction factors: for the calculation of contents, multiply the peak areas of the following impurities by the corresponding correction factor: impurity A = 4.0; impurity B = 1.5; impurity E = 2.3; impurity F = 0.5; impurity I = 1.7;
- any impurity: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (c) (0.2 per cent), and not more than

1 such peak has an area greater than the area of the principal peak in the chromatogram obtained with reference solution (c) (0.1 per cent);

- total: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.5 per cent);
- if any of the above limits are exceeded and if a peak occurs with a relative retention of about 2.5 (impurity H or I), the identity of this peak has to be clarified by use of a UV spectrum recorded with a diode array detector; if this spectrum is different from the one obtained with reference solution (e), no correction factor is applied;
- disregard limit: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.05 per cent).

Loss on drying (2.2.32): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 100-105 °C for 3 h.

ASSAY

Dissolve 0.350 g under an atmosphere of nitrogen in 50 ml of *alcohol R* and add 1.0 ml of 0.1 M *hydrochloric acid*. Carry out a potentiometric titration (2.2.20), using 0.1 M *sodium hydroxide*. Read the volume added between the 2 points of inflexion.

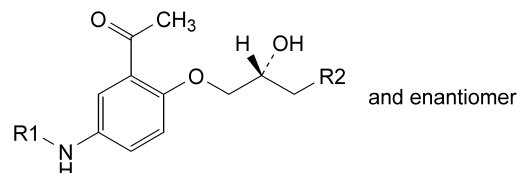
1 ml of 0.1 M *sodium hydroxide* is equivalent to 41.60 mg of $C_{20}H_{34}ClN_3O_4$.

STORAGE

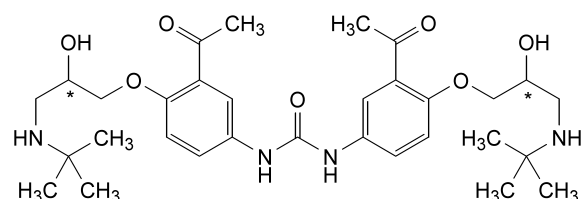
Protected from light.

IMPURITIES

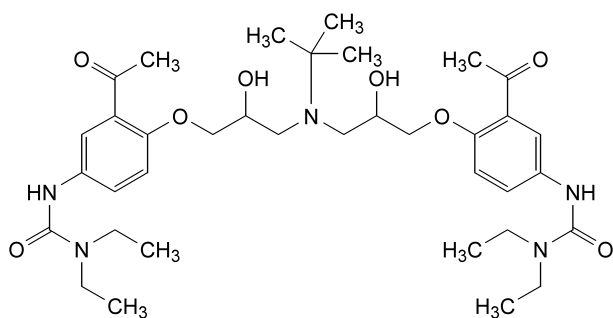
Specified impurities: A, B, C, D, E, F, G, H, I.



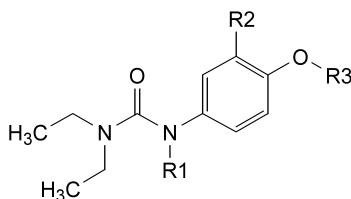
- A. $R_1 = H$, $R_2 = NH-C(CH_3)_3$: 1-[5-amino-2-[(2*RS*)-3-[(1,1-dimethylethyl)amino]-2-hydroxypropoxy]phenyl]ethanone,
- C. $R_1 = CO-NH-C(CH_3)_3$, $R_2 = NH-C(CH_3)_3$: 1-[3-acetyl-4-[(2*RS*)-3-[(1,1-dimethylethyl)amino]-2-hydroxypropoxy]phenyl]-3-(1,1-dimethylethyl)urea,
- D. $R_1 = CO-N(C_2H_5)_2$, $R_2 = N(C_2H_5)_2$: 3-[3-acetyl-4-[(2*RS*)-3-(diethylamino)-2-hydroxypropoxy]phenyl]-1,1-diethylurea,
- H. $R_1 = CO-N(C_2H_5)_2$, $R_2 = Br$: 3-[3-acetyl-4-[(2*RS*)-3-bromo-2-hydroxypropoxy]phenyl]-1,1-diethylurea (bromhydrin compound),



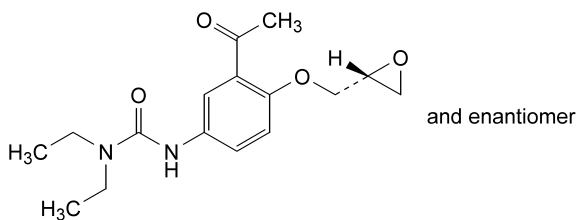
- B. 1,3-bis[3-acetyl-4-[(1,1-dimethylethyl)amino]-2-hydroxypropoxy]phenyl]urea,



- E. 1,1'-[[[(1,1-dimethylethyl)imino]bis[(2-hydroxypropane-1,3-diyl)oxy(3-acetyl-1,4-phenylene)]]bis(3,3-diethylurea),



- F. R1 = R3 = H, R2 = CO-CH₃: 3-(3-acetyl-4-hydroxyphenyl)-1,1-diethylurea,
I. R1 = CO-CH₃, R2 = H, R3 = C₂H₅: 1-acetyl-1-(4-ethoxyphenyl)-3,3-diethylurea,



- G. 3-[3-acetyl-4-[(*RS*)-oxiranyl]methoxy]phenyl]-1,1-diethylurea.

01/2005:0887

CELLULOSE ACETATE

Cellulosi acetas

DEFINITION

Partly or completely *O*-acetylated cellulose.

Content: 29.0 per cent to 44.8 per cent of acetyl groups (C₂H₃O) (dried substance) and 90.0 per cent to 110.0 per cent of the acetyl content stated on the label (dried substance).

CHARACTERS

Appearance: white, yellowish-white or greyish-white powder or granules, hygroscopic.

Solubility: practically insoluble in water, soluble in acetone, in formic acid and in a mixture of equal volumes of methanol and methylene chloride, practically insoluble in alcohol.

IDENTIFICATION

Infrared absorption spectrophotometry (2.2.24).

Comparison: *Ph. Eur. reference spectrum of cellulose acetate.*

TESTS

Free acid: maximum 0.1 per cent, calculated as acetic acid (dried substance).

To 5.00 g in a 250 ml conical flask, add 150 ml of *carbon dioxide-free water R*, insert the stopper, swirl the suspension

gently and allow to stand for 3 h. Filter, wash the flask and the filter with *carbon dioxide-free water R*. Combine the filtrate and washings. Add 0.1 ml of *phenolphthalein solution R1*. Titrate with 0.01 *M sodium hydroxide* until a faint pink colour is obtained.

1 ml of 0.01 *M sodium hydroxide* is equivalent to 0.6005 mg of free acid, calculated as acetic acid.

Heavy metals (2.4.8): maximum 10 ppm.

2.0 g complies with limit test D. Prepare the standard using 2 ml of *lead standard solution (10 ppm Pb) R*.

Loss on drying (2.2.32): maximum 5.0 per cent, determined on 1.000 g by drying in an oven at 100–105 °C for 3 h.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

Microbial contamination. Total viable aerobic count (2.6.12) not more than 10³ micro-organisms per gram of which not more than 10² fungi per gram determined by plate count. It complies with the tests for *Escherichia coli* and *Salmonella* (2.6.13).

ASSAY

Cellulose acetate containing not more than 42.0 per cent of acetyl groups

To 2.000 g in a 500 ml conical flask add 100 ml of *acetone R* and 10 ml of *water R*. Close the flask and stir with a magnetic stirrer until dissolution is complete. Add 30.0 ml of 1 *M sodium hydroxide* with constant stirring. Close the flask and stir with a magnetic stirrer for 30 min. Add 100 ml of *water R* at 80 °C, washing down the sides of the flask, stir for 2 min and cool to room temperature. Titrate with 0.5 *M sulphuric acid*, using 0.1 ml of *phenolphthalein solution R* as indicator. Carry out a blank titration.

Calculate the percentage content of acetyl groups from the expression:

$$\frac{4.305 (n_2 - n_1)}{(100 - d) \times m} \times 100$$

d = loss on drying as a percentage,

m = mass of the substance to be examined, in grams,

*n*₁ = number of millilitres of 0.5 *M sulphuric acid* used in the test,

*n*₂ = number of millilitres of 0.5 *M sulphuric acid* used in the blank titration.

Cellulose acetate containing more than 42.0 per cent of acetyl groups

To 2.000 g in a 500 ml conical flask add 30 ml of *dimethyl sulphoxide R* and 100 ml of *acetone R*. Close the flask and stir with a magnetic stirrer for 16 h. Add 30.0 ml of 1 *M sodium hydroxide* with constant stirring. Close the flask and stir with a magnetic stirrer for 6 min. Allow to stand without stirring for 60 min. Add 100 ml of *water R* at 80 °C, washing down the sides of the flask, stir for 2 min and cool to room temperature. Titrate with 0.5 *M hydrochloric acid*, using 0.1 ml of *phenolphthalein solution R*. Add 0.5 ml of 0.5 *M hydrochloric acid* in excess, stir for 5 min and allow to stand for 30 min. Titrate with 0.5 *M sodium hydroxide*, until a persistent pink colour is obtained, stirring with a magnetic stirrer. Calculate the net number of millimoles of 0.5 *M sodium hydroxide* consumed, taking the mean of 2 blank titrations into consideration.

Calculate the percentage content of acetyl groups from the expression:

$$\frac{4.305 \times n}{(100 - d) \times m} \times 100$$

- d = loss on drying as a percentage,
 m = mass of the substance to be examined, in grams,
 n = net number of millimoles of 0.5 M sodium hydroxide.

STORAGE

In an airtight container.

LABELLING

The label states the nominal percentage content of acetyl groups.

01/2005:1406

CELLULOSE ACETATE BUTYRATE

Cellulosi acetas butyras

DEFINITION

Cellulose acetate butyrate is partly or completely *O*-acetylated and *O*-butyrylated cellulose. It contains not less than 2.0 per cent and not more than 30.0 per cent of acetyl groups (C_2H_3O) and not less than 16.0 per cent and not more than 53.0 per cent of butyryl groups (C_4H_7O) calculated with reference to the dried substance. Both the acetyl content and the butyryl content are not less than 90.0 per cent and not more than 110.0 per cent of those stated on the label, calculated with reference to the dried substance.

CHARACTERS

A white, yellowish-white or greyish-white powder or granules, slightly hygroscopic, practically insoluble in water and in alcohol, soluble in acetone, in formic acid and in a mixture of equal volumes of methanol and methylene chloride.

IDENTIFICATION

- A. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the *Ph. Eur. reference spectrum of cellulose acetate butyrate*. The intensities of the bands may vary according to the degree of substitution.
 B. It complies with the limits of the assay.

TESTS

Acidity. To 5.00 g in a 250 ml conical flask, add 150 ml of *carbon dioxide-free water R*, insert the stopper, swirl the suspension gently and allow to stand for 3 h. Filter, wash the flask and the filter with *carbon dioxide-free water R*. Combine the filtrate and washings. Add 0.1 ml of *phenolphthalein solution R1*. Not more than 3.0 ml of 0.01 M sodium hydroxide is required to change the colour of the indicator.

Heavy metals (2.4.8). 1.0 g complies with limit test F for heavy metals (20 ppm). Prepare the standard using 2 ml of *lead standard solution (10 ppm Pb) R*.

Loss on drying (2.2.32). Not more than 2.0 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C for 3 h.

Total ash (2.4.16). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Examine by liquid chromatography (2.2.29).

Test solution. To 1.000 g in a 500 ml conical flask, add 100 ml of *acetone R* and 10 ml of *water R*. Close the flask and stir with a magnetic stirrer until dissolution is complete. Add 30.0 ml of 1 M sodium hydroxide with constant stirring. Close the flask and stir with a magnetic stirrer for 30 min. Add 100 ml of hot *water R* at 80 °C, washing down the sides of the flask and stir for 2 min. Cool, centrifuge or filter the suspension and wash the residue with *water R*. Combine the filtrate and washings, adjust the pH to 3 (2.2.3) using *dilute phosphoric acid R* and dilute to 500.0 ml with *water R*.

Reference solution. Dissolve 0.200 g of *glacial acetic acid R* and 0.400 g of *butyric acid R* in *water R*, adjust the pH to 3 (2.2.3) using *dilute phosphoric acid R* and dilute to 500.0 ml with *water R*.

The chromatographic procedure may be carried out using:

- a column 0.25 m long and 4.6 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (5 µm),
- as mobile phase at a flow rate of 1.2 ml/min a mixture of 5 volumes of *methanol R* and 95 volumes of *phosphate buffer solution pH 3.0 R1*, changing after 30 min, by linear gradient elution over 5 min, to a mixture of 20 volumes of *methanol R* and 80 volumes of *phosphate buffer solution pH 3.0 R1* and eluting for a further 25 min. Restore the initial solvent mixture for 1 min,
- as detector a spectrophotometer set at 210 nm.

Inject successively 20 µl of the reference solution and 20 µl of the test solution.

Calculate the percentage contents of acetic acid and butyric acid in the substance to be examined from the peak areas in the chromatograms obtained with the two solutions.

To calculate the percentage contents of acetyl (C_2H_3O) and of butyryl (C_4H_7O) groups, multiply the percentage contents of acetic acid and of butyric acid by 0.717 and 0.807, respectively.

STORAGE

Store in an airtight container.

LABELLING

The label states the nominal percentage content of acetyl and butyryl groups.

01/2005:0314

CELLULOSE ACETATE PHTHALATE

Cellulosi acetas phthalas

DEFINITION

Partly *O*-acetylated and *O*-phthalylated cellulose.

Content:

- phthaloyl groups ($C_8H_5O_3$, relative mass of the group 149.1): 30.0 per cent to 36.0 per cent (anhydrous and acid-free substance).
- acetyl groups (C_2H_3O , relative mass of the group 43.05): 21.5 per cent to 26.0 per cent (anhydrous and acid-free substance).

CHARACTERS

Appearance: white, free-flowing powder or colourless flakes, hygroscopic.

Solubility: practically insoluble in water, freely soluble in acetone, soluble in diethylene glycol, practically insoluble in ethanol and in methylene chloride. It dissolves in dilute solutions of alkalis.

IDENTIFICATION

Infrared absorption spectrophotometry (2.2.24).

Comparison: Ph. Eur. reference spectrum of cellulose acetate phthalate.

TESTS

Viscosity (2.2.9): 45 mPa·s to 90 mPa·s for the apparent viscosity determined at 25 °C.

Dissolve 15 g, calculated with reference to the anhydrous substance, in 85 g of a mixture of 1 part of *water R* and 249 parts of *acetone R*.

Free acid: maximum 3.0 per cent, calculated as phthalic acid (anhydrous substance).

Shake 3.0 g for 2 h with 100 ml of a 50 per cent V/V solution of *methanol R* and filter. Wash the flask and the filter with 2 quantities, each of 10 ml, of a mixture of a 50 per cent V/V solution of *methanol R*. Combine the filtrate and washings, add *phenolphthalein solution R* and titrate with 0.1 M *sodium hydroxide* until a faint pink colour is obtained. Carry out a blank titration.

1 ml of 0.1 M *sodium hydroxide* is equivalent to 8.3 mg of free acid, calculated as phthalic acid.

Heavy metals (2.4.8): maximum 10 ppm.

2.0 g complies with limit test C. Prepare the standard using 2 ml of *lead standard solution (10 ppm Pb) R*.

Water (2.5.12): maximum 5.0 per cent, determined on 0.500 g.

Carry out the test using a mixture of 2 volumes of *methylene chloride R* and 3 volumes of *ethanol R*.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Phthaloyl groups. Dissolve 1.000 g in 50 ml of a mixture of 2 volumes of *acetone R* and 3 volumes of *alcohol R*. Add 0.1 ml of *phenolphthalein solution R* and titrate with 0.1 M *sodium hydroxide* until a faint pink colour is obtained. Carry out a blank titration.

Calculate the percentage content of phthaloyl groups (*P*) from the expression:

$$\frac{14\,900n}{(100 - a)(100 - S)m} - \frac{179.5S}{(100 - S)}$$

- a* = percentage content of water,
m = mass of the substance to be examined, in grams,
n = number of millilitres of 0.1 M *sodium hydroxide* used,
S = percentage content of free acid (see Tests).

Acetyl groups. To 0.100 g add 25.0 ml of 0.1 M *sodium hydroxide* and heat on a water-bath under a reflux condenser for 30 min. Cool, add 0.1 ml of *phenolphthalein solution R* and titrate with 0.1 M *hydrochloric acid* until the colour is discharged. Carry out a blank titration.

Calculate the percentage content of acetyl groups from the expression:

$$\left[\frac{4300(n_2 - n_1)}{(100 - a)(100 - S)m} - \frac{51.8S}{(100 - S)} \right] - 0.578P$$

- a* = percentage content of water,
m = mass of the substance to be examined, in grams,
*n*₁ = number of millilitres of 0.1 M *hydrochloric acid* used in the test,
*n*₂ = number of millilitres of 0.1 M *hydrochloric acid* used in the blank titration,
P = percentage content of phthaloyl groups,
S = percentage content of free acid (see Tests).

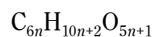
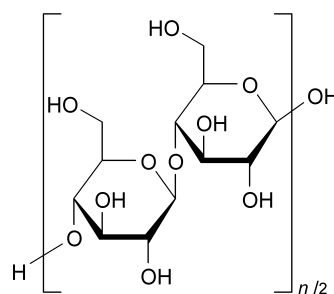
STORAGE

In an airtight container.

01/2005:0316

CELLULOSE, MICROCRYSTALLINE

Cellulosum microcristallinum



DEFINITION

Purified, partly depolymerised cellulose prepared by treating alpha-cellulose, obtained as a pulp from fibrous plant material, with mineral acids.

CHARACTERS

Appearance: white or almost white, fine or granular powder.

Solubility: practically insoluble in water, in acetone, in ethanol, in toluene, in dilute acids and in a 50 g/l solution of sodium hydroxide.

IDENTIFICATION

- A. Place about 10 mg on a watch-glass and disperse in 2 ml of *iodinated zinc chloride solution R*. The substance becomes violet-blue.
- B. The degree of polymerisation is not more than 350. Transfer 1.300 g to a 125 ml conical flask. Add 25.0 ml of *water R* and 25.0 ml of *cupriethylenediamine hydroxide solution R*. Immediately purge the solution with *nitrogen R*, insert the stopper and shake until completely dissolved. Transfer 7.0 ml of the solution to a suitable capillary viscometer (2.2.9). Equilibrate the solution at 25 ± 0.1 °C for at least 5 min. Record the

flow time t_1 in seconds, between the 2 marks on the viscometer. Calculate the kinematic viscosity ν_1 of the solution using the formula:

$$t_1 (k_1)$$

where k_1 is the viscometer constant.

Dilute a suitable volume of *cupriethylenediamine hydroxide solution R* with an equal volume of *water R* and measure the flow time t_2 using a suitable capillary viscometer. Calculate the kinematic viscosity ν_2 of the solvent using the formula:

$$t_2 (k_2)$$

where k_2 is the viscometer constant.

Determine the relative viscosity η_{rel} of the substance to be examined using the formula:

$$\nu_1 / \nu_2$$

Determine the intrinsic viscosity $[\eta]_c$ by interpolation, using the intrinsic viscosity table (Table 0316-1).

Calculate the degree of polymerisation P using the formula:

$$\frac{95 [\eta]_c}{m [(100 - b) / 100]}$$

where m is the mass in grams of the substance to be examined and b is the loss on drying as a percentage.

TESTS

Solubility. Dissolve 50 mg in 10 ml of *ammoniacal solution of copper tetrammine R*. It dissolves completely, leaving no residue.

pH (2.2.3): 5.0 to 7.5 for the supernatant liquid.

Shake 5 g with 40 ml of *carbon dioxide-free water R* for 20 min and centrifuge.

Table 0316-1. – *Intrinsic viscosity table*

Intrinsic viscosity $[\eta]_c$ at different values of relative viscosity η_{rel}										
η_{rel}	0.00	0.01	0.02	0.03	0.04	0.05	0.06	0.07	0.08	0.09
1.1	0.098	0.106	0.115	0.125	0.134	0.143	0.152	0.161	0.170	0.180
1.2	0.189	0.198	0.207	0.216	0.225	0.233	0.242	0.250	0.259	0.268
1.3	0.276	0.285	0.293	0.302	0.310	0.318	0.326	0.334	0.342	0.350
1.4	0.358	0.367	0.375	0.383	0.391	0.399	0.407	0.414	0.422	0.430
1.5	0.437	0.445	0.453	0.460	0.468	0.476	0.484	0.491	0.499	0.507
1.6	0.515	0.522	0.529	0.536	0.544	0.551	0.558	0.566	0.573	0.580
1.7	0.587	0.595	0.602	0.608	0.615	0.622	0.629	0.636	0.642	0.649
1.8	0.656	0.663	0.670	0.677	0.683	0.690	0.697	0.704	0.710	0.717
1.9	0.723	0.730	0.736	0.743	0.749	0.756	0.762	0.769	0.775	0.782
2.0	0.788	0.795	0.802	0.809	0.815	0.821	0.827	0.833	0.840	0.846
2.1	0.852	0.858	0.864	0.870	0.876	0.882	0.888	0.894	0.900	0.906
2.2	0.912	0.918	0.924	0.929	0.935	0.941	0.948	0.953	0.959	0.965
2.3	0.971	0.976	0.983	0.988	0.994	1.000	1.006	1.011	1.017	1.022
2.4	1.028	1.033	1.039	1.044	1.050	1.056	1.061	1.067	1.072	1.078
2.5	1.083	1.089	1.094	1.100	1.105	1.111	1.116	1.121	1.126	1.131
2.6	1.137	1.142	1.147	1.153	1.158	1.163	1.169	1.174	1.179	1.184
2.7	1.190	1.195	1.200	1.205	1.210	1.215	1.220	1.225	1.230	1.235
2.8	1.240	1.245	1.250	1.255	1.260	1.265	1.270	1.275	1.280	1.285
2.9	1.290	1.295	1.300	1.305	1.310	1.314	1.319	1.324	1.329	1.333
3.0	1.338	1.343	1.348	1.352	1.357	1.362	1.367	1.371	1.376	1.381
3.1	1.386	1.390	1.395	1.400	1.405	1.409	1.414	1.418	1.423	1.427
3.2	1.432	1.436	1.441	1.446	1.450	1.455	1.459	1.464	1.468	1.473
3.3	1.477	1.482	1.486	1.491	1.496	1.500	1.504	1.508	1.513	1.517
3.4	1.521	1.525	1.529	1.533	1.537	1.542	1.546	1.550	1.554	1.558
3.5	1.562	1.566	1.570	1.575	1.579	1.583	1.587	1.591	1.595	1.600
3.6	1.604	1.608	1.612	1.617	1.621	1.625	1.629	1.633	1.637	1.642
3.7	1.646	1.650	1.654	1.658	1.662	1.666	1.671	1.675	1.679	1.683

Intrinsic viscosity $[\eta]_c$ at different values of relative viscosity η_{rel}										
η_{rel}	$[\eta]_c$									
	0.00	0.01	0.02	0.03	0.04	0.05	0.06	0.07	0.08	0.09
3.8	1.687	1.691	1.695	1.700	1.704	1.708	1.712	1.715	1.719	1.723
3.9	1.727	1.731	1.735	1.739	1.742	1.746	1.750	1.754	1.758	1.762
4.0	1.765	1.769	1.773	1.777	1.781	1.785	1.789	1.792	1.796	1.800
4.1	1.804	1.808	1.811	1.815	1.819	1.822	1.826	1.830	1.833	1.837
4.2	1.841	1.845	1.848	1.852	1.856	1.859	1.863	1.867	1.870	1.874
4.3	1.878	1.882	1.885	1.889	1.893	1.896	1.900	1.904	1.907	1.911
4.4	1.914	1.918	1.921	1.925	1.929	1.932	1.936	1.939	1.943	1.946
4.5	1.950	1.954	1.957	1.961	1.964	1.968	1.971	1.975	1.979	1.982
4.6	1.986	1.989	1.993	1.996	2.000	2.003	2.007	2.010	2.013	2.017
4.7	2.020	2.023	2.027	2.030	2.033	2.037	2.040	2.043	2.047	2.050
4.8	2.053	2.057	2.060	2.063	2.067	2.070	2.073	2.077	2.080	2.083
4.9	2.087	2.090	2.093	2.097	2.100	2.103	2.107	2.110	2.113	2.116
5.0	2.119	2.122	2.125	2.129	2.132	2.135	2.139	2.142	2.145	2.148
5.1	2.151	2.154	2.158	2.160	2.164	2.167	2.170	2.173	2.176	2.180
5.2	2.183	2.186	2.190	2.192	2.195	2.197	2.200	2.203	2.206	2.209
5.3	2.212	2.215	2.218	2.221	2.224	2.227	2.230	2.233	2.236	2.240
5.4	2.243	2.246	2.249	2.252	2.255	2.258	2.261	2.264	2.267	2.270
5.5	2.273	2.276	2.279	2.282	2.285	2.288	2.291	2.294	2.297	2.300
5.6	2.303	2.306	2.309	2.312	2.315	2.318	2.320	2.324	2.326	2.329
5.7	2.332	2.335	2.338	2.341	2.344	2.347	2.350	2.353	2.355	2.358
5.8	2.361	2.364	2.367	2.370	2.373	2.376	2.379	2.382	2.384	2.387
5.9	2.390	2.393	2.396	2.400	2.403	2.405	2.408	2.411	2.414	2.417
6.0	2.419	2.422	2.425	2.428	2.431	2.433	2.436	2.439	2.442	2.444
6.1	2.447	2.450	2.453	2.456	2.458	2.461	2.464	2.467	2.470	2.472
6.2	2.475	2.478	2.481	2.483	2.486	2.489	2.492	2.494	2.497	2.500
6.3	2.503	2.505	2.508	2.511	2.513	2.516	2.518	2.521	2.524	2.526
6.4	2.529	2.532	2.534	2.537	2.540	2.542	2.545	2.547	2.550	2.553
6.5	2.555	2.558	2.561	2.563	2.566	2.568	2.571	2.574	2.576	2.579
6.6	2.581	2.584	2.587	2.590	2.592	2.595	2.597	2.600	2.603	2.605
6.7	2.608	2.610	2.613	2.615	2.618	2.620	2.623	2.625	2.627	2.630
6.8	2.633	2.635	2.637	2.640	2.643	2.645	2.648	2.650	2.653	2.655
6.9	2.658	2.660	2.663	2.665	2.668	2.670	2.673	2.675	2.678	2.680
7.0	2.683	2.685	2.687	2.690	2.693	2.695	2.698	2.700	2.702	2.705
7.1	2.707	2.710	2.712	2.714	2.717	2.719	2.721	2.724	2.726	2.729
7.2	2.731	2.733	2.736	2.738	2.740	2.743	2.745	2.748	2.750	2.752
7.3	2.755	2.757	2.760	2.762	2.764	2.767	2.769	2.771	2.774	2.776
7.4	2.779	2.781	2.783	2.786	2.788	2.790	2.793	2.795	2.798	2.800
7.5	2.802	2.805	2.807	2.809	2.812	2.814	2.816	2.819	2.821	2.823
7.6	2.826	2.828	2.830	2.833	2.835	2.837	2.840	2.842	2.844	2.847
7.7	2.849	2.851	2.854	2.856	2.858	2.860	2.863	2.865	2.868	2.870
7.8	2.873	2.875	2.877	2.879	2.881	2.884	2.887	2.889	2.891	2.893

Intrinsic viscosity $[\eta]_c$ at different values of relative viscosity η_{rel}										
η_{rel}	$[\eta]_c$									
	0.00	0.01	0.02	0.03	0.04	0.05	0.06	0.07	0.08	0.09
7.9	2.895	2.898	2.900	2.902	2.905	2.907	2.909	2.911	2.913	2.915
8.0	2.918	2.920	2.922	2.924	2.926	2.928	2.931	2.933	2.935	2.937
8.1	2.939	2.942	2.944	2.946	2.948	2.950	2.952	2.955	2.957	2.959
8.2	2.961	2.963	2.966	2.968	2.970	2.972	2.974	2.976	2.979	2.981
8.3	2.983	2.985	2.987	2.990	2.992	2.994	2.996	2.998	3.000	3.002
8.4	3.004	3.006	3.008	3.010	3.012	3.015	3.017	3.019	3.021	3.023
8.5	3.025	3.027	3.029	3.031	3.033	3.035	3.037	3.040	3.042	3.044
8.6	3.046	3.048	3.050	3.052	3.054	3.056	3.058	3.060	3.062	3.064
8.7	3.067	3.069	3.071	3.073	3.075	3.077	3.079	3.081	3.083	3.085
8.8	3.087	3.089	3.092	3.094	3.096	3.098	3.100	3.102	3.104	3.106
8.9	3.108	3.110	3.112	3.114	3.116	3.118	3.120	3.122	3.124	3.126
9.0	3.128	3.130	3.132	3.134	3.136	3.138	3.140	3.142	3.144	3.146
9.1	3.148	3.150	3.152	3.154	3.156	3.158	3.160	3.162	3.164	3.166
9.2	3.168	3.170	3.172	3.174	3.176	3.178	3.180	3.182	3.184	3.186
9.3	3.188	3.190	3.192	3.194	3.196	3.198	3.200	3.202	3.204	3.206
9.4	3.208	3.210	3.212	3.214	3.215	3.217	3.219	3.221	3.223	3.225
9.5	3.227	3.229	3.231	3.233	3.235	3.237	3.239	3.241	3.242	3.244
9.6	3.246	3.248	3.250	3.252	3.254	3.256	3.258	3.260	3.262	3.264
9.7	3.266	3.268	3.269	3.271	3.273	3.275	3.277	3.279	3.281	3.283
9.8	3.285	3.287	3.289	3.291	3.293	3.295	3.297	3.298	3.300	3.302
9.9	3.304	3.305	3.307	3.309	3.311	3.313	3.316	3.318	3.320	3.321
Intrinsic viscosity $[\eta]_c$ at different values of relative viscosity η_{rel}										
η_{rel}	$[\eta]_c$									
	0.0	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9
10	3.32	3.34	3.36	3.37	3.39	3.41	3.43	3.45	3.46	3.48
11	3.50	3.52	3.53	3.55	3.56	3.58	3.60	3.61	3.63	3.64
12	3.66	3.68	3.69	3.71	3.72	3.74	3.76	3.77	3.79	3.80
13	3.80	3.83	3.85	3.86	3.88	3.89	3.90	3.92	3.93	3.95
14	3.96	3.97	3.99	4.00	4.02	4.03	4.04	4.06	4.07	4.09
15	4.10	4.11	4.13	4.14	4.15	4.17	4.18	4.19	4.20	4.22
16	4.23	4.24	4.25	4.27	4.28	4.29	4.30	4.31	4.33	4.34
17	4.35	4.36	4.37	4.38	4.39	4.41	4.42	4.43	4.44	4.45
18	4.46	4.47	4.48	4.49	4.50	4.52	4.53	4.54	4.55	4.56
19	4.57	4.58	4.59	4.60	4.61	4.62	4.63	4.64	4.65	4.66

Conductivity (2.2.38). The conductivity of the test solution does not exceed the conductivity of the water by more than $75 \mu\text{Scm}^{-1}$.

Use as test solution the supernatant liquid obtained in the test for pH. Measure the conductivity of the supernatant liquid after a stable reading has been obtained and measure the conductivity of the water used to prepare the test solution.

Ether-soluble substances: maximum 0.05 per cent (5 mg) for the difference between the weight of the residue and the weight obtained from a blank determination.

Place 10.0 g in a column about 20 mm in internal diameter and pass 50 ml of *peroxide-free ether R* through the column. Evaporate the eluate to dryness and weigh. Carry out a blank determination under the same conditions.

Water-soluble substances: maximum 0.25 per cent (12.5 mg) for the difference between the weight of the residue and the weight obtained from a blank determination.

Shake 5.0 g with 80 ml of *water R* for 10 min. Filter with the aid of vacuum into a tared flask. Evaporate to dryness on a

water-bath. Dry at 100-105 °C for 1 h and weigh. Carry out a blank determination under the same conditions.

Heavy metals (2.4.8): maximum 10 ppm.

2.0 g complies with limit test C. Prepare the standard using 2 ml of *lead standard solution* (10 ppm Pb) R.

Loss on drying (2.2.32): maximum 7.0 per cent, determined on 1.000 g by drying in an oven at 100-105 °C for 3 h.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

Microbial contamination. Total viable aerobic count (2.6.12) not more than 10³ micro-organisms per gram and with a limit for fungi of 10² per gram, determined by plate count. It complies with the tests for *Escherichia coli*, for *Pseudomonas aeruginosa*, for *Staphylococcus aureus* and for *Salmonella* (2.6.13).

Solubility: practically insoluble in water, slightly soluble in a 50 g/l solution of sodium hydroxide, practically insoluble in acetone, in ethanol, in toluene, in dilute acids and in most organic solvents.

IDENTIFICATION

A. Place about 10 mg on a watch-glass and disperse in 2 ml of *iodinated zinc chloride solution* R. The substance becomes violet-blue.

B. The degree of polymerisation is 440 to 2250.

Transfer 0.250 g to a 125 ml conical flask. Add 25.0 ml of *water* R and 25.0 ml of *cupriethylenediamine hydroxide solution* R. Immediately purge the solution with *nitrogen* R, insert the stopper and shake until completely dissolved. Transfer 7.0 ml of the solution to a suitable capillary viscometer (2.2.9). Equilibrate the solution at 25 ± 0.1 °C for at least 5 min. Record the flow time *t*₁ in seconds, between the 2 marks on the viscometer. Calculate the kinematic viscosity *ν*₁ of the solution using the formula:

$$t_1 (k_1)$$

where *k*₁ is the viscometer constant.

Dilute a suitable volume of *cupriethylenediamine hydroxide solution* R with an equal volume of *water* R and measure the flow time *t*₂ using a suitable capillary viscometer. Calculate the kinematic viscosity *ν*₂ of the solvent using the formula:

$$t_2 (k_2)$$

where *k*₂ is the viscometer constant.

Determine the relative viscosity *η*_{rel} of the substance to be examined using the formula:

$$\nu_1/\nu_2$$

Determine the intrinsic viscosity [*η*]_c by interpolation, using the intrinsic viscosity table (Table 0315.-1).

Calculate the degree of polymerisation, *P*, using the formula:

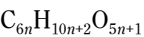
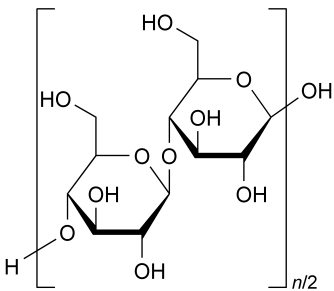
$$\frac{95 [\eta]_c}{m [(100 - b) / 100]}$$

where *m* is the mass in grams of the substance to be examined and *b* is the loss on drying as a percentage.

01/2005:0315

CELLULOSE, POWDERED

Cellulosi pulvis



DEFINITION

Purified, mechanically disintegrated cellulose prepared by processing alpha-cellulose obtained as a pulp from fibrous plant material.

CHARACTERS

Appearance: white or almost white, fine or granular powder.

Table 0315.-1. – *Intrinsic viscosity table*

Intrinsic viscosity [<i>η</i>] _c at different values of relative viscosity <i>η</i> _{rel}										
<i>η</i> _{rel}	0.00	0.01	0.02	0.03	[<i>η</i>] _c 0.04	0.05	0.06	0.07	0.08	0.09
1.1	0.098	0.106	0.115	0.125	0.134	0.143	0.152	0.161	0.170	0.180
1.2	0.189	0.198	0.207	0.216	0.225	0.233	0.242	0.250	0.259	0.268
1.3	0.276	0.285	0.293	0.302	0.310	0.318	0.326	0.334	0.342	0.350
1.4	0.358	0.367	0.375	0.383	0.391	0.399	0.407	0.414	0.422	0.430
1.5	0.437	0.445	0.453	0.460	0.468	0.476	0.484	0.491	0.499	0.507
1.6	0.515	0.522	0.529	0.536	0.544	0.551	0.558	0.566	0.573	0.580
1.7	0.587	0.595	0.602	0.608	0.615	0.622	0.629	0.636	0.642	0.649
1.8	0.656	0.663	0.670	0.677	0.683	0.690	0.697	0.704	0.710	0.717
1.9	0.723	0.730	0.736	0.743	0.749	0.756	0.762	0.769	0.775	0.782

Intrinsic viscosity $[\eta]_c$ at different values of relative viscosity η_{rel}										
η_{rel}	$[\eta]_c$									
	0.00	0.01	0.02	0.03	0.04	0.05	0.06	0.07	0.08	0.09
2.0	0.788	0.795	0.802	0.809	0.815	0.821	0.827	0.833	0.840	0.846
2.1	0.852	0.858	0.864	0.870	0.876	0.882	0.888	0.894	0.900	0.906
2.2	0.912	0.918	0.924	0.929	0.935	0.941	0.948	0.953	0.959	0.965
2.3	0.971	0.976	0.983	0.988	0.994	1.000	1.006	1.011	1.017	1.022
2.4	1.028	1.033	1.039	1.044	1.050	1.056	1.061	1.067	1.072	1.078
2.5	1.083	1.089	1.094	1.100	1.105	1.111	1.116	1.121	1.126	1.131
2.6	1.137	1.142	1.147	1.153	1.158	1.163	1.169	1.174	1.179	1.184
2.7	1.190	1.195	1.200	1.205	1.210	1.215	1.220	1.225	1.230	1.235
2.8	1.240	1.245	1.250	1.255	1.260	1.265	1.270	1.275	1.280	1.285
2.9	1.290	1.295	1.300	1.305	1.310	1.314	1.319	1.324	1.329	1.333
3.0	1.338	1.343	1.348	1.352	1.357	1.362	1.367	1.371	1.376	1.381
3.1	1.386	1.390	1.395	1.400	1.405	1.409	1.414	1.418	1.423	1.427
3.2	1.432	1.436	1.441	1.446	1.450	1.455	1.459	1.464	1.468	1.473
3.3	1.477	1.482	1.486	1.491	1.496	1.500	1.504	1.508	1.513	1.517
3.4	1.521	1.525	1.529	1.533	1.537	1.542	1.546	1.550	1.554	1.558
3.5	1.562	1.566	1.570	1.575	1.579	1.583	1.587	1.591	1.595	1.600
3.6	1.604	1.608	1.612	1.617	1.621	1.625	1.629	1.633	1.637	1.642
3.7	1.646	1.650	1.654	1.658	1.662	1.666	1.671	1.675	1.679	1.683
3.8	1.687	1.691	1.695	1.700	1.704	1.708	1.712	1.715	1.719	1.723
3.9	1.727	1.731	1.735	1.739	1.742	1.746	1.750	1.754	1.758	1.762
4.0	1.765	1.769	1.773	1.777	1.781	1.785	1.789	1.792	1.796	1.800
4.1	1.804	1.808	1.811	1.815	1.819	1.822	1.826	1.830	1.833	1.837
4.2	1.841	1.845	1.848	1.852	1.856	1.859	1.863	1.867	1.870	1.874
4.3	1.878	1.882	1.885	1.889	1.893	1.896	1.900	1.904	1.907	1.911
4.4	1.914	1.918	1.921	1.925	1.929	1.932	1.936	1.939	1.943	1.946
4.5	1.950	1.954	1.957	1.961	1.964	1.968	1.971	1.975	1.979	1.982
4.6	1.986	1.989	1.993	1.996	2.000	2.003	2.007	2.010	2.013	2.017
4.7	2.020	2.023	2.027	2.030	2.033	2.037	2.040	2.043	2.047	2.050
4.8	2.053	2.057	2.060	2.063	2.067	2.070	2.073	2.077	2.080	2.083
4.9	2.087	2.090	2.093	2.097	2.100	2.103	2.107	2.110	2.113	2.116
5.0	2.119	2.122	2.125	2.129	2.132	2.135	2.139	2.142	2.145	2.148
5.1	2.151	2.154	2.158	2.160	2.164	2.167	2.170	2.173	2.176	2.180
5.2	2.183	2.186	2.190	2.192	2.195	2.197	2.200	2.203	2.206	2.209
5.3	2.212	2.215	2.218	2.221	2.224	2.227	2.230	2.233	2.236	2.240
5.4	2.243	2.246	2.249	2.252	2.255	2.258	2.261	2.264	2.267	2.270
5.5	2.273	2.276	2.279	2.282	2.285	2.288	2.291	2.294	2.297	2.300
5.6	2.303	2.306	2.309	2.312	2.315	2.318	2.320	2.324	2.326	2.329
5.7	2.332	2.335	2.338	2.341	2.344	2.347	2.350	2.353	2.355	2.358
5.8	2.361	2.364	2.367	2.370	2.373	2.376	2.379	2.382	2.384	2.387
5.9	2.390	2.393	2.396	2.400	2.403	2.405	2.408	2.411	2.414	2.417
6.0	2.419	2.422	2.425	2.428	2.431	2.433	2.436	2.439	2.442	2.444

Intrinsic viscosity $[\eta]_c$ at different values of relative viscosity η_{rel}										
η_{rel}	$[\eta]_c$									
	0.00	0.01	0.02	0.03	0.04	0.05	0.06	0.07	0.08	0.09
6.1	2.447	2.450	2.453	2.456	2.458	2.461	2.464	2.467	2.470	2.472
6.2	2.475	2.478	2.481	2.483	2.486	2.489	2.492	2.494	2.497	2.500
6.3	2.503	2.505	2.508	2.511	2.513	2.516	2.518	2.521	2.524	2.526
6.4	2.529	2.532	2.534	2.537	2.540	2.542	2.545	2.547	2.550	2.553
6.5	2.555	2.558	2.561	2.563	2.566	2.568	2.571	2.574	2.576	2.579
6.6	2.581	2.584	2.587	2.590	2.592	2.595	2.597	2.600	2.603	2.605
6.7	2.608	2.610	2.613	2.615	2.618	2.620	2.623	2.625	2.627	2.630
6.8	2.633	2.635	2.637	2.640	2.643	2.645	2.648	2.650	2.653	2.655
6.9	2.658	2.660	2.663	2.665	2.668	2.670	2.673	2.675	2.678	2.680
7.0	2.683	2.685	2.687	2.690	2.693	2.695	2.698	2.700	2.702	2.705
7.1	2.707	2.710	2.712	2.714	2.717	2.719	2.721	2.724	2.726	2.729
7.2	2.731	2.733	2.736	2.738	2.740	2.743	2.745	2.748	2.750	2.752
7.3	2.755	2.757	2.760	2.762	2.764	2.767	2.769	2.771	2.774	2.776
7.4	2.779	2.781	2.783	2.786	2.788	2.790	2.793	2.795	2.798	2.800
7.5	2.802	2.805	2.807	2.809	2.812	2.814	2.816	2.819	2.821	2.823
7.6	2.826	2.828	2.830	2.833	2.835	2.837	2.840	2.842	2.844	2.847
7.7	2.849	2.851	2.854	2.856	2.858	2.860	2.863	2.865	2.868	2.870
7.8	2.873	2.875	2.877	2.879	2.881	2.884	2.887	2.889	2.891	2.893
7.9	2.895	2.898	2.900	2.902	2.905	2.907	2.909	2.911	2.913	2.915
8.0	2.918	2.920	2.922	2.924	2.926	2.928	2.931	2.933	2.935	2.937
8.1	2.939	2.942	2.944	2.946	2.948	2.950	2.952	2.955	2.957	2.959
8.2	2.961	2.963	2.966	2.968	2.970	2.972	2.974	2.976	2.979	2.981
8.3	2.983	2.985	2.987	2.990	2.992	2.994	2.996	2.998	3.000	3.002
8.4	3.004	3.006	3.008	3.010	3.012	3.015	3.017	3.019	3.021	3.023
8.5	3.025	3.027	3.029	3.031	3.033	3.035	3.037	3.040	3.042	3.044
8.6	3.046	3.048	3.050	3.052	3.054	3.056	3.058	3.060	3.062	3.064
8.7	3.067	3.069	3.071	3.073	3.075	3.077	3.079	3.081	3.083	3.085
8.8	3.087	3.089	3.092	3.094	3.096	3.098	3.100	3.102	3.104	3.106
8.9	3.108	3.110	3.112	3.114	3.116	3.118	3.120	3.122	3.124	3.126
9.0	3.128	3.130	3.132	3.134	3.136	3.138	3.140	3.142	3.144	3.146
9.1	3.148	3.150	3.152	3.154	3.156	3.158	3.160	3.162	3.164	3.166
9.2	3.168	3.170	3.172	3.174	3.176	3.178	3.180	3.182	3.184	3.186
9.3	3.188	3.190	3.192	3.194	3.196	3.198	3.200	3.202	3.204	3.206
9.4	3.208	3.210	3.212	3.214	3.215	3.217	3.219	3.221	3.223	3.225
9.5	3.227	3.229	3.231	3.233	3.235	3.237	3.239	3.241	3.242	3.244
9.6	3.246	3.248	3.250	3.252	3.254	3.256	3.258	3.260	3.262	3.264
9.7	3.266	3.268	3.269	3.271	3.273	3.275	3.277	3.279	3.281	3.283
9.8	3.285	3.287	3.289	3.291	3.293	3.295	3.297	3.298	3.300	3.302
9.9	3.304	3.305	3.307	3.309	3.311	3.313	3.316	3.318	3.320	3.321

Intrinsic viscosity $[\eta]_c$ at different values of relative viscosity η_{rel}										
η_{rel}	0.0	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9
10	3.32	3.34	3.36	3.37	3.39	3.41	3.43	3.45	3.46	3.48
11	3.50	3.52	3.53	3.55	3.56	3.58	3.60	3.61	3.63	3.64
12	3.66	3.68	3.69	3.71	3.72	3.74	3.76	3.77	3.79	3.80
13	3.80	3.83	3.85	3.86	3.88	3.89	3.90	3.92	3.93	3.95
14	3.96	3.97	3.99	4.00	4.02	4.03	4.04	4.06	4.07	4.09
15	4.10	4.11	4.13	4.14	4.15	4.17	4.18	4.19	4.20	4.22
16	4.23	4.24	4.25	4.27	4.28	4.29	4.30	4.31	4.33	4.34
17	4.35	4.36	4.37	4.38	4.39	4.41	4.42	4.43	4.44	4.45
18	4.46	4.47	4.48	4.49	4.50	4.52	4.53	4.54	4.55	4.56
19	4.57	4.58	4.59	4.60	4.61	4.62	4.63	4.64	4.65	4.66

TESTS

Solubility. Dissolve 50 mg in 10 ml of *ammoniacal solution of copper tetrammine R*. It dissolves completely, leaving no residue.

pH (2.2.3): 5.0 to 7.5 for the supernatant liquid.

Mix 10 g with 90 ml of *carbon dioxide-free water R* and allow to stand with occasional stirring for 1 h.

Ether-soluble substances: maximum 0.15 per cent (15 mg) for the difference between the weight of the residue and the weight obtained from a blank determination.

Place 10.0 g in a column about 20 mm in internal diameter and pass 50 ml of *peroxide-free ether R* through the column. Evaporate the eluate to dryness and weigh. Carry out a blank determination under the same conditions.

Water-soluble substances: maximum 1.5 per cent (75.0 mg) for the difference between the weight of the residue and the weight obtained from a blank determination.

Shake 5.0 g with 80 ml of *water R* for 10 min. Filter with the aid of vacuum into a tared flask. Evaporate to dryness on a water-bath. Dry at 100-105 °C for 1 h and weigh. Carry out a blank determination under the same conditions.

Heavy metals (2.4.8): maximum 10 ppm.

2.0 g complies with limit test C. Prepare the standard using 2 ml of *lead standard solution (10 ppm Pb) R*.

Loss on drying (2.2.32): maximum 6.5 per cent, determined on 1.000 g by drying in an oven at 100-105 °C for 3 h.

Sulphated ash (2.4.14): maximum 0.3 per cent, determined on 1.0 g.

Microbial contamination. Total viable aerobic count (2.6.12) not more than 10^3 micro-organisms per gram and with a limit for fungi of 10^2 per gram, determined by plate count. It complies with the tests for *Escherichia coli*, for *Pseudomonas aeruginosa*, for *Staphylococcus aureus* and for *Salmonella* (2.6.13).

CHARACTERS

Centaury has a very bitter taste.

It has the macroscopic and microscopic characters described under identification tests A and B.

IDENTIFICATION

A. The hollow cylindrical, light green to dark brown stem has longitudinal ridges, and is branched only in its upper part. The sessile leaves are entire, decussately arranged, and have an ovate to lanceolate lamina, up to about 3 cm long. Both surfaces are glabrous and green to brownish-green. The inflorescence is diaxially branched. The tubular calyx is green and has five lanceolate, acuminate teeth. The corolla consists of a whitish tube divided in five elongated lanceolate pink lobes, about 5 mm to 8 mm long. Five stamens are present attached to the top of the corolla tube. The ovary is superior and has a short style, a broad bifid stigma and numerous ovules. Cylindrical capsules, about 7 mm to 10 mm long, with small brown markedly rough seeds are frequently present.

B. Reduce to a powder (355). The powder is greenish-yellow to brownish. Examine under a microscope, using *chloral hydrate solution R*. The powder shows numerous fragments of stem containing sclerenchymatous fibres and narrow vessels with bordered pits or spiral of reticulate thickening, as well as rectangular pitted cells of the pith and medullary rays; fragments of leaves with sinuous epidermal cells and striated cuticle, especially over the margins and surrounding the stomata, anisocytic stomata (2.8.3) and cells of the mesophyll with crystals of calcium oxalate of various types; fragments of calyx and corolla, those of the calyx with straight-walled epidermal cells, epidermis of the corolla with obtuse papillae and radially striated cuticle; parts of the endothecium with reticulate or ridge-shaped wall thickenings; triangularly rounded to elliptical, yellow pollen grains, about 30 µm in diameter, with a finely grained exine and three germinal pores; fragments of the wall of the fruit capsule composed of crossed layers of fusiform cells; small yellowish-brown seeds with a raised reticulate, dark brown structure formed by the coarse lateral walls of their epidermis.

C. Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel F₂₅₄ plate R*.

Test solution. To 1.0 g of the powdered drug (355) add 20 ml of *methanol R* and heat under a reflux condenser for 10 min. Cool and filter.

Reference solution. Dissolve 10 mg of *rutin R* in 10 ml of *methanol R*.

01/2005:1301

CENTAURY

Centaurii herba

DEFINITION

Centaury consists of the whole or cut dried flowering aerial parts of *Centaureum erythraea* Rafn (*C. minus* Moench, *C. umbellatum* Gilib., *Erythraea centaurium* (L.) Pers.).

Apply to the plate as bands 30 µl of the test solution and 10 µl of the reference solution. Develop over a path of 12 cm using a mixture of 16 volumes of *anhydrous acetic acid R*, 16 volumes of *water R* and 68 volumes of *ethyl acetate R*. Allow the plate to dry in a current of cold air and develop again in the same manner. Allow the plate to dry in a current of cold air. Examine the plate in ultraviolet light at 254 nm. The chromatogram obtained with the test solution shows as the main quenching zone a strong zone (swertiamarin) similar in position to the quenching zone of rutin in the chromatogram obtained with the reference solution; further weaker quenching zones may also be present. Spray the plate with *anisaldehyde solution R*. Examine in daylight whilst heating at 100 °C to 105 °C for 5 min to 10 min. The chromatogram obtained with the reference solution shows a yellowish-brown zone (rutin). The chromatogram obtained with the test solution shows: a violet-brown zone (swertiamarin); a little above this zone, a faint yellowish-brown zone; and above this a few, very faint, mostly grey zones leading to a reddish-violet zone at the solvent front. Examine the plate in ultraviolet light at 365 nm. The chromatogram obtained with the test solution shows: a strong brown to brownish-yellow fluorescent zone (swertiamarin); a little above this zone, a blue to yellowish-green fluorescent zone; and above this a few faint blue to yellow fluorescent zones, leading to a faint reddish-violet fluorescent zone at the solvent front. Below the zone due to swertiamarin occur zones of intense light green to yellowish-green fluorescence as well as a number of faint brown fluorescent zones.

TESTS

Foreign matter (2.8.2). Not more than 3 per cent.

Bitterness value. Not less than 2000. The bitterness value is determined by comparison with quinine hydrochloride, the bitterness value of which is set at 200 000; the bitterness value is the reciprocal of the dilution that still has a bitter taste.

Quinine hydrochloride stock solution. Dissolve 0.100 g of *quinine hydrochloride R* in *water R* and dilute to 100.0 ml with the same solvent. Dilute 1.0 ml of the solution to 100.0 ml with *water R*.

Centaury extract. To 1.0 g of powdered drug (355) add 1000 ml of boiling *water R*. Heat on a water-bath for 30 min, stirring continuously. Allow to cool and dilute to 1000 ml with *water R*. Shake vigorously and filter, discarding the first 20 ml of filtrate.

Prepare a series of dilutions by placing in the first tube 4.2 ml of quinine hydrochloride stock solution and increasing the volume by 0.2 ml in each subsequent tube to a total of 5.8 ml; dilute the contents of each tube to 10.0 ml with *water R*. Determine as follows the dilution with the lowest concentration that still has a bitter taste. Take 10.0 ml of the weakest solution into the mouth and pass it from side to side over the back of the tongue for 30 s. If the solution is not found to be bitter, spit it out and wait for 1 min. Rinse the mouth with *water R*. After 10 min, use the next dilution in order of increasing concentration.

Calculate the correction factor *k* from the expression:

$$\frac{5.00}{n}$$

n = number of millilitres of quinine hydrochloride stock solution in the dilution of lowest concentration that is bitter.

Dilute 10/*k* ml of the centaury extract to 20.0 ml with *water R*. 10.0 ml of this dilution has a bitter taste.

Loss on drying (2.2.32). Not more than 10.0 per cent, determined on 1.000 g of powdered drug (355) by drying in an oven at 100 °C to 105 °C for 2 h.

Total ash (2.4.16). Not more than 6.0 per cent.

STORAGE

Store protected from light.

01/2005:1498

CENTELLA

Centellae asiaticae herba

DEFINITION

Centella consists of the dried, fragmented aerial parts of *Centella asiatica* (L.) Urban. It contains not less than 6.0 per cent of total triterpenoid derivatives, expressed as asiaticoside (C₄₈H₇₈O₁₉; *M_r* 959.15), calculated with reference to the dried drug.

CHARACTERS

It has the macroscopic and microscopic characters described under identification tests A and B.

The leaves are very variable in size; the petiole is usually 5 to 10, sometimes 15, times longer than the lamina, which is 10 mm to 40 mm long and 20 mm to 40 mm, sometimes up to 70 mm, wide.

IDENTIFICATION

- A. The leaves are alternate, sometimes grouped together at the nodes, reniform or orbicular or oblong-elliptic and have palmate nervation, usually with seven veins, and a crenate margin. Young leaves show a few trichomes on the lower surface while adult leaves are glabrous. The inflorescence, if present, is a single umbel which usually consists of three flowers, rarely two or four; the flowers are very small (about 2 mm) pentamerous and have an inferior ovary; the fruit, a brownish-grey, orbicular cremocarp, up to 5 mm long, is very flattened laterally and has seven to nine prominent curved ridges.
- B. Reduce the drug to a powder (355). The powder is greenish-grey. Examine under a microscope using *chloral hydrate solution R*. The powder shows numerous fragments of leaf epidermis with polygonal cells having an irregularly striated cuticle, and paracytic stomata (2.8.3) that are more numerous in the lower epidermis; fragments of petiole epidermis with elongated cells; uniseriate, long, flexuous unicellular covering trichomes, occasionally multicellular; young leaves; spiral vessels; resiniferous canals; calcium oxalate prisms and macles up to 40 µm in diameter; bundles of narrow septate fibres from the stem; fragments of the fruit: layers of wide cells in a parquetry arrangement, annular vessels, parenchyma cells containing simple or compound starch granules.
- C. Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel G plate R*.

Test solution. To 5.0 g of the powdered drug (355) add 50 ml of *alcohol (30 per cent V/V) R*; heat to boiling under a reflux condenser and centrifuge.

Reference solution. Dissolve 5 mg of *asiaticoside R* in *methanol R* and dilute to 10 ml with the same solvent.

Apply to the plate as bands 10 µl of each solution. Develop over a path of 15 cm using a mixture of 11 volumes of *acetic acid R*, 11 volumes of *formic acid R*, 27 volumes

of *water R* and 100 volumes of *ethyl acetate R*. Allow the plate to dry in air. Spray with *anisaldehyde solution R* and heat at 100 °C to 105 °C. Examine in daylight. The chromatograms obtained with the reference and the test solutions show in the lower third a greenish-blue zone (asiaticoside). The chromatogram obtained with the test solution shows also below this zone a violet zone (madecassoside); near the solvent front it shows a light blue zone (asiatic acid) and just below a pinkish-violet zone (madecassic acid); in the lower half it shows brown, grey and brownish-green zones between the starting point and the zone due to madecassoside, and other brownish-yellow or light yellow zones above the zone due to asiaticoside.

TESTS

Foreign matter (2.8.2). Not more than 7 per cent of which not more than 5 per cent of underground organs and not more than 2 per cent of other foreign matter.

Loss on drying (2.2.32). Not more than 10.0 per cent, determined on 1.000 g of the powdered drug by drying in an oven at 100 °C to 105 °C for 2 h.

Total ash (2.4.16). Not more than 12.0 per cent.

ASSAY

Examine by liquid chromatography (2.2.29).

Test solution. Place 5.0 g of the powdered drug (355) in a cellulose fingerstall in a continuous extraction apparatus (Soxhlet type). Add 100 ml of *methanol R* and heat for 8 h. Cool and dilute the extract to 100.0 ml with *methanol R*. Filter through a 0.45 µm filter. Dilute 2.0 ml of the filtrate and dilute to 20.0 ml with *methanol R*.

Reference solution. Dissolve 20.0 mg of *asiaticoside R* in *methanol R*, if necessary using sonication, and dilute to 20.0 ml with the same solvent. Dilute 2.0 ml of the solution to 100.0 ml with *methanol R*.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (5 µm),
- as mobile phase at a flow rate of 1.0 ml/min:
Mobile phase A. Acetonitrile for chromatography *R*,
Mobile phase B. Dilute 3 ml of *phosphoric acid R* to 1000 ml with water *R*,

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)
0 - 65	22	78
65 - 66	55	45
66 - 76	95	5
76 - 85	22	78

- as detector a spectrophotometer set at 200 nm.

Inject 20 µl of each solution.

Calculate the response factor of asiaticoside from the expression:

$$R_f = \frac{A_1 \times V_1 \times 100}{m_1 \times \text{HPLC}_P}$$

A_1 = area of the peak corresponding to asiaticoside in the chromatogram obtained with the reference solution,

V_1 = volume of the reference solution, in millilitres,

m_1 = mass of asiaticoside in the reference solution, in milligrams,

HPLC_P = purity determined for asiaticoside.

Calculate the mean response factor for asiaticoside from the expression:

$$\overline{R_f} = \frac{\sum_{i=1}^N R_{fi}}{N}$$

$\sum_{i=1}^N R_{fi}$ = sum of response factors of asiaticoside for the chromatograms obtained with the reference solution,

N = number of injections of reference solution ($N = 4$, at least).

Calculate the percentage content of total triterpenoid derivatives, expressed as asiaticoside from the expression:

$$\frac{V}{m} \left[\frac{A + (B \times 1.017) + (C \times 0.526) + (D \times 0.509)}{\overline{R_f}} \right]$$

V = volume of the test solution, in millilitres,

m = mass of the substance to be examined in the test solution, in milligrams,

A = area of the peak corresponding to asiaticoside in the chromatogram with the test solution,

B = area of the peak corresponding to madecassoside in the chromatogram with the test solution,

C = area of the peak corresponding to madecassic acid in the chromatogram with the test solution,

D = area of the peak corresponding to asiatic acid in the chromatogram with the test solution,

$\overline{R_f}$ = mean response factor of asiaticoside.

Relative retention with reference to the solvent:

madecassoside = about 5.8; asiaticoside = about 8.1; madecassic acid = about 17.6; asiatic acid = about 21.7.

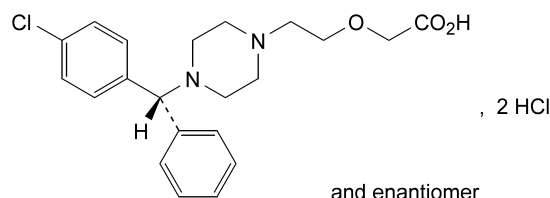
STORAGE

Store protected from light.

01/2005:1084

CETIRIZINE DIHYDROCHLORIDE

Cetirizini dihydrochloridum



$\text{C}_{21}\text{H}_{27}\text{Cl}_3\text{N}_2\text{O}_3$

M_r 461.8

DEFINITION

(*RS*)-2-[2-[4-[(4-chlorophenyl)phenylmethyl]piperazin-1-yl]ethoxy]acetic acid dihydrochloride.

Content: 99.0 per cent to 100.5 per cent (dried substance).

CHARACTERS

Appearance: white or almost white powder.

Solubility: freely soluble in water, practically insoluble in acetone and in methylene chloride.

IDENTIFICATION

First identification: B, D.

Second identification: A, C, D.

A. Dissolve 20.0 mg in 50 ml of a 10.3 g/l solution of *hydrochloric acid R* and dilute to 100.0 ml with the same acid. Dilute 10.0 ml of the solution to 100.0 ml with a 10.3 g/l solution of *hydrochloric acid R*. Examined between 210 nm and 350 nm (2.2.25), the solution shows an absorption maximum at 231 nm. The specific absorbance at the maximum is 359 to 381.

B. Infrared absorption spectrophotometry (2.2.24).

Preparation: discs.

Comparison: *cetirizine dihydrochloride CRS*.

C. Thin-layer chromatography (2.2.27).

Test solution. Dissolve 10 mg of the substance to be examined in *water R* and dilute to 5 ml with the same solvent.

Reference solution (a). Dissolve 10 mg of *cetirizine dihydrochloride CRS* in *water R* and dilute to 5 ml with the same solvent.

Reference solution (b). Dissolve 10 mg of *chlorphenamine maleate CRS* in *water R* and dilute to 5 ml with the same solvent. To 1 ml of the solution add 1 ml of reference solution (a).

Plate: TLC silica gel GF₂₅₄ plate *R*.

Mobile phase: *ammonia R*, *methanol R*, *methylene chloride R* (1:10:90 V/V/V).

Application: 5 µl.

Development: over 2/3 of the plate.

Drying: in a current of cold air.

Detection: examine in ultraviolet light at 254 nm.

System suitability: reference solution (b):

- the chromatogram obtained shows 2 clearly separated spots.

Results: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a).

D. It gives reaction (a) of chlorides (2.3.1).

TESTS

Solution S. Dissolve 1.0 g in *carbon dioxide-free water R* and dilute to 20 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and not more intensely coloured than reference solution BY₇ (2.2.2, *Method II*).

pH (2.2.3): 1.2 to 1.8 for solution S.

Related substances. Liquid chromatography (2.2.29).

Test solution. Dissolve 20.0 mg of the substance to be examined in the mobile phase and dilute to 100.0 ml with the mobile phase.

Reference solution (a). Dissolve 5.0 mg of *cetirizine dihydrochloride CRS* and 5.0 mg of *cetirizine impurity A CRS* in the mobile phase and dilute to 25.0 ml with the mobile phase. Dilute 1.0 ml of the solution to 100.0 ml with the mobile phase.

Reference solution (b). Dilute 2.0 ml of the test solution to 50.0 ml with the mobile phase. Dilute 5.0 ml of this solution to 100.0 ml with the mobile phase.

Column:

- **size:** $l = 0.25$ m, $\varnothing = 4.6$ mm,
- **stationary phase:** *silica gel for chromatography R* (5 µm).

Mobile phase: *dilute sulphuric acid R*, *water R*, *acetonitrile R* (0.4:6.6:93 V/V/V).

Flow rate: 1 ml/min.

Detection: spectrophotometer at 230 nm.

Injection: 20 µl.

Run time: 3 times the retention time of cetirizine.

System suitability: reference solution (a):

- **resolution:** minimum 3 between the peaks due to cetirizine and impurity A,
- **symmetry factors:** maximum 2.0.

Limits:

- **impurities A, B, C, D, E, F:** for each impurity, not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent),
- **any other impurity:** for each impurity, not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent),
- **total:** not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.3 per cent),
- **disregard limit:** 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.02 per cent).

Loss on drying (2.2.32): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 100–105 °C.

Sulphated ash (2.4.14): maximum 0.2 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.100 g in 70 ml of a mixture of 30 volumes of *water R* and 70 volumes of *acetone R*. Titrate with 0.1 M *sodium hydroxide* to the second point of inflexion. Determine the end-point potentiometrically (2.2.20). Carry out a blank titration.

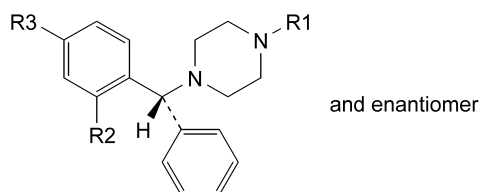
1 ml of 0.1 M *sodium hydroxide* is equivalent to 15.39 mg of C₂₁H₂₇Cl₃N₂O₃.

STORAGE

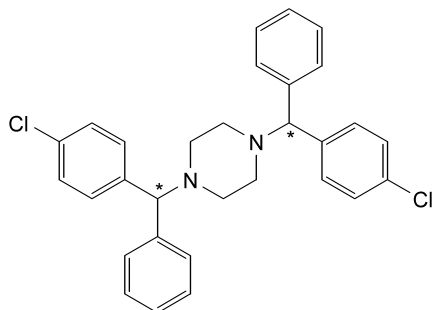
Protected from light.

IMPURITIES

Specified impurities: A, B, C, D, E, F.



- A. R1 = R2 = H, R3 = Cl: (RS)-1-[(4-chlorophenyl)phenylmethyl]piperazine,
- B. R1 = CH₂-CO₂H, R2 = H, R3 = Cl: (RS)-2-[4-[(4-chlorophenyl)phenylmethyl]piperazin-1-yl]acetic acid,
- C. R1 = CH₂-CH₂-O-CH₂-CO₂H, R2 = Cl, R3 = H: (RS)-2-[2-[4-[(2-chlorophenyl)phenylmethyl]piperazin-1-yl]ethoxy]acetic acid,
- E. R1 = CH₂-[CH₂-O-CH₂]₂-CO₂H, R2 = H, R3 = Cl: (RS)-2-[2-[2-[4-[(4-chlorophenyl)phenylmethyl]piperazin-1-yl]ethoxy]ethoxy]acetic acid (ethoxycetirizine),
- F. R1 = CH₂-CH₂-O-CH₂-CO₂H, R2 = R3 = H: [2-[4-(diphenylmethyl)piperazin-1-yl]ethoxy]acetic acid,



- D. 1,4-bis[(4-chlorophenyl)phenylmethyl]piperazine.

01/2005:0702

CETOSTEARYL ALCOHOL

Alcohol cetylicus et stearylicus

DEFINITION

Cetostearyl alcohol is a mixture of solid aliphatic alcohols. It contains not less than 40.0 per cent of stearyl alcohol (C₁₈H₃₈O; *M_r* 270.5) and the sum of the contents of cetyl alcohol (C₁₆H₃₄O; *M_r* 242.4) and of stearyl alcohol is not less than 90.0 per cent.

CHARACTERS

White or pale yellow, wax-like mass, plates, flakes or granules, practically insoluble in water, soluble in alcohol (90 per cent *V/V*) and in light petroleum; when melted, it is miscible with fatty oils, with liquid paraffin and with melted wool fat.

IDENTIFICATION

Examine the chromatograms obtained in the assay. The 2 principal peaks in the chromatogram obtained with the test solution have similar retention times to the principal peaks in the chromatograms obtained with reference solution (a) and reference solution (b), respectively.

TESTS

Appearance of solution. Dissolve 0.50 g in 20 ml of boiling alcohol *R*. The solution is clear (2.2.1) and not more intensely coloured than reference solution B₆ (2.2.2, *Method II*).

Melting point (2.2.14): 49 °C to 56 °C.

Acid value (2.5.1). Not more than 1.0.

Hydroxyl value (2.5.3, *Method A*): 208 to 228.

Iodine value (2.5.4). Not more than 2.0, determined on 2.00 g, dissolved in 25 ml of chloroform *R*.

Saponification value (2.5.6). Not more than 2.0.

ASSAY

Examine by gas chromatography (2.2.28).

Test solution. Dissolve 0.100 g of the substance to be examined in ethanol *R* and dilute to 10.0 ml with the same solvent.

Reference solution (a). Dissolve 60.0 mg of cetyl alcohol CRS in ethanol *R* and dilute to 10.0 ml with the same solvent.

Reference solution (b) Dissolve 40.0 mg of stearyl alcohol CRS in ethanol *R* and dilute to 10.0 ml with the same solvent.

Reference solution (c). Mix 1 ml of reference solution (a) and 1 ml of reference solution (b) and dilute to 10.0 ml with ethanol *R*.

The chromatographic procedure may be carried out using:

- a stainless steel column 3 m long and 4 mm in internal diameter packed with *diatomaceous earth for gas chromatography R* impregnated with 10 per cent *m/m* of poly(dimethyl)siloxane *R*,
- nitrogen for chromatography *R* as the carrier gas at a flow rate of 30 ml/min,
- a flame-ionisation detector,

maintaining the temperature of the column at 200 °C and that of the injection port and the detector at 250 °C.

Inject separately 2 µl of each solution. Adjust the flow rate so that the resolution between the 2 principal peaks in the chromatogram obtained with the test solution is not less than 1.25. The test is not valid unless the chromatogram obtained with reference solution (c) shows 2 principal peaks with a signal-to-noise ratio of at least 5. Determine the content of cetyl alcohol and of stearyl alcohol from the chromatogram obtained with the test solution, using the normalisation procedure and identifying the peaks by comparison with the chromatograms obtained with reference solution (a) and reference solution (b), respectively.

01/2005:0801

CETOSTEARYL ALCOHOL (TYPE A), EMULSIFYING

Alcohol cetylicus et stearylicus emulsificans A

DEFINITION

Emulsifying cetostearyl alcohol (type A) is a mixture which contains not less than 80.0 per cent of cetostearyl alcohol and not less than 7.0 per cent of sodium cetostearyl sulphate, both calculated with reference to the anhydrous substance. A suitable buffer may be added.

CHARACTERS

White or pale yellow, wax-like mass, plates, flakes or granules, soluble in hot water giving an opalescent solution, practically insoluble in cold water, slightly soluble in alcohol.

IDENTIFICATION

First identification: B, C, D.

Second identification: A, C.

- A. Examine by thin-layer chromatography (2.2.27), using a *TLC silanised silica gel plate R*.

Test solution (a). Dissolve 0.1 g of the substance to be examined in 10 ml of *trimethylpentane R*, heating on a water-bath. Shake with 2 ml of *alcohol (70 per cent V/V) R* and allow to separate. Use the lower layer as test solution (b). Dilute 1 ml of the upper layer to 8 ml with *trimethylpentane R*.

Test solution (b). Use the lower layer obtained in the preparation of test solution (a).

Reference solution (a). Dissolve 40 mg of *cetostearyl alcohol R* in 10 ml of *trimethylpentane R*.

Reference solution (b). Dissolve 20 mg of *sodium cetostearyl sulphate R* in 10 ml of *alcohol (70 per cent V/V) R*, heating on a water-bath.

Apply to the plate 2 µl of each solution. Develop over a path of 12 cm using a mixture of 20 volumes of *water R*, 40 volumes of *acetone R* and 40 volumes of *methanol R*. Allow the plate to dry in air and spray with a 50 g/l solution of *phosphomolybdic acid R* in *alcohol R*. Heat at 120 °C until spots appear (about 3 h). The 2 principal spots in the chromatogram obtained with test solution (a) are similar in position and colour to the principal spots in the chromatogram obtained with reference solution (a). 2 of the spots in the chromatogram obtained with test solution (b) are similar in position and colour to the principal spots in the chromatogram obtained with reference solution (b).

- B. Examine the chromatograms obtained in the assay. The retention times of the 2 principal peaks in the chromatogram obtained with test solution (b) are similar to those of the 2 principal peaks in the chromatogram obtained with the reference solution.
- C. It gives a yellow colour to a non-luminous flame.
- D. To 0.3 g add 20 ml of *ethanol R* and heat on a water-bath with shaking. Filter the mixture immediately, evaporate to dryness and take up the residue in 7 ml of *water R*. To 1 ml of the solution add 0.1 ml of a 1 g/l solution of *methylene blue R*, 2 ml of *dilute sulphuric acid R* and 2 ml of *methylene chloride R* and shake. A blue colour develops in the lower layer.

TESTS

Acid value (2.5.1). Not more than 2.0.

Iodine value (2.5.4). Not more than 3.0, determined on 2.00 g dissolved in 25 ml of *methylene chloride R*.

Saponification value (2.5.6). Not more than 2.0.

Water (2.5.12). Not more than 3.0 per cent, determined on 2.50 g by the semi-micro determination of water.

ASSAY

Cetostearyl alcohol. Examine by gas chromatography (2.2.28).

Internal standard solution. Dissolve 0.60 g of *heptadecanol CRS* in *ethanol R* and dilute to 150 ml with the same solvent.

Test solution (a). Dissolve 0.300 g of the substance to be examined in 50 ml of the internal standard solution, add 50 ml of *water R* and shake with 4 quantities, each of 25 ml, of *pentane R*, adding *sodium chloride R*, if necessary, to facilitate the separation of the layers. Combine the organic layers. Wash with 2 quantities, each of 30 ml, of *water R*, dry over *anhydrous sodium sulphate R* and filter.

Test solution (b). Dissolve 0.300 g of the substance to be examined in 50 ml of *ethanol R*, add 50 ml of *water R* and shake with 4 quantities, each of 25 ml, of *pentane R*, adding *sodium chloride R*, if necessary, to facilitate the separation of the layers. Combine the organic layers. Wash with 2 quantities, each of 30 ml, of *water R*, dry over *anhydrous sodium sulphate R* and filter.

Reference solution. Dissolve 50 mg of *cetyl alcohol CRS* and 50 mg of *stearyl alcohol CRS* in *ethanol R* and dilute to 10 ml with the same solvent.

The chromatographic procedure may be carried out using:

- a fused-silica column 25 m long and 0.25 mm in internal diameter coated with *poly(dimethyl)siloxane R*,
- *nitrogen for chromatography R* as the carrier gas at a flow rate of 1 ml/min,
- a flame-ionisation detector,
- a split ratio of 1:100,

with the following temperature programme:

	Time (min)	Temperature (°C)	Rate (°C/min)	Comment
Column	0 - 20	150 → 250	5	linear gradient
Injection port		250		
Detector		250		

The substances are eluted in the following order: cetyl alcohol, heptadecanol (internal standard) and stearyl alcohol.

Inject 1 µl of test solution (a) and 1 µl of test solution (b). If the chromatogram obtained with test solution (b) shows a peak with the same retention time as the peak corresponding to the internal standard in the chromatogram obtained with test solution (a), calculate the ratio:

$$r = \frac{S_{ci}}{S_i}$$

S_{ci} = area of the peak corresponding to cetyl alcohol in the chromatogram obtained with test solution (b),

S_i = area of the peak with the same retention time as the peak corresponding to the internal standard in the chromatogram obtained with test solution (a).

If r is less than 300, calculate the corrected area $S_{Ha(corr)}$ of the peak corresponding to the internal standard in the chromatogram obtained with test solution (a):

$$S_{Ha(corr)} = S'_{Ha} - \frac{S_i \times S_c}{S_{ci}}$$

S'_{Ha} = area of the peak corresponding to the internal standard in the chromatogram obtained with test solution (a),

S_c = area of the peak corresponding to cetyl alcohol in the chromatogram obtained with test solution (a).

Inject, under the same conditions, equal volumes of the reference solution and of test solution (a). Identify the peaks in the chromatogram obtained with test solution (a) by comparing their retention times with those of the peaks in the chromatogram obtained with the reference solution and determine the area of each peak.

Calculate the percentage content of cetyl alcohol using the expression:

$$S_A \frac{100 \times m_H}{S_{Ha(corr)} \times m}$$

- S_A = area of the peak corresponding to cetyl alcohol in the chromatogram obtained with test solution (a),
- m_H = mass of the internal standard in test solution (a), in milligrams,
- $S_{Ha(corr)}$ = corrected area of the peak corresponding to the internal standard in the chromatogram obtained with test solution (a),
- m = mass of the substance to be examined in test solution (a), in milligrams.

Calculate the percentage content of stearyl alcohol using the expression:

$$S_B \frac{100 \times m_H}{S_{Ha(corr)} \times m}$$

- S_B = area of the peak corresponding to stearyl alcohol in the chromatogram obtained with test solution (a).

The percentage content of cetostearyl alcohol corresponds to the sum of the percentage content of cetyl alcohol and of stearyl alcohol.

Sodium cetostearyl sulphate. Disperse 0.300 g in 25 ml of *methylene chloride R*. Add 50 ml of *water R* and 10 ml of *dimidium bromide-sulphan blue mixed solution R*. Titrate with 0.004 M *benzethonium chloride*, using sonication and heating and allowing the layers to separate before each addition, until the colour of the lower layer changes from pink to grey.

1 ml of 0.004 M *benzethonium chloride* is equivalent to 1.434 mg of sodium cetostearyl sulphate.

LABELLING

The label states, where applicable, the name and concentration of any added buffer.

01/2005:0802

CETOSTEARYL ALCOHOL (TYPE B), EMULSIFYING

Alcohol cetylicus et stearylicus emulsificans B

DEFINITION

Emulsifying cetostearyl alcohol (type B) is a mixture which contains not less than 80.0 per cent of cetostearyl alcohol and not less than 7.0 per cent of sodium laurilsulfate, both calculated with reference to the anhydrous substance. A suitable buffer may be added.

CHARACTERS

White or pale yellow, wax-like mass, plates, flakes or granules, soluble in hot water giving an opalescent solution, practically insoluble in cold water, slightly soluble in alcohol.

IDENTIFICATION

First identification: B, C, D.

Second identification: A, C.

- A. Examine by thin-layer chromatography (2.2.27), using a *TLC silanised silica gel plate R*.

Test solution (a). Dissolve 0.1 g of the substance to be examined in 10 ml of *trimethylpentane R*, heating on a water-bath. Shake with 2 ml of *alcohol (70 per*

cent V/V) R and allow to separate. Use the lower layer as test solution (b). Dilute 1 ml of the upper layer to 8 ml with *trimethylpentane R*.

Test solution (b). Use the lower layer obtained in the preparation of test solution (a).

Reference solution (a). Dissolve 40 mg of *cetostearyl alcohol R* in 10 ml of *trimethylpentane R*.

Reference solution (b). Dissolve 20 mg of *sodium laurilsulfate R* in 10 ml of *alcohol (70 per cent V/V) R*, heating on a water-bath.

Apply to the plate 2 µl of each solution. Develop over a path of 12 cm using a mixture of 20 volumes of *water R*, 40 volumes of *acetone R* and 40 volumes of *methanol R*. Allow the plate to dry in air and spray with a 50 g/l solution of *phosphomolybdic acid R* in *alcohol R*. Heat at 120 °C until spots appear (about 3 h). The 2 principal spots in the chromatogram obtained with test solution (a) are similar in position and colour to the principal spots in the chromatogram obtained with reference solution (a). 1 of the spots in the chromatogram obtained with test solution (b) is similar in position and colour to the principal spot in the chromatogram obtained with reference solution (b).

- B. Examine the chromatograms obtained in the assay. The retention times of the 2 principal peaks in the chromatogram obtained with test solution (b) are similar to those of the 2 principal peaks in the chromatogram obtained with the reference solution.
- C. It gives a yellow colour to a non-luminous flame.
- D. To 0.3 g add 20 ml of *ethanol R* and heat to boiling on a water-bath with shaking. Filter the mixture immediately, evaporate to dryness and take up the residue in 7 ml of *water R*. To 1 ml of the solution add 0.1 ml of a 1 g/l solution of *methylene blue R*, 2 ml of *dilute sulphuric acid R* and 2 ml of *methylene chloride R* and shake. A blue colour develops in the lower layer.

TESTS

Acid value (2.5.1). Not more than 2.0.

Iodine value (2.5.4). Not more than 3.0, determined on 2.00 g dissolved in 25 ml of *methylene chloride R*.

Saponification value (2.5.6). Not more than 2.0.

Water (2.5.12). Not more than 3.0 per cent, determined on 2.50 g by the semi-micro determination of water.

ASSAY

Cetostearyl alcohol. Examine by gas chromatography (2.2.28).

Internal standard solution. Dissolve 0.60 g of *heptadecanol CRS* in *ethanol R* and dilute to 150 ml with the same solvent.

Test solution (a). Dissolve 0.300 g of the substance to be examined in 50 ml of the internal standard solution, add 50 ml of *water R* and shake with 4 quantities, each of 25 ml, of *pentane R*, adding *sodium chloride R*, if necessary, to facilitate the separation of the layers. Combine the organic layers. Wash with 2 quantities, each of 30 ml, of *water R*, dry over *anhydrous sodium sulphate R* and filter.

Test solution (b). Dissolve 0.300 g of the substance to be examined in 50 ml of *ethanol R*, add 50 ml of *water R* and shake with 4 quantities, each of 25 ml, of *pentane R*, adding *sodium chloride R*, if necessary, to facilitate the separation of the layers. Combine the organic layers. Wash with 2 quantities, each of 30 ml, of *water R*, dry over *anhydrous sodium sulphate R* and filter.

Reference solution. Dissolve 50 mg of *cetyl alcohol CRS* and 50 mg of *stearyl alcohol CRS* in *ethanol R* and dilute to 10 ml with the same solvent.

The chromatographic procedure may be carried out using:

- a fused-silica column 25 m long and 0.25 mm in internal diameter coated with *poly(dimethyl)siloxane R*,
- *nitrogen for chromatography R* as the carrier gas at a flow rate of 1 ml/min,
- a flame-ionisation detector,
- a split ratio of 1:100,

with the following temperature programme:

	Time (min)	Temperature (°C)	Rate (°C/min)	Comment
Column	0 - 20	150 → 250	5	linear gradient
Injection port		250		
Detector		250		

The substances are eluted in the following order: cetyl alcohol, heptadecanol (internal standard) and stearyl alcohol.

Inject 1 µl of test solution (a) and 1 µl of test solution (b). If the chromatogram obtained with test solution (b) shows a peak with the same retention time as the peak corresponding to the internal standard in the chromatogram obtained with test solution (a), calculate the ratio:

$$r = \frac{S_{ci}}{S_i}$$

S_{ci} = area of the peak corresponding to cetyl alcohol in the chromatogram obtained with test solution (b),

S_i = area of the peak with the same retention time as the peak corresponding to the internal standard in the chromatogram obtained with test solution (a).

If r is less than 300, calculate the corrected area $S_{Ha(corr)}$ of the peak corresponding to the internal standard in the chromatogram obtained with test solution (a):

$$S_{Ha(corr)} = S'_{Ha} - \frac{S_i \times S_c}{S_{ci}}$$

S'_{Ha} = area of the peak corresponding to the internal standard in the chromatogram obtained with test solution (a),

S_c = area of the peak corresponding to cetyl alcohol in the chromatogram obtained with test solution (a).

Inject, under the same conditions, equal volumes of the reference solution and of test solution (a). Identify the peaks in the chromatogram obtained with test solution (a) by comparing their retention times with those of the peaks in the chromatogram obtained with the reference solution and determine the area of each peak.

Calculate the percentage content of cetyl alcohol using the expression:

$$S_A \frac{100 \times m_H}{S_{Ha(corr)} \times m}$$

S_A = area of the peak corresponding to cetyl alcohol in the chromatogram obtained with test solution (a),

m_H = mass of the internal standard in test solution (a), in milligrams,

$S_{Ha(corr)}$ = corrected area of the peak corresponding to the internal standard in the chromatogram obtained with test solution (a),

m = mass of the substance to be examined in test solution (a), in milligrams.

Calculate the percentage content of stearyl alcohol using the expression:

$$S_B \frac{100 \times m_H}{S_{Ha(corr)} \times m}$$

S_B = area of the peak corresponding to stearyl alcohol in the chromatogram obtained with test solution (a).

The percentage content of cetostearyl alcohol corresponds to the sum of the percentage content of cetyl alcohol and of stearyl alcohol.

Sodium laurilsulfate. Disperse 0.300 g in 25 ml of *methylene chloride R*. Add 50 ml of *water R* and 10 ml of *dimidium bromide-sulphan blue mixed solution R*. Titrate with 0.004 M *benzethonium chloride*, using sonication and heating, and allowing the layers to separate before each addition, until the colour of the lower layer changes from pink to grey.

1 ml of 0.004 M *benzethonium chloride* is equivalent to 1.154 mg of sodium laurilsulfate.

LABELLING

The label states, where applicable, the name and concentration of any added buffer.

01/2005:1085

CETOSTEARYL ISONONANOATE

Cetostearyl isononanoas

DEFINITION

Cetostearyl isononanoate is a mixture of esters of cetostearyl alcohol with isononanoic acid, mainly 3,5,5-trimethylhexanoic acid.

CHARACTERS

A clear, colourless or slightly yellowish liquid, practically insoluble in water, soluble in alcohol and in light petroleum, miscible with fatty oils and with liquid paraffins.

It has a viscosity of 15 mPas to 30 mPas, a relative density of 0.85 to 0.86 and a refractive index of 1.44 to 1.45.

IDENTIFICATION

- On cooling, turbidity occurs below 15 °C.
- It complies with the test for saponification value (see Tests).
- Examine by infrared absorption spectrophotometry (2.2.24), comparing with the *Ph. Eur. reference spectrum of cetostearyl isononanoate*.

TESTS

Appearance. The substance to be examined is clear (2.2.1) and not more intensely coloured than reference solution Y₆ (2.2.2, Method I).

Acid value (2.5.1). Not more than 1.0, determined on 5.0 g.

Hydroxyl value (2.5.3, Method A). Not more than 5.0.

Iodine value (2.5.4). Not more than 1.0.

Saponification value (2.5.6): 135 to 148, determined on 1.0 g.

Heavy metals (2.4.8). 2.0 g complies with limit test D for heavy metals (10 ppm). Prepare the standard using 2 ml of *lead standard solution* (10 ppm Pb) R.

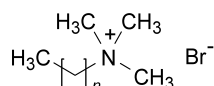
Water (2.5.12). Not more than 0.2 per cent, determined on 10.0 g by the semi-micro determination of water.

Total ash (2.4.16). Not more than 0.2 per cent, determined on 2.0 g.

01/2005:0378

CETRIMIDE

Cetrimidum



DEFINITION

Cetrimide consists of trimethyltetradecylammonium bromide and may contain smaller amounts of dodecyl- and hexadecyl-trimethylammonium bromides.

Content: 96.0 per cent to 101.0 per cent of alkyltrimethylammonium bromides, calculated as $\text{C}_{17}\text{H}_{38}\text{BrN}$ (M_r 336.4) (dried substance).

CHARACTERS

Appearance: white or almost white, voluminous, free-flowing powder.

Solubility: freely soluble in water and in alcohol.

IDENTIFICATION

- Dissolve 0.25 g in *alcohol* R and dilute to 25.0 ml with the same solvent. At wavelengths from 260 nm to 280 nm, the absorbance (2.2.25) of the solution has a maximum of 0.05.
- Dissolve about 5 mg in 5 ml of *buffer solution pH 8.0* R. Add about 10 mg of *potassium ferricyanide* R. A yellow precipitate is formed. Prepare a blank in the same manner but omitting the substance to be examined: a yellow solution is observed but no precipitate is formed.
- Solution S (see Tests) froths copiously when shaken.
- Thin-layer chromatography (2.2.27).

Test solution. Dissolve 0.10 g of the substance to be examined in *water* R and dilute to 5 ml with the same solvent.

Reference solution. Dissolve 0.10 g of *trimethyltetradecylammonium bromide CRS* in *water* R and dilute to 5 ml with the same solvent.

Plate: TLC silica gel F_{254} silanised plate R.

Mobile phase: *acetone* R, 270 g/l solution of *sodium acetate* R, *methanol* R (20:35:45 V/V/V).

Application: 1 µl.

Development: over a path of 12 cm.

Drying: in a current of hot air.

Detection: allow to cool; expose the plate to iodine vapour and examine in daylight.

Result: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

- It gives reaction (a) of bromides (2.3.1).

TESTS

Solution S. Dissolve 2.0 g in *carbon dioxide-free water* R and dilute to 100 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and colourless (2.2.2, *Method II*).

Acidity or alkalinity. To 50 ml of solution S add 0.1 ml of *bromocresol purple solution* R. Not more than 0.1 ml of 0.1 M *hydrochloric acid* or 0.1 M *sodium hydroxide* is required to change the colour of the indicator.

Amines and amine salts. Dissolve 5.0 g in 30 ml of a mixture of 1 volume of 1 M *hydrochloric acid* and 99 volumes of *methanol* R and add 100 ml of 2-propanol R. Pass a stream of *nitrogen* R slowly through the solution. Gradually add 15.0 ml of 0.1 M *tetrabutylammonium hydroxide* and record the potentiometric titration curve (2.2.20). If the curve shows 2 points of inflexion, the volume of titrant added between the 2 points is not greater than 2.0 ml.

Loss on drying (2.2.32): maximum 2.0 per cent, determined on 1.000 g by drying in an oven at 100-105 °C for 2 h.

Sulphated ash (2.4.14): maximum 0.5 per cent, determined on 1.0 g.

ASSAY

Dissolve 2.000 g in *water* R and dilute to 100.0 ml with the same solvent. Transfer 25.0 ml of the solution to a separating funnel, add 25 ml of *chloroform* R, 10 ml of 0.1 M *sodium hydroxide* and 10.0 ml of a freshly prepared 50 g/l solution of *potassium iodide* R. Shake, allow to separate and discard the chloroform layer. Shake the aqueous layer with 3 quantities, each of 10 ml, of *chloroform* R and discard the chloroform layers. Add 40 ml of *hydrochloric acid* R, allow to cool and titrate with 0.05 M *potassium iodate* until the deep brown colour is almost discharged. Add 2 ml of *chloroform* R and continue the titration, shaking vigorously, until the colour of the chloroform layer no longer changes. Carry out a blank titration on a mixture of 10.0 ml of the freshly prepared 50 g/l solution of *potassium iodide* R, 20 ml of *water* R and 40 ml of *hydrochloric acid* R.

1 ml of 0.05 M *potassium iodate* is equivalent to 33.64 mg of $\text{C}_{17}\text{H}_{38}\text{BrN}$.

01/2005:0540

CETYL ALCOHOL

Alcohol cetylicus

DEFINITION

Cetyl alcohol is a mixture of solid alcohols consisting mainly of hexadecanol ($\text{CH}_3(\text{CH}_2)_{14}\text{CH}_2\text{OH}$; M_r 242.4).

CHARACTERS

White, unctuous mass, powder, flakes or granules, practically insoluble in water, freely to sparingly soluble in alcohol; when melted, it is miscible with vegetable and animal oils, with liquid paraffin and with melted wool fat.

IDENTIFICATION

- Melting point (2.2.14): 46 °C to 52 °C.
- Hydroxyl value (2.5.3, *Method A*): 218 to 238.

TESTS

Appearance of solution. Dissolve 0.5 g in boiling *alcohol* R, allow to cool and dilute to 20 ml with the same solvent. The solution is clear (2.2.1) and not more intensely coloured than reference solution B₆ (2.2.2, *Method II*).

Acid value (2.5.1). Not more than 1.0.

Iodine value (2.5.4). Not more than 2.0, determined on 2.00 g dissolved in 25 ml of *chloroform R*.

Saponification value (2.5.6). Not more than 2.0.

01/2005:1906

CETYL PALMITATE

Cetyl palmitas

DEFINITION

Mixture of C₁₄-C₁₈ esters of lauric, myristic, palmitic and stearic acids ("Cetyl esters wax").

Content (expressed as hexadecyl hexadecanoate): 10.0 per cent to 20.0 per cent for Cetyl palmitate 15, 60.0 per cent to 70.0 per cent for Cetyl palmitate 65 and not less than 90.0 per cent for Cetyl palmitate 95.

CHARACTERS

Appearance: white, waxy plates, flakes or powder.

Solubility: practically insoluble in water, soluble in boiling ethanol and in methylene chloride, slightly soluble in light petroleum, practically insoluble in ethanol.

mp: about 45 °C for Cetyl palmitate 15 and Cetyl palmitate 65 and about 52 °C for Cetyl palmitate 95.

IDENTIFICATION

- It complies with the limits of the assay and the chromatogram obtained with the test solution shows the typical main peak(s).
- It complies with the test for saponification value (see Tests).

TESTS

Appearance of solution. The solution is not more intensely coloured than reference solution Y₆ (2.2.2, *Method II*).

Dissolve 4.0 g in *methylene chloride R* and dilute to 20 ml with the same solvent.

Acid value (2.5.1): maximum 4.0.

Dissolve 10.0 g in 50 ml of the solvent mixture described by heating under reflux on a water-bath for 5 min.

Hydroxyl value (2.5.3, *Method A*): maximum 20.0.

Iodine value (2.5.4): maximum 2.0.

Saponification value (2.5.6): 105 to 120.

Heat under reflux for 2 h.

Alkaline impurities. Dissolve with gentle heating 2.0 g in a mixture of 1.5 ml of *alcohol R* and 3 ml of *toluene R*. Add 0.05 ml of a 0.4 g/l solution of *bromophenol blue R* in *alcohol R*. Not more than 0.4 ml of 0.01 M *hydrochloric acid* is required to change the colour of the solution to yellow.

Nickel (2.4.27): maximum 1 ppm.

Water (2.5.12): maximum 0.3 per cent, determined on 1.0 g using a mixture of equal volumes of *anhydrous methanol R* and *methylene chloride R* as solvent.

Total ash (2.4.16): maximum 0.2 per cent, determined on 1.0 g.

ASSAY

Gas chromatography (2.2.28): use the normalisation procedure.

Test solution. Dissolve 20.0 mg of the substance to be examined in *hexane R* and dilute to 20.0 ml with the same solvent.

Reference solution (a). Dissolve 20.0 mg of *cetyl palmitate 95 CRS* in *hexane R* and dilute to 20.0 ml with the same solvent.

Reference solution (b). Dissolve 20.0 mg of *cetyl palmitate 15 CRS* in *hexane R* and dilute to 20.0 ml with the same solvent.

Column:

- **material:** stainless steel,
- **size:** *l* = 10 m, Ø = 0.53 mm,
- **stationary phase:** *poly(dimethyl)siloxane R* (film thickness: 2.65 µm).

Carrier gas: *helium for chromatography R*.

Flow rate: 6.5 ml/min.

Split ratio: 1:10.

Temperature:

	Time (min)	Temperature (°C)
Column	0 - 10	100 → 300
	10 - 15	300
Injection port		350
Detector		350

Detection: flame ionisation.

Injection: 1 µl.

Relative retention with reference to cetyl palmitate (retention time = about 9 min): cetyl alcohol = about 0.3; palmitic acid = about 0.4; lauric ester = about 0.8; myristic ester = about 0.9; stearic ester = about 1.1.

System suitability: reference solution (b):

- **resolution:** minimum of 1.5 between the peaks due to cetyl palmitate and cetyl stearate.

STORAGE

At a temperature not exceeding 25 °C.

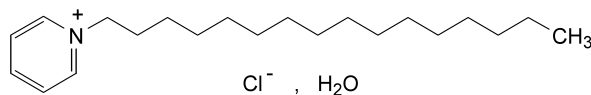
LABELLING

The label states the type of cetyl palmitate.

01/2005:0379

CETYL PYRIDINIUM CHLORIDE

Cetylpyridinii chloridum

C₂₁H₃₈ClN, H₂OM_r 358.0

DEFINITION

Cetylpyridinium chloride contains not less than 96.0 per cent and not more than the equivalent of 101.0 per cent of 1-hexadecylpyridinium chloride, calculated with reference to the anhydrous substance.

CHARACTERS

A white powder, slightly soapy to the touch, soluble in water and in alcohol. An aqueous solution froths copiously when shaken.

IDENTIFICATION

First identification: B, D.

Second identification: A, C, D.

A. Dissolve 0.10 g in *water R* and dilute to 100.0 ml with the same solvent. Dilute 5.0 ml of this solution to 100.0 ml with *water R*. Examined between 240 nm and 300 nm (2.2.25), the solution shows an absorption maximum at 259 nm and 2 shoulders at about 254 nm and at about 265 nm. The specific absorbance at the maximum is 126 to 134, calculated with reference to the anhydrous substance.

B. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *cetylpyridinium chloride CRS*. Examine the substances in the solid state.

C. To 5 ml of *dilute sodium hydroxide solution R* add 0.1 ml of *bromophenol blue solution R1* and 5 ml of *chloroform R* and shake. The chloroform layer is colourless. Add 0.1 ml of solution S (see Tests) and shake. The chloroform layer becomes blue.

D. Solution S gives reaction (a) of chlorides (2.3.1).

TESTS

Solution S. Dissolve 1.0 g in *carbon dioxide-free water R* and dilute to 100 ml with the same solvent.

Appearance of solution. Solution S is not more opalescent than reference suspension II (2.2.1) and is colourless (2.2.2, *Method II*).

Acidity. To 50 ml of solution S add 0.1 ml of *phenolphthalein solution R*. Not more than 2.5 ml of 0.02 M *sodium hydroxide* is required to change the colour of the indicator.

Amines and amine salts. Dissolve 5.0 g with heating in 20 ml of a mixture of 3 volumes of 1 M *hydrochloric acid* and 97 volumes of *methanol R* and add 100 ml of 2-propanol *R*. Pass a stream of *nitrogen R* slowly through the solution. Gradually add 12.0 ml of 0.1 M *tetrabutylammonium hydroxide* and record the potentiometric titration curve (2.2.20). If the curve shows 2 points of inflexion, the volume of titrant added between the two points is not greater than 5.0 ml. If the curve shows no point of inflexion, the substance to be examined does not comply with the test. If the curve shows one point of inflexion, repeat the test but add 3.0 ml of a 25.0 g/l solution of *dimethyldodecylamine R* in 2-propanol *R* before the titration. If the titration curve after the addition of 12.0 ml of the titrant shows only one point of inflexion, the substance to be examined does not comply with the test.

Water (2.5.12): 4.5 per cent to 5.5 per cent, determined on 0.300 g by the semi-micro determination of water.

Sulphated ash (2.4.14). Not more than 0.2 per cent, determined on 1.0 g.

ASSAY

Dissolve 2.00 g in *water R* and dilute to 100.0 ml with the same solvent. Transfer 25.0 ml of the solution to a separating funnel, add 25 ml of *chloroform R*, 10 ml of 0.1 M *sodium hydroxide* and 10.0 ml of a freshly prepared 50 g/l solution of *potassium iodide R*. Shake well, allow to separate and discard the chloroform layer. Shake the aqueous layer with three quantities, each of 10 ml, of *chloroform R* and discard the chloroform layers. To the aqueous layer add 40 ml of *hydrochloric acid R*, allow to cool and titrate with 0.05 M *potassium iodate* until the deep-brown colour is almost discharged. Add 2 ml of *chloroform R* and continue the titration, shaking vigorously, until the chloroform layer no longer changes colour. Carry out a blank titration on a mixture of 10.0 ml of the freshly prepared 50 g/l solution of *potassium iodide R*, 20 ml of *water R* and 40 ml of *hydrochloric acid R*.

1 ml of 0.05 M *potassium iodate* is equivalent to 34.0 mg of $C_{21}H_{38}ClN$.

01/2005:0380

CHAMOMILE FLOWER, ROMAN

Chamomillae romanae flos

DEFINITION

Roman chamomile flower consists of the dried flower-heads of the cultivated double variety of *Chamaemelum nobile* (L.) All. (*Anthemis nobilis* L.). It contains not less than 7 ml/kg of essential oil.

CHARACTERS

It consists of flower-heads with a white to yellowish-grey colour, being composed of solitary hemispherical capitula, made up of a solid conical receptacle bearing the florets, each subtended by a transparent small palea.

It has a strong and characteristic odour.

It has the macroscopic and microscopic characters described under identification tests A and B.

IDENTIFICATION

- The capitula have a diameter of 8 mm to 20 mm; the receptacle is solid; the base of the receptacle is surrounded by an involucre consisting of 2 or 3 rows of compact and imbricated bracts with scarious margins. Most florets are ligulate, but a few pale yellow tubular florets occur in the central region. Ligulate florets are white, dull, lanceolate and reflexed with a dark brown, inferior ovary, a filiform style and a bifid stigma; tubular florets have a five-toothed corolla tube, 5 syngenesious, epipetalous stamens and a gynoeceum similar to that of the ligulate florets.
- Separate the capitulum into its different parts. Examine under a microscope using *chloral hydrate solution R*. All parts of the flower-heads are covered with numerous small yellow glistening glandular trichomes. The involucre bracts and paleae have epidermal cells in longitudinal rows, sclerified at the base and they are covered with conical trichomes, about 500 µm long, each composed of 3 or 4 very short base cells and a long, bent, terminal cell about 20 µm wide. The corolla of the ligulate flowers consists of papillary cells with cuticular striations. The ovaries of both kinds of florets have at their base a sclerous ring consisting of a single row of cells. The receptacle and the ovaries contain small clusters of calcium oxalate. The pollen grains have a diameter of about 35 µm and are rounded and triangular with 3 germinal pores and a spiny exine.
- Examine by thin-layer chromatography (2.2.27), using a suitable silica gel as the coating substance.
Test solution. To 0.5 g of the powdered drug (710) add 10 ml of *methanol R* and heat with shaking in a water-bath at 60 °C for 5 min. Allow to cool and filter.
Reference solution. Dissolve 2.5 mg of *apigenin R* and 2.5 mg of *apigenin-7-glucoside R* in 10 ml of *methanol R*. Apply to the plate as bands 10 µl of each solution. Develop over a path of 10 cm using a mixture of 17 volumes of *glacial acetic acid R*, 17 volumes of *water R* and 66 volumes of *butanol R*. Dry the plate at 100-105 °C for 5 min and spray the warm plate with a 10 g/l solution of *diphenylboric acid aminoethyl ester R* in *methanol R*, using about 10 ml for a plate 200 mm square. Spray the plate with the same volume of a 50 g/l solution of *macrogol 400 R* in *methanol R*. Allow to stand for about

30 min and examine in ultraviolet light at 365 nm. The chromatogram obtained with the reference solution shows in the upper third a zone of yellowish-green fluorescence (apigenin) and in the middle third a zone of yellowish fluorescence (apigenin-7-glucoside). The chromatogram obtained with the test solution shows a zone of yellowish-green fluorescence corresponding in position and fluorescence to the zone due to apigenin and a zone of yellowish fluorescence corresponding in position and fluorescence to the zone due to apigenin-7-glucoside in the chromatogram obtained with the reference solution; above the apigenin-7-glucoside zone there is a zone of brownish fluorescence (luteolin); immediately below the apigenin-7-glucoside zone there is a zone of light brownish fluorescence (apiin); immediately below the apiin zone there is a zone of bright blue fluorescence and below this zone a zone of bright blue fluorescence; other faint zones may be present.

TESTS

Diameter of the flower-heads. Not more than 3 per cent of flower-heads have a diameter smaller than 8 mm.

Deteriorated flower-heads. Brown or darkened flower-heads are absent.

Loss on drying (2.2.32): maximum 11.0 per cent, determined on 1.000 g of the powdered drug (355) by drying in an oven at 100-105 °C for 2 h.

Total ash (2.4.16). Not more than 8.0 per cent.

ASSAY

Carry out the determination of essential oils in vegetable drugs (2.8.12). Use 20.0 g of whole drug, a 500 ml round-bottomed flask, 250 ml of *water R* as the distillation liquid and 0.50 ml of *xylene R* in the graduated tube. Distil at a rate of 3-3.5 ml/min for 3 h.

STORAGE

Store protected from light.

01/2005:0313

CHARCOAL, ACTIVATED

Carbo activatus

DEFINITION

Activated charcoal is obtained from vegetable matter by suitable carbonisation processes intended to confer a high adsorption power.

CHARACTERS

A black, light powder free from grittiness, practically insoluble in all usual solvents.

IDENTIFICATION

- A. When heated to redness it burns slowly without a flame.
- B. It complies with the test for adsorption power (see Tests).

TESTS

Solution S. To 2.0 g in a conical flask with a ground-glass neck add 50 ml of *dilute hydrochloric acid R*. Boil gently under a reflux condenser for 1 h, filter and wash the filter with *dilute hydrochloric acid R*. Evaporate the combined filtrate and washings to dryness on a water-bath, dissolve the residue in 0.1 M *hydrochloric acid* and dilute to 50.0 ml with the same acid.

Acidity or alkalinity. To 2.0 g add 40 ml of *water R* and boil for 5 min. Cool, restore to the original mass with *carbon dioxide-free water R* and filter. Reject the first 20 ml of the filtrate. To 10 ml of the filtrate add 0.25 ml of *bromothymol blue solution R1* and 0.25 ml of 0.02 M *sodium hydroxide*. The solution is blue. Not more than 0.75 ml of 0.02 M *hydrochloric acid* is required to change the colour of the indicator to yellow.

Acid-soluble substances. To 1.0 g add 25 ml of *dilute nitric acid R* and boil for 5 min. Filter whilst hot through a sintered-glass filter (10) and wash with 10 ml of hot *water R*. Evaporate the combined filtrate and washings to dryness on a water-bath, add to the residue 1 ml of *hydrochloric acid R*, evaporate to dryness again and dry the residue to constant mass at 100 °C to 105 °C. The residue weighs not more than 30 mg (3 per cent).

Alkali-soluble coloured substances. To 0.25 g add 10 ml of *dilute sodium hydroxide solution R* and boil for 1 min. Cool, filter and dilute the filtrate to 10 ml with *water R*. The solution is not more intensely coloured than reference solution GY₄ (2.2.2, *Method II*).

Alcohol-soluble substances. To 2.0 g add 50 ml of *alcohol R* and boil under a reflux condenser for 10 min. Filter immediately, cool, and dilute to 50 ml with *alcohol R*. The filtrate is not more intensely coloured than reference solution Y₆ or BY₆ (2.2.2, *Method II*). Evaporate 40 ml of the filtrate to dryness and dry to constant mass at 100 °C to 105 °C. The residue weighs not more than 8 mg (0.5 per cent).

Fluorescent substances. In an intermittent-extraction apparatus, treat 10.0 g with 100 ml of *cyclohexane R1* for 2 h. Collect the liquid and dilute to 100 ml with *cyclohexane R1*. Examine in ultraviolet light at 365 nm. The fluorescence of the solution is not more intense than that of a solution of 83 µg of *quinine R* in 1000 ml of 0.005 M *sulphuric acid* examined in the same manner.

Sulphides. To 1.0 g in a conical flask add 5 ml of *hydrochloric acid R1* and 20 ml of *water R*. Heat to boiling. The fumes released do not turn *lead acetate paper R* brown.

Copper. Not more than 25 ppm of Cu, determined by atomic absorption spectrometry (2.2.23, *Method I*).

Test solution. Use solution S.

Reference solutions. Prepare the reference solutions using *copper standard solution (0.1 per cent Cu) R* and diluting with 0.1 M *hydrochloric acid*.

Measure the absorbance at 325.0 nm using a copper hollow-cathode lamp as source of radiation and an air-acetylene flame.

Lead. Not more than 10 ppm of Pb, determined by atomic absorption spectrometry (2.2.23, *Method I*).

Test solution. Use solution S.

Reference solutions. Prepare the reference solutions using *lead standard solution (100 ppm Pb) R* and diluting with 0.1 M *hydrochloric acid*.

Measure the absorbance at 283.3 nm using a lead hollow-cathode lamp as source of radiation and an air-acetylene flame. Depending on the apparatus the line at 217.0 nm may be used.

Zinc. Not more than 25 ppm of Zn, determined by atomic absorption spectrometry (2.2.23, *Method I*).

Test solution. Use solution S.

Reference solutions. Prepare the reference solutions using *zinc standard solution (100 ppm Zn) R* and diluting with 0.1 M *hydrochloric acid*.

Measure the absorbance at 214.0 nm using a zinc hollow-cathode lamp as source of radiation and an air-acetylene flame.

Loss on drying (2.2.32). Not more than 15 per cent, determined on 1.00 g by drying in an oven at 120 °C for 4 h.

Sulphated ash (2.4.14). Not more than 5.0 per cent, determined on 1.0 g.

Adsorption power. To 0.300 g in a 100 ml ground-glass-stoppered conical flask add 25.0 ml of a freshly prepared solution of 0.5 g of *phenazone R* in 50 ml of *water R*. Shake thoroughly for 15 min. Filter and reject the first 5 ml of filtrate. To 10.0 ml of the filtrate add 1.0 g of *potassium bromide R* and 20 ml of *dilute hydrochloric acid R*. Using 0.1 ml of *methyl red solution R* as indicator, titrate with 0.0167 M *potassium bromate* until the red colour is discharged. Titrate slowly (1 drop every 15 s) towards the end of the titration. Carry out a blank titration using 10.0 ml of the phenazone solution.

Calculate the quantity of phenazone adsorbed per 100 g of activated charcoal from the expression:

$$\frac{2.353(a - b)}{m}$$

a = number of millilitres of 0.0167 M *potassium bromate* used for the blank,

b = number of millilitres of 0.0167 M *potassium bromate* used for the test,

m = mass in grams of the substance to be examined.

Not less than 40 g of phenazone is adsorbed per 100 g of activated charcoal, calculated with reference to the dried substance.

Microbial contamination. Total viable aerobic count (2.6.12) not more than 10³ micro-organisms per gram, determined by plate count.

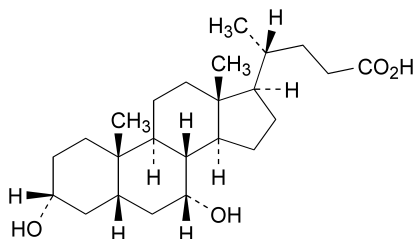
STORAGE

Store in an airtight container.

01/2005:1189

CHENODEOXYCHOLIC ACID

Acidum chenodeoxycholicum



C₂₄H₄₀O₄

*M*_r 392.6

DEFINITION

Chenodeoxycholic acid contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of 3α,7α-dihydroxy-5β-cholan-24-oic acid, calculated with reference to the dried substance.

CHARACTERS

A white or almost white powder, very slightly soluble in water, freely soluble in alcohol, soluble in acetone, slightly soluble in methylene chloride.

IDENTIFICATION

First identification: A.

Second identification: B, C.

- Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *chenodeoxycholic acid CRS*. Examine the substances prepared as discs using *potassium bromide R*.
- Examine the chromatograms obtained in the test for related substances. The principal spot in the chromatogram obtained with test solution (b) is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).
- Dissolve about 10 mg in 1 ml of *sulphuric acid R*. Add 0.1 ml of *formaldehyde solution R* and allow to stand for 5 min. Add 5 ml of *water R*. The suspension obtained is greenish-blue.

TESTS

Specific optical rotation (2.2.7). Dissolve 0.500 g in *methanol R* and dilute to 25.0 ml with the same solvent. The specific optical rotation is + 11.0 to + 13.0, calculated with reference to the dried substance.

Related substances. Examine by thin-layer chromatography (2.2.27), using a suitable silica gel as the coating substance.

Test solution (a). Dissolve 0.40 g of the substance to be examined in a mixture of 1 volume of *water R* and 9 volumes of *acetone R* and dilute to 10 ml with the same mixture of solvents.

Test solution (b). Dilute 1 ml of test solution (a) to 10 ml with a mixture of 1 volume of *water R* and 9 volumes of *acetone R*.

Reference solution (a). Dissolve 40 mg of *chenodeoxycholic acid CRS* in a mixture of 1 volume of *water R* and 9 volumes of *acetone R* and dilute to 10 ml with the same mixture of solvents.

Reference solution (b). Dissolve 20 mg of *lithocholic acid CRS* in a mixture of 1 volume of *water R* and 9 volumes of *acetone R* and dilute to 10 ml with the same mixture of solvents. Dilute 2 ml of the solution to 100 ml with a mixture of 1 volume of *water R* and 9 volumes of *acetone R*.

Reference solution (c). Dissolve 20 mg of *ursodeoxycholic acid CRS* in a mixture of 1 volume of *water R* and 9 volumes of *acetone R* and dilute to 50 ml with the same mixture of solvents.

Reference solution (d). Dissolve 20 mg of *cholic acid CRS* in a mixture of 1 volume of *water R* and 9 volumes of *acetone R* and dilute to 100 ml with the same mixture of solvents.

Reference solution (e). Dilute 0.5 ml of test solution (a) to 20 ml with a mixture of 1 volume of *water R* and 9 volumes of *acetone R*. Dilute 1 ml of the solution to 10 ml with a mixture of 1 volume of *water R* and 9 volumes of *acetone R*.

Reference solution (f). Dissolve 10 mg of *chenodeoxycholic acid CRS* in reference solution (c) and dilute to 25 ml with the same solution.

Apply separately to the plate 5 µl of each solution. Develop in an unsaturated tank over a path of 15 cm using a mixture of 1 volume of *glacial acetic acid R*, 30 volumes of *acetone R* and 60 volumes of *methylene chloride R*. Dry the plate at 120 °C for 10 min. Spray the plate immediately with a 47.6 g/l solution of *phosphomolybdic acid R* in a mixture of 1 volume of *sulphuric acid R* and 20 volumes of *glacial acetic acid R* and heat again at 120 °C until blue spots appear on a lighter background. In the chromatogram obtained with test solution (a): any spot corresponding to lithocholic acid is not more intense than the principal spot in the chromatogram obtained with reference solution (b).

(0.1 per cent); any spot corresponding to ursodeoxycholic acid is not more intense than the principal spot in the chromatogram obtained with reference solution (c) (1 per cent); any spot corresponding to cholic acid is not more intense than the principal spot in the chromatogram obtained with reference solution (d) (0.5 per cent); any spot apart from the principal spot and any spots corresponding to lithocholic acid, ursodeoxycholic acid and cholic acid, is not more intense than the principal spot in the chromatogram obtained with reference solution (e) (0.25 per cent). The test is not valid unless the chromatogram obtained with reference solution (f) shows two clearly separated principal spots.

Heavy metals (2.4.8). 1.0 g complies with limit test C for heavy metals (20 ppm). Prepare the standard using 2 ml of lead standard solution (10 ppm Pb) R.

Loss on drying (2.2.32). Not more than 1.5 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C.

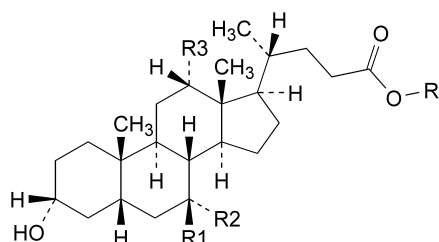
Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.350 g in 50 ml of *alcohol* R, previously neutralised to 0.2 ml of *phenolphthalein solution* R. Add 50 ml of *water* R and titrate with 0.1 M *sodium hydroxide* until a pink colour is obtained.

1 ml of 0.1 M *sodium hydroxide* is equivalent to 39.26 mg of C₂₄H₄₀O₄.

IMPURITIES



- A. R = H, R₁ = OH, R₂ = H, R₃ = H: ursodeoxycholic acid,
- B. R = H, R₁ = H, R₂ = OH, R₃ = OH: 3 α ,7 α ,12 α -trihydroxy-5 β -cholan-24-oic acid (cholic acid),
- C. R = H, R₁ = H, R₂ = H, R₃ = H: 3 α -hydroxy-5 β -cholan-24-oic acid (lithocholic acid),
- D. R = H, R₁ = OH, R₂ = H, R₃ = OH: 3 α ,7 β ,12 α -trihydroxy-5 β -cholan-24-oic acid (ursocholic acid),
- E. R = H, R₁ = H, R₂ = H, R₃ = OH: 3 α ,12 α -dihydroxy-5 β -cholan-24-oic acid (deoxycholic acid),
- F. R = H, R₁+R₂ = O, R₃ = H: 3 α -hydroxy-7-oxo-5 β -cholan-24-oic acid,
- G. R = CH₃, R₁ = OH, R₂ = H, R₃ = H: methyl 3 α ,7 β -dihydroxy-5 β -cholan-24-oate.

01/2005:1774

CHITOSAN HYDROCHLORIDE

Chitosani hydrochloridum

DEFINITION

Chitosan hydrochloride is the chloride salt of an unbranched binary heteropolysaccharide consisting of the two units *N*-acetyl-D-glucosamine and D-glucosamine, obtained by

partial deacetylation of chitin normally leading to a degree of deacetylation of 70.0 per cent to 95.0 per cent. Chitin is extracted from the shells of shrimp and crab.

PRODUCTION

The animals from which chitosan hydrochloride is derived must fulfil the requirements for the health of animals suitable for human consumption to the satisfaction of the competent authority. It must have been shown to what extent the method of production allows inactivation or removal of any contamination by viruses or other infectious agents.

CHARACTERS

Appearance: white or almost white fine powder.

Solubility: sparingly soluble in water, practically insoluble in ethanol.

IDENTIFICATION

A. Infrared absorption spectrophotometry (2.2.24).

Preparation: discs.

Comparison: chitosan hydrochloride CRS.

B. It gives reaction (a) of chlorides (2.3.1).

C. Dilute 50 ml of solution S (see Tests) to 250 ml with a 25 per cent V/V solution of *ammonia* R. A voluminous gelatinous mass is formed.

D. To 10 ml of solution S add 90 ml of *acetone* R. A voluminous gelatinous mass is formed.

TESTS

Solution S. Dissolve 1.0 g in 100 ml of *water* R and stir vigorously for 20 min with a mechanical stirrer.

Appearance of solution. Solution S is not more opalescent than reference suspension II (2.2.1) and not more intensely coloured than reference solution BY₅ (2.2.2, *Method II*).

Matter insoluble in water: maximum 0.5 per cent.

Add 2.00 g to 400.0 ml of *water* R while stirring until no further dissolution takes place. Transfer the solution to a 2 litre beaker, add 200 ml of *water* R. Boil the solution gently for 2 h, covering the beaker during the operation. Filter through a sintered-glass filter (40) wash the residue with water and dry to constant weight in an oven at 100-105 °C. The residue weighs a maximum of 10 mg.

pH (2.2.3): 4.0 to 6.0 for solution S.

Viscosity (2.2.10): 80 per cent to 120 per cent of the value stated on the label, determined on solution S.

Determine the viscosity using a rotating viscometer at 20 °C with a spindle rotating at 20 r/min, using a suitable spindle for the range of the expected viscosity.

Degree of deacetylation

Test solution. Dissolve 0.250 g in *water* R and dilute to 50.0 ml with the same solvent stirring vigorously. Dilute 1.0 ml of the solution to 100.0 ml with *water* R. Measure the absorbance (2.2.25) from 200 nm to 205 nm as the first derivative of the absorbance curve. Determine the pH of the solution.

Reference solutions. Prepare solutions of 1.0 µg/ml, 5.0 µg/ml, 15.0 µg/ml and 35.0 µg/ml of *N*-acetylglucosamine R in *water* R. Measure the absorbance (2.2.25) from 200 nm to 205 nm of each solution as first derivative of the absorption curve. Make a standard curve by plotting the first derivative at 202 nm as a function of the concentration of *N*-acetylglucosamine, and calculate the slope of the curve by least squares linear regression. Use the standard curve to determine the equivalent amount of *N*-acetylglucosamine for the substance to be examined.

Calculate the degree of deacetylation (molar) using the expression:

$$\frac{100 \times M_1 \times (C_1 - C_2)}{(M_1 \times C_1) - [(M_1 - M_3) \times C_2]}$$

- C_1 = concentration of chitosan hydrochloride in the test solution in micrograms per millilitre,
 C_2 = concentration of *N*-acetylglucosamine in the test solution, as determined from the standard curve prepared using the reference solution in micrograms per millilitre,
 M_1 = 203 (relative molecular mass of *N*-acetylglucosamine unit ($C_8H_{13}NO_5$) in polymer),
 M_3 = relative molecular mass of chitosan hydrochloride.

M_3 is calculated from the pH in solution, assuming a pK_a value of 6.8, using the following expressions:

$$M_3 = f \times M_2 + (1 - f) \times (M_2 + 36.5)$$

$$f = \frac{p}{1 + p}$$

$$p = 10^{(pH - pK_a)}$$

- M_2 = 161 (relative molecular mass of deacetylated unit (glucosamine) ($C_6H_{11}NO_4$) in polymer).

Chlorides: 10.0 per cent to 20.0 per cent.

Introduce 0.200 g into a 250 ml borosilicate flask fitted with a reflux condenser. Add 40 ml of a mixture of 1 volume of *nitric acid R* and 2 volumes of *water R*. Boil gently under a reflux condenser for 5 min. Cool and add 25 ml of *water R* through the condenser. Add 16.0 ml of 0.1 M *silver nitrate*, shake vigorously and titrate with 0.1 M *ammonium thiocyanate*, using 1 ml of *ferric ammonium sulphate solution R2* as indicator, and shaking vigorously towards the end-point. Carry out a blank titration.

1 ml of 0.1 M *silver nitrate* is equivalent to 3.55 mg of Cl.

Heavy metals (2.4.8): maximum 40 ppm.

1.0 g complies with limit test F. Prepare the standard using 4 ml of *lead standard solution* (10 ppm Pb) R.

Loss on drying (2.2.32): maximum 10 per cent, determined on 1.000 g by drying in an oven at 100–105 °C.

Sulphated ash (2.4.14): maximum 1.0 per cent, determined on 1.0 g.

STORAGE

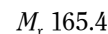
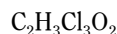
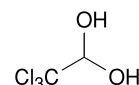
At a temperature of 2–8 °C, protected from moisture and light.

LABELLING

The label states the nominal viscosity in millipascal seconds for a 10 g/l solution in *water R*.

CHLORAL HYDRATE

Chlorali hydras



DEFINITION

Chloral hydrate contains not less than 98.5 per cent and not more than the equivalent of 101.0 per cent of 2,2,2-trichloroethane-1,1-diol.

CHARACTERS

Colourless, transparent crystals, very soluble in water, freely soluble in alcohol.

IDENTIFICATION

- A. To 10 ml of solution S (see Tests) add 2 ml of *dilute sodium hydroxide solution R*. The mixture becomes cloudy and, when heated, gives off an odour of chloroform.
 B. To 1 ml of solution S add 2 ml of *sodium sulphide solution R*. A yellow colour develops which quickly becomes reddish-brown. On standing for a short time, a red precipitate may be formed.

TESTS

Solution S. Dissolve 3.0 g in *carbon dioxide-free water R* and dilute to 30 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and colourless (2.2.2, *Method II*).

pH (2.2.3). The pH of solution S is 3.5 to 5.5.

Chloral alcoholate. Warm 1.0 g with 10 ml of *dilute sodium hydroxide solution R*, filter the supernatant solution and add 0.05 M *iodine* dropwise until a yellow colour is obtained. Allow to stand for 1 h. No precipitate is formed.

Chlorides (2.4.4). 5 ml of solution S diluted to 15 ml with *water R* complies with the limit test for chlorides (100 ppm).

Heavy metals (2.4.8). 10 ml of solution S diluted to 20 ml with *water R* complies with limit test A for heavy metals (20 ppm). Prepare the standard using *lead standard solution* (1 ppm Pb) R.

Non-volatile residue. Evaporate 2.000 g on a water-bath. The residue weighs not more than 2 mg (0.1 per cent).

ASSAY

Dissolve 4.000 g in 10 ml of *water R* and add 40.0 ml of 1 M *sodium hydroxide*. Allow to stand for exactly 2 min and titrate with 0.5 M *sulphuric acid*, using 0.1 ml of *phenolphthalein solution R* as indicator. Titrate the neutralised solution with 0.1 M *silver nitrate*, using 0.2 ml of *potassium chromate solution R* as indicator. Calculate the number of millilitres of 1 M *sodium hydroxide* used by deducting from the volume of 1 M *sodium hydroxide*, added at the beginning of the titration, the volume of 0.5 M *sulphuric acid* used in the first titration and two-fifteenths of the volume of 0.1 M *silver nitrate* used in the second titration.

1 ml of 1 M *sodium hydroxide* is equivalent to 0.1654 g of $C_2H_3Cl_3O_2$.

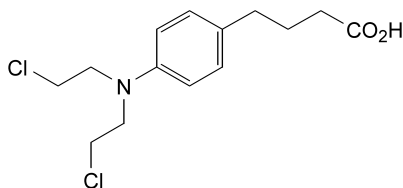
STORAGE

Store in an airtight container.

01/2005:0137

CHLORAMBUCIL

Chlorambucilum

 $C_{14}H_{19}Cl_2NO_2$ M_r 304.2**DEFINITION**

Chlorambucil contains not less than 98.5 per cent and not more than the equivalent of 101.0 per cent of 4-4-[di(2-chloroethyl)amino]phenylbutyric acid, calculated with reference to the anhydrous substance.

CHARACTERS

A white, crystalline powder, practically insoluble in water, freely soluble in acetone and in alcohol.

IDENTIFICATION

First identification: A, B.

Second identification: A, C, D.

- A. Melting point (2.2.14): 64 °C to 67 °C.
- B. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *chlorambucil CRS*.
- C. To 0.4 g add 10 ml of *dilute hydrochloric acid R*, mix and allow to stand for 30 min, shaking from time to time. Filter and wash the precipitate with 2 quantities, each of 10 ml, of *water R*. To 10 ml of the combined filtrate and washings add 0.5 ml of *potassium tetraiodomercurate solution R*. A pale-brown precipitate is formed. To another 10 ml of the combined filtrate and washings add 0.2 ml of *potassium permanganate solution R*. The colour of the latter is discharged immediately.
- D. Dissolve 50 mg in 5 ml of *acetone R* and dilute to 10 ml with *water R*. Add 0.05 ml of *dilute nitric acid R* and 0.2 ml of *silver nitrate solution R2*. No opalescence is produced immediately. Heat the solution on a water-bath; an opalescence develops.

TESTS

Related substances. Examine by thin-layer chromatography (2.2.27), using *silica gel GF₂₅₄ R* as the coating substance. Carry out all operations as rapidly as possible protected from light. Prepare the solutions immediately before use.

Test solution. Dissolve 0.2 g of the substance to be examined in *acetone R* and dilute to 10 ml with the same solvent.

Reference solution (a). Dilute 1 ml of the test solution to 50 ml with *acetone R*.

Reference solution (b). Dilute 25 ml of reference solution (a) to 100 ml with *acetone R*.

Apply separately to the plate 5 µl of each solution. Develop over a path of 10 cm using a mixture of 20 volumes of *methyl ethyl ketone R*, 20 volumes of *heptane R*, 25 volumes of *methanol R* and 40 volumes of *toluene R*. Examine in ultraviolet light at 254 nm. Any spot in the chromatogram obtained with the test solution, apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (a) (2.0 per cent) and

at most 1 such spot is more intense than the spot in the chromatogram obtained with reference solution (b) (0.5 per cent).

Water (2.5.12). Not more than 0.5 per cent, determined on 1.000 g by the semi-micro determination of water.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.200 g in 10 ml of *acetone R* and add 10 ml of *water R*. Titrate with 0.1 M *sodium hydroxide*, using 0.1 ml of *phenolphthalein solution R* as indicator.

1 ml of 0.1 M *sodium hydroxide* is equivalent to 30.42 mg of $C_{14}H_{19}Cl_2NO_2$.

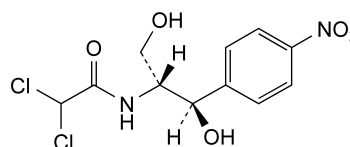
STORAGE

Store protected from light.

01/2005:0071

CHLORAMPHENICOL

Chloramphenicolum

 $C_{11}H_{12}Cl_2N_2O_5$ M_r 323.1**DEFINITION**

Chloramphenicol is 2,2-dichloro-*N*-(1*R*,2*R*)-2-hydroxy-1-(hydroxymethyl)-2-(4-nitrophenyl)ethyl]acetamide, produced by the growth of certain strains of *Streptomyces venezuelae* in a suitable medium. It is normally prepared by synthesis. It contains not less than 98.0 per cent and not more than the equivalent of 102.0 per cent of $C_{11}H_{12}Cl_2N_2O_5$, calculated with reference to the dried substance.

CHARACTERS

A white, greyish-white or yellowish-white, fine, crystalline powder or fine crystals, needles or elongated plates, slightly soluble in water, freely soluble in alcohol and in propylene glycol.

A solution in ethanol is dextrorotatory and a solution in ethyl acetate is laevorotatory.

IDENTIFICATION

First identification: A, B.

Second identification: A, C, D, E.

A. Melting point (2.2.14): 149 °C to 153 °C.

B. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *chloramphenicol CRS*.

C. Examine the chromatograms obtained in the test for related substances. The principal spot in the chromatogram obtained with 1 µl of the test solution is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a).

D. Dissolve about 10 mg in 1 ml of *alcohol (50 per cent V/V) R*, add 3 ml of a 10 g/l solution of *calcium chloride R* and 50 mg of *zinc powder R* and heat on a water-bath for 10 min. Filter the hot solution and allow to cool. Add 0.1 ml of *benzoyl chloride R* and shake for

1 min. Add 0.5 ml of *ferric chloride solution R1* and 2 ml of *chloroform R* and shake. The aqueous layer is coloured light violet-red to purple.

- E. To 50 mg in a porcelain crucible add 0.5 g of *anhydrous sodium carbonate R*. Heat over an open flame for 10 min. Allow to cool. Take up the residue with 5 ml of *dilute nitric acid R* and filter. To 1 ml of the filtrate add 1 ml of *water R*. The solution gives reaction (a) of chlorides (2.3.1).

TESTS

Acidity or alkalinity. To 0.1 g add 20 ml of *carbon dioxide-free water R*, shake and add 0.1 ml of *bromothymol blue solution R1*. Not more than 0.1 ml of 0.02 M *hydrochloric acid* or 0.02 M *sodium hydroxide* is required to change the colour of the indicator.

Specific optical rotation (2.2.7). Dissolve 1.50 g in *ethanol R* and dilute to 25.0 ml with the same solvent. The specific optical rotation is + 18.5 to + 20.5.

Related substances. Examine by thin-layer chromatography (2.2.27), using *silica gel GF₂₅₄ R* as the coating substance.

Test solution. Dissolve 0.10 g of the substance to be examined in *acetone R* and dilute to 10 ml with the same solvent.

Reference solution (a). Dissolve 0.10 g of *chloramphenicol CRS* in *acetone R* and dilute to 10 ml with the same solvent.

Reference solution (b). Dilute 0.5 ml of reference solution (a) to 100 ml with *acetone R*.

Apply separately to the plate 1 µl and 20 µl of the test solution, 1 µl of reference solution (a) and 20 µl of reference solution (b). Develop over a path of 15 cm using a mixture of 1 volume of *water R*, 10 volumes of *methanol R* and 90 volumes of *chloroform R*. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. Any spot in the chromatogram obtained with 20 µl of the test solution, apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.5 per cent).

Chlorides (2.4.4). To 1.00 g add 20 ml of *water R* and 10 ml of *nitric acid R* and shake for 5 min. Filter through a filter paper previously washed by filtering 5 ml portions of *water R* until 5 ml of filtrate no longer becomes opalescent on addition of 0.1 ml of *nitric acid R* and 0.1 ml of *silver nitrate solution R1*. 15 ml of the filtrate complies with the limit test for chlorides (100 ppm).

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 2.0 g.

Pyrogens (2.6.8). If intended for use in the manufacture of a parenteral dosage form without a further appropriate procedure for the removal of pyrogens, it complies with the test for pyrogens. Inject per kilogram of the rabbit's mass 2.5 ml of a solution containing per millilitre 2 mg of the substance to be examined.

ASSAY

Dissolve 0.100 g in *water R* and dilute to 500.0 ml with the same solvent. Dilute 10.0 ml of this solution to 100.0 ml with *water R*. Measure the absorbance (2.2.25) at the maximum at 278 nm.

Calculate the content of $C_{27}H_{42}Cl_2N_2O_6$ taking the specific absorbance to be 297.

STORAGE

Store protected from light. If the substance is sterile, store in a sterile, airtight, tamper-proof container.

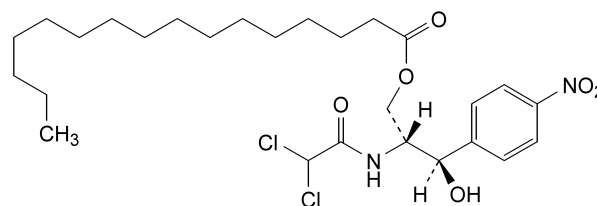
LABELLING

The label states, where applicable, that the substance is apyrogenic.

01/2005:0473

CHLORAMPHENICOL PALMITATE

Chloramphenicoli palmitas


 $C_{27}H_{42}Cl_2N_2O_6$
 M_r 561.6

DEFINITION

Chloramphenicol palmitate contains not less than 98.0 per cent and not more than the equivalent of 102.0 per cent of (2*R*,3*R*)-2-[(dichloroacetyl)amino]-3-hydroxy-3-(4-nitrophenyl)propyl hexadecanoate, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, fine, unctuous powder, practically insoluble in water, freely soluble in acetone, sparingly soluble in alcohol, very slightly soluble in hexane.

It melts at 87 °C to 95 °C.

It shows polymorphism and the thermodynamically stable form has low bioavailability following oral administration.

IDENTIFICATION

- A. Examine by thin-layer chromatography (2.2.27), using *silanised silica gel H R* as the coating substance.

Test solution. Dissolve 50 mg of the substance to be examined in a mixture of 1 ml of 1 M *sodium hydroxide* and 5 ml of *acetone R* and allow to stand for 30 min. Add 1.1 ml of 1 M *hydrochloric acid* and 3 ml of *acetone R*.

Reference solution (a). Dissolve 10 mg of *chloramphenicol CRS* in *acetone R* and dilute to 5 ml with the same solvent.

Reference solution (b). Dissolve 10 mg of *palmitic acid R* in *acetone R* and dilute to 5 ml with the same solvent.

Reference solution (c). Dissolve 10 mg of the substance to be examined in *acetone R* and dilute to 5 ml with the same solvent.

Apply to the plate 4 µl of each solution. Develop over a path of 15 cm using a mixture of 30 volumes of a 100 g/l solution of *ammonium acetate R* and 70 volumes of *alcohol R*. Allow the plate to dry in air and spray with a solution containing 0.2 g/l of *dichlorofluorescein R* and 0.1 g/l of *rhodamine B R* in *alcohol R*. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. The chromatogram obtained with the test solution shows three spots corresponding in position to the principal spots in the chromatograms obtained with reference solutions (a), (b) and (c).

- B. Dissolve 0.2 g in 2 ml of *pyridine R*, add 2 ml of a 100 g/l solution of *potassium hydroxide R* and heat on a water-bath. A red colour is produced.

C. Dissolve 10 mg in 5 ml of *alcohol R* and add 4.5 ml of *dilute sulphuric acid R* and 50 mg of *zinc powder R*. Allow to stand for 10 min and if necessary decant the supernatant liquid or filter. Cool the solution in iced water and add 0.5 ml of *sodium nitrite solution R*. Allow to stand for 2 min and add 1 g of *urea R*, 2 ml of *strong sodium hydroxide solution R* and 1 ml of *β-naphthol solution R*. A red colour develops.

TESTS

Acidity. Dissolve 1.0 g in 5 ml of a mixture of equal volumes of *alcohol R* and *ether R*, warming to 35 °C. Add 0.2 ml of *phenolphthalein solution R*. Not more than 0.4 ml of 0.1 M *sodium hydroxide* is required to produce a pink colour persisting for 30 s.

Specific optical rotation (2.2.7). Dissolve 1.25 g in *ethanol R* and dilute to 25.0 ml with the same solvent. The specific optical rotation is + 22.5 to + 25.5.

Free chloramphenicol. Not more than 450 ppm. Dissolve 1.0 g, with gentle heating, in 80 ml of *xylene R*. Cool and shake with three quantities, each of 15 ml, of *water R*. Dilute the combined aqueous extracts to 50 ml with *water R* and shake with 10 ml of *toluene R*. Allow to separate and discard the toluene layer. Centrifuge a portion of the aqueous layer and measure the absorbance (*A*) (2.2.25) at the maximum at 278 nm using as the compensation liquid a blank solution having an absorbance not greater than 0.05.

Calculate the content of free chloramphenicol in parts per million from the expression:

$$\frac{A \times 10^4}{5.96}$$

Related substances. Examine by thin-layer chromatography (2.2.27), using *silica gel GF₂₅₄ R* as the coating substance.

Test solution. Dissolve 0.1 g of the substance to be examined in *acetone R* and dilute to 10 ml with the same solvent.

Reference solution (a). Dissolve 20 mg of *chloramphenicol palmitate isomer CRS* in *acetone R* and dilute to 10 ml with the same solvent. Dilute 1 ml of this solution to 10 ml with *acetone R*.

Reference solution (b). Dissolve 20 mg of *chloramphenicol dipalmitate CRS* in *acetone R* and dilute to 10 ml with the same solvent. Dilute 1 ml of this solution to 10 ml with *acetone R*.

Reference solution (c). Dissolve 5 mg of *chloramphenicol CRS* in *acetone R* and dilute to 10 ml with the same solvent. Dilute 1 ml of this solution to 10 ml with *acetone R*.

Apply to the plate 10 µl of each solution. Develop over a path of 15 cm using a mixture of 10 volumes of *methanol R*, 40 volumes of *chloroform R* and 50 volumes of *cyclohexane R*. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. In the chromatogram obtained with the test solution, any spots corresponding to chloramphenicol palmitate isomer and chloramphenicol dipalmitate are not more intense than the corresponding spots in the chromatograms obtained with reference solutions (a) and (b) respectively (2.0 per cent) and any spot, apart from the principal spot and the spots corresponding to chloramphenicol palmitate isomer and chloramphenicol dipalmitate, is not more intense than the principal spot in the chromatogram obtained with reference solution (c) (0.5 per cent).

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by heating at 80 °C over *diphosphorus pentoxide R* at a pressure not exceeding 0.1 kPa for 3 h.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

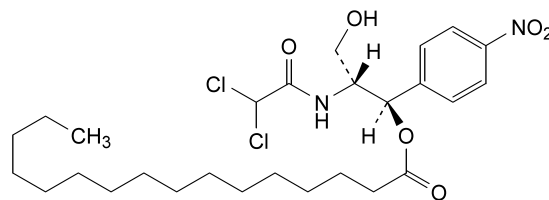
Dissolve 90.0 mg in *alcohol R* and dilute to 100.0 ml with the same solvent. Dilute 10.0 ml of this solution to 250.0 ml with *alcohol R*. Measure the absorbance (2.2.25) of the solution at the maximum at 271 nm.

Calculate the content of C₂₇H₄₂Cl₂N₂O₆ taking the specific absorbance to be 178.

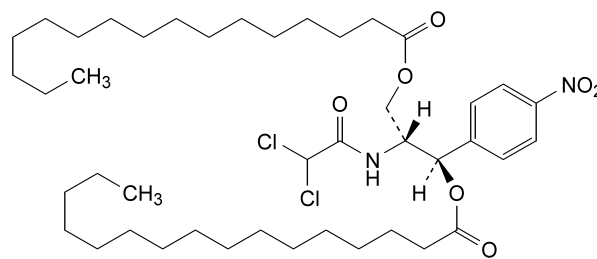
STORAGE

Store protected from light.

IMPURITIES



A. (1*R*,2*R*)-2-[(dichloroacetyl)amino]-3-hydroxy-1-(4-nitrophenyl)propyl hexadecanoate (chloramphenicol palmitate isomer),

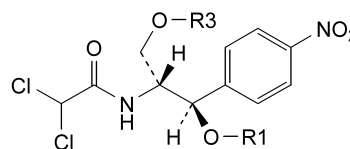


B. (1*R*,2*R*)-2-[(dichloroacetyl)amino]-1-(4-nitrophenyl)propane-1,3-diyl bis(hexadecanoate) (chloramphenicol dipalmitate).

01/2005:0709

CHLORAMPHENICOL SODIUM SUCCINATE

Chloramphenicoli natrii succinas



1 isomer : R1 = CO-CH₂-CH₂-CO₂Na, R3 = H

3 isomer : R1 = H, R3 = CO-CH₂-CH₂-CO₂Na

C₁₅H₁₅Cl₂N₂NaO₈

M_r 445.2

DEFINITION

Chloramphenicol sodium succinate, a mixture in variable proportions of sodium (2*R*,3*R*)-2-[(dichloroacetyl)amino]-3-hydroxy-3-(4-nitrophenyl)propyl butanedioate (3 isomer) and of sodium (1*R*,2*R*)-2-[(dichloroacetyl)amino]-3-hydroxy-1-(4-nitrophenyl)propyl butanedioate (1 isomer), contains not less than 98.0 per cent and not more than the equivalent of 102.0 per cent of C₁₅H₁₅Cl₂N₂NaO₈, calculated with reference to the anhydrous substance.

CHARACTERS

A white or yellowish-white powder, hygroscopic, very soluble in water, freely soluble in alcohol.

IDENTIFICATION

A. Examine by thin-layer chromatography (2.2.27), using *silica gel GF₂₅₄* R as the coating substance.

Test solution. Dissolve 20 mg of the substance to be examined in 2 ml of *acetone* R.

Reference solution (a). Dissolve 20 mg of *chloramphenicol sodium succinate* CRS in 2 ml of *acetone* R.

Reference solution (b). Dissolve 20 mg of *chloramphenicol* CRS in 2 ml of *acetone* R.

Apply separately to the plate 2 µl of each solution.

Develop over a path of 15 cm using a mixture of 1 volume of *dilute acetic acid* R, 14 volumes of *methanol* R and 85 volumes of *chloroform* R. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. The two principal spots in the chromatogram obtained with the test solution are similar in position and size to those in the chromatogram obtained with reference solution (a). Their positions are different from that of the principal spot in the chromatogram obtained with reference solution (b).

B. Dissolve about 10 mg in 1 ml of *alcohol (50 per cent V/V)* R, add 3 ml of a 10 g/l solution of *calcium chloride* R and 50 mg of *zinc powder* R and heat on a water-bath for 10 min. Filter the hot solution and allow to cool. Add 0.1 ml of *benzoyl chloride* R and shake for 1 min. Add 0.5 ml of *ferric chloride solution* R1 and 2 ml of *chloroform* R and shake. The upper layer is light violet-red to purple.

C. Dissolve 50 mg in 1 ml of *pyridine* R. Add 0.5 ml of *dilute sodium hydroxide solution* R and 1.5 ml of *water* R. Heat in a water-bath for 3 min. A red colour develops. Add 2 ml of *nitric acid* R and cool under running water. Add 1 ml of 0.1 M *silver nitrate*. A white precipitate is formed slowly.

D. It gives reaction (a) of sodium (2.3.1).

TESTS

pH (2.2.3). Dissolve 2.50 g in *carbon dioxide-free water* R and dilute to 10 ml with the same solvent. The pH of the solution is 6.4 to 7.0.

Specific optical rotation (2.2.7). Dissolve 0.50 g in *water* R and dilute to 10.0 ml with the same solvent. The specific optical rotation is + 5.0 to + 8.0, calculated with reference to the anhydrous substance.

Chloramphenicol and chloramphenicol disodium disuccinate. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 25.0 mg of the substance to be examined in the mobile phase and dilute to 100.0 ml with the mobile phase.

Reference solution (a). Dissolve 10.0 mg of *chloramphenicol* CRS in the mobile phase and dilute to 100.0 ml with the mobile phase (*solution a* †). Dilute 5.0 ml of this solution to 100.0 ml with the mobile phase.

Reference solution (b). Dissolve 10.0 mg of *chloramphenicol disodium disuccinate* CRS in the mobile phase and dilute to 100.0 ml with the mobile phase (*solution b* †). Dilute 5.0 ml of this solution to 100.0 ml with the mobile phase.

Reference solution (c). Dissolve 25 mg of the substance to be examined in the mobile phase, add 5 ml of solution (a') and 5 ml of solution (b') and dilute to 100 ml with the mobile phase.

The chromatographic procedure may be carried out using:

- a column 0.25 m long and 4.6 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography* R (5 µm),
- as mobile phase at a flow rate of 1.0 ml/min a mixture of 5 volumes of a 20 g/l solution of *phosphoric acid* R, 40 volumes of *methanol* R and 55 volumes of *water* R,
- as detector a spectrophotometer set at 275 nm,
- a 20 µl fixed-loop injector.

Inject the test solution and each of the reference solutions. The test is not valid unless, in the chromatogram obtained with reference solution (c), the two peaks corresponding to those in the chromatograms obtained with reference solutions (a) and (b) are clearly separated from the peaks corresponding to the two principal peaks in the chromatogram obtained with the test solution. If necessary, adjust the methanol content of the mobile phase.

In the chromatogram obtained with the test solution, the area of any peak corresponding to chloramphenicol is not greater than the area of the principal peak in the chromatogram obtained with reference solution (a) (2.0 per cent); the area of any peak corresponding to chloramphenicol disodium disuccinate is not greater than the area of the principal peak in the chromatogram obtained with reference solution (b) (2.0 per cent).

Water (2.5.12). Not more than 2.0 per cent, determined on 0.500 g by the semi-micro determination of water.

Pyrogens (2.6.8). If intended for use in the manufacture of a parenteral dosage form without a further appropriate procedure for removal of pyrogens, it complies with the test for pyrogens. Inject per kilogram of the rabbit's mass 2.5 ml of a solution in *water for injections* R containing 2 mg of the substance to be examined per millilitre.

ASSAY

Dissolve 0.200 g in *water* R and dilute to 500.0 ml with the same solvent. Dilute 5.0 ml of this solution to 100.0 ml with *water* R. Measure the absorbance (2.2.25) at the maximum at 276 nm.

Calculate the content of C₁₅H₁₅Cl₂N₂NaO₈, taking the specific absorbance to be 220.

STORAGE

Store in an airtight container, protected from light. If the substance is sterile, store in a sterile, airtight, tamper-proof container, protected from light.

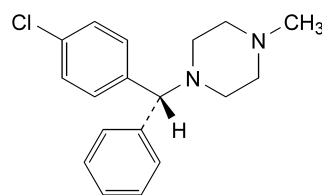
LABELLING

The label states, where applicable, that the substance is apyrogenic.

01/2005:1086

CHLORCYCLIZINE HYDROCHLORIDE

Chlorcyclizini hydrochloridum



and enantiomer, HCl

C₁₈H₂₂Cl₂N₂M_r 337.3

DEFINITION

Chlorcyclizine hydrochloride contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of (*RS*)-1-[(4-chlorophenyl)phenylmethyl]-4-methylpiperazine hydrochloride, calculated with reference to the dried substance.

CHARACTERS

A white, crystalline powder, freely soluble in water and in methylene chloride, soluble in alcohol.

IDENTIFICATION

First identification: B, D.

Second identification: A, C, D.

- Dissolve 10.0 mg in a 5 g/l solution of *sulphuric acid R* and dilute to 100.0 ml with the same acid. Dilute 10.0 ml of the solution to 100.0 ml with a 5 g/l solution of *sulphuric acid R*. Examined between 215 nm and 300 nm (2.2.25), the solution shows an absorption maximum at 231 nm. The specific absorbance at the maximum is 475 to 525, calculated with reference to the dried substance.
- Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *chlorcyclizine hydrochloride CRS*. Examine the substances prepared as discs.
- Examine the chromatograms obtained in the test for related substances (see Tests). The principal spot in the chromatogram obtained with test solution (b) is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a).
- It gives reaction (a) of chlorides (2.3.1).

TESTS

Appearance of solution. Dissolve 0.5 g in *water R* and dilute to 10 ml with the same solvent. The solution is clear (2.2.1) and colourless (2.2.2, *Method II*).

pH (2.2.3). Dissolve 0.10 g in *carbon dioxide-free water R* and dilute to 10 ml with the same solvent. The pH of the solution is 5.0 to 6.0.

Related substances. Examine by thin-layer chromatography (2.2.27), using a plate coated with a suitable silica gel.

Test solution (a). Dissolve 0.20 g of the substance to be examined in *methanol R* and dilute to 10 ml with the same solvent.

Test solution (b). Dilute 5 ml of test solution (a) to 100 ml with *methanol R*.

Reference solution (a). Dissolve 10 mg of *chlorcyclizine hydrochloride CRS* in *methanol R* and dilute to 10 ml with the same solvent.

Reference solution (b). Dissolve 5 mg of *methylpiperazine R* in *methanol R* and dilute to 50 ml with the same solvent.

Reference solution (c). Dilute 1 ml of test solution (b) to 25 ml with *methanol R*.

Reference solution (d). Dissolve 10 mg of *hydroxyzine hydrochloride CRS* and 10 mg of *chlorcyclizine hydrochloride CRS* in *methanol R* and dilute to 10 ml with the same solvent.

Apply separately to the plate 10 µl of each solution and develop over a path of 15 cm using a mixture of 2 volumes of *concentrated ammonia R*, 13 volumes of *methanol R* and 85 volumes of *methylene chloride R*. Allow the plate to dry in air and expose it to iodine vapour for 10 min. In the chromatogram obtained with test solution (a): any spot corresponding to methylpiperazine is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.5 per cent); any spot, apart from the principal

spot and any spot corresponding to methylpiperazine, is not more intense than the spot in the chromatogram obtained with reference solution (c) (0.2 per cent). The test is not valid unless the chromatogram obtained with reference solution (d) shows two clearly separated spots.

Loss on drying (2.2.32). Not more than 1.0 per cent, determined on 1.000 g by drying in an oven at 130 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

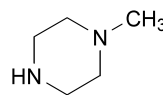
Dissolve 0.200 g in a mixture of 1 ml of 0.1 *M hydrochloric acid* and 50 ml of *methanol R*. Carry out a potentiometric titration (2.2.20), using 0.1 *M sodium hydroxide*. Read the volume added between the two points of inflexion.

1 ml of 0.1 *M sodium hydroxide* is equivalent to 33.73 mg of C₁₈H₂₂Cl₂N₂.

STORAGE

Store protected from light.

IMPURITIES

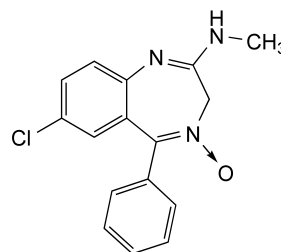


A. *N*-methylpiperazine.

01/2005:0656

CHLORDIAZEPOXIDE

Chlordiazepoxidum



C₁₆H₁₄ClN₃O

M_r 299.8

DEFINITION

7-Chloro-*N*-methyl-5-phenyl-3*H*-1,4-benzodiazepin-2-amine 4-oxide.

Content: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

Appearance: almost white or light yellow, crystalline powder.

Solubility: practically insoluble in water, sparingly soluble in alcohol.

IDENTIFICATION

Infrared absorption spectrophotometry (2.2.24).

Comparison: *chlordiazepoxide CRS*.

TESTS

Related substances. Liquid chromatography (2.2.29). *Carry out the test protected from bright light and prepare the solutions immediately before use.*

Test solution. Dissolve 20.0 mg of the substance to be examined in the mobile phase and dilute to 100.0 ml with the mobile phase.

Reference solution (a). Dilute 1.0 ml of the test solution to 100.0 ml with the mobile phase. Dilute 2.0 ml of this solution to 10.0 ml with the mobile phase.

Reference solution (b). Dissolve 5 mg of *nitrazepam R* in the mobile phase, add 25.0 ml of the test solution and dilute to 100.0 ml with the mobile phase. Dilute 2.0 ml of this solution to 50.0 ml with the mobile phase.

Reference solution (c). Dissolve 4.0 mg of *aminochlorobenzophenone R* in the mobile phase and dilute to 100.0 ml with the mobile phase. Dilute 1.0 ml of the solution to 100.0 ml with the mobile phase.

Column:

- **size:** $l = 0.15$ m, $\varnothing = 4.6$ mm,
- **stationary phase:** octadecylsilyl silica gel for chromatography *R* (5 μ m).

Mobile phase: acetonitrile *R*, water *R* (50:50 *V/V*).

Flow rate: 1.0 ml/min.

Detection: spectrophotometer at 254 nm.

Injection: 10 μ l.

Run time: 6 times the retention time of chlordiazepoxide.

Retention time: nitrazepam = about 3.1 min;
chlordiazepoxide = about 3.6 min.

Relative retention with reference to chlordiazepoxide:
impurity A = about 0.7; impurity B = about 2.3;
impurity C = about 3.9.

System suitability: reference solution (b):

- **resolution:** minimum 2.0 between the peaks due to nitrazepam and chlordiazepoxide.

Limits:

- **impurities A, B:** for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent),
- **impurity C:** not more than the area of the principal peak in the chromatogram obtained with reference solution (c) (0.2 per cent),
- **any other impurity:** not more than half the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent),
- **total:** not more than 2.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent),
- **disregard limit:** 0.25 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

Loss on drying (2.2.32): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 100–105 °C.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.250 g, with heating if necessary, in 80 ml of *anhydrous acetic acid R*. Titrate with 0.1 *M* perchloric acid, determining the end-point potentiometrically (2.2.20).

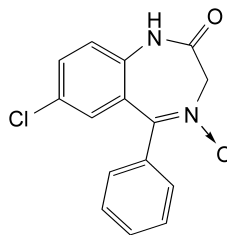
1 ml of 0.1 *M* perchloric acid is equivalent to 29.98 mg of $C_{16}H_{14}ClN_3O$.

STORAGE

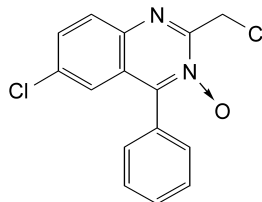
Protected from light.

IMPURITIES

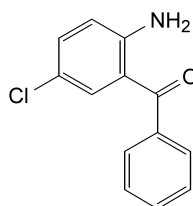
Specified impurities: A, B, C.



A. 7-chloro-5-phenyl-1,3-dihydro-2*H*-1,4-benzodiazepin-2-one 4-oxide,



B. 6-chloro-2-(chloromethyl)-4-phenylquinazoline 3-oxide,

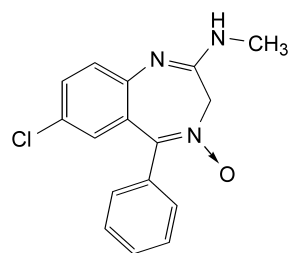


C. (2-amino-5-chlorophenyl)phenylmethanone (aminochlorobenzophenone).

01/2005:0474

CHLORDIAZEPOXIDE HYDROCHLORIDE

Chlordiazepoxidi hydrochloridum



$C_{16}H_{15}Cl_2N_3O$

M_r 336.2

DEFINITION

7-Chloro-*N*-methyl-5-phenyl-3*H*-1,4-benzodiazepin-2-amine 4-oxide hydrochloride.

Content: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

Appearance: white or slightly yellow, crystalline powder, slightly hygroscopic.

Solubility: soluble in water, sparingly soluble in alcohol.

IDENTIFICATION

A. Infrared absorption spectrophotometry (2.2.24).

Comparison: *chlordiazepoxide hydrochloride CRS*.

B. Dissolve 50 mg in 5 ml of *water R*, add 1 ml of *dilute ammonia R1*, mix, allow to stand for 5 min and filter. Acidify the filtrate with *dilute nitric acid R*. The solution gives reaction (a) of chlorides (2.3.1).

TESTS

Appearance of solution. The solution is clear (2.2.1) and not more intensely coloured than reference solution GY₆ (2.2.2, Method II).

Dissolve 2.5 g in carbon dioxide-free water R and dilute to 25 ml with the same solvent.

Related substances. Liquid chromatography (2.2.29). Carry out the following operations protected from bright light and prepare the solutions immediately before use.

Test solution. Dissolve 20.0 mg of the substance to be examined in the mobile phase and dilute to 100.0 ml with the mobile phase.

Reference solution (a). Dilute 1.0 ml of the test solution to 100.0 ml with the mobile phase. Dilute 2.0 ml of this solution to 10.0 ml with the mobile phase.

Reference solution (b). Dissolve 5 mg of nitrazepam R in the mobile phase, add 25.0 ml of the test solution and dilute to 100.0 ml with the mobile phase. Dilute 2.0 ml of this solution to 50.0 ml with the mobile phase.

Reference solution (c). Dissolve 4.0 mg of aminochlorobenzophenone R in the mobile phase and dilute to 100.0 ml with the mobile phase. Dilute 1.0 ml of this solution to 100.0 ml with the mobile phase.

Column:

- size: $l = 0.15$ m, $\varnothing = 4.6$ mm,
- stationary phase: octadecylsilyl silica gel for chromatography R (5 μ m).

Mobile phase: acetonitrile R, water R (50:50 V/V).

Flow rate: 1.0 ml/min.

Detection: spectrophotometer at 254 nm.

Injection: 10 μ l.

Run time: 6 times the retention time of chlordiazepoxide.

Retention time: nitrazepam = about 3.1 min;
chlordiazepoxide = about 3.6 min.

Relative retention with reference to chlordiazepoxide:
impurity A = about 0.7; impurity B = about 2.3;
impurity C = about 3.9.

System suitability: reference solution (b):

- resolution: minimum 2.0 between the peaks due to nitrazepam and chlordiazepoxide.

Limits:

- impurities A, B: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent),
- impurity C: not more than the area of the principal peak in the chromatogram obtained with reference solution (c) (0.2 per cent),
- any other impurity: not more than half the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent),
- total: not more than 2.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent),
- disregard limit: 0.25 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

Loss on drying (2.2.32): maximum 0.5 per cent, determined on 1.000 g by drying *in vacuo* at 60 °C for 4 h.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.250 g in 80 ml of *anhydrous acetic acid* R, heating if necessary. Cool and add 10 ml of *mercuric acetate solution* R. Titrate with 0.1 M *perchloric acid*, determining the end-point potentiometrically (2.2.20).

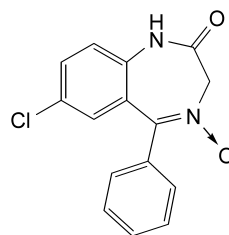
1 ml of 0.1 M *perchloric acid* is equivalent to 33.62 mg of C₁₆H₁₅Cl₂N₃O.

STORAGE

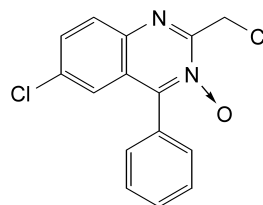
In an airtight container, protected from light.

IMPURITIES

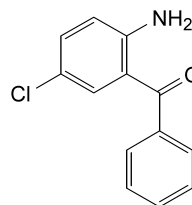
Specified impurities: A, B, C.



A. 7-chloro-5-phenyl-1,3-dihydro-2H-1,4-benzodiazepin-2-one 4-oxide,



B. 6-chloro-2-(chloromethyl)-4-phenylquinazoline 3-oxide,

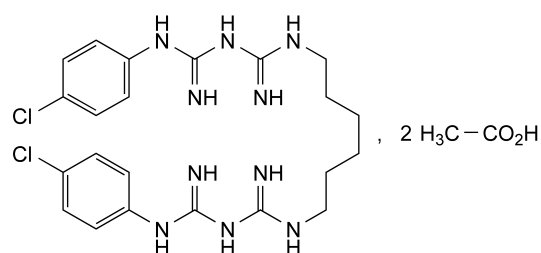


C. (2-amino-5-chlorophenyl)phenylmethanone (aminochlorobenzophenone).

01/2005:0657

CHLORHEXIDINE DIACETATE

Chlorhexidini diacetatas



C₂₆H₃₈Cl₂N₁₀O₄

M_r 625.6

DEFINITION

Chlorhexidine diacetate contains not less than 98.0 per cent and not more than the equivalent of 101.0 per cent of 1,1'-(hexane-1,6-diyl)bis[5-(4-chlorophenyl)biguanide] diacetate, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, microcrystalline powder, sparingly soluble in water, soluble in alcohol, slightly soluble in glycerol and in propylene glycol.

IDENTIFICATION

First identification: A.

Second identification: B, C, D.

- A. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *chlorhexidine diacetate CRS*.
- B. Dissolve about 5 mg in 5 ml of a warm 10 g/l solution of *cetrimide R* and add 1 ml of *strong sodium hydroxide solution R* and 1 ml of *bromine water R*. A deep red colour is produced.
- C. Dissolve 0.3 g in 10 ml of a mixture of equal volumes of *hydrochloric acid R* and *water R*. Add 40 ml of *water R*, filter if necessary and cool in ice water. Make alkaline to *titan yellow paper R* by adding dropwise and with stirring *strong sodium hydroxide solution R* and add 1 ml in excess. Filter, wash the precipitate with *water R* until the washings are free from alkali and recrystallise from *alcohol (70 per cent V/V) R*. Dry at 100 °C to 105 °C. The residue melts (2.2.14) at 132 °C to 136 °C.
- D. It gives reaction (a) of acetates (2.3.1).

TESTS

Chloroaniline. Dissolve 0.20 g of the substance to be examined in 25 ml of *water R* with shaking if necessary. Add 1 ml of *hydrochloric acid R* and dilute to 30 ml with *water R*. Add rapidly and with thorough mixing after each addition: 2.5 ml of *dilute hydrochloric acid R*, 0.35 ml of *sodium nitrite solution R*, 2 ml of a 50 g/l solution of *ammonium sulphamate R*, 5 ml of a 1.0 g/l solution of *naphthylethylenediamine dihydrochloride R* and 1 ml of *alcohol R*, dilute to 50.0 ml with *water R* and allow to stand for 30 min. Any reddish-blue colour in the solution is not more intense than that in a standard prepared at the same time in the same manner using a mixture of 10.0 ml of a 0.010 g/l solution of *chloroaniline R* in *dilute hydrochloric acid R* and 20 ml of *dilute hydrochloric acid R* instead of the solution of the substance to be examined (500 ppm).

Related substances. Examined by liquid chromatography (2.2.29).

Test solution. Dissolve 0.200 g of the substance to be examined in the mobile phase and dilute to 100 ml with the mobile phase.

Reference solution (a). Dissolve 15 mg of *chlorhexidine for performance test CRS* in the mobile phase and dilute to 10.0 ml with the mobile phase.

Reference solution (b). Dilute 2.5 ml of the test solution to 100 ml with the mobile phase.

Reference solution (c). Dilute 2.0 ml of reference solution (b) to 10 ml with the mobile phase. Dilute 1.0 ml of this solution to 10 ml with the mobile phase.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.2 m long and 4 mm in internal diameter, packed with *octadecylsilyl silica gel for chromatography R* (5 µm),
- as mobile phase at a flow-rate of 1.0 ml/min, a solution of 2.0 g of *sodium octanesulphonate R* in a mixture of 120 ml of *glacial acetic acid R*, 270 ml of *water R* and 730 ml of *methanol R*,

– as detector a spectrophotometer set at 254 nm.

Equilibrate the column with the mobile phase for at least 1 h. Adjust the sensitivity of the system so that the height of the principal peak in the chromatogram obtained with 10 µl of reference solution (b) is at least 50 per cent of the full scale of the recorder.

Inject 10 µl of reference solution (a). The test is not valid unless the resulting chromatogram is similar to the specimen chromatogram provided with *chlorhexidine for performance test CRS* in that the peaks due to impurity-A and impurity-B precede that due to chlorhexidine. If necessary, adjust the concentration of acetic acid in the mobile phase (increasing the concentration decreases the retention times).

Inject separately 10 µl of the test solution and 10 µl each of reference solutions (b) and (c). Record the chromatograms of reference solutions (b) and (c) until the peak due to chlorhexidine has been eluted and record the chromatogram of the test solution for six times the retention time of the peak due to chlorhexidine. In the chromatogram obtained with the test solution, the sum of the areas of all the peaks, apart from the principal peak is not greater than the area of the principal peak in the chromatogram obtained with reference solution (b) (2.5 per cent). Disregard any peak with a relative retention time of 0.25 or less with respect to the principal peak and any peak whose area is less than that of the principal peak in the chromatogram obtained with reference solution (c).

Loss on drying (2.2.32). Not more than 3.5 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C.

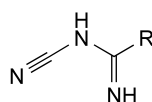
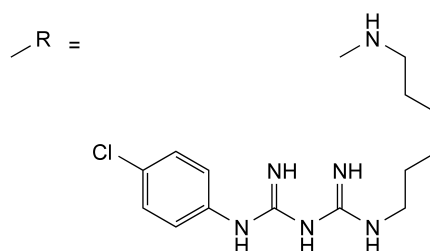
Sulphated ash (2.4.14). Not more than 0.15 per cent, determined on 1.0 g.

ASSAY

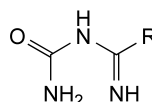
Dissolve 0.140 g in 100 ml of *anhydrous acetic acid R* and titrate with 0.1 M *perchloric acid*. Determine the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *perchloric acid* is equivalent to 15.64 mg of $C_{26}H_{38}Cl_2N_{10}O_4$.

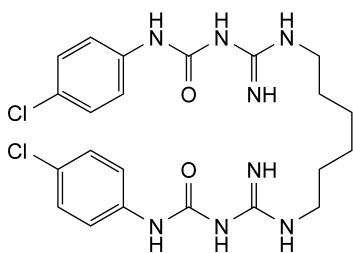
IMPURITIES



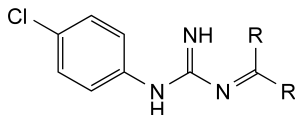
A. 1-(4-chlorophenyl)-5-[6-(3-cyanoguanidino)hexyl]biguanide,



B. [[[6-[5-(4-chlorophenyl)guanidino]hexyl]amino]iminomethyl]urea,



C. 1,1'-[hexane-1,6-diylbis[imino(iminocarbonyl)]]bis[3-(4-chlorophenyl)urea],

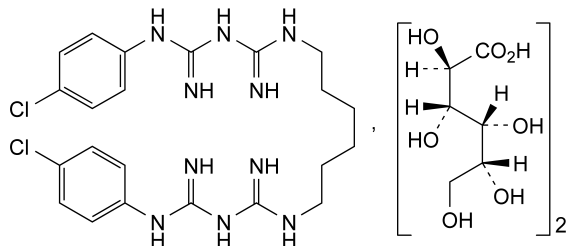


D. 1,1'-[[(4-chlorophenyl)amino]iminomethyl]imino]methylene]bis[imino(hexane-1,6-diyl)]bis[5-(4-chlorophenyl)biguanide].

01/2005:0658
corrected

CHLORHEXIDINE DIGLUCONATE SOLUTION

Chlorhexidini digluconatis solutio



$C_{34}H_{54}Cl_2N_{10}O_{14}$

M_r 898

DEFINITION

Chlorhexidine digluconate solution is an aqueous solution which contains not less than 190 g/l and not more than 210 g/l of 1,1'-[hexane-1,6-diyl]bis[5-(4-chlorophenyl)biguanide] di-D-gluconate.

CHARACTERS

An almost colourless or pale-yellowish liquid, miscible with water, with not more than 3 parts of acetone and with not more than 5 parts of ethanol (96 per cent).

IDENTIFICATION

First identification: A, B.

Second identification: B, C, D.

A. To 1 ml add 40 ml of *water R*, cool in iced water, make alkaline to *titan yellow paper R* by adding dropwise and with stirring *strong sodium hydroxide solution R* and add 1 ml in excess. Filter, wash the precipitate with *water R* until the washings are free from alkali and recrystallise from *alcohol (70 per cent V/V) R*. Dry at 100 °C to 105 °C. Examine the residue by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *chlorhexidine CRS*.

B. Examine by thin-layer chromatography (2.2.27), using *silica gel G R* as the coating substance.

Test solution. Dilute 10.0 ml of the solution to be examined to 50 ml with *water R*.

Reference solution. Dissolve 25 mg of *calcium gluconate CRS* in 1 ml of *water R*.

Apply separately to the plate 5 µl of each solution. Develop over a path of 10 cm using a mixture of 10 volumes of *ethyl acetate R*, 10 volumes of *concentrated ammonia R*, 30 volumes of *water R* and 50 volumes of *alcohol R*. Dry the plate at 100 °C for 20 min, allow to cool and spray with a 50 g/l solution of *potassium dichromate R* in a 40 per cent *m/m* solution of *sulphuric acid R*. After 5 min, the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

C. To 1 ml add 40 ml of *water R*, cool in iced water, make alkaline to *titan yellow paper R* by adding dropwise and with stirring *strong sodium hydroxide solution R* and add 1 ml in excess. Filter, wash the precipitate with *water R* until the washings are free from alkali and recrystallise from *alcohol (70 per cent V/V) R*. Dry at 100 °C to 105 °C. The residue melts (2.2.14) at 132 °C to 136 °C.

D. To 0.05 ml add 5 ml of a 10 g/l solution of *cetrimide R*, 1 ml of *strong sodium hydroxide solution R* and 1 ml of *bromine water R*; a deep red colour is produced.

TESTS

Relative density (2.2.5): 1.06 to 1.07.

pH (2.2.3). Dilute 5.0 ml to 100 ml with *carbon dioxide-free water R*. The pH of the solution is 5.5 to 7.0.

Chloroaniline. Dilute 2.0 ml to 100 ml with *water R*. To 10 ml of the solution add 2.5 ml of *dilute hydrochloric acid R* and dilute to 20 ml with *water R*. Add rapidly and with thorough mixing after each addition: 0.35 ml of *sodium nitrite solution R*, 2 ml of a 50 g/l solution of *ammonium sulphamate R*, 5 ml of a 1 g/l solution of *naphthylethylenediamine dihydrochloride R*, 1 ml of *alcohol R*, dilute to 50.0 ml with *water R* and allow to stand for 30 min.

Any reddish-blue colour in the solution is not greater than that in a standard prepared at the same time in the same manner using a mixture of 10.0 ml of a 0.010 g/l solution of *chloroaniline R* in *dilute hydrochloric acid R* and 10 ml of *water R* instead of the dilution of the solution to be examined (0.25 per cent with reference to chlorhexidine digluconate at a nominal concentration of 200 g/l).

Related substances. Examined by liquid chromatography (2.2.29).

Test solution. Dilute 5.0 ml of the sample to be examined to 50.0 ml with the mobile phase. Dilute 5.0 ml of this solution to 50.0 ml with the mobile phase.

Reference solution (a). Dissolve 15 mg of *chlorhexidine for performance test CRS* in the mobile phase and dilute to 10.0 ml with the mobile phase.

Reference solution (b). Dilute 3.0 ml of the test solution to 100 ml with the mobile phase.

Reference solution (c). Dilute 1.0 ml of reference solution (b) to 50 ml with the mobile phase.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.2 m long and 4 mm in internal diameter, packed with *octadecylsilyl silica gel for chromatography R* (5 µm),
- as mobile phase at a flow-rate of 1.0 ml/min, a solution of 2.0 g of *sodium octanesulphonate R* in a mixture of 120 ml of *glacial acetic acid R*, 270 ml of *water R* and 730 ml of *methanol R*,
- as detector a spectrophotometer set at 254 nm.

Equilibrate the column with mobile phase for at least 1 hour. Adjust the sensitivity of the system so that the height of the principal peak in the chromatogram obtained with 10 µl of reference solution (b) is at least 50 per cent of the full scale of the recorder.

Inject 10 µl of reference solution (a). The test is not valid unless the resulting chromatogram is similar to the specimen chromatogram provided with *chlorhexidine for performance test CRS* in that the peaks due to impurity A and impurity B precede that due to chlorhexidine. If necessary, adjust the concentration of acetic acid in the mobile phase (increasing the concentration decreases the retention times).

Inject separately 10 µl of the test solution and 10 µl each of reference solutions (b) and (c). Record the chromatograms of reference solutions (b) and (c) until the peak due to chlorhexidine has been eluted and record the chromatogram of the test solution for six times the retention time of the peak due to chlorhexidine. In the chromatogram obtained with the test solution, the sum of the areas of the peaks, apart from the principal peak is not greater than the area of the principal peak in the chromatogram obtained with reference solution (b) (3.0 per cent). Disregard any peak with a relative retention time of 0.25 or less with respect to the principal peak and any peak whose area is less than that of the principal peak in the chromatogram obtained with reference solution (c).

ASSAY

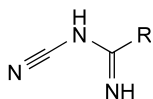
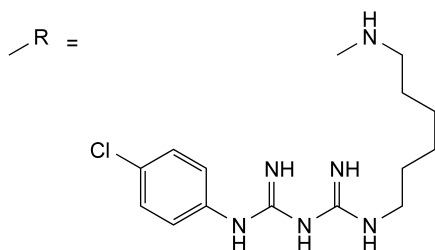
Determine the density (2.2.5) of the solution to be examined. Transfer 1.00 g to a 250 ml beaker and add 50 ml of *anhydrous acetic acid R*. Titrate with 0.1 M *perchloric acid*. Determine the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *perchloric acid* is equivalent to 22.44 mg of $C_{22}H_{32}Cl_4N_{10}O_{14}$.

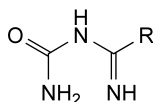
STORAGE

Store protected from light.

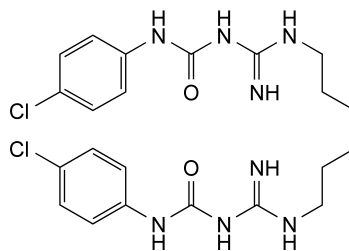
IMPURITIES



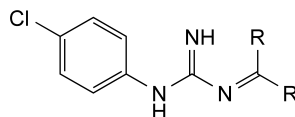
A. 1-(4-chlorophenyl)-5-[6-(3-cyanoguanidino)hexyl]biguanide,



B. [[[(6-[5-(4-chlorophenyl)guanidino]hexyl)amino]iminomethyl]urea],



C. 1,1'-[hexane-1,6-diylbis[imino(iminocarbonyl)]]bis[3-(4-chlorophenyl)urea],

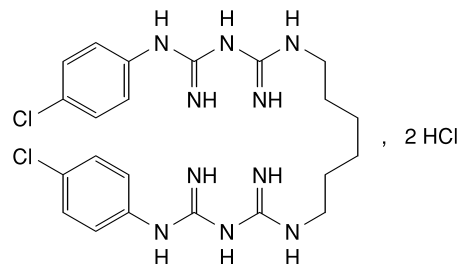


D. 1,1'-[[[(4-chlorophenyl)amino]iminomethyl]imino]methylene]bis[imino(hexane-1,6-diyl)]bis[5-(4-chlorophenyl)biguanide].

01/2005:0659

CHLORHEXIDINE DIHYDROCHLORIDE

Chlorhexidini dihydrochloridum



$C_{22}H_{32}Cl_4N_{10}$

M_r 578.4

DEFINITION

Chlorhexidine dihydrochloride contains not less than 98.0 per cent and not more than the equivalent of 101.0 per cent of 1,1'-(hexane-1,6-diyl)bis[5-(4-chlorophenyl)biguanide] dihydrochloride, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, crystalline powder, sparingly soluble in water and in propylene glycol, very slightly soluble in alcohol.

IDENTIFICATION

First identification: A, D.

Second identification: B, C, D.

- Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *chlorhexidine dihydrochloride CRS*.
- Dissolve about 5 mg in 5 ml of a warm 10 g/l solution of *cetrimide R* and add 1 ml of *strong sodium hydroxide solution R* and 1 ml of *bromine water R*. A dark red colour is produced.
- Dissolve 0.3 g in 10 ml of a mixture of equal volumes of *hydrochloric acid R* and *water R*. Add 40 ml of *water R*, filter if necessary and cool in iced water. Make alkaline to *titan yellow paper R* by adding dropwise, and with stirring, *strong sodium hydroxide solution R* and add 1 ml in excess. Filter, wash the precipitate with *water R*.

until the washings are free from alkali and recrystallise from *alcohol* (70 per cent V/V) *R*. Dry at 100 °C to 105 °C. The residue melts (2.2.14) at 132 °C to 136 °C.

D. It gives reaction (a) of chlorides (2.3.1).

TESTS

Chloroaniline. To 0.20 g of the substance to be examined add 1 ml of *hydrochloric acid R*, shake for about 30 s, dilute to 30 ml with *water R* and shake until a clear solution is obtained. Add rapidly and with thorough mixing after each addition: 2.5 ml of *dilute hydrochloric acid R*, 0.35 ml of *sodium nitrite solution R*, 2 ml of a 50 g/l solution of *ammonium sulphamate R*, 5 ml of a 1.0 g/l solution of *naphthylethylenediamine dihydrochloride R* and 1 ml of *alcohol R*; dilute to 50.0 ml with *water R* and allow to stand for 30 min. Any reddish-blue colour in the solution is not more intense than that in a standard prepared at the same time and in the same manner using a mixture of 10.0 ml of a 0.010 g/l solution of *chloroaniline R* in *dilute hydrochloric acid R* and 20 ml of *dilute hydrochloric acid R* instead of the solution of the substance to be examined (500 ppm).

Related substances. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 0.200 g of the substance to be examined in the mobile phase and dilute to 100 ml with the mobile phase.

Reference solution (a). Dissolve 15 mg of *chlorhexidine for performance test CRS* in the mobile phase and dilute to 10.0 ml with the mobile phase.

Reference solution (b). Dilute 2.5 ml of the test solution to 100 ml with the mobile phase.

Reference solution (c). Dilute 2.0 ml of reference solution (b) to 10 ml with the mobile phase. Dilute 1.0 ml of the solution to 10 ml with the mobile phase.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.2 m long and 4 mm in internal diameter, packed with *octadecylsilyl silica gel for chromatography R* (5 µm),
- as mobile phase at a flow rate of 1.0 ml/min, a solution of 2.0 g of *sodium octanesulphonate R* in a mixture of 120 ml of *glacial acetic acid R*, 270 ml of *water R* and 730 ml of *methanol R*,
- as detector a spectrophotometer set at 254 nm.

Equilibrate the column with the mobile phase for at least 1 h. Adjust the sensitivity of the system so that the height of the principal peak in the chromatogram obtained with 10 µl of reference solution (b) is at least 50 per cent of the full scale of the recorder.

Inject 10 µl of reference solution (a). The test is not valid unless the resulting chromatogram is similar to the specimen chromatogram provided with *chlorhexidine for performance test CRS* in that the peaks due to impurity A and impurity B precede that due to chlorhexidine. If necessary, adjust the concentration of acetic acid in the mobile phase (increasing the concentration decreases the retention times).

Inject 10 µl of the test solution and 10 µl each of reference solutions (b) and (c). Record the chromatograms of reference solutions (b) and (c) until the peak due to chlorhexidine is eluted and record the chromatogram of the test solution for six times the retention time of the peak due to chlorhexidine. In the chromatogram obtained with the test solution, the sum of the areas of the peaks, apart from the principal peak, is not greater than the area of the principal peak in the

chromatogram obtained with reference solution (b) (2.5 per cent). Disregard any peak with a relative retention time of 0.25 or less, with respect to chlorhexidine, and any peak with an area less than that of the principal peak in the chromatogram obtained with reference solution (c).

Loss on drying (2.2.32). Not more than 1.0 per cent, determined on 1.000 g by drying in an oven at 100–105 °C.

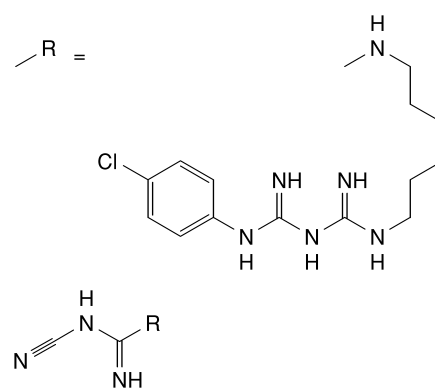
Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

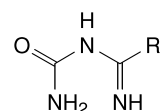
Dissolve 100.0 mg in 5 ml of *anhydrous formic acid R* and add 70 ml of *acetic anhydride R*. Titrate with 0.1 *M perchloric acid*, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 *M perchloric acid* is equivalent to 14.46 mg of $C_{22}H_{32}Cl_4N_{10}$.

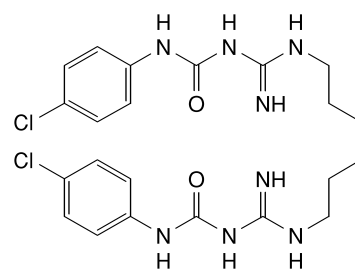
IMPURITIES



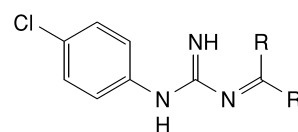
A. 1-(4-chlorophenyl)-5-[6-(3-cyanoguanidino)hexyl]biguanide,



B. [[[6-[5-(4-chlorophenyl)guanidino]hexyl]amino]iminomethyl]urea,



C. 1,1'-[hexane-1,6-diylbis[imino(iminocarbonyl)]]bis[3-(4-chlorophenyl)urea],



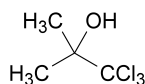
D. 1,1'-[(((4-chlorophenyl)amino]iminomethyl)imino)methylene]bis[imino(hexane-1,6-diyl)]bis[5-(4-chlorophenyl)biguanide].

01/2005:0382 STORAGE

In an airtight container.

CHLOROBUTANOL, ANHYDROUS

Chlorobutanolum anhydricum

 $C_4H_7Cl_3O$ M_r 177.5**DEFINITION**

Anhydrous chlorobutanol contains not less than 98.0 per cent and not more than the equivalent of 101.0 per cent of 1,1,1-trichloro-2-methylpropan-2-ol, calculated with reference to the anhydrous substance.

CHARACTERS

A white, crystalline powder or colourless crystals, sublimes readily, slightly soluble in water, very soluble in alcohol, soluble in glycerol (85 per cent).

It melts at about 95 °C, determined without previous drying.

IDENTIFICATION

- Add about 20 mg to a mixture of 1 ml of *pyridine R* and 2 ml of *strong sodium hydroxide solution R*. Heat in a water-bath and shake. Allow to stand. The pyridine layer becomes red.
- Add about 20 mg to 5 ml of *ammoniacal silver nitrate solution R* and warm slightly. A black precipitate is formed.
- To about 20 mg add 3 ml of *1 M sodium hydroxide* and shake to dissolve. Add 5 ml of *water R* and then, slowly, 2 ml of *iodinated potassium iodide solution R*. A yellowish precipitate is formed.
- It complies with the test for water (see Tests).

TESTS

Solution S. Dissolve 5 g in *alcohol R* and dilute to 10 ml with the same solvent.

Appearance of solution. Solution S is not more opalescent than reference suspension II (2.2.1) and not more intensely coloured than reference solution BY₅ (2.2.2, *Method II*).

Acidity. To 4 ml of solution S add 15 ml of *alcohol R* and 0.1 ml of *bromothymol blue solution R1*. Not more than 1.0 ml of *0.01 M sodium hydroxide* is required to change the colour of the indicator to blue.

Chlorides (2.4.4). Dissolve 0.17 g in 5 ml of *alcohol R* and dilute to 15 ml with *water R*. The solution complies with the limit test for chlorides (300 ppm). When preparing the standard, replace the 5 ml of *water R* by 5 ml of *alcohol R*.

Water (2.5.12). Not more than 1.0 per cent, determined on 2.00 g by the semi-micro determination of water.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

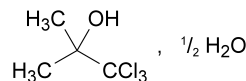
ASSAY

Dissolve 0.100 g in 20 ml of *alcohol R*. Add 10 ml of *dilute sodium hydroxide solution R*, heat in a water-bath for 5 min and cool. Add 20 ml of *dilute nitric acid R*, 25.0 ml of *0.1 M silver nitrate* and 2 ml of *dibutyl phthalate R* and shake vigorously. Add 2 ml of *ferric ammonium sulphate solution R2* and titrate with *0.1 M ammonium thiocyanate* until an orange colour is obtained.

1 ml of *0.1 M silver nitrate* is equivalent to 5.92 mg of $C_4H_7Cl_3O$.

CHLOROBUTANOL HEMIHYDRATE

Chlorobutanolum hemihydricum

 $C_4H_7Cl_3O \cdot \frac{1}{2}H_2O$ M_r 186.5**DEFINITION**

Chlorobutanol hemihydrate contains not less than 98.0 per cent and not more than the equivalent of 101.0 per cent of 1,1,1-trichloro-2-methylpropan-2-ol, calculated with reference to the anhydrous substance.

CHARACTERS

A white, crystalline powder or colourless crystals, sublimes readily, slightly soluble in water, very soluble in alcohol, soluble in glycerol (85 per cent).

It melts at about 78 °C, determined without previous drying.

IDENTIFICATION

- Add about 20 mg to a mixture of 1 ml of *pyridine R* and 2 ml of *strong sodium hydroxide solution R*. Heat in a water-bath and shake. Allow to stand. The pyridine layer becomes red.
- Add about 20 mg to 5 ml of *ammoniacal silver nitrate solution R* and warm slightly. A black precipitate is formed.
- To about 20 mg add 3 ml of *1 M sodium hydroxide* and shake to dissolve. Add 5 ml of *water R* and then, slowly, 2 ml of *iodinated potassium iodide solution R*. A yellowish precipitate is formed.
- It complies with the test for water (see Tests).

TESTS

Solution S. Dissolve 5 g in *alcohol R* and dilute to 10 ml with the same solvent.

Appearance of solution. Solution S is not more opalescent than reference suspension II (2.2.1) and not more intensely coloured than reference solution BY₅ (2.2.2, *Method II*).

Acidity. To 4 ml of solution S add 15 ml of *alcohol R* and 0.1 ml of *bromothymol blue solution R1*. Not more than 1.0 ml of *0.01 M sodium hydroxide* is required to change the colour of the indicator to blue.

Chlorides (2.4.4). To 1 ml of solution S add 4 ml of *alcohol R* and dilute to 15 ml with *water R*. The solution complies with the limit test for chlorides (100 ppm). When preparing the standard, replace the 5 ml of *water R* by 5 ml of *alcohol R*.

Water (2.5.12). 4.5 per cent to 5.5 per cent, determined on 0.300 g by the semi-micro determination of water.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.100 g in 20 ml of *alcohol R*. Add 10 ml of *dilute sodium hydroxide solution R*, heat in a water-bath for 5 min and cool. Add 20 ml of *dilute nitric acid R*, 25.0 ml of *0.1 M silver nitrate* and 2 ml of *dibutyl phthalate R* and

shake vigorously. Add 2 ml of *ferric ammonium sulphate solution R2* and titrate with 0.1 M *ammonium thiocyanate* until an orange colour is obtained.

1 ml of 0.1 M *silver nitrate* is equivalent to 5.92 mg of C_7H_7ClO .

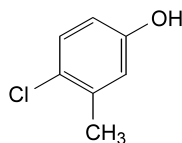
STORAGE

In an airtight container.

01/2005:0384

CHLOROCRESOL

Chlorocresolum



C_7H_7ClO

M_r 142.6

DEFINITION

Chlorocresol contains not less than 98.0 per cent and not more than the equivalent of 101.0 per cent of 4-chloro-3-methylphenol.

CHARACTERS

A white or almost white, crystalline powder or compacted crystalline masses supplied as pellets or colourless or white crystals, slightly soluble in water, very soluble in alcohol, freely soluble in fatty oils. It dissolves in solutions of alkali hydroxides.

IDENTIFICATION

- Melting point (2.2.14): 64 °C to 67 °C.
- To 0.1 g add 0.2 ml of *benzoyl chloride R* and 0.5 ml of *dilute sodium hydroxide solution R*. Shake vigorously until a white, crystalline precipitate is formed. Add 5 ml of *water R* and filter. The precipitate, recrystallised from 5 ml of *methanol R* and dried at 70 °C, melts (2.2.14) at 85 °C to 88 °C.
- To 5 ml of solution S (see Tests) add 0.1 ml of *ferric chloride solution R1*. A bluish colour is produced.

TESTS

Solution S. To 3.0 g, finely powdered, add 60 ml of *carbon dioxide-free water R*, shake for 2 min and filter.

Appearance of solution. Dissolve 1.25 g in *alcohol R* and dilute to 25 ml with the same solvent. The solution is clear (2.2.1) and not more intensely coloured than reference solution BY₆ (2.2.2, *Method II*).

Acidity. To 10 ml of solution S add 0.1 ml of *methyl red solution R*. The solution is orange or red. Not more than 0.2 ml of 0.01 M *sodium hydroxide* is required to produce a pure yellow colour.

Related substances. Examine by gas chromatography (2.2.28).

Test solution. Dissolve 1.0 g of the substance to be examined in *acetone R* and dilute to 100 ml with the same solvent.

The chromatographic procedure may be carried out using:

- a glass column 1.80 m long and 3 mm to 4 mm in internal diameter packed with *silanised diatomaceous earth for gas chromatography R* impregnated with 3 per cent *m/m* to 5 per cent *m/m* of *polymethylphenylsiloxane R*,

- nitrogen for chromatography R* as the carrier gas at a flow rate of 30 ml/min,

- a flame-ionisation detector,

maintaining the temperature of the column at 125 °C, that of the injection port at 210 °C and that of the detector at 230 °C. Continue the chromatography for three times the period of time (about 8 min) required for the appearance of the peak corresponding to chlorocresol. In the chromatogram obtained, the sum of the areas of the peaks apart from that corresponding to chlorocresol does not exceed 1 per cent of the total area of the peaks. Disregard the peak corresponding to the solvent.

Non-volatile matter. Evaporate 2.0 g to dryness on a water-bath and dry the residue at 100 °C to 105 °C. The residue weighs not more than 2 mg (0.1 per cent).

ASSAY

In a ground-glass-stoppered flask, dissolve 70.0 mg in 30 ml of *glacial acetic acid R*. Add 25.0 ml of 0.0167 M *potassium bromate*, 20 ml of a 150 g/l solution of *potassium bromide R* and 10 ml of *hydrochloric acid R*. Allow to stand protected from light for 15 min. Add 1 g of *potassium iodide R* and 100 ml of *water R*. Titrate with 0.1 M *sodium thiosulphate*, shaking vigorously and using 1 ml of *starch solution R*, added towards the end of the titration, as indicator. Carry out a blank titration.

1 ml of 0.0167 M *potassium bromate* is equivalent to 3.565 mg of C_7H_7ClO .

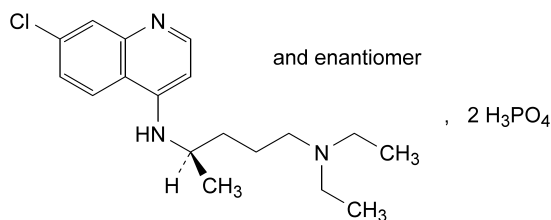
STORAGE

Store protected from light.

01/2005:0544

CHLOROQUINE PHOSPHATE

Chloroquini phosphas



$C_{18}H_{32}ClN_3O_8P_2$

M_r 515.9

DEFINITION

Chloroquine phosphate contains not less than 98.5 per cent and not more than the equivalent of 101.0 per cent of *N*⁴-(7-chloroquinolin-4-yl)-*N*²,*N*²-diethylpentane-1,4-diamine bis(dihydrogen phosphate), calculated with reference to the dried substance.

CHARACTERS

A white or almost white, crystalline powder, hygroscopic, freely soluble in water, very slightly soluble in alcohol and in methanol.

It exists in 2 forms, one of which melts at about 195 °C and the other at about 218 °C.

IDENTIFICATION

First identification: B, D.

Second identification: A, C, D.

- A. Dissolve 0.100 g in *water R* and dilute to 100.0 ml with the same solvent. Dilute 1.0 ml of this solution to 100.0 ml with *water R*. Examined between 210 nm and 370 nm (2.2.25), the solution shows absorption maxima at 220 nm, 235 nm, 256 nm, 329 nm and 342 nm. The specific absorbances at the maxima are respectively 600 to 660, 350 to 390, 300 to 330, 325 to 355 and 360 to 390.
- B. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with the base isolated from *chloroquine sulphate CRS*. Record the spectra using solutions prepared as follows: dissolve separately 0.1 g of the substance to be examined and 80 mg of the reference substance in 10 ml of *water R*, add 2 ml of *dilute sodium hydroxide solution R* and shake with 2 quantities, each of 20 ml, of *methylene chloride R*; combine the organic layers, wash with *water R*, dry over *anhydrous sodium sulphate R*, evaporate to dryness and dissolve the residues separately, each in 2 ml of *methylene chloride R*.
- C. Dissolve 25 mg in 20 ml of *water R* and add 8 ml of *picric acid solution R1*. The precipitate, washed with *water R*, with *alcohol R* and finally with *methylene chloride R*, melts (2.2.14) at 206–209 °C.
- D. Dissolve 0.1 g in 10 ml of *water R*, add 2 ml of *dilute sodium hydroxide solution R* and shake with 2 quantities, each of 20 ml, of *methylene chloride R*. The aqueous layer, acidified by the addition of *nitric acid R*, gives reaction (b) of phosphates (2.3.1).

TESTS

Solution S. Dissolve 2.5 g in *carbon dioxide-free water R* and dilute to 25 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and not more intensely coloured than reference solution BY₅ or GY₅ (2.2.2, *Method II*).

pH (2.2.3). The pH of solution S is 3.8 to 4.3.

Related substances. Examine by thin-layer chromatography (2.2.27), using *silica gel GF₂₅₄ R* as the coating substance.

Test solution. Dissolve 0.50 g of the substance to be examined in *water R* and dilute to 10 ml with the same solvent.

Reference solution (a). Dilute 1 ml of the test solution to 100 ml with *water R*.

Reference solution (b). Dilute 5 ml of reference solution (a) to 10 ml with *water R*.

Apply to the plate 2 µl of each solution. Develop over a path of 12 cm using a mixture of 10 volumes of *diethylamine R*, 40 volumes of *cyclohexane R* and 50 volumes of *chloroform R*. Allow the plate to dry in air. Examine in ultraviolet light at 254 nm. Any spot in the chromatogram obtained with the test solution, apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (a) (1.0 per cent) and not more than one such spot is more intense than the spot in the chromatogram obtained with reference solution (b) (0.5 per cent).

Heavy metals (2.4.8). Dissolve 2.0 g in 10 ml of *water R*. Add 5 ml of *concentrated ammonia R* and shake with 40 ml of *methylene chloride R*. Filter the aqueous layer and neutralise the filtrate with *glacial acetic acid R*. Heat on a water-bath to eliminate methylene chloride, allow to cool and dilute to 20.0 ml with *water R*. 12 ml of this solution complies with limit test A for heavy metals (20 ppm). Prepare the standard using *lead standard solution (2 ppm Pb R)*.

Loss on drying (2.2.32): maximum 2.0 per cent, determined on 1.000 g by drying in an oven at 100–105 °C.

ASSAY

Dissolve 0.200 g in 50 ml of *anhydrous acetic acid R*. Titrate with 0.1 M *perchloric acid* determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *perchloric acid* is equivalent to 25.79 mg of C₁₈H₂₈ClN₃O₄S·H₂O.

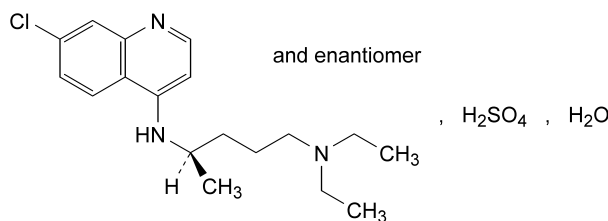
STORAGE

In an airtight container, protected from light.

01/2005:0545

CHLOROQUINE SULPHATE

Chloroquini sulfas

C₁₈H₂₈ClN₃O₄S·H₂OM_r 436.0

DEFINITION

Chloroquine sulphate contains not less than 98.5 per cent and not more than the equivalent of 101.0 per cent of *N*⁴-(7-chloroquinolin-4-yl)-*N*¹,*N*²-diethylpentane-1,4-diamine sulphate, calculated with reference to the anhydrous substance.

CHARACTERS

A white or almost white, crystalline powder, freely soluble in water and in methanol, very slightly soluble in alcohol.

It melts at about 208 °C (instantaneous method).

IDENTIFICATION

First identification: B, D.

Second identification: A, C, D.

- A. Dissolve 0.100 g in *water R* and dilute to 100.0 ml with the same solvent. Dilute 1.0 ml of this solution to 100.0 ml with *water R*. Examined between 210 nm and 370 nm (2.2.25), the solution shows absorption maxima at 220 nm, 235 nm, 256 nm, 329 nm and 342 nm. The specific absorbances at the maxima are respectively 730 to 810, 430 to 470, 370 to 410, 400 to 440 and 430 to 470.
- B. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with the base isolated from *chloroquine sulphate CRS*. Record the spectra using solutions prepared as follows: dissolve separately 0.1 g of the substance to be examined and of the reference substance in 10 ml of *water R*, add 2 ml of *dilute sodium hydroxide solution R* and shake with two quantities, each of 20 ml, of *chloroform R*; combine the chloroform layers, wash with *water R*, dry over *anhydrous sodium sulphate R*, evaporate to dryness and dissolve the residues separately each in 2 ml of *chloroform R*.
- C. Dissolve 25 mg in 20 ml of *water R* and add 8 ml of *picric acid solution R1*. The precipitate, washed with *water R*, with *alcohol R* and finally with *ether R*, melts (2.2.14) at 206 °C to 209 °C.
- D. It gives reaction (a) of sulphates (2.3.1).

TESTS

Solution S. Dissolve 2.0 g in *carbon dioxide-free water R* and dilute to 25 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and not more intensely coloured than reference solution BY₅ or GY₅ (2.2.2, *Method II*).

pH (2.2.3). The pH of solution S is 4.0 to 5.0.

Related substances. Examine by thin-layer chromatography (2.2.27), using *silica gel GF₂₅₄ R* as the coating substance.

Test solution. Dissolve 0.50 g of the substance to be examined in *water R* and dilute to 10 ml with the same solvent.

Reference solution (a). Dilute 1 ml of the test solution to 100 ml with *water R*.

Reference solution (b). Dilute 5 ml of reference solution (a) to 10 ml with *water R*.

Apply separately to the plate 2 µl of each solution. Develop over a path of 12 cm using a mixture of 10 volumes of *diethylamine R*, 40 volumes of *cyclohexane R* and 50 volumes of *chloroform R*. Allow the plate to dry in air. Examine in ultraviolet light at 254 nm. Any spot in the chromatogram obtained with the test solution, apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (a) (1.0 per cent) and not more than one such spot is more intense than the spot in the chromatogram obtained with reference solution (b) (0.5 per cent).

Heavy metals (2.4.8). Dissolve 2.0 g in 10 ml of *water R*. Add 5 ml of *concentrated ammonia R* and shake with 40 ml of *ether R*. Filter the aqueous layer and neutralise the filtrate with *glacial acetic acid R*. Heat on a water-bath to eliminate ether, allow to cool and dilute to 20.0 ml with *water R*. 12 ml of this solution complies with limit test A for heavy metals (20 ppm). Prepare the standard using *lead standard solution (2 ppm Pb) R*.

Water (2.5.12): 3.0 per cent to 5.0 per cent, determined on 0.500 g by the semi-micro determination of water.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.400 g in 50 ml of *anhydrous acetic acid R*. Titrate with 0.1 M *perchloric acid* determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *perchloric acid* is equivalent to 41.8 mg of C₇H₆ClN₃O₄S₂.

STORAGE

Store in an airtight container, protected from light.

DEFINITION

Chlorothiazide contains not less than 98.0 per cent and not more than the equivalent of 102.0 per cent of 6-chloro-2H-1,2,4-benzothiadiazine-7-sulphonamide 1,1-dioxide, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, crystalline powder, very slightly soluble in water, sparingly soluble in acetone, slightly soluble in alcohol. It dissolves in dilute solutions of alkali hydroxides.

IDENTIFICATION

First identification: B, C.

Second identification: A, C, D.

A. Dissolve 80.0 mg in 100 ml of 0.1 M *sodium hydroxide* and dilute to 1000.0 ml with *water R*. Dilute 10.0 ml of the solution to 100.0 ml with 0.01 M *sodium hydroxide*. Examined between 220 nm and 320 nm (2.2.25), the solution shows 2 absorption maxima, at 225 nm and 292 nm, and a shoulder at about 310 nm. The specific absorbances at the maxima are 725 to 800 and 425 to 455, respectively.

B. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *chlorothiazide CRS*.

C. Examine by thin-layer chromatography (2.2.27), using *silica gel GF₂₅₄ R* as the coating substance.

Test solution. Dissolve 25 mg of the substance to be examined in *acetone R* and dilute to 5 ml with the same solvent.

Reference solution. Dissolve 25 mg of *chlorothiazide CRS* in *acetone R* and dilute to 5 ml with the same solvent.

Apply to the plate 2 µl of each solution. Develop over a path of 10 cm using *ethyl acetate R*. Dry the plate in a current of air and examine in ultraviolet light at 254 nm. The principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with the reference solution.

D. To 0.1 g add a pellet of *sodium hydroxide R* and heat strongly. Gas is evolved which turns *red litmus paper R* blue. After cooling, take up the residue with 10 ml of *dilute hydrochloric acid R*. Gas is evolved which turns *lead acetate paper R* black.

TESTS

Solution S. To 1.0 g of the powdered substance to be examined add 50 ml of *water R*, shake for 2 min and filter.

Acidity or alkalinity. To 10 ml of solution S add 0.2 ml of 0.01 M *sodium hydroxide* and 0.15 ml of *methyl red solution R*. The solution is yellow. Not more than 0.4 ml of 0.01 M *hydrochloric acid* is required to change the colour of the indicator to red.

Related substances. Examine by thin-layer chromatography (2.2.27), using *silica gel G R* as the coating substance.

Test solution. Dissolve 25 mg of the substance to be examined in *acetone R* and dilute to 5 ml with the same solvent.

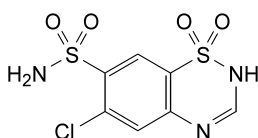
Reference solution. Dilute 1 ml of the test solution to 100 ml with *acetone R*.

Apply to the plate 5 µl of each solution. Develop over a path of 15 cm using a mixture of 15 volumes of *2-propanol R* and 85 volumes of *ethyl acetate R*. Dry the plate in a current of air until the solvents have evaporated (about 10 min) and spray with a mixture of equal volumes of *alcoholic solution*

01/2005:0385

CHLOROTHIAZIDE

Chlorothiazidum

C₇H₆ClN₃O₄S₂M_r 295.7

of *sulphuric acid R* and *alcohol R*; use about 10 ml for a plate 200 mm square and spray in small portions, allowing the solvent to evaporate each time to avoid excessive wetting. Heat at 100–105 °C for 30 min and immediately place the plate above, but not in contact with, 10 ml of a saturated solution of *sodium nitrite R* in a glass tank. Carefully add 0.5 ml of *sulphuric acid R* to the sodium nitrite solution, close the tank, and allow to stand for 15 min. Remove the plate, heat in a ventilated oven at 40 °C for 15 min and spray with 3 quantities, each of 5 ml, of a freshly prepared 5 g/l solution of *naphthylethylenediamine dihydrochloride R* in *alcohol R*. Examine the plate by transmitted light. Any spot in the chromatogram obtained with the test solution, apart from the principal spot, is not more intense than the spot in the chromatogram obtained with the reference solution (1.0 per cent).

Chlorides (2.4.4). 15 ml of solution S complies with the limit test for chlorides (160 ppm).

Heavy metals (2.4.8). 1.0 g complies with limit test C for heavy metals (20 ppm). Prepare the standard using 2 ml of *lead standard solution (10 ppm Pb) R*.

Loss on drying (2.2.32). Not more than 1.0 per cent, determined on 1.000 g by drying in an oven at 100–105 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

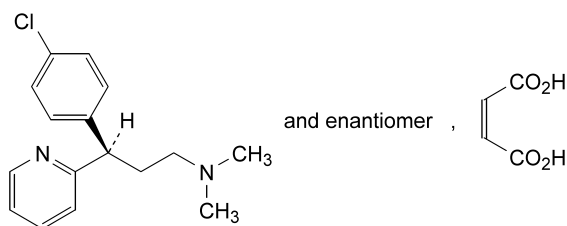
Dissolve 0.250 g in 50 ml of *dimethylformamide R*. Titrate with 0.1 M *tetrabutylammonium hydroxide in 2-propanol* determining the end-point potentiometrically (2.2.20) at the first point of inflexion. Carry out a blank titration.

1 ml of 0.1 M *tetrabutylammonium hydroxide in 2-propanol* is equivalent to 29.57 mg of $C_{20}H_{23}ClN_2O_4$.

01/2005:0386

CHLORPHENAMINE MALEATE

Chlorphenamini maleas



$C_{20}H_{23}ClN_2O_4$

M_r 390.9

DEFINITION

Chlorphenamine maleate contains not less than 98.0 per cent and not more than the equivalent of 101.0 per cent of (3*RS*)-3-(4-chlorophenyl)-*N,N*-dimethyl-3-(pyridin-2-yl)propan-1-amine hydrogen (*Z*)-butenedioate, calculated with reference to the dried substance.

CHARACTERS

A white, crystalline powder, freely soluble in water, soluble in alcohol.

IDENTIFICATION

First identification: A, C.

Second identification: A, B, D, E.

A. Melting point (2.2.14): 132 °C to 135 °C.

- B. Dissolve 30.0 mg in 0.1 M *hydrochloric acid* and dilute to 100.0 ml with the same acid. Dilute 10.0 ml of this solution to 100.0 ml with 0.1 M *hydrochloric acid*. Examined between 230 nm and 350 nm (2.2.25), the solution shows an absorption maximum at 265 nm. The specific absorbance at the maximum is 200 to 220.
- C. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *chlorphenamine maleate CRS*. Examine the substances prepared as discs.
- D. Dissolve 0.1 g in 10 ml of *water R* and add dropwise and with shaking 25 ml of *picric acid solution R*. Collect the precipitate on a sintered-glass filter. The precipitate, washed with 3 ml of *alcohol R*, recrystallised from a mixture of equal volumes of *alcohol R* and *water R* and dried at 100 °C to 105 °C, melts (2.2.14) at 196 °C to 200 °C.
- E. To 0.2 g add 3 ml of *water R* and 1 ml of *strong sodium hydroxide solution R*. Shake with three quantities, each of 5 ml, of *ether R*. To 0.1 ml of the aqueous layer add a solution of 10 mg of *resorcinol R* in 3 ml of *sulphuric acid R*. Heat on a water-bath for 15 min. No colour develops. To the remainder of the aqueous layer add 2 ml of *bromine solution R*. Heat in a water-bath for 15 min, heat to boiling and cool. To 0.2 ml of the solution add a solution of 10 mg of *resorcinol R* in 3 ml of *sulphuric acid R* and heat on a water-bath for 15 min. A blue colour develops.

TESTS

Solution S. Dissolve 2.0 g in *water R* and dilute to 20 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and not more intensely coloured than reference solution BY₆ (2.2.2, *Method II*).

Related substances. Examine by thin-layer chromatography (2.2.27), using *silica gel GF₂₅₄ R* as the coating substance.

Test solution. Dissolve 0.5 g of the substance to be examined in *chloroform R* and dilute to 10 ml with the same solvent.

Reference solution. Dilute 1 ml of the test solution to 50 ml with *chloroform R*. Dilute 1 ml of this solution to 10 ml with *chloroform R*.

Apply to the plate 10 µl of each solution. Develop over a path of 12 cm using a mixture of 10 volumes of *diethylamine R*, 40 volumes of *chloroform R* and 50 volumes of *cyclohexane R*. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. Any spot in the chromatogram obtained with the test solution, apart from the principal spot, is not more intense than the spot in the chromatogram obtained with the reference solution (0.2 per cent). Disregard any spots remaining on the starting-line.

Heavy metals (2.4.8). 1.0 g complies with limit test C for heavy metals (20 ppm). Prepare the standard using 2 ml of *lead standard solution (10 ppm Pb) R*.

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.00 g by drying in an oven at 100 °C to 105 °C for 4 h.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.150 g in 25 ml of *anhydrous acetic acid R*. Titrate with 0.1 M *perchloric acid* determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *perchloric acid* is equivalent to 19.54 mg of $C_{20}H_{23}ClN_2O_4$.

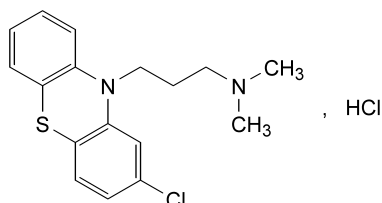
STORAGE

Store protected from light.

01/2005:0475

CHLORPROMAZINE HYDROCHLORIDE

Chlorpromazini hydrochloridum


 $C_{17}H_{20}Cl_2N_2S$
 M_r 355.3

DEFINITION

Chlorpromazine hydrochloride contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of 3-(2-chloro-10*H*-phenothiazin-10-yl)-*N,N*-dimethylpropan-1-amine hydrochloride, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, crystalline powder, very soluble in water, freely soluble in alcohol. It decomposes on exposure to air and light.

It melts at about 196 °C.

IDENTIFICATION

First identification: B, C, D.

Second identification: A, C, D.

- Prepare the solutions protected from bright light and measure the absorbances immediately. Dissolve 50.0 mg in 0.1 *M* hydrochloric acid and dilute to 500.0 ml with the same acid. Dilute 5.0 ml of the solution to 100.0 ml with 0.1 *M* hydrochloric acid. Examined between 230 nm and 340 nm (2.2.25), the solution shows two absorption maxima, at 254 nm and 306 nm respectively. The specific absorbance at the maximum at 254 nm is 890 to 960.
- Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with chlorpromazine hydrochloride CRS. Examine the substances as 60 g/l solutions in methylene chloride *R* using a 0.1 mm cell.
- It complies with the identification test for phenothiazines by thin-layer chromatography (2.3.3).
- It gives reaction (b) of chlorides (2.3.1).

TESTS

pH (2.2.3). Dissolve 1.0 g in carbon dioxide-free water *R* and dilute to 10 ml with the same solvent. The pH of the freshly prepared solution is 3.5 to 4.5.

Related substances. Carry out the test protected from bright light.

Examine by thin-layer chromatography (2.2.27), using silica gel GF₂₅₄ *R* as the coating substance.

Test solution. Dissolve 0.2 g of the substance to be examined in a mixture of 5 volumes of diethylamine *R* and 95 volumes of methanol *R* and dilute to 10 ml with the same mixture of solvents. Prepare immediately before use.

Reference solution. Dilute 1 ml of the test solution to 200 ml with a mixture of 5 volumes of diethylamine *R* and 95 volumes of methanol *R*.

Apply to the plate 10 µl of each solution. Develop over a path of 15 cm using a mixture of 10 volumes of acetone *R*, 10 volumes of diethylamine *R* and 80 volumes of cyclohexane *R*. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. Any spot in the chromatogram obtained with the test solution, apart from the principal spot, is not more intense than the spot in the chromatogram obtained with the reference solution (0.5 per cent). Disregard any spot at the starting point.

Heavy metals (2.4.8). 1.0 g complies with limit test C for heavy metals (10 ppm). Prepare the standard using 1 ml of lead standard solution (10 ppm Pb) *R*.

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.250 g in a mixture of 5.0 ml of 0.01 *M* hydrochloric acid and 50 ml of alcohol *R*. Carry out a potentiometric titration (2.2.20), using 0.1 *M* sodium hydroxide. Read the volume of 0.1 *M* sodium hydroxide added between the two points of inflexion.

1 ml of 0.1 *M* sodium hydroxide is equivalent to 35.53 mg of C₁₇H₂₀Cl₂N₂S.

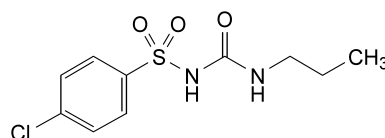
STORAGE

Store in an airtight container, protected from light.

01/2005:1087

CHLORPROPAMIDE

Chlorpropamidum


 $C_{10}H_{13}ClN_2O_3S$
 M_r 276.7

DEFINITION

Chlorpropamide contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of 1-[(4-chlorophenyl)sulphonyl]-3-propylurea, calculated with reference to the dried substance.

CHARACTERS

A white, crystalline powder, practically insoluble in water, freely soluble in acetone and in methylene chloride, soluble in alcohol. It dissolves in dilute solutions of alkali hydroxides. It shows polymorphism.

IDENTIFICATION

First identification: C, D.

Second identification: A, B, D.

A. Melting point (2.2.14): 126 °C to 130 °C.

B. Dissolve 0.10 g in methanol *R* and dilute to 50.0 ml with the same solvent. Dilute 5.0 ml of the solution to 100.0 ml with 0.01 *M* hydrochloric acid. Dilute 10.0 ml of the solution to 100.0 ml with 0.01 *M* hydrochloric

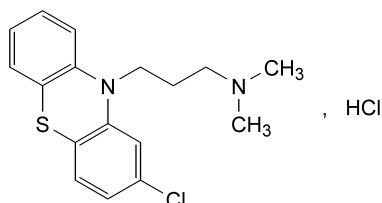
STORAGE

Store protected from light.

01/2005:0475

CHLORPROMAZINE HYDROCHLORIDE

Chlorpromazini hydrochloridum


 $C_{17}H_{20}Cl_2N_2S$
 M_r 355.3

DEFINITION

Chlorpromazine hydrochloride contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of 3-(2-chloro-10*H*-phenothiazin-10-yl)-*N,N*-dimethylpropan-1-amine hydrochloride, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, crystalline powder, very soluble in water, freely soluble in alcohol. It decomposes on exposure to air and light.

It melts at about 196 °C.

IDENTIFICATION

First identification: B, C, D.

Second identification: A, C, D.

- Prepare the solutions protected from bright light and measure the absorbances immediately. Dissolve 50.0 mg in 0.1 *M* hydrochloric acid and dilute to 500.0 ml with the same acid. Dilute 5.0 ml of the solution to 100.0 ml with 0.1 *M* hydrochloric acid. Examined between 230 nm and 340 nm (2.2.25), the solution shows two absorption maxima, at 254 nm and 306 nm respectively. The specific absorbance at the maximum at 254 nm is 890 to 960.
- Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with chlorpromazine hydrochloride CRS. Examine the substances as 60 g/l solutions in methylene chloride *R* using a 0.1 mm cell.
- It complies with the identification test for phenothiazines by thin-layer chromatography (2.3.3).
- It gives reaction (b) of chlorides (2.3.1).

TESTS

pH (2.2.3). Dissolve 1.0 g in carbon dioxide-free water *R* and dilute to 10 ml with the same solvent. The pH of the freshly prepared solution is 3.5 to 4.5.

Related substances. Carry out the test protected from bright light.

Examine by thin-layer chromatography (2.2.27), using silica gel GF₂₅₄ *R* as the coating substance.

Test solution. Dissolve 0.2 g of the substance to be examined in a mixture of 5 volumes of diethylamine *R* and 95 volumes of methanol *R* and dilute to 10 ml with the same mixture of solvents. Prepare immediately before use.

Reference solution. Dilute 1 ml of the test solution to 200 ml with a mixture of 5 volumes of diethylamine *R* and 95 volumes of methanol *R*.

Apply to the plate 10 µl of each solution. Develop over a path of 15 cm using a mixture of 10 volumes of acetone *R*, 10 volumes of diethylamine *R* and 80 volumes of cyclohexane *R*. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. Any spot in the chromatogram obtained with the test solution, apart from the principal spot, is not more intense than the spot in the chromatogram obtained with the reference solution (0.5 per cent). Disregard any spot at the starting point.

Heavy metals (2.4.8). 1.0 g complies with limit test C for heavy metals (10 ppm). Prepare the standard using 1 ml of lead standard solution (10 ppm Pb) *R*.

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.250 g in a mixture of 5.0 ml of 0.01 *M* hydrochloric acid and 50 ml of alcohol *R*. Carry out a potentiometric titration (2.2.20), using 0.1 *M* sodium hydroxide. Read the volume of 0.1 *M* sodium hydroxide added between the two points of inflexion.

1 ml of 0.1 *M* sodium hydroxide is equivalent to 35.53 mg of $C_{17}H_{20}Cl_2N_2S$.

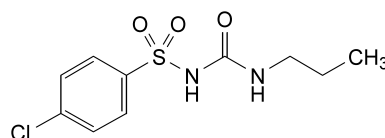
STORAGE

Store in an airtight container, protected from light.

01/2005:1087

CHLORPROPAMIDE

Chlorpropamidum


 $C_{10}H_{13}ClN_2O_3S$
 M_r 276.7

DEFINITION

Chlorpropamide contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of 1-[(4-chlorophenyl)sulphonyl]-3-propylurea, calculated with reference to the dried substance.

CHARACTERS

A white, crystalline powder, practically insoluble in water, freely soluble in acetone and in methylene chloride, soluble in alcohol. It dissolves in dilute solutions of alkali hydroxides. It shows polymorphism.

IDENTIFICATION

First identification: C, D.

Second identification: A, B, D.

A. Melting point (2.2.14): 126 °C to 130 °C.

B. Dissolve 0.10 g in methanol *R* and dilute to 50.0 ml with the same solvent. Dilute 5.0 ml of the solution to 100.0 ml with 0.01 *M* hydrochloric acid. Dilute 10.0 ml of the solution to 100.0 ml with 0.01 *M* hydrochloric

acid. Examined between 220 nm and 350 nm (2.2.25), the solution shows an absorption maximum at 232 nm. The specific absorption at the maximum is 570 to 630.

- C. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *chlorpropamide CRS*. Examine the substances prepared as discs. If the spectra obtained show differences, dissolve the substance to be examined and the reference substance in *methylene chloride R*, evaporate to dryness and record the new spectra using the residues.
- D. Heat 0.1 g with 2 g of *anhydrous sodium carbonate R* until a dull red colour appears for 10 min. Allow to cool, extract the residue with about 5 ml of *water R*, dilute to 10 ml with *water R* and filter. The solution gives the reaction (a) of chloride (2.3.1).

TESTS

Related substances. Examine by thin-layer chromatography (2.2.27), using a suitable silica gel as the coating substance.

Test solution. Dissolve 0.50 g of the substance to be examined in *acetone R* and dilute to 10 ml with the same solvent.

Reference solution (a). Dissolve 15 mg of *4-chlorobenzenesulphonamide R* (chlorpropamide impurity A) in *acetone R* and dilute to 100 ml with the same solvent.

Reference solution (b). Dissolve 15 mg of *chlorpropamide impurity B CRS* in *acetone R* and dilute to 100 ml with the same solvent.

Reference solution (c). Dilute 0.3 ml of the test solution to 100 ml with *acetone R*.

Reference solution (d). Dilute 5 ml of reference solution (c) to 15 ml with *acetone R*.

Reference solution (e). Dissolve 0.10 g of the substance to be examined, 5 mg of *4-chlorobenzenesulphonamide R* and 5 mg of *chlorpropamide impurity B CRS* in *acetone R* and dilute to 10 ml with the same solvent.

Apply to the plate 5 µl of each solution. Develop over a path of 15 cm using a mixture of 11.5 volumes of *concentrated ammonia R*, 30 volumes of *cyclohexane R*, 50 volumes of *methanol R* and 100 volumes of *methylene chloride R*. Allow the plate to dry in a current of cold air, heat at 110 °C for 10 min. At the bottom of a chromatographic tank, place an evaporating dish containing a mixture of 1 volume of *hydrochloric acid R*, 1 volume of *water R* and 2 volumes of a 50 g/l solution of *potassium permanganate R*, close the tank and allow to stand for 15 min. Place the dried hot plate in the tank and close the tank. Leave the plate in contact with the chlorine vapour for 2 min. Withdraw the plate and place it in a current of cold air until the excess of chlorine is removed and an area of coating below the points of application does not give a blue colour with a drop of *potassium iodide and starch solution R*. Spray with *potassium iodide and starch solution R*. In the chromatogram obtained with the test solution: any spot corresponding to impurity A is not more intense than the spot in the chromatogram obtained with reference solution (a) (0.3 per cent); any spot corresponding to impurity B is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.3 per cent); any spot, apart from the principal spot and any spot corresponding to impurity A and B, is not more intense than the spot in the chromatogram obtained with reference solution (c) (0.3 per cent); not more than two such spots are more intense than the spot in the chromatogram obtained with reference solution (d) (0.1 per cent). The test is not valid unless the chromatogram obtained with reference

solution (e) shows three clearly separated spots with approximate R_f values of 0.4, 0.6 and 0.9 corresponding to chlorpropamide, impurity A and impurity B respectively.

Heavy metals (2.4.8). Dissolve 2.0 g in a mixture of 15 volumes of *water R* and 85 volumes of *acetone R* and dilute to 20 ml with the same mixture of solvents. 12 ml of solution complies with limit test B for heavy metals (20 ppm). Prepare the standard using lead standard solution (2 ppm Pb) prepared by diluting *lead standard solution (100 ppm Pb) R* with a mixture of 15 volumes of *water R* and 85 volumes of *acetone R*.

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

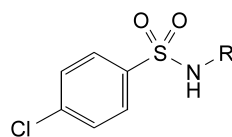
Dissolve 0.250 g in 50 ml of *alcohol R* previously neutralised using *phenolphthalein solution R1* as indicator and add 25 ml of *water R*. Titrate with 0.1 M *sodium hydroxide* until a pink colour is obtained.

1 ml of 0.1 M *sodium hydroxide* is equivalent to 27.67 mg of $C_{10}H_{13}ClN_2O_3S$.

STORAGE

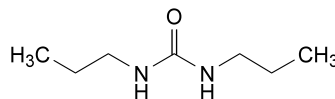
Store protected from light.

IMPURITIES



A. R = H: 4-chlorobenzenesulphonamide,

C. R = CO-NH₂: [(4-chlorophenyl)sulphonyl]urea.

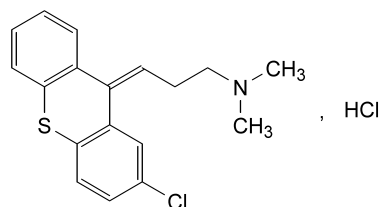


B. 1,3-dipropylurea,

01/2005:0815

CHLORPROTHIXENE HYDROCHLORIDE

Chlorprothixeni hydrochloridum



$C_{18}H_{19}Cl_2NS$

M_r 352.3

DEFINITION

(Z)-3-(2-Chloro-9H-thioxanthen-9-ylidene)-N,N-dimethylpropan-1-amine hydrochloride.

Content: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

Appearance: white or almost white, crystalline powder.

Solubility: soluble in water and in alcohol, slightly soluble in methylene chloride.

mp: about 220 °C.

IDENTIFICATION

First identification: A, E.

Second identification: B, C, D, E.

A. Infrared absorption spectrophotometry (2.2.24).

Preparation: dissolve 0.25 g in 10 ml of *water R*. Add 1 ml of *dilute sodium hydroxide solution R*. Shake with 20 ml of *methylene chloride R*. Separate the organic layer and wash with 5 ml of *water R*. Evaporate the organic layer to dryness and dry the residue at 40-50 °C. Examine the residues prepared as discs.

Comparison: *chlorprothixene hydrochloride CRS*.

B. Dissolve 0.2 g in a mixture of 5 ml of *dioxan R* and 5 ml of a 1.5 g/l solution of *sodium nitrite R*. Add 0.8 ml of *nitric acid R*. After 10 min add the solution to 20 ml of *water R*. 1 h later filter the precipitate formed. The filtrate is used immediately for identification test C. Dissolve the precipitate by warming in about 15 ml of *alcohol R* and add the solution to 10 ml of *water R*. Filter and dry the precipitate at 100-105 °C for 2 h. The melting point (2.2.14) is 152 °C to 154 °C.

C. To 1 ml of the filtrate obtained in identification test B, add 0.2 ml of a suspension of 50 mg of *fast red B salt R* in 1 ml of *alcohol R*. Add 1 ml of 0.5 M *alcoholic potassium hydroxide*. A dark red colour is produced. Carry out a blank test.

D. Dissolve about 20 mg in 2 ml of *nitric acid R*. A red colour is produced. Add 5 ml of *water R* and examine in ultraviolet light at 365 nm. The solution shows green fluorescence.

E. It gives reaction (a) of chlorides (2.3.1).

TESTS

Solution S. Dissolve 0.25 g in *carbon dioxide-free water R* and dilute to 25 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and colourless (2.2.2, *Method II*).

pH (2.2.3): 4.4 to 5.2 for solution S.

Related substances. Liquid chromatography (2.2.29). *Carry out the test protected from bright light.*

Test solution. Dissolve 20.0 mg of the substance to be examined in the mobile phase and dilute to 20.0 ml with the mobile phase.

Reference solution (a). Dissolve 20.0 mg of *chlorprothixene hydrochloride CRS* (with a defined content of *E*-isomer) in the mobile phase and dilute to 20.0 ml with the mobile phase.

Reference solution (b). Dilute 2.0 ml of the test solution to 100.0 ml with the mobile phase. Dilute 3.0 ml of this solution to 20.0 ml with the mobile phase.

Column:

- **size:** $l = 0.12$ m, $\varnothing = 4.0$ mm,
- **stationary phase:** base-deactivated octadecylsilyl silica gel for chromatography *R* (3 µm or 5 µm).

Mobile phase: solution containing 6.0 g/l of *potassium dihydrogen phosphate R*, 2.9 g/l of *sodium laurilsulfate R* and 9 g/l of *tetrabutylammonium bromide R* in a mixture of 50 volumes of *methanol R*, 400 volumes of *acetonitrile R* and 550 volumes of *distilled water R*.

Flow rate: 1.5 ml/min.

Detection: spectrophotometer at 254 nm.

Equilibration: for about 30 min with the mobile phase.

Injection: 20 µl.

Run time: twice the retention time of chlorprothixene.

Relative retention with reference to chlorprothixene: impurity E = about 1.55.

System suitability: reference solution (a):

- **retention time:** chlorprothixene = about 10 min,
- **relative retention** with reference to chlorprothixene: *E*-isomer = about 1.35.

Limits:

- ***E*-isomer:** not more than 2.0 per cent, calculated from the area of the corresponding peak in the chromatogram obtained with reference solution (a) and taking into account the assigned content of this isomer in *chlorprothixene hydrochloride CRS*,
- **impurity E:** not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.3 per cent taking into account a response factor of 3),
- **any other impurity:** not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.3 per cent),
- **total of any other impurity:** not more than 2.33 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.7 per cent),
- **disregard limit:** 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.03 per cent).

Heavy metals (2.4.8): maximum 20 ppm.

1.0 g complies with limit test F. Prepare the standard using 2 ml of *lead standard solution (10 ppm Pb) R*.

Loss on drying (2.2.32): maximum 0.5 per cent, determined on 1.000 g by drying *in vacuo* at 60 °C for 3 h.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.300 g in a mixture of 5.0 ml of 0.01 M *hydrochloric acid* and 50 ml of *alcohol R*. Carry out a potentiometric titration (2.2.20), using 0.1 M *sodium hydroxide*. Read the volume added between the 2 points of inflexion.

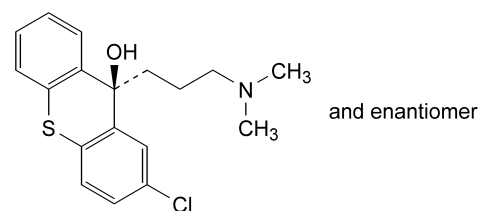
1 ml of 0.1 M *sodium hydroxide* is equivalent to 35.23 mg of $C_{18}H_{19}Cl_2NS$.

STORAGE

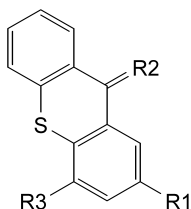
Protected from light.

IMPURITIES

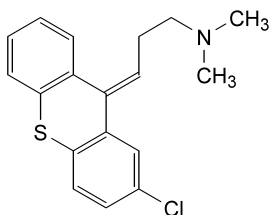
Specified impurities: A, B, C, D, E, F.



A. (RS)-2-chloro-9-[3-(dimethylamino)propyl]-9H-thioxanthen-9-ol,



- B. R1 = H, R2 = CH-CH₂-CH₂-N(CH₃)₂, R3 = H: *N,N*-dimethyl-3-(9*H*-thioxanthen-9-ylidene)propan-1-amine,
- C. R1 = Cl, R2 = CH-CH₂-CH₂-NH-CH₃, R3 = H: (*Z*)-3-(2-chloro-9*H*-thioxanthen-9-ylidene)-*N*-methylpropan-1-amine,
- D. R1 = H, R2 = CH-CH₂-CH₂-N(CH₃)₂, R3 = Cl: (*Z*)-3-(4-chloro-9*H*-thioxanthen-9-ylidene)-*N,N*-dimethylpropan-1-amine,
- E. R1 = Cl, R2 = O, R3 = H: 2-chloro-9*H*-thioxanthen-9-one,

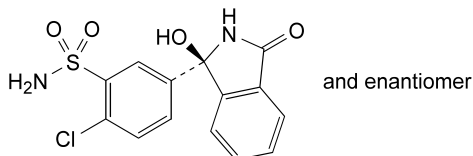


- F. (*E*)-3-(2-chloro-9*H*-thioxanthen-9-ylidene)-*N,N*-dimethylpropan-1-amine (*E*-isomer).

01/2005:0546

CHLORTALIDONE

Chlortalidonum

C₁₄H₁₁ClN₂O₄SM_r 338.8

DEFINITION

Chlortalidone contains not less than 98.0 per cent and not more than the equivalent of 102.0 per cent of 2-chloro-5-[(1*R*S)-1-hydroxy-3-oxo-2,3-dihydro-1*H*-isoindol-1-yl]benzenesulphonamide, calculated with reference to the dried substance.

CHARACTERS

A white or yellowish-white powder, practically insoluble in water, soluble in acetone and in methanol, slightly soluble in alcohol, practically insoluble in methylene chloride. It dissolves in dilute solutions of the alkali hydroxides.

It melts at about 220 °C, with decomposition.

IDENTIFICATION

First identification: B.

Second identification: A, C, D, E.

- A. Dissolve 50.0 mg in *alcohol R* and dilute to 50.0 ml with the same solvent. Dilute 10.0 ml of this solution to 100.0 ml with *alcohol R*. Examined between 230 nm and 340 nm (2.2.25), the solution shows 2 absorption maxima, at 275 nm and 284 nm. The ratio of the absorbance measured at the absorption maximum at 284 nm to that measured at the absorption maximum at 275 nm is 0.73 to 0.88.

- B. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *chlortalidone CRS*. Examine the substances prepared as discs using *potassium bromide R*.

- C. Examine by thin-layer chromatography (2.2.27), using *silica gel GF₂₅₄ R* as the coating substance.

Test solution. Dissolve 10 mg of the substance to be examined in *acetone R* and dilute to 10 ml with the same solvent.

Reference solution (a). Dissolve 10 mg of *chlortalidone CRS* in *acetone R* and dilute to 10 ml with the same solvent.

Reference solution (b). Dissolve 10 mg of *chlortalidone CRS* and 10 mg of *hydrochlorothiazide CRS* in *acetone R* and dilute to 10 ml with the same solvent.

Apply separately to the plate 5 µl of each solution. Develop over a path of 10 cm using a mixture of 1.5 volumes of *water R* and 98.5 volumes of *ethyl acetate R*. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. The principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a). The test is not valid unless the chromatogram obtained with reference solution (b) shows 2 clearly separated spots.

- D. Dissolve about 10 mg in 1 ml of *sulphuric acid R*. An intense yellow colour develops.
- E. It complies with the test for optical rotation (see Tests).

TESTS

Appearance of solution. Dissolve 1.0 g in *dilute sodium hydroxide solution R* and dilute to 10 ml with the same solution. The solution is clear (2.2.1) and not more intensely coloured than intensity 6 of the range of reference solutions of the most appropriate colour (2.2.2, *Method II*).

Acidity. Dissolve 1.0 g with heating in a mixture of 25 ml of *acetone R* and 25 ml of *carbon dioxide-free water R*. Cool. Titrate with 0.1 *M sodium hydroxide*, determining the end-point potentiometrically (2.2.20). Not more than 0.75 ml of 0.1 *M sodium hydroxide* is required.

Optical rotation (2.2.7). Dissolve 0.20 g in *methanol R* and dilute to 20 ml with the same solvent. The angle of optical rotation is −0.15° to +0.15°.

Related substances. Examine by thin-layer chromatography (2.2.27), using as the coating substance a suitable silica gel with a fluorescent indicator having an optimal intensity at 254 nm.

Test solution. Dissolve 0.2 g of the substance to be examined in a mixture of 1 volume of *water R* and 4 volumes of *acetone R* and dilute to 5 ml with the same mixture of solvents.

Reference solution (a). Dissolve 20 mg of *chlortalidone impurity B CRS* and 20 mg of *chlortalidone CRS* in a mixture of 1 volume of *water R* and 4 volumes of *acetone R* and dilute to 50 ml with the same mixture of solvents.

Reference solution (b). Dilute 1 ml of the test solution to 200 ml with a mixture of 1 volume of *water R* and 4 volumes of *acetone R*.

Apply separately to the plate 5 µl of each solution. Develop over a path of 15 cm using a mixture of 5 volumes of *toluene R*, 10 volumes of *xylene R*, 20 volumes of *concentrated ammonia R*, 30 volumes of *dioxan R* and 30 volumes of *2-propanol R*. Allow the plate to dry in a current of warm air and examine in ultraviolet light at 254 nm. In the chromatogram obtained with the test solution: any spot corresponding to impurity B is not more

01/2005:0173

intense than the corresponding spot in the chromatogram obtained with reference solution (a) (1 per cent); and any spot, apart from the principal spot and the spot corresponding to impurity B, is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.5 per cent). The test is not valid unless the chromatogram obtained with reference solution (a) shows 2 clearly separated spots.

Chlorides (2.4.4). Triturate 0.3 g, add 30 ml of *water R*, shake for 5 min and filter. 15 ml of the filtrate complies with the limit test for chlorides (350 ppm). Prepare the standard using 10 ml of *chloride standard solution (5 ppm Cl) R*.

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 100–105 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

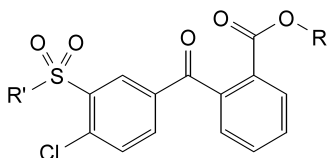
ASSAY

Dissolve 0.200 g in 50 ml of *acetone R*. In an atmosphere of nitrogen, titrate with 0.1 *M tetrabutylammonium hydroxide*, determining the end-point potentiometrically (2.2.20). Carry out a blank titration.

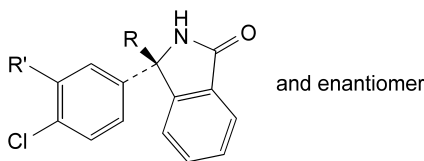
1 ml of 0.1 *M tetrabutylammonium hydroxide* is equivalent to 33.88 mg of $C_{14}H_{11}ClN_2O_4S$.

IMPURITIES

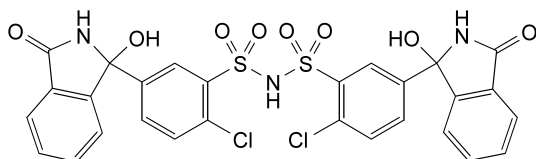
Specified impurities: A, B, C, D, E, F, G, H, I.



- A. $R = H$, $R' = OH$: 2-(4-chloro-3-sulphobenzoyl)benzoic acid,
 B. $R = H$, $R' = NH_2$: 2-(4-chloro-3-sulphamoylbenzoyl)benzoic acid,
 C. $R = C_2H_5$, $R' = NH_2$: ethyl 2-(4-chloro-3-sulphamoylbenzoyl)benzoate,
 I. $R = CH(CH_3)_2$, $R' = NH_2$: 1-methylethyl 2-(4-chloro-3-sulphamoylbenzoyl)benzoate,



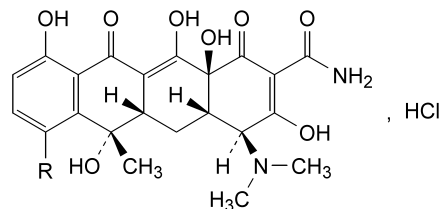
- D. $R = OC_2H_5$, $R' = SO_2NH_2$: 2-chloro-5-[(1*RS*)-1-ethoxy-3-oxo-2,3-dihydro-1*H*-isoindol-1-yl]benzenesulphonamide,
 E. $R = H$, $R' = SO_2NH_2$: 2-chloro-5-[(1*RS*)-3-oxo-2,3-dihydro-1*H*-isoindol-1-yl]benzenesulphonamide,
 G. $R = OH$, $R' = Cl$: (3*RS*)-3-(3,4-dichlorophenyl)-3-hydroxy-2,3-dihydro-1*H*-isoindol-1-one,
 H. $R = OCH(CH_3)_2$, $R' = SO_2NH_2$: 2-chloro-5-[(1*RS*)-1-(1-methylethoxy)-3-oxo-2,3-dihydro-1*H*-isoindol-1-yl]benzenesulphonamide,



- F. bis[2-chloro-5-(1-hydroxy-3-oxo-2,3-dihydro-1*H*-isoindol-1-yl)benzenesulfonyl]amine.

CHLORTETRACYCLINE HYDROCHLORIDE

Chlortetracyclini hydrochloridum



Compound	R	Molecular formula	M_r
Chlortetracycline hydrochloride	Cl	$C_{22}H_{24}Cl_2N_2O_8$	515.3
Tetracycline hydrochloride	H	$C_{22}H_{25}ClN_2O_8$	480.9

DEFINITION

Mixture of antibiotics, the main component being the hydrochloride of (4*S*,4*aS*,5*aS*,6*S*,12*aS*)-7-chloro-4-(dimethylamino)-3,6,10,12,12*a*-pentahydroxy-6-methyl-1,11-dioxo-1,4,4*a*,5,5*a*,6,11,12*a*-octahydrotetracycline-2-carboxamide (chlortetracycline hydrochloride), a substance produced by the growth of certain strains of *Streptomyces aureofaciens* or obtained by any other means.

Content:

- $C_{22}H_{24}Cl_2N_2O_8$: minimum 89.5 per cent (anhydrous substance),
- $C_{22}H_{25}ClN_2O_8$: maximum 8.0 per cent (anhydrous substance),
- 94.5 per cent to 102.0 per cent for the sum of the contents of chlortetracycline hydrochloride and tetracycline hydrochloride (anhydrous substance).

CHARACTERS

Appearance: yellow powder.

Solubility: slightly soluble in water and in alcohol. It dissolves in solutions of alkali hydroxides and carbonates.

IDENTIFICATION

- A. Thin-layer chromatography (2.2.27).

Test solution. Dissolve 5 mg of the substance to be examined in *methanol R* and dilute to 10 ml with the same solvent.

Reference solution (a). Dissolve 5 mg of *chlortetracycline hydrochloride CRS* in *methanol R* and dilute to 10 ml with the same solvent.

Reference solution (b). Dissolve 5 mg of *chlortetracycline hydrochloride CRS*, 5 mg of *doxycycline R* and 5 mg of *demeclocycline hydrochloride R* in *methanol R* and dilute to 10 ml with the same solvent.

Plate: TLC octadecylsilyl silica gel F_{254} plate *R*.

Mobile phase: mix 20 volumes of *acetonitrile R*, 20 volumes of *methanol R* and 60 volumes of a 63 g/l solution of *oxalic acid R* previously adjusted to pH 2 with *concentrated ammonia R*.

Application: 1 μ l.

Development: over 3/4 of the plate.

Drying: in air.

Detection: examine in ultraviolet light at 254 nm.

System suitability: the chromatogram obtained with reference solution (b) shows 3 clearly separated spots.

Results: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a).

- B. To about 2 mg add 5 ml of *sulphuric acid R*. A deep blue colour develops which becomes bluish-green. Add the solution to 2.5 ml of *water R*. The colour becomes brownish.
- C. It gives reaction (a) of chlorides (2.3.1).

TESTS

pH (2.2.3): 2.3 to 3.3.

Dissolve 0.1 g in 10 ml of *carbon dioxide-free water R*, heating slightly.

Specific optical rotation (2.2.7): – 235 to – 250 (anhydrous substance).

Dissolve 0.125 g in *water R* and dilute to 50.0 ml with the same solvent.

Absorbance (2.2.25): maximum 0.40 at 460 nm.

Dissolve 0.125 g in *water R* and dilute to 25.0 ml with the same solvent.

Related substances. Liquid chromatography (2.2.29). Prepare the solutions immediately before use.

Test solution. Dissolve 25.0 mg of the substance to be examined in 0.01 M *hydrochloric acid* and dilute to 25.0 ml with the same acid.

Reference solution (a). Dissolve 25.0 mg of *chlortetracycline hydrochloride CRS* in 0.01 M *hydrochloric acid* and dilute to 25.0 ml with the same acid.

Reference solution (b). Dissolve 10.0 mg of *4-epichlortetracycline hydrochloride CRS* in 0.01 M *hydrochloric acid* and dilute to 25.0 ml with the same acid.

Reference solution (c). Dissolve 20.0 mg of *tetracycline hydrochloride CRS* in 0.01 M *hydrochloric acid* and dilute to 25.0 ml with the same acid.

Reference solution (d). Mix 5.0 ml of reference solution (a) and 10.0 ml of reference solution (b) and dilute to 25.0 ml with 0.01 M *hydrochloric acid*.

Reference solution (e). Mix 5.0 ml of reference solution (b) and 5.0 ml of reference solution (c) and dilute to 50.0 ml with 0.01 M *hydrochloric acid*.

Reference solution (f). Dilute 1.0 ml of reference solution (c) to 20.0 ml with 0.01 M *hydrochloric acid*. Dilute 5.0 ml of this solution to 200.0 ml with 0.01 M *hydrochloric acid*.

Column:

- size: $l = 0.25$ m, $\varnothing = 4.6$ mm,
- stationary phase: *octylsilyl silica gel for chromatography R* (5 μ m),
- temperature: 35 °C.

Mobile phase: to 500 ml of *water R*, add 50 ml of *perchloric acid solution R*, shake and add 450 ml of *dimethyl sulphoxide R*,

Flow rate: 1 ml/min.

Detection: spectrophotometer at 280 nm.

Injection: 20 μ l; inject the test solution and reference solutions (d), (e) and (f).

System suitability: reference solution (d):

- **resolution:** minimum 2.0 between the peaks due to impurity A and to chlortetracycline; if necessary, adjust the dimethyl sulphoxide content in the mobile phase,
- **symmetry factor:** maximum 1.3 for the peak due to chlortetracycline.

Limits:

- **impurity A:** not more than the area of the corresponding peak in the chromatogram obtained with reference solution (e) (4.0 per cent),
- **total of other impurities eluting between the solvent peak and the peak corresponding to chlortetracycline:** not more than 0.25 times the area of the peak due to impurity A in the chromatogram obtained with reference solution (e) (1.0 per cent),
- **disregard limit:** area of the principal peak in the chromatogram obtained with reference solution (f) (0.1 per cent).

Heavy metals (2.4.8): maximum 50 ppm.

0.5 g complies with limit test C. Prepare the standard using 2.5 ml of *lead standard solution (10 ppm Pb) R*.

Water (2.5.12): maximum 2.0 per cent, determined on 0.300 g.

Sulphated ash (2.4.14): maximum 0.5 per cent, determined on 1.0 g.

Bacterial endotoxins (2.6.14): less than 1 IU/mg, if intended for use in the manufacture of parenteral dosage forms without a further appropriate procedure for the removal of bacterial endotoxins.

ASSAY

Liquid chromatography (2.2.29) as described in the test for related substances with the following modification.

Injection: test solution and reference solutions (a) and (e).

Calculate the percentage content of $C_{22}H_{24}Cl_2N_2O_8$ using the chromatogram obtained with reference solution (a). Calculate the percentage content of $C_{22}H_{25}ClN_2O_8$ using the chromatogram obtained with reference solution (e).

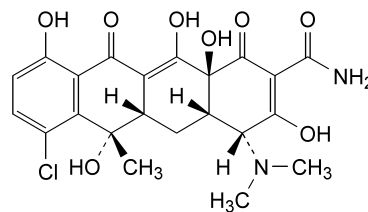
STORAGE

Protected from light. If the substance is sterile, store in a sterile, airtight, tamper-proof container.

LABELLING

The label states, where applicable, that the substance is free from bacterial endotoxins.

IMPURITIES



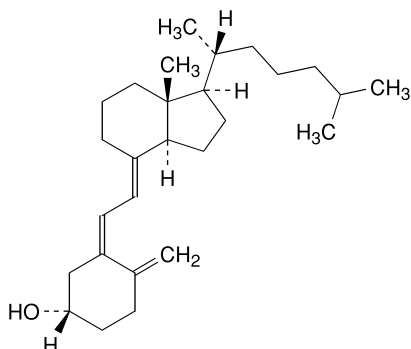
- A. (4*R*,4*aS*,5*aS*,6*S*,12*aS*)-7-chloro-4-(dimethylamino)-3,6,10,12,12*a*-pentahydroxy-6-methyl-1,11-dioxo-1,4,4*a*,5,5*a*,6,11,12*a*-octahydrotetracene-2-carboxamide (4-epichlortetracycline),

- B. demeclocycline.

01/2005:0072

CHOLECALCIFEROL

Cholecalciferolum

 $C_{27}H_{44}O$ M_r 384.6

DEFINITION

Cholecalciferol contains not less than 97.0 per cent and not more than the equivalent of 103.0 per cent of (5*Z*,7*E*)-9,10-secocholesta-5,7,10(19)-trien-3 β -ol.

1 milligram of cholecalciferol is equivalent to 40 000 IU of antirachitic activity (vitamin D) in rats.

CHARACTERS

White or almost white crystals, practically insoluble in water, freely soluble in alcohol, soluble in fatty oils. It is sensitive to air, heat and light. Solutions in volatile solvents are unstable and are to be used immediately.

A reversible isomerisation to pre-cholecalciferol takes place in solution, depending on temperature and time. The activity is due to both compounds.

IDENTIFICATION

Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *cholecalciferol CRS*. Examine the substances prepared as discs.

TESTS

Specific optical rotation (2.2.7). Dissolve 0.200 g rapidly and without heating in *aldehyde-free alcohol R* and dilute to 25.0 ml with the same solvent. The specific optical rotation, determined within 30 min of preparing the solution, is + 105 to + 112.

ASSAY

Carry out the operations as rapidly as possible, avoiding exposure to actinic light and air.

Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 10.0 mg of the substance to be examined without heating in 10.0 ml of *toluene R* and dilute to 100.0 ml with the mobile phase.

Reference solution (a). Dissolve 10.0 mg of *cholecalciferol CRS* without heating in 10.0 ml of *toluene R* and dilute to 100.0 ml with the mobile phase.

Reference solution (b). Dilute 1.0 ml of *cholecalciferol for performance test CRS* to 5.0 ml with the mobile phase. Heat in a water-bath at 90 °C under a reflux condenser for 45 min and cool.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4.6 mm in internal diameter packed with a suitable silica gel (5 μ m),
- as mobile phase at a flow rate of 2 ml/min a mixture of 3 volumes of *pentanol R* and 997 volumes of *hexane R*,
- as detector a spectrophotometer set at 254 nm.

An automatic injection device or a sample loop is recommended. Inject a suitable volume of reference solution (b). Adjust the sensitivity of the system so that the height of the principal peak is at least 50 per cent of the full scale of the recorder. Inject reference solution (b) 6 times. When the chromatograms are recorded in the prescribed conditions, the approximate relative retention times with reference to cholecalciferol are 0.4 for pre-cholecalciferol and 0.5 for *trans*-cholecalciferol. The relative standard deviation of the response for cholecalciferol is not greater than 1 per cent and the resolution between the peaks due to pre-cholecalciferol and *trans*-cholecalciferol is not less than 1.0. If necessary adjust the proportions of the constituents and the flow rate of the mobile phase to obtain this resolution.

Inject a suitable volume of reference solution (a). Adjust the sensitivity of the system so that the height of the principal peak is at least 50 per cent of the full scale of the recorder. Inject the same volume of the test solution and record the chromatogram in the same manner.

Calculate the percentage content of cholecalciferol from the expression:

$$\frac{m'}{m} \times \frac{S_D}{S'_D} \times 100$$

m = mass of the substance to be examined in the test solution, in milligrams,

m' = mass of *cholecalciferol CRS* in reference solution (a), in milligrams,

S_D = area (or height) of the peak due to cholecalciferol in the chromatogram obtained with the test solution,

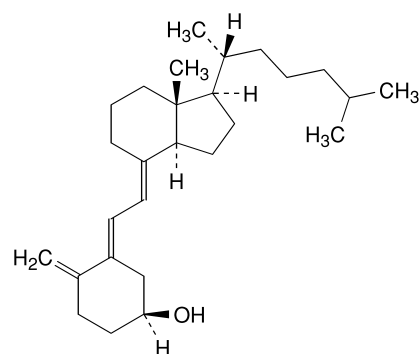
S'_D = area (or height) of the peak due to cholecalciferol in the chromatogram obtained with reference solution (a).

STORAGE

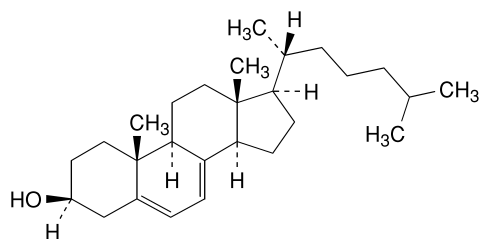
Store in an airtight container, under nitrogen, protected from light, at a temperature between 2 °C and 8 °C.

The contents of an opened container are to be used immediately.

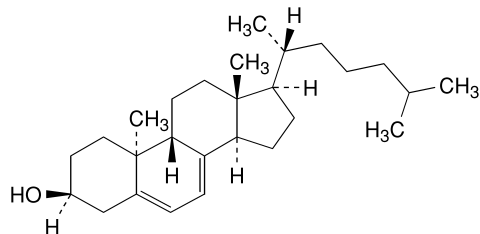
IMPURITIES



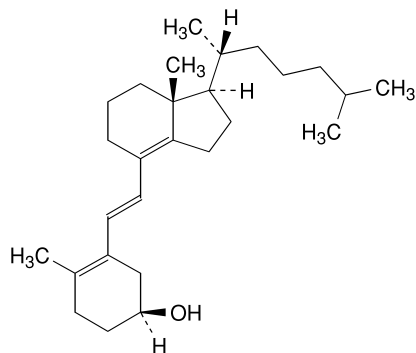
A. (5*E*,7*E*)-9,10-secocholesta-5,7,10(19)-trien-3 β -ol (*trans*-cholecalciferol, *trans*-vitamin D₃),



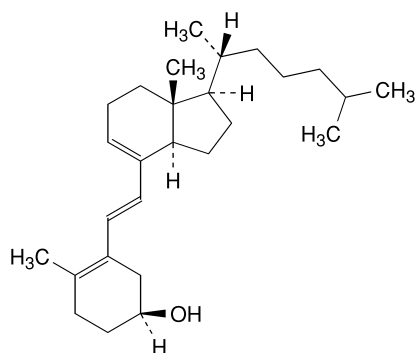
B. cholesta-5,7-dien-3 β -ol (7,8-didehydrocholesterol, provitamin D₃),



C. 9 β ,10 α -cholesta-5,7-dien-3 β -ol (lumisterol₃),



D. (6*E*)-9,10-secocholesta-5(10),6,8(14)-trien-3 β -ol (iso-tachysterol₃),



E. (6*E*)-9,10-secocholesta-5(10),6,8-trien-3 β -ol (tachysterol₃).

01/2005:0575

CHOLECALCIFEROL CONCENTRATE (OILY FORM)

Cholecalciferolum densatum oleosum

DEFINITION

Cholecalciferol concentrate (oily form) consists of a solution of *Cholecalciferol* (0072) in a suitable vegetable fatty oil, authorised by the competent authority.

The content of cholecalciferol stated on the label is not less than 500 000 IU/g and the concentrate contains not less than 90.0 per cent and not more than 110.0 per cent of the content stated on the label. The concentrate may contain suitable stabilisers such as antioxidants.

CHARACTERS

A clear, yellow liquid, practically insoluble in water, slightly soluble in ethanol, miscible with solvents of fats.

Partial solidification may occur, depending on the temperature.

IDENTIFICATION

First identification: A, C.

Second identification: A, B.

A. Examine by thin-layer chromatography (2.2.27), using a TLC silica gel G plate R.

Test solution. Dissolve an amount of the substance to be examined corresponding to 400 000 IU in *ethylene chloride R* containing 10 g/l of *squalane R* and 0.1 g/l of *butylhydroxytoluene R* and dilute to 4 ml with the same solvent. Prepare immediately before use.

Reference solution (a). Dissolve 10 mg of *cholecalciferol CRS* in *ethylene chloride R* containing 10 g/l of *squalane R* and 0.1 g/l of *butylhydroxytoluene R* and dilute to 4 ml with the same solvent. Prepare immediately before use.

Reference solution (b). Dissolve 10 mg of *ergocalciferol CRS* in *ethylene chloride R* containing 10 g/l of *squalane R* and 0.1 g/l of *butylhydroxytoluene R* and dilute to 4 ml with the same solvent. Prepare immediately before use.

Apply to the plate 20 μ l of each solution. Develop immediately, protected from light, over a path of 15 cm using a mixture of equal volumes of *cyclohexane R* and *peroxide-free ether R*, the mixture containing 0.1 g/l of *butylhydroxytoluene R*. Allow the plate to dry in air and spray with *sulphuric acid R*. Compare the principal spot in the chromatogram obtained with the test solution with the principal spot in each of the chromatograms obtained with reference solution (a) and reference solution (b), respectively. The chromatogram obtained with the test solution shows immediately a bright yellow principal spot which rapidly becomes orange-brown, then gradually greenish-grey and remains so for 10 min. This spot is similar in position, colour and size to the spot in the chromatogram obtained with reference solution (a). The chromatogram obtained with reference solution (b) shows immediately at the same level an orange principal spot which gradually becomes reddish-brown and remains so for 10 min.

B. Prepare a solution in *cyclohexane R* containing the equivalent of about 400 IU/ml. Examined between 250 nm and 300 nm (2.2.25), the solution shows an absorption maximum at 267 nm.

C. Examine the chromatograms obtained in the assay. The principal peak in the chromatogram obtained with the test solution has a similar retention time to the principal peak in the chromatogram obtained with reference solution (a).

TESTS

Acid value (2.5.1). Not more than 2.0, determined on 5.0 g dissolved in 25 ml of the prescribed mixture of solvents.

Peroxide value (2.5.5). Not more than 20.

ASSAY

Carry out the assay as rapidly as possible, avoiding exposure to actinic light and air.

Examine by liquid chromatography (2.2.29).

Test solution. Dissolve a quantity of the preparation to be examined, weighed with an accuracy of 0.1 per cent, equivalent to about 400 000 IU, in 10.0 ml of *toluene R* and dilute to 100.0 ml with the mobile phase.

Reference solution (a). Dissolve 10.0 mg of *cholecalciferol CRS* without heating in 10.0 ml of *toluene R* and dilute to 100.0 ml with the mobile phase.

Reference solution (b). Dilute 1.0 ml of *cholecalciferol for performance test CRS* to 5.0 ml with the mobile phase. Heat in a water-bath at 90 °C under a reflux condenser for 45 min and cool.

Reference solution (c). Dissolve 0.10 g of *cholecalciferol CRS* without heating in *toluene R* and dilute to 100.0 ml with the same solvent.

Reference solution (d). Dilute 5.0 ml of reference solution (c) to 50.0 ml with the mobile phase. Keep the solution in iced water.

Reference solution (e). Place 5.0 ml of reference solution (c) in a volumetric flask, add about 10 mg of *butylhydroxytoluene R* and displace air from the flask with *nitrogen R*. Heat in a water-bath at 90 °C under a reflux condenser protected from light and under *nitrogen R* for 45 min. Cool and dilute to 50.0 ml with the mobile phase.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4.6 mm in internal diameter packed with a suitable silica gel (5 µm),
- as mobile phase at a flow rate of 2 ml/min a mixture of 3 volumes of *pentanol R* and 997 volumes of *hexane R*,
- as detector a spectrophotometer set at 254 nm.

An automatic injection device or a sample loop is recommended. Inject a suitable volume of reference solution (b). Adjust the sensitivity of the system so that the height of the principal peak is at least 50 per cent of the full scale of the recorder. Inject reference solution (b) 6 times. When the chromatograms are recorded in the prescribed conditions, the approximate relative retention times with reference to cholecalciferol are 0.4 for pre-cholecalciferol and 0.5 for *trans*-cholecalciferol. The relative standard deviation of the response for cholecalciferol is not greater than 1 per cent and the resolution between the peaks due to pre-cholecalciferol and *trans*-cholecalciferol is not less than 1.0. If necessary adjust the proportions of the constituents and the flow rate of the mobile phase to obtain this resolution.

Inject a suitable volume of reference solution (d) and of reference solution (e).

Calculate the conversion factor (*f*) from the expression:

$$f = \frac{K - L}{M}$$

K = area (or height) of the peak due to cholecalciferol in the chromatogram obtained with reference solution (d),

L = area (or height) of the peak due to cholecalciferol in the chromatogram obtained with reference solution (e),

M = area (or height) of the peak due to pre-cholecalciferol in the chromatogram obtained with reference solution (e).

The value of *f* determined in duplicate on different days may be used during the entire procedure.

Inject a suitable volume of reference solution (a). Adjust the sensitivity of the system so that the height of the principal peak is at least 50 per cent of the full scale of the recorder. Inject the same volume of the test solution and record the chromatogram in the same manner.

Calculate the content of cholecalciferol in International Units per gram from the expression:

$$\frac{m'}{V'} \times \frac{V}{m} \times \frac{S_D + (f \times S_p)}{S'_D} \times 40\,000 \times 1000$$

m = mass of the preparation to be examined in the test solution, in milligrams,

m' = mass of *cholecalciferol CRS* in reference solution (a), in milligrams,

V = volume of the test solution (100 ml),

V' = volume of reference solution (a) (100 ml),

S_D = area (or height) of the peak due to cholecalciferol in the chromatogram obtained with the test solution,

S'_D = area (or height) of the peak due to cholecalciferol in the chromatogram obtained with reference solution (a),

S_p = area (or height) of the peak due to pre-cholecalciferol in the chromatogram obtained with the test solution,

f = conversion factor.

STORAGE

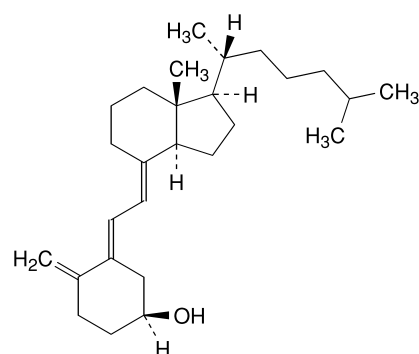
Store in an airtight, well-filled container, protected from light. The contents of an opened container are to be used as soon as possible; any unused part is to be protected by an atmosphere of nitrogen.

LABELLING

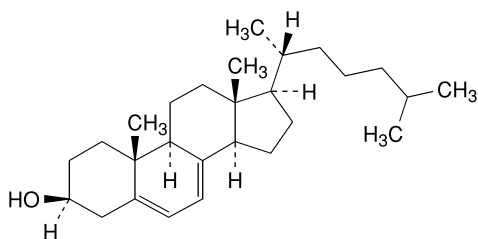
The label states:

- the number of International Units per gram,
- the method of restoring the solution if partial solidification occurs,
- the name of any added stabilisers.

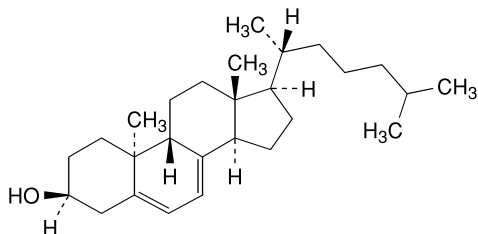
IMPURITIES



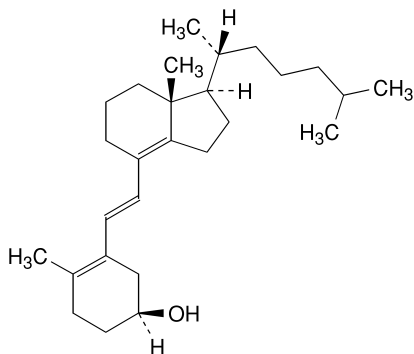
A. (5*E*,7*E*)-9,10-secocholesta-5,7,10(19)-trien-3β-ol (*trans*-cholecalciferol, *trans*-vitamin D₃),



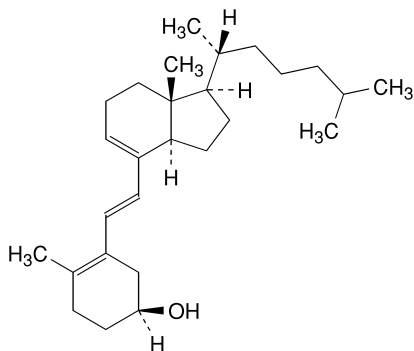
B. cholesta-5,7-dien-3 β -ol (7,8-di dehydrocholesterol, provitamin D₃),



C. (9 β ,10 α)-cholesta-5,7-dien-3 β -ol (lumisterol₃),



D. (6E)-9,10-secholesta-5(10),6,8(14)-trien-3 β -ol (iso-tachysterol₃),



E. (6E)-9,10-secholesta-5(10),6,8-trien-3 β -ol (tachysterol₃).

01/2005:0574

CHOLECALCIFEROL CONCENTRATE (POWDER FORM)

Cholecalciferoli pulvis

DEFINITION

Cholecalciferol concentrate (powder form) is obtained by dispersing an oily solution of *Cholecalciferol* (0072) in an appropriate matrix which is usually based on a combination of gelatin and carbohydrates of suitable quality, authorised by the competent authority.

The declared content of cholecalciferol is not less than 100 000 IU/g and the concentrate contains not less than 90.0 per cent and not more than 110.0 per cent of the content stated on the label. The concentrate may contain suitable stabilisers such as antioxidants.

CHARACTERS

White or yellowish-white, small particles which, depending on their formulation, may be practically insoluble in water or may swell or form a dispersion.

IDENTIFICATION

First identification: A, C.

Second identification: A, B.

A. Examine by thin-layer chromatography (2.2.27), using a TLC silica gel G plate R.

Test solution. Place 10.0 ml of the test solution prepared for the assay in a suitable flask and evaporate to dryness under reduced pressure by swirling in a water-bath at 40 °C. Cool under running water and restore atmospheric pressure with *nitrogen R*. Dissolve the residue immediately in 0.4 ml of *ethylene chloride R* containing 10 g/l of *squalane R* and 0.1 g/l of *butylhydroxytoluene R*. Prepare immediately before use.

Reference solution (a). Dissolve 10 mg of *cholecalciferol CRS* in *ethylene chloride R* containing 10 g/l of *squalane R* and 0.1 g/l of *butylhydroxytoluene R* and dilute to 4 ml with the same solvent. Prepare immediately before use.

Reference solution (b). Dissolve 10 mg of *ergocalciferol CRS* in *ethylene chloride R* containing 10 g/l of *squalane R* and 0.1 g/l of *butylhydroxytoluene R* and dilute to 4 ml with the same solvent. Prepare immediately before use.

Apply to the plate 20 μ l of each solution. Develop immediately, protected from light, over a path of 15 cm using a mixture of equal volumes of *cyclohexane R* and *peroxide-free ether R*, the mixture containing 0.1 g/l of *butylhydroxytoluene R*. Allow the plate to dry in air and spray with *sulphuric acid R*. Compare the principal spot in the chromatogram obtained with the test solution with the principal spot in each of the chromatograms obtained with reference solution (a) and reference solution (b), respectively. The chromatogram obtained with the test solution shows immediately a bright-yellow principal spot which rapidly becomes orange-brown, then gradually greenish-grey and remains so for 10 min. This spot is similar in position, colour and size to the spot in the chromatogram obtained with reference solution (a). The chromatogram obtained with reference solution (b) shows immediately at the same level an orange principal spot which gradually becomes reddish-brown and remains so for 10 min.

B. Place 5.0 ml of the test solution prepared for the assay in a suitable flask and evaporate to dryness under reduced pressure by swirling in a water-bath at 40 °C. Cool under running water and restore atmospheric pressure with *nitrogen R*. Dissolve the residue immediately in 50.0 ml of *cyclohexane R*. Examined between 250 nm and 300 nm (2.2.25), the solution shows an absorption maximum at 265 nm.

C. Examine the chromatograms obtained in the assay. The principal peak in the chromatogram obtained with the test solution has a similar retention time to the principal peak in the chromatogram obtained with reference solution (a).

ASSAY

Carry out the assay as rapidly as possible, avoiding exposure to actinic light and air.

Examine by liquid chromatography (2.2.29).

Test solution. Introduce into a saponification flask a quantity of the preparation to be examined, weighed with an accuracy of 0.1 per cent, equivalent to about 100 000 IU. Add 5 ml of *water R*, 20 ml of *ethanol R*, 1 ml of *sodium ascorbate solution R* and 3 ml of a freshly prepared 50 per cent *m/m* solution of *potassium hydroxide R*. Heat under a reflux condenser on a water-bath for 30 min. Cool rapidly under running water. Transfer the liquid to a separating funnel with the aid of two quantities, each of 15 ml, of *water R*, one quantity of 10 ml of *alcohol R* and two quantities, each of 50 ml, of *pentane R*. Shake vigorously for 30 s. Allow to stand until the two layers are clear. Transfer the lower aqueous-alcoholic layer to a second separating funnel and shake with a mixture of 10 ml of *alcohol R* and 50 ml of *pentane R*. After separation, transfer the aqueous-alcoholic layer to a third separating funnel and the pentane layer to the first separating funnel, washing the second separating funnel with two quantities, each of 10 ml, of *pentane R* and adding the washings to the first separating funnel. Shake the aqueous-alcoholic layer with 50 ml of *pentane R* and add the pentane layer to the first funnel. Wash the pentane layer with two quantities, each of 50 ml, of a freshly prepared 30 g/l solution of *potassium hydroxide R* in *alcohol (10 per cent V/V) R*, shaking vigorously, then wash with successive quantities, each of 50 ml, of *water R* until the washings are neutral to phenolphthalein. Transfer the washed pentane extract to a ground-glass-stoppered flask. Evaporate the contents of the flask to dryness under reduced pressure by swirling in a water-bath at 40 °C. Cool under running water and restore atmospheric pressure with *nitrogen R*. Dissolve the residue immediately in 5.0 ml of *toluene R* and add 20.0 ml of the mobile phase to obtain a solution containing about 4000 IU/ml.

Reference solution (a). Dissolve 10.0 mg of *cholecalciferol CRS* without heating in 10.0 ml of *toluene R* and dilute to 100.0 ml with the mobile phase.

Reference solution (b). Dilute 1.0 g of *cholecalciferol for performance test CRS* to 5.0 ml with the mobile phase. Heat in a water-bath at 90 °C under a reflux condenser for 45 min and cool.

Reference solution (c). Dissolve 0.10 g of *cholecalciferol CRS* without heating in *toluene R* and dilute to 100.0 ml with the same solvent.

Reference solution (d). Dilute 5.0 ml of reference solution (c) to 50.0 ml with the mobile phase. Keep the solution in iced water.

Reference solution (e). Place 5.0 ml of reference solution (c) in a volumetric flask, add about 10 mg of *butylhydroxytoluene R* and displace air from the flask with *nitrogen R*. Heat in a water-bath at 90 °C under a reflux condenser protected from light and under *nitrogen R* for 45 min. Cool and dilute to 50.0 ml with the mobile phase.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4.6 mm in internal diameter packed with a suitable silica gel (5 µm),
- as mobile phase at a flow rate of 2 ml/min, a mixture of 3 volumes of *pentanol R* and 997 volumes of *hexane R*,
- as detector a spectrophotometer set at 254 nm.

An automatic injection device or a sample loop is recommended. Inject a suitable volume of reference solution (b). Adjust the sensitivity of the system so that the

height of the principal peak is at least 50 per cent of the full scale of the recorder. Inject reference solution (b) 6 times. When the chromatograms are recorded in the prescribed conditions, the approximate relative retention times with reference to cholecalciferol are 0.4 for pre-cholecalciferol and 0.5 for *trans*-cholecalciferol. The relative standard deviation of the response for cholecalciferol is not greater than 1 per cent and the resolution between the peaks due to pre-cholecalciferol and *trans*-cholecalciferol is not less than 1.0. If necessary adjust the proportions of the constituents and the flow rate of the mobile phase to obtain this resolution.

Inject a suitable volume of reference solution (d) and of reference solution (e).

Calculate the conversion factor (*f*) from the expression:

$$f = \frac{K - L}{M}$$

K = area (or height) of the peak due to cholecalciferol in the chromatogram obtained with reference solution (d),

L = area (or height) of the peak due to cholecalciferol in the chromatogram obtained with reference solution (e),

M = area (or height) of the peak due to pre-cholecalciferol in the chromatogram obtained with reference solution (e).

The value of *f* determined in duplicate on different days may be used during the entire procedure.

Inject a suitable volume of reference solution (a). Adjust the sensitivity of the system so that the height of the principal peak is at least 50 per cent of the full scale of the recorder. Inject the same volume of the test solution and record the chromatogram in the same manner.

Calculate the content of cholecalciferol in International Units per gram from the expression:

$$\frac{m'}{V'} \times \frac{V}{m} \times \frac{S_D + (f \times S_p)}{S'_D} \times 40\,000 \times 1000$$

m = mass of the substance to be examined in the test solution, in milligrams,

m' = mass of *cholecalciferol CRS* in reference solution (a), in milligrams,

V = volume of the test solution (25 ml),

V' = volume of reference solution (a) (100 ml),

S_D = area (or height) of the peak due to cholecalciferol in the chromatogram obtained with the test solution,

S'_D = area (or height) of the peak due to cholecalciferol in the chromatogram obtained with reference solution (a),

S_p = area (or height) of the peak due to pre-cholecalciferol in the chromatogram obtained with the test solution,

f = conversion factor.

STORAGE

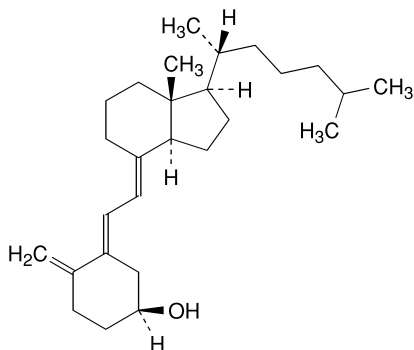
Store in an airtight, well-filled container, protected from light. The contents of an opened container are to be used as soon as possible; any unused part is to be protected by an atmosphere of nitrogen.

LABELLING

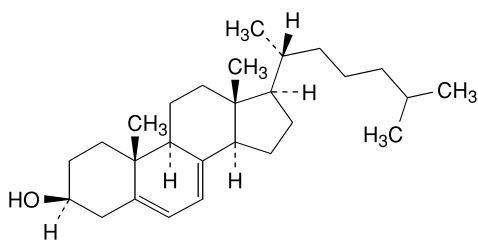
The label states:

- the number of International Units per gram,
- the name of any added stabilisers.

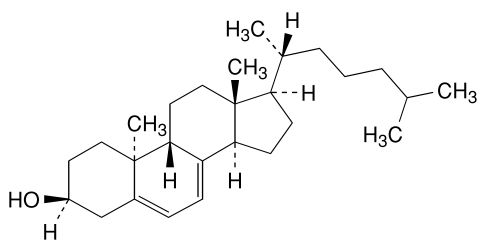
IMPURITIES



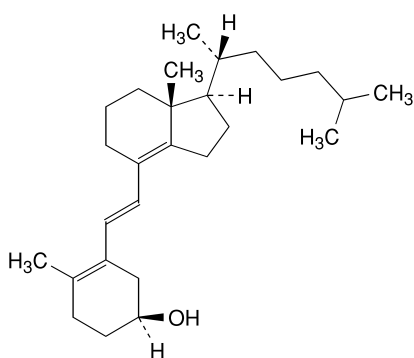
A. (5*E*,7*E*)-9,10-secocholesta-5,7,10(19)-trien-3β-ol (*trans*-cholecalciferol, *trans*-vitamin D₃),



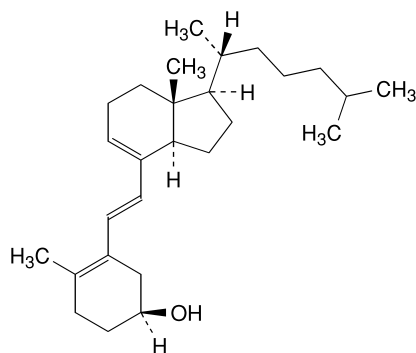
B. cholesta-5,7-dien-3β-ol (7,8-di dehydrocholesterol, provitamin D₃),



C. (9β,10α)-cholesta-5,7-dien-3β-ol (lumisterol₃),



D. (6*E*)-9,10-secocholesta-5(10),6,8(14)-trien-3β-ol (iso-tachysterol₃),



E. (6*E*)-9,10-secocholesta-5(10),6,8-trien-3β-ol (tachysterol₃).

01/2005:0598

CHOLECALCIFEROL CONCENTRATE (WATER-DISPERSIBLE FORM)

Cholecalciferolum in aqua dispergibile

DEFINITION

Cholecalciferol concentrate (water-dispersible form) consists of a solution of *Cholecalciferol* (0072) in a suitable vegetable fatty oil, authorised by the competent authority, to which suitable solubilisers have been added.

The declared content of cholecalciferol is not less than 100 000 IU/g and the concentrate contains not less than 90.0 per cent and not more than 115.0 per cent of the content stated on the label. The concentrate may contain suitable stabilisers such as antioxidants.

CHARACTERS

A slightly yellowish liquid of variable opalescence and viscosity. Highly concentrated solutions may become cloudy at low temperatures or form a gel at room temperature.

IDENTIFICATION

First identification: A, C, D.

Second identification: A, B, D.

A. Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel G plate R*.

Test solution. Place 10.0 ml of the test solution prepared for the assay in a suitable flask and evaporate to dryness under reduced pressure by swirling in a water-bath at 40 °C. Cool under running water and restore atmospheric pressure with *nitrogen R*. Dissolve the residue immediately in 0.4 ml of *ethylene chloride R* containing 10 g/l of *squalane R* and 0.1 g/l of *butylhydroxytoluene R*. Prepare immediately before use.

Reference solution (a). Dissolve 10 mg of *cholecalciferol CRS* in *ethylene chloride R* containing 10 g/l of *squalane R* and 0.1 g/l of *butylhydroxytoluene R* and dilute to 4 ml with the same solvent. Prepare immediately before use.

Reference solution (b). Dissolve 10 mg of *ergocalciferol CRS* in *ethylene chloride R* containing 10 g/l of *squalane R* and 0.1 g/l of *butylhydroxytoluene R* and dilute to 4 ml with the same solvent. Prepare immediately before use.

Apply to the plate 20 µl of each solution. Develop immediately, protected from light, over a path of 15 cm using a mixture of equal volumes of *cyclohexane R* and *peroxide-free ether R*, the mixture containing 0.1 g/l of *butylhydroxytoluene R*. Allow the plate to dry in air and spray with *sulphuric acid R*. Compare the principal spot

in the chromatogram obtained with the test solution with the principal spot in each of the chromatograms obtained with reference solution (a) and reference solution (b).

The chromatogram obtained with the test solution shows immediately a bright-yellow principal spot which rapidly becomes orange-brown, then gradually greenish-grey and remains so for 10 min. This spot is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a). The chromatogram obtained with reference solution (b) shows immediately at the same level an orange principal spot which gradually becomes reddish-brown and remains so for 10 min.

- B. Place 5.0 ml of the test solution prepared for the assay in a suitable flask and evaporate to dryness under reduced pressure by swirling in a water-bath at 40 °C. Cool under running water and restore atmospheric pressure with *nitrogen R*. Dissolve the residue immediately in 50.0 ml of *cyclohexane R*. Examined between 250 nm and 300 nm (2.2.25), the solution shows an absorption maximum at 265 nm.
- C. Examine the chromatograms obtained in the assay. The principal peak in the chromatogram obtained with the test solution has a similar retention time to the principal peak in the chromatogram obtained with reference solution (a).
- D. Mix about 1 g with 10 ml of *water R* previously warmed to 50 °C and cool to 20 °C. Immediately after cooling, a uniform, slightly opalescent and slightly yellow dispersion is obtained.

ASSAY

Carry out the assay as rapidly as possible, avoiding exposure to actinic light and air.

Examine by liquid chromatography (2.2.29).

Test solution. Introduce into a saponification flask a quantity of the preparation to be examined, weighed with an accuracy of 0.1 per cent, equivalent to about 100 000 IU. Add 5 ml of *water R*, 20 ml of *ethanol R*, 1 ml of *sodium ascorbate solution R* and 3 ml of a freshly prepared 50 per cent *m/m* solution of *potassium hydroxide R*. Heat under a reflux condenser on a water-bath for 30 min. Cool rapidly under running water. Transfer the liquid to a separating funnel with the aid of two quantities, each of 15 ml, of *water R*, one quantity of 10 ml of *alcohol R* and two quantities, each of 50 ml, of *pentane R*. Shake vigorously for 30 s. Allow to stand until the two layers are clear. Transfer the aqueous-alcoholic layer to a second separating funnel and shake with a mixture of 10 ml of *alcohol R* and 50 ml of *pentane R*. After separation, transfer the aqueous-alcoholic layer to a third separating funnel and the pentane layer to the first separating funnel, washing the second separating funnel with two quantities, each of 10 ml, of *pentane R* and adding the washings to the first separating funnel. Shake the aqueous-alcoholic layer with 50 ml of *pentane R* and add the pentane layer to the first funnel. Wash the pentane layer with two quantities, each of 50 ml, of a freshly prepared 30 g/l solution of *potassium hydroxide R* in *alcohol (10 per cent V/V) R*, shaking vigorously, and then wash with successive quantities, each of 50 ml, of *water R* until the washings are neutral to phenolphthalein. Transfer the washed pentane extract to a ground-glass-stoppered flask. Evaporate the contents of the flask to dryness under reduced pressure by swirling in a water-bath at 40 °C. Cool under running water and restore atmospheric pressure with *nitrogen R*. Dissolve the residue immediately in 5.0 ml of *toluene R* and add 20.0 ml of the mobile phase to obtain a solution containing about 4000 IU/ml.

Reference solution (a). Dissolve 10.0 mg of *cholecalciferol CRS* without heating in 10.0 ml of *toluene R* and dilute to 100.0 ml with the mobile phase.

Reference solution (b). Dilute 1.0 g of *cholecalciferol for performance test CRS* to 5.0 ml with the mobile phase. Heat in a water-bath at 90 °C under a reflux condenser for 45 min and cool.

Reference solution (c). Dissolve 0.10 g of *cholecalciferol CRS* without heating in *toluene R* and dilute to 100.0 ml with the same solvent.

Reference solution (d). Dilute 5.0 ml of reference solution (c) to 50.0 ml with the mobile phase. Keep the solution in iced water.

Reference solution (e). Place 5.0 ml of reference solution (c) in a volumetric flask, add about 10 mg of *butylhydroxytoluene R* and displace air from the flask with *nitrogen R*. Heat in a water-bath at 90 °C under a reflux condenser protected from light and under *nitrogen R* for 45 min. Cool and dilute to 50.0 ml with the mobile phase.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4.6 mm in internal diameter packed with a suitable silica gel (5 µm),
- as mobile phase at a flow rate of 2 ml/min a mixture of 3 volumes of *pentanol R* and 997 volumes of *hexane R*,
- as detector a spectrophotometer set at 254 nm.

An automatic injection device or a sample loop is recommended. Inject a suitable volume of reference solution (b). Adjust the sensitivity of the system so that the height of the principal peak is at least 50 per cent of the full scale of the recorder. Inject reference solution (b) 6 times. When the chromatograms are recorded in the prescribed conditions, the approximate relative retention times with reference to cholecalciferol are 0.4 for pre-cholecalciferol and 0.5 for *trans*-cholecalciferol. The relative standard deviation of the response for cholecalciferol is not greater than 1 per cent and the resolution between the peaks due to pre-cholecalciferol and *trans*-cholecalciferol is not less than 1.0. If necessary adjust the proportions of the constituents and the flow rate of the mobile phase to obtain this resolution.

Inject a suitable volume of reference solution (d) and of reference solution (e).

Calculate the conversion factor (*f*) from the expression:

$$f = \frac{K - L}{M}$$

K = area (or height) of the peak due to cholecalciferol in the chromatogram obtained with reference solution (d),

L = area (or height) of the peak due to cholecalciferol in the chromatogram obtained with reference solution (e),

M = area (or height) of the peak due to pre-cholecalciferol in the chromatogram obtained with reference solution (e).

The value of *f* determined in duplicate on different days may be used during the entire procedure.

Inject a suitable volume of reference solution (a). Adjust the sensitivity of the system so that the height of the principal peak is at least 50 per cent of the full scale of the recorder. Inject the same volume of the test solution and record the chromatogram in the same manner.

Calculate the content of cholecalciferol in International Units per gram from the expression:

$$\frac{m'}{V'} \times \frac{V}{m} \times \frac{S_D + (f \times S_p)}{S'_D} \times 40\,000 \times 1000$$

- m = mass of the preparation to be examined in the test solution, in milligrams,
- m' = mass of *cholecalciferol CRS* in reference solution (a), in milligrams,
- V = volume of the test solution (25 ml),
- V' = volume of reference solution (a) (100 ml),
- S_D = area (or height) of the peak due to cholecalciferol in the chromatogram obtained with the test solution,
- S'_D = area (or height) of the peak due to cholecalciferol in the chromatogram obtained with reference solution (a),
- S_p = area (or height) of the peak due to pre-cholecalciferol in the chromatogram obtained with the test solution,
- f = conversion factor.

STORAGE

Store in an airtight, well-filled container, protected from light, at the temperature stated on the label.

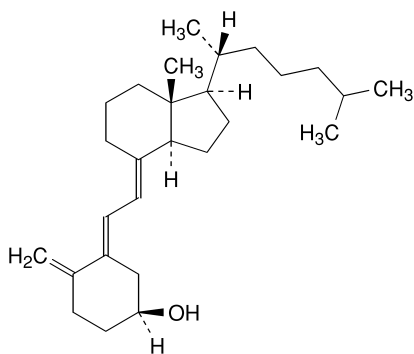
Once the container has been opened, its contents are to be used as soon as possible; any part of the contents not used at once is to be protected by an atmosphere of inert gas.

LABELLING

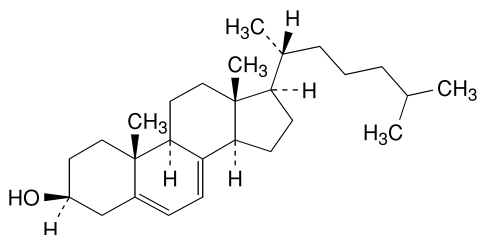
The label states:

- the number of International Units per gram,
- the name of the principal solubiliser or solubilisers used and the name of any added stabilisers,
- the storage temperature.

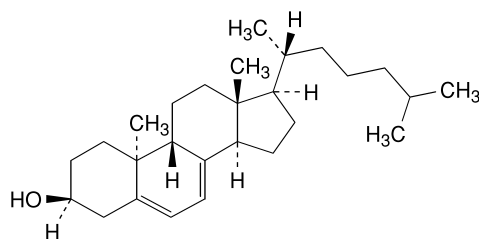
IMPURITIES



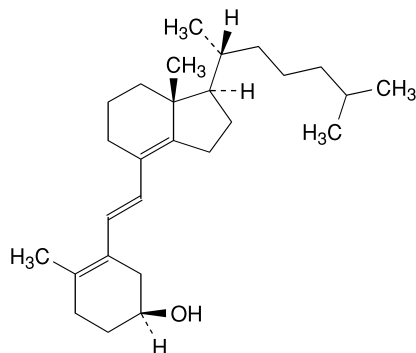
A. (5*E*,7*E*)-9,10-secocholesta-5,7,10(19)-trien-3β-ol (*trans*-cholecalciferol, *trans*-vitamin D₃),



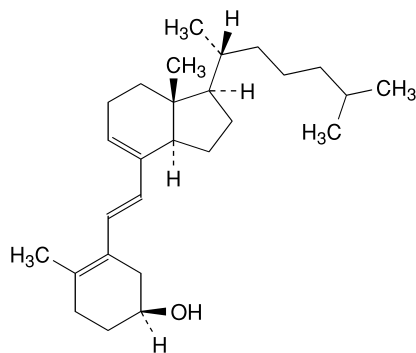
B. cholesta-5,7-dien-3β-ol (7,8-di dehydrocholesterol, provitamin D₃),



C. (9β,10α)-cholesta-5,7-dien-3β-ol (lumisterol₃),



D. (6*E*)-9,10-secocholesta-5(10),6,8(14)-trien-3β-ol (iso-tachysterol₃),

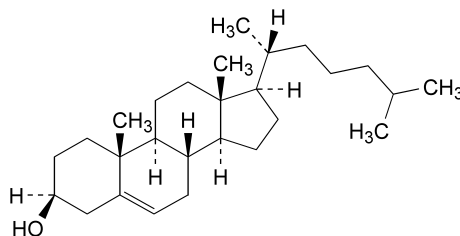


E. (6*E*)-9,10-secocholesta-5(10),6,8-trien-3β-ol (tachysterol₃).

01/2005:0993

CHOLESTEROL

Cholesterolum



C₂₇H₄₆O

M_r 386.7

DEFINITION

Cholesterol contains not less than 95.0 per cent of cholest-5-en-3β-ol and not less than 97.0 per cent and not more than 103.0 per cent of total sterols, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, crystalline powder, practically insoluble in water, sparingly soluble in acetone and in alcohol. It is sensitive to light.

IDENTIFICATION

- A. Melting point (2.2.14): 147 °C to 150 °C.
- B. Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel G plate R*. Prepare the solutions immediately before use.
- Test solution.** Dissolve 10 mg of the substance to be examined in *ethylene chloride R* and dilute to 5 ml with the same solvent.
- Reference solution.** Dissolve 10 mg of *cholesterol CRS* in *ethylene chloride R* and dilute to 5 ml with the same solvent.
- Apply to the plate 20 µl of each solution. Develop immediately, protected from light, over a path of 15 cm using a mixture of 33 volumes of *ethyl acetate R* and 66 volumes of *toluene R*. Allow the plate to dry in air and spray 3 times with *antimony trichloride solution R*. Examine the chromatograms 3–4 min after spraying. The principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.
- C. Dissolve about 5 mg in 2 ml of *methylene chloride R*. Add 1 ml of *acetic anhydride R*, 0.01 ml of *sulphuric acid R* and shake. A pink colour is produced which rapidly changes to red, then to blue and finally to brilliant green.

TESTS

Solubility in alcohol. In a stoppered flask, dissolve 0.5 g in 50 ml of *alcohol R* at 50 °C. Allow to stand for 2 h. No deposit or turbidity is formed.

Acidity. Dissolve 1.0 g in 10 ml of *ether R*, add 10.0 ml of 0.1 M *sodium hydroxide* and shake for about 1 min. Heat gently to eliminate ether and then boil for 5 min. Cool, add 10 ml of *water R* and 0.1 ml of *phenolphthalein solution R* as indicator and titrate with 0.1 M *hydrochloric acid* until the pink colour just disappears, stirring the solution vigorously throughout the titration. Carry out a blank titration. The difference between the volumes of 0.1 M *hydrochloric acid* required to change the colour of the indicator in the blank and in the test is not more than 0.3 ml.

Loss on drying (2.2.32). Not more than 0.3 per cent, determined on 1.000 g by drying *in vacuo* at 60 °C for 4 h.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Examine by gas chromatography (2.2.28), using *pregnenolone isobutyrate CRS* as the internal standard.

Internal standard solution. Dissolve 0.100 g of *pregnenolone isobutyrate CRS* in *heptane R* and dilute to 100.0 ml with the same solvent.

Test solution. Dissolve 25.0 mg of the substance to be examined in the internal standard solution and dilute to 25.0 ml with the same solution.

Reference solution. Dissolve 25.0 mg of *cholesterol CRS* in the internal standard solution and dilute to 25.0 ml with the same solution.

The chromatographic procedure may be carried out using:

- a fused-silica column 30 m long and 0.25 mm in internal diameter coated with *poly(dimethyl)siloxane R* (film thickness 0.25 µm),
- *helium for chromatography R* as the carrier gas with a split ratio of 1:25 at a flow rate of 2 ml/min,
- a flame-ionisation detector,

maintaining the temperature of the column at 275 °C, that of the injection port at 285 °C and that of the detector at 300 °C.

Inject 1.0 µl of each solution. The assay is not valid unless the resolution between the peaks due to pregnenolone isobutyrate and cholest-5-en-3β-ol in the chromatogram obtained with the reference solution is at least 10.0.

Calculate the percentage content of cholest-5-en-3β-ol, using the declared content of cholest-5-en-3β-ol in *cholesterol CRS*. Calculate the percentage content of total sterols by adding together the contents of cholest-5-en-3β-ol and other substances with a retention time less than or equal to 1.5 times the retention time of cholest-5-en-3β-ol. Disregard the peaks due to the internal standard and the solvent.

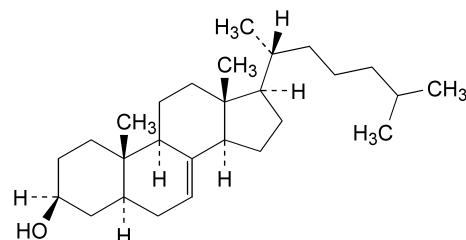
STORAGE

Store protected from light.

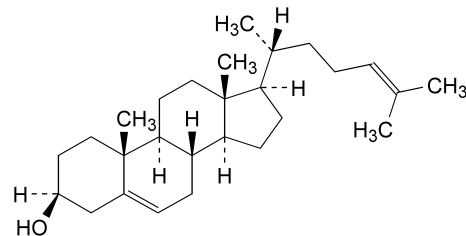
LABELLING

The label states the source material for the production of cholesterol (for example bovine brain and spinal cord, wool fat or chicken eggs).

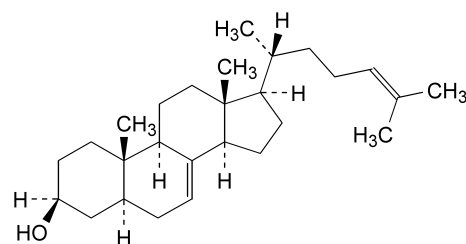
IMPURITIES



A. 5α-cholest-7-en-3β-ol (lathosterol),



B. cholesta-5,24-dien-3β-ol (desmosterol),



C. 5α-cholesta-7,24-dien-3β-ol.

01/2005:0476

CHYMOTRYPSIN

Chymotrypsinum

DEFINITION

Chymotrypsin is a proteolytic enzyme obtained by the activation of chymotrypsinogen extracted from the pancreas of beef (*Bos taurus* L.). It has an activity not less than

5.0 microkatal per milligram. In solution it has maximal enzymic activity at about pH 8; the activity is reversibly inhibited at pH 3, at which pH it is most stable.

PRODUCTION

The animals from which chymotrypsin is derived must fulfil the requirements for the health of animals suitable for human consumption to the satisfaction of the competent authority; furthermore the tissues used shall not include any specified risk material as defined by any relevant international or, where appropriate, national legislation. It must have been shown to what extent the method of production allows inactivation or removal of any contamination by viruses or other infectious agents.

The method of manufacture is validated to demonstrate that the product, if tested, would comply with the following test:

Histamine (2.6.10). Not more than 1 µg (calculated as histamine base) per 5 microkatal of chymotrypsin activity. Before carrying out the test, heat the solution of the substance to be examined on a water-bath for 30 min.

CHARACTERS

A white, crystalline or amorphous powder, sparingly soluble in water; the amorphous form is hygroscopic.

IDENTIFICATION

A. Dilute 1 ml of solution S (see Tests) to 10 ml with *water R*. In a depression in a white spot plate, mix 0.05 ml of this solution with 0.2 ml of substrate solution. A purple colour develops.

Substrate solution. To 24.0 mg of *acetyltyrosine ethyl ester R* add 0.2 ml of *alcohol R*, and swirl until solution is effected. Add 2.0 ml of 0.067 M phosphate buffer solution pH 7.0 *R* and 1 ml of *methyl red mixed solution R* and dilute to 10.0 ml with *water R*.

B. Dilute 0.5 ml of solution S to 5 ml with *water R*. Add 0.10 ml of a 20 g/l solution of *tosylphenylalanylchloromethane R* in *alcohol R*. Adjust to pH 7.0 and shake for 2 h. In a depression in a white spot plate, mix 0.05 ml of this solution with 0.2 ml of the substrate solution (see Identification test A). No colour develops within 3 min of mixing.

TESTS

Solution S. Dissolve 0.10 g in *carbon dioxide-free water R* and dilute to 10.0 ml with the same solvent.

Appearance of solution. Solution S is not more opalescent than reference suspension II (2.2.1).

pH (2.2.3). The pH of solution S is 3.0 to 5.0.

Absorbance (2.2.25). Dissolve 30.0 mg in 0.001 M *hydrochloric acid* and dilute to 100.0 ml with the same acid. The solution shows an absorption maximum at 281 nm and a minimum at 250 nm. The specific absorbance at the maximum is 18.5 to 22.5 and that at the minimum is not greater than 8.

Trypsin. Transfer to a depression in a white spot plate 0.05 ml of *tris(hydroxymethyl)aminomethane buffer solution pH 8.1 R* and 0.1 ml of solution S. Add 0.2 ml of substrate solution (test solution). At the same time and in the same manner, prepare a reference solution using the substance to be examined to which not more than 1 per cent *m/m* of *trypsin BRP* has been added. Start a timer. No colour appears in the test solution within 3 min to 5 min after the addition of the substrate solution. A purple colour is produced in the control solution.

Substrate solution. To 98.5 mg of *tosylarginine methyl ester hydrochloride R*, suitable for assaying trypsin, add

5 ml of *tris(hydroxymethyl)aminomethane buffer solution pH 8.1 R* and swirl to dissolve. Add 2.5 ml of *methyl red mixed solution R* and dilute to 25.0 ml with *water R*.

Loss on drying (2.2.32). Not more than 5.0 per cent, determined on 0.100 g by drying at 60 °C at a pressure not exceeding 0.7 kPa for 2 h.

ASSAY

The activity of chymotrypsin is determined by comparing the rate at which it hydrolyses *acetyltyrosine ethyl ester R* with the rate at which *chymotrypsin BRP* hydrolyses the same substrate under the same conditions.

Apparatus. Use a reaction vessel of about 30 ml capacity provided with:

- a device that will maintain a temperature of 25.0 ± 0.1 °C,
- a stirring device, for example a magnetic stirrer,
- a lid with holes for the insertion of electrodes, the tip of a burette, a tube for the admission of nitrogen and the introduction of reagents.

An automatic or manual titration apparatus may be used. For the latter the burette is graduated in 0.005 ml and the pH meter is provided with a wide scale and glass-calomel electrodes.

Test solution. Dissolve 25.0 mg of the substance to be examined in 0.001 M *hydrochloric acid* and dilute to 250.0 ml with the same acid.

Reference solution. Dissolve 25.0 mg of *chymotrypsin BRP* in 0.001 M *hydrochloric acid* and dilute to 250.0 ml with the same acid.

Store the solutions at 0 °C to 5 °C. Warm 1 ml of each solution to about 25 °C over 15 min and use 50 µl of each solution (corresponding to about 25 nanokatal) for each titration. Carry out the titration in an atmosphere of nitrogen. Transfer 10.0 ml of 0.01 M *calcium chloride solution R* to the reaction vessel and, while stirring, add 0.35 ml of 0.2 M *acetyltyrosine ethyl ester solution R*. When the temperature is steady at 25.0 ± 0.1 °C (after about 5 min) adjust the pH to exactly 8.0 with 0.02 M *sodium hydroxide*. Add 50 µl of the test solution (equivalent to about 5 µg of the substance to be examined) and start a timer. Maintain the pH at 8.0 by the addition of 0.02 M *sodium hydroxide*, noting the volume added every 30 s. Calculate the volume of 0.02 M *sodium hydroxide* used per second between 30 s and 210 s. Carry out a titration in the same manner using the reference solution and calculate the volume of 0.02 M *sodium hydroxide* used per second.

Calculate the activity in microkatal per milligram using the expression:

$$\frac{m' \times V}{m \times V'} \times A$$

m = mass of the substance to be examined, in milligrams,

m' = mass of *chymotrypsin BRP*, in milligrams,

V = volume of 0.02 M *sodium hydroxide* used per second by the test solution,

V' = volume of 0.02 M *sodium hydroxide* used per second by the reference solution,

A = activity of *chymotrypsin BRP*, in microkatal per milligram.

STORAGE

Store in an airtight container at 2 °C to 8 °C, protected from light.

LABELLING

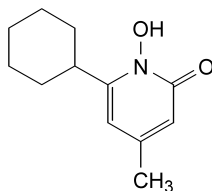
The label states:

- the quantity of chymotrypsin and the total activity in microkatal per container,
- for the amorphous substance, that it is hygroscopic.

01/2005:1407

CICLOPIROX

Ciclopiroxum

C₁₂H₁₇NO₂M_r 207.3

DEFINITION

Ciclopirox contains not less than 98.0 per cent and not more than the equivalent of 101.0 per cent of 6-cyclohexyl-1-hydroxy-4-methylpyridin-2(1*H*)-one, calculated with reference to the dried substance.

CHARACTERS

A white or yellowish-white, crystalline powder, slightly soluble in water, freely soluble in ethanol and in methylene chloride.

IDENTIFICATION

First identification: B.

Second identification: A, C.

- A. Melting point (2.2.14): 140 °C to 145 °C.
- B. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *ciclopirox CRS*.
- C. Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel F₂₅₄ plate R*.

Test solution. Dissolve 20 mg of the substance to be examined in *methanol R* and dilute to 10 ml with the same solvent.

Reference solution. Dissolve 20 mg of *ciclopirox CRS* in *methanol R* and dilute to 10 ml with the same solvent.

Before use predevelop the plate with a mixture of 10 volumes of *concentrated ammonia R*, 15 volumes of *water R* and 75 volumes of *alcohol R* until the solvent front has migrated to the top of the plate. Allow the plate to dry in air for 5 min.

Apply to the plate 10 µl of each solution. Develop over a path of 15 cm using a mixture of 10 volumes of *concentrated ammonia R*, 15 volumes of *water R* and 75 volumes of *alcohol R*. Allow the plate to dry in air for 10 min and examine in ultraviolet light at 254 nm. The principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with the reference solution. Spray the plate with a 20 g/l solution of *ferric chloride R* in *ethanol R*. The principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

TESTS

Appearance of solution. Dissolve 2.0 g in *methanol R* and dilute to 10 ml with the same solvent. The solution is clear (2.2.1) and not more intensely coloured than reference solution Y₅ (2.2.2, *Method II*).

Related substances. Examine by liquid chromatography (2.2.29). Carry out the operations avoiding exposure to actinic light. All materials which are in direct contact with the substance to be examined like column materials, reagents, solvents and others should contain only very low amounts of extractable metal cations.

Solvent mixture. Mix 1 volume of *acetonitrile R* and 9 volumes of the mobile phase.

Test solution. Dissolve 30.0 mg of the substance to be examined in 15 ml of the solvent mixture. If necessary, use an ultrasonic bath. Dilute to 20.0 ml with the solvent mixture.

Reference solution (a). Dissolve 15.0 mg of *ciclopirox impurity A CRS* and 15.0 mg of *ciclopirox impurity B CRS* in the solvent mixture and dilute to 10.0 ml with the same solvent mixture.

Reference solution (b). Dilute 1.0 ml of reference solution (a) to 200.0 ml with the solvent mixture.

Reference solution (c). Dilute 2.0 ml of reference solution (b) to 10.0 ml with the solvent mixture.

Reference solution (d). Mix 5 ml of reference solution (a) with 5 ml of the test solution.

The chromatographic procedure may be carried out using:

- a stainless steel column 80 mm long and 4 mm in internal diameter packed with *nitrile silica gel for chromatography R2* (5 µm),
- as rinsing solution a mixture of 1 volume of *glacial acetic acid R*, 1 volume of *acetylacetone R*, 500 volumes of *acetonitrile R* and 500 volumes of *water R*,
- as mobile phase at a flow rate of 0.7 ml/min a mixture of 0.1 ml of *glacial acetic acid R*, 230 ml of *acetonitrile R* and 770 ml of a 0.96 g/l solution of *sodium edetate R*. If the retention time of the principal peak in the chromatogram obtained with the test solution is not between 8 min and 11 min adjust the ratio of the 0.96 g/l solution of sodium edetate: acetonitrile accordingly,
- as detector a spectrophotometer set at 220 nm and 298 nm.

In order to ensure desorption of disruptive metal ions, every new column is to be rinsed with the rinsing solution over a period of not less than 15 h and then with the mobile phase for not less than 5 h at a flow rate of 0.2 ml/min.

Inject 10 µl each of the solvent mixture, the test solution and the reference solutions (b), (c) and (d) and record the chromatograms at 220 nm and 298 nm. Continue the chromatography of the test solution for 2.5 times the retention time of the principal peak.

The relative retention times are:

- impurity A: 0.5
- impurity C: 0.9
- ciclopirox: 1
- impurity B: 1.3

The test is not valid unless: in the chromatogram obtained with reference solution (d), the resolution between the peaks corresponding to impurity B and ciclopirox is not less than 2.0; the chromatogram obtained with reference solution (c) shows at 298 nm a peak corresponding to impurity B with a signal-to-noise ratio of not less than three; in the chromatogram obtained with the test solution the symmetry factor of the principal peak is between 0.8 and 2.0.

01/2005:1302

In the chromatogram obtained with the test solution at 220 nm, the area of the peak due to impurity A is not greater than the area of the corresponding peak in the chromatogram obtained with reference solution (b) at the same wavelength (0.5 per cent). In the chromatogram obtained with the test solution at 298 nm, the area of any peak apart from the principal peak is not greater than the area of the peak due to impurity B in the chromatogram obtained with reference solution (b) at the same wavelength (0.5 per cent); the sum of all peak areas at 298 nm, apart from the principal peak and any peak due to impurity B, is not greater than the area of the peak due to impurity B in the chromatogram obtained with reference solution (b) (0.5 per cent). At 298 nm disregard any peak due to the solvent and any peak with an area less than half the area of the peak due to impurity B in the chromatogram obtained with reference solution (c) at the same wavelength.

Heavy metals (2.4.8). 2.0 g complies with limit test C for heavy metals (10 ppm). Prepare the standard using 2 ml of *lead standard solution* (10 ppm Pb) R.

Loss on drying (2.2.32). Not more than 1.5 per cent, determined on 1.000 g by drying *in vacuo* at 60 °C over *diphosphorus pentoxide* R.

Sulphated ash (2.4.14). Not more than 0.1 per cent determined on 1.0 g.

ASSAY

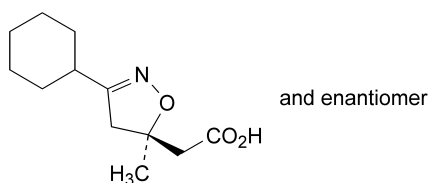
Dissolve 0.150 g in 20 ml of *methanol* R. Add 20 ml of *water* R and titrate with 0.1 M *sodium hydroxide*, determining the end-point potentiometrically (2.2.20). Carry out a blank titration.

1 ml of 0.1 M *sodium hydroxide* is equivalent to 20.73 mg of $C_{12}H_{17}NO_2$.

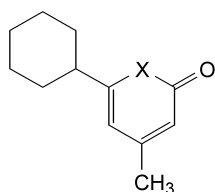
STORAGE

Store protected from light.

IMPURITIES



A. (RS)-2-(3-cyclohexyl-5-methyl-4,5-dihydroisoxazol-5-yl)acetic acid,

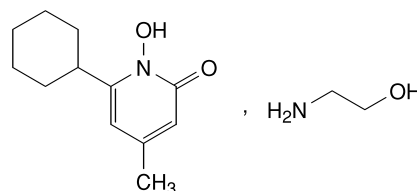


B. X = O: 6-cyclohexyl-4-methyl-2H-pyran-2-one,

C. X = NH: 6-cyclohexyl-4-methylpyridin-2(1H)-one.

CICLOPIROX OLAMINE

Ciclopirox olaminum


 $C_{14}H_{24}N_2O_3$
 M_r 268.4

DEFINITION

6-Cyclohexyl-1-hydroxy-4-methylpyridin-2(1H)-one and 2-aminoethanol.

Content:

- ciclopirox ($C_{12}H_{17}NO_2$; M_r 207.3): 76.0 per cent to 78.5 per cent (dried substance),
- 2-aminoethanol (C_2H_7NO ; M_r 61.1): 22.2 per cent to 23.3 per cent (dried substance).

CHARACTERS

Appearance: white or pale yellow, crystalline powder.

Solubility: sparingly soluble in water, very soluble in alcohol and in methylene chloride, slightly soluble in ethyl acetate, practically insoluble in cyclohexane.

It shows polymorphism.

IDENTIFICATION

First identification: A.

Second identification: B.

A. Infrared absorption spectrophotometry (2.2.24).

Comparison: *ciclopirox olamine* CRS.

If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in the minimum volume of *ethyl acetate* R, evaporate to dryness on a water-bath and record new spectra using the residues.

B. Thin-layer chromatography (2.2.27).

Test solution. Dissolve 25 mg of the substance to be examined in *methanol* R and dilute to 10 ml with the same solvent.

Reference solution. Dissolve 25 mg of *ciclopirox olamine* CRS in *methanol* R and dilute to 10 ml with the same solvent.

Plate: TLC silica gel F_{254} plate R.

Before use wash 2 plates by allowing a mixture of 10 volumes of *concentrated ammonia* R, 15 volumes of *water* R and 75 volumes of *ethanol* R to migrate until the solvent front has reached the top of the plate. Allow the plates to dry in air for 5 min.

Mobile phase: *concentrated ammonia* R, *water* R, *ethanol* R (10:15:75 V/V/V).

Application: 10 μ l.

Development: over a path of 15 cm.

Drying: in air for 10 min.

Detection A: examine in ultraviolet light at 254 nm.

Results A: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with the reference solution.

Detection B: spray one plate with *ferric chloride solution R3*.

Results B: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

Detection C: spray the second plate with *ninhydrin solution R*. Heat at 110 °C until the spots appear.

Results C: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

TESTS

Appearance of solution. The solution is clear (2.2.1) and not more intensely coloured than reference solution BY₇ (2.2.2, *Method II*).

Dissolve 2.0 g in *methanol R* and dilute to 20 ml with the same solvent.

pH (2.2.3): 8.0 to 9.0.

Dissolve 1.0 g in *carbon dioxide-free water R* and dilute to 100 ml with the same solvent.

Related substances. Liquid chromatography (2.2.29). Carry out the operations avoiding exposure to actinic light. All materials which are in direct contact with the substance to be examined, such as column materials, reagents, solvents, etc. should contain only small amounts of extractable metal cations.

Test solution. Dissolve 40.0 mg of the substance to be examined (corresponding to about 30 mg of ciclopirox) in a mixture of 20 µl of *anhydrous acetic acid R*, 2 ml of *acetonitrile R*, and 15 ml of the mobile phase. If necessary, use an ultrasonic bath. Dilute to 20.0 ml with the mobile phase.

Reference solution (a). Dissolve 15.0 mg of *ciclopirox impurity A CRS* and 15.0 mg of *ciclopirox impurity B CRS* in a mixture of 1 ml of *acetonitrile R* and 7 ml of the mobile phase. Dilute to 10.0 ml with the mobile phase.

Reference solution (b). Dilute 1.0 ml of reference solution (a) to 200.0 ml with a mixture of 1 volume of *acetonitrile R* and 9 volumes of the mobile phase.

Reference solution (c). Dilute 2.0 ml of reference solution (b) to 10.0 ml with a mixture of 1 volume of *acetonitrile R* and 9 volumes of the mobile phase.

Reference solution (d). Mix 5 ml of reference solution (a) with 5 ml of the test solution.

Column:

- size: $l = 80$ mm, $\varnothing = 4$ mm,
- stationary phase: *nitrile silica gel for chromatography R* (5 µm),
- rinsing solution: a mixture of 1 volume of *anhydrous acetic acid R*, 1 volume of *acetylacetone R*, 500 volumes of *acetonitrile R* and 500 volumes of *water R*.

Mobile phase: a mixture of 0.1 volumes of *anhydrous acetic acid R*, 230 volumes of *acetonitrile R* and 770 volumes of a 0.96 g/l solution of *sodium edetate R*. If the retention time of the principal peak in the chromatogram obtained with the test solution is not between 8 min and 11 min adjust the ratio of the 0.96 g/l solution of sodium edetate to acetonitrile accordingly.

Flow rate: 0.7 ml/min.

Detection: variable wavelength spectrophotometer capable of operating at 220 nm and 298 nm.

In order to ensure desorption of interfering metal ions, a new column is to be rinsed with the rinsing solution over a period of at least 15 h and then with the mobile phase for at least 5 h at a flow rate of 0.2 ml/min.

Injection: 10 µl; inject the test solution and reference solutions (b), (c) and (d).

Run time: 2.5 times the retention time of ciclopirox.

Relative retention with reference to ciclopirox:

impurity A = about 0.5; impurity C = about 0.9;

impurity B = about 1.3.

System suitability:

- **resolution:** minimum of 2.0 between the peaks corresponding to impurity B and ciclopirox in the chromatogram obtained with reference solution (d),
- **signal-to-noise ratio:** minimum of 10 for the peak corresponding to impurity B in the chromatogram obtained with reference solution (c) at 298 nm,
- **symmetry factor:** 0.8 to 2.0 for the principal peak in the chromatogram obtained with the test solution.

Limits:

- **impurity A at 220 nm:** not more than the area of the corresponding peak in the chromatogram obtained with reference solution (b) at the same wavelength (0.5 per cent),
- **any impurity at 298 nm:** not more than the area of the peak due to impurity B in the chromatogram obtained with reference solution (b) at the same wavelength (0.5 per cent),
- **total at 298 nm apart from impurity B:** not more than the area of the peak due to impurity B in the chromatogram obtained with reference solution (b) (0.5 per cent),
- **disregard limit at 298 nm:** area of the peak due to impurity B in the chromatogram obtained with reference solution (c) at the same wavelength (0.1 per cent).

Heavy metals (2.4.8): maximum 20 ppm.

1.0 g complies with limit test C. Prepare the standard using 2 ml of *lead standard solution (10 ppm Pb) R*.

Loss on drying (2.2.32): maximum 1.5 per cent, determined on 1.000 g by drying under high vacuum.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

2-Aminoethanol. Dissolve 0.250 g in 25 ml of *anhydrous acetic acid R*. Titrate with 0.1 M *perchloric acid*, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *perchloric acid* is equivalent to 6.108 mg of C₂H₇NO.

Ciclopirox. Dissolve 0.200 g in 2 ml of *methanol R*. Add 38 ml of *water R*, swirl and titrate immediately with 0.1 M *sodium hydroxide*, determining the end-point potentiometrically (2.2.20). Carry out a blank titration.

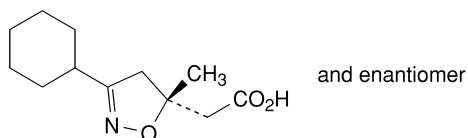
Use 0.1 M *sodium hydroxide*, the titre of which has been determined under the conditions prescribed above using 0.100 g of *benzoic acid RV*.

1 ml of 0.1 M *sodium hydroxide* is equivalent to 20.73 mg of C₁₂H₁₇NO₂.

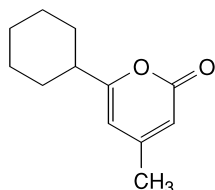
STORAGE

Protected from light.

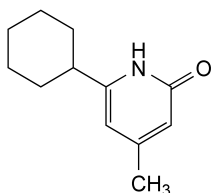
IMPURITIES



- A. (RS)-2-(3-cyclohexyl-5-methyl-4,5-dihydroisoxazol-5-yl)acetic acid,



- B. 6-cyclohexyl-4-methyl-2H-pyran-2-one,

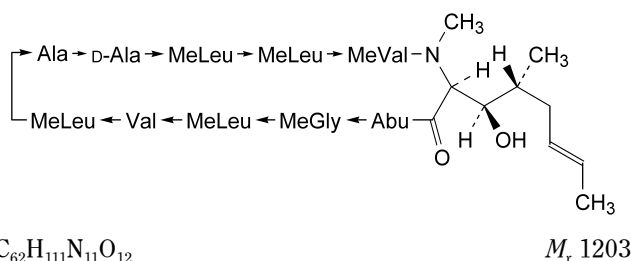


- C. 6-cyclohexyl-4-methylpyridin-2(1H)-one.

01/2005:0994
corrected

CICLOSPORIN

Ciclosporinum



DEFINITION

Ciclosporin contains not less than 98.5 per cent and not more than the equivalent of 101.5 per cent of cyclo[[*(2S,3R,4R,6E)*-3-hydroxy-4-methyl-2-(methylamino)oct-6-enoyl]-L-2-aminobutanoyl-N-methylglycyl-N-methyl-L-leucyl-L-valyl-N-methyl-L-leucyl-L-alanyl-D-alanyl-N-methyl-L-leucyl-N-methyl-L-leucyl-N-methyl-L-valyl], calculated with reference to the dried substance. Ciclosporin is a substance produced by *Beauveria nivea* (*Tolypocladium inflatum* Gams) or obtained by any other means.

CHARACTERS

A white or almost white powder, practically insoluble in water, freely soluble in ethanol and in methylene chloride.

IDENTIFICATION

- Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *ciclosporin CRS*.
- Examine the chromatograms obtained in the assay. The principal peak in the chromatogram obtained with the test solution is similar in retention time to the principal peak in the chromatogram obtained with reference solution (a).

TESTS

Appearance of solution. Dissolve 1.5 g in *ethanol R* and dilute to 15 ml with the same solvent. The solution is clear (2.2.1) and not more intensely coloured than reference solution Y_5 , BY_5 or R_7 (2.2.2, *Method II*).

Specific optical rotation (2.2.7). Dissolve 0.125 g in *methanol R* and dilute to 25.0 ml with the same solvent. The specific optical rotation is -185 to -193 , calculated with reference to the dried substance.

Related substances. Examine by liquid chromatography (2.2.29) as prescribed under Assay. Inject separately the test solution and reference solution (b). Continue the chromatography for 1.7 times the retention time of the principal peak. In the chromatogram obtained with the test solution: the area of any peak, apart from the principal peak, is not greater than 0.7 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.7 per cent); the sum of the areas of all the peaks, apart from the principal peak, is not greater than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (1.5 per cent). Disregard any peak due to the solvent and any peak with an area less than 0.05 times that of the principal peak in the chromatogram with reference solution (b).

Heavy metals (2.4.8). The residue obtained in the test for loss on drying complies with limit test C for heavy metals (20 ppm). Prepare the standard using 2 ml of *lead standard solution* (10 ppm *Pb*) *R*.

Loss on drying (2.2.32). Not more than 2.0 per cent, determined on 1.000 g at 60 °C at a pressure not exceeding 15 Pa for 3 h.

Bacterial endotoxins (2.6.14): less than 0.84 IU/mg, if intended for use in the manufacture of parenteral dosage forms without a further appropriate procedure for the removal of bacterial endotoxins. Dissolve 50 mg of the substance to be examined in a mixture of 280 mg of *alcohol R* and 650 mg of *polyoxyethylated castor oil R* and dilute to the required concentration using water LAL.

ASSAY

Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 30.0 mg of the substance to be examined in a mixture of equal volumes of *acetonitrile R* and *water R* and dilute to 25.0 ml with the same mixture of solvents.

Reference solution (a). Dissolve 30.0 mg of *ciclosporin CRS* in a mixture of equal volumes of *acetonitrile R* and *water R* and dilute to 25.0 ml with the same mixture of solvents.

Reference solution (b). Dilute 2.0 ml of reference solution (a) to 200.0 ml with a mixture of equal volumes of *acetonitrile R* and *water R*.

Reference solution (c). Dissolve the contents of a vial of *ciclosporin for system suitability CRS* in 5.0 ml of the mobile phase.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (3 µm to 5 µm); the column is connected to the injection port by a steel capillary tube about 1 m long and having an internal diameter of 0.25 mm,
- as mobile phase at a flow rate of about 1.5 ml per minute a mixture of 1 volume of *phosphoric acid R*, 50 volumes of *1,1-dimethylethyl methyl ether R*, 430 volumes of *acetonitrile R* and 520 volumes of *water R*,
- as detector a spectrophotometer set at 210 nm,

– a 20 µl loop injector,

maintaining the temperature of the column and of the steel capillary tube at 80 °C.

Inject reference solution (c). The test is not valid unless the peak-to-valley ratio is minimum 1.4 where H_p = height above the baseline of the peak due to ciclosporin U and H_v = height above the baseline of the lowest point of the curve separating this peak from the peak due to ciclosporin. Adjust the ratio of 1,1 dimethylethylmethyl ether to acetonitrile, if necessary. The test is not valid unless the retention time of the principal peak is 25 min to 30 min. Adjust the ratio of acetonitrile to water, if necessary. Inject reference solution (a) 6 times. The test is not valid unless the relative standard deviation of the area of the principal peak is at most 1.0 per cent. Inject alternately the test solution and reference solution (a).

Calculate the percentage content of ciclosporin.

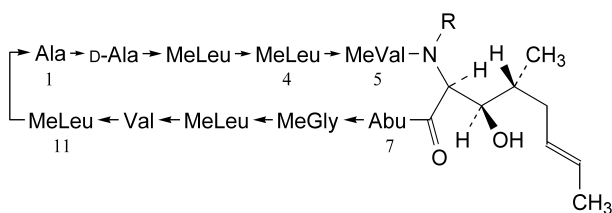
STORAGE

Store in an airtight container, protected from light. If the substance is sterile, store in a sterile, airtight, tamper-proof container.

LABELLING

The label states, where applicable, that the substance is free from bacterial endotoxins.

IMPURITIES



- A. different ciclosporins [difference with ciclosporin ($R = CH_3$: ciclosporin A)]: ciclosporin B [7-L-Ala]; ciclosporin C [7-L-Thr]; ciclosporin D [7-L-Val]; ciclosporin E [5-L-Val]; ciclosporin G [7-(L-2-aminopentanoyl)]; ciclosporin H [5-D-MeVal]; ciclosporin L [$R = H$]; ciclosporin T [4-L-Leu]; ciclosporin U [11-L-Leu]; ciclosporin V [1-L-Abu],

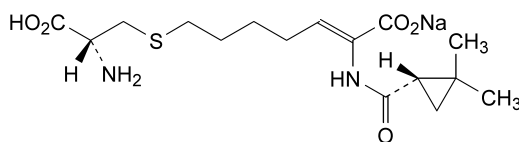


- B. [6-[(2S,3R,4R)-3-hydroxy-4-methyl-2-(methylamino)octanoic acid]]ciclosporin A,
C. isociclosporin A.

01/2005:1408

CILASTATIN SODIUM

Cilastatinum natricum



$C_{16}H_{25}N_2NaO_5S$

M_r 380.4

DEFINITION

Cilastatin sodium contains not less than 98.0 per cent and not more than the equivalent of 101.5 per cent of sodium (Z)-7-[(R)-2-amino-2-carboxyethyl]sulphonyl]-2-[[[(1S)-2,2-dimethylcyclopropyl]carbonyl]amino]hept-2-enoate, calculated with reference to the anhydrous and solvent-free substance.

CHARACTERS

A white or light yellow amorphous powder, hygroscopic, very soluble in water and in methanol, soluble in dimethyl sulphoxide, slightly soluble in ethanol, practically insoluble in acetone and in methylene chloride.

IDENTIFICATION

- A. It complies with the test for specific optical rotation (see Tests).
B. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *cilastatin sodium CRS*.
C. It gives reaction (a) of sodium (2.3.1).

TESTS

Solution S. Dissolve 1.0 g in *carbon dioxide-free water R* and dilute to 100 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and not more intensely coloured than reference solution Y_6 (2.2.2, *Method II*).

pH (2.2.3). The pH of solution S is 6.5 to 7.5.

Specific optical rotation (2.2.7). Dissolve 0.250 g in a mixture of 1 volume of *hydrochloric acid R* and 120 volumes of *methanol R* and dilute to 25.0 ml with the same mixture of solvents. The specific optical rotation is + 41.5 to + 44.5, calculated with reference to the anhydrous and solvent-free substance.

Related substances. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 32.0 mg of the substance to be examined in *water R* and dilute to 20.0 ml with the same solvent.

Reference solution (a). Dilute 2.0 ml of the test solution to 100.0 ml with *water R*. Dilute 5.0 ml of this solution to 100.0 ml with *water R*.

Reference solution (b). Dilute 5.0 ml the test solution to 100.0 ml with *water R*. Dilute 2.0 ml of this solution to 20.0 ml with *water R*.

Reference solution (c). Dissolve 16 mg of the substance to be examined in *dilute hydrogen peroxide solution R* and dilute to 10.0 ml with the same solvent. Allow to stand for 30 min. Dilute 1 ml of this solution to 100 ml with *water R*.

Reference solution (d). Dissolve 32 mg of *mesityl oxide R* in 100 ml of *water R*. Dilute 1 ml of the solution to 50 ml with *water R*.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4.6 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (5 µm),
- as mobile phase at a flow rate of 2.0 ml/min, the following mixtures:

Mobile phase A. Prepare a mixture of 300 volumes of *acetonitrile R* and 700 volumes of a 0.1 per cent V/V solution of *phosphoric acid R* in *water R*,

Mobile phase B. A 0.1 per cent V/V solution of *phosphoric acid R* in *water R*,

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)
	15	85
0 - 30	15 → 100	85 → 0
30 - 46	100	0
46 - 56	100 → 15	0 → 85

– as detector a spectrophotometer set at 210 nm,

– a 20 µl loop injector,

maintaining the temperature of the column at 50 °C.

Equilibrate the column with a mixture of 15 per cent V/V of mobile phase A and 85 per cent V/V of mobile phase B. Inject separately each solution. Adjust the sensitivity of the system so that the height of the principal peak in the chromatogram obtained with reference solution (b) is at least 15 per cent of the full scale of the recorder.

The test is not valid unless, the chromatogram obtained with reference solution (c) shows three principal peaks: the first two peaks (cilastatin impurity A) may elute without being completely resolved and the mass distribution ratio of the third peak (cilastatin) is at least ten; in the chromatogram obtained with reference solution (a), the principal peak has a signal-to-noise ratio of at least 5.0.

In the chromatogram obtained with the test solution, the area of any peak, apart from the principal peak, is not greater than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent); the sum of the areas of all peaks, apart from the principal peak, is not greater than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (1 per cent). Disregard any peak due to the solvent, any peak with an area less than that of the principal peak in the chromatogram obtained with reference solution (a) and any peak corresponding to the principal peak in the chromatogram obtained with reference solution (d).

Mesityl oxide, acetone and methanol. Not more than 1.0 per cent *m/m* of acetone, 0.5 per cent *m/m* of methanol and 0.4 per cent *m/m* of mesityl oxide. Examine by gas chromatography (2.2.28) using *propanol R* as the internal standard.

Internal standard solution. Dissolve 0.5 ml of *propanol R* in *water R* and dilute to 1000 ml with the same solvent.

Test solution. Dissolve 0.200 g of the substance to be examined in *water R*, add 2.0 ml of the internal standard solution and dilute to 10.0 ml with *water R*.

Reference solution. Dissolve 2.0 ml of *acetone R*, 0.5 ml of *methanol R* and 0.5 ml of *mesityl oxide R* in *water R* and dilute to 1000 ml with the same solvent. To 2.0 ml of this solution add 2.0 ml of the internal standard solution and dilute to 10.0 ml with *water R*. This solution contains 316 µg of acetone, 79 µg of methanol and 86 µg of mesityl oxide per millilitre.

The chromatographic procedure may be carried out using:

- a fused-silica column 30 m long and 0.53 mm in internal diameter coated with *macrogol 20 000 R* (film thickness 1.0 µm),
- *helium for chromatography R* as the carrier gas at a flow rate of 9 ml/min,
- a flame-ionisation detector,

with the following temperature programme:

	Time (min)	Temperature (°C)	Rate (°C/min)	Comment
Column	0 - 2.5	50	–	isothermal
	2.5 - 5	50 → 70	8	linear gradient
	5 - 5.5	70	–	isothermal
Injection port		160		
Detector		220		

Inject 1 µl of the reference solution and then 1 µl of the test solution. Calculate the percentage contents of acetone, methanol and mesityl oxide using the following expression:

$$\left(\frac{C}{W}\right) \times \left(\frac{R_u}{R_s}\right)$$

in which *C* is the concentration in µg/ml of the solvent in the reference solution, *W* is the quantity in milligrams of cilastatin sodium in the test solution and *R_u* and *R_s* are the ratios of the corresponding solvent peak areas to the propanol peak area in the test solution and the reference solution, respectively.

Heavy metals (2.4.8). 1.0 g complies with limit test C for heavy metals (20 ppm). Prepare the standard using 2.0 ml of *lead standard solution (10 ppm Pb) R*.

Water (2.5.12). Not more than 2.0 per cent, determined on 0.50 g by the semi-micro determination of water.

Bacterial endotoxins (2.6.14): less than 0.17 IU/mg, if intended for use in the manufacture of parenteral dosage forms without a further appropriate procedure for the removal of bacterial endotoxins.

ASSAY

Dissolve 0.300 g in 30 ml of *methanol R* and add 5 ml of *water R*. Add 0.1 M *hydrochloric acid* to a pH of about 3.0. Carry out a potentiometric titration (2.2.20), using 0.1 M *sodium hydroxide*. Three jumps of potential are observed. Titrate to the third equivalence point.

1 ml of 0.1 M *sodium hydroxide* is equivalent to 19.02 mg of C₁₆H₂₅N₂NaO₅S.

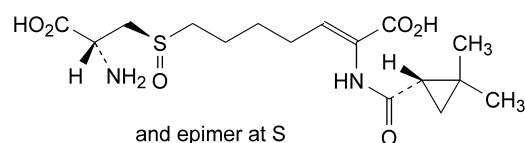
STORAGE

Store in an airtight container, at a temperature not exceeding 8 °C. If the substance is sterile, store in a sterile, airtight, tamper-proof container.

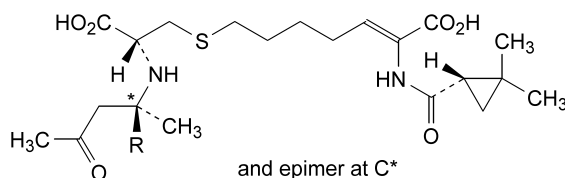
LABELLING

The label states, where applicable, that the substance is free from bacterial endotoxins.

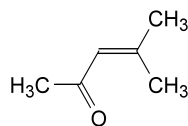
IMPURITIES



A. (Z)-7-[(RS)-[(R)-2-amino-2-carboxyethyl]sulphonyl]-2-[[[(1S)-2,2-dimethylcyclopropyl]carbonyl]amino]hept-2-enoic acid,



- B. R = H: (Z)-7-[[[(R)-2-[[[(1S)-1-methyl-3-oxobutyl]amino]-2-carboxyethyl]sulphonyl]-2-[[[(1S)-2,2-dimethylcyclopropyl]carbonyl]amino]hept-2-enoic acid,
- C. R = CH₃: (Z)-7-[[[(R)-2-[(1,1-dimethyl-3-oxobutyl)amino]-2-carboxyethyl]sulphonyl]-2-[[[(1S)-2,2-dimethylcyclopropyl]carbonyl]amino]hept-2-enoic acid,

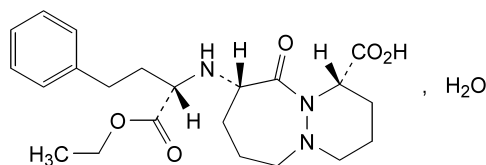


- D. 4-methylpent-3-en-2-one (mesityl oxide).

01/2005:1499
corrected

CILAZAPRIL

Cilazaprilum



C₂₂H₃₁N₃O₅·H₂O

M_r 435.5

DEFINITION

Cilazapril contains not less than 98.5 per cent and not more than the equivalent of 101.5 per cent of (1S,9S)-9-[[[(1S)-1-(ethoxycarbonyl)-3-phenylpropyl]amino]-10-oxooctahydro-6H-pyridazino[1,2-a][1,2]diazepine-1-carboxylic acid, calculated with reference to the anhydrous substance.

CHARACTERS

A white or almost white, crystalline powder, slightly soluble in water, freely soluble in methanol and in methylene chloride.

IDENTIFICATION

- A. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *cilazapril CRS*.
- B. It complies with the test for specific optical rotation (see Tests).

TESTS

Specific optical rotation (2.2.7). Dissolve 0.200 g in 0.067 M phosphate buffer solution pH 7.0 R, with the aid of ultrasound if necessary and dilute to 50.0 ml with the same buffer solution. The specific optical rotation is –383 to –399, determined at 365 nm and calculated with reference to the anhydrous substance.

Impurity A. Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel plate R*.

Test solution. Dissolve 0.20 g of the substance to be examined in *methanol R* and dilute to 5.0 ml with the same solvent.

Reference solution (a). Dissolve 2 mg of *cilazapril impurity A CRS* in *methanol R* and dilute to 50.0 ml with the same solvent.

Reference solution (b). Dissolve 5 mg of *cilazapril impurity A CRS* and 5 mg of the substance to be examined in *methanol R* and dilute to 10.0 ml with the same solvent.

Apply to the plate 5 µl of each solution. Develop over a path of 10 cm using a mixture of 5 volumes of *glacial acetic acid R*, 5 volumes of *water R*, 15 volumes of *hexane R*, 15 volumes of *methanol R* and 60 volumes of *ethyl acetate R*. Dry the plate in a current of cold air for 10 min. Spray the plate with a freshly prepared mixture of 1 volume of *potassium iodobismuthate solution R* and 10 volumes of *dilute acetic acid R* and then with *dilute hydrogen peroxide solution R*. Any spot corresponding to impurity A in the chromatogram obtained with the test solution is not more intense than the spot in the chromatogram obtained with reference solution (a) (0.1 per cent). The test is not valid unless the chromatogram obtained with reference solution (b) shows 2 clearly separated spots.

Other related substances. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 25.0 mg of the substance to be examined in the mobile phase and dilute to 50.0 ml with the mobile phase.

Reference solution (a). Dilute 1.0 ml of the test solution to 50.0 ml with the mobile phase. Dilute 5.0 ml of this solution to 20.0 ml with the mobile phase.

Reference solution (b). Dissolve 5.0 mg of *cilazapril impurity D CRS* in the test solution and dilute to 10.0 ml with the same solution.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4.6 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (5 µm),
- as mobile phase at a flow rate of 1.0 ml/min a mixture prepared as follows: mix 10 volumes of *triethylamine R* and 750 volumes of *water R*, adjust to pH 2.30 with *phosphoric acid R*, and add 200 volumes of *tetrahydrofuran R*,
- as detector a spectrophotometer set at 214 nm.

Inject 20 µl of reference solution (a) and 20 µl of reference solution (b). Adjust the sensitivity of the system so that the height of the principal peak in the chromatogram obtained with reference solution (a) is at least 50 per cent of the full scale of the recorder. The test is not valid unless the resolution between the peaks corresponding to cilazapril and impurity D in the chromatogram obtained with reference solution (b) is at least 2.5 and the symmetry factor of the peak due to cilazapril is not more than 3.0.

Inject 20 µl of the test solution and 20 µl of reference solution (a). Continue the chromatography for twice the retention time of the principal peak. When impurity A is present (relative retention time of 4 to 5), it may be necessary to continue the chromatography until it is eluted. When the chromatograms are recorded in the prescribed conditions, the retention times relative to cilazapril are: impurity B about 0.6; impurity D about 0.9; impurity C about 1.6. In the chromatogram obtained with the test solution: the area of any peak corresponding to impurity D is not greater than 0.4 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent); the area of any peak corresponding to impurity B is not greater than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent); the area of any peak corresponding to impurity C is not greater than 0.2 times the area of the principal peak in the chromatogram

01/2005:0756

obtained with reference solution (a) (0.1 per cent); the area of any peak, apart from the principal peak and the peaks corresponding to impurities B, C and D, is not greater than 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent); the sum of the areas of all the peaks, apart from the principal peak, is not greater than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (1 per cent). Disregard any peak with an area less than 0.1 times that of the principal peak in the chromatogram obtained with reference solution (a) and any peak due to impurity A.

Water (2.5.12): 3.5 per cent to 5.0 per cent, determined on 0.300 g by the semi-micro determination of water.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.300 g in 10 ml of *ethanol R* and add 50 ml of *water R*. Titrate with 0.1 M *sodium hydroxide*, determining the end-point potentiometrically (2.2.20). Carry out a blank titration.

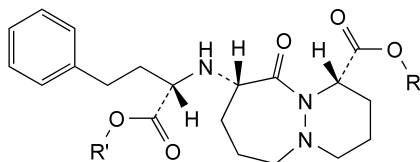
1 ml of 0.1 M *sodium hydroxide* is equivalent to 41.75 mg of $C_{10}H_{16}N_6S$.

STORAGE

Protected from light.

IMPURITIES

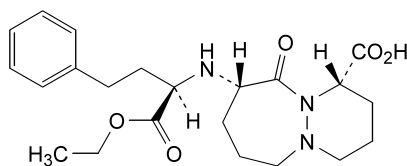
Specified impurities: A, B, C, D.



A. $R = C(CH_3)_3$, $R' = C_6H_5$: 1,1-dimethylethyl (1*S*,9*S*)-9-[(*S*)-1-(ethoxycarbonyl)-3-phenylpropyl]amino]-10-oxooctahydro-6*H*-pyridazino[1,2-*a*][1,2]diazepine-1-carboxylate,

B. $R = R' = H$: (1*S*,9*S*)-9-[(*S*)-1-carboxy-3-phenylpropyl]amino]-10-oxooctahydro-6*H*-pyridazino[1,2-*a*][1,2]diazepine-1-carboxylic acid,

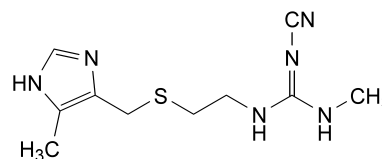
C. $R = R' = C_2H_5$: ethyl (1*S*,9*S*)-9-[(*S*)-1-(ethoxycarbonyl)-3-phenylpropyl]amino]-10-oxooctahydro-6*H*-pyridazino[1,2-*a*][1,2]diazepine-1-carboxylate,



D. (1*S*,9*S*)-9-[(*R*)-1-(ethoxycarbonyl)-3-phenylpropyl]amino]-10-oxooctahydro-6*H*-pyridazino[1,2-*a*][1,2]diazepine-1-carboxylic acid.

CIMETIDINE

Cimetidinum



$C_{10}H_{16}N_6S$

M_r 252.3

DEFINITION

Cimetidine contains not less than 98.5 per cent and not more than the equivalent of 101.5 per cent of 2-cyano-1-methyl-3-[2-[(5-methyl-1*H*-imidazol-4-yl)methyl]sulphonyl]ethyl]guanidine, calculated with reference to the dried substance.

CHARACTERS

A white or almost white powder, slightly soluble in water, soluble in alcohol, practically insoluble in methylene chloride. It dissolves in dilute mineral acids.

It shows polymorphism.

IDENTIFICATION

First identification: B.

Second identification: A, C, D.

- Melting point (2.2.14): 139 °C to 144 °C. If necessary, dissolve the substance to be examined in 2-propanol *R*, evaporate to dryness and determine the melting point again.
- Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *cimetidine CRS*. If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in 2-propanol *R*, evaporate to dryness and record the spectra again.
- Examine the chromatograms obtained in the test for related substances. The principal spot in the chromatogram obtained with test solution (b) is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (d).
- Dissolve about 1 mg in a mixture of 1 ml of *ethanol R* and 5 ml of a freshly prepared 20 g/l solution of *citric acid R* in *acetic anhydride R*. Heat in a water-bath for 10 min to 15 min. A reddish-violet colour develops.

TESTS

Appearance of solution. Dissolve 3.0 g in 12 ml of 1 M *hydrochloric acid* and dilute to 20 ml with *water R*. The solution is clear (2.2.1) and not more intensely coloured than reference solution Y_5 (2.2.2, *Method II*).

Related substances. Examine by thin-layer chromatography (2.2.27), using *silica gel GF₂₅₄ R* as the coating substance.

Test solution (a). Dissolve 0.50 g of the substance to be examined in *methanol R* and dilute to 10 ml with the same solvent.

Test solution (b). Dilute 1 ml of test solution (a) to 10 ml with *methanol R*.

Reference solution (a). Dilute 1 ml of test solution (a) to 100 ml with *methanol R*. Dilute 20 ml of this solution to 100 ml with *methanol R*.

Reference solution (b). Dilute 5 ml of reference solution (a) to 10 ml with *methanol R*.

Reference solution (c). Dilute 5 ml of reference solution (b) to 10 ml with *methanol R*.

01/2005:1500

Reference solution (d). Dissolve 10 mg of *cimetidine CRS* in 2 ml of *methanol R*.

A. Apply separately to the plate 4 µl of each solution. Allow the plate to stand for 15 min in the chromatographic tank saturated with vapour from the mobile phase which consists of a mixture of 15 volumes of *concentrated ammonia R*, 20 volumes of *methanol R* and 65 volumes of *ethyl acetate R* and develop immediately over a path of 15 cm using the same mixture of solvents. Dry the plate in a stream of cold air, expose to iodine vapour until maximum contrast of the spots has been obtained and examine in ultraviolet light at 254 nm. Any spot in the chromatogram obtained with test solution (a), apart from the principal spot, is not more intense than the principal spot in the chromatogram obtained with reference solution (a) (0.2 per cent) and not more than two such spots are more intense than the principal spot in the chromatogram obtained with reference solution (b) (0.1 per cent). The test is not valid unless the chromatogram obtained with reference solution (c) shows a clearly visible spot.

B. Apply separately to the plate 4 µl of each solution. Develop over a path of 15 cm using a mixture of 8 volumes of *concentrated ammonia R*, 8 volumes of *methanol R* and 84 volumes of *ethyl acetate R*. Dry the plate in a stream of cold air, expose to iodine vapour until maximum contrast of the spots has been obtained and examine in ultraviolet light at 254 nm. Any spot in the chromatogram obtained with the test solution (a), apart from the principal spot, is not more intense than the principal spot in the chromatogram obtained with reference solution (a) (0.2 per cent) and not more than two such spots are more intense than the principal spot in the chromatogram obtained with reference solution (b) (0.1 per cent). The test is not valid unless the chromatogram obtained with reference solution (c) shows a clearly visible spot.

Heavy metals (2.4.8). 1.0 g complies with limit test C for heavy metals (20 ppm). Prepare the standard using 2 ml of *lead standard solution (10 ppm Pb) R*.

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C.

Sulphated ash (2.4.14). Not more than 0.2 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.200 g in 60 ml of *anhydrous acetic acid R*. Titrate with 0.1 M *perchloric acid* determining the end-point potentiometrically (2.2.20).

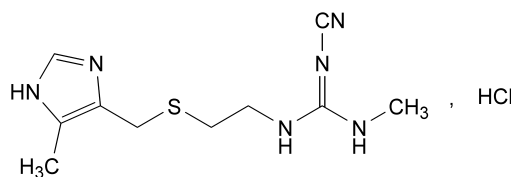
1 ml of 0.1 M *perchloric acid* is equivalent to 25.23 mg of $C_{10}H_{16}N_6S$.

STORAGE

Store in an airtight container, protected from light.

CIMETIDINE HYDROCHLORIDE

Cimetidini hydrochloridum


 $C_{10}H_{17}ClN_6S$
 M_r 288.8

DEFINITION

Cimetidine hydrochloride contains not less than 98.5 per cent and not more than the equivalent of 101.5 per cent of 2-cyano-1-methyl-3-[2-[(5-methyl-1H-imidazol-4-yl)methyl]sulphanyl]ethyl]guanidine hydrochloride, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, crystalline powder, freely soluble in water, sparingly soluble in ethanol.

IDENTIFICATION

First identification: B, E.

Second identification: A, C, D, E.

- Dissolve 70 mg in 0.2 M *sulphuric acid* and dilute to 100.0 ml with the same acid. Dilute 2.0 ml of the solution to 100.0 ml with 0.2 M *sulphuric acid*. Measure the absorbance (2.2.25) at the absorption maximum at 218 nm. The specific absorbance at the maximum is 650 to 705.
- Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *cimetidine hydrochloride CRS*.
- Examine the chromatograms obtained in the test for related substances. The principal spot in the chromatogram obtained with test solution (b) is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (d).
- Dissolve about 1 mg in a mixture of 1 ml of *ethanol R* and 5 ml of a freshly prepared 20 g/l solution of *citric acid R* in *acetic anhydride R*. Heat on a water-bath for 10 min to 15 min. A reddish-violet colour develops.
- It gives reaction (a) of chlorides (2.3.1).

TESTS

Appearance of solution. Dissolve 3.0 g in 12 ml of 1 M *hydrochloric acid* and dilute to 20 ml with *water R*. The solution is clear (2.2.1) and not more intensely coloured than reference solution Y_5 (2.2.2, *Method II*).

pH (2.2.3). Dissolve 100 mg in *carbon dioxide-free water R* and dilute to 10.0 ml with the same solvent. The pH of the solution is 4.0 to 5.0.

Related substances. Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel GF₂₅₄ plate R*.

Test solution (a). Dissolve 0.50 g of the substance to be examined in *methanol R* and dilute to 10 ml with the same solvent.

Test solution (b). Dilute 1 ml of test solution (a) to 10 ml with *methanol R*.

Reference solution (a). Dilute 2 ml of test solution (b) to 100 ml with *methanol R*.

Reference solution (b). Dilute 5 ml of reference solution (a) to 10 ml with *methanol R*.

Reference solution (c). Dilute 5 ml of reference solution (b) to 10 ml with *methanol R*.

Reference solution (d). Dissolve 10 mg of *cimetidine hydrochloride CRS* in 2 ml of *methanol R*.

A. Apply to the plate 4 µl of each solution. Allow the plate to stand for 15 min in a chromatographic tank saturated with vapour from the mobile phase, which consists of a mixture of 15 volumes of *concentrated ammonia R*, 20 volumes of *methanol R* and 65 volumes of *ethyl acetate R*, and develop immediately over a path of 15 cm using the same mixture of solvents. Dry the plate in a stream of cold air, expose to iodine vapour until maximum contrast of the spots has been obtained and examine in ultraviolet light at 254 nm. Any spot in the chromatogram obtained with test solution (a), apart from the principal spot, is not more intense than the principal spot in the chromatogram obtained with reference solution (a) (0.2 per cent) and at most two such spots are more intense than the principal spot in the chromatogram obtained with reference solution (b) (0.1 per cent). The test is not valid unless the chromatogram obtained with reference solution (c) shows a clearly visible spot.

B. Apply to the plate 4 µl of each solution. Develop over a path of 15 cm using a mixture of 8 volumes of *concentrated ammonia R*, 8 volumes of *methanol R* and 84 volumes of *ethyl acetate R*. Dry the plate in a stream of cold air, expose to iodine vapour until maximum contrast of the spots has been obtained and examine in ultraviolet light at 254 nm. Any spot in the chromatogram obtained with test solution (a), apart from the principal spot, is not more intense than the principal spot in the chromatogram obtained with reference solution (a) (0.2 per cent) and at most two such spots are more intense than the principal spot in the chromatogram obtained with reference solution (b) (0.1 per cent). The test is not valid unless the chromatogram obtained with reference solution (c) shows a clearly visible spot.

Heavy metals (2.4.8). 1.0 g complies with limit test C for heavy metals (20 ppm). Prepare the standard using 2 ml of *lead standard solution (10 ppm Pb) R*.

Loss on drying (2.2.32). Not more than 1.0 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C.

Sulphated ash (2.4.14). Not more than 0.2 per cent, determined on 1.0 g.

ASSAY

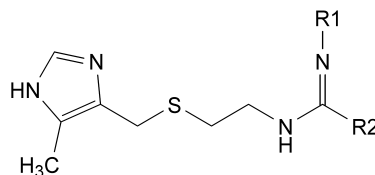
Dissolve 0.200 g of the substance to be examined in a mixture of 5 ml of 0.01 M *hydrochloric acid* and 50 ml of *alcohol R*. Carry out a potentiometric titration (2.2.20), using 0.1 M *sodium hydroxide*. Read the volume added between the two points of inflexion.

1 ml of 0.1 M *sodium hydroxide* is equivalent to 28.88 mg of $C_{10}H_{17}ClN_3O_2$.

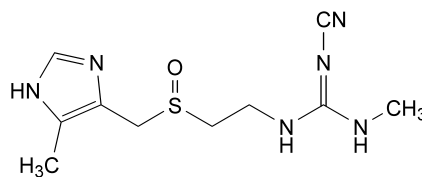
STORAGE

Store in an airtight container, protected from light.

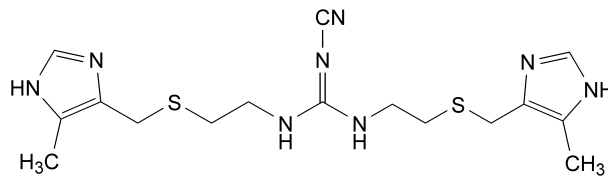
IMPURITIES



- A. R1 = CN, R2 = SCH₃: 3-cyano-2-methyl-1-[2-[(5-methyl-1H-imidazol-4-yl)methyl]sulphanyl]ethyl]isothiourea,
 B. R1 = CN, R2 = OCH₃: 3-cyano-2-methyl-1-[2-[(5-methyl-1H-imidazol-4-yl)methyl]sulphanyl]ethyl]isourea,
 C. R1 = CONH₂, R2 = NHCH₃: 1-[(methylamino)[[2-[(5-methyl-1H-imidazol-4-yl)methyl]sulphanyl]ethyl]amino]methylene]urea,
 D. R1 = H, R2 = NHCH₃: 1-methyl-3-[2-[(5-methyl-1H-imidazol-4-yl)methyl]sulphanyl]ethyl]guanidine,



- E. 2-cyano-1-methyl-3-[2-[(5-methyl-1H-imidazol-4-yl)methyl]sulphanyl]ethyl]guanidine,

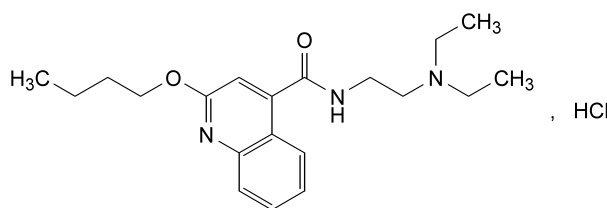


- F. 2-cyano-1,3-bis[2-[(5-methyl-1H-imidazol-4-yl)methyl]sulphanyl]ethyl]guanidine.

01/2005:1088

CINCHOCAINE HYDROCHLORIDE

Cinchocaini hydrochloridum



$C_{20}H_{30}ClN_3O_2$

M_r 379.9

DEFINITION

Cinchocaine hydrochloride contains not less than 98.5 per cent and not more than the equivalent of 101.0 per cent of 2-butoxy-N-[2-(diethylamino)ethyl]quinoline-4-carboxamide hydrochloride, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, crystalline powder or colourless crystals, hygroscopic, very soluble in water, freely soluble in acetone, in alcohol and in methylene chloride. It agglomerates very easily.

IDENTIFICATION

First identification: B, E.

Second identification: A, C, D, E.

- A. Dissolve 60.0 mg in 1 M hydrochloric acid and dilute to 100 ml with the same acid. Dilute 2 ml of the solution to 100 ml with 1 M hydrochloric acid. Examined between 220 nm and 350 nm (2.2.25), the solution shows two absorption maxima, at 246 nm and 319 nm. The ratio of the absorbance measured at 246 nm to that measured at 319 nm is 2.7 to 3.0.
- B. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *cinchocaine hydrochloride CRS*. Examine the substances prepared as discs using *potassium chloride R*.
- C. Examine the chromatograms obtained in the test for related substances. The principal spot in the chromatogram obtained with test solution (b) is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a).
- D. Dissolve 0.5 g in 5 ml of *water R*. Add 1 ml of *dilute ammonia R2*. A white precipitate is formed. Filter, wash the precipitate with five quantities, each of 10 ml, of *water R* and dry in a desiccator. It melts at 64 °C to 66 °C (2.2.14).
- E. It gives reaction (a) of chlorides (2.3.1).

TESTS

Solution S. Dissolve 5.0 g in *carbon dioxide-free water R* prepared from *distilled water R*, and dilute to 50 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and not more intensely coloured than reference solution Y_6 (2.2.2, *Method II*).

pH (2.2.3). Dilute 10 ml of solution S to 50 ml with *carbon dioxide-free water R*. The pH of the solution is 5.0 to 6.0.

Related substances. Examine by thin-layer chromatography (2.2.27), using as the coating substance a suitable silica gel with a fluorescent indicator having an optimal intensity at 254 nm.

Test solution (a). Dissolve 0.20 g of the substance to be examined in *methanol R* and dilute to 5 ml with the same solvent.

Test solution (b). Dilute 1 ml of test solution (a) to 10 ml with *methanol R*.

Reference solution (a). Dissolve 20 mg of *cinchocaine hydrochloride CRS* in *methanol R* and dilute to 5 ml with the same solvent.

Reference solution (b). Dilute 1 ml of test solution (b) to 20 ml with *methanol R*.

Reference solution (c). Dilute 1 ml of test solution (b) to 50 ml with *methanol R*.

Reference solution (d). Dissolve 20 mg of *benzocaine CRS* in *methanol R* and dilute to 5 ml with the same solvent. Dilute 1 ml of the solution and 1 ml of reference solution (a) to 20 ml with *methanol R*.

Apply separately to the plate 5 µl of each solution. Develop over a path of 15 cm using a mixture of 1 volume of *ammonia R*, 5 volumes of *methanol R*, 30 volumes of *acetone R* and 50 volumes of *toluene R*. Dry the plate in a current of warm air for 15 min. Examine in ultraviolet light at 254 nm. Any spot in the chromatogram obtained with test solution (a), apart from the principal spot, is not more intense than the principal spot in the chromatogram obtained with reference solution (b) (0.5 per cent) and at most one such spot is more intense than the spot in the chromatogram obtained with reference solution (c) (0.2 per cent). The test is not valid unless the chromatogram obtained with reference solution (d) shows two clearly separated spots.

Heavy metals (2.4.8). 12 ml of solution S complies with limit test A for heavy metals (20 ppm). Prepare the standard using *lead standard solution (2 ppm Pb) R*.

Loss on drying (2.2.32). Not more than 2.0 per cent, determined on 0.500 g by drying *in vacuo* at 60 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

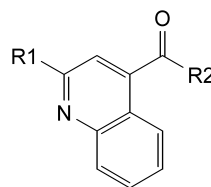
Dissolve 0.300 g in a mixture of 15.0 ml of 0.01 M hydrochloric acid and 50 ml of *alcohol R*. Carry out a potentiometric titration (2.2.20), using 0.1 M sodium hydroxide. Read the volume added between the two points of inflexion.

1 ml of 0.1 M sodium hydroxide is equivalent to 37.99 mg of $C_{20}H_{30}ClN_3O_2$.

STORAGE

Store in an airtight container, protected from light.

IMPURITIES



- A. $R1 = Cl$, $R2 = NH-[CH_2]_2-N(C_2H_5)_2$: 2-chloro-*N*-[2-(diethylamino)ethyl]quinoline-4-carboxamide,
- B. $R1 = R2 = OH$: 2-hydroxyquinoline-4-carboxylic acid,
- C. $R1 = OH$, $R2 = NH-[CH_2]_2-N(C_2H_5)_2$: *N*-[2-(diethylamino)ethyl]-2-hydroxyquinoline-4-carboxamide,
- D. $R1 = O-[CH_2]_3-CH_3$, $R2 = OH$: 2-butoxyquinoline-4-carboxylic acid.

01/2005:0174

CINCHONA BARK

Cinchonae cortex

DEFINITION

Whole or cut, dried bark of *Cinchona pubescens* Vahl (*Cinchona succirubra* Pavon), of *C. calisaya* (Weddell), of *C. ledgeriana* (Moens ex Trimen) or of its varieties or hybrids.

Content: minimum 6.5 per cent of total alkaloids, of which 30 per cent to 60 per cent consists of quinine-type alkaloids (dried drug).

CHARACTERS

Intense bitter, somewhat astringent taste.

Macroscopic and microscopic characters described under identification tests A and B.

IDENTIFICATION

- A. The stem and branch bark is supplied in quilled or curved pieces 2 mm to 6 mm thick. The outer surface is dull brownish-grey or grey and frequently bears lichens; it is usually rough, marked with transverse fissures and longitudinally furrowed or wrinkled; exfoliation of the outer surface occurs in some varieties. The inner surface is striated and deep reddish-brown; the fracture is short in the outer part and fibrous in the inner part.

- B. Reduce to a powder (355). The powder is reddish-brown. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters: thin-walled cork cells filled with reddish-brown contents; yellow, spindle-shaped striated phloem fibres up to 90 µm in diameter and up to 1300 µm in length, very thick-walled with an uneven lumen and with conspicuous, funnel-shaped pits; parenchymatous idioblasts filled with microprisms of calcium oxalate. Examine under a microscope using a 50 per cent *V/V* solution of *glycerol R*. The powder shows a few starch granules 6 µm to 10 µm in diameter; mostly simple but occasionally with 2 or 3 components.

C. Thin-layer chromatography (2.2.27).

Test solution. To 0.10 g of the powdered drug (180) in a test-tube add 0.1 ml of *concentrated ammonia R* and 5 ml of *methylene chloride R*. Shake vigorously occasionally during 30 min and filter. Evaporate the filtrate to dryness on a water-bath and dissolve the residue in 1 ml of *ethanol R*.

Reference solution. Dissolve 17.5 mg of *quinine R*, 2.5 mg of *quinidine R*, 10 mg of *cinchonine R* and 10 mg of *cinchonidine R* in 5 ml of *ethanol R*.

Plate: TLC silica gel plate *R*.

Mobile phase: *diethylamine R*, *ethyl acetate R*, *toluene R* (10:20:70 *V/V/V*).

Application: 10 µl, as bands.

Development: twice over a path of 15 cm.

Drying: at 100-105 °C then allow to cool.

Detection A: spray with *anhydrous formic acid R* and allow to dry in air. Examine in ultraviolet light at 365 nm.

Results A: see below the sequence of the zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other fluorescent zones are present in the chromatogram obtained with the test solution.

Top of the plate	
Quinidine: a distinct blue fluorescent zone	A distinct blue fluorescent zone (quinidine)
Quinine: a distinct blue fluorescent zone	A distinct blue fluorescent zone (quinine)
Reference solution	Test solution

Detection B: spray with *iodoplatinate reagent R*.

Results B: see below the sequence of the zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other zones are present in the chromatogram obtained with the test solution.

Top of the plate	
Cinchonine: a violet zone which becomes violet-grey	A violet zone, which becomes violet-grey (cinchonine)
Quinidine: a violet zone which becomes violet-grey	A violet zone, which becomes violet-grey (quinidine)
Cinchonidine: an intense dark blue zone	An intense dark blue zone (cinchonidine)
Quinine: a violet zone which becomes violet-grey	A violet zone, which becomes violet-grey (quinine)
Reference solution	Test solution

TESTS

Foreign matter (2.8.2): maximum 2 per cent *m/m*.

Total ash (2.4.16): maximum 6.0 per cent.

Loss on drying (2.2.32): maximum 10 per cent, determined on 1.000 g of the powdered drug (355) by drying in an oven at 100-105 °C for 2 h.

ASSAY

Test solution. In a 250 ml conical flask mix 1.000 g of the powdered drug (180) with 10 ml of *water R* and 7 ml of *dilute hydrochloric acid R*. Heat in a water-bath for 30 min, allow to cool and add 25 ml of *methylene chloride R*, 50 ml of *ether R* and 5 ml of a 200 g/l solution of *sodium hydroxide R*. Shake the mixture repeatedly for 30 min, add 3 g of powdered *tragacanth R* and shake until the mixture becomes clear. Filter through a plug of absorbent cotton and rinse the flask and the cotton with 5 quantities, each of 20 ml, of a mixture of 1 volume of *methylene chloride R* and 2 volumes of *ether R*. Combine the filtrate and washings, evaporate to dryness and dissolve the residue in 10.0 ml of *ethanol R*. Evaporate 5.0 ml of the solution to dryness, dissolve the residue in 0.1 *M* *hydrochloric acid* and dilute to 1000.0 ml with the same acid.

Reference solutions. Dissolve separately 30.0 mg of *quinine R* and 30.0 mg of *cinchonine R* in 0.1 *M* *hydrochloric acid* and dilute each solution to 1000.0 ml with the same acid.

Measure the absorbances (2.2.25) of the 3 solutions at 316 nm and 348 nm using 0.1 *M* *hydrochloric acid* as the compensation liquid.

Calculate the percentage content of alkaloids from the following equations:

$$x = \frac{[A_{316} \times A_{348c}] - [A_{316c} \times A_{348}]}{[A_{316q} \times A_{348c}] - [A_{316c} \times A_{348q}]} \times \frac{100}{m} \times \frac{2}{1000}$$

$$y = \frac{[A_{316} \times A_{348q}] - [A_{316q} \times A_{348}]}{[A_{316c} \times A_{348q}] - [A_{316q} \times A_{348c}]} \times \frac{100}{m} \times \frac{2}{1000}$$

m = mass of the drug used, in grams,

x = percentage content of quinine-type alkaloids,

y = percentage content of cinchonine-type alkaloids,

*A*₃₁₆ = absorbance of the test solution at 316 nm,

*A*₃₄₈ = absorbance of the test solution at 348 nm,

*A*_{316c} = absorbance of the reference solution containing cinchonine at 316 nm, corrected to a concentration of 1 mg/1000 ml,

*A*_{316q} = absorbance of the reference solution containing quinine at 316 nm, corrected to a concentration of 1 mg/1000 ml,

*A*_{348c} = absorbance of the reference solution containing cinchonine at 348 nm, corrected to a concentration of 1 mg/1000 ml,

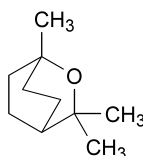
*A*_{348q} = absorbance of the reference solution containing quinine at 348 nm, corrected to a concentration of 1 mg/1000 ml.

Calculate the content of total alkaloids, (*x* + *y*), and the relative content of quinine-type alkaloids, from the following expression:

$$\frac{100x}{x + y}$$

CINEOLE

Cineolum

C₁₀H₁₈OM_r 154.3

DEFINITION

1,3,3-Trimethyl-2-oxabicyclo[2.2.2]octane.

CHARACTERS

Appearance: clear colourless liquid.*Solubility*: practically insoluble in water, miscible with alcohol and with methylene chloride.

It solidifies at about 0.5 °C.

IDENTIFICATION

A. It complies with the test for refractive index (see Tests).

B. Thin-layer chromatography (2.2.27).

Test solution. Dilute 1 ml of solution S (see Tests) to 25 ml with *alcohol R*.*Reference solution*. Mix 80 mg of *cineole CRS* with *alcohol R* and dilute to 10 ml with the same solvent.*Plate*: TLC silica gel plate *R*.*Mobile phase*: *ethyl acetate R*, *toluene R* (10:90 V/V).*Application*: 2 µl.*Development*: over 2/3 of the plate.*Drying*: in a current of cold air.*Detection*: spray with *anisaldehyde solution R*, heat at 100-105 °C for 5 min.*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.C. To 0.1 ml add 4 ml of *sulphuric acid R*. An orange-red colour develops. Add 0.2 ml of *formaldehyde solution R*. The colour changes to deep brown.

TESTS

Solution S. Dilute 2.00 g to 10.0 ml with *alcohol R*.**Appearance of solution**. Solution S is clear (2.2.1) and colourless (2.2.2, *Method I*).**Chiral impurities**. The optical rotation (2.2.7) of solution S is -0.10° to +0.10°.**Refractive index** (2.2.6): 1.456 to 1.460.**Related substances**. Gas chromatography (2.2.28).*Internal standard solution*. Dissolve 1.0 g of *camphor R* in *heptane R* and dilute to 200 ml with the same solvent.*Test solution (a)*. Dissolve 2.5 g of the substance to be examined in *heptane R* and dilute to 25.0 ml with the same solvent.*Test solution (b)*. Dissolve 2.5 g of the substance to be examined in *heptane R*, add 5.0 ml of the internal standard solution and dilute to 25.0 ml with *heptane R*.01/2005:1973 *Reference solution (a)*. To 2.0 ml of test solution (a) add 20.0 ml of the internal standard solution and dilute to 100.0 ml with *heptane R*.*Reference solution (b)*. Dissolve 50 mg of *1,4-cineole R* and 50 mg of the substance to be examined in *heptane R* and dilute to 50.0 ml with the same solvent.*Column*:– *size*: *l* = 30 m, Ø = 0.25 mm,– *stationary phase*: *macrogol 20 000 R* (film thickness 0.25 µm).*Carrier gas*: *helium for chromatography R*.*Linear velocity*: 45 cm/s.*Split-ratio*: 1:70.*Temperature*:

	Time (min)	Temperature (°C)
Column	0 - 10	50
	10 - 35	50 → 100
	35 - 45	100 → 200
	45 - 55	200
Injection port		220
Detector		250

Detection: flame ionisation.*Injection*: 1 µl.*System suitability*: reference solution (b):– *resolution*: minimum 10 between the peaks due to impurity A and to cineole.*Limits*:

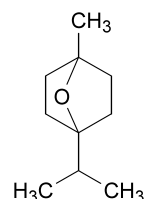
- *total*: calculate the ratio (*R*) of the area of the peak due to cineole to the area of the peak due to the internal standard from the chromatogram obtained with reference solution (a); from the chromatogram obtained with test solution (b), calculate the ratio of the sum of the areas of any peaks, apart from the principal peak and the peak due to the internal standard, to the area of the peak due to internal standard: this ratio is not greater than *R* (2 per cent),
- *disregard limit*: 0.025 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

Residue on evaporation: maximum 0.1 per cent.To 2.0 g add 5 ml of *water R*, evaporate to dryness on a water-bath and dry at 100-105 °C for 1 h. The residue weighs a maximum of 2 mg.

STORAGE

In an airtight container, protected from light.

IMPURITIES



A. 1-methyl-4-(1-methylethyl)-7-oxabicyclo[2.2.1]heptane (1,4-cineole).

01/2005:0387 ASSAY

CINNAMON**Cinnamomi cortex****DEFINITION**

Cinnamon consists of the dried bark, freed from the outer cork and the underlying parenchyma, of the shoots grown on cut stock of *Cinnamomum zeylanicum* Nees. It contains not less than 12 ml/kg of essential oil.

CHARACTERS

Cinnamon has a characteristic, aromatic odour.

It has the macroscopic and microscopic characters described under identification tests A and B.

IDENTIFICATION

- A. The bark is about 0.2 mm to 0.8 mm thick and occurs in closely-packed compound quills made up of single or double quills. The outer surface is smooth, yellowish-brown with faint scars marking the position of leaves and axillary buds and has fine, whitish and wavy longitudinal striations. The inner surface is slightly darker and longitudinally striated. The fracture is short and fibrous.
- B. Reduce to a powder (355). The powder is yellowish to reddish-brown. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters: groups of rounded sclereids with pitted, channelled and moderately thickened walls; numerous colourless single fibres, often whole with narrow lumen and thickened, lignified walls and few pits; small acicular crystals of calcium oxalate. Examine under a microscope using a 50 per cent V/V solution of *glycerol R*; the powder shows abundant starch granules. Cork fragments are absent or very rare.
- C. Examine by thin-layer chromatography (2.2.27), using *silica gel GF₂₅₄ R* as the coating substance.
- Test solution.* Shake 0.1 g of powdered drug (500) with 2 ml of *methylene chloride R* for 15 min. Filter and evaporate the filtrate carefully almost to dryness on a water-bath. Dissolve the residue in 0.4 ml of *toluene R*.
- Reference solution.* Dissolve 50 µl of *cinnamic aldehyde R* and 10 µl of *eugenol R* in *toluene R* and dilute to 10 ml with the same solvent.
- Apply separately to the plate as bands 20 mm by 3 mm, 10 µl of each solution. Develop over a path of 10 cm using *methylene chloride R*. Allow the plate to dry in air, examine in ultraviolet light at 254 nm and mark the quenching zones. Examine the plate in ultraviolet light at 365 nm and mark the fluorescent zones. In ultraviolet light at 254 nm, the chromatograms obtained with the test and reference solutions show a quenching zone (cinnamaldehyde) in the median part and just above it a weaker quenching zone (eugenol). In ultraviolet light at 365 nm, the chromatogram obtained with the test solution shows a zone of light-blue fluorescence (*o*-methoxy-cinnamaldehyde) just below the zone corresponding to cinnamaldehyde. Spray with *phloroglucinol solution R*. The zone corresponding to cinnamaldehyde is yellowish-brown and the zone corresponding to *o*-methoxycinnamaldehyde is violet.

TESTS

Total ash (2.4.16). Not more than 6.0 per cent.

ASSAY

Carry out the determination of essential oils in vegetable drugs (2.8.12). Use a 500 ml flask, 200 ml of 0.1 M *hydrochloric acid* as the distillation liquid and 0.50 ml of *xylene R* in the graduated tube. Powder the drug (710) and proceed immediately with the determination, using 20.0 g. Distil at a rate of 2.5-3.5 ml/min for 3 h.

STORAGE

Store protected from light.

01/2005:1501

CINNAMON BARK OIL, CEYLON**Cinnamomi zeylanicii corticis aetheroleum****DEFINITION**

Ceylon cinnamon bark oil is obtained by steam distillation of the bark of the shoots of *Cinnamomum zeylanicum* Nees (*C. Verum* J.S. Presl.).

CHARACTERS

A clear, mobile, light yellow liquid becoming reddish over time, with a characteristic odour reminiscent of cinnamic aldehyde.

IDENTIFICATION

First identification: B.

Second identification: A.

- A. Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel plate R*.

Test solution. Dissolve 1 ml of the substance to be examined in *acetone R* and dilute to 10 ml with the same solvent.

Reference solution. Dissolve 50 µl of *trans-cinnamic aldehyde R*, 10 µl of *eugenol R*, 10 µl of *linalol R* and 10 µl of *β-caryophyllene R* in *alcohol R* and dilute to 10 ml with the same solvent.

Apply to the plate, as bands, 10 µl of each solution. Develop over a path of 15 cm using a mixture of 10 volumes of *methanol R* and 90 volumes of *toluene R*. Allow the plate to dry in air and spray with *anisaldehyde solution R*. Examine in daylight while heating at 100-105 °C for 5-10 min. The chromatogram obtained with the test solution shows zones similar in position and colour to those in the chromatogram obtained with the reference solution.

- B. Examine the chromatograms obtained in the test for chromatographic profile. The retention times of the principal peaks in the chromatogram obtained with the test solution are similar to those in the chromatogram obtained with the reference solution. Safrole, coumarin and cineole may be absent from the chromatogram obtained with the test solution.

TESTS

Relative density (2.2.5): 1.000 to 1.030.

Refractive index (2.2.6): 1.572 to 1.591.

Optical rotation (2.2.7): -2° to +1°.

Chromatographic profile. Examine by gas chromatography (2.2.28).

Test solution. The substance to be examined.

Reference solution. Dissolve 10 µl of *cineole R*, 10 µl of *linalol R*, 10 µl of *β-caryophyllene R*, 10 µl of *safrole R*, 100 µl of *trans-cinnamic aldehyde R*, 10 µl of *eugenol R*, 20 mg of *coumarin R*, 10 µl of *trans-2-methoxycinnamaldehyde R* and 10 µl of *benzyl benzoate R* in 1 ml of *acetone R*.

The chromatographic procedure may be carried out using:

- a fused-silica column 60 m long and about 0.25 mm in internal diameter coated with *macrogol 20 000 R* as the bonded phase,
- *helium for chromatography R* as the carrier gas at a flow rate of 1.5 ml/min,
- a flame-ionisation detector,
- a split ratio of 1:100,

with the following temperature programme:

	Time (min)	Temperature (°C)	Rate (°C/min)	Comment
Column	0 - 10	60	–	isothermal
	10 - 75	60 → 190	2	linear gradient
	75 - 200	190	–	isothermal
Injection port		200		
Detector		240		

Inject 0.2 µl of the reference solution. When the chromatogram is recorded in the prescribed conditions, the components elute in the order indicated in the composition of the reference solution. Depending on the operating conditions and the state of the column, coumarin may elute before or after *trans-2-methoxycinnamaldehyde*. Record the retention times of these substances.

The test is not valid unless the resolution between the peaks corresponding to linalol and *β-caryophyllene* is at least 1.5.

Inject 0.2 µl of the test solution. Using the retention times determined from the chromatogram obtained with the reference solution, locate the components of the reference solution in the chromatogram obtained with the test solution. Determine the percentage content of each of these components by the normalisation procedure.

The percentages are within the following ranges:

- *cineole*: less than 3.0 per cent,
- *linalol*: 1.0 per cent to 6.0 per cent,
- *β-caryophyllene*: 1.0 per cent to 4.0 per cent,
- *safrole*: less than 0.5 per cent,
- *trans-cinnamic aldehyde*: 55 per cent to 75 per cent,
- *eugenol*: less than 7.5 per cent,
- *coumarin*: less than 0.5 per cent,
- *trans-2-methoxycinnamaldehyde*: 0.1 per cent to 1.0 per cent,
- *benzyl benzoate*: less than 1.0 per cent.

STORAGE

Store in a well-filled, airtight container, protected from light and heat.

01/2005:1608

CINNAMON LEAF OIL, CEYLON

Cinnamomi zeylanici folii aetheroleum

DEFINITION

Oil obtained by steam distillation of the leaves of *Cinnamomum verum* J.S. Presl.

CHARACTERS

Clear, mobile, reddish-brown to dark brown liquid, with a characteristic odour reminiscent of eugenol.

IDENTIFICATION

First identification: *B*.

Second identification: *A*.

A. Thin-layer chromatography (2.2.27).

Test solution. Dilute 1 ml of the substance to be examined in *acetone R* and dilute to 10 ml with the same solvent.

Reference solution. Dilute about 50 µl of *trans-cinnamic aldehyde R*, 10 µl of *eugenol R*, 10 µl of *linalol R* and 10 µl of *β-caryophyllene R* in *alcohol R* and dilute to 10 ml with the same solvent.

Plate: *TLC silica gel plate R*.

Mobile phase: *methanol R*, *toluene R* (10:90 *V/V*).

Application: 10 µl, as bands.

Development: over a path of 15 cm.

Drying: in air.

Detection: spray with *anisaldehyde solution R*. Examine in day light while heating at 100-105 °C for 5-10 min.

Results: the zones in the chromatogram obtained with the test solution are similar in position and colour to those in the chromatogram obtained with the reference solution. The zone due to *trans-cinnamic aldehyde* may be very faint or absent.

B. Examine the chromatogram obtained in the test for chromatographic profile.

Results: the characteristic peaks in the chromatogram obtained with the test solution are similar in retention time to those in the chromatogram obtained with the reference solution. The peaks corresponding to *cineole*, *safrole*, *trans-cinnamic aldehyde*, *cinnamyl acetate* and *coumarin* may be absent in the chromatogram obtained with the test solution.

TESTS

Relative density (2.2.5): 1.030 to 1.059.

Refractive index (2.2.6): 1.527 to 1.540.

Optical rotation (2.2.7): –2.5° to +2.0°.

Chromatographic profile. Gas chromatography (2.2.28): use the normalisation procedure.

Test solution. The substance to be examined.

Reference solution. Dissolve 10 µl of *cineole R*, 10 µl of *linalol R*, 10 µl of *β-caryophyllene R*, 10 µl of *safrole R*, 10 µl of *trans-cinnamic aldehyde R*, 10 µl of *cinnamyl acetate R*, 100 µl of *eugenol R* and 10 mg of *coumarin R* in 1 ml of *acetone R*.

Column:

- **material:** fused silica,
- **size:** *l* = 60 m, Ø = 0.25 mm,
- **stationary phase:** *macrogol 20 000 R*.

Carrier gas: *helium for chromatography R*.

Flow rate: 1.5 ml/min.

Split ratio: 1/100.

Temperature:

	Time (min)	Temperature (°C)
Column	0 - 10	45
	10 - 78	45 → 180
	78 - 88	180
Injection port		200
Detector		240

Detection: flame ionisation.

Injection: 0.2 µl.

Elution order: the order indicated in the composition of the reference solution. Record the retention times of these substances.

System suitability: reference solution:

- **resolution:** minimum of 1.5 between the peaks due to linalol and β-caryophyllene.

Using the retention times determined from the chromatogram obtained with the reference solution, locate the components of the reference solution in the chromatogram obtained with the test solution.

Determine the percentage content of these components.

The percentages are within the following ranges:

- **cineole:** less than 1.0 per cent,
- **linalol:** 1.5 per cent to 3.5 per cent,
- **β-caryophyllene:** 1.5 per cent to 7.0 per cent,
- **safrole:** less than 3.0 per cent,
- **trans-cinnamic aldehyde:** less than 3.0 per cent,
- **cinnamyl acetate:** less than 2.0 per cent,
- **eugenol:** 70 per cent to 85 per cent,
- **coumarin:** less than 1.0 per cent.

STORAGE

In a well-filled, airtight container, protected from light and heat.

01/2005:1819

CINNAMON TINCTURE**Cinnamomi corticis tinctura****DEFINITION**

Tincture produced from *Cinnamon* (0387).

PRODUCTION

The tincture is produced from 1 part of the drug and 5 parts of ethanol (70 per cent V/V) by an appropriate procedure.

CHARACTERS

Appearance: clear, brownish-red liquid, with a characteristic odour.

IDENTIFICATION

Thin-layer chromatography (2.2.27).

Test solution. Place 10 ml of the tincture to be examined, 10 ml of *saturated sodium chloride solution R* and 5 ml of *toluene R* in a ground glass-stoppered tube. Shake for 2 min and centrifuge for 10 min. Use the organic layer.

Reference solution. Dissolve 5 µl of *eugenol R*, 25 µl of *trans-cinnamic aldehyde R* and 5 µl of *trans-2-methoxycinnamaldehyde R* in *toluene R* and dilute to 10 ml with the same solvent.

Plate: TLC silica gel G plate *R*.

Mobile phase: *methylene chloride R*.

Application: 20 µl, as bands.

Development: over a path of 10 cm.

Drying: in air.

Detection A: examine in ultraviolet light at 365 nm.

Results A: see below the sequence of the zones present in the chromatograms obtained with the reference solution and the test solution.

Top of the plate	
<i>Trans</i> -2-methoxycinnamaldehyde: a light blue fluorescent zone	A light blue fluorescent zone (<i>trans</i> -2-methoxycinnamaldehyde)
	A greenish fluorescent zone (above the starting line)
Reference solution	Test solution

Detection B: spray with a 200 g/l solution of *phosphomolybdic acid R* in *ethanol R*. Examine in daylight while heating at 100-105 °C for 5-10 min.

Results B: see below the sequence of the zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other zones may be present in the chromatogram obtained with the test solution.

Top of the plate	
Eugenol: a blue zone	A blue zone (eugenol)
<i>Trans</i> -cinnamic aldehyde: a blue zone	A blue zone (<i>trans</i> -cinnamic aldehyde)
<i>Trans</i> -2-methoxycinnamaldehyde: an orange-brown zone (the colour fades away)	A weak orange-brown zone (<i>trans</i> -2-methoxycinnamaldehyde)
	2 or 3 blue zones above the starting line
Reference solution	Test solution

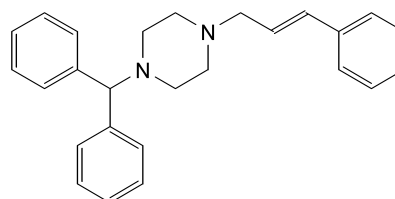
TESTS

Ethanol (2.9.10): 64 per cent V/V to 70 per cent V/V.

Methanol and 2-propanol (2.9.11): maximum 0.05 per cent V/V of methanol and maximum 0.05 per cent V/V of 2-propanol.

Dry residue (2.8.16): minimum 1.5 per cent *m/m*, determined on 5.0 g.

01/2005:0816

CINNARIZINE**Cinnarizinium**C₂₆H₂₈N₂M_r 368.5

DEFINITION

Cinnarizine contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of (*E*)-1-(diphenylmethyl)-4-(3-phenylprop-2-enyl)piperazine, calculated with reference to the dried substance.

CHARACTERS

A white or almost white powder, practically insoluble in water, freely soluble in methylene chloride, soluble in acetone, slightly soluble in alcohol and in methanol.

IDENTIFICATION

First identification: A, B.

Second identification: A, C, D.

- A. Melting point (2.2.14): 118 °C to 122 °C.
- B. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *cinnarizine CRS*. Examine the substances prepared as discs.
- C. Examine by thin-layer chromatography (2.2.27), using as the coating substance a suitable octadecylsilyl silica gel with a fluorescent indicator having an optimal intensity at 254 nm.
- Test solution.* Dissolve 10 mg of the substance to be examined in *methanol R* and dilute to 20 ml with the same solvent.
- Reference solution (a).* Dissolve 10 mg of *cinnarizine CRS* in *methanol R* and dilute to 20 ml with the same solvent.
- Reference solution (b).* Dissolve 10 mg of *cinnarizine CRS* and 10 mg of *flunarizine dihydrochloride CRS* in *methanol R* and dilute to 20 ml with the same solvent.
- Apply to the plate 5 µl of each solution. Develop in an unsaturated tank over a path of 15 cm using a mixture of 20 volumes of 1 M *sodium chloride*, 30 volumes of *methanol R* and 50 volumes of *acetone R*. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. The principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a). The test is not valid unless the chromatogram obtained with reference solution (b) shows two clearly separated spots.
- D. Dissolve 0.2 g of *anhydrous citric acid R* in 10 ml of *acetic anhydride R* in a water-bath at 80 °C and maintain the temperature of the water-bath at 80 °C for 10 min. Add about 20 mg of the substance to be examined. A purple colour develops.

TESTS

Appearance of solution. Dissolve 0.5 g in *methylene chloride R* and dilute to 20 ml with the same solvent. The solution is clear (2.2.1) and not more intensely coloured than reference solution BY₇ (2.2.2, *Method II*).

Acidity or alkalinity. Suspend 0.5 g in 15 ml of *water R*. Boil for 2 min. Cool and filter. Dilute the filtrate to 20 ml with *carbon dioxide-free water R*. To 10 ml add 0.1 ml of *phenolphthalein solution R* and 0.25 ml of 0.01 M *sodium hydroxide*. The solution is pink. To 10 ml add 0.1 ml of *methyl red solution R* and 0.25 ml of 0.01 M *hydrochloric acid*. The solution is red.

Related substances. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 25.0 mg of the substance to be examined in *methanol R* and dilute to 10.0 ml with the same solvent.

Reference solution (a). Dissolve 12.5 mg of *cinnarizine CRS* and 15.0 mg of *flunarizine dihydrochloride CRS* in *methanol R* and dilute to 100.0 ml with the same solvent. Dilute 1.0 ml of this solution to 20.0 ml with *methanol R*.

Reference solution (b). Dilute 1.0 ml of the test solution to 100.0 ml with *methanol R*. Dilute 5.0 ml of this solution to 20.0 ml with *methanol R*.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.1 m long and 4.0 mm in internal diameter packed with *base-deactivated octadecylsilyl silica gel for chromatography R* (3 µm),
- as mobile phase at a flow rate of 1.5 ml/min a gradient programme using the following conditions:

Mobile phase A. A 10 g/l solution of *ammonium acetate R*,

Mobile phase B. A 0.2 per cent V/V solution of *glacial acetic acid R* in *acetonitrile R*,

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)	Comment
0 – 20	75 → 10	25 → 90	linear gradient
20 – 25	10	90	isocratic elution
25 – 30	75	25	switch to initial eluent composition
30 = 0	75	25	restart gradient

- as detector a spectrophotometer set at 230 nm.

Equilibrate the column for a least 30 min at the initial eluent composition.

Adjust the sensitivity of the system so that the height of the principal peak in the chromatogram obtained with 10 µl of reference solution (b) is at least 50 per cent of the full scale of the recorder. If necessary, adjust the concentration of glacial acetic acid in mobile phase B to obtain a horizontal base-line in the chromatogram.

Inject 10 µl of reference solution (a). When the chromatogram is recorded in the prescribed conditions, the retention times are: cinnarizine about 11 min and flunarizine about 11.5 min. The test is not valid unless the resolution between the peaks corresponding to cinnarizine and flunarizine is at least 5.0. If necessary, adjust the time programme for the gradient elution.

Inject 10 µl of *methanol R* as a blank, 10 µl of the test solution and 10 µl of reference solution (b). In the chromatogram obtained with the test solution: the area of any peak, apart from the principal peak, is not greater than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.25 per cent); the sum of the areas of the peaks, apart from the principal peak, is not greater than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent). Disregard any peak due to the blank and any peak with an area less than 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (b).

Heavy metals (2.4.8). Dissolve 1.0 g in a mixture of 15 volumes of *water R* and 85 volumes of *acetone R*. Add dilute hydrochloric acid *R* until dissolution is complete. Dilute to 20 ml with the same mixture of *water R* and *acetone R*. 12 ml of the solution complies with limit test B for heavy metals (20 ppm). Prepare the standard using 10 ml of lead standard solution (1 ppm Pb) obtained by diluting lead standard solution (100 ppm Pb) *R* with the mixture of *water R* and *acetone R*.

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven *in vacuo* at 60 °C for 4 h.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

01/2005:2013

ASSAY

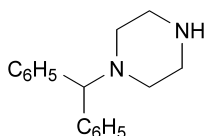
Dissolve 0.150 g in 50 ml of a mixture of 1 volume of *anhydrous acetic acid R* and 7 volumes of *ethyl methyl ketone R*. Titrate with 0.1 M *perchloric acid*, using 0.2 ml of *naphtholbenzein solution R* as indicator.

1 ml of 0.1 M *perchloric acid* is equivalent to 18.43 mg of $C_{26}H_{28}N_2$.

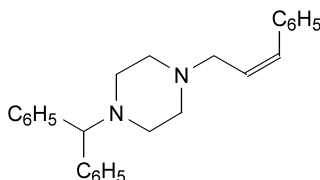
STORAGE

Store protected from light.

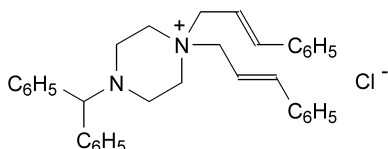
IMPURITIES



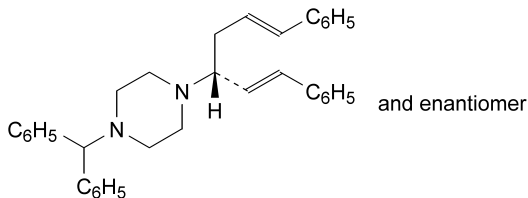
A. 1-(diphenylmethyl)piperazine,



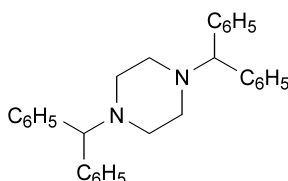
B. (Z)-1-(diphenylmethyl)-4-(3-phenylprop-2-enyl)piperazine,



C. (4-(diphenylmethyl)-1,1-bis[(E)-3-phenylprop-2-enyl]piperazinium chloride,



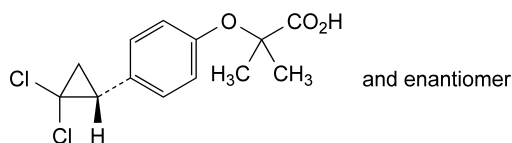
D. 1-(diphenylmethyl)-4-[(1RS,3E)-4-phenyl-1-[(E)-2-phenylethenyl]but-3-enyl]piperazine,



E. 1,4-bis(diphenylmethyl)piperazine.

CIPROFIBRATE

Ciprofibratum


 $C_{13}H_{14}Cl_2O_3$
 M_r 289.2

DEFINITION

2-[4-[(1RS)-2,2-Dichlorocyclopropyl]phenoxy]-2-methylpropanoic acid.

Content: 99.0 per cent to 101.0 per cent (anhydrous substance).

CHARACTERS

Appearance: white or slightly yellow, crystalline powder.

Solubility: practically insoluble in water, freely soluble in anhydrous ethanol, soluble in toluene.

mp: about 115 °C.

IDENTIFICATION

Infrared absorption spectrophotometry (2.2.24).

Comparison: ciprofibrate CRS.

TESTS

Appearance of solution. The solution is clear (2.2.1) and not more intensely coloured than reference solution BY₄ (2.2.2, Method II).

Dissolve 1.0 g in *anhydrous ethanol R* and dilute to 10.0 ml with the same solvent.

Related substances. Liquid chromatography (2.2.29).

Test solution. Dissolve 0.125 g of the substance to be examined in a mixture of equal volumes of *acetonitrile R* and *water R* and dilute to 50 ml with the same mixture of solvents.

Reference solution (a). Dilute 1.0 ml of the test solution to 100.0 ml with a mixture of equal volumes of *acetonitrile R* and *water R*. Dilute 1.0 ml of this solution to 10.0 ml with a mixture of equal volumes of *acetonitrile R* and *water R*.

Reference solution (b). Dissolve the contents of a vial of *ciprofibrate for system suitability CRS* in 2.0 ml of a mixture of equal volumes of *acetonitrile R* and *water R*.

Column:

- *size:* $l = 0.15$ m, $\varnothing = 4.6$ mm,
- *stationary phase:* octylsilyl silica gel for chromatography R (5 μ m).

Mobile phase:

- *mobile phase A:* 1.36 g/l solution of *potassium dihydrogen phosphate R* adjusted to pH 2.2 with *phosphoric acid R*,
- *mobile phase B:* *acetonitrile R*,

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)
0 - 30	75 → 30	25 → 70
30 - 40	30	70
40 - 42	30 → 75	70 → 25

Flow rate: 1.5 ml/min.

Detection: spectrophotometer at 230 nm.

Injection: 10 µl.

Identification of impurities: use the chromatogram supplied with *ciprofibrate* for *system suitability CRS* to identify the peaks due to impurities A, B, C, D and E.

Relative retention with reference to ciprofibrate (retention time = about 18 min): impurity A = about 0.7; impurity B = about 0.8; impurity C = about 0.95; impurity D = about 1.3; impurity E = about 1.5.

System suitability: reference solution (b):

- **resolution:** baseline separation between the peaks due to impurity C and ciprofibrate.

Limits:

- **correction factor:** for the calculation of content, multiply the peak area of impurity A by 2.3,
- **impurities A, C, D:** for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent),
- **impurity B:** not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent),
- **impurity E:** not more than 8 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.8 per cent),
- **any other impurity:** for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent),
- **total of other impurities:** not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent),
- **disregard limit:** 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

Chlorides (2.4.4): maximum 350 ppm.

To 0.190 g add 20 ml of *water R* and treat in an ultrasonic bath for 8 min. Filter. 15 ml of the filtrate complies with the test.

Water (2.5.12): maximum 0.5 per cent, determined on 1.000 g.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.250 g in a mixture of 20 ml of *water R* and 40 ml of *anhydrous ethanol R*. Titrate with 0.1 M *sodium hydroxide*, determining the end-point potentiometrically (2.2.20).

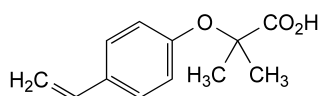
1 ml of 0.1 M *sodium hydroxide* is equivalent to 28.92 mg of $C_{17}H_{18}FN_3O_3$.

STORAGE

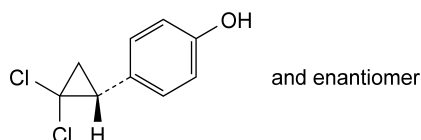
In an airtight container, protected from light.

IMPURITIES

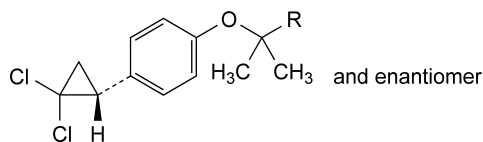
Specified impurities: A, B, C, D, E.



A. 2-(4-ethenylphenoxy)-2-methylpropanoic acid,



B. 4-[(1R)-2,2-dichlorocyclopropyl]phenol,



C. R = CH_2OH : 2-[4-[(1R)-2,2-dichlorocyclopropyl]phenoxy]-2-methylpropan-1-ol,

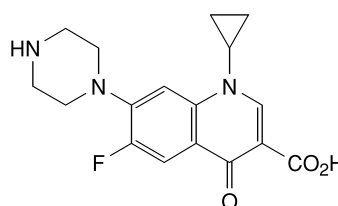
D. R = $CO-OCH_3$: methyl 2-[4-[(1R)-2,2-dichlorocyclopropyl]phenoxy]-2-methylpropanoate,

E. R = $CO-OC_2H_5$: ethyl 2-[4-[(1R)-2,2-dichlorocyclopropyl]phenoxy]-2-methylpropanoate.

01/2005:1089
corrected

CIPROFLOXACIN

Ciprofloxacinum



$C_{17}H_{18}FN_3O_3$

M_r 331.4

DEFINITION

1-Cyclopropyl-6-fluoro-4-oxo-7-(piperazin-1-yl)-1,4-dihydroquinoline-3-carboxylic acid.

Content: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

Appearance: almost white or pale yellow, crystalline powder, slightly hygroscopic.

Solubility: practically insoluble in water, very slightly soluble in ethanol and in methylene chloride.

IDENTIFICATION

Infrared absorption spectrophotometry (2.2.24).

Comparison: *ciprofloxacin CRS*.

TESTS

Appearance of solution. The solution is clear (2.2.1) and not more intensely coloured than reference solution GY_5 (2.2.2, *Method II*).

Dissolve 0.25 g in 0.1 M *hydrochloric acid* and dilute to 20 ml with the same solvent.

Impurity A. Thin-layer chromatography (2.2.27).

Test solution. Dissolve 50 mg of the substance to be examined in *dilute ammonia R1* and dilute to 5 ml with the same solvent.

Reference solution. Dissolve 10 mg of *ciprofloxacin impurity A CRS* in a mixture of 0.1 ml of *dilute ammonia R1* and 90 ml of *water R* and dilute to 100 ml with *water R*. Dilute 2 ml of the solution to 10 ml with *water R*.

Plate: *TLC silica gel F₂₅₄ plate R*.

Application: 5 µl.

At the bottom of a chromatographic tank, place an evaporating dish containing 50 ml of *concentrated ammonia R*. Expose the plate to the ammonia vapour for 15 min in the closed tank. Withdraw the plate, transfer to a second chromatographic tank and proceed with development.

Mobile phase: acetonitrile *R*, concentrated ammonia *R*, methanol *R*, methylene chloride *R* (10:20:40:40 V/V/V/V).

Development: over 3/4 of the plate.

Drying: in air.

Detection: examine in ultraviolet light at 254 nm.

Limit:

- **impurity A:** any spot corresponding to impurity A is not more intense than the principal spot in the chromatogram obtained with the reference solution (0.2 per cent).

Related substances. Liquid chromatography (2.2.29).

Test solution. To 25.0 mg of the substance to be examined add 0.2 ml of dilute phosphoric acid *R* and dilute to 50.0 ml with the mobile phase and treat in an ultrasonic bath until a clear solution is obtained.

Reference solution (a). Dilute 1.0 ml of the test solution to 100.0 ml with the mobile phase. Dilute 1.0 ml of this solution to 5.0 ml with the mobile phase.

Reference solution (b). Dissolve 5 mg of ciprofloxacin hydrochloride for peak identification CRS in the mobile phase and dilute to 10.0 ml with the mobile phase.

Column:

- **size:** $l = 0.25$ m, $\varnothing = 4.6$ mm;
- **stationary phase:** base-deactivated octadecylsilyl silica gel for chromatography *R* (5 μ m);
- **temperature:** 40 °C.

Mobile phase: mix 13 volumes of acetonitrile *R* and 87 volumes of a 2.45 g/l solution of phosphoric acid *R*, previously adjusted to pH 3.0 with triethylamine *R*.

Flow rate: 1.5 ml/min.

Detection: spectrophotometer at 278 nm.

Injection: 50 μ l.

Run time: twice the retention time of ciprofloxacin.

Relative retention with reference to ciprofloxacin (retention time = about 9 min): impurity E = about 0.4; impurity F = about 0.5; impurity B = about 0.6; impurity C = about 0.7; impurity D = about 1.2.

System suitability: reference solution (b):

- **resolution:** minimum 1.3 between the peaks due to impurity B and impurity C.

Limits:

- **correction factors:** for the calculation of contents, multiply the peak areas of the following impurities by the corresponding correction factor: impurity B = 0.7; impurity C = 0.6; impurity D = 1.4; impurity E = 6.7; use the chromatogram obtained with reference solution (b) and the type chromatogram supplied with the CRS to identify the corresponding peaks;
- **impurities B, C, D, E:** for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent);
- **any other impurity:** not more than half the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent);
- **total:** not more than 2.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent);
- **disregard limit:** 0.25 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

Heavy metals (2.4.8): maximum 20 ppm.

Dissolve 0.5 g in dilute acetic acid *R* and dilute to 30 ml with the same solvent. Add 2 ml of water *R* instead of 2 ml

of buffer solution pH 3.5 *R*. The filtrate complies with limit test E. Prepare the standard using 10 ml of lead standard solution (1 ppm Pb) *R*.

Loss on drying (2.2.32): maximum 1.0 per cent, determined on 1.000 g by drying under vacuum at 120 °C.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g in a platinum crucible.

ASSAY

Dissolve 0.300 g in 80 ml of glacial acetic acid *R*. Titrate with 0.1 M perchloric acid, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M perchloric acid is equivalent to 33.14 mg of C₁₇H₁₈FN₃O₃.

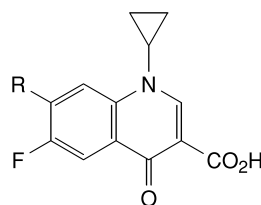
STORAGE

In an airtight container, protected from light.

IMPURITIES

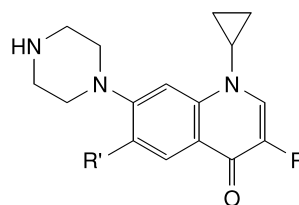
Specified impurities: A, B, C, D, E.

Other detectable impurities: F.



A. R = Cl: 7-chloro-1-cyclopropyl-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (fluoroquinolonic acid),

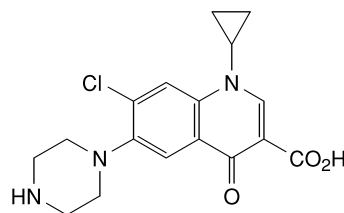
C. R = NH-(CH₂)₂-NH₂: 7-[(2-aminoethyl)amino]-1-cyclopropyl-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (ethylenediamine compound),



B. R = CO₂H, R' = H: 1-cyclopropyl-4-oxo-7-(piperazin-1-yl)-1,4-dihydroquinoline-3-carboxylic acid (desfluoro compound),

E. R = H, R' = F: 1-cyclopropyl-6-fluoro-7-(piperazin-1-yl)quinolin-4(1H)-one (decarboxylated compound),

F. R = CO₂H, R' = OH: 1-cyclopropyl-6-hydroxy-4-oxo-7-(piperazin-1-yl)-1,4-dihydroquinoline-3-carboxylic acid,

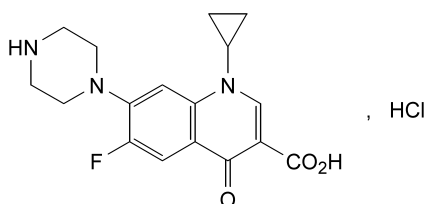


D. 7-chloro-1-cyclopropyl-4-oxo-6-(piperazin-1-yl)-1,4-dihydroquinoline-3-carboxylic acid.

01/2005:0888 Limit:

CIPROFLOXACIN HYDROCHLORIDE

Ciprofloxacinum hydrochloridum

 $C_{17}H_{19}ClFN_3O_3$ M_r 367.8

DEFINITION

1-Cyclopropyl-6-fluoro-4-oxo-7-(piperazin-1-yl)-1,4-dihydroquinoline-3-carboxylic acid hydrochloride.

Content: 98.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

Appearance: pale yellow, crystalline powder, slightly hygroscopic.

Solubility: soluble in water, slightly soluble in methanol, very slightly soluble in ethanol, practically insoluble in acetone, in ethyl acetate and in methylene chloride.

IDENTIFICATION

A. Infrared absorption spectrophotometry (2.2.24).

Preparation: discs.

Comparison: ciprofloxacin hydrochloride CRS.

B. 0.1 g gives reaction (b) of chlorides (2.3.1).

TESTS

Solution S. Dissolve 0.5 g in *carbon dioxide-free water R* and dilute to 20 ml with the same solvent.

Appearance of solution. The solution is clear (2.2.1) and not more intensely coloured than reference solution GY₅ (2.2.2, *Method II*).

Dilute 10 ml of solution S to 20 ml with *carbon dioxide-free water R*.

pH (2.2.3): 3.5 to 4.5 for solution S.

Impurity A. Thin-layer chromatography (2.2.27).

Test solution. Dissolve 50 mg of the substance to be examined in *water R* and dilute to 5 ml with the same solvent.

Reference solution. Dissolve 10 mg of ciprofloxacin impurity A CRS in a mixture of 0.1 ml of *dilute ammonia R1* and 90 ml of *water R* and dilute to 100 ml with *water R*. Dilute 2 ml of the solution to 10 ml with *water R*.

Plate: TLC silica gel F₂₅₄ plate R.

Application: 5 µl.

At the bottom of a chromatographic tank, place an evaporating dish containing 50 ml of *concentrated ammonia R*. Expose the plate to the ammonia vapour for 15 min in the closed tank. Withdraw the plate, transfer to a second chromatographic tank and proceed with development.

Mobile phase: acetonitrile R, concentrated ammonia R, methanol R, methylene chloride R (10:20:40:40 V/V/V/V).

Development: over 3/4 of the plate.

Drying: in air.

Detection: examine in ultraviolet light at 254 nm.

– *impurity A:* any spot corresponding to impurity A is not more intense than the principal spot in the chromatogram obtained with the reference solution (0.2 per cent).

Related substances. Liquid chromatography (2.2.29).

Test solution. Dissolve 25.0 mg of the substance to be examined in the mobile phase and dilute to 50.0 ml with the mobile phase.

Reference solution (a). Dissolve 25.0 mg of ciprofloxacin hydrochloride CRS in the mobile phase and dilute to 50.0 ml with the mobile phase.

Reference solution (b). Dissolve 5 mg of ciprofloxacin hydrochloride for peak identification CRS in the mobile phase and dilute to 10.0 ml with the mobile phase.

Reference solution (c). Dilute 1.0 ml of the test solution to 50.0 ml with the mobile phase. Dilute 1.0 ml of this solution to 10.0 ml with the mobile phase.

Column:

- *size:* $l = 0.25$ m, $\varnothing = 4.6$ mm;
- *stationary phase:* base-deactivated octadecylsilyl silica gel for chromatography R (5 µm);
- *temperature:* 40 °C.

Mobile phase: mix 13 volumes of acetonitrile R and 87 volumes of a 2.45 g/l solution of phosphoric acid R, previously adjusted to pH 3.0 with triethylamine R.

Flow rate: 1.5 ml/min.

Detection: spectrophotometer at 278 nm.

Injection: 50 µl.

Run time: twice the retention time of ciprofloxacin.

Relative retention with reference to ciprofloxacin (retention time = about 9 min): impurity E = about 0.4; impurity F = about 0.5; impurity B = about 0.6; impurity C = about 0.7; impurity D = about 1.2.

System suitability: reference solution (b):

- *resolution:* minimum 1.3 between the peaks due to impurity B and impurity C.

Limits:

- *correction factors:* for the calculation of contents, multiply the peak areas of the following impurities by the corresponding correction factor: impurity B = 0.7; impurity C = 0.6; impurity D = 1.4; impurity E = 6.7; use the chromatogram obtained with reference solution (b) and the type chromatogram supplied with the CRS to identify the corresponding peaks;
- *impurities B, C, D, E:* for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (c) (0.2 per cent);
- *any other impurity:* not more than half the area of the principal peak in the chromatogram obtained with reference solution (c) (0.1 per cent);
- *total:* not more than 2.5 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.5 per cent);
- *disregard limit:* 0.25 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.05 per cent).

Heavy metals (2.4.8): maximum 20 ppm.

Dissolve 0.25 g in *water R* and dilute to 30 ml with the same solvent. Carry out the prefiltration. The filtrate complies with limit test E. Prepare the standard using 5 ml of *lead standard solution (1 ppm Pb) R*.

Water (2.5.12): maximum 6.7 per cent, determined on 0.200 g.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g in a platinum crucible.

ASSAY

Liquid chromatography (2.2.29) as described in the test for related substances with the following modifications.

Injection: 10 µl; inject the test solution and reference solution (a).

Calculate the percentage content of $C_{17}H_{19}ClFN_3O_3$.

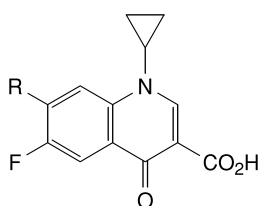
STORAGE

In an airtight container, protected from light.

IMPURITIES

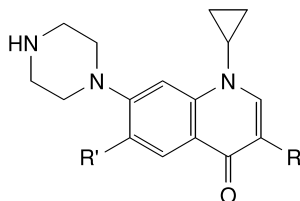
Specified impurities: A, B, C, D, E.

Other detectable impurities: F.



A. R = Cl: 7-chloro-1-cyclopropyl-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (fluoroquinolonic acid),

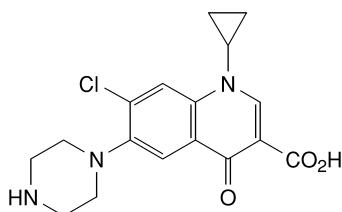
C. R = $NH-[CH_2]_2-NH_2$: 7-[(2-aminoethyl)amino]-1-cyclopropyl-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (ethylenediamine compound),



B. R = CO_2H , R' = H: 1-cyclopropyl-4-oxo-7-(piperazin-1-yl)-1,4-dihydroquinoline-3-carboxylic acid (desfluoro compound),

E. R = H, R' = F: 1-cyclopropyl-6-fluoro-7-(piperazin-1-yl)quinolin-4(1H)-one (decarboxylated compound),

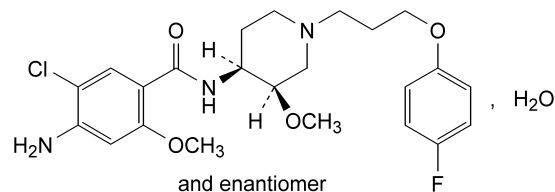
F. R = CO_2H , R' = OH: 1-cyclopropyl-6-hydroxy-4-oxo-7-(piperazin-1-yl)-1,4-dihydroquinoline-3-carboxylic acid,



D. 7-chloro-1-cyclopropyl-4-oxo-6-(piperazin-1-yl)-1,4-dihydroquinoline-3-carboxylic acid.

CISAPRIDE MONOHYDRATE

Cisapridum monohydricum



$C_{23}H_{29}ClFN_3O_4 \cdot H_2O$

M_r 484.0

DEFINITION

Cisapride monohydrate contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of 4-amino-5-chloro-*N*-[(3*RS*,4*SR*)-1-[3-(4-fluorophenoxy)propyl]-3-methoxypiperidin-4-yl]-2-methoxybenzamide, calculated with reference to the anhydrous substance.

CHARACTERS

A white or almost white powder, practically insoluble in water, freely soluble in dimethylformamide, soluble in methylene chloride, sparingly soluble in methanol.

It shows polymorphism.

IDENTIFICATION

Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *cisapride monohydrate CRS*. Examine the substances prepared as discs. If the spectra obtained show differences, dissolve the substance to be examined and the reference substance separately in the minimum volume of *methanol R*, evaporate to dryness in a current of air and record new spectra using the residues.

TESTS

Solution S. Dissolve 0.20 g in *methylene chloride R* and dilute to 20.0 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and not more intensely coloured than reference solution BY₆ (2.2.2, Method II).

Optical rotation (2.2.7). The angle of optical rotation, determined on solution S, is -0.1° to $+0.1^\circ$.

Related substances. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 0.100 g of the substance to be examined in *methanol R* and dilute to 10.0 ml with the same solvent.

Reference solution (a). Dissolve 5.0 mg of *cisapride monohydrate CRS* and 40.0 mg of *haloperidol CRS* in *methanol R* and dilute to 100.0 ml with the same solvent.

Reference solution (b). Dilute 5.0 ml of the test solution to 100.0 ml with *methanol R*. Dilute 1.0 ml of this solution to 10.0 ml with *methanol R*.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.1 m long and 4.0 mm in internal diameter packed with *base-deactivated octadecylsilyl silica gel for chromatography R* (3 µm),
- as mobile phase at a flow rate of 1.2 ml/min:
Mobile phase A. A 20 g/l solution of *tetrabutylammonium hydrogen sulphate R*,
Mobile phase B. *Methanol R*,

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)	Comment
0 - 20	80 → 55	20 → 45	linear gradient
20 - 21	55 → 5	45 → 95	switch to next step
21 - 25	5	95	isocratic
25 - 26	5 → 80	95 → 20	switch to initial eluent composition
26 - 30	80	20	re-equilibration
30 - 0	80	20	restart gradient

— as detector a spectrophotometer set at 275 nm.

Equilibrate the column with the initial mobile phase composition for at least 5 min.

Adjust the sensitivity of the system so that the height of the principal peak in the chromatogram obtained with 10 µl of reference solution (b) is at least 50 per cent of the full scale of the recorder.

Inject 10 µl of reference solution (a). When the chromatogram is recorded in the prescribed conditions, the retention times are: cisapride about 15 min and haloperidol about 16 min. The test is not valid unless the resolution between the peaks due to cisapride and haloperidol is at least 2.5. If necessary, adjust the concentration of methanol in the mobile phase or adjust the time programme for the linear gradient.

Inject separately 10 µl of *methanol R* as a blank, 10 µl of the test solution and 10 µl of reference solution (b). In the chromatogram obtained with the test solution: the area of any peak, apart from the principal peak, is not greater than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent); the sum of the areas of all the peaks, apart from the principal peak, is not greater than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (1 per cent). Disregard any peak due to the solvent (blank) and any peak with an area less than 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (b).

Water (2.5.12): 3.4 per cent to 4.0 per cent, determined on 0.500 g by the semi-micro determination of water.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g in a platinum crucible.

ASSAY

Dissolve 0.350 g in 70 ml of a mixture of 1 volume of *anhydrous acetic acid R* and 7 volumes of *methyl ethyl ketone R* and titrate with 0.1 M *perchloric acid*. Determine the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *perchloric acid* is equivalent to 46.60 mg of $C_{27}H_{35}ClFN_3O_{10}$.

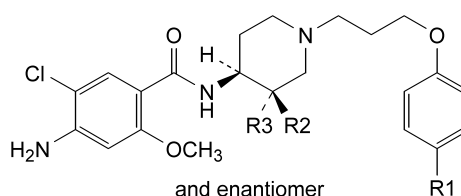
STORAGE

Store protected from light.

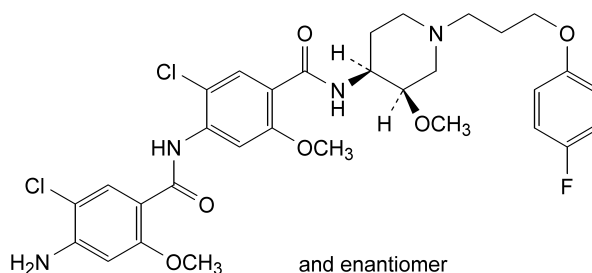
IMPURITIES

Specified impurities: A, B, C, E.

Other detectable impurities: D.



- A. R1 = R3 = H, R2 = OCH₃: 4-amino-5-chloro-2-methoxy-*N*[(3*RS*,4*SR*)-3-methoxy-1-(3-phenoxypropyl)piperidin-4-yl]benzamide,
- B. R1 = F, R2 = R3 = H: 4-amino-5-chloro-*N*[[1-[3-(4-fluorophenoxy)propyl]piperidin-4-yl]-2-methoxybenzamide,
- C. R1 = F, R2 = H, R3 = OCH₃: 4-amino-5-chloro-*N*[(3*RS*,4*RS*)-1-[3-(4-fluorophenoxy)propyl]-3-methoxypiperidin-4-yl]-2-methoxybenzamide,
- E. R1 = F, R2 = OH, R3 = H: 4-amino-5-chloro-*N*[(3*RS*,4*SR*)-1-[3-(4-fluorophenoxy)propyl]-3-hydroxypiperidin-4-yl]-2-methoxybenzamide,

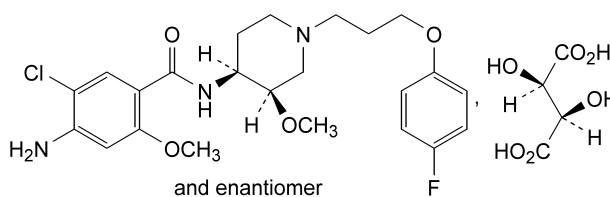


- D. 4-[(4-amino-5-chloro-2-methoxybenzoyl)amino]-5-chloro-*N*[(3*RS*,4*SR*)-1-[3-(4-fluorophenoxy)propyl]-3-methoxypiperidin-4-yl]-2-methoxybenzamide.

01/2005:1503

CISAPRIDE TARTRATE

Cisapridi tartras



$C_{27}H_{35}ClFN_3O_{10}$

M_r 616.0

DEFINITION

Cisapride tartrate contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of 4-amino-5-chloro-*N*[(3*RS*,4*SR*)-1-[3-(4-fluorophenoxy)propyl]-3-methoxypiperidin-4-yl]-2-methoxybenzamide (2*R*,3*R*)-2,3-dihydroxybutanedioate, calculated with reference to the dried substance.

CHARACTERS

A white or almost white powder, slightly soluble in water, freely soluble in dimethylformamide, slightly soluble in methanol, very slightly soluble in alcohol.

It shows polymorphism.

IDENTIFICATION

- A. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *cisapride tartrate CRS*. Examine the substances prepared

as discs. If the spectra obtained show differences, dissolve the substance to be examined and the reference substance separately in the minimum volume of *alcohol R*, evaporate to dryness in a current of air and record new spectra using the residues.

- B. It complies with the test for specific optical rotation (see Tests).

TESTS

Specific optical rotation (2.2.7). Dissolve 0.100 g in *methanol R* and dilute to 25.0 ml with the same solvent. The specific optical rotation is + 5.0 to + 10.0, calculated with reference to the dried substance.

Related substances. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 0.100 g of the substance to be examined in a mixture of 10 volumes of *water R* and 90 volumes of *methanol R*, with gentle warming if necessary, and dilute to 10.0 ml with the same mixture of solvents.

Reference solution (a). Dissolve 5.0 mg of *cisapride tartrate CRS* and 30.0 mg of *haloperidol CRS* in a mixture of 10 volumes of *water R* and 90 volumes of *methanol R* and dilute to 100.0 ml with the same mixture of solvents.

Reference solution (b). Dilute 1.0 ml of the test solution to 100.0 ml with a mixture of 10 volumes of *water R* and 90 volumes of *methanol R*. Dilute 5.0 ml of the solution to 20.0 ml with the same mixture of solvents.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.1 m long and 4.0 mm in internal diameter packed with *base-deactivated octadecylsilyl silica gel for chromatography R* (3 µm),
- as mobile phase at a flow rate of 1.2 ml/min:

Mobile phase A. A 20 g/l solution of *tetrabutylammonium hydrogen sulphate R*,

Mobile phase B. *Methanol R*,

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)	Comment
0 - 20	80 → 55	20 → 45	linear gradient
20 - 21	55 → 5	45 → 95	switch to next step
21 - 25	5	95	isocratic
25 - 26	5 → 80	95 → 20	switch to initial eluent composition
26 - 30	80	20	re-equilibration
30 = 0	80	20	restart gradient

- as detector a spectrophotometer set at 275 nm.

Equilibrate the column with the initial mobile phase composition for at least 5 min.

Adjust the sensitivity of the system so that the height of the principal peak in the chromatogram obtained with 10 µl of reference solution (b) is at least 50 per cent of the full scale of the recorder.

Inject 10 µl of reference solution (a). When the chromatogram is recorded in the prescribed conditions, the retention times are: cisapride about 15 min and haloperidol about 16 min. The test is not valid unless the resolution between the peaks due to cisapride and haloperidol is at least 2.5. If necessary, adjust the concentration of methanol in the mobile phase or adjust the time programme for the linear gradient.

Inject separately 10 µl of a mixture of 10 volumes of *water R* and 90 volumes of *methanol R* as a blank, 10 µl of the test solution and 10 µl of reference solution (b). In the

chromatogram obtained with the test solution: the area of any peak, apart from the principal peak, is not greater than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.25 per cent); the sum of the areas of all the peaks, apart from the principal peak, is not greater than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent). Disregard any peak due to the solvent (blank) and any peak with an area less than 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (b).

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g in a platinum crucible.

ASSAY

Dissolve 0.500 g in 70 ml of *anhydrous acetic acid R* and titrate with 0.1 M *perchloric acid*. Determine the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *perchloric acid* is equivalent to 61.60 mg of $C_{27}H_{35}ClFN_3O_{10}$.

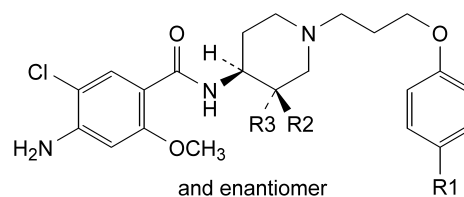
STORAGE

Store protected from light.

IMPURITIES

Specified impurities: A, C.

Other detectable impurities: B, D, E.

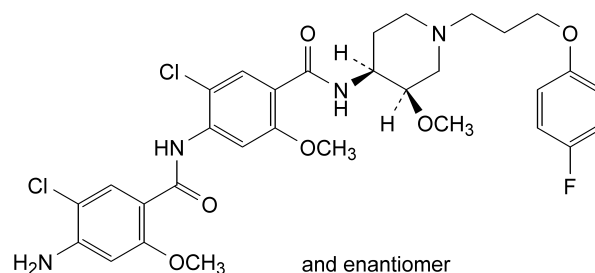


A. R1 = R3 = H, R2 = OCH₃: 4-amino-5-chloro-2-methoxy-*N*[(3*RS*,4*SR*)-3-methoxy-1-(3-phenoxypropyl)piperidin-4-yl]benzamide,

B. R1 = F, R2 = R3 = H: 4-amino-5-chloro-*N*[1-[3-(4-fluorophenoxy)propyl]piperidin-4-yl]-2-methoxybenzamide,

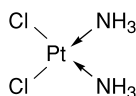
C. R1 = F, R2 = H, R3 = OCH₃: 4-amino-5-chloro-*N*[(3*RS*,4*RS*)-1-[3-(4-fluorophenoxy)propyl]-3-methoxypiperidin-4-yl]-2-methoxybenzamide,

E. R1 = F, R2 = OH, R3 = H: 4-amino-5-chloro-*N*[(3*RS*,4*SR*)-1-[3-(4-fluorophenoxy)propyl]-3-hydroxypiperidin-4-yl]-2-methoxybenzamide.



D. 4-[(4-amino-5-chloro-2-methoxybenzoyl)amino]-5-chloro-*N*[(3*RS*,4*SR*)-1-[3-(4-fluorophenoxy)propyl]-3-methoxypiperidin-4-yl]-2-methoxybenzamide,

01/2005:0599

CISPLATIN**Cisplatinum**[PtCl₂(NH₃)₂]*M_r* 300.0**DEFINITION**

Cisplatin contains not less than 97.0 per cent and not more than the equivalent of 102.0 per cent of *cis*-diamminedi-chloroplatinum (II).

CHARACTERS

A yellow powder or yellow or orange-yellow crystals, slightly soluble in water, sparingly soluble in dimethylformamide, practically insoluble in alcohol.

It decomposes with blackening at about 270 °C.

Carry out identification test B, the tests (except that for silver) and the assay protected from light.

IDENTIFICATION

First identification: A, B.

Second identification: B, C.

- A. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *cisplatin CRS*. Examine the substances prepared as discs in *potassium bromide R*.
- B. Examine the chromatograms obtained in the test for related substances. The principal spot in the chromatogram obtained with test solution (a) is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).
- C. Add 50 mg to 2 ml of *dilute sodium hydroxide solution R* in a glass dish. Evaporate to dryness. Dissolve the residue in a mixture of 0.5 ml of *nitric acid R* and 1.5 ml of *hydrochloric acid R*. Evaporate to dryness. The residue is orange. Dissolve the residue in 0.5 ml of *water R* and add 0.5 ml of *ammonium chloride solution R*. A yellow, crystalline precipitate is formed.

TESTS

Solution S1. Dissolve 25 mg in a 9 g/l solution of *sodium chloride R* prepared with *carbon dioxide-free water R* and dilute to 25 ml with the same solvent.

Solution S2. Dissolve 0.20 g in *dimethylformamide R* and dilute to 10 ml with the same solvent.

Appearance of solution S1. Solution S1 is clear (2.2.1) and not more intensely coloured than reference solution GY₅ (2.2.2, *Method II*).

Appearance of solution S2. Solution S2 is clear (2.2.1).

pH (2.2.3). The pH of solution S1, measured immediately after preparation, is 4.5 to 6.0.

Related substances. Examine by thin-layer chromatography (2.2.27), using *cellulose for chromatography R1* as the coating substance. Activate the plate by heating at 150 °C for 1 h.

Test solution (a). Dilute 1 ml of solution S2 to 10 ml with *dimethylformamide R*.

Test solution (b). Use solution S2.

Reference solution (a). Dissolve 10 mg of *cisplatin CRS* in 5 ml of *dimethylformamide R*.

Reference solution (b). Dilute 1 ml of solution S2 to 50 ml with *dimethylformamide R*.

Apply separately to the plate 2.5 µl of test solution (a), 2.5 µl of reference solution (a), 5 µl of test solution (b) and 5 µl of reference solution (b). Develop over a path of 15 cm using a mixture of 10 volumes of *acetone R* and 90 volumes of *dimethylformamide R*. Allow the plate to dry in air and spray with a 50 g/l solution of *stannous chloride R* in a mixture of equal volumes of *dilute hydrochloric acid R* and *water R*. After 1 h, the chromatogram obtained with test solution (b) shows no spot with an *R_f* value less than that of the principal spot and any spot with an *R_f* value greater than that of the principal spot is not more intense than the spot in the chromatogram obtained with reference solution (b).

Silver. Not more than 250 ppm of Ag, determined by atomic absorption spectrometry (2.2.23, *Method I*).

Test solution. Dissolve 0.100 g of the substance to be examined in 15 ml of *nitric acid R*, heating to 80 °C. Cool and dilute to 25.0 ml with *water R*.

Reference solutions. To suitable volumes (10 ml to 30 ml) of *silver standard solution (5 ppm Ag) R* add 50 ml of *nitric acid R* and dilute to 100.0 ml with *water R*.

Measure the absorbance at 328 nm using a silver hollow-cathode lamp as source of radiation, a fuel-lean air-acetylene flame and, preferably, a spectral slit width of 0.5 nm. Carry out a blank determination.

ASSAY

Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 50.0 mg of the substance to be examined in a 9 g/l solution of *sodium chloride R* and dilute to 100.0 ml with the same solvent.

Reference solution. Dissolve 50.0 mg of *cisplatin CRS* in a 9 g/l solution of *sodium chloride R* and dilute to 100.0 ml with the same solvent.

The chromatographic procedure may be carried out using:

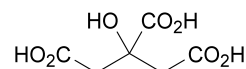
- a column 0.25 m long and 4.6 mm in internal diameter packed with *strong-anion-exchange silica gel for chromatography R* (10 µm),
- as mobile phase at a flow rate of 1.2 ml/min a mixture of 10 volumes of a 9 g/l solution of *sodium chloride R* and 90 volumes of *methanol R*,
- as detector a spectrophotometer set at 220 nm.

Use a sample loop. Inject separately 20 µl of the test solution and 20 µl of the reference solution.

STORAGE

Store in an airtight container, protected from light.

01/2005:0455

CITRIC ACID, ANHYDROUS**Acidum citricum anhydricum***C₆H₈O₇**M_r* 192.1**DEFINITION**

Anhydrous citric acid contains not less than 99.5 per cent and not more than the equivalent of 100.5 per cent of 2-hydroxypropane-1,2,3-tricarboxylic acid, calculated with reference to the anhydrous substance.

CHARACTERS

A white, crystalline powder, colourless crystals or granules, very soluble in water, freely soluble in alcohol. It melts at about 153 °C with decomposition.

IDENTIFICATION

First identification: B, E.

Second identification: A, C, D, E.

- Dissolve 1 g in 10 ml of *water R*. The solution is strongly acidic (2.2.4).
- Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *anhydrous citric acid CRS* after drying both the substance to be examined and the reference substance at 100-105 °C for 2 h.
- Add about 5 mg to a mixture of 1 ml of *acetic anhydride R* and 3 ml of *pyridine R*. A red colour develops.
- Dissolve 0.5 g in 5 ml of *water R*, neutralise using 1 M *sodium hydroxide* (about 7 ml), add 10 ml of *calcium chloride solution R* and heat to boiling. A white precipitate is formed.
- It complies with the test for water (see Tests).

TESTS

Appearance of solution. Dissolve 2.0 g in *water R* and dilute to 10 ml with the same solvent. The solution is clear (2.2.1) and not more intensely coloured than reference solution Y₇, BY₇ or GY₇ (2.2.2, *Method II*).

Readily carbonisable substances. To 1.0 g in a cleaned test tube add 10 ml of *sulphuric acid R* and immediately heat the mixture in a water-bath at 90 ± 1 °C for 60 min. Immediately cool rapidly. The solution is not more intensely coloured than a mixture of 1 ml of red primary solution and 9 ml of yellow primary solution (2.2.2, *Method I*).

Oxalic acid. Dissolve 0.80 g in 4 ml of *water R*. Add 3 ml of *hydrochloric acid R* and 1 g of *zinc R* in granules. Boil for 1 min. Allow to stand for 2 min. Transfer the supernatant liquid to a test-tube containing 0.25 ml of a 10 g/l solution of *phenylhydrazine hydrochloride R* and heat to boiling. Cool rapidly, transfer to a graduated cylinder and add an equal volume of *hydrochloric acid R* and 0.25 ml of a 50 g/l solution of *potassium ferricyanide R*. Shake and allow to stand for 30 min. Any pink colour in the solution is not more intense than that in a standard prepared at the same time in the same manner using 4 ml of a 0.1 g/l solution of *oxalic acid R* (360 ppm, calculated as anhydrous oxalic acid).

Sulphates (2.4.13). Dissolve 2.0 g in *distilled water R* and dilute to 30 ml with the same solvent. The solution complies with the limit test for sulphates (150 ppm).

Aluminium (2.4.17). If intended for use in the manufacture of dialysis solutions, it complies with the test for aluminium. Dissolve 20 g in 100 ml of *water R* and add 10 ml of *acetate buffer solution pH 6.0 R*. The solution complies with the limit test for aluminium (0.2 ppm). Use as the reference solution a mixture of 2 ml of *aluminium standard solution (2 ppm Al) R*, 10 ml of *acetate buffer solution pH 6.0 R* and 98 ml of *water R*. To prepare the blank use a mixture of 10 ml of *acetate buffer solution pH 6.0 R* and 100 ml of *water R*.

Heavy metals (2.4.8). Dissolve 5.0 g in several portions in 39 ml of *dilute sodium hydroxide solution R* and dilute to 50 ml with *distilled water R*. 12 ml complies with limit test A for heavy metals (10 ppm). Prepare the standard using *lead standard solution (1 ppm Pb) R*.

Water (2.5.12). Not more than 1.0 per cent, determined on 2.000 g by the semi-micro determination of water.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

Bacterial endotoxins (2.6.14): less than 0.5 IU/mg, if intended for use in the manufacture of parenteral dosage forms without a further appropriate procedure for the removal of bacterial endotoxins.

ASSAY

Dissolve 0.550 g in 50 ml of *water R*. Titrate with 1 M *sodium hydroxide*, using 0.5 ml of *phenolphthalein solution R* as indicator.

1 ml of 1 M *sodium hydroxide* is equivalent to 64.03 mg of C₆H₈O₇.

LABELLING

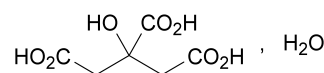
The label states:

- where applicable, that the substance is free from bacterial endotoxins,
- where applicable, that the substance is intended for use in the manufacture of dialysis solutions.

01/2005:0456

CITRIC ACID MONOHYDRATE

Acidum citricum monohydricum

C₆H₈O₇·H₂OM_r 210.1

DEFINITION

Citric acid monohydrate contains not less than 99.5 per cent and not more than the equivalent of 100.5 per cent of 2-hydroxypropane-1,2,3-tricarboxylic acid, calculated with reference to the anhydrous substance.

CHARACTERS

A white, crystalline powder, colourless crystals or granules, efflorescent, very soluble in water, freely soluble in alcohol.

IDENTIFICATION

First identification: B, E.

Second identification: A, C, D, E.

- Dissolve 1 g in 10 ml of *water R*. The solution is strongly acidic (2.2.4).
- Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *citric acid monohydrate CRS* after drying both the substance to be examined and the reference substance at 100-105 °C for 2 h.
- Add about 5 mg to a mixture of 1 ml of *acetic anhydride R* and 3 ml of *pyridine R*. A red colour develops.
- Dissolve 0.5 g in 5 ml of *water R*, neutralise using 1 M *sodium hydroxide* (about 7 ml), add 10 ml of *calcium chloride solution R* and heat to boiling. A white precipitate is formed.
- It complies with the test for water (see Tests).

TESTS

Appearance of solution. Dissolve 2.0 g in *water R* and dilute to 10 ml with the same solvent. The solution is clear (2.2.1) and not more intensely coloured than reference solution Y₇, BY₇ or GY₇ (2.2.2, *Method II*).

01/2005:1609

CITRONELLA OIL

Citronellae aetheroleum

Readily carbonisable substances. To 1.0 g in a cleaned test tube add 10 ml of *sulphuric acid R* and immediately heat the mixture in a water-bath at 90 ± 1 °C for 60 min. Immediately cool rapidly. The solution is not more intensely coloured than a mixture of 1 ml of red primary solution and 9 ml of yellow primary solution (2.2.2, *Method I*).

Oxalic acid. Dissolve 0.80 g in 4 ml of *water R*. Add 3 ml of *hydrochloric acid R* and 1 g of *zinc R* in granules. Boil for 1 min. Allow to stand for 2 min. Transfer the supernatant liquid to a test-tube containing 0.25 ml of a 10 g/l solution of *phenylhydrazine hydrochloride R* and heat to boiling. Cool rapidly, transfer to a graduated cylinder and add an equal volume of *hydrochloric acid R* and 0.25 ml of a 50 g/l solution of *potassium ferricyanide R*. Shake and allow to stand for 30 min. Any pink colour in the solution is not more intense than that in a standard prepared at the same time in the same manner using 4 ml of a 0.1 g/l solution of *oxalic acid R* (360 ppm, calculated as anhydrous oxalic acid).

Sulphates (2.4.13). Dissolve 2.0 g in *distilled water R* and dilute to 30 ml with the same solvent. The solution complies with the limit test for sulphates (150 ppm).

Aluminium (2.4.17). If intended for use in the manufacture of dialysis solutions, it complies with the test for aluminium. Dissolve 20 g in 100 ml of *water R* and add 10 ml of *acetate buffer solution pH 6.0 R*. The solution complies with the limit test for aluminium (0.2 ppm). Use as the reference solution a mixture of 2 ml of *aluminium standard solution (2 ppm Al) R*, 10 ml of *acetate buffer solution pH 6.0 R* and 98 ml of *water R*. To prepare the blank use a mixture of 10 ml of *acetate buffer solution pH 6.0 R* and 100 ml of *water R*.

Heavy metals (2.4.8). Dissolve 5.0 g in several portions in 39 ml of *dilute sodium hydroxide solution R* and dilute to 50 ml with *distilled water R*. 12 ml complies with limit test A for heavy metals (10 ppm). Prepare the standard using *lead standard solution (1 ppm Pb) R*.

Water (2.5.12): 7.5 per cent to 9.0 per cent, determined on 0.500 g by the semi-micro determination of water.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

Bacterial endotoxins (2.6.14): less than 0.5 IU/mg, if intended for use in the manufacture of parenteral dosage forms without a further appropriate procedure for the removal of bacterial endotoxins.

ASSAY

Dissolve 0.550 g in 50 ml of *water R*. Titrate with 1 M *sodium hydroxide*, using 0.5 ml of *phenolphthalein solution R* as indicator.

1 ml of 1 M *sodium hydroxide* is equivalent to 64.03 mg of $C_6H_8O_7$.

STORAGE

In an airtight container.

LABELLING

The label states:

- where applicable, that the substance is free from bacterial endotoxins,
- where applicable, that the substance is intended for use in the manufacture of dialysis solutions.

DEFINITION

Oil obtained by steam distillation from the fresh or partially dried aerial parts of *Cymbopogon winterianus* Jowitt.

CHARACTERS

Pale yellow to brown-yellow liquid, with a very strong odour of citronellal.

IDENTIFICATION

First identification: B.

Second identification: A.

A. Thin-layer chromatography (2.2.27).

Test solution. Dilute 0.1 g of citronella oil in 10.0 ml of *alcohol R*.

Reference solution. Dilute 20 µl of *citronellal R* in 10.0 ml of *alcohol R*.

Plate: TLC silica gel plate R.

Mobile phase: ethyl acetate R, toluene R (10:90 V/V).

Application: 5 µl, as bands.

Development: over a path of 15 cm.

Drying: in air.

Detection: spray with anisaldehyde solution R and heat at 100–105 °C for 10 min. Examine in ultraviolet light at 365 nm.

Result: see below the sequence of the zones present in the chromatograms obtained with the reference and test solutions. Furthermore, other zones are present in the chromatogram obtained with the test solution.

Top of the plate	
Citronellal: a violet zone	A zone similar in colour to the citronellal zone An orange zone (citronellol-geraniol)
Reference solution	Test solution

B. Examine the chromatograms obtained in the test for chromatographic profile.

Results: the characteristic peaks in the chromatogram obtained with the test solution are similar in retention time to those in the chromatogram obtained with the reference solution. Neral and geranial may be absent in the chromatogram obtained with the test solution.

TESTS

Relative density (2.2.5): 0.881 to 0.895.

Refractive index (2.2.6): 1.463 to 1.475.

Optical rotation (2.2.7): -4° to $+1.5^\circ$.

Chromatographic profile. Gas chromatography (2.2.28): use the normalisation procedure.

Test solution. The substance to be examined.

Reference solution. Dilute 25 µl of *limonene R*, 100 µl of *citronellal R*, 25 µl of *citronellyl acetate R*, 25 µl of *citral R*, 25 µl of *geranyl acetate R*, 25 µl of *citronellol R* and 100 µl of *geraniol R* in 5 ml of *hexane R*.

Column:

- *material:* fused silica,
- *size:* $l = 60$ m, $\varnothing = 0.25$ mm,
- *stationary phase:* *macrogol 20 000 R* (0.2 µm).

Carrier gas: helium for chromatography R.

Flow rate: 1.0 ml/min.

Split ratio: 1:100.

Temperature:

	Time (min)	Temperature (°C)
Column	0 - 2	80
	2 - 26	80 → 150
	26 - 42	150 → 185
	42 - 49	185 → 250
Injection port		260
Detector		260

Detection: flame ionisation.

Injection: 1 µl of the reference solution, 0.2 µl of the test solution.

Elution order: the order indicated in the composition of the reference solution. Record the retention times of these substances.

System suitability: reference solution:

- resolution: minimum of 1.2 between the peaks due to geranyl acetate and citronellol.

Using the retention times determined from the chromatogram obtained with the reference solution, locate the components of the reference solution in the chromatogram obtained with the test solution.

Determine the percentage content of each of these components.

The percentages are within the following values:

- limonene: 1.0 per cent to 5.0 per cent,
- citronellal: 30.0 per cent to 45.0 per cent,
- citronellyl acetate: 2.0 per cent to 4.0 per cent,
- neral: less than 2.0 per cent,
- geranial: less than 2.0 per cent,
- geranyl acetate: 3.0 per cent to 8.0 per cent,
- citronellol: 9.0 per cent to 15.0 per cent,
- geraniol: 20.0 per cent to 25.0 per cent.

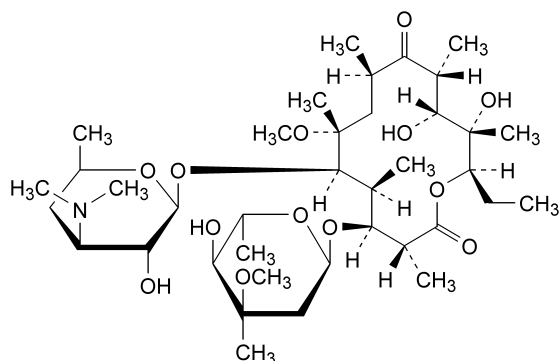
STORAGE

In a well-filled container, protected from light.

01/2005:1651
corrected

CLARITHROMYCIN

Clarithromycinum



C₃₈H₆₉NO₁₃

M_r 748

DEFINITION

(3*R*,4*S*,5*S*,6*R*,7*R*,9*R*,11*R*,12*R*,13*S*,14*R*)-4-[(2,6-Dideoxy-3-*C*-methyl-3-*O*-methyl-α-*L*-ribo-hexopyranosyl)oxy]-14-ethyl-12,13-dihydroxy-7-methoxy-3,5,7,9,11,13-hexamethyl-6-[[3,4,6-trideoxy-3-(dimethylamino)-β-*D*-xylo-hexopyranosyl]oxy]oxacyclotetradecane-2,10-dione (6-*O*-methylerythromycin A).

Content: 96.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

Appearance: white or almost white, crystalline powder.

Solubility: practically insoluble in water, soluble in acetone and in methylene chloride, slightly soluble in methanol.

IDENTIFICATION

Infrared absorption spectrophotometry (2.2.24).

Comparison: clarithromycin CRS.

TESTS

Solution S. Dissolve 0.500 g in methylene chloride R and dilute to 50.0 ml with the same solvent.

Appearance of solution. Solution S is clear or not more opalescent than reference suspension II (2.2.1) and not more intensely coloured than reference solution Y₇ (2.2.2, Method II).

Specific optical rotation (2.2.7): −94 to −102 (anhydrous substance), determined on solution S.

Related substances. Liquid chromatography (2.2.29).

Test solution. Dissolve 75.0 mg of the substance to be examined in 25 ml of acetonitrile R1 and dilute to 50.0 ml with water R.

Reference solution (a). Dissolve 75.0 mg of clarithromycin CRS in 25 ml of acetonitrile R1 and dilute to 50.0 ml with water R.

Reference solution (b). Dilute 5.0 ml of reference solution (a) to 100.0 ml with a mixture of equal volumes of acetonitrile R1 and water R.

Reference solution (c). Dilute 1.0 ml of reference solution (b) to 10.0 ml with a mixture of equal volumes of acetonitrile R1 and water R.

Reference solution (d). Dissolve 15.0 mg of clarithromycin for peak identification CRS in 5.0 ml of acetonitrile R1 and dilute to 10.0 ml with water R.

Blank solution. Dilute 25.0 ml of acetonitrile R1 to 50.0 ml with water R and mix.

Column:

- size: *l* = 0.10 m, Ø = 4.6 mm,
- stationary phase: octadecylsilyl silica gel for chromatography R (3.5 µm),
- temperature: 40 °C.

Mobile phase:

- mobile phase A: a 4.76 g/l solution of potassium dihydrogen phosphate R adjusted to pH 4.4 with dilute phosphoric acid R or a 45 g/l solution of potassium hydroxide R, filtered through a C18 filtration kit,
- mobile phase B: acetonitrile R1,

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)
0 - 32	75 → 40	25 → 60
32 - 34	40	60
34 - 36	40 → 75	60 → 25
36 - 42	75	25

Flow rate: 1.1 ml/min.

Detection: spectrophotometer at 205 nm.

Injection: 10 µl; inject the blank solution, the test solution and reference solutions (b), (c) and (d).

Relative retention with reference to clarithromycin (retention time = about 11 min): impurity I = about 0.38; impurity A = about 0.42; impurity J = about 0.63; impurity L = about 0.74; impurity B = about 0.79; impurity M = about 0.81; impurity C = about 0.89; impurity D = about 0.96; impurity N = about 1.15; impurity E = about 1.27; impurity O = about 1.31; impurity F = about 1.33; impurity P = about 1.35; impurity K = about 1.59; impurity G = about 1.72; impurity H = about 1.82.

System suitability:

- *symmetry factor*: maximum 1.7 for the peak due to clarithromycin in the chromatogram obtained with reference solution (b),
- *peak-to-valley ratio*: minimum 3.0, where H_p = height above the baseline of the peak due to impurity D and H_v = height above the baseline of the lowest point of the curve separating this peak from the peak due to clarithromycin in the chromatogram obtained with reference solution (d).

Limits:

- *correction factors*: for the calculation of contents, multiply the peak areas of the following impurities by the corresponding correction factor: impurity G = 0.27; impurity H = 0.15; use the chromatogram supplied with clarithromycin for peak identification CRS to identify the peaks;
- *any impurity*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (c) (1.0 per cent), and not more than 4 such peaks have an area greater than 0.8 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.4 per cent);
- *total*: not more than 7 times the area of the principal peak in the chromatogram obtained with reference solution (c) (3.5 per cent);
- *disregard limit*: 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.1 per cent); disregard the peaks eluting before impurity I and after impurity H.

Heavy metals (2.4.8): maximum 20 ppm.

Dissolve 1.0 g in a mixture of 15 volumes of *water R* and 85 volumes of *dioxan R* and dilute to 20 ml with the same mixture of solvents. 12 ml of the solution complies with limit test B. Prepare the standard using lead standard solution (1 ppm Pb) obtained by diluting *lead standard solution (100 ppm Pb) R* with a mixture of 15 volumes of *water R* and 85 volumes of *dioxan R*.

Water (2.5.12): maximum 2.0 per cent, determined on 0.500 g.

Sulphated ash (2.4.14): maximum 0.2 per cent, determined on 0.5 g.

ASSAY

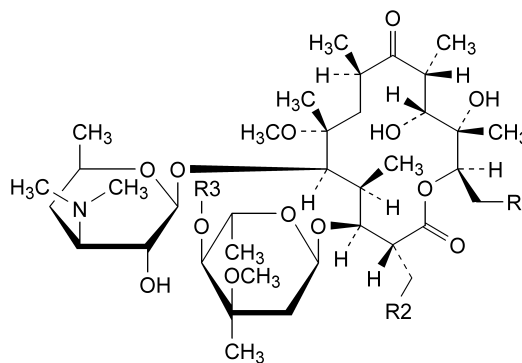
Liquid chromatography (2.2.29) as described in the test for related substances with the following modifications.

Injection: test solution and reference solution (a).

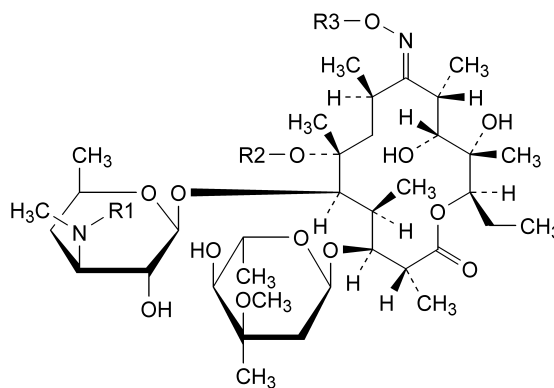
Calculate the percentage content of $C_{38}H_{69}NO_{13}$.

IMPURITIES

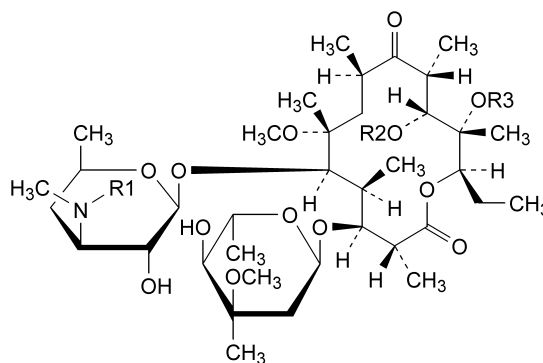
Specified impurities: A, B, C, D, E, F, G, H, I, J, K, L, M, N, O, P.



- A. R1 = CH₃, R2 = OH, R3 = H: 2-demethyl-2-(hydroxymethyl)-6-*O*-methylerythromycin A (clarithromycin F),
 B. R1 = R2 = R3 = H: 6-*O*-methyl-15-norerythromycin A,
 P. R1 = R3 = CH₃, R2 = H: 4',6-di-*O*-methylerythromycin A,



- C. R1 = R2 = CH₃, R3 = H: 6-*O*-methylerythromycin A (*E*)-9-oxime,
 G. R1 = R2 = R3 = CH₃: 6-*O*-methylerythromycin A (*E*)-9-(*O*-methyloxime),
 J. R1 = CH₃, R2 = R3 = H: erythromycin A (*E*)-9-oxime,
 M. R1 = R3 = H, R2 = CH₃: 3'-*N*-demethyl-6-*O*-methylerythromycin A (*E*)-9-oxime,



- D. R1 = R2 = R3 = H: 3'-*N*-demethyl-6-*O*-methylerythromycin A,
 E. R1 = R2 = CH₃, R3 = H: 6,11-di-*O*-methylerythromycin A,
 F. R1 = R3 = CH₃, R2 = H: 6,12-di-*O*-methylerythromycin A,
 H. R1 = CHO, R2 = R3 = H: 3'-*N*-demethyl-3'-*N*-formyl-6-*O*-methylerythromycin A,

01/2005:1850

CLARY SAGE OIL

Salviae sclareae aetheroleum

DEFINITION

Essential oil obtained by steam distillation from the fresh or dried flowering stems of *Salvia sclarea* L.

CHARACTERS

Appearance: colourless to brownish-yellow liquid, usually pale yellow, with a characteristic odour.

IDENTIFICATION

First identification: B.

Second identification: A.

A. Thin-layer chromatography (2.2.27).

Test solution. Dissolve 1 ml of the substance to be examined in *toluene R* and dilute to 10 ml with the same solvent.

Reference solution. Dissolve 60 µl of *linalol R*, 200 µl of *linalyl acetate R* and 60 µl of α -terpineol *R* in *toluene R* and dilute to 10 ml with the same solvent.

Plate: TLC silica gel plate *R*.

Mobile phase: *ethyl acetate R*, *toluene R* (5:95 V/V).

Application: 5 µl of the test solution and 10 µl of the reference solution, as bands.

Development: over a path of 15 cm.

Drying: in air.

Detection: spray with *vanillin reagent R* and heat at 100–105 °C for 5–10 min; examine in daylight within 5 min.

Results: see below the sequence of the zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other faint zones are present in the chromatogram obtained with the test solution.

Top of the plate	
α -Terpineol: a dark violet zone	A dark violet zone
Linalyl acetate: a dark violet zone	A dark violet zone
Linalol: a dark violet zone	A dark violet zone
Reference solution	Test solution

B. Examine the chromatograms obtained in the test for chromatographic profile.

Results: the chromatogram obtained with the test solution shows 5 peaks similar in position to the 5 peaks in the chromatogram obtained with the reference solution. The 2 peaks corresponding to α - and β -thujone may be absent.

TESTS

Relative density (2.2.5): 0.890 to 0.908.

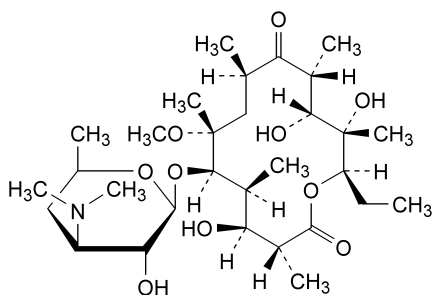
Refractive index (2.2.6): 1.456 to 1.466.

Optical rotation (2.2.7): -26° to -10° .

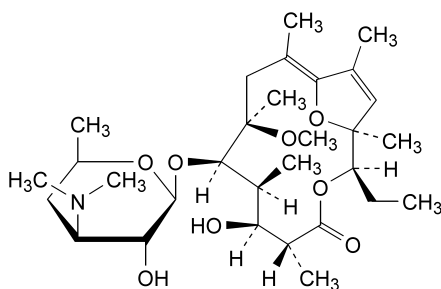
Acid value (2.5.1): maximum 1.0.

Chromatographic profile. Gas chromatography (2.2.28): use the normalisation procedure.

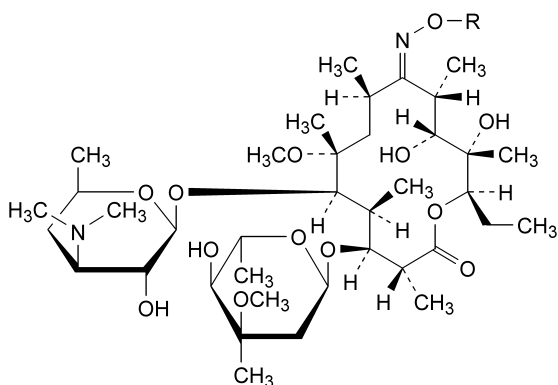
Test solution. The substance to be examined.



I. 3-O-decladinosyl-6-O-methylerythromycin A,

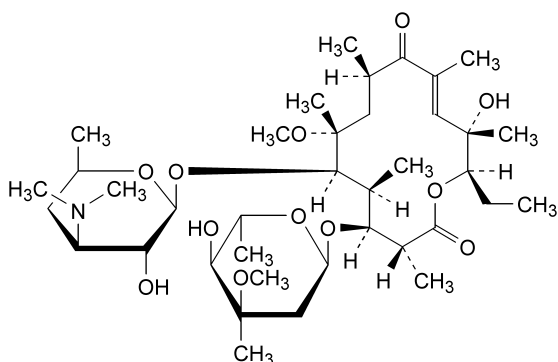


K. (1*S*,2*R*,5*R*,6*S*,7*S*,8*R*,9*R*,11*Z*)-2-ethyl-6-hydroxy-9-methoxy-1,5,7,9,11,13-hexamethyl-8-[[3,4,6-trideoxy-3-(dimethylamino)- β -D-xylo-hexopyranosyl]oxy]-3,15-dioxabicyclo[10.2.1]pentadeca-11,13-dien-4-one (3-O-decladinosyl-8,9:10,11-dianhydro-6-O-methylerythromycin A-9,12-hemiketal,



L. R = H: 6-O-methylerythromycin A (*Z*)-9-oxime,

O. R = CH₃: 6-O-methylerythromycin A (*Z*)-9-(*O*-methyloxime),



N. (10*E*)-10,11-didehydro-11-deoxy-6-O-methylerythromycin A.

01/2005:1714

Reference solution. To 1 g of *hexane R*, add 5 µl of *thujone R*, 5 µl of *linalol R*, 100 µl of *linalyl acetate R*, 10 µl of *α-terpineol R* and 25 mg (± 20 per cent) of *sclareol R*. Mix thoroughly by stirring.

Column:

- **material:** fused silica,
- **size:** $l = 30$ m (a film thickness of 1 µm may be used) to 60 m (a film thickness of 0.2 µm may be used), $\varnothing = 0.25$ -0.53 mm,
- **stationary phase:** *macrogol 20 000 R*.

Carrier gas: *helium for chromatography R*.

Split ratio: 1:100.

Temperature:

	Time (min)	Temperature (°C)
Column	0 - 10	60
	10 - 75	60 → 190
	75 - 120	190
Injection port		220
Detector		240

Detection: flame ionisation.

Injection: 0.2 µl.

Elution order: order indicated in the composition of the reference solution. Record the retention times of these substances.

System suitability: reference solution:

- **resolution:** minimum 1.5 between the peaks due to linalol and linalyl acetate,

Using the retention times determined from the chromatogram obtained with the reference solution, locate the components of the reference solution in the chromatogram obtained with the test solution (disregard any peak due to hexane). *Thujone R* is a mixture of α - and β -thujone. α -Thujone elutes before β -thujone under the described conditions.

Determine the percentage content of each of these components.

Also determine the percentage content of *germacrene-D*. The *germacrene-D* peak can be identified in the chromatogram obtained with the test solution by its relative retention of 1.23 with reference to linalol under the described operating conditions.

The percentages are within the following ranges:

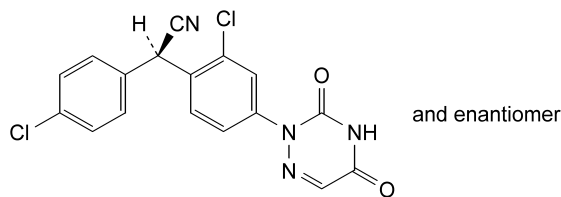
- α and β -thujone: less than 0.2 per cent,
- linalol: 6.5 per cent to 24 per cent,
- linalyl acetate: 56 per cent to 78 per cent,
- α -terpineol: less than 5.0 per cent,
- *germacrene-D*: 1.0 per cent to 12 per cent,
- *sclareol*: 0.4 per cent to 2.6 per cent.

STORAGE

In an airtight container, protected from light, at a temperature not exceeding 25 °C.

CLAZURIL FOR VETERINARY USE

Clazurilum ad usum veterinarium



$C_{17}H_{10}Cl_2N_4O_2$

M_r 373.2

DEFINITION

(2*RS*)-[2-Chloro-4-(3,5-dioxo-4,5-dihydro-1,2,4-triazin-2(3*H*)-yl)phenyl](4-chlorophenyl)acetonitrile.

Content: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

Appearance: white or light yellow powder.

Solubility: practically insoluble in water, freely soluble in dimethylformamide, slightly soluble in alcohol and in methylene chloride.

IDENTIFICATION

A. Melting point (2.2.14): 199 °C to 203 °C.

B. Infrared absorption spectrophotometry (2.2.24).

Comparison: *Ph. Eur. reference spectrum of clazuril.*

TESTS

Related substances. Liquid chromatography (2.2.29).

Test solution. Dissolve 20.0 mg of the substance to be examined in a mixture of equal volumes of *tetrahydrofuran R* and *water R* and dilute to 20.0 ml with the same mixture of solvents.

Reference solution (a). Dissolve 5 mg of *clazuril for system suitability CRS* in a mixture of equal volumes of *tetrahydrofuran R* and *water R* and dilute to 5.0 ml with the same mixture of solvents.

Reference solution (b). Dilute 1.0 ml of the test solution to 100.0 ml with a mixture of equal volumes of *tetrahydrofuran R* and *water R*. Dilute 2.0 ml of this solution to 10.0 ml with a mixture of equal volumes of *tetrahydrofuran R* and *water R*.

Column:

- **size:** $l = 0.10$ m, $\varnothing = 4.6$ mm,
- **stationary phase:** *octadecylsilyl silica gel for chromatography R* (3 µm),
- **temperature:** 35 °C.

Mobile phase:

- **mobile phase A:** mix 100 volumes of a 7.7 g/l solution of *ammonium acetate R* adjusted to pH 6.2 with a 10 per cent V/V solution of *anhydrous formic acid R*, 150 volumes of *acetonitrile R* and 750 volumes of *water R*,
- **mobile phase B:** mix 100 volumes of a 7.7 g/l solution of *ammonium acetate R* adjusted to pH 6.2 with a 10 per cent V/V solution of *anhydrous formic acid R*, 850 volumes of *acetonitrile R* and 50 volumes of *water R*,

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)
0 - 20	100 → 0	0 → 100
20 - 25	0	100
25 - 30	0 → 100	100 → 0
30 - 40	100	0

Flow rate: 1.0 ml/min.

Detection: spectrophotometer at 230 nm.

Injection: 5 µl.

System suitability: reference solution (a):

- *peak-to-valley ratio*: minimum 1.5, where H_p = height above the baseline of the peak due to impurity G and H_v = height above the baseline of the lowest point of the curve separating this peak from the peak due to clazuril,
- the chromatogram obtained is concordant with the chromatogram supplied with *clazuril for system suitability CRS*.

Limits:

- *correction factors*: for the calculation of contents, multiply the peak areas of the following impurities by the corresponding correction factor: impurity G = 1.4; impurity H = 0.8;
- *any impurity*: not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent);
- *total*: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.6 per cent);
- *disregard limit*: 0.25 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent); disregard the peaks due to the solvents.

Loss on drying (2.2.32): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 100–105 °C for 4 h.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

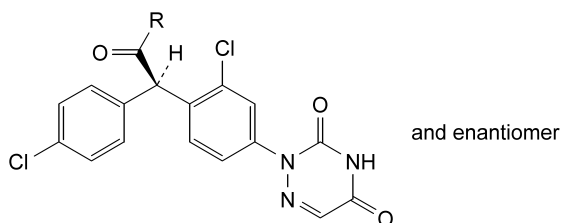
Dissolve about 0.260 g in 35 ml of *tetrahydrofuran R* and add 35 ml of *water R*. Titrate with 0.1 M *sodium hydroxide*, determining the end-point potentiometrically (2.2.20). Carry out a blank titration.

1 ml of 0.1 M *sodium hydroxide* is equivalent to 37.32 mg of $C_{17}H_{10}Cl_2N_4O_2$.

STORAGE

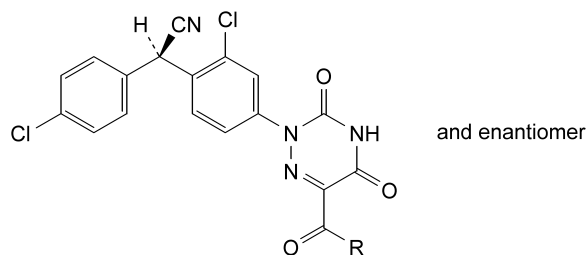
Protected from light.

IMPURITIES



- A. R = OH: (2RS)-[2-chloro-4-(3,5-dioxo-4,5-dihydro-1,2,4-triazin-2(3H)-yl)phenyl]-(4-chlorophenyl)acetic acid,

- C. R = NH₂: (2RS)-2-[2-chloro-4-(3,5-dioxo-4,5-dihydro-1,2,4-triazin-2(3H)-yl)phenyl]-2-(4-chlorophenyl)acetamide,

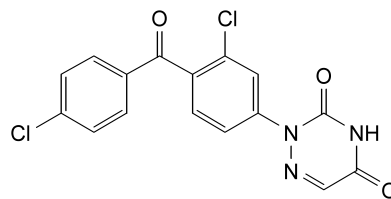


- B. R = NH₂: 2-[3-chloro-4-[(RS)-(4-chlorophenyl)cyanomethyl]phenyl]-3,5-dioxo-2,3,4,5-tetrahydro-1,2,4-triazine-6-carboxamide,

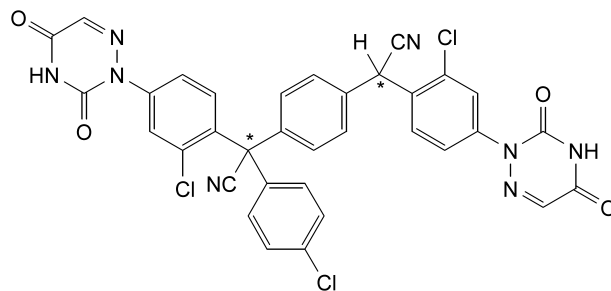
- D. R = N(CH₃)₂: 2-[3-chloro-4-[(RS)-(4-chlorophenyl)cyanomethyl]phenyl]-N,N-dimethyl-3,5-dioxo-2,3,4,5-tetrahydro-1,2,4-triazine-6-carboxamide,

- E. R = OCH₃: methyl 2-[3-chloro-4-[(RS)-(4-chlorophenyl)cyanomethyl]phenyl]-3,5-dioxo-2,3,4,5-tetrahydro-1,2,4-triazine-6-carboxylate,

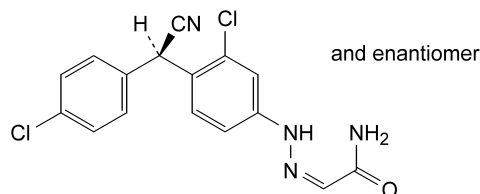
- F. R = OC₂H₅: ethyl 2-[3-chloro-4-[(RS)-(4-chlorophenyl)cyanomethyl]phenyl]-3,5-dioxo-2,3,4,5-tetrahydro-1,2,4-triazine-6-carboxylate,



- G. 2-[3-chloro-4-(4-chlorobenzoyl)phenyl]-1,2,4-triazine-3,5(2H,4H)-dione,



- H. [2-chloro-4-(3,5-dioxo-4,5-dihydro-1,2,4-triazin-2(3H)-yl)phenyl][4-[[2-chloro-4-(3,5-dioxo-4,5-dihydro-1,2,4-triazin-2(3H)-yl)phenyl]cyanomethyl]phenyl]-(4-chlorophenyl)acetonitrile,

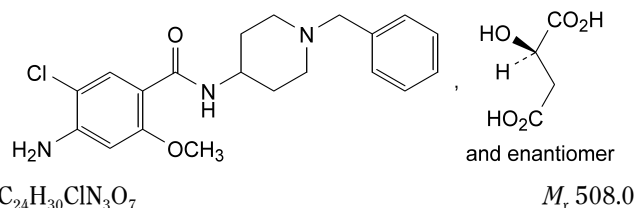


- I. (Z)-2-[[3-chloro-4-[(RS)-(4-chlorophenyl)cyanomethyl]phenyl]diazanylidene]acetamide.

01/2005:1303

CLEBOPRIDE MALATE

Clebopridi malas



DEFINITION

Clebopride malate contains not less than 98.5 per cent and not more than the equivalent of 101.0 per cent of 4-amino-*N*-(1-benzylpiperidin-4-yl)-5-chloro-2-methoxybenzamide acid (*RS*)-2-hydroxybutanedioate, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, crystalline powder, sparingly soluble in water and in methanol, slightly soluble in ethanol, practically insoluble in methylene chloride.

It melts at about 164 °C, with decomposition.

IDENTIFICATION

First identification: B, C.

Second identification: A, C, D.

- Dissolve 20.0 mg in *water R* and dilute to 100.0 ml with the same solvent. Dilute 10.0 ml of the solution to 100.0 ml with *water R*. Examined between 230 nm and 350 nm (2.2.25), the solution shows two absorption maxima, at 270 nm and 307 nm. The specific absorbances at the maxima are 252 to 278 and 204 to 226, respectively.
- Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *clebopride malate CRS*. Examine the substances prepared as discs.
- Dissolve 20 mg in 1 ml of *sulphuric acid R*, add 1 ml of *β-naphthol solution R1* and mix. The solution examined in daylight has a yellow colour with blue fluorescence.
- Examine by thin-layer chromatography (2.2.27), using as the coating substance a suitable silica gel with a fluorescent indicator having an optimal intensity at 254 nm.

Test solution. Dissolve 5 mg of the substance to be examined in *ethanol R* and dilute to 10 ml with the same solvent.

Reference solution (a). Dissolve 5 mg of *clebopride malate CRS* in *ethanol R* and dilute to 10 ml with the same solvent.

Reference solution (b). Dissolve 5 mg of *clebopride malate CRS* and 5 mg of *metoclopramide hydrochloride CRS* in *ethanol R* and dilute to 10 ml with the same solvent.

Apply to the plate as bands 10 mm by 3 mm, 5 µl of each solution. Develop over a path of 15 cm using a mixture of 2 volumes of *concentrated ammonia R*, 14 volumes of *acetone R*, 14 volumes of *methanol R* and 70 volumes of *toluene R*. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. The principal band in the chromatogram obtained with the test solution is similar in position and size to the principal band in the

chromatogram obtained with the reference solution (a). The identification is not valid unless the chromatogram obtained with reference solution (b) shows two clearly separated bands.

TESTS

Solution S. Dissolve 1.0 g in *carbon dioxide-free water R* and dilute to 100.0 ml with the same solvent.

Appearance of solution. Examined immediately after preparation, solution S is clear (2.2.1) and colourless (2.2.2, *Method I*).

pH (2.2.3). The pH of solution S is 3.8 to 4.2.

Related substances. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 0.10 g of the substance to be examined in the mobile phase and dilute to 100.0 ml with the mobile phase.

Reference solution (a). Dilute 1.0 ml of the test solution to 100.0 ml with the mobile phase. Dilute 1.0 ml of this solution to 10.0 ml with the mobile phase.

Reference solution (b). Dissolve 10.0 mg of *clebopride malate CRS* and 10.0 mg of *metoclopramide hydrochloride CRS* in the mobile phase and dilute to 100.0 ml with the mobile phase. Dilute 1.0 ml of this solution to 10.0 ml with the mobile phase.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.12 m long and 4.0 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (5 µm),
- as mobile phase at a flow rate of 1 ml/min a mixture of 20 volumes of *acetonitrile R* and 80 volumes of a 1 g/l solution of *heptane sulphonate sodium R* adjusted to pH 2.5 with *phosphoric acid R*,
- as detector a spectrophotometer set at 215 nm.

Equilibrate the column with the mobile phase for 30 min. Inject 20 µl of reference solution (b). Adjust the sensitivity of the system so that the heights of the peaks in the chromatogram obtained are at least 30 per cent of the full scale of the recorder. The test is not valid unless the retention time of the second peak (clebopride) is about 15 min and the relative retention time of the first peak is about 0.45. Inject 20 µl of the test solution and 20 µl of reference solution (a). Continue the chromatography of the test solution for twice the retention time of the principal peak. In the chromatogram obtained with the test solution: the area of any peak, apart from the principal peak and the two peaks eluting within 2 min, is not greater than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent); the sum of the areas of all peaks, apart from the principal peak and the two peaks eluting within 2 min, is not greater than three times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.3 per cent). Disregard any peak with an area less than 0.25 times that of the principal peak in the chromatogram obtained with reference solution (a).

Chlorides. Prepare the solutions at the same time.

Test solution. Dissolve 0.530 g of the substance to be examined in 20.0 ml of *anhydrous acetic acid R*, add 6 ml of *dilute nitric acid R* and dilute to 50.0 ml with *water R*.

Reference solution. To 1.5 ml of 0.001 M *hydrochloric acid*, add 20.0 ml of *anhydrous acetic acid R* and 6 ml of *dilute nitric acid R* and dilute to 50.0 ml with *water R*.

Transfer separately both solutions recently prepared to test tubes. Add to each tube 1 ml of *silver nitrate solution R2*. Allow to stand for 5 min protected from light. Examine the

01/2005:1190

tubes laterally against a black background. Any opalescence in the test solution is not more intense than that in the reference solution (100 ppm).

Sulphates. Prepare the solutions at the same time.

Test solution. Dissolve 3.00 g of the substance to be examined in 20.0 ml of *glacial acetic acid R*, heating gently if necessary. Allow to cool and dilute to 50.0 ml with *water R*.

Reference solution. To 9 ml of *sulphate standard solution (10 ppm SO₄) R1*, add 6 ml of *glacial acetic acid R*.

Into two test tubes introduce 1.5 ml of *sulphate standard solution (10 ppm SO₄) R1* and add 1 ml of a 250 g/l solution of *barium chloride R*. Shake and allow to stand for 1 min. To one of the tubes add 15 ml of the test solution and to the other one add 15 ml of the reference solution.

After 5 min, any opalescence in the tube containing the test solution is not more intense than that containing the reference solution (100 ppm).

Heavy metals (2.4.8). 1.0 g complies with limit test D for heavy metals (20 ppm). Prepare the standard using 2 ml of *lead standard solution (10 ppm Pb) R*.

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

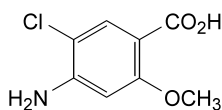
Dissolve 0.400 g in 50 ml of *anhydrous acetic acid R*. Titrate with 0.1 M *perchloric acid*, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *perchloric acid* is equivalent to 50.80 mg of C₂₄H₃₀ClN₃O₇.

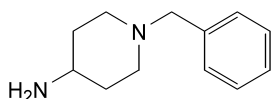
STORAGE

Store protected from light.

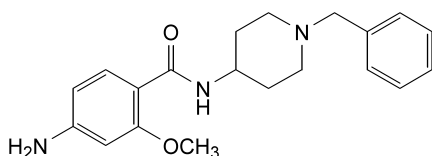
IMPURITIES



A. 4-amino-5-chloro-2-methoxybenzoic acid,



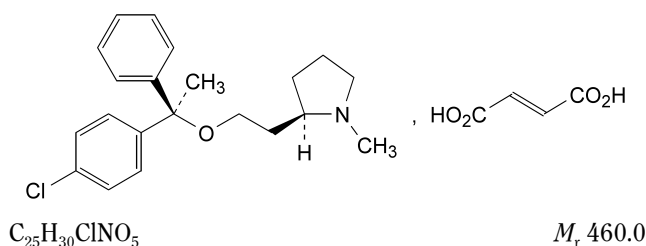
B. 1-benzylpiperidin-4-amine,



C. 4-amino-N-(1-benzylpiperidin-4-yl)-2-methoxybenzamide.

CLEMASTINE FUMARATE

Clemastini fumaras



DEFINITION

Clemastine fumarate contains not less than 98.5 per cent and not more than the equivalent of 101.0 per cent of (2*R*)-2-[2-[(*R*)-1-(4-chlorophenyl)-1-phenylethoxy]ethyl]-1-methylpyrrolidine (*E*)-butenedioate, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, crystalline powder, very slightly soluble in water, sparingly soluble in alcohol (70 per cent V/V), slightly soluble in alcohol (50 per cent V/V) and in methanol.

IDENTIFICATION

First identification: A, B.

Second identification: A, C, D.

- It complies with the test for specific optical rotation (see Tests).
- Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *clemastine fumarate CRS*.
- Examine the chromatograms obtained in the test for related substances (see Tests). The principal spot in the chromatogram obtained with test solution (b) is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).
- Examine by thin-layer chromatography (2.2.27), using *silica gel G R* as the coating substance.

Test solution. Dissolve 40 mg of the substance to be examined in *methanol R* and dilute to 2 ml with the same solvent.

Reference solution. Dissolve 50 mg of *fumaric acid CRS* in *alcohol R* and dilute to 10 ml with the same solvent.

Apply separately to the plate 5 µl of each solution. Develop over a path of 15 cm using a mixture of 5 volumes of *water R*, 25 volumes of *anhydrous formic acid R* and 70 volumes of *di-isopropyl ether R*. Dry the plate at 100 °C to 105 °C for 30 min, allow to cool and spray with a 16 g/l solution of *potassium permanganate R*. Examine in daylight. In the chromatogram obtained with test solution the spot with the highest *R_f* value is similar in position, colour and size to the spot in the chromatogram obtained with the reference solution.

TESTS

Solution S. Dissolve 0.500 g of substance to be examined in *methanol R* and dilute to 50.0 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and not more intensely coloured than reference solution BY₇ (2.2.2, *Method II*).

pH (2.2.3). Suspend 1.0 g of the substance to be examined in 10 ml of *carbon dioxide-free water R*. The pH of the suspension is 3.2 to 4.2.

Specific optical rotation (2.2.7). The specific optical rotation is + 15.0 to + 18.0, determined on solution S and calculated with reference to the dried substance.

Related substances. Examine by thin-layer chromatography (2.2.27), using *silica gel G R* as the coating substance.

Test solution (a). Dissolve 0.100 g of the substance to be examined in *methanol R* and dilute to 5.0 ml with the same solvent.

Test solution (b). Dilute 1.0 ml of test solution (a) to 10.0 ml with *methanol R*.

Reference solution (a). Dissolve 20.0 mg of *clemastine fumarate CRS* in *methanol R* and dilute to 10.0 ml with the same solvent.

Reference solution (b). Dilute 1.5 ml of test solution (b) to 50.0 ml with *methanol R*.

Reference solution (c). Dilute 0.5 ml of test solution (b) to 50.0 ml with *methanol R*.

Reference solution (d). Dissolve 10.0 mg of *diphenhydramine hydrochloride CRS* in 5.0 ml of reference solution (a).

Apply separately to the plate 5 µl of each solution. Develop over a path of 15 cm using a mixture of 1 volume of *concentrated ammonia R*, 20 volumes of *methanol R* and 80 volumes of *tetrahydrofuran R*. Dry the plate in a current of cold air for 5 min, spray with a freshly prepared mixture of 1 volume of *potassium iodobismuthate solution R* and 10 volumes of *dilute acetic acid R* and then with *dilute hydrogen peroxide solution R*. Cover the plate immediately with a glass plate of the same size and examine the chromatograms after 2 min. Any spot in the chromatogram obtained with test solution (a), apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.3 per cent) and at most four such spots are more intense than the spot in the chromatogram obtained with reference solution (c) (0.1 per cent). Disregard any spot remaining at the starting point (fumaric acid). The test is not valid unless the chromatogram obtained with reference solution (d) shows two clearly separated spots.

1-(4-Chlorophenyl)-1-phenylethanol. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 20 mg of the substance to be examined in a mixture of 25 volumes of *acetonitrile R* and 75 volumes of a 10 g/l solution of *ammonium dihydrogen phosphate R* and dilute to 100 ml with the same mixture of solvents.

Reference solution (a). Dissolve 6 mg of *1-(4-chlorophenyl)-1-phenylethanol CRS* in a mixture of 25 volumes of *acetonitrile R* and 75 volumes of a 10 g/l solution of *ammonium dihydrogen phosphate R* and dilute to 100 ml with the same mixture of solvents.

Reference solution (b). Dilute 1 ml of reference solution (a) to 100 ml with a mixture of 25 volumes of *acetonitrile R* and 75 volumes of a 10 g/l solution of *ammonium dihydrogen phosphate R*.

Reference solution (c). Dissolve 10 mg of the substance to be examined in a mixture of 25 volumes of *acetonitrile R* and 75 volumes of a 10 g/l solution of *ammonium dihydrogen phosphate R* and dilute to 100 ml with the same mixture of solvents. To 1 ml of the solution add 1 ml of reference solution (a) and dilute to 100 ml with a mixture of 25 volumes of *acetonitrile R* and 75 volumes of a 10 g/l solution of *ammonium dihydrogen phosphate R*.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.1 m long and 4.6 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (5 µm),
- as mobile phase at a flow rate of 1 ml/min a mixture of 0.1 volumes of *phosphoric acid R*, 45 volumes of *acetonitrile R* and 55 volumes of a 10 g/l solution of *ammonium dihydrogen phosphate R*,
- as detector a spectrophotometer set at 220 nm.

Inject 100 µl of each solution. The test is not valid unless the resolution between the peaks due to clemastine and 1-(4-chlorophenyl)-1-phenylethanol in the chromatogram obtained with reference solution (c) is greater than 2.2. In the chromatogram obtained with the test solution: the area of any peak corresponding to 1-(4-chlorophenyl)-1-phenylethanol is not greater than the area of the peak in the chromatogram obtained with reference solution (b) (0.3 per cent).

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C for 6 h.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

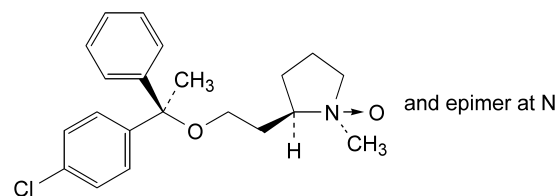
Dissolve 0.350 g of the substance to be examined in 60 ml of *anhydrous acetic acid R*. Titrate with 0.1 M *perchloric acid*, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *perchloric acid* is equivalent to 46.00 mg of $C_{25}H_{30}ClNO_5$.

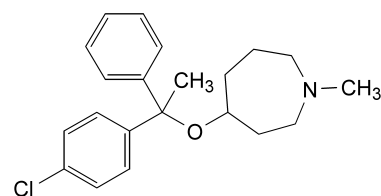
IMPURITIES

Specified impurities: A, B, C.

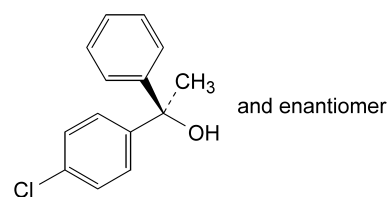
Other detectable impurities: D.



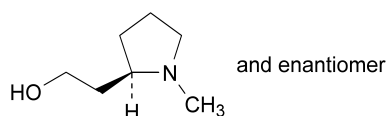
A. (1*RS*,2*R*)-2-[2-[(*R*)-1-(4-chlorophenyl)-1-phenylethoxy]ethyl]-1-methylpyrrolidine 1-oxide,



B. 4-[1-(4-chlorophenyl)-1-phenylethoxy]-1-methylazepane,



C. (*R*)-1-(4-chlorophenyl)-1-phenylethanol,

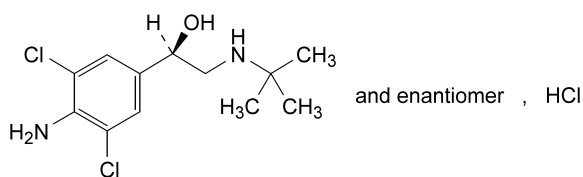


D. 2-[(*RS*)-1-methylpyrrolidin-2-yl]ethanol.

01/2005:1409

CLENBUTEROL HYDROCHLORIDE

Clenbuteroli hydrochloridum



$C_{12}H_{19}Cl_3N_2O$

M_r 313.7

DEFINITION

Clenbuterol hydrochloride contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of (*RS*)-1-(4-amino-3,5-dichlorophenyl)-2-[(1,1-dimethylethyl)amino]ethanol hydrochloride, calculated with reference to the anhydrous substance.

CHARACTERS

A white or almost white, crystalline powder, soluble in water and in alcohol, slightly soluble in acetone.

It melts at about 173 °C, with decomposition.

IDENTIFICATION

First identification: A, C.

Second identification: B, C.

- A. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *clenbuterol hydrochloride CRS*.
- B. Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel F₂₅₄ plate R*.

Test solution. Dissolve 10 mg of the substance to be examined in 10 ml of *methanol R*.

Reference solution. Dissolve 10 mg of *clenbuterol hydrochloride CRS* in 10 ml of *methanol R*.

Apply to the plate 10 µl of each solution. Develop over a path of 10 cm using a mixture of 0.15 volumes of *ammonia R*, 10 volumes of *ethanol R* and 15 volumes of *toluene R*. Allow the plate to dry in air. Spray with a 10 g/l solution of *sodium nitrite R* in 1 M *hydrochloric acid* and dip after 10 min in a 4 g/l solution of *naphthylethylenediamine dihydrochloride R* in *methanol R*. Allow the plate to dry in air. The principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

C. It gives reaction (a) of chlorides (2.3.1).

TESTS

Solution S. Dissolve 0.5 g in 10 ml of *carbon dioxide-free water R*.

Appearance of solution. Solution S is not more opalescent than reference suspension II (2.2.1) and not more intensely coloured than reference solution Y₆ (2.2.2, *Method II*).

pH (2.2.3). The pH of solution S is 5.0 to 7.0.

Optical rotation (2.2.7). Dissolve 0.30 g in *water R* and dilute to 10.0 ml with the same solvent. If necessary filter. The angle of optical rotation is −0.10° to +0.10°.

Related substances. Examine by liquid chromatography (2.2.29).

Test solution. Disperse 100.0 mg of the substance to be examined in the mobile phase and dilute to 50.0 ml with the mobile phase.

Reference solution (a). Dilute 0.1 ml of the test solution to 100.0 ml with *water R*.

Reference solution (b). Dissolve 10 mg of *clenbuterol impurity B CRS* in 20 ml of the mobile phase, add 5 ml of the test solution and dilute to 50.0 ml with the mobile phase.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.125 m long and 4 mm in internal diameter, packed with *end-capped octadecylsilyl silica gel for chromatography R* (5 µm),
- as mobile phase at a flow rate of 0.5 ml/min a mixture of 200 volumes of *acetonitrile R*, 200 volumes of *methanol R* and 600 volumes of a solution prepared as follows: dissolve 3.0 g of *sodium decanesulphonate R* and 5.0 g of *potassium dihydrogen phosphate R* in 900 ml of *water R*, adjust to pH 3.0 with *dilute phosphoric acid R* and dilute to 1000 ml with *water R*,
- as detector a spectrophotometer set at 215 nm, maintaining the temperature of the column at 40 °C.

Inject 5 µl of reference solution (a) and 5 µl of reference solution (b). Adjust the sensitivity of the system so that the height of the principal peak in the chromatogram obtained with reference solution (a) is at least 50 per cent of the full scale of the recorder. When the chromatogram are recorded in the prescribed conditions, the retention time of clenbuterol is about 11 min. The test is not valid until in the chromatogram obtained with reference solution (b) the resolution between the peaks corresponding to impurity B and clenbuterol is at least 2.5.

Inject 5 µl of the test solution and continue the chromatography for 1.5 times the retention time of the principal peak. In the chromatogram obtained with the test solution: the area of any peak apart from the principal peak is not greater than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent) and the sum of the areas of all the peaks apart of the principal peak is not greater than 2 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent). Disregard any peak with an area less than 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (a).

Water (2.5.12). Not more than 1.0 per cent, determined on 0.500 g by the semi-micro determination of water.

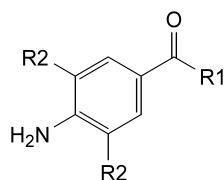
Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.250 g in 50 ml of *alcohol R* and add 5.0 ml of 0.01 M *hydrochloric acid*. Titrate with 0.1 M *sodium hydroxide*, determining the end-point potentiometrically (2.2.20). Read the volume added between the two points of inflexion.

1 ml of 0.1 M *sodium hydroxide* is equivalent to 31.37 mg of $C_{12}H_{19}Cl_3N_2O$.

IMPURITIES

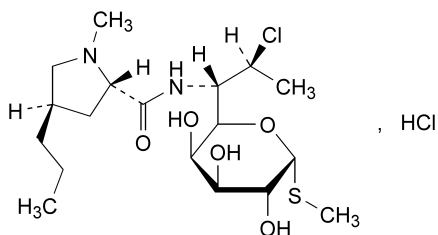


- A. R1 = H, R2 = Cl: 4-amino-3,5-dichlorobenzaldehyde,
 B. R1 = CH₂-NH-C(CH₃)₃, R2 = Cl: 1-(4-amino-3,5-dichlorophenyl)-2-[(1,1-dimethylethyl)amino]ethanone (clenbuterol-ketone),
 C. R1 = CH₃, R2 = Cl: 1-(4-amino-3,5-dichlorophenyl)ethanone,
 D. R1 = CH₃, R2 = H: 1-(4-aminophenyl)ethanone,
 E. R1 = CH₂Br, R2 = Cl: 1-(4-amino-3,5-dichlorophenyl)-2-bromoethanone.

01/2005:0582

CLINDAMYCIN HYDROCHLORIDE

Clindamycini hydrochloridum

C₁₈H₃₄Cl₂N₂O₅SM_r 461.5

DEFINITION

Methyl 7-chloro-6,7,8-trideoxy-6-[[[(2S,4R)-1-methyl-4-propylpyrrolidin-2-yl]carbonyl]amino]-1-thio-L-threo-α-D-galacto-octopyranoside hydrochloride. It contains a variable quantity of water.

Content: 84.0 per cent to 93.0 per cent of clindamycin (C₁₈H₃₃ClN₂O₅S) (anhydrous substance).

CHARACTERS

Appearance: white or almost white, crystalline powder.

Solubility: very soluble in water, slightly soluble in alcohol.

IDENTIFICATION

First identification: A, D.

Second identification: B, C, D.

A. Infrared absorption spectrophotometry (2.2.24).

Comparison: clindamycin hydrochloride CRS.

B. Thin-layer chromatography (2.2.27).

Test solution. Dissolve 10 mg of the substance to be examined in *methanol* R and dilute to 10 ml with the same solvent.

Reference solution (a). Dissolve 10 mg of *clindamycin hydrochloride* CRS in *methanol* R and dilute to 10 ml with the same solvent.

Reference solution (b). Dissolve 10 mg of *clindamycin hydrochloride* CRS and 10 mg of *lincomycin hydrochloride* CRS in *methanol* R and dilute to 10 ml with the same solvent.

Plate: TLC silica gel G plate R.

Mobile phase: mix 19 volumes of 2-propanol R, 38 volumes of a 150 g/l solution of *ammonium acetate* R adjusted to pH 9.6 with *ammonia* R, and 43 volumes of *ethyl acetate* R.

Application: 5 µl.

Development: over a path of 15 cm using the upper layer of the mobile phase.

Drying: in air.

Detection: spray with a 1 g/l solution of *potassium permanganate* R.

System suitability: the chromatogram obtained with reference solution (b) shows 2 clearly separated spots.

Results: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).

C. Dissolve about 10 mg in 2 ml of *dilute hydrochloric acid* R and heat on a water-bath for 3 min. Add 3 ml of *sodium carbonate solution* R and 1 ml of a 20 g/l solution of *sodium nitroprusside* R. A violet-red colour develops.

D. Dissolve 0.1 g in *water* R and dilute to 10 ml with the same solvent. The solution gives reaction (a) of chlorides (2.3.1).

TESTS

pH (2.2.3): 3.0 to 5.0.

Dissolve 1.0 g in *carbon dioxide-free water* R and dilute to 10 ml with the same solvent.

Specific optical rotation (2.2.7): + 135 to + 150 (anhydrous substance).

Dissolve 1.000 g in *water* R and dilute to 25.0 ml with the same solvent.

Related substances. Liquid chromatography (2.2.29).

Test solution. Dissolve 50.0 mg of the substance to be examined in the mobile phase and dilute to 50.0 ml with the mobile phase.

Reference solution (a). Dissolve 50.0 mg of *clindamycin hydrochloride* CRS in the mobile phase and dilute to 50.0 ml with the mobile phase.

Reference solution (b). Dilute 2.0 ml of the test solution to 100.0 ml with the mobile phase.

Column:

- **size:** *l* = 0.25 m, Ø = 4.6 mm,
- **stationary phase:** octadecylsilyl silica gel for chromatography R (5 µm).

Mobile phase: mix 45 volumes of *acetonitrile* R and 55 volumes of a 6.8 g/l solution of *potassium dihydrogen phosphate* R adjusted to pH 7.5 with a 250 g/l solution of *potassium hydroxide* R.

Flow rate: 1 ml/min.

Detection: spectrophotometer at 210 nm.

Injection: 20 µl.

Run time: twice the retention time of clindamycin.

System suitability: reference solution (a):

- **relative retentions** with reference to clindamycin (retention time = about 10 min): impurity A = about 0.4; impurity B = about 0.65; impurity C = about 0.8.

Limits:

- **impurity B:** not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (2.0 per cent),

- *impurity C*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (4.0 per cent),
- *any other impurity*: not more than half the area of the principal peak in the chromatogram obtained with reference solution (b) (1 per cent),
- *total*: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (b) (6.0 per cent),
- *disregard limit*: 0.025 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

Water (2.5.12): 3.0 per cent to 6.0 per cent, determined on 0.500 g.

Sulphated ash (2.4.14): maximum 0.5 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (2.2.29) as described in the test for related substances with the following modifications.

Injection: 20 µl; inject the test solution and reference solution (a).

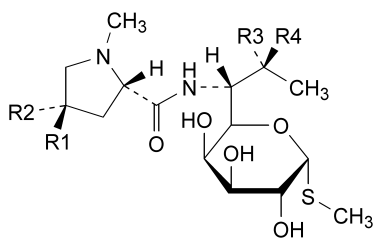
System suitability: reference solution (a):

- *repeatability*: maximum relative standard deviation of 0.85 per cent for 6 replicate injections.

STORAGE

In an airtight container.

IMPURITIES

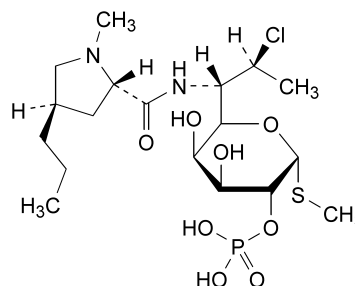


- R1 = CH₂-CH₂-CH₃, R2 = R4 = H, R3 = OH: methyl 6,8-dideoxy-6-[[[(2S,4R)-1-methyl-4-propylpyrrolidin-2-yl]carbonyl]amino]-1-thio-D-erythro-α-D-galacto-octopyranoside (lincomycin),
- R1 = R3 = H, R2 = C₂H₅, R4 = Cl: methyl 7-chloro-6,7,8-trideoxy-6-[[[(2S,4S)-4-ethyl-1-methylpyrrolidin-2-yl]carbonyl]amino]-1-thio-L-threo-α-D-galacto-octopyranoside (clindamycin B),
- R1 = CH₂-CH₂-CH₃, R2 = R4 = H, R3 = Cl: methyl 7-chloro-6,7,8-trideoxy-6-[[[(2S,4R)-1-methyl-4-propylpyrrolidin-2-yl]carbonyl]amino]-1-thio-D-erythro-α-D-galacto-octopyranoside (7-epiclindamycin).

01/2005:0996

CLINDAMYCIN PHOSPHATE

Clindamycini phosphas

C₁₈H₃₄ClN₂O₈PSM_r 505.0

DEFINITION

Clindamycin phosphate contains not less than 95.0 per cent and not more than the equivalent of 100.5 per cent of methyl 7-chloro-6,7,8-trideoxy-6-[[[(2S,4R)-1-methyl-4-propylpyrrolidin-2-yl]carbonyl]amino]-1-thio-L-threo-α-D-galacto-octopyranoside 2-(dihydrogen phosphate), calculated with reference to the anhydrous substance.

CHARACTERS

A white or almost white powder, slightly hygroscopic, freely soluble in water, very slightly soluble in alcohol, practically insoluble in methylene chloride.

It shows polymorphism.

IDENTIFICATION

First identification: A, D.

Second identification: B, C, D.

- Examine by infrared spectrophotometry (2.2.24), comparing with the spectrum obtained with *clindamycin phosphate CRS*. In two separate tubes place 50 mg of the substance to be examined and 50 mg of *clindamycin phosphate CRS*. Add 0.2 ml of *water R* and heat until completely dissolved. Evaporate to dryness under reduced pressure and dry at 100 °C to 105 °C for 2 h. Examine the substances prepared as discs using *potassium bromide R*.

- Examine by thin-layer chromatography (2.2.27), using *silica gel H R* as the coating substance.

Test solution. Dissolve 20 mg of the substance to be examined in *methanol R* and dilute to 10 ml with the same solvent.

Reference solution (a). Dissolve 20 mg of *clindamycin phosphate CRS* in *methanol R* and dilute to 10 ml with the same solvent.

Reference solution (b). Dissolve 10 mg of *lincomycin hydrochloride CRS* in 5 ml of reference solution (a).

Apply separately to the plate 5 µl of each solution. Develop over a path of 12 cm using a mixture of 20 volumes of *glacial acetic acid R*, 20 volumes of *water R* and 60 volumes of *butanol R*. Dry the plate at 100 °C to 105 °C for 30 min and spray with a 1 g/l solution of *potassium permanganate R*. The principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a). The test is not valid unless the chromatogram obtained with reference solution (b) shows two principal spots.

C. Dissolve about 10 mg in 2 ml of *dilute hydrochloric acid R* and heat in a water-bath for 3 min. Add 4 ml of *sodium carbonate solution R* and 1 ml of a 20 g/l solution of *sodium nitroprusside R*. Prepare a standard in the same manner using *clindamycin phosphate CRS*. The colour in the test solution corresponds to that in the standard.

D. Boil 0.1 g under a reflux condenser with a mixture of 5 ml of *strong sodium hydroxide solution R* and 5 ml of *water R* for 90 min. Cool and add 5 ml of *nitric acid R*. Extract with three quantities, each of 15 ml, of *methylene chloride R* and discard the extracts. Filter the upper layer through a paper filter. The filtrate gives reaction (b) of phosphates (2.3.1).

TESTS

Solution S. Dissolve 1.00 g in *carbon dioxide-free water R*. Heat gently if necessary. Cool and dilute to 25.0 ml with *carbon dioxide-free water R*.

Appearance of the solution. Solution S is clear (2.2.1) and colourless (2.2.2, *Method II*).

pH (2.2.3). Dilute 5.0 ml of solution S to 20 ml with *carbon dioxide-free water R*. The pH of the solution is 3.5 to 4.5.

Specific optical rotation (2.2.7). Dissolve 0.250 g in *water R* and dilute to 25.0 ml with the same solvent. The specific optical rotation is + 115 to + 130, calculated with reference to the anhydrous substance.

Related substances. Examine by liquid chromatography (2.2.29), as described under Assay. Inject reference solution (c). Adjust the sensitivity of the detector so that the height of the principal peak is not less than 50 per cent of the full scale of the recorder. Inject the test solution and continue the chromatography for the retention time of clindamycin: the area of any peak, apart from the principal peak and any peak due to the solvent, is not greater than 2.5 times the area of the peak corresponding to clindamycin phosphate in the chromatogram obtained with reference solution (c) (2.5 per cent) and the sum of the areas of all the peaks, apart from the principal peak and any peak due to the solvent, is not greater than four times the area of the peak corresponding to clindamycin phosphate in the chromatogram obtained with reference solution (c) (4.0 per cent). Disregard any peak with an area less than 10.0 per cent of that of the principal peak in the chromatogram obtained with reference solution (c).

Water (2.5.12). Not more than 6.0 per cent, determined on 0.250 g by the semi-micro determination of water.

Bacterial endotoxins (2.6.14): less than 0.6 IU/mg, if intended for use in the manufacture of parenteral dosage forms without a further appropriate procedure for removal of bacterial endotoxins.

ASSAY

Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 75.0 mg of the substance to be examined in the mobile phase and dilute to 25.0 ml with the mobile phase.

Reference solution (a). Dissolve 75.0 mg of *clindamycin phosphate CRS* in the mobile phase and dilute to 25.0 ml with the mobile phase.

Reference solution (b). Dissolve 5.0 mg of *lincomycin hydrochloride CRS* and 15.0 mg of *clindamycin hydrochloride CRS* in 5.0 ml of reference solution (a) and dilute to 100.0 ml with the mobile phase.

Reference solution (c). Dilute 1.0 ml of reference solution (a) to 100.0 ml with the mobile phase.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4.6 mm in internal diameter packed with *octylsilyl silica gel for chromatography R* (5 µm to 10 µm),
- as mobile phase at a flow rate of 1.0 ml/min a mixture of 200 ml of *acetonitrile R* and 800 ml of a 13.6 g/l solution of *potassium dihydrogen phosphate R*, previously adjusted to pH 2.5 with *phosphoric acid R*,
- as detector a spectrophotometer set at 210 nm,
- a 20 µl loop injector.

Inject reference solution (b). Adjust the sensitivity of the detector so that the height of the peaks is not less than 50 per cent of the full scale of the recorder. The assay is not valid unless the first peak (lincomycin) is clearly separated from the solvent peak and the resolution between the second peak (clindamycin phosphate) and the third peak (clindamycin) is at least 6.0. Adjust the concentration of acetonitrile in the mobile phase, if necessary. The assay is not valid unless the symmetry factor of the clindamycin phosphate peak is at most 1.5. Inject reference solution (a) six times. The test is not valid unless the relative standard deviation of the peak area for clindamycin phosphate is at most 1.0 per cent. If necessary adjust the integrator parameters. Inject alternately the test solution and reference solution (a).

Calculate the percentage content of clindamycin phosphate.

STORAGE

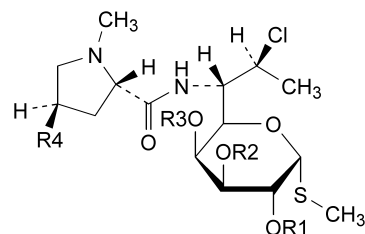
Store in an airtight container, at a temperature not exceeding 30 °C. If the substance is sterile, store in a sterile, airtight, tamper-proof container.

LABELLING

The label states, where applicable, that the substance is free from bacterial endotoxins.

IMPURITIES

A. lincomycin,



B. R1 = PO₃H₂, R2 = R3 = H, R4 = C₂H₅: clindamycin B 2-(dihydrogen phosphate),

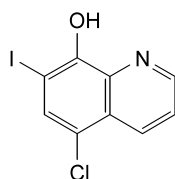
C. R1 = R3 = H, R2 = PO₃H₂, R4 = C₃H₇: clindamycin 3-(dihydrogen phosphate),

D. R1 = R2 = H, R3 = PO₃H₂, R4 = C₃H₇: clindamycin 4-(dihydrogen phosphate),

E. R1 = R2 = R3 = H, R4 = C₃H₇: clindamycin.

CLIOQUINOL

Clioquinolum

C₉H₅ClINOM_r 305.5

DEFINITION

5-Chloro-7-iodoquinolin-8-ol.

Content: 98.0 per cent to 102.0 per cent (dried substance).

CHARACTERS

Appearance: almost white, light yellow, brownish-yellow or yellowish-grey powder.*Solubility*: practically insoluble in water, sparingly soluble in methylene chloride, very slightly soluble or slightly soluble in ethanol (96 per cent).

IDENTIFICATION

First identification: B.*Second identification*: A, C, D.

A. Dissolve 40.0 mg in *methanol R* and dilute to 100.0 ml with the same solvent. Dilute 10.0 ml to 100.0 ml with *methanol R* (solution A). Examined between 280 nm and 350 nm (2.2.25), solution A shows an absorption maximum at 321 nm. Dilute 10.0 ml of solution A to 100.0 ml with *methanol R* (solution B). Examined between 230 nm and 280 nm, solution B shows an absorption maximum at 255 nm. The specific absorbance at this absorption maximum is 1530 to 1660.

B. Infrared absorption spectrophotometry (2.2.24).

Preparation: discs of *potassium bromide R*.*Comparison*: *clioquinol CRS*.

C. When heated, violet fumes are produced.

D. Dissolve about 1 mg in 5 ml of *ethanol (96 per cent) R*. Add 0.05 ml of *ferric chloride solution R1*. A dark green colour develops.

TESTS

Acidity or alkalinity. Shake 0.5 g with 10 ml of *carbon dioxide-free water R* and filter. To the filtrate add 0.2 ml of *phenolphthalein solution R*. The solution is colourless. Not more than 0.5 ml of 0.01 M *sodium hydroxide* is required to change the colour of the indicator to pink.

Related substances. Liquid chromatography (2.2.29).

Test solution. Dissolve 50.0 mg of the substance to be examined in *methanol R* and dilute to 50.0 ml with the same solvent, heating gently if necessary. Dilute 10.0 ml of the solution to 25.0 ml with the mobile phase.

Reference solution (a). Dissolve 20.0 mg of *5-chloroquinolin-8-ol R*, 10.0 mg of *5,7-dichloroquinolin-8-ol R*, 5 mg of the substance to be examined and 10.0 mg of *5,7-diiodoquinolin-8-ol R* in *methanol R*, heating gently if necessary and dilute to 20.0 ml with the same solvent. Dilute 4.0 ml of the solution to 50.0 ml with the mobile phase.

Reference solution (b). Dilute 1.0 ml of reference solution (a) to 10.0 ml with the mobile phase.

01/2005:2111 *Reference solution (c).* Dilute 1.0 ml of the test solution to 100.0 ml with the mobile phase. Dilute 1.0 ml of this solution to 20.0 ml with the mobile phase.

Column:

- *size*: $l = 0.15$ m, $\varnothing = 3.9$ mm,
- *stationary phase*: *octylsilyl silica gel for chromatography R* (5 μ m).

Mobile phase: dissolve 0.50 g of *sodium edetate R* in 350 ml of *water R*, add 4.0 ml of *hexylamine R* and mix. Adjust to pH 3.0 with *phosphoric acid R*. Add 600 ml of *methanol R* and dilute to 1000 ml with *water R*.

Flow rate: 1.3 ml/min.*Detection*: spectrophotometer at 254 nm.*Injection*: 20 μ l.*Run time*: 4 times the retention time of clioquinol.

Relative retention with reference to clioquinol (retention time = about 10 min): impurity A = about 0.4; impurity B = about 0.7; impurity C = about 1.3.

System suitability: reference solution (a):

- *resolution*: minimum 3.0 between the peaks due to clioquinol and impurity C.

Limits:

- *impurity A*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (b) (2.0 per cent),
- *impurity B*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (b) (1.0 per cent),
- *impurity C*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (b) (1.0 per cent),
- *any other impurity*: for each impurity, not more than twice the area of the principal peak in the chromatogram obtained with reference solution (c) (0.1 per cent),
- *total of the nominal contents of impurities A, B, C and any other impurities*: maximum 3.0 per cent,
- *disregard limit*: the area of the principal peak in the chromatogram obtained with reference solution (c) (0.05 per cent).

Halides: maximum 140 ppm, expressed as chlorides.

Shake 0.5 g with 25 ml of *water R* for 1 min and filter. To the filtrate add 0.5 ml of *dilute nitric acid R* and 0.5 ml of *silver nitrate solution R2*. Allow to stand for 5 min. Any opalescence is not more intense than that in a standard prepared at the same time by adding 0.5 ml of *silver nitrate solution R2* to 25 ml of *water R* containing 0.2 ml of 0.01 M *hydrochloric acid* and 0.5 ml of *dilute nitric acid R*.

Loss on drying (2.2.32): maximum 0.5 per cent, determined on 1.000 g by drying over *diphosphorus pentoxide R* at a pressure not exceeding 0.7 kPa for 24 h.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.200 g in 20 ml of *acetic anhydride R* and add 30 ml of *glacial acetic acid R*. Titrate with 0.1 M *perchloric acid*, determining the end-point potentiometrically (2.2.20).

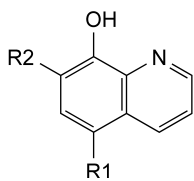
1 ml of 0.1 M *perchloric acid* is equivalent to 30.55 mg of total quinolines, calculated as clioquinol.

STORAGE

Protected from light.

IMPURITIES

Specified impurities: A, B, C.

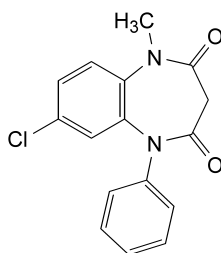


- A. R1 = Cl, R2 = H: 5-chloroquinolin-8-ol,
 B. R1 = R2 = Cl: 5,7-dichloroquinolin-8-ol,
 C. R1 = R2 = I: 5,7-diiodoquinolin-8-ol.

01/2005:1974
corrected

CLOBAZAM

Clobazamum



$C_{16}H_{13}ClN_2O_2$

M_r 300.7

DEFINITION

7-Chloro-1-methyl-5-phenyl-1,5-dihydro-3H-1,5-benzodiazepine-2,4-dione.

Content: 97.0 per cent to 103.0 per cent (dried substance).

CHARACTERS

Appearance: white or almost white, crystalline powder.

Solubility: slightly soluble in water, freely soluble in methylene chloride, sparingly soluble in alcohol.

IDENTIFICATION

Infrared absorption spectrophotometry (2.2.24).

Comparison: Ph. Eur. reference spectrum of clobazam.

TESTS

Related substances. Liquid chromatography (2.2.29).

Test solution. Dissolve 10.0 mg of the substance to be examined in the mobile phase and dilute to 50.0 ml with the mobile phase.

Reference solution (a). Dissolve 5.0 mg of clobazam impurity A CRS in the mobile phase and dilute to 50.0 ml with the mobile phase. Dilute 1.0 ml of the solution to 100.0 ml with the mobile phase.

Reference solution (b). Dissolve 5 mg of chlordiazepoxide CRS and 5 mg of clonazepam CRS in the mobile phase and dilute to 50 ml with the mobile phase. Dilute 1 ml of the solution to 100 ml with the mobile phase.

Reference solution (c). Dilute 1.0 ml of the test solution to 200.0 ml with the mobile phase.

Column:

- size: $l = 0.25$ m, $\varnothing = 4.6$ mm,
- stationary phase: octadecylsilyl silica gel for chromatography R (5 μ m).

Mobile phase: acetonitrile R, water R (40:60 V/V).

Flow rate: 1 ml/min.

Detection: spectrophotometer at 230 nm.

Injection: 20 μ l.

Run time: 5 times the retention time of clobazam.

Retention time: clobazam = about 15 min.

System suitability: reference solution (b):

- resolution: minimum 1.3 between the peaks due to chlordiazepoxide and clonazepam.

Limits:

- impurity A: not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent),
- any other impurity: not more than 0.4 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.2 per cent),
- total of other impurities: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (c) (1.0 per cent),
- disregard limit: 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.05 per cent).

Loss on drying (2.2.32): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 100-105 °C.

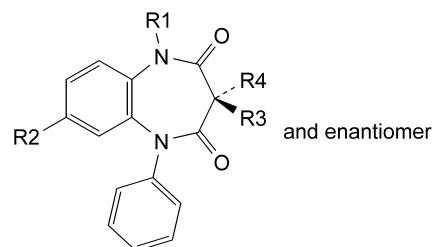
Sulphated ash (2.4.14): maximum 0.1 per cent, determined on the residue obtained in the test for loss on drying.

ASSAY

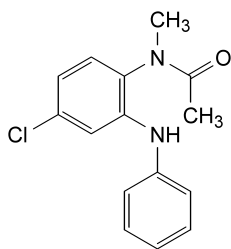
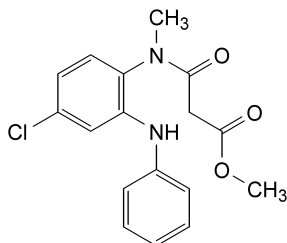
Dissolve 50.0 mg in alcohol R and dilute to 100.0 ml with the same solvent. Dilute 2.0 ml of the solution to 250.0 ml with alcohol R. Measure the absorbance (2.2.25) at the maximum at 232 nm.

Calculate the content of $C_{16}H_{13}ClN_2O_2$ taking the specific absorbance to be 1380.

IMPURITIES



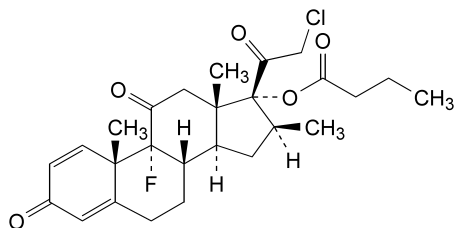
- A. R1 = R3 = R4 = H, R2 = Cl: 7-chloro-5-phenyl-1,5-dihydro-3H-1,5-benzodiazepine-2,4-dione,
 B. R1 = CH₃, R2 = R3 = R4 = H: 1-methyl-5-phenyl-1,5-dihydro-3H-1,5-benzodiazepine-2,4-dione,
 C. R1 = R3 = CH₃, R2 = Cl, R4 = H: (3R)-7-chloro-1,3-dimethyl-5-phenyl-1,5-dihydro-3H-1,5-benzodiazepine-2,4-dione,
 D. R1 = R3 = R4 = CH₃, R2 = Cl: 7-chloro-1,3,3-trimethyl-5-phenyl-1,5-dihydro-3H-1,5-benzodiazepine-2,4-dione,

E. *N*-[4-chloro-2-(phenylamino)phenyl]-*N*-methylacetamide,

F. methyl 3-[[4-chloro-2-(phenylamino)phenyl]methylamino]-3-oxopropanoate.

01/2005:1090
corrected**CLOBETASONE BUTYRATE**

Clobetasoni butyras

 $C_{26}H_{32}ClFO_5$ M_r 479.0**DEFINITION**

Clobetasone butyrate contains not less than 97.0 per cent and not more than the equivalent of 102.0 per cent of 21-chloro-9-fluoro-16β-methyl-3,11,20-trioxopregna-1,4-dien-17-yl butanoate, calculated with reference to the dried substance.

CHARACTERS

A white or almost white powder, practically insoluble in water, freely soluble in acetone and in methylene chloride, slightly soluble in alcohol.

It melts at about 178 °C.

IDENTIFICATION

- A. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *clobetasone butyrate* CRS. Examine the substances prepared as discs.
- B. Examine by thin-layer chromatography (2.2.27), using as the coating substance a suitable silica gel with a fluorescent indicator having an optimal intensity at 254 nm.

Test solution. Dissolve 10 mg of the substance to be examined in a mixture of equal volumes of *methanol R* and *methylene chloride R* and dilute to 10 ml with the same mixture of solvents.

Reference solution (a). Dissolve 10 mg of *clobetasone butyrate* CRS in a mixture of equal volumes of *methanol R* and *methylene chloride R* and dilute to 10 ml with the same mixture of solvents.

Reference solution (b). Dissolve 10 mg of *clobetasol propionate R* in a mixture of equal volumes of *methanol R* and *methylene chloride R* and dilute to 10 ml with the same mixture of solvents. Dilute 5 ml of this solution to 10 ml with reference solution (a).

Apply separately to the plate 5 µl of each solution. Develop over a path of 15 cm using a mixture of equal volumes of *cyclohexane R* and *methyl acetate R*. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. The principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a). The test is not valid unless the chromatogram obtained with reference solution (b) shows two clearly separated spots.

- C. Mix about 5 mg with 45 mg of *heavy magnesium oxide R* and ignite in a crucible until an almost white residue is obtained (usually less than 5 min). Allow to cool, add 1 ml of *water R*, 0.05 ml of *phenolphthalein solution RI* and about 1 ml of *dilute hydrochloric acid R* to render the solution colourless. Filter. To a freshly prepared mixture of 0.1 ml *alizarin S solution R* and 0.1 ml of *zirconyl nitrate solution R*, add 1.0 ml of the filtrate. Mix, allow to stand for 5 min and compare the colour of the solution with that of a blank prepared in the same manner. The colour of the test solution is yellow and that of the blank is red.

TESTS

Specific optical rotation (2.2.7). Dissolve 0.250 g in *dioxan R* and dilute to 25.0 ml with the same solvent. The specific optical rotation is + 127 to + 133, calculated with reference to the dried substance.

Related substances. Prepare the solutions immediately before use.

Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 50.0 mg of the substance to be examined in 5.0 ml of *ethanol R* and dilute to 50.0 ml with the mobile phase.

Reference solution (a). Dissolve 2 mg of *clobetasone butyrate* CRS and 1.5 mg of *clobetasol propionate R* in 5 ml of *ethanol R* and dilute to 100 ml with the mobile phase.

Reference solution (b). Dilute 1.0 ml of the test solution to 100.0 ml with the mobile phase.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.20 m long and 4.6 mm in internal diameter, packed with *octadecylsilyl silica gel for chromatography R* (5 µm),
 - as mobile phase at a flow rate of 1 ml/min a mixture of 45 volumes of *ethanol R* and 55 volumes of *water R*,
 - as detector a spectrophotometer set at 241 nm,
- maintaining the temperature of the column at 60 °C. Inject 20 µl of reference solution (a). When using a recorder, adjust the sensitivity of the system so that the height of the two principal peaks are at least 50 per cent of the full scale of the recorder. The test is not valid unless the resolution between the first peak (*clobetasol propionate*) and the second peak (*clobetasone butyrate*) is at least five.
- Inject 20 µl of the test solution and 20 µl of reference solution (b). Continue the chromatography for 2.5 times the retention time of the principal peak. In the chromatogram obtained with the test solution: the area of any peak, apart

from the principal peak, is not greater than the area of the principal peak in the chromatogram obtained with reference solution (b) (1.0 per cent) and not more than one such peak has an area greater than half the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent); the sum of the areas of all the peaks, apart from the principal peak, is not greater than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (1.5 per cent). Disregard any peak with an area less than 0.05 times the area of the principal peak in the chromatogram obtained with reference solution (b).

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C.

ASSAY

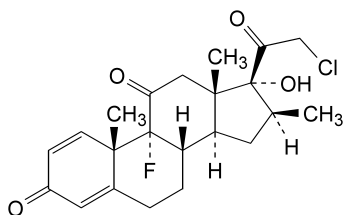
Dissolve 20.0 mg in *alcohol R* and dilute to 100.0 ml with the same solvent. Dilute 5.0 ml of the solution to 50.0 ml with *alcohol R*. Measure the absorbance (2.2.25) at the maximum at 235 nm.

Calculate the content of $C_{26}H_{32}ClFO_5$, taking the specific absorbance to be 327.

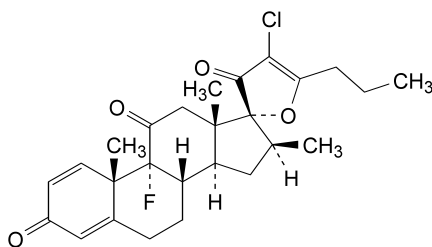
STORAGE

Store protected from light.

IMPURITIES



A. clobetasone,

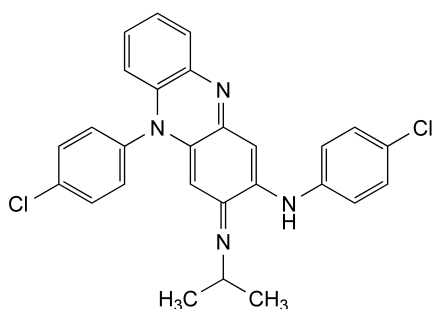


B. (17R)-4'-chloro-9-fluoro-16β-methyl-5'-propylspiro[androst-1,4-diene-17,2'(3'H)furan]-3,3',11-trione.

01/2005:2054

CLOFAZIMINE

Clofaziminum



$C_{27}H_{22}Cl_2N_4$

M_r 473.4

DEFINITION

N,5-Bis(4-chlorophenyl)-3-[(1-methylethyl)imino]-3,5-dihydrophenazin-2-amine.

Content: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

Appearance: reddish-brown, fine powder.

Solubility: practically insoluble in water, soluble in methylene chloride, very slightly soluble in ethanol (96 per cent).

It shows polymorphism.

IDENTIFICATION

First identification: A.

Second identification: B, C.

A. Infrared absorption spectrophotometry (2.2.24).

Comparison: *clofazimine CRS*.

If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in *methylene chloride R*, evaporate to dryness and record new spectra using the residues.

B. Thin-layer chromatography (2.2.27).

Test solution. Dissolve 10 mg of the substance to be examined in *methylene chloride R* and dilute to 10 ml with the same solvent.

Reference solution. Dissolve 10 mg of *clofazimine CRS* in *methylene chloride R* and dilute to 10 ml with the same solvent.

Plate: *TLC silica gel GF₂₅₄ plate R*.

Mobile phase: *propanol R*, *methylene chloride R* (6:85 V/V).

Application: 5 µl.

First development: over 2/3 of the plate.

Drying: horizontally in air for 5 min.

Second development: over 2/3 of the plate.

Drying: in air for 5 min.

Detection: examine in ultraviolet light at 254 nm.

Results: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with the reference solution.

C. Dissolve 2 mg in 3 ml of *acetone R* and add 0.1 ml of *hydrochloric acid R*. An intense violet colour is produced. Add 0.5 ml of a 200 g/l solution of *sodium hydroxide R*; the colour changes to orange-red.

TESTS

Related substances. Liquid chromatography (2.2.29).

Prepare the solutions immediately before use.

Test solution. Dissolve 50 mg of the substance to be examined in the mobile phase and dilute to 100 ml with the mobile phase.

Reference solution (a). Dilute 1.0 ml of the test solution to 100.0 ml with the mobile phase. Dilute 1.0 ml of this solution to 10.0 ml with the mobile phase.

Reference solution (b). Dissolve 5.0 mg of *clofazimine for system suitability CRS* in the mobile phase and dilute to 10.0 ml with the mobile phase.

Column:

— **size:** $l = 0.25$ m, $\varnothing = 4.6$ mm,

— **stationary phase:** *octylsilyl silica gel for chromatography R* (5 µm).

Mobile phase: dissolve 2.25 g of *sodium laurilsulfate R*, 0.85 g of *tetrabutylammonium hydrogen sulphate R* and 0.885 g of *disodium hydrogen phosphate R* in *water R*. Adjust to pH 3.0 with *dilute phosphoric acid R* and dilute to 500 ml with *water R*. Mix 35 volumes of this solution and 65 volumes of *acetonitrile R*.

Flow rate: 1 ml/min.

Detection: spectrophotometer at 280 nm.

Injection: 20 µl.

Run time: 3 times the retention time of clofazimine.

Identification of impurities: use the chromatogram supplied with *clofazimine for system suitability CRS* to identify the peak due to impurity B.

Relative retention with reference to clofazimine (retention time = about 15 min): impurity A = about 0.7; impurity B = about 0.8.

System suitability: reference solution (b):

- **resolution:** baseline separation between the peaks due to impurity B and clofazimine.

Limits:

- **impurity A:** not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent),
- **impurity B:** not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.3 per cent),
- **any other impurity:** for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent),
- **total:** not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent),
- **disregard limit:** 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

Heavy metals (2.4.8): maximum 10 ppm.

2.0 g complies with limit test C. Prepare the reference solution using 2 ml of *lead standard solution (10 ppm Pb) R*.

Loss on drying (2.2.32): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 100–105 °C.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

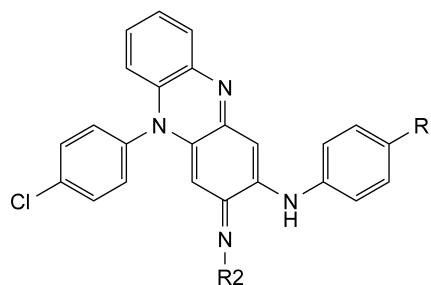
ASSAY

Dissolve 0.400 g in 5 ml of *methylene chloride R* and add 20 ml of *acetone R* and 5 ml of *anhydrous acetic acid R*. Titrate with 0.1 M *perchloric acid*, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *perchloric acid* is equivalent to 47.34 mg of $C_{27}H_{22}Cl_2N_4$.

IMPURITIES

Specified impurities: A, B.

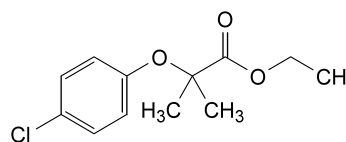


- A. R1 = Cl, R2 = H: *N*,5-bis(4-chlorophenyl)-3-imino-3,5-dihydrophenazin-2-amine,
- B. R1 = H, R2 = $CH(CH_3)_2$: 5-(4-chlorophenyl)-3-[(1-methylethyl)imino]-*N*-phenyl-3,5-dihydrophenazin-2-amine.

01/2005:0318

CLOFIBRATE

Clofibratum



$C_{12}H_{15}ClO_3$

M_r 242.7

DEFINITION

Clofibrate is ethyl 2-(4-chlorophenoxy)-2-methylpropionate.

CHARACTERS

A clear, almost colourless liquid, very slightly soluble in water, miscible with alcohol.

IDENTIFICATION

- A. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *clofibrate CRS*.
- B. Dissolve 0.10 g in *methanol R* and dilute to 100.0 ml with the same solvent. Dilute 10.0 ml of the solution to 100.0 ml with *methanol R* (solution a). Examined between 250 nm and 350 nm (2.2.25), solution (a) shows two absorption maxima, at 280 nm and at 288 nm. The specific absorbances at the maxima are about 44 and 31, respectively. Dilute 10.0 ml of solution (a) to 100.0 ml with *methanol R*. Examined between 220 nm and 250 nm, the solution shows an absorption maximum at 226 nm. The specific absorbance at the maximum is about 460.

TESTS

Refractive index (2.2.6): 1.500 to 1.505.

Relative density (2.2.5): 1.138 to 1.147.

Acidity. To 1.0 g add 10 ml of *ethanol R* and 0.1 ml of *phenol red solution R*. Not more than 1.0 ml of 0.01 M *sodium hydroxide* is required to change the colour of the indicator.

Volatile related substances. Examine by gas chromatography (2.2.28).

Test solution. To 10.0 g of the substance to be examined add a mixture of 10 ml of *dilute sodium hydroxide solution R* and 10 ml of *water R*. Shake, separate the lower (organic) layer, wash with 5 ml of *water R* and add the washings to the aqueous layer. Dry the organic layer with *anhydrous sodium sulphate R* and use as the test solution. Reserve the aqueous layer for the test for 4-chlorophenol.

Reference solution (a). Dissolve 0.12 g of the substance to be examined in *chloroform R* and dilute to 100.0 ml with the same solvent. Dilute 1.0 ml of the solution to 10.0 ml with *chloroform R*.

Reference solution (b). Dissolve 0.12 g of *methyl 2-(4-chlorophenoxy)-2-methylpropionate CRS* in the substance to be examined and dilute to 10.0 ml with the same solvent. Dilute 1.0 ml of the solution to 10.0 ml with the substance to be examined. Dilute 1.0 ml of this solution to 10.0 ml with the substance to be examined.

The chromatographic procedure may be carried out using:

- a column 1.5 m long and 4 mm in internal diameter packed with either: *silanised diatomaceous earth for gas chromatography R* (250 µm to 420 µm) impregnated with 30 per cent *m/m* of *poly(dimethyl)siloxane R*; or *silanised diatomaceous earth for gas chromatography R* (150 µm to 180 µm) impregnated with 10 per cent *m/m* of *poly(dimethyl)siloxane R*,
- *nitrogen for chromatography R* as the carrier gas,
- a flame-ionisation detector.

Maintain the temperature of the column at 185 °C. Inject 2 µl of each solution.

In the chromatogram obtained with the test solution, the sum of the areas of the peaks, except that corresponding to clofibrate, is not greater than ten times the area of the peak corresponding to clofibrate in the chromatogram obtained with reference solution (a) (0.1 per cent). In the chromatogram obtained with reference solution (b), measure from the baseline the height (*A*) of the peak corresponding to methyl 2-(4-chlorophenoxy)-2-methylpropionate and the height (*B*) of the lowest point of the curve separating this peak from the peak corresponding to clofibrate (see Figure 0318-1). The test is not valid unless *A* is equal to at least 30 per cent of the recorder-chart scale and *A* – *B* is greater than 75 per cent of *A*.

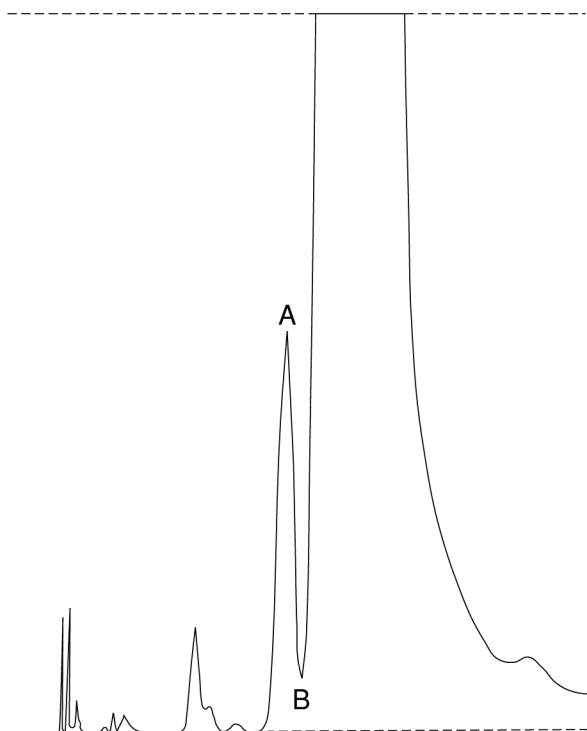


Figure 0318-1. – Typical chromatogram for the test for volatile related substances

4-Chlorophenol. Examine by gas chromatography (2.2.28).

Test solution. Shake the aqueous layer reserved in the test for volatile related substances with two quantities, each

of 5 ml, of *chloroform R* and discard the organic layers. Acidify the aqueous layer by the dropwise addition of *hydrochloric acid R*. Shake with three quantities, each of 3 ml, of *chloroform R*. Combine the organic layers and dilute to 10.0 ml with *chloroform R*.

Reference solution. Dissolve 0.25 g of *chlorophenol R* in *chloroform R* and dilute to 100.0 ml with the same solvent. Dilute 1.0 ml of the solution to 100.0 ml with *chloroform R*.

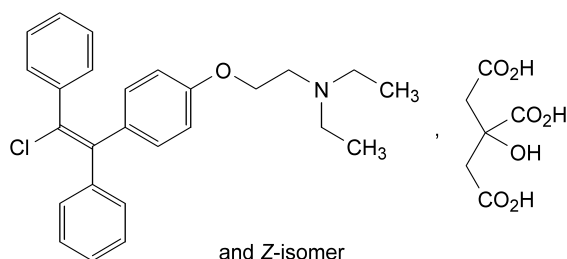
The chromatographic procedure may be carried out as described in the test for volatile related substances. Inject 2 µl of each solution.

The area of any peak corresponding to 4-chlorophenol in the chromatogram obtained with the test solution is not greater than the area of the peak corresponding to 4-chlorophenol in the chromatogram obtained with the reference solution (25 ppm).

01/2005:0997

CLOMIFENE CITRATE

Clomifeni citras



$C_{32}H_{36}ClNO_8$

M_r 598.1

DEFINITION

Clomifene citrate contains not less than 98.0 per cent and not more than the equivalent of 101.0 per cent of a mixture of the *E*- and *Z*-isomers of 2-[4-(2-chloro-1,2-diphenylethenyl)phenoxy]-*N,N*-diethylethanamine dihydrogen citrate, calculated with reference to the anhydrous substance.

CHARACTERS

A white or pale yellow, crystalline powder, slightly soluble in water, sparingly soluble in alcohol.

IDENTIFICATION

- Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *clomifene citrate CRS*. Examine the substances as discs prepared using *potassium bromide R*.
- Dissolve about 5 mg in 5 ml of a mixture of 1 volume of *acetic anhydride R* and 5 volumes of *pyridine R* and heat in a water-bath. A deep red colour is produced.

TESTS

Prepare the solutions protected from light in brown-glass vessels. Ensure minimum exposure of the solutions to daylight until they are required for chromatography.

Related substances. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 12.5 mg of the substance to be examined in the mobile phase and dilute to 10.0 ml with the mobile phase.

Reference solution (a). Dissolve 12.5 mg of *clomifene citrate for performance test CRS* in the mobile phase and dilute to 10.0 ml with the mobile phase.

Reference solution (b). Dilute 1.0 ml of the test solution to 50.0 ml with the mobile phase.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4.6 mm internal diameter packed with *butylsilyl silica gel for chromatography R* (5 µm),
- as mobile phase at a flow rate of 1.2 ml/min a mixture prepared as follows: mix 400 ml of *acetonitrile R* with 600 ml of *water R* and add 8.0 ml of *diethylamine R*; adjust the mixture to pH 6.2 by the addition of about 1 ml to 2 ml of *phosphoric acid R* taking care to reduce progressively the volume of each addition as the required pH is approached,
- as detector a spectrophotometer set at 233 nm.

Equilibrate the column with the mobile phase at a flow rate of 1.2 ml/min for about 1 h.

Adjust the sensitivity of the system so that the height of the principal peak in the chromatogram obtained with 10 µl of reference solution (b) is not less than 50 per cent of the full scale of the recorder.

Inject 10 µl of reference solution (a). Continue the chromatography for twice the retention time of the principal peak. Measure the height (*A*) above the base-line of the peak due to impurity A and the height (*B*) above the base-line of the lowest point of the curve separating this peak from the peak due to clomifene. The test is not valid unless *A* is greater than fifteen times *B* and the chromatogram obtained resembles the reference chromatogram. If necessary, adjust the concentration of acetonitrile in the mobile phase.

Inject separately 10 µl of the test solution and 10 µl of reference solution (b). Continue the chromatography for four times the retention time of the principal peak. In the chromatogram obtained with the test solution: the area of any peak due to impurity A is not greater than the area of the principal peak in the chromatogram obtained with reference solution (b) (2.0 per cent); the area of any other peak, apart from the principal peak, is not greater than half the area of the principal peak in the chromatogram obtained with reference solution (b) (1.0 per cent); the sum of the areas of all the peaks, apart from the principal peak, is not greater than 1.25 times the area of the principal peak in the chromatogram obtained with reference solution (b) (2.5 per cent). Disregard any peak with a retention time relative to the clomifene peak of 0.2 or less and any peak with an area less than 0.025 times the area of the principal peak in the chromatogram obtained with reference solution (b).

Z-isomer: 30.0 per cent to 50.0 per cent, determined by liquid chromatography (2.2.29).

Test solution. Dissolve 25 mg of the substance to be examined in 25 ml of 0.1 M *hydrochloric acid*, add 5 ml of 1 M *sodium hydroxide* and shake with three quantities, each of 25 ml, of *ethanol-free chloroform R*. Wash the combined extracts with 10 ml of *water R*, dry over *anhydrous sodium sulphate R* and dilute to 100 ml with *ethanol-free chloroform R*. To 20 ml of the solution add 0.1 ml of *triethylamine R* and dilute to 100 ml with *hexane R*.

Reference solution. Dissolve 25 mg of *clomifene citrate CRS* in 25 ml of 0.1 M *hydrochloric acid*, add 5 ml of 1 M *sodium hydroxide* and shake with three quantities, each of 25 ml, of *ethanol-free chloroform R*. Wash the combined extracts with 10 ml of *water R*, dry over *anhydrous sodium sulphate R* and dilute to 100 ml with *ethanol-free chloroform R*. To 20 ml of the solution add 0.1 ml of *triethylamine R* and dilute to 100 ml with *hexane R*.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.3 m long and 4 mm in internal diameter packed with *silica gel for chromatography R* (10 µm),
- as mobile phase at a flow rate of 2 ml/min a mixture of 1 volume of *triethylamine R*, 200 volumes of *ethanol-free chloroform R* and 800 volumes of *hexane R*,
- as detector a spectrophotometer set at 302 nm.

Equilibrate the column with the mobile phase for about 2 h. Inject 50 µl of the reference solution. The chromatogram obtained shows a peak due to the *E*-isomer just before a peak due to the *Z*-isomer. The test is not valid unless the resolution between the peaks corresponding to the *E*- and *Z*-isomers is at least 1.0. If necessary, adjust the relative proportions of ethanol-free chloroform and hexane in the mobile phase. Measure the area of the peak due to the *Z*-isomer in the chromatograms obtained with the test solution and the reference solution. Calculate the content of the *Z*-isomer as a percentage of the total clomifene citrate present using the declared content of *clomifene citrate CRS*.

Water (2.5.12). Not more than 1.0 per cent, determined on 1.000 g by the semi-micro determination of water.

ASSAY

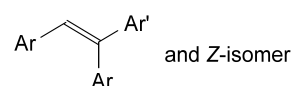
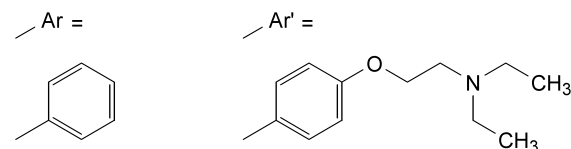
Dissolve 0.500 g in 50 ml of *anhydrous acetic acid R*. Titrate with 0.1 M *perchloric acid* determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *perchloric acid* is equivalent to 59.81 mg of C₃₂H₃₆ClNO₈.

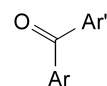
STORAGE

Store protected from light.

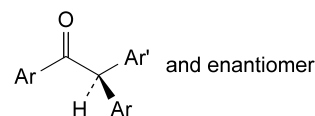
IMPURITIES



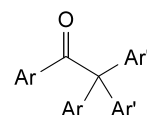
A. 2-[4-(1,2-diphenylethenyl)phenoxy]-*N,N*-diethylethanamine,



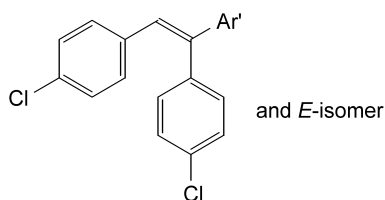
B. [4-[2-(diethylamino)ethoxy]phenyl]phenylmethanone,



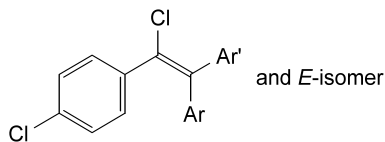
C. (2*RS*)-2-[4-[2-(diethylamino)ethoxy]phenyl]-1,2-diphenylethanone,



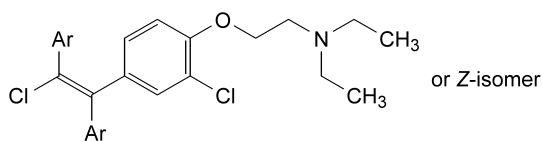
D. 2,2-bis[4-[2-(diethylamino)ethoxy]phenyl]-1,2-diphenylethanone,



E. 2-[4-[1,2-bis(4-chlorophenyl)ethenyl]phenoxy]-*N,N*-diethylethanamine,



F. 2-[4-[2-chloro-2-(4-chlorophenyl)-1-phenylethenyl]phenoxy]-*N,N*-diethylethanamine,

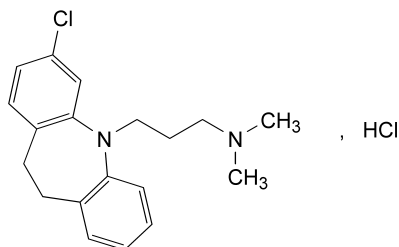


GH. 2-[2-chloro-4-(2-chloro-1,2-diphenylethenyl)phenoxy]-*N,N*-diethylethanamine (G. higher-melting point isomer; H. lower-melting point isomer).

01/2005:0889

CLOMIPRAMINE HYDROCHLORIDE

Clomipramini hydrochloridum


 $C_{19}H_{24}Cl_2N_2$
 M_r 351.3

DEFINITION

3-(3-Chloro-10,11-dihydro-5*H*-dibenzo[*b,f*]azepin-5-yl)-*N,N*-dimethylpropan-1-amine hydrochloride.

Content: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

Appearance: white or slightly yellow, crystalline powder, slightly hygroscopic.

Solubility: freely soluble in water and in methylene chloride, soluble in alcohol.

It shows polymorphism.

IDENTIFICATION

First identification: B, E.

Second identification: A, C, D, E.

A. Melting point (2.2.14): 191 °C to 195 °C.

B. Infrared absorption spectrophotometry (2.2.24).

Preparation: discs of *potassium bromide R*. The transmittance at about 2000 cm⁻¹ (5 µm) is at least 65 per cent without compensation.

Comparison: clomipramine hydrochloride CRS.

C. Thin-layer chromatography (2.2.27). Prepare the solutions immediately before use and protected from light.

Test solution. Dissolve 20 mg of the substance to be examined in *methanol R* and dilute to 10 ml with the same solvent.

Reference solution. Dissolve 20 mg of *clomipramine hydrochloride CRS* in *methanol R* and dilute to 10 ml with the same solvent.

Plate: TLC silica gel G plate R.

Mobile phase: concentrated ammonia R, acetone R, ethyl acetate R (5:25:75 V/V/V).

Application: 5 µl.

Development: over a path of 15 cm.

Drying: in air.

Detection: spray with a 5 g/l solution of *potassium dichromate R* in a 20 per cent V/V solution of *sulphuric acid R*. Examine immediately.

Results: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

D. Dissolve about 5 mg in 2 ml of *nitric acid R*. An intense blue colour develops.

E. Dissolve about 50 mg in 5 ml of *water R* and add 1 ml of *dilute ammonia R1*. Mix, allow to stand for 5 min and filter. Acidify the filtrate with *dilute nitric acid R*. The solution gives reaction (a) of chlorides (2.3.1).

TESTS

Solution S. Dissolve 2.0 g in *carbon dioxide-free water R* and dilute to 20 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and not more intensely coloured than reference solution Y₅ (2.2.2, Method D).

pH (2.2.3): 3.5 to 5.0 for solution S.

Related substances. Liquid chromatography (2.2.29).

Prepare the solutions immediately before use and protected from light.

Test solution. Dissolve 20.0 mg of the substance to be examined in a mixture of 25 volumes of mobile phase B and 75 volumes of mobile phase A and dilute to 10.0 ml with the same mixture of mobile phases.

Reference solution (a). Dissolve 22.6 mg of *imipramine hydrochloride CRS*, 4.0 mg of *clomipramine impurity C CRS*, 4.0 mg of *clomipramine impurity D CRS* and 2.0 mg of *clomipramine impurity F CRS* in a mixture of 25 volumes of mobile phase B and 75 volumes of mobile phase A and dilute to 100.0 ml with the same mixture of mobile phases. Dilute 1.0 ml of this solution to 10.0 ml with a mixture of 25 volumes of mobile phase B and 75 volumes of mobile phase A.

Reference solution (b). Dilute 1.0 ml of the test solution to 100.0 ml with a mixture of 25 volumes of mobile phase B and 75 volumes of mobile phase A.

Reference solution (c). Dissolve 10.0 mg of *clomipramine hydrochloride CRS* and 3.0 mg of *clomipramine impurity C CRS* in a mixture of 25 volumes of mobile phase B and 75 volumes of mobile phase A and dilute to 20.0 ml with the same mixture of mobile phases. Dilute 1.0 ml of this solution to 10.0 ml with a mixture of 25 volumes of mobile phase B and 75 volumes of mobile phase A.

Column:

— size: *l* = 0.25 m, Ø = 4.6 mm,

- *stationary phase*: cyanopropylsilyl silica gel for chromatography *R* (5 µm),
- *temperature*: 30 °C.

Mobile phase:

- *mobile phase A*: dissolve 1.2 g of *sodium dihydrogen phosphate R* in *water R*, add 1.1 ml of *nonylamine R*, adjust to pH 3.0 with *phosphoric acid R* and dilute to 1000 ml with *water R*,
- *mobile phase B*: *acetonitrile R*.

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)
0 - 10	75	25
10 - 20	75 → 65	25 → 35
20 - 32	65	35
32 - 34	65 → 75	35 → 25
34 - 44	75	25

Flow rate: 1.5 ml/min.

Detection: spectrophotometer at 254 nm.

Injection: 20 µl.

Relative retentions with reference to clomipramine (retention time = about 8 min): impurity A = about 0.5; impurity B = about 0.7; impurity C = about 0.9; impurity D = about 1.7; impurity E = about 2.5; impurity F = about 3.4; impurity G = about 4.3.

System suitability: reference solution (c):

- *resolution*: minimum 3.0 between the peaks due to clomipramine and to impurity C.

Limits:

- *impurity B*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (a) (1.0 per cent),
- *impurity C, D*: for each impurity, not more than the area of the corresponding peak in the chromatogram obtained with reference solution (a) (0.2 per cent),
- *impurity F*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (a) (0.1 per cent),
- *any other impurity*: not more than 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent),
- *total of other impurities*: not more than 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent),
- *total*: not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (1.0 per cent),
- *disregard limit*: 0.01 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.01 per cent).

Heavy metals (2.4.8): maximum 20 ppm.

2.0 g complies with limit test C. Prepare the standard using 4 ml of *lead standard solution* (10 ppm Pb) *R*.

Loss on drying (2.2.32): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 100-105 °C.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

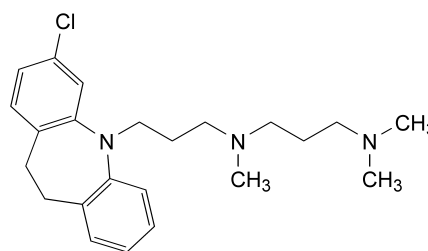
ASSAY

Dissolve 0.250 g in 50 ml of *alcohol R* and add 5.0 ml of 0.01 *M hydrochloric acid*. Carry out a potentiometric titration (2.2.20), using 0.1 *M sodium hydroxide*. Read the volume added between the 2 points of inflexion.

1 ml of 0.1 *M sodium hydroxide* is equivalent to 35.13 mg of C₁₉H₂₄Cl₂N₂.

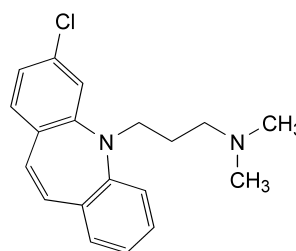
STORAGE

In an airtight container, protected from light.

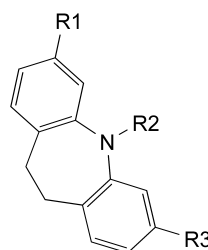
IMPURITIES

A. *N*-[3-(3-chloro-10,11-dihydro-5*H*-dibenzo[*b,f*]azepin-5-yl)propyl]-*N,N',N'*-trimethylpropane-1,3-diamine,

B. imipramine,



C. 3-(3-chloro-5*H*-dibenzo[*b,f*]azepin-5-yl)-*N,N*-dimethylpropan-1-amine,



D. R1 = R3 = Cl, R2 = CH₂-CH₂-CH₂-N(CH₃)₂: 3-(3,7-dichloro-10,11-dihydro-5*H*-dibenzo[*b,f*]azepin-5-yl)-*N,N*-dimethylpropan-1-amine,

E. R1 = R2 = R3 = H: 10,11-dihydro-5*H*-dibenzo[*b,f*]azepine (iminodibenzyl),

F. R1 = Cl, R2 = R3 = H: 3-chloro-10,11-dihydro-5*H*-dibenzo[*b,f*]azepine,

G. R1 = Cl, R2 = CH₂-CH=CH₂, R3 = H: 3-chloro-5-(prop-2-enyl)-10,11-dihydro-5*H*-dibenzo[*b,f*]azepine.

01/2005:0890 *Detection*: spectrophotometer at 254 nm.*Injection*: 10 µl.*Run time*: 3 times the retention time of clonazepam.*Relative retention* with reference to clonazepam (retention time = about 7 min): impurity B = about 2.1; impurity A = about 2.4.*System suitability*: reference solution (b):

- *resolution*: minimum 1.8 between the peaks due to flunitrazepam and to clonazepam.

Limits:

- *impurity A*: not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent),
- *impurity B*: not more than the area of the principal peak in the chromatogram obtained with reference solution (c) (0.1 per cent)
- *any other impurity*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent),
- *total*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent),
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

Loss on drying (2.2.32): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 100-105 °C for 4 h.**Sulphated ash** (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

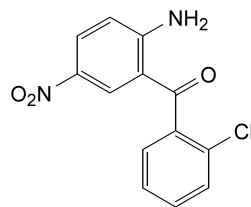
ASSAY

Dissolve 0.275 g in 50 ml of *acetic anhydride R*. Titrate with 0.1 M *perchloric acid*, determining the end-point potentiometrically (2.2.20).1 ml of 0.1 M *perchloric acid* is equivalent to 31.57 mg of C₁₅H₁₀ClN₃O₃.

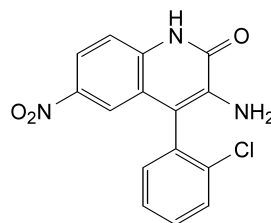
STORAGE

Protected from light.

IMPURITIES

Specified impurities: A, B.

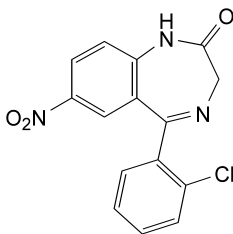
A. (2-amino-5-nitrophenyl)(2-chlorophenyl)methanone,



B. 3-amino-4-(2-chlorophenyl)-6-nitroquinolin-2(1H)-one.

CLONAZEPAM

Clonazepamum

C₁₅H₁₀ClN₃O₃*M_r* 315.7

DEFINITION

5-(2-Chlorophenyl)-7-nitro-1,3-dihydro-2H-1,4-benzodiazepin-2-one.

Content: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

Appearance: slightly yellowish, crystalline powder.*Solubility*: practically insoluble in water, slightly soluble in alcohol and in methanol.

mp: about 239 °C.

IDENTIFICATION

Infrared absorption spectrophotometry (2.2.24).

Comparison: *Ph. Eur. reference spectrum of clonazepam*.

TESTS

Related substances. Liquid chromatography (2.2.29). Carry out the test protected from light and prepare the solutions immediately before use.*Solvent mixture*: tetrahydrofuran *R*, methanol *R*, water *R* (10:42:48 V/V/V).*Test solution*. Dissolve 0.100 g of the substance to be examined in methanol *R* and dilute to 20.0 ml with the same solvent. Dilute 1.0 ml to 10.0 ml with the solvent mixture.*Reference solution (a)*. Dilute 1.0 ml of the test solution to 100.0 ml with the solvent mixture. Dilute 1.0 ml of the solution to 10.0 ml with the solvent mixture.*Reference solution (b)*. Dissolve 5 mg of the substance to be examined and 5 mg of flunitrazepam *R* in the solvent mixture and dilute to 100.0 ml with the solvent mixture.*Reference solution (c)*. Dissolve 1.0 mg of clonazepam impurity B CRS in the solvent mixture and dilute to 20.0 ml with the solvent mixture. Dilute 1.0 ml of the solution to 100.0 ml with the solvent mixture.*Column*:

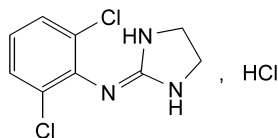
- *size*: *l* = 0.15 m, Ø = 4.6 mm,
- *stationary phase*: end-capped octylsilyl silica gel for chromatography *R* (5 µm).

Mobile phase: mix 10 volumes of tetrahydrofuran *R*, 42 volumes of methanol *R* and 48 volumes of a 6.6 g/l solution of ammonium phosphate *R* previously adjusted to pH 8.0 with a 40 g/l solution of sodium hydroxide *R* or dilute phosphoric acid *R*.*Flow rate*: 1.0 ml/min.

01/2005:0477

CLONIDINE HYDROCHLORIDE

Clonidini hydrochloridum

 $C_9H_{10}Cl_3N_3$ M_r 266.6**DEFINITION**

Clonidine hydrochloride contains not less than 98.5 per cent and not more than the equivalent of 101.0 per cent of 2,6-dichloro-*N*-(imidazolidin-2-ylidene)aniline, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, crystalline powder, soluble in water and in ethanol.

IDENTIFICATION

First identification: B, D.

Second identification: A, C, D.

- A. Dissolve 30.0 mg in 0.01 *M* hydrochloric acid and dilute to 100.0 ml with the same acid. Examined between 245 nm and 350 nm (2.2.25), the solution shows two absorption maxima, at 272 nm and at 279 nm, and a point of inflexion at 265 nm. The specific absorbances at the maxima are about 18 and about 16, respectively.
- B. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with clonidine hydrochloride CRS.
- C. Examine the chromatograms obtained in the test for related substances. The principal spot in the chromatogram obtained with test solution (b) is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).
- D. It gives reaction (a) of chlorides (2.3.1).

TESTS

Solution S. Dissolve 1.25 g in carbon dioxide-free water *R* and dilute to 25 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and not more intensely coloured than reference solution Y_7 (2.2.2, Method II).

pH (2.2.3). The pH of solution S is 4.0 to 5.0.

Related substances. Examine by thin-layer chromatography (2.2.27), using silica gel *G R* as the coating substance.

Test solution (a). Dissolve 0.1 g of the substance to be examined in methanol *R* and dilute to 10 ml with the same solvent.

Test solution (b). Dilute 1 ml of test solution (a) to 10 ml with methanol *R*.

Reference solution (a). Dissolve 10 mg of clonidine hydrochloride CRS in methanol *R* and dilute to 10 ml with the same solvent.

Reference solution (b). Dilute 1 ml of test solution (a) to 10 ml with methanol *R*. Dilute 5 ml of this solution to 100 ml with methanol *R*.

Apply to the plate 10 µl of each solution. Shake a mixture of 10 volumes of glacial acetic acid *R*, 40 volumes of butanol *R* and 50 volumes of water *R*. Allow to separate. Filter the upper layer and use the filtrate as the mobile phase. Develop

over a path of 15 cm. Allow the plate to dry in air and spray with potassium iodobismuthate solution *R2*. Allow the plate to dry in air for 1 h, spray again with potassium iodobismuthate solution *R2* and then immediately spray with a 50 g/l solution of sodium nitrite *R*. Any spot in the chromatogram obtained with test solution (a), apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.5 per cent).

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C.

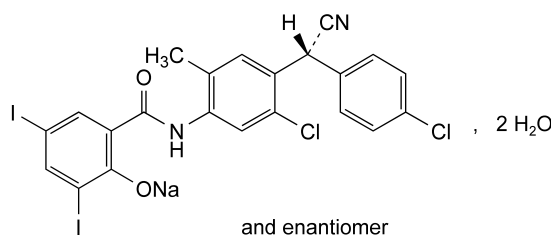
Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.200 g in 70 ml of alcohol *R*. Titrate with 0.1 *M* ethanolic sodium hydroxide determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 *M* sodium hydroxide is equivalent to 26.66 mg of $C_9H_{10}Cl_3N_3$.

01/2005:1716

CLOSADEL SODIUM DIHYDRATE FOR VETERINARY USEClosantelum natricum dihydricum
ad usum veterinarium $C_{22}H_{13}Cl_2I_2N_2NaO_2 \cdot 2H_2O$ M_r 721**DEFINITION**

N-[5-Chloro-4-[(*RS*)-(4-chlorophenyl)cyanomethyl]-2-methylphenyl]-2-hydroxy-3,5-diiodobenzamide sodium salt dihydrate.

Content: 98.5 per cent to 101.5 per cent (anhydrous substance).

CHARACTERS

Appearance: yellow powder, slightly hygroscopic.

Solubility: very slightly soluble in water, freely soluble in ethanol (96 per cent), soluble in methanol.

It shows polymorphism.

IDENTIFICATION

Infrared absorption spectrophotometry (2.2.24).

Preparation: discs without recrystallisation.

Comparison: closantel sodium dihydrate CRS.

TESTS

Appearance of solution. The solution is clear (2.2.1) and not more intensely coloured than reference solution GY_4 (2.2.2, Method II).

Dissolve 0.50 g in ethanol (96 per cent) *R* and dilute to 50 ml with the same solvent.

Related substances. Liquid chromatography (2.2.29). Prepare the solutions immediately before use and protect from light.

Test solution. Dissolve 0.100 g of the substance to be examined in *methanol R* and dilute to 10.0 ml with the same solvent.

Reference solution (a). Dissolve 10 mg of *closantel for system suitability CRS* (containing impurities A to J) in *methanol R* and dilute to 1.0 ml with the same solvent.

Reference solution (b). Dilute 1.0 ml of the test solution to 100.0 ml with *methanol R*. Dilute 5.0 ml of this solution to 25.0 ml with *methanol R*.

Column:

- size: $l = 0.10$ m, $\varnothing = 4.6$ mm,
- stationary phase: base-deactivated octadecylsilyl silica gel for chromatography R (3 μ m),
- temperature: 35 °C.

Mobile phase:

- mobile phase A: to 100 ml of a 7.7 g/l solution of ammonium acetate R previously adjusted to pH 4.3 with acetic acid R, add 50 ml of acetonitrile R and 850 ml of water R,
- mobile phase B: to 100 ml of a 7.7 g/l solution of ammonium acetate R previously adjusted to pH 4.3 with acetic acid R, add 50 ml of water R and 850 ml of acetonitrile R,

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)
0 - 2	50	50
2 - 22	50 → 20	50 → 80
22 - 27	20	80
27 - 28	20 → 50	80 → 50
28 - 32	50	50

Flow rate: 1.5 ml/min.

Detection: spectrophotometer at 240 nm.

Injection: 10 μ l.

Relative retention with reference to closantel (retention time = about 16 min): impurity A = about 0.07; impurity B = about 0.48; impurity C = about 0.62; impurity D = about 0.65; impurity E = about 0.82; impurity F = about 0.89; impurity G = about 0.93; impurity H = about 1.13; impurity I = about 1.16; impurity J = about 1.55.

System suitability: reference solution (a):

- resolution: baseline separation between the peaks due to impurity G and closantel,
- the chromatogram obtained is similar to the chromatogram supplied with *closantel for system suitability CRS*.

Limits:

- correction factors: for the calculation of contents, multiply the peak areas of the following impurities by the corresponding correction factor: impurity A = 1.5; impurity B = 1.3;
- impurity G: not more than 2.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent);
- impurities F, H, I: for each impurity, not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.3 per cent);
- impurities A, B, C, D, E, J: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent);
- any other impurity: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent);
- total: not more than 7.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (1.5 per cent);
- disregard limit: 0.25 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

Water (2.5.12): 4.8 per cent to 5.8 per cent, determined on 0.250 g.

Use a mixture of 1 volume of *dimethylformamide R* and 4 volumes of *methanol R* as the solvent.

ASSAY

Dissolve 0.500 g in 50 ml of a mixture of 1 volume of *anhydrous acetic acid R* and 7 volumes of *methyl ethyl ketone R*. Titrate with 0.1 M *perchloric acid*, determining the end-point potentiometrically (2.2.20).

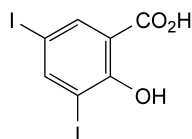
1 ml of 0.1 M *perchloric acid* is equivalent to 68.5 mg of $C_{22}H_{13}Cl_2I_2N_2NaO_2$.

STORAGE

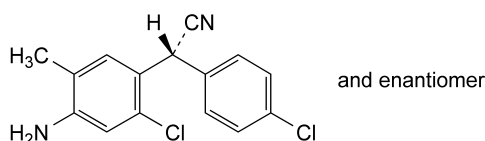
In an airtight container, protected from light.

IMPURITIES

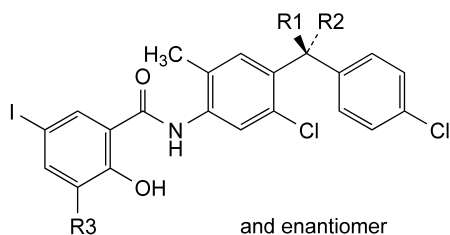
Specified impurities: A, B, C, D, E, F, G, H, I, J.



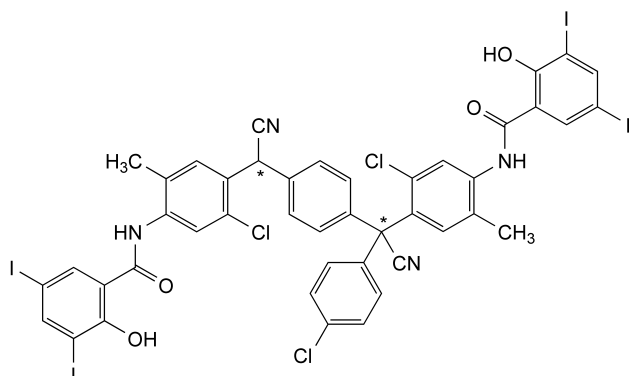
A. 2-hydroxy-3,5-diiodobenzoic acid,



B. (2RS)-(4-amino-2-chloro-5-methylphenyl)(4-chlorophenyl)ethanenitrile,



- C. R1 = H, R2 = CO₂H, R3 = I: (2*RS*)-[2-chloro-4-[(2-hydroxy-3,5-diiodobenzoyl)amino]-5-methylphenyl](4-chlorophenyl)acetic acid,
- D. R1 = H, R2 = CONH₂, R3 = I: *N*-[4-[(1*RS*)-2-amino-1-(4-chlorophenyl)-2-oxoethyl]-5-chloro-2-methylphenyl]-2-hydroxy-3,5-diiodobenzamide,
- E. R1 = H, R2 = CN, R3 = Cl: 3-chloro-*N*-[5-chloro-4-[(*RS*)-(4-chlorophenyl)cyanomethyl]-2-methylphenyl]-2-hydroxy-5-iodobenzamide,
- F. R1 + R2 = O, R3 = I: *N*-[5-chloro-4-(4-chlorobenzoyl)-2-methylphenyl]-2-hydroxy-3,5-diiodobenzamide,
- G. R1 = H, R2 = C(=NH)OCH₃, R3 = I: methyl (2*RS*)-2-[2-chloro-4-[(2-hydroxy-3,5-diiodobenzoyl)amino]-5-methylphenyl]-2-(4-chlorophenyl)acetimidate,
- H. R1 = H, R2 = CO-OCH₃, R3 = I: methyl (2*RS*)-[2-chloro-4-[(2-hydroxy-3,5-diiodobenzoyl)amino]-5-methylphenyl](4-chlorophenyl)acetate,
- I. R1 = R3 = H, R2 = CN: *N*-[5-chloro-4-[(*RS*)-(4-chlorophenyl)cyanomethyl]-2-methylphenyl]-2-hydroxy-5-iodobenzamide,

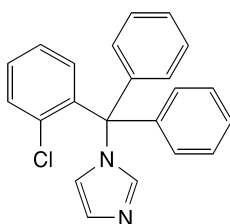


- J. *N*-[5-chloro-4-[[4-[[2-chloro-4-[(2-hydroxy-3,5-diiodobenzoyl)amino]-5-methylphenyl]cyanomethyl]phenyl](4-chlorophenyl)cyanomethyl]-2-methylphenyl]-2-hydroxy-3,5-diiodobenzamide.

01/2005:0757

CLOTRIMAZOLE

Clotrimazolum

C₂₂H₁₇ClN₂M_r 344.8

DEFINITION

Clotrimazole contains not less than 98.5 per cent and not more than the equivalent of 100.5 per cent of 1-[(2-chlorophenyl)diphenylmethyl]-1*H*-imidazole, calculated with reference to the dried substance.

CHARACTERS

A white or pale yellow, crystalline powder, practically insoluble in water, soluble in alcohol and in methylene chloride.

IDENTIFICATION

First identification: B.

Second identification: A, C, D.

- A. Melting point (2.2.14): 141 °C to 145 °C.
- B. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *clotrimazole CRS*.
- C. Examine before spraying in ultraviolet light at 254 nm, the chromatograms obtained in the test for (2-chlorophenyl)diphenylmethanol. The principal spot in the chromatogram obtained with test solution (b) is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a).
- D. Dissolve about 10 mg in 3 ml of *sulphuric acid R*. The solution is pale yellow. Add 10 mg of *mercuric oxide R* and 20 mg of *sodium nitrite R*. Allow to stand with occasional shaking. An orange colour develops, becoming orange-brown.

TESTS

Appearance of solution. Dissolve 1.25 g in *alcohol R* and dilute to 25 ml with the same solvent. The solution is clear (2.2.1) and not more intensely coloured than reference solution BY₆ (2.2.2, *Method II*).

(2-Chlorophenyl)diphenylmethanol. Examine by thin-layer chromatography (2.2.27), using *silica gel GF₂₅₄ R* as the coating substance.

Test solution (a). Dissolve 0.50 g of the substance to be examined in *alcohol R* and dilute to 5 ml with the same solvent.

Test solution (b). Dilute 1 ml of test solution (a) to 10 ml with *alcohol R*.

Reference solution (a). Dissolve 50 mg of *clotrimazole CRS* in *alcohol R* and dilute to 5 ml with the same solvent.

Reference solution (b). Dissolve 10 mg of (2-chlorophenyl)diphenylmethanol *CRS* in *alcohol R* and dilute to 5 ml with the same solvent. Dilute 1 ml of the solution to 10 ml with *alcohol R*.

Apply separately to the plate 10 µl of each solution. Develop over a path of 15 cm using a mixture of 0.5 volumes of *concentrated ammonia R1*, 10 volumes of *propanol R* and 90 volumes of *toluene R*. Allow the plate to dry in air. Spray with a 10 per cent *V/V* solution of *sulphuric acid R* in *alcohol R* and heat at 100 °C to 105 °C for 30 min. Any spot corresponding to (2-chlorophenyl)diphenylmethanol in the chromatogram obtained with test solution (a) is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.2 per cent).

Imidazole. Examine by thin-layer chromatography (2.2.27), using *silica gel G R* as the coating substance.

Test solution. Dissolve 0.50 g of the substance to be examined in *alcohol R* and dilute to 10 ml with the same solvent.

Reference solution. Dissolve 10 mg of *imidazole R* in *alcohol R* and dilute to 10 ml with the same solvent. Dilute 1 ml of the solution to 10 ml with *alcohol R*.

Apply separately to the plate 10 µl of each solution. Develop over a path of 15 cm using a mixture of 0.5 volumes of *concentrated ammonia R1*, 10 volumes of *propanol R* and 90 volumes of *toluene R*. Allow the plate to dry in air. At the bottom of a chromatography tank, place an evaporating dish containing a mixture of 1 volume of *hydrochloric acid R1*, 1 volume of *water R* and 2 volumes of a 15 g/l solution of *potassium permanganate R*, close the tank and allow to stand for 15 min. Place the dried plate in the tank and close the tank. Leave the plate in contact with the chlorine vapour for 5 min. Withdraw the plate and place it in a current of cold air until the excess of chlorine is removed and an area of coating below the points of application does not give a blue colour with a drop of *potassium iodide and starch solution R*. Spray with *potassium iodide and starch solution R*. Any spot corresponding to imidazole in the chromatogram obtained with the test solution is not more intense than the spot in the chromatogram obtained with the reference solution (0.2 per cent).

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.300 g in 80 ml of *anhydrous acetic acid R*. Titrate with 0.1 M *perchloric acid* using 0.3 ml of *naphtholbenzein solution R* as indicator until the colour changes from brownish-yellow to green.

1 ml of 0.1 M *perchloric acid* is equivalent to 34.48 mg of $C_{22}H_{17}ClN_2$.

STORAGE

Store protected from light.

upper part of the hypanthium. The head is globular and dome-shaped, composed of four imbricated petals that enclose numerous incurved stamens and a short, erect style with a nectary disc at the base. The hypanthium exudes essential oil when indented with the finger-nail.

B. Reduce to a powder (355). The powder is dark brown and has the odour and taste of the unground drug. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters: fragments of the hypanthium showing the epidermis and underlying parenchyma containing large oil glands; short fibres occurring singly or in small groups, with thickened, lignified walls and few pits; abundant fragments of parenchyma containing cluster crystals of calcium oxalate; numerous triangular pollen grains about 15 µm in diameter with three pores in the angles. Starch granules are absent.

C. Examine by thin-layer chromatography (2.2.27), using *silica gel GF₂₅₄ R* as the coating substance.

Test solution. Shake 0.1 g of the powdered drug (500) with 2 ml of *methylene chloride R* for 15 min. Filter and carefully evaporate the filtrate to dryness on a water-bath. Dissolve the residue in 2 ml of *toluene R*.

Reference solution. Dissolve 20 µl of *eugenol R* in 2 ml of *toluene R*.

Apply to the plate as bands 20 mm by 3 mm, 10 µl of the reference solution and 20 µl of the test solution. Develop in an unsaturated tank over a path of 10 cm using *toluene R*. Allow the plate to stand for 5 min and develop again in the same manner. Allow the plate to dry in air, examine in ultraviolet light at 254 nm and mark the quenching zones. In the chromatogram obtained with the test solution there is in the median part a quenching zone (eugenol) similar in position to the quenching zone in the chromatogram obtained with the reference solution and there may be a weak quenching zone (acetyleugenol) just below the zone corresponding to eugenol. Spray the plate with *anisaldehyde solution R* using 10 ml for a plate 200 mm square and examine in daylight while heating at 100 °C to 105 °C for 5 min to 10 min. The zones corresponding to eugenol in the chromatograms obtained with the test and reference solutions have a strong brownish-violet colour and the zone corresponding to acetyleugenol in the chromatogram obtained with the test solution is faint violet-blue. In the chromatogram obtained with the test solution there are other coloured zones, particularly a faint red zone in the lower part and a reddish-violet zone (caryophyllene) in the upper part.

01/2005:0376

CLOVE

Caryophylli flos

DEFINITION

Clove consists of the whole flower buds of *Syzygium aromaticum* (L.) Merril et L. M. Perry (*Eugenia caryophyllus* (C. Spreng.) Bull. et Harr.) dried until they become reddish-brown. It contains not less than 150 ml/kg of essential oil.

CHARACTERS

Clove has a characteristic, aromatic odour.

It has the macroscopic and microscopic characters described under identification tests A and B.

IDENTIFICATION

A. The flower bud is reddish-brown and consists of a quadrangular stalked portion, the hypanthium, 10 mm to 12 mm long and 2 mm to 3 mm in diameter, surmounted by four divergent lobes of sepals which surround a globular head 4 mm to 6 mm in diameter. A bilocular ovary containing numerous ovules is situated in the

TESTS

Foreign matter (2.8.2). Not more than 6 per cent of peduncles, petioles and fruits, not more than 2 per cent of deteriorated cloves and not more than 0.5 per cent of other foreign matter.

Total ash (2.4.16). Not more than 7.0 per cent.

ASSAY

Carry out the determination of essential oils in vegetable drugs (2.8.12). Use a 250 ml flask, 100 ml of *water R* as the distillation liquid and 0.50 ml of *xylene R* in the graduated tube. Grind 5.0 g of the drug with 5.0 g of *diatomaceous earth R* to form a fine, homogeneous powder and proceed immediately with the determination using 4.0 g of the mixture. Distil at a rate of 2.5 ml/min to 3.5 ml/min for 2 h.

STORAGE

Store protected from light.

01/2005:1091 **Chromatographic profile.** Examine by gas chromatography (2.2.28).

CLOVE OIL

Caryophylli floris aetheroleum

DEFINITION

Clove oil is obtained by steam distillation from the dried flower buds of *Syzygium aromaticum* (L.) Merrill et L. M. Perry (*Eugenia caryophyllus* C. Spreng. Bull. et Harr.).

CHARACTERS

A clear, yellow liquid which becomes brown when exposed to air, miscible with methylene chloride, toluene and fatty oils.

IDENTIFICATION

First identification: B.

Second identification: A.

- A. Examine by thin-layer chromatography (2.2.27) using a suitable silica gel with a fluorescent indicator having an optimal intensity at 254 nm as the coating substance.

Test solution. Dissolve 20 µl of the substance to be examined in 2.0 ml of *toluene R*.

Reference solution. Dissolve 15 µl of *eugenol R* and 15 µl of *acetyleneugenol R* in 2.0 ml of *toluene R*.

Apply separately to the plate, as bands, 20 µl of the test solution and 15 µl of the reference solution. Develop in an unsaturated tank over a path of 10 cm using *toluene R*. Allow the plate to stand for 5 min and develop again in the same manner. Allow the plate to dry in air, examine in ultraviolet light at 254 nm and mark the quenching zones. The chromatogram obtained with the test solution shows in the medium part a quenching zone (eugenol) which corresponds in position to the quenching zone in the chromatogram obtained with the reference solution; there is a weak quenching zone (acetyleneugenol) just below the quenching zone of eugenol which corresponds in position to the zone of acetyleneugenol in the chromatogram obtained with the reference solution. Spray the plate with *anisaldehyde solution R* and examine in daylight while heating at 100 °C to 105 °C for 5 to 10 min. The zones corresponding to eugenol in the chromatogram obtained with the test and reference solutions have a strong brownish-violet colour and the zone corresponding to acetyleneugenol in the chromatogram obtained with the test solution is faint violet-blue. In the chromatogram obtained with the test solution there are other coloured zones particularly a faint red zone in the lower part and a reddish-violet zone (β-caryophyllene) in the upper part.

- B. Examine the chromatograms obtained in the test for chromatographic profile. The chromatogram obtained with the test solution shows three main peaks similar in retention time to the three peaks obtained with the reference solution.

TESTS

Relative density (2.2.5): 1.030 to 1.063.

Refractive index (2.2.6): 1.528 to 1.537.

Angle of optical rotation (2.2.7): 0° to -2°.

Fatty oils and resinified essential oils (2.8.7). It complies with the test for fatty oils and resinified oils.

Solubility in alcohol (2.8.10). 1.0 ml is soluble in 2.0 ml or more of *alcohol* (70 per cent V/V) *R*.

Test solution. Dissolve 0.2 g of the substance to be examined in 10 g of *hexane R*.

Reference solution. Dissolve 7 mg of β-caryophyllene *R*, 80 mg of *eugenol R* and 4 mg of *acetyleneugenol R* in 10 g of *hexane R*.

The chromatographic procedure may be carried out using:

- a fused silica capillary column 60 m long and about 0.25 mm in internal diameter, coated with *macrogol* 20 000 *R*,
- *helium for chromatography R* as the carrier gas at a flow rate of 1.5 ml per min,
- a flame-ionisation detector,
- a split ratio of 1/100,

maintaining the temperature of the column at 60 °C for 8 min, then raising the temperature at a rate of 3 °C per min to 180 °C and maintaining at 180 °C for 5 min and maintaining the temperature of the injection port and that of the detector at 270 °C.

Inject about 1.0 µl of the reference solution. Identify the eluted components following the order indicated in the composition of the reference solution. Record the retention times of these substances.

The test is not valid unless: the number of theoretical plates is at least 30 000, calculated from the β-caryophyllene peak at 110 °C and the resolution between the peak corresponding to eugenol and that corresponding to acetyleneugenol is at least 1.5.

Inject about 1.0 µl of the substance to be examined. Using the retention times determined from the chromatogram obtained with the reference solution, locate the components of the reference solution on the chromatogram obtained with the test solution. Disregard the peak due to the solvent.

Determine the percentage content of each of the three components by the normalisation procedure. The contents are within the following ranges:

- β-caryophyllene: 5.0 per cent to 14.0 per cent,
- *eugenol*: 75.0 per cent to 88.0 per cent,
- *acetyleneugenol*: 4.0 per cent to 15.0 per cent.

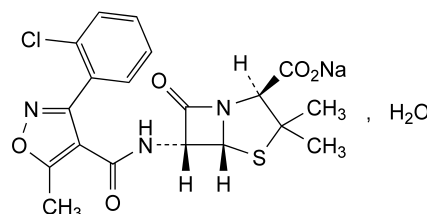
STORAGE

Store in a well-filled, airtight container, protected from light and heat.

01/2005:0661

CLOXACILLIN SODIUM

Cloxacillinum natricum



C₁₉H₁₇ClN₃NaO₅S·H₂O

*M*_r 475.9

DEFINITION

Cloxacillin sodium contains not less than 95.0 per cent and not more than the equivalent of 101.0 per cent of sodium (2*S*,5*R*,6*R*)-6-[[[3-(2-chlorophenyl)-5-oxo-2,3-dihydro-4H-1,2,4-oxadiazol-4-yl]acetyl]amino]-3,3-dimethyl-2-thioxo-1,2,3,4-tetrahydro-1H-2H-pyridine-2-carboxylate sodium salt.

methylisoxazol-4-yl]carbonyl]amino]-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylate, calculated with reference to the anhydrous substance.

CHARACTERS

A white or almost white, crystalline powder, hygroscopic, freely soluble in water and in methanol, soluble in alcohol.

IDENTIFICATION

First identification: A, D.

Second identification: B, C, D.

- A. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *cloxacillin sodium CRS*. Examine the substances prepared as discs.
- B. Examine by thin-layer chromatography (2.2.27), using *silanised silica gel H R* as the coating substance.
Test solution. Dissolve 25 mg of the substance to be examined in 5 ml of *water R*.
Reference solution (a). Dissolve 25 mg of *cloxacillin sodium CRS* in 5 ml of *water R*.
Reference solution (b). Dissolve 25 mg of each of *cloxacillin sodium CRS*, *dicloxacillin sodium CRS* and *flucloxacillin sodium CRS* in 5 ml of *water R*.
Apply separately to the plate 1 µl of each solution. Develop over a path of 15 cm using a mixture of 30 volumes of *acetone R* and 70 volumes of a 154 g/l solution of *ammonium acetate R*, adjusted to pH 5.0 with *glacial acetic acid R*. Allow the plate to dry in air and expose it to iodine vapour until the spots appear. Examine in daylight. The principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a). The test is not valid unless the chromatogram obtained with reference solution (b) shows three clearly separated spots.
- C. Place about 2 mg in a test-tube about 150 mm long and 15 mm in diameter. Moisten with 0.05 ml of *water R* and add 2 ml of *sulphuric acid-formaldehyde reagent R*. Mix the contents of the tube by swirling; the colour of the solution is slightly greenish-yellow. Place the test-tube in a water-bath for 1 min; the solution becomes yellow.
- D. It gives reaction (a) of sodium (2.3.1).

TESTS

Solution S. Dissolve 2.50 g in *carbon dioxide-free water R* and dilute to 25.0 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1). The absorbance (2.2.25) of solution S measured at 430 nm is not greater than 0.04.

pH (2.2.3). The pH of solution S is 5.0 to 7.0.

Specific optical rotation (2.2.7). Dissolve 0.250 g in *water R* and dilute to 25.0 ml with the same solvent. The specific optical rotation is + 160 to + 169, calculated with reference to the anhydrous substance.

Related substances. Examine by liquid chromatography (2.2.29) as prescribed under Assay. Inject test solution (a) and continue the chromatography for five times the retention time of the principal peak. Inject reference solution (b). In the chromatogram obtained with test solution (a): the area of any peak, apart from the principal peak, is not greater than the area of the principal peak in the chromatogram obtained with reference solution (b) (1 per cent); the sum of

the areas of all the peaks, apart from the principal peak, is not greater than five times the area of the principal peak in the chromatogram obtained with reference solution (b) (5 per cent). Disregard any peak with an area less than 0.05 times the area of the principal peak in the chromatogram obtained with reference solution (b).

***N,N*-Dimethylaniline** (2.4.26, *Method B*). Not more than 20 ppm.

2-Ethylhexanoic acid (2.4.28). Not more than 0.8 per cent *m/m*.

Water (2.5.12): 3.0 per cent to 4.5 per cent, determined on 0.300 g by the semi-micro determination of water.

Bacterial endotoxins (2.6.14): less than 0.40 IU/mg, if intended for use in the manufacture of parenteral dosage forms without a further appropriate procedure for the removal of bacterial endotoxins.

ASSAY

Examine by liquid chromatography (2.2.29).

Test solution (a). Dissolve 50.0 mg of the substance to be examined in the mobile phase and dilute to 50.0 ml with the mobile phase.

Test solution (b). Dilute 5.0 ml of test solution (a) to 50.0 ml with the mobile phase.

Reference solution (a). Dissolve 50.0 mg of *cloxacillin sodium CRS* in the mobile phase and dilute to 50.0 ml with the mobile phase. Dilute 5.0 ml of the solution to 50.0 ml with the mobile phase.

Reference solution (b). Dilute 5.0 ml of test solution (b) to 50.0 ml with the mobile phase.

Reference solution (c). Dissolve 5 mg of *flucloxacillin sodium CRS* and 5 mg of *cloxacillin sodium CRS* in the mobile phase and dilute to 50.0 ml with the mobile phase.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (5 µm),
- as mobile phase at a flow rate of 1.0 ml/min a mixture of 25 volumes of *acetonitrile R* and 75 volumes of a 2.7 g/l solution of *potassium dihydrogen phosphate R* adjusted to pH 5.0 with *dilute sodium hydroxide solution R*,
- as detector a spectrophotometer set at 225 nm,
- a 20 µl loop injector.

Inject reference solution (c). Adjust the sensitivity of the system so that the heights of the principal peaks in the chromatogram obtained are at least 50 per cent of the full scale of the recorder. The test is not valid unless the resolution between the first peak (cloxacillin) and the second peak (flucloxacillin) is at least 2.5. Inject reference solution (a) six times. The test is not valid unless the relative standard deviation of the peak area for cloxacillin is at most 1.0 per cent. Inject alternately test solution (b) and reference solution (a).

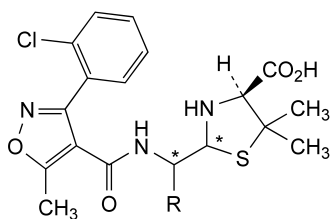
STORAGE

Store in an airtight container, at a temperature not exceeding 25 °C. If the substance is sterile, store in a sterile, airtight, tamper-proof container.

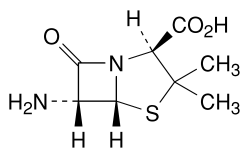
LABELLING

The label states, where applicable, that the substance is free from bacterial endotoxins.

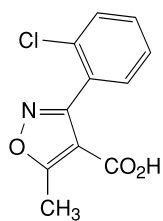
IMPURITIES



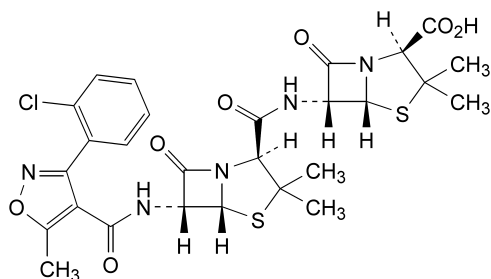
- A. R = CO₂H: (4*S*)-2-[carboxy[[[3-(2-chlorophenyl)-5-methylisoxazol-4-yl]carbonyl]amino]methyl]-5,5-dimethylthiazolidine-4-carboxylic acid (penicilloic acids of cloxacillin),
- B. R = H: (2*RS*,4*S*)-2-[[[3-(2-chlorophenyl)-5-methylisoxazol-4-yl]carbonyl]amino]methyl]-5,5-dimethylthiazolidine-4-carboxylic acid (penilloic acids of cloxacillin),



- C. (2*S*,5*R*,6*R*)-6-amino-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid (6-aminopenicillanic acid),



- D. 3-(2-chlorophenyl)-5-methylisoxazole-4-carboxylic acid,

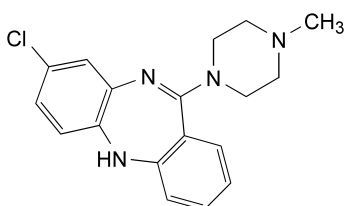


- E. (2*S*,5*R*,6*R*)-6-[[[(2*S*,5*R*,6*R*)-6-[[[3-(2-chlorophenyl)-5-methylisoxazol-4-yl]carbonyl]amino]-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carbonyl]amino]-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid (6-APA cloxacillin amide).

01/2005:1191

CLOZAPINE

Clozapinum

C₁₈H₁₉ClN₄M_r 326.8

DEFINITION

Clozapine contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of 8-chloro-11-(4-methylpiperazin-1-yl)-5*H*-dibenzo[*b,e*][1,4]diazepine, calculated with reference to the dried substance.

CHARACTERS

A yellow, crystalline powder, practically insoluble in water, freely soluble in methylene chloride, soluble in alcohol. It dissolves in dilute acetic acid.

IDENTIFICATION

- A. Melting point (2.2.14): 182 °C to 186 °C.
- B. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *clozapine CRS*. Examine the substances prepared as discs.

TESTS

Related substances. Examine by thin-layer chromatography (2.2.27), using as the coating substance a suitable silica gel with a fluorescent indicator having an optimal intensity at 254 nm. Before use, develop the plate with a mixture of 25 volumes of *methanol R* and 75 volumes of *methylene chloride R*. Allow the plate to dry in air for 15 min.

Test solution (a). Dissolve 0.20 g of the substance to be examined in *methylene chloride R* and dilute to 5 ml with the same solvent.

Test solution (b). Dilute 1 ml of test solution (a) to 10 ml with *methylene chloride R*.

Reference solution (a). Dissolve 20 mg of *clozapine CRS* in *methylene chloride R* and dilute to 5 ml with the same solvent.

Reference solution (b). Dilute 1.5 ml of test solution (b) to 50 ml with *methylene chloride R*.

Reference solution (c). Dilute 1 ml of test solution (b) to 50 ml with *methylene chloride R*.

Reference solution (d). Dilute 5 ml of reference solution (c) to 10 ml with *methylene chloride R*.

Reference solution (e). Dissolve 10 mg of *clozapine CRS* and 10 mg of *oxazepam CRS* in *methylene chloride R* and dilute to 5 ml with the same solvent.

Apply separately to the plate 5 µl of each solution. Develop over a path of 10 cm using a mixture of 25 volumes of *methanol R* and 75 volumes of *methylene chloride R*. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. Any spot in the chromatogram obtained with test solution (a), apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.3 per cent); at most one such spot is more intense than the spot in the chromatogram obtained with reference solution (c) (0.2 per cent); and at most two such spots are more intense than the spot in the chromatogram obtained with reference solution (d) (0.1 per cent). The test is not valid unless the chromatogram obtained with reference solution (e) shows two clearly separated spots and the chromatogram obtained with reference solution (d) shows a clearly visible spot.

Heavy metals (2.4.8). 1.0 g complies with limit test C for heavy metals (20 ppm). Prepare the standard using 2 ml of *lead standard solution (10 ppm Pb) R*.

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying at 100 °C to 105 °C.

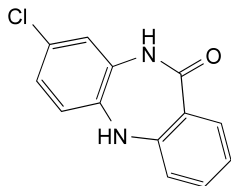
Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

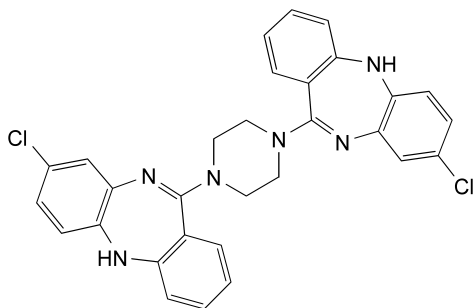
Dissolve 0.100 g in 50 ml of *anhydrous acetic acid R*. Titrate with 0.1 M perchloric acid, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M perchloric acid is equivalent to 16.34 mg of $C_{18}H_{19}ClN_4$.

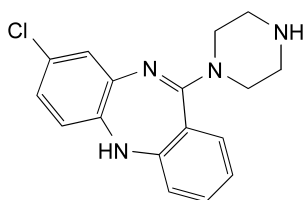
IMPURITIES



A. 8-chloro-5,10-dihydro-11H-dibenzo[*b,e*][1,4]diazepin-11-one,



B. 11,11'-(piperazin-1,4-diyl)bis(8-chloro-5H-dibenzo[*b,e*][1,4]diazepine),

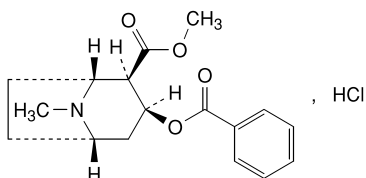


C. 8-chloro-11-(piperazin-1-yl)-5H-dibenzo[*b,e*][1,4]diazepine.

01/2005:0073

COCAINE HYDROCHLORIDE

Cocaini hydrochloridum



$C_{17}H_{22}ClNO_4$

M_r 339.8

DEFINITION

Methyl (1*R*,2*R*,3*S*,5*S*)-3-(benzoyloxy)-8-methyl-8-azabicyclo[3.2.1]octane-2-carboxylate hydrochloride.

Content: 98.5 per cent to 101.0 per cent (dried substance).

CHARACTERS

Appearance: white, crystalline powder or colourless crystals.

Solubility: very soluble in water, freely soluble in alcohol, slightly soluble in methylene chloride.

mp: about 197 °C, with decomposition.

IDENTIFICATION

First identification: B, D.

Second identification: A, C, D, E.

- Dissolve 20.0 mg in 0.01 M hydrochloric acid and dilute to 100.0 ml with the same acid. Dilute 5.0 ml of the solution to 50.0 ml with 0.01 M hydrochloric acid. Examined between 220 nm and 350 nm (2.2.25), the solution shows 2 absorption maxima, at 233 nm and 273 nm. The specific absorbance at 233 nm is 378 to 402.
- Infrared absorption spectrophotometry (2.2.24).
Comparison: Ph. Eur. reference spectrum of cocaine hydrochloride.
- Dissolve 0.1 g in 5 ml of water *R* and add 1 ml of dilute ammonia *R*2. A white precipitate is formed. Initiate crystallisation by scratching the wall of the tube with a glass rod. The crystals, washed with water *R* and dried *in vacuo*, melt (2.2.14) at 96 °C to 99 °C.
- It gives reaction (a) of chlorides (2.3.1).
- It gives the reaction of alkaloids (2.3.1).

TESTS

Solution S. Dissolve 0.5 g in water *R* and dilute to 25 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and colourless (2.2.2, *Method II*).

Acidity. To 10 ml of solution S add 0.05 ml of methyl red solution *R*. Not more than 0.2 ml of 0.02 M sodium hydroxide is required to change the colour of the indicator.

Specific optical rotation (2.2.7): –70 to –73 (dried substance).

Dissolve 0.50 g in water *R* and dilute to 20.0 ml with the same solvent.

Readily carbonisable substances. To 0.2 g add 2 ml of sulphuric acid *R*. After 15 min, the solution is not more intensely coloured than reference solution BY₅ (2.2.2, *Method I*).

Related substances. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 25.0 mg of the substance to be examined in the mobile phase and dilute to 50.0 ml with the mobile phase.

Reference solution (a). Dilute 1.0 ml of the test solution to 50.0 ml with the mobile phase. Dilute 5.0 ml of this solution to 100.0 ml with the mobile phase.

Reference solution (b). Dissolve 25 mg of the substance to be examined in 0.01 M sodium hydroxide and dilute to 10.0 ml with the same solvent. Dilute 1.0 ml of the solution to 10.0 ml with 0.01 M sodium hydroxide. Allow the solution to stand for 15 min.

Column:

- size: $l = 0.15$ m, $\varnothing = 4.6$ mm,
- stationary phase: end-capped octadecylsilyl silica gel for chromatography *R* (5 μ m) with a specific surface area of 335 m²/g, a pore size of 10 nm and a carbon loading of 19.1 per cent,
- temperature: 35 °C.

Mobile phase: triethylamine *R*, tetrahydrofuran *R*, acetonitrile *R*, water *R* (0.5:100:430:479.5 V/V/V/V).

Flow rate: 1 ml/min.

Detection: spectrophotometer at 216 nm.

Injection: 20 μ l.

Relative retention with reference to cocaine (retention time = about 7.4 min): degradation product = about 0.7.

System suitability: reference solution (b):

01/2005:1410

- *resolution*: minimum of 5 between the peaks due to cocaine and to the degradation product.

Limits:

- *any impurity eluting after the principal peak*: not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent),
- *total*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent),
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

Loss on drying (2.2.32): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 100–105 °C.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on the residue from the test for loss on drying.

ASSAY

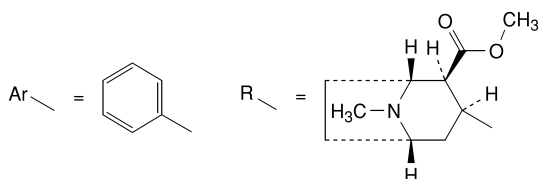
Dissolve 0.250 g in a mixture of 5.0 ml of 0.01 M hydrochloric acid and 50 ml of alcohol R. Carry out a potentiometric titration (2.2.20), using 0.1 M sodium hydroxide. Read the volume added between the 2 points of inflexion.

1 ml of 0.1 M sodium hydroxide is equivalent to 33.98 mg of C₁₇H₂₂ClNO₄.

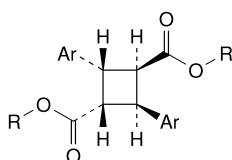
STORAGE

Protected from light.

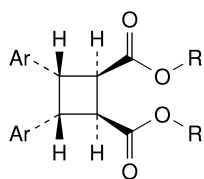
IMPURITIES



- A. methyl (1R,2R,3S,5S)-8-methyl-3-[(E)-3-phenylpropenoyl]oxy]-8-azabicyclo[3.2.1]octane-2-carboxylate (cinnamoylcocaine),



- B. bis[(1R,2R,3S,5S)-2-(methoxycarbonyl)-8-methyl-8-azabicyclo[3.2.1]oct-3-yl] (1R,2C,3t,4t)-2,4-diphenylcyclobutane-1,3-dicarboxylate (α-truxilline),



- C. bis[(1R,2R,3S,5S)-2-(methoxycarbonyl)-8-methyl-8-azabicyclo[3.2.1]oct-3-yl] (1R,2C,3t,4t)-3,4-diphenylcyclobutane-1,2-dicarboxylate (β-truxilline).

COCONUT OIL, REFINED

Cocois oleum raffinatum

DEFINITION

Refined coconut oil is the fatty oil obtained from the dried, solid part of the endosperm of *Cocos nucifera* L., then refined.

CHARACTERS

A white or almost white, unctuous mass, practically insoluble in water, freely soluble in methylene chloride and in light petroleum (bp: 65 °C to 70 °C), very slightly soluble in alcohol.

The refractive index is about 1.449, determined at 40 °C.

IDENTIFICATION

- A. It complies with the test for melting point (see Tests).
B. It complies with the test for composition of fatty acids (see Tests).

TESTS

Melting point (2.2.14): 23 °C to 26 °C.

Acid value (2.5.1). Not more than 0.5, determined on 20.0 g.

Peroxide value (2.5.5). Not more than 5.0.

Unsaponifiable matter (2.5.7). Not more than 1.0 per cent, determined on 5.0 g.

Alkaline impurities in fatty oils (2.4.19). It complies with the test for alkaline impurities in fatty oils.

Composition of fatty acids (2.4.22, Method B). Coconut oil is melted under gentle heating to a homogeneous liquid prior to sampling.

Reference solution. Dissolve 15.0 mg of *tricaproin* CRS, 80.0 mg of *tristearin* CRS, 0.150 g of *tricaprin* CRS, 0.200 g of *tricaprylin* CRS, 0.450 g of *trimyristin* CRS and 1.25 g of *trilaurin* CRS in a mixture of 2 volumes of methylene chloride R and 8 volumes of heptane R and dilute to 50 ml with the same mixture of solvents heating at 45 °C to 50 °C. Transfer 2 ml to a 10 ml centrifuge tube with a screw cap and evaporate the solvent in a current of nitrogen R. Dissolve with 1 ml of heptane R and 1 ml of dimethyl carbonate R and mix vigorously under gentle heating (50 °C to 60 °C). Add, while still warm, 1 ml of a 12 g/l solution of sodium R in anhydrous methanol R, prepared with the necessary precautions and mix vigorously for about 5 min. Add 3 ml of distilled water R and mix vigorously for about 30 s. Centrifuge for 15 min at 1500 g. Inject 1 µl of the organic phase.

Calculate the percentage content of each fatty acid using the following expression:

$$\frac{A_{x,s,c}}{\sum A_{x,s,c}} \times 100 \text{ per cent } m/m$$

$A_{x,s,c}$ is the corrected peak area of each fatty acid in the test solution:

$$A_{x,s,c} = A_{x,s} \times R_c$$

R_c is the relative correction factor:

$$R_c = \frac{m_{x,r} \times A_{1,r}}{A_{x,r} \times m_{1,r}}$$

for the peaks corresponding to caproic, caprylic, capric, lauric and myristic acid methyl esters.

- $m_{x,r}$ = mass of tricaproin, tricaprylin, tricaprin, trilaurin or trimyristin in the reference solution, in milligrams,
- $m_{1,r}$ = mass of tristearin in the reference solution, in milligrams,
- $A_{x,r}$ = area of the peaks corresponding to caproic, caprylic, capric, lauric and myristic acid methyl esters in the reference solution,
- $A_{1,r}$ = area of the peak corresponding to stearic acid methyl ester in the reference solution,
- $A_{x,s}$ = area of peaks corresponding to any specified or unspecified fatty acid methyl esters,
- R_c = 1 for peaks corresponding to each of the remaining specified fatty acid methyl esters or any unspecified fatty acid methyl ester.

The fatty acid fraction of the oil has the following composition:

- *caproic acid* (R_{Rt} 0.11): not more than 1.5 per cent,
- *caprylic acid* (R_{Rt} 0.23): 5.0 per cent to 11.0 per cent,
- *capric acid* (R_{Rt} 0.56): 4.0 per cent to 9.0 per cent,
- *lauric acid* (R_{Rt} 0.75): 40.0 per cent to 50.0 per cent,
- *myristic acid* (R_{Rt} 0.85): 15.0 per cent to 20.0 per cent,
- *palmitic acid* (R_{Rt} 0.93): 7.0 per cent to 12.0 per cent,
- *stearic acid* (R_{Rt} 1.00): 1.5 per cent to 5.0 per cent,
- *oleic acid and isomers* (R_{Rt} 1.01): 4.0 per cent to 10.0 per cent,
- *linoleic acid* (R_{Rt} 1.03): 1.0 per cent to 3.0 per cent,
- *linolenic acid* (R_{Rt} 1.06): not more than 0.2 per cent,
- *arachidic acid* (R_{Rt} 1.10): not more than 0.2 per cent,
- *eicosenoic acid* (R_{Rt} 1.11): not more than 0.2 per cent.

STORAGE

Store in a well-filled container, protected from light.

C. It complies with the test for composition of fatty acids and fatty alcohols (see Tests).

TESTS

Appearance. The substance to be examined is not more intensely coloured than reference solution Y₅ (2.2.2, *Method I*).

Acid value (2.5.1). Not more than 0.5, determined on 5.0 g.

Hydroxyl value (2.5.3, *Method A*). Not more than 5.0.

Iodine value (2.5.4). Not more than 1.0.

Saponification value (2.5.6): 160 to 173.

Composition of fatty acids and fatty alcohols (2.4.22, *Method C*). Use the chromatogram obtained with the following reference solution for identification of the peaks corresponding to the fatty alcohols.

Reference solution. Dissolve the following amounts of the substances listed in Table 1411.-1 in 10 ml of *heptane R*.

Table 1411.-1

Substance	Amount (mg)
<i>Methyl caproate R</i>	10
<i>Methyl caprylate R</i>	90
<i>Methyl caprate R</i>	50
<i>Methyl laurate R</i>	20
<i>Methyl myristate R</i>	10
<i>Methyl palmitate R</i>	10
<i>Methyl stearate R</i>	10
<i>Capric alcohol R</i>	10
<i>Lauryl alcohol R</i>	100
<i>Myristyl alcohol R</i>	40
<i>Cetyl alcohol CRS</i>	30
<i>Stearyl alcohol CRS</i>	20

Consider the sum of the areas of the peaks due to the fatty acids listed below to be equal to 100 and the sum of the areas of the peaks due to the fatty alcohols listed below to be equal to 100.

The fatty-acid fraction of the substance has the following composition:

- *caproic acid*: not more than 2.0 per cent,
- *caprylic acid*: 50.0 per cent to 80.0 per cent,
- *capric acid*: 20.0 per cent to 50.0 per cent,
- *lauric acid*: not more than 3.0 per cent,
- *myristic acid*: not more than 1.0 per cent.

The fatty-alcohol fraction of the substance has the following composition:

- *capric alcohol*: not more than 3.0 per cent,
- *lauryl alcohol*: 48.0 per cent to 59.0 per cent,
- *myristyl alcohol*: 18.0 per cent to 25.0 per cent,
- *cetyl alcohol*: 6.0 per cent to 12.0 per cent,
- *stearyl alcohol*: 9.0 per cent to 16.0 per cent.

Water (2.5.12). Not more than 0.1 per cent, determined on 5.00 g by the semi-micro determination of water.

Total ash (2.4.16). Not more than 0.1 per cent, determined on 1.0 g.

01/2005:1411

COCOYL CAPRYLOCAPRATE

Cocoylis caprylocapras

DEFINITION

Cocoyl caprylocaprate is a mixture of esters of saturated C₁₂ to C₁₈ alcohols with caprylic (octanoic) and capric (decanoic) acids obtained by the reaction of these acids with vegetable saturated fatty alcohols.

CHARACTERS

A slightly yellowish liquid, practically insoluble in water, miscible with alcohol and with liquid paraffin.

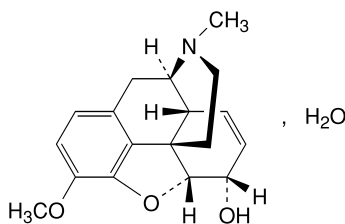
It has a relative density of about 0.86, a refractive index of about 1.445 and a viscosity of about 11 mPas.

IDENTIFICATION

- A. Freezing point (2.2.18): not more than 15 °C.
- B. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *cocoyl caprylocaprate CRS*.

CODEINE

Codeinum

 $C_{18}H_{21}NO_3 \cdot H_2O$ M_r 317.4

DEFINITION

7,8-Didehydro-4,5 α -epoxy-3-methoxy-17-methylmorphinan-6 α -ol.

Content: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

Appearance: white or almost white, crystalline powder or colourless crystals.

Solubility: soluble in boiling water, freely soluble in alcohol.

IDENTIFICATION

First identification: A, C.

Second identification: A, B, D, E.

A. Melting point (2.2.14): 155 °C to 159 °C.

B. To 2.0 ml of solution S (see Tests) add 50 ml of *water R* then 10 ml of 1 M *sodium hydroxide* and dilute to 100.0 ml with *water R*. Examined between 250 nm and 350 nm (2.2.25), the solution shows only 1 absorption maximum at 284 nm. The specific absorbance at the absorption maximum is about 50 (dried substance).

C. Infrared absorption spectrophotometry (2.2.24).

Preparation: dried substance prepared as a disc of *potassium bromide R*.

Comparison: *Ph. Eur. reference spectrum of codeine*.

D. To about 10 mg add 1 ml of *sulphuric acid R* and 0.05 ml of *ferric chloride solution R2* and heat on a water-bath. A blue colour develops. Add 0.05 ml of *nitric acid R*. The colour changes to red.

E. It gives the reaction of alkaloids (2.3.1).

TESTS

Solution S. Dissolve 50 mg in *carbon dioxide-free water R* and dilute to 10.0 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and colourless (2.2.2, *Method II*).

Specific optical rotation (2.2.7): –142 to –146 (dried substance).

Dissolve 0.50 g in *alcohol R* and dilute to 25.0 ml with the same solvent.

Related substances. Liquid chromatography (2.2.29).

Test solution. Dissolve 0.100 g of the substance to be examined and 0.100 g of *sodium octanesulphonate R* in the mobile phase and dilute to 10.0 ml with the mobile phase.

Reference solution (a). Dissolve 5.0 mg of *codeine impurity A CRS* in the mobile phase and dilute to 5.0 ml with the mobile phase.

01/2005:0076 *Reference solution (b)*. Dilute 1.0 ml of reference solution (a) to 20.0 ml with the mobile phase.

Reference solution (c). Dilute 1.0 ml of the test solution to 50.0 ml with the mobile phase. Dilute 5.0 ml of this solution to 100.0 ml with the mobile phase.

Reference solution (d). Dilute 0.5 ml of the test solution to 5.0 ml with reference solution (a).

Column:

- *size*: $l = 0.25$ m, $\varnothing = 4.6$ mm,
- *stationary phase*: end-capped octylsilyl silica gel for chromatography *R* (5 μ m).

Mobile phase: dissolve 1.08 g of *sodium octanesulphonate R* in a mixture of 20 ml of *glacial acetic acid R* and 250 ml of *acetonitrile R* and dilute to 1000 ml with *water R*.

Flow rate: 2 ml/min.

Detection: spectrophotometer at 245 nm.

Injection: 10 μ l.

Run time: 10 times the retention time of codeine.

Relative retention with reference to codeine (retention time = about 6 min): impurity B = about 0.6; impurity E = about 0.7; impurity A = about 2.0; impurity C = about 2.3; impurity D = about 3.6.

System suitability: reference solution (d):

- *resolution*: minimum 3 between the peaks due to codeine and impurity A.

Limits:

- *correction factor*: for the calculation of content, multiply the peak area of impurity C by 0.25,
- *impurity A*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (1.0 per cent),
- *impurities B, C, D, E*: for each impurity, not more than twice the area of the principal peak in the chromatogram obtained with reference solution (c) (0.2 per cent),
- *any other impurity*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (c) (0.1 per cent),
- *total of impurities other than A*: not more than 10 times the area of the principal peak in the chromatogram obtained with reference solution (c) (1.0 per cent),
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.05 per cent).

Loss on drying (2.2.32): 4.0 per cent to 6.0 per cent, determined on 1.000 g by drying in an oven at 100–105 °C.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.250 g in 10 ml of *anhydrous acetic acid R*. Add 20 ml of *dioxan R*. Titrate with 0.1 M *perchloric acid* using 0.05 ml of *crystal violet solution R* as indicator.

1 ml of 0.1 M *perchloric acid* is equivalent to 29.94 mg of $C_{18}H_{21}NO_3$.

STORAGE

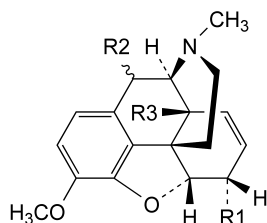
Protected from light.

IMPURITIES

Specified impurities: A, B, C, D, E.

Other detectable impurities: F, G.

01/2005:1412

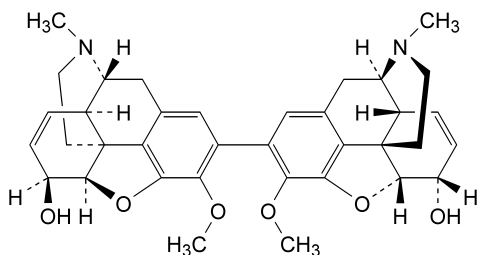


A. R1 = OCH₃, R2 = R3 = H: 7,8-didehydro-4,5α-epoxy-3,6α-dimethoxy-17-methylmorphinan (methylcodeine),

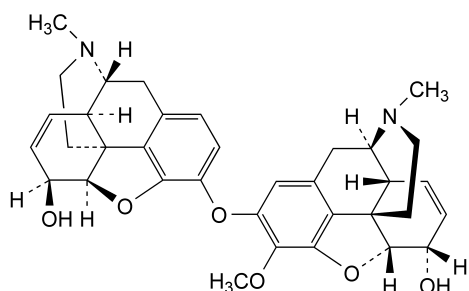
E. R1 = R2 = OH, R3 = H: 7,8-didehydro-4,5α-epoxy-3-methoxy-17-methylmorphinan-6α,10-diol,

F. R1 = R3 = OH, R2 = H: 7,8-didehydro-4,5α-epoxy-3-methoxy-17-methylmorphinan-6α,14-diol,

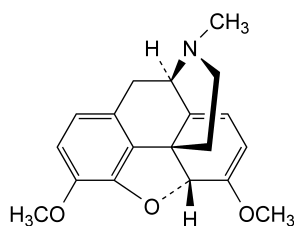
B. morphine,



C. 7,7',8,8'-tetrahydro-4,5α:4',5'α-diepoxy-3,3'-dimethoxy-17,17'-dimethyl-2,2'-bimorphinan-6α,6'α-diol (codeine dimer),



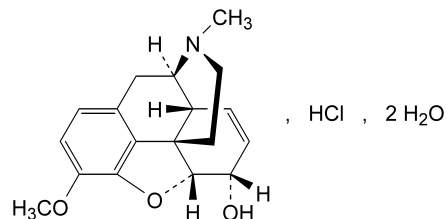
D. 7,8-didehydro-2-[(7,8-didehydro-4,5α-epoxy-6α-hydroxy-17-methylmorphinan-3-yl)oxy]-4,5α-epoxy-3-methoxy-17-methylmorphinan-6α-ol (3-O-(codein-2-yl)morphine),



G. 6,7,8,14-tetrahydro-4,5α-epoxy-3,6-dimethoxy-17-methylmorphinan (thebaine).

CODEINE HYDROCHLORIDE DIHYDRATE

Codeini hydrochloridum dihydricum



C₁₈H₂₂ClNO₃·2H₂O

M_r 371.9

DEFINITION

7,8-Didehydro-4,5α-epoxy-3-methoxy-17-methylmorphinan-6α-ol hydrochloride dihydrate.

Content: 99.0 per cent to 101.0 per cent (anhydrous substance).

CHARACTERS

Appearance: white or almost white, crystalline powder or small, colourless crystals.

Solubility: soluble in water, slightly soluble in ethanol, practically insoluble in cyclohexane.

IDENTIFICATION

First identification: A, D.

Second identification: B, C, D, E.

A. Infrared absorption spectrophotometry (2.2.24).

Comparison: Ph. Eur. reference spectrum of codeine hydrochloride dihydrate.

B. To 5 ml of solution S (see Tests) add 1 ml of a mixture of equal volumes of *strong sodium hydroxide solution R* and *water R* and initiate crystallisation, if necessary, by scratching the wall of the tube with a glass rod and cooling in iced water. Wash the precipitate with *water R* and dry at 100-105 °C. It melts (2.2.15) at 155 °C to 159 °C.

C. To about 10 mg add 1 ml of *sulphuric acid R* and 0.05 ml of *ferric chloride solution R2* and heat on a water-bath. A blue colour develops. Add 0.05 ml of *nitric acid R*. The colour changes to red.

D. Solution S gives reaction (a) of chlorides (2.3.1).

E. It gives the reaction of alkaloids (2.3.1).

TESTS

Solution S. Dissolve 2.00 g in *carbon dioxide-free water R* prepared from *distilled water R* and dilute to 50.0 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and not more intensely coloured than reference solution Y₆ (2.2.2, *Method II*).

Acidity or alkalinity. To 5 ml of solution S add 5 ml of *carbon dioxide-free water R*. Add 0.05 ml of *methyl red solution R* and 0.2 ml of 0.02 M *hydrochloric acid*; the solution is red. Add 0.4 ml of 0.02 M *sodium hydroxide*; the solution becomes yellow.

Specific optical rotation (2.2.7): −117 to −121 (anhydrous substance).

Dilute 5.0 ml of solution S to 10.0 ml with *water R*.

Related substances. Liquid chromatography (2.2.29).

Test solution. Dissolve 0.100 g of the substance to be examined and 0.100 g of *sodium octanesulphonate R* in the mobile phase and dilute to 10.0 ml with the mobile phase.

Reference solution (a). Dissolve 5.0 mg of *codeine impurity A CRS* in the mobile phase and dilute to 5.0 ml with the mobile phase.

Reference solution (b). Dilute 1.0 ml of reference solution (a) to 20.0 ml with the mobile phase.

Reference solution (c). Dilute 1.0 ml of the test solution to 50.0 ml with the mobile phase. Dilute 5.0 ml of this solution to 100.0 ml with the mobile phase.

Reference solution (d). Dilute 0.5 ml of the test solution to 5.0 ml with reference solution (a).

Column:

- **size:** $l = 0.25$ m, $\varnothing = 4.6$ mm,
- **stationary phase:** end-capped octylsilyl silica gel for chromatography *R* (5 μ m).

Mobile phase: dissolve 1.08 g of *sodium octanesulphonate R* in a mixture of 20 ml of *glacial acetic acid R* and 250 ml of *acetonitrile R* and dilute to 1000 ml with *water R*.

Flow rate: 2 ml/min.

Detection: spectrophotometer at 245 nm.

Injection: 10 μ l.

Run time: 10 times the retention time of codeine.

Relative retention with reference to codeine (retention time = about 6 min): impurity B = about 0.6; impurity E = about 0.7; impurity A = about 2.0; impurity C = about 2.3; impurity D = about 3.6.

System suitability: reference solution (d):

- **resolution:** minimum 3 between the peaks due to codeine and impurity A.

Limits:

- **correction factor:** for the calculation of content, multiply the peak area of impurity C by 0.25,
- **impurity A:** not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (1.0 per cent),
- **impurities B, C, D, E:** for each impurity, not more than twice the area of the principal peak in the chromatogram obtained with reference solution (c) (0.2 per cent),
- **any other impurity:** for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (c) (0.1 per cent),
- **total of impurities other than A:** not more than 10 times the area of the principal peak in the chromatogram obtained with reference solution (c) (1.0 per cent),
- **disregard limit:** 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.05 per cent).

Sulphates (2.4.13): maximum 0.1 per cent.

Dilute 5 ml of solution S to 20 ml with *distilled water R*. 15 ml of the solution complies with the limit test for sulphates.

Water (2.5.12): 8.0 per cent to 10.5 per cent, determined on 0.250 g.

ASSAY

Dissolve 0.300 g in a mixture of 5 ml of 0.01 *M hydrochloric acid R* and 30 ml of *alcohol R*. Carry out a potentiometric titration (2.2.20), using 0.1 *M sodium hydroxide*. Read the volume added between the 2 points of inflexion.

1 ml of 0.1 *M sodium hydroxide* is equivalent to 33.59 mg of $C_{18}H_{22}ClNO_3$.

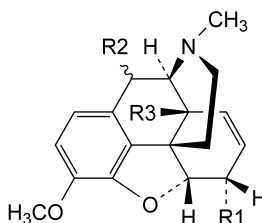
STORAGE

Protected from light.

IMPURITIES

Specified impurities: A, B, C, D, E.

Other detectable impurities: F, G.

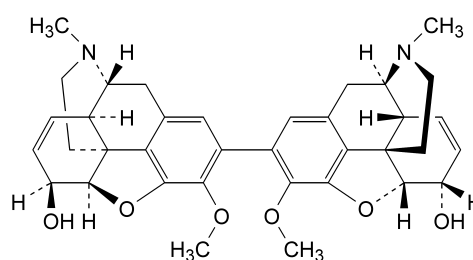


A. $R_1 = OCH_3$, $R_2 = R_3 = H$: 7,8-didehydro-4,5 α -epoxy-3,6 α -dimethoxy-17-methylmorphinan (methylcodeine),

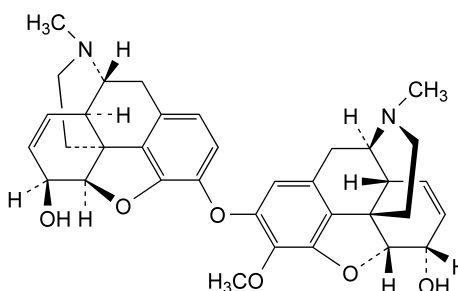
E. $R_1 = R_2 = OH$, $R_3 = H$: 7,8-didehydro-4,5 α -epoxy-3-methoxy-17-methylmorphinan-6 α ,10-diol,

F. $R_1 = R_3 = OH$, $R_2 = H$: 7,8-didehydro-4,5 α -epoxy-3-methoxy-17-methylmorphinan-6 α ,14-diol,

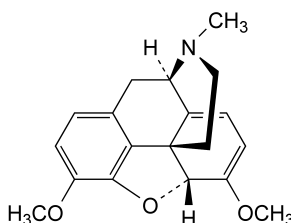
B. morphine,



C. 7,7',8,8'-tetrahydro-4,5 α :4',5' α -diepoxy-3,3'-dimethoxy-17,17'-dimethyl-2,2'-bimorphinan-6 α ,6' α -diol (codeine dimer),



D. 7,8-didehydro-2-[(7,8-didehydro-4,5 α -epoxy-6 α -hydroxy-17-methylmorphinan-3-yl)oxy]-4,5 α -epoxy-3-methoxy-17-methylmorphinan-6 α -ol (3-O-(codein-2-yl)morphine),

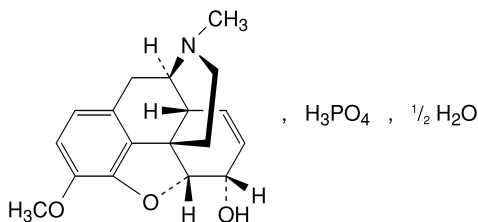


G. 6,7,8,14-tetrahydro-4,5 α -epoxy-3,6-dimethoxy-17-methylmorphinan (thebaine).

01/2005:0074 pH (2.2.3): 4.0 to 5.0 for solution S.

**CODEINE PHOSPHATE
HEMIHYDRATE**

Codeini phosphas hemihydricus

 $C_{18}H_{24}NO_7P \cdot \frac{1}{2}H_2O$ M_r 406.4**DEFINITION**

7,8-Didehydro-4,5α-epoxy-3-methoxy-17-methylmorphinan-6α-ol phosphate hemihydrate.

Content: 98.5 per cent to 101.0 per cent (dried substance).

CHARACTERS

Appearance: white or almost white, crystalline powder or small, colourless crystals.

Solubility: freely soluble in water, slightly soluble or very slightly soluble in alcohol.

IDENTIFICATION

First identification: B, E, F.

Second identification: A, C, D, E, F, G.

A. Dilute 1.0 ml of solution S (see Tests) to 100.0 ml with *water R*. To 25.0 ml of this solution add 25 ml of *water R* then 10 ml of 1 M *sodium hydroxide* and dilute to 100.0 ml with *water R*. Examined between 250 nm and 350 nm (2.2.25), the solution shows only 1 absorption maximum, at 284 nm. The specific absorbance at the absorption maximum is about 38 (dried substance).

B. Infrared absorption spectrophotometry (2.2.24).

Preparation: dissolve 0.20 g in 4 ml of *water R*. Add 1 ml of a mixture of equal volumes of *strong sodium hydroxide solution R* and *water R* and initiate crystallisation, if necessary, by scratching the wall of the tube with a glass rod and cooling in iced water. Wash the precipitate with *water R* and dry at 100-105 °C. Examine the dried precipitate prepared as discs using *potassium bromide R*.
Comparison: Ph. Eur. reference spectrum of codeine.

C. Dissolve 0.20 g in 4 ml of *water R*. Add 1 ml of a mixture of equal volumes of *strong sodium hydroxide solution R* and *water R* and initiate crystallisation, if necessary, by scratching the wall of the tube with a glass rod and cooling in iced water. The precipitate, washed with *water R* and dried at 100-105 °C, melts (2.2.14) at 155 °C to 159 °C.

D. To about 10 mg add 1 ml of *sulphuric acid R* and 0.05 ml of *ferric chloride solution R2* and heat on a water-bath. A blue colour develops. Add 0.05 ml of *nitric acid R*. The colour changes to red.

E. It complies with the test for loss on drying (see Tests).

F. Solution S gives reaction (a) of phosphates (2.3.1).

G. It gives the reaction of alkaloids (2.3.1).

TESTS

Solution S. Dissolve 1.00 g in *carbon dioxide-free water R* prepared from *distilled water R* and dilute to 25.0 ml with the same solvent.

pH (2.2.3): 4.0 to 5.0 for solution S.

Specific optical rotation (2.2.7): –98 to –102 (dried substance).

Dilute 5.0 ml of solution S to 10.0 ml with *water R*.

Related substances. Liquid chromatography (2.2.29).

Test solution. Dissolve 0.100 g of the substance to be examined and 0.100 g of *sodium octanesulphonate R* in the mobile phase and dilute to 10.0 ml with the mobile phase.

Reference solution (a). Dissolve 5.0 mg of *codeine impurity A CRS* in the mobile phase and dilute to 5.0 ml with the mobile phase.

Reference solution (b). Dilute 1.0 ml of reference solution (a) to 20.0 ml with the mobile phase.

Reference solution (c). Dilute 1.0 ml of the test solution to 50.0 ml with the mobile phase. Dilute 5.0 ml of this solution to 100.0 ml with the mobile phase.

Reference solution (d). Dilute 0.5 ml of the test solution to 5.0 ml with reference solution (a).

Column:

– *size:* $l = 0.25$ m, $\varnothing = 4.6$ mm,

– *stationary phase:* end-capped octylsilyl silica gel for chromatography R (5 µm).

Mobile phase: dissolve 1.08 g of *sodium octanesulphonate R* in a mixture of 20 ml of *glacial acetic acid R* and 250 ml of *acetonitrile R* and dilute to 1000 ml with *water R*.

Flow rate: 2 ml/min.

Detection: spectrophotometer at 245 nm.

Injection: 10 µl.

Run time: 10 times the retention time of codeine.

Relative retention with reference to codeine (retention time = about 6 min): impurity B = about 0.6; impurity E = about 0.7; impurity A = about 2.0; impurity C = about 2.3; impurity D = about 3.6.

System suitability: reference solution (d):

– *resolution:* minimum 3 between the peaks due to codeine and impurity A.

Limits:

– *correction factor:* for the calculation of content, multiply the peak area of impurity C by 0.25,

– *impurity A:* not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (1.0 per cent),

– *impurities B, C, D, E:* for each impurity, not more than twice the area of the principal peak in the chromatogram obtained with reference solution (c) (0.2 per cent),

– *any other impurity:* for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (c) (0.1 per cent),

– *total of impurities other than A:* not more than 10 times the area of the principal peak in the chromatogram obtained with reference solution (c) (1.0 per cent),

– *disregard limit:* 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.05 per cent).

Sulphates (2.4.13): maximum 0.1 per cent.

Dilute 5 ml of solution S to 20 ml with *distilled water R*. 15 ml of the solution complies with the limit test for sulphates.

Loss on drying (2.2.32): 1.5 per cent to 3.0 per cent, determined on 1.000 g by drying in an oven at 100-105 °C.

ASSAY

Dissolve 0.350 g in a mixture of 10 ml of *anhydrous acetic acid R* and 20 ml of *dioxan R*. Titrate with 0.1 M *perchloric acid* using 0.05 ml of *crystal violet solution R* as indicator.

1 ml of 0.1 M *perchloric acid* is equivalent to 39.74 mg of $C_{18}H_{24}NO_7P$.

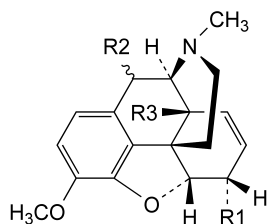
STORAGE

Protected from light.

IMPURITIES

Specified impurities: A, B, C, D, E.

Other detectable impurities: F, G.

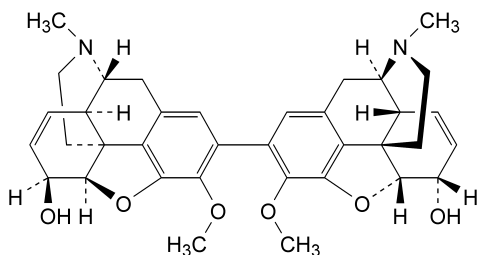


A. R1 = OCH₃, R2 = R3 = H: 7,8-didehydro-4,5 α -epoxy-3,6 α -dimethoxy-17-methylmorphinan (methylcodeine),

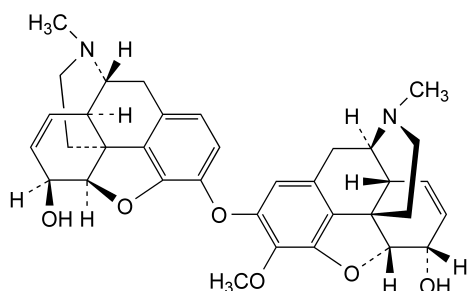
E. R1 = R2 = OH, R3 = H: 7,8-didehydro-4,5 α -epoxy-3-methoxy-17-methylmorphinan-6 α ,10-diol,

F. R1 = R3 = OH, R2 = H: 7,8-didehydro-4,5 α -epoxy-3-methoxy-17-methylmorphinan-6 α ,14-diol,

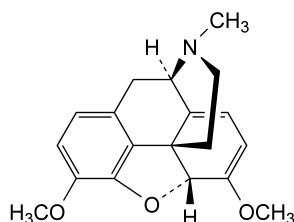
B. morphine,



C. 7,7',8,8'-tetrahydro-4,5 α :4',5' α -diepoxy-3,3'-dimethoxy-17,17'-dimethyl-2,2'-bimorphinan-6 α ,6' α -diol (codeine dimer),



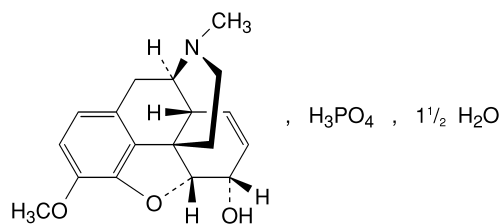
D. 7,8-didehydro-2-[(7,8-didehydro-4,5 α -epoxy-6 α -hydroxy-17-methylmorphinan-3-yl)oxy]-4,5 α -epoxy-3-methoxy-17-methylmorphinan-6 α -ol (3-*O*-(codein-2-yl)morphine),



G. 6,7,8,14-tetrahydro-4,5 α -epoxy-3,6-dimethoxy-17-methylmorphinan (thebaine).

CODEINE PHOSPHATE
SESQUIHYDRATE

Codeini phosphas sesquihydricus



$C_{18}H_{24}NO_7P \cdot 1\frac{1}{2}H_2O$

M_r 424.4

DEFINITION

7,8-Didehydro-4,5 α -epoxy-3-methoxy-17-methylmorphinan-6 α -ol phosphate sesquihydrate.

Content: 98.5 per cent to 101.0 per cent (dried substance).

CHARACTERS

Appearance: white or almost white, crystalline powder or small, colourless crystals.

Solubility: freely soluble in water, slightly soluble in alcohol.

IDENTIFICATION

First identification: B, E, F.

Second identification: A, C, D, E, F, G.

A. Dilute 1.0 ml of solution S (see Tests) to 100.0 ml with *water R*. To 25.0 ml of this solution add 25 ml of *water R* then 10 ml of 1 M *sodium hydroxide* and dilute to 100.0 ml with *water R*. Examined between 250 nm and 350 nm (2.2.25), the solution shows only 1 absorption maximum, at 284 nm. The specific absorbance at the absorption maximum is about 38 (dried substance).

B. Infrared absorption spectrophotometry (2.2.24).

Preparation: dissolve 0.20 g in 4 ml of *water R*. Add 1 ml of a mixture of equal volumes of *strong sodium hydroxide solution R* and *water R* and initiate crystallisation, if necessary, by scratching the wall of the tube with a glass rod and cooling in iced water. Wash the precipitate with *water R* and dry at 100-105 °C. Examine the dried precipitate prepared as discs using *potassium bromide R*.

Comparison: *Ph. Eur.* reference spectrum of codeine.

C. Dissolve 0.20 g in 4 ml of *water R*. Add 1 ml of a mixture of equal volumes of *strong sodium hydroxide solution R* and *water R* and initiate crystallisation, if necessary, by scratching the wall of the tube with a glass rod and cooling in iced water. The precipitate, washed with *water R* and dried at 100-105 °C, melts (2.2.14) at 155 °C to 159 °C.

D. To about 10 mg add 1 ml of *sulphuric acid R* and 0.05 ml of *ferric chloride solution R2* and heat on a water-bath. A blue colour develops. Add 0.05 ml of *nitric acid R*. The colour changes to red.

E. It complies with the test for loss on drying (see Tests).

F. Solution S gives reaction (a) of phosphates (2.3.1).

G. It gives the reaction of alkaloids (2.3.1).

TESTS

Solution S. Dissolve 1.00 g in *carbon dioxide-free water R* prepared from *distilled water R* and dilute to 25.0 ml with the same solvent.

pH (2.2.3): 4.0 to 5.0 for solution S.

Specific optical rotation (2.2.7): -98 to -102 (dried substance).

Dilute 5.0 ml of solution S to 10.0 ml with *water R*.

Related substances. Liquid chromatography (2.2.29).

Test solution. Dissolve 0.100 g of the substance to be examined and 0.100 g of *sodium octanesulphonate R* in the mobile phase and dilute to 10.0 ml with the mobile phase.

Reference solution (a). Dissolve 5.0 mg of *codeine impurity A CRS* in the mobile phase and dilute to 5.0 ml with the mobile phase.

Reference solution (b). Dilute 1.0 ml of reference solution (a) to 20.0 ml with the mobile phase.

Reference solution (c). Dilute 1.0 ml of the test solution to 50.0 ml with the mobile phase. Dilute 5.0 ml of this solution to 100.0 ml with the mobile phase.

Reference solution (d). Dilute 0.5 ml of the test solution to 5.0 ml with reference solution (a).

Column:

- **size:** $l = 0.25$ m, $\varnothing = 4.6$ mm,
- **stationary phase:** *end-capped octylsilyl silica gel for chromatography R* (5 μ m).

Mobile phase: dissolve 1.08 g of *sodium octanesulphonate R* in a mixture of 20 ml of *glacial acetic acid R* and 250 ml of *acetonitrile R* and dilute to 1000 ml with *water R*.

Flow rate: 2 ml/min.

Detection: spectrophotometer at 245 nm.

Injection: 10 μ l.

Run time: 10 times the retention time of codeine.

Relative retention with reference to codeine (retention time = about 6 min): *impurity B* = about 0.6; *impurity E* = about 0.7; *impurity A* = about 2.0; *impurity C* = about 2.3; *impurity D* = about 3.6.

System suitability: reference solution (d):

- **resolution:** minimum 3 between the peaks due to codeine and *impurity A*.

Limits:

- **correction factor:** for the calculation of content, multiply the peak area of *impurity C* by 0.25,
- **impurity A:** not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (1.0 per cent),
- **impurities B, C, D, E:** for each impurity, not more than twice the area of the principal peak in the chromatogram obtained with reference solution (c) (0.2 per cent),
- **any other impurity:** for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (c) (0.1 per cent),
- **total of impurities other than A:** not more than 10 times the area of the principal peak in the chromatogram obtained with reference solution (c) (1.0 per cent),
- **disregard limit:** 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.05 per cent).

Sulphates (2.4.13): maximum 0.1 per cent.

Dilute 5 ml of solution S to 20 ml with *distilled water R*. 15 ml of the solution complies with the limit test for sulphates.

Loss on drying (2.2.32): 5.0 per cent to 7.5 per cent, determined on 0.500 g by drying in an oven at 100–105 °C.

ASSAY

Dissolve 0.350 g in a mixture of 10 ml of *anhydrous acetic acid R* and 20 ml of *dioxan R*. Titrate with 0.1 M *perchloric acid* using 0.05 ml of *crystal violet solution R* as indicator.

1 ml of 0.1 M *perchloric acid* is equivalent to 39.74 mg of $C_{18}H_{24}NO_7P$.

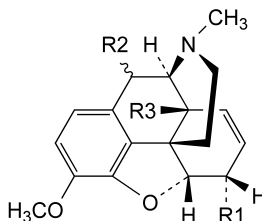
STORAGE

Protected from light.

IMPURITIES

Specified impurities: A, B, C, D, E.

Other detectable impurities: F, G.

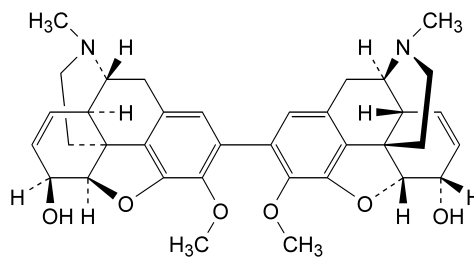


A. $R_1 = OCH_3$, $R_2 = R_3 = H$: 7,8-didehydro-4,5 α -epoxy-3,6 α -dimethoxy-17-methylmorphinan (methylcodeine),

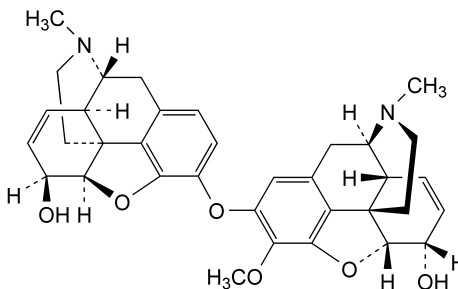
E. $R_1 = R_2 = OH$, $R_3 = H$: 7,8-didehydro-4,5 α -epoxy-3-methoxy-17-methylmorphinan-6 α ,10-diol,

F. $R_1 = R_3 = OH$, $R_2 = H$: 7,8-didehydro-4,5 α -epoxy-3-methoxy-17-methylmorphinan-6 α ,14-diol,

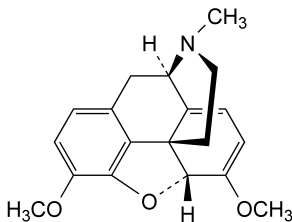
B. morphine,



C. 7,7',8,8'-tetrahydro-4,5 α :4',5' α -diepoxy-3,3'-dimethoxy-17,17'-dimethyl-2,2'-bimorphinan-6 α ,6' α -diol (codeine dimer),



D. 7,8-didehydro-2-[(7,8-didehydro-4,5 α -epoxy-6 α -hydroxy-17-methylmorphinan-3-yl)oxy]-4,5 α -epoxy-3-methoxy-17-methylmorphinan-6 α -ol (3-O-(codein-2-yl)morphine),

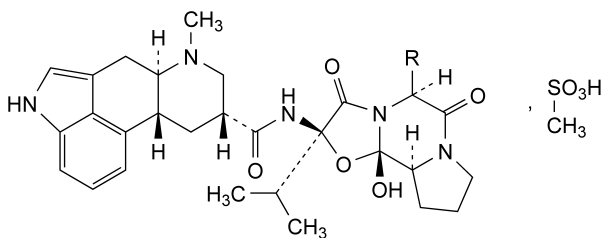


G. 6,7,8,14-tetrahydro-4,5 α -epoxy-3,6-dimethoxy-17-methylmorphinan (thebaine).

01/2005:2060

CODERGOCRINE MESILATE

Codergocrini mesilas



Name	Mol. Formula	M_r	R
dihydroergocornine mesilate	$C_{32}H_{45}N_5O_8S$	660	
dihydroergocristine mesilate	$C_{36}H_{45}N_5O_8S$	708	
α -dihydroergocryptine mesilate	$C_{33}H_{47}N_5O_8S$	674	
β -dihydroergocryptine mesilate	$C_{33}H_{47}N_5O_8S$	674	

DEFINITION

A mixture of:

- (6a*R*,9*R*,10a*R*)-*N*[(2*R*,5*S*,10a*S*,10b*S*)-10b-hydroxy-2,5-bis(1-methylethyl)-3,6-dioxooctahydro-8*H*-oxazolo[3,2-*a*]pyrrolo[2,1-*c*]pyrazin-2-yl]-7-methyl-4,6,6a,7,8,9,10,10a-octahydroindolo[4,3-*fg*]quinoline-9-carboxamide methanesulphonate (dihydroergocornine mesilate);
- (6a*R*,9*R*,10a*R*)-*N*[(2*R*,5*S*,10a*S*,10b*S*)-5-benzyl-10b-hydroxy-2-(1-methylethyl)-3,6-dioxooctahydro-8*H*-oxazolo[3,2-*a*]pyrrolo[2,1-*c*]pyrazin-2-yl]-7-methyl-4,6,6a,7,8,9,10,10a-octahydroindolo[4,3-*fg*]quinoline-9-carboxamide methanesulphonate (dihydroergocristine mesilate);
- (6a*R*,9*R*,10a*R*)-*N*[(2*R*,5*S*,10a*S*,10b*S*)-10b-hydroxy-2-(1-methylethyl)-5-(2-methylpropyl)-3,6-dioxooctahydro-8*H*-oxazolo[3,2-*a*]pyrrolo[2,1-*c*]pyrazin-2-yl]-7-methyl-4,6,6a,7,8,9,10,10a-octahydroindolo[4,3-*fg*]quinoline-9-carboxamide methanesulphonate (α -dihydroergocryptine mesilate);
- (6a*R*,9*R*,10a*R*)-*N*[(2*R*,5*S*,10a*S*,10b*S*)-10b-hydroxy-2-(1-methylethyl)-5-(1*R**S*)-1-methylpropyl]-3,6-dioxooctahydro-8*H*-oxazolo[3,2-*a*]pyrrolo[2,1-*c*]pyrazin-2-yl]-7-methyl-4,6,6a,7,8,9,10,10a-octahydroindolo[4,3-*fg*]quinoline-9-carboxamide methanesulphonate (β -dihydroergocryptine mesilate or epicriptine mesilate).

Content: 98.0 per cent to 102.0 per cent (dried substance).

PRODUCTION

The production method must be evaluated to determine the potential for formation of alkyl mesilates, which is particularly likely to occur if the reaction medium contains

lower alcohols. Where necessary, the production method is validated to demonstrate that alkyl mesilates are not detectable in the final product.

CHARACTERS

Appearance: white or yellowish powder.

Solubility: sparingly soluble in water, sparingly soluble to soluble in ethanol (96 per cent), slightly soluble in methylene chloride.

IDENTIFICATION

A. Thin-layer chromatography (2.2.27).

Test solution. Dissolve 0.20 g of the substance to be examined in a mixture of 1 volume of *methanol R* and 9 volumes of *methylene chloride R* and dilute to 5 ml with the same mixture of solvents.

Reference solution. Dissolve 0.20 g of *methanesulphonic acid R* in a mixture of 1 volume of *methanol R* and 9 volumes of *methylene chloride R* and dilute to 5 ml with the same mixture of solvents.

Plate: TLC silica gel plate *R*.

Mobile phase: water *R*, concentrated ammonia *R*, butanol *R*, acetone *R* (5:10:20:65 V/V/V/V).

Application: 10 μ l.

Development: over 2/3 of the plate.

Drying: in a current of cold air for not more than 1 min.

Detection: spray with a 1 g/l solution of *bromocresol purple R* in *methanol R*, adjusted to a violet-red colour with 0.05 ml of *dilute ammonia R1*.

Drying: in a current of hot air at 100 °C.

Results: the principal spot in the chromatogram obtained with the test solution is similar in position and colour to the principal spot in the chromatogram obtained with the reference solution.

B. Examine the chromatograms obtained in the test for composition.

Results: the 4 principal peaks in the chromatogram obtained with the test solution are similar in retention time and size to the 4 principal peaks in the chromatogram obtained with the reference solution.

TESTS

pH (2.2.3): 4.2 to 5.2.

Dissolve 0.10 g in *carbon dioxide-free water R* and dilute to 20 ml with the same solvent.

Composition. Liquid chromatography (2.2.29): use the normalisation procedure.

Test solution. Dissolve 20 mg of the substance to be examined in a mixture of 1 volume of *anhydrous ethanol R* and 2 volumes of a 10 g/l solution of *tartaric acid R* and dilute to 10 ml with the same mixture of solvents.

Reference solution. Dissolve 20 mg of *codergocrine mesilate CRS* in a mixture of 1 volume of *anhydrous ethanol R* and 2 volumes of a 10 g/l solution of *tartaric acid R* and dilute to 10 ml with the same mixture of solvents.

Column:

- size: $l = 0.15$ m, $\varnothing = 4.6$ mm,
- stationary phase: octadecylsilyl silica gel for chromatography *R* (5 μ m).

Mobile phase: triethylamine *R*, acetonitrile *R*, water *R* (2.5:25:75 V/V/V).

Flow rate: 1.5 ml/min.

Detection: spectrophotometer at 280 nm.

Injection: 20 μ l.

Run time: 20 min.

Elution order: dihydroergocornine, α -dihydroergocryptine, dihydroergocristine, β -dihydroergocryptine.

System suitability: test solution:

- resolution: minimum 3 between any 2 consecutive principal peaks.

Composition:

- dihydroergocornine: 30.0 per cent to 35.0 per cent,
- α -dihydroergocryptine: 20.0 per cent to 25.0 per cent,
- dihydroergocristine: 30.0 per cent to 35.0 per cent,
- β -dihydroergocryptine: 10.0 per cent to 13.0 per cent,
- disregard limit: 1.0 per cent.

Related substances. Thin-layer chromatography (2.2.27).

Perform the test as rapidly as possible and protected from direct light. Prepare the test solution last and immediately before application on the plate.

Test solution. Dissolve 0.40 g of the substance to be examined in a mixture of 1 volume of *methanol R* and 9 volumes of *methylene chloride R* and dilute to 5.0 ml with the same mixture of solvents.

Reference solution (a). Dissolve 40 mg of *dihydroergocristine mesilate CRS* in a mixture of 1 volume of *methanol R* and 9 volumes of *methylene chloride R* and dilute to 10.0 ml with the same mixture of solvents. Dilute 3.0 ml of the solution to 50.0 ml with a mixture of 1 volume of *methanol R* and 9 volumes of *methylene chloride R*.

Reference solution (b). To 2.0 ml of reference solution (a), add 1.0 ml of a mixture of 1 volume of *methanol R* and 9 volumes of *methylene chloride R*.

Reference solution (c). To 1.0 ml of reference solution (a), add 2.0 ml of a mixture of 1 volume of *methanol R* and 9 volumes of *methylene chloride R*.

Reference solution (d). To 1.0 ml of reference solution (a), add 5.0 ml of a mixture of 1 volume of *methanol R* and 9 volumes of *methylene chloride R*.

Plate: TLC silica gel plate *R*.

Mobile phase: concentrated ammonia *R*, *methanol R*, *ethyl acetate R*, *methylene chloride R* (1:3:50:50 V/V/V/V).

Application: 10 μ l.

Drying: in the dark for 2 min after the application of the last solution.

First development: in an unsaturated tank, over 2/3 of the plate.

Drying: in a current of cold air for not more than 1 min.

Second development: in an unsaturated tank, over 2/3 of the plate; use freshly prepared mobile phase.

Drying: in a current of cold air for not more than 1 min.

Detection: spray thoroughly with *dimethylaminobenzaldehyde solution R7* and dry in a current of hot air until the spot in the chromatogram obtained with reference solution (d) is clearly visible.

System suitability: test solution:

- the chromatogram shows at least 3 separated secondary spots.

Limits:

- any impurity: any spots, apart from the principal spot, are not more intense than the spot in the chromatogram obtained with reference solution (a) (0.3 per cent); not more than 4 such spots are more intense than the spot in the chromatogram obtained with reference solution (c) (0.1 per cent) and 2 of these may be more intense than the spot in the chromatogram obtained with reference solution (b) (0.2 per cent).

Loss on drying (2.2.32): maximum 5.0 per cent, determined on 0.500 g by drying at 120 °C under high vacuum.

ASSAY

Dissolve 0.500 g in 60 ml of *pyridine R*. Pass a stream of *nitrogen R* over the surface of the solution and titrate with 0.1 M *tetrabutylammonium hydroxide*, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *tetrabutylammonium hydroxide* is equivalent to 68.04 mg of *codergocrine mesilate* (average M_r = 680).

STORAGE

Protected from light.

01/2005:1192

COD-LIVER OIL (TYPE A)

Iecoris aselli oleum A

DEFINITION

Purified fatty oil obtained from the fresh livers of *Gadus morhua* L. and other species of *Gadidae*, solid substances being removed by cooling and filtering. A suitable antioxidant may be added.

Content: 600 IU (180 μ g) to 2500 IU (750 μ g) of vitamin A per gram and 60 IU (1.5 μ g) to 250 IU (6.25 μ g) of vitamin D₃ per gram.

CHARACTERS

Appearance: clear, yellowish, viscous liquid.

Solubility: practically insoluble in water, slightly soluble in alcohol, miscible with light petroleum.

IDENTIFICATION

First identification: A, B, C.

Second identification: C, D.

A. In the assay for vitamin A using method A, the test solution shows an absorption maximum (2.2.25) at 325 ± 2 nm. In the assay for vitamin A using method B, the chromatogram obtained with the test solution shows a peak corresponding to the peak of all-*trans*-retinol in the chromatogram obtained with the reference solution.

B. In the assay for vitamin D₃, the chromatogram obtained with test solution (a) shows a peak corresponding to the peak of *cholecalciferol* in the chromatogram obtained with reference solution (b).

C. It complies with the test for composition of fatty acids (see Tests).

D. To 0.1 g add 0.5 ml of *methylene chloride R* and 1 ml of *antimony trichloride solution R*. Mix. A deep blue colour develops in about 10 s.

TESTS

Colour: not more intensely coloured than a reference solution prepared as follows: to 3.0 ml of red primary solution add 25.0 ml of yellow primary solution and dilute to 50.0 ml with a 10 g/l solution of *hydrochloric acid R* (2.2.2, Method II).

Relative density (2.2.5): 0.917 to 0.930.

Refractive index (2.2.6): 1.477 to 1.484.

Acid value (2.5.1): maximum 2.0.

Anisidine value (2.5.36): maximum 30.0.

Iodine value (2.5.4, Method B): 150 to 180.

Use *starch solution R2*.

Peroxide value (2.5.5, Method B): maximum 10.0.

Unsaponifiable matter (2.5.7): maximum 1.5 per cent, determined on 2.0 g, and extracting with 3 quantities, each of 50 ml, of *peroxide-free ether R*.

Stearin. 10 ml remains clear after cooling in iced water for 3 h.

Composition of fatty acids. Gas chromatography (2.2.28).

Trivial name of fatty acid	Nomenclature	Lower limit area (per cent)	Upper limit area (per cent)
<i>Saturated fatty acids:</i>			
Myristic acid	14:0	2.0	6.0
Palmitic acid	16:0	7.0	14.0
Stearic acid	18:0	1.0	4.0
<i>Mono-unsaturated fatty acids:</i>			
Palmitoleic acid	16:1 n-7	4.5	11.5
cis-Vaccenic acid	18:1 n-7	2.0	7.0
Oleic acid	18:1 n-9	12.0	21.0
Gadoleic acid	20:1 n-11	1.0	5.5
Gondoic acid	20:1 n-9	5.0	17.0
Erucic acid	22:1 n-9	0	1.5
Cetoleic acid (22:1 n-11)	22:1 n-11+13	5.0	12.0
<i>Poly-unsaturated fatty acids:</i>			
Linoleic acid	18:2 n-6	0.5	3.0
α-Linolenic acid	18:3 n-3	0	2.0
Morocctic acid	18:4 n-3	0.5	4.5
Timnodonic (eicosapentaenoic) acid (EPA)	20:5 n-3	7.0	16.0
Cervonic (docosahexaenoic) acid (DHA)	22:6 n-3	6.0	18.0

Test solution. Introduce about 0.45 g of the substance to be examined into a 10 ml volumetric flask, dissolve in *hexane R* containing 50 mg of *butylhydroxytoluene R* per litre and dilute to 10.0 ml with the same solvent. Transfer 2.0 ml of the solution into a quartz tube and evaporate the solvent with a gentle current of *nitrogen R*. Add 1.5 ml of a 20 g/l solution of *sodium hydroxide R* in *methanol R*, cover with *nitrogen R*, cap tightly with a polytetrafluoroethylene lined cap, mix and heat in a water-bath for 7 min. Cool, add 2 ml of *boron trichloride-methanol solution R*, cover with *nitrogen R*, cap tightly, mix and heat in a water-bath for 30 min. Cool to 40-50 °C, add 1 ml of *trimethylpentane R*, cap and vortex or shake vigorously for at least 30 s. Immediately add 5 ml of *saturated sodium chloride solution R*, cover with *nitrogen R*, cap and vortex or shake thoroughly for at least 15 s. Allow the upper layer to become clear and transfer to a separate tube. Shake the methanol layer once more with 1 ml of *trimethylpentane R* and combine the trimethylpentane extracts. Wash the combined extracts with 2 quantities, each of 1 ml, of *water R* and dry over *anhydrous sodium sulphate R*. Prepare 2 solutions for each sample.

Column:

- **material:** fused silica,
- **size:** $l = 30$ m, $\varnothing = 0.25$ mm,
- **stationary phase:** *macrogol 20 000 R* (film thickness 0.25 µm).

Carrier gas: *hydrogen for chromatography R* or *helium for chromatography R*, where oxygen scrubber is applied.

Split ratio: 1:200.

Temperature:

	Time (min)	Temperature (°C)
Column	0 - 55	170 → 225
	55 - 75	225
Injection port		250
Detection		280

Detection: flame ionisation.

Injection: 1 µl, twice.

System suitability:

- the 15 fatty acids to be tested are satisfactorily identified from the chromatogram shown in Figure 1192.-1,
- injection of a mixture of equal amounts of *methyl palmitate R*, *methyl stearate R*, *methyl arachidate R* and *methyl behenate R* give area percentages of 24.4, 24.8, 25.2 and 25.6 (± 0.5 per cent), respectively,
- **resolution:** minimum of 1.3 between the peaks due to methyl oleate and methyl *cis*-vaccenate; the resolution between the pair due to methyl gadoleate and methyl gondoate is sufficient for purposes of identification and area measurement.

Calculate the area per cent for each fatty acid methyl ester from the expression:

$$\frac{A_x}{A_t} \times 100$$

A_x = peak area of fatty acid x ,

A_t = sum of the peak areas (up to C22:6 n-3).

The calculation is not valid unless:

- the total area is based only on peaks due to solely fatty acids methyl esters,
- the number of fatty acid methyl ester peaks exceeding 0.05 per cent of the total area is at least 24,
- the 24 largest peaks of the methyl esters account for more than 90 per cent of the total area. (These correspond to, in common elution order: 14:0, 15:0, 16:0, 16:1 n-7, 16:4 n-1, 18:0, 18:1 n-9, 18:1 n-7, 18:2 n-6, 18:3 n-3, 18:4 n-3, 20:1 n-11, 20:1 n-9, 20:1 n-7, 20:2 n-6, 20:4 n-6, 20:3 n-3, 20:4 n-3, 20:5 n-3, 22:1 n-11, 22:1 n-9, 21:5 n-3, 22:5 n-3, 22:6 n-3).

ASSAY

Vitamin A. Carry out the test as rapidly as possible, avoiding exposure to actinic light and air, oxidising agents, oxidation catalysts (for example, copper and iron) and acids.

Use method A. If method A is found not to be valid, use method B.

METHOD A

Ultraviolet absorption spectrophotometry (2.2.25).

Test solution. To 1.00 g in a round-bottomed flask, add 3 ml of a freshly prepared 50 per cent *m/m* solution of *potassium hydroxide R* and 30 ml of *ethanol R*. Boil under reflux in a current of *nitrogen R* for 30 min. Cool rapidly and add 30 ml of *water R*. Extract with 50 ml of *ether R*. Repeat the extraction 3 times and discard the lower layer after complete separation. Wash the combined upper layers with 4 quantities, each of 50 ml, of *water R* and evaporate to dryness under a gentle current of *nitrogen R* at a temperature not exceeding 30 °C or in a rotary evaporator at a temperature not exceeding 30 °C under

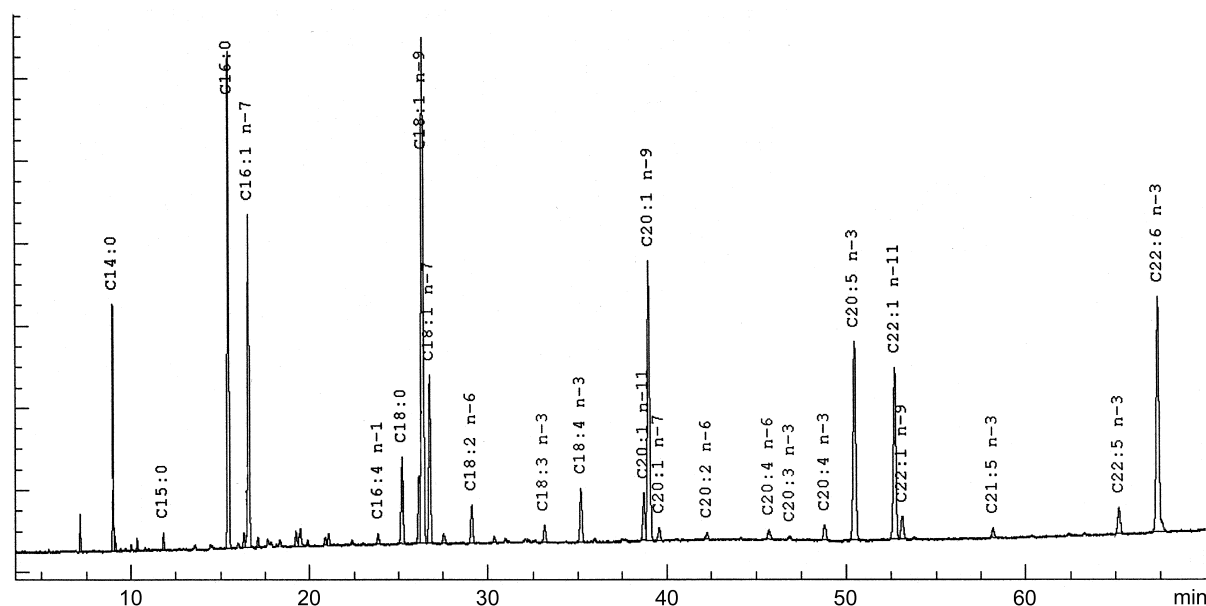


Figure 1192-1. – Chromatogram for the test for composition of fatty acids of cod-liver oil (type A)

reduced pressure (water ejector). Dissolve the residue in sufficient *2-propanol R1* to give an expected concentration of vitamin A equivalent to 10-15 IU/ml.

Measure the absorbances of the solution at 300 nm, 310 nm, 325 nm and 334 nm and at the wavelength of maximum absorption with a suitable spectrophotometer in 1 cm specially matched cells, using *2-propanol R1* as the compensation liquid.

Calculate the content of vitamin A, as all-*trans*-retinol, in International Units per gram from the expression:

$$A_{325} \times \frac{1830}{100m} \times V$$

A_{325} = absorbance at 325 nm,

m = mass of the substance to be examined, in grams,

V = total volume of solution containing 10-15 IU of vitamin A per millilitre,

1830 = conversion factor for the specific absorbance of all-*trans*-retinol, in International Units.

The above expression can be used only if A_{325} has a value of not greater than $A_{325, \text{corr}}/0.970$ where $A_{325, \text{corr}}$ is the corrected absorbance at 325 nm and is given by the equation:

$$A_{325, \text{corr}} = 6.815A_{325} - 2.555A_{310} - 4.260A_{334}$$

A designates the absorbance at the wavelength indicated by the subscript.

If A_{325} has a value greater than $A_{325, \text{corr}}/0.970$, calculate the content of vitamin A from the following expression:

$$A_{325, \text{corr}} \times \frac{1830}{100m} \times V$$

The assay is not valid unless:

- the wavelength of maximum absorption lies between 323 nm and 327 nm,
- the absorbance at 300 nm relative to that at 325 nm is at most 0.73.

METHOD B

Liquid chromatography (2.2.29).

Test solution. To 2.00 g in a round-bottomed flask, add 5 ml of a freshly prepared 100 g/l solution of *ascorbic acid R* and 10 ml of a freshly prepared 800 g/l solution of *potassium hydroxide R* and 100 ml of *ethanol R*. Boil under a reflux condenser on a water-bath for 15 min. Add 100 ml of a 10 g/l solution of *sodium chloride R* and cool. Transfer the solution to a 500 ml separating funnel rinsing the round-bottomed flask with about 75 ml of a 10 g/l solution of *sodium chloride R* and then with 150 ml of a mixture of equal volumes of *light petroleum R1* and *ether R*. Shake for 1 min. When the layers have separated completely, discard the lower layer and wash the upper layer, first with 50 ml of a 30 g/l solution of *potassium hydroxide R* in a 10 per cent V/V solution of *ethanol R* and then with 3 quantities, each of 50 ml, of a 10 g/l solution of *sodium chloride R*. Filter the upper layer through 5 g of *anhydrous sodium sulphate R* on a fast filter paper into a 250 ml flask suitable for a rotary evaporator. Wash the funnel with 10 ml of fresh extraction mixture, filter and combine the upper layers. Distil them at a temperature not exceeding 30 °C under reduced pressure (water ejector) and fill with *nitrogen R* when evaporation is completed. Alternatively evaporate the solvent under a gentle current of *nitrogen R* at a temperature not exceeding 30 °C. Dissolve the residue in *2-propanol R*, transfer to a 25 ml volumetric flask and dilute to 25 ml with *2-propanol R*. Gentle heating in an ultrasonic bath may be required. (A large fraction of the white residue is cholesterol, constituting approximately 50 per cent of the unsaponifiable matter of cod-liver oil).

Reference solution (a). Prepare a solution of *retinol acetate CRS* in *2-propanol R1* so that 1 ml contains about 1000 IU of all-*trans*-retinol.

The exact concentration of reference solution (a) is assessed by ultraviolet absorption spectrophotometry (2.2.25). Dilute reference solution (a) with *2-propanol R1* to a presumed concentration of 10-15 IU/ml and measure the absorbance at 326 nm in matched 1 cm cells using *2-propanol R1* as the compensation liquid.

Calculate the content of vitamin A in International Units per millilitre of reference solution (a) from the following expression, taking into account the assigned content of *retinol acetate CRS*:

$$A_{326} \times \frac{1900 \times V_2}{100 \times V_1}$$

A_{326} = absorbance at 326 nm,

V_1 = volume of reference solution (a) used,

V_2 = volume of the diluted solution,

1900 = conversion factor for the specific absorbance of *retinol acetate CRS*, in International Units.

Reference solution (b). Proceed as described for the test solution but using 2.00 ml of reference solution (a) in place of the substance to be examined.

The exact concentration of reference solution (b) is assessed by ultraviolet absorption spectrophotometry (2.2.25). Dilute reference solution (b) with *2-propanol R1* to a presumed concentration of 10-15 IU/ml of all-*trans*-retinol and measure the absorbance at 325 nm in matched 1 cm cells using *2-propanol R1* as the compensation liquid.

Calculate the content of all-*trans*-retinol in International Units per millilitre of reference solution (b) from the expression:

$$A_{325} \times \frac{1830 \times V_3}{100 \times V_4}$$

A_{325} = absorbance at 325 nm,

V_3 = volume of the diluted solution,

V_4 = volume of reference solution (b) used,

1830 = conversion factor for the specific absorbance of all-*trans*-retinol, in International Units.

Column:

- size: $l = 0.25$ m, $\varnothing = 4.6$ mm,
- stationary phase: octadecylsilyl silica gel for chromatography *R* (film thickness 5-10 μ m).

Mobile phase: water *R*, methanol *R* (3:97 V/V).

Flow rate: 1 ml/min.

Detection: spectrophotometer at 325 nm.

Injection: 10 μ l; inject in triplicate the test solution and reference solution (b).

Retention time: all-*trans*-retinol = 5 ± 1 min.

System suitability:

- the chromatogram obtained with the test solution shows a peak due to that of all-*trans*-retinol in the chromatogram obtained with reference solution (b),
- when using the method of standard additions to the test solution there is greater than 95 per cent recovery of the added *retinol acetate CRS*,
- the recovery of all-*trans*-retinol in reference solution (b) as assessed by direct absorption spectrophotometry is greater than 95 per cent.

Calculate the content of vitamin A using the following expression:

$$A_1 \times \frac{C \times V}{A_2} \times \frac{1}{m}$$

A_1 = area of the peak due to all-*trans*-retinol in the chromatogram obtained with the test solution,

A_2 = area of the peak due to all-*trans*-retinol in the chromatogram obtained with reference solution (b),

C = concentration of *retinol acetate CRS* in reference solution (a) as assessed prior to the saponification, in International Units per millilitre (= 1000 IU/ml),

V = volume of reference solution (a) treated (2.00 ml),

m = mass of the substance to be examined in the test solution (2.00 g).

Vitamin D₃. Liquid chromatography (2.2.29). Carry out the assay as rapidly as possible, avoiding exposure to actinic light and air.

Internal standard solution. Dissolve 0.50 mg of *ergocalciferol CRS* in 100 ml of *ethanol R*.

Test solution (a). To 4.00 g in a round-bottomed flask, add 5 ml of a freshly prepared 100 g/l solution of *ascorbic acid R*, 10 ml of a freshly prepared 800 g/l solution of *potassium hydroxide R* and 100 ml of *ethanol R*. Boil under a reflux condenser on a water-bath for 30 min. Add 100 ml of a 10 g/l solution of *sodium chloride R* and cool the solution to room temperature. Transfer the solution to a 500 ml separating funnel rinsing the round-bottomed flask with about 75 ml of a 10 g/l solution of *sodium chloride R* and then with 150 ml of a mixture of equal volumes of *light petroleum R1* and *ether R*. Shake for 1 min. When the layers have separated completely, discard the lower layer and wash the upper layer, first with 50 ml of a 30 g/l solution of *potassium hydroxide R* in a 10 per cent V/V solution of *ethanol R*, and then with 3 quantities, each of 50 ml, of a 10 g/l solution of *sodium chloride R*. Filter the upper layer through 5 g of *anhydrous sodium sulphate R* on a fast filter paper into a 250 ml flask suitable for a rotary evaporator. Wash the funnel with 10 ml of fresh extraction mixture, filter and combine the upper layers. Distil them at a temperature not exceeding 30 °C under reduced pressure (water ejector) and fill with *nitrogen R* when evaporation is completed. Alternatively evaporate the solvent under a gentle current of *nitrogen R* at a temperature not exceeding 30 °C. Dissolve the residue in 1.5 ml of the mobile phase described under Purification. Gentle heating in an ultrasonic bath may be required. (A large fraction of the white residue is cholesterol, constituting approximately 50 per cent m/m of the unsaponifiable matter of cod-liver oil).

Test solution (b). To 4.00 g add 2.0 ml of the internal standard solution and proceed as described for test solution (a).

Reference solution (a). Dissolve 0.50 mg of *cholecalciferol CRS* in 100.0 ml of *ethanol R*.

Reference solution (b). In a round-bottomed flask, add 2.0 ml of reference solution (a) and 2.0 ml of the internal standard solution and proceed as described for test solution (a).

PURIFICATION

Column:

- size: $l = 0.25$ m, $\varnothing = 4.6$ mm,
- stationary phase: nitrile silica gel for chromatography *R* (film thickness 10 μ m).

Mobile phase: isoamyl alcohol *R*, hexane *R* (1.6:98.4 V/V).

Flow rate: 1.1 ml/min.

Detection: spectrophotometer at 265 nm.

Inject 350 μ l of reference solution (b). Collect the eluate from 2 min before until 2 min after the retention time of *cholecalciferol*, in a ground-glass-stoppered tube containing 1 ml of a 1 g/l solution of *butylhydroxytoluene R* in

hexane R. Repeat the procedure with test solutions (a) and (b). Evaporate the eluates obtained from reference solution (b) and from test solutions (a) and (b), separately, to dryness at a temperature not exceeding 30 °C under a gentle current of *nitrogen R*. Dissolve each residue in 1.5 ml of *acetonitrile R*.

DETERMINATION

Column:

- size: $l = 0.15$ m, $\varnothing = 4.6$ mm,
- stationary phase: *octadecylsilyl silica gel for chromatography R* (film thickness 5 µm).

Mobile phase: *phosphoric acid R*, 96 per cent V/V solution of *acetonitrile R* (0.2:99.8 V/V).

Flow rate: 1.0 ml/min.

Detection: spectrophotometer at 265 nm.

Injection: 2 quantities not exceeding 200 µl of each of the 3 solutions obtained under Purification.

System suitability:

- resolution: minimum 1.4 between the peaks corresponding to ergocalciferol and cholecalciferol in the chromatogram obtained with reference solution (b),
- when using the method of standard additions to test solution (a) there is greater than 95 per cent recovery of the added *cholecalciferol CRS* when due consideration has been given to correction by the internal standard.

Calculate the content of vitamin D₃ in International Units per gram using the following expression, taking into account the assigned content of *cholecalciferol CRS*:

$$\frac{A_2}{A_6} \times \frac{A_3}{A_4 - \left[\frac{A_5}{A_1} \right] \times A_2} \times \frac{m_2}{m_1} \times \frac{V_2}{V_1} \times 40$$

- m_1 = mass of the sample in test solution (b) in grams,
 m_2 = total mass of *cholecalciferol CRS* used for the preparation of reference solution (a) in micrograms (500 µg),
 A_1 = area (or height) of the peak due to cholecalciferol in the chromatogram obtained with test solution (a),
 A_2 = area (or height) of the peak due to cholecalciferol in the chromatogram obtained with test solution (b),
 A_3 = area (or height) of the peak due to ergocalciferol in the chromatogram obtained with reference solution (b),
 A_4 = area (or height) of the peak due to ergocalciferol in the chromatogram obtained with test solution (b),
 A_5 = area (or height) of a possible peak in the chromatogram obtained with test solution (a) with the same retention time as the peak co-eluting with ergocalciferol in test solution (b),
 A_6 = area (or height) of the peak due to cholecalciferol in the chromatogram obtained with reference solution (b),
 V_1 = total volume of reference solution (a) (100 ml),
 V_2 = volume of reference solution (a) used for preparing reference solution (b) (2.0 ml).

STORAGE

In an airtight and well-filled container, protected from light. If no antioxidant is added, store under an inert gas.

Once the container has been opened, its contents are used as soon as possible and any part of the contents not used at once is protected by an atmosphere of inert gas.

LABELLING

The label states:

- the number of International Units of vitamin A,
- the number of International Units of vitamin D₃,
- the name and concentration of any added antioxidant.

01/2005:1193

COD-LIVER OIL (TYPE B)

Iecoris aselli oleum B

DEFINITION

Purified fatty oil obtained from the fresh livers of *Gadus morhua* L. and other species of *Gadidae*, solid substances being removed by cooling and filtering. A suitable antioxidant may be added.

Content: 600 IU (180 µg) to 2500 IU (750 µg) of vitamin A per gram and 60 IU (1.5 µg) to 250 IU (6.25 µg) of vitamin D₃ per gram.

CHARACTERS

Appearance: clear, yellowish, viscous liquid.

Solubility: practically insoluble in water, slightly soluble in alcohol, miscible with light petroleum.

IDENTIFICATION

First identification: A, B, C.

Second identification: C, D.

- In the assay for vitamin A using method A, the test solution shows an absorption maximum (2.2.25) at 325 ± 2 nm. In the assay for vitamin A using method B, the chromatogram obtained with the test solution shows a peak corresponding to the peak of all-*trans*-retinol in the chromatogram obtained with the reference solution.
- In the assay for vitamin D₃, the chromatogram obtained with test solution (a) shows a peak corresponding to the peak of cholecalciferol in the chromatogram obtained with reference solution (b).
- It complies with the test for composition of fatty acids (see Tests).
- To 0.1 g add 0.5 ml of *methylene chloride R* and 1 ml of *antimony trichloride solution R*. Mix. A deep blue colour develops in about 10 s.

TESTS

Colour: not more intensely coloured than a reference solution prepared as follows: to 3.0 ml of red primary solution add 25.0 ml of yellow primary solution and dilute to 50.0 ml with a 10 g/l solution of *hydrochloric acid R* (2.2.2, Method II).

Relative density (2.2.5): 0.917 to 0.930.

Refractive index (2.2.6): 1.477 to 1.484.

Acid value (2.5.1): maximum 2.0.

Iodine value (2.5.4, Method B): 150 to 180.

Use *starch solution R2*.

Peroxide value (2.5.5, Method B): maximum 10.0.

Unsaponifiable matter (2.5.7): maximum 1.5 per cent, determined on 2.0 g and extracting with 3 quantities, each of 50 ml, of *peroxide-free ether R*.

Stearin. 10 ml remains clear after cooling in iced water for 3 h.

Composition of fatty acids. Gas chromatography (2.2.28).

Trivial name of fatty acid	Nomenclature	Lower limit area (per cent)	Upper limit area (per cent)
<i>Saturated fatty acids:</i>			
Myristic acid	14:0	2.0	6.0
Palmitic acid	16:0	7.0	14.0
Stearic acid	18:0	1.0	4.0
<i>Mono-unsaturated fatty acids:</i>			
Palmitoleic acid	16:1 n-7	4.5	11.5
<i>cis</i> -Vaccenic acid	18:1 n-7	2.0	7.0
Oleic acid	18:1 n-9	12.0	21.0
Gadoleic acid	20:1 n-11	1.0	5.5
Gondoic acid	20:1 n-9	5.0	17.0
Erucic acid	22:1 n-9	0	1.5
Cetoleic acid (22:1 n-11)	22:1 n-11+13	5.0	12.0
<i>Poly-unsaturated fatty acids:</i>			
Linoleic acid	18:2 n-6	0.5	3.0
α -Linolenic acid	18:3 n-3	0	2.0
Moroctic acid	18:4 n-3	0.5	4.5
Timnodonic (eicosapentaenoic) acid (EPA)	20:5 n-3	7.0	16.0
Cervonic (docosahexaenoic) acid (DHA)	22:6 n-3	6.0	18.0

Test solution. Introduce about 0.45 g of the substance to be examined into a 10 ml volumetric flask, dissolve in *hexane R* containing 50 mg of *butylhydroxytoluene R* per litre and dilute to 10.0 ml with the same solvent. Transfer 2.0 ml of the solution into a quartz tube and evaporate the solvent with a gentle current of *nitrogen R*. Add 1.5 ml of a 20 g/l solution of *sodium hydroxide R* in *methanol R*, cover with *nitrogen R*, cap tightly with a polytetrafluoroethylene lined cap, mix and heat in a water-bath for 7 min. Cool, add 2 ml of *boron trichloride-methanol solution R*, cover with *nitrogen R*, cap tightly, mix and heat in a water-bath for 30 min. Cool to 40-50 °C, add 1 ml of *trimethylpentane R*, cap and vortex or shake vigorously for at least 30 s. Immediately add 5 ml of *saturated sodium chloride solution R*, cover with *nitrogen R*, cap and vortex or shake thoroughly for at least 15 s. Allow the upper layer to become clear and transfer to a separate tube. Shake the methanol layer once more with 1 ml of *trimethylpentane R* and combine the trimethylpentane extracts. Wash the combined extracts with 2 quantities, each of 1 ml, of *water R* and dry over *anhydrous sodium sulphate R*. Prepare 2 solutions for each sample.

Column:

- **material:** fused silica,
- **size:** $l = 30$ m, $\varnothing = 0.25$ mm,
- **stationary phase:** *macrogol 20 000 R* (film thickness 0.25 μ m).

Carrier gas: *hydrogen for chromatography R* or *helium for chromatography R*, where oxygen scrubber is applied.

Split ratio: 1:200.

Temperature:

	Time (min)	Temperature (°C)
Column	0 - 55	170 → 225
	55 - 75	225
Injection port		250
Detector		280

Detection: flame ionisation.

Injection: 1 μ l, twice.

System suitability:

- the 15 fatty acids to be tested are satisfactorily identified from the chromatogram shown in Figure 1193-1.
- injection of a mixture of equal amounts of *methyl palmitate R*, *methyl stearate R*, *methyl arachidate R*, and *methyl behenate R* give area percentages of 24.4, 24.8, 25.2 and 25.6 (± 0.5 per cent), respectively,
- **resolution:** minimum of 1.3 between the peaks due to methyl oleate and methyl *cis*-vaccenate; the resolution between the pair due to methyl gadoleate and methyl gondoate is sufficient for purposes of identification and area measurement.

Calculate the area per cent for each fatty acid methyl ester from the expression:

$$\frac{A_x}{A_t} \times 100$$

A_x = peak area of fatty acid x ,

A_t = sum of the peak areas (up to C22:6 n-3).

The calculation is not valid unless:

- the total area is based only on peaks due to solely fatty acids methyl esters,
- the number of fatty acid methyl ester peaks exceeding 0.05 per cent of the total area is at least 24,
- the 24 largest peaks of the methyl esters account for more than 90 per cent of the total area. (These correspond to, in common elution order: 14:0, 15:0, 16:0, 16:1 n-7, 16:4 n-1, 18:0, 18:1 n-9, 18:1 n-7, 18:2 n-6, 18:3 n-3, 18:4 n-3, 20:1 n-11, 20:1 n-9, 20:1 n-7, 20:2 n-6, 20:4 n-6, 20:3 n-3, 20:4 n-3, 20:5 n-3, 22:1 n-11, 22:1 n-9, 21:5 n-3, 22:5 n-3, 22:6 n-3).

ASSAY

Vitamin A. Carry out the test as rapidly as possible, avoiding exposure to actinic light and air, oxidising agents, oxidation catalysts (for example, copper and iron) and acids.

Use method A. If method A is found not to be valid, use method B.

METHOD A

Ultraviolet absorption spectrophotometry (2.2.25).

Test solution. To 1.00 g in a round-bottomed flask, add 3 ml of a freshly prepared 50 per cent *m/m* solution of *potassium hydroxide R* and 30 ml of *ethanol R*. Boil under reflux in a current of *nitrogen R* for 30 min. Cool rapidly and add 30 ml of *water R*. Extract with 50 ml of *ether R*. Repeat the extraction 3 times and discard the lower layer after complete separation. Wash the combined upper layers with 4 quantities, each of 50 ml, of *water R* and evaporate to dryness under a gentle current of *nitrogen R* at a temperature not exceeding 30 °C or in a rotary evaporator at a temperature not exceeding 30 °C under

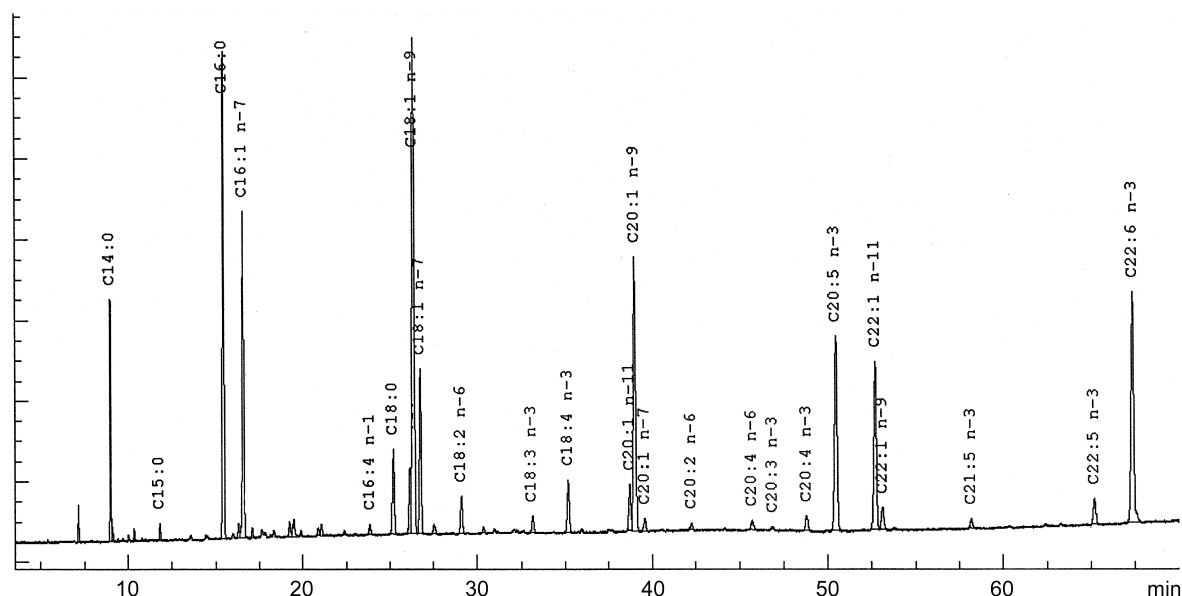


Figure 1193-1. – Chromatogram for the test for composition of fatty acids of cod-liver oil (type B)

reduced pressure (water ejector). Dissolve the residue in sufficient *2-propanol R1* to give an expected concentration of vitamin A equivalent to 10-15 IU/ml.

Measure the absorbances of the solution at 300 nm, 310 nm, 325 nm and 334 nm and at the wavelength of maximum absorption with a suitable spectrophotometer in 1 cm specially matched cells, using *2-propanol R1* as the compensation liquid.

Calculate the content of vitamin A, as all-*trans*-retinol, in International Units per gram from the expression:

$$A_{325} \times \frac{1830}{100m} \times V$$

A_{325} = absorbance at 325 nm,

m = mass of the substance to be examined, in grams,

V = total volume of solution containing 10-15 IU of vitamin A per millilitre,

1830 = conversion factor for the specific absorbance of all-*trans*-retinol, in International Units.

The above expression can be used only if A_{325} has a value of not greater than $A_{325, \text{corr}}/0.970$ where $A_{325, \text{corr}}$ is the corrected absorbance at 325 nm and is given by the equation:

$$A_{325, \text{corr}} = 6.815A_{325} - 2.555A_{310} - 4.260A_{334}$$

A designates the absorbance at the wavelength indicated by the subscript.

If A_{325} has a value greater than $A_{325, \text{corr}}/0.970$, calculate the content of vitamin A from the expression:

$$A_{325, \text{corr}} \times \frac{1830}{100m} \times V$$

The assay is not valid unless:

- the wavelength of maximum absorption lies between 323 nm and 327 nm,
- the absorbance at 300 nm relative to that at 325 nm is at most 0.73.

METHOD B

Liquid chromatography (2.2.29).

Test solution. To 2.00 g in a round-bottomed flask, add 5 ml of a freshly prepared 100 g/l solution of *ascorbic acid R* and 10 ml of a freshly prepared 800 g/l solution of *potassium*

hydroxide R and 100 ml of *ethanol R*. Boil under a reflux condenser on a water-bath for 15 min. Add 100 ml of a 10 g/l solution of *sodium chloride R* and cool. Transfer the solution to a 500 ml separating funnel rinsing the round-bottomed flask with about 75 ml of a 10 g/l solution of *sodium chloride R* and then with 150 ml of a mixture of equal volumes of *light petroleum R1* and *ether R*. Shake for 1 min. When the layers have separated completely, discard the lower layer and wash the upper layer, first with 50 ml of a 30 g/l solution of *potassium hydroxide R* in a 10 per cent V/V solution of *ethanol R* and then with 3 quantities, each of 50 ml, of a 10 g/l solution of *sodium chloride R*. Filter the upper layer through 5 g of *anhydrous sodium sulphate R* on a fast filter paper into a 250 ml flask suitable for a rotary evaporator. Wash the funnel with 10 ml of fresh extraction mixture, filter and combine the upper layers. Distil them at a temperature not exceeding 30 °C under reduced pressure (water ejector) and fill with *nitrogen R* when evaporation is completed. Alternatively evaporate the solvent under a gentle current of *nitrogen R* at a temperature not exceeding 30 °C. Dissolve the residue in *2-propanol R*, transfer to a 25 ml volumetric flask and dilute to 25 ml with *2-propanol R*. Gentle heating in an ultrasonic bath may be required. (A large fraction of the white residue is cholesterol, constituting approximately 50 per cent of the unsaponifiable matter of cod-liver oil).

Reference solution (a). Prepare a solution of *retinol acetate CRS* in *2-propanol R1* so that 1 ml contains about 1000 IU of all-*trans*-retinol.

The exact concentration of reference solution (a) is assessed by ultraviolet absorption spectrophotometry (2.2.25). Dilute reference solution (a) with *2-propanol R1* to a presumed concentration of 10-15 IU/ml and measure the absorbance at 326 nm in matched 1 cm cells using *2-propanol R1* as the compensation liquid.

Calculate the content of vitamin A in International Units per millilitre of reference solution (a) using the following expression, taking into account the assigned content of *retinol acetate CRS*:

$$A_{326} \times \frac{1900 \times V_2}{100 \times V_1}$$

A_{326} = absorbance at 326 nm,

V_1 = volume of reference solution (a) used,

- V_2 = volume of the diluted solution,
 1900 = conversion factor for the specific absorbance of *retinol acetate CRS*, in International Units.

Reference solution (b). Proceed as described for the test solution but using 2.00 ml of reference solution (a) in place of the substance to be examined.

The exact concentration of reference solution (b) is assessed by ultraviolet absorption spectrophotometry (2.2.25). Dilute reference solution (b) with *2-propanol R1* to a presumed concentration of 10-15 IU/ml of all-*trans*-retinol and measure the absorbance at 325 nm in matched 1 cm cells using *2-propanol R1* as the compensation liquid.

Calculate the content of all-*trans*-retinol in International Units per millilitre of reference solution (b) from the expression:

$$A_{325} \times \frac{1830 \times V_3}{100 \times V_4}$$

- A_{325} = absorbance at 325 nm,
 V_3 = volume of the diluted solution,
 V_4 = volume of reference solution (b) used,
 1830 = conversion factor for the specific absorbance of all-*trans*-retinol, in International Units.

Column:

- size: $l = 0.25$ m, $\emptyset = 4.6$ mm,
- stationary phase: *octadecylsilyl silica gel for chromatography R* (film thickness 5-10 μ m).

Mobile phase: *water R*, *methanol R* (3:97 V/V).

Flow rate: 1 ml/min.

Detection: spectrophotometer at 325 nm.

Injection: 10 μ l; inject in triplicate the test solution and reference solution (b).

Retention time: all-*trans*-retinol = 5 ± 1 min.

System suitability:

- the chromatogram obtained with the test solution shows a peak due to that of all-*trans*-retinol in the chromatogram obtained with reference solution (b),
- when using the method of standard additions to the test solution there is greater than 95 per cent recovery of the added *retinol acetate CRS*,
- the recovery of all-*trans*-retinol in reference solution (b) as assessed by direct absorption spectrophotometry is greater than 95 per cent.

Calculate the content of vitamin A using the following expression:

$$A_1 \times \frac{C \times V}{A_2} \times \frac{1}{m}$$

- A_1 = area of the peak due to all-*trans*-retinol in the chromatogram obtained with the test solution,
 A_2 = area of the peak due to all-*trans*-retinol in the chromatogram obtained with reference solution (b),
 C = concentration of *retinol acetate CRS* in reference solution (a) as assessed prior to the saponification, in International Units per millilitre (= 1000 IU/ml),
 V = volume of reference solution (a) treated (2.00 ml),
 m = mass of the substance to be examined in the test solution (2.00 g).

Vitamin D₃. Liquid chromatography (2.2.29). Carry out the assay as rapidly as possible, avoiding exposure to actinic light and air.

Internal standard solution. Dissolve 0.50 mg of *ergocalciferol CRS* in 100 ml of *ethanol R*.

Test solution (a). To 4.00 g in a round-bottomed flask, add 5 ml of a freshly prepared 100 g/l solution of *ascorbic acid R*, 10 ml of a freshly prepared 800 g/l solution of *potassium hydroxide R* and 100 ml of *ethanol R*. Boil under a reflux condenser on a water-bath for 30 min. Add 100 ml of a 10 g/l solution of *sodium chloride R* and cool the solution to room temperature. Transfer the solution to a 500 ml separating funnel rinsing the round-bottomed flask with about 75 ml of a 10 g/l solution of *sodium chloride R* and then with 150 ml of a mixture of equal volumes of *light petroleum R1* and *ether R*. Shake for 1 min. When the layers have separated completely, discard the lower layer and wash the upper layer, first with 50 ml of a 30 g/l solution of *potassium hydroxide R* in a 10 per cent V/V solution of *ethanol R*, and then with 3 quantities, each of 50 ml, of a 10 g/l solution of *sodium chloride R*. Filter the upper layer through 5 g of *anhydrous sodium sulphate R* on a fast filter paper into a 250 ml flask suitable for a rotary evaporator. Wash the funnel with 10 ml of fresh extraction mixture, filter and combine the upper layers. Distil them at a temperature not exceeding 30 °C under reduced pressure (water ejector) and fill with *nitrogen R* when evaporation is completed. Alternatively evaporate the solvent under a gentle current of *nitrogen R* at a temperature not exceeding 30 °C. Dissolve the residue in 1.5 ml of the mobile phase described under Purification. Gentle heating in an ultrasonic bath may be required. (A large fraction of the white residue is cholesterol, constituting approximately 50 per cent *m/m* of the unsaponifiable matter of cod-liver oil).

Test solution (b). To 4.00 g add 2.0 ml of the internal standard solution and proceed as described for test solution (a).

Reference solution (a). Dissolve 0.50 mg of *cholecalciferol CRS* in 100.0 ml of *ethanol R*.

Reference solution (b). In a round-bottomed flask, add 2.0 ml of reference solution (a) and 2.0 ml of the internal standard solution and proceed as described for test solution (a).

PURIFICATION

Column:

- size: $l = 0.25$ m, $\emptyset = 4.6$ mm,
- stationary phase: *nitrile silica gel for chromatography R* (film thickness 10 μ m).

Mobile phase: *isoamyl alcohol R*, *hexane R* (1.6:98.4 V/V).

Flow rate: 1.1 ml/min.

Detection: spectrophotometer at 265 nm.

Inject 350 μ l of reference solution (b). Collect the eluate from 2 min before until 2 min after the retention time of *cholecalciferol*, in a ground-glass-stoppered tube containing 1 ml of a 1 g/l solution of *butylhydroxytoluene R* in *hexane R*. Repeat the procedure with test solutions (a) and (b). Evaporate the eluates obtained from reference solution (b) and from test solutions (a) and (b), separately, to dryness at a temperature not exceeding 30 °C under a gentle current of *nitrogen R*. Dissolve each residue in 1.5 ml of *acetonitrile R*.

DETERMINATION

Column:

- size: $l = 0.15$ m, $\emptyset = 4.6$ mm,
- stationary phase: *octadecylsilyl silica gel for chromatography R* (film thickness 5 μ m).

Mobile phase: phosphoric acid R, a 96 per cent V/V solution of acetonitrile R (0.2:99.8 V/V).

Flow rate: 1.0 ml/min.

Detection: spectrophotometer at 265 nm.

Injection: 2 quantities not exceeding 200 µl of each of the 3 solutions obtained under Purification.

System suitability:

- **resolution:** minimum 1.4 between the peaks due to ergocalciferol and cholecalciferol in the chromatogram obtained with reference solution (b),
- when using the method of standard additions to test solution (a) there is greater than 95 per cent recovery of the added *cholecalciferol* CRS when due consideration has been given to correction by the internal standard.

Calculate the content of vitamin D₃ in International Units per gram using the following expression, taking into account the assigned content of *cholecalciferol* CRS:

$$\frac{A_2}{A_6} \times \frac{A_3}{A_4 - \left[\frac{A_5}{A_1} \right] \times A_2} \times \frac{m_2}{m_1} \times \frac{V_2}{V_1} \times 40$$

- m_1 = mass of the sample in test solution (b) in grams,
 m_2 = total mass of *cholecalciferol* CRS used for the preparation of reference solution (a) in micrograms (500 µg),
 A_1 = area (or height) of the peak due to cholecalciferol in the chromatogram obtained with test solution (a),
 A_2 = area (or height) of the peak due to cholecalciferol in the chromatogram obtained with test solution (b),
 A_3 = area (or height) of the peak due to ergocalciferol in the chromatogram obtained with reference solution (b),
 A_4 = area (or height) of the peak due to ergocalciferol in the chromatogram obtained with test solution (b),
 A_5 = area (or height) of a possible peak in the chromatogram obtained with test solution (a) with the same retention time as the peak co-eluting with ergocalciferol in test solution (b),
 A_6 = area (or height) of the peak due to cholecalciferol in the chromatogram obtained with reference solution (b),
 V_1 = total volume of reference solution (a) (100 ml),
 V_2 = volume of reference solution (a) used for preparing reference solution (b) (2.0 ml).

STORAGE

In an airtight and well-filled container, protected from light. If no antioxidant is added, store under an inert gas.

Once the container has been opened, its contents are used as soon as possible and any part of the contents not used at once is protected by an atmosphere of inert gas.

LABELLING

The label states:

- the number of International Units of vitamin A,
- the number of International Units of vitamin D₃,
- the name and concentration of any added antioxidant.

COLA

Colae semen

DEFINITION

Cola consists of the whole or fragmented dried seeds, freed from the testa, of *Cola nitida* (Vent.) Schott et Endl. (*C. vera* K. Schum.) and its varieties, as well as of *Cola acuminata* (P. Beauv.) Schott et Endl. (*Sterculia acuminata* P. Beauv.). It contains not less than 1.5 per cent of caffeine (M_r 194.2), calculated with reference to the dried drug.

CHARACTERS

It has the macroscopic and microscopic characters described under identification tests A and B.

IDENTIFICATION

- A. The kernels have an oblong, somewhat obtuse, sub-tetragonal shape, with deformations resulting from mutual pressure inside the fruit; they vary in size and mass, ranging from 5 g to 15 g; the outside is hard, smooth and very dark brown, the inside is more reddish-brown. In *C. nitida* and its varieties, the kernels are divided in two parts, almost plano-convex, corresponding to the cotyledons and usually occurring separated in the commercial drug; the cotyledons are 3 cm to 4 cm long, 2 cm to 2.5 cm wide and 1 cm to 2 cm thick. In *C. acuminata*, the cotyledons are smaller and divided into four to six irregular parts.
- B. Reduce to a powder (355). The powder is reddish-brown. Examine under a microscope using a 50 per cent V/V solution of *glycerol* R. The powder shows numerous ovoid or reniform starch granules, 5 µm to 25 µm in size, with concentric striations and a stellate, slightly eccentric hilum; fragments of cotyledon tissue showing large, thick-walled, reddish polygonal cells filled with starch granules; occasional fragments of the external epidermis of the cotyledons.
- C. Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel F₂₅₄ plate* R.
- Test solution.** To 1.0 g of the powdered drug (355), add 5 ml of *alcohol* (60 per cent V/V) R. Shake mechanically at 40 °C for 30 min and filter.

Reference solution (a). Dissolve 25 mg of *caffeine* R in 10 ml of *alcohol* (60 per cent V/V) R.

Reference solution (b). Dissolve 50 mg of *theobromine* R in 10 ml of a mixture of 10 volumes of *water* R, 13 volumes of *methanol* R and 77 volumes of *ethyl acetate* R. Filter.

Apply to the plate as bands 20 µl of each solution. Develop over a path of 10 cm using a mixture of 10 volumes of *water* R, 13 volumes of *methanol* R and 77 volumes of *ethyl acetate* R. Allow the plate to dry in air for 5 min. Examine in ultraviolet light at 254 nm. The chromatogram obtained with the test solution shows two principal quenching zones which are similar in position to the zones in the chromatograms obtained with reference solutions (a) and (b), respectively. Spray the plates with a mixture of equal volumes of *alcohol* R and *hydrochloric acid* R and then with a solution prepared immediately before use by dissolving 1 g of *iodine* R and 1 g of *potassium iodide* R in 100 ml of *alcohol* R. The chromatogram obtained with the test solution shows a reddish-brown principal zone similar in position and colour to the zone in the chromatogram obtained with reference solution (a).

TESTS

01/2005:0758

Foreign matter (2.8.2). It complies with the test for foreign matter.

Loss on drying (2.2.32). Not more than 12.0 per cent, determined on 2.00 g of the powdered drug (355) by drying in an oven at 100–105 °C for 2 h.

Total ash (2.4.16). Not more than 9.0 per cent.

ASSAY

Examine by liquid chromatography (2.2.29).

Test solution. To 1.00 g (m_1) of the powdered drug (355), add 50 ml of *methanol R*. Heat the mixture under a reflux condenser on a water-bath for 30 min. Allow to cool and filter. Rinse the filter with 10 ml of *methanol R*. Take up the residue with 50 ml of *methanol R*. Proceed as before. Combine the filtrates and the washings in a 200.0 ml volumetric flask and dilute to 200.0 ml with *methanol R*. Transfer 20.0 ml of this solution into a round-bottomed flask and evaporate to dryness under reduced pressure. Take up the residue with the mobile phase, transfer to a 50.0 ml volumetric flask and dilute to 50.0 ml with the mobile phase.

Reference solution. In a 100.0 ml volumetric flask, dissolve 30.0 mg (m_2) of *caffeine R* and 15.0 mg of *theobromine R* in the mobile phase and dilute to 100.0 ml with the mobile phase. Transfer 10.0 ml of this solution to a 100.0 ml volumetric flask and dilute to 100.0 ml with the mobile phase.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4.6 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (5 µm),
- as mobile phase at a flow rate of 1 ml/min a mixture of 25 volumes of *methanol R* and 75 volumes of *water R*,
- as detector a spectrophotometer set at 272 nm,
- a loop injector.

Inject appropriate volumes of each solution. The test is not valid unless: in the chromatogram obtained with the reference solution, the resolution between the peaks corresponding respectively to caffeine and theobromine is at least 2.5. If necessary, adjust the volume of *water R* in the mobile phase.

Calculate the caffeine content using the expression:

$$\frac{m_2 \times A_1 \times 50}{m_1 \times A_2}$$

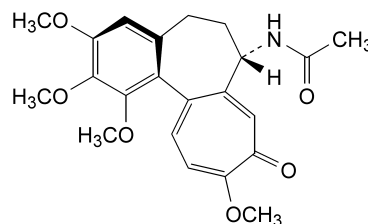
- A_1 = area of the peak due to caffeine in the chromatogram obtained with the test solution,
- A_2 = area of the peak due to caffeine in the chromatogram obtained with the reference solution,
- m_1 = mass of the drug to be examined in the test solution, in grams,
- m_2 = mass of *caffeine R* in the reference solution, in grams.

STORAGE

Store protected from light.

COLCHICINE

Colchicinum

C₂₂H₂₅NO₆ M_r 399.4

DEFINITION

(-)-N-[(7S,12aS)-1,2,3,10-Tetramethoxy-9-oxo-5,6,7,9-tetrahydrobenzo[a]heptalen-7-yl]acetamide.

Content: 97.0 per cent to 102.0 per cent (anhydrous and ethyl acetate-free substance).

CHARACTERS

Appearance: yellowish-white, amorphous or crystalline powder.

Solubility: very soluble in water, rapidly recrystallising from concentrated solutions as the sesquihydrate, freely soluble in alcohol, practically insoluble in cyclohexane.

IDENTIFICATION

First identification: B.

Second identification: A, C, D.

- A. Dissolve 5 mg in *alcohol R* and dilute to 100.0 ml with the same solvent. Dilute 5.0 ml of the solution to 25.0 ml with *alcohol R*. Examined between 230 nm and 400 nm (2.2.25), the solution shows 2 absorption maxima, at 243 nm and 350 nm. The ratio of the absorbance measured at 243 nm to that measured at 350 nm is 1.7 to 1.9.
- B. Infrared absorption spectrophotometry (2.2.24).
Preparation: discs of *potassium bromide R*.
Comparison: *colchicine CRS*.
- C. To 0.5 ml of solution S (see Tests) add 0.5 ml of *dilute hydrochloric acid R* and 0.15 ml of *ferric chloride solution R1*. The solution is yellow and becomes dark green on boiling for 30 s. Cool, add 2 ml of *methylene chloride R* and shake. The organic layer is greenish-yellow.
- D. Dissolve about 30 mg in 1 ml of *alcohol R* and add 0.15 ml of *ferric chloride solution R1*. A brownish-red colour develops.

TESTS

Solution S. Dissolve 0.10 g in *water R* and dilute to 20 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and not more intensely coloured than reference solution GY₃ (2.2.2, Method II).

Acidity or alkalinity. To 10 ml of solution S add 0.1 ml of *bromothymol blue solution R1*. Either the solution does not change colour or it becomes green. Not more than 0.1 ml of 0.01 M *sodium hydroxide* is required to change the colour of the indicator to blue.

Specific optical rotation (2.2.7): -235 to -250 (anhydrous and ethyl acetate-free substance).

Dissolve 50.0 mg in *alcohol R* and dilute to 10.0 ml with the same solvent.

Related substances. Liquid chromatography (2.2.29).

Test solution. Dissolve 20.0 mg of the substance to be examined in a mixture of equal volumes of *methanol R* and *water R* and dilute to 20.0 ml with the same mixture of solvents.

Reference solution (a). Dissolve 20.0 mg of *colchicine for system suitability CRS* in a mixture of equal volumes of *methanol R* and *water R* and dilute to 20.0 ml with the same mixture of solvents.

Reference solution (b). Dilute 1.0 ml of the test solution to 100.0 ml with a mixture of equal volumes of *methanol R* and *water R*.

Reference solution (c). Dilute 1 ml of reference solution (b) to 20.0 ml with a mixture of equal volumes of *methanol R* and *water R*.

Column:

- size: $l = 0.25$ m, $\varnothing = 4.6$ mm,
- stationary phase: octylsilyl silica gel for chromatography *R1* (5 μ m).

Mobile phase: mix 450 volumes of a 6.8 g/l solution of *potassium dihydrogen phosphate R* and 530 volumes of *methanol R*. After cooling to room temperature, adjust the volume to 1000 ml with *methanol R*. Adjust the apparent pH to 5.5 with *dilute phosphoric acid R*.

Flow rate: 1 ml/min.

Detection: spectrophotometer at 254 nm.

Injection: 20 μ l.

Run time: 3 times the retention time of colchicine.

Relative retention with reference to colchicine (retention time = about 7 min): impurity D = about 0.4; impurity E = about 0.7; impurity B = about 0.8; impurity A = about 0.94; impurity C = about 1.2.

System suitability: reference solution (a):

Peak-to-valley ratio: minimum 2, where H_p = height above the baseline of the peak due to impurity A and H_v = height above the baseline of the lowest point of the curve separating this peak from the peak due to colchicine.

Limits:

- **impurity A:** not more than 3.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (3.5 per cent),
- **any other impurity:** not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (1 per cent),
- **total:** not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (5 per cent),
- **disregard limit:** area of the principal peak in the chromatogram obtained with reference solution (c) (0.05 per cent).

Colchicine: maximum 0.2 per cent.

Dissolve 50 mg in *water R* and dilute to 5 ml with the same solvent. Add 0.1 ml of *ferric chloride solution R1*. The solution is not more intensely coloured than a mixture of 1 ml of red primary solution, 2 ml of yellow primary solution and 2 ml of blue primary solution (2.2.2, *Method II*).

Chloroform (2.4.24): maximum 500 ppm.

Ethyl acetate (2.4.24): maximum 6.0 per cent *m/m*.

Water (2.5.12): maximum 2.0 per cent, determined on 0.500 g.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 0.5 g.

ASSAY

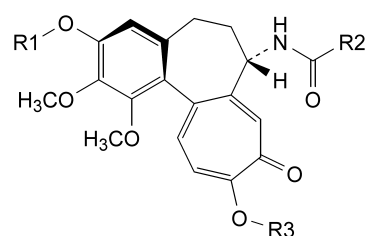
Dissolve 0.250 g with gentle heating in a mixture of 10 ml of *acetic anhydride R* and 20 ml of *toluene R*. Titrate with 0.1 *M perchloric acid*, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 *M perchloric acid* is equivalent to 39.94 mg of $C_{22}H_{25}NO_6$.

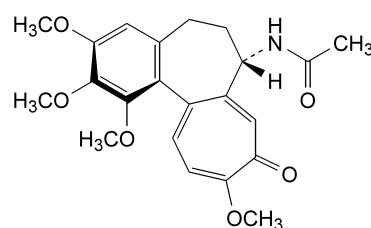
STORAGE

Protected from light.

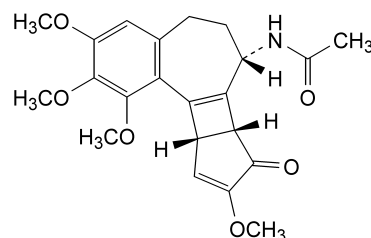
IMPURITIES



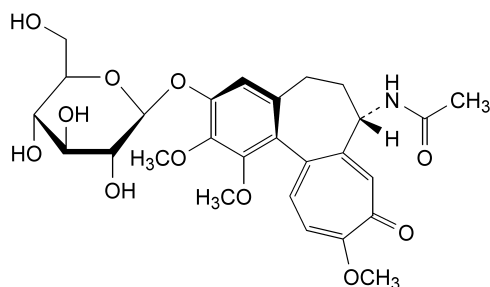
- A. $R1 = R3 = CH_3$, $R2 = H$: *N*[(7*S*,12*aS*)-1,2,3,10-tetramethoxy-9-oxo-5,6,7,9-tetrahydrobenzo[*a*]heptalen-7-yl]formamide (*N*-deacetyl-*N*-formylcolchicine),
- E. $R1 = H$, $R2 = R3 = CH_3$: *N*[(7*S*,12*aS*)-3-hydroxy-1,2,10-trimethoxy-9-oxo-5,6,7,9-tetrahydrobenzo[*a*]heptalen-7-yl]acetamide (3-*O*-demethylcolchicine),
- F. $R1 = R2 = CH_3$, $R3 = H$: *N*[(7*S*,12*aS*)-10-hydroxy-1,2,3-trimethoxy-9-oxo-5,6,7,9-tetrahydrobenzo[*a*]heptalen-7-yl]acetamide (colchicine),



- B. (-)-*N*[(7*S*,12*aR*)-1,2,3,10-tetramethoxy-9-oxo-5,6,7,9-tetrahydrobenzo[*a*]heptalen-7-yl]acetamide (conformational isomer),



- C. *N*[(7*S*,7*bR*,10*aS*)-1,2,3,9-tetramethoxy-8-oxo-5,6,7,7*b*,8,10*a*-hexahydrobenzo[*a*]cyclopenta[3,4]-cyclobuta[1,2-*c*]cyclohepten-7-yl]acetamide (β -lumicolchicine),



D. *N*-[(7*S*,12*aS*)-3-(β-*D*-glucopyranosyloxy)-1,2,10-trimethoxy-9-oxo-5,6,7,9-tetrahydrobenzo[*a*]heptalen-7-yl]acetamide (colchicoside).

01/2005:1775

COLESTYRAMINE

Colestyraminum

DEFINITION

Strongly basic anion-exchange resin in chloride form, consisting of styrene-divinylbenzene copolymer with quaternary ammonium groups.

Nominal exchange capacity: 1.8 g to 2.2 g of sodium glycocholate per gram (dried substance).

CHARACTERS

Appearance: white or almost white, fine powder, hygroscopic.

Solubility: insoluble in water, in methylene chloride and in ethanol (96 per cent).

IDENTIFICATION

A. Infrared absorption spectrophotometry (2.2.24).

Comparison: colestyramine CRS.

B. It complies with the test for chlorides (see Tests).

TESTS

pH (2.2.3): 4.0 to 6.0.

Suspend 0.100 g in 10 ml of *water R* and allow to stand for 10 min.

Dialysable quaternary amines: maximum 500 ppm, expressed as benzyltrimethylammonium chloride.

Test solution. Place a 25 cm piece of cellulose dialysis tubing having a molecular weight cut-off of 12 000-14 000 and an inflated diameter of 3-6 cm (flat width of 5-9 cm) in *water R* to hydrate until pliable, appropriately sealing one end. Introduce 2.0 g of the substance to be examined into the tube and add 10 ml of *water R*. Seal the tube and completely immerse it in 100 ml of *water R* in a suitable vessel and stir the liquid for 16 h to effect dialysis. Use the dialysate as test solution.

Reference solution. Prepare the reference solution in a similar manner but using 10 ml of a freshly prepared 0.1 g/l solution of *benzyltrimethylammonium chloride R* instead of the substance to be examined.

Transfer 5.0 ml of the test solution to a separating funnel and add 5 ml of a 3.8 g/l solution of *disodium tetraborate R*, 1 ml of a solution containing 1.5 g/l of *bromothymol blue R* and 4.05 g/l of *sodium carbonate R* and 10 ml of *chloroform R*. Shake the mixture vigorously for 1 min, allow the phases to separate and transfer the clear organic layer to a 25 ml volumetric flask. Repeat the extraction with a further 10 ml of *chloroform R*, combine the organic layers and dilute to 25 ml with *chloroform R*. Measure the absorbance (2.2.25) of

the solution at the absorption maximum at 420 nm, using as compensation liquid a solution prepared in the same manner but using 5.0 ml of *water R* instead of the test solution.

Repeat the operation using 5.0 ml of the reference solution.

The absorbance obtained with the test solution is not greater than that obtained with the reference solution.

Impurity A. Liquid chromatography (2.2.29).

Test solution. Shake 5.0 g with 10 ml of *acetone R* for 30 min. Centrifuge and use the supernatant liquid.

Reference solution (a). Dissolve 5 mg of *styrene R* in *acetone R* and dilute to 100.0 ml with the same solvent. Dilute 1.0 ml to 100.0 ml with *acetone R*.

Reference solution (b). Dissolve 0.35 ml of *styrene R* in *acetone R* and dilute to 100.0 ml with the same solvent. Dilute 1.0 ml to 100.0 ml with *acetone R*.

Reference solution (c). Dissolve 0.35 ml of *toluene R* in *acetone R* and dilute to 100.0 ml with the same solvent.

Reference solution (d). Mix 1.0 ml of reference solution (b) and 1.0 ml of reference solution (c) with *acetone R* and dilute to 100.0 ml with the same solvent.

Column:

- **size:** $l = 0.30$ m, $\varnothing = 3.9$ mm,
- **stationary phase:** *octadecylsilyl silica gel for chromatography R* (10 μ m) with a specific surface area of 330 m²/g and a pore size of 12.5 nm.

Mobile phase: *acetonitrile R*, *water R* (50:50 V/V).

Flow rate: 2.0 ml/min.

Detection: spectrophotometer at 254 nm.

Injection: 20 μ l of test solution, reference solutions (a) and (d).

System suitability: reference solution (d):

- **resolution:** minimum 1.5 between the peaks due to impurity A and toluene.

Limit:

- **impurity A:** not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (1 ppm).

Chloride: 13.0 per cent to 17.0 per cent (dried substance).

To 0.2 g add 100 ml of *water R* and 50 mg of *potassium nitrate R*. Add, with stirring, 2 ml of *nitric acid R* and titrate with 0.1 *M silver nitrate*, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 *M silver nitrate* is equivalent to 3.55 mg of Cl.

Heavy metals (2.4.8): maximum 20 ppm.

1.0 g complies with limit test F. Prepare the reference solution using 2 ml of *lead standard solution (10 ppm Pb) R*.

Loss on drying (2.2.32): maximum 12 per cent, determined on 1.000 g by drying in an oven at 70 °C over *diphosphorus pentoxide R* at a pressure not exceeding 7 kPa for 16 h.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Exchange capacity. Liquid chromatography (2.2.29).

Solution A. Dissolve 1.500 g of *sodium glycocholate R* in a solution containing 4 g/l of *potassium dihydrogen phosphate R* and 12 g/l of *dipotassium hydrogen phosphate R* and dilute to 100.0 ml with the same solution.

Test solution. Add 20.0 ml of solution A to a quantity of the substance to be examined equivalent to about 0.100 g of the dried substance. Shake mechanically for 2 h and centrifuge for 15 min. Dilute 5.0 ml of the supernatant liquid to 50.0 ml with *water R*.

Reference solution (a). Dilute 4.0 ml of solution A to 100.0 ml with *water R*.

Reference solution (b). Dissolve 60 mg of *sodium glycocholate R* and 30 mg of *sodium taurodeoxycholate R* in *water R* and dilute to 100 ml with the same solvent. Dilute 1 ml of the solution to 10 ml with *water R*.

Column:

- **size:** $l = 0.25$ m, $\varnothing = 4.6$ mm,
- **stationary phase:** octadecylsilyl silica gel for chromatography *R* (5 μ m).

Mobile phase: mix 35 volumes of *acetonitrile R* and 65 volumes of a 10.9 g/l solution of *potassium dihydrogen phosphate R* adjusted to pH 3.0 with *phosphoric acid R*.

Flow rate: 1.5 ml/min.

Detection: spectrophotometer at 214 nm.

Injection: 50 μ l.

Run time: twice the retention time of glycocholate.

System suitability: reference solution (b):

- **resolution:** minimum 1.5 between the peaks due to glycocholate and taurodeoxycholate.

Calculate the nominal exchange capacity using the following expression:

$$\frac{(2.5 A_1 - A_2) \times m_1 \times 0.73}{12.5 \times A_1 \times m_2}$$

- A_1 = area of the peak due to glycocholate in the chromatogram obtained with reference solution (a),
- A_2 = area of the peak due to glycocholate in the chromatogram obtained with the test solution,
- m_1 = mass, in milligrams, of *sodium glycocholate R* used in the preparation of solution A,
- m_2 = mass, in milligrams, of the dried substance to be examined used in the preparation of the test solution,
- 0.73 = correction factor to convert the true exchange capacity to the conventionally used nominal exchange capacity.

STORAGE

In an airtight container.

IMPURITIES

Specified impurities: A.

A. styrene.

01/2005:0319

COLISTIMETHATE SODIUM

Colistimethatum natricum

DEFINITION

Colistimethate sodium is prepared from colistin by the action of formaldehyde and sodium hydrogen sulphite. The potency is not less than 11 500 IU/mg, calculated with reference to the dried substance.

CHARACTERS

A white or almost white powder, hygroscopic, very soluble in water, slightly soluble in alcohol, practically insoluble in acetone.

IDENTIFICATION

A. Examine by thin-layer chromatography (2.2.27), using *silica gel G R* as the coating substance.

Test solution. Dissolve 5 mg of the substance to be examined in 1 ml of a mixture of equal volumes of *hydrochloric acid R* and *water R*. Heat at 135 °C in a sealed tube for 5 h. Evaporate to dryness on a water-bath and continue the heating until the hydrochloric acid has evaporated. Dissolve the residue in 0.5 ml of *water R*.

Reference solution (a). Dissolve 20 mg of *leucine R* in *water R* and dilute to 10 ml with the same solvent.

Reference solution (b). Dissolve 20 mg of *threonine R* in *water R* and dilute to 10 ml with the same solvent.

Reference solution (c). Dissolve 20 mg of *phenylalanine R* in *water R* and dilute to 10 ml with the same solvent.

Reference solution (d). Dissolve 20 mg of *serine R* in *water R* and dilute to 10 ml with the same solvent.

Carry out the following procedures protected from light.

Apply to the plate as 10 mm bands 5 μ l of each solution. Place the plate in the chromatographic tank so that it is not in contact with the mobile phase consisting of a mixture of 25 volumes of *water R* and 75 volumes of *phenol R*. Leave the plate to become impregnated with the vapour of the solvent for at least 12 h. Develop over a path of 12 cm using the same mobile phase. Dry the plate at 100–105 °C and spray with *ninhydrin solution R1*. Heat at 110 °C for 5 min. The chromatogram obtained with the test solution shows zones corresponding to those in the chromatograms obtained with reference solutions (a) and (b), but shows no zones corresponding to those in the chromatograms obtained with reference solutions (c) and (d). The chromatogram obtained with the test solution also shows a zone with a very low R_f value (2,4-diaminobutyric acid).

B. Dissolve about 5 mg in 3 ml of *water R*. Add 3 ml of *dilute sodium hydroxide solution R*. Shake and add 0.5 ml of a 10 g/l solution of *copper sulphate R*. A violet colour is produced.

C. Dissolve about 50 mg in 1 ml of 1 M *hydrochloric acid* and add 0.5 ml of 0.01 M *iodine*. The solution is decolourised and gives reaction (a) of sulphates (2.3.1).

D. It gives reaction (b) of sodium (2.3.1).

TESTS

Appearance of solution. Dissolve 0.16 g in 10 ml of *water R*. The solution is clear (2.2.1).

pH (2.2.3). Dissolve 0.1 g in *carbon dioxide-free water R* and dilute to 10 ml with the same solvent. The pH of the solution, measured after 30 min, is 6.5 to 8.5.

Specific optical rotation (2.2.7). Dissolve 1.25 g in *water R* and dilute to 25.0 ml with the same solvent. The specific optical rotation is –46 to –51, calculated with reference to the dried substance.

Free colistin. Dissolve 80 mg in 3 ml of *water R*. Add 0.1 ml of a 100 g/l solution of *silicotungstic acid R*; 10 s to 20 s after addition of the reagent, the solution is not more opalescent than reference suspension II (2.2.1).

Total sulphite. *Work in a fume cupboard.* Dissolve 0.100 g in 50 ml of *water R* and add 5 ml of a 100 g/l solution of *sodium hydroxide R* and 0.3 g of *potassium cyanide R*. Boil gently for 3 min and then cool. Neutralise with 0.5 M *sulphuric acid* using 0.2 ml of *methyl orange solution R* as indicator. Add an excess of 0.5 ml of the acid and 0.2 g of *potassium iodide R*. Titrate with 0.05 M *iodine* using 1 ml of *starch solution R* as indicator. The volume of 0.05 M *iodine* used in the titration is 5.5 ml to 7.0 ml.

Reference solution (a). Dissolve 25.0 mg of *colistin sulphate CRS* in 40 ml of *water R* and dilute to 50.0 ml with *acetonitrile R*.

Reference solution (b). Dilute 1.0 ml of reference solution (a) to 100.0 ml with a mixture of 20 volumes of *acetonitrile R* and 80 volumes of *water R*.

Column:

- size: $l = 0.15$ m, $\varnothing = 4.6$ mm,
- stationary phase: *end-capped octadecylsilyl silica gel for chromatography R* (3.5 μ m),
- temperature: 30 °C.

Mobile phase: mix 22 volumes of *acetonitrile R* and 78 volumes of a solution prepared as follows: dissolve 4.46 g of *anhydrous sodium sulphate R* in 900 ml of *water R*, add 2.5 ml of *phosphoric acid R* and dilute to 1000 ml with *water R* (pH 2.3 to 2.5).

Flow rate: 1.0 ml/min.

Detection: spectrophotometer at 215 nm.

Injection: 20 μ l.

Run time: 1.5 times the retention time of colistin E1.

Relative retention with reference to colistin E1 (retention time = about 16 min): colistin E2 = about 0.45; colistin E3 = about 0.5; colistin E1-I = about 0.8; colistin E1-7MOA = about 1.1.

System suitability: reference solution (a):

- **resolution:** minimum 8.0 between the peaks due to colistin E2 and colistin E1, minimum 6.0 between the peaks due to colistin E2 and colistin E1-I, minimum 2.5 between the peaks due to colistin E1-I and colistin E1, minimum 1.5 between the peaks due to colistin E1 and colistin E1-7MOA,
- the chromatogram obtained is concordant with the chromatogram supplied with *colistin sulphate CRS*.

Limits:

- **any impurity:** maximum 4.0 per cent,
- **total:** maximum 23.0 per cent,
- **disregard limit:** the area of the peak due to colistin E1 in the chromatogram obtained with reference solution (b); disregard the peaks due to colistin E2, E3, E1-I, E1 and E1-7MOA.

Sulphate: 16.0 per cent to 18.0 per cent (dried substance).

Dissolve 0.250 g in 100 ml of *water R* and adjust to pH 11 using *concentrated ammonia R*. Add 10.0 ml of 0.1 M *barium chloride* and about 0.5 mg of *phthalein purple R*. Titrate with 0.1 M *sodium edetate*, adding 50 ml of *alcohol R* when the colour of the solution begins to change and continuing the titration until the violet-blue colour disappears.

1 ml of 0.1 M *barium chloride* is equivalent to 9.606 mg of SO_4 .

Loss on drying (2.2.32): maximum 3.5 per cent, determined on 1.000 g by drying at 60 °C over *diphosphorus pentoxide R* at a pressure not exceeding 670 Pa for 3 h.

Sulphated ash (2.4.14): maximum 1.0 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (2.2.29) as described in the test for related substances with the following modification.

Injection: test solution and reference solution (a).

Calculate the percentage content of the sum of colistins E2, E3, E1-I, E1 and E1-7MOA, the percentage content of colistin E3, the percentage content of colistin E1-I and

the percentage content of colistin E1-7MOA using the chromatogram obtained with reference solution (a) and the declared contents of *colistin sulphate CRS*.

STORAGE

In an airtight container, protected from light.

01/2005:1862

COLOPHONY

Colophonium

DEFINITION

Residue remaining after distillation of the volatile oil from the oleoresin obtained from various species of *Pinus*.

CHARACTERS

Macroscopic characters described under identification A.

IDENTIFICATION

A. Translucent, pale yellow to brownish-yellow, angular, irregularly-shaped, brittle, glassy pieces of different sizes the surfaces of which bear conchoidal markings.

B. Thin-layer chromatography (2.2.27).

Test solution. Dissolve 1 g in 10 ml of *methanol R* by gently warming.

Reference solution. Dissolve 10 mg of *thymol R* and 10 mg of *linalol R* in 10 ml of *methanol R*.

Plate: *TLC silica gel plate R*.

Mobile phase: *methylene chloride R*.

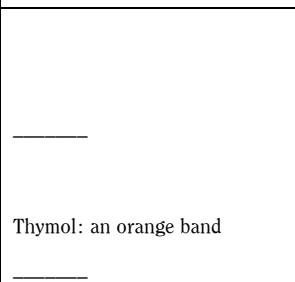
Application: 10 μ l, as bands.

Development: over a path of 15 cm.

Drying: in air.

Detection: spray with *anisaldehyde solution R* and heat at 100-105 °C for 10 min; examine in daylight.

Results: see below the sequence of the zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other coloured zones are present in the chromatogram obtained with the test solution.

Top of the plate	
 Thymol: an orange band Linalol: a purple band	A purple band
	A purple band
	2 purple bands
	Sequence of narrow purple bands
Reference solution	Test solution
	Purple extended baseline band

TESTS

Acid value (2.5.1): 145 to 180, determined on 1.0 g.

Foreign matter (2.8.2): maximum 2 per cent.

Total ash (2.4.16): maximum 0.2 per cent.

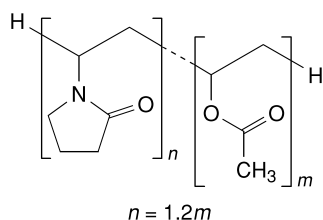
STORAGE

Do not reduce to a powder.

01/2005:0891 *Reference solution.* Dissolve 0.140 g of *acetaldehyde ammonia trimer trihydrate R* in *water R* and dilute to 200.0 ml with the same solvent. Dilute 1.0 ml of this solution to 100.0 ml with *phosphate buffer solution pH 9.0 R*.

COPOVIDONE

Copovidonum



$(C_6H_9NO)_n, (C_4H_6O_2)_m$

$M_r (111.1)_n + (86.1)_m$

DEFINITION

Copovidone is a copolymer of 1-ethenylpyrrolidin-2-one and ethenyl acetate in the mass proportion 3:2.

Content:

- nitrogen (N; A_r 14.01): 7.0 per cent to 8.0 per cent (dried substance),
- ethenyl acetate $C_4H_6O_2$; M_r 86.10): 35.3 per cent to 42.0 per cent (dried substance).

K-value: 90.0 per cent to 110.0 per cent of the value stated on the label.

CHARACTERS

Aspect: white or yellowish-white powder or flakes, hygroscopic.

Solubility: freely soluble in water, in alcohol and in methylene chloride.

IDENTIFICATION

First identification: A.

Second identification: B, C.

A. Infrared absorption spectrophotometry (2.2.24).

Comparison: Ph. Eur. reference spectrum of copovidone.

B. To 1 ml of solution S (see Tests) add 5 ml of *water R* and 0.2 ml of 0.05 M iodine. A red colour appears.

C. Dissolve 0.7 g of *hydroxylamine hydrochloride R* in 10 ml of *methanol R*, add 20 ml of a 40 g/l solution of *sodium hydroxide R* and filter if necessary. To 5 ml of the solution add 0.1 g of the substance to be examined and boil for 2 min. Transfer 50 µl to a filter paper and add 0.1 ml of a mixture of equal volumes of *ferric chloride solution R1* and *hydrochloric acid R*. A violet colour appears.

TESTS

Solution S. Dissolve 10 g in *water R* and dilute to 100 ml with the same solvent. Add the substance to be examined to the *water R* in small portions with constant stirring.

Appearance of solution. Solution S is not more opalescent than reference suspension III (2.2.1) and not more intensely coloured than reference solution B₅, R₅ or BY₅ (2.2.2, Method II).

Aldehydes: maximum 500 ppm, expressed as acetaldehyde.

Test solution. Dissolve 1.0 g of the substance to be examined in *phosphate buffer solution pH 9.0 R* and dilute to 100.0 ml with the same solvent. Stopper the flask and heat at 60 °C for 1 h. Allow to cool.

Into 3 identical spectrophotometric cells with a path length of 1 cm, introduce separately 0.5 ml of the test solution, 0.5 ml of the reference solution and 0.5 ml of *water R* (blank). To each cell, add 2.5 ml of *phosphate buffer solution pH 9.0 R* and 0.2 ml of *nicotinamide-adenine dinucleotide solution R*. Mix and stopper tightly. Allow to stand at 22 ± 2 °C for 2-3 min and measure the absorbance (2.2.25) of each solution at 340 nm, using *water R* as the compensation liquid. To each cell, add 0.05 ml of *aldehyde dehydrogenase solution R*, mix and stopper tightly. Allow to stand at 22 ± 2 °C for 5 min. Measure the absorbance of each solution at 340 nm using *water R* as compensation liquid. Determine the content of aldehydes using the expression:

$$\frac{(A_{t2} - A_{t1}) - (A_{b2} - A_{b1})}{(A_{s2} - A_{s1}) - (A_{b2} - A_{b1})} \times \frac{100\,000 \times C}{m}$$

A_{t1} = absorbance of the test solution before the addition of aldehyde dehydrogenase,

A_{t2} = absorbance of the test solution after the addition of aldehyde dehydrogenase,

A_{s1} = absorbance of the reference solution before the addition of aldehyde dehydrogenase,

A_{s2} = absorbance of the reference solution after the addition of aldehyde dehydrogenase,

A_{b1} = absorbance of the blank before the addition of aldehyde dehydrogenase,

A_{b2} = absorbance of the blank after the addition of aldehyde dehydrogenase,

m = mass of povidone, in grams, calculated with reference to the dried substance,

C = concentration (mg/ml), of acetaldehyde in the reference solution, calculated from the weight of the acetaldehyde ammonia trimer trihydrate with the factor 0.72.

Peroxides: maximum 400 ppm, expressed as H_2O_2 .

Dilute 10 ml of solution S to 25 ml with *water R*. Add 2 ml of *titanium trichloride-sulphuric acid reagent R* and allow to stand for 30 min. The absorbance (2.2.25) of the solution, measured at 405 nm using a mixture of 25 ml of a 40 g/l solution of the substance to be examined and 2 ml of a 13 per cent V/V solution of *sulphuric acid R* as the compensation liquid, is not greater than 0.35.

Hydrazine. Thin-layer chromatography (2.2.27). Use freshly prepared solutions.

Test solution. To 25 ml of solution S add 0.5 ml of a 50 g/l solution of *salicylaldehyde R* in *methanol R*, mix and heat in a water-bath at 60 °C for 15 min. Allow to cool, add 2.0 ml of *xylene R*, shake for 2 min and centrifuge. Use the clear supernatant layer.

Reference solution. Dissolve 9 mg of *salicylaldehydeazine R* in *xylene R* and dilute to 100 ml with the same solvent. Dilute 1 ml of this solution to 10 ml with *xylene R*.

Plate: TLC silanised silica gel plate R.

Mobile phase: *water R*, *methanol R* (20:80 V/V).

Application: 10 µl.

Development: over a path of 15 cm.

Drying: in air.

Detection: examine in ultraviolet light at 365 nm.

Limit:

- *hydrazine*: any spot corresponding to salicylaldehyde azine in the chromatogram obtained with the test solution is not more intense than the spot in the chromatogram obtained with the reference solution (1 ppm).

Monomers: maximum 0.1 per cent.

Dissolve 10.0 g in 30 ml of *methanol R* and add slowly 20.0 ml of *iodine bromide solution R*. Allow to stand for 30 min protected from light with repeated shaking. Add 10 ml of a 100 g/l solution of *potassium iodide R* and titrate with 0.1 M *sodium thiosulphate* until a yellow colour is obtained. Continue titration dropwise until the solution becomes colourless. Carry out a blank titration. Not more than 1.8 ml of 0.1 M *sodium thiosulphate* is used.

Impurity A. Liquid chromatography (2.2.29).

Test solution. Dissolve 100 mg of the substance to be examined in *water R* and dilute to 50.0 ml with the same solvent.

Reference solution. Dissolve 100 mg of 2-pyrrolidone *R* in *water R* and dilute to 100 ml with the same solvent. Dilute 1.0 ml to 100.0 ml with *water R*.

Precolumn:

- *size*: $l = 0.025$ m, $\varnothing = 4$ mm,
- *stationary phase*: end-capped octadecylsilyl silica gel for chromatography *R* (5 μ m).

Column:

- *size*: $l = 0.25$ m, $\varnothing = 4$ mm,
- *stationary phase*: spherical aminohexadecylsilyl silica gel for chromatography *R* (5 μ m),
- *temperature*: 30 °C.

Mobile phase: *water R*, adjusted to pH 2.4 with *phosphoric acid R*.

Flow rate: 1 ml/min.

Detection: spectrophotometer at 205 nm. A detector is placed between the precolumn and the analytical column. A second detector is placed after the analytical column.

Injection: 10 μ l. When impurity A has left the precolumn (after about 1.2 min) switch the flow directly from the pump to the analytical column. Before the next chromatogram is run, wash the precolumn by reversed flow.

Limit:

- *impurity A*: not more than the area of the principal peak obtained with the reference solution (0.5 per cent).

Heavy metals (2.4.8): maximum 20 ppm.

12 ml of solution S complies with limit test A. Prepare the standard using *lead standard solution* (2 ppm Pb) *R*.

Loss on drying (2.2.32): maximum 5.0 per cent, determined on 0.500 g by drying in an oven at 100–105 °C.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

Viscosity, expressed as *K*-value. Dilute 5.0 ml of solution S to 50.0 ml with *water R*. Allow to stand for 1 h and determine the viscosity (2.2.9) of the solution at 25 ± 0.1 °C using viscometer No. 1 with a minimum flow time of 100 s. Calculate the *K*-value from the expression:

$$\frac{1.5 \log \eta - 1}{0.15 + 0.003c} + \frac{\sqrt{300c \log \eta + (c + 1.5c \log \eta)^2}}{0.15c + 0.003c^2}$$

c = percentage concentration (g/100 ml) of the substance to be examined, calculated with reference to the dried substance,

η = viscosity of the solution relative to that of water.

ASSAY

Ethenyl acetate. Determine the saponification value (2.5.6) on 2.00 g of the substance to be examined. Multiply the result obtained by 0.1534 to obtain the percentage content of the ethenyl acetate component.

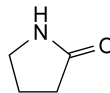
Nitrogen. Carry out the determination of nitrogen (2.5.9) using 30.0 mg of the substance to be examined and 1 g of a mixture of 3 parts of *copper sulphate R* and 997 parts of *dipotassium sulphate R*, heating until a clear, light green solution is obtained and then for a further 45 min.

STORAGE

In an airtight container.

LABELLING

The label states the *K*-value.

IMPURITIES

A. pyrrolidin-2-one (2-pyrrolidone).

01/2005:0893

COPPER SULPHATE, ANHYDROUS**Cupri sulfas anhydricus**

CuSO₄

M_r 159.6

DEFINITION

Anhydrous copper sulphate contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of CuSO₄, calculated with reference to the dried substance.

CHARACTERS

A greenish-grey powder, very hygroscopic, freely soluble in water, slightly soluble in methanol, practically insoluble in alcohol.

IDENTIFICATION

- Add several drops of *dilute ammonia R2* to 1 ml of solution S (see Tests). A blue precipitate is formed on further addition of *dilute ammonia R2*, the precipitate dissolves and a dark blue colour is produced.
- It complies with the test for loss on drying (see Tests).
- Dilute 1 ml of solution S to 5 ml with *water R*. The solution gives reaction (a) of sulphates (2.3.1).

TESTS

Solution S. Dissolve 1.6 g in *water R* and dilute to 50 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1).

Chlorides (2.4.4). Dilute 10 ml of solution S to 15 ml with *water R*. The solution complies with the limit test for chlorides (150 ppm). Examine the tubes laterally against a black background.

Iron. Not more than 150 ppm of Fe, determined by atomic absorption spectrometry (2.2.23, *Method I*).

Test solution. Dissolve 0.32 g in 10 ml of *water R*, add 2.5 ml of *lead-free nitric acid R* and dilute to 25.0 ml with *water R*.

Reference solutions. Prepare the reference solutions using *iron standard solution (20 ppm Fe) R*, adding 2.5 ml of *lead-free nitric acid R* and diluting to 25.0 ml with *water R*.

Measure the absorbance at 248.3 nm using an iron hollow-cathode lamp as a source of radiation and an air-butane flame.

Lead. Not more than 80 ppm of Pb, determined by atomic absorption spectrometry (2.2.23, *Method I*).

Test solution. Dissolve 1.6 g in 10 ml of *water R*, add 2.5 ml of *lead-free nitric acid R* and dilute to 25.0 ml with *water R*.

Reference solutions. Prepare the reference solutions using *lead standard solution (100 ppm Pb) R*, adding 2.5 ml of *lead-free nitric acid R* and diluting to 25.0 ml with *water R*.

Measure the absorbance at 217.0 nm using a lead hollow-cathode lamp as a source of radiation and an air-butane flame.

Loss on drying (2.2.32). Not more than 1.0 per cent, determined on 0.500 g by drying in an oven at 250 °C.

ASSAY

Dissolve 0.125 g in 50 ml of *water R*. Add 2 ml of *sulphuric acid R* and 3 g of *potassium iodide R*. Titrate with 0.1 M *sodium thiosulphate*, using 1 ml of *starch solution R*, added towards the end of the titration, as indicator.

1 ml of 0.1 M *sodium thiosulphate* is equivalent to 15.96 mg of CuSO_4 .

STORAGE

Store in an airtight container.

B. It complies with the test for loss on drying (see Tests).

C. Dilute 1 ml of solution S to 5 ml with *water R*. The solution gives reaction (a) of sulphates (2.3.1).

TESTS

Solution S. Dissolve 5 g in *water R* and dilute to 100 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1).

Chlorides (2.4.4). Dilute 10 ml of solution S to 15 ml with *water R*. The solution complies with the limit test for chlorides (100 ppm). Examine the tubes laterally against a black background.

Iron. Not more than 100 ppm of Fe, determined by atomic absorption spectrometry (2.2.23, *Method I*).

Test solution. Dissolve 0.5 g in 10 ml of *water R*, add 2.5 ml of *lead-free nitric acid R* and dilute to 25.0 ml with *water R*.

Reference solutions. Prepare the reference solutions using *iron standard solution (20 ppm Fe) R*, adding 2.5 ml of *lead-free nitric acid R* and diluting to 25.0 ml with *water R*.

Measure the absorbance at 248.3 nm using an iron hollow-cathode lamp as a source of radiation and an air-butane flame.

Lead. Not more than 50 ppm of Pb, determined by atomic absorption spectrometry (2.2.23, *Method I*).

Test solution. Dissolve 2.5 g in 10 ml of *water R*, add 2.5 ml of *lead-free nitric acid R* and dilute to 25.0 ml with *water R*.

Reference solutions. Prepare the reference solutions using *lead standard solution (100 ppm Pb) R*, adding 2.5 ml of *lead-free nitric acid R* and diluting to 25.0 ml with *water R*.

Measure the absorbance at 217.0 nm using a lead hollow-cathode lamp as a source of radiation and an air-butane flame.

Loss on drying (2.2.32): 35.0 per cent to 36.5 per cent, determined on 0.500 g by drying in an oven at 250 °C.

ASSAY

Dissolve 0.200 g in 50 ml of *water R*. Add 2 ml of *sulphuric acid R* and 3 g of *potassium iodide R*. Titrate with 0.1 M *sodium thiosulphate*, adding 1 ml of *starch solution R* towards the end of the titration.

1 ml 0.1 M *sodium thiosulphate* is equivalent to 24.97 mg of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$.

01/2005:0894

COPPER SULPHATE PENTAHYDRATE

Cupri sulfas pentahydricus

 $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ M_r 249.7

DEFINITION

Copper sulphate pentahydrate contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$.

CHARACTERS

A blue, crystalline powder or transparent, blue crystals, freely soluble in water, soluble in methanol, practically insoluble in alcohol.

IDENTIFICATION

A. Add several drops of *dilute ammonia R2* to 1 ml of solution S (see Tests). A blue precipitate is formed on further addition of *dilute ammonia R2*, the precipitate dissolves and a dark blue colour is produced.

01/2005:1304

CORIANDER

Coriandri fructus

DEFINITION

Coriander consists of the dried cremocarp of *Coriandrum sativum* L. It contains not less than 3 ml/kg of essential oil, calculated with reference to the dried drug.

CHARACTERS

The cremocarp is brown or light brown and is more or less spherical, about 1.5 mm to 5 mm in diameter, or oval form 2 mm to 6 mm long.

It has the macroscopic and microscopic characters described under identification tests A and B.

IDENTIFICATION

A. The mericarps are usually tightly connected. The cremocarp is glabrous and has ten wavy, slightly raised primary ridges and eight straight, more prominent

01/2005:1820

secondary ridges. The stylopod crowns the apex. The mericarps are concave on the internal surface. A small fragment of the pedicel may be present.

- B. Reduce to a powder (355). The powder is brown. Examine under a microscope using *chloral hydrate solution R*. The powder shows numerous oil droplets; fragments of endosperm with small thick-walled regular cells containing microcrystals and microrosettes of calcium oxalate and oil droplets; fragments of endocarp with very narrow cells having a parquetry arrangement and usually associated with a layer of thin-walled rectangular sclereids of the mesocarp; fragments from the sclerenchymatous layer of the mesocarp with short, strongly thickened, pitted, fusiform cells occurring in layers with the cells of adjacent layers approximately at right angles to one another; fragments of parenchyma with small, thick-walled cells; occasional fragments of vascular bundles.
- C. Examine by thin-layer chromatography (2.2.27), using a suitable silica gel as the coating substance.
- Test solution.** Shake 0.50 g of the freshly powdered drug (355) with 5.0 ml of *hexane R* for 2 min to 3 min and filter over 2 g of *anhydrous sodium sulphate R*.
- Reference solution.** Dissolve 15 µl of *linalol R* and 25 µl of *olive oil R* in 5.0 ml of *hexane R* immediately before use.
- Apply to the plate as bands 20 µl of the test solution and 10 µl of the reference solution. Develop over a path of 10 cm using a mixture of 5 volumes of *ethyl acetate R* and 95 volumes of *toluene R*. Allow the plate to dry in air and develop again in the same conditions. Allow the plate to dry in air. Spray the plate with *anisaldehyde solution R* and examine in daylight while heating at 100 °C to 105 °C for 5 min to 10 min. The chromatogram obtained with the reference solution shows in the lower half a violet to greyish-violet zone (linalol) and in the upper half a bluish-violet zone (triglycerides). The chromatogram obtained with the test solution shows zones similar in position and colour to the zones in the chromatogram obtained with the reference solution. Several violet-grey to brownish zones, including the zone corresponding to geraniol, are between the starting point and the zone due to linalol in the chromatogram obtained with the reference solution. It may also show several faint violet-grey zones between the zone due to triglycerides and that due to linalol in the chromatogram obtained with the reference solution.

TESTS

Foreign matter (2.8.2). It complies with the test for foreign matter. None of the cremocarps show perforations due to animals.

Loss on drying (2.2.32). Not more than 10.0 per cent, determined on 1.000 g of the powdered drug (355) by drying in an oven at 100 °C to 105 °C for 2 h.

Total ash (2.4.16). Not more than 8.0 per cent.

ASSAY

Carry out the determination of essential oils in vegetable drugs (2.8.12). Use a 500 ml round-bottomed flask, 200 ml of *water R* as the distillation liquid and 0.5 ml of *xylene R* in the graduated tube. Reduce the drug to a coarse powder and immediately use 30.0 g for the determination. Distil at a rate of 2 ml/min to 3 ml/min for 2 h.

STORAGE

Store protected from light.

CORIANDER OIL

Coriandri aetheroleum

DEFINITION

Essential oil obtained by steam distillation from the fruits of *Coriandrum sativum* L.

CHARACTERS

Appearance: clear, colourless or pale yellow liquid.

It has a characteristic spicy odour.

IDENTIFICATION

First identification: B.

Second identification: A.

A. Examine by thin-layer chromatography (2.2.27).

Test solution. Dissolve 10 µl of the substance to be examined in 1.0 ml of *toluene R*.

Reference solution. Dissolve 10 µl *linalol R* and 2 µl of *geranyl acetate R* in 1.0 ml of *toluene R*.

Plate: TLC silica gel plate R.

Mobile phase: *ethyl acetate R*, *toluene R* (5:95 V/V).

Application: 10 µl as bands.

Development: over a path of 10 cm.

Drying: in air.

Detection: spray with *anisaldehyde solution R* and heat at 100-105 °C for 10-15 min. Examine immediately in daylight.

Results: see below the sequence of the zones present in the chromatograms obtained with the reference solution and the test solution.

Top of the plate	
Geranyl acetate: a violet-blue zone	A violet-blue zone (geranyl acetate)
Linalol: an intense violet zone	An intense violet zone (linalol) A violet-blue zone (geraniol)
Reference solution	Test solution

B. Examine the chromatograms obtained in the test for chromatographic profile.

Results: the characteristic peaks in the chromatogram obtained with the test solution are similar in retention time to those in the chromatogram obtained with the reference solution.

TESTS

Relative density (2.2.5): 0.860 to 0.880.

Refractive index (2.2.6): 1.462 to 1.470.

Optical rotation (2.2.7): + 7° to + 13°.

Acid value (2.5.1): maximum 3.0, determined on 5.00 g of the substance to be examined.

Chromatographic profile. Gas chromatography (2.2.28): use the normalisation procedure.

Test solution. The substance to be examined.

Reference solution (a). Dissolve 10 µl of α -pinene R, 10 µl of limonene R, 10 µl of γ -terpinene R, 10 µl of *p*-cymene R, 10 mg of camphor R, 20 µl of linalol R, 10 µl of α -terpineol R, 10 µl of geranyl acetate R and 10 µl of geraniol R in 1 ml of hexane R.

Reference solution (b). Dissolve 5 µl of geraniol R in hexane R and dilute to 10 ml with the same solvent.

Column:

- **material:** fused silica,
- **size:** *l* = 60 m, Ø = 0.25 mm,
- **stationary phase:** macrogol 20 000 R (film thickness 0.25 µm).

Carrier gas: helium for chromatography R.

Flow rate: 1 ml/min.

Split ratio: 1:65.

Temperature:

	Time (min)	Temperature (°C)
Column	0 - 10 10 - 75 75 - 120	60 60 → 190 190
Injection port		220
Detector		240

Detection: flame ionisation.

Injection: 0.2 µl.

Elution order: order indicated in the composition of reference solution (a). Record the retention times of these substances.

System suitability: reference solution (a):

- **resolution:** minimum 1.5 between the peaks due to linalol and camphor.

Using the retention times determined from the chromatogram obtained with reference solution (a), locate the components of reference solution (a) in the chromatogram obtained with the test solution.

Determine the percentage content of each of these components. The percentages are within the following ranges:

- **α -pinene:** 3.0 per cent to 7.0 per cent,
- **limonene:** 1.5 per cent to 5.0 per cent,
- **γ -terpinene:** 1.5 per cent to 8.0 per cent,
- ***p*-cymene:** 0.5 per cent to 4.0 per cent,
- **camphor:** 3.0 per cent to 6.0 per cent,
- **linalol:** 65.0 per cent to 78.0 per cent,
- **α -terpineol:** 0.1 per cent to 1.5 per cent,
- **geranyl acetate:** 0.5 per cent to 4.0 per cent,
- **geraniol:** 0.5 per cent to 3.0 per cent,
- **disregard limit:** area of the peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

Chiral purity. Gas chromatography (2.2.28).

Test solution. Dissolve 0.02 g of the substance to be examined in pentane R and dilute to 10 ml with the same solvent.

Reference solution. Dissolve 10 µl of linalol R and 5 mg of borneol R in pentane R and dilute to 10 ml with the same solvent.

Column:

- **material:** fused silica,
- **size:** *l* = 25 m, Ø = 0.25 mm,
- **stationary phase:** modified β -cyclodextrin for chiral chromatography R (film thickness 0.25 µm).

Carrier gas: helium for chromatography R.

Flow rate: 1.3 ml/min.

Split ratio: 1:30.

Temperature:

	Time (min)	Temperature (°C)
Column	0 - 65	50 → 180
Injection port		230
Detector		230

Detection: flame ionisation.

Injection: 1 µl.

System suitability: reference solution:

- **resolution:** minimum 5.5 between the peaks due to (*R*)-linalol (1st peak) and (*S*)-linalol (2nd peak) and minimum 2.9 between the peaks due to (*S*)-linalol and borneol (3rd peak).

Limit: calculate the percentage content of (*R*)-linalol from the expression:

$$\frac{A_R}{A_S + A_R} \times 100$$

A_S = area of the peak due to (*S*)-linalol,

A_R = area of the peak due to (*R*)-linalol.

- **(*R*)-linalol:** maximum 14 per cent.

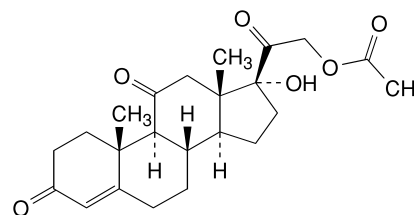
STORAGE

In a well-filled, airtight container, protected from light and at a temperature not exceeding 25 °C.

01/2005:0321

CORTISONE ACETATE

Cortisoni acetat



$C_{23}H_{30}O_6$

M_r 402.5

DEFINITION

Cortisone acetate contains not less than 97.0 per cent and not more than the equivalent of 103.0 per cent of 17-hydroxy-3,11,20-trioxopregn-4-en-21-yl acetate, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, crystalline powder, practically insoluble in water, freely soluble in methylene chloride, soluble in dioxan, sparingly soluble in acetone, slightly soluble in alcohol and in methanol.

It shows polymorphism.

IDENTIFICATION

First identification: A, B.

Second identification: C, D, E.

- A. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *cortisone acetate CRS*. If the spectra obtained in the solid state show differences, record new spectra using 50 g/l solutions of the substance to be examined and of the reference substance in *methylene chloride R* in a 0.2 mm cell.

- B. Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel F₂₅₄ plate R*.

Test solution. Dissolve 10 mg of the substance to be examined in a mixture of 1 volume of *methanol R* and 9 volumes of *methylene chloride R* and dilute to 10 ml with the same mixture of solvents.

Reference solution (a). Dissolve 20 mg of *cortisone acetate CRS* in a mixture of 1 volume of *methanol R* and 9 volumes of *methylene chloride R* and dilute to 20 ml with the same mixture of solvents.

Reference solution (b). Dissolve 10 mg of *hydrocortisone acetate R* in reference solution (a) and dilute to 10 ml with reference solution (a).

Apply to the plate 5 µl of each solution. Prepare the mobile phase by adding a mixture of 1.2 volumes of *water R* and 8 volumes of *methanol R* to a mixture of 15 volumes of *ether R* and 77 volumes of *methylene chloride R*. Develop over a path of 15 cm. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. The principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a). Spray with *alcoholic solution of sulphuric acid R*. Heat at 120 °C for 10 min or until the spots appear. Allow to cool. Examine the plate in daylight and in ultraviolet light at 365 nm. The principal spot in the chromatogram obtained with the test solution is similar in position, colour in daylight, fluorescence in ultraviolet light at 365 nm and size to the principal spot in the chromatogram obtained with reference solution (a). The test is not valid unless the chromatogram obtained with reference solution (b) shows two clearly separated spots.

- C. Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel F₂₅₄ plate R*.

Test solution (a). Dissolve 25 mg of the substance to be examined in *methanol R* with gentle heating and dilute to 5 ml with the same solvent. This solution is also used to prepare test solution (b). Dilute 2 ml of the solution to 10 ml with *methylene chloride R*.

Test solution (b). Transfer 2 ml of the solution obtained during preparation of test solution (a) to a 15 ml glass tube with a ground-glass stopper or a polytetrafluoroethylene cap. Add 10 ml of *saturated methanolic potassium hydrogen carbonate solution R* and immediately pass a stream of *nitrogen R* briskly through the solution for 5 min. Stopper the tube. Heat in a water-bath at 45 °C protected from light for 2.5 h. Allow to cool.

Reference solution (a). Dissolve 25 mg of *cortisone acetate CRS* in *methanol R* with gentle heating and dilute to 5 ml with the same solvent. This solution is also used to prepare reference solution (b). Dilute 2 ml of the solution to 10 ml with *methylene chloride R*.

Reference solution (b). Transfer 2 ml of the solution obtained during preparation of reference solution (a) to a 15 ml glass tube with a ground-glass stopper or a polytetrafluoroethylene cap. Add 10 ml of *saturated methanolic potassium hydrogen carbonate solution R* and immediately pass a stream of *nitrogen R* briskly

through the solution for 5 min. Stopper the tube. Heat in a water-bath at 45 °C protected from light for 2.5 h. Allow to cool.

Apply to the plate 5 µl of each solution. Prepare the mobile phase by adding a mixture of 1.2 volumes of *water R* and 8 volumes of *methanol R* to a mixture of 15 volumes of *ether R* and 77 volumes of *methylene chloride R*. Develop over a path of 15 cm. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. The principal spot in each of the chromatograms obtained with the test solutions is similar in position and size to the principal spot in the chromatogram obtained with the corresponding reference solution. Spray with *alcoholic solution of sulphuric acid R* and heat at 120 °C for 10 min or until the spots appear. Allow to cool. Examine the plate in daylight and in ultraviolet light at 365 nm. The principal spot in each of the chromatograms obtained with the test solutions is similar in position, colour in daylight, fluorescence in ultraviolet light at 365 nm and size to the principal spot in the chromatogram obtained with the corresponding reference solution. The principal spots in the chromatograms obtained with test solution (b) and reference solution (b) have an *R_f* value distinctly lower than that of the principal spots in the chromatograms obtained with test solution (a) and reference solution (a).

- D. Add about 2 mg to 2 ml of *sulphuric acid R* and shake to dissolve. Within 5 min, a faint yellow colour develops. Add the solution to 10 ml of *water R* and mix. The colour is discharged and a clear solution remains.

- E. About 10 mg gives the reaction of acetyl (2.3.1).

TESTS

Specific optical rotation (2.2.7). Dissolve 0.250 g in *dioxan R* and dilute to 25.0 ml with the same solvent. The specific optical rotation is + 211 to + 220, calculated with reference to the dried substance.

Related substances. Examine by liquid chromatography (2.2.29). *Prepare the solutions immediately before use.*

Test solution. Dissolve 25.0 mg of the substance to be examined in *acetonitrile R* and dilute to 10.0 ml with the same solvent.

Reference solution (a). Dissolve 2 mg of *cortisone acetate CRS* and 2 mg of *hydrocortisone acetate CRS* in *acetonitrile R* and dilute to 100.0 ml with the same solvent.

Reference solution (b). Dilute 1.0 ml of the test solution to 100.0 ml with *acetonitrile R*.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4.6 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (5 µm),
- as mobile phase at a flow rate of 1 ml/min a mixture prepared as follows: in a 1000 ml volumetric flask mix 400 ml of *acetonitrile R* with 550 ml of *water R* and allow to equilibrate; adjust the volume to 1000 ml with *water R* and mix again,
- as detector a spectrophotometer set at 254 nm.

Equilibrate the column with the mobile phase at a flow rate of 1 ml/min for about 30 min.

Adjust the sensitivity of the system so that the height of the principal peak in the chromatogram obtained with 20 µl of reference solution (b) is at least 50 per cent of the full scale of the recorder.

Inject 20 µl of reference solution (a). When the chromatograms are recorded in the prescribed conditions the retention times are: hydrocortisone acetate about

10 min and cortisone acetate about 12 min. The test is not valid unless the resolution between the peaks due to hydrocortisone acetate and cortisone acetate is at least 4.2; if necessary, adjust the concentration of acetonitrile in the mobile phase.

Inject 20 µl of the test solution, 20 µl of reference solution (b) and 20 µl of acetonitrile as a blank. Continue the chromatography for twice the retention time of the principal peak. In the chromatogram obtained with the test solution: the area of any peak apart from the principal peak, is not greater than half the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent); the sum of the areas of all the peaks apart from the principal peak, is not greater than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (1.5 per cent). Disregard any peak with an area less than 0.05 times the area of the principal peak in the chromatogram obtained with reference solution (b).

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 0.500 g by drying in an oven at 100-105 °C.

ASSAY

Dissolve 0.100 g in *alcohol R* and dilute to 100.0 ml with the same solvent. Dilute 2.0 ml of the solution to 100.0 ml with *alcohol R*. Measure the absorbance (2.2.25) at the maximum of 237 nm.

Calculate the content of $C_{23}H_{30}O_6$ taking the specific absorbance to be 395.

STORAGE

Store protected from light.

IMPURITIES

A. hydrocortisone acetate.

01/2005:0036

COTTON, ABSORBENT

Lanugo gossypii absorbens

DEFINITION

Absorbent cotton consists of new fibres or good quality combers obtained from the seed-coat of various species of the genus *Gossypium* L., cleaned, purified, bleached and carefully carded. It may not contain any compensatory colouring matter.

CHARACTERS

It is white and is composed of fibres of average length not less than 10 mm, determined by a suitable method, and contains not more than traces of leaf residue, pericarp, seed-coat or other impurities. It offers appreciable resistance when pulled. It does not shed any appreciable quantity of dust when gently shaken.

IDENTIFICATION

- Examined under a microscope, each fibre is seen to consist of a single cell, up to about 4 cm long and up to 40 µm wide, in the form of a flattened tube with thick and rounded walls and often twisted.
- When treated with *iodinated zinc chloride solution R*, the fibres become violet.
- To 0.1 g add 10 ml of *zinc chloride-formic acid solution R*. Heat to 40 °C and allow to stand for 2 h 30 min, shaking occasionally. It does not dissolve.

TESTS

Solution S. Place 15.0 g in a suitable vessel, add 150 ml of *water R*, close the vessel and allow to macerate for 2 h. Decant the solution, squeeze the residual liquid carefully from the sample with a glass rod and mix. Reserve 10 ml of the solution for the test for surface-active substances and filter the remainder.

Acidity or alkalinity. To 25 ml of solution S add 0.1 ml of *phenolphthalein solution R* and to another 25 ml add 0.05 ml of *methyl orange solution R*. Neither solution is pink.

Foreign fibres. Examined under a microscope, it is seen to consist exclusively of typical cotton fibres, except that occasionally a few isolated foreign fibres may be present.

Fluorescence. Examine a layer about 5 mm in thickness under ultraviolet light at 365 nm. It displays only a slight brownish-violet fluorescence and a few yellow particles. It shows no intense blue fluorescence, apart from that which may be shown by a few isolated fibres.

Neps. Spread about 1 g evenly between 2 colourless transparent plates each 10 cm square. Examine for neps by transmitted light and compare with *Absorbent cotton RM*. The product to be examined is not more neppy than the standard.

Absorbency

Apparatus. A dry cylindrical copper wire basket 8.0 cm high and 5.0 cm in diameter. The wire of which the basket is constructed is about 0.4 mm in diameter, the mesh is 1.5 cm to 2.0 cm wide and the mass of the basket is 2.7 ± 0.3 g.

Sinking time. Not more than 10 s. Weigh the basket to the nearest centigram (m_1). Take a total of 5.00 g in approximately equal quantities from 5 different places in the product to be examined, place loosely in the basket and weigh the filled basket to the nearest centigram (m_2). Fill a beaker 11 cm to 12 cm in diameter to a depth of 10 cm with water at about 20 °C. Hold the basket horizontally and drop it from a height of about 10 mm into the water. Measure with a stopwatch the time taken for the basket to sink below the surface of the water. Calculate the result as the average of 3 tests.

Water-holding capacity. Not less than 23.0 g of water per gram. After the sinking time has been measured, remove the basket from the water, allow it to drain for exactly 30 s suspended in a horizontal position over the beaker, transfer it to a tared beaker (m_3) and weigh to the nearest centigram (m_4). Calculate the water-holding capacity per gram of absorbent cotton using the following expression:

$$\frac{m_4 - (m_2 + m_3)}{m_2 - m_1}$$

Calculate the result as the average of 3 tests.

Ether-soluble substances. Not more than 0.50 per cent. In an extraction apparatus, extract 5.00 g with *ether R* for 4 h at a rate of at least 4 extractions per hour. Evaporate the ether extract and dry the residue to constant mass at 100 °C to 105 °C.

Extractable colouring matter. In a narrow percolator, slowly extract 10.0 g with *alcohol R* until 50 ml of extract is obtained. The liquid obtained is not more intensely coloured (2.2.2, *Method II*) than reference solution Y_5 , GY_6 or a reference solution prepared as follows: to 3.0 ml of blue primary solution add 7.0 ml of hydrochloric acid (10 g/l HCl). Dilute 0.5 ml of this solution to 10.0 ml with hydrochloric acid (10 g/l HCl).

Surface-active substances. Introduce the 10 ml portion of solution S reserved before filtration into a 25 ml graduated ground-glass-stoppered cylinder with an external diameter of 20 mm and a wall thickness of not greater than 1.5 mm, previously rinsed 3 times with *sulphuric acid R* and then with *water R*. Shake vigorously 30 times in 10 s, allow to stand for 1 min and repeat the shaking. After 5 min, any foam present must not cover the entire surface of the liquid.

Water-soluble substances. Not more than 0.50 per cent. Boil 5.000 g in 500 ml of *water R* for 30 min, stirring frequently. Replace the water lost by evaporation. Decant the liquid, squeeze the residual liquid carefully from the sample with a glass rod and mix. Filter the liquid whilst hot. Evaporate 400 ml of the filtrate (corresponding to 4/5 of the mass of the sample taken) and dry the residue to constant mass at 100 °C to 105 °C.

Loss on drying (2.2.32). Not more than 8.0 per cent, determined on 5.000 g by drying in an oven at 100 °C to 105 °C.

Sulphated ash (2.4.14). Not more than 0.40 per cent. Introduce 5.00 g into a previously heated and cooled, tared crucible. Heat cautiously over a naked flame and then carefully to dull redness at 600 °C. Allow to cool, add a few drops of *dilute sulphuric acid R*, then heat and incinerate until all the black particles have disappeared. Allow to cool. Add a few drops of *ammonium carbonate solution R*. Evaporate and incinerate carefully, allow to cool and weigh again. Repeat the incineration for periods of 5 min to constant mass.

STORAGE

Store in a dust-proof package in a dry place.

01/2005:1305

COTTONSEED OIL, HYDROGENATED

Gossypii oleum hydrogenatum

DEFINITION

Hydrogenated cottonseed oil is the product obtained by refining and hydrogenation of oil obtained from seeds of cultivated plants of various varieties of *Gossypium hirsutum* L. or of other species of *Gossypium*. The product consists mainly of triglycerides of palmitic and stearic acids.

CHARACTERS

A white mass or powder which melts to a clear, pale yellow liquid when heated, practically insoluble in water, freely soluble in methylene chloride and in toluene, very slightly soluble in alcohol.

IDENTIFICATION

- It complies with the test for melting point (see Tests).
- It complies with the test for foreign fatty oils (see Tests).

TESTS

Melting point (2.2.14): 57 °C to 70 °C.

Acid value (2.5.1). Not more than 0.5, determined on 10.0 g. Dissolve the substance to be examined in 50 ml of a hot mixture of equal volumes of *alcohol R* and *toluene R*, previously neutralised with 0.1 M *potassium hydroxide* using 0.5 ml of *phenolphthalein solution RI* as indicator. Titrate the solution immediately while still hot.

Peroxide value (2.5.5). Not more than 5.0.

Unsaponifiable matter (2.5.7). Not more than 1.0 per cent, determined on 5.0 g.

Alkaline impurities. Dissolve by gentle heating 2.0 g of the substance to be examined in a mixture of 1.5 ml of *alcohol R* and 3 ml of *toluene R*. Add 0.05 ml of a 0.4 g/l solution of *bromophenol blue R* in *alcohol R*. Not more than 0.4 ml of 0.01 M *hydrochloric acid* is required to change the colour to yellow.

Composition of fatty acids (2.4.22, Method A).

The chromatographic procedure may be carried out using:

- a fused-silica capillary column 25 m long and 0.25 mm in internal diameter coated on the inner wall with *poly(cyanopropyl)siloxane R* (film thickness 0.2 µm),
- *helium for chromatography R* as the carrier gas at a flow rate of 0.65 ml/min,
- a flame-ionisation detector,
- a split injector (1:100),

maintaining the temperature of the column at 180 °C for 35 min and that of the injection port and the detector at 250 °C.

The fatty acid fraction of the oil has the following composition:

- *saturated fatty acids of chain length less than C₁₄*: not more than 0.2 per cent,
- *myristic acid*: not more than 1.0 per cent,
- *palmitic acid*: 19.0 per cent to 26.0 per cent,
- *stearic acid*: 68.0 per cent to 80.0 per cent,
- *oleic acid and isomers* (C_{18:1} equivalent chain length on *poly(cyanopropyl)siloxane* 18.5 to 18.8): not more than 4.0 per cent,
- *linoleic acid and isomers* (C_{18:2} equivalent chain length on *poly(cyanopropyl)siloxane* 19.4 to 19.8): not more than 1.0 per cent,
- *arachidic acid*: not more than 1.0 per cent,
- *behenic acid*: not more than 1.0 per cent,
- *lignoceric acid*: not more than 0.5 per cent.

Nickel. Not more than 1 ppm of Ni, determined by atomic absorption spectrometry (2.2.23, Method II).

Test solution. Introduce 5.0 g of the substance to be examined into a platinum or silica crucible tared after ignition. Cautiously heat and introduce into the substance a wick formed from twisted ashless filter paper. Ignite the wick. When the substance ignites, stop heating. After combustion, ignite in a muffle furnace at about 600 °C. Continue the incineration until white ash is obtained. After cooling, take up the residue with two quantities, each of 2 ml, of *dilute hydrochloric acid R* and transfer into a 25 ml graduated flask. Add 0.3 ml of *nitric acid R* and dilute to 25.0 ml with *distilled water R*.

Reference solutions. Prepare three reference solutions by adding 1.0 ml, 2.0 ml and 4.0 ml of *nickel standard solution* (0.2 ppm Ni) *R* to 2.0 ml portions of the test solution, diluting to 10.0 ml with *distilled water R*.

Measure the absorbance at 232 nm using a nickel hollow-cathode lamp as a source of radiation, a graphite furnace as an atomic generator and *argon R* as the carrier gas.

STORAGE

Store protected from light.

01/2005:1306 DEFINITION

COUCH GRASS RHIZOME**Graminis rhizoma****DEFINITION**

Whole or cut, washed and dried rhizome of *Agropyron repens* (L.) Beauv. (*Elymus repens* (L.) Gould); the adventitious roots are removed.

CHARACTERS

Macroscopic and microscopic characters described under identification tests A and B.

IDENTIFICATION

- A. The shiny yellowish, light brown or yellowish-brown pieces of the rhizome are 2 mm to 3 mm thick and longitudinally furrowed. At the nodes are the remains of very thin, more or less branched roots and whitish or brownish scale-like leaves; the internodes, up to 6 cm long, are furrowed and hollow inside. The transverse section of the nodes shows a yellowish medulla.
- B. Reduce to a powder (355). The powder is whitish-yellow. Examine under a microscope, using *chloral hydrate solution R*. The powder shows the following diagnostic characters: fragments of the epidermis covered with a thick cuticle and composed of rectangular and elongated thick-walled cells with pitted slightly wavy walls which alternate with small rounded to almost square cells; U-shaped thickened endodermic cells; numerous fragments of moderately thickened fibres and groups of vessels with slit-shaped pits or with spiral and annular thickening.

TESTS

Cynodon dactylon, Imperata cylindrica. Examine under a microscope using *iodine solution R1*. No blue starch grains are visible.

Foreign matter (2.8.2): maximum 15 per cent of greyish-black pieces of rhizome in the cut drug.

Water-soluble extractive: minimum 25 per cent.

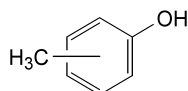
To 5.0 g of the powdered drug (355) add 200 ml of boiling *water R*. Allow to stand for 10 min, shaking occasionally. Allow to cool, dilute to 200.0 ml with *water R* and filter. Evaporate 20.0 ml of the filtrate to dryness on a water-bath. Dry the residue in an oven at 100-105 °C. The residue weighs a minimum of 0.125 g.

Loss on drying (2.2.32): maximum 12.0 per cent, determined on 1.000 g of the powdered drug (355) by drying in an oven at 100-105 °C for 2 h.

Total ash (2.4.16): maximum 5.0 per cent.

Ash insoluble in hydrochloric acid (2.8.1): maximum 1.5 per cent.

01/2005:1628

CRESOL, CRUDE**Cresolum crudum**C₇H₈OM_r 108.1

Mixture of 2-, 3- and 4-methylphenol.

CHARACTERS

Appearance: colourless or pale brown liquid.

Solubility: sparingly soluble in water, miscible with alcohol and with methylene chloride.

IDENTIFICATION

- A. To 0.5 ml add 300 ml of *water R*, mix and filter. To 10 ml of the filtrate add 1 ml of *ferric chloride solution R1*. A blue colour is produced.
- B. To 10 ml of the filtrate obtained in identification test A, add 1 ml of *bromine water R*. A pale yellow flocculent precipitate is produced.
- C. It complies with the test for relative density (see Tests).

TESTS

Solution S. To 2.5 g of the substance to be examined add 50 ml of *water R*, shake for 1 min and filter through a moistened filter.

Acidity or alkalinity. To 10 ml of solution S add 0.1 ml of *methyl red solution R* and 0.2 ml of 0.01 M *sodium hydroxide*. The solution is yellow. Add 0.3 ml of 0.01 M *hydrochloric acid*. The solution is red.

Relative density (2.2.5): 1.029 to 1.044.

Distillation range (2.2.11): a maximum of 2.0 per cent V/V distils below 188 °C and a minimum of 80 per cent V/V distils between 195 °C and 205 °C.

Sulphur compounds. Place 20 ml in a small conical flask. Over the mouth of the flask fix a piece of filter paper moistened with *lead acetate solution R*. Heat on a water-bath for 5 min. Not more than a light yellow colour is produced on the filter paper.

Residue on evaporation: maximum 0.1 per cent.

Evaporate 2.0 g to dryness on a water-bath and dry at 100-105 °C for 1 h. The residue weighs not more than 2 mg.

STORAGE

Protected from light.

01/2005:0985

CROSCARMELLOSE SODIUM**Carmellosum natricum conexum****DEFINITION**

Croscarmellose sodium (cross-linked sodium carboxymethylcellulose) is the sodium salt of a cross-linked, partly *O*-carboxymethylated cellulose.

CHARACTERS

A white or greyish-white powder, practically insoluble in acetone, in ethanol and in toluene.

IDENTIFICATION

- A. Shake 1 g with 100 ml of a solution containing 4 ppm of *methylene blue R* and allow to settle. The substance to be examined absorbs the methylene blue and settles as a blue, fibrous mass.
- B. Shake 1 g with 50 ml of *water R*. Transfer 1 ml of the mixture to a test-tube, add 1 ml of *water R* and 0.05 ml of a freshly prepared 40 g/l solution of *α-naphthol R* in

methanol R. Incline the test-tube and add carefully 2 ml of *sulphuric acid R* down the side so that it forms a lower layer. A reddish-violet colour develops at the interface.

C. It gives reaction (a) of sodium (2.3.1).

TESTS

pH (2.2.3). Shake 1 g with 100 ml of *carbon dioxide-free water R* for 5 min. The pH of the suspension is 5.0 to 7.0.

Degree of substitution. Place 1.000 g in a 500 ml conical flask, add 300 ml of a 100 g/l solution of *sodium chloride R*, 25.0 ml of 0.1 M *sodium hydroxide*, stopper the flask and allow to stand for 5 min, shaking occasionally. Add 0.05 ml of *m-cresol purple solution R* and about 15 ml of 0.1 M *hydrochloric acid* from a burette. Insert the stopper and shake. If the solution is violet, add 0.1 M *hydrochloric acid* in 1 ml portions until the solution becomes yellow, shaking after each addition. Titrate with 0.1 M *sodium hydroxide* until the colour turns to violet.

Calculate the number of milliequivalents (*M*) of base required for the neutralisation equivalent to 1 g of dried substance.

Calculate the degree of acid carboxymethyl substitution (*A*) from the expression:

$$\frac{1150M}{(7102 - 412M - 80C)}$$

C = sulphated ash as a percentage.

Calculate the degree of sodium carboxymethyl substitution (*S*) from the expression:

$$\frac{(162 + 58A)C}{(7102 - 80C)}$$

The degree of substitution is the sum of *A* + *S* and it is between 0.60 and 0.85, calculated with reference to the dried substance.

Sodium chloride and sodium glycollate. The sum of the percentage contents of sodium chloride and sodium glycollate is not more than 0.5 per cent, calculated with reference to the dried substance.

Sodium chloride. Place 5.00 g in a 250 ml conical flask, add 50 ml of *water R* and 5 ml of *strong hydrogen peroxide solution R* and heat on a water-bath for 20 min, stirring occasionally to ensure total hydration. Cool, add 100 ml of *water R* and 10 ml of *nitric acid R*. Titrate with 0.05 M *silver nitrate* determining the end-point potentiometrically (2.2.20) using a silver indicator electrode and a double-junction reference electrode containing a 100 g/l solution of *potassium nitrate R* in the outer jacket and a standard filling solution in the inner jacket, and stirring constantly.

1 ml of 0.05 M *silver nitrate* is equivalent to 2.922 mg of NaCl.

Sodium glycollate. Place a quantity of the substance to be examined equivalent to 0.500 g of the dried substance in a 100 ml beaker. Add 5 ml of *glacial acetic acid R* and 5 ml of *water R* and stir to ensure total hydration (about 15 min). Add 50 ml of *acetone R* and 1 g of *sodium chloride R*. Stir for several minutes to ensure complete precipitation of the carboxymethylcellulose. Filter through a fast filter paper impregnated with *acetone R* into a volumetric flask, rinse the beaker and filter with 30 ml of *acetone R* and dilute the filtrate to 100.0 ml with the same solvent. Allow to stand for 24 h without shaking. Use the clear supernatant to prepare the test solution.

Prepare the reference solutions as follows: in a 100 ml volumetric flask, dissolve 0.100 g of *glycollic acid R*, previously dried *in vacuo* over *diphosphorus pentoxide R*,

in *water R* and dilute to 100.0 ml with the same solvent. Use the solution within 30 days. Transfer 1.0 ml, 2.0 ml, 3.0 ml and 4.0 ml of the solution to separate volumetric flasks; dilute the contents of each flask to 5.0 ml with *water R*, add 5 ml of *glacial acetic acid R*, dilute to 100.0 ml with *acetone R* and mix.

Transfer 2.0 ml of the test solution and 2.0 ml of each of the reference solutions to separate 25 ml volumetric flasks. Heat the uncovered flasks for 20 min on a water-bath to eliminate acetone. Allow to cool and add 5.0 ml of 2,7-dihydroxynaphthalene solution *R* to each flask. Mix, add a further 15.0 ml of 2,7-dihydroxynaphthalene solution *R* and mix again. Close the flasks with aluminium foil and heat on a water-bath for 20 min. Cool and dilute to 25.0 ml with *sulphuric acid R*.

Measure the absorbance (2.2.25) of each solution at 540 nm. Prepare a blank using 2.0 ml of a solution containing 5 per cent V/V each of *glacial acetic acid R* and *water R* in *acetone R*. Prepare a standard curve using the absorbances obtained with the reference solutions. From the standard curve and the absorbance of the test solution, determine the mass *a*, in milligrams, of glycollic acid in the substance to be examined, and calculate the content of sodium glycollate from the expression:

$$\frac{10 \times 1.29 \times a}{(100 - b)m}$$

1.29 = the factor converting glycollic acid to sodium glycollate,

b = loss on drying as a percentage,

m = mass of the substance to be examined, in grams.

Water-soluble substances. Not more than 10.0 per cent. Disperse 10.00 g in 800.0 ml of *water R* and stir for 1 min every 10 min during the first 30 min. Allow to stand for 1 h and centrifuge, if necessary. Decant 200.0 ml of the supernatant liquid onto a fast filter paper in a vacuum filtration funnel, apply vacuum and collect 150.0 ml of the filtrate. Evaporate to dryness and dry the residue at 100 °C to 105 °C for 4 h.

Heavy metals (2.4.8). To the residue obtained in the determination of the sulphated ash add 1 ml of *hydrochloric acid R* and evaporate on a water-bath. Take up the residue in 20 ml of *water R*. 12 ml of the solution complies with limit test A for heavy metals (10 ppm). Prepare the standard using *lead standard solution (1 ppm Pb) R*.

Loss on drying (2.2.32). Not more than 10.0 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C for 6 h.

Sulphated ash (2.4.14): 14.0 per cent to 28.0 per cent, determined on 2.000 g, using a mixture of equal volumes of *sulphuric acid R* and *water R*, and calculated with reference to the dried substance.

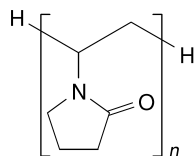
Settling volume. Place 75 ml of *water R* in a 100 ml graduated cylinder and add 1.5 g of the substance to be examined in 0.5 g portions, shaking vigorously after each addition. Dilute to 100.0 ml with *water R* and shake again until the substance is homogeneously distributed. Allow to stand for 4 h. The settling volume is between 10.0 ml and 30.0 ml.

Microbial contamination. Total viable aerobic count (2.6.12) not more than 10³ bacteria and 10² fungi per gram, determined by plate count. It complies with the test for *Escherichia coli* (2.6.13).

01/2005:0892

CROSOVIDONE

Crosopovidonum

 $(C_6H_9NO)_n$ M_r (111.1)_n

DEFINITION

Crosopovidone is a cross-linked homopolymer of 1-ethenylpyrrolidin-2-one. It is available in different degrees of powder fineness (type A and type B).

Content: 11.0 per cent to 12.8 per cent of nitrogen (N; A_r 14.01) (dried substance).

CHARACTERS

Appearance: white or yellowish-white powder or flakes, hygroscopic.

Solubility: practically insoluble in water, in alcohol and in methylene chloride.

IDENTIFICATION

A. Infrared absorption spectrophotometry (2.2.24).

Comparison: *Ph. Eur. reference spectrum of crosopovidone.*

B. Suspend 1 g in 10 ml of *water R*, add 0.1 ml of 0.05 M iodine and shake for 30 s. Add 1 ml of *starch solution R* and shake. No blue colour develops within 30 s.

C. To 10 ml of *water R*, add 0.1 g and shake. A suspension is formed and no clear solution is obtained within 15 min.

D. Weigh a suitable quantity of the substance to be examined (for example 10 mg to 100 mg) and suspend it in 10.0 ml of *water R*, adding a wetting agent. Observe under a microscope at a suitable magnification using a calibrated ocular micrometer. If the majority of particles are in the range 50 µm to 300 µm, the product is classified as type A. If almost all the particles are below 50 µm, the product is classified as type B.

TESTS

Peroxides. Type A: maximum 400 ppm expressed as H_2O_2 ; type B: maximum 1000 ppm expressed as H_2O_2 .

Suspend 2.0 g in 50 ml of *water R*. To 25 ml of this suspension add 2 ml of *titanium trichloride-sulphuric acid reagent R*. Allow to stand for 30 min and filter. The absorbance (2.2.25) of the filtrate, measured at 405 nm using a mixture of 25 ml of a filtered 40 g/l suspension of the substance to be examined and 2 ml of a 13 per cent V/V solution of *sulphuric acid R* as the compensation liquid has a maximum of 0.35.

For type B use 10 ml of the suspension diluted to 25 ml with *water R* for the test.

Water-soluble substances: maximum 1.0 per cent.

Transfer 25.0 g to a 400 ml beaker, add 200 ml of *water R* and stir for 1 h using a magnetic stirrer. Transfer the suspension to a 250.0 ml volumetric flask, rinsing with *water R*, and dilute to volume with the same solvent. Allow the bulk of the solids to settle. Filter about 100 ml of the almost clear supernatant liquid through a 0.45 µm membrane filter, protected by superimposing a 3 µm membrane filter. While filtering, stir the liquid above the filter manually or by means

of a mechanical stirrer, taking care not to damage the filter. Transfer 50.0 ml of the clear filtrate to a tared 100 ml beaker, evaporate to dryness and dry at 105–110 °C for 3 h. The residue weighs a maximum of 50 mg.

Impurity A. Liquid chromatography (2.2.29).

Test solution. Suspend 1.250 g in 50.0 ml of *methanol R* and shake for 60 min. Leave the bulk to settle and filter through a 0.2 µm filter.

Reference solution (a). Dissolve 50 mg of 1-vinylpyrrolidin-2-one *R* in *methanol R* and dilute to 100.0 ml with the same solvent. Dilute 1.0 ml of the solution to 100.0 ml with *methanol R*. Dilute 5.0 ml of this solution to 100.0 ml with the mobile phase.

Reference solution (b). Dissolve 10 mg of 1-vinylpyrrolidin-2-one *R* and 0.50 g of *vinyl acetate R* in *methanol R* and dilute to 100 ml with the same solvent. Dilute 1.0 ml of the solution to 100.0 ml with the mobile phase.

Precolumn:

- **size:** $l = 0.025$ m, $\varnothing = 4$ mm,
- **stationary phase:** octadecylsilyl silica gel for chromatography *R* (5 µm).

Column:

- **size:** $l = 0.25$ m, $\varnothing = 4$ mm,
- **stationary phase:** octadecylsilyl silica gel for chromatography *R* (5 µm),
- **temperature:** 40 °C.

Mobile phase: acetonitrile *R*, water *R* (10:90 V/V).

Flow rate: adjusted so that the retention time of the peak corresponding to impurity A is about 10 min.

Detection: spectrophotometer at 235 nm.

Injection: 50 µl. After each injection of the test solution, wash the precolumn by passing the mobile phase backward, at the same flow rate as applied in the test, for 30 min.

System suitability:

- **resolution:** minimum of 2.0 between the peaks corresponding to impurity A and to vinyl acetate in the chromatogram obtained with reference solution (b),
- **repeatability:** maximum relative standard deviation of 2.0 per cent after 5 injections of reference solution (a).

Limits:

- **impurity A:** not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (10 ppm).

Heavy metals (2.4.8): maximum 10 ppm.

2.0 g complies with limit test D. Prepare the standard using 2 ml of *lead standard solution* (10 ppm Pb) *R*.

Loss on drying (2.2.32): maximum 5.0 per cent, determined on 0.500 g by drying in an oven at 100–105 °C.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Place 100.0 mg of the substance to be examined (m mg) in a combustion flask, add 5 g of a mixture of 1 g of *copper sulphate R*, 1 g of *titanium dioxide R* and 33 g of *dipotassium sulphate R*, and 3 glass beads. Wash any adhering particles from the neck into the flask with a small quantity of *water R*. Add 7 ml of *sulphuric acid R*, allowing it to run down the sides of the flask, and mix the contents by rotation. Close the mouth of the flask loosely, for example by means of a glass bulb with a short stem, to avoid excessive loss of sulphuric acid. Heat gradually at first, then increase the temperature until there is vigorous

boiling with condensation of sulphuric acid in the neck of the flask; precautions are to be taken to prevent the upper part of the flask from becoming overheated. Continue the heating for 45 min. Cool, dissolve the solid material by cautiously adding to the mixture 20 ml of *water R*, cool again and place in a steam-distillation apparatus. Add 30 ml of *strong sodium hydroxide solution R* through the funnel, rinse the funnel cautiously with 10 ml of *water R* and distil immediately by passing steam through the mixture. Collect 80-100 ml of distillate in a mixture of 30 ml of a 40 g/l solution of *boric acid R* and 0.05 ml of *bromocresol green-methyl red solution R* and enough *water R* to cover the tip of the condenser. Towards the end of the distillation lower the receiver so that the tip of the condenser is above the surface of the acid solution and rinse the end part of the condenser with a small quantity of *water R*. Titrate the distillate with 0.025 *M* sulphuric acid until the colour of the solution changes from green through pale greyish-blue to pale greyish-red-purple (n_1 ml of 0.025 *M* sulphuric acid). Repeat the test using about 100 mg of *glucose R* in place of the substance to be examined (n_2 ml of 0.025 *M* sulphuric acid).

$$\text{Percentage content of nitrogen} = \frac{0.7004(n_1 - n_2)}{m} \times 100$$

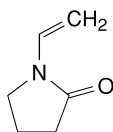
STORAGE

In an airtight container.

LABELLING

The label states the type (type A or type B).

IMPURITIES

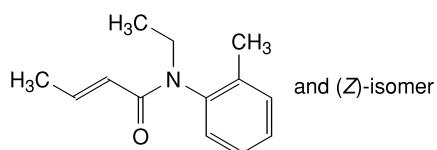


A. 1-ethenylpyrrolidin-2-one (1-vinylpyrrolidin-2-one).

01/2005:1194

CROTAMITON

Crotamitonum



$C_{13}H_{17}NO$

M_r 203.3

DEFINITION

Crotamiton contains not less than 96.0 per cent and not more than the equivalent of 102.0 per cent of *N*-ethyl-*N*-(2-methylphenyl)but-2-enamide, calculated as the sum of the *E*- and *Z*-isomers, and not more than 15.0 per cent of the *Z*-isomer.

CHARACTERS

A colourless or pale yellow, oily liquid, slightly soluble in water, miscible with alcohol. At low temperatures it may partly or completely solidify.

IDENTIFICATION

First identification: B.

Second identification: A, C, D.

- Dissolve 25.0 mg in *cyclohexane R* and dilute to 100.0 ml with the same solvent. Dilute 1.0 ml of the solution to 10.0 ml with *cyclohexane R*. Examined between 220 nm and 300 nm (2.2.25), the solution shows an absorption maximum at 242 nm. The specific absorbance at the maximum is 300 to 330.
- Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *crotamiton CRS*.
- Examine by thin-layer chromatography (2.2.27), using as the coating substance a suitable silica gel with a fluorescent indicator having an optimal intensity at 254 nm.

Test solution. Dissolve 25 mg of the substance to be examined in *ethanol R* and dilute to 10 ml with the same solvent.

Reference solution. Dissolve 25 mg of *crotamiton CRS* in *ethanol R* and dilute to 10 ml with the same solvent.

Apply separately to the plate 5 μ l of each solution. Develop over a path of 15 cm using the mixture prepared as follows: shake 98 volumes of *methylene chloride R* with 2 volumes of *concentrated ammonia R*, dry over *anhydrous sodium sulphate R*, filter and mix 97 volumes of the filtrate with 3 volumes of *2-propanol R*. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. The principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with the reference solution.

- To 10 ml of a saturated solution add a few drops of a 3 g/l solution of *potassium permanganate R*. A brown colour is produced and a brown precipitate is formed on standing.

TESTS

Relative density (2.2.5): 1.006 to 1.011.

Refractive index (2.2.6): 1.540 to 1.542.

Free amines. Dissolve 5.00 g in 16 ml of *methylene chloride R* and add 4.0 ml of *glacial acetic acid R*. Add 0.1 ml of *metanil yellow solution R* and 1.0 ml of 0.02 *M* *perchloric acid*. The solution is red-violet (500 ppm as ethylaminotoluene).

Chlorides. Boil 5.0 g under a reflux condenser for 1 h with 25 ml of *alcohol R* and 5 ml of a 200 g/l solution of *sodium hydroxide R*. Cool, add 5 ml of *water R* and shake with 25 ml of *ether R*. Dilute the lower layer to 20 ml with *water R*; add 5 ml of *nitric acid R*, dilute to 50 ml with *water R* and add 1 ml of a freshly prepared 50 g/l solution of *silver nitrate R*. Any opalescence in the solution is not more intense than that in a mixture of 1 ml of a freshly prepared 50 g/l solution of *silver nitrate R* and a solution prepared by diluting 5 ml of a 200 g/l solution of *sodium hydroxide R* to 20 ml with *water R* and adding 1.5 ml of 0.01 *M* *hydrochloric acid*, 5 ml of *nitric acid R* and sufficient *water R* to produce 50 ml (100 ppm).

Related substances. Examine by liquid chromatography (2.2.29) as described under Assay.

Inject 20 μ l each of test solution (a), reference solution (b) and reference solution (c). Continue the chromatography for 2.5 times the retention time of the principal peak. In the chromatogram obtained with test solution (a): the area of any peak corresponding to crotamiton impurity A is not greater than the area of the corresponding peak in the chromatogram obtained with reference solution (b) (3 per cent); the sum of the areas of any peaks, apart from the

principal peak, the peak corresponding to the *Z*-isomer and any peak corresponding to crotamiton impurity A, is not greater than the sum of the areas of the peaks corresponding to the *Z*- and *E*-isomers in the chromatogram obtained with reference solution (c) (1 per cent). Disregard any peak with an area less than 0.02 times the area of the principal peak in the chromatogram obtained with reference solution (c).

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Examine by liquid chromatography (2.2.29).

Test solution (a). Dissolve 50.0 mg of the substance to be examined in the mobile phase and dilute to 100.0 ml with the mobile phase.

Test solution (b). Dilute 1.0 ml of test solution (a) to 20.0 ml with the mobile phase.

Reference solution (a). Dissolve 50.0 mg of *crotamiton CRS* in the mobile phase and dilute to 100.0 ml with the mobile phase. Dilute 1.0 ml of the solution to 20.0 ml with the mobile phase.

Reference solution (b). Dissolve 15.0 mg of *crotamiton impurity A CRS* in the mobile phase and dilute to 20.0 ml with the mobile phase. Dilute 1.0 ml of the solution to 50.0 ml with the mobile phase.

Reference solution (c). Dilute 1.0 ml of test solution (a) to 100.0 ml with the mobile phase.

Reference solution (d). Dissolve 15 mg of *crotamiton impurity A CRS* in the mobile phase and dilute to 100 ml with the mobile phase. Dilute 1 ml of the solution to 10 ml with test solution (a).

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4 mm in internal diameter packed with *silica gel for chromatography R* (5 µm),
- as mobile phase at a flow rate of 1.0 ml/min a mixture of 8 volumes of *tetrahydrofuran R* and 92 volumes of *cyclohexane R*,
- as detector a spectrophotometer set at 242 nm.

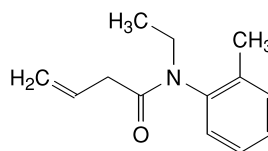
Inject 20 µl of reference solution (b) and 20 µl of reference solution (d). When the chromatograms are recorded in the prescribed conditions, the retention times relative to the principal peak (*E*-isomer) are: *Z*-isomer about 0.5 and crotamiton impurity A about 0.8. Adjust the sensitivity of the system so that the height of the principal peak in the chromatogram obtained with reference solution (b) is at least 70 per cent of the full scale of the recorder. The test is not valid unless, in the chromatogram obtained with reference solution (d), the resolution between the peaks corresponding to impurity A and the *E*-isomer is at least 4.5.

Inject alternately test solution (b) and reference solution (a). Calculate the percentage content of $C_{13}H_{17}NO$ from the sum of the areas of the peaks due to the *Z*- and *E*-isomers in the chromatograms obtained. Calculate the content of the *Z*-isomer, as a percentage of the total content of the *E*- and *Z*-isomers, from the chromatogram obtained with test solution (b).

STORAGE

Protected from light.

IMPURITIES

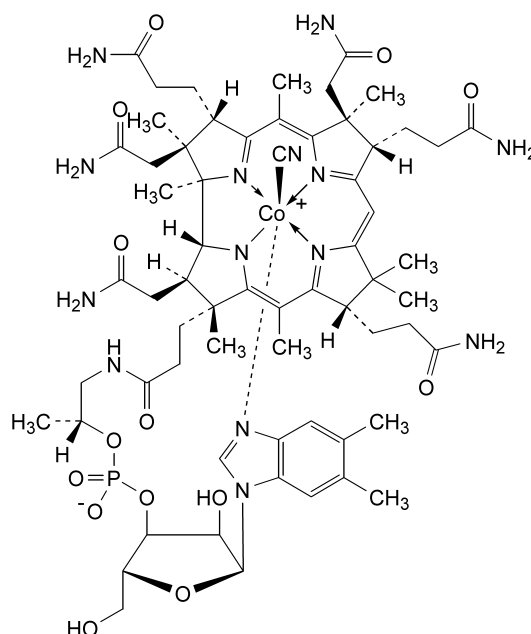


A. *N*-ethyl-*N*-(2-methylphenyl)but-3-enamide.

01/2005:0547

CYANOCOBALAMIN

Cyanocobalaminum



$C_{63}H_{88}CoN_{14}O_{14}P$

M_r 1355

DEFINITION

Cyanocobalamin contains not less than 96.0 per cent and not more than the equivalent of 102.0 per cent of α -(5,6-dimethylbenzimidazol-1-yl)cobamide cyanide, calculated with reference to the dried substance.

CHARACTERS

A dark-red, crystalline powder or dark-red crystals, sparingly soluble in water and in alcohol, practically insoluble in acetone. The anhydrous substance is very hygroscopic.

IDENTIFICATION

- A. Dissolve 2.5 mg in *water R* and dilute to 100.0 ml with the same solvent. Examined between 260 nm and 610 nm (2.2.25), the solution shows 3 absorption maxima, at 278 nm, 361 nm and at 547 nm to 559 nm. The ratio of the absorbance at the maximum at 361 nm to that at the maximum at 547 nm to 559 nm is 3.15 to 3.45. The ratio of the absorbance at the maximum at 361 nm to that at the maximum at 278 nm is 1.70 to 1.90.

- B. Carry out the test protected from light.

Examine by thin-layer chromatography (2.2.27), using *silica gel G R* as the coating substance.

Test solution. Dissolve 2 mg of the substance to be examined in 1 ml of a mixture of equal volumes of *alcohol R* and *water R*.

Reference solution. Dissolve 2 mg of *cyanocobalamin CRS* in 1 ml of a mixture of equal volumes of *alcohol R* and *water R*.

Apply to the plate 10 µl of each solution. Develop in a non-saturated tank over a path of 12 cm using a mixture of 9 volumes of *dilute ammonia R1*, 30 volumes of *methanol R* and 45 volumes of *methylene chloride R*. Allow the plate to dry in air. Examine in daylight. The principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

TESTS

Related substances. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 10.0 mg of the substance to be examined in the mobile phase and dilute to 10.0 ml with the same mixture of solvents. Use within 1 h.

Reference solution (a). Dilute 3.0 ml of the test solution to 100.0 ml with the mobile phase. Use within 1 h.

Reference solution (b). Dilute 5.0 ml of the test solution to 50.0 ml with the mobile phase. Dilute 1.0 ml of this solution to 100.0 ml with the mobile phase. Use within 1 h.

Reference solution (c). Dissolve 25 mg of the substance to be examined in 10 ml of *water R*, warming if necessary. Allow to cool and add 5 ml of a 1.0 g/l solution of *chloramine R* and 0.5 ml of 0.05 M *hydrochloric acid*. Dilute to 25 ml with *water R*. Shake and allow to stand for 5 min. Dilute 1 ml of this solution to 10 ml with the mobile phase and inject immediately.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4 mm in internal diameter packed with *octylsilyl silica gel for chromatography R* (5 µm),
- as the mobile phase at a flow rate of 0.8 ml/min a mixture prepared as follows: mix 26.5 volumes of *methanol R* and 73.5 volumes of a 10 g/l solution of *disodium hydrogen phosphate R* adjusted to pH 3.5 using *phosphoric acid R* and use within 2 days,
- a spectrophotometer set at 361 nm as detector,
- a loop injector.

Inject separately 20 µl of each solution and continue the chromatography for 3 times the retention time of cyanocobalamin. In the chromatogram obtained with the test solution, the sum of the areas of any peaks apart from the principal peak is not greater than the area of the principal peak in the chromatogram obtained with reference solution (a) (3 per cent). Disregard any peak whose area is less than that of the principal peak in the chromatogram obtained with reference solution (b). The test is not valid unless the chromatogram obtained with reference solution (c) shows 2 principal peaks, the resolution between these peaks is not less than 2.5 and the chromatogram obtained with reference solution (b) shows one principal peak with a signal-to-noise ratio of not less than 5.

Loss on drying (2.2.32). Not more than 12.0 per cent, determined on 20.00 mg by drying *in vacuo* at 100–105 °C for 2 h.

ASSAY

Dissolve 25.00 mg in *water R* and dilute to 1000.0 ml with the same solvent. Measure the absorbance (2.2.25) of the solution at the maximum at 361 nm. Calculate the content of $C_{63}H_{88}CoN_{14}O_{14}P$, taking the specific absorbance to be 207.

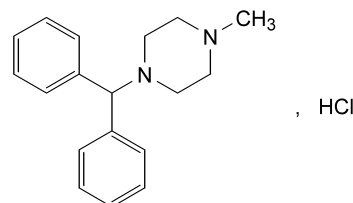
STORAGE

Store in an airtight container, protected from light.

01/2005:1092

CYCLIZINE HYDROCHLORIDE

Cyclizini hydrochloridum



$C_{18}H_{23}ClN_2$

M_r 302.8

DEFINITION

Cyclizine hydrochloride contains not less than 98.5 per cent and not more than the equivalent of 101.0 per cent of 1-(diphenylmethyl)-4-methylpiperazine hydrochloride, calculated with reference to the dried substance.

CHARACTERS

A white, crystalline powder, slightly soluble in water and in alcohol.

IDENTIFICATION

First identification: B, E.

Second identification: A, C, D, E.

- A. Dissolve 20.0 mg in a 5 g/l solution of *sulphuric acid R* and dilute to 100.0 ml with the same acid solution (solution A). Examined between 240 nm and 350 nm (2.2.25), solution A shows two absorption maxima, at 258 nm and 262 nm. The ratio of the absorbance measured at the maximum at 262 nm to that measured at the maximum at 258 nm is 1.0 to 1.1. Dilute 10.0 ml of solution A to 100.0 ml with a 5 g/l solution of *sulphuric acid R* (solution B). Examined between 210 nm and 240 nm, solution B shows an absorption maximum at 225 nm. The specific absorbance at the maximum is 370 to 410. Verify the resolution of the apparatus (2.2.25); the test is not valid unless the ratio of the absorbances is at least 1.7.
- B. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *cyclizine hydrochloride CRS*. Examine the substances prepared as discs using *potassium chloride R*.
- C. Examine the chromatograms obtained in the test for related substances. The principal spot in the chromatogram obtained with test solution (b) is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).
- D. Dissolve 0.5 g in 10 ml of *alcohol (60 per cent V/V) R* using heat, if necessary. Cool in iced water. Add 1 ml of *dilute sodium hydroxide solution R* and 10 ml of *water R*. Filter, wash the precipitate with *water R* and dry it at 60 °C at a pressure not exceeding 0.7 kPa for 2 h. The melting point (2.2.14) is 105 °C to 108 °C.
- E. It gives reaction (a) of chlorides (2.3.1).

TESTS

pH (2.2.3). Dissolve 0.5 g in a mixture of 40 volumes of *alcohol R* and 60 volumes of *carbon dioxide-free water R* and dilute to 25 ml with the same mixture of solvents. The pH of the solution is 4.5 to 5.5.

01/2005:1093

Related substances. Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel plate R*. Prepare the solutions immediately before use.

Test solution (a). Dissolve 0.20 g of the substance to be examined in *methanol R* and dilute to 10 ml with the same solvent.

Test solution (b). Dilute 5 ml of test solution (a) to 100 ml with *methanol R*.

Reference solution (a). Dissolve 10 mg of *cyclizine hydrochloride CRS* in *methanol R* and dilute to 10 ml with the same solvent.

Reference solution (b). Dissolve 5 mg of *methylpiperazine R* in *methanol R* and dilute to 50 ml with the same solvent.

Reference solution (c). Dilute 1 ml of test solution (b) to 10 ml with *methanol R*.

Reference solution (d). Dissolve 10 mg of *cyclizine hydrochloride CRS* and 10 mg of *hydroxyzine hydrochloride CRS* in *methanol R* and dilute to 10 ml with the same solvent.

Apply to the plate 20 µl of each solution. Develop over a path of 15 cm using a mixture of 2 volumes of *concentrated ammonia R*, 13 volumes of *methanol R* and 85 volumes of *methylene chloride R*. Allow the plate to dry in air for 30 min. Expose the plate to iodine vapour for 10 min. In the chromatogram obtained with test solution (a): any spot corresponding to 1-methylpiperazine is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.5 per cent); any spot, apart from the principal spot and any spot corresponding to 1-methylpiperazine, is not more intense than the principal spot in the chromatogram obtained with reference solution (c) (0.5 per cent). The test is not valid unless the chromatogram obtained with reference solution (d) shows two clearly separated spots.

Loss on drying (2.2.32). Not more than 1.0 per cent, determined on 1.000 g by drying in an oven at 130 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

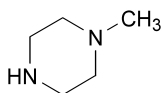
Dissolve 0.200 g in 15 ml of *anhydrous formic acid R* and add 40 ml of *acetic anhydride R*. Titrate with 0.1 M *perchloric acid*, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *perchloric acid* is equivalent to 15.14 mg of $C_{17}H_{26}ClNO_3$.

STORAGE

Store protected from light.

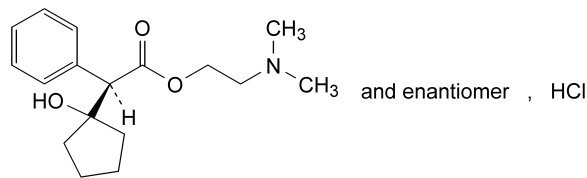
IMPURITIES



A. 1-methylpiperazine.

CYCLOPENTOLATE HYDROCHLORIDE

Cyclopentolati hydrochloridum



$C_{17}H_{26}ClNO_3$

M_r 327.9

DEFINITION

Cyclopentolate hydrochloride contains not less than 98.5 per cent and not more than the equivalent of 101.5 per cent of 2-(dimethylamino)ethyl (R,S)-2-(1-hydroxycyclopentyl)-2-phenylacetate hydrochloride, calculated with reference to the dried substance.

CHARACTERS

A white, crystalline powder, very soluble in water, freely soluble in alcohol.

IDENTIFICATION

First identification: B, D.

Second identification: A, C, D.

- Melting point (2.2.14): 135 °C to 141 °C.
- Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *cyclopentolate hydrochloride CRS*. Examine the substances as discs prepared using *potassium chloride R*. If the spectra obtained show differences, dissolve the substance to be examined and the reference substance separately in *alcohol R*, evaporate to dryness and record new spectra using the residues.
- Examine the chromatograms obtained in the test for related substances. The principal spot in the chromatogram obtained with test solution (b) is similar in position, fluorescence and size to the principal spot in the chromatogram obtained with reference solution (b).
- It gives reaction (a) of chlorides (2.3.1).

TESTS

pH (2.2.3). Dissolve 0.2 g in *carbon dioxide-free water R* and dilute to 20 ml with the same solvent. The pH of the solution is 4.5 to 5.5.

Related substances. Examine by thin-layer chromatography (2.2.27), using a suitable silica gel as the coating substance.

Test solution (a). Dissolve 0.20 g of the substance to be examined in *alcohol R* and dilute to 5 ml with the same solvent.

Test solution (b). Dilute 1 ml of test solution (a) to 20 ml with *alcohol R*.

Reference solution (a). Dilute 1 ml of test solution (a) to 200 ml with *alcohol R*.

Reference solution (b). Dissolve 10 mg of *cyclopentolate hydrochloride CRS* in *alcohol R* and dilute to 5 ml with the same solvent.

Apply separately to the plate 10 µl of each solution. Develop over a path of 15 cm using a mixture of 5 volumes of *concentrated ammonia R*, 15 volumes of *water R*, 30 volumes of *butyl acetate R* and 50 volumes of *2-propanol R*. Allow

the plate to dry in air. Spray with *an alcoholic solution of sulphuric acid R* and heat the plate at 120 °C for 30 min. Examine in ultraviolet light at 365 nm. Any spot in the chromatogram obtained with test solution (a), apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (a) (0.5 per cent).

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C for 4 h.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

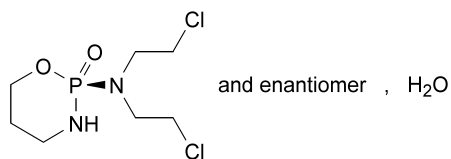
Dissolve 0.250 g in a mixture of 1.0 ml of 0.1 M hydrochloric acid and 50 ml of alcohol R. Carry out a potentiometric titration (2.2.20), using 0.1 M sodium hydroxide. Read the volume added between the two points of inflexion.

1 ml of 0.1 M sodium hydroxide is equivalent to 32.79 mg of $C_{7}H_{15}Cl_2N_2O_2P$.

01/2005:0711

CYCLOPHOSPHAMIDE

Cyclophosphamidum


 $C_7H_{15}Cl_2N_2O_2P \cdot H_2O$
 M_r 279.1

DEFINITION

Cyclophosphamide contains not less than 98.0 per cent and not more than the equivalent of 102.0 per cent of (2*RS*)-*N,N*-bis(2-chloroethyl)tetrahydro-2*H*-1,3,2-oxazaphosphorin-2-amine 2-oxide, calculated with reference to the anhydrous substance.

CHARACTERS

A white or almost white, crystalline powder, soluble in water, freely soluble in alcohol.

IDENTIFICATION

First identification: B.

Second identification: A, C, D.

- Determine the melting point (2.2.14) of the substance to be examined. Mix equal parts of the substance to be examined and *cyclophosphamide CRS* and determine the melting point of the mixture. The difference between the melting points (which are about 51 °C) is not greater than 2 °C.
- Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *cyclophosphamide CRS*.
- Examine the chromatograms obtained in the test for related substances. The principal spot in the chromatogram obtained with test solution (b) is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).

- Dissolve 0.1 g in 10 ml of *water R* and add 5 ml of *silver nitrate solution R1*; the solution remains clear. Boil, a white precipitate is formed which dissolves in *concentrated ammonia R* and is reprecipitated on the addition of *dilute nitric acid R*.

TESTS

Solution S. Dissolve 0.50 g in *carbon dioxide-free water R* and dilute to 25.0 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and not more intensely coloured than reference solution Y_6 (2.2.2, *Method II*).

pH (2.2.3). The pH of solution S is 4.0 to 6.0, determined immediately after preparation of the solution.

Related substances. Examine by thin-layer chromatography (2.2.27), using *silica gel G R* as the coating substance.

Test solution (a). Dissolve 0.10 g of the substance to be examined in *alcohol R* and dilute to 5 ml with the same solvent.

Test solution (b). Dilute 1 ml of test solution (a) to 10 ml with *alcohol R*.

Reference solution (a). Dissolve 10 mg of *cyclophosphamide CRS* in *alcohol R* and dilute to 5 ml with the same solvent.

Reference solution (b). Dilute 0.1 ml of test solution (a) to 10 ml with *alcohol R*.

Apply separately to the plate 10 µl of each solution. Develop over a path of 15 cm using a mixture of 2 volumes of *anhydrous formic acid R*, 4 volumes of *acetone R*, 12 volumes of *water R* and 80 volumes of *methyl ethyl ketone R*. Dry the plate in a current of warm air and heat at 110 °C for 10 min. At the bottom of a chromatography tank, place an evaporating dish containing a 50 g/l solution of *potassium permanganate R* and add an equal volume of *hydrochloric acid R*. Place the plate whilst still hot in the tank and close the tank. Leave the plate in contact with the chlorine gas for 2 min. Withdraw the plate and place it in a current of cold air until the excess of chlorine is removed and an area of coating below the points of application gives at most a very faint blue colour with a drop of *potassium iodide and starch solution R*. Avoid prolonged exposure to cold air. Spray with *potassium iodide and starch solution R* and allow to stand for 5 min. Any spot in the chromatogram obtained with test solution (a), apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (b) (1.0 per cent). Disregard any spot remaining at the starting-point.

Chlorides (2.4.4). Dissolve 0.15 g in *water R* and dilute to 15 ml with the same solvent. The freshly prepared solution complies with the limit test for chlorides (330 ppm).

Phosphates (2.4.11). Dissolve 0.10 g in *water R* and dilute to 100 ml with the same solvent. The solution complies with the limit test for phosphates (100 ppm).

Heavy metals (2.4.8). 1.0 g complies with limit test C for heavy metals (20 ppm). Prepare the standard using 2 ml of *lead standard solution (10 ppm Pb) R*.

Water (2.5.12): 6.0 per cent to 7.0 per cent, determined on 0.300 g by the semi-micro determination of water.

ASSAY

Dissolve 0.100 g in 50 ml of a 1 g/l solution of *sodium hydroxide R* in *ethylene glycol R* and boil under a reflux condenser for 30 min. Allow to cool and rinse the condenser with 25 ml of *water R*. Add 75 ml of *2-propanol R*, 15 ml

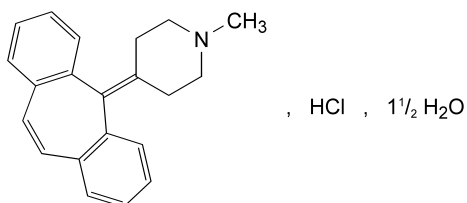
of *dilute nitric acid R*, 10.0 ml of *0.1 M silver nitrate* and 2.0 ml of *ferric ammonium sulfate solution R2* and titrate with *0.1 M ammonium thiocyanate*.

1 ml of *0.1 M silver nitrate* is equivalent to 13.05 mg of $C_{21}H_{22}ClN$.

01/2005:0817

CYPROHEPTADINE HYDROCHLORIDE

Cyproheptadini hydrochloridum


 $C_{21}H_{22}ClN \cdot 1\frac{1}{2} \text{H}_2\text{O}$
 M_r 350.9

DEFINITION

Cyproheptadine hydrochloride contains not less than 98.5 per cent and not more than the equivalent of 101.0 per cent of 4-(5*H*-dibenzo[*a,d*]cyclohepten-5-ylidene)-1-methylpiperidine hydrochloride, calculated with reference to the anhydrous substance.

CHARACTERS

A white or slightly yellow, crystalline powder, slightly soluble in water, freely soluble in methanol, sparingly soluble in alcohol.

IDENTIFICATION

First identification: B, D.

Second identification: A, C, D.

A. Dissolve 50.0 mg in *alcohol R* and dilute to 50.0 ml with the same solvent. Dilute 2.0 ml of the solution to 100.0 ml with *alcohol R*. Examined between 230 nm and 320 nm (2.2.25), the solution shows an absorption maximum at 286 nm. The specific absorbance at the maximum is 335 to 365, calculated with reference to the anhydrous substance.

B. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *cyproheptadine hydrochloride CRS*. Examine the substances as mulls in *liquid paraffin R*.

C. Examine by thin-layer chromatography (2.2.27), using *silica gel GF₂₅₄ R* as the coating substance.

Test solution. Dissolve 25 mg of the substance to be examined in *methanol R* and dilute to 25 ml with the same solvent.

Reference solution (a). Dissolve 10 mg of *cyproheptadine hydrochloride CRS* in *methanol R* and dilute to 10 ml with the same solvent.

Reference solution (b). Dissolve 10 mg of *imipramine hydrochloride CRS* in *methanol R* and dilute to 10 ml with the same solvent. Dilute 1 ml to 2 ml with reference solution (a).

Apply to the plate 2 µl of each solution. Develop over a path of 15 cm using a mixture of 5 volumes of *diethylamine R*, 20 volumes of *ether R* and 75 volumes of *cyclohexane R*. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. The principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a). The test is not valid unless the chromatogram obtained with reference solution (b) shows 2 clearly separated principal spots.

D. A saturated solution gives reaction (b) of chlorides (2.3.1).

TESTS

Acidity. Dissolve 0.10 g in *water R* and dilute to 25 ml with the same solvent. Add 0.1 ml of *methyl red solution R*. Not more than 0.15 ml of *0.01 M sodium hydroxide* is required to change the colour of the indicator.

Related substances. Examine by thin-layer chromatography (2.2.27), using *silica gel G R* as the coating substance.

Test solution. Dissolve 50 mg of the substance to be examined in a mixture of 1 volume of *methanol R* and 9 volumes of *methylene chloride R* and dilute to 5 ml with the same mixture of solvents.

Reference solution (a). Dissolve 10 mg of *dibenzocycloheptene CRS* (5*H*-dibenzo[*a,d*]cycloheptene) in a mixture of 1 volume of *methanol R* and 9 volumes of *methylene chloride R* and dilute to 50 ml with the same mixture of solvents. Dilute 1 ml to 10 ml with a mixture of 1 volume of *methanol R* and 9 volumes of *methylene chloride R*.

Reference solution (b). Dilute 1 ml of the test solution to 100 ml with a mixture of 1 volume of *methanol R* and 9 volumes of *methylene chloride R*. Dilute 1 ml to 10 ml with a mixture of 1 volume of *methanol R* and 9 volumes of *methylene chloride R*.

Apply to the plate 10 µl of each solution. Develop over a path of 15 cm using a mixture of 10 volumes of *methanol R* and 90 volumes of *methylene chloride R*. Allow the plate to dry in air and spray with *alcoholic solution of sulphuric acid R*. Heat at 110 °C for 30 min and examine in ultraviolet light at 365 nm while hot. In the chromatogram obtained with the test solution: any spot corresponding to dibenzocycloheptene is not more intense than the spot in the chromatogram obtained with reference solution (a) (0.2 per cent); any spot apart from the principal spot and any spot corresponding to dibenzocycloheptene is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.1 per cent).

Water (2.5.12): 7.0 per cent to 9.0 per cent, determined on 0.200 g.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

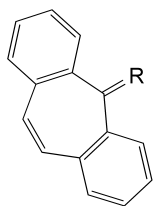
Dissolve 0.250 g in a mixture of 5.0 ml of *0.01 M hydrochloric acid* and 50 ml of *alcohol R*. Carry out a potentiometric titration (2.2.20), using *0.1 M sodium hydroxide*. Read the volume added between the 2 points of inflexion.

1 ml of *0.1 M sodium hydroxide* is equivalent to 32.39 mg of $C_{21}H_{22}ClN$.

STORAGE

Store protected from light.

IMPURITIES

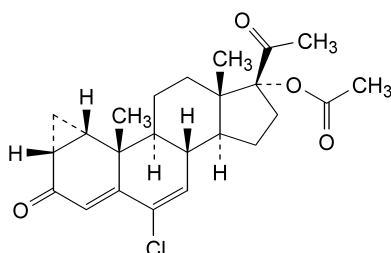


- A. R = H₂: dibenzo[*a,d*]cycloheptene,
 B. R = O: 5*H*-dibenzo[*a,d*]cyclohepten-5-one
 (dibenzosuberone).

01/2005:1094

CYPROTERONE ACETATE

Cyproteroni acetat

C₂₄H₂₉ClO₄M_r 416.9

DEFINITION

Cyproterone acetate contains not less than 97.0 per cent and not more than the equivalent of 103.0 per cent of 6-chloro-3,20-dioxo-1β,2β-dihydro-3'*H*-cyclopropa[1,2]pregna-1,4,6-trien-17-yl acetate, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, crystalline powder, practically insoluble in water, very soluble in methylene chloride, freely soluble in acetone, soluble in methanol, sparingly soluble in ethanol.

It melts at about 210 °C.

IDENTIFICATION

First identification: A.

Second identification: B, C, D, E.

- A. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *cyproterone acetate CRS*.
 B. Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel F₂₅₄ plate R*.

Test solution. Dissolve 20 mg of the substance to be examined in *methylene chloride R* and dilute to 10 ml with the same solvent.

Reference solution. Dissolve 10 mg of *cyproterone acetate CRS* in *methylene chloride R* and dilute to 5 ml with the same solvent.

Apply to the plate 5 µl of each solution. Develop over a path of 15 cm using a mixture of equal volumes of *cyclohexane R* and *ethyl acetate R*. Allow the plate to dry in air. Repeat the development. Allow the plate to dry in air. Examine in ultraviolet light at 254 nm. The principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with the reference solution.

- C. To about 1 mg add 2 ml of *sulphuric acid R* and heat on a water-bath for 2 min. A red colour develops. Cool. Add the solution cautiously to 4 ml of *water R* and shake. The solution becomes violet.
 D. Incinerate about 30 mg with 0.3 g of *anhydrous sodium carbonate R* over a naked flame for about 10 min. Cool and dissolve the residue in 5 ml of *dilute nitric acid R*. Filter. To 1 ml of the filtrate add 1 ml of *water R*. The solution gives reaction (a) of chlorides (2.3.1).
 E. It gives the reaction of acetyl (2.3.1).

TESTS

Specific optical rotation (2.2.7). Dissolve 0.25 g in *acetone R* and dilute to 25.0 ml with the same solvent. The specific optical rotation is + 152 to + 157, calculated with reference to the dried substance.

Related substances. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 10.0 mg of the substance to be examined in *acetonitrile R* and dilute to 10.0 ml with the same solvent.

Reference solution (a). Dilute 1.0 ml of the test solution to 100.0 ml with *acetonitrile R*.

Reference solution (b). Dissolve 5 mg of *medroxyprogesterone acetate CRS* in *acetonitrile R* and dilute to 50.0 ml with the same solvent. Dilute 1.0 ml of the solution to 10.0 ml with reference solution (a).

The chromatographic procedure may be carried out using:

- a stainless steel column 0.125 m long and 4.6 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (3 µm),
- as mobile phase at a flow rate of 1.5 ml/min a mixture of 40 volumes of *acetonitrile R* and 60 volumes of *water R*,
- as detector a spectrophotometer set at 254 nm.

Inject 20 µl of reference solution (a) and 20 µl of reference solution (b). Adjust the sensitivity of the system so that the height of the principal peak in the chromatogram obtained with reference solution (a) is at least 50 per cent of the full scale of the recorder. The test is not valid unless, in the chromatogram obtained with reference solution (b), the resolution between the peak corresponding to cyproterone acetate and the peak corresponding to medroxyprogesterone acetate is at least 3.0.

Inject 20 µl of the test solution. Continue the chromatography for twice the retention time of cyproterone acetate. In the chromatogram obtained with the test solution, the sum of the areas of all the peaks, apart from the principal peak, is not greater than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent). Disregard any peak with an area less than 0.05 times that of the principal peak in the chromatogram obtained with reference solution (a).

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying at 80 °C at a pressure not exceeding 0.7 kPa.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

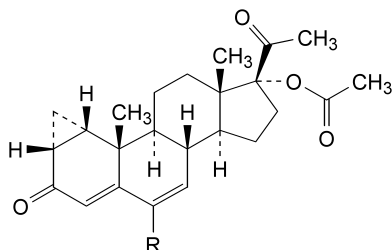
Dissolve 50.0 mg in *methanol R* and dilute to 50.0 ml with the same solvent. Dilute 1.0 ml of the solution to 100.0 ml with *methanol R*. Measure the absorbance (2.2.25) at the maximum at 282 nm.

Calculate the content of C₂₄H₂₉ClO₄ taking the specific absorbance to be 414.

STORAGE

Store protected from light.

IMPURITIES

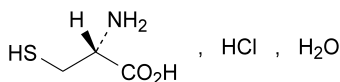


- A. R = H: 3,20-dioxo-1 β ,2 β -dihydro-3'*H*-cyclopropa[1,2]pregna-1,4,6-trien-17-yl acetate,
- B. R = OCH₃: 6-methoxy-3,20-dioxo-1 β ,2 β -dihydro-3'*H*-cyclopropa[1,2]pregna-1,4,6-trien-17-yl acetate.

01/2005:0895

CYSTEINE HYDROCHLORIDE MONOHYDRATE

Cysteinum hydrochloridum monohydricum

C₃H₈ClNO₂S·H₂O*M*_r 175.6

DEFINITION

Cysteine hydrochloride monohydrate contains not less than 98.5 per cent and not more than the equivalent of 101.0 per cent of (2*R*)-2-amino-3-sulfanylpropanoic acid hydrochloride, calculated with reference to the dried substance.

CHARACTERS

A white, crystalline powder or colourless crystals, freely soluble in water, slightly soluble in alcohol.

IDENTIFICATION

First identification: A, B, E.

Second identification: A, C, D, E.

- It complies with the test for specific optical rotation (see Tests).
- Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *cysteine hydrochloride monohydrate CRS*. Examine the substances prepared as discs.
- Examine the chromatograms obtained in the test for ninhydrin-positive substances. The principal spot in the chromatogram obtained with test solution (b) is similar in position, colour, and size to the principal spot in the chromatogram obtained with reference solution (b).
- Dissolve about 5 mg in 1 ml of *dilute sodium hydroxide solution R*. Add 1 ml of a 30 g/l solution of *sodium nitroprusside R*. An intense violet colour develops which becomes brownish-red and then orange. Add 1 ml of *hydrochloric acid R*. The solution becomes green.
- It gives reaction (a) of chlorides (2.3.1).

TESTS

Solution S. Dissolve 2.5 g in *distilled water R* and dilute to 50 ml with the same solvent.

Appearance of solution. Dilute 10 ml of solution S to 20 ml with *water R*. The solution is clear (2.2.1) and not more intensely coloured than reference solution BY₆ (2.2.2, Method II).

Specific optical rotation (2.2.7). Dissolve 2.00 g in *hydrochloric acid R1* and dilute to 25.0 ml with the same acid. The specific optical rotation is + 5.5 to + 7.0, calculated with reference to the dried substance.

Ninhydrin-positive substances. Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel plate R*.

Test solution (a). Dissolve 0.20 g of the substance to be examined in *water R* and dilute to 10 ml with the same solvent. To 5 ml of the solution add 5 ml of a 40 g/l solution of *N-ethylmaleimide R* in *alcohol R*. Allow to stand for 5 min.

Test solution (b). Dilute 1 ml of test solution (a) to 50 ml with *water R*.

Reference solution (a). Dissolve 20 mg of *cysteine hydrochloride monohydrate CRS* in *water R* and dilute to 10 ml with the same solvent. Add 10 ml of a 40 g/l solution of *N-ethylmaleimide R* in *alcohol R*. Allow to stand for 5 min.

Reference solution (b). Dilute 2 ml of reference solution (a) to 10 ml with *water R*.

Reference solution (c). Dilute 5 ml of test solution (b) to 20 ml with *water R*.

Reference solution (d). Dissolve 10 mg of *tyrosine CRS* in 10 ml of reference solution (a) and dilute to 25 ml with *water R*.

Apply separately to the plate 5 μ l of each test solution and reference solutions (b), (c), and (d). Develop over a path of 15 cm using a mixture of 20 volumes of *glacial acetic acid R*, 20 volumes of *water R* and 60 volumes of *butanol R*. Dry the plate at 80 °C for 30 min. Spray with *ninhydrin solution R* and heat at 100 °C to 105 °C for 15 min. Any spot in the chromatogram obtained with test solution (a), apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (c) (0.5 per cent). The test is not valid unless the chromatogram obtained with reference solution (d) shows 2 clearly separated principal spots.

Sulphates (2.4.13). Dilute 10 ml of solution S to 15 ml with *distilled water R*. The solution complies with the limit test for sulphates (300 ppm).

Ammonium (2.4.1). 50 mg complies with limit test B for ammonium (200 ppm). Prepare the standard using 0.1 ml of *ammonium standard solution (100 ppm NH₄) R*.

Iron (2.4.9). In a separating funnel, dissolve 0.50 g in 10 ml of *dilute hydrochloric acid R*. Shake with 3 quantities, each of 10 ml, of *methyl isobutyl ketone R1*, shaking for 3 min each time. To the combined organic layers add 10 ml of *water R* and shake for 3 min. The aqueous layer complies with the limit test for iron (20 ppm).

Heavy metals (2.4.8). Dissolve 2.0 g in *water R*. Adjust to pH 3 to 4 with *concentrated ammonia R* and dilute to 20 ml with *water R*. 12 ml of the solution complies with limit test A for heavy metals (10 ppm). Prepare the standard using *lead standard solution (1 ppm Pb) R*.

Loss on drying (2.2.32): 8.0 per cent to 12.0 per cent, determined on 1.000 g by drying at a pressure not exceeding 0.7 kPa for 24 h.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

In a ground-glass stoppered flask dissolve 0.300 g of the substance to be examined and 4 g of *potassium iodide R* in 20 ml of *water R*. Cool the solution in iced water and add 3 ml of *hydrochloric acid R1* and 25.0 ml of 0.05 *M iodine*. Stopper the flask and allow to stand in the dark for 20 min. Titrate with 0.1 *M sodium thiosulphate* using 3 ml of *starch solution R*, added towards the end of the titration, as indicator. Carry out a blank titration.

1 ml of 0.05 *M iodine* is equivalent to 15.76 mg of $C_6H_{12}N_2O_4S_2$.

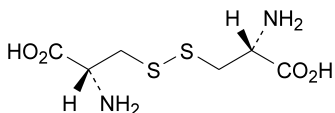
STORAGE

Store protected from light.

01/2005:0998

CYSTINE

Cystinum


 $C_6H_{12}N_2O_4S_2$
 M_r 240.3

DEFINITION

Cystine contains not less than 98.5 per cent and not more than the equivalent of 101.0 per cent of 3,3'-disulfanedibis[(2*R*)-2-aminopropanoic acid], calculated with reference to the dried substance.

CHARACTERS

A white, crystalline powder, practically insoluble in water and in alcohol. It dissolves in dilute solutions of alkali hydroxides.

IDENTIFICATION

First identification: A, B.

Second identification: A, C, D.

- It complies with the test for specific optical rotation (see Tests).
- Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *cystine CRS*. Examine the substances prepared as discs.
- Examine the chromatograms obtained in the test for ninhydrin-positive substances. The principal spot in the chromatogram obtained with test solution (b) is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).
- To 0.1 g carefully add 1 ml of *strong hydrogen peroxide solution R* and 0.1 ml of *ferric chloride solution R1*. Allow to cool. Add 1 ml of *dilute hydrochloric acid R* and 5 ml of *water R*. Add 1 ml of *barium chloride solution R1*. Turbidity or a white precipitate develops within 3 min.

TESTS

Appearance of solution. Dissolve 1.0 g in *dilute hydrochloric acid R* and dilute to 10 ml with the same acid. The solution is clear (2.2.1) and not more intensely coloured than reference solution Y₇ (2.2.2, *Method II*).

Specific optical rotation (2.2.7). Dissolve 0.50 g in 1 *M hydrochloric acid* and dilute to 25.0 ml with the same acid. The specific optical rotation is -218 to -224, calculated with reference to the dried substance.

Ninhydrin-positive substances. Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel plate R*.

Test solution (a). Dissolve 0.10 g of the substance to be examined in 1 *M hydrochloric acid* and dilute to 10 ml with the same acid.

Test solution (b). Dilute 1 ml of test solution (a) to 50 ml with *water R*.

Reference solution (a). Dissolve 10 mg of *cystine CRS* in 1 ml of 1 *M hydrochloric acid* and dilute to 50 ml with *water R*.

Reference solution (b). Dilute 2 ml of test solution (b) to 20 ml with *water R*.

Reference solution (c). Dissolve 10 mg of *cystine CRS* and 10 mg of *arginine hydrochloride CRS* in 1 ml of 1 *M hydrochloric acid* and dilute to 25 ml with *water R*.

Apply separately to the plate 5 µl of each solution. Develop over a path of 15 cm using a mixture of 30 volumes of *concentrated ammonia R* and 70 volumes of 2-propanol *R*. Allow the plate to dry in air. Spray with *ninhydrin solution R* and heat at 100 °C to 105 °C for 15 min. Any spot in the chromatogram obtained with test solution (a), apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.2 per cent). The test is not valid unless the chromatogram obtained with reference solution (c) shows two clearly separated spots.

Chlorides (2.4.4). Dissolve 0.25 g in 5 ml of *dilute nitric acid R* and dilute to 15 ml with *water R*. The solution, without further addition of nitric acid, complies with the limit test for chlorides (200 ppm).

Sulphates (2.4.13). Dissolve 0.5 g in 5 ml of *dilute hydrochloric acid R* and dilute to 15 ml with *distilled water R*. The solution complies with the limit test for sulphates (300 ppm).

Ammonium (2.4.1). 0.10 g complies with limit test B for ammonium (200 ppm). Prepare the standard using 0.2 ml of *ammonium standard solution (100 ppm NH₄) R*.

Iron (2.4.9). In a separating funnel, dissolve 1.0 g in 10 ml of *dilute hydrochloric acid R*. Shake with three quantities, each of 10 ml, of *methyl isobutyl ketone R1*, shaking for 3 min each time. To the combined organic layers add 10 ml of *water R* and shake for 3 min. The aqueous layer complies with the limit test for iron (10 ppm).

Heavy metals (2.4.8). 2.0 g complies with limit test D for heavy metals (10 ppm). Prepare the standard using 2 ml of *lead standard solution (10 ppm Pb) R*.

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

In a flask with a ground-glass stopper, dissolve 0.100 g in a mixture of 2 ml of *dilute sodium hydroxide solution R* and 10 ml of *water R*. Add 10 ml of a 200 g/l solution of *potassium bromide R*, 50.0 ml of 0.0167 *M potassium bromate* and 15 ml of *dilute hydrochloric acid R*. Stopper the flask and cool in iced water. Allow to stand in the dark for 10 min. Add 1.5 g of *potassium iodide R*. After 1 min, titrate with 0.1 *M sodium thiosulphate*, using 2 ml of *starch solution R*, added towards the end-point, as indicator. Carry out a blank titration.

1 ml of 0.0167 *M potassium bromate* is equivalent to 2.403 mg of $C_6H_{12}N_2O_4S_2$.

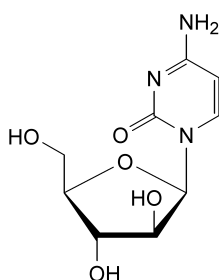
STORAGE

Store protected from light.

01/2005:0760

CYTARABINE

Cytarabinum


 $C_9H_{13}N_3O_5$
 M_r 243.2

DEFINITION

Cytarabine contains not less than 99.0 per cent and not more than the equivalent of 100.5 per cent of 4-amino-1- β -D-arabinofuranosylpyrimidin-2(1*H*)-one, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, crystalline powder, freely soluble in water, very slightly soluble in alcohol and in methylene chloride.

It melts at about 215 °C.

IDENTIFICATION

- Dissolve 20.0 mg in 0.1 *M* hydrochloric acid and dilute to 100.0 ml with the same acid. Dilute 5.0 ml of the solution to 100.0 ml with 0.1 *M* hydrochloric acid. Examined between 230 nm and 350 nm (2.2.25), the solution shows an absorption maximum at 281 nm. The specific absorbance at the maximum is 540 to 570.
- Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *cytarabine CRS*. Examine the substances prepared as discs.
- Examine the chromatograms obtained in the test for related substances in ultraviolet light at 254 nm. The principal spot in the chromatogram obtained with test solution (b) is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a).

TESTS

Appearance of solution. Dissolve 1.0 g in *water R* and dilute to 10 ml with the same solvent. The solution is clear (2.2.1) and not more intensely coloured than reference solution Y_5 (2.2.2, *Method II*).

Specific optical rotation (2.2.7). Dissolve 0.250 g in *water R* and dilute to 25.0 ml with the same solvent. The specific optical rotation is + 154 to + 160, calculated with reference to the dried substance.

Related substances. Examine by thin-layer chromatography (2.2.27), using *silica gel GF₂₅₄ R* as the coating substance.

Test solution (a). Dissolve 0.25 g of the substance to be examined in *water R* and dilute to 5 ml with the same solvent.

Test solution (b). Dilute 2 ml of test solution (a) to 50 ml with *water R*.

Reference solution (a). Dissolve 10 mg of *cytarabine CRS* in *water R* and dilute to 5 ml with the same solvent.

Reference solution (b). Dilute 0.5 ml of test solution (a) to 100 ml with *water R*.

Reference solution (c). Dissolve 20 mg of *uridine R* and 20 mg of *uracil arabinoside CRS* in *methanol R* and dilute to 10 ml with the same solvent.

Apply separately to the plate 5 μ l of each solution. Develop over a path of 15 cm using a mixture of 15 volumes of *water R*, 20 volumes of *acetone R* and 65 volumes of *methyl ethyl ketone R*. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. Any spot in the chromatogram obtained with test solution (a), apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.5 per cent). The test is not valid unless the chromatogram obtained with reference solution (c) shows two clearly separated spots.

Loss on drying (2.2.32). Not more than 1.0 per cent, determined on 0.250 g by drying over *diphosphorus pentoxide R* at 60 °C at a pressure of 0.2 kPa to 0.7 kPa for 3 h.

Sulphated ash (2.4.14). Not more than 0.5 per cent, determined on 1.0 g.

ASSAY

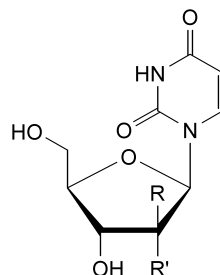
Dissolve 0.200 g in 60 ml of *anhydrous acetic acid R*, warming if necessary. Titrate with 0.1 *M* perchloric acid determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 *M* perchloric acid is equivalent to 24.32 mg of $C_9H_{13}N_3O_5$.

STORAGE

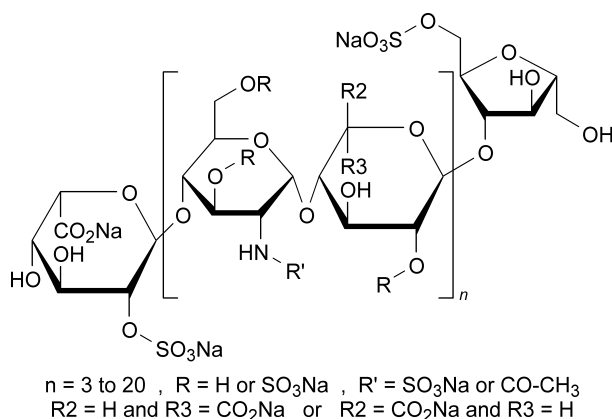
Store in an airtight container, protected from light.

IMPURITIES



- $R = OH$, $R' = H$: 1- β -D-arabinofuranosylpyrimidine-2,4(1*H*, 3*H*)-dione (uracil arabinoside),
- $R = H$, $R' = OH$: 1- β -D-ribofuranosylpyrimidine-2,4(1*H*, 3*H*)-dione (uridine).

01/2005:1195

DALTEPARIN SODIUM**Dalteparinum natricum****DEFINITION**

Dalteparin sodium is the sodium salt of a low-molecular-mass heparin that is obtained by nitrous acid depolymerisation of heparin from porcine intestinal mucosa. The majority of the components have a 2-*O*-sulpho- α -L-idopyranosuronic acid structure at the non-reducing end and a 6-*O*-sulpho-2,5-anhydro-D-mannitol structure at the reducing end of their chain.

Dalteparin sodium complies with the monograph on Low-molecular-mass heparins (0828) with the modifications and additional requirements below:

The mass-average molecular mass ranges between 5600 and 6400, with a characteristic value of about 6000.

The degree of sulphatation is 2.0 to 2.5 per disaccharide unit.

The potency is not less than 110 IU and not more than 210 IU of anti-factor Xa activity per milligram, calculated with reference to the dried substance. The anti-factor IIa activity is not less than 35 IU and not more than 100 IU/mg, calculated with reference to the dried substance. The ratio of anti-factor Xa activity to anti-factor IIa activity is between 1.9 and 3.2.

PRODUCTION

Dalteparin sodium is produced by a validated manufacturing and purification procedure under conditions designed to minimise the presence of N-NO groups.

The manufacturing procedure must have been shown to reduce any contamination by N-NO groups to approved limits using an appropriate, validated quantification method.

IDENTIFICATION

Carry out identification test C as described in the monograph on *Low-molecular-mass heparins (0828)*. The following requirements apply:

The mass-average molecular mass ranges between 5600 and 6400. The mass percentage of chains lower than 3000 is not more than 13.0 per cent. The mass percentage of chains higher than 8000 ranges between 15.0 per cent and 25.0 per cent.

TESTS

Appearance of solution. Dissolve 1 g in 10 ml of *water R*. The solution is clear (2.2.1) and not more intensely coloured than intensity 5 of the range of reference solutions of the most appropriate colour (2.2.2, *Method II*).

Nitrite. Not more than 5 ppm. Examine by liquid chromatography (2.2.29). *Rinse all volumetric flasks at least three times with water R before the preparation of the solutions.*

Test solution. Dissolve 80.0 mg of the substance to be examined in *water R* and dilute to 10.0 ml with the same solvent. Allow to stand for at least 30 min.

Reference solution (a). Dissolve 60.0 mg of *sodium nitrite R* in *water R* and dilute to 1000.0 ml with the same solvent.

For the preparation of reference solution (b), use a pipette previously rinsed with reference solution (a).

Reference solution (b). Dilute 1.00 ml of reference solution (a) to 50.0 ml with *water R*.

Before preparing reference solutions (c), (d) and (e), rinse all pipettes with reference solution (b).

Reference solution (c). Dilute 1.00 ml of reference solution (b) to 100.0 ml with *water R* (1 ppm nitrite).

Reference solution (d). Dilute 3.00 ml of reference solution (b) to 100.0 ml with *water R* (3 ppm nitrite).

Reference solution (e). Dilute 5.00 ml of reference solution (b) to 100.0 ml with *water R* (5 ppm nitrite).

The chromatographic procedure may be carried out using:

- a column 0.125 m long and 4.3 mm in internal diameter packed with a strong anion-exchange resin,
- as mobile phase at a flow rate of 1.0 ml/min a solution consisting of 13.61 g of *sodium acetate R* dissolved in *water R*, adjusted to pH 4.3 with *phosphoric acid R* and diluted to 1000 ml with *water R*,
- as detector an appropriate electrochemical device with the following characteristics and settings: a suitable working electrode, a detector potential of + 1.00 V versus Ag/AgCl reference electrode and a detector sensitivity of 0.1 μ A full scale.

Inject 100 μ l of reference solution (d). When the chromatograms are recorded in the prescribed conditions, the retention time for nitrite is 3.3 to 4.0 min. The test is not valid unless:

- the number of theoretical plates calculated for the nitrite peak is at least 7000 per metre per column (dalteparin sodium will block the binding sites of the stationary phase, which will cause shorter retention times and lower separation efficiency for the analyte; the initial performance of the column may be partially restored using a 58 g/l solution of *sodium chloride R* at a flow rate of 1.0 ml/min for 1 h; after regeneration the column is rinsed with 200 ml to 400 ml of *water R*),
- the symmetry factor for the nitrite peak is less than three,
- the relative standard deviation of the peak area for nitrite obtained from six injections is less than 3.0 per cent.

Inject 100 μ l each of reference solutions (c) and (e). The test is not valid unless:

- the correlation factor for a linear relationship between concentration and response for reference solutions (c), (d) and (e) is at least 0.995,
- the signal-to-noise ratio for reference solution (c) is not less than 5 (if the noise level is too high, electrode recalibration is recommended),
- a blank injection of *water R* does not give rise to spurious peaks.

Inject 100 μ l of the test solution. Calculate the content of nitrite from the peak areas in the chromatogram obtained with reference solutions (c), (d) and (e).

Boron. Not more than 1 ppm, determined by inductively coupled plasma atomic emission spectroscopy.

Boron is determined by measurement of the emission from an inductively coupled plasma (ICP) at a wavelength specific to boron. The emission line at 249.733 nm is used. Use an appropriate apparatus, whose settings have been optimised as directed by the manufacturer.

Test solution. Dissolve 0.2500 g of the substance to be examined in about 2 ml of *water for chromatography R*, add 100 µl of *nitric acid R* and dilute to 10.00 ml with the same solvent.

Reference solution (a). Prepare a 1 per cent V/V solution of *nitric acid R* in *water for chromatography R* (blank).

Reference solution (b). Prepare a 11.4 µg/ml solution of *boric acid R* in a 1 per cent V/V solution of *nitric acid R* in *water for chromatography R* (STD_{cal}).

Reference solution (c). Dissolve 0.2500 g of a reference dalteparin sodium with no detectable boron in about 2 ml of *water for chromatography R*, add 100 µl of *nitric acid R* and dilute to 10.00 ml with the same solvent (STD₀).

Reference solution (d). Dissolve 0.2500 g of a reference dalteparin sodium with no boron detected in about 2 ml of a 1 per cent V/V solution of *nitric acid R* in *water for chromatography R*, add 10 µl of a 5.7 mg/ml solution of *boric acid R* and dilute to 10.00 ml with the same solvent (STD₁). This solution contains 1 µg/ml of boron.

Calculate the content of boron in the substance to be examined, using the following correction factor:

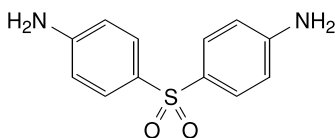
$$f = \frac{(\text{STD}_1 - \text{STD}_0) \times 2}{(\text{STD}_{\text{cal}} - \text{blank})}$$

Loss on drying (2.2.32). Not more than 5.0 per cent, determined on 1.000 g by drying in an oven at 60 °C over *diphosphorus pentoxide R* at a pressure not exceeding 670 Pa for 3 h.

01/2005:0077

DAPSONE

Dapsonum

C₁₂H₁₂N₂O₂SM_r 248.3

DEFINITION

Dapsone contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of 4,4'-sulphonyldianiline, calculated with reference to the dried substance.

CHARACTERS

A white or slightly yellowish-white, crystalline powder, very slightly soluble in water, freely soluble in acetone, sparingly soluble in alcohol. It dissolves freely in dilute mineral acids.

IDENTIFICATION

- A. Melting point (2.2.14): 175 °C to 181 °C.
- B. Dissolve 50.0 mg in *methanol R* and dilute to 100.0 ml with the same solvent. Dilute 1.0 ml of this solution to 100.0 ml with *methanol R*. Examined between 230 nm and 350 nm (2.2.25), the solution shows 2 absorption maxima, at 260 nm and 295 nm. The specific absorbances at these maxima are 700 to 760 and 1150 to 1250, respectively.
- C. Examine the chromatograms obtained in the test for related substances. The principal spot in the chromatogram obtained with test solution (b) is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).

TESTS

Related substances. Examine by thin-layer chromatography (2.2.27), using *silica gel G R* as the coating substance.

Test solution (a). Dissolve 0.10 g of the substance to be examined in *methanol R* and dilute to 10 ml with the same solvent.

Test solution (b). Dilute 1 ml of test solution (a) to 10 ml with *methanol R*.

Reference solution (a). Dissolve 10 mg of *dapsone CRS* in *methanol R* and dilute to 10 ml with the same solvent.

Reference solution (b). Dilute 1 ml of test solution (b) to 10 ml with *methanol R*.

Reference solution (c). Dilute 2 ml of reference solution (b) to 10 ml with *methanol R*.

Apply separately to the plate 1 µl of test solution (b), 1 µl of reference solution (a), 10 µl of test solution (a), 10 µl of reference solution (b) and 10 µl of reference solution (c). Develop in an unsaturated tank over a path of 15 cm using a mixture of 1 volume of *concentrated ammonia R*, 6 volumes of *methanol R*, 20 volumes of *ethyl acetate R* and 20 volumes of *heptane R*. Allow the plate to dry in air. Spray the plate with a 1 g/l solution of *4-dimethylaminocinnamaldehyde R* in a mixture of 1 volume of *hydrochloric acid R* and 99 volumes of *alcohol R*. Examine in daylight. Any spot in the chromatogram obtained with test solution (a), apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (b) (1.0 per cent) and not more than 2 such spots are more intense than the spot in the chromatogram obtained with reference solution (c) (0.2 per cent).

Loss on drying (2.2.32). Not more than 1.5 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.100 g in 50 ml of *dilute hydrochloric acid R*. Carry out the determination of primary aromatic amino-nitrogen (2.5.8).

1 ml of 0.1 M *sodium nitrite* is equivalent to 12.42 mg of C₁₂H₁₂N₂O₂S.

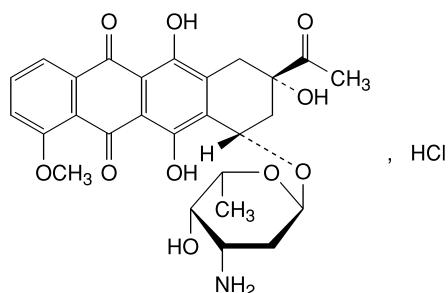
STORAGE

Store protected from light.

01/2005:0662

DAUNORUBICIN HYDROCHLORIDE

Daunorubicini hydrochloridum

 $C_{27}H_{30}ClNO_{10}$ M_r 564.0

DEFINITION

(8S,10S)-8-Acetyl-10-[(3-amino-2,3,6-trideoxy- α -L-lyxo-hexopyranosyl)oxy]-6,8,11-trihydroxy-1-methoxy-7,8,9,10-tetrahydrotetracene-5,12-dione hydrochloride.

Substance produced by certain strains of *Streptomyces coeruleorubidus* or of *Streptomyces peucetius* or obtained by any other means.

Content: 95.0 per cent to 102.0 per cent (anhydrous and solvent-free substance).

PRODUCTION

It is produced by methods of manufacture designed to eliminate or minimise the presence of histamine.

CHARACTERS

Appearance: crystalline, orange-red powder, hygroscopic.

Solubility: freely soluble in water and in methanol, slightly soluble in alcohol, practically insoluble in acetone.

IDENTIFICATION

A. Infrared absorption spectrophotometry (2.2.24).

Comparison: daunorubicin hydrochloride CRS.

B. Dissolve about 10 mg in 0.5 ml of *nitric acid R*, add 0.5 ml of *water R* and heat over a flame for 2 min. Allow to cool and add 0.5 ml of *silver nitrate solution R1*. A white precipitate is formed.

TESTS

pH (2.2.3): 4.5 to 6.5.

Dissolve 50 mg in *carbon dioxide-free water R* and dilute to 10 ml with the same solvent.

Related substances. Liquid chromatography (2.2.29). Prepare the solutions immediately before use.

Test solution. Dissolve 50.0 mg of the substance to be examined in the mobile phase and dilute to 50.0 ml with the mobile phase.

Reference solution (a). Dissolve 50.0 mg of *daunorubicin hydrochloride CRS* in the mobile phase and dilute to 50.0 ml with the mobile phase.

Reference solution (b). Dissolve 10 mg of *doxorubicin hydrochloride CRS* and 10 mg of *epirubicin hydrochloride CRS* in the mobile phase and dilute to 100.0 ml with the mobile phase. Dilute 1.0 ml of the solution to 10.0 ml with the mobile phase.

Reference solution (c). Dissolve 5.0 mg of *daunorubicinone CRS* and 5.0 mg of *doxorubicin hydrochloride CRS* in the mobile phase and dilute to 100.0 ml with the mobile phase. Dilute 1.0 ml of the solution to 10.0 ml with the mobile phase.

Reference solution (d). Dilute 1.0 ml of reference solution (a) to 200.0 ml with the mobile phase.

Column:

- **size:** $l = 0.25$ m, $\varnothing = 4.0$ mm,
- **stationary phase:** end-capped octadecylsilyl silica gel for chromatography *R* (5 μ m).

Mobile phase: mixture of equal volumes of *acetonitrile R* and a solution containing 2.88 g/l of *sodium laurilsulfate R* and 2.25 g/l of *phosphoric acid R*.

Flow rate: 1 ml/min.

Detection: spectrophotometer at 254 nm.

Injection: 5 μ l; inject the test solution and reference solutions (b), (c) and (d).

Run time: twice the retention time of daunorubicin.

Relative retention with reference to daunorubicin (retention time = about 15 min): impurity A = about 0.4; impurity D = about 0.5; epirubicin = about 0.6; impurity B = about 0.7.

System suitability: reference solution (b):

- **resolution:** minimum of 2.0 between the peaks due to impurity D and epirubicin.

Limits:

- **impurity A:** not more than the area of the corresponding peak in the chromatogram obtained with reference solution (c) (0.5 per cent),
- **impurity B:** not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (d) (1.5 per cent),
- **impurity D:** not more than the area of the corresponding peak in the chromatogram obtained with reference solution (c) (0.5 per cent),
- **any other impurity:** not more than the area of the principal peak in the chromatogram obtained with reference solution (d) (0.5 per cent),
- **total of other impurities:** not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (d) (2.5 per cent),
- **disregard limit:** 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (d) (0.05 per cent).

Butanol (2.4.24, *System B*): maximum 1.0 per cent.

Water (2.5.12): maximum 3.0 per cent, determined on 0.100 g.

Bacterial endotoxins (2.6.14): less than 4.3 IU/mg, if intended for use in the manufacture of parenteral dosage forms without a further appropriate procedure for the removal of bacterial endotoxins.

ASSAY

Liquid chromatography (2.2.29) as described in the test for related substances.

Injection: test solution and reference solution (a).

Calculate the percentage content of $C_{27}H_{30}ClNO_{10}$.

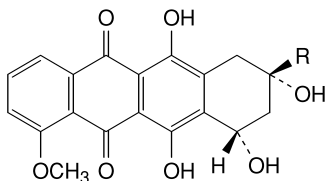
STORAGE

In an airtight container, protected from light. If the substance is sterile, store in a sterile, airtight, tamper-proof container.

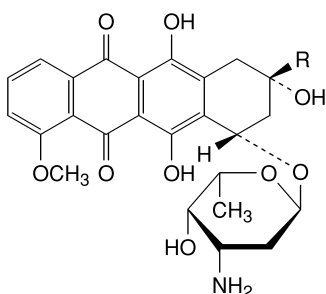
LABELLING

The label states, where applicable, that the substance is free from bacterial endotoxins.

IMPURITIES



- A. R = CO-CH₃: (8*S*,10*S*)-8-acetyl-6,8,10,11-tetrahydroxy-1-methoxy-7,8,9,10-tetrahydrotetracene-5,12-dione (daunorubicin aglycone, daunorubicinone),
- E. R = CHOH-CH₃: (8*S*,10*S*)-6,8,10,11-tetrahydroxy-8-[(1*RS*)-1-hydroxyethyl]-1-methoxy-7,8,9,10-tetrahydrotetracene-5,12-dione (13-dihydrodaunorubicinone),



- B. R = CHOH-CH₃: (8*S*,10*S*)-10-[(3-amino-2,3,6-trideoxy-α-*L*-xylo-hexopyranosyl)oxy]-6,8,11-trihydroxy-8-[(1*RS*)-1-hydroxyethyl]-1-methoxy-7,8,9,10-tetrahydrotetracene-5,12-dione (daunorubicinol),
- C. R = CH₂-CO-CH₃: (8*S*,10*S*)-10-[(3-amino-2,3,6-trideoxy-α-*L*-xylo-hexopyranosyl)oxy]-6,8,11-trihydroxy-1-methoxy-8-(2-oxopropyl)-7,8,9,10-tetrahydrotetracene-5,12-dione (feudomycin B),
- D. R = CO-CH₂-OH: doxorubicin,
- F. R = CO-CH₂-CH₃: (8*S*,10*S*)-10-[(3-amino-2,3,6-trideoxy-α-*L*-xylo-hexopyranosyl)oxy]-6,8,11-trihydroxy-1-methoxy-8-propanoyl-7,8,9,10-tetrahydrotetracene-5,12-dione (8-ethyl-daunorubicin).

01/2005:1307

DECYL OLEATE

Decylis oleas

DEFINITION

Decyl oleate is a mixture consisting of decyl esters of fatty acids, mainly oleic acid. A suitable antioxidant may be added.

CHARACTERS

A clear, pale yellow or colourless liquid, practically insoluble in water, miscible with alcohol, with methylene chloride and with light petroleum (40 °C to 60 °C).

IDENTIFICATION

- A. It complies with the test for relative density (see Tests).
- B. It complies with the test for saponification value (see Tests).
- C. It complies with the test for content of oleic acid (see Tests).

TESTS

Relative density (2.2.5): 0.860 to 0.870.

Acid value (2.5.1): Not more than 1.0, determined on 10.0 g.

Iodine value (2.5.4): 55 to 70.

Peroxide value (2.5.5): Not more than 10.0.

Saponification value (2.5.6): 130 to 140, determined on 2.0 g.

Content of oleic acid (2.4.22, Method A). The fatty acid fraction of the substance contains at least 60.0 per cent of oleic acid.

Water (2.5.12): Not more than 1.0 per cent, determined on 1.00 g by the semi-micro determination of water.

Total ash (2.4.16): Not more than 0.1 per cent, determined on 2.0 g.

STORAGE

Store protected from light.

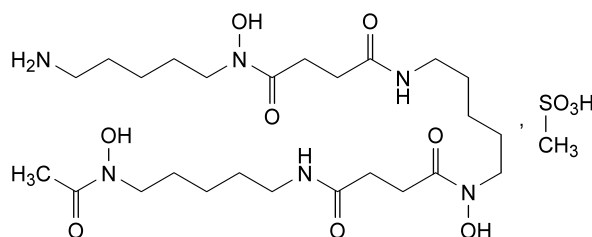
LABELLING

The label states, where applicable, the name and concentration of any added antioxidant.

01/2005:0896

DEFEROXAMINE MESILATE

Deferoxamini mesilas

C₂₆H₅₂N₆O₁₁SM_r 657

DEFINITION

Deferoxamine mesilate contains not less than 98.0 per cent and not more than the equivalent of 102.0 per cent expressed as *N*'-[5-[[4-[[5-(acetylhydroxyamino)pentyl]amino]-4-oxobutanoyl]hydroxyamino]pentyl]-*N*-(5-aminopentyl)-*N*-hydroxybutanediamide methanesulfonate, calculated with reference to the anhydrous substance.

PRODUCTION

The production method must be evaluated to determine the potential for formation of alkyl mesilates, which is particularly likely to occur if the reaction medium contains lower alcohols. Where necessary, the production method is validated to demonstrate that alkyl mesilates are not detectable in the final product.

CHARACTERS

A white or almost white powder, freely soluble in water, slightly soluble in methanol, very slightly soluble in alcohol.

IDENTIFICATION

First identification: A, D.

Second identification: B, C, D.

- A. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *deferoxamine mesilate CRS*. Examine the substances prepared as discs. If the spectra obtained show

differences, dissolve the substance to be examined and the reference substance separately in *alcohol R*, evaporate to dryness and record new spectra using the residues.

- B. Dissolve about 5 mg in 5 ml of *water R*. Add 2 ml of a 5 g/l solution of *trisodium phosphate dodecahydrate R* and 0.5 ml of a 25 g/l solution of *sodium naphthoquinonesulphonate R*. A brownish-black colour develops.
- C. The titrated solution obtained in the Assay (solution (a)) is brownish-red. To 10 ml of solution (a) add 3 ml of *ether R*. Shake. The organic layer is colourless. To 10 ml of solution (a) add 3 ml of *benzyl alcohol R*. Shake. The organic layer is brownish-red.
- D. Dissolve 0.1 g in 5 ml of *dilute hydrochloric acid R*. Add 1 ml of *barium chloride solution R2*. The solution is clear. In a porcelain crucible, mix 0.1 g with 1 g of *anhydrous sodium carbonate R*, heat and ignite over a naked flame. Allow to cool. Dissolve the residue in 10 ml of *water R*, heating if necessary, and filter. The filtrate gives reaction (a) of sulphates (2.3.1).

TESTS

Solution S. Dissolve 2.5 g in *carbon dioxide-free water R* prepared from *distilled water R* and dilute to 25 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and not more intensely coloured than reference solution Y₅ (2.2.2, *Method II*).

pH (2.2.3). The pH of freshly prepared solution S is 3.7 to 5.5.

Related substances. Examine by liquid chromatography (2.2.29). Prepare the solutions immediately before use, protected from light.

Test solution. Dissolve 50.0 mg of the substance to be examined in the mobile phase and dilute to 50.0 ml with the mobile phase.

Reference solution (a). Dissolve 10.0 mg of *desferoxamine mesilate CRS* in the mobile phase and dilute to 10.0 ml with the mobile phase.

Reference solution (b). Dilute 1.0 ml of the test solution to 25.0 ml with the mobile phase.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4.6 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (10 µm),
- as mobile phase at a flow rate of 2 ml/min a solution prepared as follows: dissolve 1.32 g of *ammonium phosphate R* and 0.37 g of *sodium edetate R* in 950 ml of *water R*; adjust to pH 2.8 with *phosphoric acid R* (about 3–4 ml) and add 55 ml of *tetrahydrofuran R*,
- as detector a spectrophotometer set at 220 nm.

Inject 20 µl of reference solution (a). If a recorder is used, adjust the sensitivity of the detector so that the height of the peak with a relative retention time of about 0.8 is not less than 15 per cent of the full scale of the recorder. The test is not valid unless the resolution between the peak with the relative retention time of about 0.8 and the principal peak is at least 1.0.

Inject 20 µl of the test solution and 20 µl of reference solution (b). Continue the chromatography for 3 times the retention time of desferoxamine. In the chromatogram

obtained with the test solution: the area of any peak, apart from the principal peak, is not greater than the area of the principal peak in the chromatogram obtained with reference solution (b) (4.0 per cent); the sum of the area of any such peaks is not greater than 1.75 times the area of the principal peak in the chromatogram obtained with reference solution (b) (7.0 per cent). Disregard any peak with an area less than 0.02 times that of the principal peak in the chromatogram obtained with reference solution (b).

Chlorides (2.4.4). Dilute 2 ml of solution S to 20 ml with *water R*. 15 ml of the solution complies with the limit test for chlorides (330 ppm).

Sulphates (2.4.13). Dilute 5 ml of solution S to 20 ml with *distilled water R*. 15 ml of the solution complies with the limit test for sulphates (400 ppm).

Heavy metals (2.4.8). 2.0 g complies with limit test C for heavy metals (10 ppm). Prepare the standard using 2 ml of *lead standard solution (10 ppm Pb) R*.

Water (2.5.12). Not more than 2.0 per cent, determined on 1.000 g by the semi-micro determination of water.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

Bacterial endotoxins (2.6.14): less than 0.025 IU/mg, if intended for use in the manufacture of parenteral dosage forms without a further appropriate procedure for the removal of bacterial endotoxins.

ASSAY

Dissolve 0.500 g in 25 ml of *water R*. Add 4 ml of 0.05 M *sulphuric acid*. Titrate with 0.1 M *ferric ammonium sulphate*. Towards the end of the titration, titrate uniformly and at a rate of about 0.2 ml/min. Determine the end-point potentiometrically (2.2.20) using a platinum indicator electrode and a calomel reference electrode. Retain the titrated solution for identification test C.

1 ml of 0.1 M *ferric ammonium sulphate* is equivalent to 65.68 mg of C₂₆H₅₂N₆O₁₁S.

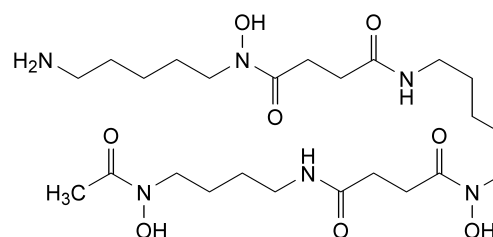
STORAGE

Store protected from light, at a temperature of 2 °C to 8 °C. If the substance is sterile, store in a sterile, airtight, tamper-proof container.

LABELLING

The label states, where applicable, that the substance is free from bacterial endotoxins.

IMPURITIES



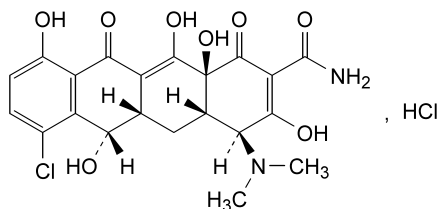
A. *N'*-[5-[4-[4-(acetylhydroxyamino)butyl]amino]-4-oxobutanoyl]hydroxyamino]pentyl]-*N*-(5-aminopentyl)-*N*-hydroxybutanediamide (desferrioxamine A₁),

B. other desferrioxamines.

01/2005:0176 TESTS

DEMECLOCYCLINE HYDROCHLORIDE

Demeclocyclini hydrochloridum



$C_{21}H_{22}Cl_2N_2O_8$

M_r 501.3

DEFINITION

(4S,4aS,5aS,6S,12aS)-7-Chloro-4-(dimethylamino)-3,6,10,12,12a-pentahydroxy-1,11-dioxo-1,4,4a,5,5a,6,11,12a-octahydrotetracene-2-carboxamide hydrochloride.

Substance produced by certain strains of *Streptomyces aureofaciens* or obtained by any other means.

Content: 89.5 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

Appearance: yellow powder.

Solubility: soluble or sparingly soluble in water, slightly soluble in alcohol, very slightly soluble in acetone. It dissolves in solutions of alkali hydroxides and carbonates.

IDENTIFICATION

A. Thin-layer chromatography (2.2.27).

Test solution. Dissolve 5 mg of the substance to be examined in *methanol R* and dilute to 10 ml with the same solvent.

Reference solution (a). Dissolve 5 mg of *demeclocycline hydrochloride CRS* in *methanol R* and dilute to 10 ml with the same solvent.

Reference solution (b). Dissolve 5 mg of *demeclocycline hydrochloride CRS*, 5 mg of *chlortetracycline hydrochloride R* and 5 mg of *tetracycline hydrochloride R* in *methanol R* and dilute to 10 ml with the same solvent.

Plate: TLC octadecylsilyl silica gel F_{254} plate *R*.

Mobile phase: mix 20 volumes of *acetonitrile R*, 20 volumes of *methanol R* and 60 volumes of a 63 g/l solution of *oxalic acid R* previously adjusted to pH 2 with *concentrated ammonia R*.

Application: 1 μ l.

Development: over 3/4 of the plate.

Drying: in air.

Detection: examine in ultraviolet light at 254 nm.

System suitability: the chromatogram obtained with reference solution (b) shows 3 clearly separated spots.

Results: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a).

B. To about 2 mg add 5 ml of *sulphuric acid R*. A violet colour develops. Add the solution to 2.5 ml of *water R*. The colour becomes yellow.

C. It gives reaction (a) of chlorides (2.3.1).

pH (2.2.3): 2.0 to 3.0.

Dissolve 0.1 g in *carbon dioxide-free water R* and dilute to 10 ml with the same solvent.

Specific optical rotation (2.2.7): –248 to –263 (anhydrous substance).

Dissolve 0.250 g in 0.1 M *hydrochloric acid* and dilute to 25.0 ml with the same acid.

Specific absorbance (2.2.25): 340 to 370 determined at the maximum at 385 nm (anhydrous substance).

Dissolve 10.0 mg in 0.01 M *hydrochloric acid* and dilute to 100.0 ml with the same acid. To 10.0 ml of the solution add 12 ml of *dilute sodium hydroxide solution R* and dilute to 100.0 ml with *water R*.

Related substances. Liquid chromatography (2.2.29). Prepare the solutions immediately before use.

Test solution. Dissolve 25.0 mg of the substance to be examined in 0.01 M *hydrochloric acid* and dilute to 25.0 ml with the same acid.

Reference solution (a). Dissolve 25.0 mg of *demeclocycline hydrochloride CRS* in 0.01 M *hydrochloric acid* and dilute to 25.0 ml with the same acid.

Reference solution (b). Dissolve 5.0 mg of *4-epidemeclocycline hydrochloride CRS* in 0.01 M *hydrochloric acid* and dilute to 25.0 ml with the same acid.

Reference solution (c). Mix 1.0 ml of reference solution (a) and 5.0 ml of reference solution (b) and dilute to 25.0 ml with 0.01 M *hydrochloric acid*.

Reference solution (d). Dilute 5.0 ml of reference solution (a) to 100.0 ml with 0.01 M *hydrochloric acid*.

Column:

- size: $l = 0.25$ m, $\varnothing = 4.6$ mm,
- stationary phase: *styrene-divinylbenzene copolymer R* (8 μ m),
- temperature: 60 °C,

Mobile phase: weigh 80.0 g of *2-methyl-2-propanol R* and transfer to a 1000 ml volumetric flask with the aid of 200 ml of *water R*; add 100 ml of a 35 g/l solution of *dipotassium hydrogen phosphate R* adjusted to pH 9.0 with *dilute phosphoric acid R*, 150 ml of a 10 g/l solution of *tetrabutylammonium hydrogen sulphate R* adjusted to pH 9.0 with *dilute sodium hydroxide solution R* and 10 ml of a 40 g/l solution of *sodium edetate R* adjusted to pH 9.0 with *dilute sodium hydroxide solution R*; dilute to 1000 ml with *water R*.

Flow rate: 1 ml/min.

Detection: spectrophotometer at 254 nm.

Injection: 20 μ l; inject the test solution and reference solutions (c) and (d).

System suitability: reference solution (c):

- resolution: minimum of 2.8 between the peaks due to impurity B (1st peak) and demeclocycline (2nd peak); if necessary, adjust the 2-methyl-2-propanol content of the mobile phase or lower the pH of the mobile phase,
- symmetry factor: maximum 1.25 for the peak due to demeclocycline.

Limits:

- any impurity: not more than the area of the principal peak in the chromatogram obtained with reference solution (d) (5.0 per cent), and not more than 1 such peak has an area greater than 0.8 times the area of the principal peak in the chromatogram obtained with reference solution (d) (4.0 per cent),

- *total*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (d) (10.0 per cent),
- *disregard limit*: 0.02 times the area of the principal peak in the chromatogram obtained with reference solution (d) (0.1 per cent).

Heavy metals (2.4.8): maximum 50 ppm.

0.5 g complies with limit test C. Prepare the standard using 2.5 ml of *lead standard solution* (10 ppm Pb) R.

Water (2.5.12): maximum 3.0 per cent, determined on 1.000 g.

Sulphated ash (2.4.14): maximum 0.5 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (2.2.29) as described in the test for related substances with the following modification.

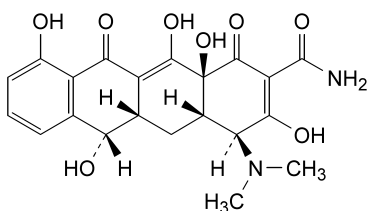
Injection: test solution and reference solution (a).

Calculate the percentage content of $C_{21}H_{22}Cl_2N_2O_8$.

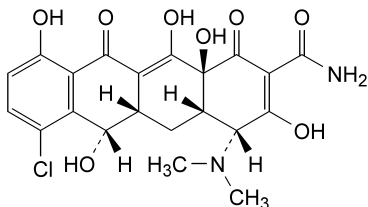
STORAGE

Protected from light.

IMPURITIES



- A. (4*S*,4*aS*,5*aS*,6*S*,12*aS*)-4-(dimethylamino)-3,6,10,12,12a-pentahydroxy-1,11-dioxo-1,4,4a,5,5a,6,11,12a-octahydrotetracycline-2-carboxamide (demethyltetracycline),

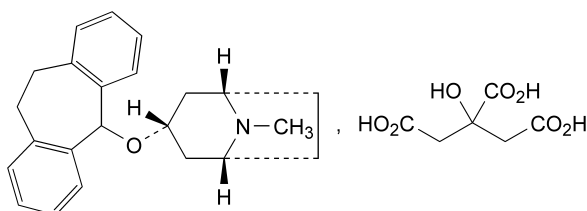


- B. (4*R*,4*aS*,5*aS*,6*S*,12*aS*)-7-chloro-4-(dimethylamino)-3,6,10,12,12a-pentahydroxy-1,11-dioxo-1,4,4a,5,5a,6,11,12a-octahydrotetracycline-2-carboxamide (4-epidemeclocycline).

01/2005:1308

DEPTROPINE CITRATE

Deptropini citras



$C_{29}H_{35}NO_8$

M_r 525.6

DEFINITION

Deptropine citrate contains not less than 98.0 per cent and not more than the equivalent of 101.0 per cent of (1*R*,3*r*,5*S*)-3-(10,11-dihydro-5*H*-dibenzo[*a,d*][7]annulen-5-yloxy)-8-methyl-8-azabicyclo[3.2.1]octane dihydrogen citrate, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, microcrystalline powder, very slightly soluble in water and in ethanol, practically insoluble in methylene chloride.

It melts at about 170 °C, with decomposition.

IDENTIFICATION

First identification: A.

Second identification: B, C, D, E.

- Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *deptropine citrate CRS*.
- Examine the chromatograms obtained in the test for related substances. The principal spot in the chromatogram obtained with test solution (b) is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (b).
- To about 1 mg add 0.5 ml of *sulphuric acid R*. A stable red-orange colour develops.
- Dissolve about 1 mg in 0.25 ml of *perchloric acid R* and warm gently until the solution becomes turbid. Add 5 ml of *glacial acetic acid R*; a pink colour with an intense green fluorescence appears.
- To about 5 mg add 1 ml of *acetic anhydride R* and 5 ml of *pyridine R*. A purple colour develops.

TESTS

pH (2.2.3). Suspend 0.25 g in *carbon dioxide-free water R*, dilute to 25 ml with the same solvent and filter. The pH of the solution is 3.7 to 4.5.

Related substances. Examine by thin-layer chromatography (2.2.27), using as the coating substance a suitable silica gel with a fluorescent indicator having an optimal intensity at 254 nm.

Test solution (a). Dissolve 0.10 g of the substance to be examined in *methanol R* and dilute to 10 ml with the same solvent.

Test solution (b). Dilute 1 ml of test solution (a) to 10 ml with *methanol R*.

Reference solution (a). Dilute 1.0 ml of test solution (a) to 100.0 ml with *methanol R*.

Reference solution (b). Dissolve 20 mg of *deptropine citrate CRS* in *methanol R* and dilute to 2 ml with the same solvent. Dilute 1 ml of the solution to 10 ml with *methanol R*.

Reference solution (c). Dissolve 5 mg of *tropine CRS* in *methanol R* and dilute to 100.0 ml with the same solvent.

Reference solution (d). Dissolve 10 mg of *deptropine citrate CRS* and 10 mg of *tropine CRS* in *methanol R* and dilute to 25 ml with the same solvent.

Apply to the plate 40 µl of each solution. Develop over a path of 10 cm using a mixture of 8 volumes of *concentrated ammonia R* and 92 volumes of *butanol R*. Dry the plate at 100 °C to 105 °C until the ammonia has completely evaporated. Examine in ultraviolet light at 254 nm. Any spot in the chromatogram obtained with test solution (a), apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (a) (1 per cent). Spray with *dilute potassium iodobismuthate solution R* and then with a 10 g/l solution

of *sodium nitrite R*. Expose the plate to iodine vapours. Examine in daylight and in ultraviolet light at 254 nm. In the chromatogram obtained with test solution (a): any spot corresponding to tropine is not more intense than the spot in the chromatogram obtained with reference solution (c) (0.5 per cent); any spot, apart from the principal spot and any spot corresponding to tropine, is not more intense than the spot in the chromatogram obtained with reference solution (a) (1 per cent). The test is not valid unless the chromatogram obtained with reference solution (d) shows two clearly separated spots.

Heavy metals (2.4.8). 1.0 g complies with limit test C for heavy metals (20 ppm). Prepare the standard using 2 ml of *lead standard solution* (10 ppm Pb) *R*.

Loss on drying (2.2.32). Not more than 2.0 per cent, determined on 1.000 g by drying in an oven at 100-105 °C for 4 h.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

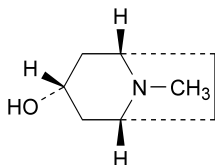
Dissolve 0.400 g in 50 ml of *anhydrous acetic acid R*. Titrate with 0.1 M *perchloric acid*, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *perchloric acid* is equivalent to 52.56 mg of $C_{29}H_{35}NO_8$.

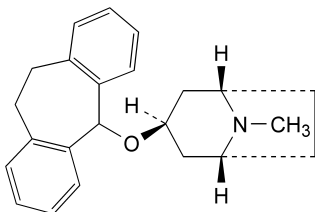
STORAGE

Store protected from light.

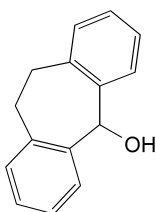
IMPURITIES



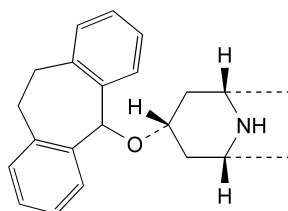
A. (1*R*,3*r*,5*S*)-8-methyl-8-azabicyclo[3.2.1]octan-3-ol (tropine),



B. (1*R*,3*s*,5*S*)-3-(10,11-dihydro-5*H*-dibenzo[*a,d*][7]annulen-5-yloxy)-8-methyl-8-azabicyclo[3.2.1]octane (pseudodeptropine),



C. 10,11-dihydro-5*H*-dibenzo[*a,d*][7]annulen-5-ol (dibenzocycloheptadienol),

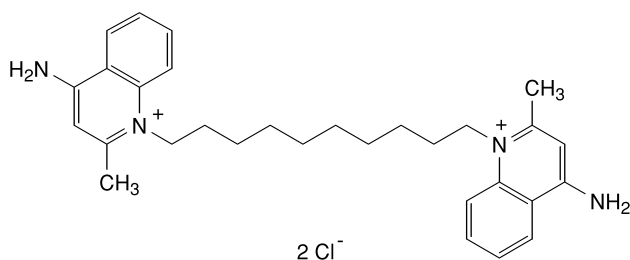


D. (1*R*,3*r*,5*S*)-3-(10,11-dihydro-5*H*-dibenzo[*a,d*][7]annulen-5-yloxy)-8-azabicyclo[3.2.1]octane (demethyldeptropine).

01/2005:1413

DEQUALINIUM CHLORIDE

Dequalinii chloridum



$C_{30}H_{40}Cl_2N_4$

M_r 527.6

DEFINITION

Dequalinium chloride contains not less than 95.0 per cent and not more than the equivalent of 101.0 per cent of 1,1'-(decane-1,10-diyl)bis(4-amino-2-methylquinolinium) dichloride, calculated with reference to the dried substance.

CHARACTERS

A white or yellowish-white powder, hygroscopic, slightly soluble in water and in alcohol.

IDENTIFICATION

First identification: B, E.

Second identification: A, C, D, E.

- Dissolve about 10 mg in *water R* and dilute to 100 ml with the same solvent. Dilute 10 ml of the solution to 100 ml with *water R*. Examined between 230 nm and 350 nm (2.2.25), the solution shows 2 absorption maxima, at 240 nm and 326 nm and a shoulder at 336 nm. The ratio of the absorbance measured at the maximum at 240 nm to that measured at the maximum at 326 nm is 1.56 to 1.80 and the ratio of the absorbance measured at the maximum at 326 nm to that measured at the shoulder at 336 nm is 1.12 to 1.30.
- Examine by infrared absorption spectrophotometry between 600 cm^{-1} and 2000 cm^{-1} (2.2.24), comparing with the spectrum obtained with *dequalinium chloride CRS*.
- To 5 ml of solution S (see Tests) add 5 ml of *potassium ferricyanide solution R*. A yellow precipitate is formed.
- To 10 ml of solution S add 1 ml of *dilute nitric acid R*. A white precipitate is formed. Filter and reserve the filtrate for identification test E.
- The filtrate from identification test D gives reaction (a) of chlorides (2.3.1).

TESTS

Solution S. Dissolve 0.2 g in 90 ml of *carbon dioxide-free water R*, heating if necessary, and dilute to 100 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and colourless (2.2.2, Method II).

Acidity or alkalinity. To 5 ml of solution S add 0.1 ml of *bromothymol blue solution R1*. Not more than 0.2 ml of 0.01 M hydrochloric acid or 0.01 M sodium hydroxide is required to change the colour of the indicator.

Related substances. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 10.0 mg of the substance to be examined in the mobile phase and dilute to 10.0 ml with the mobile phase.

Reference solution (a). Dissolve 10.0 mg of *dequalinium chloride for performance test CRS* in the mobile phase and dilute to 10.0 ml with the mobile phase.

Reference solution (b). Dissolve 10.0 mg of *dequalinium chloride CRS* in the mobile phase and dilute to 10.0 ml with the mobile phase. Dilute 1.0 ml to 50.0 ml with the mobile phase.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4.6 mm in internal diameter packed with an *end-capped octadecylsilyl silica gel for chromatography R*,
- as mobile phase at a flow rate of 1.5 ml/min the following solution: dissolve 2 g of *sodium hexanesulphonate R* in 300 ml of *water R*. Adjust to pH 4.0 with *acetic acid R* and add 700 ml of *methanol R*,
- as detector a spectrophotometer set at 240 nm.

Adjust the sensitivity of the system so that the height of the peak due to impurity B in the chromatogram obtained with 10 µl of reference solution (a) is at least 25 per cent of the full scale of the recorder. Measure the height (*A*) above the baseline of the peak due to impurity B and the height (*B*) above the baseline at the lowest point of the curve separating this peak from the peak due to dequalinium chloride. The test is not valid unless *A* is greater than twice *B*. If necessary, adjust the concentration of methanol in the mobile phase.

Inject 10 µl of the test solution and 10 µl of reference solution (b). Continue the chromatography of the test solution for 5 times the retention time of the peak due to dequalinium chloride. In the chromatogram obtained with the test solution: the area of any peak due to impurity A is not greater than half the area of the principal peak in the chromatogram obtained with reference solution (b) (1 per cent); and the sum of the areas of all the peaks, apart from the principal peak, is not greater than 5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (10 per cent). Disregard any peak with an area less than 0.025 times the area of the principal peak in the chromatogram obtained with reference solution (b).

Readily carbonisable substances. Dissolve 20 mg in 2 ml of *sulphuric acid R*. After 5 min the solution is not more intensely coloured than reference solution BY₄ (2.2.2, Method I).

Loss on drying (2.2.32). Not more than 7.0 per cent, determined on 1.000 g by drying at 100–105 °C at a pressure not exceeding 0.7 kPa.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

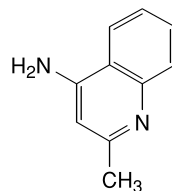
In order to avoid overheating in the reaction medium, mix thoroughly throughout and stop the titration immediately after the end-point has been reached.

Dissolve 0.200 g in 5 ml of *anhydrous formic acid R* and add 50 ml of *acetic anhydride R*. Titrate with 0.1 M perchloric acid, determining the end-point potentiometrically (2.2.20). 1 ml of 0.1 M perchloric acid is equivalent to 26.38 mg of C₃₀H₄₀Cl₂N₄.

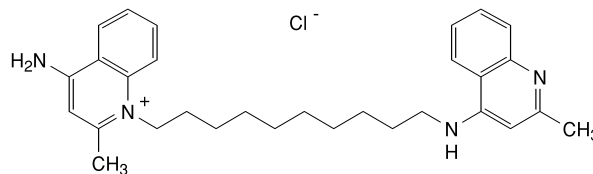
STORAGE

In an airtight container.

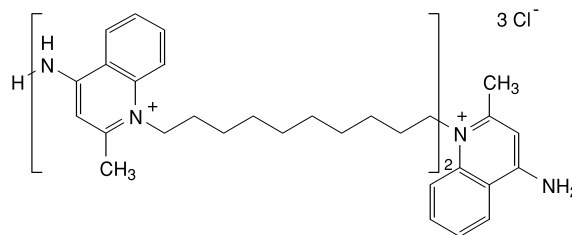
IMPURITIES



A. 2-methylquinolin-4-amine,



B. 4-amino-1-[10-[(2-methylquinolin-4-yl)amino]decyl]-2-methylquinolinium chloride,

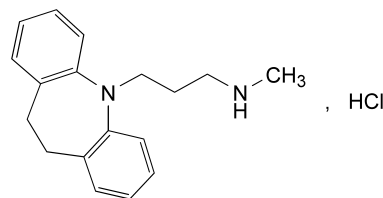


C. 1-[10-(4-amino-2-methylquinolinio)decyl]-4-[[10-(4-amino-2-methylquinolinio)decyl]amino]-2-methylquinolinium trichloride.

01/2005:0481

DESIPRAMINE HYDROCHLORIDE

Desipramini hydrochloridum



C₁₈H₂₃ClN₂

M_r 302.8

DEFINITION

Desipramine hydrochloride contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of 3-(10,11-dihydro-5H-dibenzo[*b,f*]azepin-5-yl)-*N*-methylpropan-1-amine hydrochloride, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, crystalline powder, soluble in water and in alcohol.

It melts at about 214 °C.

IDENTIFICATION

First identification: B, E.

Second identification: A, C, D, E.

- A. Dissolve 40.0 mg in 0.01 M hydrochloric acid and dilute to 100.0 ml with the same acid. Dilute 5.0 ml of the solution to 100.0 ml with 0.01 M hydrochloric acid. Examined between 230 nm and 350 nm (2.2.25), the solution shows an absorption maximum at 251 nm and a shoulder at 270 nm. The specific absorbance at the maximum is 255 to 285.
- B. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *desipramine hydrochloride CRS*.
- C. Examine the chromatograms obtained in the test for related substances. The principal spot in the chromatogram obtained with test solution (b) is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).
- D. Dissolve about 50 mg in 3 ml of *water R* and add 0.05 ml of a 25 g/l solution of *quinhydrone R* in *methanol R*. An intense pink colour develops within about 15 min.
- E. To 0.5 ml of solution S (see Tests) add 1.5 ml of *water R*. The solution gives reaction (a) of chlorides (2.3.1).

TESTS

Solution S. Dissolve 1.25 g in *carbon dioxide-free water R*, warming to not more than 30 °C if necessary, and dilute to 25 ml with the same solvent.

Appearance of solution. Solution S, examined immediately after preparation, is not more intensely coloured than reference solution BY₆ (2.2.2, *Method II*).

Acidity or alkalinity. To 10 ml of solution S add 0.1 ml of *methyl red solution R* and 0.3 ml of 0.01 M sodium hydroxide. The solution is yellow. Not more than 0.5 ml of 0.01 M hydrochloric acid is required to change the colour of the indicator to red.

Related substances. Carry out the test protected from bright light. Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel plate R*.

Test solution (a). Dissolve 0.10 g of the substance to be examined in a mixture of equal volumes of *ethanol R* and *methylene chloride R* and dilute to 10 ml with the same mixture of solvents. Prepare immediately before use.

Test solution (b). Dilute 1 ml of test solution (a) to 10 ml with a mixture of equal volumes of *ethanol R* and *methylene chloride R*.

Reference solution (a). Dissolve 25 mg of *desipramine hydrochloride CRS* in a mixture of equal volumes of *ethanol R* and *methylene chloride R* and dilute to 25 ml with the same mixture of solvents. Prepare immediately before use.

Reference solution (b). Dilute 1 ml of reference solution (a) to 50 ml with a mixture of equal volumes of *ethanol R* and *methylene chloride R*.

Apply to the plate 5 µl of each solution. Develop over a path of 7 cm using a mixture of 1 volume of *water R*, 10 volumes of *anhydrous acetic acid R* and 10 volumes of *toluene R*. Dry the plate in a current of air for 10 min, spray with a 5 g/l solution of *potassium dichromate R* in a mixture of 1 volume of *sulphuric acid R* and 4 volumes of *water R* and examine immediately. Any spot in the chromatogram obtained with test solution (a), apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.2 per cent).

Heavy metals (2.4.8). 2.0 g complies with limit test C for heavy metals (20 ppm). Prepare the standard using 4 ml of *lead standard solution (10 ppm Pb) R*.

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.2500 g in a mixture of 5 ml of 0.01 M hydrochloric acid and 50 ml of *alcohol R*. Carry out a potentiometric titration (2.2.20), using 0.1 M sodium hydroxide. Read the volume added between the two points of inflexion.

1 ml of 0.1 M sodium hydroxide is equivalent to 30.28 mg of C₄₇H₇₄O₁₉.

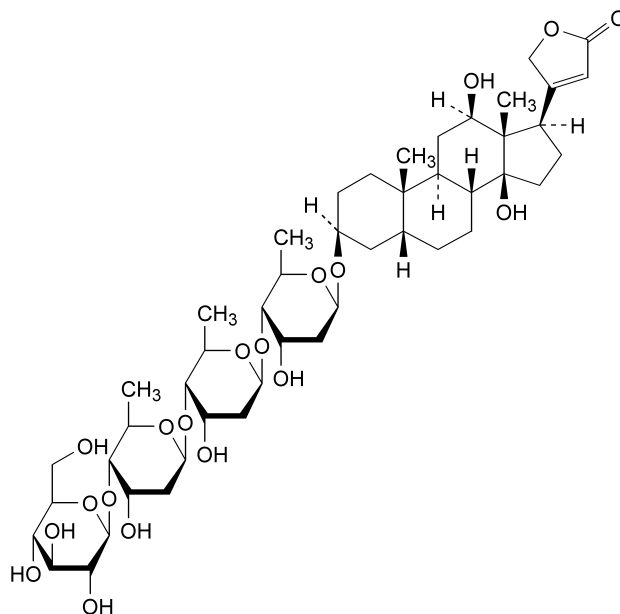
STORAGE

Store protected from light.

01/2005:0482

DESLANOSIDE

Deslanosidum

C₄₇H₇₄O₁₉M_r 943

DEFINITION

Deslanoside contains not less than 95.0 per cent and not more than the equivalent of 105.0 per cent of 3β-[(O-β-D-glucopyranosyl-(1→4)-O-2,6-dideoxy-β-D-ribo-hexopyranosyl-(1→4)-O-2,6-dideoxy-β-D-ribo-hexopyranosyl)-(1→4)-2,6-dideoxy-β-D-ribo-hexopyranosyl]oxy]-12β,14-dihydroxy-5β,14β-card-20(22)-enolide, calculated with reference to the dried substance.

CHARACTERS

A white, crystalline or finely crystalline powder, hygroscopic, practically insoluble in water, very slightly soluble in alcohol. In an atmosphere of low relative humidity, it loses water.

IDENTIFICATION

First identification: A.

Second identification: B, C, D.

- A. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *deslanoside CRS*. When comparing the spectra, special attention is given to the absence of a distinct absorption maximum at about 1260 cm^{-1} and to the intensity of the absorption maximum at about 1740 cm^{-1} . Examine the substances in discs prepared by dissolving 1 mg of the substance to be examined or 1 mg of the reference substance in 0.3 ml of *methanol R* and triturating with about 0.4 g of dry, finely powdered *potassium bromide R* until the mixture is uniform and completely dry.
- B. Examine the chromatograms obtained in the test for related substances. The principal band in the chromatogram obtained with test solution (b) is similar in position, colour and size to the principal band in the chromatogram obtained with reference solution (a).
- C. Suspend about 0.5 mg in 0.2 ml of *alcohol (60 per cent V/V) R*. Add 0.1 ml of *dinitrobenzoic acid solution R* and 0.1 ml of *dilute sodium hydroxide solution R*. A violet colour develops.
- D. Dissolve about 5 mg in 5 ml of *glacial acetic acid R* and add 0.05 ml of *ferric chloride solution R1*. Cautiously add 2 ml of *sulphuric acid R*, avoiding mixing the two liquids. Allow to stand; a brown but not reddish ring develops at the interface and a greenish-yellow, then bluish-green colour diffuses from it to the upper layer.

TESTS

Solution S. Dissolve 0.20 g in a mixture of equal volumes of *chloroform R* and *methanol R* and dilute to 10 ml with the same mixture of solvents.

Appearance of solution. Solution S is clear (2.2.1) and colourless (2.2.2, *Method II*).

Specific optical rotation (2.2.7). Dissolve 0.200 g in *anhydrous pyridine R* and dilute to 10.0 ml with the same solvent. The specific optical rotation is $+6.5$ to $+8.5$, calculated with reference to the dried substance.

Related substances. Examine by thin-layer chromatography (2.2.27), using *silica gel G R* as the coating substance.

Test solution (a). Use solution S.

Test solution (b). Dilute 1 ml of test solution (a) to 10 ml with a mixture of equal volumes of *chloroform R* and *methanol R*.

Reference solution (a). Dissolve 20 mg of *deslanoside CRS* in a mixture of equal volumes of *chloroform R* and *methanol R* and dilute to 10 ml with the same mixture of solvents.

Reference solution (b). Dilute 2.5 ml of reference solution (a) to 10 ml with a mixture of equal volumes of *chloroform R* and *methanol R*.

Reference solution (c). Dilute 1 ml of reference solution (a) to 10 ml with a mixture of equal volumes of *chloroform R* and *methanol R*.

Apply separately to the plate as 10 mm bands 5 μl of each solution. Develop immediately over a path of 15 cm using a mixture of 3 volumes of *water R*, 36 volumes of *methanol R* and 130 volumes of *methylene chloride R*. Dry the plate in a current of warm air, spray with a mixture of 5 volumes of *sulphuric acid R* and 95 volumes of *alcohol R* and heat at $140\text{ }^{\circ}\text{C}$ for 15 min. Examine in daylight. In the chromatogram obtained with test solution (a), any band, apart from the principal band, is not more intense than the band in the chromatogram obtained with reference solution (b) (2.5 per cent) and at most two such bands are more intense than the band in the chromatogram obtained with reference solution (c) (1.0 per cent).

Loss on drying (2.2.32). Not more than 5.0 per cent, determined on 0.500 g by drying *in vacuo* at $100\text{ }^{\circ}\text{C}$ to $105\text{ }^{\circ}\text{C}$.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on the residue obtained in the test for loss on drying.

ASSAY

Dissolve 50.0 mg in *alcohol R* and dilute to 50.0 ml with the same solvent. Dilute 5.0 ml of this solution to 100.0 ml with *alcohol R*. Prepare a reference solution in the same manner, using 50.0 mg of *deslanoside CRS* (undried). To 5.0 ml of each solution add 3.0 ml of *alkaline sodium picrate solution R* and allow to stand protected from bright light in a water-bath at $20 \pm 1\text{ }^{\circ}\text{C}$ for 40 min. Measure the absorbance (2.2.25) of each solution at the maximum at 484 nm, using as the compensation liquid a mixture of 3.0 ml of *alkaline sodium picrate solution R* and 5.0 ml of *alcohol R* prepared at the same time.

Calculate the content of $\text{C}_{47}\text{H}_{74}\text{O}_{19}$ from the absorbances measured and the concentrations of the solutions.

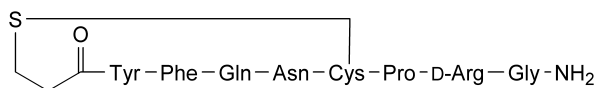
STORAGE

Store in an airtight, glass container, protected from light, at a temperature below $10\text{ }^{\circ}\text{C}$.

01/2005:0712

DESMOPRESSIN

Desmopressinum


 $\text{C}_{46}\text{H}_{64}\text{N}_{14}\text{O}_{12}\text{S}_2$
 $M_r\ 1069$

DEFINITION

Desmopressin is a synthetic cyclic nonapeptide having an antidiuretic action. It contains not less than 95.0 per cent and not more than the equivalent of 105.0 per cent of the peptide $\text{C}_{46}\text{H}_{64}\text{N}_{14}\text{O}_{12}\text{S}_2$, calculated with reference to the anhydrous, acetic acid-free substance. It is available as an acetate.

CHARACTERS

A white, fluffy powder, soluble in water, in alcohol and in glacial acetic acid.

IDENTIFICATION

Examine the chromatograms obtained in the assay. The retention time and size of the principal peak in the chromatogram obtained with the test solution are approximately the same as those of the principal peak in the chromatogram obtained with the reference solution.

TESTS

Specific optical rotation (2.2.7). Dissolve 10.0 mg in a 1 per cent *V/V* solution of *glacial acetic acid R* and dilute to 5.0 ml with the same acid. The specific optical rotation is -72 to -82 , calculated with reference to the anhydrous, acetic acid-free substance.

Amino acids. Examine by means of an amino-acid analyser. Standardise the apparatus with a mixture containing equimolar amounts of ammonia, glycine and the L-form of the following amino acids:

lysine	threonine	alanine	leucine
histidine	serine	valine	tyrosine
arginine	glutamic acid	methionine	phenylalanine
aspartic acid	proline	isoleucine	

together with half the equimolar amount of L-cystine. For the validation of the method, an appropriate internal standard, such as *DL-norleucine R*, is used.

Test solution. Place 1.0 mg of the substance to be examined in a rigorously cleaned hard-glass tube 100 mm long and 6 mm in internal diameter. Add a suitable amount of a 50 per cent *V/V* solution of *hydrochloric acid R*. Immerse the tube in a freezing mixture at -5°C , reduce the pressure to below 133 Pa and seal. Heat at 110°C to 115°C for 16 h. Cool, open the tube, transfer the contents to a 10 ml flask with the aid of five quantities, each of 0.2 ml, of *water R* and evaporate to dryness over *potassium hydroxide R* under reduced pressure. Take up the residue in *water R* and evaporate to dryness over *potassium hydroxide R* under reduced pressure; repeat these operations once. Take up the residue in a buffer solution suitable for the amino-acid analyser used and dilute to a suitable volume with the same buffer solution. Apply a suitable volume to the amino-acid analyser.

Express the content of each amino acid in moles. Calculate the relative proportions of the amino acids, taking one-sixth of the sum of the number of moles of aspartic acid, glutamic acid, proline, glycine, arginine and phenylalanine as equal to one. The values fall within the following limits: aspartic acid 0.95 to 1.05; glutamic acid 0.95 to 1.05; proline 0.95 to 1.05; glycine 0.95 to 1.05; arginine 0.95 to 1.05; phenylalanine 0.95 to 1.05; tyrosine 0.70 to 1.05; half-cystine 0.30 to 1.05. Lysine, isoleucine and leucine are absent; not more than traces of other amino acids are present.

Related peptides. Examine by liquid chromatography (2.2.29) as described under Assay, following the elution conditions shown in the table below, at a flow rate of 1.5 ml/min:

Time (min)	Mobile phase A (per cent <i>V/V</i>)	Mobile phase B (per cent <i>V/V</i>)	Comment
0 - 4	76	24	isocratic
4 - 18	76 → 58	24 → 42	linear gradient
18 - 35	58 → 48	42 → 52	linear gradient
35 - 40	48 → 76	52 → 24	switch to initial eluent composition
40 - 50	76	24	re-equilibration

Inject 50 µl of the resolution solution and identify the peaks due to desmopressin and oxytocin (first and second peaks, respectively). If necessary, adjust the concentration of acetonitrile in the mobile phase to obtain a retention time of about 16 min for the desmopressin peak. The test is not valid unless the resolution between the peaks corresponding to desmopressin and oxytocin is at least 1.5.

Inject 50 µl of the test solution. In the chromatogram obtained the area of any peak, apart from the principal peak, is not greater than 0.5 per cent of the total area of the peaks; the sum of the areas of all the peaks, apart from the principal peak, is not greater than 1.5 per cent of the total

area of the peaks. Disregard any peak due to the solvent and any peak with an area less than 0.05 per cent of that of the principal peak.

Acetic acid (2.5.34): 3.0 per cent to 8.0 per cent.

Test solution. Dissolve 20.0 mg of the substance to be examined in a mixture of 5 volumes of mobile phase B and 95 volumes of mobile phase A and dilute to 10.0 ml with the same mixture of solvents.

Water (2.5.32). Not more than 6.0 per cent, determined by the micro determination of water.

Bacterial endotoxins (2.6.14): less than 500 IU/mg, if intended for use in the manufacture of parenteral dosage forms without a further appropriate procedure for the removal of bacterial endotoxins.

ASSAY

Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 1.0 mg of the substance to be examined in 2.0 ml of *water R*.

Reference solution. Dissolve the contents of a vial of *desmopressin CRS* in *water R* to obtain a concentration of 0.5 mg/ml.

Resolution solution. Dissolve the contents of a vial of *oxytocin/desmopressin validation mixture CRS* in 500 µl of *water R*.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.12 m long and 4.0 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (5 µm),
- as mobile phase at a flow rate of 2.0 ml/min a mixture of 60 volumes of mobile phase A and 40 volumes of mobile phase B:

Mobile phase A. 0.067 M phosphate buffer solution pH 7.0 *R*; filter and degas,

Mobile phase B. Mix equal volumes of mobile phase A and of *acetonitrile for chromatography R*; filter and degas,

- as detector a spectrophotometer set at 220 nm.

Inject 50 µl of the resolution solution and identify the peaks due to desmopressin and oxytocin (first and second peaks, respectively). If necessary, adjust the concentration of acetonitrile in the mobile phase to obtain a retention time of about 5 min for the desmopressin peak. The test is not valid unless the resolution between the peaks corresponding to desmopressin and oxytocin is at least 1.5.

Inject 50 µl of the test solution and 50 µl of the reference solution.

Calculate the content of desmopressin ($\text{C}_{46}\text{H}_{64}\text{N}_{14}\text{O}_{12}\text{S}_2$) from the peak areas in the chromatograms obtained with the test solution and the reference solution and the declared content of $\text{C}_{46}\text{H}_{64}\text{N}_{14}\text{O}_{12}\text{S}_2$ in *desmopressin CRS*.

STORAGE

Store in an airtight container, protected from light, at a temperature of 2°C to 8°C . If the substance is sterile, store in a sterile, airtight, tamper-proof container.

LABELLING

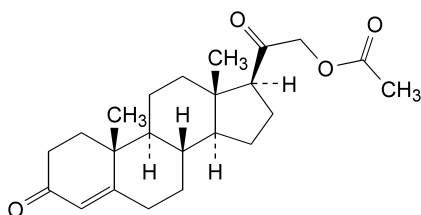
The label states:

- the mass of peptide per container,
- where applicable, that the substance is free from bacterial endotoxins.

01/2005:0322

DESOXYCORTONE ACETATE

Desoxycortoni acetat

 $C_{23}H_{32}O_4$ M_r 372.5

DEFINITION

Desoxycortone acetate contains not less than 97.0 per cent and not more than the equivalent of 103.0 per cent of 3,20-dioxopregn-4-en-21-yl acetate, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, crystalline powder or colourless crystals, practically insoluble in water, freely soluble in methylene chloride, soluble in acetone, sparingly soluble in alcohol, slightly soluble in propylene glycol and in fatty oils.

IDENTIFICATION

First identification: B, C.

Second identification: A, C, D, E.

- A. Melting point (2.2.14): 157 °C to 161 °C.
- B. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *desoxycortone acetate CRS*.
- C. Examine by thin-layer chromatography (2.2.27), using as the coating substance a suitable silica gel with a fluorescent indicator having an optimal intensity at 254 nm.

Test solution. Dissolve 10 mg of the substance to be examined in a mixture of 1 volume of *methanol R* and 9 volumes of *methylene chloride R* and dilute to 10 ml with the same mixture of solvents.

Reference solution (a). Dissolve 20 mg of *desoxycortone acetate CRS* in a mixture of 1 volume of *methanol R* and 9 volumes of *methylene chloride R* and dilute to 20 ml with the same mixture of solvents.

Reference solution (b). Dissolve 10 mg of *cortisone acetate R* in reference solution (a) and dilute to 10 ml with reference solution (a).

Apply separately to the plate 5 µl of each solution. Prepare the mobile phase by adding a mixture of 1.2 volumes of *water R* and 8 volumes of *methanol R* to a mixture of 15 volumes of *ether R* and 77 volumes of *methylene chloride R*. Develop over a path of 15 cm. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. The principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a). Spray with *alcoholic solution of sulphuric acid R*. Heat at 120 °C for 10 min or until the spots appear. Allow to cool. Examine the plate in daylight and in ultraviolet light at 365 nm. The principal spot in the chromatogram obtained with the test solution is similar in position, colour in daylight, fluorescence in ultraviolet light at 365 nm and size to the principal spot

in the chromatogram obtained with reference solution (a). The test is not valid unless the chromatogram obtained with reference solution (b) shows two clearly separated spots.

- D. Add about 2 mg to 2 ml of *sulphuric acid R* and shake to dissolve. Within 5 min, a yellow colour develops. Add the solution to 2 ml of *water R* and shake. The resulting solution is dichroic showing an intense blue colour by transparency, and red fluorescence which is particularly intense in ultraviolet light at 365 nm.
- E. About 10 mg gives the reaction of acetyl (2.3.1).

TESTS

Specific optical rotation (2.2.7). Dissolve 0.250 g in *dioxan R* and dilute to 25.0 ml with the same solvent. The specific optical rotation is + 171 to + 179, calculated with reference to the dried substance.

Related substances. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 25.0 mg of the substance to be examined in the mobile phase and dilute to 10.0 ml with the mobile phase.

Reference solution (a). Dissolve 2 mg of *desoxycortone acetate CRS* and 2 mg of *betamethasone 17-valerate CRS* in the mobile phase and dilute to 200.0 ml with the mobile phase.

Reference solution (b). Dilute 1.0 ml of the test solution to 200.0 ml with the mobile phase.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4.6 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (5 µm),
- as mobile phase at a flow rate of 1 ml/min a mixture prepared as follows: in a 1000 ml volumetric flask mix 350 ml of *water R* with 600 ml of *acetonitrile R* and allow to equilibrate; adjust the volume to 1000 ml with *water R* and mix again,
- as detector a spectrophotometer set at 254 nm.

Equilibrate the column with the mobile phase at a flow rate of 1 ml/min for about 30 min.

Adjust the sensitivity of the system so that the height of the principal peak in the chromatogram obtained with 20 µl of reference solution (b) is not less than 50 per cent of the full scale of the recorder.

Inject 20 µl of reference solution (a). When the chromatograms are recorded in the prescribed conditions, the retention times are: betamethasone 17-valerate, about 7.5 min and desoxycortone acetate, about 9.5 min. The test is not valid unless the resolution between the peaks due to betamethasone 17-valerate and desoxycortone acetate is at least 4.5; if necessary, adjust the concentration of acetonitrile in the mobile phase.

Inject separately 20 µl of the test solution and 20 µl of reference solution (b). Continue the chromatography for three times the retention time of the principal peak. In the chromatogram obtained with the test solution, the sum of the areas of all the peaks, apart from the principal peak, is not greater than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent). Disregard any peak with an area less than 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (b).

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 0.500 g by drying in an oven at 100 °C to 105 °C.

ASSAY

Dissolve 0.100 g in *alcohol R* and dilute to 100.0 ml with the same solvent. Dilute 2.0 ml to 100.0 ml with *alcohol R*. Measure the absorbance (2.2.25) at the maximum at 240 nm.

Calculate the content of $C_{23}H_{32}O_4$ taking the specific absorbance to be 450.

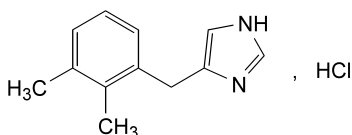
STORAGE

Store protected from light.

01/2005:1414
corrected

DETOMIDINE HYDROCHLORIDE FOR VETERINARY USE

Detomidini hydrochloridum ad usum
veterinarium



$C_{12}H_{15}ClN_2$

M_r 222.7

DEFINITION

Detomidine hydrochloride for veterinary use contains not less than 98.5 per cent and not more than the equivalent of 101.5 per cent of 4-(2,3-dimethylbenzyl)-1H-imidazole hydrochloride, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, hygroscopic, crystalline powder, soluble in water, freely soluble in alcohol, very slightly soluble in methylene chloride, practically insoluble in acetone.

It melts at about 160 °C.

IDENTIFICATION

A. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *detomidine hydrochloride CRS*. Examine the substances prepared as discs. If the spectra obtained show differences, dry the substance to be examined and the reference substance in an oven at 100 °C to 105 °C and record new spectra.

B. It gives reaction (a) of chlorides (2.3.1).

TESTS

Appearance of solution. Dissolve 0.25 g in *water R* and dilute to 25 ml with the same solvent. The solution is clear (2.2.1) and colourless (2.2.2, *Method II*).

Related substances. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 25.0 mg of the substance to be examined in 20 ml of the mobile phase and dilute to 50.0 ml with the mobile phase.

Reference solution (a). Dilute 0.20 ml of the test solution to 100.0 ml with the mobile phase.

Reference solution (b). Dissolve 1 mg of *detomidine impurity B CRS* in the mobile phase and dilute to 100 ml with the mobile phase. Dilute 1 ml of the solution to 10 ml with reference solution (a).

The chromatographic procedure may be carried out using:

- a stainless steel column 0.15 m long and 4.6 mm in internal diameter packed with *octylsilyl silica gel for chromatography R* (5 µm),
- as mobile phase at a flow rate of 1 ml/min a mixture of 35 volumes of *acetonitrile R* and 65 volumes of a 2.64 g/l solution of *ammonium phosphate R*,
- as detector a spectrophotometer set at 220 nm.

When the chromatograms are recorded in the prescribed conditions, the retention time of detomidine is about 7 min and the relative retention times of impurities A, B and C with respect to detomidine are about 0.4, 2.0 and 3.0, respectively. Inject 20 µl of reference solution (a). Adjust the sensitivity of the system so that the height of the principal peak in the chromatogram obtained is at least 50 per cent of the full scale of the recorder. Inject 20 µl of reference solution (b). The test is not valid unless: the resolution between the peaks corresponding to detomidine and to impurity B is at least 5.

Inject 20 µl of the test solution. Continue the chromatography for four times the retention time of the principal peak. Multiply the area of any peak (corresponding to the impurity C and its diastereoisomer) eluting with a relative retention time of about 3, by the correction factor 2.7. The sum of the areas of such peaks is not greater than 2.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent); the area of any other peak apart from the principal peak and the peak corresponding to the impurity C is not greater than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent); the sum of the areas of all the peaks apart from the principal peak is not greater than five times the area of the principal peak in the chromatogram obtained with reference solution (a) (1 per cent). Disregard any peak with an area less than 0.25 times the area of the principal peak in the chromatogram obtained with reference solution (a).

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in oven at 100 °C to 105 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

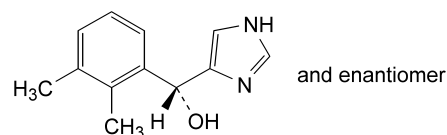
Dissolve 0.170 g in 50 ml of *alcohol R*. Add 5.0 ml of 0.01 M *hydrochloric acid*. Carry out a potentiometric titration (2.2.20), using 0.1 M *sodium hydroxide*. Read the volume added between the two points of inflection.

1 ml of 0.1 M *sodium hydroxide* corresponds to 22.27 mg of $C_{12}H_{15}ClN_2$.

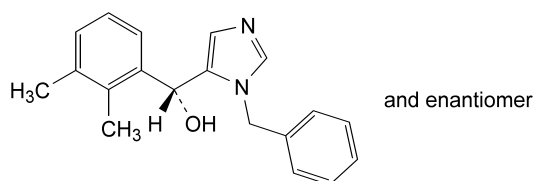
STORAGE

Store in an airtight container.

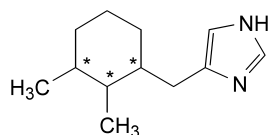
IMPURITIES



A. (RS)-(2,3-dimethylphenyl)(1H-imidazol-4-yl)methanol,



- B. (RS)-(1-benzyl-1H-imidazol-5-yl)(2,3-dimethylphenyl)methanol,



- C. 4-[(2,3-dimethylcyclohexyl)methyl]-1H-imidazole.

01/2005:1095

DEVIL'S CLAW ROOT

Harpagophyti radix

DEFINITION

Devil's claw root consists of the cut and dried tuberous, secondary roots of *Harpagophytum procumbens* D.C. and/or *H. zeyheri* L. Decne. It contains not less than 1.2 per cent of harpagoside ($C_{24}H_{30}O_{11}$; M_r 494.5), calculated with reference to the dried drug.

CHARACTERS

Devil's claw root is greyish-brown to dark brown and it has a bitter taste.

It has the macroscopic and microscopic characters described in identification tests A and B.

IDENTIFICATION

- A. It consists of thick, fan-shaped or rounded slices or of roughly crushed discs. The darker outer surface is traversed by tortuous longitudinal wrinkles. The paler cut surface shows a dark cambial zone and xylem bundles distinctly aligned in radial rows. The central cylinder shows fine concentric striations. Seen under a lens, the cut surface presents yellow to brownish-red granules.
- B. Reduce to a powder (355). The powder is brownish-yellow. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters: fragments of cork layer consisting of yellowish-brown, thin-walled cells; fragments of cortical parenchyma consisting of large, thin-walled cells, sometimes containing reddish-brown granular inclusions and isolated yellow droplets; fragments of reticulately thickened vessels and tracheidal vessels with associated lignified parenchyma from the central cylinder; small needles and crystals of calcium oxalate are present in the parenchyma. The powder may show rectangular or polygonal pitted sclereids with dark reddish-brown contents. With a solution of phloroglucinol in hydrochloric acid, the parenchyma turns green.
- C. Examine by thin-layer chromatography (2.2.27), using a suitable silica gel as the coating substance.

Test solution. Heat on a water-bath at 60 °C for 10 min 1.0 g of the powdered drug (355) with 10 ml of *methanol R*. Filter and reduce the filtrate to about 2 ml under reduced pressure at a temperature not exceeding 40 °C.

Reference solution. Dissolve 1 mg of *harpagoside R* in 1 ml of *methanol R*.

Apply to the plate as bands 20 µl of each solution.

Develop over a path of 10 cm using a mixture of 8 volumes of *water R*, 15 volumes of *methanol R* and 77 volumes of *ethyl acetate R*. Dry the plate in a current of warm air. Examine in ultraviolet light at 254 nm. The chromatograms obtained with the test solution and the reference solution both show in the middle a quenching zone corresponding to harpagoside. The chromatogram obtained with the test solution shows other distinct bands, mainly above the zone corresponding to harpagoside. Spray with a 10 g/l solution of *phloroglucinol R* in *alcohol R* and then with *hydrochloric acid R*. Dry the plate at 80 °C for 5-10 min. In the chromatograms obtained with the reference solution and the test solution the zone corresponding to harpagoside is green. The chromatogram obtained with the test solution also shows several yellow to brown zones below and above the zone corresponding to harpagoside.

TESTS

Starch. Examine the powdered drug (355) under a microscope using *water R*. Add *iodine solution R1*. No blue colour develops.

Foreign matter (2.8.2). It complies with the test for foreign matter.

Loss on drying (2.2.32). Not more than 12.0 per cent, determined on 1.000 g of the powdered drug (355) by drying in an oven at 100-105 °C.

Total ash (2.4.16). Not more than 10.0 per cent.

ASSAY

Examine by liquid chromatography (2.2.29) using *methyl cinnamate R* as the internal standard.

Internal standard solution. Dissolve 0.130 g of *methyl cinnamate R* in 50 ml of *methanol R* and dilute to 100.0 ml with the same solvent.

Test solution. To 0.500 g of the powdered drug (355) add 50 ml of *methanol R*. Shake for 1 h and filter. Transfer the filter with the residue to a 100 ml flask, add 50 ml of *methanol R* and heat under a reflux condenser for 1 h. Cool and filter. Rinse the flask and the filter with 2 quantities, each of 5 ml, of *methanol R*. Combine the filtrate and the rinsing solution and evaporate to dryness under reduced pressure at a temperature not exceeding 40 °C. Take up the residue with 3 quantities, each of 5 ml, of *methanol R* and filter the extracts into a 25 ml volumetric flask. Whilst washing the filter, dilute to 25.0 ml with *methanol R*. To 10.0 ml of this solution add 1.0 ml of the internal standard solution and dilute to 25.0 ml with *methanol R*.

Reference solution. Dilute 0.5 ml of the reference solution described in Identification test C to 2.0 ml with *methanol R*.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.10 m long and 4 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (5 µm),
- as mobile phase at a flow rate of 1.5 ml/min a mixture of equal volumes of *methanol R* and *water R*,
- as detector a spectrophotometer set at 278 nm,
- a 10 µl loop injector.

Inject the test solution. Adjust the sensitivity of the detector so that the height of the peak due to methyl cinnamate is about 50 per cent of the full scale of the recorder.

Determine the retention time of harpagoside using 10 µl of the reference solution examined under the same conditions as the test solution.

Calculate the percentage content of harpagoside from the expression:

$$\frac{m_2 \times F_1 \times 7.622}{F_2 \times m_1}$$

- m_1 = mass of the drug, in grams,
 m_2 = mass of *methyl cinnamate R*, in grams in the internal standard solution,
 F_1 = area of the peak corresponding to harpagoside in the chromatogram obtained with the test solution,
 F_2 = area of the peak corresponding to methyl cinnamate in the chromatogram obtained with the test solution.

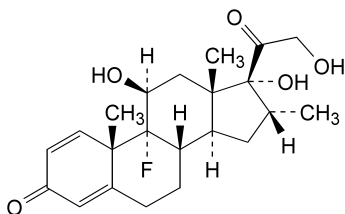
STORAGE

Store protected from light.

01/2005:0388

DEXAMETHASONE

Dexamethasonum



$C_{22}H_{29}FO_5$

M_r 392.5

DEFINITION

Dexamethasone contains not less than 97.0 per cent and not more than the equivalent of 103.0 per cent of 9-fluoro-11 β ,17,21-trihydroxy-16 α -methylpregna-1,4-diene-3,20-dione, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, crystalline powder, practically insoluble in water, sparingly soluble in ethanol, slightly soluble in methylene chloride.

IDENTIFICATION

First identification: B, C.

Second identification: A, C, D, E.

- A. Dissolve 10.0 mg in *ethanol R* and dilute to 100.0 ml with the same solvent. Place 2.0 ml of the solution in a stoppered test tube, add 10.0 ml of *phenylhydrazine-sulphuric acid solution R*, mix and heat in a water-bath at 60 °C for 20 min. Cool immediately. The absorbance (2.2.25) of the solution at the maximum at 419 nm is not less than 0.4.
- B. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *dexamethasone CRS*.
- C. Examine by thin-layer chromatography (2.2.27), using as the coating substance a suitable silica gel with a fluorescent indicator having an optimal intensity at 254 nm.

Test solution. Dissolve 10 mg of the substance to be examined in a mixture of 1 volume of *methanol R* and 9 volumes of *methylene chloride R* and dilute to 10 ml with the same mixture of solvents.

Reference solution (a). Dissolve 20 mg of *dexamethasone CRS* in a mixture of 1 volume of *methanol R* and 9 volumes of *methylene chloride R* and dilute to 20 ml with the same mixture of solvents.

Reference solution (b). Dissolve 10 mg of *betamethasone CRS* in reference solution (a) and dilute to 10 ml with the same solution.

Apply to the plate 5 μ l of each solution. Develop over a path of 15 cm, using a mixture of 5 volumes of *butanol R* saturated with *water R*, 10 volumes of *toluene R* and 85 volumes of *ether R*. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. The principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a). Spray with *alcoholic solution of sulphuric acid R*. Heat at 120 °C for 10 min or until the spots appear. Allow to cool. Examine the chromatograms in daylight and in ultraviolet light at 365 nm. The principal spot in the chromatogram obtained with the test solution is similar in position, colour in daylight, fluorescence in ultraviolet light at 365 nm and size to the principal spot in the chromatogram obtained with reference solution (a). The test is not valid unless the chromatogram obtained with reference solution (b) shows 2 spots which may, however, not be completely separated.

- D. Add about 2 mg to 2 ml of *sulphuric acid R* and shake to dissolve. Within 5 min, a faint reddish-brown colour develops. Add the solution to 10 ml of *water R* and mix. The colour is discharged.
- E. Mix about 5 mg with 45 mg of *heavy magnesium oxide R* and ignite in a crucible until an almost white residue is obtained (usually less than 5 min). Allow to cool, add 1 ml of *water R*, 0.05 ml of *phenolphthalein solution R1* and about 1 ml of *dilute hydrochloric acid R* to render the solution colourless. Filter. To a freshly prepared mixture of 0.1 ml of *alizarin S solution R* and 0.1 ml of *zirconyl nitrate solution R*, add 1.0 ml of the filtrate. Mix, allow to stand for 5 min and compare the colour of the solution with that of a blank prepared in the same manner. The test solution is yellow and the blank is red.

TESTS

Specific optical rotation (2.2.7). Dissolve 0.250 g in *dioxan R* and dilute to 25.0 ml with the same solvent. The specific optical rotation is + 75 to + 80, calculated with reference to the dried substance.

Related substances. Examine by liquid chromatography (2.2.29).

Test solution. Place 25.0 mg of the substance to be examined in a 10.0 ml volumetric flask, add 1.5 ml of *acetonitrile R* and then 5 ml of mobile phase A. Mix with the aid of an ultrasonic bath until complete dissolution and dilute to 10.0 ml with mobile phase A.

Reference solution (a). Dissolve 2 mg of *dexamethasone CRS* and 2 mg of *methylprednisolone CRS* in mobile phase A and dilute to 100.0 ml with the same mobile phase.

Reference solution (b). Dilute 1.0 ml of the test solution to 100.0 ml with mobile phase A.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4.6 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (5 μ m),

01/2005:0548

- as mobile phase at a flow rate of 2.5 ml/min a linear gradient programme using the following conditions:

Mobile phase A. In a 1000 ml volumetric flask, mix 250 ml of *acetonitrile R* with 700 ml of *water R* and allow to equilibrate; adjust the volume to 1000 ml with *water R* and mix again,

Mobile phase B. *Acetonitrile R*,

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)	Comment
0	100	0	isocratic
15	100 → 0	0 → 100	begin linear gradient
40	0	100	end chromatogram, return to 100 A
41	100	0	begin equilibration with A
46 = 0	100	0	end equilibration, begin next chromatogram

- as detector a spectrophotometer set at 254 nm, maintaining the temperature of the column at 45 °C.

Equilibrate the column for at least 30 min with mobile phase B at a flow rate of 2.5 ml/min and then with mobile phase A for 5 min. For subsequent chromatograms, use the conditions described from 40.0 min to 46.0 min.

Adjust the sensitivity of the system so that the height of the principal peak in the chromatogram obtained with 20 µl of reference solution (b) is at least 50 per cent of the full scale of the recorder.

Inject 20 µl of reference solution (a). When the chromatograms are recorded in the prescribed conditions, the retention times are: methylprednisolone about 11.5 min and dexamethasone about 13 min. The test is not valid unless the resolution between the peaks corresponding to methylprednisolone and dexamethasone is at least 2.8; if necessary, adjust the concentration of acetonitrile in mobile phase A.

Inject 20 µl of mobile phase A as a blank, 20 µl of the test solution and 20 µl of reference solution (b). Record the chromatogram of the test solution for twice the retention time of the principal peak. In the chromatogram obtained with the test solution: the area of any peak, apart from the principal peak, is not greater than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent); the sum of the areas of all the peaks, apart from the principal peak, is not greater than the area of the principal peak in the chromatogram obtained with reference solution (b) (1 per cent). Disregard any peak due to the blank and any peak with an area less than 0.05 times the area of the principal peak in the chromatogram obtained with reference solution (b).

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 0.500 g by drying in an oven at 100-105 °C.

ASSAY

Dissolve 0.100 g in *alcohol R* and dilute to 100.0 ml with the same solvent. Dilute 2.0 ml of the solution to 100.0 ml with *alcohol R*. Measure the absorbance (2.2.25) at the maximum at 238.5 nm.

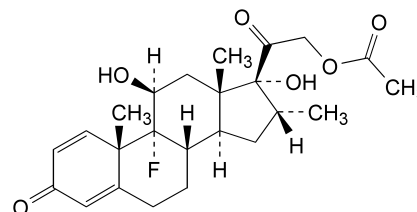
Calculate the content of $C_{22}H_{29}FO_5$ taking the specific absorbance to be 394.

STORAGE

Protected from light.

DEXAMETHASONE ACETATE

Dexamethasoni acetat


 $C_{24}H_{31}FO_6$
 M_r 434.5

DEFINITION

Dexamethasone acetate contains not less than 97.0 per cent and not more than the equivalent of 103.0 per cent of 9-fluoro-11β,17-dihydroxy-16α-methyl-3,20-dioxopregna-1,4-dien-21-yl acetate, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, crystalline powder, practically insoluble in water, freely soluble in acetone and in alcohol, slightly soluble in methylene chloride.

Dexamethasone acetate shows polymorphism.

IDENTIFICATION

First identification: B, C.

Second identification: A, C, D, E, F.

- Dissolve 10.0 mg in *ethanol R* and dilute to 100.0 ml with the same solvent. Place 2.0 ml of this solution in a ground-glass-stoppered tube, add 10.0 ml of *phenylhydrazine-sulphuric acid solution R*, mix and heat in a water-bath at 60 °C for 20 min. Cool immediately. The absorbance (2.2.25) of the solution at the maximum at 419 nm is not less than 0.35.
- Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *dexamethasone acetate CRS*. If the spectra obtained in the solid state with the substance to be examined and the reference substance show differences, record further spectra using saturated solutions (about 30 g/l) of the substance to be examined and of the reference substance in *chloroform R* in a 0.2 mm cell.
- Examine by thin-layer chromatography (2.2.27), using as the coating substance a suitable silica gel with a fluorescent indicator having an optimal intensity at 254 nm.

Test solution. Dissolve 10 mg of the substance to be examined in a mixture of 1 volume of *methanol R* and 9 volumes of *methylene chloride R* and dilute to 10 ml with the same mixture of solvents.

Reference solution (a). Dissolve 20 mg of *dexamethasone acetate CRS* in a mixture of 1 volume of *methanol R* and 9 volumes of *methylene chloride R* and dilute to 20 ml with the same mixture of solvents.

Reference solution (b). Dissolve 10 mg of *cortisone acetate R* in reference solution (a) and dilute to 10 ml with the same solution.

Apply separately to the plate 5 µl of each solution. Prepare the mobile phase by adding a mixture of 1.2 volumes of *water R* and 8 volumes of *methanol R* to a mixture of 15 volumes of *ether R* and 77 volumes of *methylene chloride R*. Develop over a path of 15 cm.

Allow the plate to dry in air and examine in ultraviolet light at 254 nm. The principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a). Spray with *alcoholic solution of sulphuric acid R*. Heat at 120 °C for 10 min or until the spots appear. Allow to cool. Examine the plate in daylight and in ultraviolet light at 365 nm. The principal spot in the chromatogram obtained with the test solution is similar in position, colour in daylight, fluorescence in ultraviolet light at 365 nm and size to the principal spot in the chromatogram obtained with reference solution (a). The test is not valid unless the chromatogram obtained with reference solution (b) shows two clearly separated spots.

- D. Add about 2 mg to 2 ml of *sulphuric acid R* and shake to dissolve. Within 5 min, a faint reddish-brown colour develops. Add the solution to 10 ml of *water R* and mix. The colour is discharged and a clear solution remains.
- E. Mix about 5 mg with 45 mg of *heavy magnesium oxide R* and ignite in a crucible until an almost white residue is obtained (usually less than 5 min). Allow to cool, add 1 ml of *water R*, 0.05 ml of *phenolphthalein solution R1* and about 1 ml of *dilute hydrochloric acid R* to render the solution colourless. Filter. To a freshly prepared mixture of 0.1 ml of *alizarin S solution R* and 0.1 ml of *zirconyl nitrate solution R*, add 1.0 ml of the filtrate. Mix, allow to stand for 5 min and compare the colour of the solution with that of a blank prepared in the same manner. The test solution is yellow and the blank is red.
- F. About 10 mg gives the reaction of acetyl (2.3.1).

TESTS

Specific optical rotation (2.2.7). Dissolve 0.250 g in *dioxan R* and dilute to 25.0 ml with the same solvent. The specific optical rotation is + 84 to + 90, calculated with reference to the dried substance.

Related substances. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 25.0 mg of the substance to be examined in about 4 ml of *acetonitrile R* and dilute to 10.0 ml with *water R*.

Reference solution (a). Dissolve 2 mg of *dexamethasone acetate CRS* and 2 mg of *betamethasone acetate CRS* in the mobile phase and dilute to 100.0 ml with the mobile phase.

Reference solution (b). Dilute 1.0 ml of the test solution to 100.0 ml with the mobile phase.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4.6 mm in internal diameter, packed with *octadecylsilyl silica gel for chromatography R* (5 µm),
- as mobile phase at a flow rate of 1 ml/min a mixture prepared as follows: in a 1000 ml volumetric flask mix 380 ml of *acetonitrile R* with 550 ml of *water R* and allow to equilibrate; dilute to 1000 ml with *water R* and mix again,
- as detector a spectrophotometer set at 254 nm.

Equilibrate the column with the mobile phase at a flow rate of 1 ml/min for about 30 min.

Adjust the sensitivity of the system so that the height of the principal peak in the chromatogram obtained with 20 µl of reference solution (b) is not less than 50 per cent of the full scale of the recorder.

Inject 20 µl of reference solution (a). When the chromatograms are recorded in the prescribed conditions, the retention times are: betamethasone acetate, about

19 min and dexamethasone acetate, about 22 min. The test is not valid unless the resolution between the peaks due to betamethasone acetate and dexamethasone acetate is at least 3.3; if necessary, adjust the concentration of acetonitrile in the mobile phase.

Inject separately 20 µl of the test solution and 20 µl of reference solution (b). Continue the chromatography for 1.5 times the retention time of the principal peak. In the chromatogram obtained with the test solution: the area of any peak, apart from the principal peak, is not greater than half the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent); the sum of the areas of all such peaks is not greater than the area of the principal peak in the chromatogram obtained with reference solution (b) (1.0 per cent). Disregard any peak with an area less than 0.05 times the area of the principal peak in the chromatogram obtained with reference solution (b).

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 0.500 g by drying *in vacuo* in an oven at 100 °C to 105 °C.

ASSAY

Dissolve 0.100 g in *alcohol R* and dilute to 100.0 ml with the same solvent. Dilute 2.0 ml to 100.0 ml with *alcohol R*. Measure the absorbance (2.2.25) at the maximum at 238.5 nm.

Calculate the content of $C_{24}H_{31}FO_6$ taking the specific absorbance to be 357.

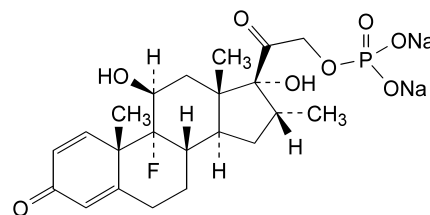
STORAGE

Store protected from light.

01/2005:0549

DEXAMETHASONE SODIUM PHOSPHATE

Dexamethasoni natrii phosphas



$C_{22}H_{28}FNa_2O_8P$

M_r 516.4

DEFINITION

Dexamethasone sodium phosphate contains not less than 97.0 per cent and not more than the equivalent of 103.0 per cent of 9-fluoro-11β,17-dihydroxy-16α-methyl-3,20-dioxopregna-1,4-dien-21-yl disodium phosphate, calculated with reference to the anhydrous, ethanol-free substance.

CHARACTERS

A white or almost white powder, very hygroscopic, freely soluble in water, slightly soluble in alcohol, practically insoluble in methylene chloride.

It shows polymorphism.

IDENTIFICATION

First identification: B, C.

Second identification: A, C, D, E, F.

A. Dissolve 10.0 mg in 5 ml of *water R* and dilute to 100.0 ml with *ethanol R*. Place 2.0 ml of this solution in a ground-glass-stoppered tube, add 10.0 ml of *phenylhydrazine-sulphuric acid solution R*, mix and heat in a water-bath at 60 °C for 20 min. Cool immediately. The absorbance (2.2.25) of the solution measured at the maximum at 419 nm is at least 0.20.

B. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *dexamethasone sodium phosphate CRS*. If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in the minimum volume of *alcohol R*, evaporate to dryness on a water-bath and record new spectra using the residues.

C. Examine by thin-layer chromatography (2.2.27), using as the coating substance a suitable silica gel with a fluorescent indicator having an optimal intensity at 254 nm.

Test solution. Dissolve 10 mg of the substance to be examined in *methanol R* and dilute to 10 ml with the same solvent.

Reference solution (a). Dissolve 20 mg of *dexamethasone sodium phosphate CRS* in *methanol R* and dilute to 20 ml with the same solvent.

Reference solution (b). Dissolve 10 mg of *prednisolone sodium phosphate CRS* in reference solution (a) and dilute to 10 ml with the same solution.

Apply separately to the plate 5 µl of each solution. Develop over a path of 15 cm, using a mixture of 20 volumes of *glacial acetic acid R*, 20 volumes of *water R* and 60 volumes of *butanol R*. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. The principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a). Spray with *alcoholic solution of sulphuric acid R*. Heat at 120 °C for 10 min or until the spots appear. Allow to cool. Examine in daylight and in ultraviolet light at 365 nm. The principal spot in the chromatogram obtained with the test solution is similar in position, colour in daylight, fluorescence in ultraviolet light at 365 nm and size to the principal spot in the chromatogram obtained with reference solution (a). The test is not valid unless the chromatogram obtained with reference solution (b) shows two spots which may, however, not be completely separated.

D. Add about 2 mg to 2 ml of *sulphuric acid R* and shake to dissolve. Within 5 min, a faint yellowish-brown colour develops. Add the solution to 10 ml of *water R* and mix. The colour fades and a clear solution remains.

E. Mix about 5 mg with 45 mg of *heavy magnesium oxide R* and ignite in a crucible until an almost white residue is obtained (usually less than 5 min). Allow to cool, add 1 ml of *water R*, 0.05 ml of *phenolphthalein solution R1* and about 1 ml of *dilute hydrochloric acid R* to render the solution colourless. Filter. To a freshly prepared mixture of 0.1 ml of *alizarin S solution R* and 0.1 ml of *zirconyl nitrate solution R*, add 1.0 ml of the filtrate. Mix, allow to stand for 5 min and compare the colour of the solution with that of a blank prepared in the same manner. The test solution is yellow and the blank is red.

F. To 40 mg add 2 ml of *sulphuric acid R* and heat gently until white fumes are evolved, add *nitric acid R* dropwise, continue the heating until the solution is almost colourless and cool. Add 2 ml of *water R*, heat until white fumes are again evolved, cool, add 10 ml of

water R and neutralise to *red litmus paper R* with *dilute ammonia R1*. The solution gives reaction (a) of sodium (2.3.1) and reaction (b) of phosphates (2.3.1).

TESTS

Solution S. Dissolve 1.0 g in *carbon dioxide-free water R* and dilute to 20 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and not more intensely coloured than reference solution B₇ (2.2.2, Method II).

pH (2.2.3). Dilute 1 ml of solution S to 5 ml with *carbon dioxide-free water R*. The pH of the solution is 7.5 to 9.5.

Specific optical rotation (2.2.7). Dissolve 0.250 g in *water R* and dilute to 25.0 ml with the same solvent. The specific optical rotation is + 75 to + 83, calculated with reference to the anhydrous, ethanol-free substance.

Related substances. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 25.0 mg of the substance to be examined in the mobile phase and dilute to 10.0 ml with the mobile phase.

Reference solution (a). Dissolve 2 mg of *dexamethasone sodium phosphate CRS* and 2 mg of *betamethasone sodium phosphate CRS* in the mobile phase and dilute to 100.0 ml with the mobile phase.

Reference solution (b). Dilute 1.0 ml of the test solution to 100.0 ml with the mobile phase.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4.6 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (5 µm),
- as mobile phase at a flow rate of 1 ml/min a mixture prepared as follows: in a 250 ml conical flask, weigh 1.360 g of *potassium dihydrogen phosphate R* and 0.600 g of *hexylamine R*, mix and allow to stand for 10 min and then dissolve in 182.5 ml of *water R*; add 67.5 ml of *acetonitrile R*, mix and filter (0.45 µm),
- as detector a spectrophotometer set at 254 nm.

Equilibrate the column with the mobile phase at a flow rate of 1 ml/min for about 45 min.

Adjust the sensitivity of the system so that the height of the principal peak in the chromatogram obtained with 20 µl of reference solution (b) is at least 50 per cent of the full scale of the recorder.

Inject 20 µl of reference solution (a). When the chromatograms are recorded in the prescribed conditions, the retention times are: betamethasone sodium phosphate, about 12.5 min and dexamethasone sodium phosphate about 14 min. The test is not valid unless the resolution between the peaks corresponding to betamethasone sodium phosphate and dexamethasone sodium phosphate is at least 2.2. If necessary, adjust slightly the concentration of acetonitrile or increase the concentration of water in the mobile phase.

Inject separately 20 µl of the test solution and 20 µl of reference solution (b). Continue the chromatography for twice the retention time of the principal peak. In the chromatogram obtained with the test solution: the area of any peak, apart from the principal peak, is not greater than half the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent); the sum of the areas of all such peaks is not greater than the area of the principal peak in the chromatogram obtained with reference solution (b) (1 per cent). Disregard any peak with an area less than 0.05 times that of the principal peak in the chromatogram obtained with reference solution (b).

Inorganic phosphates. Dissolve 50 mg in *water R* and dilute to 100 ml with the same solvent. To 10 ml of this solution add 5 ml of *molybdovanadic reagent R*, mix and allow to stand for 5 min. Any yellow colour in the solution is not more intense than that in a standard prepared at the same time in the same manner using 10 ml of *phosphate standard solution (5 ppm PO₄) R* (1 per cent).

Ethanol. Not more than 3.0 per cent *m/m*, determined by gas chromatography (2.2.28), using *propanol R* as the internal standard.

Internal standard solution. Dilute 1.0 ml of *propanol R* to 100.0 ml with *water R*.

Test solution. Dissolve 0.50 g of the substance to be examined in 5.0 ml of the internal standard solution and dilute to 10.0 ml with *water R*.

Reference solution. Dilute 1.0 g of *ethanol R* to 100.0 ml with *water R*. To 2.0 ml of the solution add 5.0 ml of the internal standard solution and dilute to 10.0 ml with *water R*. The chromatographic procedure may be carried out using:

- a column 1 m long and 3.2 mm in internal diameter packed with *ethylvinylbenzene-divinylbenzene copolymer R1* (150 µm to 180 µm),
 - *nitrogen for chromatography R* as the carrier gas at a flow rate of 30 ml/min,
 - a flame-ionisation detector,
- maintaining the temperature of the column at 150 °C, that of the injection port at 250 °C and that of the detector at 280 °C. Inject 2 µl of each solution.

Ethanol and water. Determine the water content using 0.200 g by the semi-micro determination of water (2.5.12). Add the percentage content of water and the percentage content of ethanol obtained in the test for ethanol. The sum of the percentage contents of water and ethanol is not greater than 13.0 per cent *m/m*.

ASSAY

Dissolve 0.100 g in *water R* and dilute to 100.0 ml with the same solvent. Dilute 10.0 ml of the solution to 500.0 ml with *water R*. Measure the absorbance (2.2.25) at the maximum at 241.5 nm.

Calculate the content of C₂₂H₂₈FN₂O₈P taking the specific absorbance to be 303.

STORAGE

Store in an airtight container, protected from light.

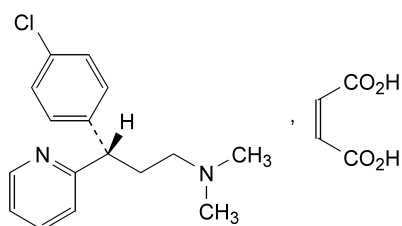
IMPURITIES

- A. dexamethasone,
- B. betamethasone sodium phosphate.

01/2005:1196

DEXCHLORPHENIRAMINE MALEATE

Dexchlorpheniramine maleate



C₂₀H₂₃ClN₂O₄

M_r 390.9

DEFINITION

Dexchlorpheniramine maleate contains not less than 98.0 per cent and not more than the equivalent of 100.5 per cent of (3*S*)-3-(4-chlorophenyl)-*N,N*-dimethyl-3-(pyridin-2-yl)propan-1-amine (*Z*)-butanedioate, calculated with reference to the dried substance.

CHARACTERS

A white crystalline powder, very soluble in water, freely soluble in alcohol, in methanol and in methylene chloride.

IDENTIFICATION

First identification: A, C, E.

Second identification: A, B, D, E.

- A. It complies with the test for specific optical rotation (see Tests).
- B. Melting point (2.2.14): 110 °C to 115 °C.
- C. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *dexchlorpheniramine maleate CRS*. Examine the substances prepared as discs using *potassium bromide R*.
- D. Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel F₂₅₄ plate R*.

Test solution. Dissolve 0.10 g of the substance to be examined in *methanol R* and dilute to 5.0 ml with the same solvent.

Reference solution. Dissolve 56 mg of *maleic acid R* in *methanol R* and dilute to 10 ml with the same solvent.

Apply to the plate 5 µl of each solution. Develop over a path of 12 cm using a mixture of 3 volumes of *water R*, 7 volumes of *anhydrous formic acid R*, 20 volumes of *methanol R* and 70 volumes of *di-isopropyl ether R*. Allow the plate to dry in a current of air for a few minutes and examine in ultraviolet light at 254 nm. The chromatogram obtained with the test solution shows two clearly separated spots. The upper spot is similar in position and size to the spot in the chromatogram obtained with the reference solution.

- E. To 0.15 g in a porcelain crucible add 0.5 g of *anhydrous sodium carbonate R*. Heat over an open flame for 10 min. Allow to cool. Take up the residue with 10 ml of *dilute nitric acid R* and filter. To 1 ml of the filtrate add 1 ml of *water R*. The solution gives reaction (a) of chlorides (2.3.1).

TESTS

Solution S. Dissolve 2.0 g in *water R* and dilute to 20.0 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and not more intensely coloured than reference solution BY₆ (2.2.2, Method II).

pH (2.2.3). Dissolve 0.20 g in 20 ml of *water R*. The pH of the solution is 4.5 to 5.5.

Specific optical rotation (2.2.7): + 22 to + 23, determined on solution S and calculated with reference to the dried substance.

Related substances. Examine by gas chromatography (2.2.28).

Test solution. Dissolve 10.0 mg in 1.0 ml of *methylene chloride R*.

Reference solution. Dissolve 5.0 mg of *brompheniramine maleate CRS* in 0.5 ml of *methylene chloride R* and add 0.5 ml of the test solution. Dilute 0.5 ml of this solution to 50.0 ml with *methylene chloride R*.

The chromatographic procedure may be carried out using:

- a glass column 2.3 m long and 2 mm in internal diameter packed with acid- and base- washed *silanised diatomaceous earth for gas chromatography R* (135 µm to 175 µm) impregnated with 3 per cent *m/m* of a mixture of 50 per cent of poly(dimethyl)siloxane and 50 per cent of poly(diphenyl)siloxane,
- *nitrogen for chromatography R* as the carrier gas at a flow rate of 20 ml/min,
- a flame-ionisation detector,

maintaining the temperature of the column at 205 °C and that of the injection port and of the detector at 250 °C.

Inject 1 µl of the reference solution. The test is not valid unless, in the chromatogram obtained with the reference solution, the resolution between the two peaks corresponding to dexchlorpheniramine and brompheniramine is at least 1.5. Inject 1 µl of the test solution. Continue the chromatography for at least 2.5 times the retention time of the principal peak. In the chromatogram obtained with the test solution: none of the peaks, apart from the principal peak, has an area greater than 0.8 times the area of the peak corresponding to dexchlorpheniramine in the chromatogram obtained with the reference solution (0.4 per cent); the sum of the areas of all the peaks, apart from the principal peak, is not greater than twice the area of the peak corresponding to dexchlorpheniramine in the chromatogram obtained with the reference solution (1 per cent).

Enantiomeric purity. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 10.0 mg in 3 ml of *water R*. Add a few drops of *concentrated ammonia R* until an alkaline reaction is produced. Shake with 5 ml of *methylene chloride R*. Separate the layers. Evaporate the lower, methylene chloride layer to an oily residue on a water-bath. Dissolve the oily residue in *2-propanol R* and dilute to 10.0 ml with the same solvent.

Reference solution (a). Dissolve 10.0 mg of *dexchlorpheniramine maleate CRS* in 3 ml of *water R*. Add a few drops of *concentrated ammonia R* until an alkaline reaction is produced. Shake with 5 ml of *methylene chloride R*. Separate the layers. Evaporate the lower, methylene chloride layer to an oily residue on a water-bath. Dissolve the oily residue in *2-propanol R* and dilute to 10.0 ml with the same solvent.

Reference solution (b). Dissolve 10.0 mg of *chlorphenamine maleate CRS* in 3 ml of *water R*. Add a few drops of *concentrated ammonia R* until an alkaline reaction is produced. Shake with 5 ml of *methylene chloride R*. Separate the layers. Evaporate the lower, methylene chloride layer to an oily residue on a water-bath. Dissolve the oily residue in *2-propanol R* and dilute to 10.0 ml with the same solvent.

Reference solution (c). Dilute 1.0 ml of the test solution to 50 ml with *2-propanol R*.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4.6 mm in internal diameter packed with *amylose derivative of silica gel for chromatography R*,
- as mobile phase at a flow rate of 1 ml/min a mixture of 3 volumes of *diethylamine R*, 20 volumes of *2-propanol R* and 980 volumes of *hexane R*,
- as detector a spectrophotometer set at 254 nm.

Under these conditions the peak of the (*S*)-isomer appears first.

Inject 10 µl of each solution. The test is not valid unless: in the chromatogram obtained with reference solution (b), the resolution between the peaks corresponding to the (*R*)-enantiomer and to the (*S*)-enantiomer is at least 1.5; the retention times of the principal peaks in the chromatograms obtained with the test solution and reference solution (a) are identical ((*S*)-enantiomer). In the chromatogram obtained with the test solution, the area of the peak corresponding to the (*R*)-enantiomer is not greater than the area of the principal peak in the chromatogram obtained with reference solution (c) (2 per cent) and the area of any peak, apart from the principal peak and any peak corresponding to the (*R*)-enantiomer, is not greater than 0.25 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.5 per cent).

Heavy metals (2.4.8). 1.0 g complies with limit test C for heavy metals (20 ppm). Prepare the standard using 2 ml of *lead standard solution (10 ppm Pb) R*.

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 65 °C for 4 h.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

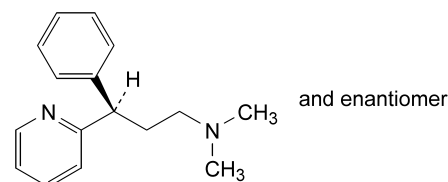
Dissolve 0.150 g in 25 ml of *anhydrous acetic acid R*. Titrate with 0.1 M *perchloric acid*, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *perchloric acid* is equivalent to 19.54 mg of C₂₀H₂₃ClN₂O₄.

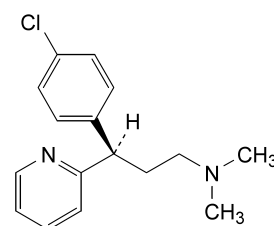
STORAGE

Store protected from light.

IMPURITIES



A. (3*RS*)-*N,N*-dimethyl-3-phenyl-3-(pyridin-2-yl)propan-1-amine,

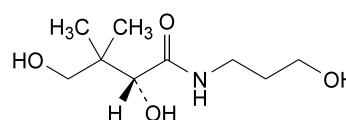


B. (3*R*)-3-(4-chlorophenyl)-*N,N*-dimethyl-3-(pyridin-2-yl)propan-1-amine.

01/2005:0761

DEXPANTHENOL

Dexpanthenolum



C₉H₁₉NO₄

*M*_r 205.3

DEFINITION

Dexpanthenol contains not less than 98.0 per cent and not more than the equivalent of 101.0 per cent of (2*R*)-2,4-dihydroxy-*N*-(3-hydroxypropyl)-3,3-dimethylbutanamide, calculated with reference to the anhydrous substance.

CHARACTERS

A colourless or slightly yellowish, viscous hygroscopic liquid, or a white or almost white, crystalline powder, very soluble in water, freely soluble in ethanol (96 per cent).

IDENTIFICATION

First identification: A, B.

Second identification: A, C, D.

- A. It complies with the test for specific optical rotation (see Tests).
- B. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *dexpanthenol CRS*. Examine the substances using discs prepared as follows: dissolve the substance to be examined and the reference substance separately in 1.0 ml of *anhydrous ethanol R* to obtain a concentration of 5 mg/ml. Place dropwise 0.5 ml of this solution on a disc of *potassium bromide R*. Dry the disc at 100–105 °C for 15 min.
- C. Examine the chromatograms obtained in the test for 3-aminopropanol. The principal spot in the chromatogram obtained with test solution (b) is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).
- D. To 1 ml of solution S (see Tests) add 1 ml of *dilute sodium hydroxide solution R* and 0.1 ml of *copper sulphate solution R*. A blue colour develops.

TESTS

Solution S. Dissolve 2.500 g in *carbon dioxide-free water R* and dilute to 50.0 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and not more intensely coloured than reference solution B₆ (2.2.2, *Method II*).

pH (2.2.3). The pH of solution S is not greater than 10.5.

Specific optical rotation (2.2.7). The specific optical rotation is + 29.0 to + 32.0, determined on solution S and calculated with reference to the anhydrous substance.

3-Aminopropanol. Examine by thin-layer chromatography (2.2.27), using *silica gel G R* as the coating substance.

Test solution (a). Dissolve 0.25 g of the substance to be examined in *anhydrous ethanol R* and dilute to 5 ml with the same solvent.

Test solution (b). Dilute 1 ml of test solution (a) to 10 ml with *anhydrous ethanol R*.

Reference solution (a). Dissolve the contents of a vial of *dexpanthenol CRS* in 1.0 ml of *anhydrous ethanol R* to obtain a concentration of 5 mg/ml.

Reference solution (b). Dissolve 25 mg of 3-aminopropanol *R* in *anhydrous ethanol R* and dilute to 100 ml with the same solvent.

Apply separately to the plate 10 µl of each solution. Develop over a path of 15 cm using a mixture of 20 volumes of *concentrated ammonia R*, 25 volumes of *methanol R* and 55 volumes of *butanol R*. Allow the plate to dry in air, spray with a 100 g/l solution of *trichloroacetic acid R* in *methanol R* and heat at 150 °C for 10 min. Spray with a 1 g/l solution of *ninhydrin R* in *methanol R* and heat at 120 °C until a colour appears. Any spot due to 3-aminopropanol

in the chromatogram obtained with test solution (a) is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.5 per cent).

Heavy metals (2.4.8). 12 ml of solution S complies with limit test A for heavy metals (20 ppm). Prepare the reference solution using *lead standard solution (1 ppm Pb) R*.

Water (2.5.12). Not more than 1.0 per cent, determined on 1.000 g.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

To 0.400 g add 50.0 ml of 0.1 *M perchloric acid*. Boil under a reflux condenser for 5 h protected from humidity. Allow to cool. Add 50 ml of *dioxan R* by rinsing the condenser, protected from humidity. Add 0.2 ml of *naphtholbenzein solution R* and titrate with 0.1 *M potassium hydrogen phthalate* until the colour changes from green to yellow. Carry out a blank titration.

1 ml of 0.1 *M perchloric acid* is equivalent to 20.53 mg of C₉H₁₉NO₄.

STORAGE

In an airtight container.

01/2005:1506

DEXTRAN 1 FOR INJECTION

Dextranum 1 ad iniectabile

DEFINITION

Dextran 1 for injection is a low molecular weight fraction of dextran, consisting of a mixture of isomaltooligosaccharides. The average relative molecular mass is about 1000.

PRODUCTION

It is obtained by hydrolysis and fractionation of dextrans produced by fermentation of sucrose using *Leuconostoc mesenteroides* strain NRRL B-512 = CIP 78.59 or substrains thereof (for example *L. mesenteroides* B-512 F = NCTC 10817).

It is prepared in conditions designed to minimise the risk of microbial contamination.

CHARACTERS

A white or almost white powder, hygroscopic, very soluble in water, very slightly soluble in alcohol.

IDENTIFICATION

- A. Dissolve 3.000 g in *water R*, heat on a water-bath and dilute to 100.0 ml with the same solvent. The specific optical rotation (2.2.7) is + 148 to + 164, calculated with reference to the dried substance. Dry an aliquot of the solution first on a water-bath and then to constant weight *in vacuo* at 70 °C. Calculate the dextran content after correction for the content of sodium chloride.
- B. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *dextran 1 CRS*. Prepare the discs as follows: to 1–2 mg add one or a few drops of *water R*; grind in an agate mortar for 1–2 min; add about 300 mg of *potassium bromide R* and mix to a slurry (do not grind); dry *in vacuo* at 40 °C for 15 min, crush the residue (if it is not dry, dry for another 15 min). Prepare a disc using *potassium bromide R*. Run the infrared spectrum with a blank disc using *potassium bromide R* in the reference beam.

C. It complies with the test for molecular-mass distribution (see Tests).

TESTS

Solution S. Dissolve 7.5 g in *carbon dioxide-free water R*, heat on a water-bath and dilute to 50 ml with the same solvent.

Absorbance (2.2.25). Measure the absorbance of solution S at 375 nm. The absorbance is not more than 0.12.

Acidity or alkalinity. To 10 ml of solution S add 0.1 ml of *phenolphthalein solution R*. The solution is colourless. Add 0.2 ml of 0.01 M *sodium hydroxide*. The solution is pink. Add 0.4 ml of 0.01 M *hydrochloric acid*. The solution is colourless. Add 0.1 ml of *methyl red solution R*. The solution is red or orange.

Nitrogen-containing substances. Carry out the determination of nitrogen by sulphuric acid digestion (2.5.9), using 0.200 g and heating for 2 h. Collect the distillate in a mixture of 0.5 ml of *bromocresol green solution R*, 0.5 ml of *methyl red solution R* and 20 ml of *water R*. Titrate with 0.01 M *hydrochloric acid*. Not more than 0.15 ml of 0.01 M *hydrochloric acid* is required to change the colour of the indicator (110 ppm N).

Sodium chloride. Not more than 1.5 per cent. Accurately weigh 3.5 g and dissolve in 100 ml of *water R*. Add 0.3 ml of *potassium chromate solution R* and titrate with 0.1 M *silver nitrate* until the yellowish-white colour changes to reddish-brown.

1 ml of 0.1 M *silver nitrate* is equivalent to 5.844 mg of NaCl.

Molecular-mass distribution. The average molecular mass (M_w) is 850 to 1150. The fraction with less than 3 units of glucose is less than 15 per cent, the fraction with more than 9 units of glucose is less than 20 per cent.

Examine by size-exclusion chromatography (2.2.30).

Test solution. Dissolve 6.0-6.5 mg of the substance to be examined in 1.0 ml of the mobile phase.

Reference solution (a). Dissolve 6.0-6.5 mg of *dextran 1 CRS* in 1.0 ml of the mobile phase.

Reference solution (b). Dissolve the content of an ampoule of *isomaltooligosaccharide CRS* in 1 ml of the mobile phase, and mix. This corresponds to approximately 45 µg of isomaltotriose (3 glucose units), approximately 45 µg of isomaltotetraose (4 glucose units), and approximately 60 µg of isomaltopentaose (5 glucose units).

The chromatographic procedure may be carried out using:

- 2 columns, 30 cm long and 10 mm in internal diameter, in series, prepacked with a packing material of dextran covalently bound to highly cross-linked porous agarose beads, allowing resolution of oligosaccharides in the molecular mass range of 180 to 3000, kept at a temperature of 20-25 °C,
- as mobile phase at a flow rate of 0.07-0.08 ml/min maintained constant to ± 1 per cent, a 2.92 g/l solution of *sodium chloride R*,
- as detector a differential refractometer.

Inject 100 µl of reference solution (b) and record the chromatogram for definition of the positions of isomaltotriose, isomaltotetraose and sodium chloride. Inject 100 µl of the test solution and 100 µl of reference solution (a) and record the chromatograms. Determine the peak areas. Disregard any peak due to sodium chloride.

Calculate the average relative molecular mass M_w and the amount of the fraction with less than 3 and more than 9 glucose units, of *dextran 1 CRS* and of the substance to be examined. The test is not valid unless the values obtained for *dextran 1 CRS* are within the values stated on the label.

$$M_w = \sum w_i \times m_i$$

M_w = average molecular mass of the dextran,

m_i = molecular mass of oligosaccharide i ,

w_i = weight proportion of oligosaccharide i .

Use the following molecular mass values for the calculation:

Oligosaccharide i	m_i
glucose	180
isomaltose	342
isomaltotriose	504
isomaltotetraose	666
isomaltopentaose	828
isomaltohexaose	990
isomaltoheptaose	1152
isomaltooctaose	1314
isomaltotonaose	1476
isomaltodecaose	1638
isomaltoundecaose	1800
isomaltododecaose	1962
isomaltotridecaose	2124
isomaltotetradecaose	2286
isomaltopentadecaose	2448
isomaltohexadecaose	2610
isomaltoheptadecaose	2772
isomaltooctadecaose	2934
isomaltotonaadecaose	3096

Heavy metals (2.4.8). Dilute 20 ml of solution S to 30 ml with *water R*. 12 ml of this solution complies with limit test A (10 ppm). Prepare the reference solution using *lead standard solution (1 ppm Pb) R*.

Loss on drying (2.2.32). Not more than 5.0 per cent, determined on 5.000 g by drying in an oven at 100-105 °C for 5 h.

Bacterial endotoxins (2.6.14): less than 25 IU/g.

Microbial contamination. Total viable aerobic count (2.6.12) not more than 10² micro-organisms per gram, determined by plate-count. It complies with the test for *Escherichia coli* (2.6.13).

01/2005:0999

DEXTRAN 40 FOR INJECTION

Dextranum 40 ad iniectabile

DEFINITION

Dextran 40 for injection is a mixture of polysaccharides, principally of the α-1,6-glucan type.

The average relative molecular mass is about 40 000.

PRODUCTION

It is obtained by hydrolysis and fractionation of dextrans produced by fermentation of sucrose using *Leuconostoc mesenteroides* strain NRRL B-512 = CIP 78.59 or substrains thereof (for example *L. mesenteroides* B-512F = NCTC 10817).

It is prepared in conditions designed to minimise the risk of microbial contamination.

CHARACTERS

A white or almost white powder, very soluble in water, very slightly soluble in alcohol.

IDENTIFICATION

- A. Dissolve 1.0 g in *water R*, heating on a water-bath, and dilute to 50.0 ml with the same solvent. The specific optical rotation (2.2.7) of the solution is + 195 to + 201, calculated with reference to the dried substance.
- B. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *dextran CRS*.
- C. It complies with the test for molecular-mass distribution (see Tests).

TESTS

Solution S. Dissolve 5.0 g in *distilled water R*, heating on a water-bath, and dilute to 50 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and colourless (2.2.2, *Method II*).

Acidity or alkalinity. To 10 ml of solution S add 0.1 ml of *phenolphthalein solution R*. The solution remains colourless. Add 0.2 ml of 0.01 M *sodium hydroxide*. The solution is red. Add 0.4 ml of 0.01 M *hydrochloric acid*. The solution is colourless. Add 0.1 ml of *methyl red solution R*. The solution is red or orange.

Nitrogen-containing substances. Carry out the determination of nitrogen by sulphuric acid digestion (2.5.9), using 0.200 g and heating for 2 h. Collect the distillate in a mixture of 0.5 ml of *bromocresol green solution R*, 0.5 ml of *methyl red solution R* and 20 ml of *water R*. Titrate with 0.01 M *hydrochloric acid*. Not more than 0.15 ml of 0.01 M *hydrochloric acid* is required to change the colour of the indicator (110 ppm N).

Residual solvents. Examine by gas chromatography (2.2.28), using *propanol R* as internal standard.

Test solution. Dissolve 5 g of the substance to be examined in 100 ml of *water R* and distil. Collect the first 45 ml of the distillate, add 1 ml of a 25 g/l solution of *propanol R* and dilute to 50 ml with *water R*.

Reference solution. Mix 0.5 ml of a 25 g/l solution of *ethanol R*, 0.5 ml of a 25 g/l solution of *propanol R* and 0.5 ml of a 2.5 g/l solution of *methanol R* and dilute to 25.0 ml with *water R*.

The chromatographic procedure may be carried out using:

- a stainless steel column 1.8 m long and 2 mm in internal diameter packed with *ethylvinylbenzene-divinylbenzene copolymer R* (125–150 µm),
- *nitrogen for chromatography R* as the carrier gas at a flow rate of 25 ml/min,
- a flame-ionisation detector,

maintaining the temperature of the column at 190 °C, that of the injection port at 240 °C and that of the detector at 210 °C. Inject the chosen volume of each solution. In the chromatogram obtained with the test solution, the area of any peak corresponding to ethanol or methanol is not greater than the area of the corresponding peak in the

chromatogram obtained with the reference solution (0.5 per cent of ethanol and 0.05 per cent of methanol) and the sum of the areas of any peaks, apart from the peaks corresponding to ethanol, methanol and the internal standard, is not greater than the area of the peak corresponding to the internal standard (0.5 per cent calculated as propanol).

Molecular-mass distribution (2.2.39). The average molecular mass (M_w) is 35 000 to 45 000. The average molecular mass of the 10 per cent high fraction is not more than 110 000. The average molecular mass of the 10 per cent low fraction is not less than 7000.

Heavy metals (2.4.8). 12 ml of solution S complies with limit test A (10 ppm). Prepare the standard using *lead standard solution (1 ppm Pb) R*.

Loss on drying (2.2.32). Not more than 7.0 per cent, determined on 0.200 g by heating in an oven at 105 ± 2 °C for 5 h.

Sulphated ash (2.4.14). Not more than 0.3 per cent, determined on 0.50 g.

Bacterial endotoxins (2.6.14): less than 10 IU/g.

Microbial contamination. Total viable aerobic count (2.6.12) not more than 10² micro-organisms per gram, determined by plate-count. It complies with the test for *Escherichia coli* (2.6.13).

01/2005:1000

DEXTRAN 60 FOR INJECTION

Dextranum 60 ad iniectionabile

DEFINITION

Dextran 60 for injection is a mixture of polysaccharides, principally of the α-1,6-glucan type.

The average relative molecular mass is about 60 000.

PRODUCTION

It is obtained by hydrolysis and fractionation of dextrans produced by fermentation of sucrose using *Leuconostoc mesenteroides* strain NRRL B-512 = CIP 78.59 or substrains thereof (for example *L. mesenteroides* B-512F = NCTC 10817).

It is prepared in conditions designed to minimise the risk of microbial contamination.

CHARACTERS

A white or almost white powder, very soluble in water, very slightly soluble in alcohol.

IDENTIFICATION

- A. Dissolve 1.0 g in *water R*, heating on a water-bath, and dilute to 50.0 ml with the same solvent. The specific optical rotation (2.2.7) of the solution is + 195 to + 201, calculated with reference to the dried substance.
- B. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *dextran CRS*.
- C. It complies with the test for molecular-mass distribution (see Tests).

TESTS

Solution S. Dissolve 5.0 g in *distilled water R*, heating on a water-bath, and dilute to 50 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and colourless (2.2.2, *Method II*).

01/2005:1001

DEXTRAN 70 FOR INJECTION

Dextranum 70 ad iniectabile

Acidity or alkalinity. To 10 ml of solution S add 0.1 ml of *phenolphthalein solution R*. The solution remains colourless. Add 0.2 ml of 0.01 M sodium hydroxide. The solution is red. Add 0.4 ml of 0.01 M hydrochloric acid. The solution is colourless. Add 0.1 ml of *methyl red solution R*. The solution is red or orange.

Nitrogen-containing substances. Carry out the determination of nitrogen by sulphuric acid digestion (2.5.9), using 0.200 g and heating for 2 h. Collect the distillate in a mixture of 0.5 ml of *bromocresol green solution R*, 0.5 ml of *methyl red solution R* and 20 ml of *water R*. Titrate with 0.01 M hydrochloric acid. Not more than 0.15 ml of 0.01 M hydrochloric acid is required to change the colour of the indicator (110 ppm N).

Residual solvents. Examine by gas chromatography (2.2.28), using *propanol R* as internal standard.

Test solution. Dissolve 5 g of the substance to be examined in 100 ml of *water R* and distil. Collect the first 45 ml of the distillate, add 1 ml of a 25 g/l solution of *propanol R* and dilute to 50 ml with *water R*.

Reference solution. Mix 0.5 ml of a 25 g/l solution of *ethanol R*, 0.5 ml of a 25 g/l solution of *propanol R* and 0.5 ml of a 2.5 g/l solution of *methanol R* and dilute to 25.0 ml with *water R*.

The chromatographic procedure may be carried out using:

- a stainless steel column 1.8 m long and 2 mm in internal diameter packed with *ethylvinylbenzene-divinylbenzene copolymer R* (125-150 µm),
- *nitrogen for chromatography R* as the carrier gas at a flow rate of 25 ml/min,
- a flame-ionisation detector,

maintaining the temperature of the column at 190 °C, that of the injection port at 240 °C and that of the detector at 210 °C. Inject the chosen volume of each solution. In the chromatogram obtained with the test solution, the area of any peak corresponding to ethanol or methanol is not greater than the area of the corresponding peak in the chromatogram obtained with the reference solution (0.5 per cent of ethanol and 0.05 per cent of methanol) and the sum of the areas of any peaks, apart from the peaks corresponding to ethanol, methanol and the internal standard, is not greater than the area of the peak corresponding to the internal standard (0.5 per cent calculated as propanol).

Molecular-mass distribution (2.2.39). The average molecular mass (M_w) is 54 000 to 66 000. The average molecular mass of the 10 per cent high fraction is not more than 180 000. The average molecular mass of the 10 per cent low fraction is not less than 14 000.

Heavy metals (2.4.8). 12 ml of solution S complies with limit test A (10 ppm). Prepare the standard using *lead standard solution* (1 ppm Pb) *R*.

Loss on drying (2.2.32). Not more than 7.0 per cent, determined on 0.200 g by heating in an oven at 105 ± 2 °C for 5 h.

Sulphated ash (2.4.14). Not more than 0.3 per cent, determined on 0.50 g.

Bacterial endotoxins (2.6.14): less than 16 IU/g.

Microbial contamination. Total viable count (2.6.12) not more than 10^2 micro-organisms per gram, determined by plate-count. It complies with the test for *Escherichia coli* (2.6.13).

DEFINITION

Dextran 70 for injection is a mixture of polysaccharides, principally of the α -1,6-glucan type.

The average relative molecular mass is about 70 000.

PRODUCTION

It is obtained by hydrolysis and fractionation of dextrans produced by fermentation of sucrose using *Leuconostoc mesenteroides* strain NRRL B-512 = CIP 78.59 or substrains thereof (for example *L. mesenteroides* B-512F = NCTC 10817).

It is prepared in conditions designed to minimise the risk of microbial contamination.

CHARACTERS

A white or almost white powder, very soluble in water, very slightly soluble in alcohol.

IDENTIFICATION

- A. Dissolve 1.0 g in *water R*, heating on a water-bath, and dilute to 50.0 ml with the same solvent. The specific optical rotation (2.2.7) of the solution is + 195 to + 201, calculated with reference to the dried substance.
- B. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *dextran CRS*.
- C. It complies with the test for molecular-mass distribution (see Tests).

TESTS

Solution S. Dissolve 5.0 g in *distilled water R*, heating on a water-bath, and dilute to 50 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and colourless (2.2.2, *Method II*).

Acidity or alkalinity. To 10 ml of solution S add 0.1 ml of *phenolphthalein solution R*. The solution remains colourless. Add 0.2 ml of 0.01 M sodium hydroxide. The solution is red. Add 0.4 ml of 0.01 M hydrochloric acid. The solution is colourless. Add 0.1 ml of *methyl red solution R*. The solution is red or orange.

Nitrogen-containing substances. Carry out the determination of nitrogen by sulphuric acid digestion (2.5.9), using 0.200 g and heating for 2 h. Collect the distillate in a mixture of 0.5 ml of *bromocresol green solution R*, 0.5 ml of *methyl red solution R* and 20 ml of *water R*. Titrate with 0.01 M hydrochloric acid. Not more than 0.15 ml of 0.01 M hydrochloric acid is required to change the colour of the indicator (110 ppm N).

Residual solvents. Examine by gas chromatography (2.2.28), using *propanol R* as internal standard.

Test solution. Dissolve 5 g of the substance to be examined in 100 ml of *water R* and distil. Collect the first 45 ml of the distillate, add 1 ml of a 25 g/l solution of *propanol R* and dilute to 50 ml with *water R*.

Reference solution. Mix 0.5 ml of a 25 g/l solution of *ethanol R*, 0.5 ml of a 25 g/l solution of *propanol R* and 0.5 ml of a 2.5 g/l solution of *methanol R* and dilute to 25.0 ml with *water R*.

The chromatographic procedure may be carried out using:

- a stainless steel column 1.8 m long and 2 mm in internal diameter packed with *ethylvinylbenzene-divinylbenzene copolymer R* (125–150 µm),
- *nitrogen for chromatography R* as the carrier gas at a flow rate of 25 ml/min,
- a flame-ionisation detector,

maintaining the temperature of the column at 190 °C, that of the injection port at 240 °C and that of the detector at 210 °C. Inject the chosen volume of each solution. In the chromatogram obtained with the test solution, the area of any peak corresponding to ethanol or methanol is not greater than the area of the corresponding peak in the chromatogram obtained with the reference solution (0.5 per cent of ethanol and 0.05 per cent of methanol) and the sum of the areas of any peaks, apart from the peaks corresponding to ethanol, methanol and the internal standard, is not greater than the area of the peak corresponding to the internal standard (0.5 per cent calculated as propanol).

Molecular-mass distribution (2.2.39). The average molecular mass (M_w) is 64 000 to 76 000. The average molecular mass of the 10 per cent high fraction is not more than 185 000. The average molecular mass of the 10 per cent low fraction is not less than 15 000.

Heavy metals (2.4.8). 12 ml of solution S complies with limit test A (10 ppm). Prepare the standard using *lead standard solution (1 ppm Pb) R*.

Loss on drying (2.2.32). Not more than 7.0 per cent, determined on 0.200 g by heating in an oven at 105 ± 2 °C for 5 h.

Sulphated ash (2.4.14). Not more than 0.3 per cent, determined on 0.50 g.

Bacterial endotoxins (2.6.14): less than 16 IU/g.

Microbial contamination. Total viable count (2.6.12) not more than 10^2 micro-organisms per gram, determined by plate-count. It complies with the test for *Escherichia coli* (2.6.13).

TESTS

pH (2.2.3). Disperse 5.0 g in 100 ml of *carbon dioxide-free water R*. The pH is 2.0 to 8.0.

Chlorides. Dissolve 2.5 g in 50 ml of boiling *water R*, dilute to 100 ml with *water R* and filter. Dilute 1 ml of the filtrate to 15 ml, add 1 ml of *dilute nitric acid R*, pour the mixture as a single addition into 1 ml of *silver nitrate solution R2* and allow to stand for 5 min protected from light. When viewed transversely against a black background any opalescence produced is not more intense than that obtained by treating a mixture of 10 ml of *chloride standard solution (5 ppm Cl) R* and 5 ml of *water R*, prepared in the same manner (0.2 per cent).

Reducing sugars. To a quantity of dextrin equivalent to 2.0 g (dried substance) add 100 ml of *water R*, shake for 30 min, dilute with *water R* to 200.0 ml and filter. To 10.0 ml of alkaline *cupri-tartaric solution R* add 20.0 ml of the filtrate, mix, and heat on a hot plate adjusted to bring the solution to boil within 3 min. Boil for 2 min, and cool immediately. Add 5 ml of a 300 g/l solution of *potassium iodide R* and 10 ml of *1 M sulphuric acid*, mix, and titrate immediately with *0.1 M sodium thiosulphate*, using *starch solution R*, added towards the end of the titration, as indicator. Repeat the procedure beginning with “To 10.0 ml of...”, using, in place of the filtrate, 20.0 ml of a 1 g/l solution of *glucose R*, accurately prepared. Perform a blank titration. ($V_B - V_U$) is not greater than ($V_B - V_S$), in which V_B , V_U and V_S are the number of millilitres of *0.1 M sodium thiosulphate* consumed in the titrations of the blank, the dextrin and the glucose, respectively (10 per cent, calculated as glucose $C_6H_{12}O_6$).

Heavy metals (2.4.8). 1.0 g complies with limit test C for heavy metals (20 ppm). Prepare the standard using 2 ml of *lead standard solution (10 ppm Pb) R*.

Loss on drying (2.2.32). Not more than 13.0 per cent, determined on 1.000 g by drying at 130 °C to 135 °C for 90 min.

Sulphated ash (2.4.14). Not more than 0.5 per cent, determined on 1.0 g.

01/2005:1507

DEXTRIN

Dextrinum

DEFINITION

Dextrin is maize, potato or cassava starch partly hydrolysed and modified by heating with or without the presence of acids, alkalis or pH control agents.

CHARACTERS

White or almost white, free-flowing powder, very soluble in boiling water forming a mucilaginous solution, slowly soluble in cold water, practically insoluble in alcohol.

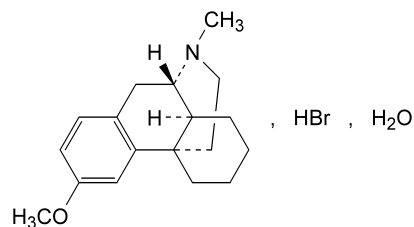
IDENTIFICATION

- Suspend 1 g in 50 ml of *water R*, boil for 1 min and cool. To 1 ml of the solution add 0.05 ml of *iodine solution R1*. A dark blue to reddish-brown colour is produced which disappears on heating.
- Centrifuge 5 ml of the mucilage obtained in identification test A. To the upper layer add 2 ml of *dilute sodium hydroxide solution R* and, dropwise with shaking, 0.5 ml of *copper sulphate solution R* and boil. A red precipitate is produced.
- It is very soluble in boiling *water R*, forming a mucilaginous solution.

01/2005:0020

DEXTROMETHORPHAN HYDROBROMIDE

Dextromethorphan hydrobromidum


 $C_{18}H_{26}BrNO \cdot H_2O$
 M_r 370.3

DEFINITION

ent-3-Methoxy-17-methylmorphinan hydrobromide monohydrate.

Content: 99.0 per cent to 101.0 per cent (anhydrous substance).

CHARACTERS

Appearance: almost white, crystalline powder.

Solubility: sparingly soluble in water, freely soluble in alcohol.

mp: about 125 °C, with decomposition.

IDENTIFICATION

First identification: A, B, D.

Second identification: A, C, D.

A. It complies with the test for specific optical rotation (see Tests).

B. Infrared absorption spectrophotometry (2.2.24).

Preparation: discs.

Comparison: dextromethorphan hydrobromide CRS.

C. Thin-layer chromatography (2.2.27).

Test solution. Dissolve 25 mg of the substance to be examined in *methanol R* and dilute to 10 ml with the same solvent.

Reference solution. Dissolve 25 mg of *dextromethorphan hydrobromide CRS* in *methanol R* and dilute to 10 ml with the same solvent.

Plate: TLC silica gel G plate *R*.

Mobile phase: concentrated ammonia *R*, methylene chloride *R*, methanol *R*, ethyl acetate *R*, toluene *R* (2:10:13:20:55 V/V/V/V/V).

Application: 5 µl.

Development: over 2/3 of the plate.

Drying: in air.

Detection: spray with *potassium iodobismuthate solution R2*.

Results: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with the reference solution.

D. It gives reaction (a) of bromides (2.3.1).

TESTS

Solution S. Dissolve 1.0 g in *alcohol R* and dilute to 20 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and colourless (2.2.2, *Method II*).

Acidity or alkalinity. Dissolve 0.4 g in *carbon dioxide-free water R* with gentle heating, cool and dilute to 20 ml with the same solvent. Add 0.1 ml of *methyl red solution R* and 0.2 ml of 0.01 M *sodium hydroxide*. The solution is yellow. Not more than 0.4 ml of 0.01 M *hydrochloric acid* is required to change the colour of the indicator to red.

Specific optical rotation (2.2.7). + 28 to + 30 (anhydrous substance).

Dissolve 0.200 g in 0.1 M *hydrochloric acid* and dilute to 10.0 ml with the same acid.

Related substances. Liquid chromatography (2.2.29).

Test solution. Dissolve 10.0 mg of the substance to be examined in the mobile phase and dilute to 10.0 ml with the mobile phase.

Reference solution (a). Dissolve 2 mg of *dextromethorphan impurity A CRS* in 2 ml of the test solution and dilute to 25.0 ml with the mobile phase.

Reference solution (b). Dilute 1.0 ml of the test solution to 200.0 ml with the mobile phase.

Column:

– **size:** $l = 0.25$ m, $\varnothing = 4.6$ mm,

– **stationary phase:** octadecylsilyl silica gel for chromatography *R* (5 µm).

Mobile phase: dissolve 3.11 g of *docusate sodium R* in a mixture of 400 ml of *water R* and 600 ml of *acetonitrile R*. Add 0.56 g of *ammonium nitrate R*. Adjust to apparent pH 2.0 with *glacial acetic acid R*.

Flow rate: 1.0 ml/min.

Detection: spectrophotometer at 280 nm.

Injection: 20 µl.

Run time: twice the retention time of dextromethorphan.

Relative retention with reference to dextromethorphan (retention time = about 21.9 min): impurity B = about 0.44; impurity C = about 0.85; impurity D = about 0.90; impurity A = about 1.13.

System suitability: reference solution (a):

– **resolution:** minimum 1.5 between the peaks due to impurity A and dextromethorphan.

Limits:

– **correction factor:** for the calculation of content, multiply the peak area of impurity C by 0.2,

– **any impurity:** not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent), and not more than 1 such peak has an area greater than half the area of the principal peak in the chromatogram obtained with reference solution (b) (0.25 per cent),

– **total:** not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (1 per cent),

– **disregard limit:** 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

N,N-Dimethylaniline: maximum 10 ppm.

Dissolve 0.5 g with heating in 20 ml of *water R*. Allow to cool, add 2 ml of *dilute acetic acid R* and 1 ml of a 10 g/l solution of *sodium nitrite R* and dilute to 25 ml with *water R*. The solution is not more intensely coloured than a reference solution prepared at the same time in the same manner using 20 ml of a 0.25 mg/l solution of *dimethylaniline R*.

Water (2.5.12): 4.0 per cent to 5.5 per cent, determined on 0.200 g.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

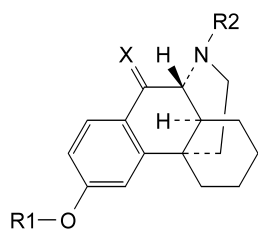
Dissolve 0.300 g in a mixture of 5.0 ml of 0.01 M *hydrochloric acid* and 20 ml of *alcohol R*. Titrate with 0.1 M *sodium hydroxide*, determining the end-point potentiometrically (2.2.20). Read the volume added between the 2 points of inflexion.

1 ml of 0.1 M *sodium hydroxide* is equivalent to 35.23 mg of $C_{18}H_{26}BrNO$.

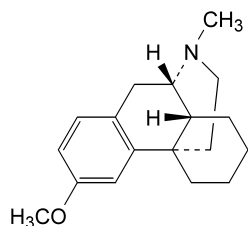
STORAGE

Protected from light.

IMPURITIES



- A. R1 = CH₃, R2 = H, X = H₂: *ent*-3-methoxymorphinan,
 B. R1 = H, R2 = CH₃, X = H₂: *ent*-17-methylmorphinan-3-ol,
 C. R1 = R2 = CH₃, X = O: *ent*-3-methoxy-17-methylmorphinan-10-one,

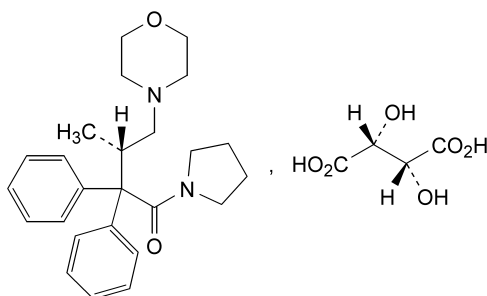


- D. *ent*-(14*S*)-3-methoxy-17-methylmorphinan.

01/2005:0021

DEXTROMORAMIDE TARTRATE

Dextromoramidi tartras

C₂₉H₃₈N₂O₈M_r 542.6

DEFINITION

Dextromoramide tartrate contains not less than 98.0 per cent and not more than the equivalent of 101.0 per cent of 1-[(3*S*)-3-methyl-4-(morpholin-4-yl)-2,2-diphenylbutanoyl]pyrrolidine hydrogen (2*R*,3*R*)-2,3-dihydroxybutanedioate, calculated with reference to the dried substance.

CHARACTERS

A white, amorphous or crystalline powder, soluble in water, sparingly soluble in alcohol.

It melts at about 190 °C, with slight decomposition.

IDENTIFICATION

- A. Dissolve 75 mg in 1 M hydrochloric acid and dilute to 100.0 ml with the same acid. Examined between 230 nm and 350 nm (2.2.25), the solution shows 3 absorption maxima, at 254 nm, 259 nm and 264 nm. The specific absorbances at the maxima are about 6.9, 7.7 and 6.5, respectively.
 B. Dissolve about 50 mg in water *R* and dilute to 10 ml with the same solvent. To 2 ml of the solution add 3 ml of ammoniacal silver nitrate solution *R* and heat on a water-bath. A grey or black precipitate is formed.

C. It gives reaction (b) of tartrates (2.3.1).

TESTS

pH (2.2.3). Dissolve 0.2 g in carbon dioxide-free water *R* and dilute to 20 ml with the same solvent. The pH of the solution is 3.0 to 4.0.

Specific optical rotation (2.2.7). Dissolve 0.50 g in 0.1 M hydrochloric acid and dilute to 10.0 ml with the same acid. The specific optical rotation is + 21 to + 23.

Related substances. Examine by thin-layer chromatography (2.2.27), using silica gel *G R* as the coating substance.

Test solution. Dissolve 0.2 g of the substance to be examined in methanol *R* and dilute to 10 ml with the same solvent.

Reference solution. Dilute 1 ml of the test solution to 100 ml with methanol *R*.

Apply separately to the plate 10 µl of each solution. Develop over a path of 15 cm using methanol *R*. Allow the plate to dry in air and spray with dilute potassium iodobismuthate solution *R*. Any spot in the chromatogram obtained with the test solution, apart from the principal spot, is not more intense than the spot in the chromatogram obtained with the reference solution (1.0 per cent).

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.00 g by drying in an oven at 100 °C to 105 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

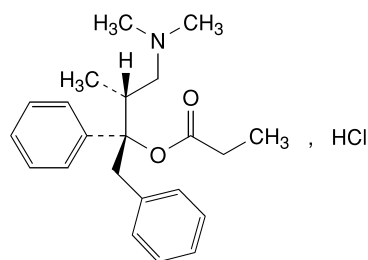
Dissolve 0.250 g in 30 ml of anhydrous acetic acid *R*. Titrate with 0.05 M perchloric acid using 0.15 ml of naphtholbenzein solution *R* as indicator.

1 ml of 0.05 M perchloric acid is equivalent to 27.13 mg of C₂₉H₃₈N₂O₈.

01/2005:0713

DEXTROPROPOXYPHENE HYDROCHLORIDE

Dextropropoxypheni hydrochloridum

C₂₂H₃₀ClNO₂M_r 375.9

DEFINITION

Dextropropoxyphene hydrochloride contains not less than 98.5 per cent and not more than the equivalent of 101.0 per cent of (1*S*,2*R*)-1-benzyl-3-(dimethylamino)-2-methyl-1-phenylpropyl propanoate hydrochloride, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, crystalline powder, very soluble in water, freely soluble in alcohol.

It melts at about 165 °C.

IDENTIFICATION

First identification: A, C, D.

Second identification: A, B, D.

- A. It complies with the test for specific optical rotation (see Tests).
- B. Dissolve 50.0 mg in 0.01 M hydrochloric acid and dilute to 100.0 ml with the same acid. Examined between 220 nm and 360 nm (2.2.25), the solution shows 3 absorption maxima, at 252 nm, 257 nm and 263 nm and 2 shoulders, at 240 nm and 246 nm. The ratio of the absorbance at the maximum at 257 nm to that at the maximum at 252 nm is 1.22 to 1.28. The ratio of the absorbance at the maximum at 257 nm to that at the maximum at 263 nm is 1.29 to 1.35. The test is not valid unless, in the test for resolution (2.2.25), the ratio of the absorbances is at least 1.5.
- C. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the *Ph. Eur. reference spectrum of dextropropoxyphene hydrochloride*.
- D. Solution S (see Tests) gives reaction (a) of chlorides (2.3.1).

TESTS

Solution S. Dissolve 1.5 g in carbon dioxide-free water R and dilute to 30 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and colourless (2.2.2, Method II).

Acidity or alkalinity. Dilute 10 ml of solution S to 25 ml with carbon dioxide-free water R. To 10 ml of this solution, add 0.1 ml of methyl red solution R and 0.2 ml of 0.01 M sodium hydroxide. The solution is yellow. Add 0.4 ml of 0.01 M hydrochloric acid. The solution is red.

Specific optical rotation (2.2.7). Dissolve 0.100 g in water R and dilute to 10.0 ml with the same solvent. The specific optical rotation is + 52 to + 57.

Related substances. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 50.0 mg of the substance to be examined in the mobile phase and dilute to 10.0 ml with the mobile phase.

Reference solution (a). Dilute 0.50 ml of the test solution to 100.0 ml with the mobile phase.

Reference solution (b). Dissolve 50.0 mg of the substance to be examined in 2.5 ml of 2 M alcoholic potassium hydroxide. Add 2.5 ml of water R and boil under a reflux condenser for 30 min. Add 2.5 ml of dilute hydrochloric acid R and dilute to 50 ml with the mobile phase.

The chromatographic procedure may be carried out using:

- a column 0.125 m long and 4.6 mm in internal diameter packed with silica gel for chromatography R (5 µm),
- a guard column packed with a suitable silica gel, equilibrated with the mobile phase and placed between the column and the injection device,
- as mobile phase at a flow rate of 1.0 ml/min a mixture of 50 volumes of 0.2 M phosphate buffer solution pH 7.5 R, 84 volumes of tetrahydrofuran R, 350 volumes of methanol R and 516 volumes of water R, containing 0.9 g/l of cetyltrimethylammonium bromide R,
- as detector a spectrophotometer set at 220 nm,
- a loop injector.

Equilibrate the chromatographic system by passage of the mobile phase for 16 h (the mobile phase may be recirculated after 6 h).

Inject 20 µl of each solution and record the chromatograms for twice the retention time of the principal peak. The test is not valid unless:

- the chromatogram obtained with reference solution (a) shows a peak with a signal-to-noise ratio of at least 5,
- the chromatogram obtained with reference solution (b) shows 2 peaks with a resolution of not less than 2.0.

In the chromatogram obtained with the test solution, the area of any peak, apart from the principal peak, is not greater than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent).

Heavy metals (2.4.8). 12 ml of solution S complies with limit test A (20 ppm). Prepare the standard using lead standard solution (1 ppm Pb) R.

Loss on drying (2.2.32). Not more than 1.0 per cent, determined on 1.000 g by drying in an oven at 100–105 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

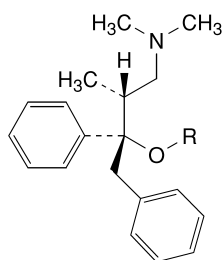
Dissolve 0.270 g in 60 ml of acetic anhydride R. Titrate with 0.1 M perchloric acid, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M perchloric acid is equivalent to 37.59 mg of C₂₂H₃₀ClNO₂.

STORAGE

Protected from light.

IMPURITIES

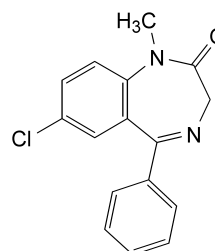


- A. R = H: (2S,3R)-4-(dimethylamino)-1,2-diphenyl-3-methylbutan-2-ol,
- B. R = CO-CH₃: (1S,2R)-1-benzyl-3-(dimethylamino)-2-methyl-1-phenylpropyl acetate.

01/2005:0022

DIAZEPAM

Diazepamum

C₁₆H₁₃ClN₂OM_r 284.7

DEFINITION

Diazepam contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of 7-chloro-1-methyl-5-phenyl-1,3-dihydro-2H-1,4-benzodiazepin-2-one, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, crystalline powder, very slightly soluble in water, soluble in alcohol.

IDENTIFICATION

- A. Melting point (2.2.14): 131 °C to 135 °C.
- B. *Protect the solutions from bright light and measure the absorbances immediately.* Dissolve 25 mg in a 5 g/l solution of *sulphuric acid R* in *methanol R* and dilute to 250.0 ml with the same acid solution (solution A). Dilute 5.0 ml of solution A to 100.0 ml with a 5 g/l solution of *sulphuric acid R* in *methanol R*. Examined between 230 nm and 330 nm (2.2.25), the solution shows 2 absorption maxima, at 242 nm and 285 nm. The specific absorbance at the maximum at 242 nm is about 1020. Dilute 25.0 ml of solution A to 100.0 ml with a 5 g/l solution of *sulphuric acid R* in *methanol R*. Examined between 325 nm and 400 nm, the solution shows a single absorption maximum at 366 nm. The specific absorbance at the maximum is 140 to 155.
- C. Dissolve about 10 mg in 3 ml of *sulphuric acid R*. The solution shows greenish-yellow fluorescence in ultraviolet light at 365 nm.
- D. To 80 mg in a porcelain crucible add 0.3 g of *anhydrous sodium carbonate R*. Heat over an open flame for 10 min. Allow to cool. Take up the residue with 5 ml of *dilute nitric acid R* and filter. To 1 ml of the filtrate add 1 ml of *water R*. The solution gives reaction (a) of chlorides (2.3.1).

TESTS

Related substances and decomposition products. Carry out the test protected from bright light. Examine by thin-layer chromatography (2.2.27), using *silica gel GF₂₅₄ R* as the coating substance.

Test solution. Dissolve 1.0 g of the substance to be examined in *acetone R* and dilute to 10 ml with the same solvent. Prepare immediately before use.

Reference solution. Dilute 1 ml of the test solution to 100 ml with *acetone R*. Dilute 1 ml of this solution to 10 ml with *acetone R*.

Apply separately to the plate 5 µl of each solution. Develop over a path of 12 cm using a mixture of equal volumes of *ethyl acetate R* and *hexane R*. Allow the plate to dry and examine in ultraviolet light at 254 nm. Any spot in the chromatogram obtained with the test solution, apart from the principal spot, is not more intense than the spot in the chromatogram obtained with the reference solution (0.1 per cent).

Heavy metals (2.4.8). 2.0 g complies with limit test C for heavy metals (20 ppm). Prepare the standard using 4 ml of *lead standard solution* (10 ppm Pb) *R*.

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying at 60 °C *in vacuo* for 4 h.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.500 g in 50 ml of *acetic anhydride R*. Using 0.3 ml of *Nile blue A solution R* as indicator, titrate with 0.1 M *perchloric acid* until a yellowish-green colour is obtained.

1 ml of 0.1 M *perchloric acid* is equivalent to 28.47 mg of C₁₆H₁₃ClN₂O.

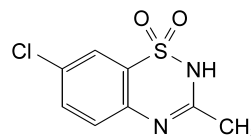
STORAGE

Store protected from light.

01/2005:0550

DIAZOXIDE

Diazoxidum

C₈H₇ClN₂O₂SM_r 230.7

DEFINITION

Diazoxide contains not less than 98.0 per cent and not more than the equivalent of 101.0 per cent of 7-chloro-3-methyl-2H-1,2,4-benzothiadiazine 1,1-dioxide, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, fine or crystalline powder, practically insoluble in water, freely soluble in dimethylformamide, slightly soluble in alcohol. It is very soluble in dilute solutions of the alkali hydroxides.

IDENTIFICATION

First identification: B.

Second identification: A, C, D.

- A. Dissolve 50.0 mg in 5 ml of 1 M *sodium hydroxide* and dilute to 50.0 ml with *water R*. Dilute 1.0 ml of this solution to 100.0 ml with 0.1 M *sodium hydroxide*. Examined between 230 nm and 350 nm (2.2.25), the solution shows an absorption maximum at 280 nm and a shoulder at 304 nm. The specific absorbance at the maximum is 570 to 610.
- B. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *diazoxide CRS*. Examine the substances prepared as discs using *potassium bromide R*.
- C. Examine the chromatograms obtained in the test for related substances in ultraviolet light at 254 nm. The principal spot in the chromatogram obtained with test solution (b) is similar in position and size to the principal spot in the chromatogram obtained with reference solution (b).
- D. Dissolve about 20 mg in a mixture of 5 ml of *hydrochloric acid R* and 10 ml of *water R*. Add 0.1 g of *zinc powder R*. Boil for 5 min, cool and filter. To the filtrate add 2 ml of a 1 g/l solution of *sodium nitrite R* and mix. Allow to stand for 1 min and add 1 ml of a 5 g/l solution of *naphthylethylenediamine dihydrochloride R*. A red or violet-red colour develops.

TESTS

Appearance of solution. Dissolve 0.4 g in 2 ml of 1 M *sodium hydroxide* and dilute to 20 ml with *water R*. The solution is clear (2.2.1) and not more intensely coloured than reference solution Y₇ (2.2.2, *Method II*).

Acidity or alkalinity. To 0.5 g of the powdered substance to be examined add 30 ml of *carbon dioxide-free water R*, shake for 2 min and filter. To 10 ml of the filtrate add 0.2 ml of 0.01 M *sodium hydroxide* and 0.15 ml of *methyl red*

solution R. The solution is yellow. Not more than 0.4 ml of 0.01 M hydrochloric acid is required to change the colour of the indicator to red.

Related substances. Examine by thin-layer chromatography (2.2.27), using silica gel GF₂₅₄ R as the coating substance.

Test solution (a). Dissolve 0.1 g of the substance to be examined in a mixture of 0.5 ml of 1 M sodium hydroxide and 1 ml of methanol R and dilute to 5 ml with methanol R.

Test solution (b). Dilute 1 ml of test solution (a) to 5 ml with a mixture of 1 volume of 1 M sodium hydroxide and 9 volumes of methanol R.

Reference solution (a). Dilute 0.5 ml of test solution (a) to 100 ml with a mixture of 1 volume of 1 M sodium hydroxide and 9 volumes of methanol R.

Reference solution (b). Dissolve 20 mg of diazoxide CRS in a mixture of 0.5 ml of 1 M sodium hydroxide and 1 ml of methanol R and dilute to 5 ml with methanol R.

Apply separately to the plate 5 µl of each solution. Develop over a path of 15 cm using a mixture of 7 volumes of concentrated ammonia R, 25 volumes of methanol R and 68 volumes of chloroform R. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. Any spot in the chromatogram obtained with test solution (a), apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (a) (0.5 per cent).

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C for 2 h.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

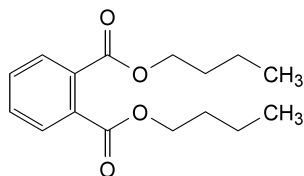
Dissolve 0.200 g with gentle heating in 50 ml of a mixture of 1 volume of water R and 2 volumes of dimethylformamide R. Titrate with 0.1 M sodium hydroxide, determining the end-point potentiometrically (2.2.20). Carry out a blank titration.

1 ml of 0.1 M sodium hydroxide is equivalent to 23.07 mg of C₈H₇ClN₂O₂S.

01/2005:0762

DIBUTYL PHTHALATE

Dibutylis phthalas



C₁₆H₂₂O₄

M_r 278.3

DEFINITION

Dibutyl phthalate contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent m/m of dibutyl benzene-1,2-dicarboxylate.

CHARACTERS

A clear, oily liquid, colourless or very slightly yellow, practically insoluble in water, miscible with alcohol.

IDENTIFICATION

First identification: B, C.

Second identification: A, D, E.

A. Relative density (2.2.5): 1.043 to 1.048.

B. Refractive index (2.2.6): 1.490 to 1.495.

C. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with dibutyl phthalate CRS.

D. Examine by thin-layer chromatography (2.2.27), using silica gel GF₂₅₄ R as the coating substance.

Test solution. Dissolve 50 mg of the substance to be examined in ether R and dilute to 10 ml with the same solvent.

Reference solution. Dissolve 50 mg of dibutyl phthalate CRS in ether R and dilute to 10 ml with the same solvent.

Apply separately to the plate 10 µl of each solution. Develop over a path of 15 cm using a mixture of 30 volumes of heptane R and 70 volumes of ether R. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. The principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with the reference solution.

E. To about 0.1 ml add 0.25 ml of sulphuric acid R and 50 mg of resorcinol R. Heat in a water-bath for 5 min. Allow to cool. Add 10 ml of water R and 1 ml of strong sodium hydroxide solution R. The solution becomes yellow or brownish-yellow and shows a green fluorescence.

TESTS

Appearance. The substance to be examined is clear (2.2.1) and not more intensely coloured than reference solution Y₆ (2.2.2, Method II).

Acidity. Dissolve 20.0 g in 50 ml of alcohol R previously neutralised to phenolphthalein solution R1. Add 0.2 ml of phenolphthalein solution R1. Not more than 0.50 ml of 0.1 M sodium hydroxide is required to change the colour of the indicator.

Related substances. Examine by gas chromatography (2.2.28), using bibenzyl R as internal standard.

Internal standard solution. Dissolve 60 mg of bibenzyl R in methylene chloride R and dilute to 20 ml with the same solvent.

Test solution (a). Dissolve 1.0 g of the substance to be examined in methylene chloride R and dilute to 20.0 ml with the same solvent.

Test solution (b). Dissolve 1.0 g of the substance to be examined in methylene chloride R, add 2.0 ml of the internal standard solution and dilute to 20.0 ml with methylene chloride R.

Reference solution. To 1.0 ml of test solution (a) add 10.0 ml of the internal standard solution and dilute to 100.0 ml with methylene chloride R.

The chromatographic procedure may be carried out using:

- a glass column 1.5 m long and 4 mm in internal diameter, packed with silanised diatomaceous earth for gas chromatography R (150 µm to 180 µm) impregnated with 3 per cent m/m of polymethylphenylsiloxane R,
- nitrogen for chromatography R as the carrier gas at a flow rate of 30 ml per minute,
- a flame-ionisation detector,

maintaining the temperature of the column at 190 °C and that of the injection port and of the detector at 225 °C.

Inject 1 µl of the reference solution. The substances elute in the following order: bibenzyl and dibutyl phthalate. Adjust the sensitivity of the detector so that the height of the bibenzyl peak is not less than 70 per cent of the full scale of the recorder. The test is not valid unless the resolution between the peaks corresponding to bibenzyl and dibutyl phthalate is at least 12.

Inject 1 µl of test solution (a). In the chromatogram obtained, verify that there is no peak with the same retention time as the internal standard.

Inject separately 1 µl of test solution (b) and the reference solution. Continue the chromatography for 3 times the retention time of dibutyl phthalate, which is about 12 min. From the chromatogram obtained with the reference solution, calculate the ratio (*R*) of the area of the peak due to dibutyl phthalate to the area of the peak due to the internal standard. From the chromatogram obtained with test solution (b), calculate the ratio of the sum of the areas of any peaks, apart from the principal peak, the peak due to the internal standard and the peak due to the solvent, to the area of the peak due to the internal standard: this ratio is not greater than *R* (1.0 per cent).

Water (2.5.12). Not more than 0.2 per cent, determined on 10.00 g by the semi-micro determination of water.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Introduce 0.750 g into a 250 ml borosilicate glass flask. Add 25.0 ml of 0.5 *M* alcoholic potassium hydroxide and a few glass beads. Heat in a water-bath under a reflux condenser for 1 h. Add 1 ml of phenolphthalein solution R1 and titrate immediately with 0.5 *M* hydrochloric acid until the colour changes from red to colourless. Carry out a blank titration. Calculate the volume of potassium hydroxide used in the saponification.

1 ml of 0.5 *M* alcoholic potassium hydroxide is equivalent to 69.59 mg of C₁₆H₂₂O₄.

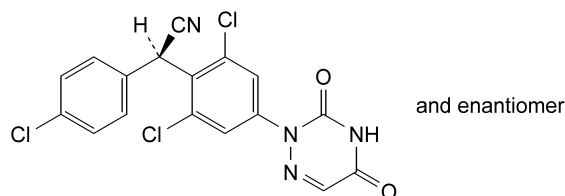
STORAGE

Store in an airtight container.

01/2005:1718

DICLAZURIL FOR VETERINARY USE

Diclazurilum ad usum veterinarium



C₁₇H₉Cl₃N₄O₂

M_r 407.6

DEFINITION

(*RS*)-(4-Chlorophenyl)[2,6-dichloro-4-(3,5-dioxo-4,5-dihydro-1,2,4-triazin-2(3*H*)-yl)phenyl]acetone nitrile.

Content: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

Appearance: white or light yellow powder.

Solubility: practically insoluble in water, sparingly soluble in dimethylformamide, practically insoluble in alcohol and methylene chloride.

IDENTIFICATION

Infrared absorption spectrophotometry (2.2.24).

Comparison: *Ph. Eur. reference spectrum of diclazuril.*

TESTS

Related substances. Liquid chromatography (2.2.29).

Test solution. Dissolve 20.0 mg of the substance to be examined in dimethylformamide *R* and dilute to 20.0 ml with the same solvent.

Reference solution (a). Dissolve 5 mg of diclazuril for system suitability CRS in dimethylformamide *R* and dilute to 5.0 ml with the same solvent.

Reference solution (b). Dilute 1.0 ml of the test solution to 100.0 ml with dimethylformamide *R*. Dilute 5.0 ml of the solution to 20.0 ml with dimethylformamide *R*.

Column:

- **size:** *l* = 0.10 m, Ø = 4.6 mm,
- **stationary phase:** base-deactivated octadecylsilyl silica gel for chromatography *R* (3 µm),
- **temperature:** 35 °C.

Mobile phase:

- **mobile phase A:** mix 10 volumes of a 6.3 g/l solution of ammonium formate *R* adjusted to pH 4.0 with anhydrous formic acid *R*, 15 volumes of acetonitrile *R* and 75 volumes of water *R*,
- **mobile phase B:** mix 10 volumes of a 6.3 g/l solution of ammonium formate *R* adjusted to pH 4.0 with anhydrous formic acid *R*, 85 volumes of acetonitrile *R* and 5 volumes of water *R*,

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)
0 - 20	100 → 0	0 → 100
20 - 25	0	100
25 - 26	0 → 100	100 → 0
26 - 36	100	0

Flow rate: 1.0 ml/min.

Detection: spectrophotometer at 230 nm.

Injection: 5 µl.

System suitability: reference solution (a):

- **peak-to-valley ratio:** minimum of 1.5, where *H_p* = height above the baseline of the peak due to impurity D and *H_v* = height above the baseline of the lowest point of the curve separating this peak from the peak due to diclazuril.

Limits:

- **correction factors:** for the calculation of contents, multiply the peak areas of the following impurities by the corresponding correction factor: impurity D = 1.9; impurity H = 1.4,
- **impurity D:** not more than 0.4 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent),
- **any other impurity:** not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.25 per cent),
- **total:** not more than 4 times the area of the principal peak in the chromatogram obtained with reference solution (b) (1.0 per cent),
- **disregard limit:** 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

Loss on drying (2.2.32): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 100-105 °C for 4 h.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.150 g in 75 ml of *dimethylformamide R*. Carry out a potentiometric titration (2.2.20), using 0.1 M *tetrabutylammonium hydroxide*. Read the volume added at the second inflexion point. Carry out a blank titration.

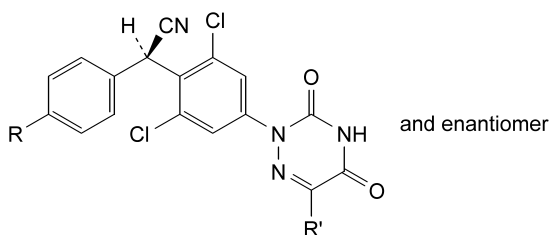
1 ml of 0.1 M *tetrabutylammonium hydroxide* is equivalent to 20.38 mg of $C_{17}H_{13}Cl_2N_4O_2$.

STORAGE

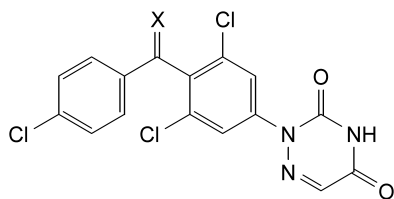
Protected from light.

IMPURITIES

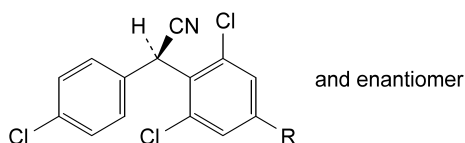
Specified impurities: A, B, C, D, E, F, G, H, I.



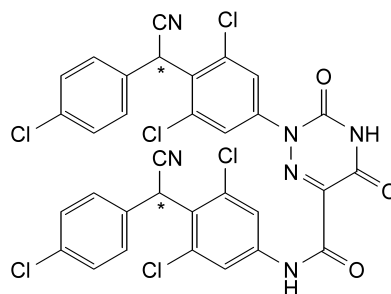
- A. R = Cl, R' = CO₂H: 2-[3,5-dichloro-4-[(*RS*)-(4-chlorophenyl)cyanomethyl]phenyl]-3,5-dioxo-2,3,4,5-tetrahydro-1,2,4-triazine-6-carboxylic acid,
- B. R = OH, R' = H: (*RS*)-[2,6-dichloro-4-(3,5-dioxo-4,5-dihydro-1,2,4-triazin-2(3*H*)-yl)phenyl](4-hydroxyphenyl)acetoneitrile,
- C. R = Cl, R' = CONH₂: 2-[3,5-dichloro-4-[(*RS*)-(4-chlorophenyl)cyanomethyl]phenyl]-3,5-dioxo-2,3,4,5-tetrahydro-1,2,4-triazine-6-carboxamide,
- G. R = Cl, R' = CO-O-[CH₂]₃-CH₃: butyl 2-[3,5-dichloro-4-[(*RS*)-(4-chlorophenyl)cyanomethyl]phenyl]-3,5-dioxo-2,3,4,5-tetrahydro-1,2,4-triazine-6-carboxylate,



- D. X = O: 2-[3,5-dichloro-4-(4-chlorobenzoyl)phenyl]-1,2,4-triazine-3,5-(2*H*,4*H*)-dione,
- F. X = H₂: 2-[3,5-dichloro-4-(4-chlorobenzyl)phenyl]-1,2,4-triazine-3,5-(2*H*,4*H*)-dione,



- E. R = NH₂: (*RS*)-(4-amino-2,6-dichlorophenyl)-(4-chlorophenyl)acetoneitrile,
- H. R = H: (*RS*)-(4-chlorophenyl)-(2,6-dichlorophenyl)acetoneitrile,

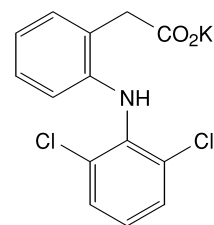


- I. *N*,2-bis[3,5-dichloro-4-[(4-chlorophenyl)cyanomethyl]phenyl]-3,5-dioxo-2,3,4,5-tetrahydro-1,2,4-triazine-6-carboxamide.

01/2005:1508

DICLOFENAC POTASSIUM

Diclofenacum kalicum



$C_{14}H_{10}Cl_2KNO_2$

M_r 334.2

DEFINITION

Potassium diclofenac contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of potassium [2-[(2,6-dichlorophenyl)amino]phenyl]acetate, calculated with reference to the dried substance.

CHARACTERS

A white or slightly yellowish, crystalline powder, slightly hygroscopic, sparingly soluble in water, freely soluble in methanol, soluble in alcohol, slightly soluble in acetone.

IDENTIFICATION

First identification: A, D.

Second identification: B, C, D.

- A. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *diclofenac potassium CRS*. Examine the substances prepared as discs.
- B. Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel GF₂₅₄ plate R*.

Test solution. Dissolve 25 mg of the substance to be examined in *methanol R* and dilute to 5 ml with the same solvent.

Reference solution (a). Dissolve 25 mg of *diclofenac potassium CRS* in *methanol R* and dilute to 5 ml with the same solvent.

Reference solution (b). Dissolve 10 mg of *indometacin R* in reference solution (a) and dilute to 2 ml with the same solution.

Apply to the plate 5 µl of each solution. Develop over a path of 10 cm using a mixture of 10 volumes of *concentrated ammonia R*, 10 volumes of *methanol R* and 80 volumes of *ethyl acetate R*. Allow the plate to dry in air. Examine in ultraviolet light at 254 nm. The principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the

chromatogram obtained with reference solution (a). The test is not valid unless the chromatogram obtained with reference solution (b) shows 2 clearly separated spots.

- C. Dissolve about 10 mg in 10 ml of *alcohol R*. To 1 ml of the solution add 0.2 ml of a mixture, prepared immediately before use, of equal volumes of a 6 g/l solution of *potassium ferricyanide R* and a 9 g/l solution of *ferric chloride R*. Allow to stand protected from light for 5 min. Add 3 ml of a 10 g/l solution of *hydrochloric acid R*. Allow to stand protected from light for 15 min. A blue colour develops and a precipitate is formed.
- D. Suspend 0.5 g of the substance to be examined in 10 ml of *water R*. Stir, add *water R* until the substance is dissolved. Add 2 ml of *hydrochloric acid R1*, stir for 1 h and filter with the aid of vacuum. Neutralise the solution with *sodium hydroxide solution R*. The solution gives reaction (b) of potassium (2.3.1).

TESTS

Appearance of solution. Dissolve 1.25 g in *methanol R* and dilute to 25.0 ml with the same solvent. The solution is clear (2.2.1). The absorbance (2.2.25) measured at 440 nm is not greater than 0.05.

Related substances. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 50.0 mg of the substance to be examined in *methanol R* and dilute to 50.0 ml with the same solvent.

Reference solution (a). Dilute 2.0 ml of the test solution to 100.0 ml with *methanol R*. Dilute 1.0 ml of the solution to 10.0 ml with *methanol R*.

Reference solution (b). Dissolve 1.0 mg of *diclofenac impurity A CRS* in *methanol R*, add 1.0 ml of the test solution and dilute to 200.0 ml with *methanol R*.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4.6 mm in internal diameter packed with *end-capped octylsilyl silica gel for chromatography R* (5 µm),
- as mobile phase at a flow rate of 1 ml/min a mixture of 34 volumes of a solution containing 0.5 g/l of *phosphoric acid R* and 0.8 g/l of *sodium dihydrogen phosphate R* adjusted to pH 2.5 with *phosphoric acid R*, and 66 volumes of *methanol R*,
- as detector a spectrophotometer set at 254 nm.

Inject 20 µl of reference solution (b). When the chromatograms are recorded in the prescribed conditions, the retention times are: diclofenac, about 25 min and impurity A, about 12 min. Adjust the sensitivity of the system so that the height of the peaks in the chromatogram obtained is at least 50 per cent of the full scale of the recorder. The test is not valid unless in the chromatogram obtained the resolution between the peaks corresponding to diclofenac and impurity A is not less than 6.5.

Inject 20 µl of the test solution and 20 µl of reference solution (a). Continue the chromatography for 1.5 times the retention time of the principal peak in the chromatogram obtained with the test solution. In the chromatogram obtained with the test solution: the area of any peak, apart from the principal peak, is not greater than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent); the sum of the areas of all the peaks, apart from the principal peak, is not greater than 2.5 times that of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent). Disregard any peak with an area less than 0.25 times the area of the principal peak in the chromatogram obtained with reference solution (a).

Heavy metals (2.4.8). 2.0 g complies with limit test C (10 ppm). Use a quartz crucible. Prepare the standard using 2 ml of *lead standard solution* (10 ppm Pb) *R*.

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 100–105 °C for 3 h.

ASSAY

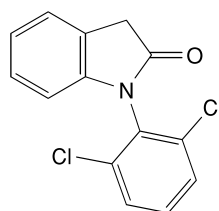
Dissolve 0.250 g in 30 ml of *anhydrous acetic acid R*. Titrate with 0.1 M *perchloric acid*, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *perchloric acid* is equivalent to 33.42 mg of C₁₄H₁₀Cl₂KNO₂.

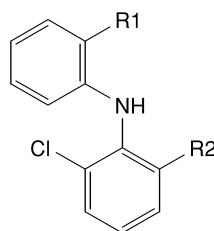
STORAGE

In an airtight container, protected from light.

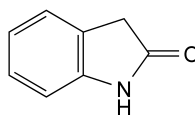
IMPURITIES



- A. 1-(2,6-dichlorophenyl)-1,3-dihydro-2H-indol-2-one,



- B. R1 = CHO, R2 = Cl: 2-[(2,6-dichlorophenyl)amino]benzaldehyde,
- C. R1 = CH₂OH, R2 = Cl: [2-[(2,6-dichlorophenyl)amino]phenyl]methanol,
- D. R1 = CH₂-CO₂H, R2 = Br: 2-[2-[(2-bromo-6-chlorophenyl)amino]phenyl]acetic acid,

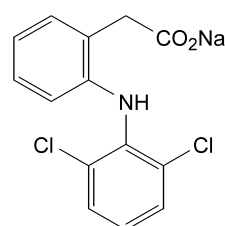


- E. 1,3-dihydro-2H-indol-2-one.

01/2005:1002

DICLOFENAC SODIUM

Diclofenacum natricum



C₁₄H₁₀Cl₂NNaO₂

M_r 318.1

DEFINITION

Diclofenac sodium contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of sodium 2-[(2,6-dichlorophenyl)amino]phenyl]acetate, calculated with reference to the dried substance.

CHARACTERS

A white or slightly yellowish, crystalline powder, slightly hygroscopic, sparingly soluble in water, freely soluble in methanol, soluble in alcohol, slightly soluble in acetone.

It melts at about 280 °C, with decomposition.

IDENTIFICATION

First identification: A, D.

Second identification: B, C, D.

A. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *diclofenac sodium CRS*. Examine the substances prepared as discs.

B. Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel GF₂₅₄ plate R*.

Test solution. Dissolve 25 mg of the substance to be examined in *methanol R* and dilute to 5 ml with the same solvent.

Reference solution (a). Dissolve 25 mg of *diclofenac sodium CRS* in *methanol R* and dilute to 5 ml with the same solvent.

Reference solution (b). Dissolve 10 mg of *indometacin R* in reference solution (a) and dilute to 2 ml with the same solution.

Apply to the plate 5 µl of each solution. Develop over a path of 10 cm using a mixture of 10 volumes of *concentrated ammonia R*, 10 volumes of *methanol R* and 80 volumes of *ethyl acetate R*. Allow the plate to dry in air. Examine in ultraviolet light at 254 nm. The principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a). The test is not valid unless the chromatogram obtained with reference solution (b) shows 2 clearly separated spots.

C. Dissolve about 10 mg in 10 ml of *alcohol R*. To 1 ml of the solution add 0.2 ml of a mixture, prepared immediately before use, of equal volumes of a 6 g/l solution of *potassium ferricyanide R* and a 9 g/l solution of *ferric chloride R*. Allow to stand protected from light for 5 min. Add 3 ml of a 10 g/l solution of *hydrochloric acid R*. Allow to stand, protected from light, for 15 min. A blue colour develops and a precipitate is formed.

D. Dissolve 60 mg in 0.5 ml of *methanol R* and add 0.5 ml of *water R*. The solution gives reaction (b) of sodium (2.3.1).

TESTS

Appearance of solution. Dissolve 1.25 g in *methanol R* and dilute to 25.0 ml with the same solvent. The solution is clear (2.2.1). The absorbance (2.2.25) measured at 440 nm is not greater than 0.05.

Related substances. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 50.0 mg of the substance to be examined in *methanol R* and dilute to 50.0 ml with the same solvent.

Reference solution (a). Dilute 2.0 ml of the test solution to 100.0 ml with *methanol R*. Dilute 1.0 ml of the solution to 10.0 ml with *methanol R*.

Reference solution (b). Dissolve 1.0 mg of *diclofenac impurity A CRS* in *methanol R*, add 1.0 ml of the test solution and dilute to 200.0 ml with *methanol R*.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4.6 mm in internal diameter packed with *end-capped octylsilyl silica gel for chromatography R* (5 µm),
- as mobile phase at a flow rate of 1 ml/min a mixture of 34 volumes of a solution containing 0.5 g/l of *phosphoric acid R* and 0.8 g/l of *sodium dihydrogen phosphate R* adjusted to pH 2.5 with *phosphoric acid R*, and 66 volumes of *methanol R*,
- as detector a spectrophotometer set at 254 nm.

Inject 20 µl of reference solution (b). When the chromatograms are recorded in the prescribed conditions, the retention times are about 25 min for diclofenac and about 12 min for impurity A. Adjust the sensitivity of the system so that the height of the peaks in the chromatogram obtained with reference solution (b) is not less than 50 per cent of the full scale of the recorder. Continue the chromatography for 1.5 times the retention time of diclofenac. The test is not valid unless in the chromatogram obtained the resolution between the peaks corresponding to diclofenac and impurity A is at least 6.5.

Inject 20 µl of the test solution and 20 µl of reference solution (a). In the chromatogram obtained with the test solution: the area of any peak, apart from the principal peak, is not greater than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent); the sum of the areas of all the peaks apart from the principal peak is not greater than 2.5 times that of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent). Disregard any peak with an area less than 0.25 times the area of the principal peak in the chromatogram obtained with reference solution (a).

Heavy metals (2.4.8). 2.0 g complies with limit test C (10 ppm). Use a quartz crucible. Prepare the standard using 2 ml of *lead standard solution (10 ppm Pb) R*.

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 100-105 °C for 3 h.

ASSAY

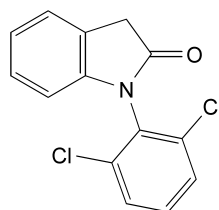
Dissolve 0.250 g in 30 ml of *anhydrous acetic acid R*. Titrate with 0.1 M *perchloric acid*, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *perchloric acid* is equivalent to 31.81 mg of C₁₄H₁₀Cl₂NNaO₂.

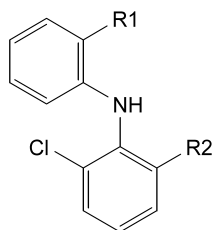
STORAGE

In an airtight container, protected from light.

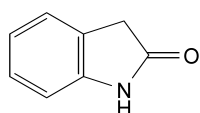
IMPURITIES



A. 1-(2,6-dichlorophenyl)-1,3-dihydro-2H-indol-2-one,



- B. R1 = CHO, R2 = Cl: 2-[(2,6-dichlorophenyl)amino]benzaldehyde,
 C. R1 = CH₂OH, R2 = Cl: [2-[(2,6-dichlorophenyl)amino]phenyl]methanol,
 D. R1 = CH₂-CO₂H, R2 = Br: 2-[2-[(2-bromo-6-chlorophenyl)amino]phenyl]acetic acid,

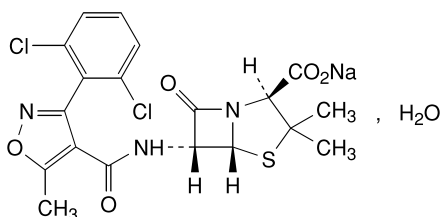


- E. 1,3-dihydro-2H-indol-2-one.

01/2005:0663

DICLOXACILLIN SODIUM

Dicloxacillinum natricum

C₁₉H₁₆Cl₂N₃NaO₅S·H₂OM_r 510.3

DEFINITION

Dicloxacillin sodium contains not less than 95.0 per cent and not more than the equivalent of 101.0 per cent of sodium (2S,5R,6R)-6-[[[3-(2,6-dichlorophenyl)-5-methylisoxazol-4-yl]carbonyl]amino]-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylate, calculated with reference to the anhydrous substance.

CHARACTERS

A white or almost white, crystalline powder, hygroscopic, freely soluble in water, soluble in alcohol and in methanol.

IDENTIFICATION

First identification: A, D.

Second identification: B, C, D.

- A. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *dicloxacillin sodium CRS*. Examine the substances prepared as discs.
 B. Examine by thin-layer chromatography (2.2.27), using *silanised silica gel H R* as the coating substance.
Test solution. Dissolve 25 mg of the substance to be examined in 5 ml of *water R*.
Reference solution (a). Dissolve 25 mg of *dicloxacillin sodium CRS* in 5 ml of *water R*.
Reference solution (b). Dissolve 25 mg each of *cloxacillin sodium CRS*, *dicloxacillin sodium CRS* and *flucloxacillin sodium CRS* in 5 ml of *water R*.

Apply to the plate 1 µl of each solution. Develop over a path of 15 cm using a mixture of 30 volumes of *acetone R* and 70 volumes of a 154 g/l solution of *ammonium acetate R* the pH of which has been adjusted to 5.0 with *glacial acetic acid R*. Allow the plate to dry in air and expose it to iodine vapour until the spots appear. Examine in daylight. The principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a). The test is not valid unless the chromatogram obtained with reference solution (b) shows three clearly separated spots.

- C. Place about 2 mg in a test-tube about 150 mm long and 15 mm in diameter. Moisten with 0.05 ml of *water R* and add 2 ml of *sulphuric acid-formaldehyde reagent R*. Mix the contents of the tube by swirling; the colour of the solution is slightly greenish-yellow. Place the test-tube in a water-bath for 1 min; a yellow colour develops.
 D. It gives reaction (a) of sodium (2.3.1).

TESTS

Solution S. Dissolve 2.50 g in *carbon dioxide-free water R* and dilute to 25.0 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1). The absorbance (2.2.25) of solution S measured at 430 nm is not greater than 0.04.

pH (2.2.3). The pH of solution S is 5.0 to 7.0.

Specific optical rotation (2.2.7). Dissolve 0.250 g in *water R* and dilute to 25.0 ml with the same solvent. The specific optical rotation is + 128 to + 143, calculated with reference to the anhydrous substance.

Related substances. Examine by liquid chromatography (2.2.29) as prescribed under Assay. Inject reference solution (b). Adjust the sensitivity of the system so that the height of the principal peak in the chromatogram obtained is at least 50 per cent of the full scale of the recorder. Inject test solution (a) and continue the chromatography for five times the retention time of the principal peak. In the chromatogram obtained with test solution (a): the area of any peak, apart from the principal peak, is not greater than the area of the principal peak in the chromatogram obtained with reference solution (b) (1 per cent); the sum of the areas of all peaks, apart from the principal peak, is not greater than five times the area of the principal peak in the chromatogram obtained with reference solution (b) (5 per cent). Disregard any peak with an area less than 0.05 times the area of the principal peak in the chromatogram obtained with reference solution (b).

N,N-Dimethylaniline (2.4.26, *Method B*). Not more than 20 ppm.

2-Ethylhexanoic acid (2.4.28). Not more than 0.8 per cent *m/m*.

Water (2.5.12): 3.0 per cent to 4.5 per cent, determined on 0.300 g by the semi-micro determination of water.

Pyrogens (2.6.8). If intended for use in the manufacture of parenteral dosage forms without a further appropriate procedure for the removal of pyrogens, it complies with the test for pyrogens. Inject per kilogram of the rabbit's mass 1 ml of a solution in *water for injections R* containing 20 mg of the substance to be examined per millilitre.

ASSAY

Examine by liquid chromatography (2.2.29).

Test solution (a). Dissolve 50.0 mg of the substance to be examined in the mobile phase and dilute to 50.0 ml with the mobile phase.

Test solution (b). Dilute 5.0 ml of test solution (a) to 50.0 ml with the mobile phase.

Reference solution (a). Dissolve 50.0 mg of *dicloxacillin sodium CRS* in the mobile phase and dilute to 50.0 ml with the mobile phase. Dilute 5.0 ml of the solution to 50.0 ml with the mobile phase.

Reference solution (b). Dilute 5.0 ml of test solution (b) to 50.0 ml with the mobile phase.

Reference solution (c). Dissolve 5 mg of *flucloxacillin sodium CRS* and 5 mg of *dicloxacillin sodium CRS* in the mobile phase and dilute to 50.0 ml with the mobile phase.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (5 µm),
- as mobile phase at a flow rate of 1.0 ml/min a mixture of 75 volumes of a 2.7 g/l solution of *potassium dihydrogen phosphate R* adjusted to pH 5.0 with *dilute sodium hydroxide solution R* and 25 volumes of *acetonitrile R*,
- as detector a spectrophotometer set at 225 nm,
- a 20 µl loop injector.

Inject reference solution (c). When the chromatograms are recorded in the prescribed conditions, the retention time of dicloxacillin is about 10 min. Adjust the sensitivity of the system so that the heights of the principal peaks in the chromatogram obtained are at least 50 per cent of the full scale of the recorder. The test is not valid unless in the chromatogram obtained, the resolution between the first peak (flucloxacillin) and the second peak (dicloxacillin) is at least 2.5. Inject reference solution (a) six times. The test is not valid unless the relative standard deviation of the peak area for dicloxacillin is at most 1.0 per cent. Inject alternately test solution (b) and reference solution (a).

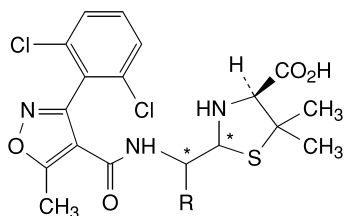
STORAGE

Store in an airtight container, at a temperature not exceeding 25 °C. If the substance is sterile, store in a sterile, airtight, tamper-proof container.

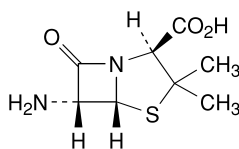
LABELLING

The label states, where applicable, that the substance is apyrogenic.

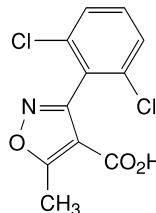
IMPURITIES



- A. R = CO₂H: (4S)-2-[carboxy[[[3-(2,6-dichlorophenyl)-5-methylisoxazol-4-yl]carbonyl]amino]methyl]-5,5-dimethylthiazolidine-4-carboxylic acid (penicilloic acids of dicloxacillin),
- B. R = H: (2RS,4S)-2-[[[3-(2,6-dichlorophenyl)-5-methylisoxazol-4-yl]carbonyl]amino]methyl]-5,5-dimethylthiazolidine-4-carboxylic acid (penilloic acids of dicloxacillin),



- C. (2S,5R,6R)-6-amino-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid (6-aminopenicillanic acid),

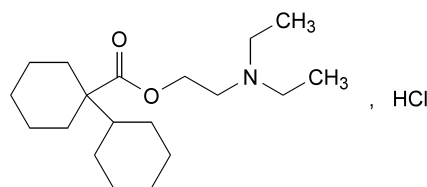


- D. 3-(2,6-dichlorophenyl)-5-methylisoxazole-4-carboxylic acid.

01/2005:1197

DICYCLOVERINE HYDROCHLORIDE

Dicycloverini hydrochloridum

C₁₉H₃₆ClNO₂M_r 346.0

DEFINITION

Dicycloverine hydrochloride contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of 2-(diethylamino)ethyl bicyclohexyl-1-carboxylate hydrochloride, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, crystalline powder, soluble in water, freely soluble in alcohol and in methylene chloride.

It shows polymorphism.

IDENTIFICATION

First identification: A, D.

Second identification: B, C, D.

- A. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *dicycloverine hydrochloride CRS*. Examine the substances prepared as discs using *potassium chloride R*. If the spectra obtained show differences, dissolve the substance to be examined and the reference substance separately in *acetone R*, evaporate to dryness and record new spectra using the residues.
- B. Examine the chromatograms obtained in the test for related substances. The principal spot in the chromatogram obtained with test solution (b) is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (b).
- C. To 3 ml of a 1.0 g/l solution of *sodium laurilsulfate R* add 5 ml of *methylene chloride R* and 0.05 ml of a 2.5 g/l solution of *methylene blue R*, mix gently and allow to stand; the lower layer is blue. Add 2 ml of a 20 g/l

solution of the substance to be examined, mix gently and allow to stand; the upper layer is blue and the lower layer is colourless.

D. It gives reaction (a) of chlorides (2.3.1).

TESTS

pH (2.2.3). Dissolve 0.5 g in *water R* and dilute to 50 ml with the same solvent. The pH of the solution is 5.0 to 5.5.

Related substances. Examine by thin-layer chromatography (2.2.27), using a suitable silica gel as the coating substance.

Test solution (a). Dissolve 0.25 g of the substance to be examined in *methanol R* and dilute to 5 ml with the same solvent.

Test solution (b). Dilute 1 ml of test solution (a) to 50 ml with *methanol R*.

Reference solution (a). Dilute 1 ml of test solution (b) to 10 ml with *methanol R*.

Reference solution (b). Dissolve 10 mg of *dicycloverine hydrochloride CRS* in *methanol R* and dilute to 10 ml with the same solvent.

Reference solution (c). Dissolve 5 mg of *tropicamide CRS* in reference solution (b) and dilute to 5 ml with the same solution.

Apply separately to the plate 10 µl of each solution. Develop over a path of 15 cm using a mixture of 5 volumes of *concentrated ammonia R*, 10 volumes of *ethyl acetate R*, 10 volumes of *water R* and 75 volumes of *propanol R*. Dry the plate in a current of warm air. Spray with *dilute potassium iodobismuthate solution R*. Any spot in the chromatogram obtained with test solution (a), apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (a) (0.2 per cent). The test is not valid unless the chromatogram obtained with reference solution (c) shows two clearly separated spots.

Loss on drying (2.2.32). Not more than 1.0 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C.

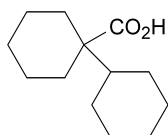
Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.300 g in a mixture of 5.0 ml of 0.01 M *hydrochloric acid* and 50 ml of *alcohol R*. Carry out a potentiometric titration (2.2.20), using 0.1 M *sodium hydroxide*. Read the volume added between the two points of inflexion.

1 ml of 0.1 M *sodium hydroxide* is equivalent to 34.60 mg of $C_{19}H_{18}O_2$.

IMPURITIES

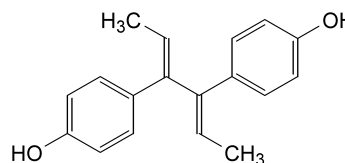


A. bicyclohexyl-1-carboxylic acid.

01/2005:0483

DIENESTROL

Dienestrolum



$C_{18}H_{18}O_2$

M_r 266.3

DEFINITION

Dienestrol contains not less than 98.5 per cent and not more than the equivalent of 101.5 per cent of (*E,E*)-4,4'-(1,2-diethylidene-ethylene)diphenol, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, crystalline powder, practically insoluble in water, freely soluble in acetone and in alcohol. It dissolves in dilute solutions of the alkali hydroxides.

IDENTIFICATION

First identification: A, D.

Second identification: B, C, D.

- Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *dienestrol CRS*. Examine the substances prepared as discs.
- Examine the chromatograms obtained in the test for related substances. The principal spot in the chromatogram obtained with test solution (b) is similar in position, colour and size to the spot in the chromatogram obtained with reference solution (a).
- Dissolve about 1 mg in 5 ml of *glacial acetic acid R*, add 1 ml of a 1 per cent V/V solution of *bromine R* in *glacial acetic acid R* and heat in a water-bath for 2 min. Place 0.5 ml of this solution in a dry test-tube, add 0.5 ml of *ethanol R*, mix and add 10 ml of *water R*. A reddish-violet colour is produced. Add 5 ml of *chloroform R*, shake vigorously and allow to separate. The chloroform layer is red and the aqueous layer is almost colourless.
- Dissolve about 0.5 mg in 0.2 ml of *glacial acetic acid R*, add 1 ml of *phosphoric acid R* and heat on a water-bath for 3 min. A reddish-violet colour is produced.

TESTS

Melting range. Determined by the capillary method (2.2.14), the melting point is 227 °C to 234 °C. The temperature interval between the formation of a definite meniscus in the melt and the disappearance of the last particle does not exceed 3 °C.

Related substances. Examine by thin-layer chromatography (2.2.27), using *silica gel G R* as the coating substance.

Test solution (a). Dissolve 0.2 g of the substance to be examined in 2 ml of *alcohol R*.

Test solution (b). Dilute 1 ml of test solution (a) to 20 ml with *alcohol R*.

Reference solution (a). Dissolve 25 mg of *dienestrol CRS* in *alcohol R* and dilute to 5 ml with the same solvent.

Reference solution (b). Dilute 1 ml of reference solution (a) to 10 ml with *alcohol R*.

Reference solution (c). Dissolve 10 mg of *diethylstilbestrol CRS* in 2 ml of *alcohol R*. To 1 ml of this solution add 1 ml of reference solution (a).

Apply separately to the plate 1 µl of each solution. Develop over a path of 15 cm using a mixture of 10 volumes of *diethylamine R* and 90 volumes of *toluene R*. Allow the plate to dry in air, spray with *alcoholic solution of sulphuric acid R* and heat at 120 °C for 10 min. Any spot in the chromatogram obtained with test solution (a), apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.5 per cent). The test is not valid unless the chromatogram obtained with reference solution (c) shows at least two clearly separated spots having approximately the same intensity.

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 25.0 mg in *ethanol R* and dilute to 100.0 ml with the same solvent. To 5.0 ml of this solution add 10 ml of *ethanol R* and dilute to 250.0 ml with 0.1 M *sodium hydroxide*. Prepare a reference solution in the same manner using 25.0 mg of *dienestrol CRS*. Measure the absorbance (2.2.25) of the solutions at the maximum at 245 nm. Calculate the content of $C_{18}H_{18}O_2$ from the measured absorbances and the concentrations of the solutions.

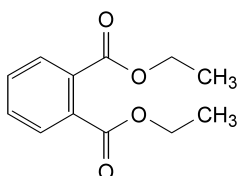
STORAGE

Store protected from light.

01/2005:0897

DIETHYL PHTHALATE

Diethylis phthalas



$C_{12}H_{14}O_4$

M_r 222.2

DEFINITION

Diethyl phthalate contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent *m/m* of diethyl benzene-1,2-dicarboxylate.

CHARACTERS

A clear, oily liquid, colourless or very slightly yellow, practically insoluble in water, miscible with alcohol.

IDENTIFICATION

First identification: B, C.

Second identification: A, D, E.

A. Relative density (2.2.5): 1.117 to 1.121.

B. Refractive index (2.2.6): 1.500 to 1.505.

C. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *diethyl phthalate CRS*. Examine as thin films.

D. Examine by thin-layer chromatography (2.2.27), using *silica gel GF₂₅₄ R* as the coating substance.

Test solution. Dissolve 50 mg of the substance to be examined in *ether R* and dilute to 10 ml with the same solvent.

Reference solution. Dissolve 50 mg of *diethyl phthalate CRS* in *ether R* and dilute to 10 ml with the same solvent.

Apply separately to the plate 10 µl of each solution. Develop over a path of 15 cm using a mixture of 30 volumes of *heptane R* and 70 volumes of *ether R*. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. The principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with the reference solution.

E. To about 0.1 ml add 0.25 ml of *sulphuric acid R* and 50 mg of *resorcinol R*. Heat on a water-bath for 5 min. Allow to cool. Add 10 ml of *water R* and 1 ml of *strong sodium hydroxide solution R*. The solution becomes yellow or brownish-yellow and shows green fluorescence.

TESTS

Appearance. The substance to be examined is clear (2.2.1) and not more intensely coloured than reference solution Y_6 (2.2.2, Method II).

Acidity. Dissolve 20.0 g in 50 ml of *alcohol R* previously neutralised to *phenolphthalein solution R1*. Add 0.2 ml of *phenolphthalein solution R1*. Not more than 0.1 ml of 0.1 M *sodium hydroxide* is required to change the colour of the indicator to pink.

Related substances. Examine by gas chromatography (2.2.28), using *naphthalene R* as the internal standard.

Internal standard solution. Dissolve 60 mg of *naphthalene R* in *methylene chloride R* and dilute to 20 ml with the same solvent.

Test solution (a). Dissolve 1.0 g of the substance to be examined in *methylene chloride R* and dilute to 20.0 ml with the same solvent.

Test solution (b). Dissolve 1.0 g of the substance to be examined in *methylene chloride R*, add 2.0 ml of the internal standard solution and dilute to 20.0 ml with *methylene chloride R*.

Reference solution. To 1.0 ml of test solution (a) add 10.0 ml of the internal standard solution and dilute to 100.0 ml with *methylene chloride R*.

The chromatographic procedure may be carried out using:

- a glass column 2 m long and 2 mm in internal diameter packed with *silanised diatomaceous earth for gas chromatography R* (150 µm to 180 µm) impregnated with 3 per cent *m/m* of *polymethylphenylsiloxane R*,
- *nitrogen for chromatography R* as the carrier gas at a flow rate of 30 ml/min,
- a flame-ionisation detector,

maintaining the temperature of the column at 150 °C and that of the injection port and of the detector at 225 °C.

Inject 1 µl of the reference solution. The substances are eluted in the following order: naphthalene and diethyl phthalate. Adjust the sensitivity of the detector so that the height of the peak due to naphthalene is not less than 50 per cent of the full scale of the recorder. The test is not valid unless the resolution between the peaks corresponding to naphthalene and diethyl phthalate is at least 10.

Inject 1 µl of test solution (a). In the chromatogram obtained, verify that there is no peak with the same retention time as the internal standard.

Inject separately 1 µl of test solution (b) and 1 µl of the reference solution. Continue the chromatography for three times the retention time of diethyl phthalate. From the chromatogram obtained with the reference solution, calculate the ratio (*R*) of the area of the peak due to diethyl phthalate to the area of the peak due to the internal standard. From the chromatogram obtained with test solution (b), calculate the ratio of the sum of the areas of any peaks, apart from the principal peak and the peaks due to the internal standard and the solvent, to the area of the peak due to the internal standard; this ratio is not greater than *R* (1.0 per cent).

Water (2.5.12). Not more than 0.2 per cent, determined on 5.0 g by the semi-micro determination of water.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Introduce 0.750 g into a 250 ml borosilicate glass flask. Add 25.0 ml of 0.5 *M* alcoholic potassium hydroxide and a few glass beads. Boil on a water-bath under a reflux condenser for 1 h. Add 1 ml of phenolphthalein solution R1 and titrate immediately with 0.5 *M* hydrochloric acid. Carry out a blank titration. Calculate the volume of 0.5 *M* alcoholic potassium hydroxide used in the saponification.

1 ml of 0.5 *M* alcoholic potassium hydroxide is equivalent to 55.56 mg of C₁₂H₁₄O₄.

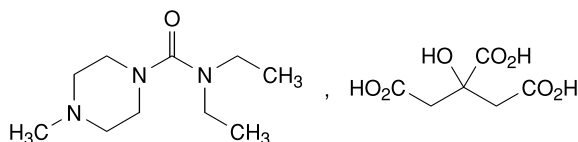
STORAGE

Store in an airtight container.

01/2005:0271

DIETHYLCARBAMAZINE CITRATE

Diethylcarbamazini citras



C₁₆H₂₉N₃O₈

M_r 391.4

DEFINITION

Diethylcarbamazine citrate contains not less than 98.0 per cent and not more than the equivalent of 101.0 per cent of *N,N*-diethyl-4-methylpiperazine-1-carboxamide dihydrogen 2-hydroxypropane-1,2,3-tricarboxylate, calculated with reference to the dried substance.

CHARACTERS

A white, crystalline powder, slightly hygroscopic, very soluble in water, soluble in alcohol, practically insoluble in acetone.

It melts at about 138 °C, with decomposition.

IDENTIFICATION

First identification: A, C.

Second identification: B, C.

- Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with diethylcarbamazine citrate CRS.
- Examine the chromatograms obtained in the test for dimethylpiperazine and methylpiperazine. The principal spot in the chromatogram obtained with the test solution

corresponds in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).

- Dissolve 0.1 g in 5 ml of *water R*. The solution gives the reaction of citrates (2.3.1).

TESTS

Solution S. Shake 2.5 g with *water R* until dissolved and dilute to 25 ml with the same solvent.

Appearance of solution. Solution S is not more opalescent than reference suspension II (2.2.1) and is not more intensely coloured than reference solution BY₆ (2.2.2, *Method II*).

Dimethylpiperazine and methylpiperazine. Examine by thin-layer chromatography (2.2.27), using *silica gel G R* as the coating substance.

Test solution. Dissolve 0.5 g of the substance to be examined in *methanol R* and dilute to 10 ml with the same solvent.

Reference solution (a). Dissolve 0.1 g of diethylcarbamazine citrate CRS in *methanol R* and dilute to 2.0 ml with the same solvent.

Reference solution (b). Dissolve 10 mg of methylpiperazine *R* in *methanol R* and dilute to 100 ml with the same solvent.

Reference solution (c). Dissolve 10 mg of dimethylpiperazine *R* in *methanol R* and dilute to 100 ml with the same solvent.

Apply to the plate 10 µl of each solution. Develop over a path of 12 cm using a mixture of 5 volumes of concentrated ammonia *R*, 30 volumes of methyl ethyl ketone *R* and 65 volumes of *methanol R*. Dry the plate at 100-105 °C and expose to iodine vapour for 30 min. In the chromatogram obtained with the test solution, any spots corresponding to methylpiperazine and dimethylpiperazine are not more intense than the spots in the chromatograms obtained with reference solutions (b) and (c) respectively (0.2 per cent).

Heavy metals (2.4.8). 12 ml of solution S complies with limit test A for heavy metals (20 ppm). Prepare the standard using 10 ml of lead standard solution (2 ppm Pb) *R*.

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.0 g by drying *in vacuo* at 60 °C for 4 h.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.350 g in 25 ml of *anhydrous acetic acid R* and add 25 ml of *acetic anhydride R*. Using 0.2 ml of *crystal violet solution R* as indicator, titrate with 0.1 *M* perchloric acid until a greenish-blue colour is obtained.

1 ml of 0.1 *M* perchloric acid is equivalent to 39.14 mg of C₁₆H₂₉N₃O₈.

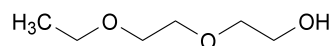
STORAGE

Store in an airtight container.

01/2005:1198

DIETHYLENE GLYCOL MONOETHYL ETHER

Diethylenglycoli monoethylicum aetherum



C₆H₁₄O₃

M_r 134.2

DEFINITION

2-(2-Ethoxyethoxy)ethanol, produced by condensation of ethylene oxide and alcohol, followed by distillation.

CHARACTERS

Appearance: clear, colourless, hygroscopic liquid.

Solubility: miscible with water, with acetone and with alcohol, miscible in certain proportions with vegetable oils, not miscible with mineral oils.

Relative density: about 0.991.

IDENTIFICATION

A. Refractive index (2.2.6): 1.426 to 1.428.

B. Infrared absorption spectrophotometry (2.2.24).

Comparison: *Ph. Eur. reference spectrum of diethylene glycol monoethyl ether.*

TESTS

Acid value (2.5.1): maximum 0.1.

Mix 30.0 ml with 30 ml of *alcohol R* previously neutralised with 0.1 M *potassium hydroxide* using *phenolphthalein solution R* as indicator. Titrate with 0.01 M *alcoholic potassium hydroxide*.

Peroxide value (2.5.5): maximum 8.0, determined on 2.00 g.

Related substances. Gas chromatography (2.2.28).

Internal standard solution. Dilute 1.00 g of *decane R* to 100.0 ml with *methanol R*.

Test solution. To 5.00 g of the substance to be examined, add 0.1 ml of the internal standard solution and dilute to 10.0 ml with *methanol R*.

Reference solution (a). Dilute 25.0 mg of *ethylene glycol monomethyl ether R*, 80.0 mg of *ethylene glycol monoethyl ether R*, 0.310 g of *ethylene glycol R* and 0.125 g of *diethylene glycol R* to 100.0 ml with *methanol R*. To 1.0 ml of this solution add 0.1 ml of the internal standard solution and dilute to 10.0 ml with *methanol R*.

Reference solution (b). Dilute 25.0 mg of *ethylene glycol monoethyl ether R* and 25.0 mg of *ethylene glycol R* to 100.0 ml with *methanol R*. Dilute 1.0 ml of this solution to 5.0 ml with *methanol R*.

Reference solution (c). Dilute 1.00 g of the substance to be examined to 100.0 ml with *methanol R*. To 1.0 ml of this solution add 0.1 ml of the internal standard solution and dilute to 10.0 ml with *methanol R*.

Column:

- **material:** fused silica,
- **size:** $l = 30$ m, $\varnothing = 0.32$ mm,
- **stationary phase:** *poly(cyanopropyl)(7)(phenyl)(7)methyl(86)siloxane R* (film thickness 1 μ m).

Carrier gas: *nitrogen for chromatography R* or *helium for chromatography R*.

Flow rate: 2.0 ml/min.

Split ratio: 1:80.

Temperature:

	Time (min)	Temperature (°C)
Column	0 - 1	120
	1 - 10	120 → 225
	10 - 12	225
Injection port		275
Detector		250

Detection: flame ionisation.

Injection: 0.5 μ l.

Relative retentions with reference to diethylene glycol monoethyl ether (retention time = about 4 min): ethylene glycol monomethyl ether = about 0.4; ethylene glycol monoethyl ether = about 0.5; ethylene glycol = about 0.55; diethylene glycol = about 1.1.

System suitability:

- **resolution:** minimum 3.0 between the peaks due to ethylene glycol monoethyl ether and to ethylene glycol in the chromatogram obtained with reference solution (b),
- **signal-to-noise ratio:** minimum 3.0 for the peak due to ethylene glycol monomethyl ether in the chromatogram obtained with reference solution (a),

Limits (take into account the impurity/internal standard peak area ratio):

- **ethylene glycol monomethyl ether:** not more than the area of the corresponding peak in the chromatogram obtained with reference solution (a) (50 ppm),
- **ethylene glycol monoethyl ether:** not more than the area of the corresponding peak in the chromatogram obtained with reference solution (a) (160 ppm),
- **ethylene glycol:** not more than the area of the corresponding peak in the chromatogram obtained with reference solution (a) (620 ppm),
- **diethylene glycol:** not more than the area of the corresponding peak in the chromatogram obtained with reference solution (a) (250 ppm),
- **total:** not more than the area of the principal peak in the chromatogram obtained with reference solution (c) (0.2 per cent).

Ethylene oxide. Head-space gas chromatography (2.2.28).

Test solution. To 1.00 g of the substance to be examined in a vial, add 50 μ l of *water R*.

Reference solution. To 1.00 g of the substance to be examined in a vial, add 50 μ l of *ethylene oxide solution R4* and close tightly.

Column:

- **material:** fused silica,
- **size:** $l = 30$ m, $\varnothing = 0.32$ mm,
- **stationary phase:** *poly(cyanopropyl)(7)(phenyl)(7)methyl(86)siloxane R* (film thickness 1 μ m).

Carrier gas: *helium for chromatography R*.

Flow rate: 1.1 ml/min.

Static head-space conditions which may be used:

- **equilibration temperature:** 80 °C,
- **equilibration time:** 45 min,
- **transfer line temperature:** 110 °C,
- **pressurisation time:** 2 min,
- **injection time:** 12 s.

Temperature:

	Time (min)	Temperature (°C)
Column	0 - 5	40
	5 - 18	40 → 200
Injection port		150
Detector		250

Detection: flame ionisation.

Injection: 1.0 ml.

The peak due to ethylene oxide is identified by injecting solutions of ethylene oxide of increasing concentration.

Determine the content of ethylene oxide (ppm) in the substance to be examined using the following expression:

$$\frac{S_T \times C}{(S_S \times M_T) - (S_T \times M_S)}$$

S_T = area of the peak corresponding to ethylene oxide in the chromatogram obtained with the test solution,

S_S = area of the peak corresponding to ethylene oxide in the chromatogram obtained with the reference solution,

M_T = mass of the substance to be examined in the test solution, in grams,

M_S = mass of the substance to be examined in the reference solution, in grams,

C = mass of added ethylene oxide in the reference solution, in micrograms.

Limit:

– *ethylene oxide*: maximum 1 ppm.

Water (2.5.12): maximum 0.1 per cent, determined on 10.0 g.

STORAGE

Under an inert gas, in an airtight container.

LABELLING

The label states that the substance is stored under an inert gas.

01/2005:1415

DIETHYLENE GLYCOL MONOPALMITOSTEARATE

Diethylenglycoli monopalmitostearas

DEFINITION

Diethylene glycol monopalmitostearate is a mixture of diethylene glycol mono- and diesters of stearic and palmitic acids. It contains not less than 45.0 per cent of monoesters produced from the condensation of diethylene glycol and stearic acid 50 of vegetable or animal origin.

CHARACTERS

A white or almost white, waxy solid, practically insoluble in water, soluble in acetone and in hot alcohol.

IDENTIFICATION

- It complies with the test for melting point (see Tests).
- It complies with the test for composition of fatty acids (see Tests).
- It complies with the assay (monoesters content).

TESTS

Melting point (2.2.15): 43 °C to 50 °C.

Acid value (2.5.1). Not more than 4.0, determined on 10.0 g.

Iodine value (2.5.4). Not more than 3.0.

Saponification value (2.5.6): 150 to 170, determined on 2.0 g.

Composition of fatty acids (2.4.22, Method A). The fatty acid fraction has the following composition:

- *stearic acid*: 40.0 per cent to 60.0 per cent,
- *sum of contents of palmitic acid and stearic acid*: not less than 90.0 per cent.

Free diethylene glycol. Not more than 8.0 per cent, determined as prescribed under Assay.

Total ash (2.4.16). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Determine the free diethylene glycol content and monoesters content by size-exclusion chromatography (2.2.30).

Test solution. Into a 15 ml flask, weigh about 0.2 g (*m*), to the nearest 0.1 mg. Add 5.0 ml of *tetrahydrofuran R* and shake to dissolve. Heat gently, if necessary. Reweigh the flask and calculate the total mass of solvent and substance (*M*).

Reference solutions. Into four 15 ml flasks, respectively weigh, to the nearest 0.1 mg, about 2.5 mg, 5.0 mg, 10.0 mg and 20.0 mg of *diethylene glycol R*. Add 5.0 ml of *tetrahydrofuran R* and shake to dissolve. Weigh the flasks again and calculate the concentration of diethylene glycol in milligrams per gram for each reference solution.

The chromatographic procedure may be carried out using:

- a gel-permeation column 0.6 m long and 7 mm in internal diameter packed with *styrene-divinylbenzene copolymer R* (particle diameter 5 µm and pore size 10 nm),
- as mobile phase at a flow rate of 1 ml/min *tetrahydrofuran R*,
- a differential refractive index detector.

Inject 40 µl of each solution. When the chromatograms are recorded in the prescribed conditions, the retention times relative to diethylene glycol are about 0.84 for the monoesters and about 0.78 for the diesters. From the calibration curve obtained with the reference solutions determine the concentration (*C*) in milligrams per gram of diethylene glycol in the test solution.

Calculate the percentage content of free diethylene glycol in the substance to be examined using the following expression:

$$\frac{C \times M}{m \times 10}$$

From the peak area of the monoesters (*A*) and the diesters (*B*), calculate the percentage content of monoesters using the following expression:

$$\frac{A}{A + B} \times (100 - D)$$

D = the percentage content of free diethylene glycol and the percentage content of free fatty acids.

Calculate the percentage content of free fatty acids using the expression:

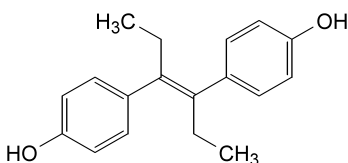
$$\frac{I_A \times 270}{561.1}$$

I_A = acid value.

STORAGE

Store protected from light.

01/2005:0484

DIETHYLSTILBESTROL**Diethylstilbestrolum** $C_{18}H_{20}O_2$ M_r 268.4**DEFINITION**

Diethylstilbestrol contains not less than 97.0 per cent and not more than the equivalent of 101.0 per cent of (*E*)-4,4'-(1,2-diethylethene-1,2-diyl)diphenol, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, crystalline powder, practically insoluble in water, freely soluble in alcohol. It dissolves in solutions of the alkali hydroxides.

It melts at about 172 °C.

IDENTIFICATION

First identification: B, D.

Second identification: A, C, D.

- A. Examined between 230 nm and 450 nm (2.2.25), the irradiated solution of the substance to be examined prepared as prescribed in the assay shows two absorption maxima, at 292 nm and 418 nm.
- B. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *diethylstilbestrol CRS*. Examine the substances prepared as discs.
- C. Examine the chromatograms obtained in the test for mono- and dimethyl ethers. The principal spot in the chromatogram obtained with test solution (b) is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).
- D. Dissolve about 0.5 mg in 0.2 ml of *glacial acetic acid R*, add 1 ml of *phosphoric acid R* and heat on a water-bath for 3 min. A deep-yellow colour develops.

TESTS

4,4'-Dihydroxystilbene and related ethers. Dissolve 0.100 g in *ethanol R* and dilute to 10.0 ml with the same solvent. The absorbance (2.2.25) of the solution measured at 325 nm is not greater than 0.50.

Mono- and dimethyl ethers. Examine by thin-layer chromatography (2.2.27), using *silica gel G R* as the coating substance.

Test solution (a). Dissolve 0.2 g of the substance to be examined in 2 ml of *alcohol R*.

Test solution (b). Dilute 1 ml of test solution (a) to 20 ml with *alcohol R*.

Reference solution (a). Dissolve 10 mg of *diethylstilbestrol CRS* in 2 ml of *alcohol R*.

Reference solution (b). Dissolve 5 mg of *diethylstilbestrol monomethyl ether CRS* in *alcohol R* and dilute to 10 ml with the same solvent.

Reference solution (c). Dissolve 5 mg of *diethylstilbestrol dimethyl ether CRS* in *alcohol R* and dilute to 10 ml with the same solvent.

Reference solution (d). Dissolve 10 mg of *dienestrol CRS* in 2 ml of *alcohol R*. To 1 ml of this solution add 1 ml of reference solution (a).

Apply to the plate 1 µl of each solution. Develop over a path of 15 cm using a mixture of 10 volumes of *diethylamine R* and 90 volumes of *toluene R*. Allow the plate to dry in air, spray with *alcoholic solution of sulphuric acid R* and heat at 120 °C for 10 min. In the chromatogram obtained with test solution (a), any spots corresponding to diethylstilbestrol monomethyl ether and diethylstilbestrol dimethyl ether are not more intense than the spots in the chromatograms obtained with reference solutions (b) and (c) respectively (0.5 per cent). Diethylstilbestrol gives one or sometimes two spots. The test is not valid unless the chromatogram obtained with reference solution (d) shows at least two clearly separated spots having approximately the same intensity.

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

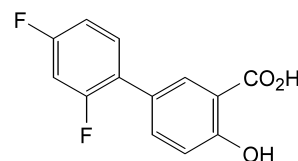
Dissolve 20.0 mg in *ethanol R* and dilute to 100.0 ml with the same solvent. Dilute 10.0 ml of the solution to 100.0 ml with *ethanol R*. To 25.0 ml of the resulting solution add 25.0 ml of a solution of 1 g of *dipotassium hydrogen phosphate R* in 55 ml of *water R*. Prepare in the same manner a reference solution using 20.0 mg of *diethylstilbestrol CRS*. Transfer an equal volume of each solution to separate 1 cm quartz cells and close the cells; place the two cells about 5 cm from a low-pressure, short-wave 2 W to 20 W mercury lamp and irradiate for about 5 min. Measure the absorbance (2.2.25) of the irradiated solutions at the maximum at 418 nm, using *water R* as the compensation liquid. Continue the irradiation for successive periods of 3 min to 15 min, depending on the power of the lamp, and repeat the measurement of the absorbances at 418 nm until the maximum absorbance (about 0.7) is obtained. If necessary, adjust the geometry of the irradiation apparatus to obtain a maximum, reproducible absorbance at 418 nm.

Calculate the content of $C_{18}H_{20}O_2$ from the measured absorbances and the concentrations of the solutions.

STORAGE

Store protected from light.

01/2005:0818

DIFLUNISAL**Diflunisalum** $C_{13}H_8F_2O_3$ M_r 250.2**DEFINITION**

Diflunisal contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of 2',4'-difluoro-4-hydroxybiphenyl-3-carboxylic acid, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, crystalline powder, practically insoluble in water, soluble in alcohol. It dissolves in dilute solutions of alkali hydroxides.

IDENTIFICATION

First identification: B.

Second identification: A, C, D.

- A. Dissolve 10 mg in a 0.3 per cent V/V solution of *hydrochloric acid R* in *methanol R* and dilute to 100.0 ml with the same acid solution. Dilute 2.0 ml of the solution to 10.0 ml with a 0.3 per cent V/V solution of *hydrochloric acid R* in *methanol R*. Examined between 230 nm and 350 nm (2.2.25), the solution shows two absorption maxima, at 251 nm and 315 nm. The ratio of the absorbance measured at the maximum at 251 nm to that measured at the maximum at 315 nm is 4.2 to 4.6.
- B. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *diflunisal CRS*. Examine the substances prepared as discs. If the spectra obtained show differences, dissolve the substance to be examined and the reference substance separately in *alcohol R*, evaporate to dryness and record new spectra using the residues.
- C. Dissolve about 2 mg in 10 ml of *alcohol R* and add 0.1 ml of *ferric chloride solution R1*. A violet-red colour is produced.
- D. Mix about 5 mg with 45 mg of *heavy magnesium oxide R* and ignite in a crucible until an almost white residue is obtained (usually less than 5 min). Allow to cool, add 1 ml of *water R*, 0.05 ml of *phenolphthalein solution R1* and about 1 ml of *dilute hydrochloric acid R* to render the solution colourless. Filter. Add 1.0 ml of the filtrate to a freshly prepared mixture of 0.1 ml of *alizarin S solution R* and 0.1 ml of *zirconyl nitrate solution R*. Mix, allow to stand for 5 min and compare the colour of the solution with that of a blank prepared in the same manner. The test solution is yellow and the blank is red.

TESTS

Appearance of solution. Dissolve 0.5 g in *alcohol R* and dilute to 50 ml with the same solvent. The solution is clear (2.2.1) and not more intensely coloured than reference solution Y₇ (2.2.2, *Method II*).

Related substances

- A. Examine by thin-layer chromatography (2.2.27), using *silica gel GF₂₅₄ R* as the coating substance.

Test solution. Dissolve 0.20 g of the substance to be examined in *methanol R* and dilute to 10 ml with the same solvent.

Reference solution (a). Dissolve 30 mg of *biphenyl-4-ol R* in *methanol R* and dilute to 100 ml with the same solvent. Dilute 1 ml of the solution to 10 ml with *methanol R*.

Reference solution (b). Dissolve 20 mg of *biphenyl-4-ol R* in *methanol R*, add 1 ml of the test solution and dilute to 10 ml with *methanol R*.

Apply to the plate 10 µl of each solution. Develop over a path of 15 cm using a mixture of 10 volumes of *glacial acetic acid R*, 20 volumes of *acetone R* and 70 volumes of *methylene chloride R*. Dry the plate in a current of warm air and examine in ultraviolet light at 254 nm. Any

spot in the chromatogram obtained with the test solution, apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (a) (0.15 per cent). The test is not valid unless the chromatogram obtained with reference solution (b) shows two clearly separated principal spots.

- B. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 50.0 mg of the substance to be examined in the reference solution and dilute to 10.0 ml with the same solution.

Reference solution. Dissolve 55.0 mg of *fluoranthene R* in a mixture of 1 volume of *water R* and 4 volumes of *acetonitrile R* and dilute to 100.0 ml with the same mixture of solvents. Dilute 1.0 ml to 100.0 ml with a mixture of 1 volume of *water R* and 4 volumes of *acetonitrile R*.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (10 µm),
- as mobile phase at a flow rate of 2 ml/min a mixture of 2 volumes of *glacial acetic acid R*, 25 volumes of *methanol R*, 55 volumes of *water R* and 70 volumes of *acetonitrile R*,
- as detector a spectrophotometer set at 254 nm.

Inject 20 µl of the reference solution and adjust the sensitivity of the detector so that the height of the principal peak in the chromatogram obtained is not less than 10 per cent of the full scale of the recorder. Inject separately 20 µl of the test solution and 20 µl of the reference solution. Continue the chromatography for three times the retention time of fluoranthene. In the chromatogram obtained with the test solution, the sum of the areas of any peaks, apart from the principal peak, with a retention time greater than that of fluoranthene is not greater than the area of the principal peak in the chromatogram obtained with the reference solution (0.1 per cent). Disregard any peak with an area less than 0.05 times that of the principal peak in the chromatogram obtained with the reference solution.

Heavy metals (2.4.8). 2.0 g complies with limit test C for heavy metals (10 ppm). Use a platinum crucible. Prepare the standard using 2 ml of *lead standard solution (10 ppm Pb) R*.

Loss on drying (2.2.32). Not more than 0.3 per cent, determined on 1.000 g by drying at 60 °C at a pressure not exceeding 700 Pa for 2 h.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g in a platinum crucible.

ASSAY

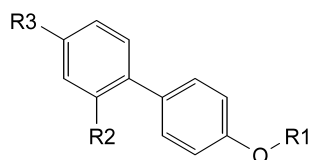
Dissolve 0.200 g in 40 ml of *methanol R*. Add 5 ml of *water R* and 0.2 ml of *phenol red solution R*. Titrate with 0.1 M *sodium hydroxide* until the colour changes from yellow to reddish-violet.

1 ml of 0.1 M *sodium hydroxide* is equivalent to 25.02 mg of C₁₃H₈F₂O₃.

STORAGE

Store protected from light.

IMPURITIES



- A. R1 = R2 = R3 = H: biphenyl-4-ol,
 B. R1 = H, R2 = R3 = F: 2',4'-difluorobiphenyl-4-ol,
 C. R1 = CO-CH₃, R2 = R3 = F: 2',4'-difluorobiphenyl-4-yl acetate,
 D. condensation products.

01/2005:0117

DIGITALIS LEAF

Digitalis purpureae folium

DEFINITION

Digitalis leaf consists of the dried leaf of *Digitalis purpurea* L. It contains not less than 0.3 per cent of cardenolic glycosides, expressed as digitoxin (*M_r* 765), and calculated with reference to the drug dried at 100 °C to 105 °C.

CHARACTERS

Digitalis leaf has a faint but characteristic odour. The whole leaf is about 10 cm to 40 cm long and 4 cm to 15 cm wide. The lamina is ovate lanceolate to broadly ovate. The winged petiole is from one quarter as long as to equal in length to the lamina.

It has the macroscopic and microscopic characters described in identification tests A and B.

IDENTIFICATION

- A. The leaf is brittle and often occurs broken. The upper surface is green and the lower surface is greyish-green. The apex is subacute and the margin is irregularly crenate, dentate or serrate. The base is decurrent. The venation is pinnate, the lateral veins being prominent especially on the lower surface, leaving the midrib at about 45° and anastomosing near the margin; a veinlet terminates in each tooth of the margin and the lower veins run down the winged petiole. The upper surface is rugose and pubescent; the lower surface shows a network of raised veinlets and is densely pubescent.
- B. Reduce to a powder (355). Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters: epidermal cells with anticlinal walls which are straight or slightly sinuous on the upper surface and markedly sinuous on the lower surface; the cuticle is smooth. Trichomes are of two types: uniseriate, bluntly pointed non-glandular, usually of three to five cells, often with one or more collapsed cells, walls mostly finely warty or faintly striated; glandular trichomes usually with a unicellular, sometimes a multicellular uniseriate stalk and a unicellular or bicellular or exceptionally tetracellular head. Anomocytic stomata (2.8.3) are absent or very rare on the upper surface, numerous on the lower surface. Calcium oxalate crystals and sclerenchyma are absent.
- C. Examine by thin-layer chromatography (2.2.27), using *silica gel G R* as the coating substance.
Test solution. To 1.0 g of the powdered drug (180) add a mixture of 20 ml of *alcohol (50 per cent V/V) R* and 10 ml of *lead acetate solution R*. Boil for 2 min, allow to cool and centrifuge. Shake the supernatant solution with

two quantities, each of 15 ml, of *chloroform R*; separate the two layers by centrifugation if necessary. Dry the chloroform layers over *anhydrous sodium sulphate R* and filter. Evaporate 10 ml of the solution to dryness on a water-bath and dissolve the residue in 1 ml of a mixture of equal volumes of *chloroform R* and *methanol R*.

Reference solution. Dissolve 5 mg of *purpureaglycoside A CRS*, 2 mg of *purpureaglycoside B CRS*, 5 mg of *digitoxin R* and 2 mg of *gitoxin R* in a mixture of equal volumes of *chloroform R* and *methanol R* and dilute to 10 ml with the same mixture of solvents.

Apply separately to the plate as bands 2 cm by 0.3 cm 20 µl of each solution. Develop over a path of 10 cm using a mixture of 7.5 volumes of *water R*, 10 volumes of *methanol R* and 75 volumes of *ethyl acetate R*. Allow the solvents to evaporate. Spray with a mixture of 2 volumes of a 10 g/l solution of *chloramine R* and 8 volumes of a 250 g/l solution of *trichloroacetic acid R* in *alcohol R*. Heat at 100 °C to 105 °C for 10 min. Examine in ultraviolet light at 365 nm. The chromatogram obtained with the reference solution shows a zone of light-blue fluorescence in the lower part of the chromatogram, corresponding to purpureaglycoside B, and just above it a zone of brownish-yellow fluorescence, corresponding to purpureaglycoside A. A zone of light-blue fluorescence, corresponding to gitoxin, appears in the middle of the chromatogram and above it a zone of brownish-yellow fluorescence, corresponding to digitoxin. The zones in the chromatogram obtained with the test solution are similar in position, colour and size to the zones in the chromatogram obtained with the reference solution. Other zones of fluorescence may also appear in the chromatogram obtained with the test solution.

- D. Evaporate 5 ml of the chloroformic solution obtained in identification test C to dryness on a water-bath. To the residue add 2 ml of *dinitrobenzoic acid solution R* and 1 ml of 1 M *sodium hydroxide*. A reddish-violet colour develops within 5 min.
- E. Evaporate 5 ml of the chloroformic solution obtained in identification test C to dryness on a water-bath. To the residue add 3 ml of *xanthidrol solution R* and heat on a water-bath for 3 min. A red colour develops.

TESTS

Foreign matter (2.8.2). There are no leaves with few or no trichomes and epidermal cells showing, in surface view, beaded anticlinal walls (*Digitalis lanata*).

Loss on drying (2.2.32). Not more than 6.0 per cent, determined on 1.000 g of the powdered drug (355) by drying in an oven at 100 °C to 105 °C.

Total ash (2.4.16). Not more than 12.0 per cent.

Ash insoluble in hydrochloric acid (2.8.1). Not more than 5.0 per cent.

ASSAY

Shake 0.250 g of the powdered drug (180) with 50.0 ml of *water R* for 1 h. Add 5.0 ml of a 150 g/l solution of *lead acetate R*, shake and after a few minutes add 7.5 ml of a 40 g/l solution of *disodium hydrogen phosphate R*. Filter through a pleated filter paper. Heat 50.0 ml of the filtrate with 5 ml of hydrochloric acid (150 g/l HCl) under a reflux condenser on a water-bath for 1 h. Transfer to a separating funnel, rinse the flask with two quantities, each of 5 ml, of *water R* and shake with three quantities, each of 25 ml, of *chloroform R*. Dry the combined chloroform layers over *anhydrous sodium sulphate R* and dilute to 100.0 ml with *chloroform R*. Evaporate 40.0 ml of the chloroformic

solution to dryness, dissolve the residue in 7 ml of *alcohol* (50 per cent V/V) *R* and add 2 ml of *dinitrobenzoic acid solution R* and 1 ml of 1 *M* *sodium hydroxide*. At the same time prepare a reference solution as follows. Dissolve 50.0 mg of *digitoxin CRS* in *alcohol R* and dilute to 50.0 ml with the same solvent. Dilute 5.0 ml of the solution to 50.0 ml with *alcohol R*. To 5.0 ml of the resulting solution add 25 ml of *water R* and 3 ml of hydrochloric acid (150 g/l HCl). Heat the solution under a reflux condenser on a water-bath for 1 h and complete the preparation as described above. Measure the absorbance (2.2.25) of the two solutions at 540 nm several times during the first 12 min until the maximum is reached, using as the compensation liquid a mixture of 7 ml of *alcohol* (50 per cent V/V) *R*, 2 ml of *dinitrobenzoic acid solution R* and 1 ml of 1 *M* *sodium hydroxide*.

From the absorbances measured and the concentrations of the solutions, calculate the content of cardenolic glycosides, expressed as digitoxin.

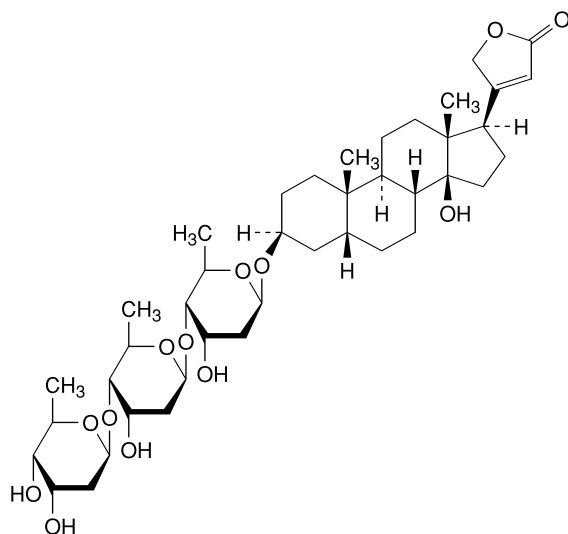
STORAGE

Store protected from light and moisture.

01/2005:0078

DIGITOXIN

Digitoxinum



$C_{41}H_{64}O_{13}$

M_r 765

DEFINITION

Digitoxin contains not less than 95.0 per cent and not more than the equivalent of 103.0 per cent of 3β-[(O-2,6-dideoxy-β-D-ribo-hexopyranosyl-(1→4)-O-2,6-dideoxy-β-D-ribo-hexopyranosyl-(1→4)-2,6-dideoxy-β-D-ribo-hexopyranosyl)oxy]-14-hydroxy-5β,14β-card-20(22)-enolide, calculated with reference to the dried substance.

CHARACTERS

A white or almost white powder, practically insoluble in water, freely soluble in a mixture of equal volumes of methanol and methylene chloride, slightly soluble in alcohol and in methanol.

IDENTIFICATION

First identification: A.

Second identification: B, C, D.

- Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *digitoxin CRS*.
- Examine the chromatograms obtained in the test for related substances. The principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).
- Suspend about 0.5 mg in 0.2 ml of *alcohol* (60 per cent V/V) *R*. Add 0.1 ml of *dinitrobenzoic acid solution R* and 0.1 ml of *dilute sodium hydroxide solution R*. A violet colour develops.
- Dissolve about 0.5 mg in 1 ml of *glacial acetic acid R*, heating gently, allow to cool and add 0.05 ml of *ferric chloride solution R1*. Cautiously add 1 ml of *sulphuric acid R*, avoiding mixing the two liquids. A brown ring develops at the interface and on standing a green, then blue colour passes to the upper layer.

TESTS

Appearance of solution. Dissolve 50 mg in a mixture of equal volumes of *methanol R* and *methylene chloride R* and dilute to 10 ml with the same mixture of solvents. The solution is clear (2.2.1) and colourless (2.2.2, *Method I*).

Specific optical rotation (2.2.7). Dissolve 0.25 g in *chloroform R* and dilute to 10.0 ml with the same solvent. The specific optical rotation is + 16.0 to + 18.5.

Related substances. Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel G plate R*.

Test solution. Dissolve 20 mg of the substance to be examined in a mixture of equal volumes of *methanol R* and *methylene chloride R* and dilute to 2 ml with the same mixture of solvents.

Reference solution (a). Dissolve 20 mg of *digitoxin CRS* in a mixture of equal volumes of *methanol R* and *methylene chloride R* and dilute to 2 ml with the same mixture of solvents.

Reference solution (b). Dilute 0.5 ml of reference solution (a) to 50 ml with a mixture of equal volumes of *methanol R* and *methylene chloride R*.

Reference solution (c). Dissolve 10 mg of *gitoxin CRS* with stirring in a mixture of equal volumes of *methanol R* and *methylene chloride R* and dilute to 50 ml with the same mixture of solvents.

Reference solution (d). Dilute 1 ml of reference solution (b) to 2 ml with a mixture of equal volumes of *methanol R* and *methylene chloride R*.

Reference solution (e). Mix 1 ml of reference solution (a) and 1 ml of reference solution (c).

Apply to the plate 5 µl of each solution. Develop immediately over a path of 15 cm using a mixture of 15 volumes of *methanol R*, 40 volumes of *cyclohexane R* and 90 volumes of *methylene chloride R*. Dry the plate in a stream of cold air for 5 min. Repeat the development and dry the plate in a stream of cold air for 5 min. Spray with a mixture of 1 volume of *sulphuric acid R* and 9 volumes of *alcohol R* and heat at 130 °C for 15 min. Examine in daylight.

Gitoxin. Any spot corresponding to gitoxin in the chromatogram obtained with the test solution is not more intense than the spot in the chromatogram obtained with reference solution (c) (2.0 per cent).

Other glycosides. Any spot in the chromatogram obtained with the test solution, apart from the principal spot and the spot corresponding to gitoxin, is not more intense than the spot in the chromatogram obtained with reference solution (b) (1.0 per cent).

The test is not valid unless the chromatogram obtained with reference solution (e) shows clearly separated spots corresponding to digitoxin, gitoxin and other glycosides and the spot in the chromatogram obtained with reference solution (d) is clearly visible.

Loss on drying (2.2.32). Not more than 1.5 per cent, determined on 0.500 g by drying in an oven at 100 °C to 105 °C for 2 h.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on the residue from the test for loss on drying.

ASSAY

Dissolve 40.0 mg in *alcohol R* and dilute to 50.0 ml with the same solvent. Dilute 5.0 ml of the solution to 100.0 ml with *alcohol R*. Prepare a reference solution in the same manner, using 40.0 mg of *digitoxin CRS*. To 5.0 ml of each solution add 3.0 ml of *alkaline sodium picrate solution R*, allow to stand protected from bright light for 30 min and measure the absorbance (2.2.25) of each solution at the maximum at 495 nm, using as the compensation liquid a mixture of 5.0 ml of *alcohol R* and 3.0 ml of *alkaline sodium picrate solution R* prepared at the same time.

Calculate the content of $C_{41}H_{64}O_{13}$ from the absorbances measured and the concentrations of the solutions.

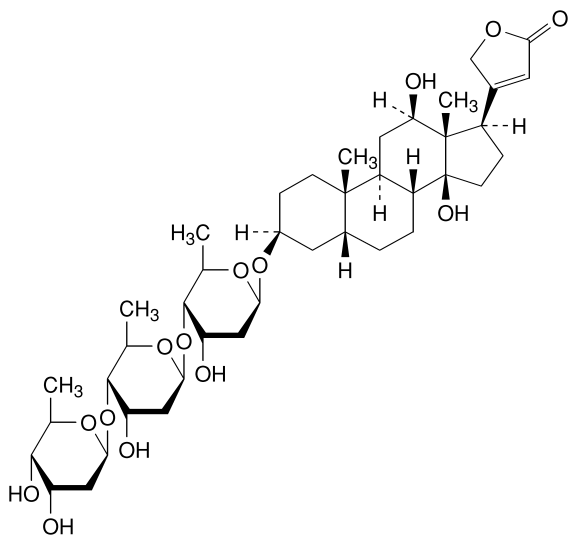
STORAGE

Store protected from light.

01/2005:0079

DIGOXIN

Digoxinum



$C_{41}H_{64}O_{14}$

M_r 781

DEFINITION

Digoxin contains not less than 95.0 per cent and not more than the equivalent of 103.0 per cent of $3\beta-[(O-2,6-dideoxy-\beta-D-ribo-hexopyranosyl-(1\rightarrow4)-O-2,6-dideoxy-\beta-D-ribo-hexopyranosyl-(1\rightarrow4)-2,6-dideoxy-\beta-D-ribo-hexopyranosyl)oxy]-12\beta,14-dihydroxy-5\beta-card-20(22)-enolide$, calculated with reference to the dried substance.

CHARACTERS

A white or almost white powder or colourless crystals, practically insoluble in water, freely soluble in a mixture of equal volumes of methanol and methylene chloride, slightly soluble in alcohol.

IDENTIFICATION

First identification: A.

Second identification: B, C, D.

- Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *digoxin CRS*.
- Examine the chromatograms obtained in the test for related substances. The principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).
- Suspend about 0.5 mg in 0.2 ml of *alcohol (60 per cent V/V) R*. Add 0.1 ml of *dinitrobenzoic acid solution R* and 0.1 ml of *dilute sodium hydroxide solution R*. A violet colour develops.
- Dissolve about 0.5 mg in 1 ml of *glacial acetic acid R*, heating gently, allow to cool and add 0.05 ml of *ferric chloride solution R1*. Cautiously add 1 ml of *sulphuric acid R* avoiding mixing the two liquids. A brown ring develops at the interface and on standing a green, then blue colour passes to the upper layer.

TESTS

Appearance of solution. Dissolve 50 mg in a mixture of equal volumes of *methanol R* and *methylene chloride R* and dilute to 10 ml with the same mixture of solvents. The solution is clear (2.2.1) and colourless (2.2.2, *Method I*).

Specific optical rotation (2.2.7). Dissolve 0.20 g in *anhydrous pyridine R* and dilute to 10.0 ml with the same solvent. The specific optical rotation is + 10.0 to + 13.0, calculated with reference to the dried substance.

Related substances. Examine by thin-layer chromatography (2.2.27), using *kieselguhr G R* as the coating substance. Impregnate the plate by placing it in a closed chromatographic tank containing the necessary quantity of a mixture of 10 volumes of *formamide R* and 90 volumes of *acetone R* so that the plate dips about 5 mm into the liquid. When the impregnation mixture has risen at least 15 cm from the lower edge of the plate, remove the plate and allow the solvent to evaporate for 30 min. Use the plate immediately.

Test solution. Dissolve 50 mg of the substance to be examined in a mixture of equal volumes of *methanol R* and *methylene chloride R* and dilute to 5 ml with the same mixture of solvents.

Reference solution (a). Dissolve 20 mg of *digoxin CRS* in a mixture of equal volumes of *methanol R* and *methylene chloride R* and dilute to 2 ml with the same mixture of solvents.

Reference solution (b). Dilute 1 ml of reference solution (a) to 50 ml with a mixture of equal volumes of *methanol R* and *methylene chloride R*.

Reference solution (c). Dilute 2 ml of reference solution (b) to 4 ml with a mixture of equal volumes of *methanol R* and *methylene chloride R*.

Reference solution (d). Dissolve 5 mg of *digitoxin CRS* in a mixture of equal volumes of *methanol R* and *methylene chloride R* and dilute to 50 ml with the same mixture of solvents.

Reference solution (e). Dissolve 5 mg of *gitoxin CRS* in a mixture of equal volumes of *methanol R* and *methylene chloride R* and dilute to 25 ml with the same mixture of solvents.

Apply separately to the plate 2 µl of each solution. Develop over a path of 12 cm using a mixture of 4 volumes of *formamide R*, 50 volumes of *methyl ethyl ketone R* and 50 volumes of *xylene R*. Dry the plate in a current of cold

01/2005:1310
corrected

air until only the lower edge is still visibly moist. Repeat the development and dry the plate at 115 °C for 20 min. Allow to cool, spray with a mixture of 1 volume of a freshly prepared 30 g/l solution of *chloramine R* and 15 volumes of a 250 g/l solution of *trichloroacetic acid R* in *alcohol R* and heat at 115 °C for 5 min. Examine in ultraviolet light at 365 nm. Any spot corresponding to digitoxin in the chromatogram obtained with the test solution is not more intense than the spot in the chromatogram obtained with reference solution (d) (1.0 per cent). Any spot corresponding to gitoxin in the chromatogram obtained with the test solution is not more intense than the spot in the chromatogram obtained with reference solution (e) (2.0 per cent). Any spot in the chromatogram obtained with the test solution, apart from the principal spot and spots corresponding to digitoxin and gitoxin, is not more intense than the spot in the chromatogram obtained with reference solution (b) (2.0 per cent) and at most one such spot is more intense than the spot in the chromatogram obtained with reference solution (c) (1.0 per cent).

Loss on drying (2.2.32). Not more than 1.0 per cent, determined on 0.500 g by drying *in vacuo*.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on the residue from the test for loss on drying.

ASSAY

Dissolve 40.0 mg in *alcohol R*, heating if necessary, and dilute to 50.0 ml with the same solvent. Dilute 5.0 ml of the solution to 100.0 ml with *alcohol R*. Prepare a reference solution in the same manner, using 40.0 mg of *digoxin CRS*. To 5.0 ml of each solution add 3.0 ml of *alkaline sodium picrate solution R*, allow to stand protected from bright light for 30 min and measure the absorbance (2.2.25) of each solution at the maximum at 495 nm, using as the compensation liquid a mixture of 5.0 ml of *alcohol R* and 3.0 ml of *alkaline sodium picrate solution R* prepared at the same time.

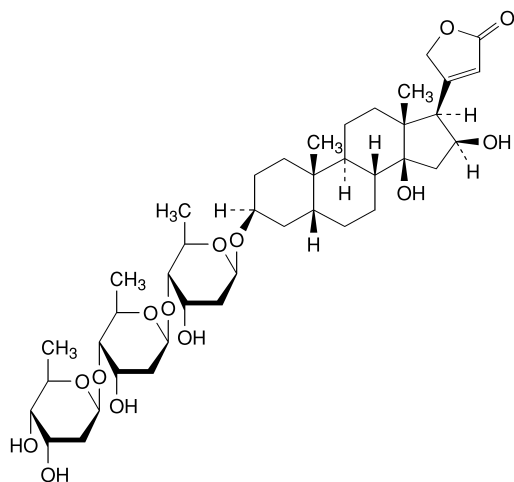
Calculate the content of $C_{41}H_{64}O_{14}$ from the absorbances measured and the concentrations of the solutions.

STORAGE

Store protected from light.

IMPURITIES

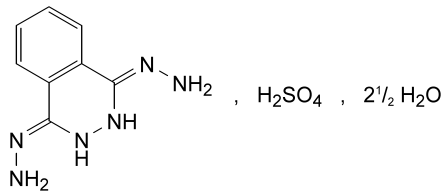
A. digitoxin,



B. 3β-[(O-2,6-dideoxy-β-D-ribo-hexopyranosyl-(1→4)-O-2,6-dideoxy-β-D-ribo-hexopyranosyl-(1→4)-2,6-dideoxy-β-D-ribo-hexopyranosyl)oxy]-14,16β-dihydroxy-5β-card-20(22)-enolide (gitoxin).

DIHYDRALAZINE SULPHATE, HYDRATED

Dihydralazini sulfas hydricus



$C_8H_{12}N_6O_4S \cdot 2\frac{1}{2}H_2O$

M_r 333.3

DEFINITION

Hydrated dihydralazine sulphate contains not less than 98.0 per cent and not more than the equivalent of 102.0 per cent of (phthalazine-1,4(2*H*,3*H*)-diylidene)dihydrazine sulphate, calculated with reference to the dried substance.

CHARACTERS

A white or slightly yellow, crystalline powder, slightly soluble in water, practically insoluble in ethanol. It dissolves in dilute mineral acids.

IDENTIFICATION

- Examine by infrared absorption spectrophotometry (2.2.24), comparing with the *Ph. Eur. reference spectrum of dihydralazine sulphate hydrated*.
- Dissolve about 50 mg in 5 ml of *dilute hydrochloric acid R*. The solution gives reaction (a) of sulphates (2.3.1).

TESTS

Appearance of solution. Dissolve 0.20 g in *dilute nitric acid R* and dilute to 10 ml with the same acid. The solution is clear (2.2.1) and not more intensely coloured than reference solution BY₆ (2.2.2, *Method II*).

Related substances. Liquid chromatography (2.2.29). Prepare the solutions immediately before use.

Test solution. Dissolve 50.0 mg of the substance to be examined in a 6 g/l solution of *glacial acetic acid R* and dilute to 50.0 ml with the same solvent.

Reference solution (a). Dilute 1.0 ml of the test solution to 100.0 ml with the mobile phase containing 0.5 g/l of *sodium edetate R*. Dilute 1.0 ml of this solution to 10.0 ml with the mobile phase containing 0.5 g/l of *sodium edetate R*.

Reference solution (b). Dilute 1.0 ml of the test solution to 50.0 ml with the mobile phase containing 0.5 g/l of *sodium edetate R*.

Reference solution (c). Dissolve 5 mg of *dihydralazine for system suitability CRS* in a 6 g/l solution of *glacial acetic acid R* and dilute to 5.0 ml with the same solvent.

Column:

- size: $l = 0.25$ m, $\varnothing = 4.6$ mm,
- stationary phase: nitrile silica gel for chromatography *R* (5 µm).

Mobile phase: to 22 volumes of *acetonitrile R* add 78 volumes of a solution containing 1.44 g/l of *sodium laurilsulfate R* and 0.75 g/l of *tetrabutylammonium bromide R* and adjust to pH 3.0 with 0.05 *M* *sulphuric acid*.

Flow rate: 1.5 ml/min.

Detection: spectrophotometer at 230 nm.

Injection: 20 µl.

Run time: twice the retention time of dihydralazine.

Relative retention with reference to dihydralazine: impurity A = about 0.8.

System suitability: reference solution (c):

- the peaks due to impurity A and dihydralazine are baseline separated as in the chromatogram supplied with *dihydralazine for system suitability CRS*.

Limits:

- impurity A:** not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (2 per cent),
- impurity C:** not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent),
- any other impurity:** for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent),
- total of impurities other than A:** not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent),
- disregard limit:** 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (a).

Hydrazine. Examine by liquid chromatography (2.2.29).

Prepare the solutions immediately before use.

Test solution. Dissolve 40.0 mg of *hydrazine sulphate R* in *water R* and dilute to 100.0 ml with the same solvent. Dilute 1.0 ml of the solution to 25.0 ml with *water R*. To 0.50 ml of the solution, add 0.200 g of the substance to be examined and dissolve in 6 ml of *dilute hydrochloric acid R*, then dilute to 10.0 ml with *water R*. In a centrifuge tube with a ground-glass stopper, place immediately 0.50 ml of the solution and 2.0 ml of a 60 g/l solution of *benzaldehyde R* in a mixture of equal volumes of *methanol R* and *water R*. Shake for 90 s. Add 1.0 ml of *water R* and 5.0 ml of *heptane R*. Shake for 1 min and centrifuge. Use the upper layer.

Reference solution. Dissolve 40.0 mg of *hydrazine sulphate R* in *water R* and dilute to 100.0 ml with the same solvent. Dilute 1.0 ml of the solution to 25.0 ml with *water R*. To 0.50 ml of the solution, add 6 ml of *dilute hydrochloric acid R* and dilute to 10.0 ml with *water R*. In a centrifuge tube with a ground-glass stopper, place 0.50 ml of the solution and 2.0 ml of a 60 g/l solution of *benzaldehyde R* in a mixture of equal volumes of *methanol R* and *water R*. Shake for 90 s. Add 1.0 ml of *water R* and 5.0 ml of *heptane R*. Shake for 1 min and centrifuge. Use the upper layer.

Blank solution. Prepare in the same manner as for the reference solution but replacing the 0.50 ml of hydrazine sulphate solution by 0.50 ml of *water R*.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4.6 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (5 µm),
- as mobile phase at a flow rate of 1 ml/min a mixture of 30 volumes of a 0.3 g/l solution of *sodium edetate R* and 70 volumes of *acetonitrile R*,
- as detector a spectrophotometer set at 305 nm.

Inject 20 µl each of the test solution, the reference solution and the blank solution. Comparing the chromatograms obtained with the reference solution and the blank solution, identify the peak of benzaldehyde azine (benzalazine) corresponding to hydrazine having a retention time relative to the principal peak (benzaldehyde) of about 1.8. In the chromatogram obtained with the test solution, the area of the peak corresponding to benzaldehyde azine is not

greater than twice that of the corresponding peak in the chromatogram obtained with the reference solution (10 ppm of hydrazine).

Iron (2.4.9). To the residue obtained in the test for sulphated ash add 0.2 ml of *sulphuric acid R* and heat carefully until the acid is almost completely eliminated. Allow to cool and dissolve the residue with heating in 5.5 ml of *hydrochloric acid R1*. Filter the hot solution through a filter previously washed 3 times with *dilute hydrochloric acid R*. Wash the crucible and the filter with 5 ml of *water R*. Combine the filtrate and the washings and neutralise with about 3.5 ml of *strong sodium hydroxide solution R*. Adjust the pH to between 3 and 4 with *acetic acid R* and dilute to 20 ml with *water R*. The solution complies with the limit test for iron (20 ppm). Prepare the standard with 5 ml of *iron standard solution (2 ppm Fe) R* and 5 ml of *water R*.

Loss on drying (2.2.32): 13.0 per cent to 15.0 per cent, determined on 1.000 g by drying in an oven at 50 °C at a pressure not exceeding 0.7 kPa for 5 h.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

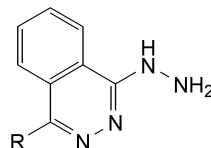
ASSAY

Dissolve 60.0 mg in 25 ml of *water R*. Add 35 ml of *hydrochloric acid R* and titrate slowly with 0.05 M *potassium iodate*, determining the end-point potentiometrically (2.2.20), using a calomel reference electrode and a platinum indicator electrode.

1 ml of 0.05 M *potassium iodate* is equivalent to 7.208 mg of C₈H₁₂N₆O₄S.

IMPURITIES

Specified impurities: A, B, C.



A. R = NH₂: 4-hydrazinophthalazin-1-amine,

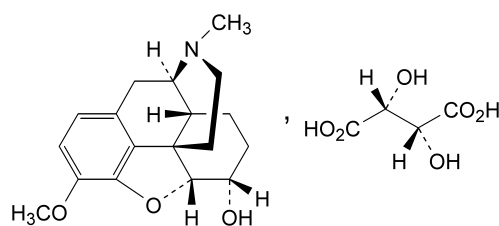
C. R = H:(phthalazin-1-yl)hydrazine (hydralazine),

B. H₂N-NH₂: hydrazine.

01/2005:1776

DIHYDROCODEINE HYDROGEN TARTRATE

Dihydrocodeini hydrogenotartras



C₂₂H₂₉NO₉

M_r 451.5

DEFINITION

4,5α-Epoxy-3-methoxy-17-methylmorphinan-6α-ol hydrogen (2R,3R)-2,3-dihydroxybutanedioate.

Content: 98.5 per cent to 101.0 per cent (anhydrous substance).

CHARACTERS

Appearance: white or almost white, crystalline powder.

Solubility: freely soluble in water, sparingly soluble in alcohol, practically insoluble in cyclohexane.

IDENTIFICATION

First identification: A.

Second identification: B, C, D.

A. Infrared absorption spectrophotometry (2.2.24).

Comparison: Ph. Eur. reference spectrum of dihydrocodeine hydrogen tartrate.

B. To about 0.1 g add 1 ml of sulphuric acid R and 0.05 ml of ferric chloride solution R1 and heat on a water-bath. A brownish-yellow colour develops. Add 0.05 ml of dilute nitric acid R. The colour does not become red.

C. To 1 ml of solution S (see Tests) add 5 ml of picric acid solution R. Heat on a water-bath until a clear solution is obtained. Allow to cool. A precipitate is formed. Filter, wash with 5 ml of water R and dry at 100–105 °C. The crystals melt (2.2.14) at 220 °C to 223 °C.

D. It gives reaction (b) of tartrates (2.3.1).

TESTS

Solution S. Dissolve 2.50 g in carbon dioxide-free water R and dilute to 25.0 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and not more intensely coloured than reference solution BY₅ (2.2.2, Method II).

pH (2.2.3): 3.2 to 4.2 for solution S.

Specific optical rotation (2.2.7): –70.5 to –73.5 (anhydrous substance).

Dilute 10.0 ml of solution S to 20.0 ml with water R.

Related substances. Liquid chromatography (2.2.29).

Test solution. Dissolve 10.0 mg of the substance to be examined in the mobile phase and dilute to 10.0 ml with the mobile phase.

Reference solution (a). Dissolve 2.0 mg of codeine phosphate R in 2.0 ml of the test solution and dilute to 25.0 ml with the mobile phase.

Reference solution (b). Dilute 1.0 ml of the test solution to 200 ml with the mobile phase.

Column:

- size: $l = 0.25$ m, $\varnothing = 4.6$ mm,
- stationary phase: octylsilyl silica gel for chromatography R (5 µm).

Mobile phase: to 1.0 g of sodium heptanesulphonate R, add 10.0 ml of glacial acetic acid R and 4.0 ml of a solution of 5.0 ml of triethylamine R diluted to 25.0 ml with a mixture of equal volumes of water R and acetonitrile R. Add 170 ml of acetonitrile R and dilute to 1000 ml with water R.

Flow rate: 1 ml/min.

Detection: spectrophotometer at 284 nm.

Injection: 20 µl.

Run time: 5 times the retention time of dihydrocodeine.

Retention time: dihydrocodeine = about 14 min.

System suitability: reference solution (a):

- resolution: minimum of 2 between the peaks due to dihydrocodeine and to impurity A.

Limits:

- impurity A: not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent),
- any other peak: not more than 0.6 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.3 per cent),
- total: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (1 per cent); disregard any peak due to tartaric acid (relative retention with reference to dihydrocodeine = about 0.25),
- disregard limit: 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

Water (2.5.12): maximum 0.7 per cent, determined on 1.00 g.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.350 g in 60 ml of anhydrous acetic acid R. Titrate with 0.1 M perchloric acid determining the end-point potentiometrically (2.2.20).

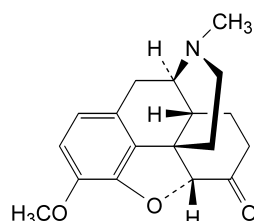
1 ml of 0.1 M perchloric acid is equivalent to 45.15 mg of C₂₂H₂₉NO₉.

STORAGE

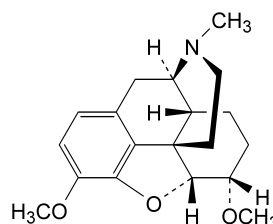
Protected from light.

IMPURITIES

- A. codeine,
B. morphine,



- C. 4,5-epoxy-3-methoxy-17-methylmorphinan-6-one (hydrocodone),

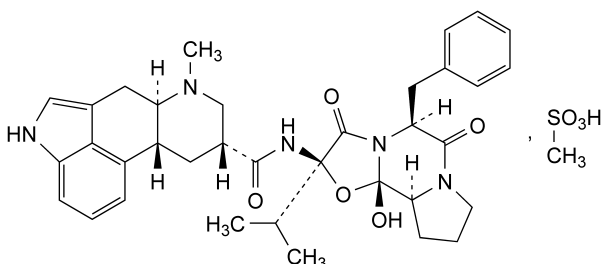


- D. 4,5-epoxy-3,6-dimethoxy-17-methylmorphinan (tetrahydrothebaine).

01/2005:1416 C. Thin-layer chromatography (2.2.27).

DIHYDROERGOCRISTINE MESILATE

Dihydroergocristini mesilas

 $C_{36}H_{45}N_5O_8S$ M_r 708

DEFINITION

(6a*R*,9*R*,10a*R*)-*N*-(2*R*,5*S*,10a*S*,10b*S*)-5-Benzyl-10b-hydroxy-2-(1-methylethyl)-3,6-dioxo-octahydro-8*H*-oxazolo[3,2-*a*]pyrrolo[2,1-*c*]pyrazin-2-yl]-7-methyl-4,6,6a,7,8,9,10,10a-octahydroindolo[4,3-*fg*]quinoline-9-carboxamide methanesulphonate.

Content: 98.0 per cent to 102.0 per cent (dried substance).

PRODUCTION

The production method must be evaluated to determine the potential for formation of alkyl mesilates, which is particularly likely to occur if the reaction medium contains lower alcohols. Where necessary, the production method is validated to demonstrate that alkyl mesilates are not detectable in the final product.

CHARACTERS

Appearance: white or almost white, fine crystalline powder.

Solubility: slightly soluble in water, soluble in methanol.

IDENTIFICATION

A. Infrared absorption spectrophotometry (2.2.24).

Preparation: discs.

Comparison: dihydroergocristine mesilate CRS.

B. Thin-layer chromatography (2.2.27).

Test solution. Dissolve 0.10 g of the substance to be examined in a mixture of 1 volume of *methanol R* and 9 volumes of *methylene chloride R* and dilute to 5 ml with the same mixture of solvents.

Reference solution. Dissolve 0.10 g of *dihydroergocristine mesilate CRS* in a mixture of 1 volume of *methanol R* and 9 volumes of *methylene chloride R* and dilute to 5 ml with the same mixture of solvents.

Plate: TLC silica gel F_{254} plate *R*.

Mobile phase: concentrated ammonia *R*, dimethylformamide *R*, ether *R* (2:15:85 V/V/V).

Application: 5 µl.

Development: over 2/3 of the plate protected from light.

Drying: in a current of cold air for 5 min.

Detection: spray with dimethylaminobenzaldehyde solution *R7* and dry in a current of hot air for 2 min.

Results: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

Test solution. Dissolve 0.20 g of the substance to be examined in a mixture of 1 volume of *methanol R* and 9 volumes of *methylene chloride R* and dilute to 5 ml with the same mixture of solvents.

Reference solution. Dissolve 0.20 g of *methanesulphonic acid R* in a mixture of 1 volume of *methanol R* and 9 volumes of *methylene chloride R* and dilute to 5 ml with the same mixture of solvents. Dilute 1 ml of the solution to 10 ml with a mixture of 1 volume of *methanol R* and 9 volumes of *methylene chloride R*.

Plate: TLC silica gel F_{254} plate *R*.

Mobile phase: water *R*, concentrated ammonia *R*, butanol *R*, acetone *R* (5:10:20:65 V/V/V/V).

Application: 10 µl.

Development: over a path of 10 cm protected from light.

Drying: in a current of cold air for not more than 1 min.

Detection: spray with a 1 g/l solution of *bromocresol purple R* in *methanol R*, adjusting the colour to violet-red with one drop of *dilute ammonia R1* and dry the plate in a current of hot air at 100 °C.

Results: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

TESTS

Appearance of solution. The solution is clear (2.2.1) and not more intensely coloured than reference solution B₇ (2.2.2, Method II).

Dissolve 0.50 g in *methanol R* and dilute to 25.0 ml with the same solvent.

pH (2.2.3): 4.0 to 5.0.

Dissolve 0.10 g in *carbon dioxide-free water R* and dilute to 20 ml with the same solvent.

Specific optical rotation (2.2.7): −37 to −43 (dried substance).

Dissolve 0.250 g in *anhydrous pyridine R* and dilute to 25.0 ml with the same solvent.

Related substances. Liquid chromatography (2.2.29). Carry out the test and preparation of the solutions protected from bright light.

Test solution. Dissolve 75.0 mg of the substance to be examined in 10 ml of *acetonitrile R*. Add 10 ml of a 1.0 g/l solution of *phosphoric acid R* and dilute to 50.0 ml with *water R*.

Reference solution. Dissolve 20.0 mg of *codergocrine mesilate CRS* in 10 ml of *acetonitrile R*. Add 10 ml of a 1.0 g/l solution of *phosphoric acid R* and dilute to 50.0 ml with *water R*. Dilute 6.0 ml of the solution to 50.0 ml with a mixture of 20 volumes of *acetonitrile R*, 20 volumes of a 1.0 g/l solution of *phosphoric acid R* and 60 volumes of *water R*.

Column:

- size: $l = 0.25$ m, $\varnothing = 4.6$ mm,
- stationary phase: octadecylsilyl silica gel for chromatography *R* (5 µm) with a pore size of 10 nm and a carbon loading of 19 per cent.

Mobile phase:

- mobile phase A: mix 100 volumes of *acetonitrile R* with 900 volumes of *water R* and add 10 volumes of *triethylamine R*,

- *mobile phase B*: mix 100 volumes of *water R* with 900 volumes of *acetonitrile R* and add 10 volumes of *triethylamine R*.

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)
0 - 5	75	25
5 - 20	75 → 25	25 → 75
20 - 22	25 → 75	75 → 25
22 - 30	75	25

Flow rate: 1.2 ml/min.

Detection: spectrophotometer at 280 nm.

Injection: 10 µl.

Relative retention with reference to dihydroergocristine (retention time = about 13.7 min): impurity F = about 0.8; impurity H = about 0.9; impurity I = about 1.02.

System suitability: reference solution:

- the chromatogram shows 4 peaks,
- *resolution*: minimum 1 between the peaks corresponding to dihydroergocristine and impurity I.

Limits:

- *any impurity*: not more than the area of the peak corresponding to dihydroergocristine in the chromatogram obtained with the reference solution (1 per cent),
- *total*: not more than twice the area of the peak corresponding to dihydroergocristine in the chromatogram obtained with the reference solution (2 per cent),
- *disregard limit*: 0.1 times the area of the peak corresponding to dihydroergocristine in the chromatogram obtained with the reference solution (0.1 per cent).

Loss on drying (2.2.32): maximum 3.0 per cent, determined on 0.500 g by drying under high vacuum at 80 °C.

ASSAY

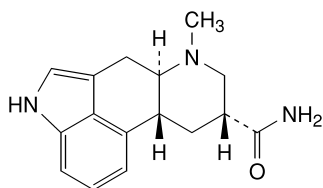
Dissolve 0.300 g in 60 ml of *pyridine R*. Pass a stream of *nitrogen R* over the surface of the solution and titrate with 0.1 M *tetrabutylammonium hydroxide*, determining the end-point potentiometrically (2.2.20). Note the volume used at the second point of inflexion.

1 ml of 0.1 M *tetrabutylammonium hydroxide* is equivalent to 35.39 mg of C₃₆H₄₅N₅O₈S.

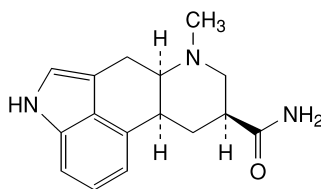
STORAGE

Store protected from light.

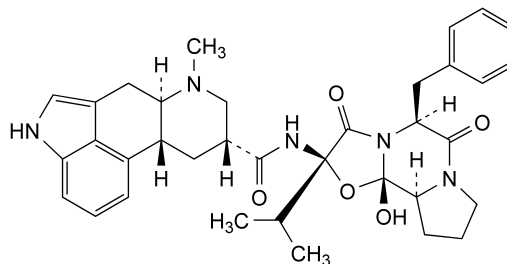
IMPURITIES



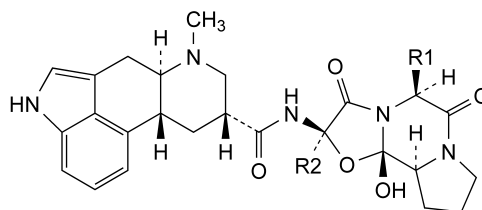
- A. (6aR,9R,10aR)-7-methyl-4,6,6a,7,8,9,10,10a-octahydroindolo[4,3-fg]quinoline-9-carboxamide (6-methylergoline-8β-carboxamide),



- B. (6aR,9S,10aS)-7-methyl-4,6,6a,7,8,9,10,10a-octahydroindolo[4,3-fg]quinoline-9-carboxamide (6-methylisoergoline-8α-carboxamide),



- C. (6aR,9R,10aR)-N-[(2S,5S,10aS,10bS)-5-benzyl-10b-hydroxy-2-(1-methylethyl)-3,6-dioxooctahydro-8H-oxazolo[3,2-a]pyrrolo[2,1-c]pyrazin-2-yl]-7-methyl-4,6,6a,7,8,9,10,10a-octahydroindolo[4,3-fg]quinoline-9-carboxamide (2'-epidihydroergocristine),



- D. R1 = CH(CH₃)₂, R2 = CH₃: (6aR,9R,10aR)-N-[(2R,5S,10aS,10bS)-10b-hydroxy-2-methyl-5-(1-methylethyl)-3,6-dioxooctahydro-8H-oxazolo[3,2-a]pyrrolo[2,1-c]pyrazin-2-yl]-7-methyl-4,6,6a,7,8,9,10,10a-octahydroindolo[4,3-fg]quinoline-9-carboxamide (dihydroergosine),

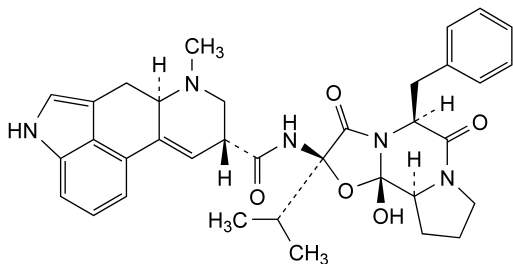
- E. R1 = CH₂-C₆H₅, R2 = CH₃: (6aR,9R,10aR)-N-[(2R,5S,10aS,10bS)-5-benzyl-10b-hydroxy-2-methyl-3,6-dioxooctahydro-8H-oxazolo[3,2-a]pyrrolo[2,1-c]pyrazin-2-yl]-7-methyl-4,6,6a,7,8,9,10,10a-octahydroindolo[4,3-fg]quinoline-9-carboxamide (dihydroergotamine),

- F. R1 = R2 = CH(CH₃)₂: (6aR,9R,10aR)-N-[(2R,5S,10aS,10bS)-10b-hydroxy-2,5-bis(1-methylethyl)-3,6-dioxooctahydro-8H-oxazolo[3,2-a]pyrrolo[2,1-c]pyrazin-2-yl]-7-methyl-4,6,6a,7,8,9,10,10a-octahydroindolo[4,3-fg]quinoline-9-carboxamide (dihydroergocornine),

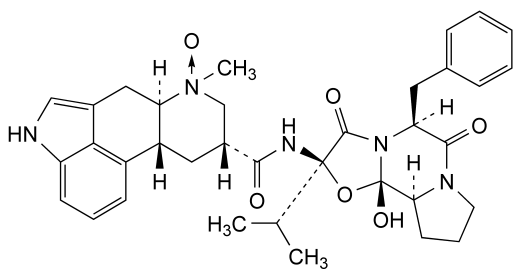
- G. R1 = CH₂-C₆H₅, R2 = CH₂-CH₃: (6aR,9R,10aR)-N-[(2R,5S,10aS,10bS)-5-benzyl-2-ethyl-10b-hydroxy-3,6-dioxooctahydro-8H-oxazolo[3,2-a]pyrrolo[2,1-c]pyrazin-2-yl]-7-methyl-4,6,6a,7,8,9,10,10a-octahydroindolo[4,3-fg]quinoline-9-carboxamide (dihydroergostine),

- H. R1 = CH₂-CH(CH₃)₂, R2 = CH(CH₃)₂: (6aR,9R,10aR)-N-[(2R,5S,10aS,10bS)-10b-hydroxy-2-(1-methylethyl)-5-(2-methylpropyl)-3,6-dioxooctahydro-8H-oxazolo[3,2-a]pyrrolo[2,1-c]pyrazin-2-yl]-7-methyl-4,6,6a,7,8,9,10,10a-octahydroindolo[4,3-fg]quinoline-9-carboxamide (α-dihydroergocryptine),

- I. $R_1 = C^*H(CH_3)-CH_2-CH_3$, $R_2 = CH(CH_3)_2$: (6a*R*,9*R*,10a*R*)-*N*[(2*R*,5*S*,10a*S*,10b*S*)-10b-hydroxy-2-(1-methylethyl)-5-[(1*RS*-1-methylpropyl)-3,6-dioxooctahydro-8*H*-oxazolo[3,2-*a*]pyrrolo[2,1-*c*]pyrazin-2-yl]-7-methyl-4,6,6a,7,8,9,10,10a-octahydroindolo[4,3-*fg*]quinoline-9-carboxamide (β-dihydroergocryptine or epicriptine),
- J. $R_1 = CH_2-C_6H_5$, $R_2 = C^*H(CH_3)-CH_2-CH_3$: (6a*R*,9*R*,10a*R*)-*N*[(2*R*,5*S*,10a*S*,10b*S*)-5-benzyl-10b-hydroxy-2-[(1*RS*)-1-methylpropyl]-3,6-dioxooctahydro-8*H*-oxazolo[3,2-*a*]pyrrolo[2,1-*c*]pyrazin-2-yl]-7-methyl-4,6,6a,7,8,9,10,10a-octahydroindolo[4,3-*fg*]quinoline-9-carboxamide (dihydroergosedmine),



- K. (6a*R*,9*R*,10a*R*)-*N*[(2*R*,5*S*,10a*S*,10b*S*)-5-benzyl-10b-hydroxy-2-(1-methylethyl)-3,6-dioxooctahydro-8*H*-oxazolo[3,2-*a*]pyrrolo[2,1-*c*]pyrazin-2-yl]-7-methyl-4,6,6a,7,8,9-hexahydroindolo[4,3-*fg*]quinoline-9-carboxamide (ergocristine),

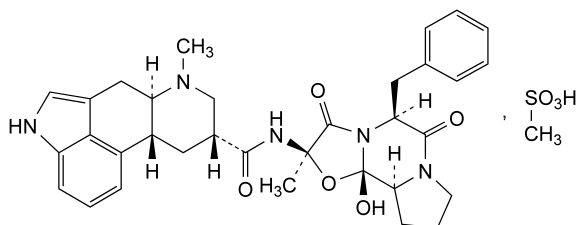


- L. (6a*R*,7*RS*,9*R*,10a*R*)-*N*[(2*R*,5*S*,10a*S*,10b*S*)-5-benzyl-10b-hydroxy-2-(1-methylethyl)-3,6-dioxooctahydro-8*H*-oxazolo[3,2-*a*]pyrrolo[2,1-*c*]pyrazin-2-yl]-7-methyl-4,6,6a,7,8,9,10,10a-octahydroindolo[4,3-*fg*]quinoline-9-carboxamide 7-oxide (dihydroergocristine 6-oxide).

01/2005:0551

DIHYDROERGOTAMINE MESILATE

Dihydroergotamini mesilas

 $C_{34}H_{41}N_5O_8S$ M_r 680

DEFINITION

(6a*R*,9*R*,10a*R*)-*N*[(2*R*,5*S*,10a*S*,10b*S*)-5-Benzyl-10b-hydroxy-2-methyl-3,6-dioxooctahydro-8*H*-oxazolo[3,2-*a*]pyrrolo[2,1-*c*]pyrazin-2-yl]-7-methyl-4,6,6a,7,8,9,10,10a-octahydroindolo[4,3-*fg*]quinoline-9-carboxamide methanesulphonate.

Content: 98.0 per cent to 101.0 per cent (dried substance).

PRODUCTION

The production method must be evaluated to determine the potential for formation of alkyl mesilates, which is particularly likely to occur if the reaction medium contains lower alcohols. Where necessary, the production method is validated to demonstrate that alkyl mesilates are not detectable in the final product.

CHARACTERS

Appearance: white or almost white, crystalline powder or colourless crystals.

Solubility: slightly soluble in water, sparingly soluble in methanol, slightly soluble in alcohol.

IDENTIFICATION

First identification: B, C.

Second identification: A, C, D.

- A. Dissolve 5.0 mg in *methanol R* and dilute to 100.0 ml with the same solvent. Examined between 250 nm and 350 nm (2.2.25), the solution shows 2 absorption maxima, at 281 nm and 291 nm, and a shoulder at 275 nm. Above 320 nm the absorbance is negligible. The specific absorbance at the absorption maximum at 281 nm is 95 to 105 (dried substance).

- B. Infrared absorption spectrophotometry (2.2.24).

Preparation: discs.

Comparison: *dihydroergotamine mesilate CRS*.

- C. Examine the chromatograms obtained in the test for related substances.

Results: the principal spot in the chromatogram obtained with test solution (b) is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).

- D. To 0.1 g add 5 ml of *dilute hydrochloric acid R* and shake for about 5 min. Filter and add 1 ml of *barium chloride solution RI*. The filtrate remains clear. Mix 0.1 g with 0.4 g of powdered *sodium hydroxide R*, heat to fusion and continue to heat for 1 min. Cool, add 5 ml of *water R*, boil and filter. Acidify the filtrate by the addition of *hydrochloric acid RI* and filter again. The filtrate gives reaction (a) of sulphates (2.3.1).

TESTS

Appearance of solution. The solution is clear (2.2.1) and not more intensely coloured than reference solution Y₇ or BY₇ (2.2.2, *Method II*).

Dissolve 0.10 g in a mixture of 0.1 ml of a 70 g/l solution of *methanesulphonic acid R* and 50 ml of *water R*.

pH (2.2.3): 4.4 to 5.4.

Dissolve 0.10 g in *carbon dioxide-free water R* and dilute to 100 ml with the same solvent.

Specific optical rotation (2.2.7): −42 to −47 (dried substance).

Dissolve 0.250 g in *anhydrous pyridine R* and dilute to 25.0 ml with the same solvent.

Related substances. Thin-layer chromatography (2.2.27). Prepare the reference solutions and the test solutions immediately before use and in the order indicated below.

Reference solution (a). Dissolve 10.0 mg of *dihydroergotamine mesilate CRS* in a mixture of 1 volume of *methanol R* and 9 volumes of *methylene chloride R* and dilute to 5 ml with the same mixture of solvents.

01/2005:0600

Reference solution (b). Dilute 2.5 ml of reference solution (a) to 50 ml with a mixture of 1 volume of *methanol R* and 9 volumes of *methylene chloride R*.

Reference solution (c). Dilute 2 ml of reference solution (b) to 5 ml with a mixture of 1 volume of *methanol R* and 9 volumes of *methylene chloride R*.

Test solution (a). Dissolve 0.10 g of the substance to be examined in a mixture of 1 volume of *methanol R* and 9 volumes of *methylene chloride R* and dilute to 5 ml with the same mixture of solvents.

Test solution (b). Dilute 1 ml of test solution (a) to 10 ml with a mixture of 1 volume of *methanol R* and 9 volumes of *methylene chloride R*.

Plate: TLC silica gel *G* plate *R*.

Mobile phase: concentrated ammonia *R*, *methanol R*, *ethyl acetate R*, *methylene chloride R* (1:6:50:50 *V/V/V/V*).

Application: 5 µl.

Development: protected from light over a path of 15 cm. Dry the plate in a current of cold air for not longer than 1 min. Repeat the development protected from light over a path of 15 cm using a freshly prepared amount of the mobile phase.

Drying: in a current of cold air.

Detection: spray abundantly with *dimethylaminobenzaldehyde solution R7* and dry in a current of hot air for about 2 min.

Limits: test solution (a):

- **any impurity:** any spot, apart from the principal spot, is not more intense than the principal spot in the chromatogram obtained with reference solution (b) (0.5 per cent) and not more than 2 such spots are more intense than the principal spot in the chromatogram obtained with reference solution (c) (0.2 per cent).

Loss on drying (2.2.32): maximum 4.0 per cent, determined on 0.500 g by drying at 100–105 °C at a pressure not exceeding 0.1 kPa for 5 h.

ASSAY

Dissolve 0.500 g in a mixture of 10 ml of *anhydrous acetic acid R* and 70 ml of *acetic anhydride R*. Titrate with 0.1 *M* *perchloric acid*, determining the end-point potentiometrically (2.2.20).

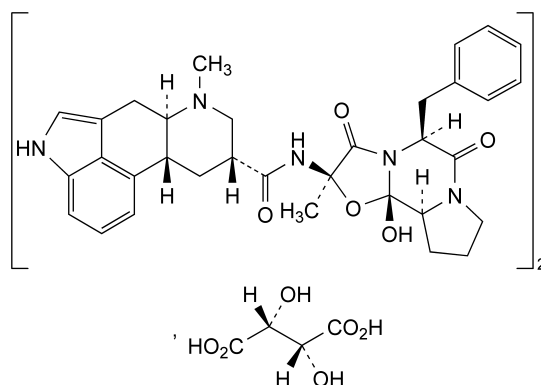
1 ml of 0.1 *M* *perchloric acid* is equivalent to 68.00 mg of $C_{34}H_{41}N_5O_8S$.

STORAGE

Protected from light.

DIHYDROERGOTAMINE TARTRATE

Dihydroergotamini tartras


 $C_{70}H_{80}N_{10}O_{16}$
 M_r 1317

DEFINITION

Bis[(6a*R*,9*R*,10a*R*)-*N*[(2*R*,5*S*,10a*S*,10b*S*)-5-benzyl-10b-hydroxy-2-methyl-3,6-dioxooctahydro-8*H*-oxazolo[3,2-*a*]pyrrolo[2,1-*c*]pyrazin-2-yl]-7-methyl-4,6,6a,7,8,9,10,10a-octahydroindolo[4,3-*fg*]quinoline-9-carboxamide] (2*R*,3*R*)-2,3-dihydroxybutanedioate.

Content: 98.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

Appearance: white or almost white, crystalline powder or colourless crystals.

Solubility: very slightly soluble in water, sparingly soluble in alcohol.

IDENTIFICATION

First identification: B, C.

Second identification: A, C, D.

A. Dissolve 5.0 mg in *methanol R* and dilute to 100.0 ml with the same solvent. Examined between 250 nm and 350 nm (2.2.25), the solution shows 2 absorption maxima, at 281 nm and 291 nm, and a shoulder at 275 nm. Above 320 nm the absorbance is negligible. The specific absorbance at the maximum at 281 nm is 95 to 115 (dried substance).

B. Infrared absorption spectrophotometry (2.2.24).

Preparation: discs.

Comparison: *dihydroergotamine tartrate CRS*.

C. Examine the chromatograms obtained in the test for related substances.

Results: the principal spot in the chromatogram obtained with test solution (b) is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).

D. Suspend about 15 mg in 1 ml of *water R*. 0.1 ml of the suspension gives reaction (b) of tartrates (2.3.1).

TESTS

Appearance of solution. The solution is clear (2.2.1) and not more intensely coloured than reference solution Y_7 or BY_7 (2.2.2, *Method II*).

Dissolve 0.1 g in *alcohol* (85 per cent *V/V*) *R* warming carefully in a water-bath at 40 °C and dilute to 50 ml with the same solvent.

pH (2.2.3): 4.0 to 5.5 for the clear supernatant.

01/2005:0485

Suspend 50 mg in 50 ml of *carbon dioxide-free water R* and shake for 10 min. Allow to stand.

Specific optical rotation (2.2.7): -52 to -57 (dried substance).

Dissolve 0.250 g in *anhydrous pyridine R* and dilute to 25.0 ml with the same solvent.

Related substances. Thin-layer chromatography (2.2.27). Prepare the reference solutions and the test solutions immediately before use and in the order indicated.

Reference solution (a). Dissolve 20 mg of *dihydroergotamine tartrate CRS* in a mixture of 1 volume of *methanol R* and 9 volumes of *chloroform R* and dilute to 10 ml with the same mixture of solvents.

Reference solution (b). Dilute 2.5 ml of reference solution (a) to 50 ml with a mixture of 1 volume of *methanol R* and 9 volumes of *chloroform R*.

Reference solution (c). Dilute 2 ml of reference solution (b) to 5 ml with a mixture of 1 volume of *methanol R* and 9 volumes of *chloroform R*.

Test solution (a). Dissolve 0.10 g of the substance to be examined in a mixture of 1 volume of *methanol R* and 9 volumes of *chloroform R* and dilute to 5 ml with the same mixture of solvents.

Test solution (b). Dilute 1 ml of test solution (a) to 10 ml with a mixture of 1 volume of *methanol R* and 9 volumes of *chloroform R*.

Plate: TLC silica gel G plate *R*.

Mobile phase: concentrated ammonia *R*, methanol *R*, ethyl acetate *R*, methylene chloride *R* (1:6:50:50 V/V/V/V).

Application: 5 μ l.

Development: protected from light over a path of 15 cm. Dry the plate in a current of cold air for not longer than 1 min. Repeat the development protected from light over a path of 15 cm using a freshly prepared amount of the mobile phase.

Drying: in a current of cold air.

Detection: spray the plate abundantly with *dimethylaminobenzaldehyde solution R7* and dry in a current of hot air for about 2 min.

Limits: in the chromatogram obtained with test solution (a):

- **any impurity:** any spot, apart from the principal spot, is not more intense than the principal spot in the chromatogram obtained with reference solution (b) (0.5 per cent) and not more than 2 such spots are more intense than the principal spot in the chromatogram obtained with reference solution (c) (0.2 per cent).

Loss on drying (2.2.32): maximum 5.0 per cent, determined on 0.200 g by drying in an oven at 100–105 °C.

ASSAY

Dissolve 0.250 g in 50 ml of *anhydrous acetic acid R*. Titrate with 0.05 M *perchloric acid*, determining the end-point potentiometrically (2.2.20).

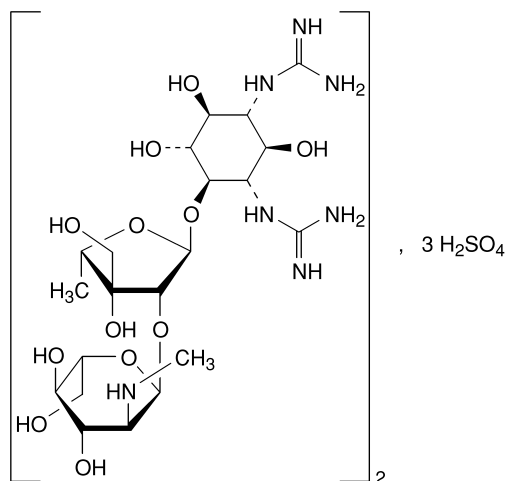
1 ml of 0.05 M *perchloric acid* is equivalent to 32.93 mg of $C_{42}H_{88}N_{14}O_{36}S_3$.

STORAGE

Protected from light.

DIHYDROSTREPTOMYCIN SULPHATE FOR VETERINARY USE

Dihydrostreptomycini sulfas
ad usum veterinarium


 $C_{42}H_{88}N_{14}O_{36}S_3$
 M_r 1461

DEFINITION

Dihydrostreptomycin sulphate for veterinary use is bis[*N,N'*-bis(aminoiminomethyl)-4-*O*-[5-deoxy-2-*O*-[2-deoxy-2-(methylamino)- α -L-glucopyranosyl]-3-*C*-(hydroxymethyl)- α -L-lyxofuranosyl]-D-streptamine] tris(sulphate), the sulphate of a substance obtained by catalytic hydrogenation of streptomycin or by any other means. Stabilisers may be added. The potency is not less than 730 IU/mg, calculated with reference to the dried substance.

PRODUCTION

The method of manufacture is validated to demonstrate that the product if tested would comply with the following test:

Abnormal toxicity (2.6.9). Inject into each mouse 1 mg dissolved in 0.5 ml of *water for injections R*.

CHARACTERS

A white or almost white powder, freely soluble in water, practically insoluble in acetone, in alcohol and in methanol. It may be hygroscopic.

IDENTIFICATION

A. Examine by thin-layer chromatography (2.2.27) using a plate coated with a 0.75 mm layer of the following mixture: mix 0.3 g of *carbomer R* with 240 ml of *water R* and allow to stand, with moderate shaking, for 1 h; adjust to pH 7 by the gradual addition, with continuous shaking, of *dilute sodium hydroxide solution R* and add 30 g of *silica gel H R*.

Heat the plate at 110 °C for 1 h, allow to cool and use immediately.

Test solution. Dissolve 10 mg of the substance to be examined in *water R* and dilute to 10 ml with the same solvent.

Reference solution (a). Dissolve 10 mg of *dihydrostreptomycin sulphate CRS* in *water R* and dilute to 10 ml with the same solvent.

Reference solution (b). Dissolve 10 mg of *dihydrostreptomycin sulphate CRS*, 10 mg of *kanamycin monosulphate CRS* and 10 mg of *neomycin sulphate CRS* in *water R* and dilute to 10 ml with the same solvent.

Apply separately to the plate 10 µl of each solution. Develop over a path of 15 cm using a 70 g/l solution of *potassium dihydrogen phosphate R*. Dry the plate in a current of warm air and spray with a mixture of equal volumes of a 2 g/l solution of *1,3-dihydroxynaphthalene R* in *alcohol R* and a 460 g/l solution of *sulphuric acid R*. Heat at 150 °C for 5-10 min. The principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a). The test is not valid unless the chromatogram obtained with reference solution (b) shows 3 clearly separated spots.

- B. Dissolve 0.1 g in 2 ml of *water R*, add 1 ml of α -*naphthol solution R* and 2 ml of a mixture of equal volumes of *strong sodium hypochlorite solution R* and *water R*. A red colour develops.
- C. Dissolve 10 mg in 5 ml of *water R* and add 1 ml of 1 M *hydrochloric acid*. Heat in a water-bath for 2 min. Add 2 ml of a 5 g/l solution of α -*naphthol R* in 1 M *sodium hydroxide* and heat in a water-bath for 1 min. A violet-pink colour is produced.
- D. It gives reaction (a) of sulphates (2.3.1).

TESTS

Solution S. Dissolve 2.5 g in *carbon dioxide-free water R* and dilute to 10 ml with the same solvent.

Appearance of solution. Solution S is not more intensely coloured than intensity 5 of the range of reference solutions of the most appropriate colour (2.2.2, *Method II*). Allow to stand protected from light at a temperature of about 20 °C for 24 h; solution S is not more opalescent than reference suspension II (2.2.1).

pH (2.2.3). The pH of solution S is 5.0 to 7.0.

Specific optical rotation (2.2.7). Dissolve 0.200 g in *water R* and dilute to 10.0 ml with the same solvent. The specific optical rotation is –83 to –91, calculated with reference to the dried substance.

Methanol. Not more than 0.2 per cent, determined by gas chromatography (2.2.28).

Test solution. Dissolve 1.00 g of the substance to be examined in *water R* and dilute to 25.0 ml with the same solvent.

Reference solution. Dilute 8.0 mg of *methanol R* to 100 ml with *water R*.

The chromatographic procedure may be carried out using:

- a column 1.5 m to 2.0 m long and 2.0 mm to 4.0 mm in internal diameter packed with *ethylvinylbenzene-divinylbenzene copolymer R* (150-180 µm),
- *nitrogen for chromatography R* as the carrier gas at a constant flow rate of 30-40 ml/min,
- a flame-ionisation detector.

Maintain the column at a constant temperature between 120 °C and 140 °C and the injection port and the detector at a temperature at least 50 °C higher than that of the column. The area of the peak corresponding to methanol in the chromatogram obtained with the test solution is not greater than the area of the peak in the chromatogram obtained with the reference solution.

Streptomycin. Dissolve 0.100 g in *water R* and dilute to 5.0 ml with the same solvent. Add 5.0 ml of 0.2 M *sodium hydroxide* and heat in a water-bath for exactly 10 min. Cool in ice for exactly 5 min, add 3 ml of a 15 g/l solution of *ferric ammonium sulphate R* in 0.25 M *sulphuric acid*, dilute to 25.0 ml with *water R* and mix (test solution). Prepare at the same time and in the same manner a reference solution using 10 mg of *streptomycin sulphate CRS* in 50 ml of *water R*. Using 5.0 ml of this solution, operate as for the test solution. Exactly 20 min after addition of the ferric ammonium sulphate solution, measure the absorbance (2.2.25) of each solution at the maximum at 525 nm in a 2 cm cell, using as the compensation liquid a solution prepared at the same time and in the same manner as the test solution but omitting the substance to be examined. The absorbance of the test solution is not greater than that of the reference solution (1 per cent).

Heavy metals (2.4.8). 1.0 g complies with limit test C for heavy metals (20 ppm). Prepare the standard using 2 ml of *lead standard solution (10 ppm Pb) R*.

Loss on drying (2.2.32). Not more than 5.0 per cent, determined on 1.000 g by drying at 60 °C over *diphosphorus pentoxide R* at a pressure not exceeding 0.1 kPa for 4 h.

Sulphated ash (2.4.14). Not more than 1.0 per cent, determined on 1.0 g.

Sulphate. 18.0 per cent to 21.5 per cent of sulphate (SO₄), calculated with reference to the dried substance. Dissolve 0.250 g in 100 ml of *water R* and adjust the solution to pH 11 using *concentrated ammonia R*. Add 10.0 ml of 0.1 M *barium chloride* and about 0.5 mg of *phthalein purple R*. Titrate with 0.1 M *sodium edetate*, adding 50 ml of *alcohol R* when the colour of the solution begins to change and continue the titration until the violet-blue colour disappears.

1 ml of 0.1 M *barium chloride* is equivalent to 9.606 mg of sulphate (SO₄).

Bacterial endotoxins (2.6.14): less than 0.50 IU/mg, if intended for use in the manufacture of parenteral dosage forms without a further appropriate procedure for removal of bacterial endotoxins.

ASSAY

Carry out the microbiological assay of antibiotics (2.7.2).

STORAGE

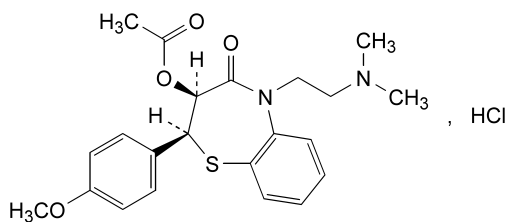
In an airtight container, protected from light. If the substance is sterile, store in a sterile, airtight, tamper-proof container.

LABELLING

The label states:

- where applicable, the name and quantity of any added stabiliser,
- where applicable, that the substance is free from bacterial endotoxins.

01/2005:1004

DILTIAZEM HYDROCHLORIDE**Diltiazemi hydrochloridum** $C_{22}H_{27}ClN_2O_4S$ M_r 451.0**DEFINITION**

Diltiazem hydrochloride contains not less than 98.5 per cent and not more than the equivalent of 101.0 per cent of (2*S*,3*S*)-5-[2-(dimethylamino)ethyl]-2-(4-methoxyphenyl)-4-oxo-2,3,4,5-tetrahydro-1,5-benzothiazepin-3-yl acetate hydrochloride, calculated with reference to the dried substance.

CHARACTERS

A white, crystalline powder, freely soluble in water, in methanol and in methylene chloride, slightly soluble in ethanol. It melts at about 213 °C with decomposition.

IDENTIFICATION

First identification: A, D.

Second identification: B, C, D.

A. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *diltiazem hydrochloride CRS*. Examine the substances prepared as discs.

B. Examine by thin-layer chromatography (2.2.27), using as the coating substance a suitable silica gel with a fluorescent indicator having an optimal intensity at 254 nm.

Test solution. Dissolve 0.10 g of the substance to be examined in *methylene chloride R* and dilute to 10 ml with the same solvent.

Reference solution. Dissolve 0.10 g of *diltiazem hydrochloride CRS* in *methylene chloride R* and dilute to 10 ml with the same solvent.

Apply to the plate 10 µl of each solution. Develop over a path of 10 cm using a mixture of 1 volume of *acetic acid R*, 3 volumes of *water R*, 10 volumes of *methylene chloride R* and 12 volumes of *ethanol R*. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. The principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with the reference solution.

C. Dissolve 50 mg in 5 ml of *water R*. Add 1 ml of *ammonium reineckate solution R*. A pink precipitate is produced.

D. It gives reaction (a) of chlorides (2.3.1).

TESTS

Solution S. Dissolve 1.00 g in *carbon-dioxide free water R* and dilute to 20.0 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and colourless (2.2.2, *Method II*).

pH (2.2.3). Dilute 2.0 ml of solution S to 10.0 ml with *carbon dioxide-free water R*. The pH of the solution is 4.3 to 5.3.

Specific optical rotation (2.2.7). Dilute 5.0 ml of solution S to 25.0 ml with *water R*. The specific optical rotation is + 115 to + 120, calculated with reference to the dried substance.

Related substances. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 50.0 mg of the substance to be examined in the mobile phase and dilute to 200.0 ml with the mobile phase.

Reference solution(a). Dissolve 50.0 mg of *diltiazem hydrochloride CRS* in the mobile phase and dilute to 200.0 ml with the mobile phase.

Reference solution (b). Dissolve 3 mg of *diltiazem impurity A CRS* in the mobile phase and dilute to 10 ml with the mobile phase. To 1 ml of this solution, add 1.2 ml of reference solution (a) and dilute to 100.0 ml with the mobile phase.

Reference solution(c). Dilute 0.3 ml of the test solution to 100.0 ml with the mobile phase.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.10 m long and 4.6 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (3 µm),
- as mobile phase at a flow rate of 1.5 ml/min a mixture of 5 volumes of *ethanol R*, 25 volumes of *acetonitrile R* and 70 volumes of a solution containing, in 1 litre, 6.8 g of *potassium dihydrogen phosphate R* and 0.1 ml of *N,N-dimethyloctylamine R*, adjusted to pH 4.5 using *dilute phosphoric acid R*,
- as detector a spectrophotometer set at 240 nm,
- a loop injector.

Inject separately 20 µl of each solution. Adjust the sensitivity of the detector so that the height of the principal peak in the chromatogram obtained with reference solution (c) is at least 10 per cent of the full scale of the recorder. Continue the chromatography for five times the retention time of the principal peak. The test is not valid unless in the chromatogram obtained with reference solution (b), the resolution between the peaks due to diltiazem impurity A and diltiazem is at least 4.0 and the symmetry factors are not more than 2.0. If necessary, adjust the concentration of *N,N-dimethyloctylamine* in the mobile phase. In the chromatogram obtained with the test solution, the sum of the areas of any peaks, apart from the principal peak, is not greater than the area of the principal peak in the chromatogram obtained with reference solution (c) (0.3 per cent). Disregard any peak with an area less than 0.025 times that of the peak in the chromatogram obtained with reference solution (c).

Heavy metals (2.4.8). Dissolve 2.0 g in *water R* and dilute to 20.0 ml with the same solvent. 12 ml of the solution complies with limit test A for heavy metals (10 ppm). Prepare the standard using *lead standard solution (1 ppm Pb) R*.

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 100-105 °C for 2 h.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

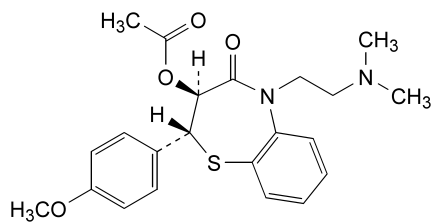
Dissolve 0.400 g in a mixture of 2 ml of *anhydrous formic acid R* and 60 ml of *acetic anhydride R* and titrate with 0.1 M *perchloric acid*, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *perchloric acid* is equivalent to 45.1 mg of $C_{22}H_{27}ClN_2O_4S$.

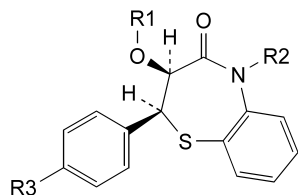
STORAGE

Store in an airtight container, protected from light.

IMPURITIES



- A. (2*R*,3*S*)-5-[2-(dimethylamino)ethyl]-2-(4-methoxyphenyl)-4-oxo-2,3,4,5-tetrahydro-1,5-benzothiazepin-3-yl acetate,

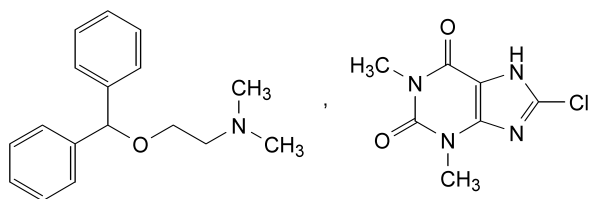


- B. R1 = CO-CH₃, R2 = H, R3 = OCH₃: (2*S*,3*S*)-2-(4-methoxyphenyl)-4-oxo-2,3,4,5-tetrahydro-1,5-benzothiazepin-3-yl acetate,
 C. R1 = CO-CH₃, R2 = CH₂-CH₂-N(CH₃)₂, R3 = OH: (2*S*,3*S*)-5-[2-(dimethylamino)ethyl]-2-(4-hydroxyphenyl)-4-oxo-2,3,4,5-tetrahydro-1,5-benzothiazepin-3-yl acetate,
 D. R1 = CO-CH₃, R2 = CH₂-CH₂-NH-CH₃, R3 = OCH₃: (2*S*,3*S*)-2-(4-methoxyphenyl)-5-[2-(methylamino)ethyl]-4-oxo-2,3,4,5-tetrahydro-1,5-benzothiazepin-3-yl acetate,
 E. R1 = R2 = H, R3 = OCH₃: (2*S*,3*S*)-3-hydroxy-2-(4-methoxyphenyl)-2,3-dihydro-1,5-benzothiazepin-4(5*H*)-one,
 F. R1 = H, R2 = CH₂-CH₂-N(CH₃)₂, R3 = OCH₃: (2*S*,3*S*)-5-[2-(dimethylamino)ethyl]-3-hydroxy-2-(4-methoxyphenyl)-2,3-dihydro-1,5-benzothiazepin-4(5*H*)-one.

01/2005:0601

DIMENHYDRINATE

Dimenhydrinatum

C₂₄H₂₈ClN₅O₃M_r 470.0

DEFINITION

Dimenhydrinate contains not less than 53.0 per cent and not more than 55.5 per cent of diphenhydramine [2-(diphenylmethoxy)-*N,N*-dimethylethylamine, C₁₇H₂₁NO, M_r 255.4] and not less than 44.0 per cent and not more than 46.5 per cent of 8-chlorotheophylline (8-chloro-3,7-dihydro-1,3-dimethyl-1*H*-purine-2,6-dione, C₇H₇ClN₄O₂, M_r 214.6), both calculated with reference to the dried substance.

CHARACTERS

A white, crystalline powder or colourless crystals, slightly soluble in water, freely soluble in alcohol.

IDENTIFICATION

First identification: C.

Second identification: A, B, D.

- A. Melting point (2.2.14): 102 °C to 106 °C.
 B. Dissolve 0.1 g in a mixture of 3 ml of *water R* and 3 ml of *alcohol R*, add 6 ml of *water R* and 1 ml of *dilute hydrochloric acid R* and cool in iced water for 30 min, scratching the wall of the tube with a glass rod if necessary to initiate crystallisation. Dissolve about 10 mg of the precipitate obtained in 1 ml of *hydrochloric acid R*, add 0.1 g of *potassium chlorate R* and evaporate to dryness in a porcelain dish. A reddish residue is obtained which becomes violet-red when exposed to ammonia vapour.
 C. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *dimenhydrinate CRS*.
 D. Dissolve 0.2 g in 10 ml of *alcohol R*. Add 10 ml of *picric acid solution R* and initiate crystallisation by scratching the wall of the tube with a glass rod. The precipitate, washed with *water R* and dried at 100 °C to 105 °C, melts (2.2.14) at 130 °C to 134 °C.

TESTS

Appearance of solution. Dissolve 1.0 g in *alcohol R* and dilute to 20 ml with the same solvent. The solution is clear (2.2.1) and colourless (2.2.2, *Method II*).

pH (2.2.3). To 0.4 g add 20 ml of *carbon dioxide-free water R*, shake for 2 min and filter. The pH of the filtrate is 7.1 to 7.6.

Theophylline and substances related to diphenhydramine. Examine by thin-layer chromatography (2.2.27), using *silica gel GF₂₅₄ R* as the coating substance.

Test solution. Dissolve 0.40 g of the substance to be examined in *methylene chloride R* and dilute to 10 ml with the same solvent.

Reference solution (a). Dissolve 20 mg of *theophylline R* in *methylene chloride R* and dilute to 100 ml with the same solvent.

Reference solution (b). Dilute 5 ml of the test solution to 100 ml with *methylene chloride R*. Dilute 10 ml of this solution to 100 ml with *methylene chloride R*.

Apply separately to the plate 5 µl of each solution. Develop over a path of 15 cm using a mixture of 1 volume of *concentrated ammonia R*, 9 volumes of *methanol R* and 90 volumes of *methylene chloride R*. Dry the plate in a current of cold air and examine in ultraviolet light at 254 nm. Any spot corresponding to theophylline in the chromatogram obtained with the test solution is not more intense than the spot in the chromatogram obtained with reference solution (a) (0.5 per cent). Spray with *potassium iodobismuthate solution R*. Allow the plate to dry in air and spray with *dilute hydrogen peroxide solution R*. Any spot in the chromatogram obtained with the test solution, apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.5 per cent). Disregard any spot extending from the starting point to an *R_f* of about 0.1.

Heavy metals (2.4.8). Dissolve 2.5 g in a mixture of 15 volumes of *water R* and 85 volumes of *acetone R* and dilute to 25 ml with the same mixture of solvents. The solution complies with limit test B for heavy metals (20 ppm). Prepare the standard using lead standard solution (2 ppm Pb) prepared by diluting *lead standard solution (100 ppm Pb) R* with a mixture of 15 volumes of *water R* and 85 volumes of *acetone R*.

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying *in vacuo*.

Sulphated ash (2.4.14). Not more than 0.2 per cent, determined on 1.0 g.

ASSAY

Diphenhydramine. Dissolve 0.200 g in 60 ml of *anhydrous acetic acid R*. Titrate with 0.1 M *perchloric acid*, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *perchloric acid* is equivalent to 25.54 mg of $C_{17}H_{21}NO$.

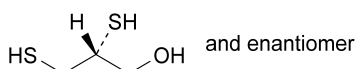
8-Chlorotheophylline. To 0.800 g add 50 ml of *water R*, 3 ml of *dilute ammonia R1* and 0.6 g of *ammonium nitrate R* and heat on a water-bath for 5 min. Add 25.0 ml of 0.1 M *silver nitrate* and continue heating on a water-bath for 15 min with frequent swirling. Cool, add 25 ml of *dilute nitric acid R* and dilute to 250.0 ml with *water R*. Filter and discard the first 25 ml of the filtrate. Using 5 ml of *ferric ammonium sulphate solution R2* as indicator, titrate 100.0 ml of the filtrate with 0.1 M *ammonium thiocyanate* until a yellowish-brown colour is obtained.

1 ml of 0.1 M *silver nitrate* is equivalent to 21.46 mg of $C_7H_7ClN_4O_2$.

01/2005:0389

DIMERCAPROL

Dimercaprolum

 $C_3H_8OS_2$ M_r 124.2

DEFINITION

Dimercaprol contains not less than 98.5 per cent and not more than the equivalent of 101.5 per cent of (2*RS*)-2,3-disulfanylmethylpropan-1-ol.

CHARACTERS

A clear, colourless or slightly yellow liquid, soluble in water and in arachis oil, miscible with alcohol and with benzyl benzoate.

IDENTIFICATION

- Dissolve 0.05 ml in 2 ml of *water R*. Add 1 ml of 0.05 M *iodine*. The colour of the iodine is discharged immediately.
- Dissolve 0.1 ml in 5 ml of *water R* and add 2 ml of *copper sulphate solution R*. A bluish-black precipitate is formed which quickly becomes dark grey.
- In a ground-glass-stoppered tube, suspend 0.6 g of *sodium bismuthate R*, previously heated to 200 °C for 2 h, in a mixture of 2.8 ml of *dilute phosphoric acid R* and 6 ml of *water R*. Add 0.2 ml of the substance to be examined, mix and allow to stand for 10 min with frequent shaking. To 1 ml of the supernatant liquid add 5 ml of a 4 g/l solution of *chromotropic acid, sodium salt R* in *sulphuric acid R* and mix. Heat for 15 min in a water-bath. A violet-red colour develops.

TESTS

Appearance. It is clear (2.2.1) and not more intensely coloured than reference solution B₆ or BY₆ (2.2.2, *Method II*).

Acidity or alkalinity. Dissolve 0.2 g in *carbon dioxide-free water R* and dilute to 10 ml with the same solvent. Add 0.25 ml of *bromocresol green solution R* and 0.3 ml of 0.01 M *hydrochloric acid*. The solution is yellow. Not more than 0.5 ml of 0.01 M *sodium hydroxide* is required to change the colour of the indicator to blue.

Refractive index (2.2.6): 1.568 to 1.574.

Halides. To 2.0 g add 25 ml of *alcoholic potassium hydroxide solution R* and boil under a reflux condenser for 2 h. Eliminate the alcohol by evaporation in a stream of hot air, add 20 ml of *water R* and cool. Add 40 ml of *water R* and 10 ml of *strong hydrogen peroxide solution R*, boil gently for 10 min, cool and filter rapidly. Add 10 ml of *dilute nitric acid R* and 5.0 ml of 0.1 M *silver nitrate*. Using 2 ml of *ferric ammonium sulphate solution R2* as indicator, titrate with 0.1 M *ammonium thiocyanate* until a reddish-yellow colour is obtained. Carry out a blank titration. The difference between the titration volumes is not greater than 1.0 ml.

ASSAY

Dissolve 0.100 g in 40 ml of *methanol R*. Add 20 ml of 0.1 M *hydrochloric acid* and 50.0 ml of 0.05 M *iodine*. Allow to stand for 10 min and titrate with 0.1 M *sodium thiosulphate*. Carry out a blank titration.

1 ml of 0.05 M *iodine* is equivalent to 6.21 mg of $C_3H_8OS_2$.

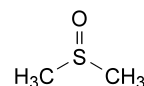
STORAGE

Store in a well-filled, airtight container, protected from light, at a temperature of 2 °C to 8 °C.

01/2005:0763

DIMETHYL SULFOXIDE

Dimethylis sulfoxidum

 C_2H_6OS M_r 78.1

DEFINITION

Dimethyl sulfoxide is sulphinylbismethane.

CHARACTERS

A colourless liquid or colourless crystals, hygroscopic, miscible with water and with alcohol.

IDENTIFICATION

First identification: C.

Second identification: A, B, D.

- It complies with the test for relative density (see Tests).
- It complies with the test for refractive index (see Tests).
- Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *dimethyl sulfoxide CRS*.
- Dissolve 50 mg of *nickel chloride R* in 5 ml of the substance to be examined. The solution is greenish-yellow. Heat in a water-bath at 50 °C. The colour changes to green or bluish-green. Cool. The colour changes to greenish-yellow.

TESTS

Acidity. Dissolve 50.0 g in 100 ml of *carbon dioxide-free water R*. Add 0.1 ml of *phenolphthalein solution R1*. Not more than 5.0 ml of 0.01 M *sodium hydroxide* is required to produce a pink colour.

Relative density (2.2.5): 1.100 to 1.104.

Refractive index (2.2.6): 1.478 to 1.479.

Freezing point (2.2.18). Not less than 18.3 °C.

Absorbance (2.2.25). Purge with *nitrogen R* for 15 min. The absorbance, measured using *water R* as the compensation liquid, is not more than 0.30 at 275 nm and not more than 0.20 at both 285 nm and 295 nm. Examined between 270 nm and 350 nm, the substance to be examined shows no absorption maximum.

Related substances. Examine by gas chromatography (2.2.28), using *bibenzyl R* as the internal standard.

Internal standard solution. Dissolve 0.125 g of *bibenzyl R* in *acetone R* and dilute to 50 ml with the same solvent.

Test solution (a). Dissolve 5.0 g of the substance to be examined in *acetone R* and dilute to 10.0 ml with the same solvent.

Test solution (b). Dissolve 5.0 g of the substance to be examined in *acetone R*, add 1.0 ml of the internal standard solution and dilute to 10.0 ml with *acetone R*.

Reference solution. Dissolve 50.0 mg of the substance to be examined and 50 mg of *dimethyl sulphone R* in *acetone R*, add 10.0 ml of the internal standard solution and dilute to 100.0 ml with *acetone R*.

The chromatographic procedure may be carried out using:

- a glass column 1.5 m long and 4 mm in internal diameter, packed with *diatomaceous earth for gas chromatography R* (125 µm to 180 µm) impregnated with 10 per cent *m/m* of *polyethyleneglycol adipate R*,
- *nitrogen for chromatography R* as the carrier gas at a flow rate of 30 ml/min,
- a flame-ionisation detector,

maintaining the temperature of the column at 165 °C and that of the injection port and of the detector at 190 °C.

Inject 1 µl of the reference solution and adjust the sensitivity of the system so that the heights of the three peaks, apart from the solvent peak, are not less than 70 per cent of the full scale of the recorder. The substances elute in the following order: dimethyl sulfoxide, dimethyl sulphone, bibenzyl. The test is not valid unless, in the chromatogram obtained with the reference solution, the resolution between the peaks due to dimethyl sulfoxide and dimethyl sulphone is at least 3.

Inject 1 µl of test solution (a). In the chromatogram obtained, verify that there is no peak with the same retention time as the internal standard.

Inject 1 µl of test solution (b) and 1 µl of the reference solution. Continue the chromatography for four times the retention time of dimethyl sulfoxide, which is about 5 min. From the chromatogram obtained with the reference solution, calculate the ratio (*R*) of the area of the peak due to dimethyl sulfoxide to the area of the peak due to the internal standard. From the chromatogram obtained with test solution (b), calculate the ratio of the sum of the areas of any peaks, apart from the principal peak, the peak due to the internal standard and the peak due to the solvent, to the area of the peak due to the internal standard; this ratio is not greater than *R* (0.1 per cent).

Water (2.5.12). Not more than 0.2 per cent, determined on 10.0 g by the semi-micro determination of water.

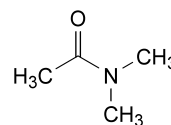
STORAGE

Store in an airtight, glass container, protected from light.

01/2005:1667

DIMETHYLACETAMIDE

Dimethylacetamidum



C_4H_9NO

M_r 87.1

DEFINITION

N,N-Dimethylacetamide.

CHARACTERS

Appearance: clear, colourless, slightly hygroscopic liquid.

Solubility: miscible with water, with alcohol, and with most common organic solvents.

bp: about 165 °C.

IDENTIFICATION

First identification: *C*.

Second identification: *A, B, D*.

A. Relative density (2.2.5): 0.941 to 0.944.

B. Refractive index (2.2.6): 1.435 to 1.439.

C. Infrared absorption spectrophotometry (2.2.24).

Preparation: films.

Comparison: *dimethylacetamide CRS*.

D. Dilute 50 mg with 1 ml of *methanol R*. Add 1 ml of a 15 g/l solution of *hydroxylamine hydrochloride R* and mix. Add 1 ml of *dilute sodium hydroxide solution R*, mix and allow to stand for 30 min. Add 1 ml of *dilute hydrochloric acid R* and add 1 ml of a 100 g/l solution of *ferric chloride R* in 0.1 *M* *hydrochloric acid*. A reddish-brown colour develops, reaching a maximum intensity after about 5 min.

TESTS

Appearance. The substance to be examined is clear (2.2.1) and not more intensely coloured than reference solution *Y*₇ (2.2.2, *Method II*).

Acidity. Dilute 50 ml with 50 ml of *water R* previously adjusted with 0.02 *M* *potassium hydroxide* or 0.02 *M* *hydrochloric acid* to a bluish-green colour, using 0.5 ml of *bromothymol blue solution R1* as indicator. Not more than 5.0 ml of 0.02 *M* *potassium hydroxide* is required to restore the initial (bluish-green) colour.

Alkalinity. To 50 ml add 50 ml of *water R* previously adjusted with 0.02 *M* *potassium hydroxide* or 0.02 *M* *hydrochloric acid* to a yellow colour, using 0.5 ml of *bromothymol blue solution R1* as indicator. Titrate with 0.02 *M* *hydrochloric acid*. Not more than 0.5 ml of 0.02 *M* *hydrochloric acid* is required to restore the initial (yellow) colour.

Related substances. Gas chromatography (2.2.28): use the normalisation procedure.

Test solution. The substance to be examined.

Reference solution (a). Dilute a mixture of 1 ml of the substance to be examined and 1 ml of *dimethylformamide R* to 20 ml with *methylene chloride R*.

Reference solution (b). Dilute 1 ml of the substance to be examined to 20.0 ml with *methylene chloride R*. Dilute 0.1 ml of the solution to 10.0 ml with *methylene chloride R*.

Column:

- *material*: fused silica,
- *size*: $l = 30$ m, $\varnothing = 0.32$ mm,
- *stationary phase*: *macrogol 20 000 R* (film thickness 1 μ m).

Carrier gas: nitrogen for chromatography R.

Linear velocity: 30 cm/s.

Split ratio: 1:20.

Temperature:

	Time (min)	Temperature (°C)
Column	0 - 15	80 → 200
Injection port		250
Detector		250

Detection: flame ionisation.

Injection: 0.5 μ l.

System suitability:

- *resolution*: minimum 5.0 between the peaks due to dimethylacetamide and dimethylformamide in the chromatogram obtained with reference solution (a),
- *signal-to-noise ratio*: minimum 10 for the principal peak in the chromatogram obtained with reference solution (b).

Limits:

- *any impurity*: maximum 0.1 per cent,
- *total*: maximum 0.3 per cent,
- *disregard limit*: area of the peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

Heavy metals (2.4.8): maximum 10 ppm.

Dilute 4.0 g to 20.0 ml with *water R*. 12 ml of the solution complies with limit test A. Prepare the standard using *lead standard solution (2 ppm Pb) R*.

Non-volatile matter: maximum 20 ppm.

Evaporate 50 g to dryness using a rotary evaporator at a pressure not exceeding 1 kPa and on a water-bath. Dry the residue in an oven at 170–175 °C. The residue weighs not more than 1 mg.

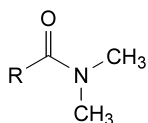
Water (2.5.32): maximum 0.1 per cent, determined on 0.100 g.

STORAGE

In an airtight container, protected from light.

IMPURITIES

A. acetic acid,



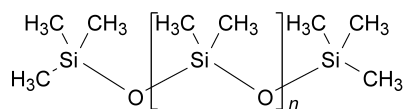
B. R = H: *N,N*-dimethylformamide,

C. R = C₂H₅: *N,N*-dimethylpropanamide,

D. R = CH₂-CH₂-CH₃: *N,N*-dimethylbutanamide.

DIMETICONE

Dimeticonum



DEFINITION

Dimeticone is a poly(dimethylsiloxane) obtained by hydrolysis and polycondensation of dichlorodimethylsilane and chlorotrimethylsilane. Different grades exist which are distinguished by a number indicating the nominal viscosity placed after the name.

Their degree of polymerisation ($n = 20$ to 400) is such that their kinematic viscosities are nominally between 20 mm²s^{−1} and 1300 mm²s^{−1}.

Dimeticones with a nominal viscosity of 50 mm²s^{−1} or lower are intended for external use only.

CHARACTERS

Clear, colourless liquids of various viscosities, practically insoluble in water, very slightly soluble to practically insoluble in ethanol, miscible with ethyl acetate, with methyl ethyl ketone and with toluene.

IDENTIFICATION

- It is identified by its kinematic viscosity at 25 °C (see Tests).
- Examine by infrared absorption spectrophotometry (2.2.24) comparing with the spectrum obtained with *dimeticone CRS*. The region of the spectrum from 850 cm^{−1} to 750 cm^{−1} is not taken into account.
- Heat 0.5 g in a test-tube over a small flame until white fumes begin to appear. Invert the tube over a 2nd tube containing 1 ml of a 1 g/l solution of *chromotropic acid, sodium salt R* in *sulphuric acid R* so that the fumes reach the solution. Shake the 2nd tube for about 10 s and heat on a water-bath for 5 min. The solution is violet.
- In a platinum crucible, prepare the sulphated ash (2.4.14) using 50 mg. The residue is a white powder that gives the reaction of silicates (2.3.1).

TESTS

Acidity. To 2.0 g add 25 ml of a mixture of equal volumes of *ethanol R* and *ether R*, previously neutralised to 0.2 ml of *bromothymol blue solution R1* and shake. Not more than 0.15 ml of 0.01 M *sodium hydroxide* is required to change the colour of the solution to blue.

Viscosity (2.2.9). Determine the kinematic viscosity at 25 °C. For dimeticones, the kinematic viscosity is not less than 90 per cent and not more than 110 per cent of the nominal viscosity stated on the label.

Mineral oils. Place 2 g in a test-tube and examine in ultraviolet light at 365 nm. The fluorescence is not more intense than that of a solution containing 0.1 ppm of *quinine sulphate R* in 0.005 M *sulphuric acid* examined in the same conditions.

Phenylated compounds. Dissolve 5.0 g with shaking in 10 ml of *cyclohexane R*. At wavelengths from 250 nm to 270 nm, the absorbance (2.2.25) of the solution is not greater than 0.2.

Heavy metals. Mix 1.0 g with *methylene chloride R* and dilute to 20 ml with the same solvent. Add 1.0 ml of a freshly prepared 0.02 g/l solution of *dithizone R* in *methylene chloride R*, 0.5 ml of *water R* and 0.5 ml of a mixture of 1 volume of *dilute ammonia R2* and 9 volumes of a 2 g/l solution of *hydroxylamine hydrochloride R*. At the same time, prepare a standard as follows: to 20 ml of *methylene chloride R* add 1.0 ml of a freshly prepared 0.02 g/l solution of *dithizone R* in *methylene chloride R*, 0.5 ml of *lead standard solution (10 ppm Pb) R* and 0.5 ml of a mixture of 1 volume of *dilute ammonia R2* and 9 volumes of a 2 g/l solution of *hydroxylamine hydrochloride R*. Immediately shake each solution vigorously for 1 min. Any red colour in the test solution is not more intense than that in the standard (5 ppm).

Volatile matter. For dimeticones with a nominal viscosity greater than 50 mm²s⁻¹, not more than 0.3 per cent, determined on 1.00 g by heating in an oven at 150 °C for 2 h. Carry out the test using a dish 60 mm in diameter and 10 mm deep.

LABELLING

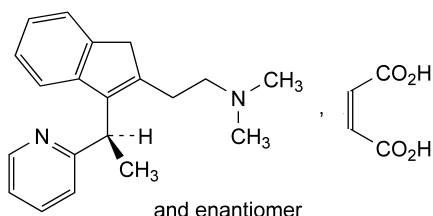
The label states:

- the nominal viscosity by a number placed after the name of the product,
- where applicable, that the product is intended for external use.

01/2005:1417

DIMETINDENE MALEATE

Dimetindeni maleas



C₂₄H₂₈N₂O₄

*M*_r 408.5

DEFINITION

Dimetindene maleate contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of *N,N*-dimethyl-2-[3-[(*RS*)-1-(pyridin-2-yl)ethyl]-1*H*-inden-2-yl]ethanamine (*Z*)-butenedioate, calculated with reference to the dried substance.

CHARACTERS

A white to almost white, crystalline powder, slightly soluble in water, soluble in methanol.

IDENTIFICATION

Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *dimetindene maleate CRS*. Examine the substance prepared as discs.

TESTS

Solution S. Dissolve 0.20 g in *methanol R* and dilute to 20.0 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and not more intensely coloured than Y₆ (2.2.2, *Method II*).

Optical rotation (2.2.7). Determined on solution S, the angle of optical rotation is −0.10° to + 0.10°.

Related substances. Examine by gas chromatography (2.2.28).

Test solution. Dissolve 50.0 mg of the substance to be examined in a mixture of equal volumes of *acetone R* and *methylene chloride R* and dilute to 5.0 ml with the same mixture of solvents.

Reference solution (a). Dilute 1 ml of the test solution to 100.0 ml with a mixture of equal volumes of *acetone R* and *methylene chloride R*.

Reference solution (b). Dissolve 5.0 mg of 2-ethylpyridine *R* in a mixture of equal volumes of *acetone R* and *methylene chloride R* and dilute to 50.0 ml with the same mixture of solvents. Dilute 10.0 ml of this solution to 100.0 ml with the same mixture of solvents.

The chromatographic procedure may be carried out using:

- a fused-silica column 30 m long and 0.32 mm in internal diameter coated with *polymethylphenylsiloxane R* (film thickness 0.25 µm),
- *helium for chromatography R* as the carrier gas at a linear velocity of about 30 cm/s,
- a flame-ionisation detector,

maintaining the temperature of the column at 60 °C for 1 min, then raising the temperature at a rate of 6 °C/min to 260 °C and maintaining at 260 °C for 12 min. Maintain the temperature of the injection port at 240 °C and that of the detector at 260 °C.

Inject via a split injector with a split flow of 30 ml/min for 1.3 times the retention time of the principal peak.

Inject 2 µl of reference solution (a). The symmetry factor of the principal peak in the chromatogram obtained is not greater than 1.3.

Inject 2 µl of the test solution and 2 µl of reference solution (b). In the chromatogram obtained with the test solution the area of the peak corresponding to 2-ethylpyridine is not greater than the area of the peak in the chromatogram obtained with reference solution (b) (0.1 per cent).

In the chromatogram obtained with the test solution the area of any peak, apart from the principal peak and any peak appearing during the first 8 min (2-ethylpyridine, the solvent mixture and maleic acid), is not greater than 0.2 times the area of the peak in the chromatogram with reference solution (a) (0.2 per cent) and the sum of the areas of all peaks is not greater than 0.5 times the area of the peak in the chromatogram obtained with reference solution (a) (0.5 per cent). Disregard any peak with an area less than 0.05 times the area of the principal peak in the chromatogram obtained with the reference solution (a).

Loss on drying (2.2.32). Not more than 0.1 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C for 2 h.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.150 g in 80 ml of *anhydrous acetic acid R*. Titrate with 0.1 *M perchloric acid*, determining the end-point potentiometrically (2.2.20).

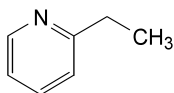
1 ml of 0.1 *M perchloric acid* is equivalent to 20.43 mg of C₂₄H₂₈N₂O₄.

STORAGE

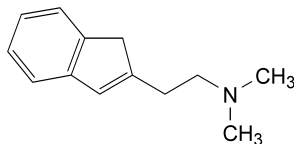
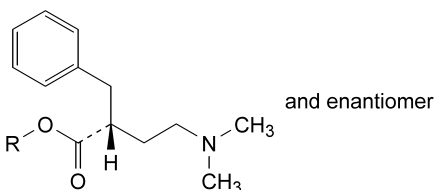
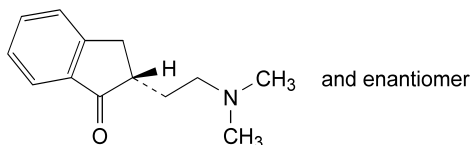
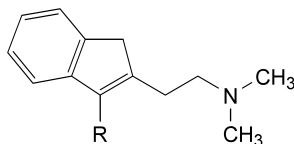
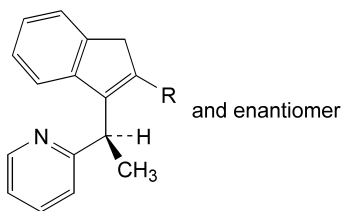
Store protected from light.

IMPURITIES

01/2005:1312

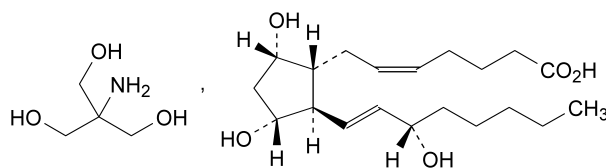


A. 2-ethylpyridine,

B. 2-(1*H*-inden-2-yl)-*N,N*-dimethylethanamine,C. R = C₂H₅: ethyl (2*RS*)-2-benzyl-4-(dimethylamino)butanoate,D. R = H: (2*RS*)-2-benzyl-4-(dimethylamino)butanoic acid,E. (2*RS*)-2-[2-(dimethylamino)ethyl]indan-1-one,F. R = [CH₂]₃-CH₃: 2-(3-butyl-1*H*-inden-2-yl)-*N,N*-dimethylethanamine,G. R = C₆H₅: *N,N*-dimethyl-2-(3-phenyl-1*H*-inden-2-yl)ethanamine,H. R = CH = CH₂: 2-[(1*RS*)-1-(2-ethenyl-1*H*-inden-3-yl)ethyl]pyridine,I. R = CH₂-CH₂-NH-CH₃: *N*-methyl-2-[3-[(1*RS*)-1-(pyridin-2-yl)ethyl]-1*H*-inden-2-yl]ethanamine.

DINOPROST TROMETAMOL

Dinoprostum trometamolum

C₂₄H₄₅NO₈M_r 475.6

DEFINITION

Dinoprost trometamol contains not less than 96.0 per cent and not more than the equivalent of 102.0 per cent of trometamol (*Z*)-7-[(1*R*,2*R*,3*R*,5*S*)-3,5-dihydroxy-2-[(*E*)-(3*S*)-3-hydroxyoct-1-enyl]cyclopentyl]hept-5-enoate (PGF_{2α}), calculated with reference to the anhydrous substance.

CHARACTERS

A white or almost white powder, very soluble in water, freely soluble in alcohol, practically insoluble in acetonitrile.

IDENTIFICATION

A. Dissolve 0.100 g in *alcohol R* and dilute to 10.0 ml with the same solvent. The specific optical rotation (2.2.7) is + 19 to + 26, calculated with reference to the anhydrous substance.

B. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *dinoprost trometamol CRS*.

TESTS

Related substances. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 10.0 mg of the substance to be examined in a mixture of 23 volumes of *acetonitrile R* and 77 volumes of *water R* and dilute to 10.0 ml with the same mixture of solvents.

Reference solution (a). *Degradation of dinoprost trometamol to impurity B.* Dissolve 1 mg of the substance to be examined in 1 ml of the mobile phase and heat the solution on a water-bath at 85 °C for 5 min and cool.

Reference solution (b). Dilute 2.0 ml of the test solution to 20.0 ml with a mixture of 23 volumes of *acetonitrile R* and 77 volumes of *water R*. Dilute 2.0 ml of the solution to 20.0 ml with a mixture of 23 volumes of *acetonitrile R* and 77 volumes of *water R*.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.15 m long and 3.9 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R1* (5 µm) with a pore size of 10 nm and a carbon loading of 19 per cent,
- as mobile phase at a flow rate of 1 ml/min a mixture prepared as follows: dissolve 2.44 g of *sodium dihydrogen phosphate R* in *water R* and dilute to 1000 ml with *water R*; adjust the pH to 2.5 with *phosphoric acid R* (about 0.6 ml); mix 770 ml of the buffer solution with 230 ml of *acetonitrile R1*,
- as detector a spectrophotometer set at 200 nm.

Adjust the sensitivity of the system so that the height of the principal peak in the chromatogram obtained with 20 µl of reference solution (b) is 25 per cent to 50 per cent of the full scale of the recorder.

Inject 20 µl of reference solution (a). When the chromatograms are recorded in the prescribed conditions, the retention times are: (15*R*)-PGF_{2α} (impurity B) about 55 min; 5,6-*trans*-PGF_{2α} (impurity A) about 60 min and dinoprost about 66 min. Continue the chromatography for 2.5 times the retention time of the principal peak (to elute degradation products formed during heating). The test is not valid unless the resolution between the peaks corresponding to impurity A and impurity B is at least 1.5 and the resolution between the peaks corresponding to impurity A and dinoprost is at least 2.0; the symmetry factor for the peaks corresponding to impurity A and impurity B is less than 1.2. If necessary, adjust the composition of the mobile phase by increasing the concentration of acetonitrile to decrease the retention times.

Inject separately 20 µl of the test solution and 20 µl of reference solution (b). Continue the chromatography of the test solution for 10 min after the elution of the principal peak. In the chromatogram obtained with the test solution the area of any peak corresponding to impurity A is not greater than twice the area of the principal peak obtained with reference solution (b) (2 per cent); the area of any other peak, apart from the principal peak and any peak corresponding to impurity A, is not greater than 1.5 times the area of the principal peak obtained with reference solution (b) (1.5 per cent) and not more than one such peak has an area greater than half the area of the principal peak obtained with reference solution (b) (0.5 per cent). The sum of the areas of all the peaks, apart from the principal peak and the peak corresponding to impurity A, is not greater than twice the area of the principal peak obtained with reference solution (b) (2 per cent). Disregard any peak due to the mobile phase and any peak due to trometamol (retention time about 1.5 min) and also any peak with an area of less than 0.05 times the area of the principal peak obtained with reference solution (b).

Water (2.5.12). Not more than 1.0 per cent, determined on 0.500 g by the semi-micro determination of water.

ASSAY

Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 10.0 mg of the substance to be examined in a mixture of 23 volumes of *acetonitrile R* and 77 volumes of *water R* and dilute to 10.0 ml with the same mixture.

Reference solution. Dissolve 10.0 mg of *dinoprost trometamol CRS* in a mixture of 23 volumes of *acetonitrile R* and 77 volumes of *water R* and dilute to 10.0 ml with the same mixture.

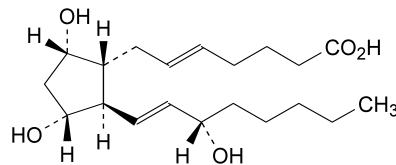
The chromatographic procedure may be carried out using:

- a stainless steel column 0.15 m long and 3.9 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R1* (5 µm) with a pore size of 10 nm and a carbon loading of 19 per cent,
- as mobile phase at a flow rate of 1 ml/min a mixture prepared as follows: dissolve 2.44 g of *sodium dihydrogen phosphate R* in *water R* and dilute to 1000 ml with *water R*; adjust the pH to 2.5 with *phosphoric acid R* (about 0.6 ml); mix 730 ml of the buffer solution with 270 ml of *acetonitrile R1*,
- as detector a spectrophotometer set at 200 nm.

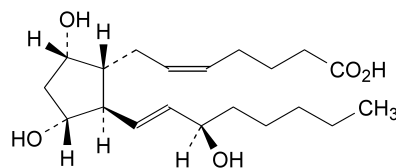
Adjust the sensitivity of the system so that the height of the principal peak in the chromatogram obtained with 20 µl of the reference solution is 70 per cent to 90 per cent of the full scale of the recorder. When the chromatogram is recorded under the conditions described above, the retention time is about 23 min for dinoprost.

Inject the reference solution six times. The test is not valid unless the relative standard deviation of the peak area for dinoprost is at most 2.0 per cent. Inject 20 µl of the test solution. Calculate the percentage of dinoprost trometamol.

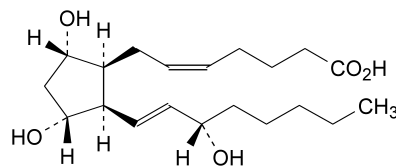
IMPURITIES



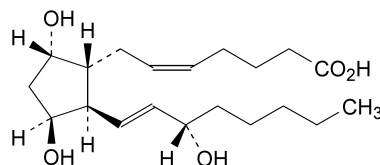
- A. (*E*)-7-[(1*R*,2*R*,3*R*,5*S*)-3,5-dihydroxy-2-[(*E*)-(3*S*)-3-hydroxyoct-1-enyl]cyclopentyl]hept-5-enoic acid ((5*E*)-PGF_{2α}; 5,6-*trans*-PGF_{2α}),



- B. (*Z*)-7-[(1*R*,2*R*,3*R*,5*S*)-3,5-dihydroxy-2-[(*E*)-(3*R*)-3-hydroxyoct-1-enyl]cyclopentyl]hept-5-enoic acid ((15*R*)-PGF_{2α}; 15-*epi*PGF_{2α}),



- C. (*Z*)-7-[(1*S*,2*R*,3*R*,5*S*)-3,5-dihydroxy-2-[(*E*)-(3*S*)-3-hydroxyoct-1-enyl]cyclopentyl]hept-5-enoic acid ((8*S*)-PGF_{2α}; 8-*epi*PGF_{2α}),

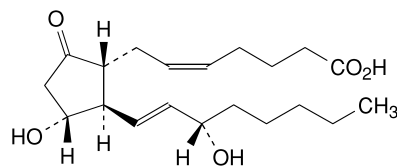


- D. (*Z*)-7-[(1*R*,2*R*,3*S*,5*S*)-3,5-dihydroxy-2-[(*E*)-(3*S*)-3-hydroxyoct-1-enyl]cyclopentyl]hept-5-enoic acid (11β-PGF_{2α}; 11-*epi*PGF_{2α}).

01/2005:1311

DINOPROSTONE

Dinoprostonium



C₂₀H₃₂O₅

M_r 352.5

DEFINITION

(*Z*)-7-[(1*R*,2*R*,3*R*)-3-hydroxy-2-[(*E*)-(3*S*)-3-hydroxyoct-1-enyl]-5-oxocyclopentyl]hept-5-enoic acid (PGE₂).

Content: 95.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

Appearance: white or almost white, crystalline powder or colourless crystals.

Solubility: practically insoluble in water, very soluble in methanol, freely soluble in alcohol.

The substance degrades at room temperature.

IDENTIFICATION

A. Specific optical rotation (2.2.7): -82 to -90 (anhydrous substance).

Immediately before use, dissolve 50.0 mg in *alcohol R* and dilute to 10.0 ml with the same solvent.

B. Infrared absorption spectrophotometry (2.2.24).

Comparison: *dinoprostone CRS*.

TESTS

Prepare the solutions immediately before use.

Related substances. Liquid chromatography (2.2.29).

Test solution (a). Dissolve 10.0 mg of the substance to be examined in a 58 per cent *V/V* solution of *methanol R2* and dilute to 2.0 ml with the same solvent.

Test solution (b). Dissolve 20.0 mg of the substance to be examined in a 58 per cent *V/V* solution of *methanol R2* and dilute to 20.0 ml with the same solvent.

Reference solution (a). Dissolve 1 mg of *dinoprostone CRS* and 1 mg of *dinoprostone impurity C CRS* in a 58 per cent *V/V* solution of *methanol R2* and dilute to 10.0 ml with the same solvent. Dilute 4.0 ml of the solution to 10.0 ml with a 58 per cent *V/V* solution of *methanol R2*.

Reference solution (b). Dilute 0.5 ml of test solution (a) to 10.0 ml with a 58 per cent *V/V* solution of *methanol R2*. Dilute 1.0 ml of the solution to 10.0 ml with a 58 per cent *V/V* solution of *methanol R2*.

Reference solution (c). In order to prepare *in situ* the degradation compounds (impurity D and impurity E), dissolve 1 mg of the substance to be examined in 100 μ l of 1 M *sodium hydroxide* (the solution becomes brownish-red), wait 4 min, add 150 μ l of 1 M *acetic acid* (yellowish-white opalescent solution) and dilute to 5.0 ml with a 58 per cent *V/V* solution of *methanol R2*.

Reference solution (d). Dissolve 20 mg of *dinoprostone CRS* in a 58 per cent *V/V* solution of *methanol R2* and dilute to 20.0 ml with the same solvent.

Column:

- **size:** $l = 0.25$ m, $\varnothing = 4.6$ mm,
- **stationary phase:** end-capped octadecylsilyl silica gel for chromatography *R*,
- **temperature:** 30 °C.

Mobile phase: mix 42 volumes of a 0.2 per cent *V/V* solution of *acetic acid R* and 58 volumes of *methanol R2*.

Flow rate: 1.0 ml/min.

Detection: spectrophotometer at 210 nm.

Injection: 20 μ l; inject test solution (a) and reference solutions (a), (b) and (c).

Relative retention with reference to *dinoprostone* (retention time = about 18 min): impurity C = about 1.2; impurity D = about 1.8; impurity E = about 2.0.

System suitability: reference solution (a):

- **resolution:** minimum of 3.8 between the peaks due to *dinoprostone* and to impurity C. If necessary adjust the concentration of the acetic acid solution and/or methanol (increase the concentration of the acetic acid solution

to increase the retention time for *dinoprostone* and impurity C and increase the concentration of methanol to decrease the retention time for both compounds).

Limits:

- **correction factors:** for the calculation of contents, multiply the peak areas of the following impurities by the corresponding correction factor: impurity D = 0.2; impurity E = 0.7,
- **impurity C:** not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (b) (1.5 per cent),
- **impurity D:** not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (1 per cent),
- **impurity E:** not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent),
- **any other impurity:** not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent),
- **total of other impurities:** not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (1 per cent),
- **disregard limit:** 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

If any peak with a relative retention to *dinoprostone* of about 0.8 is greater than 0.5 per cent or if the total of other impurities is greater than 1.0 per cent, record the chromatogram of test solution (a) with a detector set at 230 nm. If the area of the peak at 230 nm is twice the area of the peak at 210 nm, multiply the area at 210 nm by 0.2 (correction factor for impurity F).

Water (2.5.12): maximum 0.5 per cent, determined on 0.50 g.

ASSAY

Prepare the solutions immediately before use.

Liquid chromatography (2.2.29) as described in the test for related substances.

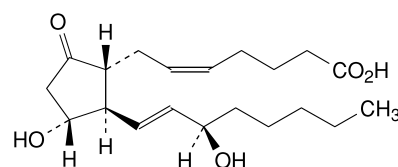
Injection: test solution (b) and reference solution (d).

Calculate the percentage content of $C_{20}H_{32}O_5$.

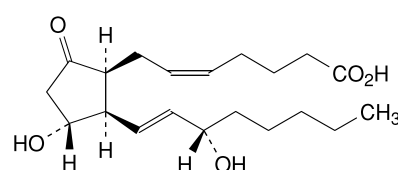
STORAGE

At a temperature not exceeding -15 °C.

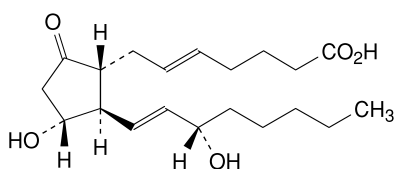
IMPURITIES



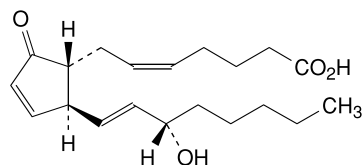
A. (Z)-7-[(1R,2R,3R)-3-hydroxy-2-[(E)-(3R)-3-hydroxyoct-1-enyl]-5-oxocyclopentyl]hept-5-enoic acid (15-epiPGE₂; (15R)-PGE₂),



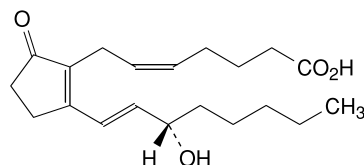
B. (Z)-7-[(1S,2R,3R)-3-hydroxy-2-[(E)-(3S)-3-hydroxyoct-1-enyl]-5-oxocyclopentyl]hept-5-enoic acid (8-epiPGE₂; (8S)-PGE₂),



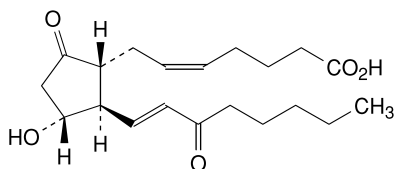
- C. (E)-7-[(1R,2R,3R)-3-hydroxy-2-[(E)-(3S)-3-hydroxyoct-1-enyl]-5-oxocyclopentyl]hept-5-enoic acid (5-*trans*-PGE₂; (5*E*)-PGE₂),



- D. (Z)-7-[(1R,2S)-2-[(E)-(3S)-3-hydroxyoct-1-enyl]-5-oxocyclopent-3-enyl]hept-5-enoic acid (PGA₂),



- E. (Z)-7-[2-[(E)-(3S)-3-hydroxyoct-1-enyl]-5-oxocyclopent-1-enyl]hept-5-enoic acid (PGB₂),

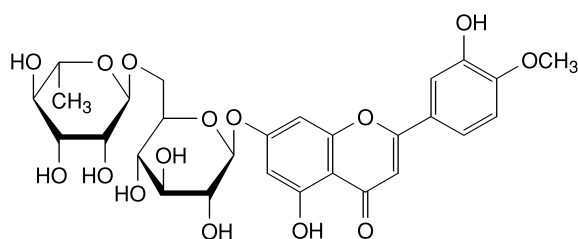


- F. (Z)-7-[(1R,2R,3R)-3-hydroxy-2-[(E)-3-oxooct-1-enyl]-5-oxocyclopentyl]hept-5-enoic acid (15-oxo-PGE₂; 15-keto-PGE₂).

01/2005:1611

DIOSMIN

Diosminum

C₂₈H₃₂O₁₅M_r 609

DEFINITION

7-[6-O-(6-Deoxy-α-L-mannopyranosyl)-β-D-glucopyranosyl]oxy]-5-hydroxy-2-(3-hydroxy-4-methoxyphenyl)-4H-1-benzopyran-4-one.

Substance obtained through iodine-assisted oxidation of (2*S*)-7-[6-O-(6-deoxy-α-L-mannopyranosyl)-β-D-glucopyranosyl]oxy]-5-hydroxy-2-(3-hydroxy-4-methoxyphenyl)-2,3-dihydro-4*H*-1-benzopyran-4-one (hesperidin) of natural origin.

Content: 90.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

Appearance: greyish-yellow or light yellow hygroscopic powder.

Solubility: practically insoluble in water, soluble in dimethyl sulphoxide, practically insoluble in alcohol. It dissolves in dilute solutions of alkali hydroxides.

IDENTIFICATION

- A. Infrared absorption spectrophotometry (2.2.24).

Comparison: *diosmin CRS*.

- B. Examine the chromatograms obtained in the assay.

Results: the principal peak in the chromatogram obtained with the test solution is similar in retention time and size to the principal peak in the chromatogram obtained with reference solution (a).

TESTS

Iodine: maximum 0.1 per cent.

Determine the total content of iodine by potentiometry, using an iodide-selective electrode (2.2.36), after oxygen combustion (2.5.10).

Test solution. Wrap 0.100 g of the substance to be examined in a piece of filter paper and place it in a sample carrier. Introduce into the flask 50 ml of a 0.2 g/l solution of *hydrazine R*. Flush the flask with oxygen for 10 min. Ignite the filter paper. Stir the contents of the flask immediately after the end of the combustion to dissolve completely the combustion products. Continue stirring for 1 h.

Reference solution. Dilute 2.0 ml of a 16.6 g/l solution of *potassium iodide R* to 100.0 ml with *water R*. Dilute 10.0 ml of the solution to 100.0 ml with *water R*.

Introduce into a beaker 30 ml of a 200 g/l solution of *potassium nitrate R* in 0.1 *M nitric acid*. Immerse the electrodes and stir for 10 min. The potential of the solution (*nT*₁) must remain stable. Add 1 ml of the test solution and measure the potential (*nT*₂).

Introduce into a beaker 30 ml of a 200 g/l solution of *potassium nitrate R* in 0.1 *M nitric acid*. Immerse the electrodes and stir for 10 min. The potential of the solution must remain stable (*nR*₁). Add 80 µl of the reference solution and measure the potential (*nR*₂).

The absolute value |*nT*₂ - *nT*₁| is not higher than the absolute value |*nR*₂ - *nR*₁|.

Related substances. Liquid chromatography (2.2.29).

Test solution. Dissolve 25.0 mg of the substance to be examined in *dimethyl sulphoxide R* and dilute to 25.0 ml with the same solvent.

Reference solution (a). Dissolve 25.0 mg of *diosmin CRS* in *dimethyl sulphoxide R* and dilute to 25.0 ml with the same solvent.

Reference solution (b). Dilute 5.0 ml of reference solution (a) to 100.0 ml with *dimethyl sulphoxide R*.

Reference solution (c). Dissolve 5.0 mg of *diosmin for system suitability CRS* in *dimethyl sulphoxide R* and dilute to 5.0 ml with the same solvent.

Column:

- **size:** *l* = 0.10 m, Ø = 4.6 mm,
- **stationary phase:** octadecylsilyl silica gel for chromatography *R* (3 µm),
- **temperature:** 40 °C.

Mobile phase: acetonitrile *R*, glacial acetic acid *R*, methanol *R*, water *R* (2:6:28:66 V/V/V/V).

Flow rate: 1.5 ml/min.

Detection: spectrophotometer at 275 nm.

Injection: 10 µl loop injector; inject the test solution and reference solutions (b) and (c).

Run time: 6 times the retention time of diosmin.

Relative retention with reference to diosmin (retention time = about 4.6 min): impurity A = about 0.5, impurity B = about 0.6, impurity C = about 0.8, impurity D = about 2.2, impurity E = about 2.6, impurity F = about 4.5.

System suitability: reference solution (c):

- **resolution:** minimum of 2.5 between the peaks due to impurities B and C.

Limits:

- **correction factors:** for the calculation of contents, multiply the peak areas of the following impurities by the corresponding correction factor: impurity A = 0.38; impurity F = 0.61,
- **impurity A:** not more than 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (b) (1 per cent),
- **impurity B:** not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (5 per cent),
- **impurity C:** not more than 0.6 times the area of the principal peak in the chromatogram obtained with reference solution (b) (3 per cent),
- **impurity E:** not more than 0.6 times the area of the principal peak in the chromatogram obtained with reference solution (b) (3 per cent),
- **impurity F:** not more than 0.6 times the area of the principal peak in the chromatogram obtained with reference solution (b) (3 per cent),
- **any other impurity:** not more than 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (b) (1 per cent),
- **total of other impurities and impurity A:** not more than 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (b) (1 per cent),
- **total:** not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (10 per cent),
- **disregard limit:** 0.02 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent).

Heavy metals (2.4.8): maximum 20 ppm.

2.0 g complies with limit test C. Prepare the standard using 4.0 ml of *lead standard solution* (10 ppm Pb) R.

Water (2.5.12): maximum 6.0 per cent, determined on 0.300 g.

Sulphated ash (2.4.14): maximum 0.2 per cent, determined on 1.0 g.

ASSAY

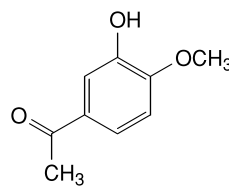
Liquid chromatography (2.2.29), as described in the test for related substances.

Injection: test solution and reference solution (a).

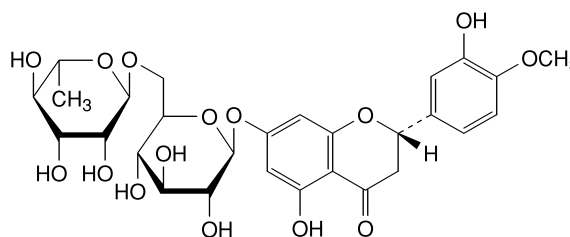
STORAGE

In an airtight container.

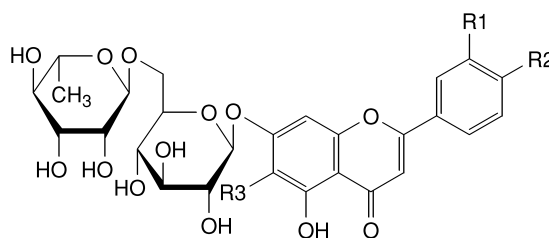
IMPURITIES



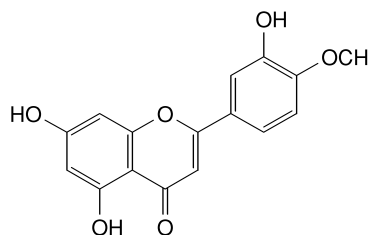
- A. 1-(3-hydroxy-4-methoxyphenyl)ethanone (acetoisovanillone),



- B. (2S)-7-[[6-O-(6-deoxy-α-L-mannopyranosyl)-β-D-glucopyranosyl]oxy]-5-hydroxy-2-(3-hydroxy-4-methoxyphenyl)-2,3-dihydro-4H-1-benzopyran-4-one (hesperidin),



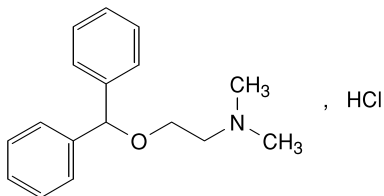
- C. R1 = R3 = H, R2 = OH: 7-[[6-O-(6-deoxy-α-L-mannopyranosyl)-β-D-glucopyranosyl]oxy]-5-hydroxy-2-(4-hydroxyphenyl)-4H-1-benzopyran-4-one (isorhoifin),
- D. R1 = OH, R2 = OCH₃, R3 = I: 7-[[6-O-(6-deoxy-α-L-mannopyranosyl)-β-D-glucopyranosyl]oxy]-5-hydroxy-2-(3-hydroxy-4-methoxyphenyl)-6-iodo-4H-1-benzopyran-4-one (6-iododiosmin),
- E. R1 = R3 = H, R2 = OCH₃: 7-[[6-O-(6-deoxy-α-L-mannopyranosyl)-β-D-glucopyranosyl]oxy]-5-hydroxy-2-(4-methoxyphenyl)-4H-1-benzopyran-4-one (linarin),



- F. 5,7-dihydroxy-2-(3-hydroxy-4-methoxyphenyl)-4H-1-benzopyran-4-one (diosmetin).

01/2005:0023
corrected**DIPHENHYDRAMINE
HYDROCHLORIDE**

Diphenhydramini hydrochloridum

 $C_{17}H_{22}ClNO$ M_r 291.8**DEFINITION**2-(Diphenylmethoxy)-*N,N*-dimethylethanamine hydrochloride.*Content*: 99.0 per cent to 101.0 per cent (dried substance).**CHARACTERS***Appearance*: white or almost white, crystalline powder.*Solubility*: very soluble in water, freely soluble in alcohol.**IDENTIFICATION***First identification*: C, D.*Second identification*: A, B, D.

A. Melting point (2.2.14): 168 °C to 172 °C.

B. Dissolve 50 mg in *alcohol R* and dilute to 100.0 ml with the same solvent. Examined between 230 nm and 350 nm, the solution shows 3 absorption maxima (2.2.25), at 253 nm, 258 nm and 264 nm. The ratio of the absorbance measured at the maximum at 258 nm to that measured at the maximum at 253 nm is 1.1 to 1.3. The ratio of the absorbance measured at the maximum at 258 nm to that measured at the maximum at 264 nm is 1.2 to 1.4.

C. Infrared absorption spectrophotometry (2.2.24).

Preparation: discs.*Comparison*: diphenhydramine hydrochloride CRS.

D. It gives the reactions of chlorides (2.3.1).

TESTS**Solution S.** Dissolve 1.0 g in *carbon dioxide-free water R* and dilute to 20 ml with the same solvent.**Appearance of solution.** Solution S and a fivefold dilution of solution S are clear (2.2.1). Solution S is not more intensely coloured than reference solution BY₆ (2.2.2, *Method II*).**Acidity or alkalinity.** To 10 ml of solution S add 0.15 ml of *methyl red solution R* and 0.25 ml of 0.01 M *hydrochloric acid*. The solution is pink. Not more than 0.5 ml of 0.01 M *sodium hydroxide* is required to change the colour of the indicator to yellow.**Related substances.** Liquid chromatography (2.2.29).*Test solution.* Dissolve 70 mg of the substance to be examined in the mobile phase and dilute to 20.0 ml with the

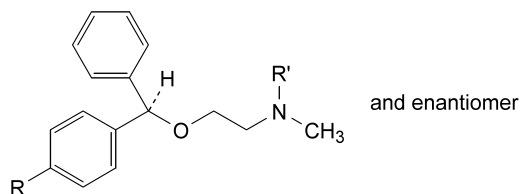
mobile phase. Dilute 2.0 ml of the solution to 10.0 ml with the mobile phase.

Reference solution (a). Dilute 1.0 ml of the test solution to 10.0 ml with the mobile phase. Dilute 1.0 ml of this solution to 20.0 ml with the mobile phase.*Reference solution (b).* Dissolve 5 mg of *diphenhydramine impurity A CRS* and 5 mg of *diphenylmethanol R* in the mobile phase and dilute to 10.0 ml with the mobile phase. To 2.0 ml of this solution add 1.5 ml of the test solution and dilute to 10.0 ml with the mobile phase.*Column*:– *size*: $l = 0.25$ m, $\varnothing = 4.6$ mm,– *stationary phase*: base-deactivated octylsilyl silica gel for chromatography R (5 µm).*Mobile phase*: mix 35 volumes of *acetonitrile R* and 65 volumes of a 5.4 g/l solution of *potassium dihydrogen phosphate R* adjusted to pH 3.0 using *phosphoric acid R*.*Flow rate*: 1.2 ml/min.*Detection*: spectrophotometer at 220 nm.*Injection*: 10 µl.*Run time*: 7 times the retention time of diphenhydramine.*Relative retention* with reference to diphenhydramine (retention time = about 6 min): impurity A = about 0.9; impurity B = about 1.5; impurity C = about 1.8; impurity D = about 2.6; impurity E = about 5.1.*System suitability*: reference solution (b):– *resolution*: minimum 2.0 between the peaks due to diphenhydramine and to impurity A.*Limits*:– *correction factor*: for the calculation of content, multiply the peak area of impurity D by 0.7,– *impurity A*: not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent),– *any other impurity*: not more than 0.6 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.3 per cent),– *total*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (1.0 per cent),– *disregard limit*: 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).**Loss on drying** (2.2.32): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 100–105 °C.**Sulphated ash** (2.4.14): maximum 0.1 per cent, determined on 1.0 g.**ASSAY**Dissolve 0.250 g in 50 ml of *alcohol R* and add 5.0 ml of 0.01 M *hydrochloric acid*. Carry out a potentiometric titration (2.2.20), using 0.1 M *sodium hydroxide*. Read the volume added between the 2 points of inflexion.1 ml of 0.1 M *sodium hydroxide* is equivalent to 29.18 mg of $C_{17}H_{22}ClNO$.**STORAGE**

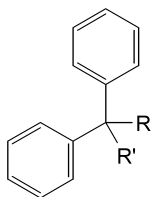
Protected from light.

IMPURITIES

Specified impurities: A, B, C, D, E.



- A. $R = R' = H$: 2-(diphenylmethoxy)-*N*-methylethanamine,
 B. $R = R' = CH_3$: 2-[(*RS*)-(4-methylphenyl)phenylmethoxy]-*N,N*-dimethylethanamine,
 C. $R = Br, R' = CH_3$: 2-[(*RS*)-(4-bromophenyl)phenylmethoxy]-*N,N*-dimethylethanamine,

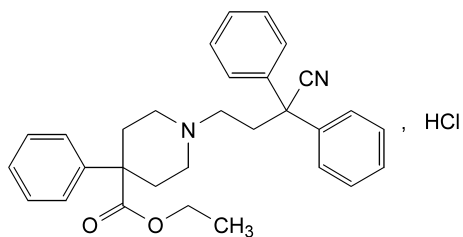


- D. $R = OH, R' = H$: diphenylmethanol (benzhydrol),
 E. $R + R' = O$: diphenylmethanone (benzophenone).

01/2005:0819

DIPHENOXYLATE HYDROCHLORIDE

Diphenoxylati hydrochloridum

 $C_{30}H_{33}ClN_2O_2$ M_r 489.1

DEFINITION

Diphenoxylate hydrochloride contains not less than 98.0 per cent and not more than the equivalent of 102.0 per cent of ethyl 1-(3-cyano-3,3-diphenylpropyl)-4-phenylpiperidine-4-carboxylate hydrochloride, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, crystalline powder, very slightly soluble in water, freely soluble in methylene chloride, sparingly soluble in alcohol.

It melts at about 220 °C, with decomposition.

IDENTIFICATION

- A. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the *Ph. Eur.* reference spectrum of diphenoxylate hydrochloride.

- B. Dissolve about 30 mg in 5 ml of *methanol R*. Add 0.25 ml of *nitric acid R* and 0.4 ml of *silver nitrate solution R1*. Shake and allow to stand. A curdled precipitate is formed. Centrifuge and rinse the precipitate with three quantities, each of 2 ml, of *methanol R*. Carry out this operation rapidly and protected from bright light. Suspend the precipitate in 2 ml of *water R* and add 1.5 ml of *ammonia R*. The precipitate dissolves easily.

TESTS

Appearance of solution. Dissolve 1.0 g in *methylene chloride R* and dilute to 10 ml with the same solvent. The solution is clear (2.2.1) and not more intensely coloured than reference solution Y_6 (2.2.2, *Method II*).

Related substances. Examine by thin-layer chromatography (2.2.27), using a plate coated with a suitable octadecylsilyl silica gel (5 µm) with a fluorescent indicator having an optimal intensity at 254 nm.

Test solution. Dissolve 1.0 g in a mixture of 1 volume of *methanol R* and 2 volumes of *methylene chloride R* and dilute to 20 ml with the same mixture of solvents.

Reference solution (a). Dilute 0.5 ml of the test solution to 100 ml with a mixture of 1 volume of *methanol R* and 2 volumes of *methylene chloride R*.

Reference solution (b). Dissolve 0.50 g of the substance to be examined in 25 ml of a 15 g/l solution of *potassium hydroxide R* in *methanol R* and add 1 ml of *water R*. Heat on a water-bath under a reflux condenser for 4 h. Cool and add 25 ml of 0.5 M *hydrochloric acid*. Shake with 100 ml of *methylene chloride R*. Evaporate the lower layer to dryness on a water-bath. Dissolve the residue in 10 ml of a mixture of 1 volume of *methanol R* and 2 volumes of *methylene chloride R*, add 10 ml of the test solution and dilute to 25 ml with a mixture of 1 volume of *methanol R* and 2 volumes of *methylene chloride R*.

Apply separately to a plate (100 mm square) 1 µl of each solution. Develop in an unsaturated tank over a path of 7 cm using a mixture of 10 volumes of *methanol R*, 30 volumes of a 59 g/l solution of *sodium chloride R* and 60 volumes of *dioxan R*. Allow the plate to dry in an oven at 160 °C for 15 min and place the hot plate in a closed tank containing about 20 ml of *fuming nitric acid R* for 30 min. Remove the plate and heat it again at 160 °C for 15 min. Allow to cool and examine immediately in ultraviolet light at 254 nm. Any spot in the chromatogram obtained with the test solution, apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (a) (0.5 per cent). The test is not valid unless the chromatogram obtained with reference solution (b) shows two clearly separated principal spots.

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

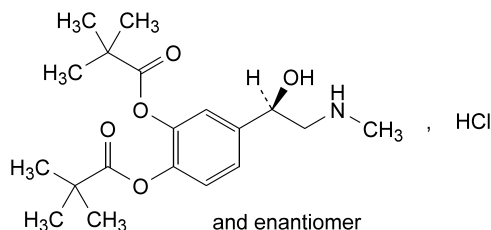
Dissolve 0.400 g in 40 ml of *alcohol R* and add 5.0 ml of 0.01 M *hydrochloric acid*. Carry out a potentiometric titration (2.2.20), using 0.1 M *ethanolic sodium hydroxide*. Read the volume added between the two points of inflexion.

1 ml of 0.1 M *ethanolic sodium hydroxide* is equivalent to 48.91 mg of $C_{30}H_{33}ClN_2O_2$.

STORAGE

Store protected from light.

01/2005:1719 Injection: 10 µl.

DIPIVEFRINE HYDROCHLORIDE**Dipivefrini hydrochloridum** $C_{19}H_{30}ClNO_5$ M_r 387.9**DEFINITION**

Hydrochloride of 4-[(1*RS*)-1-hydroxy-2-(methylamino)ethyl]-1,2-phenylene bis(2,2-dimethylpropanoate).

Content: 97.5 per cent to 102.0 per cent (dried substance).

CHARACTERS

Appearance: white or almost white, crystalline powder.

Solubility: freely soluble in water, very soluble in methanol, freely soluble in ethanol (96 per cent) and in methylene chloride.

mp: about 160 °C.

IDENTIFICATION

A. Infrared absorption spectrophotometry (2.2.24).

Preparation: discs.

Comparison: dipivefrine hydrochloride CRS.

B. It gives reaction (a) of chlorides (2.3.1).

TESTS

Impurities A and B. Liquid chromatography (2.2.29).

Test solution. Dissolve 0.100 g of the substance to be examined in 0.01 M hydrochloric acid and dilute to 10.0 ml with the same acid.

Reference solution. Dissolve 10.0 mg of adrenaline R and 10.0 mg of adrenalone hydrochloride R in 0.01 M hydrochloric acid and dilute to 100.0 ml with the same acid. Dilute 1.0 ml of this solution to 10.0 ml with 0.01 M hydrochloric acid. Protect this solution from light.

Column:

- *size*: $l = 0.15$ m, $\varnothing = 4.6$ mm,
- *stationary phase*: end-capped polar-embedded octadecylsilyl amorphous organosilica polymer R (5 µm).

Mobile phase:

- *mobile phase A*: 0.1 per cent V/V solution of anhydrous formic acid R,
- *mobile phase B*: methanol R2, acetonitrile R (40:60 V/V),

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)
0 - 3	100	0
3 - 5	100 → 40	0 → 60
5 - 10	40	60
10 - 11	40 → 100	60 → 0
11 - 25	100	0

Flow rate: 1 ml/min.

Detection: spectrophotometer at 260 nm.

Retention times: impurity A = about 2.2 min; impurity B = about 3.2 min.

System suitability: reference solution:

- *resolution*: minimum 2.0 between the peaks due to impurity A and impurity B.

Limits:

- *impurity A*: not more than the area of the corresponding peak in the chromatogram obtained with the reference solution (0.1 per cent),
- *impurity B*: not more than the area of the corresponding peak in the chromatogram obtained with the reference solution (0.1 per cent).

Related substances. Liquid chromatography (2.2.29).

Solvent mixture. Mix 40 volumes of methanol R2 and 60 volumes of acetonitrile R. Mix 55 volumes of this mixture and 45 volumes of 0.01 M hydrochloric acid.

Test solution. Dissolve 50.0 mg of the substance to be examined in the solvent mixture and dilute to 5.0 ml with the solvent mixture.

Reference solution (a). Dilute 1.0 ml of the test solution to 100.0 ml with the solvent mixture.

Reference solution (b). Dissolve 5 mg of dipivefrine for system suitability CRS (containing impurities C, D and E) in the solvent mixture and dilute to 2.0 ml with the solvent mixture.

Reference solution (c). Dissolve 5.0 mg of dipivefrine hydrochloride CRS in the solvent mixture and dilute to 2.0 ml with the solvent mixture. Dilute 1.0 ml of the solution to 25.0 ml with the solvent mixture.

Column:

- *size*: $l = 0.15$ m, $\varnothing = 4.6$ mm,
- *stationary phase*: end-capped polar-embedded octadecylsilyl amorphous organosilica polymer R (5 µm).

Mobile phase: mix 45 volumes of a 0.7 g/l solution of concentrated ammonia R adjusted to pH 10.0 with dilute acetic acid R and 55 volumes of a mixture of 40 volumes of methanol R2 and 60 volumes of acetonitrile R.

Flow rate: 1 ml/min.

Detection: spectrophotometer at 260 nm.

Injection: 10 µl.

Run time: 2.5 times the retention time of dipivefrine.

Relative retention with reference to dipivefrine (retention time = about 7 min): impurities C and D = about 0.4; impurity E = about 1.3; impurity F = about 2.0.

System suitability: reference solution (b):

- *resolution*: minimum 3.0 between the peaks due to dipivefrine and impurity E.

Limits:

- *correction factors*: for the calculation of contents, multiply the peak areas of the following impurities by the corresponding correction factor: impurities C and D = 0.5; impurity E = 0.06;
- *sum of impurities C and D*: not more than 0.3 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.3 per cent);
- *impurities E, F*: for each impurity, not more than 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent);
- *any other impurity*: for each impurity, not more than 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent);

- *total*: not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent);
- *disregard limit*: 0.05 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent), disregard any peak with a mass distribution ratio of less than 0.5.

Loss on drying (2.2.32): maximum 1.0 per cent, determined on 1.000 g by drying *in vacuo* at 60 °C for 6 h.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

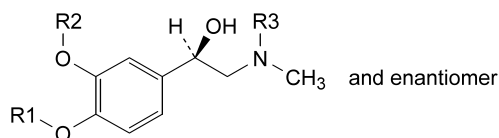
Liquid chromatography (2.2.29) as described in the test for related substances with the following modification.

Injection: 20 µl of reference solutions (a) and (c).

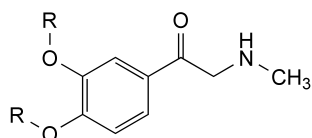
Calculate the percentage content of $C_{19}H_{30}ClNO_5$ using the chromatograms obtained with reference solutions (a) and (c) and the declared content of *dipivefrine hydrochloride CRS*.

IMPURITIES

Specified impurities: A, B, C, D, E, F.



- A. R1 = R2 = R3 = H: 4-[(1*RS*)-1-hydroxy-2-(methylamino)ethyl]benzene-1,2-diol ((±)-adrenaline),
- C. R1 = R3 = H, R2 = CO-C(CH₃)₃: 2-hydroxy-5-[(1*RS*)-1-hydroxy-2-(methylamino)ethyl]phenyl 2,2-dimethylpropanoate,
- D. R1 = CO-C(CH₃)₃, R2 = R3 = H: 2-hydroxy-4-[(1*RS*)-1-hydroxy-2-(methylamino)ethyl]phenyl 2,2-dimethylpropanoate,
- F. R1 = R2 = CO-C(CH₃)₃, R3 = C₂H₅: 4-[(1*RS*)-2-(ethylmethylamino)-1-hydroxyethyl]-1,2-phenylene bis(2,2-dimethylpropanoate),

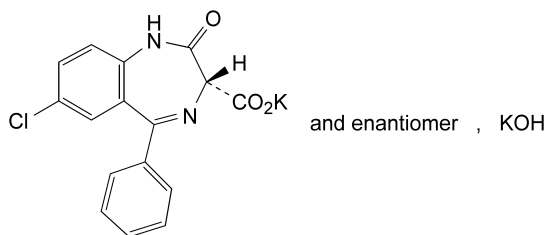


- B. R = H: 1-(3,4-dihydroxyphenyl)-2-(methylamino)ethanone (adrenalone),
- E. R = CO-C(CH₃)₃: 4-[(methylamino)acetyl]-1,2-phenylene bis(2,2-dimethylpropanoate) (adrenalone dipivalate ester).

01/2005:0898

DIPOTASSIUM CLORAZEPATE

Dikalii clorazepas



$C_{16}H_{11}ClK_2N_2O_4$

M_r 408.9

DEFINITION

Potassium (3*RS*)-7-chloro-2-oxo-5-phenyl-2,3-dihydro-1*H*-1,4-benzodiazepine-3-carboxylate compound with potassium hydroxide (1:1).

Content: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

Appearance: white or light yellow, crystalline powder.

Solubility: freely soluble to very soluble in water, very slightly soluble in alcohol, practically insoluble in methylene chloride.

Solutions in water and in alcohol are unstable and are to be used immediately.

IDENTIFICATION

First identification: B, E.

Second identification: A, C, D, E.

- A. Dissolve 10.0 mg in a 0.3 g/l solution of *potassium carbonate R* and dilute to 100.0 ml with the same solution (solution A). Dilute 10.0 ml of solution A to 100.0 ml with a 0.3 g/l solution of *potassium carbonate R* (solution B). Examined between 280 nm and 350 nm (2.2.25), solution A shows a broad absorption maximum at about 315 nm. The specific absorbance at the absorption maximum at 315 nm is 49 to 56. Examined between 220 nm and 280 nm (2.2.25), solution B shows an absorption maximum at 230 nm. The specific absorbance at the absorption maximum at 230 nm is 800 to 870.
- B. Infrared absorption spectrophotometry (2.2.24).
Preparation: discs.
Comparison: *Ph. Eur. reference spectrum of dipotassium clorazepate*.
- C. Dissolve about 20 mg in 2 ml of *sulphuric acid R*. Observed in ultraviolet light at 365 nm, the solution shows yellow fluorescence.
- D. Dissolve 0.5 g in 5 ml of *water R*. Add 0.1 ml of *thymol blue solution R*. The solution is violet-blue.
- E. Place 1.0 g in a crucible and add 2 ml of *dilute sulphuric acid R*. Heat at first on a water-bath, then ignite until all black particles have disappeared. Allow to cool. Take up the residue with *water R* and dilute to 20 ml with the same solvent. The solution gives reaction (b) of potassium (2.3.1).

TESTS

Appearance of solution. The solution is clear (2.2.1) and not more intensely coloured than reference solution GY₅ (2.2.2, *Method II*).

Rapidly dissolve 2.0 g with shaking in *water R* and dilute to 20.0 ml with the same solvent. Observe immediately.

Related substances. Thin-layer chromatography (2.2.27). *Prepare the solutions immediately before use and carry out the test protected from light.*

Test solution. Dissolve 0.20 g of the substance to be examined in *water R* and dilute to 5.0 ml with the same solvent. Shake immediately with 2 quantities, each of 5.0 ml, of *methylene chloride R*. Combine the organic layers and dilute to 10.0 ml with *methylene chloride R*.

Reference solution (a). Dissolve 10 mg of *aminochlorobenzophenone R* in *methylene chloride R* and dilute to 100.0 ml with the same solvent. Dilute 5.0 ml of the solution to 25.0 ml with *methylene chloride R*.

Reference solution (b). Dissolve 5 mg of *nordazepam CRS* in *methylene chloride R* and dilute to 25.0 ml with the same solvent. Dilute 5.0 ml of the solution to 25.0 ml with *methylene chloride R*.

Reference solution (c). Dilute 10.0 ml of reference solution (b) to 20.0 ml with *methylene chloride R*.

Reference solution (d). Dissolve 5 mg of *nordazepam CRS* and 5 mg of *nitrazepam CRS* in *methylene chloride R* and dilute to 25 ml with the same solvent.

Plate: TLC silica gel F_{254} plate *R*.

Mobile phase: acetone *R*, *methylene chloride R* (15:85 V/V).

Application: 5 µl.

Development: over 2/3 of the plate.

Drying: in air.

Detection A: examine in ultraviolet light at 254 nm.

System suitability: the chromatogram obtained with reference solution (d) shows 2 clearly separated spots.

Limits A:

- **impurity B:** any spot due to impurity B is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.2 per cent),
- **any other impurity:** any spot, apart from any spot due to impurity B, is not more intense than the spot in the chromatogram obtained with reference solution (c) (0.1 per cent).

Detection B: spray with a freshly prepared 10 g/l solution of *sodium nitrite R* in *dilute hydrochloric acid R*. Dry in a current of warm air and spray with a 4 g/l solution of *naphthylethylenediamine dihydrochloride R* in *alcohol R*.

Limits B:

- **impurity A:** any spot due to impurity A is not more intense than the spot in the chromatogram obtained with reference solution (a) (0.1 per cent).

Loss on drying (2.2.32): maximum 0.5 per cent, determined on 1.000 g by drying *in vacuo* at 60 °C for 4 h.

ASSAY

Dissolve 0.130 g in 10 ml of *anhydrous acetic acid R*. Add 30 ml of *methylene chloride R*. Titrate with 0.1 M *perchloric acid*, determining the 2 points of inflexion by potentiometry (2.2.20).

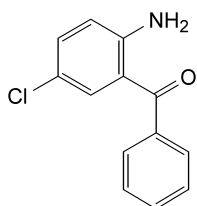
At the 2nd point of inflexion, 1 ml of 0.1 M *perchloric acid* is equivalent to 13.63 mg of $C_{16}H_{11}ClK_2N_2O_4$.

STORAGE

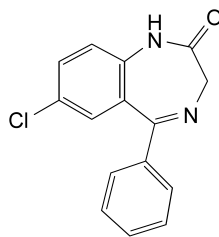
In an airtight container, protected from light.

IMPURITIES

Specified impurities: A, B.



- A. (2-amino-5-chlorophenyl)phenylmethanone (aminochlorobenzophenone),



- B. 7-chloro-5-phenyl-1,3-dihydro-2H-1,4-benzodiazepin-2-one (nordazepam).

01/2005:1003

DIPOTASSIUM PHOSPHATE

Dikalii fosphas



M_r 174.2

DEFINITION

Dipotassium phosphate contains not less than 98.0 per cent and not more than the equivalent of 101.0 per cent of K_2HPO_4 , calculated with reference to the dried substance.

CHARACTERS

A white powder or colourless crystals, very hygroscopic, very soluble in water, very slightly soluble in alcohol.

IDENTIFICATION

- A. Solution S (see Tests) is slightly alkaline (2.2.4).
B. Solution S gives reaction (b) of phosphates (2.3.1).
C. Solution S gives reaction (a) of potassium (2.3.1).

TESTS

Solution S. Dissolve 5.0 g in *distilled water R* and dilute to 50 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and colourless (2.2.2, *Method II*).

Reducing substances. Heat on a water-bath for 5 min a mixture of 5 ml of solution S, 5 ml of *dilute sulphuric acid R* and 0.25 ml of 0.02 M *potassium permanganate*. The solution remains faintly pink.

Monopotassium phosphate. Calculated from the number of millilitres of 1 M *hydrochloric acid* (10.0 ml) and of 1 M *sodium hydroxide* (n_1 ml and n_2 ml) used in the assay, the ratio:

$$\frac{n_2 - 10}{10 - n_1}$$

does not exceed 0.025 (2.5 per cent).

Chlorides (2.4.4). To 2.5 ml of solution S add 10 ml of *dilute nitric acid R* and dilute to 15 ml with *water R*. The solution complies with the limit test for chlorides (200 ppm).

Sulphates (2.4.13). To 1.5 ml of solution S add 2 ml of *dilute hydrochloric acid R* and dilute to 15 ml with *distilled water R*. The solution complies with the limit test for sulphates (0.1 per cent).

Arsenic (2.4.2). 5 ml of solution S complies with limit test A for arsenic (2 ppm).

Iron (2.4.9). 10 ml of solution S complies with the limit test for iron (10 ppm).

Heavy metals (2.4.8). Dissolve 2.0 g in 8 ml of *water R*. Acidify with about 6 ml of *dilute hydrochloric acid R* (pH 3 to 4) and dilute to 20 ml with *water R*. 12 ml of the solution complies with limit test A for heavy metals (10 ppm). Prepare the standard using *lead standard solution (1 ppm Pb) R*.

Sodium. If intended for use in the manufacture of parenteral dosage forms, it contains not more than 0.1 per cent of Na, determined by atomic emission spectrometry (2.2.22, *Method I*).

Test solution. Dissolve 1.00 g of the substance to be examined in *water R* and dilute to 100.0 ml with the same solvent.

Reference solutions. Prepare the reference solutions using *sodium standard solution (200 ppm Na) R*, diluted as necessary with *water R*.

Measure the emission intensity at 589 nm.

Loss on drying (2.2.32). Not more than 2.0 per cent, determined on 1.000 g by drying in an oven at 125 °C to 130 °C.

Bacterial endotoxins (2.6.14): less than 1.1 IU/mg, if intended for use in the manufacture of parenteral dosage forms without a further appropriate procedure for the removal of bacterial endotoxins.

ASSAY

Dissolve 0.800 g (*m*) in 40 ml of *carbon dioxide-free water R* and add 10.0 ml of *1 M hydrochloric acid*. Using *1 M sodium hydroxide*, titrate potentiometrically (2.2.20) to the first inflexion point of the pH curve (n_1 ml). Continue the titration to the second inflexion point of the curve (total volume of *1 M sodium hydroxide* required, n_2 ml).

Calculate the percentage content of K_2HPO_4 from the expression:

$$\frac{1742(10 - n_1)}{m(100 - d)}$$

d = percentage loss on drying.

STORAGE

Store in an airtight container.

LABELLING

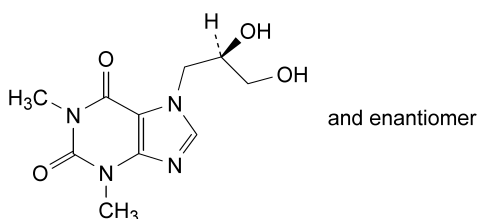
The label states:

- where applicable, that the substance is suitable for use in the manufacture of parenteral dosage forms,
- where applicable, that the substance is free from bacterial endotoxins.

01/2005:0486

DIPROPHYLLINE

Diprophyllinum



$C_{10}H_{14}N_4O_4$

M_r 254.2

DEFINITION

Diprophylline contains not less than 98.5 per cent and not more than the equivalent of 101.0 per cent of 7-[(2*RS*)-2,3-dihydroxypropyl]-1,3-dimethyl-3,7-dihydro-1*H*-purine-2,6-dione, calculated with reference to the dried substance.

CHARACTERS

A white, crystalline powder, freely soluble in water, slightly soluble in alcohol.

IDENTIFICATION

First identification: B, C.

Second identification: A, C, D.

A. Melting point (2.2.14): 160 °C to 165 °C.

B. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *diprophylline CRS*. Examine the substances as discs prepared using 0.5 mg to 1 mg of the substance to be examined in 0.3 g of *potassium bromide R*.

C. Dissolve 1 g in 5 ml of *acetic anhydride R* and boil under a reflux condenser for 15 min. Allow to cool and add 100 ml of a mixture of 20 volumes of *ether R* and 80 volumes of *light petroleum R*. Cool in iced water for at least 20 min, shaking from time to time. Filter, wash the precipitate with a mixture of 20 volumes of *ether R* and 80 volumes of *light petroleum R*, recrystallise from *alcohol R* and dry *in vacuo*. The crystals melt (2.2.14) at 142 °C to 148 °C.

D. It gives the reaction of xanthines (2.3.1).

TESTS

Solution S. Dissolve 2.5 g in *carbon dioxide-free water R* and dilute to 50 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and colourless (2.2.2, *Method II*).

Acidity or alkalinity. To 10 ml of solution S add 0.25 ml of *bromothymol blue solution R1*. The solution is yellow or green. Not more than 0.4 ml of 0.01 *M sodium hydroxide* is required to change the colour of the indicator to blue.

Related substances. Examine by thin-layer chromatography (2.2.27), using *silica gel HF₂₅₄ R* as the coating substance.

Test solution. Dissolve 0.3 g of the substance to be examined in a mixture of 20 volumes of *water R* and 30 volumes of *methanol R* and dilute to 10 ml with the same mixture of solvents. *Prepare immediately before use.*

Reference solution (a). Dilute 1 ml of the test solution to 100 ml with *methanol R*.

Reference solution (b). Dilute 0.2 ml of the test solution to 100 ml with *methanol R*.

Reference solution (c). Dissolve 10 mg of *theophylline R* in *methanol R*, add 0.3 ml of the test solution and dilute to 10 ml with *methanol R*.

Apply to the plate 10 µl of each solution. Develop over a path of 15 cm using a mixture of 1 volume of *concentrated ammonia R*, 10 volumes of *ethanol R* and 90 volumes of *chloroform R*. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. Any spot in the chromatogram obtained with the test solution, apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (a) (1 per cent) and at most one such spot is more intense than the spot in the chromatogram obtained with reference solution (b) (0.2 per cent). The test is not valid unless the chromatogram obtained with reference solution (c) shows two clearly separated spots.

Chlorides (2.4.4). Dilute 2.5 ml of solution S to 15 ml with *water R*. The solution complies with the limit test for chlorides (400 ppm).

Heavy metals (2.4.8). 12 ml of solution S complies with limit test A for heavy metals (20 ppm). Prepare the standard using *lead standard solution (1 ppm Pb) R*.

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

In order to avoid overheating in the reaction medium, mix thoroughly throughout and stop the titration immediately after the end-point has been reached.

Dissolve 0.200 g in 3.0 ml of *anhydrous formic acid R* and add 50.0 ml of *acetic anhydride R*. Titrate with 0.1 M *perchloric acid*, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *perchloric acid* is equivalent to 25.42 mg of $C_{24}H_{40}N_8O_4$.

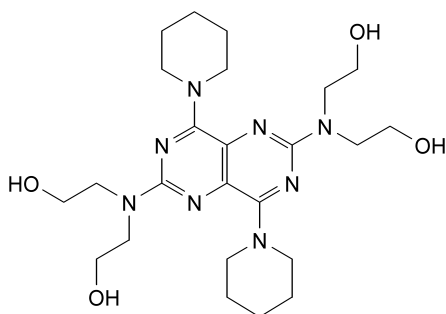
STORAGE

Store protected from light.

01/2005:1199

DIPYRIDAMOLE

Dipyridamolum



$C_{24}H_{40}N_8O_4$

M_r 504.6

DEFINITION

Dipyridamole contains not less than 98.5 per cent and not more than the equivalent of 101.5 per cent of 2,2',2'',2'''-[4,8-di(piperidin-1-yl)pyrimido[5,4-*d*]pyrimidine-2,6-diyl]dinitrilo]tetraethanol, calculated with reference to the dried substance.

CHARACTERS

A bright yellow, crystalline powder, practically insoluble in water, freely soluble in acetone, soluble in ethanol. It dissolves in dilute solutions of mineral acids.

IDENTIFICATION

First identification: C.

Second identification: A, B, D.

A. Melting point (2.2.14): 162 °C to 168 °C.

B. Dissolve 10 mg in a mixture of 1 volume of 0.1 M *hydrochloric acid* and 9 volumes of *methanol R* and dilute to 50.0 ml with the same mixture of solvents. Dilute 5.0 ml of this solution to 100.0 ml with a mixture of 1 volume of 0.1 M *hydrochloric acid* and 9 volumes of *methanol R*. Examined between 220 nm and 350 nm

(2.2.25), the solution shows two absorption maxima at 232 nm and 284 nm. The ratio of the absorbance measured at the maximum at 284 nm to that measured at the maximum at 232 nm is 1.25 to 1.45.

C. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *dipyridamole CRS*. Examine the substances as discs prepared using *potassium bromide R*.

D. Dissolve about 5 mg in a mixture of 0.1 ml of *nitric acid R* and 2 ml of *sulphuric acid R*. An intense violet colour is produced.

TESTS

Related substances. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 10.0 mg in the mobile phase and dilute to 20.0 ml with the mobile phase.

Reference solution (a). Dilute 1.0 ml of the test solution to 20.0 ml with the mobile phase. Dilute 5.0 ml of this solution to 50.0 ml with the mobile phase.

Reference solution (b). Dissolve 10.0 mg of *diltiazem hydrochloride CRS* in the mobile phase and dilute to 10.0 ml with the mobile phase. Dilute 1.0 ml of this solution to 20.0 ml with reference solution (a).

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4.6 mm in internal diameter packed with *octylsilyl silica gel for chromatography R* (5 µm),
- as mobile phase at a flow rate of 1.3 ml/min a mixture prepared as follows: dissolve 0.504 g of *potassium dihydrogen phosphate R* in 370 ml of *water R* and adjust to pH 3.0 with *phosphoric acid R*; add 80 ml of *acetonitrile R* and 550 ml of *methanol R*,
- as detector a spectrophotometer set at 290 nm, maintaining the temperature of the column at 30 °C.

Inject 20 µl of each solution and continue the chromatography of the test solution for nine times the retention time of dipyridamole. The test is not valid unless, in the chromatogram obtained with reference solution (b), the resolution between the peaks corresponding respectively to diltiazem and dipyridamole is at least 2.0. In the chromatogram obtained with the test solution: the area of any peak, apart from the principal peak is not greater than the area of the peak in the chromatogram obtained with reference solution (a) (0.5 per cent); and the sum of the areas of all the peaks, apart from the principal peak, is not greater than twice the area of the peak in the chromatogram obtained with reference solution (a) (1 per cent). Disregard any peak with an area less than 0.1 times that of the peak in the chromatogram obtained with reference solution (a).

Chlorides (2.4.4). To 0.250 g add 10 ml of *water R* and strongly shake. Filter, rinse the filter with 5 ml of *water R* and dilute to 15 ml with *water R*. The combined filtrates comply with the limit test for chlorides (200 ppm).

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

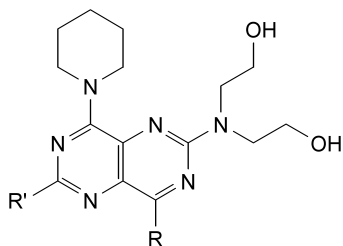
Dissolve 0.400 g in 70 ml of *methanol R*. Titrate with 0.1 M *perchloric acid*, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *perchloric acid* is equivalent to 50.46 mg of $C_{24}H_{40}N_8O_4$.

STORAGE

Store protected from light.

IMPURITIES

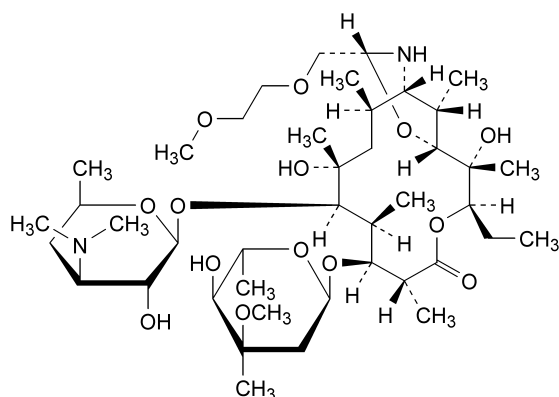


- A. $R = R' = \text{NC}_5\text{H}_{10}$: 2,2'-[[4,6,8-tri(piperidin-1-yl)pyrimido[5,4-*d*]pyrimidin-2-yl]nitrilo]diethanol,
- B. $R = R' = \text{N}(\text{CH}_2\text{-CH}_2\text{OH})_2$: 2,2',2'',2''',2''''',2'''''-[[8-(piperidin-1-yl)pyrimido[5,4-*d*]pyrimidine-2,4,6-triyl]trinitrilo]hexaethanol,
- C. $R = \text{NC}_5\text{H}_{10}$, $R' = \text{Cl}$: 2,2'-[[2-chloro-4,8-di(piperidin-1-yl)pyrimido[5,4-*d*]pyrimidin-6-yl]nitrilo]diethanol.

01/2005:1313

DIRITHROMYCIN

Dirithromycinum

 $\text{C}_{42}\text{H}_{78}\text{N}_2\text{O}_{14}$ M_r 835

DEFINITION

Dirithromycin is (1*R*,2*S*,3*R*,6*R*,7*S*,8*S*,9*R*,10*R*,12*R*,13*S*,15*R*,17*S*)-9-[[3-(dimethylamino)-3,4,6-trideoxy-β-*D*-xylo-hexopyranosyl]oxy]-3-ethyl-2,10-dihydroxy-15-[(2-methoxyethoxy)methyl]-2,6,8,10,12,17-hexamethyl-7-[(3-*C*-methyl-3-*O*-methyl-2,6-dideoxy-α-*L*-ribo-hexopyranosyl)oxy]-4,16-dioxo-14-azabicyclo[11.3.1]heptadecan-5-one (or (9*S*)-9,11-[iminol[(1*R*)-2-(2-methoxyethoxy)ethylidene]oxy]-9-deoxo-11-deoxyerythromycin). The sum of the percentage contents of $\text{C}_{42}\text{H}_{78}\text{N}_2\text{O}_{14}$ and dirithromycin 15*S*-epimer is not less than 96.0 per cent and not more than the equivalent of 102.0 per cent, calculated with reference to the anhydrous substance.

CHARACTERS

A white or almost white powder, very slightly soluble in water, very soluble in methanol and in methylene chloride. It shows polymorphism.

IDENTIFICATION

- A. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *dirithromycin CRS*. Examine the substance prepared as discs.

- B. Examine the chromatograms obtained in the assay.

The retention time and size of the principal peak in the chromatogram obtained with test solution (a) are similar to those of the principal peak in the chromatogram obtained with reference solution (a).

TESTS

Related substances. Examine by liquid chromatography (2.2.29), as described under Assay. Inject 10 µl of reference solution (b). Adjust the sensitivity of the system so that the height of the principal peak is at least 50 per cent of the full scale of the recorder. Inject 10 µl of test solution (b) and continue the chromatography for three times the retention time of the principal peak. In the chromatogram obtained with test solution (b): the area of any peak corresponding to impurity A is not greater than 0.75 times the area of the principal peak in the chromatogram obtained with reference solution (b) (1.5 per cent); the area of any peak, apart from the principal peak and any peak corresponding to impurity A and any peak corresponding to 15*S*-epimer, is not greater than half the area of the principal peak in the chromatogram obtained with reference solution (b) (1 per cent).

Dirithromycin 15*S*-epimer. Not more than 1.5 per cent, determined by liquid chromatography (2.2.29), as described under Assay. Inject 10 µl of reference solution (b). Adjust the sensitivity of the system so that the height of the principal peak is at least 50 per cent of the full scale of the recorder. Inject reference solution (b) six times. The test is not valid unless the relative standard deviation of the peak area for dirithromycin is at most 5.0 per cent. Inject alternately test solution (b) and reference solution (b). Calculate the percentage content of dirithromycin 15*S*-epimer using the chromatogram obtained with reference solution (b).

Acetonitrile (2.4.24, *System A*). Not more than 0.1 per cent. Prepare the solutions using *dimethylformamide R* instead of *water R*.

Sample solution. Dissolve 0.200 g of the substance to be examined in *dimethylformamide R* and dilute to 20.0 ml with the same solvent.

The following static head-space injection conditions may be used:

- equilibration temperature: 120 °C,
- equilibration time: 60 min,
- transfer-line temperature: 125 °C.

Heavy metals (2.4.8). Dissolve 1.0 g in 20 ml of a mixture of equal volumes of *methanol R* and *water R*. 12 ml of the solution complies with limit test B for heavy metals (20 ppm). Prepare the standard using lead standard solution (1 ppm Pb) obtained by diluting *lead standard solution (100 ppm Pb) R* with a mixture of equal volumes of *methanol R* and *water R*.

Water (2.5.12). Not more than 1.0 per cent, determined on 1.00 g by the semi-micro determination of water.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Examine by liquid chromatography (2.2.29).

Solvent mixture. Mix 30 volumes of *methanol R* and 70 volumes of *acetonitrile R*.

Test solution (a). Dissolve 20.0 mg of the substance to be examined in the solvent mixture and dilute to 10.0 ml with the solvent mixture.

Test solution (b). Dissolve 0.10 g of the substance to be examined in the solvent mixture and dilute to 10.0 ml with the solvent mixture.

Reference solution (a). Dissolve 20.0 mg of *dirithromycin CRS* in the solvent mixture and dilute to 10.0 ml with the solvent mixture.

Reference solution (b). Dilute 5.0 ml of reference solution (a) to 50.0 ml with the solvent mixture.

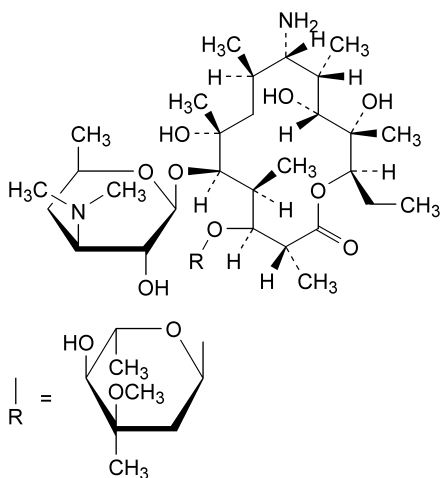
Reference solution (c). Dissolve 20 mg of *dirithromycin CRS* in the mobile phase and dilute to 10 ml with the mobile phase. Allow to stand for 24 h before use.

The chromatographic procedure may be carried out using:

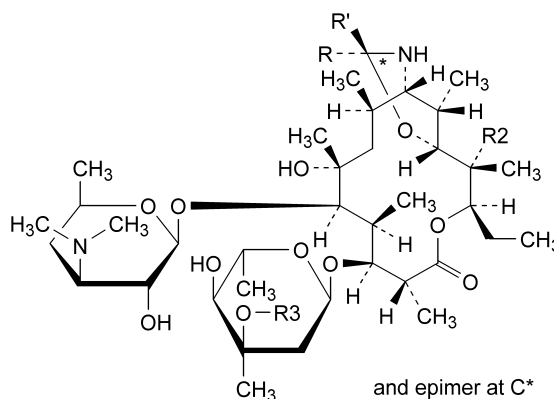
- a stainless steel column 0.25 m long and 4.6 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (5 µm),
- as mobile phase at a flow rate of 2.0 ml/min a mixture of: 9 volumes of *water R*; 19 volumes of *methanol R*; 28 volumes of a solution containing 1.9 g/l of *potassium dihydrogen phosphate R* and 9.1 g/l of *dipotassium hydrogen phosphate R* adjusted to pH 7.5 if necessary with a 100 g/l solution of *potassium hydroxide R*; 44 volumes of *acetonitrile R*,
- as detector a spectrophotometer set at 205 nm,

maintaining the temperature of the column at 40 °C. Inject 10 µl of reference solution (a). Adjust the sensitivity of the system so that the height of the principal peak in the chromatogram obtained is at least 50 per cent of the full scale of the recorder. Inject 10 µl of reference solution (c). When the chromatogram is recorded in the prescribed conditions, the retention times relative to dirithromycin are: impurity A about 0.7 and 15*S*-epimer about 1.1. The test is not valid unless, in the chromatogram obtained with reference solution (c), the resolution between the peaks corresponding to dirithromycin and its 15*S*-epimer is at least 2.0 (if necessary adjust the concentration of the organic modifiers in the mobile phase). Inject reference solution (a) six times. The test is not valid unless the relative standard deviation of the peak area for dirithromycin is at most 1.0 per cent. Inject alternately test solution (a) and reference solution (a).

IMPURITIES



- A. (9*S*)-9-amino-9-deoxyerythromycin,
- B. R = H: (9*S*)-9-amino-3-de(2,6-dideoxy-3-*C*-methyl-3-*O*-methyl- α -*L*-ribo-hexopyranosyl)-9-deoxyerythromycin,

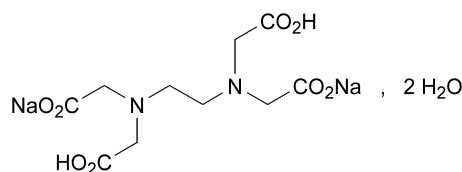


- C. R = CH₂-O-CH₂-CH₂-O-CH₃, R' = H, R₂ = H, R₃ = CH₃: (9*S*)-9,11-[imino[(1*RS*)-2-(2-methoxyethoxy)ethylidene]oxy]-9-deoxy-11,12-dideoxyerythromycin (dirithromycin B),
- D. R = CH₂-O-CH₂-CH₂-O-CH₃, R' = H, R₂ = OH, R₃ = H: (9*S*)-9,11-[imino[(1*RS*)-2-(2-methoxyethoxy)ethylidene]oxy]-3'-*O*-demethyl-9-deoxy-11-deoxyerythromycin (dirithromycin C),
- E. R = CH₃, R' = CH₃, R₂ = OH, R₃ = CH₃: 9,11-[imino(1-methylethylidene)oxy]-9-deoxy-11-deoxyerythromycin.

01/2005:0232

DISODIUM EDETATE

Dinatrii edetas



C₁₀H₁₄N₂Na₂O₈·2H₂O

M_r 372.2

DEFINITION

Disodium dihydrogen (ethylenedinitrilo)tetraacetate dihydrate.

Content: 98.5 per cent to 101.0 per cent.

CHARACTERS

Appearance: white, crystalline powder.

Solubility: soluble in water, practically insoluble in alcohol.

IDENTIFICATION

First identification: A, B, D.

Second identification: B, C, D.

A. Infrared absorption spectrophotometry (2.2.24).

Preparation: discs.

Comparison: *disodium edetate CRS*.

B. Dissolve 2 g in 25 ml of *water R*, add 6 ml of *lead nitrate solution R*, shake and add 3 ml of *potassium iodide solution R*. No yellow precipitate is formed. Make alkaline to *red litmus paper R* by the addition of *dilute ammonia R2*. Add 3 ml of *ammonium oxalate solution R*. No precipitate is formed.

C. Dissolve 0.5 g in 10 ml of *water R* and add 0.5 ml of *calcium chloride solution R*. Make alkaline to *red litmus paper R* by the addition of *dilute ammonia R2* and add 3 ml of *ammonium oxalate solution R*. No precipitate is formed.

D. It gives the reactions of sodium (2.3.1).

TESTS

Solution S. Dissolve 5.0 g in *carbon dioxide-free water R* and dilute to 100 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and colourless (2.2.2, *Method II*).

pH (2.2.3): 4.0 to 5.5 for solution S.

Impurity A. Liquid chromatography (2.2.29). Carry out the test protected from light.

Solvent mixture. Dissolve 10.0 g of *ferric sulphate pentahydrate R* in 20 ml of 0.5 M sulphuric acid and add 780 ml of *water R*. Adjust to pH 2.0 with 1 M sodium hydroxide and dilute to 1000 ml with *water R*.

Test solution. Dissolve 0.100 g of the substance to be examined in the solvent mixture and dilute to 25.0 ml with the solvent mixture.

Reference solution. Dissolve 40.0 mg of *nitrilotriacetic acid R* in the solvent mixture and dilute to 100.0 ml with the solvent mixture. To 1.0 ml of the solution add 0.1 ml of the test solution and dilute to 100.0 ml with the solvent mixture.

Column:

- size: $l = 0.10$ m, $\varnothing = 4.6$ mm,
- stationary phase: spherical graphitised carbon for chromatography R1 (5 μ m) with a specific surface area of 120 m²/g and a pore size of 25 nm.

Mobile phase: dissolve 50.0 mg of *ferric sulphate pentahydrate R* in 50 ml of 0.5 M sulphuric acid and add 750 ml of *water R*. Adjust to pH 1.5 with 0.5 M sulphuric acid or 1 M sodium hydroxide, add 20 ml of *ethylene glycol R* and dilute to 1000 ml with *water R*.

Flow rate: 1 ml/min.

Detection: spectrophotometer at 273 nm.

Injection: 20 μ l; filter the solutions and inject immediately.

Run time: 4 times the retention time of the iron complex of impurity A.

Retention times: iron complex of impurity A = about 5 min; iron complex of edetic acid = about 10 min.

System suitability: reference solution:

- resolution: minimum 7 between the peaks due to the iron complex of impurity A and the iron complex of edetic acid,
- signal-to-noise ratio: minimum 50 for the peak due to impurity A.

Limit:

- impurity A: not more than the area of the corresponding peak in the chromatogram obtained with the reference solution (0.1 per cent).

Iron (2.4.9): maximum 80 ppm.

Dilute 2.5 ml of solution S to 10 ml with *water R*. Add 0.25 g of *calcium chloride R* to the test solution and the standard before the addition of the *thioglycollic acid R*.

Heavy metals (2.4.8): maximum 20 ppm.

1.0 g complies with limit test D. Prepare the standard using 2 ml of *lead standard solution* (10 ppm Pb) R.

ASSAY

Dissolve 0.300 g in *water R* and dilute to 300 ml with the same solvent. Add 2 g of *hexamethylenetetramine R* and 2 ml of *dilute hydrochloric acid R*. Titrate with 0.1 M *lead nitrate*, using about 50 mg of *xenol orange triturate R* as indicator.

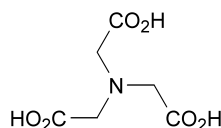
1 ml of 0.1 M *lead nitrate* is equivalent to 37.22 mg of $C_{10}H_{14}N_2Na_2O_8 \cdot 2H_2O$.

STORAGE

Protected from light.

IMPURITIES

Specified impurities: A.



A. nitrilotriacetic acid.

01/2005:1509
corrected

DISODIUM PHOSPHATE, ANHYDROUS

Dinatrii phosphas anhydricus

Na_2HPO_4

M_r 142.0

DEFINITION

Anhydrous disodium phosphate contains not less than 98.0 per cent and not more than the equivalent of 101.0 per cent of Na_2HPO_4 , calculated with reference to the dried substance.

CHARACTERS

A white powder, hygroscopic, soluble in water, practically insoluble in alcohol.

IDENTIFICATION

- Solution S (see Tests) is slightly alkaline (2.2.4).
- It complies with the test for loss on drying (see Tests).
- Solution S (see Tests) gives reaction (b) of phosphates (2.3.1).
- Solution S (see Tests) gives reaction (a) of sodium (2.3.1).

TESTS

Solution S. Dissolve 5.0 g in *distilled water R* and dilute to 100.0 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and colourless (2.2.2, *Method II*).

Reducing substances. To 10 ml of solution S add 5 ml of *dilute sulphuric acid R* and 0.25 ml of 0.02 M *potassium permanganate* and heat on a water-bath for 5 min. The solution retains a slight red colour.

Monosodium phosphate. Calculated from the number of millilitres of 1 M *hydrochloric acid* (25 ml) and of 1 M *sodium hydroxide* (n_1 ml and n_2 ml) used in the assay, the ratio:

$$\frac{n_2 - 25}{25 - n_1}$$

does not exceed 0.025.

Chlorides (2.4.4). 5 ml of solution S diluted to 15 ml with *dilute nitric acid R* complies with the limit test for chlorides (200 ppm).

Sulphates (2.4.13). To 6 ml of solution S add 2 ml of *dilute hydrochloric acid R* and dilute to 15 ml with *distilled water R*. The solution complies with the limit test for sulphates (500 ppm).

Arsenic (2.4.2). 10 ml of solution S complies with limit test A for arsenic (2 ppm).

Iron (2.4.9). 10 ml of solution S complies with the limit test for iron (20 ppm).

Heavy metals (2.4.8). 12 ml of solution S complies with limit test A for heavy metals (10 ppm). Prepare the standard using 5 ml of *lead standard solution (1 ppm Pb) R* and 5 ml of *water R*.

Loss on drying (2.2.32). Not more than 1.0 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C for 4 h.

ASSAY

Dissolve 1.600 g (*m*) in 25.0 ml of *carbon dioxide-free water R* and add 25.0 ml of *1 M hydrochloric acid*. Using *1 M sodium hydroxide*, titrate potentiometrically (2.2.20) to the first inflexion point (n_1 ml). Continue the titration to the second inflexion point (total volume of *1 M sodium hydroxide* required, n_2 ml).

Calculate the percentage content of Na_2HPO_4 from the expression:

$$\frac{1420 (25 - n_1)}{m (100 - d)}$$

d = percentage loss on drying.

STORAGE

Store in an airtight container.

01/2005:0602

DISODIUM PHOSPHATE DIHYDRATE

Dinatrii phosphas dihydricus

$\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$

M_r 178.0

DEFINITION

Disodium phosphate dihydrate contains not less than 98.0 per cent and not more than the equivalent of 101.0 per cent of Na_2HPO_4 , calculated with reference to the dried substance.

CHARACTERS

A white or almost white powder or colourless crystals, soluble in water, practically insoluble in alcohol.

IDENTIFICATION

- Solution S (see Tests) is slightly alkaline (2.2.4).
- It complies with the test for loss on drying (see Tests).
- Solution S gives reaction (b) of phosphates (2.3.1).
- Solution S gives reaction (a) of sodium (2.3.1).

TESTS

Solution S. Dissolve 5.0 g in *distilled water R* and dilute to 100 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and colourless (2.2.2, *Method II*).

Reducing substances. To 5 ml of solution S add 5 ml of *dilute sulphuric acid R* and 0.25 ml of *0.02 M potassium permanganate* and heat on a water-bath for 5 min. The solution retains a slight red colour.

Monosodium phosphate. Calculated from the number of millilitres of *1 M hydrochloric acid* (25 ml) and of *1 M sodium hydroxide* (n_1 ml and n_2 ml) used in the assay, the ratio:

$$\frac{n_2 - 25}{25 - n_1}$$

does not exceed 0.025.

Chlorides (2.4.4). To 2.5 ml of solution S add 10 ml of *dilute nitric acid R* and dilute to 15 ml with *water R*. The solution complies with the limit test for chlorides (400 ppm).

Sulphates (2.4.13). To 3 ml of solution S add 2 ml of *dilute hydrochloric acid R* and dilute to 15 ml with *distilled water R*. The solution complies with the limit test for sulphates (0.1 per cent).

Arsenic (2.4.2). 5 ml of solution S complies with limit test A for arsenic (4 ppm).

Iron (2.4.9). 5 ml of solution S diluted to 10 ml with *water R* complies with the limit test for iron (40 ppm).

Heavy metals (2.4.8). 12 ml of solution S complies with limit test A for heavy metals (20 ppm). Prepare the standard using *lead standard solution (1 ppm Pb) R*.

Loss on drying (2.2.32): 19.5 per cent to 21.0 per cent, determined on 1.000 g by drying in an oven at 130 °C.

ASSAY

Dissolve 2.000 g (*m* g) in 50 ml of *water R* and add 25.0 ml of *1 M hydrochloric acid*. Using *1 M sodium hydroxide*, titrate potentiometrically (2.2.20) to the first inflexion point of the pH curve (n_1 ml). Continue the titration to the second inflexion point of the curve (total volume of *1 M sodium hydroxide* required, n_2 ml).

Calculate the percentage content of Na_2HPO_4 from the expression:

$$\frac{1420 (25 - n_1)}{m (100 - d)}$$

d = percentage loss on drying.

01/2005:0118

DISODIUM PHOSPHATE DODECAHYDRATE

Dinatrii phosphas dodecahydricus

$\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$

M_r 358.1

DEFINITION

Disodium phosphate dodecahydrate contains not less than 98.0 per cent and not more than the equivalent of 101.0 per cent of Na_2HPO_4 , calculated with reference to the anhydrous substance.

CHARACTERS

Colourless, transparent crystals, very efflorescent, very soluble in water, practically insoluble in alcohol.

IDENTIFICATION

- Solution S (see Tests) gives the reactions of phosphates (2.3.1).
- Solution S gives the reactions of sodium (2.3.1).

TESTS

01/2005:1006

Solution S. Dissolve 5.0 g in *distilled water R* and dilute to 50 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and colourless (2.2.2, *Method II*).

Reducing substances. To 5 ml of solution S add 5 ml of *dilute sulphuric acid R* and 0.25 ml of 0.02 M *potassium permanganate* and heat on a water-bath for 5 min. The solution retains a slight red colour.

Monosodium phosphate. Calculated from the number of millilitres of 1 M *hydrochloric acid* (25 ml) and of 1 M *sodium hydroxide* (n_1 ml and n_2 ml) used in the assay, the ratio:

$$\frac{n_2 - 25}{25 - n_1}$$

does not exceed 0.025.

Chlorides (2.4.4). To 2.5 ml of solution S add 10 ml of *dilute nitric acid R* and dilute to 15 ml with *water R*. The solution complies with the limit test for chlorides (200 ppm).

Sulphates (2.4.13). To 3 ml of solution S add 2 ml of *dilute hydrochloric acid R* and dilute to 15 ml with *distilled water R*. The solution complies with the limit test for sulphates (500 ppm).

Arsenic (2.4.2). 5 ml of solution S complies with limit test A for arsenic (2 ppm).

Iron (2.4.9). 5 ml of solution S diluted to 10 ml with *water R* complies with the limit test for iron (20 ppm).

Heavy metals (2.4.8). 12 ml of solution S complies with limit test A for heavy metals (10 ppm). Prepare the standard using *lead standard solution* (1 ppm Pb) *R*.

Water (2.5.12). 57.0 per cent to 61.0 per cent, determined on 50.0 mg by the semi-micro determination of water. Use a mixture of 10 volumes of *anhydrous methanol R* and 40 volumes of *formamide R* as solvent.

ASSAY

Dissolve 4.00 g (m g) in 25 ml of *water R* and add 25.0 ml of 1 M *hydrochloric acid*. Using 1 M *sodium hydroxide*, titrate potentiometrically (2.2.20) to the first inflexion point of the pH curve (n_1 ml). Continue the titration to the second inflexion point of the curve (total volume of 1 M *sodium hydroxide* required, n_2 ml).

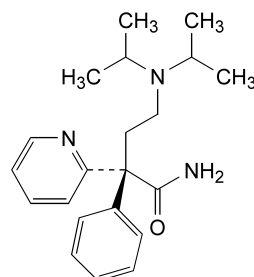
Calculate the percentage content of Na_2HPO_4 from the expression:

$$\frac{1420 (25 - n_1)}{m (100 - d)}$$

d = percentage water content.

DISOPYRAMIDE

Disopyramidum



and enantiomer

$\text{C}_{21}\text{H}_{29}\text{N}_3\text{O}$

M_r 339.5

DEFINITION

Disopyramide contains not less than 98.5 per cent and not more than the equivalent of 101.5 per cent of (2*RS*)-4-[bis(1-methylethyl)amino]-2-phenyl-2-(pyridin-2-yl)butanamide, calculated with reference to the dried substance.

CHARACTERS

A white or almost white powder, slightly soluble in water, freely soluble in methylene chloride, soluble in alcohol.

IDENTIFICATION

First identification: B.

Second identification: A, C.

- Dissolve 40.0 mg in a 5 g/l solution of *sulphuric acid R* in *methanol R* and dilute to 100.0 ml with the same solution. Dilute 5.0 ml of this solution to 50.0 ml with a 5 g/l solution of *sulphuric acid R* in *methanol R*. Examined between 240 nm and 350 nm (2.2.25), the solution shows an absorption maximum at 269 nm and a shoulder at 263 nm. The specific absorbance at the maximum is 190 to 210.
- Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *disopyramide CRS*. Examine the substances as discs prepared by placing 50 μl of a 50 g/l solution in *methylene chloride R* on a disc of *potassium bromide R*. Dry the discs at 60 °C for 1 h before use.
- Examine the chromatograms obtained in the test for related substances in ultraviolet light at 254 nm. The principal spot in the chromatogram obtained with test solution (b) is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a). Spray with *dilute potassium iodobismuthate solution R*. Examine in daylight. The principal spot in the chromatogram obtained with test solution (b) is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).

TESTS

Related substances. Examine by thin-layer chromatography (2.2.27), using *silica gel GF₂₅₄ R* as the coating substance.

Test solution (a). Dissolve 0.20 g of the substance to be examined in *methanol R* and dilute to 10 ml with the same solvent.

Test solution (b). Dilute 1 ml of test solution (a) to 10 ml with *methanol R*.

Reference solution (a). Dissolve 20 mg of *disopyramide CRS* in *methanol R* and dilute to 10 ml with the same solvent.

01/2005:1005

Reference solution (b). Dilute 0.5 ml of test solution (b) to 20 ml with *methanol R*.

Apply to the plate 10 µl of each solution. Develop over a path of 15 cm using a mixture of 1 volume of *concentrated ammonia R*, 30 volumes of *acetone R* and 30 volumes of *cyclohexane R*. Dry the plate in a current of warm air and examine in ultraviolet light at 254 nm. Any spot in the chromatogram obtained with test solution (a), apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.25 per cent).

Heavy metals (2.4.8). 2.0 g complies with limit test C for heavy metals (10 ppm). Prepare the standard using 2 ml of *lead standard solution (10 ppm Pb) R*.

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying at 80 °C over *diphosphorus pentoxide R* at a pressure not exceeding 0.7 kPa for 2 h.

Sulphated ash (2.4.14). Not more than 0.2 per cent, determined on 1.0 g.

ASSAY

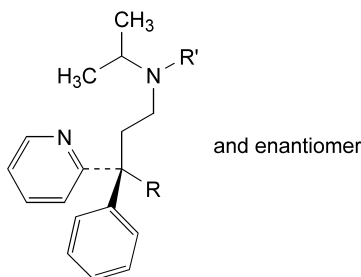
Dissolve 0.130 g in 30 ml of *anhydrous acetic acid R*. Add 0.2 ml of *naphtholbenzein solution R*. Titrate with 0.1 M *perchloric acid* until the colour changes from yellow to green.

1 ml of 0.1 M *perchloric acid* is equivalent to 16.97 mg of $C_{21}H_{29}N_3O_5$.

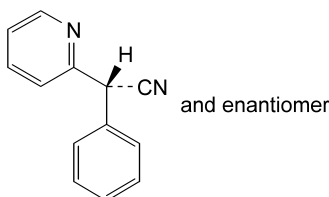
STORAGE

Store protected from light.

IMPURITIES



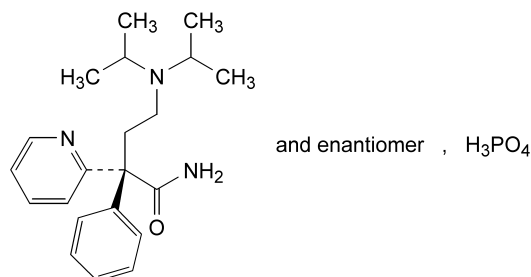
- A. $R = CN$, $R' = CH(CH_3)_2$: (2RS)-4-[bis(1-methylethyl)amino]-2-phenyl-2-(pyridin-2-yl)butanenitrile (di-isopyronitrile),
- B. $R = H$, $R' = CH(CH_3)_2$: (3RS)-N,N-bis(1-methylethyl)-3-phenyl-3-(pyridin-2-yl)propan-1-amine,
- C. $R = CO-NH_2$, $R' = H$: (2RS)-4-[(1-methylethyl)amino]-2-phenyl-2-(pyridin-2-yl)butanamide,



- D. (RS)-phenyl(pyridin-2-yl)acetonitrile (pyronitrile).

DISOPYRAMIDE PHOSPHATE

Disopyramidi phosphas


 $C_{21}H_{32}N_3O_5P$
 M_r 437.5

DEFINITION

Disopyramide phosphate contains not less than 98.0 per cent and not more than the equivalent of 102.0 per cent of (2RS)-4-[bis(1-methylethyl)amino]-2-phenyl-2-(pyridin-2-yl)butanamide dihydrogen phosphate, calculated with reference to the dried substance.

CHARACTERS

A white or almost white powder, soluble in water, sparingly soluble in alcohol, practically insoluble in methylene chloride.

IDENTIFICATION

First identification: B.

Second identification: A, C, D.

- A. Dissolve 50.0 mg in a 5 g/l solution of *sulphuric acid R* in *methanol R* and dilute to 100.0 ml with the same solution. Dilute 5.0 ml of this solution to 50.0 ml with a 5 g/l solution of *sulphuric acid R* in *methanol R*. Examined between 240 nm and 350 nm (2.2.25), the solution shows an absorption maximum at 269 nm and a shoulder at 263 nm. The specific absorbance at the maximum is 147 to 163.
- B. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *disopyramide phosphate CRS*. Examine the substances prepared as discs.
- C. Examine the chromatograms obtained in the test for related substances in ultraviolet light at 254 nm. The principal spot in the chromatogram obtained with test solution (b) is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a). Spray with *dilute potassium iodobismuthate solution R*. Examine in daylight. The principal spot in the chromatogram obtained with test solution (b) is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).
- D. Solution S (see Tests) gives reaction (a) of phosphates (2.3.1).

TESTS

Solution S. Dissolve 1.0 g in *carbon dioxide-free water R* and dilute to 20 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and colourless (2.2.2, *Method II*).

pH (2.2.3). The pH of solution S is 4.0 to 5.0.

01/2005:0603

Related substances. Examine by thin-layer chromatography (2.2.27), using silica gel GF_{254} R as the coating substance.

Test solution (a). Dissolve 0.25 g of the substance to be examined in *methanol R* and dilute to 10 ml with the same solvent.

Test solution (b). Dilute 1 ml of test solution (a) to 10 ml with *methanol R*.

Reference solution (a). Dissolve 25 mg of *disopyramide phosphate CRS* in *methanol R* and dilute to 10 ml with the same solvent.

Reference solution (b). Dilute 1 ml of test solution (b) to 20 ml with *methanol R*.

Apply to the plate 10 µl of each solution. Develop over a path of 15 cm using a mixture of 1 volume of *concentrated ammonia R*, 30 volumes of *acetone R* and 30 volumes of *cyclohexane R*. Dry the plate in a current of warm air and examine in ultraviolet light at 254 nm. Any spot in the chromatogram obtained with test solution (a), apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.5 per cent).

Heavy metals (2.4.8). 2.0 g complies with limit test C for heavy metals (10 ppm). Prepare the standard using 2 ml of *lead standard solution (10 ppm Pb) R*.

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 100–105 °C.

ASSAY

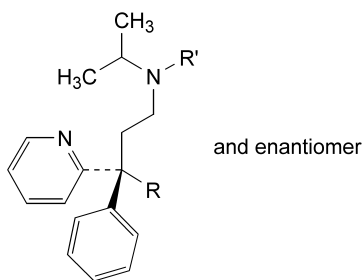
Dissolve 0.180 g in 30 ml of *anhydrous acetic acid R*. Add 0.2 ml of *naphtholbenzein solution R*. Titrate with 0.1 M *perchloric acid* until the colour changes from yellow to green.

1 ml of 0.1 M *perchloric acid* is equivalent to 21.88 mg of $C_{10}H_{20}N_2S_4$.

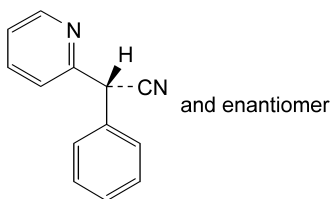
STORAGE

Store protected from light.

IMPURITIES



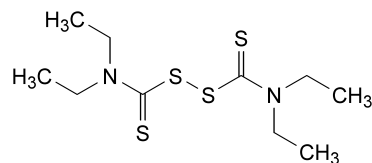
- A. $R = CN$, $R' = CH(CH_3)_2$: (2*RS*)-4-[bis(1-methylethyl)amino]-2-phenyl-2-(pyridin-2-yl)butanenitrile (di-isopyronitrile),
- B. $R = H$, $R' = CH(CH_3)_2$: (3*RS*)-*N,N*-bis(1-methylethyl)-3-phenyl-3-(pyridin-2-yl)propan-1-amine,
- C. $R = CO-NH_2$, $R' = H$: (2*RS*)-4-[(1-methylethyl)amino]-2-phenyl-2-(pyridin-2-yl)butanamide,



- D. (*RS*)-phenyl(pyridin-2-yl)acetonitrile (pyronitrile).

DISULFIRAM

Disulfiramum


 $C_{10}H_{20}N_2S_4$
 M_r 296.5

DEFINITION

Disulfiram contains not less than 98.5 per cent and not more than the equivalent of 101.0 per cent of tetraethyldisulfanedicarbothioamide, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, crystalline powder, practically insoluble in water, freely soluble in methylene chloride, sparingly soluble in alcohol.

IDENTIFICATION

First identification: A, B.

Second identification: A, C, D.

- A. Melting point (2.2.14): 70 °C to 73 °C.
- B. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *disulfiram CRS*. Examine the substances prepared as discs.
- C. Examine the chromatograms obtained in the test for related substances. The principal spot in the chromatogram obtained with test solution (b) is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a).
- D. Dissolve about 10 mg in 10 ml of *methanol R*. Add 2 ml of a 0.5 g/l solution of *cupric chloride R* in *methanol R*. A yellow colour develops which becomes greenish-yellow.

TESTS

Related substances. Examine by thin-layer chromatography (2.2.27), using as the coating substance a suitable silica gel with a fluorescent indicator having an optimal intensity at 254 nm.

Test solution (a). Dissolve 0.20 g of the substance to be examined in *ethyl acetate R* and dilute to 10 ml with the same solvent.

Test solution (b). Dilute 1 ml of test solution (a) to 10 ml with *ethyl acetate R*.

Reference solution (a). Dissolve 10 mg of *disulfiram CRS* in *ethyl acetate R* and dilute to 5 ml with the same solvent.

Reference solution (b). Dilute 1 ml of test solution (b) to 20 ml with *ethyl acetate R*.

Apply to the plate 10 µl of each solution. Develop over a path of 15 cm using a mixture of 30 volumes of *butyl acetate R* and 70 volumes of *hexane R*. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. Any spot in the chromatogram obtained with test solution (a), apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.5 per cent).

Diethyldithiocarbamate. Dissolve 0.20 g in 10 ml of *peroxide-free ether R*, add 5 ml of *buffer solution pH 8.0 R* and shake vigorously. Discard the upper layer and wash the

lower layer with 10 ml of *peroxide-free ether R*. Add to the lower layer 0.2 ml of a 4 g/l solution of *copper sulphate R* and 5 ml of *cyclohexane R*. Shake. Any yellow colour in the upper layer is not more intense than that of a standard prepared at the same time using 0.2 ml of a freshly prepared 0.15 g/l solution of *sodium diethyldithiocarbamate R* (150 ppm).

Heavy metals (2.4.8). 1.0 g complies with limit test C for heavy metals (20 ppm). Prepare the standard using 2 ml of *lead standard solution (10 ppm Pb) R*.

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying *in vacuo* at 50 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

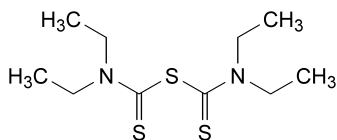
ASSAY

Dissolve 0.450 g in 80 ml of *acetone R* and add 20 ml of a 20 g/l solution of *potassium nitrate R*. Titrate with 0.1 M *silver nitrate*. Determine the end-point potentiometrically (2.2.20), using a silver electrode and a silver-silver chloride double-junction electrode saturated with potassium nitrate. 1 ml of 0.1 M *silver nitrate* is equivalent to 59.30 mg of $C_{10}H_{20}N_2S_4$.

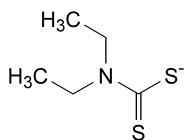
STORAGE

Store protected from light.

IMPURITIES



A. diethylthiocarbamic thioanhydride (sulfiram),

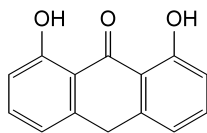


B. diethyldithiocarbamate.

01/2005:1007

DITHRANOL

Dithranolum



$C_{14}H_{10}O_3$

M_r 226.2

DEFINITION

Dithranol contains not less than 98.5 per cent and not more than the equivalent of 101.0 per cent of 1,8-dihydroxyanthracen-9(10H)-one, calculated with reference to the dried substance.

CHARACTERS

A yellow or brownish-yellow, crystalline powder, practically insoluble in water, soluble in methylene chloride, sparingly soluble in acetone, slightly soluble in alcohol. It dissolves in dilute solutions of alkali hydroxides.

Carry out all tests protected from bright light and use freshly prepared solutions.

IDENTIFICATION

First identification: A, B.

Second identification: A, C, D.

A. It melts (2.2.14) at 178 °C to 182 °C.

B. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *dithranol CRS*.

C. Examine by thin-layer chromatography (2.2.27), using *silica gel H R* as the coating substance.

Test solution. Dissolve 10 mg of the substance to be examined in *methylene chloride R* and dilute to 10 ml with the same solvent.

Reference solution (a). Dissolve 10 mg of *dithranol CRS* in *methylene chloride R* and dilute to 10 ml with the same solvent.

Reference solution (b). Dissolve about 5 mg of *dantron R* in 5 ml of reference solution (a).

Apply to the plate 10 µl of each solution. Develop over a path of 12 cm using a mixture of equal volumes of *hexane R* and *methylene chloride R*. Allow the plate to dry in air. Place the plate in a tank saturated with ammonia vapour until the spots appear. Examine in daylight. The principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a). The test is not valid unless the chromatogram obtained with reference solution (b) shows 2 clearly separated spots.

D. To 5 mg add 0.1 g of *anhydrous sodium acetate R* and 1 ml of *acetic anhydride R*. Boil for 30 s. Add 20 ml of *alcohol R*. Examined in ultraviolet light at 365 nm, the solution shows a blue fluorescence.

TESTS

Related substances

A. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 0.200 g of the substance to be examined in 20 ml of *methylene chloride R*, add 1.0 ml of *glacial acetic acid R* and dilute to 100.0 ml with *hexane R*.

Reference solution. Dissolve 5.0 mg each of *anthrone R*, *dantron R*, *dithranol impurity C CRS* and *dithranol CRS* in *methylene chloride R* and dilute to 5.0 ml with the same solvent. To 1.0 ml of the solution, add 19.0 ml of *methylene chloride R* and 1.0 ml of *glacial acetic acid R*, and dilute to 50.0 ml with *hexane R*.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4.6 mm in internal diameter packed with *silica gel for chromatography R* (5 µm),
- as mobile phase at a flow rate of 2 ml/min a mixture of 1 volume of *glacial acetic acid R*, 5 volumes of *methylene chloride R* and 82 volumes of *hexane R*,
- as detector a spectrophotometer set at 260 nm.

Inject 20 µl of each solution. Continue the chromatography for 1.5 times the retention time of dithranol impurity C. Adjust the sensitivity of the system so that the height of the principal peak in the chromatogram obtained with the reference solution is about 70 per cent of the full scale of the recorder. When the chromatograms are recorded in the prescribed conditions, the substances are eluted in

the following sequence: dithranol, dantron, anthrone, dithranol impurity C. The test is not valid unless, in the chromatogram obtained with the reference solution, the resolution between the peaks due to dithranol and dantron is greater than 2.0. In the chromatogram obtained with the test solution: the area of any peak corresponding to anthrone, dantron or dithranol impurity C is not greater than that of the corresponding peak in the chromatogram obtained with the reference solution (1 per cent); the area of any peak apart from the principal peak and any peaks due to anthrone, dantron and dithranol impurity C is not greater than that of the peak due to dithranol in the chromatogram obtained with the reference solution (1 per cent).

B. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 25.0 mg of the substance to be examined in 5 ml of *tetrahydrofuran R* and dilute to 25.0 ml with the mobile phase.

Reference solution. Dissolve 5.0 mg of *dithranol impurity D CRS* and 5.0 mg of *dithranol CRS* in 5 ml of *tetrahydrofuran R* and dilute to 10.0 ml with the mobile phase. Dilute 1.0 ml of the solution to 20.0 ml with the mobile phase.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.20 m long and 4.6 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (5 µm),
- as mobile phase at a flow rate of 0.9 ml/min a mixture of 2.5 volumes of *glacial acetic acid R*, 40 volumes of *tetrahydrofuran R* and 60 volumes of *water R*,
- as detector a spectrophotometer set at 254 nm.

Inject 20 µl of each solution. Continue the chromatography for 3 times the retention time of the peak due to dithranol. Adjust the sensitivity of the system so that the height of the principal peak in the chromatogram obtained with the reference solution is about 50 per cent of the full scale of the recorder. The test is not valid unless, in the chromatogram obtained with the reference solution, the resolution between the peaks due to dithranol impurity D and dithranol is greater than 2.5. In the chromatogram obtained with the test solution, the area of any peak corresponding to dithranol impurity Dis not greater than that of the corresponding peak in the chromatogram obtained with the reference solution (2.5 per cent).

The total content of related substances as determined in tests A and B is not more than 3.0 per cent.

Chlorides (2.4.4). Shake 1.0 g with 20 ml of *water R* for 1 min and filter. Dilute 10 ml of the filtrate to 15 ml with *water R*. The solution complies with the limit test for chlorides (100 ppm).

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 100–105 °C

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

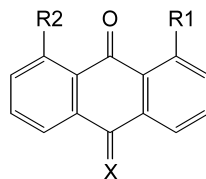
Dissolve 0.200 g in 50 ml of *anhydrous pyridine R*. Titrate with 0.1 M *tetrabutylammonium hydroxide* under *nitrogen R*. Determine the end-point potentiometrically (2.2.20), using a glass indicator electrode and a calomel reference electrode containing, as the electrolyte, a saturated solution of *potassium chloride R* in *methanol R*.

1 ml of 0.1 M *tetrabutylammonium hydroxide* is equivalent to 22.62 mg of C₁₄H₁₀O₃.

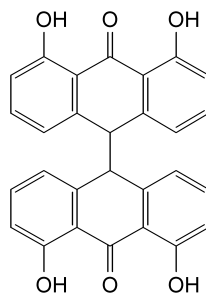
STORAGE

Store protected from light.

IMPURITIES



- A. R1 = R2 = H, X = H₂: anthracen-9(10*H*)-one (anthrone),
 B. R1 = R2 = OH, X = O: 1,8-dihydroxyanthracene-9,10-dione (dantron),
 D. R1 = OH, R2 = H, X = H₂: 1-hydroxyanthracen-9(10*H*)-one,

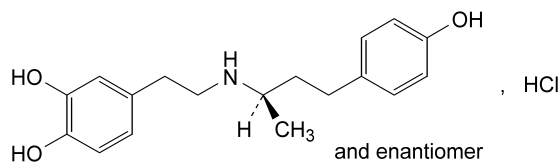


- C. C. 4,4',5,5'-tetrahydroxy-9,9'-bianthracenyl-10,10'(9*H*, 9'*H*)-dione.

01/2005:1200

DOBUTAMINE HYDROCHLORIDE

Dobutamini hydrochloridum



C₁₈H₂₄ClNO₃

M_r 337.9

DEFINITION

Dobutamine hydrochloride contains not less than 98.5 per cent and not more than the equivalent of 101.0 per cent of (*RS*)-4-[2-[[3-(4-hydroxyphenyl)-1-methylpropyl]amino]ethyl]benzene-1,2-diol hydrochloride, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, crystalline powder, sparingly soluble in water, soluble in methanol, sparingly soluble in alcohol.

IDENTIFICATION

First identification: C, E.

Second identification: A, B, D, E.

- A. Melting point (2.2.14): 189 °C to 192 °C.
 B. Dissolve 20.0 mg in *methanol R* and dilute to 100.0 ml with the same solvent. Dilute 10.0 ml of the solution to 100.0 ml with *methanol R*. Examined between 220 nm and 300 nm (2.2.25), the solution shows two absorption maxima, at 223 nm and 281 nm. The ratio of the absorbance measured at the maximum at 281 nm to that measured at the maximum at 223 nm is 0.34 to 0.36.

- C. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *dobutamine hydrochloride CRS*. Examine the substances prepared as discs.
- D. Examine by thin-layer chromatography (2.2.27), using *silica gel G R* as the coating substance.

Test solution. Dissolve 10 mg in a mixture of equal volumes of *glacial acetic acid R* and *methanol R* and dilute to 10 ml with the same mixture of solvents.

Reference solution (a). Dissolve 10.0 mg of *dobutamine hydrochloride CRS* in a mixture of equal volumes of *glacial acetic acid R* and *methanol R* and dilute to 10 ml with the same mixture of solvents.

Reference solution (b). Dissolve 5.0 mg of *dopamine hydrochloride CRS* in 5 ml of the test solution.

Apply to the plate 10 µl of each solution. Develop over a path of 15 cm using a mixture of 5 volumes of *water R*, 15 volumes of *glacial acetic acid R*, 30 volumes of *ether R* and 45 volumes of *butanol R*. Dry the plate and spray with a 1 g/l solution of *potassium permanganate R*. The principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a). The test is not valid unless the chromatogram obtained with reference solution (b) shows two clearly separated spots.

- E. It gives reaction (a) of chlorides (2.3.1) using a mixture of equal volumes of *methanol R* and *water R*.

TESTS

Acidity or alkalinity. Dissolve 0.1 g in *water R* with gentle heating and dilute to 10 ml with the same solvent. Add 0.1 ml of *methyl red solution R* and 0.2 ml of 0.01 M *sodium hydroxide*. The solution is yellow. Add 0.4 ml of 0.01 M *hydrochloric acid*. The solution is red.

Optical rotation (2.2.7). Dissolve 0.50 g in *methanol R* and dilute to 10.0 ml with the same solvent. The angle of optical rotation is -0.05° to $+0.05^\circ$.

Absorbance (2.2.25). Dissolve 0.5 g in a mixture of equal volumes of *methanol R* and of *water R* with heating, if necessary, at 30 °C to 35 °C and dilute to 25 ml with the same mixture of solvents. Cool quickly. Measure the absorbance immediately at 480 nm. The absorbance is not greater than 0.04.

Related substances. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 0.10 g in a mixture of 35 volumes of mobile phase B and 65 volumes of mobile phase A and dilute to 20.0 ml with the same mixture of mobile phases.

Reference solution (a). Dilute 4.0 ml of the test solution to 100.0 ml with a 0.05 g/l solution of *anisaldehyde R* in a mixture of 35 volumes of mobile phase B and 65 volumes of mobile phase A. Dilute 1.0 ml of the solution to 10.0 ml with a mixture of 35 volumes of mobile phase B and 65 volumes of mobile phase A.

Reference solution (b). Dilute 5.0 ml of the test solution to 100.0 ml with a mixture of 35 volumes of mobile phase B and 65 volumes of mobile phase A. Dilute 1.0 ml of the solution to 10.0 ml with a mixture of 35 volumes of mobile phase B and 65 volumes of mobile phase A.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.15 m long and 4.6 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (5 µm),

- as mobile phase at a flow rate of 1 ml/min:

Mobile phase A. Dissolve 2.60 g of *sodium octanesulphonate R* in 1000 ml of *water R* and add 3 ml of *triethylamine R*; adjust to pH 2.5 with *phosphoric acid R*,

Mobile phase B. A mixture of 18 volumes of *acetonitrile R* and 82 volumes of *methanol R*,

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)
0 - 5	65	35
5 - 20	65 → 20	35 → 80
20 - 25	20	80

- as detector a spectrophotometer set at 280 nm.

Inject 20 µl of reference solution (a). The test is not valid unless the resolution between the peaks corresponding to *dobutamine hydrochloride* and *anisaldehyde* is at least 4.0. Inject 20 µl of the test solution and 20 µl of reference solution (b). In the chromatogram obtained with the test solution: any peak apart from the principal peak is not greater than the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent); and the sum of the areas of all the peaks apart from the principal peak is not greater than twice the area of the principal peak in the chromatogram obtained with the reference solution (b) (1 per cent). Disregard any peak due to the solvent and any peak with an area less than 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (b).

Heavy metals (2.4.8). 2.0 g complies with limit test C for heavy metals (10 ppm). Prepare the standard using 2 ml of *lead standard solution (10 ppm Pb) R*.

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

In order to avoid overheating in the reaction medium, mix thoroughly throughout and stop the titration immediately after the end-point has been reached.

Dissolve 0.250 g in 10 ml of *anhydrous formic acid R*. Add 50 ml of *acetic anhydride R*. Titrate with 0.1 M *perchloric acid*, determining the end-point potentiometrically (2.2.20).

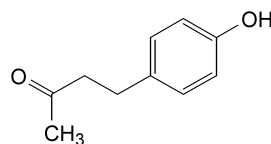
1 ml of 0.1 M *perchloric acid* is equivalent to 33.79 mg of $C_{18}H_{24}ClNO_3$.

STORAGE

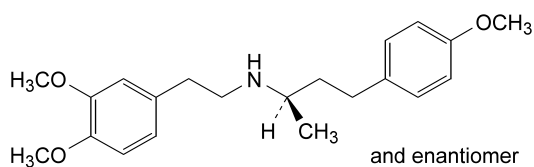
Store protected from light.

IMPURITIES

A. dopamine,



B. 4-(4-hydroxyphenyl)butan-2-one,

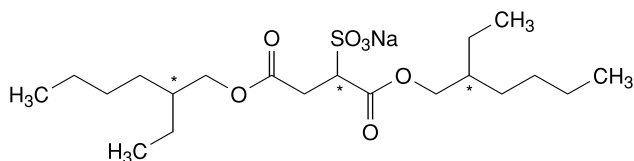


C. (2*RS*)-*N*-[2-(3,4-dimethoxyphenyl)ethyl]-4-(4-methoxyphenyl)butan-2-amine.

01/2005:1418

DOCUSATE SODIUM

Natrii docusas


 $C_{20}H_{37}NaO_7S$
 M_r 444.6

DEFINITION

Sodium 1,4-bis[(2-ethylhexyl)oxy]-1,4-dioxobutane-2-sulphonate.

Content: 98.0 to 101.0 per cent (anhydrous substance).

CHARACTERS

Appearance: white or almost white, waxy masses or flakes, hygroscopic.

Solubility: sparingly soluble in water, freely soluble in alcohol and in methylene chloride.

IDENTIFICATION

A. Infrared absorption spectrophotometry (2.2.24).

Preparation: place about 3 mg of the substance to be examined on a sodium chloride plate, add 0.05 ml of *acetone R* and immediately cover with another sodium chloride plate. Rub the plates together to dissolve the substance to be examined, slide the plates apart and allow the acetone to evaporate.

Comparison: *Ph. Eur. reference spectrum of docusate sodium*.

B. In a crucible, ignite 0.75 g in the presence of *dilute sulphuric acid R*, until an almost white residue is obtained. Allow to cool and take up the residue with 5 ml of *water R*. Filter. 2 ml of the filtrate gives reaction (a) of sodium (2.3.1).

TESTS

Alkalinity. Dissolve 1.0 g in 100 ml of a mixture of equal volumes of *methanol R* and *water R*, previously neutralised to *methyl red solution R*. Add 0.1 ml of *methyl red solution R*. Not more than 0.2 ml of 0.1 *M hydrochloric acid* is required to change the colour of the indicator to red.

Related non-ionic substances. Gas chromatography (2.2.28).

Internal standard solution. Dissolve 10 mg of *methyl behenate R* in *hexane R* and dilute to 50 ml with the same solvent.

Test solution (a). Dissolve 0.10 g of the substance to be examined in 2.0 ml of the internal standard solution and dilute to 5.0 ml with *hexane R*. Pass the solution, at a rate of about 1.5 ml/min, through a column 10 mm in internal diameter, packed with 5 g of *basic aluminium oxide R* and previously washed with 25 ml of *hexane R*. Elute with 5 ml of *hexane R* and discard the eluate. Elute with 20 ml

of a mixture of equal volumes of *ether R* and *hexane R*. Evaporate the eluate to dryness and dissolve the residue in 2.0 ml of *hexane R*.

Test solution (b). Prepare as described for test solution (a) but dissolving 0.10 g of the substance to be examined in *hexane R*, diluting to 5.0 ml with the same solvent, and using a new column.

Reference solution. Dilute 2.0 ml of the internal standard solution to 5.0 ml with *hexane R*.

Column:

- *material*: glass,
- *size*: $l = 2$ m, $\varnothing = 2$ mm,
- *stationary phase*: silanised diatomaceous earth for gas chromatography *R* impregnated with 3 per cent *m/m* of polymethylphenylsiloxane *R*.

Carrier gas: nitrogen for chromatography *R*.

Flow rate: 30 ml/min.

Temperature:

- *column*: 230 °C,
- *injection port and detector*: 280 °C.

Detection: flame ionisation.

Injection: 1 µl.

Run time: 2.5 times the retention time of the internal standard.

System suitability: there is no peak with the same retention time as the internal standard in the chromatogram obtained with test solution (b).

Limits: test solution (a):

- *any impurity*: for each impurity, not more than the area of the peak due to the internal standard (0.4 per cent).

Chlorides: maximum 350 ppm.

Dissolve 5.0 g in 50 ml of *alcohol (50 per cent V/V) R* and add 0.1 ml of *potassium dichromate solution R*. Not more than 0.5 ml of 0.1 *M silver nitrate* is required to change the colour of the indicator from yellow to orange.

Sodium sulphate: maximum 2 per cent.

Dissolve 0.25 g in 40 ml of a mixture of 20 volumes of *water R* and 80 volumes of 2-propanol *R*. Adjust to pH between 2.5 and 4.0 using *perchloric acid solution R*. Add 0.4 ml of *naphtharson solution R* and 0.1 ml of a 0.125 g/l solution of *methylene blue R*. Not more than 1.5 ml of 0.025 *M barium perchlorate* is required to change the colour of the indicator from yellowish-green to yellowish-pink.

Heavy metals (2.4.8): maximum 10 ppm.

Dissolve 4.0 g in *alcohol (80 per cent V/V) R* and dilute to 20 ml with the same solvent. 12 ml of the solution complies with limit test B. Prepare the standard using lead standard solution (2 ppm Pb) obtained by diluting *lead standard solution (100 ppm Pb) R* with *alcohol (80 per cent V/V) R*.

Water (2.5.12): maximum 3.0 per cent, determined on 0.250 g.

ASSAY

To 1.000 g in a 250 ml conical flask fitted with a reflux condenser add 25.0 ml of 0.5 *M alcoholic potassium hydroxide* and heat on a water-bath under reflux for 45 min. Allow to cool. Add 0.25 ml of *phenolphthalein solution R1* and titrate with 0.5 *M hydrochloric acid* until the red colour disappears. Carry out a blank titration.

1 ml of 0.5 *M hydrochloric acid* is equivalent to 0.1112 g of $C_{20}H_{37}NaO_7S$.

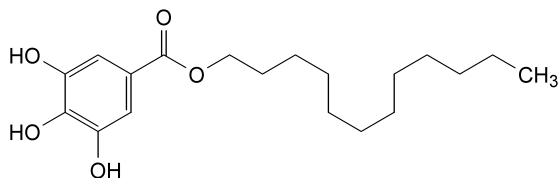
STORAGE

In an airtight container.

01/2005:2078

DODECYL GALLATE

Dodecylis gallas

 $C_{19}H_{30}O_5$ M_r 338.4**DEFINITION**

Dodecyl 3,4,5-trihydroxybenzoate.

Content: 97.0 per cent to 103.0 per cent (dried substance).**CHARACTERS***Appearance*: white or almost white, crystalline powder.*Solubility*: very slightly soluble or practically insoluble in water, freely soluble in ethanol (96 per cent), slightly soluble in methylene chloride.**IDENTIFICATION****A. Melting point (2.2.14).**

Determine the melting point of the substance to be examined. Mix equal parts of the substance to be examined and *dodecyl gallate CRS* and determine the melting point of the mixture. The difference between the melting points (which are about 96 °C) is not greater than 2 °C.

B. Examine the chromatograms obtained in the test for impurity A.

Results: the principal spot in the chromatogram obtained with test solution (b) is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).

TESTS**Impurity A.** Thin-layer chromatography (2.2.27).

Test solution (a). Dissolve 0.20 g of the substance to be examined in *acetone R* and dilute to 10 ml with the same solvent.

Test solution (b). Dilute 1.0 ml of test solution (a) to 20 ml with *acetone R*.

Reference solution (a). Dissolve 10 mg of *dodecyl gallate CRS* in *acetone R* and dilute to 10 ml with the same solvent.

Reference solution (b). Dissolve 20 mg of *gallic acid R* in *acetone R* and dilute to 20 ml with the same solvent.

Reference solution (c). Dilute 1.0 ml of reference solution (b) to 10 ml with *acetone R*.

Reference solution (d). Dilute 1.0 ml of reference solution (b) to 5 ml with test solution (a).

Plate: TLC silica gel plate *R*.

Mobile phase: anhydrous formic acid *R*, ethyl formate *R*, toluene *R* (10:40:50 V/V/V).

Application: 5 µl of test solutions (a) and (b) and reference solutions (a), (c) and (d).

Development: over 2/3 of the plate.

Drying: in air for 10 min.

Detection: spray with a mixture of 1 volume of *ferric chloride solution R1* and 9 volumes of *ethanol (96 per cent) R*.

System suitability: reference solution (d):

- the chromatogram shows 2 clearly separated principal spots.

Limit: test solution (a):

- *impurity A*: any spot due to impurity A is not more intense than the spot in the chromatogram obtained with reference solution (c) (0.5 per cent).

Chlorides (2.4.4): maximum 100 ppm.

To 1.65 g add 50 ml of *water R*. Shake for 5 min. Filter. 15 ml of the filtrate complies with the test.

Heavy metals (2.4.8): maximum 10 ppm.

2.0 g complies with limit test C. Prepare the reference solution using 2 ml of *lead standard solution (10 ppm Pb) R*.

Loss on drying (2.2.32): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 70 °C.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.100 g in *methanol R* and dilute to 250.0 ml with the same solvent. Dilute 5.0 ml of the solution to 200.0 ml with *methanol R*. Measure the absorbance (2.2.25) at the absorption maximum at 275 nm.

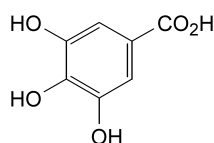
Calculate the content of $C_{19}H_{30}O_5$ taking the specific absorbance to be 321.

STORAGE

In a non-metallic container, protected from light.

IMPURITIES

Specified impurities: A.



A. 3,4,5-trihydroxybenzoic acid (gallic acid).

01/2005:1510

DOG ROSE

Rosae pseudo-fructus

DEFINITION

Dog rose consists of the rose hips made up by the receptacle and the remains of the dried sepals of *Rosa canina* L., *R. pendulina* L. and other *Rosa* species, with the achenes removed. It contains not less than 0.3 per cent of ascorbic acid ($C_6H_8O_6$; M_r 176.1), calculated with reference to the dried drug.

CHARACTERS

It has the macroscopic and microscopic characters described under identification tests A and B.

IDENTIFICATION

- It consists of fragments of the fleshy, hollow, urceolate receptacle, bearing the remains of the reduced sepals, light pink to orange-pink, the convex outer surface shiny and strongly wrinkled; bearing on its lighter inner surface abundant bristle-like hairs.
- Reduce to a powder (355). The powder is orange-yellow. Examine under a microscope using *chloral hydrate solution R*. The powder shows numerous fragments of receptacle, the outer epidermis with orange-yellow

contents and a thick cuticle, the inner epidermis composed of thin-walled cells containing cluster crystals and occasional prisms of calcium oxalate; scattered lignified cells, isodiametric, with thickened and pitted walls forming the trichome bases; abundant long, unicellular trichomes, up to 2 mm long and 30 µm to 45 µm thick, tapering towards each end, walls heavily thickened and with a waxy cuticle which may show fissures in a spiral arrangement; numerous oily orange-yellow globules.

C. Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel F₂₅₄ plate R*.

Test solution. To 5 g of the powdered drug (355) add 25 ml of *alcohol R*. Shake for 30 min and filter.

Reference solution. Dissolve 10 mg of *ascorbic acid R* in 5.0 ml of *alcohol (60 per cent V/V) R*.

Apply to the plate 20 µl of the test solution and 2 µl of the reference solution. Develop over a path of 15 cm using a mixture of 5 volumes of *acetic acid R*, 5 volumes of *acetone R*, 20 volumes of *methanol R* and 70 volumes of *toluene R*. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. The chromatogram obtained with the test solution shows a quenching zone similar in position to the principal zone in the chromatogram obtained with the reference solution. Spray with a 0.2 g/l solution of *dichlorophenolindophenol, sodium salt R* in *alcohol R*. Examine in daylight. The chromatogram obtained with the test solution shows a white zone on a pink background similar in position and colour to the principal zone in the chromatogram obtained with the reference solution. The chromatogram also shows an intense orange-yellow zone near the solvent front and a yellow zone in the upper third (carotenoids).

TESTS

Foreign matter (2.8.2). Not more than 1 per cent.

Loss on drying (2.2.32). Not more than 10.0 per cent, determined on 1.000 g of the powdered drug (355) by drying in an oven at 100–105 °C.

Total ash (2.4.16). Not more than 7.0 per cent.

ASSAY

Test solution. In a round-bottomed flask, weigh 0.500 g of the freshly powdered drug (710). Add a solution of 1.0 g of *oxalic acid R* in 50.0 ml of *methanol R*. Boil under a reflux condenser for 10 min, and cool in iced water until the temperature reaches 15 °C to 20 °C. Filter. Transfer 2.0 ml of the filtrate to a 50 ml conical flask. Add successively, with gentle shaking after each addition, 2.0 ml of *dichlorophenolindophenol standard solution R* and then, exactly 60 s later, 0.5 ml of a 100 g/l solution of *thiourea R* in *alcohol (50 per cent V/V) R* and 0.7 ml of *dinitrophenylhydrazine-sulphuric acid solution R*. Heat under a reflux condenser at 50 °C for 75 min, and place immediately in iced water for 5 min. Add dropwise 5.0 ml of a mixture of 12 ml of *water R* and 50 ml of *sulphuric acid R*, taking care to carry out the addition over a period of not less than 90 s and not more than 120 s while maintaining vigorous stirring in iced water. Allow to stand for 30 min at room temperature and measure the absorbance (2.2.25) at 520 nm using solution A as compensation liquid.

Solution A. Treat 2.0 ml of the filtrate obtained during the preparation of the test solution as described but adding the *dinitrophenylhydrazine-sulphuric acid solution R* just before the absorbance is measured.

Reference solution. Dissolve 40.0 mg of *ascorbic acid R* in a freshly prepared 20 g/l solution of *oxalic acid R* in *methanol R* and dilute to 100.0 ml with the same

solvent. Dilute 5.0 ml of the solution to 100.0 ml with the methanolic solution of oxalic acid. Treat 2.0 ml of the solution as described above for the filtrate obtained during the preparation of the test solution. Measure the absorbance (2.2.25) at 520 nm using solution B as compensation liquid.

Solution B. Treat 2.0 ml of the reference solution as described above for solution A.

Calculate the percentage content of ascorbic acid from the expression:

$$\frac{2.5 \times A_1 \times m_2}{A_2 \times m_1}$$

A_1 = absorbance of the test solution,

A_2 = absorbance of the reference solution,

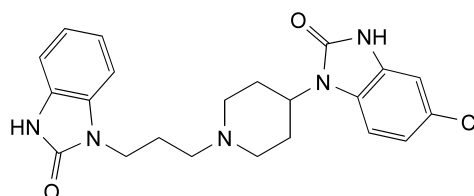
m_1 = mass of the substance to be examined, in grams,

m_2 = mass of ascorbic acid used, in grams.

01/2005:1009

DOMPERIDONE

Domperidonum



$C_{22}H_{24}ClN_5O_2$

M_r 425.9

DEFINITION

Domperidone contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of 5-chloro-1-[1-[3-(2-oxo-2,3-dihydro-1H-benzimidazol-1-yl)propyl]piperidin-4-yl]-1,3-dihydro-2H-benzimidazol-2-one, calculated with reference to the dried substance.

CHARACTERS

A white or almost white powder, practically insoluble in water, soluble in dimethylformamide, slightly soluble in alcohol and in methanol.

IDENTIFICATION

First identification: A, B.

Second identification: A, C, D.

A. Melting point (2.2.14): 244 °C to 248 °C.

B. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *domperidone CRS*. Examine the substances prepared as discs.

C. Examine by thin-layer chromatography (2.2.27), using a suitable octadecylsilyl silica gel as the coating substance.

Test solution. Dissolve 20 mg of the substance to be examined in *methanol R* and dilute to 10 ml with the same solvent.

Reference solution (a). Dissolve 20 mg of *domperidone CRS* in *methanol R* and dilute to 10 ml with the same solvent.

Reference solution (b). Dissolve 20 mg of *domperidone CRS* and 20 mg of *droperidol CRS* in *methanol R* and dilute to 10 ml with the same solvent.

Apply separately to the plate 5 µl of each solution. Develop over a path of 15 cm using a mixture of 20 volumes of *ammonium acetate solution R*, 40 volumes of *dioxan R* and 40 volumes of *methanol R*. Dry the plate in a current of warm air for 15 min and expose it to iodine vapour until the spots appear. Examine in daylight. The principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a). The test is not valid unless the chromatogram obtained with reference solution (b) shows two clearly separated spots.

D. It gives the reaction of non-nitrogen substituted barbiturates (2.3.1).

TESTS

Appearance of solution. Dissolve 0.20 g in *dimethylformamide R* and dilute to 20.0 ml with the same solvent. The solution is clear (2.2.1) and not more intensely coloured than reference solution Y₆ (2.2.2, Method II).

Related substances. Examine by liquid chromatography (2.2.29). Prepare the solutions immediately before use.

Test solution. Dissolve 0.10 g of the substance to be examined in *dimethylformamide R* and dilute to 10.0 ml with the same solvent.

Reference solution (a). Dissolve 10.0 mg of *domperidone CRS* and 15.0 mg of *droperidol CRS* in *dimethylformamide R* and dilute to 100.0 ml with the same solvent.

Reference solution (b). Dilute 1.0 ml of the test solution to 100.0 ml with *dimethylformamide R*. Dilute 5.0 ml of this solution to 20.0 ml with *dimethylformamide R*.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.1 m long and 4.6 mm in internal diameter packed with *base-deactivated octadecylsilyl silica gel for chromatography R* (3 µm),
- as mobile phase at a flow rate of 1.5 ml/min a mixture of 3 volumes *methanol R* and 7 volumes of a 5 g/l solution of *ammonium acetate R*, changing by linear gradient to *methanol R* over 10 min, followed by elution with *methanol R* for 2 min,
- as detector a spectrophotometer set at 280 nm.

Equilibrate the column for at least 30 min with *methanol R* and then equilibrate at the initial mobile phase composition for at least 5 min.

Adjust the sensitivity of the system so that the height of the principal peak in the chromatogram obtained with 10 µl of reference solution (b) is at least 50 per cent of the full scale of the recorder.

Inject 10 µl of reference solution (a). When the chromatogram is recorded in the prescribed conditions, the retention times are: domperidone about 6.5 min; droperidol about 7 min. The test is not valid unless the resolution between the peaks due to domperidone and droperidol is at least 2.0. If necessary, adjust the concentration of methanol in the mobile phase or adjust the time programme for the linear gradient.

Inject separately 10 µl of *dimethylformamide R* as a blank, 10 µl of the test solution and 10 µl of reference solution (b). In the chromatogram obtained with the test solution: the area of any peak, apart from the principal peak, is not greater than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.25 per cent); the sum of the areas of all peaks, apart from the principal peak, is not greater than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per

cent). Disregard any peak in the chromatogram obtained with the blank run and any peak with an area less than 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (b).

Heavy metals (2.4.8). 1.0 g complies with limit test D for heavy metals (20 ppm). Prepare the standard using 2 ml of *lead standard solution (10 ppm Pb) R*.

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

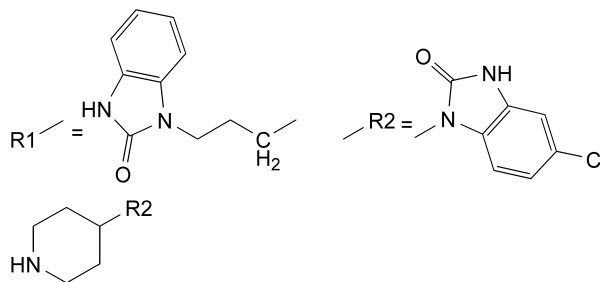
Dissolve 0.300 g in 50 ml of a mixture of 1 volume of *anhydrous acetic acid R* and 7 volumes of *methyl ethyl ketone R*. Titrate with 0.1 M *perchloric acid* until the colour changes from orange-yellow to green using 0.2 ml of *naphtholbenzein solution R* as indicator.

1 ml of 0.1 M *perchloric acid* is equivalent to 42.59 mg of C₂₂H₂₄ClN₅O₂.

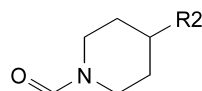
STORAGE

Store protected from light.

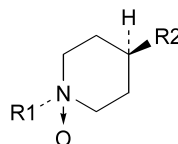
IMPURITIES



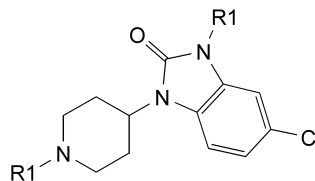
A. 5-chloro-1-(piperidin-4-yl)-1,3-dihydro-2H-benzimidazol-2-one,



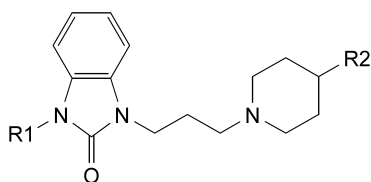
B. 4-(5-chloro-2-oxo-2,3-dihydro-1H-benzimidazol-1-yl)-1-formylpiperidine,



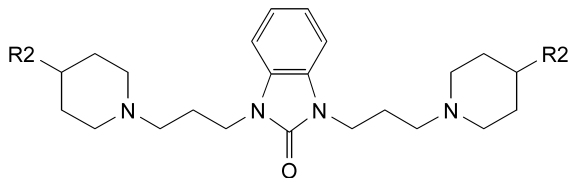
C. *cis*-4-(5-chloro-2-oxo-2,3-dihydro-1H-benzimidazol-1-yl)-1-[3-(2-oxo-2,3-dihydro-1H-benzimidazol-1-yl)propyl]piperidine 1-oxide,



D. 5-chloro-3-[3-(2-oxo-2,3-dihydro-1H-benzimidazol-1-yl)propyl]-1-[1-[3-(2-oxo-2,3-dihydro-1H-benzimidazol-1-yl)propyl]piperidin-4-yl]-1,3-dihydro-2H-benzimidazol-2-one,



- E. 1-[3-[4-(5-chloro-2-oxo-2,3-dihydro-1*H*-benzimidazol-1-yl)piperidin-1-yl]propyl]-3-[3-(2-oxo-2,3-dihydro-1*H*-benzimidazol-1-yl)propyl]-1,3-dihydro-2*H*-benzimidazol-2-one,

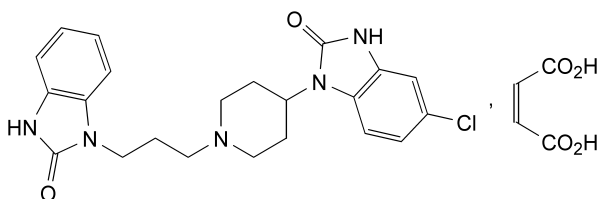


- F. 1,3-bis[3-[4-(5-chloro-2-oxo-2,3-dihydro-1*H*-benzimidazol-1-yl)piperidin-1-yl]propyl]-1,3-dihydro-2*H*-benzimidazol-2-one.

01/2005:1008
corrected

DOMPERIDONE MALEATE

Domperidoni maleas



C₂₆H₂₈ClN₅O₆

M_r 542.0

DEFINITION

Domperidone maleate contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of 5-chloro-1-[1-[3-(2-oxo-2,3-dihydro-1*H*-benzimidazol-1-yl)propyl]piperidin-4-yl]-1,3-dihydro-2*H*-benzimidazol-2-one hydrogen (*Z*)-butenedioate, calculated with reference to the dried substance.

CHARACTERS

A white or almost white powder, very slightly soluble in water, sparingly soluble in dimethylformamide, slightly soluble in methanol, very slightly soluble in alcohol.

It shows polymorphism.

IDENTIFICATION

First identification: A.

Second identification: B, C.

- A. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with domperidone maleate CRS. Examine the substances prepared as discs. If the spectra obtained show differences, dissolve the substance to be examined and the reference substance separately in the minimum volume of 2-propanol R, evaporate to dryness on a water-bath and record new spectra using the residues.

- B. Examine by thin-layer chromatography (2.2.27), using a suitable octadecylsilyl silica gel as the coating substance.

Test solution. Dissolve 20 mg of the substance to be examined in methanol R and dilute to 10 ml with the same solvent.

Reference solution (a). Dissolve 20 mg of domperidone maleate CRS in methanol R and dilute to 10 ml with the same solvent.

Reference solution (b). Dissolve 20 mg of domperidone maleate CRS and 20 mg of droperidol CRS in methanol R and dilute to 10 ml with the same solvent.

Apply to the plate 5 µl of each solution. Develop over a path of 15 cm using a mixture of 20 volumes of ammonium acetate solution R, 40 volumes of dioxan R and 40 volumes of methanol R. Dry the plate in a current of warm air for 15 min and expose it to iodine vapour until the spots appear. Examine in daylight. The principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a). The test is not valid unless the chromatogram obtained with reference solution (b) shows two clearly separated spots.

- C. Triturate 0.1 g with a mixture of 1 ml of strong sodium hydroxide solution R and 3 ml of water R. Shake with three quantities, each of 5 ml, of ether R. To 0.1 ml of the aqueous layer add a solution of 10 mg of resorcinol R in 3 ml of sulphuric acid R. Heat on a water-bath for 15 min. No colour develops. To the remainder of the aqueous layer add 2 ml of bromine solution R. Heat on a water-bath for 15 min and then heat to boiling. Cool. To 0.1 ml of the solution add a solution of 10 mg of resorcinol R in 3 ml of sulphuric acid R. Heat on a water-bath for 15 min. A violet colour develops.

TESTS

Appearance of solution. Dissolve 0.20 g in dimethylformamide R and dilute to 20.0 ml with the same solvent. The solution is clear (2.2.1) and not more intensely coloured than reference solution Y₆ (2.2.2, Method II).

Related substances. Examine by liquid chromatography (2.2.29). Prepare the solutions immediately before use.

Test solution. Dissolve 0.10 g of the substance to be examined in dimethylformamide R and dilute to 10.0 ml with the same solvent.

Reference solution (a). Dissolve 10.0 mg of domperidone maleate CRS and 15.0 mg of droperidol CRS in dimethylformamide R and dilute to 100.0 ml with the same solvent.

Reference solution (b). Dilute 1.0 ml of the test solution to 100.0 ml with dimethylformamide R. Dilute 5.0 ml of this solution to 20.0 ml with dimethylformamide R.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.1 m long and 4.6 mm in internal diameter packed with base-deactivated octadecylsilyl silica gel for chromatography R (3 µm),
- as mobile phase at a flow rate of 1.5 ml per minute of a mixture of 3 volumes of methanol R and 7 volumes of a 5 g/l solution of ammonium acetate R, changing by linear gradient to methanol R over 10 min, followed by elution with methanol R for 2 min,
- as detector a spectrophotometer set at 280 nm.

Equilibrate the column for at least 30 min with methanol R and then equilibrate at the initial mobile phase composition for at least 5 min.

Adjust the sensitivity of the system so that the height of the principal peak in the chromatogram obtained with 10 µl of reference solution (b) is at least 50 per cent of the full scale of the recorder.

Inject 10 µl of reference solution (a). When the chromatogram is recorded in the prescribed conditions, the retention times are: domperidone maleate, about 6.5 min and droperidol, about 7 min. The test is not valid unless the resolution between the peaks due to domperidone maleate and droperidol is at least 2.0. If necessary, adjust the concentration of methanol in the mobile phase or adjust the time programme for the linear gradient.

Inject 10 µl of *dimethylformamide R* as a blank, 10 µl of the test solution and 10 µl of reference solution (b). In the chromatogram obtained with the test solution: the area of any peak, apart from the principal peak, is not greater than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.25 per cent); the sum of the areas of all peaks, apart from the principal peak, is not greater than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent). Disregard any peak in the chromatogram obtained with the blank run, any peak due to maleic acid at the beginning of the chromatogram and any peak with an area less than 0.2 times that of the principal peak in the chromatogram obtained with reference solution (b).

Heavy metals (2.4.8). 1.0 g complies with limit test D for heavy metals (20 ppm). Prepare the standard using 2 ml of *lead standard solution (10 ppm Pb) R*.

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

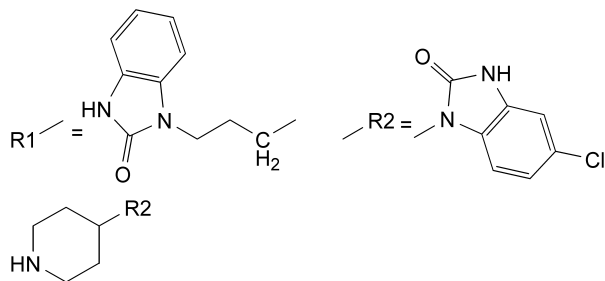
Dissolve 0.400 g in 50 ml of *anhydrous acetic acid R*. Using 0.2 ml of *naphtholbenzein solution R* as indicator, titrate with 0.1 M *perchloric acid* until the colour changes from orange-yellow to green.

1 ml of 0.1 M *perchloric acid* is equivalent to 54.20 mg of C₂₆H₂₈ClN₅O₆.

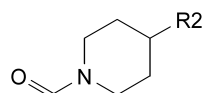
STORAGE

Store protected from light.

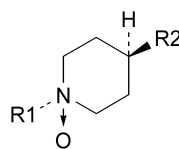
IMPURITIES



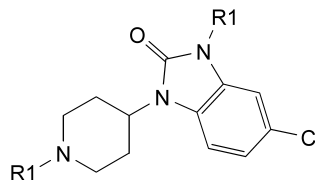
- A. 5-chloro-1-(piperidin-4-yl)-1,3-dihydro-2H-benzimidazol-2-one,



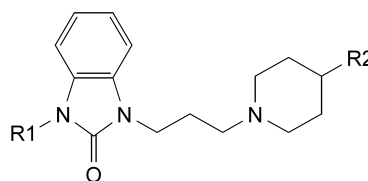
- B. 4-(5-chloro-2-oxo-2,3-dihydro-1H-benzimidazol-1-yl)-1-formylpiperidine,



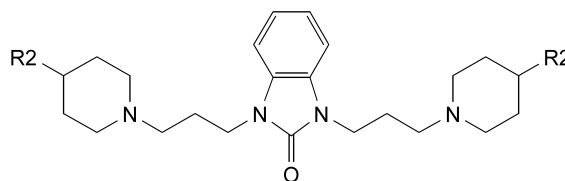
- C. *cis*-4-(5-chloro-2-oxo-2,3-dihydro-1H-benzimidazol-1-yl)-1-[3-(2-oxo-2,3-dihydro-1H-benzimidazol-1-yl)propyl]piperidine 1-oxide,



- D. 5-chloro-3-[3-(2-oxo-2,3-dihydro-1H-benzimidazol-1-yl)propyl]-1-[1-[3-(2-oxo-2,3-dihydro-1H-benzimidazol-1-yl)propyl]piperidin-4-yl]-1,3-dihydro-2H-benzimidazol-2-one,



- E. 1-[3-[4-(5-chloro-2-oxo-2,3-dihydro-1H-benzimidazol-1-yl)piperidin-1-yl]propyl]-3-[3-(2-oxo-2,3-dihydro-1H-benzimidazol-1-yl)propyl]-1,3-dihydro-2H-benzimidazol-2-one,

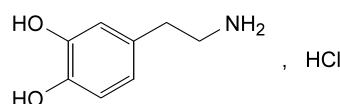


- F. 1,3-bis[3-[4-(5-chloro-2-oxo-2,3-dihydro-1H-benzimidazol-1-yl)piperidin-1-yl]propyl]-1,3-dihydro-2H-benzimidazol-2-one.

01/2005:0664

DOPAMINE HYDROCHLORIDE

Dopamini hydrochloridum



C₈H₁₂ClNO₂

M_r 189.6

DEFINITION

Dopamine hydrochloride contains not less than 98.0 per cent and not more than the equivalent of 102.0 per cent of 4-(2-aminoethyl)benzene-1,2-diol hydrochloride, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, crystalline powder, freely soluble in water, soluble in alcohol, sparingly soluble in acetone and in methylene chloride.

IDENTIFICATION

First identification: B, E.

Second identification: A, C, D, E.

- A. Dissolve 40.0 mg in 0.1 M hydrochloric acid and dilute to 100.0 ml with the same acid. Dilute 10.0 ml of the solution to 100.0 ml with 0.1 M hydrochloric acid. Examined between 230 nm and 350 nm (2.2.25), the solution shows an absorption maximum at 280 nm. The specific absorbance at the maximum is 136 to 150.
- B. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with dopamine hydrochloride CRS. Examine the substances as discs prepared using potassium chloride R.
- C. Dissolve about 5 mg in a mixture of 5 ml of 1 M hydrochloric acid and 5 ml of water R. Add 0.1 ml of sodium nitrite solution R containing 100 g/l of ammonium molybdate R. A yellow colour develops which becomes red on the addition of strong sodium hydroxide solution R.
- D. Dissolve about 2 mg in 2 ml of water R and add 0.2 ml of ferric chloride solution R2. A green colour develops which changes to bluish-violet on the addition of 0.1 g of hexamethylenetetramine R.
- E. It gives reaction (a) of chlorides (2.3.1).

TESTS

Appearance of solution. Dissolve 0.4 g in water R and dilute to 10 ml with the same solvent. The solution is clear (2.2.1) and not more intensely coloured than reference solution B₆ or Y₆ (2.2.2, Method II).

Acidity or alkalinity. Dissolve 0.5 g in carbon dioxide-free water R and dilute to 10 ml with the same solvent. Add 0.1 ml of methyl red solution R and 0.75 ml of 0.01 M sodium hydroxide. The solution is yellow. Add 1.5 ml of 0.01 M hydrochloric acid. The solution is red.

Related substances. Examine by thin-layer chromatography (2.2.27), using silica gel G R as the coating substance.

Test solution. Dissolve 0.15 g of the substance to be examined in methanol R and dilute to 5 ml with the same solvent.

Reference solution (a). Dissolve 7.5 mg of 4-O-methyldopamine hydrochloride R in methanol R and dilute to 100 ml with the same solvent.

Reference solution (b). Dissolve 7.5 mg each of 3-O-methyldopamine hydrochloride R and 4-O-methyldopamine hydrochloride R in methanol R and dilute to 100 ml with the same solvent.

Apply to the plate 10 µl of each solution. Develop over a path of 15 cm using a mixture of 2 volumes of anhydrous formic acid R, 7 volumes of water R, 36 volumes of methanol R and 52 volumes of chloroform R. Allow the plate to dry in air for 15 min. Spray evenly and abundantly with a mixture of equal volumes of potassium ferricyanide solution R and ferric chloride solution R1, prepared immediately before use. Any spot in the chromatogram obtained with the test solution with an R_f value higher than that of the principal spot is not more intense than the spot in the chromatogram obtained with reference solution (a) (0.25 per cent). The test is not valid unless the chromatogram obtained with reference solution (b) shows two clearly separated spots.

Heavy metals (2.4.8). 1.0 g complies with limit test C for heavy metals (20 ppm). Prepare the standard using 2 ml of lead standard solution (10 ppm Pb) R.

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C for 2 h.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

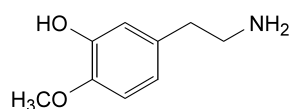
In order to avoid overheating in the reaction medium, mix thoroughly throughout and stop the titration immediately after the end-point has been reached.

Dissolve 0.1500 g in 10 ml of anhydrous formic acid R. Add 50 ml of acetic anhydride R. Titrate with 0.1 M perchloric acid, determining the end-point potentiometrically (2.2.20). 1 ml of 0.1 M perchloric acid is equivalent to 18.96 mg of C₈H₁₂ClNO₂.

STORAGE

Store in an airtight container, protected from light.

IMPURITIES

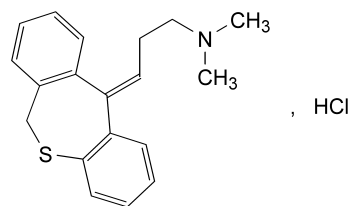


- A. 5-(2-aminoethyl)-2-methoxyphenol (4-O-methyldopamine).

01/2005:1314

DOSULEPIN HYDROCHLORIDE

Dosulepini hydrochloridum

C₁₉H₂₂ClNSM_r 331.9

DEFINITION

(E)-3-(Dibenzo[b,e]thiepin-11(6H)-ylidene)-N,N-dimethylpropan-1-amine hydrochloride.

Content: 98.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

Appearance: white or faintly yellow, crystalline powder.

Solubility: freely soluble in water, in alcohol and in methylene chloride.

IDENTIFICATION

First identification: B, D.

Second identification: A, C, D.

- A. Dissolve 25.0 mg in a 1 g/l solution of hydrochloric acid R in methanol R and dilute to 100.0 ml with the same solution. Dilute 2.0 ml to 50.0 ml with a 1 g/l solution of hydrochloric acid R in methanol R. Examined between 220 nm and 350 nm (2.2.25), the solution shows 2 absorption maxima at 231 nm and 306 nm and a shoulder at about 260 nm. The specific absorbance at the maximum at 231 nm is 660 to 730.
- B. Infrared absorption spectrophotometry (2.2.24).

Preparation: discs.

Comparison: dosulepin hydrochloride CRS.

- C. Dissolve about 1 mg in 5 ml of sulphuric acid R. A dark red colour is produced.
- D. It gives reaction (b) of chlorides (2.3.1).

TESTS

Appearance of solution. The solution is clear (2.2.1) and not more intensely coloured than reference solution Y₅ (2.2.2, Method II).

Dissolve 1 g in *water R* and dilute to 20 ml with the same solvent.

pH (2.2.3): 4.2 to 5.2.

Dissolve 1 g in *carbon dioxide-free water R* and dilute to 10 ml with the same solvent.

Impurity E and related substances. Liquid chromatography (2.2.29). Prepare the solutions immediately before use and protect from light.

Test solution. Dissolve 50.0 mg of the substance to be examined in 5 ml of *methanol R* and dilute to 100.0 ml with the mobile phase.

Reference solution (a). Dissolve 12.5 mg of *dosulepin impurity A CRS* in 5 ml of *methanol R* and dilute to 50.0 ml with the mobile phase. Dilute 0.5 ml to 100.0 ml with the mobile phase.

Reference solution (b). Dissolve 10.0 mg of *dosulepin hydrochloride CRS* in 5 ml of *methanol R* and dilute to 20.0 ml with the mobile phase.

Column:

- **size:** $l = 0.25$ m, $\varnothing = 4.6$ mm,
- **stationary phase:** nitrile silica gel for chromatography R1 (5 μ m),
- **temperature:** 35 °C.

Mobile phase: 0.83 per cent V/V solution of *perchloric acid R*, *propanol R*, *methanol R*, *water R* (1:10:30:60 V/V/V/V).

Flow rate: 1 ml/min.

Detection: spectrophotometer at 229 nm.

Injection: 5 μ l.

Run time: 2.5 times the retention time of dosulepin (*E*-isomer).

Relative retention with reference to dosulepin (*E*-isomer; retention time = about 25 min): impurity E = about 0.9.

System suitability: reference solution (b):

- **peak-to-valley ratio:** minimum 4, where H_p = height above the baseline of the peak due to impurity E and H_v = height above the baseline of the lowest point of the curve separating this peak from the peak due to dosulepin (*E*-isomer).

Limits:

- **impurity E:** not more than 5 per cent of the sum of the areas of the peak due to impurity E and the principal peak in the chromatogram obtained with the test solution,
- **impurity A:** not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.25 per cent),
- **any other impurity:** not more than 0.4 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent),
- **total of other impurities and impurity A:** not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent),

- **disregard limit:** 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

Heavy metals (2.4.8): maximum 20 ppm.

1.0 g complies with limit test C. Prepare the standard using 2 ml of *lead standard solution* (10 ppm Pb) *R*.

Loss on drying (2.2.32): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 100–105 °C.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

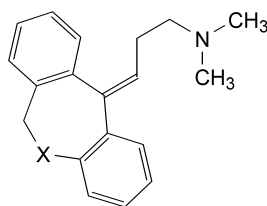
Dissolve 0.250 g in a mixture of 5 ml of *anhydrous acetic acid R* and 35 ml of *acetic anhydride R*. Titrate with 0.1 M *perchloric acid*, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *perchloric acid* is equivalent to 33.19 mg of C₁₉H₂₂CIN₃.

STORAGE

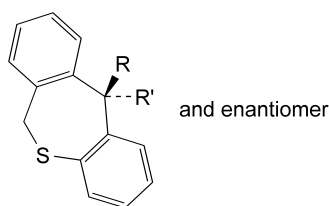
Protected from light.

IMPURITIES



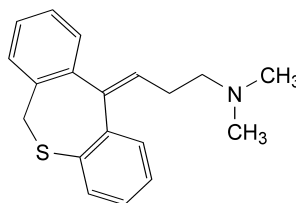
A. X = SO: (*E*)-3-(5-oxo-5 λ^4 -dibenzo[*b,e*]thiepin-11(6*H*)-ylidene)-*N,N*-dimethylpropan-1-amine,

D. X = SO₂: (*E*)-3-(5,5-dioxo-5 λ^6 -dibenzo[*b,e*]thiepin-11(6*H*)-ylidene)-*N,N*-dimethylpropan-1-amine,



B. R + R' = O: dibenzo[*b,e*]thiepin-11(6*H*)-one,

C. R = OH, R' = [CH₂]₃-N(CH₃)₂: (11*RS*)-11-[3-(dimethylamino)propyl]-6,11-dihydrodibenzo[*b,e*]thiepin-11-ol,

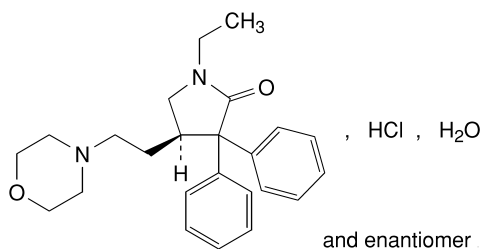


E. (*Z*)-3-(dibenzo[*b,e*]thiepin-11(6*H*)-ylidene)-*N,N*-dimethylpropan-1-amine.

01/2005:1201 **Optical rotation** (2.2.7): -0.10° to $+0.10^{\circ}$, determined on solution S.

DOXAPRAM HYDROCHLORIDE

Doxaprami hydrochloridum



$C_{24}H_{31}ClN_2O_2 \cdot H_2O$

M_r 433.0

DEFINITION

(4*RS*)-1-Ethyl-4-[2-(morpholin-4-yl)ethyl]-3,3-diphenylpyrrolidin-2-one hydrochloride.

Content: 98.0 per cent to 100.5 per cent (dried substance).

CHARACTERS

Appearance: white or almost white, crystalline powder.

Solubility: soluble in water, in alcohol and in methylene chloride.

IDENTIFICATION

First identification: A, C.

Second identification: B, C.

A. Infrared absorption spectrophotometry (2.2.24).

Preparation: discs.

Comparison: doxapram hydrochloride CRS.

B. Thin-layer chromatography (2.2.27).

Test solution. Dissolve 10 mg of the substance to be examined in *methanol R* and dilute to 10 ml with the same solvent.

Reference solution. Dissolve 10 mg of *doxapram hydrochloride CRS* in *methanol R* and dilute to 10 ml with the same solvent.

Plate: TLC silica gel plate *R*.

Mobile phase: solution of *ammonia R* containing 17 g/l of NH_3 , 2-propanol *R*, 2-methylpropanol *R* (10:10:80 V/V/V).

Application: 10 μ l.

Development: over a path of 15 cm.

Drying: in air.

Detection: spray with *dilute potassium iodobismuthate solution R* and examine immediately.

Results: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

C. It gives reaction (a) of chlorides (2.3.1).

TESTS

Solution S. Dissolve 2.500 g in *carbon dioxide-free water R* and dilute to 50.0 ml with the same solvent.

Appearance of solution. The solution is clear (2.2.1) and colourless (2.2.2, *Method II*).

Dilute 10 ml of solution S to 25 ml with *water R*.

pH (2.2.3): 3.5 to 5.0.

Dilute 5 ml of solution S to 25 ml with *carbon dioxide-free water R*.

Related substances. Liquid chromatography (2.2.29). *Prepare the solutions immediately before use.*

Test solution. Dissolve 10.0 mg of the substance to be examined in the mobile phase and dilute to 10.0 ml with the mobile phase.

Reference solution (a). Dilute 1.0 ml of the test solution to 100.0 ml with the mobile phase.

Reference solution (b). Dilute 1.0 ml of reference solution (a) to 5.0 ml with the mobile phase.

Reference solution (c). Dissolve 5 mg of *doxapram impurity B CRS* in the mobile phase and dilute to 5.0 ml with the mobile phase. To 1.0 ml of the solution, add 1.0 ml of the test solution and dilute to 100.0 ml with the mobile phase.

Column:

- **size:** $l = 0.25$ m, $\varnothing = 4.6$ mm,
- **stationary phase:** spherical *end-capped octadecylsilyl silica gel for chromatography R* (5 μ m) with a carbon loading of 14 per cent, a specific surface area of 350 m²/g and a pore size of 10 nm.

Mobile phase: mix 50 volumes of *acetonitrile R* and 50 volumes of a 0.82 g/l solution of *sodium acetate R* adjusted to pH 4.5 with *glacial acetic acid R*.

Flow rate: 1.5 ml/min.

Detection: spectrophotometer at 214 nm.

Injection: 20 μ l.

Run time: 4 times the retention time of doxapram.

Retention time: doxapram = about 6 min.

System suitability: reference solution (c):

- **resolution:** minimum of 3.0 between the peaks corresponding to doxapram and to impurity B.

Limits:

- **any impurity:** not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent),
- **total:** not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (1.0 per cent),
- **disregard limit:** 0.05 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

Heavy metals (2.4.8): maximum 20 ppm.

Dissolve 2.0 g in a mixture of 15 volumes of *water R* and 85 volumes of *methanol R* and dilute to 20 ml with the same mixture of solvents. 12 ml of the solution complies with limit test B. Prepare the standard using lead standard solution (2 ppm Pb) obtained by diluting *lead standard solution (100 ppm Pb) R* with a mixture of 15 volumes of *water R* and 85 volumes of *methanol R*.

Loss on drying (2.2.32): 3.0 per cent to 4.5 per cent, determined on 1.000 g by drying in an oven at 100–105 $^{\circ}$ C.

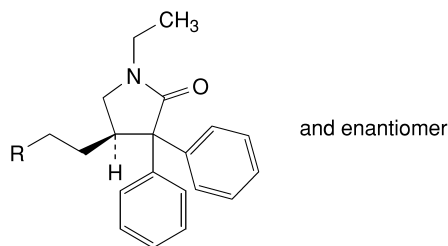
Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.300 g in a mixture of 10 ml of 0.01 *M hydrochloric acid* and 50 ml of *alcohol R*. Carry out a potentiometric titration (2.2.20) using 0.1 *M sodium hydroxide*. Read the volume added between the 2 points of inflexion.

1 ml of 0.1 *M sodium hydroxide* is equivalent to 41.50 mg of $C_{24}H_{31}ClN_2O_2$.

IMPURITIES

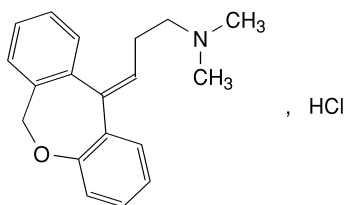


- A. R = Cl: (4RS)-4-(2-chloroethyl)-1-ethyl-3,3-diphenylpyrrolidin-2-one,
 B. R = NH-CH₂-CH₂-OH: (4RS)-1-ethyl-4-[2-[(2-hydroxyethyl)amino]ethyl]-3,3-diphenylpyrrolidin-2-one.

01/2005:1096
corrected

DOXEPIN HYDROCHLORIDE

Doxepini hydrochloridum



C₁₉H₂₂ClNO

M_r 315.8

DEFINITION

Doxepin hydrochloride is (*E*)-3-(dibenzo[*b,e*]oxepin-11(6*H*)-ylidene)-*N,N*-dimethylpropan-1-amine hydrochloride. It contains not less than 98.0 per cent and not more than the equivalent of 101.0 per cent of C₁₉H₂₂ClNO, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, crystalline powder, freely soluble in water, in alcohol and in methylene chloride.

IDENTIFICATION

First identification: C, E.

Second identification: A, B, D, E.

- A. Melting point (2.2.14): 185 °C to 191 °C.
 B. Dissolve 50.0 mg in a 1 g/l solution of *hydrochloric acid R* in *methanol R* and dilute to 100.0 ml with the same acid solution. Dilute 5.0 ml to 50.0 ml with a 1 g/l solution of *hydrochloric acid R* in *methanol R*. Examined between 230 nm and 350 nm (2.2.25), the solution shows an absorption maximum at 297 nm. The specific absorbance at the maximum is 128 to 142.
 C. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the *Ph. Eur. reference spectrum of doxepin hydrochloride*.
 D. Dissolve about 5 mg in 2 ml of *sulphuric acid R*. A dark red colour is produced.
 E. Solution S (see Tests) gives reaction (a) of chlorides (2.3.1).

TESTS

Solution S. Dissolve 1.5 g in *carbon dioxide-free water R* and dilute to 30 ml with the same solvent.

Appearance of solution. Dilute 10 ml of solution S to 25 ml with *water R*. The solution is clear (2.2.1) and colourless (2.2.2, *Method II*).

Acidity. To 10 ml of solution S add 0.1 ml of *methyl red solution R*. Not more than 0.1 ml of 0.1 M *sodium hydroxide* is required to change the colour of the indicator to yellow.

Related substances. Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel F₂₅₄ plate R* (2-10 µm) with a concentration zone.

Test solution. Dissolve 0.10 g of the substance to be examined in *methanol R* and dilute to 10 ml with the same solvent.

Reference solution (a). Dissolve 10.0 mg of *doxepin impurity A CRS* in *methanol R*, add 1 ml of the test solution and dilute to 10 ml with *methanol R*. Dilute 1.0 ml of this solution to 50 ml with *methanol R*.

Reference solution (b). Dissolve 10.0 mg of *doxepin impurity B CRS* in *methanol R*, add 1 ml of the test solution and dilute to 10 ml with *methanol R*. Dilute 1.0 ml of this solution to 50 ml with *methanol R*.

Reference solution (c). Dissolve 10.0 mg of *doxepin impurity B CRS* in *methanol R* and dilute to 200 ml with the same solvent.

Apply to one plate (plate A) 2 µl of the test solution and 2 µl of reference solution (a). Develop over a path of 5 cm using a mixture of 30 volumes of *methyl ethyl ketone R* and 60 volumes of *heptane R*. Doxepin remains on the starting line.

Apply to another plate (plate B) 2 µl of the test solution and 2 µl of reference solutions (b) and (c). Develop over a path of 5 cm using a mixture of 10 volumes of *methanol R* and 90 volumes of *methylene chloride R*.

Allow the plates to dry in a current of air. Spray with a solution prepared as follows: dissolve 20 g of *zinc chloride R* in 30 ml of *glacial acetic acid R*, add 3 ml of *phosphoric acid R* and 0.80 ml of *strong hydrogen peroxide solution R* and dilute to 60 ml with *water R*. Heat the plates at 120 °C for 15 min and examine immediately in ultraviolet light at 365 nm.

Plate A. In the chromatogram obtained with the test solution: any spot corresponding to impurity A is not more intense than the corresponding spot in the chromatogram obtained with reference solution (a) (0.2 per cent); any spot, apart from the principal spot and the spot corresponding to impurity A, is not more intense than the spots in the chromatogram obtained with reference solution (a) (0.2 per cent).

Plate B. In the chromatogram obtained with the test solution: any spot corresponding to impurity C (*R_f* about 0.12) is not more intense than the spot in the chromatogram obtained with reference solution (c) (0.5 per cent); any spot corresponding to impurity B is not more intense than the corresponding spot in the chromatogram obtained with reference solution (b) (0.2 per cent); any spot, apart from the principal spot, the spot corresponding to impurity B or the spot corresponding to impurity C, is not more intense than the spots in the chromatogram obtained with reference solution (b) (0.2 per cent).

The test is not valid unless the chromatograms obtained with reference solutions (a) and (b) show clearly visible and separated principal spots.

Z-Isomer. 13.0 per cent to 18.5 per cent of the *Z*-isomer, determined by liquid chromatography (2.2.29).

Test solution. Dissolve 20.0 mg of the substance to be examined in the mobile phase and dilute to 20.0 ml with the

mobile phase. Dilute 1.0 ml of this solution to 10.0 ml with the mobile phase.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.12 m long and 4 mm in internal diameter packed with spherical *octylsilyl silica gel for chromatography R* (5 µm) with a specific surface area of 220 m²/g and a pore size of 80 nm,
- as mobile phase at a flow rate of 1 ml/min a mixture of 30 volumes of *methanol R* and 70 volumes of a 30 g/l solution of *sodium dihydrogen phosphate R*, previously adjusted to pH 2.5 with *phosphoric acid R*,
- as detector a spectrophotometer set at 254 nm, maintaining the temperature of the column at 50 °C.

Inject 20 µl of the test solution. Adjust the sensitivity of the system so that the height of the principal peak is at least 50 per cent of the full scale of the recorder. The test is not valid unless the resolution between the first peak (*E*-isomer) and the second peak (*Z*-isomer) is at least 1.5.

The ratio of the area of the peak due to the *E*-isomer to the area of the peak due to the *Z*-isomer is 4.4 to 6.7.

Heavy metals (2.4.8). 1.0 g complies with limit test D (20 ppm). Prepare the standard using 2 ml of *lead standard solution (10 ppm Pb) R*.

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 100–105 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

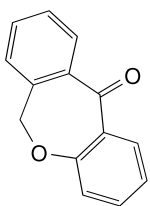
Dissolve 0.250 g in a mixture of 5 ml of *anhydrous acetic acid R* and 35 ml of *acetic anhydride R*. Titrate with 0.1 M *perchloric acid* until the colour changes from blue to green, using 0.2 ml of *crystal violet solution R* as indicator.

1 ml of 0.1 M *perchloric acid* is equivalent to 31.58 mg of C₂₇H₂₂ClNO₁₁.

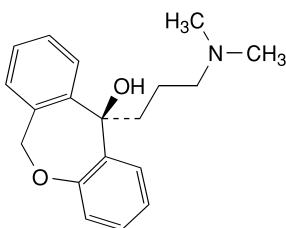
STORAGE

Protected from light.

IMPURITIES

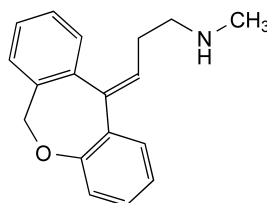


A. dibenzo[*b,e*]oxepin-11(6*H*)-one,

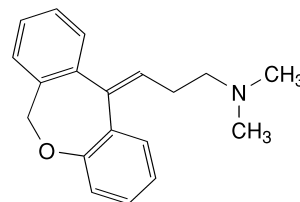


and enantiomer

B. (11*RS*)-11-[3-(dimethylamino)propyl]-6,11-dihydrodibenzo[*b,e*]oxepin-11-ol,



C. (*E*)-3-(dibenzo[*b,e*]oxepin-11(6*H*)-ylidene)-*N*-methylpropan-1-amine,

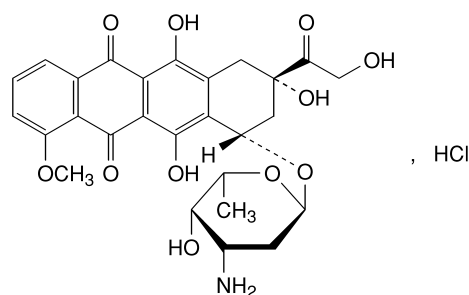


D. (*Z*)-3-(dibenzo[*b,e*]oxepin-11(6*H*)-ylidene)-*N,N*-dimethylpropan-1-amine.

01/2005:0714

DOXORUBICIN HYDROCHLORIDE

Doxorubicini hydrochloridum



C₂₇H₃₀ClNO₁₁

M_r 580.0

DEFINITION

(8*S*,10*S*)-10-[(3-Amino-2,3,6-trideoxy-α-*L*-lyxo-hexopyranosyl)oxy]-6,8,11-trihydroxy-8-(hydroxyacetyl)-1-methoxy-7,8,9,10-tetrahydrotetracene-5,12-dione hydrochloride.

Substance produced by certain strains of *Streptomyces coeruleorubidus* or *Streptomyces peucetius* or obtained by any other means.

Content: 98.0 per cent to 102.0 per cent (anhydrous, solvent-free substance).

CHARACTERS

Appearance: orange-red, crystalline powder, hygroscopic.

Solubility: soluble in water, slightly soluble in methanol.

IDENTIFICATION

A. Infrared absorption spectrophotometry (2.2.24).

Comparison: *doxorubicin hydrochloride CRS*.

B. Dissolve about 10 mg in 0.5 ml of *nitric acid R*, add 0.5 ml of *water R* and heat over a flame for 2 min. Allow to cool and add 0.5 ml of *silver nitrate solution R1*. A white precipitate is formed.

TESTS

pH (2.2.3): 4.0 to 5.5.

Dissolve 50 mg in *carbon dioxide-free water R* and dilute to 10 ml with the same solvent.

Related substances. Liquid chromatography (2.2.29).
Prepare the solutions immediately before use.

Test solution (a). Dissolve 50.0 mg of the substance to be examined in the mobile phase and dilute to 50.0 ml with the mobile phase.

Test solution (b). Dilute 10.0 ml of test solution (a) to 100.0 ml with the mobile phase.

Reference solution (a). Dissolve 10.0 mg of *doxorubicin hydrochloride CRS* and 10 mg of *epirubicin hydrochloride CRS* in the mobile phase and dilute to 50.0 ml with the mobile phase. Dilute 10.0 ml of the solution to 100.0 ml with the mobile phase.

Reference solution (b). Dilute 5.0 ml of reference solution (a) to 20.0 ml with the mobile phase.

Reference solution (c). Dissolve 50.0 mg of *doxorubicin hydrochloride CRS* in the mobile phase and dilute to 50.0 ml with the mobile phase. Dilute 10.0 ml of the solution to 100.0 ml with the mobile phase.

Column:

- size: $l = 0.25$ m, $\varnothing = 4.0$ mm,
- stationary phase: end-capped octadecylsilyl silica gel for chromatography R (5 μ m).

Mobile phase: mix equal volumes of acetonitrile R and a solution containing 2.88 g/l of sodium laurilsulfate R and 2.25 g/l of phosphoric acid R.

Flow rate: 1 ml/min.

Detection: spectrophotometer at 254 nm.

Injection: 5 μ l; inject test solution (a) and reference solutions (a) and (b).

Run time: 3.5 times the retention time of doxorubicin.

Retention time: doxorubicin = about 8 min.

System suitability: reference solution (a):

- resolution: minimum of 2.0 between the peaks due to doxorubicin and to epirubicin.

Limits:

- any impurity: not more than the area of the peak corresponding to doxorubicin in the chromatogram obtained with reference solution (b) (0.5 per cent),
- disregard limit: 0.1 times the area of the peak corresponding to doxorubicin in the chromatogram obtained with reference solution (b) (0.05 per cent).

Ethanol (2.4.24, System B): maximum 1.0 per cent.

Water (2.5.12): maximum 4.0 per cent, determined on 0.100 g.

Bacterial endotoxins (2.6.14): less than 2.2 IU/mg, if intended for use in the manufacture of parenteral dosage forms without a further appropriate procedure for the removal of bacterial endotoxins.

ASSAY

Liquid chromatography (2.2.29) as described in the test for related substances.

Injection: test solution (b) and reference solution (c).

Calculate the percentage content of $C_{27}H_{30}ClNO_{11}$.

STORAGE

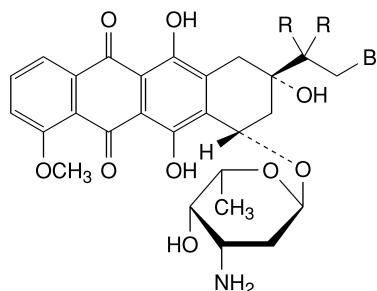
In an airtight container. If the substance is sterile, store in a sterile, airtight, tamper-proof container.

LABELLING

The label states, where applicable, that the substance is free from bacterial endotoxins.

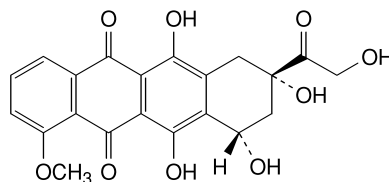
IMPURITIES

A. daunorubicin,



B. R = OCH₃: (8S,10S)-10[(3-amino-2,3,6-trideoxy-α-L-lyxohexopyranosyl)oxy]-8-(2-bromo-1,1-dimethoxyethyl)-6,8,11-trihydroxy-1-methoxy-7,8,9,10-tetrahydrotetracene-5,12-dione,

C. R + R = O: (8S,10S)-10[(3-amino-2,3,6-trideoxy-α-L-lyxohexopyranosyl)oxy]-8-(bromoacetyl)-6,8,11-trihydroxy-1-methoxy-7,8,9,10-tetrahydrotetracene-5,12-dione,

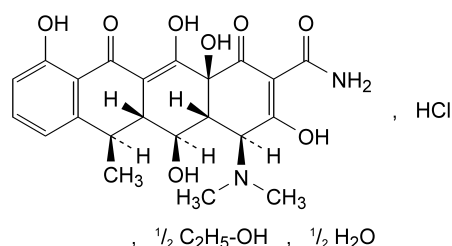


D. (8S,10S)-6,8,10,11-tetrahydroxy-8-(hydroxyacetyl)-1-methoxy-7,8,9,10-tetrahydrotetracene-5,12-dione (doxorubicin aglycone, doxorubicinone).

01/2005:0272

DOXYCYCLINE HYCLATE

Doxycyclini hyclas



$(C_{22}H_{25}ClN_2O_8), 1/2 C_2H_6O, 1/2 H_2O$

M_r 512.9

DEFINITION

Hydrochloride hemiethanol hemihydrate of (4S,4aR,5S,5aR,6R,12aS)-4-(dimethylamino)-3,5,10,12,12a-pentahydroxy-6-methyl-1,11-dioxo-1,4,4a,5,5a,6,11,12a-octahydrotetracene-2-carboxamide.

Substance obtained from oxytetracycline or metacycline or by any other means.

Content: 95.0 per cent to 102.0 per cent of $C_{22}H_{25}ClN_2O_8$ (anhydrous and ethanol-free substance).

CHARACTERS

Appearance: yellow, crystalline powder, hygroscopic.

Solubility: freely soluble in water and in methanol, sparingly soluble in ethanol (96 per cent). It dissolves in solutions of alkali hydroxides and carbonates.

IDENTIFICATION

A. Examine the chromatograms obtained in the assay.

Results: the principal peak in the chromatogram obtained with the test solution is similar in retention time and size to the principal peak in the chromatogram obtained with reference solution (a).

B. To about 2 mg add 5 ml of *sulphuric acid R*. A yellow colour develops.

C. It gives reaction (a) of chlorides (2.3.1).

TESTS

pH (2.2.3): 2.0 to 3.0.

Dissolve 0.1 g in *carbon dioxide-free water R* and dilute to 10 ml with the same solvent.

Specific optical rotation (2.2.7): – 105 to – 120 (anhydrous and ethanol-free substance).

Dissolve 0.250 g in a mixture of 1 volume of 1 *M hydrochloric acid* and 99 volumes of *methanol R* and dilute to 25.0 ml with the same mixture of solvents. Carry out the measurement within 5 min of preparing the solution.

Specific absorbance (2.2.25): 300 to 335, determined at the absorption maximum at 349 nm (anhydrous and ethanol-free substance).

Dissolve 25.0 mg in a mixture of 1 volume of 1 *M hydrochloric acid* and 99 volumes of *methanol R* and dilute to 25.0 ml with the same mixture of solvents. Dilute 1.0 ml of the solution to 100.0 ml with a mixture of 1 volume of 1 *M hydrochloric acid* and 99 volumes of *methanol R*. Carry out the measurement within 1 h of preparing the solution.

Light-absorbing impurities. The absorbance (2.2.25), determined at 490 nm is maximum 0.07 (anhydrous and ethanol-free substance).

Dissolve 0.10 g in a mixture of 1 volume of 1 *M hydrochloric acid* and 99 volumes of *methanol R* and dilute to 10.0 ml with the same mixture of solvents. Carry out the measurement within 1 h of preparing the solution.

Related substances. Liquid chromatography (2.2.29). Prepare the solutions immediately before use.

Test solution. Dissolve 20.0 mg of the substance to be examined in 0.01 *M hydrochloric acid* and dilute to 25.0 ml with the same acid.

Reference solution (a). Dissolve 20.0 mg of *doxycycline hyclate CRS* in 0.01 *M hydrochloric acid* and dilute to 25.0 ml with the same acid.

Reference solution (b). Dissolve 20.0 mg of 6-epidoxycycline hydrochloride CRS in 0.01 *M hydrochloric acid* and dilute to 25.0 ml with the same acid.

Reference solution (c). Dissolve 20.0 mg of *metacycline hydrochloride CRS* in 0.01 *M hydrochloric acid* and dilute to 25.0 ml with the same acid.

Reference solution (d). Mix 4.0 ml of reference solution (a), 1.5 ml of reference solution (b) and 1.0 ml of reference solution (c) and dilute to 25.0 ml with 0.01 *M hydrochloric acid*.

Reference solution (e). Mix 2.0 ml of reference solution (b) and 2.0 ml of reference solution (c) and dilute to 100.0 ml with 0.01 *M hydrochloric acid*.

Column:

- size: $l = 0.25$ m, $\varnothing = 4.6$ mm,
- stationary phase: *styrene-divinylbenzene copolymer R* (8 μ m),
- temperature: 60 °C.

Mobile phase: weigh 60.0 g of 2-methyl-2-propanol *R* and transfer to a 1000 ml volumetric flask with the aid of 200 ml of *water R*; add 400 ml of *buffer solution pH 8.0 R*, 50 ml of a 10 g/l solution of *tetrabutylammonium hydrogen sulphate R* adjusted to pH 8.0 with *dilute sodium hydroxide solution R* and 10 ml of a 40 g/l solution of *sodium edetate R* adjusted to pH 8.0 with *dilute sodium hydroxide solution R*; dilute to 1000.0 ml with *water R*.

Flow rate: 1.0 ml/min.

Detection: spectrophotometer at 254 nm.

Injection: 20 μ l of the test solution and reference solutions (d) and (e).

Relative retention with reference to doxycycline: impurity E = about 0.2; impurity D = about 0.3; impurity C = about 0.5; impurity F = about 1.2.

System suitability: reference solution (d):

- resolution: minimum 1.25 between the peaks due to impurity B (1st peak) and impurity A (2nd peak) and minimum 2.0 between the peaks due to impurity A and doxycycline (3rd peak); if necessary, adjust the 2-methyl-2-propanol content in the mobile phase;
- symmetry factor: maximum 1.25 for the peak due to doxycycline.

Limits:

- impurity A: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (e) (2.0 per cent),
- impurity B: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (e) (2.0 per cent),
- impurities C, D, E, F: for each impurity, not more than 0.25 times the area of the peak due to impurity A in the chromatogram obtained with reference solution (e) (0.5 per cent),
- any other impurity: for each impurity, not more than 0.25 times the area of the peak due to impurity A in the chromatogram obtained with reference solution (e) (0.5 per cent),
- disregard limit: 0.05 times the area of the peak due to impurity A in the chromatogram obtained with reference solution (e) (0.1 per cent).

Ethanol. Gas chromatography (2.2.28).

Internal standard solution. Dilute 0.50 ml of *propanol R* to 1000.0 ml with *water R*.

Test solution (a). Dissolve 0.10 g of the substance to be examined in *water R* and dilute to 10.0 ml with the same solvent.

Test solution (b). Dissolve 0.10 g of the substance to be examined in the internal standard solution and dilute to 10.0 ml with the same solution.

Reference solution. Dilute 0.50 ml of *ethanol R* to 100.0 ml with the internal standard solution. Dilute 1.0 ml of this solution to 10.0 ml with the internal standard solution.

Column:

- size: $l = 1.5$ m, $\varnothing = 4.0$ mm,
- stationary phase: *ethylvinylbenzene-divinylbenzene copolymer R* (150–180 μ m).

Carrier gas: *nitrogen for chromatography R*.

Temperature:

- column: 135 °C,
- injection port and detector: 150 °C.

Detection: flame ionisation.

Calculate the content of ethanol taking the density (2.2.5) at 20 °C to be 0.790 g/ml.

Limit:

– *ethanol*: 4.3 per cent to 6.0 per cent.

Heavy metals (2.4.8): maximum 50 ppm.

0.5 g complies with limit test C. Prepare the reference solution using 2.5 ml of *lead standard solution* (10 ppm Pb) R.

Water (2.5.12): 1.4 per cent to 2.8 per cent, determined on 1.20 g.

Sulphated ash (2.4.14): maximum 0.4 per cent, determined on 1.0 g.

Bacterial endotoxins (2.6.14): less than 1.14 IU/mg, if intended for use in the manufacture of parenteral dosage forms without a further appropriate procedure for the removal of bacterial endotoxins.

ASSAY

Liquid chromatography (2.2.29) as described in the test for related substances with the following modification.

Injection: test solution and reference solution (a).

Calculate the percentage content of $C_{22}H_{25}ClN_2O_8$ ($M_r = 480.9$).

STORAGE

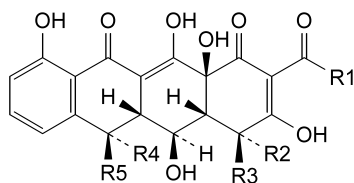
In an airtight container, protected from light. If the substance is sterile, store in a sterile, airtight, tamper-proof container.

LABELLING

The label states, where applicable, that the substance is free from bacterial endotoxins.

IMPURITIES

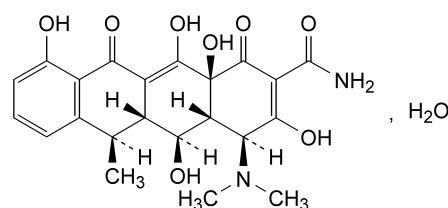
Specified impurities: A, B, C, D, E, F.



- A. $R_1 = NH_2$, $R_2 = R_5 = H$, $R_3 = N(CH_3)_2$, $R_4 = CH_3$:
(4*S*,4*aR*,5*S*,5*aR*,6*S*,12*aS*)-4-(dimethylamino)-3,5,10,12,12*a*-pentahydroxy-6-methyl-1,11-dioxo-1,4,4*a*,5,5*a*,6,11,12*a*-octahydrotetracene-2-carboxamide (6-epidoxycycline),
- B. $R_1 = NH_2$, $R_2 = H$, $R_3 = N(CH_3)_2$, $R_4 + R_5 = CH_2$:
(4*S*,4*aR*,5*S*,5*aR*,12*aS*)-4-(dimethylamino)-3,5,10,12,12*a*-pentahydroxy-6-methylene-1,11-dioxo-1,4,4*a*,5,5*a*,6,11,12*a*-octahydrotetracene-2-carboxamide (metacycline),
- C. $R_1 = NH_2$, $R_2 = N(CH_3)_2$, $R_3 = R_4 = H$, $R_5 = CH_3$:
(4*R*,4*aR*,5*S*,5*aR*,6*R*,12*aS*)-4-(dimethylamino)-3,5,10,12,12*a*-pentahydroxy-6-methyl-1,11-dioxo-1,4,4*a*,5,5*a*,6,11,12*a*-octahydrotetracene-2-carboxamide (4-epidoxycycline),
- D. $R_1 = NH_2$, $R_2 = N(CH_3)_2$, $R_3 = R_5 = H$, $R_4 = CH_3$:
(4*R*,4*aR*,5*S*,5*aR*,6*S*,12*aS*)-4-(dimethylamino)-3,5,10,12,12*a*-pentahydroxy-6-methyl-1,11-dioxo-1,4,4*a*,5,5*a*,6,11,12*a*-octahydrotetracene-2-carboxamide (4-*epi*-6-epidoxycycline),
- E. $R_1 = NH_2$, $R_2 = H$, $R_3 = N(CH_3)_2$, $R_4 = OH$, $R_5 = CH_3$:
oxytetracycline,
- F. $R_1 = CH_3$, $R_2 = R_4 = H$, $R_3 = N(CH_3)_2$, $R_5 = CH_3$:
(4*S*,4*aR*,5*S*,5*aR*,6*R*,12*aS*)-2-acetyl-4-(dimethylamino)-3,5,10,12,12*a*-pentahydroxy-6-methyl-4*a*,5*a*,6,12*a*-tetrahydrotetracene-1,11-(4*H*,5*H*)-dione (2-acetyl-2-decarbamoxyldoxycycline).

DOXYCYCLINE MONOHYDRATE

Doxycyclinum monohydricum



$C_{22}H_{24}N_2O_8 \cdot H_2O$

M_r 462.5

DEFINITION

(4*S*,4*aR*,5*S*,5*aR*,6*R*,12*aS*)-4-(Dimethylamino)-3,5,10,12,12*a*-pentahydroxy-6-methyl-1,11-dioxo-1,4,4*a*,5,5*a*,6,11,12*a*-octahydrotetracene-2-carboxamide monohydrate.

Substance obtained from oxytetracycline or metacycline or by any other means.

Content: 95.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

Appearance: yellow, crystalline powder.

Solubility: very slightly soluble in water and in alcohol. It dissolves in dilute solutions of mineral acids and in solutions of alkali hydroxides and carbonates.

IDENTIFICATION

A. Examine the chromatograms obtained in the assay.

Results: the principal peak in the chromatogram obtained with the test solution is similar in retention time and size to the principal peak in the chromatogram obtained with reference solution (a).

B. To about 2 mg add 5 ml of *sulphuric acid* R. A yellow colour develops.

C. Dissolve 25 mg in a mixture of 0.2 ml of *dilute nitric acid* R and 1.8 ml of *water* R. The solution does not give reaction (a) of chlorides (2.3.1).

TESTS

pH (2.2.3): 5.0 to 6.5.

Suspend 0.1 g in *carbon dioxide-free water* R and dilute to 10 ml with the same solvent.

Specific optical rotation (2.2.7): –113 to –130 (anhydrous substance).

Dissolve 0.250 g in a mixture of 0.5 volumes of *hydrochloric acid* R and 99.5 volumes of *methanol* R and dilute to 25.0 ml with the same mixture of solvents. Carry out the measurement within 5 min of preparing the solution.

Specific absorbance (2.2.25): 325 to 363 determined at the maximum at 349 nm (anhydrous substance).

Dissolve 25.0 mg in a mixture of 0.5 volumes of *hydrochloric acid* R and 99.5 volumes of *methanol* R and dilute to 50.0 ml with the same mixture of solvents. Dilute 2.0 ml of the solution to 100.0 ml with a mixture of 0.5 volumes of 1 *M* *hydrochloric acid* and 99.5 volumes of *methanol* R. Carry out the measurement within 1 h of preparing the solution.

Light-absorbing impurities. The absorbance (2.2.25) determined at 490 nm has a maximum of 0.07 (anhydrous substance).

Dissolve 0.10 g in a mixture of 0.5 volumes of *hydrochloric acid* R and 99.5 volumes of *methanol* R and dilute to

10.0 ml with the same mixture of solvents. Carry out the measurement within 1 h of preparing the solution.

Related substances. Liquid chromatography (2.2.29). Prepare the solutions immediately before use.

Test solution. Dissolve 20.0 mg of the substance to be examined in 0.01 M hydrochloric acid and dilute to 25.0 ml with the same acid.

Reference solution (a). Dissolve 20.0 mg of *doxycycline hyclate* CRS in 0.01 M hydrochloric acid and dilute to 25.0 ml with the same acid.

Reference solution (b). Dissolve 20.0 mg of 6-epidoxycycline hydrochloride CRS in 0.01 M hydrochloric acid and dilute to 25.0 ml with the same acid.

Reference solution (c). Dissolve 20.0 mg of *metacycline hydrochloride* CRS in 0.01 M hydrochloric acid and dilute to 25.0 ml with the same acid.

Reference solution (d). Mix 4.0 ml of reference solution (a), 1.5 ml of reference solution (b) and 1.0 ml of reference solution (c) and dilute to 25.0 ml with 0.01 M hydrochloric acid.

Reference solution (e). Mix 2.0 ml of reference solution (b) and 2.0 ml of reference solution (c) and dilute to 100.0 ml with 0.01 M hydrochloric acid.

Column:

- size: $l = 0.25$ m, $\varnothing = 4.6$ mm,
- stationary phase: styrene-divinylbenzene copolymer R (8 μ m),
- temperature: 60 °C.

Mobile phase: weigh 60.0 g of 2-methyl-2-propanol R and transfer into a 1000 ml volumetric flask with the aid of 200 ml of water R; add 400 ml of buffer solution pH 8.0 R, 50 ml of a 10 g/l solution of tetrabutylammonium hydrogen sulphate R adjusted to pH 8.0 with dilute sodium hydroxide solution R and 10 ml of a 40 g/l solution of sodium edetate R adjusted to pH 8.0 with dilute sodium hydroxide solution R; dilute to 1000.0 ml with water R.

Flow rate: 1.0 ml/min.

Detection: spectrophotometer at 254 nm.

Injection: 20 μ l; inject the test solution and reference solutions (d) and (e).

Relative retention with reference to doxycycline: impurity E = about 0.2; impurity D = about 0.3; impurity C = about 0.5; impurity F = about 1.2.

System suitability: reference solution (d):

- resolution: minimum 1.25 between the peaks due to impurity B (1st peak) and impurity A (2nd peak) and minimum 2.0 between the peaks due to impurity A and doxycycline (3rd peak); if necessary, adjust the 2-methyl-2-propanol content in the mobile phase,
- symmetry factor: maximum 1.25 for the peak due to doxycycline.

Limits:

- impurity A: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (e) (2.0 per cent),
- impurity B: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (e) (2.0 per cent),

- any other impurity: not more than 0.25 times the area of the peak due to impurity A in the chromatogram obtained with reference solution (e) (0.5 per cent),
- disregard limit: 0.05 times the area of the peak due to impurity A in the chromatogram obtained with reference solution (e) (0.1 per cent).

Heavy metals (2.4.8): maximum 50 ppm.

0.5 g complies with limit test C. Prepare the standard using 2.5 ml of lead standard solution (10 ppm Pb) R.

Water (2.5.12): 3.6 per cent to 4.6 per cent, determined on 0.200 g.

Sulphated ash (2.4.14): maximum 0.4 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (2.2.29) as described in the test for related substances with the following modification.

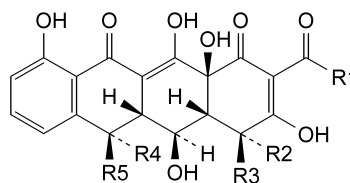
Injection: test solution and reference solution (a).

Calculate the percentage content of $C_{22}H_{24}N_2O_8$.

STORAGE

Protected from light.

IMPURITIES

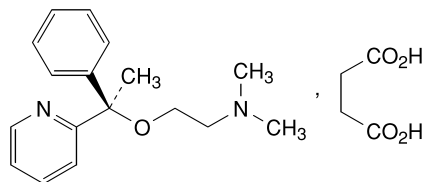


- R1 = NH₂, R2 = R5 = H, R3 = N(CH₃)₂, R4 = CH₃: (4S,4aR,5S,5aR,6S,12aS)-4-(dimethylamino)-3,5,10,12,12a-pentahydroxy-6-methyl-1,11-dioxo-1,4,4a,5,5a,6,11,12a-octahydrotetracene-2-carboxamide (6-epidoxycycline),
- R1 = NH₂, R2 = H, R3 = N(CH₃)₂, R4 + R5 = CH₂: (4S,4aR,5S,5aR,12aS)-4-(dimethylamino)-3,5,10,12,12a-pentahydroxy-6-methylene-1,11-dioxo-1,4,4a,5,5a,6,11,12a-octahydrotetracene-2-carboxamide (metacycline),
- R1 = NH₂, R2 = N(CH₃)₂, R3 = R4 = H, R5 = CH₃: (4R,4aR,5S,5aR,6R,12aS)-4-(dimethylamino)-3,5,10,12,12a-pentahydroxy-6-methyl-1,11-dioxo-1,4,4a,5,5a,6,11,12a-octahydrotetracene-2-carboxamide (4-epidoxycycline),
- R1 = NH₂, R2 = N(CH₃)₂, R3 = R5 = H, R4 = CH₃: (4R,4aR,5S,5aR,6S,12aS)-4-(dimethylamino)-3,5,10,12,12a-pentahydroxy-6-methyl-1,11-dioxo-1,4,4a,5,5a,6,11,12a-octahydrotetracene-2-carboxamide (4-epi-6-epidoxycycline),
- R1 = NH₂, R2 = H, R3 = N(CH₃)₂, R4 = OH, R5 = CH₃: oxytetracycline,
- R1 = CH₃, R2 = R4 = H, R3 = N(CH₃)₂, R5 = CH₃: (4S,4aR,5S,5aR,6R,12aS)-2-acetyl-4-(dimethylamino)-3,5,10,12,12a-pentahydroxy-6-methyl-4a,5a,6,12a-tetrahydrotetracene-1,11(4H,5H)-dione (2-acetyl-2-decarbamoyleldoxycycline).

01/2005:1589

DOXYLAMINE HYDROGEN SUCCINATE

Doxylamini hydrogenosuccinas



and enantiomer

 $C_{21}H_{28}N_2O_5$
 M_r 388.5

DEFINITION

N,N-dimethyl-2-[(1*RS*)-1-phenyl-1-(pyridin-2-yl)ethoxy]ethanamine hydrogen butanedioate.

Content: 99.0 per cent to 101.0 per cent (anhydrous substance).

CHARACTERS

Appearance: a white or almost white powder.

Solubility: very soluble in water, freely soluble in alcohol.

IDENTIFICATION

First identification: C.

Second identification: A, B.

A. Melting point (2.2.14): 103 °C to 108 °C.

B. Dissolve 0.200 g in 0.1 M hydrochloric acid and dilute to 100.0 ml with the same solvent. Dilute 1.0 ml of the solution to 100.0 ml with 0.1 M hydrochloric acid. Examined between 230 nm and 350 nm (2.2.25), the solution shows an absorption maximum at 262 nm. The specific absorbance at the maximum is 229 to 243 (anhydrous substance).

C. Infrared absorption spectrophotometry (2.2.24).

Comparison: Ph. Eur. reference spectrum of doxylamine hydrogen succinate.

TESTS

Appearance of solution. The solution is clear (2.2.1) and colourless (2.2.2, Method II).

Dissolve 0.4 g of the substance to be examined in water R and dilute to 20 ml with the same solvent.

Optical rotation (2.2.7): - 0.10° to + 0.10°.

Dissolve 2.50 g of the substance to be examined in water R and dilute to 25.0 ml with the same solvent.

Related substances. Gas chromatography (2.2.28).

Test solution. Dissolve 0.650 g of the substance to be examined in 20 ml of 0.1 M hydrochloric acid. Add 3 ml of a 100 g/l solution of sodium hydroxide R and extract 3 times with 25 ml of methylene chloride R. Combine the methylene chloride extracts and filter using hydrophobic phase-separation filter paper. Rinse the filter with 10 ml of methylene chloride R and combine the rinsings with the methylene chloride extracts. Evaporate the solvent under reduced pressure at a temperature not exceeding 40 °C. Dissolve the residue in 20.0 ml of ethanol R.

Reference solution (a). Dilute 1.0 ml of the test solution to 200.0 ml with ethanol R.

Reference solution (b). Dissolve 40 mg of doxylamine impurity A CRS and 40 mg of 2-benzoylpyridine R in ethanol R and dilute to 20 ml with the same solvent. Dilute 1 ml of this solution to 20 ml with ethanol R.

Column:

- **material:** fused silica,
- **size:** $l = 30$ m, $\varnothing = 0.53$ mm,
- **stationary phase:** poly(dimethyl)(diphenyl)siloxane R (film thickness 1.5 μ m).

Carrier gas: helium for chromatography R.

Flow rate: 7 ml/min.

Temperature:

	Time (min)	Temperature (°C)
Column	0 - 12	160 → 220
	12 - 27	220
Injection port		250
Detector		250

Detection: flame ionisation.

Injection: 1 μ l.

System suitability: reference solution (b):

- **resolution:** minimum 1.5 between the peaks due to impurity A and impurity D.

Limits:

- **any impurity:** not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent),
- **total:** not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (1 per cent),
- **disregard limit:** 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

Water (2.5.12): maximum 0.5 per cent, determined on 2.00 g.

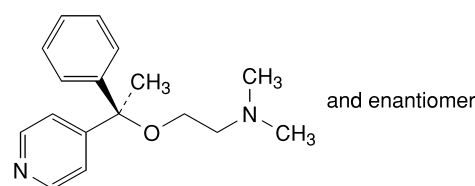
Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

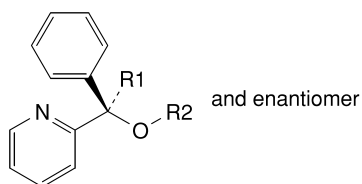
Dissolve 0.150 g in 50 ml of anhydrous acetic acid R. Titrate with 0.1 M perchloric acid, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M perchloric acid is equivalent to 19.43 mg of $C_{21}H_{28}N_2O_5$.

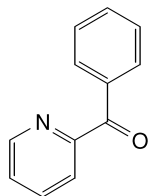
IMPURITIES



A. *N,N*-dimethyl-2-[(1*RS*)-1-phenyl-1-(pyridin-4-yl)ethoxy]ethanamine,



- B. R1 = CH₃, R2 = H: (1*RS*)-1-phenyl-1-(pyridin-2-yl)ethanol,
 C. R1 = H, R2 = CH₂-CH₂-N(CH₃)₂: *N,N*-dimethyl-2-[(*RS*)-1-phenyl(pyridin-2-yl)methoxy]ethanamine,

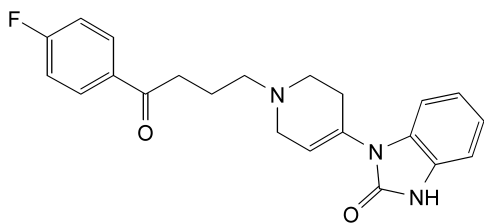


- D. phenyl(pyridin-2-yl)methanone (2-benzoylpyridine).

01/2005:1010

DROPERIDOL

Droperidolum

C₂₂H₂₂FN₃O₂M_r 379.4

DEFINITION

Droperidol contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of 1-[1-[4-(4-fluorophenyl)-4-oxobutyl]-1,2,3,6-tetrahydropyridin-4-yl]-1,3-dihydro-2*H*-benzimidazol-2-one, calculated with reference to the dried substance.

CHARACTERS

A white or almost white powder, practically insoluble in water, freely soluble in dimethylformamide and in methylene chloride, sparingly soluble in alcohol.

It shows polymorphism.

IDENTIFICATION

First identification: A.

Second identification: B, C, D.

- A. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *droperidol CRS*. Examine the substances prepared as discs. If the spectra obtained show differences, dissolve the substance to be examined and the reference substance separately in the minimum volume of *acetone R*, evaporate to dryness on a water-bath and record new spectra using the residues.

- B. Examine by thin-layer chromatography (2.2.27), using *silica gel GF₂₅₄ R* as the coating substance.

Test solution. Dissolve 30 mg of the substance to be examined in a mixture of 1 volume of *acetone R* and 9 volumes of *methanol R* and dilute to 10 ml with the same mixture of solvents.

Reference solution (a). Dissolve 30 mg of *droperidol CRS* in a mixture of 1 volume of *acetone R* and 9 volumes of *methanol R* and dilute to 10 ml with the same mixture of solvents.

Reference solution (b). Dissolve 30 mg of *droperidol CRS* and 30 mg of *benperidol CRS* in a mixture of 1 volume of *acetone R* and 9 volumes of *methanol R* and dilute to 10 ml with the same mixture of solvents.

Apply to the plate 10 µl of each solution. Develop over a path of 15 cm using a mixture of 1 volume of *acetone R* and 9 volumes of *methanol R*. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. The principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a). The test is not valid unless the chromatogram obtained with reference solution (b) shows 2 clearly separated spots.

- C. Dissolve about 10 mg in 5 ml of *ethanol R*. Add 0.5 ml of *dinitrobenzene solution R* and 0.5 ml of *2 M alcoholic potassium hydroxide R*. A violet colour is produced and becomes brownish-red after 20 min.
- D. Mix about 5 mg with 45 mg of *heavy magnesium oxide R* and ignite in a crucible until an almost white residue is obtained (usually less than 5 min). Allow to cool, add 1 ml of *water R*, 0.05 ml of *phenolphthalein solution R1* and about 1 ml of *dilute hydrochloric acid R* to render the solution colourless. Filter. To a freshly prepared mixture of 0.1 ml of *alizarin S solution R* and 0.1 ml of *zirconyl nitrate solution R*, add 1.0 ml of the filtrate. Mix, allow to stand for 5 min and compare the colour of the solution with that of a blank prepared in the same manner. The test solution is yellow and the blank is red.

TESTS

Appearance of solution. Dissolve 0.20 g in *methylene chloride R* and dilute to 20.0 ml with the same solvent. The solution is clear (2.2.1) and not more intensely coloured than reference solution BY₅ (2.2.2, *Method II*).

Related substances. Examine by liquid chromatography (2.2.29). *Prepare the solutions immediately before use.*

Test solution. Dissolve 0.10 g of the substance to be examined in *dimethylformamide R* and dilute to 10.0 ml with the same solvent.

Reference solution (a). Dissolve 2.5 mg of *droperidol CRS* and 2.5 mg of *benperidol CRS* in *dimethylformamide R* and dilute to 100.0 ml with the same solvent.

Reference solution (b). Dilute 1.0 ml of the test solution to 100.0 ml with *dimethylformamide R*. Dilute 5.0 ml of this solution to 20.0 ml with *dimethylformamide R*.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.10 m long and 4.6 mm in internal diameter packed with *base-deactivated octadecylsilyl silica gel for chromatography R* (3 µm),
- as mobile phase at a flow rate of 1.5 ml/min:
 - mobile phase A: acetonitrile R,*
 - mobile phase B: 10 g/l solution of tetrabutylammonium hydrogen sulphate R1,*

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)
0 - 15	0 → 40	100 → 60
15 - 20	40	60
20 - 25	40 → 0	60 → 100

- as detector a spectrophotometer set at 275 nm.

Adjust the sensitivity of the system so that the height of the principal peak in the chromatogram obtained with 10 µl of reference solution (b) is at least 50 per cent of the full scale of the recorder.

Inject 10 µl of reference solution (a). When the chromatogram is recorded in the prescribed conditions, the retention times are: benperidol about 6.5 min and droperidol about 7 min. The test is not valid unless the resolution between the peaks due to droperidol and benperidol is at least 2.0. If necessary, adjust the final concentration of acetonitrile in the mobile phase or adjust the time programme for the linear gradient. Inject 10 µl of *dimethylformamide R* as a blank, 10 µl of the test solution and 10 µl of reference solution (b). In the chromatogram obtained with the test solution: the area of any peak, apart from the principal peak, is not greater than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.25 per cent); the sum of the areas of all the peaks, apart from the principal peak, is not greater than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent). Disregard any peak obtained with the blank and any peak with an area less than 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (b).

Heavy metals (2.4.8). 1.0 g complies with limit test D for heavy metals (20 ppm). Prepare the standard using 2 ml of *lead standard solution (10 ppm Pb) R*.

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 100–105 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

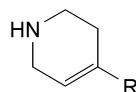
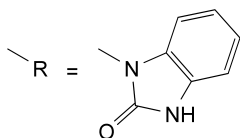
Dissolve 0.300 g in 50 ml of a mixture of 1 volume of *anhydrous acetic acid R* and 7 volumes of *methyl ethyl ketone R*. Using 0.2 ml of *naphtholbenzein solution R*, titrate with 0.1 M *perchloric acid* until the colour changes from orange-yellow to green.

1 ml of 0.1 M *perchloric acid* is equivalent to 37.94 mg of C₂₂H₂₂FN₃O₂.

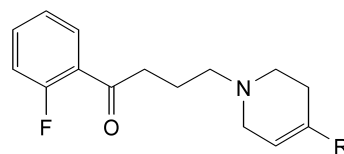
STORAGE

Store protected from light.

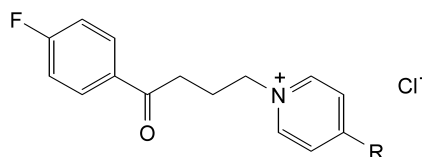
IMPURITIES



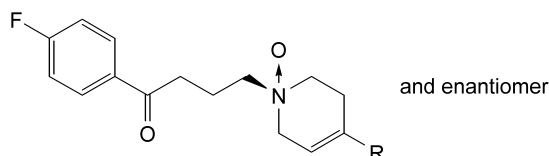
- A. 1-(1,2,3,6-tetrahydropyridin-4-yl)-1,3-dihydro-2*H*-benzimidazol-2-one,



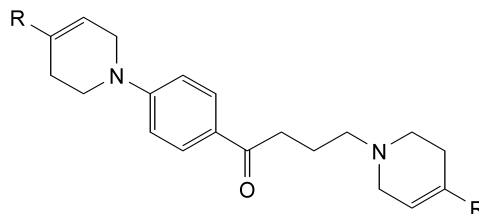
- B. 1-[1-[4-(2-fluorophenyl)-4-oxobutyl]-1,2,3,6-tetrahydropyridin-4-yl]-1,3-dihydro-2*H*-benzimidazol-2-one,



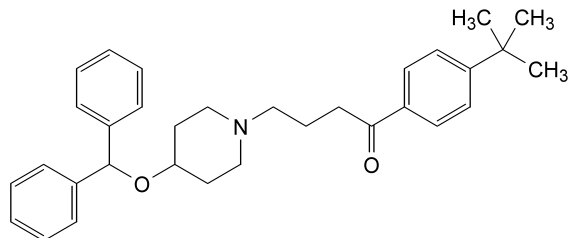
- C. 1-[4-(4-fluorophenyl)-4-oxobutyl]-4-(2-oxo-2,3-dihydro-1*H*-benzimidazol-1-yl)pyridinium chloride,



- D. (1*RS*)-1-[4-(4-fluorophenyl)-4-oxobutyl]-4-(2-oxo-2,3-dihydro-1*H*-benzimidazol-1-yl)-1,2,3,6-tetrahydropyridine 1-oxide,



- E. 1-[1-[4-[4-(2-oxo-2,3-dihydro-1*H*-benzimidazol-1-yl)-3,6-dihydropyridin-1(2*H*)-yl]-1-oxobutyl]phenyl]-1,2,3,6-tetrahydropyridin-4-yl]-1,3-dihydro-2*H*-benzimidazol-2-one.

01/2005:2015
corrected**EBASTINE****Ebastinum** $C_{32}H_{39}NO_2$ M_r 469.7**DEFINITION**

1-[4-(1,1-Dimethylethyl)phenyl]-4-[4-(diphenylmethoxy)piperidin-1-yl]butan-1-one.

Content: 99.0 per cent to 101.0 per cent (anhydrous substance).

CHARACTERS

Appearance: white or almost white, crystalline powder.

Solubility: practically insoluble in water, very soluble in methylene chloride, sparingly soluble in methanol.

mp: about 86 °C.

IDENTIFICATION

Infrared absorption spectrophotometry (2.2.24).

Comparison: Ph. Eur. reference spectrum of ebastine.

TESTS

Related substances. Liquid chromatography (2.2.29). *Keep the solutions protected from light.*

Solution A. Mix 65 volumes of *acetonitrile R* and 35 volumes of a 1.1 g/l solution of *phosphoric acid R* adjusted to pH 5.0 with a 40 g/l solution of *sodium hydroxide R*.

Test solution. Dissolve 0.125 g of the substance to be examined in solution A and dilute to 50.0 ml with the same solution.

Reference solution (a). Dissolve 5.0 mg of *ebastine impurity C CRS* and 5.0 mg of *ebastine impurity D CRS* in solution A and dilute to 20.0 ml with the same solution. Dilute 1.0 ml of the solution to 100.0 ml with solution A.

Reference solution (b). Dilute 1.0 ml of the test solution to 100.0 ml with solution A. Dilute 1.0 ml of this solution to 10.0 ml with solution A.

Column:

- *size:* $l = 0.25$ m, $\varnothing = 4.6$ mm,
- *stationary phase:* nitrile silica gel for chromatography R (5 μ m).

Mobile phase: mix 35 volumes of *acetonitrile R* and 65 volumes of a 1.1 g/l solution of *phosphoric acid R* adjusted to pH 5.0 with a 40 g/l solution of *sodium hydroxide R*. Adjust the percentage of acetonitrile to between 30 per cent V/V and 40 per cent V/V so that the retention time of ebastine is about 110 min.

Flow rate: 1 ml/min.

Detection: spectrophotometer at 210 nm.

Injection: 10 μ l.

Run time: 1.4 times the retention time of ebastine.

Relative retention with reference to ebastine:

impurity A = about 0.04; impurity B = about 0.05; impurity D = about 0.20; impurity C = about 0.22; impurity F = about 0.42; impurity G = about 0.57; impurity E = about 1.14.

System suitability: reference solution (a):

- **resolution:** minimum 2.0 between the peaks due to impurity D and impurity C.

Limits:

- **impurities A, B, C, D, E, F, G:** for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent),
- **any other impurity:** for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent),
- **total:** not more than 4 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.4 per cent),
- **disregard limit:** 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

Sulphates (2.4.13): maximum 100 ppm.

Suspend 2.5 g in 25 ml of *dilute nitric acid R*. Boil under a reflux condenser for 10 min. Cool and filter. 15 ml of the filtrate complies with the limit test for sulphates.

Water (2.5.12): maximum 0.5 per cent, determined on 0.500 g.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

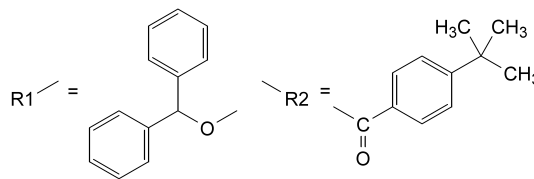
ASSAY

Dissolve 0.350 g in 50 ml of *anhydrous acetic acid R*. Titrate with 0.1 M *perchloric acid*, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *perchloric acid* is equivalent to 46.97 mg of $C_{32}H_{39}NO_2$.

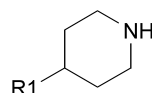
STORAGE

Protected from light.

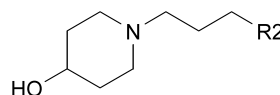
IMPURITIES

A. R1-H: diphenylmethanol (benzhydrol),

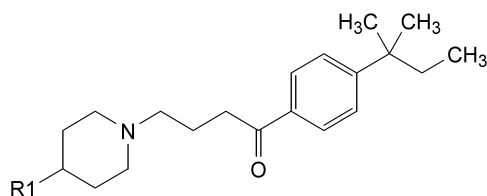
B. R2-CH₃: 1-[4-(1,1-dimethylethyl)phenyl]ethanone,



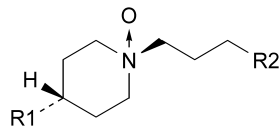
C. 4-(diphenylmethoxy)piperidine,



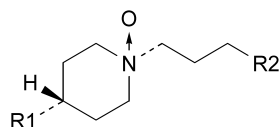
D. 1-[4-(1,1-dimethylethyl)phenyl]-4-(4-hydroxypiperidin-1-yl)butan-1-one,



- E. 1-[4-(1,1-dimethylpropyl)phenyl]-4-[4-(diphenylmethoxy)piperidin-1-yl]butan-1-one,



- F. 1-[4-(1,1-dimethylethyl)phenyl]-4-[cis-4-(diphenylmethoxy)-1-oxidopiperidin-1-yl]butan-1-one,

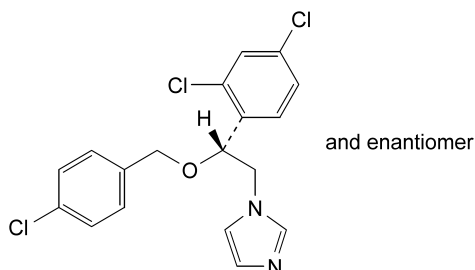


- G. 1-[4-(1,1-dimethylethyl)phenyl]-4-[trans-4-(diphenylmethoxy)-1-oxidopiperidin-1-yl]butan-1-one.

01/2005:2049
corrected

ECONAZOLE

Econazolum



$C_{18}H_{15}Cl_3N_2O$

M_r 381.7

DEFINITION

1-[(2*RS*)-2-[(4-Chlorobenzyl)oxy]-2-(2,4-dichlorophenyl)ethyl]-1*H*-imidazole.

Content: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

Appearance: white or almost white powder.

Solubility: practically insoluble in water, very soluble in alcohol and in methylene chloride.

IDENTIFICATION

A. Melting point (2.2.14): 88 °C to 92 °C.

B. Infrared absorption spectrophotometry (2.2.24).

Comparison: Ph. Eur. reference spectrum of econazole.

TESTS

Related substances. Liquid chromatography (2.2.29).

Test solution. Dissolve 0.100 g of the substance to be examined in *methanol R* and dilute to 10.0 ml with the same solvent.

Reference solution (a). Dissolve 10 mg of *econazole* for system suitability CRS in *methanol R* and dilute to 1.0 ml with the same solvent.

Reference solution (b). Dilute 1.0 ml of the test solution to 20.0 ml with *methanol R*. Dilute 1.0 ml of this solution to 25.0 ml with *methanol R*.

Column:

- size: $l = 0.10$ m, $\varnothing = 4.6$ mm,
- stationary phase: base-deactivated octadecylsilyl silica gel for chromatography R (3 μ m),
- temperature: 35 °C.

Mobile phase:

- mobile phase A: mix 20 volumes of *methanol R* and 80 volumes of a 0.77 g/l solution of ammonium acetate R,
- mobile phase B: *methanol R*, *acetonitrile R* (40:60 V/V),

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)
0 - 25	60 → 10	40 → 90
25 - 27	10	90
27 - 28	10 → 60	90 → 40
28 - 33	60	40

Flow rate: 1.5 ml/min.

Detection: spectrophotometer at 225 nm.

Injection: 10 μ l.

Relative retention with reference to econazole (retention time = about 15 min): impurity A = about 0.2; impurity B = about 0.6; impurity C = about 1.1.

System suitability: reference solution (a):

- **peak-to-valley ratio:** minimum 1.5, where H_p = height above the baseline of the peak due to impurity C and H_v = height above the baseline of the lowest point of the curve separating this peak from the peak due to econazole.

Limits:

- **correction factor:** for the calculation of content, multiply the peak area of impurity A by 1.4,
- **impurities A, B, C:** for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent),
- **total:** not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.3 per cent),
- **disregard limit:** 0.25 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

Loss on drying (2.2.32): maximum 0.5 per cent, determined on 1.000 g by drying *in vacuo* at 60 °C for 4 h.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.300 g in 75 ml of *anhydrous acetic acid R*. Titrate with 0.1 M *perchloric acid*, determining the end-point potentiometrically (2.2.20). Carry out a blank titration.

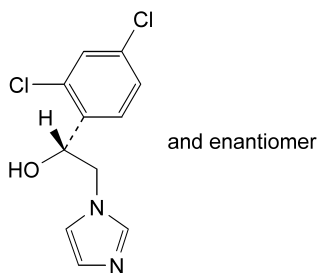
1 ml of 0.1 M *perchloric acid* is equivalent to 38.17 mg of $C_{18}H_{15}Cl_3N_2O$.

STORAGE

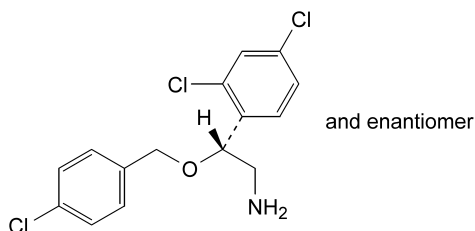
Protected from light.

IMPURITIES

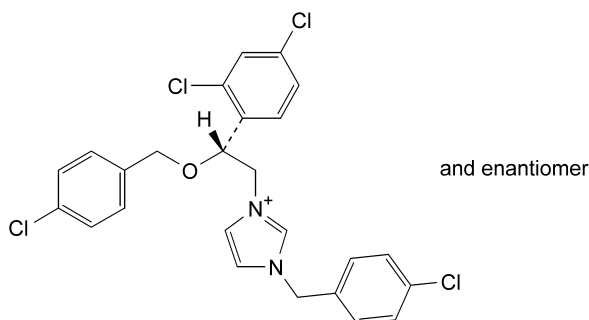
Specified impurities: A, B, C.



A. (1RS)-1-(2,4-dichlorophenyl)-2-(1H-imidazol-1-yl)ethanol,



B. (2RS)-2-[(4-chlorobenzyl)oxy]-2-(2,4-dichlorophenyl)ethanamine,

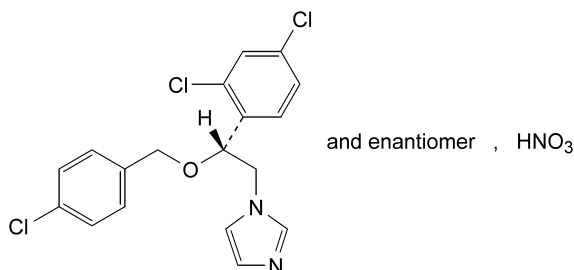


C. 1-(4-chlorobenzyl)-3-[(2RS)-2-[(4-chlorobenzyl)oxy]-2-(2,4-dichlorophenyl)ethyl]imidazolium.

01/2005:0665
corrected

ECONAZOLE NITRATE

Econazoli nitras



$C_{18}H_{16}Cl_3N_3O_4$

M_r 444.7

DEFINITION

1-[(2RS)-2-[(4-Chlorobenzyl)oxy]-2-(2,4-dichlorophenyl)ethyl]-1H-imidazole nitrate.

Content: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

Appearance: white or almost white crystalline powder.

Solubility: very slightly soluble in water, soluble in methanol, sparingly soluble in methylene chloride, slightly soluble in alcohol.

mp: about 165 °C, with decomposition.

IDENTIFICATION

Infrared absorption spectrophotometry (2.2.24).

Comparison: Ph. Eur. reference spectrum of econazole nitrate.

TESTS

Related substances. Liquid chromatography (2.2.29).

Test solution. Dissolve 0.100 g of the substance to be examined in methanol R and dilute to 10.0 ml with the same solvent.

Reference solution (a). Dissolve 10 mg of econazole for system suitability CRS in methanol R and dilute to 1.0 ml with the same solvent.

Reference solution (b). Dilute 1.0 ml of the test solution to 20.0 ml with methanol R. Dilute 1.0 ml of the solution to 25.0 ml with methanol R.

Column:

- size: $l = 0.10$ m, $\varnothing = 4.6$ mm,
- stationary phase: base-deactivated octadecylsilyl silica gel for chromatography R (3 μ m),
- temperature: 35 °C.

Mobile phase:

- mobile phase A: mix 20 volumes of methanol R and 80 volumes of a 0.77 g/l solution of ammonium acetate R,
- mobile phase B: methanol R, acetonitrile R (40:60 V/V),

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)
0 - 25	60 → 10	40 → 90
25 - 27	10	90
27 - 28	10 → 60	90 → 40
28 - 33	60	40

Flow rate: 1.5 ml/min.

Detection: spectrophotometer at 225 nm.

Injection: 10 μ l.

Relative retention with reference to econazole (retention time = about 15 min): impurity A = about 0.2; impurity B = about 0.6; impurity C = about 1.1.

System suitability: reference solution (a):

- peak-to-valley ratio: minimum of 1.5, where H_p = height above the baseline of the peak due to impurity C, and H_v = height above the baseline of the lowest point of the curve separating this peak from the peak due to econazole.

Limits:

- correction factor: for the calculation of content, multiply the peak area of impurity A by 1.4,
- impurities A, B, C: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent),
- total: not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.3 per cent),
- disregard limit: 0.25 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent); disregard the peak due to the nitrate ion at the beginning of the chromatogram.

01/2005:1612

Loss on drying (2.2.32): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 100-105 °C for 4 h.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.400 g in 50 ml of *anhydrous acetic acid R*. Titrate with 0.1 M *perchloric acid*, determining the end-point potentiometrically (2.2.20). Carry out a blank titration.

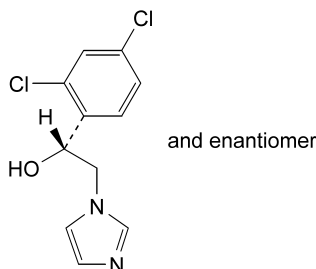
1 ml of 0.1 M *perchloric acid* is equivalent to 44.47 mg of $C_{18}H_{16}Cl_3N_3O_4$.

STORAGE

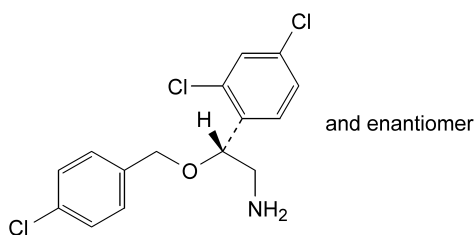
Protected from light.

IMPURITIES

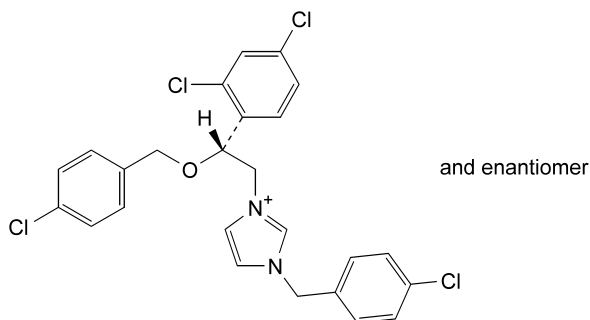
Specified impurities: A, B, C.



A. (1*RS*)-1-(2,4-dichlorophenyl)-2-(1*H*-imidazol-1-yl)ethanol,



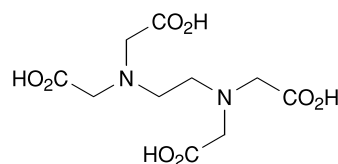
B. (2*RS*)-2-[(4-chlorobenzyl)oxy]-2-(2,4-dichlorophenyl)ethanamine,



C. 1-(4-chlorobenzyl)-3-[(2*RS*)-2-[(4-chlorobenzyl)oxy]-2-(2,4-dichlorophenyl)ethyl]imidazolium.

EDETIC ACID

Acidum edeticum


 $C_{10}H_{16}N_2O_8$
 M_r 292.2

DEFINITION

(Ethylenedinitrilo)tetraacetic acid.

Content: 98.0 per cent to 101.0 per cent.

CHARACTERS

Appearance: white, crystalline powder or colourless crystals.

Solubility: practically insoluble in water and in alcohol. It dissolves in dilute solutions of alkali hydroxides.

IDENTIFICATION

First identification: A.

Second identification: B, C.

A. Infrared absorption spectrophotometry (2.2.24).

Preparation: discs, after drying the substance to be examined in an oven at 100-105 °C for 2 h.

Comparison: *sodium edetate R*, treated as follows: dissolve 0.25 g of *sodium edetate R* in 5 ml of *water R*, add 1.0 ml of *dilute hydrochloric acid R*. Filter, wash the residue with 2 quantities, each of 5 ml, of *water R* and dry the residue in an oven at 100-105 °C for 2 h.

B. To 5 ml of *water R* add 0.1 ml of *ammonium thiocyanate solution R* and 0.1 ml of *ferric chloride solution R1* and mix. The solution is red. Add 0.5 ml of solution S (see Tests). The solution becomes yellowish.

C. To 10 ml of solution S add 0.5 ml of *calcium chloride solution R*. Make alkaline to *red litmus paper R* by the addition of *dilute ammonia R2* and add 3 ml of *ammonium oxalate solution R*. No precipitate is formed.

TESTS

Solution S. Dissolve 5.0 g in 20 ml of *dilute sodium hydroxide solution R* and dilute to 100 ml with *water R*.

Appearance of solution. Solution S is clear (2.2.1) and colourless (2.2.2, *Method II*).

Impurity A. Liquid chromatography (2.2.29). Carry out the test protected from light.

Solvent mixture. Dissolve 10.0 g of *ferric sulphate pentahydrate R* in 20 ml of 0.5 M *sulphuric acid* and add 780 ml of *water R*. Adjust to pH 2.0 with 1 M *sodium hydroxide* and dilute to 1000 ml with *water R*.

Test solution. Dissolve 0.100 g of the substance to be examined in 1.0 ml of 1 M *sodium hydroxide* and dilute to 25.0 ml with the solvent mixture.

Reference solution. Dissolve 40.0 mg of *nitrilotriacetic acid R* in the solvent mixture and dilute to 100.0 ml with the solvent mixture. To 1.0 ml of the solution add 0.1 ml of the test solution and dilute to 100.0 ml with the solvent mixture.

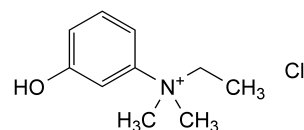
Column:

- *size:* $l = 0.10$ m, $\varnothing = 4.6$ mm,
- *stationary phase:* spherical *graphitised carbon for chromatography R1* (5 μ m) with a specific surface area of 120 m²/g and a pore size of 25 nm.

01/2005:2106

EDROPHONIUM CHLORIDE

Edrophonii chloridum

 $C_{10}H_{16}ClNO$ M_r 201.7

DEFINITION

N-Ethyl-3-hydroxy-*N,N*-dimethylanilinium chloride.*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

Appearance: white or almost white, crystalline powder.*Solubility*: very soluble in water, freely soluble in ethanol (96 per cent), practically insoluble in methylene chloride.

IDENTIFICATION

A. Infrared absorption spectrophotometry (2.2.24).

Comparison: edrophonium chloride CRS.

B. It gives reaction (a) of chlorides (2.3.1).

TESTS

Appearance of solution. The solution is clear (2.2.1) and colourless (2.2.2, *Method II*).Dissolve 0.5 g in *water R* and dilute to 25 ml with the same solvent.**pH** (2.2.3): 4.0 to 5.0.Dissolve 1.0 g in *carbon dioxide-free water R* and dilute to 10.0 ml with the same solvent.**Related substances.** Liquid chromatography (2.2.29).*Test solution.* Dissolve 50.0 mg in *water R* and dilute to 50.0 ml with the same solvent.*Reference solution (a).* Dissolve 10.0 mg of 3-dimethylaminophenol *R* in *acetonitrile R* and dilute to 10.0 ml with the same solvent.*Reference solution (b).* Mix 1.0 ml of the test solution and 1.0 ml of reference solution (a) and dilute to 100.0 ml with *water R*. Dilute 10.0 ml of this solution to 100.0 ml with *water R*.*Column*:

- *size*: $l = 0.25$ m, $\varnothing = 4.6$ mm,
- *stationary phase*: styrene-divinylbenzene copolymer *R* (8–10 μ m).

Mobile phase: mix 10 volumes of *acetonitrile R* and 90 volumes of a 7.7 g/l solution of tetramethylammonium bromide *R* previously adjusted to pH 3.0 with *phosphoric acid R*.*Flow rate*: 1 ml/min.*Detection*: spectrophotometer at 281 nm.*Injection*: 20 μ l.*Run time*: twice the retention time of edrophonium.*Relative retention* with reference to edrophonium (retention time = about 3.8 min): impurity A = about 1.3.*System suitability*: reference solution (b):

- *resolution*: minimum 2.0 between the peaks due to edrophonium and impurity A.

Mobile phase: dissolve 50.0 mg of ferric sulphate pentahydrate *R* in 50 ml of 0.5 *M* sulphuric acid and add 750 ml of *water R*. Adjust to pH 1.5 with 0.5 *M* sulphuric acid or 1 *M* sodium hydroxide, add 20 ml of ethylene glycol *R* and dilute to 1000 ml with *water R*.

Flow rate: 1 ml/min.*Detection*: spectrophotometer at 273 nm.*Injection*: 20 μ l; filter the solutions and inject immediately.*Run time*: 4 times the retention time of the iron complex of impurity A.*Retention time*: iron complex of impurity A = about 5 min; iron complex of edetic acid = about 10 min.*System suitability*: reference solution:

- *resolution*: minimum 7 between the peaks due to the iron complex of impurity A and the iron complex of edetic acid,
- *signal-to-noise ratio*: minimum 50 for the peak due to impurity A.

Limit:

- *impurity A*: not more than the area of the corresponding peak in the chromatogram obtained with the reference solution (0.1 per cent).

Chlorides (2.4.4): maximum 200 ppm.

To 10 ml of solution S add 8 ml of *nitric acid R* and stir for 10 min. A precipitate is formed. Filter and wash the filter with *water R*. Collect the filtrate and the washings and dilute to 20 ml with *water R*. Dilute 10 ml of this solution to 15 ml with *water R*.

Iron (2.4.9): maximum 80 ppm.

Dilute 2.5 ml of solution S to 10 ml with *water R* and add 0.25 g of *calcium chloride R* before adding the *thioglycollic acid R*. Allow to stand for 5 min. Also add 0.25 g of *calcium chloride R* to the standard.

Heavy metals (2.4.8): maximum 20 ppm.

1.0 g complies with limit test D. Use a fused-silica crucible. If necessary moisten the residue on ignition with *nitric acid R* and ignite until a white residue is obtained. Prepare the standard using 2 ml of *lead standard solution (10 ppm Pb) R*.

Sulphated ash (2.4.14): maximum 0.2 per cent, determined on 1.0 g.

ASSAY

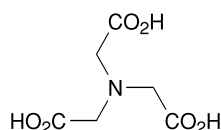
Dissolve 0.250 g in 2.0 ml of *dilute sodium hydroxide solution R* and dilute to 300 ml with *water R*. Add 2 g of *hexamethylenetetramine R* and 2 ml of *dilute hydrochloric acid R*. Titrate with 0.1 *M* *zinc sulphate* using about 50 mg of *xylene orange triturate R* as indicator.

1 ml of 0.1 *M* *zinc sulphate* corresponds to 29.22 mg of $C_{10}H_{16}N_2O_8$.

STORAGE

Protected from light.

IMPURITIES

Specified impurities: A.

A. nitritotriacetic acid.

Limits:

- **impurity A:** not more than the area of the corresponding peak in the chromatogram obtained with reference solution (b) (0.1 per cent),
- **any other impurity:** for each impurity, not more than the area of the peak due to edrophonium in the chromatogram obtained with reference solution (b) (0.1 per cent),
- **total:** not more than 5 times the area of the peak due to edrophonium in the chromatogram obtained with reference solution (b) (0.5 per cent),
- **disregard limit:** 0.5 times the area of the peak due to edrophonium in the chromatogram obtained with reference solution (b) (0.05 per cent).

Loss on drying (2.2.32): maximum 0.5 per cent, determined on 1.000 g by drying in a desiccator over *diphosphorus pentoxide R* at a pressure not exceeding 0.7 kPa for 24 h.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

Bacterial endotoxins (2.6.14): less than 8.3 IU/mg.

ASSAY

Dissolve 0.150 g in 60 ml of a mixture of equal volumes of *acetic anhydride R* and *anhydrous acetic acid R*. Titrate with 0.1 M *perchloric acid*, determining the end-point potentiometrically (2.2.20).

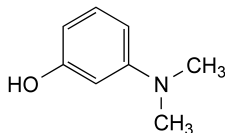
1 ml of 0.1 M *perchloric acid* is equivalent to 20.17 mg of $C_{10}H_{16}ClNO$.

STORAGE

Protected from light.

IMPURITIES

Specified impurities: A.



A. 3-(dimethylamino)phenol.

01/2005:1217

ELDER FLOWER**Sambuci flos****DEFINITION**

Elder flower consists of the dried flowers of *Sambucus nigra* L. It contains not less than 0.80 per cent of flavonoids, calculated as isoquercitroside ($C_{21}H_{20}O_{12}$; M_r 464.4) with reference to the dried drug.

CHARACTERS

It has the macroscopic and microscopic characters described under identification tests A and B.

IDENTIFICATION

- A. The flower, about 5 mm in diameter, has three small bracts (hand lens) and may have a peduncle. The five-toothed calyx is small; the corolla is light yellow, with five broadly oval petals fused at their bases into a tube. The filaments of the five yellow stamens alternate with the petals. The corolla is often isolated or attached to the stamens, to which it is fused at the base. The ovary is inferior with three locules and it bears a short style with three obtuse stigmata.

- B. Reduce to a powder (355). The powder is greenish-yellow. Examine under a microscope using *chloral hydrate solution R*. The powder shows numerous spherical, sometimes ellipsoidal, pollen grains about 30 µm in diameter, with three germinal pores and very finely pitted exine; calyx epidermal cells with a striated cuticle and occasional unicellular marginal teeth from the basal region; corolla fragments with numerous small globules of volatile oil, those of the upper epidermis with slightly thickened and beaded walls and a striated cuticle; mesophyll cells of petals and sepals with idioblasts containing numerous sandy crystals of calcium oxalate.
- C. Examine in ultraviolet light at 365 nm the chromatograms obtained in the test for *Sambucus ebulus*. The chromatogram obtained with the test solution shows an intense zone of light blue fluorescence due to chlorogenic acid, a zone of orange fluorescence due to rutin as well as a zone of orange fluorescence due to isoquercitrin, which appears slightly above the zone due to hyperoside in the chromatogram obtained with the reference solution. A zone of greenish-blue fluorescence appears in the chromatogram obtained with the test solution a little below the caffeic acid zone in the chromatogram obtained with the reference solution. Additional faint fluorescent zones may be present. In daylight, only the orange fluorescent zones due to rutin and isoquercitroside in the chromatogram obtained with the test solution are clearly visible.

TESTS

Foreign matter (2.8.2). Determined on 10 g, not more than 8 per cent of fragments of coarse pedicels and other foreign matter; not more than 15 per cent of discoloured, brown flowers.

Sambucus ebulus L. Examine by thin-layer chromatography (2.2.27), using a suitable silica gel as the coating substance.

Test solution. To 0.5 g of the powdered drug (355) add 10 ml of *methanol R* and heat in a water bath at 65 °C for 5 min, shaking frequently. Allow to cool and filter. Dilute the filtrate to 10 ml with *methanol R*.

Reference solution. Dissolve 1 mg of *caffeic acid R*, 1 mg of *chlorogenic acid R*, 2.5 mg of *hyperoside R* and 2.5 mg of *rutin R* in 10 ml of *methanol R*.

Apply separately to the plate, as bands, 10 µl of each solution. Develop over a path of 15 cm using a mixture of 10 volumes of *anhydrous formic acid R*, 10 volumes of *water R*, 30 volumes of *methyl ethyl ketone R* and 50 volumes of *ethyl acetate R*. Dry the plate at 100-105 °C and spray it while still warm with a 10 g/l solution of *diphenylboric acid aminoethyl ester R* in *methanol R*. Subsequently spray the plate with a 50 g/l solution of *macrogol 400 R* in *methanol R*. Allow the plate to dry in air for 30 min and examine in ultraviolet light at 365 nm. The chromatogram obtained with the reference solution shows in the lower half, with increasing R_f values, the zone of orange fluorescence due to rutin, the zone of light blue fluorescence due to chlorogenic acid and the zone of orange-yellow to orange-brown fluorescence due to hyperoside. The upper third presents a zone of greenish-blue fluorescence due to caffeic acid. The chromatogram obtained with the test solution does not show a pink zone below the zone due to rutin in the chromatogram obtained with the reference solution.

Loss on drying (2.2.32). Not more than 10.0 per cent, determined on 1.000 g of the powdered drug (355) by drying in an oven at 100-105 °C for 2 h.

Total ash (2.4.16). Not more than 10.0 per cent.

ASSAY

Stock solution. In a 100 ml round-bottomed flask, introduce 0.600 g of the powdered drug (355), add 1 ml of a 5 g/l solution of *hexamethylenetetramine R*, 20 ml of *acetone R* and 2 ml of *hydrochloric acid R1*. Boil the mixture under a reflux condenser for 30 min. Filter the liquid through a plug of absorbent cotton into a flask. Add the absorbent cotton to the residue in the round-bottomed flask and extract with two quantities, each of 20 ml, of *acetone R*, each time boiling under a reflux condenser for 10 min. Allow to cool, filter each extract through the plug of absorbent cotton into the flask. After cooling, filter the combined acetone extracts through a paper-filter into a volumetric flask, dilute to 100.0 ml with *acetone R* by rinsing the flask and the paper-filter. Introduce 20.0 ml of the solution into a separating funnel, add 20 ml of *water R* and shake the mixture with one quantity of 15 ml and then three quantities, each of 10 ml, of *ethyl acetate R*. Combine the ethyl acetate extracts in a separating funnel, wash with two quantities, each of 50 ml, of *water R*, and filter the extracts over 10 g of *anhydrous sodium sulphate R* into a volumetric flask and dilute to 50.0 ml with *ethyl acetate R*.

Test solution. To 10.0 ml of the stock solution add 1 ml of *aluminium chloride reagent R* and dilute to 25.0 ml with a 5 per cent V/V solution of *glacial acetic acid R* in *methanol R*.

Compensation solution. Dilute 10.0 ml of the stock solution to 25.0 ml with a 5 per cent V/V solution of *glacial acetic acid R* in *methanol R*.

Measure the absorbance (2.2.25) of the test solution after 30 min, by comparison with the compensation solution at 425 nm. Calculate the percentage content of flavonoids, calculated as isoquercitroside, from the expression:

$$\frac{A \times 1.25}{m}$$

i.e. taking the specific absorbance of isoquercitroside to be 500.

A = absorbance at 425 nm,

m = mass of the substance to be examined, in grams.

STORAGE

Store protected from light.

01/2005:1419
corrected

ELEUTHEROCOCCUS

Eleutherococci radix

DEFINITION

Dried, whole or cut underground organs of *Eleutherococcus senticosus* (Rupr. et Maxim.) Maxim.

Content: minimum 0.08 per cent for the sum of eleutheroside B (*M_r* 372.4) and eleutheroside E (*M_r* 742.7).

CHARACTERS

Macroscopic and microscopic characters described under identification tests A and B.

IDENTIFICATION

A. The rhizome is knotty, of irregular cylindrical shape, 1.5 cm to 4.0 cm in diameter; the surface is rugged, longitudinally wrinkled and greyish-brown to blackish-brown; the bark, about 2 mm thick, closely adheres to the xylem; the heartwood is light brown and the sapwood is pale yellow; the fracture shows short thin

fibres in the bark and is coarsely fibrous, especially in the internal part of the xylem. The lower surface bears numerous cylindrical and knotty roots, 3.5 cm to 15 cm long and 0.3 cm to 1.5 cm in diameter; with a smooth, greyish-brown to blackish-brown surface; the bark is about 0.5 mm thick, closely adhering to the pale yellow xylem; the fracture is slightly fibrous; in places where the outer layer has been removed, the outer surface is yellowish-brown.

- B. Reduce to a powder (355). The powder is yellowish-brown. Examine under a microscope, using *chloral hydrate solution R*. The powder shows numerous groups of thick-walled, lignified fibres; fragments of reticulate and bordered pitted vessels with a wide lumen; groups of secretory canals, up to 20 µm in diameter with brown contents; parenchymatous cells containing cluster crystals of calcium oxalate 10 µm to 50 µm in diameter. Examine under a microscope, using a 50 per cent V/V solution of *glycerol R*. The powder shows small starch granules, rounded to slightly angular in outline, single compounds or with 2 or 3 components.
- C. Thin-layer chromatography (2.2.27).

Test solution. To 1.0 g of the powdered drug (355) add 10 ml of *alcohol (50 per cent V/V) R* and boil under reflux for 1 h. Cool and filter. Evaporate the filtrate to dryness on a water-bath. Dissolve the residue in 2.5 ml of a mixture of 5 volumes of *water R* and 20 volumes of *alcohol (50 per cent V/V) R* and filter.

Reference solution. Dissolve 2.0 mg of *esculin R* and 2.0 mg of *catalpol R* in 20 ml of a mixture of 2 volumes of *water R* and 8 volumes of *alcohol (50 per cent V/V) R*.

Plate: TLC silica gel plate *R*.

Mobile phase: *water R*, *methanol R*, *methylene chloride R* (4:30:70 V/V/V).

Application: 20 µl, as bands.

Development: over a path of 10 cm.

Drying: in air.

Detection A: examine in ultraviolet light at 365 nm.

Results A: the chromatogram obtained with the reference solution shows in the upper half a blue fluorescent zone (esculin).

Detection B: spray with *anisaldehyde solution R* and examine in daylight while heating at 100-105 °C for 5-10 min.

Results B: see below the sequence of the zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other faint zones are present in the chromatogram obtained with the test solution.

Top of the plate	
Esculin: a blue fluorescent zone (marked at 365 nm) Catalpol: a violet-brown zone	A brown zone (eleutheroside B)
	A reddish-brown zone (eleutheroside E)
	2 brown zones
Reference solution	Test solution

TESTS

Foreign matter (2.8.2): maximum 3 per cent.

Loss on drying (2.2.32): maximum 10.0 per cent, determined on 1.000 g of the powdered drug (355) by drying in an oven at 100-105 °C for 2 h.

Total ash (2.4.16): maximum 8.0 per cent.

ASSAY

Liquid chromatography (2.2.29).

Reference solution (a). Dissolve 10 mg of *ferulic acid R* in a mixture of equal volumes of *methanol R* and *water R* and dilute to 20.0 ml with the same mixture of solvents.

Reference solution (b). Dissolve 10 mg of *caffeic acid R* in a mixture of equal volumes of *methanol R* and *water R* and dilute to 20.0 ml with the same mixture of solvents.

Reference solution (c). Transfer 1 ml of reference solution (a) to a 25 ml volumetric flask and dilute to 25.0 ml with a mixture of equal volumes of *methanol R* and *water R*. Filter through a nylon filter (pore size 0.45 µm).

Reference solution (d). Transfer 1 ml of reference solution (a) and 1 ml of reference solution (b) in a mixture of equal volumes of *methanol R* and *water R* and dilute to 25.0 ml with the same mixture of solvents. Filter through a nylon filter (pore size 0.45 µm).

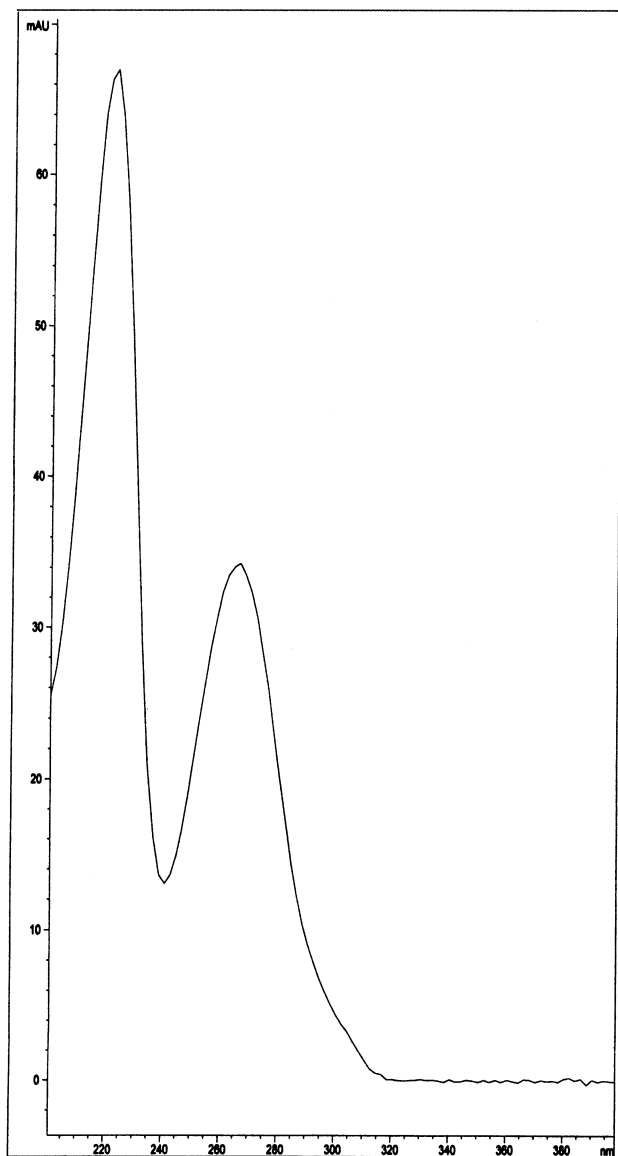


Figure 1419.-1. – UV spectrum of eleutheroside B for the assay of eleutherococcus

Test solution. To 0.500 g of the powdered drug (355) in a 100 ml round-bottomed flask, add 30 ml of a mixture of equal volumes of *alcohol R* and *water R*. Heat in a water-bath at 60 °C for 30 min. Allow to cool and filter through a sintered-glass filter. Collect the liquid in a 250 ml round-bottomed flask. Repeat this operation twice, using the residue obtained in the filtration step instead of the powdered drug. Add both fractions of supernatant liquid to the 250 ml round-bottomed flask. Evaporate under reduced pressure until about 10 ml of supernatant liquid is left in the flask. Transfer the supernatant liquid quantitatively to a 20.0 ml volumetric flask and dilute to 20.0 ml with a mixture of equal volumes of *alcohol R* and *water R*. Filter through a nylon filter (pore size 0.45 µm).

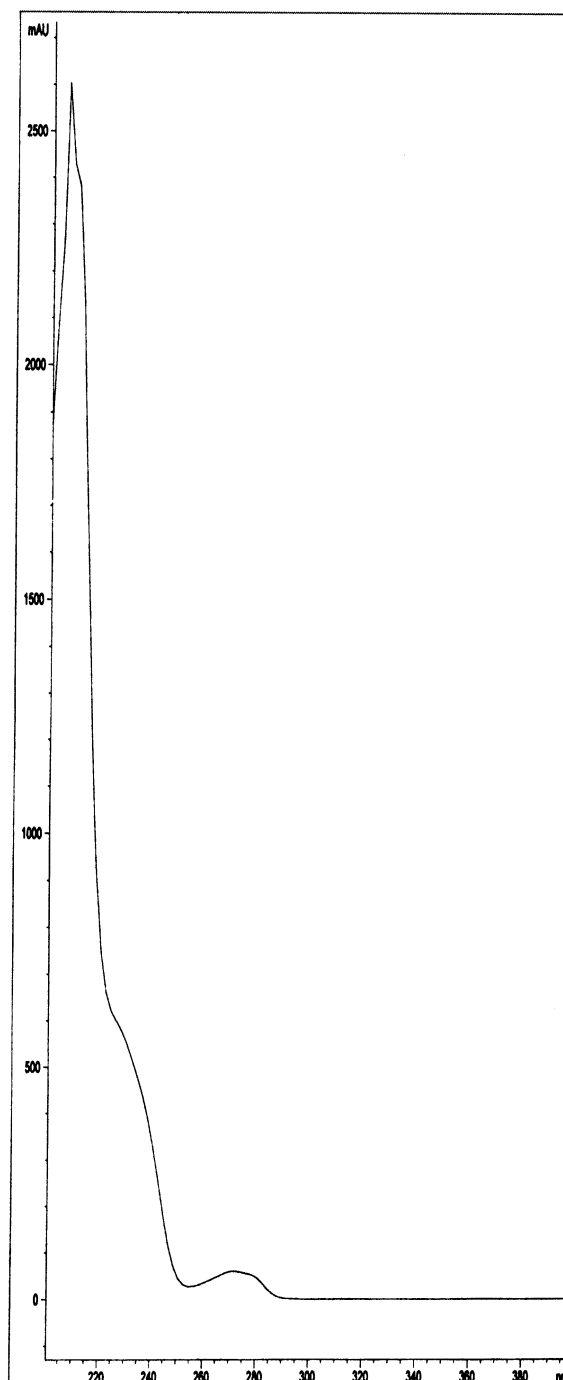


Figure 1419.-2. – UV spectrum of eleutheroside E for the assay of eleutherococcus

Precolumn:

- size: $l = 0.04$ m, $\varnothing = 4.6$ mm,
- stationary phase: octadecylsilyl silica gel for chromatography R (5 μ m).

Column:

- size: $l = 0.25$ m, $\varnothing = 4.6$ mm,
- stationary phase: octadecylsilyl silica gel for chromatography R (5 μ m).

Mobile phase:

- mobile phase A: phosphoric acid R, water R (0.5:99.5 V/V),
- mobile phase B: acetonitrile for chromatography R,

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)
0 - 5	90	10
5 - 27	90 $\rightarrow$ 80	10 $\rightarrow$ 20
27 - 30	80 $\rightarrow$ 50	20 $\rightarrow$ 50
30 - 35	50	50
35 - 40	50 $\rightarrow$ 90	50 $\rightarrow$ 10

Flow rate: 1.0 ml/min.

Detection: spectrophotometer at 220 nm.

Injection: 20 μ l of the test solution and reference solutions (c) and (d).

Retention time: eleutheroside B = about 10 min; eleutheroside E = about 22 min.

Locate the peaks due to eleutheroside B and eleutheroside E using the UV spectra shown in Figures 1419-1 and 1419-2.

System suitability: reference solution (d):

- resolution: minimum 15 between the peaks due to caffeic acid and ferulic acid.

Calculate the total percentage content of eleutheroside B and eleutheroside E from the expression:

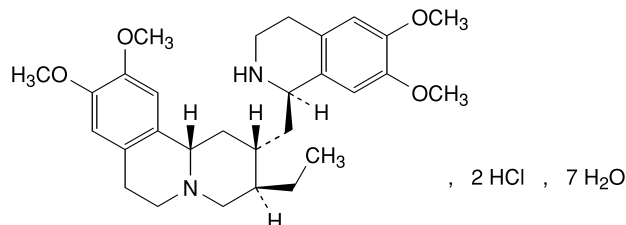
$$\frac{(A_B \times C \times 0.73 \times 2)}{(A_R \times m)} + \frac{(A_E \times C \times 1.90 \times 2)}{(A_R \times m)}$$

- A_B = area of the peak due to eleutheroside B in the chromatogram obtained with the test solution,
- A_E = area of the peak due to eleutheroside E in the chromatogram obtained with the test solution,
- A_R = area of the peak due to ferulic acid in the chromatogram obtained with reference solution (c),
- C = concentration of ferulic acid in reference solution (c), in micrograms per millilitre,
- m = mass of the drug to be examined, in milligrams.

01/2005:0080

EMETINE HYDROCHLORIDE HEPTAHYDRATE

Emetini hydrochloridum heptahydricum



$C_{29}H_{42}Cl_2N_2O_4 \cdot 7H_2O$

M_r 680

DEFINITION

Emetine hydrochloride heptahydrate contains not less than 98.0 per cent and not more than the equivalent of 102.0 per cent of (2S,3R,11bS)-2-[[[(1R)-6,7-dimethoxy-1,2,3,4-tetrahydroisoquinolin-1-yl]methyl]-3-ethyl-9,10-dimethoxy-1,3,4,6,7,11b-hexahydro-2H-benzo[a]quinolizine dihydrochloride, calculated with reference to the dried substance.

CHARACTERS

A white or slightly yellowish, crystalline powder, freely soluble in water and in alcohol.

IDENTIFICATION

First identification: A, E.

Second identification: B, C, D, E.

- Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with emetine hydrochloride CRS.
- Examine the chromatograms obtained in the test for related substances in ultraviolet light at 365 nm. The principal spot in the chromatogram obtained with the test solution is similar in position, fluorescence and size to the spot in the chromatogram obtained with reference solution (a).
- Dissolve about 10 mg in 2 ml of dilute hydrogen peroxide solution R, add 1 ml of hydrochloric acid R and heat. An orange colour develops.
- Sprinkle about 5 mg on the surface of 1 ml of sulphomolybdic reagent R2. A bright-green colour develops.
- It gives reaction (a) of chlorides (2.3.1).

TESTS

Solution S. Dissolve 1.25 g in carbon dioxide-free water R and dilute to 25 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and not more intensely coloured than reference solution Y₅ or BY₅ (2.2.2, Method II).

pH (2.2.3). Dilute 4 ml of solution S to 10 ml with carbon dioxide-free water R. The pH of the solution is 4.0 to 6.0.

Specific optical rotation (2.2.7). Dissolve in water R a quantity of the substance to be examined corresponding to 1.250 g of dried substance and dilute to 25.0 ml with the same solvent. The specific optical rotation is + 16 to + 19, calculated with reference to the dried substance.

01/2005:0081

Related substances. Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel G plate R*. Prepare the solutions immediately before use.

Test solution. Dissolve 50 mg of the substance to be examined in *methanol R* containing 1 per cent V/V of *dilute ammonia R2* and dilute to 100 ml with the same solvent.

Reference solution (a). Dissolve 50 mg of *emetine hydrochloride CRS* in *methanol R* containing 1 per cent V/V of *dilute ammonia R2* and dilute to 100 ml with the same solvent.

Reference solution (b). Dissolve 10 mg of *isoemetine hydrobromide CRS* in *methanol R* containing 1 per cent V/V of *dilute ammonia R2* and dilute to 100 ml with the same solvent. Dilute 5 ml of this solution to 50 ml with *methanol R* containing 1 per cent V/V of *dilute ammonia R2*.

Reference solution (c). Dissolve 10 mg of *cephaeline hydrochloride CRS* in *methanol R* containing 1 per cent V/V of *dilute ammonia R2* and dilute to 100 ml with the same solvent. Dilute 5 ml of this solution to 50 ml with *methanol R* containing 1 per cent V/V of *dilute ammonia R2*.

Reference solution (d). Dilute 1 ml of reference solution (a) to 100 ml with *methanol R* containing 1 per cent V/V of *dilute ammonia R2*.

Reference solution (e). To 1 ml of reference solution (a) add 1 ml of reference solution (b) and 1 ml of reference solution (c).

Apply to the plate 10 µl of the test solution and each of reference solutions (a), (b), (c) and (d) and 30 µl of reference solution (e). Develop over a path of 15 cm using a mixture of 0.5 volumes of *diethylamine R*, 2 volumes of *water R*, 5 volumes of *methanol R*, 20 volumes of *ethylene glycol monomethyl ether R* and 100 volumes of *chloroform R*. Allow the plate to dry in air until the solvent has evaporated. Spray in a well ventilated fume-cupboard with *chloroformic solution of iodine R* and heat at 60 °C for 15 min. Examine in ultraviolet light at 365 nm. In the chromatogram obtained with the test solution, any spots corresponding to isoemetine and cephaeline are not more intense than the spots in the chromatograms obtained with reference solutions (b) and (c) respectively (2.0 per cent); any spot, apart from the principal spot and the spots corresponding to isoemetine and cephaeline, is not more intense than the spot in the chromatogram obtained with reference solution (d) (1.0 per cent). The test is not valid unless the chromatogram obtained with reference solution (e) shows three clearly separated spots.

Loss on drying (2.2.32). 15.0 per cent to 19.0 per cent, determined on 1.00 g by drying in an oven at 100 °C to 105 °C for 3 h.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.200 g in a mixture of 5.0 ml of 0.01 M *hydrochloric acid* and 50 ml of *alcohol R*. Carry out a potentiometric titration (2.2.20), using 0.1 M *sodium hydroxide*. Read the volume added between the 2 points of inflexion.

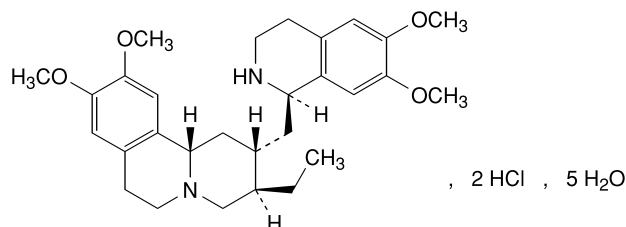
1 ml of 0.1 M *sodium hydroxide* is equivalent to 27.68 mg of $C_{29}H_{42}Cl_2N_2O_4 \cdot 5H_2O$.

STORAGE

Store protected from light.

EMETINE HYDROCHLORIDE PENTAHYDRATE

Emetini hydrochloridum pentahydricum



$C_{29}H_{42}Cl_2N_2O_4 \cdot 5H_2O$

M_r 644

DEFINITION

Emetine hydrochloride pentahydrate contains not less than 98.0 per cent and not more than the equivalent of 102.0 per cent of (2*S*,3*R*,11*bS*)-2-[[*(1R*)-6,7-dimethoxy-1,2,3,4-tetrahydroisoquinolin-1-yl]methyl]-3-ethyl-9,10-dimethoxy-1,3,4,6,7,11*b*-hexahydro-2*H*-benzo[*a*]quinolizine dihydrochloride, calculated with reference to the dried substance.

CHARACTERS

A white or slightly yellowish, crystalline powder, freely soluble in water and in alcohol.

IDENTIFICATION

First identification: A, E.

Second identification: B, C, D, E.

- Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *emetine hydrochloride CRS*.
- Examine the chromatograms obtained in the test for related substances in ultraviolet light at 365 nm. The principal spot in the chromatogram obtained with the test solution is similar in position, fluorescence and size to the spot in the chromatogram obtained with reference solution (a).
- Dissolve about 10 mg in 2 ml of *dilute hydrogen peroxide solution R*, add 1 ml of *hydrochloric acid R* and heat. An orange colour develops.
- Sprinkle about 5 mg on the surface of 1 ml of *sulphomolybdic reagent R2*. A bright-green colour develops.
- It gives reaction (a) of chlorides (2.3.1).

TESTS

Solution S. Dissolve 1.25 g in *carbon dioxide-free water R* and dilute to 25 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and not more intensely coloured than reference solution Y_5 or BY_5 (2.2.2, *Method II*).

pH (2.2.3). Dilute 4 ml of solution S to 10 ml with *carbon dioxide-free water R*. The pH of the solution is 4.0 to 6.0.

Specific optical rotation (2.2.7). Dissolve in *water R* a quantity of the substance to be examined corresponding to 1.250 g of dried substance and dilute to 25.0 ml with the same solvent. The specific optical rotation is + 16 to + 19, calculated with reference to the dried substance.

01/2005:1420

Related substances. Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel G plate R*. Prepare the solutions immediately before use.

Test solution. Dissolve 50 mg of the substance to be examined in *methanol R* containing 1 per cent V/V of *dilute ammonia R2* and dilute to 100 ml with the same solvent.

Reference solution (a). Dissolve 50 mg of *emetine hydrochloride CRS* in *methanol R* containing 1 per cent V/V of *dilute ammonia R2* and dilute to 100 ml with the same solvent.

Reference solution (b). Dissolve 10 mg of *isoemetine hydrobromide CRS* in *methanol R* containing 1 per cent V/V of *dilute ammonia R2* and dilute to 100 ml with the same solvent. Dilute 5 ml of this solution to 50 ml with *methanol R* containing 1 per cent V/V of *dilute ammonia R2*.

Reference solution (c). Dissolve 10 mg of *cephaeline hydrochloride CRS* in *methanol R* containing 1 per cent V/V of *dilute ammonia R2* and dilute to 100 ml with the same solvent. Dilute 5 ml of this solution to 50 ml with *methanol R* containing 1 per cent V/V of *dilute ammonia R2*.

Reference solution (d). Dilute 1 ml of reference solution (a) to 100 ml with *methanol R* containing 1 per cent V/V of *dilute ammonia R2*.

Reference solution (e). To 1 ml of reference solution (a) add 1 ml of reference solution (b) and 1 ml of reference solution (c).

Apply to the plate 10 µl of the test solution and each of reference solutions (a), (b), (c) and (d) and 30 µl of reference solution (e). Develop over a path of 15 cm using a mixture of 0.5 volumes of *diethylamine R*, 2 volumes of *water R*, 5 volumes of *methanol R*, 20 volumes of *ethylene glycol monomethyl ether R* and 100 volumes of *chloroform R*. Allow the plate to dry in air until the solvent has evaporated. Spray in a well ventilated fume-cupboard with *chloroformic solution of iodine R* and heat at 60 °C for 15 min. Examine in ultraviolet light at 365 nm. In the chromatogram obtained with the test solution, any spots corresponding to isoemetine and cephaeline are not more intense than the spots in the chromatograms obtained with reference solutions (b) and (c) respectively (2.0 per cent); any spot, apart from the principal spot and the spots corresponding to isoemetine and cephaeline, is not more intense than the spot in the chromatogram obtained with reference solution (d) (1.0 per cent). The test is not valid unless the chromatogram obtained with reference solution (e) shows three clearly separated spots.

Loss on drying (2.2.32). 11.0 per cent to 15.0 per cent, determined on 1.00 g by drying in an oven at 100 °C to 105 °C for 3 h.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.200 g in a mixture of 5.0 ml of 0.01 M *hydrochloric acid* and 50 ml of *alcohol R*. Carry out a potentiometric titration (2.2.20), using 0.1 M *sodium hydroxide*. Read the volume added between the two points of inflexion.

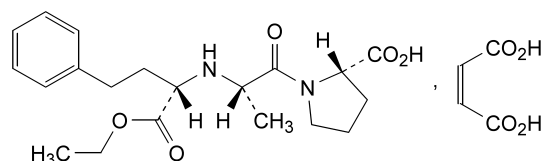
1 ml of 0.1 M *sodium hydroxide* is equivalent to 27.68 mg of $C_{29}H_{42}Cl_2N_2O_4$.

STORAGE

Store protected from light.

ENALAPRIL MALEATE

Enalaprili maleas


 $C_{24}H_{32}N_2O_9$
 M_r 492.5

DEFINITION

Enalapril maleate contains not less than 98.5 per cent and not more than the equivalent of 101.5 per cent of (2S)-1-[(2S)-2-[(1S)-1-(ethoxycarbonyl)-3-phenylpropyl]amino]propanoyl]pyrrolidine-2-carboxylic acid (Z)-butenedioate, calculated with reference to the dried substance.

CHARACTERS

A white or almost white crystalline powder, sparingly soluble in water, freely soluble in methanol, practically insoluble in methylene chloride. It dissolves in dilute solutions of alkali hydroxides.

IDENTIFICATION

First identification: B.

Second identification: A, C, D.

- Melting point (2.2.14): 143 °C to 145 °C.
- Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *enalapril maleate CRS*.
- Dissolve about 30 mg in 3 ml of *water R*. Add 1 ml of *bromine water R* and heat on a water bath until bromine has disappeared completely and cool. To 0.2 ml of this solution, add 3 ml of a 3 g/l solution of *resorcinol R* in *sulphuric acid R* and heat on a water bath for 15 min. A reddish-brown colour develops.
- To about 30 mg, add 0.5 ml of a 100 g/l solution of *hydroxylamine hydrochloride R* in *methanol R* and 1.0 ml of a 100 g/l solution of *potassium hydroxide R* in *alcohol R*. Heat to boiling, allow to cool and acidify with *dilute hydrochloric acid R*. Add 0.2 ml of *ferric chloride solution R1* diluted 1 to 10; a reddish-brown colour appears.

TESTS

Solution S. Dissolve 0.25 g in *carbon dioxide-free water R* and dilute to 25.0 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and colourless (2.2.2, *Method II*).

pH (2.2.3). The pH of solution S is 2.4 to 2.9.

Specific optical rotation (2.2.7): −48 to −51, determined on solution S and calculated with reference to the dried substance.

Related substances. Examine by liquid chromatography (2.2.29).

Buffer solution A. Dissolve 2.8 g of *sodium dihydrogen phosphate monohydrate R* in 950 ml of *water R*. Adjust to pH 2.5 with *phosphoric acid R* and dilute to 1000 ml with *water R*.

Buffer solution B. Dissolve 2.8 g of *sodium dihydrogen phosphate monohydrate R* in 950 ml of *water R*. Adjust to pH 6.8 with *strong sodium hydroxide solution R* and dilute to 1000 ml with *water R*.

Dissolution mixture. Mix 50 ml of *acetonitrile R1* and 950 ml of buffer solution A.

Test solution. Dissolve 30.0 mg of the substance to be examined in the dissolution mixture and dilute to 100.0 ml with the same mixture.

Reference solution (a). Dilute 1.0 ml of the test solution to 100.0 ml with the dissolution mixture.

Reference solution (b). Dissolve 3.0 mg of *enalapril for system suitability CRS* in the dissolution mixture and dilute to 10.0 ml with the same mixture.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.15 m long and 4.1 mm in internal diameter packed with *styrene-divinylbenzene copolymer R* (5 µm),

- as mobile phase at a flow rate of 1.4 ml/min,

Mobile phase A. Mix 50 ml of *acetonitrile R1* and 950 ml of buffer solution B,

Mobile phase B. Mix 340 ml of buffer solution B and 660 ml of *acetonitrile R1*,

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)
0 - 20	95 → 40	5 → 60
20 - 25	40	60
25 - 26	40 → 95	60 → 5
26 - 30	95	5

- as detector a spectrophotometer set at 215 nm, maintaining the temperature of the column at 70 °C.

Inject 50 µl of reference solution (b). When the chromatogram is recorded in the prescribed conditions, the retention times are: enalapril about 11 min and impurity A about 12 min. The test is not valid unless the resolution between the peaks corresponding to enalapril and impurity A is at least 1.5. Inject 50 µl of the test solution and 50 µl of reference solution (a). In the chromatogram obtained with the test solution: the area of any peak corresponding to impurity A is not greater than that of the principal peak in the chromatogram obtained with reference solution (a) (1.0 per cent); the area of any peak apart from the principal peak and any peak corresponding to impurity A, is not greater than 0.3 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.3 per cent) and the sum of the areas of any such peaks is not greater than the area of the principal peak in the chromatogram obtained with reference solution (a) (1.0 per cent). Disregard any peak with an area less than 0.05 times that of the principal peak in the chromatogram obtained with reference solution (a).

Heavy metals (2.4.8). 2.0 g complies with limit test C (10 ppm). Use 2 ml of *lead standard solution (10 ppm Pb) R*.

Loss on drying (2.2.32). Not more than 1.0 per cent determined on 1.000 g by heating in an oven at 100-105 °C for 3 h.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.100 g in *carbon dioxide-free water R* and dilute to 30 ml with the same solvent. Titrate with 0.1 M *sodium hydroxide* determining the end point potentiometrically (2.2.20). Titrate to the second point of inflexion.

1 ml of 0.1 M *sodium hydroxide* is equivalent to 16.42 mg of C₂₄H₃₂N₂O₉.

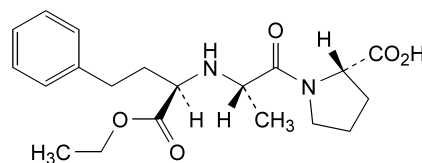
STORAGE

Store protected from light.

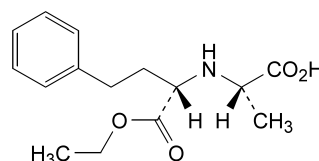
IMPURITIES

Specified impurities: A, B, C, D, E, H.

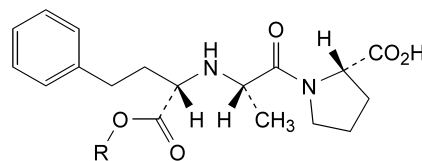
Other detectable impurities: F, G, I.



A. (2S)-1-[(2S)-2-[(1R)-1-(ethoxycarbonyl)-3-phenylpropyl]amino]propanoyl]pyrrolidine-2-carboxylic acid,



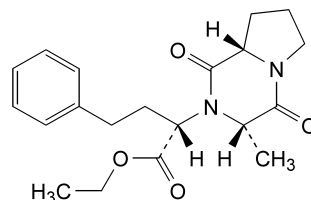
B. (2S)-2-[(1S)-1-(ethoxycarbonyl)-3-phenylpropyl]amino]propanoic acid,



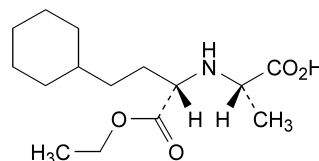
C. R = H: (2S)-1-[(2S)-2-[(1S)-1-carboxy-3-phenylpropyl]amino]propanoyl]pyrrolidine-2-carboxylic acid,

E. R = CH₂-CH₂-C₆H₅: (2S)-1-[(2S)-2-[(1S)-3-phenyl-1-[(2-phenylethoxy)carbonyl]propyl]amino]propanoyl]pyrrolidine-2-carboxylic acid,

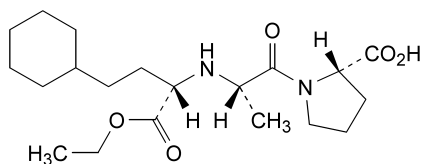
F. R = C₄H₉: (2S)-1-[(2S)-2-[(1S)-1-(butoxycarbonyl)-3-phenylpropyl]amino]propanoyl]pyrrolidine-2-carboxylic acid,



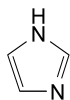
D. ethyl (2S)-2-[(3S,8aS)-3-methyl-1,4-dioxooctahydropyrrolo[1,2-a]pyrazin-2-yl]-4-phenylbutanoate,



G. (2S)-2-[(1S)-3-cyclohexyl-1-(ethoxycarbonyl)propyl]amino]propanoic acid,



H. (2*S*)-1-[(2*S*)-2-[[[(1*S*)-3-cyclohexyl-1-(ethoxycarbonyl)propyl]amino]propanoyl]pyrrolidine-2-carboxylic acid,

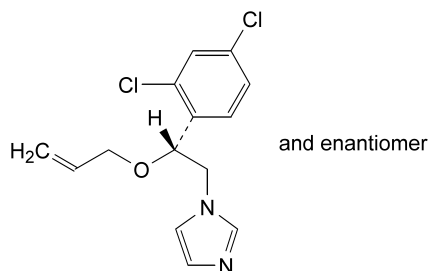


I. 1*H*-imidazole.

01/2005:1720

ENILCONAZOLE FOR VETERINARY USE

Enilconazolum ad usum veterinarium



$C_{14}H_{14}Cl_2N_2O$

M_r 297.2

DEFINITION

1-[(2*RS*)-2-(2,4-Dichlorophenyl)-2-(prop-2-enyloxy)ethyl]-1*H*-imidazole.

Content: 98.5 per cent to 101.5 per cent (dried substance).

CHARACTERS

Appearance: clear, yellowish, oily liquid or solid mass.

Solubility: very slightly soluble in water, freely soluble in alcohol, in methanol and in toluene.

IDENTIFICATION

Infrared absorption spectrophotometry (2.2.24).

Comparison: enilconazole CRS.

TESTS

Optical rotation (2.2.7): -0.10° to $+0.10^\circ$.

Dissolve 0.1 g in *methanol R* and dilute to 10 ml with the same solvent.

Related substances. Gas chromatography (2.2.28). Prepare the solutions immediately before use and protect from light.

Test solution. Dissolve 0.100 g of the substance to be examined in *toluene R* and dilute to 100.0 ml with the same solvent.

Reference solution (a). Dissolve 10.0 mg of enilconazole CRS and 10.0 mg of enilconazole impurity E CRS in *toluene R* and dilute to 100.0 ml with the same solvent.

Reference solution (b). Dilute 5.0 ml of the test solution to 100.0 ml with *toluene R*. Dilute 1.0 ml of this solution to 10.0 ml with *toluene R*.

Column:

- *material*: fused silica,
- *size*: $l = 25$ m, $\varnothing = 0.32$ mm,
- *stationary phase*: chemically bonded poly(dimethyl)(diphenyl)siloxane R (film thickness 0.52 μ m).

Carrier gas: helium for chromatography R.

Flow rate: 1.3 ml/min.

Split ratio: 1:38.

Temperature:

	Time (min)	Temperature ($^\circ$ C)
Column	0 - 6.4 6.4 - 14	100 $\rightarrow$ 260 260
Injection port	-	250
Detector	-	300

Detection: flame ionisation.

Injection: 2 μ l.

Relative retentions with reference to enilconazole (retention time = about 10 min): impurity A = about 0.6; impurity B = about 0.7; impurity C = about 0.8; impurity D = about 0.9; impurity F = about 1.1.

System suitability: reference solution (a):

- *resolution*: minimum 2.5 between the peaks due to enilconazole and impurity E.

Limits:

- *any impurity*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (1.0 per cent), and not more than one such peak has an area greater than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent),
- *total*: not more than 4 times the area of the principal peak in the chromatogram obtained with reference solution (b) (2.0 per cent),
- *disregard limit*: 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

Loss on drying (2.2.32): maximum 0.5 per cent, determined on 1.000 g by drying *in vacuo* at 40 $^\circ$ C for 4 h.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

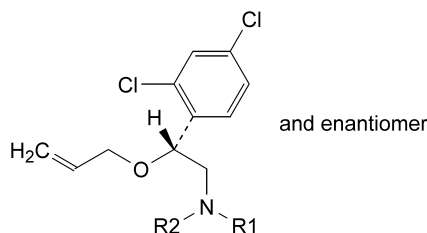
Dissolve 0.230 g in 50 ml of a mixture of 1 volume of *anhydrous acetic acid R* and 7 volumes of *methyl ethyl ketone R*. Titrate with 0.1 *M perchloric acid* using 0.2 ml of *naphtholbenzein solution R* as indicator.

1 ml of 0.1 *M perchloric acid* is equivalent to 29.72 mg of $C_{14}H_{14}Cl_2N_2O$.

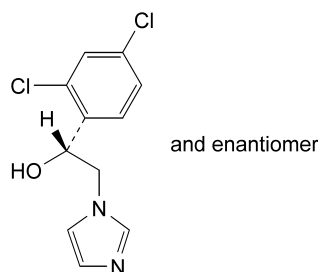
STORAGE

In an airtight container, protected from light.

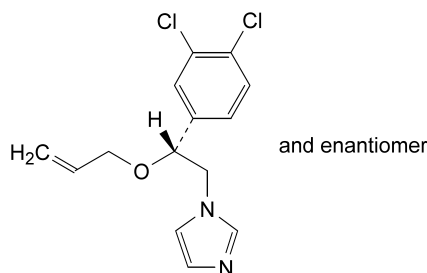
IMPURITIES



- A. $R_1 = R_2 = H$: (2*RS*)-2-(2,4-dichlorophenyl)-2-(prop-2-en-1-yloxy)ethanamine,
 B. $R_1 = H$, $R_2 = CH_2CH=CH_2$: *N*-(2*RS*)-2-(2,4-dichlorophenyl)-2-(prop-2-en-1-yloxy)ethylprop-2-en-1-amine,
 C. $R_1 = CHO$, $R_2 = H$: *N*-(2*RS*)-2-(2,4-dichlorophenyl)-2-(prop-2-en-1-yloxy)ethylformamide,
 D. $R_1 = CHO$, $R_2 = CH_2CH=CH_2$: *N*-(2*RS*)-2-(2,4-dichlorophenyl)-2-(prop-2-en-1-yloxy)ethyl-*N*-(prop-2-en-1-yl)formamide,



- E. (1*RS*)-1-(2,4-dichlorophenyl)-2-(1*H*-imidazol-1-yl)ethanol,

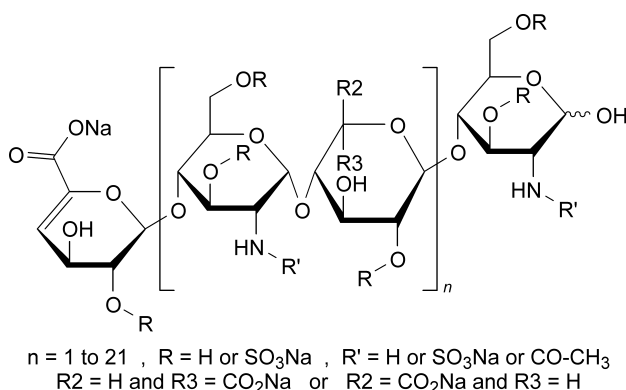


- F. 1-[(2*RS*)-2-(3,4-dichlorophenyl)-2-(prop-2-en-1-yloxy)ethyl]-1*H*-imidazole.

01/2005:1097

ENOXAPARIN SODIUM

Enoxaparinum natricum



DEFINITION

Enoxaparin sodium is the sodium salt of a low-molecular-mass heparin that is obtained by alkaline depolymerisation of the benzyl ester derivative of heparin from porcine intestinal mucosa. The majority of the components have a 4-enopyranose uronate structure at the non-reducing end of their chain.

Enoxaparin sodium complies with the monograph on Heparins, low-molecular-mass (0828) with the modifications and additional requirements below.

The mass-average molecular mass ranges between 3500 and 5500 with a characteristic value of about 4500.

The degree of sulphatation is about 2 per disaccharide unit.

The potency is not less than 90 IU and not more than 125 IU of anti-factor Xa activity per milligram, calculated with reference to the dried substance. The ratio of anti-factor Xa activity to anti-factor IIa activity is between 3.3 and 5.3.

IDENTIFICATION

Carry out identification test C as described in the monograph on *Heparins, low-molecular-mass (0828)*. The following requirements apply.

The mass-average molecular mass ranges between 3500 and 5500. The mass percentage of chains lower than 2000 ranges between 12.0 per cent and 20.0 per cent. The mass percentage of chains between 2000 and 8000 ranges between 68.0 per cent and 88.0 per cent.

TESTS

Appearance of solution. Dissolve 1.0 g in 10 ml of *water R*. The solution is clear (2.2.1) and not more intensely coloured than intensity 5 of the range of reference solutions of the most appropriate colour (2.2.2, *Method II*).

Absorbance (2.2.25). Dissolve 50.0 mg in 100 ml of 0.01 *M hydrochloric acid*. The specific absorbance at 231 nm is 14.0 to 20.0, calculated with reference to the dried substance.

Benzyl alcohol. Not more than 0.1 per cent *m/m*, determined by liquid chromatography (2.2.29).

Internal standard solution. Prepare a 1 g/l solution of 3,4-dimethylphenol *R* in *methanol R*.

Test solution. Dissolve about 0.500 g of the substance to be examined in 5.0 ml of 1 *M sodium hydroxide*. Allow to stand for 1 h. Add 1.0 ml of *glacial acetic acid R* and 1.0 ml of the internal standard solution and dilute to 10.0 ml with *water R*.

Reference solution. Prepare a 0.25 g/l solution of *benzyl alcohol R* in *water R*. Mix 0.50 ml of this solution with 1.0 ml of the internal standard solution and dilute to 10.0 ml with *water R*.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.15 m long and 4.6 mm in internal diameter packed with *octylsilyl silica gel for chromatography R* (5 μ m) equipped with a guard column 20 mm long and 4.6 mm in internal diameter, packed with the same material,
- as mobile phase at a flow rate of 1 ml/min a mixture of 5 volumes of *methanol R*, 15 volumes of *acetonitrile R* and 80 volumes of *water R*,
- as detector a spectrophotometer set at 256 nm.

From the chromatogram obtained with the reference solution, calculate the ratio (R_1) of the height of the peak due to benzyl alcohol to the height of the peak due to the internal standard. From the chromatogram obtained with the test solution, calculate the ratio (R_2) of the height of the peak due to benzyl alcohol to the height of the peak due to the internal standard.

Calculate the content (*m/m*) of benzyl alcohol from the following expression:

$$\frac{0.0125 \times R_2}{m \times R_1}$$

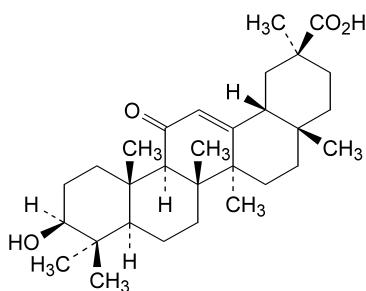
m = mass of the substance to be examined in grams.

Sodium. 11.3 to 13.5 per cent of Na calculated with reference to the dried substance and determined by atomic absorption spectrometry (2.2.23, *Method I*).

01/2005:1511

ENOXOLONE

Enoxolonum

C₃₀H₄₆O₄*M_r* 470.7

DEFINITION

(20β)-3β-Hydroxy-11-oxo-olean-12-en-29-oic acid.

Content: 98.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

Appearance: white or almost white crystalline powder.

Solubility: practically insoluble in water, soluble in ethanol, sparingly soluble in methylene chloride.

It shows polymorphism.

IDENTIFICATION

First identification: A.

Second identification: B, C.

A. Examine by infrared absorption spectrophotometry (2.2.24).

Comparison: enoxolone CRS.

If the spectra obtained in the solid state show differences, dissolve 0.2 g of the substance to be examined and 0.2 g of the reference substance separately in 6 ml of *ethanol R*. Boil under a reflux condenser for 1 h and add 6 ml of *water R*. A precipitate is formed. Cool to about 10 °C and filter with the aid of vacuum. Wash the precipitate with 10 ml of *alcohol R*, dry in an oven at 80 °C and record new spectra.

B. Thin-layer chromatography (2.2.27).

Test solution. Dissolve 10 mg of the substance to be examined in *methylene chloride R* and dilute to 10 ml with the same solvent.

Reference solution. Dissolve 10 mg of *enoxolone CRS* in *methylene chloride R* and dilute to 10 ml with the same solvent.

Plate: TLC silica gel plate *R*.

Mobile phase: glacial acetic acid *R*, acetone *R*, methylene chloride *R* (5:10:90 V/V/V).

Application: 5 µl.

Development: over 2/3 of the plate.

Drying: in air for 5 min.

Detection: spray with *anisaldehyde solution R* and heat at 100-105 °C for 10 min.

Results: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

C. Dissolve 50 mg in 10 ml of *methylene chloride R*. To 2 ml of this solution, add 1 ml of *acetic anhydride R* and 0.3 ml of *sulphuric acid R*. A pink colour is produced.

TESTS

Appearance of solution. The solution is clear (2.2.1) and not more intensely coloured than reference solution Y₆ (2.2.2, *Method II*).

Dissolve 0.1 g in *ethanol R* and dilute to 10 ml with the same solvent.

Specific optical rotation (2.2.7): + 145 to + 154 (dried substance).

Dissolve 0.50 g in *dioxan R* and dilute to 50.0 ml with the same solvent.

Related substances. Liquid chromatography (2.2.29).

Test solution. Dissolve 0.10 g of the substance to be examined in the mobile phase and dilute to 100.0 ml with the mobile phase.

Reference solution (a). Dilute 2.0 ml of the test solution to 100.0 ml with the mobile phase.

Reference solution (b). Dilute 5.0 ml of reference solution (a) to 100.0 ml with the mobile phase.

Reference solution (c). Dissolve 0.1 g of *18α-glycyrrhetinic acid R* in *tetrahydrofuran R* and dilute to 100.0 ml with the same solvent. To 2.0 ml of the solution, add 2.0 ml of the test solution and dilute to 100.0 ml with the mobile phase.

Column:

- **size:** *l* = 0.25 m, Ø = 4.6 mm,
- **stationary phase:** octadecylsilyl silica gel for chromatography *R* (5 µm),
- **temperature:** 30 °C.

Mobile phase: mix 430 volumes of *tetrahydrofuran R* and 570 volumes of a 1.36 g/l solution of *sodium acetate R* adjusted to pH 4.8 with *glacial acetic acid R*.

Flow rate: 0.8 ml/min.

Detection: spectrophotometer at 250 nm.

Injection: 20 µl loop injector; inject the test solution and the reference solutions.

Run time: 4 times the retention time of enoxolone.

System suitability:

- **resolution:** minimum of 2.0 between the peaks due to enoxolone and to *18α-glycyrrhetinic acid* in the chromatogram obtained with reference solution (c).

Limits:

- **any impurity:** not more than 7 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.7 per cent),
- **total:** not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (2.0 per cent),
- **disregard limit:** 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

Heavy metals (2.4.8): maximum 20 ppm.

1.0 g complies with limit test F. Prepare the standard using 2 ml of *lead standard solution* (10 ppm Pb) *R*.

Loss on drying (2.2.32): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 100–105 °C for 4 h.

Sulphated ash (2.4.14): maximum 0.2 per cent, determined on 1.0 g.

ASSAY

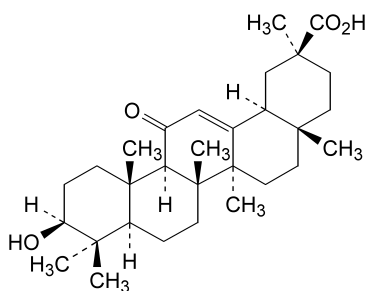
Dissolve 0.330 g in 40 ml of *dimethylformamide R*. Titrate with 0.1 M *tetrabutylammonium hydroxide*, determining the end-point potentiometrically (2.2.20). Carry out a blank titration.

1 ml of 0.1 M *tetrabutylammonium hydroxide* is equivalent to 47.07 mg of $C_{30}H_{46}O_4$.

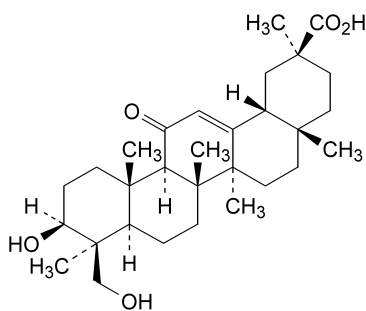
STORAGE

Protected from light.

IMPURITIES



A. (20β)-3β-hydroxy-11-oxo-18α-olean-12-en-29-oic acid,

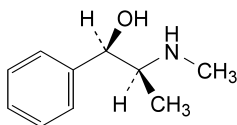


B. (4β,20β)-3β,23-dihydroxy-11-oxo-olean-12-en-29-oic acid.

01/2005:0488

EPHEDRINE, ANHYDROUS

Ephedrinum anhydricum



$C_{10}H_{15}NO$

M_r 165.2

DEFINITION

Anhydrous ephedrine contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of (1*R*,2*S*)-2-methylamino-1-phenylpropan-1-ol, calculated with reference to the anhydrous substance.

CHARACTERS

A white, crystalline powder or colourless crystals, soluble in water, very soluble in alcohol.

It melts at about 36 °C.

IDENTIFICATION

First identification: B, D.

Second identification: A, C, D, E.

- It complies with the test for specific optical rotation (see Tests).
- Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with the base isolated from *ephedrine hydrochloride CRS*. Examine the substances in discs prepared as follows: dissolve 40 mg of the substance to be examined in 1 ml of *water R*, add 1 ml of *dilute sodium hydroxide solution R* and 4 ml of *chloroform R* and shake; dry the organic layer over 0.2 g of *anhydrous sodium sulphate R*; prepare a blank disc using about 0.3 g of *potassium bromide R*; apply dropwise to the disc 0.1 ml of the organic layer, allowing the solvent to evaporate between applications; dry the disc at 50 °C for 2 min. Repeat the operations using 50 mg of *ephedrine hydrochloride CRS*.
- Examine the chromatograms obtained in the test for related substances. The principal spot in the chromatogram obtained with test solution (b) is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).
- Dissolve about 10 mg in 1 ml of *water R*. Add 0.2 ml of *strong sodium hydroxide solution R* and 0.2 ml of *copper sulphate solution R*. A violet colour is produced. Add 2 ml of *ether R* and shake. The ether layer is purple and the aqueous layer blue.
- It complies with the test for water (see Tests).

TESTS

Appearance of solution. Dissolve 0.25 g in *water R* and dilute to 10 ml with the same solvent. The solution is clear (2.2.1) and colourless (2.2.2, *Method II*).

Specific optical rotation (2.2.7). Dissolve 2.25 g in 15 ml of *dilute hydrochloric acid R* and dilute to 50.0 ml with *water R*. The specific optical rotation is –41 to –43, calculated with reference to the anhydrous substance.

Related substances. Examine by thin-layer chromatography (2.2.27), using *silica gel G R* as the coating substance.

Test solution (a). Dissolve 0.2 g of the substance to be examined in *methanol R* and dilute to 10 ml with the same solvent.

Test solution (b). Dilute 1 ml of test solution (a) to 10 ml with *methanol R*.

Reference solution (a). Dissolve 25 mg of *ephedrine hydrochloride CRS* in *methanol R* and dilute to 10 ml with the same solvent.

Reference solution (b). Dilute 1.0 ml of test solution (a) to 200 ml with *methanol R*.

Apply separately to the plate 10 µl of each solution. Develop over a path of 15 cm using a mixture of 5 volumes of *chloroform R*, 15 volumes of *concentrated ammonia R* and 80 volumes of *2-propanol R*. Allow the plate to dry in air and spray with *ninhydrin solution R*. Heat at 110 °C for 5 min. Any spot in the chromatogram obtained with test solution (a), apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.5 per cent). Disregard any spot of lighter colour than the background.

Chlorides. Dissolve 0.17 g in 10 ml of *water R*. Add 5 ml of *dilute nitric acid R* and 0.5 ml of *silver nitrate solution R1*. Allow to stand for 2 min, protected from bright light. Any opalescence in the solution is not more intense than that in a standard prepared at the same time and in the same

manner using 10 ml of *chloride standard solution* (5 ppm *Cl*) *R*, 5 ml of *dilute nitric acid R* and 0.5 ml of *silver nitrate solution R1* (290 ppm).

Water (2.5.12). Not more than 0.5 per cent, determined on 2.000 g by the semi-micro determination of water.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.200 g in 5 ml of *alcohol R* and add 20.0 ml of 0.1 *M hydrochloric acid*. Using 0.05 ml of *methyl red solution R* as indicator, titrate with 0.1 *M sodium hydroxide* until a yellow colour is obtained.

1 ml of 0.1 *M hydrochloric acid* is equivalent to 16.52 mg of $C_{10}H_{15}NO$.

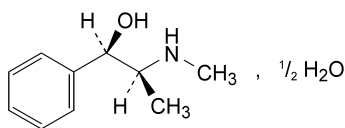
STORAGE

Store protected from light.

01/2005:0489

EPHEDRINE HEMIHYDRATE

Ephedrinum hemihydricum



$C_{10}H_{15}NO \cdot \frac{1}{2}H_2O$

M_r 174.2

DEFINITION

Ephedrine hemihydrate contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of (1*R*,2*S*)-2-(methylamino)-1-phenylpropan-1-ol, calculated with reference to the anhydrous substance.

CHARACTERS

A white, crystalline powder or colourless crystals, soluble in water, very soluble in alcohol.

It melts at about 42 °C, determined without previous drying.

IDENTIFICATION

First identification: B, D.

Second identification: A, C, D, E.

A. It complies with the test for specific optical rotation (see Tests).

B. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with the base isolated from *ephedrine hydrochloride CRS*. Examine the substances in discs prepared as follows: dissolve 40 mg of the substance to be examined in 1 ml of *water R*, add 1 ml of *dilute sodium hydroxide solution R* and 4 ml of *chloroform R* and shake; dry the organic layer over 0.2 g of *anhydrous sodium sulphate R*; prepare a blank disc using about 0.3 g of *potassium bromide R*; apply dropwise to the disc 0.1 ml of the organic layer, allowing the solvent to evaporate between applications; dry the disc at 50 °C for 2 min. Repeat the operations using 50 mg of *ephedrine hydrochloride CRS*.

- C. Examine the chromatograms obtained in the test for related substances. The principal spot in the chromatogram obtained with test solution (b) is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).
- D. Dissolve about 10 mg in 1 ml of *water R*. Add 0.2 ml of *strong sodium hydroxide solution R* and 0.2 ml of *copper sulphate solution R*. A violet colour is produced. Add 2 ml of *ether R* and shake. The ether layer is purple and the aqueous layer blue.
- E. It complies with the test for water (see Tests).

TESTS

Appearance of solution. Dissolve 0.25 g in *water R* and dilute to 10 ml with the same solvent. The solution is clear (2.2.1) and colourless (2.2.2, *Method II*).

Specific optical rotation (2.2.7). Dissolve 2.25 g in 15 ml of *dilute hydrochloric acid R* and dilute to 50.0 ml with *water R*. The specific optical rotation is –41 to –43, calculated with reference to the anhydrous substance.

Related substances. Examine by thin-layer chromatography (2.2.27), using *silica gel G R* as the coating substance.

Test solution (a). Dissolve 0.2 g of the substance to be examined in *methanol R* and dilute to 10 ml with the same solvent.

Test solution (b). Dilute 1 ml of test solution (a) to 10 ml with *methanol R*.

Reference solution (a). Dissolve 25 mg of *ephedrine hydrochloride CRS* in *methanol R* and dilute to 10 ml with the same solvent.

Reference solution (b). Dilute 1.0 ml of test solution (a) to 200 ml with *methanol R*.

Apply separately to the plate 10 µl of each solution. Develop over a path of 15 cm using a mixture of 5 volumes of *chloroform R*, 15 volumes of *concentrated ammonia R* and 80 volumes of *2-propanol R*. Allow the plate to dry in air and spray with *ninhydrin solution R*. Heat at 110 °C for 5 min. Any spot in the chromatogram obtained with test solution (a), apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.5 per cent). Disregard any spot of lighter colour than the background.

Chlorides. Dissolve 0.18 g in 10 ml of *water R*. Add 5 ml of *dilute nitric acid R* and 0.5 ml of *silver nitrate solution R1*. Allow to stand for 2 min, protected from bright light. Any opalescence in the solution is not more intense than that in a standard prepared at the same time and in the same manner using 10 ml of *chloride standard solution* (5 ppm *Cl*) *R*, 5 ml of *dilute nitric acid R* and 0.5 ml of *silver nitrate solution R1* (280 ppm).

Water (2.5.12): 4.5 per cent to 5.5 per cent, determined on 0.300 g by the semi-micro determination of water.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

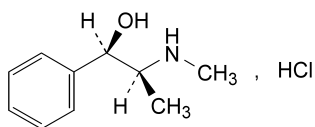
Dissolve 0.200 g in 5 ml of *alcohol R* and add 20.0 ml of 0.1 *M hydrochloric acid*. Using 0.05 ml of *methyl red solution R* as indicator, titrate with 0.1 *M sodium hydroxide* until a yellow colour is obtained.

1 ml of 0.1 *M hydrochloric acid* is equivalent to 16.52 mg of $C_{10}H_{15}NO$.

STORAGE

Store protected from light.

01/2005:0487

EPHEDRINE HYDROCHLORIDE**Ephedrini hydrochloridum** $C_{10}H_{16}ClNO$ M_r 201.7**DEFINITION**(1*R*,2*S*)-2-(Methylamino)-1-phenylpropan-1-ol hydrochloride.*Content*: 99.0 per cent to 101.0 per cent (dried substance).**CHARACTERS***Appearance*: white, crystalline powder or colourless crystals.*Solubility*: freely soluble in water, soluble in alcohol.*mp*: about 219 °C.**IDENTIFICATION***First identification*: B, E.*Second identification*: A, C, D, E.

A. Specific optical rotation (see Tests).

B. Infrared absorption spectrophotometry (2.2.24).

Comparison: ephedrine hydrochloride CRS.

C. Thin-layer chromatography (2.2.27).

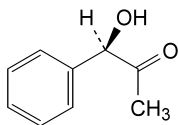
Test solution. Dissolve 20 mg of the substance to be examined in *methanol R* and dilute to 10 ml with the same solvent.*Reference solution*. Dissolve 10 mg of *ephedrine hydrochloride CRS* in *methanol R* and dilute to 5 ml with the same solvent.*Plate*: TLC silica gel plate *R*.*Mobile phase*: *methylene chloride R*, concentrated *ammonia R*, 2-*propanol R* (5:15:80 V/V/V).*Application*: 10 µl.*Development*: over 2/3 of the plate.*Drying*: in air.*Detection*: spray with *ninhydrin solution R*; heat at 110 °C for 5 min.*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.D. To 0.1 ml of solution S (see Tests) add 1 ml of *water R*, 0.2 ml of *copper sulphate solution R* and 1 ml of *strong sodium hydroxide solution R*. A violet colour is produced. Add 2 ml of *methylene chloride R* and shake. The lower (organic) layer is dark grey and the upper (aqueous) layer is blue.E. To 5 ml of solution S (see Tests) add 5 ml of *water R*. The solution gives reaction (a) of chlorides (2.3.1).**TESTS****Solution S**. Dissolve 5.00 g in *distilled water R* and dilute to 50.0 ml with the same solvent.**Appearance of solution**. Solution S is clear (2.2.1) and colourless (2.2.2, *Method II*).**Acidity or alkalinity**. To 10 ml of solution S add 0.1 ml of *methyl red solution R* and 0.2 ml of 0.01 *M sodium hydroxide*. The solution is yellow. Add 0.4 ml of 0.01 *M hydrochloric acid*. The solution is red.**Specific optical rotation** (2.2.7): –33.5 to –35.5 (dried substance).Dilute 12.5 ml of solution S to 25.0 ml with *water R*.**Related substances**. Liquid chromatography (2.2.29).*Test solution*. Dissolve 75 mg of the substance to be examined in the mobile phase and dilute to 10 ml with the mobile phase.*Reference solution (a)*. Dilute 2.0 ml of the test solution to 100.0 ml with the mobile phase. Dilute 1.0 ml of this solution to 10.0 ml with the mobile phase.*Reference solution (b)*. Dissolve 5 mg of the substance to be examined and 5 mg of *pseudoephedrine hydrochloride CRS* in the mobile phase and dilute to 50 ml with the mobile phase.*Column*:– *size*: $l = 0.25$ m, $\varnothing = 4.6$ mm,– *stationary phase*: spherical *phenylsilyl silica gel for chromatography R* (5 µm).*Mobile phase*: mix 6 volumes of *methanol R* and 94 volumes of a 11.6 g/l solution of *ammonium acetate R* adjusted to pH 4.0 with *glacial acetic acid R*.*Flow rate*: 1.0 ml/min.*Detection*: spectrophotometer at 257 nm.*Injection*: 20 µl.*Run time*: 2.5 times the retention time of ephedrine.*Relative retention* with reference to ephedrine (retention time = about 8 min): impurity B = about 1.1; impurity A = about 1.4.*System suitability*: reference solution (b):– *resolution*: minimum 2.0 between the peaks due to ephedrine and impurity B.*Limits*:– *correction factor*: for the calculation of content, multiply the peak area of impurity A by 0.4,– *impurity A*: not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent),– *any other impurity*: for each impurity, not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent),– *total of impurities other than A*: not more than 2.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent),– *disregard limit*: 0.25 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).**Sulphates** (2.4.13): maximum 100 ppm, determined on 15 ml of solution S.**Loss on drying** (2.2.32): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 100–105 °C.**Sulphated ash** (2.4.14): maximum 0.1 per cent, determined on 1.0 g.**ASSAY**Dissolve 0.150 g in 50 ml of *alcohol R* and add 5.0 ml of 0.01 *M hydrochloric acid*. Carry out a potentiometric titration (2.2.20), using 0.1 *M sodium hydroxide*. Read the volume added between the 2 points of inflexion.1 ml of 0.1 *M sodium hydroxide* is equivalent to 20.17 mg of $C_{10}H_{16}ClNO$.**STORAGE**

Protected from light.

IMPURITIES

Specified impurities: A.

Other detectable impurities: B.



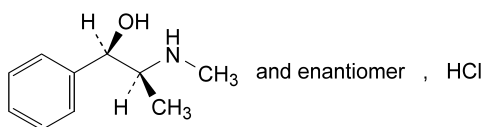
A. (–)-(1R)-1-hydroxy-1-phenylpropan-2-one,

B. pseudoephedrine.

01/2005:0715

EPHEDRINE HYDROCHLORIDE,
RACEMIC

Ephedrini racemici hydrochloridum

 $C_{10}H_{16}ClNO$ M_r 201.7

DEFINITION

Racemic ephedrine hydrochloride contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of (1R,2S)-2-(methylamino)-1-phenylpropan-1-ol hydrochloride, calculated with reference to the dried substance.

CHARACTERS

A white, crystalline powder or colourless crystals, freely soluble in water, soluble in alcohol.

It melts at about 188 °C.

IDENTIFICATION

First identification: B, E.

Second identification: A, C, D, E.

- It complies with the test for angle of optical rotation (see Tests).
- Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *racemic ephedrine hydrochloride CRS*. Examine the substances prepared as discs.
- Examine the chromatograms obtained in the test for related substances. The principal spot in the chromatogram obtained with test solution (b) is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).
- To 0.1 ml of solution S (see Tests) add 1 ml of *water R*, 0.2 ml of *copper sulphate solution R* and 1 ml of *strong sodium hydroxide solution R*. A violet colour is produced. Add 2 ml of *ether R* and shake. The ether layer is purple and the aqueous layer is blue.
- To 5 ml of solution S add 5 ml of *water R*. The solution gives reaction (a) of chlorides (2.3.1).

TESTS

Solution S. Dissolve 5.00 g in *distilled water R* and dilute to 50.0 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and colourless (2.2.2, *Method II*).

Acidity or alkalinity. To 10 ml of solution S add 0.1 ml of *methyl red solution R* and 0.1 ml of 0.01 M *sodium hydroxide*; the solution is yellow. Add 0.2 ml of 0.01 M *hydrochloric acid*; the solution is red.

Angle of optical rotation (2.2.7): + 0.2° to – 0.2°, determined on solution S.

Related substances. Examine by thin-layer chromatography (2.2.27), using *silica gel G R* as the coating substance.

Test solution (a). Dissolve 0.20 g of the substance to be examined in *methanol R* and dilute to 10 ml with the same solvent.

Test solution (b). Dilute 1 ml of test solution (a) to 10 ml with *methanol R*.

Reference solution (a). Dissolve 20 mg of *racemic ephedrine hydrochloride CRS* in *methanol R* and dilute to 10 ml with the same solvent.

Reference solution (b). Dilute 1 ml of test solution (a) to 200 ml with *methanol R*.

Apply separately to the plate 10 µl of each solution. Develop over a path of 15 cm using a mixture of 5 volumes of *chloroform R*, 15 volumes of *concentrated ammonia R* and 80 volumes of *2-propanol R*. Allow the plate to dry in air. Spray with *ninhydrin solution R* and heat at 110 °C for 5 min. Any spot in the chromatogram obtained with test solution (a), apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.5 per cent). Disregard any spot of lighter colour than the background.

Sulphates (2.4.13). 15 ml of solution S complies with the limit test for sulphates (100 ppm).

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.170 g in 30 ml of *alcohol R*. Add 5.0 ml of 0.01 M *hydrochloric acid*. Carry out a potentiometric titration (2.2.20), using 0.1 M *sodium hydroxide*. Read the volume added between the two points of inflexion.

1 ml of 0.1 M *sodium hydroxide* corresponds to 20.17 mg of $C_{10}H_{16}ClNO$.

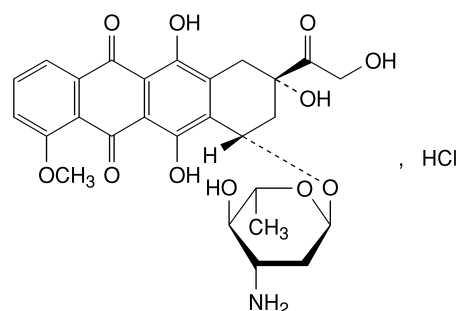
STORAGE

Store protected from light.

01/2005:1590

EPIRUBICIN HYDROCHLORIDE

Epirubicini hydrochloridum

 $C_{27}H_{30}ClNO_{11}$ M_r 580.0

DEFINITION

(8S,10S)-10-[(3-Amino-2,3,6-trideoxy- α -L-arabino-hexopyranosyl)oxy]-6,8,11-trihydroxy-8-(hydroxyacetyl)-1-methoxy-7,8,9,10-tetrahydrotetracene-5,12-dione hydrochloride.

Substance obtained by chemical transformation of a substance produced by certain strains of *Streptomyces peuceii*us.

Content: 97.0 per cent to 102.0 per cent (anhydrous and acetone-free substance).

CHARACTERS

Appearance: orange-red powder.

Solubility: soluble in water and in methanol, slightly soluble in anhydrous ethanol, practically insoluble in acetone.

IDENTIFICATION

A. Infrared absorption spectrophotometry (2.2.24).

Comparison: *epirubicin hydrochloride CRS*.

B. Examine the chromatograms obtained in the assay.

Results: the principal peak in the chromatogram obtained with the test solution is similar in retention time to the principal peak in the chromatogram obtained with reference solution (a).

C. Dissolve about 10 mg in 0.5 ml of *nitric acid R*, add 0.5 ml of *water R* and heat over a flame for 2 min. Allow to cool and add 0.5 ml of *silver nitrate solution R1*. A white precipitate is formed.

TESTS

pH (2.2.3): 4.0 to 5.5.

Dissolve 50 mg in *carbon dioxide-free water R* and dilute to 10 ml with the same solvent.

Related substances. Liquid chromatography (2.2.29).

Test solution. Dissolve 25.0 mg of the substance to be examined in the mobile phase and dilute to 25.0 ml with the mobile phase.

Reference solution (a). Dissolve 25.0 mg of *epirubicin hydrochloride CRS* in the mobile phase and dilute to 25.0 ml with the mobile phase.

Reference solution (b). Dissolve 10 mg of *epirubicin hydrochloride CRS* and 10 mg of *doxorubicin hydrochloride CRS* in the mobile phase and dilute to 100 ml with the mobile phase.

Reference solution (c). Dissolve 10 mg of *doxorubicin hydrochloride CRS* in a mixture of 5 ml of *water R* and 5 ml of *phosphoric acid R*. Allow to stand for 30 min. Adjust to pH 2.6 with an 80 g/l solution of *sodium hydroxide R*. Add 15 ml of *acetonitrile R* and 10 ml of *methanol R*. Mix.

Reference solution (d). Dilute 1.0 ml of the test solution to 100.0 ml with the mobile phase.

Column:

- **size:** $l = 0.25$ m, $\varnothing = 4.6$ mm,
- **stationary phase:** trimethylsilyl silica gel for chromatography *R* (6 μ m),
- **temperature:** 35 °C.

Mobile phase: mix 17 volumes of *methanol R*, 29 volumes of *acetonitrile R* and 54 volumes of a solution containing 3.7 g/l of *sodium laurilsulfate R* and 2.8 per cent V/V of *dilute phosphoric acid R*.

Flow rate: 2.5 ml/min.

Detection: spectrophotometer at 254 nm.

Injection: 10 μ l of the test solution and reference solutions (b), (c) and (d).

Run time: 3.5 times the retention time of epirubicin.

Identification of impurities: use the 2nd most abundant peak present in the chromatogram obtained with reference solution (c) to identify impurity A.

Relative retention with reference to epirubicin (retention time = about 9.5 min): impurity A = about 0.3; impurity B = about 0.4; impurity C = about 0.8; impurity E = about 1.1; impurity D = about 1.5; impurity F = about 1.7; impurity G = about 2.1.

System suitability: reference solution (b):

- **resolution:** minimum 2.0 between the peaks due to impurity C and epirubicin.

Limits:

- **correction factor:** for the calculation of content, multiply the peak area of impurity A by 0.7,
- **impurity A:** not more than the area of the principal peak in the chromatogram obtained with reference solution (d) (1.0 per cent),
- **impurity C:** not more than the area of the principal peak in the chromatogram obtained with reference solution (d) (1.0 per cent),
- **any other impurity:** for each impurity, not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (d) (0.5 per cent),
- **total:** not more than twice the area of the principal peak in the chromatogram obtained with reference solution (d) (2.0 per cent),
- **disregard limit:** 0.05 times the area of the principal peak in the chromatogram obtained with reference solution (d) (0.05 per cent).

Acetone (2.4.24): maximum 1.5 per cent.

Water (2.5.12): maximum 4.0 per cent, determined on 0.100 g.

Bacterial endotoxins (2.6.14): less than 1.1 IU/mg, if intended for use in the manufacture of parenteral dosage forms without a further appropriate procedure for removal of bacterial endotoxins.

ASSAY

Liquid chromatography (2.2.29) as described in the test for related substances with the following modification.

Injection: test solution and reference solution (a).

Calculate the percentage content of $C_{27}H_{30}ClNO_{11}$.

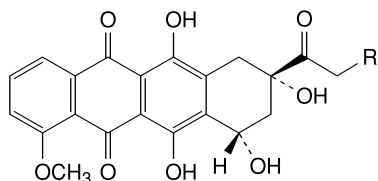
STORAGE

In an airtight container, protected from light, at a temperature of 2 °C to 8 °C. If the substance is sterile, store in a sterile, airtight, tamper-proof container.

LABELLING

The label states, where applicable, that the substance is free from bacterial endotoxins.

IMPURITIES

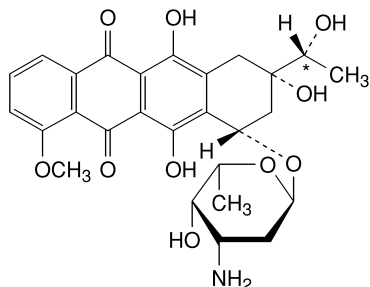


A. R = OH: (8*S*,10*S*)-6,8,10,11-tetrahydroxy-8-(hydroxyacetyl)-1-methoxy-7,8,9,10-tetrahydrotetracene-5,12-dione (doxorubicinone),

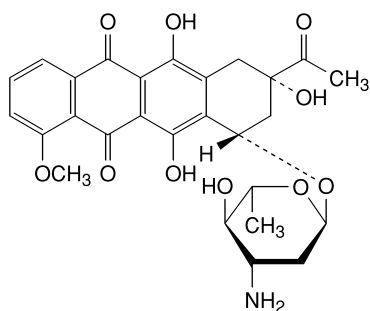
B. R = H: (8*S*,10*S*)-8-acetyl-6,8,10,11-tetrahydroxy-1-methoxy-7,8,9,10-tetrahydrotetracene-5,12-dione (daunorubicinone),

C. doxorubicin,

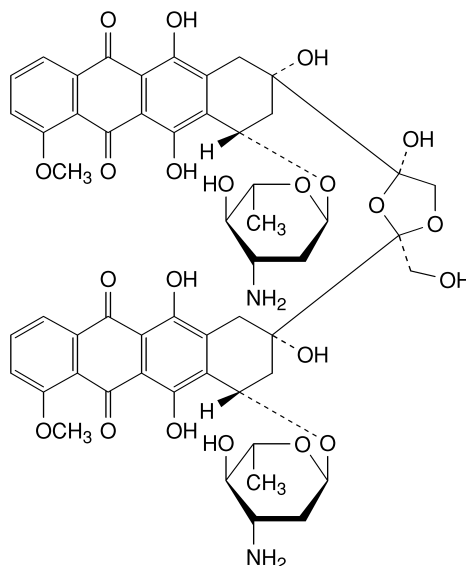
D. daunorubicin,



E. (8*S*,10*S*)-10-[(3-amino-2,3,6-trideoxy- α -*L*-lyxo-hexopyranosyl)oxy]-6,8,11-trihydroxy-8-[(1*RS*)-1-hydroxyethyl]-1-methoxy-7,8,9,10-tetrahydrotetracene-5,12-dione (dihydrodaunorubicin),



F. (8*S*,10*S*)-8-acetyl-10-[(3-amino-2,3,6-trideoxy- α -*L*-arabino-hexopyranosyl)oxy]-6,8,11-trihydroxy-1-methoxy-7,8,9,10-tetrahydrotetracene-5,12-dione (*epi*-daunorubicin),



G. 8,8'-[(2*R*,4*R*)-4-hydroxy-2-(hydroxymethyl)-1,3-dioxolan-2,4-diyl]bis[(8*S*,10*S*)-10-[(3-amino-2,3,6-trideoxy- α -*L*-arabino-hexopyranosyl)oxy]-6,8,11-trihydroxy-1-methoxy-7,8,9,10-tetrahydrotetracene-5,12-dione] (epirubicin dimer).

01/2005:1825

EQUISETUM STEM

Equiseti herba

DEFINITION

Whole or cut, dried sterile aerial parts of *Equisetum arvense* L.

Content: minimum 0.3 per cent of total flavonoids expressed as isoquercitroside ($C_{21}H_{20}O_{12}$; M_r 464.4) (dried drug).

CHARACTERS

Macroscopic and microscopic characters described under identification tests A and B.

IDENTIFICATION

- A. It consists of fragments of grooved stems and linear leaves, light green to greenish-grey. They are rough to the touch, brittle and crunchy when crushed. The main stems are about 0.8 mm to 4.5 mm in diameter, hollow, jointed at the nodes which occur at intervals of about 1.5 cm to 4.5 cm; distinct vertical grooves are present on the internodes, ranging in number from 4 to 14 or more. Verticils of widely spaced and erect branches, usually simple, each about 1 mm thick with 2 to 4 longitudinal grooves, occur at the nodes. The leaves are small, linear, verticillate at each node, concrescent at the base, they form a toothed sheath around the stem; with the number of teeth corresponding to the number of grooves on the stem. Each tooth, often brown, is lanceolate-triangular. The lowest internode of each branch is longer than the sheath of the stem it belongs to.
- B. Reduce to a powder (355). The powder is greenish-grey. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters: fragments of the epidermis in surface view, composed of rectangular cells with wavy walls and paracytic stomata (2.8.3) with the 2 subsidiary cells covering the guard cells and having conspicuous radial ridges; in transverse sectional view the epidermis is crenate, with the protuberances formed from the contiguous walls of 2 adjacent, U-shaped cells. Fragments

of large-celled parenchyma and groups of long, non-lignified fibres with narrow lumens; scattered small, lignified vessels with spiral or annular thickening.

- C. Examine the chromatograms obtained in the test for other *Equisetum* species and hybrids.

Results: see below the sequence of the zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other fluorescent zones may be present in the chromatogram obtained with the test solution.

Top of the plate	
Caffeic acid: a greenish-blue fluorescent zone	2 red fluorescent zones
_____	2 greenish-blue fluorescent zones
_____	An orange fluorescent zone
Hyperoside: an orange fluorescent zone	2 greenish-blue fluorescent zones
_____	_____
Rutin: an orange fluorescent zone	_____
Reference solution	Test solution

TESTS

Foreign matter (2.8.2): maximum 5 per cent of stems from other *Equisetum* species and hybrids and maximum 2 per cent of other foreign matter.

Other *Equisetum* species and hybrids. Thin-layer chromatography (2.2.27).

Test solution. To 1.0 g of the powdered drug (355) add 10 ml of *methanol R*. Heat in a water-bath at 60 °C for 10 min with occasional shaking. Allow to cool. Filter.

Reference solution. Dissolve 1.0 mg of *caffeic acid R*, 2.5 mg of *hyperoside R* and 2.5 mg of *rutin R* in 10 ml of *methanol R*.

Plate: TLC silica gel plate *R*.

Mobile phase: *anhydrous formic acid R*, *glacial acetic acid R*, *water R*, *ethyl acetate R* (7.5:7.5:18:67 V/V/V/V).

Application: 10 µl, as bands.

Development: over a path of 10 cm.

Drying: at 100-105 °C.

Detection: spray the warm plate with a 10 g/l solution of *diphenylboric acid aminoethyl ester R* in *methanol R*. Then spray with a 50 g/l solution of *macrogol 400 R* in *methanol R*. Allow the plate to dry in air for 30 min. Examine in ultraviolet light at 365 nm.

Results: the chromatogram obtained with the test solution shows no yellow or greenish-yellow fluorescent zone shortly above the starting line.

Loss on drying (2.2.32): maximum 10 per cent, determined on 1.000 g of the powdered drug (355) by drying in an oven at 100-105 °C for 2 h.

Ash insoluble in hydrochloric acid (2.8.1): minimum 3.0 per cent and maximum 15.0 per cent.

Total ash (2.4.16): minimum 12.0 per cent and maximum 27.0 per cent.

ASSAY

Stock solution. In a 100 ml round-bottomed flask, introduce 0.800 g of the powdered drug (355), add 1 ml of a 5 g/l solution of *hexamethylenetetramine R*, 20 ml of *acetone R* and 2 ml of *hydrochloric acid R1*. Boil the mixture under a reflux condenser for 30 min. Filter the liquid through a plug of absorbent cotton into a flask. Add the absorbent cotton to the residue in the round-bottomed flask and extract with 2 quantities, each of 20 ml, of *acetone R*, each time boiling under a reflux condenser for 10 min. Allow to cool and filter each extract through a plug of absorbent cotton into the flask. After cooling, filter the combined acetone extracts through a filter paper into a volumetric flask and dilute to 100.0 ml with *acetone R* by rinsing the flask and the filter paper. Introduce 20.0 ml of the solution into a separating funnel, add 20 ml of *water R* and shake the mixture with 1 quantity of 15 ml and then 3 quantities, each of 10 ml, of *ethyl acetate R*. Combine the ethyl acetate extracts in a separating funnel, wash with 2 quantities, each of 50 ml, of *water R*, and filter the extracts over 10 g of *anhydrous sodium sulphate R* into a volumetric flask. Dilute to 50.0 ml with *ethyl acetate R*.

Test solution. To 10.0 ml of the stock solution add 1 ml of *aluminium chloride reagent R* and dilute to 25.0 ml with a 5 per cent V/V solution of *glacial acetic acid R* in *methanol R*.

Compensation solution. Dilute 10.0 ml of the stock solution to 25.0 ml with a 5 per cent V/V solution of *glacial acetic acid R* in *methanol R*.

Measure the absorbance (2.2.25) of the test solution after 30 min, by comparison with the compensation solution at 425 nm. Calculate the percentage content of flavonoids, calculated as isoquercitroside, from the expression:

$$\frac{A \times 1.25}{m}$$

i.e. taking the specific absorbance of isoquercitroside to be 500,

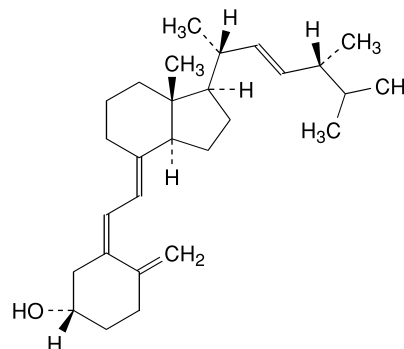
A = absorbance at 425 nm,

m = mass of the substance to be examined, in grams.

01/2005:0082

ERGOCALCIFEROL

Ergocalciferolum



$C_{28}H_{44}O$

M_r 396.7

DEFINITION

Ergocalciferol contains not less than 97.0 per cent and not more than the equivalent of 103.0 per cent of (5Z,7E,22E)-9,10-secoergosta-5,7,10(19),22-tetraen-3β-ol.

1 mg of ergocalciferol is equivalent to 40 000 IU of antirachitic activity (vitamin D) in rats.

CHARACTERS

A white or slightly yellowish, crystalline powder or white or almost white crystals, practically insoluble in water, freely soluble in alcohol, soluble in fatty oils. It is sensitive to air, heat and light. Solutions in volatile solvents are unstable and are to be used immediately.

A reversible isomerisation to pre-ergocalciferol takes place in solution, depending on temperature and time. The activity is due to both compounds.

IDENTIFICATION

Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *ergocalciferol CRS*. Examine the substances prepared as discs.

TESTS

Specific optical rotation (2.2.7). Dissolve 0.200 g rapidly and without heating in *aldehyde-free alcohol R* and dilute to 25.0 ml with the same solvent. The specific optical rotation, determined within 30 min of preparing the solution, is + 103 to + 107.

Reducing substances. Dissolve 0.1 g in *aldehyde-free alcohol R* and dilute to 10.0 ml with the same solvent. Add 0.5 ml of a 5 g/l solution of *tetrazolium blue R* in *aldehyde-free alcohol R* and 0.5 ml of *dilute tetramethylammonium hydroxide solution R*. Allow to stand for exactly 5 min and add 1.0 ml of *glacial acetic acid R*. Prepare a reference solution at the same time and in the same manner using 10.0 ml of a solution containing 0.2 µg/ml of *hydroquinone R* in *aldehyde-free alcohol R*. Measure the absorbance (2.2.25) of the two solutions at 525 nm using as the compensation liquid 10.0 ml of *aldehyde-free alcohol R* treated in the same manner. The absorbance of the test solution is not greater than that of the reference solution (20 ppm).

Ergosterol. Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel G plate R*.

Test solution. Dissolve 0.25 g of the substance to be examined in *ethylene chloride R* containing 10 g/l of *squalane R* and 0.1 g/l of *butylhydroxytoluene R* and dilute to 5 ml with the same solvent. Prepare immediately before use.

Reference solution (a). Dissolve 0.10 g of *ergocalciferol CRS* in *ethylene chloride R* containing 10 g/l of *squalane R* and 0.1 g/l of *butylhydroxytoluene R* and dilute to 2 ml with the same solvent. Prepare immediately before use.

Reference solution (b). Dissolve 5 mg of *ergosterol CRS* in *ethylene chloride R* containing 10 g/l of *squalane R* and 0.1 g/l of *butylhydroxytoluene R* and dilute to 50 ml with the same solvent. Prepare immediately before use.

Reference solution (c). Mix equal volumes of reference solution (a) and reference solution (b). Prepare immediately before use.

Apply to the plate 10 µl of the test solution, 10 µl of reference solution (a), 10 µl of reference solution (b) and 20 µl of reference solution (c). Develop immediately, protected from light, over a path of 15 cm using a mixture of equal volumes of *cyclohexane R* and *peroxide-free ether R*, the mixture containing 0.1 g/l of *butylhydroxytoluene R*. Allow the plate to dry in air and spray three times with *antimony trichloride solution R1*. Examine the chromatograms for 3 min to 4 min

after spraying. The principal spot in the chromatogram obtained with the test solution is initially orange-yellow and then becomes brown. In the chromatogram obtained with the test solution, any slowly appearing violet spot (corresponding to ergosterol) immediately below the principal spot is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.2 per cent). There is no spot in the chromatogram obtained with the test solution that does not correspond to one of the spots in the chromatograms obtained with reference solutions (a) and (b). The test is not valid unless the chromatogram obtained with reference solution (c) shows two clearly separated spots.

ASSAY

Carry out the operations as rapidly as possible, avoiding exposure to actinic light and air.

Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 10.0 mg of the substance to be examined without heating in 10.0 ml of *toluene R* and dilute to 100.0 ml with the mobile phase.

Reference solution (a). Dissolve 10.0 mg of *ergocalciferol CRS* without heating in 10.0 ml of *toluene R* and dilute to 100.0 ml with the mobile phase.

Reference solution (b). Dilute 1.0 ml of *cholecalciferol for performance test CRS* to 5.0 ml with the mobile phase. Heat in a water-bath at 90 °C under a reflux condenser for 45 min and cool.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4.6 mm in internal diameter packed with a suitable silica gel (5 µm),
- as mobile phase at a flow rate of 2 ml/min a mixture of 3 volumes of *pentanol R* and 997 volumes of *hexane R*,
- as detector a spectrophotometer set at 254 nm.

An automatic injection device or a sample loop is recommended. Inject a suitable volume of reference solution (b). Adjust the sensitivity of the system so that the height of the principal peak is at least 50 per cent of the full scale of the recorder. Inject reference solution (b) 6 times. When the chromatograms are recorded in the prescribed conditions, the approximate relative retention times with reference to cholecalciferol are 0.4 for pre-cholecalciferol and 0.5 for *trans*-cholecalciferol. The relative standard deviation of the response for cholecalciferol is not greater than 1 per cent and the resolution between the peaks corresponding to pre-cholecalciferol and *trans*-cholecalciferol is not less than 1.0. If necessary adjust the proportions of the constituents and the flow rate of the mobile phase to obtain this resolution.

Inject a suitable volume of reference solution (a). Adjust the sensitivity of the system so that the height of the principal peak is at least 50 per cent of the full scale of the recorder. Inject the same volume of the test solution and record the chromatogram in the same manner.

Calculate the percentage content of ergocalciferol from the expression:

$$\frac{m'}{m} \times \frac{S_D}{S'_D} \times 100$$

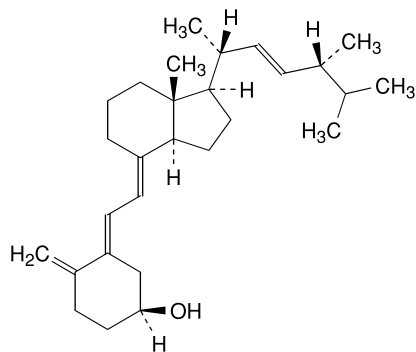
- m* = mass of the substance to be examined in the test solution, in milligrams,
- m'* = mass of *ergocalciferol CRS* in reference solution (a), in milligrams,

- S_D = area (or height) of the peak due to ergocalciferol in the chromatogram obtained with the test solution,
- S'_D = area (or height) of the peak due to ergocalciferol in the chromatogram obtained with reference solution (a).

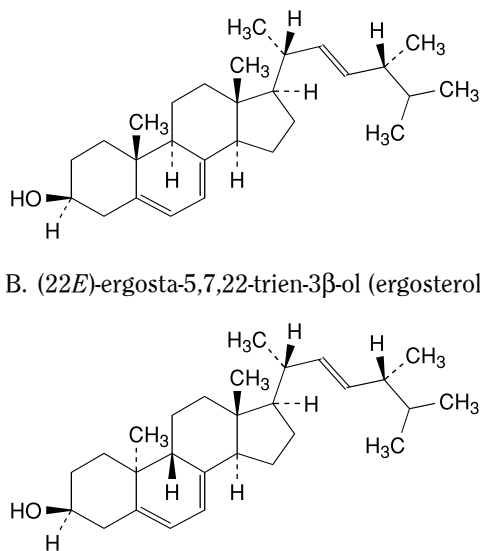
STORAGE

Store in an airtight container, under nitrogen, protected from light, at a temperature between 2 °C and 8 °C. The contents of an opened container are to be used immediately.

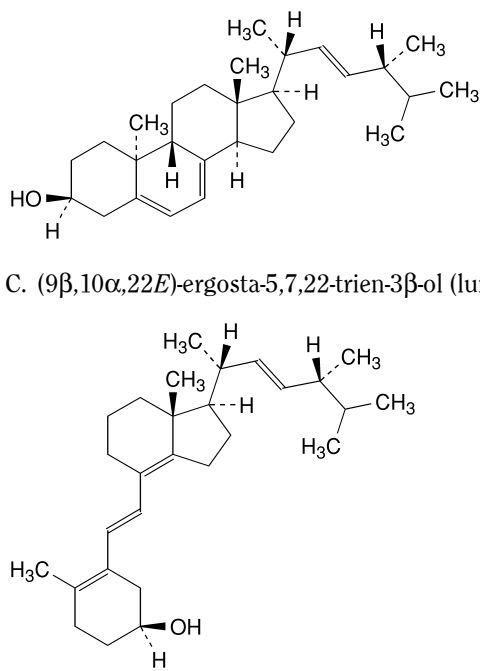
IMPURITIES



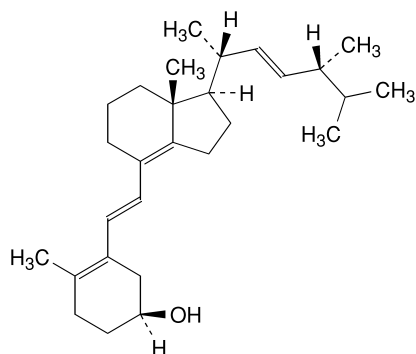
A. (5*E*,7*E*,22*E*)-9,10-secoergosta-5,7,10(19),22-tetraen-3β-ol (*trans*-vitamin D₂),



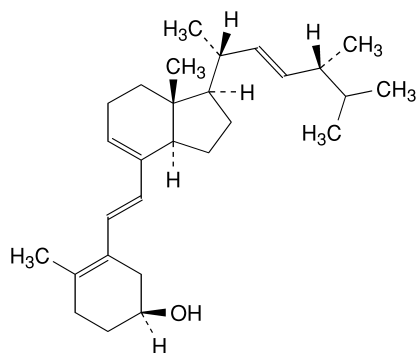
B. (22*E*)-ergosta-5,7,22-trien-3β-ol (ergosterol),



C. (9β,10α,22*E*)-ergosta-5,7,22-trien-3β-ol (lumisterol₂),



D. (6*E*,22*E*)-9,10-secoergosta-5(10),6,8(14),22-tetraen-3β-ol (iso-tachysterol₂),

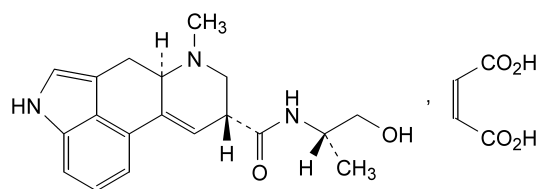


E. (6*E*,22*E*)-9,10-secoergosta-5(10),6,8,22-tetraen-3β-ol (tachysterol₂).

01/2005:0223

ERGOMETRINE MALEATE

Ergometrini maleas

 $C_{23}H_{27}N_3O_6$ M_r 441.5

DEFINITION

Ergometrine maleate contains not less than 98.0 per cent and not more than the equivalent of 101.0 per cent of (6*aR*,9*R*)-*N*[(*S*)-2-hydroxy-1-methylethyl]-7-methyl-4,6,6*a*,7,8,9-hexahydro-indolo[4,3-*fg*]quinoline-9-carboxamide maleate, calculated with reference to the dried substance.

CHARACTERS

A white or slightly coloured, crystalline powder, sparingly soluble in water, slightly soluble in alcohol.

IDENTIFICATION

First identification: B, C.

Second identification: A, C, D, E.

- Dissolve 30 mg in 0.01 *M* hydrochloric acid and dilute to 100.0 ml with the same acid. Dilute 10.0 ml of the solution to 100.0 ml with 0.01 *M* hydrochloric acid. Examined between 250 nm and 360 nm (2.2.25), the solution shows an absorption maximum at 311 nm and a minimum at 265 nm to 272 nm. The specific absorbance at the maximum is 175 to 195.
- Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *ergometrine maleate* CRS. Examine the substances prepared as discs.
- Examine the chromatograms obtained in the test for related substances. The principal spot in the chromatogram obtained with test solution (b) is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).
- To 0.1 ml of solution S (see Tests) add 1 ml of *glacial acetic acid R*, 0.05 ml of *ferric chloride solution R1* and 1 ml of *phosphoric acid R* and heat in a water-bath at 80 °C. After about 10 min, a blue or violet colour develops which becomes more intense on standing.

E. Dissolve 0.1 g in a mixture of 0.5 ml of *dilute sulphuric acid R* and 2.5 ml of *water R*. Add 5 ml of *ether R* and 1 ml of *strong sodium hydroxide solution R* and shake. Separate the aqueous layer and shake with two quantities, each of 5 ml, of *ether R*. To 0.1 ml of the aqueous layer add a solution of 10 mg of *resorcinol R* in 3 ml of *sulphuric acid R*. Heat on a water-bath for 15 min. No colour develops. To the rest of the aqueous layer add 1 ml of *bromine water R*. Heat on a water-bath for 10 min, then heat to boiling and cool. To 0.2 ml of this solution add a solution of 10 mg of *resorcinol R* in 3 ml of *sulphuric acid R*. Heat on a water-bath for 15 min. A pinkish-violet colour develops.

TESTS

Solution S. Dissolve 0.100 g, without heating and protected from light, in 9 ml of *carbon dioxide-free water R* and dilute to 10.0 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and not more intensely coloured than reference solution Y₅ or BY₅ (2.2.2, *Method II*).

pH (2.2.3). The pH of solution S is 3.6 to 4.4.

Specific optical rotation (2.2.7): + 50 to + 56, determined on solution S and calculated with reference to the dried substance.

Related substances. Examine by thin-layer chromatography (2.2.27), using *silica gel G R* as the coating substance. Carry out all operations as rapidly as possible, protected from light. Prepare the test and reference solutions immediately before use.

Test solution (a). Dissolve 50 mg of the substance to be examined in a mixture of 1 volume of *concentrated ammonia R* and 9 volumes of *alcohol (80 per cent V/V) R* and dilute to 5.0 ml with the same mixture of solvents.

Test solution (b). Dilute 1.0 ml of test solution (a) to 10.0 ml with a mixture of 1 volume of *concentrated ammonia R* and 9 volumes of *alcohol (80 per cent V/V) R*.

Reference solution (a). Dissolve 10 mg of *ergometrine maleate CRS* in a mixture of 1 volume of *concentrated ammonia R* and 9 volumes of *alcohol (80 per cent V/V) R* and dilute to 10.0 ml with the same mixture of solvents.

Reference solution (b). Dilute 5.0 ml of reference solution (a) to 50.0 ml with a mixture of 1 volume of *concentrated ammonia R* and 9 volumes of *alcohol (80 per cent V/V) R*.

Reference solution (c). To 2.0 ml of reference solution (b) add 2.0 ml of a mixture of 1 volume of *concentrated ammonia R* and 9 volumes of *alcohol (80 per cent V/V) R*.

Apply separately to the plate 5 µl of each solution. Develop immediately over a path of 14 cm using a mixture of 3 volumes of *water R*, 25 volumes of *methanol R* and 75 volumes of *chloroform R*. Dry the plate in a current of cold air and spray with *dimethylaminobenzaldehyde solution R7*. Dry the plate in a current of warm air for about 2 min. Any spot in the chromatogram obtained with test solution (a), apart from the principal spot, is not more intense than the principal spot in the chromatogram obtained with reference solution (b) (1.0 per cent) and at most one such spot is more intense than the principal spot in the chromatogram obtained with reference solution (c) (0.5 per cent).

Loss on drying (2.2.32). Not more than 2.0 per cent, determined on 0.20 g by drying over *diphosphorus pentoxide R* at 80 °C at a pressure not exceeding 2.7 kPa for 2 h.

ASSAY

Dissolve 0.150 g in 40 ml of *anhydrous acetic acid R*. Titrate with 0.05 M *perchloric acid*, determining the end-point potentiometrically (2.2.20).

1 ml of 0.05 M *perchloric acid* is equivalent to 22.07 mg of C₂₃H₂₇N₃O₆.

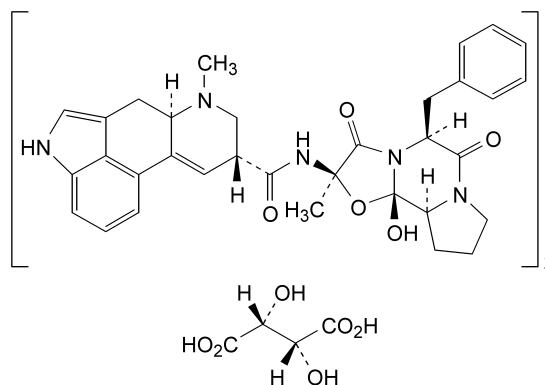
STORAGE

Store in an airtight, glass container, protected from light, at a temperature of 2 °C to 8 °C.

01/2005:0224

ERGOTAMINE TARTRATE

Ergotamini tartras

C₇₀H₇₆N₁₀O₁₆M_r 1313

DEFINITION

Ergotamine tartrate contains not less than 98.0 per cent and not more than the equivalent of 101.0 per cent of bis[(6aR,9R)-N-[(2R,5S,10aS,10bS)-5-benzyl-10b-hydroxy-2-methyl-3,6-dioxo-octahydro-8H-oxazolo[3,2-a]pyrrolo[2,1-c]pyrazin-2-yl]-7-methyl-4,6,6a,7,8,9-hexahydroindolo[4,3-fg]quinoline-9-carboxamide] tartrate, calculated with reference to the dried substance. It may contain two molecules of methanol of crystallisation.

CHARACTERS

A white or almost white, crystalline powder or colourless crystals, slightly hygroscopic, slightly soluble in alcohol. Aqueous solutions slowly become cloudy owing to hydrolysis; this may be prevented by the addition of tartaric acid.

IDENTIFICATION

First identification: B, C.

Second identification: A, C, D, E.

A. Dissolve 50 mg in 0.01 M *hydrochloric acid* and dilute to 100.0 ml with the same acid. Dilute 10.0 ml of the solution to 100.0 ml with 0.01 M *hydrochloric acid*. Examined between 250 nm and 360 nm (2.2.25), the solution shows an absorption maximum at 311 nm to 321 nm and a minimum at 265 nm to 275 nm. The specific absorbance at the maximum is 118 to 128, calculated with reference to the dried substance.

B. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *ergotamine tartrate CRS*. Examine the substances as discs prepared as follows: triturate the substance to be examined and the reference substance separately with 0.2 ml of *methanol R* and then with *potassium bromide R* as prescribed in the general method.

- C. Examine for not more than 1 min in ultraviolet light at 365 nm the chromatograms obtained in the test for related substances. The principal spot in the chromatogram obtained with test solution (b) is similar in position and fluorescence to the principal spot in the chromatogram obtained with reference solution (a). After spraying with *dimethylaminobenzaldehyde solution R7*, examine in daylight. The principal spot in the chromatogram obtained with test solution (b) is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).
- D. To 0.1 ml of solution S (see Tests) add 1 ml of *glacial acetic acid R*, 0.05 ml of *ferric chloride solution R1* and 1 ml of *phosphoric acid R* and heat in a water-bath at 80 °C. After about 10 min, a blue or violet colour develops which becomes more intense on standing.
- E. Dissolve about 10 mg in 1.0 ml of 0.1 M *sodium hydroxide*. Transfer to a separating funnel and shake with 5 ml of *methylene chloride R*. Discard the organic layer. Neutralise the aqueous layer with a few drops of *dilute hydrochloric acid R*. 0.1 ml of this solution gives reaction (b) of tartrates (2.3.1). Pour the reaction mixture into 1 ml of *water R* to observe the colour change to red or brownish-red.

TESTS

Carry out all operations as rapidly as possible, protected from light.

Solution S. Triturate 30 mg finely with about 15 mg of *tartaric acid R* and dissolve with shaking in 6 ml of *water R*.

Appearance of solution. Solution S is clear (2.2.1) and not more intensely coloured than reference solution Y₆ (2.2.2, Method II).

pH (2.2.3). Shake 10 mg, finely powdered, with 4 ml of *carbon dioxide-free water R*. The pH of the suspension is 4.0 to 5.5.

Specific optical rotation (2.2.7). Dissolve 0.40 g in 40 ml of a 10 g/l solution of *tartaric acid R*. Add 0.5 g of *sodium hydrogen carbonate R* cautiously in several portions and mix thoroughly. Shake with four quantities, each of 10 ml, of *chloroform R* previously washed with five quantities of *water R*, each of 50 ml per 100 ml of *chloroform R*. Combine the organic layers. Filter through a small filter moistened with *chloroform R* previously washed as described above. Dilute the filtrate to 50.0 ml with *chloroform R* previously washed as described above. Measure the angle of rotation.

Determine the amount of ergotamine base in the chloroformic solution as follows: to 25.0 ml of the solution add 50 ml of *anhydrous acetic acid R* and titrate with 0.05 M *perchloric acid*, determining the end-point potentiometrically (2.2.20).

1 ml of 0.05 M *perchloric acid* is equivalent to 29.08 mg of C₃₃H₃₅N₅O₅.

The specific optical rotation is –154 to –165, calculated from the angle of rotation and the concentration of ergotamine base.

Related substances. Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel G plate R*. Prepare the reference solutions and the test solutions immediately before use and in the order indicated below.

Reference solution (a). Dissolve 10 mg of *ergotamine tartrate CRS* in a mixture of 1 volume of *methanol R* and 9 volumes of *methylene chloride R* and dilute to 10.0 ml with the same mixture of solvents.

Reference solution (b). Dilute 7.5 ml of reference solution (a) to 50.0 ml with a mixture of 1 volume of *methanol R* and 9 volumes of *methylene chloride R*.

Reference solution (c). To 2.0 ml of reference solution (b) add 4.0 ml of a mixture of 1 volume of *methanol R* and 9 volumes of *methylene chloride R*.

Test solution (a). Dissolve 50 mg of the substance to be examined in a mixture of 1 volume of *methanol R* and 9 volumes of *methylene chloride R* and dilute to 5.0 ml with the same mixture of solvents.

Test solution (b). Dilute 1.0 ml of test solution (a) to 10.0 ml with a mixture of 1 volume of *methanol R* and 9 volumes of *methylene chloride R*.

Apply immediately to the plate 5 µl of each reference solution and then 5 µl of each test solution. Expose the starting points immediately to ammonia vapour and for exactly 20 s by moving the starting line from side to side above a beaker 55 mm high and 45 mm in diameter containing about 20 ml of *concentrated ammonia R*. Dry the starting line in a current of cold air for exactly 20 s. Develop immediately over a path of 17 cm using a mixture of 5 volumes of *ethanol R*, 10 volumes of *methylene chloride R*, 15 volumes of *dimethylformamide R* and 70 volumes of *ether R*. Dry the plate in a current of cold air for about 2 min. Examine for not more than 1 min in ultraviolet light at 365 nm for the identification. Spray the plate abundantly with *dimethylaminobenzaldehyde solution R7* and dry in a current of warm air for about 2 min. Any spot in the chromatogram obtained with test solution (a), apart from the principal spot, is not more intense than the principal spot in the chromatogram obtained with reference solution (b) (1.5 per cent) and at most one such spot is more intense than the principal spot in the chromatogram obtained with reference solution (c) (0.5 per cent).

Loss on drying (2.2.32). Not more than 6.0 per cent, determined on 0.100 g by drying *in vacuo* at 95 °C for 6 h.

ASSAY

Dissolve 0.200 g in 40 ml of *anhydrous acetic acid R*. Titrate with 0.05 M *perchloric acid*, determining the end-point potentiometrically (2.2.20).

1 ml of 0.05 M *perchloric acid* is equivalent to 32.84 mg of C₇₀H₇₆N₁₀O₁₆.

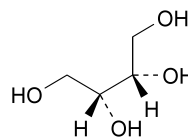
STORAGE

Store in an airtight, glass container, protected from light, at a temperature of 2 °C to 8 °C.

01/2005:1803

ERYTHRITOL

Erythritolum

C₄H₁₀O₄M_r 122.1

DEFINITION

(2R,3S)-Butane-1,2,3,4-tetrol (*meso*-erythritol).

Content: 96.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

Appearance: white or almost white, crystalline powder or free-flowing granules.

Solubility: freely soluble in water, very slightly soluble in alcohol.

IDENTIFICATION

A. Melting point (2.2.14): 119 °C to 122 °C.

B. Infrared absorption spectrophotometry (2.2.24).

Comparison: erythritol CRS.

TESTS

Appearance of solution. The solution is clear (2.2.1) and colourless (2.2.2, *Method II*).

Dissolve 5.0 g in *water R* and dilute to 50 ml with the same solvent.

Conductivity (2.2.38): maximum 20 $\mu\text{S}\cdot\text{cm}^{-1}$.

Dissolve 20.0 g in *carbon dioxide-free water R* prepared from *distilled water R* and dilute to 100.0 ml with the same solvent. Measure the conductivity of the solution, while gently stirring with a magnetic stirrer.

Related substances. Liquid chromatography (2.2.29).

Test solution. Dissolve 0.50 g of the substance to be examined in *water R* and dilute to 10.0 ml with the same solvent.

Reference solution (a). Dissolve 0.50 g of *erythritol CRS* in *water R* and dilute to 10.0 ml with the same solvent.

Reference solution (b). Dilute 2.0 ml of the test solution to 100.0 ml with *water R*.

Reference solution (c). Dilute 5.0 ml of reference solution (b) to 100.0 ml with *water R*.

Reference solution (d). Dissolve 1.0 g of *erythritol R* and 1.0 g of *glycerol R* in *water R* and dilute to 20.0 ml with the same solvent.

Column:

- *size:* $l = 0.3 \text{ m}$, $\varnothing = 7.8 \text{ mm}$,
- *stationary phase:* cation-exchange resin *R* (9 μm),
- *temperature:* 70 °C.

Mobile phase: 0.01 per cent *V/V* solution of *sulphuric acid R*.

Flow rate: 0.8 ml/min.

Detection: refractometer maintained at a constant temperature.

Injection: 20 μl ; inject the test solution and reference solutions (b), (c) and (d).

Run time: 3 times the retention time of erythritol.

Relative retention with reference to erythritol (retention time = about 11 min): impurity A = about 0.77; impurity B = about 0.90; impurity C = about 0.94; impurity D = about 1.10.

System suitability: reference solution (d):

- *resolution:* minimum 2 between the peaks due to erythritol and impurity D.

Limits:

- *any impurity:* not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (2.0 per cent),
- *total:* not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (2.0 per cent),
- *disregard limit:* area of the principal peak in the chromatogram obtained with reference solution (c) (0.1 per cent).

Lead (2.4.10): maximum 0.5 ppm.

Water (2.5.12): maximum 0.5 per cent, determined on 1.00 g.

Microbial contamination. Total viable aerobic count (2.6.12) not more than 10^3 bacteria and 10^2 fungi per gram, determined by plate count. It complies with the tests for *Escherichia coli* and *Salmonella* (2.6.13). If intended for use in the manufacture of parenteral dosage forms, the total viable aerobic count (2.6.12) is not more than 10^2 bacteria and 10^2 fungi per gram, determined by plate count.

Bacterial endotoxins (2.6.14). If intended for use in the manufacture of parenteral dosage forms without a further appropriate procedure for the removal of bacterial endotoxins:

- less than 4 IU/g for parenteral dosage forms having a concentration of 100 g/l or less of erythritol,
- less than 2.5 IU/g for parenteral dosage forms having a concentration of more than 100 g/l of erythritol.

ASSAY

Liquid chromatography (2.2.29) as described in the test for related substances with the following modification.

Injection: test solution and reference solution (a).

Calculate the percentage content of erythritol using the chromatogram obtained with reference solution (a) and the declared content of *erythritol CRS*.

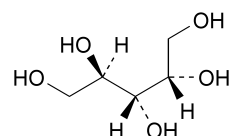
LABELLING

The label states where applicable, that the substance is suitable for use in the manufacture of parenteral dosage forms.

IMPURITIES

A. maltitol,

B. sorbitol,



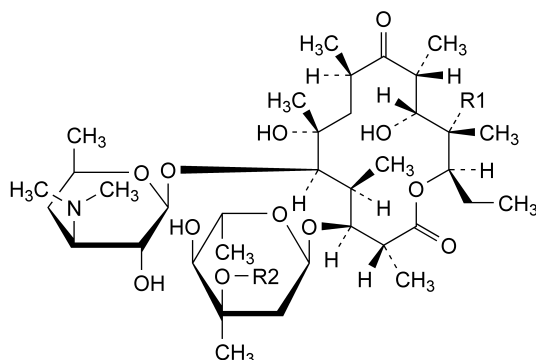
C. (2*R*,3*S*,4*S*)-pentane-1,2,3,4,5-pentol (*meso*-ribitol),

D. glycerol.

01/2005:0179

ERYTHROMYCIN

Erythromycinum



Erythromycin	Mol. Formula	M _r	R1	R2
A	C ₃₇ H ₆₇ NO ₁₃	734	OH	CH ₃
B	C ₃₇ H ₆₇ NO ₁₂	718	H	CH ₃
C	C ₃₆ H ₆₅ NO ₁₃	720	OH	H

DEFINITION

Mixture of macrolide antibiotics produced by a strain of *Streptomyces erythreus*, the main component being (3*R*,4*S*,5*S*,6*R*,7*R*,9*R*,11*R*,12*R*,13*S*,14*R*)-4-[(2,6-dideoxy-3-*C*-methyl-3-*O*-methyl- α -*L*-ribo-hexopyranosyl)oxy]-14-ethyl-7,12,13-trihydroxy-3,5,7,9,11,13-hexamethyl-6-[(3,4,6-trideoxy-3-dimethylamino- β -*D*-xylo-hexopyranosyl)-oxy]oxacyclotetradecane-2,10-dione (erythromycin A).

Content:

- sum of the contents of erythromycin A, erythromycin B and erythromycin C: 93.0 per cent to 102.0 per cent (anhydrous substance),
- erythromycin B: maximum 5.0 per cent,
- erythromycin C: maximum 5.0 per cent.

CHARACTERS

Appearance: white or slightly yellow powder or colourless or slightly yellow crystals, slightly hygroscopic.

Solubility: slightly soluble in water (the solubility decreases as the temperature rises), freely soluble in alcohol, soluble in methanol.

IDENTIFICATION

First identification: A.

Second identification: B, C, D.

A. Infrared absorption spectrophotometry (2.2.24).

Comparison: erythromycin CRS.

Disregard any band in the region from 1980 cm⁻¹ to 2050 cm⁻¹.

If the spectra obtained show differences, dissolve 50 mg of the substance to be examined and of the reference substance separately in 1.0 ml of *methylene chloride R*, dry at 60 °C at a pressure not exceeding 670 Pa for 3 h and record new spectra using the residues.

B. Thin-layer chromatography (2.2.27).

Test solution. Dissolve 10 mg of the substance to be examined in *methanol R* and dilute to 10 ml with the same solvent.

Reference solution (a). Dissolve 10 mg of *erythromycin A CRS* in *methanol R* and dilute to 10 ml with the same solvent.

Reference solution (b). Dissolve 20 mg of *spiramycin CRS* in *methanol R* and dilute to 10 ml with the same solvent.

Plate: TLC silica gel G plate *R*.

Mobile phase: mix 4 volumes of *2-propanol R*, 8 volumes of a 150 g/l solution of *ammonium acetate R* previously adjusted to pH 9.6 with *ammonia R* and 9 volumes of *ethyl acetate R*. Allow to settle and use the upper layer.

Application: 10 μ l.

Development: over 2/3 of the plate.

Drying: in air.

Detection: spray with *anisaldehyde solution R1* and heat at 110 °C for 5 min.

Results: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a) and its position and colour are different from those of the spots in the chromatogram obtained with reference solution (b).

- C. To about 5 mg add 5 ml of a 0.2 g/l solution of *xanthidrol R* in a mixture of 1 volume of *hydrochloric acid R* and 99 volumes of *acetic acid R* and heat on a water-bath. A red colour develops.
- D. Dissolve about 10 mg in 5 ml of *hydrochloric acid R1* and allow to stand for 10-20 min. A yellow colour develops.

TESTS

Specific optical rotation (2.2.7): –71 to –78 (anhydrous substance).

Dissolve 1.00 g in *ethanol R* and dilute to 50.0 ml with the same solvent. The specific optical rotation is determined at least 30 min after preparing the solution.

Related substances. Liquid chromatography (2.2.29).

Test solution. Dissolve 40.0 mg of the substance to be examined in a mixture of 1 volume of *methanol R* and 3 volumes of *phosphate buffer solution pH 7.0 R1* and dilute to 10.0 ml with the same mixture of solvents.

Reference solution (a). Dissolve 40.0 mg of *erythromycin A CRS* in a mixture of 1 volume of *methanol R* and 3 volumes of *phosphate buffer solution pH 7.0 R1* and dilute to 10.0 ml with the same mixture of solvents.

Reference solution (b). Dissolve 10.0 mg of *erythromycin B CRS* and 10.0 mg of *erythromycin C CRS* in a mixture of 1 volume of *methanol R* and 3 volumes of *phosphate buffer solution pH 7.0 R1* and dilute to 50.0 ml with the same mixture of solvents.

Reference solution (c). Dissolve 5 mg of *N-demethyl-erythromycin A CRS* in reference solution (b). Add 1.0 ml of reference solution (a) and dilute to 25 ml with reference solution (b).

Reference solution (d). Dilute 3.0 ml of reference solution (a) to 100.0 ml with a mixture of 1 volume of *methanol R* and 3 volumes of *phosphate buffer solution pH 7.0 R1*.

Reference solution (e). Transfer 40 mg of *erythromycin A CRS* to a glass vial and spread evenly such that it forms a layer not more than about 1 mm thick. Heat at 130 °C for 4 h. Allow to cool and dissolve in a mixture of 1 volume of *methanol R* and 3 volumes of *phosphate buffer solution pH 7.0 R1* and dilute to 10 ml with the same mixture of solvents.

Column:

- **size:** $l = 0.25$ m, $\varnothing = 4.6$ mm,
- **stationary phase:** *styrene-divinylbenzene copolymer R* (8 μ m) with a pore size of 100 nm,
- **temperature:** 70 °C using a water-bath for the column and at least one-third of the tubing preceding the column.

Mobile phase: to 50 ml of a 35 g/l solution of *dipotassium hydrogen phosphate R* adjusted to pH 9.0 ± 0.05 with *dilute phosphoric acid R*, add 400 ml of *water R*, 165 ml of *2-methyl-2-propanol R* and 30 ml of *acetonitrile R*, and dilute to 1000 ml with *water R*.

Flow rate: 2.0 ml/min.

Detection: spectrophotometer at 215 nm.

Injection: 100 μ l; inject the test solution and reference solutions (c), (d) and (e).

Run time: 5 times the retention time of erythromycin A.

Relative retention with reference to erythromycin A (retention time = about 15 min): impurity A = about 0.3; impurity B = about 0.45; erythromycin C = about 0.5; impurity C = about 0.9; impurity D = about 1.4; impurity F = about 1.5; erythromycin B = about 1.8; impurity E = about 4.3.

System suitability: reference solution (c):

- **resolution:** minimum 0.8 between the peaks due to impurity B and erythromycin C and minimum 5.5 between the peaks due to impurity B and erythromycin A. If necessary, adjust the concentration of 2-methyl-2-propanol in the mobile phase or reduce the flow rate to 1.5 ml or 1.0 ml/min.

Limits:

- **correction factors:** for the calculation of contents, multiply the peak areas of the following impurities (use the chromatogram obtained with reference solution (e) to identify them) by the corresponding correction factor: impurity E = 0.09; impurity F = 0.15,
- **any impurity:** not more than the area of the principal peak in the chromatogram obtained with reference solution (d) (3.0 per cent),
- **total:** not more than 2.3 times the area of the principal peak in the chromatogram obtained with reference solution (d) (7.0 per cent),
- **disregard limit:** 0.02 times the area of the principal peak in the chromatogram obtained with reference solution (d) (0.06 per cent); disregard the peaks due to erythromycin B and erythromycin C.

Thiocyanate: maximum 0.3 per cent.

Prepare the solutions immediately before use and protect from actinic light.

Compensation liquid. Dilute 1.0 ml of a 90 g/l solution of *ferric chloride R* to 50.0 ml with *methanol R*.

Test solution. Dissolve 0.100 g (m g) of the substance to be examined in 20 ml of *methanol R*, add 1.0 ml of a 90 g/l solution of *ferric chloride R* and dilute to 50.0 ml with *methanol R*.

Prepare 2 independent reference solutions.

Reference solution. Dissolve 0.100 g of *potassium thiocyanate R*, previously dried at 105 °C for 1 h, in *methanol R* and dilute to 50.0 ml with the same solvent. Dilute 5.0 ml to 50.0 ml with *methanol R*. To 5.0 ml of this solution, add 1.0 ml of a 90 g/l solution of *ferric chloride R* and dilute to 50.0 ml with *methanol R*.

Measure the absorbances (2.2.25) of each reference solution (A_1 , A_2) and of the test solution (A) at the maximum (about 492 nm).

Suitability value:

$$S = \frac{m_2 \times A_1}{m_1 \times A_2}$$

m_1 , m_2 = mass of the potassium thiocyanate used to prepare the respective reference solutions, in grams.

The test is not valid unless S is not less than 0.985 and not more than 1.015.

Calculate the percentage content of thiocyanate from the expression:

$$\frac{A \times 58.08 \times 0.5}{m \times 97.18} \times \left(\frac{m_1}{A_1} + \frac{m_2}{A_2} \right)$$

58.08 = relative molecular mass of the thiocyanate moiety,

97.18 = relative molecular mass of potassium thiocyanate.

Water (2.5.12): maximum 6.5 per cent, determined on 0.200 g.

Use a 100 g/l solution of *imidazole R* in *anhydrous methanol R* as the solvent.

Sulphated ash (2.4.14): maximum 0.2 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (2.2.29) as described in the test for related substances with the following modifications.

Injection: test solution and reference solutions (a) and (b).

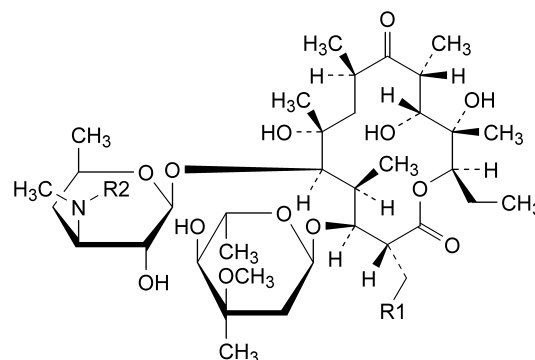
System suitability: reference solution (a):

- **repeatability:** maximum relative standard deviation of 1.2 per cent for 6 replicate injections.

Calculate the percentage content of erythromycin A using the chromatogram obtained with reference solution (a). Calculate the percentage contents of erythromycin B and erythromycin C using the chromatogram obtained with reference solution (b).

STORAGE

Protected from light.

IMPURITIES

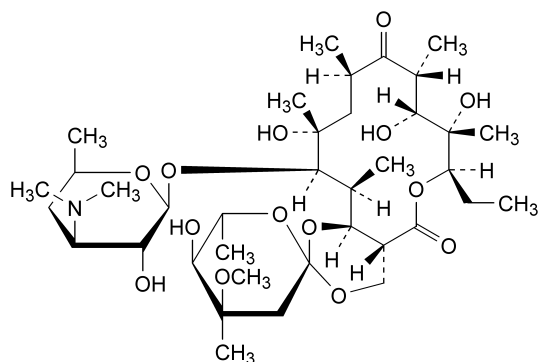
A. R1 = OH, R2 = CH₃: erythromycin F,

B. R1 = R2 = H: *N*-demethylerythromycin A,

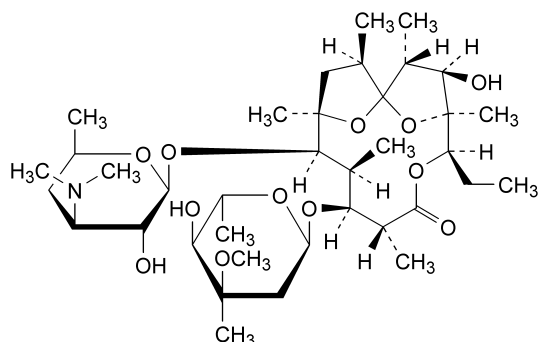
01/2005:0552

ERYTHROMYCIN ESTOLATE

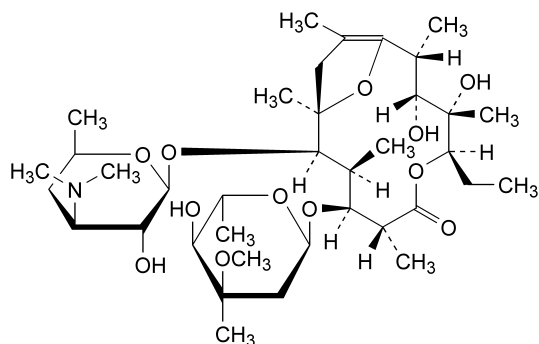
Erythromycini estolas



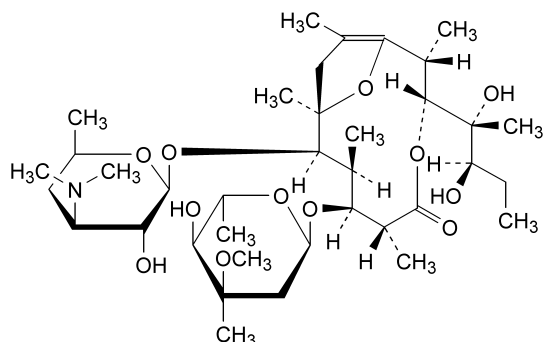
C. erythromycin E,



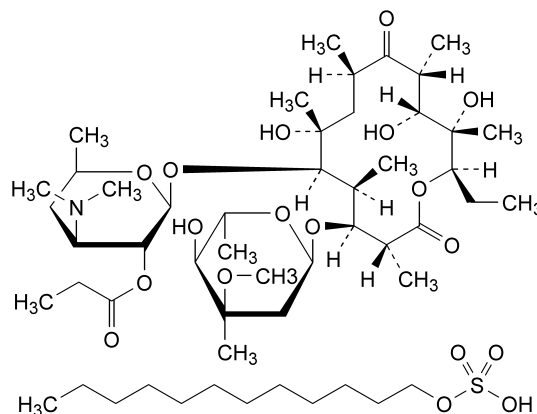
D. anhydroerythromycin A,



E. erythromycin A enol ether,



F. pseudoerythromycin A enol ether.

 $C_{52}H_{97}NO_{18}S$ M_r 1056

DEFINITION

Erythromycin estolate (or erythromycin 2'-propionate dodecyl sulphate) is (3*R*,4*S*,5*S*,6*R*,7*R*,9*R*,11*R*,12*R*,13*S*,14*R*)-4-[(2,6-dideoxy-3-*C*-methyl-3-*O*-methyl- α -L-*ribo*-hexopyranosyl)oxy]-14-ethyl-7,12,13-trihydroxy-3,5,7,9,11,13-hexamethyl-6-[[3,4,6-trideoxy-3-(dimethylamino)-2-*O*-propionyl- β -D-*xylo*-hexopyranosyl]oxy]oxacyclotetradecane-2,10-dione dodecyl sulphate. The potency is not less than 610 IU/mg, calculated with reference to the anhydrous substance.

CHARACTERS

A white, crystalline powder, practically insoluble in water, freely soluble in alcohol, soluble in acetone. It is practically insoluble in dilute hydrochloric acid.

IDENTIFICATION

First identification: A, D.

Second identification: B, C, D.

- Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *erythromycin estolate CRS*.
- Examine the chromatograms obtained in the test for related substances. The principal spot in the chromatogram obtained with test solution (b) is similar in position and colour to the principal spot in the chromatogram obtained with reference solution (a). The test is not valid unless the chromatogram obtained with reference solution (b) shows two clearly separated spots.
- Suspend about 3 mg in 2 ml of *dilute sulphuric acid R*. Add 0.1 ml of a 0.1 g/l solution of *methylene blue R* and 2 ml of *chloroform R* and shake. The chloroform layer is blue.
- Dissolve about 10 mg in 5 ml of *hydrochloric acid R1* and allow to stand for 10 min to 20 min. A yellow colour develops.

TESTS

pH (2.2.3). Suspend 0.4 g in 10 ml of *carbon dioxide-free water R*. Shake for 5 min and allow to stand. The pH of the clear supernatant liquid is 5.5 to 7.0.

Related substances. Examine by thin-layer chromatography (2.2.27), using *silica gel G R* as the coating substance.

01/2005:0274

Test solution (a). Dissolve 40 mg of the substance to be examined in *acetone R* and dilute to 10 ml with the same solvent.

Test solution (b). Dilute 2.5 ml of test solution (a) to 10 ml with *acetone R*.

Reference solution (a). Dissolve 10 mg of *erythromycin estolate CRS* in *acetone R* and dilute to 10 ml with the same solvent.

Reference solution (b). Dissolve 10 mg of *erythromycin estolate CRS* and 10 mg of *erythromycin ethylsuccinate CRS* in *acetone R* and dilute to 10 ml with the same solvent.

Reference solution (c). Dissolve 8 mg of *erythromycin CRS* in *acetone R* and dilute to 100 ml with the same solvent.

Apply separately to the plate 10 µl of each solution. Develop over a path of 15 cm using a mixture of 1 volume of a 150 g/l solution of *ammonium acetate R* previously adjusted to pH 7.0, 15 volumes of *alcohol R* and 85 volumes of *chloroform R*. Allow the plate to dry in air and spray with *anisaldehyde solution R*. Heat at 110 °C for 5 min and allow to cool. Any spot in the chromatogram obtained with test solution (a), apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (c) (2.0 per cent).

Dodecyl sulphate. 23.0 per cent to 25.5 per cent of $C_{12}H_{26}O_4S$, calculated with reference to the anhydrous substance. Dissolve 0.500 g of the substance to be examined in 25 ml of *dimethylformamide R*. Titrate with 0.1 M *sodium methoxide* using 0.05 ml of a 3 g/l solution of *thymol blue R* in *methanol R* as indicator.

1 ml of 0.1 M *sodium methoxide* is equivalent to 26.64 mg of $C_{12}H_{26}O_4S$.

Water (2.5.12). Not more than 4.0 per cent, determined on 0.300 g by the semi-micro determination of water. Use a 100 g/l solution of *imidazole R* in *anhydrous methanol R* as the solvent.

Sulphated ash (2.4.14). Not more than 0.5 per cent, determined on 0.5 g.

ASSAY

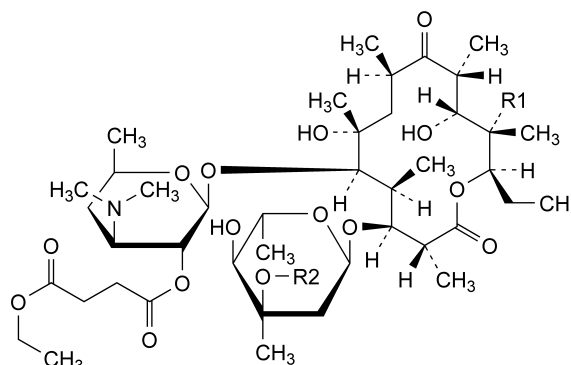
Dissolve 40.0 mg in 40 ml of *methanol R*, add 20 ml of *phosphate buffer solution pH 7.0 R* and dilute to 100.0 ml with *water R*. Maintain at 60 °C for 3 h and allow to cool. Carry out the microbiological assay of antibiotics (2.7.2). Use *erythromycin CRS* as the reference substance.

STORAGE

Store in an airtight container, protected from light, at a temperature below 30 °C.

ERYTHROMYCIN ETHYLSUCCINATE

Erythromycini ethylsuccinas



Ethylsuccinate compound	Mol. Formula	M_r	R1	R2
Erythromycin A	$C_{43}H_{75}NO_{16}$	862	OH	CH_3
Erythromycin B	$C_{43}H_{75}NO_{15}$	846	H	CH_3
Erythromycin C	$C_{42}H_{73}NO_{16}$	848	OH	H

DEFINITION

Main component: (3R,4S,5S,6R,7R,9R,11R,12R,13S,14R)-4-[(2,6-dideoxy-3-C-methyl-3-O-methyl- α -L-ribo-hexopyranosyl)oxy]-14-ethyl-7,12,13-trihydroxy-3,5,7,9,11,13-hexamethyl-6-[[3,4,6-trideoxy-3-(dimethylamino)-2-O-(4-ethoxy-4-oxobutanoyl)- β -D-xyllo-hexopyranosyl]oxy]oxacyclotetradecane-2,10-dione (erythromycin A ethylsuccinate).

Content:

- sum of erythromycin A, erythromycin B and erythromycin C: minimum 78.0 per cent (anhydrous substance),
- erythromycin B: maximum 5.0 per cent (anhydrous substance),
- erythromycin C: maximum 5.0 per cent (anhydrous substance).

CHARACTERS

Appearance: white, crystalline powder, hygroscopic.

Solubility: practically insoluble in water, freely soluble in acetone, in ethanol and in methanol.

IDENTIFICATION

Infrared absorption spectrophotometry (2.2.24).

Comparison: *erythromycin ethylsuccinate CRS*.

TESTS

Specific optical rotation (2.2.7): -70 to -82 (anhydrous substance).

Dissolve 0.100 g in *acetone R* and dilute to 10.0 ml with the same solvent. Measure the angle of rotation at least 30 min after preparing the solution.

Related substances. Liquid chromatography (2.2.29).

Hydrolysis solution. A 20 g/l solution of *dipotassium hydrogen phosphate R* adjusted to pH 8.0 with *phosphoric acid R*.

Test solution. Dissolve 0.115 g of the substance to be examined in 25 ml of *methanol R*. Add 20 ml of the hydrolysis solution, mix and allow to stand at room temperature for at least 12 h. Dilute to 50.0 ml with the hydrolysis solution.

Reference solution (a). Dissolve 40.0 mg of erythromycin A CRS in 10 ml of methanol R and dilute to 20.0 ml with the hydrolysis solution.

Reference solution (b). Dissolve 10.0 mg of erythromycin B CRS and 10.0 mg of erythromycin C CRS in 50 ml of methanol R. Add 5.0 ml of reference solution (a) and dilute to 100.0 ml with the hydrolysis solution.

Reference solution (c). Dissolve 2 mg of *N*-demethylethylerythromycin A CRS in 20 ml of reference solution (b).

Reference solution (d). Dilute 3.0 ml of reference solution (a) to 100.0 ml with a mixture of equal volumes of methanol R and the hydrolysis solution.

Reference solution (e). Dissolve 40 mg of erythromycin A CRS, previously heated at 130 °C for 3 h, in 10 ml of methanol R and dilute to 20 ml with the hydrolysis solution.

Column:

- size: $l = 0.25$ m, $\varnothing = 4.6$ mm,
- stationary phase: styrene-divinylbenzene copolymer R (8 μ m) with a pore size of 100 nm,
- temperature: 70 °C using a water-bath for the column and at least one third of the tubing preceding the column.

Mobile phase: to 50 ml of a 35 g/l solution of dipotassium hydrogen phosphate R adjusted to pH 8.0 with dilute phosphoric acid R, add 400 ml of water R, 165 ml of 2-methyl-2-propanol R and 30 ml of acetonitrile R, and dilute to 1000 ml with water R.

Flow rate: 2.0 ml/min.

Detection: spectrophotometer at 215 nm.

Injection: 200 μ l; inject the test solution and reference solutions (a), (c), (d) and (e).

Run time: 5 times the retention time of erythromycin A; begin integration after the hydrolysis peak.

Relative retention with reference to erythromycin A (retention time = about 15 min): hydrolysis peak = less than 0.3; impurity B = about 0.45; erythromycin C = about 0.5; impurity C = about 0.9; impurity G = about 1.3; impurity D = about 1.4; impurity F = about 1.5; erythromycin B = about 1.8; impurity E = about 4.3.

System suitability: reference solution (c):

- resolution: minimum 0.8 between the peaks due to impurity B and to erythromycin C and minimum 5.5 between the peaks due to impurity B and to erythromycin A.

Limits:

- correction factors: for the calculation of contents, multiply the peak areas of the following impurities by the corresponding correction factor: impurity E = 0.09; impurity F = 0.15; impurity G = 0.14; use the chromatogram obtained with reference solution (e) to identify the peaks due to impurities E and F.
- any impurity: not more than the area of the principal peak in the chromatogram obtained with reference solution (d) (3.0 per cent).

- total: not more than 1.67 times the area of the principal peak in the chromatogram obtained with reference solution (d) (5.0 per cent).
- disregard limit: 0.02 times the area of the principal peak in the chromatogram obtained with reference solution (d) (0.06 per cent).

Free erythromycin. Liquid chromatography (2.2.29).

Test solution. Dissolve 0.250 g of the substance to be examined in acetonitrile R and dilute to 50.0 ml with the same solvent.

Reference solution. Dissolve 75.0 mg of erythromycin A CRS in acetonitrile R and dilute to 50.0 ml with the same solvent. Dilute 5.0 ml of the solution to 25.0 ml with acetonitrile R.

Column:

- size: $l = 0.25$ m, $\varnothing = 4.6$ mm,
- stationary phase: octylsilyl silica gel for chromatography R (5 μ m).

Mobile phase: mix 35 volumes of acetonitrile R and 65 volumes of a solution containing 3.4 g/l of potassium dihydrogen phosphate R and 2.0 g/l of triethylamine R, adjusted to pH 3.0 with dilute phosphoric acid R.

Flow rate: 1 ml/min.

Detection: spectrophotometer at 195 nm.

Injection: 20 μ l.

Run time: twice the retention time of erythromycin A (retention time = about 8 min) for the reference solution and twice the retention time of erythromycin ethylsuccinate (retention time = about 24 min) for the test solution.

Limit:

- free erythromycin: not more than the area of the principal peak in the chromatogram obtained with the reference solution (6.0 per cent).

Water (2.5.12): maximum 3.0 per cent, determined on 0.30 g.

Use a 100 g/l solution of imidazole R in anhydrous methanol R as the solvent.

Sulphated ash (2.4.14): maximum 0.3 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (2.2.29) as described in the test for related substances.

Injection: inject the test solution and reference solutions (a) and (b).

System suitability: reference solution (a):

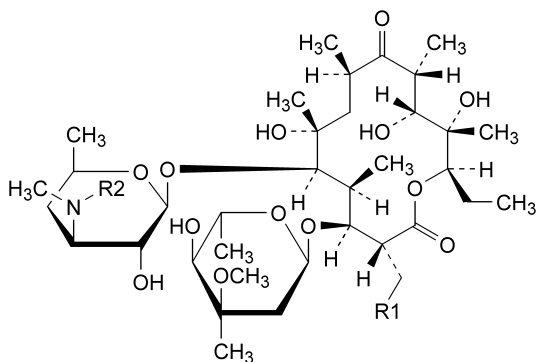
- relative standard deviation: maximum 1.2 per cent for 6 replicate injections.

Calculate the percentage content of erythromycin A using the chromatogram obtained with reference solution (a). Calculate the percentage contents of erythromycin B and erythromycin C using the chromatogram obtained with reference solution (b).

STORAGE

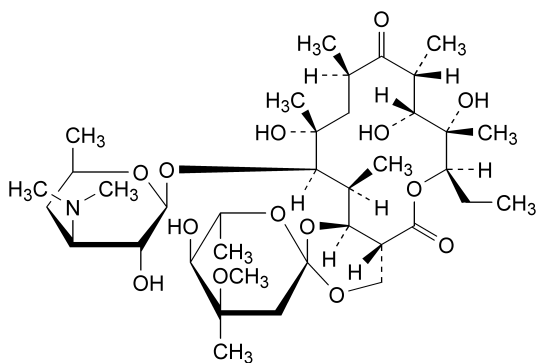
In an airtight container, protected from light.

IMPURITIES

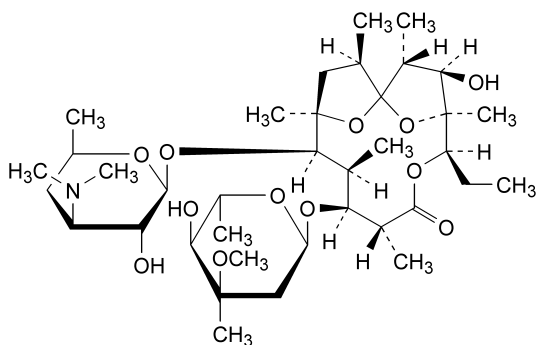


A. R1 = OH, R2 = CH₃: erythromycin F,

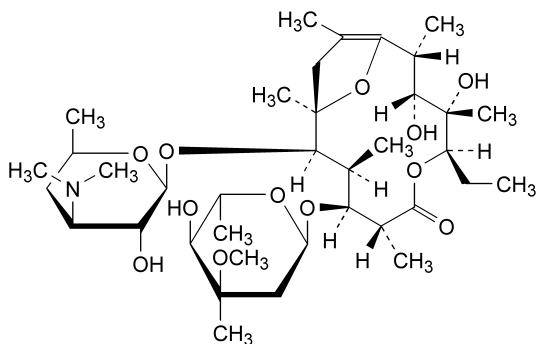
B. R1 = R2 = H: *N*-demethylerythromycin A,



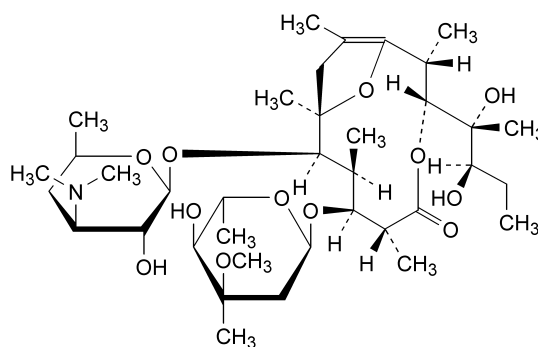
C. erythromycin E,



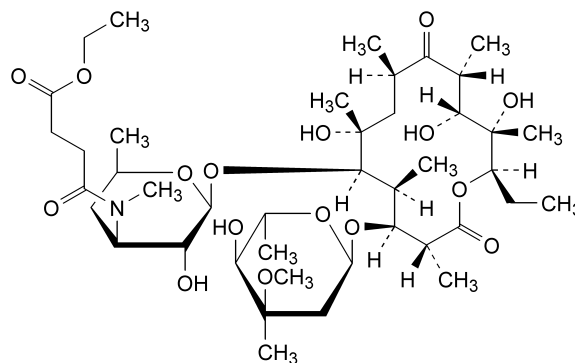
D. anhydroerythromycin A,



E. erythromycin A enol ether,



F. pseudoerythromycin A enol ether,

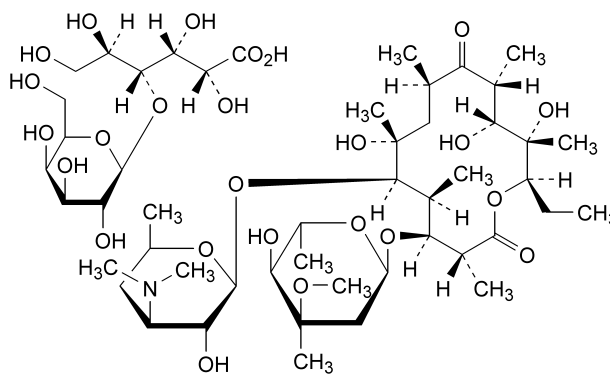


G. erythromycin *N*-ethylsuccinate.

01/2005:1098

ERYTHROMYCIN LACTOBIONATE

Erythromycini lactobionas



C₄₉H₈₉NO₂₅

*M*_r 1092

DEFINITION

Erythromycin lactobionate is a mixture of lactobionates of macrolide antibiotics. The main component is (3*R*,4*S*,5*S*,6*R*,7*R*,9*R*,11*R*,12*R*,13*S*,14*R*)-4-[(2,6-dideoxy-3-*C*-methyl-3-*O*-methyl- α -*L*-ribo-hexopyranosyl)oxy]-14-ethyl-7,12,13-trihydroxy-3,5,7,9,11,13-hexamethyl-6-[[[3,4,6-trideoxy-3-(dimethylamino)- β -*D*-xylo-hexopyranosyl]oxy]oxacyclotetradecane-2,10-dione 4-*O*- β -*D*-galactopyranosyl-*D*-gluconate. The sum of the content of erythromycin A lactobionate, of erythromycin B lactobionate and of erythromycin C lactobionate is not less than 93.0 per cent and not more than the equivalent of 100.5 per cent, calculated with reference to the anhydrous substance.

CHARACTERS

A white or slightly yellow powder, hygroscopic, soluble in water, freely soluble in ethanol and in methanol, very slightly soluble in acetone and in methylene chloride.

IDENTIFICATION

- A. Examine by thin-layer chromatography (2.2.27) using *silica gel H R* as the coating substance.

Test solution. Dissolve 30 mg of the substance to be examined in *methanol R* and dilute to 10 ml with the same solvent.

Reference solution (a). Dissolve 20 mg of *erythromycin A CRS* in *methanol R* and dilute to 10 ml with the same solvent.

Reference solution (b). Dissolve 10 mg of *lactobionic acid R* in *water R* and dilute to 10 ml with the same solvent.

Apply separately to the plate 5 µl of each solution. Develop over a path of 15 cm using a mixture of 3 volumes of *glacial acetic acid R*, 10 volumes of *water R* and 90 volumes of *methanol R*. Allow the plate to dry in air, spray with a 5 g/l solution of *potassium permanganate R* in 1 M *sodium hydroxide* and heat at 110 °C for 5 min. The chromatogram obtained with the test solution shows two spots, one of which corresponds in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a) and the other to the principal spot in the chromatogram obtained with reference solution (b).

- B. To about 5 mg add 5 ml of a 0.2 g/l solution of *xanthidrol R* in a mixture of 1 volume of *hydrochloric acid R* and 99 volumes of *acetic acid R*. A red colour develops.
- C. Dissolve about 10 mg in 5 ml of *hydrochloric acid R1*. A yellowish-green colour develops.

TESTS

Appearance of solution. Dissolve 1.0 g in 20 ml of *water R*. The solution is clear (2.2.1) and colourless (2.2.2, *Method II*).

pH (2.2.3). Dissolve 0.50 g in *carbon dioxide-free water R* and dilute to 25 ml with the same solvent. The pH of the solution is 6.5 to 7.5.

Related substances. Examine by liquid chromatography (2.2.29), as described under Assay. The content of erythromycin B lactobionate and that of erythromycin C lactobionate is not greater than 5.0 per cent. Inject reference solution (d). Inject the test solution and continue the chromatography for five times the retention time of erythromycin A. In the chromatogram obtained with the test solution, the area of any peak, apart from the peaks corresponding to erythromycin A, erythromycin B or erythromycin C, is not greater than the area of the principal peak in the chromatogram obtained with reference solution (d) (3.0 per cent).

Free lactobionic acid. Not more than 1.0 per cent of $C_{12}H_{22}O_{12}$, calculated with reference to the anhydrous substance. Dissolve 0.400 g in 50 ml of *water R*. Titrate with 0.1 M *sodium hydroxide*, determining the end-point potentiometrically (2.2.20). Calculate the volume of 0.1 M *sodium hydroxide* required per gram of the substance to be examined (n_1 ml). Dissolve 0.500 g in 40 ml of *anhydrous acetic acid R* and titrate with 0.1 M *perchloric*

acid, determining the end-point potentiometrically (2.2.20). Calculate the volume of 0.1 M *perchloric acid* required per gram of the substance to be examined (n_2 ml).

Calculate the percentage content of $C_{12}H_{22}O_{12}$ from the expression:

$$3.580 (n_1 - n_2)$$

Water (2.5.12). Not more than 5.0 per cent, determined on 0.200 g by the semi-micro determination of water. Use a 100 g/l solution of *imidazole R* in *anhydrous methanol R* as the solvent.

Sulphated ash (2.4.14). Not more than 0.5 per cent, determined on 1.0 g.

Pyrogens (2.6.8). If intended for use in the manufacture of parenteral dosage forms without a further appropriate procedure for the removal of pyrogens, it complies with the test for pyrogens. Inject per kilogram of the rabbit's mass 1 ml of a solution in *water for injections R* containing 7.4 mg of the substance to be examined per millilitre (equivalent to 5 mg of erythromycin).

ASSAY

Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 60.0 mg of the substance to be examined in a mixture of 1 volume of *methanol R* and 3 volumes of *phosphate buffer solution pH 7.0 R* and dilute to 10.0 ml with the same mixture of solvents.

Reference solution (a). Dissolve 40.0 mg of *erythromycin A CRS* in a mixture of 1 volume of *methanol R* and 3 volumes of *phosphate buffer solution pH 7.0 R* and dilute to 10.0 ml with the same mixture of solvents.

Reference solution (b). Dissolve 10.0 mg of *erythromycin B CRS* and 10.0 mg of *erythromycin C CRS* in a mixture of 1 volume of *methanol R* and 3 volumes of *phosphate buffer solution pH 7.0 R* and dilute to 50.0 ml with the same mixture of solvents.

Reference solution (c). Dissolve 5 mg of *N-demethyl-erythromycin A CRS* in reference solution (b). Add 1.0 ml of reference solution (a) and dilute to 25 ml with reference solution (b).

Reference solution (d). Dilute 3.0 ml of reference solution (a) to 100.0 ml with a mixture of 1 volume of *methanol R* and 3 volumes of *phosphate buffer solution pH 7.0 R*.

The test solution and the reference solutions can be used within one day if stored at 5 °C.

The chromatographic procedure may be carried out using:

- a column 0.25 m long and 4.6 mm in internal diameter packed with *styrene-divinylbenzene copolymer R* (8 µm to 10 µm) with a pore size of 100 nm,
- as mobile phase at a flow rate of 2.0 ml/min a solution prepared as follows: to 50 ml of a 35 g/l solution of *dipotassium hydrogen phosphate R* adjusted to pH 9.0 with *dilute phosphoric acid R*, add 400 ml of *water R*, 165 ml of *2-methyl-2-propanol R* and 30 ml of *acetonitrile R*, and dilute to 1000 ml with *water R*,
- as detector a spectrophotometer set at 215 nm,
- a 100 µl injector,
- an electronic integrator,

maintaining the column at 70 °C, preferably using a water-bath. Inject reference solution (c). Adjust the sensitivity of the system so that the height of the peaks is at least 25 per cent of the full scale of the recorder. The substances are eluted in the following order: *N-demethylerythromycin A*, erythromycin C,

erythromycin A and erythromycin B. The assay is not valid unless the resolution between the peaks corresponding to *N*-demethylethromycin A and erythromycin C is at least 0.8 and the resolution between the peaks corresponding to *N*-demethylethromycin A and erythromycin A is at least 5.5. If necessary, adjust the concentration of 2-methyl-2-propanol in the mobile phase or reduce the flow rate to 1.5 ml/min or 1.0 ml/min. Inject reference solution (a) six times. The assay is not valid unless the relative standard deviation of the area of the peak due to erythromycin A is at most 2.0 per cent. Inject alternately the test solution and reference solutions (a) and (b).

Calculate the percentage content of erythromycin A using the chromatogram obtained with reference solution (a). Express the result as erythromycin A lactobionate by multiplying the percentage content of erythromycin A by 1.4877. Calculate the percentage contents of erythromycin B and erythromycin C using the chromatogram obtained with reference solution (b). Express the result as erythromycin B lactobionate and as erythromycin C lactobionate by multiplying by 1.4877.

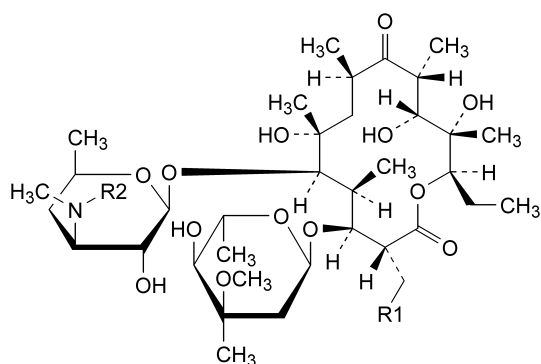
STORAGE

Store in an airtight container at a temperature not exceeding 25 °C. If the substance is sterile, store in a sterile, airtight, tamper-proof container.

LABELLING

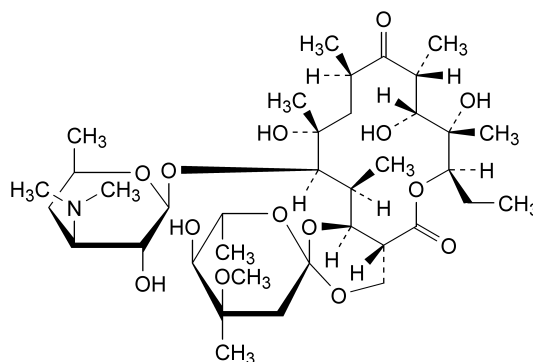
The label states, where applicable, that the substance is free from pyrogens.

IMPURITIES

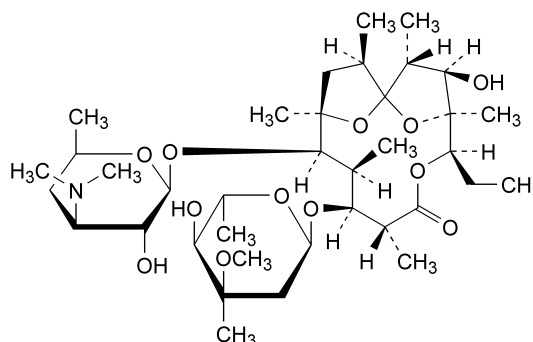


A. R1 = OH, R2 = CH₃: erythromycin F,

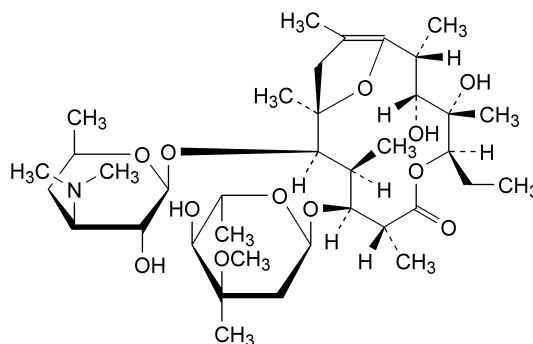
B. R1 = R2 = H: *N*-demethylethromycin A,



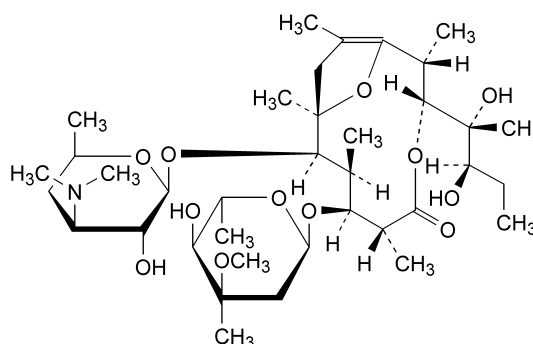
C. erythromycin E,



D. anhydroerythromycin A,



E. erythromycin A enol ether,

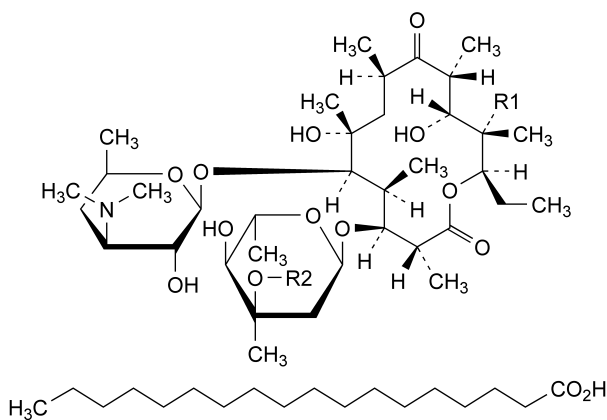


F. pseudoerythromycin A enol ether.

01/2005:0490

ERYTHROMYCIN STEARATE

Erythromycini stearas



Erythromycin	Mol. Formula	R1	R2
A	C ₅₅ H ₁₀₃ NO ₁₅	OH	CH ₃
B	C ₅₅ H ₁₀₃ NO ₁₄	H	CH ₃
C	C ₅₄ H ₁₀₁ NO ₁₅	OH	H

C₅₅H₁₀₃NO₁₅M_r 1018

DEFINITION

A mixture of the stearates of erythromycin and stearic acid. The main component is the octadecanoate of (3*R*,4*S*,5*S*,6*R*,7*R*,9*R*,11*R*,12*R*,13*S*,14*R*)-4-[(2,6-dideoxy-3-*C*-methyl-3-*O*-methyl- α -*L*-ribo-hexopyranosyl)oxy]-14-ethyl-7,12,13-trihydroxy-3,5,7,9,11,13-hexamethyl-6-[[3,4,6-trideoxy-3-(dimethylamino)- β -*D*-xylo-hexopyranosyl]oxy]oxacyclotetradecane-2,10-dione (erythromycin A stearate).

Content:

- sum of the contents of erythromycin A, erythromycin B and erythromycin C: minimum 60.5 per cent (anhydrous substance),
- erythromycin B: maximum 5.0 per cent,
- erythromycin C: maximum 5.0 per cent.

CHARACTERS

Appearance: white, crystalline powder.

Solubility: practically insoluble in water, soluble in acetone and in methanol.

Solutions may be opalescent.

IDENTIFICATION

A. Infrared absorption spectrophotometry (2.2.24).

Comparison: erythromycin stearate CRS.

B. Thin-layer chromatography (2.2.27).

Test solution. Dissolve 28 mg of the substance to be examined in methanol *R* and dilute to 10 ml with the same solvent.

Reference solution (a). Dissolve 20 mg of erythromycin A CRS in methanol *R* and dilute to 10 ml with the same solvent.

Reference solution (b). Dissolve 10 mg of stearic acid *R* in methanol *R* and dilute to 10 ml with the same solvent.

Plate: TLC silica gel *G* plate *R*.

Mobile phase: mix 4 volumes of 2-propanol *R*, 8 volumes of a 150 g/l solution of ammonium acetate *R* previously adjusted to pH 9.6 with ammonia *R* and 9 volumes of ethyl acetate *R*. Allow to settle and use the upper layer.

Application: 5 μ l.

Development: over 2/3 of the plate.

Drying: in air.

Detection A: spray with a solution containing 0.2 g/l of dichlorofluorescein *R* and 0.1 g/l of rhodamine B *R* in alcohol *R*. Maintain the plate for a few seconds in the vapour above a water-bath. Examine in ultraviolet light at 365 nm.

Results A: the chromatogram obtained with the test solution shows 2 spots, one of which corresponds in position to the principal spot in the chromatogram obtained with reference solution (a) and the other to the principal spot in the chromatogram obtained with reference solution (b).

Detection B: spray the plate with anisaldehyde solution *R1*. Heat at 110 °C for 5 min and examine in daylight.

Results B: the spot in the chromatogram obtained with the test solution corresponds in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).

TESTS

Free stearic acid: maximum 14.0 per cent (anhydrous substance) of C₁₈H₃₆O₂.

Dissolve 0.400 g in 50 ml of methanol *R*. Titrate with 0.1 *M* sodium hydroxide, determining the end-point potentiometrically (2.2.20). Calculate the volume of 0.1 *M* sodium hydroxide required per gram of the substance to be examined (n_1 ml). Dissolve 0.500 g in 30 ml of methylene chloride *R*. If the solution is opalescent, filter and shake the residue with 3 quantities, each of 25 ml, of methylene chloride *R*. Filter, if necessary, and rinse the filter with methylene chloride *R*. Reduce the volume of the combined filtrate and rinsings to 30 ml by evaporation on a water-bath. Add 50 ml of glacial acetic acid *R* and titrate with 0.1 *M* perchloric acid, determining the end-point potentiometrically (2.2.20). Calculate the volume of 0.1 *M* perchloric acid required per gram of the substance to be examined (n_2 ml).

Calculate the percentage content of C₁₈H₃₆O₂ from the expression:

$$2.845 (n_1 - n_2) \times \frac{100}{100 - h}$$

h = percentage water content.

Related substances. Liquid chromatography (2.2.29).

Test solution. Dissolve 55.0 mg of the substance to be examined in 5.0 ml of methanol *R* and dilute to 10.0 ml with buffer solution pH 8.0 *R1*. Centrifuge and use the clear solution.

Reference solution (a). Dissolve 40.0 mg of erythromycin A CRS in 5.0 ml of methanol *R* and dilute to 10.0 ml with buffer solution pH 8.0 *R1*.

Reference solution (b). Dissolve 10.0 mg of erythromycin B CRS and 10.0 mg of erythromycin C CRS in 25.0 ml of methanol *R* and dilute to 50.0 ml with buffer solution pH 8.0 *R1*.

Reference solution (c). Dissolve 5 mg of *N*-demethylerythromycin A CRS in reference solution (b). Add 1.0 ml of reference solution (a) and dilute to 25 ml with reference solution (b).

Reference solution (d). Dilute 3.0 ml of reference solution (a) to 100.0 ml with a mixture of equal volumes of *methanol R* and *buffer solution pH 8.0 R1*.

Reference solution (e). Transfer 40 mg of *erythromycin A CRS* to a glass vial and spread evenly such that it forms a layer not more than about 1 mm thick. Heat at 130 °C for 4 h. Allow to cool and dissolve in a mixture of 1 volume of *methanol R* and 3 volumes of *buffer solution pH 8.0 R1* and dilute to 10 ml with the same mixture of solvents.

Column:

- **size:** $l = 0.25$ m, $\varnothing = 4.6$ mm,
- **stationary phase:** *styrene-divinylbenzene copolymer R* (8 μ m) with a pore size of 100 nm,
- **temperature:** 70 °C using a water-bath for the column and at least one third of the tubing preceding the column.

Mobile phase: to 50 ml of a 35 g/l solution of *dipotassium hydrogen phosphate R* adjusted to pH 9.0 ± 0.05 with *dilute phosphoric acid R*, add 400 ml of *water R*, 165 ml of *2-methyl-2-propanol R* and 30 ml of *acetonitrile R*, and dilute to 1000 ml with *water R*.

Flow rate: 2.0 ml/min.

Detection: spectrophotometer at 215 nm.

Injection: 100 μ l; inject the test solution and reference solutions (c), (d) and (e).

Run time: 5 times the retention time of erythromycin A.

Relative retention with reference to erythromycin A (retention time = about 15 min): impurity A = about 0.3; impurity B = about 0.45; erythromycin C = about 0.5; impurity C = about 0.9; impurity D = about 1.4; impurity F = about 1.5; erythromycin B = about 1.8; impurity E = about 4.3.

System suitability: reference solution (c):

- **resolution:** minimum 0.8 between the peaks due to impurity B and to erythromycin C and minimum 5.5 between the peaks due to impurity B and to erythromycin A. If necessary, adjust the concentration of 2-methyl-2-propanol in the mobile phase or reduce the flow rate to 1.5 ml/min or 1.0 ml/min.

Limits:

- **correction factors:** for the calculation of contents, multiply the peak areas of the following impurities (use the chromatogram obtained with reference solution (e) to identify them) by the corresponding correction factor: impurity E = 0.09; impurity F = 0.15,
- **any impurity:** not more than the area of the principal peak in the chromatogram obtained with reference solution (d) (3 per cent),
- **total:** not more than twice the area of the principal peak in the chromatogram obtained with reference solution (d) (6 per cent),
- **disregard limit:** 0.02 times the area of the principal peak in the chromatogram obtained with reference solution (d) (0.06 per cent); disregard the peaks due to erythromycin B and to erythromycin C.

Water (2.5.12): maximum 4.0 per cent, determined on 0.300 g.

Use a 100 g/l solution of *imidazole R* in *anhydrous methanol R* as the solvent.

Sulphated ash (2.4.14): maximum 0.5 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (2.2.29) as described in the test for related substances with the following modifications.

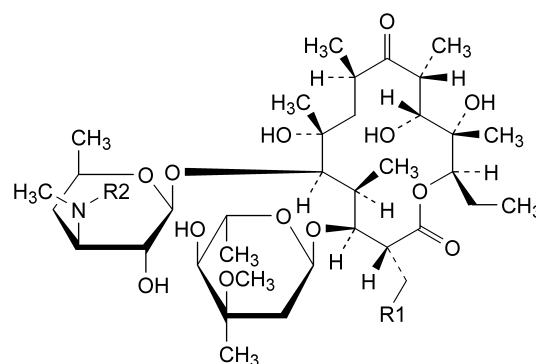
Injection: test solution and reference solutions (a) and (b).

System suitability: reference solution (a):

- **repeatability:** maximum relative standard deviation of 1.2 per cent for 6 replicate injections.

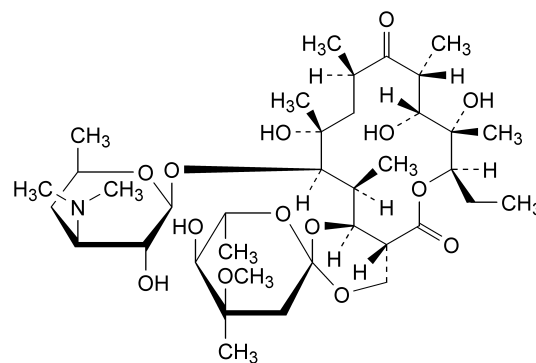
Calculate the percentage content of erythromycin A using the chromatogram obtained with reference solution (a). Calculate the percentage contents of erythromycin B and erythromycin C using the chromatogram obtained with reference solution (b).

IMPURITIES

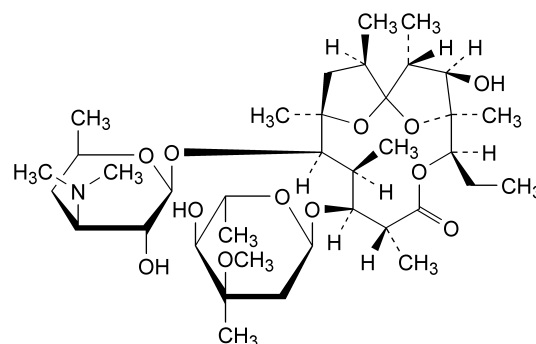


A. R1 = OH, R2 = CH₃: erythromycin F,

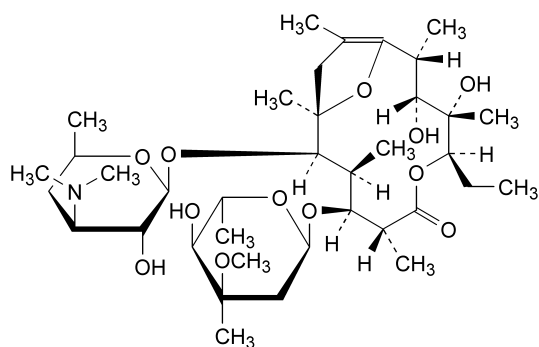
B. R1 = R2 = H: *N*-demethylerythromycin A,



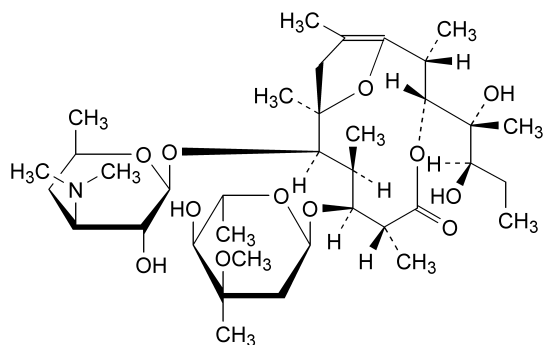
C. erythromycin E,



D. anhydroerythromycin A,



E. erythromycin A enol ether,



F. pseudoerythromycin A enol ether.

01/2005:1316
corrected

ERYTHROPOIETIN CONCENTRATED SOLUTION

Erythropoietini solutio concentrata

APPRLICDSR	VLERYLLEAK	EAENITTGCA
EHCSLNENIT	VPDTKVNIFYA	WKRMEVGQQA
VEVWQGLALL	SEAVLRGQAL	LVNSSQPWEP
LQLHVDKAVS	GLRSLTTLLR	ALGAQKEAIS
PPDAASAAPL	RTITADTFRK	LFRVYSNFLR
GKLKLYTGEA	CRTGD	

 M_r approx. 30 600

DEFINITION

Erythropoietin concentrated solution is a solution containing a family of closely-related glycoproteins which are indistinguishable from the naturally occurring human erythropoietin (urinary erythropoietin) in terms of amino acid sequence (165 amino acids) and average glycosylation pattern, at a concentration of 0.5 mg/ml to 10 mg/ml. It may also contain buffer salts and other excipients. It has a potency of not less than 100 000 IU/mg of active substance determined using the conditions described under Assay and in the test for protein.

PRODUCTION

Erythropoietin is produced in rodent cells *in vitro* by a method based on recombinant DNA technology.

Prior to batch release, the following tests are carried out on each batch of the final product, unless exemption has been granted by the competent authority.

Host cell-derived proteins: the limit is approved by the competent authority.

Host cell- and vector-derived DNA: the limit is approved by the competent authority.

CHARACTERS

Appearance: clear or slightly turbid colourless solution.

IDENTIFICATION

A. It gives the appropriate response when examined using the conditions described under Assay.

B. Capillary zone electrophoresis (2.2.47)

Test solution. Dilute the substance to be examined with *water R* to obtain a concentration of 1 mg/ml. Desalt 0.25 ml of the solution by passage through a micro-concentrator cartridge provided with a membrane with a molecular mass cut-off of not more than 10 000. Add 0.2 ml of *water R* to the sample and desalt again. Repeat the desalting procedure once more. Dilute the sample with *water R*, determine its protein concentration as described under Tests and adjust to a concentration of approximately 1 mg/ml with *water R*.

Reference solution. Dissolve the contents of a vial of *erythropoietin BRP* in 0.25 ml of *water R*. Proceed with desalting as described for the test solution.

Capillary:

- **material:** uncoated fused silica,
- **size:** effective length = about 100 cm, $\varnothing$ = 50 μ m,
- Temperature:** 35 °C.

CZE buffer concentrate (0.1 M sodium chloride, 0.1 M tricine, 0.1 M sodium acetate). Dissolve 0.584 g of sodium chloride *R*, 1.792 g of tricine *R* and 0.820 g of anhydrous sodium acetate *R* in *water R* and dilute to 100.0 ml with the same solvent.

1 M putrescine solution. Dissolve 0.882 g of putrescine *R* in 10 ml of *water R*. Distribute in 0.5 ml aliquots.

CZE buffer (0.01 M tricine, 0.01 M sodium chloride, 0.01 M sodium acetate, 7 M urea, 2.5 mM putrescine). Dissolve 21.0 g of urea *R* in 25 ml of *water R* by warming in a water-bath at 30 °C. Add 5.0 ml of CZE buffer concentrate and 125 μ l of 1 M putrescine solution. Dilute to 50.0 ml with *water R*. Using dilute acetic acid *R*, adjust to pH 5.55 at 30 °C and filter through a 0.45 μ m membrane filter.

Detection: spectrophotometer at 214 nm.

Set the autosampler to store the samples at 4 °C during analysis.

Preconditioning of the capillary: rinse the capillary for 60 min with 0.1 M sodium hydroxide filtered through a 0.45 μ m membrane filter and for 60 min with CZE buffer. Apply voltage for 12 h (20 kV).

Between-run rinsing: rinse the capillary for 10 min with *water R*, for 5 min with 0.1 M sodium hydroxide filtered through a 0.45 μ m membrane filter and for 10 min with CZE buffer.

Injection: under pressure or vacuum.

Migration: apply a field strength of 143 V/cm (15.4 kV for capillaries of 107 cm total length) for 80 min, using CZE buffer as the electrolyte in both buffer reservoirs.

System suitability: in the electropherogram obtained with the reference solution, a pattern of well separated peaks corresponding to the peaks in the *Ph. Eur. reference*

electropherogram of erythropoietin (Figure 1316.-1) is seen, and the largest peak is at least 50 times greater than the baseline noise. If necessary, adjust the sample load to give peaks of sufficient height. Identify the peaks corresponding to isoforms 1 to 8. The peak corresponding to isoform 1 is detected; the resolution between the peaks corresponding to isoforms 5 and 6 is not less than 1. Repeat the separation at least 3 times. The baseline is stable, showing little drift, and the distribution of peaks is qualitatively and quantitatively similar to the distribution of peaks in the *Ph. Eur. reference electropherogram of erythropoietin*. The relative standard deviation of the migration time of the peak corresponding to isoform 2 is less than 2 per cent.

Limits: identify the peaks corresponding to isoforms 1 to 8 in the electropherogram obtained with the test solution by comparison with the electropherogram obtained with the reference solution. Calculate the percentage content of each isoform from the corresponding peak area. The percentages are within the following ranges:

Isoform number	Content (per cent)
1	0 - 15
2	0 - 15
3	5 - 20
4	10 - 35
5	15 - 40
6	10 - 35
7	0 - 20
8	0 - 15

C. Polyacrylamide gel electrophoresis and immunoblotting

(a) Polyacrylamide gel electrophoresis (2.2.31)

Gel dimensions: 0.75 mm thick, about 16 cm square.

Resolving gel: 12 per cent acrylamide.

Sample buffer. SDS-PAGE sample buffer (concentrated) R.

Test solution (a). Dilute the preparation to be examined in *water R* to obtain a concentration of 1.0 mg/ml. To 1 volume of this solution add 1 volume of sample buffer.

Test solution (b). Dilute the preparation to be examined in *water R* to obtain a concentration of 0.1 mg/ml. To 1 volume of this solution add 1 volume of sample buffer.

Reference solution (a). Dissolve the contents of a vial of *erythropoietin BRP* in 0.25 ml of *water R*. To 1 volume of this solution add 1 volume of sample buffer.

Reference solution (b). Dissolve the contents of a vial of *erythropoietin BRP* in *water R* and dilute with the same solvent to obtain a concentration of 0.1 mg/ml. To 1 volume of this solution add 1 volume of sample buffer.

Reference solution (c). A solution of molecular mass markers suitable for calibrating SDS-polyacrylamide gels in the range of 10-70 kDa.

Reference solution (d). A solution of pre-stained molecular mass markers suitable for calibrating SDS-polyacrylamide gels in the range of 10-70 kDa and suitable for the electrotransfer to an appropriate membrane.

Sample treatment: boil for 2 min.

Application: 20 µl, in the following order: reference solution (c), reference solution (a), test solution (a), empty well, reference solution (b), test solution (b), reference solution (d).

At the end of the separation, remove the gel-cassette from the apparatus, and cut the gel into 2 parts: the first part containing reference solution (c), reference solution (a) and test solution (a); the second part containing reference solution (b), test solution (b) and reference solution (d).

Detection: Coomassie staining on the first part of the gel.

System suitability: reference solution (c).

Validation criteria are met.

Results: the electropherogram obtained with test solution (a) shows a single diffuse band corresponding in position and intensity to the single band seen in the electropherogram obtained with reference solution (a).

(b) Immunoblotting

Transfer the second part of the gel onto a membrane suitable for the immobilisation of proteins, using commercially available electrotransfer equipment and following the manufacturer's instructions. After electrotransfer, incubate the membrane in a neutral isotonic buffer containing a suitable blocking agent (for example, 50 g/l of dried milk or 10 per cent V/V foetal calf serum), for 1-2 h, followed by incubation for 1-14 h in the same blocking solution with a suitable dilution of either a polyclonal or monoclonal anti-erythropoietin antibody. Detect erythropoietin-bound antibody using a suitable enzyme- or radiolabelled antibody (for example, an alkaline phosphatase-conjugated second antibody). The precise details of blocking agents, concentrations and incubation times should be optimised using the principles set out in *Immunochemical methods* (2.7.1).

System suitability: in the electropherogram obtained with reference solution (d), the molecular mass markers are resolved on the membrane into discrete bands, with a linear relationship between distance migrated and $\log_{10}$ of the molecular mass.

Results: the electropherogram obtained with test solution (b) shows a single broad band corresponding in position and intensity to the single band seen in the electropherogram obtained with reference solution (b).

D. Peptide mapping

Test solution. Dilute the preparation to be examined in *tris-acetate buffer solution pH 8.5 R* to a concentration of 1.0 mg/ml. Equilibrate the solution in *tris-acetate buffer solution pH 8.5 R* using a suitable procedure (dialysis against *tris-acetate buffer solution pH 8.5 R*, or membrane filtration using the procedure described under Identification B, but reconstituting the desalted sample with *tris-acetate buffer solution pH 8.5 R*, are suitable). Transfer the dialysed solution to a polypropylene centrifuge tube. Freshly prepare a solution of *trypsin for peptide mapping R* at a concentration of 1 mg/ml in *water R*, and add 5 µl to 0.25 ml of the dialysed solution. Cap the tube and place in a water-bath at 37 °C for 18 h. Remove the sample from the water-bath and stop the reaction immediately by freezing.

Reference solution. Dissolve the contents of a vial of *erythropoietin BRP* in 0.25 ml of *water R*. Prepare as for the test solution, ensuring that all procedures are carried out simultaneously, and under identical conditions.

Examine the 2 tryptic digests by liquid chromatography (2.2.29).

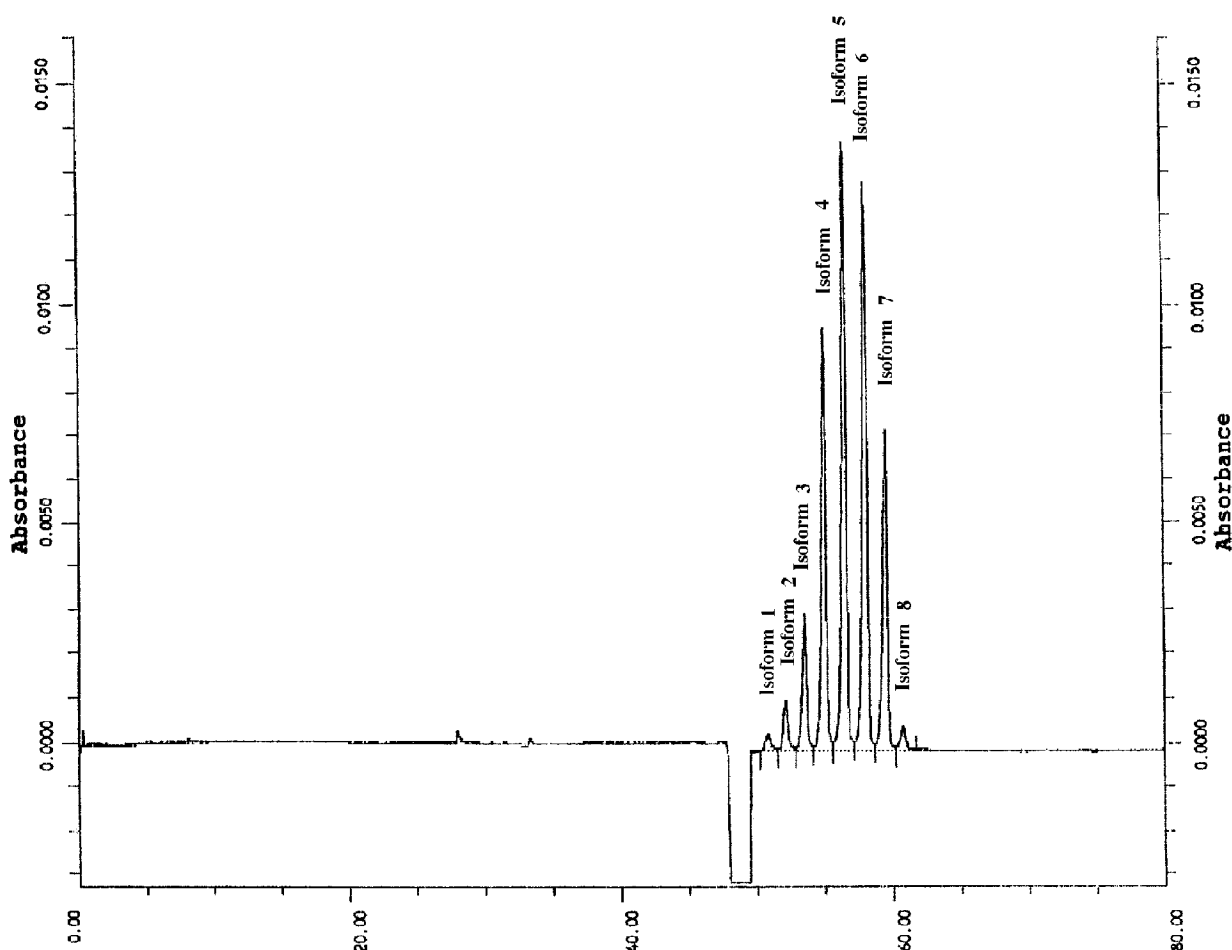


Figure 1316.-1. – Ph. Eur. reference electropherogram of erythropoietin

Column:

- size: $l = 0.25$ m, $\varnothing = 4.6$ mm,
- stationary phase: butylsilyl silica gel R (5-10 μ m).

Mobile phase:

- mobile phase A: 0.06 per cent V/V solution of trifluoroacetic acid R,
- mobile phase B: to 100 ml of water R add 0.6 ml of trifluoroacetic acid R and dilute to 1000 ml with of acetonitrile for chromatography R,

Time (min)	Flow rate (ml/min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)
0 - 10	0.75	100	0
10 - 125	0.75	100 $\rightarrow$ 39	0 $\rightarrow$ 61
125 - 135	1.25	39 $\rightarrow$ 17	61 $\rightarrow$ 83
135 - 145	1.25	17 $\rightarrow$ 0	83 $\rightarrow$ 100
145 - 150	1.25	100	0

Detection: spectrophotometer at 214 nm.

Equilibration: at initial conditions for at least 15 min. Carry out a blank run using the above-mentioned gradient.

Injection: 50 μ l.

System suitability: the chromatogram obtained with each solution is qualitatively similar to the chromatogram of erythropoietin digest supplied with erythropoietin BRP.

Results: the profile of the chromatogram obtained with the test solution corresponds to that of the chromatogram obtained with the reference solution.

E. N-terminal sequence analysis

The first 15 amino acids are: Alanine - Proline - Proline - Arginine - Leucine - Isoleucine - (no recovered peak) - Aspartic acid - Serine - Arginine - Valine - Leucine - Glutamic acid - Arginine - Tyrosine.

Perform the Edman degradation using an automated solid-phase sequencer, operated in accordance with the manufacturer's instructions.

Desalt the equivalent of 50 μ g of erythropoietin. For example, dilute a volume of the substance to be examined equivalent to 50 μ g of the active substance in 1 ml of a 0.1 per cent V/V solution of trifluoroacetic acid R. Pre-wash a C18 reverse-phase sample preparation cartridge according to the instructions supplied and equilibrate the cartridge in a 0.1 per cent V/V solution of trifluoroacetic acid R. Apply the sample to the cartridge, and wash successively with a 0.1 per cent V/V solution of trifluoroacetic acid R containing 0 per cent, 10 per cent and 50 per cent V/V of acetonitrile R according to the manufacturer's instructions. Lyophilise the 50 per cent V/V acetonitrile R eluate.

Redissolve the desalted sample in 50 μ l of a 0.1 per cent V/V solution of trifluoroacetic acid R and couple to a sequencing cartridge using the protocol provided by the manufacturer. Run 15 sequencing cycles, using the reaction conditions for proline when running the second and third cycles.

Identify the phenylthiohydantoin (PTH)-amino acids released at each sequencing cycle by reverse-phase liquid chromatography. The procedure may be carried out using the column and reagents recommended by the manufacturer of the sequencing equipment for the separation of PTH-amino-acids.

The separation procedure is calibrated using:

- the mixture of PTH-amino acids provided by the manufacturer of the sequencer, with the gradient conditions adjusted as indicated to achieve optimum resolution of all amino acids,
- a sample obtained from a blank sequencing cycle obtained as recommended by the equipment manufacturer.

TESTS

Protein (2.5.33, *Method I*): 80 per cent to 120 per cent of the stated concentration.

Test solution. Dilute the preparation to be examined in a 4 g/l solution of *ammonium hydrogen carbonate R*.

Record the absorbance spectrum between 250 nm and 400 nm. Measure the value at the absorbance maximum (276–280 nm), after correction for any light scattering, measured up to 400 nm. Calculate the concentration of erythropoietin using a specific absorbance value of 7.43.

Dimers and related substances of higher molecular mass. Size-exclusion chromatography (2.2.30).

Test solution. Dilute the preparation to be examined in the mobile phase to obtain a concentration of 0.2 mg/ml.

Reference solution. To 0.02 ml of the test solution add 0.98 ml of the mobile phase.

Column:

- *size:* $l = 0.6$ m, $\varnothing = 7.5$ mm,
- *stationary phase:* *hydrophilic silica gel for chromatography R*, of a grade suitable for fractionation of globular proteins in the molecular mass range of 20 000 to 200 000.

Mobile phase: dissolve 1.15 g of *anhydrous disodium hydrogen phosphate R*, 0.2 g of *potassium dihydrogen phosphate R* and 23.4 g of *sodium chloride R* in 1 litre of *water R* (1.5 mM potassium dihydrogen phosphate, 8.1 mM disodium hydrogen phosphate, 0.4 M sodium chloride, pH 7.4); adjust the pH to 7.4 if necessary.

Flow rate: 0.5 ml/min.

Detection: spectrophotometer at 214 nm.

Injection: 100 µl.

Run time: minimum 1 h.

System suitability: the area of the principal peak in the chromatogram obtained with the reference solution is 1.5 to 2.5 per cent of the area of the principal peak in the chromatogram obtained with the test solution.

Limit:

- *total of any peaks eluted before the principal peak:* not more than the area of the principal peak in the chromatogram obtained with the reference solution (2 per cent).

Sialic acids: minimum 10 mol of sialic acids (calculated as *N*-acetylneuraminic acid) per mole of erythropoietin.

Test solution (a). Dilute the preparation to be examined in the mobile phase used in the test for dimers and related substances of higher molecular mass to obtain a concentration of 0.3 mg/ml.

Test solution (b). To 0.5 ml of test solution (a) add 0.5 ml of the mobile phase used in the test for dimers and related substances of higher molecular mass.

Reference solution (a). Dissolve a suitable amount of *N*-acetylneuraminic acid *R* in *water R* to obtain a concentration of 0.1 mg/ml.

Reference solution (b). To 0.8 ml of reference solution (a) add 0.2 ml of *water R*.

Reference solution (c). To 0.6 ml of reference solution (a) add 0.4 ml of *water R*.

Reference solution (d). To 0.4 ml of reference solution (a) add 0.6 ml of *water R*.

Reference solution (e). To 0.2 ml of reference solution (a) add 0.8 ml of *water R*.

Reference solution (f). Use *water R*.

Carry out the test in triplicate. Transfer 100 µl of each of the test and reference solutions to 10 ml glass test tubes. To each tube add 1.0 ml of *resorcinol reagent R*. Stopper the tubes and incubate at 100 °C for 30 min. Cool on ice. To each tube, add 2.0 ml of a mixture of 12 volumes of *butanol R* and 48 volumes of *butyl acetate R*. Mix vigorously, and allow the 2 phases to separate. Ensuring that the upper phase is completely clear, remove the upper phase, taking care to exclude completely any of the lower phase. Measure the absorbance (2.2.25) of all samples at 580 nm. Using the calibration curve generated by the reference solutions, determine the content of sialic acids in each of the 2 test solutions and calculate the mean. Calculate the number of moles of sialic acids per mole of erythropoietin assuming that the relative molecular mass of erythropoietin is 30 600 and that the relative molecular mass of *N*-acetylneuraminic acid is 309.

System suitability:

- the individual replicates agree to within ± 10 per cent of each other,
- the value obtained from reference solution (a) is between 1.5 and 2.5 times that obtained with test solution (a).

Bacterial endotoxins (2.6.14): less than 20 IU in the volume that contains 100 000 IU of erythropoietin.

ASSAY

The activity of the preparation is compared with that of *erythropoietin BRP* and expressed in International Units (IU).

The estimated potency is not less than 80 per cent and not more than 125 per cent of the stated potency. The confidence limits of the estimated potency ($P = 0.95$) are not less than 64 per cent and not more than 156 per cent of the stated potency.

Carry out the determination of potency by Method A or B.

A. In polycythaemic mice

The activity of the preparation is estimated by examining, under given conditions, its effect in stimulating the incorporation of ^{59}Fe into circulating red blood cells of mice made polycythaemic by exposure to reduced atmospheric pressure.

The following schedule, using treatment in a hypobaric chamber, has been found to be suitable.

Induce polycythaemia in female mice of the same strain, weighing 16 g to 18 g. Place the mice in a hypoxic chamber and reduce the pressure to 0.6 atmospheres. After 3 days at 0.6 atmospheres, further reduce the pressure to 0.4–0.5 atmospheres and maintain the animals at this pressure for a further 11 days (the partial vacuum is interrupted daily for a maximum of 1 h at about 11:00 a.m., in order to clean the cages and feed the animals). At the end of the specified period, return the mice to normal atmospheric conditions. Randomly distribute the mice into cages, each containing 6 animals, and mark them.

Test solution (a). Dilute the substance to be examined in *phosphate-albumin buffered saline pH 7.2 R1* to obtain a concentration of 0.2 IU/ml.

Test solution (b). Mix equal volumes of test solution (a) and *phosphate-albumin buffered saline pH 7.2 R1*.

Test solution (c). Mix equal volumes of test solution (b) and *phosphate-albumin buffered saline pH 7.2 R1*.

Reference solution (a). Dissolve *erythropoietin BRP* in *phosphate-albumin buffered saline pH 7.2 R1* to obtain a concentration of 0.2 IU/ml.

Reference solution (b). Mix equal volumes of reference solution (a) and *phosphate-albumin buffered saline pH 7.2 R1*.

Reference solution (c). Mix equal volumes of reference solution (b) and *phosphate-albumin buffered saline pH 7.2 R1*.

Radiolabelled ferric [⁵⁹Fe] chloride solution, concentrated. Use a commercially available solution of [⁵⁹Fe]ferric chloride (approximate specific activity: 100-1000 MBq/mg of Fe).

Radiolabelled [⁵⁹Fe]ferric chloride solution. Dilute the concentrated radiolabelled [⁵⁹Fe]ferric chloride solution in *sodium citrate buffer solution pH 7.8 R* to obtain a solution with an activity of 3.7×10^4 Bq/ml.

The concentrations of the test solutions and reference solutions may need to be modified, based on the response range of the animals used.

3 days after returning the animals to atmospheric pressure, inject each animal subcutaneously with 0.2 ml of one of the solutions. The 6 animals in each cage must each receive one of the 6 different treatments (3 test solutions and 3 reference solutions), and the order of injection must be separately randomised for each cage. A minimum of 8 cages is recommended. 2 days after injection of the test or reference solution, inject each animal intraperitoneally with 0.2 ml of radiolabelled [⁵⁹Fe]ferric chloride solution. The order of the injections must be the same as that of the erythropoietin injections, and the time interval between administration of the erythropoietin and the radiolabelled ferric chloride solution must be the same for each animal. After a further 48 h, anaesthetise each animal by injection of a suitable anaesthetic, record body weights and withdraw blood samples (0.65 ml) into haematocrit capillaries from the bifurcation of the aorta. After determining the packed cell volume for each sample, measure the radioactivity.

Calculate the response (percentage of iron-59 in total circulating blood) for each mouse using the expression:

$$\frac{A_s \times M \times 7.5}{A_t \times V_s}$$

- A_s = radioactivity in the sample,
 A_t = total radioactivity injected,
 7.5 = total blood volume as per cent body weight,
 M = body weight, in grams,
 V_s = sample volume.

Calculate the potency by the usual statistical methods for a parallel line assay. Eliminate from the calculation any animal where the packed cell volume is less than 54 per cent, or where the body weight is more than 24 g.

B. In normocythaemic mice

The assay is based on the measurement of stimulation of reticulocyte production in normocythaemic mice.

The assay may be carried out using the following procedure:

Test solution (a). Dilute the substance to be examined in *phosphate-albumin buffered saline pH 7.2 R1* to obtain a concentration of 80 IU/ml.

Test solution (b). Mix equal volumes of test solution (a) and *phosphate-albumin buffered saline pH 7.2 R1*.

Test solution (c). Mix equal volumes of test solution (b) and *phosphate-albumin buffered saline pH 7.2 R1*.

Reference solution (a). Dissolve *erythropoietin BRP* in *phosphate-albumin buffered saline pH 7.2 R1* to obtain a concentration of 80 IU/ml.

Reference solution (b). Mix equal volumes of reference solution (a) and *phosphate-albumin buffered saline pH 7.2 R1*.

Reference solution (c). Mix equal volumes of reference solution (b) and *phosphate-albumin buffered saline pH 7.2 R1*.

The exact concentrations of the test solutions and reference solutions may need to be modified, based on the response range of the animals used.

At the beginning of the assay procedure, randomly distribute mice of a suitable age and strain (8-week old B6D2F1 mice are suitable) into 6 cages. A minimum of 8 mice per cage is recommended. Inject each animal subcutaneously with 0.5 ml of the appropriate treatment (one solution per cage) and put the animal in a new cage. Combine the mice in such a way that each cage housing the treated mice contains one mouse out of the 6 different treatments (3 test solutions and 3 reference solutions, 6 mice per cage). 4 days after the injections, collect blood samples from the animals and determine the number of reticulocytes using a suitable procedure.

The following method may be employed:

The volume of blood, dilution procedure and fluorescent reagent may need to be modified to ensure maximum development and stability of fluorescence.

Colorant solution, concentrated. Use a solution of thiazole orange suitable for the determination of reticulocytes. Prepare at a concentration twice that necessary for the analysis.

Proceed with the following dilution steps. Dilute whole blood 500-fold in the buffer used to prepare the colorant solution. Dilute this solution 2-fold in the concentrated colorant solution. After staining for 3-10 min, determine the reticulocyte count microfluorometrically in a flow cytometer. The percentage of reticulocytes is determined using a biparametric histogram: number of cells/red fluorescence (620 nm).

Calculate the potency by the usual statistical methods for a parallel line assay.

STORAGE

In an airtight container at a temperature below -20°C . Avoid repeated freezing and thawing.

LABELLING

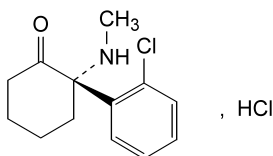
The label states:

- the erythropoietin content in milligrams per millilitre,
- the activity in International Units per millilitre,
- the name and the concentration of any other excipients.

01/2005:1742 Column:

ESKETAMINE HYDROCHLORIDE

Esketamini hydrochloridum

 $C_{13}H_{17}Cl_2NO$ M_r 274.2**DEFINITION**

(2S)-2-(2-Chlorophenyl)-2-(methylamino)cyclohexanone hydrochloride.

Content: 99.0 per cent to 101.0 per cent.

CHARACTERS*Appearance*: white, crystalline powder.*Solubility*: freely soluble in water and in methanol, soluble in alcohol.**IDENTIFICATION**

A. Specific optical rotation (2.2.7): + 85.0 to + 95.0.

Dilute 12.5 ml of solution S (see Tests) to 40.0 ml with water R.

B. Infrared absorption spectrophotometry (2.2.24).

Comparison: Ph. Eur. reference spectrum of esketamine hydrochloride.

C. It gives reaction (a) of chlorides (2.3.1).

TESTS**Solution S.** Dissolve 8.0 g in carbon dioxide-free water R and dilute to 50.0 ml with the same solvent.**Appearance of solution.** Solution S is clear (2.2.1) and colourless (2.2.2, Method II).**pH** (2.2.3): 3.5 to 4.5.

Dilute 12.5 ml of solution S to 20 ml with carbon dioxide-free water R.

Impurity D. Liquid chromatography (2.2.29).*Test solution.* Dissolve 25.0 mg of the substance to be examined in water R and dilute to 100.0 ml with the same solvent.*Reference solution (a).* Dissolve 5 mg of esketamine impurity D CRS in water R, add 20 ml of the test solution and dilute to 50 ml with water R. Dilute 10 ml of this solution to 100 ml with water R.*Reference solution (b).* Dilute 5.0 ml of the test solution to 25.0 ml with water R. Dilute 5.0 ml of this solution to 50.0 ml with water R.*Reference solution (c).* Dilute 2.5 ml of reference solution (b) to 10.0 ml with water R. Dilute 1.0 ml of this solution to 10.0 ml with water R.*Precolumn*:

- size: $l = 0.01$ m, $\emptyset = 3.0$ mm,
- stationary phase: silica gel AGP for chiral chromatography R (5 μ m),
- temperature: 30 °C.

- size: $l = 0.125$ m, $\emptyset = 4.6$ mm,
- stationary phase: silica gel AGP for chiral chromatography R (5 μ m),
- temperature: 30 °C.

Mobile phase: mix 16 volumes of methanol R and 84 volumes of a 6.8 g/l solution of potassium dihydrogen phosphate R previously adjusted to pH 7.0 with potassium hydroxide R.*Flow rate*: 0.8 ml/min.*Detection*: spectrophotometer at 215 nm.*Injection*: 20 μ l.*Run time*: 20 min.*Relative retention* with reference to esketamine (retention time = about 10 min): impurity D = about 1.3.*System suitability*:

- resolution: minimum 2.0 between the peaks due to esketamine and impurity D in the chromatogram obtained with reference solution (a),
- signal-to-noise ratio: minimum 3 for the principal peak in the chromatogram obtained with reference solution (c).

Limit:

- impurity D: not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (2.0 per cent).

Related substances. Liquid chromatography (2.2.29).*Test solution.* Dissolve 50.0 mg of the substance to be examined in the mobile phase and dilute to 50.0 ml with the mobile phase.*Reference solution (a).* Dissolve 5 mg of ketamine impurity A CRS in the mobile phase (using ultrasound, if necessary) and dilute to 10 ml with the mobile phase. To 1 ml of the solution add 0.5 ml of the test solution and dilute to 100 ml with the mobile phase. Prepare immediately before use.*Reference solution (b).* Dilute 1.0 ml of the test solution to 10.0 ml with the mobile phase. Dilute 1.0 ml of this solution to 20.0 ml with the mobile phase.*Column*:

- size: $l = 0.125$ m, $\emptyset = 4.0$ mm,
- stationary phase: spherical octadecylsilyl silica gel for chromatography R (5 μ m).

Mobile phase: dissolve 0.95 g of sodium hexanesulphonate R in 1000 ml of a mixture of 25 volumes of acetonitrile R and 75 volumes of water R and add 4 ml of acetic acid R.*Flow rate*: 1.0 ml/min.*Detection*: spectrophotometer at 215 nm.*Injection*: 20 μ l.*Run time*: 10 times the retention time of esketamine.*Relative retention* with reference to esketamine: impurity A = about 1.6; impurity B = about 3.3; impurity C = about 4.6.*System suitability*: reference solution (a):

- retention time: esketamine = 3.0 min to 4.5 min,
- resolution: minimum 1.5 between the peaks due to impurity A and esketamine.

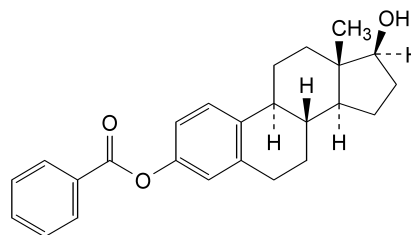
Limits:

- impurities A, B, C: for each impurity, not more than 0.4 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent),

01/2005:0139

ESTRADIOL BENZOATE

Estradioli benzoas

 $C_{25}H_{28}O_3$ M_r 376.5

DEFINITION

17 β -Hydroxyestra-1,3,5(10)-trien-3-yl benzoate.

Content: 97.0 per cent to 103.0 per cent (dried substance).

CHARACTERS

Appearance: almost white, crystalline powder or colourless crystals.**Solubility:** practically insoluble in water, freely soluble in methylene chloride, sparingly soluble in acetone, slightly soluble in methanol.

It shows polymorphism.

IDENTIFICATION

Infrared absorption spectrophotometry (2.2.24).

Comparison: estradiol benzoate CRS.If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in *acetone R*, evaporate to dryness and record new spectra using the residues.

TESTS

Specific optical rotation (2.2.7): + 55.0 to + 59.0 (dried substance).Dissolve 0.250 g in *acetone R* and dilute to 25.0 ml with the same solvent.**Related substances.** Liquid chromatography (2.2.29).**Test solution.** Dissolve 20 mg of the substance to be examined in *acetonitrile R* and dilute to 10.0 ml with the same solvent.**Reference solution (a).** Dissolve 5 mg of *estradiol benzoate impurity E CRS* in 5 ml of *acetonitrile R*, add 2.5 ml of the test solution and dilute to 10 ml with *acetonitrile R*.**Reference solution (b).** Dilute 1.0 ml of the test solution to 100.0 ml with *acetonitrile R*.**Column:**

- size: $l = 0.25$ m, $\varnothing = 4.6$ mm,
- stationary phase: octylsilyl silica gel for chromatography *R* (5 μ m).

Mobile phase:

- mobile phase A: *water R*, *acetonitrile R* (40:60 V/V),
- mobile phase B: *acetonitrile R*,

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)
0 - t_R	100	0
$t_R - (t_R + 1)$	100 $\rightarrow$ 10	0 $\rightarrow$ 90
$(t_R + 1) - (t_R + 10)$	10	90

 t_R = retention time of impurity E

- **any other impurity:** for each impurity, not more than 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent),
- **total:** not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent),
- **disregard limit:** 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent).

Heavy metals (2.4.8): maximum 20 ppm.Dilute 12.5 ml of solution S to 20 ml with *water R*. 12 ml of the solution complies with limit test A. Prepare the standard using *lead standard solution* (2 ppm Pb) *R*.**Sulphated ash** (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

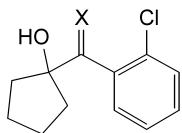
Dissolve 0.200 g in 50 ml of *methanol R* and add 1.0 ml of 0.1 M *hydrochloric acid*. Carry out a potentiometric titration (2.2.20), using 0.1 M *sodium hydroxide*. Read the volume added between the 2 points of inflexion.1 ml of 0.1 M *sodium hydroxide* is equivalent to 27.42 mg of $C_{13}H_{17}Cl_2NO$.

STORAGE

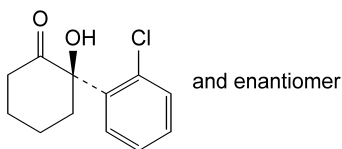
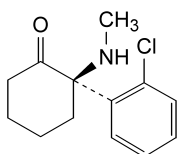
Protected from light.

IMPURITIES

Specified impurities: A, B, C, D.

A. X = N-CH₃: 1-[(2-chlorophenyl)(methylimino)methyl]cyclopentanol,

C. X = O: (2-chlorophenyl)(1-hydroxycyclopentyl)methanone,

B. (2*RS*)-2-(2-chlorophenyl)-2-hydroxycyclohexanone,D. (2*R*)-2-(2-chlorophenyl)-2-(methylamino)cyclohexanone ((*R*)-ketamine).

Flow rate: 1.0 ml/min.

Detection: spectrophotometer at 230 nm.

Injection: 10 µl.

Elution order: impurity A, impurity F, estradiol benzoate, impurity E, impurity B, impurity D, impurity C.

Relative retention with reference to estradiol benzoate: impurity C = about 1.5.

System suitability: reference solution (a):

- resolution: minimum 2.0 between the peaks due to estradiol benzoate and impurity E.

Limits:

- correction factor: for the calculation of content, multiply the peak area of impurity C by 0.7,
- any impurity: for each impurity, not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent),
- total: not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (1.5 per cent),
- disregard limit: 0.05 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

Loss on drying (2.2.32): maximum 0.5 per cent, determined on 0.5 g by drying in an oven at 100–105 °C for 3 h.

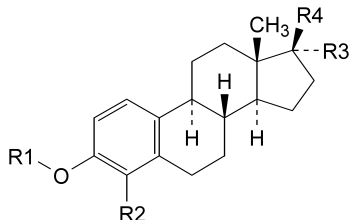
ASSAY

Dissolve 25.0 mg in *anhydrous ethanol R* and dilute to 250.0 ml with the same solvent. Dilute 10.0 ml of the solution to 100.0 ml with *anhydrous ethanol R*. Measure the absorbance (2.2.25) at the absorption maximum at 231 nm.

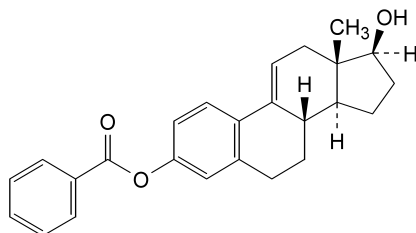
Calculate the content of $C_{25}H_{28}O_3$ taking the specific absorbance to be 500.

IMPURITIES

Specified impurities: A, B, C, D, E, F.



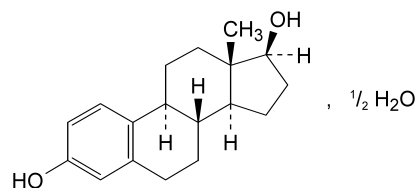
- A. $R_1 = R_2 = R_3 = H, R_4 = OH$: estradiol,
 B. $R_1 = CO-C_6H_5, R_2 = CH_3, R_3 = H, R_4 = OH$:
 17β-hydroxy-4-methylestra-1,3,5(10)-trien-3-yl benzoate,
 C. $R_1 = CO-C_6H_5, R_2 = R_3 = H, R_4 = O-CO-C_6H_5$:
 estra-1,3,5(10)-triene-3,17β-diyl dibenzoate,
 D. $R_1 = R_2 = R_3 = H, R_4 = O-CO-C_6H_5$: 3-hydroxyestra-1,3,
 5(10)-trien-17β-yl benzoate,
 E. $R_1 = CO-C_6H_5, R_2 = R_4 = H, R_3 = OH$:
 17α-hydroxyestra-1,3,5(10)-trien-3-yl benzoate,



- F. 17β-hydroxyestra-1,3,5(10),9(11)-tetraen-3-yl benzoate.

ESTRADIOL HEMIHYDRATE

Estradiolum hemihydricum



$C_{18}H_{24}O_2 \cdot \frac{1}{2}H_2O$

M_r 281.4

DEFINITION

Estra-1,3,5(10)-triene-3,17β-diol hemihydrate.

Content: 97.0 per cent to 103.0 per cent (anhydrous substance).

CHARACTERS

Appearance: white or almost white, crystalline powder or colourless crystals.

Solubility: practically insoluble in water, soluble in acetone, sparingly soluble in alcohol, slightly soluble in methylene chloride.

IDENTIFICATION

First identification: B.

Second identification: A, C, D, E.

A. Melting point (2.2.14): 175 °C to 180 °C.

B. Infrared absorption spectrophotometry (2.2.24).

Comparison: estradiol hemihydrate CRS.

C. Thin-layer chromatography (2.2.27).

Test solution. Dissolve 50 mg of the substance to be examined in *methanol R* and dilute to 50 ml with the same solvent.

Reference solution (a). Dissolve 50 mg of *estradiol hemihydrate CRS* in *methanol R* and dilute to 50 ml with the same solvent.

Reference solution (b). Dissolve 25 mg of *ethinylestradiol CRS* in reference solution (a) and dilute to 25 ml with reference solution (a).

Plate: TLC silica gel plate R.

Mobile phase: alcohol R, toluene R (20:80 V/V).

Application: 5 µl.

Development: over 3/4 of the plate.

Drying: in air until the solvent has evaporated.

Detection: heat at 110 °C for 10 min. Spray the hot plate with *alcoholic solution of sulphuric acid R*. Heat again at 110 °C for 10 min. Allow to cool. Examine in daylight and in ultraviolet light at 365 nm.

System suitability: the chromatogram obtained with reference solution (b) shows 2 spots which may however not be completely separated.

Results: the principal spot in the chromatogram obtained with the test solution is similar in position, colour in daylight, fluorescence in ultraviolet light at 365 nm and size to the principal spot in the chromatogram obtained with reference solution (a).

- D. To about 1 mg add 0.5 ml of freshly prepared *sulphomolybdic reagent R2*. A blue colour develops which in ultraviolet light at 365 nm has an intense green

fluorescence. Add 1 ml of *sulphuric acid R* and 9 ml of *water R*. The colour becomes pink with a yellowish fluorescence.

E. It complies with the test for water (see Tests).

TESTS

Specific optical rotation (2.2.7): + 76.0 to + 83.0 (anhydrous substance).

Dissolve 0.250 g in *alcohol R* and dilute to 25.0 ml with the same solvent.

Related substances. Liquid chromatography (2.2.29).

Test solution. Dissolve 25.0 mg of the substance to be examined in 10 ml of *acetonitrile R* and dilute to 25.0 ml with *methanol R2*.

Reference solution (a). Dilute 1.0 ml of the test solution to 100.0 ml with the mobile phase. Dilute 2.0 ml of the solution to 10.0 ml with the mobile phase.

Reference solution (b). Dissolve 2 mg of *17 α -estradiol R* in 5.0 ml of *acetonitrile R*. Mix 2.0 ml of this solution with 1.0 ml of the test solution and dilute to 5.0 ml with the mobile phase.

Reference solution (c). Mix equal volumes of a 1 mg/ml solution of the substance to be examined in *methanol R2* and of a 1 mg/ml solution of *2,3-dichloro-5,6-dicyanobenzoquinone R* in *methanol R2*. Allow to stand for 30 min before injection.

Reference solution (d). Dissolve 5 mg of *estradiol for peak identification CRS* (estradiol hemihydrate spiked with impurities A, B and C at about 0.5 per cent) in 2 ml of *acetonitrile R* and dilute to 5 ml with *methanol R2*.

Column:

- size: $l = 0.25$ m, $\varnothing = 4.6$ mm,
- stationary phase: end-capped octadecylsilyl silica gel for chromatography R (5 μ m).

Mobile phase: to 400 ml of *acetonitrile R* add 50 ml of *methanol R2* and 400 ml of *water R*; allow to stand for 10 min, dilute to 1000 ml with *water R* and mix again.

Flow rate: 1 ml/min.

Detection: spectrophotometer at 280 nm.

Equilibration: about 60 min.

Injection: 20 μ l.

Run time: twice the retention time of the principal peak.

Relative retention with reference to estradiol (retention time = about 13 min): impurity D = about 0.9; impurity B = about 1.1; impurity A = about 1.4; impurity C = about 1.9.

System suitability: reference solution (b):

- resolution: minimum 2.5 between the peaks due to estradiol and impurity B.

Limits:

- correction factor: for the calculation of content, multiply the peak area of impurity D by 0.4 (use the chromatogram obtained with reference solution (c) to identify this peak),
- impurities A, B, C, D: for each impurity, not more than 1.5 times the area of the principal peak obtained with reference solution (a) (0.3 per cent),
- any other impurity: for each impurity, not more than 0.5 times the area of the principal peak obtained with reference solution (a) (0.1 per cent),

- total: not more than 2.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent),
- disregard limit: 0.25 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

Water (2.5.12): 2.9 per cent to 3.5 per cent, determined on 0.500 g.

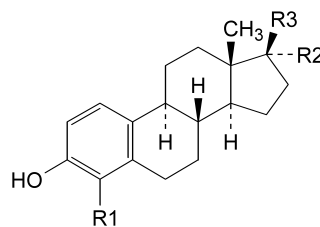
ASSAY

Dissolve 20.0 mg in *alcohol R* and dilute to 100.0 ml with the same solvent. Dilute 5.0 ml of the solution to 50.0 ml with 0.1 M sodium hydroxide. Allow to cool to room temperature. Measure the absorbance (2.2.25) of the solution at the maximum at 238 nm.

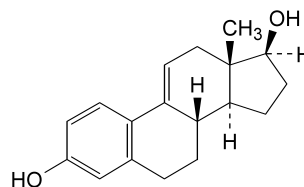
Calculate the content of $C_{18}H_{24}O_2$ taking the specific absorbance to be 335.

IMPURITIES

Specified impurities: A, B, C, D.



- A. $R_1 = H$, $R_2 + R_3 = O$: 3-hydroxyestra-1,3,5(10)-trien-17-one (estrone),
- B. $R_1 = R_3 = H$, $R_2 = OH$: estra-1,3,5(10)-triene-3,17 α -diol (17 α -estradiol),
- C. $R_1 = CH_3$, $R_2 = H$, $R_3 = OH$: 4-methylestra-1,3,5(10)-triene-3,17 β -diol,

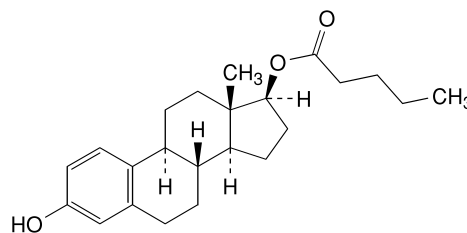


- D. estra-1,3,5(10),9(11)-tetraene-3,17 β -diol.

01/2005:1614

ESTRADIOL VALERATE

Estradioli valeras



$C_{23}H_{32}O_3$

M_r 356.5

DEFINITION

3-Hydroxyestra-1,3,5(10)-trien-17 β -yl pentanoate.

Content: 97.0 per cent to 103.0 per cent (dried substance).

CHARACTERS

Appearance: white or almost white, crystalline powder or colourless crystals.

Solubility: practically insoluble in water, soluble in alcohol. mp: about 145 °C.

IDENTIFICATION

Infrared absorption spectrophotometry (2.2.24).

Comparison: estradiol valerate CRS.

TESTS

Solution S. Dissolve 0.500 g in *methanol R* and dilute to 20.0 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and colourless (2.2.2, *Method II*).

Specific optical rotation (2.2.7): + 41 to + 47 (dried substance), determined on solution S.

Related substances. Liquid chromatography (2.2.29).

Solvent mixture. Mix 15 volumes of *water R* and 135 volumes of *acetonitrile R*.

Test solution. Dissolve 0.100 g of the substance to be examined in the solvent mixture and dilute to 10.0 ml with the solvent mixture.

Reference solution (a). Dissolve 2 mg of *estradiol valerate CRS* and 2 mg of *estradiol butyrate CRS* in the solvent mixture and dilute to 10 ml with the solvent mixture.

Reference solution (b). Dilute 0.5 ml of the test solution to 100.0 ml with the solvent mixture.

Column:

- size: $l = 0.25$ m, $\varnothing = 4.6$ mm,
- stationary phase: *octadecylsilyl silica gel for chromatography R* (5 μ m),
- temperature: 40 °C.

Mobile phase:

- mobile phase A: *water R*,
- mobile phase B: *acetonitrile R*,

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)
0 - 15	40 $\rightarrow$ 0	60 $\rightarrow$ 100
15 - 25	0	100
25 - 30	40	60
30 = 0	40	60

Flow rate: 1.0 ml/min.

Detection: spectrophotometer at 220 nm.

Injection: 10 μ l.

Relative retention with reference to estradiol valerate (retention time = about 12 min): impurity F = about 0.9.

System suitability: reference solution (a):

- resolution: minimum of 5.0 between the peaks due to impurity F and to estradiol valerate.

Limits:

- any impurity: not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent),
- total: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (1.0 per cent),
- disregard limit: 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

Loss on drying (2.2.32): maximum 1.0 per cent, determined on 0.500 g by drying in an oven at 100-105 °C for 3 h.

ASSAY

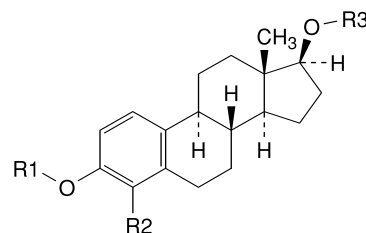
Dissolve 25.0 mg in *alcohol R* and dilute to 250.0 ml with the same solvent. Measure the absorbance (2.2.25) at the maximum at 280 nm.

Calculate the content of $C_{23}H_{32}O_3$ taking the specific absorbance to be 58.0.

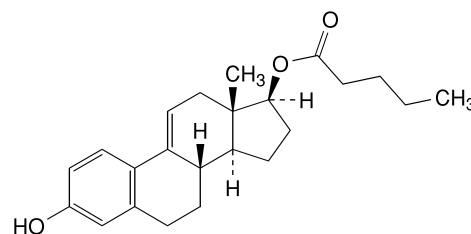
STORAGE

Protected from light.

IMPURITIES



- A. $R_1 = R_2 = R_3 = H$: estradiol,
 B. $R_1 = CO-[CH_2]_3-CH_3$, $R_2 = R_3 = H$: 17 β -hydroxyestra-1,3,5(10)-trien-3-yl pentanoate,
 D. $R_1 = H$, $R_2 = CH_3$, $R_3 = CO-[CH_2]_3-CH_3$: 3-hydroxy-4-methylestra-1,3,5(10)-trien-17 β -yl pentanoate,
 E. $R_1 = R_3 = CO-[CH_2]_3-CH_3$, $R_2 = H$: estra-1,3,5(10)-trien-3,17 β -diyl dipentanoate,
 F. $R_1 = R_2 = H$, $R_3 = CO-[CH_2]_2-CH_3$: 3-hydroxyestra-1,3,5(10)-trien-17 β -yl butanoate (estradiol butyrate),

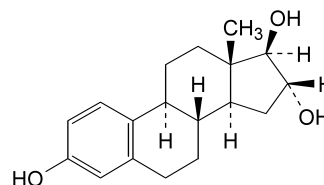


- C. 3-hydroxyestra-1,3,5(10),9(11)-tetraen-17 β -yl pentanoate.

01/2005:1203

ESTRIOL

Estriolum



$C_{18}H_{24}O_3$

M_r 288.4

DEFINITION

Estriol contains not less than 97.0 per cent and not more than the equivalent of 103.0 per cent of estra-1,3,5(10)-triene-3,16 α ,17 β -triol, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, crystalline powder, practically insoluble in water, sparingly soluble in alcohol.

It melts about 282 °C.

IDENTIFICATION

- A. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *estriol CRS*.
- B. Examine by thin-layer chromatography (2.2.27), using a suitable silica gel as the coating substance.

Test solution. Dissolve 10 mg of the substance to be examined in *methanol R* and dilute to 10 ml with the same solvent.

Reference solution (a). Dissolve 10 mg of *estriol CRS* in *methanol R* and dilute to 10 ml with the same solvent.

Reference solution (b). Dissolve 5 mg of *estradiol hemihydrate CRS* in reference solution (a) and dilute to 5 ml with the same solvent.

Apply to the plate 5 µl of each solution. Develop over a path of 15 cm using a mixture of 20 volumes of *alcohol R* and 80 volumes of *toluene R*. Allow the plate to dry in air. Spray the plate with *alcoholic solution of sulphuric acid R*. Heat the plate at 100 °C for 10 min or until the spots appear. Allow to cool. Examine in daylight and ultraviolet light at 365 nm. The principal spot in the chromatogram obtained with the test solution is similar in position, colour in daylight, fluorescence in ultraviolet light at 365 nm and size to the principal spot in the chromatogram obtained with the reference solution (a). The test is not valid unless the chromatogram obtained with reference solution (b) shows 2 clearly separated spots.

TESTS

Specific optical rotation (2.2.7). Dissolve 80 mg in *ethanol R* and dilute to 10 ml with the same solvent. The specific optical rotation is + 60 to + 65, calculated with reference to the dried substance.

Related substances. Examine by liquid chromatography (2.2.29).

Solvent mixture. A mixture of 20 volumes of *2-propanol R1* and 80 volumes of *heptane R*.

Test solution. Dissolve 20.0 mg of the substance to be examined in 5 ml of *2-propanol R1* and dilute to 20.0 ml with the solvent mixture.

Reference solution (a). Dissolve 5 mg of *estriol CRS* and 2.0 mg of *estriol impurity A CRS* in 5 ml of *2-propanol R1* and dilute to 10.0 ml with the solvent mixture. Dilute 1.0 ml of the solution to 20.0 ml with the solvent mixture.

Reference solution (b). Dilute 1.0 ml of the test solution to 10.0 ml with the solvent mixture. Dilute 1.0 ml of the solution to 10.0 ml with the same solvent mixture.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.15 m long and 4.0 mm in internal diameter packed with *diol silica gel for chromatography R* (5 µm),

- as mobile phase at a flow rate of 1.2 ml/min a linear gradient programme using the following conditions:

Mobile phase A. Heptane R,

Mobile phase B. 2-Propanol R1,

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)	Comment
0 - 10	95 → 88	5 → 12	linear gradient
10 - 20	88	12	isocratic
20 - 30	88 → 95	12 → 5	switch to initial eluent composition
30 - 35	95	5	equilibration
35 = 0	95	5	restart gradient

- as detector a spectrophotometer set at 280 nm, maintaining the temperature of the column at 40 °C.

Equilibrate the column with a mixture of 20 per cent V/V of *2-propanol R1* in *heptane R* until a stable baseline is obtained.

Adjust the sensitivity of the system so that the height of the principal peak in the chromatogram obtained with 20 µl of reference solution (b) is about 25 per cent of the full scale of the recorder.

Inject 20 µl of reference solution (a). When the chromatograms are recorded in the prescribed conditions, the retention times are: estriol about 19 min and estriol impurity A about 21 min. The test is not valid unless the resolution between the peaks corresponding to estriol and estriol impurity A is at least 2.2. If the retention times increase or the resolution decreases, wash the column first with *acetone R* and then with *heptane R*.

Inject separately 20 µl of the solvent mixture as a blank, 20 µl of the test solution and 20 µl of each of the reference solutions (a) and (b). In the chromatogram obtained with the test solution: the area of any peak corresponding to estriol impurity A is not greater than half the area of the peak corresponding to estriol impurity A in the chromatogram obtained with reference solution (a) (0.5 per cent) and any other peak, apart from the principal peak, is not greater than half the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent); the sum of the areas of all the peaks, apart from the principal peak and the peak corresponding to estriol impurity A, is not greater than the area of the peak in the chromatogram obtained with reference solution (b) (1 per cent). Disregard any peak due to the blank and any peak with an area less than 0.05 times the area of the principal peak in the chromatogram obtained with reference solution (b).

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 100-105 °C for 3 h.

ASSAY

Dissolve 25.0 mg in *alcohol R* and dilute to 50.0 ml with the same solvent. Dilute 10.0 ml of this solution to 50.0 ml with the same solvent. Measure the absorbance (2.2.25) at the maximum at 281 nm.

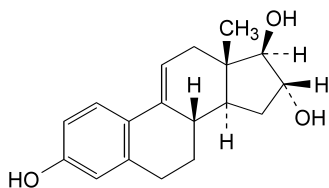
Calculate the content of C₁₈H₂₄O₃ taking the specific absorbance to be 72.5.

IMPURITIES

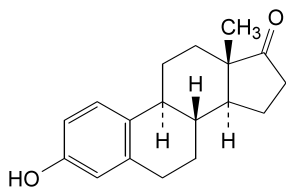
01/2005:1512

Specified impurities: A, B, C, D, E, F, G.

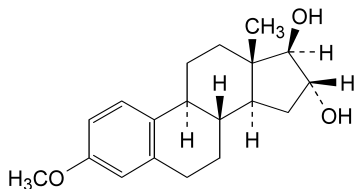
Other detectable impurities: H, I.



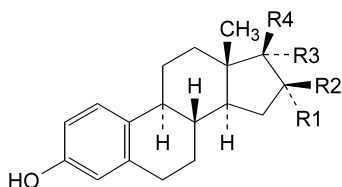
A. estra-1,3,5(10),9(11)-tetraene-3,16α,17β-triol (9,11-didehydroestradiol),



B. 3-hydroxyestra-1,3,5(10)-trien-17-one (estrone),



C. 3-methoxyestra-1,3,5(10)-triene-16α,17β-diol (estriol 3-methyl ether),



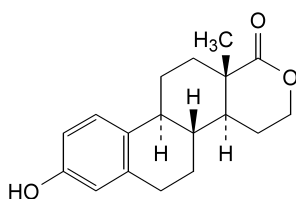
D. R1 = R2 = R3 = H, R4 = OH: estradiol,

E. R1 = R3 = OH, R2 = R4 = H: estra-1,3,5(10)-triene-3,16α,17α-triol (17-epi-estriol),

F. R1 = R3 = H, R2 = R4 = OH: estra-1,3,5(10)-triene-3,16β,17β-triol (16-epi-estriol),

G. R1 = R4 = H, R2 = R3 = OH: estra-1,3,5(10)-triene-3,16β,17α-triol (16,17-epi-estriol),

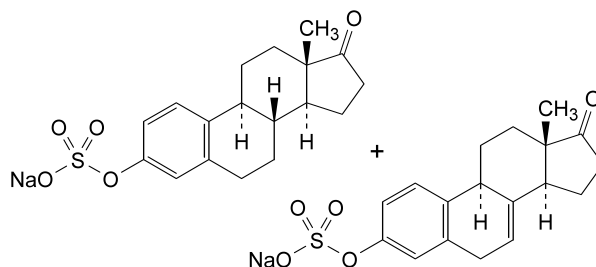
H. R1 = OH, R2 = H, R3 + R4 = O: 3,16α-dihydroxyestra-1,3,5(10)-trien-17-one,



I. 3-hydroxy-17-oxa-D-homoestra-1,3,5(10)-trien-17a-one.

ESTROGENS, CONJUGATED

Estrogeni coniuncti

 $C_{18}H_{21}O_5NaS + C_{18}H_{19}O_5NaS$ $M_r 372.4 + 370.4$

DEFINITION

Conjugated estrogens are a mixture of various conjugated forms of estrogens obtained from the urine of pregnant mares or by synthesis, dispersed in a suitable powdered diluent.

The two principal components are 17-oxoestra-1,3,5(10)-trien-3-yl sodium sulphate (sodium estrone sulphate) and 17-oxoestra-1,3,5(10),7-tetraen-3-yl sodium sulphate (sodium equilin sulphate). Concomitants are sodium 17α-estradiol sulphate, sodium 17α-dihydroequilin sulphate and sodium 17β-dihydroequilin sulphate.

Conjugated estrogens contain not less than 52.5 per cent and not more than 61.5 per cent of sodium estrone sulphate, not less than 22.5 per cent and not more than 30.5 per cent of sodium equilin sulphate, not less than 2.5 per cent and not more than 9.5 per cent of sodium 17α-estradiol sulphate, not less than 13.5 per cent and not more than 19.5 per cent of sodium 17α-dihydroequilin sulphate, not less than 0.5 per cent and not more than 4.0 per cent of sodium 17β-dihydroequilin sulphate. The total of sodium estrone sulphate and sodium equilin sulphate is not less than 79.5 per cent and not more than 88.0 per cent.

All percentages are related to the labelled content.

CHARACTERS

An almost white or brownish, amorphous powder.

IDENTIFICATION

A. Examine the chromatograms obtained in the assay.

The retention times and sizes of the 2 principal peaks corresponding to estrone and equilin in the chromatogram obtained with test solution (a) are approximately the same as those of the 2 principal peaks in the chromatogram obtained with reference solution (a).

B. Examine the chromatogram obtained with test solution (b) in the chromatographic profile. The chromatogram exhibits additional peaks corresponding to 17α-estradiol, 17α-dihydroequilin and 17β-dihydroequilin, at relative retentions with reference to 3-O-methylestrone (internal standard) of about 0.24, 0.30 and 0.35 respectively.

TESTS

Chromatographic profile. Carry out the test as prescribed in the assay with the following additional information.

Test solution (b). Prepare the test solution as described in the assay, do not add the sulphatase and use 6.0 ml of the upper layer instead of 3.0 ml. Prepare a blank in the same manner.

Reference solution (b). Prepare the reference solution as described in the assay. Dilute tenfold with *ethanol R* before adding the internal standard.

Inject 1 µl of reference solution (a). Measure the areas of the peaks due to 17α-dihydroequilin, estrone and 3-O-methylestrone, with relative retentions with reference to 3-O-methylestrone of about 0.30, 0.80 and 1 respectively.

Inject 1 µl of test solution (a). Locate the peaks with relative retentions with reference to 3-O-methylestrone of 1 and about 0.24, 0.29, 0.30, 0.35, 0.56, 0.64, 0.90 and 1.3 and measure their areas.

Calculate the percentage content of the components occurring as sodium sulphate salts using expression (1) below.

Inject 1 µl of reference solution (b). Measure the areas of the peaks due to estrone and 3-O-methylestrone, with relative retentions with reference to 3-O-methylestrone of about 0.80 and 1 respectively.

Inject 1 µl of test solution (b). Locate the peaks with relative retentions with reference to 3-O-methylestrone of about 0.30, 0.80 and 0.87 and measure the sum of the areas.

Calculate the percentage content of 17α-dihydroequilin, estrone and equilin occurring as free steroids using expression (2):

$$\frac{S'_A \times S_I \times m_R \times 137.8 \times 1000}{S_R \times S'_I \times m \times LC} \quad (1)$$

$$\frac{S'_{FS} \times S_I \times m_E \times 100 \times 1000}{S_E \times S'_I \times m \times LC} \quad (2)$$

S_I = area of the peak due to the internal standard in the chromatogram obtained with the corresponding reference solution,

S'_I = area of the peak due to the internal standard in the chromatogram obtained with the corresponding test solution,

S_R = area of the peak due to the reference substance (Table 1512.-1) in the chromatogram obtained with the corresponding reference solution,

S'_A = area of the peak due to the analyte in the chromatogram obtained with the corresponding test solution,

m_R = mass of the reference substance (Table 1512.-1) in the corresponding reference solution, in milligrams,

m = mass of the substance to be examined in the corresponding test solution, in milligrams,

S'_{FS} = sum of the areas of the peaks due to 17α-dihydroequilin, estrone and equilin in the chromatogram obtained with the corresponding test solution,

S_E = area of the peak due to *estrone CRS* in the chromatogram obtained with the corresponding reference solution,

m_E = mass of *estrone CRS* in the corresponding reference solution, in milligrams,

LC = labelled content, in milligrams per gram.

The percentages are within the following ranges:

- *sodium 17α-estradiol sulphate*: 2.5 to 9.5 per cent,
- *sodium 17α-dihydroequilin sulphate*: 13.5 to 19.5 per cent,
- *sodium 17β-dihydroequilin sulphate*: 0.5 to 4.0 per cent,
- *sodium 17β-estradiol sulphate*: not more than 2.25 per cent,
- *sodium 17α-dihydroequilenin sulphate*: not more than 3.25 per cent,
- *sodium 17β-dihydroequilenin sulphate*: not more than 2.75 per cent,
- *sodium 8,9-didehydroestrone sulphate*: not more than 6.25 per cent,
- *sodium equilenin sulphate*: not more than 5.5 per cent,
- *total estrone, equilin and 17α-dihydroequilin*: not more than 1.3 per cent.

ASSAY

Examine by gas chromatography (2.2.28), using *3-O-methylestrone R* as the internal standard.

Internal standard solution. Dissolve 8 mg of *3-O-methylestrone R* in 10.0 ml of *ethanol R*. Dilute 2.0 ml of this solution to 10.0 ml with *ethanol R*.

Acetate buffer solution pH 5.2. Dissolve 10 g of *sodium acetate R* in 100 ml of *water R* and add 10 ml of *dilute acetic acid R*. Dilute with *water R* to 500 ml and adjust to pH 5.2 ± 0.1.

Test solution (a). Considering the labelled content, transfer an accurately weighed quantity corresponding to about 2 mg of conjugated estrogens to a 50 ml centrifuge tube containing 15 ml of acetate buffer solution pH 5.2 and 1 g of *barium chloride R*. Cap the tube tightly and shake for 30 min. If necessary, adjust the pH of the solution to

Table 1512.-1

Relative retention (to 3-O-methylestrone)	Analyte	Quantified with reference to CRS	Present as
0.24	17α-estradiol	17α-dihydroequilin CRS	sodium sulphate
0.29	17β-estradiol	estrone CRS	sodium sulphate
0.30	17α-dihydroequilin	17α-dihydroequilin CRS	free steroid, sodium sulphate (assay)
0.35	17β-dihydroequilin	17α-dihydroequilin CRS	sodium sulphate
0.56	17α-dihydroequilenin	estrone CRS	sodium sulphate
0.64	17β-dihydroequilenin	estrone CRS	sodium sulphate
0.80	estrone	estrone CRS	free steroid, sodium sulphate (assay)
0.87	equilin	equilin CRS	free steroid, sodium sulphate (assay)
0.90	8,9-didehydroestrone	estrone CRS	sodium sulphate
1	3-O-methylestrone	(internal standard)	
1.3	equilenin	estrone CRS	sodium sulphate

5.0 ± 0.5 with *acetic acid R* or a 120 g/l solution of *sodium acetate R*. Sonicate for 30 s, then shake for 30 min. Add a suitable sulphatase preparation equivalent to 2500 units and shake mechanically for 10 min in a water-bath at 50 ± 1 °C. Swirl the tube by hand, then shake mechanically for 10 min in the water-bath. Allow to cool. Add 15.0 ml of *ethylene chloride R* to the mixture, immediately cap the tube tightly and shake for 15 min. Centrifuge for 10 min or until the lower layer is clear. Draw out the organic layer to a screw cap tube and add 5 g of *anhydrous sodium sulphate R* and shake. Allow the solution to stand until clear. Protect the solution from any loss due to evaporation. Transfer 3.0 ml of the clear solution to a suitable centrifuge tube fitted with a screw cap. Add 1.0 ml of the internal standard solution. Evaporate the mixture to dryness with the aid of a stream of *nitrogen R* maintaining the temperature below 50 °C. To the dry residue add 15 µl of *anhydrous pyridine R* and 65 µl of *N,O-bis(trimethylsilyl)trifluoroacetamide R* containing 1 per cent *chlorotrimethylsilane R*. Immediately cap the tube tightly, mix thoroughly and allow to stand for 15 min. Add 0.5 ml of *toluene R* and mix mechanically.

Reference solution (a). Dissolve separately 8 mg of *estrone CRS*, 7 mg of *equilin CRS* and 5 mg of *17α-dihydroequilin CRS* in 10.0 ml of *ethanol R*. Dilute together 2.0 ml, 1.0 ml and 1.0 ml respectively of these solutions to 10.0 ml with *ethanol R*. Transfer 1.0 ml of this solution and 1.0 ml of the internal standard solution to a centrifuge tube fitted with a screw-cap. Evaporate the mixture to dryness with the aid of a stream of *nitrogen R*, maintaining the temperature below 50 °C. To the dry residue add 15 µl of *anhydrous pyridine R* and 65 µl of *N,O-bis(trimethylsilyl)trifluoroacetamide R* containing 1 per cent *chlorotrimethylsilane R*. Immediately cap the tube tightly, mix and allow to stand for 15 min. Add 0.5 ml of *toluene R*.

The chromatographic procedure may be carried out using:

- a fused-silica column 15 m long and 0.25 mm in internal diameter coated with *poly[(cyanopropyl)(methyl)](phenyl)(methyl)siloxane R* (film thickness 0.25 µm),
- *hydrogen for chromatography R* as the carrier gas at a flow rate of 2 ml/min,
- a flame-ionisation detector,
- a split ratio of 1:20 to 1:30,

maintaining the temperature of the column at 220 °C and that of the injection port and of the detector at 260 °C. Set the temperature and the flow rate of the carrier gas in such a manner that the required resolution is achieved.

Inject 1 µl of reference solution (a). The relative retentions with reference to 3-*O*-methylestrone are about 0.30, 0.80, 0.87, and 1 for 17α-dihydroequilin, estrone, equilin and 3-*O*-methylestrone, respectively.

The test is not valid unless the resolution between estrone and equilin is at least 1.2. The relative standard deviation of the ratio of the peak area due to estrone to that of the internal standard, obtained from at least 6 injections, is not more than 2.0 per cent.

Inject 1 µl of reference solution (a). Measure the areas of the peaks due to estrone or equilin and *O*-methylestrone. Inject 1 µl of test solution (a). Measure the areas of the peaks due to estrone, equilin and 3-*O*-methylestrone.

Calculate the percentage content of sodium estrone sulphate and sodium equilin sulphate using expression (1).

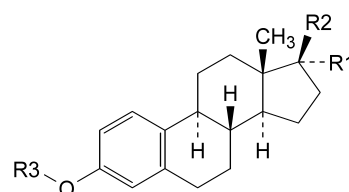
LABELLING

The label states:

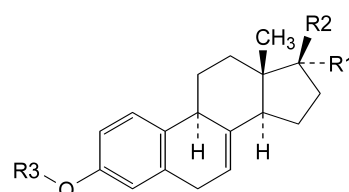
- the name of the substance,

- the content of the substance,
- the nature of the diluent.

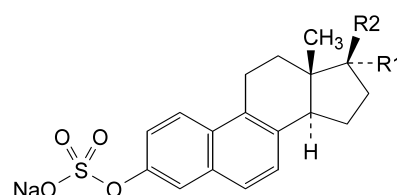
IMPURITIES AND CONCOMITANTS



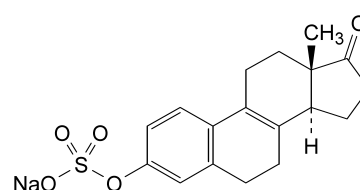
- A. R1 = OH, R2 = H, R3 = SO₃Na: 17α-hydroxyestra-1,3,5(10)-trien-3-yl sodium sulphate (sodium 17α-estradiol sulphate),
- D. R1 = H, R2 = OH, R3 = SO₃Na: 17β-hydroxyestra-1,3,5(10)-trien-3-yl sodium sulphate (sodium 17β-estradiol sulphate),
- I. R1 + R2 = O, R3 = H: 3-hydroxyestra-1,3,5(10)-trien-17-one (estrone),



- B. R1 = OH, R2 = H, R3 = SO₃Na: 17α-hydroxyestra-1,3,5(10),7-tetraen-3-yl sodium sulphate (sodium 17α-dihydroequilin sulphate),
- C. R1 = H, R2 = OH, R3 = SO₃Na: 17β-hydroxyestra-1,3,5(10),7-tetraen-3-yl sodium sulphate (sodium 17β-dihydroequilin sulphate),
- J. R1 + R2 = O, R3 = H: 3-hydroxyestra-1,3,5(10),7-tetraen-17-one (equilin),
- K. R1 = OH, R2 = R3 = H: estra-1,3,5(10),7-tetraene-3,17α-diol (17α-dihydroequilin),



- E. R1 = OH, R2 = H: 17α-hydroxyestra-1,3,5(10),6,8-pentaen-3-yl sodium sulphate (sodium 17α-dihydroequilenin sulphate),
- F. R1 = H, R2 = OH: 17β-hydroxyestra-1,3,5(10),6,8-pentaen-3-yl sodium sulphate (sodium 17β-dihydroequilenin sulphate),
- H. R1 + R2 = O: 17-oxoestra-1,3,5(10),6,8-pentaen-3-yl sodium sulphate (sodium equilenin sulphate),

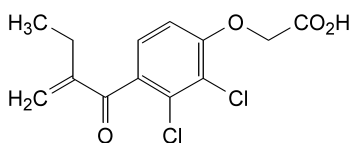


- G. 17-oxoestra-1,3,5(10),8-tetraen-3-yl sodium sulphate (sodium 8,9-didehydroestrone sulphate).

01/2005:0457 *Mobile phase: glacial acetic acid R, ethyl acetate R, methylene chloride R (20:50:60 V/V/V).*

ETACRYNIC ACID

Acidum etacrynicum



$C_{13}H_{12}Cl_2O_4$

M_r 303.1

DEFINITION

[2,3-Dichloro-4-(2-ethylpropenoyl)phenoxy]acetic acid.

Content: 98.0 per cent to 102.0 per cent (dried substance).

CHARACTERS

Appearance: white or almost white, crystalline powder.

Solubility: very slightly soluble in water, freely soluble in alcohol. It dissolves in ammonia and in dilute solutions of alkali hydroxides and carbonates.

IDENTIFICATION

First identification: C.

Second identification: A, B, D, E.

A. Melting point (2.2.14): 121 °C to 124 °C.

B. Dissolve 50.0 mg in a mixture of 1 volume of a 103 g/l solution of *hydrochloric acid R* and 99 volumes of *methanol R* and dilute to 100.0 ml with the same mixture of solvents. Dilute 10.0 ml of this solution to 100.0 ml with a mixture of 1 volume of a 103 g/l solution of *hydrochloric acid R* and 99 volumes of *methanol R*. Examined between 230 nm and 350 nm (2.2.25), the solution shows an absorption maximum at 270 nm and a shoulder at about 285 nm. The specific absorbance at the maximum at 270 nm is 110 to 120.

C. Infrared absorption spectrophotometry (2.2.24).

Preparation: discs.

Comparison: *etacrynic acid CRS*.

D. Dissolve about 30 mg in 2 ml of *aldehyde-free alcohol R*. Dissolve 70 mg of *hydroxylamine hydrochloride R* in 0.1 ml of *water R*, add 7 ml of *alcoholic potassium hydroxide solution R* and dilute to 10 ml with *aldehyde-free alcohol R*. Allow to stand and add 1 ml of the supernatant liquid to the solution of the substance to be examined. Heat the mixture on a water-bath for 3 min. After cooling, add 3 ml of *water R* and 0.15 ml of *hydrochloric acid R*. Examined in ultraviolet light at 254 nm, the mixture shows an intense blue fluorescence.

E. Dissolve about 25 mg in 2 ml of a 42 g/l solution of *sodium hydroxide R* and heat in a water-bath for 5 min. Cool and add 0.25 ml of a mixture of equal volumes of *water R* and *sulphuric acid R*. Add 0.5 ml of a 100 g/l solution of *chromotropic acid, sodium salt R* and, carefully, 2 ml of *sulphuric acid R*. An intense violet colour is produced.

TESTS

Related substances. Thin-layer chromatography (2.2.27).

Test solution. Dissolve 0.2 g of the substance to be examined in *alcohol R* and dilute to 10 ml with the same solvent.

Reference solution. Dilute 0.3 ml of the test solution to 100 ml with *alcohol R*.

Plate: TLC silica gel F_{254} plate *R*.

Application: 10 µl.

Development: over 2/3 of the plate.

Drying: in air.

Detection: examine in ultraviolet light at 254 nm.

Limits:

– *any impurity:* any spots, apart from the principal spot, are not more intense than the spot in the chromatogram obtained with the reference solution (0.3 per cent).

Heavy metals (2.4.8): maximum 20 ppm.

1.0 g complies with limit test F. Prepare the standard using 2 ml of *lead standard solution (10 ppm Pb) R*.

Loss on drying (2.2.32): maximum 0.5 per cent, determined on 2.000 g by drying at 60 °C over *diphosphorus pentoxide R* at a pressure of 0.1-0.5 kPa.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

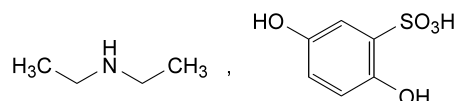
Dissolve 0.250 g in 100 ml of *methanol R* and add 5 ml of *water R*. Titrate with 0.1 M *sodium hydroxide*, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *sodium hydroxide* is equivalent to 30.31 mg of $C_{13}H_{12}Cl_2O_4$.

01/2005:1204

ETAMSYLATE

Etamsylatum



$C_{10}H_{17}NO_5S$

M_r 263.3

DEFINITION

Etamsylate contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of *N*-ethylethanamine 2,5-dihydroxybenzenesulphonate, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, crystalline powder, very soluble in water, freely soluble in methanol, soluble in ethanol, practically insoluble in methylene chloride.

It shows polymorphism.

IDENTIFICATION

First identification: B.

Second identification: A, C, D.

A. Melting point (2.2.14): 127 °C to 134 °C.

B. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *etamsylate CRS*. Examine the substances prepared as discs.

C. Dissolve 0.100 g in *water R* and dilute to 200.0 ml with the same solvent. Dilute 5.0 ml of the solution to 100.0 ml with *water R*. Examined immediately between 210 nm and 350 nm (2.2.25), the solution shows two absorption, maxima at 221 nm and 301 nm. The specific absorbance at the maximum at 301 nm is 145 to 151.

01/2005:1591

D. In a test-tube, introduce 2 ml of freshly prepared solution S (see Tests) and 0.5 g of *sodium hydroxide R*. Warm the mixture and place near the open end of the tube a wet strip of *red litmus paper R*. The colour of the paper becomes blue.

TESTS

Solution S. Dissolve 10.0 g in *carbon dioxide-free water R* and dilute to 100 ml with the same solvent.

Appearance of solution. Solution S, when freshly prepared, is clear (2.2.1) and colourless (2.2.2, *Method II*).

pH (2.2.3). The pH of solution S is 4.5 to 5.6.

Hydroquinone. Examine by thin-layer chromatography (2.2.27), using as the coating substance a suitable silica gel with a fluorescent indicator having an optimal intensity at 254 nm.

Test solution. Dissolve 2.0 g of the substance to be examined in *water R* and dilute to 10 ml with the same solvent.

Reference solution. Dissolve 10 mg of *hydroquinone R* in *water R* and dilute to 50 ml with the same solvent.

Apply to the plate 10 µl of each solution and dry the starting points in a current of cool air. Develop over a path of 15 cm using a mixture of 20 volumes of *methylene chloride R*, 30 volumes of *methyl acetate R* and 50 volumes of *ethyl acetate R*. Dry the plate in a current of hot air and examine in ultraviolet light at 254 nm. Any spot corresponding to hydroquinone in the chromatogram obtained with the test solution is not more intense than the principal spot in the chromatogram obtained with the reference solution (0.1 per cent).

Heavy metals (2.4.8). 1.0 g complies with limit test C for heavy metals (15 ppm). Prepare the standard using 1.5 ml of *lead standard solution (10 ppm Pb) R*.

Iron (2.4.9). 10 ml of solution S complies with the limit test for iron (10 ppm).

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven *in vacuo* at 60 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

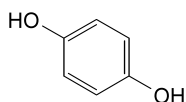
Dissolve 0.200 g in a mixture of 10 ml of *water R* and 40 ml of *dilute sulphuric acid R*. Titrate with 0.1 M *cerium sulphate*, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *cerium sulphate* is equivalent to 13.16 mg of $C_{18}H_{21}N_3O_4$.

STORAGE

Store in an airtight container, protected from light.

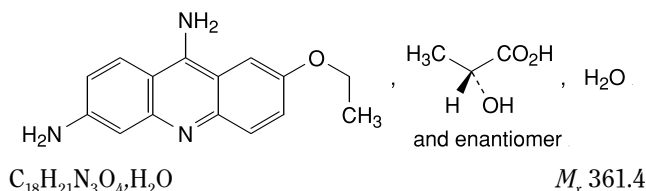
IMPURITIES



A. benzene-1,4-diol (hydroquinone).

ETHACRIDINE LACTATE MONOHYDRATE

Ethacridini lactas monohydricus



DEFINITION

7-Ethoxyacridine-3,9-diamine (2*RS*)-2-hydroxypropanoate.

Content: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

Appearance: yellow crystalline powder.

Solubility: sparingly soluble in water, very slightly soluble in alcohol, practically insoluble in methylene chloride.

IDENTIFICATION

First identification: A.

Second identification: B, C, D.

A. Infrared absorption spectrophotometry (2.2.24).

Comparison: *ethacridine lactate monohydrate CRS*.

B. Mix 0.1 ml of solution S (see Tests) and 100 ml of *water R*. The solution is greenish-yellow and shows a strong green fluorescence in ultraviolet light at 365 nm. Add 5 ml of 1 M *hydrochloric acid*. The fluorescence remains.

C. To 0.5 ml of solution S add 1.0 ml of *water R*, 0.1 ml of a 10 g/l solution of *cobalt chloride R* and 0.1 ml of a 50 g/l solution of *potassium ferrocyanide R*. The solution is green.

D. To 50 ml of solution S add 10 ml of *dilute sodium hydroxide solution R*. Filter. To 5 ml of the filtrate, add 1 ml of *dilute sulphuric acid R*. 5 ml of the solution obtained gives the reaction of lactates (2.3.1).

TESTS

Solution S. Dissolve 2.0 g in *carbon dioxide-free water R* and dilute to 100.0 ml with the same solvent.

pH (2.2.3): 5.5 to 7.0 for solution S.

Related substances. Liquid chromatography (2.2.29).

Test solution. Dissolve 10.0 mg of the substance to be examined in the mobile phase and dilute to 25.0 ml with the mobile phase.

Reference solution (a). Dilute 1.0 ml of test solution to 100.0 ml with the mobile phase.

Reference solution (b). Dilute 1.0 ml of reference solution (a) to 10.0 ml with the mobile phase.

Column:

- size: $l = 0.25$ m, $\varnothing = 4.6$ mm,
- stationary phase: octadecylsilyl silica gel for chromatography R (5 µm).

Mobile phase: dissolve 1.0 g of *sodium octanesulphonate R* in a mixture of 300 ml of *acetonitrile R* and 700 ml of *phosphate buffer solution pH 2.8 R*.

Flow rate: 1 ml/min.

Detection: spectrophotometer at 268 nm.

Injection: 10 µl.

Run time: 3 times the retention time of ethacridine.

Retention time: ethacridine = about 15 min.

Limits:

- **any impurity:** not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.3 per cent),
- **total:** not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (1 per cent),
- **disregard limit:** 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

Heavy metals (2.4.8): maximum 50 ppm.

1.0 g complies with limit test F. Prepare the standard using 5.0 ml of *lead standard solution* (10 ppm Pb) R.

Loss on drying (2.2.32): 4.5 per cent to 5.5 per cent, determined on 1.000 g by drying in an oven *in vacuo* at 100–105 °C.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

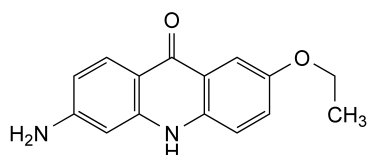
Dissolve 0.270 g in 5.0 ml of *anhydrous formic acid* R. Add 60.0 ml of *acetic anhydride* R and titrate with 0.1 M *perchloric acid*, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *perchloric acid* is equivalent to 34.34 mg of C₁₈H₂₁N₃O₄.

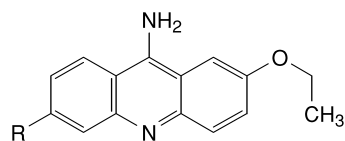
STORAGE

Protected from light.

IMPURITIES



A. 6-amino-2-ethoxyacridin-9(10H)-one,



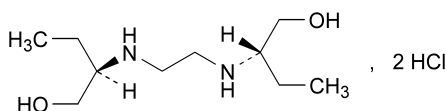
B. R = Cl: 6-chloro-2-ethoxyacridin-9-amine,

C. R = O-CH₂-CH₂-OH: 2-[(9-amino-7-ethoxyacridin-3-yl)oxy]ethanol.

01/2005:0553

ETHAMBUTOL HYDROCHLORIDE

Ethambutoli hydrochloridum



C₁₀H₂₆Cl₂N₂O₂

M_r 277.2

DEFINITION

Ethambutol hydrochloride contains not less than 97.0 per cent and not more than the equivalent of 101.0 per cent of 2,2'-(ethylenediimino)bis[(2S)-butan-1-ol] dihydrochloride, calculated with reference to the dried substance.

CHARACTERS

A white, crystalline powder, freely soluble in water, soluble in alcohol.

It melts at about 202 °C.

IDENTIFICATION

First identification: A, D.

Second identification: B, C, D.

- Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *ethambutol hydrochloride* CRS. Examine the substances prepared as discs.
- Examine the chromatograms obtained in the test for 2-aminobutanol. The principal spot in the chromatogram obtained with test solution (b) is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (b).
- Dissolve 0.1 g in 10 ml of *water* R and add 0.2 ml of *copper sulphate solution* R. Add 0.5 ml of *dilute sodium hydroxide solution* R. A blue colour is produced.
- It gives reaction (a) of chlorides (2.3.1).

TESTS

pH (2.2.3). Dissolve 0.2 g in 10 ml of *carbon dioxide-free water* R. The pH of the solution is 3.7 to 4.0.

2-Aminobutanol. Examine by thin-layer chromatography (2.2.27), using *silica gel G* R as the coating substance.

Test solution (a). Dissolve 0.50 g of the substance to be examined in *methanol* R and dilute to 10 ml with the same solvent.

Test solution (b). Dilute 1 ml of test solution (a) to 10 ml with *methanol* R.

Reference solution (a). Dissolve 50 mg of 2-aminobutanol R in *methanol* R and dilute to 10 ml with the same solvent. Dilute 1 ml of this solution to 10 ml with *methanol* R.

Reference solution (b). Dissolve 50 mg of *ethambutol hydrochloride* CRS in *methanol* R and dilute to 10 ml with the same solvent.

Apply separately to the plate 2 µl of each solution. Develop over a path of 15 cm using a mixture of 10 volumes of *concentrated ammonia* R, 15 volumes of *water* R and 75 volumes of *methanol* R. Allow the plate to dry in air, heat at 110 °C for 10 min, cool and spray with *ninhydrin solution* R1. Heat the plate at 110 °C for 5 min. Any spot corresponding to 2-aminobutanol in the chromatogram obtained with test solution (a) is not more intense than the spot in the chromatogram obtained with reference solution (a) (1.0 per cent).

Heavy metals (2.4.8). 2.0 g complies with limit test C for heavy metals (10 ppm). Prepare the standard using 2 ml of *lead standard solution* (10 ppm Pb) R.

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 0.500 g by drying in an oven at 100 °C to 105 °C for 3 h.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

To 70 ml of *dilute ammonia R2* add 4.0 ml of *copper sulphate solution R*, mix, add 5.0 ml of *dilute sodium hydroxide solution R* and dilute to 100 ml with *water R*. Dissolve 0.100 g of the substance to be examined in 20 ml of this solution and dilute to 25.0 ml with the same solution. Prepare a reference solution in the same manner using 0.100 g of *ethambutol hydrochloride CRS*. Measure the angle of optical rotation (2.2.7) of the solutions at 436 nm. Calculate the content of $C_{10}H_{26}Cl_2N_2O_2$ from the angles of optical rotation measured and the concentrations of the solutions.

STORAGE

Store in an airtight container.

01/2005:1317

ETHANOL (96 PER CENT)

Ethanolum (96 per centum)

DEFINITION

Content:

- ethanol (C_2H_6O ; M_r 46.07): 95.1 per cent V/V (92.6 per cent m/m) to 96.9 per cent V/V (95.2 per cent m/m) at 20 °C, calculated from the relative density using the alcoholimetric tables (5.5),
- water.

CHARACTERS

Appearance: colourless, clear, volatile, flammable liquid, hygroscopic.

Solubility: miscible with water and with methylene chloride.

It burns with a blue, smokeless flame.

bp: about 78 °C.

IDENTIFICATION

First identification: A, B.

Second identification: A, C, D.

A. It complies with the test for relative density (see Tests).

B. Infrared absorption spectrophotometry (2.2.24).

Comparison: Ph. Eur. reference spectrum ethanol (96 per cent).

C. Mix 0.1 ml with 1 ml of a 10 g/l solution of *potassium permanganate R* and 0.2 ml of *dilute sulphuric acid R* in a test-tube. Cover immediately with a filter paper moistened with a freshly prepared solution containing 0.1 g of *sodium nitroprusside R* and 0.5 g of *piperazine hydrate R* in 5 ml of *water R*. After a few minutes, an intense blue colour appears on the paper and becomes paler after 10-15 min.

D. To 0.5 ml add 5 ml of *water R*, 2 ml of *dilute sodium hydroxide solution R*, then slowly add 2 ml of 0.05 *M* *iodine*. A yellow precipitate is formed within 30 min.

TESTS

Appearance. It is clear (2.2.1) and colourless (2.2.2, *Method II*) when compared with *water R*. Dilute 1.0 ml to 20 ml with *water R*. After standing for 5 min, the dilution remains clear (2.2.1) when compared with *water R*.

Acidity or alkalinity. To 20 ml add 20 ml of *carbon dioxide-free water R* and 0.1 ml of *phenolphthalein solution R*. The solution is colourless. Add 1.0 ml of 0.01 *M* *sodium hydroxide*. The solution is pink (30 ppm, expressed as acetic acid).

Relative density (2.2.5): 0.805 to 0.812.

Absorbance (2.2.25): maximum 0.40 at 240 nm, 0.30 between 250 nm and 260 nm and 0.10 between 270 nm and 340 nm.

Examine between 235 nm and 340 nm, in a 5 cm cell using *water R* as the compensation liquid. The absorption curve is smooth.

Volatile impurities. Gas chromatography (2.2.28).

Test solution (a). The substance to be examined.

Test solution (b). Add 150 µl of 4-methylpentan-2-ol *R* to 500.0 ml of the substance to be examined.

Reference solution (a). Dilute 100 µl of *anhydrous methanol R* to 50.0 ml with the substance to be examined. Dilute 5.0 ml of the solution to 50.0 ml with the substance to be examined.

Reference solution (b). Dilute 50 µl of *anhydrous methanol R* and 50 µl of *acetaldehyde R* to 50.0 ml with the substance to be examined. Dilute 100 µl of the solution to 10.0 ml with the substance to be examined.

Reference solution (c). Dilute 150 µl of *acetal R* to 50.0 ml with the substance to be examined. Dilute 100 µl of the solution to 10.0 ml with the substance to be examined.

Reference solution (d). Dilute 100 µl of *benzene R* to 100.0 ml with the substance to be examined. Dilute 100 µl of the solution to 50.0 ml with the substance to be examined.

Column:

- *material*: fused silica,
- *size*: $l = 30$ m, $\varnothing = 0.32$ mm,
- *stationary phase*: poly[(cyanopropyl)(phenyl)][dimethylsiloxane *R* (film thickness 1.8 µm).

Carrier gas: helium for chromatography *R*.

Linear velocity: 35 cm/s.

Split ratio: 1:20.

Temperature:

	Time (min)	Temperature (°C)
Column	0 - 12	40
	12 - 32	40 → 240
	32 - 42	240
Injection port		200
Detector		280

Detection: flame ionisation.

Injection: 1 µl.

System suitability: reference solution (b):

- *resolution*: minimum 1.5 between the first peak (acetaldehyde) and the second peak (methanol).

Limits:

- *methanol* in the chromatogram obtained with test solution (a): not more than half the area of the corresponding peak in the chromatogram obtained with reference solution (a) (200 ppm V/V),
- *acetaldehyde + acetal*: maximum 10 ppm V/V , expressed as acetaldehyde.

Calculate the sum of the contents of acetaldehyde and acetal in parts per million (V/V) using the following expression:

$$\frac{10 \times A_E}{A_T - A_E} + \frac{30 \times C_E}{C_T - C_E}$$

A_E = area of the acetaldehyde peak in the chromatogram obtained with test solution (a),

A_T = area of the acetaldehyde peak in the chromatogram obtained with reference solution (b),

C_E = area of the acetal peak in the chromatogram obtained with test solution (a),

C_T = area of the acetal peak in the chromatogram obtained with reference solution (c).

– *benzene*: maximum 2 ppm V/V.

Calculate the content of benzene in parts per million (V/V) using the following expression:

$$\frac{2B_E}{B_T - B_E}$$

B_E = area of the benzene peak in the chromatogram obtained with the test solution (a),

B_T = area of the benzene peak in the chromatogram obtained with reference solution (d).

If necessary, the identity of benzene can be confirmed using another suitable chromatographic system (stationary phase with a different polarity).

– *total of other impurities* in the chromatogram obtained with test solution (b): not more than the area of the peak due to 4-methylpentan-2-ol in the chromatogram obtained with test solution (b) (300 ppm),

– *disregard limit*: 0.03 times the area of the peak corresponding to 4-methylpentan-2-ol in the chromatogram obtained with test solution (b) (9 ppm).

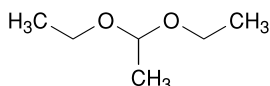
Residue on evaporation: maximum 25 ppm *m/V*.

Evaporate 100 ml to dryness on a water-bath and dry at 100–105 °C for 1 h. The residue weighs a maximum of 2.5 mg.

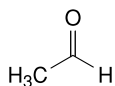
STORAGE

Protected from light.

IMPURITIES



A. 1,1-diethoxyethane (acetal),



B. acetaldehyde,

C. acetone,

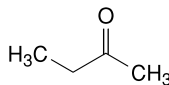


D. benzene,

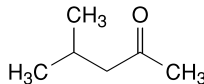


E. cyclohexane,

F. $\text{CH}_3\text{-OH}$: methanol,



G. butan-2-one (methyl ethyl ketone),

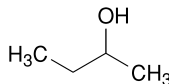


H. 4-methylpentan-2-one (methyl isobutyl ketone),

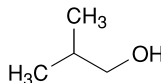
I. $\text{CH}_3\text{-(CH}_2)_2\text{-OH}$: propanol,

J. propan-2-ol (isopropyl alcohol),

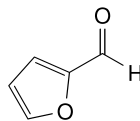
K. $\text{CH}_3\text{-(CH}_2)_3\text{-OH}$: butanol,



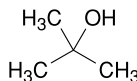
L. butan-2-ol,



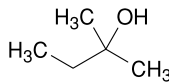
M. 2-methylpropanol (isobutanol),



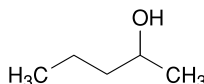
N. furane-2-carbaldehyde (furfural),



O. 2-methylpropan-2-ol (1,1-dimethylethyl alcohol),



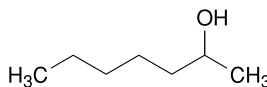
P. 2-methylbutan-2-ol,



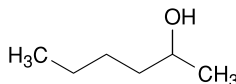
Q. pentan-2-ol,

R. $\text{CH}_3\text{-(CH}_2)_4\text{-OH}$: pentanol,

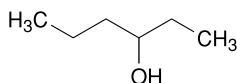
S. $\text{CH}_3\text{-(CH}_2)_5\text{-OH}$: hexanol,



T. heptan-2-ol,



U. hexan-2-ol,

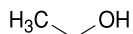


V. hexan-3-ol.

01/2005:1318

ETHANOL, ANHYDROUS

Ethanolum anhydricum

 C_2H_6O M_r 46.07

DEFINITION

Content: not less than 99.5 per cent V/V of C_2H_6O (99.2 per cent m/m), at 20 °C, calculated from the relative density using the alcoholimetric tables (5.5).

CHARACTERS

Appearance: colourless, clear, volatile, flammable liquid, hygroscopic.

Solubility: miscible with water and with methylene chloride. It burns with a blue, smokeless flame.

bp: about 78 °C.

IDENTIFICATION

First identification: A, B.

Second identification: A, C, D.

A. It complies with the test for relative density (see Tests).

B. Infrared absorption spectrophotometry (2.2.24).

Comparison: Ph. Eur. reference spectrum of anhydrous ethanol.

C. Mix 0.1 ml with 1 ml of a 10 g/l solution of *potassium permanganate R* and 0.2 ml of *dilute sulphuric acid R* in a test-tube. Cover immediately with a filter paper moistened with a freshly prepared solution containing 0.1 g of *sodium nitroprusside R* and 0.5 g of *piperazine hydrate R* in 5 ml of *water R*. After a few minutes, an intense blue colour appears on the paper and becomes paler after 10-15 min.

D. To 0.5 ml add 5 ml of *water R*, 2 ml of *dilute sodium hydroxide solution R*, then slowly add 2 ml of 0.05 *M iodine*. A yellow precipitate is formed within 30 min.

TESTS

Appearance. It is clear (2.2.1) and colourless (2.2.2, *Method II*) when compared with *water R*. Dilute 1.0 ml to 20 ml with *water R*. After standing for 5 min, the dilution remains clear (2.2.1) when compared with *water R*.

Acidity or alkalinity. To 20 ml add 20 ml of *carbon dioxide-free water R* and 0.1 ml of *phenolphthalein solution R*. The solution is colourless. Add 1.0 ml of 0.01 *M sodium hydroxide*. The solution is pink (30 ppm, expressed as acetic acid).

Relative density (2.2.5): 0.790 to 0.793.

Absorbance (2.2.25): maximum 0.40 at 240 nm, 0.30 between 250 nm and 260 nm, and 0.10 between 270 nm and 340 nm.

Examined between 235 nm and 340 nm in a 5 cm cell using *water R* as the compensation liquid. The absorption curve is smooth.

Volatile impurities. Gas chromatography (2.2.28).

Test solution (a). The substance to be examined.

Test solution (b). Add 150 µl of *4-methylpentan-2-ol R* to 500.0 ml of the substance to be examined.

Reference solution (a). Dilute 100 µl of *anhydrous methanol R* to 50.0 ml with the substance to be examined. Dilute 5.0 ml of the solution to 50.0 ml with the substance to be examined.

Reference solution (b). Dilute 50 µl of *anhydrous methanol R* and 50 µl of *acetaldehyde R* to 50.0 ml with the substance to be examined. Dilute 100 µl of the solution to 10.0 ml with the substance to be examined.

Reference solution (c). Dilute 150 µl of *acetal R* to 50.0 ml with the substance to be examined. Dilute 100 µl of the solution to 10.0 ml with the substance to be examined.

Reference solution (d). Dilute 100 µl of *benzene R* to 100.0 ml with the substance to be examined. Dilute 100 µl of the solution to 50.0 ml with the substance to be examined.

Column:

- **material:** fused silica,
- **size:** $l = 30$ m, $\varnothing = 0.32$ mm,
- **stationary phase:** poly[(cyanopropyl)(phenyl)]dimethylsiloxane *R* (film thickness 1.8 µm).

Carrier gas: helium for chromatography *R*.

Linear velocity: 35 cm/s.

Split ratio: 1:20.

Temperature:

	Time (min)	Temperature (°C)
Column	0 - 12	40
	12 - 32	40 → 240
	32 - 42	240
Injection port		200
Detector		280

Detection: flame ionisation.

Injection: 1 µl.

System suitability: reference solution (b):

- **resolution:** minimum 1.5 between the first peak (acetaldehyde) and the second peak (methanol).

Limits:

- **methanol:** in the chromatogram obtained with test solution (a): not more than half the area of the corresponding peak in the chromatogram obtained with reference solution (a) (200 ppm V/V).
- **acetaldehyde + acetal:** maximum of 10 ppm V/V , expressed as acetaldehyde.

Calculate the sum of the contents of acetaldehyde and acetal in parts per million (V/V) using the following expression:

$$\frac{10 \times A_E}{A_T - A_E} + \frac{30 \times C_E}{C_T - C_E}$$

A_E = area of the acetaldehyde peak in the chromatogram obtained with test solution (a),

A_T = area of the acetaldehyde peak in the chromatogram obtained with reference solution (b),

C_E = area of the acetal peak in the chromatogram obtained with test solution (a),

C_T = area of the acetal peak in the chromatogram obtained with reference solution (c).

- *benzene*: maximum 2 ppm V/V .

Calculate the content of benzene in parts per million (V/V) using the following expression:

$$\frac{2B_E}{B_T - B_E}$$

B_E = area of the benzene peak in the chromatogram obtained with the test solution (a),

B_T = area of the benzene peak in the chromatogram obtained with reference solution (d).

If necessary, the identity of benzene can be confirmed using another suitable chromatographic system (stationary phase with a different polarity).

- *total of other impurities* in the chromatogram obtained with test solution (b): not more than the area of the peak due to 4-methylpentan-2-ol in the chromatogram obtained with test solution (b) (300 ppm),
- *disregard limit*: 0.03 times the area of the peak corresponding to 4-methylpentan-2-ol in the chromatogram obtained with test solution (b) (9 ppm).

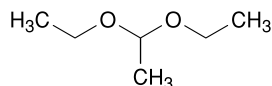
Residue on evaporation: maximum 25 ppm m/V .

Evaporate 100 ml to dryness on a water-bath and dry at 100–105 °C for 1 h. The residue weighs a maximum of 2.5 mg.

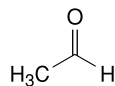
STORAGE

Protected from light.

IMPURITIES



- A. 1,1-diethoxyethane (acetal),



- B. acetaldehyde,

- C. acetone,

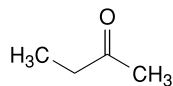


- D. benzene,

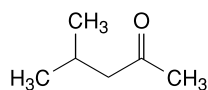


- E. cyclohexane,

- F. $\text{CH}_3\text{-OH}$: methanol,



- G. butan-2-one (methyl ethyl ketone),

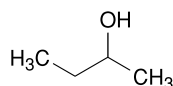


- H. 4-methylpentan-2-one (methyl isobutyl ketone),

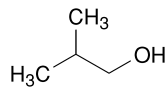
- I. $\text{CH}_3\text{-(CH}_2)_2\text{-OH}$: propanol,

- J. propan-2-ol (isopropyl alcohol),

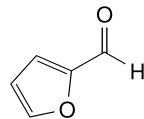
- K. $\text{CH}_3\text{-(CH}_2)_3\text{-OH}$: butanol,



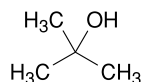
- L. butan-2-ol,



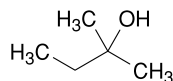
- M. 2-methylpropanol (isobutanol),



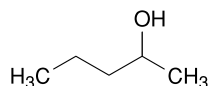
- N. furan-2-carbaldehyde (furfural),



- O. 2-methylpropan-2-ol (1,1-dimethylethyl alcohol),



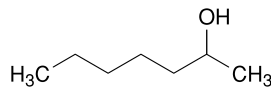
- P. 2-methylbutan-2-ol,



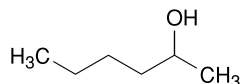
- Q. pentan-2-ol,

- R. $\text{CH}_3\text{-(CH}_2)_4\text{-OH}$: pentanol,

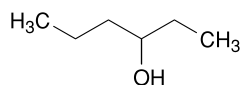
- S. $\text{CH}_3\text{-(CH}_2)_5\text{-OH}$: hexanol,



- T. heptan-2-ol,



- U. hexan-2-ol,

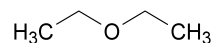


- V. hexan-3-ol.

01/2005:0650

ETHER

Aether



$\text{C}_4\text{H}_{10}\text{O}$

M_r 74.1

DEFINITION

Ether is diethyl ether which may contain a suitable non-volatile antioxidant at a suitable concentration.

CHARACTERS

A clear, colourless liquid, volatile, highly flammable, soluble in water, miscible with alcohol, with methylene chloride and with fatty oils.

IDENTIFICATION

- A. It complies with the test for relative density (see Tests).
 B. It complies with the test for distillation range (see Tests).

TESTS

Acidity. To 20 ml of *alcohol R* add 0.25 ml of *bromothymol blue solution R1* and, dropwise, 0.02 M *sodium hydroxide* until a blue colour persists for 30 s. Add 25 ml of the substance to be examined, shake and add, dropwise, 0.02 M *sodium hydroxide* until the blue colour reappears and persists for 30 s. Not more than 0.4 ml of 0.02 M *sodium hydroxide* is required.

Relative density (2.2.5): 0.714 to 0.716.

Distillation range (2.2.11). *Do not distil if the substance to be examined does not comply with the test for peroxides.* It distils completely between 34.0 °C and 35.0 °C. Carry out the test using a suitable heating device and taking care to avoid directly heating the flask above the level of the liquid.

Non-volatile matter. *After ensuring that the substance to be examined complies with the test for peroxides,* evaporate 50 ml to dryness on a water-bath and dry the residue in an oven at 100 °C to 105 °C. The residue weighs not more than 1 mg (20 mg/l).

Substances with a foreign odour. Moisten a disc of filter paper 80 mm in diameter with 5 ml of the substance to be examined and allow to evaporate. No foreign odour is perceptible immediately after the evaporation.

Aldehydes. To 10.0 ml in a ground-glass-stoppered cylinder add 1 ml of *alkaline potassium tetraiodomercurate solution R* and shake for 10 s. Allow to stand for 5 min, protected from light. The lower layer may show yellow or reddish-brown opalescence but not grey or black opalescence.

Peroxides. Place 8 ml of *potassium iodide and starch solution R* in a 12 ml ground-glass-stoppered cylinder about 15 mm in diameter. Fill completely with the substance to be examined, mix and allow to stand protected from light for 5 min. No colour develops.

Water (2.5.12). Not more than 2 g/l, determined on 20 ml by the semi-micro determination of water.

STORAGE

Store in an airtight container, protected from light, at a temperature of 8 °C to 15 °C.

LABELLING

The label states, where appropriate, the name and concentration of any added non-volatile antioxidant.

DEFINITION

Anaesthetic ether is diethyl ether which may contain a suitable non-volatile antioxidant at an appropriate concentration.

CHARACTERS

A clear, colourless liquid, volatile, very mobile, highly flammable, soluble in 15 parts of water, miscible with alcohol and with fatty oils.

IDENTIFICATION

- A. It complies with the test for relative density (see Tests).
 B. It complies with the test for distillation range (see Tests).

TESTS

Relative density (2.2.5): 0.714 to 0.716.

Distillation range (2.2.11). *Do not distil if the substance to be examined does not comply with the test for peroxides.* It distils completely between 34.0 °C and 35.0 °C. Carry out the test using a suitable heating device and taking care to avoid directly heating the flask above the level of the liquid.

Acids. To 20 ml of *alcohol R* add 0.25 ml of *bromothymol blue solution R1* and, dropwise, 0.02 M *sodium hydroxide* until a blue colour persists for 30 s. Add 25 ml of the substance to be examined, shake and add, dropwise, 0.02 M *sodium hydroxide* until the blue colour reappears and persists for 30 s. Not more than 0.4 ml of 0.02 M *sodium hydroxide* is required.

Acetone and aldehydes. To 10.0 ml in a ground-glass-stoppered cylinder add 1 ml of *alkaline potassium tetra-iodomercurate solution R* and shake for 10 s. Allow to stand for 5 min, protected from light. The lower layer shows only a slight opalescence.

If the substance to be examined does not comply with the test, distil 40 ml, *after ensuring that the substance to be examined complies with the test for peroxides*, until only 5 ml remains. Collect the distillate in a receiver cooled by placing in a bath of iced water and repeat the test described above using 10.0 ml of the distillate.

Peroxides. Place 8 ml of *potassium iodide and starch solution R* in a 12 ml ground-glass-stoppered cylinder about 15 mm in diameter. Fill completely with the substance to be examined, shake vigorously and allow to stand protected from light for 30 min. No colour is produced.

Non-volatile matter. *After ensuring that the substance to be examined complies with the test for peroxides,* evaporate 50 ml to dryness on a water-bath and dry the residue at 100 °C to 105 °C. The residue weighs not more than 1 mg (20 mg/l).

Substances with a foreign odour. Moisten a disc of filter paper 80 mm in diameter with 5 ml of the substance to be examined and allow to evaporate. No foreign odour is perceptible immediately after the evaporation.

Water (2.5.12). Not more than 2 g/l, determined on 20 ml by the semi-micro determination of water.

STORAGE

Store in an airtight container, protected from light, at a temperature of 8 °C to 15 °C. The contents of a partly filled container may deteriorate rapidly.

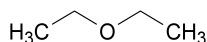
LABELLING

The label states, where appropriate, the name and concentration of any added non-volatile antioxidant.

01/2005:0367

ETHER, ANAESTHETIC

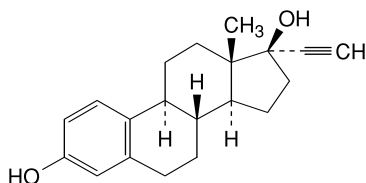
Aether anaestheticus

C₄H₁₀OM_r 74.1

01/2005:0140

ETHINYLESTRADIOL

Ethinylestradiolum

 $C_{20}H_{24}O_2$ M_r 296.4

DEFINITION

19-Nor-17 α -pregna-1,3,5(10)-trien-20-yne-3,17-diol.

Content: 97.0 per cent to 102.0 per cent (dried substance).

CHARACTERS

Appearance: white or slightly yellowish-white, crystalline powder.**Solubility:** practically insoluble in water, freely soluble in alcohol. It dissolves in dilute alkaline solutions.

IDENTIFICATION

A. Infrared absorption spectrophotometry (2.2.24).

Comparison: *ethinylestradiol CRS*.If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance in *methanol R*, evaporate to dryness and record new spectra using the residues.

B. Thin-layer chromatography (2.2.27).

Test solution. Dissolve 25 mg of the substance to be examined in a mixture of 1 volume of *methanol R* and 9 volumes of *methylene chloride R* and dilute to 25 ml with the same mixture of solvents.**Reference solution.** Dissolve 25 mg of *ethinylestradiol CRS* in a mixture of 1 volume of *methanol R* and 9 volumes of *methylene chloride R* and dilute to 25 ml with the same mixture of solvents.**Plate:** TLC silica gel G plate *R*.**Mobile phase:** *alcohol R*, *toluene R* (10:90 V/V).**Application:** 5 μ l.**Development:** over a path of 15 cm.**Drying:** in air until the solvent has evaporated.**Detection:** heat at 110 °C for 10 min, spray the hot plate with *alcoholic solution of sulphuric acid R* and heat again at 110 °C for 10 min. Examine in daylight and in ultraviolet light at 365 nm.**Results:** the principal spot in the chromatogram obtained with the test solution is similar in position, colour, fluorescence and size to the principal spot in the chromatogram obtained with the reference solution.

TESTS

Specific optical rotation (2.2.7): -27 to -30 (dried substance).Dissolve 1.25 g in *pyridine R* and dilute to 25.0 ml with the same solvent.**Related substances.** Liquid chromatography (2.2.29).**Test solution.** Dissolve 0.10 g of the substance to be examined in the mobile phase and dilute to 100.0 ml with the mobile phase.**Reference solution (a).** Dissolve 10 mg of *estradiol R* in the mobile phase, add 10.0 ml of the test solution and dilute to 50.0 ml with the mobile phase. Dilute 1.0 ml of this solution to 10.0 ml with the mobile phase.**Reference solution (b).** Dilute 10.0 ml of the test solution to 50.0 ml with the mobile phase. Dilute 1.0 ml of this solution to 20.0 ml with the mobile phase.**Column:**– **size:** $l = 0.15$ m, $\varnothing = 4.6$ mm,– **stationary phase:** *octadecylsilyl silica gel for chromatography R* (5 μ m).**Mobile phase:** *acetonitrile R*, *water R* (45:55 V/V).**Flow rate:** 1 ml/min.**Detection:** spectrophotometer at 280 nm.**Injection:** 20 μ l.**Run time:** 2.5 times the retention time of ethinylestradiol.**Relative retention** with reference to ethinylestradiol (retention time = about 4.6 min): impurity D = about 0.76, impurity B = about 0.94.**System suitability:** reference solution (a):– **resolution:** minimum 3.5 between the peaks due to impurity D and ethinylestradiol.**Limits:**– **impurity B:** not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (1.0 per cent),– **any other impurity:** not more than 0.25 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.25 per cent),– **total of other impurities:** not more than half the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent)– **disregard limit:** 0.05 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent)**Loss on drying** (2.2.32): maximum 1.0 per cent, determined on 0.500 g by drying in an oven at 100–105 °C for 3 h.

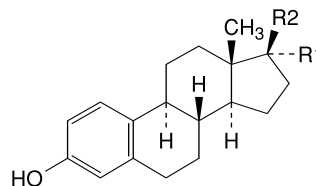
ASSAY

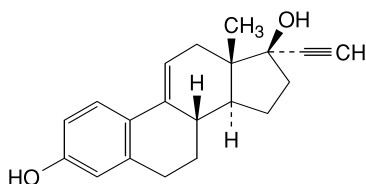
Dissolve 0.200 g in 40 ml of *tetrahydrofuran R* and add 5 ml of a 100 g/l solution of *silver nitrate R*. Titrate with 0.1 M *sodium hydroxide*, determining the end-point potentiometrically (2.2.20). Carry out a blank titration.1 ml of 0.1 M *sodium hydroxide* is equivalent to 29.64 mg of $C_{20}H_{24}O_2$.

STORAGE

Protected from light.

IMPURITIES

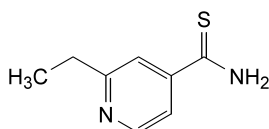
A. $R_1 = OH$, $R_2 = C\equiv CH$: 19-norpregna-1,3,5(10)-trien-20-yne-3,17-diol (17 β -ethinylestradiol),C. $R_1 + R_2 = O$: 3-hydroxyestra-1,3,5(10)-trien-17-one (estrone),D. $R_1 = H$, $R_2 = OH$: estradiol,

B. 19-nor-17 α -pregna-1,3,5(10),9(11)-tetraen-20-yne-3,17-diol.

01/2005:0141

ETHIONAMIDE

Ethionamidum

 $C_8H_{10}N_2S$ M_r 166.2

DEFINITION

Ethionamide contains not less than 98.5 per cent and not more than the equivalent of 101.0 per cent of 2-ethylpyridine-4-carbothioamide, calculated with reference to the dried substance.

CHARACTERS

A yellow, crystalline powder or small, yellow crystals, practically insoluble in water, soluble in methanol, sparingly soluble in alcohol.

IDENTIFICATION

First identification: A, C.

Second identification: A, B, D.

- A. Melting point (2.2.14): 158 °C to 164 °C.
- B. Dissolve 10.0 mg in *methanol R* and dilute to 100.0 ml with the same solvent. Dilute 10.0 ml of the solution to 100.0 ml with *methanol R*. Examined between 230 nm and 350 nm (2.2.25), the solution shows an absorption maximum at 290 nm. The specific absorbance at the maximum is 380 to 440.
- C. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *ethionamide CRS*.
- D. Dissolve about 10 mg in 5 ml of *methanol R*. Add 5 ml of *silver nitrate solution R2*. A dark-brown precipitate is formed.

TESTS

Appearance of solution. Dissolve 0.5 g in 10 ml of *methanol R*, heating to about 50 °C. Allow to cool to room temperature. The solution is not more opalescent than reference suspension II (2.2.1).

Acidity. Dissolve 2.0 g in 20 ml of *methanol R*, heating to about 50 °C, and add 20 ml of *water R*. Cool slightly while shaking until crystallisation begins and then allow to cool to room temperature. Add 60 ml of *water R* and 0.2 ml of *cresol red solution R*. Not more than 0.2 ml of 0.1 M *sodium hydroxide* is required to change the colour of the indicator to red.

Related substances. Examine by thin-layer chromatography (2.2.27), using *silica gel GF₂₅₄ R* as the coating substance.

Test solution. Dissolve 0.2 g of the substance to be examined in *acetone R* and dilute to 10 ml with the same solvent.

Reference solution (a). Dilute 0.5 ml of the test solution to 100 ml with *acetone R*.

Reference solution (b). Dilute 0.2 ml of the test solution to 100 ml with *acetone R*.

Apply separately to the plate 10 μ l of each solution. Develop over a path of 15 cm using a mixture of 10 volumes of *methanol R* and 90 volumes of *chloroform R*. Allow the plate to dry in air. Examine in ultraviolet light at 254 nm. Any spot in the chromatogram obtained with the test solution, apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (a) (0.5 per cent) and at most 1 such spot is more intense than the spot in the chromatogram obtained with reference solution (b) (0.2 per cent).

Heavy metals (2.4.8). 1.0 g complies with limit test D for heavy metals (20 ppm). Prepare the standard using 2 ml of *lead standard solution (10 ppm Pb) R*.

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.00 g by drying in an oven at 100 °C to 105 °C for 3 h.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

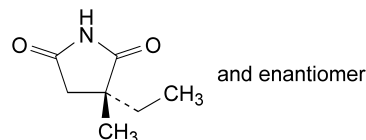
Dissolve 0.150 g in 50 ml of *anhydrous acetic acid R*. Titrate with 0.1 M *perchloric acid*, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *perchloric acid* is equivalent to 16.62 mg of $C_8H_{10}N_2S$.

01/2005:0764

ETHOSUXIMIDE

Ethosuximidum

 $C_7H_{11}NO_2$ M_r 141.2

DEFINITION

(*RS*)-3-Ethyl-3-methylpyrrolidine-2,5-dione.

Content: 99.0 per cent to 101.0 per cent (anhydrous substance).

CHARACTERS

Appearance: white or almost white powder or waxy solid.

Solubility: freely soluble in water, very soluble in alcohol and in methylene chloride.

It shows polymorphism.

IDENTIFICATION

First identification: A, C.

Second identification: A, B, D, E.

- A. Melting point (2.2.14): 45 °C to 50 °C.
- B. Dissolve 50.0 mg in *alcohol R* and dilute to 50.0 ml with the same solvent. Examined between 230 nm and 300 nm (2.2.25), the solution shows an absorption maximum at 248 nm. The specific absorbance at the maximum is 8 to 9.
- C. Infrared absorption spectrophotometry (2.2.24).
Preparation: discs of *potassium bromide R*.

Comparison: ethosuximide CRS.

If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in *methylene chloride R*, evaporate to dryness and record new spectra using the residues.

- D. Dissolve 0.1 g in 3 ml of *methanol R*. Add 0.05 ml of a 100 g/l solution of *cobalt chloride R* and 0.05 ml of a 100 g/l solution of *calcium chloride R* and add 0.1 ml of *dilute sodium hydroxide solution R*. A purple colour develops and no precipitate is formed.
- E. To about 10 mg add 10 mg of *resorcinol R* and 0.2 ml of *sulphuric acid R*. Heat at 140 °C for 5 min and cool. Add 5 ml of *water R* and 2 ml of *concentrated ammonia R1*. A brown colour is produced. Add about 100 ml of *water R*. A green fluorescence is produced.

TESTS

Solution S. Dissolve 2.5 g in *water R* and dilute to 25 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and colourless (2.2.2, Method II).

Cyanide. Liquid chromatography (2.2.29).

Test solution. Dissolve 0.50 g of the substance to be examined in *water R* and dilute to 10.0 ml with the same solvent.

Reference solution (a). Dissolve 0.125 g of *potassium cyanide R* in *water R* and dilute to 50.0 ml with the same solvent. Dilute 1.0 ml to 100.0 ml with *water R*. Dilute 0.5 ml of this solution to 10.0 ml with *water R*.

Reference solution (b). Dissolve 0.50 g of the substance to be examined in *water R*, add 0.5 ml of reference solution (a) and dilute to 10.0 ml with *water R*.

Column:

- *size:* $l = 0.075$ m, $\varnothing = 7.5$ mm,
- *stationary phase:* spherical *weak anion exchange resin* (10 μ m).

Mobile phase: dissolve 2.1 g of *lithium hydroxide R* and 85 mg of *sodium edetate R* in *water for chromatography R* and dilute to 1000.0 ml with the same solvent.

Flow rate: 2.0 ml/min.

Detection: electrochemical detector (direct amperometry) with a silver working electrode, a silver-silver chloride reference electrode, held at + 0.05 V oxidation potential, and a detector sensitivity of 20 nA full scale.

Injection: 20 μ l; inject the test solution and reference solution (b).

System suitability: reference solution (b):

- *peak-to-valley ratio:* minimum 3, where H_p = height above the baseline of the peak due to cyanide and H_v = height above the baseline of the lowest point of the curve separating this peak from the peak due to ethosuximide.

Limit:

- *cyanide:* not more than half the height of the corresponding peak in the chromatogram obtained with reference solution (b) (0.5 ppm).

Related substances. Gas chromatography (2.2.28).

Internal standard solution. Dissolve 20 mg of *myristyl alcohol R* in *ethanol R* and dilute to 10.0 ml with the same solvent.

Test solution. Dissolve 1.00 g of the substance to be examined in *ethanol R*, add 1.0 ml of the internal standard solution and dilute to 20.0 ml with *ethanol R*.

Reference solution (a). Dissolve 20.0 mg of *ethosuximide impurity A CRS* in *ethanol R* and dilute to 10.0 ml with the same solvent. To 0.5 ml of the solution, add 1.0 ml of the internal standard solution and dilute to 20.0 ml with *ethanol R*.

Reference solution (b). Dissolve 0.500 g of the substance to be examined in *ethanol R* and dilute to 10.0 ml with the same solvent. Dilute 1.0 ml to 50.0 ml with *ethanol R*. To 2.0 ml of this solution, add 1.0 ml of the internal standard solution and dilute to 20.0 ml with *ethanol R*.

Column:

- *material:* fused silica,
- *size:* $l = 30$ m, $\varnothing = 0.25$ mm,
- *stationary phase:* *poly(cyanopropyl)(phenylmethyl)siloxane R* (film thickness 0.25 μ m).

Carrier gas: *helium for chromatography R*.

Flow rate: 1 ml/min.

Split ratio: 1:67.

Temperature:

- *column:* 175 °C,
- *injection port and detector:* 240 °C.

Detection: flame ionisation.

Injection: 1 μ l.

Run time: 1.5 times the retention time of ethosuximide.

Relative retentions with reference to the internal standard (retention time = about 8 min): impurity A = about 0.7; ethosuximide = about 1.1.

System suitability: reference solution (b):

- *resolution:* minimum 5 between the peaks due to the internal standard and to ethosuximide.

Limits:

- *impurity A:* calculate the ratio (R) of the area of the peak due to impurity A to the area of the peak due to the internal standard from the chromatogram obtained with reference solution (a); from the chromatogram obtained with the test solution, calculate the ratio of the area of any peak due to impurity A to the area of the peak due to the internal standard: this ratio is not greater than R (0.1 per cent),
- *any other impurity:* calculate the ratio (R) of half the area of the peak due to ethosuximide to the area of the peak due to the internal standard from the chromatogram obtained with reference solution (b); from the chromatogram obtained with the test solution, calculate the ratio of the area of any peak, apart from the principal peak and the peaks due to impurity A and to the internal standard, to the area of the peak due to the internal standard: this ratio is not greater than R (0.1 per cent),
- *total:* calculate the ratio (R) of the area of the peak due to ethosuximide to the area of the peak due to the internal standard from the chromatogram obtained with reference solution (b); from the chromatogram obtained with the test solution, calculate the ratio of the sum of the areas of any peaks, apart from the principal peak and the peak due to the internal standard, to the area of the peak due to the internal standard: this ratio is not greater than R (0.2 per cent),
- *disregard limit:* calculate the ratio (R) of 0.25 times the area of the peak due to impurity A to the area of the peak due to the internal standard from the chromatogram obtained with reference solution (a); from the chromatogram obtained with the test solution, calculate the ratio of the area of any peak, apart from the principal peak and the peak due to the internal

standard, to the area of the peak due to the internal standard: disregard any peak which has a ratio less than *R* (0.025 per cent).

Heavy metals (2.4.8): maximum 10 ppm.

12 ml of solution *S* complies with limit test *A*. Prepare the standard using *lead standard solution* (1 ppm *Pb*) *R*.

Water (2.5.12): maximum 0.5 per cent, determined on 1.00 g.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.120 g in 20 ml of *dimethylformamide R* and carry out a potentiometric titration (2.2.20) using 0.1 *M* *tetrabutylammonium hydroxide*. Protect the solution from atmospheric carbon dioxide throughout the titration. Carry out a blank titration.

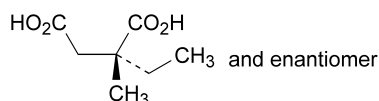
1 ml of 0.1 *M* *tetrabutylammonium hydroxide* is equivalent to 14.12 mg of $C_7H_{11}NO_2$.

STORAGE

Protected from light.

IMPURITIES

Specified impurities: A.

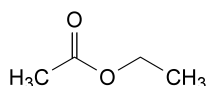


A. (2*RS*)-2-ethyl-2-methylbutanedioic acid.

01/2005:0899

ETHYL ACETATE

Ethylis acetas



$C_4H_8O_2$

M_r 88.1

DEFINITION

Ethyl acetate is ethyl ethanoate.

CHARACTERS

A clear, colourless liquid, volatile, soluble in water, miscible with acetone, with alcohol and with methylene chloride.

IDENTIFICATION

First identification: B.

Second identification: A, C, D.

A. Boiling point (2.2.12): 76 °C to 78 °C.

B. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the *Ph. Eur. reference spectrum of ethyl acetate*.

C. It gives the reaction of acetyl (2.3.1).

D. It gives the reaction of esters (2.3.1).

TESTS

Appearance of solution. A mixture of 1 ml of the substance to be examined and 15 ml of *water R* is clear (2.2.1) and colourless (2.2.2, *Method II*).

Acidity. To 10 ml of *alcohol R* add 0.1 ml of *phenolphthalein solution R* and 0.01 *M* *sodium hydroxide* until the colour changes to pink. Add 5.5 ml of the substance to be examined and 0.25 ml of 0.02 *M* *sodium hydroxide*. The solution remains pink for not less than 15 s.

Relative density (2.2.5): 0.898 to 0.902.

Refractive index (2.2.6): 1.370 to 1.373.

Reaction with sulphuric acid. Carefully add 2 ml to 10 ml of *sulphuric acid R*. After 15 min, the interface between the two liquids is not coloured.

Related substances. Examine by gas chromatography (2.2.28).

Test solution. The substance to be examined.

The chromatographic procedure may be carried out using:

- a glass column 2 m long and 2 mm in internal diameter, packed with *ethylvinylbenzene-divinylbenzene copolymer R* (136 µm to 173 µm),
- *nitrogen for chromatography R* as the carrier gas at a flow rate of 30 ml/min,
- a flame-ionisation detector,

raising the temperature at a rate of 8 °C/min from 90 °C to 240 °C and maintaining at 240 °C for 8 min and maintaining the temperature of the injection port and that of the detector at 240 °C.

Inject 1 µl of the test solution. In the chromatogram obtained with the test solution, the sum of the areas of any peaks apart from the principal peak is not greater than 0.2 per cent of the area of the principal peak.

Residue on evaporation. Evaporate 100.0 g to dryness on a water-bath and dry in an oven at 100 °C to 105 °C. The residue weighs not more than 3 mg (30 ppm).

Water (2.5.12). Not more than 0.1 per cent, determined on 10.0 ml by the semi-micro determination of water.

STORAGE

Store protected from light, at a temperature not exceeding 30 °C.

IMPURITIES

- A. methyl acetate,
- B. ethanol,
- C. methanol.

01/2005:1319

ETHYL OLEATE

Ethylis oleas

DEFINITION

Ethyl oleate is a mixture consisting of the ethyl esters of fatty acids, mainly oleic acid. A suitable antioxidant may be added.

CHARACTERS

A clear, pale yellow or colourless liquid, practically insoluble in water, miscible with alcohol, with methylene chloride and with light petroleum (40 °C to 60 °C).

IDENTIFICATION

- A. It complies with the test for relative density (see Tests).
- B. It complies with the test for saponification value (see Tests).
- C. It complies with the test of oleic acid (see Tests).

TESTS

Relative density (2.2.5): 0.866 to 0.874.

Acid value (2.5.1). Not more than 0.5, determined on 10.0 g.

Iodine value (2.5.4): 75 to 90.

Peroxide value (2.5.5). Not more than 10.0.

Saponification value (2.5.6): 177 to 188, determined on 2.0 g.

Oleic acid (2.4.22, *Method A*). The fatty acid fraction of the substance contains at least 60.0 per cent of oleic acid.

Water (2.5.12). Not more than 1.0 per cent, determined on 1.00 g by the semi-micro determination of water.

Total ash (2.4.16). Not more than 0.1 per cent, determined on 2.0 g.

STORAGE

Store protected from light.

LABELLING

The label states, where applicable, the name and concentration of any added antioxidant.

sodium carbonate solution R; the substance partly dissolves (solution B). Add at the same time to solution A and solution B 5 ml of *aminopyrazolone solution R* and 1 ml of *potassium ferricyanide solution R* and mix. Solution B is yellow to orange-brown. Solution A is orange to red, the colour being clearly more intense than any similar colour which may be obtained with solution B.

TESTS

Solution S. Dissolve 1.0 g in *alcohol R* and dilute to 10 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and not more intensely coloured than reference solution BY₆ (2.2.2, *Method II*).

Acidity. To 2 ml of solution S add 3 ml of *alcohol R*, 5 ml of *carbon dioxide-free water R* and 0.1 ml of *bromocresol green solution R*. Not more than 0.1 ml of 0.1 M *sodium hydroxide* is required to change the colour of the indicator to blue.

Related substances. Thin-layer chromatography (2.2.27).

Test solution (a). Dissolve 0.10 g of the substance to be examined in *acetone R* and dilute to 10 ml with the same solvent.

Test solution (b). Dilute 1 ml of test solution (a) to 10 ml with *acetone R*.

Reference solution (a). Dilute 0.5 ml of test solution (a) to 100 ml with *acetone R*.

Reference solution (b). Dissolve 10 mg of *ethyl parahydroxybenzoate CRS* in *acetone R* and dilute to 10 ml with the same solvent.

Reference solution (c). Dissolve 10 mg of *methyl parahydroxybenzoate R* in 1 ml of test solution (a) and dilute to 10 ml with *acetone R*.

Plate: suitable octadecylsilyl silica gel with a fluorescent indicator having an optimal intensity at 254 nm as the coating substance.

Mobile phase: *glacial acetic acid R*, *water R*, *methanol R* (1:30:70 V/V/V).

Application: 2 µl.

Development: over a path of 15 cm.

Drying: in air.

Detection: examine in ultraviolet light at 254 nm.

System suitability: the chromatogram obtained with reference solution (c) shows 2 clearly separated principal spots.

Limits:

- *any impurity:* any spot in the chromatogram obtained with test solution (a), apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (a) (0.5 per cent).

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

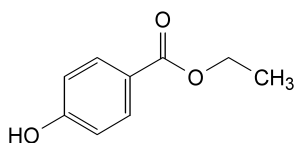
To 1.000 g add 20.0 ml of 1 M *sodium hydroxide*. Heat at about 70 °C for 1 h. Cool rapidly in an ice bath. Prepare a blank in the same manner. Carry out the titration on the solutions at room temperature. Titrate the excess sodium hydroxide with 0.5 M *sulphuric acid*, continuing the titration until the second point of inflexion and determining the end-point potentiometrically (2.2.20).

1 ml of 1 M *sodium hydroxide* is equivalent to 166.2 mg of C₉H₁₀O₃.

01/2005:0900

ETHYL PARAHYDROXYBENZOATE

Ethylis parahydroxybenzoas

C₉H₁₀O₃M_r 166.2

DEFINITION

Ethyl 4-hydroxybenzoate.

Content: 98.0 per cent to 102.0 per cent.

CHARACTERS

Appearance: white or almost white, crystalline powder or colourless crystals.

Solubility: very slightly soluble in water, freely soluble in alcohol and in methanol.

IDENTIFICATION

First identification: A, B.

Second identification: A, C, D.

A. Melting point (2.2.14): 115 °C to 118 °C.

B. Infrared absorption spectrophotometry (2.2.24).

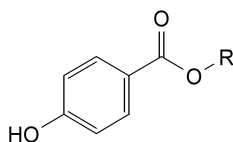
Comparison: *ethyl parahydroxybenzoate CRS*.

C. Examine the chromatograms obtained in the test for related substances.

Results: the principal spot in the chromatogram obtained with test solution (b) is similar in position and size to the principal spot in the chromatogram obtained with reference solution (b).

D. To about 10 mg in a test-tube add 1 ml of *sodium carbonate solution R*, boil for 30 s and cool (solution A). To a further 10 mg in a similar test-tube add 1 ml of

IMPURITIES



- A. R = H: 4-hydroxybenzoic acid,
 B. R = CH₃: methyl 4-hydroxybenzoate,
 C. R = CH₂-CH₂-CH₃: propyl 4-hydroxybenzoate,
 D. R = CH₂-CH₂-CH₂-CH₃: butyl 4-hydroxybenzoate.

01/2005:0822

ETHYLCELLULOSE

Ethylcellulosum

DEFINITION

Partly *O*-ethylated cellulose.

Content: 44.0 per cent to 51.0 per cent of ethoxy (-OC₂H₅) groups (dried substance).

CHARACTERS

Appearance: white or yellowish-white powder or granular powder, odourless or almost odourless.

Solubility: practically insoluble in water, soluble in methylene chloride and in a mixture of 20 g of alcohol and 80 g of toluene, slightly soluble in ethyl acetate and in methanol, practically insoluble in glycerol (85 per cent) and in propylene glycol. The solutions may show a slight opalescence.

IDENTIFICATION

- A. Infrared absorption spectrophotometry (2.2.24).

Comparison: Ph. Eur. reference spectrum of ethylcellulose.

- B. It complies with the limits of the assay.

TESTS

Acidity or alkalinity. To 0.5 g add 25 ml of *carbon dioxide-free water R* and shake for 15 min. Filter through a sintered-glass filter (40). To 10 ml add 0.1 ml of *phenolphthalein solution R* and 0.5 ml of 0.01 M *sodium hydroxide*. The solution is pink. To 10 ml add 0.1 ml of *methyl red solution R* and 0.5 ml of 0.01 M *hydrochloric acid*. The solution is red.

Viscosity (2.2.9). The viscosity, determined at 25 °C and expressed in mPas, is not less than 80.0 per cent and not more than 120.0 per cent of that stated on the label for a nominal viscosity greater than 6 mPas; and not less than 75.0 per cent and not more than 140.0 per cent of that stated on the label for a nominal viscosity not greater than 6 mPas. Shake a quantity of the substance to be examined equivalent to 5.00 g of the dried substance with 95 g of a mixture of 20 g of *alcohol R* and 80 g of *toluene R* until the substance is dissolved. Determine the viscosity using a capillary viscometer.

Acetaldehyde: maximum 100 ppm.

Introduce 3.0 g into a 250 ml conical flask with a ground-glass stopper, add 10 ml of *water R* and stir mechanically for 1 h. Allow to stand for 24 h, filter and dilute the filtrate to 100.0 ml with *water R*. Transfer 5.0 ml to a 25 ml volumetric flask, add 5 ml of a 0.5 g/l solution of *methylbenzothiazolone hydrazone hydrochloride R* and

heat in a water-bath at 60 °C for 5 min. Add 2 ml of *ferric chloride-sulphamic acid reagent R* and heat again at 60 °C for 5 min. Cool and dilute to 25.0 ml with *water R*. The solution is not more intensely coloured than a standard prepared at the same time and in the same manner using instead of the 5.0 ml of filtrate, 5.0 ml of a reference solution prepared by diluting 3.0 ml of *acetaldehyde standard solution* (100 ppm C₂H₄O) *R1* to 100.0 ml with *water R*.

Chlorides (2.4.4): maximum 0.1 per cent.

Disperse 0.250 g in 50 ml of *water R*, heat to boiling and allow to cool, shaking occasionally. Filter and discard the first 10 ml of the filtrate. Dilute 10 ml of the filtrate to 15 ml with *water R*. The solution complies with the limit test for chlorides.

Heavy metals (2.4.8): maximum 20 ppm.

1.0 g complies with limit test C. Prepare the standard using 2 ml of *lead standard solution* (10 ppm Pb) *R*.

Loss on drying (2.2.32): maximum 3.0 per cent, determined on 1.000 g by drying in an oven at 100-105 °C for 2 h.

Sulphated ash (2.4.14): maximum 0.5 per cent, determined on 1.0 g.

ASSAY

Gas chromatography (2.2.28).

CAUTION: *hydriodic acid* and its reaction by-products are highly toxic. Perform all steps for preparation of the test and reference solutions in a properly functioning hood.

Internal standard solution. Dilute 120 µl of *toluene R* to 10 ml with *o-xylene R*.

Test solution. Transfer 50.0 mg of the substance to be examined, 50.0 mg of *adipic acid R* and 2.0 ml of the internal standard solution into a suitable 5 ml thick-walled reaction vial with a pressure-tight septum-type closure. Cautiously add 2.0 ml of *hydriodic acid R*, immediately close the vial tightly and weigh the contents and the vial accurately. Shake the vial for 30 s, heat to 125 °C for 10 min, allow to cool for 2 min, shake again for 30 s and heat to 125 °C for 10 min. Afterwards allow to cool for 2 min and repeat shaking and heating for a third time. Allow the vial to cool for 45 min and reweigh. If the loss is greater than 10 mg, discard the mixture and prepare another. Use the upper layer.

Reference solution. Transfer 100.0 mg of *adipic acid R*, 4.0 ml of the internal standard solution and 4.0 ml of *hydriodic acid R* into a suitable 10 ml thick-walled reaction vial with a pressure-tight septum-type closure. Close the vial tightly and weigh the vial and contents accurately. Afterwards inject 50 µl of *iodoethane R* through the septum with a syringe, weigh the vial again and calculate the mass of *iodoethane* added, by difference. Shake well and allow the layers to separate.

Column:

- *material:* stainless steel,
- *size:* *l* = 5.0 m, Ø = 2 mm,
- *stationary phase:* *diatomaceous earth for gas chromatography R* (150-180 µm) impregnated with 3 per cent *m/m* of *poly(dimethyl)siloxane R*.

Carrier gas: *nitrogen for chromatography R*.

Flow rate: 15 ml/min.

Temperature:

- *column:* 80 °C,
- *injection port and detector:* 200 °C.

Detection: flame ionisation.

Injection: 1 µl of the upper layer of the test solution and 1 µl of the upper layer of the reference solution.

Relative retention with reference to toluene: iodoethane = about 0.6; *o*-xylene = about 2.3.

System suitability: reference solution:

- **resolution:** minimum 2.0 between the peaks due to iodoethane and toluene.

Calculate the percentage content of ethoxy groups from the following expression:

$$\frac{Q_1 \times m_2 \times 45.1 \times 100 \times 100}{2 \times Q_2 \times m_1 \times 156.0 \times (100 - d)}$$

- Q_1 = ratio of iodoethane peak area to toluene peak area in the chromatogram obtained with the test solution,
- Q_2 = ratio of iodoethane peak area to toluene peak area in the chromatogram obtained with the reference solution,
- m_1 = mass of the substance to be examined used in the test solution, in milligrams,
- m_2 = mass of iodoethane used in the reference solution, in milligrams,
- d = percentage loss on drying.

LABELLING

The label states the nominal viscosity in millipascal seconds for a 5 per cent *m/m* solution.

01/2005:1421

ETHYLENE GLYCOL MONOPALMITOSTEARATE

Ethylenglycoli monopalmitostearas

DEFINITION

Mixture of ethylene glycol mono- and diesters of stearic and palmitic acids, produced from the condensation of ethylene glycol and stearic acid 50 of vegetable or animal origin (see *Stearic acid* (1474)).

Content: minimum of 50.0 per cent of monoesters.

CHARACTERS

Appearance: white or almost white, waxy solid.

Solubility: practically insoluble in water, soluble in acetone and in hot alcohol.

IDENTIFICATION

- It complies with the test for melting point (see Tests).
- It complies with the test for composition of fatty acids (see Tests).
- It complies with the assay (monoesters content).

TESTS

Melting point (2.2.15): 54 °C to 60 °C.

Acid value (2.5.1): maximum 3.0, determined on 10.0 g.

Iodine value (2.5.4): maximum 3.0.

Saponification value (2.5.6): 170 to 195, determined on 2.0 g.

Composition of fatty acids (2.4.22, Method A). The fatty acid fraction has the following composition:

- *stearic acid*: 40.0 per cent to 60.0 per cent,
- *sum of contents of palmitic acid and stearic acid*: minimum 90.0 per cent.

Free ethylene glycol: maximum 5.0 per cent, determined as prescribed under Assay.

Total ash (2.4.16): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Size-exclusion chromatography (2.2.30).

Test solution. Into a 15 ml flask, weigh about 0.2 g (*m*), to the nearest 0.1 mg. Add 5.0 ml of *tetrahydrofuran R* and shake to dissolve. Heat gently, if necessary. Reweigh the flask and calculate the total mass of solvent and substance (*M*).

Reference solutions. Into four 15 ml flasks, weigh, to the nearest 0.1 mg, about 2.5 mg, 5.0 mg, 10.0 mg and 20.0 mg of *ethylene glycol R*. Add 5.0 ml of *tetrahydrofuran R* and shake to dissolve. Weigh the flasks again and calculate the concentration of ethylene glycol in milligrams per gram for each reference solution.

Column:

- **size:** *l* = 0.6 m, Ø = 7 mm,
- **stationary phase:** *styrene-divinylbenzene copolymer R* (particle diameter 5 µm and pore size 10 nm).

Mobile phase: *tetrahydrofuran R*.

Flow rate: 1 ml/min.

Detection: differential refractometer.

Injection: 40 µl.

Relative retention with reference to ethylene glycol: diesters = about 0.76, monoesters = about 0.83.

Limits:

- **free ethylene glycol:** from the calibration curve obtained with the reference solutions, determine the concentration (*C*) in milligrams per gram in the test solution and calculate the percentage content in the substance to be examined using the following expression:

$$\frac{C \times M}{m \times 10}$$

- **monoesters:** calculate the percentage content of monoesters using the following expression:

$$\frac{A}{A + B} \times (100 - D)$$

- A = area of the peak due to the monoesters,
- B = area of the peak due to the diesters,
- D = percentage content of free ethylene glycol + percentage content of free fatty acids which may be determined using the following expression:
- $$\frac{I_A \times 270}{561.1}$$
- I_A = acid value.

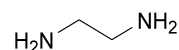
STORAGE

Protected from light.

01/2005:0716

ETHYLENEDIAMINE

Ethylendiaminum



$\text{C}_2\text{H}_8\text{N}_2$

M_r 60.1

DEFINITION

Ethylenediamine contains not less than 98.0 per cent and not more than the equivalent of 101.0 per cent of ethane-1,2-diamine.

CHARACTERS

A clear, colourless or slightly yellow liquid, hygroscopic, miscible with water and with ethanol. On exposure to air, white fumes are evolved. On heating, it evaporates completely.

IDENTIFICATION

- A. Relative density (2.2.5): 0.895 to 0.905.
 B. Boiling point (2.2.12): 116 °C to 118 °C.
 C. To 0.2 ml add 0.5 ml of *acetic anhydride R*. Boil. A crystalline mass forms after cooling, which dissolves in 5 ml of *2-propanol R* with heating. Cool the solution and add 5 ml of *ether R*. If necessary, initiate crystallisation by scratching the walls of the test-tube with a glass rod. Filter through a sintered-glass filter, wash with several portions of *ether R* and dry at 100 °C to 105 °C. The residue melts (2.2.14) at 173 °C to 177 °C.

TESTS

Solution S. Mix 10 g with *carbon dioxide-free water R* and dilute to 100 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and not more intensely coloured than the reference solution BY₆ (2.2.2, *Method II*).

Carbonate. A mixture of 4 ml of solution S and 6 ml of *calcium hydroxide solution R* is not more opalescent than reference suspension II (2.2.1).

Chlorides (2.4.4). To 5 ml of solution S add 5 ml of *dilute nitric acid R* and dilute to 15 ml with *water R*. This solution complies with the limit test for chlorides (100 ppm).

Ammonia and other bases. Dissolve 1.2 g in 20 ml of *alcohol R* and add, dropwise with stirring, 4.5 ml of *hydrochloric acid R*. Evaporate to dryness on a water-bath, breaking up any resulting cake with a glass rod, and dry at 100 °C to 105 °C for 1 h.

1 g of the residue is equivalent to 0.4518 g of C₂H₈N₂. The percentage content of C₂H₈N₂ found in this test does not vary by more than 0.5 from the percentage content determined in the assay.

Iron (2.4.9). 10 ml of solution S complies with the limit test for iron (10 ppm).

Heavy metals (2.4.8). 12 ml of solution S complies with limit test A for heavy metals (10 ppm). Prepare the standard using *lead standard solution (1 ppm Pb) R*.

Residue on evaporation. Evaporate 5.00 g to dryness on a water-bath and dry at 100 °C to 105 °C for 1 h. The residue weighs not more than 15 mg (0.3 per cent).

ASSAY

Place 25.0 ml of *1 M hydrochloric acid* and 0.2 ml of *methyl red mixed solution R* in a flask. Add 0.600 g of the substance to be examined. Titrate with *1 M sodium hydroxide* until the colour changes from violet-red to green.

1 ml of *1 M hydrochloric acid* is equivalent to 30.05 mg of C₂H₈N₂.

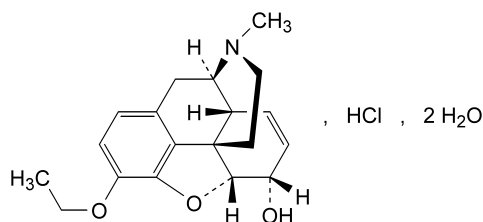
STORAGE

Store in an airtight container, protected from light.

01/2005:0491

ETHYLMORPHINE HYDROCHLORIDE

Ethylmorphini hydrochloridum

C₁₉H₂₄ClNO₃·2H₂OM_r 385.9

DEFINITION

7,8-Didehydro-4,5α-epoxy-3-ethoxy-17-methylmorphinan-6α-ol hydrochloride dihydrate.

Content: 99.0 per cent to 101.0 per cent (anhydrous substance).

CHARACTERS

Appearance: white or almost white, crystalline powder.

Solubility: soluble in water and in alcohol, insoluble in cyclohexane.

IDENTIFICATION

First identification: A, D.

Second identification: B, C, D.

A. Infrared absorption spectrophotometry (2.2.24).

Comparison: Ph. Eur. reference spectrum of *ethylmorphine hydrochloride*.

B. In a test-tube, dissolve 0.5 g in 6 ml of *water R* and add 15 ml of *0.1 M sodium hydroxide*. Scratch the wall of the tube with a glass rod. A white, crystalline precipitate is formed. Collect the precipitate, wash and dissolve in 20 ml of *water R* heated to 80 °C. Filter and cool in iced water. The crystals, after drying *in vacuo* for 12 h, melt (2.2.14) at 85 °C to 89 °C.

C. To about 10 mg add 1 ml of *sulphuric acid R* and 0.05 ml of *ferric chloride solution R2*. Heat on a water-bath. A blue colour develops. Add 0.05 ml of *nitric acid R*. The colour becomes red.

D. Solution S (see Tests) gives reaction (a) of chlorides (2.3.1).

TESTS

Solution S. Dissolve 0.500 g in *carbon dioxide-free water R* and dilute to 25.0 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and not more intensely coloured than reference solution BY₆ (2.2.2, *Method II*).

Acidity or alkalinity. To 10 ml of solution S add 0.05 ml of *methyl red solution R* and 0.2 ml of *0.02 M hydrochloric acid*, the solution is red. Add 0.4 ml of *0.02 M sodium hydroxide*, the solution becomes yellow.

Specific optical rotation (2.2.7): −102 to −105 (anhydrous substance), determined on solution S.

Related substances. Liquid chromatography (2.2.29).

Test solution. Dissolve 50.0 mg of the substance to be examined in the mobile phase and dilute to 20.0 ml with the mobile phase.

Reference solution (a). Dilute 1.0 ml of the test solution to 25.0 ml with the mobile phase. Dilute 1.0 ml of this solution to 20.0 ml with the mobile phase.

Reference solution (b). Dissolve 12.5 mg of *codeine R* in the mobile phase and dilute to 5.0 ml with the mobile phase.

Reference solution (c). Dilute 0.5 ml of reference solution (b) to 100.0 ml with the mobile phase.

Reference solution (d). To 1.0 ml of the test solution, add 1.0 ml of reference solution (b) and dilute to 50.0 ml with the mobile phase.

Column:

- **size:** $l = 0.25$ m, $\varnothing = 4.6$ mm,
- **stationary phase:** octylsilyl silica gel for chromatography *R* (5 μ m),
- **temperature:** 30 °C.

Mobile phase: add 1.25 g of *sodium heptanesulphonate R* to a mixture of 12.5 ml of *glacial acetic acid R* and 5 ml of a 20 per cent *V/V* solution of *triethylamine R* in a mixture of equal volumes of *methanol R* and *water R*. Dilute to 1000 ml with *water R*. To 550 ml of this solution add 450 ml of *methanol R*.

Flow rate: 1 ml/min.

Detection: spectrophotometer at 230 nm.

Injection: 10 μ l.

Run time: 4 times the retention time of ethylmorphine.

Relative retention with reference to ethylmorphine (retention time = about 6.2 min): impurity B = about 0.7; impurity C = about 0.8; impurity D = about 1.3; impurity A = about 2.5.

System suitability: reference solution (d):

- **resolution:** minimum 5 between the peaks due to ethylmorphine and impurity C.

Limits:

- **correction factor:** for the calculation of content, multiply the peak area of impurity D by 0.4,
- **impurities A, B, D:** for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent),
- **impurity C:** not more than the area of the principal peak in the chromatogram obtained with reference solution (c) (0.5 per cent),
- **any other impurity:** for each impurity, not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent),
- **total of impurities other than C:** not more than 2.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent),
- **disregard limit:** 0.25 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

Water (2.5.12): 8.0 per cent to 10.0 per cent, determined on 0.250 g.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.300 g in a mixture of 5 ml of 0.01 *M* hydrochloric acid and 30 ml of *alcohol R*. Carry out a potentiometric titration (2.2.20), using 0.1 *M* sodium hydroxide. Read the volume added between the 2 points of inflexion.

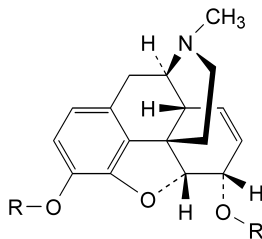
1 ml of 0.1 *M* sodium hydroxide is equivalent to 34.99 mg of $C_{19}H_{24}ClNO_3$.

STORAGE

Protected from light.

IMPURITIES

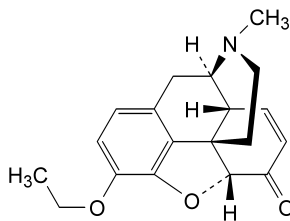
Specified impurities: A, B, C, D.



A. $R = R' = C_2H_5$: 7,8-didehydro-4,5 α -epoxy-3,6 α -diethoxy-17-methylmorphinan,

B. $R = R' = H$: morphine,

C. $R = CH_3$, $R' = H$: codeine,

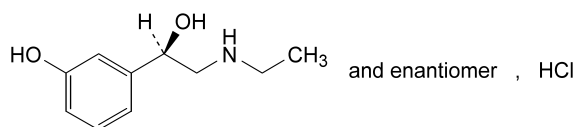


D. 7,8-didehydro-4,5 α -epoxy-3-ethoxy-17-methylmorphinan-6-one (ethylmorphinone).

01/2005:1205

ETILEFRINE HYDROCHLORIDE

Etilefrini hydrochloridum



$C_{10}H_{16}ClNO_2$

M_r 217.7

DEFINITION

(1*RS*)-2-(Ethylamino)-1-(3-hydroxyphenyl)ethanol hydrochloride.

Content: 98.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

Appearance: white or almost white, crystalline powder or colourless crystals.

Solubility: freely soluble in water, soluble in ethanol (96 per cent), practically insoluble in methylene chloride.

IDENTIFICATION

First identification: B, E.

Second identification: A, C, D, E.

A. Melting point (2.2.14): 118 °C to 122 °C.

B. Infrared absorption spectrophotometry (2.2.24).

Preparation: discs of *potassium chloride R*.

Comparison: *etilefrine hydrochloride CRS*.

C. Thin-layer chromatography (2.2.27).

Prepare the solutions protected from bright light and develop the chromatograms protected from light.

Test solution. Dissolve 25 mg of the substance to be examined in *methanol R* and dilute to 5 ml with the same solvent.

Reference solution (a). Dissolve 25 mg of *etilefrine hydrochloride CRS* in *methanol R* and dilute to 5 ml with the same solvent.

Reference solution (b). Dissolve 10 mg of *phenylephrine hydrochloride CRS* in 2 ml of reference solution (a) and dilute to 10 ml with *methanol R*.

Plate: TLC silica gel plate *R*.

Mobile phase: concentrated ammonia *R*, *methanol R*, *methylene chloride R* (5:25:70 V/V/V).

Application: 5 µl.

Development: over a path of 15 cm.

Drying: in a current of warm air.

Detection: spray with a 10 g/l solution of *potassium permanganate R*; examine in daylight after 15 min.

System suitability: reference solution (b):

- the chromatogram shows 2 clearly separated spots.

Results: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).

- D. To 0.2 ml of solution S (see Tests), add 1 ml of *water R*, 0.1 ml of *copper sulphate solution R* and 1 ml of *strong sodium hydroxide solution R*. A blue colour is produced. Add 2 ml of *ether R* and shake. The upper layer is colourless.

- E. Dilute 1 ml of solution S to 10 ml with *water R*. The solution gives reaction (a) of chlorides (2.3.1).

TESTS

Solution S. Dissolve 2.50 g in *carbon dioxide-free water R* prepared from *distilled water R* and dilute to 50.0 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and colourless (2.2.2, Method II).

Acidity or alkalinity. Dilute 4 ml of solution S to 10 ml with *carbon dioxide-free water R*. Add 0.1 ml of *methyl red solution R* and 0.2 ml of 0.01 M *sodium hydroxide*. The solution is yellow. Not more than 0.4 ml of 0.01 M *hydrochloric acid* is required to change the colour of the indicator to red.

Optical rotation (2.2.7): -0.10° to $+0.10^\circ$, determined on solution S.

Related substances. Liquid chromatography (2.2.29).

Prepare the solutions immediately before use.

Test solution. Dissolve 50.0 mg of the substance to be examined in *water R* and dilute to 50.0 ml with the same solvent.

Reference solution (a). Dilute 1.0 ml of the test solution to 10.0 ml with *water R*. Dilute 1.0 ml of this solution to 50.0 ml with *water R*.

Reference solution (b). Dissolve 10.0 mg of *etilefrine impurity A CRS* in *water R* and dilute to 50.0 ml with the same solvent. Dilute 1.0 ml of the solution to 50.0 ml with *water R*.

Reference solution (c). To 10.0 ml of reference solution (a) add 5.0 ml of reference solution (b) and dilute to 20.0 ml with *water R*.

Column:

- size: $l = 0.25$ m, $\varnothing = 4.6$ mm,
- stationary phase: octylsilyl silica gel for chromatography *R* (5 µm).

Mobile phase: mix 35 volumes of *acetonitrile R* and 65 volumes of a 1.1 g/l solution of *sodium laurilsulfate R* adjusted to pH 2.3 with *phosphoric acid R*.

Flow rate: 1 ml/min.

Detection: spectrophotometer at 220 nm.

Injection: 20 µl.

Run time: 5 times the retention time of etilefrine.

Relative retention with reference to etilefrine (retention time = about 9 min): impurity E = about 0.5; impurity C = about 0.8; impurity B = about 0.9; impurity A = about 1.2; impurity F = about 1.7; impurity D = about 4.5.

System suitability: reference solution (c):

- resolution: minimum 2.5 between the peaks due to etilefrine and impurity A.

Limits:

- impurity A: not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.4 per cent),
- impurities B, C, D, E: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent),
- any other impurity: for each impurity, not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent),
- sum of impurities other than A: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (1 per cent),
- disregard limit: 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.02 per cent); disregard any peak due to the solvent.

Sulphates (2.4.13): maximum 200 ppm, determined on 15 ml of solution S.

Heavy metals (2.4.8): maximum 20 ppm.

Dissolve 2.0 g in 20 ml of *water R*. 12 ml of the solution complies with limit test A. Prepare the reference solution using *lead standard solution (2 ppm Pb) R*.

Loss on drying (2.2.32): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 100–105 °C.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.150 g in a mixture of 20 ml of *anhydrous acetic acid R* and 50 ml of *acetic anhydride R*. Titrate with 0.1 M *perchloric acid*, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *perchloric acid* is equivalent to 21.77 mg of $C_{10}H_{16}ClNO_2$.

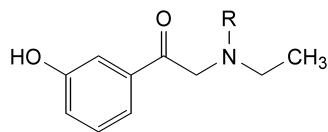
STORAGE

In an airtight container, protected from light.

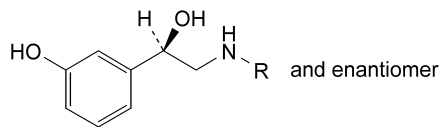
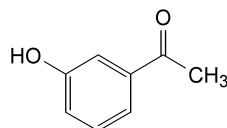
IMPURITIES

Specified impurities: A, B, C, D, E.

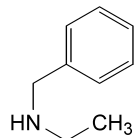
Other detectable impurities: F.



A. R = H: 2-(ethylamino)-1-(3-hydroxyphenyl)ethanone (etilefrone),

D. R = CH₂-C₆H₅: 2-(benzylethylamino)-1-(3-hydroxyphenyl)ethanone (benzyletilefrone),B. R = CH₃: (1*RS*)-1-(3-hydroxyphenyl)-2-(methylamino)ethanol (phenylephrine),C. R = H: (1*RS*)-2-amino-1-(3-hydroxyphenyl)ethanol (norfenefrine),

E. 1-(3-hydroxyphenyl)ethanone (3-hydroxyacetophenone),

F. *N*-benzylethanamine (benzylethylamine).

Second identification: A, C.

A. Melting point (2.2.14): 144 °C to 150 °C.

B. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *etodolac CRS*.C. Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel GF₂₅₄ plate R* previously activated by heating at 105 °C for 1 h.*Test solution.* Dissolve 10 mg of the substance to be examined in *acetone R* and dilute to 10 ml with the same solvent.*Reference solution.* Dissolve 10 mg of *etodolac CRS* in *acetone R* and dilute to 10 ml with the same solvent.Place the plate in an unsaturated chamber containing a mixture of 20 volumes of a 25 g/l solution of *ascorbic acid R* and 80 volumes of *methanol R*. Allow the solution to ascend 1 cm above the line of application on the plate, remove the plate and allow it to dry for at least 30 min.

Apply separately to the plate 10 µl of each solution.

Develop over a path of 15 cm using a mixture of 0.5 volumes of *glacial acetic acid R*, 30 volumes of *ethanol R* and 70 volumes of *toluene R*. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. The principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with the reference solution.

TESTS

Optical rotation (2.2.7). Dissolve 2.50 g in *methanol R* and dilute to 25.0 ml with the same solvent. The angle of optical rotation is -0.10° to +0.10°.**Related substances.** Examine by liquid chromatography (2.2.29).*Test solution.* Dissolve 50.0 mg of the substance to be examined in *methanol R* and dilute to 50.0 ml with the same solvent.*Reference solution (a).* Dilute 5.0 ml of the test solution to 50.0 ml with *methanol R*. Dilute 5.0 ml of this solution to 100.0 ml with *methanol R*.*Reference solution (b).* Dissolve 1.0 mg of *etodolac impurity H CRS* and 1.0 mg of *etodolac CRS* in *methanol R* and dilute to 50 ml with the same solvent.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.15 m long and 4.6 mm in internal diameter, packed with *octadecylsilyl silica gel for chromatography R* (5 µm),
- as mobile phase at a flow rate of 1.5 ml/min:

Mobile phase A. A mixture of 312 volumes of *methanol R* and 688 volumes of a solution prepared as follows: dissolve 13.6 g of *potassium dihydrogen phosphate R* in 900 ml of *water R*, adjust the pH to 7.0 with a 300 g/l solution of *potassium hydroxide R* and dilute to 1000 ml with *water R*,

Mobile phase B. *Acetonitrile R*,

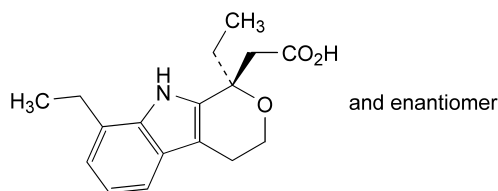
Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)	Comment
0 - 20	90 → 80	10 → 20	linear gradient
20 - 40	80 → 50	20 → 50	linear gradient
40 - 45	90	10	switch to initial conditions
45 - 55	90	10	re-equilibration

— as detector a spectrophotometer set at 225 nm, maintaining the temperature of the column at 40 °C.

01/2005:1422

ETODOLAC

Etodolacum

C₁₇H₂₁NO₃M_r 287.4

DEFINITION

Etodolac contains not less than 98.0 per cent and not more than the equivalent of 102.0 per cent of 2-[(1*RS*)-1,8-diethyl-1,3,4,9-tetrahydropyrano[3,4-*b*]indol-1-yl]acetic acid, calculated with reference to the anhydrous substance.

CHARACTERS

A white or almost white, crystalline powder, practically insoluble in water, freely soluble in acetone and in ethanol.

IDENTIFICATION

First identification: B.

Inject 10 µl of reference solution (a). Adjust the sensitivity of the system so that the height of the principal peak in the chromatogram obtained is at least 50 per cent of the full scale of the recorder. Inject 10 µl of reference solution (b). When the chromatogram is recorded in the prescribed conditions, the retention times are: impurity H about 8 min and etodolac about 9 min. The test is not valid unless the resolution between the peaks due to impurity H and etodolac is at least 5.0.

Inject separately 10 µl of the test solution and 10 µl of reference solution (a). In the chromatogram obtained with the test solution the area of any peak, apart from the principal peak, is not greater than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent); the sum of the areas of all of the peaks apart from the principal peak is not greater than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (1 per cent). Disregard any peak with an area less than 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (a).

Chlorides. Not more than 300 ppm. Dissolve 1.0 g of the substance to be examined in 60 ml of *methanol R*, add 10 ml of *water R* and 20 ml of *dilute nitric acid R*. Titrate with 0.01 M *silver nitrate*, determining the end-point potentiometrically (2.2.20).

1 ml of 0.01 M *silver nitrate* is equivalent to 0.3545 mg Cl.

Heavy metals (2.4.8). 2.0 g complies with limit test C for heavy metals (10 ppm). Prepare the standard using 2 ml of *lead standard solution (10 ppm Pb) R*.

Water (2.5.12). Not more than 0.5 per cent, determined on 1.000 g by the semi-micro determination of water.

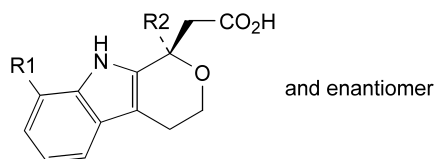
Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.250 g in 60 ml of *methanol R*. Titrate with 0.1 M *tetrabutylammonium hydroxide* determining the end-point potentiometrically (2.2.20). Carry out a blank titration.

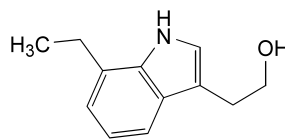
1 ml of 0.1 M *tetrabutylammonium hydroxide* is equivalent to 28.74 mg of C₁₇H₂₁NO₃.

IMPURITIES

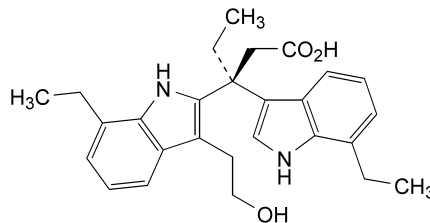


- A. R1 = H, R2 = CH₂-CH₃: 2-[(1*RS*)-1-ethyl-1,3,4,9-tetrahydropyrano[3,4-*b*]indol-1-yl]acetic acid (8-desethyl etodolac),
- B. R1 = CH₃, R2 = CH₂-CH₃: 2-[(1*RS*)-1-ethyl-8-methyl-1,3,4,9-tetrahydropyrano[3,4-*b*]indol-1-yl]acetic acid (8-methyl etodolac),
- C. R1 = CH₂-CH₃, R2 = CH₃: 2-[(1*RS*)-8-ethyl-1-methyl-1,3,4,9-tetrahydropyrano[3,4-*b*]indol-1-yl]acetic acid (1-methyl etodolac),
- D. R1 = CH(CH₃)₂, R2 = CH₂-CH₃: 2-[(1*RS*)-1-ethyl-8-(1-methylethyl)-1,3,4,9-tetrahydropyrano[3,4-*b*]indol-1-yl]acetic acid (8-isopropyl etodolac),
- E. R1 = CH₂-CH₂-CH₃, R2 = CH₂-CH₃: 2-[(1*RS*)-1-ethyl-8-propyl-1,3,4,9-tetrahydropyrano[3,4-*b*]indol-1-yl]acetic acid (8-propyl etodolac),

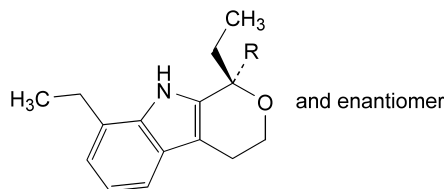
- F. R1 = CH₂-CH₃, R2 = CH(CH₃)₂: 2-[(1*RS*)-8-ethyl-1-(1-methylethyl)-1,3,4,9-tetrahydropyrano[3,4-*b*]indol-1-yl]acetic acid (1-isopropyl etodolac),
- G. R1 = CH₂-CH₃, R2 = CH₂-CH₂-CH₃: 2-[(1*RS*)-8-ethyl-1-propyl-1,3,4,9-tetrahydropyrano[3,4-*b*]indol-1-yl]acetic acid (1-propyl etodolac),



- H. 2-(7-ethylindol-3-yl)ethanol,

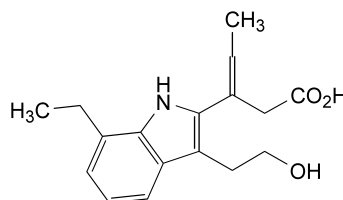


- I. (3*RS*)-3-[7-ethyl-3-(2-hydroxyethyl)indol-2-yl]-3-(7-ethylindol-3-yl)pentanoic acid (etodolac dimer),



- J. R = CH₃: (1*RS*)-1,8-diethyl-1-methyl-1,3,4,9-tetrahydropyrano[3,4-*b*]indole (decarboxy etodolac),

- K. R = CH₂-CO-O-CH₃: methyl 2-[(1*RS*)-1,8-diethyl-1,3,4,9-tetrahydropyrano[3,4-*b*]indol-1-yl]acetate (etodolac methyl ester),

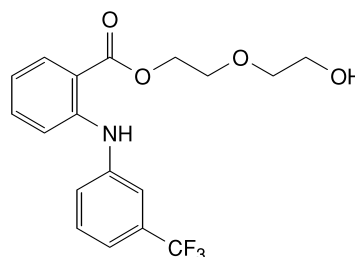


- L. (*EZ*)-3-[7-ethyl-3-(2-hydroxyethyl)-1*H*-indol-2-yl]pent-3-enoic acid.

01/2005:1513

ETOFENAMATE

Etofenamatum

C₁₈H₁₈F₃NO₄M_r 369.4

DEFINITION

2-(2-Hydroxyethoxy)ethyl 2-[[3-(trifluoromethyl)phenyl]amino]benzoate.

Content: 98.5 per cent to 101.5 per cent (anhydrous substance).

CHARACTERS

Appearance: yellowish viscous liquid.

Solubility: practically insoluble in water, miscible with alcohol and with ethyl acetate.

IDENTIFICATION

Infrared absorption spectrophotometry (2.2.24).

Comparison: etofenamate CRS.

Preparation: films.

TESTS

Appearance. The substance to be examined is clear (2.2.1) and not more intensely coloured than reference solution GY₁ (2.2.2, Method II).

Impurity F. Gas chromatography (2.2.28).

Internal standard: tetradecane R.

Solution A. Dissolve 6.0 mg of tetradecane R in hexane R and dilute to 10.0 ml with the same solvent.

Solution B. To 6.0 mg of diethylene glycol R in a 10 ml volumetric flask add 3 ml of *N*-methyltrimethylsilyl-trifluoroacetamide R and heat for 30 min at 50 °C. After cooling dilute to 10.0 ml with *N*-methyltrimethylsilyl-trifluoroacetamide R.

Test solution. To 0.200 g of the substance to be examined add 10 µl of solution A. Add 2 ml of *N*-methyltrimethylsilyl-trifluoroacetamide R and heat for 30 min at 50 °C.

Reference solution. To 2.0 ml of *N*-methyltrimethylsilyl-trifluoroacetamide R add 10 µl of solution A and 10 µl of solution B.

Column:

- size: $l = 25$ m, $\varnothing = 0.20$ mm,
- stationary phase: poly(dimethyl)(diphenyl)siloxane R (film thickness 0.33 µm).

Carrier gas: hydrogen for chromatography R.

Flow rate: 0.9 ml/min.

Temperature:

	Time (min)	Temperature (°C)	Rate (°C/min)
Column	0 - 13	60 → 150	7
	13 - 19	150 → 300	25
	19 - 34	300	
Injection port		150	
Detector		300	

Detection: flame ionisation.

Injection: 0.2 µl.

Limit:

- impurity F: maximum 0.1 per cent.

Related substances. Liquid chromatography (2.2.29).

Test solution. Dissolve 50.0 mg of the substance to be examined in 30 ml of methanol R and dilute to 50.0 ml with water R.

Reference solution (a). Dissolve 10.0 mg of etofenamate impurity G CRS in methanol R and dilute to 20.0 ml with the same solvent. Dilute 0.2 ml of the solution to 50.0 ml with a mixture of 40 volumes of water R and 60 volumes of methanol R.

Reference solution (b). Dilute 0.2 ml of the test solution to 100.0 ml with a mixture of 40 volumes of water R and 60 volumes of methanol R.

Reference solution (c). To 5.0 ml of reference solution (a), add 5.0 ml of reference solution (b).

Reference solution (d). Dissolve 10.0 mg of etofenamate for peak identification CRS (contains etofenamate spiked with about 1 per cent of impurities A, B, C, D and E) in 6.0 ml of methanol R and dilute to 10.0 ml with water R.

Column:

- size: $l = 0.10$ m, $\varnothing = 4.0$ mm,
- stationary phase: octadecylsilyl silica gel for chromatography R (3 µm),
- temperature: 40 °C.

Mobile phase:

- mobile phase A: dissolve 1.3 g of ammonium phosphate R and 4.0 g of tetrabutylammonium hydroxide R in 900 ml of water R. Adjust the pH to 8.0 with dilute phosphoric acid R and dilute to 1000 ml with water R,
- mobile phase B: methanol R,

Time	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)
0 - 13	40	60
13 - 20	40 → 10	60 → 90
20 - 25	10	90
25 - 26	10 → 40	90 → 60
26 - 31	40	60

Flow rate: 1.2 ml/min.

Detection: spectrophotometer at 286 nm.

Injection: 20 µl.

Relative retention with reference to etofenamate (retention time = about 13 min): impurity A = about 0.2; impurity C = about 0.7; impurity G = about 0.85; impurity E = about 1.5; impurity B = about 1.6; impurity D = about 1.7.

System suitability: reference solution (c):

- resolution: minimum of 2.3 between the peaks due to impurity G and to etofenamate.

Limits:

- correction factors: for the calculation of contents, multiply the peak areas of the following impurities by the corresponding correction factor: impurity A = 0.62; impurity C = 0.45; impurity D = 0.77,
- impurity A: not more than 1.25 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.25 per cent),
- impurity B: not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent),
- impurity C: not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent),
- impurity D: not more than 2.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent),

01/2005:0492

- **impurity E**: not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent),
- **impurity G**: not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent),
- **any other impurity**: not more than half the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent),
- **total**: not more than 6 times the area of the principal peak in the chromatogram obtained with reference solution (b) (1.2 per cent),
- **disregard limit**: 0.25 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

Heavy metals (2.4.8): maximum 10 ppm.

2.0 g complies with limit test C. Prepare the standard using 2 ml of *lead standard solution* (10 ppm Pb) R.

Water (2.5.12): maximum 0.5 per cent, determined on 1.00 g.

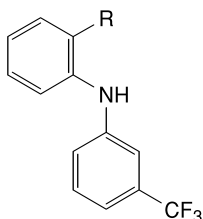
Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

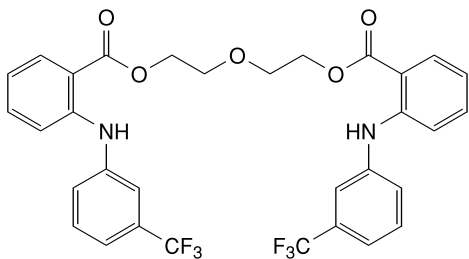
To 3.000 g add 20 ml of *2-propanol* R and 20.0 ml of *1 M sodium hydroxide* and heat under reflux for 2 h. Add 0.1 ml of *bromothymol blue solution* R1. Titrate after cooling with *1 M hydrochloric acid* until the colour disappears. Carry out a blank titration.

1 ml of *1 M sodium hydroxide* is equivalent to 0.3694 g of $C_{18}H_{18}F_3NO_4$.

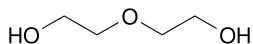
IMPURITIES



- A. R = CO_2H : 2-[[3-(trifluoromethyl)phenyl]amino]benzoic acid (flufenamic acid),
- B. R = $CO-O-C_4H_9$: butyl 2-[[3-(trifluoromethyl)phenyl]amino]benzoate (butyl flufenamate),
- C. R = H: *N*-phenyl-3-(trifluoromethyl)aniline,
- E. R = $CO-[O-CH_2-CH_2]_3-CH_2-CH_3$: 2-(2-butoxyethoxy)ethyl 2-[[3-(trifluoromethyl)phenyl]amino]benzoate,
- G. R = $CO-O-CH_2-CH_2-OH$: 2-hydroxyethyl 2-[[3-(trifluoromethyl)phenyl]amino]benzoate,



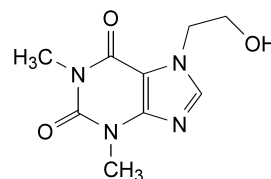
- D. 2,2'-oxybis(ethylene) bis[2-[[3-(trifluoromethyl)phenyl]amino]benzoate],



- F. 2,2'-oxydiethanol.

ETOFYLLINE

Etofyllinum



$C_9H_{12}N_4O_3$

M_r 224.2

DEFINITION

Etofylline contains not less than 98.5 per cent and not more than the equivalent of 101.0 per cent of 7-(2-hydroxyethyl)-1,3-dimethyl-3,7-dihydro-1H-purine-2,6-dione, calculated with reference to the dried substance.

CHARACTERS

A white, crystalline powder, soluble in water, slightly soluble in alcohol.

IDENTIFICATION

First identification: B, C.

Second identification: A, C, D.

- A. Melting point (2.2.14): 161 °C to 166 °C.
- B. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *etofylline CRS*. Examine the substances as discs prepared using 0.5 mg to 1 mg of the substance to be examined in 0.3 g of *potassium bromide* R.
- C. Dissolve 1 g in 5 ml of *acetic anhydride* R and boil under a reflux condenser for 15 min. Allow to cool and add 100 ml of a mixture of 20 volumes of *ether* R and 80 volumes of *light petroleum* R. Cool in iced water for at least 20 min, shaking from time to time. Filter, wash the precipitate with a mixture of 20 volumes of *ether* R and 80 volumes of *light petroleum* R, recrystallise from *alcohol* R and dry *in vacuo*. The crystals melt (2.2.14) at 101 °C to 105 °C.
- D. It gives the reaction of xanthines (2.3.1).

TESTS

Solution S. Dissolve 2.5 g in *carbon dioxide-free water* R and dilute to 50 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and colourless (2.2.2, *Method II*).

Acidity or alkalinity. To 10 ml of solution S add 0.25 ml of *bromothymol blue solution* R1. The solution is yellow or green. Not more than 0.4 ml of 0.01 M *sodium hydroxide* is required to change the colour of the indicator to blue.

Related substances. Examine by thin-layer chromatography (2.2.27), using *silica gel* HF_{254} R as the coating substance.

Test solution. Dissolve 0.3 g of the substance to be examined in a mixture of 20 volumes of *water* R and 30 volumes of *methanol* R and dilute to 10 ml with the same mixture of solvents. Prepare immediately before use.

Reference solution (a). Dilute 1 ml of the test solution to 100 ml with *methanol* R.

Reference solution (b). Dilute 0.2 ml of the test solution to 100 ml with *methanol* R.

Reference solution (c). Dissolve 10 mg of *theophylline R* in *methanol R*, add 0.3 ml of the test solution and dilute to 10 ml with *methanol R*.

Apply to the plate 10 µl of each solution. Develop over a path of 15 cm using a mixture of 1 volume of *concentrated ammonia R*, 10 volumes of *ethanol R* and 90 volumes of *chloroform R*. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. Any spot in the chromatogram obtained with the test solution, apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (a) (1 per cent) and at most one such spot is more intense than the spot in the chromatogram obtained with reference solution (b) (0.2 per cent). The test is not valid unless the chromatogram obtained with reference solution (c) shows two clearly separated spots.

Chlorides (2.4.4). Dilute 2.5 ml of solution S to 15 ml with *water R*. The solution complies with the limit test for chlorides (400 ppm).

Heavy metals (2.4.8). 12 ml of solution S complies with limit test A for heavy metals (20 ppm). Prepare the standard using *lead standard solution (1 ppm Pb) R*.

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

In order to avoid overheating in the reaction medium, mix thoroughly throughout and stop the titration immediately after the end-point has been reached.

Dissolve 0.200 g in 3.0 ml of *anhydrous formic acid R* and add 50.0 ml of *acetic anhydride R*. Titrate with 0.1 M *perchloric acid*, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *perchloric acid* is equivalent to 22.42 mg of C₉H₁₂N₄O₃.

STORAGE

Store protected from light.

IDENTIFICATION

- Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *etomidate CRS*.
- It complies with the test for specific optical rotation (see Tests).

TESTS

Solution S. Dissolve 0.25 g in *ethanol R* and dilute to 25.0 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and colourless (2.2.2, *Method II*).

Specific optical rotation (2.2.7): + 67 to + 70, determined on solution S and calculated with reference to the dried substance.

Related substances. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 0.100 g of the substance to be examined in a mixture of 50 volumes of *ethanol R* and 50 volumes of *water R* and dilute to 10.0 ml with the same mixture of solvents.

Reference solution (a). Dissolve 5.0 mg of *etomidate CRS* and 5.0 mg of *etomidate impurity B CRS* in a mixture of 50 volumes of *ethanol R* and 50 volumes of *water R* and dilute to 250.0 ml with the same mixture of solvents.

Reference solution (b). Dilute 1.0 ml of the test solution to 100.0 ml with a mixture of 50 volumes of *ethanol R* and 50 volumes of *water R*. Dilute 5.0 ml of the solution to 25.0 ml with a mixture of 50 volumes of *ethanol R* and 50 volumes of *water R*.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.1 m long and 4.6 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (3 µm),
- as mobile phase at a flow rate of 2.0 ml/min, the following linear gradient programme:

Mobile phase A. A 5 g/l solution of *ammonium carbonate R*,

Mobile phase B. *Acetonitrile R*,

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)	Comment
0 - 5	90 → 30	10 → 70	linear gradient
5 - 6	30 → 10	70 → 90	linear gradient
6 - 10	10	90	isocratic
10 - 11	10 → 90	90 → 10	linear gradient
11 - 15	90	10	re-equilibration

- as detector a spectrophotometer set at 235 nm.

Equilibrate the column for at least 30 min with *acetonitrile R* and then equilibrate at the initial eluent composition for at least 5 min.

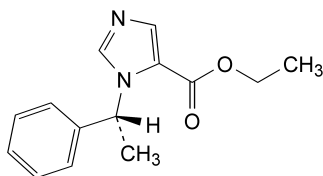
Adjust the sensitivity of the system so that the height of the principal peak in the chromatogram obtained with 10 µl of reference solution (b) is at least 50 per cent of the full scale of the recorder.

Inject 10 µl of reference solution (a). When the chromatograms are recorded in the prescribed conditions, the retention times are: impurity B about 4.5 min; etomidate about 5.0 min. The test is not valid unless the resolution between the peaks due to impurity B and etomidate is at least 5.0. If necessary, adjust the concentration of *ammonium carbonate R* in the mobile phase or the time programme of the linear gradient.

01/2005:1514

ETOMIDATE

Etomidatum



C₁₄H₁₆N₂O₂

*M*_r 244.3

DEFINITION

Etomidate contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of ethyl 1-[(1*R*)-1-phenylethyl]-1*H*-imidazole-5-carboxylate, calculated with reference to the dried substance.

CHARACTERS

A white or almost white powder, very slightly soluble in water, freely soluble in alcohol and in methylene chloride. It melts at about 68 °C.

Inject 10 µl of the test solution and 10 µl of reference solution (b). In the chromatogram obtained with the test solution: the area of any peak, apart from the principal peak, is not greater than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent) and the sum of the areas of any such peaks is not greater than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.3 per cent). Disregard any peak with an area less than 0.25 times the area of the principal peak in the chromatogram obtained with reference solution (b).

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying *in vacuo* at 40 °C for 4 h.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

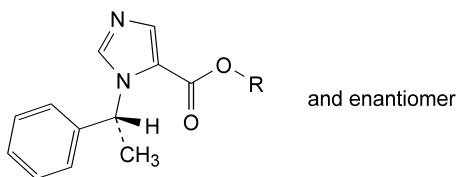
Dissolve 0.200 g in 50 ml of a mixture of 1 volume of *anhydrous acetic acid R* and 7 volumes of *methyl ethyl ketone R* and titrate with 0.1 M *perchloric acid* using 0.2 ml of *naphtholbenzein solution R* as indicator.

1 ml of 0.1 M *perchloric acid* is equivalent to 24.43 mg of $C_{14}H_{16}N_2O_2$.

STORAGE

Protected from light.

IMPURITIES

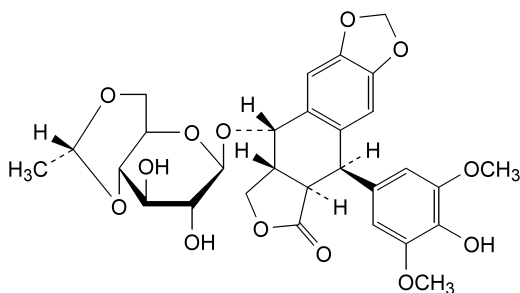


- A. R = H: 1-[(1*RS*)-1-phenylethyl]-1*H*-imidazole-5-carboxylic acid,
- B. R = CH₃: methyl 1-[(1*RS*)-1-phenylethyl]-1*H*-imidazole-5-carboxylate (metomidate),
- C. R = CH(CH₃)₂: 1-methylethyl 1-[(1*RS*)-1-phenylethyl]-1*H*-imidazole-5-carboxylate.

01/2005:0823

ETOPOSIDE

Etoposidum



$C_{29}H_{32}O_{13}$

M_r 588.6

DEFINITION

(5*R*,5*aR*,8*aR*,9*S*)-9-[[4,6-*O*[(*R*)-Ethylidene]-β-*D*-glucopyranosyl]oxy]-5-(4-hydroxy-3,5-dimethoxyphenyl)-5,8,8*a*,9-tetrahydroisobenzofuro[5,6-*f*][1,3]benzodioxol-6(5*aH*)-one.

Content: 98.0 per cent to 101.0 per cent (anhydrous substance).

CHARACTERS

Appearance: white or almost white crystalline powder.

Solubility: practically insoluble in water, sparingly soluble in methanol, slightly soluble in alcohol and in methylene chloride.

IDENTIFICATION

First identification: A, B.

Second identification: C, D.

A. Specific optical rotation (see Tests).

B. Infrared absorption spectrophotometry (2.2.24).

Comparison: *etoposide CRS*.

C. Thin-layer chromatography (2.2.27).

Test solution. Dissolve 10 mg of the substance to be examined in a mixture of 1 volume of *methanol R* and 9 volumes of *methylene chloride R* and dilute to 2 ml with the same mixture of solvents.

Reference solution. Dissolve 10 mg of *etoposide CRS* in a mixture of 1 volume of *methanol R* and 9 volumes of *methylene chloride R* and dilute to 2 ml with the same mixture of solvents.

Plate: plate with *silica gel H R* as coating substance.

Mobile phase: *water R*, *glacial acetic acid R*, *acetone R*, *methylene chloride R* (1.5:8:20:100 V/V/V/V).

Application: 5 µl as 10 mm bands.

Development: immediately, over a path of 17 cm.

Drying: in a current of warm air for 5 min.

Detection: spray with a mixture of 1 volume of *sulphuric acid R* and 9 volumes of *alcohol R* and heat at 140 °C for 15 min. Cover the plate immediately with a glass plate of the same size. Examine in daylight.

Results: the principal band in the chromatogram obtained with the test solution is similar in position, colour and size to the principal band in the chromatogram obtained with the reference solution.

D. In a test-tube dissolve about 5 mg in 5 ml of *glacial acetic acid R* and add 0.05 ml of *ferric chloride solution R1*. Mix and cautiously add 2 ml of *sulphuric acid R*. Avoid mixing the 2 layers. Allow to stand for about 30 min; a pink to reddish-brown ring develops at the interface and the upper layer is yellow.

TESTS

Appearance of solution. The solution is clear (2.2.1) and not more intensely coloured than reference solution Y₆ or BY₆ (2.2.2, *Method II*).

Dissolve 0.6 g in a mixture of 1 volume of *methanol R* and 9 volumes of *methylene chloride R* and dilute to 20 ml with the same mixture of solvents.

Specific optical rotation (2.2.7): –106 to –114 (anhydrous substance).

Dissolve 50 mg in a mixture of 1 volume of *methanol R* and 9 volumes of *methylene chloride R* and dilute to 10.0 ml with the same mixture of solvents.

Related substances. Liquid chromatography (2.2.29).

Test solution (a). Dissolve 40 mg of the substance to be examined in a mixture of equal volumes of mobile phase A and mobile phase B and dilute to 10.0 ml with the same mixture of mobile phases.

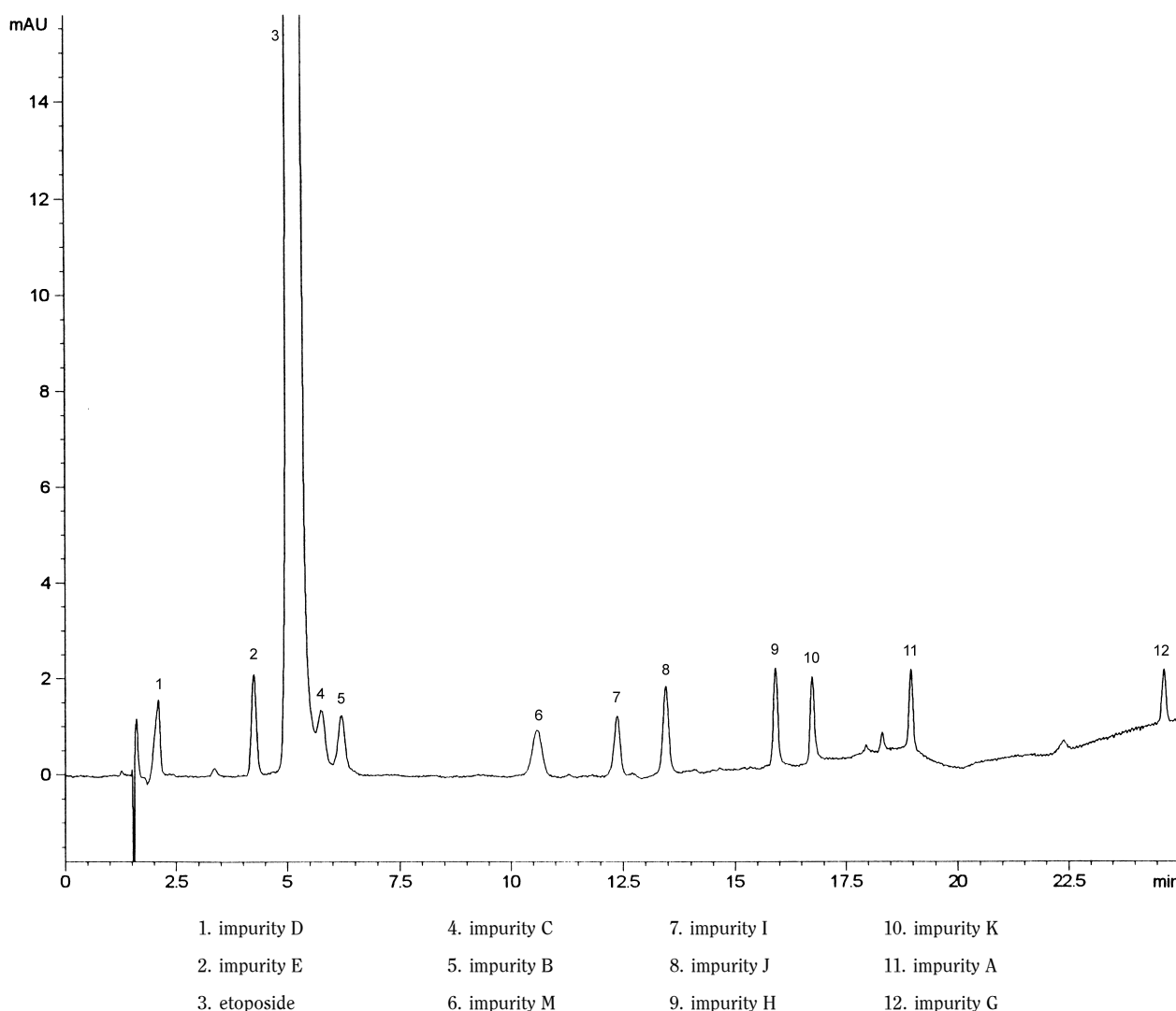


Figure 0823.-1. – Chromatogram for the test for related substances of etoposide

Test solution (b). Dissolve 50.0 mg of the substance to be examined in a mixture of equal volumes of mobile phase A and mobile phase B and dilute to 50.0 ml with the same mixture of mobile phases.

Reference solution (a). Dilute 1.0 ml of test solution (a) to 10.0 ml with a mixture of equal volumes of mobile phase A and mobile phase B. Dilute 1.0 ml of this solution to 20.0 ml with a mixture of equal volumes of mobile phase A and mobile phase B.

Reference solution (b). Dilute 4.0 ml of reference solution (a) to 10.0 ml with a mixture of equal volumes of mobile phase A and mobile phase B.

Reference solution (c). Dissolve 50.0 mg of *etoposide CRS* in a mixture of equal volumes of mobile phase A and mobile phase B and dilute to 50.0 ml with the same mixture of mobile phases.

Reference solution (d). To 10 ml of test solution (b), add 0.1 ml of a 4 per cent V/V solution of *glacial acetic acid R* and 0.1 ml of *phenolphthalein solution R*. Add 1 M *sodium hydroxide* until the solution becomes faintly pink (about 0.15 ml). After 15 min, add 0.1 ml of a 4 per cent V/V solution of *glacial acetic acid R*.

Column:

- size: $l = 0.125$ m, $\varnothing = 4.6$ mm,
- stationary phase: *octadecylsilyl silica gel for chromatography R* (5 μ m),
- temperature: 40 °C.

Mobile phase:

- mobile phase A: *triethylamine R*, *anhydrous formic acid R*, *water R* (1:1:998 V/V/V),
- mobile phase B: *triethylamine R*, *anhydrous formic acid R*, *acetonitrile R* (1:1:998 V/V/V),

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)
0 - 7	75	25
7 - 23	75 → 27	25 → 73
23 - 25	27 → 75	73 → 25
25 - 40	75	25

Flow rate: 1 ml/min.

Detection: spectrophotometer at 285 nm.

Injection: 10 μ l; inject test solution (a) and reference solutions (a), (b) and (d).

Retention times: the retention times and the elution order of the peaks are similar to those shown in the chromatogram (Figure 0823.-1).

System suitability: reference solution (d): continue the chromatography until the peak due to phenolphthalein is eluted.

- the chromatogram shows 2 principal peaks corresponding to etoposide and to impurity B. Disregard any peak due to phenolphthalein.

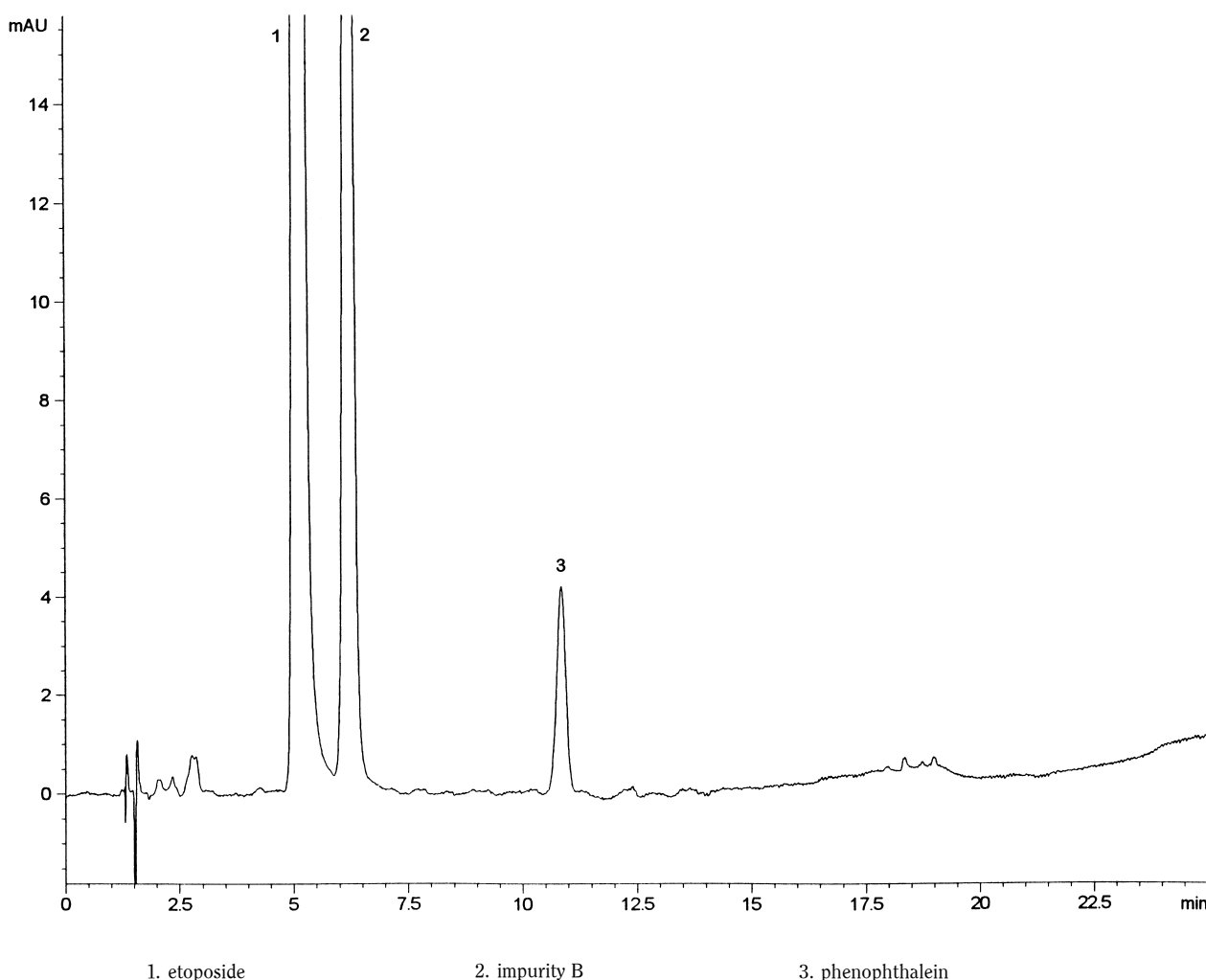


Figure 0823.-2. – Chromatogram for the test for related substances of etoposide: reference solution (d)

- **resolution**: minimum 3.0 between the peaks due to etoposide and to impurity B.

If necessary, increase slightly the proportion of mobile phase A during the isocratic phase of the gradient. When the chromatograms are recorded under the prescribed conditions, the retention times of the peaks in the chromatogram obtained with reference solution (d) are similar to those shown in the chromatogram (Figure 0823.-2).

Limits:

- **any impurity**: not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent) and not more than 2 such peaks have an area greater than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent),
- **total**: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (1 per cent),
- **disregard limit**: 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (a). Disregard any peak due to the solvent.

Heavy metals (2.4.8): maximum 20 ppm.

1.0 g complies with limit test C. Prepare the reference solution using 2 ml of *lead standard solution* (10 ppm Pb) R.

Water (2.5.12): maximum 6.0 per cent, determined on 0.250 g.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (2.2.29) as described in the test for related substances with the following modifications.

Injection: test solution (b) and reference solution (c).

System suitability:

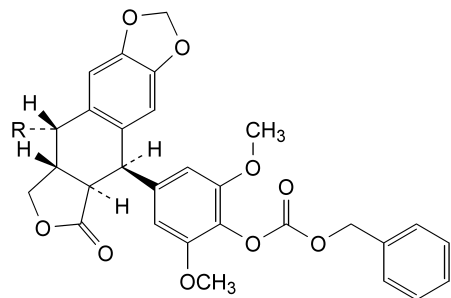
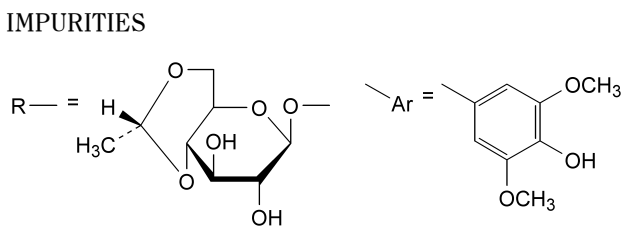
- **repeatability**: maximum relative standard deviation of 1.0 per cent after 6 injections of reference solution (c).

Calculate the percentage content of $C_{29}H_{32}O_{13}$ from the areas of the peaks and the declared content of *etoposide CRS*.

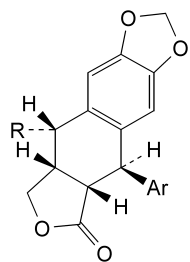
STORAGE

In an airtight container.

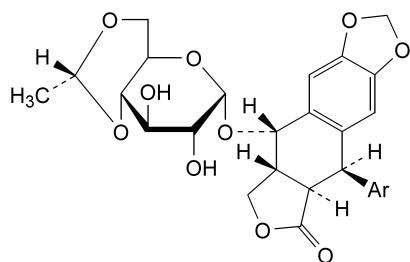
IMPURITIES



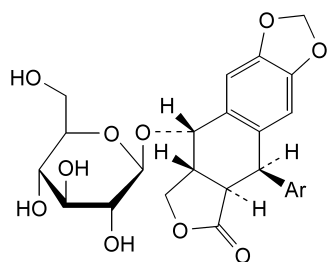
A. (5*R*,5*aR*,8*aR*,9*S*)-5-[4-[(benzyloxy)carbonyl]oxy]-3,5-dimethoxyphenyl]-9-[[4,6-*O*-[(*R*)-ethylidene]-β-D-glucopyranosyl]oxy]-5,8,8*a*,9-tetrahydroisobenzofuro[5,6-*f*][1,3]benzodioxol-6(5*aH*)-one (4'-carbobenzoyloxyethylidene-lignan P),



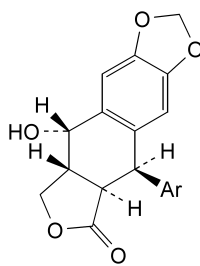
B. (5*R*,5*aS*,8*aR*,9*S*)-9-[[4,6-*O*-[(*R*)-ethylidene]-β-D-glucopyranosyl]oxy]-5-(4-hydroxy-3,5-dimethoxyphenyl)-5,8,8*a*,9-tetrahydroisobenzofuro[5,6-*f*][1,3]benzodioxol-6(5*aH*)-one (picroethylidene-lignan P; *cis*-etoposide),



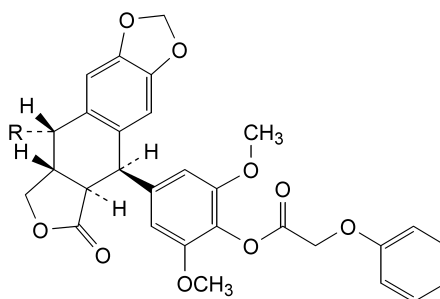
C. (5*R*,5*aR*,8*aR*,9*S*)-9-[[4,6-*O*-[(*R*)-ethylidene]-α-D-glucopyranosyl]oxy]-5-(4-hydroxy-3,5-dimethoxyphenyl)-5,8,8*a*,9-tetrahydroisobenzofuro[5,6-*f*][1,3]benzodioxol-6(5*aH*)-one (α-etoposide),



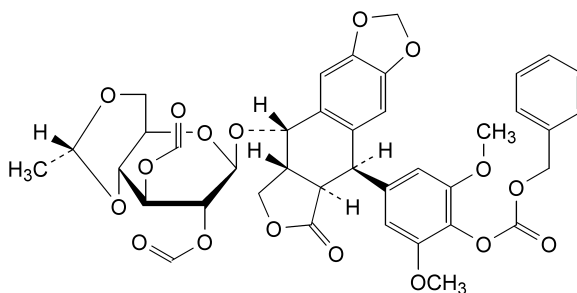
D. (5*R*,5*aR*,8*aR*,9*S*)-9-(β-D-glucopyranosyloxy)-5-(4-hydroxy-3,5-dimethoxyphenyl)-5,8,8*a*,9-tetrahydroisobenzofuro[5,6-*f*][1,3]benzodioxol-6(5*aH*)-one (lignan P),



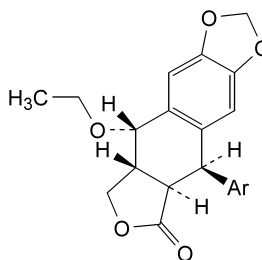
E. (5*R*,5*aR*,8*aR*,9*S*)-9-hydroxy-5-(4-hydroxy-3,5-dimethoxyphenyl)-5,8,8*a*,9-tetrahydroisobenzofuro[5,6-*f*][1,3]benzodioxol-6(5*aH*)-one (4'-demethylepipodophyllotoxin),



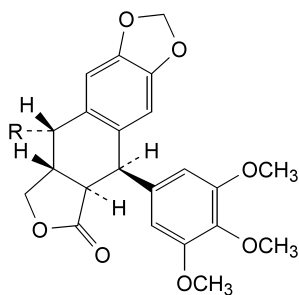
F. (5*R*,5*aR*,8*aR*,9*S*)-9-[[4,6-*O*-[(*R*)-ethylidene]-β-D-glucopyranosyl]oxy]-5-[4-(phenoxyacetyl)oxy]-3,5-dimethoxyphenyl]-5,8,8*a*,9-tetrahydroisobenzofuro[5,6-*f*][1,3]benzodioxol-6(5*aH*)-one (4'-phenoxyacetyletoposide),



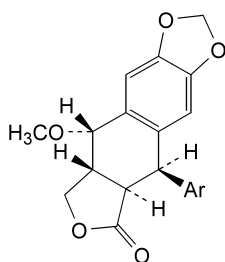
G. (5*R*,5*aR*,8*aR*,9*S*)-5-[4-[(benzyloxy)carbonyl]oxy]-3,5-dimethoxyphenyl]-9-[[4,6-*O*-[(*R*)-ethylidene]-2,3-di-*O*-formyl-β-D-glucopyranosyl]oxy]-5,8,8*a*,9-tetrahydroisobenzofuro[5,6-*f*][1,3]benzodioxol-6(5*aH*)-one (4'-carbobenzoyoxydiformylethylidene-lignan P),



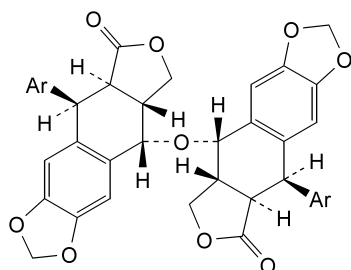
H. (5*R*,5*aR*,8*aR*,9*S*)-9-ethoxy-5-(4-hydroxy-3,5-dimethoxyphenyl)-5,8,8*a*,9-tetrahydroisobenzofuro[5,6-*f*][1,3]benzodioxol-6(5*aH*)-one (4'-*O*-demethyl-1-*O*-ethylepipodophyllotoxin),



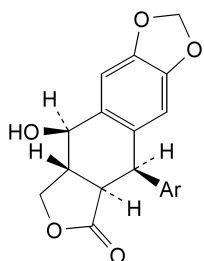
- I. (5*R*,5*aR*,8*aR*,9*S*)-9-[[4,6-*O*-[(*R*)-ethylidene]-β-D-glucopyranosyl]oxy]-5-(3,4,5-trimethoxyphenyl)-5,8,8*a*,9-tetrahydroisobenzofuro[5,6-*f*][1,3]benzodioxol-6(5*aH*)-one (4-*O*-methylethylidene-lignan P),



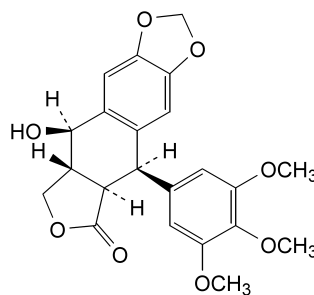
- J. (5*R*,5*aR*,8*aR*,9*S*)-5-(4-hydroxy-3,5-dimethoxyphenyl)-9-methoxy-5,8,8*a*,9-tetrahydroisobenzofuro[5,6-*f*][1,3]benzodioxol-6(5*aH*)-one (4'-*O*-demethyl-1-*O*-methylepipodophyllotoxin),



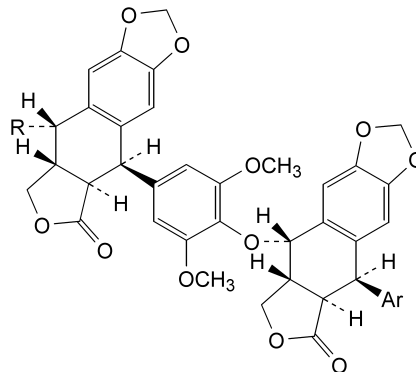
- K. 9,9'-oxybis[(5*R*,5*aR*,8*aR*,9*S*)-5-(4-hydroxy-3,5-dimethoxyphenyl)-5,8,8*a*,9-tetrahydroisobenzofuro[5,6-*f*][1,3]benzodioxol-6(5*aH*)-one] (di-4'-*O*-demethylepipodophyllotoxin),



- L. (5*R*,5*aR*,8*aR*,9*R*)-9-hydroxy-5-(4-hydroxy-3,5-dimethoxyphenyl)-5,8,8*a*,9-tetrahydroisobenzofuro[5,6-*f*][1,3]benzodioxol-6(5*aH*)-one (4'-*O*-demethylpodophyllotoxin),



- M. (5*R*,5*aR*,8*aR*,9*R*)-9-hydroxy-5-(3,4,5-trimethoxyphenyl)-5,8,8*a*,9-tetrahydroisobenzofuro[5,6-*f*][1,3]benzodioxol-6(5*aH*)-one (podophyllotoxin),



- N. (5*R*,5*aR*,8*aR*,9*S*)-9-[[4,6-*O*-[(*R*)-ethylidene]-β-D-glucopyranosyl]oxy]-5-[4-[(5*R*,5*aR*,8*aR*,9*S*)-5-(4-hydroxy-3,5-dimethoxyphenyl)-6-oxo-5,5*a*,6,8,8*a*,9-hexahydroisobenzofuro[5,6-*f*][1,3]benzodioxol-9-yl]oxy]-3,5-dimethoxyphenyl]-5,8,8*a*,9-tetrahydroisobenzofuro[5,6-*f*][1,3]benzodioxol-6(5*aH*)-one.

01/2005:1320

EUCALYPTUS LEAF

Eucalypti folium

DEFINITION

Eucalyptus leaf consists of the whole or cut dried leaves of older branches of *Eucalyptus globulus* Labill. The whole drug contains not less than 20 ml/kg of essential oil and the cut drug not less than 15 ml/kg of essential oil, both calculated with reference to the anhydrous drug.

CHARACTERS

The drug has an aromatic odour of cineole.

It has the macroscopic and microscopic characters described under identification tests A and B.

IDENTIFICATION

- A. The leaves which are mainly greyish-green and relatively thick are elongated, elliptical and slightly sickle-shaped and usually up to 25 cm in length, and up to 5 cm in width. The petiole is twisted, strongly wrinkled and is 2 cm to 3 cm, rarely 5 cm, in length. The coriaceous, stiff leaves are entire and glabrous and have a yellowish-green mid-rib. Lateral veins anastomose near the margin to a continuous line. The margin is even and somewhat thickened. On both surfaces are minute, irregularly distributed, warty dark brown spots. Small oil glands may be seen in transmitted light.
- B. Reduce to a powder (355). The powder is greyish-green. Examine under a microscope, using *chloral hydrate solution R*. The powder shows fragments of glabrous

lamina with small thick-walled epidermal cells bearing a thick cuticle, numerous anomocytic stomata (2.8.3) of more than 80 µm in diameter and occasionally groups of brown cork cells, 300 µm in diameter and brownish-black in their centre; fragments of isobilateral mesophyll with two or three layers of palisade parenchyma on each side and in the centre several layers of spongy mesophyll with elongated cells with the same orientation as the palisade cells and containing prisms and cluster crystals of calcium oxalate; fragments of mesophyll containing large schizogenous oil glands.

- C. Examine by thin-layer chromatography (2.2.27), using a suitable silica gel as the coating substance.

Test solution. Shake 0.5 g of the freshly powdered drug (355) with 5 ml of *toluene R* for 2 min to 3 min and filter over about 2 g of *anhydrous sodium sulphate R*.

Reference solution. Dissolve 50 µl of *cineole R* in *toluene R* and dilute to 5 ml with the same solvent.

Apply to the plate as bands 10 µl of each solution. Develop over a path of 15 cm using a mixture of 10 volumes of *ethyl acetate R* and 90 volumes of *toluene R*. Allow the plate to dry in air and spray the plate with *anisaldehyde solution R*. Examine in daylight while heating at 100 °C to 105 °C for 5 min to 10 min. The chromatogram obtained with the reference solution shows in the middle a zone corresponding to cineole. The main zone in the chromatogram obtained with the test solution is similar in position and colour to the zone corresponding to cineole in the chromatogram obtained with the reference solution, it also shows an intense violet zone (hydrocarbons) near the solvent front and there may also be other fainter zones.

TESTS

Foreign matter (2.8.2). Not more than 3 per cent of dark and brown leaves, not more than 5 per cent of stems and not more than 2 per cent of other foreign matter. Cordate or ovate sessile leaves of young branches, with numerous glands on both sides, visible as points in transmitted light, are not present. Determine by using 30 g of the drug.

Water (2.2.13). Not more than 100 ml/kg, determined on 20.0 g of powdered drug (355) by distillation.

Total ash (2.4.16). Not more than 6.0 per cent.

ASSAY

Carry out the determination of essential oil in vegetable drugs (2.8.12). Use 10.0 g of the cut drug immediately before determination, a 500 ml round-bottomed flask, 200 ml of *water R* and 100 ml of *glycerol R* as the distillation liquid and 0.5 ml of *xylene R* in the graduated tube. Distil at a rate of 2 ml/min to 3 ml/min for 2 h.

STORAGE

Store protected from light.

01/2005:0390

EUCALYPTUS OIL

Eucalypti aetheroleum

DEFINITION

Eucalyptus oil is obtained by steam distillation and rectification from the fresh leaves or the fresh terminal branchlets of various species of *Eucalyptus* rich in 1,8-cineole. The species mainly used are *Eucalyptus globulus* Labill., *Eucalyptus polybractea* R.T. Baker and *Eucalyptus smithii* R.T. Baker.

CHARACTERS

A colourless or pale yellow liquid with an aromatic and camphoraceous odour and a pungent and camphoraceous taste.

IDENTIFICATION

First identification: B.

Second identification: A.

- A. Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel plate R*.

Test solution. Dissolve 0.1 g of the substance to be examined in *toluene R* and dilute to 10 ml with the same solvent.

Reference solution. Dissolve 50 µl of *cineole R* in *toluene R* and dilute to 5 ml with the same solvent.

Apply to the plate as bands 10 µl of each solution. Develop over a path of 15 cm using a mixture of 10 volumes of *ethyl acetate R* and 90 volumes of *toluene R*. Allow the plate to dry in air and spray with *anisaldehyde solution R* and examine in daylight while heating at 100-105 °C for 5-10 min. The chromatogram obtained with the reference solution shows in the middle a zone due to cineole. The chromatogram obtained with the test solution shows a main zone similar in position and colour to the zone due to cineole in the chromatogram obtained with the reference solution. Other weaker zones may be present.

- B. Examine the chromatograms obtained in the test for chromatographic profile. The chromatogram obtained with the test solution shows 5 peaks similar in retention time to the 5 peaks in the chromatogram obtained with the reference solution.

TESTS

Relative density (2.2.5): 0.906 to 0.927.

Refractive index (2.2.6): 1.458 to 1.470.

Optical rotation (2.2.7): 0° to + 10°.

Solubility in alcohol (2.8.10). It is soluble in 5 volumes of *ethanol* (70 per cent V/V) *R*.

Aldehydes. Place 10 ml in a glass-stoppered tube 25 mm in diameter and 150 mm long. Add 5 ml of *toluene R* and 4 ml of *alcoholic hydroxylamine solution R*. Shake vigorously and titrate immediately with 0.5 M *potassium hydroxide in alcohol* (60 per cent V/V) until the red colour changes to yellow. Continue the titration with shaking; the end-point is reached when the pure yellow colour of the indicator is permanent in the lower layer after shaking vigorously for 2 min and allowing separation to take place. The reaction is complete in about 15 min. Repeat the titration using a further 10 ml of the substance to be examined and, as a reference solution for the end-point, the titrated liquid from the first determination to which has been added 0.5 ml of 0.5 M *potassium hydroxide in alcohol* (60 per cent V/V). Not more than 2.0 ml of 0.5 M *potassium hydroxide in alcohol* (60 per cent V/V) is required in the second titration.

Chromatographic profile. Examine by gas chromatography (2.2.28).

Test solution. The substance to be examined.

Reference solution. Dissolve 80 µl of *α-pinene R*, 10 µl of *β-pinene R*, 10 µl of *α-phellandrene R*, 10 µl of *limonene R*, 0.8 ml of *cineole R* and 10 mg of *camphor R* in 10 ml of *acetone R*.

The chromatographic procedure may be carried out using:

- a fused-silica column 60 m long and about 0.25 mm in internal diameter coated with *macrogol 20 000 R* as the bonded phase,

- *helium for chromatography R* as the carrier gas at a flow rate of 1.5 ml/min,
- a flame-ionisation detector,
- a split ratio of 1:100,

maintaining the temperature of the column at 60 °C for 5 min, then raising the temperature at a rate of 5 °C/min to 200 °C and maintaining at 200 °C for 5 min; maintaining the temperature of the injection port and that of the detector at 220 °C.

Inject about 0.5 µl of the reference solution. When the chromatogram is recorded in the prescribed conditions the components elute in the order indicated in the composition of the reference solution. Record the retention times of these substances.

The assay is not valid unless: the number of theoretical plates calculated for the peak due to limonene at 110 °C is at least 30 000; the resolution between the peaks corresponding to limonene and cineole is at least 1.5.

Inject about 0.5 µl of the test solution. Using the retention times determined from the chromatogram obtained with the reference solution, locate the components of the reference solution on the chromatogram obtained with the test solution.

Determine the percentage content of these components by the normalisation procedure.

The percentages are within the following ranges:

- *α-pinene*: traces to 9.0 per cent,
- *β-pinene*: less than 1.5 per cent,
- *α-phellandrene*: less than 1.5 per cent,
- *limonene*: traces to 12.0 per cent,
- *1,8-cineole*: minimum 70.0 per cent,
- *camphor*: less than 0.1 per cent.

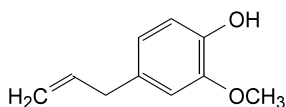
STORAGE

In a well-filled, airtight container, protected from light and at a temperature not exceeding 25 °C.

01/2005:1100

EUGENOL

Eugenolum



C₁₀H₁₂O₂

*M*_r 164.2

DEFINITION

Eugenol is 2-methoxy-4-(prop-2-enyl)phenol.

CHARACTERS

A colourless or pale yellow, clear liquid, darkening on exposure to air, with a strong odour of clove, practically insoluble in water, freely soluble in alcohol (70 per cent V/V), practically insoluble in glycerol, miscible with glacial acetic acid, with alcohol, with fatty oils and with methylene chloride.

IDENTIFICATION

First identification: B.

Second identification: A, C, D.

A. It complies with the test for refractive index (see Tests).

- B. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *eugenol CRS*.
- C. Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel F₂₅₄ plate R*.

Test solution. Dissolve 50 µl of the substance to be examined in *alcohol R* and dilute to 25 ml with the same solvent.

Reference solution. Dissolve 50 µl of *eugenol CRS* in *alcohol R* and dilute to 25 ml with the same solvent.

Apply to the plate 5 µl of each solution. Develop over a path of 15 cm using a mixture of 10 volumes of *ethyl acetate R* and 90 volumes of *toluene R*. Dry the plate in a current of cold air and examine in ultraviolet light at 254 nm. The principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with the reference solution. Spray with *anisaldehyde solution R*. Heat at 100-105 °C for 10 min. The principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

- D. Dissolve 0.05 ml in 2 ml of *alcohol R* and add 0.1 ml of *ferric chloride solution R1*. A dark green colour is produced which changes to yellowish-green within 10 min.

TESTS

Relative density (2.2.5): 1.066 to 1.070.

Refractive index (2.2.6): 1.540 to 1.542.

Dimeric and oligomeric compounds. Dissolve 0.150 g of the substance to be examined in *ethanol R* and dilute to 100.0 ml with the same solvent. The absorbance of the solution (2.2.25) at 330 nm is not greater than 0.25.

Related substances. Examine by gas chromatography (2.2.28).

Test solution. Dissolve 1.00 g of the substance to be examined in *ethanol R* and dilute to 5.0 ml with the same solvent.

Reference solution (a). Dilute 1.0 ml of the test solution to 100.0 ml with *ethanol R*.

Reference solution (b). Dissolve 50 mg of *vanillin R* in 1 ml of the test solution and dilute to 5 ml with *ethanol R*.

The chromatographic procedure may be carried out using:

- a fused-silica capillary column 30 m long and 0.25 mm in internal diameter coated with a film of *polymethylphenylsiloxane R* (film thickness 0.25 µm),
- *helium for chromatography R* as the carrier gas at a flow rate of 1 ml/min,
- a flame-ionisation detector,
- a split ratio of 1:40,

	Time (min)	Temperature (°C)	Rate (°C/min)	Comment
Column	0 - 2	80		isothermal
	2 - 27	80 → 280	8	linear gradient
	27 - 47	280		isothermal
Injection port		250		
Detector		280		

Inject 1 µl of the test solution and 1 µl each of reference solutions (a) and (b). The test is not valid unless in the chromatogram obtained with reference solution (b), the relative retention time of the peak due to vanillin is at least 1.1 with respect to the peak due to eugenol. Calculate the percentage content of related substances from the areas

of the peaks in the chromatogram obtained with the test solution by the normalisation procedure; disregard the solvent peak and any peak with an area less than 0.05 times that of the principal peak in the chromatogram obtained with reference solution (a). The content of related substances with a relative retention time greater than 2.0 in respect to the main peak is not greater than 1.0 per cent; the content of any related substances is not greater than 0.5 per cent; the total content of related substances is not greater than 3.0 per cent.

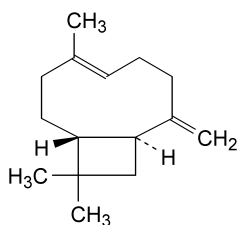
Hydrocarbons. Dissolve 1 ml in 5 ml of *dilute sodium hydroxide solution R* and add 30 ml of *water R* in a stoppered test-tube. Examined immediately, the solution is yellow and clear (2.2.1).

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

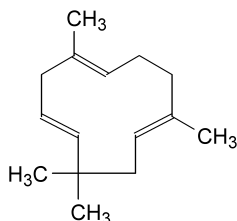
STORAGE

Store in a well-filled container, protected from light.

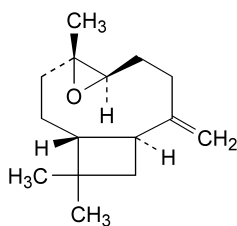
IMPURITIES



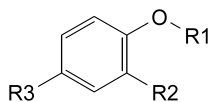
- A. (1*R*,4*E*,9*S*)-4,11,11-trimethyl-8-methylenebicyclo[7.2.0]undec-4-ene (β -caryophyllene),



- B. (1*E*,4*E*,8*E*)-2,6,6,9-tetramethylcycloundeca-1,4,8-triene (α -humulene, α -caryophyllene),



- C. (1*R*,4*R*,6*R*,10*S*)-4,12,12-trimethyl-9-methylene-5-oxatricyclo[8.2.0.0^{4,6}]dodecane (β -caryophyllene oxide),



- D. R1 = H, R2 = H, R3 = CH₂-CH=CH₂: 4-(prop-2-enyl)phenol,

- E. R1 = CH₃, R2 = OCH₃, R3 = CH₂-CH=CH₂: 1,2-dimethoxy-4-(prop-2-enyl)benzene (eugenol methyl ether),

- F. R1 = H, R2 = OCH₃, R3 = CH=CH-CH₃ (*cis*): 2-methoxy-4-[(*Z*)-prop-1-enyl]phenol (*cis*-isoeugenol),

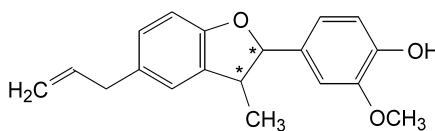
- G. R1 = H, R2 = OCH₃, R3 = CH=CH-CH₃ (*trans*): 2-methoxy-4-[(*E*)-prop-1-enyl]phenol (*trans*-isoeugenol),

- H. R1 = H, R2 = OCH₃, R3 = CHO: 4-hydroxy-3-methoxybenzaldehyde (vanillin),

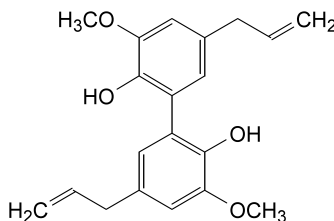
- I. R1 = CO-CH₃, R2 = OCH₃, R3 = CH₂-CH=CH₂: 2-methoxy-4-(prop-2-enyl)phenyl acetate (acetyleneugenol),

- J. R1 = H, R2 = OCH₃, R3 = CO-CH=CH₂: 1-(4-hydroxy-3-methoxyphenyl)prop-2-enone,

- K. R1 = H, R2 = OCH₃, R3 = CH=CH-CHO: (*E*)-3-(4-hydroxy-3-methoxyphenyl)prop-2-enal (*trans*-coniferyl aldehyde),

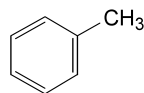


- L. 2-methoxy-4-[3-methyl-5-(prop-2-enyl)-2,3-dihydrobenzofuran-2-yl]phenol (dehydrodiisoeugenol),



- M. 3,3'-dimethoxy-5,5'-bis(prop-2-enyl)biphenyl-2,2'-diol (dehydrodieugenol),

- N. O. two further unknown dimeric compounds,

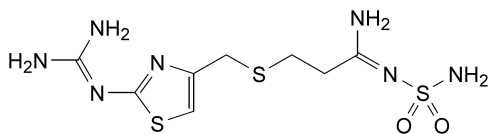


- P. toluene.

01/2005:1012

FAMOTIDINE

Famotidinum

 $C_8H_{15}N_7O_2S_3$ M_r 337.5

DEFINITION

3-[[[2-[(Diaminomethylene)amino]thiazol-4-yl]methyl]sulphonyl]-N'-sulphamoylpropanimidamide.

Content: 98.5 per cent to 101.5 per cent (dried substance).

CHARACTERS

Appearance: white or yellowish-white, crystalline powder or crystals.

Solubility: very slightly soluble in water, freely soluble in glacial acetic acid, very slightly soluble in anhydrous ethanol, practically insoluble in ethyl acetate. It dissolves in dilute mineral acids.

It shows polymorphism.

IDENTIFICATION

Infrared absorption spectrophotometry (2.2.24).

Preparation: discs.

Comparison: famotidine CRS.

If the spectra obtained show differences, suspend 0.10 g of the substance to be examined and 0.10 g of the reference substance separately in 5 ml of *water R*. Heat to boiling and allow to cool, scratching the wall of the tube with a glass rod to initiate crystallisation. Filter, wash the crystals with 2 ml of iced *water R* and dry in an oven at 80 °C at a pressure not exceeding 670 Pa for 1 h. Record new spectra using the residues.

TESTS

Appearance of solution. Dissolve 0.20 g in a 50 g/l solution of *hydrochloric acid R*, heating to 40 °C if necessary, and dilute to 20 ml with the same acid. The solution is clear (2.2.1) and not more intensely coloured than reference solution BY₇ (2.2.2, *Method II*).

Related substances. Liquid chromatography (2.2.29).

Test solution. Dissolve 12.5 mg of the substance to be examined in mobile phase A and dilute to 25.0 ml with mobile phase A.

Reference solution (a). Dilute 1.0 ml of the test solution to 10.0 ml with mobile phase A. Dilute 1.0 ml of this solution to 100.0 ml with mobile phase A.

Reference solution (b). Dissolve 2.5 mg of *famotidine impurity D CRS* in *methanol R* and dilute to 10.0 ml with the same solvent. To 1.0 ml of the solution add 0.50 ml of the test solution and dilute to 100.0 ml with mobile phase A.

Reference solution (c). Dissolve 5.0 mg of *famotidine for system suitability CRS* (famotidine containing impurities A, B, C, D, E, F, G) in mobile phase A and dilute to 10.0 ml with mobile phase A.

Column:

– *size*: $l = 0.25$ m, $\varnothing = 4.6$ mm,

– *stationary phase*: end-capped octadecylsilyl silica gel for chromatography *R* (5 µm),

– *temperature*: 50 °C.

Mobile phase:

– *mobile phase A*: mix 6 volumes of *methanol R*, 94 volumes of *acetonitrile R* and 900 volumes of a 1.882 g/l solution of *sodium hexanesulphonate R* previously adjusted to pH 3.5 with *acetic acid R*,

– *mobile phase B*: *acetonitrile R*,

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)	Flow rate (ml/min)
0 - 23	100 → 96	0 → 4	1
23 - 27	96	4	1 → 2
27 - 47	96 → 78	4 → 22	2
47 - 48	78 → 100	22 → 0	2
48 - 54	100	0	2 → 1

Detection: spectrophotometer at 265 nm.

Injection: 20 µl.

Relative retention with reference to famotidine (retention time = about 21 min): impurity D = about 1.1; impurity C = about 1.2; impurity G = about 1.4; impurity F = about 1.5; impurity A = about 1.6; impurity B = about 2.0; impurity E = about 2.1.

System suitability:

- the chromatogram obtained with reference solution (c) is similar to the chromatogram supplied with *famotidine for system suitability CRS*;
- *retention time*: famotidine = 19-23 min in all the chromatograms; impurity E = maximum 48 min in the chromatogram obtained with reference solution (c);
- *resolution*: minimum 3.5 between the peaks due to famotidine and impurity D in the chromatogram obtained with reference solution (b).

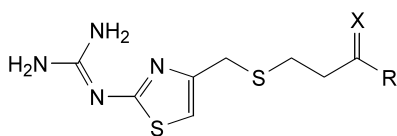
Limits:

- *correction factors*: for the calculation of contents, multiply the peak areas of the following impurities by the corresponding correction factor: impurity A = 1.9; impurity B = 2.5; impurity C = 1.9; impurity F = 1.7; impurity G = 1.4;
- *impurities A, G*: for each impurity, not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent);
- *impurities B, C, D, E*: for each impurity, not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.3 per cent), and not more than 3 such peaks have an area greater than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent);
- *impurity F*: not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent);
- *any other impurity*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) for the peaks eluting by 25 min, and not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) for the peaks eluting after 25 min (0.1 per cent);
- *total*: not more than 10 times the area of the principal peak in the chromatogram obtained with reference solution (a) (1.0 per cent);
- *disregard limit*: 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (a).

01/2005:1013

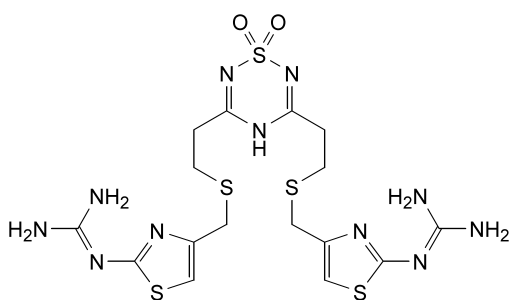
Heavy metals (2.4.8): maximum 10 ppm.2.0 g complies with limit test D. Prepare the reference solution using 2 ml of *lead standard solution* (10 ppm Pb) R.**Loss on drying** (2.2.32): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 80 °C at a pressure not exceeding 670 Pa for 5 h.**Sulphated ash** (2.4.14): maximum 0.1 per cent, determined on 1.0 g.**ASSAY**Dissolve 0.120 g in 60 ml of *anhydrous acetic acid* R. Titrate with 0.1 M *perchloric acid*, determining the end-point potentiometrically (2.2.20).1 ml of 0.1 M *perchloric acid* is equivalent to 16.87 mg of C₈H₁₅N₇O₂S₃.**STORAGE**

Protected from light.

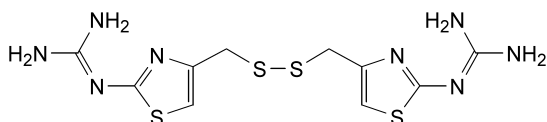
IMPURITIES*Specified impurities: A, B, C, D, E, F, G.*A. R = NH₂, X = NH: 3-[[[2-[(diaminomethylene)amino]thiazol-4-yl]methyl]sulphanyl]propanimidamide,C. R = NH-SO₂-NH₂, X = O: 3-[[[2-[(diaminomethylene)amino]thiazol-4-yl]methyl]sulphanyl]-N-sulphamoylpropanamide,D. R = NH₂, X = O: 3-[[[2-[(diaminomethylene)amino]thiazol-4-yl]methyl]sulphanyl]propanamide,

F. R = OH, X = O: 3-[[[2-[(diaminomethylene)amino]thiazol-4-yl]methyl]sulphanyl]propanoic acid,

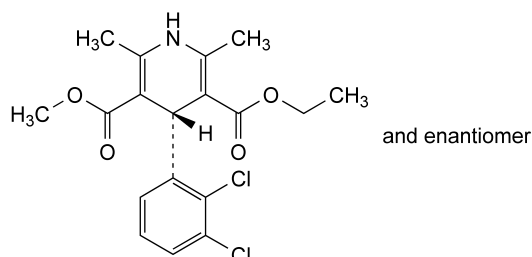
G. R = NH-CN, X = NH: N-cyano-3-[[[2-[(diaminomethylene)amino]thiazol-4-yl]methyl]sulphanyl]propanimidamide,



B. 3,5-bis[2-[[[2-[(diaminomethylene)amino]thiazol-4-yl]methyl]sulphanyl]ethyl]-4H-1,2,4,6-thiatriazine 1,1-dioxide,



E. 2,2'-[disulphanediyl]bis(methylenethiazole-4,2-diyl)]diguanidine.

FELODIPINE**Felodipinum**C₁₈H₁₉Cl₂NO₄M_r 384.3**DEFINITION**Felodipine contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of ethyl methyl (4*RS*)-4-(2,3-dichlorophenyl)-2,6-dimethyl-1,4-dihydropyridine-3,5-dicarboxylate, calculated with reference to the dried substance.**CHARACTERS**

A white or light yellow, crystalline powder, practically insoluble in water, freely soluble in acetone, in ethanol, in methanol and in methylene chloride.

IDENTIFICATION*First identification: B.**Second identification: A, C, D.*A. Dissolve 50 mg in *methanol* R and dilute to 100 ml with the same solvent. Dilute 3 ml to 100 ml with *methanol* R. Examined between 220 nm and 400 nm (2.2.25), the solution shows two absorption maxima, at 238 nm and 361 nm. The ratio of the absorbance measured at the maximum at 361 nm to that measured at the maximum at 238 nm is 0.34 to 0.36.B. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *felodipine CRS*. Examine the substances prepared as discs.

C. Examine by thin-layer chromatography (2.2.27), using as the coating substance a suitable silica gel with a fluorescent indicator having an optimal intensity at 254 nm.

Test solution. Dissolve 10 mg of the substance to be examined in *methanol* R and dilute to 10 ml with the same solvent.*Reference solution (a).* Dissolve 10 mg of *felodipine CRS* in *methanol* R and dilute to 10 ml with the same solvent.*Reference solution (b).* Dissolve 5 mg of *nifedipine CRS* in reference solution (a) and dilute to 5 ml with the same solution.Apply separately to the plate 5 µl of each solution. Develop over a path of 15 cm using a mixture of 40 volumes of *ethyl acetate* R and 60 volumes of *cyclohexane* R. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. The principal spot in the chromatogram obtained with the test solution is similar in position, fluorescence and size to the spot in the chromatogram obtained with reference solution (a). The test is not valid unless the chromatogram obtained with reference solution (b) shows two clearly separated spots.

D. Dissolve 150 mg in a mixture of 25 ml of *2-methyl-2-propanol R* and 25 ml of *perchloric acid solution R*. Add 10 ml of *0.1 M cerium sulphate*, allow to stand for 15 min, add 3.5 ml of *strong sodium hydroxide solution R* and neutralise with *dilute sodium hydroxide solution R*. Shake with 25 ml of *methylene chloride R*. Evaporate the lower layer to dryness on a water-bath under nitrogen (the residue is also used in the test for related substances). Dissolve about 20 mg of the residue in *methanol R* and dilute to 50 ml with the same solvent. Dilute 2 ml to 50 ml with *methanol R*. Examined between 220 nm and 400 nm (2.2.25), the solution shows an absorption maximum at 273 nm.

TESTS

Solution S. Dissolve 1.00 g in *methanol R* and dilute to 20.0 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1).

Absorbance (2.2.25). The absorbance of solution S at 440 nm is not greater than 0.10.

Related substances. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 25.0 mg of the substance to be examined in the mobile phase and dilute to 50.0 ml with the mobile phase.

Reference solution (a). Dilute 1.0 ml of the test solution to 100.0 ml with the mobile phase.

Reference solution (b). Dilute 1.0 ml of reference solution (a) to 10.0 ml with the mobile phase.

Reference solution (c). Dissolve 50.0 mg of the residue obtained in identification test D (impurity A) and 25.0 mg of *felodipine CRS* in the mobile phase and dilute to 50.0 ml with the mobile phase. Dilute 1.0 ml to 100.0 ml with the mobile phase. Dilute 1.0 ml of this solution to 10.0 ml with the mobile phase.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.125 m to 0.15 m long and 4 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (5 µm),
- as mobile phase at a flow rate of 1 ml/min a mixture of 20 volumes of *methanol R*, 40 volumes of *acetonitrile R* and 40 volumes of a phosphate buffer solution pH 3.0, containing 0.8 g/l of *phosphoric acid R* and 8 g/l of *sodium dihydrogen phosphate R*,
- as detector a spectrophotometer set at 254 nm.

Inject 20 µl of reference solution (c). Adjust the sensitivity of the system so that the heights of the two peaks are not less than 20 per cent of the full scale of the recorder. The test is not valid unless the resolution between the first peak (impurity A) and the second peak (felodipine) is at least 2.5. Two other peaks may be observed corresponding to impurity B and impurity C. The substances elute in the following order: impurity B, impurity A, felodipine and impurity C.

Inject 20 µl of the test solution, 20 µl of reference solution (a) and 20 µl of reference solution (b). Continue the chromatography for twice the retention time of felodipine, which is about 12 min. In the chromatogram obtained with the test solution: the sum of the areas of any peaks corresponding to impurity B and impurity C is not greater than the area of the principal peak in the chromatogram obtained with reference solution (a) (1.0 per cent); the area of any peak apart from the principal peak and any peaks corresponding to impurity B and impurity C is not greater than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent) and the

sum of the areas of such peaks is not greater than three times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.3 per cent). Disregard any peak with an area less than 0.2 times that of the principal peak in the chromatogram obtained with reference solution (b).

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C for 3 h.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

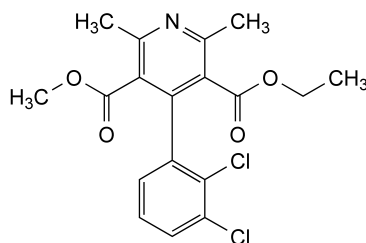
Dissolve 0.160 g in a mixture of 25 ml of *2-methyl-2-propanol R* and 25 ml of *perchloric acid solution R*. Add 0.05 ml of *ferroin R*. Titrate with *0.1 M cerium sulphate* until the pink colour disappears. Titrate slowly towards the end of the titration.

1 ml of *0.1 M cerium sulphate* is equivalent to 19.21 mg of $C_{18}H_{19}Cl_2NO_4$.

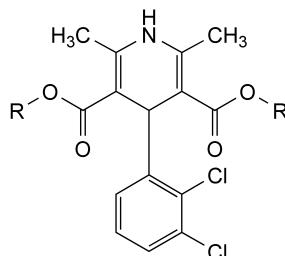
STORAGE

Store protected from light.

IMPURITIES



A. ethyl methyl 4-(2,3-dichlorophenyl)-2,6-dimethylpyridine-3,5-dicarboxylate,



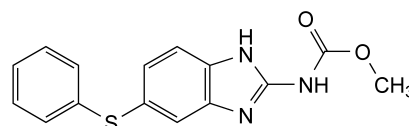
B. R = CH₃: dimethyl 4-(2,3-dichlorophenyl)-2,6-dimethyl-1,4-dihydropyridine-3,5-dicarboxylate,

C. R = C₂H₅: diethyl 4-(2,3-dichlorophenyl)-2,6-dimethyl-1,4-dihydropyridine-3,5-dicarboxylate.

01/2005:1208

FENBENDAZOLE FOR VETERINARY USE

Fenbendazolum ad usum veterinarium



$C_{15}H_{13}N_3O_2S$

M_r 299.4

DEFINITION

Fenbendazole for veterinary use contains not less than 98.0 per cent and not more than the equivalent of 101.0 per cent of methyl [5-(phenylsulphonyl)-1*H*-benzimidazol-2-yl]carbamate, calculated with reference to the dried substance.

CHARACTERS

A white or almost white powder, practically insoluble in water, sparingly soluble in dimethylformamide, very slightly soluble in methanol.

IDENTIFICATION

Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *fenbendazole CRS*. Examine the substances prepared as discs.

TESTS

Related substances. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 50.0 mg of the substance to be examined in 10.0 ml of *hydrochloric methanol R*.

Reference solution (a). Dissolve 50.0 mg of *fenbendazole CRS* in 10.0 ml of *hydrochloric methanol R*. Dilute 1.0 ml of this solution to 200.0 ml with *methanol R*. Dilute 5.0 ml of this second solution to 10.0 ml with *hydrochloric methanol R*.

Reference solution (b). Dissolve 10.0 mg of *fenbendazole impurity A CRS* in 100.0 ml of *methanol R*. Dilute 1.0 ml of this solution to 10.0 ml with *hydrochloric methanol R*.

Reference solution (c). Dissolve 10.0 mg of *fenbendazole impurity B CRS* in 100.0 ml of *methanol R*. Dilute 1.0 ml of this solution to 10.0 ml with *hydrochloric methanol R*.

Reference solution (d). Dissolve 10.0 mg of *fenbendazole CRS* and 10.0 mg of *mebendazole CRS* in 100.0 ml of *methanol R*. Dilute 1.0 ml of this solution to 10.0 ml with *hydrochloric methanol R*.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4.6 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (5 µm),
- as mobile phase at a flow rate of 1 ml/min:

Mobile phase A. Mix 1 volume of *anhydrous acetic acid R*, 30 volumes of *methanol R* and 70 volumes of *water R*,

Mobile phase B. Mix 1 volume of *anhydrous acetic acid R*, 30 volumes of *water R* and 70 volumes of *methanol R*,

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)	Comment
0 - 10	100 → 0	0 → 100	linear gradient
10 - 40	0	100	isocratic
40 - 50	0 → 100	100 → 0	re-equilibration

- as detector a spectrophotometer set at 280 nm.

When the chromatograms are recorded in the prescribed conditions, the retention time for fenbendazole is about 19 min. Inject separately 10 µl of each solution. The test is not valid unless, in the chromatogram obtained with reference solution (d), the resolution between the peaks corresponding to fenbendazole and mebendazole is at least 1.5.

In the chromatogram obtained with the test solution: the area of the peaks corresponding to impurity A and impurity B is not greater than 2.5 times the area of the corresponding peak in the chromatograms obtained with reference

solution (b) and reference solution (c) (0.5 per cent); the area of any peak, apart from the principal peak and the peaks corresponding to impurity A and impurity B respectively, is not greater than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent); the sum of the areas of all the peaks, apart from the principal peak, is not greater than 4 times the area of the principal peak in the chromatogram obtained with reference solution (a) (1 per cent). Disregard any peak with an area less than 0.2 times that of the principal peak in the chromatogram obtained with reference solution (a).

Heavy metals (2.4.8). 1.0 g complies with limit test C for heavy metals (20 ppm). Prepare the standard using 2 ml of *lead standard solution (10 ppm Pb) R*.

Loss on drying (2.2.32). Not more than 1.0 per cent, determined on 1.000 g by drying in an oven at 100-105 °C for 3 h.

Sulphated ash (2.4.14). Not more than 0.3 per cent, determined on 1.0 g.

ASSAY

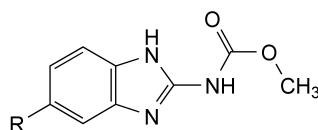
Dissolve 0.200 g in 30 ml of *anhydrous acetic acid R*, warming gently if necessary. Cool and titrate with 0.1 *M perchloric acid*, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 *M perchloric acid* is equivalent to 29.94 mg of C₁₅H₁₃N₃O₂S.

STORAGE

Protected from light.

IMPURITIES



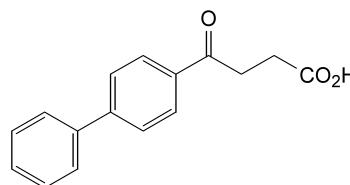
A. R = H: methyl (1*H*-benzimidazol-2-yl)carbamate,

B. R = Cl: methyl (5-chloro-1*H*-benzimidazol-2-yl)carbamate.

01/2005:1209

FENBUFEN

Fenbufenum

C₁₆H₁₄O₃M_r 254.3

DEFINITION

Fenbufen contains not less than 98.5 per cent and not more than the equivalent of 101.0 per cent of 4-(biphenyl-4-yl)-4-oxobutanoic acid, calculated with reference to the dried substance.

CHARACTERS

A white, fine, crystalline powder, very slightly soluble in water, slightly soluble in acetone, in alcohol and in methylene chloride.

IDENTIFICATION

First identification: B.

Second identification: A, C.

- A. Melting point (2.2.14): 186 °C to 189 °C.
- B. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *fenbufen CRS*.
- C. Examine by thin-layer chromatography (2.2.27), using as the coating substance a suitable silica gel with a fluorescent indicator having an optimal intensity at 254 nm.

Test solution. Dissolve 10 mg of the substance to be examined in *methylene chloride R* and dilute to 10 ml with the same solvent.

Reference solution (a). Dissolve 10 mg of *fenbufen CRS* in *methylene chloride R* and dilute to 10 ml with the same solvent.

Reference solution (b). Dissolve 10 mg of *ketoprofen CRS* in *methylene chloride R* and dilute to 10 ml with the same solvent. To 5 ml of the solution, add 5 ml of reference solution (a).

Apply to the plate 10 µl of each solution. Develop over a path of 15 cm using a mixture of 5 volumes of *anhydrous acetic acid R*, 25 volumes of *ethyl acetate R* and 75 volumes of *hexane R*. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. The principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a). The test is not valid unless the chromatogram obtained with reference solution (b) shows two clearly separated spots.

TESTS

Related substances. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 50.0 mg of the substance to be examined in a mixture of 40 volumes of *dimethylformamide R* and 60 volumes of mobile phase A and dilute to 10.0 ml with the same mixture of solvents.

Reference solution (a). Dilute 0.5 ml of the test solution to 50.0 ml with a mixture of 40 volumes of *dimethylformamide R* and 60 volumes of mobile phase A. Dilute 1.0 ml of the solution to 10.0 ml with a mixture of 40 volumes of *dimethylformamide R* and 60 volumes of mobile phase A.

Reference solution (b). Dissolve 25 mg of *fenbufen CRS* and 6 mg of *ketoprofen CRS* in a mixture of 40 volumes of *dimethylformamide R* and 60 volumes of mobile phase A and dilute to 10 ml with the same mixture of solvents. Dilute 1 ml of the solution to 100 ml with a mixture of 40 volumes of *dimethylformamide R* and 60 volumes of mobile phase A.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.125 m long and 4.0 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (5 µm),
- as mobile phase at a flow rate of 2 ml/min the following solutions:

Mobile phase A. 32 volumes of *acetonitrile R* and 68 volumes of a mixture of 1 volume of *glacial acetic acid R* and 55 volumes of *water R*,

Mobile phase B. 45 volumes of *acetonitrile R* and 55 volumes of a mixture of 1 volume of *glacial acetic acid R* and 55 volumes of *water R*,

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)	Comment
0 – 15	100	0	isocratic
15 – 20	100 → 0	0 → 100	linear gradient
20 – 35	0	100	isocratic
35 – 40	0 → 100	100 → 0	linear gradient
40 – 45	100	0	re-equilibration

– as detector a spectrophotometer set at 254 nm.

Inject 20 µl of reference solution (a) and 20 µl of reference solution (b). Adjust the sensitivity of the system so that the height of the principal peak in the chromatogram obtained with reference solution (a) is at least 50 per cent of the full scale of the recorder. The test is not valid unless, in the chromatogram obtained with reference solution (b), the resolution between the peaks corresponding to ketoprofen and to fenbufen is at least 5.0.

Inject 20 µl of the test solution. In the chromatogram obtained with the test solution: the area of any peak, apart from the principal peak, is not greater than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent); the sum of the areas of any peaks, apart from the principal peak, is not greater than five times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent). Disregard any peak due to the solvent and any peak with an area less than 0.2 times that of the principal peak in the chromatogram obtained with reference solution (a).

Heavy metals (2.4.8). 1.0 g complies with limit test C for heavy metals (20 ppm). Prepare the standard using 2 ml of *lead standard solution (10 ppm Pb) R*.

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 100–105 °C for 3 h.

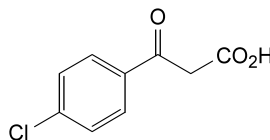
Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

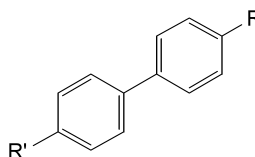
Dissolve 0.200 g in 75 ml of *acetone R* previously neutralised with *phenolphthalein solution R1* and add 50 ml of *water R*. Add 0.2 ml of *phenolphthalein solution R1* and titrate with 0.1 M *sodium hydroxide*. Carry out a blank titration.

1 ml of 0.1 M *sodium hydroxide* is equivalent to 25.43 mg of $C_{16}H_{14}O_3$.

IMPURITIES



A. 3-(4-chlorophenyl)-3-oxopropanoic acid,



B. R = CO-CH=CH-CO₂H, R' = H: 4-(biphenyl-4-yl)-4-oxobut-2-enoic acid,

C. R = R' = H: biphenyl,

D. R = CO-CH₂-CH₂-CO₂H, R' = OH: 4-(4'-hydroxybiphenyl-4-yl)-4-oxobutanoic acid.

01/2005:0824 TESTS

FENNEL, BITTER**Foeniculi amari fructus****DEFINITION**

Bitter fennel consists of the dry, cremocarps and mericarps of *Foeniculum vulgare* Miller sp. *vulgare* var. *vulgare*. It contains not less than 40 ml/kg of essential oil, calculated with reference to the anhydrous drug. The oil contains not less than 60.0 per cent of anethole and not less than 15.0 per cent of fenchone.

CHARACTERS

Bitter fennel is greenish-brown, brown or green.

It has the macroscopic and microscopic characters described under identification tests A and B.

IDENTIFICATION

- A. The fruit of bitter fennel is a cremocarp, of almost cylindrical shape with a rounded base and a narrower summit crowned with a large stylopod. It is generally 3 mm to 12 mm long and 3 mm to 4 mm wide. The mericarps, usually free, are glabrous. Each bears five prominent slightly crenated ridges. When cut transversely, four vittae on the dorsal surface and two on the commissural surface may be seen with a lens.
- B. Reduce to a powder (355). The powder is greyish-brown to greyish-yellow. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters: yellow fragments of wide secretory canals, often made up of yellowish-brown-walled polygonal secretory cells, frequently associated with a layer of thin-walled transversely elongated cells 2 µm to 9 µm wide, having a parquetry arrangement; reticulate parenchyma of the mesocarp; numerous fibre bundles from the ridges, often accompanied by narrow spiral vessels; very numerous endosperm fragments containing aleurone grains and very small calcium oxalate microrosette crystals, as well as some fibre bundles from the carpophore.
- C. Examine by thin-layer chromatography (2.2.27), using *silica gel GF₂₅₄ R* as the coating substance.

Test solution. Shake 0.3 g of the freshly powdered drug (1400) with 5.0 ml of *methylene chloride R* for 15 min. Filter and carefully evaporate the filtrate to dryness on a water-bath at 60 °C. Dissolve the residue in 0.5 ml of *toluene R*.

Reference solution. Dissolve 50 µl of *anethole R* and 10 µl of *fenchone R* in 5.0 ml of *hexane R*.

Apply separately to the plate, as bands 20 mm by 3 mm, 10 µl of each solution. Develop over a path of 10 cm using a mixture of 20 volumes of *hexane R* and 80 volumes of *toluene R*. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. The chromatograms show in the central part a quenching zone corresponding to anethole. Spray the plate with *sulphuric acid R* and heat at 140 °C for 5 min to 10 min until a yellow zone corresponding to fenchone appears in the lower third of the chromatograms. Anethole appears as a violet band in the central part. The chromatogram obtained with the test solution also shows a reddish-brown zone in its upper third (terpenes).

TESTS

Estragole. The essential oil obtained in the assay contains not more than 5.0 per cent of estragole.

Operate as described in the assay for anethole and fenchone, using the following reference solution.

Reference solution. Dissolve 5 mg of *estragole R* in 0.5 ml of *xylene R*.

Determine the content of estragole by normalisation.

Foreign matter (2.8.2). Not more than 1.5 per cent of peduncles and not more than 1.5 per cent of other foreign matter.

Water (2.2.13). Not more than 80 ml/kg, determined by distillation on 20.0 g of the powdered drug (710).

Total ash (2.4.16). Not more than 10.0 per cent.

ASSAY

Essential oil. Carry out the determination of essential oils in vegetable drugs (2.8.12). Use a 500 ml round-bottomed flask and 200 ml of *water R* as the distillation liquid. Reduce the drug to a coarse powder (1400) and immediately use 5.0 g for the determination. Introduce 0.50 ml of *xylene R* in the graduated tube. Distil at a rate of 2 ml/min to 3 ml/min for 2 h.

Anethole and fenchone. Examine by gas chromatography (2.2.28).

Test solution. Dilute the mixture of essential oil and *xylene R* obtained in the determination of essential oil to 5.0 ml with *xylene R* and by rinsing the apparatus.

Reference solution. Dissolve 5 mg of *fenchone R* and 5 mg of *anethole R* in 0.5 ml of *xylene R*.

The chromatographic procedure may be carried out using:

- a capillary column 30 m to 60 m long and 0.3 mm in internal diameter coated with *macrogol 20 000 R*,
- *nitrogen for chromatography R* as the carrier gas at a flow rate of 0.40 ml/min and split at a ratio of 1 to 200,
- a flame-ionisation detector,

maintaining the temperature of the column at 60 °C for 4 min, then raising the temperature linearly at a rate of 5 °C per minute to 170 °C and maintaining at 170 °C for 15 min and maintaining the temperature of the injection port at 220 °C and that of the detector at 270 °C.

Inject 1 µl of the reference solution. Identify and record the retention times of the components, which are eluted in the order indicated in the composition of the reference solution.

Inject 1 µl of the test solution. Determine the contents of anethole and fenchone by normalisation.

STORAGE

Store protected from light and moisture.

01/2005:0825

FENNEL, SWEET**Foeniculi dulcis fructus****DEFINITION**

Sweet fennel consists of the dry, cremocarps and mericarps of *Foeniculum vulgare* Miller sp. *vulgare* var. *dulce* (Miller) Thellung. It contains not less than 20 ml/kg of essential oil, calculated with reference to the anhydrous drug. The oil contains not less than 80.0 per cent of anethole.

CHARACTERS

Sweet fennel is pale green or pale yellowish-brown.

It has the macroscopic and microscopic characters described under identification tests A and B.

IDENTIFICATION

- A. The fruit of sweet fennel is a cremocarp of almost cylindrical shape with a rounded base and a narrowed summit crowned with a large stylopod. It is generally 3 mm to 12 mm long and 3 mm to 4 mm wide. The mericarps, usually free, are glabrous. Each bears five prominent slightly carenated ridges. When cut transversely, four vittae on the dorsal surface and two on the commissural surface may be seen with a lens.
- B. Reduce to a powder (355). The powder is greyish-brown to greyish-yellow. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters: yellow fragments of wide secretory canals, often made up of yellowish-brown-walled polygonal secretory cells, frequently associated with a layer of thin-walled transversely elongated cells 2 µm to 9 µm wide, having a parquetry arrangement; reticulate parenchyma of the mesocarp; numerous fibre bundles from the ridges, often accompanied by narrow spiral vessels; very numerous endosperm fragments containing aleurone grains and very small calcium oxalate microrosette crystals, as well as some fibre bundles from the carpophore.
- C. Examine by thin-layer chromatography (2.2.27), using *silica gel GF₂₅₄ R* as the coating substance.

Test solution. Shake 0.3 g of the freshly powdered drug (1400) with 5.0 ml of *methylen chloride R* for 15 min. Filter and carefully evaporate the filtrate to dryness on a water-bath at 60 °C. Dissolve the residue in 0.5 ml of *toluene R*.

Reference solution. Dissolve 60 µl of *anethole R* in 5.0 ml of *hexane R*.

Apply separately to the plate, as bands 20 mm by 3 mm, 10 µl of each solution. Develop over a path of 10 cm using a mixture of 20 volumes of *hexane R* and 80 volumes of *toluene R*. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. The chromatograms show in the central part a quenching zone corresponding to anethole. Spray the plate with *sulphuric acid R* and heat at 140 °C for 5 min. Examine in daylight. The chromatograms show in the central part a violet band corresponding to anethole. The chromatogram obtained with the test solution also shows a reddish-brown zone in its upper third (terpenes).

TESTS

Estragole and fenchone. The essential oil obtained in the assay contains not more than 10.0 per cent of estragole and not more than 7.5 per cent of fenchone.

Proceed as described in the assay for anethole, using the following reference solution.

Reference solution. Dissolve 5 mg of *estragole R* and 5 mg of *fenchone R* in 0.5 ml of *xylene R*.

Determine the contents of estragole and fenchone by normalisation.

Foreign matter (2.8.2). Not more than 1.5 per cent of peduncles and not more than 1.5 per cent of other foreign matter.

Water (2.2.13). Not more than 80 ml/kg, determined by distillation on 20.0 g of the powdered drug (710).

Total ash (2.4.16). Not more than 10.0 per cent.

ASSAY

Essential oil. Carry out the determination of essential oils in vegetable drugs (2.8.12). Use a 500 ml round-bottomed flask and 200 ml of *water R* as the distillation liquid. Reduce the drug to a coarse powder (1400) and immediately use 10.0 g for the determination. Introduce 0.50 ml of *xylene R* in the graduated tube. Distil at a rate of 2 ml/min to 3 ml/min for 2 h.

Anethole. Examine by gas chromatography (2.2.28).

Test solution. Dilute the mixture of essential oil and *xylene R* obtained in the determination of the essential oil to 5.0 ml with *xylene R* and by rinsing the apparatus.

Reference solution. Dissolve 5 mg of *anethole R* in 0.5 ml of *xylene R*.

The chromatographic procedure may be carried out using:

- a capillary column 30 m to 60 m long and 0.3 mm in internal diameter coated with *macrogol 20 000 R*,
- *nitrogen for chromatography R* as the carrier gas at a flow rate of 0.40 ml/min and split at a ratio of 1 to 200,
- a flame-ionisation detector,

maintaining the temperature of the column at 60 °C for 4 min, then raising the temperature linearly at a rate of 5 °C per minute to 170 °C and maintaining at 170 °C for 15 min and maintaining the temperature of the injection port at 220 °C and that of the detector at 270 °C.

Inject 1 µl of each solution. Determine the content of anethole by normalisation.

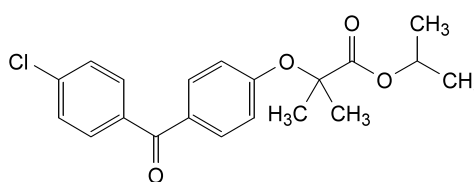
STORAGE

Store protected from light and moisture.

01/2005:1322

FENOFIBRATE

Fenofibratum



C₂₀H₂₁ClO₄

M_r 360.8

DEFINITION

Fenofibrate contains not less than 98.5 per cent and not more than the equivalent of 101.0 per cent of 1-methylethyl 2-[4-(4-chlorobenzoyl)phenoxy]-2-methylpropanoate, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, crystalline powder, practically insoluble in water, very soluble in methylene chloride, slightly soluble in alcohol.

IDENTIFICATION

- A. Melting point (2.2.14): 79 °C to 82 °C.
- B. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *fenofibrate CRS*. Examine the substances prepared as discs.

TESTS

Solution S. To 5.0 g, add 25 ml of *distilled water R* and heat at 50 °C for 10 min. Cool and dilute to 50.0 ml with the same solvent. Filter. Use the filtrate as solution S.

Appearance of solution. Dissolve 0.50 g in *acetone R* and dilute to 10.0 ml with the same solvent. The solution is clear (2.2.1) and not more intensely coloured than reference solution BY₆ (2.2.2, *Method II*).

Acidity. Dissolve 1.0 g in 50 ml of *alcohol R* previously neutralised using 0.2 ml of *phenolphthalein solution R1*. Not more than 0.2 ml of 0.1 M *sodium hydroxide* is required to change the colour of the indicator to pink.

Related substances. Examine by liquid chromatography (2.2.29) as described under Assay.

Inject 20 µl of reference solution (b). Adjust the sensitivity of the system so that the height of the peaks in the chromatogram obtained is at least 20 per cent of the full scale of the recorder. When the chromatograms are recorded in the prescribed conditions, the relative retention times are: impurity A about 0.34, impurity B about 0.36, impurity C about 0.50, impurity D about 0.65, impurity E about 0.80, impurity F about 0.85 and impurity G about 1.35. The test is not valid unless the resolution between the peaks corresponding to impurity A and impurity B is at least 1.5.

Inject 20 µl of reference solution (b) and 20 µl of the test solution. Continue the chromatography of the test solution for twice the retention time of fenofibrate. In the chromatogram obtained with the test solution, the area of any peak corresponding to impurity A, impurity B or impurity G is not greater than that of the corresponding peak in the chromatogram obtained with reference solution (b) (0.1 per cent for impurities A and B and 0.2 per cent for impurity G); the area of any peak, apart from the principal peak and any peaks corresponding to impurities A, B and G, is not greater than the area of the peak corresponding to fenofibrate in the chromatogram obtained with reference solution (b) (0.1 per cent); the sum of the areas of all the peaks, apart from the principal peak, is not greater than five times the area of the peak corresponding to fenofibrate in the chromatogram obtained with reference solution (b) (0.5 per cent). Disregard any peak with an area less than 0.1 times that of the peak due to fenofibrate in the chromatogram obtained with reference solution (b).

Halides expressed as chlorides (2.4.4). To 5 ml of solution S add 10 ml of *distilled water R*. The solution complies with the limit test for chlorides (100 ppm).

Sulphates (2.4.13). 15 ml of solution S complies with the limit test for sulphates (100 ppm).

Heavy metals (2.4.8). 1.0 g complies with limit test C for heavy metals (20 ppm). Prepare the standard using 2 ml of *lead standard solution (10 ppm Pb R)*.

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying *in vacuo* at 60 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 0.100 g of the substance to be examined in the mobile phase and dilute to 100.0 ml with the mobile phase.

Reference solution (a). Dissolve 25.0 mg of *fenofibrate CRS* in the mobile phase and dilute to 25.0 ml with the mobile phase.

Reference solution (b). Dissolve 10.0 mg of *fenofibrate CRS*, 10.0 mg of *fenofibrate impurity A CRS*, 10.0 mg of *fenofibrate impurity B CRS* and 20.0 mg of *fenofibrate impurity G CRS* in the mobile phase and dilute to 100.0 ml with the mobile phase. Dilute 1.0 ml of this solution to 100.0 ml with the mobile phase.

The chromatographic procedure may be carried out using:

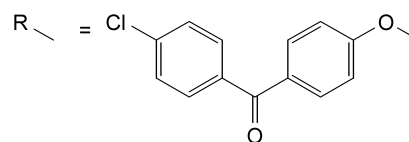
- a stainless steel column 0.25 m long and 4.0 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (5 µm),
- as mobile phase at a flow rate of 1 ml/min a mixture of 30 volumes of *water R* acidified to pH 2.5 with *phosphoric acid R* and 70 volumes of *acetonitrile R*,
- as detector a spectrophotometer set at 286 nm.

Inject 5 µl of reference solution (b). Adjust the sensitivity of the system so that the height of the peaks in the chromatogram is at least 50 per cent of the full scale of the recorder. Inject 5 µl of reference solution (a) six times. The assay is not valid unless the relative standard deviation of the peak area for fenofibrate is at most 1.0 per cent. Inject 5 µl of the test solution and 5 µl of reference solution (a).

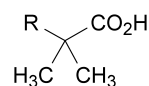
STORAGE

Store protected from light.

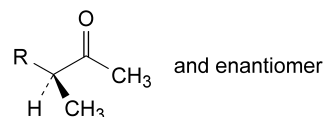
IMPURITIES



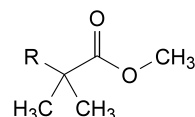
A. R-H: (4-chlorophenyl)(4-hydroxyphenyl)methanone,



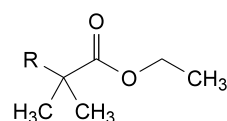
B. 2-[4-(4-chlorobenzoyl)phenoxy]-2-methylpropanoic acid (fenofibric acid),



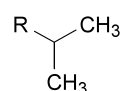
C. (3*RS*)-3-[4-(4-chlorobenzoyl)phenoxy]butan-2-one,



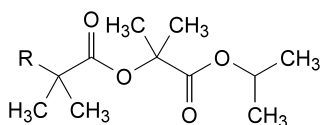
D. methyl 2-[4-(4-chlorobenzoyl)phenoxy]-2-methylpropanoate,



E. ethyl 2-[4-(4-chlorobenzoyl)phenoxy]-2-methylpropanoate,



F. (4-chlorophenyl)[4-(1-methylethoxy)phenyl]methanone,

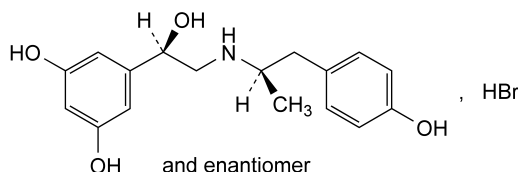


- G. 1-methylethyl 2-[[2-[4-(4-chlorobenzoyl)phenoxy]-2-methylpropanoyl]oxy]-2-methylpropanoate.

01/2005:0901

FENOTEROL HYDROBROMIDE

Fenoteroli hydrobromidum

C₁₇H₂₂BrNO₄M_r 384.3

DEFINITION

(1*RS*)-1-(3,5-dihydroxyphenyl)-2-[[1*RS*)-2-(4-hydroxyphenyl)-1-methylethyl]amino]ethanol hydrobromide.

Content: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

Appearance: white, crystalline powder.

Solubility: soluble in water and in alcohol.

IDENTIFICATION

First identification: B, E.

Second identification: A, C, D, E.

- A. Dissolve 50.0 mg in *dilute hydrochloric acid R1* and dilute to 50.0 ml with the same acid. Dilute 5.0 ml to 50.0 ml with *dilute hydrochloric acid R1*. Examined between 230 nm and 350 nm (2.2.25), the solution shows an absorption maximum at 275 nm and a shoulder at about 280 nm. The specific absorbance at the maximum is 80 to 86.

- B. Infrared absorption spectrophotometry (2.2.24).

Preparation: discs.

Comparison: Ph. Eur. reference spectrum of fenoterol hydrobromide.

- C. Thin-layer chromatography (2.2.27).

Test solution. Dissolve 10 mg of the substance to be examined in *alcohol R* and dilute to 10 ml with the same solvent.

Reference solution. Dissolve 10 mg of *fenoterol hydrobromide CRS* in *alcohol R* and dilute to 10 ml with the same solvent.

Plate: TLC silica gel G plate R.

Mobile phase: concentrated ammonia R, water R, aldehyde-free methanol R (1.5:10:90 V/V/V).

Application: 2 µl.

Development: over a path of 15 cm.

Drying: in air.

Detection: spray with a 10 g/l solution of *potassium permanganate R*.

Results: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

- D. Dissolve about 10 mg in a 20 g/l solution of *disodium tetraborate R* and dilute to 50 ml with the same solution. Add 1 ml of a 10 g/l solution of *aminopyrazolone R*, 10 ml of a 2 g/l solution of *potassium ferricyanide R* and 10 ml of *methylene chloride R*. Shake and allow to separate. A reddish-brown colour develops in the lower layer.

- E. It gives reaction (a) of bromides (2.3.1).

TESTS

Solution S. Dissolve 2.00 g in *carbon dioxide-free water R* and dilute to 50.0 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and not more intensely coloured than reference solution Y₇ (2.2.2, *Method II*).

pH (2.2.3): 4.2 to 5.2 for solution S.

Phenone: maximum 0.2 per cent. The absorbance (2.2.25) of solution S at 330 nm has a maximum of 0.42.

Diastereoisomers. Liquid chromatography (2.2.29). *Prepare the solutions immediately before use.*

Test solution. Dissolve 25.0 mg of the substance to be examined in *water R* and dilute to 10.0 ml with the same solvent.

Reference solution. Dissolve 25.0 mg of *fenoterol hydrobromide CRS* in *water R* and dilute to 10.0 ml with the same solvent.

Column:

- *size*: *l* = 0.25 m, Ø = 4.6 mm,
- *stationary phase*: octadecylsilyl silica gel for chromatography R (5 µm to 10 µm).

Mobile phase: to a mixture of 1 volume of a 9 g/l solution of *potassium dihydrogen phosphate R* and 69 volumes of a 24 g/l solution of *disodium hydrogen phosphate R*, adjusted to pH 8.5 using *phosphoric acid R*, add 30 volumes of *methanol R*.

Flow rate: 1 ml/min.

Detection: spectrophotometer at 280 nm.

Injection: 20 µl loop injector.

Sensitivity: the height of the peak due to the diastereoisomers eluting immediately after the principal peak is not less than 10 per cent of the full scale of the recorder.

System suitability: reference solution:

- the height of the trough separating the peak due to the diastereoisomers from the principal peak is less than 4 per cent of the full scale of the recorder,
- the retention time of the principal peak is less than 20 min.

Limits:

Calculate the content of diastereoisomers by determining the height of a perpendicular dropped from the apex of the peak to a line drawn from the trough between the 2 peaks to the baseline, and taking into account the declared content of diastereoisomers in *fenoterol hydrobromide CRS* (4.0 per cent).

Iron (2.4.9): maximum 5 ppm.

Dissolve the residue from the test for sulphated ash in 2.5 ml of *dilute hydrochloric acid R* and dilute to 10 ml with *water R*. The solution complies with the limit test for iron.

Loss on drying (2.2.32): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 100–105 °C.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

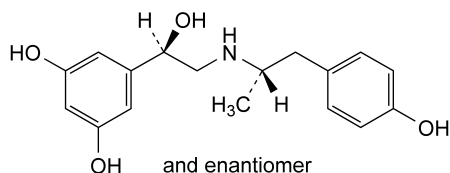
Dissolve 0.600 g in 50 ml of *water R* and add 5 ml of *dilute nitric acid R*, 25.0 ml of 0.1 M *silver nitrate* and 2 ml of *ferric ammonium sulphate solution R2*. Shake and titrate with 0.1 M *ammonium thiocyanate* until an orange colour is obtained. Carry out a blank titration.

1 ml of 0.1 M *silver nitrate* is equivalent to 38.43 mg of $C_{17}H_{22}BrNO_4$.

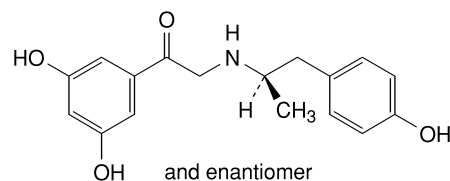
STORAGE

Protected from light.

IMPURITIES



A. (1*RS*)-1-(3,5-dihydroxyphenyl)-2-[(1*RS*)-2-(4-hydroxyphenyl)-1-methylethyl]amino]ethanol,

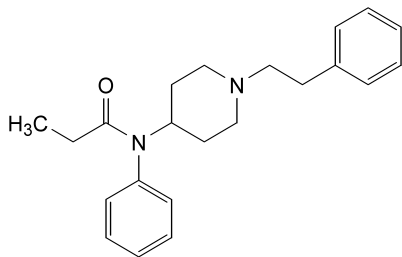


B. 1-(3,5-dihydroxyphenyl)-2-[(1*RS*)-2-(4-hydroxyphenyl)-1-methylethyl]amino]ethanone (phenone).

01/2005:1210

FENTANYL

Fentanylum



$C_{22}H_{28}N_2O$

M_r 336.5

DEFINITION

Fentanyl contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of *N*-phenyl-*N*-[1-(2-phenylethyl)piperidin-4-yl]propanamide, calculated with reference to the dried substance.

CHARACTERS

A white or almost white powder, practically insoluble in water, freely soluble in alcohol and in methanol. It shows polymorphism.

IDENTIFICATION

Examine by infrared absorption spectrophotometry (2.2.24), comparing with the *Ph. Eur. reference spectrum of fentanyl*. If the spectrum obtained in the solid state shows differences, dissolve the substance to be examined in the minimum volume of *ethanol R*, evaporate to dryness at room temperature under an air-stream and record the spectrum again using the residue.

TESTS

Related substances. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 0.100 g of the substance to be examined in *methanol R* and dilute to 10.0 ml with the same solvent.

Reference solution (a). In order to prepare the *in situ* degradation compound (fentanyl impurity D), dissolve 10 mg of the substance to be examined in 10.0 ml of *dilute hydrochloric acid R*. Heat on a water-bath under a reflux condenser for 4 h. Neutralise with 10.0 ml of *dilute sodium hydroxide solution R*. Evaporate to dryness on a water-bath. Cool and take up the residue in 10 ml of *methanol R*. Filter.

Reference solution (b). Dilute 1.0 ml of the test solution to 100.0 ml with *methanol R*. Dilute 5.0 ml of this solution to 20.0 ml with *methanol R*.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.1 m long and 4.6 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (3 μ m),
- as mobile phase at a flow rate of 1.5 ml/min the following mixtures:

Mobile phase A. A 5 g/l solution of *ammonium carbonate R* in a mixture of 10 volumes of *tetrahydrofuran R* and 90 volumes of *water R*,
Mobile phase B. *Acetonitrile R*,

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)	Comment
0 - 15	90 → 40	10 → 60	linear gradient
15 - 20	40	60	isocratic elution
20 - 25	90	10	switch to initial eluent composition
20 = 0	90	10	restart gradient

- as detector a spectrophotometer set at 220 nm.

Equilibrate the column for at least 30 min with *acetonitrile R* and then equilibrate at the initial eluent composition for at least 5 min.

Adjust the sensitivity of the system so that the height of the principal peak in the chromatogram obtained with 10 μ l of reference solution (b) is at least 50 per cent of the full scale of the recorder.

Inject 10 μ l of reference solution (a). When the chromatograms are recorded in the prescribed conditions, the retention times are: fentanyl about 10 min and fentanyl impurity D about 12 min. The test is not valid unless the resolution between the peaks corresponding to fentanyl and fentanyl impurity D is at least 8.0. If necessary, adjust the concentration of acetonitrile in the mobile phase or adjust the time programme for the linear gradient elution.

Inject separately 10 μ l of *methanol R* as a blank, 10 μ l of the test solution and 10 μ l of reference solution (b). In the chromatogram obtained with the test solution: the area of any peak, apart from the principal peak, is not greater than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.25 per cent); the sum of the areas of all peaks, apart from the principal peak, is not greater than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent). Disregard any peak obtained with the blank run and any peak with an area less than 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (b).

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying *in vacuo* at 50 °C.

ASSAY

01/2005:1103

Dissolve 0.200 g in 50 ml of a mixture of 1 volume of *anhydrous acetic acid R* and 7 volumes of *methyl ethyl ketone R* and titrate with 0.1 M *perchloric acid*, using 0.2 ml of *naphtholbenzein solution R* as indicator.

1 ml of 0.1 M *perchloric acid* is equivalent to 33.65 mg of $C_{22}H_{28}N_2O$.

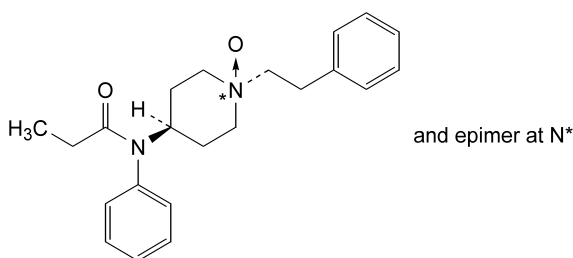
STORAGE

Store protected from light.

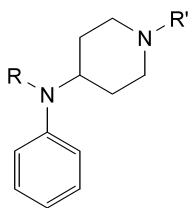
IMPURITIES

Specified impurities: A, B, C, D.

Other detectable impurities: E, F, G.



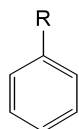
A. *N*-phenyl-*N*-[*cis,trans*-1-oxido-1-(2-phenylethyl)piperidin-4-yl]propanamide,



B. R = CO-C₂H₅, R' = H: *N*-phenyl-*N*-(piperidin-4-yl)propanamide,

C. R = CO-CH₃, R' = CH₂-CH₂-C₆H₅: *N*-phenyl-*N*-[1-(2-phenylethyl)piperidin-4-yl]acetamide,

D. R = H, R' = CH₂-CH₂-C₆H₅: *N*-phenyl-1-(2-phenylethyl)piperidin-4-amine,



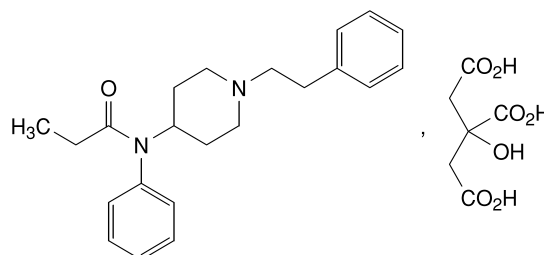
E. R = CHO: benzaldehyde,

F. R = NH₂: aniline (phenylamine),

G. R = NH-CO-C₂H₅: *N*-phenylpropanamide.

FENTANYL CITRATE

Fentanyli citras



$C_{28}H_{36}N_2O_8$

M_r 528.6

DEFINITION

Fentanyl citrate contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of *N*-phenyl-*N*-[1-(2-phenylethyl)piperidin-4-yl]propanamide dihydrogen 2-hydroxypropane-1,2,3-tricarboxylate, calculated with reference to the dried substance.

CHARACTERS

A white or almost white powder, soluble in water, freely soluble in methanol, sparingly soluble in alcohol.

It melts at about 152 °C with decomposition.

IDENTIFICATION

Examine by infrared absorption spectrophotometry (2.2.24), comparing with the *Ph. Eur. reference spectrum of fentanyl citrate*.

TESTS

Appearance of solution. Dissolve 0.2 g of the substance to be examined in *water R* and dilute to 20 ml with the same solvent. The solution is clear (2.2.1) and colourless (2.2.2, *Method II*).

Related substances. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 0.100 g of the substance to be examined in *methanol R* and dilute to 10.0 ml with the same solvent.

Reference solution (a). In order to prepare the *in situ* degradation compound (impurity D), dissolve 10 mg of the substance to be examined in 10.0 ml of *dilute hydrochloric acid R*. Heat on a water-bath under a reflux condenser for 4 h. Neutralise with 10.0 ml of *dilute sodium hydroxide solution R*. Evaporate to dryness on a water-bath. Cool and take up the residue in 10 ml of *methanol R*. Filter.

Reference solution (b). Dilute 1.0 ml of the test solution to 100.0 ml with *methanol R*. Dilute 5.0 ml of the solution to 20.0 ml with *methanol R*.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.1 m long and 4.6 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (3 µm),
- as mobile phase at a flow rate of 1.5 ml/min a gradient programme using the following conditions:

Mobile phase A. A 5 g/l solution of *ammonium carbonate R* in a mixture of 10 volumes of *tetrahydrofuran R* and 90 volumes of *water R*,

Mobile phase B. *Acetonitrile R*,

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)	Comment
0 - 15	90 → 40	10 → 60	linear gradient
15 - 20	40	60	isocratic elution
20 - 25	90	10	switch to initial eluent composition
25 = 0	90	10	restart gradient

— as detector a spectrophotometer set at 220 nm.

Equilibrate the column for at least 30 min with *acetonitrile R* and then equilibrate at the initial eluent composition for at least 5 min. Adjust the sensitivity of the system so that the height of the principal peak in the chromatogram obtained with 10 µl of reference solution (b) is at least 50 per cent of the full scale of the recorder.

Inject 10 µl of reference solution (a). When the chromatograms are recorded in the prescribed conditions, the retention times are: fentanyl about 10 min; impurity D about 12 min. The test is not valid unless the resolution between the peaks corresponding to fentanyl and impurity D is at least 8.0. If necessary, adjust the concentration of acetonitrile in the mobile phase or adjust the time programme for the linear gradient elution.

Inject separately 10 µl of *methanol R* as a blank, 10 µl of the test solution and 10 µl of reference solution (b). In the chromatogram obtained with the test solution: the area of any peak, apart from the principal peak, is not greater than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.25 per cent); the sum of the areas of all peaks, apart from the principal peak, is not greater than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent). Disregard any peak obtained with the blank run and any peak with a retention time relative to the principal peak of 0.05 or less and any peak with an area less than 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (b).

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying *in vacuo* at 60 °C.

ASSAY

Dissolve 0.300 g in 50 ml of a mixture of 1 volume of *anhydrous acetic acid R* and 7 volumes of *methyl ethyl ketone R*. Titrate with 0.1 M *perchloric acid* using 0.2 ml of *naphtholbenzein solution R* as indicator.

1 ml of 0.1 M *perchloric acid* is equivalent to 52.86 mg of C₂₈H₃₆N₂O₈.

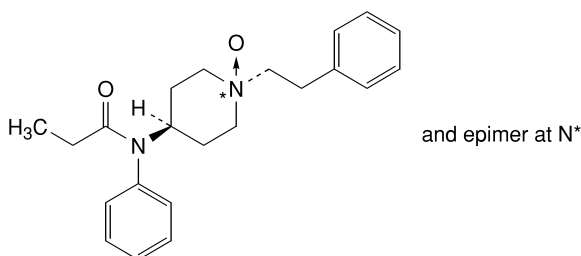
STORAGE

Store protected from light.

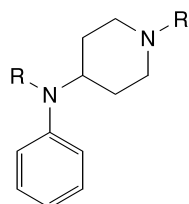
IMPURITIES

Specified impurities: A, B, C, D.

Other detectable impurities: E.



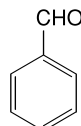
A. *N*-phenyl-*N*-[*cis,trans*-1-oxido-1-(2-phenylethyl)piperidin-4-yl]propanamide,



B. R = CO-C₂H₅, R' = H: *N*-phenyl-*N*-(piperidin-4-yl)propanamide,

C. R = CO-CH₃, R' = CH₂-CH₂-C₆H₅: *N*-phenyl-*N*-[1-(2-phenylethyl)piperidin-4-yl]acetamide,

D. R = H, R' = CH₂-CH₂-C₆H₅: *N*-phenyl-1-(2-phenylethyl)piperidin-4-amine,

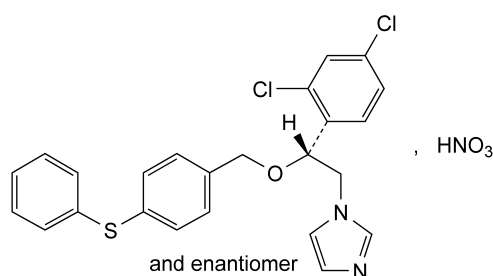


E. benzaldehyde.

01/2005:1211

FENTICONAZOLE NITRATE

Fenticonazoli nitras



C₂₄H₂₁Cl₂N₃O₄S

M_r 518.4

DEFINITION

Fenticonazole nitrate contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of 1-[(2*RS*)-2-(2,4-dichlorophenyl)-2-[[4-(phenylsulphonyl)benzyl]oxy]ethyl]-1*H*-imidazole nitrate, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, crystalline powder, practically insoluble in water, freely soluble in methanol and in dimethylformamide, sparingly soluble in ethanol.

IDENTIFICATION

First identification: C, D.

Second identification: A, B, D.

A. Melting point (2.2.14): 134 °C to 137 °C.

B. Dissolve 20.0 mg in *ethanol R* and dilute to 100.0 ml with the same solvent. Dilute 1.0 ml of this solution to 10.0 ml with *ethanol R*. Examined between 230 nm and 350 nm (2.2.25), the solution shows an absorption maximum at 252 nm, a shoulder at about 270 nm and an absorption minimum at 236 nm. The specific absorption at the maximum is 260 to 280.

C. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *fenticonazole nitrate CRS*.

D. It gives the reaction of nitrates (2.3.1).

TESTS

Optical rotation (2.2.7). Dissolve 0.10 g in *methanol R* and dilute to 10.0 ml with the same solvent. The angle of optical rotation is -0.10° to $+0.10^\circ$.

Related substances. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 25.0 mg of the substance to be examined in the mobile phase and dilute to 25.0 ml with the mobile phase.

Reference solution (a). Dilute 1.0 ml of the test solution to 200.0 ml with the mobile phase.

Reference solution (b). Dilute 10.0 ml of reference solution (a) to 25.0 ml with the mobile phase.

Reference solution (c). Dilute 1.0 ml of reference solution (a) to 10.0 ml with the mobile phase.

Reference solution (d). To 5 ml of the test solution add 5.0 mg of *fenticonazole impurity D CRS*, dissolve in the mobile phase and dilute to 100.0 ml with the mobile phase. Dilute 2.0 ml of this solution to 10.0 ml with the mobile phase.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (5 μm to 10 μm),
- as mobile phase at a flow rate of 1.0 ml/min a mixture of 70 volumes of *acetonitrile R* and 30 volumes of a phosphate buffer solution prepared by dissolving 3.4 g of *potassium dihydrogen phosphate R* in 900 ml of *water R*, adjusting to pH 3.0 with *phosphoric acid R* and diluting to 1000 ml with *water R*,
- as detector a spectrophotometer set at 229 nm.

Inject 10 μl of reference solution (b). Adjust the sensitivity of the system so that the height of the peak due to fenticonazole is at least 10 per cent of the full scale of the recorder.

Inject separately 10 μl of reference solution (c) and 10 μl of reference solution (d). The test is not valid unless in the chromatogram obtained with reference solution (d), the resolution between the peak due to fenticonazole impurity D and fenticonazole is at least 2.0. The test is not valid unless in the chromatogram obtained with reference solution (c) the signal-to-noise ratio is at least five.

Inject separately 10 μl of the test solution and 10 μl of reference solution (a). Record the chromatogram obtained with the test solution for 5.5 times the retention time of the principal peak. In the chromatogram obtained with the test solution: the area of any peak apart from the principal peak and the nitric ion peak (which corresponds to the dead volume of the column), is not greater than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent) and the sum of the areas of such peaks is not greater than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent). Disregard any peak with an area less than that of the principal peak in the chromatogram obtained with reference solution (c).

Toluene. Not more than 100 ppm, determined by head-space gas chromatography (2.2.28), using the standard additions method.

Test solution. Disperse 0.2 g of the substance to be examined in a 10 ml vial with 5 ml of *water R*.

Reference solution. Mix 4 mg of *toluene R* with *water R* and dilute to 1000 ml. Place 5 ml of this solution in a 10 ml vial.

The chromatographic procedure may be carried out using:

- a column 25 m long and 0.32 mm in internal diameter coated with *poly(cyanopropyl)(7)phenyl(7)methyl(86)siloxane R* (film thickness 1.2 μm),
 - *helium for chromatography R* as the carrier gas, split ratio 1:25, column head pressure 40 kPa,
 - a flame-ionisation detector,
- maintaining the temperature of the column at 80 $^\circ\text{C}$, that of the injection port at 180 $^\circ\text{C}$ and that of the detector at 220 $^\circ\text{C}$. Maintain each solution at 90 $^\circ\text{C}$ for 1 h, and transfer onto the column 1 ml of the vapour phase.

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying *in vacuo* at 60 $^\circ\text{C}$.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

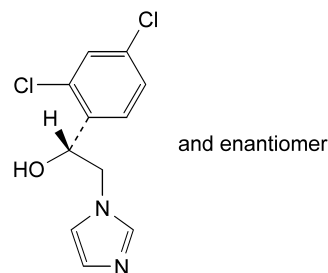
Dissolve 0.450 g in 50 ml of a mixture of equal volumes of *anhydrous acetic acid R* and *methyl ethyl ketone R*. Titrate with 0.1 M *perchloric acid*, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *perchloric acid* is equivalent to 51.84 mg of $\text{C}_{24}\text{H}_{21}\text{Cl}_2\text{N}_3\text{O}_4\text{S}$.

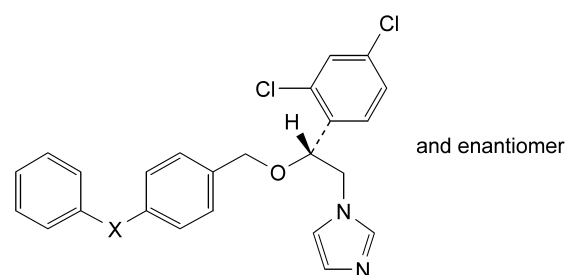
STORAGE

Store protected from light.

IMPURITIES

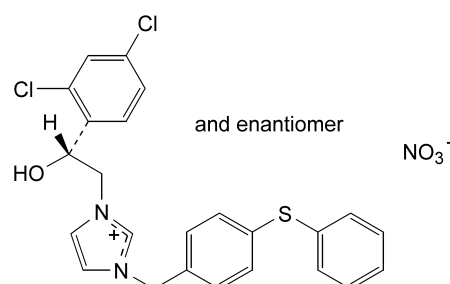


A. (RS)-1-(2,4-dichlorophenyl)-2-(1H-imidazol-1-yl)ethanol,

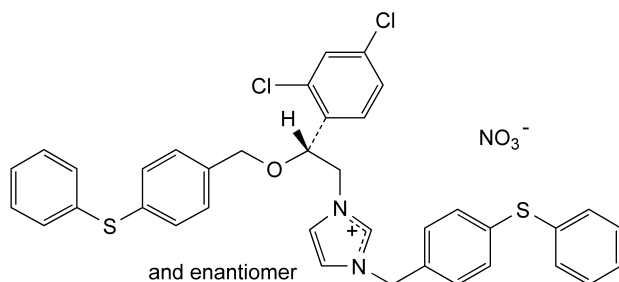


B. X = SO: 1-[(2RS)-2-(2,4-dichlorophenyl)-2-[[4-(phenylsulphonyl)benzyl]oxy]ethyl]-1H-imidazole,

C. X = SO₂: 1-[(2RS)-2-(2,4-dichlorophenyl)-2-[[4-(phenylsulphonyl)benzyl]oxy]ethyl]-1H-imidazole,



D. (RS)-1-[2-(2,4-dichlorophenyl)-2-hydroxyethyl]-3-[4-(phenylsulphonyl)benzyl]imidazolium nitrate,



- E. (RS)-1-[2-(2,4-dichlorophenyl)-2-[4-(phenylsulphanyl)benzyloxy]ethyl]-3-[4-(phenylsulphanyl)benzyl]imidazolium nitrate.

01/2005:1323

FENUGREEK

Trigonellae foenugraeci semen

DEFINITION

Fenugreek consists of the dried, ripe seeds of *Trigonella foenum-graecum* L.

CHARACTERS

Fenugreek has a strong characteristic aromatic odour.

It has the macroscopic and microscopic characters described under identification tests A and B.

IDENTIFICATION

- A. The seed is hard, flattened, brown to reddish-brown and more or less rhomboidal with rounded edges. It is 3 mm to 5 mm long, 2 mm to 3 mm wide and 1.5 mm to 2 mm thick. The widest surfaces are marked by a groove that divides the seed into two unequal parts. The smaller part contains the radicle; the larger part contains the cotyledons.
- B. Reduce to a powder (355). The powder is yellowish-brown. Examine under a microscope using *chloral hydrate solution R*. The powder shows fragments of the testa in sectional view with thick cuticle covering lageniform epidermal cells, with an underlying hypodermis of large cells, narrower at the upper end and constricted in the middle, with bar-like thickenings of the radial walls; yellowish-brown fragments of the epidermis in surface view, composed of small, polygonal cells with thickened and pitted walls, frequently associated with the hypodermal cells, circular in outline with thickened and closely beaded walls; fragments of the hypodermis viewed from below, composed of polygonal cells whose bar-like thickenings extend to the upper and lower walls; parenchyma of the testa with elongated, rectangular cells with slightly thickened and beaded walls; fragments of endosperm with irregularly thickened, sometimes elongated cells, containing mucilage.
- C. Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel F₂₅₄ plate R*.
Test solution. Place 1.0 g of the powdered drug (710) in a 25 ml conical flask and add 5.0 ml of *methanol R*. Heat in a water-bath at 65 °C for 5 min. Cool and filter.
Reference solution. Dissolve 3.0 mg of *trigonelline hydrochloride R* in 1.0 ml of *methanol R*.
 Apply to the plate as bands 20 µl of the test solution and 10 µl of the reference solution. Develop over a path of 10 cm using a mixture of 30 volumes of *water R* and 70 volumes of *methanol R*. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. The

chromatogram obtained with the test solution shows in its lower half a quenching zone similar in position and fluorescence to the zone in the chromatogram obtained with the reference solution. Spray with *potassium iodobismuthate solution R2*. The chromatogram obtained with the test solution shows an intense orange-red zone similar in position and colour to the zone in the chromatogram obtained with the reference solution. It also shows in its upper half a broad, light brownish-yellow zone (triglycerides).

TESTS

Foreign matter (2.8.2). It complies with the test for foreign matter.

Swelling index (2.8.4). Not less than 6, determined on the powdered drug (710).

Loss on drying (2.2.32). Not more than 12.0 per cent, determined on 1.000 g of the powdered drug by drying in an oven at 100 °C to 105 °C for 2 h.

Total ash (2.4.16). Not more than 5.0 per cent.

STORAGE

Store protected from light.

01/2005:1515

FERRIC CHLORIDE HEXAHYDRATE

Ferri chloridum hexahydricum

FeCl₃·6H₂OM_r 270.3

DEFINITION

Ferric chloride hexahydrate contains not less than 98.0 per cent and not more than 102.0 per cent of FeCl₃·6H₂O.

CHARACTERS

Crystalline mass or orange-yellow to brownish-yellow crystals, very hygroscopic, very soluble in water and in alcohol, freely soluble in glycerol.

IDENTIFICATION

- A. It gives reaction (a) of chlorides (2.3.1).
 B. It gives reaction (c) of iron (2.3.1).

TESTS

Solution S. Dissolve 10 g in *distilled water R* and dilute to 100 ml with the same solvent.

Acidity. In a suitable polyethylene container, dissolve 3.0 g of *potassium fluoride R* in 15 ml of *water R*. Titrate with 0.1 M *sodium hydroxide* using 0.1 ml of *phenolphthalein solution R* as indicator until a pink colour is obtained. Add 10 ml of solution S and allow to stand for 3 h. Filter and use 12.5 ml of the filtrate. Not more than 0.30 ml of 0.1 M *sodium hydroxide* is required to change the colour of the indicator to pink.

Free chlorine. Heat 5 ml of solution S. The vapour does not turn *starch iodide paper R* blue.

Sulphates (2.4.13). Heat 15 ml of solution S on a water-bath and add 5 ml of *strong sodium hydroxide solution R*. Allow to cool and filter. Neutralise the filtrate to *blue litmus paper R* using *hydrochloric acid R1* and evaporate to 15 ml. The solution complies with the limit test for sulphates (100 ppm).

Ferrous ions. To 10 ml of solution S, add 1 ml of *water R*, 0.05 ml of *potassium ferricyanide solution R* followed by 4 ml of *phosphoric acid R*. After 10 min, any blue colour

in the solution is not more intense than that in a standard prepared at the same time and in the same manner using 10 ml of *water R* and 1 ml of a freshly prepared 0.250 g/l solution of *ferrous sulphate R* (50 ppm Fe).

Heavy metals (2.4.8). Dissolve 1.0 g in 10 ml of *hydrochloric acid R1*. Add 2 ml of *strong hydrogen peroxide solution R*, then evaporate to 5 ml. Allow to cool and dilute to 20 ml with *hydrochloric acid R1* and transfer the solution to a separating funnel. Shake three times, for 3 min each time, with 20 ml of *methyl isobutyl ketone R1*. Separate the lower phase, reduce to half its volume by evaporation and dilute to 25 ml with *water R*. Neutralise 10 ml of the solution with *dilute ammonia R1* to *red litmus paper R* and dilute to 20 ml with *water R*. 12 ml of the solution complies with limit test A for heavy metals (50 ppm). Prepare the standard using *lead standard solution (1 ppm Pb) R*.

ASSAY

In a conical flask with a ground-glass stopper, dissolve 0.200 g in 20 ml of *water R*. Add 10 ml of *dilute hydrochloric acid R* and 2 g of *potassium iodide R*. Allow the stoppered flask to stand for 1 h protected from light. Titrate with 0.1 M *sodium thiosulphate*, using 5 ml of *starch solution R* as indicator, added towards the end of the titration.

1 ml of 0.1 M *sodium thiosulphate* is equivalent to 27.03 mg of $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$.

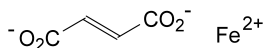
STORAGE

Store in an airtight container, protected from light.

01/2005:0902

FERROUS FUMARATE

Ferrosi fumaras



$\text{C}_4\text{H}_2\text{FeO}_4$

M_r 169.9

DEFINITION

Ferrous fumarate contains not less than 93.0 per cent and not more than the equivalent of 101.0 per cent of iron(II) (*E*)-butenedioate, calculated with reference to the dried substance.

CHARACTERS

A fine, reddish-orange or reddish-brown powder, slightly soluble in water, very slightly soluble in alcohol.

IDENTIFICATION

A. Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel F₂₅₄ plate R*.

Test solution. To 1.0 g add 25 ml of a mixture of equal volumes of *hydrochloric acid R* and *water R* and heat on a water-bath for 15 min. Cool and filter. Use the filtrate for identification test C. Wash the residue with 50 ml of a mixture of 1 volume of *dilute hydrochloric acid R* and 9 volumes of *water R* and discard the washings. Dry the residue at 100–105 °C. Dissolve 20 mg of the residue in *acetone R* and dilute to 10 ml with the same solvent.

Reference solution. Dissolve 20 mg of *fumaric acid CRS* in *acetone R* and dilute to 10 ml with the same solvent.

Apply to the plate 5 µl of each solution. Develop in an unsaturated tank over a path of 10 cm using a mixture of 12 volumes of *anhydrous formic acid R*, 16 volumes

of *methylene chloride R*, 32 volumes of *butanol R* and 44 volumes of *heptane R*. Dry the plate at 105 °C for 15 min and examine in ultraviolet light at 254 nm. The principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with the reference solution.

- B. Mix 0.5 g with 1 g of *resorcinol R*. To 0.5 g of the mixture in a crucible add 0.15 ml of *sulphuric acid R* and heat gently. A dark red semi-solid mass is formed. Add the mass, with care, to 100 ml of *water R*. An orange-yellow colour develops and the solution shows no fluorescence.
- C. The filtrate obtained for the test solution in identification test A gives reaction (a) of iron (2.3.1).

TESTS

Solution S. Dissolve 2.0 g of the substance to be examined in a mixture of 10 ml of *lead-free hydrochloric acid R* and 80 ml of *water R*, heating slightly if necessary. Allow to cool, filter if necessary and dilute to 100 ml with *water R*.

Sulphates (2.4.13). Heat 0.15 g with 8 ml of *dilute hydrochloric acid R* and 20 ml of *distilled water R*. Cool in iced water, filter and dilute to 30 ml with *distilled water R*. 15 ml of this solution complies with the limit test for sulphates (0.2 per cent).

Arsenic (2.4.2). Mix 1.0 g with 15 ml of *water R* and 15 ml of *sulphuric acid R*. Warm to precipitate the fumaric acid completely. Cool and add 30 ml of *water R*. Filter. Wash the precipitate with *water R*. Dilute the combined filtrate and washings to 125 ml with *water R*. 25 ml of the solution complies with limit test A for arsenic (5 ppm).

Ferric ion. Not more than 2.0 per cent of ferric ion.

In a flask with a ground-glass stopper, dissolve 3.0 g in a mixture of 10 ml of *hydrochloric acid R* and 100 ml of *water R* by heating rapidly to boiling. Boil for 15 s. Cool rapidly, add 3 g of *potassium iodide R*, stopper the flask and allow to stand protected from light for 15 min. Add 2 ml of *starch solution R* as indicator. Titrate the liberated iodine with 0.1 M *sodium thiosulphate*. Carry out a blank test. The difference between the volumes used in the two titrations corresponds to the amount of iodine liberated by ferric ion.

1 ml of 0.1 M *sodium thiosulphate* is equivalent to 5.585 mg of ferric ion.

Cadmium. Not more than 10 ppm of Cd, determined by atomic absorption spectrometry (2.2.23, *Method I*).

Test solution. Use solution S.

Reference solutions. Prepare the solutions using *cadmium standard solution (0.1 per cent Cd) R* and diluting with a 10 per cent V/V solution of *lead-free hydrochloric acid R*.

Measure the absorbance at 228.8 nm using a cadmium hollow-cathode lamp as source of radiation and an air-acetylene flame.

Chromium. Not more than 200 ppm of Cr, determined by atomic absorption spectrometry (2.2.23, *Method I*).

Test solution. Use solution S.

Reference solutions. Prepare the solutions using *chromium standard solution (0.1 per cent Cr) R* and diluting with a 10 per cent V/V solution of *lead-free hydrochloric acid R*.

Measure the absorbance at 357.9 nm using a chromium hollow-cathode lamp as source of radiation and an air-acetylene flame.

Lead. Not more than 20 ppm of Pb, determined by atomic absorption spectrometry (2.2.23, *Method I*).

Test solution. Use solution S.

Reference solutions. Prepare the solutions using *lead standard solution (10 ppm Pb) R* and diluting with a 10 per cent V/V solution of *lead-free hydrochloric acid R*.

Measure the absorbance at 283.3 nm using a lead hollow-cathode lamp as the source of radiation and an air-acetylene flame.

Mercury. Not more than 1 ppm of Hg, determined by atomic absorption spectrometry (2.2.23, *Method I*).

Test solution. Use solution S.

Reference solutions. Prepare the solutions using *mercury standard solution (10 ppm Hg) R* and diluting with a 25 per cent V/V solution of *lead-free hydrochloric acid R*.

Following the recommendations of the manufacturer, introduce 5 ml of solution S or 5 ml of the reference solutions into the reaction vessel of the cold-vapour mercury assay accessory, add 10 ml of *water R* and 1 ml of *stannous chloride solution R1*.

Measure the absorbance at 253.7 nm using a mercury hollow-cathode lamp as the source of radiation.

Nickel. Not more than 200 ppm of Ni, determined by atomic absorption spectrometry (2.2.23, *Method I*).

Test solution. Use solution S.

Reference solutions. Prepare the solutions using *nickel standard solution (10 ppm Ni) R* and diluting with a 10 per cent V/V solution of *lead-free hydrochloric acid R*.

Measure the absorbance at 232 nm using a nickel hollow-cathode lamp as source of radiation and an air-acetylene flame.

Zinc. Not more than 500 ppm of Zn, determined by atomic absorption spectrometry (2.2.23, *Method I*).

Test solution. Use solution S diluted ten times.

Reference solutions. Prepare the solutions using *zinc standard solution (10 ppm Zn) R* and diluting with a 1 per cent V/V solution of *lead-free hydrochloric acid R*.

Measure the absorbance at 213.9 nm using a zinc hollow-cathode lamp as source of radiation and an air-acetylene flame.

Loss on drying (2.2.32). Not more than 1.0 per cent, determined on 1.000 g by drying in an oven at 100-105 °C.

ASSAY

Dissolve with slight heating 0.150 g in 7.5 ml of *dilute sulphuric acid R*. Cool and add 25 ml of *water R*. Add 0.1 ml of *ferroin R*. Titrate immediately with 0.1 M *cerium sulphate* until the colour changes from orange to light bluish-green.

1 ml of 0.1 M *cerium sulphate* is equivalent to 16.99 mg of C₁₂H₂₂FeO₁₄.

STORAGE

Store in an airtight container, protected from light.

DEFINITION

Ferrous gluconate is iron(II) di(D-gluconate). It contains not less than 11.8 per cent and not more than 12.5 per cent of iron(II), calculated with reference to the dried substance.

CHARACTERS

A greenish-yellow to grey powder or granules, freely but slowly soluble in water giving a greenish-brown solution, more readily soluble in hot water, practically insoluble in alcohol.

IDENTIFICATION

A. Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel G plate R*.

Test solution. Dissolve 20 mg of the substance to be examined in 2 ml of *water R*, heating if necessary in a water-bath at 60 °C.

Reference solution. Dissolve 20 mg of *ferrous gluconate CRS* in 2 ml of *water R*, heating if necessary in a water-bath at 60 °C.

Apply separately to the plate 5 µl of each solution. Develop over a path of 10 cm using a mixture of 10 volumes of *ethyl acetate R*, 10 volumes of *concentrated ammonia R*, 30 volumes of *water R* and 50 volumes of *alcohol R*. Dry the plate at 100 °C to 105 °C for 20 min, allow to cool and spray with a 50 g/l solution of *potassium dichromate R* in a 40 per cent m/m solution of *sulphuric acid R*. After 5 min, the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

B. 1 ml of solution S (see Tests) gives reaction (a) of iron (2.3.1).

TESTS

Solution S. Dissolve 5.0 g in *carbon dioxide-free water R* prepared from *distilled water R* and heated to about 60 °C, cool and dilute to 50 ml with *carbon dioxide-free water R* prepared from *distilled water R*.

Appearance of solution. Dilute 2 ml of solution S to 10 ml with *water R*. Examined against the light, the solution is clear (2.2.1).

pH (2.2.3). The pH of solution S, measured 3 h to 4 h after preparation, is 4.0 to 5.5.

Sucrose and reducing sugars. Dissolve 0.5 g in 10 ml of warm *water R* and add 1 ml of *dilute ammonia R1*. Pass *hydrogen sulphide R* through the solution and allow to stand for 30 min. Filter and wash the precipitate with two quantities, each of 5 ml, of *water R*. Acidify the combined filtrate and washings to *blue litmus paper R* with *dilute hydrochloric acid R* and add 2 ml in excess. Boil until the vapour no longer darkens *lead acetate paper R* and continue boiling, if necessary, until the volume is reduced to about 10 ml. Cool, add 15 ml of *sodium carbonate solution R*, allow to stand for 5 min and filter. Dilute the filtrate to 100 ml with *water R*. To 5 ml of this solution add 2 ml of *cupri-tartaric solution R* and boil for 1 min. Allow to stand for 1 min. No red precipitate is formed.

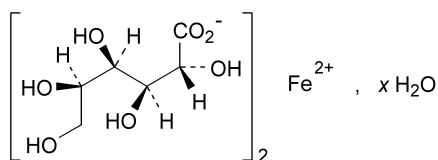
Chlorides (2.4.4). 0.8 ml of solution S diluted to 15 ml with *water R* complies with the limit test for chlorides (0.06 per cent).

Oxalates. Dissolve 5.0 g in a mixture of 10 ml of *dilute sulphuric acid R* and 40 ml of *water R*. Shake the solution with 50 ml of *ether R* for 5 min. Separate the aqueous layer and shake it with 20 ml of *ether R* for 5 min. Combine the ether layers, evaporate to dryness and dissolve the residue in 15 ml of *water R*. Filter, boil the filtrate until the volume

01/2005:0493

FERROUS GLUCONATE

Ferrosi gluconas



C₁₂H₂₂FeO₁₄·xH₂O

M_r 446.1 (anhydrous substance)

01/2005:0083

is reduced to 5 ml and add 1 ml of *dilute acetic acid R* and 1.5 ml of *calcium chloride solution R*. Allow to stand for 30 min. No precipitate is formed.

Sulphates (2.4.13). To 3.0 ml of solution S add 3 ml of *acetic acid R* and dilute to 15 ml with *distilled water R*. The solution complies with the limit test for sulphates (500 ppm). Examine the solutions against the light.

Arsenic (2.4.2). 0.5 g complies with limit test A for arsenic (2 ppm).

Barium. Dilute 10 ml of solution S to 50 ml with *distilled water R* and add 5 ml of *dilute sulphuric acid R*. Allow to stand for 5 min. Any opalescence in the solution is not more intense than that in a mixture of 10 ml of solution S and 45 ml of *distilled water R*.

Ferric ion. In a ground-glass-stoppered flask, dissolve 5.00 g in a mixture of 10 ml of *hydrochloric acid R* and 100 ml of *carbon dioxide-free water R*. Add 3 g of *potassium iodide R*, close the flask and allow to stand protected from light for 5 min. Titrate with 0.1 M *sodium thiosulphate*, using 0.5 ml of *starch solution R*, added towards the end of the titration, as indicator. Carry out a blank titration. Not more than 9.0 ml of 0.1 M *sodium thiosulphate* is used in the test (1.0 per cent).

Heavy metals (2.4.8). Thoroughly mix 2.5 g with 0.5 g of *magnesium oxide R1* in a silica crucible. Ignite to dull redness until a homogeneous mass is obtained. Heat at 800 °C for about 1 h, allow to cool and take up the residue in 20 ml of hot *hydrochloric acid R*. Allow to cool. Transfer the liquid to a separating funnel and shake for 3 min with three quantities, each of 20 ml, of methyl isobutyl ketone saturated with hydrochloric acid (prepared by shaking 100 ml of freshly distilled *methyl isobutyl ketone R* with 1 ml of *hydrochloric acid R*). Allow to stand, separate the aqueous layer, reduce to half its volume by boiling, allow to cool and dilute to 25 ml with *water R*. Neutralise 10 ml of this solution to *red litmus paper R* using *dilute ammonia R1* and dilute to 20 ml with *water R*. 12 ml of the solution complies with limit test A for heavy metals (20 ppm). Prepare the standard using *lead standard solution (1 ppm Pb) R*.

Loss on drying (2.2.32): 5.0 per cent to 10.5 per cent, determined on 0.500 g by drying in an oven at 100 °C to 105 °C for 5 h.

Microbial contamination. Total viable aerobic count (2.6.12) not more than 10³ micro-organisms per gram, determined by plate count.

ASSAY

Dissolve 0.5 g of *sodium hydrogen carbonate R* in a mixture of 30 ml of *dilute sulphuric acid R* and 70 ml of *water R*. When the effervescence stops, dissolve in the solution 1.00 g of the substance to be examined with gentle shaking. Using 0.1 ml of *ferroin R* as indicator, titrate with 0.1 M *ammonium and cerium nitrate* until the red colour disappears.

1 ml of 0.1 M *ammonium and cerium nitrate* is equivalent to 5.585 mg of iron(II).

STORAGE

Store protected from light.

FERROUS SULPHATE HEPTAHYDRATE

Ferrosi sulfas heptahydricus

FeSO₄·7H₂O

M_r 278.0

DEFINITION

Content: 98.0 per cent to 105.0 per cent.

CHARACTERS

Appearance: light green, crystalline powder or bluish-green crystals, efflorescent in air.

Solubility: freely soluble in water, very soluble in boiling water, practically insoluble in alcohol.

Ferrous sulphate is oxidised in moist air, becoming brown.

IDENTIFICATION

- A. It gives the reaction of sulphates (2.3.1).
- B. It gives reaction (a) of iron (2.3.1).
- C. It complies with the limits of the assay.

TESTS

Solution S. Dissolve 2.5 g in *carbon dioxide-free water R*, add 0.5 ml of *dilute sulphuric acid R* and dilute to 50 ml with *water R*.

Appearance of solution. Solution S is not more opalescent than reference suspension II (2.2.1).

pH (2.2.3): 3.0 to 4.0.

Dissolve 0.5 g in *carbon dioxide-free water R* and dilute to 10 ml with the same solvent.

Chlorides (2.4.4): maximum 300 ppm.

Dilute 3.3 ml of solution S to 10 ml with *water R* and add 5 ml of *dilute nitric acid R*. The solution complies with the limit test for chlorides. Prepare the standard using a mixture of 10 ml of *chloride standard solution (5 ppm Cl) R* and 5 ml of *dilute nitric acid R*. Use 0.15 ml of *silver nitrate solution R2* in this test.

Ferric ions: maximum 0.5 per cent.

In a ground-glass-stoppered flask, dissolve 5.00 g in a mixture of 10 ml of *hydrochloric acid R* and 100 ml of *carbon dioxide-free water R*. Add 3 g of *potassium iodide R*, close the flask and allow to stand in the dark for 5 min. Titrate the liberated iodine with 0.1 M *sodium thiosulphate*, using 0.5 ml of *starch solution R*, added towards the end of the titration, as indicator. Carry out a blank test in the same conditions. Not more than 4.5 ml of 0.1 M *sodium thiosulphate* is used, taking into account the blank titration.

Manganese: maximum 0.1 per cent.

Dissolve 1.0 g in 40 ml of *water R*, add 10 ml of *nitric acid R* and boil until red fumes are evolved. Add 0.5 g of *ammonium persulphate R* and boil for 10 min. Discharge any pink colour by adding dropwise a 50 g/l solution of *sodium sulphite R* and boil until any odour of sulphur dioxide is eliminated. Add 10 ml of *water R*, 5 ml of *phosphoric acid R* and 0.5 g of *sodium periodate R*, boil for 1 min and allow to cool. The solution is not more intensely coloured than a standard prepared at the same time in the same manner using 1.0 ml of 0.02 M *potassium permanganate* and adding the same volumes of the same reagents.

Zinc: maximum 500 ppm.

To 5 ml of solution A obtained in the test for heavy metals add 1 ml of *potassium ferrocyanide solution R* and dilute

to 13 ml with *water R*. After 5 min, any turbidity produced is not more intense than that of a standard prepared at the same time by mixing 10 ml of *zinc standard solution (10 ppm Zn) R*, 2 ml of *hydrochloric acid R1* and 1 ml of *potassium ferrocyanide solution R*.

Heavy metals (2.4.8): maximum 50 ppm.

Dissolve 1.0 g in 10 ml of *hydrochloric acid R1*, add 2 ml of *strong hydrogen peroxide solution R* and boil until the volume is reduced to 5 ml. Allow to cool, dilute to 20 ml with *hydrochloric acid R1*, transfer the solution to a separating funnel and shake for 3 min with 3 quantities, each of 20 ml, of methyl isobutyl ketone saturated with hydrochloric acid (prepared by shaking 100 ml of freshly distilled *methyl isobutyl ketone R* with 1 ml of *hydrochloric acid R1*). Allow to stand, separate the aqueous layer and reduce to half its volume by boiling, allow to cool and dilute to 25 ml with *water R* (solution A). Neutralise 10 ml of solution A to *litmus paper R* using *dilute ammonia R1* and dilute to 20 ml with *water R*. 12 ml of the solution complies with limit test A. Prepare the standard using *lead standard solution (1 ppm Pb) R*.

ASSAY

Dissolve 2.5 g of *sodium hydrogen carbonate R* in a mixture of 150 ml of *water R* and 10 ml of *sulphuric acid R*. When the effervescence ceases add to the solution 0.500 g of the substance to be examined and dissolve with gentle shaking. Add 0.1 ml of *ferroin R* and titrate with 0.1 M *ammonium and cerium nitrate* until the red colour disappears.

1 ml of 0.1 M *ammonium and cerium nitrate* is equivalent to 27.80 mg of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$.

STORAGE

In an airtight container.

01/2005:1516

FEVERFEW

Tanacetum parthenii herba

DEFINITION

Feverfew consists of the dried, whole or fragmented aerial parts of *Tanacetum parthenium* (L.) Schultz Bip. It contains not less than 0.20 per cent of parthenolide ($\text{C}_{15}\text{H}_{20}\text{O}_3$; M_r 248.3), calculated with reference to the dried drug.

CHARACTERS

Fewerfew has a camphoraceous odour.

It has the macroscopic and microscopic characters described under identification tests A and B.

IDENTIFICATION

A. The leafy, more or less branched stem has a diameter of up to 5 mm; it is almost quadrangular, channelled longitudinally and slightly pubescent. The leaves are ovate, 2 cm to 5 cm long, sometimes up to 10 cm, yellowish-green, petiolate and alternate. They are pinnate or bipinnate, deeply divided into five to nine segments, each with a coarsely crenate margin and an obtuse apex. Both surfaces are somewhat pubescent and the midrib is prominent on the lower surface. When present, the flowering heads are 12 mm to 22 mm in diameter with long pedicels; they are clustered into broad corymbs consisting of five to thirty flower-heads. The hemispherical involucre is 6 mm to 8 mm wide and consists of many overlapping bracts, which are rather narrow, are obtuse and scarious and have membranous

margins. The central flowers are yellow, hermaphrodite, tube-shaped with five teeth and have five stamens inserted in the corolla; the filaments of the stamens are separate from each other but the anthers are fused into a tube through which passes the style, bearing two stigmatic branches. The peripheral flowers are female and have a white three-toothed ligule, 2 mm to 7 mm long. The fruit is an achene, 1.2 mm to 1.5 mm long, brown when ripe, with five to ten white longitudinal ribs. It is glandular and bears a short, crenate, membranous crown.

B. Reduce to a powder (355). The powder is yellowish-green. Examine under a microscope using *chloral hydrate solution R*. The powder shows numerous large, multicellular, uniseriate covering trichomes consisting of a rhomboidal basal cell, three to five smaller, thick-walled rectangular cells and a very long, flat, slender terminal cell, often curved at a right angle to the axis of the basal cell; glandular trichomes with a short, biseriate, two to four celled stalk and a biseriate head of four cells around which the cuticle forms a bladder-like covering; epidermal cells with a very sinuous anticlinal walls, a striated cuticle and anomocytic stomata; numerous spirally and annularly thickened vessels; stratified parenchyma and collenchyma. Fragments of disc florets containing pale yellow amorphous masses and small rosette crystals of calcium oxalate may be present; spherical pollen grains about 25 µm in diameter, with three pores and a spiny exine may be present.

C. Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel plate R*.

Test solution. To 1 g of the powdered drug (355) add 20 ml of *methanol R*. Heat in a water-bath at 60 °C for 15 min. Allow to cool and filter. Evaporate to dryness under reduced pressure and dissolve the residue in 2 ml of *methanol R*.

Reference solution. Dissolve 5 mg of *parthenolide R* in *methanol R* and dilute to 5 ml with the same solvent.

Apply to the plates as bands 20 µl of each solution. Develop over a path of 10 cm using a mixture of 15 volumes of *acetone R* and 85 volumes of *toluene R*. Allow the plate to dry in air. Spray with a 5 g/l solution of *vanillin R* in a mixture of 20 volumes of *ethanol R* and 80 volumes of *sulphuric acid R*. After 5 min examine in daylight. The chromatogram obtained with the test solution shows in its central part a blue principal zone that is similar in position, colour and size to the principal zone in the chromatogram obtained with the reference solution, and somewhat below the principal zone it may show a second blue zone. It also shows, one or two blue zones in its lower third. Other violet zones may be present.

TESTS

Foreign matter (2.8.2). Not more than 10.0 per cent of stem having a diameter exceeding 5 mm, and not more than 2.0 per cent of other foreign matter.

Loss on drying (2.2.32). Not more than 10.0 per cent, determined on 1.000 g of the powdered drug (355) by drying in an oven at 100 °C to 105 °C for 2 h.

Total ash (2.4.16). Not more than 12.0 per cent.

ASSAY

Examine by liquid chromatography (2.2.29).

Test solution. Completely reduce about 50 g to a powder (355). After homogenisation, introduce 1.00 g of the powdered drug into a flask and add 40 ml of *methanol R*. Heat in a water-bath at 60 °C for 10 min. Allow to cool

and filter. Rinse the filter with 15 ml of *methanol R*. Take up the residue with 40 ml of *methanol R*. Repeat the operation. Collect the filtrates and rinsings and evaporate to dryness under reduced pressure. Take up the residue with *methanol R* and dilute to 20.0 ml with the same solvent. Dilute 10.0 ml of this solution to 50.0 ml with the mobile phase. Filter (0.45 µm).

Reference solution. Dissolve 5.0 mg of *parthenolide R* in *methanol R* and dilute to 10.0 ml with the same solvent. Dilute 2.0 ml of the solution to 50.0 ml with the mobile phase.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4.6 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (5 µm),
- as mobile phase at a flow rate of 1 ml/min a mixture of 40 volumes of *acetonitrile R* and 60 volumes of *water R*,
- as detector a spectrophotometer set at 220 nm,

Inject 20 µl of each solution. The retention time of parthenolide is about 11.5 min.

Calculate the percentage content of parthenolide from the expression:

$$\frac{A_1 \times m_2 \times 40}{A_2 \times m_1}$$

A_1 = area corresponding to the principal compound in the chromatogram obtained with the test solution,

A_2 = area corresponding to the principal compound in the chromatogram obtained with the reference solution,

m_1 = mass of the drug used in the test solution, in grams,

m_2 = mass of parthenolide in the reference solution, in grams.

STORAGE

Store protected from light.

01/2005:0903
corrected

FIBRIN SEALANT KIT

Fibrini glutinum

DEFINITION

Fibrin sealant kit is essentially composed of 2 components, namely fibrinogen concentrate (component 1), a protein fraction containing human fibrinogen and a preparation containing human thrombin (component 2). A fibrin clot is rapidly formed when the 2 thawed or reconstituted components are mixed. Other ingredients (for example, human coagulation factor XIII, a fibrinolysis inhibitor or calcium ions) and stabilisers (for example, *Human albumin solution (0255)*) may be added. No antimicrobial preservative is added.

Human constituents are obtained from plasma that complies with the requirements of the monograph on *Human plasma for fractionation (0853)*. No antibiotic is added to the plasma used.

When thawed or reconstituted as stated on the label, component 1 contains not less than 40 g/l of clottable protein; the thrombin activity of component 2 varies over a wide range (approximately 4-1000 IU/ml).

PRODUCTION

The method of preparation includes a step or steps that have been shown to remove or to inactivate known agents of infection; if substances are used for inactivation of viruses during production, the subsequent purification procedure must be validated to demonstrate that the concentration of these substances is reduced to a suitable level and any residues are such as not to compromise the safety of the preparation for patients.

Constituents or mixtures of constituents are passed through a bacteria-retentive filter and distributed aseptically into sterile containers. Containers of freeze-dried constituents are closed under vacuum or filled with oxygen-free nitrogen or other suitable inert gas before being closed. In either case, they are closed so as to exclude micro-organisms.

If the human coagulation factor XIII content in component 1 is greater than 10 units/ml, the assay of factor XIII is carried out.

CHARACTERS

Freeze-dried constituents are hygroscopic, white or pale yellow powders or friable solids. Frozen constituents are colourless or pale yellow, opaque solids. Liquid constituents are colourless or pale yellow.

For the freeze-dried or frozen constituents, reconstitute or thaw as stated on the label immediately before carrying out the identification and the tests, except those for solubility and water.

Component 1 (fibrinogen concentrate)

IDENTIFICATION

- A. It complies with the limits of the assay of fibrinogen.
- B. It complies with the limits of the assay of factor XIII (where applicable).

TESTS

pH (2.2.3): 6.5 to 8.0.

Solubility. Freeze-dried concentrates dissolve within 20 min in the volume of solvent for reconstitution and at the temperature stated on the label, forming an almost colourless, clear or slightly turbid solution.

Stability of solution. No gel formation appears at room temperature during 120 min following thawing or reconstitution.

Water. Determined by a suitable method, such as the semi-microdetermination (2.5.12), loss on drying (2.2.32) or near infrared spectrophotometry (2.2.40), the water content is within the limits approved by the competent authority.

Sterility (2.6.1). It complies with the test for sterility.

ASSAY

Fibrinogen (clottable protein). The estimated content in milligrams of clottable protein is not less than 70 per cent and not more than 130 per cent of the content stated on the label.

Mix 0.2 ml of the reconstituted preparation with 2 ml of a suitable buffer solution (pH 6.6-7.4) containing sufficient *human thrombin R* (approximately 3 IU/ml) and calcium (0.05 mol/l). Maintain at 37 °C for 20 min, separate the precipitate by centrifugation (5000 g, 20 min), wash thoroughly with a 9 g/l solution of *sodium chloride R* and determine the protein as nitrogen by sulphuric acid digestion (2.5.9). Calculate the protein content by multiplying the result by 6.0. If for a particular preparation this method cannot be applied, use another validated method for determination of fibrinogen.

Factor XIII. Where the label indicates that the human coagulation factor XIII activity is greater than 10 units/ml, the estimated activity is not less than 80 per cent and not more than 120 per cent of the activity stated on the label.

Make at least 3 suitable dilutions of thawed or reconstituted component 1 and of human normal plasma (reference preparation) using as diluent coagulation factor XIII deficient plasma or another suitable diluent. Add to each dilution suitable amounts of the following reagents:

- activator reagent, containing bovine or human thrombin, a suitable buffer, calcium chloride and a suitable inhibitor such as Gly-Pro-Arg-Pro-Ala-NH₂ which inhibits clotting of the sample but does not prevent coagulation factor XIII activation by thrombin,
- detection reagent, containing a suitable factor XIIIa-specific peptide substrate, such as Leu-Gly-Pro-Gly-Glu-Ser-Lys-Val-Ile-Gly-NH₂ and glycine ethyl ester as 2nd substrate in a suitable buffer solution,
- NADH reagent, containing glutamate dehydrogenase, α-ketoglutarate and NADH in a suitable buffer solution.

After mixing, the absorbance changes ($\Delta A/\text{min}$) are measured at a wavelength of 340 nm, after the linear phase of the reaction is reached.

1 unit of factor XIII is equal to the activity of 1 ml of human normal plasma.

Calculate the activity of the test preparation by the usual statistical methods (5.3, for example). The confidence limits ($P = 0.95$) are not less than 80 per cent and not more than 125 per cent of the estimated activity.

Component 2 (thrombin preparation)

IDENTIFICATION

It complies with the limits of the assay of thrombin.

TESTS

pH (2.2.3): 5.0 to 8.0.

Solubility. Freeze-dried preparations dissolve within 5 min in the volume of solvent for reconstitution stated on the label, forming a colourless, clear or slightly turbid solution.

Water. Determined by a suitable method, such as the semi-microdetermination (2.5.12), loss on drying (2.2.32) or near infrared spectrophotometry (2.2.40), the water content is within the limits approved by the competent authority.

Sterility (2.6.1). It complies with the test for sterility.

ASSAY

Thrombin. The estimated activity is not less than 80 per cent and not more than 125 per cent of the activity stated on the label.

If necessary, dilute the reconstituted preparation to be examined to approximately 2·20 IU of thrombin per millilitre using as diluent a suitable buffer pH 7.3–7.5, such as *imidazole buffer solution pH 7.3 R* containing 10 g/l of *human albumin R* or *bovine albumin R*. To a suitable volume of the dilution, add a suitable volume of fibrinogen solution (1 g/l of clottable protein) warmed to 37 °C and start measurement of the clotting time immediately. Repeat the procedure with each of at least 3 dilutions, in the range stated above, of a reference preparation of thrombin, calibrated in International Units. Calculate the activity of the test preparation by the usual statistical methods (5.3, for example). The confidence limits ($P = 0.95$) are not less than 80 per cent and not more than 125 per cent of the estimated activity.

STORAGE

Protected from light and, for freeze-dried components, in an airtight container.

LABELLING

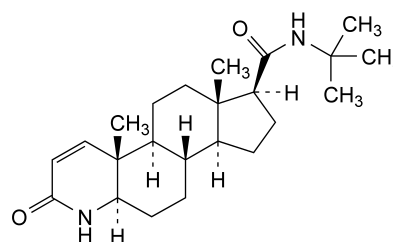
The label states:

- the amount of fibrinogen (milligrams of clottable protein), thrombin (International Units) per container, and coagulation factor XIII, if this is greater than 10 units/ml,
- where applicable, the name and volume of solvent to be used to reconstitute the components.

01/2005:1615

FINASTERIDE

Finasteridum



C₂₃H₃₆N₂O₂

M_r 372.6

DEFINITION

N-(1,1-Dimethylethyl)-3-oxo-4-aza-5α-androst-1-ene-17β-carboxamide.

Content: 98.0 per cent to 102.0 per cent (dried substance).

CHARACTERS

Appearance: white or almost white, crystalline powder.

Solubility: practically insoluble in water, freely soluble in ethanol and in methylene chloride.

It shows polymorphism.

IDENTIFICATION

Infrared absorption spectrophotometry (2.2.24).

Comparison: *finasteride CRS*.

If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in *methanol R*, evaporate to dryness and record new spectra using the residues.

TESTS

Specific optical rotation (2.2.7): + 12.0 to + 14.0 (dried substance).

Dissolve 0.250 g in *methanol R* and dilute to 25.0 ml with the same solvent.

Related substances. Liquid chromatography (2.2.29).

Test solution (a). Dissolve 25.0 mg of the substance to be examined in a mixture of equal volumes of *acetonitrile R* and *water R* and dilute to 50.0 ml with the same mixture of solvents.

Test solution (b). Dissolve 0.100 g of the substance to be examined in a mixture of equal volumes of *acetonitrile R* and *water R* and dilute to 10.0 ml with the same mixture of solvents.

Reference solution (a). Dissolve 25.0 mg of *finasteride CRS* in a mixture of equal volumes of *acetonitrile R* and *water R* and dilute to 50.0 ml with the same mixture of solvents.

Reference solution (b). Dissolve 50.0 mg of *finasteride* for *system suitability CRS* in a mixture of equal volumes of *acetonitrile R* and *water R* and dilute to 5.0 ml with the same mixture of solvents.

Reference solution (c). Dilute 2.0 ml of test solution (b) to 100.0 ml in a mixture of equal volumes of *acetonitrile R* and *water R*. Dilute 1.0 ml of this solution to 10.0 ml with a mixture of equal volumes of *acetonitrile R* and *water R*.

Column:

- **size:** $l = 0.25$ m, $\varnothing = 4.0$ mm,
 - **stationary phase:** *end-capped octadecylsilyl silica gel for chromatography R* (5 μ m) with a ratio of specific surface area (m^2g^{-1})/carbon-percentage less than 20,
 - **temperature:** 60 °C.
- Mobile phase:** *acetonitrile R*, *tetrahydrofuran R*, *water R* (10:10:80 V/V/V).

Flow rate: 1.5 ml/min.

Detection: spectrophotometer at 210 nm.

Injection: 15 μ l; inject test solution (b) and reference solutions (b) and (c).

Run time: twice the retention time of *finasteride*.

Relative retention with reference to *finasteride* (retention time = about 28 min): impurity A = about 0.94; impurity B = about 1.22; impurity C = about 1.36.

System suitability: reference solution (b):

- **peak-to-valley ratio:** minimum 2.5, where H_p = height above the baseline of the peak due to impurity A, and H_v = height above the baseline of the lowest point of the curve separating this peak from the peak due to *finasteride*.
- Limits:**
- **impurity A:** maximum 0.3 per cent, calculated from the area of the corresponding peak in the chromatogram obtained with reference solution (b) and taking into account the assigned value of impurity A in *finasteride* for *system suitability CRS*,
 - **impurity B:** not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.3 per cent),
 - **impurity C:** not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.3 per cent),
 - **any other impurity:** not more than half the area of the principal peak in the chromatogram obtained with reference solution (c) (0.1 per cent),
 - **total:** not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.6 per cent),
 - **disregard limit:** 0.25 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.05 per cent).

Loss on drying (2.2.32): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 100–105 °C.

ASSAY

Liquid chromatography (2.2.29) as described in the test for related substances.

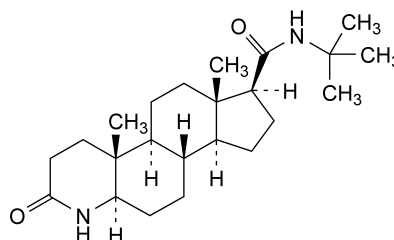
Injection: test solution (a) and reference solution (a).

Calculate the percentage content of $\text{C}_{23}\text{H}_{36}\text{N}_2\text{O}_2$.

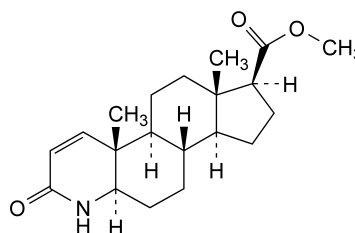
STORAGE

Protected from light.

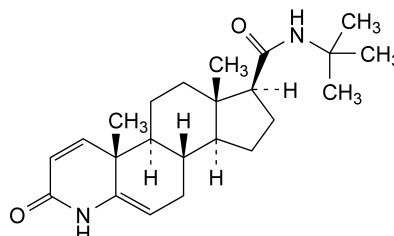
IMPURITIES



A. *N*-(1,1-dimethylethyl)-3-oxo-4-aza-5 α -androstane-17 β -carboxamide (dihydrofinasteride),



B. methyl 3-oxo-4-aza-5 α -androst-1-ene-17 β -carboxylate (Δ -1-aza ester),



C. *N*-(1,1-dimethylethyl)-3-oxo-4-azaandrosta-1,5-diene-17 β -carboxamide (Δ -1,5-aza amide).

01/2005:1912
corrected

FISH OIL, RICH IN OMEGA-3-ACIDS

Piscis oleum omega-3 acidis abundans

DEFINITION

Purified, winterised and deodorised fatty oil obtained from fish of the families *Engraulidae*, *Carangidae*, *Clupeidae*, *Osmeridae*, *Scombridae* and *Ammodytidae*. The omega-3 acids are defined as the following acids: *alpha*-linolenic acid (C18:3 n-3), moroctic acid (C18:4 n-3), eicosatetraenoic acid (C20:4 n-3), timnodonic (eicosapentaenoic) acid (C20:5 n-3; EPA), heneicosapentaenoic acid (C21:5 n-3), clupanodonic acid (C22:5 n-3) and cervonic (docosahexaenoic) acid (C22:6 n-3; DHA).

Content:

- EPA, expressed as triglycerides: minimum 13.0 per cent,
- DHA, expressed as triglycerides: minimum 9.0 per cent,
- total omega-3-acids, expressed as triglycerides: minimum 28.0 per cent.

Authorised antioxidants in concentrations not exceeding the levels specified by the competent authorities may be added.

CHARACTERS

Appearance: pale yellow liquid.

Solubility: practically insoluble in water, very soluble in acetone and in heptane, slightly soluble in ethanol.

IDENTIFICATION

Examine the chromatograms obtained in the assay for EPA and DHA.

Results: the peaks due to eicosapentaenoic acid methyl ester and to docosahexaenoic acid methyl ester in the chromatogram obtained with test solution (b) are similar in retention time to the corresponding peaks in the chromatogram obtained with reference solution (a).

TESTS

Appearance. The substance to be examined is not more intensely coloured than a reference solution prepared as follows: to 3.0 ml of red primary solution add 25.0 ml of yellow primary solution and dilute to 50.0 ml with a 10 g/l solution of *hydrochloric acid R* (2.2.2, *Method II*).

Absorbance (2.2.25): maximum 0.70 at 233 nm.

Dilute 0.300 g of the substance to be examined to 50.0 ml with *trimethylpentane R*. Dilute 2.0 ml of this solution to 50.0 ml with *trimethylpentane R*.

Acid value (2.5.1): maximum 0.5, determined on 20.0 g in 50 ml of the prescribed mixture of solvents.

Anisidine value: maximum 30.0.

The anisidine value is defined as 100 times the absorbance measured in a 1 cm cell filled with a solution containing 1 g of the substance to be examined in 100 ml of a mixture of solvents and reagents according to the method described below.

Carry out the operations as rapidly as possible, avoiding exposure to actinic light.

Test solution (a). Dilute 0.500 g of the substance to be examined to 25.0 ml with *trimethylpentane R*.

Test solution (b). To 5.0 ml of test solution (a) add 1.0 ml of a 2.5 g/l solution of *p-anisidine R* in *glacial acetic acid R*, shake and store protected from light.

Reference solution. To 5.0 ml of *trimethylpentane R* add 1.0 ml of a 2.5 g/l solution of *p-anisidine R* in *glacial acetic acid R*, shake and store protected from light.

Measure the absorbance (2.2.25) of test solution (a) at 350 nm using *trimethylpentane R* as the compensation liquid. Measure the absorbance of test solution (b) at 350 nm exactly 10 min after its preparation, using the reference solution as the compensation liquid.

Calculate the anisidine value from the expression:

$$\frac{25 \times (1.2 A_s - A_b)}{m}$$

A_s = absorbance of test solution (b),

A_b = absorbance of test solution (a),

m = mass of the substance to be examined in test solution (a), in grams.

Peroxide value (2.5.5, *Method A*): maximum 10.0.

Unsaponifiable matter (2.5.7): maximum 1.5 per cent, determined on 5.0 g.

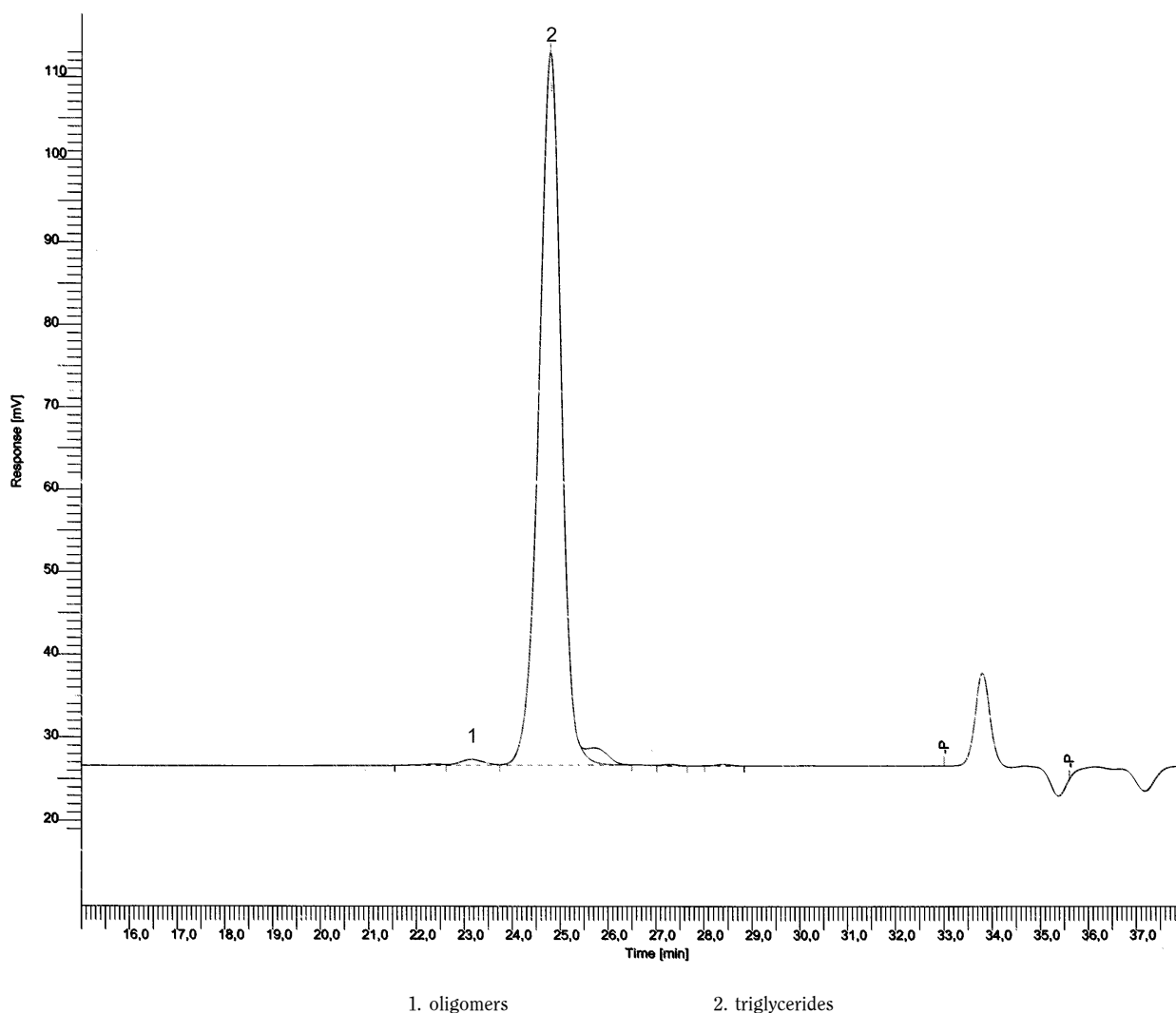


Figure 1912.-1. – Chromatogram of the test for oligomers in fish oil rich in omega-3 acids

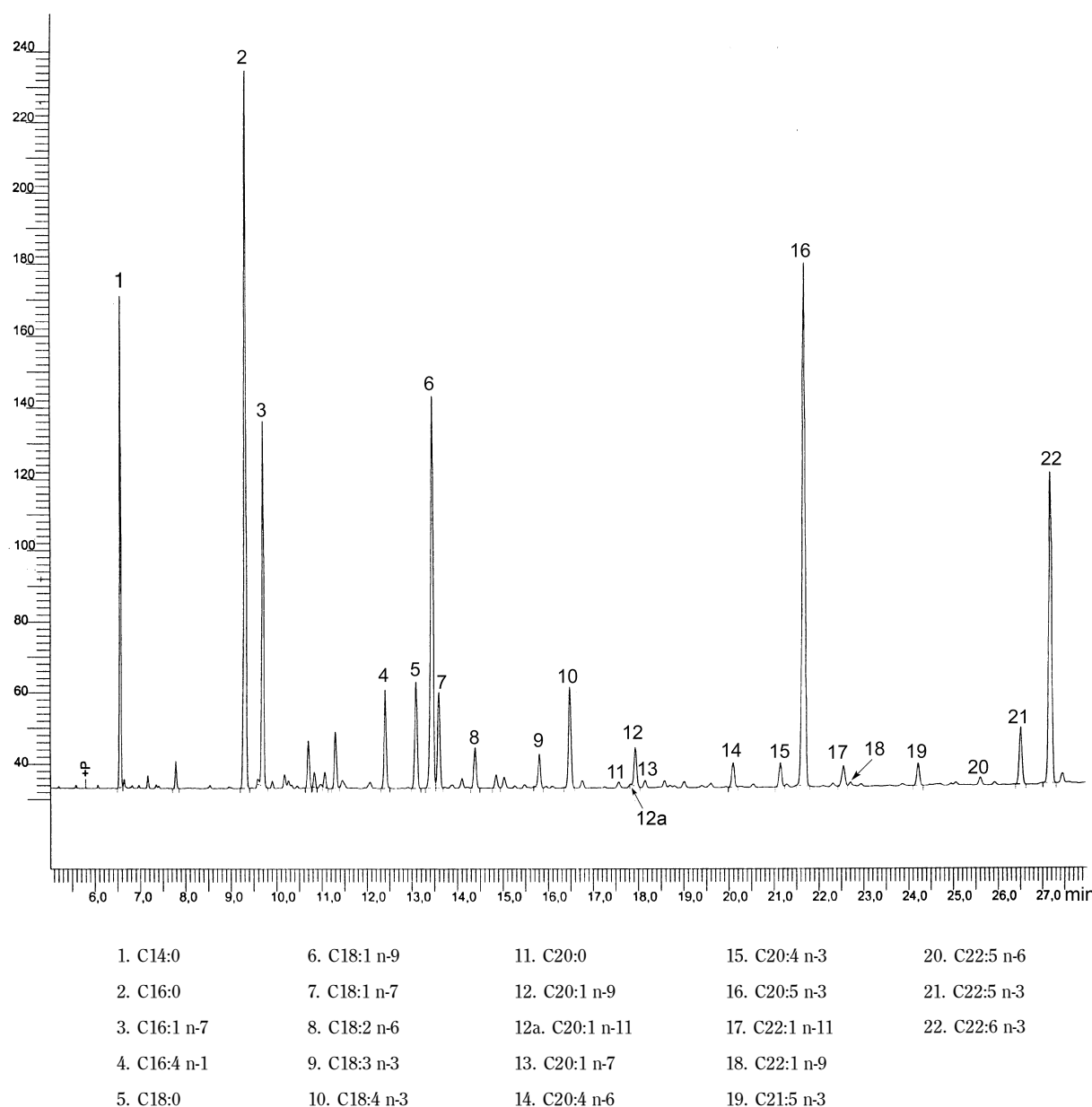


Figure 1912.-2. – Chromatogram for the assay of total omega-3 acids in fish oil rich in omega-3 acids

Stearin. 100 ml remains clear after cooling at 0 °C for 3 h.

Oligomers. Size-exclusion chromatography (2.2.30).

Test solution. Dilute 10.0 mg of the substance to be examined to 10.0 ml with *tetrahydrofuran R*.

Reference solution. In a 100 ml volumetric flask dissolve 50 mg of *monodocosahexaenoin R*, 30 mg of *didocosahexaenoin R* and 20 mg of *tridocosahexaenoin R* in *tetrahydrofuran R* and dilute to 100.0 ml with the same solvent.

Column 1:

- size: $l = 0.3$ m, $\varnothing = 7.8$ mm,
- stationary phase: *styrene-divinylbenzene copolymer R* (7 μ m) with a pore size of 10 nm.

Columns 2 and 3, placed closest to the injector:

- size: $l = 0.3$ m, $\varnothing = 7.8$ mm,
- stationary phase: *styrene-divinylbenzene copolymer R* (7 μ m) with a pore size of 50 nm.

Mobile phase: *tetrahydrofuran R*.

Flow rate: 0.8 ml/min.

Detection: differential refractometer.

Injection: 40 μ l.

System suitability: reference solution:

- **elution order:** tridocosahexaenoin, didocosahexaenoin, monodocosahexaenoin.
- **resolution:** minimum of 2.0 between the peaks due to monodocosahexaenoin and to didocosahexaenoin and minimum of 1.0 between the peaks due to didocosahexaenoin and to tridocosahexaenoin.

Identify the peaks from the chromatogram (Figure 1912.-1). Calculate the percentage content of oligomers using the following expression:

$$\frac{B}{A} \times 100$$

A = sum of the areas of all the peaks in the chromatogram,

B = area of the peak with a retention time less than the retention time of the triglyceride peak.

Limit:

- **oligomers:** maximum 1.5 per cent.

ASSAY

EPA and DHA (2.4.29). See Figure 1912.-2.

Total omega-3-acids (2.4.29). See Figure 1912.-2.

STORAGE

In an airtight, well-filled container, protected from light, under inert gas.

LABELLING

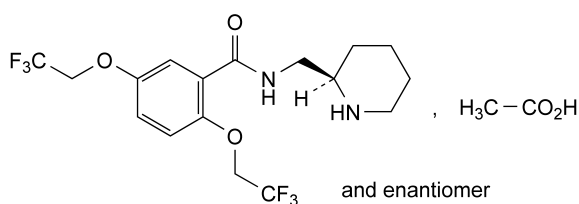
The label states:

- the concentration of EPA, DHA and total omega-3-acids, expressed as triglycerides,
- the name and the concentration of any added antioxidant.

01/2005:1324

FLECAINIDE ACETATE

Flecainidi acetat

C₁₉H₂₄F₆N₂O₅M_r 474.4

DEFINITION

Flecainide acetate contains not less than 98.0 per cent and not more than the equivalent of 101.0 per cent of *N*[(*RS*)-(piperidin-2-ylmethyl)]-2,5-bis(2,2,2-trifluoroethoxy)benzamide acetate, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, crystalline powder, very hygroscopic, soluble in water and in ethanol. It is freely soluble in dilute acetic acid and practically insoluble in dilute hydrochloric acid.

IDENTIFICATION

First identification: A, C.

Second identification: A, B, D.

- Melting point (2.2.14): 146 °C to 152 °C. The melting range is not greater than 3 °C.
- Dissolve 50 mg in *alcohol R* and dilute to 50.0 ml with the same solvent. Dilute 5.0 ml of the solution to 50.0 ml with the same solvent. Examined between 230 nm and 350 nm (2.2.25), the solution shows an absorption maximum at 298 nm. The specific absorbance at the maximum is 61 to 65.
- Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *flecainide acetate CRS*.
- It gives reaction (b) of acetates (2.3.1).

TESTS

Appearance of solution. Dissolve 0.25 g in *water R*, add 0.05 ml of *glacial acetic acid R* and dilute to 10 ml with *water R*. The solution is clear (2.2.1) and colourless (2.2.2, *Method II*).

pH (2.2.3). Dissolve 0.25 g in *carbon dioxide-free water R* and dilute to 10 ml with the same solvent. The pH of the solution is 6.7 to 7.1.

Related substances. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 0.25 g of the substance to be examined in *methanol R* and dilute to 25.0 ml with the same solvent.

Reference solution (a). Dilute 5.0 ml of the test solution to 100.0 ml with *methanol R*. Dilute 1.0 ml of the solution to 10.0 ml with the same solvent.

Reference solution (b). Dissolve 25 mg of *flecainide acetate CRS* and 25 mg of *flecainide impurity A CRS* in *methanol R* and dilute to 25.0 ml with the same solvent.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.15 m long and 4.6 mm in internal diameter packed with *octylsilyl silica gel for chromatography R* (5 µm),
- as mobile phase at a flow rate of 2 ml/min:

Mobile phase A. Mix 2 ml of *concentrated ammonia R*, 4 ml of *triethylamine R* and 985 ml of *water R*. Add 6 ml of *phosphoric acid R* and adjust to pH 2.8 with *concentrated ammonia R*,

Mobile phase B. *Acetonitrile R*,

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)	Comment
	90	10	equilibration
0 - 12	90 → 30	10 → 70	linear gradient
12 - 17	30	70	isocratic
17 - 19	30 → 90	70 → 10	linear gradient
19 - 21	90	10	re-equilibration

- as detector a spectrophotometer set at 300 nm,
- a loop injector.

If a suitable baseline cannot be obtained, use another grade of triethylamine. Inject 20 µl of reference solution (b). The test is not valid unless the resolution between the two peaks in the chromatogram obtained is at least four. Inject 20 µl of the test solution and 20 µl of reference solution (a).

In the chromatogram obtained with the test solution, the area of any peak apart from the principal peak is not greater than 0.4 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent) and the sum of the areas of all the peaks apart from the principal peak is not greater than the area of the peak in the chromatogram obtained with reference solution (a) (0.5 per cent). Disregard any peak with an area less than 0.02 times that of the principal peak in the chromatogram obtained with reference solution (a).

Impurity B. Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel F₂₅₄ plate R*.

Test solution. Dissolve 0.10 g of the substance to be examined in *methanol R* and dilute to 2 ml with the same solvent.

Reference solution. Dissolve 10 mg of *flecainide impurity B CRS* in *methanol R* and dilute to 100 ml with the same solvent (solution A). Dissolve 0.10 g of *flecainide acetate CRS* in solution A and dilute to 2 ml with the same solution.

Apply separately to the plate 5 µl of each solution. Develop over a path of 10 cm using a freshly prepared mixture of 5 volumes of *concentrated ammonia R* and 95 volumes of *acetone R*. Dry the plate at 100 °C to 105 °C until the ammonia has evaporated and examine in ultraviolet light at 254 nm to establish the position of the flecainide spot. Then spray with a freshly prepared 2 g/l solution of *ninhydrin R* in *methanol R* and heat at 100 °C to 110 °C for 2 min

to 5 min and examine in daylight. In the chromatogram obtained with the test solution, any spot corresponding to impurity B is not more intense than the spot corresponding to impurity B in the chromatogram obtained with the reference solution (0.2 per cent). The test is not valid unless the chromatogram obtained with the reference solution shows two clearly separated spots.

Heavy metals (2.4.8). 1.0 g complies with limit test C for heavy metals (20 ppm). Prepare the standard using 2 ml of *lead standard solution* (10 ppm Pb) R.

Loss on drying (2.2.32). Not more than 0.5 per cent determined on 1.000 g by drying in an oven at 60 °C under a pressure not exceeding 0.6 kPa for 2 h.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g in a platinum crucible.

ASSAY

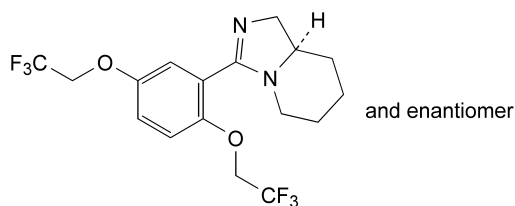
Dissolve 0.400 g in 25 ml of *anhydrous acetic acid* R. Titrate with 0.1 M *perchloric acid*, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *perchloric acid* is equivalent to 47.44 mg of $C_{19}H_{24}F_6N_2O_5$.

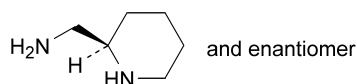
STORAGE

Store protected from light.

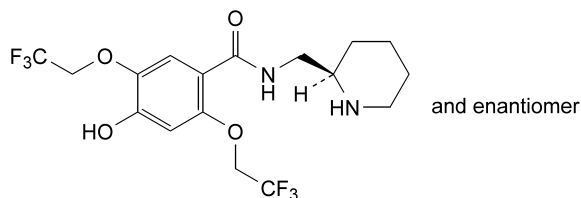
IMPURITIES



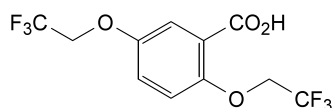
- A. (8aRS)-3-[2,5-bis(2,2,2-trifluoroethoxy)phenyl]-1,5,6,7,8,8a-hexahydroimidazo[1,5-a]pyridine,



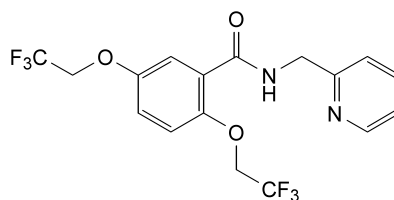
- B. (RS)-(piperidin-2-yl)methanamine,



- C. (RS)-4-hydroxy-N-(piperidin-2-ylmethyl)-2,5-bis(2,2,2-trifluoroethoxy)benzamide,



- D. 2,5-bis(2,2,2-trifluoroethoxy)benzoic acid,

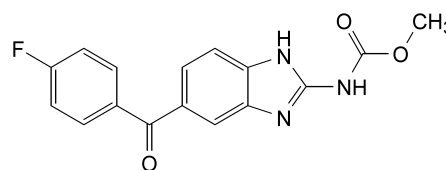


- E. N-(pyridin-2-ylmethyl)-2,5-bis(2,2,2-trifluoroethoxy)benzamide.

01/2005:1721

FLUBENDAZOLE

Flubendazolum



$C_{16}H_{12}FN_3O_3$

M_r 313.3

DEFINITION

Methyl [5-(4-fluorobenzoyl)-1H-benzimidazol-2-yl]carbamate

Content: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

Appearance: white or almost white powder.

Solubility: practically insoluble in water, in alcohol and in methylene chloride.

It shows polymorphism.

IDENTIFICATION

Infrared absorption spectrophotometry (2.2.24), without recrystallisation.

Comparison: flubendazole CRS.

TESTS

Related substances. Liquid chromatography (2.2.29).

Test solution. Dissolve 0.100 g of the substance to be examined in *dimethylformamide* R and dilute to 100.0 ml with the same solvent.

Reference solution (a). Dissolve 5 mg of flubendazole for system suitability CRS in *dimethylformamide* R and dilute to 5.0 ml with the same solvent.

Reference solution (b). Dilute 1.0 ml of the test solution to 100.0 ml with *dimethylformamide* R. Dilute 5.0 ml of this solution to 20.0 ml with *dimethylformamide* R.

Column:

- size: $l = 0.10$ m, $\varnothing = 4.6$ mm,
- stationary phase: base-deactivated octadecylsilyl silica gel for chromatography R (3 μ m),
- temperature: 40 °C.

Mobile phase:

- mobile phase A: 7.5 g/l solution of ammonium acetate R,
- mobile phase B: acetonitrile R,

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)
0 - 15	90 → 75	10 → 25
15 - 30	75 → 45	25 → 55
30 - 32	45 → 10	55 → 90
32 - 37	10	90
37 - 38	10 → 90	90 → 10
38 - 42	90	10

Flow rate: 1.2 ml/min.

Detection: spectrophotometer at 250 nm.

Injection: 10 µl.

System suitability: reference solution (a):

- the chromatogram obtained is similar to the chromatogram supplied with *flubendazole* for system suitability CRS.

Limits:

- correction factors: for the calculation of contents, multiply the peak areas of the following impurities by the corresponding correction factor: impurity A = 1.4; impurity C = 1.3; impurity D = 1.3; impurity G = 1.4,
- impurities A, B, C, D, E, G: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.25 per cent),
- impurity F: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent),
- any other impurity with a relative retention between 1.2 and 1.3: not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.25 per cent),
- total: not more than 6 times the area of the principal peak in the chromatogram obtained with reference solution (b) (1.5 per cent),
- disregard limit: 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

Loss on drying (2.2.32): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 100-105 °C, for 4 h.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.250 g in 3 ml of *anhydrous formic acid R* and add 50 ml of a mixture of 1 volume of *anhydrous acetic acid R* and 7 volumes of *methyl ethyl ketone R*. Titrate with 0.1 M *perchloric acid*, determining the end-point potentiometrically (2.2.20).

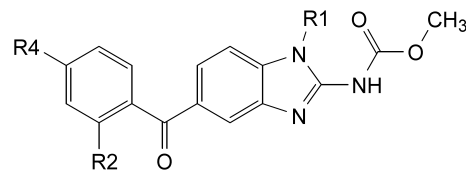
1 ml of 0.1 M *perchloric acid* is equivalent to 31.33 mg of C₁₆H₁₂FN₃O₃.

STORAGE

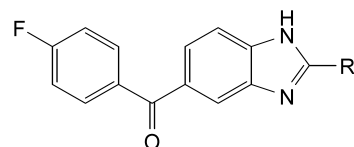
Protected from light.

IMPURITIES

Specified impurities: A, B, C, D, E, F, G.



- A. R1 = R2 = H, R4 = NH-CHO: methyl [5-[4-(formylamino)benzoyl]-1H-benzimidazol-2-yl]carbamate,
 E. R1 = R4 = H, R2 = F: methyl [5-(2-fluorobenzoyl)-1H-benzimidazol-2-yl]carbamate,
 F. R1 = CH₃, R2 = H, R4 = F: methyl [5-(4-fluorobenzoyl)-1-methyl-1H-benzimidazol-2-yl]carbamate,
 G. R1 = R2 = H, R4 = O-CH(CH₃)₂: methyl [5-[4-(1-methylethoxy)benzoyl]-1H-benzimidazol-2-yl]carbamate,

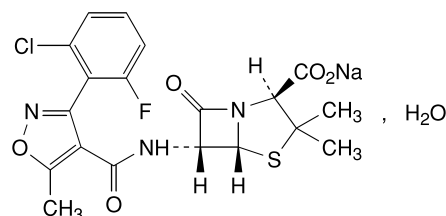


- B. R = NH₂: (2-amino-1H-benzimidazol-5-yl)(4-fluorophenyl)methanone,
 C. R = OH: (4-fluorophenyl)(2-hydroxy-1H-benzimidazol-5-yl)methanone,
 D. R = H: (1H-benzimidazol-5-yl)(4-fluorophenyl)methanone.

01/2005:0668

FLUCLOXACILLIN SODIUM

Flucloxacillinum natricum



C₁₉H₁₆ClFN₃NaO₅S·H₂O

M_r 493.9

DEFINITION

Flucloxacillin sodium contains not less than 95.0 per cent and not more than the equivalent of 101.0 per cent of sodium (2S,5R,6R)-6-[[[3-(2-chloro-6-fluorophenyl)-5-methylisoxazol-4-yl]carbonyl]amino]-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylate, calculated with reference to the anhydrous substance.

CHARACTERS

A white or almost white, crystalline powder, hygroscopic, freely soluble in water and in methanol, soluble in alcohol.

IDENTIFICATION

First identification: A, D.

Second identification: B, C, D.

A. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *flucloxacillin sodium CRS*.

B. Examine by thin-layer chromatography (2.2.27), using *silanised silica gel H R* as the coating substance.

Test solution. Dissolve 25 mg of the substance to be examined in 5 ml of *water R*.

Reference solution (a). Dissolve 25 mg of *flucloxacillin sodium CRS* in 5 ml of *water R*.

Reference solution (b). Dissolve 25 mg of *cloxacillin sodium CRS*, 25 mg of *dicloxacillin sodium CRS* and 25 mg of *flucloxacillin sodium CRS* in 5 ml of *water R*.

Apply separately to the plate 1 µl of each solution. Develop over a path of 15 cm using a mixture of 30 volumes of *acetone R* and 70 volumes of a 154 g/l solution of *ammonium acetate R* adjusted to pH 5.0 with *glacial acetic acid R*. Allow the plate to dry in air and expose it to iodine vapour until the spots appear. Examine in daylight. The principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a). The test is not valid unless the chromatogram obtained with reference solution (b) shows three clearly separated spots.

- C. Place about 2 mg in a test-tube about 150 mm long and 15 mm in diameter. Moisten with 0.05 ml of *water R* and add 2 ml of *sulphuric acid-formaldehyde reagent R*. Mix the contents of the tube by swirling; the colour of the solution is slightly greenish-yellow. Place the test-tube in a water-bath for 1 min; the solution becomes yellow.
- D. It gives reaction (a) of sodium (2.3.1).

TESTS

Solution S. Dissolve 2.50 g in *carbon dioxide-free water R* and dilute to 25.0 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1). The absorbance (2.2.25) of solution S measured at 430 nm is not greater than 0.04.

pH (2.2.3). The pH of solution S is 5.0 to 7.0.

Specific optical rotation (2.2.7). Dissolve 0.250 g in *water R* and dilute to 25.0 ml with the same solvent. The specific optical rotation is + 158 to + 168, calculated with reference to the anhydrous substance.

Related substances. Examine by liquid chromatography (2.2.29) as prescribed under Assay. Inject test solution (a) and continue the chromatography for five times the retention time of the principal peak. Inject reference solution (b). In the chromatogram obtained with test solution (a): the area of any peak, apart from the principal peak, is not greater than the area of the principal peak in the chromatogram obtained with reference solution (b) (1 per cent); the sum of the areas of all peaks, apart from the principal peak, is not greater than five times the area of the principal peak in the chromatogram obtained with reference solution (b) (5 per cent). Disregard any peak with an area less than 0.05 times the area of the principal peak in the chromatogram obtained with reference solution (b).

***N,N*-Dimethylaniline (2.4.26, Method B).** Not more than 20 ppm.

2-Ethylhexanoic acid (2.4.28). Not more than 0.8 per cent *m/m*.

Water (2.5.12): 3.0 per cent to 4.5 per cent, determined on 0.300 g by the semi-micro determination of water.

Pyrogens (2.6.8). If intended for use in the manufacture of parenteral dosage forms without a further appropriate procedure for the removal of pyrogens, it complies with the test for pyrogens. Inject per kilogram of the rabbit's mass 1 ml of a solution in *water for injections R* containing 20 mg of the substance to be examined per millilitre.

ASSAY

Examine by liquid chromatography (2.2.29).

Test solution (a). Dissolve 50.0 mg of the substance to be examined in the mobile phase and dilute to 50.0 ml with the mobile phase.

Test solution (b). Dilute 5.0 ml of test solution (a) to 50.0 ml with the mobile phase.

Reference solution (a). Dissolve 50.0 mg of *flucloxacillin sodium CRS* in the mobile phase and dilute to 50.0 ml with the mobile phase. Dilute 5.0 ml of the solution to 50.0 ml with the mobile phase.

Reference solution (b). Dilute 5.0 ml of reference solution (a) to 50.0 ml with the mobile phase.

Reference solution (c). Dissolve 5 mg of *flucloxacillin sodium CRS* and 5 mg of *cloxacillin sodium CRS* in the mobile phase and dilute to 50.0 ml with the mobile phase.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (5 µm),
- as mobile phase at a flow rate of 1 ml/min a mixture of 25 volumes of *acetonitrile R* and 75 volumes of a 2.7 g/l solution of *potassium dihydrogen phosphate R*; adjust to pH 5.0 with *dilute sodium hydroxide solution R*,
- as detector a spectrophotometer set at 225 nm,
- a 20 µl loop injector.

Inject reference solution (c). Adjust the sensitivity of the system so that the heights of the principal peaks in the chromatogram obtained are at least 50 per cent of the full scale of the recorder. The test is not valid unless the resolution between the first peak (cloxacillin) and the second peak (flucloxacillin) is at least 2.5. Inject reference solution (a) six times. The test is not valid unless the relative standard deviation of the peak area for flucloxacillin is at most 1.0 per cent. Inject alternately test solution (b) and reference solution (a).

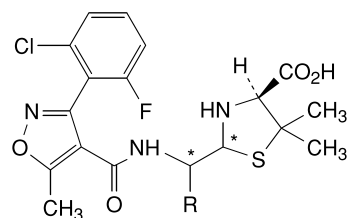
STORAGE

Store in an airtight container, at a temperature not exceeding 25 °C. If the substance is sterile, store in a sterile, airtight, tamper-proof container.

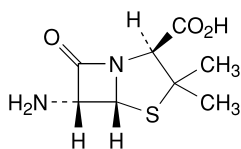
LABELLING

The label states, where applicable, that the substance is apyrogenic.

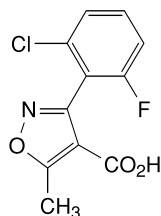
IMPURITIES



- A. R = CO₂H: (4*S*)-2-[carboxy[[[3-(2-chloro-6-fluorophenyl)-5-methylisoxazol-4-yl]carbonyl]amino]methyl]-5,5-dimethylthiazolidine-4-carboxylic acid (penicilloic acids of flucloxacillin),
- B. R = H: (2*RS*,4*S*)-2-[[[[3-(2-chloro-6-fluorophenyl)-5-methylisoxazol-4-yl]carbonyl]amino]methyl]-5,5-dimethylthiazolidine-4-carboxylic acid (penilloic acids of flucloxacillin),



- C. (2*S*,5*R*,6*R*)-6-amino-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid (6-aminopenicillanic acid),

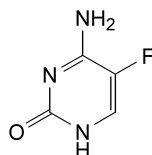


- D. 3-(2-chloro-6-fluorophenyl)-5-methylisoxazole-4-carboxylic acid.

01/2005:0766

FLUCYTOSINE

Flucytosinum

C₄H₄FN₃OM_r 129.1

DEFINITION

Flucytosine contains not less than 98.5 per cent and not more than the equivalent of 101.0 per cent of 4-amino-5-fluoropyrimidin-2(1*H*)-one, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, crystalline powder, sparingly soluble in water, slightly soluble in ethanol.

IDENTIFICATION

First identification: A.

Second identification: B, C, D.

- Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *flucytosine CRS*.
- Examine the chromatograms obtained in the test for related substances. The principal spot in the chromatogram obtained with test solution (b) is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a).
- Mix about 5 mg with 45 mg of *heavy magnesium oxide R* and ignite in a crucible until an almost white residue is obtained (usually less than 5 min). Allow to cool, add 1 ml of *water R*, 0.05 ml of *phenolphthalein solution R1* and about 1 ml of *dilute hydrochloric acid R* to render the solution colourless. Filter and add to the filtrate a freshly prepared mixture of 0.1 ml of *alizarin S solution R* and 0.1 ml of *zirconyl nitrate solution R*. Mix, allow to stand for 5 min and compare the colour of the solution with that of a blank prepared in the same manner. The colour of the solution changes from red to yellow.

- To 5 ml of solution S (see Tests) add 0.15 ml of *bromine water R* and shake. The colour of the solution is discharged.

TESTS

Solution S. Dissolve 0.5 g in *carbon dioxide-free water R* and dilute to 50 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and not more intensely coloured than reference solution BY₇ or Y₇ (2.2.2, *Method II*).

Related substances. Examine by thin-layer chromatography (2.2.27), using a suitable silica gel as the coating substance.

Test solution (a). Dissolve 50 mg of the substance to be examined in a mixture of 10 volumes of *water R* and 15 volumes of *methanol R* and dilute to 5 ml with the same mixture of solvents.

Test solution (b). Dilute 1 ml of test solution (a) to 10 ml with a mixture of 10 volumes of *water R* and 15 volumes of *methanol R*.

Reference solution (a). Dissolve 10 mg of *flucytosine CRS* in a mixture of 10 volumes of *water R* and 15 volumes of *methanol R* and dilute to 10 ml with the same mixture of solvents.

Reference solution (b). Dilute 1 ml of test solution (b) to 100 ml with a mixture of 10 volumes of *water R* and 15 volumes of *methanol R*.

Reference solution (c). Dissolve 5 mg of *fluorouracil CRS* in 5 ml of reference solution (a).

Apply separately to the plate 10 µl of each solution. Develop over a path of 12 cm in an unsaturated tank using a mixture of 1 volume of *anhydrous formic acid R*, 15 volumes of *water R*, 25 volumes of *methanol R* and 60 volumes of *ethyl acetate R*. Allow the solvents to evaporate. At the bottom of a chromatography tank place an evaporating dish containing a mixture of 2 volumes of a 15 g/l solution of *potassium permanganate R*, 1 volume of *hydrochloric acid R1* and 1 volume of *water R*, close the tank and allow to stand for 15 min. Place the dried plate in the tank and close the tank. Leave the plate in contact with the chlorine vapour for 5 min. Withdraw the plate and place it in a current of cold air until the excess of chlorine is removed and an area of the coating below the points of application does not give a blue colour with a drop of *potassium iodide and starch solution R*. Spray with *potassium iodide and starch solution R*. Examine the plate in daylight. Any spot in the chromatogram obtained with test solution (a), apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.1 per cent). The test is not valid unless the chromatogram obtained with reference solution (c) shows two clearly separated spots.

Fluoride. Not more than 200 ppm. Carry out a potentiometric determination of fluoride ion, using a fluoride-selective indicator electrode and a silver-silver chloride reference electrode.

Prepare and store all solutions in plastic containers.

Buffer solution. Dissolve 58 g of *sodium chloride R* in 500 ml of *water R*. Add 57 ml of *glacial acetic acid R* and 200 ml of a 100 g/l solution of *cyclohexylenedinitrilotetra-acetic acid R* in 1 *M sodium hydroxide*. Adjust the pH to 5.0 to 5.5 with a 200 g/l solution of *sodium hydroxide R* and dilute to 1000.0 ml with *water R*.

Test solution. Dissolve 1.00 g of the substance to be examined in *water R* and dilute to 100.0 ml with the same solvent.

Reference solutions. Dissolve 4.42 g of *sodium fluoride R*, previously dried at 120 °C for 2 h, in 300 ml of *water R* and dilute to 1000.0 ml with the same solvent (solution (a): 1.9 g/l of fluoride). Prepare three reference solutions by dilution of solution (a) 1 in 100, 1 in 1000 and 1 in 10 000.

To 20.0 ml of each reference solution, add 10.0 ml of the buffer solution and stir with a magnetic stirrer. Introduce the electrodes into the solution and allow to stand for 5 min with constant stirring. Determine the potential difference between the electrodes. Plot on semi-logarithmic graph paper the potential difference obtained for each solution as a function of concentration of fluoride. Using exactly the same conditions, determine the potential difference obtained with the test solution and calculate the content of fluoride.

Heavy metals (2.4.8). 1.0 g complies with limit test C for heavy metals (20 ppm). Use *aplatinum crucible*. Prepare the standard using 2 ml of *lead standard solution (10 ppm Pb) R*.

Loss on drying (2.2.32). Not more than 1.0 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g in a platinum crucible.

ASSAY

Dissolve 0.100 g in 40 ml of *anhydrous acetic acid R* and add 100 ml of *acetic anhydride R*. Titrate with 0.1 M *perchloric acid* determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *perchloric acid* is equivalent to 12.91 mg of $C_{23}H_{31}FN_3O_6$.

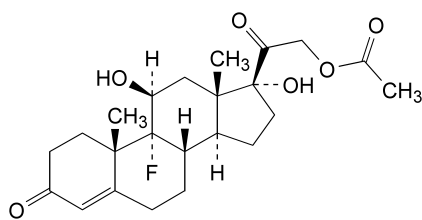
STORAGE

Store protected from light.

01/2005:0767

FLUDROCORTISONE ACETATE

Fludrocortisoni acetat



$C_{23}H_{31}FN_3O_6$

M_r 422.5

DEFINITION

Fludrocortisone acetate contains not less than 97.0 per cent and not more than the equivalent of 103.0 per cent of 9-fluoro-11 β ,17-dihydroxy-3,20-dioxopregn-4-en-21-yl acetate, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, crystalline powder, practically insoluble in water, sparingly soluble in ethanol.

IDENTIFICATION

First identification: A, B.

Second identification: C, D, E.

A. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *fludrocortisone acetate CRS*. If the spectra obtained in the solid state show differences, dissolve the substance

to be examined and the reference substance separately in the minimum volume of *acetone R*, evaporate to dryness and record the spectra again using the residues.

B. Examine by thin-layer chromatography (2.2.27), using as the coating substance a suitable silica gel with a fluorescent indicator having an optimal intensity at 254 nm.

Test solution. Dissolve 10 mg of the substance to be examined in a mixture of 1 volume of *methanol R* and 9 volumes of *methylene chloride R* and dilute to 10 ml with the same mixture of solvents.

Reference solution (a). Dissolve 10 mg of *fludrocortisone acetate CRS* in a mixture of 1 volume of *methanol R* and 9 volumes of *methylene chloride R* and dilute to 10 ml with the same mixture of solvents.

Reference solution (b). Dissolve 5 mg of *cortisone acetate CRS* in 5 ml of reference solution (a).

Apply separately to the plate 5 μ l of each solution. Prepare the mobile phase by adding a mixture of 1.2 volumes of *water R* and 8 volumes of *methanol R* to a mixture of 15 volumes of *ether R* and 77 volumes of *methylene chloride R*. Develop over a path of 15 cm. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. The principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a). Spray the plate with *alcoholic solution of sulphuric acid R*. Heat at 120 °C for 10 min or until the spots appear. Allow to cool. Examine in daylight and in ultraviolet light at 365 nm. The principal spot in the chromatogram obtained with the test solution is similar in position, colour in daylight, fluorescence in ultraviolet light at 365 nm and size to the principal spot in the chromatogram obtained with reference solution (a). The test is not valid unless the chromatogram obtained with reference solution (b) shows two clearly separated spots.

C. Examine by thin-layer chromatography (2.2.27), using as the coating substance a suitable silica gel with a fluorescent indicator having an optimal intensity at 254 nm.

Test solution (a). Dissolve 25 mg of the substance to be examined in *methanol R* and dilute to 5 ml with the same solvent. (This solution is also used to prepare test solution (b)). Dilute 2 ml of the solution to 10 ml with *methylene chloride R*.

Test solution (b). Transfer 2 ml of the solution obtained during preparation of test solution (a) to a 15 ml glass tube with a ground-glass stopper or a polytetrafluoroethylene cap. Add 10 ml of *saturated methanolic potassium hydrogen carbonate solution R* and immediately pass a stream of *nitrogen R* through the solution for 5 min. Stopper the tube. Heat on a water-bath at 45 °C protected from light for 2 h 30 min. Allow to cool.

Reference solution (a). Dissolve 25 mg of *fludrocortisone acetate CRS* in *methanol R* and dilute to 5 ml with the same solvent. (This solution is also used to prepare reference solution (b)). Dilute 2 ml of the solution to 10 ml with *methylene chloride R*.

Reference solution (b). Transfer 2 ml of the solution obtained during preparation of reference solution (a) to a 15 ml glass tube with a ground-glass stopper or a polytetrafluoroethylene cap. Add 10 ml of *saturated methanolic potassium hydrogen carbonate solution R* and immediately pass a stream of *nitrogen R* through the solution for 5 min. Stopper the tube. Heat on a water bath at 45 °C protected from light for 2 h 30 min. Allow to cool.

Apply separately to the plate 5 µl of each solution. Prepare the mobile phase by adding a mixture of 1.2 volumes of *water R* and 8 volumes of *methanol R* to a mixture of 15 volumes of *ether R* and 77 volumes of *methylene chloride R*. Develop over a path of 15 cm. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. The principal spot in each of the chromatograms obtained with the test solutions is similar in position and size to the principal spot in the chromatogram obtained with the corresponding reference solution. Spray with *alcoholic solution of sulphuric acid R*. Heat at 120 °C for 10 min or until the spots appear. Allow to cool. Examine in daylight and in ultraviolet light at 365 nm. The principal spot in each of the chromatograms obtained with the test solutions is similar in position, colour in daylight, fluorescence in ultraviolet light at 365 nm and size to the principal spot in the chromatogram obtained with the corresponding reference solution. The principal spots in the chromatograms obtained with test solution (b) and reference solution (b) have R_f values distinctly lower than those of the principal spots in the chromatograms obtained with test solution (a) and reference solution (a).

D. Mix about 5 mg with 45 mg of *heavy magnesium oxide R* and ignite in a crucible until an almost white residue is obtained (usually less than 5 min). Allow to cool, add 1 ml of *water R*, 0.05 ml of *phenolphthalein solution R1* and about 1 ml of *dilute hydrochloric acid R* to render the solution colourless. Filter and add to the filtrate a freshly prepared mixture of 0.1 ml of *alizarin S solution R* and 0.1 ml of *zirconyl nitrate solution R*. Mix, allow to stand for 5 min and compare the colour of the solution with that of a blank prepared in the same manner. The colour of the solution changes from red to yellow.

E. About 10 mg gives the reaction of acetyl (2.3.1).

TESTS

Specific optical rotation (2.2.7). Dissolve 0.250 g in *dioxan R* and dilute to 25.0 ml with the same solvent. The specific optical rotation is + 148 to + 156, calculated with reference to the dried substance.

Related substances. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 20.0 mg of the substance to be examined in the mobile phase and dilute to 10.0 ml with the mobile phase.

Reference solution (a). Dissolve 2.0 mg of *fludrocortisone acetate CRS* and 2.0 mg of *hydrocortisone acetate CRS* in the mobile phase and dilute to 50.0 ml with the mobile phase.

Reference solution (b). Dilute 1.0 ml of the test solution to 50.0 ml with the mobile phase.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.2 m long and 4.6 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R*,
- as mobile phase at a flow rate of 1 ml/min a mixture of 35 volumes of *tetrahydrofuran R* and 65 volumes of *water R*,
- as detector a spectrophotometer set at 254 nm.

Adjust the sensitivity so that the height of the principal peak in the chromatogram obtained with reference solution (b) is 70 per cent to 90 per cent of the full scale of the recorder.

Equilibrate the column with the mobile phase at a flow rate of 1 ml/min for about 30 min.

Inject 20 µl of reference solution (a). When the chromatograms are recorded in the conditions described above the retention times are: hydrocortisone acetate about

8.5 min and fludrocortisone acetate about 10 min. The test is not valid unless the resolution between the peaks corresponding to hydrocortisone acetate and fludrocortisone acetate is at least 1.0; if this resolution is not achieved, adjust slightly the concentration of tetrahydrofuran in the mobile phase. Increasing the concentration of tetrahydrofuran reduces the retention times.

Inject separately 20 µl of the test solution and 20 µl of reference solution (b). Continue the chromatography for twice the retention time of the principal peak. In the chromatogram obtained with the test solution: the area of any peak, apart from the principal peak, is not greater than half the area of the principal peak in the chromatogram obtained with reference solution (b) (1.0 per cent) and the sum of the areas of all such peaks, is not greater than 0.75 times the area of the principal peak in the chromatogram obtained with reference solution (b) (1.5 per cent). Disregard any peak due to the solvent and any peak with an area less than 0.025 times the area of the principal peak in the chromatogram obtained with reference solution (b).

Loss on drying (2.2.32). Not more than 1.0 per cent, determined on 0.500 g by drying in an oven at 100 °C to 105 °C.

ASSAY

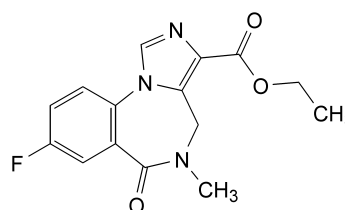
Dissolve 10.0 mg of the substance to be examined in *alcohol R* and dilute to 100.0 ml with the same solvent. Dilute 5.0 ml of this solution to 50.0 ml with *alcohol R*. Measure the absorbance (2.2.25) at the maximum at 238 nm.

Calculate the content of $C_{23}H_{31}FO_6$ taking the specific absorbance to be 405.

01/2005:1326

FLUMAZENIL

Flumazenilum



$C_{15}H_{14}FN_3O_3$

M_r 303.3

DEFINITION

Ethyl 8-fluoro-5-methyl-6-oxo-5,6-dihydro-4H-imidazo[1,5-a][1,4]benzodiazepine-3-carboxylate.

Content: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

Appearance: white or almost white, crystalline powder.

Solubility: very slightly soluble in water, freely soluble in methylene chloride, sparingly soluble in methanol.

mp: 198 °C to 202 °C.

IDENTIFICATION

Infrared absorption spectrophotometry (2.2.24).

Comparison: *Ph. Eur. reference spectrum of flumazenil.*

TESTS

Appearance of solution. The solution is clear (2.2.1) and is not more intensely coloured than reference solution BY₇ (2.2.2, Method II).

Dissolve 0.10 g in *methanol R* and dilute to 10 ml with the same solvent.

Impurity C: maximum 1 per cent.

Dissolve 0.10 g in 0.5 ml of *methylene chloride R* and dilute to 10 ml with *butanol R*. To 5.0 ml of this solution add 2.0 ml of *ninhydrin solution R* and heat in a water-bath at 95 °C for 15 min. Any blue-purple colour in the solution is not more intense than that in a standard prepared at the same time and in the same manner using 5.0 ml of a 0.1 g/l solution of *dimethylformamide diethylacetal R* in *butanol R*.

Related substances. Liquid chromatography (2.2.29).

Test solution. Dissolve 50.0 mg of the substance to be examined in 5 ml of *methanol R* and dilute to 25.0 ml with the mobile phase.

Reference solution (a). Dissolve 2.0 mg of *flumazenil impurity B CRS* and 2.0 mg of the substance to be examined in the mobile phase and dilute to 25.0 ml with the mobile phase. Dilute 2.0 ml of this solution to 25.0 ml with the mobile phase.

Reference solution (b). Dilute 10.0 ml of the test solution to 100.0 ml with the mobile phase. Dilute 1.0 ml of this solution to 100.0 ml with the mobile phase.

Column:

- *size:* $l = 0.25$ m, $\varnothing = 4.6$ mm,
- *stationary phase:* end-capped octadecylsilyl silica gel for chromatography R (5 μ m).

Mobile phase: to 800 ml of *water R* adjusted to pH 2.0 with *phosphoric acid R*, add 130 ml of *methanol R* and 70 ml of *tetrahydrofuran R* and mix.

Flow rate: 1 ml/min.

Detection: spectrophotometer at 230 nm.

Injection: 20 μ l.

Run time: 3 times the retention time of flumazenil.

Relative retention with reference to flumazenil (retention time = about 14 min): impurity A = about 0.4; impurity D = about 0.5; impurity E = about 0.6; impurity B = about 0.7; impurity F = about 2.4.

System suitability: reference solution (a):

- *resolution:* minimum 3.0 between the peaks due to impurity B and flumazenil.

Limits:

- *impurity B:* not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent),
- *any other impurity:* for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent),
- *total:* not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent),
- *disregard limit:* 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

Loss on drying (2.2.32): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 100–105 °C.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g in a platinum crucible.

ASSAY

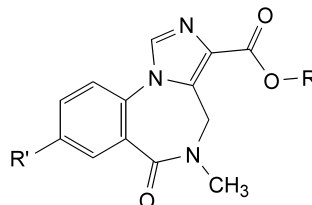
Dissolve 0.250 g in 50 ml of a mixture of 2 volumes of *acetic anhydride R* and 3 volumes of *anhydrous acetic acid R*. Titrate with 0.1 M *perchloric acid*, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *perchloric acid* is equivalent to 30.33 mg of $C_{15}H_{14}FN_3O_3$.

IMPURITIES

Specified impurities: B, C.

Other detectable impurities: A, D, E, F.

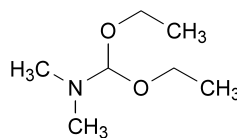


A. R = H, R' = F: 8-fluoro-5-methyl-6-oxo-5,6-dihydro-4*H*-imidazo[1,5-*a*][1,4]benzodiazepine-3-carboxylic acid,

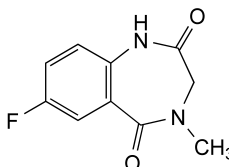
B. R = C_2H_5 , R' = OH: ethyl 8-hydroxy-5-methyl-6-oxo-5,6-dihydro-4*H*-imidazo[1,5-*a*][1,4]benzodiazepine-3-carboxylate,

E. R = C_2H_5 , R' = H: ethyl 5-methyl-6-oxo-5,6-dihydro-4*H*-imidazo[1,5-*a*][1,4]benzodiazepine-3-carboxylate,

F. R = C_2H_5 , R' = Cl: ethyl 8-chloro-5-methyl-6-oxo-5,6-dihydro-4*H*-imidazo[1,5-*a*][1,4]benzodiazepine-3-carboxylate,



C. diethoxy-*N,N*-dimethylmethanamine,

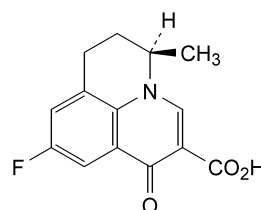


D. 7-fluoro-4-methyl-3,4-dihydro-1*H*-1,4-benzodiazepine-2,5-dione.

01/2005:1517

FLUMEQUINE

Flumequinum



and enantiomer

$C_{14}H_{12}FNO_3$

M_r 261.3

DEFINITION

Flumequine contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of (RS)-9-fluoro-5-methyl-1-oxo-6,7-dihydro-1*H*,5*H*-benzo[*i*,*j*]quinolizine-2-carboxylic acid, calculated with reference to the dried substance.

CHARACTERS

A microcrystalline white powder, practically insoluble in water, freely soluble in dilute solutions of alkali hydroxides, sparingly soluble in methylene chloride, very slightly soluble in methanol.

IDENTIFICATION

First identification: A, B.

Second identification: B, C, D.

- A. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with flumequine CRS.
- B. It complies with the test for optical rotation (see Tests).
- C. Examine by thin-layer chromatography (2.2.27), using a TLC silica gel F_{254} plate R.

Test solution. Dissolve 5 mg of the substance to be examined in 10 ml of methylene chloride R.

Reference solution. Dissolve 5 mg of flumequine CRS in 10 ml of methylene chloride R.

Apply to the plate 5 μ l of each solution. Develop over a path of 15 cm using a mixture of 10 volumes of ammonia R, 10 volumes of water R and 90 volumes of alcohol R. Allow the plate to dry in air. Examine in ultraviolet light at 254 nm. The principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with the reference solution.

- D. Mix about 5 mg with 45 mg of heavy magnesium oxide R and ignite in a crucible until an almost white residue is obtained (usually less than 5 min). Allow to cool, add 1 ml of water R, 0.05 ml of phenolphthalein solution R1 and about 2 ml of dilute hydrochloric acid R to render the solution colourless. Filter and add to the filtrate a freshly prepared mixture of 0.1 ml of alizarin S solution R and 0.1 ml of zirconyl nitrate solution R. Mix, allow to stand for 5 min and compare the colour of the solution with that of a blank prepared in the same manner. The colour of the test solution changes from red to yellow. The colour of the blank solution remains red.

TESTS

Solution S. Dissolve 5.00 g in 0.5 M sodium hydroxide and dilute to 50.0 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and not more intensely coloured than reference solution BY₅ (2.2.2, Method II).

Optical rotation (2.2.7). The angle of optical rotation, determined on solution S, is -0.10° to $+0.10^\circ$.

Related substances. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 35.0 mg of the substance to be examined in dimethylformamide R and dilute to 100.0 ml with the same solvent.

Reference solution (a). Dissolve 5.0 mg of flumequine CRS and 5.0 mg of flumequine impurity B CRS in dimethylformamide R and dilute to 100.0 ml with the same solvent.

Reference solution (b). Dilute 1.0 ml of the test solution to 200.0 ml with dimethylformamide R.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.15 m long and 4.6 mm in internal diameter packed with octadecylsilyl silica gel for chromatography R (5 μ m),
- as mobile phase at a flow rate of 0.8 ml/min a mixture of 49 volumes of methanol R and 51 volumes of a 1.36 g/l solution of potassium dihydrogen phosphate R,
- as detector a spectrophotometer set at 313 nm,
- a 10 μ l loop injector.

Inject reference solution (a). When the chromatograms are recorded in the prescribed conditions, the retention times are: impurity B, about 11 min and flumequine, about 13 min. The test is not valid unless the resolution between the peaks corresponding to flumequine and impurity B is at least 2.0.

Inject separately dimethylformamide R as a blank, the test solution and reference solution (b). Continue the chromatography of the test solution for three times the retention time of flumequine. When the chromatograms are recorded in the prescribed conditions, the relative retention time for impurity A is about 0.67 with respect to the flumequine peak. In the chromatogram obtained with the test solution: the area of any peak, apart from the principal peak, is not greater than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent); the sum of the areas of all the peaks, apart from the principal peak, is not greater than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (1 per cent). Disregard any peak obtained with dimethylformamide and any peak with an area less than 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (b).

Heavy metals (2.4.8). 2.0 g complies with limit test C for heavy metals (10 ppm). Prepare the standard using 2 ml of lead standard solution (10 ppm Pb) R.

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C for 3 h.

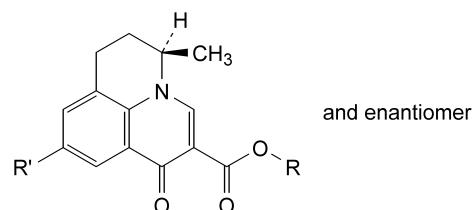
Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g in a platinum crucible.

ASSAY

Dissolve 0.500 g in 50 ml of dimethylformamide R. Titrate with 0.1 M tetrabutylammonium hydroxide, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M tetrabutylammonium hydroxide is equivalent to 26.13 mg of C₁₄H₁₂FNO₃.

IMPURITIES

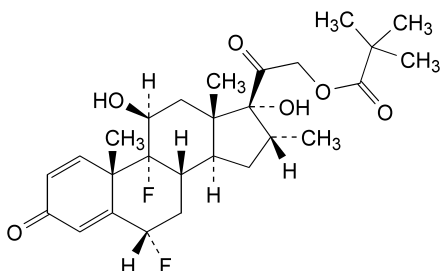


- A. R = R' = H: (RS)-5-methyl-1-oxo-6,7-dihydro-1*H*,5*H*-benzo[*i*,*j*]quinolizine-2-carboxylic acid (defluoroflumequine),
- B. R = C₂H₅, R' = F: ethyl (RS)-9-fluoro-5-methyl-1-oxo-6,7-dihydro-1*H*,5*H*-benzo[*i*,*j*]quinolizine-2-carboxylate (flumequine ethyl ester).

01/2005:1327

FLUMETASONE PIVALATE

Flumetasoni pivalas

 $C_{27}H_{36}F_2O_6$ M_r 494.6

DEFINITION

Flumetasone pivalate contains not less than 97.0 per cent and not more than the equivalent of 103.0 per cent of 6 α ,9-difluoro-11 β ,17-dihydroxy-16 α -methyl-3,20-dioxopregna-1,4-dien-21-yl 2,2-dimethylpropanoate, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, crystalline powder, practically insoluble in water, sparingly soluble in acetone, slightly soluble in alcohol and in methylene chloride.

It shows polymorphism.

IDENTIFICATION

First identification: A, B.

Second identification: B, C, D.

- A. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *flumetasone pivalate CRS*. If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in *acetone R*, evaporate to dryness on a water-bath and record new spectra using the residues.
- B. Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel F₂₅₄ plate R*.

Test solution. Dissolve 10 mg of the substance to be examined in *acetone R* and dilute to 10 ml with the same solvent.

Reference solution (a). Dissolve 10 mg of *flumetasone pivalate CRS* in *acetone R* and dilute to 10 ml with the same solvent.

Reference solution (b). Dissolve 10 mg of *desoxycortone acetate CRS* in *acetone R* and dilute to 10 ml with the same solvent. Dilute 5 ml of the solution to 10 ml with reference solution (a).

Apply to the plate 5 μ l of each solution. Prepare the mobile phase by adding a mixture of 1.2 volumes of *water R* and 8 volumes of *methanol R* to a mixture of 15 volumes of *ether R* and 77 volumes of *methylene chloride R*. Develop over a path of 15 cm. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. The principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a). Spray the plate with *alcoholic solution of sulphuric acid R*. Heat at 120 °C for 10 min or until the spots appear. Allow to cool. Examine in daylight and in ultraviolet light at 365 nm. The principal spot in the chromatogram obtained with the test solution is similar

in position, colour in daylight, fluorescence in ultraviolet light at 365 nm and size to the principal spot in the chromatogram obtained with reference solution (a). The test is not valid unless the chromatogram obtained with reference solution (b) shows two clearly separated spots.

- C. Add about 2 mg to 2 ml of a mixture of 0.5 ml of *water R* and 1.5 ml of *sulphuric acid R* and shake to dissolve. Within 5 min, a pink colour develops. Add the solution to 10 ml of *water R* and mix. The colour fades and a clear solution remains.
- D. Mix about 5 mg with 45 mg of *heavy magnesium oxide R* and ignite in a crucible until an almost white residue is obtained (usually less than 5 min). Allow to cool, add 1 ml of *water R*, 0.05 ml of *phenolphthalein solution RI* and about 1 ml of *dilute hydrochloric acid R* to render the solution colourless. Filter. To a freshly prepared mixture of 0.1 ml of *alizarin S solution R* and 0.1 ml of *zirconyl nitrate solution R* add 1.0 ml of the filtrate. Mix, allow to stand for 5 min and compare the colour of the solution with that of a blank prepared in the same manner. The test solution is yellow and the blank is red.

TESTS

Solution S. Dissolve 0.50 g in *acetone R* and dilute to 25.0 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and not more intensely coloured than reference solution BY₆ (2.2.2, Method II).

Specific optical rotation (2.2.7): + 69 to + 77, determined on solution S and calculated with reference to the dried substance.

Related substances. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 25.0 mg of the substance to be examined in the mobile phase and dilute to 25.0 ml with the mobile phase. Dilute 5.0 ml of the solution to 50.0 ml with the mobile phase.

Reference solution (a). Dissolve 10 mg of *dexamethasone pivalate CRS* in the mobile phase and dilute to 100.0 ml with the mobile phase. Take 5.0 ml of the solution, add 5.0 ml of the test solution, mix and dilute to 50.0 ml with the mobile phase.

Reference solution (b). Dilute 2.0 ml of the test solution to 100.0 ml with the mobile phase.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4.6 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (5 μ m),
- as mobile phase at a flow rate of 0.6 ml/min a mixture of 5 volumes of *tetrahydrofuran R*, 30 volumes of *acetonitrile R*, 30 volumes of *water R* and 35 volumes of *methanol R*,
- as detector a spectrophotometer set at 254 nm.

Inject 20 μ l of reference solution (b). Adjust the sensitivity of the system so that the height of the principal peak in the chromatogram obtained with reference solution (b) is at least 50 per cent of the full scale of the recorder.

Inject 20 μ l of reference solution (a). When the chromatograms are recorded in the prescribed conditions, the relative retention time of dexamethasone pivalate, relative to flumetasone pivalate, is about 1.1. The test is not valid unless in the chromatogram obtained with reference solution (a) the resolution between the peaks corresponding to dexamethasone pivalate and flumetasone pivalate is at least 2.8. If necessary adjust the concentration of tetrahydrofuran in the mobile phase.

Inject 20 µl of the test solution. Continue the chromatography for 1.5 times the retention time of the principal peak. In the chromatogram obtained with the test solution, the area of any peak, apart from the principal peak, is not greater than 0.75 times the area of the principal peak in the chromatogram obtained with reference solution (b) (1.5 per cent); the sum of the areas of any peaks, apart from the principal peak, is not greater than the area of the principal peak in the chromatogram obtained with reference solution (b) (2 per cent). Disregard any peak with an area less than 0.025 times the area of the principal peak in the chromatogram obtained with reference solution (b).

Loss on drying (2.2.32). Not more than 1.0 per cent, determined on 0.500 g by drying in an oven at 100 °C to 105 °C for 4 h.

ASSAY

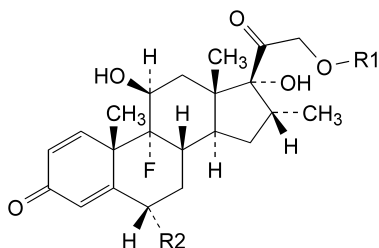
Dissolve 50.0 mg in *alcohol R* and dilute to 100.0 ml with the same solvent. Dilute 2.0 ml of the solution to 100.0 ml with *alcohol R*. Measure the absorbance (2.2.25) at the maximum at 239 nm.

Calculate the content of $C_{27}H_{36}F_2O_6$ taking the specific absorbance to be 336.

STORAGE

Store protected from light.

IMPURITIES

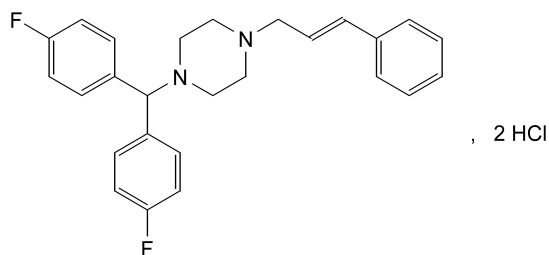


- A. R1 = H, R2 = F: 6α,9-difluoro-11β,17,21-trihydroxy-16α-methylpregna-1,4-diene-3,20-dione (flumetasone),
 B. R1 = CO-CH₃, R2 = F: 6α,9-difluoro-11β,17-dihydroxy-16α-methyl-3,20-dioxopregna-1,4-dien-21-yl acetate (flumetasone acetate),
 C. R1 = CO-C(CH₃)₃, R2 = H: 9-fluoro-11β,17-dihydroxy-16α-methyl-3,20-dioxopregna-1,4-dien-21-yl 2,2-dimethylpropanoate (dexamethasone pivalate),
 D. R1 = CO-C(CH₃)₃, R2 = Cl: 6α-chloro-9-fluoro-11β,17-dihydroxy-16α-methyl-3,20-dioxopregna-1,4-dien-21-yl 2,2-dimethylpropanoate (chlordexamethasone pivalate).

01/2005:1722

FLUNARIZINE DIHYDROCHLORIDE

Flunarizini dihydrochloridum



$C_{26}H_{28}Cl_2F_2N_2$

M_r 477.4

DEFINITION

1-[Bis(4-fluorophenyl)methyl]-4-[(2*E*)-3-phenylprop-2-enyl]piperazine dihydrochloride.

Content: 99.0 per cent to 101.5 per cent (dried substance).

CHARACTERS

Appearance: white or almost white powder, hygroscopic.

Solubility: slightly soluble in water, sparingly soluble in methanol, slightly soluble in alcohol and in methylene chloride.

mp: about 208 °C, with decomposition.

IDENTIFICATION

A. Infrared absorption spectrophotometry (2.2.24).

Comparison: *Ph. Eur. reference spectrum of flunarizine dihydrochloride.*

B. Dissolve 25 mg in 2 ml of *methanol R* and add 0.5 ml of *water R*. The solution gives reaction (a) of chlorides (2.3.1).

TESTS

Related substances. Liquid chromatography (2.2.29).

Prepare the solutions immediately before use and protect from light.

Test solution. Dissolve 0.100 g of the substance to be examined in *methanol R* and dilute to 10.0 ml with the same solvent.

Reference solution (a). Dissolve 10 mg of *flunarizine dihydrochloride* for system suitability CRS in *methanol R* and dilute to 1.0 ml with the same solvent.

Reference solution (b). Dilute 1.0 ml of the test solution to 100.0 ml with *methanol R*. Dilute 5.0 ml of this solution to 20.0 ml with *methanol R*.

Column:

- size: $l = 0.10$ m, $\varnothing = 4.6$ mm,
- stationary phase: base-deactivated octadecylsilyl silica gel for chromatography R (3 µm).

Mobile phase:

- mobile phase A: solution containing 23.8 g/l of *tetrabutylammonium hydrogen sulphate R* and 7 g/l of *ammonium acetate R*,
- mobile phase B: *acetonitrile R*,

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)
0 - 12	80 → 40	20 → 60
12 - 15	40	60
15 - 16	40 → 80	60 → 20
16 - 20	80	20

Flow rate: 1.5 ml/min.

Detection: spectrophotometer at 230 nm.

Injection: 10 µl.

System suitability: reference solution (a):

- peak-to-valley ratio: minimum 1.5, where H_p = height above the baseline of the peak due to impurity C and H_v = height above the baseline of the lowest point of the curve separating this peak from the peak due to flunarizine,
- the chromatogram obtained is concordant with the chromatogram supplied with *flunarizine dihydrochloride* for system suitability CRS.

Limits:

- **correction factor:** for the calculation of content, multiply the peak area of impurity A by 1.5,
- **impurities A, D:** for each impurity, not more than 0.4 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent),
- **impurity B:** not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent),
- **impurity C:** not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.25 per cent),
- **any other impurity:** for each impurity, not more than 0.4 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent),
- **total:** not more than 4 times the area of the principal peak in the chromatogram obtained with reference solution (b) (1.0 per cent),
- **disregard limit:** 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

Loss on drying (2.2.32): maximum 5.0 per cent, determined on 1.000 g by drying in an oven at 100–105 °C for 4 h.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g in a platinum crucible.

ASSAY

Dissolve 0.200 g in 70 ml of *alcohol R*. Carry out a potentiometric titration (2.2.20), using 0.1 M *sodium hydroxide*. Read the volume added at the second point of inflexion. Carry out a blank titration.

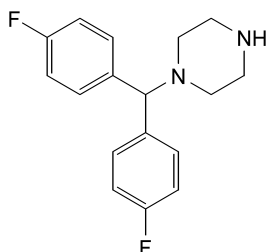
1 ml of 0.1 M *sodium hydroxide* is equivalent to 23.87 mg of $C_{16}H_{12}FN_3O_3$.

STORAGE

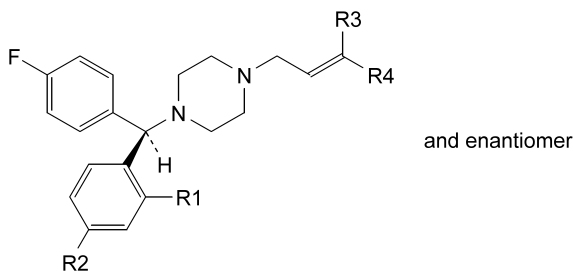
In an airtight container, protected from light.

IMPURITIES

Specified impurities: A, B, C, D.



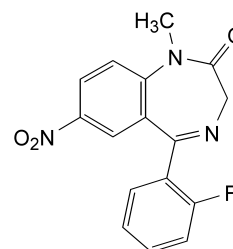
A. 1-[bis(4-fluorophenyl)methyl]piperazine,



B. R1 = R2 = R3 = H, R4 = C_6H_5 : 1-[(RS)-(4-fluorophenyl)phenylmethyl]-4-[(2E)-3-phenylprop-2-enyl]piperazine,

- C. R1 = F, R2 = R3 = H, R4 = C_6H_5 : 1-[(RS)-(2-fluorophenyl)(4-fluorophenyl)methyl]-4-[(2E)-3-phenylprop-2-enyl]piperazine,
- D. R1 = R4 = H, R2 = F, R3 = C_6H_5 : 1-[bis(4-fluorophenyl)methyl]-4-[(2Z)-3-phenylprop-2-enyl]piperazine.

01/2005:0717

FLUNITRAZEPAM**Flunitrazepamum**
 $C_{16}H_{12}FN_3O_3$
 M_r 313.3
DEFINITION

5-(2-Fluorophenyl)-1-methyl-7-nitro-1,3-dihydro-2H-1,4-benzodiazepin-2-one.

Content: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

Appearance: white or yellowish, crystalline powder.

Solubility: practically insoluble in water, soluble in acetone, slightly soluble in alcohol.

IDENTIFICATION

Infrared absorption spectrophotometry (2.2.24).

Comparison: *Ph. Eur.* reference spectrum of flunitrazepam.

TESTS

Related substances. Liquid chromatography (2.2.29).

Prepare the solutions immediately before use.

Test solution. Dissolve 100.0 mg of the substance to be examined in 10 ml of *acetonitrile R* and dilute to 50.0 ml with the mobile phase.

Reference solution (a). Dilute 1.0 ml of the test solution to 100.0 ml with the mobile phase. Dilute 5.0 ml of this solution to 50.0 ml with the mobile phase.

Reference solution (b). Dissolve 4 mg of the substance to be examined and 4 mg of *nitrazepam R* in 5 ml of *acetonitrile R* and dilute to 20.0 ml with the mobile phase. Dilute 1.0 ml of the solution to 20.0 ml with the mobile phase.

Column:

- **size:** $l = 0.15$ m, $\varnothing = 4.6$ mm,
- **stationary phase:** octadecylsilyl silica gel for chromatography *R* (5 μ m).

Mobile phase: *methanol R*, *acetonitrile R*, *water R* (50:305:645 V/V/V).

Flow rate: 1.0 ml/min.

Detection: spectrophotometer at 254 nm.

Injection: 20 μ l.

Run time: 6 times the retention time of flunitrazepam.

Relative retention with reference to flunitrazepam (retention time = about 11 min): impurity A = about 0.2; impurity B = about 0.6; impurity C = about 2.3; impurity D = about 4.0.

System suitability: reference solution (b):

- **resolution:** minimum 4.0 between the peaks due to nitrazepam and flunitrazepam.

Limits:

- **correction factor:** for the calculation of content, multiply the peak area of impurity C by 2.44,
- **any impurity:** not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent),
- **total:** not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.3 per cent),
- **disregard limit:** 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

Loss on drying (2.2.32): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 100–105 °C.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

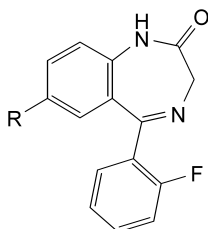
Dissolve 0.250 g in 20 ml of *anhydrous acetic acid R* and add 50 ml of *acetic anhydride R*. Titrate with 0.1 M *perchloric acid*, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *perchloric acid* is equivalent to 31.33 mg of $C_{16}H_{12}FN_3O_3$.

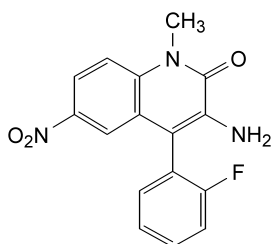
STORAGE

Protected from light.

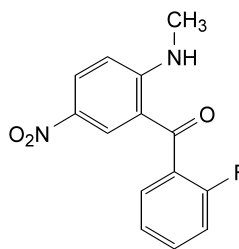
IMPURITIES



- A. R = NH₂: 7-amino-5-(2-fluorophenyl)-1,3-dihydro-2H-1,4-benzodiazepin-2-one (7-aminodemethylflunitrazepam),
- B. R = NO₂: 5-(2-fluorophenyl)-7-nitro-1,3-dihydro-2H-1,4-benzodiazepin-2-one (demethylflunitrazepam),



- C. 3-amino-4-(2-fluorophenyl)-1-methyl-6-nitroquinolin-2(1H)-one,

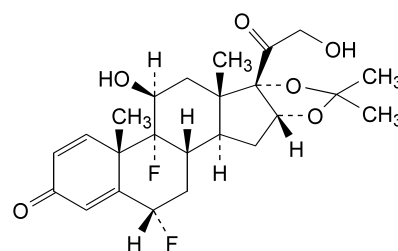


D. (2-fluorophenyl)[2-(methylamino)-5-nitrophenyl]methanone.

01/2005:0494

FLUOCINOLONE ACETONIDE

Fluocinoloni acetonidum



$C_{24}H_{30}F_2O_6$

M_r 452.5

DEFINITION

6 α ,9-Difluoro-11 β ,21-dihydroxy-16 α ,17-(1-methylethylidene-dioxy)pregna-1,4-diene-3,20-dione.

Content: 97.0 per cent to 103.0 per cent (dried substance).

CHARACTERS

Appearance: white or almost white, crystalline powder.

Solubility: practically insoluble in water, soluble in acetone and in ethanol.

It shows polymorphism.

IDENTIFICATION

- A. Infrared absorption spectrophotometry (2.2.24).

Comparison: *fluocinolone acetonide CRS*.

If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in *ethanol R*, evaporate to dryness and record new spectra using the residues.

- B. Examine the chromatograms obtained in the test for related substances.

Results: the principal peak in the chromatogram obtained with the reference solution (b) is similar in retention time to the peak due to *fluocinolone acetonide CRS* in the chromatogram obtained with the reference solution (a).

TESTS

Specific optical rotation (2.2.7): + 100 to + 104 (dried substance).

Dissolve 0.100 g in *ethanol R* and dilute to 10.0 ml with the same solvent.

Related substances. Liquid chromatography (2.2.29). *Carry out the test protected from light.*

Test solution. Dissolve 25.0 mg of the substance to be examined in *acetonitrile R* and dilute to 10.0 ml with the same solvent.

Reference solution (a). Dissolve 2.5 mg of *fluocinolone acetonide CRS* and 2.5 mg of *triamcinolone acetonide R* in 45 ml of *acetonitrile R* and dilute to 100.0 ml with *water R*.

Reference solution (b). Dilute 1.0 ml of the test solution to 100.0 ml with *acetonitrile R*.

Column:

- **size:** $l = 0.25$ m, $\varnothing = 4.6$ mm,
- **stationary phase:** *base-deactivated end-capped octadecylsilyl silica gel for chromatography R* (5 μ m).

Mobile phase: mix 450 ml of *acetonitrile R* with 500 ml of *water R* and allow to equilibrate; adjust the volume to 1000.0 ml with *water R* and mix again.

Flow rate: 1 ml/min.

Detection: spectrophotometer at 238 nm.

Injection: 20 μ l.

Run time: 4 times the retention time of fluocinolone acetonide.

Retention times: triamcinolone acetonide = about 8.5 min; fluocinolone acetonide = about 10 min.

System suitability:

- **resolution:** minimum of 3.0 between the peaks due to triamcinolone acetonide and fluocinolone acetonide in the chromatogram obtained with reference solution (a).

Limits:

- **any impurity:** not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (1 per cent) and not more than 1 such peak has an area greater than half the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent),
- **total:** not more than 2.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (2.5 per cent),
- **disregard limit:** 0.05 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

Loss on drying (2.2.32): maximum 1.0 per cent, determined on 1.000 g by drying in an oven at 100–105 °C for 3 h.

ASSAY

Protect the solutions from light throughout the assay.

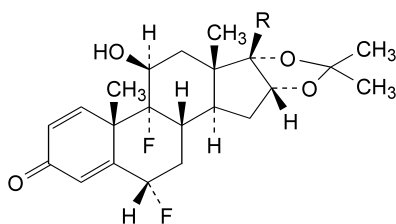
Dissolve 50.0 mg in *alcohol R* and dilute to 50.0 ml with the same solvent. Dilute 2.0 ml of this solution to 100.0 ml with *alcohol R*. Measure the absorbance (2.2.25) at the maximum at 238 nm.

Calculate the content of $C_{27}H_{30}F_2O_6$ taking the specific absorbance to be 355.

STORAGE

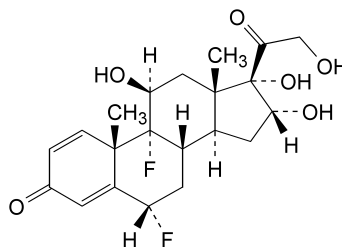
Protected from light.

IMPURITIES

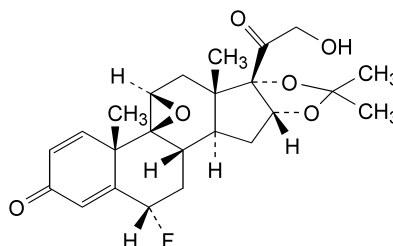


- A. R = CO-CO₂H: 6 α ,9-difluoro-11 β -hydroxy-16 α ,17-(1-methylethylidenedioxy)-3,20-dioxopregna-1,4-dien-21-oic acid,

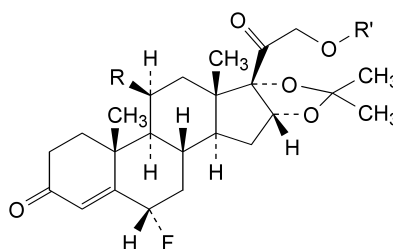
- B. R = CO₂H: 6 α ,9-difluoro-11 β -hydroxy-16 α ,17-(1-methylethylidenedioxy)-3-oxoandrosta-1,4-diene-17 β -carboxylic acid,
D. R = CO-CH=O: 6 α ,9-difluoro-11 β -hydroxy-16 α ,17-(1-methylethylidenedioxy)-3,20-dioxopregna-1,4-dien-21-al,



- C. 6 α ,9-difluoro-11 β ,16 α ,17,21-tetrahydroxypregna-1,4-diene-3,20-dione (fluocinolone),



- E. 9,11 β -epoxy-6 α -fluoro-21-hydroxy-16 α ,17-(1-methylethylidenedioxy)-9 β -pregna-1,4-diene-3,20-dione,

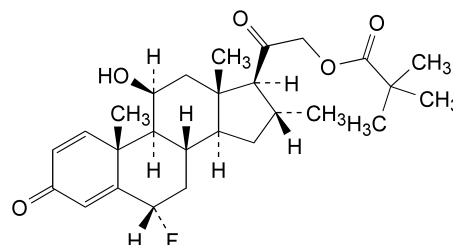


- F. R = R' = H: 6 α -fluoro-21-hydroxy-16 α ,17-(1-methylethylidenedioxy)pregn-4-ene-3,20-dione,
G. R = OH, R' = CO-CH₃: 6 α -fluoro-11 β -hydroxy-16 α ,17-(1-methylethylidenedioxy)-3,20-dioxopregn-4-en-21-yl acetate.

01/2005:1212

FLUCORTOLONE PIVALATE

Fluocortoloni pivalas



$C_{27}H_{37}FO_5$

M_r 460.6

DEFINITION

Fluocortolone pivalate contains not less than 97.0 per cent and not more than the equivalent of 103.0 per cent of 6 α -fluoro-11 β -hydroxy-16 α -methyl-3,20-dioxopregna-1,4-dien-21-yl 2,2-dimethylpropanoate calculated with reference to the dried substance.

CHARACTERS

A white or almost white, crystalline powder, practically insoluble in water, freely soluble in methylene chloride and in dioxan, sparingly soluble in alcohol.

IDENTIFICATION

First identification: A, B.

Second identification: B, C, D.

A. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *fluocortolone pivalate CRS*.

B. Examine by thin-layer chromatography (2.2.27), using as the coating substance a suitable silica gel with a fluorescent indicator having an optimal intensity at 254 nm.

Test solution. Dissolve 10 mg of the substance to be examined in a mixture of 1 volume of *methanol R* and 9 volumes of *methylene chloride R* and dilute to 10 ml with the same mixture of solvents.

Reference solution (a). Dissolve 20 mg of *fluocortolone pivalate CRS* in a mixture of 1 volume of *methanol R* and 9 volumes of *methylene chloride R* and dilute to 20 ml with the same mixture of solvents.

Reference solution (b). Dissolve 10 mg of *norethisterone CRS* in reference solution (a) and dilute to 10 ml with the same solution.

Apply separately to the plate 5 µl of each solution. Prepare the mobile phase by adding a mixture of 1.2 volumes of *water R* and 8 volumes of *methanol R* to a mixture of 15 volumes of *ether R* and 77 volumes of *methylene chloride R*. Develop over a path of 15 cm. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. The principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a). Spray with *alcoholic solution of sulphuric acid R*. Heat at 120 °C for 10 min or until the spots appear. Allow to cool. Examine the plate in daylight and in ultraviolet light at 365 nm. The principal spot in the chromatogram obtained with the test solution is similar in position, colour in daylight, fluorescence in ultraviolet light at 365 nm and size to the principal spot in the chromatogram obtained with reference solution (a). The test is not valid unless the chromatogram obtained with reference solution (b) shows two clearly separated spots.

C. To about 1 mg add 2 ml of a mixture of 2 volumes of *glacial acetic acid R* and 3 volumes of *sulphuric acid R* and heat for 1 minute on a water-bath. A red colour is produced. Add 5 ml of *water R*, the colour changes to violet-red.

D. Mix about 5 mg with 45 mg of *heavy magnesium oxide R* and ignite in a crucible until an almost white residue is obtained (usually less than 5 min). Allow to cool, add 1 ml of *water R*, 0.05 ml of *phenolphthalein solution R1* and about 1 ml of *dilute hydrochloric acid R* to render the solution colourless. Filter and add to the filtrate a freshly prepared mixture of 0.1 ml of *alizarin S solution R* and 0.1 ml of *zirconyl nitrate solution R*. Mix, allow to stand

for 5 min and compare the colour of the solution with that of a blank prepared in the same manner. The test solution is yellow and the blank is red.

TESTS

Specific optical rotation (2.2.7). Dissolve 0.25 g in *dioxan R* and dilute to 25.0 ml with the same solvent. The specific optical rotation is + 100 to + 105, calculated with reference to the dried substance.

Related substances. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 10.0 mg of the substance to be examined in *acetonitrile R* and dilute to 10.0 ml with the same solvent.

Reference solution (a). Dilute 1.0 ml of the test solution to 100.0 ml with *acetonitrile R*.

Reference solution (b). Dissolve 2 mg of *fluocortolone pivalate CRS* and 2 mg of *prednisolone hexanoate CRS* in *acetonitrile R* and dilute to 100 ml with the same solvent.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4.6 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (5 µm),
- as mobile phase at a flow rate of 1.5 ml/min a mixture of 25 volumes of *methanol R*, 30 volumes of *acetonitrile R* and 32 volumes of *water R*,
- as detector a spectrophotometer set at 243 nm.

Inject 20 µl of reference solution (a). Adjust the sensitivity of the system so that the height of the principal peak in the chromatogram obtained is at least 50 per cent of the full scale of the recorder. Inject 20 µl of reference solution (b). The test is not valid unless, in the chromatogram obtained, the resolution between the two principal peaks is at least 5.0. Inject 20 µl of the test solution. Continue the chromatography for twice the retention time of fluocortolone pivalate. In the chromatogram obtained with the test solution: the area of any peak, apart from the principal peak, is not greater than the area of the principal peak in the chromatogram obtained with reference solution (a) (1 per cent) and the sum of the areas of any peaks, apart from the principal peak, is not greater than 2.0 times the area of the principal peak in the chromatogram obtained with reference solution (a) (2 per cent). Disregard any peak due to the solvent and any peak with an area less than 0.025 times that of the principal peak in the chromatogram obtained with reference solution (a).

Loss on drying (2.2.32). Not more than 1.0 per cent, determined on 1.000 g by drying in an oven at 100-105 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

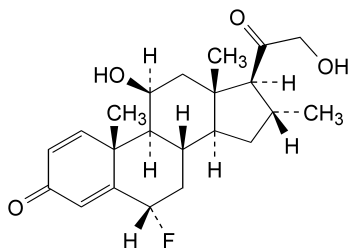
Dissolve 30.0 mg in *ethanol R* and dilute to 100.0 ml with the same solvent. Dilute 5.0 ml of the solution to 100.0 ml with *ethanol R*. Measure the absorbance (2.2.25) at the maximum at 242 nm.

Calculate the content of $C_{27}H_{37}FO_5$ taking the specific absorbance to be 350.

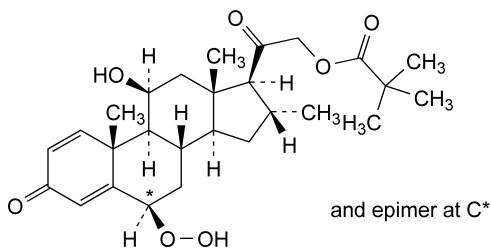
STORAGE

Store protected from light.

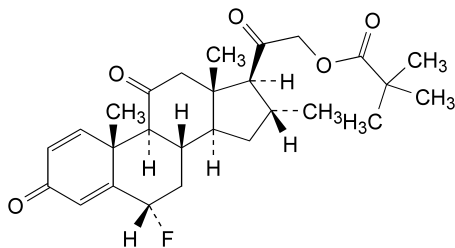
IMPURITIES



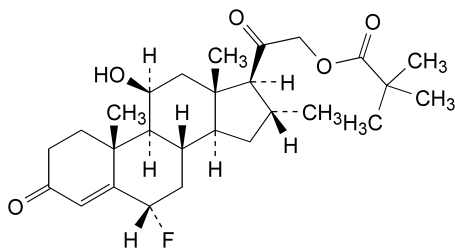
- A. 6α-fluoro-11β,21-dihydroxy-16α-methylpregna-1,4-diene-3,20-dione (fluocortolone),



- B. 6-hydroperoxy-11β-hydroxy-16α-methyl-3,20-dioxopregna-1,4-dien-21-yl 2,2-dimethylpropanoate,



- C. 6α-fluoro-16α-methyl-3,11,20-trioxopregna-1,4-dien-21-yl 2,2-dimethylpropanoate,

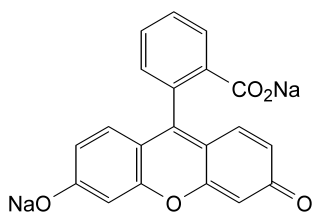


- D. 6α-fluoro-11β-hydroxy-16α-methyl-3,20-dioxopregna-4-en-21-yl 2,2-dimethylpropanoate.

01/2005:1213

FLUORESC EIN SODIUM

Fluoresceinum natricum

 $C_{20}H_{10}Na_2O_5$ M_r 376.3

DEFINITION

Fluorescein sodium contains not less than 95.0 per cent and not more than the equivalent of 103.0 per cent of disodium 2-(3-oxo-6-oxido-3*H*-xanthen-9-yl)benzoate, calculated with reference to the dried substance.

CHARACTERS

An orange-red, fine powder, hygroscopic, freely soluble in water, soluble in alcohol, practically insoluble in hexane and in methylene chloride.

IDENTIFICATION

First identification: B, D.

Second identification: A, C, D.

- Dilute 0.1 ml of solution S (see Tests) to 10 ml with *water R*. The solution shows yellowish-green fluorescence. The fluorescence disappears on addition of 0.1 ml of *dilute hydrochloric acid R* and reappears on addition of 0.2 ml of *dilute sodium hydroxide solution R*.
- Examine by infrared absorption spectrophotometry (2.2.24), comparing with the *Ph. Eur. reference spectrum of fluorescein sodium*. Examine the substance prepared as a disc.
- The absorption by a piece of filter paper of 0.05 ml of the solution prepared for identification A (before the addition of *dilute hydrochloric acid R*) colours the paper yellow. On exposing the moist paper to bromine vapour for 1 min and then to ammonia vapour the colour becomes deep pink.
- Ignite 0.1 g in a porcelain crucible. Dissolve the residue in 5 ml of *water R* and filter. 2 ml of the filtrate gives reaction (a) of sodium (2.3.1).

TESTS

Solution S. Dissolve 1.0 g in *carbon dioxide-free water R* prepared from *distilled water R* and dilute to 50 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and orange-yellow with yellowish-green fluorescence.

pH (2.2.3). The pH of solution S is 7.0 to 9.0.

Related substances and resorcinol. Examine by thin-layer chromatography (2.2.27), using as the coating substance a suitable silica gel.

Test solution (a). Dissolve 0.10 g of the substance to be examined in a 10 g/l solution of *hydrochloric acid R* in *methanol R* and dilute to 10 ml with the same acidic solution.

Test solution (b). Dissolve 0.250 g of the substance to be examined in 5 ml of *water R*. Transfer to a separating funnel and wash with 3 ml of *water R*. Add 2 ml of *buffer solution pH 8.0 R* and 2.5 g of *sodium chloride R*. Shake until the sodium chloride dissolves. Shake the solution obtained with two quantities, each of 25 ml, of *peroxide-free ether R*. Dry the ether layers over *anhydrous sodium sulphate R* and evaporate to dryness using a rotary evaporator. Dissolve the residue in 10 ml of a 10 g/l solution of *hydrochloric acid R* in *methanol R*.

Reference solution (a). Dilute 0.5 ml of test solution (a) to 100 ml with a 10 g/l solution of *hydrochloric acid R* in *methanol R*.

Reference solution (b). Dilute 1 ml of test solution (a) to 50 ml with a 10 g/l solution of *hydrochloric acid R* in *methanol R*. Dilute 1 ml of the solution to 10 ml with a 10 g/l solution of *hydrochloric acid R* in *methanol R*.

Reference solution (c). Dissolve 25 mg of *resorcinol R* in *methanol R* and dilute to 100 ml with the same solvent.

Reference solution (d). Dilute 10 ml of reference solution (c) to 20 ml with *water R*.

Reference solution (e). To 5 ml of reference solution (c) add 1 ml of test solution (a) and dilute to 10 ml with *methanol R*.

Apply separately to the plate 5 µl of test solutions (a) and (b) and 5 µl of reference solutions (a), (b), (d) and (e). Develop over a path of 15 cm using a mixture of 10 volumes of *methanol R* and 90 volumes of *methylene chloride R*. Allow the plate to dry in air. Examine in ultraviolet light at 365 nm. Expose the plate to iodine vapour for 30 min and examine in daylight. In the chromatogram obtained with test solution (a): any spot, apart from the principal spot, is not more intense than the principal spot in the chromatogram obtained with reference solution (a) (0.5 per cent) and not more than one such spot is more intense than the principal spot in the chromatogram obtained with reference solution (b) (0.2 per cent). In the chromatogram obtained with test solution (b) any spot corresponding to resorcinol is not more intense than the principal spot in the chromatogram obtained with reference solution (d) (0.5 per cent). The test is not valid unless the chromatogram obtained with reference solution (e) shows two clearly separated spots. The spots corresponding to resorcinol are visible only after exposing the plate to iodine vapour.

Dimethylformamide. Examine by gas chromatography (2.2.28) using *dimethylacetamide R* as the internal standard.

Internal standard solution. Dilute 20 µl of *dimethylacetamide R* to 100 ml with *water R*.

Test solution (a). Dissolve 1.0 g of the substance to be examined in 10 ml of *water R*. Add, with gently stirring, 10 ml of a 60 g/l solution of *hydrochloric acid R*. Allow to stand for 15 min and centrifuge. Dissolve 0.10 g of *trisodium phosphate dodecahydrate R* in 5 ml of the supernatant.

Test solution (b). Dissolve 1.0 g of the substance to be examined in 10 ml of the internal standard solution. Add, with gentle stirring, 10 ml of a 60 g/l solution of *hydrochloric acid R*. Allow to stand for 15 min and centrifuge. Dissolve 0.10 g of *trisodium phosphate dodecahydrate R* in 5 ml of the supernatant.

Reference solution. Dilute 20 µl of *dimethylformamide R* to 10 ml with *water R*. Dilute 1 ml of the solution to 10 ml with *water R*. Add 10 ml of the internal standard solution and mix.

The chromatographic procedure may be carried out using:

- a glass column 1.5 m long and 4 mm in internal diameter packed with *silanised diatomaceous earth for gas chromatography R* (135 µm to 175 µm) impregnated with 10 per cent *m/m* of *macrogol 1000 R*,
- *nitrogen for chromatography R* as the carrier gas at a flow rate of 40 ml/min,
- a flame-ionisation detector,

maintaining the temperature of the column at 120 °C and that of the injection port and of the detector at 170 °C.

Inject 2 µl of each solution. In the chromatogram obtained with test solution (b), the ratio of the area of any peak corresponding to dimethylformamide to the area of the peak due to the internal standard is not greater than the corresponding ratio in the chromatogram obtained with the reference solution (0.2 per cent).

Chlorides (2.4.4). To 10 ml of solution S add 90 ml of *water R* and 1 ml of *dilute nitric acid R*, wait for at least 10 min and filter. 10 ml of the filtrate diluted to 15 ml with *water R* complies with the limit test for chlorides (0.25 per cent).

Sulphates (2.4.13). To 5 ml of solution S add 90 ml of *distilled water R*, 2.5 ml of *dilute hydrochloric acid R*, dilute to 100 ml with *distilled water R* and filter. 15 ml of the filtrate complies with the limit test for sulphates (1.0 per cent).

Zinc. Dilute 5 ml of solution S to 10 ml with *water R*. Add 2 ml of *hydrochloric acid R1*, filter and add 0.1 ml of *potassium ferrocyanide solution R*. No turbidity or precipitate is formed immediately.

Loss on drying (2.2.32). Not more than 10.0 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C.

ASSAY

Dissolve 50.0 mg in *water R* and dilute to 500.0 ml with the same solvent. Dilute 5.0 ml of this solution to 200.0 ml with *buffer solution pH 8.0 R*. Measure the absorbance (2.2.25) at the maximum at 492 nm.

Calculate the content of $C_{20}H_{10}Na_2O_5$ taking the specific absorbance to be 2050.

STORAGE

Store in an airtight container, protected from light.

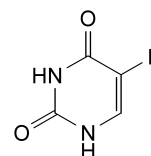
IMPURITIES

A. benzene-1,3-diol (resorcinol).

01/2005:0611

FLUOROURACIL

Fluorouracilum



$C_4H_3FN_2O_2$

M_r 130.1

DEFINITION

Fluorouracil contains not less than 98.5 per cent and not more than the equivalent of 101.0 per cent of 5-fluoropyrimidine-2,4(1*H*,3*H*)-dione, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, crystalline powder, sparingly soluble in water, slightly soluble in alcohol.

IDENTIFICATION

First identification: A.

Second identification: B, C.

- A. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *fluorouracil CRS*.
- B. Examine the chromatograms obtained in the test for related substances in ultraviolet light at 254 nm. The principal spot in the chromatogram obtained with test solution (b) is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a).
- C. In a test-tube, heat 0.5 ml of *chromic acid cleansing mixture R* in a naked flame until white fumes appear in the upper part of the tube. The solution wets the side of the tube and there is no appearance of greasiness. Add

about 2 mg of the substance to be examined and heat again in a naked flame until white fumes appear. The solution does not wet the sides of the tube.

01/2005:1104

TESTS

Solution S. Dissolve 0.5 g in *carbon dioxide-free water R* and dilute to 50 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and not more intensely coloured than reference solution BY₇ or Y₇ (2.2.2, *Method II*).

pH (2.2.3). The pH of solution S is 4.5 to 5.0.

Related substances. Examine by thin-layer chromatography (2.2.27), using *silica gel GF₂₅₄ R* as the coating substance.

Test solution (a). Dissolve 0.10 g of the substance to be examined in a mixture of equal volumes of *methanol R* and *water R* and dilute to 10.0 ml with the same mixture of solvents.

Test solution (b). Dilute 2 ml of test solution (a) to 10 ml with a mixture of equal volumes of *methanol R* and *water R*.

Reference solution (a). Dissolve 20 mg of *fluorouracil CRS* in a mixture of equal volumes of *methanol R* and *water R* and dilute to 10 ml with the same mixture of solvents.

Reference solution (b). Dilute 2.5 ml of reference solution (a) to 200 ml with a mixture of equal volumes of *methanol R* and *water R*.

Reference solution (c). Dissolve 5 mg of *5-hydroxyuracil R* in a mixture of equal volumes of *methanol R* and *water R* and dilute to 200 ml with the same mixture of solvents.

Apply to the plate 10 µl of each solution. Develop over a path of 12 cm using a mixture of 15 volumes of *methanol R*, 15 volumes of *water R* and 70 volumes of *ethyl acetate R*. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. Any spot in the chromatogram obtained with test solution (a), apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.25 per cent). Spray with a freshly prepared 5 g/l solution of *fast blue B salt R* and subsequently with 0.1 M *sodium hydroxide*. Any spot corresponding to 5-hydroxyuracil in the chromatogram obtained with test solution (a) is not more intense than the spot in the chromatogram obtained with reference solution (c) (0.25 per cent).

Heavy metals (2.4.8). Use a *platinum crucible*. 1.0 g complies with limit test C for heavy metals (20 ppm). Prepare the standard using 2 ml of *lead standard solution* (10 ppm Pb) R.

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying *in vacuo* at 80 °C for 4 h.

Sulphated ash (2.4.14). Use a *platinum crucible*. Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.1000 g in 80 ml of *dimethylformamide R*, warming gently. Cool and titrate with 0.1 M *tetrabutylammonium hydroxide*, using 0.25 ml of a 10 g/l solution of *thymol blue R* in *dimethylformamide R* as indicator. Carry out a blank titration.

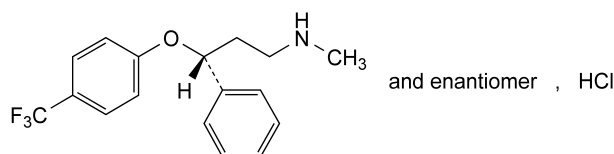
1 ml of 0.1 M *tetrabutylammonium hydroxide* is equivalent to 13.01 mg of C₁₇H₁₉ClF₃NO.

STORAGE

Store protected from light.

FLUOXETINE HYDROCHLORIDE

Fluoxetini hydrochloridum

C₁₇H₁₉ClF₃NOM_r 345.8

DEFINITION

Fluoxetine hydrochloride contains not less than 98.0 per cent and not more than the equivalent of 102.0 per cent of (3*RS*)-*N*-methyl-3-phenyl-3-[4-(trifluoromethyl)phenoxy]propan-1-amine hydrochloride, calculated with reference to the anhydrous, acetonitrile-free substance.

CHARACTERS

A white or almost white, crystalline powder, sparingly soluble in water, freely soluble in methanol, sparingly soluble in methylene chloride.

IDENTIFICATION

A. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *fluoxetine hydrochloride CRS*. Examine the substances prepared as discs.

B. It gives reaction (a) of chlorides (2.3.1).

TESTS

Solution S. Dissolve 2.0 g in a mixture of 15 volumes of *water R* and 85 volumes of *methanol R* and dilute to 100 ml with the same mixture of solvents.

Appearance of solution. Solution S is clear (2.2.1) and colourless (2.2.2, *Method II*).

pH (2.2.3). Dissolve 0.20 g in *carbon dioxide-free water R* and dilute to 20 ml with the same solvent. The pH of the solution is 4.5 to 6.5.

Optical rotation (2.2.7): −0.05° to +0.05°, determined on solution S.

Related substances. Examine by liquid chromatography (2.2.29) as prescribed under Assay, setting the spectrophotometer at 215 nm.

Test solution (a). Dissolve 55.0 mg of the substance to be examined in the mobile phase and dilute to 10.0 ml with the mobile phase.

Test solution (b). Dilute 2.0 ml of test solution (a) to 10.0 ml with the mobile phase.

Reference solution. Dissolve 22.0 mg of *fluoxetine hydrochloride CRS* in 10.0 ml of 0.5 M *sulphuric acid*. Heat at about 85 °C for 3 h. Allow to cool. The resulting solution contains considerable quantities of impurity A and 4-trifluoromethylphenol. To 0.4 ml of the solution, add 28.0 mg of *fluoxetine hydrochloride CRS* and about 1 mg of *fluoxetine impurity B CRS* and of *fluoxetine impurity C CRS* and dilute to 25.0 ml with the mobile phase. When the chromatograms are recorded in the prescribed conditions, the retention times relative to fluoxetine are: impurity A about 0.24, impurity B about 0.27 and impurity C about 0.94.

Inject 10 µl of the reference solution.

The test is not valid unless: in the chromatogram obtained with the reference solution, the retention time of the peak due to fluoxetine is 10 min to 18 min; the retention time of the peak due to 4-trifluoromethylphenol is not greater than 35 min; the h/v ratio is not greater than 1.1 (h is the distance between the top of the peak due to impurity C and the baseline; v is the distance between the top of the peak due to impurity C and the lowest point of the valley defined between the peak due to impurity C and the peak due to fluoxetine). If the ratio is greater than 1.1, reduce the volume of methanol and increase the volume of the solution of triethylamine in the mobile phase.

Inject 10 µl of test solution (a) and 10 µl of test solution (b). Continue the chromatography for 3 times the retention time of fluoxetine.

In the chromatogram obtained with test solution (b), the area of any peak due to impurity C is not greater than 0.0015 times the area of the principal peak (0.15 per cent).

In the chromatogram obtained with test solution (a): the areas of any peaks due to impurity A and impurity B are not greater than 0.0125 times the area of the principal peak in the chromatogram obtained with test solution (b) (0.25 per cent); none of the peaks, except the principal peak and the peaks due to impurity A and impurity B, has an area greater than 0.005 times the area of the principal peak in the chromatogram obtained with test solution (b) (0.1 per cent); the sum of the areas of all the peaks, apart from the principal peak, is not greater than 0.025 times the area of the principal peak in the chromatogram obtained with test solution (b) (0.5 per cent).

Disregard any peak with an area less than 0.0025 times that of the principal peak in the chromatogram obtained with test solution (b).

Acetonitrile. Not more than 0.1 per cent, determined by gas chromatography (2.2.28).

Test solution. Dissolve 50 mg of the substance to be examined in *dimethylformamide R* and dilute to 5.0 ml with the same solvent.

Reference solution. To 1.0 g of *acetonitrile R*, add *dimethylformamide R*, mix and dilute to 100.0 ml with the same solvent. Dilute 1.0 ml of the solution to 1000.0 ml with *dimethylformamide R*.

The chromatographic procedure may be carried out using:

- a fused-silica capillary column 30 m long and about 0.53 mm in internal diameter coated with *macrogol 20 000 R* (1 µm thick),
- *helium for chromatography R* as the carrier gas at a flow rate of 10 ml/min,
- a flame-ionisation detector,

maintaining the temperature of the column at 35 °C for 2 min then raising the temperature at a rate of 15 °C/min to 220 °C and maintaining at 220 °C for 10 min and maintaining the temperature of the injection port and that of the detector at 250 °C.

Inject 1 µl of the test solution, 1 µl of the reference solution and 1 µl of the dissolution solvent. In the chromatogram obtained with the reference solution, note the retention time of acetonitrile.

In the chromatogram obtained with the dissolution solvent, verify that there is no peak with the same retention time as acetonitrile.

The area of the peak due to acetonitrile in the chromatogram obtained with the test solution is not greater than that of the corresponding peak in the chromatogram obtained with the reference solution.

Heavy metals (2.4.8). 1.0 g complies with limit test C (20 ppm). Prepare the standard using 2 ml of *lead standard solution (10 ppm Pb) R*.

Water (2.5.12). Not more than 0.5 per cent, determined on 1.00 g by the semi-micro determination of water.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 55.0 mg of the substance to be examined in the mobile phase and dilute to 50.0 ml with the mobile phase. Dilute 10.0 ml of the solution to 100.0 ml with the mobile phase.

Reference solution. Dissolve 55.0 mg of *fluoxetine hydrochloride CRS* in the mobile phase and dilute to 50.0 ml with the mobile phase. Dilute 10.0 ml of the solution to 100.0 ml with the mobile phase.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4.6 mm in internal diameter packed with *octylsilyl silica gel for chromatography R* (5 µm),
- as mobile phase at a flow rate of 1 ml/min a mixture of 8 volumes of *methanol R*, 30 volumes of *tetrahydrofuran R* and 62 volumes of a solution of *triethylamine R* prepared as follows: to 10 ml of *triethylamine R*, add 980 ml of *water R*, mix and adjust to pH 6.0 with *phosphoric acid R* (about 4.5 ml) and dilute to 1000 ml with *water R*,
- as detector a spectrophotometer set at 227 nm.

When using a recorder, adjust the sensitivity of the system so that the height of the principal peak in the chromatogram obtained with the reference solution is at least 50 per cent of the full scale of the recorder.

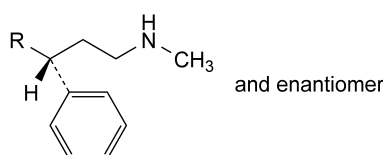
Adjust the volumes of methanol and the solution of triethylamine in the mobile phase so that the retention time of fluoxetine is between 10 min and 18 min.

The assay is not valid unless the symmetry factor calculated at 10 per cent of the height of the peak due to fluoxetine is at most 2.0.

Inject 10 µl of the test solution and 10 µl of the reference solution.

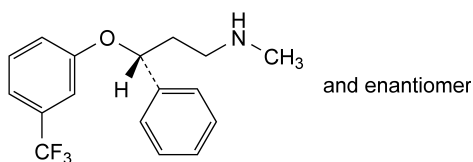
Calculate the content of fluoxetine hydrochloride ($C_{17}H_{19}ClF_3NO$) from the area of the peaks in the chromatograms obtained with the test solution and the reference solution using the stated content of $C_{17}H_{19}ClF_3NO$ in *fluoxetine hydrochloride CRS* and correcting for the content of water and of acetonitrile in the substance to be examined.

IMPURITIES



A. R = OH: (1*RS*)-3-(methylamino)-1-phenylpropan-1-ol,

B. R = H: *N*-methyl-3-phenylpropan-1-amine,

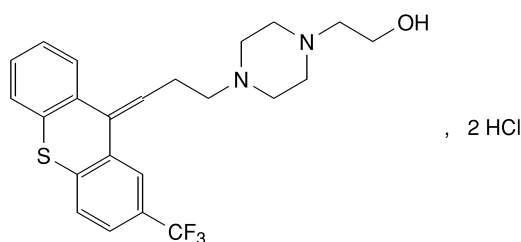


C. (3*RS*)-*N*-methyl-3-phenyl-3-[3-(trifluoromethyl)phenoxy]propan-1-amine.

01/2005:1693

FLUPENTIXOL DIHYDROCHLORIDE

Flupentixoli dihydrochloridum



$C_{23}H_{27}Cl_2F_3N_2OS$

M_r 507.4

DEFINITION

2-[4-[3-[(*EZ*)-2-(trifluoromethyl)-9*H*-thioxanthen-9-ylidene]propyl]piperazin-1-yl]ethanol dihydrochloride.

Content:

- flupentixol dihydrochloride: 98.0 per cent to 101.5 per cent (dried substance),
- *Z*-isomer: 42.0 per cent to 52.0 per cent.

CHARACTERS

Appearance: white or almost white powder.

Solubility: very soluble in water, soluble in alcohol, practically insoluble in methylene chloride.

IDENTIFICATION

First identification: A, D.

Second identification: B, C, D.

A. Infrared absorption spectrophotometry (2.2.24).

Comparison: flupentixol dihydrochloride CRS.

B. Thin-layer chromatography (2.2.27).

Test solution. Dissolve 20 mg of the substance to be examined in methanol *R* and dilute to 10 ml with the same solvent.

Reference solution. Dissolve 20 mg of flupentixol dihydrochloride CRS in methanol *R* and dilute to 10 ml with the same solvent.

Plate: TLC silica gel F_{254} plate *R*.

Mobile phase: water *R*, diethylamine *R*, methyl ethyl ketone *R* (1:4:95 V/V/V).

Application: 2 μ l.

Development: twice over a path of 15 cm.

Drying: in air.

Detection A: examine in ultraviolet light at 254 nm.

Results A: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with the reference solution. Doubling of the spot may be observed in both chromatograms.

Detection B: spray with alcoholic solution of sulphuric acid *R*; heat at 110 °C for 5 min and allow to cool; examine in ultraviolet light at 365 nm.

Results B: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with the reference solution. Doubling of the spot may be observed in both chromatograms.

C. Mix about 5 mg with 45 mg of heavy magnesium oxide *R* and ignite in a crucible until an almost white residue is obtained (usually less than 5 min). Allow to cool, add 1 ml of water *R*, 0.05 ml of phenolphthalein solution *R1* and about 1 ml of dilute hydrochloric acid *R* to render the solution colourless. Filter and add to the filtrate a freshly prepared mixture of 0.1 ml of alizarin *S* solution *R* and 0.1 ml of zirconyl nitrate solution *R*. Mix, allow to stand for 5 min and compare the colour of the solution with that of a blank prepared in the same manner. The test solution is yellow. The blank is red.

D. It gives reaction (a) of chlorides (2.3.1).

TESTS

Appearance of solution. The solution is clear (2.2.1) and not more intensely coloured than reference solution GY₆ (2.2.2, Method II).

Dissolve 2.0 g of the substance to be examined in water *R* and dilute to 20 ml with the same solvent.

pH (2.2.3): 2.0 to 3.0.

Dissolve 0.5 g in carbon dioxide-free water *R* and dilute to 50 ml with the same solvent.

Related substances. Thin-layer chromatography (2.2.27). Carry out the test protected from light and prepare the solutions immediately before use.

Test solution (a). Dissolve 0.40 g of the substance to be examined in alcohol *R* and dilute to 20 ml with the same solvent.

Test solution (b). Dilute 2.0 ml of test solution (a) to 20.0 ml with alcohol *R*.

Reference solution (a). Dilute 1.0 ml of test solution (b) to 50.0 ml with alcohol *R*.

Reference solution (b). Dilute 2.0 ml of reference solution (a) to 20.0 ml with alcohol *R*.

Reference solution (c). Dissolve 10 mg of flupentixol impurity D CRS in alcohol *R*, add 0.5 ml of test solution (a) and dilute to 20.0 ml with alcohol *R*.

Plate: TLC silica gel F_{254} plate *R*.

Mobile phase: diethylamine *R*, toluene *R*, ethyl acetate *R* (10:20:70 V/V/V).

Application: 5 μ l.

Development: in an unsaturated tank over a path of 10 cm.

Drying: in air.

Detection: spray with alcoholic solution of sulphuric acid *R*, heat at 110 °C for 5 min and allow to cool; examine in ultraviolet light at 365 nm. Doubling of the spot due to flupentixol may be observed.

System suitability: the chromatogram obtained with reference solution (c) shows 2 clearly separated spots.

Limits:

- in the chromatogram obtained with test solution (a): any spots, apart from the principal spot, are not more intense than the spot, or spots in the chromatogram obtained with reference solution (a) (0.2 per cent),

- in the chromatogram obtained with test solution (b): any spots, apart from the principal spot, are not more intense than the spot or spots in the chromatogram obtained with reference solution (b) (0.2 per cent).

Impurity F. Liquid chromatography (2.2.29). Carry out the test protected from light and prepare the solutions immediately before use.

Test solution. Dissolve 20.0 mg of the substance to be examined in the mobile phase and dilute to 20.0 ml with the mobile phase.

Reference solution. Dissolve 10.0 mg of flupentixol dihydrochloride CRS and 10.0 mg of flupentixol impurity F CRS in the mobile phase and dilute to 100.0 ml with the mobile phase. Dilute 1.0 ml of the solution to 20.0 ml with the mobile phase.

Column:

- size: $l = 0.125$ m, $\varnothing = 4.6$ mm,
- stationary phase: octylsilyl silica gel for chromatography R (3 μ m)

Mobile phase: mix 10 volumes of acetonitrile R, 55 volumes of methanol R and 35 volumes of a solution containing 8.72 g/l of potassium dihydrogen phosphate R, 0.37 g/l of anhydrous disodium hydrogen phosphate R and 0.77 g/l of dodecyltrimethylammonium bromide R.

Flow rate: 1.0 ml/min.

Detection: spectrophotometer at 270 nm.

Injection: 20 μ l.

System suitability: reference solution:

- resolution: minimum 2.0 between the 2nd of the peaks due to impurity F and the 1st of the peaks due to flupentixol. Peak splitting may not always occur.

Limit:

- impurity F: not more than the area of the corresponding peak or peaks in the chromatogram obtained with the reference solution (0.5 per cent).

Heavy metals (2.4.8): maximum 20 ppm.

1.0 g complies with limit test C. Prepare the standard using 2 ml of lead standard solution (10 ppm Pb) R.

Loss on drying (2.2.32): maximum 2.0 per cent, determined on 1.000 g by drying in an oven at 100–105 °C.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g in a platinum crucible.

ASSAY

Flupentixol dihydrochloride. Dissolve 0.200 g in 30 ml of alcohol R. Carry out a potentiometric titration (2.2.20), using 0.1 M sodium hydroxide. Read the volume added between the 2 points of inflexion.

1 ml of 0.1 M sodium hydroxide is equivalent to 50.74 mg of C₂₃H₂₇Cl₂F₃N₂OS.

Z-Isomer. Liquid chromatography (2.2.29).

Test solution. Dissolve 20.0 mg of the substance to be examined in the mobile phase and dilute to 50.0 ml with the mobile phase.

Reference solution. Dissolve 20.0 mg of flupentixol dihydrochloride CRS in the mobile phase and dilute to 50.0 ml with the mobile phase.

Column:

- size: $l = 0.25$ m, $\varnothing = 4.0$ mm,

- stationary phase: silica gel for chromatography R (5 μ m).

Mobile phase: water R, concentrated ammonia R, 2-propanol R, heptane R (2:4:150:850 V/V/V/V).

Flow rate: 1.5 ml/min.

Detection: spectrophotometer at 254 nm.

Injection: 20 μ l.

System suitability: reference solution:

- resolution: minimum 3.0 between the peaks due to Z-isomer (1st peak) and to E-isomer (2nd peak).

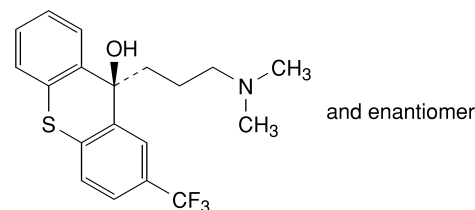
Results:

- calculate the percentage content of Z-isomer taking into account the assigned content of Z-isomer in flupentixol dihydrochloride CRS,
- calculate the ratio of the area of the peak due to the E-isomer to the area of the peak due to the Z-isomer: this ratio is 0.9 to 1.4.

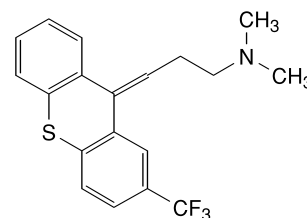
STORAGE

Protected from light.

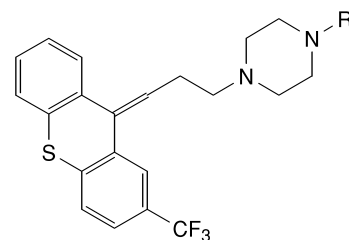
IMPURITIES



A. (9RS)-9-[3-(dimethylamino)propyl]-2-(trifluoromethyl)-9H-thioxanthen-9-ol,



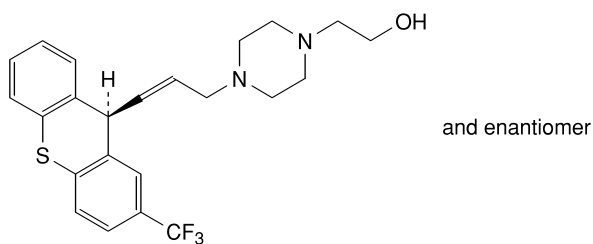
B. N,N-dimethyl-3-[(EZ)-2-(trifluoromethyl)-9H-thioxanthen-9-ylidene]propan-1-amine,



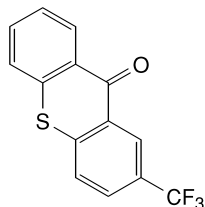
C. R = H: 1-[3-[(EZ)-2-(trifluoromethyl)-9H-thioxanthen-9-ylidene]propyl]piperazine,

D. R = CH₂-CH₂-O-CH₂-CH₂-OH: 2-[2-[4-[3-[(EZ)-2-(trifluoromethyl)-9H-thioxanthen-9-ylidene]propyl]piperazin-1-yl]ethoxy]ethanol,

E. R = CH₂-CH₂-O-CO-CH₃: 2-[4-[3-[(EZ)-2-(trifluoromethyl)-9H-thioxanthen-9-ylidene]propyl]piperazin-1-yl]ethyl acetate,



F. 2-[4-[(*EZ*)-3-[(9*RS*)-2-(trifluoromethyl)-9*H*-thioxanthen-9-yl]prop-2-enyl]piperazin-1-yl]ethanol,

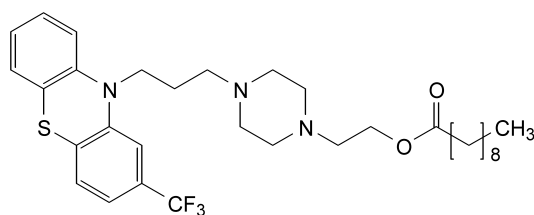


G. 2-(trifluoromethyl)-9*H*-thioxanthen-9-one.

01/2005:1014

FLUPHENAZINE DECANOATE

Fluphenazini decanoas



$C_{32}H_{44}F_3N_3O_2S$

M_r 591.8

DEFINITION

2-[4-[3-[2-(Trifluoromethyl)-10*H*-phenothiazin-10-yl]propyl]piperazin-1-yl]ethyl decanoate.

Content: 98.5 per cent to 101.5 per cent (dried substance).

CHARACTERS

Appearance: pale yellow, viscous liquid or yellow solid.

Solubility: practically insoluble in water, very soluble in ethanol and in methylene chloride, freely soluble in methanol.

IDENTIFICATION

First identification: B, C.

Second identification: A, C.

A. Dissolve 50.0 mg in *methanol R* and dilute to 100.0 ml with the same solvent. Dilute 1.0 ml to 50.0 ml with *methanol R*. Examined between 230 nm and 350 nm (2.2.25), the solution shows an absorption maximum at 260 nm and a broad absorption maximum at about 310 nm. The specific absorbance at the maximum at 260 nm is 570 to 630.

B. Infrared absorption spectrophotometry (2.2.24).

Preparation: apply 50 µl of a 25 g/l solution in *methylene chloride R* to a disc of *potassium bromide R*. Dry the discs at 60 °C for 1 h before use.

Comparison: fluphenazine decanoate CRS.

C. Thin-layer chromatography (2.2.27).

Test solution. Dissolve 10 mg of the substance to be examined in *methanol R* and dilute to 10 ml with the same solvent.

Reference solution (a). Dissolve 10 mg of fluphenazine decanoate CRS in *methanol R* and dilute to 10 ml with the same solvent.

Reference solution (b). Dissolve 5 mg of fluphenazine enantate CRS in reference solution (a) and dilute to 5 ml with the same solution.

Plate: TLC octadecylsilyl silica gel F_{254} plate R.

Mobile phase: concentrated ammonia R1, water R, *methanol R* (1:4:95 V/V/V).

Application: 2 µl.

Development: over a path of 8 cm.

Detection: examine in ultraviolet light at 254 nm.

System suitability: the chromatogram obtained with reference solution (b) shows 2 clearly separated spots.

Results: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a).

TESTS

Related substances. Liquid chromatography (2.2.29). Carry out the test protected from light and prepare the solutions immediately before use.

Test solution. Dissolve 10.0 mg of the substance to be examined in *acetonitrile R* and dilute to 50.0 ml with the same solvent.

Reference solution (a). Dissolve 5 mg of fluphenazine octanoate CRS and 5 mg of fluphenazine enantate CRS in *acetonitrile R* and dilute to 50 ml with the same solvent.

Reference solution (b). Dilute 5.0 ml of the test solution to 100.0 ml with a mixture of 5 volumes of mobile phase A and 95 volumes of mobile phase B. Dilute 1.0 ml of this solution to 10.0 ml with a mixture of 5 volumes of mobile phase A and 95 volumes of mobile phase B.

Reference solution (c). Dissolve 11.7 mg of fluphenazine dihydrochloride CRS and 5.0 mg of fluphenazine sulphoxide CRS in a mixture of 5 volumes of *water R* and 95 volumes of *acetonitrile R* and dilute to 100.0 ml with the same mixture of solvents. Dilute 1.0 ml to 50.0 ml with a mixture of 5 volumes of *water R* and 95 volumes of *acetonitrile R*.

Column:

- size: $l = 0.25$ m, $\varnothing = 4.6$ mm,
- stationary phase: spherical octadecylsilyl silica gel for chromatography R (5 µm).

Mobile phase:

- mobile phase A: 10 g/l solution of ammonium carbonate R adjusted to pH 7.5 with dilute hydrochloric acid R,
- mobile phase B: mobile phase A, *acetonitrile R*, *methanol R* (7.5:45:45 V/V/V),

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)
0 - 7	20	80
7 - 17	20 → 0	80 → 100
17 - 80	0	100
80 - 81	0 → 20	100 → 80

Flow rate: 1.0 ml/min.

Detection: spectrophotometer at 260 nm.

01/2005:1015

Equilibration: at least 30 min with the mobile phase at the initial composition.

Injection: 10 µl.

Relative retention with reference to fluphenazine decanoate (retention time = about 34 min): impurity A = about 0.13; impurity B = about 0.33; impurity C = about 0.76; impurity D = about 0.82.

System suitability: reference solution (a):

- **resolution:** minimum 6 between the peaks due to impurity C and impurity D.

Limits:

- **impurity A:** not more than the area of the corresponding peak in the chromatogram obtained with reference solution (c) (0.5 per cent),
- **impurity B:** not more than the area of the corresponding peak in the chromatogram obtained with reference solution (c) (1.0 per cent),
- **any other impurity:** not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent),
- **total:** not more than 2.0 per cent,
- **disregard limit for any other impurity:** 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

Heavy metals (2.4.8): maximum 20 ppm.

1.0 g complies with limit test C. Prepare the standard using 2 ml of *lead standard solution* (10 ppm Pb) R.

Loss on drying (2.2.32): maximum 1.0 per cent, determined on 1.000 g by drying in an oven at 60 °C at a pressure not exceeding 0.7 kPa for 3 h.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g in a platinum crucible.

ASSAY

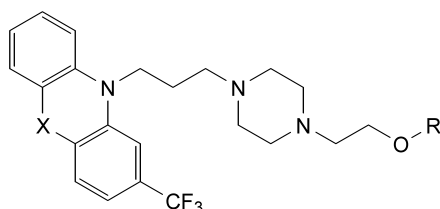
Dissolve 0.250 g in 30 ml of *glacial acetic acid* R. Using 0.05 ml of *crystal violet solution* R as indicator, titrate with 0.1 M *perchloric acid* until the colour changes from violet to green.

1 ml of 0.1 M *perchloric acid* is equivalent to 29.59 mg of C₂₉H₃₈F₃N₃O₂S.

STORAGE

Protected from light.

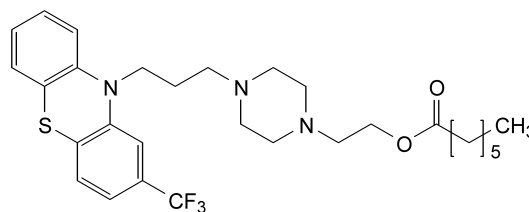
IMPURITIES



- X = SO, R = H: fluphenazine S-oxide,
- X = S, R = H: fluphenazine,
- X = S, R = CO-[CH₂]₅-CH₃: fluphenazine enantate,
- X = S, R = CO-[CH₂]₆-CH₃: fluphenazine octanoate,
- X = S, R = CO-[CH₂]₇-CH₃: fluphenazine nonanoate,
- X = S, R = CO-[CH₂]₉-CH₃: fluphenazine undecanoate,
- X = S, R = CO-[CH₂]₁₀-CH₃: fluphenazine dodecanoate.

FLUPHENAZINE ENANTATE

Fluphenazini enantas

C₂₉H₃₈F₃N₃O₂SM_r 549.7

DEFINITION

2-[4-[3-[2-(Trifluoromethyl)-10H-phenothiazin-10-yl]propyl]piperazin-1-yl]ethyl heptanoate.

Content: 98.5 per cent to 101.5 per cent (dried substance).

CHARACTERS

Appearance: pale yellow, viscous liquid or yellow solid.

Solubility: practically insoluble in water, very soluble in ethanol and in methylene chloride, freely soluble in methanol.

IDENTIFICATION

First identification: B, C.

Second identification: A, C.

A. Dissolve 50.0 mg in *methanol* R and dilute to 100.0 ml with the same solvent. Dilute 1.0 ml to 50.0 ml with *methanol* R. Examined between 230 nm and 350 nm (2.2.25), the solution shows an absorption maximum at 260 nm and a broad absorption maximum at about 310 nm. The specific absorbance at the maximum at 260 nm is 610 to 670.

B. Infrared absorption spectrophotometry (2.2.24).

Preparation: apply 50 µl of a 25 g/l solution in *methylene chloride* R to a disc of *potassium bromide* R. Dry the discs at 60 °C for 1 h before use.

Comparison: *fluphenazine enantate* CRS.

C. Thin-layer chromatography (2.2.27).

Test solution. Dissolve 10 mg of the substance to be examined in *methanol* R and dilute to 10 ml with the same solvent.

Reference solution (a). Dissolve 10 mg of *fluphenazine enantate* CRS in *methanol* R and dilute to 10 ml with the same solvent.

Reference solution (b). Dissolve 5 mg of *fluphenazine decanoate* CRS in reference solution (a) and dilute to 5 ml with the same solution.

Plate: *TLC octadecylsilyl silica gel F₂₅₄* plate R.

Mobile phase: concentrated ammonia R1, water R, *methanol* R (1:4:95 V/V/V).

Application: 2 µl.

Development: over a path of 8 cm.

Detection: examine in ultraviolet light at 254 nm.

System suitability: the chromatogram obtained with reference solution (b) shows 2 clearly separated spots.

Results: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a).

TESTS

Related substances. Liquid chromatography (2.2.29). Carry out the test protected from light and prepare the solutions immediately before use.

Test solution. Dissolve 10.0 mg of the substance to be examined in *acetonitrile R* and dilute to 50.0 ml with the same solvent.

Reference solution (a). Dissolve 5 mg of *fluphenazine octanoate CRS* and 5 mg of *fluphenazine enantate CRS* in *acetonitrile R* and dilute to 50 ml with the same solvent.

Reference solution (b). Dilute 5.0 ml of the test solution to 100.0 ml with a mixture of 5 volumes of mobile phase A and 95 volumes of mobile phase B. Dilute 1.0 ml of this solution to 10.0 ml with a mixture of 5 volumes of mobile phase A and 95 volumes of mobile phase B.

Reference solution (c). Dissolve 5.0 mg of *fluphenazine sulphoxide CRS* in *acetonitrile R* and dilute to 100.0 ml with the same solvent. Dilute 1.0 ml to 50.0 ml with *acetonitrile R*.

Column:

- size: $l = 0.25$ m, $\varnothing = 4.6$ mm,
- stationary phase: spherical octadecylsilyl silica gel for chromatography *R* (5 μ m).

Mobile phase:

- mobile phase A: 10 g/l solution of ammonium carbonate *R* adjusted to pH 7.5 with dilute hydrochloric acid *R*,
- mobile phase B: mobile phase A, *acetonitrile R*, methanol *R* (7.5:45:45 V/V/V),

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)
0 - 7	20	80
7 - 17	20 $\rightarrow$ 0	80 $\rightarrow$ 100
17 - 80	0	100
80 - 81	0 $\rightarrow$ 20	100 $\rightarrow$ 80

Flow rate: 1.0 ml/min.

Detection: spectrophotometer at 260 nm.

Equilibration: at least 30 min with the mobile phase at the initial composition.

Injection: 10 μ l.

Relative retention with reference to fluphenazine enantate (retention time = about 25 min): impurity A = about 0.2; impurity D = about 1.1.

System suitability: reference solution (a):

- resolution: minimum 6 between the peaks due to fluphenazine enantate and impurity D.

Limits:

- impurity A: not more than the area of the principal peak in the chromatogram obtained with reference solution (c) (0.5 per cent),
- any other impurity: not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent),
- total: not more than 1.6 per cent,
- disregard limit for any other impurity: 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

Heavy metals (2.4.8): maximum 20 ppm.

1.0 g complies with limit test C. Prepare the standard using 2 ml of lead standard solution (10 ppm Pb) *R*.

Loss on drying (2.2.32): maximum 1.0 per cent, determined on 1.000 g by drying in an oven at 60 °C at a pressure not exceeding 0.7 kPa for 3 h.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g in a platinum crucible.

ASSAY

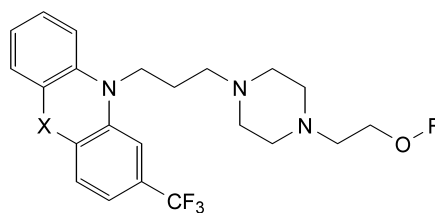
Dissolve 0.250 g in 30 ml of *glacial acetic acid R*. Using 0.05 ml of *crystal violet solution R* as indicator titrate with 0.1 M *perchloric acid* until the colour changes from violet to green.

1 ml of 0.1 M *perchloric acid* is equivalent to 27.49 mg of $C_{29}H_{38}F_3N_3O_2S$.

STORAGE

Protected from light.

IMPURITIES

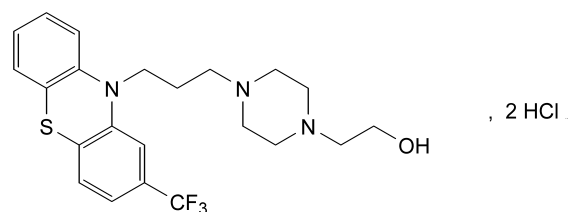


- A. X = SO, R = H: fluphenazine S-oxide,
 B. X = S, R = H: fluphenazine,
 C. X = S, R = CO-[CH₂]₈-CH₃: fluphenazine decanoate,
 D. X = S, R = CO-[CH₂]₆-CH₃: fluphenazine octanoate,
 E. X = S, R = CO-[CH₂]₇-CH₃: fluphenazine nonanoate,
 F. X = S, R = CO-[CH₂]₉-CH₃: fluphenazine undecanoate,
 G. X = S, R = CO-[CH₂]₁₀-CH₃: fluphenazine dodecanoate.

01/2005:0904

FLUPHENAZINE HYDROCHLORIDE

Fluphenazini hydrochloridum


 $C_{22}H_{28}Cl_2F_3N_3OS$
 M_r 510.5

DEFINITION

Fluphenazine hydrochloride contains not less than 98.5 per cent and not more than the equivalent of 101.0 per cent of 2-[4-[3-[2-(trifluoromethyl)-10H-phenothiazin-10-yl]propyl]piperazin-1-yl]ethanol dihydrochloride, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, crystalline powder, freely soluble in water, slightly soluble in alcohol and in methylene chloride.

IDENTIFICATION

First identification: B, E.

Second identification: A, C, D, E.

- A. Dissolve 50.0 mg in *methanol R* and dilute to 100.0 ml with the same solvent. Dilute 2.0 ml to 100.0 ml with *methanol R*. Examined between 230 nm and 350 nm (2.2.25), the solution shows an absorption maximum at 260 nm and a broad absorption maximum at about 310 nm. The specific absorbance at the maximum at 260 nm is 630 to 700.
- B. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *fluphenazine hydrochloride CRS*.
- C. Examine by thin-layer chromatography (2.2.27), using *silica gel GF₂₅₄ R* as the coating substance.
Test solution. Dissolve 20 mg of the substance to be examined in *methanol R* and dilute to 10 ml with the same solvent.
Reference solution (a). Dissolve 20 mg of *fluphenazine hydrochloride CRS* in *methanol R* and dilute to 10 ml with the same solvent.
Reference solution (b). Dissolve 10 mg of *perphenazine CRS* in reference solution (a) and dilute to 5 ml with the same solution.
 Apply to the plate 2 µl of each solution. Develop twice over a path of 15 cm using a mixture of 1 volume of *water R*, 4 volumes of *diethylamine R* and 95 volumes of *methyl ethyl ketone R*. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. The principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a). The test is not valid unless the chromatogram obtained with reference solution (b) shows two clearly separated principal spots.
- D. Mix about 5 mg with 45 mg of *heavy magnesium oxide R* and ignite in a crucible until an almost white residue is obtained (usually less than 5 min). Allow to cool, add 1 ml of *water R*, 0.05 ml of *phenolphthalein solution R1* and about 1 ml of *dilute hydrochloric acid R* to render the solution colourless. Filter. Add 1.0 ml of the filtrate to a freshly prepared mixture of 0.1 ml of *alizarin S solution R* and 0.1 ml of *zirconyl nitrate solution R*. Mix, allow to stand for 5 min and compare the colour of the solution with that of a blank prepared in the same manner. The test solution is yellow and the blank is red.
- E. It gives reaction (a) of chlorides (2.3.1).

TESTS

pH (2.2.3). Dissolve 0.5 g in 10 ml of *carbon dioxide-free water R*. The pH of the solution is 1.9 to 2.4.

Related substances. Carry out the test protected from light and prepare the solutions immediately before use. Examine by thin-layer chromatography (2.2.27), using *silica gel GF₂₅₄ R* as the coating substance.

Test solution. Dissolve 0.20 g of the substance to be examined in a mixture of 5 volumes of *diethylamine R* and 95 volumes of *methanol R* and dilute to 10 ml with the same mixture of solvents.

Reference solution (a). Dilute 1 ml of the test solution to 100 ml with a mixture of 5 volumes of *diethylamine R* and 95 volumes of *methanol R*.

Reference solution (b). Dilute 5 ml of reference solution (a) to 10 ml with a mixture of 5 volumes of *diethylamine R* and 95 volumes of *methanol R*.

Apply to the plate 10 µl of each solution. Develop over a path of 12 cm using a mixture of 10 volumes of *acetone R*, 10 volumes of *diethylamine R* and 80 volumes of *cyclohexane R*. Allow the plate to dry in air and examine in

ultraviolet light at 254 nm. Any spot in the chromatogram obtained with the test solution, apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (a) (1.0 per cent) and at most one such spot is more intense than the spot in the chromatogram obtained with reference solution (b) (0.5 per cent).

Heavy metals (2.4.8). 1.0 g complies with limit test C for heavy metals (20 ppm). Prepare the standard using 2 ml of *lead standard solution (10 ppm Pb) R*.

Loss on drying (2.2.32). Not more than 1.0 per cent, determined on 0.500 g by drying in an oven at 65 °C for 3 h.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

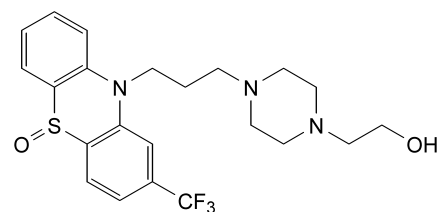
Dissolve 0.220 g in a mixture of 10 ml of *anhydrous formic acid R* and 40 ml of *acetic anhydride R*. Titrate with 0.1 M *perchloric acid*, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *perchloric acid* is equivalent to 25.52 mg of C₂₂H₂₈Cl₂F₃N₃OS.

STORAGE

Store protected from light.

IMPURITIES

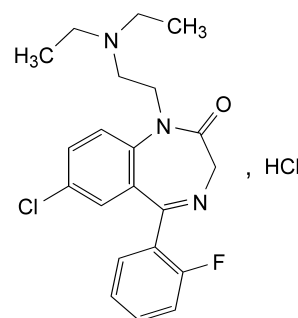


A. fluphenazine S-oxide.

01/2005:0905

FLURAZEPAM MONOHYDROCHLORIDE

Flurazepami monohydrochloridum



C₂₁H₂₄Cl₂FN₃O

M_r 424.3

DEFINITION

7-Chloro-1-[2-(diethylamino)ethyl]-5-(2-fluorophenyl)-1,3-dihydro-2H-1,4-benzodiazepin-2-one monohydrochloride.

Content: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

Appearance: white or almost white, crystalline powder.

Solubility: very soluble in water, freely soluble in alcohol.

IDENTIFICATION

A. Infrared absorption spectrophotometry (2.2.24).

Comparison: Ph. Eur. reference spectrum of flurazepam monohydrochloride.

B. It gives reaction (a) of chlorides (2.3.1).

TESTS

pH (2.2.3): 5.0 to 6.0.

Dissolve 0.50 g in carbon dioxide-free water R and dilute to 10 ml with the same solvent.

Related substances. Liquid chromatography (2.2.29). Prepare the solutions immediately before use.

Test solution. Dissolve 50.0 mg of the substance to be examined in the mobile phase and dilute to 50.0 ml with the mobile phase.

Reference solution (a). Dilute 1.0 ml of the test solution to 100.0 ml with the mobile phase. Dilute 5.0 ml of this solution to 50.0 ml with the mobile phase.

Reference solution (b). Dissolve 5 mg of the substance to be examined and 5 mg of oxazepam R in 10 ml of acetonitrile R and dilute to 50.0 ml with the mobile phase.

Column:

- size: $l = 0.15$ m, $\varnothing = 4.6$ mm,
- stationary phase: base-deactivated octylsilyl silica gel for chromatography R (5 μ m).

Mobile phase: mix 350 volumes of acetonitrile R and 650 volumes of a 10.5 g/l solution of potassium dihydrogen phosphate R and adjust to pH 6.1 with a 40 g/l solution of sodium hydroxide R.

Flow rate: 1.0 ml/min.

Detection: spectrophotometer at 239 nm.

Injection: 20 μ l.

Run time: 6 times the retention time of flurazepam.

Relative retention with reference to flurazepam (retention time = about 7 min): impurity C = about 1.5; impurity B = about 1.9; impurity A = about 2.4.

System suitability: reference solution (b):

- resolution: minimum of 4.5 between the peaks due to flurazepam and to oxazepam.

Limits:

- *correction factors:* for the calculation of contents, multiply the peak areas of the following impurities by the corresponding correction factor: impurity B = 0.61; impurity C = 0.65,
- *any impurity:* not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent),
- *total:* not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.3 per cent),
- *disregard limit:* 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

Fluorides (2.4.5): maximum 500 ppm.

0.10 g complies with the limit test for fluorides.

Loss on drying (2.2.32): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 100–105 °C for 4 h.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

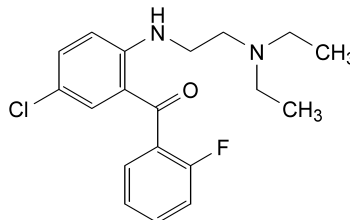
Dissolve 0.350 g in a mixture of 1.0 ml of 0.1 M hydrochloric acid and 50 ml of alcohol R. Carry out a potentiometric titration (2.2.20), using 0.1 M sodium hydroxide. Read the volume added between the 2 points of inflexion.

1 ml of 0.1 M sodium hydroxide is equivalent to 42.43 mg of $C_{21}H_{24}Cl_2FN_3O$.

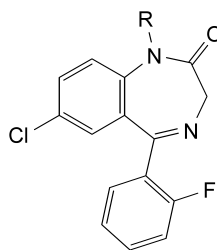
STORAGE

Protected from light.

IMPURITIES



A. [5-chloro-2-[[2-(diethylamino)ethyl]amino]phenyl](2-fluorophenyl)methanone,



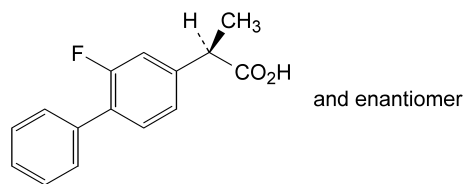
B. R = H: 7-chloro-5-(2-fluorophenyl)-1,3-dihydro-2H-1,4-benzodiazepin-2-one,

C. R = CHOH-CH_3 : 7-chloro-5-(2-fluorophenyl)-1-[(1R)-1-hydroxyethyl]-1,3-dihydro-2H-1,4-benzodiazepin-2-one.

01/2005:1519

FLURBIPROFEN

Flurbiprofenum



and enantiomer

 $C_{15}H_{13}FO_2$
 M_r 244.3

DEFINITION

Flurbiprofen contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of (2RS)-2-(2-fluorobiphenyl-4-yl)propanoic acid, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, crystalline powder, practically insoluble in water, freely soluble in alcohol and in methylene chloride. It dissolves in aqueous solutions of alkali hydroxides and carbonates.

IDENTIFICATION

First identification: C, D.

Second identification: A, B, D.

- A. Melting point (2.2.14): 114 °C to 117 °C.
- B. Dissolve 0.10 g in 0.1 M sodium hydroxide and dilute to 100.0 ml with the same alkaline solution. Dilute 1.0 ml of the solution to 100.0 ml with 0.1 M sodium hydroxide. Examined between 230 nm and 350 nm (2.2.25), the solution shows an absorption maximum at 247 nm. The specific absorbance at the maximum is 780 to 820.
- C. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with flurbiprofen CRS.
- D. Mix about 5 mg with 45 mg of heavy magnesium oxide R and ignite in a crucible until an almost white residue is obtained (usually less than 5 min). Allow to cool, add 1 ml of water R, 0.05 ml of phenolphthalein solution R1 and about 1 ml of dilute hydrochloric acid R to render the solution colourless. Filter. To a freshly prepared mixture of 0.1 ml of alizarin S solution R and 0.1 ml of zirconyl nitrate solution R add 1.0 ml of the filtrate. Mix, allow to stand for 5 min and compare the colour of the solution with that of a blank prepared in the same manner. The test solution is yellow and the blank is red.

TESTS

Appearance of solution. Dissolve 1.0 g in methanol R and dilute to 10 ml with the same solvent. The solution is clear (2.2.1) and colourless (2.2.2, Method I).

Optical rotation (2.2.7). Dissolve 0.50 g in methanol R and dilute to 20.0 ml with the same solvent. The angle of optical rotation is -0.1° to $+0.1^{\circ}$.

Related substances. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 0.20 g of the substance to be examined in a mixture of 45 volumes of acetonitrile R and 55 volumes of water R and dilute to 100.0 ml with the same mixture of solvents.

Reference solution (a). Dilute 1.0 ml of the test solution to 50.0 ml with a mixture of 45 volumes of acetonitrile R and 55 volumes of water R. Dilute 1.0 ml of the solution to 10.0 ml with a mixture of 45 volumes of acetonitrile R and 55 volumes of water R.

Reference solution (b). Dissolve 10.0 mg of flurbiprofen impurity A CRS in a mixture of 45 volumes of acetonitrile R and 55 volumes of water R and dilute to 100.0 ml with the same mixture of solvents. Dilute 10.0 ml of this solution to 100.0 ml with a mixture of 45 volumes of acetonitrile R and 55 volumes of water R.

Reference solution (c). Dissolve 10 mg of the substance to be examined in a mixture of 45 volumes of acetonitrile R and 55 volumes of water R and dilute to 100.0 ml with the same mixture of solvents. Dilute 1.0 ml of the solution to 10.0 ml with reference solution (b).

The chromatographic procedure may be carried out using:

- a stainless steel column 0.15 m long and 3.9 mm in internal diameter packed with octadecylsilyl silica gel for chromatography R (5 µm),
- as mobile phase at a flow rate of 1 ml/min a mixture of 5 volumes of glacial acetic acid R, 35 volumes of acetonitrile R and 60 volumes of water R,
- as detector a spectrophotometer set at 254 nm.

Inject 10 µl of reference solution (c). Adjust the sensitivity of the system so that the heights of the two principal peaks in the chromatogram obtained are at least 40 per cent of

the full scale of the recorder. The test is not valid unless the resolution between the peak corresponding to impurity A and the peak corresponding to flurbiprofen is at least 1.5.

Inject 10 µl of the test solution and of reference solutions (a) and (b). Continue the chromatography for twice the retention time of flurbiprofen. In the chromatogram obtained with the test solution the area of any peak corresponding to impurity A is not greater than the area of the peak in the chromatogram obtained with reference solution (b) (0.5 per cent); the area of any peak, apart from the principal peak and the peak due to impurity A, is not greater than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent) and the sum of the areas of any such peaks is not greater than five times the area of the principal peak in the chromatogram obtained with reference solution (a) (1.0 per cent). Disregard any peak with an area less than 0.1 times that of the principal peak in the chromatogram obtained with reference solution (a) (0.02 per cent).

Heavy metals (2.4.8). Dissolve 2.0 g in a mixture of 10 volumes of water R and 90 volumes of methanol R and dilute to 20 ml with the same mixture of solvents. 12 ml of the solution complies with limit test B for heavy metals (10 ppm). Prepare the standard using lead standard solution (1 ppm Pb) obtained by diluting lead standard solution (100 ppm Pb) R with a mixture of 10 volumes of water R and 90 volumes of methanol R.

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying at 60 °C at a pressure not exceeding 0.7 kPa for 3 h.

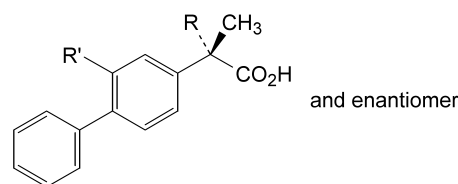
Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g in a platinum crucible.

ASSAY

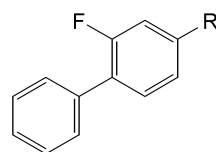
Dissolve 0.200 g in 50 ml of alcohol R. Titrate with 0.1 M sodium hydroxide, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M sodium hydroxide is equivalent to 24.43 mg of C₁₅H₁₃FO₂.

IMPURITIES



- A. R = R' = H: (2*RS*)-2-(biphenyl-4-yl)propanoic acid,
- B. R = CH(CH₃)-CO₂H: 2-(2-fluorobiphenyl-4-yl)-2,3-dimethylbutanedioic acid,
- C. R = OH, R' = F: (2*RS*)-2-(2-fluorobiphenyl-4-yl)-2-hydroxypropanoic acid,

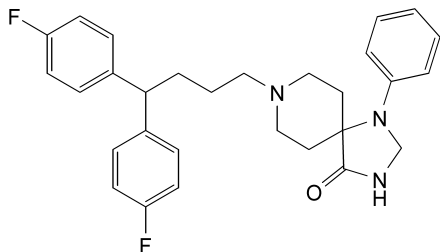


- D. R = CO-CH₃: 1-(2-fluorobiphenyl-4-yl)ethanone,
- E. R = CO₂H: 2-fluorobiphenyl-4-carboxylic acid.

01/2005:1723 – stationary phase: octadecylsilyl silica gel for chromatography R (3 µm).

FLUSPIRILENE

Fluspirilenum



C₂₉H₃₁F₂N₃O

M_r 475.6

DEFINITION

8-[4,4-bis(4-Fluorophenyl)butyl]-1-phenyl-1,3,8-triazaspiro[4.5]decan-4-one.

Content: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

Appearance: white or almost white powder.

Solubility: practically insoluble in water, soluble in methylene chloride, slightly soluble in alcohol.

It shows polymorphism.

IDENTIFICATION

Infrared absorption spectrophotometry (2.2.24).

Preparation: discs.

Comparison: fluspirilene CRS.

If the spectra obtained show differences, dissolve the substance to be examined and the reference substance separately in *methylene chloride R*, gently evaporate to dryness and record new spectra using the residues.

TESTS

Appearance of solution. The solution is clear (2.2.1) and colourless (2.2.2, Method II).

Dissolve 0.25 g in 25 ml of *methylene chloride R*.

Related substances. Liquid chromatography (2.2.29).

Test solution. Dissolve 0.100 g of the substance to be examined in *dimethylformamide R* and dilute to 10.0 ml with the same solvent.

Reference solution (a). Dissolve 5.0 mg of *fluspirilene impurity C CRS* in *dimethylformamide R*, add 0.5 ml of the test solution and dilute to 100.0 ml with *dimethylformamide R*.

Reference solution (b). Dilute 1.0 ml of the test solution to 20.0 ml with *dimethylformamide R*. Dilute 1.0 ml of this solution to 25.0 ml with *dimethylformamide R*.

Column:

– size: *l* = 0.15 m, Ø = 4.6 mm,

Mobile phase:

– mobile phase A: 13.6 g/l solution of *tetrabutylammonium hydrogen sulphate R*,

– mobile phase B: *acetonitrile R*,

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)
0 - 15	75 → 70	25 → 30
15 - 20	70	30
20 - 22	70 → 0	30 → 100
22 - 30	0	100
30 - 31	0 → 75	100 → 25
31 - 40	75	25

Flow rate: 1.2 ml/min.

Detection: spectrophotometer at 250 nm.

Injection: 10 µl.

Relative retention with reference to fluspirilene (retention time = about 15 min): impurity A = about 0.8; impurity B = about 0.93; impurity C = 0.97.

System suitability: reference solution (a):

– resolution: minimum 2.2 between the peaks due to impurity C and fluspirilene.

Limits:

- impurities A, B, C: for each impurity, not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.3 per cent),
- any other impurity: not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent),
- total: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.6 per cent),
- disregard limit: 0.25 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

Loss on drying (2.2.32): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 100-105 °C for 4 h.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g in a platinum crucible.

ASSAY

Dissolve 0.350 g in 50 ml of a mixture of 1 volume of *anhydrous acetic acid R* and 7 volumes of *methyl ethyl ketone R*. Titrate with 0.1 M *perchloric acid*, determining the end-point potentiometrically (2.2.20). Carry out a blank titration.

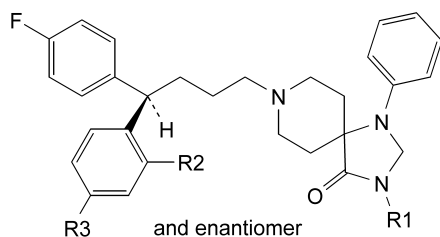
1 ml of 0.1 M *perchloric acid* is equivalent to 47.56 mg of C₂₉H₃₁F₂N₃O.

STORAGE

Protected from light.

IMPURITIES

Specified impurities: A, B, C.

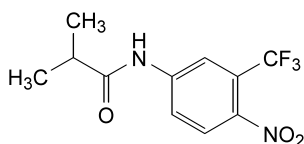


- A. R1 = R2 = R3 = H: 8-[(4*RS*)-4-(4-fluorophenyl)-4-phenylbutyl]-1-phenyl-1,3,8-triazaspiro[4.5]decan-4-one,
- B. R1 = R3 = H, R2 = F: 8-[(4*RS*)-4-(2-fluorophenyl)-4-(4-fluorophenyl)butyl]-1-phenyl-1,3,8-triazaspiro[4.5]decan-4-one,
- C. R1 = CH₂OH, R2 = H, R3 = F: 8-[4,4-bis(4-fluorophenyl)butyl]-3-(hydroxymethyl)-1-phenyl-1,3,8-triazaspiro[4.5]decan-4-one.

01/2005:1423

FLUTAMIDE

Flutamidum

C₁₁H₁₁F₃N₂O₃M_r 276.2

DEFINITION

Flutamide contains not less than 97.0 per cent and not more than the equivalent of 103.0 per cent of 2-methyl-*N*-[4-nitro-3-(trifluoromethyl)phenyl]propanamide, calculated with reference to the dried substance.

CHARACTERS

A pale yellow, crystalline powder, practically insoluble in water, freely soluble in acetone and in alcohol.

It melts at about 112 °C.

IDENTIFICATION

Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *flutamide CRS*.

TESTS

Related substances. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 20.0 mg of the substance to be examined in the mobile phase and dilute to 20.0 ml with the mobile phase.

Reference solution (a). Dissolve 2 mg of *flutamide CRS* and 2 mg of *flutamide impurity C CRS* in the mobile phase and dilute to 50.0 ml with the mobile phase. Dilute 1.0 ml of this solution to 20.0 ml with the mobile phase.

Reference solution (b). Dilute 1.0 ml of the test solution to 50.0 ml with the mobile phase. Dilute 2.0 ml of this solution to 20.0 ml with the mobile phase.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4.0 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (5 µm),
- as mobile phase at a flow rate of 0.5 ml/min a mixture of equal volumes of *acetonitrile R* and *water R*,
- as detector a spectrophotometer set at 240 nm.

Inject 20 µl of reference solution (b). Adjust the sensitivity of the system so that the height of the principal peak in the chromatogram obtained is not less than 50 per cent of the full scale of the recorder.

Inject 20 µl of reference solution (a). When the chromatogram is recorded in the prescribed conditions, the retention times are: flutamide about 19 min and impurity C about 14 min. The test is not valid unless the resolution between the peaks corresponding to impurity C and flutamide is at least 10.5.

Inject 20 µl of the test solution and 20 µl of reference solution (b). Continue the chromatography for 1.5 times the retention time of the principal peak. In the chromatogram obtained with the test solution: the area of the peak corresponding to impurity C, with a retention time of about 0.72 relative to flutamide, is not greater than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.3 per cent); the area of any other peak apart from the principal peak and the peak corresponding to impurity C is not greater than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent); the sum of the areas of the peaks apart from the principal peak is not greater than 2.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent). Disregard any peak with an area less than 0.25 times the area of the principal peak in the chromatogram obtained with reference solution (b).

Heavy metals (2.4.8). 1.0 g complies with limit test C for heavy metals (20 ppm). Prepare the standard using 2 ml of *lead standard solution* (10 ppm Pb) *R*.

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying *in vacuo* at 60 °C for 3 h.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

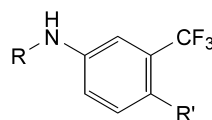
Dissolve 25.0 mg in *methanol R* and dilute to 25.0 ml with the same solvent. Dilute 2.0 ml of this solution to 100.0 ml with *methanol R*. Measure the absorbance (2.2.25) at the maximum at 295 nm.

Calculate the content of C₁₁H₁₁F₃N₂O₃ taking the specific absorbance to be 295.

STORAGE

Store protected from light.

IMPURITIES



- A. R = H, R' = NO₂: 4-nitro-3-(trifluoromethyl)aniline,
- B. R = CO-CH₃, R' = NO₂: *N*-[4-nitro-3-(trifluoromethyl)phenyl]acetamide,
- C. R = CO-CH₂-CH₃, R' = NO₂: *N*-[4-nitro-3-(trifluoromethyl)phenyl]propanamide,
- D. R = R' = H: 3-(trifluoromethyl)aniline,

Temperature:

	Time (min)	Temperature (°C)
Column	0 - 3.5	60
	3.5 - 7.5	60 → 180
	7.5 - 10.5	180
Injection port		150
Detector		250

Detection: flame ionisation.

Injection: 0.1 µl.

Limit:

– acetone: maximum 1.0 per cent *m/m*.

Water (2.5.12): maximum 0.5 per cent determined on 0.250 g.

Use as solvent a mixture of equal volumes of *chloroform R* and *methanol R*.

ASSAY

Liquid chromatography (2.2.29).

Test solution. Dissolve 20.0 mg of the substance to be examined in the mobile phase and dilute to 50.0 ml with the mobile phase. Dilute 1.0 ml to 10.0 ml with the mobile phase.

Reference solution (a). Dissolve 20.0 mg of *fluticasone propionate CRS* in the mobile phase and dilute to 50.0 ml with the mobile phase.

Reference solution (b). Dilute 1.0 ml of reference solution (a) to 10.0 ml with the mobile phase.

Reference solution (c). Dissolve 4.0 mg of *fluticasone impurity D CRS* in the mobile phase and dilute to 50.0 ml with the mobile phase. To 1.0 ml of this solution, add 1.0 ml of reference solution (a) and dilute to 10.0 ml with the mobile phase.

Column:

- size: *l* = 0.25 m, Ø = 4.6 mm,
- stationary phase: octadecylsilyl silica gel for chromatography *R* (5 µm),
- temperature: 40 °C.

Mobile phase: mix 15 volumes of *acetonitrile R*, 35 volumes of a 1.15 g/l solution of *ammonium dihydrogen phosphate R* adjusted to pH 3.5 and 50 volumes of *methanol R*.

Flow rate: 1.5 ml/min.

Detection: spectrophotometer at 239 nm.

Injection: 20 µl; inject the test solution and reference solutions (b) and (c).

System suitability: reference solution (c):

- resolution: minimum 1.5 between the peaks due to impurity D and to fluticasone propionate.

If necessary, adjust the ratio of acetonitrile to methanol in the mobile phase.

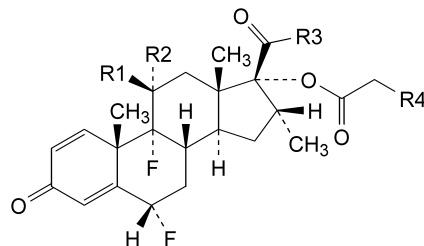
Calculate the percentage content of $C_{25}H_{31}F_3O_5S$ using the chromatograms obtained with the test solution and reference solution (b), and the declared content of *fluticasone propionate CRS*.

STORAGE

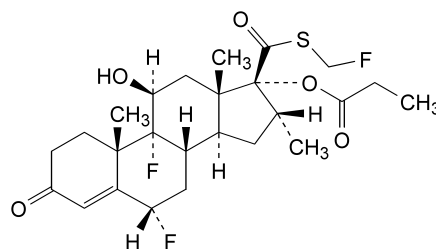
Protected from light.

IMPURITIES

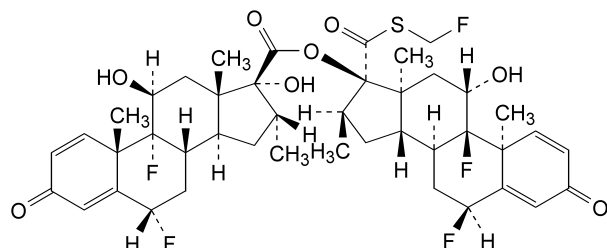
Specified impurities: A, B, C, D, E, F, G, H, I.



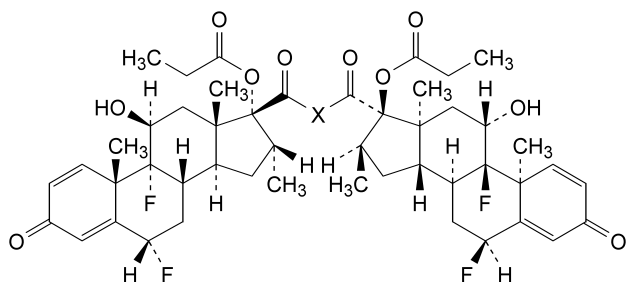
- A. R1 = R3 = OH, R2 = H, R4 = CH₃: 6α,9-difluoro-11β-hydroxy-16α-methyl-3-oxo-17-(propanoyloxy)androsta-1,4-diene-17β-carboxylic acid,
- B. R1 = OH, R2 = H, R3 = S-OH, R4 = CH₃: [[6α,9-difluoro-11β-hydroxy-16α-methyl-3-oxo-17-(propanoyloxy)androsta-1,4-dien-17β-yl]carbonyl]sulphenic acid,
- C. R1 = OH, R2 = R4 = H, R3 = S-CH₂-F: 6α,9-difluoro-17-[(fluoromethyl)sulphonyl]carbonyl]-11β-hydroxy-16α-methyl-3-oxoandrosta-1,4-dien-17α-yl acetate,
- D. R1 = OH, R2 = H, R3 = S-CH₃, R4 = CH₃: 6α,9-difluoro-17-[(methylsulphonyl)carbonyl]-11β-hydroxy-16α-methyl-3-oxoandrosta-1,4-dien-17α-yl propanoate,
- F. R1 + R2 = O, R3 = S-CH₂-F, R4 = CH₃: 6α,9-difluoro-17-[(fluoromethyl)sulphonyl]carbonyl]-16α-methyl-3,11-dioxoandrosta-1,4-dien-17α-yl propanoate,



- E. 6α,9-difluoro-17-[(fluoromethyl)sulphonyl]carbonyl]-11β-hydroxy-16α-methyl-3-oxoandrosta-1,4-dien-17α-yl propanoate,



- G. 6α,9-difluoro-17-[(fluoromethyl)sulphonyl]carbonyl]-11β-hydroxy-16α-methyl-3-oxoandrosta-1,4-dien-17α-yl 6α,9-difluoro-11β,17-dihydroxy-16α-methyl-3-oxoandrosta-1,4-diene-17β-carboxylate,

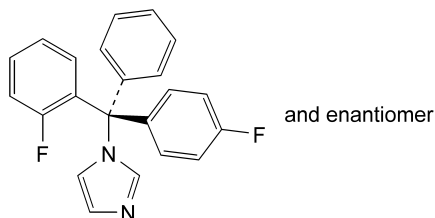


- H. X = S-S: 17,17'-(disulphanediyl)dicarbonylbis(6 α ,9-difluoro-11 β -hydroxy-16 α -methyl-3-oxoandrosta-1,4-dien-17 α -yl) dipropanoate,
- I. X = S-S-S: 17,17'-(trisulphanediyl)dicarbonylbis(6 α ,9-difluoro-11 β -hydroxy-16 α -methyl-3-oxoandrosta-1,4-dien-17 α -yl) dipropanoate.

01/2005:1424

FLUTRIMAZOLE

Flutrimazolum

C₂₂H₁₆F₂N₂M_r 346.4

DEFINITION

Flutrimazole contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of (RS)-1-[(2-fluorophenyl)(4-fluorophenyl)phenylmethyl]-1H-imidazole, calculated with reference to the dried substance.

CHARACTERS

A white or almost white powder, practically insoluble in water, freely soluble in tetrahydrofuran and soluble in methanol.

IDENTIFICATION

First identification: B.

Second identification: A, C, D.

- A. Melting point (2.2.14): 161 °C to 166 °C.
- B. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *flutrimazole CRS*. Examine the substances prepared as discs.
- C. Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel F₂₅₄ plate R*. Heat the plate at 110 °C for 1 h.

Test solution. Dissolve 20 mg of the substance to be examined in *acetone R* and dilute to 10 ml with the same solvent.

Reference solution (a). Dissolve 20 mg of *flutrimazole CRS* in *acetone R* and dilute to 10 ml with the same solvent.

Reference solution (b). Dissolve 20 mg of *flutrimazole CRS* and 10 mg of *metronidazole benzoate CRS* in *acetone R* and dilute to 10 ml with the same solvent.

Apply separately to the plate 10 μ l of each solution. Develop over a path corresponding to two-thirds of the height of the plate using a mixture of 10 volumes of *2-propanol R* and 90 volumes of *ethyl acetate R*. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. The principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a). The test is not valid unless the chromatogram obtained with reference solution (b) shows two clearly separated spots.

- D. Mix about 5 mg with 45 mg of *heavy magnesium oxide R* and ignite in a crucible until an almost white residue is obtained (usually less than 5 min). Allow to cool, add 1 ml of *water R*, 0.05 ml of *phenolphthalein solution R1* and about 1 ml of *dilute hydrochloric acid R* to render the solution colourless. Filter. Add 1.0 ml of the filtrate to a freshly prepared mixture of 0.1 ml of *alizarin S solution R* and 0.1 ml of *zirconyl nitrate solution R*. Mix, allow to stand for 5 min and compare the colour of the solution with that of a blank prepared in the same manner. The test solution is yellow and the blank is red.

TESTS

Solution S. Dissolve 1.00 g in *methanol R* and dilute to 50.0 ml with the same solvent.

Appearance of solution. Solution S is not more opalescent than reference suspension II (2.2.1) and not more intensely coloured than reference solution Y₇ (2.2.2, *Method II*).

Optical rotation (2.2.7). The angle of optical rotation, determined on solution S, is -0.05° to +0.05°.

Related substances. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 40.0 mg of the substance to be examined in the mobile phase and dilute to 50.0 ml with the mobile phase.

Reference solution (a). Dissolve 25.0 mg of *imidazole CRS* in the mobile phase and dilute to 50.0 ml with the mobile phase. Dilute 10.0 ml of this solution to 50.0 ml with the mobile phase.

Reference solution (b). Dissolve 30.0 mg of *flutrimazole impurity B CRS* in the mobile phase and dilute to 100.0 ml with the mobile phase.

Reference solution (c). Mix 2.0 ml of reference solution (a) and 2.0 ml of reference solution (b) and dilute to 50.0 ml with the mobile phase.

Reference solution (d). Dilute 10.0 ml of reference solution (c) to 50.0 ml with the mobile phase.

Reference solution (e). Mix 2.0 ml of the test solution and 10.0 ml of reference solution (c) and dilute to 50.0 ml with the mobile phase.

Reference solution (f). Dilute 1.0 ml of the test solution to 100.0 ml with the mobile phase. Dilute 1.0 ml of this solution to 10.0 ml with the mobile phase.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.2 m long and 4.6 mm in internal diameter packed with *octylsilyl silica gel for chromatography R* (5 μ m),
- as mobile phase at a flow rate of 1.3 ml/min a mixture of 40 volumes of 0.03 M *phosphate buffer solution pH 7.0 R* and 60 volumes of *acetonitrile R*,
- as detector a spectrophotometer set at 220 nm.

Inject 20 μ l of reference solution (e). The test is not valid unless the resolution between the first peak (imidazole) and the second peak (impurity B) is at least 2.0 and the resolution

01/2005:0067

between the second peak and the third peak (flutrimazole) is at least 1.5. The symmetry factor of the first peak and the second peak is not greater than 2.0.

Inject separately 20 µl of the test solution, 20 µl of reference solution (d) and 20 µl of reference solution (f). Continue the chromatography for 2.5 times the retention time of the principal peak.

In the chromatogram obtained with the test solution:

- the area of the peak due to imidazole is not greater than the corresponding area in the chromatogram obtained with reference solution (d) (0.1 per cent);
- the area of the peak due to impurity B is not greater than the corresponding area in the chromatogram obtained with reference solution (d) (0.3 per cent);
- the area of any peak, apart from the principal peak and any peak corresponding to impurity B, is not greater than the area of the principal peak in the chromatogram obtained with reference solution (f) (0.1 per cent); the sum of the areas of any such peaks is not greater than three times the area of the principal peak in the chromatogram obtained with reference solution (f) (0.3 per cent). Disregard any peak with an area less than half the area of the principal peak in the chromatogram obtained with reference solution (f).

Heavy metals (2.4.8). 2.0 g complies with limit test F for heavy metals (10 ppm). Use a platinum crucible. Prepare the standard using 2 ml of *lead standard solution* (10 ppm Pb) R.

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g in a platinum crucible.

ASSAY

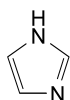
Dissolve 0.300 g in 50 ml of *anhydrous acetic acid* R. Titrate with 0.1 M *perchloric acid* determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *perchloric acid* is equivalent to 34.64 mg of C₁₉H₁₉N₇O₆.

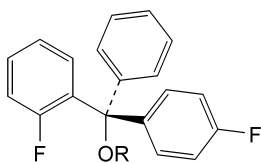
STORAGE

Store protected from light.

IMPURITIES



A. imidazole,

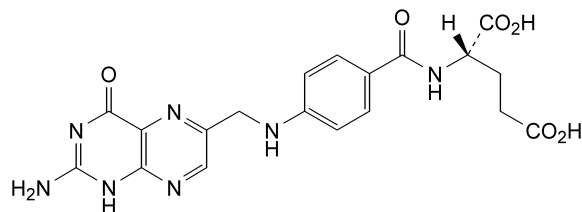


B. R = H: (RS)-(2-fluorophenyl)(4-fluorophenyl)phenylmethanol,

C. R = CH₃: (RS)-(2-fluorophenyl)(4-fluorophenyl)methoxyphenylmethane.

FOLIC ACID

Acidum folicum



C₁₉H₁₉N₇O₆

M_r 441.4

DEFINITION

(2S)-2-[[4-[[[(2-amino-4-oxo-1,4-dihydropteridin-6-yl)methyl]amino]benzoyl]amino]pentanedioic acid.

Content: 96.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

Appearance: yellowish or orange, crystalline powder.

Solubility: practically insoluble in water and in most organic solvents. It dissolves in dilute acids and in alkaline solutions.

IDENTIFICATION

First identification: A, B.

Second identification: A, C.

A. Specific optical rotation (2.2.7): + 18 to + 22 (anhydrous substance).

Dissolve 0.25 g in 0.1 M *sodium hydroxide* and dilute to 25.0 ml with the same solvent.

B. Examine the chromatograms obtained in the assay.

Results: the principal peak in the chromatogram obtained with the test solution is similar in retention time to the principal peak in the chromatogram obtained with reference solution (a).

C. Thin-layer chromatography (2.2.27).

Test solution. Dissolve 50 mg of the substance to be examined in a mixture of 2 volumes of *concentrated ammonia* R and 9 volumes of *methanol* R and dilute to 100 ml with the same mixture of solvents.

Reference solution. Dissolve 50 mg of *folic acid CRS* in a mixture of 2 volumes of *concentrated ammonia* R and 9 volumes of *methanol* R and dilute to 100 ml with the same mixture of solvents.

Plate: TLC silica gel G plate R.

Mobile phase: *concentrated ammonia* R, *propanol* R, *alcohol* R (20:20:60 V/V/V).

Application: 2 µl.

Development: over 3/4 of the plate.

Drying: in air.

Detection: ultraviolet light at 365 nm.

Results: the principal spot in the chromatogram obtained with the test solution is similar in position, fluorescence and size to the principal spot in the chromatogram obtained with the reference solution.

TESTS

Related substances. Liquid chromatography (2.2.29).

Test solution. Dissolve 0.100 g of the substance to be examined in 5 ml of a 28.6 g/l solution of *sodium carbonate* R and dilute to 100.0 ml with the mobile phase.

Dilute 2.0 ml of this solution to 10.0 ml with the mobile phase.

Reference solution (a). Dissolve 0.100 g of *folic acid CRS* in 5 ml of a 28.6 g/l solution of *sodium carbonate R* and dilute to 100.0 ml with the mobile phase. Dilute 2.0 ml of this solution to 10.0 ml with the mobile phase.

Reference solution (b). Dissolve 20 mg of *pteroic acid R* in 5 ml of a 28.6 g/l solution of *sodium carbonate R* and dilute to 100.0 ml with the mobile phase. Mix 1.0 ml of this solution with 1.0 ml of reference solution (a) and dilute to 100.0 ml with the mobile phase.

Reference solution (c). Dilute 2.0 ml of the test solution to 20.0 ml with the mobile phase. Dilute 1.0 ml of this solution to 20.0 ml with the mobile phase.

Reference solution (d). Dissolve 10.0 mg of *N*-(4-aminobenzoyl)-L-glutamic acid *R* in 1 ml of a 28.6 g/l solution of *sodium carbonate R* and dilute to 100.0 ml with the mobile phase. Dilute 1.0 ml of this solution to 100.0 ml with the mobile phase.

Reference solution (e). Dissolve 12.0 mg of *pteroic acid R* in 1 ml of a 28.6 g/l solution of *sodium carbonate R* and dilute to 100.0 ml with the mobile phase. Dilute 1.0 ml of this solution to 100.0 ml with the mobile phase.

Column:

- **size:** $l = 0.25$ m, $\varnothing = 4.0$ mm,
- **stationary phase:** spherical *octylsilyl silica gel for chromatography R* (5 μ m) with a carbon loading of 12.5 per cent, a specific surface of 350 m²/g and a pore size of 10 nm.

Mobile phase: mix 12 volumes of *methanol R* and 88 volumes of a solution containing 11.16 g/l *potassium dihydrogen phosphate R* and 5.50 g/l of *dipotassium hydrogen phosphate R* solution.

Flow rate: 0.6 ml/min.

Detection: spectrophotometer at 280 nm.

Injection: 5 μ l; inject the test solution and reference solutions (b), (c) (d) and (e).

Run time: 3 times the retention time of folic acid.

Relative retention with reference to folic acid (retention time = about 8.5 min): impurity A = about 0.5; impurity B = about 0.6; impurity C = about 0.9; impurity E = about 1.27; impurity D = about 1.33; impurity F = about 2.2.

System suitability: reference solution (b):

- **resolution:** minimum 4.0 between the peaks due to folic acid and to impurity D.

Limits:

- **impurity A:** not more than the area of the principal peak in the chromatogram obtained with reference solution (d) (0.5 per cent),
- **impurity D:** not more than the area of the principal peak in the chromatogram obtained with reference solution (e) (0.6 per cent),
- **any other impurity:** not more than the area of the principal peak in the chromatogram obtained with reference solution (c) (0.5 per cent),
- **total of other impurities:** not more than 2 times the area of the principal peak in the chromatogram obtained with reference solution (c) (1.0 per cent),
- **disregard limit:** 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.05 per cent).

Water (2.5.12): 5.0 per cent to 8.5 per cent, determined on 0.150 g.

Sulphated ash (2.4.14): maximum 0.2 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (2.2.29) as described in the test for related substances with the following modification.

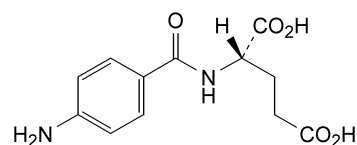
Injection: test solution and reference solution (a).

STORAGE

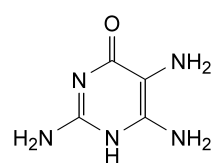
Protected from light.

IMPURITIES

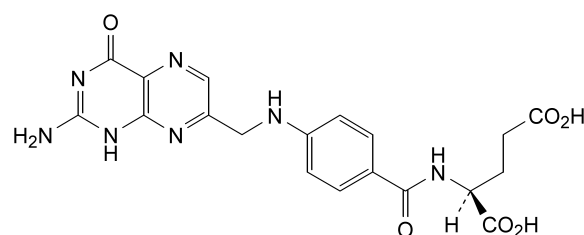
Specified impurities: A, B, C, D, E, F.



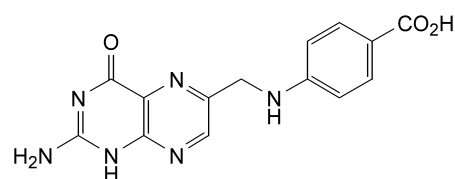
A. (2S)-2-[(4-aminobenzoyl)amino]pentanedioic acid (*N*-(4-aminobenzoyl)-L-glutamic acid),



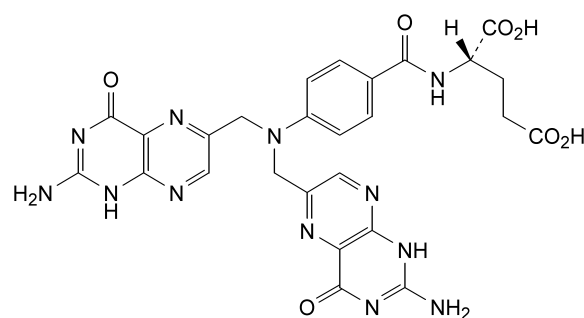
B. 2,5,6-triaminopyrimidin-4(1H)-one,



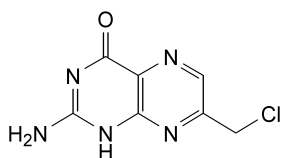
C. (2S)-2-[[4-[(2-amino-4-oxo-1,4-dihydropteridin-7-yl)methyl]amino]benzoyl]amino]pentanedioic acid (isofolic acid),



D. 4-[(2-amino-4-oxo-1,4-dihydropteridin-6-yl)methyl]amino]benzoic acid (pteroic acid),



E. (2S)-2-[[4-bis[(2-amino-4-oxo-1,4-dihydropteridin-6-yl)methyl]amino]benzoyl]amino]pentanedioic acid (6-pterinylfolic acid),

F. 2-amino-7-(chloromethyl)pteridin-4(1*H*)-one.

01/2005:0826

FORMALDEHYDE SOLUTION (35 PER CENT)

Formaldehydi solutio (35 per centum)

DEFINITION

Formaldehyde solution (35 per cent) contains not less than 34.5 per cent *m/m* and not more than 38.0 per cent *m/m* of formaldehyde (CH_2O ; M_r 30.03) with methanol as stabiliser.

CHARACTERS

A clear, colourless liquid, miscible with water and with alcohol. It may be cloudy after storage.

IDENTIFICATION

- Dilute 1 ml of solution S (see Tests) to 10 ml with *water R*. To 0.05 ml of the solution add 1 ml of a 15 g/l solution of *chromotropic acid, sodium salt R*, 2 ml of *water R* and 8 ml of *sulphuric acid R*. A violet-blue or violet-red colour develops within 5 min.
- To 0.1 ml of solution S add 10 ml of *water R*. Add 2 ml of a 10 g/l solution of *phenylhydrazine hydrochloride R*, prepared immediately before use, 1 ml of *potassium ferricyanide solution R* and 5 ml of *hydrochloric acid R*. An intense red colour is formed.
- Mix 0.5 ml with 2 ml of *water R* and 2 ml of *silver nitrate solution R2* in a test-tube. Add *dilute ammonia R2* until slightly alkaline. Heat on a water-bath. A grey precipitate or a silver mirror is formed.
- It complies with the limits of the assay.

TESTS

Solution S. Dilute 10 ml, filtered if necessary, to 50 ml with *carbon dioxide-free water R*.

Appearance of solution. Solution S is colourless (2.2.2, *Method II*).

Acidity. To 10 ml of solution S add 1 ml of *phenolphthalein solution R*. Not more than 0.4 ml of 0.1 *M* sodium hydroxide is required to change the colour of the indicator to red.

Methanol: 9.0 per cent *V/V* to 15.0 per cent *V/V*, determined by gas chromatography (2.2.28), using *ethanol R1* as the internal standard.

Internal standard solution. Dilute 10 ml of *ethanol R1* to 100 ml with *water R*.

Test solution. To 10.0 ml of the solution to be examined add 10.0 ml of the internal standard solution and dilute to 100.0 ml with *water R*.

Reference solution. To 1.0 ml of *methanol R* add 10.0 ml of the internal standard solution and dilute to 100.0 ml with *water R*.

The chromatographic procedure may be carried out using:

- a glass column 1.5 m to 2.0 m long and 2 mm to 4 mm in internal diameter packed with *ethylvinylbenzene-divinylbenzene copolymer R* (150 μm to 180 μm),

- nitrogen for chromatography R* as the carrier gas at a flow rate of 30 ml/min to 40 ml/min,
 - a flame-ionisation detector,
- maintaining the temperature of the column at 120 °C and that of the injection port and of the detector at 150 °C.

Inject 1 μl of the reference solution. Adjust the sensitivity of the detector so that the heights of the peaks in the chromatogram obtained are at least 50 per cent of the full scale of the recorder. The test is not valid unless the resolution between the peaks corresponding to methanol and ethanol is at least 2.0. Inject separately 1 μl of the test solution and 1 μl of the reference solution. Calculate the percentage content of methanol.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Into a 100 ml volumetric flask containing 2.5 ml of *water R* and 1 ml of *dilute sodium hydroxide solution R*, introduce 1.000 g of the solution to be examined, shake and dilute to 100.0 ml with *water R*. To 10.0 ml of the solution add 30.0 ml of 0.05 *M* iodine. Mix and add 10 ml of *dilute sodium hydroxide solution R*. After 15 min, add 25 ml of *dilute sulphuric acid R* and 2 ml of *starch solution R*. Titrate with 0.1 *M* sodium thiosulphate.

1 ml of 0.05 *M* iodine is equivalent to 1.501 mg of CH_2O .

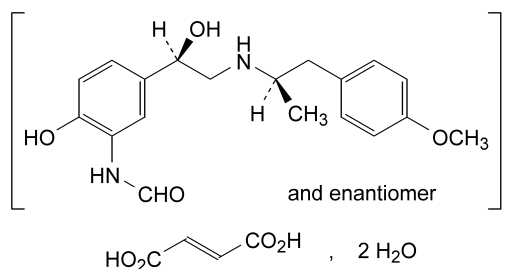
STORAGE

Store protected from light, at a temperature of 15 °C to 25 °C.

01/2005:1724

FORMOTEROL FUMARATE DIHYDRATE

Formoteroli fumaras dihydricus

 $\text{C}_{42}\text{H}_{52}\text{N}_4\text{O}_{12} \cdot 2\text{H}_2\text{O}$ M_r 841

DEFINITION

N-[2-Hydroxy-5-[(1*RS*)-1-hydroxy-2-[(1*RS*)-2-(4-methoxyphenyl)-1-methylethylamino]ethyl]phenyl]formamide (*E*)-butenedioate dihydrate.

Content: 98.5 per cent to 101.5 per cent (anhydrous substance).

CHARACTERS

Appearance: white or almost white or slightly yellow powder.

Solubility: slightly soluble in water, soluble in methanol, slightly soluble in 2-propanol, practically insoluble in acetonitrile.

IDENTIFICATION

Infrared absorption spectrophotometry (2.2.24).

Comparison: formoterol fumarate dihydrate CRS.

TESTS

pH (2.2.3): 5.5 to 6.5.

Dissolve 20 mg in *carbon dioxide-free water R* while heating to about 40 °C, allow to cool and dilute to 20 ml with the same solvent.

Optical rotation (2.2.7): -0.10° to $+0.10^{\circ}$.

Dissolve 0.25 g in *methanol R* and dilute to 25.0 ml with the same solvent.

Related substances. Liquid chromatography (2.2.29).

Solution A. Dissolve 6.10 g of *sodium dihydrogen phosphate monohydrate R* and 1.03 g of *disodium hydrogen phosphate dihydrate R* in *water R* and dilute to 1000 ml with the same solvent. The pH is 6.0 ± 0.1 .

Solvent mixture: *acetonitrile R*, solution A (16:84 V/V).

Test solution. Dissolve 20.0 mg of the substance to be examined in the solvent mixture and dilute to 100.0 ml with the solvent mixture. *Inject within 4 h of preparation, or within 24 h if stored protected from light at 4 °C.*

Reference solution (a). Dissolve 5 mg of *formoterol fumarate for system suitability CRS* (containing impurities A, B, C, D, E, F, G) in the solvent mixture and dilute to 25.0 ml with the solvent mixture.

Reference solution (b). Dilute 1.0 ml of the test solution to 25.0 ml with the solvent mixture. Dilute 1.0 ml of this solution to 20.0 ml with the solvent mixture.

Column:

- **size:** $l = 0.15$ m, $\emptyset = 4.6$ mm,
- **stationary phase:** spherical *octylsilyl silica gel for chromatography R3* (5 μ m) with a pore size of 8 nm.

Mobile phase:

- **mobile phase A:** *acetonitrile R1*;
- **mobile phase B:** dissolve 3.73 g of *sodium dihydrogen phosphate monohydrate R* and 0.35 g of *phosphoric acid R* in *water R* and dilute to 1000 ml with the same solvent; the pH is 3.1 ± 0.1 ;

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)
0 - 10	16	84
10 - 37	16 $\rightarrow$ 70	84 $\rightarrow$ 30
37 - 40	70 $\rightarrow$ 16	30 $\rightarrow$ 84
40 - 55	16	84

Flow rate: 1.0 ml/min.

Detection: spectrophotometer at 214 nm.

Injection: 20 μ l, inject the solvent mixture until a repeatable profile is obtained.

Identification of impurities: use the chromatogram obtained with reference solution (a) and the chromatogram supplied with *formoterol for system suitability CRS* to identify the peaks.

Relative retention with reference to formoterol (retention time = about 12 min): impurity G = about 0.4; impurity A = about 0.5; impurity B = about 0.7; impurity C = about 1.2; impurity D = about 1.3; impurity E = about 1.8; impurity F = about 2.0; impurity H = about 2.2.

System suitability: reference solution (a):

- **resolution:** minimum 1.5 between the peaks due to impurity G and impurity A.

- **peak-to-valley ratio:** minimum 2.5, where H_p = height above the baseline of the peak due to impurity C and H_v = height above the baseline of the lowest point of the curve separating this peak from the peak due to formoterol.

Limits:

- **correction factor:** for the calculation of content, multiply the peak area of impurity A by 1.75,
- **impurity A:** not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.3 per cent),
- **impurities B, C, D, F:** for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent),
- **impurity E:** not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent),
- **any other impurity:** for each impurity, not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent),
- **total:** not more than 2.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent),
- **disregard limit:** 0.25 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

Impurity I. Liquid chromatography (2.2.29).

Test solution. Dissolve 5.0 mg of the substance to be examined in *water R* and dilute to 50.0 ml with the same solvent. Sonicate if necessary.

Reference solution (a). Dissolve 5.0 mg of *formoterol for impurity I identification CRS* in *water R* and dilute to 50.0 ml with the same solvent. Sonicate if necessary.

Reference solution (b). Dilute 1.0 ml of the test solution to 20.0 ml with *water R*. Dilute 1.0 ml of this solution to 25.0 ml with *water R*.

Column:

- **size:** $l = 0.15$ m, $\emptyset = 4.6$ mm,
- **stationary phase:** *octadecyl vinyl polymer for chromatography R*.

Mobile phase: mix 12 volumes of *acetonitrile R1* with 88 volumes of a 5.3 g/l solution of *tripotassium phosphate trihydrate R* previously adjusted to pH 12.0 ± 0.1 with a 280 g/l solution of *potassium hydroxide R* or *phosphoric acid R*.

Flow rate: 0.5 ml/min.

Detection: spectrophotometer at 225 nm.

Injection: 20 μ l.

Elution order: formoterol, impurity I.

System suitability: reference solution (a):

- **peak-to-valley ratio:** minimum 3.5, where H_p = height above the baseline of the peak due to impurity I and H_v = height above the baseline of the lowest point of the curve separating this peak from the peak due to formoterol.

Limit:

- **impurity I:** not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.3 per cent),

Water (2.5.12): 4.0 per cent to 5.0 per cent, determined on 0.100 g.

ASSAY

Dissolve 0.350 g in 50 ml of *anhydrous acetic acid R*. Titrate with 0.1 M *perchloric acid*, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *perchloric acid* is equivalent to 40.24 mg of $C_{42}H_{52}N_4O_{12}$.

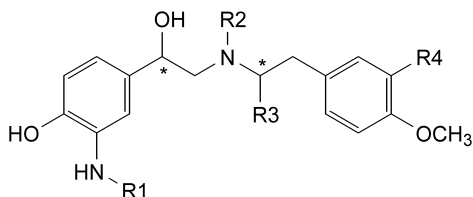
STORAGE

Protected from light.

IMPURITIES

Specified impurities: A, B, C, D, E, F, I.

Other detectable impurities: G, H.



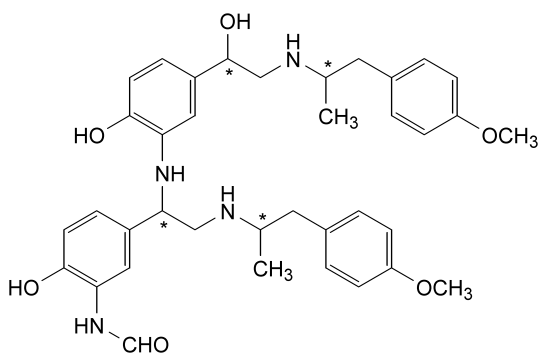
A. $R_1 = R_2 = R_4 = H, R_3 = CH_3$: 1-(3-amino-4-hydroxyphenyl)-2-[[2-(4-methoxyphenyl)-1-methylethyl]amino]ethanol,

B. $R_1 = CHO, R_2 = R_3 = R_4 = H$: *N*-[2-hydroxy-5-[(1*RS*)-1-hydroxy-2-[[2-(4-methoxyphenyl)ethyl]amino]ethyl]phenyl]formamide,

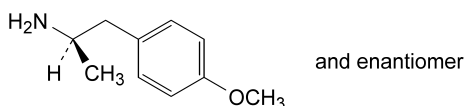
C. $R_1 = CO-CH_3, R_2 = R_4 = H, R_3 = CH_3$: *N*-[2-hydroxy-5-[1-hydroxy-2-[[2-(4-methoxyphenyl)-1-methylethyl]amino]ethyl]phenyl]acetamide,

D. $R_1 = CHO, R_2 = R_3 = CH_3, R_4 = H$: *N*-[2-hydroxy-5-[1-hydroxy-2-[methyl[2-(4-methoxyphenyl)-1-methylethyl]amino]ethyl]phenyl]formamide,

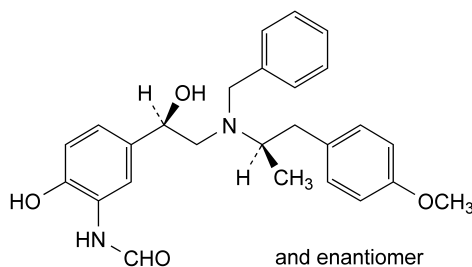
E. $R_1 = CHO, R_2 = H, R_3 = R_4 = CH_3$: *N*-[2-hydroxy-5-[1-hydroxy-2-[[2-(4-methoxy-3-methylphenyl)-1-methylethyl]amino]ethyl]phenyl]formamide,



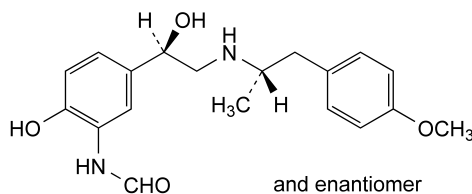
F. *N*-[2-hydroxy-5-[1-[[2-hydroxy-5-[1-hydroxy-2-[[2-(4-methoxyphenyl)-1-methylethyl]amino]ethyl]phenyl]amino]-2-[[2-(4-methoxyphenyl)-1-methylethyl]amino]ethyl]phenyl]formamide,



G. (2*RS*)-1-(4-methoxyphenyl)propan-2-amine,



H. *N*-[5-[(1*RS*)-2-[benzyl[(1*RS*)-2-(4-methoxyphenyl)-1-methylethyl]amino]-1-hydroxyethyl]-2-hydroxyphenyl]formamide (monobenzyl analogue),

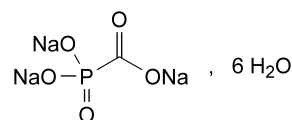


I. *N*-[2-hydroxy-5-[(1*RS*)-1-hydroxy-2-[(1*SR*)-2-(4-methoxyphenyl)-1-methylethyl]amino]ethyl]phenyl]formamide (diastereoisomer).

01/2005:1520
corrected

FOSCARNET SODIUM HEXAHYDRATE

Foscarnetum natricum hexahydricum



$CNa_3O_5P, 6H_2O$

M_r 300.0

DEFINITION

Foscarnet sodium hexahydrate contains not less than 98.5 per cent and not more than the equivalent of 101.0 per cent of trisodium phosphonatoformate, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, crystalline powder, soluble in water, practically insoluble in alcohol.

IDENTIFICATION

- A. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *foscarnet sodium hexahydrate CRS*.
B. It gives reaction (a) of sodium (2.3.1).

TESTS

Solution S. Dissolve 0.5 g in *carbon dioxide-free water R* and dilute to 25 ml with the same solvent.

Appearance of solution. Solution S is not more opalescent than reference suspension I (2.2.1) and is colourless (2.2.2, *Method II*).

pH (2.2.3). The pH of solution S is 9.0 to 11.0.

Related substances. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 25 mg of the substance to be examined in the mobile phase and dilute to 10.0 ml with the mobile phase.

Reference solution (a). Dilute 1.0 ml of the test solution to 50.0 ml with mobile phase. Dilute 1.0 ml of this solution to 10.0 ml with the mobile phase.

Reference solution (b). Dissolve 5.0 mg of foscarnet impurity B CRS in the mobile phase, add 2.0 ml of the test solution and dilute to 50.0 ml with the mobile phase.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.10 m long and 4.6 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (3 µm),
- as mobile phase at a flow rate of 1.0 ml/min a solution prepared as follows: dissolve 3.22 g of *sodium sulphate decahydrate R* in *water R*, add 3 ml of *glacial acetic acid R*, 6 ml of a 44.61 g/l solution of *sodium pyrophosphate R* and dilute to 1000 ml with *water R* (solution A); dissolve 3.22 g of *sodium sulphate decahydrate R* in *water R*, add 6.8 g of *sodium acetate R* and 6 ml of a 44.61 g/l solution of *sodium pyrophosphate R* and dilute to 1000 ml with *water R* (solution B). Mix about 700 ml of solution A and about 300 ml of solution B to obtain a solution of pH 4.4. To 1000 ml of this solution, add 0.25 g of *tetrahexylammonium hydrogen sulphate R* and 100 ml of *methanol R*.
- as detector a spectrophotometer set at 230 nm.

Inject 20 µl of reference solution (b). Continue the chromatography for 2.5 times the retention time of foscarnet. The test is not valid unless the resolution between the peaks due to foscarnet and impurity B is at least 7.

Inject 20 µl of the test solution and 20 µl of reference solution (a). In the chromatogram obtained with the test solution: the area of any peak, apart from the principal peak, is not greater than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent); the sum of the areas of all the peaks, apart from the principal peak, is not greater than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.4 per cent). Disregard any peak with a relative retention time less than 0.6 and any peak with an area less than 0.2 times that of the peak in the chromatogram obtained with reference solution (a).

Impurity D. Examine by gas chromatography (2.2.28).

Test solution. Dissolve 0.25 g of the substance to be examined in 9.0 ml of 0.1 M *acetic acid* using a magnetic stirrer. Add 1.0 ml of *ethanol R* and mix.

Reference solution. Dissolve 25 mg of *triethyl phosphonoformate R* in *ethanol R* and dilute to 100 ml with the same solvent. Dilute 1 ml of the solution to 10 ml with *ethanol R*.

The chromatographic procedure may be carried out using:

- a fused-silica column 25 m long and 0.31 mm in internal diameter coated with *poly(dimethyl)(diphenyl)(divinyl)siloxane R* (film thickness 0.5 µm),
- *helium for chromatography R* as the carrier gas,
- a flame-ionisation detector,
- a split mode injector of 1:20,

raising the temperature of the column from 100 °C to 180 °C at a rate of 10 °C per minute and maintaining the temperature of the injection port at 200 °C and that of the detector at 250 °C.

Inject 3 µl of each solution.

In the chromatogram obtained with the test solution the area of any peak due to impurity D is not greater than the area of the peak in the chromatogram obtained with reference solution (0.1 per cent).

Phosphate and phosphite. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 60.0 mg of the substance to be examined in *water R* and dilute to 25.0 ml with the same solvent.

Reference solution (a). Dissolve 28 mg of *sodium dihydrogen phosphate monohydrate R* in *water R* and dilute to 100 ml with the same solvent.

Reference solution (b). Dissolve 43 mg of *sodium phosphite pentahydrate R* in *water R* and dilute to 100 ml with the same solvent.

Reference solution (c). Dilute 1.0 ml of reference solution (a) and 1.0 ml of reference solution (b) to 25 ml with *water R*.

Reference solution (d). Dilute 3 ml of reference solution (a) and 3 ml of reference solution (b) to 25 ml with *water R*.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.05 m long and 4.6 mm in internal diameter packed with an *anion exchange resin R*,
- as mobile phase at a flow rate of 1.4 ml/min a solution prepared as follows: dissolve 0.102 g of *potassium hydrogen phthalate R* in *water R*, add 2.5 ml of 1 M *nitric acid* and dilute to 1000 ml with *water R*,
- as detector a spectrophotometer set at 290 nm (indirect detection).

Inject 20 µl of reference solution (d). The test is not valid unless: the resolution between the peaks due to phosphate (first peak) and phosphite is at least 2.0; the principal peak has a signal-to-noise ratio of at least ten.

Inject 20 µl of the test solution and 20 µl of reference solution (c). In the chromatogram obtained with the test solution: the area of any peak due to phosphate and phosphite is not greater than the area of the corresponding peaks in the chromatogram obtained with reference solution (c) (0.3 per cent of phosphate and 0.3 per cent of phosphite).

Heavy metals. Dissolve 1.25 g in 12.5 ml of 1 M *hydrochloric acid*. Warm on a water-bath for 3 min and cool to room temperature. Transfer to a beaker and adjust the pH to about 3.5 with *dilute ammonia R1* and dilute to 25 ml with *water R* (solution A). To 12 ml of solution A, add 2.0 ml of *buffer solution pH 3.5 R*. Rapidly pour the mixture into a test tube containing one drop of *sodium sulphide solution R*. The solution is not more intensely coloured than a standard prepared simultaneously and in the same manner pouring a mixture of 5.0 ml of *lead standard solution (1 ppm Pb) R*, 5.0 ml of *water R*, 2.0 ml of solution A and 2.0 ml of *buffer solution pH 3.5 R* into a test tube containing one drop of *sodium sulphide solution R* (10 ppm).

Loss on drying (2.2.32): 35.0 per cent to 37.0 per cent, determined on 1.000 g by drying in an oven at 150 °C.

Bacterial endotoxins (2.6.14): less than 83.3 IU/g, if intended for use in the manufacture of parenteral dosage forms without a further appropriate procedure for the removal of bacterial endotoxins.

ASSAY

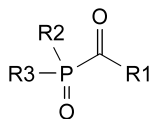
Dissolve 0.200 g in 50 ml of *water R*. Titrate with 0.05 M *sulphuric acid*, determining the end-point potentiometrically (2.2.20) at the first inflexion point.

1 ml of 0.05 M *sulphuric acid* is equivalent to 19.20 mg of CNa₃O₅P.

STORAGE

Store protected from light.

IMPURITIES

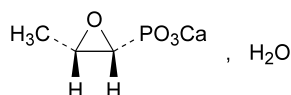


- A. R1 = OC₂H₅, R2 = R3 = ONa: disodium (ethoxycarbonyl)phosphonate,
- B. R1 = R2 = ONa, R3 = OC₂H₅: disodium (ethoxyoxydophosphanyl)formate,
- C. R1 = R2 = OC₂H₅, R3 = ONa: ethyl sodium (ethoxycarbonyl)phosphonate,
- D. R1 = R2 = R3 = OC₂H₅: methyl (diethoxyphosphoryl)formate.

01/2005:1328

FOSFOMYCIN CALCIUM

Fosfomycinum calcium

C₃H₅CaO₄P·H₂OM_r 194.1

DEFINITION

Calcium fosfomycin contains not less than 95.0 per cent and not more than the equivalent of 101.0 per cent of calcium (2*R*,3*S*)-(3-methyloxiran-2-yl)phosphonate, calculated with reference to the anhydrous substance.

CHARACTERS

A white or almost white powder, slightly soluble in water, practically insoluble in acetone, in methanol and in methylene chloride.

IDENTIFICATION

First identification: A, D.

Second identification: B, C, D.

- A. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the *Ph. Eur. reference spectrum of fosfomycin calcium*. Examine the substance as discs prepared using *potassium bromide R*.
- B. Dissolve about 0.1 g in 3 ml of a 25 per cent V/V solution of *perchloric acid R*. Add 1 ml of 0.1 M *sodium periodate* and heat on a water-bath for 30 min. Allow to cool and add 50 ml of *water R*. Neutralise with a saturated solution of *sodium hydrogen carbonate R* and add 1 ml of a freshly prepared 400 g/l solution of *potassium iodide R*. Prepare a blank at the same time and in the same manner. The test solution remains colourless and the blank is orange.
- C. To about 8 mg of the substance to be examined, add 2 ml of *water R*, 1 ml of *perchloric acid R* and 2 ml of 0.1 M *sodium periodate*. Heat on a water-bath for 10 min and add, without cooling, 1 ml of *ammonium molybdate solution R5* and 1 ml of *aminohydroxynaphthalenesulphonic acid solution R*. Allow to stand for 30 min. A blue colour develops.

D. It gives reaction (a) of calcium (2.3.1).

TESTS

pH (2.2.3). Dissolve 20 mg in *carbon dioxide-free water R* and dilute to 20.0 ml with the same solvent. The pH of the solution is 8.1 to 9.6.

Specific optical rotation (2.2.7). Dissolve 2.5 g in a 125 g/l solution of *sodium edetate R* previously adjusted to pH 8.5 with *strong sodium hydroxide solution R*, and dilute to 50.0 ml with the same solution. Measured at 405 nm using a mercury lamp, the specific optical rotation is –11.0 to –13.0, calculated with reference to the anhydrous substance.

Calcium 1,2-(dihydroxypropyl)phosphonate. Not more than 1.5 per cent. Into a glass-stoppered flask, dissolve 0.200 g of the substance to be examined in 100.0 ml of *water R*. Add 50 ml of 0.5 M *phthalate buffer solution pH 6.4 R* and 5.0 ml of 0.005 M *sodium periodate*, close and shake. Allow to stand protected from light for 90 min. Add 10 ml of a freshly prepared 400 g/l solution of *potassium iodide R*, close and shake for 2 min. Titrate with 0.0025 M *sodium arsenite* until the yellow colour almost disappears. Add 2 ml of *starch solution R* and titrate slowly until the colour is completely discharged. Carry out a blank test under the same conditions. Calculate the percentage content of C₃H₇CaO₅P from the expression:

$$\frac{(n_1 - n_2) \times c \times 97}{m(100 - H)} \times 100$$

- m* = quantity of the substance to be examined, in milligrams,
- n*₁ = volume of 0.0025 M *sodium arsenite* used in the blank titration,
- n*₂ = volume of 0.0025 M *sodium arsenite* used in the titration of the test solution,
- c* = molarity of the sodium arsenite solution,
- H* = percentage content of water.

Chlorides (2.4.4). Dissolve 0.500 g in *water R*, add 2 ml of *nitric acid R* and dilute to 50 ml with the same acid. To 2.5 ml of the solution add 12.5 ml of *water R*. The solution complies with the limit test for chlorides (0.2 per cent).

Heavy metals (2.4.8). Dissolve 2.5 g of the substance to be examined in 6 ml of *glacial acetic acid R* and dilute to 25.0 ml with *water R*. 12 ml of the solution complies with limit test A for heavy metals (20 ppm). Prepare the standard using *lead standard solution (2 ppm Pb) R*.

Water (2.5.12): 8.5 per cent to 11.5 per cent, determined on 0.250 g by the semi-micro determination of water. Use as the solvent a mixture of 1 volume of *pyridine R* and 3 volumes of *ethylene glycol R*.

ASSAY

Into a glass-stoppered flask, dissolve 0.120 g of the substance to be examined in 20.0 ml of 0.1 M *sodium periodate*. Add 5 ml of a 50 per cent V/V solution of *perchloric acid R* and shake. Heat in a water-bath at 37 °C for 105 min. Add 50 ml of *water R* and immediately adjust to pH 6.4 with a saturated solution of *sodium hydrogen carbonate R*. Add 10 ml of a freshly prepared 400 g/l solution of *potassium iodide R*, close and allow to stand for 2 min. Titrate with 0.1 M *sodium arsenite* until the yellow colour almost disappears. Add 2 ml of *starch solution R* and titrate slowly until the colour is completely discharged. Carry out a blank test under the same conditions.

Calculate the percentage content of $C_3H_5CaO_4P$ from the expression:

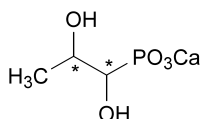
$$\frac{(n_1 - n_2) \times c \times 88 \times 100}{m(100 - H)} \times 100 - G$$

- m = quantity of the substance to be examined, in milligrams,
 n_1 = volume of 0.1 M sodium arsenite used in the blank titration,
 n_2 = volume of 0.1 M sodium arsenite used in the titration of the test solution,
 c = molarity of the sodium arsenite solution,
 G = percentage content of calcium 1,2-(dihydroxypropyl)phosphonate,
 H = percentage content of water.

STORAGE

Store in an airtight container, protected from light.

IMPURITIES

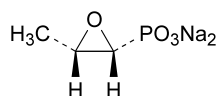


- A. calcium (1,2-dihydroxypropyl)phosphonate.

01/2005:1329

FOSFOMYCIN SODIUM

Fosfomycinum natricum



$C_3H_5Na_2O_4P$

M_r 182.0

DEFINITION

Fosfomycin sodium contains not less than 95.0 per cent and not more than the equivalent of 101.0 per cent of disodium (2R,3S)-(3-methyloxiran-2-yl)phosphonate, calculated with reference to the anhydrous substance.

CHARACTERS

A white or almost white, very hygroscopic powder, very soluble in water, sparingly soluble in methanol, practically insoluble in ethanol and in methylene chloride.

IDENTIFICATION

First identification: A, D.

Second identification: B, C, D.

- A. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the *Ph. Eur. reference spectrum of fosfomycin sodium*. Examine the substance as discs prepared using *potassium bromide R*.
 B. Dissolve about 0.1 g in 3 ml of a 25 per cent V/V solution of *perchloric acid R*. Add 1 ml of 0.1 M sodium periodate and heat on a water-bath for 30 min. Allow to cool and add 50 ml of *water R*. Neutralise with a saturated solution of *sodium hydrogen carbonate R* and add 1 ml of a freshly prepared 400 g/l solution of *potassium iodide R*. Prepare a blank at the same time and in the same manner. The test solution remains colourless and the blank is orange.

- C. To about 8 mg of the substance to be examined, add 2 ml of *water R*, 1 ml of *perchloric acid R* and 2 ml of 0.1 M sodium periodate. Heat on a water-bath for 10 min and add, without cooling, 1 ml of *ammonium molybdate solution R5* and 1 ml of *aminohydroxynaphthalenesulphonic acid solution R*. Allow to stand for 30 min. A blue colour develops.

- D. It gives reaction (a) of sodium (2.3.1).

TESTS

Solution S. Dissolve 5.0 g in *carbon dioxide-free water R* and dilute to 50.0 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and not more intensely coloured than reference solution B₉ (2.2.2, *Method II*).

pH (2.2.3). Dilute 10 ml of solution S to 20 ml with *carbon dioxide-free water R*. The pH of the solution is 9.0 to 10.5.

Specific optical rotation (2.2.7). Dissolve 2.5 g in *water R* and dilute to 50.0 ml with the same solvent. Measured at 405 nm using a mercury lamp, the specific optical rotation is –13.0 to –15.0, calculated with reference to the anhydrous substance.

Disodium 1,2-(dihydroxypropyl)phosphonate. Not more than 1.0 per cent. Into a glass-stoppered flask, dissolve 0.200 g of the substance to be examined in 100.0 ml of *water R*. Add 50 ml of 0.5 M *phthalate buffer solution pH 6.4 R* and 5.0 ml of 0.005 M *sodium periodate*, close and shake. Allow to stand protected from light for 90 min. Add 10 ml of a freshly prepared 400 g/l solution of *potassium iodide R*, close and shake for 2 min. Titrate with 0.0025 M *sodium arsenite* until the yellow colour almost disappears. Add 2 ml of *starch solution R* and titrate slowly until the colour is completely discharged. Carry out a blank test under the same conditions. Calculate the percentage content of $C_3H_7Na_2O_5P$ from the expression:

$$\frac{(n_1 - n_2) \times c \times 100}{m(100 - H)} \times 100$$

- m = quantity of the substance to be examined, in milligrams,
 n_1 = volume of 0.0025 M sodium arsenite used in the blank titration,
 n_2 = volume of 0.0025 M sodium arsenite used in the titration of the test solution,
 c = molarity of the sodium arsenite solution,
 H = percentage content of water.

Heavy metals (2.4.8). 12 ml of solution S complies with limit test A for heavy metals (20 ppm). Prepare the standard using *lead standard solution (2 ppm Pb) R*.

Water (2.5.12). Not more than 1.0 per cent, determined on 0.50 g by the semi-micro determination of water. Use as the solvent a mixture of 1 volume of *pyridine R* and 3 volumes of *ethylene glycol R*.

Bacterial endotoxins (2.6.14): less than 0.083 IU/mg, if intended for use in the manufacture of parenteral dosage forms without a further appropriate procedure for removal of bacterial endotoxins.

ASSAY

Into a glass-stoppered flask, dissolve 0.120 g of the substance to be examined in 20.0 ml of 0.1 M *sodium periodate*. Add 5 ml of a 50 per cent V/V solution of *perchloric acid R* and shake. Heat on a water-bath at 37 °C for 105 min. Add 50 ml of *water R* and immediately adjust to pH 6.4 with a saturated

solution of *sodium hydrogen carbonate R*. Add 10 ml of a freshly prepared 400 g/l solution of *potassium iodide R*, close and allow to stand for 2 min. Titrate with 0.1 M *sodium arsenite* until the yellow colour almost disappears. Add 2 ml of *starch solution R* and titrate slowly until the colour is completely discharged. Carry out a blank test under the same conditions.

Calculate the percentage content of $C_3H_5Na_2O_4P$ from the expression:

$$\frac{(n_1 - n_2) \times c \times 91 \times 100}{m(100 - H)} \times 100 - G$$

- m = quantity of the substance to be examined, in milligrams,
 n_1 = volume of 0.1 M *sodium arsenite* used in the blank titration,
 n_2 = volume of 0.1 M *sodium arsenite* used in the titration of the test solution,
 c = molarity of the sodium arsenite solution,
 G = percentage content of disodium 1,2-(dihydroxypropyl)phosphonate,
 H = percentage content of water.

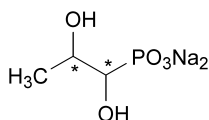
STORAGE

Store in an airtight container, protected from light. If the substance is sterile, store in a sterile, airtight, tamper-proof container.

LABELLING

The label states, where applicable, that the substance is free from bacterial endotoxins.

IMPURITIES

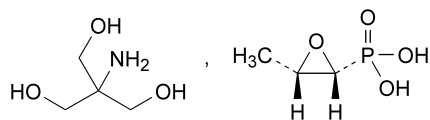


- A. disodium (1,2-dihydroxypropyl)phosphonate.

01/2005:1425

FOSFOMYCIN TROMETAMOL

Fosfomycinum trometamol



$C_7H_{18}NO_7P$

M_r 259.2

DEFINITION

Fosfomycin trometamol contains not less than 98.0 per cent and not more than the equivalent of 102.0 per cent of 1,3-dihydroxy-2-(hydroxymethyl)propan-2-aminium (2*R*,3*S*)-(3-methyloxiran-2-yl)phosphonate, calculated with reference to the anhydrous substance.

CHARACTERS

A white or almost white powder, hygroscopic, very soluble in water, slightly soluble in alcohol and in methanol, practically insoluble in acetone.

IDENTIFICATION

First identification: A.

Second identification: B, C.

- A. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *fosfomycin trometamol CRS*.
- B. Examine by thin-layer chromatography (2.2.27), using *cellulose for chromatography R* as the coating substance.
Test solution. Dissolve 50 mg of the substance to be examined in *water R* and dilute to 10 ml with the same solvent.
Reference solution. Dissolve 50 mg of *fosfomycin trometamol CRS* in *water R* and dilute to 10 ml with the same solvent.
 Apply to the plate 10 µl of each solution. Develop over a path of 15 cm using a mixture of 10 volumes of *concentrated ammonia R*, 20 volumes of *water R* and 70 volumes of *2-propanol R*. Dry the plate in a current of warm air and expose it to iodine vapour until the spots appear. The principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.
- C. To about 15 mg of the substance to be examined, add 2 ml of *water R*, 1 ml of *perchloric acid R* and 2 ml of 0.1 M *sodium periodate*. Heat on a water-bath for 10 min and add, without cooling, 1 ml of *ammonium molybdate solution R5* and 1 ml of *aminohydroxynaphthalenesulphonic acid solution R*. Allow to stand for 30 min. A blue colour develops.

TESTS

Solution S. Dissolve 1.00 g in *carbon dioxide-free water R* and dilute to 20.0 ml with the same solvent.

pH (2.2.3). The pH of solution S is 3.5 to 5.5.

Specific optical rotation (2.2.7). Measured at 365 nm using a mercury lamp, the specific optical rotation is -13.5 to -12.5 , determined on solution S and calculated with reference to the anhydrous substance.

Related substances. Examine by liquid chromatography (2.2.29) as prescribed under Assay.

Inject 5 µl of the test solution, 5 µl of reference solution (b) and 5 µl of the blank solution. Continue the chromatography for twice the retention time of the peak due to fosfomycin. In the chromatogram obtained with the test solution: the area of any peak corresponding to impurity A is not greater than the area of the corresponding peak in the chromatogram obtained with reference solution (b) (0.5 per cent, calculated as trometamol salts); the area of any peak, apart from the principal peak, two peaks corresponding to trometamol and any peak corresponding to impurity A, is not greater than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent, calculated as trometamol salts). The sum of the areas of all the peaks, apart from the principal peak and the two peaks corresponding to trometamol, is not greater than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (1 per cent, calculated as trometamol salts). Disregard any peak due to the blank and any peak with an area less than 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (b).

Phosphates. Dissolve 0.1 g in 3 ml of *dilute nitric acid R* and dilute to 10 ml with *water R*. To 5 ml of this test solution add 5 ml of *water R* and 5 ml of *molybdovanadic reagent R*. Shake vigorously. After 5 min, any colour in the test solution

is not more intense than that in a standard prepared at the same time in the same manner, using 5 ml of *phosphate standard solution* (5 ppm PO_4) R (500 ppm).

Heavy metals (2.4.8). Dissolve 2.0 g in *water* R and dilute to 20 ml with the same solvent. 12 ml of the solution complies with limit test A for heavy metals (10 ppm). Prepare the standard using *lead standard solution* (1 ppm Pb) R.

Water (2.5.12). Not more than 0.5 per cent, determined on 0.500 g by the semi-micro determination of water.

ASSAY

Examine the substance by liquid chromatography (2.2.29). Prepare the solutions immediately before use.

Test solution. Dissolve 0.60 g of the substance to be examined in the mobile phase and dilute to 5.0 ml with the mobile phase.

Reference solution (a). Dissolve 0.60 g of *fosfomycin trometamol CRS* in the mobile phase and dilute to 5.0 ml with the mobile phase.

Reference solution (b). Dissolve 8.7 mg of *fosfomycin trometamol impurity A CRS* (disodium salt) in the mobile phase and dilute to 20.0 ml with the mobile phase.

Reference solution (c). Dissolve 5 mg of *fosfomycin trometamol impurity A CRS* (disodium salt) and 10 mg of *fosfomycin trometamol CRS* in the mobile phase and dilute to 5 ml with the mobile phase.

Blank solution. A 0.3 g/l solution of *anhydrous disodium hydrogen phosphate* R in the mobile phase.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4.6 mm in internal diameter packed with *aminopropylsilyl silica gel for chromatography* R (5 μm),
- as mobile phase at a flow rate of 1 ml/min a 10.89 g/l solution of *potassium dihydrogen phosphate* R,
- as detector a differential refractometer maintaining the temperature at 35 °C.

When the chromatograms are recorded in the prescribed conditions, the relative retention times (to fosfomycin) are about 0.3 for the two peaks corresponding to trometamol and 0.8 for impurity A.

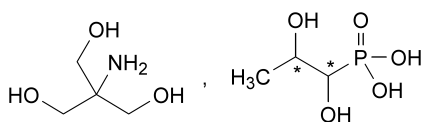
Inject 5 μl of reference solution (c). The test is not valid unless the resolution between the peaks corresponding to fosfomycin and impurity A is at least 1.5. Inject reference solution (a) six times. The test is not valid unless the relative standard deviation of the peak area for fosfomycin is at most 1.0 per cent. Inject alternately the test solution and reference solution (a).

Calculate the percentage content of fosfomycin trometamol.

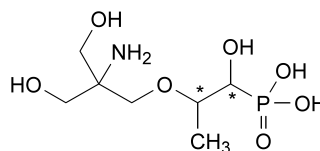
STORAGE

Store in an airtight container.

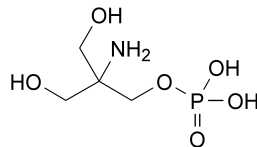
IMPURITIES



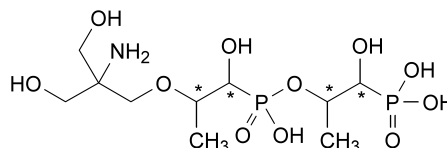
- A. 1,3-dihydroxy-2-(hydroxymethyl)propan-2-aminium (1,2-dihydroxypropyl)phosphonate,



- B. [2-[2-amino-3-hydroxy-2-(hydroxymethyl)propoxy]-1-hydroxypropyl]phosphonic acid,



- C. 2-amino-3-hydroxy-2-(hydroxymethyl)propyl dihydrogenphosphate (trometamol phosphoric ester),

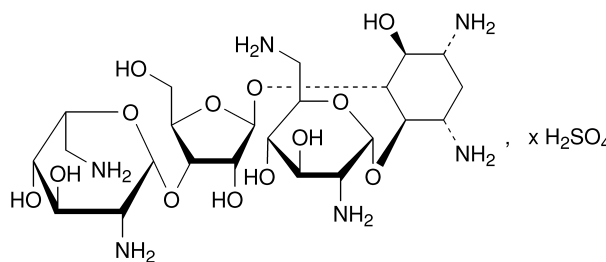


- D. [2-[[[2-[2-amino-3-hydroxy-2-(hydroxymethyl)propoxy]-1-hydroxypropyl]hydroxyphosphoryl]oxy]-1-hydroxypropyl]phosphonic acid (trometamoyloxy fosfomycin dimer).

01/2005:0180
corrected

FRAMYCETIN SULPHATE

Framycetini sulfas



$\text{C}_{23}\text{H}_{46}\text{N}_6\text{O}_{13} \cdot x\text{H}_2\text{SO}_4$

M_r 615 (base)

DEFINITION

Sulphate of 2-deoxy-4-O-(2,6-diamino-2,6-dideoxy- α -D-glucopyranosyl)-5-O-[3-O-(2,6-diamino-2,6-dideoxy- β -L-idopyranosyl)- β -D-ribofuranosyl]-D-streptamine (neomycin B), a substance produced by the growth of selected strains of *Streptomyces fradiae* or *Streptomyces decaris* or obtained by any other means.

Content: minimum of 630 IU/mg (dried substance).

CHARACTERS

Appearance: white or yellowish-white powder, hygroscopic.

Solubility: freely soluble in water, very slightly soluble in alcohol, practically insoluble in acetone.

IDENTIFICATION

- A. Examine the chromatograms obtained in the test for related substances.

Results:

- the retention time of the principal peak in the chromatogram obtained with the test solution is approximately the same as that of the principal peak in the chromatogram obtained with reference solution (a),
- it complies with the limit given for impurity C.

B. It gives reaction (a) of sulphates (2.3.1).

TESTS

pH (2.2.3): 6.0 to 7.0.

Dissolve 0.1 g in *carbon dioxide-free water R* and dilute to 10 ml with the same solvent.

Specific optical rotation (2.2.7): + 52.5 to + 55.5 (dried substance).

Dissolve 1.00 g in *water R* and dilute to 10.0 ml with the same solvent

Related substances. Liquid chromatography (2.2.29).

Test solution. Dissolve 25.0 mg of the substance to be examined in the mobile phase and dilute to 50.0 ml with the mobile phase.

Reference solution (a). Dissolve the contents of a vial of *framycetin sulphate CRS* in the mobile phase and dilute with the mobile phase to obtain a solution containing 0.5 mg/ml.

Reference solution (b). Dilute 3.0 ml of reference solution (a) to 100.0 ml with the mobile phase.

Reference solution (c). Dilute 1.0 ml of reference solution (a) to 100.0 ml with the mobile phase.

Reference solution (d). Dissolve the contents of a vial of *neamine CRS* (corresponding to 0.5 mg) in the mobile phase and dilute to 100.0 ml with the mobile phase.

Reference solution (e). Dissolve 10 mg of *neomycin sulphate CRS* in the mobile phase and dilute to 100.0 ml with the mobile phase.

Column:

- **size:** $l = 0.25$ m, $\varnothing = 4.6$ mm,
- **stationary phase:** base-deactivated octadecylsilyl silica gel for chromatography *R* (5 μ m),
- **temperature:** 25 °C.

Mobile phase: mix 20.0 ml of *trifluoroacetic acid R*, 6.0 ml of *carbonate-free sodium hydroxide solution R* and 500 ml of *water R*, allow to equilibrate, dilute to 1000 ml with *water R* and degas.

Flow rate: 0.7 ml/min.

Post-column solution: *carbonate-free sodium hydroxide solution R* diluted 1 in 25 previously degassed, which is added pulse-less to the column effluent using a 375 μ l polymeric mixing coil.

Flow rate: 0.5 ml/min.

Detection: pulsed amperometric detector with a gold working electrode, a silver-silver chloride reference electrode and a stainless steel auxiliary electrode which is the cell body, held at respectively 0.00 V detection, + 0.80 V oxidation and – 0.60 V reduction potentials, with pulse durations according to the instrument used.

Injection: 10 μ l.

Run time: 1.5 times the retention time of neomycin B.

Relative retention with reference to neomycin B (retention time = about 10 min): impurity A = about 0.65; impurity C = about 0.9; impurity G = about 1.1.

System suitability:

- **resolution:** minimum 2.0 between the peaks due to impurity C and to neomycin B in the chromatogram obtained with reference solution (e); if necessary, adjust the volume of the carbonate-free sodium hydroxide solution in the mobile phase,
- **signal-to-noise ratio:** minimum 10 for the principal peak in the chromatogram obtained with reference solution (c).

Limits:

- **impurity A:** not more than the area of the principal peak in the chromatogram obtained with reference solution (d) and taking into account the declared content of *neamine CRS* (1.0 per cent),
- **impurity C:** not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (3.0 per cent),
- **total of other impurities:** not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (3.0 per cent),
- **disregard limit:** area of the principal peak in the chromatogram obtained with reference solution (c) (1.0 per cent).

Sulphate: 27.0 per cent to 31.0 per cent (dried substance).

Dissolve 0.250 g in 100 ml of *water R* and adjust the solution to pH 11 using *concentrated ammonia R*. Add 10.0 ml of 0.1 M *barium chloride* and about 0.5 mg of *phthalein purple R*. Titrate with 0.1 M *sodium edetate* adding 50 ml of *alcohol R* when the colour of the solution begins to change and continuing the titration until the violet-blue colour disappears.

1 ml of 0.1 M *barium chloride* is equivalent to 9.606 mg of SO_4 .

Loss on drying (2.2.32): maximum 8.0 per cent, determined on 1.000 g by drying at 60 °C over *diphosphorus pentoxide R* at a pressure not exceeding 0.7 kPa for 3 h.

Sulphated ash (2.4.14): maximum 1.0 per cent, determined on 1.0 g.

Sterility (2.6.1). If intended for introduction into body cavities without a further appropriate sterilisation procedure, it complies with the test for sterility.

Bacterial endotoxins (2.6.14, *Method D*): less than 1.3 IU/mg if intended for introduction into body cavities without a further appropriate procedure for the removal of bacterial endotoxins.

ASSAY

Carry out the microbiological assay of antibiotics (2.7.2). Use *framycetin sulphate CRS* as the reference substance.

STORAGE

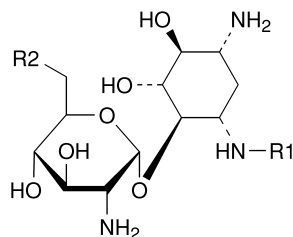
In an airtight container, protected from light. If the substance is intended for introduction into body cavities, store in a sterile, tamper-proof container.

LABELLING

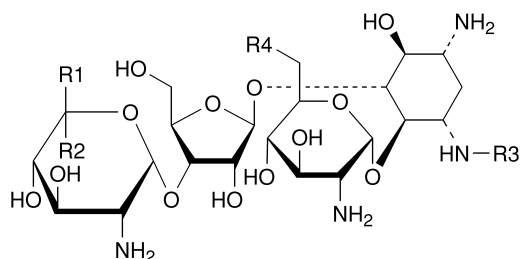
The label states:

- where applicable, that the substance is sterile,
- where applicable, that the substance is free from bacterial endotoxins.

IMPURITIES



- A. R1 = H, R2 = NH₂: 2-deoxy-4-*O*-(2,6-diamino-2,6-dideoxy- α -D-glucopyranosyl)-D-streptamine (neamine or neomycin A-LP),
- B. R1 = CO-CH₃, R2 = NH₂: 3-*N*-acetyl-2-deoxy-4-*O*-(2,6-diamino-2,6-dideoxy- α -D-glucopyranosyl)-D-streptamine (3-acetylneamine),
- D. R1 = H, R2 = OH: 4-*O*-(2-amino-2-deoxy- α -D-glucopyranosyl)-2-deoxy-D-streptamine (paromamine or neomycin D),



- C. R1 = CH₂-NH₂, R2 = R3 = H, R4 = NH₂: 2-deoxy-4-*O*-(2,6-diamino-2,6-dideoxy- α -D-glucopyranosyl)-5-*O*-[3-*O*-(2,6-diamino-2,6-dideoxy- α -D-glucopyranosyl)- β -D-ribofuranosyl]-D-streptamine (neomycin C),
- E. R1 = R3 = H, R2 = CH₂-NH₂, R4 = OH: 4-*O*-(2-amino-2-deoxy- α -D-glucopyranosyl)-2-deoxy-5-*O*-[3-*O*-(2,6-diamino-2,6-dideoxy- β -L-idopyranosyl)- β -D-ribofuranosyl]-D-streptamine (paromomycin I or neomycin E),
- F. R1 = CH₂-NH₂, R2 = R3 = H, R4 = OH: 4-*O*-(2-amino-2-deoxy- α -D-glucopyranosyl)-2-deoxy-5-*O*-[3-*O*-(2,6-diamino-2,6-dideoxy- α -D-glucopyranosyl)- β -D-ribofuranosyl]-D-streptamine (paromomycin II or neomycin F),
- G. R1 = H, R2 = CH₂-NH₂, R3 = CO-CH₃, R4 = NH₂: 3-*N*-acetyl-2-deoxy-4-*O*-(2,6-diamino-2,6-dideoxy- α -D-glucopyranosyl)-5-*O*-[3-*O*-(2,6-diamino-2,6-dideoxy- β -L-idopyranosyl)- β -D-ribofuranosyl]-D-streptamine (neomycin B-LP).

01/2005:0025

FRANGULA BARK

Frangulae cortex

DEFINITION

Frangula bark consists of the dried, whole or fragmented bark of the stems and branches of *Rhamnus frangula* L. (*Frangula alnus* Miller). It contains not less than 7.0 per cent of glucofrangulins, expressed as glucofrangulin A (C₂₇H₃₀O₁₄; M_r 578.5) and calculated with reference to the dried drug.

CHARACTERS

It has the macroscopic and microscopic characters described under Identification tests A and B.

IDENTIFICATION

- A. The bark occurs in curved, almost flat or rolled fragments or in single or double quilled pieces usually 0.5 mm to 2 mm thick and variable in length and width. The greyish-brown or dark brown outer surface is wrinkled longitudinally and covered with numerous greyish, transversely elongated lenticels; when the outer layers are removed, a dark red layer is exposed. The orange-brown to reddish-brown inner surface is smooth and bears fine longitudinal striations; it becomes red when treated with alkali. The fracture is short, fibrous in the inner part.
- B. Reduce to a powder (355). The powder is yellowish or reddish-brown. Examine under a microscope using *chloral hydrate solution R*. The powdered drug shows: numerous phloem fibres, partially lignified, in groups with crystal sheaths containing calcium oxalate prisms; reddish-brown fragments of cork; fragments of parenchyma containing calcium oxalate cluster crystals. Sclereids are absent.
- C. Examine the chromatogram obtained in the test for "Other species of *Rhamnus*; anthrones" in daylight. The chromatogram obtained with the test solution shows two orange brown zones (glucofrangulins) in the lower third and two to four red zones (frangulins, not always clearly separated, and above them frangula-emodin) in the upper third.
- D. To about 50 mg of the powdered drug (180) add 25 ml of *dilute hydrochloric acid R* and heat the mixture on a water-bath for 15 min. Allow to cool, shake with 20 ml of *ether R* and discard the aqueous layer. Shake the ether layer with 10 ml of *dilute ammonia R1*. The aqueous layer becomes reddish-violet.

TESTS

Other species of *Rhamnus*; anthrones. Examine by thin-layer chromatography (2.2.27), using a suitable silica gel as the coating substance.

Test solution. To 0.5 g of the powdered drug (180) add 5 ml of *alcohol (70 per cent V/V) R* and heat to boiling. Cool and centrifuge. Decant the supernatant solution immediately and use within 30 min.

Reference solution. Dissolve 20 mg of *barbaloin R* in *alcohol (70 per cent V/V) R* and dilute to 10 ml with the same solvent.

Apply separately to the plate, as bands, 10 μ l of each solution. Develop over a path of 10 cm using a mixture of 13 volumes of *water R*, 17 volumes of *methanol R* and 100 volumes of *ethyl acetate R*. Allow the plate to dry for 5 min, spray with a 50 g/l solution of *potassium hydroxide R* in *alcohol (50 per cent V/V) R*, and heat at 100-105 °C for 15 min. Examine in ultraviolet light at 365 nm. The chromatogram obtained with the reference solution shows a brownish-yellow zone corresponding to barbaloin in the central part. The chromatogram obtained with the test solution shows no zones of intense yellow fluorescence and no zone of orange to reddish fluorescence similar in position to the zone of barbaloin in the chromatogram obtained with the reference solution.

Apply to another plate, as a band, 10 μ l of the test solution and develop as described above. Allow the plate to dry for not longer than 5 min and spray immediately with a 5 g/l solution of *nitrotetrazolium blue R* in *methanol R*. Examine the chromatogram immediately. No violet or greyish-blue zones appear.

Foreign matter (2.8.2). Not more than 1 per cent.

Loss on drying (2.2.32). Not more than 10.0 per cent, determined on 1.000 g of the powdered drug (355) by drying in an oven at 100-105 °C for 2 h.

Total ash (2.4.16). Not more than 6.0 per cent.

ASSAY

Carry out the assay protected from bright light.

In a tared, round-bottomed flask with a ground-glass neck, weigh 0.250 g of powdered drug (180). Add 25.0 ml of a 70 per cent V/V solution of *methanol R*; mix and weigh again. Heat in a water-bath under a reflux condenser for 15 min. Allow to cool, weigh and adjust to the original mass with a 70 per cent V/V solution of *methanol R*. Filter and transfer 5.0 ml of the filtrate to a separating funnel. Add 50 ml of *water R* and 0.1 ml of *hydrochloric acid R*. Shake with five quantities, each of 20 ml, of *light petroleum R*. Allow the layers to separate and transfer the aqueous layer to a 100 ml volumetric flask. Combine the light petroleum layers and wash with two quantities, each of 15 ml, of *water R*. Use this water for washing the separating funnel and add it to the aqueous solution in the volumetric flask. Add 5 ml of a 50 g/l solution of *sodium carbonate R* and dilute to 100.0 ml with *water R*. Discard the light petroleum layer. Transfer 40.0 ml of the aqueous solution to a 200 ml round-bottomed flask with a ground-glass neck. Add 20 ml of a 200 g/l solution of *ferric chloride R* and heat under a reflux condenser for 20 min in a water-bath with the water level above that of the liquid in the flask. Add 2 ml of *hydrochloric acid R* and continue heating for 20 min, shaking frequently, until the precipitate is dissolved. Allow to cool, transfer the mixture to a separating funnel and shake with three quantities, each of 25 ml, of *ether R*, previously used to rinse the flask. Combine the ether extracts and wash with two quantities, each of 15 ml, of *water R*. Transfer the ether layer to a volumetric flask and dilute to 100.0 ml with *ether R*. Evaporate 20.0 ml carefully to dryness and dissolve the residue in 10.0 ml of a 5 g/l solution of *magnesium acetate R* in *methanol R*. Measure the absorbance (2.2.25) at 515 nm using *methanol R* as the compensation liquid. Calculate the percentage of glucofrangulins, expressed as glucofrangulin A, from the expression:

$$\frac{A \times 3.06}{m}$$

i.e. taking the specific absorbance of glucofrangulin A to be 204.

A = absorbance at 515 nm,

m = mass of the sample, in grams.

STORAGE

Store protected from light.

01/2005:1214

FRANGULA BARK DRY EXTRACT, STANDARDISED

Frangulae corticis extractum siccum normatum

DEFINITION

Standardised frangula bark dry extract is produced from *Frangula bark* (0025). It contains not less than 15.0 per cent and not more than 30.0 per cent of glucofrangulins, expressed as glucofrangulin A ($C_{27}H_{30}O_{14}$; M_r 578.5) and

calculated with reference to the dried extract. The measured content does not deviate from that stated on the label by more than ± 10 per cent.

PRODUCTION

The extract is produced from the drug and ethanol (50 to 80 per cent V/V) by an appropriate procedure.

CHARACTERS

A yellowish-brown, fine powder.

IDENTIFICATION

A. Examine by thin-layer chromatography (2.2.27), using a suitable silica gel as the coating substance.

Test solution. To 0.05 g add 5 ml of *alcohol* (70 per cent V/V) *R* and heat to boiling. Cool and centrifuge. Decant the supernatant solution immediately and use within 30 min.

Reference solution. Dissolve 20 mg of *barbaloin R* in *alcohol* (70 per cent V/V) *R* and dilute to 10 ml with the same solvent.

Apply separately to the plate, as bands, 10 μ l of each solution. Develop over a path of 10 cm using a mixture of 13 volumes of *water R*, 17 volumes of *methanol R* and 100 volumes of *ethyl acetate R*. Allow the plate to dry for 5 min, then spray with a 50 g/l solution of *potassium hydroxide R* in *alcohol* (50 per cent V/V) *R* and heat at 100-105 °C for 15 min. Examine immediately after heating. The chromatogram obtained with the reference solution shows a reddish-brown zone in the median third corresponding to barbaloin. The chromatogram obtained with the test solution shows two orange-brown zones (glucofrangulins) in the lower third and two to four red zones (frangulins, not always clearly separated, and above them frangula-emodin) in the upper third.

B. To about 25 mg add 25 ml of *dilute hydrochloric acid R* and heat the mixture on a water-bath for 15 min. Allow to cool, shake with 20 ml of *ether R* and discard the aqueous layer. Shake the ether layer with 10 ml of *dilute ammonia R1*. The aqueous layer becomes reddish-violet.

TESTS

Loss on drying (2.8.17): maximum 5.0 per cent.

Microbial contamination. Total viable aerobic count (2.6.12) not more than 10^4 per gram of which not more than 10^2 fungi per gram, determined by plate count. It complies with the test for *Escherichia coli* and *Salmonella* (2.6.13).

ASSAY

Carry out the assay protected from bright light.

In a tared round-bottomed flask with a ground-glass neck, weigh 0.100 g of the preparation to be examined. Add 25.0 ml of a 70 per cent V/V solution of *methanol R*, mix and weigh again. Heat the flask in a water-bath under a reflux condenser at 70 °C for 15 min. Allow to cool, weigh and adjust to the original mass with a 70 per cent V/V solution of *methanol R*. Filter and transfer 5.0 ml of the filtrate to a separating funnel. Add 50 ml of *water R* and 0.1 ml of *hydrochloric acid R*. Shake with five quantities, each of 20 ml, of *light petroleum R1*. Allow the layers to separate and transfer the aqueous layer to a 100 ml volumetric flask. Combine the light petroleum layers and wash with two quantities, each of 15 ml, of *water R*. Use this water for washing the separating funnel and add it to the aqueous solution in the volumetric flask. Add 5 ml of a 50 g/l solution of *sodium carbonate R* and dilute to 100.0 ml with *water R*. Discard the light petroleum layer. Transfer 40.0 ml of the aqueous solution to a 200 ml round-bottomed flask with a

ground-glass neck. Add 20 ml of a 200 g/l solution of *ferric chloride R* and heat under a reflux condenser for 20 min in a water-bath with the water level above that of the liquid in the flask. Add 2 ml of *hydrochloric acid R* and continue heating for 20 min, shaking frequently, until the precipitate is dissolved. Allow to cool, transfer the mixture to a separating funnel and shake with three quantities, each of 25 ml, of *ether R*, previously used to rinse the flask. Combine the ether extracts and wash with two quantities, each of 15 ml, of *water R*. Transfer the ether layer to a volumetric flask and dilute to 100.0 ml with *ether R*. Evaporate 20.0 ml carefully to dryness and dissolve the residue in 10.0 ml of a 5 g/l solution of *magnesium acetate R* in *methanol R*. Measure the absorbance (2.2.25) at 515 nm using *methanol R* as the compensation liquid.

Calculate the percentage of glucofrangulins, expressed as glucofrangulin A from the expression:

$$\frac{A \times 3.06}{m}$$

i.e. taking the specific absorbance of glucofrangulin A to be 204, calculated on the basis of the specific absorbance of barbaloin,

A = absorbance at 515 nm,

m = mass of the preparation to be examined, in grams.

STORAGE

Store in an airtight container, protected from light.

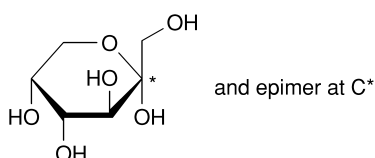
LABELLING

The label states the content of glucofrangulins.

01/2005:0188

FRUCTOSE

Fructosum



$C_6H_{12}O_6$

M_r 180.2

DEFINITION

Fructose is (-)-D-arabino-hex-2-ulopyranose. The substance described in this monograph is not necessarily suitable for parenteral use.

CHARACTERS

A white, crystalline powder, with a very sweet taste, very soluble in water, soluble in alcohol.

IDENTIFICATION

A. Examine by thin-layer chromatography (2.2.27), using *silica gel G R* as the coating substance.

Test solution. Dissolve 10 mg of the substance to be examined in a mixture of 2 volumes of *water R* and 3 volumes of *methanol R* and dilute to 20 ml with the same mixture of solvents.

Reference solution (a). Dissolve 10 mg of *fructose CRS* in a mixture of 2 volumes of *water R* and 3 volumes of *methanol R* and dilute to 20 ml with the same mixture of solvents.

Reference solution (b). Dissolve 10 mg each of *fructose CRS*, *glucose CRS*, *lactose CRS* and *sucrose CRS* in a mixture of 2 volumes of *water R* and 3 volumes of *methanol R* and dilute to 20 ml with the same mixture of solvents.

Apply separately to the plate 2 µl of each solution and thoroughly dry the starting points. Develop over a path of 15 cm using a mixture of 10 volumes of *water R*, 15 volumes of *methanol R*, 25 volumes of *anhydrous acetic acid R* and 50 volumes of *ethylene chloride R*. The solvents should be measured accurately since a slight excess of water produces cloudiness. Dry the plate in a current of warm air. Repeat the development immediately, after renewing the mobile phase. Dry the plate in a current of warm air and spray evenly with a solution of 0.5 g of *thymol R* in a mixture of 5 ml of *sulphuric acid R* and 95 ml of *alcohol R*. Heat at 130 °C for 10 min. The principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a). The test is not valid unless the chromatogram obtained with reference solution (b) shows four clearly separated spots.

- B. Dissolve 0.1 g in 10 ml of *water R*. Add 3 ml of *cupri-tartaric solution R* and heat. A red precipitate is formed.
- C. To 1 ml of solution S (see Tests) add 9 ml of *water R*. To 1 ml of the solution add 5 ml of *hydrochloric acid R* and heat to 70 °C. A brown colour develops.
- D. Dissolve 5 g in *water R* and dilute to 10 ml with the same solvent. To 0.5 ml of the solution add 0.2 g of *resorcinol R* and 9 ml of *dilute hydrochloric acid R* and heat on a water-bath for 2 min. A red colour develops.

TESTS

Solution S. Dissolve 10.0 g in *distilled water R* and dilute to 100 ml with the same solvent.

Appearance of solution. Dissolve 5.0 g in *water R* and dilute to 10 ml with the same solvent. The solution is clear (2.2.1). Add 10 ml of *water R*. The solution is colourless (2.2.2, *Method II*).

Acidity or alkalinity. Dissolve 6.0 g in 25 ml of *carbon dioxide-free water R* and add 0.3 ml of *phenolphthalein solution R*. The solution is colourless. Not more than 0.15 ml of 0.1 M *sodium hydroxide* is required to change the colour of the indicator to pink.

Specific optical rotation (2.2.7). Dissolve 10.0 g in 80 ml of *water R*, add 0.2 ml of *dilute ammonia R1*, allow to stand for 30 min and dilute to 100.0 ml with *water R*. The specific optical rotation is -91.0 to -93.5, calculated with reference to the anhydrous substance.

Foreign sugars. Dissolve 5.0 g in *water R* and dilute to 10 ml with the same solvent. To 1 ml of the solution add 9 ml of *alcohol R*. Any opalescence in the solution is not more intense than that in a mixture of 1 ml of the initial solution and 9 ml of *water R*.

5-Hydroxymethylfurfural and related compounds. To 5 ml of solution S add 5 ml of *water R*. The absorbance (2.2.25) measured at 284 nm is not greater than 0.32.

Barium. To 10 ml of solution S add 1 ml of *dilute sulphuric acid R*. When examined immediately and after 1 h, any opalescence in the solution is not more intense than that in a mixture of 1 ml of *distilled water R* and 10 ml of solution S.

Lead in sugars (2.4.10). It complies with the limit test for lead in sugars (0.5 ppm).

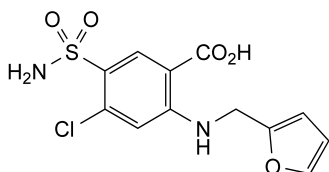
Water (2.5.12). Not more than 0.5 per cent, determined on 1.00 g by the semi-micro determination of water.

Sulphated ash (2.4.14). Not more than 0.1 per cent. Dissolve 5.0 g in 10 ml of *water R*, add 2 ml of *sulphuric acid R*, evaporate to dryness on a water-bath and ignite to constant mass.

01/2005:0391

FUROSEMIDE

Furosemidum

C₁₂H₁₁ClN₂O₅SM_r 330.7

DEFINITION

Furosemide contains not less than 98.5 per cent and not more than the equivalent of 101.0 per cent of 4-chloro-2-[(furan-2-ylmethyl)amino]-5-sulphamoylbenzoic acid, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, crystalline powder, practically insoluble in water, soluble in acetone, sparingly soluble in alcohol, practically insoluble in methylene chloride. It dissolves in dilute solutions of alkali hydroxides.

It melts at about 210 °C, with decomposition.

IDENTIFICATION

First identification: B.

Second identification: A, C.

- Dissolve 50 mg in a 4 g/l solution of *sodium hydroxide R* and dilute to 100 ml with the same alkaline solution. Dilute 1 ml of the solution to 100 ml with a 4 g/l solution of *sodium hydroxide R*. Examined between 220 nm and 350 nm (2.2.25), the solution shows three absorption maxima, at 228 nm, 270 nm and 333 nm. The ratio of the absorbance measured at the maximum at 270 nm to that measured at the maximum at 228 nm is 0.52 to 0.57.
- Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *furosemide CRS*.
- Dissolve about 25 mg in 10 ml of *alcohol R*. To 5 ml add 10 ml of *water R*. To 0.2 ml of this solution add 10 ml of *dilute hydrochloric acid R* and heat under a reflux condenser for 15 min. Allow to cool and add 18 ml of 1 M *sodium hydroxide* and 1 ml of a 5 g/l solution of *sodium nitrite R*. Allow to stand for 3 min, add 2 ml of a 25 g/l solution of *sulphamic acid R* and mix. Add 1 ml of a 5 g/l solution of *naphthylethylenediamine dihydrochloride R*. A violet-red colour develops.

TESTS

Related substances. Examine by liquid chromatography (2.2.29). Prepare the solutions immediately before use and protect from light.

Test solution. Dissolve 50.0 mg of the substance to be examined in the mobile phase and dilute to 50.0 ml with the mobile phase.

Reference solution (a). Dissolve 20.0 mg of *furosemide impurity A CRS* in the mobile phase and dilute to 20.0 ml with the mobile phase.

Reference solution (b). Dilute a mixture of 1.0 ml of the test solution and 1.0 ml of reference solution (a) to 20.0 ml with the mobile phase. Dilute 1.0 ml of this solution to 20.0 ml with the mobile phase.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4.6 mm in internal diameter packed with *octylsilyl silica gel for chromatography R* (5 µm),
- as mobile phase at a flow rate of 1 ml per minute a mixture prepared as follows: dissolve 0.2 g of *potassium dihydrogen phosphate R* and 0.25 g of *cetrimide R* in 70 ml of *water R*; adjust to pH 7.0 with *ammonia R* and add 30 ml of *propanol R*,
- as detector a spectrophotometer set at 238 nm.

Inject 20 µl of reference solution (b). Adjust the sensitivity of the system so that the heights of the two peaks in the chromatogram obtained are not less than 20 per cent of the full scale of the recorder. The test is not valid unless the resolution between the first peak (*furosemide impurity A*) and the second peak (*furosemide*) is at least 4.

Inject 20 µl of the test solution. Continue the chromatography for three times the retention time of the principal peak. In the chromatogram obtained with the test solution: the area of any peak, apart from the principal peak, is not greater than the area of the first peak in the chromatogram obtained with reference solution (b) (0.25 per cent); the sum of the areas of all the peaks, apart from the principal peak, is not greater than twice the area of the first peak in the chromatogram obtained with reference solution (b) (0.5 per cent). Disregard any peak with an area less than 0.1 times the area of the first peak in the chromatogram obtained with reference solution (b).

Chlorides (2.4.4). To 0.5 g add a mixture of 0.2 ml of *nitric acid R* and 30 ml of *water R* and shake for 5 min. Allow to stand for 15 min and filter. 15 ml of the filtrate complies with the limit test for chlorides (200 ppm).

Sulphates (2.4.13). To 1.0 g add a mixture of 0.2 ml of *acetic acid R* and 30 ml of *distilled water R* and shake for 5 min. Allow to stand for 15 min and filter. 15 ml of the filtrate complies with the limit test for sulphates (300 ppm).

Heavy metals (2.4.8). 1.0 g complies with limit test C for heavy metals (20 ppm). Prepare the standard using 2 ml of *lead standard solution (10 ppm Pb) R*.

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

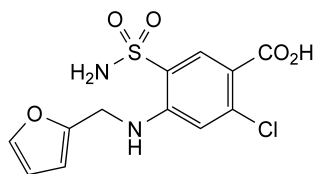
Dissolve 0.250 g in 20 ml of *dimethylformamide R*. Titrate with 0.1 M *sodium hydroxide* using 0.2 ml of *bromothymol blue solution R2*. Carry out a blank titration.

1 ml of 0.1 M *sodium hydroxide* is equivalent to 33.07 mg of C₁₂H₁₁ClN₂O₅S.

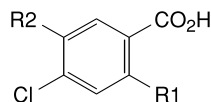
STORAGE

Store protected from light.

IMPURITIES



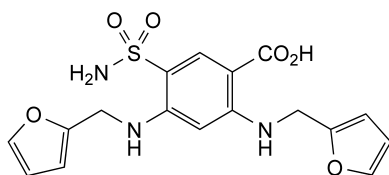
A. 2-chloro-4-[(furan-2-ylmethyl)amino]-5-sulphamoylbenzoic acid,



B. R1 = Cl, R2 = SO₂NH₂: 2,4-dichloro-5-sulphamoylbenzoic acid,

C. R1 = NH₂, R2 = SO₂NH₂: 2-amino-4-chloro-5-sulphamoylbenzoic acid,

E. R1 = Cl, R2 = H: 2,4-dichlorobenzoic acid,

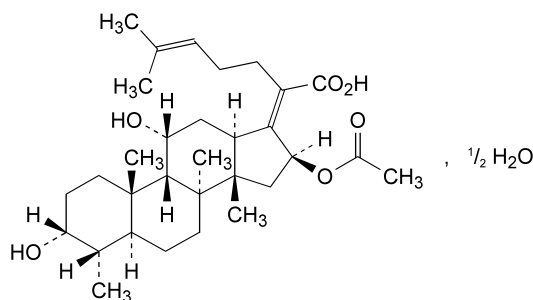


D. 2,4-bis[(furan-2-ylmethyl)amino]-5-sulphamoylbenzoic acid.

01/2005:0798

FUSIDIC ACID

Acidum fusidicum



C₃₁H₄₈O₆ · 1/2 H₂O

M_r 525.7

DEFINITION

Fusidic acid is *ent*-(17*Z*)-16α-(acetyloxy)-3β,11β-dihydroxy-4β,8,14-trimethyl-18-nor-5β,10α-cholesta-17(20),24-dien-21-oic acid, an antimicrobial substance produced by the growth of certain strains of *Fusidium coccineum* or by any other means. It contains not less than 97.5 per cent and not more than the equivalent of 101.0 per cent of C₃₁H₄₈O₆, calculated with reference to the anhydrous substance.

CHARACTERS

A white or almost white, crystalline powder, practically insoluble in water, freely soluble in alcohol.

IDENTIFICATION

- Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with the *Ph. Eur. reference spectrum of fusidic acid*.
- Examine by thin-layer chromatography (2.2.27), using *silica gel HF₂₅₄ R* as the coating substance.

Test solution. Dissolve 20 mg of the substance to be examined in *methanol R* and dilute to 10 ml with the same solvent.

Reference solution. Dissolve 24 mg of *diethanolamine fusidate CRS* in *methanol R* and dilute to 10 ml with the same solvent.

Apply to the plate 10 µl of each solution. Develop over a path of 15 cm using a mixture of 2.5 volumes of *methanol R*, 10 volumes of *glacial acetic acid R*, 10 volumes of *cyclohexane R* and 80 volumes of *chloroform R*. Dry the plate in a current of hot air. Examine in ultraviolet light at 254 nm. The principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with the reference solution.

TESTS

Related substances. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 50 mg of the substance to be examined in the mobile phase and dilute to 10.0 ml with the mobile phase.

Reference solution (a). Dissolve 5 mg of *3-ketofusidic acid CRS* in 5 ml of the mobile phase. To 1.0 ml of this solution add 0.20 ml of the test solution and dilute to 20.0 ml with the mobile phase.

Reference solution (b). Dilute 20 µl of the test solution to 100.0 ml with the mobile phase.

The chromatographic procedure may be carried out using:

- a steel column 0.125 m to 0.15 m long and 4 mm to 5 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (5 µm),
- as mobile phase at a flow rate of 2 ml/min a mixture of 10 volumes of *methanol R*, 20 volumes of a 10 g/l solution of *phosphoric acid R*, 20 volumes of *water R* and 50 volumes of *acetonitrile R*,
- as detector a spectrophotometer set at 235 nm,
- a 20 µl loop injector.

Continue the chromatography for at least 3.5 times the retention time of the principal peak. In the chromatogram obtained with the test solution, the sum of the areas of the peaks, apart from the principal peak and the solvent peak, is not greater than twice the area of the peak corresponding to fusidic acid in the chromatogram obtained with reference solution (a) (2.0 per cent). Disregard any peak with an area less than that of the principal peak in the chromatogram obtained with reference solution (b). The test is not valid unless: the resolution between the peaks corresponding to 3-ketofusidic acid and fusidic acid in the chromatogram obtained with reference solution (a) is at least 2.5; the principal peak in the chromatogram obtained with reference solution (b) has a signal-to-noise ratio of at least 3.

Water (2.5.12): 1.4 per cent to 2.0 per cent, determined on 0.50 g by the semi-micro determination of water.

Sulphated ash (2.4.14). Not more than 0.2 per cent, determined on 1.0 g.

ASSAY

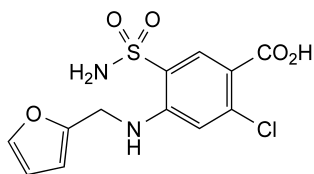
Dissolve 0.500 g in 10 ml of *alcohol R*. Add 0.5 ml of *phenolphthalein solution R*. Titrate with 0.1 M *sodium hydroxide* until a pink colour is obtained.

1 ml of 0.1 M *sodium hydroxide* is equivalent to 51.67 mg of C₃₁H₄₈O₆.

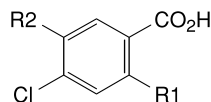
STORAGE

Store protected from light, at a temperature of 2 °C to 8 °C.

IMPURITIES



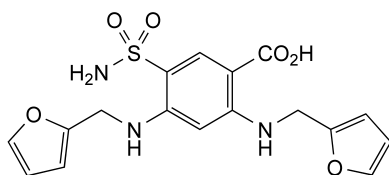
A. 2-chloro-4-[(furan-2-ylmethyl)amino]-5-sulphamoylbenzoic acid,



B. R1 = Cl, R2 = SO₂NH₂: 2,4-dichloro-5-sulphamoylbenzoic acid,

C. R1 = NH₂, R2 = SO₂NH₂: 2-amino-4-chloro-5-sulphamoylbenzoic acid,

E. R1 = Cl, R2 = H: 2,4-dichlorobenzoic acid,

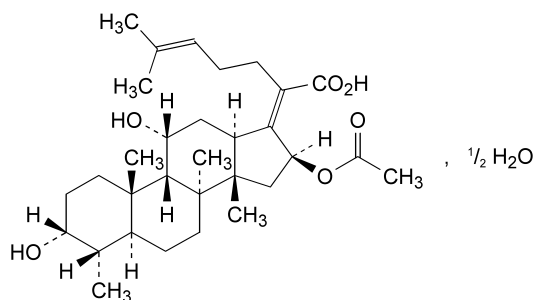


D. 2,4-bis[(furan-2-ylmethyl)amino]-5-sulphamoylbenzoic acid.

01/2005:0798

FUSIDIC ACID

Acidum fusidicum



C₃₁H₄₈O₆, 1/2 H₂O

M_r 525.7

DEFINITION

Fusidic acid is *ent*-(17*Z*)-16 α -(acetyloxy)-3 β ,11 β -dihydroxy-4 β ,8,14-trimethyl-18-nor-5 β ,10 α -cholesta-17(20),24-dien-21-oic acid, an antimicrobial substance produced by the growth of certain strains of *Fusidium coccineum* or by any other means. It contains not less than 97.5 per cent and not more than the equivalent of 101.0 per cent of C₃₁H₄₈O₆, calculated with reference to the anhydrous substance.

CHARACTERS

A white or almost white, crystalline powder, practically insoluble in water, freely soluble in alcohol.

IDENTIFICATION

- Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with the *Ph. Eur. reference spectrum of fusidic acid*.
- Examine by thin-layer chromatography (2.2.27), using *silica gel HF₂₅₄ R* as the coating substance.

Test solution. Dissolve 20 mg of the substance to be examined in *methanol R* and dilute to 10 ml with the same solvent.

Reference solution. Dissolve 24 mg of *diethanolamine fusidate CRS* in *methanol R* and dilute to 10 ml with the same solvent.

Apply to the plate 10 μ l of each solution. Develop over a path of 15 cm using a mixture of 2.5 volumes of *methanol R*, 10 volumes of *glacial acetic acid R*, 10 volumes of *cyclohexane R* and 80 volumes of *chloroform R*. Dry the plate in a current of hot air. Examine in ultraviolet light at 254 nm. The principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with the reference solution.

TESTS

Related substances. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 50 mg of the substance to be examined in the mobile phase and dilute to 10.0 ml with the mobile phase.

Reference solution (a). Dissolve 5 mg of *3-ketofusidic acid CRS* in 5 ml of the mobile phase. To 1.0 ml of this solution add 0.20 ml of the test solution and dilute to 20.0 ml with the mobile phase.

Reference solution (b). Dilute 20 μ l of the test solution to 100.0 ml with the mobile phase.

The chromatographic procedure may be carried out using:

- a steel column 0.125 m to 0.15 m long and 4 mm to 5 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (5 μ m),
- as mobile phase at a flow rate of 2 ml/min a mixture of 10 volumes of *methanol R*, 20 volumes of a 10 g/l solution of *phosphoric acid R*, 20 volumes of *water R* and 50 volumes of *acetonitrile R*,
- as detector a spectrophotometer set at 235 nm,
- a 20 μ l loop injector.

Continue the chromatography for at least 3.5 times the retention time of the principal peak. In the chromatogram obtained with the test solution, the sum of the areas of the peaks, apart from the principal peak and the solvent peak, is not greater than twice the area of the peak corresponding to fusidic acid in the chromatogram obtained with reference solution (a) (2.0 per cent). Disregard any peak with an area less than that of the principal peak in the chromatogram obtained with reference solution (b). The test is not valid unless: the resolution between the peaks corresponding to 3-ketofusidic acid and fusidic acid in the chromatogram obtained with reference solution (a) is at least 2.5; the principal peak in the chromatogram obtained with reference solution (b) has a signal-to-noise ratio of at least 3.

Water (2.5.12): 1.4 per cent to 2.0 per cent, determined on 0.50 g by the semi-micro determination of water.

Sulphated ash (2.4.14). Not more than 0.2 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.500 g in 10 ml of *alcohol R*. Add 0.5 ml of *phenolphthalein solution R*. Titrate with 0.1 M *sodium hydroxide* until a pink colour is obtained.

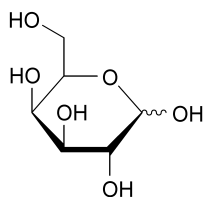
1 ml of 0.1 M *sodium hydroxide* is equivalent to 51.67 mg of C₃₁H₄₈O₆.

STORAGE

Store protected from light, at a temperature of 2 °C to 8 °C.

GALACTOSE

Galactosum


 $C_6H_{12}O_6$
 M_r 180.2

DEFINITION

Galactose is D-galactopyranose.

CHARACTERS

A white, crystalline or finely granulated powder, freely soluble or soluble in water, very slightly soluble in alcohol.

IDENTIFICATION

First identification: A.

Second identification: B, C.

- A. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *galactose CRS*. Examine the substances prepared as discs.
- B. Examine by thin-layer chromatography (2.2.27), using a suitable silica gel as the coating substance.

Test solution. Dissolve 10 mg of the substance to be examined in a mixture of 2 volumes of *water R* and 3 volumes of *methanol R* and dilute to 20 ml with the same mixture of solvents.

Reference solution (a). Dissolve 10 mg of *galactose CRS* in a mixture of 2 volumes of *water R* and 3 volumes of *methanol R* and dilute to 20 ml with the same mixture of solvents.

Reference solution (b). Dissolve 10 mg of *galactose CRS*, 10 mg of *glucose CRS* and 10 mg of *lactose CRS* in a mixture of 2 volumes of *water R* and 3 volumes of *methanol R* and dilute to 20 ml with the same mixture of solvents.

Apply to the plate 2 µl of each solution and thoroughly dry the starting points. Develop in an unsaturated tank over a path of 15 cm using a mixture of 15 volumes of *water R* and 85 volumes of *propanol R*. Dry the plate in a current of warm air. Spray uniformly with a solution of 0.5 g of *thymol R* in a mixture of 5 ml of *sulphuric acid R* and 95 ml of *alcohol R*. Heat in an oven at 130 °C for 10 min. The principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a). The test is not valid unless the chromatogram obtained with reference solution (b) shows three clearly separated spots.

- C. Dissolve 0.1 g in 10 ml of *water R*. Add 3 ml of *cupri-tartaric solution R* and heat. An orange to red precipitate is formed.

TESTS

Solution S. Dissolve, with heating in a water-bath at 50 °C, 10.0 g in *carbon dioxide-free water R* prepared from *distilled water R* and dilute to 50 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and not more intensely coloured than reference solution B₈ (2.2.2, *Method II*).

01/2005:1215 Acidity or alkalinity. To 30 ml of solution S add 0.3 ml of *phenolphthalein solution R*. The solution is colourless. Not more than 1.5 ml of 0.01 M *sodium hydroxide* is required to change the colour of the indicator to pink.

Specific optical rotation (2.2.7). Dissolve 10.00 g in 80 ml of *water R* and add 0.2 ml of *dilute ammonia R1*. Allow to stand for 30 min and dilute to 100.0 ml with *water R*. The specific optical rotation is + 78.0 to + 81.5, calculated with reference to the anhydrous substance.

Barium. Dilute 5 ml of solution S to 10 ml with *distilled water R*. Add 1 ml of *dilute sulphuric acid R*. When examined immediately and after 1 h, any opalescence in the solution is not more intense than that in a mixture of 5 ml of solution S and 6 ml of *distilled water R*.

Lead (2.4.10). It complies with the limit test for lead in sugars (0.5 ppm).

Water (2.5.12). Not more than 1.0 per cent, determined on 1.00 g by the semi-micro determination of water.

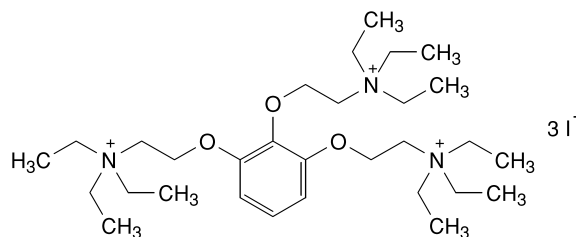
Sulphated ash. To 5 ml of solution S add 2 ml of *sulphuric acid R*, evaporate to dryness on a water-bath and ignite to constant mass. The residue weighs not more than 1 mg (0.1 per cent).

Microbial contamination. Total viable aerobic count (2.6.12) not more than 10² micro-organisms per gram.

01/2005:0181

GALLAMINE TRIETHIODIDE

Gallamini triethiodidum


 $C_{30}H_{60}I_3N_3O_3$
 M_r 892

DEFINITION

Gallamine triethiodide contains not less than 98.0 per cent and not more than the equivalent of 101.0 per cent of 2,2',2''-[benzene-1,2,3-triyltris(oxy)]tris(*N,N,N*-triethylethanaminium) triiodide, calculated with reference to the dried substance.

CHARACTERS

A white or almost white powder, hygroscopic, very soluble in water, slightly soluble in alcohol, practically insoluble in methylene chloride.

IDENTIFICATION

First identification: B, D.

Second identification: A, C, D.

- A. Dissolve 50 mg in 0.01 M *hydrochloric acid* and dilute to 50.0 ml with the same solvent. Dilute 1.0 ml of the solution to 100.0 ml with 0.01 M *hydrochloric acid*. Examined between 220 nm and 350 nm (2.2.25), the solution shows an absorption maximum at 225 nm. The specific absorbance at the maximum is 500 to 550.

- B. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *gallamine triethiodide CRS*.

- C. To 5 ml of solution S (see Tests) add 1 ml of *potassium tetraiodomercurate solution R*. A yellow precipitate is formed.
- D. Dilute 0.5 ml of solution S to 2 ml with *water R*. Add 0.2 ml of *dilute nitric acid R*. The solution gives reaction (a) of iodides (2.3.1).

TESTS

Solution S. Dissolve 0.6 g in *water R* and dilute to 30 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and immediately after preparation is not more intensely coloured than reference solution Y₇ (2.2.2, *Method II*).

Acidity or alkalinity. To 50 ml of *water R* add 0.2 ml of *methyl red solution R*. Add either 0.01 M *sulphuric acid* or 0.02 M *sodium hydroxide* until an orange-yellow colour is obtained. Add 1.0 g of the substance to be examined and dissolve by shaking. Not more than 0.2 ml of 0.01 M *sulphuric acid* or 0.02 M *sodium hydroxide* is required to restore the orange-yellow colour.

Related substances. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 30.0 mg of the substance to be examined in the mobile phase and dilute to 50.0 ml with the mobile phase.

Reference solution. Dilute 1.0 ml of the test solution to 100.0 ml with the mobile phase.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4.6 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (5 µm),
- as mobile phase at a flow rate of 1 ml/min a solution prepared as follows: dissolve 14 g of *sodium perchlorate R* in 850 ml of *phosphate buffer pH 3.0 R* and add 150 ml of *methanol R*,
- as detector a spectrophotometer set at 205 nm.

When the chromatograms are recorded in the prescribed conditions, the retention times relative to triethylgallamine as perchlorate (about 40 min) are: impurity A about 0.45, impurity B about 0.50, impurity C about 0.65, impurity D about 0.75, impurity E about 0.85 and impurity F about 0.90.

Inject separately 20 µl of the test solution and 20 µl of the reference solution. Continue the chromatography for 1.5 times the retention time of triethylgallamine as perchlorate.

In the chromatogram obtained with the test solution: the area of any peak, apart from the principal peak, is not greater than the area of the principal peak in the chromatogram obtained with the reference solution (1 per cent) and the sum of the areas of such peaks is not greater than twice the area of the principal peak in the chromatogram obtained with the reference solution (2 per cent). Disregard the peak due to iodide with a retention time of zero.

Loss on drying (2.2.32). Not more than 1.5 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

In order to avoid overheating in the reaction medium, mix thoroughly throughout and stop the titration immediately after the end-point has been reached.

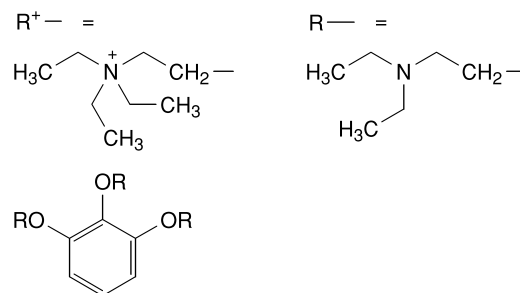
Dissolve 0.270 g in a mixture of 5.0 ml of *anhydrous formic acid R* and 50.0 ml of *acetic anhydride R*. Titrate with 0.1 M *perchloric acid*, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *perchloric acid* is equivalent to 29.72 mg of C₃₀H₆₀I₃N₃O₃.

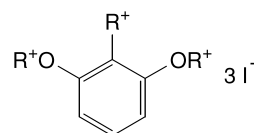
STORAGE

Store in an airtight container, protected from light.

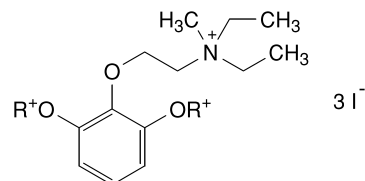
IMPURITIES



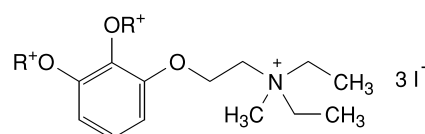
- A. 2,2',2''-[benzene-1,2,3-triyltris(oxy)]tris(*N,N*-diethylethanamine),



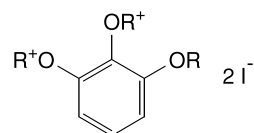
- B. 2,2'-[2-[2-(triethylammonio)ethyl]-1,3-phenylenebis(oxy)]bis(*N,N,N*-triethylethanaminium) triiodide,



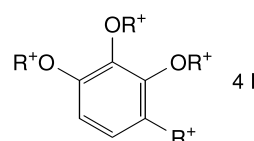
- C. 2,2'-[2-[2-(diethylmethylammonio)ethoxy]-1,3-phenylenebis(oxy)]bis(*N,N,N*-triethylethanaminium) triiodide,



- D. 2,2'-[3-[2-(diethylmethylammonio)ethoxy]-1,2-phenylenebis(oxy)]bis(*N,N,N*-triethylethanaminium) triiodide,



- E. 2,2'-[3-[2-(diethylamino)ethoxy]-1,2-phenylenebis(oxy)]bis(*N,N,N*-triethylethanaminium) diiodide,



- F. 2,2',2''-[4-[2-(triethylammonio)ethyl]benzene-1,2,3-triyltris(oxy)]tris(*N,N,N*-triethylethanaminium) tetraiodide.

01/2005:1216

GARLIC POWDER

Allii sativi bulbi pulvis

DEFINITION

Garlic powder is produced from the bulbs of *Allium sativum* L., cut, freeze-dried or dried at a temperature not exceeding 65 °C and powdered. It contains not less than 0.45 per cent of allicin (C₆H₁₀OS₂; *M_r* 162.3), calculated with reference to the dried drug.

CHARACTERS

A light yellowish powder.

It shows the microscopic characters described under Identification test A.

IDENTIFICATION

A. Examine under a microscope using *chloral hydrate solution R*. The powder shows numerous fragments of parenchyma and groups of spiral or annular vessels accompanied by thin-walled parenchyma.

B. Examine by thin-layer chromatography (2.2.27), using a suitable silica gel as the coating substance.

Test solution. To 1.0 g of garlic powder add 5.0 ml of *methanol R*, shake for 60 s and filter.

Reference solution. Dissolve 5 mg of *alanine R* in 10 ml of *water R* and dilute to 20 ml with *methanol R*.

Apply separately to the plate, as bands, 20 µl of the test solution and 10 µl of the reference solution. Develop over a path of 10 cm using a mixture of 20 volumes of *glacial acetic acid R*, 20 volumes of *propanol R*, 20 volumes of *water R* and 40 volumes of *ethanol R*. Allow the plate to dry in air. Spray with a 2 g/l solution of *ninhydrin R* in a mixture of 5 volumes of *glacial acetic acid R* and 95 volumes of *butanol R* and heat at 105 °C to 110 °C for 5 min to 10 min. Examine in daylight. The chromatogram obtained with the reference solution shows a violet zone (alanine) in its central third. The chromatogram obtained with the test solution shows a violet or brownish-red zone similar in position to that in the chromatogram obtained with the reference solution and corresponding to alliin; above and below this zone are other, generally fainter, violet zones.

TESTS

Starch. Examine the powdered drug under a microscope using *water R*. Add *iodine solution R1*. No blue colour develops.

Loss on drying (2.2.32). Not more than 7.0 per cent, determined on 1.000 g of the powdered drug by drying in an oven at 100-105 °C.

Total ash (2.4.16). Not more than 5.0 per cent.

ASSAY

Examine by liquid chromatography (2.2.29), using *butyl parahydroxybenzoate R* as the internal standard. Carry out the assay as quickly as possible.

Internal standard solution. Dissolve 20.0 mg of *butyl parahydroxybenzoate R* in 100.0 ml of a mixture of equal volumes of *methanol R* and *water R*.

Test solution. To 0.800 g of garlic powder add 20.0 ml of *water R* and homogenise the mixture in an ultrasonic bath at 4 °C for 5 min. Allow to stand at room temperature for 30 min. Then centrifuge for 30 min. Dilute 10.0 ml of the supernatant to 25.0 ml with a mixture of 40 volumes of a

1 per cent *V/V* solution of *anhydrous formic acid R* and 60 volumes of *methanol R* (stock solution). Shake and centrifuge for 5 min. Place 0.50 ml of the internal standard solution in a volumetric flask and dilute to 10.0 ml with the stock solution.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4 mm in internal diameter packed with silanised *octadecylsilyl silica gel for chromatography R* (5 µm) combined with a stainless steel precolumn 20 mm long and 4 mm in internal diameter packed with silanised *octadecylsilyl silica gel for chromatography R* (5 µm),
- as mobile phase at a flow rate of 0.8 ml/min a mixture of 40 volumes of a 1 per cent *V/V* solution of *anhydrous formic acid R* and 60 volumes of *methanol R*,
- a loop injector,
- as detector a spectrophotometer set at 254 nm.

Inject 1 µl of the internal standard solution. Inject 10 µl of the test solution. Adjust the sensitivity of the system so that the height of the peak due to butyl parahydroxybenzoate in the chromatogram obtained with the test solution is about 50 per cent of the full scale of the recorder.

Calculate the percentage of allicin from the expression:

$$\frac{S_1 \times m_2 \times 22.75}{S_2 \times m_1}$$

*S*₁ = area of the peak corresponding to allicin (most prominent peak),

*S*₂ = area of the peak corresponding to butyl parahydroxybenzoate in the chromatogram obtained with the test solution,

*m*₁ = mass of the drug, in grams,

*m*₂ = mass of butyl parahydroxybenzoate in grams in 100.0 ml of the internal standard solution. 1 mg of butylparahydroxybenzoate corresponds to 8.65 mg of allicin.

STORAGE

Store protected from light.

01/2005:0330

GELATIN

Gelatina

DEFINITION

Purified protein obtained either by partial acid hydrolysis (type A), partial alkaline hydrolysis (type B) or enzymatic hydrolysis of collagen from animals (including fish and poultry); it may also be a mixture of different types.

The hydrolysis leads to gelling or non-gelling product grades. Both product grades are covered by this monograph.

Gelatin described in this monograph is not suitable for parenteral use or for other special purposes.

CHARACTERS

Appearance: faintly yellow or light yellowish-brown, solid, usually occurring as translucent sheets, shreds, granules or powder.

Solubility: practically insoluble in common organic solvents; gelling grades swell in cold water and give on heating a colloidal solution which on cooling forms a more or less firm gel.

The isoelectric point is a relevant quality parameter for use of gelatin in different applications: for type A gelatin it is typically between pH 6.0 and pH 9.5 and for type B gelatin it is typically between pH 4.7 and pH 5.6. These ranges cover a variety of different gelatins and for specific applications a narrower tolerance is usually applied.

Different gelatins form aqueous solutions that vary in clarity and colour. For a particular application, a suitable specification for clarity and colour is usually applied.

IDENTIFICATION

- A. To 2 ml of solution S (see Tests) add 0.05 ml of *copper sulphate solution R*. Mix and add 0.5 ml of *dilute sodium hydroxide solution R*. A violet colour is produced.
- B. To 0.5 g in a test-tube add 10 ml of *water R*. Allow to stand for 10 min, heat at 60 °C for 15 min and keep the tube upright at 0 °C for 6 h. Invert the tube; the contents immediately flow out for non-gelling grades and do not flow out immediately for gelling grades.

TESTS

Solution S. Dissolve 1.00 g in *carbon dioxide-free water R* at about 55 °C, dilute to 100 ml with the same solvent and keep the solution at this temperature to carry out the tests.

pH (2.2.3): 3.8 to 7.6 for solution S.

Conductivity (2.2.38): maximum 1 mS·cm⁻¹, determined on a 1.0 per cent solution at 30 ± 1.0 °C.

Sulphur dioxide (2.5.29): maximum 50 ppm.

Peroxides: maximum 10 ppm, determined using *peroxide test strips R*.

Peroxidase transfers oxygen from peroxides to an organic redox indicator which is converted to a blue oxidation product. The intensity of the colour obtained is proportional to the quantity of peroxide and can be compared with a colour scale provided with the test strips, to determine the peroxide concentration.

Suitability test. Dip a test strip for 1 s into *hydrogen peroxide standard solution (10 ppm H₂O₂) R*, such that the reaction zone is properly wetted. Remove the test strip, shake off excess liquid and compare the reaction zone after 15 s with the colour scale provided with the test strips used. The colour must match that of the 10 ppm concentration, otherwise the test is invalid.

Test. Weigh 20.0 ± 0.1 g of the substance to be tested in a beaker and add 80.0 ± 0.2 ml of *water R*. Stir to moisten all gelatin and allow the sample to stand at room temperature for 1-3 h. Cover the beaker with a watch-glass. Place the beaker for 20 ± 5 min in a water bath at 65 ± 2 °C to dissolve the sample. Stir the contents of the beaker with a glass rod to achieve a homogeneous solution. Dip a test strip for 1 s into the test solution, such that the reaction zone is properly wetted. Remove the test strip, shake off excess liquid and compare the reaction zone after 15 s with the colour scale provided with the test strips used. Multiply the concentration read from the colour scale by a factor of 5 to calculate the concentration in parts per million of peroxide in the test substance.

Gel strength (Bloom value): 80 to 120 per cent of the labelled nominal value.

The gel strength is expressed as the mass in grams necessary to produce the force which, applied to a plunger 12.7 mm in diameter, makes a depression 4 mm deep in a gel having a concentration of 6.67 per cent *m/m* and matured at 10 °C.

Apparatus. Texture analyser or gelometer with:

- a cylindrical piston 12.7 ± 0.1 mm in diameter with a plane pressure surface with a sharp bottom edge,
- a bottle 59 ± 1 mm in internal diameter and 85 mm high.

Adjust the apparatus according to the manufacturer's manual. Settings are: distance 4 mm, test speed 0.5 mm/s.

Method. Perform the test in duplicate. Place 7.5 g of the substance to be tested in each bottle. Add 105 ml of *water R*, place a watch-glass over each bottle and allow to stand for 1-4 h. Heat in a water-bath at 65 ± 2 °C for 15 min. While heating, gently stir with a glass rod. Ensure that the solution is uniform and that any condensed water on the inner walls of the bottle is incorporated. Allow to cool at room temperature for 15 min and transfer the bottles to a thermostatically controlled bath at 10.0 ± 0.1 °C, and fitted with a device to ensure that the platform on which the bottles stand is perfectly horizontal. Close the bottles with a rubber stopper and allow to stand for 17 ± 1 h. Remove the sample bottles from the bath and quickly wipe the water from the exterior of the bottle. Centre consecutively the 2 bottles on the platform of the apparatus so that the plunger contacts the sample as nearly at its midpoint as possible and start the measurement. Report the result as the average of the 2 measurements.

Iron: maximum 30 ppm.

Atomic absorption spectrometry (2.2.23, *Method I*).

Test solution. To 5.00 g of the substance to be examined, in a conical flask, add 10 ml of *hydrochloric acid R*. Close the flask and place in a water-bath at 75-80 °C for 2 h. Allow to cool and adjust the content of the flask to 100.0 g with *water R*.

Reference solutions. Prepare the reference solutions using *iron standard solution (8 ppm Fe) R*, diluted as necessary with *water R*.

Wavelength: 248.3 nm.

Chromium: maximum 10 ppm.

Atomic absorption spectrometry (2.2.23, *Method I*).

Test solution. Test solution described in the test for iron.

Reference solutions. Prepare the reference solutions using *chromium standard solution (100 ppm Cr) R*, diluted if necessary with *water R*.

Wavelength: 357.9 nm.

Zinc: maximum 30 ppm.

Atomic absorption spectrometry (2.2.23, *Method I*).

Test solution. Test solution described in the test for iron.

Reference solutions. Prepare the reference solutions using *zinc standard solution (10 ppm Zn) R*, diluted if necessary with *water R*.

Wavelength: 213.9 nm.

Loss on drying (2.2.32): maximum 15.0 per cent, determined on 1.000 g, by drying in an oven at 100-105 °C.

Microbial contamination. Total viable aerobic count (2.6.12) not more than 10³ micro-organisms per gram, determined by plate count. It complies with the tests for *Escherichia coli* and *Salmonella* (2.6.13).

STORAGE

Protect from heat and moisture.

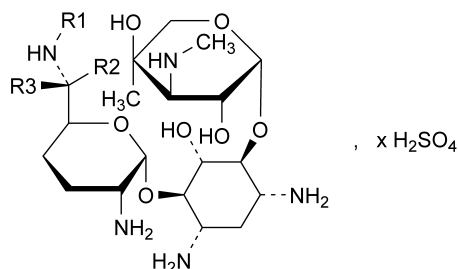
LABELLING

The label states the gel strength (Bloom value) or that it is a non-gelling grade.

01/2005:0331
corrected

GENTAMICIN SULPHATE

Gentamicini sulfas



Gentamicin	Mol. Formula	R1	R2	R3
C1	C ₂₁ H ₄₃ N ₅ O ₇	CH ₃	CH ₃	H
C1a	C ₁₉ H ₃₉ N ₅ O ₇	H	H	H
C2	C ₂₀ H ₄₁ N ₅ O ₇	H	CH ₃	H
C2a	C ₂₀ H ₄₁ N ₅ O ₇	H	H	CH ₃
C2b	C ₂₀ H ₄₁ N ₅ O ₇	CH ₃	H	H

DEFINITION

Mixture of the sulphates of antimicrobial substances produced by *Micromonospora purpurea*, the main components being gentamicins C1, C1a, C2, C2a and C2b.

Content: minimum 590 IU/mg (anhydrous substance).

CHARACTERS

Appearance: white or almost white, hygroscopic powder.

Solubility: freely soluble in water, practically insoluble in alcohol.

IDENTIFICATION

First identification: C, D.

Second identification: A, B, D.

A. Dissolve about 10 mg in 1 ml of *water R* and add 5 ml of a 400 g/l solution of *sulphuric acid R*. Heat on a water-bath for 100 min, cool and dilute to 25 ml with *water R*. Examined between 240 nm and 330 nm (2.2.25), the solution shows no absorption maximum.

B. Thin-layer chromatography (2.2.27).

Test solution. Dissolve 25 mg of the substance to be examined in *water R* and dilute to 5 ml with the same solvent.

Reference solution. Dissolve 25 mg of *gentamicin sulphate CRS* in *water R* and dilute to 5 ml with the same solvent.

Plate: TLC silica gel plate *R*.

Mobile phase: the lower layer of a mixture of equal volumes of *concentrated ammonia R*, *methanol R* and *methylene chloride R*.

Application: 10 µl.

Development: over 2/3 of the plate.

Drying: in air.

Detection: spray with *ninhydrin solution R1* and heat at 110 °C for 5 min.

Results: the 3 principal spots in the chromatogram obtained with the test solution are similar in position, colour and size to the 3 principal spots in the chromatogram obtained with the reference solution.

C. Examine the chromatograms obtained in the test for composition.

Results: the chromatogram obtained with the test solution shows 5 principal peaks having the same retention times as the 5 principal peaks in the chromatogram obtained with reference solution (a).

D. It gives reaction (a) of sulphates (2.3.1).

TESTS

Solution S. Dissolve 0.8 g in *carbon dioxide-free water R* and dilute to 20 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and not more intensely coloured than intensity 6 of the range of reference solutions of the most appropriate colour (2.2.2, *Method II*).

pH (2.2.3): 3.5 to 5.5 for solution S.

Specific optical rotation (2.2.7): + 107 to + 121 (anhydrous substance).

Dissolve 2.5 g in *water R* and dilute to 25.0 ml with the same solvent.

Composition. Liquid chromatography (2.2.29): use the normalisation procedure taking into account only the peaks due to gentamicins C1, C1a, C2, C2a and C2b; use the chromatogram supplied with *gentamicin sulphate CRS* to identify the corresponding peaks.

Test solution. Dissolve 50 mg of the substance to be examined in the mobile phase and dilute to 100.0 ml with the mobile phase.

Reference solution (a). Dissolve the content of a vial of *gentamicin sulphate CRS* in the mobile phase and dilute with the mobile phase to obtain a solution containing 0.5 mg/ml.

Reference solution (b). Dilute 5.0 ml of reference solution (a) to 100.0 ml with the mobile phase.

Column:

- *size*: $l = 0.25$ m, $\varnothing = 4.6$ mm,
- *stationary phase*: *styrene-divinylbenzene copolymer R* (8 µm) with a pore size of 100 nm,
- *temperature*: 55 °C.

Mobile phase: a mixture prepared with *carbon dioxide-free water R* containing 60 g/l of *anhydrous sodium sulphate R*, 1.75 g/l of *sodium octanesulphonate R*, 8 ml/l of *tetrahydrofuran R*, 50 ml/l of 0.2 M *potassium dihydrogen phosphate R* previously adjusted to pH 3.0 with *dilute phosphoric acid R* and degassed.

Flow rate: 1.0 ml/min.

Post-column solution: a *carbonate-free sodium hydroxide solution R* diluted 1 to 25, previously degassed, which is added pulse-less to the column effluent using a 375 µl polymeric mixing coil.

Flow rate: 0.3 ml/min.

Detection: pulsed amperometric detector or equivalent with a gold indicator electrode, a silver-silver chloride reference electrode, and a stainless steel auxiliary electrode which is the cell body, held at respectively + 0.05 V detection, + 0.75 V oxidation and -0.15 V reduction potentials, with pulse durations according to the instrument used.

Injection: 20 µl.

Run time: 1.2 times the retention time of gentamicin C1.

System suitability: reference solution (a):

- **peak-to-valley ratio:** minimum 2.0 where H_p = height above the baseline of the peak due to gentamicin C2a, and H_v = height above the baseline of the lowest point of the curve separating this peak from the peak due to gentamicin C2.

Limits:

- **gentamicin C1:** 20.0 per cent to 40.0 per cent,
- **gentamicin C1a:** 10.0 per cent to 30.0 per cent,
- **sum of gentamicins C2, C2a, and C2b:** 40.0 per cent to 60.0 per cent,
- **disregard limit:** the area of the peak due to gentamicin C1a in the chromatogram obtained with reference solution (b).

Related substances. Liquid chromatography (2.2.29) as described in the test for composition.

Limits (for related substances eluting before gentamicin C1a):

- **any impurity:** maximum 3.0 per cent,
- **total:** maximum 10.0 per cent.

Methanol (2.4.24, *System B*): maximum 1.0 per cent.

Sulphate: 32.0 per cent to 35.0 per cent (anhydrous substance).

Dissolve 0.250 g in 100 ml of *distilled water R* and adjust the solution to pH 11 using *concentrated ammonia R*. Add 10.0 ml of 0.1 M *barium chloride* and about 0.5 mg of *phthalein purple R*. Titrate with 0.1 M *sodium edetate*, adding 50 ml of *alcohol R* when the colour of the solution begins to change and continue the titration until the violet-blue colour disappears.

1 ml of 0.1 M *barium chloride* is equivalent to 9.606 mg of SO_4 .

Water (2.5.12): maximum 15.0 per cent, determined on 0.300 g.

Sulphated ash (2.4.14): maximum 1.0 per cent, determined on 0.50 g.

Bacterial endotoxins (2.6.14): less than 0.71 IU/mg, if intended for use in the manufacture of parenteral dosage forms without a further appropriate procedure for the removal of bacterial endotoxins.

ASSAY

Carry out the microbiological assay of antibiotics (2.7.2).

STORAGE

In an airtight container. If the substance is sterile, store in a sterile, airtight, tamper-proof container.

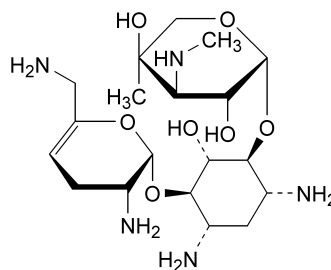
LABELLING

The label states, where applicable, that the substance is free from bacterial endotoxins.

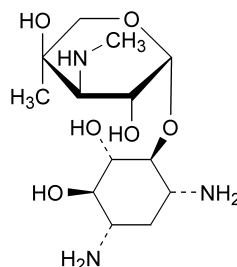
IMPURITIES

Specified impurities: A, B, C.

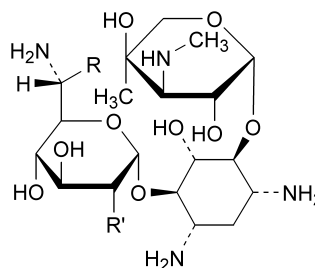
Other detectable impurities: D, E.



A. 2-deoxy-4-O-[3-deoxy-4-C-methyl-3-(methylamino)-β-L-arabinopyranosyl]-6-O-(2,6-diamino-2,3,4,6-tetra-deoxy-α-D-glycero-hex-4-enopyranosyl)-L-streptamine (sisomicin),

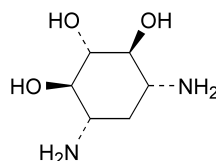


B. 2-deoxy-4-O-[3-deoxy-4-C-methyl-3-(methylamino)-β-L-arabinopyranosyl]-L-streptamine (garamine),



C. R = CH_3 , R' = OH: 4-O-(6-amino-6,7-dideoxy-D-glycero-α-D-glucopyranosyl)-2-deoxy-6-O-[3-deoxy-4-C-methyl-3-(methylamino)-β-L-arabinopyranosyl]-D-streptamine (gentamicin B₁),

D. R = H, R' = NH_2 : 2-deoxy-4-O-[3-deoxy-4-C-methyl-3-(methylamino)-β-L-arabinopyranosyl]-6-O-(2,6-diamino-2,6-dideoxy-α-D-glucopyranosyl)-L-streptamine,



E. 2-deoxystreptamine.

01/2005:0392

GENTIAN ROOT

Gentianae radix

DEFINITION

Dried, fragmented underground organs of *Gentiana lutea* L.

CHARACTERS

Gentian root occurs as single or branched subcylindrical pieces of various lengths and usually 10 mm to 40 mm thick but occasionally up to 80 mm thick at the crown.

Macroscopic and microscopic characters described under identification tests A and B.

Characteristic odour and strong and persistent bitter taste.

IDENTIFICATION

- A. The surface is brownish-grey, and the colour of a transverse section is yellowish to reddish-yellow, but not reddish-brown. The root is longitudinally wrinkled and bears occasional rootlet scars. The branches of the rhizome frequently bear a terminal bud and are always encircled by closely arranged leaf scars. The rhizome and root are brittle when dry and break with a short fracture but they absorb moisture readily to become flexible. The smoothed, transversely cut surface shows a bark, occupying about one-third of the radius, separated by the well-marked cambium from an indistinctly radiate and mainly parenchymatous xylem.
- B. Reduce to a powder (355). The powder is light brown or yellowish-brown. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters: fragments of the subero-phellodermic layer, consisting of thin-walled yellowish-brown cork cells and thick-walled collenchyma (phello-derm); fragments of cortical and ligneous parenchymatous cells with moderately thickened walls containing droplets of oil and small prisms and minute needles of calcium oxalate; fragments of lignified vessels with spiral or reticulate thickening.
- C. Thin-layer chromatography (2.2.27).

Test solution. To 1.0 g of the powdered drug (355) add 25 ml of *methanol R*, shake for 15 min and filter. Evaporate the filtrate to dryness under reduced pressure, at a temperature not exceeding 50 °C. Take up the residue with small quantities of *methanol R* so as to obtain 5 ml of a solution, which may contain a sediment.

Reference solution. Dissolve 5 mg of *phenazone R* and 5 mg of *hyperoside R* in 10 ml of *methanol R*.

Plate: TLC silica gel *F*₂₅₄ plate *R*.

Mobile phase: *water R*, *anhydrous formic acid R*, *ethyl formate R* (4:8:88 V/V/V).

Application: 20 µl, as bands.

Development: over a path of 8 cm, in an unsaturated tank.

Drying: in air.

Detection A: examine in ultraviolet light at 254 nm.

Results A: see below the sequence of the zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other zones may be present in the chromatogram obtained with the test solution.

Top of the plate	
Phenazone: a quenching zone	A prominent quenching zone
	A weak quenching zone (amarogentin)
Hyperoside: a quenching zone	A prominent quenching zone (gentiopicroside)
Reference solution	Test solution

Detection B: spray with a 10 per cent V/V solution of *potassium hydroxide R* in *methanol R* and then with a freshly prepared 2 g/l solution of *fast blue B salt R* in a mixture of *ethanol R* and *water R* (50:50 V/V). Examine in daylight.

Results B: see below the sequence of the zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other zones may be present in the chromatogram obtained with the test solution.

Top of the plate	
	A prominent dark violet zone
	A violet-red zone (amarogentin)
Hyperoside: a brownish-red zone	A weak light brown zone (gentiopicroside)
Reference solution	Test solution

TESTS

Chromatography. Examine the chromatograms obtained in identification test C, detection B.

Results: the chromatogram obtained with the test solution does not show violet zones immediately above the zone due to amarogentine (other species of *Gentiana*).

Bitterness value (2.8.15): minimum 10 000.

Water-soluble extractive: minimum 33 per cent.

To 5.0 g of powdered drug (710) add 200 ml of boiling *water R*. Allow to stand for 10 min, shaking occasionally. Allow to cool, dilute to 200.0 ml with *water R* and filter. Evaporate 20.0 ml of the filtrate to dryness on a water-bath. Dry the residue in an oven at 100-105 °C. The residue weighs a minimum of 0.165 g.

Total ash (2.4.16): maximum 6.0 per cent.

01/2005:1870

GENTIAN TINCTURE

Gentianae tinctura

DEFINITION

Tincture produced from *Gentian root (0392)*.

PRODUCTION

The tincture is produced from 1 part of the comminuted drug and 5 parts of ethanol (70 per cent V/V) by a suitable procedure.

CHARACTERS

Appearance: yellowish-brown or reddish-brown liquid. It has a strong bitter taste.

IDENTIFICATION

Thin-layer chromatography (2.2.27).

Test solution. The tincture to be examined.

Reference solution. Dissolve 5 mg of *phenazone R* and 5 mg of *hyperoside R* in 10 ml of *methanol R*.

Plate: TLC silica gel *F*₂₅₄ plate *R*.

Mobile phase: *water R*, *anhydrous formic acid R*, *ethyl formate R* (4:8:88 V/V/V).

Application: 20 µl, as bands.

Development: over a path of 8 cm, in an unsaturated tank.

Drying: in air.

Detection A: examine in ultraviolet light at 254 nm.

Monographs
F-H

Results A: see below the sequence of the zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other zones may be present in the chromatogram obtained with the test solution.

Top of the plate	
Phenazone: a quenching zone	A prominent quenching zone
_____	A weak quenching zone (amarogentin)
_____	_____
Hyperoside: a quenching zone	A prominent quenching zone (gentiopicroside)
Reference solution	Test solution

Detection B: spray with a 10 per cent V/V solution of *potassium hydroxide R* in *methanol R* and then with a freshly prepared 2 g/l solution of *fast blue B salt R* in a mixture of *ethanol R* and *water R* (50:50 V/V). Examine in daylight.

Results B: see below the sequence of the zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other zones may be present in the chromatogram obtained with the test solution.

Top of the plate	
_____	A prominent dark violet zone
_____	A violet-red zone (amarogentin)
_____	_____
Hyperoside: a brownish-red zone	A weak light brown zone (gentiopicroside)
Reference solution	Test solution

TESTS

Ethanol content (2.9.10): 62 per cent V/V to 67 per cent V/V.

Bitterness value (2.8.15): minimum 1000.

Dry residue (2.8.16): minimum 5.0 per cent m/m, determined on 3.00 g.

01/2005:1522

GINGER

Zingiberis rhizoma

DEFINITION

Ginger consists of the dried, whole or cut rhizome of *Zingiber officinale* Roscoe, with the cork removed, either completely or from the wide flat surfaces only. Whole or cut, it contains not less than 15 ml/kg of essential oil, calculated with reference to the anhydrous drug.

CHARACTERS

Ginger has a characteristic aromatic odour and a spicy and burning taste.

It has the macroscopic and microscopic characters described under identification tests A and B.

IDENTIFICATION

A. The rhizome is laterally compressed, bearing short, flattened, obovate oblique branches on the upper side, each sometimes having a depressed scar at the apex;

the whole rhizomes are about 5 cm to 10 cm long, 1.5 cm to 3 cm or 4 cm wide and 1 cm to 1.5 cm thick, sometimes split longitudinally. The scraped rhizome with a light-brown external surface shows longitudinal striations and occasional loose fibres; the outer surface of the unscraped rhizome varies from pale to dark brown and is more or less covered with cork which shows conspicuous, narrow, longitudinal and transverse ridges; the cork readily exfoliates from the lateral surfaces but persists between the branches. The fracture is short and starchy with projecting fibres. The smoothed transversely cut surface exhibits a narrow cortex separated by an endodermis from a much wider stele; it shows numerous, scattered, fibrovascular bundles and abundant scattered oleoresin cells with yellow contents. The unscraped rhizome shows, in addition, an outer layer of dark brown cork.

B. Reduce to a powder (355). The powder is pale yellow to brownish. Examine under a microscope using *chloral hydrate solution R*. The powder shows groups of large, thin-walled, septate fibres, with one wall frequently dentate; fairly large vessels with reticulate thickening and often accompanied by narrow, thin-walled cells containing brown pigment; abundant thin-walled parenchyma of the ground tissue, some cells containing brown oleoresin; fragments of brown cork, usually seen in surface view. Examine under a microscope using a 50 per cent V/V solution of *glycerol R*. The powder shows abundant starch granules, simple, flattened, oblong to oval or irregular, up to about 50 µm long and 25 µm wide, with a small point hilum situated at the narrower end; occasional granules show faint, transverse striations.

C. Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel plate R*.

Test solution. To 1.0 g of the powdered drug (710) add 5 ml of *methanol R*. Shake for 15 min and filter.

Reference solution. Dissolve 10 µl of *citral R* and 10 mg of *resorcinol R* in 10 ml of *methanol R*. Prepare the solution immediately before use.

Apply to the plate as bands, 20 µl of each solution. Develop over a path of 15 cm in an unsaturated chromatographic tank using a mixture of 40 volumes of *hexane R* and 60 volumes of *ether R*. Allow the plate to dry in air. Spray the plate with a 10 g/l solution of *vanillin R* in *sulphuric acid R* and examine in daylight while heating at 100 °C to 105 °C for 10 min. The chromatogram obtained with the reference solution shows in the lower half an intense red zone (resorcinol) and in the upper half two violet zones (citral). The chromatogram obtained with the test solution shows below the resorcinol zone in the chromatogram obtained with the reference solution two intense violet zones (gingerols) and in the middle, between the resorcinol and citral zones in the chromatogram obtained with the reference solution, two other less intense violet zones (shogaols). Other zones may be present.

TESTS

Foreign matter (2.8.2). It complies with the test for foreign matter.

Water (2.2.13). Not more than 100 ml/kg, determined by distillation on 20.0 g of the powdered drug (710).

Total ash (2.4.16). Not more than 6.0 per cent.

ASSAY

Carry out the determination of essential oils in vegetable drugs (2.8.12). Use 20.0 g of the freshly, coarsely powdered drug, a 1000 ml round-bottomed flask, 10 drops of *liquid*

paraffin R or other antifoam, 500 ml of *water R* as distillation liquid and 0.5 ml of *xylene R* in the graduated tube. Distil at a rate of 2 ml/min to 3 ml/min for 4 h.

STORAGE

Store protected from light.

01/2005:1828

GINKGO LEAF

Ginkgo folium

DEFINITION

Whole or fragmented, dried leaf of *Ginkgo biloba* L.

Content: not less than 0.5 per cent of flavonoids, calculated as flavone glycosides (M_r 757) (dried drug).

CHARACTERS

Ginkgo leaf is greyish or yellowish-green or yellowish-brown. Macroscopic and microscopic characters described under identification tests A and B.

IDENTIFICATION

- A. The upper surface of ginkgo leaf is slightly darker than the lower surface. The petioles of the leaf are about 4 cm to 9 cm long. The lamina is about 4 cm to 10 cm wide, fan-shaped, usually bilobate or sometimes undivided. Both surfaces are smooth, and the venation dichotomous, the veins appearing to radiate from the base; they are equally prominent on both surfaces. The distal margin is incised, irregularly and to different degrees, and irregularly lobate or emarginate. The lateral margins are entire and taper towards the base.
- B. Reduce to a powder (355). The powder is greyish or yellowish-green or yellowish-brown. Examine under a microscope using *chloral hydrate solution R*. The powder shows irregularly-shaped fragments of the lamina in surface view, the upper epidermis consisting of elongated cells with irregularly sinuous walls, the lower epidermal cells smaller, with a finely striated cuticle and each cell shortly papillose; stomata about 60 µm, large, deeply sunken with 6 to 8 subsidiary cells, are more numerous in the lower epidermis; abundant large cluster crystals of calcium oxalate of various sizes in the mesophyll; fragments of fibro-vascular tissue from the petiole and veins.
- C. Thin-layer chromatography (2.2.27).
Test solution. To 2.0 g of the powdered drug (710) add 10 ml of *methanol R*. Heat in a water-bath at 65 °C for 10 min. Shake frequently. Allow to cool to room temperature and filter.
Reference solution. Dissolve 1.0 mg of *chlorogenic acid R* and 3.0 mg of *rutin R* in 20 ml of *methanol R*.
Plate: TLC silica gel plate *R*.
Mobile phase: *anhydrous formic acid R*, *glacial acetic acid R*, *water R*, *ethyl acetate R* (7.5:7.5:17.5:67.5 V/V/V/V).
Application: 20 µl, as bands.
Development: over a path of 17 cm.
Drying: at 100-105 °C.
Detection: spray the warm plate with a 10 g/l solution of *diphenylboric acid aminoethyl ester R* in *methanol R*. Subsequently spray with the same volume of a 50 g/l

solution of *macrogol 400 R* in *methanol R*. Allow the plate to dry in air for about 30 min. Examine in ultraviolet light at 365 nm.

Results: see below the sequence of the zones present in the chromatograms obtained with the reference and test solutions. Furthermore, other weaker fluorescent zones may be present in the chromatogram obtained with the test solution.

Top of the plate	
Chlorogenic acid: a light blue fluorescent zone Rutin: a yellowish-brown fluorescent zone	A yellowish-brown fluorescent zone A green fluorescent zone 2 yellowish-brown fluorescent zones An intense light blue fluorescent zone sometimes overlapped by a greenish-brown fluorescent zone
	A green fluorescent zone 2 yellowish-brown fluorescent zones A green fluorescent zone A yellowish-brown fluorescent zone
Reference solution	Test solution

TESTS

Foreign matter (2.8.2): maximum 5 per cent of stems and 2 per cent of other foreign matter.

Loss on drying (2.2.32): maximum 11.0 per cent, determined on 1.000 g of the powdered drug (355) by drying in an oven at 100-105 °C for 2 h.

Total ash (2.4.16): maximum 11.0 per cent.

ASSAY

Flavonoids. Liquid chromatography (2.2.29).

Test solution. Heat 2.500 g of the powdered drug (710) in 50 ml of a 60 per cent V/V solution of *acetone R* under a reflux condenser for 30 min. Filter and collect the filtrate. Extract the drug residue a second time in the same manner, using 40 ml of a 60 per cent V/V solution of *acetone R* and filter. Collect the filtrates and dilute to 100.0 ml with a 60 per cent V/V solution of *acetone R*. Evaporate 50.0 ml of the solution to eliminate the acetone and transfer to a 50.0 ml vial, rinsing with 30 ml of *methanol R*. Add 4.4 ml of *hydrochloric acid R1*, dilute to 50.0 ml with *water R* and centrifuge. Place 10 ml of the supernatant liquid in a 10 ml brown-glass vial. Close with a rubber seal and an aluminium cap and heat on a water-bath for 25 min. Allow to cool to room temperature.

Reference solution. Dissolve 10.0 mg of *quercetin dihydrate R* in 20 ml of *methanol R*. Add 15.0 ml of *dilute hydrochloric acid R* and 5 ml of *water R* and dilute to 50.0 ml with *methanol R*.

Column:

- **stationary phase:** octadecylsilyl silica gel for chromatography *R* (5 µm),
- **size:** l = 0.125 m, Ø = 4 mm,
- **temperature:** 25 °C.

Mobile phase:

- **mobile phase A:** 0.3 g/l solution of *phosphoric acid R* adjusted to pH 2.0,
- **mobile phase B:** *methanol R*,

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)
0 - 1	60	40
1 - 20	60 → 45	40 → 55
20 - 21	45 → 0	55 → 100
21 - 25	0	100

Flow rate: 1.0 ml/min.

Detector: spectrophotometer at 370 nm.

Injection: 10 µl.

Relative retention with reference to quercetin (retention time = about 12.5 min): kaempferol = about 1.4; isorhamnetin = about 1.5.

System suitability:

- *resolution:* minimum 1.5 between the peaks due to kaempferol and to isorhamnetin.

Do not take into account peaks eluting before the quercetin peak or after the isorhamnetin peak in the chromatogram obtained with the test solution.

Calculate the percentage content of flavonoids, expressed as flavone glycosides, from the expression:

$$2 \times \frac{F_1 \times m_1 \times 2.514 \times p}{F_2 \times m_2}$$

- F_1 = sum of the areas of all the considered peaks in the chromatogram obtained with the test solution,
- F_2 = area of the peak corresponding to quercetin in the chromatogram obtained with the reference solution,
- m_1 = mass of quercetin used to prepare the reference solution, in grams,
- m_2 = mass of the drug to be examined used to prepare the test solution, in grams,
- p = percentage content of anhydrous quercetin in *quercetin dihydrate R*.

01/2005:1523

GINSENG

Ginseng radix

DEFINITION

Ginseng consists of the whole or cut dried root of *Panax ginseng* C. A. Meyer. It contains not less than 0.40 per cent of combined ginsenosides Rg1 (C₄₂H₇₂O₁₄·2H₂O; M_r 837) and Rb1 (C₅₄H₉₂O₂₃·3H₂O; M_r 1163), calculated with reference to the dried drug.

CHARACTERS

It has the macroscopic and microscopic characters described under identification tests A and B.

IDENTIFICATION

- A. The principal root is fusiform or cylindrical, sometimes branched, up to about 20 cm long and 2.5 cm in diameter, and may be curved or markedly re-curved. The surface is pale yellow to cream and shows longitudinal ridges; stem scars may be seen at the crown. The fracture is short. The transversely-cut surface shows a wide outer zone with scattered orange-red resin canals and a finely radiate inner region. The rootlets, numerous in the lower part, are fine with a small diameter.

- B. Reduce to a powder (355). Examine under a microscope using *chloral hydrate solution R*. The light yellow powder shows abundant fragments of thin-walled parenchymatous cells and fragments of large secretory canals containing yellowish-brown resin. The powder occasionally shows non-lignified tracheids and partially-lignified vessels with spiral or reticulate thickening, occurring singly or in small groups, and scattered cluster crystals of calcium oxalate. Examine under a microscope using a mixture of equal volumes of *glycerol R* and *water R*. The starch granules are very abundant, simple or two or three compound, and range from 1 µm to 10 µm in diameter.
- C. Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel plate R*.

Test solution. Boil 1.0 g of the powdered drug (355) under a reflux condenser with 10 ml of a 70 per cent V/V solution of *methanol R* for 15 min. Filter after cooling and dilute to 10.0 ml with *methanol R*.

Reference solution. Dissolve 5.0 mg of *aescin R* and 5.0 mg of *arbutin R* in 1 ml of *methanol R*.

Apply to the plate as bands 20 µl of each solution. Develop over a path of 10 cm, in an unsaturated tank, using the upper layer of a mixture of 25 ml of *ethyl acetate R*, 50 ml of *water R* and 100 ml of *butanol R*, which has been allowed to separate for 10 min. Allow the plate to dry in air. Spray with *anisaldehyde solution R* and heat at 105 °C to 110 °C for 5 min to 10 min. Examine in daylight. The chromatogram obtained with the reference solution shows in the upper third a brown zone corresponding to arbutin, and in the lower third a grey zone corresponding to aescin. Between these two zones, a little below the zone corresponding to arbutin in the chromatogram obtained with the reference solution, the chromatogram obtained with the test solution shows two violet-grey zones due to ginsenoside Rg1 (upper zone) and to ginsenoside Re (lower zone). The violet-grey zone due to ginsenoside Rb1 is similar in position to the grey zone due to aescin in the chromatogram obtained with the reference solution. Between the zones corresponding to ginsenoside Rb1 and Re other, less intense, bands are present; the zone closest to the starting point corresponds to ginsenoside Rc. Other zones are visible in the lower third of the chromatogram.

TESTS

Foreign matter (2.8.2). It complies with the test for foreign matter.

Panax quinquefolium. Examine the chromatograms obtained in the assay. The chromatogram obtained with the test solution shows the peak corresponding to ginsenoside Rf. In the case of a substitution by *Panax quinquefolium* no peak corresponding to ginsenoside Rf is present.

Loss on drying (2.2.32). Not more than 10.0 per cent, determined on 1.000 g of the powdered drug (355) by drying in an oven at 100 °C to 105 °C.

Total ash (2.4.16). Not more than 7.0 per cent.

Ash insoluble in hydrochloric acid (2.8.1). Not more than 1.0 per cent.

ASSAY

Examine by liquid chromatography (2.2.29).

Test solution. Reduce about 50 g to a powder (355). Place 1.00 g of the powdered drug and 70 ml of a 50 per cent V/V solution of *methanol R* in a 250 ml round-bottomed flask. After adding a few grains of pumice, boil on a water-bath under a reflux condenser for 1 h. After cooling, centrifuge

and collect the supernatant liquid. Treat the residue as described above. Mix the collected liquids and evaporate to dryness under reduced pressure at a temperature not exceeding 60 °C. Take up the residue with 10 ml of a buffer solution containing 3.5 g of *sodium dihydrogen phosphate R* and 7.2 g of *potassium dihydrogen phosphate R* in 1000 ml of *water R* (solution A).

Wash a cartridge containing about 360 mg of a suitable *octadecylsilyl silica gel for chromatography R* with 5 ml of *methanol R* followed by 20 ml of *water R*. Apply 5 ml of solution A to the cartridge. Elute with 20 ml of *water R*, followed by 15 ml of a 30 per cent V/V solution of *methanol R*. Discard the eluates. Elute with 20 ml of *methanol R*. Evaporate the eluate to dryness and take up the residue with 2 ml of *methanol R*. Discard the eluates after checking that no ginsenosides are present, otherwise repeat the assay with a cartridge from a different manufacturer.

Reference solution. Dissolve 3.0 mg of *ginsenoside Rb1 R*, 3.0 mg of *ginsenoside Rg1 R* and 3.0 mg of *ginsenoside Rf R*, accurately weighed, in *methanol R* and dilute to 10 ml with the same solvent.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.10 m long and 4.6 mm in internal diameter packed with *aminopropylsilyl silica gel for chromatography R*,
- as mobile phase at a flow rate of 2 ml/min:
Mobile phase A. Acetonitrile R,
Mobile phase B. Water R,

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)
0 - 14	90	10
14 - 18	90 → 80	10 → 20
18 - 55	80	20
55 - 60	90	10

- a 20 µl loop injector,
- as detector a spectrophotometer set at 203 nm.

Inject the reference solution and adjust the sensitivity of the system so that the heights of the peaks due to ginsenosides are about 50 per cent of the full scale of the recorder. The test is not valid unless the resolution between the peaks corresponding to ginsenoside Rf and ginsenoside Rg1 is at least 1.0.

Inject the test solution.

Calculate the percentage content of ginsenosides Rb1 and Rg1 from the expression:

$$\frac{A_1 \times m_2 \times 40}{m_1 \times A_{Rb1}} + \frac{A_2 \times m_3 \times 40}{m_1 \times A_{Rg1}}$$

- A_1 = area of the peak due to ginsenoside Rb1 in the chromatogram obtained with the test solution,
 A_2 = area of the peak due to ginsenoside Rg1 in the chromatogram obtained with the test solution,
 A_{Rb1} = area of the peak due to ginsenoside Rb1 in the chromatogram obtained with the reference solution,
 A_{Rg1} = area of the peak due to ginsenoside Rg1 in the chromatogram obtained with the reference solution,
 m_1 = mass of the drug to be examined, in grams,
 m_2 = mass of ginsenoside Rb1, in grams,
 m_3 = mass of ginsenoside Rg1, in grams.

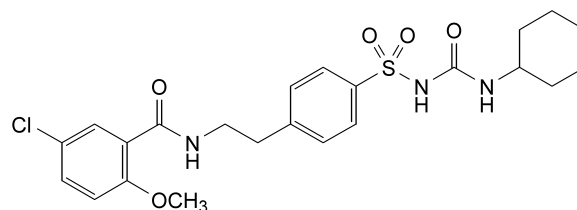
STORAGE

Store protected from light.

01/2005:0718

GLIBENCLAMIDE

Glibenclamidum



$C_{23}H_{28}ClN_3O_5S$

M_r 494.0

DEFINITION

1-[[4-[2-[(5-Chloro-2-methoxybenzoyl)amino]ethyl]phenyl]sulfonyl]-3-cyclohexylurea.

Content: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

Appearance: white or almost white, crystalline powder.

Solubility: practically insoluble in water, sparingly soluble in methylene chloride, slightly soluble in alcohol and in methanol.

IDENTIFICATION

First identification: A, C.

Second identification: A, B, D, E.

A. Melting point (2.2.14): 169 °C to 174 °C.

B. Dissolve 50.0 mg in *methanol R*, with the aid of ultrasound if necessary, and dilute to 50.0 ml with the same solvent. To 10.0 ml of the solution add 1.0 ml of a 103 g/l solution of *hydrochloric acid R* and dilute to 100.0 ml with *methanol R*. Examined between 230 nm and 350 nm (2.2.25), the solution shows an absorption maximum at 300 nm and a less intense maximum at 275 nm. The specific absorbances at the maxima are 61 to 65 and 27 to 32, respectively.

C. Infrared absorption spectrophotometry (2.2.24).

Preparation: discs of *potassium bromide R*.

Comparison: *glibenclamide CRS*.

If the spectra obtained show differences, moisten separately the substance to be examined and the reference substance with *methanol R*, triturate, dry at 100-105 °C and record the spectra again.

D. Thin-layer chromatography (2.2.27).

Test solution. Dissolve 10 mg of the substance to be examined in a mixture of equal volumes of *methanol R* and *methylene chloride R* and dilute to 10 ml with the same mixture of solvents.

Reference solution. Dissolve 10 mg of *glibenclamide CRS* in a mixture of equal volumes of *methanol R* and *methylene chloride R* and dilute to 10 ml with the same mixture of solvents.

Plate: *TLC silica gel GF₂₅₄ plate R*.

Mobile phase: *alcohol R*, *glacial acetic acid R*, *cyclohexane R*, *methylene chloride R* (5:5:45:45 V/V/V/V).

Application: 10 µl.

Development: over a path of 10 cm.

Drying: in air.

Detection: examine in ultraviolet light at 254 nm.

Results: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with the reference solution.

- E. Dissolve 20 mg in 2 ml of *sulphuric acid R*. The solution is colourless and shows blue fluorescence in ultraviolet light at 365 nm. Dissolve 0.1 g of *chloral hydrate R* in the solution. Within about 5 min, the colour changes to deep yellow and, after about 20 min, develops a brownish tinge.

TESTS

Related substances. Liquid chromatography (2.2.29).

Test solution. Dissolve 25.0 mg of the substance to be examined in *methanol R* and dilute to 10.0 ml with the same solvent. Prepare immediately before use.

Reference solution (a). Dissolve 5.0 mg of *glibenclamide impurity A CRS* and 5.0 mg of *glibenclamide impurity B CRS* in *methanol R* and dilute to 100.0 ml with the same solvent. Dilute 5.0 ml of the solution to 20.0 ml with *methanol R*.

Reference solution (b). Dilute 2.0 ml of the test solution to 100.0 ml with *methanol R*. Dilute 5.0 ml of this solution to 50.0 ml with *methanol R*.

Reference solution (c). Dissolve 5 mg of *gliclazide CRS* in *methanol R*, add 2 ml of the test solution and dilute to 100 ml with *methanol R*. Dilute 1 ml of this solution to 10 ml with *methanol R*.

Column:

- **size:** $l = 0.10$ m, $\varnothing = 4.6$ mm,
- **stationary phase:** spherical base-deactivated end-capped octadecylsilyl silica gel for chromatography *R* (3 μ m),
- **temperature:** 35 °C.

Mobile phase:

- **mobile phase A:** mix 20 ml of a 101.8 g/l solution of freshly distilled *triethylamine R* adjusted to pH 3.0 using *phosphoric acid R*, and 50 ml of *acetonitrile R*; dilute to 1000 ml with *water R*,
- **mobile phase B:** mobile phase A, *water R*, *acetonitrile R* (20:65:915 V/V/V),

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)
0 - 15	45	55
15 - 30	45 → 5	55 → 95
30 - 40	5	95
40 - 41	5 → 45	95 → 55
41 - 55	45	55

Flow rate: 0.8 ml/min.

Detection: spectrophotometer at 230 nm.

Injection: 10 μ l.

Relative retention with reference to glibenclamide (retention time = about 5 min): impurity A = about 0.5; impurity B = about 0.6.

System suitability: reference solution (c):

- **resolution:** minimum 5.0 between the peaks due to glibenclamide and gliclazide.

Limits:

- **impurity A:** not more than the area of the corresponding peak in the chromatogram obtained with reference solution (a) (0.5 per cent),
- **impurity B:** not more than the area of the corresponding peak in the chromatogram obtained with reference solution (a) (0.5 per cent),

- **any other impurity:** not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent), and not more than 2 such peaks have an area greater than half the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent),
- **total of other impurities:** not more than 2.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent),
- **disregard limit:** 0.25 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

Heavy metals (2.4.8): maximum 20 ppm.

1.0 g complies with limit test D. Prepare the standard using 2 ml of *lead standard solution* (10 ppm Pb) *R*.

Loss on drying (2.2.32): maximum 1.0 per cent, determined on 1.000 g by drying in an oven at 100-105 °C.

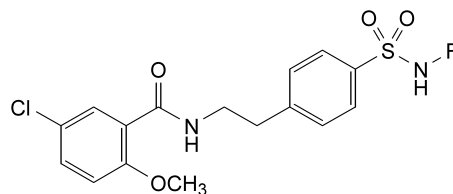
Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.400 g with heating in 100 ml of *alcohol R*. Titrate with 0.1 M *sodium hydroxide*, using 1.0 ml of *phenolphthalein solution R* as indicator, until a pink colour is obtained.

1 ml of 0.1 M *sodium hydroxide* is equivalent to 49.40 mg of $C_{23}H_{28}ClN_3O_5S$.

IMPURITIES



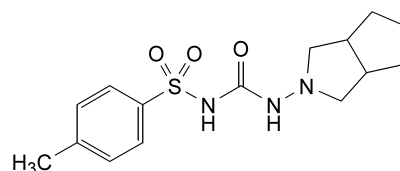
A. R = H: 5-chloro-2-methoxy-*N*-[2-(4-sulphamoylphenyl)ethyl]benzamide,

B. R = CO-OCH₃: methyl [[4-[2-[(5-chloro-2-methoxybenzoyl)amino]ethyl]phenyl]sulphonyl]carbamate.

01/2005:1524

GLICLAZIDE

Gliclazidum



$C_{15}H_{21}N_3O_3S$

M_r 323.4

DEFINITION

Gliclazide contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of 1-(hexahydrocyclopenta[c]pyrrol-2(1*H*)-yl)-3-[(4-methylphenyl)sulphonyl]urea, calculated with reference to the dried substance.

CHARACTERS

A white or almost white powder, practically insoluble in water, freely soluble in methylene chloride, sparingly soluble in acetone, slightly soluble in alcohol.

IDENTIFICATION

Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *gliclazide CRS*. Examine the substances prepared as discs.

TESTS

Related substances. Examine by liquid chromatography (2.2.29). Prepare the solutions immediately before use.

Test solution. Dissolve 50.0 mg of the substance to be examined in 23 ml of *acetonitrile R* and dilute to 50.0 ml with *water R*.

Reference solution (a). Dilute 1.0 ml of the test solution to 100.0 ml with a mixture of 45 volumes of *acetonitrile R* and 55 volumes of *water R*. Dilute 10.0 ml of the solution to 100.0 ml with a mixture of 45 volumes of *acetonitrile R* and 55 volumes of *water R*.

Reference solution (b). Dissolve 5 mg of the substance to be examined and 15 mg of *gliclazide impurity F CRS* in 23 ml of *acetonitrile R* and dilute to 50 ml with *water R*. Dilute 1 ml of the solution to 20 ml with a mixture of 45 volumes of *acetonitrile R* and 55 volumes of *water R*.

Reference solution (c). Dissolve 10.0 mg of *gliclazide impurity F CRS* in 45 ml of *acetonitrile R* and dilute to 100.0 ml with *water R*. Dilute 1.0 ml of the solution to 100.0 ml with a mixture of 45 volumes of *acetonitrile R* and 55 volumes of *water R*.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4 mm in internal diameter packed with *octylsilyl silica gel for chromatography R* (5 µm),
- as mobile phase at a flow rate of 0.9 ml/min a mixture of 0.1 volumes of *triethylamine R*, 0.1 volumes of *trifluoroacetic acid R*, 45 volumes of *acetonitrile R* and 55 volumes of *water R*,
- as detector a spectrophotometer set at 235 nm.

Inject 20 µl of reference solution (b). Adjust the sensitivity of the system so that the heights of the 2 principal peaks in the chromatogram obtained with reference solution (b) are at least 50 per cent of the full scale of the recorder. The test is not valid unless in the chromatogram obtained the resolution between the two principal peaks is at least 1.8.

Inject 20 µl of the test solution and 20 µl each of reference solutions (a) and (c). Continue the chromatography of the test solution for twice the retention time of gliclazide. In the chromatogram obtained with the test solution: the area of any peak corresponding to impurity F is not greater than the area of the corresponding peak in the chromatogram obtained with reference solution (c) (0.1 per cent); the area of any peaks, apart from the principal peak and the peak due to impurity F, is not greater than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent); the sum of the areas of any such peaks is not greater than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent). Disregard any peak with an area less than 0.2 times that of the principal peak in the chromatogram obtained with reference solution (a).

Impurity B. Examine by liquid chromatography (2.2.29) as described in the test for related substances.

Test solution. Dissolve 0.400 g in 2.5 ml of *dimethyl sulphoxide R* and dilute to 10.0 ml with *water R*. Stir for 10 min, store at 4 °C for 30 min and filter.

Reference solution (a). Dissolve 20.0 mg of *gliclazide impurity B CRS* in *dimethyl sulphoxide R* and dilute to 100.0 ml with the same solvent. To 1.0 ml of the solution, add 12 ml of *dimethyl sulphoxide R* and dilute to 50.0 ml with *water R*.

Reference solution (b). To 1.0 ml of reference solution (a), add 12 ml of *dimethyl sulphoxide R* and dilute to 50.0 ml with *water R*.

Inject 50 µl of the test solution and 50 µl of reference solution (b). In the chromatogram obtained with the test solution, the area of any peak corresponding to impurity B is not greater than the area of the corresponding peak in the chromatogram obtained with reference solution (b) (2 ppm).

Heavy metals (2.4.8). 1.5 g complies with limit test F for heavy metals (10 ppm). Prepare the standard using 1.5 ml of *lead standard solution (10 ppm Pb) R*.

Loss on drying (2.2.32). Not more than 0.25 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C for 2 h.

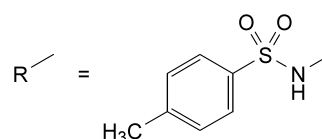
Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

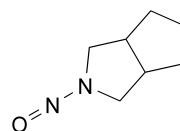
Dissolve 0.250 g in 50 ml of *anhydrous acetic acid R*. Titrate with 0.1 M *perchloric acid*, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *perchloric acid* is equivalent to 32.34 mg of $C_{15}H_{21}N_3O_3S$.

IMPURITIES

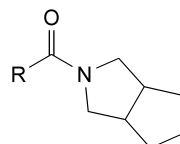


A. R-H: 4-methylbenzenesulphonamide,

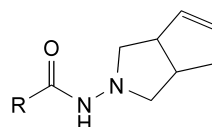


B. 2-nitroso-octahydrocyclopenta[c]pyrrole,

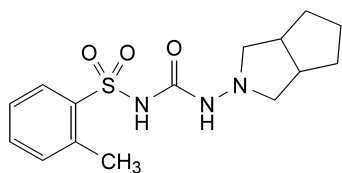
C. R-CO-O-C₂H₅: ethyl [(4-methylphenyl)sulphonyl]carbamate,



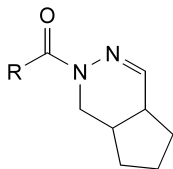
D. *N*[(4-methylphenyl)sulphonyl]hexahydrocyclopenta[c]pyrrol-2(1*H*)-carboxamide,



E. 1-[(4-methylphenyl)sulphonyl]-3-(3a,4,6a-tetrahydrocyclopenta[c]pyrrol-2(1*H*)-yl)urea,



- F. 1-(hexahydrocyclopenta[c]pyrrol-2(1H)-yl)-3-[(2-methylphenyl)sulphonyl]urea,

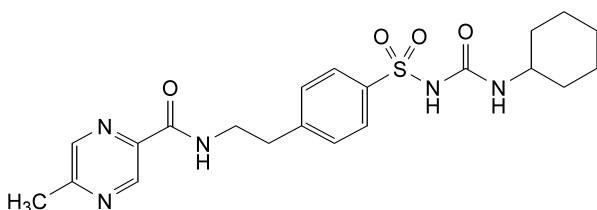


- G. N-[(4-methylphenyl)sulphonyl]-1,4a,5,6,7,7a-hexahydro-2H-cyclopenta[d]pyridazine-2-carboxamide.

01/2005:0906

GLIPIZIDE

Glipizidum

 $C_{21}H_{27}N_5O_4S$ M_r 445.5

DEFINITION

Glipizide contains not less than 98.0 per cent and not more than the equivalent of 102.0 per cent of 1-cyclohexyl-3-[[4-[2-[(5-methylpyrazin-2-yl)carbonyl]amino]ethyl]phenyl]sulphonyl]urea, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, crystalline powder, practically insoluble in water, soluble in methylene chloride, sparingly soluble in acetone, practically insoluble in alcohol. It dissolves in dilute solutions of alkali hydroxides.

IDENTIFICATION

First identification: B.

Second identification: A, C, D.

- Dissolve about 2 mg in *methanol R* and dilute to 100 ml with the same solvent. Examined between 220 nm and 350 nm (2.2.25), the solution shows two absorption maxima, at 226 nm and 274 nm. The ratio of the absorbance measured at the maximum of 226 nm to that measured at the maximum at 274 nm is 2.0 to 2.4.
- Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *glipizide CRS*. Examine the substances prepared as discs.
- Examine the chromatograms obtained in the test for related substances in ultraviolet light at 254 nm. The principal spot in the chromatogram obtained with test solution (b) is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a).

- Dissolve 50 mg in 5 ml of *dioxan R*. Add 1 ml of a 5 g/l solution of *fluorodinitrobenzene R* in *dioxan R* and boil for 2-3 min. A yellow colour is produced.

TESTS

Related substances. Examine by thin-layer chromatography (2.2.27), using *silica gel GF₂₅₄ R* as the coating substance.

Test solution (a). Dissolve 0.20 g of the substance to be examined in a mixture of equal volumes of *methanol R* and of *methylene chloride R* and dilute to 10 ml with the same mixture of solvents.

Test solution (b). Dilute 1 ml of test solution (a) to 20 ml with a mixture of equal volumes of *methanol R* and *methylene chloride R*.

Reference solution (a). Dissolve 10 mg of *glipizide CRS* in a mixture of equal volumes of *methanol R* and *methylene chloride R* and dilute to 10 ml with the same mixture of solvents.

Reference solution (b). Dissolve 5 mg of *glipizide impurity A CRS* in a mixture of equal volumes of *methanol R* and *methylene chloride R* and dilute to 50 ml with the same mixture of solvents.

Reference solution (c). Dilute 0.5 ml of test solution (a) to 100 ml with a mixture of equal volumes of *methanol R* and *methylene chloride R*.

Reference solution (d). Dilute 4 ml of reference solution (c) to 10 ml with a mixture of equal volumes of *methanol R* and *methylene chloride R*.

Reference solution (e). Dilute 5 ml of test solution (a) to 10 ml with reference solution (b).

Apply to the plate 10 µl of each solution. Develop over a path of 15 cm using a mixture of 25 volumes of *anhydrous formic acid R*, 25 volumes of *ethyl acetate R* and 50 volumes of *methylene chloride R*. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. In the chromatogram obtained with test solution (a): any spot corresponding to *glipizide impurity A* is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.5 per cent); any spot apart from the principal spot and the spot corresponding to *glipizide impurity A* is not more intense than the spot in the chromatogram obtained with reference solution (c) (0.5 per cent) and not more than two such spots are more intense than the spot in the chromatogram obtained with reference solution (d) (0.2 per cent). The test is not valid unless the chromatogram obtained with reference solution (e) shows two clearly separated spots.

Cyclohexylamine. Not more than 100 ppm, determined by gas chromatography (2.2.28), using *decane R* as internal standard.

Internal standard solution. Dissolve 25 mg of *decane R* in *hexane R* and dilute to 100 ml with the same solvent. Dilute 5 ml of this solution to 50 ml with *hexane R*.

Test solution (a). Dissolve 3.0 g of the substance to be examined in 50 ml of a 12 g/l solution of *sodium hydroxide R* and shake with two quantities, each of 5.0 ml, of *hexane R*. Use the combined upper layers.

Test solution (b). Dissolve 3.0 g of the substance to be examined in 50 ml of a 12 g/l solution of *sodium hydroxide R* and shake with two quantities, each of 5.0 ml, of the internal standard solution. Use the combined upper layers.

Reference solution. Dissolve 30.0 mg of *cyclohexylamine R* in a 17.5 g/l solution of *hydrochloric acid R* and dilute to 100.0 ml with the same acid. To 1.0 ml of this solution add 50 ml of a 12 g/l solution of *sodium hydroxide R* and shake with two quantities, each of 5.0 ml, of the internal standard solution. Use the combined upper layers.

The chromatographic procedure may be carried out using:

01/2005:0612

- a glass column 1.5 m long and 4 mm in internal diameter packed with *silanised diatomaceous earth for gas chromatography R* impregnated with 10 per cent *m/m* of *macrogol 20 000 R* and 4 per cent *m/m* of *potassium hydroxide R*,
- *nitrogen for chromatography R* as the carrier gas at a flow rate of 30 ml/min,
- a flame-ionisation detector,

maintaining the temperature of the column at 80 °C and that of the injection port and of the detector at 120 °C.

Inject 1 μ l of the reference solution and adjust the sensitivity of the detector so that the heights of the first peak (decane) and the second peak (cyclohexylamine) are not less than 50 per cent of the full scale of the recorder. The test is not valid unless the resolution between these two peaks is at least 2. Inject 1 μ l of test solution (a). In the chromatogram obtained, verify that there is no peak with the same retention time as that of the internal standard. Inject separately 1 μ l of test solution (b) and 1 μ l of the reference solution. From the chromatogram obtained with the reference solution, calculate the ratio (R) of the area of the peak due to cyclohexylamine to the area of the peak due to the internal standard. From the chromatogram obtained with test solution (b), calculate the ratio of the area of any peak corresponding to cyclohexylamine to the area of the peak due to the internal standard: this ratio is not greater than R .

Heavy metals (2.4.8). 2.0 g complies with limit test C for heavy metals (20 ppm). Prepare the standard using 4 ml of *lead standard solution (10 ppm Pb) R*.

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 100-105 °C.

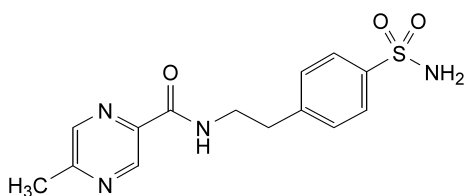
Sulphated ash (2.4.14). Not more than 0.2 per cent, determined on 1.0 g.

ASSAY

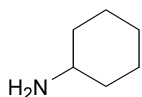
Dissolve 0.400 g in 50 ml of *dimethylformamide R*. Titrate with 0.1 M *lithium methoxide* using 0.2 ml of *quinaldine red solution R* as indicator until the colour changes from red to colourless.

1 ml of 0.1 M lithium methoxide is equivalent to 44.55 mg of $C_{21}H_{27}N_5O_4S$.

IMPURITIES



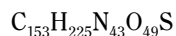
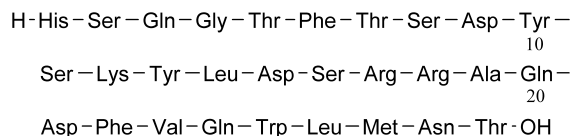
- A. 5-methyl-N-[2-(4-sulphamoylphenyl)ethyl]pyrazine-2-carboxamide,



- B. cyclohexanamine.

GLUCAGON

Glucagonum

 $M_{\mathrm{r}} 3482$

DEFINITION

Glucagon is a polypeptide hormone obtained from beef or pork pancreas and which increases the blood-glucose concentration by promoting rapid breakdown of liver glycogen. The potency is not less than 1 IU/mg, calculated with reference to the dried substance. Glucagon is prepared in conditions designed to minimise microbial contamination.

CHARACTERS

A white or almost white powder, practically insoluble in water and in most organic solvents. It dissolves in dilute mineral acids and in dilute solutions of the alkali hydroxides.

IDENTIFICATION

- A. It causes a rise of blood-glucose concentration in the test animals when injected as prescribed in the assay.
- B. Examine the electropherograms obtained in the test for related substances. The principal band in the electropherogram obtained with test solution (a) corresponds in position to the principal band in the electropherogram obtained with reference solution (a).

TESTS

Absorbance. Dissolve 2.5 mg in 0.01 M hydrochloric acid and dilute to 10.0 ml with the same acid. The specific absorbance (2.2.25) determined at the maximum at 276 nm is 21 to 25, calculated with reference to the dried substance.

Related substances. Examine by polyacrylamide gel electrophoresis (2.2.31), using rod gels 75 mm long and 5 mm in diameter and, as buffer, *tris-glycine buffer solution pH 8.3 R*. The electrode in the upper reservoir is the cathode and that in the lower reservoir is the anode.

Use the following gel mixture: mix 1 volume of a solution containing in 100 ml 36.6 mg of *tris(hydroxymethyl)aminomethane R*, 0.23 ml of *tetramethylethylenediamine R* and 48.0 ml of 1 M *hydrochloric acid* and 2 volumes of a solution containing in 100 ml 0.735 g of *methylene-bisacrylamide R* and 30.0 g of *acrylamide R*. Add sufficient *urea R* to give a concentration of 480 g/l in the final solution and dilute to 7 volumes with *water R*. If necessary, heat to not more than 40 °C to dissolve the urea. Degas the solution and add 1 volume of a 5.6 g/l solution of *ammonium persulphate R*.

Test solution (a). Dissolve 10 mg of the substance to be examined in 0.5 ml of 0.01 M sodium hydroxide.

Test solution (b). Dilute 0.25 ml of test solution (a) to 5 ml with 0.01 M sodium hydroxide.

Reference solution (a). Dissolve a quantity of *glucagon CRS* equivalent to 5 IU in 0.01 M sodium hydroxide and dilute to 25 ml with the same solvent.

Reference solution (b). Dilute 8 ml of reference solution (a) to 10 ml with 0.01 M sodium hydroxide.

Reference solution (c). Dilute 6 ml of reference solution (a) to 10 ml with 0.01 M sodium hydroxide.

Reference solution (d). Dilute 4 ml of reference solution (a) to 10 ml with 0.01 M sodium hydroxide.

Reference solution (e). Dilute 2 ml of reference solution (a) to 10 ml with 0.01 M sodium hydroxide.

Apply 100 µl of each solution to the surface of a gel. After the addition of the buffer, add 0.2 ml of *bromophenol blue solution R*. Allow electrophoresis to take place with a constant current of 1 mA per tube for 30 min and then increase the current to 3 mA per tube. Immerse the gels in a 125 g/l solution of *trichloroacetic acid R* for at least 1 h. For each 10 ml of trichloroacetic acid solution used add 0.5 ml of a 2.5 g/l solution of *acid blue 90 R* and allow to stand for 12 h. Remove the staining solution and wash twice with a mixture of 1 volume of *acetic acid R* and 4 volumes of *water R*, discard the washings and store the gels in a similar mixture of *acetic acid R* and *water R*. Examine the electropherograms using a cold-light illuminator. In the electropherograms obtained with test solution (a), any band, other than the bromophenol blue band, that migrates between two and three times the distance of the principal band is not more intense than the principal band in the electropherogram obtained with reference solution (e). In the electropherogram obtained with test solution (b), any band that migrates immediately in front of the principal band is not more intense than the principal band in the electropherogram obtained with reference solution (a). The test is not valid unless a band is seen in the electropherogram obtained with reference solution (e) and a gradation of intensity of staining is seen in the electropherograms obtained with reference solutions (a) to (e).

Nitrogen: 16.0 per cent to 18.5 per cent, determined by the method of sulphuric acid digestion (2.5.9) and calculated with reference to the dried substance.

Zinc. Not more than 0.15 per cent of Zn, determined by atomic absorption spectrometry (2.2.23, *Method I*).

Test solution. Dissolve 50.0 mg of the substance to be examined in 0.01 M *hydrochloric acid* and dilute to 25.0 ml with the same acid. Dilute if necessary with 0.01 M *hydrochloric acid* to obtain a zinc concentration of 0.4 µg/ml to 1.6 µg/ml.

Reference solutions. Use solutions containing 0.10 µg, 0.40 µg, 0.80 µg, 1.00 µg, 1.20 µg and 1.60 µg of Zn per millilitre, freshly prepared by diluting *zinc standard solution (5 mg/ml Zn) R* with 0.01 M *hydrochloric acid*.

Measure the absorbance at 213.9 nm using a zinc hollow-cathode lamp as the source of radiation and an air-acetylene flame of suitable composition (for example, 2 litres of acetylene and 11 litres of air per minute).

Loss on drying (2.2.32). Not more than 10.0 per cent, determined on 50.0 mg by drying at 105 °C for 24 h.

ASSAY

The potency of glucagon is determined by comparing, in given conditions, the hyperglycaemic effect it produces with that produced by the International Standard or by a reference preparation calibrated in International Units.

The International Unit is the specific hyperglycaemic activity contained in a stated amount of the International Standard which consists of a quantity of freeze-dried glucagon to which lactose and sodium chloride have been added. The equivalence in International units of the International Standard is stated by the World Health Organisation.

The estimated potency is not less than 80 per cent and not more than 125 per cent of the stated potency. The confidence limits ($P = 0.95$) of the estimated potency are not less than 64 per cent and not more than 156 per cent of the stated potency.

Reference solution. Dissolve the entire contents of an ampoule of the reference preparation in 2 ml of a 9 g/l solution of *sodium chloride R* adjusted to pH 3.0 with *hydrochloric acid R* and dilute with the same solvent to obtain a solution containing, for example, 0.1 IU/ml. Store the solution at 2 °C to 8 °C and use within 2 days.

Use in the test healthy rabbits, the difference in mass between the heaviest and the lightest being not greater than 1.2 kg (for example, rabbits with body masses in the range 1.8 kg to 2.8 kg). Keep the rabbits in the laboratory under uniform conditions on a uniform diet for at least 1 week before use in the assay. Handle the rabbits with care to avoid undue excitement.

Inject intramuscularly into each rabbit 48 h before the test 1 ml of a 25 g/l solution of cortisone acetate and withhold all food but not water from 16 h before each test day until after the withdrawal of the last blood sample on that day. Distribute the rabbits at random into four equal groups of not fewer than four rabbits.

Prepare two dilutions of the reference solution with a concentration ratio of 1 to 4, for example 0.006 IU/ml (reference dilution 1) and 0.024 IU/ml (reference dilution 2). Use as diluent a 9 g/l solution of *sodium chloride R* adjusted to pH 3.0 with *hydrochloric acid R*. Use the same diluent to dissolve the substance to be examined and to prepare two dilutions, one of which (test dilution 1) is presumed, on the basis of the stated potency to contain the same number of units per millilitre as reference dilution 1 and the other (test dilution 2) the same number of units per millilitre as reference dilution 2. Inject 1.0 ml of each dilution subcutaneously giving the doses in the order indicated in the following table, the second injection being made approximately 24 h after the first injection.

Group of rabbits	First injection	Second injection
1	reference dilution 1	test dilution 2
2	reference dilution 2	test dilution 1
3	test dilution 1	reference dilution 2
4	test dilution 2	reference dilution 1

Take a suitable blood sample from the marginal ear vein of each rabbit 1 h after each injection and determine the blood-glucose concentration in each sample. The optimal time depends on the strain. It is important that for a given assay a definite and exactly identical time interval be scrupulously respected for each rabbit.

Calculate the potency by the usual statistical methods for the twin cross-over assay.

STORAGE

Store in an airtight container, at a temperature below 8 °C and preferably at –20 °C.

LABELLING

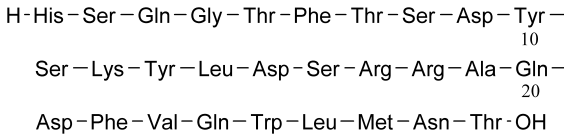
The label states:

- the number of units per container,
- the number of units per milligram,
- the storage conditions.

01/2005:1635

GLUCAGON, HUMAN

Glucagonum humanum



C₁₅₃H₂₂₅N₄₃O₄₉S M_r 3483

DEFINITION

Polypeptide having the same structure (29 amino-acids) as the hormone produced by the α-cells of the human pancreas which increases the blood-glucose concentration by promoting rapid breakdown of liver glycogen.

Content: 92.5 per cent to 105.0 per cent (anhydrous substance).

PRODUCTION

Human glucagon is produced by a method based on recombinant DNA (rDNA) technology. During the course of product development it must be demonstrated that the manufacturing process produces a product having a biological activity of not less than 1 IU/mg using a suitable validated bioassay, based on hyperglycaemia measurement.

Host-cell-derived proteins: the limit is approved by the competent authority.

Host-cell- and vector-derived DNA: the limit is approved by the competent authority.

CHARACTERS

Appearance: white or almost white powder.

Solubility: practically insoluble in water and in most organic solvents, soluble in dilute mineral acids and in dilute solutions of alkali hydroxides.

IDENTIFICATION

A. Peptide mapping. Liquid chromatography (2.2.29).

Test solution. Prepare a 10 mg/ml solution of the substance to be examined in 0.01 M hydrochloric acid. Mix 200 µl of the solution with 800 µl of 0.1 M ammonium carbonate buffer solution pH 10.3 R (diluted stock solution). Freshly prepare a 2.0 mg/ml solution of α-chymotrypsin for peptide mapping R in 0.1 M ammonium carbonate buffer solution pH 10.3 R and add 25 µl to the diluted stock solution of the substance to be examined. Place the test solution in a closed vial at 37 °C for 2 h. Remove the vial and stop the reaction immediately by the addition of 120 µl of glacial acetic acid R.

Reference solution. Prepare at the same time and in the same manner as for the test solution but using human glucagon CRS instead of the substance to be examined.

Column:

- size: l = 0.05 m, Ø = 4 mm,
- stationary phase: octadecylsilyl silica gel for chromatography R (5 µm).

Mobile phase:

- mobile phase A: mix 500 µl of trifluoroacetic acid R and 1000 ml of water R,
- mobile phase B: mix 500 µl of trifluoroacetic acid R with 600 ml of ethanol R and add 400 ml of water R,

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)
0 - 35	100 → 53	0 → 47
35 - 45	53 → 0	47 → 100
45 - 46	0 → 100	100 → 0
46 - 75	100	0

Flow rate: 1.0 ml/min.

Detection: spectrophotometer at 215 nm.

Equilibration: mobile phase A for at least 15 min.

Injection: 10 µl.

Results: the profile of the chromatogram obtained with the test solution corresponds to that of the chromatogram obtained with the reference solution.

B. Examine the chromatograms obtained in the assay. The principal peak in the chromatogram obtained with the test solution is similar in retention time to the principal peak in the chromatogram obtained with the reference solution.

TESTS

Specific absorbance (2.2.25): 21 to 25, determined at the maximum at 276 nm (anhydrous substance).

Dissolve 2.5 mg in 0.01 M hydrochloric acid and dilute to 10.0 ml with the same solvent.

Deamidated glucagon. Liquid chromatography (2.2.29): use the normalisation procedure.

Test solution. Dissolve the substance to be examined in 0.01 M hydrochloric acid to obtain a concentration of 1.0 mg/ml.

Resolution solution. Dissolve the substance to be examined in 0.1 M hydrochloric acid to obtain a concentration of 1.0 mg/ml. Incubate in an oven at 60 °C for 2 h. Immediately after degradation, adjust to pH 2.5 with 1 M sodium hydroxide.

Column:

- material: glass,
- size: l = 0.05 m, Ø = 5 mm,
- stationary phase: anion exchange resin R2.

Mobile phase:

- mobile phase A: mix 1000 ml of tris-hydrochloride buffer solution pH 8.3 R and 1000 ml of ethanol R,
- mobile phase B: dissolve 29.2 g of sodium chloride R in 1000 ml of tris-hydrochloride buffer solution pH 8.3 R; add 1000 ml of ethanol R,

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)
0 - 4	100	0
4 - 30	100 → 78	0 → 22
30 - 34	78 → 45	22 → 55
34 - 38	45 → 20	55 → 80
38 - 40	20 → 100	80 → 0
40 - 60	100	0

Flow rate: 0.6 ml/min.

Detection: spectrophotometer at 230 nm.

Equilibration: mobile phase A for at least 15 min.

Injection: 60 µl.

System suitability: resolution solution:

- retention time: glucagon = about 10 min; 4 deamidated forms: between 15 min and 40 min,

- **resolution**: baseline separation of the 4 deamidated forms and glucagon.

Limit:

- **total of the 4 deamidated forms**: maximum 0.5 per cent, calculated from the peaks eluting between 10 min and 40 min.

Related proteins. Liquid chromatography (2.2.29): use the normalisation procedure.

2.8 M urea solution. Dissolve 16.8 g of *urea R* in 0.01 M *hydrochloric acid* and dilute to 100 ml with the same solvent.

Test solution. Dissolve the substance to be examined in 0.01 M *hydrochloric acid* to obtain a concentration of 1.0 mg/ml. Maintain the solution at 2–8 °C and use within 24 h.

Reference solution. Dissolve the contents of a vial of *human glucagon CRS* in 0.01 M *hydrochloric acid* to obtain a concentration of 1.0 mg/ml. Maintain the solution at 2–8 °C and use within 24 h.

Resolution solution. Dissolve 10 mg of the substance to be examined in 10 ml of 2.8 M urea solution. Adjust to pH 7 with 1 M *sodium hydroxide* and place the sealed vial at about 50 °C for about 2 h. Cool and adjust to pH 2.5 with 1 M *hydrochloric acid*. Maintain the solution at 2–8 °C and use within 2 h.

Column:

- **size**: $l = 0.25$ m, $\varnothing = 4.6$ mm,
- **stationary phase**: octadecylsilyl silica gel for chromatography *R* (5 µm) with a pore size of 30 nm,
- **temperature**: 45 °C.

Mobile phase:

- **mobile phase A**: dissolve 14.2 g of *anhydrous sodium sulphate R* in 400 ml of *water R*; add 1.35 ml of *phosphoric acid R* and adjust to pH 2.5 (2.2.3) with *ethanolamine R*; add 100 ml of *acetonitrile for chromatography R*,
- **mobile phase B**: *acetonitrile for chromatography R*, *water R* (40:60 V/V),

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)
0 - 23	57	43
23 - 29	57 → 10	43 → 90
29 - 30	10	90
30 - 31	10 → 57	90 → 43
31 - 75	57	43

Flow rate: 1.0 ml/min.

Detection: spectrophotometer at 214 nm.

Injection: 25 µl; inject the test solution and the resolution solution.

System suitability: resolution solution:

- **retention time**: glucagon = about 20 min; carbamoylglucagon = about 22 min,
- **resolution**: minimum 1.3 between the peaks due to glucagon and carbamoylglucagon,
- **symmetry factor**: 0.6 to 1 for the peak due to glucagon.

Limit:

- **total of all impurities**: maximum 2.5 per cent.

Water (2.5.12): maximum 10 per cent, determined on 20.0 mg.

Bacterial endotoxins (2.6.14): less than 10 IU/mg.

ASSAY

Liquid chromatography (2.2.29) as described in the test for related proteins with the following modifications.

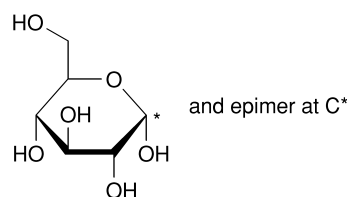
Injection: test solution and reference solution.

Calculate the content of human glucagon ($C_{153}H_{225}N_{43}O_{49}S$) from the declared content of $C_{153}H_{225}N_{43}O_{49}S$ in *human glucagon CRS*.

STORAGE

In an airtight container, protected from light, at a temperature lower than – 15 °C.

01/2005:0177

GLUCOSE, ANHYDROUS**Glucosum anhydricum** $C_6H_{12}O_6$ M_r 180.2**DEFINITION**

Anhydrous glucose is (+)-D-glucopyranose.

CHARACTERS

A white, crystalline powder, with a sweet taste, freely soluble in water, sparingly soluble in alcohol.

IDENTIFICATION

- Specific optical rotation (see Tests): + 52.5 to + 53.3.
- Examine by thin-layer chromatography (2.2.27), using silica gel *G R* as the coating substance.

Test solution. Dissolve 10 mg of the substance to be examined in a mixture of 2 volumes of *water R* and 3 volumes of *methanol R* and dilute to 20 ml with the same mixture of solvents.

Reference solution (a). Dissolve 10 mg of *glucose CRS* in a mixture of 2 volumes of *water R* and 3 volumes of *methanol R* and dilute to 20 ml with the same mixture of solvents.

Reference solution (b). Dissolve 10 mg each of *fructose CRS*, *glucose CRS*, *lactose CRS* and *sucrose CRS* in a mixture of 2 volumes of *water R* and 3 volumes of *methanol R* and dilute to 20 ml with the same mixture of solvents.

Apply separately to the plate 2 µl of each solution and thoroughly dry the starting points. Develop over a path of 15 cm using a mixture of 10 volumes of *water R*, 15 volumes of *methanol R*, 25 volumes of *anhydrous acetic acid R* and 50 volumes of *ethylene chloride R*. The solvents should be measured accurately since a slight excess of water produces cloudiness. Dry the plate in a current of warm air. Repeat the development immediately, after renewing the mobile phase. Dry the plate in a current of warm air and spray evenly with a solution of 0.5 g of *thymol R* in a mixture of 5 ml of *sulphuric acid R* and 95 ml of *alcohol R*. Heat at 130 °C for 10 min. The principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with

reference solution (a). The test is not valid unless the chromatogram obtained with reference solution (b) shows four clearly separated spots.

- C. Dissolve 0.1 g in 10 ml of *water R*. Add 3 ml of *cupri-tartaric solution R* and heat. A red precipitate is formed.

TESTS

Solution S. Dissolve 10.0 g in *distilled water R* and dilute to 100 ml with the same solvent.

Appearance of solution. Dissolve 10.0 g in 15 ml of *water R*. The solution is clear (2.2.1), odourless, and not more intensely coloured than reference solution BY₇ (2.2.2, *Method II*).

Acidity or alkalinity. Dissolve 6.0 g in 25 ml of *carbon dioxide-free water R* and add 0.3 ml of *phenolphthalein solution R*. The solution is colourless. Not more than 0.15 ml of 0.1 M *sodium hydroxide* is required to change the colour of the indicator to pink.

Specific optical rotation (2.2.7). Dissolve 10.0 g in 80 ml of *water R*, add 0.2 ml of *dilute ammonia R1*, allow to stand for 30 min and dilute to 100.0 ml with *water R*. The specific optical rotation is + 52.5 to + 53.3, calculated with reference to the anhydrous substance.

Foreign sugars, soluble starch, dextrins. Dissolve 1.0 g by boiling in 30 ml of *alcohol (90 per cent V/V) R*. Cool; the appearance of the solution shows no change.

Sulphites. Dissolve 5.0 g in 40 ml of *water R*, add 2.0 ml of 0.1 M *sodium hydroxide* and dilute to 50.0 ml with *water R*. To 10.0 ml of the solution, add 1 ml of a 310 g/l solution of *hydrochloric acid R*, 2.0 ml of *decolorised fuchsin solution R1* and 2.0 ml of a 0.5 per cent V/V solution of *formaldehyde R*. Allow to stand for 30 min and measure the absorbance (2.2.25) at the maximum at 583 nm. Prepare a standard as follows. Dissolve 76 mg of *sodium metabisulphite R* in *water R* and dilute to 50.0 ml with the same solvent. Dilute 5.0 ml of this solution to 100.0 ml with *water R*. To 3.0 ml of this solution add 4.0 ml of 0.1 M *sodium hydroxide* and dilute to 100.0 ml with *water R*. Immediately add to 10.0 ml of this solution 1 ml of a 310 g/l solution of *hydrochloric acid R*, 2.0 ml of *decolorised fuchsin solution R1* and 2.0 ml of a 0.5 per cent V/V solution of *formaldehyde R*. Allow to stand for 30 min and measure the absorbance at the maximum at 583 nm. Use as compensation liquid for both measurements a solution prepared in the same manner using 10.0 ml of *water R*. The absorbance of the test solution is not greater than that of the standard (15 ppm of SO₂).

Chlorides (2.4.4). 4 ml of solution S diluted to 15 ml with *water R* complies with the limit test for chlorides (125 ppm).

Sulphates (2.4.13). 7.5 ml of solution S diluted to 15 ml with *distilled water R* complies with the limit test for sulphates (200 ppm).

Arsenic (2.4.2). 1.0 g complies with limit test A for arsenic (1 ppm).

Barium. To 10 ml of solution S add 1 ml of *dilute sulphuric acid R*. When examined immediately and after 1 h, any opalescence in the solution is not more intense than that in a mixture of 1 ml of *distilled water R* and 10 ml of solution S.

Calcium (2.4.3). 5 ml of solution S diluted to 15 ml with *distilled water R* complies with the limit test for calcium (200 ppm).

Lead in sugars (2.4.10). It complies with the limit test for lead in sugars (0.5 ppm).

Water (2.5.12). Not more than 1.0 per cent, determined on 0.50 g by the semi-micro determination of water.

Sulphated ash (2.4.14). Not more than 0.1 per cent. Dissolve 5.0 g in 5 ml of *water R*, add 2 ml of *sulphuric acid R*, evaporate to dryness on a water-bath and ignite to constant mass. If necessary, repeat the heating with *sulphuric acid R*.

Pyrogens (2.6.8). If intended for use in large-volume preparations for parenteral use, the competent authority may require that it comply with the test for pyrogens carried out as follows. Inject per kilogram of the rabbit's mass 10 ml of a solution containing 50 mg per millilitre of the substance to be examined in *water for injections R*.

LABELLING

The label states where applicable, that the substance is apyrogenic.

01/2005:1330

GLUCOSE, LIQUID

Glucosum liquidum

DEFINITION

Liquid glucose is an aqueous solution containing a mixture of glucose, oligosaccharides and polysaccharides obtained by hydrolysis of starch. It contains not less than 70.0 per cent of dry matter. The degree of hydrolysis expressed as dextrose equivalent (DE) is not less than 20 (nominal value).

CHARACTERS

A clear, colourless or brown, viscous liquid, miscible with water. The substance may partly or totally solidify at room temperature and liquefies again when heated to 50 °C.

IDENTIFICATION

- Dissolve 0.1 g in 2.5 ml of *water R* and heat with 2.5 ml of *cupri-tartaric solution R*. A red precipitate is formed.
- Dip, for 1 s, a suitable stick with a reactive pad containing glucose-oxidase, peroxidase and a hydrogen-donating substance, such as tetramethylbenzidine, in a 5 g/l solution of the substance to be examined. Observe the colour of the reactive pad; within 60 s the colour changes from yellow to green or blue.
- It is a clear, colourless or brown, viscous liquid, miscible with water. The substance may partly or totally solidify at room temperature and liquefies again when heated to 50 °C.
- It complies with the test for dextrose equivalent (see Tests).

TESTS

Solution S. Dissolve 25.0 g in *carbon dioxide-free water R* and dilute to 50.0 ml with the same solvent.

pH (2.2.3). The pH of a mixture of 1 ml of a 223.6 g/l solution of *potassium chloride R* and 30 ml of solution S is 4.0 to 6.0.

Sulphur dioxide (2.5.29). Not more than 20 ppm. Not more than 400 ppm if intended for the production of lozenges or pastilles obtained by high boiling techniques, provided that the final product does not contain more than 50 ppm of sulphur dioxide.

Heavy metals (2.4.8). Dilute 2 ml of solution S to 30 ml with *water R*. The solution complies with limit test E for heavy metals (10 ppm). Prepare the standard using 10 ml of *lead standard solution (1 ppm Pb) R*.

Loss on drying (2.2.32). Not more than 30.0 per cent, determined on 1.000 g. Triturate the sample with 3.000 g of *kieselguhr G R*, previously dried at 80 °C under reduced pressure for 2 h, and dry at 80 °C under reduced pressure for 2 h.

Sulphated ash (2.4.14). Not more than 0.5 per cent, determined on 1.0 g.

Dextrose equivalent. Weigh an amount of the substance to be examined equivalent to 2.85-3.15 g of reducing carbohydrates, calculated as dextrose equivalent, into a 500 ml volumetric flask. Dissolve in *water R* and dilute to 500.0 ml with the same solvent. Transfer the solution to a 50 ml burette.

Pipette 25.0 ml of *cupri-tartaric solution R* into a 250 ml flask and add 18.5 ml of the test solution from the burette, mix and add boiling chips. Place the flask on a hot plate, previously adjusted so that the solution begins to boil after 2 min ± 15 s. Leave to boil for exactly 120 s, add 1 ml of a 1 g/l solution of *methylene blue R* and titrate with the test solution (V_1) until the blue colour disappears. Maintain the solution at boiling throughout the titration.

Standardise the cupri-tartaric solution using a 6.00 g/l solution of *glucose R* (V_0).

Calculate the dextrose equivalent (DE) from the equation:

$$DE = \frac{300 \times V_0 \times 100}{V_1 \times M \times D}$$

V_0 = total volume of glucose standard solution, in millilitres,

V_1 = total volume of test solution, in millilitres,

M = mass of the sample, in grams,

D = percentage content of dry matter in the substance.

The dextrose equivalent (DE) is within 10 per cent of the nominal value.

LABELLING

The label states the dextrose equivalent (DE) (= nominal value).

B. Dip, for 1 s, a suitable stick with a reactive pad containing glucose-oxidase, peroxidase and a hydrogen-donating substance, such as tetramethylbenzidine, in a 5 g/l solution of the substance to be examined. Observe the colour of the reactive pad; within 60 s the colour changes from yellow to green or blue.

C. It is a powder or granules.

D. It complies with the test for dextrose equivalent (see Tests).

TESTS

Solution S. Dissolve 12.5 g in *carbon dioxide-free water R* and dilute to 50.0 ml with the same solvent.

pH (2.2.3). The pH of a mixture of 1 ml of a 223.6 g/l solution of *potassium chloride R* and 30 ml of solution S is 4.0 to 7.0.

Sulphur dioxide (2.5.29). Not more than 20 ppm.

Heavy metals (2.4.8). Dilute 4 ml of solution S to 30 ml with *water R*. The solution complies with limit test E (10 ppm). Prepare the standard using 10 ml of *lead standard solution (1 ppm Pb) R*.

Loss on drying (2.2.32). Not more than 6.0 per cent, determined on 10.00 g by drying in an oven at 100-105 °C.

Sulphated ash (2.4.14). Not more than 0.5 per cent, determined on 1.0 g.

Dextrose equivalent. Weigh an amount of the substance to be examined equivalent to 2.85-3.15 g of reducing carbohydrates, calculated as dextrose equivalent, into a 500 ml volumetric flask. Dissolve in *water R* and dilute to 500.0 ml with the same solvent. Transfer the solution to a 50 ml burette.

Pipette 25.0 ml of *cupri-tartaric solution R* into a 250 ml flask and add 18.5 ml of the test solution from the burette, mix and add boiling chips. Place the flask on a hot plate, previously adjusted so that the solution begins to boil after 2 min ± 15 s. Allow to boil for exactly 120 s, add 1 ml of a 1 g/l solution of *methylene blue R* and titrate with the test solution (V_1) until the blue colour disappears. Maintain the solution at boiling throughout the titration.

Standardise the cupri-tartaric solution using a 6.00 g/l solution of *glucose R* (V_0).

Calculate the dextrose equivalent (DE) from the equation:

$$DE = \frac{300 \times V_0 \times 100}{V_1 \times M \times D}$$

V_0 = total volume of glucose standard solution, in millilitres,

V_1 = total volume of test solution, in millilitres,

M = mass of the sample, in grams,

D = percentage content of dry matter in the substance.

The dextrose equivalent (DE) is within 10 per cent of the nominal value.

Microbial contamination. Total viable aerobic count (2.6.12) not more than 10^3 bacteria and 10^2 fungi per gram, determined by plate count. It complies with the tests for *Escherichia coli* and *Salmonella* (2.6.13).

LABELLING

The label states the dextrose equivalent (DE) (= nominal value).

01/2005:1525

GLUCOSE, LIQUID, SPRAY-DRIED

Glucosum liquidum dispersione desiccatum

DEFINITION

Spray-dried liquid glucose is a mixture of glucose, oligosaccharides and polysaccharides, obtained by the partial hydrolysis of starch. The degree of hydrolysis, expressed as dextrose equivalent (DE), is not less than 20 (nominal value).

CHARACTERS

A white or almost white, slightly hygroscopic powder or granules, freely soluble in water.

IDENTIFICATION

A. Dissolve 0.1 g in 2.5 ml of *water R* and heat with 2.5 ml of *cupri-tartaric solution R*. A red precipitate is formed.

out as follows. Inject per kilogram of the rabbit's mass 10 ml of a solution containing 55 mg per millilitre of the substance to be examined in *water for injections R*.

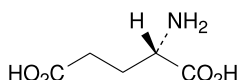
LABELLING

The label states where applicable, that the substance is apyrogenic.

01/2005:0750

GLUTAMIC ACID

Acidum glutamicum



$C_5H_9NO_4$

M_r 147.1

DEFINITION

Glutamic acid contains not less than 98.5 per cent and not more than the equivalent of 100.5 per cent of (2S)-2-aminopentanedioic acid, calculated with reference to the dried substance.

CHARACTERS

A white, crystalline powder or colourless crystals, freely soluble in boiling water, slightly soluble in cold water, practically insoluble in acetic acid, in acetone and in alcohol.

IDENTIFICATION

First identification: A, B.

Second identification: A, C, D.

- It complies with the test for specific optical rotation (see Tests).
- Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *glutamic acid CRS*. Examine the substances prepared as discs. If the spectra obtained show differences, dissolve the substance to be examined and the reference substance separately in the minimum quantity of *water R*, evaporate to dryness at 60 °C and record new spectra using the residues.
- Examine the chromatograms obtained in the test for ninhydrin-positive substances. The principal spot in the chromatogram obtained with test solution (b) is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).
- To 2.0 ml of solution S (see Tests) add 0.1 ml of *phenolphthalein solution R* and 3.0 ml to 3.5 ml of 1 M *sodium hydroxide* to change the colour of the indicator to red. Add a mixture of 3 ml of *formaldehyde solution R*, 3 ml of *carbon dioxide-free water R* and 0.1 ml of *phenolphthalein solution R*, to which sufficient 1 M *sodium hydroxide* has been added to produce a pink colour. The solution is decolourised. Add 1 M *sodium hydroxide* until a red colour is produced. The total volume of 1 M *sodium hydroxide* used is 4.0 ml to 4.7 ml.

TESTS

Solution S. Dissolve 5.00 g in 1 M *hydrochloric acid* with gentle heating, and dilute to 50.0 ml with the same acid.

Appearance of solution. Solution S is clear (2.2.1) and colourless (*Method II*, 2.2.2).

Specific optical rotation (2.2.7): + 30.5 to + 32.5, determined on solution S and calculated with reference to the dried substance.

Ninhydrin-positive substances. Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel plate R*.

Test solution (a). Dissolve 0.10 g of the substance to be examined in 5 ml of *dilute ammonia R2* and dilute to 10 ml with *water R*.

Test solution (b). Dilute 1 ml of test solution (a) to 50 ml with *water R*.

Reference solution (a). Dissolve 10 mg of *glutamic acid CRS* in *water R* and dilute to 50 ml with the same solvent.

Reference solution (b). Dilute 5 ml of test solution (b) to 20 ml with *water R*.

Reference solution (c). Dissolve 10 mg of *glutamic acid CRS* and 10 mg of *aspartic acid CRS* in *water R* and dilute to 25 ml with the same solvent.

Apply to the plate 5 µl of each solution. Dry the plate in a current of air for 15 min. Develop over a path of 15 cm using a mixture of 20 volumes of *glacial acetic acid R*, 20 volumes of *water R* and 60 volumes of *butanol R*. Allow the plate to dry in air, spray with *ninhydrin solution R* and heat at 100-105 °C for 15 min. Any spot in the chromatogram obtained with test solution (a), apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.5 per cent). The test is not valid unless the chromatogram obtained with reference solution (c) shows 2 clearly separated spots.

Chlorides (2.4.4). Dissolve 0.25 g in 3 ml of *dilute nitric acid R* and dilute to 15 ml with *water R*. The solution, to which 1 ml of *water R* is added instead of *dilute nitric acid R*, complies with the limit test for chlorides (200 ppm).

Sulphates (2.4.13). Dilute 5 ml of solution S to 15 ml with *distilled water R*. The solution complies with the limit test for sulphates (300 ppm).

Ammonium (2.4.1). 50 mg complies with limit test B for ammonium (200 ppm). Prepare the standard using 0.1 ml of *ammonium standard solution (100 ppm NH₄) R*.

Iron (2.4.9). In a separating funnel, dissolve 1.0 g in 10 ml of *dilute hydrochloric acid R*. Shake with 3 quantities, each of 10 ml, of *methyl isobutyl ketone R1*, shaking for 3 min each time. To the combined organic layers add 10 ml of *water R* and shake for 3 min. The aqueous layer complies with the limit test for iron (10 ppm).

Heavy metals (2.4.8). 2.0 g complies with limit test D for heavy metals (10 ppm). Prepare the standard using 2 ml of *lead standard solution (10 ppm Pb) R*.

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 100-105 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.130 g in 50 ml of *carbon dioxide-free water R* with gentle heating. Cool. Using 0.1 ml of *bromothymol blue solution R1* as indicator, titrate with 0.1 M *sodium hydroxide* until the colour changes from yellow to blue.

1 ml of 0.1 M *sodium hydroxide* is equivalent to 14.71 mg of $C_5H_9NO_4$.

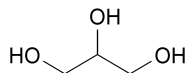
STORAGE

Protected from light.

01/2005:0496
corrected

GLYCEROL

Glycerolum

 $C_3H_8O_3$ M_r 92.1

DEFINITION

Propane-1,2,3-triol.

Content: 98.0 per cent *m/m* to 101.0 per cent *m/m* (anhydrous substance).

CHARACTERS

Aspect: syrupy liquid, unctuous to the touch, colourless or almost colourless, clear, very hygroscopic.*Solubility*: miscible with water and with alcohol, slightly soluble in acetone, practically insoluble in fatty oils and in essential oils.

IDENTIFICATION

First identification: A, B.*Second identification*: A, C, D.

A. It complies with the test for refractive index (see Tests).

B. Infrared absorption spectrophotometry (2.2.24).

Preparation: to 5 ml add 1 ml of *water R* and mix carefully.*Comparison*: *Ph. Eur. reference spectrum of glycerol* (85 per cent).C. Mix 1 ml with 0.5 ml of *nitric acid R*. Superimpose 0.5 ml of *potassium dichromate solution R*. A blue ring develops at the interface of the liquids. Within 10 min, the blue colour does not diffuse into the lower layer.D. Heat 1 ml with 2 g of *potassium hydrogen sulphate R* in an evaporating dish. Vapours (acrolein) are evolved which blacken filter paper impregnated with *alkaline potassium tetraiodomercurate solution R*.

TESTS

Solution S. Dilute 100.0 g to 200.0 ml with *carbon dioxide-free water R*.**Appearance of solution**. Solution S is clear (2.2.1). Dilute 10 ml of solution S to 25 ml with *water R*. The solution is colourless (2.2.2, *Method II*).**Acidity or alkalinity**. To 50 ml of solution S add 0.5 ml of *phenolphthalein solution R*. The solution is colourless. Not more than 0.2 ml of 0.1 M *sodium hydroxide* is required to change the colour of the indicator to pink.**Refractive index** (2.2.6): 1.470 to 1.475.**Aldehydes**: maximum 10 ppm.Place 7.5 ml of solution S in a ground-glass-stoppered flask and add 7.5 ml of *water R* and 1.0 ml of *decolorised pararosaniline solution R*. Close the flask and allow to stand for 1 h at a temperature of 25 ± 1 °C. The absorbance (2.2.25) of the solution measured at 552 nm is not greater than that of a standard prepared at the same time and in the same manner using 7.5 ml of *formaldehyde standard solution* (5 ppm CH_2O) *R* and 7.5 ml of *water R*. The test is not valid unless the standard is pink.**Esters**. Add 10.0 ml of 0.1 M *sodium hydroxide* to the final solution obtained in the test for acidity or alkalinity. Boil under a reflux condenser for 5 min. Cool. Add 0.5 ml of *phenolphthalein solution R* and titrate with 0.1 M *hydrochloric acid*. Not less than 8.0 ml of 0.1 M *hydrochloric acid* is required to change the colour of the indicator.**Impurity A and related substances**. Gas chromatography (2.2.28).*Test solution*. Dilute 10.0 ml of solution S to 100.0 ml with *water R*.*Reference solution (a)*. Dilute 10.0 g of *glycerol R1* to 20.0 ml with *water R*. Dilute 10.0 ml of the solution to 100.0 ml with *water R*.*Reference solution (b)*. Dissolve 1.000 g of *diethylene glycol R* in *water R* and dilute to 100.0 ml with the same solvent.*Reference solution (c)*. Dilute 1.0 ml of reference solution (b) to 10.0 ml with reference solution (a). Dilute 1.0 ml of this solution to 20.0 ml with reference solution (a).*Reference solution (d)*. Mix 1.0 ml of the test solution and 5.0 ml of reference solution (b) and dilute to 100.0 ml with *water R*. Dilute 1.0 ml of this solution to 10.0 ml with *water R*.*Reference solution (e)*. Dilute 5.0 ml of reference solution (b) to 100.0 ml with *water R*.*Column*:

- *size*: $l = 30$ m, $\varnothing = 0.53$ mm,
- *stationary phase*: 6 per cent polycyanopropylphenyl siloxane and 94 per cent of polydimethylsiloxane.

Carrier gas: *helium for chromatography R*.*Split ratio*: 1:10.*Linear velocity*: 38 cm/s.*Temperature*:

	Time (min)	Temperature (°C)
Column	0	100
	0 - 16	100 → 220
	16 - 20	220
Injection port		220
Detector		250

Detection: flame ionisation.*Injection*: 0.5 µl.*Elution order*: impurity A, glycerol.*System suitability*: reference solution (d):

- *resolution*: minimum 7.0 between the peaks due to impurity A and glycerol.

Limits:

- *impurity A*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (c) (0.1 per cent),
- *any other impurity with a retention time less than the retention time of glycerol*: not more than the area of the peak due to impurity A in the chromatogram obtained with reference solution (c) (0.1 per cent),
- *total of all impurities with retention times greater than the retention time of glycerol*: not more than 5 times the area of the peak due to impurity A in the chromatogram obtained with reference solution (c) (0.5 per cent),
- *disregard limit*: 0.05 times the area of the peak due to impurity A in the chromatogram obtained with reference solution (e) (0.05 per cent).

Halogenated compounds: maximum 35 ppm.

01/2005:0497
corrected

To 10 ml of solution S add 1 ml of *dilute sodium hydroxide solution R*, 5 ml of *water R* and 50 mg of *halogen-free nickel-aluminium alloy R*. Heat on a water-bath for 10 min, allow to cool and filter. Rinse the flask and the filter with *water R* until 25 ml of filtrate is obtained. To 5 ml of the filtrate add 4 ml of *alcohol R*, 2.5 ml of *water R*, 0.5 ml of *nitric acid R* and 0.05 ml of *silver nitrate solution R2* and mix. Allow to stand for 2 min. Any opalescence in the solution is not more intense than that in a standard prepared at the same time by mixing 7.0 ml of *chloride standard solution (5 ppm Cl) R*, 4 ml of *alcohol R*, 0.5 ml of *water R*, 0.5 ml of *nitric acid R* and 0.05 ml of *silver nitrate solution R2*.

Sugars. To 10 ml of solution S add 1 ml of *dilute sulphuric acid R* and heat on a water-bath for 5 min. Add 3 ml of carbonate-free *dilute sodium hydroxide solution R* (prepared by the method described for carbonate-free 1 M sodium hydroxide (4.2.2)), mix and add dropwise 1 ml of freshly prepared *copper sulphate solution R*. The solution is clear and blue. Continue heating on the water-bath for 5 min. The solution remains blue and no precipitate is formed.

Chlorides (2.4.4): maximum 10 ppm.

1 ml of solution S diluted to 15 ml with *water R* complies with the limit test for chlorides. Prepare the standard using 1 ml of *chloride standard solution (5 ppm Cl) R* diluted to 15 ml with *water R*.

Heavy metals (2.4.8): maximum 5 ppm.

Dilute 8 ml of solution S to 20 ml with *water R*. 12 ml of the solution complies with limit test A. Prepare the standard using *lead standard solution (1 ppm Pb) R*.

Water (2.5.12): maximum 2.0 per cent, determined on 1.000 g.

Sulphated ash (2.4.14): maximum 0.01 per cent, determined on 5.0 g after heating to boiling and ignition.

ASSAY

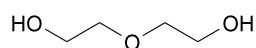
Thoroughly mix 0.075 g with 45 ml of *water R*. Add 25.0 ml of a mixture of 1 volume of 0.1 M sulphuric acid and 20 volumes of 0.1 M sodium periodate. Allow to stand protected from light for 15 min. Add 5.0 ml of a 500 g/l solution of *ethylene glycol R* and allow to stand protected from light for 20 min. Using 0.5 ml of *phenolphthalein solution R* as indicator, titrate with 0.1 M sodium hydroxide. Carry out a blank titration.

1 ml of 0.1 M sodium hydroxide is equivalent to 9.21 mg of C₃H₈O₃.

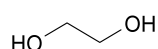
STORAGE

In an airtight container.

IMPURITIES



A. 2,2'-oxydiethanol (diethylene glycol),



B. ethane-1,2-diol (ethylene glycol),

C. propylene glycol.

GLYCEROL (85 PER CENT)

Glycerolum (85 per centum)

DEFINITION

Aqueous solution of propane-1,2,3-triol.

Content: 83.5 per cent *m/m* to 88.5 per cent *m/m* of propane-1,2,3-triol (C₃H₈O₃; *M_r* 92.1).

CHARACTERS

Aspect: syrupy liquid, unctuous to the touch, colourless or almost colourless, clear, very hygroscopic.

Solubility: miscible with water and with alcohol, slightly soluble in acetone, practically insoluble in fatty oils and in essential oils.

IDENTIFICATION

First identification: A, B.

Second identification: A, C, D.

A. It complies with the test for refractive index (see Tests).

B. Infrared absorption spectrophotometry (2.2.24).

Comparison: Ph. Eur. reference spectrum of glycerol (85 per cent).

C. Mix 1 ml with 0.5 ml of *nitric acid R*. Superimpose 0.5 ml of *potassium dichromate solution R*. A blue ring develops at the interface of the liquids. Within 10 min, the blue colour does not diffuse into the lower layer.

D. Heat 1 ml with 2 g of *potassium hydrogen sulphate R* in an evaporating dish. Vapours (acrolein) are evolved which blacken filter paper impregnated with *alkaline potassium tetraiodomercurate solution R*.

TESTS

Solution S. Dilute 117.6 g to 200.0 ml with *carbon dioxide-free water R*.

Appearance of solution. Solution S is clear (2.2.1). Dilute 10 ml of solution S to 25 ml with *water R*. The solution is colourless (2.2.2, *Method II*).

Acidity or alkalinity. To 50 ml of solution S add 0.5 ml of *phenolphthalein solution R*. The solution is colourless. Not more than 0.2 ml of 0.1 M sodium hydroxide is required to change the colour of the indicator to pink.

Refractive index (2.2.6): 1.449 to 1.455.

Aldehydes: maximum 10 ppm.

Place 7.5 ml of solution S in a ground-glass-stoppered flask and add 7.5 ml of *water R* and 1.0 ml of *decolorised pararosaniline solution R*. Close the flask and allow to stand for 1 h at a temperature of 25 ± 1 °C. The absorbance (2.2.25) of the solution measured at 552 nm is not greater than that of a standard prepared at the same time and in the same manner using 7.5 ml of *formaldehyde standard solution (5 ppm CH₂O) R* and 7.5 ml of *water R*. The test is not valid unless the standard is pink.

Esters. Add 10.0 ml of 0.1 M sodium hydroxide to the final solution obtained in the test for acidity or alkalinity. Boil under a reflux condenser for 5 min. Cool. Add 0.5 ml of *phenolphthalein solution R* and titrate with 0.1 M hydrochloric acid. Not less than 8.0 ml of 0.1 M hydrochloric acid is required to change the colour of the indicator.

Impurity A and related substances. Gas chromatography (2.2.28).

Test solution. Dilute 10.0 ml of solution S to 100.0 ml with water R.

Reference solution (a). Dilute 11.8 g of glycerol (85 per cent) R1 to 20.0 ml with water R. Dilute 10.0 ml of the solution to 100.0 ml with water R.

Reference solution (b). Dissolve 1.000 g of diethylene glycol R in water R and dilute to 100.0 ml with the same solvent.

Reference solution (c). Dilute 1.0 ml of reference solution (b) to 10.0 ml with reference solution (a). Dilute 1.0 ml of this solution to 20.0 ml with reference solution (a).

Reference solution (d). Mix 1.0 ml of the test solution and 5.0 ml of reference solution (b) and dilute to 100.0 ml with water R. Dilute 1.0 ml of this solution to 10.0 ml with water R.

Reference solution (e). Dilute 5.0 ml of reference solution (b) to 100.0 ml with water R.

Column:

- size: $l = 30$ m, $\varnothing = 0.53$ mm,
- stationary phase: 6 per cent polycyanolpropylphenyl siloxane and 94 per cent of polydimethylsiloxane.

Carrier gas: helium for chromatography R.

Split ratio: 1:10.

Linear velocity: 38 cm/s.

Temperature:

	Time (min)	Temperature (°C)
Column	0	100
	0 - 16	100 → 220
	16 - 20	220
Injection port		220
Detector		250

Detection: flame ionisation.

Injection: 0.5 µl.

Elution order: impurity A, glycerol.

System suitability: reference solution (d):

- resolution: minimum 7.0 between the peaks due to impurity A and glycerol.

Limits:

- impurity A: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (c) (0.1 per cent),
- any other impurity with a retention time less than the retention time of glycerol: not more than the area of the peak due to impurity A in the chromatogram obtained with reference solution (c) (0.1 per cent),
- total of all impurities with retention times greater than the retention time of glycerol: not more than 5 times the area of the peak due to impurity A in the chromatogram obtained with reference solution (c) (0.5 per cent),
- disregard limit: 0.05 times the area of the peak due to impurity A in the chromatogram obtained with reference solution (e) (0.05 per cent).

Halogenated compounds: maximum 30 ppm.

To 10 ml of solution S add 1 ml of dilute sodium hydroxide solution R, 5 ml of water R and 50 mg of halogen-free nickel-aluminium alloy R. Heat on a water-bath for 10 min, allow to cool and filter. Rinse the flask and the filter with water R until 25 ml of filtrate is obtained. To 5 ml of the filtrate add 4 ml of alcohol R, 2.5 ml of water R, 0.5 ml

of nitric acid R and 0.05 ml of silver nitrate solution R2 and mix. Allow to stand for 2 min. Any opalescence in the solution is not more intense than that in a standard prepared at the same time by mixing 7.0 ml of chloride standard solution (5 ppm Cl) R, 4 ml of alcohol R, 0.5 ml of water R, 0.5 ml of nitric acid R and 0.05 ml of silver nitrate solution R2.

Sugars. To 10 ml of solution S add 1 ml of dilute sulphuric acid R and heat on a water-bath for 5 min. Add 3 ml of carbonate-free dilute sodium hydroxide solution R (prepared by the method described for carbonate-free 1 M sodium hydroxide (4.2.2)), mix and add dropwise 1 ml of freshly prepared copper sulphate solution R. The solution is clear and blue. Continue heating on the water-bath for 5 min. The solution remains blue and no precipitate is formed.

Chlorides (2.4.4): maximum 10 ppm.

1 ml of solution S diluted to 15 ml with water R complies with the limit test for chlorides. Prepare the standard using 1 ml of chloride standard solution (5 ppm Cl) R diluted to 15 ml with water R.

Heavy metals (2.4.8): maximum 5 ppm.

Dilute 8 ml of solution S to 20 ml with water R. 12 ml of the solution complies with limit test A. Prepare the standard using lead standard solution (1 ppm Pb) R.

Water (2.5.12): 12.0 per cent to 16.0 per cent, determined on 0.200 g.

Sulphated ash (2.4.14): maximum 0.01 per cent, determined on 5.0 g after heating to boiling and ignition.

ASSAY

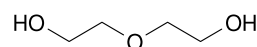
Thoroughly mix 0.075 g with 45 ml of water R. Add 25.0 ml of a mixture of 1 volume of 0.1 M sulphuric acid and 20 volumes of 0.1 M sodium periodate. Allow to stand protected from light for 15 min. Add 5.0 ml of a 500 g/l solution of ethylene glycol R and allow to stand protected from light for 20 min. Using 0.5 ml of phenolphthalein solution R as indicator, titrate with 0.1 M sodium hydroxide. Carry out a blank titration.

1 ml of 0.1 M sodium hydroxide is equivalent to 9.21 mg of $C_{31}H_{64}O_2$.

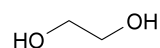
STORAGE

In an airtight container.

IMPURITIES



A. 2,2'-oxydiethanol (diethylene glycol),



B. ethane-1,2-diol (ethylene glycol),

C. propylene glycol.

01/2005:1427

GLYCEROL DIBEHENATE

Glyceroli dibehenas

DEFINITION

Mixture of diacylglycerols, mainly dibehenylglycerol, together with variable quantities of mono- and triacylglycerols, obtained by esterification of glycerol with behenic acid.

Content:

- monoacylglycerols: 13.0 per cent to 21.0 per cent,

- diacylglycerols: 40.0 per cent to 60.0 per cent,
- triacylglycerols: 21.0 per cent to 35.0 per cent.

CHARACTERS

Appearance: hard, waxy mass or powder or white or almost white, unctuous flakes.

Solubility: practically insoluble in water, soluble in methylene chloride and partly soluble in hot alcohol.

IDENTIFICATION

A. Melting point (2.2.14): 65 °C to 77 °C.

B. Thin-layer chromatography (2.2.27).

Test solution. Dissolve 1.0 g of the substance to be examined in *toluene R*, with gentle heating and dilute to 20 ml with the same solvent.

Reference solution. Dissolve 1.0 g of *glycerol dibehenate CRS* in *toluene R*, with gentle heating and dilute to 20 ml with the same solvent.

Plate: TLC silica gel plate *R*.

Mobile phase: *hexane R*, *ether R* (30:70 V/V).

Application: 10 µl.

Development: over a path of 15 cm.

Drying: in air.

Detection: spray with a 0.1 g/l solution of *rhodamine B R* in *alcohol R*. Examine in ultraviolet light at 365 nm.

Results: the spots in the chromatogram obtained with the test solution are similar in position to those in the chromatogram obtained with the reference solution.

C. It complies with the test for composition of fatty acids (see Tests).

TESTS

Acid value (2.5.1): maximum 4.0, determined on 1.0 g, using a mixture of equal volumes of *alcohol R* and *toluene R* as solvent and with gentle heating.

Iodine value (2.5.4): maximum 3.0.

Saponification value (2.5.6): 145 to 165.

Carry out the titration with heating.

Free glycerol: maximum 1.0 per cent, determined as described under Assay.

Composition of fatty acids. Gas chromatography (2.4.22, Method C), raising the temperature of the column to 240 °C.

Composition of the fatty acid fraction of the substance:

- *palmitic acid*: maximum 3.0 per cent,
- *stearic acid*: maximum 5.0 per cent,
- *arachidic acid*: maximum 10.0 per cent,
- *behenic acid*: minimum 83.0 per cent,
- *lignoceric acid*: maximum 3.0 per cent,
- *erucic acid*: maximum 3.0 per cent.

Nickel (2.4.27): maximum 1 ppm.

Water (2.5.12): maximum 1.0 per cent, determined on 1.00 g. Use *pyridine R* as the solvent.

Total ash (2.4.16): maximum 0.1 per cent, determined on 1.00 g.

ASSAY

Size-exclusion chromatography (2.2.30).

Test solution. Into a 15 ml flask, weigh about 0.2 g (*m*), to the nearest 0.1 mg. Add 5 ml of *tetrahydrofuran R*, warm the flask slightly (about 35 °C) and shake until dissolution is obtained. Reweigh the flask and calculate the total mass of solvent and substance (*M*).

Reference solutions. In four 15 ml flasks, weigh to the nearest 0.1 mg about 2 mg, 5 mg, 10 mg and 20 mg respectively of *glycerol R*. Add 5 ml of *tetrahydrofuran R* and shake until well mixed. Weigh the flasks again and calculate the concentration of glycerol in milligrams per gram for each reference solution.

Column:

- **size:** *l* = 0.6 m, Ø = 7 mm,
- **stationary phase:** *styrene-divinylbenzene copolymer R* (5 µm) with a pore size of 10 nm.

Mobile phase: *tetrahydrofuran R*.

Flow rate: 1 ml/min.

Detection: differential refractive index.

Injection: 40 µl; when injecting the test solution, maintain the flask at about 35 °C in order to avoid precipitation.

Relative retentions with reference to glycerol: monoacylglycerols = about 0.82; diacylglycerols = about 0.76; triacylglycerols = about 0.73.

From the calibration curve obtained with the reference solutions determine the concentration (*C*) in milligrams per gram of glycerol in the test solution.

Calculate the percentage content of free glycerol in the substance to be examined using the following expression:

$$\frac{C \times M}{m \times 10}$$

Calculate the percentage content of mono-, di- and triacylglycerols in the substance to be examined by the normalisation procedure.

01/2005:1428

GLYCEROL DISTEARATE

Glyceroli distearas

DEFINITION

Glycerol distearate is a mixture of diacylglycerols, mainly distearoylglycerol, together with variable quantities of mono- and triacylglycerols. It contains 8.0 per cent to 22.0 per cent of monoacylglycerols, 40.0 per cent to 60.0 per cent of diacylglycerols and 25.0 per cent to 35.0 per cent of triacylglycerols, obtained by partial glycerolysis of vegetable oils containing triacylglycerols of palmitic or stearic acid or by esterification of glycerol with stearic acid 50 (type I), stearic acid 70 (type II) or stearic acid 95 (type III) (see *Stearic acid* (1474)). The fatty acids may be of vegetable or animal origin.

CHARACTERS

A hard, waxy mass or powder or white or almost white, unctuous flakes, insoluble in water, soluble in methylene chloride, partly soluble in hot alcohol.

IDENTIFICATION

A. Melting point (2.2.14): 50 °C to 60 °C (type I and II), 50 °C to 70 °C (type III).

B. Examine by thin-layer chromatography (2.2.27), using a TLC silica gel plate *R*.

Test solution. Dissolve 1.0 g of the substance to be examined in *methylene chloride R*, with gentle heating and dilute to 20 ml with the same solvent.

Reference solution. Dissolve 1.0 g of *glycerol distearate CRS* in *methylene chloride R*, with gentle heating and dilute to 20 ml with the same solvent.

Apply to the plate 10 µl of each solution. Develop over a path of 15 cm using a mixture of 30 volumes of *hexane R* and 70 volumes of *ether R*. Allow the plate to dry in air. Spray with a 0.1 g/l solution of *rhodamine B R* in *alcohol R* and examine in ultraviolet light at 365 nm. The spots in the chromatogram obtained with the test solution are similar in position to those in the chromatogram obtained with the reference solution.

- C. It complies with the test for composition of fatty acids according to the type stated on the label (see Tests).
- D. It complies with the limits of the assay (diacylglycerol content).

TESTS

Acid value (2.5.1). Not more than 6.0, determined on 1.0 g, using a mixture of equal volumes of *alcohol R* and *toluene R* as solvent and with gentle heating.

Iodine value (2.5.4). Not more than 3.0.

Saponification value (2.5.6): 165 to 195, determined on 2.0 g. Carry out the titration with heating.

Free glycerol. Not more than 1.0 per cent, determined as described under Assay.

Composition of fatty acids (2.4.22, Method C). The fatty-acid fraction of the substance to be examined has the following composition:

	Fatty acid used for production by esterification	Composition of fatty acids
Glycerol distearate type I	Stearic acid 50	Stearic acid: 40.0 per cent to 60.0 per cent Sum of the contents of palmitic and stearic acids: not less than 90.0 per cent
Glycerol distearate type II	Stearic acid 70	Stearic acid: 60.0 per cent to 80.0 per cent Sum of palmitic and stearic acids: not less than 90.0 per cent
Glycerol distearate type III	Stearic acid 95	Stearic acid: 90.0 per cent to 99.0 per cent Sum of the contents of palmitic and stearic acids: not less than 96.0 per cent

Nickel (2.4.27). Not more than 1 ppm of Ni.

Water (2.5.12). Not more than 1.0 per cent, determined on 1.00 g by the semi-micro determination of water. Use *pyridine R* as the solvent.

Total ash (2.4.16). Not more than 0.1 per cent, determined on 1.00 g.

ASSAY

Determine the free glycerol content and the mono-, di- and triacylglycerol contents by size-exclusion chromatography (2.2.30).

Test solution. Into a 15 ml flask, weigh about 0.2 g (*m*), to the nearest 0.1 mg. Add 5 ml of *tetrahydrofuran R* and shake to dissolve. Reweigh the flask and calculate the total mass of solvent and substance (*M*).

Reference solutions. Into four 15 ml flasks, respectively weigh, to the nearest 0.1 mg, about 2 mg, 5 mg, 10 mg and 20 mg of *glycerol R*. Add 5 ml of *tetrahydrofuran R* and shake until well mixed. Weigh the flasks again and calculate the concentration of glycerol in milligrams per gram for each reference solution.

The chromatographic procedure may be carried out using:

- a gel-permeation column 0.6 m long and 7 mm in internal diameter packed with *styrene-divinylbenzene copolymer R* (particle diameter 5 µm and porosity 10 nm),
- as mobile phase at a flow rate of 1 ml/min *tetrahydrofuran R*,
- a differential refractive index detector.

Inject 40 µl of each solution. When the chromatograms are recorded in the prescribed conditions, the retention times relative to glycerol are 0.84 for the monoacylglycerols, 0.78 for the diacylglycerols and 0.75 for the triacylglycerols. From the calibration curve obtained with the reference solutions determine the concentration (*C*) in milligrams per gram of glycerol in the test solution.

Calculate the percentage content of free glycerol in the substance to be examined using the following expression:

$$\frac{C \times M}{m \times 10}$$

Calculate the percentage content of mono-, di- and triacylglycerols in the substance to be examined by the normalisation procedure.

LABELLING

The label states the type of glycerol distearate.

01/2005:1429

GLYCEROL MONOLINOLEATE

Glyceroli monolinoleas

DEFINITION

Glycerol monolinoleate is a mixture of monoacylglycerols, mainly mono-oleoyl- and monolinoleoylglycerol, together with variable quantities of di- and triacylglycerols. It contains 32.0 per cent to 52.0 per cent of monoacylglycerols, 40.0 per cent to 55.0 per cent of diacylglycerols and 5.0 per cent to 20.0 per cent of triacylglycerols, obtained by partial glycerolysis of vegetable oils mainly containing triacylglycerols of linoleic acid. A suitable antioxidant may be added.

CHARACTERS

Amber, oily liquids which may be partially solidified at room temperature, practically insoluble in water, freely soluble in methylene chloride.

IDENTIFICATION

- A. It complies with the test for iodine value (see Tests).
- B. Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel plate R*.

Test solution. Dissolve 1.0 g of the substance to be examined in *methylene chloride R* and dilute to 20 ml with the same solvent.

Reference solution. Dissolve 1.0 g of *glycerol monolinoleate CRS* in *methylene chloride R* and dilute to 20 ml with the same solvent.

Apply to the plate 10 µl of each solution. Develop over a path of 15 cm using a mixture of 30 volumes of *hexane R* and 70 volumes of *ether R*. Allow the plate to dry in air. Spray with a 0.1 g/l solution of *rhodamine B R* in *alcohol R* and examine in ultraviolet light at 365 nm. The spots in the chromatogram obtained with the test solution are similar in position to those in the chromatogram obtained with the reference solution.

C. It complies with the test for composition of fatty acids (see Tests).

TESTS

Acid value (2.5.1). Not more than 6.0, determined on 1.0 g.

Iodine value (2.5.4): 100 to 140.

Peroxide value (2.5.5, Method A). Not more than 12.0, determined on 2.0 g.

Saponification value (2.5.6): 160 to 180, determined on 2.0 g.

Free glycerol. Not more than 6.0 per cent, determined as described under Assay.

Composition of fatty acids (2.4.22, Method C). The fatty-acid fraction of the substance has the following composition:

- *palmitic acid*: 4.0 per cent to 20.0 per cent,
- *stearic acid*: not more than 6.0 per cent,
- *oleic acid*: 10.0 per cent to 35.0 per cent,
- *linoleic acid*: not less than 50.0 per cent,
- *linolenic acid*: not more than 2.0 per cent,
- *arachidic acid*: not more than 1.0 per cent,
- *eicosenoic acid*: not more than 1.0 per cent.

Water (2.5.12). Not more than 1.0 per cent, determined on 1.00 g by the semi-micro determination of water. Use as the solvent a mixture of equal volumes of *anhydrous methanol R* and *methylene chloride R*.

Total ash (2.4.16). Not more than 0.1 per cent, determined on 1.00 g.

ASSAY

Determine the free glycerol content and the mono-, di- and triacylglycerol contents by size-exclusion chromatography (2.2.30).

Test solution. Into a 15 ml flask, weigh about 0.2 g (*m*), to the nearest 0.1 mg. Add 5 ml of *tetrahydrofuran R* and shake to dissolve. Reweigh the flask and calculate the total mass of solvent and substance (*M*).

Reference solutions. Into four 15 ml flasks, respectively weigh, to the nearest 0.1 mg, about 2.5 mg, 5 mg, 10 mg and 20 mg of *glycerol R*. Add 5 ml of *tetrahydrofuran R* and shake until well mixed. Weigh the flasks again and calculate the concentration of glycerol in milligrams per gram for each reference solution.

The chromatographic procedure may be carried out using:

- a gel-permeation column 0.6 m long and 7 mm in internal diameter packed with *styrene-divinylbenzene copolymer R* (particle diameter 5 µm and porosity 10 nm),
- as mobile phase at a flow rate of 1 ml/min *tetrahydrofuran R*,
- a differential refractive index detector.

Inject 40 µl of each solution. When the chromatograms are recorded in the prescribed conditions, the retention times relative to glycerol are about 0.86 for the monoacylglycerols, about 0.80 for the diacylglycerols and about 0.76 for the triacylglycerols. From the calibration curve obtained with the reference solutions determine the concentration (*C*) in milligrams per gram of glycerol in the test solution.

Calculate the percentage content of free glycerol in the substance to be examined using the following expression:

$$\frac{C \times M}{m \times 10}$$

Calculate the percentage content of mono-, di- and triacylglycerols in the substance to be examined by the normalisation procedure.

STORAGE

Store in an airtight container, protected from light.

LABELLING

The label states the name and concentration of any added antioxidant.

01/2005:1430

GLYCEROL MONO-OLEATES

Glyceroli mono-oleates

DEFINITION

Glycerol mono-oleates are mixtures of monoacylglycerols, mainly mono-oleoylglycerol, together with variable quantities of di- and triacylglycerols. They are defined by the nominal content of monoacylglycerols and obtained by partial glycerolysis of vegetable oils mainly containing triacylglycerols of oleic acid or by esterification of glycerol by oleic acid, this fatty acid being of vegetable or animal origin.

	Nominal content of acylglycerol (per cent)		
	40	60	90
Monoacylglycerols	32.0 - 52.0	55.0 - 65.0	90.0 - 101.0
Diacylglycerols	30.0 - 50.0	15.0 - 35.0	< 10.0
Triacylglycerols	5.0 - 20.0	2.0 - 10.0	< 2.0

A suitable antioxidant may be added.

CHARACTERS

Amber, oily liquids which may be partially solidified at room temperature, practically insoluble in water, freely soluble in methylene chloride.

IDENTIFICATION

A. It complies with the test for iodine value (see Tests).

B. Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel plate R*.

Test solution. Dissolve 1.0 g of the substance to be examined in *methylene chloride R* and dilute to 20 ml with the same solvent.

Reference solution. Dissolve 1.0 g of *glycerol mono-oleate CRS* in *methylene chloride R* and dilute to 20 ml with the same solvent.

Apply to the plate 10 µl of each solution. Develop over a path of 15 cm using a mixture of 30 volumes of *hexane R* and 70 volumes of *ether R*. Allow the plate to dry in air. Spray with a 0.1 g/l solution of *rhodamine B R* in *alcohol R* and examine in ultraviolet light at 365 nm. The spots in the chromatogram obtained with the test solution are similar in position to those in the chromatogram obtained with the reference solution.

C. It complies with the limits of the assay (monoacylglycerol content).

TESTS

Acid value (2.5.1). Not more than 6.0, determined on 1.0 g.

Iodine value (2.5.4): 65.0 to 95.0.

Peroxide value (2.5.5). Not more than 12.0, determined on 2.0 g.

Saponification value (2.5.6): 150 to 170, determined on 2.0 g.

Free glycerol. Not more than 6.0 per cent, determined as described under Assay.

Composition of fatty acids (2.4.22, Method C). The fatty-acid fraction of the substance has the following composition:

- *palmitic acid*: not more than 12.0 per cent,
- *stearic acid*: not more than 6.0 per cent,
- *oleic acid*: not less than 60.0 per cent,
- *linoleic acid*: not more than 35.0 per cent,
- *linolenic acid*: not more than 2.0 per cent,
- *arachidic acid*: not more than 2.0 per cent,
- *eicosenoic acid*: not more than 2.0 per cent.

Water (2.5.12). Not more than 1.0 per cent, determined on 1.00 g by the semi-micro determination of water. Use as the solvent a mixture of equal volumes of *anhydrous methanol R* and *methylene chloride R*.

Total ash (2.4.16). Not more than 0.1 per cent, determined on 1.00 g.

ASSAY

Determine the free glycerol content and the mono-, di- and triacylglycerol contents by size-exclusion chromatography (2.2.30).

Test solution. Into a 15 ml flask, weigh about 0.2 g (*m*), to the nearest 0.1 mg. Add 5 ml of *tetrahydrofuran R* and shake to dissolve. Reweigh the flask and calculate the total mass of solvent and substance (*M*).

Reference solutions. Into four 15 ml flasks, respectively weigh, to the nearest 0.1 mg, about 2.5 mg, 5 mg, 10 mg and 20 mg of *glycerol R*. Add 5 ml of *tetrahydrofuran R* and shake until well mixed. Weigh the flasks again and calculate the concentration of glycerol in milligrams per gram for each reference solution.

The chromatographic procedure may be carried out using:

- a gel-permeation column 0.6 m long and 7 mm in internal diameter packed with *styrene-divinylbenzene copolymer R* (particle diameter 5 µm and porosity 10 nm),
- as mobile phase at a flow rate of 1 ml/min *tetrahydrofuran R*,
- a differential refractive index detector.

Inject 40 µl of each solution. When the chromatograms are recorded in the prescribed conditions, the retention times relative to glycerol are about 0.85 for the monoacylglycerols, about 0.79 for the diacylglycerols and about 0.76 for the triacylglycerols. From the calibration curve obtained with the reference solutions determine the concentration (*C*) in milligrams per gram of glycerol in the test solution.

Calculate the percentage content of free glycerol in the substance to be examined using the following expression:

$$\frac{C \times M}{m \times 10}$$

Calculate the percentage content of mono-, di- and triacylglycerols in the substance to be examined by the normalisation procedure.

STORAGE

Store in an airtight container, protected from light.

LABELLING

The label states:

- the nominal content of monoacylglycerol,

– the name and concentration of any added antioxidant.

01/2005:0495

GLYCEROL MONOSTEARATE 40-55

Glyceroli monostearas 40-55

DEFINITION

Glycerol monostearate 40-55 is a mixture of monoacylglycerols, mainly monostearoylglycerol, together with variable quantities of di- and triacylglycerols. It contains 40.0 per cent to 55.0 per cent of monoacylglycerols, 30.0 per cent to 45.0 per cent of diacylglycerols and 5.0 per cent to 15.0 per cent of triacylglycerols, obtained by partial glycerolysis of vegetable oils mainly containing triacylglycerols of palmitic or stearic acid or by esterification of glycerol with stearic acid 50 (type I), stearic acid 70 (type II) or stearic acid 95 (type III) (see *Stearic acid* (1474)). The fatty acids may be of vegetable or animal origin.

CHARACTERS

A hard, waxy mass or unctuous powder or flakes, white or almost white, practically insoluble in water, soluble in alcohol at 60 °C.

IDENTIFICATION

- A. Melting point (2.2.15): 54 °C to 64 °C. Introduce the melted substance into the capillary tubes and allow to stand for 24 h in a well-closed container.
- B. Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel plate R*.

Test solution. Dissolve 1.0 g of the substance to be examined in *methylene chloride R*, with gentle heating, and dilute to 20 ml with the same solvent.

Reference solution. Dissolve 1.0 g of *glycerol monostearate 40-55 CRS* in *methylene chloride R*, with gentle heating, and dilute to 20 ml with the same solvent.

Apply to the plate 10 µl of each solution. Develop over a path of 15 cm using a mixture of 30 volumes of *hexane R* and 70 volumes of *ether R*. Allow the plate to dry in air. Spray with a 0.1 g/l solution of *rhodamine B R* in *alcohol R* and examine in ultraviolet light at 365 nm. The spots in the chromatogram obtained with the test solution are similar in position to those in the chromatogram obtained with the reference solution.

- C. It complies with the test for composition of fatty acids according to the type stated on the label (see Tests).
- D. It complies with the limits of the assay (monoacylglycerol content).

TESTS

Acid value (2.5.1). Not more than 3.0, determined on 1.0 g, using a mixture of equal volumes of *alcohol R* and *toluene R* as solvent and heating gently.

Iodine value (2.5.4). Not more than 3.0.

Saponification value (2.5.6): 158 to 177, determined on 2.0 g. Carry out the titration with heating.

Free glycerol. Not more than 6.0 per cent, determined as described under Assay.

01/2005:1331

Composition of fatty acids (2.4.22, Method C). The fatty acid fraction of the substance to be examined has the following composition:

	Fatty acid used for production by esterification	Composition of fatty acids
Glycerol monostearate 40-55 type I	Stearic acid 50	<i>Stearic acid</i> : 40.0 per cent to 60.0 per cent <i>Sum of the contents of palmitic and stearic acids</i> : not less than 90.0 per cent
Glycerol monostearate 40-55 type II	Stearic acid 70	<i>Stearic acid</i> : 60.0 per cent to 80.0 per cent <i>Sum of palmitic and stearic acids</i> : not less than 90.0 per cent
Glycerol monostearate 40-55 type III	Stearic acid 95	<i>Stearic acid</i> : 90.0 per cent to 99.0 per cent <i>Sum of the contents of palmitic and stearic acids</i> : not less than 96.0 per cent

Nickel (2.4.27). Not more than 1 ppm of Ni.

Water (2.5.12). Not more than 1.0 per cent, determined on 1.00 g by the semi-micro determination of water. Use *pyridine R* as the solvent and heat gently.

Total ash (2.4.16). Not more than 0.1 per cent, determined on 1.00 g.

ASSAY

Determine the free glycerol content and the mono-, di- and triacylglycerol contents by size-exclusion chromatography (2.2.30).

Test solution. Into a 15 ml flask, weigh about 0.2 g (*m*), to the nearest 0.1 mg. Add 5 ml of *tetrahydrofuran R* and shake to dissolve. Reweigh the flask and calculate the total mass of solvent and substance (*M*).

Reference solutions. Into four 15 ml flasks, respectively weigh, to the nearest 0.1 mg, about 2.5 mg, 5 mg, 10 mg and 20 mg of *glycerol R*. Add 5 ml of *tetrahydrofuran R* and shake until well mixed. Weigh the flasks again and calculate the concentration of glycerol in milligrams per gram for each reference solution.

The chromatographic procedure may be carried out using:

- a gel-permeation column 0.6 m long and 7 mm in internal diameter packed with *styrene-divinylbenzene copolymer R* (particle diameter 5 µm and porosity 10 nm),
- as mobile phase at a flow rate of 1 ml/min *tetrahydrofuran R*,
- a differential refractive index detector.

Inject 40 µl of each solution. When the chromatograms are recorded in the prescribed conditions, the retention times relative to glycerol are about 0.86 for the monoacylglycerols, about 0.81 for the diacylglycerols and about 0.77 for the triacylglycerols. From the calibration curve obtained with the reference solutions determine the concentration (*C*) in milligrams per gram of glycerol in the test solution.

Calculate the percentage content of free glycerol in the substance to be examined using the following expression:

$$\frac{C \times M}{m \times 10}$$

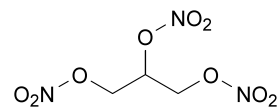
Calculate the percentage content of mono-, di- and triacylglycerols in the substance to be examined by the normalisation procedure.

LABELLING

The label states the type of glycerol monostearate 40-55.

GLYCERYL TRINITRATE SOLUTION

Glyceroli trinitratis solutio

C₃H₅N₃O₉M_r 227.1

DEFINITION

Ethanollic solution of glyceryl trinitrate.

Content: 1 per cent *m/m* to 10 per cent *m/m* of propane-1,2,3-triyl trinitrate and 96.5 per cent to 102.5 per cent of the declared content of glyceryl trinitrate stated on the label.

CHARACTERS

Appearance: clear, colourless or slightly yellow solution.

Solubility: miscible with acetone and with ethanol.

Solubility of pure glyceryl trinitrate: practically insoluble in water, freely soluble in ethanol, miscible with acetone.

IDENTIFICATION

First identification: A, C.

Second identification: B, C.

Upon diluting glyceryl trinitrate solution, care must be taken to always use anhydrous ethanol, otherwise droplets of pure glyceryl trinitrate may precipitate from the solution.

After examination, the residues and the solutions obtained in both the identification and the test sections must be heated on a water-bath for 5 min with dilute sodium hydroxide solution R.

A. Infrared absorption spectrophotometry (2.2.24).

Preparation: place 50 µl of a solution diluted, if necessary, with *ethanol R*, to contain 10 g/l of glyceryl trinitrate, on a disc of *potassium bromide R* and evaporate the solvent *in vacuo*.

Comparison: *Ph. Eur.* reference spectrum of *glyceryl trinitrate*.

B. Thin-layer chromatography (2.2.27).

Test solution. Dilute a quantity of the substance to be examined corresponding to 50 mg of glyceryl trinitrate to 100 ml with *acetone R*.

Reference solution. Dilute 0.05 ml of *glyceryl trinitrate solution CRS* to 1 ml with *acetone R*.

Plate: *TLC silica gel G plate R*.

Mobile phase: *ethyl acetate R, toluene R* (20:80 *V/V*).

Application: 5 µl.

Development: over 2/3 of the plate.

Drying: in air.

Detection: spray with freshly prepared *potassium iodide and starch solution R*. Expose the plate to ultraviolet light at 254 nm for 15 min. Examine in daylight.

Results: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

C. It complies with the limits of the assay.

TESTS

Upon diluting glyceryl trinitrate solution, care must be taken always to use anhydrous ethanol, otherwise droplets of pure glyceryl trinitrate may precipitate from the solution. After examination, the residues and the solutions obtained in both the identification and the test sections must be heated on a water-bath for 5 min with dilute sodium hydroxide solution R.

Appearance of solution. If necessary dilute the solution to be examined to a concentration of 10 g/l with ethanol R. The solution is not more intensely coloured than reference solution Y₇ (2.2.2, Method II).

Inorganic nitrates. Thin-layer chromatography (2.2.27).

Test solution. If necessary dilute the solution to be examined to a concentration of 10 g/l with ethanol R.

Reference solution. Dissolve 5 mg of potassium nitrate R in 1 ml of water R and dilute to 100 ml with alcohol R.

Plate: TLC silica gel plate R.

Mobile phase: glacial acetic acid R, acetone R, toluene R (15:30:60 V/V/V).

Application: 10 µl.

Development: over 2/3 of the plate.

Drying: in a current of air until the acetic acid is completely removed.

Detection: spray intensively with freshly prepared potassium iodide and starch solution R. Expose the plate to ultraviolet light at 254 nm for 15 min. Examine in daylight.

Limit:

- **nitrate ion:** any spot corresponding to the nitrate ion in the chromatogram obtained with the test solution is not more intense than the spot in the chromatogram obtained with the reference solution (0.5 per cent of the content of glyceryl trinitrate calculated as potassium nitrate).

Related substances. Liquid chromatography (2.2.29).

Test solution. Dissolve a quantity of the substance to be examined corresponding to 2 mg of glyceryl trinitrate in the mobile phase and dilute to 20.0 ml with the mobile phase.

Reference solution (a). Dissolve 0.10 g of glyceryl trinitrate solution CRS and a quantity of diluted pentaerythrityl tetranitrate CRS equivalent to 1.0 mg of pentaerythrityl tetranitrate in the mobile phase and dilute to 100.0 ml with the mobile phase. Sonicate and filter if necessary.

Reference solution (b). Dilute 1.0 ml of the test solution to 100.0 ml with the mobile phase.

Column:

- **size:** $l = 0.25$ m, $\varnothing = 4.6$ mm,
- **stationary phase:** octadecylsilyl silica gel for chromatography R (5 µm).

Mobile phase: acetonitrile R, water R (50:50 V/V).

Flow rate: 1 ml/min.

Detection: spectrophotometer at 210 nm.

Injection: 20 µl.

Run time: 3 times the retention time of the principal peak.

System suitability: reference solution (a):

- **resolution:** minimum 2.0 between the peaks due to glyceryl trinitrate and to pentaerythrityl tetranitrate.

Limits:

- **any impurity:** not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (1 per cent, calculated as glyceryl trinitrate),

- **total:** not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (b) (3 per cent, calculated as glyceryl trinitrate),
- **disregard limit:** 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent).

ASSAY

Test solution. Prepare a solution containing 1.0 mg of glyceryl trinitrate in 250.0 ml of methanol R.

Reference solution. Dissolve 70.0 mg of sodium nitrite R in methanol R and dilute to 250.0 ml with the same solvent. Dilute 5.0 ml of the solution to 500.0 ml with methanol R.

Into three 50 ml volumetric flasks introduce 10.0 ml of the test solution, 10.0 ml of the reference solution and 10 ml of methanol R as a blank. To each flask add 5 ml of dilute sodium hydroxide solution R, close the flask, mix and allow to stand at room temperature for 30 min. Add 10 ml of sulphanilic acid solution R and 10 ml of dilute hydrochloric acid R and mix. After exactly 4 min, add 10 ml of naphthylethylenediamine dihydrochloride solution R, dilute to volume with water R and mix. After 10 min read the absorbance (2.2.25) of the test solution and the reference solution at 540 nm using the blank solution as the compensation liquid.

Calculate the amount of glyceryl trinitrate in milligrams in the test solution from the following expression:

$$\frac{A_T \times m_S \times C}{A_R \times m_T \times 60.8 \times 100}$$

A_T = absorption of the test solution,

m_T = mass of the substance to be examined, in milligrams,

C = percentage content of sodium nitrite used as reference,

A_R = absorption of the reference solution,

m_S = mass of sodium nitrite, in milligrams.

STORAGE

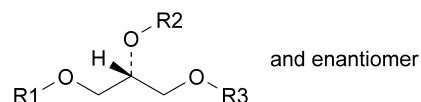
Store diluted solutions (10 g/l) protected from light, at a temperature of 2 °C to 15 °C. Store more concentrated solutions protected from light, at a temperature of 15 °C to 20 °C.

LABELLING

The label states the declared content of glyceryl trinitrate.

IMPURITIES

A. inorganic nitrates,



B. $R_1 = \text{NO}_2$, $R_2 = R_3 = \text{H}$: (2*RS*)-2,3-dihydroxypropyl nitrate,

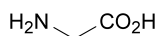
C. $R_1 = R_3 = \text{H}$, $R_2 = \text{NO}_2$: 2-hydroxy-1-(hydroxymethyl)ethyl nitrate,

D. $R_1 = R_2 = \text{NO}_2$, $R_3 = \text{H}$: (2*RS*)-3-hydroxypropane-1,2-diyl dinitrate,

E. $R_1 = R_3 = \text{NO}_2$, $R_2 = \text{H}$: 2-hydroxypropane-1,3-diyl dinitrate.

GLYCINE

Glycinum

C₂H₅NO₂M_r 75.1

DEFINITION

2-Aminoacetic acid.

Content: 98.5 per cent to 101.0 per cent (dried substance).

CHARACTERS

Appearance: white or almost white, crystalline powder.*Solubility*: freely soluble in water, very slightly soluble in alcohol.

It shows polymorphism.

IDENTIFICATION

First identification: A.*Second identification*: B, C.

A. Infrared absorption spectrophotometry (2.2.24).

Preparation: discs. Use about 1 mg for 0.4 g of *potassium bromide R*.*Comparison*: *glycine CRS*.If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in the smallest necessary quantity of *alcohol (60 per cent V/V) R*, evaporate to dryness and record the spectra again.

B. Examine the chromatograms obtained in the test for ninhydrin-positive substances.

Results: the principal spot in the chromatogram obtained with test solution (b) is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).C. Dissolve 50 mg in 5 ml of *water R*, add 1 ml of *strong sodium hypochlorite solution R* and boil for 2 min. Add 1 ml of *hydrochloric acid R* and boil for 4-5 min. Add 2 ml of *hydrochloric acid R* and 1 ml of a 20 g/l solution of *resorcinol R*, boil for 1 min and cool. Add 10 ml of *water R* and mix. To 5 ml of the solution add 6 ml of *dilute sodium hydroxide solution R*. The solution is violet with greenish-yellow fluorescence. After a few minutes, the colour becomes orange and then yellow and an intense fluorescence remains.

TESTS

Solution S. Dissolve 5.0 g in *carbon dioxide-free water R* and dilute to 50 ml with the same solvent.**Appearance of solution**. Solution S is clear (2.2.1) and not more intensely coloured than reference solution Y₇ (2.2.2, *Method II*).**pH** (2.2.3): 5.9 to 6.4.Dilute 10 ml of solution S to 20 ml with *carbon dioxide-free water R*.**Ninhydrin-positive substances**. Thin-layer chromatography (2.2.27).*Test solution (a)*. Dissolve 0.10 g of the substance to be examined in *water R* and dilute to 10.0 ml with the same solvent.01/2005:0614
corrected*Test solution (b)*. Dilute 1.0 ml of test solution (a) to 10.0 ml with *water R*.*Reference solution (a)*. Dissolve 10 mg of *glycine CRS* in *water R* and dilute to 10.0 ml with the same solvent.*Reference solution (b)*. Dilute 1.0 ml of test solution (a) to 200 ml with *water R*.*Reference solution (c)*. Dissolve 10 mg of *glycine CRS* and 10 mg of *alanine CRS* in *water R* and dilute to 25 ml with the same solvent.*Plate*: *cellulose for chromatography R* as the coating substance.*Mobile phase*: *glacial acetic acid R*, *water R*, *butanol R* (20:20:60 V/V/V).*Application*: 5 µl.*Development*: over 2/3 of the plate.*Drying*: at 80 °C for 30 min.*Detection*: spray with *ninhydrin solution R* and dry at 100-105 °C for 15 min.*System suitability*: the chromatogram obtained with reference solution (c) shows 2 clearly separated spots.*Limits*: in the chromatogram obtained with test solution (a):

- *any impurity*: any spots, apart from the principal spot, are not more intense than the principal spot in the chromatogram obtained with reference solution (b) (0.5 per cent).

Chlorides (2.4.4): maximum 75 ppm.Dissolve 0.67 g in *water R* and dilute to 15 ml with the same solvent. The solution complies with the limit test for chlorides.**Heavy metals** (2.4.8): maximum 10 ppm.12 ml of solution S complies with limit test A. Prepare the standard using *lead standard solution (1 ppm Pb) R*.**Loss on drying** (2.2.32): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 100-105 °C for 2 h.**Sulphated ash** (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 70.0 mg in 3 ml of *anhydrous formic acid R* and add 30 ml of *anhydrous acetic acid R*. Immediately after dissolution, titrate with 0.1 M *perchloric acid*, determining the end-point potentiometrically (2.2.20).1 ml of 0.1 M *perchloric acid* is equivalent to 7.51 mg of C₂H₅NO₂.01/2005:1892
corrected

GOLDENROD

Solidaginis herba

DEFINITION

Whole or cut, dried, flowering aerial parts of *Solidago gigantea* Ait or *S. canadensis* L., their varieties or hybrids and/or mixtures of these.*Content*: minimum 2.5 per cent of flavonoids, expressed as hyperoside (C₂₁H₂₀O₁₂; M_r 464.4) (dried drug).

CHARACTERS

Macroscopic and microscopic characters described under identification tests A and B.

IDENTIFICATION

- A. The stems are greenish-yellow or greenish-brown, partly tinted reddish, roundish, more or less conspicuously grooved, glabrous and smooth in the lower part, slightly or densely pubescent in the upper part. They are solid with a whitish pith.

The leaves are green, sessile, lanceolate, with a serrate margin, 8 cm to 12 cm long and about 1 cm to 3 cm wide, the upper surface is green and more or less glabrous, the lower surface is greyish-green and pubescent, especially on the veins. The inflorescence consists of a number of unilateral, curved racemes which together form a pyramidal panicle at the end of the stems.

Each capitulum has an involucre composed of linear-lanceolate, imbricated yellowish-green bracts, surrounding a single row of yellow ligulate florets about the same length as the involucre; yellow, radially arranged tubular florets, as long as, or longer, than the ligulate florets; a brownish inferior ovary surmounted by a white pappus of silky hairs.

- B. Reduce to a powder (355). The powder is greyish-green. Examine under a microscope using *chloral hydrate solution R*. The powder shows pappus bristles and their fragments, consisting of multiseriate trichomes composed of elongated cells with the tips free from the surface and forming pointed projections over the entire length; fragments of the leaf mesophyll with vascular bundles accompanied by secretory cells; fragments of the leaf epidermis with sinuous to wavy-walled cells and stomata of the anomocytic type (2.8.3); uniseriate covering trichomes with up to 5 or 6 cells, some whip-like with a thicker-walled terminal cell; fragments of the style with long, slender papillae; fragments of the stem with reticulate and spiral vessels; pollen grains, with 3 germinal pores and a spiny exine; numerous whisk-shaped hairs, a few isolated twin-hairs from the ovary, absence of multicellular trichomes with a terminal cell bent at a right angle.

- C. Thin-layer chromatography (2.2.27), as described in the test for *Solidago virgaurea* with the following modifications.

Detection: spray with a 10 g/l solution of *diphenylboric acid aminoethyl ester R* in *methanol R* and then with a 50 g/l solution of *macrogol 400 R* in *methanol R*. Allow to stand for 30 min. Examine in ultraviolet light at 365 nm.

Results: see below the sequence of the zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other zones may be present in the chromatogram obtained with the test solution.

Top of the plate	
Caffeic acid: a light blue fluorescent zone.	A bluish-green fluorescent zone.
Quercitrin: a yellowish-brown fluorescent zone.	A faint to intense yellowish-brown fluorescent zone. A more or less intense yellowish brown zone.
Chlorogenic acid: a light blue fluorescent zone.	A light blue zone and/or a yellow fluorescent zone.
Rutin: an orange fluorescent zone.	A faint to intense yellowish-brown fluorescent zone.
Reference solution	Test solution

TESTS

Foreign matter (2.8.2): maximum 5 per cent of brownish parts and maximum 2 per cent of other foreign matter.

Solidago virgaurea. Thin-layer chromatography (2.2.27).

Test solution. To 0.75 g of the powdered drug (355) add 5 ml of *methanol R* and boil in a water-bath under a reflux condenser for 10 min. Cool and filter.

Reference solution. Dissolve 1.0 mg of *chlorogenic acid R*, 1.0 mg of *caffeic acid R*, 2.5 mg of *quercitrin R* and 2.5 mg of *rutin R* in 10 ml of *methanol R*.

Plate: TLC silica gel plate *R*.

Mobile phase: *anhydrous formic acid R*, *water R*, *methyl ethyl ketone R*, *ethyl acetate R* (6:6:18:30 V/V/V/V).

Application: 20 µl of the test solution and 10 µl of the reference solution, as bands.

Development: over a path of 10 cm.

Drying: at 100-105 °C.

Detection: spray with *anisaldehyde solution R* and heat at 100-105 °C for 10 min. Examine in ultraviolet light at 365 nm.

Results: the chromatograms obtained with the reference solution and the test solution show a greyish-green fluorescent zone in the lower third (rutin). The chromatogram obtained with the test solution does not show a dark grey or dark brown zone (leiocarposide) below the zone due to rutin.

Loss on drying (2.2.32): maximum 10 per cent, determined on 0.500 g of the powdered drug (355) by drying in an oven at 100-105 °C for 2 h.

Total ash (2.4.16): maximum 7.0 per cent.

Ash insoluble in hydrochloric acid (2.8.1): maximum 1.0 per cent.

ASSAY

Stock solution. In a 100 ml round-bottomed flask, introduce 0.200 g of the powdered drug (250), add 1 ml of a 5 g/l solution of *hexamethylenetetramine R*, 20 ml of *acetone R* and 2 ml of *hydrochloric acid R1*. Boil the mixture under a reflux condenser for 30 min. Filter the liquid through a small plug of absorbent cotton into a 100 ml flask. Add the absorbent cotton to the residue in the round-bottomed flask, extract with 2 quantities, each of 20 ml of *acetone R*, each time boiling under a reflux condenser for 10 min. Allow to cool. Filter the combined acetone extracts through a filter paper into a volumetric flask. Rinse the flask and the filter paper and dilute to 100.0 ml with *acetone R*. Introduce 20.0 ml of the solution into a suitable separating funnel, add 20 ml of *water R* and shake the mixture with 1 quantity of 15 ml and then 3 quantities, each of 10 ml, of *ethyl acetate R*. Combine the ethyl acetate extracts in a separating funnel, wash twice with 50 ml of *water R* and filter the extracts over 10 g of *anhydrous sodium sulphate R* into a volumetric flask. Dilute to 50.0 ml with *ethyl acetate R*, rinsing the separating funnel and the sodium sulphate.

Test solution. To 10.0 ml of the stock solution add 1.0 ml of *aluminium chloride reagent R* and dilute to 25.0 ml with a 5 per cent V/V solution of *glacial acetic acid R* in *methanol R*.

Compensation solution. Dilute 10.0 ml of the stock solution to 25.0 ml with a 5 per cent V/V solution of *glacial acetic acid R* in *methanol R*.

Measure the absorbance of the test solution (2.2.25) at 425 nm after 30 min by comparison with the compensation solution.

Calculate the percentage content of flavonoids, expressed as hyperoside, from the expression:

$$\frac{A \times 1.25}{m}$$

i.e. taking the value of the specific absorbance of hyperoside to be 500.

A = absorbance measured at 425 nm,
 m = mass of the drug to be examined, in grams.

01/2005:1893
 corrected

GOLDENROD, EUROPEAN

Solidaginis virgaureae herba

DEFINITION

Whole or cut, dried, flowering aerial parts of *Solidago virgaurea* L.

Content: minimum 1.0 per cent of flavonoids, expressed as hyperoside ($C_{21}H_{20}O_{12}$; M_r 464.4) (dried drug).

CHARACTERS

Macroscopic and microscopic characters described under identification tests A and B.

IDENTIFICATION

- A. The stem is cylindrical, striated, the lower part often reddish-violet, sometimes entirely glabrous or pubescent with short, bent, apically directed hairs. The basal leaves are obovate to oblanceolate, with a serrate margin, and taper at the base into a long, winged petiole; the cauline leaves are alternate, smaller than the basal leaves and more elliptical in outline, with an entire or slightly toothed margin; they are sessile or with only a short petiole. Both surfaces of the leaves are glabrous or only slightly pubescent with a prominent reticulate venation on the lower surface. The capitula form a tightly packed panicle. At the base of the pedicels there are 2, small, linear bracts with scarious margins. The involucre consists of 2 to 4 rows of loosely-arranged, imbricate bracts, each bract greenish-yellow with a smooth and shiny inner surface, the outer surface hairy or glabrous, with a scarious margin. Each capitulum contains from 6 to 12 widely separated female ray florets, about twice as long as the bracts, and about 10 to 30 hermaphrodite, tubular florets. All florets are yellow. The brown, inferior ovary tapers towards the base and has a ribbed surface, covered with scattered hairs; it is surmounted by a whitish pappus composed of smooth or rough, bristly hairs.
- B. Reduce to a powder (355). The powder is light green. Examine under a microscope using *chloral hydrate solution R*. The powder shows fragments of the leaf in surface view, those of the upper epidermis composed of polygonal cells with straight, beaded walls and distinct cuticular striations, those of the lower epidermis more sinuous with fewer striations and numerous anomocytic stomata (2.8.3); occasional leaf fragments showing cells containing small, isolated cluster crystals of calcium oxalate; uniseriate, conical covering trichomes from the leaves and the bracts with up to about 10 cells, some of the shorter trichomes showing a terminal cell extended and pennant-like; occasional glandular trichomes with a 1 or 2 celled stalk and a unicellular, elongated head; rare

paired, covering trichomes from the ovary with a distinctly pitted central wall and a bifid apex; abundant pappus hairs and their fragments, multiseriate with the marginal cells overlapping outwards; groups of fibres and vascular tissue from the stems; fragments of the epidermis of the petals with striated cuticle and occasional long, biseriate glandular trichomes; pollen grains spherical, with 3 pores and a spiny exine.

- C. Thin-layer chromatography (2.2.27) as described in the test for *Solidago gigantea* and *Solidago canadensis*.

Detection: spray 1 of the 2 plates with *anisaldehyde solution R* and heat at 105-110 °C for 10 min. Examine the plate in daylight.

Results: see below the sequence of the zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other fluorescent zones may be present in the chromatogram obtained with the test solution.

Top of the plate	
Quercitrin: a yellow zone	A violet zone
Rutin: a yellow zone	A faint yellow zone (nicotiflorin)
Leiocarposide: a brownish zone	A yellow zone (rutin)
	A brownish zone (leiocarposide)
	A greenish zone
Reference solution	Test solution

TESTS

Foreign matter (2.8.2): maximum 5 per cent of brown coloured matter and maximum 2 per cent of other foreign matter.

***Solidago gigantea* and *Solidago canadensis*.** Thin-layer chromatography (2.2.27).

Test solution. To 0.75 g of the powdered drug (355) add 5 ml of *methanol R* and heat on a water-bath under a reflux condenser for 10 min. Cool and filter.

Reference solution. Dissolve 1.0 mg of *chlorogenic acid R*, 2.5 mg of *quercitrin R*, 2.5 mg of *rutin R* and 5 mg of *leiocarposide R* in 10 ml of *methanol R*.

Plate: *TLC silica gel plate R* (2 plates).

Mobile phase: *anhydrous formic acid R*, *water R*, *methylethyl ketone R*, *ethyl acetate R* (6:6:18:30 V/V/V/V).

Application: 20 µl, as bands.

Development: over a path of 10 cm.

Drying: in air.

Detection: spray 1 of the 2 plates with a 10 g/l solution of *diphenylboric acid aminoethyl ester R* in *methanol R* and then with a 50 g/l solution of *macrogol 400 R* in *methanol R*. Examine in ultraviolet light at 365 nm after 30 min.

Results: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other fluorescent zones may be present in the chromatogram obtained with the test solution. The chromatogram obtained with the test solution shows no strong orange fluorescent zone similar in position to the zone of quercitrin in the chromatogram obtained with the reference solution.

01/2005:1831

GOLDENSEAL RHIZOME

Hydrastis rhizoma

DEFINITION

Whole or cut, dried rhizome and root of *Hydrastis canadensis* L.

Content:

- hydrastine ($C_{21}H_{21}NO_6$; M_r 383.4): minimum 2.5 per cent (dried drug),
- berberine ($C_{20}H_{19}NO_5$; M_r 353.4): minimum 3.0 per cent (dried drug).

CHARACTERS

Macroscopic and microscopic characters described under identification tests A and B.

IDENTIFICATION

- A. The rhizome is tortuous and knotty, about 5 cm long and 5 mm to 10 mm thick. The surface is yellowish to brownish-grey and irregularly wrinkled, and bears the remains of numerous slender, wiry roots; stem bases and scale leaves occur on the upper surface. The fracture is short and resinous. The transversely-cut surface is yellowish-brown and shows a fairly wide bark, a ring of from about 12 to 20 widely separated xylem bundles and a large, central pith.
- B. Reduce to a powder (180). The powder is greenish-yellow. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters: abundant thin-walled parenchyma; occasional fragments of yellowish-brown cork from the rhizome and roots; groups of small vessels with conspicuous perforations in the oblique end walls and with simple or bordered, slit-shaped pits; infrequent groups of thin-walled, pitted fibres, usually found associated with the vessels; numerous ovoid or spherical, orange-brown granular masses. Examine under a microscope using a 50 per cent V/V solution of *glycerol R*. The powder shows abundant starch granules, mostly simple but sometimes compound with up to 4 components; the granules are small, spherical to ovoid, up to about 10 µm in diameter, occasionally with a small, rounded or slit-shaped hilum.
- C. Thin-layer chromatography (2.2.27).

Test solution. Sonicate for 10 min 250 mg of powdered drug (180) in 4 ml of a mixture of 20 ml of *water R* and 80 ml of *methanol R* and filter. Wash the residue twice with 2 ml of *methanol R*. Combine the solutions and dilute to 20 ml with *methanol R*.

Reference solution. Dissolve 5 mg of *hydrastine hydrochloride R* and 5 mg of *berberine chloride R* in 20 ml of *methanol R*.

Plate: TLC silica gel plate *R*.

Mobile phase: *anhydrous formic acid R*, *water R*, *ethyl acetate R* (10:10:80 V/V/V).

Application: 20 µl as bands.

Development: over a path of 15 cm.

Drying: in air.

Detection: examine in ultraviolet light at 365 nm.

Results: see below the sequence of the zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore other fluorescent zones may be present in the chromatogram obtained with the test solution.

Top of the plate	
Quercitrin: an orange fluorescent zone _____	A light blue fluorescent zone _____
Chlorogenic acid: a light blue fluorescent zone _____	A light blue fluorescent zone (chlorogenic acid) _____
Rutin: an orange fluorescent zone _____	An orange fluorescent zone (rutin) _____
Reference solution	Test solution

Loss on drying (2.2.32): maximum 12 per cent, determined on 1.000 g of the powdered drug (355) by drying in an oven at 100-105 °C for 2 h.

Total ash (2.4.16): maximum 8.0 per cent.

ASSAY

Stock solution. In a 100 ml round-bottomed flask, place 0.200 g of the powdered drug (355), add 1 ml of a 5 g/l solution of *hexamethylenetetramine R*, 20 ml of *acetone R* and 2 ml of *hydrochloric acid R1*. Boil the mixture in a water-bath under a reflux condenser for 30 min. Filter the liquid through a small plug of absorbent cotton into a 100 ml flask. Add the absorbent cotton to the residue in the round-bottomed flask and extract with 2 quantities, each of 20 ml, of *acetone R*, each time boiling under a reflux condenser for 10 min. Allow to cool. Filter the combined acetone extracts through filter paper, dilute to 100.0 ml with *acetone R*, rinsing the volumetric flask and the filter paper with acetone. Introduce 20.0 ml of the solution into a suitable separating funnel, add 20 ml of *water R* and shake the mixture with 1 quantity of 15 ml and then with 3 quantities, each of 10 ml, of *ethyl acetate R*. Combine the ethyl acetate extracts in a separating funnel, wash twice with 50 ml of *water R* and filter the extracts over 10 g of *anhydrous sodium sulphate R* into a volumetric flask. Dilute to 50.0 ml with *ethyl acetate R*, rinsing the separating funnel and the sodium sulphate.

Test solution. To 10.0 ml of the stock solution add 1.0 ml of *aluminium chloride reagent R* and dilute to 25.0 ml with a 5 per cent V/V solution of *glacial acetic acid R* in *methanol R*.

Compensation liquid. Dilute 10.0 ml of the stock solution to 25.0 ml with a 5 per cent V/V solution of *glacial acetic acid R* in *methanol R*.

After 30 min, measure the absorbance (2.2.25) of the test solution at 425 nm by comparison with the compensation liquid.

Calculate the percentage content of flavonoids, expressed as hyperoside, from the expression:

$$\frac{A \times 1.25}{m}$$

i.e. taking the specific absorbance of hyperoside to be 500.

A = measured absorbance at 425 nm,

m = mass of the drug to be examined, in grams.

Top of the plate	
Berberine: a bright yellow fluorescent zone	A bright yellow fluorescent zone (berberine)
Hydrastine: a deep blue fluorescent zone	A deep blue fluorescent zone (hydrastine)
	A bright light blue fluorescent zone (hydrastinine)
	A deep blue fluorescent zone
Reference solution	Test solution

TESTS

Foreign matter (2.8.2): maximum 2 per cent *m/m*.

Loss on drying (2.2.32): maximum 10.0 per cent, determined on 1.000 g of the powdered drug (180) by drying in an oven at 100–105 °C for 2 h.

Total ash (2.4.16): maximum 8.0 per cent.

Ash insoluble in hydrochloric acid (2.8.1): maximum 4.0 per cent.

ASSAY

Liquid chromatography (2.2.29).

Test solution. To 1.000 g of the powdered drug (355) in a 100 ml round-bottomed flask, add 50 ml of a 10 ml/l solution of *concentrated ammonia R* in *alcohol R* and boil the mixture under a reflux condenser for 30 min. Allow to cool to room temperature and filter the liquid through a plug of absorbent cotton into a flask. Add the absorbent cotton to the residue in the round-bottomed flask and repeat the extraction with a further 2 quantities, each of 30 ml of a 10 ml/l solution of *concentrated ammonia R* in *alcohol R*, each time boiling under a reflux condenser for 10 min and filtering through a plug of absorbent cotton in the same flask as previously. Filter the combined filtrates through a filter-paper into a 250 ml round-bottomed flask, and rinse flask and filter with 20 ml of a 10 ml/l solution of *concentrated ammonia R* in *alcohol R*. Evaporate the filtrate to dryness *in vacuo* in a water-bath at 55 °C. Dissolve the residue in 50.0 ml of the mobile phase. Dilute 10.0 ml of this solution to 250.0 ml with the mobile phase.

Reference solution. Dissolve 10 mg of *hydrastine hydrochloride R* and 10 mg of *berberine chloride R* in *methanol R* and dilute to 100.0 ml with the same solvent.

Column:

- **size:** *l* = 0.125 m, Ø = 4 mm,
- **stationary phase:** *end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

Mobile phase: dissolve 9.93 g of *potassium dihydrogen phosphate R* in 730 ml of *water R*, add 270 ml of *acetonitrile R* and mix.

Flow rate: 1.2 ml/min.

Detection: spectrophotometer at 235 nm.

Injection: 10 µl.

System suitability: reference solution:

- **elution order:** when the chromatograms are recorded in the prescribed conditions, the components elute in the order indicated in the composition of the reference solution; record the retention times of these substances;
- **resolution:** minimum 1.5 between the peaks due to hydrastine and berberine.

Using the retention times determined from the chromatogram obtained with the reference solution, locate the components of the reference solution on the chromatogram obtained with the test solution.

Calculate the percentage content of each alkaloid (hydrastine and berberine) from the following expression:

$$\frac{A_1 \times m_2 \times p}{A_2 \times m_1} \times 12.5$$

*A*₁ = area of the peak due to hydrastine or berberine in the chromatogram obtained with the test solution,

*A*₂ = area of the peak due to hydrastine or berberine in the chromatogram obtained with the reference solution,

*m*₁ = mass of the drug to be examined, in grams,

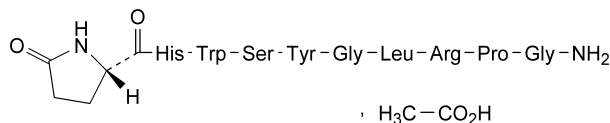
*m*₂ = mass of hydrastine hydrochloride or berberine chloride in the reference solution, in grams,

p = percentage content of hydrastine in *hydrastine hydrochloride R* or berberine in *berberine chloride R*.

01/2005:0827
corrected

GONADORELIN ACETATE

Gonadorelini acetat



C₅₇H₇₉N₁₇O₁₅

*M*_r 1242

DEFINITION

Gonadorelin acetate is the acetate form of a hypothalamic peptide that stimulates the release of follicle-stimulating hormone and luteinising hormone from the pituitary gland. It contains not less than 95.0 per cent and not more than the equivalent of 102.0 per cent of the peptide C₅₅H₇₅N₁₇O₁₃, calculated with reference to the anhydrous, acetic acid-free substance. It is obtained by chemical synthesis.

CHARACTERS

A white or slightly yellowish powder, soluble in water and in a 1 per cent *V/V* solution of glacial acetic acid, sparingly soluble in methanol.

IDENTIFICATION

- Examine the chromatograms obtained in the assay. The retention time and size of the principal peak in the chromatogram obtained with the test solution are approximately the same as those of the principal peak in the chromatogram obtained with reference solution (a).
- Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel G plate R*. Use the test solution and reference solution (a) prepared under Assay. Apply to the plate 10 µl of each solution. Develop over a path of 15 cm using a mixture of 6 volumes of *glacial acetic acid R*, 14 volumes of *water R*, 45 volumes of *methanol R* and 60 volumes of *methylene chloride R*. Allow the plate to dry in air for 5 min. At the bottom of a chromatography tank, place an evaporating dish containing a mixture of 10 ml of a 50 g/l solution of

potassium permanganate R and 3 ml of *hydrochloric acid R*, close the tank and allow to stand. Place the dried plate in the tank and close the tank. Leave the plate in contact with the chlorine vapour for 2 min. Withdraw the plate and place it in a current of cold air until the excess of chlorine is removed and an area of coating below the points of application no longer gives a blue colour with 0.05 ml of *potassium iodide and starch solution R*. Spray with *potassium iodide and starch solution R*. The principal spot in the chromatogram obtained with the test solution corresponds in position and size to the principal spot in the chromatogram obtained with reference solution (a).

TESTS

Appearance of solution. A 10 g/l solution is clear (2.2.1) and not more intensely coloured than reference solution Y₅ (2.2.2, *Method II*).

Specific optical rotation (2.2.7). Dissolve 10.0 mg in 1.0 ml of a 1 per cent V/V solution of *glacial acetic acid R*. The specific optical rotation is –54 to –66, calculated on the basis of the peptide content as determined in the assay.

Absorbance (2.2.25). Dissolve 10.0 mg in *water R* and dilute to 100.0 ml with the same solvent. The absorbance, determined at the maximum at 278 nm, corrected to a 10 mg/100 ml solution on the basis of the peptide content determined in the assay, is 0.55 to 0.61.

Amino acids. Examine by means of an amino-acid analyser. Standardise the apparatus with a mixture containing equimolar amounts of ammonia, glycine and the L-form of the following amino acids:

lysine	threonine	alanine	leucine
histidine	serine	valine	tyrosine
arginine	glutamic acid	methionine	phenylalanine
aspartic acid	proline	isoleucine	

together with half the equimolar amount of L-cystine. For the validation of the method, an appropriate internal standard, such as *DL-norleucine R*, is used.

Test solution. Place 1.0 mg of the substance to be examined in a rigorously cleaned hard-glass tube 100 mm long and 6 mm in internal diameter. Add a suitable amount of a 50 per cent V/V solution of *hydrochloric acid R*. Immerse the tube in a freezing mixture at –5 °C, reduce the pressure to below 133 Pa and seal. Heat at 110 °C to 115 °C for 16 h. Cool, open the tube, transfer the contents to a 10 ml flask with the aid of five quantities, each of 0.2 ml, of *water R* and evaporate to dryness over *potassium hydroxide R* under reduced pressure. Take up the residue in *water R* and evaporate to dryness over *potassium hydroxide R* under reduced pressure; repeat these operations once. Take up the residue in a buffer solution suitable for the amino-acid analyser used and dilute to a suitable volume with the same buffer solution. Apply a suitable volume to the amino-acid analyser.

Express the content of each amino acid in moles. Calculate the relative proportions of the amino acids, taking one-eighth of the sum of the number of moles of histidine, glutamic acid, leucine, proline, glycine, tyrosine and arginine as equal to one. The values fall within the following limits: serine 0.7 to 1.05; glutamic acid 0.95 to 1.05; proline 0.95 to 1.05; glycine 1.9 to 2.1; leucine 0.9 to 1.1; tyrosine 0.7 to 1.05; histidine 0.95 to 1.05 and arginine 0.95 to 1.05. Lysine and isoleucine are absent; not more than traces of other amino acids are present, with the exception of tryptophan.

Related substances. Examine by liquid chromatography (2.2.29) as described under Assay.

Inject 20 µl of reference solution (b). Adjust the sensitivity of the system so that the height of the principal peak in the chromatogram obtained is at least 50 per cent of the full scale of the recorder.

Inject 20 µl of the test solution. Continue the chromatography for twice the retention time of gonadorelin. In the chromatogram obtained with the test solution: the area of any peak apart from the principal peak, is not greater than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (2 per cent); the sum of the areas of the peaks, apart from the principal peak, is not greater than 5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (5 per cent). Disregard any peak with an area less than 0.05 times that of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

Acetic acid (2.5.34): 4.0 per cent to 7.5 per cent.

Test solution. Dissolve 10.0 mg of the substance to be examined in a mixture of 5 volumes of mobile phase B and 95 volumes of mobile phase A and dilute to 10.0 ml with the same mixture of solvents.

Water (2.5.12). Not more than 7.0 per cent, determined on 0.200 g by the semi-micro determination of water.

Bacterial endotoxins (2.6.14): less than 70 IU/mg, if intended for use in the manufacture of parenteral dosage forms without a further appropriate procedure for the removal of bacterial endotoxins.

ASSAY

Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 5.0 mg of the substance to be examined in *water R* and dilute to 10.0 ml with the same solvent.

Reference solution (a). Dissolve the contents of a vial of *gonadorelin CRS* in *water R* to obtain a concentration of 0.5 mg/ml.

Reference solution (b). Dilute 1.0 ml of the test solution to 100.0 ml with *water R*.

Reference solution (c). Dissolve 2.5 mg of the substance to be examined in 1 ml of 0.1 M *hydrochloric acid* and heat in a water-bath at 65 °C for 4 h. Add 1 ml of 0.1 M *sodium hydroxide* and dilute to 5.0 ml with *water R*.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.12 m long and 4.0 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (5 µm),
- as mobile phase at a flow rate of 1.5 ml/min a mixture of 13 volumes of *acetonitrile R* and 87 volumes of a 1.18 per cent V/V solution of *phosphoric acid R* (adjusted to pH 2.3 with *triethylamine R*),
- as detector a spectrophotometer set at 215 nm.

Inject 20 µl of reference solution (c). The test is not valid unless the resolution between the first and second peaks is at least 2.0.

Inject 20 µl of the test solution and 20 µl of reference solution (a).

Calculate the content of gonadorelin (C₅₅H₇₅N₁₇O₁₃) from the peak areas in the chromatograms obtained with the test solution and reference solution (a) and the declared content of C₅₅H₇₅N₁₇O₁₃ in *gonadorelin CRS*.

STORAGE

Store in an airtight container, protected from light at a temperature of 2 °C to 8 °C. If the substance is sterile, store in a sterile, airtight, tamper-proof container.

LABELLING

The label states:

- the mass of peptide in the container,
- where applicable, that the substance is free from bacterial endotoxins.

01/2005:0498

GONADOTROPHIN, CHORIONIC

Gonadotropinum chorionicum

DEFINITION

Chorionic gonadotrophin is a dry preparation of placental glycoproteins which have luteinising activity. The potency is not less than 2500 IU/mg.

PRODUCTION

Chorionic gonadotrophin is extracted from the urine of pregnant women using a suitable fractionation procedure. It is either dried under reduced pressure or freeze-dried. It is prepared in conditions designed to minimise or eliminate microbial and viral contamination. The manufacturing process must have been shown to reduce any viral contamination such as hepatitis virus or HIV by appropriate validated methods.

CHARACTERS

Appearance: white to yellowish-white, amorphous powder.

Solubility: soluble in water.

IDENTIFICATION

When administered to immature rats as prescribed in the assay, it causes an increase in the mass of the seminal vesicles and of the prostate gland.

TESTS

Water (2.5.32): maximum 5.0 per cent.

Bacterial endotoxins (2.6.14): less than 0.02 IU per IU of chorionic gonadotrophin, if intended for use in the manufacture of parenteral dosage forms without a further appropriate procedure for the removal of bacterial endotoxins.

ASSAY

The potency of chorionic gonadotrophin is estimated by comparing under given conditions its effect of increasing the mass of the seminal vesicles (or the prostate gland) of immature rats with the same effect of the International Standard of chorionic gonadotrophin or of a reference preparation calibrated in International Units.

The International Unit is the activity contained in a stated amount of the International Standard, which consists of a mixture of a freeze-dried extract of chorionic gonadotrophin from the urine of pregnant women with lactose. The equivalence in International Units of the International Standard is stated by the World Health Organisation.

Use immature male rats of the same strain, 19 to 28 days old, differing in age by not more than 3 days and having body masses such that the difference between the heaviest

and the lightest rat is not more than 10 g. Assign the rats at random to 6 equal groups of at least 5 animals. If sets of 6 litter mates are available, assign one litter mate from each set to each group and mark according to litter.

Choose 3 doses of the reference preparation and 3 doses of the preparation to be examined such that the smallest dose is sufficient to produce a positive response in some of the rats and the largest dose does not produce a maximal response in all the rats. Use doses in geometric progression and as an initial approximation total doses of 4 IU, 8 IU and 16 IU may be tried although the dose will depend on the sensitivity of the animals used, which may vary widely.

Dissolve separately the total quantities of the preparation to be examined and of the reference preparation corresponding to the daily doses to be used in sufficient *phosphate-albumin buffered saline pH 7.2 R* such that the daily dose is administered in a volume of about 0.5 ml. Add a suitable antimicrobial preservative such as 4 g/l of phenol or 0.02 g/l of thiomersal. Store the solutions at 5 ± 3 °C.

Inject subcutaneously into each rat the daily dose allocated to its group, on 4 consecutive days at the same time each day. On the fifth day, about 24 h after the last injection, kill the rats and remove the seminal vesicles. Remove any extraneous fluid and tissue and weigh the vesicles immediately. Calculate the results by the usual statistical methods, using the mass of the vesicles as the response. (The precision of the assay may be improved by a suitable correction of the organ mass with reference to the body mass of the animal from which it was taken; an analysis of covariance may be used).

The estimated potency is not less than 80 per cent and not more than 125 per cent of the stated potency. The confidence limits ($P = 0.95$) of the estimated potency are not less than 64 per cent and not more than 156 per cent of the stated potency.

STORAGE

In an airtight, tamper-proof container, protected from light at a temperature of 2 °C to 8 °C. If the substance is sterile, store in a sterile, airtight, tamper-proof container.

LABELLING

The label states:

- the number of International Units per container,
- the potency in International Units per milligram,
- where applicable, that the substance is free from bacterial endotoxins.

01/2005:0719

GONADOTROPHIN, EQUINE SERUM,
FOR VETERINARY USEGonadotropinum sericum equinum
ad usum veterinarium

DEFINITION

Equine serum gonadotrophin for veterinary use is a dry preparation of a glycoprotein fraction obtained from the serum or plasma of pregnant mares. It has follicle-stimulating and luteinising activities. The potency is not less than 1000 IU of gonadotrophin activity per milligram, calculated with reference to the anhydrous substance.

PRODUCTION

Equine serum gonadotrophin may be prepared by precipitation with alcohol (70 per cent *V/V*) and further purification by a suitable form of chromatography. It is prepared in conditions designed to minimise microbial contamination.

CHARACTERS

Appearance: white or pale grey, amorphous powder.

Solubility: soluble in water.

IDENTIFICATION

When administered as prescribed in the assay it causes an increase in the mass of the ovaries of immature female rats.

TESTS

Water (2.5.12): maximum 10.0 per cent, determined on 80 mg.

Bacterial endotoxins (2.6.14, *method C*): less than 0.035 IU per IU of equine serum gonadotrophin, if intended for use in the manufacture of parenteral dosage forms without a further appropriate procedure for the removal of bacterial endotoxins.

ASSAY

The potency of equine serum gonadotrophin is estimated by comparing under given conditions its effect of increasing the mass of the ovaries of immature female rats with the same effect of the International Standard of equine serum gonadotrophin or of a reference preparation calibrated in International Units.

The International Unit is the activity contained in a stated amount of the International Standard, which consists of a mixture of a freeze-dried extract of equine serum gonadotrophin from the serum of pregnant mares with lactose. The equivalence in International Units of the International Standard is stated by the World Health Organisation.

Use immature female rats of the same strain, 21 to 28 days old, differing in age by not more than 3 days and having masses such that the difference between the heaviest and the lightest rat is not more than 10 g. Assign the rats at random to 6 equal groups of not fewer than 5 animals. If sets of 6 litter mates are available, assign one litter mate from each set to each group and mark according to litter.

Choose 3 doses of the reference preparation and 3 doses of the preparation to be examined such that the smallest dose is sufficient to produce a positive response in some of the rats and the largest dose does not produce a maximal response in all the rats. Use doses in geometric progression: as an initial approximation total doses of 8 IU, 12 IU and 18 IU may be tried, although the dose will depend on the sensitivity of the animals used and may vary widely.

Dissolve separately the total quantities of the preparation to be examined and of the reference preparation corresponding to the doses to be used in sufficient of a sterile 9 g/l solution of *sodium chloride R* containing 1 mg/ml of *bovine albumin R* such that each single dose is administered in a volume of about 0.2 ml. Store the solutions at 5 ± 3 °C.

Inject subcutaneously into each rat the dose allocated to its group. Repeat the injections 18 h, 21 h, 24 h, 42 h and 48 h after the first injection. Not less than 40 h and not more than 72 h after the last injection, kill the rats and remove the ovaries. Remove any extraneous fluid and tissue and weigh the 2 ovaries immediately. Calculate the results by the usual statistical methods, using the combined mass of the 2 ovaries of each animal as the response.

The estimated potency is not less than 80 per cent and not more than 125 per cent of the stated potency. The confidence limits ($P = 0.95$) of the estimated potency are not less than 64 per cent and not more than 156 per cent of the stated potency.

STORAGE

In an airtight container, protected from light, at a temperature not exceeding 8 °C. If the substance is sterile, store in a sterile, airtight, tamper-proof container.

LABELLING

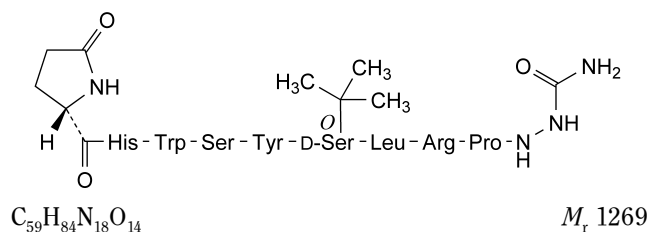
The label states:

- the potency in International Units per milligram,
- where applicable, that the substance is free from bacterial endotoxins.

01/2005:1636

GOSERELIN

Goserelinum



DEFINITION

1-Carbamoyl-2-[5-oxo-L-prolyl-L-histidyl-L-tryptophyl-L-seryl-L-tyrosyl-O-(1,1-dimethylethyl)-D-seryl-L-leucyl-L-arginyl-L-prolyl]diazane.

Synthetic nonapeptide analogue of the hypothalamic decapeptide, gonadorelin. It is obtained by chemical synthesis and is available as an acetate.

Content: 94.5 per cent to 103.0 per cent of the peptide $C_{59}H_{84}N_{18}O_{14}$ (anhydrous and acetic-acid free substance).

CHARACTERS

Appearance: white or almost white powder.

Solubility: soluble in water, freely soluble in glacial acetic acid. It dissolves in dilute solutions of mineral acids and alkali hydroxides.

IDENTIFICATION

A. Nuclear magnetic resonance spectrometry (2.2.33).

Preparation: 40 mg/ml solution of the substance to be examined in *deuterium oxide R* adjusted to pH 4.0 with *deuterated acetic acid R*.

Results: the ^{13}C , proton decoupled NMR spectrum obtained is qualitatively similar to the *Ph. Eur. reference spectrum of goserelin*.

B. Examine the chromatograms obtained in the assay.

Results: the principal peak in the chromatogram obtained with the test solution is similar in retention time and size to the principal peak in the chromatogram obtained with reference solution (a).

C. Amino acid analysis (2.2.56). For protein hydrolysis use Method 1 and for analysis use Method 1.

Express the content of each amino acid in moles. Calculate the relative proportions of the amino acids taking one sixth of the sum of the number of moles of glutamic acid, histidine, tyrosine, leucine, arginine, proline as equal to 1. The values fall within the following

limits: glutamic acid, histidine, tyrosine, leucine, arginine and proline 0.9 to 1.1; serine 1.6 to 2.2. Not more than traces of other amino acids are present, with the exception of tryptophan.

TESTS

Specific optical rotation (2.2.7): -52 to -56 (anhydrous and acetic-acid free substance).

Dissolve the substance to be examined in *water R* to obtain a concentration of 2 mg/ml.

Related substances. Liquid chromatography (2.2.29).

Test solution. Dissolve the substance to be examined in *water R* to obtain a concentration of 1.0 mg/ml.

Reference solution (a). Dissolve the contents of a vial of *goserelin CRS* in *water R* to obtain a concentration of 1.0 mg/ml.

Reference solution (b). Dilute 1.0 ml of the test solution to 100 ml with *water R*.

Reference solution (c). Dilute 1.0 ml of the test solution to 10.0 ml with *water R*.

Resolution solution (a). Dissolve the contents of a vial of *4-D-Ser-goserelin CRS* in *water R* to obtain a concentration of 0.1 mg/ml. Mix equal volumes of this solution and of reference solution (c).

Resolution solution (b). Dissolve the contents of a vial of *goserelin validation mixture CRS* with 1.0 ml of *water R*.

Column:

- **size:** $l = 0.15$ m, $\varnothing = 4.6$ mm,
- **stationary phase:** *octadecylsilyl amorphous organosilica polymer R* (3.5 μ m) with a pore size of 12.5 nm,
- **temperature:** 50-55 °C.

Mobile phase: *trifluoroacetic acid R, acetonitrile for chromatography R, water R* (0.5:200:800 V/V/V).

Flow rate: 0.7-1.2 ml/min.

Detection: spectrophotometer at 220 nm.

Injection: 10 μ l of the test solution, reference solution (b) and the resolution solutions.

Run time: 90 min.

Relative retention with reference to goserelin:
 impurity A = about 0.67; impurity C = about 0.78;
 impurity B = about 0.79; impurity D = about 0.85;
 impurity E = about 0.89; impurity F = about 0.92;
 impurity G = about 0.94; impurity H = about 0.98;
 impurity I = about 1.43; impurity J = about 1.53;
 impurity K = about 1.67; impurity L = about 1.77.

System suitability:

- **retention time:** goserelin = 40 min to 50 min in the chromatogram obtained with resolution solution (b); adjust the flow rate of the mobile phase if necessary; if adjusting the flow rate does not result in a correct retention time of the principal peak, change the composition of acetonitrile in the mobile phase to obtain the requested retention time for goserelin;
- **resolution:** minimum 7.0 between the peaks due to impurity A and goserelin in the chromatogram obtained with resolution solution (a);

- **symmetry factor:** 0.8 to 2.5 for the peaks due to impurity A and goserelin in the chromatogram obtained with resolution solution (a);
- the chromatogram obtained with resolution solution (b) is similar to the chromatogram supplied with *goserelin validation mixture CRS*. 2 peaks eluting prior to the principal peak and corresponding to impurity E and impurity G, are clearly visible. 3 peaks eluting after the principal peak are clearly visible.

Limits:

- **impurity E:** not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (1.0 per cent),
- **any other impurity:** for each impurity, not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent),
- **total:** not more than 2.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (2.5 per cent),
- **disregard limit:** 0.05 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

Acetic acid (2.5.34): 4.5 per cent to 15.0 per cent.

Test solution. Dissolve 10.0 mg of the substance to be examined in a mixture of 5 volumes of mobile phase B and 95 volumes of mobile phase A and dilute to 10.0 ml with the same mixture of mobile phases.

Water (2.5.32): maximum 10.0 per cent.

Bacterial endotoxins (2.6.14): less than 16 IU/mg, if intended for use in the manufacture of parenteral dosage forms without a further appropriate procedure for the removal of bacterial endotoxins.

ASSAY

Liquid chromatography (2.2.29) as described in the test for related substances with the following modifications.

Injection: test solution and reference solution (a).

Run time: 60 min.

Calculate the content of goserelin ($C_{59}H_{84}N_{18}O_{14}$) using the chromatograms obtained with the test solution and reference solution (a) and the declared content of $C_{59}H_{84}N_{18}O_{14}$ in *goserelin CRS*.

STORAGE

In an airtight container, protected from light, at a temperature of 2 °C to 8 °C.

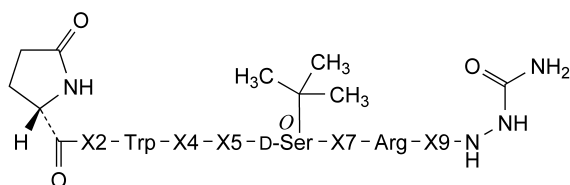
LABELLING

The label states:

- the mass of peptide in the container,
- where applicable, that the substance is free from bacterial endotoxins.

IMPURITIES

Specified impurities: A, B, C, D, E, F, G, H, I, J, K, L.



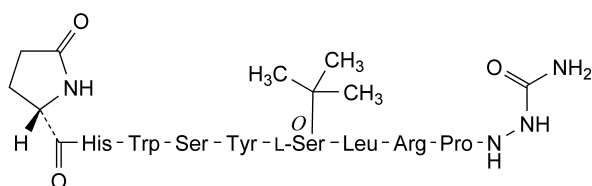
A. X2 = L-His, X4 = D-Ser, X5 = L-Tyr, X7 = L-Leu, X9 = L-Pro: [4-D-serine]goserelin,

C. X2 = L-His, X4 = L-Ser, X5 = L-Tyr, X7 = L-Leu, X9 = D-Pro: [9-D-proline]goserelin,

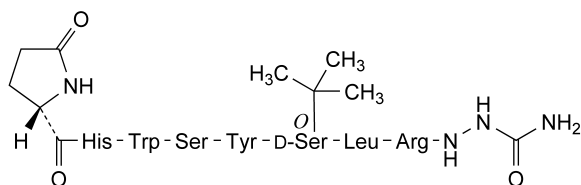
F. X2 = L-His, X4 = L-Ser, X5 = D-Tyr, X7 = L-Leu, X9 = L-Pro: [5-D-tyrosine]goserelin,

G. X2 = D-His, X4 = L-Ser, X5 = L-Tyr, X7 = L-Leu, X9 = L-Pro: [2-D-histidine]goserelin,

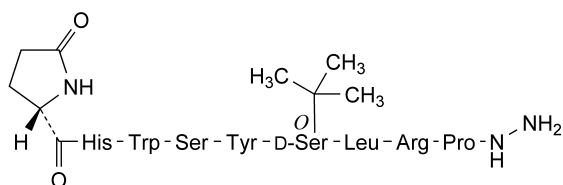
L. X2 = L-His, X4 = L-Ser, X5 = L-Tyr, X7 = D-Leu, X9 = L-Pro: [7-D-leucine]goserelin,



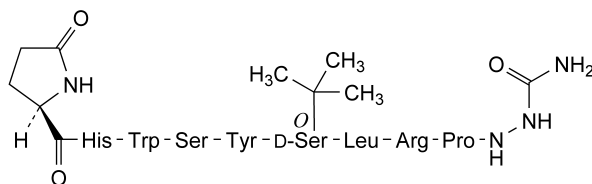
B. [6-[O-(1,1-dimethylethyl)-L-serine]]goserelin,



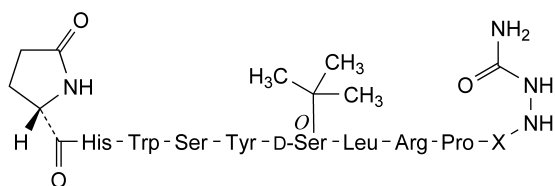
D. 1-carbamoyl-2-[5-oxo-L-prolyl-L-histidyl-L-tryptophyl-L-seryl-L-tyrosyl-O-(1,1-dimethylethyl)-D-seryl-L-leucyl-L-arginyl]diazane,



E. 5-oxo-L-prolyl-L-histidyl-L-tryptophyl-L-seryl-L-tyrosyl-O-(1,1-dimethylethyl)-D-seryl-L-leucyl-L-arginyl-L-prolinohydrazide,

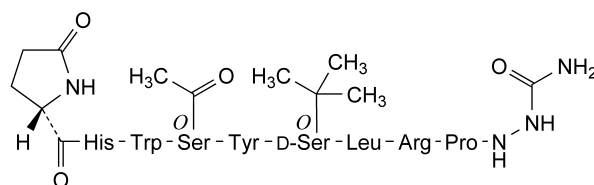


H. [1-(5-oxo-D-proline)]goserelin,



I. X = Pro-Pro: endo-8a,8b-di-L-proline-goserelin,

J. X = Pro: endo-8a-L-proline-goserelin,

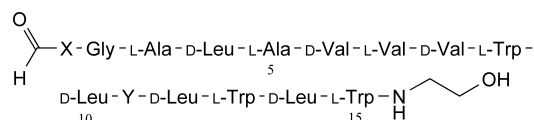


K. O⁴-acetyl-goserelin.

01/2005:0907

GRAMICIDIN

Gramicidinum



Gramicidin	X	Y	Mol. formula	<i>M_r</i>
A1	L-Val	L-Trp	C ₉₉ H ₁₄₀ N ₂₀ O ₁₇	1882
A2	L-Ile	L-Trp	C ₁₀₀ H ₁₄₂ N ₂₀ O ₁₇	1896
B1	L-Val	L-Phe	C ₉₇ H ₁₃₉ N ₁₉ O ₁₇	1843
C1	L-Val	L-Tyr	C ₉₇ H ₁₃₉ N ₁₉ O ₁₈	1859
C2	L-Ile	L-Tyr	C ₉₈ H ₁₄₁ N ₁₉ O ₁₈	1873

DEFINITION

Gramicidin consists of a family of antimicrobial linear polypeptides, usually obtained by extraction from tyrothricin, the complex isolated from the fermentation broth of *Brevibacillus brevis* Dubos. The main component is gramicidin A1, together with gramicidins A2, B1, C1 and C2 in particular.

Content: minimum 900 IU/mg (dried substance).

CHARACTERS

Appearance: white or almost white, crystalline powder, slightly hygroscopic.

Solubility: practically insoluble in water, soluble in methanol, sparingly soluble in alcohol.

mp: about 230 °C.

IDENTIFICATION

First identification: A, C.

Second identification: A, B.

A. Dissolve 0.100 g in *alcohol R* and dilute to 100.0 ml with the same solvent. Dilute 5.0 ml of this solution to 100.0 ml with *alcohol R*. Examined between 240 nm and 320 nm (2.2.25), the solution shows 2 absorption maxima, at 282 nm and 290 nm, a shoulder at about 275 nm and an absorption minimum at 247 nm. The specific absorbance at the maximum at 282 nm is 105 to 125.

B. Thin-layer chromatography (2.2.27).

Test solution. Dissolve 5 mg of the substance to be examined in 6.0 ml of *alcohol R*.

Reference solution (a). Dissolve 5 mg of *gramicidin CRS* in 6.0 ml of *alcohol R*.

Reference solution (b). Dissolve 5 mg of *tyrothricin CRS* in 6.0 ml of *alcohol R*.

Plate: TLC silica gel plate *R*.

Mobile phase: *methanol R*, *butanol R*, *water R*, *glacial acetic acid R*, *butyl acetate R* (3:9:15:24:49 V/V/V/V/V).

Application: 1 µl.

Development: over 2/3 of the plate.

Drying: in air.

Detection: dip the plate into *dimethylaminobenzaldehyde solution R2*. Heat at 90 °C until the spots appear.

System suitability: the chromatogram obtained with reference solution (b) shows 2 clearly separated spots or 2 clearly separated groups of spots.

Results: the principal spot or group of principal spots in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot or group of principal spots in the chromatogram obtained with reference solution (a) and to the spot or group of spots with the highest R_f value in the chromatogram obtained with reference solution (b).

- C. Examine the chromatograms obtained in the test for composition.

Results: the 3 principal peaks in the chromatogram obtained with the test solution are similar in retention time to the 3 principal peaks in the chromatogram obtained with reference solution (a).

TESTS

Composition. Liquid chromatography (2.2.29): use the normalisation procedure.

Test solution. Dissolve 25 mg of the substance to be examined in 10 ml of *methanol R* and dilute to 25 ml with the mobile phase.

Reference solution (a). Dissolve 25 mg of *gramicidin CRS* in 10 ml of *methanol R* and dilute to 25 ml with the mobile phase.

Reference solution (b). Dilute 1.0 ml of reference solution (a) to 50.0 ml with the mobile phase. Dilute 1.0 ml of this solution to 10.0 ml with the mobile phase.

Column:

- **size:** $l = 0.25$ m, $\varnothing = 4.6$ mm,
- **stationary phase:** *base-deactivated end-capped octadecylsilyl silica gel for chromatography R* (5 µm),
- **temperature:** 50 °C.

Mobile phase: *water R*, *methanol R* (29:71 V/V).

Flow rate: 1.0 ml/min.

Detection: spectrophotometer at 282 nm.

Injection: 20 µl.

Run time: 2.5 times the retention time of *gramicidin A1*.

Relative retention with reference to *gramicidin A1* (retention time = about 22 min): *gramicidin C1* = about 0.7; *gramicidin C2* = about 0.8; *gramicidin A2* = about 1.2; *gramicidin B1* = about 1.9.

System suitability: reference solution (a):

- **resolution:** minimum 1.5 between the peaks due to *gramicidin A1* and *gramicidin A2*,
- the chromatogram obtained is concordant with the chromatogram supplied with *gramicidin CRS*.

Composition:

- **sum of the contents of *gramicidins A1*, *A2*, *B1*, *C1* and *C2*:** minimum 95.0 per cent,
- **ratio of the content of *gramicidin A1* to the sum of the contents of *gramicidins A1*, *A2*, *B1*, *C1* and *C2*:** minimum 60.0 per cent,
- **disregard limit:** the area of the peak due to *gramicidin A1* in the chromatogram obtained with reference solution (b).

Related substances. Liquid chromatography (2.2.29) as described in the test for composition.

Limit:

- **any impurity:** maximum 2.0 per cent and not more than 1 peak is more than 1.0 per cent; disregard the peaks due to *gramicidins A1*, *A2*, *B1*, *C1* and *C2*.

Loss on drying (2.2.32): maximum 3.0 per cent, determined on 1.000 g by drying over *diphosphorus pentoxide R* at 60 °C at a pressure not exceeding 0.1 kPa for 3 h.

Sulphated ash (2.4.14): maximum 1.0 per cent, determined on 1.0 g.

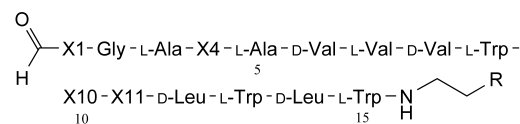
ASSAY

Carry out the microbiological assay of antibiotics (2.7.2), using the turbidimetric method. Use *gramicidin CRS* as the reference substance.

STORAGE

In an airtight container, protected from light.

IMPURITIES



Impurity	X1	X4	X10	X11	R
A	L-Val	Met	D-Leu	L-Trp	OH
B	L-Val	D-Leu	D-Leu	L-Trp	CH ₂ -OH
C	L-Ile	D-Leu	D-Leu	L-Phe	OH
D	L-Val	D-Leu	Met	L-Tyr	OH
E	L-Ile	D-Leu	D-Leu	L-Trp	CH ₂ -OH

- A. [4-methionine]*gramicidin A1*,
 B. *gramicidin A1* 3-hydroxypropyl,
 C. *gramicidin B2*,
 D. [10-methionine]*gramicidin C1*,
 E. *gramicidin A2* 3-hydroxypropyl.

01/2005:1861

GREATER CELANDINE

Chelidonii herba

DEFINITION

Dried, whole or cut aerial parts of *Chelidonium majus* L. collected during flowering.

Content: minimum 0.6 per cent of total alkaloids, expressed as *chelidonine* (C₂₀H₁₉NO₅; M_r 353.4) (dried drug).

CHARACTERS

Macroscopic and microscopic characters described under identification tests A and B.

IDENTIFICATION

- A. The stems are rounded, ribbed, yellowish to greenish-brown, somewhat pubescent, about 3 mm to 7 mm in diameter, hollow and mostly collapsed. The leaves are thin, irregularly pinnate, the leaflets ovate to oblong with coarsely dentate margins, the terminal leaflet often three-lobed; the adaxial surface is bluish-green and glabrous, the abaxial surface paler and pubescent,

especially on the veins. The flowers have 2 deeply concavo-convex sepals, readily removed, and 4 yellow, broadly ovate, spreading petals about 8 mm to 10 mm long; the stamens are numerous, yellow, and a short style arises from a superior ovary; long, capsular, immature fruits are rarely present.

- B. Reduce to a powder (355). The powder is dark greyish-green to brownish-green. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters: numerous fragments of leaves in surface view, the epidermal cells with sinuous walls; anomocytic stomata (2.8.3) occur on the abaxial surface only; covering trichomes long, uniseriate, with thin walls and usually fragmented; vascular tissue from the leaves and stems with groups of fibres, pitted and spirally thickened vessels and associated latex tubes with yellowish-brown contents; occasional fragments of the corolla with thin-walled, partly papillose cells containing numerous pale yellow droplets of oil; spherical pollen grains about 30 µm to 40 µm in diameter with 3 pores and a finely pitted exine.

- C. Thin-layer chromatography (2.2.27).

Test solution. To 0.4 g of the powdered drug (710) add 50 ml of *dilute acetic acid R*. Boil the mixture under a reflux condenser in a water-bath for 30 min. Cool and filter. To the filtrate add *concentrated ammonia R* until a strong alkaline reaction is produced. Shake with 30 ml of *methylene chloride R*. Dry the organic layer over *anhydrous sodium sulphate R*, filter and evaporate *in vacuo* to dryness. Dissolve the residue in 1.0 ml of *methanol R*.

Reference solution. Dissolve 2 mg of *papaverine hydrochloride R* and 2 mg of *methyl red R* in 10 ml of *alcohol R*.

Plate: TLC silica gel plate *R*.

Mobile phase: *anhydrous formic acid R*, *water R*, *propanol R* (1:9:90 V/V/V).

Application: 10 µl as bands.

Development: over a path of 10 cm.

Drying: in air.

Detection: spray with *potassium iodobismuthate solution R* and dry the plate in air; spray with *sodium nitrite solution R* and allow the plate to dry in air; examine in daylight.

Results: see below the sequence of the zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other weaker zones may be present in the chromatogram obtained with the test solution.

Top of the plate	
Methyl red: a red zone	A brown zone
	A brown zone
Papaverine: a greyish-brown zone	A greyish-brown zone
	A brown zone
	A brown zone
Reference solution	Test solution

TESTS

Foreign matter (2.8.2): maximum 10 per cent.

Loss on drying (2.2.32): maximum 10.0 per cent, determined on 1.000 g of the powdered drug (355) by drying in an oven at 100-105 °C for 2 h.

Total ash (2.4.16): maximum 13.0 per cent.

ASSAY

Test solution. To 0.750 g of the powdered drug (710), add 200 ml of *dilute acetic acid R* and heat on a water-bath for 30 min, shaking frequently. Cool and dilute to 250.0 ml with *dilute acetic acid R*. Filter. Discard the first 20 ml of the filtrate. To 30.0 ml of the filtrate add 6.0 ml of *concentrated ammonia R* and 100.0 ml of *methylene chloride R*. Shake for 30 min. Separate the organic layer, place 50.0 ml in a 100 ml round-bottomed flask and evaporate to dryness *in vacuo* at a temperature not exceeding 40 °C. Dissolve the residue in about 2-3 ml of *alcohol R*, warming slightly. Transfer the solution to a 25 ml volumetric flask by rinsing the round-bottomed flask with *dilute sulphuric acid R* and dilute to 25.0 ml with the same solvent. To 5.0 ml of the solution, add 5.0 ml of a 10 g/l solution of *chromotropic acid, sodium salt R* in *sulphuric acid R* in a 25 ml volumetric flask, stopper the flask and mix carefully. Dilute to 25.0 ml with *sulphuric acid R* and stopper the flask.

Compensation solution. At the same time and in the same manner, place in a 25 ml volumetric flask 5.0 ml of *dilute sulphuric acid R* and 5.0 ml of a 10 g/l solution of *chromotropic acid, sodium salt R* in *sulphuric acid R*, stopper the flask and mix carefully. Dilute to 25.0 ml with *sulphuric acid R* and stopper the flask.

Place both solutions on a water-bath for 10 min. Cool to about 20 °C and dilute if necessary to 25.0 ml with *sulphuric acid R*. Measure the absorbance (2.2.25) of the test solution at 570 nm.

Calculate the percentage content of total alkaloids, expressed as chelidonine, from the expression:

$$\frac{A \times 2.23}{m}$$

i.e. taking the specific absorbance of chelidonine to be 933.

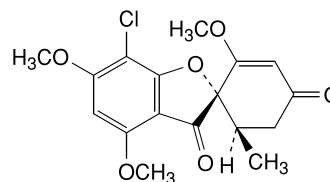
A = absorbance at 570 nm,

m = mass of the substance to be examined, in grams.

01/2005:0182

GRISEOFULVIN

Griseofulvinum



$C_{17}H_{17}ClO_6$

M_r 352.8

DEFINITION

Griseofulvin is (1'S,3-6'R)-7-chloro-2',4,6-trimethoxy-6'-methylspiro[benzofuran-2(3H),1'-[2]cyclohexene]-3,4'-dione, a substance produced by the growth of certain strains of *Penicillium griseofulvum* or obtained by any other means. It contains not less than 97.0 per cent and not more than the equivalent of 102.0 per cent of $C_{17}H_{17}ClO_6$, calculated with reference to the dried substance.

CHARACTERS

A white or yellowish-white, microfine powder, the particles of which are generally up to 5 µm in maximum dimension although larger particles which may occasionally exceed 30 µm may be present, tasteless, practically insoluble in water, freely soluble in dimethylformamide and in tetrachloroethane, slightly soluble in ethanol and in methanol.

It melts at about 220 °C.

IDENTIFICATION

- Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *griseofulvin CRS*.
- Dissolve about 5 mg in 1 ml of *sulphuric acid R* and add about 5 mg of powdered *potassium dichromate R*. A wine-red colour develops.

TESTS

Appearance of solution. Dissolve 0.75 g in *dimethylformamide R* and dilute to 10 ml with the same solvent. The solution is clear (2.2.1) and not more intensely coloured than reference solution Y₄ (2.2.2, *Method II*).

Acidity. Suspend 0.25 g in 20 ml of *alcohol R* and add 0.1 ml of *phenolphthalein solution R*. Not more than 1.0 ml of 0.02 M *sodium hydroxide* is required to change the colour of the indicator.

Specific optical rotation (2.2.7). Dissolve 0.250 g in *dimethylformamide R* and dilute to 25.0 ml with the same solvent. The specific optical rotation is + 354 to + 364, calculated with reference to the dried substance.

Related substances. Examine by gas chromatography (2.2.28), using *diphenylanthracene R* as the internal standard.

Internal standard solution. Dissolve 0.2 g of *diphenylanthracene R* in *acetone R* and dilute to 100.0 ml with the same solvent.

Test solution (a). Dissolve 0.10 g of the substance to be examined in *acetone R* and dilute to 10.0 ml with the same solvent.

Test solution (b). Dissolve 0.10 g of the substance to be examined in *acetone R*, add 1.0 ml of the internal standard solution and dilute to 10.0 ml with *acetone R*.

Reference solution. Dissolve 5.0 mg of *griseofulvin CRS* in *acetone R*, add 1.0 ml of the internal standard solution and dilute to 10.0 ml with *acetone R*.

The chromatographic procedure may be carried out using:

- a glass column 1 m long and 4 mm in internal diameter packed with *diatomaceous earth for gas chromatography R* impregnated with 1 per cent *m/m* of *poly[(cyanopropyl)(methyl)][(phenyl)(methyl)siloxane R*,
- *nitrogen for chromatography R* as the carrier gas at a flow rate of 50-60 ml/min,
- a flame-ionisation detector.

Maintain the temperature of the column at 250 °C, that of the injection port at 270 °C and that of the detector at 300 °C. Continue the chromatography for three times the period of time required for the appearance of the peak corresponding to *griseofulvin* which is about 11 min. For the chromatogram obtained with the reference solution, determine the ratio of the area of the peak corresponding to *griseofulvin* to the area of the peak corresponding to the internal standard. For the chromatogram obtained with test solution (b), determine the ratio of the area of the peak corresponding

to dechloro-*griseofulvin* (distance t_R about 0.6 times that of *griseofulvin*) to the area of the peak corresponding to the internal standard, determine also the ratio of the area of the peak corresponding to dehydro-*griseofulvin* (distance t_R about 1.4 times that of *griseofulvin*) to the area of the peak corresponding to the internal standard.

The ratios calculated from the chromatogram obtained with test solution (b) divided by the ratio calculated from the chromatogram obtained with the reference solution are less than 0.6 for dechloro-*griseofulvin* and less than 0.15 for dehydro-*griseofulvin*.

Substances soluble in light petroleum. Shake 1.0 g with 20 ml of *light petroleum R*. Boil under a reflux condenser for 10 min. Cool, filter and wash with three quantities, each of 15 ml, of *light petroleum R*. Combine the filtrate and washings, evaporate to dryness on a water-bath and dry at 100 °C to 105 °C for 1 h. The residue weighs not more than 2 mg (0.2 per cent).

Loss on drying (2.2.32). Not more than 1.0 per cent, determined on 1.00 g by drying in an oven at 100 °C to 105 °C.

Sulphated ash (2.4.14). Not more than 0.2 per cent, determined on 1.0 g.

Abnormal toxicity. To each of five healthy mice, each weighing 17 g to 22 g, administer orally a suspension of 0.1 g of the substance to be examined in 0.5 ml to 1 ml of *water R*. None of the mice dies within 48 h.

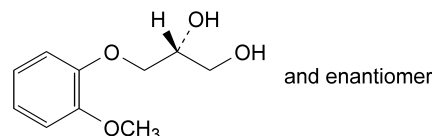
ASSAY

Dissolve 80.0 mg in *ethanol R* and dilute to 200.0 ml with the same solvent. Dilute 2.0 ml of the solution to 100.0 ml with *ethanol R*. Measure the absorbance (2.2.25) at the maximum at 291 nm. Calculate the content of C₁₇H₁₇ClO₆, taking the specific absorbance to be 686.

01/2005:0615
corrected

GUAIFENESIN

Guaifenesinum



C₁₀H₁₄O₄

M_r 198.2

DEFINITION

(2*RS*)-3-(2-Methoxyphenoxy)propane-1,2-diol.

Content: 98.0 per cent to 102.0 per cent (dried substance).

CHARACTERS

Appearance: white or almost white, crystalline powder.

Solubility: sparingly soluble in water, soluble in alcohol.

IDENTIFICATION

First identification: B.

Second identification: A, C.

A. Melting point (2.2.14): 79 °C to 83 °C.

B. Infrared absorption spectrophotometry (2.2.24).

Comparison: *guaifenesin CRS*.

C. Thin-layer chromatography (2.2.27).

Test solution. Dissolve 30 mg of the substance to be examined in *methanol R* and dilute to 10 ml with the same solvent.

Reference solution. Dissolve 30 mg of *guaifenesin CRS* in *methanol R* and dilute to 10 ml with the same solvent.

Plate: *TLC silica gel G plate R*.

Mobile phase: *methylene chloride R, propanol R* (20:80 V/V).

Application: 5 µl.

Development: over 2/3 of the plate.

Drying: in air.

Detection: spray with a mixture of equal volumes of a 10 g/l solution of *potassium ferricyanide R*, a 200 g/l solution of *ferric chloride R* and *alcohol R*.

Results: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

TESTS

Solution S. Dissolve 1.0 g in *carbon dioxide-free water R*, heating gently if necessary, and dilute to 50 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and colourless (2.2.2, *Method II*).

Acidity or alkalinity. To 10 ml of solution S add 0.05 ml of *phenolphthalein solution R1*. Not more than 0.1 ml of 0.01 M *sodium hydroxide* is required to change the colour of the indicator. To 10 ml of solution S add 0.15 ml of *methyl red solution R*. Not more than 0.1 ml of 0.01 M *hydrochloric acid* is required to change the colour of the indicator to red.

Related substances. Liquid chromatography (2.2.29).

Test solution. Dissolve 0.100 g of the substance to be examined in *acetonitrile R* and dilute to 50.0 ml with the same solvent.

Reference solution (a). Dilute 1.0 ml of the test solution to 20.0 ml with *acetonitrile R*. Dilute 1.0 ml of this solution to 10.0 ml with *acetonitrile R*.

Reference solution (b). Dissolve 10.0 mg of *guaiacol R* in *acetonitrile R* and dilute to 50.0 ml with the same solvent. Dilute 0.5 ml of this solution to 50.0 ml with *acetonitrile R*.

Reference solution (c). Dissolve 50.0 mg of *guaiacol R* in *acetonitrile R* and dilute to 50.0 ml with the same solvent. Dilute 5.0 ml of this solution to 10.0 ml with the test solution.

Column:

- size: $l = 0.25$ m, $\varnothing = 4.6$ mm,
- stationary phase: *octadecylsilyl silica gel for chromatography R* (5 µm).

Mobile phase:

- mobile phase A: *glacial acetic acid R, water R* (10:990 V/V),
- mobile phase B: *acetonitrile R*,

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)
0 - 32	80 → 50	20 → 50
32 - 33	50 → 80	50 → 20
33 - 40	80	20

Flow rate: 1 ml/min.

Detection: spectrophotometer at 276 nm.

Injection: 10 µl.

Relative retention with reference to *guaifenesin* (retention time = about 8 min): impurity B = about 0.9; impurity A = about 1.4; impurity C = about 3.1; impurity D = about 3.7.

System suitability: reference solution (c):

- resolution: minimum 3.0 between the peaks due to *guaifenesin* and impurity A.

Limits:

- impurity A: not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent),
- impurity B: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (1.0 per cent),
- any other impurity: not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent),
- total (excluding impurity B): not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (1.0 per cent),
- disregard level: 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

Chlorides and monochlorhydrins: maximum of 250 ppm.

To 10 ml of solution S add 2 ml of *dilute sodium hydroxide solution R* and heat on a water-bath for 5 min. Cool and add 3 ml of *dilute nitric acid R*. The resulting solution complies with the limit test for chlorides (2.4.4).

Heavy metals (2.4.8): maximum of 25 ppm.

Dissolve 2.0 g in a mixture of 1 volume of *water R* and 9 volumes of *alcohol R* and dilute to 25 ml with the same mixture of solvents. 12 ml of the solution complies with limit test B. Prepare the standard using lead standard solution (2 ppm Pb) prepared by diluting *lead standard solution (100 ppm Pb) R* with a mixture of 1 volume of *water R* and 9 volumes of *alcohol R*.

Loss on drying (2.2.32): maximum 0.5 per cent, determined on 1.000 g by drying *in vacuo* at 60 °C for 3 h.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

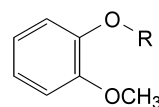
ASSAY

To 0.500 g (m g) add 10.0 ml of a freshly prepared mixture of 1 volume of *acetic anhydride R* and 7 volumes of *pyridine R*. Boil under a reflux condenser for 45 min. Cool and add 25 ml of *water R*. Using 0.25 ml of *phenolphthalein solution R* as indicator, titrate with 1 M *sodium hydroxide* (n_1 ml). Carry out a blank titration (n_2 ml).

Calculate the percentage content of $C_{10}H_{14}O_4$ from the expression:

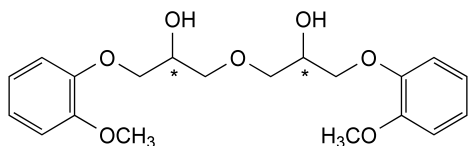
$$\frac{19.82 (n_2 - n_1)}{2m}$$

IMPURITIES

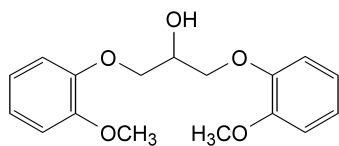


A. R = H: 2-methoxyphenol (*guaiacol*),

B. R = $CH(CH_2OH)_2$: 2-(2-methoxyphenoxy)propane-1,3-diol (B-isomer),



C. 1,1'-oxybis[3-(2-methoxyphenoxy)propan-2-ol] (bisether),

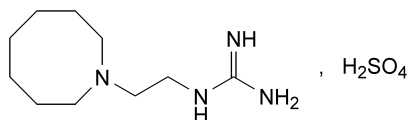


D. 1,3-bis(2-methoxyphenoxy)propan-2-ol.

01/2005:0027

GUANETHIDINE MONOSULPHATE

Guanethidini monosulfas

 $C_{10}H_{24}N_4O_4S$ M_r 296.4

DEFINITION

Guanethidine monosulphate contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of 1-[2-(hexahydroazocin-1(2*H*)-yl)ethyl]guanidine monosulphate, calculated with reference to the dried substance.

CHARACTERS

A colourless, crystalline powder, freely soluble in water, practically insoluble in alcohol.

It melts at about 250 °C, with decomposition.

IDENTIFICATION

- Dissolve about 25 mg in 25 ml of *water R*, add 20 ml of *picric acid solution R* and filter. The precipitate, washed with *water R* and dried at 100 °C to 105 °C, melts (2.2.14) at about 154 °C.
- Dissolve about 25 mg in 5 ml of *water R*. Add 1 ml of *strong sodium hydroxide solution R*, 1 ml of *α-naphthol solution R* and, dropwise with shaking, 0.5 ml of *strong sodium hypochlorite solution R*. A bright pink precipitate is formed and becomes violet-red on standing.
- It gives the reactions of sulphates (2.3.1).

TESTS

Solution S. Dissolve 0.4 g in *carbon dioxide-free water R* and dilute to 20 ml with the same solvent.

Appearance of solution. Solution S is not more intensely coloured than reference solution GY₆ (2.2.2, *Method II*).

pH (2.2.3). The pH of solution S is 4.7 to 5.5.

Oxidisable substances. In a conical, ground-glass-stoppered flask, dissolve 1.0 g in 25 ml of *water R* and add 25 ml of *dilute sodium hydroxide solution R*. Allow to stand for 10 min and add 1 g of *potassium bromide R* and 1 ml of 0.0083 *M* *potassium bromate*. Acidify with 30 ml of *dilute hydrochloric acid R*. Mix and allow to stand in the dark for 5 min. Add 2 g of *potassium iodide R* and shake. Allow to stand for 2 min and titrate the liberated iodine with 0.05 *M*

sodium thiosulphate, using *starch solution R* as indicator. Not less than 0.3 ml of 0.05 *M* *sodium thiosulphate* is required to decolorise the solution.

Heavy metals (2.4.8). 2.0 g complies with limit test C (10 ppm). Prepare the standard using 2 ml of *lead standard solution (10 ppm Pb) R*.

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.00 g by drying in an oven at 100 °C to 105 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.250 g, warming if necessary, in 30 ml of *anhydrous acetic acid R* and add 15 ml of *acetic anhydride R*. Titrate with 0.1 *M* *perchloric acid*, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 *M* *perchloric acid* is equivalent to 29.64 mg of $C_{10}H_{24}N_4O_4S$.

STORAGE

Protected from light.

01/2005:1218

GUAR

Cyamopsidis seminis pulvis

DEFINITION

Guar is obtained by grinding the endosperms of seeds of *Cyamopsis tetragonolobus* (L.) Taub. It consists mainly of guar galactomannan.

CHARACTERS

A white or almost white powder, yielding a mucilage of variable viscosity when dissolved in water, practically insoluble in alcohol.

It has the microscopic characters described under Identification test A.

IDENTIFICATION

- Examined under a microscope in *glycerol R*, the substance to be examined (125) shows pyriform to ovoid cells, usually isolated, having very thick walls around a central somewhat elongated lumen with granular contents, and smaller polyhedral cells, isolated or in clusters, with thinner walls.
- In a conical flask place 2 g, add rapidly 45 ml of *water R* and stir vigorously for 30 s. After 5-10 min a stiff gel forms which does not flow when the flask is inverted.
- Mix a suspension of 0.1 g in 10 ml of *water R* with 1 ml of a 10 g/l solution of *disodium tetraborate R*; the mixture soon gels.
- Examine by thin-layer chromatography (2.2.27), using a suitable silica gel as the coating substance.
Test solution. To 10 mg in a thick-walled, centrifuge test tube add 2 ml of a 100 g/l solution of *trifluoroacetic acid R*, shake vigorously to dissolve the forming gel, stopper the test tube and heat the mixture at 120 °C for 1 h. Centrifuge the hydrolysate, transfer the clear supernatant liquid carefully into a 50 ml flask, add 10 ml of *water R* and evaporate the solution to dryness under reduced pressure. To the resulting clear film add 0.1 ml of *water R* and 0.9 ml of *methanol R*. Centrifuge to separate the amorphous precipitate. Dilute the supernatant liquid, if necessary, to 1 ml with *methanol R*.

Reference solution. Dissolve 10 mg of *galactose R*, 10 mg of *mannose R* in 2 ml of *water R* and dilute to 20 ml with *methanol R*.

Apply separately to the plate as bands 5 µl of each solution. Develop over a path of 15 cm using a mixture of 15 volumes of *water R* and 85 volumes of *acetonitrile R*. Spray with *aminohippuric acid reagent R* and dry the plate at 120 °C for 5 min. The chromatogram obtained with the reference solution shows in the lower part, two clearly separated brownish zones (galactose and mannose in order of increasing R_f value). The chromatogram obtained with the test solution shows two zones corresponding to galactose and mannose.

TESTS

Tragacanth, sterculia gum, agar, alginates, carrageenan.

To a small amount of the substance to be examined add 0.2 ml of freshly prepared *ruthenium red solution R*. Examined under a microscope the cell walls do not stain red.

Protein. Not more than 8.0 per cent. Carry out the determination of nitrogen by the method of sulphuric acid digestion (2.5.9) using 0.170 g. Multiply the result by 6.25.

Apparent viscosity. Moisten 1.00 g calculated with reference to the dried substance with 2.5 ml of *2-propanol R*. While stirring, dilute to 100.0 ml with *water R*. After 1 h, determine the viscosity (2.2.10) using a rotating viscometer at 20 °C and a shear rate of 100 s⁻¹. The apparent viscosity is not less than 85 per cent and not more than 115 per cent of the value stated on the label.

Loss on drying (2.2.32). Not more than 15.0 per cent, determined on 1.000 g by drying in an oven at 100-105 °C for 5 h.

Total ash (2.4.16). Not more than 1.8 per cent.

Microbial contamination. Total viable aerobic count (2.6.12) not more than 10⁴ micro-organisms per gram, determined by plate count. It complies with the tests for *Escherichia coli* and for *Salmonella* (2.6.13).

LABELLING

The label states the apparent viscosity in millipascal seconds for a 10 g/l solution.

01/2005:0908

GUAR GALACTOMANNAN

Guar galactomannanum

DEFINITION

Guar galactomannan is obtained from the seeds of *Cyamopsis tetragonolobus* (L.) Taub. by grinding of the endosperms and subsequent partial hydrolysis. The main components are polysaccharides composed of D-galactose and D-mannose at molecular ratios of 1:1.4 to 1:2. The molecules consist of a linear main chain of β-(1→4)-glycosidically linked mannopyranoses and single α-(1→6)-glycosidically linked galactopyranoses.

CHARACTERS

A yellowish-white powder, soluble in cold water and in hot water, practically insoluble in organic solvents.

IDENTIFICATION

A. Mix 5 g of solution S (see Tests) with 0.5 ml of a 10 g/l solution of *disodium tetraborate R*. A gel forms within a short time.

B. Heat 20 g of solution S in a water-bath for 10 min. Allow to cool and adjust to the original mass with *water R*. The solution does not gel.

C. Examine by thin-layer chromatography (2.2.27), using *silica gel G R* as the coating substance.

Test solution. To 10 mg of the substance to be examined in a thick-walled centrifuge tube add 2 ml of a 230 g/l solution of *trifluoroacetic acid R*, shake vigorously to dissolve the forming gel, stopper the test tube and heat the mixture at 120 °C for 1 h. Centrifuge the hydrolysate, transfer the clear supernatant liquid carefully into a 50 ml flask, add 10 ml of *water R* and evaporate the solution to dryness under reduced pressure. Take up the residue in 10 ml of *water R* and evaporate again to dryness under reduced pressure. To the resulting clear film, which has no odour of acetic acid, add 0.1 ml of *water R* and 1 ml of *methanol R*. Centrifuge to separate the amorphous precipitate. Dilute the supernatant liquid, if necessary, to 1 ml with *methanol R*.

Reference solution. Dissolve 10 mg of *galactose R* and 10 mg of *mannose R* in 2 ml of *water R* and dilute to 10 ml with *methanol R*.

Apply separately to the plate, as bands 20 mm by 3 mm, 5 µl of each solution. Develop over a path of 15 cm using a mixture of 15 volumes of *water R* and 85 volumes of *acetonitrile R*. Spray with *aminohippuric acid reagent R* and heat at 120 °C for 5 min. The chromatogram obtained with the reference solution shows in the lower part two clearly separated brownish zones (galactose and mannose in order of increasing R_f value). The chromatogram obtained with the test solution shows two zones corresponding to galactose and mannose.

TESTS

Solution S. Moisten 1.0 g with 2 ml of *2-propanol R*. While stirring, dilute with *water R* to 100 g and stir until the substance is uniformly dispersed. Allow to stand for at least 1 h. If the apparent viscosity is below 200 mPas, use 3.0 g of substance instead of 1.0 g.

pH (2.2.3). The pH of solution S is 5.5 to 7.5.

Apparent viscosity. Moisten a quantity of the substance to be examined equivalent to 2.00 g of the dried substance with 2.5 ml of *2-propanol R* and, while stirring, dilute to 100.0 ml with *water R*. After 1 h, determine the viscosity (2.2.10) using a rotating viscometer at 20 °C and a shear rate of 100 s⁻¹. The apparent viscosity is not less than 75 per cent and not more than 140 per cent of the value stated on the label.

Insoluble matter. In a 250 ml flask disperse, while stirring, 1.50 g in a mixture of 1.6 ml of *sulphuric acid R* and 150 ml of *water R* and weigh. Immerse the flask in a water-bath and heat under a reflux condenser for 6 h. Adjust to the original mass with *water R*. Filter the hot solution through a tared, sintered-glass filter (160). Rinse the filter with hot *water R* and dry at 100 °C to 105 °C. The residue weighs not more than 105 mg (7.0 per cent).

Protein. Not more than 5.0 per cent. Carry out the determination of nitrogen by sulphuric acid digestion (2.5.9), using 0.400 g of substance. Multiply the result by 6.25.

Tragacanth, sterculia gum, agar, alginates and carrageenan. To a small amount of the substance to be examined, add 0.2 ml of freshly prepared *ruthenium red solution R*. Examined under a microscope, none of the structures are stained red.

Loss on drying (2.2.32). Not more than 15.0 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C for 5 h.

Total ash (2.4.16). Not more than 1.8 per cent, determined on 1.00 g after wetting with 10 ml of *water R*.

Microbial contamination. Total viable aerobic count (2.6.12) not more than 10^3 micro-organisms per gram, determined by plate-count. It complies with the tests for *Escherichia coli* and for *Salmonella* (2.6.13).

LABELLING

The label states the apparent viscosity in millipascal seconds for a 20 g/l solution.

01/2005:1167

HAEMODIALYSIS SOLUTIONS, CONCENTRATED, WATER FOR DILUTING

Aqua ad dilutionem solutionium concentratarum ad haemodialysim

The following monograph is given for information.

The analytical methods described and the limits proposed are intended to be used for validating the procedure for obtaining the water.

DEFINITION

Water for diluting concentrated haemodialysis solutions is obtained from potable water by distillation, by reverse osmosis, by ion exchange or by any other suitable method. The conditions of preparation, transfer and storage are designed to minimise the risk of chemical and microbial contamination.

When water obtained by one of the methods described above is not available, potable water may be used for home dialysis. Because the chemical composition of potable water varies considerably from one locality to another, consideration must be given to its chemical composition to enable adjustments to be made to the content of ions so that the concentrations in the diluted solution correspond to the intended use.

Attention has also to be paid to the possible presence of residues from water treatment (for example, chloramines) and volatile halogenated hydrocarbons.

For the surveillance of the quality of water for diluting concentrated haemodialysis solutions, the following methods may be used to determine the chemical composition and/or to detect the presence of possible contaminants together with suggested limits to be obtained.

CHARACTERS

Clear, colourless, tasteless liquid.

TESTS

Acidity or alkalinity. To 10 ml of the water to be examined, freshly boiled and cooled in a borosilicate glass flask, add 0.05 ml of *methyl red solution R*. The solution is not red. To 10 ml of the water to be examined add 0.1 ml of *bromothymol blue solution R1*. The solution is not blue.

Oxidisable substances. To 100 ml of the water to be examined add 10 ml of *dilute sulphuric acid R* and 0.1 ml of 0.02 M *potassium permanganate* and boil for 5 min. The solution remains faintly pink.

Total available chlorine: maximum 0.1 ppm.

In a 125 ml test-tube (A), place successively 5 ml of *buffer solution pH 6.5 R*, 5 ml of *diethylphenylenediamine sulphate solution R* and 1 g of *potassium iodide R*. In a second 125 ml test-tube (B), place successively 5 ml of *buffer solution pH 6.5 R* and 5 ml of *diethylphenylenediamine sulphate solution R*. Add as simultaneously as possible to tube A 100 ml of the water to be examined and to tube B a reference solution prepared as follows: to 1 ml of a 10 mg/l solution of *potassium iodate R*, add 1 g of *potassium iodide R* and 1 ml of *dilute sulphuric acid R*; allow to stand for 1 min, add 1 ml of *dilute sodium hydroxide solution R* and dilute to 100 ml with *water R*. Any colour in the mixture obtained with the water to be examined is not more intense than that in the mixture obtained with the reference solution.

Chlorides (2.4.4): maximum 50 ppm.

Dilute 1 ml of the water to be examined to 15 ml with *water R*. The solution complies with the limit test for chlorides.

Fluorides: maximum 0.2 ppm.

Potentiometry (2.2.36, *Method I*): use as indicator electrode a fluoride-selective solid-membrane electrode and as reference electrode a silver-silver chloride electrode.

Test solution. The water to be examined.

Reference solutions. Dilute 2.0 ml, 4.0 ml and 10.0 ml of *fluoride standard solution (1 ppm F) R* respectively to 20.0 ml with *total-ionic-strength-adjustment buffer R1*.

Carry out the measurement of each solution.

Nitrates: maximum 2 ppm.

Dilute 2 ml of the water to be examined to 100 ml with *nitrate-free water R*. Place 5 ml of the dilution in a test-tube immersed in iced water, add 0.4 ml of a 100 g/l solution of *potassium chloride R* and 0.1 ml of *diphenylamine solution R* and then, dropwise and with shaking, 5 ml of *sulphuric acid R*. Transfer the tube to a water-bath at 50 °C. Allow to stand for 15 min. Any blue colour in the solution is not more intense than that in a standard prepared at the same time and in the same manner using a mixture of 0.1 ml of *nitrate standard solution (2 ppm NO₃) R* and 4.9 ml of *nitrate-free water R*.

Sulphates (2.4.13): maximum 50 ppm.

Dilute 3 ml of the water to be examined to 15 ml with *distilled water R*. The solution complies with the limit test for sulphates.

Aluminium (2.4.17): maximum 10 µg/l.

Prescribed solution. To 400 ml of the water to be examined add 10 ml of *acetate buffer solution pH 6.0 R* and 100 ml of *water R*.

Reference solution. Mix 2 ml of *aluminium standard solution (2 ppm Al) R*, 10 ml of *acetate buffer solution pH 6.0 R* and 98 ml of *water R*.

Blank solution. Mix 10 ml of *acetate buffer solution pH 6.0 R* and 100 ml of *water R*.

Ammonium: maximum 0.2 ppm.

To 20 ml of the water to be examined in a flat-bottomed and transparent tube, add 1 ml of *alkaline potassium tetraiodomercurate solution R*. Allow to stand for 5 min. The solution is not more intensely coloured than a standard prepared at the same time and in the same manner using a mixture of 4 ml of *ammonium standard solution (1 ppm NH₄) R* and 16 ml of *ammonium-free water R*. Examine the solutions along the vertical axis of the tube.

Calcium: maximum 2 ppm.

Atomic absorption spectrometry (2.2.23, *Method I*).

Test solution. The water to be examined.

Reference solutions. Prepare reference solutions (1 ppm to 5 ppm) using *calcium standard solution (400 ppm Ca) R*.

Source: calcium hollow-cathode lamp.

Wavelength: 422.7 nm.

Atomisation device: oxidising air-acetylene flame.

Magnesium: maximum 2 ppm.

Atomic absorption spectrometry (2.2.23, *Method I*).

Test solution. Dilute 10 ml of the water to be examined to 100 ml with *distilled water R*.

Reference solutions. Prepare reference solutions (0.1 ppm to 0.5 ppm) using *magnesium standard solution (100 ppm Mg) R*.

Source: magnesium hollow-cathode lamp.

Wavelength: 285.2 nm.

Atomisation device: oxidising air-acetylene flame.

Mercury: maximum 0.001 ppm.

Atomic absorption spectrometry (2.2.23, *Method I*).

Test solution. Add 5 ml of *nitric acid R* per litre of the water to be examined. In a 50 ml borosilicate glass flask with a ground-glass-stopper, place 20 ml of the water to be examined and add 1 ml of *dilute nitric acid R* and shake. Add 0.3 ml of *bromine water R1*. Stopper the flask, shake and heat the stoppered flask at 45 °C for 4 h. Allow to cool. If the solution does not become yellow, add 0.3 ml of *bromine water R1* and re-heat at 45 °C for 4 h. Add 0.5 ml of a freshly prepared 10 g/l solution of *hydroxylamine hydrochloride R*. Shake. Allow to stand for 20 min.

Reference solutions. Use freshly prepared reference solutions (0.0005 ppm to 0.002 ppm) obtained by diluting *mercury standard solution (1000 ppm Hg) R* with a 5 per cent V/V solution of *dilute nitric acid R* and treat as described for the test solution.

To a volume of solution suitable for the instrument to be used, add *stannous chloride solution R2* equal to 1/5 of this volume. Fit immediately the device for the entrainment of the mercury vapour. Wait 20 s and pass through the device a stream of *nitrogen R* as the carrier gas.

Source: mercury hollow-cathode tube or a discharge lamp.

Wavelength: 253.7 nm.

Atomisation device: flameless system whereby the mercury can be entrained in the form of cold vapour.

Potassium: maximum 2 ppm.

Atomic emission spectrometry (2.2.22, *Method I*).

Test solution (a). Dilute 50.0 ml of the water to be examined to 100 ml with *distilled water R*. Carry out a determination using this solution. If the potassium content is more than 0.75 mg/l, further dilute the water to be examined with *distilled water R*.

Test solution (b). Take 50.0 ml of the water to be examined or, if necessary, the water to be examined diluted as described in the preparation of test solution (a). Add 1.25 ml of *potassium standard solution (20 ppm K) R* and dilute to 100.0 ml with *distilled water R*.

Reference solutions. Prepare reference solutions (0 ppm; 0.25 ppm; 0.50 ppm; 0.75 ppm; 1 ppm) using *potassium standard solution (20 ppm K) R*.

Wavelength: 766.5 nm.

Calculate the potassium content of the water to be examined in parts per million from the expression:

$$\frac{p \times n_1 \times 0.5}{n_2 - n_1}$$

p = dilution factor used for the preparation of test solution (a),

n_1 = measured value of test solution (a),

n_2 = measured value of test solution (b).

Sodium: maximum 50 ppm.

Atomic emission spectrometry (2.2.22, *Method I*).

Test solution. The water to be examined. If the sodium content is more than 10 mg/l, dilute with *distilled water R* to obtain a concentration suitable for the apparatus used.

Reference solutions. Prepare reference solutions (0 ppm; 2.5 ppm; 5.0 ppm; 7.5 ppm; 10 ppm) using *sodium standard solution (200 ppm Na) R*.

Wavelength: 589 nm.

Zinc: maximum 0.1 ppm.

Atomic absorption spectrometry (2.2.23, *Method I*): use sampling and analytical equipment free from zinc or not liable to yield zinc under the conditions of use.

Test solution. The water to be examined.

Reference solutions. Prepare reference solutions (0.05 ppm to 0.15 ppm) using *zinc standard solution (100 ppm Zn) R*.

Source: zinc hollow-cathode lamp.

Wavelength: 213.9 nm.

Atomisation device: oxidising air-acetylene flame.

Heavy metals (2.4.8): maximum 0.1 ppm.

Heat 200 ml of the water to be examined in a glass evaporating dish on a water-bath until the volume is reduced to 20 ml. 12 ml of the solution complies with limit test A. Prepare the standard using *lead standard solution (1 ppm Pb) R*.

Microbial contamination. Total viable aerobic count (2.6.12) not more than 10^2 micro-organisms per millilitre, determined by plate count.

Bacterial endotoxins (2.6.14): less than 0.25 IU/ml.

01/2005:0128

HAEMODIALYSIS, SOLUTIONS FOR

Solutiones ad haemodialysim

DEFINITION

Solutions for haemodialysis are solutions of electrolytes with a concentration close to the electrolytic composition of plasma. Glucose may be included in the formulation.

Because of the large volumes used, haemodialysis solutions are usually prepared by diluting a concentrated solution with water of suitable quality (see the monograph on *Haemodialysis solutions concentrated, water for diluting (1167)*), using for example an automatic dosing device.

Concentrated solutions for haemodialysis

Concentrated haemodialysis solutions are prepared and stored using materials and methods designed to produce solutions having as low a degree of microbial contamination as possible. In certain circumstances, it may be necessary to use sterile solutions.

During dilution and use, precautions are taken to avoid microbial contamination. Diluted solutions are to be used immediately after preparation.

Concentrated solutions for haemodialysis are supplied in:

- rigid, semi-rigid or flexible plastic containers,
- glass containers.

Three types of concentrated solutions are used:

1. Concentrated solutions with acetate or lactate

Several formulations of concentrated solutions are used. The concentrations of the components in the solutions are such that after dilution to the stated volume the concentrations of the components per litre are usually in the following ranges:

Table 0128-1.

	Expression in mmol	Expression in mEq
Sodium	130 - 145	130 - 145
Potassium	0 - 3.0	0 - 3.0
Calcium	0 - 2.0	0 - 4.0
Magnesium	0 - 1.2	0 - 2.4
Acetate or lactate	32 - 45	32 - 45
Chloride	90 - 120	90 - 120
Glucose	0 - 12.0	

Concentrated solutions with acetate or lactate are diluted before use.

2. Concentrated acidic solutions

Several formulations of concentrated solutions are used. The concentrations of the components in the solutions are such that after dilution to the stated volume and before neutralisation with sodium hydrogen carbonate the concentrations of the components per litre are usually in the following ranges:

Table 0128-2.

	Expression in mmol	Expression in mEq
Sodium	80 - 110	80 - 110
Potassium	0 - 3.0	0 - 3.0
Calcium	0 - 2.0	0 - 4.0
Magnesium	0 - 1.2	0 - 2.4
Acetic acid	2.5 - 10	2.5 - 10
Chloride	90 - 120	90 - 120
Glucose	0 - 12.0	

Sodium hydrogen carbonate must be added immediately before use to a final concentration of not more than 45 mmol/l. The concentrated solution of sodium hydrogen carbonate is supplied in a separate container. The concentrated acidic solutions and the concentrated solutions of sodium hydrogen carbonate are diluted and mixed immediately before use using a suitable device. Alternatively, solid sodium hydrogen carbonate may be used to prepare the solution.

3. Concentrated solutions without buffer

Several formulations of concentrated solutions without buffer are used. The concentrations of the components in the solutions are such that after dilution to the stated volume, the concentrations of the components per litre are usually in the following ranges:

Table 0128-3.

	Expression in mmol	Expression in mEq
Sodium	130 - 145	130 - 145
Potassium	0 - 3.0	0 - 3.0
Calcium	0 - 2.0	0 - 4.0
Magnesium	0 - 1.2	0 - 2.4
Chloride	130 - 155	130 - 155
Glucose	0 - 12.0	

Concentrated solutions without buffer are used together with parenteral administration of suitable hydrogen carbonate solutions.

IDENTIFICATION

According to the stated composition, the solution to be examined gives the following identification reactions (2.3.1):

- potassium: reaction (b);
- calcium: reaction (a);
- sodium: reaction (b);
- chlorides: reaction (a);
- lactates;
- carbonates and hydrogen carbonates;
- acetates:
 - if the solution is free from glucose, use reaction (b),
 - if the solution contains glucose, use the following method: to 5 ml of the solution to be examined add 1 ml of *hydrochloric acid R* in a test tube fitted with a stopper and a bent tube, heat and collect a few millilitres of distillate; carry out reaction (b) of acetates on the distillate;
- magnesium: to 0.1 ml of *titan yellow solution R* add 10 ml of *water R*, 2 ml of the solution to be examined and 1 ml of a 4.2 g/l solution of *sodium hydroxide R*; a pink colour is produced;
- glucose: to 5 ml of the solution to be examined, add 2 ml of *dilute sodium hydroxide solution R* and 0.05 ml of *copper sulphate solution R*; the solution is blue and clear; heat to boiling; an abundant red precipitate is formed.

TESTS

Appearance of solution. The solution to be examined is clear (2.2.1). If it does not contain glucose, it is colourless (2.2.2, *Method I*). If it contains glucose, it is not more intensely coloured than reference solution Y₇ (2.2.2, *Method I*).

Aluminium (2.4.17): maximum 0.1 mg/l.

Prescribed solution. Take 20 ml of the solution to be examined, adjust to pH 6.0 and add 10 ml of *acetate buffer solution pH 6.0 R*.

Reference solution. Mix 1 ml of *aluminium standard solution (2 ppm Al) R*, 10 ml of *acetate buffer solution pH 6.0 R* and 9 ml of *water R*.

Blank solution. Mix 10 ml of *acetate buffer solution pH 6.0 R* and 10 ml of *water R*.

Extractable volume (2.9.17). The volume measured is not less than the nominal volume stated on the label.

Sterility (2.6.1). If the label states that the concentrated haemodialysis solution is sterile, it complies with the test for sterility.

Bacterial endotoxins (2.6.14): less than 0.5 IU/ml in the solution diluted for use.

Pyrogens (2.6.8). Solutions for which a validated test for bacterial endotoxins cannot be carried out comply with the test for pyrogens. Dilute the solution to be examined with *water for injections R* to the concentration prescribed for use. Inject 10 ml of the solution per kilogram of the rabbit's mass.

ASSAY

Determine the density (2.2.5) of the concentrated solution and calculate the content in grams per litre and in millimoles per litre.

Sodium: 97.5 per cent to 102.5 per cent of the content of sodium (Na) stated on the label.

Atomic emission spectrometry (2.2.22, *Method I*).

Test solution. Dilute 5.0 ml of the solution to be examined to 100.0 ml with *water R*. Dilute 2.0 ml of this solution to 50.0 ml with *water R*. To 1.0 ml of this solution add 10 ml of *lanthanum chloride solution R* and dilute to 100.0 ml with *water R*.

Reference solutions. Into 4 identical volumetric flasks each containing 10 ml of *lanthanum chloride solution R*, introduce respectively 1.0 ml, 2.0 ml, 4.0 ml and 5.0 ml of *sodium standard solution (10 ppm Na) R* and dilute to 100.0 ml with *water R*.

Wavelength: 589.0 nm.

Potassium: 95.0 per cent to 105.0 per cent of the content of potassium (K) stated on the label.

Atomic absorption spectrometry (2.2.23, *Method I*).

Test solution. Dilute with *water R* an accurately weighed quantity of the solution to be examined to a concentration suitable for the instrument to be used. To 100 ml of this solution add 10 ml of a 22 g/l solution of *sodium chloride R*.

Reference solutions. Prepare the reference solutions using *potassium standard solution (100 ppm K) R*. To 100 ml of each reference solution add 10 ml of a 22 g/l solution of *sodium chloride R*.

Source: potassium hollow-cathode lamp.

Wavelength: 766.5 nm.

Atomisation device: air-acetylene flame.

Calcium: 95.0 per cent to 105.0 per cent of the content of calcium (Ca) stated on the label.

Atomic absorption spectrometry (2.2.23, *Method I*).

Test solution. Dilute 5.0 ml of the solution to be examined to 100.0 ml with *water R*. To 3.0 ml of this solution add 5 ml of *lanthanum chloride solution R* and dilute to 50.0 ml with *water R*.

Reference solutions. Into 4 keep volumetric flasks each containing 5 ml of *lanthanum chloride solution R*, introduce respectively 2.5 ml, 5.0 ml, 7.0 ml and 10.0 ml of *calcium standard solution (10 ppm Ca) R* and dilute to 50.0 ml with *water R*.

Source: calcium hollow-cathode lamp.

Wavelength: 422.7 nm.

Atomisation device: air-acetylene flame.

Magnesium: 95.0 per cent to 105.0 per cent of the content of magnesium (Mg) stated on the label.

Atomic absorption spectrometry (2.2.23, *Method I*).

Test solution. Dilute 5.0 ml of the solution to be examined to 100.0 ml with *water R*. To 2.0 ml of this solution add 5 ml of *lanthanum chloride solution R* and dilute to 50.0 ml with *water R*.

Reference solutions. Into 4 identical volumetric flasks each containing 5 ml of *lanthanum chloride solution R*, introduce respectively 1.0 ml, 2.0 ml, 3.0 ml and 4.0 ml of *magnesium standard solution (10 ppm Mg) R* and dilute to 50.0 ml with *water R*.

Source: magnesium hollow-cathode lamp.

Wavelength: 285.2 nm.

Atomisation device: air-acetylene flame.

Total chloride: 95.0 per cent to 105.0 per cent of the content of chloride (Cl) stated on the label.

Dilute to 50 ml with *water R* an accurately measured volume of the solution to be examined containing the equivalent of about 60 mg of chloride. Add 5 ml of *dilute nitric acid R*, 25.0 ml of *0.1 M silver nitrate* and 2 ml of *dibutyl phthalate R*. Shake. Using 2 ml of *ferric ammonium*

sulphate solution R2 as indicator, titrate with *0.1 M ammonium thiocyanate* until a reddish-yellow colour is obtained.

1 ml of *0.1 M silver nitrate* is equivalent to 3.545 mg of Cl.

Acetate: 95.0 per cent to 105.0 per cent of the content of acetate stated on the label.

To a volume of the solution to be examined, corresponding to about 0.7 mmol of acetate, add 10.0 ml of *0.1 M hydrochloric acid*. Carry out a potentiometric titration (2.2.20), using *0.1 M sodium hydroxide*. Read the volume added between the 2 points of inflexion.

1 ml of *0.1 M sodium hydroxide* is equivalent to 0.1 mmol of acetate.

Lactate: 95.0 per cent to 105.0 per cent of the content of lactate stated on the label.

To a volume of the solution to be examined, corresponding to about 0.7 mmol of lactate, add 10.0 ml of *0.1 M hydrochloric acid*. Then add 50 ml of *acetonitrile R*. Carry out a potentiometric titration (2.2.20), using *0.1 M sodium hydroxide*. Read the volume added between the 2 points of inflexion.

1 ml of *0.1 M sodium hydroxide* is equivalent to 0.1 mmol of lactate.

Sodium hydrogen carbonate: 95.0 per cent to 105.0 per cent of the content of sodium hydrogen carbonate stated on the label.

Titrate with *0.1 M hydrochloric acid* a volume of the solution to be examined corresponding to about 0.1 g of sodium hydrogen carbonate, determining the end-point potentiometrically (2.2.20).

1 ml of *0.1 M hydrochloric acid* is equivalent to 8.40 mg of NaHCO_3 .

Reducing sugars (expressed as anhydrous glucose): 95.0 per cent to 105.0 per cent of the content of glucose stated on the label.

Transfer a volume of the solution to be examined containing the equivalent of 25 mg of glucose to a 250 ml conical flask with a ground-glass neck and add 25.0 ml of *cupri-citric solution R*. Add a few grains of pumice, fit a reflux condenser, heat so that boiling occurs within 2 min and maintain boiling for exactly 10 min. Cool and add 3 g of *potassium iodide R* dissolved in 3 ml of *water R*. Carefully add, in small amounts, 25 ml of a 25 per cent *m/m* solution of *sulphuric acid R*. Titrate with *0.1 M sodium thiosulphate* using *starch solution R*, added towards the end of the titration, as indicator. Carry out a blank titration using 25.0 ml of *water R*.

Calculate the content of reducing sugars, expressed as anhydrous glucose ($\text{C}_6\text{H}_{12}\text{O}_6$), using Table 0128-4:

Table 0128-4.

Volume of <i>0.1 M sodium thiosulphate</i> (ml)	Anhydrous glucose (mg)
8	19.8
9	22.4
10	25.0
11	27.6
12	30.3
13	33.0
14	35.7
15	38.5
16	41.3

STORAGE

Store at a temperature not below 4 °C.

LABELLING

The label states:

- the formula of the concentrated solution for haemodialysis expressed in grams per litre and in millimoles per litre,
- the nominal volume of the solution in the container,
- where applicable, that the concentrated solution is sterile,
- the storage conditions,
- that the concentrated solution is to be diluted immediately before use,
- the dilution to be made,
- that the volume taken for use is to be measured accurately,
- the ionic formula for the diluted solution ready for use in millimoles per litre,
- that any unused portion of solution is to be discarded,
- where applicable, that sodium hydrogen carbonate is to be added before use.

01/2005:0861

HAEMOFILTRATION AND FOR HAEMODIAFILTRATION, SOLUTIONS FOR

Solutiones ad haemocolaturam haemodiacolaturamque

DEFINITION

Solutions for haemofiltration and for haemodiafiltration are preparations for parenteral use containing electrolytes with a concentration close to the electrolytic composition of plasma. Glucose may be included in the formulation.

Solutions for haemofiltration and for haemodiafiltration are supplied in:

- rigid or semi-rigid plastic containers,
- flexible plastic containers inside closed protective envelopes,
- glass containers.

The containers and closures comply with the requirements for containers for preparations for parenteral use (3.2. Containers).

In haemofiltration and haemodiafiltration, the following formulations are used. The concentrations of the components per litre of solution are usually in the following range:

Table 0861.-1.

	Expression in mmol	Expression in mEq
Sodium	125 - 150	125 - 150
Potassium	0 - 4.5	0 - 4.5
Calcium	1.0 - 2.5	2.0 - 5.0
Magnesium	0.25 - 1.5	0.50 - 3.0
Acetate and/or lactate and/or hydrogen carbonate	30 - 60	30 - 60
Chloride	90 - 120	90 - 120
Glucose	0 - 25	

When hydrogen carbonate is present, the solution of sodium hydrogen carbonate is supplied in a container or a separate compartment and is added to the electrolyte solution immediately before use.

In haemofiltration and in haemodiafiltration, the following formulations may also be used:

Table 0861.-2.

	Expression in mmol	Expression in mEq
Sodium	130 - 167	130 - 167
Potassium	0 - 4.0	0 - 4.0
Hydrogen carbonate	20 - 167	20 - 167
Chloride	0 - 147	0 - 147

Antioxidants such as metabisulphite salts are not added to the solutions.

IDENTIFICATION

According to the stated composition, the solution to be examined gives the following identification reactions (2.3.1):

- potassium: reaction (b);
- calcium: reaction (a);
- sodium: reaction (b);
- chlorides: reaction (a);
- acetates:
 - if the solution is free from glucose, use reaction (b),
 - if the solution contains glucose, use the following method: to 5 ml of the solution to be examined add 1 ml of *hydrochloric acid R* in a test-tube fitted with a stopper and a bent tube, heat and collect a few millilitres of distillate; carry out reaction (b) of acetates on the distillate;
- lactates;
- carbonates and hydrogen carbonates;
- magnesium: to 0.1 ml of *titan yellow solution R* add 10 ml of *water R*, 2 ml of the solution to be examined and 1 ml of 1 M *sodium hydroxide*; a pink colour is produced;
- glucose: to 5 ml of the solution to be examined, add 2 ml of *dilute sodium hydroxide solution R* and 0.05 ml of *copper sulphate solution R*; the solution is blue and clear; heat to boiling; an abundant red precipitate is formed.

TESTS

Appearance of solution. The solution is clear (2.2.1). If it does not contain glucose, it is colourless (2.2.2, *Method I*). If it contains glucose, it is not more intensely coloured than reference solution Y₇ (2.2.2, *Method I*).

pH (2.2.3). The pH of the solution is 5.0 to 7.5. If the solution contains glucose, the pH is 4.5 to 6.5. If the solution contains hydrogen carbonate, the pH is 7.0 to 8.5.

Hydroxymethylfurfural. To a volume of the solution containing the equivalent of 25 mg of glucose, add 5.0 ml of a 100 g/l solution of *p-toluidine R* in 2-propanol *R* containing 10 per cent V/V of *glacial acetic acid R* and 1.0 ml of a 5 g/l solution of *barbituric acid R*. The absorbance (2.2.25), determined at 550 nm after allowing the mixture to stand for 2 min to 3 min, is not greater than that of a standard prepared at the same time in the same manner using a solution containing 10 µg of *hydroxymethylfurfural R* in the same volume as the solution to be examined. If the solution contains hydrogen carbonate, use as the standard a solution containing 20 µg of *hydroxymethylfurfural R*.

Aluminium (2.4.17). Take 200 ml, adjust to pH 6.0 and add 10 ml of *acetate buffer solution pH 6.0 R*. The solution complies with the limit test for aluminium (10 µg/l). Use as the reference solution a mixture of 1 ml of *aluminium standard solution (2 ppm Al) R*, 10 ml of *acetate*

buffer solution pH 6.0 R and 9 ml of water R. To prepare the blank use a mixture of 10 ml of acetate buffer solution pH 6.0 R and 10 ml of water R.

Particulate contamination. Carry out the test for sub-visible particles (2.9.19) using 50 ml of solution.

Table 0861.-3.

Particles larger than	10 µm	25 µm
Maximum number of particles per millilitre	25	3

Extractable volume (2.9.17). The solution complies with the test prescribed for parenteral infusions.

Sterility (2.6.1). The solution complies with the test for sterility.

Bacterial endotoxins (2.6.14): less than 0.25 IU/ml.

Pyrogens (2.6.8). Solutions for which a validated test for bacterial endotoxins cannot be carried out comply with the test for pyrogens. Inject per kilogram of the rabbit's mass 10 ml of the solution.

ASSAY

Sodium: 97.5 per cent to 102.5 per cent of the content of sodium (Na) stated on the label, determined by atomic absorption spectrometry (2.2.23, Method II).

Test solution. If necessary, dilute the solution to be examined with water R to a concentration suitable for the instrument to be used.

Reference solutions. Prepare the reference solutions using sodium standard solution (200 ppm Na) R.

Measure the absorbance at 589.0 nm using a sodium hollow-cathode lamp as source of radiation and an air-propane or an air-acetylene flame.

Potassium. 95.0 per cent to 105.0 per cent of the content of potassium (K) stated on the label, determined by atomic absorption spectrometry (2.2.23, Method I).

Test solution. If necessary, dilute the solution to be examined with water R to a concentration suitable for the instrument to be used. To 100 ml of the solution add 10 ml of a 22 g/l solution of sodium chloride R.

Reference solutions. Prepare the reference solutions using potassium standard solution (100 ppm K) R. To 100 ml of each reference solution add 10 ml of a 22 g/l solution of sodium chloride R.

Measure the absorbance at 766.5 nm using a potassium hollow-cathode lamp as source of radiation and an air-propane or an air-acetylene flame.

Calcium. 95.0 per cent to 105.0 per cent of the content of calcium (Ca) stated on the label, determined by atomic absorption spectrometry (2.2.23, Method I).

Test solution. If necessary, dilute the solution to be examined with water R to a concentration suitable for the instrument to be used.

Reference solutions. Prepare the reference solutions using calcium standard solution (400 ppm Ca) R.

Measure the absorbance at 422.7 nm using a calcium hollow-cathode lamp as source of radiation and an air-propane or an air-acetylene flame.

Magnesium. 95.0 per cent to 105.0 per cent of the content of magnesium (Mg) stated on the label, determined by atomic absorption spectrometry (2.2.23, Method I).

Test solution. If necessary, dilute the solution to be examined with water R to a concentration suitable for the instrument to be used.

Reference solutions. Prepare the reference solutions using magnesium standard solution (100 ppm Mg) R.

Measure the absorbance at 285.2 nm using a magnesium hollow-cathode lamp as source of radiation and an air-propane or an air-acetylene flame.

Total chloride: 95.0 per cent to 105.0 per cent of the content of chloride (Cl) stated on the label. Dilute to 50 ml with water R an accurately measured volume of the solution to be examined containing the equivalent of about 60 mg of chloride. Add 5 ml of dilute nitric acid R, 25.0 ml of 0.1 M silver nitrate and 2 ml of dibutyl phthalate R. Shake. Using 2 ml of ferric ammonium sulphate solution R2 as indicator, titrate with 0.1 M ammonium thiocyanate until a reddish-yellow colour is obtained.

1 ml of 0.1 M silver nitrate is equivalent to 3.545 mg of Cl.

Acetate: 95.0 per cent to 105.0 per cent of the content of acetate stated on the label. To a volume of the solution to be examined, corresponding to about 0.7 mmol of acetate, add 10.0 ml of 0.1 M hydrochloric acid. Carry out a potentiometric titration (2.2.20), using 0.1 M sodium hydroxide. Read the volume added between the two points of inflexion.

1 ml of 0.1 M sodium hydroxide is equivalent to 0.1 mmol of acetate.

Lactate: 95.0 per cent to 105.0 per cent of the content of lactate stated on the label. To a volume of the solution to be examined, corresponding to about 0.7 mmol of lactate, add 10.0 ml of 0.1 M hydrochloric acid. Add 50 ml of acetonitrile R. Carry out a potentiometric titration (2.2.20), using 0.1 M sodium hydroxide. Read the volume added between the two points of inflexion.

1 ml of 0.1 M sodium hydroxide is equivalent to 0.1 mmol of lactate.

Sodium hydrogen carbonate: 95.0 per cent to 105.0 per cent of the content of sodium hydrogen carbonate stated on the label. Titrate with 0.1 M hydrochloric acid, a volume of the solution to be examined corresponding to about 0.1 g of sodium hydrogen carbonate, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M hydrochloric acid is equivalent to 8.40 mg of NaHCO₃.

Lactate and hydrogen carbonate: 95.0 per cent to 105.0 per cent of the content of lactates and/or hydrogen carbonates stated on the label. Examine by liquid chromatography (2.2.29).

Test solution. Solution to be examined.

Reference solution. Dissolve in 100 ml of water for chromatography R quantities of lactates and bicarbonates, accurately weighed, in order to obtain solutions having concentrations representing about 90 per cent, 100 per cent and 110 per cent of the concentrations indicated on the label.

The chromatographic procedure may be carried out using:

- a column 0.30 m long and 7.8 mm in internal diameter packed with cation exchange resin R (9 µm),
- as mobile phase at a flow rate of 0.6 ml/min 0.005 M sulphuric acid previously degassed with helium R,
- a differential refractometer detector,

maintaining the temperature of the column at 85 °C.

Inject in duplicate 20 µl of test solution and 20 µl of each reference solution. When the chromatograms are recorded in the prescribed conditions, the peaks elute in the following order: lactates then hydrogen carbonates.

Determine the concentration of lactates and hydrogen carbonates in the test solution by interpolating the peak area for lactate and the peak height for hydrogen carbonate from the linear regression curve obtained with the reference solutions.

Reducing sugars (expressed as anhydrous glucose). 95.0 per cent to 105.0 per cent of the content of glucose stated on the label. Transfer a volume of the solution to be examined containing the equivalent of 25 mg of glucose to a 250 ml conical flask with a ground-glass neck and add 25.0 ml of *cupri-citric solution R*. Add a few grains of pumice, fit a reflux condenser, heat so that boiling occurs within 2 min and boil for exactly 10 min. Cool and add 3 g of *potassium iodide R* dissolved in 3 ml of *water R*. Carefully add, in small amounts, 25 ml of a 25 per cent *m/m* solution of *sulphuric acid R*. Titrate with 0.1 M *sodium thiosulphate* using *starch solution R*, added towards the end of titration, as indicator. Carry out a blank titration using 25.0 ml of *water R*.

Calculate the content of reducing sugars expressed as anhydrous glucose ($C_6H_{12}O_6$), using Table 0861.4:

Table 0861.4.

Volume of 0.1 M sodium thiosulphate (ml)	Anhydrous glucose (mg)
8	19.8
9	22.4
10	25.0
11	27.6
12	30.3
13	33.0
14	35.7
15	38.5
16	41.3

STORAGE

Store at a temperature not lower than 4 °C.

LABELLING

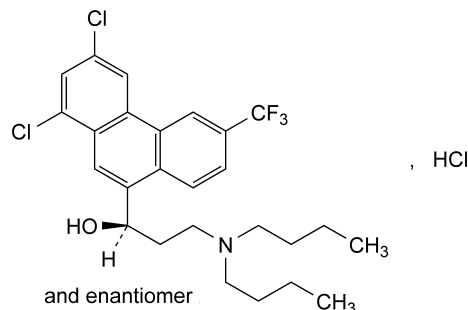
The label states:

- the formula of the solution for haemofiltration or haemodiafiltration, expressed in grams per litre and in millimoles per litre,
- the calculated osmolality, expressed in milliosmoles per litre,
- the nominal volume of the solution for haemofiltration or haemodiafiltration in the container,
- that the solution is free from bacterial endotoxins, or where applicable, that it is apyrogenic,
- the storage conditions,
- that any unused portion of solution is to be discarded.

01/2005:1979
corrected

HALOFANTRINE HYDROCHLORIDE

Halofantrini hydrochloridum



$C_{26}H_{31}Cl_3F_3NO$

M_r 536.9

DEFINITION

(1*S*)-3-(Dibutylamino)-1-[1,3-dichloro-6-(trifluoromethyl)phenanthren-9-yl]propan-1-ol hydrochloride.

Content: 97.5 per cent to 102.0 per cent (dried substance).

CHARACTERS

Appearance: white or almost white powder.

Solubility: practically insoluble in water, freely soluble in methanol, sparingly soluble in alcohol.

It shows polymorphism.

IDENTIFICATION

A. Infrared absorption spectrophotometry (2.2.24).

Comparison: halofantrine hydrochloride CRS.

If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in *methyl ethyl ketone R*, evaporate to dryness and record new spectra using the residues.

B. It gives reaction (b) of chlorides (2.3.1).

TESTS

Optical rotation (2.2.7): -0.10° to $+0.10^\circ$.

Dissolve 1.00 g in *alcohol R* and dilute to 100.0 ml with the same solvent.

Absorbance (2.2.25): maximum 0.085 at 450 nm.

Dissolve 0.200 g in *methanol R* and dilute to 10.0 ml with the same solvent.

Related substances. Liquid chromatography (2.2.29).

Test solution (a). Dissolve 40.0 mg of the substance to be examined in the mobile phase and dilute to 100.0 ml with the mobile phase.

Test solution (b). Dilute 5.0 ml of test solution (a) to 50.0 ml with the mobile phase.

Reference solution (a). Dissolve 40.0 mg of *halofantrine hydrochloride CRS* in the mobile phase and dilute to 100.0 ml with the mobile phase.

Reference solution (b). Dilute 5.0 ml of reference solution (a) to 50.0 ml with the mobile phase.

Reference solution (c). Dilute 1.0 ml of test solution (a) to 100.0 ml with the mobile phase. Dilute 5.0 ml of the solution to 50.0 ml with the mobile phase.

Reference solution (d). Dissolve 10.0 mg of *halofantrine impurity C CRS* in the mobile phase and dilute to 25 ml with the mobile phase. To 5.0 ml of the solution, add 5.0 ml of reference solution (a) and dilute to 50.0 ml with the mobile phase.

Column:

- **size:** $l = 0.30$ m, $\varnothing = 3.9$ mm,
- **stationary phase:** *octadecylsilyl silica gel for chromatography R* (10 μ m) of irregular type, with a specific surface of 330 m²/g, a pore size of 12.5 nm and a carbon loading of 9.8 per cent.

Mobile phase: mix 250 ml of a 2.0 g/l solution of *sodium hydroxide R*, previously adjusted to pH 2.5 with *perchloric acid R* and 750 ml of *acetonitrile R*.

Flow rate: 1 ml/min.

Detection: spectrophotometer at 260 nm.

Injection: 20 μ l; inject the test solution (a) and reference solutions (c) and (d).

Run time: 5 times the retention time of halofantrine which is about 6 min.

System suitability:

- **resolution:** minimum 3.3 between the peaks due to halofantrine and impurity C in the chromatogram obtained with reference solution (d).

Limits:

- **any impurity:** not more than twice the area of the principal peak in the chromatogram obtained with reference solution (c) (0.2 per cent),
- **total:** not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.5 per cent),
- **disregard limit:** 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.05 per cent).

Heavy metals (2.4.8): maximum 20 ppm.

1.0 g complies with limit test C. Prepare the standard using 2 ml of *lead standard solution (10 ppm Pb) R*.

Loss on drying (2.2.32): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 100–105 °C for 4 h.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (2.2.29) as described in the test for related substances.

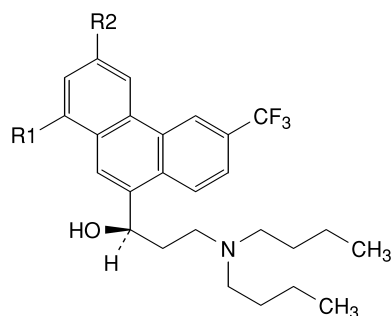
Injection: test solution (b) and reference solution (b).

Calculate the percentage content of halofantrine hydrochloride.

STORAGE

Protected from light.

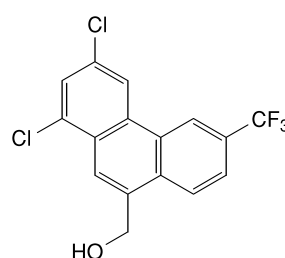
IMPURITIES



and enantiomer

A. R1 = H, R2 = Cl: (1*RS*)-1-[3-chloro-6-(trifluoromethyl)phenanthren-9-yl]-3-(dibutylamino)propan-1-ol (1-dechlorohalofantrine),

B. R1 = Cl, R2 = H: (1*RS*)-1-[1-chloro-6-(trifluoromethyl)phenanthren-9-yl]-3-(dibutylamino)propan-1-ol (3-dechlorohalofantrine),

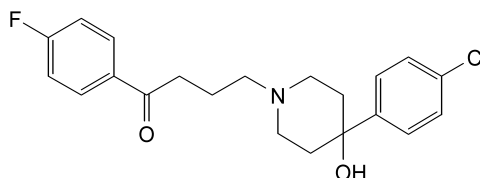


C. [1,3-dichloro-6-(trifluoromethyl)phenanthren-9-yl]methanol.

01/2005:0616

HALOPERIDOL

Haloperidolum



C₂₁H₂₃ClFNO₂

M_r 375.9

DEFINITION

Haloperidol contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of 4-[4-(4-chlorophenyl)-4-hydroxypiperidin-1-yl]-1-(4-fluorophenyl)butan-1-one, calculated with reference to the dried substance.

CHARACTERS

A white or almost white powder, practically insoluble in water, slightly soluble in alcohol, in methanol and in methylene chloride.

IDENTIFICATION

First identification: B, E.

Second identification: A, C, D, E.

A. Melting point (2.2.14): 150 °C to 153 °C.

B. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *haloperidol CRS*. Examine the substances prepared as discs.

C. Examine by thin-layer chromatography (2.2.27), using a suitable octadecylsilyl silica gel as the coating substance.

Test solution. Dissolve 10 mg of the substance to be examined in *methanol R* and dilute to 10 ml with the same solvent.

Reference solution (a). Dissolve 10 mg of *haloperidol CRS* in *methanol R* and dilute to 10 ml with the same solvent.

Reference solution (b). Dissolve 10 mg of *haloperidol CRS* and 10 mg of *bromperidol CRS* in *methanol R* and dilute to 10 ml with the same solvent.

Apply to the plate 1 µl of each solution. Develop in an unsaturated tank over a path of 15 cm using a mixture of 10 volumes of *tetrahydrofuran R*, 45 volumes of *methanol R* and 45 volumes of a 58 g/l solution of *sodium chloride R*. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. The principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a). The test is not valid unless the chromatogram obtained with reference solution (b) shows 2 spots which may, however, not be completely separated.

D. Dissolve about 10 mg in 5 ml of *ethanol R*. Add 0.5 ml of *dinitrobenzene solution R* and 0.5 ml of *2 M alcoholic potassium hydroxide R*. A violet colour is produced and becomes brownish-red after 20 min.

E. To 0.1 g in a porcelain crucible add 0.5 g of *anhydrous sodium carbonate R*. Heat over an open flame for 10 min. Allow to cool. Take up the residue with 5 ml of *dilute nitric acid R* and filter. To 1 ml of the filtrate add 1 ml of *water R*. The solution gives reaction (a) of chlorides (2.3.1).

TESTS

Appearance of solution. Dissolve 0.2 g in 20 ml of a 1 per cent V/V solution of *lactic acid R*. The solution is clear (2.2.1) and not more intensely coloured than reference solution Y₇ (2.2.2, *Method II*).

Related substances. Examine by liquid chromatography (2.2.29). Prepare the solutions immediately before use and protect from light.

Test solution. Dissolve 0.100 g of the substance to be examined in *methanol R* and dilute to 10.0 ml with the same solvent.

Reference solution (a). Dissolve 5.0 mg of *haloperidol CRS* and 2.5 mg of *bromperidol CRS* in *methanol R* and dilute to 50.0 ml with the same solvent.

Reference solution (b). Dilute 5.0 ml of the test solution to 100.0 ml with *methanol R*. Dilute 1.0 ml of this solution to 10.0 ml with *methanol R*.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.1 m long and 4.6 mm in internal diameter packed with *base-deactivated octadecylsilyl silica gel for chromatography R* (3 µm),
- as mobile phase at a flow rate of 1.5 ml/min:

Mobile phase A. A 17 g/l solution of *tetrabutylammonium hydrogen sulphate R1*,

Mobile phase B. *Acetonitrile R*,

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)	Comment
0 - 15	90 → 50	10 → 50	linear gradient
15 - 20	50	50	isocratic elution
20 - 25	90	10	switch to initial eluent composition
25 = 0	90	10	restart gradient

- as detector a spectrophotometer set at 230 nm.

Adjust the sensitivity of the system so that the height of the principal peak in the chromatogram obtained with 10 µl of reference solution (b) is at least 50 per cent of the full scale of the recorder.

Inject 10 µl of reference solution (a). When the chromatogram is recorded in the prescribed conditions, the retention times are: haloperidol about 5.5 min and bromperidol about 6 min. The test is not valid unless the resolution between the peaks due to haloperidol and bromperidol is at least 3.0. If necessary, adjust the concentration of acetonitrile in the mobile phase or adjust the time programme for the linear-gradient elution.

Inject separately 10 µl of *methanol R* as a blank, 10 µl of the test solution and 10 µl of reference solution (b). In the chromatogram obtained with the test solution: the area of any peak, apart from the principal peak, is not greater than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent); the sum of the areas of all peaks, apart from the principal peak, is not greater than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (1 per cent). Disregard any peak obtained with the blank and any peak with an area less than 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (b).

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 100-105 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g using a platinum crucible.

ASSAY

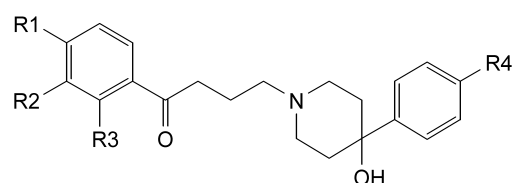
Dissolve 0.300 g in 50 ml of a mixture of 1 volume of *anhydrous acetic acid R* and 7 volumes of *methyl ethyl ketone R*. Titrate with 0.1 M *perchloric acid*, using 0.2 ml of *naphtholbenzein solution R* as indicator.

1 ml of 0.1 M *perchloric acid* is equivalent to 37.59 mg of C₂₁H₂₃ClFNO₂.

STORAGE

Store protected from light.

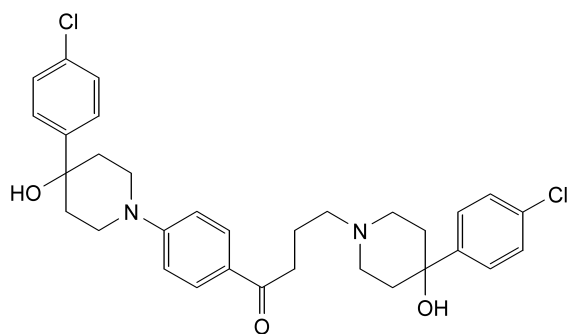
IMPURITIES



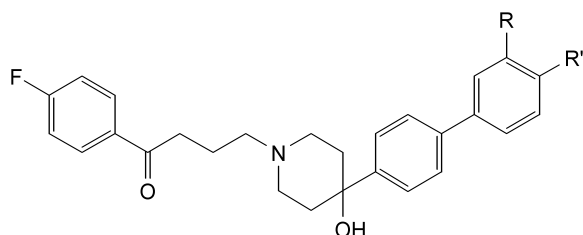
A. R1 = F, R2 = R3 = R4 = H: 1-(4-fluorophenyl)-4-(4-hydroxy-4-phenylpiperidin-1-yl)butan-1-one,

B. R1 = R2 = H, R3 = F, R4 = Cl: 4-[4-(4-chlorophenyl)-4-hydroxypiperidin-1-yl]-1-(2-fluorophenyl)butan-1-one,

C. R1 = F, R2 = C₂H₅, R3 = H, R4 = Cl: 4-[4-(4-chlorophenyl)-4-hydroxypiperidin-1-yl]-1-(3-ethyl-4-fluorophenyl)butan-1-one,



D. 4-[4-(4-chlorophenyl)-4-hydroxypiperidin-1-yl]-1-[4-(4-chlorophenyl)-4-hydroxypiperidin-1-yl]phenyl]butan-1-one,



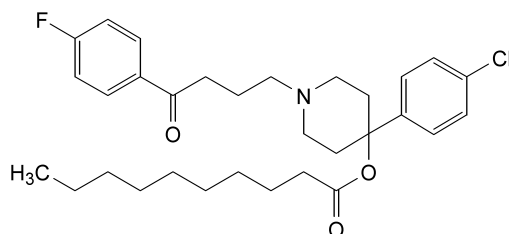
E. R = H, R' = Cl: 4-[4-(4'-chlorobiphenyl-4-yl)-4-hydroxypiperidin-1-yl]-1-(4-fluorophenyl)butan-1-one,

F. R = Cl, R' = H: 4-[4-(3'-chlorobiphenyl-4-yl)-4-hydroxypiperidin-1-yl]-1-(4-fluorophenyl)butan-1-one.

01/2005:1431

HALOPERIDOL DECANOATE

Haloperidoli decanoas



$C_{31}H_{41}ClFNO_3$

M_r 530.1

DEFINITION

Haloperidol decanoate contains not less than 98.5 per cent and not more than the equivalent of 101.0 per cent of 4-(4-chlorophenyl)-1-[4-(4-fluorophenyl)-4-oxobutyl]piperidin-4-yl decanoate, calculated with reference to the dried substance.

CHARACTERS

A white or almost white powder, practically insoluble in water, very soluble in alcohol, in methanol and in methylene chloride.

It melts at about 42 °C.

IDENTIFICATION

- Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *haloperidol decanoate CRS*. Examine the substances prepared as mulls in *liquid paraffin R*.
- To 0.1 g in a porcelain crucible add 0.5 g of *anhydrous sodium carbonate R*. Heat over an open flame for 10 min. Allow to cool. Take up the residue with 5 ml of *dilute*

nitric acid R and filter. To 1 ml of the filtrate add 1 ml of *water R*. The solution gives reaction (a) of chlorides (2.3.1).

TESTS

Appearance of solution. Dissolve 2.0 g in *methylene chloride R* and dilute to 20 ml with the same solvent. The solution is clear (2.2.1) and not more intensely coloured than reference solution B₅ (2.2.2, *Method II*).

Related substances. Examine by liquid chromatography (2.2.29). Prepare the solutions immediately before use and protect from light.

Test solution. Dissolve 0.100 g of the substance to be examined in *methanol R* and dilute to 10.0 ml with the same solvent.

Reference solution (a). Dissolve 2.5 mg of *bromperidol decanoate CRS* and 2.5 mg of *haloperidol decanoate CRS* in *methanol R* and dilute to 50.0 ml with the same solvent.

Reference solution (b). Dilute 5.0 ml of the test solution to 100.0 ml with *methanol R*. Dilute 1.0 ml of this solution to 10.0 ml with *methanol R*.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.1 m long and 4.0 mm in internal diameter packed with *base-deactivated octadecylsilyl silica gel for chromatography R* (3 µm),
- as mobile phase at a flow rate of 1.5 ml/min the following linear gradient programme:

Mobile phase A. A 27 g/l solution of *tetrabutylammonium hydrogen sulphate R*,

Mobile phase B. *Acetonitrile R*,

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)	Comment
0 - 30	80 → 40	20 → 60	linear gradient
30 - 35	40	60	isocratic elution
35 - 40	40 → 80	60 → 20	switch to initial eluent composition
40 = 0	80	20	restart gradient

- as detector a spectrophotometer set at 230 nm.

Equilibrate the column for at least 30 min with *acetonitrile R* and then equilibrate at the initial eluent composition for at least 5 min.

Adjust the sensitivity of the system so that the height of the principal peak in the chromatogram obtained with 10 µl of reference solution (b) is at least 50 per cent of the full scale of the recorder.

Inject 10 µl of reference solution (a). When the chromatogram is recorded in the prescribed conditions the retention times are: haloperidol decanoate about 24 min and bromperidol decanoate about 24.5 min. The test is not valid unless the resolution between the peaks due to haloperidol decanoate and bromperidol decanoate is at least 1.5. If necessary, adjust the gradient or the time programme for the linear gradient elution.

Inject 10 µl of *methanol R* as a blank, 10 µl of the test solution and 10 µl of reference solution (b). In the chromatogram obtained with the test solution: the area of any peak apart from the principal peak, is not greater than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent); the sum of the areas of all the peaks apart from the principal peak, is not greater than three times the area of the principal peak in the chromatogram obtained with the reference solution (b) (1.5 per cent). Disregard any peak due to the blank and

any peak with an area less than 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (b).

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying *in vacuo* at 30 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g in a platinum crucible.

ASSAY

Dissolve 0.425 g in 50 ml of a mixture of 1 volume of *anhydrous acetic acid R* and 7 volumes of *methyl ethyl ketone R*. Titrate with 0.1 M perchloric acid using 0.2 ml of *naphtholbenzein solution R* as indicator.

1 ml of 0.1 M perchloric acid is equivalent to 53.01 mg of $C_{31}H_{41}ClFNO_3$.

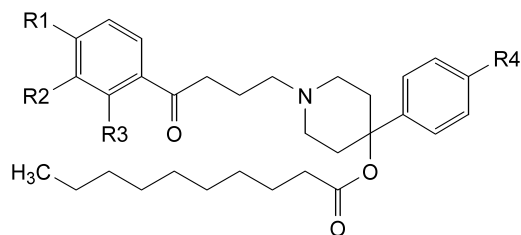
STORAGE

Store at room temperature below 25 °C, protected from light.

IMPURITIES

Specified impurities: A, B, C, D, E, F, G, H, I, J, K.

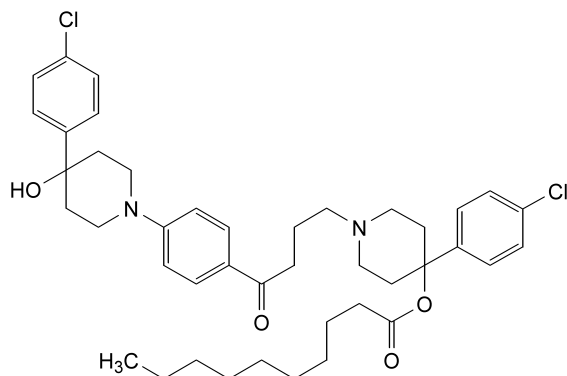
Other detectable impurities: L.



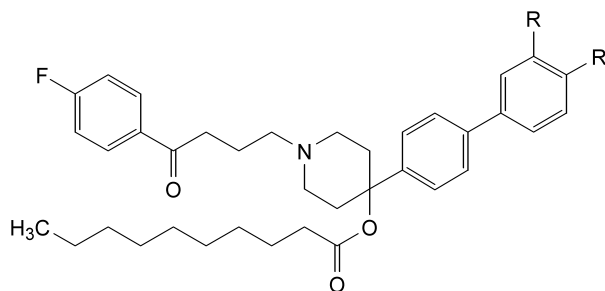
A. R1 = F, R2 = R3 = R4 = H: 1-[4-(4-fluorophenyl)-4-oxobutyl]-4-phenylpiperidin-4-yl decanoate,

B. R1 = R2 = H, R3 = F, R4 = Cl: 4-(4-chlorophenyl)-1-[4-(2-fluorophenyl)-4-oxobutyl]piperidin-4-yl decanoate,

C. R1 = F, R2 = C₂H₅, R3 = H, R4 = Cl: 4-(4-chlorophenyl)-1-[4-(3-ethyl-4-fluorophenyl)-4-oxobutyl]piperidin-4-yl decanoate,



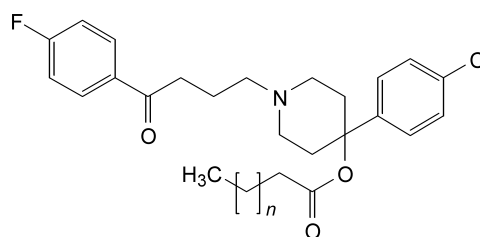
D. 4-(4-chlorophenyl)-1-[4-[4-[4-(4-chlorophenyl)-4-hydroxypiperidin-1-yl]phenyl]-4-oxobutyl]piperidin-4-yl decanoate,



E. R = H, R' = Cl: 4-(4'-chlorobiphenyl-4-yl)-1-[4-(4-fluorophenyl)-4-oxobutyl]piperidin-4-yl decanoate,

F. R = Cl, R' = H: 4-(3'-chlorobiphenyl-4-yl)-1-[4-(4-fluorophenyl)-4-oxobutyl]piperidin-4-yl decanoate,

G. haloperidol,

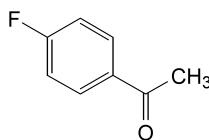


H. $n = 5$: 4-(4-chlorophenyl)-1-[4-(4-fluorophenyl)-4-oxobutyl]piperidin-4-yl octanoate,

I. $n = 6$: 4-(4-chlorophenyl)-1-[4-(4-fluorophenyl)-4-oxobutyl]piperidin-4-yl nonanoate,

J. $n = 8$: 4-(4-chlorophenyl)-1-[4-(4-fluorophenyl)-4-oxobutyl]piperidin-4-yl undecanoate,

K. $n = 9$: 4-(4-chlorophenyl)-1-[4-(4-fluorophenyl)-4-oxobutyl]piperidin-4-yl dodecanoate,

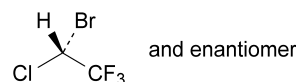


L. 1-(4-fluorophenyl)ethanone.

01/2005:0393

HALOTHANE

Halothanum



$C_2HBrClF_3$

M_r 197.4

DEFINITION

Halothane is (*RS*)-2-bromo-2-chloro-1,1,1-trifluoroethane to which 0.01 per cent *m/m* of thymol has been added.

CHARACTERS

A clear, colourless, mobile, heavy, non-flammable liquid, slightly soluble in water, miscible with ethanol and with trichloroethylene.

IDENTIFICATION

First identification: B.

Second identification: A, C.

A. It complies with the test for distillation range (see Tests).

- B. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the *Ph. Eur. reference spectrum of halothane*. Examine the substance in a 0.1 mm cell.
- C. Add 0.1 ml of the substance to be examined to 2 ml of 2-methyl-2-propanol *R* in a test-tube. Add 1 ml of copper edetate solution *R*, 0.5 ml of concentrated ammonia *R* and a mixture of 0.4 ml of strong hydrogen peroxide solution *R* and 1.6 ml of water *R* (solution a). Prepare a blank at the same time (solution b). Place both tubes in a water-bath at 50 °C for 15 min, cool and add 0.3 ml of glacial acetic acid *R*. To 1 ml of each of solutions (a) and (b) add 0.5 ml of a mixture of equal volumes of freshly prepared alizarin *S* solution *R* and zirconyl nitrate solution *R*. Solution (a) is yellow and solution (b) is red. To 1 ml of each of solutions (a) and (b) add 1 ml of buffer solution pH 5.2 *R*, 1 ml of phenol red solution *R* diluted 1 in 10 with water *R* and 0.1 ml of chloramine solution *R*. Solution (a) is bluish-violet and solution (b) is yellow. To 2 ml of each of solutions (a) and (b) add 0.5 ml of a mixture of 25 volumes of sulphuric acid *R* and 75 volumes of water *R*, 0.5 ml of acetone *R* and 0.2 ml of a 50 g/l solution of potassium bromate *R* and shake. Warm the tubes in a water-bath at 50 °C for 2 min, cool and add 0.5 ml of a mixture of equal volumes of nitric acid *R* and water *R* and 0.5 ml of silver nitrate solution *R*2. Solution (a) is opalescent and a white precipitate is formed after a few minutes; solution (b) remains clear.

TESTS

Acidity or alkalinity. To 20 ml add 20 ml of carbon dioxide-free water *R*, shake for 3 min and allow to stand. Separate the aqueous layer and add 0.2 ml of bromocresol purple solution *R*. Not more than 0.1 ml of 0.01 *M* sodium hydroxide or 0.6 ml of 0.01 *M* hydrochloric acid is required to change the colour of the indicator.

Relative density (2.2.5): 1.872 to 1.877.

Distillation range (2.2.11). It distils completely between 49.0 °C and 51.0 °C, 95 per cent distilling within a range of 1.0 °C.

Volatile related substances. Examine by gas chromatography (2.2.28), using trichlorotrifluoroethane *CRS* as the internal standard.

Test solution (a). Use the substance to be examined.

Test solution (b). Dilute 5.0 ml of trichlorotrifluoroethane *CRS* to 100.0 ml with the substance to be examined. Dilute 1.0 ml of the solution to 100.0 ml with the substance to be examined. Dilute 1.0 ml of this solution to 10.0 ml with the substance to be examined.

The chromatographic procedure may be carried out using:

- a column 2.75 m long and 5 mm in internal diameter packed with silanised diatomaceous earth for gas chromatography *R*1 (180 µm to 250 µm), the first 1.8 m being impregnated with 30 per cent *m/m* of macrogol 400 *R* and the remainder with 30 per cent *m/m* of dinonyl phthalate *R*,
 - nitrogen for chromatography *R* as the carrier gas at a flow rate of 30 ml/min,
 - a flame-ionisation detector,
- maintaining the temperature of the column at 50 °C. Inject 5 µl of test solutions (a) and (b).

In the chromatogram obtained with test solution (b), the sum of the areas of the peaks, apart from the principal peak and the peak due to the internal standard, is not greater than the area of the peak due to the internal standard, corrected if necessary for any impurity with the same retention time as the internal standard (0.005 per cent).

Bromides and chlorides. To 10 ml add 20 ml of water *R* and shake for 3 min. To 5 ml of the aqueous layer add 5 ml of water *R*, 0.05 ml of nitric acid *R* and 0.2 ml of silver nitrate solution *R*1. The solution is not more opalescent than a mixture of 5 ml of the aqueous layer and 5 ml of water *R*.

Bromine and chlorine. To 10 ml of the aqueous layer obtained in the test for bromides and chlorides add 1 ml of potassium iodide and starch solution *R*. No blue colour is produced.

Thymol. Examine by gas chromatography (2.2.28), using menthol *R* as the internal standard.

Internal standard solution. Dissolve 0.10 g of menthol *R* in methylene chloride *R* and dilute to 100.0 ml with the same solvent.

Test solution. To 20.0 ml of the substance to be examined add 5.0 ml of the internal standard solution.

Reference solution. Dissolve 20.0 mg of thymol *R* in methylene chloride *R* and dilute to 100.0 ml with the same solvent. To 20.0 ml, add 5.0 ml of the internal standard solution.

The chromatographic procedure may be carried out using:

- a fused-silica capillary column 15 m long and 0.53 mm in internal diameter coated with a 1.5 µm film of poly(dimethyl)siloxane *R*,
- nitrogen for chromatography *R* as the carrier gas at a flow rate of 15 ml/min,
- a flame-ionisation detector,

maintaining the temperature of the column at 150 °C and that of the injection port at 170 °C and of the detector at 200 °C.

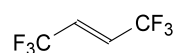
Inject separately 1.0 µl of the internal standard solution and of the test and reference solutions. In the chromatogram obtained with the test solution, the area of the peak due to thymol is not less than 75 per cent and not more than 115 per cent of the area of the corresponding peak in the chromatogram obtained with the reference solution (0.008 per cent *m/m* to 0.012 per cent *m/m*).

Non-volatile matter. Evaporate 50 ml to dryness on a water-bath and dry the residue in an oven at 100 °C to 105 °C for 2 h. The residue weighs not more than 1 mg (20 mg/l).

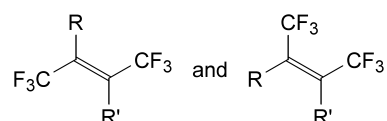
STORAGE

Store in an airtight container, protected from light, at a temperature not exceeding 25 °C. The choice of material for the container is made taking into account the particular reactivity of halothane with certain metals.

IMPURITIES

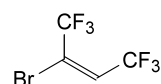


A. (*E*)-1,1,1,4,4,4-hexafluorobut-2-ene,

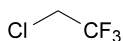


B. R = Cl, R' = H: (*EZ*)-2-chloro-1,1,1,4,4,4-hexafluorobut-2-ene (*cis* and *trans*),

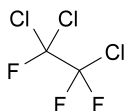
C. R = R' = Cl: (*EZ*)-2,3-dichloro-1,1,1,4,4,4-hexafluorobut-2-ene (*cis* and *trans*),



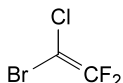
D. (*E*)-2-bromo-1,1,1,4,4,4-hexafluorobut-2-ene,



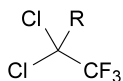
E. 2-chloro-1,1,1-trifluoroethane,



F. 1,1,2-trichloro-1,2,2-trifluoroethane,

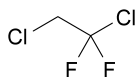


G. 1-bromo-1-chloro-2,2-difluoroethene,



H. R = H: 2,2-dichloro-1,1,1-trifluoroethane,

I. R = Br: 1-bromo-1,1-dichloro-2,2,2-trifluoroethane,



J. 1,2-dichloro-1,1-difluoroethane.

01/2005:0909

HAMAMELIS LEAF

Hamamelidis folium

DEFINITION

Hamamelis leaf consists of the whole or cut, dried leaf of *Hamamelis virginiana* L. It contains not less than 3 per cent of tannins, expressed as pyrogallol ($\text{C}_6\text{H}_6\text{O}_3$; M_r 126.1), calculated with reference to the dried drug.

CHARACTERS

It has the macroscopic and microscopic characters described under identification tests A and B.

IDENTIFICATION

A. The leaf is green or greenish-brown, often broken, crumpled and compressed into more or less compact masses. The lamina is broadly ovate to obovate; the base is oblique and asymmetric and the apex is acute or, rarely, obtuse. The margins of the lamina are roughly crenate or dentate. The venation is pinnate and prominent on the abaxial surface. Usually, four to six pairs of secondary veins are attached to the main vein, emerging at an acute angle and curving gently to the marginal points where there are fine veins often at right angles to the secondary veins.

B. Reduce to a powder (355). The powder is brownish-green. Examine under a microscope using *chloral hydrate solution R*. The powder shows fragments of adaxial epidermis with wavy anticlinal walls; abaxial epidermis with stomata mainly paracytic (2.8.3); star-shaped covering trichomes, either entire or broken, composed of four to twelve unicellular branches which are united by their bases, elongated, conical and curved, usually up to 250 µm long, thick-walled and with a clearly visible lumen with contents often brown in colour; fibres are lignified and thick-walled, isolated or in groups, and they are accompanied by a sheath of prismatic calcium oxalate crystals; small, cylindrical parenchymatous cells

of palisade; irregular-shaped cells of spongy mesophyll; sclereids, frequently enlarged at one or both ends, 150 µm to 180 µm long, whole or fragmented; fragments of annular or spiral vessels; isolated prisms of calcium oxalate.

C. Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel G plate R*.

Test solution. To 1.0 g of the powdered drug (355) add 10 ml of *alcohol (60 per cent V/V) R*, shake for 15 min and filter.

Reference solution (a). Dissolve 30 mg of *tannic acid R* in 5 ml of *alcohol (60 per cent V/V) R*.

Reference solution (b). Dissolve 5 mg of *gallic acid R* in 5 ml of *alcohol (60 per cent V/V) R*.

Apply to the plate as bands 10 µl of each solution. Develop over a path of 10 cm using a mixture of 10 volumes of *anhydrous formic acid R*, 10 volumes of *water R* and 80 volumes of *ethyl formate R*. Dry the plate at 100 °C to 105 °C for 10 min and allow to cool. Spray with *ferric chloride solution R2* until bluish-grey zones (phenolic compounds) appear. The chromatogram obtained with the test solution shows in its lower third a principal zone similar in position to the principal zone in the chromatogram obtained with reference solution (a) and, in its upper part, a narrow zone similar in position to the principal zone in the chromatogram obtained with reference solution (b). The chromatogram obtained with the test solution shows in addition several slightly coloured zones in the central part.

TESTS

Foreign matter (2.8.2). Not more than 7 per cent of stem and not more than 2 per cent of other foreign matter, determined on 50 g.

Loss on drying (2.2.32). Not more than 10.0 per cent, determined on 2.000 g of powdered drug (355) by drying in an oven at 100 °C to 105 °C for 4 h.

Total ash (2.4.16). Not more than 7.0 per cent.

Ash insoluble in hydrochloric acid (2.8.1). Not more than 2.0 per cent.

ASSAY

Carry out the determination of tannins in herbal drugs (2.8.14). Use 0.750 g of the powdered drug (180).

STORAGE

Store protected from light.

01/2005:0462

HARD FAT

Adeps solidus

DEFINITION

Hard fat consists of a mixture of triglycerides, diglycerides and monoglycerides, which may be obtained either by esterification of fatty acids of natural origin with glycerol or by transesterification of natural fats. Each type of hard fat is characterised by its melting point, its hydroxyl value and its saponification value. It contains no additives.

CHARACTERS

A white or almost white, waxy, brittle mass, practically insoluble in water, slightly soluble in ethanol. When heated to 50 °C, it melts giving a colourless or slightly yellowish liquid.

IDENTIFICATION

Examine by thin-layer chromatography (2.2.27), using *silica gel G R* as the coating substance.

Test solution. Dissolve 1.0 g of the substance to be examined in *ethylene chloride R* and dilute to 10 ml with the same solvent.

Apply to the plate 2 µl of the test solution. Develop over a path of 12 cm with a mixture of 10 volumes of *ether R* and 90 volumes of *ethylene chloride R*. Allow the plate to dry in air and expose to iodine vapour until the spots appear. Examine in daylight. The chromatogram shows a spot with an R_f value of about 0.6, corresponding to triglycerides (R_{st} 1) and spots corresponding to 1,3-diglycerides (R_{st} 0.5) and to 1,2-di-glycerides (R_{st} 0.3). A spot corresponding to 1-monoglycerides may also be visible (R_{st} 0.05).

TESTS

Alkaline impurities. Dissolve 2.00 g in a mixture of 1.5 ml of *alcohol R* and 3.0 ml of *ether R*. Add 0.05 ml of *bromophenol blue solution R*. Not more than 0.15 ml of 0.01 M *hydrochloric acid* is required to change the colour of the indicator to yellow.

Melting point (2.2.15): 30 °C to 45 °C; the melting point does not differ by more than 2 °C from the nominal value. Introduce the melted substance into the capillary tube and allow to stand at a temperature below 10 °C for 24 h.

Acid value (2.5.1). Not more than 0.5. Dissolve 5.0 g in 50 ml of the prescribed mixture of solvents.

Hydroxyl value (2.5.3, Method A). The hydroxyl value is not more than 50 and does not differ by more than 5 units from the nominal value. If the nominal value is less than 5, the hydroxyl value is not more than 5.

Iodine value (2.5.4). Not more than 3.

Peroxide value (2.5.5). Not more than 3.

Saponification value (2.5.6): 210 to 260, determined on 2.0 g. The saponification value does not differ by more than 5 per cent from the nominal value.

Unsaponifiable matter (2.5.7). Not more than 0.6 per cent, determined on 5.0 g.

Heavy metals (2.4.8). 2.0 g complies with limit test D for heavy metals (10 ppm). Prepare the standard using 2 ml of *lead standard solution (10 ppm Pb) R*.

Total ash (2.4.16). Not more than 0.05 per cent, determined on 2.00 g.

STORAGE

Store protected from light and heat.

LABELLING

The label states:

- the nominal melting point,
- the nominal hydroxyl value,
- the nominal saponification value.

01/2005:1220

HAWTHORN BERRIES

Crataegi fructus

DEFINITION

Hawthorn berries consist of the dried false fruits of *Crataegus monogyna* Jacq. (Lindm.), or *Crataegus laevigata* (Poir.) D.C. (synonym: *Crataegus oxyacantha* L.) or their hybrids or a mixture of these false fruits. They contain not

less than 1.0 per cent of procyanidins, calculated as cyanidin chloride ($C_{15}H_{11}ClO_6$; M_r 322.7) with reference to the dried drug.

CHARACTERS

The false fruit has a sweet mucilaginous taste.

It has the macroscopic and microscopic characters described under identification tests A and B.

IDENTIFICATION

A. The false fruit of *Crataegus monogyna* is obovate to globular. It is generally 6 mm to 10 mm long and 4 mm to 8 mm wide, reddish-brown to dark red. The surface is pitted or, more rarely, reticulated. The upper end of the fruit is crowned by the remains of five reflexed sepals surrounding a small sunken disc with a shallow, raised rim. The remains of the style occur in the centre of the disc with tufts of stiff, colourless hairs at the base. At the lower end of the fruit a short length of pedicel or, more frequently, a small pale circular scar where the pedicel was attached. The receptacle is fleshy and encloses a yellowish-brown, ovoid fruit with a hard, thick wall containing a single, elongated, pale brown, smooth and shiny seed.

The false fruit of *Crataegus laevigata* is up to 13 mm long. It contains two to three stony fruits, ventrally flattened, with short hairs at the top. Frequently, in the centre of the disc of the false fruit occur the remains of the two styles.

B. Reduce to a powder (355). The powder is greyish-red. Examine under a microscope using *chloral hydrate solution R*. The powder shows covering trichomes from inside the disc which are long, unicellular, frequently bent, tapering to a point, with smooth, much thickened and lignified walls, parenchymatous receptacle fragments the outer layer with red colouring matter, some cells of the inner layers containing small cluster crystals of calcium oxalate; occasional fragments including groups of sclereids and vascular strands with associated files of cells containing prisms of calcium oxalate; pericarp fragments consisting of large thick-walled sclereids with numerous pits, some of which are conspicuously branched; a few fragments of the testa having an epidermal layer composed of hexagonal, mucilaginous cells beneath which is a yellowish-brown pigment layer containing numerous elongated prisms of calcium oxalate; thin-walled parenchyma of the endosperm and cotyledons containing aleurone grains and globules of fixed oil.

C. Examine by thin-layer chromatography (2.2.27), using a suitable silica gel as the coating substance.

Test solution. To 1.0 g of the powdered drug (355) add 10 ml of *methanol R* and heat in a water bath at 65 °C for 5 min. Shake frequently. Allow to cool to room temperature and filter. Dilute the filtrate to 10 ml with *methanol R*.

Reference solution. Dissolve 2 mg of *chlorogenic acid R*, 2 mg of *caffeic acid R*, 5 mg of *hyperoside R* and 5 mg of *rutin R* in 20 ml of *methanol R*.

Apply separately to the plate, as bands, 30 µl of the test solution and 10 µl of the reference solution. Develop over a path of 15 cm using as the mobile phase a mixture of 10 volumes of *anhydrous formic acid R*, 10 volumes of *water R*, 30 volumes of *methyl ethyl ketone R* and 50 volumes of *ethyl acetate R*. Dry the plate at 100-105 °C and spray whilst hot with a 10 g/l solution of *diphenylboric acid aminoethyl ester R* in *methanol R*. Subsequently spray the plate with a 50 g/l solution

01/2005:1432

of *macrogol 400 R* in *methanol R*. Allow the plate to dry in air for 30 min and examine in ultraviolet light at 365 nm. The chromatogram obtained with the reference solution shows in the lower half, in order of increasing R_f values, the yellowish-brown fluorescent zone of rutin, the light blue fluorescent zone of chlorogenic acid and the yellowish-brown fluorescent zone of hyperoside; in the upper third appears the light blue fluorescent zone of caffeic acid. The chromatogram obtained with the test solution shows three zones similar in position and fluorescence to the zones due to chlorogenic acid, hyperoside and caffeic acid in the chromatogram obtained with the reference solution and three weak reddish fluorescent zones, one corresponding to the rutin zone in the chromatogram obtained with the reference solution and both of the others located above the hyperoside zone. Below and above the caffeic acid zone some light blue zones appear.

TESTS

Foreign matter (2.8.2). Not more than 2 per cent. Not more than 5 per cent of deteriorated false fruit. It does not contain fruits of other *Crataegus* species (*C. nigra* Waldst. et Kit., *C. pentagyna* Waldst. et Kit. ex Willd. and *C. azarolus* L.) which are characterised by the presence of more than three hard stones.

Loss on drying (2.2.32). Not more than 12.0 per cent, determined on 1.000 g of the powdered drug (355) by drying in an oven at 100-105 °C for 2 h.

Total ash (2.4.16). Not more than 5.0 per cent.

ASSAY

To 2.50 g of the powdered drug (355) add 30 ml of *alcohol* (70 per cent V/V) *R*. Heat under a reflux condenser for 30 min and filter. Wash the residue with 10.0 ml of *alcohol* (70 per cent V/V) *R*. Add to the filtrate 15.0 ml of *hydrochloric acid R1* and 10.0 ml of *water R*. Heat under a reflux condenser for 80 min. Allow to cool, filter and wash the residue with *alcohol* (70 per cent V/V) *R* until the filtrate is colourless. Dilute the filtrate to 250.0 ml with *alcohol* (70 per cent V/V) *R*. Evaporate 50.0 ml of this solution in a round-bottomed flask to about 3 ml and transfer it to a separating funnel. Rinse the round-bottomed flask sequentially with 10 ml and 5 ml of *water R* and transfer to the separating funnel. Shake the combined solution with three quantities, each of 15 ml, of *butanol R*. Combine the organic layers and dilute to 100.0 ml with *butanol R*. Measure the absorbance (2.2.25) of the solution at 545 nm.

Calculate the percentage content of procyanidins, as cyanidin chloride, from the expression:

$$\frac{A \times 500}{75 \times m}$$

i.e. taking the specific absorbance of cyanidin chloride to be 75.

A = absorbance at 545 nm,

m = mass of the substance to be examined, in grams.

STORAGE

Store protected from light.

HAWTHORN LEAF AND FLOWER

Crataegi folium cum flore

DEFINITION

Whole or cut, dried flower bearing branches of *Crataegus monogyna* Jacq. (Lindm.), *C. laevigata* (Poir.) D.C. (*C. oxyacanthoides* Thuill.) or their hybrids or, more rarely, other European *Crataegus* species including *C. pentagyna* Waldst. et Kit. ex Willd., *C. nigra* Waldst. et Kit., *C. azarolus* L.

Content: minimum 1.5 per cent of flavonoids expressed as hyperoside ($C_{21}H_{20}O_{12}$; M_r 464.4) (dried drug).

CHARACTERS

Macroscopic and microscopic characters described under identification tests A and B.

IDENTIFICATION

- A. The stems are dark brown, woody, 1-2.5 mm in diameter, bearing alternate, petiolate leaves with small, often deciduous stipules and corymbs of numerous small white flowers. The leaves are more or less deeply lobed with slightly serrate or almost entire margins; those of *C. laevigata* are pinnately lobed or pinnatifid with 3, 5 or 7 obtuse lobes, those of *C. monogyna* pinnatisect with 3 or 5 acute lobes; the adaxial surface is dark green to brownish-green, the abaxial surface is lighter greyish-green and shows a prominent, dense, reticulate venation. The leaves of *C. laevigata*, *C. monogyna* and *C. pentagyna* are glabrous or bear only isolated trichomes, those of *C. azarolus* and *C. nigra* are densely pubescent. The flowers have a brownish-green tubular calyx composed of 5 free, reflexed sepals, a corolla composed of 5 free, yellowish-white to brownish, rounded or broadly ovate and shortly unguiculate petals and numerous stamens. The ovary is fused to the calyx and consists of 1 to 5 carpels, each with a long style and containing a single ovule; in *C. monogyna* there is 1 carpel, in *C. laevigata* 2 or 3, in *C. azarolus* 2 or 3, or sometimes only 1, in *C. pentagyna* 5 or, rarely, 4.
- B. Reduce to a powder (355). The powder is yellowish-green. Examine under a microscope using *chloral hydrate solution R*. The powder shows unicellular covering trichomes, usually with a thick wall and wide lumen, almost straight to slightly curved, pitted at the base; fragments of leaf epidermis with cells which have sinuous to polygonal anticlinal walls and with large anomocytic stomata (2.8.3) surrounded by 4 to 7 subsidiary cells; parenchymatous cells of the mesophyll containing calcium oxalate clusters, usually measuring 10-20 µm, those associated with the veins containing groups of small prism crystals; fragments of petals showing rounded polygonal epidermal cells, strongly papillose, with thick walls, the cuticle of which clearly shows wavy striations; fragments of anthers showing endothecium with an arched and regularly thickened margin; fragments of stems containing collenchymatous cells, bordered pitted vessels and groups of lignified sclerenchymatous fibres with narrow lumina; numerous spherical to elliptical or triangular pollen grains up to 45 µm in diameter, with 3 germinal pores and a faintly granular exine.
- C. Thin-layer chromatography (2.2.27).

Test solution. To 1.0 g of the powdered drug (355) add 10 ml of *methanol R* and heat in a water-bath at 65 °C under a reflux condenser for 5 min. Cool and filter.

Reference solution. Dissolve 1.0 mg of *chlorogenic acid R* and 2.5 mg of *hyperoside R* in 10 ml of *methanol R*.

Plate: TLC silica gel plate *R*.

Mobile phase: *anhydrous formic acid R*, *water R*, *methyl ethyl ketone R*, *ethyl acetate R* (10:10:30:50 V/V/V/V).

Application: 20 µl as bands.

Development: over a path of 15 cm.

Drying: at 100-105 °C.

Detection: spray with a 10 g/l solution of *diphenylboric acid aminoethyl ester R* in *methanol R*. Subsequently spray with a 50 g/l solution of *macrogol 400 R* in *methanol R*. Allow the plate to dry in air for about 30 min. Examine in ultraviolet light at 365 nm.

Results: see below the sequence of the zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other fluorescent zones may be present in the chromatogram obtained with the test solution.

Top of the plate	
Hyperoside: a yellowish-orange fluorescent zone	A yellowish-green fluorescent zone (vitexin)
Chlorogenic acid: a light blue fluorescent zone	A yellowish-orange fluorescent zone (hyperoside)
	A light blue fluorescent zone (chlorogenic acid)
	A yellowish-green fluorescent zone (vitexin-2''-rhamnoside)
Reference solution	Test solution

TESTS

Foreign matter (2.8.2): maximum 8 per cent of lignified branches with a diameter greater than 2.5 mm and maximum 2 per cent of other foreign matter.

Loss on drying (2.2.32): maximum 10.0 per cent, determined on 1.000 g of powdered drug (355) by drying in an oven at 100-105 °C for 2 h.

Total ash (2.4.16): maximum 10.0 per cent.

ASSAY

Stock solution. In a 200 ml flask introduce 0.400 g of the powdered drug (250) and 40 ml of *alcohol (60 per cent V/V) R*. Heat in a water-bath at 60 °C for 10 min, shaking frequently. Allow to cool and filter through a plug of absorbent cotton into a 100 ml volumetric flask. Transfer the absorbent cotton with the drug residue back into the 200 ml flask, add 40 ml of *alcohol (60 per cent V/V) R* and heat again in a water-bath at 60 °C for 10 min, shaking frequently. Allow to cool and filter into the same 100 ml volumetric flask as previously. Rinse the 200 ml flask and filter with a further quantity of *alcohol (60 per cent V/V) R* and transfer to the same 100 ml volumetric flask. Dilute to volume with *alcohol (60 per cent V/V) R* and filter.

Test solution. Introduce 5.0 ml of the stock solution into a round-bottomed flask and evaporate to dryness under reduced pressure. Take up the residue with 8 ml of a mixture of 10 volumes of *methanol R* and 100 volumes of *glacial acetic acid R* and transfer into a 25 ml volumetric flask. Rinse the round-bottomed flask with 3 ml of a mixture of 10 volumes of *methanol R* and 100 volumes of *glacial acetic acid R* and transfer into the same 25 ml volumetric flask as previously. Add 10.0 ml of a solution containing 25.0 g/l

of *boric acid R* and 20.0 g/l of *oxalic acid R* in *anhydrous formic acid R* and dilute to 25.0 ml with *anhydrous acetic acid R*.

Compensation liquid. Introduce 5.0 ml of the stock solution into a round-bottomed flask and evaporate to dryness under reduced pressure. Take up the residue with 8 ml of a mixture of 10 volumes of *methanol R* and 100 volumes of *glacial acetic acid R* and transfer it into a 25 ml volumetric flask. Rinse the round-bottomed flask with 3 ml of a mixture of 10 volumes of *methanol R* and 100 volumes of *glacial acetic acid R* and transfer into the same 25 ml volumetric flask as previously. Add 10.0 ml of *anhydrous formic acid R* and dilute to 25.0 ml with *anhydrous acetic acid R*.

Measure the absorbance (2.2.25) of the test solution at 410 nm after 30 min.

Calculate the percentage content of total flavonoids expressed as hyperoside from the expression:

$$\frac{A \times 1.235}{m}$$

i.e. taking the value of the specific absorbance of hyperoside at 410 nm to be 405.

A = absorbance of the test solution at 410 nm,

m = mass of the drug to be examined, in grams.

01/2005:1865

HAWTHORN LEAF AND FLOWER DRY EXTRACT

Crataegi folii cum flore extractum siccum

DEFINITION

Extract produced from *Hawthorn leaf and flower (1432)*.

Content:

- for aqueous extracts: minimum 2.5 per cent of flavonoids, expressed as hyperoside ($C_{21}H_{20}O_{12}$; M_r 464.4) (dried extract);
- for hydroalcoholic extracts: minimum 6.0 per cent of flavonoids, expressed as hyperoside ($C_{21}H_{20}O_{12}$; M_r 464.4) (dried extract).

PRODUCTION

The extract is produced from the drug by a suitable procedure using either water or a hydroalcoholic solvent equivalent in strength to a minimum of 45 per cent V/V ethanol.

CHARACTERS

Appearance: light brown or greenish-brown powder.

IDENTIFICATION

Thin-layer chromatography (2.2.27).

Test solution. Suspend 0.2 g of the extract to be examined in 20 ml of *alcohol (70 per cent V/V) R* and filter.

Reference solution. Dissolve 1 mg of *chlorogenic acid R*, 2.5 mg of *hyperoside R* and 2.5 mg of *rutin R* in 10 ml of *methanol R*.

Plate: TLC silica gel plate *R*.

Mobile phase: *anhydrous formic acid R*, *water R*, *methyl ethyl ketone R*, *ethyl acetate R* (10:10:30:50 V/V/V/V).

Application: 20 µl of the test solution and 10 µl of the reference solution, as bands.

Development: over a path of 15 cm.

Drying: at 100-105 °C.

Detection: spray the plate whilst hot with a 10 g/l solution of *diphenylboric acid aminoethyl ester R* in *methanol R*; subsequently spray the plate with a 50 g/l solution of *macrogol 400 R* in *methanol R*; allow the plate to dry in air for 30 min and examine in ultraviolet light at 365 nm.

Results: see below the sequence of the zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other fluorescent zones may be present in the chromatogram obtained with the test solution.

Top of the plate	
Hyperoside: a yellowish-orange fluorescent zone	A light yellow fluorescent zone A yellowish-orange fluorescent zone (hyperoside)
Chlorogenic acid: a light blue fluorescent zone	A light blue fluorescent zone (chlorogenic acid) A yellowish-green fluorescent zone (vitexin 2''-rhamnoside)
Rutin: a yellowish-orange fluorescent zone	A yellowish-orange fluorescent zone (rutin)
Reference solution	Test solution

TESTS

Methanol (2.9.11): maximum 0.05 per cent *V/V*.

Loss on drying (2.2.32): maximum 6.0 per cent, determined on 0.500 g of the extract to be examined by drying in an oven at 100-105 °C for 2 h.

ASSAY

Stock solution. Dissolve 0.100 g of the extract to be examined in *alcohol (60 per cent V/V) R* and dilute to 100.0 ml with the same solvent.

Test solution. Introduce 5.0 ml of the stock solution into a round-bottomed flask and evaporate to dryness under reduced pressure. Dissolve the residue in 8 ml of a mixture of 10 volumes of *methanol R* and 100 volumes of *glacial acetic acid R* and transfer into a 25 ml volumetric flask. Rinse the round-bottomed flask with 3 ml of a mixture of 10 volumes of *methanol R* and 100 volumes of *glacial acetic acid R* and transfer into the 25 ml volumetric flask. Add 10.0 ml of a solution containing 25.0 g/l of *boric acid R* and 20.0 g/l of *oxalic acid R* in *anhydrous formic acid R* and dilute to 25.0 ml with *anhydrous acetic acid R*.

Compensation liquid. Introduce 5.0 ml of the stock solution into a round-bottomed flask and evaporate to dryness under reduced pressure. Dissolve the residue in 8 ml of a mixture of 10 volumes of *methanol R* and 100 volumes of *glacial acetic acid R* and transfer into a 25 ml volumetric flask. Rinse the round-bottomed flask with 3 ml of a mixture of 10 volumes of *methanol R* and 100 volumes of *glacial acetic acid R* and transfer into the 25 ml volumetric flask. Add 10.0 ml of *anhydrous formic acid R* and dilute to 25.0 ml with *anhydrous acetic acid R*.

After 30 min measure the absorbance (2.2.25) of the test solution at 410 nm by comparison with the compensation liquid.

Calculate the percentage content of total flavonoids, calculated as hyperoside, from the expression:

$$\frac{A \times 1.235}{m}$$

i.e. taking the specific absorbance to be 405.

A = absorbance at 410 nm,

m = mass of the extract to be examined, in grams.

01/2005:0332

HEPARIN CALCIUM

Heparinum calcicum

DEFINITION

Heparin calcium is a preparation containing the calcium salt of a sulphated glucosaminoglycan present in mammalian tissues. On complete hydrolysis, it liberates D-glucosamine, D-glucuronic acid, L-iduronic acid, acetic acid and sulphuric acid. It has the characteristic property of delaying the clotting of freshly shed blood. The potency of heparin calcium intended for parenteral administration is not less than 150 IU/mg, calculated with reference to the dried substance. The potency of heparin calcium not intended for parenteral administration is not less than 120 IU/mg, calculated with reference to the dried substance.

PRODUCTION

It is prepared from the lungs of oxen or from the intestinal mucosae of oxen, pigs or sheep.

It is produced by methods of manufacturing designed to minimise or eliminate microbial contamination and substances lowering blood pressure.

CHARACTERS

A white or almost white powder, moderately hygroscopic, freely soluble in water.

IDENTIFICATION

- It delays the clotting of recalcified citrated sheep plasma (see Assay).
- Dissolve 0.40 g in *water R* and dilute to 10.0 ml with the same solvent. The specific optical rotation (2.2.7) is not less than + 35.
- Examine by zone electrophoresis (2.2.31) using *agarose for electrophoresis R* as the supporting medium. To equilibrate the agarose and as electrolyte solution use a mixture of 50 ml of *glacial acetic acid R* and 800 ml of *water R* adjusted to pH 3 by addition of *lithium hydroxide R* and diluted to 1000.0 ml with *water R*.

Test solution. Dissolve 25 mg of the substance to be examined in *water R* and dilute to 10 ml with the same solvent.

Reference solution. Dilute *heparin sodium BRP* with an equal volume of *water R*.

Apply separately to the strip 2 µl to 3 µl of each solution. Pass a current of 1 mA to 2 mA per centimetre of strip width at a potential difference of 300 V for about 10 min. Stain the strips using a 1 g/l solution of *toluidine blue R* and remove the excess by washing. The ratio of the mobility of the principal band or bands in the electropherogram obtained with the test solution to the mobility of the band in the electropherogram obtained with the reference solution is 0.9 to 1.1.

- It gives the reactions of calcium (2.3.1).

TESTS

Appearance of solution. Dissolve a quantity equivalent to 50 000 IU in *water R* and dilute to 10 ml with the same solvent. The solution is clear (2.2.1) and not more intensely coloured than degree 5 of the range of reference solutions of the most appropriate colour (2.2.2, *Method II*).

pH (2.2.3). Dissolve 0.1 g in *carbon dioxide-free water R* and dilute to 10 ml with the same solvent. The pH of the solution is 5.5 to 8.0.

Protein and nucleotidic impurities. Dissolve 40 mg in 10 ml of *water R*. The absorbance (2.2.25) measured at 260 nm is not greater than 0.20 and that measured at 280 nm is not greater than 0.15.

Nitrogen. Not more than 2.5 per cent, calculated with reference to the dried substance. Carry out the determination of nitrogen by sulphuric acid digestion (2.5.9), using 0.100 g.

Calcium: 9.5 per cent to 11.5 per cent of Ca, calculated with reference to the dried substance. Determine the calcium by complexometric titration (2.5.11), using 0.200 g.

Heavy metals (2.4.8). 0.5 g complies with limit test C for heavy metals (30 ppm). Prepare the standard using 1.5 ml of *lead standard solution (10 ppm Pb) R*.

Loss on drying (2.2.32). Not more than 8.0 per cent, determined on 1.000 g by drying at 60 °C over *diphosphorus pentoxide R* at a pressure not exceeding 670 Pa for 3 h.

Sulphated ash (2.4.14): 32 per cent to 40 per cent, determined on 0.20 g and calculated with reference to the dried substance.

Bacterial endotoxins (2.6.14): less than 0.01 IU per IU of heparin, if intended for use in the manufacture of parenteral dosage forms without a further appropriate procedure for removal of bacterial endotoxins. The addition of divalent cations may be necessary in order to fulfil the validation criteria.

ASSAY

Carry out the assay of heparin (2.7.5). The estimated potency is not less than 90 per cent and not more than 111 per cent of the stated potency. The confidence limits of the estimated potency ($P = 0.95$) are not less than 80 per cent and not more than 125 per cent of the stated potency.

STORAGE

Store in an airtight container. If the substance is sterile, store in a sterile, airtight, tamper-proof container.

LABELLING

The label states:

- the number of International Units per milligram,
- the name and quantity of any added substance,
- where applicable, that the substance is free from bacterial endotoxins.

01/2005:0333

HEPARIN SODIUM

Heparinum natricum

DEFINITION

Heparin sodium is a preparation containing the sodium salt of a sulphated glucosaminoglycan present in mammalian tissues. On complete hydrolysis, it liberates D-glucosamine, D-glucuronic acid, L-iduronic acid, acetic acid and sulphuric acid. It has the characteristic property of delaying the clotting of freshly shed blood. The potency of heparin sodium intended for parenteral administration is not less than 150 IU/mg, calculated with reference to the dried

substance. The potency of heparin sodium not intended for parenteral administration is not less than 120 IU/mg, calculated with reference to the dried substance.

PRODUCTION

It is prepared from the lungs of oxen or from the intestinal mucosae of oxen, pigs or sheep.

It is produced by methods of manufacturing designed to minimise or eliminate microbial contamination and substances lowering blood pressure.

CHARACTERS

A white or almost white powder, moderately hygroscopic, freely soluble in water.

IDENTIFICATION

- It delays the clotting of recalcified citrated sheep plasma (see Assay).
- Dissolve 0.40 g in *water R* and dilute to 10.0 ml with the same solvent. The specific optical rotation (2.2.7) is not less than + 35.
- Examine by zone electrophoresis (2.2.31) using *agarose for electrophoresis R* as the supporting medium. To equilibrate the agarose and as electrolyte solution use a mixture of 50 ml of *glacial acetic acid R* and 800 ml of *water R* adjusted to pH 3 by addition of *lithium hydroxide R* and diluted to 1000.0 ml with *water R*.
Test solution. Dissolve 25 mg of the substance to be examined in *water R* and dilute to 10 ml with the same solvent.
Reference solution. Dilute *heparin sodium BRP* with an equal volume of *water R*.
Apply separately to the strip 2 µl to 3 µl of each solution. Pass a current of 1 mA to 2 mA per centimetre of strip width at a potential difference of 300 V for about 10 min. Stain the strips using a 1 g/l solution of *toluidine blue R* and remove the excess by washing. The ratio of the mobility of the principal band or bands in the electropherogram obtained with the test solution to the mobility of the band in the electropherogram obtained with the reference solution is 0.9 to 1.1.
- The residue obtained in the test for sulphated ash (see Tests) gives reaction (a) of sodium (2.3.1).

TESTS

Appearance of solution. Dissolve a quantity equivalent to 50 000 IU in *water R* and dilute to 10 ml with the same solvent. The solution is clear (2.2.1) and not more intensely coloured than intensity 5 of the range of reference solutions of the most appropriate colour (2.2.2, *Method II*).

pH (2.2.3). Dissolve 0.1 g in *carbon dioxide-free water R* and dilute to 10 ml with the same solvent. The pH of the solution is 5.5 to 8.0.

Protein and nucleotidic impurities. Dissolve 40 mg in 10 ml of *water R*. The absorbance (2.2.25) measured at 260 nm is not greater than 0.20 and that measured at 280 nm is not greater than 0.15.

Nitrogen. Not more than 2.5 per cent, calculated with reference to the dried substance. Carry out the determination of nitrogen by sulphuric acid digestion (2.5.9), using 0.100 g.

Sodium: 9.5 per cent to 12.5 per cent of Na, calculated with reference to the dried substance and determined by atomic absorption spectrometry (2.2.23, *Method I*).

Test solution. Dissolve 50 mg of the substance to be examined in 0.1 M *hydrochloric acid* containing 1.27 mg of *caesium chloride R* per millilitre and dilute to 100.0 ml with the same solvent.

Reference solutions. Prepare reference solutions containing 25 ppm, 50 ppm and 75 ppm of Na, using *sodium standard solution (200 ppm Na) R* diluted with 0.1 M hydrochloric acid containing 1.27 mg of *caesium chloride R* per millilitre.

Measure the absorbance at 330.3 nm using a sodium hollow-cathode lamp as the source of radiation and a flame of suitable composition (for example 11 litres of air and 2 litres of acetylene per minute).

Heavy metals (2.4.8). 0.5 g complies with limit test C for heavy metals (30 ppm). Prepare the standard using 1.5 ml of *lead standard solution (10 ppm Pb) R*.

Loss on drying (2.2.32). Not more than 8.0 per cent, determined on 1.000 g by drying at 60 °C over *diphosphorus pentoxide R* at a pressure not exceeding 670 Pa for 3 h.

Sulphated ash (2.4.14): 30 per cent to 43 per cent, determined on 0.20 g and calculated with reference to the dried substance.

Bacterial endotoxins (2.6.14): less than 0.01 IU per IU of heparin, if intended for use in the manufacture of parenteral dosage forms without a further appropriate procedure for removal of bacterial endotoxins.

ASSAY

Carry out the assay of heparin (2.7.5). The estimated potency is not less than 90 per cent and not more than 111 per cent of the stated potency. The confidence limits of the estimated potency ($P = 0.95$) are not less than 80 per cent and not more than 125 per cent of the stated potency.

STORAGE

Store in an airtight container. If the substance is sterile, store in a sterile, airtight, tamper-proof container.

LABELLING

The label states:

- the number of International Units per milligram,
- the name and quantity of any added substance,
- where applicable, that the substance is free from bacterial endotoxins.

01/2005:0828
corrected

HEPARINS, LOW-MOLECULAR-MASS

Heparina massae molecularis minoris

DEFINITION

Salts of sulphated glucosaminoglycans having a mass-average molecular mass less than 8000 and for which at least 60 per cent of the total mass has a molecular mass less than 8000. Low-molecular-mass heparins display different chemical structures at the reducing, or the non-reducing end of the polysaccharide chains.

The potency is not less than 70 IU of anti-factor Xa activity per milligram, calculated with reference to the dried substance. The ratio of anti-factor Xa activity to anti-factor IIa activity, determined as described under Assay, is not less than 1.5.

PRODUCTION

Low-molecular-mass heparins are produced in conditions designed to minimise microbial contamination.

Low-molecular-mass heparins are obtained by fractionation or depolymerisation of heparin of natural origin that complies with the monograph on *Heparin sodium (0333)* or *Heparin calcium (0332)*, whichever is appropriate, for parenteral use, unless otherwise justified and authorised. For each type of low-molecular-mass heparin the batch-to-batch consistency is ensured by demonstrating, for example, that the mass-average molecular mass and the mass percentage within defined molecular-mass ranges lower than 8000 are not less than 75 per cent and not more than 125 per cent of the mean value stated as type specification. The same limits apply also to the ratio of anti-factor Xa activity to anti-factor IIa activity.

Nucleotide and protein impurities of the source material. Dissolve 40 mg of the source material before fractionation in 10 ml of *water R*. The absorbance (2.2.25) measured at 260 nm and 280 nm is not greater than 0.20 and 0.15, respectively.

CHARACTERS

Appearance: white or almost white powder, hygroscopic.

Solubility: freely soluble in water.

IDENTIFICATION

A. Nuclear magnetic resonance spectrometry (2.2.33) using a pulsed (Fourier transform) spectrometer operating at 75 MHz for ^{13}C .

Test solution. Dissolve 0.200 g of the substance to be examined in a mixture of 0.2 ml of *deuterium oxide R* and 0.8 ml of *water R*.

Reference solution. Dissolve 0.200 g of the appropriate specific low-molecular-mass heparin CRS in a mixture of 0.2 ml of *deuterium oxide R* and 0.8 ml of *water R*.

Record the spectra at 40 °C, using cells 5 mm in diameter. Use *deuterated methanol R* as internal reference at $\delta = 50.0$ ppm.

Results: the spectrum obtained with the test solution is similar to that obtained with the reference solution containing the appropriate specific low-molecular-mass heparin CRS.

B. The ratio of anti-factor Xa activity to anti-factor IIa activity, determined as described under Assay, is not less than 1.5.

C. Size-exclusion chromatography (2.2.30).

Test solution. Dissolve 20 mg of the substance to be examined in 2 ml of the mobile phase.

Reference solution. Dissolve 20 mg of *low-molecular-mass heparin for calibration CRS* in 2 ml of the mobile phase.

Column:

- **size:** $l = 0.30$ m, $\varnothing = 7.5$ mm,
- **stationary phase:** appropriate porous silica beads (5 μm) with a fractionation range for proteins of approximately 15 000 to 100 000,
- **number of theoretical plates:** minimum of 20 000 per metre.

Mobile phase: 28.4 g/l solution of *anhydrous sodium sulphate R* adjusted to pH 5.0 using *dilute sulphuric acid R*.

Flow rate: 0.5 ml/min.

Detection: differential refractometer.

Injection: 25 μl .

Calibration. For detection, use a differential refractometer (RI) detector connected in series to a ultraviolet spectrophotometer set (UV) at 234 nm such that the UV monitor is connected to the column outlet, and the RI detector to the UV-monitor outlet.

It is necessary to measure the time lapse between the 2 detectors accurately, so that chromatograms from them can be aligned correctly. The retention times used in the calibration must be those from the RI detector.

The normalisation factor used to calculate the molecular weight from the RI/UV ratio is obtained as follows: calculate the total area under the UV₂₃₄ (ΣUV₂₃₄) and the RI (ΣRI) curves by numerical integration over the range of interest (i.e. excluding salt and solvent peaks at the end of the chromatogram). Calculate the ratio *r* using the following expression:

$$\frac{\sum \text{RI}}{\sum \text{UV}_{234}}$$

Calculate the factor *f* using the following expression:

$$\frac{M_{na}}{r}$$

M_{na} = the known number-average molecular mass of the *low-molecular-mass heparin for calibration CRS* found in the leaflet supplied with the CRS.

Provided the UV₂₃₄ and the RI responses are aligned, the molecular mass *M* at any point is calculated from:

$$f \frac{\text{RI}}{\text{UV}_{234}}$$

The resulting table of retention times and molecular masses may be used to derive a calibration for the chromatographic system by fitting a suitable mathematical relationship to the data. A polynomial of the third degree is recommended. *It must be stressed that the extrapolation of this fitted calibration curve to higher molecular masses is not valid.*

Inject 25 µl of the test solution and record the chromatogram for a period of time, ensuring complete elution of sample and solvent peaks.

The mass-average molecular mass is defined by the expression:

$$\frac{\sum (\text{RI}_i M_i)}{\sum \text{RI}_i}$$

RI_{*i*} = mass of substance eluting in the fraction *i*

M_i = molecular mass corresponding to fraction *i*.

The value for the mass-average molecular mass is not greater than 8000 and at least 60 per cent of the total mass has a molecular mass lower than 8000. In addition, the molecular mass parameters of a given substance do not differ by more than 25 per cent from those of the corresponding reference substance.

- D. It gives reaction (a) of sodium or the reactions of calcium (as appropriate) (2.3.1).

TESTS

pH (2.2.3): 5.5 to 8.0.

Dissolve 0.1 g in *carbon dioxide-free water R* and dilute to 10 ml with the same solvent.

Nitrogen (2.5.9): 1.5 per cent to 2.5 per cent (dried substance).

Calcium (2.5.11): 9.5 per cent to 11.5 per cent (dried substance), if prepared from heparin complying with the monograph on *Heparin calcium* (0332). Use 0.200 g.

Sodium (2.2.23, *Method I*): 9.5 per cent to 12.5 per cent (dried substance), if prepared from heparin complying with the monograph on *Heparin sodium* (0333).

Atomic absorption spectrometry.

Test solution. Dissolve 50 mg of the substance to be examined in 0.1 M *hydrochloric acid* containing 1.27 mg of *caesium chloride R* per millilitre and dilute to 100.0 ml with the same solvent.

Reference solutions. Prepare reference solutions (25 ppm, 50 ppm and 75 ppm) using *sodium standard solution* (200 ppm Na) *R* diluted with 0.1 M *hydrochloric acid* containing 1.27 mg of *caesium chloride R* per millilitre.

Source: sodium hollow-cathode lamp.

Wavelength: 330.3 nm.

Atomisation device: flame of suitable composition (for example, 11 litres of air and 2 litres of acetylene per minute).

Molar ratio of sulphate ions to carboxylate ions (2.2.38): minimum 1.8.

The sample of heparin used in this titration must be free from ionisable impurities, particularly salts.

Weigh 0.100 g of the substance to be examined taking the necessary measures to avoid the problems linked to hygroscopicity.

Take up into about 20 ml of double-glass-distilled *water R*. Cool to 4 °C and apply 2.0 ml of this solution to a pre-cooled column (approximately 10 × 1 cm), packed with a suitable *cation exchange resin R*. Wash through with double-glass-distilled *water R* into the titration vessel up to a final volume of about 10-15 ml (*the titration vessel must be just large enough to hold the electrodes from the conductivity meter, a small stirrer bar and a fine flexible tube from the outlet of a 2 ml burette*). Stir magnetically. When the conductivity reading is constant, note it and titrate with 0.05 M *sodium hydroxide* added in approximately 50 µl portions. Record the burette level and the conductivity meter reading a few seconds after each addition until the end-point is reached.

For each measured figure, calculate the number of milliequivalents of sodium hydroxide added from the volume and the known concentration of the sodium hydroxide solution. Plot on a graph the figures for conductivity (as *y*-axis) against the figures of milliequivalent of sodium hydroxide (as *x*-axis). The graph will have 3, approximately linear sections: an initial steep downward slope, a middle slight rise and a final steep rise. Estimate the best straight lines through these 3 parts of the graph. At the points where the first and second lines intersect, and where the second and third lines intersect, draw perpendiculars to the *x*-axis to estimate the milliequivalents of sodium hydroxide taken up by the sample at those points. The point where the first and second lines intersect will give the number of milliequivalents of sodium hydroxide taken up by the sulphate groups, and the point where the second and third lines intersect will give the number of milliequivalents taken up by the sulphate and carboxylate groups together. The difference between the 2 will therefore give the number of milliequivalents taken up by the carboxylate groups.

Heavy metals (2.4.8): maximum 30 ppm.

0.5 g complies with limit test C. Prepare the standard using 1.5 ml of *lead standard solution* (10 ppm Pb) *R*.

Loss on drying (2.2.32): maximum 10.0 per cent, determined on 1.000 g by drying at 60 °C over *diphosphorus pentoxide R* at a pressure not exceeding 670 Pa for 3 h.

Bacterial endotoxins (2.6.14): less than 0.01 IU per International Unit of anti-Xa activity, if intended for use in the manufacture of parenteral dosage forms without a

further appropriate procedure for the removal of bacterial endotoxins. The addition of divalent cations may be necessary to fulfil the validation criteria.

ASSAY

The anticoagulant activity of low-molecular-mass heparins is determined *in vitro* by 2 assays which determine its ability to accelerate the inhibition of factor Xa (anti-Xa assay) and thrombin, factor IIa (anti-IIa assay), by antithrombin III.

The International Units for anti-Xa and anti-IIa activity are the activities contained in a stated amount of the International Standard for low-molecular-mass heparin.

Low-molecular-mass heparin for assay BRP, calibrated in International Units by comparison with the International Standard using the 2 assays given below, is used as reference preparation.

ANTI-FACTOR Xa ACTIVITY

Reference and test solutions

Prepare 4 independent series of 4 dilutions each, of the substance to be examined and of the reference preparation of low-molecular-mass heparin in *tris(hydroxymethyl)aminomethane sodium chloride buffer solution pH 7.4 R*; the concentration range should be within 0.025 IU to 0.2 IU of anti-factor Xa activity per millilitre and the dilutions chosen should give a linear response when results are plotted as absorbance against log concentration.

Procedure

Label 16 tubes in duplicate: T₁, T₂, T₃, T₄ for the dilutions of the substance to be examined and S₁, S₂, S₃, S₄ for the dilutions of the reference preparation. To each tube add 50 µl of *antithrombin III solution R1* and 50 µl of the appropriate dilution of the substance to be examined, or the reference preparation. After each addition, mix but do not allow bubbles to form. Treating the tubes in the order S₁, S₂, S₃, S₄, T₁, T₂, T₃, T₄, T₁, T₂, T₃, T₄, S₁, S₂, S₃, S₄, allow to equilibrate at 37 °C (water-bath or heating block) for 1 min and add to each tube 100 µl of *bovine factor Xa solution R*. Incubate for exactly 1 min and add 250 µl of *chromophore substrate R1*. Stop the reaction after exactly 4 min by adding 375 µl of *acetic acid R*. Transfer the mixtures to semi-micro cuvettes and measure the absorbance (2.2.25) at 405 nm using a suitable reading device. Determine the blank amidolytic activity at the beginning and at the end of the procedure in a similar manner, using *tris(hydroxymethyl)aminomethane sodium chloride buffer solution pH 7.4 R* instead of the reference and test solutions; the 2 blank values do not differ significantly. Calculate the regression of the absorbance on log concentrations of the solutions of the substance to be examined and of the reference preparation of low-molecular-mass heparins and calculate the potency of the substance to be examined in International Units of anti-factor Xa activity per millilitre using the usual statistical methods for parallel-line assays.

ANTI-FACTOR IIa ACTIVITY

Reference and test solutions

Prepare 4 independent series of 4 dilutions each, of the substance to be examined and of the reference preparation of low molecular-mass heparin in *tris(hydroxymethyl)aminomethane sodium chloride buffer solution pH 7.4 R*; the concentration range should be within 0.015 IU to 0.075 IU of anti-factor IIa activity per millilitre and the dilutions chosen should give a linear response when results are plotted as absorbance against log concentration.

Procedure

Label 16 tubes in duplicate: T₁, T₂, T₃, T₄ for the dilutions of the substance to be examined and S₁, S₂, S₃, S₄ for the

dilutions of the reference preparation. To each tube add 50 µl of *antithrombin III solution R2* and 50 µl of the appropriate dilution of the substance to be examined or the reference preparation. After each addition, mix but do not allow bubbles to form. Treating the tubes in the order S₁, S₂, S₃, S₄, T₁, T₂, T₃, T₄, T₁, T₂, T₃, T₄, S₁, S₂, S₃, S₄, allow to equilibrate at 37 °C (water-bath or heating block) for 1 min and add to each tube 100 µl of *human thrombin solution R*. Incubate for exactly 1 min and add 250 µl of *chromophore substrate R2*. Stop the reaction after exactly 4 min by adding 375 µl of *acetic acid R*. Transfer the mixtures to semi-micro cuvettes and measure the absorbance (2.2.25) at 405 nm using a suitable reading device. Determine the blank amidolytic activity at the beginning and at the end of the procedure in a similar manner, using *tris(hydroxymethyl)aminomethane sodium chloride buffer solution pH 7.4 R* instead of the reference and test solutions; the 2 blank values do not differ significantly. Calculate the regression of the absorbance on log concentrations of the solutions of the substance to be examined and of the reference preparation of low-molecular-mass heparins, and calculate the potency of the substance to be examined in International Units of anti-factor IIa activity per millilitre using the usual statistical methods for parallel-line assays.

LABELLING

The label states:

- the number of International Units of anti-factor Xa activity per milligram,
- the number of International Units of anti-factor IIa activity per milligram,
- the mass-average molecular mass and the percentage of molecules within defined molecular mass ranges,
- where applicable, that the substance is free from bacterial endotoxins,
- where applicable, that the contents are the sodium salt,
- where applicable, that the contents are the calcium salt.

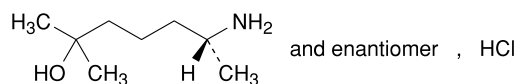
STORAGE

In an airtight tamper-proof container. If the product is sterile and free of bacterial endotoxins, store in a sterile and apyrogenic container.

01/2005:1980
corrected

HEPTAMINOL HYDROCHLORIDE

Heptaminoli hydrochloridum



C₈H₂₀ClNO

M_r 181.7

DEFINITION

(6*RS*)-6-Amino-2-methylheptan-2-ol hydrochloride.

Content: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

Appearance: white or almost white, crystalline powder.

Solubility: freely soluble in water, soluble in alcohol, practically insoluble in methylene chloride.

IDENTIFICATION

First identification: B, D.

Second identification: A, C, D.

A. To 1 ml of solution S (see Tests) add 4 ml of *water R* and 2 ml of a 200 g/l solution of *ammonium and cerium nitrate R* in 4 M *nitric acid*. An orange-brown colour develops.

B. Infrared absorption spectrophotometry (2.2.24).

Comparison: *heptaminol hydrochloride CRS*.

C. Examine the chromatograms obtained in the test for related substances.

Detection: examine in daylight.

Results: the principal spot in the chromatogram obtained with test solution (b) is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (b).

D. It gives reaction (a) of chlorides (2.3.1).

TESTS

Solution S. Dissolve 5.0 g in *carbon dioxide-free water R* and dilute to 50 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and not more intensely coloured than reference solution BY₆ (2.2.2, *Method II*).

Acidity or alkalinity. To 10 ml of solution S add 0.1 ml of *methyl red solution R* and 0.3 ml of 0.01 M *hydrochloric acid*. The solution is red. Add 0.6 ml of 0.01 M *sodium hydroxide*. The solution is yellow.

Related substances. Thin-layer chromatography (2.2.27).

Test solution (a). Dissolve 0.50 g of the substance to be examined in *methanol R* and dilute to 5.0 ml with the same solvent.

Test solution (b). Dilute 1.0 ml of test solution (a) to 10 ml with *methanol R*.

Reference solution (a). Dilute 3.0 ml of test solution (a) to 10.0 ml with *methanol R*. Dilute 1.0 ml of this solution to 50.0 ml with *methanol R*.

Reference solution (b). Dissolve 0.10 g of *heptaminol hydrochloride CRS* in *methanol R* and dilute to 10 ml with the same solvent.

Reference solution (c). Dissolve 10.0 mg of *heptaminol impurity A CRS* in *methanol R* and dilute to 5.0 ml with the same solvent.

Reference solution (d). Dilute 1.0 ml of reference solution (c) to 10.0 ml with *methanol R*.

Reference solution (e). To 2.5 ml of reference solution (c) add 0.5 ml of test solution (b) and dilute to 5 ml with *methanol R*.

Plate: TLC silica gel G plate R.

Mobile phase: concentrated ammonia R, dioxan R, 2-propanol R (10:50:50 V/V/V).

Application: 10 µl; apply test solutions (a) and (b) and reference solutions (a), (b), (d) and (e).

Development: over 2/3 of the plate.

Drying: in air.

Detection: expose the plate to iodine vapour for at least 15 h.

System suitability: the chromatogram obtained with reference solution (e) shows 2 clearly separated principal spots and the chromatogram obtained with reference solution (a) shows a single principal spot.

Limits: in the chromatogram obtained with test solution (a):

- *impurity A*: any spot corresponding to impurity A is not more intense than the spot in the chromatogram obtained with reference solution (d) (0.2 per cent),
- *any other impurity*: any spot, apart from the principal spot and any spot corresponding to impurity A is not more intense than the spot in the chromatogram obtained with reference solution (a) (0.6 per cent).

Heavy metals (2.4.8): maximum 10 ppm.

12 ml of solution S complies with limit test A. Prepare the standard using *lead standard solution (1 ppm Pb) R*.

Loss on drying (2.2.32): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 100–105 °C for 4 h.

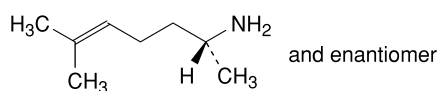
Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.140 g in 50 ml of *alcohol R* and add 5.0 ml of 0.01 M *hydrochloric acid*. Carry out a potentiometric titration (2.2.20), using 0.1 M *sodium hydroxide*. Read the volume added between the 2 points of inflexion.

1 ml of 0.1 M *sodium hydroxide* is equivalent to 18.17 mg of C₂₄H₃₈N₄O₁₀S₂.

IMPURITIES

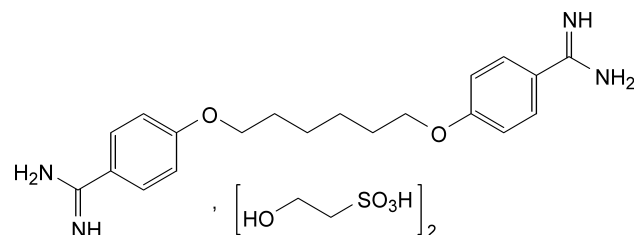


A. (2*RS*)-6-methylhept-5-en-2-amine.

01/2005:1436

HEXAMIDINE DISETIONATE

Hexamidini diisetonas



C₂₄H₃₈N₄O₁₀S₂

M_r 607

DEFINITION

Hexamidine diisetonate contains not less than 98.5 per cent and not more than the equivalent of 101.5 per cent of 4,4'-(hexane-1,6-diylidioxy)dibenzimidamide bis(2-hydroxyethanesulphonate), calculated with reference to the dried substance.

CHARACTERS

A white or slightly yellow powder, hygroscopic, sparingly soluble in water, slightly soluble in alcohol, practically insoluble in methylene chloride.

IDENTIFICATION

- Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *hexamidine diisetonate CRS*.
- Dissolve about 40 mg in 5 ml of *water R* and add dropwise with shaking, 1 ml of a 100 g/l solution of *sodium chloride R*. Allow to stand for 5 min. An abundant shimmering white precipitate forms slowly.

TESTS

Appearance of solution. Dissolve 0.5 g in *carbon dioxide-free water R* heating at about 70 °C and dilute to 10 ml with the same solvent. Allow to cool to room temperature for 10 min to 15 min. The solution is not more

opalescent than reference suspension II (2.2.1) and not more intensely coloured than intensity 6 of the range of reference solutions of the most appropriate colour (2.2.2, Method II).

Acidity or alkalinity. Dissolve 2.0 g in *water R* heating at about 50 °C and dilute to 20 ml with *water R* heating at about 50 °C. Allow to cool to about 35 °C, add 0.1 ml of *methyl red solution R*. Not more than 0.25 ml of 0.05 M *hydrochloric acid* or 0.05 M *sodium hydroxide* is required to change the colour of the indicator.

Related substances. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 20.0 mg of the substance to be examined in mobile phase A and dilute to 100.0 ml with mobile phase A.

Reference solution (a). Dilute 1.0 ml of the test solution to 100.0 ml with the mobile phase A.

Reference solution (b). Dissolve 5 mg of the substance to be examined and 5 mg of *pentamidine diisetonate CRS* in mobile phase A and dilute to 100 ml with mobile phase A. Dilute 2 ml of the solution to 5 ml with mobile phase A.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4.6 mm in internal diameter, packed with *styrene-divinylbenzene copolymer R* (8 µm),
- as mobile phase at a flow rate of 1 ml/min:

Mobile phase A. Mix 20 volumes of *acetonitrile R* and 80 volumes of a 6.8 g/l solution of *potassium dihydrogen phosphate R* adjusted to pH 3 using *phosphoric acid R*,

Mobile phase B. Mix equal volumes of *acetonitrile R* and of a 6.8 g/l solution of *potassium dihydrogen phosphate R* adjusted to pH 3 using *phosphoric acid R*,

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)	Comment
0 - 30	100 → 0	0 → 100	linear gradient
30 - 35	0	100	isocratic
35 - 40	0 → 100	100 → 0	re-equilibration

- as detector a spectrophotometer set at 263 nm.

Inject 20 µl of reference solution (a) and 20 µl of reference solution (b). Adjust the sensitivity of the system so that the height of the principal peak in the chromatogram obtained with reference solution (a) is at least 50 per cent of the full scale of the recorder. The test is not valid unless, in the chromatogram obtained with reference solution (b), the resolution between the peak corresponding respectively to hexamidine diisetonate and pentamidine diisetonate is at least 5.

Inject 20 µl of the test solution. In the chromatogram obtained, the area of any peak, apart from the principal peak, is not greater than the area of the principal peak in the chromatogram obtained with reference solution (a) (1 per cent) and not more than one such peak has an area greater than half the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent); the sum of the areas of all the peaks, apart from the principal peak, is not greater than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (1.5 per cent). Disregard any peak with an area less than 0.05 times that of the principal peak in the chromatogram obtained with reference solution (a).

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

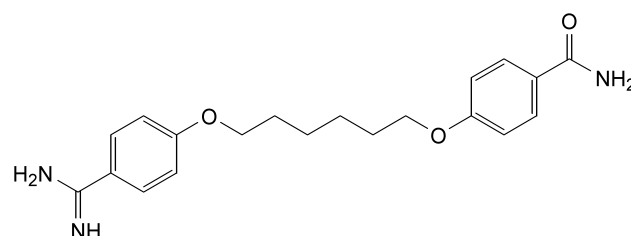
Dissolve 0.250 g in 50 ml of *dimethylformamide R*. Titrate with 0.1 M *tetrabutylammonium hydroxide* under a current of *nitrogen R*, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *tetrabutylammonium hydroxide* is equivalent to 30.35 mg of C₂₄H₃₈N₄O₁₀S₂.

STORAGE

Store in an airtight container.

IMPURITIES

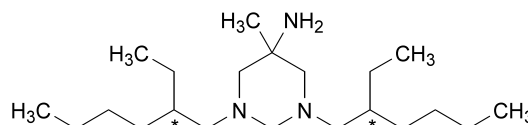


A. 4-[[6-(4-carbamimidoylphenoxy)hexyl]oxy]benzamide.

01/2005:1221

HEXETIDINE

Hexetidinum



C₂₁H₄₅N₃

M_r 339.6

DEFINITION

Hexetidine contains not less than 98.0 per cent and not more than the equivalent of 102.0 per cent of 1,3-bis(2-ethylhexyl)-5-methylhexahydropyrimidin-5-amine.

CHARACTERS

An oily liquid, colourless or slightly yellow, very slightly soluble in water, very soluble in acetone, in alcohol and in methylene chloride. It dissolves in dilute mineral acids.

IDENTIFICATION

First identification: A.

Second identification: B, C, D.

- Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *hexetidine CRS*.
- Examine the chromatograms obtained in the test for related substances. The principal spot in the chromatogram obtained with test solution (b) is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).
- To 0.2 ml add 2 ml of *sulphuric acid R* and 2 mg of *chromotropic acid, sodium salt R*. Heat in a water-bath at 60 °C. A violet colour develops.
- Dissolve 0.2 ml in 1 ml of *methylene chloride R*. Add 0.5 ml of *copper sulphate solution R*, 0.05 ml of 0.25 M *alcoholic sulphuric acid R* and 5 ml of *water R*. Shake, then allow to stand. The lower layer becomes deep blue.

TESTS

Appearance. The substance to be examined is clear (2.2.1) and not more intensely coloured than reference solution Y₅ or reference solution GY₅ (2.2.2, *Method II*).

Relative density (2.2.5): 0.864 to 0.870.

Refractive index (2.2.6): 1.461 to 1.467.

Optical rotation (2.2.7). Dissolve 1.0 g in *ethanol R* and dilute to 10.0 ml with the same solvent. The angle of optical rotation is -0.10° to $+0.10^\circ$.

Absorbance (2.2.25). Dissolve 0.50 g in *heptane R* and dilute to 50.0 ml with the same solvent. At wavelengths from 270 nm to 350 nm, the absorbance of the solution is not greater than 0.1.

Related substances. Examine by thin-layer chromatography (2.2.27), using *silica gel H R* as the coating substance. Prepare the solutions immediately before use.

Test solution (a). Dissolve 2.0 g of the substance to be examined in *heptane R* and dilute to 20 ml with the same solvent.

Test solution (b). Dilute 1 ml of test solution (a) to 10 ml with *heptane R*.

Reference solution (a). Dissolve 20 mg of *hexetidine CRS* in *heptane R* and dilute to 2 ml with the same solvent.

Reference solution (b). Dilute 1 ml of test solution (a) to 100 ml with *heptane R*.

Reference solution (c). Dilute 5 ml of reference solution (b) to 10 ml with *heptane R*.

Reference solution (d). Dissolve 10 mg of *dehydrohexetidine CRS* in test solution (a) and dilute to 10 ml with the same solution.

Apply separately to the plate 1 µl of each solution. At the bottom of a chromatography tank, place an evaporating dish containing *concentrated ammonia R1*. Place the dried plate in the tank and close the tank. Leave the plate in contact with the ammonia vapour for 15 min. Withdraw the plate and place it in a current of air to remove the ammonia vapour. Develop over a path of 15 cm using a mixture of 20 volumes of *methanol R* and 80 volumes of *toluene R*. Allow the plate to dry in air. Expose the plate to iodine vapour for 30 min. Any spot in the chromatogram obtained with test solution (a), apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (b) (1 per cent) and at most two such spots are more intense than the spot in the chromatogram obtained with reference solution (c) (0.5 per cent). The test is not valid unless the chromatogram obtained with reference solution (d) shows two clearly separated spots.

Heavy metals (2.4.8). Dissolve 2.0 g in a mixture of 15 volumes of *water R* and 85 volumes of *acetone R* and dilute to 20 ml with the same mixture of solvents. 12 ml of the solution complies with limit test B for heavy metals (10 ppm). Prepare the standard using lead standard solution (1 ppm Pb) obtained by diluting *lead standard solution* (100 ppm Pb) *R* with a mixture of 15 volumes of *water R* and 85 volumes of *acetone R*.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

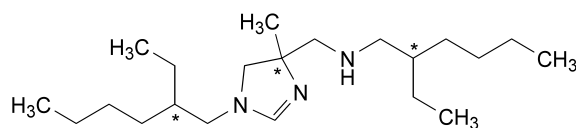
Dissolve 0.150 g in 80 ml of *anhydrous acetic acid R*. Titrate with 0.1 M *perchloric acid*, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *perchloric acid* is equivalent to 16.98 mg of C₂₁H₄₅N₃.

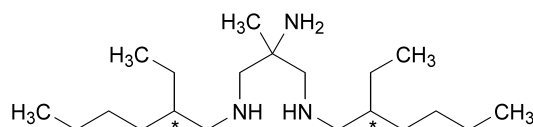
STORAGE

Store protected from light.

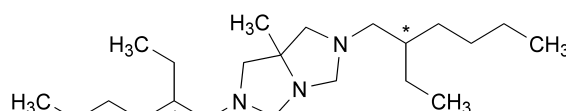
IMPURITIES



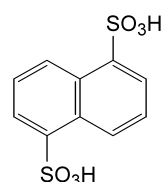
A. 2-ethyl-N-[[1-(2-ethylhexyl)-4-methyl-4,5-dihydro-1H-imidazol-4-yl]methyl]hexan-1-amine (dehydrohexetidine),



B. *N',N'*-bis(2-ethylhexyl)-2-methylpropane-1,2,3-triamine (triamine),



C. 2,6-bis(2-ethylhexyl)-7a-methylhexahydro-1H-imidazo[1,5-c]imidazole (hexedine),

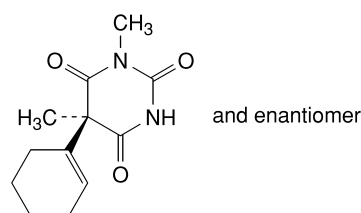


D. naphthalene-1,5-disulphonic acid.

01/2005:0183

HEXOBARBITAL

Hexobarbitalum



C₁₂H₁₆N₂O₃

*M*_r 236.3

DEFINITION

Hexobarbital contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of (5*RS*)-5-(cyclohex-1-enyl)-1,5-dimethylpyrimidine-2,4,6(1*H*, 3*H*, 5*H*)-trione, calculated with reference to the dried substance.

CHARACTERS

A white, crystalline powder, very slightly soluble in water, sparingly soluble in alcohol. It forms water-soluble compounds with alkali hydroxides and carbonates and with ammonia.

IDENTIFICATION

First identification: A, B.

Second identification: A, C, D.

- A. Determine the melting point (2.2.14) of the substance to be examined. Mix equal parts of the substance to be examined and *hexobarbital CRS* and determine the melting point of the mixture. The difference between the melting points (which are about 146 °C) is not greater than 2 °C.
- B. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *hexobarbital CRS*.
- C. Examine by thin-layer chromatography (2.2.27), using *silica gel GF₂₅₄ R* as the coating substance.
- Test solution.* Dissolve 0.1 g of the substance to be examined in *chloroform R* and dilute to 100 ml with the same solvent.
- Reference solution.* Dissolve 0.1 g of *hexobarbital CRS* in *chloroform R* and dilute to 100 ml with the same solvent.
- Apply separately to the plate 10 µl of each solution. Develop over a path of 18 cm using the lower layer of a mixture of 5 volumes of *concentrated ammonia R*, 15 volumes of *alcohol R* and 80 volumes of *chloroform R*. Examine immediately in ultraviolet light at 254 nm. The principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with the reference solution.
- D. To about 10 mg add 1.0 ml of a 10 g/l solution of *vanillin R* in *alcohol R* and 2 ml of a cooled mixture of 1 volume of *water R* and 2 volumes of *sulphuric acid R*. Shake and allow to stand for 5 min. A greenish-yellow colour develops. Heat on a water-bath for 10 min. The colour becomes dark red.

TESTS

Appearance of solution. Dissolve 1.0 g in a mixture of 4 ml of *dilute sodium hydroxide solution R* and 6 ml of *water R*. The solution is clear (2.2.1) and not more intensely coloured than reference solution Y₆ (2.2.2, *Method II*).

Acidity. Boil 1.0 g with 50 ml of *water R* for 2 min, allow to cool and filter. To 10 ml of the filtrate add 0.15 ml of *methyl red solution R*. The solution is orange-yellow. Not more than 0.1 ml of 0.1 M *sodium hydroxide* is required to produce a pure yellow colour.

Related substances. Examine by thin-layer chromatography (2.2.27), using *silica gel GF₂₅₄ R* as the coating substance.

Test solution. Dissolve 1.0 g of the substance to be examined in *chloroform R* and dilute to 100 ml with the same solvent.

Reference solution. Dilute 0.5 ml of the test solution to 100 ml with *chloroform R*.

Apply separately to the plate 20 µl of each solution. Develop over a path of 15 cm using the lower layer of a mixture of 5 volumes of *concentrated ammonia R*, 15 volumes of *alcohol R* and 80 volumes of *chloroform R*. Examine immediately in ultraviolet light at 254 nm. Any spot in the chromatogram obtained with the test solution, apart from the principal spot, is not more intense than the spot in the chromatogram obtained with the reference solution (0.5 per cent).

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.00 g by drying in an oven at 100 °C to 105 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

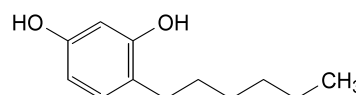
Dissolve 0.200 g in 5 ml of *pyridine R*. Add 0.5 ml of *thymolphthalein solution R* and 10 ml of *silver nitrate solution in pyridine R*. Titrate with 0.1 M *ethanolic sodium hydroxide* until a pure blue colour is obtained. Carry out a blank titration.

1 ml of 0.1 M *ethanolic sodium hydroxide* is equivalent to 23.63 mg of C₁₂H₁₆N₂O₃.

01/2005:1437

HEXYLRESORCINOL

Hexylresorcinolum

C₁₂H₁₈O₂M_r 194.3

DEFINITION

Hexylresorcinol contains not less than 98.0 per cent and not more than the equivalent of 101.0 per cent of 4-hexylbenzene-1,3-diol calculated with reference to the anhydrous substance.

CHARACTERS

A colourless, yellowish or reddish, crystalline powder or needles, turning brownish-pink on exposure to light or air, very slightly soluble in water, freely soluble in alcohol and in methylene chloride.

It shows polymorphism.

IDENTIFICATION

First identification: B.

Second identification: A, C, D.

- A. Melting point (2.2.14): 66 °C to 68 °C. Melting may occur at about 60 °C, followed by solidification and a second melting between 66 °C and 68 °C.
- B. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *hexylresorcinol CRS*. If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in *methanol R*, evaporate to dryness and record new spectra using the residues.
- C. Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel G plate R*.

Test solution. Dilute 0.1 ml of solution S (see Tests) to 10 ml with *alcohol R*.

Reference solution (a). Dissolve 10 mg of *hexylresorcinol CRS* in *alcohol R* and dilute to 10 ml with the same solvent.

Reference solution (b). Dissolve 10 mg of *hexylresorcinol CRS* and 10 mg of *resorcinol R* in *alcohol R* and dilute to 10 ml with the same solvent.

Apply to the plate 10 µl of each solution. Develop over a path corresponding to two thirds of the plate height with a mixture of equal volumes of *methyl ethyl ketone R* and *pentane R*. Allow the plate to dry in air for 5 min. Spray the plate with 3 ml of *anisaldehyde solution R* and heat at 100 °C to 105 °C for 5 min. The principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).

The test is not valid unless the chromatogram obtained with reference solution (b) shows two clearly separated principal spots.

- D. Dissolve 0.1 g in 1 ml of *alcohol R*. Add one drop of *ferric chloride solution R1*. A green colour is produced. Add *dilute ammonia R1*. The solution becomes brown.

TESTS

Solution S. Dissolve 1.0 g in *alcohol R* and dilute to 10.0 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1).

Acidity. Dissolve 0.5 g in a mixture of 25 ml of *carbon dioxide-free water R* and 25 ml of *ether R* previously neutralised to *phenolphthalein solution R1* and titrate with 0.1 M *sodium hydroxide*, shaking vigorously after each addition. Not more than 0.4 ml is required to change the colour of the solution.

Related substances. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 0.1 g of the substance to be examined in the mobile phase and dilute to 10.0 ml with the mobile phase.

Reference solution (a). Dilute 1.0 ml of the test solution to 200.0 ml with the mobile phase.

Reference solution (b). Dissolve 20.0 mg of *phenol R* in the mobile phase and dilute to 100.0 ml with the mobile phase.

Reference solution (c). Dissolve 20.0 mg of *resorcinol R* in the mobile phase and dilute to 100.0 ml with the mobile phase.

Reference solution (d). To 8.0 ml of reference solution (a) add 2.0 ml of reference solution (b), 2.0 ml of reference solution (c) and dilute to 20.0 ml with the mobile phase.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4.6 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (5 µm),
- as mobile phase at a flow rate of 1 ml/min a mixture of 25 volumes of a 3.0 g/l solution of *glacial acetic acid R*, adjusted to pH 5.9 with *dilute ammonia R1* and 75 volumes of *methanol R*,
- as detector a spectrophotometer set at 281 nm,
- a loop injector.

Inject 20 µl of reference solution (d). Adjust the sensitivity of the system so that the heights of the three principal peaks in the chromatogram obtained are at least 20 per cent of the full scale of the recorder. The test is not valid unless the resolution between the second peak (phenol) and the third peak (hexylresorcinol) is at least 5.0.

Inject 20 µl of the test solution and 20 µl of reference solution (a). Continue the chromatography of the test solution for twice the retention time of hexylresorcinol. In the chromatogram obtained with the test solution: the areas corresponding to phenol and resorcinol are not greater than the areas of the corresponding peaks in the chromatograms obtained with reference solution (d) (0.2 per cent); the area of any peak, apart from the principal peak and the peaks corresponding to phenol and resorcinol is not greater than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent); the sum of the areas of such peaks is not greater than twice the area of the principal peak obtained with reference solution (a) (1 per cent). Disregard any peak with an area less than 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (a).

Water (2.5.12). Not more than 0.5 per cent determined on 1.000 g by the semi-micro determination of water.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.100 g in 10 ml of *methanol R* in a ground-glass-stoppered flask, add 30.0 ml of 0.0167 M *potassium bromate* and 2 g of *potassium bromide R*. Shake to dissolve the substance and add 15 ml of *dilute sulphuric acid R*. Stopper the flask, shake and allow to stand in the dark for 15 min, stirring continuously. Add 5 ml of *methylene chloride R* and a solution of 1 g of *potassium iodide R* in 10 ml of *water R*, allow to stand in the dark for 15 min, stirring continuously. Titrate with 0.1 M *sodium thiosulphate*, using 1 ml of *starch solution R*, shaking thoroughly. Carry out a blank titration under the same conditions.

1 ml of 0.0167 M *potassium bromate* is equivalent to 4.857 mg of C₁₂H₁₈O₂.

STORAGE

Store in an airtight container, protected from light.

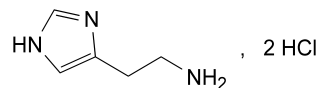
IMPURITIES

- A. phenol,
B. resorcinol.

01/2005:0143

HISTAMINE DIHYDROCHLORIDE

Histamini dihydrochloridum



C₅H₁₁Cl₂N₃

M_r 184.1

DEFINITION

Histamine dihydrochloride contains not less than 98.5 per cent and not more than the equivalent of 101.0 per cent of 2-(1H-imidazol-4-yl)ethanamine dihydrochloride, calculated with reference to the dried substance.

CHARACTERS

A white, crystalline powder or colourless crystals, hygroscopic, very soluble in water, soluble in alcohol.

IDENTIFICATION

First identification: A, D.

Second identification: B, C, D.

- A. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *histamine dihydrochloride CRS*. Examine as discs prepared using 1 mg of substance.
- B. Examine the chromatograms obtained in the test for histidine. The principal spot in the chromatogram obtained with test solution (b) is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).
- C. Dissolve 0.1 g in 7 ml of *water R* and add 3 ml of a 200 g/l solution of *sodium hydroxide R*. Dissolve 50 mg of *sulphanilic acid R* in a mixture of 0.1 ml of *hydrochloric acid R* and 10 ml of *water R* and add 0.1 ml of *sodium nitrite solution R*. Add the second solution to the first and mix. A red colour is produced.

D. It gives reaction (a) of chlorides (2.3.1).

01/2005:0144

TESTS

Solution S. Dissolve 0.5 g in *carbon dioxide-free water R* prepared from *distilled water R* and dilute to 10 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and not more intensely coloured than reference solution Y₇ (2.2.2, *Method II*).

pH (2.2.3). The pH of solution S is 2.85 to 3.60.

Histidine. Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel G plate R*.

Test solution (a). Dissolve 0.5 g of the substance to be examined in *water R* and dilute to 10 ml with the same solvent.

Test solution (b). Dilute 2 ml of test solution (a) to 10 ml with *water R*.

Reference solution (a). Dissolve 0.1 g of *histamine dihydrochloride CRS* in *water R* and dilute to 10 ml with the same solvent.

Reference solution (b). Dissolve 50 mg of *histidine monohydrochloride R* in *water R* and dilute to 100 ml with the same solvent.

Reference solution (c). Mix 1 ml of test solution (a) and 1 ml of reference solution (b).

Apply to the plate 1 µl of test solution (a), 1 µl of test solution (b), 1 µl of reference solution (a), 1 µl of reference solution (b) and 2 µl of reference solution (c). Develop over a path of 15 cm using a mixture of 5 volumes of *concentrated ammonia R*, 20 volumes of *water R* and 75 volumes of *acetonitrile R*. Dry the plate in a current of air. Repeat the development in the same direction, dry the plate in a current of air and spray with *ninhydrin solution R1*. Heat the plate at 110 °C for 10 min. Any spot corresponding to histidine in the chromatogram obtained with test solution (a) is not more intense than the spot in the chromatogram obtained with reference solution (b) (1 per cent). The test is not valid unless the chromatogram obtained with reference solution (c) shows 2 clearly separated spots.

Sulphates (2.4.13). 3 ml of solution S diluted to 15 ml with *distilled water R* complies with the limit test for sulphates (0.1 per cent).

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 0.20 g by drying in an oven at 100-105 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 0.5 g.

ASSAY

Dissolve 0.080 g in a mixture of 5.0 ml of 0.01 M *hydrochloric acid* and 50 ml of *alcohol R*. Carry out a potentiometric titration (2.2.20), using 0.1 M *sodium hydroxide*. Read the volume added between the first and third points of inflexion.

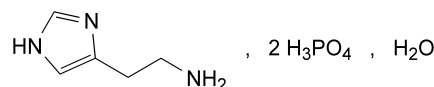
1 ml of 0.1 M *sodium hydroxide* is equivalent to 9.203 mg of C₅H₁₁Cl₂N₃.

STORAGE

Store in an airtight container, protected from light.

HISTAMINE PHOSPHATE

Histamini fosphas

C₅H₁₅N₃O₈P₂·H₂OM_r 325.2

DEFINITION

Histamine phosphate contains not less than 98.0 per cent and not more than the equivalent of 101.0 per cent of 2-(1H-imidazol-4-yl)ethanamine diphosphate, calculated with reference to the anhydrous substance.

CHARACTERS

Colourless, long prismatic crystals, freely soluble in water, slightly soluble in alcohol.

IDENTIFICATION

First identification: A, D.

Second identification: B, C, D.

- Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *histamine phosphate CRS*. Examine as discs prepared using 1 mg of substance.
- Examine the chromatograms obtained in the test for histidine. The principal spot in the chromatogram obtained with test solution (b) is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).
- Dissolve 0.1 g in 7 ml of *water R* and add 3 ml of a 200 g/l solution of *sodium hydroxide R*. Dissolve 50 mg of *sulphanilic acid R* in a mixture of 0.1 ml of *hydrochloric acid R* and 10 ml of *water R* and add 0.1 ml of *sodium nitrite solution R*. Add the second solution to the first and mix. A red colour is produced.
- It gives reaction (a) of phosphates (2.3.1).

TESTS

Solution S. Dissolve 0.5 g in *carbon dioxide-free water R* prepared from *distilled water R* and dilute to 10 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and not more intensely coloured than reference solution BY₇ (2.2.2, *Method II*).

pH (2.2.3). The pH of solution S is 3.75 to 3.95.

Histidine. Examine by thin-layer chromatography (2.2.27), using *silica gel G R* as the coating substance.

Test solution (a). Dissolve 0.5 g of the substance to be examined in *water R* and dilute to 10 ml with the same solvent.

Test solution (b). Dilute 2 ml of test solution (a) to 10 ml with *water R*.

Reference solution (a). Dissolve 0.1 g of *histamine phosphate CRS* in *water R* and dilute to 10 ml with the same solvent.

Reference solution (b). Dissolve 50 mg of *histidine monohydrochloride R* in *water R* and dilute to 100 ml with the same solvent.

Reference solution (c). Mix 1 ml of test solution (a) and 1 ml of reference solution (b).

Apply separately to the plate 1 µl of test solution (a), 1 µl of test solution (b), 1 µl of reference solution (a), 1 µl of reference solution (b) and 2 µl of reference solution (c). Develop over a path of 15 cm using a mixture of 5 volumes of *concentrated ammonia R*, 20 volumes of *water R* and 75 volumes of *acetonitrile R*. Dry the plate in a current of air. Repeat the development in the same direction, dry the plate in a current of air and spray with *ninhydrin solution R1*. Heat at 110 °C for 10 min. Any spot corresponding to histidine in the chromatogram obtained with test solution (a) is not more intense than the spot in the chromatogram obtained with reference solution (b) (1 per cent). The test is not valid unless the chromatogram obtained with reference solution (c) shows 2 clearly separated spots.

Sulphates (2.4.13). 3 ml of solution S diluted to 15 ml with *distilled water R* complies with the limit test for sulphates (0.1 per cent).

Water (2.5.12). 5.0 per cent to 6.2 per cent, determined on 0.30 g by the semi-micro determination of water.

ASSAY

Dissolve 0.140 g in 5 ml of *anhydrous formic acid R* and add 20 ml of *anhydrous acetic acid R*. Titrate with 0.1 M *perchloric acid*, determining the end-point potentiometrically (2.2.20). Carry out a blank titration. 1 ml of 0.1 M *perchloric acid* is equivalent to 15.36 mg of C₅H₁₅N₃O₈P₂.

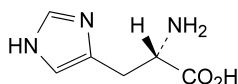
STORAGE

Store protected from light.

01/2005:0911

HISTIDINE

Histidinum



C₆H₉N₃O₂

M_r 155.2

DEFINITION

(S)-2-Amino-3-(imidazol-4-yl)propanoic acid.

Content: 98.5 per cent to 101.0 per cent (dried substance).

CHARACTERS

Appearance: white, crystalline powder or colourless crystals.

Solubility: soluble in water, very slightly soluble in alcohol.

IDENTIFICATION

First identification: A, B.

Second identification: A, C, D.

A. It complies with the test for specific optical rotation (see Tests).

B. Infrared absorption spectrophotometry (2.2.24).

Preparation: discs.

Comparison: *histidine CRS*.

If the spectra obtained show differences, dissolve the substance to be examined and the reference substance separately in the minimum volume of *water R*, evaporate to dryness at 60 °C and record new spectra using the residues.

- C. Examine the chromatograms obtained in the test for ninhydrin-positive substances. The principal spot in the chromatogram obtained with test solution (b) is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).
- D. Dissolve 0.1 g in 7 ml of *water R* and add 3 ml of a 200 g/l solution of *sodium hydroxide R*. Dissolve 50 mg of *sulphanilic acid R* in a mixture of 0.1 ml of *hydrochloric acid R* and 10 ml of *water R* and add 0.1 ml of *sodium nitrite solution R*. Add the second solution to the first and mix. An orange-red colour develops.

TESTS

Solution S. Dissolve 2.5 g in *distilled water R*, heating in a water-bath and dilute to 50 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and not more intensely coloured than reference solution BY₇ (2.2.2, *Method II*).

Specific optical rotation (2.2.7): + 11.4 to + 12.4 (dried substance).

Dissolve 2.75 g in 12.0 ml of *hydrochloric acid R1* and dilute to 25.0 ml with *water R*.

Ninhydrin-positive substances. Thin-layer chromatography (2.2.27).

Test solution (a). Dissolve 0.10 g of the substance to be examined in *water R* and dilute to 10 ml with the same solvent.

Test solution (b). Dilute 1 ml of test solution (a) to 50 ml with *water R*.

Reference solution (a). Dissolve 10 mg of *histidine CRS* in *water R* and dilute to 50 ml with the same solvent.

Reference solution (b). Dilute 5 ml of test solution (b) to 20 ml with *water R*.

Reference solution (c). Dissolve 10 mg of *histidine CRS* and 10 mg of *proline CRS* in *water R* and dilute to 25 ml with the same solvent.

Plate: TLC silica gel plate *R*.

Mobile phase: *glacial acetic acid R*, *water R*, *butanol R* (20:20:60 V/V/V).

Application: 5 µl.

Development: over 2/3 of the plate.

Drying: in air.

Detection: spray with *ninhydrin solution R* and heat at 100-105 °C for 15 min.

System suitability: the chromatogram obtained with reference solution (c) shows 2 clearly separated spots.

Limits:

- **any impurity:** any spots in the chromatogram obtained with test solution (a), apart from the principal spot, are not more intense than the spot in the chromatogram obtained with reference solution (b) (0.5 per cent).

Chlorides (2.4.4): maximum 200 ppm.

Dilute 5 ml of solution S to 15 ml with *water R*.

Sulphates (2.4.13): maximum 300 ppm.

Dilute 10 ml of solution S to 15 ml with *distilled water R*.

Ammonium (2.4.1): maximum 200 ppm, determined on 50 mg.

Prepare the standard using 0.1 ml of *ammonium standard solution (100 ppm NH₄) R*.

Iron (2.4.9): maximum 10 ppm.

In a separating funnel, dissolve 1.0 g in 10 ml of *dilute hydrochloric acid R*. Shake with 3 quantities, each of 10 ml, of *methyl isobutyl ketone R1*, shaking for 3 min each time.

To the combined organic layers add 10 ml of *water R* and shake for 3 min. The aqueous layer complies with the limit test for iron.

Heavy metals (2.4.8): maximum 10 ppm.

Dissolve 2.0 g in a mixture of 3 ml of *dilute hydrochloric acid R* and 15 ml of *water R*, with gentle warming if necessary, and dilute to 20 ml with *water R*. 12 ml of the solution complies with limit test A. Prepare the standard using *lead standard solution (1 ppm Pb) R*.

Loss on drying (2.2.32): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 100–105 °C.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.130 g in 50 ml of *water R*. Titrate with 0.1 M *hydrochloric acid*, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *hydrochloric acid* is equivalent to 15.52 mg of $C_6H_9N_3O_2$.

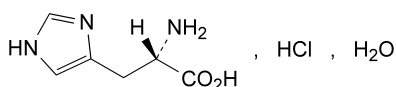
STORAGE

Protected from light.

01/2005:0910

HISTIDINE HYDROCHLORIDE MONOHYDRATE

Histidini hydrochloridum monohydricum



$C_6H_{10}ClN_3O_2 \cdot H_2O$

M_r 209.6

DEFINITION

Histidine hydrochloride monohydrate contains not less than 98.5 per cent and not more than the equivalent of 101.0 per cent of the hydrochloride of (S)-2-amino-3-(imidazol-4-yl)propanoic acid, calculated with reference to the dried substance.

CHARACTERS

A white, crystalline powder or colourless crystals, freely soluble in water, slightly soluble in alcohol.

IDENTIFICATION

First identification: A, B, C, F.

Second identification: A, B, D, E, F.

- It complies with the test for specific optical rotation (see Tests).
- It complies with the test for the pH (see Tests).
- Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *histidine hydrochloride monohydrate CRS*. Examine the substances prepared as discs.
- Examine the chromatograms obtained in the test for ninhydrin-positive substances. The principal spot in the chromatogram obtained with test solution (b) is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).
- Dissolve 0.1 g in 7 ml of *water R* and add 3 ml of a 200 g/l solution of *sodium hydroxide R*. Dissolve 50 mg of *sulphanilic acid R* in a mixture of 0.1 ml of *hydrochloric*

acid R and 10 ml of *water R* and add 0.1 ml of *sodium nitrite solution R*. Add the second solution to the first and mix. An orange-red colour develops.

F. About 20 mg gives reaction (a) of chlorides (2.3.1).

TESTS

Solution S. Dissolve 2.5 g in *carbon dioxide-free water R* prepared from *distilled water R* and dilute to 50 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and not more intensely coloured than reference solution BY₆ (2.2.2, *Method II*).

pH (2.2.3). The pH of solution S is 3.0 to 5.0.

Specific optical rotation (2.2.7). Dissolve 2.75 g in 12.0 ml of *hydrochloric acid R1* and dilute to 25.0 ml with *water R*. The specific optical rotation is + 9.2 to + 10.6, calculated with reference to the dried substance.

Ninhydrin-positive substances. Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel plate R*.

Test solution (a). Dissolve 0.10 g of the substance to be examined in *water R* and dilute to 10 ml with the same solvent.

Test solution (b). Dilute 1 ml of test solution (a) to 50 ml with *water R*.

Reference solution (a). Dissolve 10 mg of *histidine hydrochloride monohydrate CRS* in *water R* and dilute to 50 ml with the same solvent.

Reference solution (b). Dilute 5 ml of test solution (b) to 20 ml with *water R*.

Reference solution (c). Dissolve 10 mg of *histidine hydrochloride monohydrate CRS* and 10 mg of *proline CRS* in *water R* and dilute to 25 ml with the same solvent.

Apply separately to the plate 5 µl of each solution. Dry the plate in a current of air. Develop over a path of 15 cm using a mixture of 20 volumes of *glacial acetic acid R*, 20 volumes of *water R* and 60 volumes of *butanol R*. Allow the plate to dry in air. Spray with *ninhydrin solution R* and heat at 100 °C to 105 °C for 15 min. Any spot in the chromatogram obtained with test solution (a), apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.5 per cent). The test is not valid unless the chromatogram obtained with reference solution (c) shows two clearly separated principal spots.

Sulphates (2.4.13). Dilute 10 ml of solution S to 15 ml with *distilled water R*. The solution complies with the limit test for sulphates (300 ppm).

Ammonium (2.4.1). 50 mg complies with limit test B for ammonium (200 ppm). Prepare the standard using 0.1 ml of *ammonium standard solution (100 ppm NH₄) R*.

Iron (2.4.9). In a separating funnel, dissolve 1.0 g in 10 ml of *dilute hydrochloric acid R*. Shake with three quantities, each of 10 ml, of *methyl isobutyl ketone R1*, shaking for 3 min each time. To the combined organic layers add 10 ml of *water R* and shake for 3 min. The aqueous layer complies with the limit test for iron (10 ppm).

Heavy metals (2.4.8). Dissolve 2.0 g in *water R* and dilute to 20 ml with the same solvent. 12 ml of the solution complies with limit test A for heavy metals (10 ppm). Prepare the standard using *lead standard solution (1 ppm Pb) R*.

Loss on drying (2.2.32): 7.0 per cent to 10.0 per cent, determined on 1.000 g by drying in an oven at 145 °C to 150 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.160 g in 50 ml of *carbon dioxide-free water R*. Titrate with 0.1 M *sodium hydroxide*, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *sodium hydroxide* is equivalent to 19.16 mg of $C_{16}H_{22}BrNO_3$.

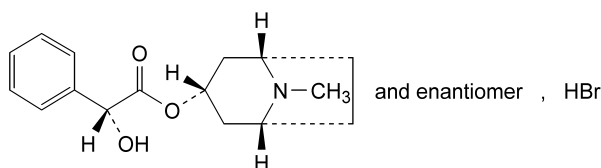
STORAGE

Store protected from light.

01/2005:0500

HOMATROPINE HYDROBROMIDE

Homatropini hydrobromidum


 $C_{16}H_{22}BrNO_3$
 M_r 356.3

DEFINITION

(1*R*,3*r*,5*S*)-8-Methyl-8-azabicyclo[3.2.1]oct-3-yl (2*RS*)-2-hydroxy-2-phenylacetate hydrobromide.

Content: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

Appearance: white, crystalline powder or colourless crystals.

Solubility: freely soluble in water, sparingly soluble in alcohol.

mp: about 215 °C, with decomposition.

IDENTIFICATION

First identification: A, C.

Second identification: B, C.

A. Infrared absorption spectrophotometry (2.2.24).

Comparison: homatropine hydrobromide CRS.

B. Dissolve 50 mg in 1 ml of *water R* and add 2 ml of *dilute acetic acid R*. Heat and add 4 ml of *picric acid solution R*. Allow to cool, shaking occasionally. Collect the crystals, wash with 2 quantities, each of 3 ml, of *iced water R* and dry at 100-105 °C. The crystals melt (2.2.14) at 182 °C to 186 °C.

C. It gives reaction (a) of bromides (2.3.1).

TESTS

Solution S. Dissolve 1.25 g in *carbon dioxide-free water R* and dilute to 25 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and colourless (2.2.2, *Method II*).

pH (2.2.3): 5.0 to 6.5 for solution S.

Related substances. Liquid chromatography (2.2.29).

Test solution. Dissolve 50.0 mg of the substance to be examined in the mobile phase and dilute to 25.0 ml with the mobile phase.

Reference solution (a). Dilute 5.0 ml of the test solution to 100.0 ml with the mobile phase. Dilute 5.0 ml of this solution to 50.0 ml with the mobile phase.

Reference solution (b). Dilute 5.0 ml of reference solution (a) to 25.0 ml with the mobile phase.

Reference solution (c). Dissolve 5.0 mg of *hyoscine hydrobromide CRS* in the mobile phase and dilute to 50.0 ml with the mobile phase. To 10.0 ml of this solution add 0.5 ml of the test solution and dilute to 100.0 ml with the mobile phase.

Column:

- *size*: $l = 0.1$ m, $\varnothing = 4.6$ mm,
- *stationary phase*: octadecylsilyl silica gel for chromatography R (3 μ m),
- *temperature*: 40 °C.

Mobile phase: mix 33 volumes of *methanol R2* and 67 volumes of a solution prepared as follows: dissolve 6.8 g of *potassium dihydrogen phosphate R* and 7.0 g of *sodium heptanesulphonate monohydrate R* in 1000 ml of *water R* and adjust to pH 2.7 with a 330 g/l solution of *phosphoric acid R*.

Flow rate: 1.5 ml/min.

Detection: spectrophotometer at 210 nm.

Injection: 10 μ l.

Run time: 3 times the retention time of homatropine.

Relative retention with reference to homatropine (retention time = about 6.8 min): impurity C = about 0.2; impurity A = about 0.9; impurity B = about 1.1; impurity D = about 1.9.

System suitability: reference solution (c):

- *resolution*: minimum 1.5 between the peaks due to homatropine and impurity B,
- *symmetry factor*: maximum 2.5 for the peak due to homatropine.

Limits:

- *impurity A*: not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent),
- *impurities B, C, D*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent),
- *any other impurity*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent),
- *total*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (1.0 per cent); disregard the peak due to the bromide ion which appears close to the peak due to the solvent,
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

Loss on drying (2.2.32): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 100-105 °C.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.300 g in a mixture of 5.0 ml of 0.01 M *hydrochloric acid* and 50 ml of *alcohol R*. Carry out a potentiometric titration (2.2.20), using 0.1 M *sodium hydroxide*. Read the volume added between the 2 points of inflexion.

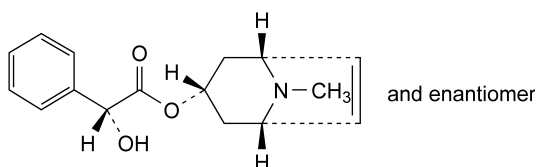
1 ml of 0.1 M *sodium hydroxide* is equivalent to 35.63 mg of $C_{16}H_{22}BrNO_3$.

STORAGE

Protected from light.

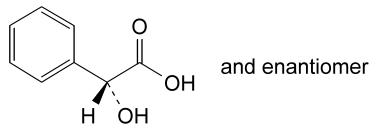
IMPURITIES

Specified impurities: A, B, C, D.



A. (1*R*,3*s*,5*S*)-8-methyl-8-azabicyclo[3.2.1]oct-6-en-3-yl (2*RS*)-2-hydroxy-2-phenylacetate (dehydrohomatropine),

B. hyoscyne,



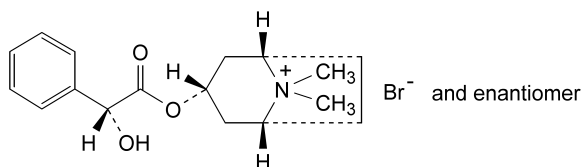
C. (2*RS*)-2-hydroxy-2-phenylacetic acid (mandelic acid),

D. atropine.

01/2005:0720

HOMATROPINE METHYLBROMIDE

Homatropini methylbromidum



C₁₇H₂₄BrNO₃

*M*_r 370.3

DEFINITION

(1*R*,3*r*,5*S*)-3-[[[(2*RS*)-2-hydroxy-2-phenylacetyl]oxy]-8,8-dimethyl-8-azoniabicyclo[3.2.1]octane bromide.

Content: 98.5 per cent to 101.0 per cent (dried substance).

CHARACTERS

Appearance: white, crystalline powder or colourless crystals.

Solubility: freely soluble in water, soluble in alcohol.

mp: about 190 °C.

IDENTIFICATION

First identification: A, C.

Second identification: B, C.

A. Infrared absorption spectrophotometry (2.2.24),

Comparison: homatropine methylbromide CRS.

B. Dissolve 50 mg in 1 ml of *water R* and add 2 ml of *dilute acetic acid R*. Heat and add 4 ml of *picric acid solution R*. Allow to cool, shaking occasionally. The crystals, washed with 2 quantities, each of 3 ml, of iced *water R* and dried at 100-105 °C melt (2.2.14) at 132 °C to 138 °C.

C. It gives reaction (a) of bromides (2.3.1).

TESTS

Solution S. Dissolve 1.25 g in *carbon dioxide-free water R* and dilute to 25 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and colourless (2.2.2, *Method II*).

pH (2.2.3): 4.5 to 6.5 for solution S.

Related substances. Liquid chromatography (2.2.29).

Solvent mixture: acetonitrile *R*, mobile phase A (9:41 *V/V*).

Test solution. Dissolve 50.0 mg of the substance to be examined in the solvent mixture and dilute to 25.0 ml with the solvent mixture.

Reference solution (a). Dilute 5.0 ml of the test solution to 100.0 ml with the solvent mixture. Dilute 5.0 ml of the solution to 50.0 ml with the solvent mixture.

Reference solution (b). Dilute 5.0 ml of reference solution (a) to 25.0 ml with the solvent mixture.

Reference solution (c). Dissolve 5.0 mg of *homatropine hydrobromide CRS* in the solvent mixture and dilute to 50.0 ml with the solvent mixture. To 10.0 ml of the solution add 0.5 ml of the test solution and dilute to 100.0 ml with the solvent mixture.

Column:

- *size*: *l* = 0.15 m, Ø = 4.6 mm,
- *stationary phase*: octadecylsilyl silica gel for chromatography *R* (3 µm),
- *temperature*: 25 °C.

Mobile phase:

- *mobile phase A*: dissolve 3.4 g of *potassium dihydrogen phosphate R* and 5.0 g of *sodium heptanesulphonate monohydrate R* in 1000 ml of *water R*, and adjust to pH 3.0 with a 330 g/l solution of *phosphoric acid R*,
- *mobile phase B*: mix 400 ml of mobile phase A and 600 ml of *acetonitrile R*,

Time (min)	Mobile phase A (per cent <i>V/V</i>)	Mobile phase B (per cent <i>V/V</i>)
0 - 2	70	30
2 - 15	70 → 30	30 → 70
15 - 20	30 → 70	70 → 30

Flow rate: 1.4 ml/min.

Detection: spectrophotometer at 210 nm.

Injection: 10 µl.

Relative retention with reference to homatropine methylbromide (retention time = about 4.8 min): impurity C = about 0.7; impurity A = about 0.9; impurity B = about 1.2; impurity D = about 1.3; impurity E = about 1.4; impurity F = about 1.7.

System suitability: reference solution (c):

- *resolution*: minimum 2.5 between the peaks due to homatropine methylbromide and impurity B,
- *symmetry factor*: maximum 2.5 for the peak due to homatropine methylbromide.

Limits:

- *impurities A, B*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent),
- *impurities C, D, E, F*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent),
- *any other impurity*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent),
- *total*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (1.0 per cent); disregard the peak due to the bromide ion which appears close to the peak due to the solvent,
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

Loss on drying (2.2.32): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 100-105 °C.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.300 g in 10 ml of *water R*. Titrate with 0.1 M *silver nitrate*. Determine the end-point potentiometrically (2.2.20), using a silver indicator electrode and a silver-silver chloride reference electrode.

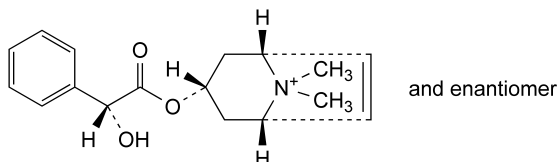
1 ml of 0.1 M *silver nitrate* is equivalent to 37.03 mg of $C_{17}H_{24}BrNO_3$.

STORAGE

Protected from light.

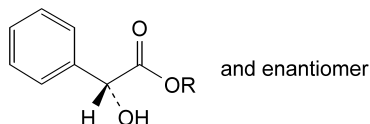
IMPURITIES

Specified impurities: A, B, C, D, E, F.



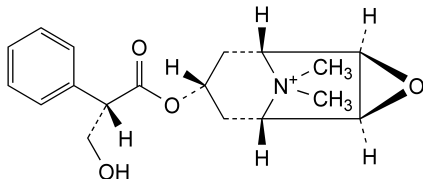
A. (1R,3s,5S)-3-[[[(2RS)-2-hydroxy-2-phenylacetyl]oxy]-8,8-dimethyl-8-azoniabicyclo[3.2.1]oct-6-ene (methyldehydrohomatropine),

B. homatropine,



C. R = H: (2RS)-2-hydroxy-2-phenylacetic acid (mandelic acid),

F. R = CH₃: methyl (2RS)-2-hydroxy-2-phenylacetate (methyl mandelate),



D. (1R,2R,4S,5S,7s)-7-[[[(2S)-3-hydroxy-2-phenylpropanoyl]oxy]-9,9-dimethyl-3-oxa-9-azoniatricyclo[3.3.1.0^{2,4}]nonane (methylhyoscine),

E. methylatropine.

- are longer and asymmetrical at the base because of a fold generally encircling an induviate fruit (achene). The ovary or rarely the fruit, the base of the bracts and especially the induvial fold, are covered with small orange-yellow glands.
- B. Reduce to a powder (355). The powder is greenish-yellow. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters: fragments of bracts and bracteoles covered by polygonal, irregular epidermal cells with wavy walls; unicellular, conical, straight or curved covering trichomes with thin, smooth walls; rare anomocytic stomata; fragments of mesophyll containing small calcium oxalate cluster crystals; many characteristic orange-yellow glandular trichomes with short, bicellular biseriate stalks, bearing a part widening into a cup, 150 µm to 250 µm in diameter, made up of a hemispherical layer of secretory cells with a cuticle that has been detached and distended by the accumulation of oleoresinous secretions; fragments of elongated sclerenchymatous cells of the testa with thick walls showing striations and numerous pits.

- C. Examine by thin-layer chromatography (2.2.27), using as the coating substance a suitable silica gel with a fluorescent indicator having an optimal intensity at 254 nm.

Test solution. To 1.0 g of the freshly powdered drug (355), add 10 ml of a mixture of 3 volumes of *water R* and 7 volumes of *methanol R*; shake for 15 min and filter.

Reference solution. Dissolve 1.0 mg of *Sudan orange R*, 2.0 mg of *curcumin R* and 2.0 mg of *dimethylaminobenzaldehyde R* in 20 ml of *methanol R*.

Apply to the plate as bands, 20 µl of each solution.

Develop over a path of 15 cm using a mixture of 2 volumes of *anhydrous acetic acid R*, 38 volumes of *ethyl acetate R* and 60 volumes of *cyclohexane R*. Allow the plate to dry in air and examine in ultraviolet light at 254 nm.

The chromatogram obtained with the reference solution shows three quenching bands; in the lower quarter is the faint curcumin band, somewhat below the middle is the dimethylaminobenzaldehyde band and above the band due to Sudan orange. The chromatogram obtained with the test solution shows a number of quenching bands similar in position to the bands in the chromatogram obtained with the reference solution; at about the level of curcumin is a faint band due to xanthohumol, near the level of dimethylaminobenzaldehyde are bands due to humulones, and near the level of Sudan orange are bands due to lupulones. Examine in ultraviolet light at 365 nm. In the chromatogram obtained with the test solution the bands due to lupulones show blue fluorescence, the bands due to humulones brown fluorescence and the band due to xanthohumol dark brown fluorescence. Spray with *dilute phosphomolybdotungstic reagent R*. Expose the plate to ammonia vapour. Examine in daylight. In the chromatogram obtained with the test solution, the bands due to humulones and to lupulones are bluish-grey and the band due to xanthohumol is greenish-grey. In the chromatogram obtained with the reference solution, the bands are bluish-grey to brownish-grey.

TESTS

Foreign matter (2.8.2). It complies with the test for foreign matter.

Matter extractable by alcohol (70 per cent V/V). To 10.0 g of the powdered drug (355) add 300 ml of *alcohol (70 per cent V/V) R* and heat for 10 min on a water bath under a reflux condenser. Allow to cool, filter and discard the first 10 ml of the filtrate. Evaporate 30.0 ml of the filtrate to

01/2005:1222

HOP STROBILE

Lupuli flos

DEFINITION

Hop strobile consists of the dried, generally whole, female inflorescences of *Humulus lupulus* L.

CHARACTERS

It has a characteristic, aromatic odour.

It has the macroscopic and microscopic characters described in identification tests A and B.

IDENTIFICATION

- A. Hop strobiles are generally isolated and 2 cm to 5 cm long, petiolate, ovoid, made up of many oval, greenish-yellow, sessile, membranous, overlapping bracts. The external bracts are flattened and symmetrical. The internal bracts

dryness on a water-bath and dry in an oven at 100 °C to 105 °C for 2 h. The mass of the residue is not less than 0.250 g (25.0 per cent).

Loss on drying (2.2.32). Not more than 10.0 per cent, determined on 1.000 g of the powdered drug (355) by drying in an oven at 100 °C to 105 °C for 2 h.

Total ash (2.4.16). Not more than 12.0 per cent.

STORAGE

Store protected from light.

01/2005:0255

HUMAN ALBUMIN SOLUTION

Albumini humani solutio

DEFINITION

Human albumin solution is an aqueous solution of protein obtained from plasma that complies with the requirements of the monograph on *Plasma for fractionation, human* (0853).

PRODUCTION

Separation of the albumin is carried out under controlled conditions, particularly of pH, ionic strength and temperature so that in the final product not less than 95 per cent of the total protein is albumin. Human albumin solution is prepared as a concentrated solution containing 150 g/l to 250 g/l of total protein or as an isotonic solution containing 35 g/l to 50 g/l of total protein. A suitable stabiliser against the effects of heat, such as sodium caprylate (sodium octanoate) or *N*-acetyltryptophan or a combination of these two, at a suitable concentration, may be added but no antimicrobial preservative is added at any stage during preparation. The solution is passed through a bacteria-retentive filter and distributed aseptically into sterile containers which are then closed so as to prevent contamination. The solution in its final container is heated to 60 ± 1.0 °C and maintained at this temperature for not less than 10 h. The containers are then incubated at 30–32 °C for not less than 14 days or at 20–25 °C for not less than 4 weeks and examined visually for evidence of microbial contamination.

CHARACTERS

A clear, slightly viscous liquid; it is almost colourless, yellow, amber or green.

IDENTIFICATION

Examine by a suitable immunoelectrophoresis technique. Using antiserum to normal human serum, compare normal human serum and the preparation to be examined, both diluted to contain 10 g/l of protein. The main component of the preparation to be examined corresponds to the main component of normal human serum. The preparation may show the presence of small quantities of other plasma proteins.

TESTS

pH (2.2.3): 6.7 to 7.3.

Dilute the preparation to be examined with a 9 g/l solution of *sodium chloride R* to obtain a solution containing 10 g/l of protein.

Total protein. Dilute the preparation to be examined with a 9 g/l solution of *sodium chloride R* to obtain a solution containing about 15 mg of protein in 2 ml. To 2.0 ml of

this solution in a round-bottomed centrifuge tube add 2 ml of a 75 g/l solution of *sodium molybdate R* and 2 ml of a mixture of 1 volume of *nitrogen-free sulphuric acid R* and 30 volumes of *water R*. Shake, centrifuge for 5 min, decant the supernatant liquid and allow the inverted tube to drain on filter paper. Determine the nitrogen in the residue by the method of sulphuric acid digestion (2.5.9) and calculate the quantity of protein by multiplying by 6.25. The preparation contains not less than 95 per cent and not more than 105 per cent of the quantity of protein stated on the label.

Protein composition. Zone electrophoresis (2.2.31).

Use strips of suitable cellulose acetate gel as the supporting medium and *barbital buffer solution pH 8.6 R1* as the electrolyte solution.

Test solution. Dilute the preparation to be examined with a 9 g/l solution of *sodium chloride R* to a protein concentration of 20 g/l.

Reference solution. Dilute *human albumin for electrophoresis BRP* with a 9 g/l solution of *sodium chloride R* to a protein concentration of 20 g/l.

To a strip apply 2.5 µl of the test solution as a 10 mm band or apply 0.25 µl per millimetre if a narrower strip is used. To another strip, apply in the same manner the same volume of the reference solution. Apply a suitable electric field such that the most rapid band migrates at least 30 mm. Treat the strips with *amido black 10B solution R* for 5 min. Decolorise with a mixture of 10 volumes of *glacial acetic acid R* and 90 volumes of *methanol R* until the background is just free of colour. Develop the transparency of the strips with a mixture of 19 volumes of *glacial acetic acid R* and 81 volumes of *methanol R*. Measure the absorbance of the bands at 600 nm in an instrument having a linear response over the range of measurement. Calculate the result as the mean of 3 measurements of each strip. In the electropherogram obtained with the test solution, not more than 5 per cent of the protein has a mobility different from that of the principal band. The test is not valid unless, in the electropherogram obtained with the reference preparation, the proportion of protein in the principal band is within the limits stated in the leaflet accompanying the reference preparation.

Molecular size distribution. Liquid chromatography (2.2.29).

Test solution. Dilute the preparation to be examined with a 9 g/l solution of *sodium chloride R* to a concentration suitable for the chromatographic system used. A concentration in the range 4 g/l to 12 g/l and injection of 50 µg to 600 µg of protein are usually suitable.

Column:

- size: $l = 0.6$ m, $\varnothing = 7.5$ mm,
- stationary phase: *hydrophilic silica gel for chromatography R*, of a grade suitable for fractionation of globular proteins with relative molecular masses in the range 10 000 to 500 000.

Mobile phase: dissolve 4.873 g of *disodium hydrogen phosphate dihydrate R*, 1.741 g of *sodium dihydrogen phosphate monohydrate R*, 11.688 g of *sodium chloride R* and 50 mg of *sodium azide R* in 1 litre of *water R*.

Flow rate: 0.5 ml/min.

Detection: spectrophotometer at 280 nm.

The peak due to polymers and aggregates is located in the part of the chromatogram representing the void volume. Disregard the peak due to the stabiliser. The area of the peak due to polymers and aggregates is not greater than 10 per cent of the total area of the chromatogram (corresponding to about 5 per cent of polymers and aggregates).

Haem. Dilute the preparation to be examined using a 9 g/l solution of *sodium chloride R* to obtain a solution containing 10 g/l of protein. The absorbance (2.2.25) of the solution measured at 403 nm using *water R* as the compensation liquid is not greater than 0.15.

Prekallikrein activator (2.6.15): maximum 35 IU/ml.

Aluminium: maximum 200 µg of Al per litre.

Atomic absorption spectrometry (2.2.23, *Method I*). Use a furnace as atomic generator. Use plastic containers for preparation of the solutions. Wash equipment in nitric acid (200 g/l HNO_3) before use.

Test solution. Use the preparation to be examined.

Validation solution. Use human albumin for aluminium validation BRP.

Reference solutions. Prepare a suitable range of reference solutions by adding suitable volumes of aluminium standard solution (10 ppm Al) *R* to known volumes of *water R*.

Dilute the solutions as necessary using nitric acid (10 g/l HNO_3) containing 1.7 g/l of *magnesium nitrate R* and 0.05 per cent V/V of *octoxinol 10 R*. Measure the absorbance at 309.3 nm. The test is not valid unless the aluminium content determined for human albumin for aluminium validation BRP is within 20 per cent of the value stated in the leaflet accompanying the reference preparation.

Potassium: maximum 0.05 mmol of K per gram of protein.

Atomic emission spectrometry (2.2.22, *Method I*).

Wavelength: 766.5 nm.

Sodium: maximum 160 mmol of Na per litre and not less than 95 per cent and not more than 105 per cent of the content of Na stated on the label. Atomic emission spectrometry (2.2.22, *Method I*).

Wavelength: 589 nm.

Sterility (2.6.1). It complies with the test for sterility.

Pyrogens (2.6.8). It complies with the test for pyrogens. For a solution containing 35 g/l to 50 g/l of protein, inject per kilogram of the rabbit's mass 10 ml of the preparation to be examined. For a solution containing 150 g/l to 250 g/l of protein, inject per kilogram of the rabbit's mass 5 ml of the preparation to be examined.

STORAGE

Protected from light.

LABELLING

The label states:

- the name of the preparation,
- the volume of the preparation,
- the content of protein expressed in grams per litre,
- the content of sodium expressed in millimoles per litre,
- that the product is not to be used if it is cloudy or if a deposit has formed,
- the name and concentration of any added substance (for example stabiliser).

01/2005:0557

HUMAN ANTI-D IMMUNOGLOBULIN

Immunoglobulinum humanum anti-D

DEFINITION

Human anti-D immunoglobulin is a liquid or freeze-dried preparation containing immunoglobulins, mainly immunoglobulin G. The preparation is intended for

intramuscular administration. It contains specific antibodies against erythrocyte D-antigen and may also contain small quantities of other blood-group antibodies. *Human normal immunoglobulin (0338)* may be added.

It complies with the monograph on *Human normal immunoglobulin (0338)*, except for the minimum number of donors and the minimum total protein content. For products prepared by a method that eliminates immunoglobulins with specificities other than anti-D, where authorised, the test for antibodies to hepatitis B surface antigen is not required.

PRODUCTION

Human anti-D immunoglobulin is preferably obtained from the plasma of donors with a sufficient titre of previously acquired anti-D antibodies. Where necessary, in order to ensure an adequate supply of human anti-D immunoglobulin, it is obtained from plasma derived from donors immunised with D-positive erythrocytes that are compatible in relevant blood group systems in order to avoid formation of undesirable antibodies.

ERYTHROCYTE DONORS

Erythrocyte donors complies with the requirements for donors prescribed in the monograph *Human plasma for fractionation (0853)*.

IMMUNISATION

Immunisation of the plasma donor is carried out under proper medical supervision. Recommendations concerning donor immunisation, including testing of erythrocyte donors, have been formulated by the World Health Organisation (*Requirements for the collection, processing and quality control of blood, blood components and plasma derivatives*, WHO Technical Report Series, No. 840, 1994 or subsequent revision).

POOLED PLASMA

To limit the potential B19 virus burden in plasma pools used for the manufacture of anti-D immunoglobulin, the plasma pool is tested for B19 virus using validated nucleic acid amplification techniques (2.6.21).

B19 virus DNA: maximum 10^4 IU/ml.

A positive control with 10^4 IU of B19 virus DNA per millilitre and, to test for inhibitors, an internal control prepared by addition of a suitable marker to a sample of the plasma pool are included in the test. The test is invalid if the positive control is non-reactive or if the result obtained with the internal control indicates the presence of inhibitors.

B19 virus DNA for NAT testing BRP is suitable for use as a positive control.

If *Human normal immunoglobulin (0338)* is added to the preparation, the plasma pool from which it is derived complies with the above requirement for B19 virus DNA.

POTENCY

Carry out the assay of human anti-D immunoglobulin (2.7.13, *Method A*). The estimated potency is not less than 90 per cent of the stated potency. The confidence limits ($P = 0.95$) are not less than 80 per cent and not more than 120 per cent of the estimated potency.

Method B or C (2.7.13) may be used for potency determination if a satisfactory correlation with the results obtained by Method A has been established for the particular product.

STORAGE

See *Human normal immunoglobulin (0338)*.

LABELLING

See *Human normal immunoglobulin (0338)*.

The label states the number of International Units per container.

01/2005:1527
corrected

HUMAN ANTI-D IMMUNOGLOBULIN FOR INTRAVENOUS ADMINISTRATION

Immunoglobulinum humanum anti-D ad usum intravenosum

DEFINITION

Human anti-D immunoglobulin for intravenous administration is a liquid or freeze-dried preparation containing immunoglobulins, mainly immunoglobulin G. It contains specific antibodies against erythrocyte D-antigen and may also contain small quantities of other blood-group antibodies. *Human normal immunoglobulin for intravenous administration (0918)* may be added.

It complies with the monograph on *Human normal immunoglobulin for intravenous administration (0918)*, except for the minimum number of donors, the minimum total protein content, the limit for osmolality and the limit for prekallikrein activator. For products prepared by a method that eliminates immunoglobulins with specificities other than anti-D: where authorised, the test for antibodies to hepatitis B surface antigen is not required; a suitable test for Fc function is carried out instead of that described in chapter 2.7.9, which is not applicable to such a product.

PRODUCTION

Human anti-D immunoglobulin is preferably obtained from the plasma of donors with a sufficient titre of previously acquired anti-D antibodies. Where necessary, in order to ensure an adequate supply of human anti-D immunoglobulin, it is obtained from plasma derived from donors immunised with D-positive erythrocytes that are compatible in relevant blood group systems in order to avoid formation of undesirable antibodies.

ERYTHROCYTE DONORS

Erythrocyte donors comply with the requirements for donors prescribed in the monograph *Human plasma for fractionation (0853)*.

IMMUNISATION

Immunisation of the plasma donor is carried out under proper medical supervision. Recommendations concerning donor immunisation, including testing of erythrocyte donors, have been formulated by the World Health Organisation (*Requirements for the collection, processing and quality control of blood, blood components and plasma derivatives*, WHO Technical Report Series, No. 840, 1994 or subsequent revision).

POOLED PLASMA

To limit the potential B19 virus burden in plasma pools used for the manufacture of anti-D immunoglobulin, the plasma pool is tested for B19 virus using validated nucleic acid amplification techniques (2.6.21).

B19 virus DNA: maximum 10^4 IU/ml.

A positive control with 10^4 IU of B19 virus DNA per millilitre and, to test for inhibitors, an internal control prepared by addition of a suitable marker to a sample of the plasma pool

are included in the test. The test is invalid if the positive control is non-reactive or if the result obtained with the internal control indicates the presence of inhibitors.

B19 virus DNA for NAT testing BRP is suitable for use as a positive control.

If *Human normal immunoglobulin for intravenous administration (0918)* is added to the preparation, the plasma pool from which it is derived complies with the above requirement for B19 virus DNA.

POTENCY

Carry out the assay of human anti-D immunoglobulin (2.7.13, *Method A*). The estimated potency is not less than 90 per cent of the stated potency. The confidence limits ($P = 0.95$) are not less than 80 per cent and not more than 120 per cent of the estimated potency.

Method B or C (2.7.13) may be used for potency determination if a satisfactory correlation with the results obtained by Method A has been established for the particular product.

STORAGE

See *Human normal immunoglobulin for intravenous administration (0918)*.

LABELLING

See *Human normal immunoglobulin for intravenous administration (0918)*.

The label states the number of International Units per container.

01/2005:0878

HUMAN ANTITHROMBIN III CONCENTRATE

Antithrombinum III humanum densatum

DEFINITION

Human antithrombin III concentrate is a preparation of a glycoprotein fraction obtained from human plasma that inactivates thrombin in the presence of an excess of heparin. It is obtained from plasma that complies with the requirements of the monograph on *Human plasma for fractionation (0853)*.

When reconstituted in the volume of solvent stated on the label, the potency is not less than 25 IU of antithrombin III per millilitre.

PRODUCTION

The method of preparation includes a step or steps that have been shown to remove or to inactivate known agents of infection; if substances are used for inactivation of viruses during production, the subsequent purification procedure must be validated to demonstrate that the concentration of these substances is reduced to a suitable level and any residues are such as not to compromise the safety of the preparation for patients.

The antithrombin III is purified and concentrated and a suitable stabiliser may be added. The specific activity is not less than 3 IU of antithrombin III per milligram of total protein, excluding albumin. The antithrombin III concentrate is passed through a bacteria-retentive filter, distributed aseptically into its final, sterile containers and immediately frozen. It is then freeze-dried and the containers are closed under vacuum or in an atmosphere of inert gas. No antimicrobial preservative is added at any stage of production.

VALIDATION TEST

It shall be demonstrated that the manufacturing process yields a product that consistently complies with the following test.

Heparin-binding fraction. Examine by agarose gel electrophoresis (2.2.31). Prepare a 10 g/l solution of *agarose for electrophoresis R* containing 15 IU of *heparin R* per millilitre in *barbital buffer solution pH 8.4 R*. Pour 5 ml of this solution onto a glass plate 5 cm square. Cool at 4 °C for 30 min. Cut 2 wells 2 mm in diameter 1 cm and 4 cm from the side of the plate and 1 cm from the cathode. Introduce into one well 5 µl of the preparation to be examined, diluted to an activity of about 1 IU of antithrombin III per millilitre. Introduce into the other well 5 µl of a solution of a marker dye such as *bromophenol blue R*. Allow the electrophoresis to proceed at 4 °C, using a constant electric field of 7 V/cm, until the dye reaches the anode.

Cut across the agarose gel 1.5 cm from that side of the plate on which the preparation to be examined was applied and remove the larger portion of the gel leaving a band 1.5 cm wide containing the material to be examined. Replace the removed portion with an even layer consisting of 3.5 ml of a 10 g/l solution of *agarose for electrophoresis R* in *barbital buffer solution pH 8.4 R*, containing a rabbit anti-human antithrombin III antiserum at a suitable concentration, previously determined, to give adequate peak heights of at least 1.5 cm. Place the plate with the original gel at the cathode so that a second electrophoretic migration can occur at right angles to the first. Allow this second electrophoresis to proceed using a constant electric field of 2 V/cm for 16 h. Cover the plates with filter paper and several layers of thick lint soaked in a 9 g/l solution of *sodium chloride R* and compress for 2 h, renewing the saline several times. Rinse with *water R*, dry the plates and stain with *acid blue 92 solution R*.

Calculate the fraction of antithrombin III bound to heparin, which is the peak closest to the anode, with respect to the total amount of antithrombin III, by measuring the area defined by the 2 precipitation peaks.

The fraction of antithrombin III able to bind to heparin is not less than 60 per cent.

CHARACTERS

A white, hygroscopic, friable solid or a powder.

Reconstitute the preparation to be examined as stated on the label immediately before carrying out the identification, the tests (except those for solubility, total protein and water), and the assay.

IDENTIFICATION

It complies with the limits of the assay.

TESTS

pH (2.2.3): 6.0 to 7.5.

Solubility. It dissolves completely under gentle swirling within 10 min in the volume of the solvent stated on the label, forming a clear or slightly turbid, colourless solution.

Osmolality (2.2.35): minimum 240 mosmol/kg.

Total protein. If necessary, dilute an accurately measured volume of the preparation to be examined with *water R* to obtain a solution containing about 15 mg of protein in 2 ml. To 2.0 ml of the solution in a round-bottomed centrifuge tube add 2 ml of a 75 g/l solution of *sodium molybdate R* and 2 ml of a mixture of 1 volume of *nitrogen-free sulphuric acid R* and 30 volumes of *water R*. Shake, centrifuge for 5 min, decant the supernatant liquid and allow the inverted tube to drain on filter paper. Determine the nitrogen in the

residue by the method of sulphuric acid digestion (2.5.9) and calculate the amount of protein by multiplying the result by 6.25.

Heparin (2.7.5): maximum 0.1 IU of heparin activity per International Unit of antithrombin III activity. It is necessary to validate the method for assay of heparin for each specific preparation to be examined to allow for interference by antithrombin III.

Water. Determined by a suitable method, such as the semi-micro determination of water (2.5.12), loss on drying (2.2.32) or near infrared spectrophotometry (2.2.40), the water content is within the limits approved by the competent authority.

Sterility (2.6.1). It complies with the test for sterility.

Pyrogens (2.6.8). It complies with the test for pyrogens. Inject per kilogram of the rabbit's mass a volume of the preparation to be examined equivalent to 50 IU of antithrombin III, calculated from the activity stated on the label.

ASSAY

Assay of human antithrombin III (2.7.17).

The estimated potency is not less than 80 per cent and not more than 120 per cent of the potency stated on the label. The confidence limits ($P = 0.95$) are not less than 90 per cent and not more than 110 per cent of the estimated potency.

STORAGE

Protected from light, in an airtight container.

LABELLING

The label states:

- the content of antithrombin III expressed in International Units per container.
- the name and volume of solvent to be used to reconstitute the preparation,
- where applicable, the amount of albumin present as a stabiliser.

01/2005:1224

HUMAN COAGULATION FACTOR VII

Factor VII coagulationis humanus

DEFINITION

Human coagulation factor VII is a plasma protein fraction that contains the single-chain glycoprotein factor VII and may also contain small amounts of the activated form, the two-chain derivative factor VIIa. It may also contain coagulation factors II, IX and X and protein C and protein S. It is obtained from human plasma that complies with the monograph on *Human plasma for fractionation* (0853).

The potency of the preparation, reconstituted as stated on the label, is not less than 15 IU of factor VII per millilitre.

PRODUCTION

The method of preparation is designed to minimise activation of any coagulation factor (to minimise potential thrombogenicity) and includes a step or steps that have been shown to remove or to inactivate known agents of infection; if substances are used for inactivation of viruses during production, the subsequent purification procedure must be validated to demonstrate that the concentration of these substances is reduced to a suitable level and that any residues are such as not to compromise the safety of the preparation for patients.

The specific activity is not less than 2 IU of factor VII per milligram of total protein, before the addition of any protein stabiliser.

The factor VII fraction is dissolved in a suitable liquid. Heparin, antithrombin and other auxiliary substances such as a stabiliser may be added. No antimicrobial preservative is added. The solution is passed through a bacteria-retentive filter, distributed aseptically into the final containers and immediately frozen. It is subsequently freeze-dried and the containers are closed under vacuum or under an inert gas.

CONSISTENCY OF THE METHOD OF PRODUCTION

The consistency of the method of production with respect to the activities of factors II, IX and X of the preparation, expressed in International Units relative to the activity of factor VII, shall be demonstrated.

The consistency of the method of production with respect to the activity of factor VIIa of the preparation shall be demonstrated. The activity of factor VIIa may be determined, for example, using a recombinant soluble tissue factor that does not activate factor VII but possesses a cofactor function specific for factor VIIa; after incubation of a mixture of the recombinant soluble tissue factor with phospholipids reagent and the dilution of the test sample in factor VII-deficient plasma, calcium chloride is added and the clotting time determined; the clotting time is inversely related to the factor VIIa activity of the test sample.

CHARACTERS

A hygroscopic powder or friable solid that may be white, pale yellow, green or blue.

Reconstitute the preparation to be examined as stated on the label immediately before carrying out the identification, tests (except those for solubility and water) and assay.

IDENTIFICATION

It complies with the limits of the assay.

TESTS

Solubility. To a container of the preparation to be examined add the volume of liquid stated on the label at the recommended temperature. The preparation dissolves completely with gentle swirling within 10 min, giving a clear or slightly opalescent solution that may be coloured.

pH (2.2.3): 6.5 to 7.5.

Osmolality (2.2.35): minimum 240 mosmol/kg.

Total protein. If necessary, dilute an accurately measured volume of the reconstituted preparation with a 9 g/l solution of *sodium chloride R* to obtain a solution expected to contain about 15 mg of protein in 2 ml. To 2.0 ml of the solution in a round-bottomed centrifuge tube add 2 ml of a 75 g/l solution of *sodium molybdate R* and 2 ml of a mixture of 1 volume of *nitrogen-free sulphuric acid R* and 30 volumes of *water R*. Shake, centrifuge for 5 min, decant the supernatant liquid and allow the inverted tube to drain on filter paper. Determine the nitrogen in the residue by the method of sulphuric acid digestion (2.5.9) and calculate the amount of protein by multiplying the result by 6.25.

Activated coagulation factors (2.6.22). For each of the dilutions, the coagulation time is not less than 150 s.

Heparin. If heparin has been added during preparation, determine the amount present by the assay of heparin in coagulation factor concentrates (2.7.12). The preparation to be examined contains not more than the amount of heparin stated on the label and in any case not more than 0.5 IU of heparin per International Unit of factor VII.

Thrombin. If the preparation to be examined contains heparin, determine the amount present as described in the test for heparin and neutralise the heparin by addition of *protamine sulphate R* (10 µg of protamine sulphate neutralises 1 IU of heparin). In each of 2 test-tubes, mix equal volumes of the reconstituted preparation and a 3 g/l solution of *fibrinogen R*. Keep one of the tubes at 37 °C for 6 h and the other at room temperature for 24 h. In a third tube, mix a volume of the fibrinogen solution with an equal volume of a solution of *human thrombin R* (1 IU/ml) and place the tube in a water-bath at 37 °C. No coagulation occurs in the tubes containing the preparation to be examined. Coagulation occurs within 30 s in the tube containing thrombin.

Factor II. Carry out the assay of human coagulation factor II (2.7.18).

The estimated content is not more than 125 per cent of the stated content. The confidence limits ($P = 0.95$) are not less than 90 per cent and not more than 111 per cent of the estimated potency.

Factor IX. Carry out the assay of human coagulation factor IX (2.7.11).

The estimated content is not more than 125 per cent of the stated content. The confidence limits ($P = 0.95$) are not less than 80 per cent and not more than 125 per cent of the estimated potency.

Factor X. Carry out the assay of human coagulation factor X (2.7.19).

The estimated content is not more than 125 per cent of the stated content. The confidence limits ($P = 0.95$) are not less than 90 per cent and not more than 111 per cent of the estimated potency.

Water. Determined by a suitable method, such as the semi-micro determination of water (2.5.12), loss on drying (2.2.32) or near-infrared spectrometry (2.2.40), the water content is within the limits approved by the competent authority.

Sterility (2.6.1). It complies with the test for sterility.

Pyrogens (2.6.8). It complies with the test for pyrogens. Inject per kilogram of the rabbit's mass a volume equivalent to not less than 30 IU of factor VII.

ASSAY

Assay of human coagulation factor VII (2.7.10).

The estimated potency is not less than 80 per cent and not more than 125 per cent of the stated potency. The confidence limits ($P = 0.95$) are not less than 80 per cent and not more than 125 per cent of the estimated potency.

STORAGE

In an airtight container, protected from light.

LABELLING

The label states:

- the number of International Units of factor VII per container,
- the maximum content of International Units of factor II, factor IX and factor X per container,
- the amount of protein per container,
- the name and quantity of any added substances, including where applicable, heparin,
- the name and volume of the liquid to be used for reconstitution,
- that the transmission of infectious agents cannot be totally excluded when medicinal products prepared from human blood or plasma are administered.

01/2005:0275

HUMAN COAGULATION FACTOR VIII

Factor VIII coagulationis humanus

DEFINITION

Human coagulation factor VIII is a plasma protein fraction that contains the glycoprotein coagulation factor VIII together with varying amounts of von Willebrand factor, depending on the method of preparation. It is prepared from human plasma that complies with the monograph on *Human plasma for fractionation* (0853).

The potency of the preparation, reconstituted as stated on the label, is not less than 20 IU of factor VIII:C per millilitre.

PRODUCTION

The method of preparation includes a step or steps that have been shown to remove or to inactivate known agents of infection; if substances are used for the inactivation of viruses during production, the subsequent purification procedure must be validated to demonstrate that the concentration of these substances is reduced to a suitable level and that any residues are such as not to compromise the safety of the preparation for patients.

The specific activity is not less than 1 IU of factor VIII:C per milligram of total protein before the addition of any protein stabiliser.

The factor VIII fraction is dissolved in a suitable liquid. Auxiliary substances such as a stabiliser may be added. No antimicrobial preservative is added. The solution is passed through a bacteria-retentive filter, distributed aseptically into the final containers and immediately frozen. It is subsequently freeze-dried and the containers are closed under vacuum or under an inert gas.

Validation test applied to products stated to have von Willebrand factor activity. For products intended for treatment of von Willebrand's disease it shall be demonstrated that the manufacturing process yields a product with a consistent composition with respect to von Willebrand factor. This composition may be characterised in a number of ways. For example, the number and the relative amount of the different multimers may be determined by sodium dodecyl sulphate (SDS) agarose gel electrophoresis (about 1 per cent agarose) with or without Western blot analysis on nitrocellulose, using a normal human plasma pool as reference; visualisation of the multimeric pattern may be performed using an immunoenzymatic technique and quantitative evaluation may be carried out by densitometric analysis or by other suitable methods.

von Willebrand factor activity. For products intended for treatment of von Willebrand's disease the von Willebrand factor activity is determined by a suitable method using a reference preparation of the same type as the preparation to be examined, calibrated against the International Standard for von Willebrand factor in plasma. Suitable methods include determination of ristocetin cofactor activity and determination of collagen-binding activity. The following method for determination of ristocetin cofactor activity is given as an example of a suitable method.

Ristocetin cofactor activity. Carry out appropriate dilutions of the preparation to be examined and of the reference preparation using as diluent a solution containing 9 g/l of *sodium chloride R* and 50 g/l of human albumin. Add to each dilution suitable amounts of a von Willebrand reagent containing stabilised human platelets and ristocetin A. Mix

on a glass plate by moving it gently in circles for 1 min. Allow to stand for a further 1 min and read the result against a dark background with side lighting. The last dilution which shows clearly visible agglutination indicates the ristocetin cofactor titre of the sample. Use diluent as a negative control.

The estimated potency is not less than 60 per cent and not more than 140 per cent of the potency approved for the particular product.

CHARACTERS

A white or pale yellow, hygroscopic powder or friable solid.

Reconstitute the preparation to be examined as stated on the label immediately before carrying out the identification, tests (except those for solubility and water) and assay.

IDENTIFICATION

It complies with the limits of the assay.

TESTS

pH (2.2.3): 6.5 to 7.5.

Solubility. To a container of the preparation to be examined add the volume of the solvent stated on the label at the recommended temperature. The preparation dissolves completely with gentle swirling within 10 min, giving a clear or slightly opalescent, colourless or slightly yellow solution.

Osmolality (2.2.35): minimum 240 mosmol/kg.

Total protein. If necessary, dilute an accurately measured volume of the preparation to be examined with a 9 g/l solution of *sodium chloride R* to obtain a solution containing about 15 mg of protein in 2 ml. To 2.0 ml of the solution in a round-bottomed centrifuge tube add 2 ml of a 75 g/l solution of *sodium molybdate R* and 2 ml of a mixture of 1 volume of *nitrogen-free sulphuric acid R* and 30 volumes of *water R*. Shake, centrifuge for 5 min, decant the supernatant liquid and allow the inverted tube to drain on filter paper. Determine the nitrogen in the residue by the method of sulphuric acid digestion (2.5.9) and calculate the amount of protein by multiplying the result by 6.25.

For some products, especially those without a protein stabiliser such as albumin, this method may not be applicable and another validated method for protein determination must therefore be performed.

Haemagglutinins anti-A and anti-B. Dilute the preparation with a 9 g/l solution of *sodium chloride R* to contain 3 IU of factor VIII:C per millilitre. Carry out the indirect determination of haemagglutinins A and B (2.6.20). The 1 to 64 dilutions do not show agglutination.

Hepatitis B surface antigen. Examine the reconstituted preparation by a suitably sensitive method such as enzyme immunoassay (2.7.1). Hepatitis B surface antigen is not detected.

Water. Determined by a suitable method, such as the semi-micro determination of water (2.5.12), loss on drying (2.2.32) or near infrared spectrophotometry (2.2.40), the water content is within the limits approved by the competent authority.

Sterility (2.6.1). It complies with the test for sterility.

Pyrogens (2.6.8). It complies with the test for pyrogens. Inject per kilogram of the rabbit's mass a volume of the preparation to be examined equivalent to not less than 50 IU of factor VIII:C.

ASSAY

Assay of human coagulation factor VIII (2.7.4).

The estimated potency is not less than 80 per cent and not more than 120 per cent of the stated potency. The confidence limits ($P = 0.95$) are not less than 80 per cent and not more than 120 per cent of the estimated potency.

STORAGE

In an airtight container, protected from light.

LABELLING

The label states:

- the number of International Units of factor VIII:C and, where applicable, of von Willebrand factor in the container,
- the amount of protein in the container,
- the name and quantity of any added substance,
- the name and volume of the liquid to be used for reconstitution,
- that the transmission of infectious agents cannot be totally excluded when medicinal products prepared from human blood or plasma are administered.

01/2005:1643

HUMAN COAGULATION FACTOR VIII (rDNA)

Factor VIII coagulationis humanus (ADNr)

DEFINITION

Human coagulation factor VIII (rDNA) is a freeze-dried preparation of glycoproteins having the same activity as coagulation factor VIII in human plasma. It acts as a cofactor of the activation of factor X in the presence of factor IXa, phospholipids and calcium ions.

Human coagulation factor VIII circulates in plasma mainly as a two-chain glycosylated protein with 1 heavy (relative molecular mass of about 200 000) and 1 light (relative molecular mass 80 000) chain held together by divalent metal ions. Human coagulation factor VIII (rDNA) is prepared as full-length factor VIII (octocog alfa), or as a shortened two-chain structure (relative molecular mass 90 000 and 80 000), in which the B-domain has been deleted from the heavy chain (moroctocog alfa).

Full-length human rDNA coagulation factor VIII contains 25 potential *N*-glycosylation sites, 19 in the B domain of the heavy chain, 3 in the remaining part of the heavy chain (relative molecular mass 90 000) and 3 in the light chain (relative molecular mass 80 000). The different products are characterised by their molecular size and post-translational modification and/or other modifications.

PRODUCTION

Human coagulation factor VIII (rDNA) is produced by recombinant DNA technology in mammalian cell culture. It is produced under conditions designed to minimise microbial contamination.

Purified bulk factor VIII (rDNA) may contain added human albumin and/or other stabilising agents, as well as other auxiliary substances to provide, for example, correct pH and osmolality.

The specific activity is not less than 2000 IU of factor VIII:C per milligram of total protein before the addition of any protein stabiliser, and varies depending on purity and the type of modification of molecular structure of factor VIII.

The quality of the bulk preparation is controlled using one or more manufacturer's reference preparations as reference.

MANUFACTURER'S REFERENCE PREPARATIONS

During development, reference preparations are established for subsequent verification of batch consistency during production, and for control of bulk and final preparation. They are derived from representative batches of purified bulk factor VIII (rDNA) that are extensively characterised by tests including those described below and whose procoagulant and other relevant functional properties have been ascertained and compared, wherever possible, with the International Standard for factor VIII concentrate. The reference preparations are suitably characterised for their intended purpose and are stored in suitably sized aliquots under conditions ensuring their stability.

PURIFIED BULK FACTOR VIII (rDNA)

The purified bulk complies with a suitable combination of the following tests for characterisation of integrity of the factor VIII (rDNA). Where any substance added during preparation of the purified bulk interferes with a test, the test is carried out before addition of that substance. Where applicable, the characterisation tests may alternatively be carried out on the finished product.

Specific biological activity or ratio of factor VIII activity to factor VIII antigen. Carry out the assay of human coagulation factor VIII (2.7.4). The protein content, or where a protein stabiliser is present, the factor VIII antigen content, is determined by a suitable method and the specific biological activity or the ratio of factor VIII activity to factor VIII antigen is calculated.

Protein composition. The protein composition is determined by a selection of appropriate characterisation techniques which may include peptide mapping, Western blots, HPLC, gel electrophoresis, capillary electrophoresis, mass spectrometry or other techniques to monitor integrity and purity. The protein composition is comparable to that of the manufacturer's reference preparation.

Molecular size distribution. Using size-exclusion chromatography (2.2.30), the molecular size distribution is comparable to that of the manufacturer's reference preparation.

Peptide mapping (2.2.55). There is no significant difference between the test protein and the manufacturer's reference preparation.

Carbohydrates/sialic acid. To monitor batch-to-batch consistency, the monosaccharide content and the degree of sialylation or the oligosaccharide profile are monitored and correspond to those of the manufacturer's reference preparation.

FINAL LOT

It complies with the requirements under Identification, Tests and Assay.

Excipients: 80 per cent to 120 per cent of the stated content, determined by a suitable method, where applicable.

CHARACTERS

Appearance: white or slightly yellow powder or friable mass.

IDENTIFICATION

- It complies with the limits of the assay.
- The distribution of characteristic peptide bands corresponds with that of the manufacturer's reference preparation (SDS-PAGE or Western blot).

TESTS

Reconstitute the preparation as stated on the label immediately before carrying out the tests (except those for solubility and water) and assay.

Solubility. It dissolves within 5 min at 20-25 °C, giving a clear or slightly opalescent solution.

pH (2.2.3): 6.5 to 7.5.

Osmolality (2.2.35): minimum 240 mosmol/kg.

Water. Determined by a suitable method, such as the semi-micro determination of water (2.5.12), loss on drying (2.2.32) or near infrared spectrophotometry (2.2.40), the water content is within the limits approved by the competent authority.

Sterility (2.6.1). It complies with the test for sterility.

Bacterial endotoxins (2.6.14): less than 3 IU in the volume that contains 100 IU of factor VIII activity.

ASSAY

Carry out the assay of human coagulation factor VIII (2.7.4).

The estimated potency is not less than 80 per cent and not more than 125 per cent of the stated potency. The confidence limits ($P = 0.95$) are not less than 80 per cent and not more than 120 per cent of the estimated potency.

STORAGE

Protected from light.

LABELLING

The label states:

- the factor VIII content in International Units,
- the name and amount of any excipient,
- the composition and volume of the liquid to be used for reconstitution.

01/2005:1223

HUMAN COAGULATION FACTOR IX

Factor IX coagulationis humanus

DEFINITION

Human coagulation factor IX is a plasma protein fraction containing coagulation factor IX, prepared by a method that effectively separates factor IX from other prothrombin complex factors (factors II, VII and X). It is obtained from human plasma that complies with the monograph on *Human plasma for fractionation* (0853).

The potency of the preparation, reconstituted as stated on the label, is not less than 20 IU of factor IX per millilitre.

PRODUCTION

The method of preparation is designed to maintain functional integrity of factor IX, to minimise activation of any coagulation factor (to minimise potential thrombogenicity) and includes a step or steps that have been shown to remove or to inactivate known agents of infection; if substances are used for inactivation of viruses during production, the subsequent purification procedure must be validated to demonstrate that the concentration of these substances is reduced to a suitable level and that any residues are such as not to compromise the safety of the preparation for patients.

The specific activity is not less than 50 IU of factor IX per milligram of total protein, before the addition of any protein stabiliser.

The factor IX fraction is dissolved in a suitable liquid. Heparin, antithrombin and other auxiliary substances such as a stabiliser may be included. No antimicrobial preservative

is added. The solution is passed through a bacteria-retentive filter, distributed aseptically into the final containers and immediately frozen. It is subsequently freeze-dried and the containers are closed under vacuum or under an inert gas.

CONSISTENCY OF THE METHOD OF PRODUCTION

The consistency of the method of production is evaluated by suitable analytical procedures that are determined during process development and which normally include:

- assay of factor IX,
- determination of activated coagulation factors,
- determination of activities of factors II, VII and X which shall be shown to be not more than 5 per cent of the activity of factor IX.

CHARACTERS

A white or pale yellow, hygroscopic powder or friable solid.

Reconstitute the preparation to be examined as stated on the label, immediately before carrying out the identification, tests (except those for solubility and water) and assay.

IDENTIFICATION

It complies with the limits of the assay.

TESTS

pH (2.2.3): 6.5 to 7.5.

Solubility. To a container of the preparation to be examined add the volume of the liquid stated on the label at the recommended temperature. The preparation dissolves completely with gentle swirling within 10 min, giving a clear or slightly opalescent, colourless solution.

Osmolality (2.2.35): minimum 240 mosmol/kg.

Total protein. If necessary, dilute an accurately measured volume of the preparation to be examined with a 9 g/l solution of *sodium chloride R*, to obtain a solution which may be expected to contain about 15 mg of protein in 2 ml. To 2.0 ml of that solution, in a round-bottomed centrifuge tube, add 2 ml of a 75 g/l solution of *sodium molybdate R* and 2 ml of a mixture of 1 volume of *nitrogen-free sulphuric acid R* and 30 volumes of *water R*. Shake, centrifuge for 5 min, decant the supernatant liquid and allow the inverted tube to drain on filter paper. Determine the nitrogen in the residue by the method of sulphuric acid digestion (2.5.9) and calculate the amount of protein by multiplying the result by 6.25.

For some products, especially those without a protein stabiliser such as albumin, this method may not be applicable. Another validated method for protein determination must therefore be performed.

Activated coagulation factors (2.6.22). If necessary, dilute the preparation to be examined to contain 20 IU of factor IX per millilitre. For each of the dilutions the coagulation time is not less than 150 s.

Heparin. If heparin has been added during preparation, determine the amount by the assay of heparin in coagulation factor concentrates (2.7.12). The preparation to be examined contains not more than the amount of heparin stated on the label and in any case not more than 0.5 IU of heparin per International Unit of factor IX.

Water. Determined by a suitable method, such as the semi-micro determination of water (2.5.12), loss on drying (2.2.32) or near infrared spectrophotometry (2.2.40), the water content is within the limits approved by the competent authority.

Sterility (2.6.1). It complies with the test for sterility.

Pyrogens (2.6.8). It complies with the test for pyrogens. Inject per kilogram of the rabbit's mass a volume equivalent to not less than 50 IU of factor IX.

ASSAY

Assay of human blood coagulation factor IX (2.7.11).

The estimated potency is not less than 80 per cent and not more than 125 per cent of the stated potency. The confidence limits ($P = 0.95$) are not less than 80 per cent and not more than 125 per cent of the estimated potency.

STORAGE

In an airtight container, protected from light.

LABELLING

The label states:

- the number of International Units of factor IX per container,
- the amount of protein per container,
- the name and quantity of any added substances including, where applicable, heparin,
- the name and volume of the liquid to be used for reconstitution,
- that the transmission of infectious agents cannot be totally excluded when medicinal products prepared from human blood or plasma are administered.

01/2005:1644

HUMAN COAGULATION FACTOR XI

Factor XI coagulationis humanus

DEFINITION

Human coagulation factor XI is a plasma protein fraction containing coagulation factor XI. It is obtained from *Human plasma for fractionation* (0853).

When reconstituted as stated on the label, the potency is not less than 50 units of factor XI per millilitre.

PRODUCTION

The method of preparation includes a step or steps that have been shown to remove or to inactivate known agents of infection; if substances are used for inactivation of viruses during production, the subsequent purification procedure must be validated to demonstrate that the concentration of these substances is reduced to a suitable level and any residues are such as not to compromise the safety of the preparation for patients.

After preparation, the factor XI fraction is dissolved in a suitable liquid. Heparin, C₁-esterase inhibitor and antithrombin III may be added. The solution is distributed into the final containers and immediately frozen. It is subsequently freeze-dried and the containers are closed under vacuum or under nitrogen. No antimicrobial preservative is added.

CHARACTERS

A white powder or friable solid.

Reconstitute the preparation to be examined as stated on the label immediately before carrying out the identification, tests (except those for solubility and water) and assay.

IDENTIFICATION

The assay of factor XI serves also to identify the preparation.

TESTS

Solubility. To a container of the preparation to be examined, add the volume of liquid stated on the label at room temperature. The preparation dissolves completely with gentle swirling within 10 min.

pH (2.2.3): 6.8 to 7.4.

Osmolality (2.2.35): minimum 240 mosmol/kg.

Total protein (2.5.33). The preparation to be examined contains not more than the amount of protein stated on the label.

Activated coagulation factors (2.6.22). For each of the dilutions, the coagulation time is not less than 150 s.

Heparin (2.7.12). If heparin has been added, the preparation to be examined contains not more than the amount of heparin stated on the label and in any case not more than 0.5 IU of heparin per unit of factor XI.

Antithrombin III (2.7.17). If antithrombin III has been added, the preparation to be examined contains not more than the amount of antithrombin III stated on the label.

C₁-esterase inhibitor. If C₁-esterase inhibitor has been added, the preparation to be examined contains not more than the amount of C₁-esterase inhibitor stated on the label.

The C₁-esterase inhibitor content of the preparation to be examined is determined by comparing its ability to inhibit C₁-esterase with the same ability of a reference preparation consisting of human normal plasma. 1 unit of C₁-esterase is equal to the activity of 1 ml of human normal plasma. Varying quantities of the preparation to be examined are mixed with an excess of C₁-esterase and the remaining C₁-esterase activity is determined using a suitable chromogenic substrate.

Method. Reconstitute the preparation as stated on the label. Prepare an appropriate series of 3 or 4 independent dilutions from 1 unit/ml of factor XI, for both the preparation to be examined and the reference preparation, using a solution containing 9 g/l of *sodium chloride R* and either 10 g/l of *human albumin R* or 10 g/l of *bovine albumin R*. Warm all solutions to 37 °C in a water bath for 1-2 min before use. Place a suitable amount of C₁-esterase solution in tubes or in microtitre plate wells and incubate at 37 °C. Add a suitable amount of one of the dilutions of the reference preparation or of the preparation to be examined and incubate at 37 °C for 5 min. Add a suitable amount of a suitable chromogenic substrate such as methoxycarbonyl-L-lysyl(e-benzyloxycarbonyl)-glycyl-L-arginine 4-nitroanilide. Read the rate of increase of absorbance ($\Delta A/\text{min}$) at 405 nm. Carry out a blank test using *tris(hydroxymethyl)aminomethane sodium chloride buffer solution pH 7.4 R* instead of the C₁-esterase and the substrate.

Calculate the C₁-esterase inhibitor content using the usual statistical methods (for example, 5.3.).

Anti-A and anti-B haemagglutinins (2.6.20). The 1 to 64 dilutions do not show agglutination.

Water. Determined by a suitable method, such as the semi-micro determination of water (2.5.12), loss on drying (2.2.32) or near infrared spectrophotometry (2.2.40), the water content is within the limits approved by the competent authority.

Sterility (2.6.1). The reconstituted preparation complies with the test for sterility.

Pyrogens (2.6.8). The reconstituted preparation complies with the test for pyrogens. Inject per kilogram of the rabbit's mass a volume of the reconstituted preparation equivalent to 100 units of factor XI, calculated from the activity stated on the label.

ASSAY

Carry out the assay of human coagulation factor XI (2.7.22).

The estimated potency is not less than 80 per cent and not more than 120 per cent of the stated potency. The confidence limits ($P = 0.95$) are not less than 80 per cent and not more than 125 per cent of the estimated potency.

STORAGE

Protected from light, at a temperature of 2 °C to 8 °C.

LABELLING

The label states:

- the number of units per container,
- the maximum amount of protein per container,
- where applicable, the amount of heparin per container,
- where applicable, the amount of antithrombin III per container,
- where applicable, the amount of C₁-esterase inhibitor per container,
- the name and volume of the liquid to be used for reconstitution.

the preparation, the requirement for specific activity applies to the fibrinogen before addition of the stabiliser. Albumin may also be obtained with fibrinogen during fractionation and a specific determination of albumin is then carried out by a suitable immunochemical method (2.7.1) and the quantity of albumin determined is subtracted from the total protein content for the calculation of the specific activity.

CHARACTERS

A white or pale yellow, hygroscopic powder or friable solid.

IDENTIFICATION

It complies with the limits of the assay.

TESTS

pH (2.2.3): 6.5 to 7.5.

Osmolality (2.2.35): minimum 240 mosmol/kg.

Solubility. Add the volume of solvent stated on the label to the contents of a container. The preparation dissolves within 30 min at 20-25 °C, forming an almost colourless, slightly opalescent solution.

Stability of solution. Allow the reconstituted solution to stand at 20-25 °C. No gel formation appears within 60 min of reconstitution.

Hepatitis B surface antigen. Examine the reconstituted preparation by an immunochemical method (2.7.1) of suitable sensitivity, such as radio-immunoassay. Hepatitis B surface antigen is not detected.

Water. Determined by a suitable method, such as the semi-micro determination of water (2.5.12), loss on drying (2.2.32) or near infrared spectrophotometry (2.2.40), the water content is within the limits approved by the competent authority.

Sterility (2.6.1). The reconstituted preparation complies with the test for sterility.

Pyrogens (2.6.8). The reconstituted preparation complies with the test for pyrogens. Inject per kilogram of the rabbit's mass a volume of the reconstituted preparation equivalent to not less than 30 mg of fibrinogen, calculated from the quantity stated on the label.

ASSAY

Mix 0.2 ml of the reconstituted preparation with 2 ml of a suitable buffer solution (pH 6.6-6.8) containing sufficient thrombin (approximately 3 IU/ml) and calcium (0.05 mol/l). Maintain at 37 °C for 20 min, separate the precipitate by centrifugation (5000 g, 20 min), wash thoroughly with a 9 g/l solution of *sodium chloride R*. Determine the nitrogen content by sulphuric acid digestion (2.5.9) and calculate the fibrinogen (clottable protein) content by multiplying the result by 6.0. The content is not less than 70 per cent and not more than 130 per cent of the amount of fibrinogen stated on the label.

STORAGE

In an airtight container, protected from light.

LABELLING

The label states:

- the amount of fibrinogen in the container,
- the name and volume of the solvent to be used to reconstitute the preparation,
- where applicable, the name and quantity of protein stabiliser in the preparation.

01/2005:0024

HUMAN FIBRINOGEN

Fibrinogenum humanum

DEFINITION

Human fibrinogen contains the soluble constituent of human plasma that is transformed to fibrin on the addition of thrombin. It is obtained from *Human plasma for fractionation* (0853). The preparation may contain auxiliary substances such as salts, buffers and stabilisers.

When dissolved in the volume of the solvent stated on the label, the solution contains not less than 10 g/l of fibrinogen.

PRODUCTION

The method of preparation includes a step or steps that have been shown to remove or to inactivate known agents of infection; if substances are used for inactivation of viruses during production, the subsequent purification procedure must be validated to demonstrate that the concentration of these substances is reduced to a suitable level and any residues are such as not to compromise the safety of the preparation for patients.

No antibiotic is added to the plasma used and no antimicrobial preservative is included in the preparation. The preparation is freeze-dried.

The method of preparation is such as to obtain fibrinogen with a specific activity (fibrinogen content with respect to total protein content) not less than 80 per cent. The fibrinogen content is determined by a suitable method such as that described under Assay and the total protein content is determined by a suitable method such as that described under Total protein in *Human albumin solution* (0255). If a protein stabiliser (for example, human albumin) is added to

01/2005:0769 POTENCY

HUMAN HEPATITIS A IMMUNOGLOBULIN

Immunoglobulinum humanum hepatitidis A

DEFINITION

Human hepatitis A immunoglobulin is a liquid or freeze-dried preparation containing immunoglobulins, mainly immunoglobulin G. The preparation is intended for intramuscular administration. It is obtained from plasma from selected donors having antibodies against hepatitis A virus. *Human normal immunoglobulin (0338)* may be added.

It complies with the monograph on *Human normal immunoglobulin (0338)*, except for the minimum number of donors and the minimum total protein content.

POTENCY

The potency is determined by comparing the antibody titre of the immunoglobulin to be examined with that of a reference preparation calibrated in International Units, using an immunoassay of suitable sensitivity and specificity (2.7.1).

The International Unit is the activity contained in a stated amount of the International Standard for anti-hepatitis A immunoglobulin. The equivalence in International Units of the International Standard is stated by the World Health Organisation.

Human hepatitis A immunoglobulin BRP is calibrated in International Units by comparison with the International Standard.

The stated potency is not less than 600 IU/ml. The estimated potency is not less than the stated potency. The confidence limits ($P = 0.95$) of the estimated potency are not less than 80 per cent and not more than 125 per cent.

STORAGE

See *Human normal immunoglobulin (0338)*.

LABELLING

See *Human normal immunoglobulin (0338)*.

The label states the number of International Units per container.

01/2005:0722

HUMAN HEPATITIS B IMMUNOGLOBULIN

Immunoglobulinum humanum hepatitidis B

DEFINITION

Human hepatitis B immunoglobulin is a liquid or freeze-dried preparation containing immunoglobulins, mainly immunoglobulin G. The preparation is intended for intramuscular administration. It is obtained from plasma from selected and/or immunised donors having antibodies against hepatitis B surface antigen. *Human normal immunoglobulin (0338)* may be added.

It complies with the monograph on *Human normal immunoglobulin (0338)*, except for the minimum number of donors and the minimum total protein content.

POTENCY

The potency is determined by comparing the antibody titre of the immunoglobulin to be examined with that of a reference preparation calibrated in International Units, using an immunoassay of suitable sensitivity and specificity (2.7.1).

The International Unit is the activity contained in a stated amount of the International Reference Preparation of hepatitis B immunoglobulin. The equivalence in International Units of the International Reference Preparation is stated by the World Health Organisation.

The stated potency is not less than 100 IU/ml. The estimated potency is not less than the stated potency. The confidence limits ($P = 0.95$) of the estimated potency are not less than 80 per cent and not more than 125 per cent.

STORAGE

See *Human normal immunoglobulin (0338)*.

LABELLING

See *Human normal immunoglobulin (0338)*.

The label states the number of International Units per container.

01/2005:1016

HUMAN HEPATITIS B IMMUNOGLOBULIN FOR INTRAVENOUS ADMINISTRATION

Immunoglobulinum humanum hepatitidis B ad usum intravenosum

DEFINITION

Human hepatitis B immunoglobulin for intravenous administration is a liquid or freeze-dried preparation containing immunoglobulins, mainly immunoglobulin G. It is obtained from plasma from selected and/or immunised donors having antibodies against hepatitis B surface antigen. *Human normal immunoglobulin for intravenous administration (0918)* may be added.

It complies with the monograph on *Human normal immunoglobulin for intravenous administration (0918)*, except for the minimum number of donors, the minimum total protein content and the limit for osmolality.

POTENCY

The potency is determined by comparing the antibody titre of the immunoglobulin to be examined with that of a reference preparation calibrated in International Units, using an immunoassay (2.7.1) of suitable sensitivity and specificity.

The International Unit is the activity contained in a stated amount of the International Reference Preparation of hepatitis B immunoglobulin. The equivalence in International Units of the International Reference Preparation is stated by the World Health Organisation.

The stated potency is not less than 50 IU/ml. The estimated potency is not less than the stated potency. The confidence limits ($P = 0.95$) are not less than 80 per cent and not more than 120 per cent of the estimated potency.

STORAGE

See *Human normal immunoglobulin for intravenous administration (0918)*.

LABELLING

See *Human normal immunoglobulin for intravenous administration (0918)*.

The label states the minimum number of International Units of hepatitis B immunoglobulin per container.

01/2005:0338

HUMAN MEASLES IMMUNOGLOBULIN

Immunoglobulinum humanum morbillicum

DEFINITION

Human measles immunoglobulin is a liquid or freeze-dried preparation containing immunoglobulins, mainly immunoglobulin G. The preparation is intended for intramuscular administration. It is obtained from plasma containing specific antibodies against the measles virus. *Human normal immunoglobulin (0338)* may be added.

It complies with the monograph on *Human normal immunoglobulin (0338)*, except for the minimum number of donors and the minimum total protein content.

POTENCY

The potency of the liquid preparation and of the freeze-dried preparation after reconstitution as stated on the label is not less than 50 IU per millilitre of neutralising antibody against measles virus.

The potency is determined by comparing the antibody titres of the immunoglobulin to be examined and of a reference preparation calibrated in International Units, using a challenge dose of measles virus in a suitable cell culture system. A method of equal sensitivity and precision may be used providing that the competent authority is satisfied that it correlates with neutralising activity for the measles virus by comparison with the reference preparation.

The International Unit is the specific neutralising activity for measles virus contained in a stated amount of the International Standard for human anti-measles serum. The equivalence in International Units of the International Reference Preparation is stated by the World Health Organisation.

Prepare serial two-fold dilutions of the immunoglobulin to be examined and of the reference preparation. Mix each dilution with an equal volume of a suspension of measles virus containing about 100 CCID₅₀ in 0.1 ml and incubate protected from light at 37 °C for 2 h. Using not fewer than six cell cultures per mixture, inoculate 0.2 ml of each mixture into each of the cell cultures allocated to that mixture and incubate for not less than 10 days. Examine the cultures for viral activity and compare the dilution containing the smallest quantity of the immunoglobulin which neutralises the virus with that of the corresponding dilution of the reference preparation.

Calculate the potency of the immunoglobulin to be examined in International Units per millilitre of neutralising antibody against measles virus.

STORAGE

See *Human normal immunoglobulin (0338)*.

LABELLING

See *Human normal immunoglobulin (0338)*.

The label states the number of International Units per container.

HUMAN NORMAL IMMUNOGLOBULIN

Immunoglobulinum humanum normale

DEFINITION

Human normal immunoglobulin is a liquid or freeze-dried preparation containing immunoglobulins, mainly immunoglobulin G (IgG). Other proteins may be present. Human normal immunoglobulin contains the IgG antibodies of normal subjects. It is intended for intramuscular injection.

Human normal immunoglobulin is obtained from plasma that complies with the requirements of the monograph on *Human plasma for fractionation (0853)*. No antibiotic is added to the plasma used.

PRODUCTION

The method of preparation includes a step or steps that have been shown to remove or to inactivate known agents of infection; if substances are used for inactivation of viruses, it shall have been shown that any residues present in the final product have no adverse effects on the patients treated with the immunoglobulin.

The product shall have been shown, by suitable tests in animals and evaluation during clinical trials, to be well tolerated when administered intramuscularly.

Human normal immunoglobulin is prepared from pooled material from at least 1000 donors by a method that has been shown to yield a product that:

- does not transmit infection;
- at a protein concentration of 160 g/l, contains antibodies for at least 2 of which (one viral and one bacterial) an International Standard or Reference Preparation is available, the concentration of such antibodies being at least 10 times that in the initial pooled material.

Human normal immunoglobulin is prepared as a stabilised solution, for example in a 9 g/l solution of sodium chloride, a 22.5 g/l solution of glycine or, if the preparation is to be freeze-dried, a 60 g/l solution of glycine. Multidose preparations contain an antimicrobial preservative. Single-dose preparations do not contain an antimicrobial preservative. Any antimicrobial preservative or stabilising agent used shall have been shown to have no deleterious effect on the final product in the amount present. The solution is passed through a bacteria-retentive filter. The preparation may subsequently be freeze-dried and the containers closed under vacuum or under an inert gas.

The stability of the preparation is demonstrated by suitable tests carried out during development studies.

CHARACTERS

The liquid preparation is clear and pale-yellow to light-brown; during storage it may show formation of slight turbidity or a small amount of particulate matter. The freeze-dried preparation is a hygroscopic, white or slightly yellow powder or solid, friable mass.

For the freeze-dried preparation, reconstitute as stated on the label immediately before carrying out the identification and the tests, except those for solubility and water.

IDENTIFICATION

Examine by a suitable immunoelectrophoresis technique. Using antiserum to normal human serum, compare normal human serum and the preparation to be examined, both diluted to contain 10 g/l of protein. The main component

of the preparation to be examined corresponds to the IgG component of normal human serum. The solution may show the presence of small quantities of other plasma proteins.

TESTS

Solubility. For the freeze-dried preparation, add the volume of the liquid stated on the label. The preparation dissolves completely within 20 min at 20–25 °C.

pH (2.2.3): 5.0 to 7.2.

Dilute the preparation to be examined with a 9 g/l solution of *sodium chloride R* to obtain a solution containing 10 g/l of protein.

Total protein. Dilute the preparation to be examined with a 9 g/l solution of *sodium chloride R* to obtain a solution containing about 15 mg of protein in 2 ml. To 2.0 ml of this solution in a round-bottomed centrifuge tube add 2 ml of a 75 g/l solution of *sodium molybdate R* and 2 ml of a mixture of 1 volume of *nitrogen-free sulphuric acid R* and 30 volumes of *water R*. Shake, centrifuge for 5 min, decant the supernatant liquid and allow the inverted tube to drain on filter paper. Determine the nitrogen in the residue by the method of sulphuric acid digestion (2.5.9) and calculate the content of protein by multiplying the result by 6.25. The preparation contains not less than 100 g/l and not more than 180 g/l of protein and not less than 90 per cent and not more than 110 per cent of the quantity of protein stated on the label.

Protein composition. Examine by zone electrophoresis (2.2.31), using strips of suitable cellulose acetate gel as the supporting medium and *barbital buffer solution pH 8.6 RI* as the electrolyte solution.

Test solution. Dilute the preparation to be examined with a 9 g/l solution of *sodium chloride R* to a protein concentration of 50 g/l.

Reference solution. Reconstitute *human immunoglobulin for electrophoresis BRP* and dilute with a 9 g/l solution of *sodium chloride R* to a protein concentration of 50 g/l.

To a strip apply 2.5 µl of the test solution as a 10 mm band or apply 0.25 µl per millimetre if a narrower strip is used. To another strip apply in the same manner the same volume of the reference solution. Apply a suitable electric field such that the albumin band of normal human serum applied on a control strip migrates at least 30 mm. Stain the strip with *amido black 10B solution R* for 5 min. Decolourise with a mixture of 10 volumes of *glacial acetic acid R* and 90 volumes of *methanol R* so that the background is just free of colour. Develop the transparency of the strips with a mixture of 19 volumes of *glacial acetic acid R* and 81 volumes of *methanol R*. Measure the absorbance of the bands at 600 nm in an instrument having a linear response over the range of measurement. Calculate the result as the mean of 3 measurements of each strip. In the electropherogram obtained with the test solution, not more than 10 per cent of the protein has a mobility different from that of the principal band. The test is not valid unless, in the electropherogram obtained with the reference preparation, the proportion of protein in the principal band is within the limits stated in the leaflet accompanying the reference preparation.

Distribution of molecular size. Liquid chromatography (2.2.29).

Test solution. Dilute the preparation to be examined with a 9 g/l solution of *sodium chloride R* to a concentration suitable for the chromatographic system used. A concentration in the range 4 g/l to 12 g/l and injection of 50 µg to 600 µg of protein are usually suitable.

Reference solution. Dilute *human immunoglobulin BRP* with a 9 g/l solution of *sodium chloride R* to the same protein concentration as the test solution.

Column:

- *size*: $l = 0.6$ m, $\varnothing = 7.5$ mm,
- *stationary phase*: *hydrophilic silica gel for chromatography R*, of a grade suitable for fractionation of globular proteins with relative molecular masses in the range 10 000 to 500 000.

Mobile phase: dissolve 4.873 g of *disodium hydrogen phosphate dihydrate R*, 1.741 g of *sodium dihydrogen phosphate monohydrate R*, 11.688 g of *sodium chloride R* and 50 mg of *sodium azide R* in 1 litre of *water R*.

Flow rate: 0.5 ml/min.

Detection: spectrophotometer at 280 nm.

In the chromatogram obtained with the reference solution, the principal peak corresponds to IgG monomer and there is a peak corresponding to dimer with a relative retention to the principal peak of about 0.85. Identify the peaks in the chromatogram obtained with the test solution by comparison with the chromatogram obtained with the reference solution; any peak with a retention time shorter than that of dimer corresponds to polymers and aggregates. The preparation to be examined complies with the test if, in the chromatogram obtained with the test solution:

- *relative retention*: for monomer and dimer, the relative retention to the corresponding peak in the chromatogram obtained with the reference solution is 1 ± 0.02 ;
- *peak area*: the sum of the peak areas of monomer and dimer represent not less than 85 per cent of the total area of the chromatogram and the sum of the peak area of polymers and aggregates represents not more than 10 per cent of the total area of the chromatogram.

Water. Determined by a suitable method, such as the semi-micro determination of water (2.5.12), loss on drying (2.2.32) or near infrared spectrophotometry (2.2.40), the water content is within the limits approved by the competent authority.

Sterility (2.6.1). It complies with the test for sterility.

Pyrogens (2.6.8). It complies with the test for pyrogens. Inject 1 ml per kilogram of the rabbit's mass.

Antibody to hepatitis B surface antigen. Not less than 0.5 IU/g of immunoglobulin, determined by a suitable immunochemical method (2.7.1).

Antibody to hepatitis A virus. If intended for use in the prophylaxis of hepatitis A, it complies with the following additional requirement. Determine the antibody content by comparison with a reference preparation calibrated in International Units, using an immunoassay of suitable sensitivity and specificity (2.7.1).

The International Unit is the activity contained in a stated amount of the International Standard for anti-hepatitis A immunoglobulin. The equivalence in International Units of the International Standard is stated by the World Health Organisation.

Human hepatitis A immunoglobulin BRP is calibrated in International Units by comparison with the International Standard.

The stated potency is not less than 100 IU/ml. The estimated potency is not less than the stated potency. The confidence limits ($P = 0.95$) of the estimated potency are not less than 80 per cent and not more than 125 per cent.

STORAGE

For the liquid preparation, store in a colourless glass container, protected from light. For the freeze-dried preparation, store in an airtight colourless glass container, protected from light.

LABELLING

The label states:

- for liquid preparations, the volume of the preparation in the container and the protein content expressed in grams per litre,
- for freeze-dried preparations, the quantity of protein in the container,
- the route of administration,
- for freeze-dried preparations, the name or composition and the volume of the reconstituting liquid to be added,
- where applicable, that the preparation is suitable for use in the prophylaxis of hepatitis A infection,
- where applicable, the anti-hepatitis A virus activity in International Units per millilitre,
- where applicable, the name and amount of antimicrobial preservative in the preparation.

- at an immunoglobulin concentration of 50 g/l, contains antibodies for at least 2 of which (one viral and one bacterial) an International Standard or Reference Preparation is available, the concentration of such antibodies being at least 3 times that in the initial pooled material,
- has a defined distribution of immunoglobulin G subclasses,
- complies with the test for Fc function of immunoglobulin (2.7.9).

Human normal immunoglobulin for intravenous administration is prepared as a stabilised solution or as a freeze-dried preparation. A stabiliser may be added. In both cases the preparation is passed through a bacteria-retentive filter. The preparation may subsequently be freeze-dried and the containers closed under vacuum or under an inert gas. No antimicrobial preservative is added either during fractionation or at the stage of the final bulk solution.

The stability of the preparation is demonstrated by suitable tests carried out during development studies.

CHARACTERS

The liquid preparation is clear or slightly opalescent and colourless or pale yellow. The freeze-dried preparation is a hygroscopic, white or slightly yellow powder or solid friable mass.

For the freeze-dried preparation, reconstitute as stated on the label immediately before carrying out the identification and the tests, except those for solubility and water.

IDENTIFICATION

Examine by a suitable immunoelectrophoresis technique. Using antiserum to normal human serum, compare normal human serum and the preparation to be examined, both diluted to contain 10 g/l of protein. The main component of the preparation to be examined corresponds to the IgG component of normal human serum. The preparation to be examined may show the presence of small quantities of other plasma proteins; if human albumin has been added as a stabiliser, it may be seen as a major component.

TESTS

Solubility. For the freeze-dried preparation, add the volume of the liquid stated on the label. The preparation dissolves completely within 30 min at 20-25 °C.

pH (2.2.3): 4.0 to 7.4.

Dilute the preparation to be examined with a 9 g/l solution of *sodium chloride R* to obtain a solution containing 10 g/l of protein.

Osmolality (2.2.35): minimum 240 mosmol/kg.

Total protein. Dilute the preparation to be examined with a 9 g/l solution of *sodium chloride R* to obtain a solution containing about 15 mg of protein in 2 ml. To 2.0 ml of this solution in a round-bottomed centrifuge tube add 2 ml of a 75 g/l solution of *sodium molybdate R* and 2 ml of a mixture of 1 volume of *nitrogen-free sulphuric acid R* and 30 volumes of *water R*. Shake, centrifuge for 5 min, decant the supernatant liquid and allow the inverted tube to drain on filter paper. Determine the nitrogen in the centrifugation residue by the method of sulphuric acid digestion (2.5.9) and calculate the content of protein by multiplying the result by 6.25. The preparation contains not less than 30 g/l of protein and not less than 90 per cent and not more than 110 per cent of the quantity of protein stated on the label.

01/2005:0918

HUMAN NORMAL IMMUNOGLOBULIN FOR INTRAVENOUS ADMINISTRATION

Immunoglobulinum humanum normale ad usum intravenosum

DEFINITION

Human normal immunoglobulin for intravenous administration is a liquid or freeze-dried preparation containing immunoglobulins, mainly immunoglobulin G (IgG). Other proteins may be present. Human normal immunoglobulin for intravenous administration contains the IgG antibodies of normal subjects. This monograph does not apply to products intentionally prepared to contain fragments or chemically modified IgG.

Human normal immunoglobulin for intravenous administration is obtained from plasma that complies with the requirements of the monograph on *Human plasma for fractionation (0853)*. No antibiotic is added to the plasma used.

PRODUCTION

The method of preparation includes a step or steps that have been shown to remove or to inactivate known agents of infection; if substances are used for inactivation of viruses, it shall have been shown that any residues present in the final product have no adverse effects on the patients treated with the immunoglobulin.

The product shall have been shown, by suitable tests in animals and evaluation during clinical trials, to be well tolerated when administered intravenously.

Human normal immunoglobulin is prepared from pooled material from not fewer than 1000 donors by a method that has been shown to yield a product that:

- does not transmit infection,

Protein composition. Examine by zone electrophoresis (2.2.31), using strips of suitable cellulose acetate gel as the supporting medium and *barbital buffer solution pH 8.6 R1* as the electrolyte solution.

Test solution. Dilute the preparation to be examined with a 9 g/l solution of *sodium chloride R* to an immunoglobulin concentration of 30 g/l.

Reference solution. Reconstitute *human immunoglobulin for electrophoresis BRP* and dilute with a 9 g/l solution of *sodium chloride R* to a protein concentration of 30 g/l.

To a strip apply 4.0 µl of the test solution as a 10 mm band or apply 0.4 µl per millimetre if a narrower strip is used. To another strip apply in the same manner the same volume of the reference solution. Apply a suitable electric field such that the albumin band of normal human serum applied on a control strip migrates at least 30 mm. Stain the strips with *amido black 10B solution R* for 5 min. Decolourise with a mixture of 10 volumes of *glacial acetic acid R* and 90 volumes of *methanol R* so that the background is just free of colour. Develop the transparency of the strips with a mixture of 19 volumes of *glacial acetic acid R* and 81 volumes of *methanol R*. Measure the absorbance of the bands at 600 nm in an instrument having a linear response over the range of measurement. Calculate the result as the mean of 3 measurements of each strip. In the electropherogram obtained with the test solution, not more than 5 per cent of protein has a mobility different from that of the principal band. This limit is not applicable if albumin has been added to the preparation as a stabiliser; for such preparations, a test for protein composition is carried out during manufacture before addition of the stabiliser. The test is not valid unless, in the electropherogram obtained with the reference preparation, the proportion of protein in the principal band is within the limits stated in the leaflet accompanying the reference preparation.

Distribution of molecular size. Liquid chromatography (2.2.29).

Test solution. Dilute the preparation to be examined with a 9 g/l solution of *sodium chloride R* to a concentration suitable for the chromatographic system used. A concentration in the range 4 g/l to 12 g/l and injection of 50 µg to 600 µg of protein are usually suitable.

Reference solution. Dilute *human immunoglobulin BRP* with a 9 g/l solution of *sodium chloride R* to the same protein concentration as the test solution.

Column:

- size: $l = 0.6$ m, $\varnothing = 7.5$ mm,
- stationary phase: *hydrophilic silica gel for chromatography R* of a grade suitable for fractionation of globular proteins with relative molecular masses in the range 10 000 to 500 000.

Mobile phase: dissolve 4.873 g of *disodium hydrogen phosphate dihydrate R*, 1.741 g of *sodium dihydrogen phosphate monohydrate R*, 11.688 g of *sodium chloride R* and 50 mg of *sodium azide R* in 1 litre of *water R*.

Flow rate: 0.5 ml/min.

Detection: spectrophotometer at 280 nm.

In the chromatogram obtained with the reference solution, the principal peak corresponds to IgG monomer and there is a peak corresponding to dimer with a relative retention to the principal peak of about 0.85. Identify the peaks in the chromatogram obtained with the test solution by comparison with the chromatogram obtained with the reference solution; any peak with a retention time shorter

than that of dimer corresponds to polymers and aggregates. The preparation to be examined complies with the test if, in the chromatogram obtained with the test solution:

- *relative retention*: for monomer and dimer, the relative retention to the corresponding peak in the chromatogram obtained with the reference solution is 1 ± 0.02 ;
- *peak area*: the sum of the peak areas of monomer and dimer represent not less than 90 per cent of the total area of the chromatogram and the sum of the peak area of polymers and aggregates represents not more than 3 per cent of the total area of the chromatogram. This requirement does not apply to products where albumin has been added as a stabiliser; for products stabilised with albumin, a test for distribution of molecular size is carried out during manufacture before addition of the stabiliser.

Anticomplementary activity (2.6.17). The consumption of complement is not greater than 50 per cent (1 CH₅₀ per milligram of immunoglobulin).

Prekallikrein activator (2.6.15): maximum 35 IU/ml, calculated with reference to a dilution of the preparation to be examined containing 30 g/l of immunoglobulin.

Anti-A and anti-B haemagglutinins (2.6.20). Carry out the tests for anti-A and anti-B haemagglutinins. If the preparation to be examined contains more than 30 g/l of immunoglobulin, dilute to this concentration before preparing the dilutions to be used in the test. The 1:64 dilutions do not show agglutination.

Water. Determined by a suitable method, such as the semi-micro determination of water (2.5.12), loss on drying (2.2.32) or near infrared spectrophotometry (2.2.40), the water content is within the limits approved by the competent authority.

Sterility (2.6.1). It complies with the test for sterility.

Pyrogens (2.6.8). It complies with the test for pyrogens. Inject per kilogram of the rabbit's mass a volume equivalent to 0.5 g of immunoglobulin but not more than 10 ml per kilogram of body mass.

Antibody to hepatitis B surface antigen: minimum 0.5 IU/g of immunoglobulin, determined by a suitable immunochemical method (2.7.1).

STORAGE

For the liquid preparation, store in a colourless glass container, protected from light, at the temperature stated on the label. For the freeze-dried preparation, store in an airtight colourless glass container, protected from light, at a temperature not exceeding 25 °C.

LABELLING

The label states:

- for liquid preparations, the volume of the preparation in the container and the protein content expressed in grams per litre,
- for freeze-dried preparations, the quantity of protein in the container,
- the amount of immunoglobulin in the container,
- the route of administration,
- for freeze-dried preparations, the name or composition and the volume of the reconstituting liquid to be added,
- the distribution of subclasses of immunoglobulin G present in the preparation,
- where applicable, the amount of albumin added as a stabiliser,
- the maximum content of immunoglobulin A.

01/2005:0853
corrected

HUMAN PLASMA FOR FRACTIONATION

Plasma humanum ad separationem

DEFINITION

Human plasma for fractionation is the liquid part of human blood remaining after separation of the cellular elements from blood collected in a receptacle containing an anticoagulant, or separated by continuous filtration or centrifugation of anticoagulated blood in an apheresis procedure; it is intended for the manufacture of plasma-derived products.

PRODUCTION

DONORS

Only a carefully selected, healthy donor who, as far as can be ascertained after medical examination, laboratory blood tests and a study of the donor's medical history, is free from detectable agents of infection transmissible by plasma-derived products may be used. Recommendations in this field are made by the Council of Europe [*Recommendation No. R (95) 15 on the preparation, use and quality assurance of blood components*, or subsequent revision] and the European Union [*Council Recommendation of 29 June 1998 on the suitability of blood and plasma donors and the screening of donated blood in the European Community* (98/463/EC)].

Immunisation of donors. Immunisation of donors to obtain immunoglobulins with specified activities may be carried out when sufficient supplies of material of suitable quality cannot be obtained from naturally immunised donors. Recommendations for such immunisation are formulated by the World Health Organisation (*Requirements for the collection, processing and quality control of blood, blood components and plasma derivatives*, WHO Technical Report Series, No. 840, 1994 or subsequent revision).

Records. Records of donors and donations made are kept in such a way that, while maintaining the required degree of confidentiality concerning the donor's identity, the origin of each donation in a plasma pool and the results of the corresponding acceptance procedures and laboratory tests can be traced.

Laboratory tests. Laboratory tests are carried out for each donation to detect the following viral markers:

1. antibodies against human immunodeficiency virus 1 (anti-HIV-1),
2. antibodies against human immunodeficiency virus 2 (anti-HIV-2),
3. hepatitis B surface antigen (HBsAg),
4. antibodies against hepatitis C virus (anti-HCV).

Pending complete harmonisation of the laboratory tests to be carried out, the competent authority may require that a test for alanine aminotransferase (ALT) also be carried out.

The test methods used are of suitable sensitivity and specificity and comply with the regulations in force. If a repeat-reactive result is found in any of these tests, the donation is not accepted.

INDIVIDUAL PLASMA UNITS

The plasma is prepared by a method that removes cells and cell debris as completely as possible. Whether prepared from whole blood or by plasmapheresis, the plasma is separated from the cells by a method designed to prevent

the introduction of micro-organisms. No antibacterial or antifungal agent is added to the plasma. The containers comply with the requirements for glass containers (3.2.1) or for plastic containers for blood and blood components (3.2.3). The containers are closed so as to prevent contamination.

If 2 or more units are pooled prior to freezing, the operations are carried out using sterile connecting devices or under aseptic conditions and using containers that have not previously been used.

When obtained by plasmapheresis, plasma intended for the recovery of proteins that are labile in plasma is frozen by cooling rapidly at -30°C or below as soon as possible and at the latest within 24 h of collection.

When obtained from whole blood, plasma intended for the recovery of proteins that are labile in plasma is separated from cellular elements and is frozen by cooling rapidly at -30°C or below as soon as possible and at the latest within 24 h of collection.

When obtained from whole blood, plasma intended solely for the recovery of proteins that are not labile in plasma is separated from cellular elements and frozen at -20°C or below as soon as possible and at the latest within 72 h of collection.

It is not intended that the determination of total protein and factor VIII shown below be carried out on each unit of plasma. They are rather given as guidelines for good manufacturing practice, the test for factor VIII being relevant for plasma intended for use in the preparation of concentrates of labile proteins.

The total protein content of a unit of plasma depends on the serum protein content of the donor and the degree of dilution inherent in the donation procedure. When plasma is obtained from a suitable donor and using the intended proportion of anticoagulant solution, a total protein content complying with the limit of 50 g/l is obtained. If a volume of blood or plasma smaller than intended is collected into the anticoagulant solution, the resulting plasma is not necessarily unsuitable for pooling for fractionation. The aim of good manufacturing practice must be to achieve the prescribed limit for all normal donations.

Preservation of factor VIII in the donation depends on the collection procedure and the subsequent handling of the blood and plasma. With good practice, 0.7 IU/ml can usually be achieved, but units of plasma with a lower activity may still be suitable for use in the production of coagulation factor concentrates. The aim of good manufacturing practice is to conserve labile proteins as much as possible.

Total protein. Carry out the test using a pool of not fewer than 10 units. Dilute the pool with a 9 g/l solution of sodium chloride R to obtain a solution containing about 15 mg of protein in 2 ml. To 2.0 ml of this solution in a round-bottomed centrifuge tube add 2 ml of a 75 g/l solution of sodium molybdate R and 2 ml of a mixture of 1 volume of nitrogen-free sulphuric acid R and 30 volumes of water R. Shake, centrifuge for 5 min, decant the supernatant liquid and allow the inverted tube to drain on filter paper. Determine the nitrogen in the residue by the method of sulphuric acid digestion (2.5.9) and calculate the protein content by multiplying the quantity of nitrogen by 6.25. The total protein content is not less than 50 g/l.

Factor VIII. Carry out the test using a pool of not fewer than 10 units. Thaw the samples to be examined, if necessary, at 37°C . Carry out the assay of factor VIII (2.7.4), using a reference plasma calibrated against the International Standard for blood coagulation factor VIII in plasma. The activity is not less than 0.7 IU/ml.

POOLED PLASMA

During the manufacture of plasma products, the first homogeneous pool of plasma (for example, after removal of cryoprecipitate) is tested for HBsAg, for hepatitis C virus antibodies and for HIV antibodies using test methods of suitable sensitivity and specificity; the pool must give negative results in these tests.

The plasma pool is also tested for hepatitis C virus RNA using a validated nucleic acid amplification technique (2.6.21). A positive control with 100 IU/ml of hepatitis C virus RNA and, to test for inhibitors, an internal control prepared by addition of a suitable marker to a sample of the plasma pool are included in the test. The test is invalid if the positive control is non-reactive or if the result obtained with the internal control indicates the presence of inhibitors. The plasma pool complies with the test if it is found non-reactive for hepatitis C virus RNA.

Hepatitis C virus RNA for NAT testing BRP is suitable for use as a positive control.

CHARACTERS

Before freezing, a clear to slightly turbid liquid without visible signs of haemolysis; it may vary in colour from light yellow to green.

STORAGE

Store and transport frozen plasma at or below -20°C ; the plasma may still be used for fractionation if the temperature is between -20°C and -15°C for not more than a total of 72 h without exceeding -15°C on more than one occasion as long as the temperature is at all times -5°C or lower.

LABELLING

The label enables each individual unit to be traced to a specific donor.

01/2005:1646
corrected

HUMAN PLASMA (POOLED AND TREATED FOR VIRUS INACTIVATION)

Plasma humanum collectum deinde conditum ad viros exstinguendos

DEFINITION

Human plasma pooled and treated for virus inactivation is a frozen or freeze-dried, sterile, non-pyrogenic preparation obtained from human plasma derived from donors belonging to the same ABO blood group. The preparation is thawed or reconstituted before use to give a solution for infusion.

The human plasma used complies with the monograph on *Human plasma for fractionation* (0853).

PRODUCTION

The units of plasma to be used are cooled to -30°C or lower within 6 h of separation of cells and in any case within 24 h of collection.

The pool is prepared by mixing units of plasma belonging to the same ABO blood group.

The pool of plasma is tested for hepatitis B surface antigen (HBsAg), for hepatitis C virus antibodies and for HIV antibodies using test methods of suitable sensitivity and specificity; the pool must give negative results in these tests.

The plasma pool is also tested for hepatitis C virus RNA using a validated nucleic acid amplification technique (2.6.21).

A positive control with 100 IU of hepatitis C virus RNA

per millilitre and, to test for inhibitors, an internal control prepared by addition of a suitable marker to a sample of the plasma pool are included in the test. The test is invalid if the positive control is non-reactive or if the result obtained with the internal control indicates the presence of inhibitors. The pool complies with the test if it is found non-reactive for hepatitis C virus RNA.

Hepatitis C virus RNA for NAT testing BRP is suitable for use as a positive control.

To limit the potential burden of B19 virus in plasma pools, the plasma pool is also tested for B19 virus using a validated nucleic acid amplification technique (2.6.21).

A positive control with 10^4 IU of B19 virus DNA per millilitre and, to test for inhibitors, an internal control prepared by addition of a suitable marker to a sample of the plasma pool are included in the test. The test is invalid if the positive control is non-reactive or if the result obtained with the internal control indicates the presence of inhibitors. The plasma pool contains not more than 10^4 IU of B19 virus DNA per millilitre.

B19 virus DNA for NAT testing BRP is suitable for use as a positive control.

The method of preparation is designed to minimise activation of any coagulation factor (to minimise potential thrombogenicity) and includes a step, or steps that have been shown to inactivate known agents of infection; if substances are used for the inactivation of viruses during production, the subsequent purification procedure must be validated to demonstrate that the concentration of these substances is reduced to a suitable level and that any residues are such as not to compromise the safety of the preparation for patients.

A typical method to inactivate enveloped viruses is the solvent-detergent process which uses treatment with a combination of tributyl phosphate and octoxinol 10; these reagents are subsequently removed by oil extraction or by solid phase extraction so that the amount in the final product is less than $2\text{ }\mu\text{g/ml}$ for tributyl phosphate and less than $5\text{ }\mu\text{g/ml}$ for octoxinol 10.

No antimicrobial preservative is added.

The solution is passed through a bacteria-retentive filter, distributed aseptically into the final containers and immediately frozen; it may subsequently be freeze-dried.

Plastic containers comply with the requirements for sterile plastic containers for human blood and blood components (3.2.3).

Glass containers comply with the requirements for glass containers for pharmaceutical use (3.2.1).

CHARACTERS

The frozen preparation, after thawing, is a clear or slightly opalescent liquid free from solid and gelatinous particles. The freeze-dried preparation is an almost white or slightly yellow powder or friable solid.

Thaw or reconstitute the preparation to be examined as stated on the label immediately before carrying out the identification, tests and assay.

IDENTIFICATION

A. Examine by electrophoresis (2.2.31) comparing with normal human plasma. The electropherograms show the same bands.

B. It complies with the test for anti-A and anti-B haemagglutinins (see Tests).

TESTS

pH (2.2.3): 6.5 to 7.6.

Osmolality (2.2.35): minimum 240 mosmol/kg.

Total protein: minimum 45 g/l.

Dilute with a 9 g/l solution of *sodium chloride R* to obtain a solution containing about 15 mg of protein in 2 ml. To 2.0 ml of this solution in a round-bottomed centrifuge tube add 2 ml of a 75 g/l solution of *sodium molybdate R* and 2 ml of a mixture of 1 volume of *nitrogen-free sulphuric acid R* and 30 volumes of *water R*. Shake, centrifuge for 5 min, decant the supernatant liquid and allow the inverted tube to drain on filter paper. Determine the nitrogen in the residue by the method of sulphuric acid digestion (2.5.9) and calculate the quantity of protein by multiplying the result by 6.25.

Activated coagulation factors (2.6.22). It complies with the test for activated coagulation factors. Carry out the test with 0.1 ml of the preparation to be examined instead of 1 to 10 and 1 to 100 dilutions. The coagulation time for the tube containing the preparation to be examined is not less than 150 s.

Anti-A and anti-B haemagglutinins (2.6.20). The presence of haemagglutinin (anti-A or anti-B) corresponds to the blood group stated on the label.

Hepatitis A virus antibodies: minimum 2 IU/ml, determined by a suitable immunochemical method (2.7.1).

Human hepatitis A immunoglobulin BRP is suitable for use as a reference preparation.

Irregular erythrocyte antibodies. The preparation to be examined does not show the presence of irregular erythrocyte antibodies when examined without dilution by an indirect antiglobulin test.

Citrate. Liquid chromatography (2.2.29).

Test solution. Dilute the preparation to be examined with an equal volume of a 9 g/l solution of *sodium chloride R*. Filter the solution using a filter with 0.45 µm pores.

Reference solution. Dissolve 0.300 g of *sodium citrate R* in *water R* and dilute to 100.0 ml with the same solvent.

Column:

- *size*: $l = 0.3$ m, $\varnothing = 7.8$ mm,
- *stationary phase*: cation exchange resin *R* (9 µm).

Mobile phase: 0.51 g/l solution of *sulphuric acid R*.

Flow rate: 0.5 ml/min.

Detection: spectrophotometer at 215 nm.

Equilibration: 15 min.

Injection: 10 µl.

Retention time: citrate = about 10 min.

Limit:

- *citrate*: maximum 25 mmol/l.

Calcium: maximum 5.0 mmol/l.

Atomic absorption spectrometry (2.2.23, *Method I*).

Source: calcium hollow-cathode lamp using a transmission band preferably of 0.5 nm.

Wavelength: 622 nm.

Atomisation device: air-acetylene or acetylene-propane flame.

Potassium: maximum 5.0 mmol/l.

Atomic emission spectrometry (2.2.22, *Method I*).

Wavelength: 766.5 nm.

Sodium: maximum 200 mmol/l.

Atomic emission spectrometry (2.2.22, *Method I*).

Wavelength: 589 nm.

Water: for the freeze-dried product: determined by a suitable method, such as the semi-micro determination of water (2.5.12), loss on drying (2.2.32) or near-infrared spectrometry (2.2.40), the water content is within the limits approved by the competent authority.

Sterility (2.6.1). It complies with the test for sterility.

Pyrogens (2.6.8). It complies with the test for pyrogens. Inject 3 ml per kilogram of the rabbit's mass.

ASSAY

Factor VIII. Carry out the assay of coagulation factor VIII (2.7.4) using a reference plasma calibrated against the International Standard for blood coagulation factor VIII in plasma.

The estimated potency is not less than 0.5 IU/ml. The confidence limits ($P = 0.95$) are not less than 80 per cent and not more than 120 per cent of the estimated potency.

Factor V. Using *imidazole buffer solution pH 7.3 R*, prepare 3 twofold dilutions of the preparation to be examined, preferably in duplicate, from 1 in 10 to 1 in 40. Test each dilution as follows: mix 0.1 ml of *plasma substrate deficient in factor V R*, 0.1 ml of the dilution to be examined, 0.1 ml of *thromboplastin R* and 0.1 ml of a 3.5 g/l solution of *calcium chloride R*; measure the coagulation times, i.e. the interval between the moment at which the calcium chloride solution is added and the first indication of the formation of fibrin, which may be observed visually or by means of a suitable apparatus.

In the same manner, determine the coagulation time of 4 twofold dilutions (1 in 10 to 1 in 80) of human normal plasma in *imidazole buffer solution pH 7.3 R*. 1 unit of factor V is equal to the activity of 1 ml of human normal plasma. Human normal plasma is prepared by pooling plasma units from not fewer than 30 donors and stored at -30°C or lower.

Check the validity of the assay and calculate the potency of the test preparation by the usual statistical methods for a parallel-line assay (for example, 5.3).

The estimated potency is not less than 0.5 IU/ml. The confidence limits ($P = 0.95$) are not less than 80 per cent and not more than 120 per cent of the estimated potency.

Factor XI. Carry out the assay of human coagulation factor XI (2.7.22) using as reference human normal plasma (see above under Factor V).

The estimated potency is not less than 0.5 IU/ml. The confidence limits ($P = 0.95$) are not less than 80 per cent and not more than 125 per cent of the estimated potency.

LABELLING

The label states:

- the ABO blood group,
- the method used for virus inactivation.

01/2005:0554

HUMAN PROTHROMBIN COMPLEX

Prothrombinum multiplex humanum

DEFINITION

Human prothrombin complex is a plasma protein fraction containing blood coagulation factor IX together with variable amounts of coagulation factors II, VII and X; the presence and proportion of these additional factors depends on the

method of fractionation. It is obtained from human plasma that complies with the monograph on *Human plasma for fractionation* (0853).

The potency of the preparation, reconstituted as stated on the label, is not less than 20 IU of factor IX per millilitre.

PRODUCTION

The method of preparation is designed to minimise activation of any coagulation factor (to minimise potential thrombogenicity) and includes a step or steps that have been shown to remove or to inactivate known agents of infection; if substances are used for inactivation of viruses during production, the subsequent purification procedure must be validated to demonstrate that the concentration of these substances is reduced to a suitable level and that any residues are such as not to compromise the safety of the preparation for patients.

The specific activity is not less than 0.6 IU of factor IX per milligram of total protein, before the addition of any protein stabiliser.

The prothrombin complex fraction is dissolved in a suitable liquid. Heparin, antithrombin and other auxiliary substances such as a stabiliser may be added. No antimicrobial preservative is added. The solution is passed through a bacteria-retentive filter, distributed aseptically into the final containers and immediately frozen. It is subsequently freeze-dried and the containers are closed under vacuum or under an inert gas.

CHARACTERS

A white or slightly coloured powder or friable solid, very hygroscopic.

Reconstitute the preparation to be examined as stated on the label immediately before carrying out the identification, tests (except those for solubility and water) and assay.

IDENTIFICATION

It complies with the limits of the assay for coagulation factor IX activity and, where applicable, those for factors II, VII and X.

TESTS

Solubility. To a container of the preparation to be examined add the volume of the liquid stated on the label at the recommended temperature. The preparation dissolves completely with gentle swirling within 10 min, giving a clear solution that may be coloured.

pH (2.2.3): 6.5 to 7.5.

Osmolality (2.2.35): minimum 240 mosmol/kg.

Total protein. If necessary, dilute an accurately measured volume of the reconstituted preparation with a 9 g/l solution of *sodium chloride R* to obtain a solution expected to contain about 15 mg of protein in 2 ml. To 2.0 ml of the solution in a round-bottomed centrifuge tube add 2 ml of a 75 g/l solution of *sodium molybdate R* and 2 ml of a mixture of 1 volume of *nitrogen-free sulphuric acid R* and 30 volumes of *water R*. Shake, centrifuge for 5 min, decant the supernatant liquid and allow the inverted tube to drain on filter paper. Determine the nitrogen in the residue by the method of sulphuric acid digestion (2.5.9) and calculate the amount of protein by multiplying the result by 6.25.

Activated coagulation factors (2.6.22). If necessary, dilute the preparation to be examined to contain 20 IU of factor IX per millilitre. For each of the dilutions, the coagulation time is not less than 150 s.

Heparin. If heparin has been added during preparation, determine the amount present by the assay of heparin in coagulation factor concentrates (2.7.12). The preparation to be examined contains not more than the amount of heparin stated on the label and in any case not more than 0.5 IU of heparin per International Unit of factor IX.

Thrombin. If the preparation to be examined contains heparin, determine the amount present as described in the test for heparin and neutralise it by addition of *protamine sulphate R* (10 µg of protamine sulphate neutralises 1 IU of heparin). In each of 2 test-tubes, mix equal volumes of the reconstituted preparation and a 3 g/l solution of *fibrinogen R*. Keep one of the tubes at 37 °C for 6 h and the other at room temperature for 24 h. In a third tube, mix a volume of the fibrinogen solution with an equal volume of a solution of *human thrombin R* (1 IU/ml) and place the tube in a water-bath at 37 °C. No coagulation occurs in the tubes containing the preparation to be examined. Coagulation occurs within 30 s in the tube containing thrombin.

Water. Determined by a suitable method, such as the semi-micro determination of water (2.5.12), loss on drying (2.2.32) or near-infrared spectrometry (2.2.40), the water content is within the limits approved by the competent authority.

Sterility (2.6.1). It complies with the test for sterility.

Pyrogens (2.6.8). It complies with the test for pyrogens. Inject per kilogram of the rabbit's mass a volume of the reconstituted preparation equivalent to not less than 30 IU of factor IX.

ASSAY

Factor IX. Carry out the assay of human coagulation factor IX (2.7.11).

The estimated potency is not less than 80 per cent and not more than 125 per cent of the stated potency. The confidence interval ($P = 0.95$) of the estimated potency is not greater than 80 per cent to 125 per cent.

Factor II. Carry out the assay of human coagulation factor II (2.7.18).

The estimated potency is not less than 80 per cent and not more than 125 per cent of the stated potency. The confidence interval ($P = 0.95$) of the estimated potency is not greater than 90 per cent to 111 per cent.

Factor VII. If the label states that the preparation contains factor VII, carry out the assay of human coagulation factor VII (2.7.10).

The estimated potency is not less than 80 per cent and not more than 125 per cent of the stated potency. The confidence interval ($P = 0.95$) of the estimated potency is not greater than 80 per cent to 125 per cent.

Factor X. Carry out the assay of human coagulation factor X (2.7.19).

The estimated potency is not less than 80 per cent and not more than 125 per cent of the stated potency. The confidence interval ($P = 0.95$) of the estimated potency is not greater than 90 per cent to 111 per cent.

STORAGE

In an airtight container, protected from light.

LABELLING

The label states:

- the number of International Units of factor IX, factor II and factor X per container,
- where applicable, the number of International Units of factor VII per container,

- where applicable, that the preparation contains protein C and/or protein S,
- the amount of protein per container,
- the name and quantity of any added substances, including where applicable, heparin,
- the name and quantity of the liquid to be used for reconstitution,
- that the transmission of infectious agents cannot be totally excluded when medicinal products prepared from human blood or plasma are administered.

01/2005:0723

HUMAN RABIES IMMUNOGLOBULIN

Immunoglobulinum humanum rabicum

DEFINITION

Human rabies immunoglobulin is a liquid or freeze-dried preparation containing immunoglobulins, mainly immunoglobulin G. The preparation is intended for intramuscular administration. It is obtained from plasma from donors immunised against rabies. It contains specific antibodies neutralising the rabies virus. *Human normal immunoglobulin (0338)* may be added.

It complies with the monograph on *Human normal immunoglobulin (0338)*, except for the minimum number of donors and the minimum total protein content.

POTENCY

The potency is determined by comparing the dose of immunoglobulin required to neutralise the infectivity of a rabies virus suspension with the dose of a reference preparation, calibrated in International Units, required to produce the same degree of neutralisation (2.7.1). The test is performed in sensitive cell cultures and the presence of unneutralised virus is revealed by immunofluorescence.

The International Unit is the specific neutralising activity for rabies virus in a stated amount of the International Standard for anti-rabies immunoglobulin. The equivalence in International Units of the International Standard is stated by the World Health Organisation.

Human rabies immunoglobulin BRP is calibrated in International Units by comparison with the International Standard.

Carry out the test in suitable sensitive cells. It is usual to use the BHK 21 cell line, grown in the medium described below, between the 18th and 30th passage levels counted from the ATCC seed lot. Harvest the cells after 2 to 4 days of growth, treat with trypsin and prepare a suspension containing 500 000 cells per millilitre (cell suspension). 10 min before using this suspension add 10 µg of *diethylamino-ethyl-dextran R* per millilitre, if necessary, to increase the sensitivity of the cells.

Use a fixed virus strain grown in sensitive cells, such as the CVS strain of rabies virus adapted to growth in the BHK 21 cell line (seed virus suspension). Estimate the titre of the seed virus suspension as follows.

Prepare a series of dilutions of the viral suspension. In the chambers of cell-culture slides (8 chambers per slide), place 0.1 ml of each dilution and 0.1 ml of medium and add 0.2 ml of the cell suspension. Incubate in an atmosphere of carbon dioxide at 37 °C for 24 h. Carry out

fixation, immunofluorescence staining and evaluation as described below. Determine the end-point titre of the seed virus suspension and prepare the working virus dilution corresponding to 100 CCID₅₀ per 0.1 ml.

For each assay, check the amount of virus used by performing a control titration: from the dilution corresponding to 100 CCID₅₀ per 0.1 ml, make three tenfold dilutions. Add 0.1 ml of each dilution to four chambers containing 0.1 ml of medium and add 0.2 ml of the cell suspension. The test is not valid unless the titre lies between 30 CCID₅₀ and 300 CCID₅₀.

Dilute the reference preparation to a concentration of 2 IU/ml using non-supplemented culture medium (stock reference dilution, stored below –80 °C). Prepare two suitable predilutions (1:8 and 1:10) of the stock reference dilution so that the dilution of the reference preparation that reduces the number of fluorescent fields by 50 per cent lies within the four dilutions of the cell-culture slide. Add 0.1 ml of the medium to each chamber, except the first in each of two rows, to which add respectively 0.2 ml of the two predilutions of the stock reference dilution transferring successively 0.1 ml to the other chambers.

Dilute the preparation to be examined 1 in 100 using non-supplemented medium (stock immunoglobulin dilution) - to reduce to a minimum errors due to viscosity of the undiluted preparation - and make three suitable predilutions so that the dilution of the preparation to be examined that reduces the number of fluorescent fields by 50 per cent lies within the four dilutions of the cell-culture slide. Add 0.1 ml of the medium to all the chambers except the first in each of three rows, to which add respectively 0.2 ml of the three predilutions of the stock immunoglobulin dilution. Prepare a series of twofold dilutions transferring successively 0.1 ml to the other chambers.

To all the chambers containing the dilutions of the reference preparation and the dilutions of the preparation to be examined, add 0.1 ml of the virus suspension corresponding to 100 CCID₅₀ per 0.1 ml (working virus dilution), shake manually, allow to stand in an atmosphere of carbon dioxide at 37 °C for 90 min, add 0.2 ml of the cell suspension, shake manually and allow to stand in an atmosphere of carbon dioxide at 37 °C for 24 h.

After 24 h, discard the medium and remove the plastic walls. Wash the cell monolayer with *phosphate buffered saline pH 7.4 R* and then with a mixture of 20 volumes of *water R* and 80 volumes of *acetone R* and fix in a mixture of 20 volumes of *water R* and 80 volumes of *acetone R* at –20 °C for 3 min. Spread on the slides *fluorescein-conjugated rabies antiserum R* ready for use. Allow to stand in an atmosphere with a high level of moisture at 37 °C for 30 min. Wash with *phosphate buffered saline pH 7.4 R* and dry. Examine twenty fields in each chamber at a magnification of 250 ×, using a microscope equipped for fluorescence readings. Note the number of fields with at least one fluorescent cell. Check the test dose used in the virus titration slide and determine the dilution of the reference preparation and the dilution of the preparation to be examined that reduce the number of fluorescent fields by 50 per cent, calculating the two or three dilutions together using probit analysis. The test is not valid unless the statistical analysis shows a significant slope of the dose-response curve and no evidence of deviation from linearity or parallelism.

The stated potency is not less than 150 IU/ml. The estimated potency is not less than the stated potency and is not greater than twice the stated potency. The confidence limits ($P = 0.95$) of the estimated potency are not less than 80 per cent and not more than 125 per cent.

CULTURE MEDIUM FOR GROWTH OF BHK 21 CELLS

01/2005:0617

Commercially available media that have a slightly different composition from that shown below may also be used.

Sodium chloride	6.4 g
Potassium chloride	0.40 g
Calcium chloride, anhydrous	0.20 g
Magnesium sulphate, heptahydrate	0.20 g
Sodium dihydrogen phosphate, monohydrate	0.124 g
Glucose monohydrate	4.5 g
Ferric nitrate, nonahydrate	0.10 mg
L-Arginine hydrochloride	42.0 mg
L-Cystine	24.0 mg
L-Histidine	16.0 mg
L-Isoleucine	52.0 mg
L-Leucine	52.0 mg
L-Lysine hydrochloride	74.0 mg
L-Phenylalanine	33.0 mg
L-Threonine	48.0 mg
L-Tryptophan	8.0 mg
L-Tyrosine	36.0 mg
L-Valine	47.0 mg
L-Methionine	15.0 mg
L-Glutamine	0.292 g
<i>D</i> -Inositol	3.60 mg
Choline chloride	2.0 mg
Folic acid	2.0 mg
Nicotinamide	2.0 mg
Calcium pantothenate	2.0 mg
Pyridoxal hydrochloride	2.0 mg
Thiamine hydrochloride	2.0 mg
Riboflavine	0.2 mg
Phenol red	15.0 mg
Sodium hydrogen carbonate	2.75 g
Water to	1000 ml

The medium is supplemented with:

Foetal calf serum (heated at 56 °C for 30 min)	10 per cent
Tryptose phosphate broth	10 per cent
Benzylpenicillin sodium	60 mg/l
Streptomycin	0.1 g/l

STORAGE

See *Human normal immunoglobulin (0338)*.

LABELLING

See *Human normal immunoglobulin (0338)*.

The label states the number of International Units per container.

HUMAN RUBELLA IMMUNOGLOBULIN

Immunoglobulinum humanum rubellae

DEFINITION

Human rubella immunoglobulin is a liquid or freeze-dried preparation containing immunoglobulins, mainly immunoglobulin G. The preparation is intended for intramuscular administration. It is obtained from plasma containing specific antibodies against rubella virus. *Human normal immunoglobulin (0338)* may be added.

It complies with the monograph on *Human normal immunoglobulin (0338)*, except for the minimum number of donors and the minimum total protein content.

POTENCY

The potency is determined by comparing the activity of the preparation to be examined in a suitable haemagglutination-inhibition test with that of a reference preparation calibrated in International Units.

The International Unit is the activity contained in a stated amount of the International Standard for anti-rubella immunoglobulin. The equivalence in International Units of the International Reference Preparation is stated by the World Health Organisation.

The estimated potency is not less than 4500 IU/ml. The confidence limits ($P = 0.95$) of the estimate of potency are not less than 50 per cent and not more than 200 per cent of the stated potency.

STORAGE

See *Human normal immunoglobulin (0338)*.

LABELLING

See *Human normal immunoglobulin (0338)*.

The label states the number of International Units per millilitre.

01/2005:0398

HUMAN TETANUS IMMUNOGLOBULIN

Immunoglobulinum humanum tetanicum

DEFINITION

Human tetanus immunoglobulin is a liquid or freeze-dried preparation containing immunoglobulins, mainly immunoglobulin G. The preparation is intended for intramuscular administration. It is obtained from plasma containing specific antibodies against the toxin of *Clostridium tetani*. *Human normal immunoglobulin (0338)* may be added.

It complies with the monograph on *Human normal immunoglobulin (0338)*, except for the minimum number of donors and the minimum total protein content.

PRODUCTION

During development, a satisfactory relationship shall be established between the potency determined by immunoassay as described under Potency and that determined by means of the following test for toxin-neutralising capacity in mice.

Toxin-neutralising capacity in mice. The potency is determined by comparing the quantity necessary to protect mice against the paralytic effects of a fixed quantity of

tetanus toxin with the quantity of a reference preparation of human tetanus immunoglobulin, calibrated in International Units, necessary to give the same protection.

The International Unit of antitoxin is the specific neutralising activity for tetanus toxin contained in a stated amount of the International Standard, which consists of freeze-dried human immunoglobulin. The equivalence in International Units of the International Standard is stated by the World Health Organisation.

Human tetanus immunoglobulin BRP is calibrated in International Units by comparison with the International Standard.

Selection of animals. Use mice weighing 16 g to 20 g.

Preparation of the test toxin. Prepare the test toxin by a suitable method from the sterile filtrate of a culture in liquid medium of *C. tetani*. The two methods shown below are given as examples and any other suitable method may be used.

(1) To the filtrate of an approximately 9-day culture add 1 to 2 volumes of *glycerol R* and store the mixture in the liquid state at a temperature slightly below 0 °C.

(2) Precipitate the toxin by addition to the filtrate of *ammonium sulphate R*, dry the precipitate *in vacuo* over *diphosphorus pentoxide R*, reduce to a powder and store dry, either in sealed ampoules or *in vacuo* over *diphosphorus pentoxide R*.

Determination of test dose of toxin (Lp/10 dose). Prepare a solution of the reference preparation in a suitable liquid such that it contains 0.5 IU of antitoxin per millilitre. If the test toxin is stored dry, reconstitute it using a suitable liquid. Prepare mixtures of the solution of the reference preparation and the test toxin such that each contains 2.0 ml of the solution of the reference preparation, one of a graded series of volumes of the test toxin and sufficient of a suitable liquid to bring the volume to 5.0 ml. Allow the mixtures to stand, protected from light, for 60 min. Using six mice for each mixture, inject a dose of 0.5 ml subcutaneously into each mouse. Observe the mice for 96 h. Mice that become paralysed may be killed. The test dose of toxin is the quantity in 0.5 ml of the mixture made with the smallest amount of toxin capable of causing, despite partial neutralisation by the reference preparation, paralysis in all six mice injected with the mixture, within the observation period.

Determination of potency of the immunoglobulin. Prepare a solution of the reference preparation in a suitable liquid such that it contains 0.5 IU of antitoxin per millilitre. Prepare a solution of the test toxin in a suitable liquid such that it contains five test doses per millilitre. Prepare mixtures of the solution of the test toxin and the immunoglobulin to be examined such that each contains 2.0 ml of the solution of the test toxin, one of a graded series of volumes of the immunoglobulin to be examined and sufficient of a suitable liquid to bring the total volume to 5.0 ml. Also prepare mixtures of the solution of the test toxin and the solution of the reference preparation such that each contains 2.0 ml of the solution of the test toxin, one of a graded series of volumes of the solution of the reference preparation centred on that volume (2.0 ml) that contains 1 IU and sufficient of a suitable liquid to bring the total volume to 5.0 ml. Allow the mixtures to stand, protected from light, for 60 min. Using six mice for each mixture, inject subcutaneously a dose of 0.5 ml into each mouse. Observe the mice for 96 h. Mice that become paralysed may be killed. The mixture that contains the largest volume of immunoglobulin that fails to protect the mice from paralysis contains 1 IU. This quantity is used to calculate the potency of the immunoglobulin in International Units per millilitre.

The test is not valid unless all the mice injected with mixtures containing 2.0 ml or less of the solution of the reference preparation show paralysis and all those injected with mixtures containing more do not.

POTENCY

The potency is determined by comparing the antibody titre of the immunoglobulin to be examined with that of a reference preparation calibrated in International Units, using an immunoassay of suitable sensitivity and specificity (2.7.1).

The International Unit is the activity contained in a stated amount of the International Standard for anti-tetanus immunoglobulin. The equivalence in International Units of the International Standard is stated by the World Health Organisation.

Human tetanus immunoglobulin BRP is calibrated in International Units by comparison with the International Standard.

The stated potency is not less than 100 IU of tetanus antitoxin per millilitre. The estimated potency is not less than the stated potency. The confidence limits ($P = 0.95$) of the estimated potency are not less than 80 per cent and not more than 125 per cent.

STORAGE

See *Human normal immunoglobulin (0338)*.

LABELLING

See *Human normal immunoglobulin (0338)*.

The label states the number of International Units per container.

01/2005:0724

HUMAN VARICELLA IMMUNOGLOBULIN

Immunoglobulinum humanum varicellae

DEFINITION

Human varicella immunoglobulin is a liquid or freeze-dried preparation containing immunoglobulins, mainly immunoglobulin G. The preparation is intended for intramuscular administration. It is obtained from plasma from selected donors having antibodies against *Herpesvirus varicellae*. *Human normal immunoglobulin (0338)* may be added.

It complies with the monograph on *Human normal immunoglobulin (0338)* except for the minimum number of donors, the minimum total protein content and, where authorised, the test for antibody to hepatitis B surface antigen.

POTENCY

The potency is determined by comparing the antibody titre of the immunoglobulin to be examined with that of a reference preparation calibrated in International Units, using an immunoassay of suitable sensitivity and specificity (2.7.1).

The International Unit is the activity contained in a stated amount of the International Standard for anti varicella-zoster. The equivalence in International Units of the International Standard is stated by the World Health Organisation.

The stated potency is not less than 100 IU/ml. The estimated potency is not less than the stated potency. The confidence limits ($P = 0.95$) of the estimated potency are not less than 80 per cent and not more than 125 per cent.

STORAGE

See *Human normal immunoglobulin (0338)*.

LABELLING

See *Human normal immunoglobulin (0338)*.

The label states the number of International Units per container.

01/2005:1528

HUMAN VARICELLA IMMUNOGLOBULIN FOR INTRAVENOUS ADMINISTRATION

Immunoglobulinum humanum varicellae ad usum intravenosum

DEFINITION

Human varicella immunoglobulin for intravenous administration is a liquid or freeze-dried preparation containing immunoglobulins, mainly immunoglobulin G. It is obtained from plasma from selected donors having antibodies against human herpesvirus 3 (varicella-zoster virus 1). *Human normal immunoglobulin for intravenous administration (0918)* may be added.

It complies with the monograph on *Human normal immunoglobulin for intravenous administration (0918)*, except for the minimum number of donors, the minimum total protein content and the limit for osmolality.

POTENCY

The potency is determined by comparing the antibody titre of the immunoglobulin to be examined with that of a reference preparation calibrated in International Units, using an immunoassay of suitable sensitivity and specificity (2.7.1).

The International Unit is the activity contained in a stated amount of the International Standard for anti varicella-zoster immunoglobulin. The equivalence in International Units of the International Standard is stated by the World Health Organisation.

The stated potency is not less than 25 IU/ml. The estimated potency is not less than the stated potency. The confidence limits ($P = 0.95$) of the estimated potency are not less than 80 per cent and not more than 125 per cent.

STORAGE

See *Human normal immunoglobulin for intravenous administration (0918)*.

LABELLING

See *Human normal immunoglobulin for intravenous administration (0918)*.

The label states the number of International Units per container.

01/2005:0912
corrected

HYALURONIDASE

Hyaluronidasum

DEFINITION

Hyaluronidase is an enzyme extracted from mammalian testes (for example bovine testes) and capable of hydrolysing mucopolysaccharides of the hyaluronic acid type. It contains

not less than 300 IU of hyaluronidase activity per milligram, calculated with reference to the dried substance. It may contain a suitable stabiliser.

PRODUCTION

The animals from which hyaluronidase is derived must fulfil the requirements for the health of animals suitable for human consumption to the satisfaction of the competent authority.

Hyaluronidase is produced using a validated method of extraction and purification in conditions designed to minimise the degree of microbial contamination; the method of preparation must have been shown to reduce any contamination by viruses or other known infectious agents to acceptable limits.

CHARACTERS

A white or yellowish-white, amorphous powder, soluble in water, practically insoluble in acetone and in ethanol.

IDENTIFICATION

A solution containing the equivalent of 100 IU of hyaluronidase in 1 ml of a 9 g/l solution of *sodium chloride R* depolymerises an equal volume of a 10 g/l solution of *sodium hyaluronate BRP* in 1 min at 20 °C as shown by a pronounced decrease in viscosity. This action is destroyed by heating the hyaluronidase at 100 °C for 30 min.

TESTS

Appearance of solution. Dissolve 0.10 g of the substance to be examined in *water R* and dilute to 10 ml. The solution is clear (2.2.1).

pH (2.2.3). Dissolve 30 mg in 10 ml of *carbon dioxide-free water R*. The pH of the solution is 4.5 to 7.5.

Loss on drying (2.2.32). Not more than 5.0 per cent, determined on 0.500 g by drying at 60 °C at a pressure not exceeding 670 Pa for 2 h.

Bacterial endotoxins (2.6.14): less than 0.2 IU per IU of hyaluronidase.

ASSAY

The activity of hyaluronidase is determined by comparing the rate at which it hydrolyses *sodium hyaluronate BRP* with the rate obtained with the International Standard, or a reference preparation calibrated in International Units, using a slope-ratio assay.

Substrate solution. To 0.10 g of *sodium hyaluronate BRP* in a 25 ml conical flask add slowly 20.0 ml of *water R* at 4 °C. The rate of addition must be slow enough to allow the substrate particles to swell (about 5 min). Maintain at 4 °C and stir for at least 12 h. Store at 4 °C and use within 4 days.

For the test solution and the reference solution, prepare the solution and carry out the dilution at 0 °C to 4 °C.

Test solution. Dissolve a suitable amount of the substance to be examined in *hyaluronidase diluent R* so as to obtain a solution containing 0.6 ± 0.3 IU of hyaluronidase per millilitre.

Reference solution. Dissolve a suitable amount of *hyaluronidase BRP* in *hyaluronidase diluent R* so as to obtain a solution containing 0.6 IU of hyaluronidase per millilitre.

In a reaction vessel, mix 1.50 ml of *phosphate buffer solution pH 6.4 R* and 1.0 ml of the substrate solution and equilibrate at 37 ± 0.1 °C. At time $t_1 = 0$ (first chronometer) add 0.50 ml of the test solution containing E_t mg of the enzyme to be examined, mix, measure the viscosity of the solution using a suitable viscometer maintained at 37 ± 0.1 °C and record

the outflow time t_2 using a second chronometer (graduated in 0.1 second intervals), several times during about 20 min (read on the first chronometer). The following viscometer has been found suitable: Ubbelohde microviscometer (DIN 51 562, Part 2), capillary type MII, viscometer constant about $0.1 \text{ mm}^2/\text{s}^2$.

Repeat the procedure using 0.50 ml of the reference solution containing E_r mg of *hyaluronidase BRP*.

Calculate the viscosity ratio from the expression:

$$\eta_r = \frac{k \times t_2}{0.6915}$$

k = the viscometer constant in mm^2/s^2 (indicated on the viscometer),

t_2 = the outflow time (in seconds) of the solution.

0.6915 = the kinematic viscosity in mm^2/s of the buffer solution at 37°C .

Since the enzymatic reaction continues during the outflow time measurements, the real reaction time equals $t_1 + t_2/2$, half of the outflow time ($t_2/2$) for which a certain measurement is valid being added to the time t_1 at which the measurement is started. Plot $(\ln \eta_r)^{-1}$ as a function of the reaction time ($t_1 + t_2/2$) in seconds. A linear relationship is obtained. Calculate the slope for the substance to be examined (b_r) and the reference preparation (b_r).

Calculate the specific activity in International Units per milligram from the expression:

$$\frac{b_t}{b_r} \times \frac{E_r}{E_t} \times A$$

A = the specific activity of *hyaluronidase BRP* in International Units per milligram.

Carry out the complete procedure at least three times and calculate the average activity of the substance to be examined.

STORAGE

Store in an airtight container at a temperature of 2°C to 8°C . If the substance is sterile, store in a sterile, tamper-proof container.

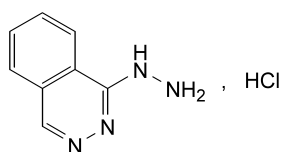
LABELLING

The label states the activity in International Units per milligram.

01/2005:0829

HYDRALAZINE HYDROCHLORIDE

Hydralazini hydrochloridum



$\text{C}_8\text{H}_9\text{ClN}_4$

M_r 196.6

DEFINITION

Hydralazine hydrochloride contains not less than 98.5 per cent and not more than the equivalent of 101.0 per cent of 1-hydrazinophthalazine hydrochloride, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, crystalline powder, soluble in water, slightly soluble in alcohol, very slightly soluble in methylene chloride.

It melts at about 275°C , with decomposition.

IDENTIFICATION

First identification: B, E.

Second identification: A, C, D, E.

- Dissolve 50 mg in *water R* and dilute to 100 ml with the same solvent. Dilute 2 ml of the solution to 100 ml with *water R*. Examined between 220 nm and 350 nm (2.2.25), the solution shows four absorption maxima, at 240 nm, 260 nm, 303 nm and 315 nm. The ratio of the absorbance measured at the maximum at 240 nm to that measured at the maximum at 303 nm is 2.0 to 2.2.
- Examine by infrared spectrophotometry (2.2.24), comparing with the spectrum obtained with *hydralazine hydrochloride CRS*. Examine the substances prepared as discs.
- Dissolve 0.5 g in a mixture of 8 ml of *dilute hydrochloric acid R* and 100 ml of *water R*. Add 2 ml of *sodium nitrite solution R*, allow to stand for 10 min and filter. The precipitate, washed with *water R* and dried at 100°C to 105°C , melts (2.2.14) at 209°C to 212°C .
- Dissolve about 10 mg in 2 ml of *water R*. Add 2 ml of a 20 g/l solution of *nitrobenzaldehyde R* in *alcohol R*. An orange precipitate is formed.
- It gives reaction (a) of chlorides (2.3.1).

TESTS

Solution S. Dissolve 0.5 g in *carbon dioxide-free water R* and dilute to 25 ml with the same solvent.

Appearance of solution. Dilute 4 ml of solution S to 20 ml with *water R*. The solution is clear (2.2.1) and not more intensely coloured than reference solution GY₆ (2.2.2, Method II).

pH (2.2.3). The pH of solution S is 3.5 to 4.2.

Related substances. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 25.0 mg of the substance to be examined in the mobile phase and dilute to 50.0 ml with the mobile phase.

Reference solution (a). Dilute 1.0 ml of the test solution to 100.0 ml with the mobile phase.

Reference solution (b). Dilute 10.0 ml of reference solution (a) to 50.0 ml with the mobile phase.

Reference solution (c). Dissolve 25.0 mg of *phthalazine R* in the mobile phase and dilute to 50.0 ml with the mobile phase. Dilute 4.0 ml of this solution to 100.0 ml with the mobile phase.

Reference solution (d). Dilute a mixture of 4.0 ml of the test solution and 10.0 ml of reference solution (c) to 100.0 ml with the mobile phase.

The solutions must be analysed within one working day.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4.6 mm in internal diameter packed with *nitrile silica gel for chromatography R1* (10 μm),
- as mobile phase at a flow rate of 1 ml/min a solution prepared as follows: to 22 volumes of *acetonitrile R* add 78 volumes of a solution containing 1.44 g of *sodium laurilsulfate R* and 0.75 g of *tetrabutylammonium bromide R* per litre and adjust the mixture to pH 3.0 with 0.05 M *sulphuric acid*,

— as detector a spectrophotometer set at 230 nm.

Inject 20 µl of reference solution (a) and adjust the sensitivity of the detector so that the height of the principal peak in the chromatogram is not less than 70 per cent of the full scale of the recorder. When the chromatograms are recorded in the prescribed conditions, the retention time of hydralazine is about 10 min to 12 min. If necessary, adjust the concentration of acetonitrile in the mobile phase. Inject 20 µl of the test solution and continue the chromatography for three times the retention time of hydralazine. Inject 20 µl of reference solution (b). The area of any peak in the chromatogram obtained with the test solution, apart from the peaks due to the solvent and to hydralazine, is not greater than the area of the peak in the chromatogram obtained with reference solution (b) (0.2 per cent).

The test is not valid unless: the chromatogram obtained with reference solution (d) shows two principal peaks and the resolution between the peaks is at least 2.5; the principal peak in the chromatogram obtained with reference solution (b) has a signal-to-noise ratio of at least 3.

Hydrazine. Examine by thin-layer chromatography (2.2.27), using *silica gel G R* as the coating substance.

Test solution. Dissolve 0.12 g of the substance to be examined in 4 ml of *water R* and add 4 ml of a 150 g/l solution of *salicylaldehyde R* in *methanol R* and 0.2 ml of *hydrochloric acid R*. Mix and keep at a temperature not exceeding 25 °C for 2 h to 4 h, until the precipitate formed has sedimented. Add 4 ml of *toluene R*, shake vigorously and centrifuge. Transfer the clear supernatant liquid to a 100 ml separating funnel, separate the toluene layer and shake vigorously, each time for 3 min, with two quantities, each of 20 ml, of a 200 g/l solution of *sodium metabisulphite R* and with two quantities, each of 50 ml, of *water R*. Separate the toluene layer which is the test solution.

Reference solution (a). Dissolve 12 mg of *hydrazine sulphate R* in *dilute hydrochloric acid R* and dilute to 100.0 ml with the same acid. Dilute 1.0 ml of this solution to 100.0 ml with *dilute hydrochloric acid R*.

Reference solution (b). Prepare the solution at the same time and in the same manner as described for the test solution, using 1.0 ml of reference solution (a) and 3 ml of *water R*.

Apply to the plate 20 µl of the test solution and 20 µl of reference solution (b). Develop over a path of 10 cm using a mixture of 10 volumes of *alcohol R* and 90 volumes of *toluene R*. Allow the plate to dry in air and examine in ultraviolet light at 365 nm. Any spot showing yellow fluorescence in the chromatogram obtained with the test solution is not more intense than the corresponding spot in the chromatogram obtained with reference solution (b) (10 ppm of hydrazine).

Heavy metals (2.4.8). 1.0 g complies with limit test C for heavy metals (20 ppm). Prepare the standard using 2 ml of *lead standard solution (10 ppm Pb R)*.

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying *in vacuo*.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 80.0 mg in 25 ml of *water R*. Add 35 ml of *hydrochloric acid R* and titrate with 0.05 M *potassium iodate*, determining the end-point potentiometrically (2.2.20), using a calomel reference electrode and a platinum indicator electrode.

1 ml of 0.05 M *potassium iodate* is equivalent to 9.832 mg of $C_8H_9ClN_4$.

STORAGE

Store protected from light.

01/2005:0002

HYDROCHLORIC ACID, CONCENTRATED

Acidum hydrochloridum concentratum

HCl

M_r 36.46

DEFINITION

Concentrated hydrochloric acid contains not less than 35.0 per cent *m/m* and not more than 39.0 per cent *m/m* of HCl.

CHARACTERS

A clear, colourless, fuming liquid, miscible with water.

It has a relative density of about 1.18.

IDENTIFICATION

- Dilute with *water R*. The solution is strongly acid (2.2.4).
- It gives the reactions of chlorides (2.3.1).
- It complies with the limits of the assay.

TESTS

Appearance of solution. To 2 ml add 8 ml of *water R*. The solution is clear (2.2.1) and colourless (2.2.2, *Method II*).

Free chlorine. To 15 ml add 100 ml of *carbon dioxide-free water R*, 1 ml of a 100 g/l solution of *potassium iodide R* and 0.5 ml of *iodide-free starch solution R*. Allow to stand in the dark for 2 min. Any blue colour disappears on the addition of 0.2 ml of 0.01 M *sodium thiosulphate* (4 ppm).

Sulphates (2.4.13). To 6.4 ml add 10 mg of *sodium hydrogen carbonate R* and evaporate to dryness on a water-bath. Dissolve the residue in 15 ml of *distilled water R*. The solution complies with the limit test for sulphates (20 ppm).

Heavy metals (2.4.8). Dissolve the residue obtained in the test for residue on evaporation in 1 ml of *dilute hydrochloric acid R* and dilute to 25 ml with *water R*. Dilute 5 ml of this solution to 20 ml with *water R*. 12 ml of the solution complies with limit test A for heavy metals (2 ppm). Prepare the standard using *lead standard solution (2 ppm Pb R)*.

Residue on evaporation. Evaporate 100.0 g to dryness on a water-bath and dry at 100 °C to 105 °C. The residue weighs not more than 10 mg (0.01 per cent).

ASSAY

Weigh accurately a ground-glass-stoppered flask containing 30 ml of *water R*. Introduce 1.5 ml of the acid and weigh again. Titrate with 1 M *sodium hydroxide*, using *methyl red solution R* as indicator.

1 ml of 1 M *sodium hydroxide* is equivalent to 36.46 mg of HCl.

STORAGE

Store in a stoppered container made of glass or another inert material, at a temperature below 30 °C.

01/2005:0003 CHARACTERS

HYDROCHLORIC ACID, DILUTE**Acidum hydrochloridum dilutum****DEFINITION**

Dilute hydrochloric acid contains 9.5 per cent *m/m* to 10.5 per cent *m/m* of HCl (M_r 36.46).

PREPARATION

To 274 g of concentrated hydrochloric acid add 726 g of *water R* and mix.

IDENTIFICATION

- A. It is strongly acid (2.2.4).
 B. It gives the reactions of chlorides (2.3.1).
 C. It complies with the limits of the assay.

TESTS

Appearance. It is clear (2.2.1) and colourless (2.2.2, *Method II*).

Free chlorine. To 60 ml add 50 ml of *carbon dioxide-free water R*, 1 ml of a 100 g/l solution of *potassium iodide R* and 0.5 ml of *iodide-free starch solution R*. Allow to stand in the dark for 2 min. Any blue colour disappears on the addition of 0.2 ml of 0.01 *M sodium thiosulphate* (1 ppm).

Sulphates (2.4.13). To 26 ml add 10 mg of *sodium hydrogen carbonate R* and evaporate to dryness on a water-bath. Dissolve the residue in 15 ml of *distilled water R*. The solution complies with the limit test for sulphates (5 ppm).

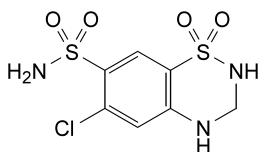
Heavy metals (2.4.8). Dissolve the residue obtained in the test for residue on evaporation in 1 ml of *dilute hydrochloric acid R* and dilute to 25 ml with *water R*. Dilute 5 ml of this solution to 20 ml with *water R*. 12 ml of the solution complies with limit test A for heavy metals (2 ppm). Prepare the standard using *lead standard solution* (2 ppm *Pb*) *R*.

Residue on evaporation. Evaporate 100.0 g to dryness on a water-bath and dry at 100 °C to 105 °C. The residue weighs not more than 10 mg (0.01 per cent).

ASSAY

To 6.00 g add 30 ml of *water R*. Titrate with 1 *M sodium hydroxide*, using *methyl red solution R* as indicator. 1 ml of 1 *M sodium hydroxide* is equivalent to 36.46 mg of HCl.

01/2005:0394

HYDROCHLOROTHIAZIDE**Hydrochlorothiazidum**C₇H₈ClN₃O₄S₂ M_r 297.7**DEFINITION**

Hydrochlorothiazide contains not less than 98.0 per cent and not more than the equivalent of 102.0 per cent of 6-chloro-3,4-dihydro-2H-1,2,4-benzothiadiazine-7-sulphonamide 1,1-dioxide, calculated with reference to the dried substance.

A white or almost white, crystalline powder, very slightly soluble in water, soluble in acetone, sparingly soluble in alcohol. It dissolves in dilute solutions of alkali hydroxides.

IDENTIFICATION

First identification: B.

Second identification: A, C, D.

A. Dissolve 50.0 mg in 10 ml of 0.1 *M sodium hydroxide* and dilute to 100.0 ml with *water R*. Dilute 2.0 ml of this solution to 100.0 ml with 0.01 *M sodium hydroxide*. Examined between 250 nm and 350 nm (2.2.25), the solution shows absorption maxima at 273 nm and at 323 nm. The ratio of the absorbance measured at the maximum at 273 nm to that measured at 323 nm is 5.4 to 5.7.

B. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *hydrochlorothiazide CRS*.

C. Examine by thin-layer chromatography (2.2.27), using as the coating substance a suitable silica gel with a fluorescent indicator having an optimal intensity at 254 nm.

Test solution. Dissolve 50 mg in *acetone R* and dilute to 10 ml with the same solvent.

Reference solution (a). Dissolve 50 mg of *hydrochlorothiazide CRS* in *acetone R* and dilute to 10 ml with the same solvent.

Reference solution (b). Dissolve 25 mg of *chlorothiazide R* in reference solution (a) and dilute to 5 ml with the same reference solution.

Apply separately to the plate 2 µl of each solution. Develop over a path of 10 cm using *ethyl acetate R*. Dry the plate in a current of air and examine in ultraviolet light at 254 nm. The principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a). The test is not valid unless the chromatogram obtained with reference solution (b) shows two clearly separated spots.

D. Gently heat about 1 mg with 2 ml of a freshly prepared 0.5 g/l solution of *chromotropic acid*, *sodium salt R* in a cooled mixture of 35 volumes of *water R* and 65 volumes of *sulphuric acid R*. A violet colour develops.

TESTS

Acidity or alkalinity. Shake 0.5 g of the powdered substance to be examined with 25 ml of *water R* for 2 min and filter. To 10 ml of the filtrate, add 0.2 ml of 0.01 *M sodium hydroxide* and 0.15 ml of *methyl red solution R*. The solution is yellow. Not more than 0.4 ml of 0.01 *M hydrochloric acid* is required to change the colour of the indicator to red.

Related substances. Examine by liquid chromatography (2.2.29).

Solvent solution. Dilute 50.0 ml of a mixture of equal volumes of *acetonitrile R* and *methanol R* to 200.0 ml with *phosphate buffer solution pH 3.2 R1*.

Test solution. Dissolve 30.0 mg of the substance to be examined in 5 ml of a mixture of equal volumes of *acetonitrile R* and *methanol R*, using sonication if necessary, and dilute to 20.0 ml with *phosphate buffer solution pH 3.2 R1*.

Reference solution (a). Dissolve 15.0 mg of *hydrochlorothiazide CRS* and 15.0 mg of *chlorothiazide CRS* in 25.0 ml of a mixture of equal volumes of *acetonitrile R*

and *methanol R*, using sonication if necessary, and dilute to 100.0 ml with *phosphate buffer solution pH 3.2 R1*. Dilute 5.0 ml to 100.0 ml with the solvent solution.

Reference solution (b). Dilute 1.0 ml of the test solution to 50.0 ml with the solvent solution. Dilute 5.0 ml of this solution to 20.0 ml with the solvent solution.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.1 m long and 4.6 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (3 µm),
- as mobile phase at a flow rate of 0.8 ml/min:

Mobile phase A. To 940 ml of *phosphate buffer solution pH 3.2 R1* add 60.0 ml of *methanol R* and 10.0 ml of *tetrahydrofuran R* and mix,

Mobile phase B. To a mixture of 500 ml of *methanol R* and 500 ml of *phosphate buffer solution pH 3.2 R1* add 50.0 ml of *tetrahydrofuran R* and mix,

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)	Comment
0 - 17	100 → 55	0 → 45	linear gradient
17 - 30	55	45	isocratic
30 - 35	55 → 100	45 → 0	linear gradient
35 - 50	100	0	isocratic
50 = 0	100	0	return to initial eluent composition

- as detector a spectrophotometer set at 224 nm.

Equilibrate the column for at least 20 min with mobile phase A. Adjust the sensitivity of the system so that the height of the principal peak in the chromatogram obtained with 10 µl of reference solution (b) is at least 50 per cent of the full scale of the recorder.

Inject 10 µl of reference solution (a). When the chromatogram is recorded in the prescribed conditions, the retention times are: chlorothiazide about 7 min and hydrochlorothiazide about 8 min. The test is not valid unless the resolution between the peaks corresponding to chlorothiazide and hydrochlorothiazide is at least 2.5. If necessary, adjust slightly the composition of the mobile phase or the time programme of the linear gradient.

Inject separately 10 µl of the solvent solution as a blank, 10 µl of the test solution and 10 µl of reference solution (b). In the chromatogram obtained with the test solution: the area of any peak, apart from the principal peak, is not greater than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent); the sum of the areas of all peaks, apart from the principal peak, is not greater than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (1 per cent). Disregard any peak due to the solvent solution and any peak with an area less than 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (b).

Chlorides (2.4.4). Dissolve 1.0 g in 25 ml of *acetone R* and dilute to 30 ml with *water R*. 15 ml complies with the limit test for chlorides (100 ppm). Prepare the standard using 5 ml of *acetone R* containing 15 per cent V/V of *water R* and 10 ml of *chloride standard solution (5 ppm Cl) R*.

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

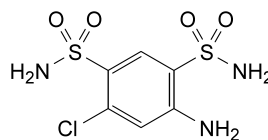
ASSAY

Dissolve 0.120 g in 50 ml of *dimethyl sulphoxide R*. Titrate with 0.1 M *tetrabutylammonium hydroxide in 2-propanol*, determining the end-point potentiometrically (2.2.20) at the second point of inflexion. Carry out a blank titration.

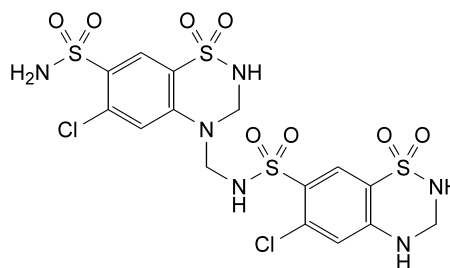
1 ml of 0.1 M *tetrabutylammonium hydroxide in 2-propanol* is equivalent to 14.88 mg of C₂₁H₃₀ClN₃O₅S₂.

IMPURITIES

A. chlorothiazide,



B. 4-amino-6-chlorobenzene-1,3-disulphonamide (salamide),

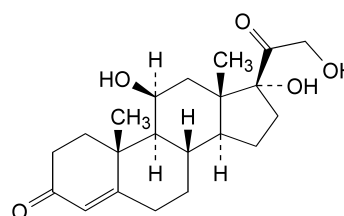


C. 6-chloro-N-[(6-chloro-7-sulphamoyl-2,3-dihydro-4H-1,2,4-benzothiadiazin-4-yl 1,1-dioxide)methyl]-3,4-dihydro-2H-1,2,4-benzothiadiazine-7-sulphonamide 1,1-dioxide.

01/2005:0335

HYDROCORTISONE

Hydrocortisonum



C₂₁H₃₀O₅

M_r 362.5

DEFINITION

Hydrocortisone contains not less than 97.0 per cent and not more than the equivalent of 103.0 per cent of 11β,17,21-trihydroxypregn-4-ene-3,20-dione, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, crystalline powder, practically insoluble in water, sparingly soluble in acetone and in alcohol, slightly soluble in methylene chloride.

It shows polymorphism.

IDENTIFICATION

First identification: A, B.

Second identification: C, D.

A. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *hydrocortisone CRS*. If the spectra obtained in the solid state show differences, dissolve the substance to be

examined and the reference substance separately in the minimum volume of *acetone R*, evaporate to dryness on a water-bath and record new spectra using the residues.

- B. Examine by thin-layer chromatography (2.2.27), using as the coating substance a suitable silica gel with a fluorescent indicator having an optimal intensity at 254 nm.

Test solution. Dissolve 10 mg of the substance to be examined in a mixture of 1 volume of *methanol R* and 9 volumes of *methylene chloride R* and dilute to 10 ml with the same mixture of solvents.

Reference solution (a). Dissolve 20 mg of *hydrocortisone CRS* in a mixture of 1 volume of *methanol R* and 9 volumes of *methylene chloride R* and dilute to 20 ml with the same mixture of solvents.

Reference solution (b). Dissolve 10 mg of *prednisolone CRS* in reference solution (a) and dilute to 10 ml with reference solution (a).

Apply to the plate 5 µl of each solution. Prepare the mobile phase by adding a mixture of 1.2 volumes of *water R* and 8 volumes of *methanol R* to a mixture of 15 volumes of *ether R* and 77 volumes of *methylene chloride R*. Develop over a path of 15 cm. Carry out a second development over a path of 15 cm using a mixture of 5 volumes of *butanol R* saturated with *water R*, 15 volumes of *toluene R* and 80 volumes of *ether R*. Allow the plate to dry in air. Examine in ultraviolet light at 254 nm. The principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a). Spray with *alcoholic solution of sulphuric acid R*. Heat at 120 °C for 10 min or until the spots appear. Allow to cool. Examine the chromatograms in daylight and in ultraviolet light at 365 nm. The principal spot in the chromatogram obtained with the test solution is similar in position, colour in daylight, fluorescence in ultraviolet light at 365 nm and size to the principal spot in the chromatogram obtained with reference solution (a). The test is not valid unless the chromatogram obtained with reference solution (b) shows two clearly separated spots.

- C. Examine by thin-layer chromatography (2.2.27), using as the coating substance a suitable silica gel with a fluorescent indicator having an optimal intensity at 254 nm.

Test solution (a). Dissolve 25 mg of the substance to be examined in *methanol R* and dilute to 5 ml with the same solvent. This solution is also used to prepare test solution (b). Dilute 2 ml of the solution to 10 ml with *methylene chloride R*.

Test solution (b). Transfer 0.4 ml of the solution obtained during preparation of test solution (a) to a glass tube 100 mm long and 20 mm in diameter and fitted with a ground-glass stopper or a polytetrafluoroethylene cap and evaporate the solvent with gentle heating under a stream of *nitrogen R*. Add 2 ml of a 15 per cent V/V solution of *glacial acetic acid R* and 50 mg of *sodium bismuthate R*. Stopper the tube and shake the suspension for 1 h in a mechanical shaker, protected from light. Add 2 ml of a 15 per cent V/V solution of *glacial acetic acid R* and filter into a 50 ml separating funnel, washing the filter with two quantities, each of 5 ml, of *water R*. Shake the clear filtrate with 10 ml of *methylene chloride R*. Wash the organic layer with 5 ml of 1 M *sodium hydroxide* and two quantities, each of 5 ml, of *water R*. Dry over *anhydrous sodium sulphate R*.

Reference solution (a). Dissolve 25 mg of *hydrocortisone CRS* in *methanol R* and dilute to 5 ml with the same solvent. This solution is also used to prepare reference solution (b). Dilute 2 ml of the solution to 10 ml with *methylene chloride R*.

Reference solution (b). Transfer 0.4 ml of the solution obtained during preparation of reference solution (a) to a glass tube 100 mm long and 20 mm in diameter and fitted with a ground-glass stopper or a polytetrafluoroethylene cap and evaporate the solvent with gentle heating under a stream of *nitrogen R*. Add 2 ml of a 15 per cent V/V solution of *glacial acetic acid R* and 50 mg of *sodium bismuthate R*. Stopper the tube and shake the suspension for 1 h in a mechanical shaker protected from light. Add 2 ml of a 15 per cent V/V solution of *glacial acetic acid R* and filter into a 50 ml separating funnel, washing the filter with two quantities, each of 5 ml, of *water R*. Shake the clear filtrate with 10 ml of *methylene chloride R*. Wash the organic layer with 5 ml of 1 M *sodium hydroxide* and two quantities, each of 5 ml, of *water R*. Dry over *anhydrous sodium sulphate R*.

Apply to the plate 5 µl of test solution (a), 5 µl of reference solution (a), 25 µl of test solution (b) and 25 µl of reference solution (b), applying the latter two in small quantities to obtain small spots. Prepare the mobile phase by adding a mixture of 1.2 volumes of *water R* and 8 volumes of *methanol R* to a mixture of 15 volumes of *ether R* and 77 volumes of *methylene chloride R*. Develop over a path of 15 cm. Carry out a second development over a path of 15 cm using a mixture of 5 volumes of *butanol R* saturated with *water R*, 15 volumes of *toluene R* and 80 volumes of *ether R*. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. The principal spot in each of the chromatograms obtained with the test solutions is similar in position and size to the principal spot in the chromatogram obtained with the corresponding reference solution. Spray with *alcoholic solution of sulphuric acid R* and heat at 120 °C for 10 min or until the spots appear. Allow to cool. Examine the plate in daylight and in ultraviolet light at 365 nm. The principal spot in each of the chromatograms obtained with the test solutions is similar in position, colour in daylight, fluorescence in ultraviolet light at 365 nm and size to the principal spot in the chromatogram obtained with the corresponding reference solution. The principal spots in the chromatograms obtained with test solution (b) and reference solution (b) have an R_f value distinctly higher than that of the principal spots in the chromatograms obtained with test solution (a) and reference solution (a).

- D. Add about 2 mg to 2 ml of *sulphuric acid R* and shake to dissolve. Within 5 min, an intense brownish-red colour develops with a green fluorescence which is particularly intense when examined in ultraviolet light at 365 nm. Add the solution to 10 ml of *water R* and mix. The colour fades and a clear solution remains. The fluorescence in ultraviolet light does not disappear.

TESTS

Specific optical rotation (2.2.7). Dissolve 0.250 g in *dioxan R* and dilute to 25.0 ml with the same solvent. The specific optical rotation is + 150 to + 156, calculated with reference to the dried substance.

Related substances. Examine by liquid chromatography (2.2.29). Prepare the solutions immediately before use.

Test solution. Dissolve 25.0 mg of the substance to be examined in 2 ml of *tetrahydrofuran R* and dilute to 10.0 ml with *water R*.

Reference solution (a). Dissolve 2 mg of *hydrocortisone CRS* and 2 mg of *prednisolone CRS* in the mobile phase and dilute to 100.0 ml with the mobile phase.

Reference solution (b). Dilute 1.0 ml of the test solution to 100.0 ml with the mobile phase.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4.6 mm in internal diameter packed with *base-deactivated end-capped octadecylsilyl silica gel for chromatography R* (5 µm),
- as mobile phase at a flow rate of 1 ml/min a mixture prepared as follows: in a 1000 ml volumetric flask mix 220 ml of *tetrahydrofuran R* with 700 ml of *water R* and allow to equilibrate; dilute to 1000 ml with *water R* and mix again,
- as detector a spectrophotometer set at 254 nm, maintaining the temperature of the column at 45 °C.

Equilibrate the column with the mobile phase at a flow rate of 1 ml/min for about 30 min.

Adjust the sensitivity of the system so that the height of the principal peak in the chromatogram obtained with 20 µl of reference solution (b) is at least 50 per cent of the full scale of the recorder.

Inject 20 µl of reference solution (a). When the chromatograms are recorded in the prescribed conditions, the retention times are: prednisolone about 14 min and hydrocortisone about 15.5 min. The test is not valid unless the resolution between the peaks due to prednisolone and hydrocortisone is at least 2.2. If necessary, adjust the concentration of *tetrahydrofuran R* in the mobile phase.

Inject separately 20 µl of the solvent mixture of the test solution as a blank, 20 µl of the test solution and 20 µl of reference solution (b). Continue the chromatography of the test solution for four times the retention time of the principal peak. In the chromatogram obtained with the test solution: the area of any peak, apart from the principal peak, is not greater than half the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent); the sum of the areas of all the peaks, apart from the principal peak, is not greater than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (1.5 per cent). Disregard any peak obtained with the blank run and any peak with an area less than 0.05 times the area of the principal peak in the chromatogram obtained with reference solution (b).

Loss on drying (2.2.32). Not more than 1.0 per cent, determined on 0.500 g by drying in an oven at 100 °C to 105 °C.

ASSAY

Dissolve 0.100 g in *alcohol R* and dilute to 100.0 ml with the same solvent. Dilute 2.0 ml of the solution to 100.0 ml with *alcohol R*. Measure the absorbance (2.2.25) at the maximum at 241.5 nm.

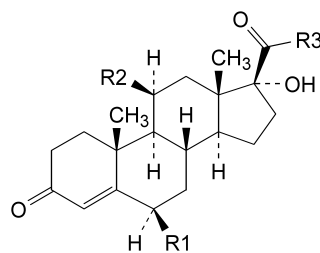
Calculate the content of $C_{21}H_{30}O_5$ taking the specific absorbance to be 440.

STORAGE

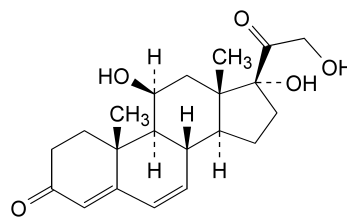
Store protected from light.

IMPURITIES

- A. prednisolone,
- B. cortisone,
- C. hydrocortisone acetate,



- D. $R_1 = R_2 = OH$, $R_3 = CH_2OH$: 6β,11β,17,21-tetrahydroxypregn-4-ene-3,20-dione (6β-hydroxyhydrocortisone),
- F. $R_1 = R_2 = H$, $R_3 = CH_2OH$: 17,21-dihydroxypregn-4-ene-3,20-dione (Reichstein's substance),
- G. $R_1 = H$, $R_2 = OH$, $R_3 = CHO$: 11β,17-dihydroxy-3,20-dioxopregn-4-en-21-al,

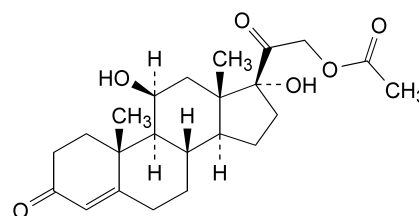


- E. 11β,17,21-trihydroxypregna-4,6-diene-3,20-dione (Δ6-hydrocortisone).

01/2005:0334

HYDROCORTISONE ACETATE

Hydrocortisoni acetas



$C_{23}H_{32}O_6$

M_r 404.5

DEFINITION

Hydrocortisone acetate contains not less than 97.0 per cent and not more than the equivalent of 103.0 per cent of 11β,17-dihydroxy-3,20-dioxopregn-4-en-21-yl acetate, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, crystalline powder, practically insoluble in water, slightly soluble in ethanol and in methylene chloride.

It melts at about 220 °C, with decomposition.

IDENTIFICATION

First identification: A, B.

Second identification: C, D, E.

- A. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *hydrocortisone acetate CRS*.
- B. Examine by thin-layer chromatography (2.2.27), using as the coating substance a suitable silica gel with a fluorescent indicator having an optimal intensity at 254 nm.

Test solution. Dissolve 10 mg of the substance to be examined in a mixture of 1 volume of *methanol R* and 9 volumes of *methylene chloride R* and dilute to 10 ml with the same mixture of solvents.

Reference solution (a). Dissolve 20 mg of *hydrocortisone acetate CRS* in a mixture of 1 volume of *methanol R* and 9 volumes of *methylene chloride R* and dilute to 20 ml with the same mixture of solvents.

Reference solution (b). Dissolve 10 mg of *cortisone acetate R* in reference solution (a) and dilute to 10 ml with reference solution (a).

Apply to the plate 5 µl of each solution. Prepare the mobile phase by adding a mixture of 1.2 volumes of *water R* and 8 volumes of *methanol R* to a mixture of 15 volumes of *ether R* and 77 volumes of *methylene chloride R*. Develop over a path of 15 cm. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. The principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a). Spray with *alcoholic solution of sulphuric acid R*. Heat at 120 °C for 10 min or until the spots appear. Allow to cool. Examine the plate in daylight and in ultraviolet light at 365 nm. The principal spot in the chromatogram obtained with the test solution is similar in position, colour in daylight, fluorescence in ultraviolet light at 365 nm and size to the principal spot in the chromatogram obtained with reference solution (a). The test is not valid unless the chromatogram obtained with reference solution (b) shows two clearly separated spots.

- C. Examine by thin-layer chromatography (2.2.27), using as the coating substance a suitable silica gel with a fluorescent indicator having an optimal intensity at 254 nm.

Test solution (a). Dissolve 25 mg of the substance to be examined in *methanol R* and dilute to 5 ml with the same solvent. This solution is also used to prepare test solution (b). Dilute 2 ml of the solution to 10 ml with *methylene chloride R*.

Test solution (b). Transfer 2 ml of the solution obtained during preparation of test solution (a) to a 15 ml glass tube with a ground-glass stopper or a polytetrafluoroethylene cap. Add 10 ml of *saturated methanolic potassium hydrogen carbonate solution R* and immediately pass a stream of *nitrogen R* briskly through the solution for 5 min. Stopper the tube. Heat in a water-bath at 45 °C protected from light for 2 h 30 min. Allow to cool.

Reference solution (a). Dissolve 25 mg of *hydrocortisone acetate CRS* in *methanol R* and dilute to 5 ml with the same solvent. This solution is also used to prepare reference solution (b). Dilute 2 ml of the solution to 10 ml with *methylene chloride R*.

Reference solution (b). Transfer 2 ml of the solution obtained during preparation of reference solution (a) to a 15 ml glass tube with a ground-glass stopper or a polytetrafluoroethylene cap. Add 10 ml of *saturated methanolic potassium hydrogen carbonate solution R* and immediately pass a stream of *nitrogen R* briskly through the solution for 5 min. Stopper the tube. Heat in a water-bath at 45 °C protected from light for 2 h 30 min. Allow to cool.

Apply to the plate 5 µl of each solution. Prepare the mobile phase by adding a mixture of 1.2 volumes of *water R* and 8 volumes of *methanol R* to a mixture of 15 volumes of *ether R* and 77 volumes of *methylene chloride R*. Develop over a path of 15 cm. Allow the plate

to dry in air and examine in ultraviolet light at 254 nm. The principal spot in each of the chromatograms obtained with the test solutions is similar in position and size to the principal spot in the chromatogram obtained with the corresponding reference solution. Spray with *alcoholic solution of sulphuric acid R* and heat at 120 °C for 10 min or until the spots appear. Allow to cool. Examine in daylight and in ultraviolet light at 365 nm. The principal spot in each of the chromatograms obtained with the test solutions is similar in position, colour in daylight, fluorescence in ultraviolet light at 365 nm and size to the principal spot in the chromatogram obtained with the corresponding reference solution. The principal spots in the chromatograms obtained with test solution (b) and reference solution (b) have an R_f value distinctly lower than that of the principal spots in the chromatograms obtained with test solution (a) and reference solution (a).

- D. Add about 2 mg to 2 ml of *sulphuric acid R* and shake to dissolve. Within 5 min an intense brownish-red colour develops with a green fluorescence which is particularly intense when viewed in ultraviolet light at 365 nm. Add the solution to 10 ml of *water R* and mix. The colour fades and the fluorescence in ultraviolet light does not disappear.

- E. About 10 mg gives the reaction of acetyl (2.3.1).

TESTS

Specific optical rotation (2.2.7). Dissolve 0.250 g in *dioxan R* and dilute to 25.0 ml with the same solvent. The specific optical rotation is + 158 to + 167, calculated with reference to the dried substance.

Related substances. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 25.0 mg of the substance to be examined in *methanol R* and dilute to 10.0 ml with the same solvent.

Reference solution (a). Dissolve 2 mg of *hydrocortisone acetate CRS* and 2 mg of *cortisone acetate R* in the mobile phase and dilute to 100.0 ml with the mobile phase.

Reference solution (b). Dilute 1.0 ml of the test solution to 100.0 ml with the mobile phase.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4.6 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (5 µm),
- as mobile phase at a flow rate of 1 ml/min a mixture prepared as follows: in a 1000 ml volumetric flask mix 400 ml of *acetonitrile R* with 550 ml of *water R* and allow to equilibrate; adjust the volume to 1000 ml with *water R* and mix again,
- as detector a spectrophotometer set at 254 nm.

Equilibrate the column with the mobile phase at a flow rate of 1 ml/min for about 30 min.

Adjust the sensitivity of the system so that the height of the principal peak in the chromatogram obtained with 20 µl of reference solution (b) is not less than 50 per cent of the full scale of the recorder.

Inject 20 µl of reference solution (a). When the chromatograms are recorded in the prescribed conditions the retention times are: hydrocortisone acetate, about 10 min and cortisone acetate, about 12 min. The test is not valid unless the resolution between the peaks due to hydrocortisone acetate and cortisone acetate is at least 4.2; if necessary, adjust the concentration of acetonitrile in the mobile phase.

Inject separately 20 µl of the test solution and 20 µl of reference solution (b). Continue the chromatography for 2.5 times the retention time of the principal peak. In the chromatogram obtained with the test solution: the area of any peak, apart from the principal peak, is not greater than the area of the principal peak in the chromatogram obtained with reference solution (b) (1.0 per cent) and not more than one such peak has an area greater than half the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent); the sum of the areas of all the peaks, apart from the principal peak, is not greater than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (1.5 per cent). Disregard any peak due to the solvent and any peak with an area less than 0.05 times the area of the principal peak in the chromatogram obtained with reference solution (b).

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 0.500 g by drying in an oven at 100 °C to 105 °C.

ASSAY

Dissolve 0.100 g in *alcohol R* and dilute to 100.0 ml with the same solvent. Dilute 2.0 ml of the solution to 100.0 ml with *alcohol R*. Measure the absorbance (2.2.25) at the maximum of 241.5 nm.

Calculate the content of $C_{23}H_{32}O_6$ taking the specific absorbance to be 395.

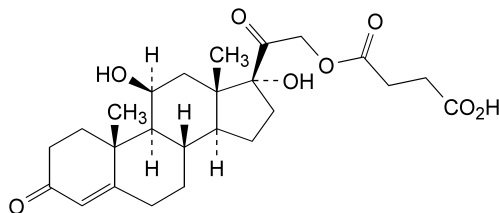
STORAGE

Store protected from light.

01/2005:0768

HYDROCORTISONE HYDROGEN SUCCINATE

Hydrocortisoni hydrogenosuccinas



$C_{25}H_{34}O_8$

M_r 462.5

DEFINITION

Hydrocortisone hydrogen succinate contains not less than 97.0 per cent and not more than the equivalent of 103.0 per cent of 11β,17-dihydroxy-3,20-dioxopregn-4-en-21-yl hydrogen butanedioate, calculated with reference to the dried substance.

CHARACTERS

A white or almost white powder, hygroscopic, practically insoluble in water, freely soluble in acetone and in ethanol. It dissolves in dilute solutions of alkali carbonates and alkali hydroxides.

IDENTIFICATION

First identification: A, B.

Second identification: C, D.

A. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *hydrocortisone hydrogen succinate CRS*. Dry the substances before use at 100 °C to 105 °C for 3 h.

B. Examine by thin-layer chromatography (2.2.27), using as the coating substance a suitable silica gel with a fluorescent indicator having an optimal intensity at 254 nm.

Test solution. Dissolve 10 mg of the substance to be examined in a mixture of 1 volume of *methanol R* and 9 volumes of *methylene chloride R* and dilute to 10 ml with the same mixture of solvents.

Reference solution (a). Dissolve 20 mg of *hydrocortisone hydrogen succinate CRS* in a mixture of 1 volume of *methanol R* and 9 volumes of *methylene chloride R* and dilute to 20 ml with the same mixture of solvents.

Reference solution (b). Dissolve 10 mg of *methylprednisolone hydrogen succinate CRS* in reference solution (a) and dilute to 10 ml with reference solution (a).

Apply separately to the plate 5 µl of each solution.

Develop over a path of 15 cm using a mixture of 0.1 volumes of *anhydrous formic acid R*, 1 volume of *ethanol R* and 15 volumes of *methylene chloride R*. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. The principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a). Spray the plate with *alcoholic solution of sulphuric acid R*. Heat at 120 °C for 10 min or until the spots appear. Allow to cool. Examine in daylight and in ultraviolet light at 365 nm. The principal spot in the chromatogram obtained with the test solution is similar in position, colour in daylight, fluorescence in ultraviolet light at 365 nm and size to the principal spot in the chromatogram obtained with reference solution (a). The test is not valid unless the chromatogram obtained with reference solution (b) shows two spots which may however not be completely separated.

C. Examine by thin-layer chromatography (2.2.27), using as the coating substance a suitable silica gel with a fluorescent indicator having an optimal intensity at 254 nm.

Test solution (a). Dissolve 25 mg of the substance to be examined in *methanol R* with gentle heating and dilute to 5 ml with the same solvent. (This solution is also used to prepare test solution (b)). Dilute 2 ml of the solution to 10 ml with *methylene chloride R*.

Test solution (b). Transfer 2 ml of the solution obtained during preparation of test solution (a) to a 15 ml glass tube with a ground-glass stopper or a polytetrafluoroethylene cap. Add 10 ml of a 0.8 g/l solution of *sodium hydroxide R* in *methanol R* and immediately pass a stream of *nitrogen R* briskly through the solution for 5 min. Stopper the tube. Heat in a water-bath at 45 °C, protected from light, for 30 min. Allow to cool.

Reference solution (a). Dissolve 25 mg of *hydrocortisone hydrogen succinate CRS* in *methanol R* with gentle heating and dilute to 5 ml with the same solvent. (This solution is also used to prepare reference solution (b)). Dilute 2 ml of the solution to 10 ml with *methylene chloride R*.

Reference solution (b). Transfer 2 ml of the solution obtained during preparation of reference solution (a) to a 15 ml glass tube with a ground-glass stopper or a polytetrafluoroethylene cap. Add 10 ml of a 0.8 g/l solution of *sodium hydroxide R* in *methanol R* and immediately pass a stream of *nitrogen R* briskly through the solution for 5 min. Stopper the tube. Heat in a water-bath at 45 °C, protected from light, for 30 min. Allow to cool.

Apply separately to the plate 5 µl of each solution. Prepare the mobile phase by adding a mixture of 1.2 volumes of *water R* and 8 volumes of *methanol R* to a mixture of 15 volumes of *ether R* and 77 volumes of *methylene chloride R*. Develop over a path of 15 cm. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. The principal spot in each of the chromatograms obtained with the test solutions is similar in position and size to the principal spot in the chromatogram obtained with the corresponding reference solution. Spray the plate with *alcoholic solution of sulphuric acid R*. Heat at 120 °C for 10 min or until the spots appear. Allow to cool. Examine in daylight and in ultraviolet light at 365 nm. The principal spot in the chromatograms obtained with the test solutions is similar in position, colour in daylight, fluorescence in ultraviolet light at 365 nm and size to the principal spot in the chromatogram obtained with the corresponding reference solution. The principal spot in each of the chromatograms obtained with test solution (b) and reference solution (b) has an R_f value distinctly higher than that of the principal spots in each of the chromatograms obtained with test solution (a) and reference solution (a).

- D. Add about 2 mg to 2 ml of *sulphuric acid R* and shake to dissolve. Within 5 min, an intense brownish-red colour develops with a green fluorescence which is particularly intense when viewed in ultraviolet light at 365 nm. Add the solution to 10 ml of *water R* and mix. The colour fades and a clear solution remains. The fluorescence in ultraviolet light does not disappear.

TESTS

Appearance of solution. Dissolve 0.10 g in 5 ml of *sodium hydrogen carbonate solution R*. The solution is clear (2.2.1).

Specific optical rotation (2.2.7). Dissolve 0.250 g in *ethanol R* and dilute to 25.0 ml with the same solvent. The specific optical rotation is + 147 to + 153, calculated with reference to the dried substance.

Related substances. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 25.0 mg of the substance to be examined in a mixture of equal volumes of *acetonitrile R* and *water R* and dilute to 10.0 ml with the same solvent.

Reference solution (a). Dissolve 2 mg of *hydrocortisone hydrogen succinate CRS* and 2 mg of *dexamethasone CRS* in 50 ml of *acetonitrile R* and dilute to 100.0 ml with *water R*.

Reference solution (b). Dilute 1.0 ml of the test solution to 100.0 ml with a mixture of equal volumes of *acetonitrile R* and *water R*.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4.6 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (5 µm),
- as mobile phase at a flow rate of 1 ml/min a mixture prepared as follows: in a 1000 ml volumetric flask mix 330 ml of *acetonitrile R* with 600 ml of *water R* and 1.0 ml of *phosphoric acid R* and allow to equilibrate; dilute to 1000 ml with *water R* and mix again,
- as detector a spectrophotometer set at 254 nm.

Equilibrate the column with the mobile phase at a flow rate of 1 ml/min for about 30 min.

Adjust the sensitivity of the system so that the height of the principal peak in the chromatogram obtained with 20 µl of reference solution (b) is not less than 50 per cent of the full scale of the recorder.

Inject 20 µl of reference solution (a). When the chromatograms are recorded in the conditions described above the retention times are: dexamethasone, about 12.5 min and hydrocortisone hydrogen succinate, about 15 min. The test is not valid unless the resolution between the peaks corresponding to dexamethasone and hydrocortisone hydrogen succinate is at least 5.0; if necessary, adjust the concentration of acetonitrile in the mobile phase.

Inject separately 20 µl of the test solution and 20 µl of reference solution (b). Continue the chromatography for twice the retention time of the principal peak. In the chromatogram obtained with the test solution: the area of any peak, apart from the principal peak, is not greater than half the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent); the sum of the areas of all the peaks, apart from the principal peak, is not greater than 0.75 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.75 per cent). Disregard any peak due to the solvent and any peak with an area less than 0.05 times the area of the principal peak in the chromatogram obtained with reference solution (b).

Loss on drying (2.2.32). Not more than 4.0 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.100 g in *alcohol R* and dilute to 100.0 ml with the same solvent. Dilute 2.0 ml to 100.0 ml with *alcohol R*. Measure the absorbance (2.2.25) at the maximum at 241.5 nm.

Calculate the content of $C_{25}H_{34}O_8$ taking the specific absorbance to be 353.

STORAGE

Store in an airtight container, protected from light.

IMPURITIES

- A. hydrocortisone,
B. hydrocortisone acetate.

01/2005:0395

HYDROGEN PEROXIDE SOLUTION (3 PER CENT)

Hydrogenii peroxidum 3 per centum

DEFINITION

Hydrogen peroxide solution (3 per cent) contains not less than 2.5 per cent *m/m* and not more than 3.5 per cent *m/m* of H_2O_2 (M_r 34.01). One volume of this solution corresponds to about ten times its volume of oxygen. A suitable stabiliser may be added.

CHARACTERS

A colourless, clear liquid.

IDENTIFICATION

- A. To 2 ml add 0.2 ml of *dilute sulphuric acid R* and 0.2 ml of 0.02 *M* *potassium permanganate*. The solution becomes colourless or slightly pink within 2 min.
B. To 0.5 ml add 1 ml of *dilute sulphuric acid R*, 2 ml of *ether R* and 0.1 ml of *potassium chromate solution R* and shake. The ether layer is blue.

C. It complies with the requirement for the content of H_2O_2 .

TESTS

Acidity. To 10 ml add 20 ml of *water R* and 0.25 ml of *methyl red solution R*. Not less than 0.05 ml and not more than 1.0 ml of 0.1 M sodium hydroxide is required to change the colour of the indicator.

Organic stabilisers. Shake 20 ml with 10 ml of *chloroform R* and then with two quantities, each of 5 ml, of *chloroform R*. Evaporate the combined chloroform layers under reduced pressure at a temperature not exceeding 25 °C and dry the residue in a desiccator. The residue weighs not more than 5 mg (250 ppm).

Non-volatile residue. Allow 10 ml to stand in a platinum dish until all effervescence has ceased. Evaporate the solution to dryness on a water-bath and dry the residue at 100 °C to 105 °C. The residue weighs not more than 20 mg (2 g/l).

ASSAY

Dilute 10.0 g to 100.0 ml with *water R*. To 10.0 ml of this solution add 20 ml of *dilute sulphuric acid R*. Titrate with 0.02 M potassium permanganate until a pink colour is obtained.

1 ml of 0.02 M potassium permanganate is equivalent to 1.701 mg of H_2O_2 or 0.56 ml of oxygen.

STORAGE

Store protected from light; if the solution does not contain a stabiliser, store at a temperature below 15 °C.

LABELLING

If the solution contains a stabiliser, the label states that the contents are stabilised. The competent authority may require that the name of the stabiliser be stated on the label.

CAUTION

It decomposes in contact with oxidisable organic matter and with certain metals and if allowed to become alkaline.

TESTS

Acidity. To 10 ml add 100 ml of *water R* and 0.25 ml of *methyl red solution R*. Not less than 0.05 ml and not more than 0.5 ml of 0.1 M sodium hydroxide is required to change the colour of the indicator.

Organic stabilisers. Shake 20 ml with 10 ml of *chloroform R* and then with two quantities, each 5 ml, of *chloroform R*. Evaporate the combined chloroform layers under reduced pressure at a temperature not exceeding 25 °C and dry the residue in a desiccator. The residue weighs not more than 10 mg (500 ppm).

Non-volatile residue. Allow 10 ml to stand in a platinum dish until all effervescence has ceased, cooling if necessary. Evaporate the solution to dryness on a water-bath and dry the residue at 100 °C to 105 °C. The residue weighs not more than 20 mg (2 g/l).

ASSAY

Dilute 1.00 g to 100.0 ml with *water R*. To 10.0 ml of this solution add 20 ml of *dilute sulphuric acid R*. Titrate with 0.02 M potassium permanganate until a pink colour is obtained.

1 ml of 0.02 M potassium permanganate is equivalent to 1.701 mg of H_2O_2 or 0.56 ml of oxygen.

STORAGE

Store protected from light; if the solution does not contain a stabiliser, store at a temperature below 15 °C.

LABELLING

If the solution contains a stabiliser, the label states that the contents are stabilised. The competent authority may require that the name of the stabiliser be stated on the label.

CAUTION

It decomposes vigorously in contact with oxidisable organic matter and with certain metals and if allowed to become alkaline.

01/2005:2099

01/2005:0396

HYDROGEN PEROXIDE SOLUTION (30 PER CENT)

Hydrogenii peroxidum 30 per centum

DEFINITION

Hydrogen peroxide solution (30 per cent) contains not less than 29.0 per cent *m/m* and not more than 31.0 per cent *m/m* of H_2O_2 (M_r 34.01). One volume of this solution corresponds to about 110 times its volume of oxygen. A suitable stabiliser may be added.

CHARACTERS

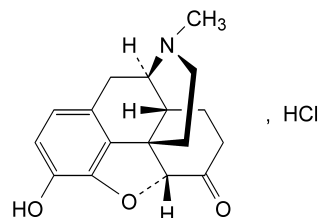
A colourless, clear liquid.

IDENTIFICATION

- To 1 ml add 0.2 ml of *dilute sulphuric acid R* and 0.25 ml of 0.02 M potassium permanganate. The solution becomes colourless with evolution of gas.
- To 0.05 ml add 2 ml of *dilute sulphuric acid R*, 2 ml of *ether R* and 0.05 ml of *potassium chromate solution R* and shake. The ether layer is blue.
- It complies with the requirement for the content of H_2O_2 .

HYDROMORPHONE HYDROCHLORIDE

Hydromorphoni hydrochloridum

 $\text{C}_{17}\text{H}_{20}\text{ClNO}_3$ M_r 321.8

DEFINITION

4,5 α -Epoxy-3-hydroxy-17-methylmorphinan-6-one hydrochloride.

Content: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

Appearance: white or almost white, crystalline powder.

Solubility: freely soluble in water, very slightly soluble in ethanol (96 per cent), practically insoluble in methylene chloride.

IDENTIFICATION

A. Infrared absorption spectrophotometry (2.2.24).

Comparison: hydromorphone hydrochloride CRS.

B. It gives reaction (a) of chlorides (2.3.1).

TESTS

Solution S. Dissolve 1.250 g in *carbon dioxide-free water R* and dilute to 25.0 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and not more intensely coloured than reference solution BY₅ (2.2.2, *Method II*).

Acidity or alkalinity. To 2 ml of solution S add 0.1 ml of *methyl red solution R*. The solution is not yellow. To 2 ml of solution S add 0.05 ml of *bromocresol green solution R*. The solution is not yellow.

Specific optical rotation (2.2.7): -136 to -140 (dried substance), determined on solution S.

Related substances. Liquid chromatography (2.2.29).

Test solution. Dissolve 0.100 g of the substance to be examined in *water R*, sonicating if necessary and dilute to 100.0 ml with the same solvent.

Reference solution (a). Dilute 1.0 ml of the test solution to 100.0 ml with *water R*. Dilute 1.0 ml of this solution to 10.0 ml with *water R*.

Reference solution (b). To 5 ml of the test solution add 5 mg of *naloxone hydrochloride dihydrate CRS* and dilute to 50 ml with *water R*.

Column:

- *size:* $l = 0.25$ m, $\varnothing = 4.6$ mm,
- *stationary phase:* base-deactivated end-capped octadecylsilyl silica gel for chromatography *R* (5 μ m).

Mobile phase: dissolve 18.29 g of *diethylamine R* and 2.88 g of *sodium laurilsulfate R* in *water R* and dilute to 1000 ml with the same solvent. Adjust 800 ml of this solution to pH 3.0 with *phosphoric acid R*. Add 100 ml of *acetonitrile R* and 100 ml of *methanol R*.

Flow rate: 1 ml/min.

Detection: spectrophotometer at 284 nm.

Injection: 20 μ l.

Run time: 4 times the retention time of hydromorphone.

Relative retention with reference to hydromorphone (retention time = about 9 min): impurity D = about 0.72; impurity B = about 0.77; impurity C = about 0.82; impurity A = about 3.2.

System suitability: reference solution (b):

- *resolution:* minimum 4.0 between the peaks due to hydromorphone and naloxone.

Limits:

- *impurity A:* not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.3 per cent),
- *impurities B, C, D:* for each impurity, not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent),
- *any other impurity:* for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent),

- *total:* not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent),
- *disregard limit:* 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

Loss on drying (2.2.32): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 100–105 °C.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on the residue obtained in the test for loss on drying.

ASSAY

Dissolve 0.250 g in 50 ml of *ethanol (96 per cent) R* and add 5.0 ml of 0.01 *M hydrochloric acid*. Carry out a potentiometric titration (2.2.20), using 0.1 *M sodium hydroxide*. Read the volume added between the 2 points of inflexion.

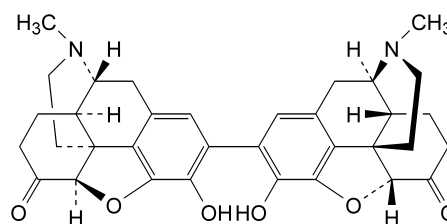
1 ml of 0.1 *M sodium hydroxide* is equivalent to 32.18 mg of $C_{17}H_{20}ClNO_3$.

STORAGE

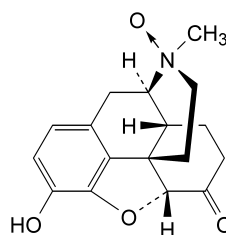
Protected from light.

IMPURITIES

Specified impurities: A, B, C, D.

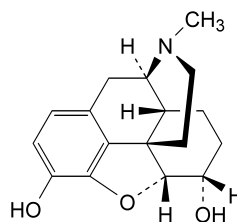


A. 4,5 α :4',5' α -diepoxy-3,3'-dihydroxy-17,17'-dimethyl-2,2'-bimorphinan-6,6'-dione (pseudohydromorphone),



B. 4,5 α -epoxy-3-hydroxy-17-methylmorphinan-6-one 17-oxide (hydromorphone *N*-oxide),

C. morphine,

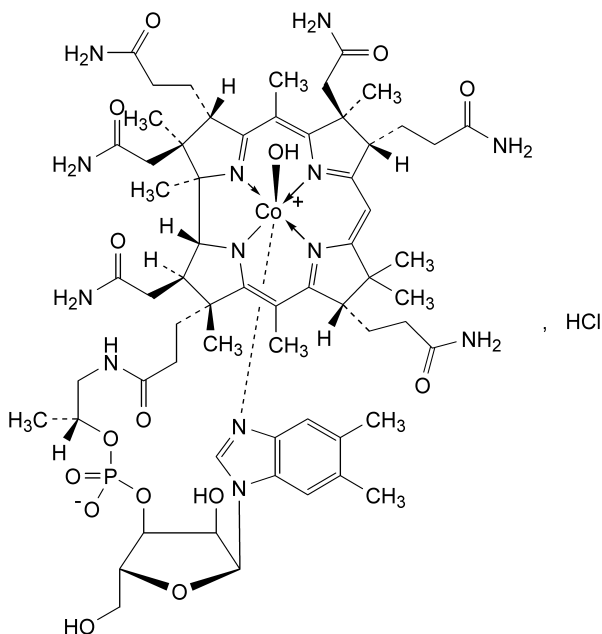


D. 4,5 α -epoxy-17-methylmorphinan-3,6 α -diol (dihydromorphine).

01/2005:0914

HYDROXOCOBALAMIN CHLORIDE

Hydroxocobalamini chloridum

 $C_{62}H_{90}ClCoN_{13}O_{15}P$ M_r 1383**DEFINITION**

Hydroxocobalamin chloride contains not less than 96.0 per cent and not more than the equivalent of 102.0 per cent of $Co\alpha$ -[α -(5,6-dimethylbenzimidazolyl)]- $Co\beta$ -hydroxocobamide chloride, calculated with reference to the dried substance.

CHARACTERS

A dark red, crystalline powder or dark red crystals, soluble in water, very hygroscopic. Some decomposition may occur on drying.

IDENTIFICATION

A. Dissolve 2.5 mg in a solution containing 0.8 per cent *V/V* of glacial acetic acid *R* and 10.9 g/l of sodium acetate *R* and dilute to 100 ml with the same solution. Examined between 260 nm and 610 nm (2.2.25), the solution shows three absorption maxima, at 274 nm, 351 nm and 525 nm. The ratio of the absorbance at the maximum at 274 nm to that at the maximum at 351 nm is 0.75 to 0.83. The ratio of the absorbance at the maximum at 525 nm to that at the maximum at 351 nm is 0.31 to 0.35.

B. Examine by thin-layer chromatography (2.2.27), using silica gel *G R* as the coating substance. Carry out the test protected from light.

Test solution. Dissolve 2 mg of the substance to be examined in 1 ml of a mixture of equal volumes of alcohol *R* and water *R*.

Reference solution. Dissolve 2 mg of hydroxocobalamin CRS in 1 ml of a mixture of equal volumes of alcohol *R* and water *R*.

Apply to the plate 10 μ l of each solution. Develop in an unlined tank over a path of 12 cm using a mixture of 25 volumes of dilute ammonia *R1* and 75 volumes of methanol *R*. Allow the plate to dry in air. Examine in daylight. The principal spot in the chromatogram

obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

C. It gives reaction (a) of chlorides (2.3.1).

TESTS

Related substances. Examine by liquid chromatography (2.2.29). Use freshly prepared solutions and protect them from bright light.

Test solution. Dissolve 10.0 mg of the substance to be examined in the mobile phase and dilute to 10.0 ml with the mobile phase.

Reference solution (a). Dilute 5.0 ml of the test solution to 100.0 ml with the mobile phase.

Reference solution (b). Dilute 1.0 ml of the test solution to 10.0 ml with the mobile phase. Dilute 1.0 ml of this solution to 100.0 ml with the mobile phase.

Reference solution (c). Dissolve 25 mg of the substance to be examined in 10 ml of water *R*, warming if necessary. Allow to cool and add 1 ml of a 20 g/l solution of chloramine *R* and 0.5 ml of 0.05 *M* hydrochloric acid. Dilute to 25 ml with water *R*. Shake and allow to stand for 5 min. Inject immediately.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4 mm in internal diameter packed with octylsilyl silica gel for chromatography *R* (5 μ m),
- as mobile phase at a flow rate of 1.5 ml/min a mixture prepared as follows: mix 19.5 volumes of methanol *R* and 80.5 volumes of a solution containing 15 g/l of citric acid *R* and 8.1 g/l of disodium hydrogen phosphate *R*,
- as detector a spectrophotometer set at 351 nm,
- a loop injector.

Inject separately 20 μ l of each solution and continue the chromatography for four times the retention time of the principal peak in the chromatogram obtained with reference solution (a). In the chromatogram obtained with the test solution, the sum of the areas of any peaks apart from the principal peak is not greater than the area of the principal peak in the chromatogram obtained with reference solution (a) (5 per cent). Disregard any peak whose area is less than that of the principal peak in the chromatogram obtained with reference solution (b). The test is not valid unless: the chromatogram obtained with reference solution (c) shows three principal peaks and the resolution between each pair of adjacent peaks is at least 3.0; the chromatogram obtained with reference solution (b) shows one principal peak with a signal-to-noise ratio of at least 5.

Loss on drying (2.2.32): 8.0 per cent to 12.0 per cent, determined on 0.400 g by drying at 100–105 °C at a pressure not exceeding 0.7 kPa.

ASSAY

Protect the solutions from light throughout the assay.

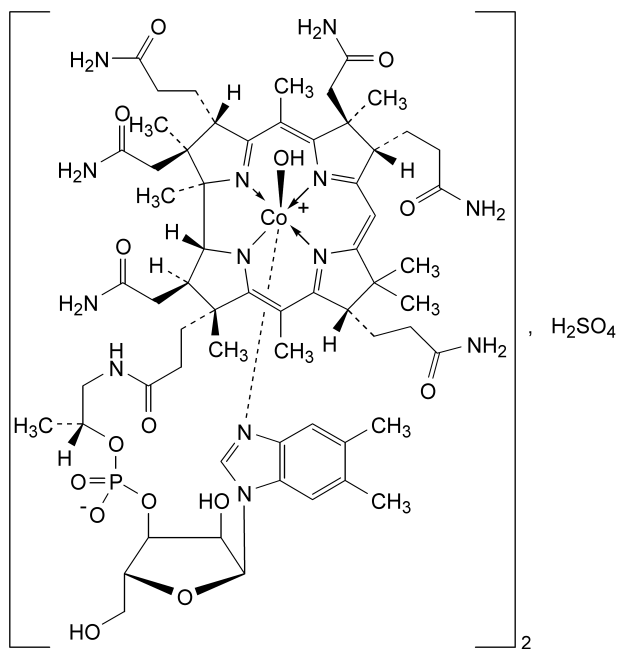
Dissolve 25.0 mg in a solution containing 0.8 per cent *V/V* of glacial acetic acid *R* and 10.9 g/l of sodium acetate *R* and dilute to 1000.0 ml with the same solvent. Measure the absorbance of the resulting solution at the maximum at 351 nm (2.2.25).

Calculate the content of $C_{62}H_{90}ClCoN_{13}O_{15}P$ taking the specific absorbance to be 190.

STORAGE

Store in an airtight container protected from light, at a temperature of 2 °C to 8 °C.

01/2005:0915

HYDROXOCOBALAMIN SULPHATE**Hydroxocobalamini sulfas** $C_{124}H_{180}Co_2N_{26}O_{34}P_2S$ M_r 2791**DEFINITION**

Hydroxocobalamin sulphate contains not less than 96.0 per cent and not more than the equivalent of 102.0 per cent of di-(Co α -[α -(5,6-dimethylbenzimidazolyl)]-Co β -hydroxocobamide) sulphate, calculated with reference to the dried substance.

CHARACTERS

A dark red, crystalline powder or dark red crystals, soluble in water, very hygroscopic. Some decomposition may occur on drying.

IDENTIFICATION

A. Dissolve 2.5 mg in a solution containing 0.8 per cent *V/V* of *glacial acetic acid R* and 10.9 g/l of *sodium acetate R* and dilute to 100 ml with the same solution. Examined between 260 nm and 610 nm (2.2.25), the solution shows three absorption maxima, at 274 nm, 351 nm and 525 nm. The ratio of the absorbance at the maximum at 274 nm to that at the maximum at 351 nm is 0.75 to 0.83. The ratio of the absorbance at the maximum at 525 nm to that at the maximum at 351 nm is 0.31 to 0.35.

B. Examine by thin-layer chromatography (2.2.27), using *silica gel G R* as the coating substance. Carry out the test protected from light.

Test solution. Dissolve 2 mg of the substance to be examined in 1 ml of a mixture of equal volumes of *alcohol R* and *water R*.

Reference solution. Dissolve 2 mg of *hydroxocobalamin CRS* in 1 ml of a mixture of equal volumes of *alcohol R* and *water R*.

Apply separately to the plate 10 μ l of each solution. Develop in an unlined tank over a path of 12 cm using a mixture of 25 volumes of *dilute ammonia R1* and 75 volumes of *methanol R*. Allow the plate to dry in

air. Examine in daylight. The principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

C. It gives reaction (a) of sulphates (2.3.1).

TESTS

Related substances. Examine by liquid chromatography (2.2.29). Use freshly prepared solutions and protect them from bright light.

Test solution. Dissolve 10.0 mg of the substance to be examined in the mobile phase and dilute to 10.0 ml with the mobile phase.

Reference solution (a). Dilute 5.0 ml of the test solution to 100.0 ml with the mobile phase.

Reference solution (b). Dilute 1.0 ml of the test solution to 10.0 ml with the mobile phase. Dilute 1.0 ml of this solution to 100.0 ml with the mobile phase.

Reference solution (c). Dissolve 25 mg of the substance to be examined in 10 ml of *water R*, warming if necessary. Allow to cool and add 1 ml of a 20 g/l solution of *chloramine R* and 0.5 ml of 0.05 M *hydrochloric acid*. Dilute to 25 ml with *water R*. Shake and allow to stand for 5 min. Inject immediately.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4 mm in internal diameter packed with *octylsilyl silica gel for chromatography R* (5 μ m),
- as mobile phase at a flow rate of 1.5 ml/min a mixture prepared as follows: mix 19.5 volumes of *methanol R* and 80.5 volumes of a solution containing 15 g/l of *citric acid R* and 8.1 g/l of *disodium hydrogen phosphate R*,
- as detector a spectrophotometer set at 351 nm,
- a loop injector.

Inject separately 20 μ l of each solution and continue the chromatography for four times the retention time of the principal peak in the chromatogram obtained with reference solution (a). In the chromatogram obtained with the test solution, the sum of the areas of any peaks apart from the principal peak is not greater than the area of the principal peak in the chromatogram obtained with reference solution (a) (5 per cent). Disregard any peak whose area is less than that of the principal peak in the chromatogram obtained with reference solution (b). The test is not valid unless: the chromatogram obtained with reference solution (c) shows three principal peaks and the resolution between each pair of adjacent peaks is at least 3.0; the chromatogram obtained with reference solution (b) shows one principal peak with a signal-to-noise ratio of at least 5.

Loss on drying (2.2.32): 8.0 per cent to 16.0 per cent, determined on 0.400 g by drying at 100 °C to 105 °C at a pressure not exceeding 0.7 kPa.

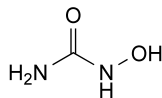
ASSAY

Protect the solutions from light throughout the assay. Dissolve 25.0 mg in a solution containing 0.8 per cent *V/V* of *glacial acetic acid R* and 10.9 g/l of *sodium acetate R* and dilute to 1000.0 ml with the same solvent. Measure the absorbance of the resulting solution at the maximum at 351 nm (2.2.25).

Calculate the content of $C_{124}H_{180}Co_2N_{26}O_{34}P_2S$ taking the specific absorbance to be 188.

STORAGE

Store in an airtight container protected from light, at a temperature of 2 °C to 8 °C.

01/2005:1616 *Limit:*
corrected**HYDROXYCARBAMIDE****Hydroxycarbamidum**CH₄N₂O₂*M*_r 76.1**DEFINITION***N*-Hydroxyurea.*Content:* 97.5 per cent to 102.0 per cent (anhydrous substance).**CHARACTERS***Appearance:* white or almost white, crystalline powder, hygroscopic.*Solubility:* freely soluble in water, practically insoluble in alcohol.

It shows polymorphism.

IDENTIFICATION

A. Infrared absorption spectrophotometry (2.2.24).

Comparison: hydroxycarbamide CRS.If the spectra obtained in the solid state show differences dissolve the substance to be examined and the reference substance separately in *alcohol R*, evaporate to dryness and record new spectra using the residues.

B. Examine the chromatograms obtained in the test for urea.

Results: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with reference solution (c).**TESTS****Urea.** Thin-layer chromatography (2.2.27).*Test solution.* Dissolve 50 mg of the substance to be examined in *water R* and dilute to 1.0 ml with the same solvent.*Reference solution (a).* Dissolve 12.5 mg of *urea R* in *water R* and dilute to 50 ml with the same solvent.*Reference solution (b).* Dissolve 5 mg of the substance to be examined and 5 mg of *urea R* in *water R* and dilute to 20 ml with the same solvent.*Reference solution (c).* Dissolve 50 mg of *hydroxycarbamide CRS* in *water R* and dilute to 1 ml with the same solvent.*Plate:* TLC silica gel plate *R*.*Mobile phase:* pyridine *R*, *water R*, *ethyl acetate R* (2:2:10 *V/V/V*).*Application:* 10 µl.*Development:* over 2/3 of the plate.*Drying:* in air.*Detection:* spray with a 10 g/l solution of *dimethylaminobenzaldehyde R* in 1 *M hydrochloric acid*.*System suitability:* the test is not valid unless the chromatogram obtained with reference solution (b) shows 2 clearly separated spots.— *urea:* any spot corresponding to urea in the chromatogram obtained with the test solution is not more intense than the spot in the chromatogram obtained with the reference solution (a) (0.5 per cent).**Related substances.** Liquid chromatography (2.2.29).*Test solution (a).* Dissolve 0.100 g of the substance to be examined in the mobile phase and dilute to 10.0 ml with the same mobile phase.*Test solution (b).* Dilute 5.0 ml of test solution (a) to 50.0 ml with the mobile phase.*Reference solution (a).* Dissolve 0.100 g of *hydroxylamine hydrochloride R* and 5 mg of the substance to be examined in the mobile phase and dilute to 10.0 ml with the mobile phase. Prepare immediately before use.*Reference solution (b).* Dilute 0.1 ml of test solution (a) to 100.0 ml with the mobile phase.*Reference solution (c).* Dissolve 0.100 g of *hydroxycarbamide CRS* in the mobile phase and dilute to 10.0 ml with the same solvent. Dilute 5.0 ml to 50.0 ml with the mobile phase.*Column:*

- *size:* *l* = 0.25 m, Ø = 4.6 mm,
- *stationary phase:* octadecylsilyl silica gel for chromatography *R* (5 µm).

Mobile phase: *methanol R*, *water R* (5:95 *V/V*).*Flow rate:* 0.5 ml/min.*Detection:* spectrophotometer at 214 nm.*Injection:* 20 µl; inject test solution (a) and reference solutions (a) and (b).*Run time:* 3 times the retention time of hydroxycarbamide which is about 5 min.*System suitability:* reference solution (a):

- *resolution:* minimum of 1.0 between the peaks due to impurity A and to hydroxycarbamide.

Limits:

- *any impurity:* not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent),
- *total:* not more than 2 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent),
- *disregard limit:* 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.02 per cent).

Chlorides (2.4.4): maximum 50 ppm.Dissolve 1.0 g in *water R* and dilute to 15 ml with the same solvent. The solution complies with the limit test for chlorides.**Heavy metals** (2.4.8): maximum 10 ppm.Dissolve 2.0 g in *water R* and dilute to 20 ml with the same solvent. 12 ml of the solution complies with limit test A. Prepare the standard using *lead standard solution (1 ppm Pb) R*.**Water** (2.5.12): maximum 0.5 per cent, determined on 2.00 g.**Sulphated ash** (2.4.14): maximum 0.1 per cent, determined on 1.0 g.**ASSAY**

Liquid chromatography (2.2.29) as described in the test for related substances.

Injection: test solution (b) and reference solution (c).

STORAGE

In an airtight container, protected from light.

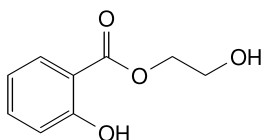
IMPURITIES

A. $\text{H}_2\text{N-OH}$: hydroxylamine.

01/2005:1225

HYDROXYETHYL SALICYLATE

Hydroxyethylis salicylas


 $\text{C}_9\text{H}_{10}\text{O}_4$
 M_r 182.2

DEFINITION

2-Hydroxyethyl 2-hydroxybenzoate.

Content: 98.0 per cent to 102.0 per cent.

CHARACTERS

Appearance: oily, colourless or almost colourless liquid, or colourless crystals.

Solubility: sparingly soluble in water, very soluble in acetone and in methylene chloride, freely soluble in alcohol.

Mp: about 21 °C.

IDENTIFICATION

First identification: A, B.

Second identification: A, C, D, E.

A. It complies with the test for refractive index (see Tests).

B. Infrared absorption spectrophotometry (2.2.24).

Preparation: thin films.

Comparison: hydroxyethyl salicylate CRS.

C. Examine the chromatograms obtained in the test for related substances.

Results: the principal spot in the chromatogram obtained with test solution (b) is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a).

D. To 1 ml of solution S (see Tests), add 1 ml of *water R* and 0.2 ml of *ferric chloride solution R2*. A violet-red colour appears which disappears immediately after the addition of 2 ml of *dilute acetic acid R*. A very faint violet colour may remain.

E. In a test tube 160 mm long, mix 1.0 g with 2.0 g of finely powdered *manganese sulphate R*. Insert 2 cm into the test-tube a strip of filter paper impregnated with a freshly prepared mixture of 1 volume of a 20 per cent V/V solution of *diethanolamine R* and 11 volumes of a 50 g/l solution of *sodium nitroprusside R* adjusted to pH 9.8 with 1 M *hydrochloric acid*. Heat the test-tube over a naked flame for 1-2 min. The filter paper becomes blue.

TESTS

Solution S. Dissolve 2.5 g in 40 ml of *alcohol R* and dilute to 50 ml with *distilled water R*.

Appearance of solution. Solution S is clear (2.2.1) and colourless (2.2.2, *Method II*).

Acidity or alkalinity. To 2 ml of solution S add 0.1 ml of *methyl red solution R* and 0.2 ml of 0.01 M *sodium hydroxide*. The solution is yellow. Add 0.3 ml of 0.01 M *hydrochloric acid*. The solution is red.

Relative density (2.2.5): 1.252 to 1.257.

Refractive index (2.2.6): 1.548 to 1.551.

Related substances. Thin-layer chromatography (2.2.27).

Test solution (a). Dissolve 0.50 g of the substance to be examined in *methanol R* and dilute to 10 ml with the same solvent.

Test solution (b). Dilute 2 ml of test solution (a) to 50 ml with *methanol R*.

Reference solution (a). Dissolve 50.0 mg of *hydroxyethyl salicylate CRS* in *methanol R* and dilute to 25 ml with the same solvent.

Reference solution (b). Dilute 2.5 ml of test solution (b) to 10 ml with *methanol R*.

Reference solution (c). Dissolve 0.10 g of *ethylene glycol R* in *methanol R* and dilute to 50 ml with the same solvent. Dilute 1.25 ml of the solution to 10 ml with *methanol R*.

Plate: TLC silica gel F_{254} plate *R*.

Mobile phase: *ethyl acetate R*, *glacial acetic acid R*, *cyclohexane R* (20:20:60 V/V/V).

Application: 10 µl.

Development: over a path of 15 cm.

Drying: in a current of cold air.

Detection A: in ultraviolet light at 254 nm.

Limits A:

- **any impurity:** any spot in the chromatogram obtained with test solution (a), apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (b) (1 per cent).

Detection B: spray the plate with *ammonium vanadate solution R* and heat at 100 °C for 10 min. Allow to cool for 10 min and examine in daylight.

Limits B: in the chromatogram obtained with test solution (a):

- **impurity B:** any spot corresponding to impurity B is not more intense than the spot in the chromatogram obtained with reference solution (c) (0.5 per cent),
- **any other impurity:** any spot, apart from the principal spot and any spot corresponding to impurity B is not more intense than the spot in the chromatogram obtained with reference solution (b) (1 per cent).

System suitability: the chromatogram obtained with reference solution (c) shows a clearly visible spot.

Chlorides (2.4.4): maximum 100 ppm.

Dilute 10 ml of solution S to 15 ml with *water R*. The solution complies with the limit test for chlorides.

Sulphates (2.4.13): maximum 250 ppm.

Dilute 12 ml of solution S to 15 ml with *distilled water R*. The solution complies with the limit test for sulphates.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

In a flask with a ground-glass stopper, dissolve 0.125 g in 30 ml of *glacial acetic acid R*. Add 10 ml of *dilute sulphuric acid R*, 1.5 g of *potassium bromide R* and 50.0 ml of 0.0167 M *potassium bromate*. Immediately close the flask and allow to stand protected from light for 15 min. Add 1.5 g of *potassium iodide R* immediately after removing the

stopper and titrate with *0.1 M sodium thiosulphate*, adding 1 ml of *starch solution R* towards the end of the titration. Carry out a blank titration.

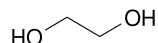
1 ml of *0.0167 M potassium bromate* is equivalent to 4.555 mg of $C_9H_{10}O_4$.

STORAGE

Protected from light.

IMPURITIES

A. salicylic acid,



B. ethane-1,2-diol (ethylene glycol).

01/2005:0336

HYDROXYETHYLCELLULOSE

Hydroxyethylcellulosum

DEFINITION

Partly *O*-(2-hydroxyethylated) cellulose.

CHARACTERS

Appearance: white, yellowish-white or greyish-white powder or granules.

Solubility: soluble in hot and cold water giving a colloidal solution, practically insoluble in acetone, in alcohol and in toluene.

IDENTIFICATION

- Heat 10 ml of solution S (see Tests) to boiling. The solution remains clear.
- To 10 ml of solution S add 0.3 ml of *dilute acetic acid R* and 2.5 ml of a 100 g/l solution of *tannic acid R*. A yellowish-white, flocculent precipitate is formed which dissolves in *dilute ammonia RI*.
- In a test-tube about 160 mm in length, thoroughly mix 1 g with 2 g of finely powdered *manganese sulphate R*. Introduce to a depth of 2 cm into the upper part of the tube a strip of filter paper impregnated with a freshly prepared mixture of 1 volume of a 200 g/l solution of *diethanolamine R* and 11 volumes of a 50 g/l solution of *sodium nitroprusside R*, adjusted to about pH 9.8 with *1 M hydrochloric acid*. Insert the tube 8 cm into a silicone-oil bath and heat at 190-200 °C. The filter paper becomes blue within 10 min. Carry out a blank test.
- Dissolve 0.2 g completely, without heating, in 15 ml of a 700 g/l solution of *sulphuric acid R*. Pour the solution with stirring into 100 ml of iced *water R* and dilute to 250 ml with iced *water R*. In a test-tube, mix thoroughly while cooling in iced water 1 ml of the solution with 8 ml of *sulphuric acid R*, added dropwise. Heat on a water-bath for exactly 3 min and immediately cool in iced water. While the mixture is cold, carefully add 0.6 ml of *ninhydrin solution R2* and mix well. Allow to stand at 25 °C. A pink colour is produced immediately and does not become violet within 100 min.

TESTS

Solution S. Disperse a quantity of the substance to be examined equivalent to 1.0 g of the dried substance in 50 ml of *carbon dioxide-free water R*. After 10 min, dilute to 100 ml with *carbon dioxide-free water R* and stir until dissolution is complete.

pH (2.2.3): 5.5 to 8.5 for solution S.

Apparent viscosity (2.2.10): 75 per cent to 140 per cent of the value stated on the label.

While stirring, introduce a quantity of the substance to be examined equivalent to 2.00 g of the dried substance into 50 g of *water R*. Dilute to 100.0 g with *water R* and stir until dissolution is complete. Determine the viscosity using a rotating viscometer at 25 °C and at a shear rate of 100 s^{-1} for substances with an expected viscosity up to 100 mPa·s, at a shear rate of 10 s^{-1} for substances with an expected viscosity between 100 mPa·s and 20 000 mPa·s and at a shear rate of 1 s^{-1} for substances with an expected viscosity above 20 000 mPa·s. If it is impossible to obtain a shear rate of exactly 1 s^{-1} , 10 s^{-1} or 100 s^{-1} respectively, use a rate slightly higher and a rate slightly lower and interpolate.

Chlorides (2.4.4): maximum 1.0 per cent.

Dilute 1 ml of solution S to 30 ml with *water R*. 15 ml of the solution complies with the limit test for chlorides.

Nitrates: maximum 3.0 per cent (dried substance), if hydroxyethylcellulose has an apparent viscosity of 1000 mPa·s or less and maximum 0.2 per cent (dried substance), if hydroxyethylcellulose has an apparent viscosity of more than 1000 mPa·s.

Determine potentiometrically (2.2.36, *Method I*) using as indicator a nitrate selective electrode and a silver-silver chloride electrode with *0.1 M ammonium sulphate* as reference electrolyte.

Prepare the solutions immediately before use.

Buffer solution. To a mixture of 50 ml of *1 M sulphuric acid* and 800 ml of *water R*, add 135 g of *potassium dihydrogen phosphate R* and dilute to 1000 ml with *water R*.

Buffered water. Dilute 80 ml of buffer solution to 2000 ml with *water R*.

Nitrate standard solution (500 ppm NO_3). Dissolve 0.8154 g of *potassium nitrate R* in 500 ml of buffered water and dilute to 1000.0 ml with the same solvent.

Test solution. Dissolve 0.50 g of the substance to be examined in buffered water and dilute to 100.0 ml with the same solvent.

Reference solutions. If hydroxyethylcellulose has an apparent viscosity of 1000 mPa·s or less, dilute 10.0 ml, 20.0 ml and 40.0 ml of nitrate standard solution (500 ppm NO_3) to 100.0 ml with buffered water and mix.

If hydroxyethylcellulose has an apparent viscosity of more than 1000 mPa·s, dilute 1.0 ml, 2.0 ml and 4.0 ml of nitrate standard solution (500 ppm NO_3) to 100.0 ml with buffered water and mix.

Carry out the measurements for each solution. Calculate the concentration of nitrates using the calibration curve.

Glyoxal: maximum 20 ppm.

Introduce 1.0 g into a test tube with a ground-glass stopper and add 10.0 ml of *ethanol R*. Stopper the tube and stir mechanically for 30 min. Centrifuge. To 2.0 ml of the supernatant liquid add 5.0 ml of a 4 g/l solution of *methylbenzothiazolone hydrazone hydrochloride R* in an 80 per cent V/V solution of *glacial acetic acid R* in *water R*. Shake to homogenise. After 2 h, the solution is not more intensely coloured than a standard prepared at the same time and in the same manner using 2.0 ml of *glyoxal standard solution* (2 ppm $\text{C}_2\text{H}_2\text{O}_2$) *R* instead of the 2.0 ml of supernatant liquid.

Ethylene oxide. Head-space gas chromatography (2.4.25).

Test preparation. Place 1.00 g of the substance to be examined in a 5 ml vial (other sizes may be used depending on the operating conditions) and add 1 ml of *water R*. It swells in water but does not dissolve.

Reference preparation (a). Place 1.00 g of the substance to be examined into an identical 5 ml vial. Add 0.2 ml of cooled *ethylene oxide solution R2* and 0.8 ml of *water R*. It swells in water but does not dissolve.

Reference preparation (b). To 0.1 ml of *ethylene oxide solution R2* in a 5 ml vial add 0.1 ml of a freshly prepared 10 mg/l solution of *acetaldehyde R*.

Close the vials immediately with a butyl rubber membrane stopper, coated with aluminium or polytetrafluoroethylene and secured with an aluminium crimped cap.

Limit:

– *ethylene oxide*: maximum 1 ppm.

2-Chloroethanol. Head-space gas chromatography (2.2.28).

Test preparation. To 50 mg of the substance to be examined in a 10 ml vial (other size may be used depending on the operating conditions), add 2 µl of *2-propanol R*. Seal the flask and mix.

Reference preparation (a). Dissolve 0.125 g of *2-chloroethanol R* and dilute to 50.0 ml with *2-propanol R*. Dilute 1.0 ml of the solution to 10.0 ml with *2-propanol R*.

Reference preparation (b). To 50 mg of the substance to be examined into an identical 10 ml vial, add 2 µl of reference solution (a). Seal the flask and mix.

Close the vials immediately with a butyl rubber membrane stopper, coated with aluminium or polytetrafluoroethylene and secured with an aluminium crimped cap.

Column:

– *size*: $l = 50$ m, $\varnothing = 0.32$ mm,

– *stationary phase*: *poly(dimethyl)siloxane R* (1.2 µm).

Carrier gas: *helium for chromatography R*.

Flow rate: 25–35 cm/s.

Split ratio: 1:10.

Static head-space conditions which may be used:

– *equilibration temperature*: 110 °C,

– *equilibration time*: 20 min,

– *temperature of injection system*: 115 °C.

Temperature:

	Time (min)	Temperature (°C)
Column	0 - 6	60
	6 - 16	60 → 110
	16 - 31	110 → 230
	31 - 36	230
Injection port		150
Detector		250

Detection: flame ionisation.

Injection: 2 ml.

Retention time: 2-chloroethanol = about 7.8 min.

Limit:

– *2-chloroethanol*: not more than 0.5 times the area of the peak due to 2-chloroethanol in reference solution (b) (10 ppm).

Heavy metals (2.4.8): maximum 20 ppm.

1.0 g complies with limit test C. Prepare the standard using 2 ml of *lead standard solution (10 ppm Pb) R*.

Loss on drying (2.2.32): maximum 10.0 per cent, determined on 1.000 g by drying in an oven at 100–105 °C for 3 h.

Sulphated ash (2.4.14): maximum 4.0 per cent, determined on 1.0 g.

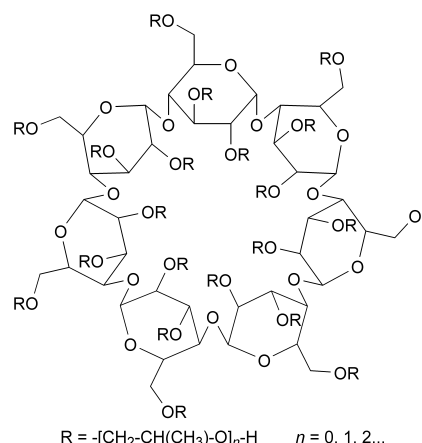
LABELLING

The label states the apparent viscosity, in millipascal seconds for a 2 per cent *m/m* solution.

01/2005:1804

HYDROXYPROPYLBETADEX

Hydroxypropylbetadexum



$C_{42}H_{70}O_{35}(C_3H_6O)_x$ with $x = 7$ MS

DEFINITION

Hydroxypropylbetadex (β-cyclodextrin, 2-hydroxypropyl ether) is a partially substituted poly(hydroxypropyl) ether of betadex. The number of hydroxypropyl groups per anhydroglucose unit, expressed as Molar Substitution (MS), is not less than 0.40 and not more than 1.50 and is within 10 per cent of the value stated on the label.

CHARACTERS

Appearance: white or almost white, amorphous or crystalline powder.

Solubility: freely soluble in water and in propylene glycol.

IDENTIFICATION

A. Infrared absorption spectrophotometry (2.2.24).

Comparison: *hydroxypropylbetadex CRS*.

Results: the spectrum obtained with the substance to be examined shows the same absorption bands as the spectrum obtained with *hydroxypropylbetadex CRS*. Due to the difference in the substitution of the substance, the intensity of some absorption bands can vary.

B. It complies with the test for appearance of solution (see Tests).

TESTS

Solution S. Dissolve 5.0 g in *carbon dioxide-free water R* prepared from *distilled water R* and dilute to 50.0 ml with the same solvent.

Appearance of solution. The solution is clear (2.2.1) and colourless (2.2.2, *Method II*) and remains so after cooling at room temperature.

Dissolve 1.0 g in 2.0 ml of *water R*, with heating.

Conductivity (2.2.38): maximum 200 µS·cm⁻¹.

Measure the conductivity of solution S, while gently stirring with a magnetic stirrer.

Related substances. Liquid chromatography (2.2.29).

Test solution. Dissolve 2.50 g of the substance to be examined in *water R* with heating, cool and dilute to 25.0 ml with the same solvent.

Reference solution (a). Dissolve 0.15 g of *betadex CRS* and 0.25 g of *propylene glycol R* in *water R* and dilute to 10.0 ml with the same solvent.

Reference solution (b). Dilute 5.0 ml of reference solution (a) to 50.0 ml with *water R*.

Precolumn:

- *stationary phase: phenylsilyl silica gel for chromatography R.*

Column:

- *size: $l = 0.30$ m, $\varnothing = 3.9$ mm,*
- *stationary phase: phenylsilyl silica gel for chromatography R,*
- *temperature: 40 °C.*

Mobile phase: water for chromatography R.

Flow rate: 1.5 ml/min.

Detection: differential refractometer, at 40 °C.

Injection: 20 µl.

Run time: 3 times the retention time of impurity A.

Relative retention with reference to impurity B (retention time = about 2.5 min): impurity A = about 4.2; hydroxypropylbetadex = about 6 for the beginning of the elution.

Hydroxypropylbetadex elutes as a very wide peak or several peaks.

System suitability: reference solution (a):

- **resolution:** minimum 4 between the peaks due to impurity A and impurity B.

Limits:

- **impurity A:** not more than the area of the corresponding peak in the chromatogram obtained with reference solution (b) (1.5 per cent),
- **impurity B:** not more than the area of the corresponding peak in the chromatogram obtained with reference solution (b) (2.5 per cent),
- **any other impurity:** not more than 0.04 times the area of the peak due to impurity B in the chromatogram obtained with reference solution (b) (0.1 per cent),
- **total of other impurities:** not more than 0.4 times the area of the peak due to impurity B in the chromatogram obtained with reference solution (b) (1.0 per cent),
- **disregard limit:** 0.02 times the area of the peak due to impurity B in the chromatogram obtained with reference solution (b) (0.05 per cent); disregard any peak eluting before impurity B and after impurity A.

Heavy metals (2.4.8): maximum 20 ppm.

12 ml of solution S complies with limit test A. Prepare the standard using *lead standard solution (2 ppm Pb) R*.

Loss on drying (2.2.32): maximum 10.0 per cent, determined on 1.000 g by drying in an oven at 120 °C for 2 h.

Molar substitution. Nuclear magnetic resonance spectrometry (2.2.33).

The Molar Substitution (*MS*) is calculated from the ratio between the signal from the 3 protons of the methyl group, which is part of the hydroxypropyl group and the signal from

the proton attached to the carbon C₁ (glycosidic proton) of the anhydroglucose units.

Use a Fourier transform nuclear magnetic resonance spectrometer of minimum frequency 250 MHz, suited to perform a proton spectrum and to carry out quantitative analysis, at a temperature of at least 25 °C.

Introduce not less than the equivalent of 10.0 mg of the substance to be examined (dried substance) into a 5 mm NMR tube, equipped with a spinner in order to record the spectrum in rotation. Add approximately 0.75 ml of *deuterium oxide R1*. Cap the tube, mix thoroughly and adapt the spinner.

Make the appropriate instrument settings (frequency, gain, digital resolution, sample rotation, shims, probe tuning, resolution/data point, receiver gain etc.) so as to obtain a suitable spectrum for quantitative analysis (good FID (Free Induction Decay), no distortion of the spectrum after Fourier transform and phase corrections). The relaxation delay must be adapted to the pulse angle in order to have sufficient relaxation of the protons concerned between 2 pulses (for example: 10 s for a 90° pulse).

Record the FID, with at least 8 scans, so as to obtain a spectral window comprised, at least, between 0 ppm and 6.2 ppm, referring to the signal of exchangeable protons (solvent) at 4.8 ppm (25 °C).

Make a zero filling of at least 3-fold in size relative to the acquisition data file and transform the FID to the spectrum without any correction of Gaussian broadening factor (GB = 0) and with a line broadening factor up to maximum 0.2 (LB ≤ 0.2). Call the integration sub-routine after phase corrections and baseline correction between 0.5 ppm and 6.2 ppm.

Measure the peak areas of the doublet from the methyl groups at 1.2 ppm (*A*₁), and of the signals of the glycosidic protons between 5 ppm and 5.4 ppm (*A*₂).

The molar substitution is obtained using the formula:

$$MS = \frac{A_1}{(3 \times A_2)}$$

*A*₁ = area of the signal due to the 3 protons of the methyl groups, which are part of the hydroxypropyl groups,

*A*₂ = area of the signals due to the glycosidic protons.

The degree of substitution is the number of hydroxypropyl groups per molecule of β-cyclodextrin and is obtained by multiplying the *MS* by 7.

Microbial contamination. Total viable aerobic count (2.6.12) not more than 10³ bacteria and 10² fungi per gram, determined by plate count. If intended for use in the manufacture of parenteral dosage forms, the total viable aerobic count is not more than 10² bacteria and 10² fungi per gram. It complies with the tests for *Escherichia coli* and *Salmonella* (2.6.13).

Bacterial endotoxins (2.6.14): less than 10 IU/g, if intended for use in the manufacture of parenteral dosage forms without a further appropriate procedure for the removal of bacterial endotoxins.

LABELLING

The label states:

- the molar substitution (*MS*),
- where applicable, that the substance is suitable for use in the manufacture of parenteral dosage forms.

IMPURITIES

- A. betadex,
B. propylene glycol.

01/2005:0337

HYDROXYPROPYLCELLULOSE

Hydroxypropylcellulosum

DEFINITION

Hydroxypropylcellulose is a partly *O*-(2-hydroxypropylated) cellulose. It may contain not more than 0.6 per cent of silica (SiO_2).

CHARACTERS

A white or yellowish-white powder or granules, hygroscopic after drying, soluble in cold water, in glacial acetic acid, in ethanol, in methanol and in propylene glycol and in a mixture of 10 parts of methanol and 90 parts of methylene chloride giving colloidal solutions, sparingly soluble or slightly soluble in acetone depending on the degree of substitution, practically insoluble in hot water, in ethylene glycol and in toluene.

IDENTIFICATION

- A. Heat 10 ml of solution S (see Tests) in a water-bath while stirring. At a temperature above 40 °C the solution becomes cloudy or a flocculent precipitate is formed. The solution becomes clear again on cooling.
- B. To 10 ml of solution S add 0.3 ml of *dilute acetic acid R* and 2.5 ml of a 100 g/l solution of *tannic acid R*. A yellowish-white, flocculent precipitate is formed which dissolves in *dilute ammonia R1*.
- C. In a test-tube about 160 mm long, thoroughly mix 1 g with 2 g of finely powdered *manganese sulphate R*. Introduce to a depth of 2 cm into the upper part of the tube a strip of filter paper impregnated with a freshly prepared mixture of 1 volume of a 20 per cent *V/V* solution of *diethanolamine R* and 11 volumes of a 50 g/l solution of *sodium nitroprusside R*, adjusted to about pH 9.8 with *1 M hydrochloric acid*. Insert the tube 8 cm into a silicone-oil bath at 190 °C to 200 °C. The filter paper becomes blue within 10 min. Carry out a blank test.
- D. Dissolve 0.2 g completely, without heating, in 15 ml of a 70 per cent *m/m* solution of *sulphuric acid R*. Pour the solution with stirring into 100 ml of iced *water R* and dilute to 250 ml with iced *water R*. In a test-tube, mix thoroughly while cooling in iced water 1 ml of the solution with 8 ml of *sulphuric acid R*, added dropwise. Heat in a water-bath for exactly 3 min and immediately cool in iced water. While the mixture is cold, carefully add 0.6 ml of *ninhydrin solution R2* and mix well. Allow to stand at 25 °C. A pink colour is produced immediately and becomes violet within 100 min.
- E. Place 1 ml of solution S on a glass plate. After evaporation of the water a thin film is formed.
- F. 0.2 g does not dissolve in 10 ml of *toluene R* but dissolves completely in 10 ml of *ethanol R*.

TESTS

Solution S. While stirring, introduce a quantity of the substance to be examined equivalent to 1.0 g of the dried substance into 50 g of *carbon dioxide-free water R* heated to 90 °C. Allow to cool, adjust the mass of the solution to 100 g with *carbon dioxide-free water R* and stir until dissolution is complete.

Appearance of solution. Solution S is not more opalescent than reference suspension III (2.2.1) and not more intensely coloured than reference solution Y_6 (2.2.2, *Method II*).

pH (2.2.3). The pH of solution S is 5.0 to 8.5.

Apparent viscosity. While stirring, introduce a quantity of the substance to be examined equivalent to 6.00 g of the dried substance into 150 g of *water R* heated to 90 °C. Stir with a propeller-type stirrer for 10 min, place the flask in a bath of iced water, continue the stirring and allow to remain in the bath of iced water for 40 min to ensure that dissolution is complete. Adjust the mass of the solution to 300 g and centrifuge the solution to expel any entrapped air. Adjust the temperature of the solution to 20 ± 0.1 °C. Determine the viscosity (2.2.10) with a rotating viscometer at 20 °C and a shear rate of 10 s^{-1} . The apparent viscosity is not less than 75 per cent and not more than 140 per cent of the value stated on the label.

For a product of low viscosity, use a quantity of the substance to be examined sufficient to prepare a solution of the concentration stated on the label.

Silica. Not more than 0.6 per cent. To the residue obtained in the test for sulphated ash add sufficient *alcohol R* to moisten the residue completely. Add 6 ml of *hydrofluoric acid R* in small portions. Evaporate to dryness at 95 °C to 105 °C, taking care to avoid loss from sputtering. Cool and rinse the wall of the platinum crucible with 6 ml of *hydrofluoric acid R*. Add 0.5 ml of *sulphuric acid R* and evaporate to dryness. Progressively increase the temperature, ignite at 900 °C, allow to cool in a desiccator and weigh. The difference between the mass of the residue obtained in the test for sulphated ash and the mass of the final residue is equal to the amount of silica in the substance to be examined.

Chlorides (2.4.4). 1 ml of solution S diluted to 15 ml with *water R* complies with the limit test for chlorides (0.5 per cent).

Heavy metals (2.4.8). 1.0 g complies with limit test C for heavy metals (20 ppm). Prepare the standard using 2 ml of *lead standard solution (10 ppm Pb) R*.

Loss on drying (2.2.32). Not more than 7.0 per cent, determined on 1.000 g by drying in an oven at 100-105 °C.

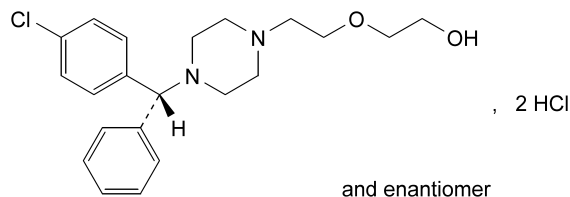
Sulphated ash (2.4.14). Not more than 1.6 per cent, determined on 1.0 g using a platinum crucible.

LABELLING

The label states:

- the apparent viscosity in millipascal seconds for a 2 per cent *m/m* solution,
- for a product of low viscosity, the concentration of the solution to be used and the apparent viscosity in millipascal seconds.
- where applicable, that the substance contains silica.

01/2005:0916

HYDROXYZINE HYDROCHLORIDE**Hydroxyzini hydrochloridum** $C_{21}H_{29}Cl_3N_2O_2$ M_r 447.8**DEFINITION**

Hydroxyzine hydrochloride contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of (RS)-2-[2-[4-[(4-chlorophenyl)phenylmethyl]piperazin-1-yl]ethoxy]ethanol dihydrochloride, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, crystalline powder, hygroscopic, freely soluble in water and in alcohol, very slightly soluble in acetone.

It melts at about 200 °C, with decomposition.

IDENTIFICATION

First identification: A, D.

Second identification: B, C, D.

- A. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *hydroxyzine hydrochloride CRS*. Examine the substances prepared as discs.
- B. Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel G plate R*.

Test solution. Dissolve 0.50 g of the substance to be examined in a mixture of equal volumes of *methanol R* and *methylene chloride R* and dilute to 10 ml with the same mixture of solvents.

Reference solution (a). Dissolve 0.50 g of *hydroxyzine hydrochloride CRS* in a mixture of equal volumes of *methanol R* and *methylene chloride R* and dilute to 10 ml with the same mixture of solvents.

Reference solution (b). Dissolve 0.50 g of *meclozine hydrochloride R* in a mixture of equal volumes of *methanol R* and *methylene chloride R* and dilute to 10 ml with the same mixture of solvents. Dilute 1 ml to 2 ml with reference solution (a).

Apply to the plate 2 µl of each solution. Develop over a path of 15 cm using a mixture of 1 volume of *concentrated ammonia R*, 24 volumes of *alcohol R* and 75 volumes of *toluene R*. Allow the plate to dry in air. Spray with *potassium iodobismuthate solution R2*. The principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a). The test is not valid unless the chromatogram obtained with reference solution (b) shows two clearly separated principal spots.

- C. Dissolve 0.1 g in *alcohol R* and dilute to 15 ml with the same solvent. Add 15 ml of a saturated solution of *picric acid R* in *alcohol R*. Allow to stand for 15 min. A precipitate is formed. Filter. Recrystallise from *alcohol R*.

Initiate crystallisation, if necessary, by scratching the wall of the tube with a glass rod. The crystals melt (2.2.14) at 189 °C to 192 °C.

D. It gives reaction (a) of chlorides (2.3.1).

TESTS

Solution S. Dissolve 2.0 g in *water R* and dilute to 20.0 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and not more intensely coloured than reference solution Y_7 (2.2.2, *Method II*).

Optical rotation (2.2.7): -0.10° to $+0.10^\circ$, determined on solution S.

Related substances. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 10.0 mg of the substance to be examined in the mobile phase and dilute to 10.0 ml with the mobile phase.

Reference solution (a). Dissolve 10.0 mg of *hydroxyzine hydrochloride CRS* in the mobile phase and dilute to 10.0 ml with the mobile phase.

Reference solution (b). Dilute 3.0 ml of the test solution to 200.0 ml with the mobile phase. Dilute 5.0 ml to 25.0 ml with the mobile phase.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.15 m long and 4.6 mm in internal diameter packed with *base-deactivated octadecylsilyl silica gel for chromatography R* (3 µm),
- as mobile phase at a flow rate of 1 ml/min a mixture prepared as follows: dissolve 0.5 g of *sodium methanesulphonate R* in a mixture of 14 volumes of *triethylamine R*, 300 volumes of *acetonitrile R* and 686 volumes of *water R*; adjust to pH 2.7 with *sulphuric acid R*,
- as detector a spectrophotometer set at 230 nm.

Inject 20 µl of reference solution (a) and 20 µl of reference solution (b). Adjust the sensitivity of the system so that the height of the principal peak in the chromatogram obtained with reference solution (b) is at least 50 per cent of the full scale of the recorder.

In the chromatogram obtained with reference solution (a), measure the height (A) above the baseline of the peak immediately before the principal peak and the height (B) above the baseline of the lowest point of the curve (B) separating this peak from the peak due to hydroxyzine. The test is not valid unless A is greater than ten times B.

Inject 20 µl of the test solution and 20 µl of reference solution (b). Continue the chromatography for 2.5 times the retention time of the principal peak. In the chromatogram obtained with the test solution: the area of any peak, apart from the principal peak, is not greater than one third of the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent); the sum of the areas of all the peaks, apart from the principal peak, is not greater than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.3 per cent). Disregard any peak with an area less than 0.1 times that of the principal peak in the chromatogram obtained with reference solution (b).

Heavy metals (2.4.8). 12 ml of solution S complies with limit test A for heavy metals (10 ppm). Prepare the standard using *lead standard solution (1 ppm Pb) R*.

Loss on drying (2.2.32). Not more than 5.0 per cent, determined on 1.000 g by drying in an oven at 100-105 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

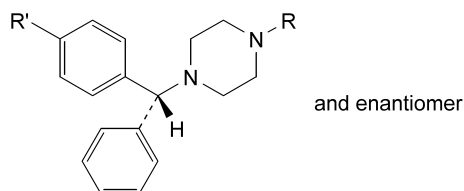
ASSAY

Dissolve 0.200 g in 10 ml of *anhydrous acetic acid R*. Add 40 ml of *acetic anhydride R*. Titrate with 0.1 M *perchloric acid*, determining the end-point potentiometrically (2.2.20). 1 ml of 0.1 M *perchloric acid* is equivalent to 22.39 mg of $C_{21}H_{29}Cl_3N_2O_2$.

STORAGE

Store in an airtight container, protected from light.

IMPURITIES

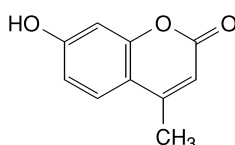


- A. R = H, R' = Cl: (RS)-1-[(4-chlorophenyl)phenylmethyl]piperazine,
- B. R = $CH_2CH_2OCH_2CH_2OH$, R' = H: 2-[2-[4-(diphenylmethyl)piperazin-1-yl]ethoxy]ethanol (decloxizine).

01/2005:1786

HYMECROMONE

Hymecromonum



$C_{10}H_8O_3$

M_r 176.2

DEFINITION

7-Hydroxy-4-methyl-2H-1-benzopyran-2-one.

Content: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

Appearance: almost white crystalline powder.

Solubility: very slightly soluble in water, sparingly soluble in methanol, slightly soluble in methylene chloride. It dissolves in dilute solutions of ammonia.

IDENTIFICATION

Infrared absorption spectrophotometry (2.2.24).

Comparison: *hymecromone CRS*.

TESTS

Absorbance (2.2.25). Dissolve 50 mg in 10 ml of *ammonium chloride buffer solution pH 10.4 R* and dilute to 100.0 ml with *water R*. To 1.0 ml of the solution, add 10 ml of *ammonium chloride buffer solution pH 10.4 R* and dilute to 100.0 ml with *water R*. Examined between 200 nm and 400 nm, the solution shows 2 absorption maxima, at 229 nm and 360 nm, and an absorption minimum at 276 nm. The specific absorbance at the maximum at 360 nm is 1020 to 1120.

Related substances. Liquid chromatography (2.2.29).

Buffer solution. To 280 ml of a 1.56 g/l solution of *sodium dihydrogen phosphate R*, add 720 ml of a 3.58 g/l solution of *disodium hydrogen phosphate R*. Adjust to pH 7 with a 100 g/l solution of *phosphoric acid R*.

Test solution. Dissolve 10 mg of the substance to be examined in the mobile phase and dilute to 10.0 ml with the mobile phase.

Reference solution (a). Dissolve 20 mg of *hymecromone CRS*, 10 mg of *hymecromone impurity A CRS* and 10 mg of *hymecromone impurity B CRS* in the mobile phase and dilute to 100.0 ml with the mobile phase.

Reference solution (b). Dilute 1.0 ml of reference solution (a) to 200.0 ml with the mobile phase.

Column:

- **size:** $l = 0.25$ m, $\varnothing = 4$ mm,
- **stationary phase:** spherical *octadecylsilyl silica gel for chromatography R* (10 μ m).

Mobile phase: *methanol R*, buffer solution (465:535 V/V).

Flow rate: 1.0 ml/min.

Detection: spectrophotometer at 270 nm.

Injection: 20 μ l.

Run time: 1.5 times the retention time of hymecromone.

Relative retention with reference to hymecromone (retention time = about 6 min): impurity A = about 0.5; impurity B = about 0.7.

System suitability: reference solution (a):

- **resolution:** minimum of 2 between the peaks due to impurity A and to impurity B and minimum of 3 between the peaks due to impurity B and to hymecromone.

Limits:

- **impurity A:** not more than the area of the corresponding peak in the chromatogram obtained with reference solution (b) (0.05 per cent),
- **impurity B:** not more than the area of the corresponding peak in the chromatogram obtained with reference solution (b) (0.05 per cent),
- **any other impurity:** not more than the area of the peak due to hymecromone in the chromatogram obtained with reference solution (b) (0.1 per cent),
- **total:** not more than twice the area of the peak due to hymecromone in the chromatogram obtained with reference solution (b) (0.2 per cent),
- **disregard limit:** 0.1 times the area of the peak due to hymecromone in the chromatogram obtained with reference solution (b) (0.01 per cent).

Heavy metals (2.4.8): maximum 10 ppm.

Dissolve 1.5 g in a mixture of 15 volumes of *water R* and 85 volumes of *dimethylformamide R* and dilute to 18 ml with the same mixture of solvents. The solution complies with limit test B. Prepare the standard using a lead standard solution (1 ppm Pb) obtained by diluting *lead standard solution* (100 ppm Pb) *R* with a mixture of 15 volumes of *water R* and 85 volumes of *dimethylformamide R*.

Loss on drying (2.2.32): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 100–105 °C for 4 h.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.100 g in 80 ml of *2-propanol R*. Titrate with 0.1 M *tetrabutylammonium hydroxide in 2-propanol* determining the end-point potentiometrically (2.2.20). Carry out a blank titration.

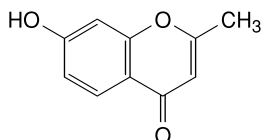
1 ml of 0.1 M tetrabutylammonium hydroxide in 2-propanol is equivalent to 17.62 mg of C₁₀H₈O₃.

STORAGE

Protected from light.

IMPURITIES

A. resorcinol,

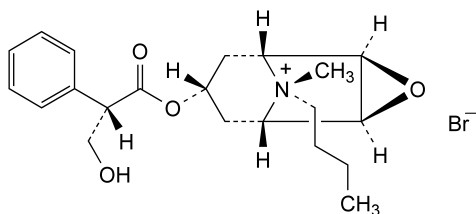


B. 7-hydroxy-2-methyl-4H-1-benzopyran-4-one.

01/2005:0737

HYOSCINE BUTYLBROMIDE

Hyoscini butylbromidum
Scopolamini butylbromidum



C₂₁H₃₀BrNO₄

M_r 440.4

DEFINITION

(1*R*,2*R*,4*S*,5*S*,7*s*,9*r*)-9-Butyl-7-[[[(2*S*)-3-hydroxy-2-phenylpropanoyl]oxy]-9-methyl-3-oxa-9-azoniatricyclo[3.3.1.0^{2,4}]nonane bromide.

Content: 98.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

Appearance: white or almost white, crystalline powder.

Solubility: freely soluble in water and in methylene chloride, sparingly soluble in anhydrous ethanol.

IDENTIFICATION

First identification: A, C, F.

Second identification: A, B, D, E, F.

A. It complies with the test for specific optical rotation (see Tests).

B. Melting point (2.2.14): 139 °C to 141 °C.

C. Infrared absorption spectrophotometry (2.2.24).

Comparison: hyoscine butylbromide CRS.

D. To about 1 mg add 0.2 ml of *nitric acid R* and evaporate to dryness on a water-bath. Dissolve the residue in 2 ml of *acetone R* and add 0.1 ml of a 30 g/l solution of *potassium hydroxide R* in *methanol R*. A violet colour develops.

E. To 5 ml of solution S (see Tests) add 2 ml of *dilute sodium hydroxide solution R*. No precipitate is formed.

F. It gives reaction (a) of bromides (2.3.1).

TESTS

Solution S. Dissolve 1.25 g in *carbon dioxide-free water R* and dilute to 25.0 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and colourless (2.2.2, *Method II*).

pH (2.2.3): 5.5 to 6.5 for solution S.

Specific optical rotation (2.2.7): –18 to –20 (dried substance), determined on solution S.

Related substances. Liquid chromatography (2.2.29).

Test solution. Dissolve 50.0 mg of the substance to be examined in the mobile phase and dilute to 10.0 ml with the mobile phase.

Reference solution (a). Dilute 1.0 ml of the test solution to 50.0 ml with the mobile phase. Dilute 5.0 ml of this solution to 50.0 ml with the mobile phase.

Reference solution (b). Dilute 10.0 ml of reference solution (a) to 20.0 ml with the mobile phase.

Reference solution (c). Dissolve 5.0 mg of *hyoscine butylbromide impurity E CRS* in the mobile phase, add 1.0 ml of the test solution and dilute to 10.0 ml with the mobile phase. Dilute 5.0 ml of this solution to 50.0 ml with the mobile phase.

Column:

- **size:** *l* = 0.125 m, Ø = 4.0 mm,
- **stationary phase:** octylsilyl silica gel for chromatography *R* (4 µm),
- **temperature:** 25 ± 1 °C.

Mobile phase: dissolve 5.8 g of *sodium dodecyl sulphate R* in a mixture of 410 ml of *acetonitrile R* and 605 ml of a 7.0 g/l solution of *potassium dihydrogen phosphate R* previously adjusted to pH 3.3 with 0.05 M *phosphoric acid*.

Flow rate: 2.0 ml/min.

Detection: spectrophotometer at 210 nm.

Injection: 10 µl.

Run time: 3.5 times the retention time of butylhyoscine.

Relative retention with reference to butylhyoscine (retention time = about 7.0 min): impurity B = about 0.1; impurity A = about 0.36; impurity C = about 0.40; impurity D = about 0.7; impurity E = about 0.8; impurity F = about 0.9; impurity G = about 3.0.

System suitability: reference solution (c):

- **resolution:** minimum 1.5 between the peaks due to butylhyoscine and impurity E,
- **symmetry factor:** maximum 2.5 for the peak due to butylhyoscine.

Limits:

- **correction factors:** for the calculation of contents, multiply the peak areas of the following impurities by the corresponding correction factor: impurity B = 0.3; impurity G = 0.6;
- **impurity A:** not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent);
- **impurities B, C, D, E, F, G:** for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent);
- **any other impurity:** for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent);
- **total:** not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.4 per cent); disregard any peak due to the bromide ion which appears close to the solvent peak;
- **disregard limit:** 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

Loss on drying (2.2.32): maximum 2.5 per cent, determined on 0.500 g by drying in an oven at 100–105 °C.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 0.5 g.

01/2005:0106

ASSAY

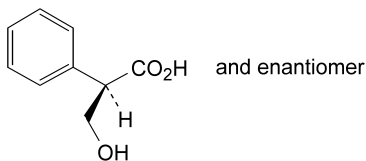
Dissolve 0.400 g in 50 ml of *water R*. Titrate with 0.1 M *silver nitrate*, determining the end-point potentiometrically (2.2.20) using a silver indicator electrode and a silver-silver chloride reference electrode.

1 ml of 0.1 M *silver nitrate* is equivalent to 44.04 mg of $C_{21}H_{30}BrNO_4$.

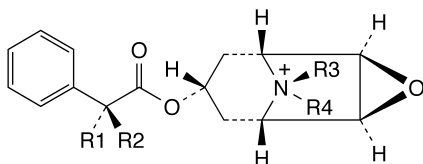
IMPURITIES

Specified impurities: A, B, C, D, E, F, G.

A. hyoscine,



B. (2*RS*)-3-hydroxy-2-phenylpropanoic acid (DL-tropic acid),

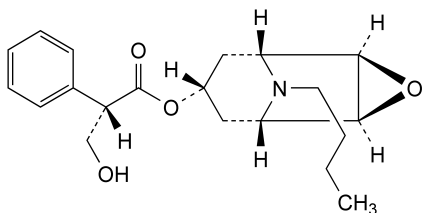


C. $R_1 = CH_2OH$, $R_2 = H$, $R_3 = R_4 = CH_3$: (1*R*,2*R*,4*S*,5*S*,7*S*)-7-[[[(2*S*)-3-hydroxy-2-phenylpropanoyl]oxy]-9,9-dimethyl-3-oxa-9-azoniatricyclo[3.3.1.0^{2,4}]nonane (methylhyoscine),

D. $R_1 = CH_2OH$, $R_2 = H$, $R_3 = CH_3$, $R_4 = CH_2CH_2CH_3$: (1*R*,2*R*,4*S*,5*S*,7*S*,9*r*)-9-butyl-7-[[[(2*S*)-3-hydroxy-2-phenylpropanoyl]oxy]-9-methyl-3-oxa-9-azoniatricyclo[3.3.1.0^{2,4}]nonane (propylhyoscine),

F. $R_1 = CH_2OH$, $R_2 = H$, $R_3 = CH_2CH_2CH_2CH_3$, $R_4 = CH_3$: (1*R*,2*R*,4*S*,5*S*,7*S*,9*s*)-9-butyl-7-[[[(2*S*)-3-hydroxy-2-phenylpropanoyl]oxy]-9-methyl-3-oxa-9-azoniatricyclo[3.3.1.0^{2,4}]nonane (pseudo-isomer),

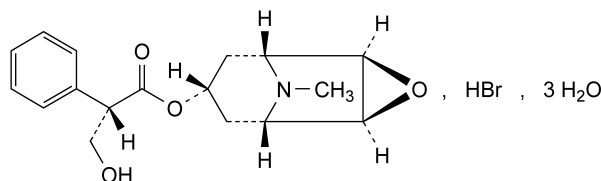
G. $R_1 + R_2 = CH_2$, $R_3 = CH_3$, $R_4 = CH_2CH_2CH_2CH_3$: (1*R*,2*R*,4*S*,5*S*,7*S*,9*r*)-9-butyl-9-methyl-7-[[[(2-phenylprop-2-enoyl)oxy]-3-oxa-9-azoniatricyclo[3.3.1.0^{2,4}]nonane (apo-*N*-butylhyoscine);



E. (1*R*,2*R*,4*S*,5*S*,7*S*)-9-butyl-3-oxa-9-azoniatricyclo[3.3.1.0^{2,4}]nonan-7-yl (2*S*)-3-hydroxy-2-phenylpropanoate (*N*-butylhyoscine).

HYOSCINE HYDROBROMIDE

Hyosini hydrobromidum
Scopolamini hydrobromidum


 $C_{17}H_{22}BrNO_4 \cdot 3H_2O$
 M_r 438.3

DEFINITION

(1*R*,2*R*,4*S*,5*S*,7*S*)-9-Methyl-3-oxa-9-azoniatricyclo[3.3.1.0^{2,4}]non-7-yl (2*S*)-3-hydroxy-2-phenylpropanoate hydrobromide trihydrate.

Content: 99.0 per cent to 101.0 per cent (anhydrous substance).

CHARACTERS

Appearance: white or almost white, crystalline powder or colourless crystals, efflorescent.

Solubility: freely soluble in water, soluble in ethanol (96 per cent).

IDENTIFICATION

First identification: B, E.

Second identification: A, C, D, E.

A. It complies with the test for specific optical rotation (see Tests).

B. Infrared absorption spectrophotometry (2.2.24).

Comparison: *hyoscine hydrobromide CRS*.

If the spectra obtained in the solid state show differences, proceed as follows: dissolve 3 mg of the substance to be examined in 1 ml of *ethanol (96 per cent) R* and evaporate to dryness on a water-bath; dissolve the residue in 0.5 ml of *methylene chloride R* and add 0.2 g of *potassium bromide R* and 15 ml of *ether R*; allow to stand for 5 min shaking frequently; decant; dry the residue on a water-bath until the solvents have evaporated; using the residue prepare a disc and dry at 100–105 °C for 3 h. Repeat the procedure with *hyoscine hydrobromide CRS* and record the spectra.

C. Dissolve about 50 mg in 5 ml of *water R* and add 5 ml of *picric acid solution R* dropwise and with shaking. The precipitate, washed with *water R* and dried at 100–105 °C for 2 h, melts (2.2.14) at 188 °C to 193 °C.

D. To about 1 mg add 0.2 ml of *fuming nitric acid R* and evaporate to dryness on a water-bath. Dissolve the residue in 2 ml of *acetone R* and add 0.1 ml of a 30 g/l solution of *potassium hydroxide R* in *methanol R*. A violet colour develops.

E. It gives reaction (a) of bromides (2.3.1).

TESTS

Solution S. Dissolve 2.50 g in *carbon dioxide-free water R* and dilute to 50.0 ml with the same solvent.

pH (2.2.3): 4.0 to 5.5 for solution S.

Specific optical rotation (2.2.7): −24 to −27 (anhydrous substance), determined on solution S.

Related substances. Liquid chromatography (2.2.29).

Test solution. Dissolve 70.0 mg of the substance to be examined in the mobile phase and dilute to 50.0 ml with the mobile phase.

Reference solution (a). Dilute 2.0 ml of the test solution to 100.0 ml with the mobile phase. Dilute 5.0 ml of this solution to 20.0 ml with the mobile phase.

Reference solution (b). Dilute 5.0 ml of reference solution (a) to 25.0 ml with the mobile phase.

Reference solution (c). Dissolve 5.0 mg of *hyoscyne hydrobromide impurity B CRS* in the mobile phase, add 5.0 ml of the test solution and dilute to 50.0 ml with the mobile phase. Dilute 1.0 ml of this solution to 10.0 ml with the mobile phase.

Column:

- size: $l = 0.125$ m, $\varnothing = 4.0$ mm,
- stationary phase: octylsilyl silica gel for chromatography R (3 μ m),
- temperature: 25 ± 1 °C.

Mobile phase: mix 330 ml of acetonitrile R with 670 ml of a 2.5 g/l solution of sodium dodecyl sulphate R previously adjusted to pH 2.5 with 3 M phosphoric acid.

Flow rate: 1.5 ml/min.

Detection: spectrophotometer at 210 nm.

Injection: 5 μ l.

Run time: 3 times the retention time of hyoscyne.

Relative retention with reference to hyoscyne (retention time = about 5.0 min): impurity D = about 0.2; impurity B = about 0.9; impurity A = about 1.3; impurity C = about 2.4.

System suitability: reference solution (c):

- resolution: minimum 1.5 between the peaks due to impurity B and hyoscyne,
- symmetry factor: maximum 2.5 for the peak due to hyoscyne.

Limits:

- correction factors: for the calculation of contents, multiply the peak areas of the following impurities by the corresponding correction factor: impurity D = 0.3; impurity C = 0.6;
- impurity B: not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent);
- impurities A, C, D: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent);
- any other impurity: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent);
- total: not more than 1.4 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.7 per cent); disregard any peak due to the bromide ion which appears close to the solvent peak;
- disregard limit: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

Water (2.5.12): 10.0 per cent to 13.0 per cent, determined on 0.20 g.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.300 g in a mixture of 5.0 ml of 0.01 M hydrochloric acid and 50 ml of ethanol (96 per cent) R. Carry out a potentiometric titration (2.2.20), using 0.1 M sodium hydroxide free from carbonate. Read the volume added between the 2 points of inflexion.

1 ml of 0.1 M sodium hydroxide is equivalent to 38.43 mg of $C_{17}H_{22}BrNO_4$.

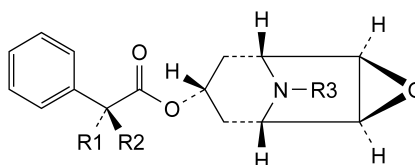
STORAGE

In a well-filled, airtight container of small capacity, protected from light.

IMPURITIES

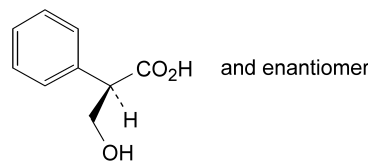
Specified impurities: A, B, C, D.

A. hyoscyamine,



B. R1 = CH_2OH , R2 = R3 = H: (1R,2R,4S,5S,7S)-3-oxa-9-azatricyclo[3.3.1.0^{2,4}]non-7-yl (2S)-3-hydroxy-2-phenylpropanoate (norhyoscyne),

C. R1 + R2 = CH_2 , R3 = CH_3 : (1R,2R,4S,5S,7S)-9-methyl-3-oxa-9-azatricyclo[3.3.1.0^{2,4}]non-7-yl 2-phenylprop-2-enoate (apohyoscyne),

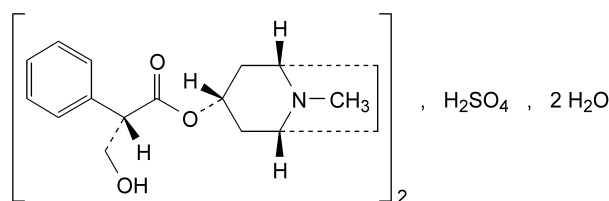


D. (2RS)-3-hydroxy-2-phenylpropanoic acid (DL-tropic acid).

01/2005:0501

HYOSCYAMINE SULPHATE

Hyoscyamini sulfas



$C_{34}H_{48}N_2O_{10}S \cdot 2H_2O$

M_r 713

DEFINITION

Bis[(1R,3r,5S)-8-methyl-8-azabicyclo[3.2.1]oct-3-yl (2S)-3-hydroxy-2-phenylpropanoate] sulphate dihydrate.

Content: 98.0 per cent to 101.0 per cent (anhydrous substance).

CHARACTERS

Appearance: white or almost white, crystalline powder or colourless needles.

Solubility: very soluble in water, sparingly soluble or soluble in ethanol (96 per cent).

IDENTIFICATION

First identification: A, B, E.

Second identification: C, D, E.

A. It complies with the test for specific optical rotation (see Tests).

B. Infrared absorption spectrophotometry (2.2.24).

Comparison: *hyoscyamine sulphate CRS*.

C. To 0.5 ml of solution S (see Tests) add 2 ml of *dilute acetic acid R* and heat. To the hot solution add 4 ml of *picric acid solution R*. Allow to cool, shaking occasionally. Collect the crystals, wash with 2 quantities, each of 3 ml, of iced *water R* and dry at 100–105 °C. The crystals melt (2.2.14) at 164 °C to 168 °C.

D. To about 1 mg add 0.2 ml of *fuming nitric acid R* and evaporate to dryness on a water-bath. Dissolve the residue in 2 ml of *acetone R* and add 0.2 ml of a 30 g/l solution of *potassium hydroxide R* in *methanol R*. A violet colour develops.

E. It gives reaction (a) of sulphates (2.3.1).

TESTS

Solution S. Dissolve 2.50 g in *water R* and dilute to 50.0 ml with the same solvent.

Appearance of solution. Solution S is not more intensely coloured than reference solution BY₆ (2.2.2, *Method II*).

pH (2.2.3): 4.5 to 6.2.

Dissolve 0.5 g in *carbon dioxide-free water R* and dilute to 25 ml with the same solvent.

Specific optical rotation (2.2.7): –24 to –29 (anhydrous substance), determined on solution S.

Related substances. Liquid chromatography (2.2.29).

Test solution. Dissolve 60.0 mg of the substance to be examined in mobile phase A and dilute to 50.0 ml with mobile phase A. Dilute 10.0 ml of the solution to 50.0 ml with mobile phase A.

Reference solution (a). Dilute 5.0 ml of the test solution to 100.0 ml with mobile phase A. Dilute 5.0 ml of this solution to 50.0 ml with mobile phase A.

Reference solution (b). Dilute 5.0 ml of reference solution (a) to 25.0 ml with mobile phase A.

Reference solution (c). Dissolve 5.0 mg of *hyoscyamine impurity E CRS* in mobile phase A, add 1.0 ml of the test solution and dilute to 20.0 ml with mobile phase A. Dilute 5.0 ml of this solution to 25.0 ml with mobile phase A.

Column:

- size: $l = 0.10$ m, $\varnothing = 4.6$ mm,
- stationary phase: *octadecylsilyl silica gel for chromatography R* (3 μ m),
- temperature: 25 ± 1 °C.

Mobile phase:

- mobile phase A: dissolve 3.5 g of *sodium dodecyl sulphate R* in 606 ml of a 7.0 g/l solution of *potassium dihydrogen phosphate R* previously adjusted to pH 3.3 with 0.05 M *phosphoric acid* and mix with 320 ml of *acetonitrile R*,

– mobile phase B: *acetonitrile R*,

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)
0 - 2.0	95	5
2.0 - 20.0	95 → 70	5 → 30
20.0 - 20.1	70 → 95	30 → 5
20.1 - 25.0	95	5

Flow rate: 1.0 ml/min.

Detection: spectrophotometer at 210 nm.

Injection: 10 μ l.

Relative retention with reference to hyoscyamine (retention time = about 10.5 min): impurity A = about 0.2; impurity B = about 0.67; impurity C = about 0.72; impurity D = about 0.8; impurity E = about 0.9; impurity F = about 1.1; impurity G = about 1.8.

System suitability: reference solution (c):

- resolution: minimum 2.5 between the peaks due to hyoscyamine and impurity E,
- symmetry factor: maximum 2.5 for the peak due to hyoscyamine.

Limits:

- correction factors: for the calculation of contents, multiply the peak areas of the following impurities by the corresponding correction factor: impurity A = 0.3; impurity G = 0.6;
- impurity E: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.3 per cent);
- impurities A, B, C, D, F, G: for each impurity, not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent);
- any other impurity: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent);
- total: not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent);
- disregard limit: 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

Water (2.5.12): 2.0 per cent to 5.5 per cent, determined on 0.500 g.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.500 g in 25 ml of *anhydrous acetic acid R*. Titrate with 0.1 M *perchloric acid*, determining the end-point potentiometrically (2.2.20).

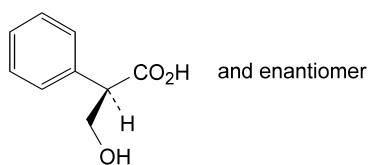
1 ml of 0.1 M *perchloric acid* is equivalent to 67.7 mg of C₃₄H₄₈N₂O₁₀S.

STORAGE

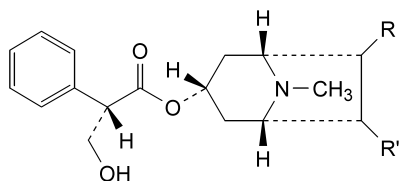
In an airtight container, protected from light.

IMPURITIES

Specified impurities: A, B, C, D, E, F, G.

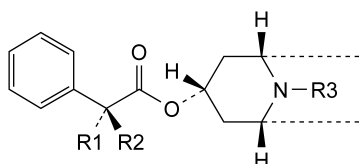


- A. (2*RS*)-3-hydroxy-2-phenylpropanoic acid (DL-tropic acid),

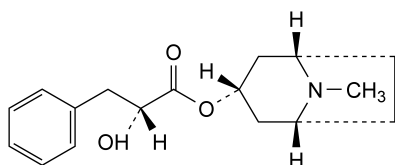


- B. R = OH, R' = H: (1*R*,3*S*,5*R*,6*RS*)-6-hydroxy-8-methyl-8-azabicyclo[3.2.1]oct-3-yl (2*S*)-3-hydroxy-2-phenylpropanoate (7-hydroxyhyoscyamine),
C. R = H, R' = OH: (1*S*,3*R*,5*S*,6*RS*)-6-hydroxy-8-methyl-8-azabicyclo[3.2.1]oct-3-yl (2*S*)-3-hydroxy-2-phenylpropanoate (6-hydroxyhyoscyamine),

- D. hyoscyine,



- E. R1 = CH₂OH, R2 = R3 = H: (1*R*,3*r*,5*S*)-8-azabicyclo[3.2.1]oct-3-yl (2*S*)-3-hydroxy-2-phenylpropanoate (norhyoscyamine),
G. R1 + R2 = CH₂, R3 = CH₃: (1*R*,3*r*,5*S*)-8-methyl-8-azabicyclo[3.2.1]oct-3-yl 2-phenylprop-2-enoate (apoatropine),



- F. (1*R*,3*r*,5*S*)-8-methyl-8-azabicyclo[3.2.1]oct-3-yl (2*R*)-2-hydroxy-3-phenylpropanoate (littorine).

- B. To 10 ml of solution S add 0.3 ml of *dilute acetic acid R* and 2.5 ml of a 100 g/l solution of *tannic acid R*. A yellowish-white, flocculent precipitate is formed which dissolves in *dilute ammonia R1*.
C. In a test-tube about 160 mm long, thoroughly mix 1 g with 2 g of finely powdered *manganese sulphate R*. Introduce to a depth of 2 cm into the upper part of the tube a strip of filter paper impregnated with a freshly prepared mixture of 1 volume of a 20 per cent V/V solution of *diethanolamine R* and 11 volumes of a 50 g/l solution of *sodium nitroprusside R*, adjusted to about pH 9.8 with 1 M *hydrochloric acid*. Insert the tube 8 cm into a silicone-oil bath at 190 °C to 200 °C. The filter paper becomes blue within 10 min. Carry out a blank test.
D. Dissolve 0.2 g completely, without heating, in 15 ml of a 70 per cent *m/m* solution of *sulphuric acid R*. Pour the solution with stirring into 100 ml of iced *water R* and dilute to 250 ml with iced *water R*. In a test-tube, mix thoroughly while cooling in iced *water R* 1 ml of the solution with 8 ml of *sulphuric acid R*, added dropwise. Heat in a water-bath for exactly 3 min and immediately cool in iced *water*. While the mixture is cold, carefully add 0.6 ml of *ninhydrin solution R2* and mix well. Allow to stand at 25 °C. A pink colour is produced immediately and becomes violet within 100 min.
E. Place 1 ml of solution S on a glass plate. After evaporation of the *water* a thin film is formed.
F. 0.2 g does not dissolve in 10 ml of *toluene R* nor in 10 ml of *ethanol R*.

TESTS

Solution S. While stirring, introduce a quantity of the substance to be examined equivalent to 1.0 g of the dried substance into 50 g of *carbon dioxide-free water R* heated to 90 °C. Allow to cool, adjust the mass of the solution to 100 g with *carbon dioxide-free water R* and stir until dissolution is complete.

Appearance of solution. Solution S is not more opalescent than reference suspension III (2.2.1) and not more intensely coloured than reference solution Y₆ (2.2.2, *Method II*).

pH (2.2.3). The pH of solution S is 5.5 to 8.0.

Apparent viscosity. While stirring, introduce a quantity of the substance to be examined equivalent to 6.00 g of the dried substance into 150 g of *water R* heated to 90 °C. Stir with a propeller-type stirrer for 10 min, place the flask in a bath of iced *water*, continue the stirring and allow to remain in the bath of iced *water* for 40 min to ensure that dissolution is complete. Adjust the mass of the solution to 300 g, and centrifuge the solution to expel any entrapped air. Adjust the temperature of the solution to 20 ± 0.1 °C. Determine the viscosity (2.2.10) with a rotating viscometer at 20 °C and a shear rate of 10 s⁻¹. The apparent viscosity is not less than 75 per cent and not more than 140 per cent of the value stated on the label.

Chlorides (2.4.4). 1 ml of solution S diluted to 15 ml with *water R* complies with the limit test for chlorides (0.5 per cent).

Heavy metals (2.4.8). 1.0 g complies with limit test C for heavy metals (20 ppm). Prepare the standard using 2 ml of *lead standard solution (10 ppm Pb) R*.

Loss on drying (2.2.32). Not more than 10.0 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C.

Sulphated ash (2.4.14). Not more than 1.0 per cent, determined on 1.0 g.

01/2005:0348

HYPROMELLOSE

Hypromellosum

DEFINITION

Hypromellose (hydroxypropylmethylcellulose) is a partly *O*-methylated and *O*-(2-hydroxypropylated) cellulose.

CHARACTERS

A white, yellowish-white or greyish-white powder or granules, hygroscopic after drying, practically insoluble in hot *water*, in acetone, in ethanol and in toluene. It dissolves in cold *water* giving a colloidal solution.

IDENTIFICATION

- A. Heat 10 ml of solution S (see Tests) in a water-bath while stirring. At a temperature above 50 °C the solution becomes cloudy or a flocculent precipitate is formed. The solution becomes clear again on cooling.

LABELLING

The label states the apparent viscosity in millipascal seconds for a 2 per cent *m/m* solution.

01/2005:0347

HYPROMELLOSE PHTHALATE

Hypromellosi phthalas

DEFINITION

Hypromellose phthalate (hydroxypropylmethylcellulose phthalate) is a monophthalic acid ester of hypromellose. It contains methoxy ($-\text{OCH}_3$) and 2-hydroxypropoxy ($-\text{OCH}_2\text{CHOHCH}_3$) groups and not less than 21.0 per cent and not more than 35.0 per cent of phthaloyl (*o*-carboxybenzoyl $\text{C}_8\text{H}_5\text{O}_3$) groups, calculated with reference to the anhydrous substance.

CHARACTERS

White or slightly off-white, free-flowing flakes or a granular powder, practically insoluble in water, soluble in a mixture of equal volumes of acetone and methanol and in a mixture of equal volumes of methanol and methylene chloride, very slightly soluble in acetone and in toluene, practically insoluble in ethanol.

IDENTIFICATION

- Examine by infrared absorption spectrophotometry (2.2.24), comparing with the *Ph. Eur. reference spectrum of hypromellose phthalate*.
- Dissolve about 40 mg in 1 ml of a mixture of equal volumes of *acetone R* and of *methanol R*. Allow the solution to flow over a glass plate and dry. A colourless, transparent film is formed.

TESTS

Free phthalic acid. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 0.20 g of the substance to be examined in about 50 ml of *acetonitrile R* with the aid of ultrasound. Add 10 ml of *water R*, cool to room temperature and dilute to 100.0 ml with *acetonitrile R*.

Reference solution. Dissolve 5.0 mg of *phthalic acid R* in 125 ml of *acetonitrile R*. Add 25 ml of *water R* and dilute to 250.0 ml with *acetonitrile R*.

The chromatographic procedure may be carried out using:

- a column 0.25 m long and 4.6 mm in internal diameter packed with octadecylsilane chemically bound to porous silica or ceramic micro-particles (5 μm to 10 μm),
- as mobile phase at a flow rate of 2 ml/min a mixture of 15 volumes of *acetonitrile R* and 85 volumes of an 8.5 g/l solution of *cyanoacetic acid R*,
- as detector a spectrophotometer set at 235 nm.

Inject 20 μl of the reference solution. Adjust the sensitivity of the system so that the height of the principal peak, apart from the peaks due to the solvent, is at least 50 per cent of the full scale of the recorder.

Inject 20 μl of the test solution. In the chromatogram obtained with the test solution, the area of any peak corresponding to phthalic acid is not greater than the area of the principal peak in the chromatogram obtained with the reference solution (1 per cent).

Chlorides. Dissolve 1.0 g in 40.0 ml of 0.2 *M sodium hydroxide*, add 0.05 ml of *phenolphthalein solution R* and dilute *nitric acid R* dropwise and with stirring until the red colour disappears. Add an additional 20.0 ml of *dilute nitric acid R* with stirring. Heat on a water-bath with stirring until the gel-like precipitate formed becomes granular. Cool and centrifuge. Separate the liquid phase and wash the residue with three quantities, each of 20 ml, of *water R*, separating the washings by centrifugation. Combine the liquid phases, filter, add 5.0 ml of 0.1 *M silver nitrate*, dilute to 200.0 ml with *water R* and mix. 50.0 ml of the solution is not more opalescent than a standard prepared by treating 0.5 ml of 0.01 *M hydrochloric acid* with 10.0 ml of 0.2 *M sodium hydroxide*, adding 7 ml of *dilute nitric acid R*, 5.0 ml of 0.1 *M silver nitrate* and diluting to 50.0 ml with *water R* (0.07 per cent).

Heavy metals (2.4.8). 2.0 g complies with limit test C for heavy metals (10 ppm). Prepare the standard using 2 ml of *lead standard solution (10 ppm Pb) R*.

Sulphated ash (2.4.14). Not more than 0.2 per cent, determined on 1.0 g.

Water (2.5.12). Not more than 5.0 per cent, determined on 0.500 g by the semi-micro determination of water using 50 ml of *anhydrous methanol R* as the solvent.

ASSAY

Dissolve 1.000 g in 50 ml of a mixture of 1 volume of *water R*, 2 volumes of *acetone R* and 2 volumes of *alcohol R*. Add 0.1 ml of *phenolphthalein solution R* and titrate with 0.1 *M sodium hydroxide* until a faint pink colour is obtained. Carry out a blank titration.

Calculate the percentage content of phthaloyl groups from the expression:

$$\frac{149n}{(100 - a)m} - 1.795S$$

a = percentage content of water,

m = mass of the substance to be examined, in grams,

n = number of millilitres of 0.1 *M sodium hydroxide* used,

S = percentage content of free phthalic acid (see Tests).

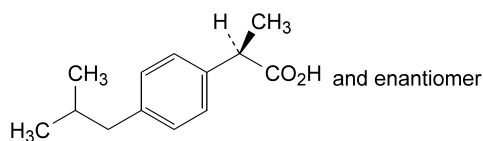
STORAGE

Store in an airtight container.

01/2005:0721 TESTS

IBUPROFEN

Ibuprofenum

 $C_{13}H_{18}O_2$ M_r 206.3

DEFINITION

(2*RS*)-2-[4-(2-Methylpropyl)phenyl]propanoic acid.*Content*: 98.5 per cent to 101.0 per cent (dried substance).

CHARACTERS

Appearance: white, crystalline powder or colourless crystals.*Solubility*: practically insoluble in water, freely soluble in acetone, in methanol and in methylene chloride. It dissolves in dilute solutions of alkali hydroxides and carbonates.

IDENTIFICATION

First identification: A, C.*Second identification*: A, B, D.

A. Melting point (2.2.14): 75 °C to 78 °C.

B. Dissolve 50.0 mg in a 4 g/l solution of *sodium hydroxide R* and dilute to 100.0 ml with the same alkaline solution. Examined between 240 nm and 300 nm (2.2.25), using a spectrophotometer with a band width of 1.0 nm and a scan speed of not more than 50 nm/min, the solution shows a shoulder at 258 nm and 2 absorption maxima, at 264 nm and 272 nm. The ratio of the absorbance measured at the maximum at 264 nm to that measured at the shoulder at 258 nm is 1.20 to 1.30. The ratio of the absorbance measured at the maximum at 272 nm to that measured at the shoulder at 258 nm is 1.00 to 1.10.

C. Infrared absorption spectrophotometry (2.2.24).

Preparation: discs.*Comparison*: *ibuprofen CRS*.

D. Thin-layer chromatography (2.2.27).

Test solution. Dissolve 50 mg of the substance to be examined in *methylene chloride R* and dilute to 10 ml with the same solvent.*Reference solution*. Dissolve 50 mg of *ibuprofen CRS* in *methylene chloride R* and dilute to 10 ml with the same solvent.*Plate*: TLC silica gel plate *R*.*Mobile phase*: *anhydrous acetic acid R*, *ethyl acetate R*, *hexane R* (5:24:71 V/V/V).*Application*: 5 µl.*Development*: over a path of 10 cm.*Drying*: at 120 °C for 30 min.*Detection*: lightly spray with a 10 g/l solution of *potassium permanganate R* in *dilute sulphuric acid R* and heat at 120 °C for 20 min. Examine in ultraviolet light at 365 nm.*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.**Solution S**. Dissolve 2.0 g in *methanol R* and dilute to 20 ml with the same solvent.**Appearance of solution**. Solution S is clear (2.2.1) and colourless (2.2.2, *Method II*).**Angle of optical rotation** (2.2.7): -0.05° to $+0.05^\circ$.Dissolve 0.50 g in *methanol R* and dilute to 20.0 ml with the same solvent.**Related substances**. Liquid chromatography (2.2.29).*Test solution*. Dissolve 20 mg of the substance to be examined in 2 ml of *acetonitrile R* and dilute to 10.0 ml with mobile phase A.*Reference solution (a)*. Dilute 1.0 ml of the test solution to 100.0 ml with mobile phase A.*Reference solution (b)*. Dissolve 20 mg of *ibuprofen CRS* in 2 ml of *acetonitrile R*, add 1.0 ml of a 0.06 g/l solution of *ibuprofen impurity B CRS* in *acetonitrile R* and dilute to 10.0 ml with mobile phase A.*Column*:

- *size*: $l = 0.15$ m, $\varnothing = 4.6$ mm,
- *stationary phase*: octadecylsilyl silica gel for chromatography *R* (5 µm).

Mobile phase:

- *mobile phase A*: mix 0.5 volumes of *phosphoric acid R*, 340 volumes of *acetonitrile R* and 600 volumes of *water R*; allow to equilibrate and dilute to 1000 volumes with *water R*,
- *mobile phase B*: *acetonitrile R*,

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)
0 - 25	100	0
25 - 55	100 → 15	0 → 85
55 - 70	15	85
70 - 75	15 → 100	85 → 0

Flow rate: 2 ml/min.*Detection*: spectrophotometer at 214 nm.

Equilibration: for about 45 min with mobile phase A.

Injection: 20 µl.*System suitability*: reference solution (b):

- *peak-to-valley ratio*: minimum of 1.5, where H_p = height above the baseline of the peak due to impurity B, and H_v = height above the baseline of the lowest point of the curve separating this peak from the peak due to ibuprofen. If necessary, adjust the concentration of acetonitrile in mobile phase A.

Limits:

- *impurity B*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (b) (0.3 per cent),
- *any other impurity*: not more than 0.3 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.3 per cent),
- *total of all impurities apart from impurity B*: not more than 0.7 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.7 per cent),
- *disregard limit*: 0.05 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

Impurity F. Gas chromatography (2.2.28): use the normalisation procedure.

Methylating solution. Dilute 1 ml of *N,N*-dimethylformamide dimethyl acetal *R* and 1 ml of pyridine *R* to 10 ml with ethyl acetate *R*.

Test solution. Weigh about 50.0 mg of the substance to be examined into a sealable vial, dissolve in 1.0 ml of ethyl acetate *R*, add 1 ml of methylating solution, seal and heat at 100 °C in a block heater for 20 min. Allow to cool. Remove the reagents under a stream of nitrogen at room temperature. Dissolve the residue in 5 ml of ethyl acetate *R*.

Reference solution (a). Dissolve 0.5 mg of ibuprofen impurity *F* CRS in ethyl acetate *R* and dilute to 10.0 ml with the same solvent.

Reference solution (b). Weigh about 50.0 mg of ibuprofen CRS into a sealable vial, dissolve in 1.0 ml of reference solution (a), add 1 ml of methylating solution, seal and heat at 100 °C in a block heater for 20 min. Allow to cool. Remove the reagents under a stream of nitrogen at room temperature. Dissolve the residue in 5 ml of ethyl acetate *R*.

Column:

- **material:** fused-silica,
- **size:** $l = 25$ m, $\varnothing = 0.53$ mm,
- **stationary phase:** macrogol 20 000 *R* (film thickness 2 μ m).

Carrier gas: helium for chromatography *R*.

Flow rate: 5.0 ml/min.

Temperature:

- **column:** 150 °C,
- **injection port:** 200 °C,
- **detector:** 250 °C.

Detection: flame-ionisation.

Injection: 1 μ l; inject the test solution and reference solution (b).

Run time: twice the retention time of ibuprofen.

System suitability:

- **relative retention** with reference to ibuprofen (retention time = about 17 min): impurity *F* = about 1.5.

Limit:

- **impurity *F*:** maximum 0.1 per cent.

Heavy metals (2.4.8): maximum 10 ppm.

12 ml of solution *S* complies with limit test *B*. Prepare the standard using lead standard solution (1 ppm Pb) prepared by diluting lead standard solution (100 ppm Pb) *R* with methanol *R*.

Loss on drying (2.2.32): maximum 0.5 per cent, determined on 1.000 g by drying *in vacuo* over diphosphorus pentoxide *R*.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

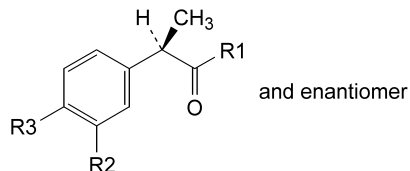
Dissolve 0.450 g in 50 ml of methanol *R*. Add 0.4 ml of phenolphthalein solution *R1*. Titrate with 0.1 *M* sodium hydroxide until a red colour is obtained. Carry out a blank titration.

1 ml of 0.1 *M* sodium hydroxide is equivalent to 20.63 mg of $C_{13}H_{18}O_2$.

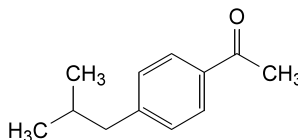
IMPURITIES

Specified impurities: *A*, *B*, *C*, *D*, *E*.

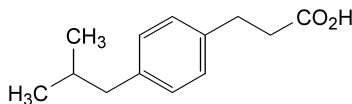
Other detectable impurities: *F*, *G*, *H*, *I*, *J*, *K*, *L*, *M*, *N*, *O*, *P*, *Q*, *R*.



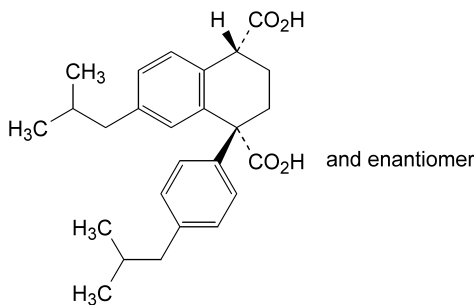
- A.** $R1 = OH$, $R2 = CH_2-CH(CH_3)_2$, $R3 = H$: (2*RS*)-2-[3-(2-methylpropyl)phenyl]propanoic acid,
- B.** $R1 = OH$, $R2 = H$, $R3 = [CH_2]_3-CH_3$: (2*RS*)-2-(4-butylphenyl)propanoic acid,
- C.** $R1 = NH_2$, $R2 = H$, $R3 = CH_2-CH(CH_3)_2$: (2*RS*)-2-[4-(2-methylpropyl)phenyl]propanamide,
- D.** $R1 = OH$, $R2 = H$, $R3 = CH_3$: (2*RS*)-2-(4-methylphenyl)propanoic acid,



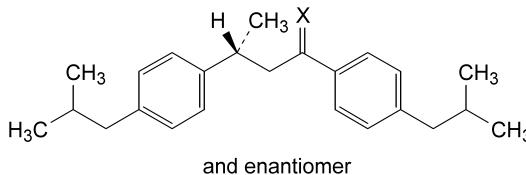
- E.** 1-[4-(2-methylpropyl)phenyl]ethanone,



- F.** 3-[4-(2-methylpropyl)phenyl]propanoic acid,

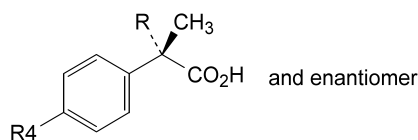


- G.** *cis*-7-(2-methylpropyl)-1-[4-(2-methylpropyl)phenyl]-1,2,3,4-tetrahydronaphthalene-1,4-dicarboxylic acid,

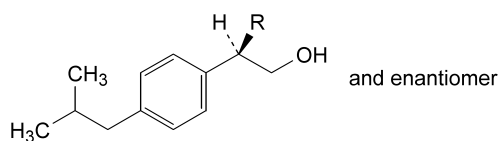


- H.** $X = O$: (3*RS*)-1,3-bis[4-(2-methylpropyl)phenyl]butan-1-one,

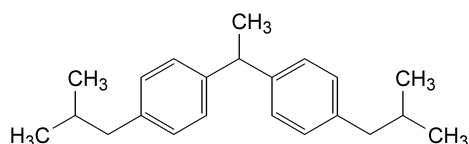
- I.** $X = H_2$: (3*RS*)-1,3-bis[4-(2-methylpropyl)phenyl]butane,



- J. R = H, R4 = CO-CH(CH₃)₂: (2*RS*)-2-[4-(2-methylpropanoyl)phenyl]propanoic acid,
 K. R = H, R4 = CHO: (2*RS*)-2-(4-formylphenyl)propanoic acid,
 L. R = H, R4 = CHOH-CH(CH₃)₂: 2-[4-(1-hydroxy-2-methylpropyl)phenyl]propanoic acid,
 M. R = OH, R4 = CH₂-CH(CH₃)₂: (2*RS*)-2-hydroxy-2-[4-(2-methylpropyl)phenyl]propanoic acid,
 N. R = H, R4 = C₂H₅: (2*RS*)-2-(4-ethylphenyl)propanoic acid,
 O. R = H, R4 = CH(CH₃)-C₂H₅: 2-[4-(1-methylpropyl)phenyl]propanoic acid,



- P. R = CH₃: (2*RS*)-2-[4-(2-methylpropyl)phenyl]propan-1-ol,
 Q. R = H: 2-[4-(2-methylpropyl)phenyl]ethanol,



- R. 1,1-bis[4-(2-methylpropyl)phenyl]ethane.

01/2005:1439

ICELAND MOSS

Lichen islandicus

DEFINITION

Iceland moss consists of the whole or cut dried thallus of *Cetraria islandica* (L.) Acharius s.l.

CHARACTERS

Iceland moss has a bitter, mucilaginous taste.

It has the macroscopic and microscopic characters described under identification tests A and B.

IDENTIFICATION

- A. The thallus, up to 15 cm long, is irregularly dichotomous and consists of glabrous, groove-shaped or almost flat, stiff, brittle bands, 0.3 cm to 1.5 cm wide and about 0.5 mm thick, sometimes serrated with the margin appearing ciliated (pseudopodia). The upper surface is greenish to greenish-brown, the lower surface is greyish-white to light brownish and shows whitish, depressed spots (so-called respiratory cavities). On the apexes of the terminal lobes, very rarely, there are brown, discoid apothecia.
- B. Reduce to a powder (355). The powder is greyish-brown. Examine under a microscope, using *chloral hydrate solution R*. The powder shows numerous fragments of the pseudoparenchyma consisting of narrow-lumened, thick-walled hyphae from the marginal layer and

wide-lumened hyphae from the adjacent layer consisting of loosely entwined hyphae, in which, in the medullary zone, greenish to brownish algae cells up to 15 µm in diameter, are embedded; occasionally marginal fragments of the thallus with tube-like or cylindrical spermatogonia, up to about 160 µm wide and up to about 400 µm long.

- C. To 1.0 g of the powdered drug (355) add 10 ml of *water R* and boil for 2 min to 3 min. The greyish-brown solution forms a gel after cooling.
- D. Examine the chromatograms obtained in the test for other lichen species. The chromatogram obtained with the test solution shows the violet to grey zone of fumaroprotocetraric acid located slightly below the zone of caffeic acid in the chromatogram obtained with the reference solution. Other fainter zones are present.

TESTS

Foreign matter (2.8.2). Not more than 5 per cent.

Other lichen species. Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel plate R*.

Test solution. To 1.0 g of the powdered drug (355) add 5 ml of *acetone R* and heat in a water-bath under a reflux condenser for 2 min to 3 min. Cool and filter.

Reference solution. Dissolve 5 mg of *anethole R* and 5 mg of *caffeic acid R* in 2 ml of *acetone R*.

Apply to the plate as bands 20 µl of the test solution and 10 µl of the reference solution. Develop over a path of 10 cm, using a mixture of 5 volumes of *acetone R*, 5 volumes of *methanol R*, 10 volumes of *glacial acetic acid R* and 80 volumes of *toluene R*. Allow the plate to dry in air and spray the plate with *anisaldehyde solution R*. Examine in daylight while heating at 100 °C to 105 °C for 5 min to 10 min. The chromatogram obtained with the reference solution shows in the lower half a greyish-blue to violet-blue zone (caffeic acid) and in the upper half a blue to blue-violet zone (anethole). The chromatogram obtained with the test solution shows no red-violet zone a little below the zone of anethole in the chromatogram obtained with the reference solution.

Loss on drying (2.2.32). Not more than 12.0 per cent, determined on 1.000 g of powdered drug (355) by drying in an oven at 100 °C to 105 °C for 2 h.

Total ash (2.4.16). Not more than 3.0 per cent.

Swelling value (2.8.4). Not less than 4.5, determined on the powdered drug (355).

STORAGE

Store in an airtight container, protected from light.

01/2005:0917

ICHTHAMMOL

Ichthammolum

DEFINITION

Ichthammol is obtained by distillation from certain bituminous schists, sulphonation of the distillate and neutralisation of the product with ammonia. It contains not less than 50.0 per cent *m/m* and not more than 56.0 per cent *m/m* of dry matter, not less than 4.5 per cent *m/m* and not more than 7.0 per cent *m/m* of total ammonia (NH₃; *M_r* 17.03) and not less than 10.5 per cent *m/m* of organically combined sulphur, calculated with reference to the dried substance; not more than 20.0 per cent *m/m* of the total sulphur is in the form of sulphate.

CHARACTERS

A dense, blackish-brown liquid, miscible with water and with glycerol, slightly soluble in alcohol, in fatty oils and in liquid paraffin. It forms homogeneous mixtures with wool fat and soft paraffin.

IDENTIFICATION

- Dissolve 1.5 g in 15 ml of *water R* (solution A). To 2 ml of solution A add 2 ml of *hydrochloric acid R*. A resinous precipitate is formed. Decant the supernatant liquid. The precipitate is partly soluble in *ether R*.
- 2 ml of solution A, obtained in identification test A, gives the reaction of ammonium salts and salts of volatile bases (2.3.1).
- Evaporate and ignite the mixture of solution A and *dilute sodium hydroxide solution R* obtained in identification test B. Take up the residue with 5 ml of *dilute hydrochloric acid R*. Gas is evolved which turns *lead acetate paper R* brown or black. Filter the solution. The filtrate gives reaction (a) of sulphates (2.3.1).

TESTS

Acidity or alkalinity. To 10.0 ml of the clear filtrate obtained in the assay of total ammonia add 0.05 ml of *methyl red solution R*. Not more than 0.2 ml of 0.02 M *hydrochloric acid* or 0.02 M *sodium hydroxide* is required to change the colour of the indicator.

Relative density (2.2.5): 1.040 to 1.085, determined on a mixture of equal volumes of the substance to be examined and *water R*.

Sulphated ash (2.4.14). Not more than 0.3 per cent, determined on 1.00 g.

ASSAY

Dry matter. Weigh 1.000 g in a tared flask containing 2 g of *sand R*, previously dried to constant mass, and a small glass rod. Heat on a water-bath for 2 h with frequent stirring and dry in an oven at 100 °C to 105 °C until two consecutive weighings do not differ by more than 2.0 mg; the second weighing is carried out after drying again for 1 h.

Total ammonia. Dissolve 2.50 g in 25 ml of warm *water R*. Rinse the solution into a 250 ml volumetric flask, add 200 ml of *sodium chloride solution R* and dilute to 250.0 ml with *water R*. Filter the solution, discarding the first 20 ml of filtrate. To 100.0 ml of the clear filtrate add 25 ml of *formaldehyde solution R*, neutralised to *phenolphthalein solution R1*. Titrate with 0.1 M *sodium hydroxide* until a faint pink colour is obtained.

1 ml of 0.1 M *sodium hydroxide* is equivalent to 1.703 mg of NH_3 .

Organically combined sulphur. Mix 0.500 g with 4 g of *anhydrous sodium carbonate R* and 3 ml of *methylene chloride R* in a porcelain crucible of about 50 ml capacity, warm and stir until all the methylene chloride has evaporated. Add 10 g of coarsely powdered *copper nitrate R*, mix thoroughly and heat the mixture very gently using a small flame. When the initial reaction has subsided, increase the temperature slightly until most of the material has blackened. Cool, place the crucible in a large beaker, add 20 ml of *hydrochloric acid R* and, when the reaction has ceased, add 100 ml of *water R* and boil until all the copper oxide has dissolved. Filter the solution, add 400 ml of *water R*, heat to boiling and add 20 ml of *barium chloride solution R1*. Allow to stand for 2 h, filter, wash with *water R*, dry and ignite at about 600 °C until two successive weighings do not differ by more than 0.2 per cent of the mass of the residue.

1 g of residue is equivalent to 0.1374 g of total sulphur.

Calculate the percentage of sulphur and subtract the percentage of sulphur in the form of sulphate.

Sulphur in the form of sulphate. Dissolve 2.000 g in 100 ml of *water R*, add 2 g of *cupric chloride R* dissolved in 80 ml of *water R* and dilute to 200.0 ml with *water R*. Shake and filter. Heat 100.0 ml of the filtrate almost to boiling, add 1 ml of *hydrochloric acid R* and 5 ml of *barium chloride solution R1* dropwise and heat on a water-bath. Filter, wash the precipitate with *water R*, dry and ignite at about 600 °C until two successive weighings do not differ by more than 0.2 per cent of the mass of the residue.

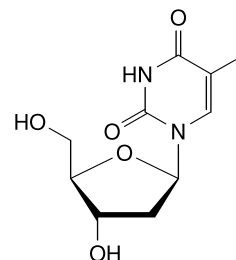
1 g of residue is equivalent to 0.1374 g of sulphur present in the form of sulphate.

Calculate the percentage of sulphur in the form of sulphate.

01/2005:0669

IDOXURIDINE

Idoxuridinum


 $\text{C}_9\text{H}_{11}\text{IN}_2\text{O}_5$
 M_r 354.1

DEFINITION

Idoxuridine contains not less than 98.0 per cent and not more than the equivalent of 101.0 per cent of 5-iodo-1-(2-deoxy-β-D-erythro-pentofuranosyl)pyrimidine-2,4(1*H*,3*H*)-dione, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, crystalline powder, slightly soluble in water and in alcohol. It dissolves in dilute solutions of alkali hydroxides.

It melts at about 180 °C, with decomposition.

IDENTIFICATION

First identification: A.

Second identification: B, C, D.

- Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *idoxuridine CRS*. Examine the substances as discs prepared using 1 mg of the substance to be examined and of the reference substance each in 0.3 g of *potassium bromide R*.
- Examine the chromatograms obtained in the test for related substances. The principal spot in the chromatogram obtained with test solution (b) is similar in position and size to the principal spot in the chromatogram obtained with reference solution (c).
- Heat about 5 mg in a test-tube over a naked flame. Violet vapour is evolved.
- Disperse about 2 mg in 1 ml of *water R* and add 2 ml of *diphenylamine solution R2*. Heat in a water-bath for 10 min. A persistent light-blue colour develops.

TESTS

Solution S. Dissolve 0.500 g in 1 M *sodium hydroxide* and dilute to 50.0 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and colourless (2.2.2, Method II).

pH (2.2.3). Dissolve 0.10 g in *carbon dioxide-free water R* and dilute to 100 ml with the same solvent. The pH of the solution is 5.5 to 6.5.

Specific optical rotation (2.2.7): + 28 to + 32, determined on solution S and calculated with reference to the dried substance.

Related substances. Examine by thin-layer chromatography (2.2.27), using as coating substance a suitable silica gel with a fluorescent indicator having an optimal intensity at 254 nm.

Test solution (a). Dissolve 0.20 g of the substance to be examined in a mixture of 1 volume of *concentrated ammonia R* and 5 volumes of *methanol R* and dilute to 5 ml with the same mixture of solvents.

Test solution (b). Dilute 1 ml of test solution (a) to 10 ml with a mixture of 1 volume of *concentrated ammonia R* and 5 volumes of *methanol R*.

Reference solution (a). Dissolve 20 mg of 5-iodouracil *R*, 20 mg of 2'-deoxyuridine *R* and 20 mg of 5-bromo-2'-deoxyuridine *R* in a mixture of 1 volume of *concentrated ammonia R* and 5 volumes of *methanol R* and dilute to 100 ml with the same mixture of solvents.

Reference solution (b). Dissolve 0.20 g of the substance to be examined in 5 ml of reference solution (a).

Reference solution (c). Dissolve 20 mg of idoxuridine CRS in a mixture of 1 volume of *concentrated ammonia R* and 5 volumes of *methanol R* and dilute to 5 ml with the same mixture of solvents.

Reference solution (d). Dilute 1 ml of test solution (b) to 20 ml with a mixture of 1 volume of *concentrated ammonia R* and 5 volumes of *methanol R*.

Apply separately to the plate 5 µl of each solution. Develop twice over a path of 15 cm using a mixture of 10 volumes of *concentrated ammonia R*, 40 volumes of *chloroform R* and 50 volumes of 2-propanol *R*, drying the plate in a current of cold air after each development. Examine in ultraviolet light at 254 nm. In the chromatogram obtained with test solution (a): any spots corresponding to 5-iodouracil, 2'-deoxyuridine and 5-bromo-2'-deoxyuridine are not more intense than the corresponding spots in the chromatogram obtained with reference solution (a) (0.5 per cent); any spot, apart from the principal spot and the spots corresponding to 5-iodouracil, 2'-deoxyuridine and 5-bromo-2'-deoxyuridine, is not more intense than the spot in the chromatogram obtained with reference solution (d) (0.5 per cent). The test is not valid unless the chromatogram obtained with reference solution (b) shows four clearly separated spots.

Iodide. Dissolve 0.25 g in 25 ml of 0.1 M sodium hydroxide, add 5 ml of dilute hydrochloric acid *R* and dilute to 50 ml with *water R*. Allow to stand for 10 min and filter. To 25 ml of the filtrate add 5 ml of dilute hydrogen peroxide solution *R* and 10 ml of *chloroform R* and shake. Any pink colour in the organic layer is not more intense than that in a standard prepared at the same time in the same manner using 1 ml of a 0.33 g/l solution of potassium iodide *R* instead of the substance to be examined (0.1 per cent).

Loss on drying (2.2.32). Not more than 1.0 per cent, determined on 1.000 g by drying *in vacuo* at 60 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.3000 g in 20 ml of dimethylformamide *R*. Titrate with 0.1 M tetrabutylammonium hydroxide, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M tetrabutylammonium hydroxide is equivalent to 35.41 mg of C₇H₁₅Cl₂N₂O₂P.

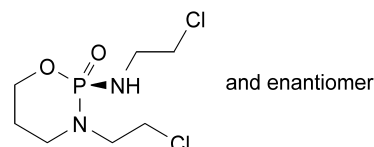
STORAGE

Store protected from light.

01/2005:1529
corrected

IFOSFAMIDE

Ifosfamidum



C₇H₁₅Cl₂N₂O₂P

M_r 261.1

DEFINITION

Ifosfamide contains not less than 98.0 per cent and not more than the equivalent of 102.0 per cent of (RS)-N,3-bis(2-chloroethyl)-1,3,2-oxazaphosphinan-2-amine 2-oxide, calculated with reference to the anhydrous substance.

CHARACTERS

A white or almost white, fine, crystalline powder, hygroscopic, soluble in water, freely soluble in methylene chloride.

IDENTIFICATION

Examine by infrared absorption spectrophotometry (2.2.24), comparing with the *Ph. Eur. reference spectrum of ifosfamide*. Examine the substance prepared as a disc.

TESTS

Solution S. Dissolve 5.0 g in *carbon dioxide-free water R* and dilute to 50.0 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and not more intensely coloured than reference solution Y₇ (2.2.2, Method II).

Acidity or alkalinity. Dilute 5 ml of solution S to 50 ml with *carbon dioxide-free water R*. To 10 ml of this solution add 0.1 ml of *methyl red solution R*. Not more than 0.1 ml of 0.01 M hydrochloric acid is required to change the colour of the indicator to red. To another 10 ml of the solution add 0.1 ml of *phenolphthalein solution R*. Not more than 0.3 ml of 0.01 M sodium hydroxide is required to change the colour of the indicator to pink.

Optical rotation (2.2.7). The angle of optical rotation, determined on solution S, is -0.10° to + 0.10°.

Related substances

A. Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel plate R*.

Test solution. Dissolve 1.00 g of the substance to be examined in a mixture of equal volumes of *methanol R* and *water R* and dilute to 10 ml with the same mixture of solvents.

Reference solution (a). Dissolve 25 mg of ifosfamide impurity A CRS and 25 mg of chloroethylamine hydrochloride *R* (impurity C) in a mixture of equal volumes of *methanol R* and *water R* and dilute to 100 ml with the same mixture of solvents.

Reference solution (b). Dissolve 15 mg of *ifosfamide impurity B CRS* in a mixture of equal volumes of *methanol R* and *water R* and dilute to 100 ml with the same mixture of solvents.

Reference solution (c). Dissolve 5 mg of *ethanolamine R* (impurity D), 20 mg of *ifosfamide impurity A CRS* and 80 mg of *chloroethylamine hydrochloride R* (impurity C) in a mixture of equal volumes of *methanol R* and *water R* and dilute to 100 ml with the same mixture of solvents.

Apply to the plate 10 µl of each solution. Develop over a path of 15 cm using a mixture of 10 volumes of *water R*, 15 volumes of *methanol R*, 25 volumes of *anhydrous acetic acid R* and 50 volumes of *methylene chloride R*. Dry the plate at 115 °C for 45 min. At the bottom of a chromatography tank, place an evaporating dish containing a 3.2 g/l solution of *potassium permanganate R* and add an equal volume of *dilute hydrochloric acid R*, close the tank and allow to stand for 10 min. Place the plate whilst still hot in the tank, avoiding contact of the stationary phase with the solution, and close the tank. Leave the plate in contact with the chlorine vapour for 20 min. Withdraw the plate and place it in a current of cold air until the excess of chlorine is removed (about 20 min) and an area of coating below the points of application does not give a blue colour with a drop of *potassium iodide and starch solution R*. Avoid prolonged exposure to cold air. Immerse the plate in a 1 g/l solution of *tetramethylbenzidine R* in *alcohol R* for 5 s. Allow the plate to dry and examine. In the chromatogram obtained with the test solution: any spot corresponding to impurity A or impurity C is not more intense than the corresponding spot in the chromatogram obtained with reference solution (a) (0.25 per cent); any spot corresponding to impurity B is not more intense than the corresponding spot in the chromatogram obtained with reference solution (b) (0.15 per cent); any other spot is not more intense than the principal spot in the chromatogram obtained with reference solution (b) (0.15 per cent). The test is not valid unless the chromatogram obtained with reference solution (c) shows 3 clearly separated spots.

B. Examine by thin-layer chromatography (2.2.27), using a TLC silica gel plate R.

Test solution. Dissolve 0.200 g of the substance to be examined in a mixture of equal volumes of *methanol R* and *methylene chloride R* and dilute to 10 ml with the same mixture of solvents.

Reference solution (a). Dissolve 5 mg of *ifosfamide impurity E CRS* and 5 mg of *ifosfamide impurity F CRS* in a mixture of equal volumes of *methanol R* and *methylene chloride R* and dilute to 100 ml with the same mixture of solvents.

Reference solution (b). Dissolve 10 mg of *ifosfamide impurity E CRS* and 10 mg of *ifosfamide CRS* in a mixture of equal volumes of *methanol R* and *methylene chloride R* and dilute to 100 ml with the same mixture of solvents.

Apply to the plate 5 µl of each solution. Develop over a path of 15 cm using a mixture of 1 volume of *methylene chloride R* and 10 volumes of *acetone R*. Dry the plate at 115 °C for 45 min. Proceed as described in Related substances test A. Any spot corresponding to impurity E or impurity F in the chromatogram obtained with the test solution is not more intense than the corresponding spot in the chromatogram obtained with reference solution (a) (0.25 per cent). The test is not valid unless the chromatogram obtained with reference solution (b) shows 2 clearly separated spots.

Chlorides (2.4.4). Dilute 5 ml of solution S to 15 ml with *water R*. The freshly prepared solution complies with the limit test for chlorides (100 ppm).

Heavy metals (2.4.8). 12 ml of solution S complies with limit test A for heavy metals (10 ppm). Prepare the standard using *lead standard solution (1 ppm Pb) R*.

Water (2.5.12). Not more than 0.5 per cent, determined on 1.00 g by the semi-micro determination of water.

ASSAY

Examine by liquid chromatography (2.2.29). Use the solutions within 24 h.

Solution A. Dissolve 50.0 mg of *ethyl parahydroxybenzoate R* in 25 ml of *alcohol R*, dilute to 100.0 ml with *water R* and mix.

Test solution. To 0.150 g of the substance to be examined add 10.0 ml of solution A and dilute to 250.0 ml with *water R*.

Reference solution. To 15.0 mg of *ifosfamide CRS* add 1.0 ml of solution A and dilute to 25.0 ml with *water R*.

The chromatography may be carried out using:

- a stainless steel column 0.25 m long and 4.6 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (5 µm),
- as mobile phase at a flow rate of 1.5 ml/min a mixture of 30 volumes of *acetonitrile R* and 70 volumes of *water R*,
- as detector a spectrophotometer set at 195 nm.

Inject 1 µl of the reference solution six times. The assay is not valid unless the resolution between the peaks due to ifosfamide and to ethyl parahydroxybenzoate is not less than 6.0 and the relative standard deviation of the peak area for ifosfamide is at most 2.0 per cent.

Inject 1 µl of the test solution. Calculate the percentage content of $C_7H_{15}Cl_2N_2O_2P$ from the area of the corresponding peak in the chromatogram obtained and the declared content of *ifosfamide CRS*.

STORAGE

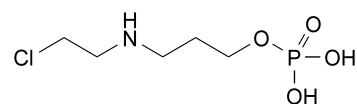
Store in an airtight container.

IMPURITIES

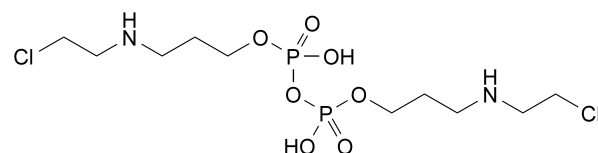
Specified impurities: A, B, C, E, F.

Other detectable impurities: D.

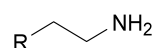
Related substances test A



A. 3-[(2-chloroethyl)amino]propyl dihydrogen phosphate,

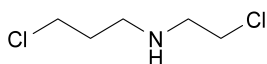


B. bis[3-[(2-chloroethyl)amino]propyl] dihydrogen diphosphate,

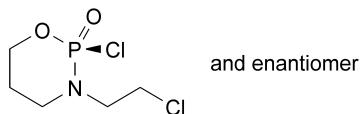


C. R = Cl: 2-chloroethanamine,

D. R = OH: 2-aminoethanol.

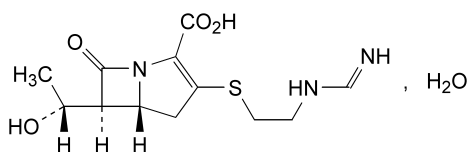
Related substances test B

E. 3-chloro-*N*-(2-chloroethyl)propan-1-amine,



F. (*RS*)-2-chloro-3-(2-chloroethyl)-1,3,2-oxazaphosphinane 2-oxide.

01/2005:1226

IMIPENEM**Imipenemum**

$C_{12}H_{17}N_3O_4S.H_2O$

M_r 317.4

DEFINITION

Imipenem contains not less than 98.0 per cent and not more than the equivalent of 101.0 per cent of (5*R*,6*S*)-6-[(*R*)-1-hydroxyethyl]-3-[[2-[(iminomethyl)amino]ethyl]sulphonyl]-7-oxo-1-azabicyclo[3.2.0]hept-2-ene-2-carboxylic acid, calculated with reference to the anhydrous substance.

CHARACTERS

A white to almost white or pale yellow powder, sparingly soluble in water, slightly soluble in methanol.

IDENTIFICATION

Examine by infrared absorption spectroscopy (2.2.24), comparing with the spectrum obtained with *imipenem CRS*.

TESTS

Appearance of solution. Dissolve 0.500 g in *phosphate buffer solution pH 7.0 R3* and dilute to 50 ml with the same solution. The solution is not more opalescent than reference suspension II (2.2.1) and not more intensely coloured than intensity 6 of the range of the reference solutions of the most appropriate colour (2.2.2, *Method II*).

pH (2.2.3). Dissolve 0.500 g in *carbon dioxide-free water R* and dilute to 100.0 ml with the same solvent. The pH of the solution is 4.5 to 7.0.

Specific optical rotation (2.2.7). Dissolve 0.125 g in *phosphate buffer solution pH 7.0 R3* and dilute to 25.0 ml with the same solution. The specific optical rotation measured at 25 °C is + 84 to + 89, calculated with reference to the anhydrous substance.

Related substances. Examine by liquid chromatography (2.2.29), as described under Assay. Inject 20 µl of reference solution (b). Adjust the sensitivity of the system so that the height of the principal peak in the chromatogram obtained is at least 50 per cent of the full scale of the recorder. Inject 20 µl of the test solution and continue the chromatography for twice the retention time of the principal peak. In the chromatogram obtained with the test solution: the area of any peak corresponding to thienamycin is not greater than that of the principal peak in the chromatogram obtained

with reference solution (b) (1 per cent); the area of any peak, apart from the principal peak and any peak corresponding to thienamycin, is not greater than 0.3 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.3 per cent); the sum of the areas of all the peaks, apart from the principal peak and any peak corresponding to thienamycin, is not greater than the area of the principal peak in the chromatogram obtained with reference solution (b) (1 per cent). Disregard any peak with an area less than 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (b).

Water (2.5.12): 5.0 per cent to 8.0 per cent, determined on 0.200 g by the semi-micro determination of water. Use an iodosulphurous reagent containing imidazole instead of pyridine and a clean container for each determination.

Sulphated ash (2.4.14). Not more than 0.2 per cent, determined on 1.0 g.

Bacterial endotoxins (2.6.14): less than 0.17 IU/mg, if intended for use in the manufacture of parenteral dosage forms without a further appropriate procedure for removal of bacterial endotoxins.

ASSAY

Examine by liquid chromatography (2.2.29).

Keep the solutions in an ice-bath and use within 8 h of preparation.

Test solution. Dissolve 40.0 mg of the substance to be examined in a mixture of 0.7 volumes of *acetonitrile R* and 99.3 volumes of a 0.135 g/l solution of *dipotassium hydrogen phosphate R* adjusted to pH 6.8 with *dilute phosphoric acid R* and dilute to 100.0 ml with the same mixture of solvents.

Reference solution (a). Dissolve 40.0 mg of *imipenem CRS* in a mixture of 0.7 volumes of *acetonitrile R* and 99.3 volumes of a 0.135 g/l solution of *dipotassium hydrogen phosphate R* adjusted to pH 6.8 with *dilute phosphoric acid R* and dilute to 100.0 ml with the same mixture of solvents.

Reference solution (b). Dilute 1.0 ml of the test solution to 100.0 ml with a mixture of 0.7 volumes of *acetonitrile R* and 99.3 volumes of a 0.135 g/l solution of *dipotassium hydrogen phosphate R* adjusted to pH 6.8 with *dilute phosphoric acid R*.

Reference solution (c). Heat at 80 °C for 5 min 20 ml of the test solution previously adjusted to pH 10 with *sodium hydroxide solution R*.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4.6 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (5 µm),
- as mobile phase at a flow rate of 1.0 ml/min a mixture of 0.7 volumes of *acetonitrile R* and 99.3 volumes of a 8.7 g/l solution of *dipotassium hydrogen phosphate R* adjusted to pH 7.3 with *dilute phosphoric acid R*,
- as detector a spectrophotometer set at 254 nm.

Inject 20 µl of reference solution (a). Adjust the sensitivity of the system so that the height of the principal peak in the chromatogram obtained is at least 50 per cent of the full scale of the recorder. Inject 20 µl of reference solution (c). When the chromatograms are recorded in the prescribed conditions, the retention time of imipenem is about 9 min and the relative retention time of thienamycin relative to imipenem is about 0.8. The assay is not valid unless the resolution between the peaks corresponding to imipenem and thienamycin is at least 3.5. Inject 20 µl of reference solution (a) six times. The test is not valid unless the relative

standard deviation of the peak area for imipenem is at most 1.0 per cent. Inject alternately the test solution and reference solution (a).

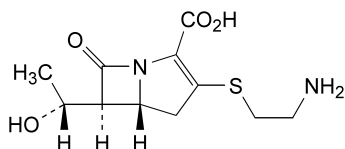
STORAGE

Store in an airtight container, at a temperature of 2 °C to 8 °C. If the substance is sterile, store in a sterile, airtight, tamper-proof container.

LABELLING

The label states, where applicable, that the substance is free from bacterial endotoxins.

IMPURITIES

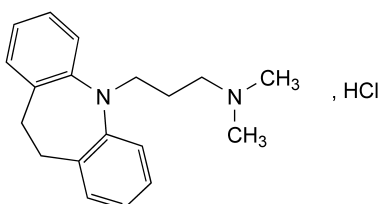


- A. (5*R*,6*S*)-3-[(2-aminoethyl)sulphonyl]-6-[(*R*)-1-hydroxyethyl]-7-oxo-1-azabicyclo[3.2.0]hept-2-ene-2-carboxylic acid (thienamycin).

01/2005:0029
corrected

IMIPRAMINE HYDROCHLORIDE

Imipramini hydrochloridum



$C_{19}H_{25}ClN_2$

M_r 316.9

DEFINITION

Imipramine hydrochloride contains not less than 98.5 per cent and not more than the equivalent of 101.0 per cent of 3-(10,11-dihydro-5*H*-dibenzo[*b,f*]azepin-5-yl)-*N,N*-dimethylpropan-1-amine hydrochloride, calculated with reference to the dried substance.

CHARACTERS

A white or slightly yellow, crystalline powder, freely soluble in water and in alcohol.

IDENTIFICATION

First identification: A, C, F.

Second identification: A, B, D, E, F.

A. Melting point (2.2.14): 170 °C to 174 °C.

B. Dissolve 20 mg in 0.01 *M* hydrochloric acid and dilute to 100.0 ml with the same acid. Dilute 1.0 ml of the solution to 10.0 ml with 0.01 *M* hydrochloric acid. Examined between 230 nm and 350 nm, the solution shows a single absorption maximum (2.2.25), at 251 nm, and a shoulder at 270 nm. The specific absorbance at the maximum is about 260.

C. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with imipramine hydrochloride CRS. Examine the substances prepared as discs.

D. Dissolve about 5 mg in 2 ml of *nitric acid R*. An intense blue colour develops.

E. Dissolve about 50 mg in 3 ml of *water R* and add 0.05 ml of a 25 g/l solution of *quinhydrone R* in *methanol R*. No red colour develops within 15 min.

F. About 20 mg gives reaction (a) of chlorides (2.3.1).

TESTS

Solution S. To 3.0 g add 20 ml of *carbon dioxide-free water R*, dissolve rapidly by shaking and triturating with a glass rod and dilute to 30 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1). Immediately after preparation, dilute solution S with an equal volume of *water R*. This solution is not more intensely coloured than reference solution BY₆ (2.2.2, *Method II*).

pH (2.2.3). The pH of solution S, measured immediately after preparation, is 3.6 to 5.0.

Related substances. Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel G plate R*.

Test solution. Dissolve 0.25 g of the substance to be examined in *methanol R* and dilute to 10 ml with the same solvent. Prepare immediately before use.

Reference solution (a). Dilute 1 ml of the test solution to 10 ml with *methanol R*. Dilute 1 ml of this solution to 50 ml with *methanol R*.

Reference solution (b). Dissolve 5 mg of *iminodibenzyl R* in *methanol R* and dilute to 100 ml with the same solvent. Prepare immediately before use.

Apply to the plate 10 µl of each solution. Develop over a path of 12 cm using a mixture of 5 volumes of *hydrochloric acid R*, 5 volumes of *water R*, 35 volumes of *glacial acetic acid R* and 55 volumes of *ethyl acetate R*. Allow the plate to dry in air for 5 min and spray with a 5 g/l solution of *potassium dichromate R* in a mixture of 1 volume of *sulphuric acid R* and 4 volumes of *water R*. Examine the plate immediately. The chromatogram obtained with the test solution shows a blue principal spot. In the chromatogram obtained with the test solution: any spot corresponding to iminodibenzyl is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.2 per cent); any spot apart from the principal spot and any spot corresponding to iminodibenzyl, is not more intense than the spot in the chromatogram obtained with reference solution (a) (0.2 per cent).

Heavy metals (2.4.8). 2.0 g complies with limit test C for heavy metals (20 ppm). Prepare the standard using 4 ml of *lead standard solution (10 ppm Pb) R*.

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.00 g by drying in an oven at 100 °C to 105 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.250 g in 50 ml of *alcohol R* and add 5.0 ml of 0.01 *M* hydrochloric acid. Carry out a potentiometric titration (2.2.20), using 0.1 *M* sodium hydroxide. Read the volume added between the 2 points of inflexion.

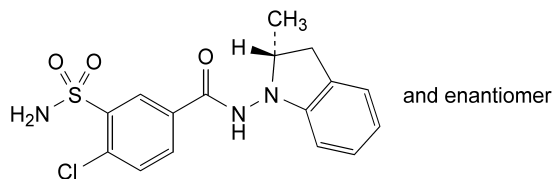
1 ml of 0.1 *M* sodium hydroxide is equivalent to 31.69 mg of $C_{19}H_{25}ClN_2$.

STORAGE

Store protected from light.

INDAPAMIDE

Indapamidum

C₁₆H₁₆ClN₃O₃SM_r 365.8

DEFINITION

Indapamide contains not less than 98.0 per cent and not more than the equivalent of 102.0 per cent of 4-chloro-*N*-(2*RS*)-2-methyl-2,3-dihydro-1*H*-indol-1-yl]-3-sulphamoylbenzamide, calculated with reference to the anhydrous substance.

CHARACTERS

A white or almost white powder, practically insoluble in water, soluble in alcohol.

IDENTIFICATION

First identification: B.

Second identification: A, C.

- Dissolve 50.0 mg in *alcohol R* and dilute to 100.0 ml with the same solvent. Dilute 2.0 ml of the solution to 100.0 ml with *alcohol R*. Examined between 220 nm and 350 nm (2.2.25), the solution shows an absorption maximum at 242 nm and two shoulders, at 279 nm and 287 nm. The specific absorbance at the maximum is 590 to 630.
- Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *indapamide CRS*. Examine the substances prepared as discs using *potassium bromide R*.
- Examine by thin-layer chromatography (2.2.27), using *silica gel GF₂₅₄ R* as the coating substance.

Test solution. Dissolve 20 mg of the substance to be examined in *alcohol R* and dilute to 10 ml with the same solvent.

Reference solution (a). Dissolve 20 mg of *indapamide CRS* in *alcohol R* and dilute to 10 ml with the same solvent.

Reference solution (b). Dissolve 10 mg of *indometacin R* in 5 ml of reference solution (a) and dilute to 10 ml with *alcohol R*.

Apply to the plate 10 µl of each solution. Develop over a path of 15 cm using a mixture of 1 volume of *glacial acetic acid R*, 20 volumes of *acetone R* and 79 volumes of *toluene R*. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. The principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a). The test is not valid unless the chromatogram obtained with reference solution (b) shows two clearly separated spots.

TESTS

Optical rotation (2.2.7). Dissolve 0.250 g in *ethanol R* and dilute to 25.0 ml with the same solvent. The angle of optical rotation is -0.02° to $+0.02^\circ$.

01/2005:1108 Related substances. Examine by liquid chromatography (2.2.29) as described under Assay.

Adjust the sensitivity of the system so that the height of the principal peak in the chromatogram obtained with reference solution (b) is not less than 15 per cent of the full scale of the recorder.

Inject 10 µl of each solution and continue the chromatography for 2.5 times the retention time of the principal peak. The test is not valid unless in the chromatogram obtained with reference solution (d): the resolution between the peak corresponding to indapamide and the peak corresponding to methylnitrosoindoline is at least 4.0; the principal peak in the chromatogram obtained with reference solution (b) has a signal-to-noise ratio of at least 6.

In the chromatogram obtained with the test solution: the area of any peak corresponding to indapamide impurity B is not greater than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.3 per cent); the area of any peak, apart from the principal peak and any peak corresponding to indapamide impurity B, is not greater than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent); the sum of the areas of all the peaks, apart from the principal peak, is not greater than five times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent). Disregard any peak with an area less than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b).

Methylnitrosoindoline. Not more than 5 ppm, determined by liquid chromatography (2.2.29). *Carry out the test protected from light.*

Test solution. Dissolve 25.0 mg of the substance to be examined in 1 ml of *acetonitrile R* and dilute to 10.0 ml with *water R*. Shake for 15 min. Allow to stand at 4 °C for 1 h and filter.

Reference solution. Dissolve 25.0 mg of the substance to be examined in 1.0 ml of a 0.125 mg/l solution of *methylnitrosoindoline CRS* in *acetonitrile R* and dilute to 10.0 ml with *water R*. Shake for 15 min. Allow to stand at 4 °C for 1 h and filter.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.15 m long and 4.6 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (5 µm),
- as mobile phase at a flow rate of 1.4 ml/min a mixture of 7 volumes of *acetonitrile R*, 20 volumes of *tetrahydrofuran R* and 73 volumes of a 1.5 g/l solution of *triethylamine R* adjusted to pH 2.8 with *phosphoric acid R*,
- as detector a spectrophotometer set at 305 nm, maintaining the temperature of the column at 30 °C.

Inject 0.1 ml of the solutions.

In the chromatogram obtained with the reference solution, measure from the baseline the height *A* of the peak corresponding to methylnitrosoindoline, appearing just before the principal peak, and the height *B* corresponding to the lowest point in the curve between this peak and the principal peak.

The test is not valid unless in the chromatogram obtained with the reference solution: the difference between *A* and *B* is at least 85 per cent of height *A*, the peak corresponding to methylnitrosoindoline appearing just before the principal peak has a signal-to-noise ratio of at least 3.

In the chromatogram obtained with the test solution, the area of any peak corresponding to methylnitrosoindoline is not greater than the difference between the areas of

the peaks corresponding to methylnitrosoindoline in the chromatograms obtained with the reference solution and the test solution.

Heavy metals (2.4.8). 2.0 g complies with limit test C for heavy metals (10 ppm). Prepare the standard using 2 ml of *lead standard solution* (10 ppm Pb) R.

Water (2.5.12). Not more than 3.0 per cent, determined on 0.10 g by the semi-micro determination of water.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Examine by liquid chromatography (2.2.29). Carry out the assay protected from light and prepare the solutions immediately before use or maintain them at 4 °C.

Test solution. Dissolve 20.0 mg of the substance to be examined in 7 ml of a mixture of equal volumes of *acetonitrile* R and *methanol* R and dilute to 20.0 ml with a 0.2 g/l solution of *sodium edetate* R.

Reference solution (a). Dissolve 30.0 mg of *indapamide impurity B* CRS in 35 ml of a mixture of equal volumes of *acetonitrile* R and *methanol* R and dilute to 100.0 ml with a 0.2 g/l solution of *sodium edetate* R. To 1.0 ml of the solution, add 35 ml of a mixture of equal volumes of *acetonitrile* R and *methanol* R and dilute to 100.0 ml with a 0.2 g/l solution of *sodium edetate* R.

Reference solution (b). Dilute 1.0 ml of the test solution to 50.0 ml with a mixture of 17.5 volumes of *acetonitrile* R, 17.5 volumes of *methanol* R and 65 volumes of a 0.2 g/l solution of *sodium edetate* R. Dilute 1.0 ml of the solution to 20.0 ml with a mixture of 17.5 volumes of *acetonitrile* R, 17.5 volumes of *methanol* R and 65 volumes of a 0.2 g/l solution of *sodium edetate* R.

Reference solution (c). Dissolve 20.0 mg of *indapamide* CRS in 7 ml of a mixture of equal volumes of *acetonitrile* R and *methanol* R and dilute to 20.0 ml with a 0.2 g/l solution of *sodium edetate* R.

Reference solution (d). Dissolve 25.0 mg of *indapamide* CRS and 45.0 mg of *methylnitrosoindoline* CRS in 17.5 ml of a mixture of equal volumes of *acetonitrile* R and *methanol* R and dilute to 50.0 ml with a 0.2 g/l solution of *sodium edetate* R.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.20 m long and 4.6 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography* R (5 µm),
- as mobile phase at a flow rate of 2 ml/min a mixture of 0.1 volumes of *glacial acetic acid* R, 17.5 volumes of *acetonitrile* R, 17.5 volumes of *methanol* R and 65 volumes of a 0.2 g/l solution of *sodium edetate* R,
- as detector a spectrophotometer set at 254 nm, maintaining the temperature of the column at 40 °C.

When the chromatograms are recorded at the prescribed conditions, the retention time of the principal peak in the chromatogram obtained with reference solution (c) is about 11 min.

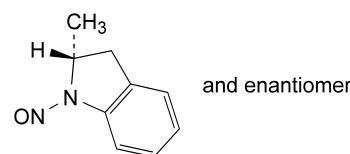
Inject reference solution (c) six times. The test is not valid unless the relative standard deviation of the peak area for indapamide is at most 1.0 per cent. If necessary, adjust the integrator parameters. Inject alternately the test solution and reference solution (c).

Calculate the per cent *m/m* content of anhydrous substance.

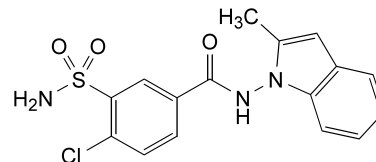
STORAGE

Store protected from light.

IMPURITIES



A. (2*RS*)-2-methyl-1-nitroso-2,3-dihydro-1*H*-indole,

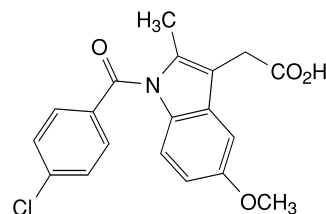


B. 4-chloro-*N*-(2-methyl-1*H*-indol-1-yl)-3-sulphamoylbenzamide.

01/2005:0092

INDOMETACIN

Indometacinum



C₁₉H₁₆ClNO₄

*M*_r 357.8

DEFINITION

Indometacin contains not less than 98.5 per cent and not more than the equivalent of 100.5 per cent of [1-(4-chlorobenzoyl)-5-methoxy-2-methylindol-3-yl]acetic acid, calculated with reference to the dried substance.

CHARACTERS

A white or yellow, crystalline powder, practically insoluble in water, sparingly soluble in alcohol.

IDENTIFICATION

First identification: A, C.

Second identification: A, B, D, E.

A. Melting point (2.2.14): 158 °C to 162 °C.

B. Dissolve 25 mg in a mixture of 1 volume of 1 *M* *hydrochloric acid* and 9 volumes of *methanol* R and dilute to 100.0 ml with the same mixture of solvents. Dilute 10.0 ml of the solution to 100.0 ml with a mixture of 1 volume of 1 *M* *hydrochloric acid* and 9 volumes of *methanol* R. Examined between 300 nm and 350 nm (2.2.25), the solution shows an absorption maximum at 318 nm. The specific absorbance at the maximum is 170 to 190.

C. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *indometacin* CRS. Examine the substances in the solid state without recrystallisation.

D. Dissolve 0.1 g in 10 ml of *alcohol* R, heating slightly if necessary. To 0.1 ml of the solution add 2 ml of a freshly prepared mixture of 1 volume of a 250 g/l solution of *hydroxylamine hydrochloride* R and 3 volumes of *dilute sodium hydroxide solution* R. Add 2 ml of *dilute hydrochloric acid* R and 1 ml of *ferric chloride solution* R2 and mix. A violet-pink colour develops.

E. To 0.5 ml of the solution in alcohol prepared in identification test D, add 0.5 ml of *dimethylaminobenzaldehyde solution R2*. A precipitate is formed that dissolves on shaking. Heat on a water-bath. A bluish-green colour is produced. Continue to heat for 5 min and cool in iced water for 2 min. A precipitate is formed and the colour changes to light greyish-green. Add 3 ml of *alcohol R*. The solution is clear and violet-pink in colour.

TESTS

Related substances. Examine by thin-layer chromatography (2.2.27), using *silica gel HF₂₅₄ R* as the coating substance. Prepare the slurry using a 46.8 g/l solution of *sodium dihydrogen phosphate R*.

Test solution. Dissolve 0.2 g of the substance to be examined in *methanol R* and dilute to 10 ml with the same solvent. Prepare immediately before use.

Reference solution. Dilute 1 ml of the test solution to 200 ml with *methanol R*.

Apply separately to the plate 10 µl of each solution. Develop over a path of 15 cm using a mixture of 30 volumes of *light petroleum R* and 70 volumes of *ether R*. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. Any spot in the chromatogram obtained with the test solution, apart from the principal spot, is not more intense than the spot in the chromatogram obtained with the reference solution (0.5 per cent).

Heavy metals (2.4.8). 2.0 g complies with limit test C for heavy metals (20 ppm). Prepare the standard using 4 ml of *lead standard solution (10 ppm Pb) R*.

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

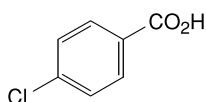
Dissolve 0.300 g in 75 ml of *acetone R*, through which *nitrogen R*, free from carbon dioxide, has been passed for 15 min. Maintain a constant stream of nitrogen through the solution. Add 0.1 ml of *phenolphthalein solution R*. Titrate with 0.1 M *sodium hydroxide*. Carry out a blank titration.

1 ml of 0.1 M *sodium hydroxide* is equivalent to 35.78 mg of C₁₉H₁₆ClNO₄.

STORAGE

Store protected from light.

IMPURITIES

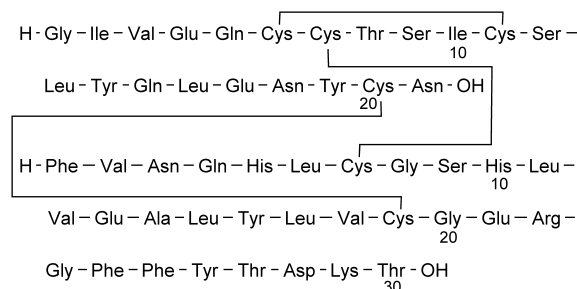


A. 4-chlorobenzoic acid.

01/2005:2084

INSULIN ASPART

Insulinum aspartum



C₂₅₆H₃₈₁N₆₅O₇₉S₆

M_r 5826

DEFINITION

28^B-L-Aspartate insulin (human).

Insulin aspart is a 2-chain peptide containing 51 amino acids. The A-chain is composed of 21 amino acids and the B-chain is composed of 30 amino acids. It is identical in primary structure to human insulin, except that it has aspartic acid instead of proline at position 28 of the B-chain. As in human insulin, insulin aspart contains 2 interchain disulphide bonds and 1 intrachain disulphide bond.

Content: 90.0 per cent to 104.0 per cent of insulin aspart C₂₅₆H₃₈₁N₆₅O₇₉S₆ plus A21Asp insulin aspart, B3Asp insulin aspart, B3isoAsp insulin aspart and B28isoAsp insulin aspart (dried substance).

By convention, for the purpose of labelling insulin aspart preparations, 0.0350 mg of insulin aspart is equivalent to 1 unit.

PRODUCTION

Insulin aspart is produced by a method based on recombinant DNA (rDNA) technology under conditions designed to minimise the degree of microbial contamination.

Prior to release the following tests are carried out on each batch of the final bulk product, unless exemption has been granted by the competent authority.

Host-cell-derived proteins. The limit is approved by the competent authority.

Single-chain precursor. The limit is approved by the competent authority. Use a suitably sensitive method.

CHARACTERS

Appearance: white or almost white powder.

Solubility: practically insoluble in ethanol (96 per cent), in methanol and in aqueous solutions with a pH around 5.1. In aqueous solutions below pH 3.5 or above pH 6.5, the solubility is greater than or equal to 25 mg/ml.

IDENTIFICATION

A. Examine the chromatograms obtained in the assay.

Results: the principal peak in the chromatogram obtained with the test solution is similar in retention time to the principal peak in the chromatogram obtained with reference solution (a).

B. Peptide mapping (2.2.55).

SELECTIVE CLEAVAGE OF THE PEPTIDE BONDS

Test solution. Prepare a 2.0 mg/ml solution of the substance to be examined in 0.01 M *hydrochloric acid* and transfer 25 µl of this solution to a clean tube. Add 100 µl of *HEPES buffer solution pH 7.5 R* and 20 µl of

a 1 mg/ml solution of *Staphylococcus aureus strain V8 protease R*. Cap the tube and incubate at 25 °C for 6 h. Stop the reaction by adding 145 µl of *sulphate buffer solution pH 2.0 R*.

Reference solution. Prepare at the same time and in the same manner as for the test solution, but using *insulin aspart CRS* instead of the substance to be examined.

CHROMATOGRAPHIC SEPARATION. Liquid chromatography (2.2.29).

Column:

- size: $l = 0.10$ m, $\varnothing = 4.6$ mm,
- stationary phase: octadecylsilyl silica gel for chromatography R (3 µm) with a pore size of 8 nm,
- temperature: 40 °C.

Mobile phase:

- mobile phase A: mix 100 ml of acetonitrile for chromatography R, 200 ml of sulphate buffer solution pH 2.0 R and 700 ml of water R; filter and degas;
- mobile phase B: mix 200 ml of sulphate buffer solution pH 2.0 R, 400 ml of acetonitrile for chromatography R and 400 ml of water R; filter and degas;

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)
0 - 60	90 → 30	10 → 70
60 - 65	30 → 0	70 → 100
65 - 70	0	100

Flow rate: 1 ml/min.

Detection: spectrophotometer at 214 nm.

Equilibration: at initial conditions for at least 15 min. Carry out a blank run using the above-mentioned gradient.

Injection: 50 µl.

System suitability:

- the chromatograms obtained with the test solution and the reference solution are qualitatively similar to the chromatogram of insulin aspart digest supplied with *insulin aspart CRS*,
- in the chromatogram obtained with the reference solution, identify the peaks due to digest fragments I, II and III:

symmetry factor: maximum 1.5, for the peaks due to fragments II and III,

resolution : minimum 8.0, between the peaks due to fragments II and III.

Results: the profile of the chromatogram obtained with the test solution corresponds to that of the chromatogram obtained with the reference solution.

NOTE: the retention times of fragments I, II and IV are the same as for human insulin. The retention time of fragment III differs from human insulin due to substitution of proline by aspartic acid.

TESTS

Impurities with molecular masses greater than that of insulin aspart. Size-exclusion chromatography (2.2.30): use the normalisation procedure.

Test solution. Prepare a solution containing 4 mg/ml of the substance to be examined in 0.01 M hydrochloric acid. Maintain the solution at 2-8 °C and use within 48 h.

Resolution solution. Use a solution of insulin (about 4 mg/ml), containing more than 0.4 per cent of high molecular mass proteins. An injectable insulin preparation,

whether a solution or a suspension, that has been clarified with a sufficient amount of 6 M hydrochloric acid, containing the indicated percentage of high molecular mass proteins, or a solution prepared from insulin, dissolved in 0.01 M hydrochloric acid may be used. Insulin containing the indicated percentage of high molecular mass proteins may be prepared by allowing insulin powder to stand at room temperature for about 10 days. Maintain the solution at 2-8 °C and use within 7 days.

Column:

- size: $l = 0.3$ m, $\varnothing = 7.8$ mm,
- stationary phase: hydrophilic silica gel for chromatography R (5-10 µm) with a pore size of 12-12.5 nm, of a grade suitable for the separation of insulin monomer from dimer and polymers.

Mobile phase: mix 15 volumes of glacial acetic acid R, 20 volumes of acetonitrile for chromatography R and 65 volumes of a 1.0 g/l solution of arginine R; filter and degas.

Flow rate: 0.5 ml/min.

Detection: spectrophotometer at 276 nm.

Equilibration: at least 3 injections of the resolution solution; the column is equilibrated when repeatable results are obtained from 2 subsequent injections.

Injection: 100 µl.

Run time: about 35 min.

Retention time: insulin aspart polymers = 13-17 min; insulin aspart dimer = about 17.5 min; insulin aspart monomer = about 20 min; salts = about 22 min.

System suitability: resolution solution:

- peak-to-valley ratio: minimum 2.0, where H_p = height above the baseline of the peak due to the dimer and H_v = height above the baseline of the lowest point of the curve separating this peak from the peak due to the monomer.

Limits: the sum of the areas of the peaks with a retention time less than that of the principal peak is not more than 0.5 per cent of the total area of the peaks. Disregard any peak with a retention time greater than that of the peak due to insulin aspart monomer.

Related proteins. Liquid chromatography (2.2.29) as described under Assay: use the normalisation procedure.

Limits:

- B28isoAsp insulin aspart: maximum 1.0 per cent,
- total of the peaks due to A21Asp insulin aspart, B3Asp insulin aspart and B3isoAsp insulin aspart: maximum 2.0 per cent,
- total of other impurities: maximum 1.5 per cent.

Loss on drying (2.2.32): maximum 10.0 per cent, determined on 0.200 g by drying in an oven at 100-105 °C for 24 h.

Sulphated ash (2.4.14): maximum 6.0 per cent, determined on 0.200 g (dried substance).

Bacterial endotoxins (2.6.14): less than 10 IU/mg, if intended for use in the manufacture of parenteral dosage forms without a further appropriate procedure for the removal of bacterial endotoxins.

ASSAY

Liquid chromatography (2.2.29).

Test solution. Dissolve the substance to be examined in 0.01 M hydrochloric acid to obtain a concentration of 4.0 mg/ml. Maintain the solution at 2-8 °C and use within 24 h.

Reference solution (a). Dissolve the contents of a vial of *insulin aspart CRS* in 0.01 M hydrochloric acid to obtain a concentration of 4.0 mg/ml. Maintain the solution at 2-8 °C and use within 48 h.

Reference solution (b). Mix equal volumes of reference solution (a) and *water R*. Maintain the solution at 2-8 °C and use within 48 h.

Resolution solution. Use an appropriate solution with a content of B3Asp insulin aspart and A21Asp insulin aspart of not less than 1 per cent. This may be achieved by storing reference solution (a) at room temperature for about 1-3 days. Maintain the solution at 2-8 °C and use within 72 h.

Column:

- **size:** $l = 0.25$ m, $\varnothing = 4$ mm,
- **stationary phase:** octadecylsilyl silica gel for chromatography R (5 µm),
- **temperature:** 40 °C.

Mobile phase:

- **mobile phase A:** dissolve 142.0 g of *anhydrous sodium sulphate R* in *water R*; add 13.5 ml of *phosphoric acid R* and dilute to 5000 ml with *water R*; adjust to pH 3.6, if necessary, with *strong sodium hydroxide solution R*; filter and degas; mix 9 volumes of the solution with 1 volume of *acetonitrile for chromatography R*; filter and degas;
- **mobile phase B:** mix equal volumes of *water R* and *acetonitrile for chromatography R*; filter and degas;

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)
0 - 35	58	42
35 - 40	58 → 20	42 → 80
40 - 45	20	80
45 - 46	20 → 58	80 → 42
46 - 60	58	42

Flow rate: 1 ml/min.

Detection: spectrophotometer at 214 nm.

Injection: 10 µl.

Relative retention with reference to insulin aspart (retention time = 20-24 min): B28isoAsp insulin aspart = about 0.9; B3Asp insulin aspart plus A21Asp insulin aspart (generally coeluted) = about 1.3; B3isoAsp insulin aspart = about 1.5.

System suitability: resolution solution:

- **resolution:** minimum 2.0 between the peak due to insulin aspart and the peak due to A21Asp insulin aspart and to B3Asp insulin aspart.

Calculate the content of insulin aspart $C_{256}H_{381}N_{65}O_{79}S_6$, plus B28isoAsp insulin aspart, A21Asp insulin aspart, B3Asp insulin aspart and B3isoAsp insulin aspart using the areas of the corresponding peaks in the chromatograms obtained with the test solution and reference solution (a) and the declared content of insulin aspart plus B28isoAsp insulin aspart, A21Asp insulin aspart, B3Asp insulin aspart and B3isoAsp insulin aspart in *insulin aspart CRS*.

STORAGE

In an airtight container, protected from light, at or below –18 °C until released by the manufacturer. When thawed, insulin aspart is stored at 5 ± 3 °C and used for manufacturing preparations within a short period of time. To avoid absorption of humidity from the air during weighing, insulin aspart must be at room temperature before opening the container.

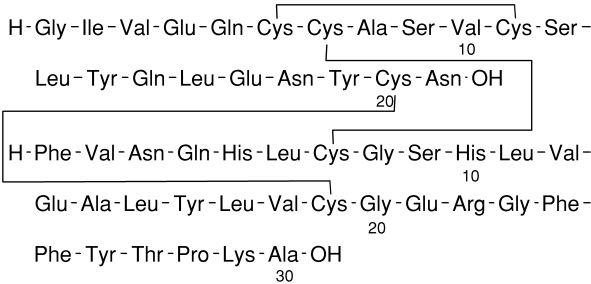
LABELLING

The label states, where applicable, that the substance is free from bacterial endotoxins.

01/2005:1637
corrected

INSULIN, BOVINE

Insulinum bovinum



$C_{254}H_{377}N_{65}O_{75}S_6$

M_r 5734

DEFINITION

Bovine insulin is the natural antidiabetic principle obtained from beef pancreas and purified. The content of bovine insulin $C_{254}H_{377}N_{65}O_{75}S_6$ plus A21 desamido bovine insulin is not less than 93.0 per cent and not more than 105.0 per cent, calculated with reference to the dried substance.

By convention, for the purpose of labelling insulin preparations, 0.0342 mg of bovine insulin is equivalent to 1 IU of insulin.

PRODUCTION

The animals from which bovine insulin is derived must fulfil the requirements for the health of animals suitable for human consumption to the satisfaction of the competent authority. The manufacturing process is validated to demonstrate suitable inactivation or removal of any contamination by viruses or other infectious agents.

CHARACTERS

Appearance: white or almost white powder.

Solubility: practically insoluble in water and in ethanol. It dissolves in dilute mineral acids and with decomposition in dilute solutions of alkali hydroxides.

IDENTIFICATION

A. Examine the chromatograms obtained in the assay.

Results: the retention time of the principal peak in the chromatogram obtained with the test solution corresponds to that of the principal peak in the chromatogram obtained with reference solution (c).

B. Peptide mapping.

Test solution. Prepare a 2.0 mg/ml solution of the substance to be examined in 0.01 M hydrochloric acid and transfer 500 µl of this solution to a clean tube. Add 2.0 ml of *HEPES buffer solution pH 7.5 R* and 400 µl of a 1 mg/ml solution of *Staphylococcus aureus strain V8 protease R*. Cap the tube and incubate at 25 °C for 6 h. Stop the reaction by adding 2.9 ml of *sulphate buffer solution pH 2.0 R*.

Reference solution. Prepare at the same time and in the same manner as for the test solution but using *bovine insulin CRS* instead of the substance to be examined.

Examine the digests by liquid chromatography (2.2.29).

Column:

- size: $l = 0.10$ m, $\varnothing = 4.6$ mm,
- stationary phase: octadecylsilyl silica gel for chromatography R (3 μm),
- temperature: 40 °C.

Mobile phase:

- mobile phase A: mix 100 ml of acetonitrile for chromatography R, 700 ml of water R and 200 ml of sulphate buffer solution pH 2.0 R; filter and degas;
- mobile phase B: mix 400 ml of acetonitrile for chromatography R, 400 ml of water R and 200 ml of sulphate buffer solution pH 2.0 R; filter and degas;

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)
0 - 60	90 → 30	10 → 70
60 - 65	30 → 0	70 → 100
65 - 70	0	100

Flow rate: 1 ml/min.

Detection: spectrophotometer at 214 nm.

Equilibration: at initial conditions for at least 15 min. Carry out a blank run using the above-mentioned gradient.

Injection: 50 μL .

System suitability: the chromatograms obtained with the test solution and the reference solution are qualitatively similar to the chromatogram of bovine insulin digest supplied with *bovine insulin CRS*. In the chromatogram obtained with the reference solution, identify the peaks due to digest fragments I, II and III. The symmetry factor of the peaks due to fragments II and III is not greater than 1.5, and the resolution between the 2 peaks is at least 1.9.

Results: the profile of the chromatogram obtained with the test solution corresponds to that of the chromatogram obtained with the reference solution.

NOTE: The retention time of fragment I is the same for porcine insulin and for human insulin. The retention times of fragments II and IV are the same for all insulins. The retention time of fragment III is the same for bovine insulin and for porcine insulin.

TESTS

Impurities with molecular masses greater than that of insulin. Size-exclusion chromatography (2.2.30): use the normalisation procedure.

Test solution. Dissolve 4 mg of the substance to be examined in 1.0 ml of 0.01 M hydrochloric acid.

Resolution solution. Use a solution of insulin (approximately 4 mg/ml), containing more than 0.4 per cent of high molecular mass proteins. An injectable insulin preparation, whether a solution or a suspension, that has been clarified with a sufficient amount of 6 M hydrochloric acid, containing the indicated percentage of high molecular mass proteins, or a solution prepared from insulin, dissolved in 0.01 M hydrochloric acid, may be used. Insulin containing the indicated percentage of high molecular mass proteins may be prepared by allowing insulin powder to stand at room temperature for about 10 days.

Maintain the solutions at 2-10 °C and use within 7 days. If an automatic injector is used, maintain the temperature at 2-10 °C.

Column:

- size: $l = 0.3$ m, $\varnothing =$ at least 7.5 mm,
- stationary phase: hydrophilic silica gel for chromatography R (5-10 μm), of a grade suitable for the separation of insulin monomer from dimer and polymers.

Mobile phase: mix of 15 volumes of glacial acetic acid R, 20 volumes of acetonitrile R and 65 volumes of a 1.0 g/l solution of arginine R; filter and degas.

Flow rate: 0.5 ml/min.

Detection: spectrophotometer at 276 nm.

Equilibration: before using a new column for chromatographic analysis, equilibrate by repeated injections of an insulin solution containing high molecular mass proteins. This can be done by at least 3 injections of the resolution solution. The column is equilibrated when repeatable results are obtained from 2 subsequent injections.

Injection: 100 μL .

Run time: about 35 min.

Retention times: polymeric insulin complexes = 13-17 min; covalent insulin dimer = about 17.5 min; insulin monomer = about 20 min; salts = about 22 min.

System suitability: resolution solution:

- peak-to-valley ratio: minimum 2.0, where H_p = height above the baseline of the peak due to the dimer and H_v = height above the baseline of the lowest point of the curve separating this peak from the peak due to the monomer.

Limits: the sum of the areas of any peaks with a retention time less than that of the principal peak is not greater than 1.0 per cent of the total area of the peaks. Disregard any peak with a retention time greater than that of the insulin peak.

Related proteins. Liquid chromatography (2.2.29) as described under Assay, following the elution conditions as described in the table below:

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)
0 - 30	42	58
30 - 44	42 → 11	58 → 89
44 - 50	11	89

Maintain the solutions at 2-10 °C and use within 24 h. Perform a system suitability test (resolution, linearity) as described under Assay. If necessary, the relative proportions of the mobile phases may be adjusted to ensure complete elution of A21 desamido porcine insulin before commencement of the gradient. The profile of the gradient may also be adjusted to ensure complete elution of all insulin related impurities.

Inject 20 μL of reference solution (c) and 20 μL of the test solution. If necessary, adjust the injection volume to between 10 μL and 20 μL in accordance with the results obtained in the test for linearity as described under Assay. Record the chromatograms for approximately 50 min. In the chromatogram obtained with reference solution (c), A21 desamido bovine insulin appears as a small peak after the principal peak and has a relative retention of about 1.3 with reference to the principal peak. In the chromatogram obtained with the test solution, the area of the peak due to A21 desamido bovine insulin is not greater than 3.0 per cent of the total area of the peaks; the sum of the areas of all the peaks, apart from those due to bovine insulin and A21 desamido bovine insulin, is not greater than 3.0 per cent of the total area of the peaks.

Bovine proinsulin-like immunoreactivity (PLI): maximum 10 ppm, calculated with reference to the dried substance.

Use a suitably sensitive immunochemical method (2.7.1) such as radio-immunoassay, using the International Reference Reagent for bovine proinsulin to calibrate the method.

Zinc: maximum 1.0 per cent, calculated with reference to the dried substance.

Atomic absorption spectrometry (2.2.23, Method I).

Test solution. Dissolve 50.0 mg of the substance to be examined in 0.01 M hydrochloric acid and dilute to 25.0 ml with the same acid. Dilute if necessary to a suitable concentration (for example, 0.4 µg to 1.6 µg of Zn per millilitre) with 0.01 M hydrochloric acid.

Reference solutions. Use solutions containing 0.40 µg, 0.80 µg, 1.00 µg, 1.20 µg and 1.60 µg of Zn per millilitre, freshly prepared by diluting zinc standard solution (5 mg/ml Zn) R with 0.01 M hydrochloric acid.

Source: zinc hollow-cathode lamp.

Wavelength: 213.9 nm.

Flame: air-acetylene flame of suitable composition (for example, 11 litres of air and 2 litres of acetylene per minute).

Loss on drying (2.2.32): maximum 10.0 per cent, determined on 0.200 g by drying in an oven at 100-105 °C for 24 h.

Sulphated ash (2.4.14): maximum 2.5 per cent, determined on 0.200 g and calculated with reference to the dried substance.

Bacterial endotoxins (2.6.14): less than 10 IU/mg, if intended for use in the manufacture of parenteral dosage forms without a further appropriate procedure for the removal of bacterial endotoxins.

ASSAY

Liquid chromatography (2.2.29).

Test solution. Dissolve 40.0 mg of the substance to be examined in 0.01 M hydrochloric acid and dilute to 10.0 ml with the same solvent.

Reference solution (a). Dissolve the contents of a vial of human insulin CRS in 0.01 M hydrochloric acid to obtain a concentration of 4.0 mg/ml.

Reference solution (b). Dissolve the contents of a vial of porcine insulin CRS in 0.01 M hydrochloric acid to obtain a concentration of 4.0 mg/ml.

Reference solution (c). Dissolve 40.0 mg of bovine insulin CRS in 10.0 ml of 0.01 M hydrochloric acid.

Reference solution (d). Dilute 1.0 ml of reference solution (c) to 10.0 ml with 0.01 M hydrochloric acid.

Resolution solution. Mix 1.0 ml of reference solution (a) and 1.0 ml of reference solution (b).

Maintain the solutions at 2-10 °C and use within 48 h. If an automatic injector is used, maintain the temperature at 2-10 °C.

Column:

- size: $l = 0.25$ m, $\varnothing = 4.6$ mm,
- stationary phase: octadecylsilyl silica gel for chromatography R (5 µm),

- temperature: 40 °C.

Mobile phase: mix 42 volumes of mobile phase A and 58 volumes of mobile phase B, adjusting the composition of the mixture if necessary.

Prepare and maintain the following solutions at a temperature of at least 20 °C:

- mobile phase A: dissolve 28.4 g of anhydrous sodium sulphate R in water R and dilute to 1000 ml with the same solvent; add 2.7 ml of phosphoric acid R; adjust to pH 2.3, if necessary, with ethanolamine R; filter and degas;
- mobile phase B: mix 550 ml of mobile phase A with 450 ml of acetonitrile R. Warm the solution to a temperature of at least 20 °C in order to avoid precipitation (mixing of mobile phase A with acetonitrile is endothermic); filter and degas.

Flow rate: 1 ml/min.

Detection: spectrophotometer at 214 nm.

System suitability:

- resolution: inject 20 µl of the resolution solution and 20 µl of reference solution (b). Record the chromatogram of the resolution solution until the peak corresponding to the principal peak in the chromatogram obtained with reference solution (b) is clearly visible. In the chromatogram obtained with the resolution solution, identify the peaks due to porcine insulin and human insulin. The test is not valid unless the resolution between the peaks corresponding to human insulin and porcine insulin is at least 1.2. If necessary, adjust the concentration of acetonitrile in the mobile phase until this resolution is achieved.
- linearity: inject 20 µl each of reference solutions (c) and (d). The test is not valid unless the area of the principal peak in the chromatogram obtained with reference solution (c) is 10 ± 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (d). If this test fails, adjust the injection volume to between 10 µl and 20 µl, in order that the responses are within the linearity range of the detector.

Injection: 20 µl of the test solution.

Calculate the content of bovine insulin $C_{254}H_{377}N_{65}O_{75}S_6$ plus A21 desamido bovine insulin from the area of the principal peak and the area of the peak corresponding to A21 desamido bovine insulin in the chromatograms obtained with the test solution and reference solution (c) and the declared content of bovine insulin plus A21 desamido bovine insulin in bovine insulin CRS.

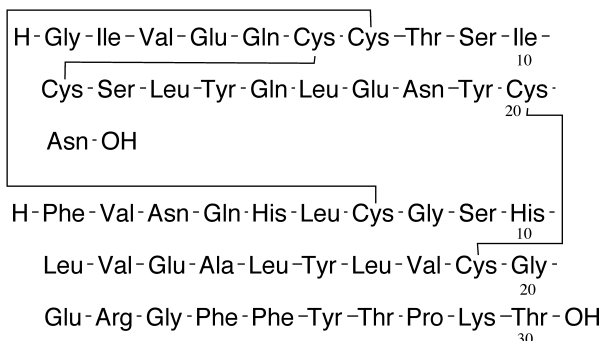
STORAGE

In an airtight container, protected from light, at -20 °C until released by the manufacturer. When thawed, insulin may be stored at 5 ± 3 °C and used for manufacturing preparations within a short period of time. To avoid absorption of humidity from the air during weighing, the insulin must be at room temperature.

LABELLING

The label states where applicable, that the substance is free from bacterial endotoxins.

01/2005:0838 B. Peptide mapping (2.2.55).

INSULIN, HUMAN**Insulinum humanum** $C_{257}H_{383}N_{65}O_{77}S_6$ M_r 5808**DEFINITION**

Human insulin is a 2-chain peptide having the structure of the antidiabetic hormone produced by the human pancreas.

Content: 95.0 per cent to 105.0 per cent of human insulin $C_{257}H_{383}N_{65}O_{77}S_6$ plus A21 desamido human insulin (dried substance).

By convention, for the purpose of labelling insulin preparations, 0.0347 mg of human insulin is equivalent to 1 IU of insulin.

PRODUCTION

Human insulin is produced either by enzymatic modification and suitable purification of insulin obtained from the pancreas of the pig or by a method based on recombinant DNA (rDNA) technology.

Human insulin is produced under conditions designed to minimise the degree of microbial contamination.

For human insulin produced by enzymatic modification of insulin obtained from the pancreas of the pig, the manufacturing process is validated to demonstrate removal of any residual proteolytic activity. The competent authority may require additional tests.

For human insulin produced by a method based on rDNA technology, prior to release the following tests are carried out on each batch of the final bulk product, unless exemption has been granted by the competent authority.

Host-cell-derived proteins. The limit is approved by the competent authority.

Single chain precursor. The limit is approved by the competent authority. Use a suitably sensitive method.

CHARACTERS

Appearance: white or almost white powder.

Solubility: practically insoluble in water and in ethanol (96 per cent). It dissolves in dilute mineral acids and with decomposition in dilute solutions of alkali hydroxides.

IDENTIFICATION

A. Examine the chromatograms obtained in the assay.

Results: the principal peak in the chromatogram obtained with the test solution is similar in retention time to the principal peak in the chromatogram obtained with reference solution (a).

SELECTIVE CLEAVAGE OF THE PEPTIDE BONDS

Test solution. Prepare a 2.0 mg/ml solution of the substance to be examined in 0.01 M hydrochloric acid and transfer 500 µl of this solution to a clean tube. Add 2.0 ml of HEPES buffer solution pH 7.5 R and 400 µl of a 1 mg/ml solution of *Staphylococcus aureus* strain V8 protease R. Cap the tube and incubate at 25 °C for 6 h. Stop the reaction by adding 2.9 ml of sulphate buffer solution pH 2.0 R.

Reference solution. Prepare at the same time and in the same manner as for the test solution but using human insulin CRS instead of the substance to be examined.

CHROMATOGRAPHIC SEPARATION. Liquid chromatography (2.2.29).

Column:

- size: $l = 0.10$ m, $\varnothing = 4.6$ mm,
- stationary phase: octadecylsilyl silica gel for chromatography R (3 µm) with a pore size of 8 nm,
- temperature: 40 °C.

Mobile phase:

- mobile phase A: mix 100 ml of acetonitrile for chromatography R, 200 ml of sulphate buffer solution pH 2.0 R and 700 ml of water R; filter and degas;
- mobile phase B: mix 200 ml of sulphate buffer solution pH 2.0 R, 400 ml of acetonitrile for chromatography R and 400 ml of water R; filter and degas;

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)
0 - 60	90 → 30	10 → 70
60 - 65	30 → 0	70 → 100
65 - 70	0	100

Flow rate: 1 ml/min.

Detection: spectrophotometer at 214 nm.

Equilibration: at initial conditions for at least 15 min. Carry out a blank run using the above-mentioned gradient.

Injection: 50 µl.

System suitability:

- the chromatograms obtained with the test solution and the reference solution are qualitatively similar to the chromatogram of human insulin digest supplied with human insulin CRS,
- in the chromatogram obtained with the reference solution, identify the peaks due to digest fragments I, II and III:

symmetry factor: maximum 1.5 for the peaks due to fragments II and III,

resolution: minimum 3.4 between the peaks due to fragments II and III.

Results: the profile of the chromatogram obtained with the test solution corresponds to that of the chromatogram obtained with the reference solution.

NOTE: the retention time of fragment I is the same for porcine insulin and for human insulin. The retention times of fragments II and IV are the same for all insulins. The retention time of fragment III is the same for bovine insulin and for porcine insulin.

TESTS

Impurities with molecular masses greater than that of insulin. Size-exclusion chromatography (2.2.30): use the normalisation procedure.

Test solution. Prepare a solution containing 4 mg/ml of the substance to be examined in 0.01 M hydrochloric acid.

Resolution solution. Use a solution of insulin (about 4 mg/ml), containing more than 0.4 per cent of high molecular mass proteins. An injectable insulin preparation, whether a solution or a suspension, that has been clarified with a sufficient amount of 6 M hydrochloric acid, containing the indicated percentage of high molecular mass proteins, or a solution prepared from insulin, dissolved in 0.01 M hydrochloric acid, may be used. Insulin containing the indicated percentage of high molecular mass proteins may be prepared by allowing insulin powder to stand at room temperature for about 10 days.

Maintain the solutions at 2-8 °C and use within 7 days. If an automatic injector is used, maintain the temperature at 2-8 °C.

Column:

- size: $l = 0.3$ m, $\varnothing =$ minimum 7.5 mm,
- stationary phase: hydrophilic silica gel for chromatography R (5-10 μ m) with a pore size of 12-12.5 nm, of a grade suitable for the separation of insulin monomer from dimer and polymers.

Mobile phase: mix 15 volumes of glacial acetic acid R, 20 volumes of acetonitrile R and 65 volumes of a 1.0 g/l solution of arginine R; filter and degas.

Flow rate: 0.5 ml/min.

Detection: spectrophotometer at 276 nm.

Equilibration: before using a new column for chromatographic analysis, equilibrate by repeated injections of an insulin solution containing high molecular mass proteins. This can be done by at least 3 injections of the resolution solution. The column is equilibrated when repeatable results are obtained from 2 subsequent injections.

Injection: 100 μ l.

Run time: about 35 min.

Retention time: polymeric insulin complexes = 13-17 min; covalent insulin dimer = about 17.5 min; insulin monomer = about 20 min; salts = about 22 min.

System suitability: resolution solution:

- peak-to-valley ratio: minimum 2.0, where H_p = height above the baseline of the peak due to the dimer and H_v = height above the baseline of the lowest point of the curve separating this peak from the peak due to the monomer.

Limits: the sum of the areas of any peaks with a retention time less than that of the principal peak is not greater than 1.0 per cent of the total area of the peaks. Disregard any peak with a retention time greater than that of the peak due to insulin.

Related proteins. Liquid chromatography (2.2.29) as described under Assay, following the elution conditions as described below:

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)
0 - 30	42	58
30 - 44	42 $\rightarrow$ 11	58 $\rightarrow$ 89
44 - 50	11	89

Maintain the solutions at 2-8 °C and use within 24 h. Perform a system suitability test (resolution, linearity) as described in the assay. If necessary, the relative proportions of the mobile phases may be adjusted to ensure complete elution of A21 desamido porcine insulin before commencement of the gradient. The profile of the gradient may also be adjusted to ensure complete elution of all insulin related impurities.

Inject 20 μ l of reference solution (a), 20 μ l of reference solution (b), 20 μ l of reference solution (c) and 20 μ l of the test solution. If necessary, adjust the injection volume to a volume between 10 μ l and 20 μ l in accordance with the results obtained in the test for linearity as described in the assay. Record the chromatograms for approximately 50 min. In the chromatogram obtained with reference solution (a), A21 desamido human insulin appears as a small peak after the principal peak and has a retention time of about 1.3 relative to the principal peak. In the chromatogram obtained with the test solution, the area of the peak due to A21 desamido human insulin is not greater than 2.0 per cent of the total area of the peaks; the sum of the areas of all peaks, apart from those due to human insulin and that due to A21 desamido human insulin, is not greater than 2.0 per cent of the total area of the peaks. For semisynthetic human insulin only: in the chromatogram obtained with the test solution, the area of any peak corresponding to the principal peak in the chromatogram obtained with reference solution (b) is not greater than the area of the corresponding peak in the chromatogram obtained with reference solution (c) (1.0 per cent of porcine insulin in human insulin).

The following test applies only to human insulin produced by enzymatic modification of porcine insulin.

Proinsulin-like immunoreactivity (PLI): maximum 10 ppm, calculated with reference to the dried substance and determined by a suitably sensitive immunochemical method (2.7.1) such as radio-immunoassay. Use the International Reference Reagent for porcine proinsulin to calibrate the method.

Zinc: maximum 1.0 per cent (dried substance).

Atomic absorption spectrometry (2.2.23, Method I).

Test solution. Dissolve 50.0 mg of the substance to be examined in 0.01 M hydrochloric acid and dilute to 25.0 ml with the same acid. Dilute if necessary to a suitable concentration (for example, 0.4-1.6 μ g of Zn per millilitre) with 0.01 M hydrochloric acid.

Reference solutions. Use solutions containing 0.40 μ g, 0.80 μ g, 1.00 μ g, 1.20 μ g and 1.60 μ g of Zn per millilitre, freshly prepared by diluting zinc standard solution (5 mg/ml Zn) R with 0.01 M hydrochloric acid.

Source: zinc hollow-cathode lamp.

Wavelength: 213.9 nm.

Atomisation device: air-acetylene flame of suitable composition (for example, 11 litres of air and 2 litres of acetylene per minute).

Loss on drying (2.2.32): maximum 10.0 per cent, determined on 0.200 g by drying in an oven at 100-105 °C for 24 h.

Sulphated ash (2.4.14): maximum 2.5 per cent, determined on 0.200 g (dried substance).

Bacterial endotoxins (2.6.14): less than 10 IU/mg, if intended for use in the manufacture of parenteral dosage forms without a further appropriate procedure for removal of bacterial endotoxins.

ASSAY

Liquid chromatography (2.2.29).

Test solution. Dissolve 40.0 mg of the substance to be examined in 0.01 M hydrochloric acid and dilute to 10.0 ml with the same solvent.

Reference solution (a). Dissolve the contents of a vial of human insulin CRS in 0.01 M hydrochloric acid to obtain a concentration of 4.0 mg/ml.

Reference solution (b). Dissolve the contents of a vial of porcine insulin CRS in 0.01 M hydrochloric acid to obtain a concentration of 4.0 mg/ml.

Reference solution (c). Dilute 1.0 ml of reference solution (b) to 50.0 ml with 0.01 M hydrochloric acid. To 1.0 ml of this solution add 1.0 ml of reference solution (a).

Reference solution (d). Dilute 1.0 ml of reference solution (a) to 10.0 ml with 0.01 M hydrochloric acid.

Resolution solution. Mix 1.0 ml of reference solution (a) and 1.0 ml of reference solution (b).

Maintain the solutions at 2-8 °C and use within 48 h. If an automatic injector is used, maintain at 2-8 °C.

Column:

- size: $l = 0.25$, $\varnothing = 4.6$ mm,
- stationary phase: octadecylsilyl silica gel for chromatography R (5 µm),
- temperature: 40 °C.

Mobile phase: mix 42 volumes of mobile phase A and 58 volumes of mobile phase B, adjusting the composition of the mixture if necessary.

Prepare and maintain the following solutions at a temperature of at least 20 °C:

- mobile phase A: dissolve 28.4 g of anhydrous sodium sulphate R in water R and dilute to 1000 ml with the same solvent; add 2.7 ml of phosphoric acid R; adjust to pH 2.3, if necessary, with ethanolamine R; filter and degas;
- mobile phase B: mix 550 ml of mobile phase A with 450 ml of acetonitrile R. Warm the solution to a temperature of at least 20 °C in order to avoid precipitation (mixing of mobile phase A with acetonitrile is endothermic); filter and degas.

Flow rate: 1 ml/min.

Detection: spectrophotometer at 214 nm.

System suitability:

- resolution: inject 20 µl of the resolution solution and 20 µl of reference solution (b). Record the chromatogram of the resolution solution until the peak corresponding to the principal peak in the chromatogram obtained with reference solution (b) is clearly visible. In the chromatogram obtained with the resolution solution, identify the peaks due to porcine insulin and human insulin. The test is not valid unless the resolution between the peaks due to human insulin and porcine insulin is at least 1.2. If necessary, adjust the concentration of acetonitrile in the mobile phase until this resolution is achieved.
- linearity: inject 20 µl each of reference solutions (a) and (d). The test is not valid unless the area of the principal peak in the chromatogram obtained with reference solution (a) is 10 ± 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (d). If this test fails, adjust the injection volume to between 10 µl and 20 µl, in order that the responses are within the linearity range of the detector.

Injection: 20 µl of the test solution and reference solution (a).

Calculate the content of human insulin $C_{257}H_{383}N_{65}O_{77}S_6$ plus A21 desamido human insulin using the areas of the corresponding peaks in the chromatograms obtained with the test solution and reference solution (a) and the declared content of human insulin plus A21 desamido human insulin in human insulin CRS.

STORAGE

In an airtight container, protected from light, at -18 °C or below, until released by the manufacturer. When thawed, insulin is stored at 5 ± 3 °C and used for manufacturing preparations within a short period of time. To avoid absorption of humidity from the air during weighing, the insulin must be at room temperature.

LABELLING

The label states:

- whether the substance is produced by enzymatic modification of porcine insulin or by rDNA technology,
- where applicable, that the substance is free from bacterial endotoxins.

01/2005:0831

INSULIN INJECTION, BIPHASIC

Insulini biphasici iniectionabilium

Biphasic insulin injection complies with the monograph on Insulin preparations, injectable (0854) with the amendments prescribed below.

DEFINITION

Biphasic insulin injection is a sterile suspension of crystals containing bovine insulin in a solution of porcine insulin.

CHARACTERS

A white suspension. When examined under a microscope, the majority of the particles are seen to be rhombohedral crystals, with a maximum dimension measured from corner to corner through the crystal greater than 10 µm but rarely exceeding 40 µm.

IDENTIFICATION

Examine the chromatograms obtained in the assay. The position of the peaks due to the two insulins in the chromatogram obtained with the test solution correspond to those of the principal peaks in the chromatogram obtained with the appropriate reference solution.

TESTS

pH (2.2.3). The pH of the suspension to be examined is 6.6 to 7.2.

Insulin in the supernatant: 22.0 per cent to 28.0 per cent of insulin in solution. Determine by the method described in the test for insulin in the supernatant in the monograph on *Insulin preparations, injectable (0854)*.

Total zinc: 26.0 µg to 37.5 µg per 100 IU of insulin. Determine by the method described in the monograph on *Insulin preparations, injectable (0854)*.

01/2005:0832 PRODUCTION

INSULIN INJECTION, BIPHASIC ISOPHANE

Insulini isophani biphasici iniectabilium

Biphasic isophane insulin injection complies with the monograph on Insulin preparations, injectable (0854) with the exception of the test for Insulin in the supernatant and with the amendments prescribed below for the other tests.

DEFINITION

Biphasic isophane insulin injection is a sterile buffered suspension of either porcine or human insulin, complexed with protamine sulphate or another suitable protamine, in a solution of insulin of the same species.

PRODUCTION

Biphasic isophane insulin injection is prepared by carrying out the procedures described in the monograph on *Insulin preparations, injectable (0854)*.

Biphasic isophane insulin injection is produced by mixing, in defined ratios, soluble insulin injection and isophane insulin injection. The defined ratios shall be demonstrated by a test method which has been approved by the competent authority to comply with the label claim.

CHARACTERS

A white suspension which on standing deposits a white sediment and leaves a colourless or almost colourless supernatant liquid; the sediment is readily resuspended by gently shaking. When examined under a microscope, the particles are seen to be rod-shaped crystals, the majority with a maximum dimension greater than 1 µm but rarely exceeding 60 µm, free from large aggregates.

IDENTIFICATION

Examine the chromatograms obtained in the Assay. The position of the peak due to insulin in the chromatogram obtained with the test solution corresponds to that of the principal peak obtained with the appropriate reference solution.

TESTS

Total zinc. Not more than 40.0 µg per 100 IU of insulin, determined as described in the monograph on *Insulin preparations, injectable (0854)*.

LABELLING

The label states in addition to the indications mentioned in the monograph on *Insulin preparations, injectable (0854)* the ratio of soluble insulin injection to isophane insulin injection used in the manufacturing process of biphasic isophane insulin injection.

01/2005:0833

INSULIN INJECTION, ISOPHANE

Insulini isophani iniectabilium

Isophane insulin injection complies with the monograph on Insulin preparations, injectable (0854) with the modifications prescribed below.

DEFINITION

Isophane insulin injection is a sterile suspension of bovine, porcine or human insulin, complexed with protamine sulphate or another suitable protamine.

PRODUCTION

Isophane insulin injection is prepared by carrying out the procedures described in the monograph on *Insulin preparations, injectable (0854)*.

The amount of protamine is based on the known isophane ratio and is not less than the equivalent of 0.3 mg and not more than the equivalent of 0.6 mg of protamine sulphate for each 100 IU of insulin in the insulin-protamine complex.

CHARACTERS

A white suspension which on standing deposits a white sediment and leaves a colourless or almost colourless supernatant liquid; the sediment is readily resuspended by gently shaking. When examined under a microscope, the particles are seen to be rod-shaped crystals, the majority with a maximum dimension greater than 1 µm but rarely exceeding 60 µm, free from large aggregates.

IDENTIFICATION

Examine the chromatograms obtained in the Assay. The position of the peak due to insulin in the chromatogram obtained with the test solution corresponds to that of the principal peak in the chromatogram obtained with the appropriate reference solution.

TESTS

Total zinc. Not more than 40.0 µg per 100 IU of insulin, determined as described in the monograph on *Insulin preparations, injectable (0854)*.

01/2005:0834

INSULIN INJECTION, SOLUBLE

Insulini solubilis iniectabilium

Soluble insulin injection complies with the monograph on Insulin preparations, injectable (0854) with the amendments prescribed below.

DEFINITION

Soluble insulin injection is a neutral, sterile solution of bovine, porcine or human insulin.

CHARACTERS

A colourless liquid, free from turbidity and foreign matter; during storage, traces of a very fine sediment may be deposited.

IDENTIFICATION

Examine the chromatograms obtained in the assay. The position of the peak due to insulin in the chromatogram obtained with the test solution corresponds to that of the principal peak obtained with the appropriate reference solution.

TESTS

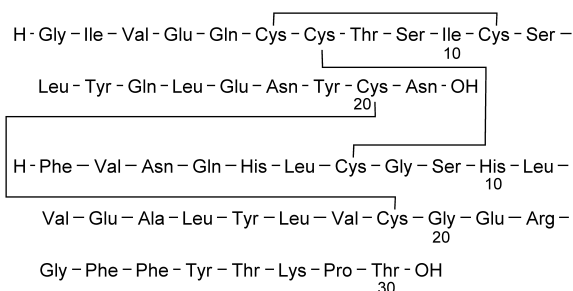
Total zinc. Not more than 40.0 µg per 100 IU of insulin.

Determine by the method described in the monograph on *Insulin preparations, injectable (0854)*.

Use the following test solution.

Test solution. Dilute a volume of the gently shaken preparation containing 200 IU to 25.0 ml with *water R*. Dilute if necessary to a suitable concentration (for example, 0.4 µg to 1.6 µg of Zn per millilitre) with *water R*.

01/2005:2085

INSULIN LISPRO**Insulinum lisprum** $C_{257}H_{383}N_{65}O_{77}S_6$ M_r 5808**DEFINITION**28^B-L-Lysine-29^B-L-proline insulin (human).

Insulin lispro is a 2-chain peptide containing 51 amino acids. The A-chain is composed of 21 amino acids and the B-chain is composed of 30 amino acids. It is identical in primary structure to human insulin, only differing in amino acid sequence at positions 28 and 29 of the B-chain. Human insulin is Pro(B28), Lys(B29), whereas insulin lispro is Lys(B28), Pro(B29). As in human insulin, insulin lispro contains 2 interchain disulphide bonds and 1 intrachain disulphide bond.

Content: 94.0 per cent to 104.0 per cent (dried substance).

By convention, for the purpose of labelling insulin lispro preparations, 0.0347 mg of insulin lispro is equivalent to 1 unit.

PRODUCTION

Insulin lispro is produced by a method based on recombinant DNA (rDNA) technology under conditions designed to minimise the degree of microbial contamination.

Prior to release the following tests are carried out on each batch of final bulk product, unless exemption has been granted by the competent authority.

Host-cell-derived proteins. The limit is approved by the competent authority.

Single-chain precursor. The limit is approved by the competent authority. Use a suitably sensitive method.

CHARACTERS

Appearance: white or almost white powder.

Solubility: practically insoluble in water and in ethanol (96 per cent). It dissolves in dilute mineral acids and with decomposition in dilute solutions of alkali hydroxides.

IDENTIFICATION

A. Examine the chromatograms obtained in the assay.

Results: the principal peak in the chromatogram obtained with the test solution is similar in retention time to the principal peak in the chromatogram obtained with the reference solution.

B. Peptide mapping (2.2.55).

SELECTIVE CLEAVAGE OF THE PEPTIDE BONDS

Test solution. Prepare a 2.0 mg/ml solution of the substance to be examined in 0.01 M hydrochloric acid and transfer 500 µl of this solution to a clean tube. Add 2.0 ml of HEPES buffer solution pH 7.5 R and 400 µl of a 1 mg/ml solution of *Staphylococcus aureus* strain

V8 protease R. Cap the tube and incubate at 25 °C for 6 h. Stop the reaction by adding 2.9 ml of sulphate buffer solution pH 2.0 R.

Reference solution. Prepare at the same time and in the same manner as for the test solution but using *insulin lispro CRS* instead of the substance to be examined.

CHROMATOGRAPHIC SEPARATION. Liquid chromatography (2.2.29).

Column:

- **size:** $l = 0.10$ m, $\varnothing = 4.6$ mm,
- **stationary phase:** octadecylsilyl silica gel for chromatography R (3 µm) with a pore size of 8 nm,
- **temperature:** 40 °C.

Mobile phase:

- **mobile phase A:** mix 100 ml of acetonitrile for chromatography R, 200 ml of sulphate buffer solution pH 2.0 R and 700 ml of water R; filter and degas;
- **mobile phase B:** mix 200 ml of sulphate buffer solution pH 2.0 R, 400 ml of acetonitrile for chromatography R and 400 ml of water R; filter and degas;

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)
0 - 60	90 → 30	10 → 70
60 - 65	30 → 0	70 → 100
65 - 70	0	100

Flow rate: 1 ml/min.

Detection: spectrophotometer at 214 nm.

Equilibration: at initial conditions for at least 15 min. Carry out a blank run using the above-mentioned gradient.

Injection: 50 µl.

System suitability:

- the chromatograms obtained with the test solution and the reference solution are qualitatively similar to the chromatogram of insulin lispro digest supplied with *insulin lispro CRS*,
- in the chromatogram obtained with the reference solution, identify the peaks due to digest fragments I, II and III:
symmetry factor: maximum 1.5 for the peaks due to fragments II and III,
resolution: minimum 8.0 between the peaks due to fragments II and III.

Results: the profile of the chromatogram obtained with the test solution corresponds to that of the chromatogram obtained with the reference solution.

NOTE: the retention times of fragments I, II and IV are the same as for human insulin. The retention time of fragment III differs from human insulin due to differences in sequence at positions 28 and 29 of the B-chain.

TESTS

Impurities with molecular masses greater than that of insulin lispro. Size-exclusion chromatography (2.2.30): use the normalisation procedure.

Test solution. Prepare a solution containing 4 mg/ml of the substance to be examined in 0.01 M hydrochloric acid. Maintain the solution at 2-8 °C and use within 48 h.

Resolution solution. Use a solution of insulin (about 4 mg/ml), containing more than 0.4 per cent of high molecular mass proteins. An injectable insulin preparation, whether a solution or a suspension, that has been clarified

with a sufficient amount of 6 M hydrochloric acid, containing the indicated percentage of high molecular mass proteins, or a solution prepared from insulin, dissolved in 0.01 M hydrochloric acid, may be used. Insulin containing the indicated percentage of high molecular mass proteins may be prepared by allowing insulin powder to stand at room temperature for about 10 days. Maintain the solution at 2-8 °C and use within 8 days.

Column:

- size: $l = 0.30$ m, $\varnothing = 7.8$ mm,
- stationary phase: hydrophilic silica gel for chromatography R (5-10 μm) with a pore size of 12-12.5 nm, of a grade suitable for the separation of insulin monomer from dimer and polymers.

Mobile phase: mix 15 volumes of glacial acetic acid R, 20 volumes of acetonitrile for chromatography R and 65 volumes of a 1.0 g/l solution of arginine R; filter and degas.

Flow rate: 0.5 ml/min.

Detection: spectrophotometer at 276 nm.

Equilibration: at least 3 injections of the resolution solution; the column is equilibrated when repeatable results are obtained for 2 subsequent injections.

Injection: 100 μl .

Run time: about 35 min.

Retention time: insulin lispro polymers = 13-17 min; insulin lispro dimer = about 17.5 min; insulin lispro monomer = about 20 min; salts = about 22 min.

System suitability: resolution solution:

- peak-to-valley ratio: minimum 2.0, where H_p = height above the baseline of the peak due to the dimer and H_v = height above the baseline of the lowest point of the curve separating this peak from the peak due to the monomer,
- symmetry factor: maximum 2.0 for the peak due to insulin lispro.

Limits: the sum of the areas of the peaks with a retention time less than that of the principal peak is not more than 0.25 per cent of the total area of the peaks. Disregard any peak with a retention time greater than that of the peak due to insulin lispro monomer.

Related proteins. Liquid chromatography (2.2.29): use the normalisation procedure.

Test solution. Dissolve 3.5 mg of the substance to be examined in 1.0 ml of 0.01 M hydrochloric acid. Maintain the solution at 2-8 °C and use within 56 h.

Resolution solution. Dissolve 3.5 mg of the substance to be examined in 1.0 ml of 0.01 M hydrochloric acid. Allow to stand at room temperature to obtain a solution containing between 0.8 per cent and 11 per cent of A21 desamido insulin lispro.

Column:

- size: $l = 0.25$ m, $\varnothing = 4.6$ mm,
- stationary phase: octadecylsilyl silica gel for chromatography R (5 μm) with a pore size of 30 nm,
- temperature: 40 °C.

Mobile phase:

- mobile phase A: mix 82 volumes of a 28.4 g/l solution of anhydrous sodium sulphate R adjusted to pH 2.3 with phosphoric acid R and 18 volumes of acetonitrile for chromatography R; filter and degas;

- mobile phase B: mix equal volumes of a 28.4 g/l solution of anhydrous sodium sulphate R adjusted to pH 2.3 with phosphoric acid R and acetonitrile for chromatography R; filter and degas;

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)
0 - 60	81	19
60 - 83	81 → 51	19 → 49
83 - 84	51 → 81	49 → 19
84 - 94	81	19

Flow rate: 1 ml/min.

Detection: spectrophotometer at 214 nm.

Injection: 20 μl .

Retention time: adjust the mobile phase composition to obtain a retention time of about 41 min for insulin lispro; A21 desamido insulin lispro elutes near the start of the gradient elution.

System suitability: resolution solution:

- resolution: minimum 1.5 between the 1st peak (insulin lispro) and the 2nd peak (A21 desamido insulin lispro),
- symmetry factor: maximum 2.0 for the peak due to insulin lispro.

Limits:

- A21 desamido insulin lispro: maximum 1.0 per cent,
- any other impurity: maximum 0.50 per cent,
- total (excluding A21): maximum 2.0 per cent.

Zinc: maximum 1.0 per cent (dried substance).

Atomic absorption spectrometry (2.2.23, Method I).

Test solution. Dissolve at least 50 mg of the substance to be examined in 0.01 M hydrochloric acid and dilute to 25 ml with the same acid. Dilute if necessary to a suitable concentration (for example 0.4-0.6 μg of Zn per millilitre) with 0.01 M hydrochloric acid.

Reference solutions. Use solutions of concentrations which bracket the expected zinc concentration of the samples, for example, 0.2-0.8 μg of Zn per millilitre, freshly prepared by diluting zinc standard solution (5 mg/ml Zn) R with 0.01 M hydrochloric acid.

Source: zinc hollow-cathode lamp.

Wavelength: 213.9 nm.

Atomisation device: air-acetylene flame of suitable composition (for example, 11 litres of air and 2 litres of acetylene per minute).

Loss on drying (2.2.32): maximum 10.0 per cent, determined on 0.200 g by drying in an oven at 100-105 °C for 16 h.

Sulphated ash (2.4.14): maximum 2.5 per cent, determined on 0.200 g (dried substance).

Bacterial endotoxins (2.6.14, Method D): less than 10 IU/mg, if intended for use in the manufacture of parenteral dosage forms without a further appropriate procedure for the removal of bacterial endotoxins.

ASSAY

Liquid chromatography (2.2.29).

Test solution. Dissolve the substance to be examined in 0.01 M hydrochloric acid to obtain a concentration of 0.8 mg/ml. Maintain the solution at 2-8 °C and use within 48 h.

Reference solution. Dissolve the contents of a vial of insulin lispro CRS in 0.01 M hydrochloric acid to obtain a concentration of 0.8 mg/ml. Maintain the solution at 2-8 °C and use within 48 h.

Resolution solution. Dissolve about 10 mg of the substance to be examined in 10 ml of 0.01 M hydrochloric acid. Allow to stand at room temperature to obtain a solution containing between 0.8 per cent and 11 per cent of A21 desamido insulin lispro. Maintain the solution at 2-8 °C and use within 14 days.

Column:

- size: $l = 0.10$ m, $\varnothing = 4.6$ mm,
- stationary phase: octadecylsilyl silica gel for chromatography R ($3\ \mu\text{m}$) with a pore size of 8 nm,
- temperature: $40\ ^\circ\text{C}$.

Mobile phase: mix 745 volumes of a 28.4 g/l solution of *anhydrous sodium sulphate R* adjusted to pH 2.3 with *phosphoric acid R* and 255 volumes of *acetonitrile for chromatography R*; filter and degas.

Flow rate: 0.8 ml/min.

Detection: spectrophotometer at 214 nm.

Injection: 20 μ l.

Retention time: insulin lispro = about 24 min.

System suitability:

- **resolution**: minimum 1.8 between the 1st peak (insulin lispro) and the 2nd peak (A21 desamido insulin lispro), in the chromatogram obtained with the resolution solution,
- **repeatability**: maximum relative standard deviation of 1.1 per cent after 3 injections of the reference solution.

Calculate the content of insulin lispro $C_{257}H_{383}N_{65}O_{77}S_6$ using the chromatograms obtained with the test solution and the reference solution and the declared content of $C_{257}H_{383}N_{65}O_{77}S_6$ in *insulin lispro CRS*.

STORAGE

In an airtight container, protected from light, at or below – 18 °C. When thawed, insulin lispro is stored and weighed under conditions defined by the manufacturer to maintain the quality attributes of the drug substance and is used for manufacturing preparations within a short period of time. To avoid absorption of humidity from the air during weighing, insulin lispro must be at room temperature before opening the container.

LABELLING

The label states, where applicable, that the substance is free from bacterial endotoxins.

DEFINITION

Porcine insulin is the natural antidiabetic principle obtained from pork pancreas and purified. The content of porcine insulin C₂₅₆H₃₈₁N₆₅O₇₆S₆ plus A21 desamido porcine insulin is not less than 95.0 per cent and not more than 105.0 per cent, calculated with reference to the dried substance.

By convention, for the purpose of labelling insulin preparations, 0.0345 mg of porcine insulin is equivalent to 1 IU of insulin.

PRODUCTION

The animals from which porcine insulin is derived must fulfil the requirements for the health of animals suitable for human consumption to the satisfaction of the competent authority. The manufacturing process is validated to demonstrate suitable inactivation or removal of any contamination by viruses or other infectious agents.

CHARACTERS

Appearance: white or almost white powder.

Solubility: practically insoluble in water and in ethanol. It dissolves in dilute mineral acids and with decomposition in dilute solutions of alkali hydroxides.

IDENTIFICATION

A. Examine the chromatograms obtained in the assay.

Results: the retention time of the principal peak in the chromatogram obtained with the test solution corresponds to that of the principal peak in the chromatogram obtained with reference solution (b).

B. Peptide mapping.

Test solution. Prepare a 2.0 mg/ml solution of the substance to be examined in 0.01 M hydrochloric acid and transfer 500 µl of this solution to a clean tube. Add 2.0 ml of HEPES buffer solution pH 7.5 R and 400 µl of a 1 mg/ml solution of *Staphylococcus aureus* strain V8 protease R. Cap the tube and incubate at 25 °C for 6 h. Stop the reaction by adding 2.9 ml of sulphate buffer solution pH 2.0 R.

Reference solution. Prepare at the same time and in the same manner as for the test solution but using *porcine insulin CRS* instead of the substance to be examined.

Examine the digests by liquid chromatography (2.2.29).

Column:

- size: $l = 0.10$ m, $\varnothing = 4.6$ mm,
- stationary phase: octadecylsilyl silica gel for chromatography R (3 μ m),
- temperature: 40 °C.

Mobile phase:

- *mobile phase A*: mix 100 ml of *acetonitrile for chromatography R*, 700 ml of *water R* and 200 ml of *sulphate buffer solution pH 2.0 R*; filter and degas;
- *mobile phase B*: mix 400 ml of *acetonitrile for chromatography R*, 400 ml of *water R* and 200 ml of *sulphate buffer solution pH 2.0 R*; filter and degas;

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)
0 - 60	90 → 30	10 → 70
60 - 65	30 → 0	70 → 100
65 - 70	0	100

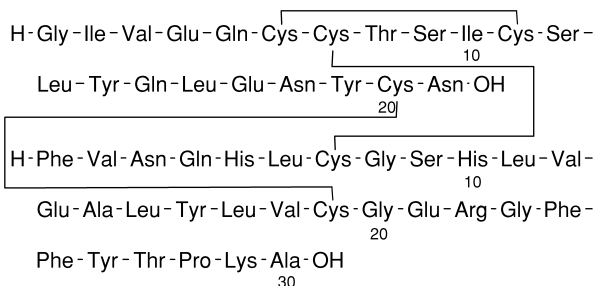
Flow rate: 1 ml/min.

Detection: spectrophotometer at 214 nm.

01/2005:1638
corrected

INSULIN, PORCINE

Insulinum porcinum


$$\text{C}_{256}\text{H}_{381}\text{N}_{65}\text{O}_{76}\text{S}_6$$

M. 5778

Equilibration: at initial conditions for at least 15 min. Carry out a blank run using the above-mentioned gradient.

Injection: 50 µl.

System suitability: the chromatograms obtained with the test solution and the reference solution are qualitatively similar to the chromatogram of porcine insulin digest supplied with *porcine insulin CRS*. In the chromatogram obtained with the reference solution, identify the peaks due to digest fragments I, II and III. The symmetry factor of the peaks due to fragments II and III is not greater than 1.5, and the resolution between the 2 peaks is at least 1.9.

Results: the profile of the chromatogram obtained with the test solution corresponds to that of the chromatogram obtained with the reference solution.

NOTE: the retention time of fragment I is the same for porcine insulin and for human insulin. The retention times of fragments II and IV are the same for all insulins. The retention time of fragment III is the same for bovine insulin and for porcine insulin.

TESTS

Impurities with molecular masses greater than that of insulin. Size-exclusion chromatography (2.2.30): use the normalisation procedure.

Test solution. Dissolve 4 mg of the substance to be examined in 1.0 ml of 0.01 M hydrochloric acid.

Resolution solution. Use a solution of insulin (approximately 4 mg/ml), containing more than 0.4 per cent of high molecular mass proteins. An injectable insulin preparation, whether a solution or a suspension, that has been clarified with a sufficient amount of 6 M hydrochloric acid, containing the indicated percentage of high molecular mass proteins, or a solution prepared from insulin, dissolved in 0.01 M hydrochloric acid, may be used. Insulin containing the indicated percentage of high molecular mass proteins may be prepared by allowing insulin powder to stand at room temperature for about 10 days.

Maintain the solutions at 2-10 °C and use within 7 days. If an automatic injector is used, maintain the temperature at 2-10 °C.

Column:

- size: $l = 0.3$ m, $\varnothing =$ at least 7.5 mm,
- stationary phase: hydrophilic silica gel for chromatography R (5-10 µm), of a grade suitable for the separation of insulin monomer from dimer and polymers.

Mobile phase: mix 15 volumes of glacial acetic acid R, 20 volumes of acetonitrile R and 65 volumes of a 1.0 g/l solution of arginine R; filter and degas.

Flow rate: 0.5 ml/min.

Detection: spectrophotometer at 276 nm.

Equilibration: before using a new column for chromatographic analysis, equilibrate by repeated injections of an insulin solution containing high molecular mass proteins. This can be done by at least 3 injections of the resolution solution. The column is equilibrated when repeatable results are obtained from 2 subsequent injections.

Injection: 100 µl.

Run time: about 35 min.

Retention times: polymeric insulin complexes = 13-17 min; covalent insulin dimer = about 17.5 min; insulin monomer = about 20 min; salts = about 22 min.

System suitability: resolution solution:

- peak-to-valley ratio: minimum 2.0, where H_p = height above the baseline of the peak due to the dimer and H_v = height above the baseline of the lowest point of the curve separating this peak from the peak due to the monomer.

Limits: the sum of the areas of any peaks with a retention time less than that of the principal peak is not greater than 1.0 per cent of the total area of the peaks. Disregard any peak with a retention time greater than that of the insulin peak.

Related proteins. Liquid chromatography (2.2.29) as described under Assay, following the elution conditions as described in the table below:

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)
0 - 30	42	58
30 - 44	42 → 11	58 → 89
44 - 50	11	89

Maintain the solutions at 2-10 °C and use within 24 h. Perform a system suitability test (resolution, linearity) as described under Assay. If necessary, the relative proportions of the mobile phases may be adjusted to ensure complete elution of A21 desamido porcine insulin before commencement of the gradient. The profile of the gradient may also be adjusted to ensure complete elution of all insulin related impurities.

Inject 20 µl of reference solution (b) and 20 µl of the test solution. If necessary, adjust the injection volume to between 10 µl and 20 µl in accordance with the results obtained in the test for linearity as described under Assay. Record the chromatograms for approximately 50 min. In the chromatogram obtained with reference solution (b), A21 desamido porcine insulin appears as a small peak after the principal peak and has a relative retention of about 1.3 with reference to the principal peak. In the chromatogram obtained with the test solution, the area of the peak due to A21 desamido porcine insulin is not greater than 2.0 per cent of the total area of the peaks; the sum of the areas of all the peaks, apart from those due to porcine insulin and A21 desamido porcine insulin, is not greater than 2.0 per cent of the total area of the peaks.

Porcine proinsulin-like immunoreactivity (PLI): maximum 10 ppm, calculated with reference to the dried substance. Use a suitably sensitive immunochemical method (2.7.1) such as radio-immunoassay, using the International Reference Reagent for porcine proinsulin to calibrate the method.

Zinc: maximum 1.0 per cent, calculated with reference to the dried substance.

Atomic absorption spectrometry (2.2.23, Method I).

Test solution. Dissolve 50.0 mg of the substance to be examined in 0.01 M hydrochloric acid and dilute to 25.0 ml with the same acid. Dilute if necessary to a suitable concentration (for example, 0.4 µg to 1.6 µg of Zn per millilitre) with 0.01 M hydrochloric acid.

Reference solutions. Use solutions containing 0.40 µg, 0.80 µg, 1.00 µg, 1.20 µg and 1.60 µg of Zn per millilitre, freshly prepared by diluting zinc standard solution (5 mg/ml Zn) R with 0.01 M hydrochloric acid.

Source: zinc hollow-cathode lamp.

Wavelength: 213.9 nm.

Flame: air-acetylene flame of suitable composition (for example, 11 litres of air and 2 litres of acetylene per minute).

Loss on drying (2.2.32): maximum 10.0 per cent, determined on 0.200 g by drying in an oven at 100-105 °C for 24 h.

Sulphated ash (2.4.14): maximum 2.5 per cent, determined on 0.200 g and calculated with reference to the dried substance.

Bacterial endotoxins (2.6.14): less than 10 IU/mg, if intended for use in the manufacture of parenteral dosage forms without a further appropriate procedure for the removal of bacterial endotoxins.

ASSAY

Liquid chromatography (2.2.29).

Test solution. Dissolve 40.0 mg of the substance to be examined in 0.01 M hydrochloric acid and dilute to 10.0 ml with the same solvent.

Reference solution (a). Dissolve the contents of a vial of human insulin CRS in 0.01 M hydrochloric acid to obtain a concentration of 4.0 mg/ml.

Reference solution (b). Dissolve the contents of a vial of porcine insulin CRS in 0.01 M hydrochloric acid to obtain a concentration of 4.0 mg/ml.

Reference solution (c). Dilute 1.0 ml of reference solution (b) to 10.0 ml with 0.01 M hydrochloric acid.

Resolution solution. Mix 1.0 ml of reference solution (a) and 1.0 ml of reference solution (b).

Maintain the solutions at 2–10 °C and use within 48 h. If an automatic injector is used, maintain the temperature at 2–10 °C.

Column:

- size: $l = 0.25$, $\varnothing = 4.6$ mm,
- stationary phase: octadecylsilyl silica gel for chromatography R (5 µm),
- temperature: 40 °C.

Mobile phase: mix 42 volumes of mobile phase A and 58 volumes of mobile phase B, adjusting the composition of the mixture if necessary.

Prepare and maintain the following solutions at a temperature of at least 20 °C:

- **mobile phase A:** dissolve 28.4 g of anhydrous sodium sulphate R in water R and dilute to 1000 ml with the same solvent; add 2.7 ml of phosphoric acid R; adjust to pH 2.3, if necessary, with ethanolamine R; filter and degas;
- **mobile phase B:** mix 550 ml of mobile phase A with 450 ml of acetonitrile R. Warm the solution to a temperature of at least 20 °C in order to avoid precipitation (mixing of mobile phase A with acetonitrile is endothermic); filter and degas.

Flow rate: 1 ml/min.

Detection: spectrophotometer at 214 nm.

System suitability:

- **resolution:** inject 20 µl of the resolution solution and 20 µl of reference solution (b). Record the chromatogram of the resolution solution until the peak corresponding to the principal peak in the chromatogram obtained with reference solution (b) is clearly visible. In the chromatogram obtained with the resolution solution, identify the peaks due to porcine insulin and human insulin. The test is not valid unless the resolution between the peaks corresponding to human insulin and porcine insulin is at least 1.2. If necessary, adjust the concentration of acetonitrile in the mobile phase until this resolution is achieved.
- **linearity:** inject 20 µl each of reference solutions (b) and (c). The test is not valid unless the area of the principal peak in the chromatogram obtained with reference solution (b) is 10 ± 0.5 times the area of the principal peak

in the chromatogram obtained with reference solution (c). If this test fails, adjust the injection volume to between 10 µl and 20 µl, in order that the responses are within the linearity range of the detector.

Injection: 20 µl of the test solution.

Calculate the content of porcine insulin $C_{256}H_{381}N_{65}O_{76}S_6$ plus A21 desamido porcine insulin from the area of the principal peak and the area of the peak corresponding to A21 desamido porcine insulin in the chromatograms obtained with the test solution and reference solution (b) and the declared content of porcine insulin plus A21 desamido porcine insulin in porcine insulin CRS.

STORAGE

In an airtight container, protected from light, at –20 °C until released by the manufacturer. When thawed, insulin may be stored at 5 ± 3 °C and used for manufacturing preparations within a short period of time. To avoid absorption of humidity from the air during weighing, the insulin must be at room temperature.

LABELLING

The label states where applicable, that the substance is free from bacterial endotoxins.

01/2005:0854

INSULIN PREPARATIONS, INJECTABLE

Praeparationes insulini iniectionabiles

Injectable insulin preparations comply with the requirements for Injections prescribed in the monograph on Parenteral preparations (0520).

DEFINITION

Injectable insulin preparations are sterile preparations of *Insulin, human (0838)*, *Insulin, bovine (1637)* or *Insulin, porcine (1638)*. They contain not less than 90.0 per cent and not more than the equivalent of 110.0 per cent of the amount of insulin stated on the label. They are either solutions or suspensions or they are prepared by combining solutions and suspensions.

PRODUCTION

The methods of preparation are designed to confer suitable properties with respect to the onset and duration of therapeutic action.

The following procedures are carried out in a suitable sequence, depending on the method of preparation:

- addition of suitable antimicrobial preservatives,
- addition of a suitable substance or substances to render the preparation isotonic with blood,
- addition of a suitable substance or substances to adjust the pH to the appropriate value,
- determination of the strength of the insulin-containing component or components followed, where necessary, by adjustment so that the final preparation contains the requisite number of International Units per millilitre,
- sterilisation by filtration of the insulin-containing component or components; once this procedure has been carried out all subsequent procedures are carried out aseptically using materials that have been sterilised by a suitable method.

In addition, where appropriate, suitable substances are added and suitable procedures carried out to confer the appropriate physical form on the insulin-containing component or

components. The final preparation is distributed aseptically into sterile containers which are closed so as to exclude microbial contamination.

TESTS

pH (2.2.3). The pH of the solution or suspension is 6.9 to 7.8, unless otherwise prescribed in the specific monograph.

Insulin in the supernatant. For injectable insulin preparations that are suspensions, not more than 2.5 per cent of the total insulin content, unless otherwise stated. Centrifuge 10 ml of the suspension at 1500 *g* for 10 min and carefully separate the supernatant liquid and the residue. Determine the insulin content of the supernatant liquid (*S*) by a suitable method, for example using the chromatographic conditions described under Assay. Calculate the percentage of the insulin in solution from the expression:

$$\frac{100S}{T}$$

where *T* is the total insulin content determined as described under the Assay.

Impurities with molecular masses greater than that of insulin. Examine by size-exclusion chromatography (2.2.30).

Test solution. Add 4 µl of 6 *M* hydrochloric acid per millilitre of the preparation to be examined, whether a suspension or a solution, to obtain a clear acid insulin solution. When sampling a suspension, agitate the material prior to sampling in order to obtain a homogeneous sample. If a suspension does not turn clear within 5 min of the initial addition of hydrochloric acid, add small aliquots of acid (less than 4 µl per millilitre) until a solution is obtained. Preparations with concentrations higher than 100 IU/ml need to be diluted with 0.01 *M* hydrochloric acid to avoid overloading the column with insulin monomer.

Resolution solution. Use a solution of insulin (approximately 4 mg/ml), containing more than 0.4 per cent of high molecular mass proteins. An injectable insulin preparation, whether a solution or a suspension, that has been clarified with a sufficient amount of 6 *M* hydrochloric acid, containing the indicated percentage of high molecular mass proteins, or a solution prepared from insulin, dissolved in 0.01 *M* hydrochloric acid, may be used. Insulin containing the indicated percentage of high molecular mass proteins may be prepared by allowing insulin powder to stand at room temperature for about ten days.

Maintain the solutions at 2 °C to 10 °C and use within 30 h (soluble insulin injection) or 7 days (other insulin preparations). If an automatic injector is used, maintain the temperature at 2 °C to 10 °C.

The chromatographic procedure may be carried out using:

- a column 0.3 m long and at least 7.5 mm in internal diameter packed with *hydrophilic silica gel for chromatography R* (5 µm to 10 µm), of a grade suitable for the separation of insulin monomer from dimers and polymers,
- as mobile phase at a flow rate of 0.5 ml/min a mixture consisting of 15 volumes of *glacial acetic acid R*, 20 volumes of *acetonitrile R* and 65 volumes of a 1.0 g/l solution of *arginine R*; filter and degas,
- as detector a spectrophotometer set at 276 nm.

Equilibration of the column. Before using a new column for chromatographic analysis, equilibrate by repeated injections of an insulin solution containing high molecular mass proteins. This can be done by at least three injections of the resolution solution. The column is equilibrated

when repeatable results are obtained from two subsequent injections. If protamine-containing samples are to be analysed, the equilibration of the column is performed using a solution containing protamine.

Inject 100 µl of the resolution solution. When the chromatograms are recorded under the prescribed conditions, the retention times are: polymeric insulin complexes or covalent insulin-protamine complex: about 13 min to 17 min, covalent insulin dimer: about 17.5 min, insulin monomer: about 20 min, salts: about 22 min. If the sample solution contains preservatives, for example methyl paraben, *m*-cresol or phenol, these compounds elute later. The test is not valid unless the resolution, defined by the ratio of the height of the dimer peak to the height above the baseline of the valley separating the monomer and dimer peaks, is at least 2.0.

Inject 100 µl of the test solution. Record the chromatogram for approximately 35 min. In the chromatogram obtained, the sum of the areas of any peak with a retention time less than that of the insulin peak is not greater than 3.0 per cent (protamine containing preparations) or 2.0 per cent (non-protamine containing preparations) of the total area of the peaks. Disregard any peak with a retention time greater than that of the insulin peak.

Related proteins. Examine by liquid chromatography (2.2.29) as described under Assay, following the elution conditions as described in the table below:

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)	Comment
0 - 30	42	58	isocratic
30 - 44	42 → 11	58 → 89	linear gradient
44 - 50	11	89	isocratic

Maintain the solutions at 2 °C to 10 °C and use within 24 h. Perform a system suitability check (resolution, linearity) as described under Assay. If necessary, the relative proportions of the mobile phases may be adjusted to ensure complete elution of A21 desamido porcine insulin before commencement of the gradient. The profile of the gradient may also be adjusted to ensure complete elution of all insulin related impurities.

Inject 20 µl of the test solution and 20 µl of either reference solution (a), for insulin preparations containing 100 IU/ml, or reference solution (b), for insulin preparations containing 40 IU/ml. If necessary, adjust the injection volume to a volume between 10 µl and 20 µl in accordance with the results obtained in the test for linearity as described under Assay. Record the chromatograms for approximately 50 min. If necessary, make further adjustments to the mobile phase in order to ensure that the antimicrobial preservatives present in the test solution are well separated from the insulin and show a shorter retention time. A small reduction in the concentration of acetonitrile increases the retention time of the insulin peaks relatively more than those of the preservatives. In the chromatogram obtained with either reference solution (a), or reference solution (b), as appropriate, A21 desamido insulin appears as a small peak after the principal peak and has a retention time of about 1.3 relative to the principal peak, due to insulin. In the chromatogram obtained with the test solution the area of the peak due to A21 desamido insulin is not greater than 5.0 per cent of the total area of the peaks; the sum of the areas of any other peaks, apart from those due to insulin and A21 desamido insulin is not greater than 6.0 per cent of the total area of the peaks. Disregard the peaks due to the preservatives and protamine (early eluting peaks).

Total zinc. Not more than the amount stated in the individual monograph, determined by atomic absorption spectrometry (2.2.23, *Method I*).

Use the following method, unless otherwise prescribed in the specific monograph.

Test solution. Shake the preparation gently and dilute a volume containing 200 IU of insulin to 25.0 ml with 0.01 M hydrochloric acid. Dilute if necessary to a suitable concentration of zinc (for example 0.4 µg to 1.6 µg of Zn per millilitre) with 0.01 M hydrochloric acid.

Reference solutions. Use solutions containing 0.40 µg, 0.80 µg, 1.00 µg, 1.20 µg and 1.60 µg of Zn per millilitre, freshly prepared by diluting *zinc standard solution* (5 mg/ml Zn) R with 0.01 M hydrochloric acid.

Measure the absorbance at 213.9 nm using a zinc hollow-cathode lamp as source of radiation and an air-acetylene flame of suitable composition (for example 11 litres of air and 2 litres of acetylene per minute).

Zinc in solution. Where applicable, not more than the amount stated in the individual monograph, determined by atomic absorption spectrometry (2.2.23, *Method I*).

Test solution. Centrifuge the preparation to be examined and dilute 1 ml of the clear supernatant liquid obtained to 25.0 ml with *water R*. Dilute if necessary to a suitable concentration of zinc (for example 0.4 µg to 1.6 µg of Zn per millilitre) with *water R*.

Reference solutions. Use solutions containing 0.40 µg, 0.80 µg, 1.00 µg, 1.20 µg and 1.60 µg of Zn per millilitre, freshly prepared by diluting *zinc standard solution* (5 mg/ml Zn) R with 0.01 M hydrochloric acid.

Measure the absorbance at 213.9 nm using a zinc hollow-cathode lamp as source of radiation and an air-acetylene flame of suitable composition (for example 11 litres of air and 2 litres of acetylene per minute).

Bacterial endotoxins (2.6.14): less than 80 IU per 100 IU of insulin.

ASSAY

Examine by liquid chromatography (2.2.29).

Test solution. Add 4 µl of 6 M hydrochloric acid per millilitre of the preparation to be examined, whether a suspension or a solution, to obtain a clear solution. When sampling a suspension, shake the material prior to sampling in order to obtain a homogeneous sample. If a suspension does not turn clear within 5 min of the initial addition of acid, add small aliquots of acid (less than 4 µl per millilitre) until a solution is obtained. For a preparation containing more than 100 IU/ml, an additional dilution with 0.01 M hydrochloric acid is necessary to avoid overloading the column.

Reference solution (a). For a preparation containing a single species of insulin, dissolve in 0.01 M hydrochloric acid, as appropriate, the contents of a vial of *human insulin CRS* or *porcine insulin CRS*, or a defined quantity of *bovine insulin CRS* to obtain a concentration of 4.0 mg/ml. For a preparation containing both bovine and porcine insulins, mix 1.0 ml of a solution containing 4.0 mg of *bovine insulin CRS* per millilitre of 0.01 M hydrochloric acid and 1.0 ml of a solution containing 4.0 mg of *porcine insulin CRS* per millilitre of 0.01 M hydrochloric acid. *Reference solution (a) is used for the assay of insulin preparations containing 100 IU/ml.*

Reference solution (b). Dilute 4.0 ml of reference solution (a) to 10.0 ml with 0.01 M hydrochloric acid. *Reference solution (b) is used for the assay of insulin preparations containing 40 IU/ml.*

Reference solution (c). Dissolve the contents of a vial of *human insulin CRS* in 0.01 M hydrochloric acid to obtain a concentration of 4.0 mg/ml.

Reference solution (d). Dissolve the contents of a vial of *porcine insulin CRS* in 0.01 M hydrochloric acid to obtain a concentration of 4.0 mg/ml.

Reference solution (e). Dilute 1.0 ml of reference solution (a) to 10.0 ml with 0.01 M hydrochloric acid.

Reference solution (f). Dilute 1.0 ml of reference solution (b) to 10.0 ml with 0.01 M hydrochloric acid.

Resolution solution. Mix 1.0 ml of reference solution (c) and 1.0 ml of reference solution (d).

Maintain the solutions at 2 °C to 10 °C and use within 48 h. If an automatic injector is used, maintain the temperature at 2 °C to 10 °C.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4.6 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (5 µm),
- as mobile phase at a flow rate of 1 ml/min the following solutions prepared and maintained at a temperature not lower than 20 °C:

Mobile phase A. Dissolve 28.4 g of *anhydrous sodium sulphate R* in *water R* and dilute to 1000 ml with the same solvent; add 2.7 ml of *phosphoric acid R*; adjust the pH to 2.3, if necessary, with *ethanolamine R*; filter and degas,

Mobile phase B. Mix 550 ml of mobile phase A with 450 ml of *acetonitrile R*. Warm the solution to a temperature not lower than 20 °C in order to avoid precipitation (mixing of mobile phase A with acetonitrile is endothermic); filter and degas,

- as detector a spectrophotometer set at 214 nm, maintaining the temperature of the column at 40 °C.

Elute with a mixture of 42 volumes of mobile phase A and 58 volumes of mobile phase B, adjusted if necessary.

Inject 20 µl of the resolution solution and 20 µl of reference solution (d). Record the chromatogram of the resolution solution until the peak corresponding to the principal peak in the chromatogram obtained with reference solution (d) is clearly visible. In the chromatogram obtained with the resolution solution, identify the peaks due to porcine insulin and human insulin. The test is not valid unless the resolution between the peaks due to human insulin and porcine insulin is at least 1.2. If necessary, adjust the concentration of acetonitrile in the mobile phase until this resolution is achieved.

Inject 20 µl of the test solution and 20 µl of either reference solutions (a) and (e), for insulin preparations containing 100 IU/ml, or 20 µl of reference solutions (b) and (f), for insulin preparations containing 40 IU/ml. If necessary, make further adjustments of the mobile phase in order to ensure that the antimicrobial preservatives present in the test solution are well separated from the insulin and show shorter retention times. A small reduction in the concentration of acetonitrile increases the retention time of the insulin peaks relatively more than those of the preservatives. If necessary, after having carried out the chromatography of a solution wash the column with a mixture of equal volumes of *acetonitrile R* and *water R* for a sufficient time to ensure elution of any interfering substances before injecting the next solution. The test is not valid unless the area of the principal peak in the chromatogram obtained with reference solution (a) or (b) is 10 ± 0.5 times the area of the principal peak in the chromatogram obtained

with reference solution (e) or (f). If this test fails, adjust the injection volume between 10 µl and 20 µl, in order to be in the linearity range of the detector.

Calculate the content of insulin plus A21 desamido insulin from the area of the peak due to the bovine, porcine or human insulin and that of any peak due to the A21 desamido insulin, using the declared content of insulin plus A21 desamido insulin in *bovine insulin CRS*, *porcine insulin CRS* or *human insulin CRS*, as appropriate. For preparations containing both bovine and porcine insulin use the sum of the areas of both the bovine and porcine insulin peaks and of the peaks due to the A21 desamido insulin⁽¹⁾ derivatives.

STORAGE

Unless otherwise prescribed, store in a sterile, airtight, tamper-proof container, protected from light, at a temperature of 2 °C to 8 °C. Insulin preparations are not to be frozen.

LABELLING

The label states:

- the potency in International Units per millilitre,
- the concentration in terms of the number of milligrams of insulin per millilitre (for preparations containing both bovine insulin and porcine insulin the concentration is stated as the combined amount of both insulins),
- where applicable, that the substance is produced by enzymatic modification of porcine insulin,
- where applicable, that the substance is produced by recombinant DNA technology,
- where applicable, the animal species of origin,
- that the preparation must not be frozen,
- where applicable, that the preparation must be resuspended before use.

01/2005:0837

INSULIN ZINC INJECTABLE SUSPENSION

Insulini zinci suspensio iniectionabilis

Insulin zinc injectable suspension complies with the monograph on Insulin preparations, injectable (0854) with the amendments prescribed below.

DEFINITION

Insulin zinc injectable suspension is a sterile neutral suspension of bovine insulin and/or porcine insulin or of human insulin with a suitable zinc salt; the insulin is in a form which is practically insoluble in water.

PRODUCTION

Insulin zinc injectable suspension is prepared by carrying out the procedures described in the monograph on *Insulin preparations, injectable (0854)*.

Insulin zinc injectable suspension is produced by mixing insulin zinc injectable suspension (crystalline) and insulin zinc injectable suspension (amorphous) in a ratio of 7 to 3.

CHARACTERS

A white suspension which on standing deposits a white sediment and leaves a colourless or almost colourless supernatant liquid; the sediment is readily resuspended

by gently shaking. When examined under a microscope, the majority of the particles are seen to be rhombohedral crystals with a maximum dimension when measured from corner to corner through the crystal greater than 10 µm but rarely exceeding 40 µm; a considerable proportion of the particles are seen to have no uniform shape and a maximum dimension rarely exceeding 2 µm.

IDENTIFICATION

Examine the chromatograms obtained in the Assay.

For preparations made from a single species of insulin (bovine, porcine or human), the position of the peak due to insulin in the chromatogram obtained with the test solution corresponds to that of the principal peak in the chromatogram obtained with the appropriate reference solution. For preparations made from a mixture of bovine and porcine insulin, the positions of the peaks due to the two insulins in the chromatogram obtained with the test solution correspond to those of the principal peaks in the chromatogram obtained with the appropriate reference solution.

TESTS

Insulin not extractable with buffered acetone solution:

63 per cent to 77 per cent of the total insulin content. Centrifuge a volume of the substance to be examined containing 200 IU of insulin and discard the supernatant liquid. Suspend the residue in 1.65 ml of *water R*, add 3.3 ml of *buffered acetone solution R*, stir for 3 min, again centrifuge, discard the supernatant liquid and repeat all the operations with the residue. Dissolve the residue using a suitable procedure, for example dissolve in 0.1 M *hydrochloric acid* to give a final volume of 2.0 ml. Determine the insulin content of the residue (*R*) and determine the total insulin content (*T*) of an equal volume of the suspension by a suitable method. Calculate the percentage of insulin not extractable with buffered acetone solution from the expression:

$$\frac{100R}{T}$$

Total zinc: 0.12 mg to 0.25 mg per 100 IU of insulin, determined as described in the monograph on *Insulin preparations, injectable (0854)*.

Zinc in solution: 20 per cent to 65 per cent of the total zinc is in the form of zinc in solution. Determine by the method described in the monograph on *Insulin preparations, injectable (0854)*.

01/2005:0835

INSULIN ZINC INJECTABLE SUSPENSION (AMORPHOUS)

Insulini zinci amorphi suspensio iniectionabilis

Insulin zinc injectable suspension (amorphous) complies with the monograph on Insulin preparations, injectable (0854) with the amendments prescribed below.

DEFINITION

Insulin zinc injectable suspension (amorphous) is a sterile neutral suspension of bovine, porcine or human insulin complexed with a suitable zinc salt; the insulin is in a form which is practically insoluble in water.

(1) 100 IU are equivalent to 3.47 mg of human insulin, to 3.45 mg of porcine insulin and to 3.42 mg of bovine insulin.

CHARACTERS

A white suspension which on standing deposits a white sediment and leaves a colourless or almost colourless supernatant liquid; the sediment is readily resuspended by gently shaking. When examined under a microscope, the particles are seen to have no uniform shape and a maximum dimension rarely exceeding 2 µm.

IDENTIFICATION

Examine the chromatograms obtained in the Assay. The position of the peak due to insulin in the chromatogram obtained with the test solution corresponds to that of the principal peak in the chromatogram obtained with the appropriate reference solution.

TESTS

Total zinc. 0.12 mg to 0.25 mg per 100 IU of insulin, determined as described in the monograph on *Insulin preparations, injectable* (0854).

Zinc in solution. 20 per cent to 65 per cent of the total zinc is in the form of zinc in solution. Determine by the method described in the monograph on *Insulin preparations, injectable* (0854).

01/2005:0836

INSULIN ZINC INJECTABLE SUSPENSION (CRYSTALLINE)

Insulini zinci cristallini suspensio iniectabilis

Insulin zinc injectable suspension (crystalline) complies with the monograph on Insulin preparations, injectable (0854) with the amendments prescribed below.

DEFINITION

Insulin zinc injectable suspension (crystalline) is a sterile neutral suspension of bovine, porcine or human insulin, complexed with a suitable zinc salt; the insulin is in a form which is practically insoluble in water.

CHARACTERS

A white suspension which on standing deposits a white sediment and leaves a colourless or almost colourless supernatant liquid; the sediment is readily resuspended by gently shaking. When examined under a microscope, the particles are seen to be rhombohedral crystals, the majority having a maximum dimension when measured from corner to corner through the crystal greater than 10 µm but rarely exceeding 40 µm.

IDENTIFICATION

Examine the chromatograms obtained in the Assay. The position of the peak due to insulin in the chromatogram obtained with the test solution corresponds to that of the principal peak in the chromatogram obtained with the appropriate reference solution.

TESTS

Insulin not extractable with buffered acetone solution. Not less than 90 per cent of the total insulin content. Centrifuge a volume of the substance to be examined containing 200 IU of insulin and discard the supernatant liquid. Suspend the residue in 1.65 ml of *water R*, add 3.3 ml of *buffered acetone solution R*, stir for 3 min, again centrifuge, discard the supernatant liquid and repeat all the operations with the residue. Dissolve the residue using a suitable procedure,

for example dissolve in 0.1 M *hydrochloric acid* to give a final volume of 2.0 ml. Determine the insulin content of the residue (*R*) and determine the total insulin content (*T*) of an equal volume of the suspension by a suitable method. Calculate the percentage of insulin not extractable with buffered acetone solution from the expression:

$$\frac{100R}{T}$$

Total zinc: 0.12 mg to 0.25 mg per 100 IU of insulin, determined as described in the monograph on *Insulin preparations, injectable* (0854).

Zinc in solution: 20 per cent to 65 per cent of the total zinc is in the form of zinc in solution. Determine by the method described in the monograph on *Insulin preparations, injectable* (0854).

01/2005:1110
corrected

INTERFERON ALFA-2 CONCENTRATED SOLUTION

Interferoni alfa-2 solutio concentrata

```

CDLPQTHSLG  SRRTLMLLAQ  MRX1ISLFSCL  KDRHDFGFPQ
EEFGNQFQKA  ETIPVLHEMI  QQIFNLFSTK  DSSAAWDETL
LDKFYTELYQ  QLNDLEACVI  QGVGVTTETPL  MKEDSILAVR
KYFQRITLYL  KEKKYSPCAW  EVVRAEIMRS  FSLTSNLQES
LRSKE

```

DEFINITION

Interferon alfa-2 concentrated solution is a solution of a protein that is produced according to the information coded by the alfa-2 sub-species of interferon alfa gene and that exerts non-specific antiviral activity, at least in homologous cells, through cellular metabolic processes involving synthesis of both ribonucleic acid and protein. Interferon alfa-2 concentrated solution also exerts antiproliferative activity. Different types of alfa-2 interferon, varying in the amino acid residue at position 23, are designated by a letter in lower case.

Designation	Residue at position 23 (X ₁)
alfa-2a	Lys
alfa-2b	Arg

This monograph applies to interferon alfa-2a and -2b concentrated solutions.

The potency of interferon alfa-2 concentrated solution is not less than 1.4×10^8 IU per milligram of protein. Interferon alfa-2 concentrated solution contains not less than 2×10^8 IU of interferon alfa-2 per millilitre.

PRODUCTION

Interferon alfa-2 concentrated solution is produced by a method based on recombinant DNA (rDNA) technology using bacteria as host cells. It is produced under conditions designed to minimise microbial contamination of the product.

Interferon alfa-2 concentrated solution complies with the following additional requirements.

Host-cell-derived proteins. The limit is approved by the competent authority.

Host-cell- or vector-derived DNA. The limit is approved by the competent authority.

CHARACTERS

A clear, colourless or slightly yellowish liquid.

IDENTIFICATION

A. It shows the expected biological activity (see Assay).

B. Examine by isoelectric focusing.

Test solution. Dilute the preparation to be examined with *water R* to a protein concentration of 1 mg/ml.

Reference solution. Prepare a 1 mg/ml solution of the appropriate *interferon alfa-2 CRS* in *water R*.

Isoelectric point calibration solution pI range 3.0 to 10.0. Prepare and use according to the manufacturer's instructions.

Use a suitable apparatus connected with a recirculating temperature controlled water-bath set at 10 °C and gels for isoelectric focusing with a pH gradient from 3.5 to 9.5. Operate the apparatus in accordance with the manufacturer's instructions. Use as the anode solution *phosphoric acid R* (98 g/l H_3PO_4) and as the cathode solution *1 M sodium hydroxide*. Samples are applied to the gel by filter papers. Place sample application filters on the gel close to the cathode.

Apply 15 µl of the test solution and 15 µl of the reference solution. Start the isoelectric focusing at 1500 V and 50 mA. Turn off the power after 30 min, remove the application filters and reconnect the power supply for 1 h. Keep the power constant during the focusing process. After focusing, immerse the gel in a suitable volume of a solution containing 115 g/l of *trichloroacetic acid R* and 34.5 g/l of *sulphosalicylic acid R* in *water R* and agitate the container gently for 60 min. Transfer the gel to a mixture of 32 volumes of *glacial acetic acid R*, 100 volumes of *ethanol R* and 268 volumes of *water R*, and soak for 5 min. Immerse the gel for 10 min in a staining solution prewarmed to 60 °C in which 1.2 g/l of *acid blue 83 R* has been added to the previous mixture of glacial acetic acid, ethanol and water. Wash the gel in several containers with the previous mixture of glacial acetic acid, ethanol and water and keep the gel in this mixture until the background is clear (12 h to 24 h). After adequate destaining, soak the gel for 1 h in a 10 per cent V/V solution of *glycerol R* in the previous mixture of glacial acetic acid, ethanol and water.

The principal bands of the electropherogram obtained with the test solution correspond in position to the principal bands of the electropherogram obtained with the reference solution. Plot the migration distances of the isoelectric point markers versus their isoelectric points and determine the isoelectric points of the principal components of the test solution and the reference solution. They do not differ by more than 0.2 pI unit. The test is not valid unless the isoelectric point markers are distributed along the entire length of the gel and the isoelectric points of the principal bands in the electropherogram obtained with the reference solution are between 5.8 and 6.3.

C. Examine the electropherograms obtained under reducing conditions in the test for impurities of molecular masses differing from that of interferon alfa-2. The principal band in the electropherogram obtained with test solution (a) corresponds in position to the principal band in the electropherogram obtained with reference solution (a).

D. Examine by peptide mapping.

Test solution. Dilute the preparation to be examined in *water R* to a protein concentration of 1.5 mg/ml. Transfer 25 µl to a polypropylene or glass tube of 1.5 ml capacity. Add 1.6 µl of *1 M phosphate buffer solution*

pH 8.0 R, 2.8 µl of a freshly prepared 1.0 mg/ml solution of *trypsin for peptide mapping R* in *water R* and 3.6 µl of *water R* and mix vigorously. Cap the tube and place it in a water-bath at 37 °C for 18 h, then add 100 µl of a 573 g/l solution of *guanidine hydrochloride R* and mix well. Add 7 µl of 154.2 g/l solution of *dithiothreitol R* and mix well. Place the capped tube in boiling water for 1 min. Cool to room temperature.

Reference solution. Prepare at the same time and in the same manner as for the test solution but use a 1.5 mg/ml solution of the appropriate *interferon alfa-2 CRS* in *water R*.

Examine by liquid chromatography (2.2.29).

The chromatographic procedure may be carried out using:

- a stainless steel column 0.10 m long and 4.6 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (5 µm) with a pore size of 30 nm,
- as mobile phase at a flow rate of 1.0 ml/min:

Mobile phase A. Dilute 1 ml of *trifluoroacetic acid R* to 1000 ml with *water R*,

Mobile phase B. To 100 ml of *water R* add 1 ml of *trifluoroacetic acid R* and dilute to 1000 ml with *acetonitrile for chromatography R*,

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)	Comment
0 - 8	100	0	isocratic
8 - 68	100 → 40	0 → 60	linear gradient
68 - 72	40	60	isocratic
72 - 75	40 → 100	60 → 0	linear gradient
75 - 80	100	0	re-equilibration

- as detector a spectrophotometer set at 214 nm, maintaining the temperature of the column at 30 °C. Equilibrate the column with mobile phase A for at least 15 min.

Inject 100 µl of the test solution and 100 µl of the reference solution. The test is not valid unless the chromatogram obtained with each solution is qualitatively similar to the chromatogram of interferon alfa-2 digest supplied with the appropriate *interferon alfa-2 CRS*. The profile of the chromatogram obtained with the test solution corresponds to that of the chromatogram obtained with the reference solution.

TESTS

Impurities of molecular masses differing from that of interferon alfa-2. Examine by SDS polyacrylamide gel electrophoresis (2.2.31). The test is performed under both reducing and non-reducing conditions, using resolving gels of 14 per cent acrylamide and silver staining as the detection method.

Sample buffer (non-reducing conditions). Mix equal volumes of *water R* and *concentrated SDS PAGE sample buffer R*.

Sample buffer (reducing conditions). Mix equal volumes of *water R* and *concentrated SDS PAGE sample buffer for reducing conditions R* containing 2-mercaptoethanol as the reducing agent.

Test solution (a). Dilute the preparation to be examined in sample buffer to a protein concentration of 0.5 mg/ml.

Test solution (b). Dilute 0.20 ml of test solution (a) to 1 ml with sample buffer.

Reference solution (a). Prepare a 0.625 mg/ml solution of the appropriate *interferon alfa-2 CRS* in sample buffer.

Reference solution (b). Dilute 0.20 ml of reference solution (a) to 1 ml with sample buffer.

Reference solution (c). Dilute 0.20 ml of reference solution (b) to 1 ml with sample buffer.

Reference solution (d). Dilute 0.20 ml of reference solution (c) to 1 ml with sample buffer.

Reference solution (e). Dilute 0.20 ml of reference solution (d) to 1 ml with sample buffer.

Reference solution (f). Use a solution of molecular mass standards suitable for calibrating SDS-PAGE gels in the range 15 kDa to 67 kDa.

Place test and reference solutions, contained in covered test-tubes, on a water-bath for 2 min.

Apply 10 µl of reference solution (f) and 50 µl of each of the other solutions to the stacking gel wells. Perform the electrophoresis under the conditions recommended by the manufacturer of the equipment. Detect proteins in the gel by silver staining.

The test is not valid unless: the validation criteria are met (2.2.31); a band is seen in the electropherogram obtained with reference solution (e); and a gradation of intensity of staining is seen in the electropherograms obtained, respectively, with test solution (a) and test solution (b) and with reference solutions (a) to (e).

The electropherogram obtained with test solution (a) under reducing conditions may show, in addition to the principal band, less intense bands with molecular masses lower than the principal band. No such band is more intense than the principal band in the electropherogram obtained with reference solution (d) (1.0 per cent) and not more than three such bands are more intense than the principal band in the electropherogram obtained with reference solution (e) (0.2 per cent).

The electropherogram obtained with test solution (a) under non-reducing conditions may show, in addition to the principal band, less intense bands with molecular masses higher than the principal band. No such band is more intense than the principal band in the electropherogram obtained with reference solution (d) (1.0 per cent) and not more than three such bands are more intense than the principal band in the electropherogram obtained with reference solution (e) (0.2 per cent).

Related proteins. Examine by liquid chromatography (2.2.29).

Test solution. Dilute the preparation to be examined with *water R* to a protein concentration of 1 mg/ml.

0.25 per cent m/m hydrogen peroxide solution. Dilute dilute hydrogen peroxide solution *R* in *water R* in order to obtain a 0.25 per cent m/m solution.

Reference solution. To a volume of the test solution, add a suitable volume of 0.25 per cent m/m hydrogen peroxide solution to give a final hydrogen peroxide concentration of 0.005 per cent m/m, and allow to stand at room temperature for 1 h, or for the length of time that will generate about 5 per cent oxidised interferon. Add 12.5 mg of *L-methionine R* per millilitre of solution. Allow to stand at room temperature for 1 h. Store the solutions for not longer than 24 h at a temperature of 2 °C to 8 °C.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4.6 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (5 µm) with a pore size of 30 nm,

- as mobile phase at a flow rate of 1.0 ml/min:

Mobile phase A. To 700 ml of *water R* add 2 ml of *trifluoroacetic acid R* and 300 ml of *acetonitrile for chromatography R*,

Mobile phase B. To 200 ml of *water R* add 2 ml of *trifluoroacetic acid R* and 800 ml of *acetonitrile for chromatography R*,

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)	Comment
0 - 1	72	28	isocratic
1 - 5	72 → 67	28 → 33	linear gradient
5 - 20	67 → 63	33 → 37	linear gradient
20 - 30	63 → 57	37 → 43	linear gradient
30 - 40	57 → 40	43 → 60	linear gradient
40 - 42	40	60	isocratic
42 - 50	40 → 72	60 → 28	linear gradient
50 - 60	72	28	re-equilibration

- as detector a spectrophotometer set at 210 nm.

Equilibrate the column with the mobile phases in the initial gradient ratio for at least 15 min. Inject 50 µl of each solution.

In the chromatograms obtained, interferon alfa-2 elutes at a retention time of about 20 min. In the chromatogram obtained with the reference solution a peak related to oxidised interferon appears at a retention time of about 0.9 relative to the principal peak. The test is not valid unless the resolution between the peaks corresponding to oxidised interferon and interferon is at least 1.0. Consider only the peaks whose retention time is 0.7 to 1.4 relative to that of the principal peak. In the chromatogram obtained with the test solution, the area of any peak, apart from the principal peak, is not greater than 3.0 per cent of the total area of all of the peaks. The sum of the areas of any peaks other than the principal peak is not greater than 5.0 per cent of the total area of all of the peaks.

Bacterial endotoxins (2.6.14): less than 100 IU in the volume that contains 1.0 mg of protein.

ASSAY

Protein

Test solution. Dilute the preparation to be examined with *water R* to obtain a concentration of about 0.5 mg/ml of interferon alfa-2.

Reference solutions. Prepare a stock solution of 0.5 mg/ml of *bovine albumin R*. Prepare eight dilutions of the stock solution containing between 3 µg/ml and 30 µg/ml of *bovine albumin R*.

Prepare 30-fold and 50-fold dilutions of the test solution. Add 1.25 ml of a mixture prepared the same day by combining 2.0 ml of a 20 g/l solution of *copper sulphate R* in *water R*, 2.0 ml of a 40 g/l solution of *sodium tartrate R* in *water R* and 96.0 ml of a 40 g/l solution of *sodium carbonate R* in 0.2 M *sodium hydroxide* to test-tubes containing 1.5 ml of *water R* (blank), 1.5 ml of the different dilutions of the test solution or 1.5 ml of the reference solutions. Mix after each addition. After approximately 10 min, add to each test-tube 0.25 ml of a mixture of equal volumes of *water R* and *phosphomolybdotungstic reagent R*. Mix after each addition. After approximately 30 min, measure the absorbance (2.2.25) of each solution at 750 nm using the blank as the compensation liquid. Draw a calibration curve

from the absorbances of the eight reference solutions and the corresponding protein contents and read from the curve the content of protein in the test solution.

Potency

The potency of interferon alfa-2 is estimated by comparing its effect to protect cells against a viral cytopathic effect with the same effect of the appropriate International Standard of human recombinant interferon alfa-2 or of a reference preparation calibrated in International Units.

The International Unit is the activity contained in a stated amount of the appropriate International Standard. The equivalence in International Units of the International Standard is stated by the World Health Organisation.

Carry out the assay by a suitable method, based on the following design.

Use, in standard culture conditions, an established cell line sensitive to the cytopathic effect of a suitable virus (a human diploid fibroblast cell line, free of microbial contamination, responsive to interferon and sensitive to encephalomyocarditis virus, is suitable).

The following cell cultures and virus have shown to be suitable: MDBK cells (ATCC No. CCL22), or Mouse L cells (NCTC clone 929; ATCC No. CCL 1) as the cell culture and vesicular stomatitis virus VSV, Indiana strain (ATCC No. VR-158) as the infective agent; or human diploid fibroblast FS-71 cells responsive to interferon as the cell culture, and encephalomyocarditis virus (ATCC No. VR-129B) as the infective agent.

Incubate in at least four series, cells with three or more different concentrations of the preparation to be examined and the reference preparation in a microtitre plate and include in each series appropriate controls of untreated cells. Choose the concentrations of the preparations such that the lowest concentration produces some protection and the largest concentration produces less than maximal protection against the viral cytopathic effect. Add at a suitable time the cytopathic virus to all wells with the exception of a sufficient number of wells in all series, which are left with uninfected control cells. Determine the cytopathic effect of virus quantitatively with a suitable method. Calculate the potency of the preparation to be examined by the usual statistical methods for a parallel line assay.

The estimated potency is not less than 80 per cent and not more than 125 per cent of the stated potency. The confidence limits of the estimated potency ($P = 0.95$) are not less than 64 per cent and not more than 156 per cent of the stated potency.

STORAGE

Store in an airtight container, protected from light, at or below -20°C .

LABELLING

The label states:

- the type of interferon (alfa-2a or alfa-2b),
- the type of production.

01/2005:1440
corrected

INTERFERON GAMMA-1b CONCENTRATED SOLUTION

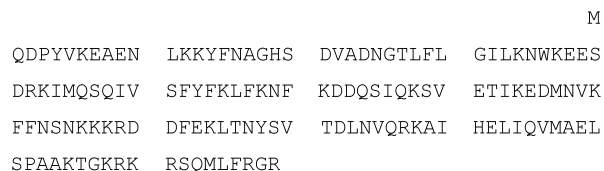
Interferoni gamma-1b solutio concentrata

$\text{C}_{734}\text{H}_{1166}\text{N}_{204}\text{O}_{216}\text{S}_5$

M_r 16 465

DEFINITION

Interferon gamma-1b concentrated solution is a solution of the *N*-terminal methionyl form of interferon gamma, a protein which is produced and secreted by human antigen-stimulated T lymphocytes in response to viral infections and various other inducers. It has specific immunomodulatory properties, such as potent phagocyte-activating effects. The protein consists of non-covalent dimers of two identical monomers. The formula of the monomer is as follows:



The potency of interferon gamma-1b is not less than 20×10^6 IU per milligram of protein. Interferon gamma-1b concentrated solution contains not less than 30×10^6 IU of interferon gamma-1b per millilitre.

PRODUCTION

Interferon gamma-1b concentrated solution is produced by a method based on recombinant DNA technology, using bacteria as host-cells. It is produced under conditions designed to minimise microbial contamination.

Interferon gamma-1b concentrated solution complies with the following additional requirements.

Host-cell derived proteins. The limit is approved by the competent authority.

Host-cell- and vector-derived DNA. The limit is approved by the competent authority.

CHARACTERS

A clear, colourless or slightly yellowish liquid.

IDENTIFICATION

- It shows the expected biological activity when tested as prescribed in the assay.
- Examine the electropherograms obtained in the test for impurities of molecular masses differing from that of interferon gamma-1b. The principal bands in the electropherogram obtained with the test solution correspond in position to the principal bands in the electropherogram obtained with reference solution (a).
- Examine by peptide mapping.

Solution A. Prepare a solution containing 1.2 g/l of *tris(hydroxymethyl)aminomethane R*, 8.2 g/l of *anhydrous sodium acetate R*, 0.02 g/l of *calcium chloride R* and adjust to pH 8.3 (2.2.3) with *dilute acetic acid R*. Add *polysorbate 20 R* to a concentration of 0.1 per cent V/V.

Test solution. Desalt a volume of the preparation to be examined containing 1 mg of protein by a suitable procedure. For example, filter in a microcentrifuge tube and reconstitute with 500 µl of solution A. Add 10 µl of a freshly prepared 1 mg/ml solution of *trypsin for peptide mapping R* in *water R* and mix gently by inversion. Incubate at 30°C to 37°C for 24 h, add 100 µl of *phosphoric acid R* per millilitre of digested sample and mix by inversion.

Reference solution. Dilute *interferon gamma-1b CRS* in *water R* to obtain a concentration of 1 mg/ml. Prepare as for the test solution, ensuring that all procedures are carried out simultaneously and under identical conditions.

Examine by liquid chromatography (2.2.29).

The chromatographic procedure may be carried out using:

- a stainless steel column, 0.15 m long and 4.6 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (10 µm),
- as mobile phase at a flow rate of 1.0 ml/min:

Mobile phase A (0.05 M sodium phosphate buffer solution pH 3.3). Solution I: dissolve 7.80 g of *sodium dihydrogen phosphate R* in *water R* and dilute to 1000.0 ml with the same solvent. Solution II: dilute 0.33 ml of *phosphoric acid R* to 100.0 ml with *water R*. Mix 920 ml of solution I and 80 ml of solution II. Adjust the pH (2.2.3) if necessary,

Mobile phase B. *Acetonitrile for chromatography R*,

with the following elution conditions (if necessary, the gradient may be modified to improve the separation of the digest):

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)
0 - 30	100 → 80	0 → 20
30 - 50	80 → 60	20 → 40
50 - 51	60 → 30	40 → 70
51 - 59	30	70
59 - 60	30 → 100	70 → 0

- as detector a spectrophotometer set at 214 nm, maintaining the temperature of the column at 40 °C.

Equilibrate the column for at least 15 min at the initial elution composition. Carry out a blank run using the above-mentioned gradient.

Inject 100 µl of the test solution and 100 µl of the reference solution. The test is not valid unless the chromatogram obtained with each solution is qualitatively similar to the chromatogram of interferon gamma-1b digest supplied with *interferon gamma-1b CRS*. The profile of the chromatogram obtained with the test solution corresponds to that of the chromatogram obtained with the reference solution.

D. Examine by N-terminal sequence analysis.

Use an automated solid-phase sequencer, operated in accordance with the manufacturer's instructions.

Equilibrate by a suitable procedure the equivalent of 100 µg of interferon gamma-1b in a 10 g/l solution of *ammonium hydrogen carbonate R*, pH 9.0.

Identify the phenylthiohydantoin (PTH)-amino acids released at each sequencing cycle by reverse-phase liquid chromatography. The procedure may be carried out using the column and reagents recommended by the manufacturer of the sequencing equipment for the separation of PTH-amino acids.

The separation procedure is calibrated using:

- the mixture of PTH-amino acids provided by the manufacturer, with the gradient conditions adjusted as indicated to achieve optimum resolution of all amino acids,
- a sample from a blank sequencing cycle, obtained as recommended by the equipment manufacturer.

The first fifteen amino acids are:

Met-Gln-Asp-Pro-Tyr-Val-Lys-Glu-Ala-Glu-Asn-Leu-Lys-Lys-Tyr.

TESTS

Appearance. The preparation to be examined is clear (2.2.1) and not more intensely coloured than reference solution Y₇ (2.2.2, *Method II*).

pH (2.2.3). The pH of the preparation to be examined is 4.5 to 5.5.

Covalent dimers and oligomers. Not greater than 2 per cent, determined by size-exclusion chromatography (2.2.30).

Test solution. Dilute the preparation to be examined with the mobile phase to a protein concentration of 0.1 mg/ml.

Reference solution (a). Dilute *interferon gamma-1b CRS* with the mobile phase to a protein concentration of 0.1 mg/ml.

Reference solution (b). Prepare a mixture of the following molecular mass standards: bovine albumin, ovalbumin, trypsinogen, lysozyme, at a concentration of 0.1 mg/ml to 0.2 mg/ml for each standard.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.3 m long and 7.8 mm in internal diameter packed with *hydrophilic silica gel for chromatography R*, of a grade suitable for fractionation of globular proteins in the molecular weight range of 10 000 to 500 000 (5 µm),
- as mobile phase at a flow rate of 1.0 ml/min a mixture prepared as follows (0.2 M sodium phosphate buffer solution pH 6.8). Solution I: dissolve 31.2 g of *sodium dihydrogen phosphate R* and 1.0 g of *sodium dodecyl sulphate R* in *water R* and dilute to 1000.0 ml with the same solvent. Solution II: dissolve 28.4 g of *anhydrous disodium hydrogen phosphate R* and 1.0 g of *sodium dodecyl sulphate R* in *water R* and dilute to 1000.0 ml with the same solvent. Mix 450 ml of solution I and 550 ml of solution II. Adjust the pH (2.2.3) if necessary,
- as detector a spectrophotometer set at 210 nm to 214 nm.

Inject 200 µl of each solution. The test is not valid unless: the molecular mass standards in reference solution (b) are well separated; the retention time of the principal peak in the chromatogram obtained with reference solution (a) is between the retention time of trypsinogen and lysozyme in the chromatogram obtained with reference solution (b).

Compare the chromatograms obtained with the test solution and with reference solution (a). There are no additional shoulders or peaks in the chromatogram obtained with the test solution compared with the chromatogram obtained with reference solution (a).

Calculate the percentage content of covalent dimers and oligomers.

Monomer and aggregates. Examine by size-exclusion chromatography (2.2.30). The content of monomer and aggregates is not greater than 2 per cent.

Solution A. Prepare a solution of the following composition: 0.59 g/l of *succinic acid R* and 40 g/l of *mannitol R*, adjusted to pH 5.0 (2.2.3) with *sodium hydroxide solution R*.

Test solution. Dilute the preparation to be examined with solution A to a protein concentration of 1 mg/ml.

Reference solution. Dilute *interferon gamma-1b CRS* with solution A to a protein concentration of 1 mg/ml.

Resolution solution. Prepare 500 µl of a mixture consisting of 0.04 mg/ml of *bovine albumin R* and 0.2 mg/ml of *interferon gamma-1b CRS* in solution A. Use this solution within 24 h of preparation.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.3 m long and 7.8 mm in internal diameter packed with *hydrophilic silica gel for chromatography R*, of a grade suitable for fractionation of globular proteins in the molecular weight range of 10 000 - 300 000 (5 µm),
- as mobile phase at a flow rate of 0.8 ml/min a 89.5 g/l solution of *potassium chloride R* (1.2 M),
- as detector a spectrophotometer set at 214 nm.

Inject 20 µl of the resolution solution. In the chromatogram obtained, the retention time of the principal peak, corresponding to the native interferon gamma-1b dimer, is about 10 min. bovine albumin elutes at a relative retention time of about 0.85, relative to the main peak. The test is not valid unless the resolution between the peaks corresponding to bovine albumin and interferon gamma-1b is at least 1.5.

Inject 20 µl of the test solution and 20 µl of the reference solution. The chromatograms obtained show principal peaks with identical retention times. Calculate the percentage content of monomer and aggregates from the peak area of the monomer peak and of peaks which elute prior to the native interferon gamma-1b peak in the chromatogram obtained with the test solution, by the normalisation procedure, disregarding any peak due to the solvent.

Deamidated and oxidised forms and heterodimers.

Examine by liquid chromatography (2.2.29). The content of deamidated and oxidised forms is not greater than 10 per cent. The content of heterodimers is not greater than 3 per cent.

Test solution. Dilute the preparation to be examined with *water R* to a protein concentration of 1 mg/ml.

Reference solution. Dilute *interferon gamma-1b CRS* with *water R* to a protein concentration of 1 mg/ml.

Resolution solution. Use *interferon gamma-1b validation solution CRS*.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.075 m long and 7.5 mm in internal diameter packed with an appropriate hydrophilic polymethacrylate, strong cation exchange gel (10 µm, 100 nm),
- as mobile phase at a flow rate of 1.2 ml/min:

Mobile phase A (0.05 M ammonium acetate buffer pH 6.5). A 3.86 g/l solution of *ammonium acetate R*, adjusted to pH 6.5 with *dilute acetic acid R*,

Mobile phase B (1.2 M ammonium acetate buffer pH 6.5). A 92.5 g/l solution of *ammonium acetate R*, adjusted to pH 6.5 with *dilute acetic acid R*,

with the following elution conditions (if necessary, the slope of the gradient may be modified to improve the separation).

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)
0 - 1	100	0
2 - 30	100 → 0	0 → 100
31 - 35	0	100
36 - 37	0 → 100	100 → 0
38 - 47	100	0

- as detector a spectrophotometer set at 280 nm, maintaining the temperature of the column at 35 °C.

Inject 25 µl of the resolution solution. In the chromatogram obtained, the retention time of the principal peak is about 26 min. Deamidated and oxidised forms co-elute at a relative

retention time of about 0.95, relative to the principal peak. The test is not valid unless the resolution, defined by the ratio of the height of the peak corresponding to the deamidated and oxidised forms to the height above the baseline of the valley separating the two peaks, is at least 1.2.

Inject 25 µl of the test solution and 25 µl of the reference solution. The chromatograms obtained show principal peaks with identical retention times. Calculate the percentage content of deamidated and oxidised interferon gamma-1b as a percentage of the area of the main peak. Heterodimers have relative retention times of 0.7 and 0.85 relative to the main peak. Calculate the percentage of heterodimers as a percentage of the sum of the areas of all peaks.

Impurities of molecular masses differing from that of interferon gamma-1b. Examine by polyacrylamide gel electrophoresis (2.2.31). The test is performed under both reducing and non-reducing conditions, using resolving gels of 15 per cent acrylamide and silver staining as the detection method.

Sample buffer (non-reducing conditions). Dissolve 3.78 g of *tris(hydroxymethyl)aminomethane R*, 10.0 g of *sodium dodecyl sulphate R* and 0.100 g of *bromophenol blue R* in *water R*. Add 50.0 ml of *glycerol R* and dilute to 80 ml with *water R*. Adjust the pH (2.2.3) to 6.8 with *hydrochloric acid R* and dilute to 100 ml with *water R*.

Sample buffer (reducing conditions). Dissolve 3.78 g of *tris(hydroxymethyl)aminomethane R*, 10.0 g of *sodium dodecyl sulphate R* and 0.100 g of *bromophenol blue R* in *water R*. Add 50.0 ml of *glycerol R* and dilute to 80 ml with *water R*. Adjust the pH (2.2.3) to 6.8 with *hydrochloric acid R* and dilute to 100 ml with *water R*. Immediately before use, add *dithiothreitol R* to a final concentration of 250 mM.

Test solution. Dilute the preparation to be examined in *water R* to a protein concentration of 1 mg/ml. Dilute 150 µl of the solution with 38 µl of sample buffer.

Reference solution (a). Prepare in the same manner as for the test solution, but using *interferon gamma-1b CRS* instead of the preparation to be examined.

Reference solution (b) (5 ng control). Mix 50 µl of a 0.01 mg/ml solution of *bovine albumin R* with 2000 µl of *water R* and 450 µl of sample buffer.

Reference solution (c) (2 ng control). Mix 20 µl of a 0.01 mg/ml solution of *bovine albumin R* with 2000 µl of *water R* and 450 µl of sample buffer.

Reference solution (d). Use a solution of molecular mass standards suitable for calibrating SDS-polyacrylamide gels in the range of 10 kDa to 70 kDa.

Leave each solution, contained in a test tube, at ambient temperature for 15 min, then store on ice.

Apply 25 µl of each solution to the stacking gel wells. Perform the electrophoresis under the conditions recommended by the manufacturer of the equipment. Detect proteins in the gel by silver staining.

The test is not valid unless: the validation criteria are met (2.2.31); a band is seen in the electropherograms obtained with reference solutions (b) and (c).

The principal band in the electropherogram obtained with the test solution is similar in intensity to the principal band in the electropherogram obtained with reference solution (a). In the electropherogram obtained with the test solution, no significant bands are observed that are not present in the electropherogram obtained with reference solution (a) (0.01 per cent). A significant band is defined as any band whose intensity is greater than or equal to that of the band in the electropherogram obtained with reference solution (c).

Norleucine. Not more than 0.2 mole of norleucine per mole of interferon gamma-1b, determined by amino acid analysis.

Test solution. Add 2.5 ml of the preparation to be examined onto a column suitable for the desalting of proteins previously equilibrated with 25 ml of a 10 per cent V/V solution of *acetic acid R*. Elute the sample with another 2.5 ml of a 10 per cent V/V solution of *acetic acid R*. Determine the protein content by measuring the absorbance of this solution as described under Protein, in the Assay section. Pipette a volume containing the equivalent of 100 µg of interferon gamma-1b into each of three reaction vials. Evaporate to dryness under reduced pressure.

Perform the hydrolysis of the three samples as follows. Add to each reaction vial 200 µl of a 50 per cent V/V solution of *hydrochloric acid R* containing 1 per cent V/V of *phenol R*, evacuate the samples, purge with nitrogen and hydrolyse in the gas phase. Heat the reaction vials at 110 °C for 22 h. After hydrolysis evaporate to dryness under reduced pressure.

Perform the derivatisation of the samples as follows. Prepare immediately before use a mixture consisting of two volumes of *ethanol R*, one volume of *water R* and one volume of *triethylamine R*. Add 50 µl of this solution to each reaction vial and shake lightly. Evaporate to dryness under reduced pressure. Add to each vial 50 µl of a mixture consisting of 7 volumes of *ethanol R*, one volume of *water R*, one volume of *triethylamine R* and one volume of *phenyl isothiocyanate R*. Shake lightly and allow to stand at room temperature for about 15 min. Evaporate to dryness under reduced pressure. Reconstitute the samples in 250 µl of mobile phase A.

Norleucine stock solution. Prepare a 250 nmol/ml solution of *DL-norleucine R* in 0.01 M *hydrochloric acid*. This solution may be kept for two months at 4 °C.

Leucine stock solution. Prepare a 250 nmol/ml solution of *leucine R* in 0.01 M *hydrochloric acid*. This solution may be kept at 4 °C for two months.

Reference solution. Mix 10 µl of norleucine stock solution with 100 µl of leucine stock solution in each of the three reaction vials. Evaporate to dryness under reduced pressure. Perform the derivatisation of the samples as described for the preparation of the test solution.

Examine by liquid chromatography (2.2.29).

The chromatographic procedure may be carried out using:

- a stainless steel column 0.15 m long and 3.9 mm in diameter packed with *octadecylsilyl silica gel for chromatography R* (4 µm),
- as mobile phase at a flow rate of 1.0 ml/min:

Mobile phase A. Mix 70 volumes of a 19 g/l solution of *sodium acetate R* containing 0.05 per cent V/V of *triethylamine R* and adjusted to pH 6.4 with *dilute acetic acid R* and 30 volumes of mobile phase B,

Mobile phase B. Mix 40 volumes of *water R* and 60 volumes of *acetonitrile R*,

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)	Comment
0 - 7	100	0	isocratic
7 - 7.1	100 → 0	0 → 100	linear gradient
7.1 - 10	0	100	washing step
10 - 10.1	0 → 100	100 → 0	linear gradient
10.1 - 15	100	0	re-equilibration

- as detector a spectrophotometer set at 254 nm,

maintaining the temperature of the column at 43 °C.

Inject 50 µl of each solution.

In the chromatograms obtained with the test solution, identify the peaks corresponding to leucine and norleucine. The retention time of norleucine is 6.2 min to 7 min.

Calculate the content of norleucine (in moles of norleucine per mole of interferon gamma-1b) from the peak areas of leucine and norleucine in the chromatograms obtained with the reference and test solutions, considering that there are 10 moles of leucine per mole of interferon gamma-1b.

Bacterial endotoxins (2.6.14): less than 5 IU in the volume that contains 20×10^6 IU of interferon gamma-1b.

ASSAY

Protein (2.2.25). Dilute the substance to be examined in *water R* to obtain a concentration of 1 mg/ml. Record the absorbance spectrum between 220 nm and 340 nm. Measure the value at the absorbance maximum of 280 nm, after correction for any light scattering due to turbidity measured at 316 nm. Calculate the concentration of interferon gamma-1b using a specific absorbance value of 7.5.

Potency. The potency of interferon gamma-1b is estimated by evaluating the increase of the expression of human-leukocyte-antigen-DR (HLA-DR) due to the interferon gamma-1b present in test solutions during cultivation of the cells, and comparing this increase with the same effect of the appropriate International Standard of human recombinant interferon gamma or of a reference preparation calibrated in International Units.

The International Unit is the activity contained in a stated amount of the appropriate International Standard. The equivalence in International Units of the International Standard is stated by the World Health Organisation.

Carry out the assay by a suitable method, based on the following design.

Use COLO 205 cells under standard culture conditions. Trypsinise a 3- to 5-day-old flask of COLO 205 cells and prepare a cell suspension at a concentration of 1.0×10^6 cells/ml.

Add 100 µl of the dilution medium to all wells of a 96-well microtitre plate. Add an additional 100 µl of this solution to the wells designed for the blanks. Add 100 µl of each solution to be tested onto the plate and carry out a series of twofold dilution steps in order to obtain a standard curve. Then add 100 µl of the cell suspension to all wells and incubate the plate under appropriate conditions for cell cultivation.

After cultivation remove the growth medium and wash and fix cells to the plate. Add an antibody able to detect HLA-DR expressed due to the presence of interferon gamma-1b and incubate under appropriate conditions. After washing the plate, incubate with an antibody conjugated to a marker enzyme which is able to detect the anti-HLA-DR antibody. After this incubation step, wash the plate and add an appropriate substrate solution. Stop the reaction. Measure the absorbance of the solution and calculate the potency of the preparation to be examined by the usual statistical methods.

The estimated specific activity is not less than 80 per cent and not more than 125 per cent of the stated potency. The confidence limits ($P = 0.95$) are not less than 70 per cent and not more than 140 per cent of the estimated potency.

STORAGE

Store in an airtight container, protected from light and at a temperature of –70 °C.

01/2005:0031

01/2005:1114

IODINE

Iodum

 I_2 M_r 253.8

DEFINITION

Iodine contains not less than 99.5 per cent and not more than the equivalent of 100.5 per cent of I.

CHARACTERS

Greyish-violet, brittle plates or fine crystals with a metallic sheen, very slightly soluble in water, soluble in alcohol, slightly soluble in glycerol, very soluble in concentrated solutions of iodides. Iodine volatilises slowly at room temperature.

IDENTIFICATION

- A. Heat a few fragments in a test-tube. Violet vapour is evolved and a bluish-black crystalline sublimate is formed.
- B. To a saturated solution add *starch solution R*. A blue colour is produced. Heat until decolourised. On cooling, the colour reappears.

TESTS

Solution S. Triturate 3.0 g with 20 ml of *water R*, filter, wash the filter with *water R* and dilute the filtrate to 30 ml with the same solvent. To the solution add 1 g of *zinc powder R*. When the solution is decolourised, filter, wash the filter with *water R* and dilute to 40 ml with the same solvent.

Bromides and chlorides. To 10 ml of solution S add 3 ml of *ammonia R* and 6 ml of *silver nitrate solution R2*. Filter, wash the filter with *water R* and dilute the filtrate to 20 ml with the same solvent. To 10 ml of the solution add 1.5 ml of *nitric acid R*. After 1 min, any opalescence in the solution is not more intense than that in a standard prepared at the same time by mixing 10.75 ml of *water R*, 0.25 ml of 0.01 M *hydrochloric acid*, 0.2 ml of *dilute nitric acid R* and 0.3 ml of *silver nitrate solution R2* (250 ppm).

Non-volatile substances. Heat 1.00 g in a porcelain dish on a water-bath until the iodine has volatilised. Dry the residue at 100 °C to 105 °C. The residue weighs not more than 1 mg (0.1 per cent).

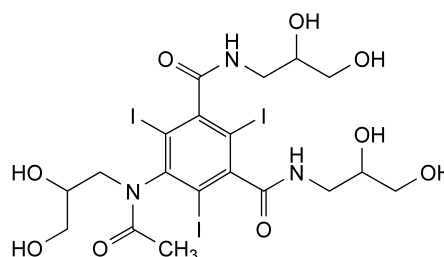
ASSAY

Introduce 0.200 g into a flask containing 1 g of *potassium iodide R* and 2 ml of *water R* and add 1 ml of *dilute acetic acid R*. When dissolution is complete, add 50 ml of *water R* and titrate with 0.1 M *sodium thiosulphate*, using *starch solution R* as indicator.

1 ml of 0.1 M *sodium thiosulphate* is equivalent to 12.69 mg of I.

IOHEXOL

Iohexolum

 $C_{19}H_{26}I_3N_3O_9$ M_r 821

DEFINITION

5-[Acetyl(2,3-dihydroxypropyl)amino]-*N,N'*-bis(2,3-dihydroxypropyl)-2,4,6-triiodobenzene-1,3-dicarboxamide.

The substance is a mixture of diastereoisomers and atropisomers.

Content: 98.0 per cent to 101.0 per cent (anhydrous substance).

CHARACTERS

Appearance: white or greyish-white, hygroscopic powder.

Solubility: very soluble in water, freely soluble in methanol, practically insoluble in methylene chloride.

IDENTIFICATION

- A. Infrared absorption spectrophotometry (2.2.24).

Comparison: *iohexol CRS*.

- B. Examine the chromatograms obtained in test A for related substances (see Tests).

Results: the principal peaks in the chromatogram obtained with reference solution (b) are similar in retention time and size to the peaks due to iohexol in the chromatogram obtained with reference solution (a).

TESTS

Solution S. Dissolve 5.0 g in *water R* and dilute to 50.0 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and not more intensely coloured than reference solution Y_7 (2.2.2, *Method II*).

Related substances

- A. Liquid chromatography (2.2.29).

NOTE: *iohexol* gives rise to 2 non-resolved peaks in the chromatogram due to endo-exo isomerism. In addition, a small peak (also due to *iohexol*) usually appears at the leading edge of the 1st principal peak. This small peak has a retention time about 1.2 min less than the 1st principal peak.

Test solution. Dissolve 0.150 g of the substance to be examined in *water R* and dilute to 100.0 ml with the same solvent.

Reference solution (a). Dissolve 15.0 mg of *iohexol CRS* and 15.0 mg of *iohexol impurity A CRS* in a mixture of 1-2 drops of *dilute sodium hydroxide solution R* and 10 ml of *water R* and dilute to 100.0 ml with *water R*. Dilute 1.0 ml of this solution to 10.0 ml with *water R*.

Reference solution (b). Dilute 1.0 ml of the test solution to 100.0 ml with *water R*.

Reference solution (c). Dissolve 5.0 mg of *iohexol* for peak identification CRS (containing impurities B, C, D and E) in *water R* and dilute to 5.0 ml with the same solvent.

Blank solution: *water R*.

Column:

- **size:** $l = 0.25$ m, $\varnothing = 4.6$ mm,
- **stationary phase:** octadecylsilyl silica gel for chromatography *R* (5 μ m).

Mobile phase:

- **mobile phase A:** *water R*;
- **mobile phase B:** *acetonitrile R*;

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)
0 - 60	99 → 87	1 → 13
60 - 65	87 → 99	13 → 1

Flow rate: 1 ml/min.

Detection: spectrophotometer at 254 nm.

Equilibration: at the initial eluent composition for at least 10 min.

Injection: 10 μ l.

Retention times: impurity A and impurity H = about 17 min; *iohexol* (peaks corresponding to *endo-exo* isomerism) = about 20 min.

System suitability: reference solution (a):

- **resolution:** minimum 5.0 between the peak due to impurity A and the 2nd and greater peak due to *iohexol*.

Limits:

- **sum of impurities B, C, D and E** (relative retention with reference to the 2nd and greater peak due to *iohexol* between 1.1 and 1.4): not more than 0.6 times the total area of the principal peaks in the chromatogram obtained with reference solution (b) (0.6 per cent); use the chromatogram obtained with reference solution (c) to identify the corresponding peaks;
- **sum of impurities A and H:** not more than 0.5 times the total area of the principal peaks in the chromatogram obtained with reference solution (b) (0.5 per cent);
- **impurities M, N, O, P, Q:** for each impurity, not more than 0.1 times the total area of the principal peaks in the chromatogram obtained with reference solution (b) (0.1 per cent);
- **any other impurity:** for each impurity, not more than 0.1 times the total area of the principal peaks in the chromatogram obtained with reference solution (b) (0.1 per cent);
- **total:** not more than 1.5 times the total area of the principal peaks in the chromatogram obtained with reference solution (b) (1.5 per cent);
- **disregard limit:** 0.03 times the total area of the principal peaks in the chromatogram obtained with reference solution (b) (0.03 per cent); disregard any peak observed with the blank.

B. Thin-layer chromatography (2.2.27).

Test solution. Dissolve 1.0 g of the substance to be examined in *water R* and dilute to 10.0 ml with the same solvent.

Reference solution (a). Dissolve 50 mg of *iohexol* impurity J CRS and 50 mg of *iohexol* CRS in *water R* and dilute to 10.0 ml with the same solvent.

Reference solution (b). Dilute 1.0 ml of the test solution to 10.0 ml with *water R*. Dilute 1.0 ml of this solution to 50.0 ml with *water R*.

Plate: TLC silica gel F_{254} plate *R*.

Preconditioning: wash the plate with the mobile phase, dry at room temperature for 30 min, then at 90 °C for 1 h.

Mobile phase: concentrated ammonia *R*, methanol *R*, 2-propanol *R*, acetone *R* (16:16:28:40 V/V/V/V).

Application: 10 μ l.

Development: over half of the plate.

Drying: in air.

Detection: examine in ultraviolet light at 254 nm.

System suitability: reference solution (a):

- the chromatogram shows 2 clearly separated spots.

Limits:

- **any impurity:** any spot in the chromatogram obtained with the test solution, apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.2 per cent).

3-Chloropropane-1,2-diol. Gas chromatography (2.2.28).

Test solution. Dissolve 1.0 g of the substance to be examined in 1.0 ml of *water R*. Shake with 4 quantities, each of 2 ml, of *methyl acetate R*. Dry the combined upper layers over *anhydrous sodium sulphate R*. Filter and concentrate to about 0.7 ml using a warm water bath at 60 °C and a stream of nitrogen and dilute to 1.0 ml with *methyl acetate R*.

Reference solution. Dissolve 0.25 g of 3-chloropropane-1,2-diol *R* in 100.0 ml of *methyl acetate R*. Dilute 1.0 ml of this solution with 100.0 ml of *methyl acetate R*.

Column:

- **material:** fused silica,
- **size:** $l = 25$ m, $\varnothing = 0.33$ mm,
- **stationary phase:** polymethylphenylsiloxane *R* (film thickness 1 μ m).

Carrier gas: helium for chromatography *R*.

Flow rate: 1 ml/min.

Temperature:

	Time (min)	Temperature (°C)
Column	0 - 2	80
	2 - 8	80 → 170
	8 - 10	170
Injection port		230
Detector		250

Detection: flame ionisation.

Injection: 2 μ l (splitless for 30 s).

System suitability: reference solution:

- **retention time:** 3-chloropropane-1,2-diol = about 8 min.

Limit:

- **3-chloropropane-1,2-diol:** not more than the area of the principal peak in the chromatogram obtained with the reference solution (25 ppm).

Free aromatic amine: maximum 500 ppm.

Test solution. Transfer 0.200 g of the substance to be examined to a 25 ml volumetric flask and dissolve in 15.0 ml of *water R*.

Reference solution. Dissolve 5.0 mg of *iohexol* impurity J CRS in *water R* and dilute to 5.0 ml with *water R*. Dilute 1.0 ml of this solution to 100.0 ml with *water R*. Mix 10.0 ml of this solution with 5.0 ml of *water R* in a 25 ml volumetric flask.

Blank solution. Transfer 15.0 ml of *water R* to a 25 ml volumetric flask.

In conducting the following steps, keep the flasks in iced water and protected as much as possible from light until all of the reagents have been added.

Place the 3 flasks containing respectively the test solution, the reference solution and the blank solution in iced water, protected from light, for 5 min. Add 1.5 ml of *hydrochloric acid R1* and mix by swirling. Add 1.0 ml of a 20 g/l solution of *sodium nitrite R*, mix and allow to stand for 4 min. Add 1.0 ml of a 40 g/l solution of *sulphamic acid R*, swirl gently until gas liberation has ceased and allow to stand for 1 min. (**CAUTION: considerable pressure is produced**). Add 1.0 ml of a freshly prepared 3 g/l solution of *naphthylethylenediamine dihydrochloride R* in a mixture of 30 volumes of *water R* and 70 volumes of *propylene glycol R* and mix. Remove the flasks from the iced water, dilute to 25.0 ml with *water R*, mix and allow to stand for 5 min. Simultaneously determine the absorbance (2.2.25) at 495 nm of the solutions obtained from the test solution and the reference solution in 5 cm cells, using the blank as the compensation liquid. The absorbance of the test solution is not greater than that of the reference solution.

Iodide: maximum 10 ppm.

Dissolve 6.000 g in *water R* and dilute to 20 ml with the same solvent. Add 2.0 ml of 0.001 M *potassium iodide*. Titrate with 0.001 M *silver nitrate*. Determine the end-point potentiometrically (2.2.20), using a silver indicator electrode and an appropriate reference electrode. Subtract the volume of titrant corresponding to the 2.0 ml of 0.001 M *potassium iodide*, determined by titrating a blank to which is added 2.0 ml of 0.001 M *potassium iodide* and use the residual value to calculate the iodide content.

1 ml of 0.001 M *silver nitrate* is equivalent to 126.9 µg of I⁻.

Ionic compounds (2.2.38): maximum 0.01 per cent *m/m* calculated as sodium chloride.

Rinse all glassware with distilled water R 5 times before use.

Test solution. Dissolve 1.0 g of the substance to be examined in *water R* and dilute to 50.0 ml with the same solvent.

Reference solution. Dissolve 20.0 mg of *sodium chloride R* in *water R* and dilute to 100.0 ml with the same solvent. Dilute 1.0 ml of this solution to 100.0 ml with *water R*.

Measure the conductivity of the test solution and the reference solution using a suitable conductivity meter. The conductivity of the test solution is not greater than that of the reference solution.

Heavy metals (2.4.8): maximum 10 ppm.

12 ml of solution S complies with limit test A. Prepare the reference solution using *lead standard solution (1 ppm Pb) R*.

Water (2.5.12): maximum 4.0 per cent, determined on 1.00 g.

ASSAY

To 0.500 g in a 125 ml round-bottomed flask add 25 ml of a 50 g/l solution of *sodium hydroxide R*, 0.5 g of *zinc powder R* and a few glass beads. Boil under a reflux condenser for 30 min. Allow to cool and rinse the condenser with 20 ml of *water R*, adding the rinsings to the flask. Filter through a sintered-glass filter and wash the filter with several quantities of *water R*. Collect the filtrate and washings. Add 5 ml of *glacial acetic acid R* and titrate immediately with 0.1 M *silver nitrate*. Determine the end-point potentiometrically (2.2.20).

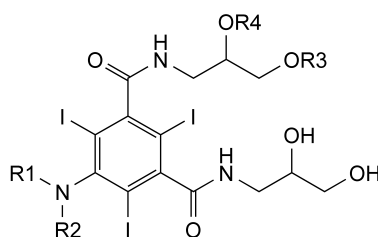
1 ml of 0.1 M *silver nitrate* is equivalent to 27.37 mg of C₁₉H₂₆I₃N₃O₉.

STORAGE

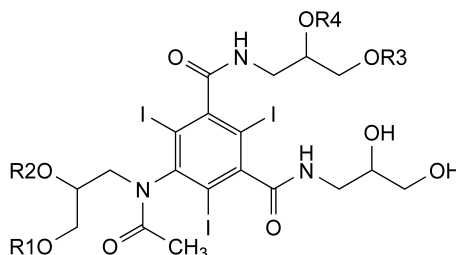
In an airtight container, protected from light and moisture.

IMPURITIES

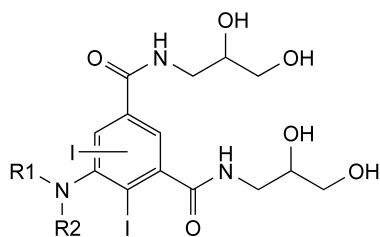
Specified impurities: A, B, C, D, E, F, G, H, I, J, K, L, M, N, O, P, Q.



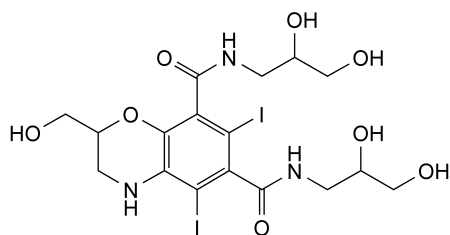
- A. R1 = CO-CH₃, R2 = R3 = R4 = H: 5-(acetylamino)-N,N'-bis(2,3-dihydroxypropyl)-2,4,6-triodobenzene-1,3-dicarboxamide,
- J. R1 = R2 = R3 = R4 = H: 5-amino-N,N'-bis(2,3-dihydroxypropyl)-2,4,6-triodobenzene-1,3-dicarboxamide,
- P. R1 = R2 = CO-CH₃, R3 = CH₂-CHOH-CH₂OH, R4 = H: 5-(diacetylamino)-N-[3-(2,3-dihydroxypropoxy)-2-hydroxypropyl]-N'-(2,3-dihydroxypropyl)-2,4,6-triodobenzene-1,3-dicarboxamide,
- Q. R1 = R2 = CO-CH₃, R3 = H, R4 = CH₂-CHOH-CH₂OH: 5-(diacetylamino)-N-[2-(2,3-dihydroxypropoxy)-3-hydroxypropyl]-N'-(2,3-dihydroxypropyl)-2,4,6-triodobenzene-1,3-dicarboxamide,



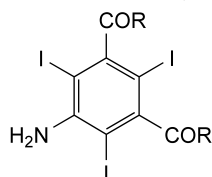
- B. R1 = CH₂-CHOH-CH₂OH, R2 = R3 = R4 = H: 5-[acetyl[3-(2,3-dihydroxypropoxy)-2-hydroxypropyl]amino]-N,N'-bis(2,3-dihydroxypropyl)-2,4,6-triodobenzene-1,3-dicarboxamide,
- C. R2 = CH₂-CHOH-CH₂OH, R1 = R3 = R4 = H: 5-[acetyl[2-(2,3-dihydroxypropoxy)-3-hydroxypropyl]amino]-N,N'-bis(2,3-dihydroxypropyl)-2,4,6-triodobenzene-1,3-dicarboxamide,
- D. R3 = CH₂-CHOH-CH₂OH, R1 = R2 = R4 = H: 5-[acetyl(2,3-dihydroxypropyl)amino]-N-[3-(2,3-dihydroxypropoxy)-2-hydroxypropyl]-N'-(2,3-dihydroxypropyl)-2,4,6-triodobenzene-1,3-dicarboxamide,
- E. R4 = CH₂-CHOH-CH₂OH, R1 = R2 = R3 = H: 5-[acetyl(2,3-dihydroxypropyl)amino]-N-[2-(2,3-dihydroxypropoxy)-3-hydroxypropyl]-N'-(2,3-dihydroxypropyl)-2,4,6-triodobenzene-1,3-dicarboxamide,
- N. R4 = CO-CH₃, R1 = R2 = R3 = H: 5-[acetyl(2,3-dihydroxypropyl)amino]-N-[2-(acetyloxy)-3-hydroxypropyl]-N'-(2,3-dihydroxypropyl)-2,4,6-triodobenzene-1,3-dicarboxamide,
- O. R3 = CO-CH₃, R1 = R2 = R4 = H: 5-[acetyl(2,3-dihydroxypropyl)amino]-N-[3-(acetyloxy)-2-hydroxypropyl]-N'-(2,3-dihydroxypropyl)-2,4,6-triodobenzene-1,3-dicarboxamide,



- F. R1 = R2 = H: 5-amino-*N,N'*-bis(2,3-dihydroxypropyl)diiodobenzene-1,3-dicarboxamide,
 G. R1 = H, R2 = CO-CH₃: 5-(acetylamino)-*N,N'*-bis(2,3-dihydroxypropyl)diiodobenzene-1,3-dicarboxamide,
 H. R1 = CH₂-CHOH-CH₂OH, R2 = CO-CH₃: 5-[acetyl(2,3-dihydroxypropyl)amino]-*N,N'*-bis(2,3-dihydroxypropyl)diiodobenzene-1,3-dicarboxamide,
 M. R1 = CH₂-CHOH-CH₂OH, R2 = H: *N,N'*-bis(2,3-dihydroxypropyl)-5-[(2,3-dihydroxypropyl)amino]diiodobenzene-1,3-dicarboxamide,



- I. *N,N'*-bis(2,3-dihydroxypropyl)-2-(hydroxymethyl)-5,7-diiodo-3,4-dihydro-2*H*-1,4-benzoxazine-6,8-dicarboxamide,

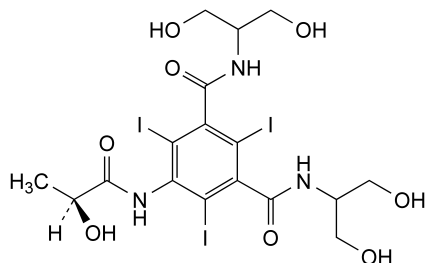


- K. R = OH: 5-amino-2,4,6-triiodobenzene-1,3-dicarboxylic acid,
 L. R = Cl: 5-amino-2,4,6-triiodobenzene-1,3-dicarbonyl dichloride.

01/2005:1115

IOPAMIDOL

Iopamidolum

C₁₇H₂₂I₃N₃O₈M_r 777

DEFINITION

N,N'-Bis[2-hydroxy-1-(hydroxymethyl)ethyl]-5-[[2*S*]-2-hydroxypropanoyl]amino]-2,4,6-triiodobenzene-1,3-dicarboxamide.

Content: 98.5 per cent to 101.0 per cent (dried substance).

CHARACTERS

Appearance: white or almost white powder.

Solubility: freely soluble in water, very slightly soluble in methanol, practically insoluble in ethanol (96 per cent) and in methylene chloride.

IDENTIFICATION

A. Infrared absorption spectrophotometry (2.2.24).

Comparison: *iopamidol CRS*.

B. It complies with the test for loss on drying (see Tests).

C. It complies with the test for specific optical rotation (see Tests).

TESTS

Appearance of solution. The solution is clear (2.2.1) and colourless (2.2.2, *Method II*).

Dissolve 1 g in *water R* and dilute to 50 ml with the same solvent.

Acidity or alkalinity. Dissolve 10.0 g in *carbon dioxide-free water R* and dilute to 100 ml with the same solvent. Not more than 0.75 ml of 0.01 *M* hydrochloric acid or 1.4 ml of 0.01 *M* sodium hydroxide is required to adjust to pH 7.0 (2.2.3).

Specific optical rotation (2.2.7): −4.6 to −5.2 (dried substance), determined at 436 nm.

Dissolve 10.0 g, with heating if necessary, in *water R* and dilute to 25.0 ml with the same solvent.

Related substances. Liquid chromatography (2.2.29).

Test solution. Dissolve 0.50 g of the substance to be examined in *water R* and dilute to 50.0 ml with the same solvent.

Reference solution (a). Dissolve 5.0 mg of *iopamidol impurity H CRS* in *water R* and dilute to 100.0 ml with the same solvent.

Reference solution (b). Dilute 2.0 ml of the test solution to 20.0 ml with *water R*. Dilute 1.0 ml of this solution to 50.0 ml with *water R*.

Reference solution (c). Add 0.1 ml of the test solution to 20 ml of reference solution (a) and dilute to 50 ml with *water R*.

Column: 2 columns coupled in series,

– **size:** *l* = 0.25 m, Ø = 4.6 mm,

– **stationary phase:** *phenylsilyl silica gel for chromatography R* (5 µm),

– **temperature:** 60 °C.

Mobile phase:

– **mobile phase A:** *water R*,

– **mobile phase B:** *acetonitrile R, water R* (50:50 V/V),

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)
0 - 18	100	0
18 - 40	100 - 62	0 - 38
40 - 45	62 - 50	38 - 50
45 - 50	50 - 100	50 - 0
50 - 60	100	0

Flow rate: 2.0 ml/min.

Detection: spectrophotometer at 240 nm.

Injection: 20 µl.

Relative retention with reference to iopamidol (retention time = about 14.6 min): impurity D = about 0.1; impurity B = about 0.6; impurities I and H = about 0.9; impurity G = about 1.1; impurity K = about 1.2; impurity C = about 1.3; impurity J = about 1.5; impurity A = about 1.8; impurity E = about 2.2; impurity F = about 2.3.

System suitability: reference solution (c):

- **resolution:** minimum 2.0 between the peaks due to impurity H and iopamidol.

Limits:

- **sum of impurities H and I:** not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent),
- **impurities A, B, C, D, E, F, G, J, K:** for each impurity, not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent),
- **any other impurity:** for each impurity, not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent),
- **total of other impurities:** not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent),
- **disregard limit:** 0.05 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.01 per cent).

Free aromatic amines: maximum 200 ppm.

Keep the solutions and reagents in iced water, protected from bright light.

Test solution. In a 25 ml volumetric flask, dissolve 0.500 g of the substance to be examined in 20.0 ml of *water R*.

Reference solution. In a 25 ml volumetric flask, mix 4.0 ml of a 25.0 mg/l solution of *iopamidol impurity A CRS* with 16.0 ml of *water R*.

Blank solution. Place 20.0 ml of *water R* in a 25 ml volumetric flask.

Place the flasks in iced water, protected from light, for 5 min. Add 1.0 ml of *hydrochloric acid R* to each flask, mix and allow to stand for 5 min. Add 1.0 ml of a 20 g/l solution of *sodium nitrite R* prepared immediately before use, mix and allow to stand for 5 min. Add 1.0 ml of a 120 g/l solution of *ammonium sulphamate R*, swirl gently until gas liberation has ceased, and allow to stand for 5 min. (**CAUTION:** *considerable pressure is produced*). Add 1.0 ml of a freshly prepared 1 g/l solution of *naphthylethylenediamine dihydrochloride R* and mix. Remove the flasks from the iced water and allow to stand for 10 min. Dilute to 25.0 ml with *water R* and mix. Measure immediately the absorbance (2.2.25) at 500 nm of the solutions obtained from the test solution and the reference solution using, as the compensation liquid, the solution obtained from the blank solution.

The absorbance of the test solution is not greater than that of the reference solution.

Free iodine: maximum 10 ppm.

Dissolve 2.0 g in 25 ml of *water R* in a ground-glass stoppered centrifuge tube. Add 5 ml of *toluene R* and 5 ml of *diluted sulphuric acid R*. Shake and centrifuge. Any red colour of

the upper layer is not more intense than that of the upper phase obtained in the same way from 22 ml of *water R*, 2 ml of *iodide standard solution (10 ppm I) R*, 5 ml of *dilute sulphuric acid R*, 1 ml of *concentrated hydrogen peroxide solution R* and 5 ml of *toluene R*.

Iodide: maximum 10 ppm.

Dissolve 6.000 g in *water R* and dilute to 20 ml with the same solvent. Add 2.0 ml of 0.001 M *potassium iodide*. Carry out a potentiometric titration (2.2.20) with 0.001 M *silver nitrate* using a silver indicator electrode and an appropriate reference electrode. Subtract the volume of titrant corresponding to the 2.0 ml of 0.001 M *potassium iodide*, determined by titrating a blank to which is added 2.0 ml of 0.001 M *potassium iodide* and use the residual value to calculate the iodide content.

1 ml of 0.001 M *silver nitrate* is equivalent to 126.9 µg of iodide.

Heavy metals (2.4.8): maximum 10 ppm.

2.0 g complies with limit test C. Prepare the reference solution using 2 ml of *lead standard solution (10 ppm Pb) R*.

Loss on drying (2.2.32): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 100-105 °C.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

Bacterial endotoxins (2.6.14): less than 1.4 IU/g, if intended for use in the manufacture of parenteral dosage forms without a further appropriate procedure for the removal of bacterial endotoxins.

ASSAY

To 0.300 g in a 250 ml round-bottomed flask add 5 ml of *strong sodium hydroxide solution R*, 20 ml of *water R*, 1 g of *zinc powder R* and a few glass beads. Boil under a reflux condenser for 30 min. Allow to cool and rinse the condenser with 20 ml of *water R*, adding the rinsings to the flask. Filter through a sintered-glass filter and wash the filter with several quantities of *water R*. Collect the filtrate and washings. Add 5 ml of *glacial acetic acid R* and titrate immediately with 0.1 M *silver nitrate*. Determine the end-point potentiometrically (2.2.20) using a suitable electrode system such as silver-silver chloride.

1 ml of 0.1 M *silver nitrate* is equivalent to 25.90 mg of $C_{17}H_{22}I_3N_3O_8$.

STORAGE

Protected from light. If the substance is sterile, store in a sterile, airtight, tamper-proof container.

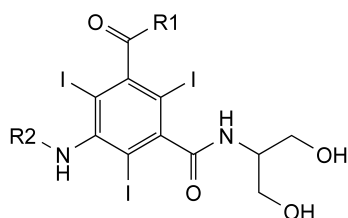
LABELLING

The label states, where applicable, that the substance is free from bacterial endotoxins.

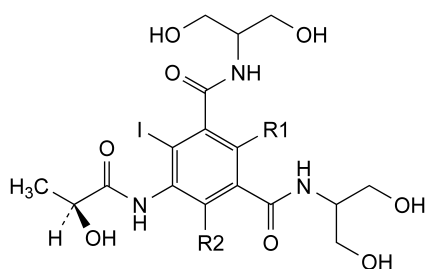
IMPURITIES

Specified impurities: A, B, C, D, E, F, G, H, I, J, K.

01/2005:0700



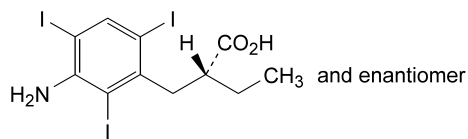
- A. R1 = NH-CH(CH₂OH)₂, R2 = H: 5-amino-*N,N'*-bis[2-hydroxy-1-(hydroxymethyl)ethyl]-2,4,6-triiodobenzene-1,3-dicarboxamide,
- B. R1 = NH-CH(CH₂OH)₂, R2 = CO-CH₂OH: 5-[(hydroxyacetyl)amino]-*N,N'*-bis[2-hydroxy-1-(hydroxymethyl)ethyl]-2,4,6-triiodobenzene-1,3-dicarboxamide,
- C. R1 = NH-CH(CH₂OH)₂, R2 = CO-CH₃: 5-(acetylamino)-*N,N'*-bis[2-hydroxy-1-(hydroxymethyl)ethyl]-2,4,6-triiodobenzene-1,3-dicarboxamide,
- D. R1 = H, R2 = CO-CHOH-CH₃: 3-[[2-hydroxy-1-(hydroxymethyl)ethyl]carbonyl]-5-[(2*S*)-2-hydroxypropanoyl]amino]-2,4,6-triiodobenzoic acid,
- E. R1 = NH-CH(CH₂OH)₂, R2 = CO-CH(CH₃)-O-CO-CH₃: (1*S*)-2-[[3,5-bis[[2-hydroxy-1-(hydroxymethyl)ethyl]carbonyl]-2,4,6-triiodophenyl]amino]-1-methyl-2-oxoethyl acetate,
- F. R1 = N(CH₃)₂, R2 = CO-CHOH-CH₃: *N'*-[2-hydroxy-1-(hydroxymethyl)ethyl]-5-[[[(2*S*)-2-hydroxypropanoyl]amino]-2,4,6-triiodo-*N,N'*-dimethylbenzene-1,3-dicarboxamide,
- G. R1 = NH-CH₂-CHOH-CH₂OH, R2 = CO-CHOH-CH₃: *N*-(2,3-dihydroxypropyl)-*N'*-[2-hydroxy-1-(hydroxymethyl)ethyl]-5-[[[(2*S*)-2-hydroxypropanoyl]amino]-2,4,6-triiodobenzene-1,3-dicarboxamide,
- J. R1 = NH-CH₂-CH₂OH, R2 = CO-CHOH-CH₃: *N*-(2-hydroxyethyl)-*N'*-[2-hydroxy-1-(hydroxymethyl)ethyl]-5-[[[(2*S*)-2-hydroxypropanoyl]amino]-2,4,6-triiodobenzene-1,3-dicarboxamide,



- H. R1 = I, R2 = Cl: 4-chloro-*N,N'*-bis[2-hydroxy-1-(hydroxymethyl)ethyl]-5-[[[(2*S*)-2-hydroxypropanoyl]amino]-2,6-diiodobenzene-1,3-dicarboxamide,
- I. R1 = Cl, R2 = I: 2-chloro-*N,N'*-bis[2-hydroxy-1-(hydroxymethyl)ethyl]-5-[[[(2*S*)-2-hydroxypropanoyl]amino]-4,6-diiodobenzene-1,3-dicarboxamide,
- K. R1 = I, R2 = H: *N,N'*-bis[2-hydroxy-1-(hydroxymethyl)ethyl]-5-[[[(2*S*)-2-hydroxypropanoyl]amino]-2,4-diiodobenzene-1,3-dicarboxamide.

IOPANOIC ACID

Acidum iopanoicum

C₁₁H₁₂I₃NO₂M_r 571

DEFINITION

Iopanoic acid contains not less than 98.5 per cent and not more than the equivalent of 101.0 per cent of (*RS*)-2-(3-amino-2,4,6-triiodobenzyl)butanoic acid, calculated with reference to the dried substance.

CHARACTERS

A white or yellowish-white powder, practically insoluble in water, soluble in ethanol and in methanol. It dissolves in dilute solutions of alkali hydroxides.

IDENTIFICATION

First identification: B.

Second identification: A, C, D.

- A. Melting point (2.2.14): about 155 °C, with decomposition.
- B. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *iopanoic acid CRS*.
- C. Examine the chromatograms obtained in the test for related substances (see Tests). Spray the plate with a 1 g/l solution of 4-dimethylaminocinnamaldehyde *R* in a mixture of 1 volume of *hydrochloric acid R* and 99 volumes of *alcohol R*. The principal spot in the chromatogram obtained with test solution (b) is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).
- D. Heat 50 mg carefully in a small porcelain dish over a flame. Violet vapour is evolved.

TESTS

Appearance of solution. Dissolve 1.0 g in 1 *M sodium hydroxide* and dilute to 20 ml with the same solvent. The solution is clear (2.2.1) and not more intensely coloured than reference solution Y₃ (2.2.2, *Method II*).

Related substances. Examine by thin-layer chromatography (2.2.27), using *silica gel GF₂₅₄ R* as the coating substance.

Test solution (a). Dissolve 1.0 g of the substance to be examined in a mixture of 3 volumes of *ammonia R* and 97 volumes of *methanol R* and dilute to 10 ml with the same mixture of solvents.

Test solution (b). Dilute 1 ml of test solution (a) to 10 ml with a mixture of 3 volumes of *ammonia R* and 97 volumes of *methanol R*.

Reference solution (a). Dissolve 50 mg of *iopanoic acid CRS* in a mixture of 3 volumes of *ammonia R* and 97 volumes of *methanol R* and dilute to 5 ml with the same mixture of solvents.

Reference solution (b). Dilute 1 ml of test solution (b) to 50 ml with a mixture of 3 volumes of *ammonia R* and 97 volumes of *methanol R*.

Apply separately to the plate 5 µl of each solution. Develop over a path of 10 cm using a mixture of 10 volumes of *concentrated ammonia R*, 20 volumes of *methanol R*,

20 volumes of *toluene R* and 50 volumes of *dioxan R*. Examine in ultraviolet light at 254 nm. Any spot in the chromatogram obtained with test solution (a), apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.2 per cent).

Halides. To 0.46 g add 10 ml of *nitric acid R* and 15 ml of *water R*. Shake for 5 min and filter. 15 ml of the filtrate complies with the limit test for chlorides (2.4.4) (180 ppm, expressed as chloride).

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C for 1 h.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

To 0.150 g in a 250 ml round-bottomed flask add 5 ml of *strong sodium hydroxide solution R*, 20 ml of *water R*, 1 g of *zinc powder R* and a few glass beads. Boil under a reflux condenser for 60 min. Allow to cool and rinse the condenser with 20 ml of *water R*, adding the rinsings to the flask. Filter through a sintered-glass filter and wash the filter with several quantities of *water R*. Collect the filtrate and washings. Add 40 ml of *dilute sulphuric acid R* and titrate immediately with 0.1 M *silver nitrate*. Determine the end-point potentiometrically (2.2.20), using a suitable electrode system such as silver-mercurous sulphate.

1 ml of 0.1 M *silver nitrate* is equivalent to 19.03 mg of $C_{11}H_9I_3N_2O_4$.

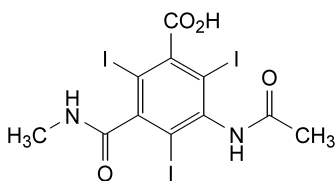
STORAGE

Store protected from light.

01/2005:0751

IOTALAMIC ACID

Acidum iotalamicum



$C_{11}H_9I_3N_2O_4$

M_r 614

DEFINITION

Iotalamic acid contains not less than 98.5 per cent and not more than the equivalent of 101.0 per cent of 3-(acetylamino)-2,4,6-tri-iodo-5-(methylcarbamoyl)benzoic acid, calculated with reference to the dried substance.

CHARACTERS

A white or almost white powder, slightly soluble in water and in alcohol. It dissolves in dilute solutions of alkali hydroxides.

IDENTIFICATION

First identification: A.

Second identification: B, C.

- Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *iotalamic acid CRS*.
- Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel GF₂₅₄ plate R*.

Test solution. Dissolve 50 mg of the substance to be examined in *methanol R* containing 3 per cent V/V of *ammonia R* and dilute to 5 ml with the same solvent.

Reference solution. Dissolve 50 mg of *iotalamic acid CRS* in *methanol R* containing 3 per cent V/V of *ammonia R* and dilute to 5 ml with the same solvent.

Apply separately to the plate 5 µl of each solution.

Develop over a path of 15 cm using a mixture of 20 volumes of *anhydrous formic acid R*, 25 volumes of *methyl ethyl ketone R* and 60 volumes of *toluene R*. Allow the plate to dry until the solvents have evaporated and examine in ultraviolet light at 254 nm. The principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with the reference solution.

- Heat 50 mg gently in a small porcelain dish over a flame. Violet vapour is evolved.

TESTS

Appearance of solution. Dissolve 1.0 g in 1 M *sodium hydroxide* and dilute to 20 ml with the same solvent. The solution is clear (2.2.1) and colourless (2.2.2, *Method II*).

Related substances. Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel GF₂₅₄ plate R*.

Test solution. Dissolve 1.0 g of the substance to be examined in *methanol R* containing 3 per cent V/V of *ammonia R* and dilute to 10 ml with the same solvent.

Reference solution (a). Dilute 1 ml of the test solution to 50 ml with *water R*. Dilute 1 ml of the solution to 10 ml with *water R*.

Reference solution (b). Dissolve 1 mg of *iotalamic acid impurity A CRS* in 5 ml of reference solution (a).

Apply to the plate 5 µl of each solution. Develop over a path of 10 cm using a mixture of 1 volume of *glacial acetic acid R*, 1 volume of *anhydrous formic acid R*, 1 volume of *methanol R*, 5 volumes of *ether R* and 10 volumes of *methylene chloride R*. Allow the plate to dry until the solvents have evaporated. Examine in ultraviolet light at 254 nm. Any spot in the chromatogram obtained with the test solution, apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (a) (0.2 per cent). The test is not valid unless the chromatogram obtained with reference solution (b) shows two clearly separated principal spots.

Halides. Dissolve 0.55 g in a mixture of 4 ml of *dilute sodium hydroxide solution R* and 15 ml of *water R*. Add 6 ml of *dilute nitric acid R* and filter. 15 ml of the filtrate complies with the limit test for chlorides (2.4.4) (150 ppm, expressed as chloride).

Impurity A. Not more than 0.05 per cent *m/m* determined by absorption spectrophotometry (2.2.25).

Test solution. Transfer 0.500 g of the substance to be examined to a 50 ml volumetric flask, add 14 ml of *water R*, shake and add 1 ml of *dilute sodium hydroxide solution R*.

Reference solution. Prepare the reference solution by mixing 10.0 ml of a 8.5 g/l solution of *sodium hydroxide R* containing 25 µg/ml of *iotalamic acid impurity A CRS* with 5 ml of *water R* in a 50 ml volumetric flask.

Blank solution. Transfer 14 ml of *water R* and 1 ml of *dilute sodium hydroxide solution R* to a 50 ml volumetric flask.

In conducting the following steps, keep the flasks in iced water and protected as far as possible from light until all of the reagents have been added.

Place all three of the flasks containing the test solution, the reference solution and the blank solution in iced water, protected from light. Add 5 ml of a 5 g/l solution of *sodium*

01/2005:2009
corrected

nitrite R and 12 ml of *dilute hydrochloric acid R*. Shake gently and allow to stand for exactly 2 min after adding the hydrochloric acid. Add 10 ml of a 20 g/l solution of *ammonium sulphamate R*. Allow to stand for 5 min shaking frequently (*CAUTION: considerable pressure is produced*), and add 0.15 ml of a 100 g/l solution of *α-naphthol R* in *alcohol R*. Shake and allow to stand for 5 min. Add 3.5 ml of *buffer solution pH 10.9 R*, mix and dilute to 50.0 ml with *water R*. Concomitantly and within 20 min determine the absorbance at 485 nm of the solutions obtained from the test solution and the reference solution in 5 cm cells, using the blank as the compensation liquid.

Calculate the content of impurity A.

Iodide. Not more than 20 ppm, determined by potentiometric titration (2.2.20). Dissolve 6.000 g in 20 ml of 1 M *sodium hydroxide*, add 10 ml of *water R* and adjust to pH 4.5 to 5.5 with *acetic acid R*. Add 2.0 ml of 0.001 M *potassium iodide*. Titrate with 0.001 M *silver nitrate* using a silver indicator electrode and an appropriate reference electrode. Subtract the volume of titrant corresponding to the 2.0 ml of 0.001 M *potassium iodide*, determined by titrating a blank to which is added 2.0 ml of 0.001 M *potassium iodide* and use the residual value to calculate the iodide content.

1 ml of 0.001 M *silver nitrate* is equivalent to 126.9 µg of iodide.

Heavy metals (2.4.8). Dissolve 2.0 g in 4 ml of *dilute sodium hydroxide solution R* and dilute to 20 ml with *water R*. 12 ml of this solution complies with limit test A for heavy metals (20 ppm). Prepare the standard using *lead standard solution (2 ppm Pb) R*.

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 0.300 g by drying in an oven at 100 °C to 105 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

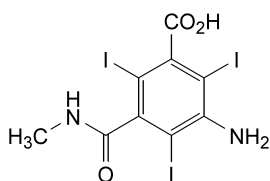
To 0.150 g in a 250 ml round-bottomed flask add 5 ml of *strong sodium hydroxide solution R*, 20 ml of *water R*, 1 g of *zinc powder R* and a few glass beads. Boil under a reflux condenser for 30 min. Allow to cool and rinse the condenser with 20 ml of *water R*, adding the rinsings to the flask. Filter through a sintered-glass filter and wash the filter with several quantities of *water R*. Collect the filtrate and washings. Add 40 ml of *dilute sulphuric acid R* and titrate immediately with 0.1 M *silver nitrate*. Determine the end-point potentiometrically (2.2.20), using a suitable electrode system such as silver-mercurous sulphate.

1 ml of 0.1 M *silver nitrate* is equivalent to 20.47 mg of $C_{11}H_9I_3N_2O_4$.

STORAGE

Store protected from light.

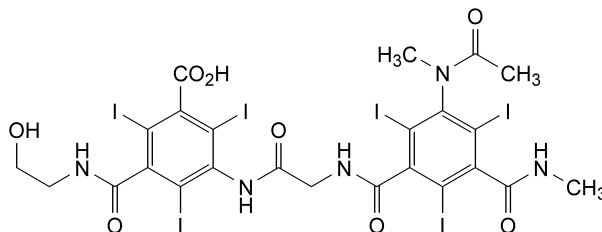
IMPURITIES



A. 3-amino-2,4,6-triiodo-5-(methylcarbamoyl)benzoic acid.

IOXAGLIC ACID

Acidum ioxaglicum



$C_{24}H_{21}I_6N_5O_8$

M_r 1269

DEFINITION

3-[[[3-(Acetylmethylamino)-2,4,6-triiodo-5-(methylcarbamoyl)benzoyl]amino]acetyl]amino]-5-[(2-hydroxyethyl)carbamoyl]-2,4,6-triiodobenzoic acid.

Content: 98.5 per cent to 101.5 per cent (anhydrous substance).

CHARACTERS

Appearance: white or almost white powder, hygroscopic.

Solubility: very slightly soluble in water, slightly soluble in alcohol, very slightly soluble in methylene chloride. It dissolves in dilute solutions of alkali hydroxides.

IDENTIFICATION

Infrared absorption spectrophotometry (2.2.24).

Comparison: ioxaglic acid CRS.

TESTS

Appearance of solution. The solution is clear (2.2.1).

Dissolve 1.0 g in a 40 g/l solution of *sodium hydroxide R* and dilute to 20 ml with the same solution.

Absorbance (2.2.25): maximum 0.18, calculated for a solution containing 40 per cent of anhydrous ioxaglic acid.

Dissolve 10.0 g in about 8 ml of a 40 g/l solution of *sodium hydroxide R*. Adjust to pH 7.2-7.6 with a 40 g/l solution of *sodium hydroxide R* or 1 M *hydrochloric acid*. Dilute to 25 ml with *water R*. Filter using a filter of 0.45 µm pore size. Measure the absorbance at 450 nm using *water R* as the compensation liquid.

Related substances. Liquid chromatography (2.2.29): use the normalisation procedure.

Test solution. Dissolve 0.10 g of the substance to be examined in about 40 ml of a mixture of 5 volumes of *acetonitrile R* and 95 volumes of *water R*. Add 0.5 ml ± 0.1 ml of a 4 g/l solution of *sodium hydroxide R* and dilute to 50.0 ml with a mixture of 5 volumes of *acetonitrile R* and 95 volumes of *water R*. Shake until dissolution is complete (using ultrasound, if necessary).

Reference solution (a). Dissolve 0.10 g of *ioxaglic acid CRS* in about 40 ml of a mixture of 5 volumes of *acetonitrile R* and 95 volumes of *water R*. Add 0.5 ml ± 0.1 ml of a 4 g/l solution of *sodium hydroxide R* and dilute to 50.0 ml with a mixture of 5 volumes of *acetonitrile R* and 95 volumes of *water R*. Shake until dissolution is complete (using ultrasound, if necessary).

Reference solution (b). Dissolve 5 mg of *ioxaglic acid impurity A CRS* in a mixture of 5 volumes of *acetonitrile R* and 95 volumes of *water R* and dilute to 50.0 ml with the

same mixture of solvents. Dilute 1.0 ml of the solution to 50.0 ml with a mixture of 5 volumes of *acetonitrile R* and 95 volumes of *water R*.

Column:

- **size:** $l = 0.25$ m, $\varnothing = 4.6$ mm,
- **stationary phase:** spherical end-capped *octylsilyl silica gel for chromatography R* (5 μ m) with a specific surface area of not less than 335 m²/g, a pore size of 10 nm and a carbon loading of not less than 12 per cent,
- **temperature:** 25 °C.

Mobile phase:

- **mobile phase A:** 136 mg/l solution of *potassium dihydrogen phosphate R* adjusted to pH 3.0 with *phosphoric acid R*,
- **mobile phase B:** *acetonitrile R*,

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)
0 - 5	95 → 90	5 → 10
5 - 40	90	10
40 - 85	90 → 70	10 → 30
85 - 115	70	30
115 - 120	70 → 50	30 → 50
120 - 125	50	50
125 - 130	50 → 95	50 → 5
130 - 140	95	5

Flow rate: 0.8 ml/min.

Detection: spectrophotometer at 242 nm.

Injection: 10 μ l.

System suitability:

- **retention time:** ioxaglic acid = about 65 min (reference solution (a)), impurity A = about 22 min (reference solution (b)),
- the chromatogram obtained with reference solution (a) is similar to the chromatogram provided with *ioxaglic acid CRS*,
- **peak-to-valley ratio:** minimum 1.3, where H_p = height above the baseline of the peak due to impurity C and H_v = height above the baseline of the lowest point of the curve separating this peak from the peak due to ioxaglic acid in the chromatogram obtained with reference solution (a).

Limit: locate the impurities by comparison with the chromatogram provided with *ioxaglic acid CRS*.

- **impurity A:** not more than 0.1 per cent,
- **impurity B:** not more than 0.3 per cent,
- **impurity C:** not more than 0.3 per cent,
- **impurity E:** not more than 0.7 per cent,
- **impurity F:** not more than 0.4 per cent,
- **impurity D (sum of the peaks D1, D2, D3 and D4):** not more than 0.7 per cent,
- **any other impurity:** not more than 0.2 per cent,
- **total:** not more than 2 per cent,
- **disregard limit:** 0.05 per cent; disregard any peak appearing at a retention time greater than 125 min.

Iodides: maximum 50 ppm.

Disperse 10.0 g in 50 ml of *water R*. Add 8 ml of 1 M *sodium hydroxide*. After dissolution and homogenisation, add 1.0 ml of *glacial acetic acid R*. Immediately titrate with 0.001 M *silver nitrate*, determining the end-point potentiometrically (2.2.20), using a silver indicator electrode and a suitable reference electrode.

1 ml of 0.001 M *silver nitrate* is equivalent to 0.1269 mg of iodides.

Heavy metals (2.4.8): maximum 10 ppm.

Dissolve 2.0 g in 4 ml of a 40 g/l solution of *sodium hydroxide R* and dilute to 20 ml with *water R*. 12 ml of the solution complies with limit test A. Prepare the standard using 1 ml of *lead standard solution (10 ppm Pb) R*.

Water (2.5.12): maximum 5.0 per cent, determined on 0.100 g.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g. Use *sulphuric acid R* instead of *dilute sulphuric acid R*.

ASSAY

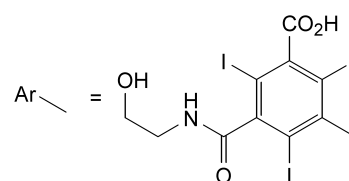
In a round-bottomed flask, place 0.100 g of the substance to be examined, add 5 ml of *strong sodium hydroxide solution R*, 20 ml of *water R*, 1 g of *zinc powder R* and a few glass beads. Fit the flask with a reflux condenser and boil for 30 min. Cool and rinse the condenser with 20 ml of *water R*. Add the rinsing liquid to the contents of the flask. Filter through a sintered-glass filter, wash the filter 3 times with 15 ml of *water R* and add the washings to the filtrate. Add 40 ml of *dilute sulphuric acid R* and titrate immediately with 0.05 M *silver nitrate*. Determine the end-point potentiometrically (2.2.20), using a suitable electrode combination such as the silver/mercurous sulphate system.

1 ml of 0.05 M *silver nitrate* is equivalent to 10.58 mg of C₂₄H₂₁I₆N₅O₈.

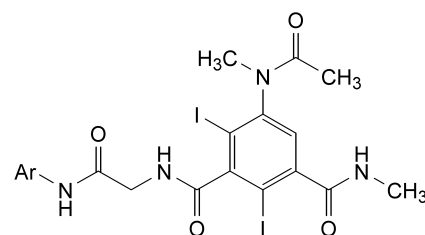
STORAGE

In an airtight container, protected from light.

IMPURITIES

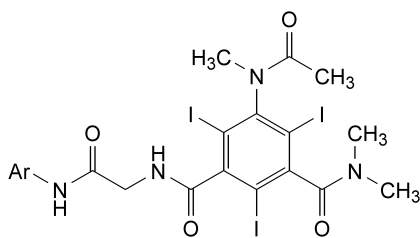


A. Ar-NH₂: 3-amino-5-[(2-hydroxyethyl)carbamoyl]-2,4,6-triiodobenzoic acid,

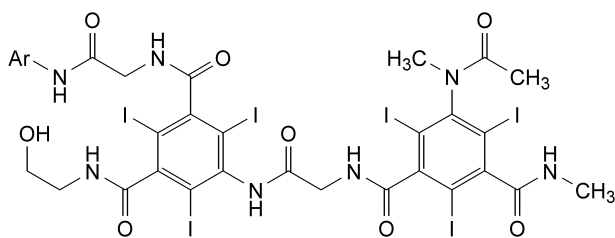


B. 3-[[[3-(acetylmethylamino)-2,6-diiodo-5-(methylcarbamoyl)benzoyl]amino]acetyl]amino]-5-[(2-hydroxyethyl)carbamoyl]-2,4,6-triiodobenzoic acid,

C. specified impurity whose structure is unknown,

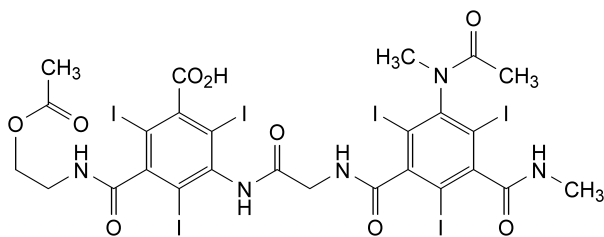


D. D1, D2, D3 and D4: 3-[[[3-(acetylmethylamino)-5-(dimethylcarbamoyl)-2,4,6-triiodobenzoyl]amino]acetyl]amino]-5-[(2-hydroxyethyl)carbamoyl]-2,4,6-triiodobenzoic acid,

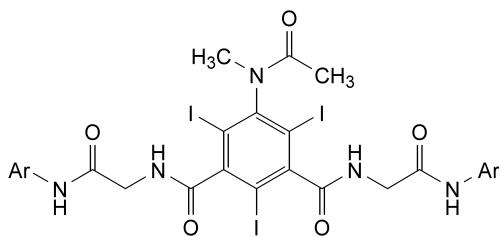


E. 3-[[[3-[[[3-(acetylmethylamino)-2,4,6-triiodo-5-(methylcarbamoyl)benzoyl]amino]acetyl]amino]-5-[(2-hydroxyethyl)carbamoyl]-2,4,6-triiodobenzoyl]amino]acetyl]amino]-5-[(2-hydroxyethyl)carbamoyl]-2,4,6-triiodobenzoic acid,

F. specified impurity whose structure is unknown,



G. 3-[[[3-(acetylmethylamino)-2,4,6-triiodo-5-(methylcarbamoyl)benzoyl]amino]acetyl]amino]-5-[[2-(acetyloxy)ethyl]carbamoyl]-2,4,6-triiodobenzoic acid,



H. 3,3'-[[5-(acetylmethylamino)-2,4,6-triiodo-1,3-phenylene]bis(carbonyliminomethylenecarbonylimino)]bis[5-[(2-hydroxyethyl)carbamoyl]-2,4,6-triiodobenzoic acid.

01/2005:1875

IPECACUANHA LIQUID EXTRACT, STANDARDISED

*Ipecacuanhae extractum fluidum
normatum*

DEFINITION

Standardised liquid extract produced from *Ipecacuanha* root (0094).

Content: minimum 1.80 per cent and maximum 2.20 per cent of total alkaloids, calculated as emetine ($C_{29}H_{40}N_2O_4$; M_r 480.7).

PRODUCTION

The extract is produced from the herbal drug and solvent of suitable strength by an appropriate procedure.

CHARACTERS

Appearance: dark-brown liquid.

IDENTIFICATION

Thin-layer chromatography (2.2.27).

Test solution. Dilute 5.0 ml of the extract to be examined to 50 ml with *alcohol* (70 per cent V/V) *R*. To 2.0 ml of this solution add 2 ml of *water R* and 0.1 ml of *concentrated ammonia R*. Add 10 ml of *ether R* and shake. Separate the upper layer, dry it over about 2 g of *anhydrous sodium sulphate R* and filter.

Reference solution. Dissolve 2.5 mg of *emetine hydrochloride CRS* and 3 mg of *cephaeline hydrochloride CRS* in *methanol R* and dilute to 10 ml with the same solvent.

Plate: TLC silica gel plate *R*.

Mobile phase: *concentrated ammonia R*, *methanol R*, *ethyl acetate R*, *toluene R* (2:15:18:65 V/V/V/V).

Application: 10 µl, as bands.

Development: over a path of 10 cm.

Drying: in air.

Detection A: spray with a 5 g/l solution of *iodine R* in *alcohol R*. Heat at 60 °C for 10 min and allow to cool for 30 min. Examine in daylight.

Results A: see below the sequence of the zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other zones may be present in the chromatogram obtained with the test solution.

Top of the plate	
_____	_____
_____	_____
Emetine: a yellow zone	A yellow zone (emetine)
Cephaeline: a light brown zone	A light brown zone (cephaeline)
Reference solution	Test solution

Detection B: examine in ultraviolet light at 365 nm.

Results B: see below the sequence of the zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other faint fluorescent zones are present in the chromatogram obtained with the test solution.

Top of the plate	
_____	_____
_____	_____
Emetine: an intense yellow fluorescent zone	An intense yellow fluorescent zone (emetine)
Cephaeline: a light blue or orange-yellow fluorescent zone	A light blue or orange-yellow fluorescent zone (cephaeline)
Reference solution	Test solution

With a liquid extract from *Cephaelis acuminata* root, the zones of emetine and cephaeline in the chromatogram obtained with the test solution are of similar size.

With a liquid extract from *Cephaelis ipecacuanha* root, the zone of emetine is much larger than the zone of cephaeline in the chromatogram obtained with the test solution.

TESTS

Ethanol (2.9.10): minimum 95 per cent and maximum 105 per cent of the quantity stated on the label.

ASSAY

Dilute 1.00 g of the extract to be examined to 10 ml with *alcohol* (70 per cent V/V) R and transfer to a chromatography column about 0.2 m long and about 15 mm in internal diameter, containing 8 g of *basic aluminium oxide* R, using a glass rod. After infiltration into the aluminium oxide layer, rinse the flask, glass rod and internal wall of the column with 3 quantities, each of 2 ml, of *alcohol* (70 per cent V/V) R. Elute in portions with 40 ml of *alcohol* (70 per cent V/V) R. Avoid disturbance or drying of the surface of the aluminium oxide layer. Collect the eluate in a 100 ml flask. Evaporate the eluate on a water-bath to about 10 ml. Allow to cool. Add 10.0 ml of 0.02 M *hydrochloric acid* and 20 ml of *carbon dioxide-free water* R. Titrate the excess acid with 0.02 M *sodium hydroxide* using 0.15 ml of *methyl red mixed solution* R as indicator.

Perform a blank assay by repeating the assay but replacing the extract to be examined with 10.0 ml of *alcohol* of the strength stated on the label.

1 ml of 0.02 M *hydrochloric acid* is equivalent to 4.807 mg of total alkaloids, calculated as *emetine*.

01/2005:0093

IPECACUANHA, PREPARED

Ipecacuanhae pulvis normatus

DEFINITION

Prepared ipecacuanha is ipecacuanha root powder (180) adjusted, if necessary, by the addition of powdered lactose or ipecacuanha root powder with a lower alkaloidal content to contain 1.9 per cent to 2.1 per cent of total alkaloids, calculated as *emetine* ($C_{29}H_{40}N_2O_4$; M_r 480.7) with reference to the dried drug.

CHARACTERS

Light grey to yellowish-brown powder with a slight odour.

IDENTIFICATION

- A. Examine under a microscope, using *chloral hydrate solution* R. The powder shows the following diagnostic characters: parenchymatous cells, raphides of calcium oxalate up to 80 µm in length either in bundles or scattered throughout the powder; fragments of tracheids and vessels usually 10 µm to 20 µm in diameter, with bordered pits; larger vessels and sclereids from the rhizome. Examine under a microscope using a 50 per cent V/V solution of *glycerol* R. The powder shows simple or two- to eight-compound starch granules contained in parenchymatous cells, the simple granules being up to 15 µm in diameter in *C. ipecacuanha* and up to 22 µm in diameter in *C. acuminata*. Examined in *glycerol* (85 per cent) R, it may be seen to contain lactose crystals.
- B. Examine by thin-layer chromatography (2.2.27), using a suitable silica gel as the coating substance.
Test solution. To 0.1 g in a test-tube add 0.05 ml of *concentrated ammonia* R and 5 ml of *ether* R and stir the mixture vigorously with a glass rod. Allow to stand for 30 min and filter.
Reference solution. Dissolve 2.5 mg of *emetine hydrochloride* CRS and 3 mg of *cephaeline hydrochloride* CRS in *methanol* R and dilute to 20 ml with the same solvent.

Apply separately to the plate as bands 10 µl of each solution. Develop over a path of 10 cm using a mixture of 2 volumes of *concentrated ammonia* R, 15 volumes of *methanol* R, 18 volumes of *ethyl acetate* R and 65 volumes of *toluene* R. Allow the plate to dry in air. Spray with a 5 g/l solution of *iodine* R in *alcohol* R and heat at 60 °C for 10 min. Examine in daylight. The chromatograms obtained with the test solution and with the reference solution show in the lower part a yellow zone corresponding to *emetine* and below it a light brown zone corresponding to *cephaeline*. Examine in ultraviolet light at 365 nm. The zone corresponding to *emetine* shows an intense yellow fluorescence and that corresponding to *cephaeline* a light blue fluorescence. The chromatogram obtained with the test solution shows also faint fluorescent zones.

With *C. acuminata* the principal zones in the chromatogram obtained with the test solution are similar in position, fluorescence and size to the zones in the chromatogram obtained with the reference solution.

With *C. ipecacuanha* the only difference is that the zone corresponding to *cephaeline* in the chromatogram obtained with the test solution is much smaller than the corresponding zone in the chromatogram obtained with the reference solution.

TESTS

Loss on drying (2.2.32). Not more than 5.0 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C.

Total ash (2.4.16). Not more than 5.0 per cent.

Ash insoluble in hydrochloric acid (2.8.1). Not more than 3.0 per cent.

ASSAY

To 7.5 g in a dry flask, add 100 ml of *ether* R and shake for 5 min. Add 5 ml of *dilute ammonia* R1, shake for 1 h, add 5 ml of *water* R and shake vigorously. Decant the ether layer into a flask through a plug of cotton. Wash the residue in the flask with two quantities, each of 25 ml, of *ether* R, decanting each portion through the same plug of cotton. Combine the ether solutions and eliminate the ether by distillation. Dissolve the residue in 2 ml of *alcohol* (90 per cent V/V) R, evaporate the alcohol to dryness and heat at 100 °C for 5 min. Dissolve the residue in 5 ml of previously neutralised *alcohol* (90 per cent V/V) R, warming on a water-bath, add 15.0 ml of 0.1 M *hydrochloric acid* and titrate the excess acid with 0.1 M *sodium hydroxide* using 0.5 ml of *methyl red mixed solution* R as indicator.

1 ml of 0.1 M *hydrochloric acid* is equivalent to 24.03 mg of total alkaloids, calculated as *emetine*.

STORAGE

Store in an airtight container, protected from light.

01/2005:0094

IPECACUANHA ROOT

Ipecacuanhae radix

DEFINITION

Ipecacuanha root consists of the fragmented and dried underground organs of *Cephaelis ipecacuanha* (Brot.) A. Rich., known as Matto Grosso ipecacuanha, or of *Cephaelis acuminata* Karsten, known as Costa Rica ipecacuanha, or of a mixture of both species. It contains not less than 2.0 per

cent of total alkaloids, calculated as emetine ($C_{29}H_{40}N_2O_4$; M_r 480.7) with reference to the dried drug. The principal alkaloids are emetine and cephaeline.

CHARACTERS

Ipecacuanha root has a slight odour.

It has the macroscopic and microscopic characters described under identification tests A and B.

IDENTIFICATION

A. *C. ipecacuanha*. The root occurs as somewhat tortuous pieces, from dark reddish-brown to very dark brown, seldom more than 15 cm long or 6 mm thick, closely annulated externally, having rounded ridges completely encircling the root; the fracture is short in the bark and splintery in the wood. The transversely cut surface shows a wide greyish bark and a small uniformly dense wood. The rhizome occurs as short lengths usually attached to roots, cylindrical, up to 2 mm in diameter, finely wrinkled longitudinally and with pith occupying approximately one-sixth of the whole diameter.

C. acuminata. The root in general resembles the root of *C. ipecacuanha*, but differs in the following particulars: it is often up to 9 mm thick; the external surface is greyish-brown or reddish-brown with transverse ridges at intervals of usually 1 mm to 3 mm, the ridges being about 0.5 mm to 1 mm wide, extending about half-way round the circumference and fading at the extremities into the general surface level.

B. Reduce to a powder (355). The powder is light grey to yellowish-brown. Examine under a microscope, using *chloral hydrate solution R*. The powder shows the following diagnostic characters: parenchymatous cells, raphides of calcium oxalate up to 80 µm in length either in bundles or scattered throughout the powder; fragments of tracheids and vessels usually 10 µm to 20 µm in diameter, with bordered pits; larger vessels and sclereids from the rhizome. Examine under a microscope using a 50 per cent V/V solution of *glycerol R*. The powder shows simple or two- to eight-compound starch granules contained in parenchymatous cells, the simple granules being up to 15 µm in diameter in *C. ipecacuanha* and up to 22 µm in diameter in *C. acuminata*.

C. Examine by thin-layer chromatography (2.2.27), using a suitable silica gel as the coating substance.

Test solution. To 0.1 g of the powdered drug (180) in a test-tube add 0.05 ml of *concentrated ammonia R* and 5 ml of *ether R* and stir the mixture vigorously with a glass rod. Allow to stand for 30 min and filter.

Reference solution. Dissolve 2.5 mg of *emetine hydrochloride CRS* and 3 mg of *cephaeline hydrochloride CRS* in *methanol R* and dilute to 20 ml with the same solvent.

Apply separately to the plate as bands 10 µl of each solution. Develop over a path of 10 cm using a mixture of 2 volumes of *concentrated ammonia R*, 15 volumes of *methanol R*, 18 volumes of *ethyl acetate R* and 65 volumes of *toluene R*. Allow the plate to dry in air. Spray with a 5 g/l solution of *iodine R* in *alcohol R* and heat at 60 °C for 10 min. Examine in daylight. The chromatograms obtained with the test solution and with the reference solution show in the lower part a yellow zone corresponding to emetine and below it a light brown zone corresponding to cephaeline. Examine in ultraviolet light at 365 nm. The zone corresponding to emetine shows an intense yellow fluorescence and that

corresponding to cephaeline a light blue fluorescence. The chromatogram obtained with the test solution shows also faint fluorescent zones.

With *C. acuminata* the principal zones in the chromatogram obtained with the test solution are similar in position, fluorescence and size to the zones in the chromatogram obtained with the reference solution.

With *C. ipecacuanha* the only difference is that the zone corresponding to cephaeline in the chromatogram obtained with the test solution is much smaller than the corresponding zone in the chromatogram obtained with the reference solution.

TESTS

Foreign matter (2.8.2). It complies with the test for foreign matter.

Loss on drying (2.2.32). Not more than 10.0 per cent, determined on 1.000 g of powdered drug (180) by drying in an oven at 100-105 °C.

Total ash (2.4.16). Not more than 5.0 per cent.

Ash insoluble in hydrochloric acid (2.8.1). Not more than 3.0 per cent.

ASSAY

To 7.5 g of the powdered drug (180) in a dry flask, add 100 ml of *ether R* and shake for 5 min. Add 5 ml of *dilute ammonia R1*, shake for 1 h, add 5 ml of *water R* and shake vigorously. Decant the ether layer into a flask through a plug of cotton. Wash the residue in the flask with 2 quantities, each of 25 ml, of *ether R*, decanting each portion through the same plug of cotton. Combine the ether solutions and eliminate the ether by distillation. Dissolve the residue in 2 ml of *alcohol (90 per cent V/V) R*, evaporate the alcohol to dryness and heat at 100 °C for 5 min. Dissolve the residue in 5 ml of previously neutralised *alcohol (90 per cent V/V) R*, warming on a water-bath, add 15.0 ml of 0.1 M *hydrochloric acid* and titrate the excess acid with 0.1 M *sodium hydroxide* using 0.5 ml of *methyl red mixed solution R* as indicator.

1 ml of 0.1 M *hydrochloric acid* is equivalent to 24.03 mg of total alkaloids, calculated as emetine.

STORAGE

Store protected from light and moisture.

01/2005:1530

IPECACUANHA TINCTURE, STANDARDISED

Ipecacuanhae tinctura normata

DEFINITION

Tincture produced from *Ipecacuanha root (0094)*.

Content: 0.18 per cent (m/m) to 0.22 per cent (m/m) of total alkaloids, calculated as emetine ($C_{29}H_{40}N_2O_4$; M_r 480.7).

PRODUCTION

The tincture is produced by a suitable procedure from the herbal drug and ethanol of suitable strength.

CHARACTERS

Appearance: yellowish-brown liquid.

IDENTIFICATION

Thin-layer chromatography (2.2.27).

Test solution. To 2.0 ml of the tincture to be examined add 2 ml of *water R* and 0.1 ml of *concentrated ammonia R*. Add 10 ml of *ether R* and shake. Separate the ether layer, dry it over about 2 g of *anhydrous sodium sulphate R* and filter.

Reference solution. Dissolve 2.5 mg of *emetine hydrochloride CRS* and 3 mg of *cephaeline hydrochloride CRS* in *methanol R* and dilute to 10 ml with the same solvent.

Plate: TLC silica gel plate *R*.

Mobile phase: *concentrated ammonia R*, *methanol R*, *ethyl acetate R*, *toluene R* (2:15:18:65 V/V/V/V).

Application: 10 µl, as bands.

Development: over a path of 10 cm.

Drying: in air.

Detection A: spray the plate with a 5 g/l solution of *iodine R* in *alcohol R* and heat at 60 °C for 10 min. Examine in daylight.

Results A: see below the sequence of the zones present in the chromatograms obtained with the reference solution and the test solution.

Top of the plate	
_____	_____
_____	_____
Emetine: a yellow zone	A yellow zone (emetine)
Cephaeline: a light brown zone	A light brown zone (cephaeline)
Reference solution	Test solution

Detection B: examine the plate in ultraviolet light at 365 nm.

Results B: see below the sequence of the zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other faint fluorescent zones are present in the chromatogram obtained with the test solution.

Top of the plate	
_____	_____
_____	_____
Emetine: an intense yellow fluorescent zone	An intense yellow fluorescent zone (emetine)
Cephaeline: a light blue or orange-yellow fluorescent zone	A light blue or orange-yellow fluorescent zone (cephaeline)
Reference solution	Test solution

With a tincture from *Cephaelis acuminata* root the zones of emetine and cephaeline in the chromatogram obtained with the test solution are similar in size.

With a tincture from *Cephaelis ipecacuanha* root the zone of emetine is much larger than the zone of cephaeline in the chromatogram obtained with the test solution.

TESTS

Ethanol (2.9.10): 95 per cent to 105 per cent of the quantity stated on the label.

ASSAY

Transfer 10.00 g of the tincture to be examined to a chromatography column about 0.2 m long and about 15 mm in internal diameter, filled with 8 g of *basic aluminium oxide R*. After infiltration into the aluminium oxide layer rinse the internal wall of the column with 3 quantities, each of 2 ml, of *alcohol (70 per cent V/V) R*. Elute in portions, with 40 ml of *alcohol (70 per cent V/V) R*. Avoid whirling or drying of the surface of the aluminium oxide layer. Collect the eluate in a 100 ml flask. Evaporate the eluate on a water-bath to about 10 ml. Allow to cool. Add 10.0 ml of 0.02 M hydrochloric acid and 20 ml of carbon dioxide-free

water R. Titrate the excess acid with 0.02 M sodium hydroxide using 0.15 ml of *methyl red mixed solution R* as indicator.

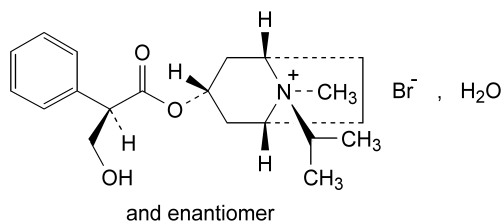
Perform a blank assay replacing the tincture to be examined with 10.0 ml of alcohol of the strength stated on the label.

1 ml of 0.02 M hydrochloric acid is equivalent to 4.807 mg of total alkaloids, calculated as emetine.

01/2005:0919

IPRATROPIUM BROMIDE

Ipratropii bromidum

C₂₀H₃₀BrNO₃·H₂OM_r 430.4

DEFINITION

(1*R*,3*r*,5*S*,8*r*)-3-[[*(2RS)*-3-Hydroxy-2-phenylpropanoyl]oxy]-8-methyl-8-(1-methylethyl)-8-azoniabicyclo[3.2.1]octane bromide monohydrate.

Content: 99.0 per cent to 100.5 per cent (anhydrous substance).

CHARACTERS

Appearance: white or almost white, crystalline powder.

Solubility: soluble in water, freely soluble in methanol, slightly soluble in alcohol.

mp: about 230 °C, with decomposition.

IDENTIFICATION

First identification: A, E.

Second identification: B, C, D, E.

A. Infrared absorption spectrophotometry (2.2.24).

Comparison: *ipratropium bromide CRS*.

B. Examine the chromatograms obtained in the test for impurity A.

Results: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).

C. To 5 ml of solution S (see Tests), add 2 ml of *dilute sodium hydroxide solution R*. No precipitate is formed.

D. To about 1 mg add 0.2 ml of *nitric acid R* and evaporate to dryness on a water-bath. Dissolve the residue in 2 ml of *acetone R* and add 0.1 ml of a 30 g/l solution of *potassium hydroxide R* in *methanol R*. A violet colour develops.

E. It gives reaction (a) of bromides (2.3.1).

TESTS

Solution S. Dissolve 0.50 g in *carbon dioxide-free water R* and dilute to 50.0 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and not more intensely coloured than reference solution GY₇ (2.2.2, Method II).

pH (2.2.3): 5.0 to 7.5 for solution S.

Impurity A. Thin-layer chromatography (2.2.27).

Test solution. Dissolve 20 mg of the substance to be examined in *methanol R* and dilute to 1.0 ml with the same solvent.

Reference solution (a). Dissolve 20 mg of *ipratropium bromide CRS* in *methanol R* and dilute to 1.0 ml with the same solvent.

Reference solution (b). Dissolve 20 mg of *methylatropine bromide CRS* in 1.0 ml of reference solution (a).

Reference solution (c). Dissolve 5 mg of *ipratropium impurity A CRS* in 100.0 ml of *methanol R*. Dilute 2.0 ml of the solution to 5.0 ml with *methanol R*.

Plate: TLC silica gel plate *R* (2-10 µm).

Mobile phase: *anhydrous formic acid R*, *water R*, *alcohol R*, *methylene chloride R* (1:3:18:18 V/V/V/V).

Application: 1 µl.

Development: over a path of 6 cm.

Drying: at 60 °C for 15 min.

Detection: spray with *potassium iodobismuthate solution R*, allow the plate to dry in air, spray with a 50 g/l solution of *sodium nitrite R* and protect immediately with a sheet of glass.

System suitability: the chromatogram obtained with reference solution (b) shows 2 clearly separated principal spots.

Limits:

- **impurity A:** any spot due to impurity A is not more intense than the principal spot in the chromatogram obtained with reference solution (c) (0.1 per cent).

Related substances. Liquid chromatography (2.2.29).

Test solution. Dissolve 0.200 g of the substance to be examined in the mobile phase and dilute to 20.0 ml with the mobile phase.

Reference solution (a). Dissolve 10.0 mg of *ipratropium bromide CRS* in the mobile phase and dilute to 20.0 ml with the mobile phase. Dilute 1.0 ml of the solution to 50.0 ml with the mobile phase.

Reference solution (b). Dissolve 5 mg of *ipratropium bromide CRS* and 5 mg of *ipratropium impurity B CRS* in 1 ml of *methanol R* and dilute to 25.0 ml with the mobile phase. Dilute 1.0 ml of the solution to 20.0 ml with the mobile phase.

Column:

- **size:** $l = 0.15$ m, $\varnothing = 3.9$ mm,
- **stationary phase:** *octadecylsilyl silica gel for chromatography R* (5 µm),
- **temperature:** 30 °C.

Mobile phase: dissolve 12.4 g of *sodium dihydrogen phosphate R* and 1.7 g of *tetrapropylammonium chloride R* in 870 ml of *water R*; adjust to pH 5.5 with a 180 g/l solution of *disodium hydrogen phosphate R* and add 130 ml of *methanol R*.

Flow rate: 1.5 ml/min.

Detection: spectrophotometer at 220 nm.

Injection: 5 µl.

Run time: 6 times the retention time of ipratropium.

Relative retention with reference to ipratropium (retention time = about 4.9 min): impurity C = about 0.7; impurity B = about 1.2; impurity D = about 1.8; impurity E = about 2.3; impurity F = about 5.1.

System suitability: reference solution (b):

- **resolution:** minimum 3.0 between the peaks due to impurity B and ipratropium,
- **symmetry factor:** maximum 2.5 for the principal peak.

Limits:

- **correction factors:** for the calculation of contents, multiply the peak areas of the following impurities by the corresponding correction factor: impurity C = 0.3; impurity D = 0.2; impurity F = 0.5;
- **impurity D:** not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent);
- **any other impurity:** not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent);
- **total:** not more than 2.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.25 per cent);
- **disregard limit:** one-third of the area of the principal peak in the chromatogram obtained with reference solution (a) (0.03 per cent).

Water (2.5.12): 3.9 per cent to 4.4 per cent, determined on 0.50 g.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

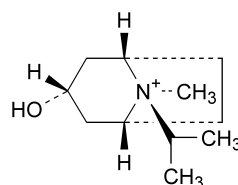
Dissolve 0.350 g in 50 ml of *water R* and add 3 ml of *dilute nitric acid R*. Titrate with 0.1 M *silver nitrate*, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *silver nitrate* is equivalent to 41.24 mg of $C_{20}H_{30}BrNO_3$.

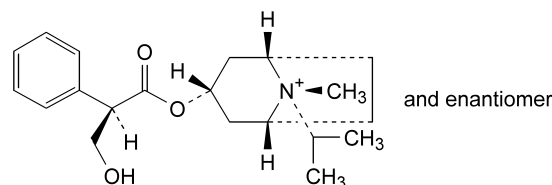
IMPURITIES

Specified impurities: A, B, C, D.

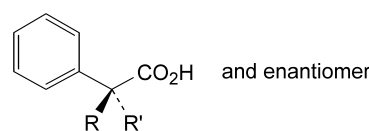
Other detectable impurities: E, F.



A. (1*R*,3*r*,5*S*,8*r*)-3-hydroxy-8-methyl-8-(1-methylethyl)-8-azoniabicyclo[3.2.1]octane,

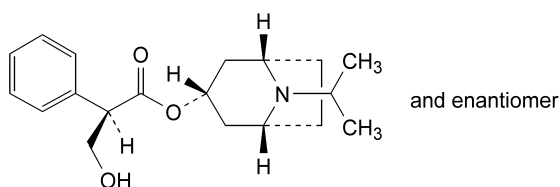


B. (1*R*,3*r*,5*S*,8*s*)-3-[(2*RS*)-3-hydroxy-2-phenylpropanoyl]oxy]-8-methyl-8-(1-methylethyl)-8-azoniabicyclo[3.2.1]octane,

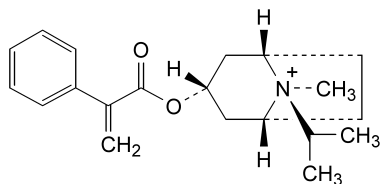


C. R = CH_2-OH , R' = H: (2*RS*)-3-hydroxy-2-phenylpropanoic acid (DL-tropic acid),

D. R + R' = CH_2 : 2-phenylpropenoic acid (atropic acid),



E. (1*R*,3*r*,5*S*)-8-(1-methylethyl)-8-azabicyclo[3.2.1]oct-3-yl (2*RS*)-3-hydroxy-2-phenylpropanoate,

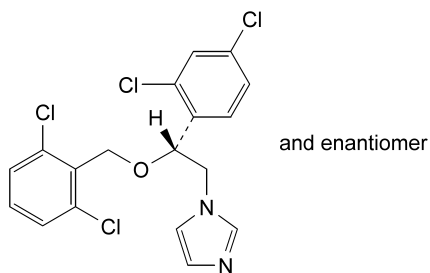


F. (1*R*,3*r*,5*S*,8*r*)-8-methyl-8-(1-methylethyl)-3-[(2-phenylpropenyl)oxy]-8-azoniabicyclo[3.2.1]octane.

01/2005:1018

ISOCONAZOLE

Isoconazolium



$C_{18}H_{14}Cl_4N_2O$

M_r 416.1

DEFINITION

Isoconazole contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of 1-[(2*RS*)-2-[(2,6-dichlorobenzyl)oxy]-2-(2,4-dichlorophenyl)ethyl]-1*H*-imidazole, calculated with reference to the dried substance.

CHARACTERS

A white or almost white powder, practically insoluble in water, very soluble in methanol, freely soluble in alcohol.

IDENTIFICATION

First identification: A, B.

Second identification: A, C, D.

- A. Melting point (2.2.14): 111 °C to 115 °C.
- B. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *isoconazole CRS*. Examine the substances prepared as discs.
- C. Examine by thin-layer chromatography (2.2.27), using a suitable octadecylsilyl silica gel as the coating substance.
- Test solution.* Dissolve 30 mg of the substance to be examined in *methanol R* and dilute to 5 ml with the same solvent.
- Reference solution (a).* Dissolve 30 mg of *isoconazole CRS* in *methanol R* and dilute to 5 ml with the same solvent.
- Reference solution (b).* Dissolve 30 mg of *isoconazole CRS* and 30 mg of *econazole nitrate CRS* in *methanol R* and dilute to 5 ml with the same solvent.

Apply separately to the plate 5 µl of each solution. Develop over a path of 15 cm using a mixture of 20 volumes of *ammonium acetate solution R*, 40 volumes of *dioxan R* and 40 volumes of *methanol R*. Dry the plate in a current of warm air for 15 min and expose it to iodine vapour until the spots appear. Examine in daylight. The principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a). The test is not valid unless the chromatogram obtained with reference solution (b) shows 2 clearly separated spots.

- D. To about 30 mg in a porcelain crucible add 0.3 g of *anhydrous sodium carbonate R*. Heat over an open flame for 10 min. Allow to cool. Take up the residue with 5 ml of *dilute nitric acid R* and filter. To 1 ml of the filtrate add 1 ml of *water R*. The solution gives reaction (a) of chlorides (2.3.1).

TESTS

Solution S. Dissolve 0.20 g in *methanol R* and dilute to 20.0 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and is not more intensely coloured than reference solution Y₆ (2.2.2, *Method II*).

Optical rotation (2.2.7). The angle of optical rotation, determined on solution S, is −0.10° to +0.10°.

Related substances. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 0.100 g of the substance to be examined in 3.2 ml of *methanol R*. Add 3.0 ml of *acetonitrile R* and dilute to 10.0 ml with a solution of *ammonium acetate R* (6.0 g in 380 ml of *water R*).

Reference solution (a). Dissolve 2.5 mg of *isoconazole CRS* and 2.5 mg of *econazole nitrate CRS* in the mobile phase and dilute to 100.0 ml with the mobile phase.

Reference solution (b). Dilute 1.0 ml of the test solution to 100.0 ml with the mobile phase. Dilute 5.0 ml of this solution to 20.0 ml with the mobile phase.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.1 m long and 4.6 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (3 µm),
- as mobile phase at a flow rate of 2 ml/min a solution of 6.0 g of *ammonium acetate R* in a mixture of 300 ml of *acetonitrile R*, 320 ml of *methanol R* and 380 ml of *water R*,
- as detector a spectrophotometer set at 235 nm.

Equilibrate the column with the mobile phase at a flow rate of 2 ml/min for about 30 min.

Adjust the sensitivity of the system so that the height of the principal peak in the chromatogram obtained with 10 µl of reference solution (b) is not less than 50 per cent of the full scale of the recorder.

Inject 10 µl of reference solution (a). When the chromatogram is recorded in the prescribed conditions, the retention times are: *econazole*, about 10 min; *isoconazole*, about 14 min. The test is not valid unless the resolution between the peaks corresponding to *econazole* and *isoconazole* is at least 5.0. If necessary, adjust the composition of the mobile phase.

Inject separately 10 µl of the test solution and 10 µl of reference solution (b). Continue the chromatography for 1.5 times the retention time of the principal peak. In the chromatogram obtained with the test solution: the area of any peak, apart from the principal peak, is not greater than the area of the principal peak in the chromatogram obtained

01/2005:1017

with reference solution (b) (0.25 per cent); the sum of the areas of all the peaks, apart from the principal peak, is not greater than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent). Disregard any peaks due to the solvent and any peak with an area less than 0.2 times that of the principal peak in the chromatogram obtained with reference solution (b).

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C for 2 h.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

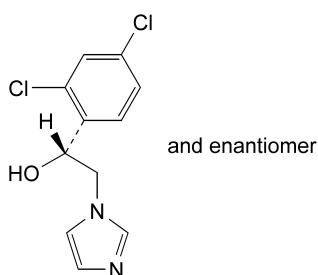
Dissolve 0.300 g in 50 ml of a mixture of 1 volume of *anhydrous acetic acid R* and 7 volumes of *methyl ethyl ketone R*. Using 0.2 ml of *naphtholbenzein solution R* as indicator, titrate with 0.1 M *perchloric acid* until the colour changes from orange-yellow to green.

1 ml of 0.1 M *perchloric acid* is equivalent to 41.61 mg of $C_{18}H_{14}Cl_4N_2O$.

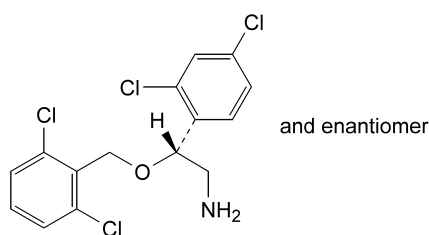
STORAGE

Store protected from light.

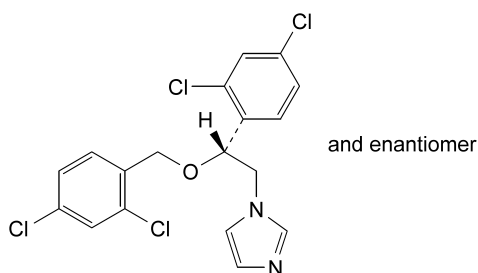
IMPURITIES



B. (1RS)-1-(2,4-dichlorophenyl)-2-(1H-imidazol-1-yl)ethanol,



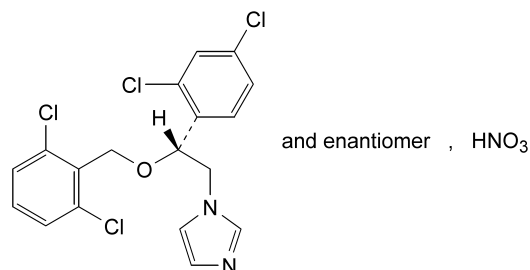
C. (2RS)-2-[(2,6-dichlorobenzyl)oxy]-2-(2,4-dichlorophenyl)ethanamine,



D. 1-[(2RS)-2-[(2,4-dichlorobenzyl)oxy]-2-(2,4-dichlorophenyl)ethyl]-1H-imidazole.

ISOCONAZOLE NITRATE

Isoconazoli nitras



$C_{18}H_{15}Cl_4N_3O_4$

M_r 479.1

DEFINITION

Isoconazole nitrate contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of 1-[(2RS)-2-[(2,6-dichlorobenzyl)oxy]-2-(2,4-dichlorophenyl)ethyl]-1H-imidazole, calculated with reference to the dried substance.

CHARACTERS

A white or almost white powder, very slightly soluble in water, soluble in methanol, slightly soluble in alcohol.

IDENTIFICATION

First identification: A, B.

Second identification: A, C, D.

A. Melting point (2.2.14): 178 °C to 182 °C.

B. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *isoconazole nitrate CRS*. Examine the substances prepared as discs.

C. Examine by thin-layer chromatography (2.2.27), using a suitable octadecylsilyl silica gel as the coating substance.

Test solution. Dissolve 30 mg of the substance to be examined in *methanol R* and dilute to 5 ml with the same solvent.

Reference solution (a). Dissolve 30 mg of *isoconazole nitrate CRS* in *methanol R* and dilute to 5 ml with the same solvent.

Reference solution (b). Dissolve 30 mg of *isoconazole nitrate CRS* and 30 mg of *econazole nitrate CRS* in *methanol R* and dilute to 5 ml with the same solvent.

Apply separately to the plate 5 µl of each solution. Develop over a path of 15 cm using a mixture of 20 volumes of *ammonium acetate solution R*, 40 volumes of *dioxan R* and 40 volumes of *methanol R*. Dry the plate in a current of warm air for 15 min and expose it to iodine vapour until the spots appear. Examine in daylight. The principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a). The test is not valid unless the chromatogram obtained with reference solution (b) shows two clearly separated spots.

D. It gives the reaction of nitrates (2.3.1).

TESTS

Solution S. Dissolve 0.20 g in *methanol R* and dilute to 20.0 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and is not more intensely coloured than reference solution Y₇ (2.2.2, Method II).

Optical rotation (2.2.7). The angle of optical rotation, determined on solution S, is -0.10° to $+0.10^{\circ}$.

Related substances. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 0.100 g of the substance to be examined in the mobile phase and dilute to 10.0 ml with the mobile phase.

Reference solution (a). Dissolve 2.5 mg of *isoconazole nitrate CRS* and 2.5 mg of *econazole nitrate CRS* in the mobile phase and dilute to 100.0 ml with the mobile phase.

Reference solution (b). Dilute 1.0 ml of the test solution to 100.0 ml with the mobile phase. Dilute 5.0 ml of this solution to 20.0 ml with the mobile phase.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.1 m long and 4.6 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (3 µm),
- as mobile phase at a flow rate of 2 ml/min a solution of 6.0 g of *ammonium acetate R* in a mixture of 300 ml of *acetonitrile R*, 320 ml of *methanol R* and 380 ml of *water R*,
- as detector a spectrophotometer set at 235 nm.

Equilibrate the column with the mobile phase at a flow rate of 2 ml/min for about 30 min.

Adjust the sensitivity of the system so that the height of the principal peak in the chromatogram obtained with 10 µl of reference solution (b) is at least 50 per cent of the full scale of the recorder.

Inject 10 µl of reference solution (a). When the chromatogram is recorded in the prescribed conditions, the retention times are: econazole, about 10 min and isoconazole, about 14 min. The test is not valid unless the resolution between the peaks due to econazole and isoconazole is not less than 5.0. If necessary, adjust the composition of the mobile phase.

Inject separately 10 µl of the test solution and 10 µl of reference solution (b). Continue the chromatography for 1.5 times the retention time of the principal peak. In the chromatogram obtained with the test solution: the area of any peak, apart from the principal peak, is not greater than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.25 per cent); the sum of the areas of all the peaks, apart from the principal peak, is not greater than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent). Disregard any peaks due to the solvent and to nitrate ion and any peak with an area less than 0.2 times that of the principal peak in the chromatogram obtained with reference solution (b).

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C for 2 h.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

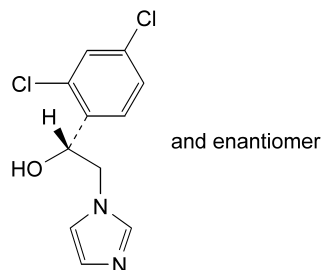
Dissolve 0.350 g in 75 ml of a mixture of 1 volume of *anhydrous acetic acid R* and 7 volumes of *methyl ethyl ketone R* and titrate with 0.1 M *perchloric acid*. Determine the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *perchloric acid* is equivalent to 47.91 mg of C₁₈H₁₅Cl₄N₃O₄.

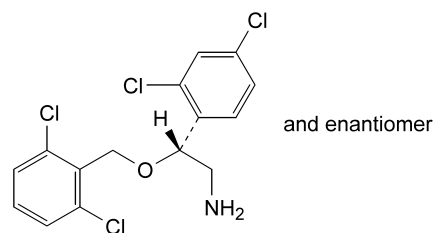
STORAGE

Store protected from light.

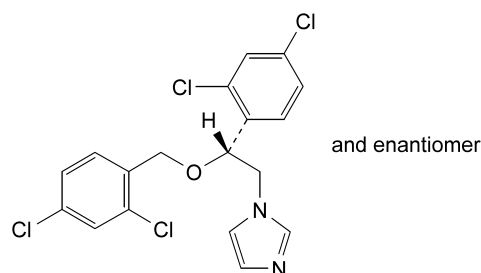
IMPURITIES



A. (1*RS*)-1-(2,4-dichlorophenyl)-2-(1*H*-imidazol-1-yl)ethanol,



B. (2*RS*)-2-[(2,6-dichlorobenzyl)oxy]-2-(2,4-dichlorophenyl)ethanamine,

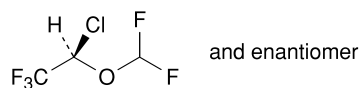


C. 1-[(2*RS*)-2-[(2,4-dichlorobenzyl)oxy]-2-(2,4-dichlorophenyl)ethyl]-1*H*-imidazole.

01/2005:1673
corrected

ISOFLURANE

Isofluranum



C₃H₂ClF₅O

*M*_r 184.5

DEFINITION

(2*RS*)-2-Chloro-2-(difluoromethoxy)-1,1,1-trifluoroethane.

CHARACTERS

Appearance: clear, colourless, mobile, heavy liquid.

Solubility: practically insoluble in water, miscible with ethanol and trichloroethylene.

bp: about 48 °C.

It is non-flammable.

IDENTIFICATION

Infrared absorption spectrophotometry (2.2.24).

Preparation: examine the substance in the gaseous state.

Comparison: *Ph. Eur. reference spectrum of isoflurane.*

TESTS

Acidity or alkalinity. To 20 ml add 20 ml of *carbon dioxide-free water R*, shake for 3 min and allow to stand. Collect the upper layer and add 0.2 ml of *bromocresol purple solution R*. Not more than 0.1 ml of 0.01 M *sodium hydroxide* or 0.6 ml of 0.01 M *hydrochloric acid* is required to change the colour of the indicator.

Related substances. Gas chromatography (2.2.28).

Test solution. The substance to be examined.

Reference solution. To 80 ml of *ethanol R*, add 1.0 ml of the substance to be examined and 1.0 ml of *acetone R*, avoiding loss by evaporation. Dilute to 100.0 ml with *ethanol R*. Dilute 1.0 ml of the solution to 100.0 ml with *ethanol R*.

Column:

- **material:** fused silica,
- **size:** $l = 30$ m, $\varnothing = 0.32$ mm,
- **stationary phase:** *macrogol 20 000 R* (film thickness 0.25 μ m).

Carrier gas: *helium for chromatography R*.

Flow rate: 1.0 ml/min.

Split ratio: 1:25.

Temperature:

- **column:** 35 °C,
- **injection port:** 150 °C,
- **detector:** 250 °C.

Detection: flame ionisation.

Injection: 1.0 μ l of each solution and 1.0 μ l of *ethanol R* as a blank.

Run time: until elution of the ethanol peak in the chromatogram obtained with the reference solution.

Relative retention with reference to isoflurane (retention time = about 3.8 min): acetone = about 0.75.

System suitability: reference solution:

- **resolution:** minimum of 5 between the peaks due to acetone and to isoflurane,
- **repeatability:** maximum relative standard deviation 15.0 per cent for the peak due to isoflurane after 3 injections.

Limits:

- **acetone:** not more than the area of the corresponding peak in the chromatogram obtained with the reference solution (0.01 per cent),
- **any other impurity:** not more than the area of the peak due to isoflurane in the chromatogram obtained with the reference solution (0.01 per cent),
- **total:** not more than 3 times the area of the peak due to isoflurane in the chromatogram obtained with the reference solution (0.03 per cent),
- **disregard limit:** 0.1 times the area of the peak due to isoflurane in the chromatogram obtained with the reference solution (0.001 per cent).

Chlorides (2.4.4): maximum 10 ppm.

To 10 ml add 10 ml of 0.01 M *sodium hydroxide* and shake for 3 min. To 5 ml of the upper layer add 10 ml of *water R*. The solution complies with the limit test for chlorides.

Fluorides: maximum 10 ppm.

Determine by potentiometry (2.2.36, *Method I*) using a fluoride-selective indicator-electrode and a silver-silver chloride reference electrode.

Test solution. To 10.0 ml in a separating funnel, add 10 ml of a mixture of 30.0 ml of *dilute ammonia R2* and 70.0 ml of *distilled water R*. Shake for 1 min and collect the upper

layer. Repeat this extraction procedure twice collecting the upper layer each time. Adjust the combined upper layers to pH 5.2 using *dilute hydrochloric acid R*. Add 5.0 ml of *fluoride standard solution (1 ppm F) R* and dilute to 50.0 ml with *distilled water R*. To 20.0 ml of the solution add 20.0 ml of *total-ionic-strength-adjustment buffer R* and dilute to 50.0 ml with *distilled water R*.

Reference solutions. To each of 5.0 ml, 4.0 ml, 3.0 ml, 2.0 ml and 1.0 ml of *fluoride standard solution (10 ppm F) R* add 20.0 ml of *total-ionic-strength-adjustment buffer R* and dilute to 50.0 ml with *distilled water R*.

Carry out the measurements on 20 ml of each solution. Calculate the concentration of fluorides using the calibration curve, taking into account the addition of fluoride to the test solution.

Non-volatile matter: maximum 200 mg/l.

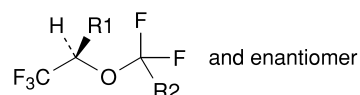
Evaporate 10.0 ml to dryness with the aid of a stream of cold air and dry the residue at 50 °C for 2 h. The residue weighs a maximum of 2.0 mg.

Water (2.5.12): maximum 1.0 mg/ml, determined on 10.0 ml.

STORAGE

In an airtight container, protected from light.

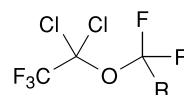
IMPURITIES



A. R1 = H, R2 = Cl: 2-(chlorodifluoromethoxy)-1,1,1-trifluoroethane,

B. R1 = R2 = H: 2-(difluoromethoxy)-1,1,1-trifluoroethane,

C. R1 = R2 = Cl: (2*RS*)-2-chloro-2-(chlorodifluoromethoxy)-1,1,1-trifluoroethane,



D. R = H: 1,1-dichloro-1-(difluoromethoxy)-2,2,2-trifluoroethane,

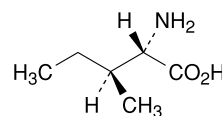
E. R = Cl: 1,1-dichloro-1-(chlorodifluoromethoxy)-2,2,2-trifluoroethane,

F. acetone.

01/2005:0770

ISOLEUCINE

Isoleucinum



$C_6H_{13}NO_2$

M_r 131.2

DEFINITION

Isoleucine contains not less than 98.5 per cent and not more than the equivalent of 101.0 per cent of (2*S*,3*S*)-2-amino-3-methylpentanoic acid, calculated with reference to the dried substance.

CHARACTERS

White or almost white, crystalline powder or flakes, sparingly soluble in water, slightly soluble in alcohol. It dissolves in dilute mineral acids and in dilute solutions of alkali hydroxides.

IDENTIFICATION

First identification: A, C.

Second identification: A, B, D.

- It complies with the test for specific optical rotation (see Tests).
- Dissolve 0.5 g in *water R* and dilute to 25 ml with the same solvent. The solution is dextrorotatory.
- Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *isoleucine CRS*. Examine the substances prepared as discs.
- Examine the chromatograms obtained in the test for ninhydrin-positive substances. The principal spot in the chromatogram obtained with test solution (b) is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).

TESTS

Appearance of solution. Dissolve 0.5 g in 1 M *hydrochloric acid* and dilute to 10 ml with the same acid. The solution is clear (2.2.1) and not more intensely coloured than reference solution BY₆ (2.2.2, *Method II*).

Specific optical rotation (2.2.7). Dissolve 1.00 g in *hydrochloric acid R1* and dilute to 25.0 ml with the same acid. The specific optical rotation is + 40.0 to + 43.0, calculated with reference to the dried substance.

Ninhydrin-positive substances. Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel plate R*.

Test solution (a). Dissolve 0.10 g of the substance to be examined in 0.1 M *hydrochloric acid* and dilute to 10 ml with the same acid.

Test solution (b). Dilute 1 ml of test solution (a) to 50 ml with *water R*.

Reference solution (a). Dissolve 10 mg of *isoleucine CRS* in 0.1 M *hydrochloric acid* and dilute to 50 ml with the same acid.

Reference solution (b). Dilute 5 ml of test solution (b) to 20 ml with *water R*.

Reference solution (c). Dissolve 10 mg of *isoleucine CRS* and 10 mg of *valine CRS* in 0.1 M *hydrochloric acid* and dilute to 25 ml with the same acid.

Apply separately to the plate 5 µl of each solution. Develop over a path of 15 cm using a mixture of 20 volumes of *glacial acetic acid R*, 20 volumes of *water R* and 60 volumes of *butanol R*. Allow the plate to dry in air, spray with *ninhydrin solution R* and heat at 100-105 °C for 15 min. Any spot in the chromatogram obtained with test solution (a), apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.5 per cent). The test is not valid unless the chromatogram obtained with reference solution (c) shows 2 clearly separated spots.

Chlorides (2.4.4). Dissolve 0.25 g in *water R* and dilute to 15 ml with the same solvent. The solution complies with the limit test for chlorides (200 ppm).

Sulphates (2.4.13). Dissolve 0.5 g in 3 ml of *dilute hydrochloric acid R* and dilute to 15 ml with *distilled water R*. The solution complies with the limit test for sulphates (300 ppm).

Ammonium (2.4.1). 50 mg complies with limit test B for ammonium (200 ppm). Prepare the standard using 0.1 ml of *ammonium standard solution (100 ppm NH₄) R*.

Iron (2.4.9). In a separating funnel, dissolve 1.0 g in 10 ml of *dilute hydrochloric acid R*. Shake with 3 quantities, each of 10 ml, of *methyl isobutyl ketone R1*, shaking for 3 min each time. To the combined organic layers add 10 ml of *water R* and shake for 3 min. The aqueous layer complies with the limit test for iron (10 ppm).

Heavy metals (2.4.8). 2.0 g complies with limit test D for heavy metals (10 ppm). Prepare the standard using 2 ml of *lead standard solution (10 ppm Pb) R*.

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 100-105 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.100 g in 3 ml of *anhydrous formic acid R*. Add 30 ml of *anhydrous acetic acid R*. Using 0.1 ml of *naphtholbenzein solution R* as indicator, titrate with 0.1 M *perchloric acid* until the colour changes from brownish-yellow to green.

1 ml of 0.1 M *perchloric acid* is equivalent to 13.12 mg of C₆H₁₃NO₂.

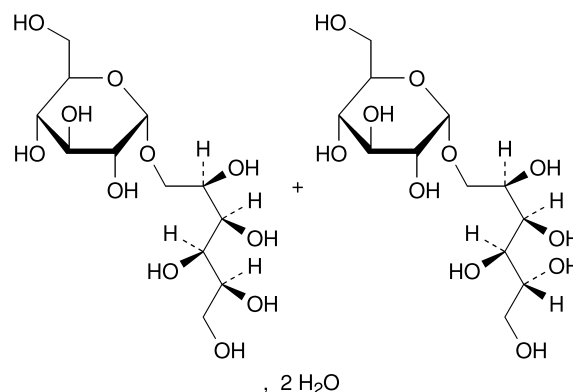
STORAGE

Store protected from light.

01/2005:1531

ISOMALT

Isomaltum

C₁₂H₂₄O₁₁M_r 344.3C₁₂H₂₄O₁₁·2H₂OM_r 380.3

DEFINITION

Isomalt contains not less than 98.0 per cent and not more than the equivalent of 102.0 per cent of a mixture of 6-O-α-D-glucopyranosyl-D-glucitol (6-O-α-D-glucopyranosyl-D-sorbitol; 1,6-GPS) and 1-O-α-D-glucopyranosyl-D-mannitol (1,1-GPM) and neither of the 2 components is less than 3.0 per cent, calculated with reference to the anhydrous substance. The percentage content of 1,6-GPS and 1,1-GPM is stated on the label.

CHARACTERS

A white or almost white powder or granules, freely soluble in water, practically insoluble in ethanol.

IDENTIFICATION

First identification: A.

Second identification: B, C.

A. Examine the chromatograms obtained in the assay. The 2 principal peaks in the chromatogram obtained with the test solution are similar in retention time to the 2 principal peaks in the chromatogram obtained with reference solution (a).

B. Examine by thin layer chromatography (2.2.27) using a *TLC silica gel F₂₅₄ plate R*.

Test solution. Dissolve 50 mg of the substance to be examined in *water R* and dilute to 10 ml with the same solvent.

Reference solution. Dissolve 50 mg of *isomalt CRS* in *water R* and dilute to 10 ml with the same solvent.

Apply to the plate 1 µl of each solution and thoroughly dry the starting points in warm air. Develop over a path of 10 cm using a mixture of 5 volumes of *acetic acid R*, 5 volumes of *propionic acid R*, 10 volumes of *water R*, 50 volumes of *ethyl acetate R* and 50 volumes of *pyridine R*. Dry the plate in a current of warm air and dip for 3 s in a 1 g/l solution of *sodium periodate R*. Dry the plate in a current of hot air. Dip the plate for 3 s in a mixture of 1 volume of *acetic acid R*, 1 volume of *anisaldehyde R*, 5 volumes of *sulphuric acid R* and 90 volumes of *ethanol R*. Dry the plate in a current of hot air until coloured spots become visible. The background colour may be brightened in warm steam. Examine in daylight. The chromatogram obtained with the reference solution shows 2 blue-grey spots with *R_f* values of about 0.13 (1,6-GPS) and 0.16 (1,1-GPM). The chromatogram obtained with the test solution show principal spots similar in position and colour to the spots in the chromatogram obtained with the reference solution.

C. To 3 ml of a freshly prepared 100 g/l solution of *pyrocatechol R* add 6 ml of *sulphuric acid R* while cooling in iced water. To 3 ml of the cooled mixture add 0.3 ml of a 100 g/l solution of the substance to be examined. Heat gently over a naked flame for about 30 s. A pink colour develops.

TESTS

Conductivity (2.2.38). Not more than 20 µS·cm⁻¹. Dissolve 20.0 g in *carbon dioxide-free water R* prepared from *distilled water R* and dilute to 100.0 ml with the same solvent. Measure the conductivity of the solution while gently stirring with a magnetic stirrer.

Reducing sugars. Dissolve 3.3 g in 10 ml of *water R* with the aid of gentle heat. Cool and add 20 ml of *cupri-citric solution R* and a few glass beads. Heat so that the boiling begins after 4 min and maintain boiling for 3 min. Cool rapidly and add 100 ml of a 2.4 per cent *V/V* solution of *glacial acetic acid R* and 20.0 ml of 0.025 *M* *iodine*. With continuous shaking, add 25 ml of a mixture of 6 volumes of *hydrochloric acid R* and 94 volumes of *water R* and, when the precipitate has dissolved, titrate the excess of iodine with 0.05 *M* *sodium thiosulphate* using 1 ml of *starch solution R* as indicator, added towards the end of the titration. Not less than 12.8 ml of 0.05 *M* *sodium thiosulphate* is required (0.3 per cent, calculated as glucose).

Related products. Examine the chromatograms obtained in the assay. In the chromatogram obtained with the test solution: the area of any peak corresponding to mannitol or sorbitol is not greater than the area of the corresponding peak in the chromatogram obtained with reference solution (b) (0.5 per cent); the area of any peak, apart from the 2 principal peaks corresponding to 1,1-GPM and 1,6-GPS and the peaks corresponding to mannitol and sorbitol, is not greater than the area of the peak due to sorbitol in the chromatogram obtained with reference solution (b) (0.5 per cent); the sum of the area of all the peaks apart from the peaks corresponding to 1,1-GPM and 1,6-GPS is not greater than 4 times the area of the peak due to sorbitol in the chromatogram obtained with reference solution (b) (2 per cent). Disregard any peak with an area less than 0.2 times that of the peak due to sorbitol in the chromatogram obtained with reference solution (b) (0.1 per cent).

Lead (2.4.10). It complies with the limit test for lead in sugars (0.5 ppm).

Nickel (2.4.15). It complies with the limit test for nickel in polyols (1 ppm).

Water (2.5.12). Not more than 7.0 per cent, determined on 0.3 g by the semi-micro determination of water. As solvent, use a mixture of 20 ml of *anhydrous methanol R* and 20 ml of *formamide R* at 50 ± 5 °C.

ASSAY

Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 1.00 g of the substance to be examined in 20 ml of *water R* and dilute to 50.0 ml with the same solvent.

Reference solution (a). Dissolve 1.00 g of *isomalt CRS* in 20 ml of *water R* and dilute to 50.0 ml with the same solvent.

Reference solution (b). Dissolve 10.0 mg of *sorbitol CRS* and 10.0 mg of *mannitol CRS* in 20 ml of *water R* and dilute to 100.0 ml with the same solvent.

The chromatographic procedure may be carried out using:

- a stainless steel precolumn 30 mm long and 4.6 mm in internal diameter and a stainless steel column 0.3 m long and 7.8 mm in internal diameter, both packed with a *strong cation-exchange resin (calcium form) R* (9 µm) and maintained at 80 ± 1 °C,
- as mobile phase at a flow rate of 0.5 ml/min degassed *water R*,
- as detector a differential refractometer maintained at a constant temperature.

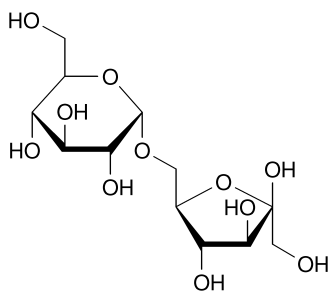
Inject 20 µl of each solution and continue the chromatography until sorbitol is completely eluted (about 25 min). When the chromatograms are recorded in the prescribed conditions, the retention time of 1,1-GPM is about 12.3 min and the relative retentions to 1,1-GPM are: 1,6-GPS about 1.2, mannitol about 1.6, sorbitol about 2.0 and isomaltulose about 0.8.

Calculate the percentage content of isomalt (1,1-GPM and 1,6-GPS) from the areas of the peaks of 1,1-GPM and 1,6-GPS and the declared content of 1,1-GPM and 1,6-GPS in *isomalt CRS*.

LABELLING

The label states the percentage content of 1,6-GPS and 1,1-GPM.

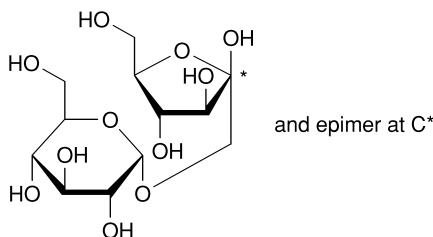
IMPURITIES



A. 6-O-α-D-glucopyranosyl-β-D-arabino-hex-2-ulofuranose (isomaltulose),

B. mannitol,

C. sorbitol,

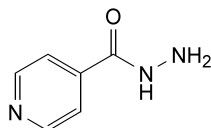


D. 1-O-α-D-glucopyranosyl-D-arabino-hex-2-ulofuranose (trehalulose).

01/2005:0146

ISONIAZID

Isoniazidum


 $C_6H_7N_3O$
 M_r 137.1

DEFINITION

Isoniazid contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of pyridine-4-carbohydrazide, calculated with reference to the dried substance.

CHARACTERS

A white, crystalline powder or colourless crystals, freely soluble in water, sparingly soluble in alcohol.

IDENTIFICATION

First identification: A, B.

Second identification: A, C.

A. Melting point (2.2.14): 170 °C to 174 °C.

B. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with isoniazid CRS.

C. Dissolve 0.1 g in 2 ml of water R and add 10 ml of a warm 10 g/l solution of vanillin R. Allow to stand and scratch the wall of the test tube with a glass rod. A yellow precipitate is formed, which, after recrystallisation from 5 ml of alcohol (70 per cent V/V) R and drying at 100 °C to 105 °C, melts (2.2.14) at 226 °C to 231 °C.

TESTS

Solution S. Dissolve 2.5 g in carbon dioxide-free water R and dilute to 50 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and not more intensely coloured than reference solution BY₇ (2.2.2, Method II).

pH (2.2.3). The pH of solution S is 6.0 to 8.0.

Hydrazine and related substances. Examine by thin-layer chromatography (2.2.27), using silica gel GF₂₅₄ R as the coating substance.

Test solution. Dissolve 1.0 g of the substance to be examined in a mixture of equal volumes of acetone R and water R and dilute to 10.0 ml with the same mixture of solvents.

Reference solution. Dissolve 50.0 mg of hydrazine sulphate R in 50 ml of water R and dilute to 100.0 ml with acetone R. To 10.0 ml of this solution add 0.2 ml of the test solution and dilute to 100.0 ml with a mixture of equal volumes of acetone R and water R.

Apply separately to the plate 5 µl of each solution and develop over a path of 15 cm using a mixture of 10 volumes of water R, 20 volumes of acetone R, 20 volumes of methanol R and 50 volumes of ethyl acetate R. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. Any spot in the chromatogram obtained with the test solution, apart from the principal spot, is not more intense than the spot in the chromatogram obtained with the reference solution (0.2 per cent). Spray the plate with dimethylaminobenzaldehyde solution R1. Examine in daylight. An additional spot, corresponding to hydrazine, appears in the chromatogram obtained with the reference solution. Any corresponding spot in the chromatogram obtained with the test solution is not more intense than the spot corresponding to hydrazine in the chromatogram obtained with the reference solution (0.05 per cent).

Heavy metals (2.4.8). 2.0 g complies with limit test C for heavy metals (10 ppm). Prepare the standard using 2 ml of lead standard solution (10 ppm Pb) R.

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.00 g by drying in an oven at 100 °C to 105 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

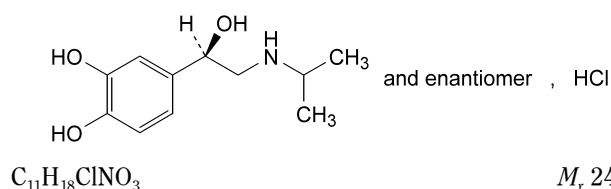
Dissolve 0.250 g in water R and dilute to 100.0 ml with the same solvent. To 20.0 ml of the solution add 100 ml of water R, 20 ml of hydrochloric acid R, 0.2 g of potassium bromide R and 0.05 ml of methyl red solution R. Titrate dropwise with 0.0167 M potassium bromate, shaking continuously, until the red colour disappears.

1 ml of 0.0167 M potassium bromate is equivalent to 3.429 mg of C₆H₇N₃O.

01/2005:1332

ISOPRENALINE HYDROCHLORIDE

Isoprenalini hydrochloridum



DEFINITION

Isoprenaline hydrochloride contains not less than 98.0 per cent and not more than the equivalent of 101.5 per cent of (1*R*S)-1-(3,4-dihydroxyphenyl)-2-[(1-methylethyl)amino]ethanol hydrochloride, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, crystalline powder, freely soluble in water, sparingly soluble in alcohol, practically insoluble in methylene chloride.

IDENTIFICATION

First identification: B, C, E.

Second identification: A, C, D, E.

- Melting point (2.2.14): 165 °C to 170 °C, with decomposition.
- Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *isoprenaline hydrochloride CRS*. Examine the substances as discs.
- It complies with the test for optical rotation (see Tests).
- To 0.1 ml of solution S (see Tests) add 0.05 ml of *ferric chloride solution R1* and 0.9 ml of *water R*. A green colour is produced. Add dropwise *sodium hydrogen carbonate solution R*. The colour becomes blue then red.
- To 0.5 ml of solution S add 1.5 ml of *water R*. The solution gives reaction (a) of chlorides (2.3.1).

TESTS

Prepare the solutions immediately before use.

Solution S. Dissolve 2.5 g in *carbon dioxide-free water R* and dilute to 25.0 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and not more intensely coloured than reference solution B₇ or BY₇ (2.2.2, *Method II*).

pH (2.2.3). The pH of a mixture of 5 ml of solution S and 5 ml of *carbon dioxide-free water R* is 4.3 to 5.5.

Optical rotation (2.2.7). The angle of optical rotation, determined on solution S, is -0.10° to +0.10°.

Related substances. Examine by liquid chromatography (2.2.29).

Test solution (a). Dissolve 50.0 mg of the substance to be examined in the mobile phase and dilute to 10.0 ml with the mobile phase.

Test solution (b). Dilute 0.5 ml of test solution (a) to 100.0 ml with the mobile phase.

Reference solution (a). Dissolve 2.5 mg of *isoprenaline hydrochloride CRS* in the mobile phase and dilute to 100.0 ml with the mobile phase.

Reference solution (b). Dissolve 2.5 mg of *orcioprenaline sulphate CRS* in the mobile phase and dilute to 100.0 ml with the mobile phase.

Reference solution (c). To 1.0 ml of test solution (b) add 1.0 ml of reference solution (b) and dilute to 20.0 ml with the mobile phase.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.125 m long and 4.0 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (5 µm),
- as mobile phase at a flow rate of 1.0 ml/min a mixture of 5 volumes of *methanol R* and 95 volumes of a 11.5 g/l solution of *phosphoric acid R*,
- as detector a spectrophotometer set at 280 nm,

- a loop injector.

Inject 20 µl of reference solution (a). Adjust the sensitivity of the system so that the height of the principal peak in the chromatogram obtained is at least 50 per cent of the full scale of the recorder. Adjust the retention time of the peak to about 3 min by varying the concentration of methanol in the mobile phase. Inject 20 µl of test solution (a) and 20 µl of reference solution (c). Continue the chromatography of test solution (a) for seven times the retention time of isoprenaline. The test is not valid unless in the chromatogram obtained with reference solution (c): the resolution between the two principal peaks is at least 3; the peak due to isoprenaline has a signal-to-noise ratio of at least 3.

In the chromatogram obtained with test solution (a): the area of any peak, apart from the principal peak, is not greater than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent) and the sum of the areas of such peaks is not greater than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (1 per cent). Disregard any peak with an area less than 0.05 times that of the principal peak in the chromatogram obtained with reference solution (a).

Loss on drying (2.2.32). Not more than 1.0 per cent, determined on 1.000 g by drying *in vacuo* at 15 °C to 25 °C for 4 h.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

In order to avoid overheating in the reaction medium, mix thoroughly throughout and stop the titration immediately after the end-point has been reached.

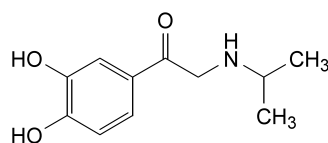
Dissolve 0.150 g in 10 ml of *anhydrous formic acid R* and add 50 ml of *acetic anhydride R*. Titrate with 0.1 M *perchloric acid*, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *perchloric acid* is equivalent to 24.77 mg of C₁₁H₁₈ClNO₃.

STORAGE

Store in an airtight container, protected from light.

IMPURITIES

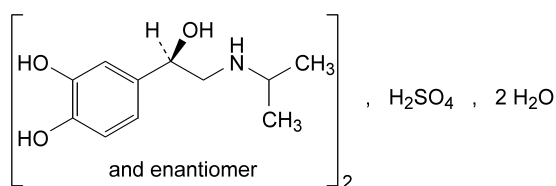


- 1-(3,4-dihydroxyphenyl)-2-[(1-methylethyl)amino]ethanone.

01/2005:0502

ISOPRENALINE SULPHATE

Isoprenalini sulfas



C₂₂H₃₆N₂O₁₀·2H₂O

M_r 556.6

DEFINITION

Isoprenaline sulphate contains not less than 98.0 per cent and not more than the equivalent of 102.0 per cent of bis[(1*R,S*)-1-(3,4-dihydroxyphenyl)-2-[(1-methylethyl)amino]ethanol] sulphate, calculated with reference to the anhydrous substance.

CHARACTERS

A white or almost white, crystalline powder, freely soluble in water, very slightly soluble in alcohol.

It melts at about 128 °C, with decomposition.

IDENTIFICATION

First identification: A, D.

Second identification: B, C, D.

- A. Dissolve 0.5 g of the substance to be examined in 1.5 ml of *water R* and add 3.5 ml of *2-propanol R*. Scratch the wall of the tube with a glass rod to initiate crystallisation. Collect the crystals and dry *in vacuo* at 60 °C over *diphosphorus pentoxide R*. Repeat the operations with *isoprenaline sulphate CRS*. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *isoprenaline sulphate CRS*.
- B. To 0.1 ml of solution S (see Tests) add 0.9 ml of *water R* and 0.05 ml of *ferric chloride solution RI*. A green colour is produced. Add dropwise *sodium hydrogen carbonate solution R*. The colour becomes blue and then red.
- C. Dilute 1 ml of solution S to 10 ml with *water R* and add 0.25 ml of *silver nitrate solution RI*. A shining, grey, fine precipitate is formed within 10 min and the solution becomes pink.
- D. Solution S gives reaction (a) of sulphates (2.3.1).

TESTS

Solution S. Dissolve 5.0 g in *carbon dioxide-free water R* and dilute to 50 ml with the same solvent. Use within 2 h of preparation.

Appearance of solution. Solution S is clear (2.2.1) and not more intensely coloured than reference solution Y₆ (2.2.2, *Method II*).

pH (2.2.3). Dilute 5 ml of solution S to 10 ml with *carbon dioxide-free water R*. The pH of the solution is 4.3 to 5.5.

Isoprenalone. Dissolve 0.20 g in 0.005 *M* sulphuric acid and dilute to 100.0 ml with the same solvent. The absorbance (2.2.25) measured at 310 nm is not greater than 0.20.

Water (2.5.12): 5.0 per cent to 7.5 per cent, determined on 0.200 g by the semi-micro determination of water.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.400 g in 20 ml of *anhydrous acetic acid R*, warming gently if necessary and add 20 ml of *methyl isobutyl ketone R*. Titrate with 0.1 *M* perchloric acid determining the end-point potentiometrically (2.2.20).

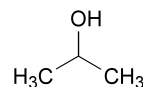
1 ml of 0.1 *M* perchloric acid is equivalent to 52.06 mg of C₂₂H₃₆N₂O₁₀S.

STORAGE

Store in an airtight container, protected from light.

ISOPROPYL ALCOHOL

Alcohol isopropylicus



C₃H₈O

M_r 60.1

DEFINITION

Propan-2-ol.

CHARACTERS

Appearance: clear, colourless liquid.

Solubility: miscible with water and with alcohol.

IDENTIFICATION

- A. Relative density (2.2.5): 0.785 to 0.789.
- B. Refractive index (2.2.6): 1.376 to 1.379.
- C. To 1 ml add 2 ml of *potassium dichromate solution R* and 1 ml of *dilute sulphuric acid R*. Boil. Vapour is produced which changes the colour of a piece of filter paper impregnated with *nitrobenzaldehyde solution R* to green. Moisten the filter paper with *dilute hydrochloric acid R*. The colour changes to blue.

TESTS

Appearance. The substance to be examined is clear (2.2.1) and colourless (2.2.2, *Method II*). Dilute 1 ml to 20 ml with *water R*. After 5 min, the solution is clear (2.2.1).

Acidity or alkalinity. Gently boil 25 ml for 5 min. Add 25 ml of *carbon dioxide-free water R* and allow to cool protected from carbon dioxide in the air. Add 0.1 ml of *phenolphthalein solution R*. The solution is colourless. Not more than 0.6 ml of 0.01 *M* sodium hydroxide is required to change the colour of the indicator to pale pink.

Absorbance (2.2.25): maximum 0.30 at 230 nm, 0.10 at 250 nm, 0.03 at 270 nm, 0.02 at 290 nm and 0.01 at 310 nm.

The absorbance is measured between 230 nm and 310 nm using *water R* as the compensation liquid. The absorption curve is smooth.

Benzene and related substances. Gas chromatography (2.2.28).

Test solution (a). The substance to be examined.

Test solution (b). Dilute 1.0 ml of *2-butanol RI* to 50.0 ml with test solution (a). Dilute 5.0 ml of the solution to 100.0 ml with test solution (a).

Reference solution (a). Dilute 0.5 ml of *2-butanol RI* and 0.5 ml of *propanol R* to 50.0 ml with test solution (a). Dilute 5.0 ml of the solution to 50.0 ml with test solution (a).

Reference solution (b). Dilute 100 µl of *benzene R* to 100.0 ml with test solution (a). Dilute 0.20 ml of the solution to 100.0 ml with test solution (a).

Column:

- *material:* fused silica,
- *size:* *l* = 30 m, Ø = 0.32 mm,
- *stationary phase:* poly[(cyanopropyl)(phenyl)][dimethylsiloxane *R*] (film thickness 1.8 µm).

Carrier gas: helium for chromatography *R*.

Auxiliary gas: nitrogen for chromatography *R* or helium for chromatography *R*.

Linear velocity: 35 cm/s.

Split ratio: 1:5.

Temperature:

	Time (min)	Temperature (°C)
Column	0 - 12	40
	12 - 32	40 → 240
	32 - 42	240
Injection port		280
Detector		280

Detection: flame ionisation.

Injection: 1 µl.

Retention time: benzene = about 10 min.

System suitability: reference solution (a):

- **resolution**: minimum of 10 between the first peak (propanol) and the second peak (2-butanol).

Limits:

- **benzene** (test solution (a)): not more than half of the area of the corresponding peak in the chromatogram obtained with reference solution (b) (2 ppm), after the sensitivity has been adjusted so that the height of the peak due to benzene in the chromatogram obtained with reference solution (b) represents at least 10 per cent of the full scale of the recorder.
- **total of impurities apart from 2-butanol** (test solution (b)): not more than 3 times the area of the peak due to 2-butanol in the chromatogram obtained with test solution (b) (0.3 per cent), after the sensitivity has been adjusted so that the height of the 2 peaks following the principal peak in the chromatogram obtained with reference solution (a) represents at least 50 per cent of the full scale of the recorder.

Peroxides. In a 12 ml test-tube with a ground-glass stopper and a diameter of about 15 mm, introduce 8 ml of *potassium iodide and starch solution R*. Fill completely with the substance to be examined. Shake vigorously and allow to stand protected from light for 30 min. No colour develops.

Non-volatile substances: maximum 20 ppm.

Evaporate 100 g to dryness on a water-bath *after having verified that it complies with the test for peroxides* and dry in an oven at 100-105 °C. The residue weighs a maximum of 2 mg.

Water (2.5.12): maximum 0.5 per cent, determined on 5.0 g.

STORAGE

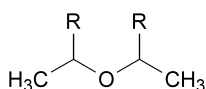
Protected from light.

IMPURITIES

A. acetone,



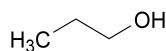
B. benzene,



C. R = CH₃: 2-(1-methylethoxy)propane (diisopropyl ether),

D. R = H: ethoxyethane (diethyl ether),

E. CH₃-OH: methanol,

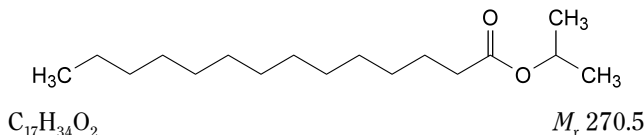


F. propan-1-ol (*n*-propanol).

01/2005:0725

ISOPROPYL MYRISTATE

Isopropylis myristas



DEFINITION

1-Methylethyl tetradecanoate together with variable amounts of other fatty acid isopropyl esters.

Content: minimum 90.0 per cent of C₁₇H₃₄O₂.

CHARACTERS

Appearance: clear, colourless, oily liquid.

Solubility: immiscible with water, miscible with alcohol, with methylene chloride, with fatty oils and with liquid paraffin.

Relative density: about 0.853.

IDENTIFICATION

First identification: B.

Second identification: A, C.

A. It complies with the test for saponification value (see Tests).

B. Examine the chromatograms obtained in the assay.

Results: the principal peak in the chromatogram obtained with the test solution is similar in retention time to the principal peak in the chromatogram obtained with the reference solution.

C. Superpose 2 ml of a 1 g/l solution in *alcohol R* on a freshly prepared solution of 20 mg of *dimethylaminobenzaldehyde R* in 2 ml of *sulphuric acid R*. After 2 min, a yellowish-red colour appears at the junction of the 2 liquids and gradually becomes red.

TESTS

Appearance of solution. The solution is clear (2.2.1) and not more intensely coloured than reference solution Y₇ (2.2.2, *Method II*).

Dissolve 2.0 g in *methanol R* and dilute to 20 ml with the same solvent.

Refractive index (2.2.6): 1.434 to 1.437.

Viscosity (2.2.9): 5 mPas to 6 mPas.

Acid value (2.5.1): maximum 1.0.

Iodine value (2.5.4): maximum 1.0.

Saponification value (2.5.6): 202 to 212.

Water (2.5.12): maximum 0.1 per cent, determined on 5.0 g.

Total ash (2.4.16): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Gas chromatography (2.2.28).

Internal standard solution. Dissolve 50.0 mg of *tricosane R* in *heptane R* and dilute to 250.0 ml with the same solvent.

Test solution. Dissolve 20.0 mg of the substance to be examined in the internal standard solution and dilute to 100.0 ml with the same solution.

Reference solution. Dissolve 20.0 mg of *isopropyl tetradecanoate CRS* in the internal standard solution and dilute to 100.0 ml with the same solution.

Column:

- **material:** fused silica,
- **size:** $l = 50$ m, $\varnothing = 0.2$ mm,
- **stationary phase:** *poly(cyanopropyl)siloxane R* (film thickness 0.2 μ m).

Carrier gas: *helium for chromatography R*.

Flow rate: 1 ml/min.

Split ratio: 1:40.

Temperature:

	Time (min)	Temperature (°C)
Column	0 - 6	125 → 185
Injection port		250
Detector		250

Detection: flame ionisation.

Injection: 2 μ l.

Calculate the percentage content of $C_{17}H_{34}O_2$ in the substance to be examined.

STORAGE

Protected from light.

B. Examine the chromatograms obtained in the assay.

Results: the principal peak in the chromatogram obtained with the test solution is similar in retention time to the principal peak in the chromatogram obtained with the reference solution.

C. Superpose 2 ml of a 1 g/l solution in *alcohol R* on a freshly prepared solution of 20 mg of *dimethylaminobenzaldehyde R* in 2 ml of *sulphuric acid R*. After 2 min, a yellowish-red colour appears at the junction of the 2 liquids which gradually becomes red.

TESTS

Appearance of solution. The solution is clear (2.2.1) and not more intensely coloured than reference solution Y_7 (2.2.2, Method II).

Dissolve 2.0 g in *methanol R* and dilute to 20 ml with the same solvent.

Refractive index (2.2.6): 1.436 to 1.440.

Viscosity (2.2.9): 5 mPa.s to 10 mPa.s.

Acid value (2.5.1): maximum 1.0.

Iodine value (2.5.4): maximum 1.0.

Saponification value (2.5.6): 183 to 193.

Water (2.5.12): maximum 0.1 per cent, determined on 5.0 g.

Total ash (2.4.16): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Gas chromatography (2.2.28).

Internal standard solution. Dissolve 50.0 mg of *tricosane R* in *heptane R* and dilute to 250.0 ml with the same solvent.

Test solution. Dissolve 20.0 mg of the substance to be examined in the internal standard solution and dilute to 100.0 ml with the same solution.

Reference solution. Dissolve 20.0 mg of *isopropyl hexadecanoate CRS* in the internal standard solution and dilute to 100.0 ml with the same solution.

Column:

- **material:** fused silica,
- **size:** $l = 50$ m, $\varnothing = 0.2$ mm,
- **stationary phase:** *poly(cyanopropyl)siloxane R* (film thickness 0.2 μ m).

Carrier gas: *helium for chromatography R*.

Flow rate: 1 ml/min.

Split ratio: 1:40.

Temperature:

	Time (min)	Temperature (°C)
Column	0 - 6	125 → 185
Injection port		250
Detector		250

Detection: flame ionisation.

Injection: 2 μ l.

Calculate the percentage content of $C_{19}H_{38}O_2$ in the substance to be examined.

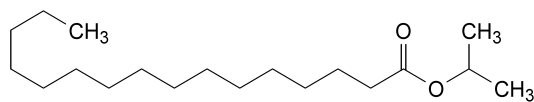
STORAGE

Protected from light.

01/2005:0839

ISOPROPYL PALMITATE

Isopropyliis palmitas



$C_{19}H_{38}O_2$

M_r 298.5

DEFINITION

1-Methylethyl hexadecanoate together with varying amounts of other fatty acid isopropyl esters.

Content: minimum 90.0 per cent of $C_{19}H_{38}O_2$.

CHARACTERS

Appearance: clear, colourless, oily liquid.

Solubility: immiscible with water, miscible with alcohol, with methylene chloride, with fatty oils and with liquid paraffin.

Relative density: about 0.854.

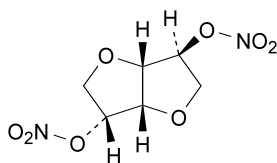
IDENTIFICATION

First identification: B.

Second identification: A, C.

A. It complies with the test for saponification value (see Tests).

01/2005:1117

ISOSORBIDE DINITRATE, DILUTED**Isosorbidi dinitras dilutus** $C_6H_8N_2O_8$ M_r 236.1**DEFINITION**

Diluted isosorbide dinitrate is a dry mixture of isosorbide dinitrate and *Lactose monohydrate* (0187) or *Mannitol* (0559). It contains not less than 95.0 per cent *m/m* and not more than 105.0 per cent *m/m* of the content of 1,4:3,6-dianhydro-D-glucitol 2,5-dinitrate stated on the label.

CAUTION: Undiluted isosorbide dinitrate may explode if subjected to percussion or excessive heat. Appropriate precautions must be taken and only very small quantities should be handled.

CHARACTERS

Undiluted isosorbide dinitrate is a fine, white, crystalline powder, very slightly soluble in water, very soluble in acetone, sparingly soluble in alcohol.

The solubility of the diluted product depends on the diluent and its concentration.

IDENTIFICATION

First identification: A, C, D.

Second identification: B, C, D.

A. Examine by infrared absorption spectrophotometry (2.2.24) the residue obtained in identification test D, comparing with the spectrum of the residue obtained in the same way with *isosorbide dinitrate CRS*. Examine the residues prepared as discs.

B. Examine by thin-layer chromatography (2.2.27), using *silica gel G R* as the coating substance.

Test solution. Shake a quantity of the substance to be examined corresponding to 10 mg of isosorbide dinitrate with 10 ml of *alcohol R* for 5 min and filter.

Reference solution. Shake a quantity of *isosorbide dinitrate CRS* corresponding to 10 mg of isosorbide dinitrate with 10 ml of *alcohol R* for 5 min and filter.

Apply to the plate 10 µl of each solution. Develop over a path of 15 cm using a mixture of 5 volumes of *methanol R* and 95 volumes of *methylene chloride R*. Dry the plate in a current of air. Spray with freshly prepared *potassium iodide and starch solution R*. Expose to ultraviolet light at 254 nm for 15 min. Examine in daylight. The principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

C. Examine by thin-layer chromatography (2.2.27), using *silica gel G R* as the coating substance.

Test solution. Shake a quantity of the substance to be examined corresponding to 0.10 g of lactose or mannitol with 10 ml of *water R*. Filter if necessary.

Reference solution (a). Dissolve 0.10 g of *lactose R* in *water R* and dilute to 10 ml with the same solvent.

Reference solution (b). Dissolve 0.10 g of *mannitol R* in *water R* and dilute to 10 ml with the same solvent.

Reference solution (c). Mix equal volumes of reference solutions (a) and (b).

Apply to the plate 1 µl of each solution and thoroughly dry the starting points. Develop over a path of 15 cm using a mixture of 10 volumes of *water R*, 15 volumes of *methanol R*, 25 volumes of *anhydrous acetic acid R* and 50 volumes of *ethylene chloride R*, measured accurately since a slight excess of water produces cloudiness. Dry the plate in a current of warm air. Repeat immediately the development after renewing the mobile phase. Dry the plate in a current of warm air. Spray with *4-aminobenzoic acid solution R*. Dry the plate in a current of cold air until the acetone is removed. Heat the plate at 100 °C for 15 min. Allow to cool and spray with a 2 g/l solution of *sodium periodate R*. Dry the plate in a current of cold air. Heat the plate at 100 °C for 15 min. The principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a) for lactose or to the principal spot in the chromatogram obtained with reference solution (b) for mannitol. The identification is not valid unless the chromatogram obtained with reference solution (c) shows two clearly separated spots.

D. Shake a quantity of the substance to be examined corresponding to 25 mg of isosorbide dinitrate with 10 ml of *acetone R*, for 5 min. Filter, evaporate to dryness at a temperature below 40 °C and dry the residue over *diphosphorus pentoxide R* at a pressure of 0.7 kPa for 16 h. The melting point (2.2.14) of the residue is 69 °C to 72 °C.

TESTS

Inorganic nitrates. Examine by thin-layer chromatography (2.2.27), using *silica gel H R* as the coating substance.

Test solution. Shake a quantity of the substance to be examined corresponding to 0.10 g of isosorbide dinitrate with 5 ml of *alcohol R* and filter.

Reference solution. Dissolve 10 mg of *potassium nitrate R* in 1 ml of *water R* and dilute to 100 ml with *alcohol R*.

Apply to the plate 10 µl of each solution. Develop over a path of 15 cm using a mixture of 15 volumes of *glacial acetic acid R*, 30 volumes of *acetone R* and 60 volumes of *toluene R*. Thoroughly dry the plate in a current of air until the acetic acid is completely removed. Spray copiously with freshly prepared *potassium iodide and starch solution R*. Expose the plate to ultraviolet light at 254 nm for 15 min. Examine in daylight. Any spot corresponding to nitrate ion in the chromatogram obtained with the test solution is not more intense than the spot in the chromatogram obtained with the reference solution (0.5 per cent, calculated as potassium nitrate).

Isosorbide 5-nitrate and isosorbide 2-nitrate. Examine by liquid chromatography (2.2.29) as described under Assay, changing the detection to 210-215 nm.

When the chromatograms are recorded in the prescribed conditions, the retention times are: isosorbide dinitrate, about 5 min; isosorbide 2-nitrate, about 8 min; isosorbide 5-nitrate about 11 min.

Inject 10 µl of reference solution (c). When a recorder is used, adjust the sensitivity of the system so that the height of the principal peak in the chromatogram obtained with reference solution (c) is not less than 20 per cent of the full scale of the recorder.

Inject 10 µl of reference solution (e). The test is not valid unless in the chromatogram obtained with reference solution (e), the resolution between the peaks corresponding to isosorbide dinitrate and isosorbide 2-nitrate is at least 6.0. Inject 10 µl of test solution (a), 10 µl of reference solution (c) and 10 µl of reference solution (d). In the chromatogram obtained with test solution (a): the area of any peak corresponding to isosorbide 2-nitrate is not greater than the area of the principal peak in the chromatogram obtained with reference solution (c) (0.5 per cent); the area of any peak corresponding to isosorbide 5-nitrate is not greater than the area of the principal peak in the chromatogram obtained with reference solution (d) (0.5 per cent).

ASSAY

Examine by liquid chromatography (2.2.29).

Test solution (a). Sonicate a quantity of the substance to be examined corresponding to 25.0 mg of isosorbide dinitrate with 20 ml of the mobile phase for 15 min and dilute to 25.0 ml with the mobile phase. Filter the solution through a suitable membrane filter.

Test solution (b). Dilute 1.0 ml of test solution (a) to 10.0 ml with the mobile phase.

Reference solution (a). Sonicate a quantity of *isosorbide dinitrate CRS* corresponding to 25.0 mg of isosorbide dinitrate with 20 ml of the mobile phase for 15 min and dilute to 25.0 ml with the mobile phase. Filter the solution through a suitable membrane filter.

Reference solution (b). Dilute 1.0 ml of reference solution (a) to 10.0 ml with the mobile phase.

Reference solution (c). Dissolve 10.0 mg of *isosorbide 2-nitrate CRS* in the mobile phase and dilute to 10.0 ml with the mobile phase. Dilute 0.1 ml of this solution to 20.0 ml with the mobile phase.

Reference solution (d). Dissolve 10.0 mg of *isosorbide mononitrate CRS* in the mobile phase and dilute to 10.0 ml with the mobile phase. Dilute 0.1 ml of this solution to 20.0 ml with the mobile phase.

Reference solution (e). Dissolve 5 mg of *isosorbide 2-nitrate CRS* in the mobile phase and dilute to 10 ml with the mobile phase. To 1 ml of this solution, add 0.5 ml of reference solution (a) and dilute to 10 ml with the mobile phase.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4.6 mm in internal diameter packed with *aminopropylmethylsilyl silica gel for chromatography R* (10 µm),
- as mobile phase at a flow rate of 1 ml/min a mixture of 15 volumes of *ethanol R* and 85 volumes of *trimethylpentane R*,
- as detector a spectrophotometer set at 230 nm.

Inject 20 µl of reference solution (b). When a recorder is used, adjust the sensitivity of the system so that the height of the principal peak in the chromatogram obtained is not less than 50 per cent of the full scale of the recorder. If the areas of the peaks from two successive injections do not agree to within 1.0 per cent, then inject a further four times and calculate, for the six injections, the relative standard deviation. The assay is not valid unless the relative standard deviation for the six injections is at most 2.0 per cent. Inject test solution (b) and reference solution (b), alternately.

Calculate the content of isosorbide dinitrate as a percentage of the declared content.

STORAGE

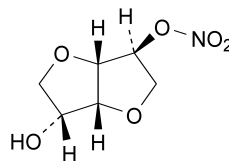
Store protected from light.

LABELLING

The label states the percentage content of isosorbide dinitrate.

IMPURITIES

A. inorganic nitrates,



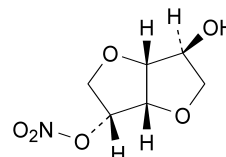
B. isosorbide 2-nitrate,

C. isosorbide mononitrate (isosorbide 5-nitrate).

01/2005:1118

ISOSORBIDE MONONITRATE, DILUTED

Isosorbidi mononitras dilutus



$C_6H_9NO_6$

M_r 191.1

DEFINITION

Diluted isosorbide mononitrate is a dry mixture of isosorbide mononitrate and *Lactose monohydrate (0187)* or *Mannitol (0559)*. It contains not less than 95.0 per cent *m/m* and not more than 105.0 per cent *m/m* of the content of 1,4:3,6-dianhydro-D-glucitol 5-nitrate stated on the label.

CHARACTERS

Undiluted isosorbide mononitrate is a white, crystalline powder, freely soluble in water, in acetone, in alcohol and in methylene chloride.

The solubility of the diluted product depends on the diluent and its concentration.

IDENTIFICATION

First identification: A, C, D.

Second identification: B, C, D.

A. Examine by infrared absorption spectrophotometry (2.2.24) the residue obtained in identification test D, comparing with the spectrum obtained with *isosorbide mononitrate CRS*. Examine the substances prepared as discs.

B. Examine by thin-layer chromatography (2.2.27), using *silica gel G R* as the coating substance.

Test solution. Shake a quantity of the substance to be examined corresponding to 10 mg of isosorbide mononitrate with 10 ml of *alcohol R* for 5 min and filter.

Reference solution. Dissolve 10 mg of *isosorbide mononitrate CRS* in *alcohol R* and dilute to 10 ml with the same solvent.

Apply to the plate 10 µl of each solution. Develop over a path of 15 cm using a mixture of 5 volumes of *methanol R* and 95 volumes of *methylene chloride R*. Dry the plate in a current of air. Spray with freshly prepared *potassium iodide and starch solution R*. Expose to ultraviolet light at 254 nm for 15 min. Examine in daylight. The principal

spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

- C. Examine by thin-layer chromatography (2.2.27), using *silica gel G R* as the coating substance.

Test solution. Shake a quantity of the substance to be examined corresponding to 0.10 g of lactose or mannitol with 10 ml of *water R*. Filter if necessary.

Reference solution (a). Dissolve 0.10 g of *lactose R* in *water R* and dilute to 10 ml with the same solvent.

Reference solution (b). Dissolve 0.10 g of *mannitol R* in *water R* and dilute to 10 ml with the same solvent.

Reference solution (c). Mix equal volumes of reference solutions (a) and (b).

Apply to the plate 1 µl of each solution and thoroughly dry the starting points. Develop over a path of 15 cm using a mixture of 10 volumes of *water R*, 15 volumes of *methanol R*, 25 volumes of *anhydrous acetic acid R* and 50 volumes of *ethylene chloride R*, measured accurately since a slight excess of water produces cloudiness. Dry the plate in a current of warm air. Repeat immediately the development after renewing the mobile phase. Dry the plate in a current of warm air. Spray with *4-aminobenzoic acid solution R*. Dry the plate in a current of cold air until the acetone is removed. Heat the plate at 100 °C for 15 min. Allow to cool and spray with a 2 g/l solution of *sodium periodate R*. Dry the plate in a current of cold air. Heat the plate at 100 °C for 15 min. The principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a) for lactose or to the principal spot in the chromatogram obtained with reference solution (b) for mannitol. The identification is not valid unless the chromatogram obtained with reference solution (c) shows two clearly separated spots.

- D. Shake a quantity of the substance to be examined corresponding to 25 mg of isosorbide mononitrate with 10 ml of *acetone R* for 5 min. Filter, evaporate to dryness at a temperature below 40 °C and dry the residue over *diphosphorus pentoxide R* at a pressure of 0.7 kPa for 16 h. The melting point (2.2.14) of the residue is 89 °C to 91 °C.

TESTS

Inorganic nitrates. Examine by thin-layer chromatography (2.2.27), using *silica gel H R* as the coating substance.

Test solution. Shake a quantity of the substance to be examined corresponding to 0.10 g of isosorbide mononitrate with 5 ml of *alcohol R* and filter.

Reference solution. Dissolve 10 mg of *potassium nitrate R* in 1 ml of *water R* and dilute to 100 ml with *alcohol R*.

Apply separately to the plate 10 µl of each solution. Develop over a path of 15 cm using a mixture of 15 volumes of *glacial acetic acid R*, 30 volumes of *acetone R* and 60 volumes of *toluene R*. Thoroughly dry the plate in a current of air until the acetic acid is completely removed. Spray copiously with freshly prepared *potassium iodide and starch solution R*. Expose the plate to ultraviolet light at 254 nm for 15 min. Examine in daylight. Any spot corresponding to nitrate ion in the chromatogram obtained with the test solution is not more intense than the spot in the chromatogram obtained with the reference solution (0.5 per cent, calculated as potassium nitrate).

Isosorbide dinitrate and isosorbide 2-nitrate. Examine by liquid chromatography (2.2.29) as described under Assay, changing the detection to 210 nm to 215 nm.

When the chromatograms are recorded in the prescribed conditions, the retention times are: isosorbide dinitrate about 5 min, isosorbide-2-nitrate about 8 min and isosorbide 5-nitrate about 11 min.

Inject 10 µl of reference solution (b). When a recorder is used, adjust the sensitivity of the system so that the height of the principal peak in the chromatogram obtained with reference solution (b) is at least 20 per cent of the full scale of the recorder.

Inject 10 µl of reference solution (d). The test is not valid unless, in the chromatogram obtained with reference solution (d), the resolution between the peaks corresponding to isosorbide 2-nitrate and isosorbide 5-nitrate is at least 4.0.

Inject 10 µl of test solution (a), 10 µl of reference solution (b) and 10 µl of reference solution (c). In the chromatogram obtained with test solution (a): the area of any peak corresponding to isosorbide 2-nitrate is not greater than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent); the area of any peak corresponding to isosorbide dinitrate is not greater than the area of the principal peak in the chromatogram obtained with reference solution (c) (0.5 per cent).

ASSAY

Examine by liquid chromatography (2.2.29).

Test solution (a). Sonicate a quantity of the substance to be examined corresponding to 25.0 mg of isosorbide mononitrate with 20 ml of the mobile phase for 15 min and dilute to 25.0 ml with the mobile phase. Filter the solution through a suitable membrane filter.

Test solution (b). Dilute 1.0 ml of test solution (a) to 10.0 ml with the mobile phase.

Reference solution (a). Dissolve 25.0 mg of *isosorbide mononitrate CRS* in the mobile phase and dilute to 25.0 ml with the mobile phase. Dilute 1.0 ml of the solution to 10.0 ml with the mobile phase.

Reference solution (b). Dissolve 10.0 mg of *isosorbide-2-nitrate CRS* in the mobile phase and dilute to 10.0 ml with the mobile phase. Dilute 0.1 ml of the solution to 20.0 ml with the mobile phase.

Reference solution (c). Sonicate a quantity of *isosorbide dinitrate CRS* corresponding to 10.0 mg of isosorbide dinitrate in 15 ml of the mobile phase for 15 min and dilute to 20.0 ml with the mobile phase. Filter the solution through a suitable membrane filter. Dilute 0.1 ml of the solution to 10.0 ml with the mobile phase.

Reference solution (d). Dissolve 5 mg of *isosorbide mononitrate CRS* and 5 mg of *isosorbide-2-nitrate CRS* in the mobile phase and dilute to 10 ml with the mobile phase. Dilute 1 ml of the solution to 10 ml with the mobile phase.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4.6 mm in internal diameter packed with *aminopropylmethylsilyl silica gel for chromatography R* (10 µm),
- as mobile phase at a flow rate of 1 ml/min a mixture of 15 volumes of *ethanol R* and 85 volumes of *trimethylpentane R*,
- as detector a spectrophotometer set at 230 nm.

Inject 20 µl of reference solution (a). When a recorder is used, adjust the sensitivity of the system so that the height of the principal peak in the chromatogram obtained with reference solution (a) is at least 50 per cent of the full scale of the recorder. If the areas of the peaks from two successive

injections do not agree to within 1.0 per cent, then inject a further four times and calculate, for the six injections, the relative standard deviation. The assay is not valid unless the relative standard deviation for the six injections is at most 2.0 per cent. Inject test solution (b) and reference solution (a), alternately.

Calculate the content of isosorbide mononitrate as a percentage of the declared content.

STORAGE

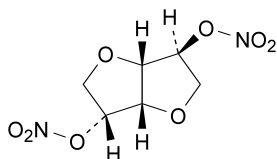
Store protected from light.

LABELLING

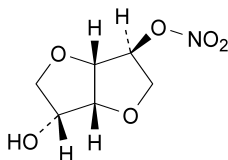
The label states the percentage content of isosorbide mononitrate.

IMPURITIES

A. inorganic nitrates,



B. isosorbide dinitrate,

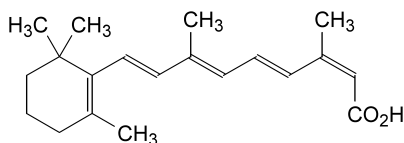


C. isosorbide 2-nitrate.

01/2005:1019

ISOTRETINOIN

Isotretinoinum



$C_{20}H_{28}O_2$

M_r 300.4

DEFINITION

Isotretinoin contains not less than 98.0 per cent and not more than the equivalent of 102.0 per cent of (2Z,4E,6E,8E)-3,7-dimethyl-9-(2,6,6-trimethylcyclohex-1-enyl)nona-2,4,6,8-tetraenoic acid, calculated with reference to the dried substance.

CHARACTERS

A yellow or light-orange, crystalline powder, practically insoluble in water, soluble in methylene chloride, slightly soluble in alcohol. It is sensitive to air, heat and light, especially in solution.

Carry out all operations as rapidly as possible and avoid exposure to actinic light; use freshly prepared solutions.

IDENTIFICATION

First identification: A, B.

Second identification: A, C, D.

A. Dissolve 75.0 mg in 5 ml of *methylene chloride R* and dilute immediately to 100.0 ml with acidified 2-propanol (prepared by diluting 1 ml of 0.01 M *hydrochloric acid R* to 1000 ml with 2-propanol R). Dilute 5.0 ml of this solution to 100.0 ml with the acidified 2-propanol (solution A). Dilute 5.0 ml of solution A to 50.0 ml with the acidified 2-propanol. Examined between 300 nm and 400 nm (2.2.25), the solution shows an absorption maximum at 354 nm. The specific absorbance at the maximum is 1290 to 1420.

B. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *isotretinoin CRS*. Examine the substances prepared as discs.

C. Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel GF₂₅₄ plate R*.

Test solution. Dissolve 10 mg of the substance to be examined in *methylene chloride R* and dilute to 10 ml with the same solvent.

Reference solution (a). Dissolve 10 mg of *isotretinoin CRS* in *methylene chloride R* and dilute to 10 ml with the same solvent.

Reference solution (b). Dissolve 10 mg of *isotretinoin CRS* and 10 mg of *tretinoin CRS* in *methylene chloride R* and dilute to 10 ml with the same solvent.

Apply separately to the plate 5 µl of each solution. Develop over a path of 15 cm using a mixture of 2 volumes of *glacial acetic acid R*, 4 volumes of *acetone R*, 40 volumes of *peroxide-free ether R* and 54 volumes of *cyclohexane R*. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. The principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a). The test is not valid unless the chromatogram obtained with reference solution (b) shows two clearly separated principal spots.

D. Dissolve about 5 mg in 2 ml of *antimony trichloride solution R*. An intense red colour develops and later becomes violet.

TESTS

Related substances. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 0.100 g of the substance to be examined in *methanol R* and dilute to 50.0 ml with the same solvent.

Reference solution (a). Dissolve 10.0 mg of *tretinoin CRS* in *methanol R* and dilute to 10.0 ml with the same solvent.

Reference solution (b). Dilute 1.0 ml of reference solution (a) to 25.0 ml with *methanol R*.

Reference solution (c). Mix 1.0 ml of reference solution (a) with 0.5 ml of the test solution and dilute to 25.0 ml with *methanol R*.

Reference solution (d). Dilute 0.5 ml of the test solution to 100.0 ml with *methanol R*.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.15 m long and 4.6 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (3 µm),
- as mobile phase at a flow rate of 1.0 ml/min a mixture of 5 volumes of *glacial acetic acid R*, 225 volumes of *water R* and 770 volumes of *methanol R*.
- as detector a spectrophotometer set at 355 nm.

01/2005:1119

Inject separately 10 µl of each of reference solutions (b), (c) and (d) and of the test solution. Adjust the sensitivity of the detector so that the height of the principal peak in the chromatogram obtained with reference solution (b) is not less than 70 per cent of the full scale of the recorder. The test is not valid unless the resolution between the peaks due to isotretinoin and tretinoin in the chromatogram obtained with reference solution (c) is at least 2.0. In the chromatogram obtained with the test solution: the area of any peak due to tretinoin is not greater than the area of the principal peak in the chromatogram obtained with reference solution (b) (2.0 per cent); the sum of the areas of any peaks, apart from the principal peak and any peak due to tretinoin, is not greater than the area of the principal peak in the chromatogram obtained with reference solution (d) (0.5 per cent).

Heavy metals (2.4.8). 0.5 g complies with limit test D for heavy metals (20 ppm). Prepare the standard using 1 ml of *lead standard solution* (10 ppm Pb) R.

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying *in vacuo* for 16 h.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.200 g in 70 ml of *acetone R*. Titrate with 0.1 M *tetrabutylammonium hydroxide* determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *tetrabutylammonium hydroxide* is equivalent to 30.04 mg of C₂₀H₂₈O₂.

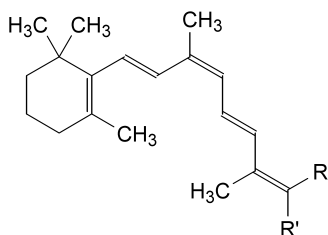
STORAGE

Store in an airtight container, protected from light, at a temperature not exceeding 25 °C.

It is recommended that the contents of an opened container be used as soon as possible and any unused part be protected by an atmosphere of an inert gas.

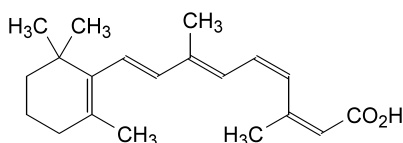
IMPURITIES

A. tretinoin,



B. R = CO₂H, R' = H: (2Z,4E,6Z,8E)-3,7-dimethyl-9-(2,6,6-trimethylcyclohex-1-enyl)nona-2,4,6,8-tetraenoic acid (9,13-di-*cis*-retinoic acid),

D. R = H, R' = CO₂H: (2E,4E,6Z,8E)-3,7-dimethyl-9-(2,6,6-trimethylcyclohex-1-enyl)nona-2,4,6,8-tetraenoic acid (9-*cis*-retinoic acid),

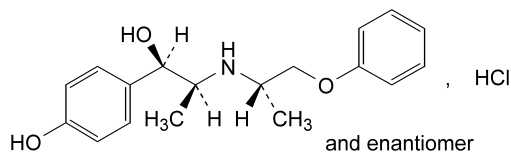


C. (2Z,4Z,6E,8E)-3,7-dimethyl-9-(2,6,6-trimethylcyclohex-1-enyl)nona-2,4,6,8-tetraenoic acid (11,13-di-*cis*-retinoic acid),

E. oxidation products of isotretinoin.

ISOXSUPRINE HYDROCHLORIDE

Isoxsuprini hydrochloridum

C₁₈H₂₄ClNO₃M_r 337.8

DEFINITION

Isoxsuprine hydrochloride contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of (1*RS*,2*SR*)-1-(4-hydroxyphenyl)-2-[(1*SR*)-1-methyl-2-phenoxyethyl]amino]propan-1-ol hydrochloride, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, crystalline powder, sparingly soluble in water and in alcohol, practically insoluble in methylene chloride.

It melts at about 205 °C, with decomposition.

IDENTIFICATION

First identification: B, E.

Second identification: A, C, D, E.

A. Dissolve 50.0 mg in 0.1 M *hydrochloric acid* and dilute to 50.0 ml with the same acid. Dilute 10.0 ml of this solution to 100.0 ml with 0.1 M *hydrochloric acid*. Examined between 230 nm and 350 nm (2.2.25), the solution shows two absorption maxima, at 269 nm and 275 nm. The specific absorbance at these maxima are 71 to 74 and 70 to 73, respectively. The test is not valid unless, in the test for resolution (2.2.25), the ratio of the absorbances is at least 1.7.

B. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *isoxsuprine hydrochloride CRS*. Examine the substances prepared as discs. If the spectra obtained show differences, dissolve 50 mg of the substance to be examined and the reference substance separately in 2 ml of *methanol R*, add 15 ml of *methylene chloride R*, evaporate to dryness and record new spectra using the residues.

C. Examine by thin-layer chromatography (2.2.27), using *silica gel G R* as the coating substance.

Test solution. Dissolve 20 mg of the substance to be examined in *methanol R* and dilute to 10 ml with the same solvent.

Reference solution. Dissolve 20 mg of *isoxsuprine hydrochloride CRS* in *methanol R* and dilute to 10 ml with the same solvent.

Apply to the plate 10 µl of each solution. Develop over a path of 12 cm using a mixture of 0.25 volumes of *concentrated ammonia R*, 15 volumes of *methanol R* and 85 volumes of *methylene chloride R*. Dry the plate in a current of warm air and spray with a 10 g/l solution of *potassium permanganate R*. The principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

- D. To 1 ml of solution S (see Tests) add 0.05 ml of *copper sulphate solution R* and 0.5 ml of *strong sodium hydroxide solution R*. The solution becomes blue. Add 1 ml of *ether R* and shake. Allow to separate. The upper layer remains colourless.

E. 2 ml of solution S gives reaction (a) of chlorides (2.3.1).

TESTS

Solution S. Dissolve 0.50 g, with gentle heating if necessary, in *carbon dioxide-free water R*, cool and dilute to 50.0 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and colourless (2.2.2, *Method II*).

pH (2.2.3). The pH of solution S is 4.5 to 6.0.

Optical rotation (2.2.7). The angle of optical rotation of solution S is -0.05° to $+0.05^\circ$.

Phenones. Dissolve 10.0 mg in *water R* and dilute to 100.0 ml with the same solvent. The absorbance (2.2.25) measured at the maximum at 310 nm is not greater than 0.10 (1.0 per cent, calculated as impurity B).

Related substances. Prepare the solutions immediately before use. Examine by gas chromatography (2.2.28), using *hexacosane R* as the internal standard.

Internal standard solution (a). Dissolve 0.1 g of *hexacosane R* in *trimethylpentane R* and dilute to 20 ml with the same solvent.

Internal standard solution (b). Dilute 1 ml of internal standard solution (a) to 50 ml with *trimethylpentane R*.

Test solution. To 10.0 mg of the substance to be examined, add 0.5 ml of *N-trimethylsilylimidazole R*. Heat to 65°C for 10 min. Allow to cool, then add 2.0 ml of the internal standard solution (b) and 2.0 ml of *water R*. Shake. Use the upper layer.

Reference solution (a). To 10.0 mg of the substance to be examined, add 0.5 ml of *N-trimethylsilylimidazole R*. Heat to 65°C for 10 min. Allow to cool, then add 2.0 ml of the internal standard solution (a) and 2.0 ml of *water R*. Shake. Dilute 1.0 ml of the upper layer to 50.0 ml with *trimethylpentane R*.

Reference solution (b). To 10.0 mg of the substance to be examined, add 0.5 ml of *N-trimethylsilylimidazole R*. Heat to 65°C for 10 min. Allow to cool, then add 2.0 ml of *trimethylpentane R* and 2.0 ml of *water R*. Shake. Use the upper layer.

The chromatographic procedure may be carried out using:

- a glass column 1.5 m long and 4 mm in internal diameter, packed with *silanised diatomaceous earth for gas chromatography R* (125–135 μm) impregnated with 3 per cent *m/m* of *poly(dimethyl)siloxane R*,
- *nitrogen for chromatography R* as the carrier gas at a flow rate of 30 ml/min,
- a flame-ionisation detector,

maintaining the temperature of the column at 195°C for 25 min, then raising the temperature at a rate of $5^\circ\text{C}/\text{min}$ to 215°C and maintaining at 215°C for 10 min, and maintaining the temperature of the injection port and that of the detector at 225°C .

Inject 1 μl of reference solution (a). The substances elute in the following order: isoxsuprine and hexacosane. Adjust the sensitivity of the detector so that the heights of the two principal peaks are not less than 50 per cent of the full scale of the recorder. The test is not valid unless, in the chromatogram obtained, the resolution between the peaks corresponding to isoxsuprine and hexacosane is at least 5.0.

Inject 1 μl of reference solution (b). In the chromatogram obtained, verify that there is no peak with the same retention time as the internal standard.

Inject 1 μl of the test solution and 1 μl of reference solution (a). From the chromatogram obtained with reference solution (a), calculate the ratio (*R*) of the area of the peak due to the trimethylsilyl derivative of isoxsuprine to the area of the peak due to the internal standard. From the chromatogram obtained with the test solution, calculate the ratio of the sum of the areas of any peaks, apart from the principal peak, the peak due to the internal standard and the peak due to the solvent, to the area of the peak due to the internal standard: this ratio is not greater than *R* (2.0 per cent).

Heavy metals (2.4.8). 1.0 g complies with limit test C for heavy metals (20 ppm). Prepare the standard using 2 ml of *lead standard solution (10 ppm Pb) R*.

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at $100\text{--}105^\circ\text{C}$.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

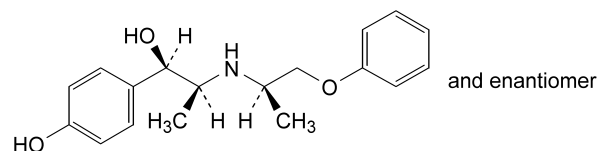
Dissolve 0.250 g in 80 ml of *alcohol R* and add 1.0 ml of 0.1 M *hydrochloric acid*. Carry out a potentiometric titration (2.2.20), using 0.1 M *sodium hydroxide*. Read the volume added between the two points of inflexion.

1 ml of 0.1 M *sodium hydroxide* is equivalent to 33.78 mg of $\text{C}_{18}\text{H}_{24}\text{ClNO}_3$.

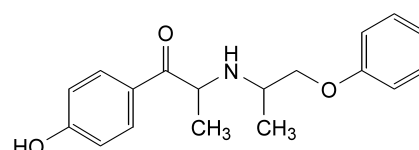
STORAGE

Store protected from light.

IMPURITIES



A. (1*RS*,2*SR*)-1-(4-hydroxyphenyl)-2-[(1*RS*)-1-methyl-2-phenoxyethyl]amino]propan-1-ol,



B. 1-(4-hydroxyphenyl)-2-[(1-methyl-2-phenoxyethyl)amino]propan-1-one.

01/2005:1334

ISPAGHULA HUSK

Plantaginis ovatae seminis tegumentum

DEFINITION

Ispaghula husk consists of the epispem and collapsed adjacent layers removed from the seeds of *Plantago ovata* Forssk. (*P. ispaghula* Roxb.).

CHARACTERS

It has the macroscopic and microscopic characters described under identification tests A and B.

IDENTIFICATION

01/2005:1333

- A. Ispaghula husk consists of pinkish-beige fragments or flakes up to about 2 mm long and 1 mm wide, some showing a light brown spot corresponding to the location of the embryo before it was removed from the seed.
- B. Reduce to a powder (355). The powder is pale yellow. Examine under a microscope using *lactic reagent R*. The powder shows mainly fragments of the episperm with polygonal cells filled with mucilage; fragments of the inner layers of the testa with brownish thin-walled cells often associated with the outer layers of the endosperm. Examine under a microscope using a 50 per cent V/V solution of *glycerol R*. The powder shows occasional starch granules, single or in groups of two to four, measuring 3 µm to 25 µm in diameter.
- C. Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel plate R*.

Test solution. To 10 mg of the powdered drug (355) in a thick-walled centrifuge tube add 2 ml of a 230 g/l solution of *trifluoroacetic acid R* and shake vigorously. Stopper the test tube and heat the mixture at 120 °C for 1 h. Centrifuge the hydrolysate, transfer the clear supernatant liquid into a 50 ml flask, add 10 ml of *water R* and evaporate the solution to dryness under reduced pressure. Take up the residue in 10 ml of *water R* and evaporate again to dryness under reduced pressure. Take up the residue with 2 ml of *methanol R*.

Reference solution (a). Dissolve 10 mg of *arabinose R* in a small quantity of *water R* and dilute to 10 ml with *methanol R*.

Reference solution (b). Dissolve 10 mg of *xylose R* in a small quantity of *water R* and dilute to 10 ml with *methanol R*.

Reference solution (c). Dissolve 10 mg of *galactose R* in a small quantity of *water R* and dilute to 10 ml with *methanol R*.

Apply to the plate, as bands, 10 µl of each solution. Develop over a path of 15 cm using a mixture of 15 volumes of *water R* and 85 volumes of *acetonitrile R*. Spray with *aminohippuric acid reagent R* and heat at 120 °C for 5 min. Examine in daylight. The chromatogram obtained with the test solution shows two orange-pink zones (arabinose and xylose) and a yellow zone (galactose) similar in position and colour to the zones in the chromatograms obtained with the reference solutions.

TESTS

Foreign matter (2.8.2). It complies with the test for foreign matter, determined on 5.0 g of the substance to be examined.

Swelling index (2.8.4). Not less than 40, determined on 0.1 g of the powdered drug (355).

Loss on drying (2.2.32). Not more than 12.0 per cent, determined on 1.000 g of the powdered drug (355) by drying in an oven at 100-105 °C for 2 h.

Total ash (2.4.16). Not more than 4.0 per cent.

STORAGE

Store in a well closed container, protected from light.

ISPAGHULA SEED

Plantaginis ovatae semen

DEFINITION

Dried ripe seeds of *Plantago ovata* Forssk. (*P. ispaghula* Roxb.).

CHARACTERS

Macroscopic and microscopic characters described under identification tests A and B.

IDENTIFICATION

- A. Ispaghula seed is pinkish-beige, smooth, boat-shaped and curved. It is 1.5 mm to 3.5 mm long, 1.5 mm to 2 mm wide and 1 mm to 1.5 mm thick. The concave surface shows in the centre a light coloured spot corresponding to the hilum. The convex surface shows a light brown spot corresponding to the location of the embryo and takes up about one quarter of the length of the seed.
- B. Reduce to a powder (355). The powder is pale brown. Examine under a microscope using *lactic reagent R*. The powder shows mainly fragments of the episperm with polygonal cells filled with mucilage; fragments of the inner layers of the testa with brownish thin-walled cells often associated with the outer layers of the endosperm; fragments of the endosperm with cells with thick cellulose walls containing aleurone grains and oil droplets; a few fragments of embryo with thin-walled cells. Examine under a microscope using a 50 per cent V/V solution of *glycerol R*. The powder shows starch granules, single or in groups of 2 to 4 and measuring 3 µm to 25 µm in diameter.
- C. Thin-layer chromatography (2.2.27).

Test solution. To 50 mg of the powdered drug (355) in a thick-walled centrifuge tube add 2 ml of a 230 g/l solution of *trifluoroacetic acid R*, and shake vigorously. Stopper the test tube and heat the mixture at 120 °C for 1 h. Centrifuge the hydrolysate, transfer the clear supernatant liquid into a 50 ml flask, add 10 ml of *water R* and evaporate the solution to dryness under reduced pressure. Take up the residue in 10 ml of *water R* and evaporate again to dryness under reduced pressure. Take up the residue in 2 ml of *methanol R*.

Reference solution (a). Dissolve 10 mg of *arabinose R* in a small quantity of *water R* and dilute to 10 ml with *methanol R*.

Reference solution (b). Dissolve 10 mg of *xylose R* in a small quantity of *water R* and dilute to 10 ml with *methanol R*.

Reference solution (c). Dissolve 10 mg of *galactose R* in a small quantity of *water R* and dilute to 10 ml with *methanol R*.

Plate: *TLC silica gel plate R*

Mobile phase: *water R*, *acetonitrile R* (15:85 V/V).

Application: 10 µl, as bands.

Development: over a path of 15 cm.

Detection: Spray with *aminohippuric acid reagent R* and heat at 120 °C for 5 min. Examine in daylight.

Results: see below the sequence of the zones present in the chromatograms obtained with the reference and the test solutions.

Top of the plate	
Xylose: an orange-pink zone	An orange-pink zone (xylose)
Arabinose: an orange-pink zone	An orange-pink zone (arabinose)
Galactose: a yellow zone	A yellow zone (galactose)
Reference solution	Test solution

TESTS

Foreign matter (2.8.2): maximum 2 per cent, determined on 10.0 g of the drug.

Swelling index (2.8.4): minimum 9.

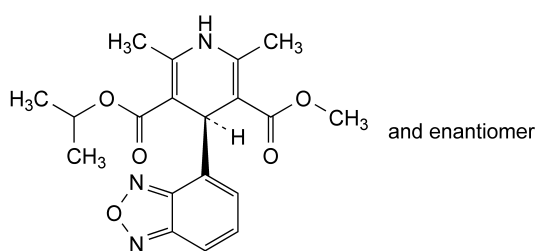
Loss on drying (2.2.32): maximum 10.0 per cent, determined on 1.000 g of the powdered drug (355) by drying in an oven at 100-105 °C for 2 h.

Total ash (2.4.16): maximum 4.0 per cent.

01/2005:2110

ISRADIPINE

Isradipinum

C₁₉H₂₁N₃O₅M_r 371.4

DEFINITION

Methyl 1-methylethyl (4*RS*)-4-(2,1,3-benzoxadiazol-4-yl)-2,6-dimethyl-1,4-dihydropyridine-3,5-dicarboxylate.

Content: 97.0 per cent to 102.0 per cent (dried substance).

CHARACTERS

Appearance: yellow, crystalline powder.

Solubility: practically insoluble in water, freely soluble in acetone, soluble in methanol.

mp: about 168 °C.

IDENTIFICATION

Infrared absorption spectrophotometry (2.2.24).

Comparison: isradipine CRS.

TESTS

Related substances. Liquid chromatography (2.2.29).

Test solution (a). Dissolve 50.0 mg of the substance to be examined in 1 ml of *methanol R*, using an ultrasonic bath if necessary, and dilute to 25.0 ml with the mobile phase.

Test solution (b). Dissolve 50.0 mg of the substance to be examined in 2 ml of *methanol R* and dilute to 250.0 ml with the mobile phase.

Reference solution (a). Dilute 1.0 ml of test solution (a) to 100.0 ml with the mobile phase. Dilute 1.0 ml of this solution to 10.0 ml with the mobile phase.

Reference solution (b). Dissolve 2 mg of the substance to be examined and 2 mg of *isradipine impurity D CRS* in the mobile phase and dilute to 10.0 ml with the mobile phase. Dilute 1.0 ml of the solution to 10.0 ml with the mobile phase.

Reference solution (c). Dissolve 50.0 mg of *isradipine CRS* in 2 ml of *methanol R* and dilute to 250.0 ml with the mobile phase.

Column:

- **size:** *l* = 0.10 m, Ø = 4.6 mm,
- **stationary phase:** octadecylsilyl silica gel for chromatography *R* (5 µm).

Mobile phase: acetonitrile *R*, tetrahydrofuran *R*, water *R* (125:270:625 V/V/V).

Flow rate: 1.2 ml/min.

Detection: spectrophotometer at 230 nm.

Injection: 20 µl of test solution (a) and reference solutions (a) and (b).

Run time: 5 times the retention time of isradipine.

Identification of impurities: use the chromatogram supplied with *isradipine CRS* to identify the peak due to impurity B.

Relative retention with reference to isradipine (retention time = about 7 min): impurity D = about 0.9; impurity B = about 1.8.

System suitability: reference solution (b):

- **resolution:** minimum 2.0 between the peaks due to isradipine and impurity D.

Limits:

- **correction factor:** for the calculation of content, multiply the peak area of impurity D by 1.4,
- **impurity B:** not more than 8 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.8 per cent),
- **impurity D:** not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent),
- **any other impurity:** for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent),
- **total:** not more than 10 times the area of the principal peak in the chromatogram obtained with reference solution (a) (1.0 per cent),
- **disregard limit:** 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

Loss on drying (2.2.32): maximum 0.2 per cent, determined on 1.000 g by drying in an oven at 100-105 °C for 4 h.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (2.2.29) as described in the test for related substances with the following modifications.

Detection: spectrophotometer at 326 nm.

Injection: test solution (b) and reference solution (c).

Run time: twice the retention time of isradipine.

Calculate the percentage content of isradipine from the areas of the peaks and the declared content of *isradipine CRS*.

STORAGE

Protected from light.

IMPURITIES

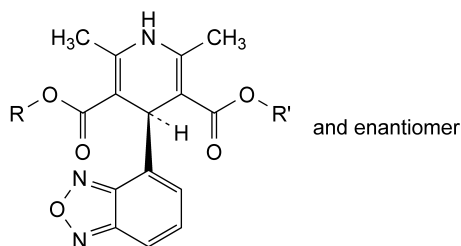
Specified impurities: B, D.

Other detectable impurities: A, C, E.

01/2005:1335

ITRACONAZOLE

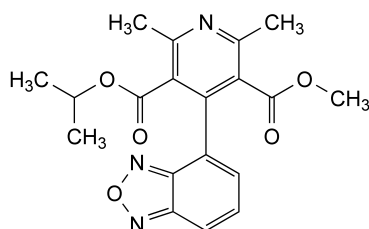
Itraconazolium



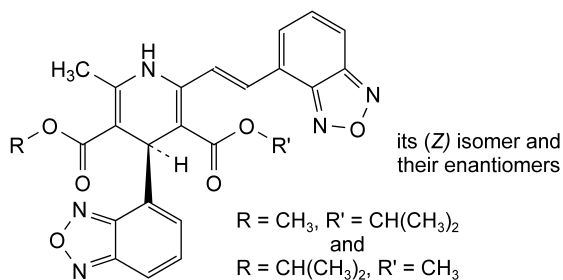
A. $R = C_2H_5$, $R' = CH_3$: ethyl methyl (4*RS*)-4-(2,1,3-benzoxadiazol-4-yl)-2,6-dimethyl-1,4-dihydropyridine-3,5-dicarboxylate,

B. $R = R' = CH(CH_3)_2$: bis(1-methylethyl) (4*RS*)-4-(2,1,3-benzoxadiazol-4-yl)-2,6-dimethyl-1,4-dihydropyridine-3,5-dicarboxylate,

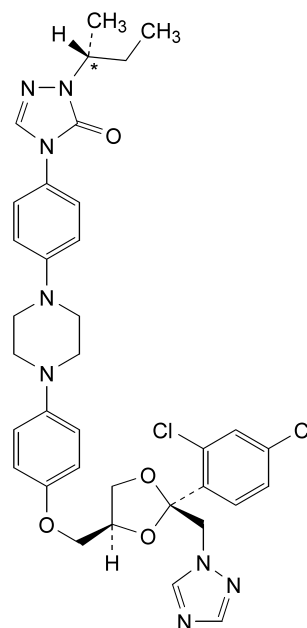
C. $R = R' = CH_3$: dimethyl (4*RS*)-4-(2,1,3-benzoxadiazol-4-yl)-2,6-dimethyl-1,4-dihydropyridine-3,5-dicarboxylate,



D. methyl 1-methylethyl 4-(2,1,3-benzoxadiazol-4-yl)-2,6-dimethylpyridine-3,5-dicarboxylate,



E. methyl 1-methylethyl (4*RS*)-4-(2,1,3-benzoxadiazol-4-yl)-2-[(*EZ*)-2-(2,1,3-benzoxadiazol-4-yl)ethenyl]-6-methyl-1,4-dihydropyridine-3,5-dicarboxylate.

 $C_{35}H_{38}Cl_2N_8O_4$ M_r 706

DEFINITION

Itraconazole contains not less than 98.5 per cent and not more than the equivalent of 101.5 per cent of 4-[4-[4-[[*cis*-2-(2,4-dichlorophenyl)-2-(1*H*-1,2,4-triazol-1-ylmethyl)-1,3-dioxolan-4-yl]methoxy]phenyl]piperazin-1-yl]phenyl]-2-[(1*RS*)-1-methylpropyl]-2,4-dihydro-3*H*-1,2,4-triazol-3-one, calculated with reference to the dried substance.

CHARACTERS

A white or almost white powder, practically insoluble in water, freely soluble in methylene chloride, sparingly soluble in tetrahydrofuran, very slightly soluble in alcohol.

IDENTIFICATION

First identification: B.

Second identification: A, C, D.

A. Melting point (2.2.14): 166 °C to 170 °C.

B. Examine by infrared spectrophotometry (2.2.24), comparing with the spectrum obtained with *itraconazole CRS*. Examine the substance prepared as discs.

C. Examine by thin-layer chromatography (2.2.27), using a suitable octadecylsilyl silica gel as the coating substance.

Test solution. Dissolve 30 mg of the substance to be examined in a mixture of equal volumes of *methanol R* and *methylene chloride R* and dilute to 5 ml with the same mixture of solvents.

Reference solution (a). Dissolve 30 mg of *itraconazole CRS* in a mixture of equal volumes of *methanol R* and *methylene chloride R* and dilute to 5 ml with the same mixture of solvents.

Reference solution (b). Dissolve 30 mg of *itraconazole CRS* and 30 mg of *ketoconazole CRS* in a mixture of equal volumes of *methanol R* and *methylene chloride R* and dilute to 5 ml with the same mixture of solvents.

Apply to the plate 5 µl of each solution. Develop in an unsaturated tank over a path of 10 cm using a mixture of 20 volumes of *ammonium acetate solution R*, 40 volumes of *dioxan R* and 40 volumes of *methanol R*. Dry the plate in a current of warm air for 15 min and expose it to iodine vapour until the spots appear. Examine in daylight. The principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a). The test is not valid unless the chromatogram obtained with reference solution (b) shows two clearly separated spots.

- D. To 30 mg in a porcelain crucible add 0.3 g of *anhydrous sodium carbonate R*. Heat over an open flame for 10 min. Allow to cool. Take up the residue with 5 ml of *dilute nitric acid R* and filter. To 1 ml of the filtrate add 1 ml of *water R*. The solution gives reaction (a) of chlorides (2.3.1).

TESTS

Solution S. Dissolve 2.0 g in *methylene chloride R* and dilute to 20.0 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and not more intensely coloured than reference solution BY₆ (2.2.2, Method II).

Optical rotation (2.2.7). The angle of optical rotation is –0.10° to +0.10°, determined on solution S.

Related substances. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 0.100 g of the substance to be examined in a mixture of equal volumes of *methanol R* and *tetrahydrofuran R* and dilute to 10.0 ml with the same mixture of solvents.

Reference solution (a). Dissolve 5.0 mg of *itraconazole CRS* and 5.0 mg of *miconazole CRS* in a mixture of equal volumes of *methanol R* and *tetrahydrofuran R* and dilute to 100.0 ml with the same mixture of solvents.

Reference solution (b). Dilute 1.0 ml of the test solution to 100.0 ml with a mixture of equal volumes of *methanol R* and *tetrahydrofuran R*. Dilute to 5.0 ml of this solution to 10.0 ml with the same mixture of solvents.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.1 m long and 4.0 mm in internal diameter packed with *base-deactivated octadecylsilyl silica gel for chromatography R* (3 µm),
- as mobile phase at a flow rate of 1.5 ml/min a gradient programme using the following conditions:

Mobile phase A. A 27.2 g/l solution of *tetrabutylammonium hydrogen sulphate R*,

Mobile phase B. *Acetonitrile R*,

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)	Comment
0 - 20	80 → 50	20 → 50	linear gradient
20 - 25	50	50	isocratic elution
25 - 30	80	20	switch to initial eluent composition
30 = 0	80	20	restart gradient

- as detector a spectrophotometer set at 225 nm.

Equilibrate the column for at least 30 min with *acetonitrile R* at a flow rate of 1.5 ml/min and then equilibrate at the initial eluent composition for at least 5 min.

Adjust the sensitivity of the system so that the height of the principal peak in the chromatogram obtained with 10 µl of reference solution (b) is at least 50 per cent of the full scale of the recorder.

Inject 10 µl of reference solution (a). When the chromatogram is recorded in the prescribed conditions, the retention times are: *miconazole* about 10.5 min and *itraconazole* about 11 min. The test is not valid unless the resolution between the peaks corresponding to *miconazole* and *itraconazole* is at least 2.0. If necessary, adjust the concentration of *acetonitrile* in the mobile phase or adjust the time programme for the linear gradient elution.

Inject separately 10 µl of the mixture of equal volumes of *methanol R* and *tetrahydrofuran R* as a blank, 10 µl of the test solution and 10 µl of reference solution (b). In the chromatogram obtained with the test solution: the area of any peak, apart from the principal peak, is not greater than that of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent); the sum of the areas of all the peaks, apart from the principal peak, is not greater than 2.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (1.25 per cent). Disregard any peak obtained with the blank run and any peak with an area less than 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (b).

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C for 4 h.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

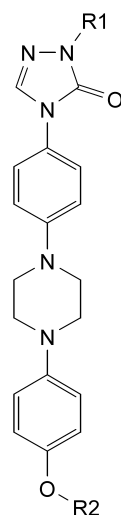
Dissolve 0.300 g in 70 ml of a mixture of 1 volume of *anhydrous acetic acid R* and 7 volumes of *methyl ethyl ketone R*. Titrate with 0.1 M *perchloric acid*, determining the end-point potentiometrically at the second point of inflexion (2.2.20).

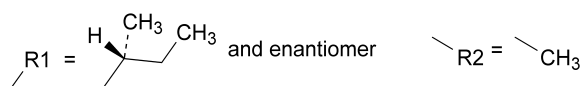
1 ml of 0.1 M *perchloric acid* is equivalent to 35.3 mg of C₃₅H₃₈Cl₂N₈O₄.

STORAGE

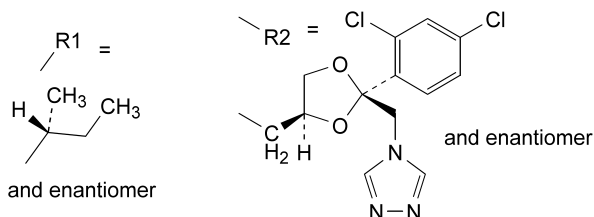
Store protected from light.

IMPURITIES

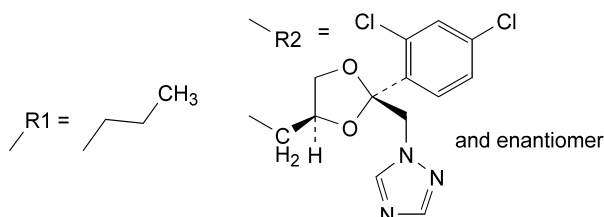




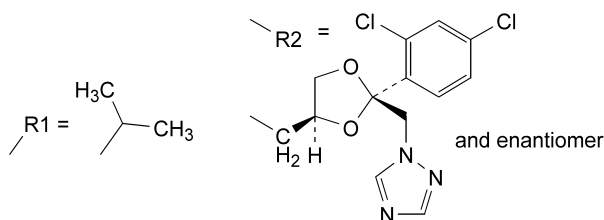
- A. 4-[4-[4-(4-methoxyphenyl)piperazin-1-yl]phenyl]-2-[(1*RS*)-1-methylpropyl]-2,4-dihydro-3*H*-1,2,4-triazol-3-one,



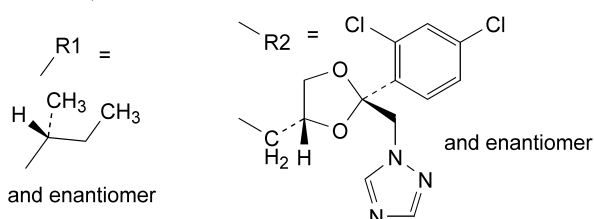
- B. 4-[4-[4-[[*cis*-2-(2,4-dichlorophenyl)-2-(4*H*-1,2,4-triazol-4-ylmethyl)-1,3-dioxolan-4-yl]methoxy]phenyl]piperazin-1-yl]phenyl]-2-[(1*RS*)-1-methylpropyl]-2,4-dihydro-3*H*-1,2,4-triazol-3-one,



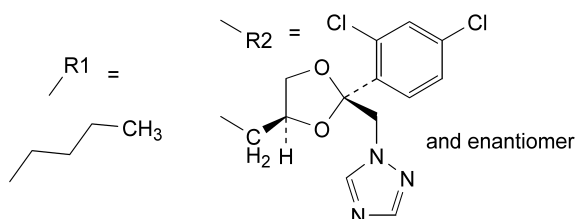
- C. 4-[4-[4-[[*cis*-2-(2,4-dichlorophenyl)-2-(1*H*-1,2,4-triazol-1-ylmethyl)-1,3-dioxolan-4-yl]methoxy]phenyl]piperazin-1-yl]phenyl]-2-propyl-2,4-dihydro-3*H*-1,2,4-triazol-3-one,



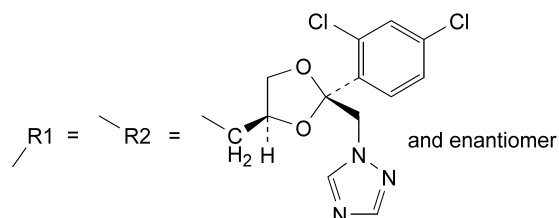
- D. 4-[4-[4-[[*cis*-2-(2,4-dichlorophenyl)-2-(1*H*-1,2,4-triazol-1-ylmethyl)-1,3-dioxolan-4-yl]methoxy]phenyl]piperazin-1-yl]phenyl]-2-(1-methylethyl)-2,4-dihydro-3*H*-1,2,4-triazol-3-one,



- E. 4-[4-[4-[[*trans*-2-(2,4-dichlorophenyl)-2-(1*H*-1,2,4-triazol-1-ylmethyl)-1,3-dioxolan-4-yl]methoxy]phenyl]piperazin-1-yl]phenyl]-2-[(1*RS*)-1-methylpropyl]-2,4-dihydro-3*H*-1,2,4-triazol-3-one,



- F. 2-butyl-4-[4-[4-[[*cis*-2-(2,4-dichlorophenyl)-2-(1*H*-1,2,4-triazol-1-ylmethyl)-1,3-dioxolan-4-yl]methoxy]phenyl]piperazin-1-yl]phenyl]-2,4-dihydro-3*H*-1,2,4-triazol-3-one,

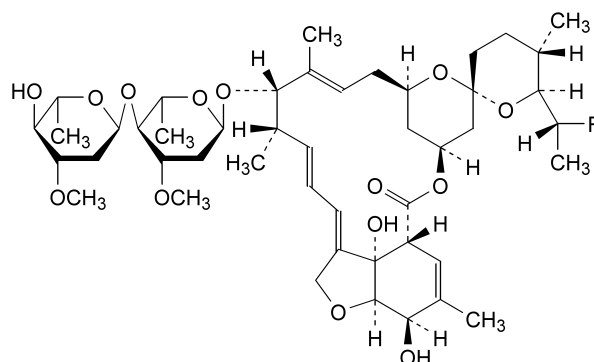


- G. 4-[4-[4-[[*cis*-2-(2,4-dichlorophenyl)-2-(1*H*-1,2,4-triazol-1-ylmethyl)-1,3-dioxolan-4-yl]methoxy]phenyl]piperazin-1-yl]phenyl]-2-[[*cis*-2-(2,4-dichlorophenyl)-2-(1*H*-1,2,4-triazol-1-ylmethyl)-1,3-dioxolan-4-yl]methyl]-2,4-dihydro-3*H*-1,2,4-triazol-3-one.

01/2005:1336
corrected

IVERMECTIN

Ivermectinum



Component	R	Molecular formula	M_r
H ₂ B _{1a}	CH ₂ -CH ₃	C ₄₈ H ₇₄ O ₁₄	875
H ₂ B _{1b}	CH ₃	C ₄₇ H ₇₂ O ₁₄	861

DEFINITION

Mixture of (2*aE*,4*E*,5'*S*,6*S*,6'*R*,7*S*,8*E*,11*R*,13*R*,15*S*,17*aR*,20*R*,20*aR*,20*bS*)-7-[[2,6-dideoxy-4-*O*-(2,6-dideoxy-3-*O*-methyl- α -L-arabino-hexopyranosyl)-3-*O*-methyl- α -L-arabino-hexopyranosyl]oxy]-20,20b-dihydroxy-5',6,8,19-tetramethyl-6'-[(1*S*)-1-methylpropyl]-3',4',5',6,6',7,10,11,14,15,17*a*,20,20*a*,20*b*-tetradecahydrospiro[11,15-methano-2*H*,13*H*,17*H*-furo[4,3,2-*pq*][2,6]benzodioxacyclooctadecene-13,2'-[2*H*]pyran]-17-one (or 5-*O*-demethyl-22,23-dihydroavermectin A_{1a}) (component H₂B_{1a}) and (2*aE*,4*E*,5'*S*,6*S*,6'*R*,7*S*,8*E*,11*R*,13*R*,15*S*,17*aR*,20*R*,20*aR*,20*bS*)-7-[[2,6-dideoxy-4-*O*-(2,6-dideoxy-3-*O*-methyl- α -L-arabino-hexopyranosyl)-3-*O*-methyl- α -L-arabino-hexopyranosyl]oxy]-20,20b-dihydroxy-5',6,8,19-tetramethyl-6'-[(1-methylethyl)-3',4',5',6,6',7,10,11,14,15,17*a*,20,20*a*,20*b*-tetradecahydrospiro[11,15-methano-2*H*,13*H*,17*H*-furo[4,3,2-*pq*][2,6]benzodioxacyclooctadecene-13,2'-[2*H*]pyran]-17-one (or 5-*O*-demethyl-25-de(1-methylpropyl)-25-(1-methylethyl)-22,23-dihydroavermectin A_{1a}) (component H₂B_{1b}).

Content:

- ivermectin (H₂B_{1a} + H₂B_{1b}): 95.0 per cent to 102.0 per cent (anhydrous and solvent-free substance),
- ratio H₂B_{1a}/(H₂B_{1a} + H₂B_{1b}) (areas by liquid chromatography): minimum 90.0 per cent.

CHARACTERS

Appearance: white or yellowish-white, crystalline powder, slightly hygroscopic.

Solubility: practically insoluble in water, freely soluble in methylene chloride, soluble in alcohol.

IDENTIFICATION

A. Infrared absorption spectrophotometry (2.2.24).

Comparison: ivermectin CRS.

B. Examine the chromatograms obtained in the assay.

Results: the retention times and sizes of the 2 principal peaks in the chromatogram obtained with the test solution are similar to those of the 2 principal peaks in the chromatogram obtained with reference solution (a).

TESTS

Appearance of solution. The solution is clear (2.2.1) and not more intensely coloured than reference solution BY₇ (2.2.2, Method II).

Dissolve 1.0 g in 50 ml of *toluene R*.

Specific optical rotation (2.2.7): –17 to –20 (anhydrous and solvent-free substance).

Dissolve 0.250 g in *methanol R* and dilute to 10.0 ml with the same solvent.

Related substances. Liquid chromatography (2.2.29).

Test solution. Dissolve 40.0 mg of the substance to be examined in *methanol R* and dilute to 50.0 ml with the same solvent.

Reference solution (a). Dissolve 40.0 mg of ivermectin CRS in *methanol R* and dilute to 50.0 ml with the same solvent.

Reference solution (b). Dilute 1.0 ml of reference solution (a) to 100.0 ml with *methanol R*.

Reference solution (c). Dilute 5.0 ml of reference solution (b) to 100.0 ml with *methanol R*.

Column:

- size: $l = 0.25$ m, $\varnothing = 4.6$ mm,
- stationary phase: octadecylsilyl silica gel for chromatography R (5 μ m).

Mobile phase: *water R*, *methanol R*, *acetonitrile R* (15:34:51 V/V/V).

Flow rate: 1 ml/min.

Detection: spectrophotometer at 254 nm.

Injection: 20 μ l.

System suitability:

- *resolution:* minimum of 3.0 between the first peak (component H₂B_{1b}) and the second peak (component H₂B_{1a}) in the chromatogram obtained with reference solution (a),
- *signal-to-noise ratio:* minimum of 10 for the principal peak in the chromatogram obtained with reference solution (c),
- *symmetry factor:* maximum of 2.5 for the principal peak in the chromatogram obtained with reference solution (a).

Limits:

- *impurity with a relative retention of 1.3 to 1.5* with reference to the principal peak: not more than 2.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (2.5 per cent),
- *any other impurity* (apart from the 2 principal peaks): not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (1 per cent),
- *total:* not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (5 per cent),

- *disregard limit:* area of the principal peak in the chromatogram obtained with reference solution (c) (0.05 per cent).

Ethanol and formamide. Gas chromatography (2.2.28).

Internal standard solution. Dilute 0.5 ml of *propanol R* to 100 ml with *water R*.

Test solution. In a centrifuge tube, dissolve 0.120 g of the substance to be examined in 2.0 ml of *m-xylene R* (if necessary heat in a water-bath at 40–50 °C). Add 2.0 ml of *water R*, mix thoroughly and centrifuge. Remove the upper layer and extract it with 2.0 ml of *water R*. Discard the upper layer and combine the aqueous layers. Add 1.0 ml of the internal standard solution. Centrifuge and discard any remaining *m-xylene*.

Reference solution (a). Dilute 3.0 g of *ethanol R* to 100.0 ml with *water R*.

Reference solution (b). Dilute 1.0 g of *formamide R* to 100.0 ml with *water R*.

Reference solution (c). Dilute 5.0 ml of reference solution (a) and 5.0 ml of reference solution (b) to 50.0 ml with *water R*. Introduce 2.0 ml of this solution into a centrifuge tube, add 2.0 ml of *m-xylene R*, mix thoroughly and centrifuge. Remove the upper layer and extract it with 2.0 ml of *water R*. Discard the upper layer and combine the aqueous layers. Add 1.0 ml of the internal standard solution. Centrifuge and discard any remaining *m-xylene*.

Reference solution (d). Dilute 10.0 ml of reference solution (a) and 10.0 ml of reference solution (b) to 50.0 ml with *water R*. Treat as prescribed for reference solution (c) (from "Introduce 2.0 ml of this solution...").

Column:

- *material:* fused silica,
- *size:* $l = 30$ m, $\varnothing = 0.53$ mm,
- *stationary phase:* macrogol 20 000 R (film thickness 1 μ m).

Carrier gas: helium for chromatography R.

Flow rate: 7.5 ml/min.

Split ratio: 1:10.

Temperature:

	Time (min)	Temperature (°C)
Column	0 - 2	50 → 80
	2 - 8	80 → 240
Injection port		220
Detector		280

Detection: flame ionisation.

Injection: 1 μ l; inject the test solution and reference solutions (c) and (d).

Limits:

- *ethanol:* maximum 5.0 per cent,
- *formamide:* maximum 3.0 per cent.

Heavy metals (2.4.8): maximum 20 ppm.

1.0 g complies with limit test C. Prepare the standard using 2 ml of *lead standard solution (10 ppm Pb) R*.

Water (2.5.12): maximum 1.0 per cent, determined on 0.50 g.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (2.2.29) as described in the test for related substances.

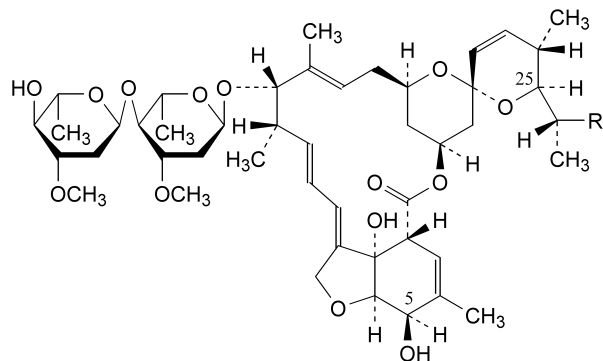
Injection: 20 µl; inject the test solution and reference solution (a).

Calculate the percentage contents of ivermectin ($H_2B_{1a} + H_2B_{1b}$) and the ratio $H_2B_{1a}/(H_2B_{1a} + H_2B_{1b})$ using the declared contents of *ivermectin CRS*.

STORAGE

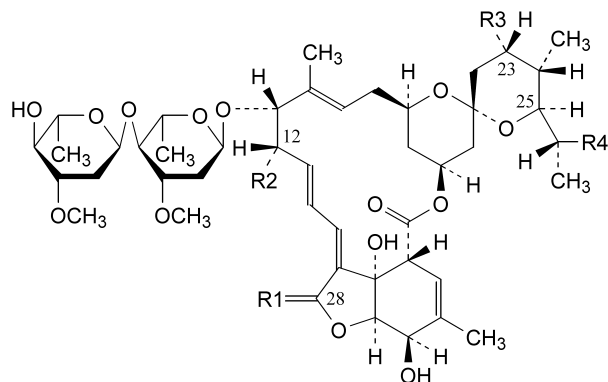
In an airtight container.

IMPURITIES



A. R = C₂H₅: 5-*O*-demethylavermectin A_{1a} (avermectin B_{1a}),

B. R = CH₃: 5-*O*-demethyl-25-de(1-methylpropyl)-25-(1-methylethyl)avermectin A_{1a} (avermectin B_{1b}),

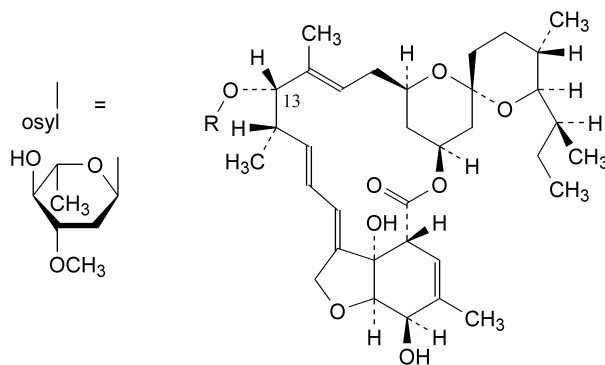


C. R1 = H₂, R2 = CH₃, R3 = OH, R4 = C₂H₅: (23*S*)-5-*O*-demethyl-23-hydroxy-22,23-dihydroavermectin A_{1a} (avermectin B_{2a}),

D. R1 = O, R2 = CH₃, R3 = H, R4 = C₂H₅: 5-*O*-demethyl-28-oxo-22,23-dihydroavermectin A_{1a} (28-oxoH₂B_{1a}),

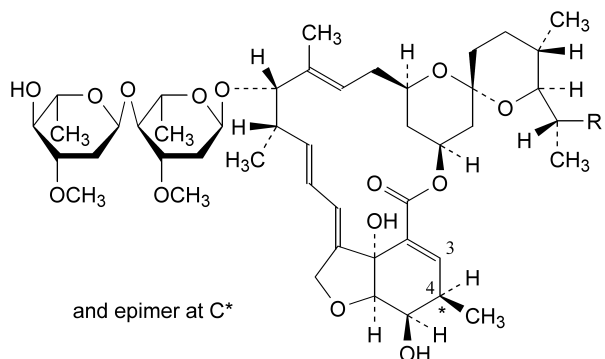
E. R1 = H₂, R2 = C₂H₅, R3 = H, R4 = C₂H₅: 5-*O*,12-didemethyl-12-ethyl-22,23-dihydroavermectin A_{1a} (12-demethyl-12-ethyl-H₂B_{1a}),

F. R1 = H₂, R2 = C₂H₅, R3 = H, R4 = CH₃: 5-*O*,12-didemethyl-25-de(1-methylpropyl)-12-ethyl-25-(1-methylethyl)-22,23-dihydroavermectin A_{1a} (12-demethyl-12-ethyl-H₂B_{1b}),



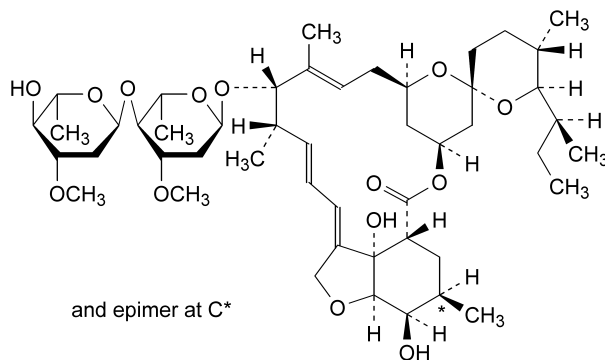
G. R = H: (6*R*,13*S*,25*R*)-5-*O*-demethyl-28-deoxy-6,28-epoxy-13-hydroxy-25-[(1*S*)-1-methylpropyl]milbemycin B (H₂B_{1a} aglycone),

H. R = osyl: 4'-*O*-de(2,6-dideoxy-3-*O*-methyl-α-*L*-arabino-hexopyranosyl)-5-*O*-demethyl-22,23-dihydroavermectin A_{1a},



I. R = C₂H₅: 2,3-didehydro-5-*O*-demethyl-3,4,22,23-tetrahydroavermectin A_{1a} (Δ^{2,3} H₂B_{1a}),

J. R = CH₃: 2,3-didehydro-5-*O*-demethyl-25-de(1-methylpropyl)-25-(1-methylethyl)-3,4,22,23-tetrahydroavermectin A_{1a} (Δ^{2,3} H₂B_{1b}),



K. (4*R*) and (4*S*)-5-*O*-demethyl-3,4,22,23-tetrahydroavermectin A_{1a} (H₄B_{1a} isomers).

01/2005:1229

JAVA TEA

Orthosiphonis folium

DEFINITION

Fragmented, dried leaves and tops of stems of *Orthosiphon stamineus* Benth. (*O. aristatus* Miq.; *O. spicatus* Bak.).

Content: minimum 0.05 per cent of sinensetin ($C_{20}H_{20}O_7$; M_r 372.4) (dried drug).

CHARACTERS

Macroscopic and microscopic characters described under identification tests A and B.

IDENTIFICATION

- A. The leaves are friable, up to 7.5 cm in length and 2.5 cm in width. The petiole is short. The lamina is oval to lanceolate, the apex acuminate and the base cuneate. The abaxial surface of the leaves is light greyish-green and the adaxial surface is dark green to brownish-green. The venation is pinnate with few secondary veins. Examined under a lens ($\times 10$), the secondary veins, after running parallel to the midrib, diverge at an acute angle. The margin is irregularly and roughly dentate, sometimes crenate and the abaxial surface is slightly curved. The petioles are thin, quadrangular, 4 mm to 8 mm long and, like the primary venation, usually violet-coloured. Occasionally, inflorescences in clusters of bluish-white to violet flowers, not yet opened, are found.
- B. Reduce to a powder (355). The powder is dark green. Examine under a microscope using *chloral hydrate solution R*. The powder shows fragments of epidermis, with cells with sinuous outlines bearing unicellular or bicellular conical covering trichomes and articulated uniseriate trichomes up to 450 μm long, consisting of 3 to 8 cells with thick pitted walls; capitate trichomes with unicellular or bicellular heads; secretory trichomes with unicellular stalks and usually tetracellular heads; diacytic stomata (2.8.3), which are more numerous on the lower epidermis.
- C. Thin-layer chromatography (2.2.27).
- Test solution.** Shake 1 g of the powdered drug (710) for 5 min with 10 ml of *methanol R* in a water-bath at 60 °C and filter the cooled solution.
- Reference solution.** Dissolve 1 mg of *sinensetin R* in *methanol R* and dilute to 20 ml with the same solvent.
- Plate:** TLC silica gel plate *R*.
- Mobile phase:** *methanol R*, *ethyl acetate R*, *toluene R* (5:40:55 V/V/V).
- Application:** 10 μl , as bands.
- Development:** over a path of 10 cm.
- Drying:** in air.
- Detection:** examine in ultraviolet light at 365 nm.
- Results:** see below the sequence of the zones present in the chromatogram obtained with the reference solution and the test solution. Furthermore, red fluorescent zones are present in the lower third and near the solvent front of the chromatogram obtained with the test solution.

Top of the plate	
	1 or 2 more or less intense blue to violet-blue fluorescent zones
Sinensetin: an intense light blue fluorescent zone	A major blue fluorescent zone (sinensetin)
	2 bluish fluorescent zones
Reference solution	Test solution

TESTS

Foreign matter (2.8.2): maximum 5 per cent of stems with a diameter greater than 1 mm; maximum 2 per cent of other foreign matter.

Loss on drying (2.2.32): maximum 11.0 per cent, determined on 1.000 g of the powdered drug (355) by drying in an oven at 100-105 °C for 2 h.

Total ash (2.4.16): maximum 12.5 per cent.

ASSAY

Liquid chromatography (2.2.29).

Test solution. Heat 2.5 g of the powdered drug (355) and 100 ml of *methylene chloride R* on a water-bath for 30 min with stirring. Filter. Collect the filtrate and repeat the operation twice, in the same manner, on the filtration residue. Combine the filtrates. Evaporate the solvent under reduced pressure. Dissolve the residue in 25.0 ml of the mobile phase, using an ultrasonic bath if necessary. Filter the solution through a nitrocellulose filter with a pore size of 0.45 μm .

Reference solution. In a 100.0 ml graduated flask dissolve 5 mg (m_2) of *sinensetin R* in 25.0 ml of the mobile phase and dilute to 100.0 ml with the mobile phase.

Column:

- size: $l = 0.25\text{ m}$, $\varnothing = 4.6\text{ mm}$,
- stationary phase: octadecylsilyl silica gel for chromatography *R* (5 μm).

Mobile phase: *tetrahydrofuran R*, *acetic acid R*, *water R*, *methanol R* (5:8:42:45 V/V/V/V).

Flow rate: 0.5 ml/min.

Detection: spectrophotometer at 258 nm.

Injection: 20 μl .

Calculate the percentage content of sinensetin from the expression:

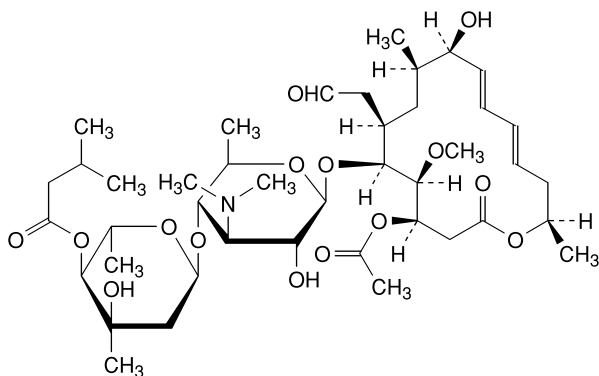
$$\frac{m_2 \times F_1 \times 25}{m_1 \times F_2}$$

- F_1 = area of the peak corresponding to sinensetin in the chromatogram obtained with the test solution,
- F_2 = area of the peak corresponding to sinensetin in the chromatogram obtained with the reference solution,
- m_1 = mass of the drug to be examined, in grams
- m_2 = mass of sinensetin in the reference solution, in grams.

01/2005:1983 **Specific optical rotation** (2.2.7): –65 to –75 (dried substance).

JOSAMYCIN

Josamycinum



$C_{42}H_{69}NO_{15}$

M_r 828

DEFINITION

Josamycin is a macrolide antibiotic produced by certain strains of *Streptomyces narbonensis* var. *josamyceticus* var. *nova*, or obtained by any other means. The main component is (4*R*,5*S*,6*S*,7*R*,9*R*,10*R*,11*E*,13*E*,16*R*)-4-(acetyloxy)-6-[[3,6-dideoxy-4-*O*-[2,6-dideoxy-3-*C*-methyl-4-*O*-(3-methylbutanoyl)-α-*L*-ribo-hexopyranosyl]-3-(dimethylamino)-β-*D*-glucopyranosyl]oxy]-10-hydroxy-5-methoxy-9,16-dimethyl-7-(2-oxoethyl)oxacyclohexadeca-11,13-dien-2-one.

Content: minimum 900 Ph. Eur. U./mg (dried substance).

CHARACTERS

Appearance: white or slightly yellowish powder, slightly hygroscopic.

Solubility: very slightly soluble in water, freely soluble in methanol and in methylene chloride, soluble in acetone.

IDENTIFICATION

First identification: A, B.

Second identification: B, C.

A. Dissolve 0.10 g in *methanol R* and dilute to 100.0 ml with the same solvent. Dilute 1.0 ml of the solution to 50.0 ml with *methanol R*. Examined between 220 nm and 350 nm (2.2.25), the solution shows an absorption maximum at 232 nm. The specific absorbance at the maximum is 330 to 370.

B. Examine the chromatograms obtained in the test for related substances.

Results: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a) and its position is different from that of the principal spot in the chromatograms obtained with reference solutions (d) and (e).

C. Dissolve about 10 mg in 5 ml of *hydrochloric acid R1* and allow to stand for 10-20 min. A pink colour develops, turning brown.

TESTS

Appearance of solution. The solution is clear (2.2.1) and not more intensely coloured than reference solution BY₄ (2.2.2, *Method II*).

Dissolve 1.0 g in *methanol R* and dilute to 10 ml with the same solvent.

Specific optical rotation (2.2.7): –65 to –75 (dried substance).

Dissolve 1.000 g in *methanol R* and dilute to 100.0 ml with the same solvent. Allow to stand for 30 min before measuring the angle of rotation.

Related substances. Thin-layer chromatography (2.2.27).

Test solution. Dissolve 0.10 g of the substance to be examined in *methanol R* and dilute to 10 ml with the same solvent.

Reference solution (a). Dissolve 0.10 g of *josamycin CRS* in *methanol R* and dilute to 10 ml with the same solvent.

Reference solution (b). Dilute 1 ml of reference solution (a) to 10 ml with *methanol R*.

Reference solution (c). Dilute 1 ml of reference solution (a) to 20 ml with *methanol R*.

Reference solution (d). Dissolve 0.10 g of *josamycin propionate CRS* in *methanol R* and dilute to 10 ml with the same solvent.

Reference solution (e). Dissolve 0.10 g of *spiramycin CRS* in *methylene chloride R* and dilute to 10 ml with the same solvent.

Reference solution (f). Dissolve 10 mg of *josamycin CRS* and 10 mg of *josamycin propionate CRS* in 1 ml of *methanol R*.

Plate: TLC silica gel G plate R.

Mobile phase: *methanol R*, *acetone R*, *ethyl acetate R*, *toluene R*, *hexane R* (8:10:20:25:30 V/V/V/V/V).

Application: 10 µl.

Development: over a path of 15 cm.

Drying: at 100 °C for 10 min.

Detection: spray with *dilute sulphuric acid R* and heat at 100 °C for 10 min.

System suitability: the chromatogram obtained with reference solution (f) shows 2 clearly separated principal spots.

Limits:

– **any impurity:** any spot, apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (b) (10 per cent) and not more than 1 such spot is more intense than the spot in the chromatogram obtained with reference solution (c) (5 per cent).

Heavy metals (2.4.8): maximum 30 ppm.

1.0 g complies with limit test C. Prepare the standard using 3 ml of *lead standard solution* (10 ppm Pb) R.

Loss on drying (2.2.32): maximum 1.0 per cent, determined on 1.000 g by drying in an oven *in vacuo* at 60 °C for 3 h.

Sulphated ash (2.4.14): maximum 0.2 per cent, determined on 1.0 g.

ASSAY

Dissolve 30.0 mg in 5 ml of *methanol R* and dilute to 100.0 ml with *water R*.

Carry out the microbiological assay of antibiotics (2.7.2).

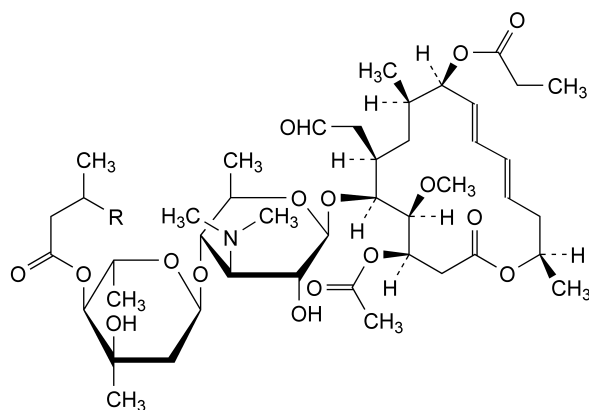
STORAGE

In an airtight container.

01/2005:1982

JOSAMYCIN PROPIONATE

Josamycini propionas



Leucomycin propionate	R	Mol. Formula	Mr
A3	CH ₃	C ₄₅ H ₇₃ NO ₁₆	884
A4	H	C ₄₄ H ₇₁ NO ₁₆	870

DEFINITION

Propionyl ester of a macrolide antibiotic produced by certain strains of *Streptomyces narbonensis* var. *josamyceticus* var. *nova*, or obtained by any other means. The main component is (4*R*,5*S*,6*S*,7*R*,9*R*,10*R*,11*E*,13*E*,16*R*)-4-(acetyloxy)-6-[[3,6-dideoxy-4-*O*-[2,6-dideoxy-3-*C*-methyl-4-*O*-(3-methylbutanoyl)-α-*L*-ribo-hexopyranosyl]-3-(dimethylamino)-β-*D*-glucopyranosyl]oxy]-5-methoxy-9,16-dimethyl-7-(2-oxoethyl)-10-(propanoyloxy)oxacyclohexadeca-11,13-dien-2-one propionate (leucomycin A3 propionate).

Content:

– minimum 843 Ph. Eur. U./mg (dried substance).

CHARACTERS

Appearance: white or slightly yellowish, crystalline, slightly hygroscopic powder.

Solubility: practically insoluble in water, freely soluble in methanol and in methylene chloride, soluble in acetone.

IDENTIFICATION

First identification: A, B.

Second identification: B, C.

Prepare solutions in methanol immediately before use.

A. Dissolve 0.10 g in *methanol R* and dilute to 100.0 ml with the same solvent. Dilute 1.0 ml of the solution to 50.0 ml with *methanol R*. Examined between 220 nm and 350 nm (2.2.25), the solution shows an absorption maximum at 231 nm. The specific absorbance at the absorption maximum is 310 to 350.

B. Thin-layer chromatography (2.2.27).

Test solution. Dissolve 10 mg of the substance to be examined in *methanol R* and dilute to 1 ml with the same solvent.

Reference solution (a). Dissolve 10 mg of *josamycin propionate CRS* in *methanol R* and dilute to 1 ml with the same solvent.

Reference solution (b). Dissolve 10 mg of *josamycin CRS* in *methanol R* and dilute to 1 ml with the same solvent.

Reference solution (c). Dissolve 10 mg of *spiramycin CRS* in *methylene chloride R* and dilute to 1 ml with the same solvent.

Reference solution (d). Mix 0.5 ml of reference solution (a) with 0.5 ml of reference solution (b).

Plate: TLC silica gel G plate *R*.

Mobile phase: *methanol R*, *acetone R*, *ethyl acetate R*, *toluene R*, *hexane R* (8:10:20:25:30 V/V/V/V/V).

Application: 10 µl.

Development: over 2/3 of the plate.

Drying: at 100 °C for 10 min.

Detection: spray with *dilute sulphuric acid R* and heat at 100 °C for 10 min.

System suitability: the chromatogram obtained with reference solution (d) shows 2 clearly separated principal spots.

Results: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a) and its position is different from that of the principal spot in the chromatograms obtained with reference solutions (b) and (c).

C. Dissolve about 10 mg in 5 ml of *hydrochloric acid R1* and allow to stand for 10-20 min. A pink colour develops, turning brown.

TESTS

Appearance of solution. The solution is clear (2.2.1) and not more intensely coloured than reference solution BY₄ (2.2.2, *Method II*).

Dissolve 1 g in *methanol R* and dilute to 10 ml with the same solvent.

Specific optical rotation (2.2.7): –65 to –75 (dried substance).

Dissolve 1.000 g in *methanol R* and dilute to 100.0 ml with the same solvent. Allow to stand for 30 min before measuring the angle of rotation.

Related substances. Liquid chromatography (2.2.29).

Test solution. Dissolve 50.0 mg of the substance to be examined in *acetonitrile for chromatography R* and dilute to 100.0 ml with the same solvent.

Reference solution (a). Dissolve 50.0 mg of *josamycin propionate CRS* in *acetonitrile for chromatography R* and dilute to 100.0 ml with the same solvent.

Reference solution (b). Dissolve 5 mg of the substance to be examined in 10 ml of *methanol R* and add 40 µl of *dilute phosphoric acid R*. Mix, allow to stand for 5 min and inject.

Reference solution (c). Dilute 2.0 ml of reference solution (a) to 100.0 ml with *acetonitrile for chromatography R*.

Column:

- size: *l* = 0.15 m, Ø = 3.9 mm,
- stationary phase: end-capped octadecylsilyl silica gel for chromatography *R* (5 µm),
- temperature: 30 °C.

Mobile phase: *acetonitrile R*, a 15.4 g/l solution of *ammonium acetate R* previously adjusted to pH 6.0 with *dilute phosphoric acid R* (60:40 V/V).

Flow rate: 1.0 ml/min.

Detection: spectrophotometer at 232 nm.

Injection: 20 µl of the test solution and reference solutions (b) and (c).

Run time: 3 times the retention time of leucomycin A3 propionate.

01/2005:1532

JUNIPER

Juniperi pseudo-fructus

Relative retention with reference to leucomycin A3 propionate (retention time = about 18 min): impurity E = about 0.2; impurity A = about 0.3; impurity B = about 0.5; leucomycin A4 propionate = about 0.7; impurity C = about 1.4; impurity D = about 2.0.

System suitability: reference solution (b):

- **resolution:** minimum 2.0 between the 2 peaks eluting with a relative retention with reference to leucomycin A3 propionate of about 0.5 and 0.7 respectively.

Limits:

- **impurity D:** not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (c),
- **impurities A, B, C, E:** for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (c),
- **any other impurity:** for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (c),
- **total:** not more than 7 times the area of the principal peak in the chromatogram obtained with reference solution (c),
- **disregard limit:** 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (c).

Loss on drying (2.2.32): maximum 1.0 per cent, determined on 1.000 g by drying in an oven *in vacuo* at 60 °C for 3 h.

Sulphated ash (2.4.14): maximum 0.2 per cent, determined on 1.0 g.

ASSAY

Dissolve 40.0 mg in 20 ml of *methanol R* and dilute to 100.0 ml with *phosphate buffer solution pH 5.6 R*.

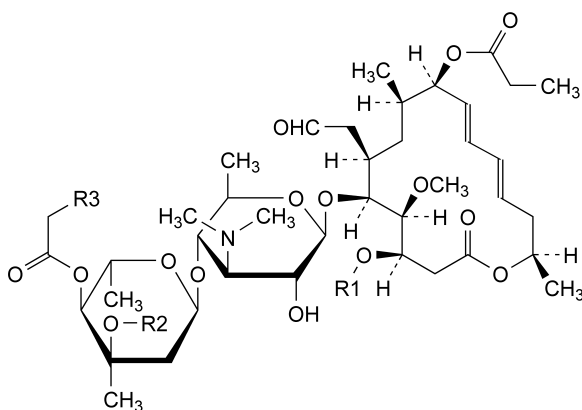
Carry out the microbiological assay of antibiotics (2.7.2).

STORAGE

In an airtight container.

IMPURITIES

Specified impurities: A, B, C, D, E.



- R1 = CO-CH₃, R2 = R3 = H: leucomycin A8 9-propionate,
- R1 = R2 = H, R3 = C₂H₅: leucomycin A5 9-propionate,
- R1 = CO-C₂H₅, R2 = H, R3 = CH(CH₃)₂: platenomycin A1 9-propionate,
- R1 = CO-CH₃, R2 = CO-C₂H₅, R3 = CH(CH₃)₂: leucomycin A3 3'',9-dipropionate,
- josamycin.

DEFINITION

Juniper consists of the dried ripe cone berry of *Juniperus communis* L. It contains not less than 10 ml/kg of essential oil, calculated with reference to the anhydrous drug.

CHARACTERS

Juniper has a strongly aromatic odour, especially if crushed. It has the macroscopic and microscopic characters described under identification tests A and B.

IDENTIFICATION

- The berry-shaped cone is globular up to 10 mm in diameter, violet-brown to blackish-brown, frequently with a bluish bloom. It consists of three fleshy scales. The apex has a three-rayed closed cleft and three not very clearly defined projections. A remnant of peduncle is frequently attached at the base. The fleshy part is crumbly and brownish. It contains three, seldom two, small, elongated, extremely hard seeds that have three sharp edges and are slightly rounded at the back, acuminate at the apex. The seeds are fused with the fleshy part of the cone berry in the lower part on the outside of their bases. Very large, oval oil glands containing sticky resin lie at the outer surface of the seeds.
- Reduce to a powder (355). The powder is brown. Examine under a microscope, using *chloral hydrate solution R*. The powder shows fragments of epidermis of the cone berry wall containing cells with thick, pitted colourless walls and brown glandular content, occasionally with anomocytic stomata (2.8.3); fragments of the three-rayed apical cleft of the cone berry with spaces and epidermal cells interlocked by papillous outgrowths; fragments of the hypodermis with collenchymatous thickened cells; fragments of the mesocarp consisting of large thin-walled parenchymatous cells, usually rounded, with large intercellular spaces and irregular, large, usually scarcely pitted, yellow idioblasts (barrel cells); fragments of schizogenous oil cells; fragments of the testa with thick-walled, pitted colourless sclereids containing one or several prism crystals of calcium oxalate; fragments of the endosperm and embryonic tissue with thin-walled cells containing fatty oil and aleurone grains.
- Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel plate R*.

Test solution. Dilute the oil-xylene mixture obtained in the assay to 5.0 ml with *hexane R*.

Reference solution. Dissolve 4.0 mg of *guaiazulene R* and 50 µl of *cineole R* in 10 ml of *hexane R*.

Apply to the plate as bands 20 µl of the test solution and 10 µl of the reference solution. Develop over a path of 15 cm using a mixture of 5 volumes of *ethyl acetate R* and 95 volumes of *toluene R*. Allow the plate to dry in air. Spray the plate with *anisaldehyde solution R* and examine in daylight while heating at 100 °C to 105 °C for 5 min to 10 min. The chromatogram obtained with the reference solution shows a red zone (guaiazulene) in the upper half and a brownish-violet to greyish-violet zone (cineole) in the lower half. The chromatogram obtained with the test solution shows a strong violet zone (mono- and sesquiterpenes) similar in position to the zone due to guaiazulene in the chromatogram obtained with the reference solution, a reddish-violet zone a little above

the zone corresponding to cineole in the chromatogram obtained with the reference solution, a greyish-violet zone (terpinen-4-ol) a little below the zone corresponding to cineole in the chromatogram obtained with the reference solution and just below it a blue zone. A faint violet zone may be present in a similar position to the zone corresponding to cineole. Further zones are present.

TESTS

Foreign matter (2.8.2). Not more than 5 per cent of unripe or discoloured cone berries and not more than 2 per cent of other foreign matter.

Water (2.2.13). Not more than 120 ml/kg, determined by distillation on 20.0 g of the crushed drug.

Total ash (2.4.16). Not more than 4.0 per cent.

ASSAY

Carry out the determination of essential oils in vegetable drugs (2.8.12). Use a 500 ml round-bottomed flask, 200 ml of *water R* as the distillation liquid and 0.5 ml of *xylene R* in the graduated tube. Crush the drug and immediately use 20.0 g for the determination. Distil at a rate of 3 ml/min to 4 ml/min for 90 min.

STORAGE

Store protected from light.

01/2005:1832

JUNIPER OIL

Iuniperi aetheroleum

DEFINITION

Essential oil obtained by steam distillation from the ripe, non-fermented berry cones of *Juniperus communis* L. A suitable antioxidant may be added.

CHARACTERS

Appearance: mobile, colourless to yellowish liquid, with a characteristic odour.

IDENTIFICATION

First identification: B.

Second identification: A.

A. Thin-layer chromatography (2.2.27).

Test solution. Dissolve 0.2 ml of the substance to be examined in 5 ml of *heptane R*.

Reference solution. Dissolve 20 mg of α -terpineol *R* and 20 μ l of *terpinen-4-ol R* in 25 ml of *heptane R*.

Plate: TLC silica gel plate *R*.

Mobile phase: *ethyl acetate R*, *toluene R* (5:95 V/V).

Application: 20 μ l, as bands.

Development: over a path of 12 cm.

Drying: in air.

Detection: spray with *anisaldehyde solution R* and heat at 100-105 °C until the zones appear. Examine immediately in daylight.

Results: see below the sequence of the zones present in the chromatograms obtained with the reference solution and the test solution.

Top of the plate	
Terpinen-4-ol: a brownish-violet zone	An intense brownish-violet zone
	A brown zone
	A violet-pink zone
	A brownish-violet zone (terpinen-4-ol)
α -Terpineol: a violet or brownish-violet zone	A violet zone
	A violet or brownish-violet zone (α -terpineol)
Reference solution	Test solution

B. Examine the chromatograms obtained in the test for chromatographic profile.

Results: the characteristic peaks in the chromatogram obtained with the test solution are similar in retention time to those in the chromatogram obtained with the reference solution.

TESTS

Relative density (2.2.5): 0.857 to 0.876.

Refractive index (2.2.6): 1.471 to 1.483.

Optical rotation (2.2.7): -15° to -0.5° .

Peroxide value (2.5.5): maximum 20.

Fatty oils and resinified essential oils (2.8.7). It complies with the test for fatty oils and resinified essential oils.

Chromatographic profile. Gas chromatography (2.2.28): use the normalisation procedure.

Test solution. Dissolve 60 mg of the substance to be examined in *trimethylpentane R* and dilute to 5.0 ml with the same solvent.

Reference solution. Mix 25 μ l each of α -pinene *R*, sabinene *R*, β -pinene *R*, β -myrcene *R*, α -phellandrene *R*, limonene *R*, *terpinen-4-ol R*, *bornyl acetate R* and β -caryophyllene *R* and dilute to 25.0 ml with *trimethylpentane R*.

Column:

- **material:** fused silica,
- **size:** $l = 30$ m (a film thickness of 1 μ m may be used) to 60 m (a film thickness of 0.2 μ m may be used), $\varnothing = 0.25$ -0.53 mm,
- **stationary phase:** *poly(dimethyl)(diphenyl)siloxane R*.

Carrier gas: *helium for chromatography R*.

Flow rate: 2.0 ml/min.

Split ratio: 1:50.

Temperature:

	Time (min)	Temperature (°C)
Column	0 - 1	60
	1 - 58	60→230
Injection port		250
Detector		250

Detection: flame ionisation.

Injection: 0.5 μ l.

Elution order: order indicated in the composition of the reference solution. Record the retention times of these substances.

System suitability: reference solution:

- **resolution:** minimum 1.5 between the peaks due to sabinene and β -pinene.

Using the retention times determined from the chromatogram obtained with the reference solution, locate the components of the reference solution in the chromatogram obtained with the test solution.

Determine the percentage content of the components. Disregard the peak due to trimethylpentane and peaks comprising less than 0.01 per cent of the total surface area. The percentages are within the following ranges:

- *α-pinene*: 20 per cent to 50 per cent,
- *sabinene*: less than 20 per cent,
- *β-pinene*: 1.0 per cent to 12 per cent
- *β-myrcene*: 1.0 per cent to 35 per cent,

- *α-phellandrene*: less than 1.0 per cent,
- *limonene*: 2.0 per cent to 12 per cent,
- *terpinen-4-ol*: 0.5 per cent to 10 per cent,
- *bornyl acetate*: less than 2.0 per cent,
- *β-caryophyllene*: less than 7.0 per cent.

STORAGE

In a well-filled, airtight container, protected from light, at a temperature not exceeding 25 °C.

LABELLING

The label states the name and concentration of any added antioxidant.

01/2005:0033

KANAMYCIN ACID SULPHATE

Kanamycini sulfas acidus

DEFINITION

Kanamycin acid sulphate is a form of kanamycin sulphate prepared by adding sulphuric acid to a solution of kanamycin monosulphate and drying by a suitable method. The potency is not less than 670 IU/mg, calculated with reference to the dried substance.

PRODUCTION

It is produced by methods of manufacture designed to eliminate or minimise substances lowering blood pressure. The method of manufacture is validated to demonstrate that the product if tested would comply with the following test:

Abnormal toxicity (2.6.9). Inject into each mouse 0.5 ml of a solution containing 2 mg per millilitre of the substance to be examined.

CHARACTERS

A white or almost white powder, hygroscopic, soluble in about 1 part of water, practically insoluble in acetone and in alcohol.

IDENTIFICATION

A. Examine by thin-layer chromatography (2.2.27), using a plate coated with a 0.75 mm layer of the following mixture: mix 0.3 g of *carbomer R* with 240 ml of *water R* and allow to stand, with moderate shaking, for 1 h; adjust to pH 7 by the gradual addition, with continuous shaking, of *dilute sodium hydroxide solution R* and add 30 g of *silica gel H R*.

Heat the plate at 110 °C for 1 h, allow to cool and use immediately.

Test solution. Dissolve 10 mg of the substance to be examined in *water R* and dilute to 10 ml with the same solvent.

Reference solution (a). Dissolve 10 mg of *kanamycin monosulphate CRS* in *water R* and dilute to 10 ml with the same solvent.

Reference solution (b). Dissolve 10 mg of *kanamycin monosulphate CRS*, 10 mg of *neomycin sulphate CRS* and 10 mg of *streptomycin sulphate CRS* in *water R* and dilute to 10 ml with the same solvent.

Apply separately to the plate 10 µl of each solution. Develop over a path of 12 cm using a 70 g/l solution of *potassium dihydrogen phosphate R*. Dry the plate in a current of warm air and spray with a mixture of equal volumes of a 2 g/l solution of *dihydroxynaphthalene R* in *alcohol R* and a 460 g/l solution of *sulphuric acid R*. Heat at 150 °C for 5 min to 10 min. The principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a). The test is not valid unless the chromatogram obtained with reference solution (b) shows 3 clearly separated spots.

B. Dissolve 0.5 g in 10 ml of *water R*. Add 10 ml of *picric acid solution R*. Initiate crystallisation if necessary by scratching the wall of the tube with a glass rod and allow to stand. Collect the crystals, wash with 20 ml of *water R* and filter. Dry at 100 °C. The crystals melt (2.2.14) at about 235 °C, with decomposition.

C. Dissolve about 50 mg in 2 ml of *water R*. Add 1 ml of a 10 g/l solution of *ninhydrin R* and heat for a few minutes on a water-bath. A violet colour develops.

D. It gives the reactions of sulphates (2.3.1).

TESTS

Solution S. Dissolve 0.20 g in *carbon dioxide-free water R* and dilute to 20.0 ml with the same solvent.

pH (2.2.3). The pH of solution S is 5.5 to 7.5.

Specific optical rotation (2.2.7). +103 to +115, determined on solution S and calculated with reference to the dried substance.

Kanamycin B. Examine by thin-layer chromatography (2.2.27), using a plate prepared as prescribed under identification test A.

Heat the plate at 110 °C for 1 h, allow to cool and use immediately.

Test solution. Dissolve 0.11 g of the substance to be examined in *water R* and dilute to 20 ml with the same solvent.

Reference solution. Dissolve 4 mg of *kanamycin B sulphate CRS* in *water R* and dilute to 20 ml with the same solvent.

Apply separately to the plate 4 µl of each solution. Develop over a path of 12 cm using a 70 g/l solution of *potassium dihydrogen phosphate R*. Dry the plate in a current of warm air and spray with *ninhydrin and stannous chloride reagent R*. Heat the plate at 110 °C for 15 min. Any spot corresponding to kanamycin B in the chromatogram obtained with the test solution is not more intense than the spot in the chromatogram obtained with the reference solution (4.0 per cent).

Loss on drying (2.2.32). Not more than 5.0 per cent, determined on 1.00 g by drying at 60 °C at a pressure not exceeding 670 Pa for 3 h.

Sulphated ash (2.4.14). Not more than 0.5 per cent, determined on 1.0 g.

Sulphate. 23.0 per cent to 26.0 per cent of sulphate (SO₄), calculated with reference to the dried substance. Dissolve 0.175 g in 100 ml of *water R* and adjust the solution to pH 11 using *concentrated ammonia R*. Add 10.0 ml of 0.1 M *barium chloride* and about 0.5 mg of *phthalein purple R*. Titrate with 0.1 M *sodium edetate* adding 50 ml of *alcohol R* when the colour of the solution begins to change and continue the titration until the violet-blue colour disappears. 1 ml of 0.1 M *barium chloride* is equivalent to 9.606 mg of sulphate (SO₄).

Pyrogens (2.6.8). If intended for use in the manufacture of parenteral dosage forms without a further appropriate procedure for the removal of pyrogens, it complies with the test for pyrogens. Inject per kilogram of the rabbit's mass 1 ml of a solution in *water for injections R* containing 10 mg per millilitre of the substance to be examined.

ASSAY

Carry out the microbiological assay of antibiotics (2.7.2). Use *kanamycin monosulphate CRS* as the reference substance.

STORAGE

If the substance is sterile, store in a sterile, tamper-proof container.

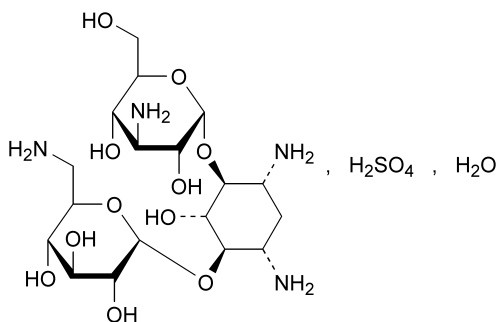
LABELLING

The label states, where applicable, that the substance is apyrogenic.

01/2005:0032

KANAMYCIN MONOSULPHATE

Kanamycini monosulfas

 $C_{18}H_{38}N_4O_{15}S \cdot H_2O$ M_r 601

DEFINITION

Kanamycin monosulphate is 6-*O*-(3-amino-3-deoxy- α -D-glucopyranosyl)-4-*O*-(6-amino-6-deoxy- α -D-glucopyranosyl)-2-deoxy-D-streptamine sulphate, an antimicrobial substance produced by the growth of certain strains of *Streptomyces kanamyceticus*. The potency is not less than 750 IU/mg, calculated with reference to the dried substance.

PRODUCTION

It is produced by methods of manufacture designed to eliminate or minimise substances lowering blood pressure. The method of manufacture is validated to demonstrate that the product if tested would comply with the following test:

Abnormal toxicity (2.6.9). Inject into each mouse 0.5 ml of a solution containing 2 mg per millilitre of the substance to be examined.

CHARACTERS

A white or almost white, crystalline powder, soluble in about 8 parts of water, practically insoluble in acetone and in alcohol.

IDENTIFICATION

A. Examine by thin-layer chromatography (2.2.27), using a plate coated with a 0.75 mm layer of the following mixture: mix 0.3 g of *carbomer R* with 240 ml of *water R* and allow to stand, with moderate shaking, for 1 h; adjust to pH 7 by the gradual addition, with continuous shaking, of *dilute sodium hydroxide solution R* and add 30 g of *silica gel H R*.

Heat the plate at 110 °C for 1 h, allow to cool and use immediately.

Test solution. Dissolve 10 mg of the substance to be examined in *water R* and dilute to 10 ml with the same solvent.

Reference solution (a). Dissolve 10 mg of *kanamycin monosulphate CRS* in *water R* and dilute to 10 ml with the same solvent.

Reference solution (b). Dissolve 10 mg of *kanamycin monosulphate CRS*, 10 mg of *neomycin sulphate CRS* and 10 mg of *streptomycin sulphate CRS* in *water R* and dilute to 10 ml with the same solvent.

Apply separately to the plate 10 μ l of each solution. Develop over a path of 12 cm using a 70 g/l solution of *potassium dihydrogen phosphate R*. Dry the plate in a current of warm air and spray with a mixture of equal volumes of a 2 g/l solution of

dihydroxynaphthalene R in *alcohol R* and a 460 g/l solution of *sulphuric acid R*. Heat at 150 °C for 5 min to 10 min. The principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a). The test is not valid unless the chromatogram obtained with reference solution (b) shows three clearly separated spots.

B. Dissolve 0.5 g in 10 ml of *water R*. Add 10 ml of *picric acid solution R*. Initiate crystallisation if necessary by scratching the wall of the tube with a glass rod and allow to stand. Collect the crystals, wash with 20 ml of *water R* and filter. Dry at 100 °C. The crystals melt (2.2.14) at about 235 °C, with decomposition.

C. Dissolve about 50 mg in 2 ml of *water R*. Add 1 ml of a 10 g/l solution of *ninhydrin R* and heat for a few minutes on a water-bath. A violet colour develops.

D. It gives the reactions of sulphates (2.3.1).

TESTS

Solution S. Dissolve 0.20 g in *carbon dioxide-free water R* and dilute to 20.0 ml with the same solvent.

pH (2.2.3). The pH of solution S is 6.5 to 8.5.

Specific optical rotation (2.2.7). +112 to +123, determined on solution S and calculated with reference to the dried substance.

Kanamycin B. Examine by thin-layer chromatography (2.2.27), using a plate prepared as prescribed under identification test A. Heat the plate at 110 °C for 1 h, allow to cool and use immediately.

Test solution. Dissolve 0.1 g of the substance to be examined in *water R* and dilute to 20 ml with the same solvent.

Reference solution. Dissolve 4 mg of *kanamycin B sulphate CRS* in *water R* and dilute to 20 ml with the same solvent.

Apply separately to the plate 4 μ l of each solution. Develop over a path of 12 cm using a 70 g/l solution of *potassium dihydrogen phosphate R*. Dry the plate in a current of warm air and spray with *ninhydrin and stannous chloride reagent R*. Heat the plate at 110 °C for 15 min. Any spot corresponding to kanamycin B in the chromatogram obtained with the test solution is not more intense than the spot in the chromatogram obtained with the reference solution.

Loss on drying (2.2.32). Not more than 1.5 per cent, determined on 1.00 g by drying at 60 °C at a pressure not exceeding 670 Pa for 3 h.

Sulphated ash (2.4.14). Not more than 0.5 per cent, determined on 1.0 g.

Sulphate. 15.0 per cent to 17.0 per cent of sulphate (SO_4), calculated with reference to the dried substance. Dissolve 0.250 g in 100 ml of *water R* and adjust the solution to pH 11 using *concentrated ammonia R*. Add 10.0 ml of 0.1 M *barium chloride* and about 0.5 mg of *phthalein purple R*. Titrate with 0.1 M *sodium edetate* adding 50 ml of *alcohol R* when the colour of the solution begins to change and continue the titration until the violet-blue colour disappears. 1 ml of 0.1 M *barium chloride* is equivalent to 9.606 mg of sulphate (SO_4).

Pyrogens (2.6.8). If intended for use in the manufacture of parenteral dosage forms without a further appropriate procedure for the removal of pyrogens, it complies with the test for pyrogens. Inject per kilogram of the rabbit's mass 1 ml of a solution in *water for injections R* containing 10 mg per millilitre of the substance to be examined.

ASSAY

Carry out the microbiological assay of antibiotics (2.7.2).

STORAGE

If the substance is sterile, store in a sterile, tamper-proof container.

LABELLING

The label states, where applicable, that the substance is apyrogenic.

01/2005:0503

KAOLIN, HEAVY

Kaolinum ponderosum

DEFINITION

Heavy kaolin is a purified, natural, hydrated aluminium silicate of variable composition.

CHARACTERS

A fine, white or greyish-white, unctuous powder, practically insoluble in water and in organic solvents.

IDENTIFICATION

- A. To 0.5 g in a metal crucible add 1 g of *potassium nitrate R* and 3 g of *sodium carbonate R* and heat until the mixture melts. Allow to cool. To the residue add 20 ml of boiling *water R*, mix and filter. Wash the residue with 50 ml of *water R*. To the residue add 1 ml of *hydrochloric acid R* and 5 ml of *water R*. Filter. To the filtrate add 1 ml of *strong sodium hydroxide solution R* and filter. To the filtrate add 3 ml of *ammonium chloride solution R*. A gelatinous white precipitate is formed.
- B. Add 2.0 g in twenty portions to 100 ml of a 10 g/l solution of *sodium laurilsulfate R* in a 100 ml graduated cylinder about 30 mm in diameter. Allow 2 min between additions for each portion to settle. Allow to stand for 2 h. The apparent volume of the sediment is not greater than 5 ml.
- C. 0.25 g gives the reaction of silicates (2.3.1).

TESTS

Solution S. To 4 g add a mixture of 6 ml of *acetic acid R* and 34 ml of *distilled water R*, shake for 1 min and filter.

Acidity or alkalinity. To 1.0 g add 20 ml of *carbon dioxide-free water R*, shake for 2 min and filter. To 10 ml of the filtrate add 0.1 ml of *phenolphthalein solution R*. The solution is colourless. Not more than 0.25 ml of 0.01 M *sodium hydroxide* is required to change the colour of the indicator to pink.

Organic impurities. Heat 0.3 g to redness in a calcination tube. The residue is only slightly more coloured than the original substance.

Adsorption power. To 1.0 g in a ground-glass-stoppered test-tube add 10.0 ml of a 3.7 g/l solution of *methylene blue R* and shake for 2 min. Allow to settle. Centrifuge and dilute the solution 1 in 100 with *water R*. The solution is not more intensely coloured than a 0.03 g/l solution of *methylene blue R*.

Swelling power. Triturate 2 g with 2 ml of *water R*. The mixture does not flow.

Substances soluble in mineral acids. To 5.0 g add 7.5 ml of *dilute hydrochloric acid R* and 27.5 ml of *water R* and boil for 5 min. Filter, wash the residue on the filter with *water R* and dilute the combined filtrate and washings to 50.0 ml

with *water R*. To 10.0 ml of the solution add 1.5 ml of *dilute sulphuric acid R*, evaporate to dryness on a water-bath and ignite. The residue weighs not more than 10 mg (1 per cent).

Chlorides (2.4.4). 2 ml of solution S diluted to 15 ml with *water R* complies with the limit test for chlorides (250 ppm).

Sulphates (2.4.13). 1.5 ml of solution S diluted to 15 ml with *distilled water R* complies with the limit test for sulphates (0.1 per cent).

Calcium (2.4.3). 4 ml of solution S diluted to 15 ml with *distilled water R* complies with the limit test for calcium (250 ppm).

Heavy metals (2.4.8). To 5 ml of the solution prepared for the test for substances soluble in mineral acids add 5 ml of *water R*, 10 ml of *hydrochloric acid R* and 25 ml of *methyl isobutyl ketone R*. Shake for 2 min. Separate the layers. Evaporate the aqueous layer to dryness on a water-bath. Dissolve the residue in 1 ml of *acetic acid R* and dilute to 25 ml with *water R*. Filter. 12 ml of the solution complies with limit test A for heavy metals (50 ppm) Prepare the standard using *lead standard solution (1 ppm Pb) R*.

Heavy kaolin intended for internal use complies with the requirements of the monograph modified as shown below for the test for heavy metals.

Heavy metals (2.4.8). To 10 ml of the solution prepared for the test for substances soluble in mineral acids add 10 ml of *water R*, 20 ml of *hydrochloric acid R* and 25 ml of *methyl isobutyl ketone R*. Shake for 2 min. Separate the layers. Evaporate the aqueous layer to dryness on a water-bath. Dissolve the residue in 1 ml of *acetic acid R* and dilute to 25 ml with *water R*. Filter. 12 ml of the solution complies with limit test A for heavy metals (25 ppm). Prepare the standard using *lead standard solution (1 ppm Pb) R*.

Microbial contamination. Total viable aerobic count (2.6.12) not more than 10^3 micro-organisms per gram, determined by plate-count.

LABELLING

The label states, where applicable, that the substance is suitable for internal use.

01/2005:1426

KELP

Fucus vel Ascophyllum

DEFINITION

Fragmented dried thallus of *Fucus vesiculosus* L. or *F. serratus* L. or *Ascophyllum nodosum* Le Jolis.

Content: minimum 0.03 per cent and maximum 0.2 per cent of total iodine (A_1 126.9) (dried drug).

CHARACTERS

Salty and mucilaginous taste, unpleasant marine odour.

Macroscopic and microscopic characters described under identification tests A and B.

IDENTIFICATION

- A. The drug consists of fragments with a corneous consistency, blackish-brown to greenish-brown, sometimes covered with whitish efflorescence. The thallus consists of a ribbon-like blade, branching dichotomously with prominent central ribs (pseudoveins). *F. vesiculosus* typically shows a foliose blade with smooth edges and bears occasional ovoid, single or paired, air vesicles. The ends of certain branches are of ovoid shape and a little widened. They bear numerous reproductive

01/2005:1020

organs (conceptacles). *F. serratus* has a foliose blade with a serrate margin and no vesicles, the branches bearing conceptacles are less swollen. The thallus of *A. nodosum* is irregularly branched, without pseudo-midrib. It shows single ovoid air vesicles; the falciform conceptacles are located at the end of small branches.

- B. Reduce to a powder (355). The powder is greenish-brown. Examine under a microscope using *chloral hydrate solution R*. The powder shows fragments of surface tissue with regular isodiametric cells with brown contents, and fragments of deep tissue with colourless, elongated cells arranged in long filaments with large mucilaginous spaces between them. Thick-walled cells in files and in closely packed groups, from the pseudovein, are sometimes visible.
- C. To 1 g of the powdered drug (355) add 20 ml of a 2 per cent V/V solution of *hydrochloric acid R*. Shake vigorously and filter. Wash the residue with 10 ml of *water R* and filter. To the residue add 10 ml of a 200 g/l solution of *sodium carbonate R*. Shake and centrifuge. Collect the supernatant liquid. Adjust to pH 1.5 using *sulphuric acid R*. A white, flocculent precipitate is slowly formed.

TESTS

Foreign matter (2.8.2): maximum 2 per cent *m/m*.

Arsenic (2.4.27): maximum 90 ppm.

Cadmium (2.4.27): maximum 4 ppm.

Lead (2.4.27): maximum 5 ppm.

Mercury (2.4.27): maximum 0.1 ppm.

Swelling index (2.8.4): minimum 6.

Loss on drying (2.2.32): maximum 15.0 per cent, determined on 1.000 g by drying in an oven at 100–105 °C, for 2 h.

Total ash (2.4.16): maximum 24 per cent.

Ash insoluble in hydrochloric acid (2.8.1): maximum 3.0 per cent.

ASSAY

TOTAL IODINE. To 1.000 g of the powdered drug, in a tall silica crucible, add 5 ml of *water R* and 5 g of *potassium hydroxide R*. Stir with a magnesium rod. Heat on a water bath. Add 1 g of *potassium carbonate R*. Mix, add the tip of the magnesium rod with the residues of the drug and dry, first on a water-bath then over an open flame. Incinerate raising the temperature progressively to not more than 600 °C. Allow to cool. Add 20 ml of *water R* and heat gently to boiling, stirring with a glass rod. Filter the hot mixture through an unpleated filter, into a conical flask. Rinse the residue with 4 quantities, each of 20 ml, of hot *water R*. Rinse the filter and the crucible with 50 ml of hot *water R*. Combine the solutions. Allow to cool. Neutralise with *dilute sulphuric acid R* in the presence of *methyl orange solution R*. Add 3 ml of *dilute sulphuric acid R* and 1 ml of *bromine water R*. The solution is yellow. After 5 min add 0.6 ml of a 50 g/l solution of *phenol R*. The solution is clear. Acidify with 5 ml of *phosphoric acid R* and add 0.2 g of *potassium iodide R*. Allow to stand for 5 min protected from light. Add 1 ml of *starch solution R* and titrate with 0.01 M *sodium thiosulphate*.

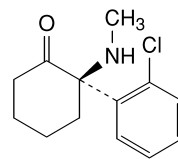
1 ml of 0.01 M *sodium thiosulphate* is equivalent to 0.2115 mg of iodine.

LABELLING

The label states the species of kelp present.

KETAMINE HYDROCHLORIDE

Ketamini hydrochloridum



and enantiomer, HCl

$C_{13}H_{17}Cl_2NO$

M_r 274.2

DEFINITION

(*RS*)-2-(2-Chlorophenyl)-2-(methyamino)cyclohexanone hydrochloride.

Content: 99.0 per cent to 101.0 per cent.

CHARACTERS

Appearance: white, crystalline powder.

Solubility: freely soluble in water and in methanol, soluble in alcohol.

mp: about 260 °C, with decomposition.

IDENTIFICATION

A. Optical rotation (see Tests).

B. Infrared absorption spectrophotometry (2.2.24).

Comparison: Ph. Eur. reference spectrum of ketamine hydrochloride.

C. It gives reaction (a) of chlorides (2.3.1).

TESTS

Solution S. Dissolve 5.0 g in *carbon dioxide-free water R* and dilute to 25.0 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and colourless (2.2.2, *Method II*).

pH (2.2.3): 3.5 to 4.1

Dilute 10 ml of solution S to 20 ml with *carbon dioxide-free water R*.

Optical rotation (2.2.7): -0.2° to $+0.2^\circ$.

Dilute 2.5 ml of solution S to 25.0 ml with *water R*.

Related substances. Liquid chromatography (2.2.29).

Test solution. Dissolve 50.0 mg of the substance to be examined in the mobile phase and dilute to 50.0 ml with the mobile phase.

Reference solution (a). Dissolve 25.0 mg of *ketamine impurity A CRS* in the mobile phase and dilute to 50.0 ml with the mobile phase (using ultrasound, if necessary). To 1.0 ml of the solution, add 0.5 ml of the test solution and dilute to 100.0 ml with the mobile phase. Prepare immediately before use.

Reference solution (b). Dilute 1.0 ml of the test solution to 10.0 ml with the mobile phase. Dilute 1.0 ml of the solution to 20.0 ml with the mobile phase.

Precolumn:

— **size:** $l = 4$ mm, $\varnothing = 4.0$ mm,

— **stationary phase:** spherical octadecylsilyl silica gel for chromatography R (5 μ m).

Column:

— **size:** $l = 0.125$ m, $\varnothing = 4.0$ mm,

— **stationary phase:** spherical octadecylsilyl silica gel for chromatography R (5 μ m).

01/2005:1746

Mobile phase: dissolve 0.95 g of sodium hexanesulphonate *R* in 1 litre of a mixture of 25 volumes of acetonitrile *R* and 75 volumes of water *R* and add 4 ml of acetic acid *R*.

Flow rate: 1.0 ml/min.

Detection: spectrophotometer at 215 nm.

Injection: 20 µl.

Run time: 10 times the retention time of ketamine.

System suitability: reference solution (a):

- **retention time:** ketamine = 3 min to 4.5 min,
- **resolution:** minimum of 1.5 between the peaks due to impurity A and to ketamine.

Limits:

- **total:** not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent),
- **disregard limit:** 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent).

Heavy metals (2.4.8): maximum 20 ppm.

Dilute 10 ml of solution S to 20 ml with water *R*. 12 ml of the solution complies with limit test A. Prepare the standard using lead standard solution (2 ppm Pb) *R*.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

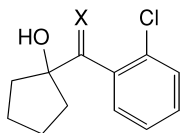
Dissolve 0.200 g in 50 ml of methanol *R* and add 1.0 ml of 0.1 M hydrochloric acid. Carry out a potentiometric titration (2.2.20), using 0.1 M sodium hydroxide. Read the volume added between the 2 points of inflexion.

1 ml of 0.1 M sodium hydroxide is equivalent to 27.42 mg of C₁₅H₁₇Cl₂NO.

STORAGE

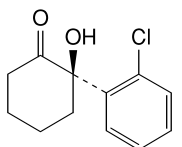
Protected from light.

IMPURITIES



A. X = N-CH₃: 1-[(2-chlorophenyl)(methylimino)methyl]cyclopentanol,

C. X = O: (2-chlorophenyl)(1-hydroxycyclopentyl)methanone,

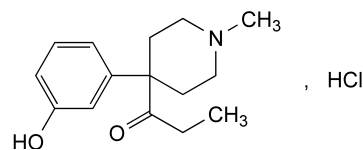


and enantiomer

B. (2*RS*)-2-(2-chlorophenyl)-2-hydroxycyclohexanone.

KETOBEMIDONE HYDROCHLORIDE

Cetobemidoni hydrochloridum



C₁₅H₂₂ClNO₂

M_r 283.8

DEFINITION

1-[4-(3-Hydroxyphenyl)-1-methylpiperidin-4-yl]propan-1-one hydrochloride.

Content: 99.0 per cent to 101.0 per cent (anhydrous substance).

CHARACTERS

Appearance: white or almost white, crystalline powder.

Solubility: freely soluble in water, soluble in alcohol, very slightly soluble in methylene chloride.

IDENTIFICATION

A. Infrared absorption spectrophotometry (2.2.24).

Comparison: *Ph. Eur.* reference spectrum of ketobemidone hydrochloride.

B. Solution S (see Tests) gives reaction (a) of chlorides (2.3.1).

TESTS

Solution S. Dissolve 0.250 g in carbon dioxide-free water *R* and dilute to 25.0 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and not more intensely coloured than reference solution B₈ (2.2.2, Method II).

pH (2.2.3): 4.5 to 5.5 for solution S.

Related substances. Liquid chromatography (2.2.29).

Solution A: 1.54 g/l solution of ammonium acetate *R* adjusted to pH 8.0 with dilute ammonia *R*1.

Test solution. Dissolve 50.0 mg of the substance to be examined in solution A and dilute to 25.0 ml with the same solution.

Reference solution (a). Dissolve 1 mg of ketobemidone impurity B CRS and 1 mg of ketobemidone impurity C CRS in solution A and dilute to 25 ml with the same solution.

Reference solution (b). Dilute 1.0 ml of the test solution to 100.0 ml with solution A. Dilute 20.0 ml of this solution to 100.0 ml with solution A.

Column:

- **size:** *l* = 0.25 m, Ø = 4.6 mm,
- **stationary phase:** phenylhexylsilyl silica gel for chromatography *R* (5 µm),
- **temperature:** 40 °C.

Mobile phase: acetonitrile *R*, solution A (20:80 V/V).

Flow rate: 1.5 ml/min.

Detection: spectrophotometer at 278 nm.

Injection: 20 µl.

Run time: 4.5 times the retention time of ketobemidone.

Relative retention with reference to ketobemidone (retention time = about 10 min): impurity A = about 0.4; impurity B = about 0.6; impurity C = about 0.7; impurity D = about 3.5; impurity E = about 4.2.

System suitability: reference solution (a):

- **resolution:** minimum 4.0 between the peaks due to impurity B and impurity C.

Limits:

- **impurities A, B, C, D:** for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent),
- **any other impurity:** for each impurity, not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent),
- **total:** not more than 3.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.7 per cent),
- **disregard limit:** 0.25 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

Water (2.5.12): maximum 1.0 per cent, determined on 0.50 g.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

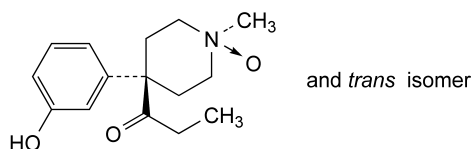
Dissolve 0.200 g in a mixture of 5.0 ml of 0.01 M hydrochloric acid and 50 ml of alcohol R. Carry out a potentiometric titration (2.2.20) using 0.1 M sodium hydroxide. Read the volume added between the 2 points of inflexion.

1 ml of 0.1 M sodium hydroxide is equivalent to 28.38 mg of $C_{15}H_{22}ClNO_2$.

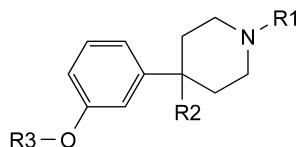
IMPURITIES

Specified impurities: A, B, C, D.

Other detectable impurities: E.



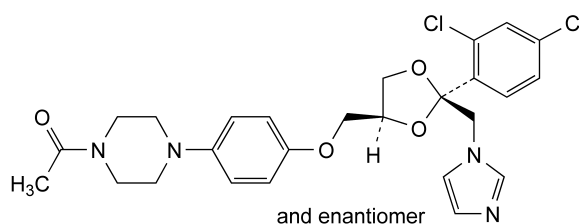
- A. 1-[4-(3-hydroxyphenyl)-1-methyl-1-oxopiperidin-4-yl]propan-1-one (*cis* and *trans* isomers),



- B. R1 = CH₃, R2 = CO-CH₃, R3 = H: 1-[4-(3-hydroxyphenyl)-1-methylpiperidin-4-yl]ethanone,
- C. R1 = R3 = H, R2 = CO-CH₂-CH₃: 1-[4-(3-hydroxyphenyl)piperidin-4-yl]propan-1-one,
- D. R1 = R3 = CH₃, R2 = CO-CH₂-CH₃: 1-[4-(3-methoxyphenyl)-1-methylpiperidin-4-yl]propan-1-one,
- E. R1 = CH₃, R2 = CN, R3 = H: 4-(3-hydroxyphenyl)-1-methylpiperidin-4-carbonitrile.

KETOCONAZOLE

Ketoconazolum



$C_{26}H_{28}Cl_2N_4O_4$

M_r 531.4

DEFINITION

Ketoconazole contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of 1-acetyl-4-[4-[[[(2*RS*,4*SR*)-2-(2,4-dichlorophenyl)-2-(1*H*-imidazol-1-ylmethyl)-1,3-dioxolan-4-yl]methoxy]phenyl]piperazine, calculated with reference to the dried substance.

CHARACTERS

A white or almost white powder, practically insoluble in water, freely soluble in methylene chloride, soluble in methanol, sparingly soluble in alcohol.

IDENTIFICATION

First identification: B.

Second identification: A, C, D.

- A. Melting point (2.2.14): 148 °C to 152 °C.
- B. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with ketoconazole CRS. Examine the substances prepared as discs.
- C. Examine by thin-layer chromatography (2.2.27), using a suitable octadecylsilyl silica gel as the coating substance.

Test solution. Dissolve 30 mg of the substance to be examined in the mobile phase and dilute to 5 ml with the mobile phase.

Reference solution (a). Dissolve 30 mg of ketoconazole CRS in the mobile phase and dilute to 5 ml with the mobile phase.

Reference solution (b). Dissolve 30 mg of ketoconazole CRS and 30 mg of econazole nitrate CRS in the mobile phase and dilute to 5 ml with the mobile phase.

Apply separately to the plate 5 µl of each solution. Develop over a path of 15 cm using a mixture of 20 volumes of ammonium acetate solution R, 40 volumes of dioxan R and 40 volumes of methanol R. Dry the plate in a current of warm air for 15 min and expose it to iodine vapour until the spots appear. Examine in daylight. The principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a). The test is not valid unless the chromatogram obtained with reference solution (b) shows two clearly separated spots.

- D. To about 30 mg in a porcelain crucible add 0.3 g of anhydrous sodium carbonate R. Heat over an open flame for 10 min. Allow to cool. Take up the residue with 5 ml of dilute nitric acid R and filter. To 1 ml of the filtrate add 1 ml of water R. The solution gives reaction (a) of chlorides (2.3.1).

TESTS

Solution S. Dissolve 1.0 g in *methylene chloride R* and dilute to 10 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and not more intensely coloured than reference solution BY₄ (2.2.2, Method II).

Optical rotation (2.2.7). The angle of optical rotation, determined on solution S, is -0.10° to $+0.10^{\circ}$.

Related substances. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 0.100 g of the substance to be examined in *methanol R* and dilute to 10.0 ml with the same solvent.

Reference solution (a). Dissolve 2.5 mg of *ketoconazole CRS* and 2.5 mg of *loperamide hydrochloride CRS* in *methanol R* and dilute to 50.0 ml with the same solvent.

Reference solution (b). Dilute 5.0 ml of the test solution to 100.0 ml with *methanol R*. Dilute 1.0 ml of this solution to 10.0 ml with *methanol R*.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.10 m long and 4.6 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (3 μ m),
- as mobile phase at a flow rate of 2 ml/min a mixture of 0.5 volumes of *acetonitrile R* and 9.5 volumes of a 3.4 g/l solution of *tetrabutylammonium hydrogen sulphate R* changing by linear-gradient elution to a mixture of 5 volumes of *acetonitrile R* and 5 volumes of a 3.4 g/l solution of *tetrabutylammonium hydrogen sulphate R* over 10 min, followed by the final elution mixture for 5 min,
- as detector a spectrophotometer set at 220 nm.

Equilibrate the column for at least 30 min with *acetonitrile R* and then equilibrate at the initial elution composition for at least 5 min.

Adjust the sensitivity of the system so that the height of the principal peak in the chromatogram obtained with 10 μ l of reference solution (b) is at least 50 per cent of the full scale of the recorder.

Inject 10 μ l of reference solution (a). When the chromatograms are recorded in the prescribed conditions, the retention times are: ketoconazole about 6 min and loperamide hydrochloride about 8 min. The test is not valid unless the resolution between the peaks corresponding to ketoconazole and loperamide hydrochloride is at least 15. If necessary, adjust the final concentration of acetonitrile in the mobile phase or adjust the time programme for the linear-gradient elution.

Inject separately 10 μ l of *methanol R* as a blank, 10 μ l of the test solution and 10 μ l of reference solution (b). In the chromatogram obtained with the test solution, the sum of the areas of all peaks, apart from the principal peak, is not greater than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent). Disregard any peak obtained with the blank and any peak with an area less than 0.1 times that of the principal peak in the chromatogram obtained with reference solution (b).

Heavy metals (2.4.8). 1.0 g complies with limit test D for heavy metals (20 ppm). Prepare the standard using 2 ml of *lead standard solution (10 ppm Pb) R*.

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

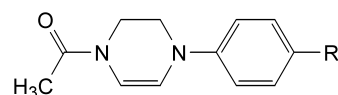
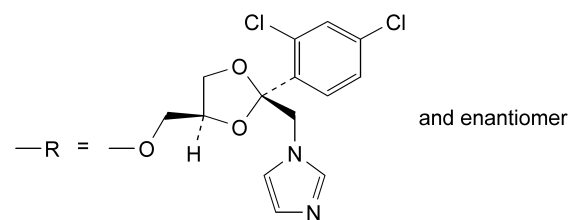
Dissolve 0.200 g in 70 ml of a mixture of 1 volume of *anhydrous acetic acid R* and 7 volumes of *methyl ethyl ketone R*. Titrate with 0.1 M *perchloric acid*, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *perchloric acid* is equivalent to 26.57 mg of C₂₆H₂₈Cl₂N₄O₄.

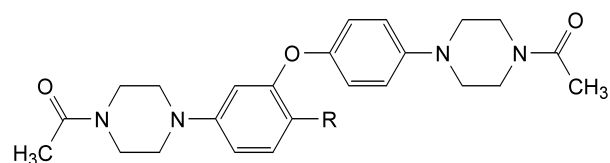
STORAGE

Store protected from light.

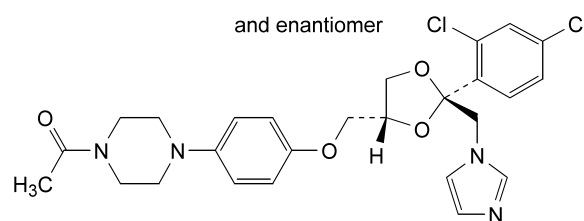
IMPURITIES



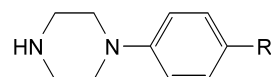
- A. 1-acetyl-4-[4-[(2RS,4SR)-2-(2,4-dichlorophenyl)-2-(1H-imidazol-1-ylmethyl)-1,3-dioxolan-4-yl]methoxy]phenyl]-1,2,3,4-tetrahydropyrazine,



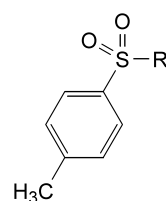
- B. 1-acetyl-4-[4-[(2RS,4SR)-2-(2,4-dichlorophenyl)-2-(1H-imidazol-1-ylmethyl)-1,3-dioxolan-4-yl]methoxy]-3-[4-(4-acetylpiperazin-1-yl)phenoxy]phenyl]piperazine,



- C. 1-acetyl-4-[4-[(2RS,4SR)-2-(2,4-dichlorophenyl)-2-(1H-imidazol-1-ylmethyl)-1,3-dioxolan-4-yl]methoxy]phenyl]piperazine,

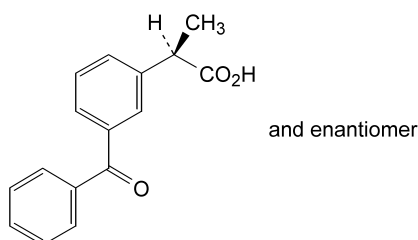


- D. 1-[4-[(2RS,4SR)-2-(2,4-dichlorophenyl)-2-(1H-imidazol-1-ylmethyl)-1,3-dioxolan-4-yl]methoxy]phenyl]piperazine.



- E. [(2RS,4SR)-2-(2,4-dichlorophenyl)-2-(1H-imidazol-1-ylmethyl)-1,3-dioxolan-4-yl]methyl 4-methylbenzenesulphonate.

01/2005:0922 TESTS

KETOPROFEN**Ketoprofenum** $C_{16}H_{14}O_3$ M_r 254.3**DEFINITION**

Ketoprofen contains not less than 99.0 per cent and not more than the equivalent of 100.5 per cent of (2*RS*)-2-(3-benzoylphenyl)propanoic acid, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, crystalline powder, practically insoluble in water, freely soluble in acetone, in ethanol (96 per cent) and in methylene chloride.

IDENTIFICATION

First identification: C.

Second identification: A, B, D.

- A. Melting point (2.2.14): 94 °C to 97 °C.
- B. Dissolve 50.0 mg in *ethanol (96 per cent) R* and dilute to 100.0 ml with the same solvent. Dilute 1.0 ml to 50.0 ml with *ethanol (96 per cent) R*. Examined between 230 nm and 350 nm (2.2.25), the solution shows an absorption maximum at 255 nm. The specific absorbance at the absorption maximum is 615 to 680.
- C. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *ketoprofen CRS*.
- D. Examine by thin-layer chromatography (2.2.27), using *silica gel GF₂₅₄ R* as the coating substance.

Test solution. Dissolve 10 mg of the substance to be examined in *acetone R* and dilute to 10 ml with the same solvent.

Reference solution (a). Dissolve 10 mg of *ketoprofen CRS* in *acetone R* and dilute to 10 ml with the same solvent.

Reference solution (b). Dissolve 10 mg of *indometacin CRS* in *acetone R* and dilute to 10 ml with the same solvent. To 1 ml of the solution add 1 ml of reference solution (a).

Apply separately to the plate 10 µl of each solution. Develop over a path of 15 cm using a mixture of 1 volume of *glacial acetic acid R*, 49 volumes of *methylene chloride R* and 50 volumes of *acetone R*. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. The principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a). The test is not valid unless the chromatogram obtained with reference solution (b) shows 2 clearly separated principal spots.

Appearance of solution. Dissolve 1.0 g in *acetone R* and dilute to 10 ml with the same solvent. The solution is clear (2.2.1) and not more intensely coloured than reference solution Y_6 (2.2.2, *Method II*).

Related substances. Prepare the solutions immediately before use. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 20.0 mg of the substance to be examined in the mobile phase and dilute to 20.0 ml with the mobile phase.

Reference solution (a). Dilute 1.0 ml of the test solution to 50.0 ml with the mobile phase. Dilute 1.0 ml to 10.0 ml with the mobile phase.

Reference solution (b). Dissolve 5.0 mg of *ketoprofen impurity A CRS* in the mobile phase and dilute to 50.0 ml with the mobile phase. Dilute 1.0 ml to 50.0 ml with the mobile phase.

Reference solution (c). Dissolve 5.0 mg of *ketoprofen impurity C CRS* in the mobile phase and dilute to 50.0 ml with the mobile phase. Dilute 1.0 ml to 50.0 ml with the mobile phase.

Reference solution (d). Dilute 1.0 ml of the test solution to 100.0 ml with the mobile phase. To 1.0 ml add 1.0 ml of reference solution (b).

The chromatographic procedure may be carried out using:

- a stainless steel column 0.15 m long and 4.6 mm in internal diameter packed with a spherical *octadecylsilyl silica gel for chromatography R* (5 µm) with a specific surface area of 350 m²/g and a pore size of 10 nm,
- as mobile phase at a flow rate of 1 ml/min a mixture of 2 volumes of *phosphate buffer solution pH 3.5 R*, freshly prepared, 43 volumes of *acetonitrile R* and 55 volumes of *water R*,
- as detector a spectrophotometer set at 233 nm,
- a loop injector.

Inject 20 µl of reference solution (d). The substances are eluted in the following order: ketoprofen and ketoprofen impurity A. Adjust the sensitivity of the detector so that the heights of the 2 principal peaks in the chromatogram obtained are not less than 50 per cent of the full scale of the recorder. The test is not valid unless the resolution between the peaks corresponding to ketoprofen and ketoprofen impurity A is at least 7.0.

Inject 20 µl of the test solution, 20 µl of reference solution (a), 20 µl of reference solution (b) and 20 µl of reference solution (c). Continue the chromatography of the test solution for 7 times the retention time of ketoprofen. The relative retentions with reference to ketoprofen (retention time = about 7 min) are as follows: impurity C = about 0.34; impurity H = about 0.39; impurity G = about 0.46; impurity E = about 0.69; impurity B = about 0.73; impurity D = about 1.35; impurity I = about 1.43; impurity A = about 1.50; impurity J = about 1.86; impurity F = about 1.95; impurity K = about 2.27; impurity L = about 2.49. In the chromatogram obtained with the test solution: the areas of any peaks corresponding to ketoprofen impurity A and ketoprofen impurity C are not greater than the areas of the principal peaks in the chromatograms obtained with reference solution (b) and reference solution (c), respectively (0.2 per cent); the area of any peak, apart from the principal peak and any peaks corresponding to ketoprofen impurity A and ketoprofen impurity C, is not greater than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent); the sum of the areas of all the peaks, apart from the principal peak and any peaks corresponding to the

2 named impurities, is not greater than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.4 per cent). Disregard any peak with an area less than 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (a).

Heavy metals (2.4.8). 2.0 g complies with limit test C for heavy metals (10 ppm). Prepare the reference solution using 2 ml of *lead standard solution* (10 ppm Pb) R.

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g at 60 °C at a pressure not exceeding 670 Pa.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

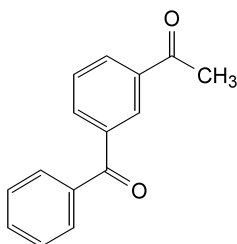
Dissolve 0.200 g in 25 ml of *ethanol* (96 per cent) R. Add 25 ml of *water* R. Titrate with 0.1 M *sodium hydroxide*, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *sodium hydroxide* is equivalent to 25.43 mg of $C_{16}H_{14}O_3$.

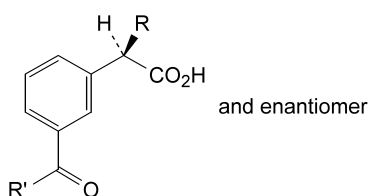
IMPURITIES

Specified impurities: A, B, C, D, E, F.

Other detectable impurities: G, H, I, J, K, L.

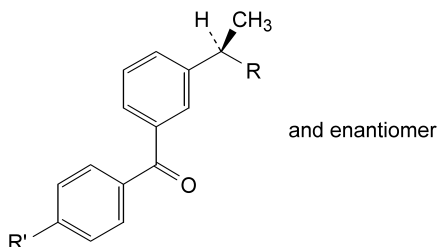


A. 1-(3-benzoylphenyl)ethanone,



B. R = H, R' = C_6H_5 : (3-benzoylphenyl)acetic acid,

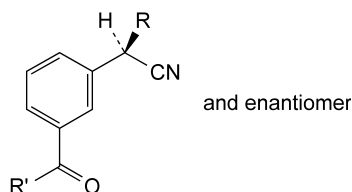
C. R = CH_3 , R' = OH: 3-[(1RS)-1-carboxyethyl]benzoic acid,



D. R = CO_2H , R' = CH_3 : (2RS)-2-[3-(4-methylbenzoyl)phenyl]propanoic acid,

E. R = $CO-NH_2$, R' = H: (2RS)-2-(3-benzoylphenyl)propanamide,

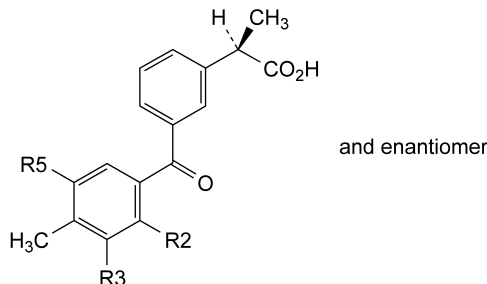
F. R = CN, R' = H: (2RS)-2-(3-benzoylphenyl)propanenitrile,



G. R = CH_3 , R' = OH: 3-[(1RS)-1-cyanoethyl]benzoic acid,

H. R = H, R' = OH: 3-(cyanomethyl)benzoic acid,

I. R = H, R' = C_6H_5 : (3-benzoylphenyl)ethanenitrile,



J. R2 = CH_3 , R3 = R5 = H: (2RS)-2-[3-(2,4-dimethylbenzoyl)phenyl]propanoic acid,

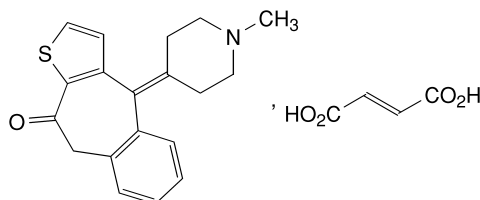
K. R2 = R3 = CH_3 , R5 = H + R2 = H, R3 = R5 = CH_3 : mixture of (2RS)-2-[3-(2,3,4-trimethylbenzoyl)phenyl]propanoic acid and (2RS)-2-[3-(3,4,5-trimethylbenzoyl)phenyl]propanoic acid,

L. R2 = R5 = CH_3 , R3 = H: (2RS)-2-[3-(2,4,5-trimethylbenzoyl)phenyl]propanoic acid.

01/2005:1592

KETOTIFEN HYDROGEN FUMARATE

Ketotifeni hydrogenofumaras



$C_{23}H_{23}NO_5S$

M_r 425.5

DEFINITION

4-(1-Methylpiperidin-4-ylidene)-4,9-dihydro-10H-benzo[4,5]cyclohepta[1,2-b]thiophen-10-one hydrogen (E)-butenedioate.

Content: 98.5 per cent to 101.0 per cent (dried substance).

CHARACTERS

Appearance: white to brownish-yellow, fine, crystalline powder.

Solubility: sparingly soluble in water, slightly soluble in methanol, very slightly soluble in acetonitrile.

IDENTIFICATION

A. Infrared absorption spectrophotometry (2.2.24).

Comparison: Ph. Eur. reference spectrum of ketotifen hydrogen fumarate.

B. Thin-layer chromatography (2.2.27).

Test solution. Dissolve 40 mg of the substance to be examined in *methanol* R and dilute to 10 ml with the same solvent.

Reference solution. Dissolve 11 mg of *fumaric acid CRS* in *methanol R* and dilute to 10 ml with the same solvent.

Plate: *cellulose for chromatography F₂₅₄ R* as the coating substance.

Mobile phase: *water R, anhydrous formic acid R, di-isopropyl ether R* (3:7:90 V/V/V).

Application: 5 µl.

Development: over a path of 17 cm.

Drying: in a current of warm air.

Detection: examine in ultraviolet light at 254 nm. Spray lightly with a 5 g/l solution of *potassium permanganate R* in a 1.4 per cent V/V solution of *sulphuric acid R*. Examine in daylight by transparency.

Results: the spot due to *fumaric acid* in the chromatogram obtained with the test solution is similar in position, colour and intensity to the principal spot in the chromatogram obtained with the reference solution.

TESTS

Appearance of solution. The solution is clear (2.2.1) and not more intensely coloured than reference solution Y₄, BY₄ or B₄ (2.2.2, *Method II*).

Dissolve 0.2 g in *methanol R* and dilute to 10 ml with the same solvent.

Related substances. Liquid chromatography (2.2.29).

Test solution. Dissolve 30.0 mg of the substance to be examined in a mixture of equal volumes of *methanol R* and *water R* and dilute to 100.0 ml with the same mixture of solvents.

Reference solution (a). Dilute 1.0 ml of the test solution to 50.0 ml with a mixture of equal volumes of *methanol R* and *water R*. Dilute 1.0 ml to 10.0 ml with a mixture of equal volumes of *methanol R* and *water R*.

Reference solution (b). Dissolve 3.0 mg of *ketotifen impurity G CRS* in 10 ml of *methanol R* and dilute to 20.0 ml with *water R*. Protect the solution from light.

Reference solution (c). To 1.5 ml of reference solution (b) add 1.0 ml of the test solution and dilute to 10.0 ml with a mixture of equal volumes of *methanol R* and *water R*. Protect the solution from light.

Reference solution (d). Dilute 0.5 ml of reference solution (b) to 50.0 ml with a mixture of equal volumes of *methanol R* and *water R*. Protect the solution from light.

Column:

- size: $l = 0.15$ m, $\varnothing = 4.0$ mm,
- stationary phase: *octadecylsilyl silica gel for chromatography R* (3 µm),
- temperature: 40 °C.

Mobile phase:

- mobile phase A: mix 175 µl of *triethylamine R* and 500 ml of *water R*,
- mobile phase B: mix 175 µl of *triethylamine R* and 500 ml of *methanol R*,

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)
0 - 12	40	60
12 - 20	40 → 10	60 → 90
20 - 25	10	90
25 - 26	10 → 40	90 → 60
26 - 31	40	60

Flow rate: 1.0 ml/min.

Detection: spectrophotometer at 297 nm.

Injection: 20 µl; inject the test solution and reference solutions (a), (c) and (d).

Relative retentions with reference to *ketotifen*:
 impurity D = about 0.31; impurity C = about 0.61;
 impurity G = about 0.86; impurity E = about 1.18;
 impurity F = about 1.36; impurity B = about 1.72;
 impurity A = about 2.15.

System suitability:

- resolution: minimum of 1.5 between the peaks due to *ketotifen* and to impurity G in the chromatogram obtained with reference solution (c),
- signal-to-noise ratio: minimum of 70 for the principal peak in the chromatogram obtained with reference solution (d).

Limits:

- correction factor: for the calculation of contents, multiply the area of the corresponding peak by the following correction factor: impurity G = 1.36,
- impurity G: not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent),
- any impurity: not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent),
- total: not more than 2.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent),
- disregard limit: 0.25 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

Loss on drying (2.2.32): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 100-105 °C for 4 h.

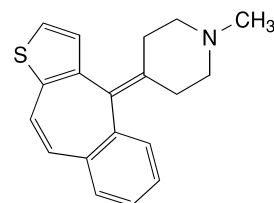
Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

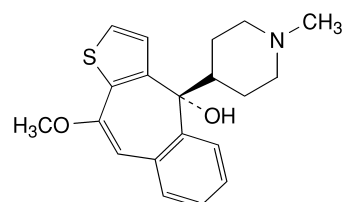
Dissolve 0.350 g in a mixture of 30 ml of *anhydrous acetic acid R* and 30 ml of *acetic anhydride R*. Titrate with 0.1 M *perchloric acid*, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *perchloric acid* is equivalent to 42.55 mg of C₂₃H₂₃NO₅S.

IMPURITIES

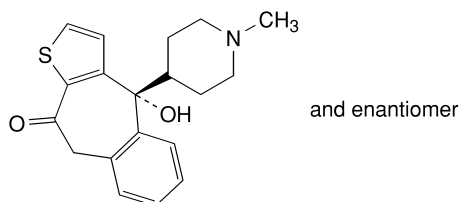


A. 4-(4*H*-benzo[4,5]cyclohepta[1,2-*b*]thiophen-4-ylidene)-1-methylpiperidine,

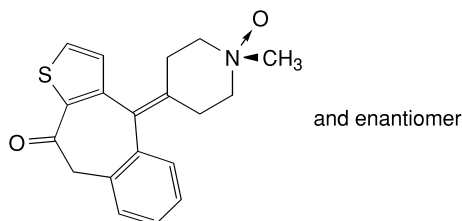


and enantiomer

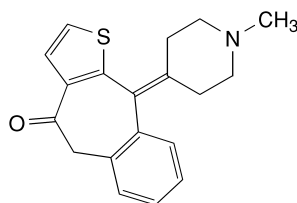
B. (4*RS*)-10-methoxy-4-(1-methylpiperidin-4-yl)-4*H*-benzo[4,5]cyclohepta[1,2-*b*]thiophen-4-ol,



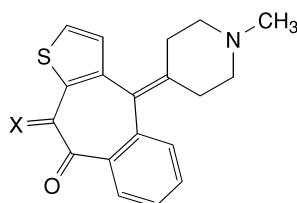
- C. (4*RS*)-4-hydroxy-4-(1-methylpiperidin-4-yl)-4,9-dihydro-10*H*-benzo[4,5]cyclohepta[1,2-*b*]thiophen-10-one,



- D. 4-[(*aRaS*)-1-methylpiperidin-4-ylidene]-4,9-dihydro-10*H*-benzo[4,5]cyclohepta[1,2-*b*]thiophen-10-one *N*-oxide (ketotifen *N*-oxide),



- E. 10-(1-methylpiperidin-4-ylidene)-5,10-dihydro-4*H*-benzo[5,6]cyclohepta[1,2-*b*]thiophen-4-one,



- F. X = H₂: 4-(1-methylpiperidin-4-ylidene)-4,10-dihydro-9*H*-benzo[4,5]cyclohepta[1,2-*b*]thiophen-9-one,
G. X = O: 4-(1-methylpiperidin-4-ylidene)-4*H*-benzo[4,5]cyclohepta[1,2-*b*]thiophen-9,10-dione.

01/2005:1885

KNOTGRASS

Polygoni avicularis herba

DEFINITION

Whole or cut dried flowering aerial parts of *Polygonum aviculare* L. *s.l.*

Content: minimum 0.30 per cent of flavonoids, expressed as hyperoside (C₂₁H₂₀O₁₂; *M_r* 464.4) (dried drug).

CHARACTERS

Macroscopic and microscopic characters described under identification tests A and B.

IDENTIFICATION

- A. The stem is 0.5 mm to 2 mm thick, branched, with nodes, cylindrical or slightly angular and longitudinally striated. It bears sessile or shortly petiolate, glabrous entire leaves,

which differ widely in shape and size. The sheath-like stipules (ochrea) are lacerate and silvery. The small axillary flowers have 5 greenish-white perianth segments, the tips of which are often coloured red. The fruits are 2 mm to 4 mm, brown to black triangular nuts, usually punctate or striate.

- B. Reduce to a powder (355). The powder is greenish-brown. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters: fragments of the leaf epidermis with polygonal to sinuous cell walls and numerous anisocytic stomata (2.8.3), with a striated cuticle; fragments of leaves and stems containing numerous calcium oxalate clusters, some of them very large; groups of thick-walled fibres from the hypodermis of the stem; globular pollen grains with smooth exine and 3 germinal pores; occasional brown fragments of the exocarp composed of cells with thick sinuous walls. Examine under a microscope using a 675 g/l solution of *potassium hydroxide R*. Heat gently. The epidermis of the leaves and a few cells of the mesophyll stain red to reddish-violet. Examine under a microscope using a 0.1 g/l solution of *ferric chloride R*. Leaf fragments are stained almost black.
- C. Thin-layer chromatography (2.2.27).

Test solution. To 1.0 g of the powdered drug (355) add 10 ml of *methanol R*. Heat the mixture under a reflux condenser, in a water-bath for 10 min. Cool and filter.

Reference solution. Dissolve 1 mg of *caffeic acid R*, 2.5 mg of *hyperoside R* and 1 mg of *chlorogenic acid R* in 10 ml of *methanol R*.

Plate: TLC silica gel plate *R*.

Mobile phase: anhydrous formic acid *R*, glacial acetic acid *R*, water *R*, ethyl acetate *R* (7:7:14:72 V/V/V/V).

Application: 20 µl, as bands.

Development: over a path of 10 cm.

Drying: at 100–105 °C.

Detection: spray with a 10 g/l solution of *diphenylboric acid aminoethyl ester R* in *methanol R* subsequently spray with a 50 g/l solution of *macrogol 400 R* in *methanol R*. Allow the plate to dry in air for about 30 min. Examine in ultraviolet light at 365 nm.

Results: see below the sequence of the fluorescent zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other fluorescent zones are present in the chromatogram obtained with the test solution.

Top of the plate	
Caffeic acid: a light blue fluorescent zone	1 or 2 blue fluorescent zones (caffeic acid)
Hyperoside: a yellowish-brown fluorescent zone	1 or 2 yellowish-green fluorescent zones A yellow fluorescent zone
Chlorogenic acid: a light blue fluorescent zone	A yellowish-brown fluorescent zone A light blue fluorescent zone (chlorogenic acid)
	A yellowish-brown fluorescent zone
Reference solution	Test solution

TESTS

Foreign matter (2.8.2): maximum 2 per cent of roots and maximum 2 per cent of other foreign matter.

Loss on drying (2.2.32): maximum 10.0 per cent, determined on 1.000 g of powdered drug (710) by drying in an oven at 100-105 °C for 2 h.

Total ash (2.4.16): maximum 10.0 per cent.

ASSAY

Stock solution. In a 100 ml round-bottomed flask, place 0.800 g of the powdered drug (355), add 1 ml of a 5 g/l solution of *hexamethylenetetramine R*, 20 ml of *acetone R* and 2 ml of *hydrochloric acid R1*. Boil the mixture under a reflux condenser for 30 min. Filter the liquid through a plug of absorbent cotton into a flask. Add the absorbent cotton to the residue in the round-bottomed flask and extract with 2 quantities, each of 20 ml, of *acetone R*, each time boiling under a reflux condenser for 10 min. Allow to cool, filter each extract through the plug of absorbent cotton into the flask. Filter the combined acetone extracts through a filter paper into a volumetric flask, dilute to 100.0 ml with *acetone R* rinsing the flask and the filter paper. Introduce 20.0 ml of the solution into a separating funnel, add 20 ml of *water R* and shake the mixture with 1 quantity of 15 ml

and then 3 quantities, each of 10 ml of *ethyl acetate R*. Combine the ethyl acetate extracts in a separating funnel and wash with 2 quantities, each of 50 ml, of *water R*. Filter the extracts over 10 g of *anhydrous sodium sulphate R* into a 50 ml volume tric flask and dilute to volume with *ethyl acetate R*.

Test solution. To 10.0 ml of the stock solution add 1 ml of *aluminium chloride reagent R* and dilute to 25.0 ml with a 5 per cent V/V solution of *glacial acetic acid R* in *methanol R*.

Compensation liquid. Dilute 10.0 ml of the stock solution to 25.0 ml with a 5 per cent V/V solution of *glacial acetic acid R* in *methanol R*.

Measure the absorbance (2.2.25) of the test solution after 30 min by comparison with the compensation liquid at 425 nm. Calculate the percentage content of flavonoids, calculated as hyperoside, from the expression:

$$\frac{A \times 1.25}{m}$$

i.e. taking the specific absorbance of hyperoside to be 500.

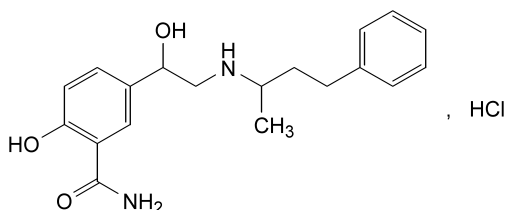
A = absorbance at 425 nm,

m = mass of the drug to be examined, in grams.

01/2005:0923 TESTS

LABETALOL HYDROCHLORIDE

Labetaloli hydrochloridum

 $C_{19}H_{25}ClN_2O_3$ M_r 364.9

DEFINITION

Labetalol hydrochloride contains not less than 98.5 per cent and not more than the equivalent of 101.0 per cent of 2-hydroxy-5-[1-hydroxy-2-[(1-methyl-3-phenylpropyl)amino]ethyl]benzamide hydrochloride, calculated with reference to the dried substance.

CHARACTERS

A white or almost white powder, sparingly soluble in water and in alcohol, practically insoluble in methylene chloride.

IDENTIFICATION

First identification: A, C, E.

Second identification: A, B, D, E.

- Determined on solution S (see Tests), the angle of optical rotation (2.2.7) is $+0.05^\circ$ to -0.05° .
- Dissolve 25.0 mg in 0.1 M hydrochloric acid and dilute to 250.0 ml with the same acid. Examined between 230 nm and 350 nm (2.2.25), the solution shows an absorption maximum at 302 nm. The specific absorbance at the maximum is 83 to 88.
- Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with labetalol hydrochloride CRS.
- Examine by thin-layer chromatography (2.2.27), using as the coating substance a suitable octadecylsilyl silica gel with a fluorescent indicator having an optimal intensity at 254 nm.

Test solution. Dissolve 10 mg of the substance to be examined in 1 ml of alcohol R.

Reference solution (a). Dissolve 10 mg of labetalol hydrochloride CRS in 1 ml of alcohol R.

Reference solution (b). Dissolve 10 mg of labetalol hydrochloride CRS and 10 mg of propranolol hydrochloride CRS in alcohol R and dilute to 5 ml with the same solvent.

Apply separately to the plate 2 μ l of each solution. Place the plate in a chromatographic tank immediately after the addition of the mobile phase consisting of a mixture of 0.5 volumes of perchloric acid R, 50 volumes of water R and 80 volumes of methanol R. Close the tank and develop over a path of 15 cm. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. The principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a). The test is not valid unless the chromatogram obtained with reference solution (b) shows two clearly separated spots.

- It gives reaction (a) of chlorides (2.3.1).

Solution S. Dissolve 0.50 g in carbon dioxide-free water R and dilute to 50 ml with the same solvent. *Solution S must be freshly prepared.*

Appearance of solution. Solution S is clear (2.2.1) and not more intensely coloured than intensity 6 of the range of reference solutions of the most appropriate colour (2.2.2, Method II).

pH (2.2.3). The pH of solution S is 4.0 to 5.0.

Diastereoisomer ratio. Examine by gas chromatography (2.2.28).

Test solution. Dissolve 2.0 mg of the substance to be examined in 1.0 ml of a 12.0 g/l solution of butylboronic acid R in anhydrous pyridine R and allow to stand for 20 min.

The chromatographic procedure may be carried out using:

- a glass column 1.5 m long and 4 mm in internal diameter packed with silanised diatomaceous earth for gas chromatography R (125 μ m to 150 μ m) impregnated with 3 per cent m/m of polymethylphenylsiloxane R,
- nitrogen for chromatography R as the carrier gas at a flow rate of 40 ml/min,
- a flame-ionisation detector,

maintaining the temperature of the column, and that of the injection port and the detector at 300 °C.

Inject 2 μ l of the test solution. Two peaks, each corresponding to a pair of diastereoisomers, appear in the chromatogram. Adjust the sensitivity of the system so that in the chromatogram obtained, the height of the taller of the diastereoisomer peaks is about 80 per cent of the full scale of the recorder. The test is not valid unless the height of the trough separating these peaks is less than 5 per cent of the full scale of the recorder. The area of each peak is not less than 45 per cent and not more than 55 per cent of the total area of the two peaks.

Related substances. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 50.0 mg of the substance to be examined in the mobile phase and dilute to 10.0 ml with the mobile phase.

Reference solution. Dilute 0.5 ml of the test solution to 100.0 ml with the mobile phase.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.15 m long and 4.6 mm in internal diameter packed with octadecylsilyl silica gel for chromatography R (5 μ m),
- as mobile phase at a flow rate of 1 ml/min a mixture of 150 ml of tetrahydrofuran R, 300 ml of methanol R, 550 ml of water R, 0.82 g of tetrabutylammonium hydrogen sulphate R, 1 g of sodium octyl sulphate R and 10 ml of a 10 per cent V/V solution of sulphuric acid R,
- as detector a spectrophotometer set at 229 nm.

Equilibrate the column with the mobile phase at a flow rate of 1 ml/min for about 30 min.

Adjust the sensitivity of the system so that the height of the principal peak in the chromatogram obtained with 20 μ l of the reference solution is at least 50 per cent of the full scale of the recorder. When the chromatograms are recorded in the prescribed conditions, the retention time of the principal peak is 10 min to 15 min. If necessary, adjust the water content of the mobile phase ensuring that the 2:1 ratio of methanol to tetrahydrofuran is maintained.

Inject separately 20 µl of each solution. Continue the chromatography for three times the retention time of the principal peak. In the chromatogram obtained with the test solution: the area of any peak, apart from the principal peak, is not greater than 0.6 times that of the principal peak in the chromatogram obtained with the reference solution (0.3 per cent); the sum of the areas of any such peaks is not greater than the area of the principal peak in the chromatogram obtained with the reference solution (0.5 per cent). Disregard any peak due to the solvent and any peak with an area less than 0.1 times the area of the principal peak in the chromatogram obtained with the reference solution.

Heavy metals (2.4.8). 1.0 g complies with limit test C for heavy metals (20 ppm). Prepare the standard using 2 ml of *lead standard solution* (10 ppm Pb) R.

Loss on drying (2.2.32). Not more than 1.0 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C at a pressure not exceeding 0.7 kPa.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

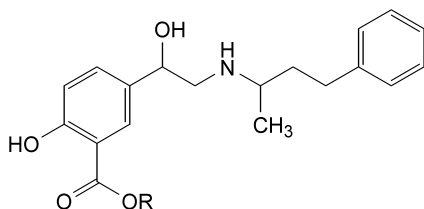
ASSAY

In order to avoid overheating in the reaction medium, mix thoroughly throughout and stop the titration immediately after the end-point has been reached.

Dissolve 0.200 g in a mixture of 10 ml of *anhydrous formic acid* R and 40 ml of *acetic anhydride* R. Titrate with 0.1 M *perchloric acid*, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *perchloric acid* is equivalent to 36.49 mg of C₁₉H₂₅ClN₂O₃.

IMPURITIES

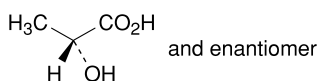


- A. R = H: 2-hydroxy-5-[1-hydroxy-2-[(1-methyl-3-phenylpropyl)amino]ethyl]benzoic acid,
B. R = CH₃: methyl 2-hydroxy-5-[1-hydroxy-2-[(1-methyl-3-phenylpropyl)amino]ethyl]benzoate.

01/2005:0458

LACTIC ACID

Acidum lacticum



C₃H₆O₃

M_r 90.1

DEFINITION

Mixture of 2-hydroxypropanoic acid, its condensation products, such as lactoyl-lactic acid and polylactic acids, and water. The equilibrium between lactic acid and polylactic acids depends on the concentration and temperature. It is usually the racemate ((*RS*)-lactic acid).

Content: 88.0 per cent *m/m* to 92.0 per cent *m/m* of C₃H₆O₃.

CHARACTERS

Appearance: colourless or slightly yellow, syrupy liquid.

Solubility: miscible with water and with alcohol.

IDENTIFICATION

- A. Dissolve 1 g in 10 ml of *water* R. The solution is strongly acidic (2.2.4).
B. Relative density (2.2.5): 1.20 to 1.21.
C. It gives the reaction of lactates (2.3.1).

TESTS

Solution S. Dissolve 5.0 g in 42 ml of 1 M *sodium hydroxide* and dilute to 50 ml with *distilled water* R.

Appearance. The substance to be examined is not more intensely coloured than reference solution Y₆ (2.2.2, *Method II*).

Ether-insoluble substances. Dissolve 1.0 g in 25 ml of *ether* R. The solution is not more opalescent than the solvent used for the test.

Sugars and other reducing substances. To 1 ml of solution S add 1 ml of 1 M *hydrochloric acid*, heat to boiling, allow to cool and add 1.5 ml of 1 M *sodium hydroxide* and 2 ml of *cupri-tartaric solution* R. Heat to boiling. No red or greenish precipitate is formed.

Methanol (2.4.24): maximum 50 ppm, if intended for use in the manufacture of parenteral dosage forms.

Citric, oxalic and phosphoric acids. To 5 ml of solution S add *dilute ammonia* R1 until slightly alkaline (2.2.4). Add 1 ml of *calcium chloride solution* R. Heat on a water-bath for 5 min. Both before and after heating, any opalescence in the solution is not more intense than that in a mixture of 1 ml of *water* R and 5 ml of solution S.

Sulphates (2.4.13): maximum 200 ppm.

7.5 ml of solution S diluted to 15 ml with *distilled water* R complies with the limit test for sulphates.

Calcium (2.4.3): maximum 200 ppm.

5 ml of solution S diluted to 15 ml with *distilled water* R complies with the limit test for calcium.

Heavy metals (2.4.8): maximum 10 ppm.

12 ml of solution S complies with limit test A. Prepare the standard using *lead standard solution* (1 ppm Pb) R.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

Bacterial endotoxins (2.6.14): less than 5 IU/g, if intended for use in the manufacture of parenteral dosage forms without a further appropriate procedure for the elimination of bacterial endotoxins.

ASSAY

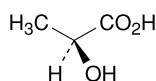
Place 1.000 g in a ground-glass-stoppered flask and add 10 ml of *water* R and 20.0 ml of 1 M *sodium hydroxide*. Close the flask and allow to stand for 30 min. Using 0.5 ml of *phenolphthalein solution* R as indicator, titrate with 1 M *hydrochloric acid* until the pink colour is discharged.

1 ml of 1 M *sodium hydroxide* is equivalent to 90.1 mg of C₃H₆O₃.

LABELLING

The label states:

- where applicable, that the substance is free from bacterial endotoxins,
- where applicable, that the substance is suitable for use in the manufacture of parenteral dosage forms.

(S)-LACTIC ACID**Acidum (S)-lacticum**C₃H₆O₃*M*_r 90.1**DEFINITION**

Mixture of (S)-2-hydroxypropanoic acid, its condensation products, such as lactoyl-lactic acid and polylactic acids, and water. The equilibrium between lactic acid and polylactic acids depends on the concentration and temperature.

Content: 88.0 per cent *m/m* to 92.0 per cent *m/m* of C₃H₆O₃, not less than 95.0 per cent of which is the (S)-enantiomer.

CHARACTERS

Appearance: colourless or slightly yellow, syrupy liquid.

Solubility: miscible with water and with alcohol.

IDENTIFICATION

- Dissolve 1 g in 10 ml of *water R*. The solution is strongly acidic (2.2.4).
- Relative density (2.2.5): 1.20 to 1.21.
- It gives the reaction of lactates (2.3.1).
- It complies with the limits of the assay.

TESTS

Solution S. Dissolve 5.0 g in 42 ml of 1 *M sodium hydroxide* and dilute to 50 ml with *distilled water R*.

Appearance. The substance to be examined is not more intensely coloured than reference solution Y₆ (2.2.2, *Method II*).

Ether-insoluble substances. Dissolve 1.0 g in 25 ml of *ether R*. The solution is not more opalescent than the solvent used for the test.

Sugars and other reducing substances. To 1 ml of solution S add 1 ml of 1 *M hydrochloric acid*, heat to boiling, allow to cool and add 1.5 ml of 1 *M sodium hydroxide* and 2 ml of *cupri-tartaric solution R*. Heat to boiling. No red or greenish precipitate is formed.

Methanol (2.4.24): maximum 50 ppm, if intended for use in the manufacture of parenteral dosage forms.

Citric, oxalic and phosphoric acids. To 5 ml of solution S add *dilute ammonia RI* until slightly alkaline (2.2.4). Add 1 ml of *calcium chloride solution R*. Heat on a water-bath for 5 min. Both before and after heating, any opalescence in the solution is not more intense than that in a mixture of 1 ml of *water R* and 5 ml of solution S.

Sulphates (2.4.13): maximum 200 ppm.

7.5 ml of solution S diluted to 15 ml with *distilled water R* complies with the limit test for sulphates.

Calcium (2.4.3): maximum 200 ppm.

5 ml of solution S diluted to 15 ml with *distilled water R* complies with the limit test for calcium.

Heavy metals (2.4.8): maximum 10 ppm.

12 ml of solution S complies with limit test A. Prepare the standard using *lead standard solution (1 ppm Pb) R*.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

01/2005:1771 Bacterial endotoxins (2.6.14): less than 5 IU/g if intended for use in the manufacture of parenteral dosage forms without a further appropriate procedure for the elimination of bacterial endotoxins.

ASSAY

Place 1.000 g in a ground-glass-stoppered flask and add 10 ml of *water R* and 20.0 ml of 1 *M sodium hydroxide*. Close the flask and allow to stand for 30 min. Using 0.5 ml of *phenolphthalein solution R* as indicator, titrate with 1 *M hydrochloric acid* until the pink colour is discharged. 1 ml of 1 *M sodium hydroxide* is equivalent to 90.1 mg of C₃H₆O₃.

(S)-enantiomer

Transfer an amount of the substance to be examined equivalent to 2.00 g of lactic acid into a round-bottomed flask, add 25 ml of 1 *M sodium hydroxide* and boil gently for 15 min. Cool down and adjust to pH 7.0 using 1 *M hydrochloric acid*. Add 5.0 g of *ammonium molybdate R*, dissolve and dilute to 50.0 ml with *water R*. Filter and measure the angle of optical rotation (2.2.7). Calculate the percentage content of (S)-enantiomer using the expression:

$$50 + \left(24.18 \times \alpha \times \frac{2.222}{m} \times \frac{90}{c} \right)$$

α = angle of optical rotation (absolute value),

m = mass of the substance to be examined, in grams,

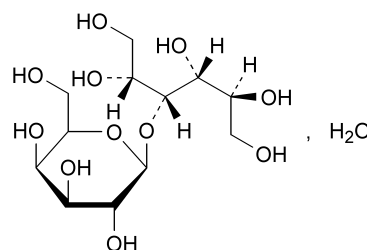
c = percentage content of C₃H₆O₃ in the substance to be examined.

The complex of (S)-lactic acid formed under these test conditions is laevorotatory.

LABELLING

The label states:

- where applicable, that the substance is free from bacterial endotoxins,
- where applicable, that the substance is suitable for use in the manufacture of parenteral dosage forms.

01/2005:1337**LACTITOL MONOHYDRATE****Lactitolum monohydricum**C₁₂H₂₄O₁₁·H₂O*M*_r 362.3**DEFINITION**

4-*O*-(β-D-galactopyranosyl)-D-glucitol.

Content: 96.5 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

Appearance: white, crystalline powder.

Solubility: very soluble in water, slightly soluble in alcohol, practically insoluble in methylene chloride

IDENTIFICATION

First identification: B.

Second identification: A, C.

A. Specific optical rotation (see Tests).

B. Infrared absorption spectrophotometry (2.2.24).

Comparison: lactitol monohydrate CRS.

C. Thin-layer chromatography (2.2.27).

Test solution. Dissolve 50 mg of the substance to be examined in *methanol R* and dilute to 20 ml with the same solvent.

Reference solution (a). Dissolve 5 mg of *lactitol monohydrate CRS* in *methanol R* and dilute to 2 ml with the same solvent.

Reference solution (b). Dissolve 5 mg of *sorbitol CRS* in 2 ml of reference solution (a) and dilute to 20 ml with *methanol R*.

Plate: TLC silica gel G plate R.

Mobile phase: *water R*, *acetonitrile R* (25:75 V/V).

Application: 2 µl.

Development: over 2/3 of the plate.

Drying: in air.

Detection: spray with 4-aminobenzoic acid solution R. Dry the plate in a current of cold air until the solvent is removed. Heat at 100 °C for 15 min. Allow to cool and spray with a 2 g/l solution of *sodium periodate R*. Dry the plate in a current of cold air. Heat at 100 °C for 15 min.

System suitability: the chromatogram obtained with reference solution (b) shows 2 clearly separated spots.

Results: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).

Column:

– size: $l = 0.30$ m, $\varnothing = 7.8$ mm,

– stationary phase: strong cation exchange resin (calcium form) R,

– temperature: 60 °C.

Mobile phase: *water R*.

Flow rate: 0.6 ml/min.

Detection: refractive index detector maintained at a constant temperature.

Injection: 100 µl; inject test solution (a) and reference solutions (b) and (c).

Run time: 2.5 times the retention time of lactitol.

Relative retention with reference to lactitol (retention time = about 13 min): impurity A = about 0.7; impurity B = about 0.8; glycerol = about 1.3; impurity C = about 1.5; impurity D = about 1.8; impurity E = about 1.9.

System suitability: reference solution (c):

– resolution: minimum 5 between the peaks due to lactitol and glycerol.

Limits:

– impurity B: not more than the area of the peak due to lactitol in the chromatogram obtained with reference solution (c) (1.0 per cent),

– total of other impurities: not more than the area of the peak due to lactitol in the chromatogram obtained with reference solution (c) (1.0 per cent),

– disregard limit: the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent); disregard any peak due to the solvent.

Reducing sugars: maximum 0.2 per cent.

Dissolve 5.0 g in 3 ml of *water R* with gentle heating. Cool and add 20 ml of *cupri-citric solution R* and a few glass beads. Heat so that boiling begins after 4 min and maintain boiling for 3 min. Cool rapidly and add 100 ml of a 2.4 per cent V/V solution of *glacial acetic acid R* and 20.0 ml of 0.025 M iodine. With continuous shaking, add 25 ml of a mixture of 6 volumes of *hydrochloric acid R* and 94 volumes of *water R*. When the precipitate has dissolved, titrate the excess of iodine with 0.05 M *sodium thiosulphate* using 1 ml of *starch solution R* added towards the end of the titration, as indicator. Not less than 12.8 ml of 0.05 M *sodium thiosulphate* is required.

Lead (2.4.10): maximum 0.5 ppm.

Nickel (2.4.15): maximum 1 ppm.

Water (2.5.12): 4.5 per cent to 5.5 per cent, determined on 0.30 g.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

Microbial contamination. Total viable aerobic count (2.6.12) not more than 10^3 micro-organisms per gram. It complies with the tests for *Escherichia coli*, *Salmonella* and *Pseudomonas aeruginosa* (2.6.13).

ASSAY

Liquid chromatography (2.2.29) as described in the test for related substances with the following modification.

Injection: test solution (b) and reference solution (a).

Calculate the percentage content of $C_{12}H_{24}O_{11}$ using the chromatograms obtained with test solution (b) and reference solution (a) and the declared content of *lactitol monohydrate CRS*.

TESTS

Solution S. Dissolve 5.000 g in *carbon dioxide-free water R* and dilute to 50.0 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and not more intensely coloured than reference solution BY₇ (2.2.2, Method II).

Acidity or alkalinity. To 10 ml of solution S add 10 ml of *carbon dioxide-free water R*. To 10 ml of this solution add 0.05 ml of *phenolphthalein solution R*. Not more than 0.2 ml of 0.01 M *sodium hydroxide* is required to change the colour of the indicator to pink. To a further 10 ml of the solution add 0.05 ml of *methyl red solution R*. Not more than 0.3 ml of 0.01 M *hydrochloric acid* is required to change the colour of the indicator to red.

Specific optical rotation (2.2.7): + 13.5 to + 15.5 (anhydrous substance), determined on solution S.

Related substances. Liquid chromatography (2.2.29).

Test solution (a). Dissolve 50.0 mg of the substance to be examined in *water R* and dilute to 10.0 ml with the same solvent.

Test solution (b). Dilute 2.0 ml of test solution (a) to 50.0 ml with *water R*.

Reference solution (a). Dissolve 5.0 mg of *lactitol monohydrate CRS* and 5 mg of *glycerol R* in *water R* and dilute to 25.0 ml with the same solvent.

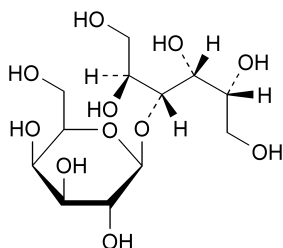
Reference solution (b). Dilute 1.0 ml of test solution (a) to 100.0 ml with *water R*. Dilute 5.0 ml of this solution to 100.0 ml with *water R*.

Reference solution (c). Dilute 2.5 ml of reference solution (a) to 10.0 ml with *water R*.

IMPURITIES

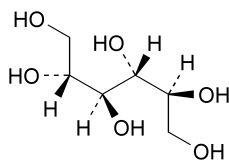
Specified impurities: A, B, C, D, E.

A. lactose,



B. lactulitol,

C. mannitol,



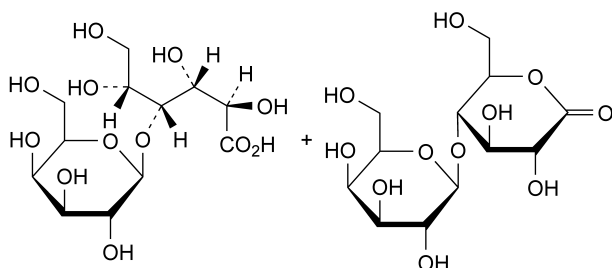
D. dulcitol (galactitol),

E. sorbitol.

01/2005:1647

LACTOBIONIC ACID

Acidum lactobionicum



$C_{12}H_{22}O_{12}$ (acid form)

M_r 358.3

$C_{12}H_{20}O_{11}$ (δ -lactone)

M_r 340.3

DEFINITION

Mixture in variable proportions of 4-O- β -D-galactopyranosyl-D-gluconic acid and 4-O- β -D-galactopyranosyl-D-glucono-1,5-lactone.

Content: 98.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

Appearance: white or almost white powder.

Solubility: freely soluble in water, slightly soluble in glacial acetic acid, in anhydrous ethanol and in methanol.

mp: about 125 °C with decomposition.

IDENTIFICATION

A. Infrared absorption spectrophotometry (2.2.24).

Comparison: lactobionic acid CRS.

If the spectra obtained show differences, dissolve the substance to be examined and the reference substance separately in *water R*, dry at 105 °C and record new spectra using the residues.

B. Thin-layer chromatography (2.2.27).

Test solution. Dissolve 10 mg of the substance to be examined in *water R* and dilute to 1 ml with the same solvent.

Reference solution. Dissolve 10 mg of *lactobionic acid CRS* in *water R* and dilute to 1 ml with the same solvent.

Plate: TLC silica gel plate *R*.

Mobile phase: concentrated ammonia *R1*, ethyl acetate *R*, *water R*, methanol *R* (2:2:2:4 V/V/V/V).

Application: 5 μ l.

Development: over 3/4 of the plate.

Detection: spray 3 times with ammonium molybdate solution *R6* and heat in an oven at 110 °C for 15 min.

Results: the principal spot in the chromatogram obtained with the test solution is similar in position and colour to the principal spot in the chromatogram obtained with the reference solution.

TESTS

Appearance of solution. The solution is clear (2.2.1) and not more intensely coloured than reference solution Y_5 (2.2.2, *Method II*).

Dissolve 3.0 g in 25 ml of *water R*.

Specific optical rotation (2.2.7): + 23.0 to + 29.0 (anhydrous substance).

Dissolve 1.0 g in 80 ml of *water R* and dilute to 100.0 ml with the same solvent. Allow to stand for 24 h.

Reducing sugars: maximum 0.2 per cent, calculated as glucose.

Dissolve 5.0 g in 25 ml of *water R* with the aid of gentle heat. Cool and add 20 ml of *cupri-citric solution R* and a few glass beads. Heat so that boiling begins after 4 min and maintain boiling for 3 min. Cool rapidly and add 100 ml of a 2.4 per cent V/V solution of *glacial acetic acid R* and 20.0 ml of 0.025 M iodine. With continuous shaking, add 25 ml of a mixture of 6 volumes of *hydrochloric acid R* and 94 volumes of *water R* and, when the precipitate has dissolved, titrate the excess of iodine with 0.05 M sodium thiosulphate using 1 ml of *starch solution R*, added towards the end of the titration, as indicator. Not less than 12.8 ml of 0.05 M sodium thiosulphate is required.

Heavy metals (2.4.8): maximum 20 ppm.

1.0 g complies with limit test E. Prepare the reference solution using 2 ml of *lead standard solution (10 ppm Pb) R*.

Water (2.5.12): maximum 5.0 per cent, determined on 0.50 g. Use a mixture of 1 volume of *formamide R* and 2 volumes of *methanol R* as solvent.

Total ash (2.4.16): maximum 0.2 per cent.

ASSAY

Dissolve 0.350 g in 50 ml of *carbon dioxide-free water R*, previously heated to 30 °C. Immediately titrate with 0.1 M sodium hydroxide and determine the 2 equivalence points potentiometrically (2.2.20).

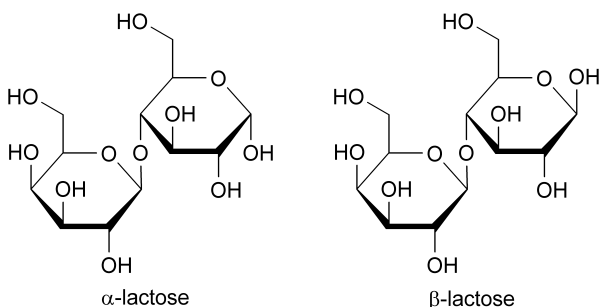
The first equivalence point (V_1) corresponds to the acid form of lactobionic acid and the second equivalence point ($V_2 - V_1$) corresponds to the δ -lactone form.

1 ml of 0.1 M sodium hydroxide is equivalent to 35.83 mg of $C_{12}H_{22}O_{12}$.

1 ml of 0.1 M sodium hydroxide is equivalent to 34.03 mg of $C_{12}H_{20}O_{11}$.

The sum of the 2 results is expressed as a percentage content of lactobionic acid.

01/2005:1061

LACTOSE, ANHYDROUS**Lactosum anhydricum** $C_{12}H_{22}O_{11}$ M_r 342.3**DEFINITION**

Anhydrous lactose is *O*-β-D-galactopyranosyl-(1→4)-β-D-glucopyranose or a mixture of *O*-β-D-galactopyranosyl-(1→4)-α-D-glucopyranose and *O*-β-D-galactopyranosyl-(1→4)-β-D-glucopyranose.

CHARACTERS

A white or almost white, crystalline powder, freely but slowly soluble in water, practically insoluble in ethanol (96 per cent).

IDENTIFICATION

First identification: A, D.

Second identification: B, C, D.

- A. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *anhydrous lactose CRS*.
- B. Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel G plate R*.

Test solution. Dissolve 10 mg of the substance to be examined in a mixture of 2 volumes of *water R* and 3 volumes of *methanol R* and dilute to 20 ml with the same mixture of solvents.

Reference solution (a). Dissolve 10 mg of *anhydrous lactose CRS* in a mixture of 2 volumes of *water R* and 3 volumes of *methanol R* and dilute to 20 ml with the same mixture of solvents.

Reference solution (b). Dissolve 10 mg each of *glucose CRS*, *anhydrous lactose CRS*, *fructose CRS* and *sucrose CRS* in a mixture of 2 volumes of *water R* and 3 volumes of *methanol R* and dilute to 20 ml with the same mixture of solvents.

Apply separately to the plate 2 µl of each solution and thoroughly dry the starting points. Develop over a path of 15 cm using a mixture of 10 volumes of *water R*, 15 volumes of *methanol R*, 25 volumes of *glacial acetic acid R* and 50 volumes of *ethylene chloride R*, measured accurately since a slight excess of water produces cloudiness. Dry the plate in a current of warm air. Repeat the development immediately, after renewing the mobile phase. Dry the plate in a current of warm air and spray evenly with a solution of 0.5 g of *thymol R* in a mixture of 5 ml of *sulphuric acid R* and 95 ml of *alcohol R*. Heat at 130 °C for 10 min. The principal spot in the chromatogram obtained with the test solution is similar

in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a). The test is not valid unless the chromatogram obtained with reference solution (b) shows 4 clearly separated spots.

- C. Dissolve 0.25 g in 5 ml of *water R*. Add 5 ml of *ammonia R* and heat in a water-bath at 80 °C for 10 min. A red colour develops.

- D. It complies with the test for water (see Tests).

TESTS

Appearance of solution. Dissolve 1.0 g in boiling *water R*, dilute to 10 ml with the same solvent. The solution is clear (2.2.1) and not more intensely coloured than reference solution BY₇ (2.2.2, *Method II*).

Acidity or alkalinity. Dissolve 6.0 g by heating in 25 ml of *carbon dioxide-free water R*, cool and add 0.3 ml of *phenolphthalein solution R*. The solution is colourless. Not more than 0.4 ml of 0.1 M *sodium hydroxide* is required to change the colour of the indicator to pink.

Specific optical rotation (2.2.7). Dissolve 10.0 g in 80 ml of *water R*, heating to 50 °C. Allow to cool and add 0.2 ml of *dilute ammonia R1*. Allow to stand for 30 min and dilute to 100.0 ml with *water R*. The specific optical rotation is + 54.4 to + 55.9, calculated with reference to the anhydrous substance.

Absorbance (2.2.25). Dissolve 1.0 g in boiling *water R* and dilute to 10.0 ml with the same solvent (solution A). The absorbance of the solution measured at 400 nm is not greater than 0.04. Dilute 1.0 ml of solution A to 10.0 ml with *water R*. Examine the solution from 210 nm to 300 nm. At wavelengths from 210 nm to 220 nm, the absorbance is not greater than 0.25. At wavelengths from 270 nm to 300 nm, the absorbance is not greater than 0.07.

Heavy metals (2.4.8). 2.0 g complies with limit test C for heavy metals (5 ppm). Prepare the reference solution using 1.0 ml of *lead standard solution (10 ppm Pb) R*.

Water (2.5.12). Not more than 1.0 per cent, determined on 0.50 g by the semi-micro determination of water, using a mixture of 1 volume of *formamide R* and 2 volumes of *methanol R* as the solvent.

Sulphated ash. Not more than 0.1 per cent. To 1.0 g add 1 ml of *sulphuric acid R*, evaporate to dryness on a water-bath and ignite to constant mass.

Microbial contamination. Total viable aerobic count (2.6.12) not more than 10² micro-organisms per gram, determined by plate-count. It complies with the test for *Escherichia coli* (2.6.13)

FUNCTIONALITY-RELATED CHARACTERISTICS

The following test are not mandatory requirements but in view of their known importance for achieving consistency in manufacture, quality and performance of medicinal products, it is recommended that suppliers should verify these characteristics and provide information on the results and analytical method applied to users. The methods indicated below have been found suitable however, other methods may be used.

Anhydrous lactose is predominantly used as a filler/diluent in solid dosage forms (compressed and powder). The following characteristics are relevant for this type of application.

Particle size distribution. Determine by laser diffraction or sieve analysis.

01/2005:0187

Bulk and tapped density (2.9.15). Determine the bulk density and the tapped density. Calculate the Hausner index using the following expression:

$$\frac{V_0}{V_f}$$

V_0 = volume of bulk substance,

V_f = volume of tapped substance.

α -Lactose and β -lactose. Gas chromatography (2.2.28).

Silylation reagent. Mix 28 volumes of *N*-trimethylsilylimidazole *R* and 72 volumes of pyridine *R*.

Test solution. Dissolve about 1 mg of the substance to be examined in 0.45 ml of dimethyl sulphoxide *R*. Add 1.8 ml of the silylation reagent. Mix gently and allow to stand for 20 min.

Reference solution. Prepare a mixture of α -lactose monohydrate *R* and β -lactose *R* having an anomeric ratio of about 1:1 based on the labelled anomeric contents of the α -lactose monohydrate and β -lactose. Dissolve about 1 mg of this mixture in 0.45 ml of dimethyl sulphoxide *R*. Add 1.8 ml of the silylation reagent. Mix gently and allow to stand for 20 min.

Column:

- **material:** glass,
- **size:** $l = 0.9$ m, $\varnothing = 4$ mm,
- **stationary phase:** silanised diatomaceous earth for gas chromatography *R* impregnated with 3 per cent *m/m* of poly[(cyanopropyl)(methyl)][(phenyl)(methyl)]siloxane *R*.

Carrier gas: helium for chromatography *R*.

Flow rate: 40 ml/min.

Temperature:

- **column:** 215 °C,
- **injection port and detector:** 275 °C.

Detection: flame ionisation.

Injection: 2 μ l.

System suitability: reference solution:

- **relative retention** with reference to β -lactose: α -lactose = about 0.7,
- **resolution:** minimum 3.0 between the peaks due to α -lactose and β -lactose.

Calculate the percentage content of α -lactose from the expression:

$$\frac{100S_a}{S_a + S_b}$$

Calculate the percentage content of β -lactose from the expression:

$$\frac{100S_b}{S_a + S_b}$$

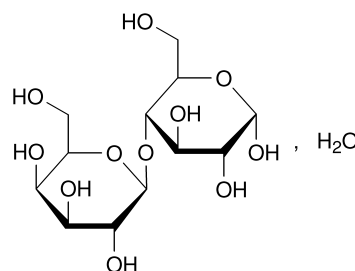
S_a = area of the peak due to α -lactose,

S_b = area of the peak due to β -lactose.

Loss on drying (2.2.32). Determine on 1.000 g by drying in an oven at 80 °C for 2 h.

LACTOSE MONOHYDRATE

Lactosum monohydricum



$C_{12}H_{22}O_{11} \cdot H_2O$

M_r 360.3

DEFINITION

Lactose monohydrate is the monohydrate of *O*- β -D-galactopyranosyl-(1 $\rightarrow$ 4)- α -D-glucopyranose.

CHARACTERS

A white or almost white, crystalline powder, freely but slowly soluble in water, practically insoluble in ethanol (96 per cent).

IDENTIFICATION

First identification: A, D.

Second identification: B, C, D.

A. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with lactose CRS.

B. Examine by thin-layer chromatography (2.2.27), using a TLC silica gel G plate *R*.

Test solution. Dissolve 10 mg of the substance to be examined in a mixture of 2 volumes of water *R* and 3 volumes of methanol *R* and dilute to 20 ml with the same mixture of solvents.

Reference solution (a). Dissolve 10 mg of lactose CRS in a mixture of 2 volumes of water *R* and 3 volumes of methanol *R* and dilute to 20 ml with the same mixture of solvents.

Reference solution (b). Dissolve 10 mg each of fructose CRS, glucose CRS, lactose CRS and sucrose CRS in a mixture of 2 volumes of water *R* and 3 volumes of methanol *R* and dilute to 20 ml with the same mixture of solvents.

Apply separately to the plate 2 μ l of each solution and thoroughly dry the starting points. Develop over a path of 15 cm using a mixture of 10 volumes of water *R*, 15 volumes of methanol *R*, 25 volumes of glacial acetic acid *R* and 50 volumes of ethylene chloride *R*, measured accurately since a slight excess of water produces cloudiness. Dry the plate in a current of warm air. Repeat the development immediately, after renewing the mobile phase. Dry the plate in a current of warm air and spray evenly with a solution of 0.5 g of thymol *R* in a mixture of 5 ml of sulphuric acid *R* and 95 ml of alcohol *R*. Heat at 130 °C for 10 min. The principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a). The test is not valid unless the chromatogram obtained with reference solution (b) shows 4 clearly separated spots.

C. Dissolve 0.25 g in 5 ml of *water R*. Add 5 ml of *ammonia R* and heat in a water-bath at 80 °C for 10 min. A red colour develops.

D. It complies with the test for water (see Tests).

TESTS

Appearance of solution. Dissolve 1.0 g in boiling *water R*, dilute to 10 ml with the same solvent. The solution is clear (2.2.1) and not more intensely coloured than reference solution BY₇ (2.2.2, Method II).

Acidity or alkalinity. Dissolve 6.0 g by heating in 25 ml of *carbon dioxide-free water R*, cool and add 0.3 ml of *phenolphthalein solution R*. The solution is colourless. Not more than 0.4 ml of 0.1 M *sodium hydroxide* is required to change the colour of the indicator to pink.

Specific optical rotation (2.2.7). Dissolve 10.0 g in 80 ml of *water R*, heating to 50 °C. Allow to cool and add 0.2 ml of *dilute ammonia R1*. Allow to stand for 30 min and dilute to 100.0 ml with *water R*. The specific optical rotation is + 54.4 to + 55.9, calculated with reference to the anhydrous substance.

Absorbance (2.2.25). Dissolve 1.0 g in boiling *water R* and dilute to 10.0 ml with the same solvent (solution A). The absorbance of the solution measured at 400 nm is not greater than 0.04. Dilute 1.0 ml of solution A to 10.0 ml with *water R*. Examine the solution from 210 nm to 300 nm. At wavelengths from 210 nm to 220 nm, the absorbance is not greater than 0.25. At wavelengths from 270 nm to 300 nm, the absorbance is not greater than 0.07.

Heavy metals (2.4.8). Dissolve 4.0 g in *water R* with warming, add 1 ml of 0.1 M *hydrochloric acid* and dilute to 20 ml with *water R*. 12 ml of the solution complies with limit test A for heavy metals (5 ppm). Prepare the reference solution using *lead standard solution* (1 ppm Pb) *R*.

Water (2.5.12): 4.5 per cent to 5.5 per cent, determined on 0.50 g by the semi-micro determination of water, using a mixture of 1 volume of *formamide R* and 2 volumes of *methanol R* as the solvent.

Sulphated ash. Not more than 0.1 per cent. To 1.0 g add 1 ml of *sulphuric acid R*, evaporate to dryness on a water-bath and ignite to constant mass.

Microbial contamination. Total viable aerobic count (2.6.12) not more than 10² micro-organisms per gram, determined by plate-count. It complies with the test for *Escherichia coli* (2.6.13).

STORAGE

In an airtight container.

FUNCTIONALITY-RELATED CHARACTERISTICS

The following test are not mandatory requirements but in view of their known importance for achieving consistency in manufacture, quality and performance of medicinal products, it is recommended that suppliers should verify these characteristics and provide information on the results and analytical method applied to users. The methods indicated below have been found suitable however, other methods may be used.

Lactose monohydrate is predominantly used as a filler/diluent in solid dosage forms (compressed and powder). The following characteristics are relevant for this type of application.

Particle size distribution. Determine by laser diffraction or sieve analysis.

Bulk and tapped density (2.9.15). Determine the bulk density and the tapped density. Calculate the Hausner Index using the following expression:

$$\frac{V_0}{V_f}$$

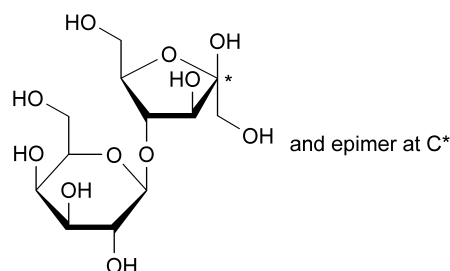
V_0 = volume of bulk substance,

V_f = volume of tapped substance.

01/2005:1230

LACTULOSE

Lactulosum



C₁₂H₂₂O₁₁

M_r 342.3

DEFINITION

Lactulose contains not less than 95.0 per cent and not more than the equivalent of 102.0 per cent of 4-O-(β-D-galactopyranosyl)-D-arabino-hex-2-ulofuranose, calculated with reference to the anhydrous substance.

CHARACTERS

A white or almost white, crystalline powder, freely soluble in water, sparingly soluble in methanol, practically insoluble in toluene.

It melts at about 168 °C.

IDENTIFICATION

First identification: B, C, D, E.

Second identification: A, C, D, E.

A. Examine by thin-layer chromatography (2.2.27), using *silica gel G R* as the coating substance.

Test solution. Dissolve 50.0 mg of the substance to be examined in *water R* and dilute to 10.0 ml with the same solvent.

Reference solution. Dissolve 50.0 mg of *lactulose CRS* in *water R* and dilute to 10.0 ml with the same solvent.

Apply separately to the plate 2 µl of each solution. Develop over a path of 15 cm using a mixture of 10 volumes of *glacial acetic acid R*, 15 volumes of a 50 g/l solution of *boric acid R*, 20 volumes of *methanol R* and 55 volumes of *ethyl acetate R*. Dry the plate at 100-105 °C for 5 min and allow to cool. Spray the plate with a 1.0 g/l solution of *1,3-dihydroxynaphthalene R* in a mixture of 10 volumes of *sulphuric acid R* and 90 volumes of *methanol R*. Heat the plate at 110 °C for 5 min. The principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

- B. Examine the chromatograms obtained in the Assay. The principal peak in the chromatogram obtained with the test solution is similar in position and size to the principal peak in the chromatogram obtained with reference solution (b).
- C. Dissolve 0.05 g in 10 ml of *water R*. Add 3 ml of *cupri-tartaric solution R* and heat. A red precipitate is formed.
- D. Dissolve 0.125 g in 5 ml of *water R*. Add 5 ml of *ammonia R*. Heat on a water-bath at 80 °C for 10 min. A red colour develops.
- E. It complies with the test for specific optical rotation (see Tests).

TESTS

Solution S. Dissolve 3.0 g in *carbon dioxide-free water R* and dilute to 50 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and not more intensely coloured than reference solution BY₅ (2.2.2, Method II).

pH (2.2.3). To 10 ml of solution S add 0.1 ml of a saturated solution of *potassium chloride R*. The pH of the solution is 3.0 to 7.0.

Specific optical rotation (2.2.7). Dissolve 1.25 g of the substance to be examined in *water R*, add 0.2 ml of *concentrated ammonia R* and dilute to 25.0 ml with *water R*. The specific optical rotation is –46.0 to –50.0, calculated with reference to the anhydrous substance.

Related substances. Examine the chromatograms obtained in the Assay. In the chromatogram obtained with the test solution, the sum of the areas of any peaks corresponding to galactose, lactose, epilactose, tagatose and fructose is not greater than the area of the peak corresponding to lactulose in the chromatogram obtained with reference solution (a) (3 per cent).

Methanol. Examine by head-space gas chromatography (2.2.28).

Internal standard solution. Mix 0.5 ml of *propanol R* with 100.0 ml of *water R*. Dilute 1.0 ml to 100.0 ml with *water R*. Dilute 5.0 ml to 50.0 ml with *water R*.

Test solution. To 79 mg of the substance to be examined in a 20 ml vial add 1.0 ml of the internal standard solution and 5 µl of a 0.1 per cent V/V solution of *methanol R*.

Reference solution. To 1.0 ml of the internal standard solution in a 20 ml vial add 5 µl of a 0.1 per cent V/V solution of *methanol R*.

The chromatographic procedure may be carried out using:

- a column 2 m long and 2 mm in internal diameter packed with *ethylvinylbenzene-divinylbenzene copolymer R* (180 µm),
- *helium for chromatography R* as the carrier gas at a flow rate of 30 ml/min,
- a flame-ionisation detector,

maintaining the temperature of the column at 140 °C, that of the injection port at 200 °C and that of the detector at 220 °C. Maintain each solution at 60 °C for 1 h, pressurise for 1 min and transfer onto the column 1 ml of the gaseous phase.

In the chromatogram obtained with the test solution, the ratio of the area of the methanol peak to that of the internal standard peak is not greater than twice the corresponding ratio for the chromatogram obtained with the reference solution (50 ppm, calculated assuming the density (2.2.5) of methanol to be 0.79 g/ml at 20 °C).

Boron. Avoid where possible the use of glassware.

Reference solution. Dissolve 50.0 mg of *boric acid R* in *water R* and dilute to 100.0 ml with the same solvent. Dilute 5.0 ml of this solution to 100.0 ml with *water R*. Keep in a well-closed polyethylene container.

In 4 polyethylene 25 ml flasks, place:

- 0.50 g of the substance to be examined dissolved in 2.0 ml of *water R* (solution A),
- 0.50 g of the substance to be examined dissolved in 1.0 ml of the reference solution and 1.0 ml of *water R* (solution B),
- 1.0 ml of the reference solution and 1.0 ml of *water R* (solution C),
- 2.0 ml of *water R* (solution D).

To each flask, add 4.0 ml of *acetate-edetate buffer solution pH 5.5 R*. Mix and add 4.0 ml of freshly prepared *azomethine H solution R*. Mix and allow to stand for 1 h. Measure the absorbance (2.2.25) of solutions A, B and C at 420 nm, using solution D as the compensation liquid. The test is not valid unless the absorbance of solution C is at least 0.25. The absorbance of solution B is not less than twice that of solution A (9 ppm of boron).

Lead (2.4.10). It complies with the limit test for lead in sugars (0.5 ppm).

Water (2.5.12). Not more than 2.5 per cent, determined on 0.500 g by the semi-micro determination of water.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

Microbial contamination. Total viable aerobic count (2.6.12) not more than 10² micro-organisms per gram, determined by plate count. It complies with the test for *Escherichia coli* (2.6.13).

ASSAY

Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 1.00 g of the substance to be examined in 10 ml of *water R*. Add 12.5 ml of *acetonitrile R* with gentle heating and dilute to 25.0 ml with *water R*.

Reference solution (a). To 3 ml of the test solution add 47.5 ml of *acetonitrile R* with gentle heating and dilute to 100.0 ml with *water R*.

Reference solution (b). Dissolve 1.00 g of *lactulose CRS* in 10 ml of *water R*. Add 12.5 ml of *acetonitrile R* with gentle heating and dilute to 25.0 ml with *water R*.

Reference solution (c). Dissolve the contents of 1 vial of *lactulose for system suitability CRS* in 1 ml of a mixture of equal volumes of *acetonitrile R* and *water R*.

The chromatographic procedure may be carried out using:

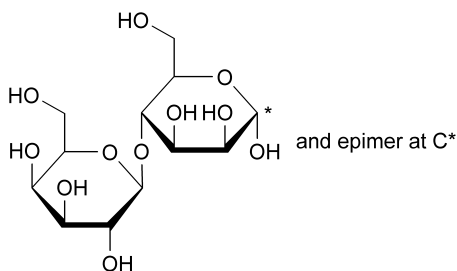
- a stainless steel column 0.05 m long and 4.6 mm in internal diameter followed by a stainless steel column 0.15 m long and 4.6 mm in internal diameter, both packed with *aminopropylsilyl silica gel for chromatography R* (3 µm) and maintained at 38 ± 1 °C,
- as mobile phase at a flow rate of 1.0 ml/min a mixture prepared as follows: dissolve 0.253 g of *sodium dihydrogen phosphate R* in 220 ml of *water R* and add 780 ml of *acetonitrile R*,
- as detector a refractometer maintained at a constant temperature.

When the chromatograms are recorded in the prescribed conditions, the retention time of lactulose is about 18.3 min and relative retentions to lactulose are about 0.38 for tagatose, 0.42 for fructose, 0.57 for galactose, 0.90 for epilactose and 1.17 for lactose.

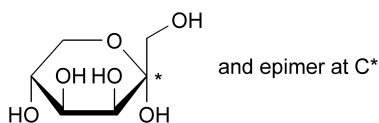
Inject 20 µl of reference solution (c). The test is not valid unless the chromatogram obtained has a similar profile to the chromatogram supplied with *lactulose for system suitability CRS* and the resolution between the peaks corresponding to lactulose and epilactose is at least 1.3. If necessary, adjust the concentration of *acetonitrile R* in the mobile phase to between 75.0 per cent V/V and 82.0 per cent V/V to achieve the prescribed resolution.

Inject separately 20 µl of the test solution and 20 µl of reference solution (b) and continue the chromatography for 2.5 times the retention time of lactulose. Calculate the percentage content of C₁₂H₂₂O₁₁ (lactulose) from the areas of the peaks and the declared content of *lactulose CRS*.

IMPURITIES



- A. 4-O-(β-D-galactopyranosyl)-D-mannopyranose (epilactose),
 B. galactose,
 C. lactose,
 D. fructose,



- E. D-*lyxo*-hex-2-ulopyranose (tagatose).

01/2005:0924

LACTULOSE, LIQUID

Lactulosum liquidum

DEFINITION

Liquid lactulose is an aqueous solution of 4-O-(β-D-galactopyranosyl)-D-*arabino*-hex-2-ulofuranose normally prepared by alkaline isomerisation of lactose. It may contain lesser amounts of other sugars including lactose, epilactose, galactose, tagatose and fructose. It contains not less than 620 g/l of lactulose (C₁₂H₂₂O₁₁; *M_r* 342.3) and not less than 95.0 per cent and not more than 105.0 per cent of the declared content of lactulose. It may contain a suitable antimicrobial preservative.

CHARACTERS

A clear, viscous liquid, colourless or pale brownish-yellow, miscible with water. It may be a supersaturated solution or may contain crystals which disappear on heating.

A 10 per cent V/V solution is laevorotatory.

IDENTIFICATION

First identification: B, C, D.

Second identification: A, C, D.

- A. Examine by thin-layer chromatography (2.2.27), using *silica gel G R* as the coating substance.

Test solution. Dilute 0.50 g of the substance to be examined to 50 ml with *water R*.

Reference solution. Dissolve 60 mg of *lactulose CRS* in *water R* and dilute to 10 ml with the same solvent.

Apply separately to the plate 2 µl of each solution. Develop over a path of 15 cm using a mixture of 10 volumes of *glacial acetic acid R*, 15 volumes of a 50 g/l solution of *boric acid R*, 20 volumes of *methanol R* and 55 volumes of *ethyl acetate R*. Dry the plate at 100-105 °C for 5 min and allow to cool. Spray the plate with a 1.0 g/l solution of *1,3-dihydroxynaphthalene R* in a mixture of 10 volumes of *sulphuric acid R* and 90 volumes of *methanol R*. Heat the plate at 110 °C for 5 min. The principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

- B. Examine the chromatograms obtained in the Assay. The retention time of the principal peak in the chromatogram obtained with the test solution is approximately the same as that of the principal peak in the chromatogram obtained with reference solution (b).
- C. To 0.1 g add 10 ml of *water R* and 3 ml of *cupri-tartaric solution R* and heat. A red precipitate is formed.
- D. To 0.25 g add 5 ml of *water R* and 5 ml of *ammonia R*. Heat in a water-bath at 80 °C for 10 min. A red colour develops.

TESTS

Solution S. Mix 10 g with *carbon dioxide-free water R* and dilute to 100 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and not more intensely coloured than reference solution BY₅ (2.2.2, *Method II*).

pH (2.2.3). To 10 ml of solution S, add 0.1 ml of a saturated solution of *potassium chloride R*. The pH of the solution is 3.0 to 7.0.

Related substances. Examine the chromatograms obtained in the Assay. In the chromatogram obtained with the test solution: the area of any peak corresponding to galactose is not greater than 3 times the area of the peak corresponding to lactulose in the chromatogram obtained with reference solution (a) (15 per cent); the area of any peak corresponding to lactose is not greater than twice the area of the peak corresponding to lactulose in the chromatogram obtained with reference solution (a) (10 per cent); the area of any peak corresponding to epilactose is not greater than twice the area of the peak corresponding to lactulose in the chromatogram obtained with reference solution (a) (10 per cent); the area of any peak corresponding to tagatose is not greater than 0.8 times the area of the peak corresponding to lactulose in the chromatogram obtained with reference solution (a) (4 per cent); the area of any peak corresponding to fructose is not greater than 0.2 times the area of the peak corresponding to lactulose in the chromatogram obtained with reference solution (a) (1 per cent).

Methanol. Examine by head-space gas chromatography (2.2.28).

Internal standard solution. Mix 0.5 ml of *propanol R* with 100.0 ml of *water R*. Dilute 1.0 ml to 100.0 ml with *water R*. Dilute 5.0 ml to 50.0 ml with *water R*.

Test solution. To 0.13 g of the substance to be examined in a 20 ml vial add 1.0 ml of the internal standard solution. Add 5 µl of a 0.1 per cent V/V solution of *methanol R*.

Reference solution. To 1.0 ml of the internal standard solution in a 20 ml vial add 5 µl of a 0.1 per cent V/V solution of *methanol R*.

The chromatographic procedure may be carried out using:

- a column 2 m long and 2 mm in internal diameter packed with *ethylvinylbenzene-divinylbenzene copolymer R* (180 µm),
- *helium for chromatography R* as the carrier gas at a flow rate of 30 ml/min,
- a flame-ionisation detector,

maintaining the temperature of the column at 140 °C, that of the injection port at 200 °C and that of the detector at 220 °C. Maintain each solution at 60 °C for 1 h, pressurise for 1 min and transfer onto the column 1 ml of the gaseous phase.

In the chromatogram obtained with the test solution, the ratio of the area of the methanol peak to that of the internal standard peak is not greater than twice the corresponding ratio for the chromatogram obtained with the reference solution (30 ppm calculated assuming the density (2.2.5) of methanol to be 0.79 g/ml at 20 °C).

Sulphites. Mix 5.0 g with 40 ml of *water R*, add 2.0 ml of 0.1 M *sodium hydroxide* and dilute to 100 ml with *water R*. To 10.0 ml of the solution, add 1.0 ml of *hydrochloric acid R1*, 2.0 ml of *decolorised fuchsin solution R1* and 2.0 ml of a 0.5 per cent V/V solution of *formaldehyde R*. Allow to stand for 30 min and measure the absorbance (2.2.25) of the solution at 583 nm using as the compensation liquid a solution prepared simultaneously and in the same manner using 10.0 ml of *water R* instead of the solution of the substance to be examined. The absorbance is not greater than that of a reference solution prepared simultaneously and in the same manner, using 10.0 ml of *sulphite standard solution* (1.5 ppm SO₂) *R* instead of the solution of the substance to be examined (30 ppm).

Boron. Avoid where possible the use of glassware.

Reference solution. Dissolve 56.0 mg of *boric acid R* in *water R* and dilute to 100.0 ml with the same solvent. Dilute 5.0 ml of this solution to 100.0 ml with *water R*. Keep in a well-closed polyethylene container.

In 4 polyethylene 25 ml flasks, place:

- 1.00 g of the substance to be examined and 1 ml of *water R* (solution A),
- 1.00 g of the substance to be examined and 1 ml of the reference solution (solution B),
- 1 ml of the reference solution and 1 ml of *water R* (solution C),
- 2 ml of *water R* (solution D).

To each flask, add 4.0 ml of *acetate-edetate buffer solution pH 5.5 R*. Mix and add 4.0 ml of freshly prepared *azomethine H solution R*. Mix and allow to stand for 1 h. Measure the absorbance (2.2.25) of solutions A, B and C at 420 nm, using solution D as the compensation liquid. The test is not valid unless the absorbance of solution C is at least 0.25. The absorbance of solution B is not less than twice that of solution A (5 ppm of B).

Lead (2.4.10). It complies with the limit test for lead in sugars (0.5 ppm, calculated with reference to the declared content of lactulose).

Sulphated ash (2.4.14). Not more than 0.2 per cent, determined on 1.5 g and calculated with reference to the declared content of lactulose.

Microbial contamination. Total viable aerobic count (2.6.12) not more than 10² micro-organisms per gram, determined by plate count. It complies with the test for *Escherichia coli* (2.6.13).

ASSAY

Examine by liquid chromatography (2.2.29).

Test solution. Mix 4.00 g of the substance to be examined with 20 ml of *water R*. Add 25.0 ml of *acetonitrile R* with gentle heating and dilute to 50.0 ml with *water R*.

Reference solution (a). To 5 ml of the test solution, add 47.5 ml of *acetonitrile R* with gentle heating and dilute to 100.0 ml with *water R*.

Reference solution (b). Dissolve 2.00 g of *lactulose CRS* in 20 ml of *water R*. Add 25.0 ml of *acetonitrile R* with gentle heating and dilute to 50.0 ml with *water R*.

Reference solution (c). Dissolve the contents of 1 vial of *lactulose for system suitability CRS* in 1 ml of a mixture of equal volumes of *acetonitrile R* and *water R*.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.05 m long and 4.6 mm in internal diameter followed by a stainless steel column 0.15 m long and 4.6 mm in internal diameter, both packed with *aminopropylsilyl silica gel for chromatography R* (3 µm) and maintained at 38 ± 1 °C,
- as mobile phase at a flow rate of 1.0 ml/min a mixture prepared as follows: dissolve 0.253 g of *sodium dihydrogen phosphate R* in 220 ml of *water R* and add 780 ml of *acetonitrile R*,
- as detector a refractometer maintained at a constant temperature.

When the chromatograms are recorded in the prescribed conditions, the retention time of lactulose is about 18 min and relative retentions to lactulose are about: 0.38 for tagatose, 0.42 for fructose, 0.57 for galactose, 0.90 for epilactose and 1.17 for lactose.

Inject 20 µl of reference solution (c). The test is not valid unless the chromatogram obtained has a similar profile to the chromatogram supplied with *lactulose for system suitability CRS* and the resolution between the peaks corresponding to lactulose and epilactose is at least 1.3. If necessary, adjust the concentration of *acetonitrile R* in the mobile phase to between 75.0 per cent V/V and 82.0 per cent V/V to achieve the prescribed resolution.

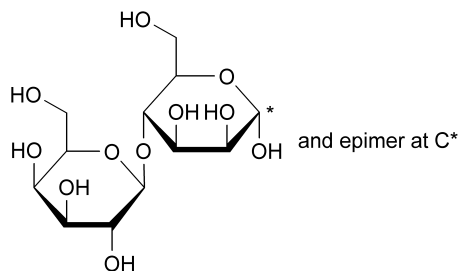
Inject separately 20 µl of the test solution and 20 µl of reference solution (b) and continue the chromatography for 2.5 times the retention time of lactulose. Calculate the percentage content of C₁₂H₂₂O₁₁ (lactulose) from the areas of the peaks and the declared content of *lactulose CRS*.

LABELLING

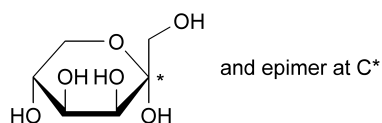
The label states:

- the declared content of lactulose,
- the name and concentration of any added antimicrobial preservative.

IMPURITIES



- A. 4-O-(β-D-galactopyranosyl)-D-mannopyranose (epilactose),
 B. galactose,
 C. lactose,
 D. fructose,



- E. D-lyxo-hex-2-ulopyranose (tagatose).

01/2005:1231

LAUROYL MACROGOLGLYCERIDES

Macrogolglyceridorum laurates

DEFINITION

Lauroyl macrogolglycerides are mixtures of monoesters, diesters and triesters of glycerol and monoesters and diesters of macrogols with a mean relative molecular mass between 300 and 1500. They are obtained by partial alcoholysis of saturated oils mainly containing triglycerides of lauric acid using macrogol or by esterification of glycerol and macrogol with saturated fatty acids or by mixing of glycerol esters and condensates of ethylene oxide with the fatty acids of these hydrogenated oils.

CHARACTERS

Pale yellow waxy solids, dispersible in hot water, freely soluble in methylene chloride.

IDENTIFICATION

- A. Examine by thin-layer chromatography (2.2.27), using a suitable silica gel as the coating substance.
Test solution. Dissolve 1.0 g of the substance to be examined in *methylene chloride R* and dilute to 20 ml with the same solvent.
 Apply to the plate 10 µl of the test solution. Develop over a path of 15 cm using a mixture of 30 volumes of *hexane R* and 70 volumes of *ether R*. Allow the plate to dry in air. Spray with a 0.1 g/l solution of *rhodamine B R* in *alcohol R* and examine in ultraviolet light at 365 nm. The chromatogram shows a spot corresponding to triglycerides with an R_f value of about 0.9 (R_{st} 1) and spots corresponding to 1,3-diglycerides (R_{st} 0.7), to 1,2-diglycerides (R_{st} 0.6), to monoglycerides (R_{st} 0.1) and to esters of macrogol (R_{st} 0).
 B. They comply with the test for hydroxyl value (see Tests).
 C. They comply with the test for fatty acid composition (see Tests).
 D. They comply with the test for saponification value (see Tests).

TESTS

Drop point (2.2.17). Introduce into the cup the substance to be examined which has been melted by heating for 1 h in an oven at 100 ± 2 °C and allow to stand for 5 h at about 5 °C. The drop point is indicated in Table 1231.-1.

Table 1231.-1

Ethylene oxide units per molecule (nominal value)	Type of macrogol	Drop point
6	300	33 to 38
8	400	36 to 41
12	600	38 to 43
32	1500	42.5 to 47.5

Acid value (2.5.1). Not more than 2.0, determined on 2.0 g.

Hydroxyl value (2.5.3, *Method A*). The ranges are presented in Table 1231.-2, determined on 1.0 g.

Table 1231.-2

Ethylene oxide units per molecule (nominal value)	Type of macrogol	Hydroxyl value
6	300	65 to 85
8	400	60 to 80
12	600	50 to 70
32	1500	36 to 56

Peroxide value (2.5.5). Not more than 6.0, determined on 2.0 g.

Saponification value (2.5.6). The ranges are presented in Table 1231.-3, determined on 2.0 g.

Table 1231.-3

Ethylene oxide units per molecule (nominal value)	Type of macrogol	Saponification value
6	300	190 to 204
8	400	170 to 190
12	600	150 to 170
32	1500	79 to 93

Alkaline impurities. Introduce 5.0 g into a test tube and carefully add a mixture, neutralised if necessary with 0.01 M *hydrochloric acid* or with 0.01 M *sodium hydroxide*, of 0.05 ml of a 0.4 g/l solution of *bromophenol blue R* in *alcohol R*, 0.3 ml of *water R* and 10 ml of *alcohol R*. Shake and allow to stand. Not more than 1.0 ml of 0.01 M *hydrochloric acid* is required to change the colour of the upper layer to yellow.

Free glycerol. Not more than 3.0 per cent. Dissolve 1.20 g in 25.0 ml of *methylene chloride R*. Heat if necessary. After cooling, add 100 ml of *water R*. Shake and add 25.0 ml of a 6 g/l solution of *periodic acid R*. Shake and allow to stand for 30 min. Add 40 ml of a 75 g/l solution of *potassium iodide R*. Allow to stand for 1 min. Add 1 ml of *starch solution R*. Titrate the iodine with 0.1 M *sodium thiosulphate*. Carry out a blank titration.

1 ml of 0.1 M *sodium thiosulphate* is equivalent to 2.3 mg of glycerol.

Composition of fatty acids (2.4.22, *Method A*). The fatty acid fraction has the following composition:

- *caprylic acid*: not more than 15.0 per cent,
- *capric acid*: not more than 12.0 per cent,
- *lauric acid*: 30.0 per cent to 50.0 per cent,

- *myristic acid*: 5.0 per cent to 25.0 per cent,
- *palmitic acid*: 4.0 per cent to 25.0 per cent,
- *stearic acid*: 5.0 per cent to 35.0 per cent.

Ethylene oxide and dioxan (2.4.25). Not more than 1 ppm of ethylene oxide and not more than 10 ppm of dioxan.

Heavy metals (2.4.8). 2.0 g complies with limit test C for heavy metals (10 ppm). Prepare the standard using 2 ml of *lead standard solution* (10 ppm Pb) R.

Water (2.5.12). Not more than 1.0 per cent, determined on 1.0 g by the semi-micro determination of water using a mixture of 30 volumes of *anhydrous methanol R* and 70 volumes of *methylene chloride R* as solvent.

Total ash (2.4.16). Not more than 0.1 per cent, determined on 1.0 g.

LABELLING

The label states the type of macrogol used (mean relative molecular mass) or the number of units of ethylene oxide per molecule (nominal value).

01/2005:1534

LAVENDER FLOWER

Lavandulae flos

DEFINITION

Lavender flower consists of the dried flower of *Lavandula angustifolia* P. Mill. (*L. officinalis* Chaix). It contains not less than 13 ml/kg of essential oil, calculated with reference to the anhydrous drug.

CHARACTERS

Lavender flower has a strongly aromatic odour.

It has the macroscopic and microscopic characters described under identification tests A and B.

IDENTIFICATION

First identification: A, B, D.

Second identification: A, B, C.

- A. The flower has a short peduncle and consists of a bluish-grey tubular calyx divided distally into four very short teeth and a small rounded lobe, a blue bilabial corolla with the upper lip bifid and the lower lip trilobate, four didynamous stamens with ovoid anthers.
- B. Reduce to a powder (355). The powder is bluish-grey. Examine under a microscope using *chloral hydrate solution R*. The powder shows covering trichomes bifurcating at one or more levels; secretory trichomes with short stalks and eight-celled heads of the *Labiatae* type; secretory trichomes with unicellular or multicellular stalks and unicellular heads; secretory trichomes with long uneven stalks and unicellular heads, separated from the peduncle by an intermediary cell with a smooth cuticle; certain such trichomes show a crown of small spheroid cells just below the insertion point of the intermediary cell on the peduncle; fragments of warty epidermis from the inner surface of the petals; fragments of calyx epidermis with sinuous-walled cells and containing prismatic crystals of calcium oxalate; spherical pollen grains which have a diameter of about 45 µm and an exine with six slit-like germinal pores and six ribbon-like groins radiating from the poles.

- C. Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel plate R*.

Test solution. To 0.5 g of the powdered drug (355) add 5 ml of *hexane R*, shake for 5 min and filter.

Reference solution. Dissolve 10 µl of *linalol R* and 10 µl of *linalyl acetate R* in 5 ml of *hexane R*.

Apply to the plate as bands 10 µl of each solution. Develop over a path of 15 cm using a mixture of 5 volumes of *ethyl acetate R* and 95 volumes of *toluene R*. Allow the plate to dry in air. Spray the plate with *anisaldehyde solution R* and examine in daylight while heating at 100 °C to 105 °C for 5 min to 10 min. The chromatogram obtained with the reference solution shows in the lower third a greyish-blue zone (linalol) and in the middle third a greyish-blue zone (linalyl acetate). The chromatogram obtained with the test solution shows zones corresponding to linalol and linalyl acetate and in the middle, between these zones, a redish-violet zone (epoxydihydrocaryophyllene). Further zones are also present.

- D. Examine the chromatograms obtained in the test for other species and varieties of lavender. The five principal peaks in the chromatogram obtained with the reference solution are similar in retention time to peaks in the chromatogram obtained with the test solution. Among them are mainly linalol and linalyl acetate peaks.

TESTS

Foreign matter (2.8.2). Not more than 3 per cent of stems and not more than 2 per cent of other foreign matter.

Other species and varieties of lavender. Examine by gas chromatography (2.2.28).

Test solution. Dilute 0.2 ml of the essential oil-xylene mixture obtained in the assay to 5 ml with *hexane R*, add 1 g of *anhydrous sodium sulphate R*, shake and use the supernatant liquid.

Reference solution. Dissolve 0.1 g of *limonene R*, 0.2 g of *cineole R*, 0.05 g of *camphor R*, 0.4 g of *linalol R*, 0.6 g of *linalyl acetate R* and 0.2 g of *α-terpineol R* in 100 ml of *hexane R*.

The chromatographic procedure may be carried out using:

- a fused-silica column about 60 m long and about 0.25 mm in internal diameter, coated with *macrogol 20 000 R*,
- *helium for chromatography R* as the carrier gas at a flow rate of 1.5 ml per/min,
- a flame-ionisation detector,
- a split ratio of 1:100,

with the following temperature programme:

	Time (min)	Temperature (°C)	Rate (°C/min)	Comment
Column	0 - 15	70	–	isothermal
	15 - 70	70 → 180	2	linear gradient
Injection port		220		
Detector		220		

Inject the same volume of each solution. When the chromatograms are recorded under the prescribed conditions, the components are eluted in the order indicated in the composition of the reference solution. Record the retention times of these substances.

The test is not valid unless: the number of theoretical plates calculated from the limonene peak at 110 °C is at least 30 000, and the resolution between the peak corresponding to limonene and that corresponding to cineole is at least 1.5.

Using the retention times determined from the chromatogram obtained with the reference solution, locate the six components of the reference solution in the chromatogram obtained with the test solution. Disregard the peaks due to hexane and xylene.

In the chromatogram obtained with the test solution the area of the peak due to camphor is not greater than 1 per cent of the total area of the peaks.

Water (2.2.13). Not more than 100 ml/kg, determined by distillation using 20.0 g of drug.

Total ash (2.4.16). Not more than 9.0 per cent.

ASSAY

Carry out the determination of essential oils in vegetable drugs (2.8.12). Use 20.0 g of the drug, a 1000 ml round-bottomed flask, 500 ml of *water R* as the distillation liquid and 0.5 ml of *xylene R* in the graduated tube. Distil at a rate of 2 ml/min to 3 ml/min for 2 h.

STORAGE

Store protected from light.

01/2005:1338

LAVENDER OIL

Lavandulae aetheroleum

DEFINITION

Essential oil obtained by steam distillation from the flowering tops of *Lavandula angustifolia* Miller (*Lavandula officinalis* Chaix).

CHARACTERS

Appearance: colourless or pale yellow, clear liquid.

It has a characteristic odour.

IDENTIFICATION

First identification: B.

Second identification: A.

A. Thin-layer chromatography (2.2.27).

Test solution. Dissolve 20 µl of the substance to be examined in 1 ml of *toluene R*.

Reference solution. Dissolve 10 µl of *linalol R* and 10 µl of *linalyl acetate R* in 1 ml of *toluene R*.

Plate: TLC silica gel plate *R*.

Mobile phase: *ethyl acetate R*, *toluene R* (5:95 V/V).

Application: 10 µl as bands.

Development: twice, 5 min apart, over a path of 10 cm.

Drying: in air.

Detection: spray with *anisaldehyde solution R* and heat at 100-105 °C for 5-10 min. Examine immediately in daylight.

Results: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other violet-red or greenish-brown zones are present in the chromatogram obtained with the test solution above the zone of linalyl acetate up to the solvent front.

Top of the plate	
	Several violet-red or greenish-brown zones
Linalyl acetate: a violet to brown zone	A violet to brown zone (linalyl acetate) A violet-red zone Possibly a weak violet-brown zone (cineole)
Linalol: a violet to brown zone	A violet to brown zone (linalol) A weak brownish-green zone Several unresolved zones
Reference solution	Test solution

B. Examine the chromatograms obtained in the test for chromatographic profile.

Results: the characteristic peaks in the chromatogram obtained with the test solution are similar in retention time to those in the chromatogram obtained with reference solution (a).

TESTS

Relative density (2.2.5): 0.878 to 0.892.

Refractive index (2.2.6): 1.455 to 1.466.

Optical rotation (2.2.7): -12.5° to -7.0° .

Acid value (2.5.1): maximum 1.0, determined on 5.0 g of the substance to be examined dissolved in 50 ml of the prescribed mixture of solvents.

Chromatographic profile. Gas chromatography (2.2.28): use the normalisation procedure.

Test solution. The substance to be examined.

Reference solution (a). Dissolve 0.1 g of *limonene R*, 0.2 g of *cineole R*, 0.2 g of *3-octanone R*, 0.05 g of *camphor R*, 0.4 g of *linalol R*, 0.6 g of *linalyl acetate R*, 0.2 g of *terpinen-4-ol R*, 0.1 g of *lavandulyl acetate R*, 0.2 g of *lavandulol R* and 0.2 g of α -*terpineol R* in 5 ml of *hexane R*.

Reference solution (b). Dissolve 5 mg of *3-octanone R* in *hexane R* and dilute to 10 ml with the same solvent.

Column:

- **material:** fused silica,
- **size:** $l = 60\text{ m}$, $\varnothing = 0.25\text{ mm}$,
- **stationary phase:** *macrogol 20 000 R* (film thickness 0.25 mm).

Carrier gas: *helium for chromatography R*.

Flow rate: 1.5 ml/min.

Split ratio: 1:100.

Temperature:

	Time (min)	Temperature (°C)
Column	0 - 15	70
	15 - 70	70 → 180
Injection port		220
Detector		220

Detection: flame ionisation.

Injection: 0.2 µl.

Elution order: order indicated in the composition of reference solution (a). Record the retention times of these substances.

System suitability: reference solution (a):

- **resolution:** minimum 1.4 between the peaks due to terpinen-4-ol and lavandulyl acetate.

Using the retention times determined from the chromatogram obtained with reference solution (a), locate the components of reference solution (a) in the chromatogram obtained with the test solution.

Determine the percentage content of each of these components. The percentages are within the following ranges:

- **limonene:** less than 1.0 per cent,
- **cineole:** less than 2.5 per cent,
- **3-octanone:** 0.1 per cent to 2.5 per cent,
- **camphor:** less than 1.2 per cent,
- **linalol:** 20.0 per cent to 45.0 per cent,
- **linalyl acetate:** 25.0 per cent to 46.0 per cent,
- **terpinen-4-ol:** 0.1 per cent to 6.0 per cent,
- **lavandulyl acetate:** more than 0.2 per cent,
- **lavandulol:** more than 0.1 per cent,
- **α-terpineol:** less than 2.0 per cent.
- **disregard limit:** area of the peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

Chiral purity. Gas chromatography (2.2.28).

Test solution. Dissolve 0.02 g of the substance to be examined in *pentane R* and dilute to 10 ml with the same solvent.

Reference solution. Dissolve 10 µl of *linalol R*, add 10 µl of *linalyl acetate R* and 5 mg of *borneol R* in *pentane R* and dilute to 10 ml with the same solvent.

Column:

- **material:** fused silica,
- **size:** *l* = 25 m, Ø = 0.25 mm,
- **stationary phase:** modified β-cyclodextrin for chiral chromatography *R* (film thickness 0.25 µm).

Carrier gas: helium for chromatography *R*.

Flow rate: 1.3 ml/min.

Split ratio: 1:30.

Temperature:

	Time (min)	Temperature (°C)
Column	0 - 65	50 → 180
Injection port		230
Detector		230

Detection: flame ionisation.

Injection: 1 µl.

System suitability: reference solution:

- **resolution:** minimum 5.5 between the peaks due to (*R*)-linalol (1st peak) and (*S*)-linalol (2nd peak), minimum 2.9 between the peaks due to (*S*)-linalol and borneol (3rd peak) and minimum 2.7 between the peaks due to (*R*)-linalyl acetate (4th peak) and (*S*)-linalyl acetate (5th peak).

Limits: calculate the percentage content of the specified (*S*)-enantiomers from the expression:

$$\frac{A_S}{A_S + A_R} \times 100$$

A_S = area of the peak due to the corresponding (*S*)-enantiomer,

A_R = area of the peak due to the corresponding (*R*)-enantiomer.

- (*S*)-linalol: maximum 12 per cent,

- (*S*)-linalyl acetate: maximum 1 per cent.

STORAGE

In a well-filled, airtight container, protected from light, at a temperature not exceeding 25 °C.

01/2005:0620
corrected

LEMON OIL

Limonis aetheroleum

DEFINITION

Essential oil obtained by suitable mechanical means, without the aid of heat, from the fresh peel of *Citrus limon* (L.) Burman fil.

CHARACTERS

Appearance: clear, mobile, pale yellow to greenish-yellow liquid with a characteristic odour. It may become cloudy at low temperatures.

IDENTIFICATION

First identification: *B*.

Second identification: *A*.

A. Thin-layer chromatography (2.2.27).

Test solution. Mix 1 ml of the substance to be examined in 1 ml of *toluene R*.

Reference solution. Dissolve 10 mg of *citropten R* and 50 µl of *citral R* in *toluene R* and dilute to 10 ml with the same solvent.

Plate: TLC silica gel GF₂₅₄ plate *R*.

Mobile phase: *ethyl acetate R*, *toluene R* (15:85 *V/V*).

Application: 10 µl, as bands.

Development: over a path of 15 cm.

Drying: in air.

Detection A: examine in ultraviolet light at 254 nm.

Results A: see below the sequence of the zones present in the chromatograms obtained with the reference solution and the test solution.

Top of the plate	
Citral: a quenching zone	A quenching zone (bergamotin)
	A quenching zone (citral)
Citropten: a light blue fluorescent zone	A dark blue zone (5-geranyloxy-7-methoxycoumarin)
	A light blue fluorescent zone (citropten)
	A quenching zone (psoralen derivative)
	A quenching zone (biakangelicin)
Reference solution	Test solution

Detection B: examine in ultraviolet light at 365 nm.

Results B: see below the sequence of the zones present in the chromatograms obtained with the reference solution and the test solution.

Top of the plate	
Citral: a quenching zone	A yellow fluorescent zone (bergamotin)
	A quenching zone (citral)
Citropten: a bright blue fluorescent zone	A bright blue fluorescent zone (5-geranyloxy-7-methoxycoumarin)
	A bright violet-blue fluorescent zone (citropten)
	A yellow fluorescent zone (psoralen derivative)
	An orange zone (biakangelicin)
Reference solution	Test solution

B. Examine the chromatograms obtained in the test for chromatographic profile.

Results: the characteristic peaks in the chromatogram obtained with the test solution are similar in retention time to those in the chromatogram obtained with the reference solution.

TESTS

Relative density (2.2.5): 0.850 to 0.858.

Refractive index (2.2.6): 1.473 to 1.476.

Optical rotation (2.2.7): + 57° to + 70°.

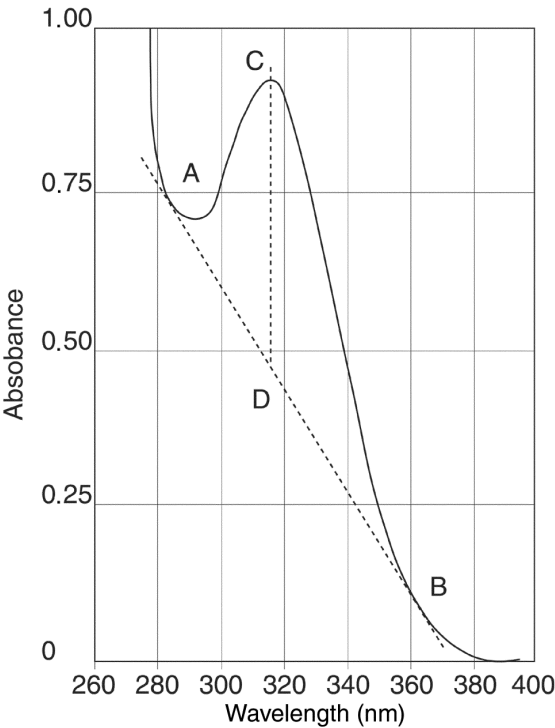


Figure 0620.-1. – Typical spectrum of lemon oil for the test for absorbance

Absorbance (2.2.25). Dissolve 0.250 g of the substance to be examined in *alcohol R*, mix and dilute to 100.0 ml with the same solvent. Measure the absorbance over the range 260 nm to 400 nm. If a manual instrument is used, measure the absorbance at 5 nm intervals from 260 nm to about 12 nm before the expected absorption maximum, then at 3 nm intervals for 3 readings and at 1 nm intervals to about 5 nm beyond the maximum and finally at 10 nm intervals to

400 nm. Plot a curve representing the absorption spectrum with the absorbances as ordinates and the wavelengths as abscissae. Draw as a baseline the tangent between A and B (Figure 0620.-1). The absorption maximum C is situated at 315 ± 3 nm. From C draw a line perpendicular to the axis of abscissae and intersecting AB at D. Deduct the absorbance corresponding to point D from that corresponding to point C. The value $C - D$ is 0.20 to 0.96 and for Italian-type lemon oil it is not less than 0.45.

Fatty oils and resinified essential oils (2.8.7). It complies with the test for fatty oils and resinified essential oils.

Chromatographic profile. Gas chromatography (2.2.28): use the normalisation procedure.

Test solution. The substance to be examined.

Reference solution. Dissolve 20 µl of *β-pinene R*, 10 µl of *sabinene R*, 100 µl of *limonene R*, 10 µl of *γ-terpinene R*, 5 µl of *β-caryophyllene R*, 20 µl of *citral R*, 5 µl of *α-terpineol R*, 5 µl of *neryl acetate R* and 5 µl of *geranyl acetate R* in 1 ml of *acetone R*.

Column:

- **material:** fused silica,
- **size:** $l = 30$ m (a film thickness of 1 µm may be used) to 60 m (a film thickness of 0.2 µm may be used), $\varnothing = 0.25$ -0.53 mm,
- **stationary phase:** *macrogol 20 000 R*.

Carrier gas: *helium for chromatography R*.

Flow rate: 1.0 ml/min.

Split ratio: 1:100.

Temperature:

	Time (min)	Temperature (°C)
Column	0 - 6	45
	6 - 21	45 → 90
	21 - 39	90 → 180
	39 - 55	180
Injection port		220
Detector		220

Detection: flame ionisation.

Injection: 0.5 µl of the reference solution and 0.2 µl of the test solution.

Elution order: order indicated in the composition of the reference solution. Record the retention times of these substances.

System suitability: reference solution:

- **resolution:** minimum 1.5 between the peaks due to *β-pinene* and *sabinene* and minimum 1.5 between the peaks due to *geranial* and *geranyl acetate*.

Using the retention times determined from the chromatogram obtained with the reference solution, locate the components of the reference solution in the chromatogram obtained with the test solution.

Determine the percentage content of these components. The percentages are within the following ranges:

- *β-pinene*: 7.0 per cent to 17.0 per cent,
- *sabinene*: 1.0 per cent to 3.0 per cent,
- *limonene*: 56.0 per cent to 78.0 per cent,
- *γ-terpinene*: 6.0 per cent to 12.0 per cent,
- *β-caryophyllene*: maximum 0.5 per cent,
- *neral*: 0.3 per cent to 1.5 per cent,
- *α-terpineol*: maximum 0.6 per cent,
- *neryl acetate*: 0.2 per cent to 0.9 per cent,
- *geranial*: 0.5 per cent to 2.3 per cent,

– *geranyl acetate*: 0.1 per cent to 0.8 per cent.

Residue on evaporation (2.8.9): 1.8 per cent to 3.6 per cent after heating on the water-bath for 4 h.

STORAGE

In a well-filled, airtight container, protected from light, at a temperature not exceeding 25 °C.

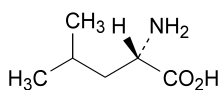
LABELLING

The label states, where applicable, that the contents are Italian-type lemon oil.

01/2005:0771

LEUCINE

Leucinum

C₆H₁₃NO₂M_r 131.2

DEFINITION

Leucine contains not less than 98.5 per cent and not more than the equivalent of 101.0 per cent of (S)-2-amino-4-methylpentanoic acid, calculated with reference to the dried substance.

CHARACTERS

White or almost white crystalline powder or shiny flakes, sparingly soluble in water, practically insoluble in alcohol. It dissolves in dilute mineral acids and in dilute solutions of alkali hydroxides.

IDENTIFICATION

First identification: A, C.

Second identification: A, B, D.

- It complies with the test for specific optical rotation (see Tests).
- Dissolve 0.50 g in *water R* and dilute to 25 ml with the same solvent. The solution is laevorotatory.
- Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *leucine CRS*. Examine the substances prepared as discs.
- Examine the chromatograms obtained in the test for ninhydrin-positive substances. The principal spot in the chromatogram obtained with test solution (b) is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).

TESTS

Appearance of solution. Dissolve 0.5 g in 1 M *hydrochloric acid* and dilute to 10 ml with the same acid. The solution is clear (2.2.1) and not more intensely coloured than reference solution BY₆ (2.2.2, *Method II*).

Specific optical rotation (2.2.7). Dissolve 1.00 g in *hydrochloric acid R1* and dilute to 25.0 ml with the same acid. The specific optical rotation is + 14.5 to + 16.5, calculated with reference to the dried substance.

Ninhydrin-positive substances. Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel plate R*.

Test solution (a). Dissolve 0.10 g of the substance to be examined in 0.1 M *hydrochloric acid* and dilute to 10 ml with the same acid.

Test solution (b). Dilute 1 ml of test solution (a) to 50 ml with *water R*.

Reference solution (a). Dissolve 10 mg of *leucine CRS* in 0.1 M *hydrochloric acid* and dilute to 50 ml with the same acid.

Reference solution (b). Dilute 5 ml of test solution (b) to 20 ml with *water R*.

Reference solution (c). Dissolve 10 mg of *leucine CRS* and 10 mg of *valine CRS* in 0.1 M *hydrochloric acid* and dilute to 25 ml with the same acid.

Apply separately to the plate 5 µl of each solution. Allow the plate to dry in air. Develop over a path of 15 cm using a mixture of 20 volumes of *glacial acetic acid R*, 20 volumes of *water R* and 60 volumes of *butanol R*. Allow the plate to dry in air, spray with *ninhydrin solution R* and heat at 100 °C to 105 °C for 15 min. Any spot in the chromatogram obtained with test solution (a), apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.5 per cent). The test is not valid unless the chromatogram obtained with reference solution (c) shows two clearly separated spots.

Chlorides (2.4.4). Dissolve 0.25 g in *water R* and dilute to 15 ml with the same solvent. The solution complies with the limit test for chlorides (200 ppm).

Sulphates (2.4.13). Dissolve 0.5 g in 3 ml of *dilute hydrochloric acid R* and dilute to 15 ml with *distilled water R*. The solution complies with the limit test for sulphates (300 ppm).

Ammonium (2.4.1). 50 mg complies with limit test B for ammonium (200 ppm). Prepare the standard using 0.1 ml of *ammonium standard solution* (100 ppm NH₄) *R*.

Iron (2.4.9). In a separating funnel, dissolve 1.0 g in 10 ml of *dilute hydrochloric acid R*. Shake with three quantities, each of 10 ml, of *methyl isobutyl ketone R1*, shaking for 3 min each time. To the combined organic layers add 10 ml of *water R* and shake for 3 min. The aqueous layer complies with the limit test for iron (10 ppm).

Heavy metals (2.4.8). 2.0 g complies with limit test D for heavy metals (10 ppm). Prepare the standard using 2 ml of *lead standard solution* (10 ppm Pb) *R*.

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

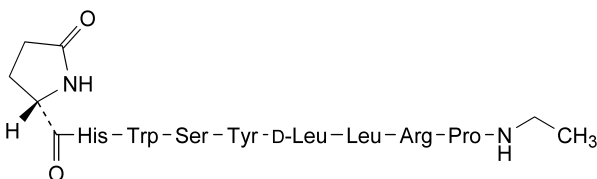
Dissolve 0.100 g in 3 ml of *anhydrous formic acid R*. Add 30 ml of *anhydrous acetic acid R*. Titrate with 0.1 M *perchloric acid* using 0.1 ml of *naphtholbenzein solution R* as indicator, until the colour changes from brownish-yellow to green.

1 ml of 0.1 M *perchloric acid* is equivalent to 13.12 mg of C₆H₁₃NO₂.

STORAGE

Store protected from light.

01/2005:1442

LEUPRORELIN**Leuprorelinum** $C_{59}H_{84}N_{16}O_{12}$ M_r 1209**DEFINITION**

5-Oxo-L-prolyl-L-histidyl-L-tryptophyl-L-seryl-L-tyrosyl-D-leucyl-L-leucyl-L-arginyl-N-ethyl-L-prolinamide.

Synthetic nonapeptide analogue of the hypothalamic peptide, gonadorelin. It is obtained by chemical synthesis and is available as an acetate.

Content: 97.0 per cent to 103.0 per cent (anhydrous and acetic acid-free substance).

CHARACTERS

Appearance: hygroscopic, white or almost white powder.

IDENTIFICATION

A. Infrared absorption spectrophotometry (2.2.24).

Preparation: discs of *potassium bromide R*.

Comparison: *Ph. Eur. reference spectrum of leuprorelin*.

B. Examine the chromatograms obtained in the assay.

Results: the principal peak in the chromatogram obtained with test solution (b) is similar in retention time and size to the principal peak in the chromatogram obtained with reference solution (b).

C. Amino acid analysis (2.2.56). For hydrolysis use Method 1 and for analysis use Method 1.

Express the content of each amino acid in moles. Calculate the relative proportions of the amino acids taking one seventh of the sum of the number of moles of histidine, glutamic acid, leucine, proline, tyrosine and arginine as equal to 1. The values fall within the following limits: serine present; glutamic acid = 0.85 to 1.1; proline = 0.85 to 1.1; leucine = 1.8 to 2.2; tyrosine = 0.85 to 1.1; histidine = 0.85 to 1.1 and arginine = 0.85 to 1.1. Not more than traces of other amino acids are present, with the exception of tryptophan.

TESTS

Specific optical rotation (2.2.7): -38.0 to -42.0 (anhydrous and acetic acid-free substance).

Dissolve the substance to be examined in a 1 per cent *V/V* solution of *glacial acetic acid R* to obtain a concentration of 10.0 mg/ml.

Related substances. Liquid chromatography (2.2.29): use the normalisation procedure.

Test solution (a). Dissolve the substance to be examined in the mobile phase to obtain a concentration of 1.0 mg/ml.

Test solution (b). Dilute 1.0 ml of test solution (a) to 20.0 ml with the mobile phase.

Reference solution (a). Dissolve *leuprorelin CRS* in the mobile phase to obtain a concentration of 1.0 mg/ml.

Reference solution (b). Dilute 1.0 ml of reference solution (a) to 20.0 ml with the mobile phase.

Resolution solution. Dilute 5.0 ml of reference solution (a) to 50.0 ml with *water R*. To 5 ml of the solution add 100 μ l of 1 *M sodium hydroxide* and shake vigorously. Heat in an oven at 100 °C for 60 min, cool immediately and add 50 μ l of *dilute phosphoric acid R*. Shake vigorously.

Column:

- **size:** $l = 0.10$ m, $\varnothing = 4.6$ mm,
- **stationary phase:** *octadecylsilyl silica gel for chromatography R* (3 μ m).

Mobile phase: dissolve about 15.2 g of *triethylamine R* in 800 ml of *water R*, adjust to pH 3.0 with *phosphoric acid R* and dilute to 1000 ml with *water R*. Add 850 ml of this solution to 150 ml of a mixture of 2 volumes of *propanol R* and 3 volumes of *acetonitrile R*.

Flow rate: 1.0–1.5 ml/min.

Detection: spectrophotometer at 220 nm.

Injection: 20 μ l of test solution (a) and the resolution solution.

Run time: 90 min.

Relative retention with reference to leuprorelin (retention time = 41–49 min): impurity E = about 0.7; impurity F = about 0.7; impurity H = about 0.78; impurity A = about 0.8; impurity B = about 0.9; impurity I = about 0.94; impurity J = about 1.09; impurity C = about 1.2; impurity G = about 1.3; impurity K = about 1.31; impurity D = about 1.5.

System suitability: resolution solution:

- **resolution:** minimum 1.5 between the peaks due to impurity B and leuprorelin.

Limits:

- **impurity D:** maximum 1.0 per cent,
- **impurities A, B, C:** for each impurity, maximum 0.5 per cent,
- **any other impurity:** for each impurity, maximum 0.5 per cent,
- **total:** maximum 2.5 per cent,
- **disregard limit:** 0.1 per cent.

Acetic acid (2.5.34): 4.7 per cent to 9.0 per cent.

Test solution. Dissolve 10.0 mg of the substance to be examined in a mixture of 5 volumes of mobile phase B and 95 volumes of mobile phase A and dilute to 10.0 ml with the same mixture of mobile phases.

Water (2.5.32): maximum 5.0 per cent.

Sulphated ash (2.4.14): maximum 0.3 per cent.

Bacterial endotoxins (2.6.14, *Method D*): less than 16.7 IU/mg, if intended for use in the manufacture of parenteral dosage forms without a further appropriate procedure for removal of bacterial endotoxins.

ASSAY

Liquid chromatography (2.2.29) as described in the test for related substances with the following modifications.

Run time: 60 min.

Injection: 20 μ l of test solution (b) and reference solution (b).

Calculate the content of leuprorelin ($C_{59}H_{84}N_{16}O_{12}$) using the areas of the peaks and the declared content of $C_{59}H_{84}N_{16}O_{12}$ in *leuprorelin CRS*.

STORAGE

In an airtight container, protected from light, at a temperature not exceeding 30 °C.

If the substance is sterile, store in a sterile, airtight, tamper-proof container.

LABELLING

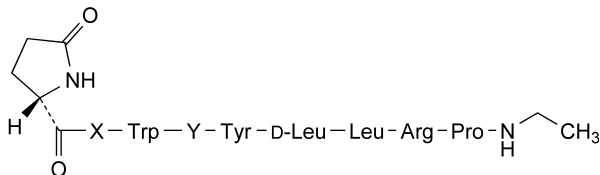
The label states:

- the mass of peptide in the container,
- where applicable, that the substance is free from bacterial endotoxins.

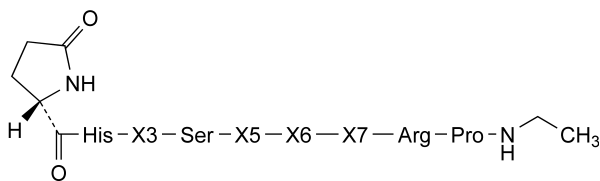
IMPURITIES

Specified impurities: A, B, C, D.

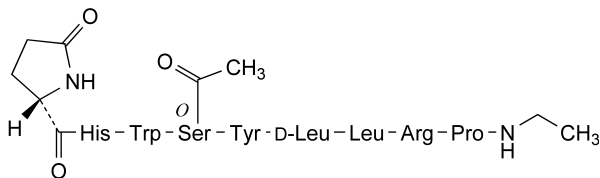
Other detectable impurities: E, F, G, H, I, J, K.



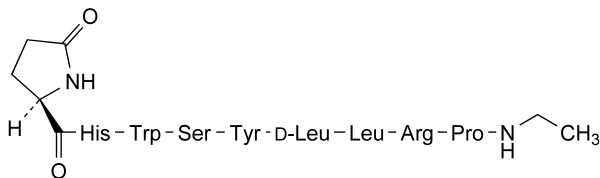
- A. X = L-His, Y = D-Ser: [4-D-serine]leuporelin,
 B. X = D-His, Y = L-Ser: [2-D-histidine]leuporelin,
 F. X = D-His, Y = D-Ser: [2-D-histidine,4-D-serine]leuporelin,



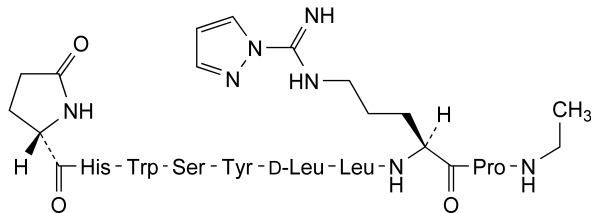
- C. X3 = L-Trp, X5 = L-Tyr, X6 = X7 = L-Leu: [6-L-leucine]leuporelin,
 E. X3 = D-Trp, X5 = L-Tyr, X6 = D-Leu, X7 = L-Leu: [3-D-tryptophane]leuporelin,
 G. X3 = L-Trp, X5 = D-Tyr, X6 = D-Leu, X7 = L-Leu: [5-D-tyrosine]leuporelin,
 H. X3 = L-Trp, X5 = L-Tyr, X6 = X7 = D-Leu: [7-D-leucine]leuporelin,



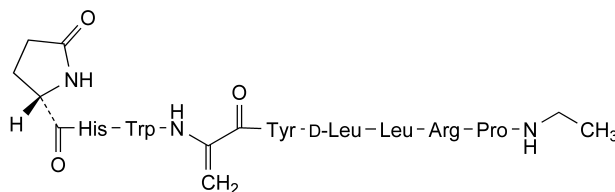
- D. [4-(O-acetyl-L-serine)]leuporelin,



- I. [1-(5-oxo-D-proline)]leuporelin,



- J. [8-[5-N-[imino(1H-pyrazol-1-yl)methyl]-L-ornithine]]leuporelin,

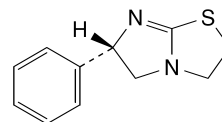


- K. [4-dehydroalanine]leuporelin.

01/2005:1728

LEVAMISOLE FOR VETERINARY USE

Levamisolum ad usum veterinarium



$C_{11}H_{12}N_2S$

M_r 204.3

DEFINITION

(6S)-6-Phenyl-2,3,5,6-tetrahydroimidazo[2,1-b]thiazole.

Content: 98.5 per cent to 101.5 per cent (anhydrous substance).

CHARACTERS

Appearance: white or almost white powder.

Solubility: slightly soluble in water, freely soluble in alcohol and in methanol.

It shows polymorphism.

IDENTIFICATION

A. It complies with the test for specific optical rotation (see Tests).

B. Infrared absorption spectrophotometry (2.2.24).

Comparison: Ph. Eur. reference spectrum of levamisole.

If the spectra show differences, dissolve the substance to be examined in *methylene chloride R*, evaporate to dryness and record a new spectrum using the residue.

TESTS

Solution S. Dissolve 2.50 g in *ethanol R* and dilute to 50.0 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and not more intensely coloured than reference solution BY₆ (2.2.2, Method II).

Specific optical rotation (2.2.7): −85 to −89 (anhydrous substance), determined on solution S.

Related substances. Liquid chromatography (2.2.29).

Prepare the solutions immediately before use, protect from light and keep below 25 °C.

Test solution. Dissolve 0.100 g of the substance to be examined in *methanol R* and dilute to 10.0 ml with the same solvent.

Reference solution (a). Dissolve 50 mg of *levamisole hydrochloride* for system suitability CRS in *methanol R*, add 0.5 ml of concentrated ammonia R and dilute to 5.0 ml with *methanol R*.

Reference solution (b). Dilute 1.0 ml of the test solution to 100.0 ml with *methanol R*. Dilute 5.0 ml of the solution to 25.0 ml with *methanol R*.

Column:

– size: $l = 0.10$ m, $\varnothing = 4.6$ mm,

- *stationary phase*: base-deactivated octadecylsilyl silica gel for chromatography R (3 µm).

Mobile phase:

- *mobile phase A*: dissolve 0.5 g of ammonium dihydrogen phosphate R in 90 ml of water R; adjust to pH 6.5 with a 40 g/l solution of sodium hydroxide R and dilute to 100 ml with water R,
- *mobile phase B*: acetonitrile R.

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)
0 - 8	90 → 30	10 → 70
8 - 10	30	70
10 - 11	30 → 90	70 → 10

Flow rate: 1.5 ml/min.

Detection: spectrophotometer at 215 nm.

Equilibration: at least 4 min with the mobile phase at the initial composition.

Injection: 10 µl.

Relative retention with reference to levamisole (retention time = about 3 min): impurity A = about 0.9; impurity B = about 1.4; impurity C = about 1.5; impurity D = about 1.6; impurity E = about 2.0.

System suitability: reference solution (a):

- the chromatogram obtained is similar to the chromatogram supplied with levamisole hydrochloride for system suitability CRS.

Limits:

- *correction factors*: for the calculation of content, multiply the peak areas of the following impurities by the corresponding correction factor: impurity A = 2.0; impurity B = 1.7; impurity C = 2.9; impurity D = 1.3; impurity E = 2.7;
- *impurities A, B, C, D, E*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent);
- *any other impurity*: not more than half the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent);
- *total*: not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.3 per cent);
- *disregard limit*: 0.25 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

Water (2.5.12): maximum 0.5 per cent, determined on 1.00 g.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

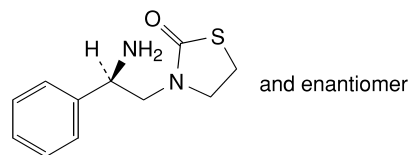
Dissolve 0.150 g in 50 ml of a mixture of 1 volume of anhydrous acetic acid R and 7 volumes of methyl ethyl ketone R. Titrate with 0.1 M perchloric acid, using 0.2 ml of naphtholbenzein solution R as indicator.

1 ml of 0.1 M perchloric acid is equivalent to 20.43 mg of C₁₁H₁₂N₂S.

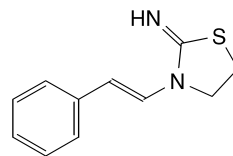
STORAGE

In an airtight container, protected from light.

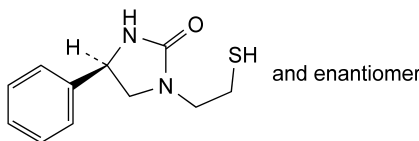
IMPURITIES



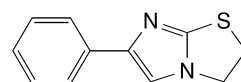
A. 3-[(2RS)-2-amino-2-phenylethyl]thiazolidin-2-one,



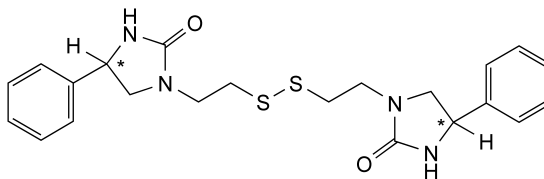
B. 3-[(E)-2-phenylethenyl]thiazolidin-2-imine,



C. (4RS)-4-phenyl-1-(2-sulphanylethyl)imidazolidin-2-one,



D. 6-phenyl-2,3-dihydroimidazo[2,1-b]thiazole,

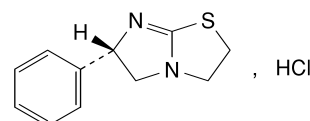


E. 1,1'-[(disulphane-1,2-diyl)bis(ethylene)]bis[(4RS)-4-phenylimidazolidin-2-one].

01/2005:0726

LEVAMISOLE HYDROCHLORIDE

Levamisoli hydrochloridum



C₁₁H₁₃ClN₂S

M_r 240.8

DEFINITION

(6S)-6-Phenyl-2,3,5,6-tetrahydroimidazo[2,1-b]thiazole hydrochloride.

Content: 98.5 per cent to 101.0 per cent (dried substance).

CHARACTERS

Appearance: white or almost white crystalline powder.

Solubility: freely soluble in water, soluble in alcohol, slightly soluble in methylene chloride.

IDENTIFICATION

- It complies with the test for specific optical rotation (see Tests).
- Infrared absorption spectrophotometry (2.2.24).
Preparation: discs.
Comparison: levamisole hydrochloride CRS.

C. It gives reaction (a) of chlorides (2.3.1).

TESTS

Solution S. Dissolve 2.50 g in *carbon dioxide-free water R* and dilute to 50.0 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and not more intensely coloured than reference solution Y₇ (2.2.2, *Method II*).

pH (2.2.3): 3.0 to 4.5 for solution S.

Specific optical rotation (2.2.7): –121 to –128 (dried substance), determined on solution S.

Related substances. Liquid chromatography (2.2.29). Prepare the solutions immediately before use, protect from light and keep below 25 °C.

Test solution. Dissolve 0.100 g of the substance to be examined in *methanol R*, add 1.0 ml of *concentrated ammonia R* and dilute to 10.0 ml with *methanol R*.

Reference solution (a). Dissolve 50 mg of *levamisole hydrochloride for system suitability CRS* in *methanol R*, add 0.5 ml of *concentrated ammonia R* and dilute to 5.0 ml with *methanol R*.

Reference solution (b). Dilute 1.0 ml of the test solution to 100.0 ml with *methanol R*. Dilute 5.0 ml of the solution to 25.0 ml with *methanol R*.

Column:

- size: *l* = 0.10 m, Ø = 4.6 mm,
- stationary phase: base-deactivated octadecylsilyl silica gel for chromatography R (3 µm).

Mobile phase:

- mobile phase A: dissolve 0.5 g of *ammonium dihydrogen phosphate R* in 90 ml of *water R*; adjust to pH 6.5 with a 40 g/l solution of *sodium hydroxide R* and dilute to 100 ml with *water R*,
- mobile phase B: *acetonitrile R*,

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)
0 - 8	90 → 30	10 → 70
8 - 10	30	70
10 - 11	30 → 90	70 → 10

Flow rate: 1.5 ml/min.

Detection: spectrophotometer at 215 nm.

Equilibration: at least 4 min with the mobile phase at the initial composition.

Injection: 10 µl.

Relative retention with reference to levamisole (retention time = about 3 min): impurity A = about 0.9; impurity B = about 1.4; impurity C = about 1.5; impurity D = about 1.6; impurity E = about 2.0.

System suitability: reference solution (a):

- the chromatogram obtained is similar to the chromatogram supplied with *levamisole hydrochloride for system suitability CRS*.

Limits:

- **correction factors:** for the calculation of contents, multiply the peak areas of the following impurities by the corresponding correction factor: impurity A = 2.0; impurity B = 1.7; impurity C = 2.9; impurity D = 1.3; impurity E = 2.7;
- **impurities A, B, C, D, E:** for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent);

- **any other impurity:** not more than half the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent);
- **total:** not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.3 per cent);
- **disregard limit:** 0.25 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

Heavy metals (2.4.8): maximum 20 ppm.

12 ml of solution S complies with limit test A. Prepare the standard using *lead standard solution (1 ppm Pb) R*.

Loss on drying (2.2.32): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 100–105 °C for 4 h.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

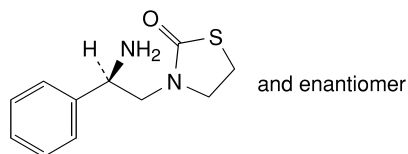
Dissolve 0.200 g in 30 ml of *alcohol R* and add 5.0 ml of 0.01 M *hydrochloric acid*. Carry out a potentiometric titration (2.2.20), using 0.1 M *sodium hydroxide*. Read the volume added between the 2 points of inflexion.

1 ml of 0.1 M *sodium hydroxide* is equivalent to 24.08 mg of C₁₁H₁₃ClN₂S.

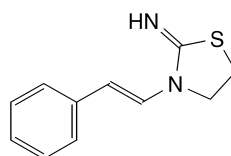
STORAGE

Protected from light.

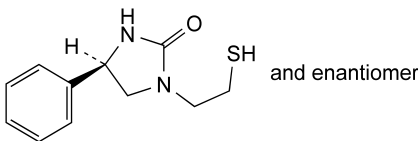
IMPURITIES



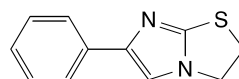
A. 3-[(2*RS*)-2-amino-2-phenylethyl]thiazolidin-2-one,



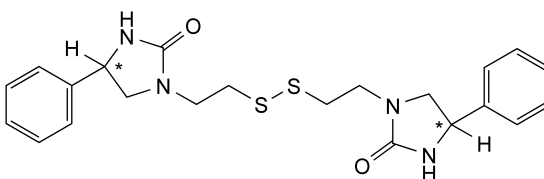
B. 3-[(*E*)-2-phenylethenyl]thiazolidin-2-imine,



C. (4*RS*)-4-phenyl-1-(2-sulphanylethyl)imidazolidin-2-one,



D. 6-phenyl-2,3-dihydroimidazo[2,1-*b*]thiazole,

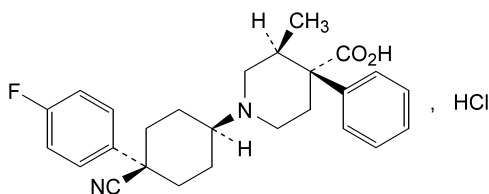


E. 1,1'-[bis(disulphane-1,2-diyl)]bis(ethylene)]bis[(4*RS*)-4-phenylimidazolidin-2-one].

01/2005:1484

LEVOCABASTINE HYDROCHLORIDE

Levocabastini hydrochloridum

 $C_{26}H_{30}ClFN_2O_2$ M_r 457.0

DEFINITION

Levocabastine hydrochloride contains not less than 98.5 per cent and not more than the equivalent of 101.5 per cent of (3*S*,4*R*)-1-[*cis*-4-cyano-4-(4-fluorophenyl)cyclohexyl]-3-methyl-4-phenylpiperidine-4-carboxylic acid monohydrochloride, calculated with reference to the dried substance.

CHARACTERS

A white or almost white powder, practically insoluble in water, sparingly soluble in methanol, slightly soluble in alcohol and in a 2 g/l solution of sodium hydroxide.

IDENTIFICATION

- Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *levocabastine hydrochloride CRS*. Examine the substances prepared as discs.
- Dissolve 50 mg in a mixture of 0.4 ml of *ammonia R* and 2 ml of *water R*. Mix, allow to stand for 5 min and filter. Acidify the filtrate with *dilute nitric acid R*. It gives reaction (a) of chlorides (2.3.1).
- It complies with the test for specific optical rotation (see Tests).

TESTS

Solution S. Dissolve 0.250 g in *methanol R* and dilute to 25.0 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and not more intensely coloured than reference solution Y_7 (2.2.2, Method II).

Specific optical rotation (2.2.7). The specific optical rotation is -102 to -106 , determined on solution S, calculated with reference to the dried substance.

Related substances. Examine by capillary electrophoresis (2.2.47). Prepare the solutions immediately before use.

Test solution. Dissolve 25.0 mg of the substance to be examined in a 2 g/l solution of *sodium hydroxide R* and dilute to 10.0 ml with the same solvent.

Reference solution (a). Dissolve 2.5 mg of *levocabastine hydrochloride CRS* and 2.5 mg of *levocabastine impurity D CRS* in a 2 g/l solution of *sodium hydroxide R* and dilute to 200.0 ml with the same solvent.

Reference solution (b). Dilute 5.0 ml of the test solution to 100.0 ml with a 2 g/l solution of *sodium hydroxide R*. Dilute 1.0 ml of the solution to 10.0 ml with a 2 g/l solution of *sodium hydroxide R*.

The micellar electrokinetic chromatographic procedure may be carried out using:

- an uncoated fused-silica capillary with the length to the detector cell of 0.5 m and 75 μ m in internal diameter;
- an electrolyte solution prepared as follows: dissolve 1.08 g of *sodium dodecyl sulphate R* and 0.650 g of *hydroxypropyl- β -cyclodextrin R* in 5 ml of *2-propanol R* and dilute to 50.0 ml with buffer solution pH 9.0 prepared as follows: dissolve 1.39 g of *boric acid R* in *water R* and adjust to pH 9.0 with 1 *M sodium hydroxide* (about 9 ml). Dilute to 100.0 ml with *water R*;
- as detector a spectrophotometer set at 214 nm;
- an injection of 5 s under a pressure of 3450 Pa;
- the following current gradient:

Time (min)	Current (μ A)
0 - 0.17	0 $\rightarrow$ 75
0.17 - 15	75 $\rightarrow$ 130
15 - 40	130
40 - 60	130 $\rightarrow$ 200

maintaining the temperature of the capillary at 50 °C.

Equilibrate the capillary for 2 min with a 2 g/l solution of *sodium hydroxide R* and then equilibrate with the electrolyte solution for at least 5 min.

Inject reference solution (a). When the electropherogram is recorded in the prescribed conditions, the migration times are: levocabastine about 28 min and impurity D about 30 min. The test is not valid unless the resolution between the peaks due to levocabastine and impurity D is at least 4. If necessary adjust the current gradient.

Inject a 2 g/l solution of *sodium hydroxide R* as a blank, the test solution and reference solution (b). In the electropherogram obtained with the test solution: the area of any peak, apart from the principal peak, is not greater than the area of the principal peak in the electropherogram obtained with reference solution (b) (0.5 per cent); the sum of the areas of all peaks, apart from the principal peak, is not greater than twice the area of the principal peak in the electropherogram obtained with reference solution (b) (1.0 per cent). Disregard any peak obtained with the blank and any peak with an area less than 0.1 times the area of the principal peak in the electropherogram obtained with reference solution (b) (0.05 per cent).

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 100-105 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g in a platinum crucible.

ASSAY

Dissolve 0.175 g in 50 ml of *alcohol R* and add 5.0 ml of 0.01 *M hydrochloric acid*. Carry out a potentiometric titration (2.2.20), using 0.1 *M sodium hydroxide*. Read the volume added between the first and third point of inflexion.

1 ml of 0.1 *M sodium hydroxide* is equivalent to 22.85 mg of $C_{26}H_{30}ClFN_2O_2$.

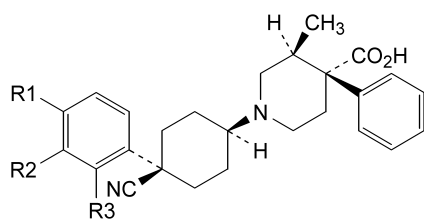
STORAGE

Store protected from light.

IMPURITIES

Specified impurities: A, B, C, D, E.

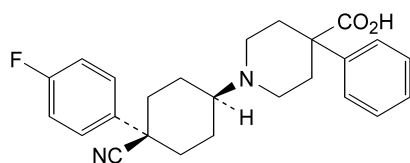
Other detectable impurities: F, G, H, I.



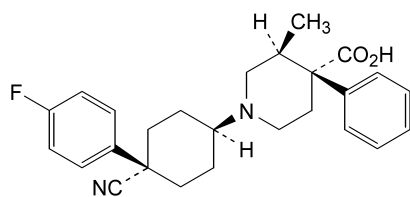
A. $R_1 = R_2 = R_3 = H$: (3*S*,4*R*)-1-(*cis*-4-cyano-4-phenylcyclohexyl)-3-methyl-4-phenylpiperidine-4-carboxylic acid,

B. $R_1 = R_2 = H$, $R_3 = F$: (3*S*,4*R*)-1-[*cis*-4-cyano-4-(2-fluorophenyl)cyclohexyl]-3-methyl-4-phenylpiperidine-4-carboxylic acid,

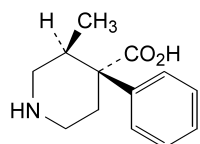
C. $R_1 = H$, $R_2 = F$, $R_3 = H$: (3*S*,4*R*)-1-[*cis*-4-cyano-4-(3-fluorophenyl)cyclohexyl]-3-methyl-4-phenylpiperidine-4-carboxylic acid,



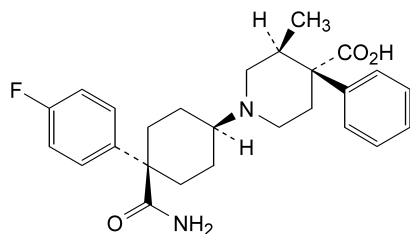
D. 1-[*cis*-4-cyano-4-(4-fluorophenyl)cyclohexyl]-4-phenylpiperidine-4-carboxylic acid,



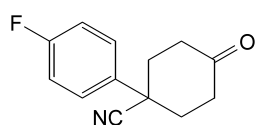
E. (3*S*,4*R*)-1-[*trans*-4-cyano-4-(4-fluorophenyl)cyclohexyl]-3-methyl-4-phenylpiperidine-4-carboxylic acid,



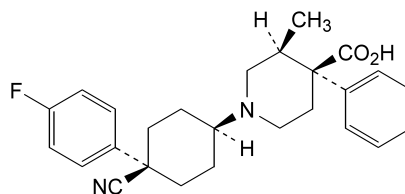
F. (3*S*,4*R*)-3-methyl-4-phenylpiperidine-4-carboxylic acid,



G. (3*S*,4*R*)-1-[*cis*-4-carbamoyl-4-(4-fluorophenyl)cyclohexyl]-3-methyl-4-phenylpiperidine-4-carboxylic acid,



H. 1-(4-fluorophenyl)-4-oxocyclohexanecarbonitrile,

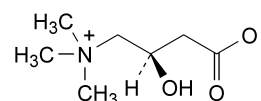


I. (3*S*,4*S*)-1-[*cis*-4-cyano-4-(4-fluorophenyl)cyclohexyl]-3-methyl-4-phenylpiperidine-4-carboxylic acid.

01/2005:1339

LEVOCARNITINE

Levocarnitinum


 $C_7H_{15}NO_3$
 M_r 161.2

DEFINITION

Levocarnitine contains not less than 98.0 per cent and not more than the equivalent of 102.0 per cent of (3*R*)-3-hydroxy-4-(trimethylammonio)butanoate, calculated with reference to the anhydrous substance.

CHARACTERS

A white, crystalline powder or colourless crystals, hygroscopic, freely soluble in water, soluble in warm alcohol, practically insoluble in acetone.

IDENTIFICATION

First identification: A, B.

Second identification: A, C.

- It complies with the test for specific optical rotation (see Tests).
- Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *levocarnitine CRS*. Examine the substances, previously dried *in vacuo* at 50 °C for 5 h, prepared as discs.
- To 1 ml of solution S (see Tests) add 9 ml of *water R*, 10 ml of *dilute sulphuric acid R* and 30 ml of *ammonium reineckate solution R*. A pink precipitate is formed. Allow to stand for 30 min. Filter and wash with *water R*, with *alcohol R* and then with *acetone R* and dry at 80 °C. The precipitate melts (2.2.14) at 147 °C to 150 °C.

TESTS

Solution S. Dissolve 5.00 g in *carbon dioxide-free water R* prepared from *distilled water R* and dilute to 50.0 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and colourless (2.2.2, *Method II*).

pH (2.2.3). Dilute 10 ml of solution S to 20 ml with *carbon dioxide-free water R*. The pH of the solution is 6.5 to 8.5.

Specific optical rotation (2.2.7): -29.0 to -32.0 , determined on solution S and calculated with reference to the anhydrous substance.

Related substances. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 0.10 g of the substance to be examined in the mobile phase and dilute to 20.0 ml with the mobile phase.

Reference solution (a). Dilute 1.0 ml of the test solution to 100.0 ml with the mobile phase. Dilute 1.0 ml of the solution to 10.0 ml with the mobile phase.

Reference solution (b). Dissolve 12.5 mg of *levocarnitine impurity A CRS* in *water R* and dilute to 50.0 ml with the same solvent. Dilute 2.0 ml of the solution to 20.0 ml with the mobile phase.

Reference solution (c). Dissolve 10.0 mg of *levocarnitine impurity A CRS* in *water R* and dilute to 10.0 ml with the same solvent. Dilute 2.0 ml of the solution to 20.0 ml with the mobile phase.

Reference solution (d). Dissolve 0.100 g of *levocarnitine CRS* in reference solution (c) and dilute to 10.0 ml with the same solution.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.30 m long and 3.9 mm in internal diameter packed with *aminopropylmethylsilyl silica gel for chromatography R* (10 µm),
- as mobile phase at a flow rate of 1 ml/min a mixture of 35 volumes of a 6.81 g/l solution of *potassium dihydrogen phosphate R* adjusted to pH 4.7 with *dilute sodium hydroxide solution R*, and 65 volumes of *acetonitrile R*,
- as detector a spectrophotometer set at 205 nm, maintaining the temperature of the column at 30 °C.

When the chromatograms are recorded under the prescribed conditions, the retention times are: about 9.6 min for levocarnitine and about 10.6 min for levocarnitine impurity A. Adjust the sensitivity of the system so that the height of the principal peak in the chromatogram obtained with reference solution (b) is at least 20 per cent of the full scale of the recorder.

Inject 25 µl of reference solution (d) and continue the chromatography for 15 min. The test is not valid unless, in the chromatogram obtained with reference solution (d), the resolution between the peaks corresponding to levocarnitine and to levocarnitine impurity A is at least 0.9.

Inject 25 µl of the test solution, 25 µl of reference solution (a) and 25 µl of reference solution (b). In the chromatogram obtained with the test solution: the area of any peak corresponding to levocarnitine impurity A is not greater than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent); the area of any peak, apart from the principal peak and the peak due to levocarnitine impurity A, is not greater than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent).

Chlorides (2.4.4). Dilute 2.5 ml of solution S to 15 ml with *water R*. The solution complies with the limit test for chlorides (200 ppm).

Sulphates (2.4.13). Dilute 5 ml of solution S to 15 ml with *distilled water R*. The solution complies with the limit test for sulphates (300 ppm).

Heavy metals (2.4.8). 12 ml of solution S complies with limit test A for heavy metals (10 ppm). Prepare the standard using *lead standard solution (1 ppm Pb) R*.

Water (2.5.12). Not more than 1.0 per cent, determined on 2.00 g by the semi-micro determination of water.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

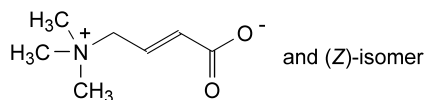
ASSAY

Dissolve 0.125 g in a mixture of 3 volumes of *anhydrous formic acid R* and 50 volumes of *anhydrous acetic acid R*. Add 0.2 ml of *crystal violet solution R*. Titrate with 0.1 M *perchloric acid* until the colour changes from violet to green. 1 ml of 0.1 M *perchloric acid* is equivalent to 16.12 mg of C₇H₁₅NO₃.

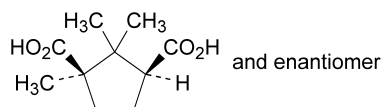
STORAGE

Store in an airtight container.

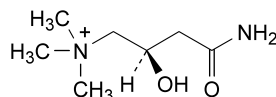
IMPURITIES



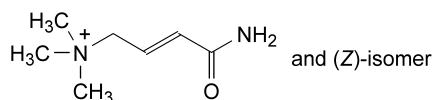
A. (*E*)- or (*Z*)-4-(trimethylammonio)but-2-enoate,



B. (1*RS*,3*SR*)-1,2,2-trimethylcyclopentane-1,3-dicarboxylic acid (camphoric acid),



C. (2*R*)-4-amino-2-hydroxy-*N,N,N*-trimethyl-4-oxobutan-1-aminium (carnitinamide),

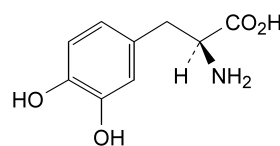


D. (*E*)- or (*Z*)-4-amino-*N,N,N*-trimethyl-4-oxobut-2-en-1-aminium.

01/2005:0038

LEVODOPA

Levodopum



C₉H₁₁NO₄

*M*_r 197.2

DEFINITION

Levodopa contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of (2*S*)-2-amino-3-(3,4-dihydroxyphenyl)propanoic acid, calculated with reference to the dried substance.

CHARACTERS

A white or slightly cream-coloured, crystalline powder, slightly soluble in water, practically insoluble in alcohol. It is freely soluble in 1 M *hydrochloric acid* and sparingly soluble in 0.1 M *hydrochloric acid*.

IDENTIFICATION

First identification: A.

Second identification: B, C, D.

- A. Examine by infrared absorption spectrophotometry (2.2.24) comparing with the spectrum obtained with *levodopa CRS*.
- B. Dissolve about 2 mg in 2 ml of *water R* and add 0.2 ml of *ferric chloride solution R2*. A green colour develops which changes to bluish-violet on the addition of 0.1 g of *hexamethylenetetramine R*.
- C. Dissolve about 5 mg in a mixture of 5 ml of 1 M *hydrochloric acid* and 5 ml of *water R*. Add 0.1 ml of *sodium nitrite solution R* containing 100 g/l of *ammonium molybdate R*. A yellow colour develops which changes to red on the addition of *strong sodium hydroxide solution R*.
- D. To about 5 mg add 1 ml of *water R*, 1 ml of *pyridine R* and about 5 mg of *nitrobenzoyl chloride R*, mix and allow to stand for 3 min. A violet colour develops which changes to pale yellow on boiling the mixture. Add, while shaking, 0.2 ml of *sodium carbonate solution R*. The violet colour reappears.

TESTS

Appearance of solution. Dissolve 1.0 g in 1 M *hydrochloric acid* and dilute to 25 ml with the same solvent. The solution is not more intensely coloured than reference solution BY₆ (2.2.2, *Method II*).

pH (2.2.3). Shake 0.10 g for 15 min with 10 ml of *carbon dioxide-free water R*. The pH of the suspension is 4.5 to 7.0.

Optical rotation (2.2.7). Dissolve a quantity equivalent to 0.200 g of the dried substance and 5 g of *hexamethylenetetramine R* in 10 ml of 1 M *hydrochloric acid* and dilute to 25.0 ml with the same acid. Allow the solution to stand protected from light for 3 h. The angle of optical rotation is -1.27° to -1.34° .

Absorbance (2.2.25). Dissolve 30.0 mg in 0.1 M *hydrochloric acid* and dilute to 100.0 ml with the same acid. Dilute 10.0 ml of this solution to 100.0 ml with 0.1 M *hydrochloric acid*. Examined between 230 nm and 350 nm, the solution shows a single absorption maximum, at 280 nm. The specific absorbance at this maximum is 137 to 147, calculated with reference to the dried substance.

Related substances. Examine by thin-layer chromatography (2.2.27), using *cellulose for chromatography R* as the coating substance.

Test solution. Dissolve 0.1 g of the substance to be examined in 5 ml of *anhydrous formic acid R* and dilute to 10 ml with *methanol R*. Prepare immediately before use.

Reference solution (a). Dilute 0.5 ml of the test solution to 100 ml with *methanol R*.

Reference solution (b). Dissolve 30 mg of *tyrosine R* in 1 ml of *anhydrous formic acid R* and dilute to 100 ml with *methanol R*. Mix 1 ml of this solution with 1 ml of the test solution.

Apply separately to the plate as bands 20 mm long 10 µl of the test solution, 10 µl of reference solution (a) and 20 µl of reference solution (b). Dry in a current of air. Develop over a path of 15 cm using a mixture of 25 volumes of *glacial acetic acid R*, 25 volumes of *water R* and 50 volumes of *butanol R*. Dry the plate in a current of warm air and spray with a freshly prepared mixture of equal volumes of a 100 g/l solution of *ferric chloride R* and a 50 g/l solution of *potassium ferricyanide R*. Examine the chromatograms immediately. Any spot in the chromatogram obtained with the test solution, apart from the principal spot is not more intense than the spot in the chromatogram obtained with reference solution (a) (0.5 per cent). The test is not valid unless the chromatogram obtained with reference

solution (b) shows, above the principal spot, a distinct spot which is more intense than the spot in the chromatogram obtained with reference solution (a).

Heavy metals (2.4.8). 2.0 g complies with limit test C for heavy metals (10 ppm). Prepare the standard using 2 ml of *lead standard solution (10 ppm Pb) R*.

Loss on drying (2.2.32). Not more than 1.0 per cent, determined on 0.50 g by drying in an oven at 100 °C to 105 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.180 g, heating if necessary in 5 ml of *anhydrous formic acid R* and add 25 ml of *anhydrous acetic acid R* and 25 ml of *dioxan R*. Titrate with 0.1 M *perchloric acid*, using 0.1 ml of *crystal violet solution R* as indicator, until a green colour is obtained.

1 ml of 0.1 M *perchloric acid* is equivalent to 19.72 mg of C₉H₁₁NO₄.

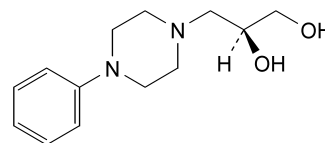
STORAGE

Store protected from light.

01/2005:1535

LEVODROPROPIZINE

Levodropropizinum

C₁₃H₂₀N₂O₂M_r 236.3

DEFINITION

(2S)-3-(4-Phenylpiperazin-1-yl)propane-1,2-diol.

Content: 98.5 per cent to 101.0 per cent (dried substance).

CHARACTERS

Appearance: white or almost white powder.

Solubility: slightly soluble in water, freely soluble in dilute acetic acid and in methanol, slightly soluble in alcohol.

IDENTIFICATION

- A. Specific optical rotation (2.2.7): -30.0 to -33.5 (dried substance).

Dissolve 1.50 g in a 21 g/l solution of *hydrochloric acid R* and dilute to 50.0 ml with the same acid.

- B. Infrared absorption spectrophotometry (2.2.24).

Comparison: *levodropropizine CRS*.

TESTS

pH (2.2.3): 9.2 to 10.2.

Suspend 2.5 g in *carbon dioxide-free water R*, heat to dissolve, cool to room temperature and dilute to 100 ml with the same solvent.

Impurity B and related substances. Liquid chromatography (2.2.29).

Test solution. Dissolve 24.0 mg of the substance to be examined in the mobile phase and dilute to 100.0 ml with the mobile phase.

Reference solution (a). Dissolve 12.0 mg of *1-phenylpiperazine R* in *methanol R* and dilute to 100.0 ml with the same solvent. Dilute 1.0 ml to 100.0 ml with the mobile phase.

Reference solution (b). Mix 0.5 ml of the test solution with 1 ml of reference solution (a) and dilute to 100 ml with the mobile phase.

Column:

- **size:** $l = 0.15$ m, $\varnothing = 4.6$ mm,
- **stationary phase:** *base-deactivated octylsilyl silica gel for chromatography R* (5 μ m).

Mobile phase: mix 12 volumes of *methanol R* and 88 volumes of a 6.81 g/l solution of *potassium dihydrogen phosphate R*, adjusted to pH 3.0 with *phosphoric acid R*.

Flow rate: 1.5 ml/min.

Detection: spectrophotometer at 254 nm.

Injection: 20 μ l.

System suitability: reference solution (b):

- **resolution:** minimum 2.0 between the peaks due to levodropropizine and to impurity B.

Limits:

- **impurity B:** not more than the area of the corresponding peak in the chromatogram obtained with reference solution (a) (0.5 per cent),
- **any other impurity:** not more than 0.2 times the area of the peak due to impurity B in the chromatogram obtained with reference solution (a) (0.1 per cent),
- **disregard limit:** 0.02 times the area of the peak due to impurity B in the chromatogram obtained with reference solution (a) (0.01 per cent).

Impurity C. Gas chromatography (2.2.28). Prepare the solutions immediately before use.

Test solution. Dissolve 0.50 g of the substance to be examined in *methylene chloride R* and dilute to 2.5 ml with the same solvent.

Reference solution (a). Dissolve 0.20 g of *glycidol R* in *methylene chloride R* and dilute to 100.0 ml with the same solvent. Dilute 0.5 ml of this solution to 100.0 ml with *methylene chloride R*.

Reference solution (b). Dissolve 0.50 g of the substance to be examined in *methylene chloride R*, add 0.5 ml of reference solution (a) and dilute to 2.5 ml with *methylene chloride R*.

Column:

- **material:** fused silica,
- **size:** $l = 30$ m, $\varnothing = 0.53$ mm,
- **stationary phase:** *poly[(cyanopropyl)(phenyl)][dimethylsiloxane R* (film thickness 3 μ m).

Carrier gas: *helium for chromatography R*.

Flow rate: 2.5 ml/min.

Split ratio: 1:8.

Temperature:

- **column:** 140 °C,
- **injection port:** 170 °C,
- **detector:** 250 °C.

Detection: flame ionisation.

Injection: 1 μ l; inject the test solution and reference solution (b).

Use a split-liner consisting of a column about 1 cm long packed with glass wool.

At the end of a series of tests, heat the column at 250 °C for 4–6 h.

Limit:

- **impurity C:** not more than half the area of the corresponding peak in the chromatogram obtained with reference solution (b) (10 ppm).

Enantiomeric purity. Liquid chromatography (2.2.29).

Test solution. Dissolve 10.0 mg of the substance to be examined in 10.0 ml of a mixture of 40 volumes of *ethanol R* and 60 volumes of *hexane R*. Dilute 1.0 ml of the solution to 50.0 ml with a mixture of 40 volumes of *ethanol R* and 60 volumes of *hexane R*.

Reference solution (a). Dissolve 10.0 mg of *levodropropizine CRS* in 10.0 ml of a mixture of 40 volumes of *ethanol R* and 60 volumes of *hexane R*. Dilute 1.0 ml of the solution to 50.0 ml with a mixture of 40 volumes of *ethanol R* and 60 volumes of *hexane R*.

Reference solution (b). Dissolve 10.0 mg of *levodropropizine impurity A CRS* in 10.0 ml of a mixture of 40 volumes of *ethanol R* and 60 volumes of *hexane R*. Dilute 1.0 ml of the solution to 50.0 ml with a mixture of 40 volumes of *ethanol R* and 60 volumes of *hexane R*.

Reference solution (c). Dilute 1.0 ml of reference solution (b) to 50.0 ml with a mixture of 40 volumes of *ethanol R* and 60 volumes of *hexane R*.

Reference solution (d). Mix 1 ml of reference solution (a) and 1 ml of reference solution (b).

Column:

- **size:** $l = 0.25$ m, $\varnothing = 4.6$ mm,
- **stationary phase:** *silica gel OD for chiral separations R*.

Mobile phase: *diethylamine R*, *ethanol R*, *hexane R* (0.2:5:95 V/V/V).

Flow rate: 0.8 ml/min.

Detection: spectrophotometer at 254 nm.

Injection: 20 μ l; inject the test solution and reference solutions (a), (c) and (d).

Elution order: impurity A, levodropropizine.

System suitability:

- **retention times:** the retention times of the principal peaks in the chromatograms obtained with the test solution and reference solution (a) are similar,
- **resolution:** minimum 2.0 between the peaks due to impurity A and to levodropropizine in the chromatogram obtained with reference solution (d).

Limit:

- **impurity A:** not more than the area of the corresponding peak in the chromatogram obtained with reference solution (c) (2 per cent).

Loss on drying (2.2.32): maximum 1.0 per cent, determined on 0.500 g by drying *in vacuo* at 60 °C over *diphosphorus pentoxide R* at a pressure of 0.15 kPa to 0.25 kPa for 4 h.

Sulphated ash (2.4.14): maximum 0.2 per cent, determined on 1.0 g.

ASSAY

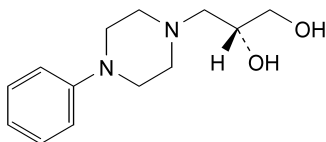
Dissolve 0.100 g in 50 ml of *anhydrous acetic acid R*. Carry out a potentiometric titration (2.2.20), using 0.1 M *perchloric acid*. Read the volume added at the second point of inflexion.

1 ml of 0.1 M *perchloric acid* is equivalent to 11.82 mg of $C_{13}H_{20}N_2O_2$.

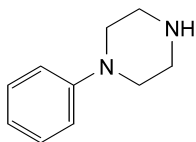
STORAGE

Protected from light.

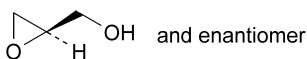
IMPURITIES



- A. (2*R*)-3-(4-phenylpiperazin-1-yl)propane-1,2-diol (dextropropizine),



- B. 1-phenylpiperazine,

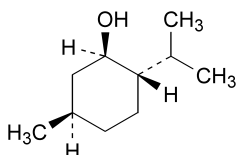


- C. [(2*RS*)-oxiran-2-yl]methanol (glycidol).

01/2005:0619

LEVOMENTHOL

Levomentholum

C₁₀H₂₀OM_r 156.3

DEFINITION

Levomenthol is (1*R*,2*S*,5*R*)-5-methyl-2-(1-methylethyl)cyclohexanol.

CHARACTERS

Prismatic or acicular, colourless, shiny crystals, practically insoluble in water, very soluble in alcohol and in light petroleum, freely soluble in fatty oils and in liquid paraffin, very slightly soluble in glycerol.

It melts at about 43 °C.

IDENTIFICATION

First identification: A, C.

Second identification: B, D.

- A. It complies with the test for specific optical rotation (see Tests).

- B. Examine by thin-layer chromatography (2.2.27), using silica gel G R as the coating substance.

Test solution. Dissolve 25 mg of the substance to be examined in methanol R and dilute to 5 ml with the same solvent.

Reference solution. Dissolve 25 mg of menthol CRS in methanol R and dilute to 5 ml with the same solvent.

Apply separately to the plate 2 µl of each solution. Develop over a path of 15 cm using a mixture of 5 volumes of ethyl acetate R and 95 volumes of toluene R. Allow the plate to dry in air until the solvents have evaporated and spray with anisaldehyde solution R. Heat at 100 °C to 105 °C for 5 min to 10 min. The principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

- C. Examine the chromatograms obtained in the test for related substances. The principal peak in the chromatogram obtained with test solution (b) is similar in position and approximate dimensions to the principal peak in the chromatogram obtained with reference solution (c).
- D. Dissolve 0.20 g in 0.5 ml of anhydrous pyridine R. Add 3 ml of a 150 g/l solution of dinitrobenzoyl chloride R in anhydrous pyridine R. Heat on a water-bath for 10 min. Add 7.0 ml of water R in small quantities with stirring and allow to stand in iced water for 30 min. A precipitate is formed. Allow to stand and decant the supernatant liquid. Wash the precipitate with two quantities, each of 5 ml, of iced water R, recrystallise from 10 ml of acetone R, wash with iced acetone R and dry at 75 °C at a pressure not exceeding 2.7 kPa for 30 min. The crystals melt (2.2.14) at 154 °C to 157 °C.

TESTS

Solution S. Dissolve 2.50 g in 10 ml of alcohol R and dilute to 25.0 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and colourless (2.2.2, Method II).

Acidity or alkalinity. Dissolve 1.0 g in alcohol R and dilute to 10 ml with the same solvent. Add 0.1 ml of phenolphthalein solution R; the solution is colourless. Not more than 0.5 ml of 0.01 M sodium hydroxide is required to change the colour of the indicator to pink.

Specific optical rotation (2.2.7): −48 to −51, determined on solution S.

Related substances. Examine by gas chromatography (2.2.28).

Test solution (a). Dissolve 0.20 g of the substance to be examined in methylene chloride R and dilute to 50.0 ml with the same solvent.

Test solution (b). Dilute 1.0 ml of test solution (a) to 10.0 ml with methylene chloride R.

Reference solution (a). Dissolve 40.0 mg of the substance to be examined and 40.0 mg of isomenthol R in methylene chloride R and dilute to 100.0 ml with the same solvent.

Reference solution (b). Dilute 0.10 ml of test solution (a) to 100.0 ml with methylene chloride R.

Reference solution (c). Dissolve 40.0 mg of menthol CRS in methylene chloride R and dilute to 100.0 ml with the same solvent.

The chromatographic procedure may be carried out using:

- a glass column 2.0 m long and 2 mm internal diameter packed with diatomaceous earth for gas chromatography R impregnated with 15 per cent m/m of macrogol 1500 R,
- nitrogen for chromatography R as the carrier gas at a flow rate of 30 ml/min,
- a flame-ionisation detector,

maintaining the temperature of the column at 120 °C, that of the injection port at 150 °C and that of the detector at 200 °C.

Inject separately 1 µl of each solution. Record the chromatograms for twice the retention time of the peak corresponding to menthol. In the chromatogram obtained with test solution (a), the sum of the areas of the peaks, apart from the principal peak, is not greater than 1 per cent of the area of the principal peak. Disregard any peak due to the solvent and any peak whose area is less than 0.05 per cent of the area of the principal peak. The test is not valid unless: in the chromatogram obtained with reference solution (a) the

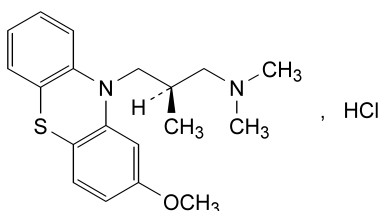
resolution between the peaks corresponding to menthol and isomenthol is not less than 1.4 and the principal peak in the chromatogram obtained with reference solution (b) has a signal-to-noise ratio of not less than 5.

Residue on evaporation. Evaporate 2.00 g on a water-bath and heat in an oven at 100 °C to 105 °C for 1 h. The residue weighs not more than 1.0 mg (0.05 per cent).

01/2005:0505

LEVOMEPRMAZINE HYDROCHLORIDE

Levomepromazini hydrochloridum


 $C_{19}H_{25}ClN_2OS$
 M_r 364.9

DEFINITION

Levomepromazine hydrochloride contains not less than 98.5 per cent and not more than the equivalent of 101.0 per cent of (2*R*)-3-(2-methoxy-10*H*-phenothiazin-10-yl)-*N,N*,2-trimethylpropan-1-amine hydrochloride, calculated with reference to the dried substance.

CHARACTERS

A white or very slightly yellow, crystalline powder, slightly hygroscopic, freely soluble in water and in alcohol. It deteriorates when exposed to air and light. It exists in two forms, one melting at about 142 °C and the other at about 162 °C.

IDENTIFICATION

- Prepare the solution protected from bright light and carry out the measurements immediately. Dissolve 50.0 mg in *water R* and dilute to 500.0 ml with the same solvent. Dilute 10.0 ml of this solution to 100.0 ml with *water R*. Examined between 230 nm and 340 nm (2.2.25), the solution shows two absorption maxima, at 250 nm and 302 nm. The specific absorbance at the maximum at 250 nm is 640 to 700.
- It complies with the identification test for phenothiazines by thin-layer chromatography (2.3.3).
- Introduce 0.2 g into a 100 ml separating funnel. Add 5 ml of *water R* and 0.5 ml of *strong sodium hydroxide solution R*. Shake vigorously with two quantities, each of 10 ml, of *ether R*. Combine the ether layers, dry over *anhydrous sodium sulphate R* and evaporate to dryness. Keep the residue at 100 °C to 105 °C for 15 min and allow to crystallise in iced water. Initiate crystallisation if necessary by scratching the wall of the flask with a glass rod. Dry the crystals at 60 °C for 2 h. The crystals melt (2.2.14) at 122 °C to 128 °C.
- It gives reaction (b) of chlorides (2.3.1).

TESTS

Solution S. Dissolve 2.50 g in *carbon dioxide-free water R* and dilute to 25.0 ml with the same solvent.

Acidity or alkalinity. To 10 ml of solution S add 0.1 ml of *bromocresol green solution R*. Not more than 0.5 ml of 0.01 *M* sodium hydroxide or 1.0 ml of 0.01 *M* hydrochloric acid is required to change the colour of the indicator.

Specific optical rotation (2.2.7): + 9.5 to + 11.5, determined on solution S and calculated with reference to the dried substance.

Related substances. Carry out the test protected from bright light. Examine by thin-layer chromatography (2.2.27), using *silica gel GF₂₅₄ R* as the coating substance.

Test solution. Dissolve 0.2 g of the substance to be examined in a mixture of 5 volumes of *diethylamine R* and 95 volumes of *methanol R* and dilute to 10 ml with the same mixture of solvents. Prepare immediately before use.

Reference solution. Dilute 0.5 ml of the test solution to 100 ml with a mixture of 5 volumes of *diethylamine R* and 95 volumes of *methanol R*.

Apply separately to the plate 10 µl of each solution. Develop over a path of 15 cm using a mixture of 10 volumes of *acetone R*, 10 volumes of *diethylamine R* and 80 volumes of *cyclohexane R*. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. Any spot in the chromatogram obtained with the test solution, apart from the principal spot, is not more intense than the spot in the chromatogram obtained with the reference solution (0.5 per cent).

Loss on drying (2.2.32). Not more than 1.0 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C for 3 h.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.300 g in 5 ml of *water R* and add 50 ml of *2-propanol R*. Titrate with 0.1 *M* sodium hydroxide, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 *M* sodium hydroxide is equivalent to 36.49 mg of $C_{19}H_{25}ClN_2OS$.

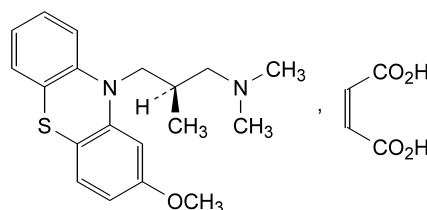
STORAGE

Store in an airtight container, protected from light.

01/2005:0925

LEVOMEPRMAZINE MALEATE

Levomepromazini maleas


 $C_{23}H_{28}N_2O_5S$
 M_r 444.6

DEFINITION

Levomepromazine maleate contains not less than 98.5 per cent and not more than the equivalent of 101.0 per cent of (2*R*)-3-(2-methoxy-10*H*-phenothiazin-10-yl)-*N,N*,2-trimethylpropan-1-amine (*Z*)-butenedioate, calculated with reference to the dried substance.

CHARACTERS

A white or slightly yellowish, crystalline powder, slightly soluble in water, sparingly soluble in methylene chloride, slightly soluble in alcohol. It deteriorates when exposed to air and light.

It melts at about 186 °C, with decomposition.

IDENTIFICATION

First identification: A, B.

Second identification: A, C, D.

- A. It complies with the test for specific optical rotation (see Tests).
- B. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *levomepromazine maleate* CRS. Examine the substances prepared as discs.
- C. It complies with the identification test for phenothiazines by thin-layer chromatography (2.3.3).
- D. Examine by thin-layer chromatography (2.2.27), using *silica gel GF₂₅₄* R as the coating substance.

Test solution. Dissolve 0.20 g of the substance to be examined in a mixture of 10 volumes of *water* R and 90 volumes of *acetone* R and dilute to 10 ml with the same mixture of solvents.

Reference solution. Dissolve 50 mg of *maleic acid* CRS in a mixture of 10 volumes of *water* R and 90 volumes of *acetone* R and dilute to 10 ml with the same mixture of solvents.

Apply separately to the plate as bands 10 mm by 2 mm 5 µl of each solution. Develop over a path of 12 cm using a mixture of 3 volumes of *water* R, 7 volumes of *anhydrous formic acid* R and 90 volumes of *di-isopropyl ether* R. Dry the plate at 120 °C for 10 min and examine in ultraviolet light at 254 nm. The chromatogram obtained with the test solution shows a band at the starting-point and another band similar in position and size to the principal band in the chromatogram obtained with the reference solution.

TESTS

pH (2.2.3). Carry out the test protected from bright light. Introduce 0.50 g into a conical flask and add 25.0 ml of *carbon dioxide-free water* R. Shake and allow the solids to settle. The pH of the supernatant solution is 3.5 to 5.5.

Specific optical rotation (2.2.7). Dissolve 1.25 g in *dimethylformamide* R and dilute to 25.0 ml with the same solvent. The specific optical rotation is –7.0 to –8.5, calculated with reference to the dried substance.

Related substances. Carry out the test protected from bright light and prepare the solutions immediately before use. Examine by thin-layer chromatography (2.2.27), using *silica gel GF₂₅₄* R as the coating substance.

Test solution. Dissolve 0.20 g of the substance to be examined in a mixture of 10 volumes of *water* R and 90 volumes of *acetone* R and dilute to 10 ml with the same mixture of solvents.

Reference solution. Dilute 0.5 ml of the test solution to 100 ml with a mixture of 10 volumes of *water* R and 90 volumes of *acetone* R.

Apply separately to the plate 10 µl of each solution. Develop over a path of 15 cm using a mixture of 10 volumes of *acetone* R, 10 volumes of *diethylamine* R and 80 volumes of *cyclohexane* R. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. Any spot in the chromatogram obtained with the test solution, apart from the principal spot, is not more intense than the spot in the chromatogram obtained with the reference solution (0.5 per cent).

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C for 3 h.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

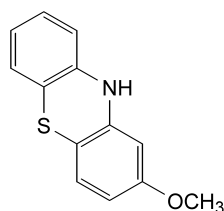
Dissolve 0.350 g in 50 ml of *anhydrous acetic acid* R. Titrate with 0.1 M *perchloric acid*, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *perchloric acid* is equivalent to 44.46 mg of C₂₃H₂₈N₂O₅S.

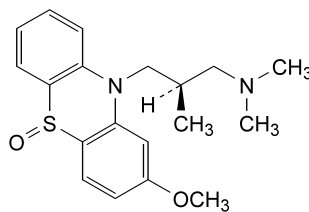
STORAGE

Store protected from light.

IMPURITIES



A. 2-methoxyphenothiazine,

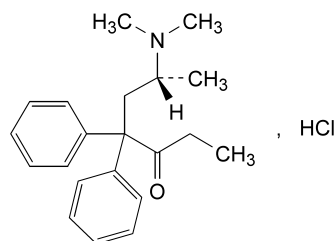


B. 10-[(2R)-3-(dimethylamino)-2-methylpropyl]-2-methoxy-10H-phenothiazine 5-oxide.

01/2005:1787

LEVOMETHADONE HYDROCHLORIDE

Levomethadoni hydrochloridum



C₂₁H₂₈ClNO

M_r 345.9

DEFINITION

(6R)-6-(Dimethylamino)-4,4-diphenylheptan-3-one hydrochloride.

Content: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

Appearance: white, crystalline powder.

Solubility: soluble in water, freely soluble in alcohol.

IDENTIFICATION

First identification: A, C, D.

Second identification: A, B, D.

A. Specific optical rotation (see Tests).

B. Melting point (2.2.14): 239 °C to 242 °C.

C. Infrared absorption spectrophotometry (2.2.24).

Comparison: Ph. Eur. reference spectrum of methadone hydrochloride.

D. Dilute 1 ml of solution S (see Tests) to 5 ml with *water R* and add 1 ml of *dilute ammonia R1*. Mix, allow to stand for 5 min and filter. The filtrate gives reaction (a) of chlorides (2.3.1).

TESTS

Solution S. Dissolve 2.50 g in *carbon dioxide-free water R* and dilute to 50.0 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and colourless (2.2.2, *Method II*).

Acidity or alkalinity. Dilute 10 ml of solution S to 25 ml with *carbon dioxide-free water R*. To 10 ml of the solution add 0.2 ml of *methyl red solution R* and 0.2 ml of 0.01 M *sodium hydroxide*. The solution is yellow. Add 0.4 ml of 0.01 M *hydrochloric acid*. The solution is red.

Specific optical rotation (2.2.7): –125 to –135 (dried substance), determined on solution S.

Related substances. Liquid chromatography (2.2.29).

Test solution. Dissolve 25.0 mg of the substance to be examined in the mobile phase and dilute to 100.0 ml with the mobile phase.

Reference solution (a). Dilute 1.0 ml of the test solution to 50.0 ml with the mobile phase. Dilute 1.0 ml of the solution to 10.0 ml with the mobile phase.

Reference solution (b). Dissolve 12.0 mg of *imipramine hydrochloride CRS* in the mobile phase and dilute to 10 ml with the mobile phase. To 1 ml of the solution add 5 ml of the test solution and dilute to 10 ml with the mobile phase.

Column:

- *size:* $l = 0.125$ m, $\varnothing = 4.6$ mm,
- *stationary phase:* octadecylsilyl silica gel for chromatography R (5 μ m),
- *temperature:* 25 °C.

Mobile phase: mix 35 volumes of *acetonitrile R* and 65 volumes of an 11.5 g/l solution of *phosphoric acid R* adjusted to pH 3.6 with *tetraethylammonium hydroxide solution R*.

Flow rate: 1.0 ml/min.

Detection: spectrophotometer at 210 nm.

Equilibration: about 30 min.

Injection: 10 μ l.

Run time: 7 times the retention time of levomethadone.

Retention time: levomethadone = about 5 min.

System suitability: reference solution (b):

- *resolution:* minimum 2.5 between the peaks due to imipramine and levomethadone.

Limits:

- *any impurity:* not more than half the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent),
- *total:* not more than 2.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent),
- *disregard limit:* 0.25 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

Dextromethadone. Liquid chromatography (2.2.29).

Test solution. Dissolve 40.0 mg of the substance to be examined in the mobile phase and dilute to 100.0 ml with the mobile phase.

Reference solution. Dilute 1.0 ml of the test solution to 10.0 ml with the mobile phase. Dilute 1.0 ml of the solution to 20.0 ml with the mobile phase.

Column:

- *size:* $l = 0.25$ m, $\varnothing = 4.6$ mm,
- *stationary phase:* 2-hydroxypropylbetadex for chromatography R (5 μ m),
- *temperature:* 10 °C.

Mobile phase: mix 1 volume of *triethylamine R* adjusted to pH 4.0 with *phosphoric acid R*, 15 volumes of *acetonitrile R* and 85 volumes of a 13.6 g/l solution of *potassium dihydrogen phosphate R*.

Flow rate: 0.7 ml/min.

Detection: spectrophotometer at 210 nm.

Equilibration: about 30 min.

Injection: 10 μ l.

Relative retention with reference to levomethadone: dextromethadone = about 1.4.

System suitability: test solution:

- *number of theoretical plates:* minimum 2000, calculated for the peak due to levomethadone,
- *tailing factor:* maximum 3 for the peak due to levomethadone.

Limit:

- *dextromethadone:* not more than the area of the principal peak in the chromatogram obtained with the reference solution (0.5 per cent).

Loss on drying (2.2.32): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 100–105 °C.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.300 g in a mixture of 40 ml of *water R* and 5 ml of *acetic acid R*. Titrate with 0.1 M *silver nitrate*. Determine the end-point potentiometrically (2.2.20), using a silver electrode.

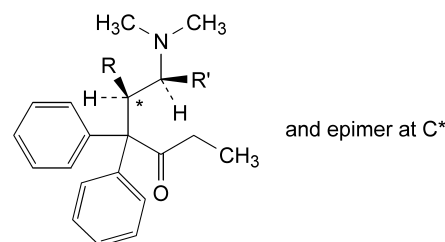
1 ml of 0.1 M *silver nitrate* is equivalent to 34.59 mg of $C_{21}H_{28}ClNO$.

STORAGE

Protected from light.

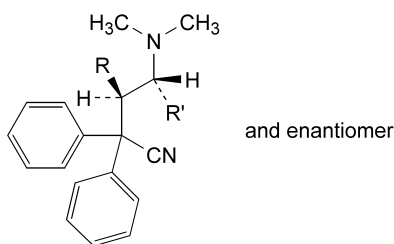
IMPURITIES

Specified impurities: A, B, C, D, E, F.



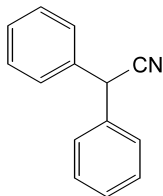
A. R = H, R' = CH₃: (6S)-6-(dimethylamino)-4,4-diphenylheptan-3-one,

D. R = CH₃, R' = H: (5RS)-6-(dimethylamino)-5-methyl-4,4-diphenylhexan-3-one,

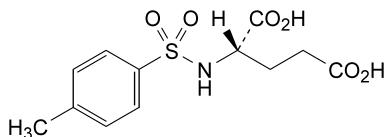


B. $R = H, R' = CH_3$: (4*RS*)-4-(dimethylamino)-2,2-diphenylpentanenitrile,

C. $R = CH_3, R' = H$: (3*RS*)-4-(dimethylamino)-3-methyl-2,2-diphenylbutanenitrile,



E. diphenylacetoneitrile,



F. (2*S*)-2-[(4-methylphenyl)sulfonyl]amino]pentanedioic acid (*N*-*p*-tosyl-L-glutamic acid).

B. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *levonorgestrel CRS*.

TESTS

Specific optical rotation (2.2.7). Dissolve 0.200 g in *chloroform R* and dilute to 10.0 ml with the same solvent. The specific optical rotation is - 30 to - 35.

Related substances. Examine by thin-layer chromatography (2.2.27), using *silica gel G R* as the coating substance.

Test solution. Dissolve 0.2 g of the substance to be examined in *chloroform R* and dilute to 10 ml with the same solvent.

Reference solution (a). Dilute 1 ml of the test solution to 10 ml with *methylene chloride R*. Dilute 1 ml of this solution to 20 ml with *methylene chloride R*.

Reference solution (b). Dilute 4 ml of reference solution (a) to 10 ml with *methylene chloride R*.

Reference solution (c) Dissolve 5 mg of *levonorgestrel CRS* and 5 mg of *ethinylestradiol CRS* in *methylene chloride R* and dilute to 50 ml with the same solvent.

Apply separately to the plate 10 µl of each solution. Develop over a path of 15 cm using a mixture of 20 volumes of *ethyl acetate R* and 80 volumes of *methylene chloride R*. Allow the plate to dry in air, spray with a 100 g/l solution of *phosphomolybdic acid R* in *alcohol R*, heat at 100 °C to 105 °C for 15 min and examine immediately. Any spot in the chromatogram obtained with the test solution, apart from the principal spot, is not more intense than the principal spot in the chromatogram obtained with reference solution (a) (0.5 per cent) and at most two such spots are more intense than the spot in the chromatogram obtained with reference solution (b) (0.2 per cent). The test is not valid unless the chromatogram obtained with reference solution (c) shows two clearly separated spots.

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 100-105 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

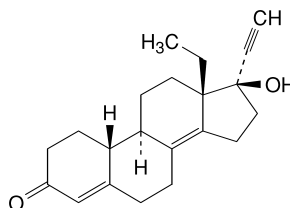
Dissolve 0.200 g in 45 ml of *tetrahydrofuran R*. Add 10 ml of a 100 g/l solution of *silver nitrate R*. After 1 min, titrate with 0.1 *M* sodium hydroxide, determining the end-point potentiometrically (2.2.20). Carry out a blank titration.

1 ml of 0.1 *M* sodium hydroxide is equivalent to 31.25 mg $C_{21}H_{28}O_2$.

STORAGE

Store protected from light.

IMPURITIES

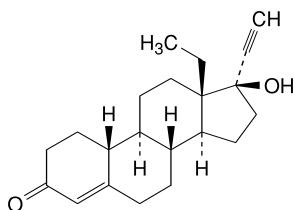


A. 13-ethyl-17-hydroxy-18,19-dinor-17α-pregna-4,8(14)-dien-20-yn-3-one,

01/2005:0926

LEVONORGESTREL

Levonorgestrelum



$C_{21}H_{28}O_2$

M_r 312.5

DEFINITION

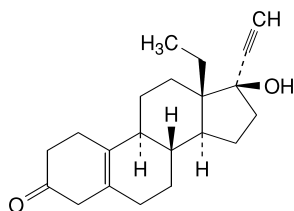
Levonorgestrel contains not less than 98.0 per cent and not more than the equivalent of 102.0 per cent of 13β-ethyl-17β-hydroxy-18,19-dinor-17α-pregna-4-en-20-yn-3-one, calculated with reference to the dried substance.

CHARACTERS

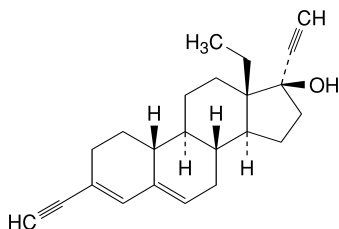
A white or almost white, crystalline powder, practically insoluble in water, sparingly soluble in methylene chloride, slightly soluble in alcohol.

IDENTIFICATION

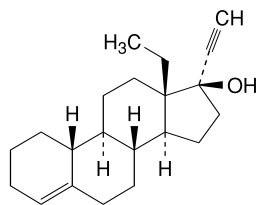
A. It complies with the test for specific optical rotation (see Tests).



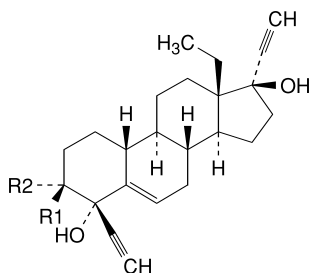
B. 13-ethyl-17-hydroxy-18,19-dinor-17 α -pregn-5(10)-en-20-yn-3-one,



C. 13-ethyl-3-ethynyl-18,19-dinor-17 α -pregna-3,5-dien-20-yn-17-ol,



D. 13-ethyl-18,19-dinor-17 α -pregn-4-en-20-yn-17-ol,



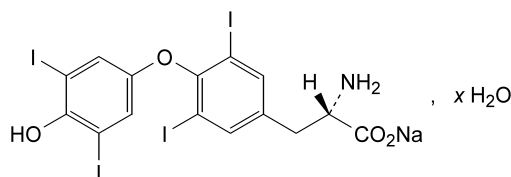
E. R1 = OH, R2 = C $\equiv$ CH: 13-ethyl-3,4-diethynyl-18,19-dinor-17 α -pregn-5-en-20-yn-3 β ,4 α ,17-triol,

F. R1 = C $\equiv$ CH, R2 = OH: 13-ethyl-3,4-diethynyl-18,19-dinor-17 α -pregn-5-en-20-yn-3 α ,4 α ,17-triol.

01/2005:0401

LEVOTHYROXINE SODIUM

Levothyroxinum natricum



C₁₅H₁₀I₄NNaO₄·xH₂O

M_r 799 (anhydrous substance)

DEFINITION

Levothyroxine sodium contains not less than 97.0 per cent and not more than the equivalent of 102.0 per cent of sodium (2*S*)-2-amino-3-[4-(4-hydroxy-3,5-diiodophenoxy)-3,5-diiodophenyl]propanoate, calculated with reference to the dried substance. It contains a variable amount of water.

CHARACTERS

An almost white or slightly brownish-yellow powder or a fine, crystalline powder, very slightly soluble in water, slightly soluble in alcohol. It dissolves in dilute solutions of alkali hydroxides.

IDENTIFICATION

First identification: A, B, E.

Second identification: A, C, D, E.

A. It complies with the test for specific optical rotation (see Tests).

B. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *levothyroxine sodium CRS*.

C. Examine by thin-layer chromatography (2.2.27), using *silica gel G R* as the coating substance.

Test solution. Dissolve 5 mg of the substance to be examined in a mixture of 5 volumes of *concentrated ammonia R* and 70 volumes of *methanol R* and dilute to 5 ml with the same mixture of solvents.

Reference solution (a). Dissolve 5 mg of *levothyroxine sodium CRS* in a mixture of 5 volumes of *concentrated ammonia R* and 70 volumes of *methanol R* and dilute to 5 ml with the same mixture of solvents.

Reference solution (b). Dissolve 5 mg of *liothyronine sodium CRS* in a mixture of 5 volumes of *concentrated ammonia R* and 70 volumes of *methanol R* and dilute to 5 ml with the same mixture of solvents. Mix 1 ml of this solution and 1 ml of the test solution.

Apply separately to the plate 5 μ l of each solution.

Develop over a path of 15 cm using a mixture of 20 volumes of *concentrated ammonia R*, 35 volumes of *2-propanol R* and 55 volumes of *ethyl acetate R*.

Allow the plate to dry in air and spray with *ninhydrin solution R*. Heat at 100 °C to 105 °C until the spots appear. Examine in daylight. The principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a). The test is not valid unless the chromatogram obtained with reference solution (b) shows two clearly separated spots.

D. To about 50 mg in a porcelain dish add a few drops of *sulphuric acid R* and heat. Violet vapour is evolved.

E. To 200 mg add 2 ml of *dilute sulphuric acid R*. Heat on a water-bath and then carefully over a naked flame, increasing the temperature gradually up to about 600 °C. Continue the ignition until most of the black particles have disappeared. Dissolve the residue in 2 ml of *water R*. The solution gives reaction (a) of sodium (2.3.1).

TESTS

Solution S. Dissolve 0.500 g in 23 ml of a gently boiling mixture of 1 volume of 1 *M* *hydrochloric acid* and 4 volumes of *alcohol R*. Cool and dilute to 25.0 ml with the same mixture of solvents.

Appearance of solution. Freshly prepared solution S is not more intensely coloured than reference solution BY₃ (2.2.2, Method II).

Specific optical rotation (2.2.7): + 16 to + 20, determined on solution S and calculated with reference to the dried substance.

Liothyronine and other related substances. Examine the chromatograms obtained in the assay. In the chromatogram obtained with test solution (a), the area of any peak corresponding to liothyronine is not greater than that of the principal peak in the chromatogram obtained with

reference solution (b) (1.0 per cent) and the sum of the areas of all the peaks apart from the principal peak and any peak corresponding to liothyronine is not greater than the area of the principal peak in the chromatogram obtained with reference solution (a) (1.0 per cent). Disregard any peak with an area less than that of the peak in the chromatogram obtained with reference solution (d).

Loss on drying (2.2.32): 6.0 per cent to 12.0 per cent, determined on 0.100 g by drying in an oven at 100 °C to 105 °C.

ASSAY

Examine by liquid chromatography (2.2.29). *Protect the solutions from light throughout the assay.*

Test solution (a). Dissolve 20.0 mg of the substance to be examined in *methanolic sodium hydroxide solution R* and dilute to 100.0 ml with the same solvent.

Test solution (b). Dilute 2.0 ml of test solution (a) to 200 ml with *methanolic sodium hydroxide solution R*.

Reference solution (a). Dissolve 20.0 mg of *levothyroxine CRS* in *methanolic sodium hydroxide solution R* and dilute to 100.0 ml with the same solvent. Dilute 2.0 ml of this solution to 200 ml with *methanolic sodium hydroxide solution R*.

Reference solution (b). Dissolve 5 mg of *liothyronine sodium CRS* in *methanolic sodium hydroxide solution R* and dilute to 50.0 ml with the same solvent. Dilute 10.0 ml of this solution to 50 ml with *methanolic sodium hydroxide solution R*. Dilute 10.0 ml of this solution to 100 ml with *methanolic sodium hydroxide solution R*.

Reference solution (c). Mix equal volumes of reference solution (a) and reference solution (b).

Reference solution (d). Dilute 1 ml of reference solution (a) to 10 ml with *methanolic sodium hydroxide solution R*.

The chromatographic procedure may be carried out using:

- a column 0.25 m long and 4 mm in internal diameter packed with *nitrile silica gel for chromatography R* (5 µm to 10 µm),
- as mobile phase at a flow rate of 1 ml/min a mixture of 1 volume of *phosphoric acid R*, 300 volumes of *acetonitrile R* and 700 volumes of *water R*,
- as detector a spectrophotometer set at 225 nm,
- a loop injector.

Inject separately 50 µl of each solution. Continue the chromatography for 3.5 times the retention time of the principal peak. The assay is not valid unless the resolution between the peaks corresponding to levothyroxine and liothyronine in the chromatogram obtained with reference solution (c) is at least 4 and the principal peak in the chromatogram obtained with reference solution (d) has a signal-to-noise ratio of at least 5. Calculate the percentage of $C_{15}H_{10}I_4NNaO_4$ from the expression:

$$\frac{S_u}{0.972S_r} \times C_r$$

S_u = area of the peak corresponding to levothyroxine in the chromatogram obtained with test solution (b);

S_r = area of the peak corresponding to levothyroxine in the chromatogram obtained with reference solution (a);

C_r = declared content of $C_{15}H_{11}I_4NO_4$ in *levothyroxine CRS*.

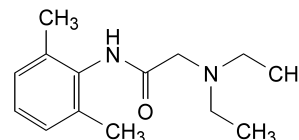
STORAGE

Store in an airtight container, protected from light, at 2 °C to 8 °C.

01/2005:0727

LIDOCAINE

Lidocainum



$C_{14}H_{22}N_2O$

M_r 234.3

DEFINITION

Lidocaine contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of 2-(diethylamino)-*N*-(2,6-dimethylphenyl)acetamide, calculated with reference to the anhydrous substance.

CHARACTERS

A white or almost white, crystalline powder, practically insoluble in water, very soluble in alcohol and in methylene chloride.

IDENTIFICATION

First identification: A, B.

Second identification: A, C, D, E.

- A. Melting point (2.2.14): 66 °C to 70 °C, determined without previous drying.
- B. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *lidocaine CRS*.
- C. Dissolve 0.20 g in a mixture of 0.5 ml of *dilute hydrochloric acid R* and 10 ml of *water R* with warming and add 10 ml of *picric acid solution R*. The precipitate, washed with *water R* and dried, melts (2.2.14) at about 230 °C, with decomposition.
- D. To about 5 mg add 0.5 ml of *fuming nitric acid R*. Evaporate to dryness on a water-bath, cool and dissolve the residue in 5 ml of *acetone R*. Add 0.2 ml of *alcoholic potassium hydroxide solution R*. A green colour is produced.
- E. Dissolve about 0.1 g in 1 ml of *alcohol R* and add 0.5 ml of a 100 g/l solution of *cobalt nitrate R*. A bluish-green precipitate is formed.

TESTS

Appearance of solution. Dissolve 1.0 g in 3 ml of *dilute hydrochloric acid R* and dilute to 10 ml with *water R*. The solution is clear (2.2.1) and colourless (2.2.2, *Method II*).

2,6-Dimethylaniline. Dissolve 0.25 g in *methanol R* and dilute to 10 ml with the same solvent. To 2 ml of the solution add 1 ml of a freshly prepared 10 g/l solution of *dimethylaminobenzaldehyde R* in *methanol R* and 2 ml of *glacial acetic acid R* and allow to stand for 10 min. Any yellow colour in the solution is not more intense than that in a standard prepared at the same time and in the same manner using 2 ml of a 2.5 mg/l solution of *2,6-dimethylaniline R* in *methanol R* (100 ppm).

Chlorides (2.4.4). Dissolve 1.4 g in a mixture of 3 ml of *dilute nitric acid R* and 12 ml of *water R*. The solution complies with the limit test for chlorides (35 ppm).

Sulphates (2.4.13). Dissolve 0.2 g in 5 ml of *alcohol R* and dilute to 20 ml with *distilled water R*. 15 ml of the solution complies with the limit test for sulphates (0.1 per cent).

Heavy metals (2.4.8). 1.0 g complies with limit test C for heavy metals (20 ppm). Prepare the standard using 2 ml of *lead standard solution (10 ppm Pb R)*.

Water (2.5.12). Not more than 1.0 per cent, determined on 1.000 g by the semi-micro determination of water.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

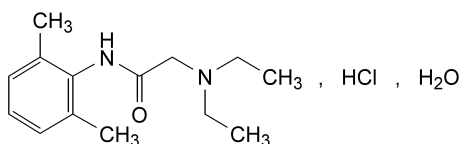
ASSAY

To 0.200 g add 50 ml of *anhydrous acetic acid R* and stir until dissolution is complete. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (2.2.20). 1 ml of *0.1 M perchloric acid* is equivalent to 23.43 mg of $C_{14}H_{23}ClN_2O$.

01/2005:0227

LIDOCAINE HYDROCHLORIDE

Lidocaini hydrochloridum

 $C_{14}H_{23}ClN_2O \cdot H_2O$ M_r 288.8

DEFINITION

Lidocaine hydrochloride contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of 2-(diethylamino)-*N*-(2,6-dimethylphenyl)acetamide hydrochloride, calculated with reference to the anhydrous substance.

CHARACTERS

A white, crystalline powder, very soluble in water, freely soluble in alcohol.

IDENTIFICATION

First identification: A, B, F.

Second identification: A, C, D, E, F.

- Melting point (2.2.14): 74 °C to 79 °C, determined without previous drying.
- Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *lidocaine hydrochloride CRS*.
- Dissolve 0.2 g in 10 ml of *water R* and add 10 ml of *picric acid solution R*. The precipitate, washed with *water R* and dried, melts (2.2.14) at about 230 °C.
- To about 5 mg add 0.5 ml of *fuming nitric acid R*. Evaporate to dryness on a water-bath, cool and dissolve the residue in 5 ml of *acetone R*. Add 0.2 ml of *alcoholic potassium hydroxide solution R*. A green colour is produced.
- To 5 ml of solution S (see Tests) add 5 ml of *water R* and make alkaline with *dilute sodium hydroxide solution R*. Collect the precipitate on a filter and wash with *water R*. Dissolve half of the precipitate in 1 ml of *alcohol R* and add 0.5 ml of a 100 g/l solution of *cobalt nitrate R*. A bluish-green precipitate is formed.
- It gives reaction (a) of chlorides (2.3.1).

TESTS

Solution S. Dissolve 1.0 g in *carbon dioxide-free water R* and dilute to 20 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and colourless (2.2.2, *Method II*).

pH (2.2.3). Dilute 1 ml of solution S to 10 ml with *carbon dioxide-free water R*. The pH of the solution is 4.0 to 5.5.

Impurity A

Solution (a). Dissolve 0.25 g of the substance to be examined in *methanol R* and dilute to 10 ml with the same solvent. This solution is used to prepare the test solution.

Solution (b). Dissolve 50 mg of *2,6-dimethylaniline R* in *methanol R* and dilute to 100 ml with the same solvent. Dilute 1 ml of the solution to 100 ml with *methanol R*. This solution is used to prepare the standard.

Using three flat-bottomed tubes, place in the first 2 ml of solution (a), in the second 1 ml of solution (b) and 1 ml of *methanol R* and in the third 2 ml of *methanol R* (used to prepare a blank). To each tube add 1 ml of a freshly prepared 10 g/l solution of *dimethylaminobenzaldehyde R* in *methanol R* and 2 ml of *glacial acetic acid R* and allow to stand at room temperature for 10 min. The intensity of the yellow colour of the test solution is between that of the blank and that of the standard (100 ppm).

Heavy metals (2.4.8). Dissolve 1.0 g in *water R* and dilute to 25 ml with the same solvent. Carry out the prefiltration. 10 ml of the prefiltrate complies with limit test E for heavy metals (5 ppm). Prepare the standard using 2 ml of *lead standard solution (1 ppm Pb R)*.

Water (2.5.12): 5.5 per cent to 7.0 per cent, determined on 0.25 g by the semi-micro determination of water.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

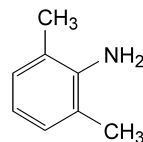
Dissolve 0.220 g in 50 ml of *alcohol R* and add 5.0 ml of *0.01 M hydrochloric acid*. Carry out a potentiometric titration (2.2.20), using *0.1 M sodium hydroxide*. Read the volume added between the 2 inflexion points.

1 ml of *0.1 M sodium hydroxide* is equivalent to 27.08 mg of $C_{14}H_{23}ClN_2O$.

STORAGE

Store protected from light.

IMPURITIES



A. 2,6-dimethylaniline.

01/2005:0957

LIME FLOWER

Tiliae flos

DEFINITION

Lime flower consists of the whole, dried inflorescence of *Tilia cordata* Miller, of *Tilia platyphyllos* Scop., of *Tilia × vulgaris* Heyne or a mixture of these.

CHARACTERS

Lime flower has a faint aromatic odour and a faint, sweet and mucilaginous taste.

It has the macroscopic and microscopic characters described under identification tests A and B.

IDENTIFICATION

A. The inflorescence is yellowish-green. The main axis of the inflorescence bears a linguiform bract, membranous, yellowish-green, practically glabrous, the central vein of which is joined for up to about half of its length with the peduncle. The inflorescence usually consists of two to seven flowers, occasionally up to sixteen. The sepals are detached easily from the perianth; they are up to 6 mm long, their abaxial surface is usually glabrous, their adaxial surface and their borders are strongly pubescent. The five spatulate, thin petals are yellowish-white, up to 8 mm long. They show fine venation and their borders only are sometimes covered with isolated trichomes. The numerous stamens are free and usually constitute five groups. The superior ovary has a pistil with a somewhat 5-lobate stigma.

B. Separate the inflorescence into its different parts. Examine under a microscope using *chloral hydrate solution R*. The adaxial epidermis of the bract shows cells with straight or slightly sinuous anticlinal walls; the abaxial epidermis shows cells with wavy-sinuous anticlinal walls and anomocytic stomata (2.8.3). Isolated cells in the mesophyll contain small calcium oxalate cluster crystals. The parenchyma of the sepals shows, particularly near the veins, numerous mucilaginous cells and cells containing small calcium oxalate clusters. The adaxial epidermis of sepals bears bent, thick-walled covering trichomes, unicellular or stellate with up to five cells. The epidermal cells of the petals show straight anticlinal walls with a striated cuticle without stomata. The parenchyma of the petals shows small calcium oxalate clusters and especially in its acuminate part mucilaginous cells. The pollen grains have a diameter of about 30 µm to 40 µm and are oval or slightly triangular with three germinal pores and a finely granulated exine. The ovary is glabrous or densely covered with trichomes, often very twisted, unicellular or stellate with two to four branches.

C. Examine by thin-layer chromatography (2.2.27), using a suitable silica gel as the coating substance.

Test solution. Shake 1.0 g of the powdered drug (355) with 10 ml of *methanol R* in a water-bath at 65 °C for 5 min. Allow to cool and filter.

Reference solution. Dissolve 2.0 mg of *caffeic acid R*, 5 mg of *hyperoside R* and 5 mg of *rutin R* in 10 ml of *methanol R*.

Apply separately to the plate as bands 10 µl of each solution. Develop over a path of 15 cm using a mixture of 10 volumes of *anhydrous formic acid R*, 10 volumes of *water R*, 30 volumes of *methyl ethyl ketone R* and 50 volumes of *ethyl acetate R*. Dry the plate at 100 °C to 105 °C and spray the warm plate with a 10 g/l solution of *diphenylboric acid aminoethyl ester R* in *methanol R*. Then spray the plate with a 50 g/l solution of *macrogol 400 R* in *methanol R*. Allow the plate to dry for about 30 min and examine in ultraviolet light at 365 nm. The chromatogram obtained with the reference solution shows in order of increasing R_f value zones corresponding to rutin and hyperoside with yellowish-orange to brownish-orange fluorescence and a zone corresponding to caffeic acid with greenish-blue fluorescence. In the chromatogram obtained with the test solution, the main zone shows brownish-yellow to orange fluorescence. This

zone is situated just above the zone of hyperoside in the chromatogram obtained with the reference solution. In daylight, this zone stands out from the other zones as the main zone. At the R_f level of rutin there is also a zone of brownish-yellow fluorescence. Below this zone, two zones of yellow fluorescence may be present. Between the zones of rutin and hyperoside, zones of orange and yellow fluorescence are visible. Between the zones of hyperoside and caffeic acid, up to five zones of yellow to orange fluorescence are present. Immediately below the zone of caffeic acid is a zone of a blue fluorescence.

TESTS

Foreign matter (2.8.2). Not more than 2 per cent, determined on 30 g. There are no inflorescences with a bract bearing at the abaxial face stellate, five- to eight-rayed trichomes and flowers having an apparent double corolla by transformation of five stamens into petal-like staminoids and having a pistil which is not lobular nor indented. 6-merous flowers occur only occasionally (*Tilia americana* L., *Tilia tomentosa* Moench).

Loss on drying (2.2.32). Not more than 12.0 per cent, determined on 1.000 g of the powdered drug (355) by drying in an oven at 100 °C to 105 °C for 2 h.

Total ash (2.4.16). Not more than 8.0 per cent.

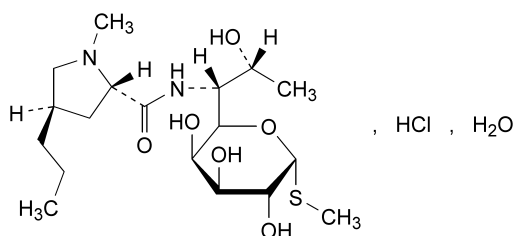
STORAGE

Store protected from light.

01/2005:0583

LINCOMYCIN HYDROCHLORIDE

Lincomycini hydrochloridum


 $C_{18}H_{35}ClN_2O_6S \cdot H_2O$
 M_r 461.0

DEFINITION

Lincomycin hydrochloride consists mainly of the methyl 6,8-dideoxy-6-[[[(2S,4R)-1-methyl-4-propylpyrrolidin-2-yl]carbonyl]amino]-1-thio-D-erythro-α-D-galactopyranoside hydrochloride, an antimicrobial substance produced by *Streptomyces lincolnensis* var. *lincolnensis* or by any other means. It contains not less than 82.5 per cent and not more than 93.0 per cent of lincomycin ($C_{18}H_{34}N_2O_6S$), calculated with reference to the anhydrous substance.

CHARACTERS

A white or almost white, crystalline powder, very soluble in water, slightly soluble in alcohol, very slightly soluble in acetone.

IDENTIFICATION

First identification: A, D.

Second identification: B, C, D.

A. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *lincomycin hydrochloride CRS*.

- B. Examine by thin-layer chromatography (2.2.27), using *silica gel G R* as the coating substance.

Test solution. Dissolve 10 mg of the substance to be examined in *methanol R* and dilute to 10 ml with the same solvent.

Reference solution (a). Dissolve 10 mg of *lincomycin hydrochloride CRS* in *methanol R* and dilute to 10 ml with the same solvent.

Reference solution (b). Dissolve 10 mg of *lincomycin hydrochloride CRS* and 10 mg of *clindamycin hydrochloride CRS* in *methanol R* and dilute to 10 ml with the same solvent.

Apply separately to the plate 5 µl of each solution. Develop over a path of 15 cm using the upper layer from a mixture of 20 volumes of *2-propanol R*, 40 volumes of a 150 g/l solution of *ammonium acetate R* previously adjusted to pH 9.6 with *ammonia R* and 45 volumes of *ethyl acetate R*. Allow the plate to dry in air and spray with a 1 g/l solution of *potassium permanganate R*. The principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a). The test is not valid unless the chromatogram obtained with reference solution (b) shows two clearly separated spots.

- C. Dissolve about 10 mg in 2 ml of *dilute hydrochloric acid R* and heat in a water-bath for 3 min. Add 3 ml of *sodium carbonate solution R* and 1 ml of a 20 g/l solution of *sodium nitroprusside R*. A violet-red colour develops.
- D. Dissolve 0.1 g in *water R* and dilute to 10 ml with the same solvent. The solution gives reaction (a) of chlorides (2.3.1).

TESTS

Solution S. Dissolve 2.0 g in *carbon dioxide-free water R* and dilute to 20 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and not more intensely coloured than reference solution Y₆ (2.2.2, Method II).

pH (2.2.3). The pH of solution S is 3.5 to 5.5.

Specific optical rotation (2.2.7). Dissolve 1.000 g in *water R* and dilute to 25.0 ml with the same solvent. The specific optical rotation is + 135 to + 150, calculated with reference to the anhydrous substance.

Lincomycin B. Examine the chromatogram obtained in the assay with test solution (a). The area of the peak due to lincomycin B, which is eluted just before lincomycin, is not more than 5 per cent of the area of the peak due to lincomycin.

Heavy metals (2.4.8). 2.0 g complies with limit test C for heavy metals (5 ppm). Prepare the standard using 1.0 ml of *lead standard solution (10 ppm Pb) R*.

Water (2.5.12). 3.1 per cent to 4.6 per cent, determined on 0.500 g by the semi-micro determination of water.

Sulphated ash (2.4.14). Not more than 0.5 per cent, determined on 1.0 g.

Bacterial endotoxins (2.6.14): less than 0.50 IU/mg, if intended for use in the manufacture of parenteral dosage forms without a further appropriate procedure for removal of bacterial endotoxins.

ASSAY

Examine by gas chromatography (2.2.28), using *dotriacontane R* as the internal standard.

Internal standard solution. Dissolve 0.200 g of *dotriacontane R* in *chloroform R* and dilute to 25.0 ml with the same solvent.

Test solution (a). Dissolve 0.100 g of the substance to be examined in a 20 g/l solution of *imidazole R* in *chloroform R* and dilute to 100.0 ml with the same solution. Shake until dissolution is complete. Place 4.0 ml of the solution in a ground-glass-stoppered 15 ml centrifuge tube. Add 1.0 ml of a mixture of 1 volume of *chlorotrimethylsilane R* and 99 volumes of *N,O-bis(trimethylsilyl)acetamide R* and swirl gently. Position the glass stopper loosely in the tube and heat at 65 °C for 30 min.

Test solution (b). Prepare as described for test solution (a) but add 10.0 ml of the internal standard solution before dissolution of the substance to be examined.

Reference solution. Prepare as described for test solution (a) using 0.100 g of *lincomycin hydrochloride CRS* instead of the substance to be examined and adding 10.0 ml of the internal standard solution before dissolution of the reference substance.

The chromatographic procedure may be carried out using:

- a glass column 1.5 m long and 3 mm in internal diameter packed with *silanised diatomaceous earth for gas chromatography R* impregnated with 3 per cent *m/m* of *poly(methylphenylsiloxane) R*,
 - *helium for chromatography R* as the carrier gas at a flow rate of about 45 ml/min,
 - a flame-ionisation detector,
- maintaining the temperature of the column at 260 °C and that of the injection port and of the detector between 260 °C and 290 °C. Inject the chosen volume of the test solutions and the reference solution.

STORAGE

Store in an airtight container at a temperature not exceeding 30 °C. If the substance is sterile, store in a sterile, airtight, tamper-proof container.

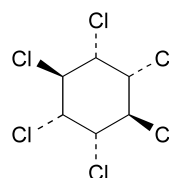
LABELLING

The label states, where applicable, that the substance is free from bacterial endotoxins.

01/2005:0772

LINDANE

Lindanum



C₆H₆Cl₆

M_r 290.8

DEFINITION

Lindane contains not less than 99.0 per cent and not more than the equivalent of 100.5 per cent of *r*-1,*c*-2,*t*-3,*c*-4,*c*-5,*t*-6-hexachlorocyclohexane.

CHARACTERS

A white or almost white, crystalline powder, practically insoluble in water, freely soluble in acetone, soluble in ethanol.

IDENTIFICATION

First identification: A, B.

Second identification: A, C, D.

- A. Melting point (2.2.14): 112 °C to 115 °C.
- B. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *lindane CRS*. Examine the substances prepared as discs.
- C. Examine the chromatograms obtained in the test for related substances. The principal spot in the chromatogram obtained with test solution (b) is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).
- D. Dissolve about 5 mg in 4 ml of *alcohol R*. Add 1 ml of 0.5 M *potassium hydroxide alcoholic*. Allow to stand for 10 min. The solution gives reaction (a) of chlorides (2.3.1).

TESTS

Appearance of solution. Dissolve 0.50 g in *acetone R* and dilute to 10 ml with the same solvent. The solution is clear (2.2.1) and not more intensely coloured than reference solution B₇ (2.2.2, *Method II*).

Related substances. Examine by thin-layer chromatography (2.2.27), using *silica gel G R* as the coating substance.

Test solution (a). Dissolve 1.0 g of the substance to be examined in *chloroform R* and dilute to 10 ml with the same solvent.

Test solution (b). Dilute 1 ml of test solution (a) to 10 ml with *chloroform R*.

Reference solution (a). Dissolve 0.1 g of *lindane CRS* in *chloroform R* and dilute to 10 ml with the same solvent.

Reference solution (b). Dilute 1 ml of test solution (b) to 10 ml with *chloroform R*.

Reference solution (c). Dissolve 10 mg of α -hexachlorocyclohexane *CRS* in test solution (a) and dilute to 5 ml with the same solvent.

Apply separately to the plate 1 µl of each solution. Develop over a path of 12 cm using a mixture of 10 volumes of *chloroform R* and 90 volumes of *cyclohexane R*. Dry the plate in a current of air, irradiate with ultraviolet light at 254 nm for 15 min and spray with a 6 g/l solution of *dicarboxidine hydrochloride R* in *alcohol (90 per cent V/V) R*. Examine in daylight. Any spot in the chromatogram obtained with test solution (a), apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (b) (1.0 per cent). The test is not valid unless the chromatogram obtained with reference solution (c) shows two clearly separated spots.

Chlorides (2.4.4). To 0.75 g, finely powdered, add 15 ml of *water R*. Boil for 1 min. Allow to cool, shaking frequently, and filter. To 10 ml of the filtrate add 3 ml of *water R* and 2 ml of *alcohol R*. The solution complies with the limit test for chlorides (100 ppm).

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

To 0.200 g add 10 ml of *alcohol R* and warm on a water-bath until dissolved. Cool, add 20 ml of 0.5 M *alcoholic potassium hydroxide* and allow to stand for 10 min, swirling frequently. Add 50 ml of *water R*, 20 ml of *dilute nitric acid R*, 25.0 ml of 0.1 M *silver nitrate* and 5 ml of *ferric ammonium sulphate solution R2*. Titrate with 0.1 M *ammonium thiocyanate* until a reddish-yellow colour is obtained. Carry out a blank titration.

1 ml of 0.1 M *silver nitrate* is equivalent to 9.694 mg of C₆H₆Cl₆.

STORAGE

Store protected from light.

01/2005:1232

LINOLEOYL MACROGOLGLYCERIDES

Macrogolglyceridum linoleates

DEFINITION

Linoleoyl macrogolglycerides are mixtures of monoesters, diesters and triesters of glycerol and monoesters and diesters of macrogols. They are obtained by partial alcoholysis of an unsaturated oil mainly containing triglycerides of linoleic acid using macrogol with a mean relative molecular mass between 300 and 400 or by esterification of glycerol and macrogol with unsaturated fatty acids or by mixing of glycerol esters and condensates of ethylene oxide with the fatty acids of this unsaturated oil.

CHARACTERS

Amber, oily liquids which may give rise to a deposit after prolonged periods at 20 °C, practically insoluble but dispersible in water, freely soluble in methylene chloride.

The viscosity at 40 °C is about 35 mPa·s, the relative density at 20 °C is about 0.95, the refractive index at 20 °C is about 1.47.

IDENTIFICATION

- A. Examine by thin-layer chromatography (2.2.27), using a suitable silica gel as the coating substance.

Test solution. Dissolve 1.0 g of the substance to be examined in *methylene chloride R* and dilute to 20 ml with the same solvent.

Apply to the plate 10 µl of the test solution. Develop over a path of 15 cm using a mixture of 30 volumes of *hexane R* and 70 volumes of *ether R*. Allow the plate to dry in air. Spray with a 0.1 g/l solution of *rhodamine B R* in *alcohol R* and examine in ultraviolet light at 365 nm. The chromatogram shows a spot corresponding to triglycerides with an *R_f* value of about 0.9 (*R_{st}* 1) and spots corresponding to 1,3-diglycerides (*R_f* 0.7), to 1,2-diglycerides (*R_f* 0.6), to monoglycerides (*R_f* 0.1) and to esters of macrogol (*R_f* 0).

- B. They comply with the test for hydroxyl value (see Tests).
- C. They comply with the test for fatty acid composition (see Tests).
- D. They comply with the test for saponification value (see Tests).

TESTS

Acid value (2.5.1). Not more than 2.0, determined on 2.0 g.

Hydroxyl value (2.5.3, *Method A*): 45 to 65, determined on 1.0 g.

Iodine value (2.5.4): 90 to 110.

Peroxide value (2.5.5). Not more than 12.0, determined on 2.0 g.

Saponification value (2.5.6): 150 to 170, determined on 2.0 g.

Alkaline impurities. Into a test-tube introduce 5.0 g and carefully add a mixture, neutralised if necessary with 0.01 M *hydrochloric acid* or with 0.01 M *sodium hydroxide*, of 0.05 ml of a 0.4 g/l solution of *bromophenol blue R* in *alcohol R*, 0.3 ml of *water R* and 10 ml of *alcohol R*.

Shake and allow to stand. Not more than 1.0 ml of 0.01 M hydrochloric acid is required to change the colour of the upper layer to yellow.

Free glycerol. Not more than 3.0 per cent. Dissolve 1.20 g in 25.0 ml of methylene chloride R. Heat if necessary. After cooling, add 100 ml of water R. Shake and add 25.0 ml of a 6 g/l solution of periodic acid R. Shake and allow to stand for 30 min. Add 40 ml of a 75 g/l solution of potassium iodide R. Allow to stand for 1 min. Add 1 ml of starch solution R. Titrate the iodine with 0.1 M sodium thiosulphate. Carry out a blank titration.

1 ml of 0.1 M sodium thiosulphate is equivalent to 2.3 mg of glycerol.

Composition of fatty acids (2.4.22, Method A). The fatty acid fraction has the following composition:

- palmitic acid: 4.0 per cent to 20.0 per cent,
- stearic acid: not more than 6.0 per cent,
- oleic acid: 20.0 per cent to 35.0 per cent,
- linoleic acid: 50.0 per cent to 65.0 per cent,
- linolenic acid: not more than 2.0 per cent,
- arachidic acid: not more than 1.0 per cent,
- eicosenoic acid: not more than 1.0 per cent.

Ethylene oxide and dioxan (2.4.25). Not more than 1 ppm of ethylene oxide and not more than 10 ppm of dioxan.

Heavy metals (2.4.8). 2.0 g complies with limit test C for heavy metals (10 ppm). Prepare the standard using 2 ml of lead standard solution (10 ppm Pb) R.

Water (2.5.12). Not more than 1.0 per cent, determined on 1.0 g by the semi-micro determination of water, use a mixture of 30 volumes of anhydrous methanol R and 70 volumes of methylene chloride R as solvent.

Total ash (2.4.16). Not more than 0.1 per cent, determined on 1.0 g.

STORAGE

Store protected from light at room temperature.

LABELLING

The label states the type of macrogol used (mean relative molecular mass) or the number of units of ethylene oxide per molecule (nominal value).

01/2005:0095

LINSEED

Lini semen

DEFINITION

Linseed consists of the dried ripe seeds of *Linum usitatissimum* L.

DESCRIPTION

The seeds have a flattened, elongated ovoid shape and are 4 mm to 6 mm long, 2 mm to 3 mm wide and 1.5 mm to 2 mm thick; one end is rounded and the other end forms an oblique point near which the hilum appears as a slight depression. The testa is dark reddish-brown, smooth and glossy but when viewed with a lens the surface is seen to be minutely pitted. The interior of the testa has a narrow, whitish endosperm and an embryo composed of 2 large, flattened, yellowish and oily cotyledons; the radicle points towards the hilum.

Examined under a microscope, the testa is composed of an epidermis of isodiametric cells with mucilaginous outer walls and suberised inner walls; within this is an

area of collenchymatous cells followed by a single layer of longitudinally elongated sclereids, each 120 µm to 190 µm long and 12 µm to 15 µm wide, with thickened and pitted walls; the testa has a hyaline layer composed of thin-walled parenchyma and the inner epidermis formed of a layer of flattened polygonal cells each containing a mass of orange-brown pigment. The endosperm and cotyledons are composed of polygonal parenchymatous cells with slightly thickened walls, containing aleurone grains up to 20 µm in diameter and globules of fatty oil; starch is absent.

The powder is greasy to the touch; it is yellowish-brown and has a slight characteristic odour and a mucilaginous and oily taste. It consists of: fragments of the outer epidermal cells of the testa filled with mucilage; sub-epidermal collenchymatous layer seen in surface view as round cells with distinct triangular intercellular spaces often in connection with groups of elongated sclereids with pitted walls; thin-walled pitted cells of the hyaline layer often remaining attached to the elongated sclereids and crossing them at approximately right angles; pigmented cells of the inner epidermis of the testa; parenchyma of the endosperm and cotyledons containing aleurone grains and fatty oil. Starch granules are absent.

TESTS

Odour and taste. The drug does not have a rancid odour or taste.

Foreign matter (2.8.2). Not more than 1.5 per cent.

Swelling index (2.8.4). Not less than 4 for the whole drug and not less than 4.5 for the powdered drug (710).

Sulphated ash (2.4.14). Not more than 6.0 per cent, determined on 1.00 g of powdered drug.

STORAGE

Store protected from light.

01/2005:1908

LINSEED OIL, VIRGIN

Lini oleum virginale

DEFINITION

Virgin oil obtained by cold expression from ripe seeds of *Linum usitatissimum* L. A suitable antioxidant may be added.

CHARACTERS

Appearance: clear, yellow or brownish-yellow liquid, on exposure to air turning dark and gradually thickening. When cooled, it becomes a soft mass at about –20 °C.

Solubility: very slightly soluble in alcohol, miscible with light petroleum.

Relative density: about 0.931.

Refractive index: about 1.480.

IDENTIFICATION

First identification: B, C.

Second identification: A, B.

A. Identification of fatty oils by thin-layer chromatography (2.3.2). The chromatogram obtained is similar to the type chromatogram for linseed oil.

B. It complies with the test for iodine value (see Tests).

C. It complies with the test for composition of fatty acids (see Tests).

TESTS

Acid value (2.5.1): maximum 4.5.

Iodine value (2.5.4): 160 to 200.

Peroxide value (2.5.5): maximum 15.0.

Saponification value (2.5.6): 188 to 195. Carry out the saponification for 1 h.

Unsaponifiable matter (2.5.7): maximum 1.5 per cent, determined on 5.0 g.

Composition of fatty acids. Gas chromatography (2.4.22, Method C). Use the calibration mixture in Table 2.4.22-3.

Composition of the fatty-acid fraction of the oil:

- *fatty acids with a chain length less than C₁₆*: maximum 1.0 per cent,
- *palmitic acid*: 3.0 per cent to 8.0 per cent,
- *palmitoleic acid* (equivalent chain length on polyethyleneglycol adipate 16.3): maximum 1.0 per cent,
- *stearic acid*: 2.0 per cent to 8.0 per cent,
- *oleic acid* (equivalent chain length on polyethyleneglycol adipate 18.3): 11.0 per cent to 35.0 per cent,
- *linoleic acid* (equivalent chain length on polyethyleneglycol adipate 18.9): 11.0 per cent to 24.0 per cent,
- *linolenic acid* (equivalent chain length on polyethyleneglycol adipate 19.7): 35.0 per cent to 65.0 per cent,
- *arachidic acid*: maximum 1.0 per cent.

Cadmium (2.4.27): maximum 0.5 ppm.

Water (2.5.32): maximum 0.1 per cent, determined on 5.00 g.

STORAGE

In an airtight container, protected from light.

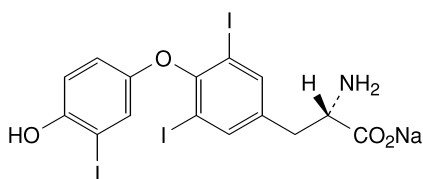
LABELLING

The label states the name and concentration of any added antioxidant.

01/2005:0728

LIOTHYRONINE SODIUM

Liothyroninum natricum

C₁₅H₁₁I₃NNaO₄M_r 673

DEFINITION

Liothyronine sodium contains not less than 95.0 per cent and not more than the equivalent of 102.0 per cent of sodium (2S)-2-amino-3-[4-(4-hydroxy-3-iodophenoxy)-3,5-diiodophenyl]propanoate, calculated with reference to the dried substance.

CHARACTERS

A white or slightly coloured powder, practically insoluble in water, slightly soluble in alcohol. It dissolves in dilute solutions of alkali hydroxides.

IDENTIFICATION

First identification: A, C, E.

Second identification: A, B, D, E.

A. It complies with the test for specific optical rotation (see Tests).

B. Dissolve 10.0 mg in 0.1 M sodium hydroxide and dilute to 100.0 ml with the same solvent. Examined between 230 nm and 350 nm (2.2.25), the solution shows an absorption maximum at 319 nm. The specific absorbance at the maximum is 63 to 69, calculated with reference to the dried substance.

C. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with liothyronine sodium CRS.

D. To about 50 mg in a porcelain dish add a few drops of sulphuric acid R and heat. Violet vapour is evolved.

E. Dissolve the residue obtained in the test for sulphated ash in 2 ml of water R. The solution gives reaction (a) of sodium (2.3.1).

TESTS

Solution S. Dissolve 0.250 g in a mixture of 1 volume of 1 M hydrochloric acid and 4 volumes of alcohol R and dilute to 25.0 ml with the same mixture of solvents.

Appearance of solution. Freshly prepared solution S is not more intensely coloured than intensity 5 of the range of reference solutions of the most appropriate colour (2.2.2, Method II).

Specific optical rotation (2.2.7). + 18.0 to + 22.0, determined on solution S and calculated with reference to the dried substance.

Levothyroxine and other related substances. Examine the chromatograms obtained in the assay. In the chromatogram obtained with test solution (a), the area of any peak corresponding to levothyroxine is not greater than that of the principal peak in the chromatogram obtained with reference solution (c) (5.0 per cent) and the sum of the areas of all the peaks apart from the principal peak and any peak corresponding to levothyroxine is not greater than half the area of the principal peak in the chromatogram obtained with reference solution (c) (2.5 per cent). Disregard any peak with an area less than that of the peak in the chromatogram obtained with reference solution (e).

Chlorides. Not more than 2.0 per cent, expressed as NaCl and calculated with reference to the dried substance.

Dissolve 0.500 g in a 2 g/l solution of sodium hydroxide R and dilute to 100 ml with the same solvent. Add 15 ml of dilute nitric acid R and titrate with 0.05 M silver nitrate, determining the end-point potentiometrically (2.2.20).

1 ml of 0.05 M silver nitrate is equivalent to 2.93 mg of NaCl.

Loss on drying (2.2.32). Not more than 4.0 per cent, determined on 0.500 g by drying in an oven at 105 °C for 2 h.

Sulphated ash (2.4.14). 9.0 per cent to 12.2 per cent, determined on 0.200 g and calculated with reference to the dried substance.

ASSAY

Examine by liquid chromatography (2.2.29).

Test solution (a). Dissolve 20.0 mg in methanolic sodium hydroxide solution R and dilute to 100.0 ml with the same solvent.

Test solution (b). Dilute 5.0 ml of test solution (a) to 200 ml with methanolic sodium hydroxide solution R.

Reference solution (a). Dissolve 20.0 mg of liothyronine sodium CRS in methanolic sodium hydroxide solution R and dilute to 100.0 ml with the same solvent.

Reference solution (b). Dilute 5.0 ml of reference solution (a) to 200 ml with *methanolic sodium hydroxide solution R*.

Reference solution (c). Dissolve 5 mg of *levothyroxine sodium CRS* in *methanolic sodium hydroxide solution R* and dilute to 50.0 ml with same solvent. Dilute 10.0 ml of this solution to 100.0 ml with *methanolic sodium hydroxide solution R*.

Reference solution (d). Mix equal volumes of reference solution (b) and reference solution (c).

Reference solution (e). Dilute 1.0 ml of reference solution (b) to 25.0 ml with *methanolic sodium hydroxide solution R*.

The chromatographic procedure may be carried out using:

- a column 0.25 m long and 4 mm in internal diameter packed with *nitrile silica gel for chromatography R* (5–10 µm),
- as mobile phase at a flow rate of 1 ml/min a mixture of 5 volumes of *phosphoric acid R*, 300 volumes of *acetonitrile R* and 700 volumes of *water R*,
- as detector a spectrophotometer set at 225 nm,
- a loop injector.

Inject separately 50 µl of each solution. Record the chromatogram obtained with test solution (a) for 5 times the retention time of the principal peak. The test is not valid unless the resolution between the peaks corresponding to *liothyronine* and *levothyroxine* in the chromatogram obtained with reference solution (d) is at least 4 and the principal peak in the chromatogram obtained with reference solution (e) has a signal-to-noise ratio of at least 5. Calculate the content of $C_{15}H_{11}I_3NNaO_4$ from the peak areas in the chromatograms obtained with test solution (b) and reference solution (b) and the declared content of $C_{15}H_{11}I_3NNaO_4$ in *liothyronine sodium CRS*.

STORAGE

Store in an airtight container, protected from light, at a temperature between 2 °C and 8 °C.

01/2005:1536

LIQUORICE ETHANOLIC LIQUID EXTRACT, STANDARDISED

Liquiritiae extractum fluidum ethanolicum normatum

DEFINITION

Standardised liquorice ethanolic liquid extract is produced from *Liquorice root* (0277). It contains not less than 3.0 per cent and not more than 5.0 per cent of glycyrrhizic acid ($C_{42}H_{62}O_{16}$; M_r 823).

PRODUCTION

The extract is produced from the drug and alcohol (70 per cent V/V), with an appropriate procedure for liquid extracts.

CHARACTERS

A dark brown, clear liquid with a faint characteristic odour and a sweet taste.

IDENTIFICATION

Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel F₂₅₄ plate R*.

Test solution. Place 1.0 g of the extract to be examined in a 50 ml round-bottomed flask, add 16.0 ml of *water R* and 4.0 ml of *hydrochloric acid R1* and heat on a water-bath under a reflux condenser for 30 min. Allow to cool and filter.

Dry the filter and the round-bottomed flask at 105 °C for 60 min. Transfer the filter to the round-bottomed flask, add 20 ml of *ether R* and heat in a water-bath at 40 °C under a reflux condenser for 5 min. Allow to cool and filter. Evaporate the filtrate to dryness and dissolve the residue in 5.0 ml of *ether R*.

Reference solution. Dissolve 5.0 mg of *glycyrrhetic acid R* and 5.0 mg of *thymol R* in 5 ml of *ether R*.

Apply to the plate, as bands, 10 µl of each solution. Develop over a path of 15 cm using a mixture of 1 volume of *concentrated ammonia R*, 9 volumes of *water R*, 25 volumes of *alcohol R* and 65 volumes of *ethyl acetate R*. Allow the plate to dry in air for 5 min and examine in ultraviolet light at 254 nm. The chromatograms obtained with the test solution and the reference solution show in the lower half a quenching zone due to glycyrrhetic acid. Spray the plate with *anisaldehyde solution R*. Heat at 100 °C to 105 °C for 5 min to 10 min and examine in daylight. The chromatogram obtained with the reference solution shows in the lower half, a violet zone (glycyrrhetic acid) and, in the upper third, a red zone (thymol). The chromatogram obtained with the test solution shows, in the lower half, a violet zone corresponding to glycyrrhetic acid in the chromatogram obtained with the reference solution and, in the upper third, below the zone of thymol in the chromatogram obtained with the reference solution, a yellow zone due to isoliquiritigenin. Further zones are present.

TESTS

Ethanol content (2.9.10). 52 per cent V/V to 65 per cent V/V.

Methanol and 2-propanol (2.9.11): maximum 0.05 per cent V/V of methanol and maximum 0.05 per cent V/V of 2-propanol.

ASSAY

Examine by liquid chromatography (2.2.29).

Test solution. Dilute 1.000 g of the liquid extract to 100 ml with a mixture of 8 volumes of *dilute ammonia R1* and 92 volumes of *water R*. Centrifuge. Dilute 2.0 ml of the supernatant to 100.0 ml with a mixture of 8 volumes of *dilute ammonia R1* and 92 volumes of *water R*.

Stock solution. Dissolve 0.130 g of *monoammonium glycyrrhizate CRS* in a mixture of 8 volumes of *dilute ammonia R1* and 92 volumes of *water R* and dilute to 100.0 ml with the same mixture of solvents.

Reference solution (a). Dilute 5.0 ml of the stock solution to 100.0 ml with a mixture of 8 volumes of *dilute ammonia R1* and 92 volumes of *water R*.

Reference solution (b). Dilute 10.0 ml of the stock solution to 100.0 ml with a mixture of 8 volumes of *dilute ammonia R1* and 92 volumes of *water R*.

Reference solution (c). Dilute 15.0 ml of the stock solution to 100.0 ml with a mixture of 8 volumes of *dilute ammonia R1* and 92 volumes of *water R*.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.10 m long and 4 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (5 µm),
- as mobile phase at a flow rate of 1.5 ml/min a mixture of 6 volumes of *glacial acetic acid R*, 30 volumes of *acetonitrile R* and 64 volumes of *water R*,
- as detector a spectrophotometer set at 254 nm.

Inject 10 µl of reference solution (c). Adjust the sensitivity of the system so that the heights of the peaks are at least 50 per cent of the full scale of the recorder. Inject each reference solution and determine the peak areas.

Establish a calibration curve with the concentrations of the reference solutions (g/100 ml) as the abscissa and the corresponding peak areas as the ordinate.

Inject 10 µl of the test solution. Using the retention times and the peak areas determined from the chromatograms obtained with the reference solutions, locate and integrate the peak due to glycyrrhizic acid in the chromatogram obtained with the test solution.

Calculate the percentage content of glycyrrhizic acid from the expression:

$$A \times \frac{5}{m} \times B \times \frac{822}{840}$$

- A* = concentration of monoammonium glycyrrhizate in the test solution, determined from the calibration curve, in g/100 ml,
B = declared percentage content of *monoammonium glycyrrhizate CRS*,
m = mass of the liquid extract in grams,
 822 = molecular mass of glycyrrhizic acid,
 840 = molecular mass of monoammonium glycyrrhizate (without any water of crystallisation).

STORAGE

Store protected from light.

01/2005:0277

LIQUORICE ROOT

Liquiritiae radix

DEFINITION

Liquorice root consists of the dried unpeeled or peeled, whole or cut root and stolons of *Glycyrrhiza glabra* L. It contains not less than 4.0 per cent of glycyrrhizic acid ($C_{42}H_{62}O_{16}$, M_r 823), calculated with reference to the dried drug.

CHARACTERS

It has the macroscopic and microscopic characters described under Identification tests A and B.

IDENTIFICATION

- A. The root has few branches. Its bark is brownish-grey to brown with longitudinal striations and bears traces of lateral roots. The cylindrical stolons are 1 cm to 2 cm in diameter; their external appearance is similar to that of the root but there are occasional small buds. The fracture of the root and the stolon is granular and fibrous. The cork layer is thin; the secondary phloem region is thick and light yellow with radial striations. The yellow xylem cylinder is compact, with a radiate structure. The stolon has a central pith, which is absent from the root. The external part of the bark is absent from the peeled root.
- B. Reduce to a powder (355). The powder is light yellow to faintly greyish. Examine under a microscope using *chloral hydrate solution R*. The powder shows fragments of yellow thick-walled fibres, 700 µm to 1200 µm long and 10 µm to 20 µm wide with a punctiform lumen, often accompanied by crystal sheaths containing prisms of calcium oxalate 10 µm to 35 µm long and 2 µm to 5 µm wide. The walls of the large vessels are yellow, 5 µm to 10 µm thick lignified and have numerous bordered pits with a slit-shaped aperture; fragments of cork consisting

of thin-walled cells and isolated prisms of calcium oxalate occur as well as fragments of parenchymatous tissue. Fragments of cork are absent from the peeled root. Examine under a microscope using a mixture of equal volumes of *glycerol R* and *water R*. The powder shows simple, round or oval starch granules, 2 µm to 20 µm in diameter.

- C. Examine by thin-layer chromatography (2.2.27), using as the coating substance a suitable silica gel with a fluorescent indicator having an optimal intensity at 254 nm.

Test solution. To 0.50 g of the powdered drug (180) in a 50 ml round-bottomed flask add 16.0 ml of *water R* and 4.0 ml of *hydrochloric acid R1* and heat on a water-bath under a reflux condenser for 30 min. Cool and filter. Dry the filter and the round-bottomed flask at 105 °C for 60 min. Place the filter in the round-bottomed flask, add 20.0 ml of *ether R* and heat in a water-bath at 40 °C under a reflux condenser for 5 min. Cool and filter. Evaporate the filtrate to dryness. Dissolve the residue in 5.0 ml of *ether R*.

Reference solution. Dissolve 5.0 mg of *glycyrrhetic acid R* and 5.0 mg of *thymol R* in 5.0 ml of *ether R*.

Apply separately to the plate as bands 10 µl of each solution. Develop over a path of 15 cm using a mixture of 1 volume of *concentrated ammonia R*, 9 volumes of *water R*, 25 volumes of *alcohol R* and 65 volumes of *ethyl acetate R*. Allow the plate to dry in air for 5 min and examine in ultraviolet light at 254 nm. The chromatograms obtained with the test solution and with the reference solution show in the lower half a quenching zone due to glycyrrhetic acid. Spray the plate with *anisaldehyde solution R*, and heat at 100-105 °C for 5 min to 10 min. Examine in daylight. The chromatogram obtained with the reference solution shows in the lower half the violet zone of glycyrrhetic acid and in the upper third the red zone of thymol. The chromatogram obtained with the test solution shows in the lower half of violet zone corresponding to the zone of glycyrrhetic acid in the chromatogram obtained with the reference solution and a yellow zone (isoliquiridigenine) in the upper third under the zone of thymol in the chromatogram obtained with the reference solution. Further zones may be present.

TESTS

Loss on drying (2.2.32). Not more than 10.0 per cent, determined on 1.000 g of powdered drug (355) by drying in an oven at 100-105 °C for 2 h.

Total ash (2.4.16). Not more than 10.0 per cent for the unpeeled drug and not more than 6.0 per cent for the peeled drug.

Ash insoluble in hydrochloric acid (2.8.1). Not more than 2.0 per cent for the unpeeled drug and not more than 0.5 per cent for the peeled drug.

ASSAY

Examine by liquid chromatography (2.2.29).

Test solution. Place 1.000 g of the powdered drug (180) in a 150 ml ground glass conical flask. Add 100.0 ml of an 8 g/l solution of *ammonia R* and treat in an ultrasonic bath for 30 min. Centrifuge a part of the supernatant layer and dilute 1.0 ml to 5.0 ml with an 8 g/l solution of *ammonia R*. Filter the solution through a filter (0.45 µm) and use the filtrate as the test solution.

Stock solution. Dissolve 0.130 g of *monoammonium glycyrrhizate CRS* in an 8 g/l solution of *ammonia R* and dilute to 100.0 ml with the same solvent.

Reference solution (a). Dilute 5.0 ml of the stock solution to 100.0 ml with an 8 g/l solution of *ammonia R*.

Reference solution (b). Dilute 10.0 ml of the stock solution to 100.0 ml with an 8 g/l solution of *ammonia R*.

Reference solution (c). Dilute 15.0 ml of the stock solution to 100.0 ml with an 8 g/l solution of *ammonia R*.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.10 m long and 4 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (5 µm),
- as mobile phase at a flow rate of 1.5 ml/min a mixture of 6 volumes of *glacial acetic acid R*, 30 volumes of *acetonitrile R* and 64 volumes of *water R*,
- as detector a spectrophotometer set at 254 nm,
- a 10 µl loop injector.

Inject reference solution (c). Adjust the sensitivity of the system so that the height of the peaks are at least 50 per cent of the full scale of the recorder. Inject each reference solution and determine the peak areas.

Establish a calibration curve with the concentration of the reference solutions (g/100 ml) as the abscissa and the corresponding areas as the ordinate.

Inject the test solution. Using the retention time and the peak area determined from the chromatograms obtained with the reference solutions, locate and integrate the peak due to glycyrrhizic acid in the chromatogram obtained with the test solution.

Calculate the percentage content of glycyrrhizic acid from the expression:

$$A \times \frac{5}{m} \times B \times \frac{822}{840}$$

- A* = concentration of monoammonium glycyrrhizate in the test solution determined from the calibration curve, in g/100 ml,
- B* = declared percentage content of *monoammonium glycyrrhizate CRS*,
- m* = mass of the drug, in grams,
- 822 = molecular weight of glycyrrhizic acid,
- 840 = molecular weight of the monoammonium glycyrrhizate (without any water of crystallisation).

STORAGE

Store protected from light.

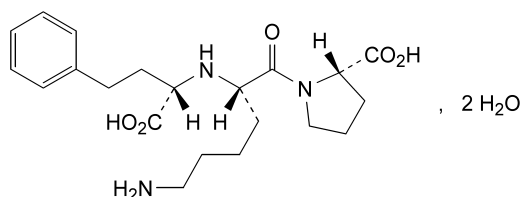
LABELLING

The label states whether the drug is peeled or unpeeled.

01/2005:1120
corrected

LISINOPRIL DIHYDRATE

Lisinoprilum dihydricum



$C_{21}H_{31}N_3O_5 \cdot 2H_2O$

M_r 441.5

DEFINITION

Lisinopril dihydrate contains not less than 98.5 per cent and not more than the equivalent of 101.5 per cent of (2S)-1-[(2S)-6-amino-2-[(1S)-1-carboxy-3-phenylpropyl]amino]hexanoyl]pyrrole-2-carboxylic acid, calculated with reference to the anhydrous substance.

CHARACTERS

A white or almost white, crystalline powder, soluble in water, sparingly soluble in methanol, practically insoluble in acetone and in ethanol.

IDENTIFICATION

Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *lisinopril dihydrate CRS*. Examine the substances prepared as discs.

TESTS

Specific optical rotation (2.2.7). Dissolve 0.5 g in *zinc acetate solution R* and dilute to 50.0 ml with the same solvent. The specific optical rotation is –43 to –47, calculated with reference to the anhydrous substance.

Related substances. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 20.0 mg of the substance to be examined in mobile phase A and dilute to 10.0 ml with the same mobile phase.

Reference solution (a). Dissolve the contents of 1 vial of *lisinopril dihydrate for performance test CRS* with 1.0 ml of mobile phase A.

Reference solution (b). Dilute 0.5 ml of the test solution to 50.0 ml with mobile phase A.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4.6 mm in internal diameter packed with *octylsilyl silica gel for chromatography R*,
- as mobile phase at a flow rate of 1.8 ml/min:
Mobile phase A. Prepare a mixture of 30 volumes of *acetonitrile R* and 970 volumes of a 3.12 g/l *sodium dihydrogen phosphate R* solution adjusted to pH 5.0 with a 50 g/l solution of *sodium hydroxide R*,
Mobile phase B. Prepare a mixture of 200 ml of *acetonitrile R* and 800 ml of a 3.12 g/l *sodium dihydrogen phosphate R* solution adjusted to pH 5.0 with a 50 g/l solution of *sodium hydroxide R*,

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)	Comment
0 - 35	100 → 70	0 → 30	linear gradient
35 - 45	70	30	isocratic
45 - 50	70 → 100	30 → 0	switch to initial eluent composition
50 = 0	100	0	restart gradient

- as detector a spectrophotometer set at 210 nm, maintaining the temperature of the column at 50 °C.

Equilibrate the column with mobile phase A for at least 30 min. Adjust the sensitivity of the system so that the height of the principal peak in the chromatogram obtained with 20 µl of reference solution (b) is at least 50 per cent of the full scale of the recorder.

Inject 20 µl of reference solution (a). The resulting chromatogram resembles that of the specimen chromatogram supplied with *lisinopril dihydrate for performance test CRS* in that the peaks due to impurity A and impurity E fall on either side of the peak due to lisinopril. Measure the heights *A1* and *A2* above the baseline of the

peaks due to impurity A and impurity E and the heights *B1* and *B2* above the baseline of the lowest points of the curve separating these peaks from the peak due to lisinopril. The test is not valid unless *A1* is greater than nine times *B1* and *A2* is greater than nine times *B2*.

If necessary, adjust the pH of the mobile phase to 4.5 with *phosphoric acid R* and repeat the chromatography. A further adjustment to pH 4.0 may be necessary with some columns before satisfactory separation of impurity A, lisinopril and impurity E is obtained. If, after adjustment, the retention time of the peak due to impurities C and D becomes extended to the point where integration becomes difficult, increase the content of mobile phase B from 30 per cent to 40 per cent over the interval from 35 min to 45 min from the start of the chromatogram. Maintain this concentration for a further 10 min. Return the concentration of mobile phase A to 100 per cent over the next 10 min prior to the next injection.

Inject 20 µl of the test solution and 20 µl of reference solution (b). In the chromatogram obtained with the test solution: the area of any peak due to impurity E is not greater than 0.3 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.3 per cent); the area of any peak, apart from the principal peak and any peak due to impurity E, is not greater than 0.3 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.3 per cent) and the sum of the areas of all such peaks is not greater than half the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent). Disregard any peak due to the solvent, any peak occurring in the first 3 minutes and any peak with an area less than 0.05 times the area of the principal peak in the chromatogram obtained with reference solution (b).

Water (2.5.12): 8.0 to 9.5 per cent, determined on 0.200 g by the semi-micro determination of water.

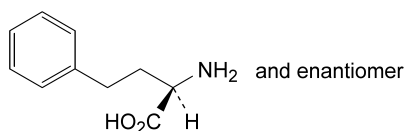
Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

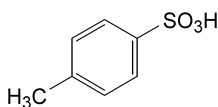
Dissolve 0.350 g in 50 ml of *distilled water R*. Titrate with 0.1 M *sodium hydroxide*, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *sodium hydroxide* is equivalent to 40.55 mg of $C_{21}H_{31}N_3O_5$.

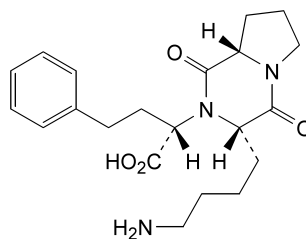
IMPURITIES



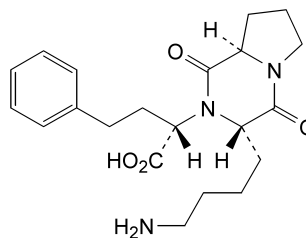
A. (2*RS*)-2-amino-4-phenylbutanoic acid,



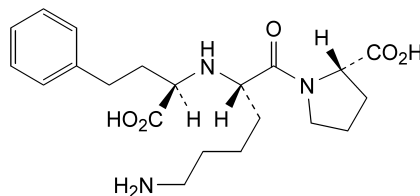
B. 4-methylbenzenesulphonic acid,



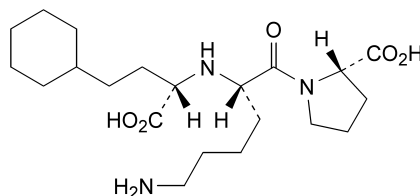
C. (2*S*)-2-[(3*S*,8*aS*)-3-(4-aminobutyl)-1,4-dioxohexahydro-pyrrolo[1,2-*a*]pyrazin-2(1*H*)-yl]-4-phenylbutanoic acid (*S,S,S*-diketopiperazine),



D. (2*S*)-2-[(3*S*,8*aR*)-3-(4-aminobutyl)-1,4-dioxohexahydro-pyrrolo[1,2-*a*]pyrazin-2(1*H*)-yl]-4-phenylbutanoic acid (*R,S,S*-diketopiperazine),



E. (2*S*)-1-[(2*S*)-6-amino-2-[[[(1*R*)-1-carboxy-3-phenylpropyl]amino]hexanoyl]pyrrole-2-carboxylic acid (lisinopril *R,S,S*-isomer),



F. (2*S*)-1-[(2*S*)-6-amino-2-[[[(1*S*)-1-carboxy-3-cyclohexylpropyl]amino]hexanoyl]pyrrole-2-carboxylic acid (cyclohexyl analogue).

01/2005:0228

LITHIUM CARBONATE

Lithii carbonas

Li_2CO_3

M_r 73.9

DEFINITION

Lithium carbonate contains not less than 98.5 per cent and not more than the equivalent of 100.5 per cent of Li_2CO_3 .

CHARACTERS

A white powder, slightly soluble in water, practically insoluble in alcohol.

IDENTIFICATION

A. When moistened with *hydrochloric acid R*, it gives a red colour to a non-luminous flame.

B. Dissolve 0.2 g in 1 ml of *hydrochloric acid R*. Evaporate to dryness on a water-bath. The residue dissolves in 3 ml of *alcohol R*.

C. It gives the reaction of carbonates (2.3.1).

TESTS

Solution S. Suspend 10.0 g in 30 ml of *distilled water R* and dissolve by the addition of 22 ml of *nitric acid R*. Add *dilute sodium hydroxide solution R* until the solution is neutral and dilute to 100 ml with *distilled water R*.

Appearance of solution. Solution S is clear (2.2.1) and colourless (2.2.2, *Method II*).

Chlorides (2.4.4). 2.5 ml of solution S diluted to 15 ml with *water R* complies with the limit test for chlorides (200 ppm).

Sulphates (2.4.13). Disperse 1.25 g in 5 ml of *distilled water R* and dissolve by adding 5 ml of *hydrochloric acid R1*. Boil for 2 min. Cool and add *dilute sodium hydroxide solution R* until neutral. Dilute to 25 ml with *distilled water R*. The solution complies with the limit test for sulphates (200 ppm).

Arsenic (2.4.2). 0.5 g complies with limit test A for arsenic (2 ppm).

Calcium (2.4.3). 5 ml of solution S diluted to 15 ml with *distilled water R* complies with the limit test for calcium (200 ppm).

Heavy metals (2.4.8). 12 ml of solution S complies with limit test A for heavy metals (20 ppm). Prepare the standard using *lead standard solution* (2 ppm Pb) *R*.

Iron (2.4.9). 5 ml of solution S diluted to 10 ml with *water R* complies with the limit test for iron (20 ppm).

Magnesium (2.4.6). Dilute 1 ml of solution S to 10 ml with *water R*. 6.7 ml of this solution diluted to 10 ml with *water R* complies with the limit test for magnesium (150 ppm).

Potassium. Not more than 300 ppm of K, determined by atomic emission spectrometry (2.2.22, *Method I*).

Test solution. Dissolve 1.0 g of the substance to be examined in 10 ml of *hydrochloric acid R1* and dilute to 50.0 ml with *water R*.

Reference solutions. Prepare the reference solutions using a solution of *potassium chloride R* containing 500 µg of K per millilitre, diluted as required.

Measure the emission intensity at 766.5 nm.

Sodium. Not more than 300 ppm of Na, determined by atomic emission spectrometry (2.2.22, *Method I*).

Test solution. Dissolve 1.0 g of the substance to be examined in 10 ml of *hydrochloric acid R1* and dilute to 50.0 ml with *water R*.

Reference solutions. Prepare the reference solutions using a solution of *sodium chloride R* containing 500 µg of Na per millilitre, diluted as required.

Measure the emission intensity at 589 nm.

ASSAY

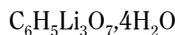
Dissolve 0.500 g in 25.0 ml of 1 M *hydrochloric acid*. Titrate with 1 M *sodium hydroxide*, using *methyl orange solution R* as indicator.

1 ml of 1 M *hydrochloric acid* is equivalent to 36.95 mg of Li_2CO_3 .

01/2005:0621

LITHIUM CITRATE

Lithii citras



M_r 282.0

DEFINITION

Lithium citrate contains not less than 98.0 per cent and not more than the equivalent of 102.0 per cent of trilithium 2-hydroxypropane-1,2,3-tricarboxylate, calculated with reference to the anhydrous substance.

CHARACTERS

A white or almost white, fine crystalline powder, freely soluble in water, slightly soluble in alcohol.

IDENTIFICATION

- When moistened with *hydrochloric acid R*, it gives a red colour to a non-luminous flame.
- Dilute 3 ml of solution S (see Tests) to 10 ml with *water R*. Add 3 ml of *potassium ferriperiodate solution R*. A white or yellowish-white precipitate is formed.
- To 1 ml of solution S add 4 ml of *water R*. The solution gives the reaction of citrates (2.3.1).

TESTS

Solution S. Dissolve 10.0 g in *carbon dioxide-free water R* prepared from *distilled water R* and dilute to 100 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and colourless (2.2.2, *Method II*).

Acidity or alkalinity. To 10 ml of solution S add 0.1 ml of *phenolphthalein solution R*. Not more than 0.2 ml of 0.1 M *hydrochloric acid* or 0.1 M *sodium hydroxide* is required to change the colour of the indicator.

Readily carbonisable substances. To 0.20 g of the powdered substance to be examined add 10 ml of *sulphuric acid R* and heat in a water-bath at $90 \pm 1^\circ\text{C}$ for 60 min. Cool rapidly. The solution is not more intensely coloured than reference solution Y₂ or GY₂ (2.2.2, *Method II*).

Chlorides (2.4.4). Dilute 5 ml of solution S to 15 ml with *water R*. The solution complies with the limit test for chlorides (100 ppm).

Oxalates. Dissolve 0.50 g in 4 ml of *water R*, add 3 ml of *hydrochloric acid R* and 1 g of granulated *zinc R* and heat on a water-bath for 1 min. Allow to stand for 2 min, decant the liquid into a test-tube containing 0.25 ml of a 10 g/l solution of *phenylhydrazine hydrochloride R* and heat to boiling. Cool rapidly, transfer to a graduated cylinder and add an equal volume of *hydrochloric acid R* and 0.25 ml of *potassium ferricyanide solution R*. Shake and allow to stand for 30 min. Any pink colour in the solution is not more intense than that in a standard prepared at the same time and in the same manner using 4 ml of a 0.05 g/l solution of *oxalic acid R* (300 ppm, calculated as anhydrous oxalate ion).

Sulphates (2.4.13). To 3 ml of solution S add 2 ml of *hydrochloric acid R1* and dilute to 17 ml with *distilled water R*. The solution complies with the limit test for sulphates (500 ppm). Prepare the standard using 15 ml of a mixture of 2 ml of *hydrochloric acid R1* and 15 ml of *sulphate standard solution* (10 ppm SO₄) *R* and compare the opalescence after 15 min.

Heavy metals (2.4.8). 12 ml of solution S complies with limit test A for heavy metals (10 ppm). Prepare the standard using *lead standard solution* (1 ppm Pb) R.

Water (2.5.12): 24.0 per cent to 27.0 per cent, determined on 0.100 g by the semi-micro determination of water. After adding the substance to be examined, stir for 15 min before titrating. Carry out a blank titration.

ASSAY

Dissolve 80.0 mg in 50 ml of *anhydrous acetic acid* R, heating to about 50 °C. Allow to cool. Titrate with 0.1 M *perchloric acid*, using 0.25 ml of *naphtholbenzein solution* R as indicator, until the colour changes from yellow to green.

1 ml of 0.1 M *perchloric acid* is equivalent to 7.00 mg of C₂₂H₂₈ClNO₂.

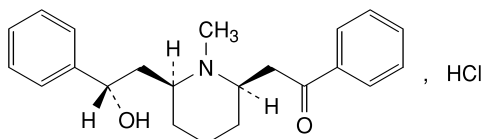
STORAGE

Store in an airtight container.

01/2005:1988
corrected

LOBELINE HYDROCHLORIDE

Lobelini hydrochloridum



C₂₂H₂₈ClNO₂

M_r 373.9

DEFINITION

2-[(2*R*,6*S*)-6-[(2*S*)-2-Hydroxy-2-phenylethyl]-1-methylpiperidin-2-yl]-1-phenylethanone hydrochloride.

Content: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

Appearance: white or almost white, microcrystalline powder.

Solubility: sparingly soluble in water, freely soluble in alcohol, soluble in methylene chloride.

IDENTIFICATION

First identification: A, B.

Second identification: B, C.

A. Infrared absorption spectrophotometry (2.2.24).

Comparison: *lobeline hydrochloride* CRS.

B. Solution S (see Tests) gives reaction (a) of chlorides (2.3.1).

C. Examine the chromatograms obtained in the test for foreign alkaloids.

Results: the principal spot in the chromatogram obtained with test solution (b) is similar in position and size to the principal spot in the chromatogram obtained with reference solution (b).

TESTS

Solution S. Dissolve 0.250 g in *carbon dioxide-free water* R prepared from *distilled water* R and dilute to 25.0 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and colourless (2.2.2, *Method II*).

pH (2.2.3): 4.6 to 6.4 for solution S.

Specific optical rotation (2.2.7): –55 to –59 (dried substance), determined on solution S.

Foreign alkaloids. Thin-layer chromatography (2.2.27).

Test solution (a). Dissolve 0.10 g of the substance to be examined in *methanol* R and dilute to 5.0 ml with the same solvent.

Test solution (b). Dilute 1 ml of test solution (a) to 10 ml with *methanol* R.

Reference solution (a). Dilute 0.1 ml of test solution (a) to 10 ml with *methanol* R.

Reference solution (b). Dissolve 10 mg of *lobeline hydrochloride* CRS in *methanol* R and dilute to 5 ml with the same solvent.

Plate: TLC silica gel GF₂₅₄ plate R.

Mobile phase: *diethylamine* R, *cyclohexane* R (10:90 V/V).

Application: 10 µl.

Development: over 2/3 of the plate.

Drying: at 120 °C.

Detection: examine in ultraviolet light at 254 nm.

Limits: in the chromatogram obtained with test solution (a):

- *any impurity*: any spot, apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (a) (1 per cent).

Related substances. Liquid chromatography (2.2.29).

Test solution. Dissolve 10.0 mg of the substance to be examined in the mobile phase and dilute to 10.0 ml with the mobile phase.

Reference solution (a). Dilute 1.0 ml of the test solution to 100.0 ml with the mobile phase. Dilute 1.0 ml of this solution to 10.0 ml with the mobile phase.

Reference solution (b). Dissolve 5 mg of *phenytoin* CRS in the mobile phase and dilute to 100.0 ml with the mobile phase. To 1 ml of the solution add 0.1 ml of the test solution and dilute to 25 ml with the mobile phase.

Column:

- *size*: *l* = 0.25 m, Ø = 4 mm,
- *stationary phase*: spherical *end-capped octylsilyl silica gel for chromatography* R (5 µm).

Mobile phase: dissolve 1.0 g of *sodium methanesulphonate* R and 2.50 g of *disodium hydrogen phosphate dihydrate* R in a mixture of 3 volumes of a 6.7 per cent V/V solution of *phosphoric acid* R, 29 volumes of *acetonitrile* R and 70 volumes of *water* R and dilute to 1000 ml with the same mixture of solvents.

Flow rate: 1.5 ml/min.

Detection: spectrophotometer at 210 nm.

Injection: 10 µl.

Run time: 2 times the retention time of lobeline which is about 17 min.

System suitability: reference solution (b):

- *resolution*: minimum 4.0 between the peaks due to phenytoin and to lobeline.

Limits:

- *any impurity*: not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent),
- *total*: maximum of 2 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent),
- *disregard level*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

Heavy metals (2.4.8). 12 ml of solution S complies with limit test A for heavy metals (10 ppm). Prepare the standard using *lead standard solution* (1 ppm Pb) R.

Water (2.5.12): 24.0 per cent to 27.0 per cent, determined on 0.100 g by the semi-micro determination of water. After adding the substance to be examined, stir for 15 min before titrating. Carry out a blank titration.

ASSAY

Dissolve 80.0 mg in 50 ml of *anhydrous acetic acid* R, heating to about 50 °C. Allow to cool. Titrate with 0.1 M *perchloric acid*, using 0.25 ml of *naphtholbenzein solution* R as indicator, until the colour changes from yellow to green.

1 ml of 0.1 M *perchloric acid* is equivalent to 7.00 mg of C₂₂H₂₈ClNO₂.

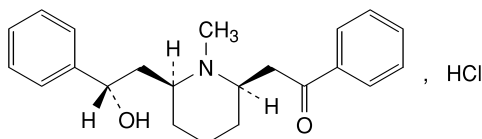
STORAGE

Store in an airtight container.

01/2005:1988
corrected

LOBELINE HYDROCHLORIDE

Lobelini hydrochloridum



C₂₂H₂₈ClNO₂

M_r 373.9

DEFINITION

2-[(2*R*,6*S*)-6-[(2*S*)-2-Hydroxy-2-phenylethyl]-1-methylpiperidin-2-yl]-1-phenylethanone hydrochloride.

Content: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

Appearance: white or almost white, microcrystalline powder.

Solubility: sparingly soluble in water, freely soluble in alcohol, soluble in methylene chloride.

IDENTIFICATION

First identification: A, B.

Second identification: B, C.

A. Infrared absorption spectrophotometry (2.2.24).

Comparison: *lobeline hydrochloride* CRS.

B. Solution S (see Tests) gives reaction (a) of chlorides (2.3.1).

C. Examine the chromatograms obtained in the test for foreign alkaloids.

Results: the principal spot in the chromatogram obtained with test solution (b) is similar in position and size to the principal spot in the chromatogram obtained with reference solution (b).

TESTS

Solution S. Dissolve 0.250 g in *carbon dioxide-free water* R prepared from *distilled water* R and dilute to 25.0 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and colourless (2.2.2, *Method II*).

pH (2.2.3): 4.6 to 6.4 for solution S.

Specific optical rotation (2.2.7): –55 to –59 (dried substance), determined on solution S.

Foreign alkaloids. Thin-layer chromatography (2.2.27).

Test solution (a). Dissolve 0.10 g of the substance to be examined in *methanol* R and dilute to 5.0 ml with the same solvent.

Test solution (b). Dilute 1 ml of test solution (a) to 10 ml with *methanol* R.

Reference solution (a). Dilute 0.1 ml of test solution (a) to 10 ml with *methanol* R.

Reference solution (b). Dissolve 10 mg of *lobeline hydrochloride* CRS in *methanol* R and dilute to 5 ml with the same solvent.

Plate: TLC silica gel GF₂₅₄ plate R.

Mobile phase: *diethylamine* R, *cyclohexane* R (10:90 V/V).

Application: 10 µl.

Development: over 2/3 of the plate.

Drying: at 120 °C.

Detection: examine in ultraviolet light at 254 nm.

Limits: in the chromatogram obtained with test solution (a):

- *any impurity*: any spot, apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (a) (1 per cent).

Related substances. Liquid chromatography (2.2.29).

Test solution. Dissolve 10.0 mg of the substance to be examined in the mobile phase and dilute to 10.0 ml with the mobile phase.

Reference solution (a). Dilute 1.0 ml of the test solution to 100.0 ml with the mobile phase. Dilute 1.0 ml of this solution to 10.0 ml with the mobile phase.

Reference solution (b). Dissolve 5 mg of *phenytoin* CRS in the mobile phase and dilute to 100.0 ml with the mobile phase. To 1 ml of the solution add 0.1 ml of the test solution and dilute to 25 ml with the mobile phase.

Column:

- *size*: *l* = 0.25 m, Ø = 4 mm,
- *stationary phase*: spherical *end-capped octylsilyl silica gel for chromatography* R (5 µm).

Mobile phase: dissolve 1.0 g of *sodium methanesulphonate* R and 2.50 g of *disodium hydrogen phosphate dihydrate* R in a mixture of 3 volumes of a 6.7 per cent V/V solution of *phosphoric acid* R, 29 volumes of *acetonitrile* R and 70 volumes of *water* R and dilute to 1000 ml with the same mixture of solvents.

Flow rate: 1.5 ml/min.

Detection: spectrophotometer at 210 nm.

Injection: 10 µl.

Run time: 2 times the retention time of lobeline which is about 17 min.

System suitability: reference solution (b):

- *resolution*: minimum 4.0 between the peaks due to phenytoin and to lobeline.

Limits:

- *any impurity*: not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent),
- *total*: maximum of 2 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent),
- *disregard level*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

Sulphates (2.4.13): maximum 0.1 per cent.

15 ml of solution S complies with the limit test for sulphates.

Loss on drying (2.2.32): maximum 1.0 per cent, determined on 1.000 g *in vacuo*.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on the residue obtained in the test for loss on drying.

ASSAY

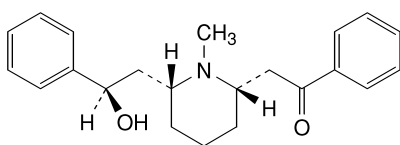
Dissolve 0.300 g in 50 ml of *alcohol R*. Add 5 ml of 0.01 M *hydrochloric acid*. Carry out a potentiometric titration (2.2.20), using 0.1 M *sodium hydroxide*. Read the volume added between the 2 points of inflexion.

1 ml of 0.1 M *sodium hydroxide* is equivalent to 37.39 mg of $C_{22}H_{28}ClNO_2$.

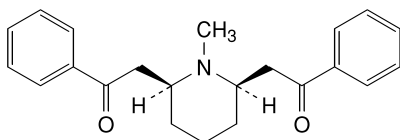
STORAGE

Protected from light.

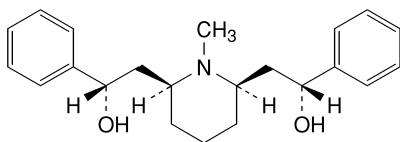
IMPURITIES



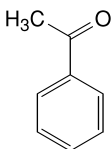
- A. 2-[(2*S*,6*R*)-6-[(2*R*)-2-hydroxy-2-phenylethyl]-1-methylpiperidin-2-yl]-1-phenylethanone ((+)-lobeline),



- B. 2,2'-[(2*R*,6*S*)-1-methylpiperidine-2,6-diyl]bis(1-phenylethanone) (lobelanine),



- C. *meso*-(1*R*,1'*S*)-2,2'-[(2*R*,6*S*)-1-methylpiperidine-2,6-diyl]bis(1-phenylethanol) (lobelanidine),



- D. acetophenone.

DEFINITION

Lomustine contains not less than 98.5 per cent and not more than the equivalent of 100.5 per cent of 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea, calculated with reference to the dried substance.

CHARACTERS

A yellow, crystalline powder, practically insoluble in water, freely soluble in acetone and in methylene chloride, soluble in alcohol.

Carry out the tests protected from light and prepare all the solutions immediately before use.

IDENTIFICATION

First identification: C.

Second identification: A, B, D, E.

A. Melting point (2.2.14): 89 °C to 91 °C.

B. Dissolve 50.0 mg in *alcohol R* and dilute to 50.0 ml with the same solvent. Dilute 2.0 ml to 100.0 ml with *alcohol R*. Examined between 220 nm and 350 nm (2.2.25), the solution shows an absorption maximum at 230 nm. The specific absorbance at the maximum is 250 to 270.

C. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *lomustine CRS*. Examine the substances prepared as discs.

D. Examine the chromatograms obtained in the test for related substances. The principal spot in the chromatogram obtained with test solution (b) is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).

E. Dissolve about 25 mg in 1 ml of *methanol R*, add 0.1 ml of *dilute sodium hydroxide solution R* and 2 ml of *water R*. Acidify by adding dropwise *dilute nitric acid R*. Filter. The filtrate gives reaction (a) of chlorides (2.3.1).

TESTS

Related substances

A. Examine by thin-layer chromatography (2.2.27), using *silica gel G R* as the coating substance.

Test solution (a). Dissolve 0.25 g of the substance to be examined in *methanol R* and dilute to 10 ml with the same solvent.

Test solution (b). Dilute 1 ml of test solution (a) to 25 ml with *methanol R*.

Reference solution (a). Dissolve 10 mg of *lomustine CRS* in *methanol R* and dilute to 10 ml with the same solvent.

Reference solution (b). Dilute 1 ml of test solution (b) to 10 ml with *methanol R*.

Reference solution (c). Dilute 1 ml of test solution (b) to 20 ml with *methanol R*.

Reference solution (d). Dissolve 10 mg of *lomustine CRS* and 10 mg of *dicyclohexylurea R* in *methanol R* and dilute to 10 ml with the same solvent.

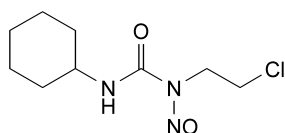
Apply separately to the plate 5 µl of each solution.

Develop over a path of 15 cm using a mixture of 20 volumes of *glacial acetic acid R* and 80 volumes of *toluene R*. Dry the plate at 110 °C for 1 h. At the bottom of a chromatography tank, place an evaporating dish containing a mixture of 1 volume of *hydrochloric acid R1*, 1 volume of *water R* and 2 volumes of a 15 g/l solution of *potassium permanganate R*, close the tank and allow to stand for 15 min. Place the dried plate in the tank and close the tank. Leave the plate in contact with the chlorine vapour for 5 min. Withdraw the plate and place it in a current of cold air until the excess of

01/2005:0928
corrected

LOMUSTINE

Lomustinum



$C_9H_{16}ClN_3O_2$

M_r 233.7

chlorine is removed and an area of coating below the points of application does not give a blue colour with a drop of *potassium iodide and starch solution R*. Spray with *potassium iodide and starch solution R*. Any spot in the chromatogram obtained with test solution (a), apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.4 per cent) and at most one such spot is more intense than the spot in the chromatogram obtained with reference solution (c) (0.2 per cent). The test is not valid unless the chromatogram obtained with reference solution (d) shows two clearly separated principal spots.

B. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 0.25 g of the substance to be examined in *methanol R* and dilute to 10.0 ml the same solvent.

Reference solution. Dilute 1.0 ml of the test solution to 100.0 ml with *methanol R*.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (5 µm to 10 µm),
- as mobile phase at a flow rate of 2 ml/min a mixture of 50 volumes of *methanol R* and 50 volumes of *water R*,
- as detector a spectrophotometer set at 230 nm,
- a loop injector.

Inject 20 µl of the reference solution. Adjust the sensitivity of the system so that the height of the principal peak in the chromatogram obtained with the reference solution is at least 50 per cent of the full scale of the recorder. Inject separately 20 µl of each solution. In the chromatogram obtained with the test solution, the sum of the areas of any peaks apart from the principal peak is not greater than the area of the principal peak in the chromatogram obtained with the reference solution (1 per cent). Disregard any peak due to the solvent and any peak with an area less than 0.05 times that of the principal peak in the chromatogram obtained with the reference solution.

Chlorides (2.4.4). Dissolve 0.24 g in 4 ml of *methanol R* and add 20 ml of *water R*. Allow to stand for 20 min and filter. To 10 ml of the filtrate, add 5 ml of *methanol R*. The solution complies with the limit test for chlorides (500 ppm). When preparing the standard, replace the 5 ml of *water R* with 5 ml of *methanol R*.

Loss on drying (2.2.32). Not more than 1.0 per cent, determined on 1.000 g by drying in a desiccator over *diphosphorus pentoxide R* at a pressure not exceeding 0.7 kPa for 24 h.

ASSAY

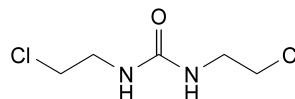
Dissolve 0.200 g in about 3 ml of *alcohol R* and add 20 ml of a 200 g/l solution of *potassium hydroxide R* and boil under a reflux condenser for 2 h. Add 75 ml of *water R* and 4 ml of *nitric acid R*. Cool and titrate with 0.1 M *silver nitrate*, determining the end-point potentiometrically (2.2.20). Carry out a blank titration.

1 ml of 0.1 M *silver nitrate* is equivalent to 23.37 mg of $C_9H_{16}ClN_3O_2$.

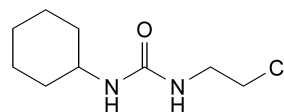
STORAGE

Store protected from light.

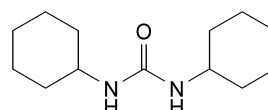
IMPURITIES



A. 1,3-bis(2-chloroethyl)urea,



B. 1-(2-chloroethyl)-3-cyclohexylurea,



C. 1,3-dicyclohexylurea.

01/2005:1537

LOOSESTRIFE

Lythri herba

DEFINITION

Loosestrife consists of the dried flowering tops, whole or cut, of *Lythrum salicaria* L. It contains not less than 5.0 per cent of tannins, expressed as pyrogallol ($C_6H_6O_3$; M_r 126.1) and calculated with reference to the dried drug.

CHARACTERS

It has the macroscopic and microscopic characters described under identification tests A and B.

IDENTIFICATION

- A.** The stems are rigid, four-angled, branching at the top, brownish-green, longitudinally wrinkled and pubescent. The leaves are opposite, decussate, rarely verticillate in threes and sometimes alternate at the inflorescence which forms a long terminal spike. The leaves are sessile, lanceolate and cordate at the base, 5 cm to 15 cm long and 1 cm to 2.5 cm wide, pubescent on the lower surface; the subsidiary veins form arcs that anastomose near the leaf margin. The flowers have a pubescent, tubular, persistent gamosepalous calyx, 4 mm to 8 mm long, consisting of 6 sepals bearing 6 small, triangular teeth alternating with 6 large acute teeth at least half as long as the tube; a polypetalous corolla consisting of 6 violet-pink petals, each expanded at the top with a wavy outline and narrowing at the base. The androecium consists of 2 verticils of 6 stamens (one verticil with short, barely emerging stamens, the other with long stamens extending well out of the corolla). The fruit, if formed, is a small capsule included in the persistent calyx.
- B.** Reduce to a powder (355). The powder is greenish-yellow. Examine under a microscope using *chloral hydrate solution R*. The powder shows unicellular or bicellular, uniseriate, thick-walled, finely pitted covering trichomes from the lower epidermis of the stem and leaf; numerous uniseriate, unicellular or bicellular, thin-walled, finely pitted covering trichomes from the calyx; transparent violet-pink fragments from the petals; numerous cluster crystals of calcium oxalate; pollen grains with 3 pores and a thin and slightly granular exine; fragments of the upper epidermis with large polygonal cells and sinuous walls; fragments of the lower epidermis with smaller polygonal cells and anomocytic stomata (2.8.3).

C. Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel plate R*.

Test solution. To 1.0 g of the powdered drug (355) add 10 ml of *methanol R* and heat in a water-bath at 65 °C for 5 min with frequent shaking. Cool and filter. Dilute the filtrate to 10 ml with *methanol R*.

Reference solution. Dissolve 0.5 mg of *chlorogenic acid R*, 1 mg of *hyperoside R*, 1 mg of *rutin R* and 1 mg of *vitexin R* in 10 ml of *methanol R*.

Apply to the plate as bands 10 µl of each solution. Develop over a path of 15 cm using a mixture of 7.5 volumes of *anhydrous acetic acid R*, 7.5 volumes of *anhydrous formic acid R*, 18 volumes of *water R* and 67 volumes of *ethyl acetate R*. Dry the plate at 100 °C to 105 °C and spray it while still warm with a 10 g/l solution of *diphenylboric acid aminoethyl ester R* in *methanol R*. Subsequently spray the plate with a 50 g/l solution of *macrogol 400 R* in *methanol R*. Allow the plate to dry in air for 30 min and examine in ultraviolet light at 365 nm. The chromatogram obtained with the reference solution shows in the lower third a yellowish-brown fluorescent zone (rutin) and in the middle third a light blue fluorescent zone (chlorogenic acid), above it a yellowish-brown fluorescent zone (hyperoside) and a green fluorescent zone (vitexin). The chromatogram obtained with the test solution shows a bright green fluorescent zone slightly above the rutin zone in the chromatogram obtained with the reference solution, a yellow fluorescent zone similar in position to the chlorogenic acid zone in the chromatogram obtained with the reference solution, a yellow fluorescent zone similar in position to the hyperoside zone in the chromatogram obtained with the reference solution and a bright green fluorescent zone corresponding to the vitexin zone in the chromatogram obtained with the reference solution.

TESTS

Foreign matter (2.8.2). It complies with the test for foreign matter.

Loss on drying (2.2.32). Not more than 12.0 per cent, determined on 1.000 g of the powdered drug (355) by drying in an oven at 100 °C to 105 °C.

Total ash (2.4.16). Not more than 7.0 per cent.

ASSAY

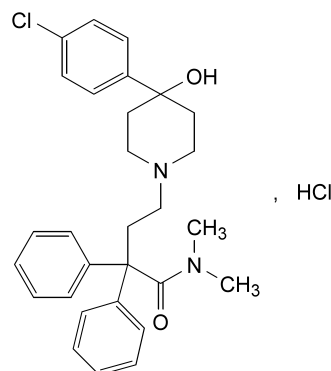
Carry out the determination of tannins in herbal drugs (2.8.14). Use 0.750 g of the powdered drug (180).

STORAGE

Store protected from light.

LOPERAMIDE HYDROCHLORIDE

Loperamidi hydrochloridum



$C_{29}H_{34}Cl_2N_2O_2$

M_r 513.5

DEFINITION

4-[4-(4-Chlorophenyl)-4-hydroxypiperidin-1-yl]-*N,N*-dimethyl-2,2-diphenylbutanamide hydrochloride.

Content: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

Appearance: white or almost white powder.

Solubility: slightly soluble in water, freely soluble in alcohol and in methanol.

It shows polymorphism.

IDENTIFICATION

Infrared absorption spectrophotometry (2.2.24).

Comparison: *loperamide hydrochloride CRS*.

If the spectra obtained show differences, dissolve the substance to be examined and the reference substance separately in the minimum volume of *methylene chloride R*, evaporate to dryness and record new spectra using the residues.

TESTS

Related substances. Liquid chromatography (2.2.29).

Test solution. Dissolve 0.100 g of the substance to be examined in *methanol R* and dilute to 10.0 ml with the same solvent.

Reference solution (a). Dissolve 10.0 mg of *loperamide hydrochloride for system suitability CRS* in *methanol R* and dilute to 1.0 ml with the same solvent.

Reference solution (b). Dilute 1.0 ml of the test solution to 20.0 ml with *methanol R*. Dilute 1.0 ml of this solution to 25.0 ml with *methanol R*.

Column:

- size: $l = 0.10$ m, $\varnothing = 4.6$ mm,
- stationary phase: base-deactivated octadecylsilyl silica gel for chromatography *R* (3 µm),
- temperature: 35 °C.

Mobile phase:

- mobile phase A: 17.0 g/l solution of tetrabutylammonium hydrogen sulphate *R1*,
- mobile phase B: acetonitrile *R*,

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)
0 - 15	90 → 30	10 → 70
15 - 17	30	70
17 - 19	30 → 90	70 → 10
19 - 24	90	10

Flow rate: 1.5 ml/min.

Detection: spectrophotometer at 220 nm.

Injection: 10 µl.

System suitability: reference solution (a):

- *peak-to-valley ratio*: minimum 1.5, where H_p = height above the baseline of the peak due to impurity G and H_v = height above the baseline of the lowest point of the curve separating this peak from the peak due to impurity H;
- *peak-to-valley ratio*: minimum 1.5, where H_p = height above the baseline of the peak due to impurity E and H_v = height above the baseline of the lowest point of the curve separating this peak from the peak due to impurity A;
- the chromatogram obtained is concordant with the chromatogram supplied with *loperamide hydrochloride* for system suitability CRS.

Limits:

- *correction factors*: for the calculation of contents, multiply the peak areas of the following impurities by the corresponding correction factor: impurity A = 1.3; impurity D = 1.7;
- *impurities A, B, C, D, E, F, G, H*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent);
- *any other impurity*: not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent);
- *total*: not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.3 per cent);
- *disregard limit*: 0.25 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

Loss on drying (2.2.32): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 100–105 °C for 4 h.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.400 g in 50 ml of *alcohol R* and add 5.0 ml of 0.01 M *hydrochloric acid*. Carry out a potentiometric titration (2.2.20), using 0.1 M *sodium hydroxide*. Read the volume added between the 2 points of inflexion.

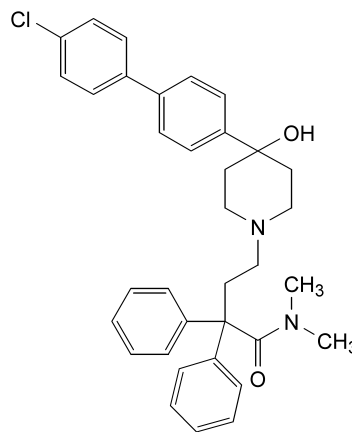
1 ml of 0.1 M *sodium hydroxide* is equivalent to 51.35 mg of $C_{29}H_{34}Cl_2N_2O_2$.

STORAGE

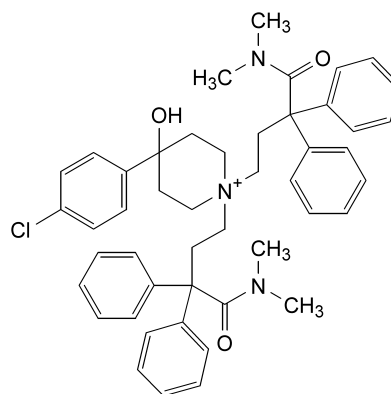
Protected from light.

IMPURITIES

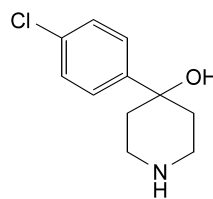
Specified impurities: A, B, C, D, E, F, G, H.



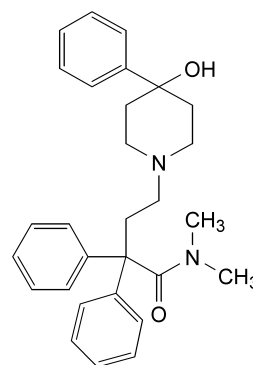
A. 4-[4-(4'-chlorobiphenyl-4-yl)-4-hydroxypiperidin-1-yl]-N,N-dimethyl-2,2-diphenylbutanamide,



B. 4-(4-chlorophenyl)-1,1-bis[4-(dimethylamino)-4-oxo-3,3-diphenylbutyl]-4-hydroxypiperidinium,



C. 4-(4-chlorophenyl)piperidin-4-ol,

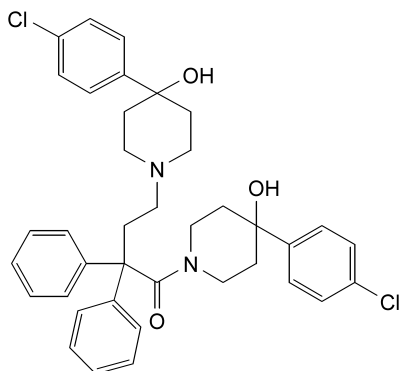


D. 4-(4-hydroxy-4-phenylpiperidin-1-yl)-N,N-dimethyl-2,2-diphenylbutanamide,

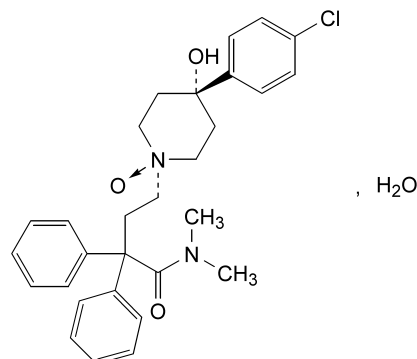
01/2005:1729

LOPERAMIDE OXIDE MONOHYDRATE

Loperamidi oxidum monohydricum



E. 4-(4-chlorophenyl)-1-[4-[4-(4-chlorophenyl)-4-hydroxypiperidin-1-yl]-2,2-diphenylbutanoyl]piperidin-4-ol,



F. loperamide oxide,

 $C_{29}H_{33}ClN_2O_3 \cdot H_2O$
 M_r 511.1
DEFINITION

4-[*trans*-4-(4-Chlorophenyl)-4-hydroxy-1-oxidopiperidin-1-yl]-*N,N*-dimethyl-2,2-diphenylbutanamide monohydrate.

Content: 99.0 per cent to 101.0 per cent (anhydrous substance).

CHARACTERS

Appearance: white or almost white powder, slightly hygroscopic.

Solubility: practically insoluble in water, freely soluble in alcohol and in methylene chloride.

mp: about 152 °C, with decomposition.

IDENTIFICATION

Infrared absorption spectrophotometry (2.2.24).

Comparison: loperamide oxide monohydrate CRS.

TESTS

Related substances. Liquid chromatography (2.2.29).

Test solution. Dissolve 0.100 g of the substance to be examined in *methanol R* and dilute to 10.0 ml with the same solvent.

Reference solution (a). Dissolve 5.0 mg of *loperamide hydrochloride CRS* in *methanol R*, add 0.5 ml of the test solution and dilute to 100.0 ml with *methanol R*.

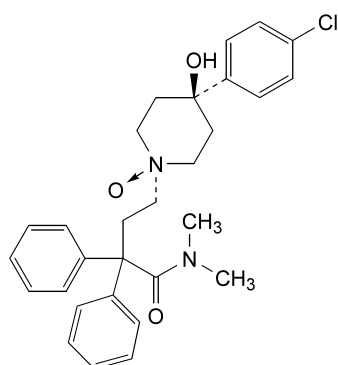
Reference solution (b). Dilute 1.0 ml of the test solution to 20.0 ml with *methanol R*. Dilute 1.0 ml of this solution to 25.0 ml with *methanol R*.

Column:

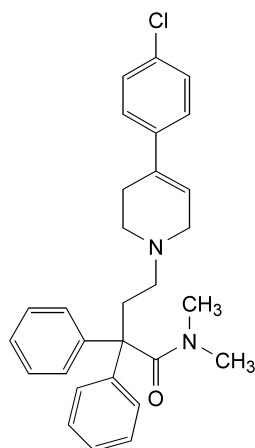
- *size*: $l = 0.10$ m, $\varnothing = 4.6$ mm,
- *stationary phase*: base-deactivated octadecylsilyl silica gel for chromatography *R* (3 μ m),
- *temperature*: 35 °C.

Mobile phase:

- *mobile phase A*: 17.0 g/l solution of tetrabutylammonium hydrogen sulphate *R1*,
- *mobile phase B*: acetonitrile *R*,



G. 4-[*cis*-4-(4-chlorophenyl)-4-hydroxy-1-oxidopiperidin-1-yl]-*N,N*-dimethyl-2,2-diphenylbutanamide,



H. 4-[4-(4-chlorophenyl)-3,6-dihydropyridin-1(2*H*)-yl]-*N,N*-dimethyl-2,2-diphenylbutanamide.

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)
0 - 15	90 → 30	10 → 70
15 - 17	30	70
17 - 19	30 → 90	70 → 10
19 - 24	90	10

Flow rate: 1.5 ml/min.

Detection: spectrophotometer at 220 nm.

Injection: 10 µl.

Relative retention with reference to loperamide oxide (retention time = about 7 min): impurity A = about 0.9; impurity B = about 1.11; impurity C = about 1.13.

System suitability: reference solution (a):

- resolution: minimum 3.8 between the peaks due to loperamide oxide and impurity A.

Limits:

- impurities A, B, C: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent),
- any other impurity: not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent),
- total: not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.3 per cent),
- disregard limit: 0.25 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

Water (2.5.12): 3.4 per cent to 4.2 per cent, determined on 0.500 g.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.350 g in 50 ml of a mixture of 1 volume of *anhydrous acetic acid R* and 7 volumes of *methyl ethyl ketone R*. Titrate with 0.1 M *perchloric acid* using 0.2 ml of *naphtholbenzein solution R* as indicator.

1 ml of 0.1 M *perchloric acid* is equivalent to 49.30 mg of $C_{29}H_{33}ClN_2O_3$.

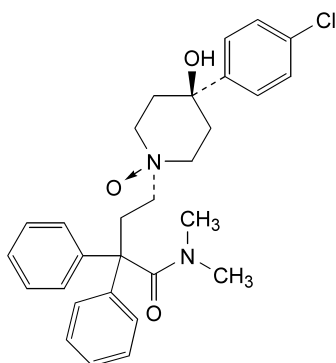
STORAGE

In an airtight container, protected from light.

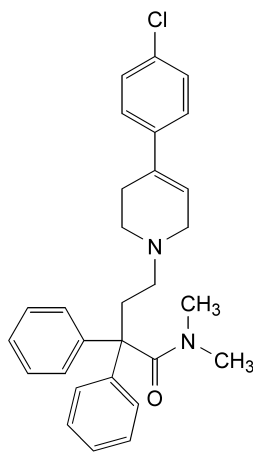
IMPURITIES

Specified impurities: A, B, C.

A. loperamide,



B. 4-[*cis*-4-(4-chlorophenyl)-4-hydroxy-1-oxopiperidin-1-yl]-*N,N*-dimethyl-2,2-diphenylbutanamide,

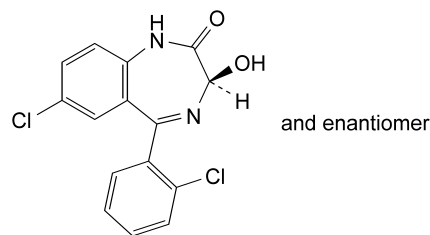


C. 4-[4-(4-chlorophenyl)-3,6-dihydropyridin-1(2*H*)-yl]-*N,N*-dimethyl-2,2-diphenylbutanamide.

01/2005:1121

LORAZEPAM

Lorazepamum



$C_{15}H_{10}Cl_2N_2O_2$

M_r 321.2

DEFINITION

Lorazepam contains not less than 98.5 per cent and not more than the equivalent of 102.0 per cent of (*RS*)-7-chloro-5-(2-chlorophenyl)-3-hydroxy-1,3-dihydro-2*H*-1,4-benzodiazepin-2-one, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, crystalline powder, practically insoluble in water, sparingly soluble in alcohol, sparingly soluble or slightly soluble in methylene chloride.

It shows polymorphism.

IDENTIFICATION

First identification: B.

Second identification: A, C.

- Dissolve 10.0 mg in *alcohol R* and dilute to 100.0 ml with the same solvent. Dilute 10.0 ml of this solution to 100.0 ml with *alcohol R*. Examined between 210 nm and 280 nm (2.2.25), the solution shows an absorption maximum at 230 nm. The specific absorbance at the maximum is 1070 to 1170.
- Examine by infrared absorption spectrophotometry between 600 cm^{-1} and 2000 cm^{-1} (2.2.24), comparing with the spectrum obtained with *lorazepam CRS*. Examine the substances as discs prepared using *potassium bromide R*.
- Examine the chromatograms obtained in the test for related substances. The principal spot in the chromatogram obtained with test solution (b) is similar in position and size to the spot in the chromatogram

obtained with reference solution (a). The test is not valid unless the chromatogram obtained with reference solution (d) shows two clearly separated spots.

TESTS

Related substances. Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel F₂₅₄* plate R. Place the plate in a chromatographic tank containing *methanol R* and allow the solvent front to migrate over a path of 17 cm. Allow the plate to dry in air and heat it at 100-105 °C for 1 h.

Test solution (a). Dissolve 0.200 g of the substance to be examined in *acetone R* and dilute to 10 ml with the same solvent.

Test solution (b). Dilute 2 ml of test solution (a) to 50 ml with *acetone R*.

Reference solution (a). Dissolve 20 mg of *lorazepam CRS* in *acetone R* and dilute to 25 ml with the same solvent.

Reference solution (b). Dilute 1 ml of test solution (b) to 20 ml with *acetone R*.

Reference solution (c). Dilute 5 ml of reference solution (b) to 10 ml with *acetone R*.

Reference solution (d). Dissolve 4 mg of *nitrazepam CRS* in *acetone R*, add 5 ml of reference solution (a) and dilute to 20 ml with *acetone R*.

Apply to the plate 20 µl of each solution and develop in the direction used for the migration of *methanol R* over a path of 12 cm with a mixture of 10 volumes of *methanol R* and 100 volumes of *methylene chloride R*. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. Any spot in the chromatogram obtained with test solution (a), apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.2 per cent) and at most one such spot is more intense than the spot in the chromatogram obtained with reference solution (c) (0.1 per cent).

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g under high vacuum at 100-105 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

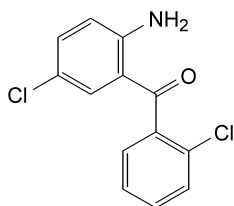
Dissolve 0.250 g in 30 ml of *dimethylformamide R*. Titrate with 0.1 M *tetrabutylammonium hydroxide*, determining the end-point potentiometrically (2.2.20). Protect the solution from atmospheric carbon dioxide throughout the titration.

1 ml of 0.1 M *tetrabutylammonium hydroxide* is equivalent to 32.12 mg of C₁₅H₁₀Cl₂N₂O₂.

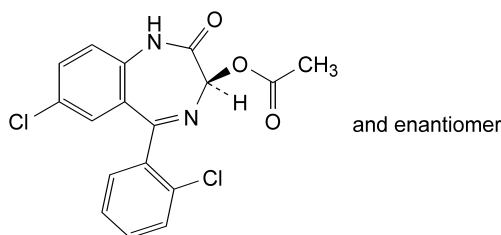
STORAGE

Store in an airtight container, protected from light.

IMPURITIES



A. (2-amino-5-chlorophenyl)(2-chlorophenyl)methanone,



B. (3*RS*)-7-chloro-5-(2-chlorophenyl)-2-oxo-2,3-dihydro-1*H*-1,4-benzodiazepin-3-yl acetate.

01/2005:1233

LOVAGE ROOT

Levistici radix

DEFINITION

Whole or cut, dried rhizome and root of *Levisticum officinale* Koch.

Content: minimum 4.0 ml/kg of essential oil for the whole drug and minimum 3.0 ml/kg of essential oil for the cut drug (dried drug).

CHARACTERS

Macroscopic and microscopic characters described under identification tests A and B.

IDENTIFICATION

- The rhizome and the large roots are often split longitudinally. The rhizome is short, up to 5 cm in diameter, light greyish-brown or yellowish-brown, simple or with several protuberances; the roots, showing little ramification, are the same colour as the rhizome; they are usually up to 1.5 cm thick and up to about 25 cm long; the fracture is usually smooth and shows a very wide yellowish-white bark and a narrow brownish-yellow wood.
- Reduce to a powder (355). The powder is brownish-yellow. Examine under a microscope using *chloral hydrate solution R*. The powder shows: cork cells, polygonal or rounded in surface view, with brown contents; abundant parenchyma, mostly thin-walled and rounded but some with thicker walls; groups of small, reticulately thickened vessels embedded in small-celled, unlignified parenchyma; fragments of larger vessels with reticulate thickening, up to 125 µm in diameter; fragments of secretory canals up to 180 µm wide. Examine under a microscope using a 50 per cent V/V solution of *glycerol R*. The powder shows starch granules, simple, rounded to ovoid, up to about 12 µm, and numerous larger, compound granules, many with several components.
- Examine the chromatograms obtained in the test for *Angelica root*.

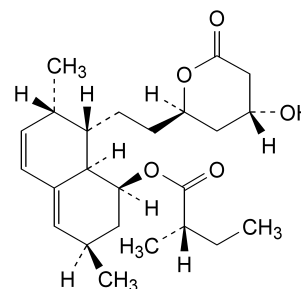
Results: see below the sequence of the zones present in the chromatograms obtained with the reference solution and the test solution.

01/2005:1538

Top of the plate	
Eugenol: (marked at 254 nm)	An intense pale blue to white fluorescent zone
Coumarin: (marked at 254 nm)	An intense pale blue to white fluorescent zone
	1 or 2 less intense pale blue to white fluorescent zones
Reference solution	Test solution

LOVASTATIN

Lovastatinum

 $C_{24}H_{36}O_5$ M_r 404.5

TESTS

Angelica root. Thin-layer chromatography (2.2.27).

Test solution. To 1.0 g of the freshly powdered drug (355) add 5 ml of *methanol R* and boil for 30 s. Cool and filter.

Reference solution. Dissolve 5 mg of *coumarin R* and 25 µl of *eugenol R* in 10 ml of *methanol R*.

Plate: TLC silica gel F_{254} plate *R*.

Mobile phase: *methylene chloride R*, *toluene R* (50:50 V/V).

Application: 20 µl, as bands.

Development: twice over a path of 10 cm.

Drying: in air.

Detection: examine in ultraviolet light at 254 nm. Mark the quenching zones due to coumarin and eugenol on the chromatogram obtained with the reference solution. Examine in ultraviolet light at 365 nm.

Results: the chromatogram obtained with the test solution shows no blue or yellow fluorescent zones in the lower third.

Foreign matter (2.8.2): maximum 3 per cent, determined on 50 g.

Loss on drying (2.2.32): maximum 12.0 per cent, determined on 1.000 g of the powdered drug (355) by drying in an oven at 100–105 °C for 2 h.

Total ash (2.4.16): maximum 8.0 per cent.

Ash insoluble in hydrochloric acid (2.8.1): maximum 2.0 per cent.

ASSAY

Carry out the determination of essential oils in vegetable drugs (2.8.12). Use a 2 litre flask, 10 drops of *liquid paraffin R*, 500 ml of *water R* as the distillation liquid and 0.50 ml of *xylene R* in the graduated tube. Reduce the drug to a powder (500) and immediately use 40.0 g for the determination. Distil at a rate of 2–3 ml/min for 4 h.

DEFINITION

Lovastatin contains not less than 97.0 per cent and not more than the equivalent of 102.0 per cent of (1*S*,3*R*,7*S*,8*S*,8*aR*)-8-[2-[(2*R*,4*R*)-4-hydroxy-6-oxotetrahydro-2*H*-pyran-2-yl]ethyl]-3,7-dimethyl-1,2,3,7,8,8*a*-hexahydronaphthalen-1-yl (2*S*)-2-methylbutanoate, calculated with reference to the dried substance.

CHARACTERS

A white or almost white crystalline powder, practically insoluble in water, soluble in acetone, sparingly soluble in ethanol.

IDENTIFICATION

- It complies with the test for specific optical rotation (see Tests).
- Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *lovastatin CRS*. Examine the substances prepared as discs.

TESTS

Appearance of solution. Dissolve 0.200 g in *acetonitrile R* and dilute to 20.0 ml with the same solvent. The solution is clear (2.2.1) and not more intensely coloured than reference solution B₆ or BY₆ (2.2.2, *Method II*).

Specific optical rotation (2.2.7). Dissolve 0.125 g in *acetonitrile R* and dilute to 25.0 ml with the same solvent. The specific optical rotation is + 325 to + 340, calculated with reference to the dried substance.

Related substances. Examine by liquid chromatography (2.2.29) as described under Assay.

Inject 10 µl of reference solution (b). Adjust the sensitivity of the system so that the height of the principal peak is at least 20 per cent of the full scale of the recorder. Inject 10 µl of test solution (a). When the chromatograms are recorded under the prescribed conditions the relative retentions are: impurity A about 0.8, impurity B about 0.6, impurity C about 1.2 and impurity D about 2.3 (retention time of lovastatin: about 7 min). In the chromatogram obtained with test solution (a): the area of any peak, apart from the principal peak, is not greater than 0.6 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.3 per cent); the sum of the areas of all peaks, apart from the principal peak, is not greater than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (1 per cent). Disregard any peak with an area less than 0.1 times the area of the principal peak.

in the chromatogram obtained with reference solution (b) (0.05 per cent).

Heavy metals (2.4.8). 1.0 g complies with limit test C for heavy metals (20 ppm). Prepare the standard using 2 ml of *lead standard solution* (10 ppm Pb) R.

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in a desiccator under high vacuum at 60 °C for 3 h.

Sulphated ash (2.4.14). Not more than 0.2 per cent, determined on 1.0 g.

ASSAY

Examine by liquid chromatography (2.2.29).

Test solution (a). Dissolve 20.0 mg of the substance to be examined in *acetonitrile* R and dilute to 50.0 ml with the same solvent.

Test solution (b). Dilute 10.0 ml of test solution (a) to 20.0 ml with *acetonitrile* R.

Reference solution (a). Dissolve 10.0 mg of *lovastatin CRS* in *acetonitrile* R and dilute to 50.0 ml with the same solvent.

Reference solution (b). Dilute 0.5 ml of test solution (a) to 100.0 ml with *acetonitrile* R.

Reference solution (c). To 5.0 ml of reference solution (a) add 1 mg of *simvastatin CRS* and dilute to 50.0 ml with *acetonitrile* R.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4.6 mm in internal diameter packed with *octylsilyl silica gel for chromatography* R (5 µm),

- as mobile phase at a flow rate of 1.5 ml/min:

Mobile phase A. *Acetonitrile* R,

Mobile phase B. A 0.1 per cent V/V solution of *phosphoric acid* R,

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)	Comment
0 - 5	60	40	isocratic
5 - 7	60 → 65	40 → 35	linear gradient
7 - 13	65 → 90	35 → 10	linear gradient
13 - 15	90	10	isocratic
15 - 17	90 → 60	10 → 40	linear gradient
17 - 20	60	40	re-equilibration

- as detector a spectrophotometer set at 238 nm.

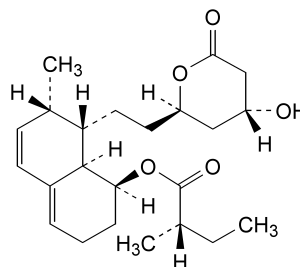
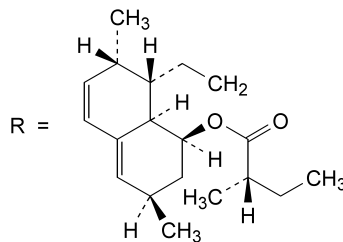
Inject 10 µl of reference solution (c). The test is not valid unless, in the chromatogram obtained, the resolution between the peaks corresponding to simvastatin and lovastatin is at least 5.0. When the chromatograms are recorded in the prescribed conditions the relative retention of simvastatin is about 1.1 (retention time of lovastatin: about 7 min). Inject 10 µl of reference solution (a). Adjust the sensitivity of the system so that the height of the principal peak is at least 50 per cent of the full scale of the recorder. Inject 10 µl of test solution (b).

Calculate the content of $C_{24}H_{36}O_5$ from the peak areas in the chromatograms obtained with test solution (b) and reference solution (a), and the declared content of $C_{24}H_{36}O_5$ in *lovastatin CRS*.

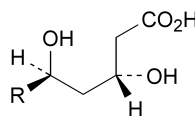
STORAGE

Store under nitrogen at a temperature of 2 °C to 8 °C.

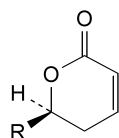
IMPURITIES



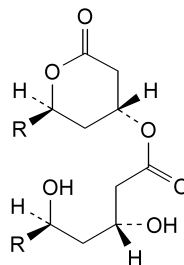
- A. (1*S*,7*S*,8*S*,8*aR*)-8-[2-[(2*R*,4*R*)-4-hydroxy-6-oxotetrahydro-2*H*-pyran-2-yl]ethyl]-7-methyl-1,2,3,7,8,8*a*-hexahydronaphthalen-1-yl (2*S*)-2-methylbutanoate (mevastatin),



- B. (3*R*,5*R*)-7-[(1*S*,2*S*,6*R*,8*S*,8*aR*)-2,6-dimethyl-8-[(2*S*)-2-methylbutanoyl]oxy]-1,2,6,7,8,8*a*-hexahydronaphthalen-1-yl]-3,5-dihydroxyheptanoic acid (hydroxyacid lovastatin),



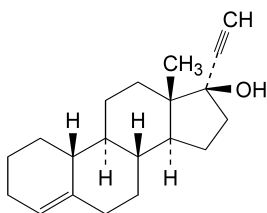
- C. (1*S*,3*R*,7*S*,8*S*,8*aR*)-3,7-dimethyl-8-[2-[(2*R*)-6-oxo-3,6-dihydro-2*H*-pyran-2-yl]ethyl]-1,2,3,7,8,8*a*-hexahydronaphthalen-1-yl (2*S*)-2-methylbutanoate (dehydrolovastatin),



- D. (2*R*,4*R*)-2-[2-[(1*S*,2*S*,6*R*,8*S*,8*aR*)-2,6-dimethyl-8-[(2*S*)-2-methylbutanoyl]oxy]-1,2,6,7,8,8*a*-hexahydronaphthalen-1-yl]ethyl]-6-oxotetrahydro-2*H*-pyran-4-yl (3*R*,5*R*)-7-[(1*S*,2*S*,6*R*,8*S*,8*aR*)-2,6-dimethyl-8-[(2*S*)-2-methylbutanoyl]oxy]-1,2,6,7,8,8*a*-hexahydronaphthalen-1-yl]-3,5-dihydroxyheptanoate (lovastatin dimer).

LYNESTRENOL

Lynestrenolum

C₂₀H₂₈OM_r 284.4

DEFINITION

Lynestrenol contains not less than 98.0 per cent and not more than the equivalent of 102.0 per cent of 19-nor-17 α -pregn-4-en-20-yn-17-ol, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, crystalline powder, practically insoluble in water, soluble in acetone and in alcohol.

IDENTIFICATION

First identification: B.

Second identification: A, C.

- A. Melting point (2.2.14): 161 °C to 165 °C.
- B. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *lynestrenol CRS*.
- C. Examine the chromatograms obtained in the test for related substances in ultraviolet light at 365 nm. The principal spot in the chromatogram obtained with test solution (b) is similar in position, fluorescence and size to the principal spot in the chromatogram obtained with reference solution (b).

TESTS

Appearance of solution. Dissolve 0.2 g in *alcohol R* and dilute to 10 ml with the same solvent. The solution is clear (2.2.1) and colourless (2.2.2, *Method II*).

Specific optical rotation (2.2.7). Dissolve 0.900 g in *alcohol R* and dilute to 25.0 ml with the same solvent. The specific optical rotation is –9.5 to –11, calculated with reference to the dried substance.

Related substances. Examine by thin-layer chromatography (2.2.27), using *silica gel G R* as the coating substance.

Test solution (a). Dissolve 0.125 g of the substance to be examined in *chloroform R* and dilute to 25 ml with the same solvent.

Test solution (b). Dilute 5 ml of test solution (a) to 10 ml with *chloroform R*.

Reference solution (a). Dilute 1 ml of test solution (a) to 100 ml with *chloroform R*. Dilute 5 ml of the solution to 10 ml with *chloroform R*.

Reference solution (b). Dissolve 25 mg of *lynestrenol CRS* in *chloroform R* and dilute to 10 ml with the same solvent.

Apply separately to the plate 10 μ l of each solution. Develop over a path of 15 cm using a mixture of 20 volumes of *acetone R* and 80 volumes of *heptane R*. Allow the plate to dry in air, spray with 0.25 M *alcoholic sulphuric acid R* and heat at 105 °C for 10 min. Examine in ultraviolet light at 365 nm. Any spot in the chromatogram obtained with

01/2005:0558

test solution (a), apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (a) (0.5 per cent).

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 0.500 g by drying in an oven at 100 °C to 105 °C.

ASSAY

Dissolve 0.150 g in 40 ml of *tetrahydrofuran R* and add 5.0 ml of a 100 g/l solution of *silver nitrate R*. Titrate with 0.1 M *sodium hydroxide*. Determine the end-point potentiometrically (2.2.20), using a glass indicator electrode and as comparison electrode a silver-silver chloride double-junction electrode with a saturated solution of *potassium nitrate R* as junction liquid. Carry out a blank titration.

1 ml of 0.1 M *sodium hydroxide* is equivalent to 28.44 mg of C₂₀H₂₈O.

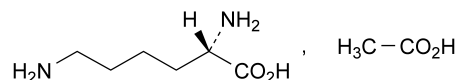
STORAGE

Store protected from light.

01/2005:2114

LYSINE ACETATE

Lysini acetat

C₈H₁₈N₂O₄M_r 206.2

DEFINITION

(2S)-2,6-Diaminohexanoic acid acetate.

Content: 98.5 per cent to 101.0 per cent (dried substance).

CHARACTERS

Appearance: white or almost white, crystalline powder or colourless crystals.

Solubility: freely soluble in water, very slightly soluble in ethanol (96 per cent).

It shows polymorphism.

IDENTIFICATION

First identification: A, B, E.

Second identification: A, C, D, E.

A. It complies with the test for specific optical rotation (see Tests).

B. Infrared absorption spectrophotometry (2.2.24).

Comparison: *lysine acetate CRS*.

If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in the minimum volume of *water R*, evaporate to dryness at 60 °C and record new spectra using the residues.

C. Examine the chromatograms obtained in the test for ninhydrin-positive substances.

Results: the principal spot in the chromatogram obtained with test solution (b) is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).

D. To 0.1 ml of solution S (see Tests) add 2 ml of *water R* and 1 ml of a 50 g/l solution of *phosphomolybdic acid R*. A yellowish-white precipitate is formed.

E. It gives reaction (a) of acetates (2.3.1).

TESTS

Solution S. Dissolve 5.0 g in *distilled water R* and dilute to 50 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and colourless (2.2.2, *Method II*).

Specific optical rotation (2.2.7): + 8.5 to + 10.0 (dried substance), determined on solution S.

Ninhydrin-positive substances. Thin-layer chromatography (2.2.27).

Test solution (a). Dissolve 0.10 g of the substance to be examined in *water R* and dilute to 10 ml with the same solvent.

Test solution (b). Dilute 1.0 ml of test solution (a) to 50 ml with *water R*.

Reference solution (a). Dissolve 10 mg of *lysine acetate CRS* in *water R* and dilute to 50 ml with the same solvent.

Reference solution (b). Dilute 5 ml of test solution (b) to 20 ml with *water R*.

Reference solution (c). Dissolve 10 mg of *lysine acetate CRS* and 10 mg of *arginine CRS* in *water R* and dilute to 25 ml with the same solvent.

Plate: TLC silica gel plate *R*.

Mobile phase: concentrated ammonia *R*, 2-propanol *R* (30:70 V/V).

Application: 5 µl.

Development: over 2/3 of the plate.

Drying: at 100-105 °C until the ammonia has evaporated.

Detection: spray with *ninhydrin solution R* and heat at 100-105 °C for 15 min.

System suitability: reference solution (c):

– the chromatogram shows 2 clearly separated spots.

Limits: test solution (a):

– *any impurity:* any spot, apart from the principal spot, is not more intense than the principal spot in the chromatogram obtained with reference solution (b) (0.5 per cent).

Chlorides (2.4.4): maximum 200 ppm.

Dilute 2.5 ml of solution S to 15 ml with *water R*.

Sulphates (2.4.13): maximum 300 ppm.

Dilute 5 ml of solution S to 15 ml with *distilled water R*.

Ammonium (2.4.1, *Method B*): maximum 200 ppm, determined on 50 mg.

Prepare the standard using 0.1 ml of *ammonium standard solution* (100 ppm NH₄) *R*.

Iron (2.4.9): maximum 30 ppm.

In a separating funnel, dissolve 0.33 g in 10 ml of *dilute hydrochloric acid R*. Shake with 3 quantities, each of 10 ml, of *methyl isobutyl ketone RI*, shaking for 3 min each time. To the combined organic layers add 10 ml of *water R* and shake for 3 min. The aqueous layer complies with the limit test for iron.

Heavy metals (2.4.8): maximum 10 ppm.

12 ml of solution S complies with limit test A. Prepare the reference solution using *lead standard solution* (1 ppm Pb) *R*.

Loss on drying (2.2.32): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 60 °C for 3 h.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 80 mg in 3 ml of *anhydrous formic acid R*. Add 50 ml of *anhydrous acetic acid R*. Titrate with 0.1 M *perchloric acid*, determining the end-point potentiometrically (2.2.20). Carry out a blank titration.

1 ml of 0.1 M *perchloric acid* is equivalent to 10.31 mg of C₈H₁₈N₂O₄.

STORAGE

Protected from light.

IMPURITIES

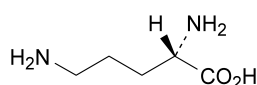
Specified impurities: A, B, C, D, E, F.

A. aspartic acid,

B. glutamic acid,

C. alanine,

D. valine,



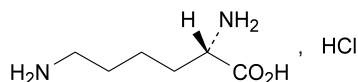
E. (2S)-2,5-diaminopentanoic acid (ornithine),

F. arginine.

01/2005:0930

LYSINE HYDROCHLORIDE

Lysini hydrochloridum



C₆H₁₅ClN₂O₂

*M*_r 182.7

DEFINITION

Lysine hydrochloride contains not less than 98.5 per cent and not more than the equivalent of 101.0 per cent of (S)-2,6-diaminohexanoic acid hydrochloride, calculated with reference to the dried substance.

CHARACTERS

A white, crystalline powder or colourless crystals, freely soluble in water, slightly soluble in alcohol.

IDENTIFICATION

First identification: A, B, E.

Second identification: A, C, D, E.

A. It complies with the test for specific optical rotation (see Tests).

B. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *lysine hydrochloride CRS*. Examine the substances prepared as discs. If the spectra obtained show differences, dissolve the substance to be examined and the reference substance separately in the minimum volume of *water R*, evaporate to dryness at 60 °C, and record new spectra using the residues.

C. Examine the chromatograms obtained in the test for ninhydrin-positive substances. The principal spot in the chromatogram obtained with test solution (b) is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).

- D. To 0.1 ml of solution S (see Tests) add 2 ml of *water R* and 1 ml of a 50 g/l solution of *phosphomolybdic acid R*. A yellowish-white precipitate is formed.
- E. To 0.1 ml of solution S add 2 ml of *water R*. The solution gives reaction (a) of chlorides (2.3.1).

TESTS

Solution S. Dissolve 5.0 g in *carbon dioxide-free water R* prepared from *distilled water R* and dilute to 50 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and not more intensely coloured than reference solution B₇ or GY₇ (2.2.2, *Method II*).

Specific optical rotation (2.2.7). Dissolve 2.00 g in *hydrochloric acid R1* and dilute to 25.0 ml with the same acid. The specific optical rotation is + 21.0 to + 22.5, calculated with reference to the dried substance.

Ninhydrin-positive substances. Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel plate R*.

Test solution (a). Dissolve 0.10 g of the substance to be examined in *water R* and dilute to 10 ml with the same solvent.

Test solution (b). Dilute 1 ml of test solution (a) to 50 ml with *water R*.

Reference solution (a). Dissolve 10 mg of *lysine hydrochloride CRS* in *water R* and dilute to 50 ml with the same solvent.

Reference solution (b). Dilute 5 ml of test solution (b) to 20 ml with *water R*.

Reference solution (c). Dissolve 10 mg of *lysine hydrochloride CRS* and 10 mg of *arginine CRS* in *water R* and dilute to 25 ml with the same solvent.

Apply separately to the plate 5 µl of each solution. Develop over a path of 15 cm using a mixture of 30 volumes of *concentrated ammonia R* and 70 volumes of *2-propanol R*. Dry the plate at 100 °C to 105 °C until the ammonia disappears completely. Spray with *ninhydrin solution R*

and heat at 100 °C to 105 °C for 15 min. Any spot in the chromatogram obtained with test solution (a), apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.5 per cent). The test is not valid unless the chromatogram obtained with reference solution (c) shows two clearly separated principal spots.

Sulphates (2.4.13). Dilute 5 ml of solution S to 15 ml with *distilled water R*. The solution complies with the limit test for sulphates (300 ppm).

Ammonium (2.4.1). 50 mg complies with limit test B for ammonium (200 ppm). Prepare the standard using 0.1 ml of *ammonium standard solution (100 ppm NH₄) R*.

Iron (2.4.9). In a separating funnel, dissolve 0.33 g in 10 ml of *dilute hydrochloric acid R*. Shake with three quantities, each of 10 ml, of *methyl isobutyl ketone R1*, shaking for 3 min each time. To the combined organic layers add 10 ml of *water R* and shake for 3 min. The aqueous layer complies with the limit test for iron (30 ppm).

Heavy metals (2.4.8). 12 ml of solution S complies with limit test A for heavy metals (10 ppm). Prepare the standard using *lead standard solution (1 ppm Pb) R*.

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.150 g in 5 ml of *anhydrous formic acid R*. Add 50 ml of *anhydrous acetic acid R*. Titrate with 0.1 M *perchloric acid*, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *perchloric acid* is equivalent to 18.27 mg of C₆H₁₅ClN₂O₂.

STORAGE

Store protected from light.

01/2005:1443

01/2005:2052

MACROGOL 6 GLYCEROL CAPRYLOCAPRATE

Macrogol 6 glyceroli caprylocapras

DEFINITION

Macrogol 6 glycerol caprylocaprate is a mixture of mainly mono- and diesters of polyoxyethylene glycerol ethers mainly with caprylic (octanoic) and capric (decanoic) acids. The average content of the ethylene oxide is 6 units per molecule. Macrogol 6 glycerol caprylocaprate may be obtained by ethoxylation of glycerol and esterification with distilled coconut or palm kernel fatty acids, or by ethoxylation of mono- and diglycerides of caprylic and capric acids.

CHARACTERS

A pale yellow liquid, partly soluble in water, freely soluble in castor oil, in glycerol, in isopropanol and in propylene glycol. It has a viscosity of about 145 mPas.

IDENTIFICATION

- A. Dissolve 1.0 g in 99 g of a mixture of 10 volumes of *2-propanol R* and 90 volumes of *water R*. Heat the solution obtained to about 40 °C. A turbidity is produced. Allow to cool until the turbidity disappears. The cloud point is between 15 °C and 35 °C.
- B. It complies with the test for saponification value (see Tests).
- C. It complies with the test for composition of fatty acids (see Tests).

TESTS

Appearance. The substance to be examined is clear (2.2.1) and not more intensely coloured than reference solution Y_2 (2.2.2, Method I).

Alkalinity. Dissolve 2.0 g in a hot mixture of 10 ml of *alcohol R* and 10 ml of *water R*. Add 0.1 ml of *bromothymol blue solution R1*. Not more than 0.5 ml of 0.1 M *hydrochloric acid* is required to change the colour of the indicator to yellow.

Acid value (2.5.1). Not more than 5.0, determined on 5.0 g.

Hydroxyl value (2.5.3, Method A): 165 to 225.

Saponification value (2.5.6): 85 to 105, determined on 2.0 g.

Composition of fatty acids (2.4.22, Method A). The fatty acid fraction has the following composition:

- *caproic acid*: not more than 2.0 per cent,
- *caprylic acid*: 50.0 per cent to 80.0 per cent,
- *capric acid*: 20.0 per cent to 50.0 per cent,
- *lauric acid*: not more than 3.0 per cent,
- *myristic acid*: not more than 1.0 per cent.

Ethylene oxide and dioxan (2.4.25). Not more than 1 ppm of ethylene oxide and not more than 10 ppm of dioxan.

Water (2.5.12). Not more than 1.0 per cent, determined on 1.00 g by the semi-micro determination of water.

Total ash (2.4.16). Not more than 0.3 per cent, determined on 1.0 g.

MACROGOL 15 HYDROXYSTEARATE

Macrogoli 15 hydroxystearas

DEFINITION

Mixture of mainly monoesters and diesters of 12-hydroxystearic acid and macrogols obtained by ethoxylation of 12-hydroxystearic acid. The number of moles of ethylene oxide reacted per mole of 12-hydroxystearic acid is 15 (nominal value). It contains free macrogols.

CHARACTERS

Appearance: yellowish, waxy mass.

Solubility: very soluble in water, soluble in alcohol, insoluble in liquid paraffin.

It solidifies at about 25 °C.

IDENTIFICATION

- A. Thin-layer chromatography (2.2.27).

Test solution. To 1.0 g add 100 ml of a 100 g/l solution of *potassium hydroxide R* and boil under a reflux condenser for 30 min. Acidify the warm solution with 20 ml of *hydrochloric acid R* and cool to room temperature. Shake the mixture with 50 ml of *ether R* and allow to stand until a separation of the layers is visible. Separate the clear upper layer, add 5 g of *anhydrous sodium sulphate R*, wait for 30 min, filter and evaporate to dryness on a water-bath. Dissolve 50 mg of the residue in 25 ml of *ether R*.

Reference solution. Dissolve 50 mg of *12-hydroxystearic acid R* in 25 ml of *methylene chloride R*.

Plate: *octadecylsilyl silica gel for chromatography R* as the coating substance.

Mobile phase: *methylene chloride R*, *glacial acetic acid R*, *acetone R* (10:40:50 V/V/V).

Application: 2 µl.

Development: over 2/3 of the plate.

Drying: in a current of cold air.

Detection: spray with a 80 g/l solution of *phosphomolybdic acid R* in *2-propanol R* and heat at 120 °C for 1-2 min.

Results: the principal spot in the chromatogram obtained with the test solution is similar in position and colour to the principal spot in the chromatogram obtained with the reference solution.

- B. Dissolve 15.0 g in 50 ml of *water R*. The viscosity (2.2.9) has a maximum of 20 mPas.
- C. It complies with the test for free macrogols (see Tests).

TESTS

Appearance of solution. The solution is not more opalescent than reference suspension III (2.2.1) and not more intensely coloured than reference solution B_6 or BY_6 (2.2.2, Method II). Dissolve 2.0 g in *water R* and dilute to 20 ml with the same solvent.

Acid value (2.5.1): maximum 1.0, determined on 2.0 g.

Hydroxyl value (2.5.3, Method A): 90 to 110.

Iodine value (2.5.4): maximum 2.0.

Peroxide value (2.5.5, Method A): maximum 5.0.

Saponification value (2.5.6): 53 to 63.

Free macrogols. Size-exclusion chromatography (2.2.30).

01/2005:2044

Test solution. Dissolve 1.20 g of the substance to be examined in the mobile phase and dilute to 250.0 ml with the mobile phase.

Reference solution (a). Dissolve about 0.4 g of *macroglol 1000 R* in the mobile phase and dilute to 250.0 ml with the mobile phase.

Reference solution (b). Dilute 50.0 ml of reference solution (a) to 100.0 ml with the mobile phase.

Precolumns (2):

- **size:** $l = 0.125$ m, $\varnothing = 4$ mm,
- **stationary phase:** spherical *octadecylsilyl silica gel for chromatography R* (5 μ m) with a pore size of 10 nm.

Column:

- **size:** $l = 0.30$ m, $\varnothing = 7.8$ mm,
- **stationary phase:** *hydroxylated polymethacrylate gel R* (6 μ m) with a pore size of 12 nm.

Connect both precolumns to the column using a 3-way valve and switch the mobile phase flow according to the following programme:

- 0-114 s: precolumn 1 and column,
- 115 s to the end: precolumn 2 and column,
- 115 s to 7 min: flow back of precolumn 1.

Mobile phase: *water R*, *methanol R* (2:8 V/V).

Flow rate: 1.1 ml/min.

Detection: refractometer.

Injection: 50 μ l.

Calculate the percentage content of free macrogols using the following expression:

$$\frac{A_1 \times m_2 \times 200}{m_1 \times (A_2 + 2A_3)}$$

- m_1 = mass of the substance to be examined in the test solution, in grams,
- m_2 = mass of *macroglol 1000 R* in reference solution (a), in grams,
- A_1 = area of the peak due to free macrogols in the substance to be examined in the chromatogram obtained with the test solution,
- A_2 = area of the peak due to macroglol 1000 in the chromatogram obtained with reference solution (a),
- A_3 = area of the peak due to macroglol 1000 in the chromatogram obtained with reference solution (b).

Limit:

- **free macrogols:** 27.0 per cent to 39.0 per cent.

Ethylene oxide and dioxan (2.4.25): maximum 1 ppm of ethylene oxide and maximum 50 ppm of dioxan.

Nickel (2.4.27): maximum 1 ppm.

Water (2.5.12): maximum 1.0 per cent, determined on 2.00 g.

Total ash (2.4.16): maximum 0.3 per cent, determined on 1.0 g.

STORAGE

In an airtight container.

MACROGOL 20 GLYCEROL MONOSTEARATE

Macroglol 20 glyceroli monostearas

DEFINITION

Macroglol 20 glycerol monostearate is obtained by ethoxylation with ethylene oxide of different types of glycerol stearates, mainly *Glycerol monostearate 40-55 (0495)*. The number of moles of ethylene oxide reacted per mole of glycerol stearate is 20 (nominal value).

CHARACTERS

Appearance: pale yellow, oily liquid or gel.

Solubility: soluble in water at 40 °C and above and in alcohol, practically insoluble in light liquid paraffin and in fatty oils.

Relative density: about 1.07.

IDENTIFICATION

- It complies with the test for hydroxyl value (see Tests).
- It complies with the test for saponification value (see Tests).
- It complies with the test for composition of fatty acids (see Tests).
- Place 1 g in a test-tube and add 0.1 ml of *sulphuric acid R*. Heat the tube until white fumes appear. The fumes turn filter paper impregnated with *alkaline potassium tetraiodomercurate solution R* black.

TESTS

Acid value (2.5.1): maximum 2.0, determined on 5.0 g.

Hydroxyl value (2.5.3, *Method B*): 65 to 85, determined on 0.350 g.

Iodine value (2.5.4): maximum 2.0.

Peroxide value (2.5.5): maximum 6.0.

Saponification value (2.5.6): 40 to 60.

Composition of fatty acids. Gas chromatography (2.4.22, *Method C*).

Composition of the fatty acid fraction of the substance:

Type of macroglol 20 glycerol monostearate	Type of glycerol stearate used	Composition of fatty acids
Type I	Type I (obtained using stearic acid 50)	<i>Stearic acid</i> : 40.0 per cent to 60.0 per cent, <i>Sum of the contents of palmitic and stearic acids</i> : minimum 90.0 per cent.
Type II	Type II (obtained using stearic acid 70)	<i>Stearic acid</i> : 60.0 per cent to 80.0 per cent, <i>Sum of the contents of palmitic and stearic acids</i> : minimum 90.0 per cent.
Type III	Type III (obtained using stearic acid 95)	<i>Stearic acid</i> : 90.0 per cent to 99.0 per cent, <i>Sum of the contents of palmitic and stearic acids</i> : minimum 96.0 per cent.

Ethylene oxide and dioxan (2.4.25, *Method A*): maximum 1 ppm of ethylene oxide and 10 ppm of dioxan.

Heavy metals (2.4.8): maximum 10 ppm.

2.0 g complies with limit test C. Prepare the standard using 2 ml of *lead standard solution (10 ppm Pb) R*.

Water (2.5.12): maximum 3.0 per cent, determined on 1.00 g.

Total ash (2.4.16): maximum 0.2 per cent, determined on 1.0 g.

STORAGE

Protected from light.

LABELLING

The label states the type of macrogol 20 glycerol monostearate.

01/2005:1123

MACROGOL CETOSTEARYL ETHER

Macrogoli aether cetostearylicus

DEFINITION

Macrogol cetostearyl ether is a mixture of ethers of mixed macrogols with linear fatty alcohols, mainly cetostearyl alcohol. It may contain some free macrogols and it contains various amounts of free cetostearyl alcohol. The amount of ethylene oxide reacted with cetostearyl alcohol is from 2 to 33 units per molecule (nominal value).

CHARACTERS

A waxy, white or yellowish-white, unctuous mass, pellets, microbeads or flakes.

Macrogol cetostearyl ether with low numbers of ethylene oxide units per molecule is practically insoluble in water, soluble in alcohol and in methylene chloride.

Macrogol cetostearyl ether with higher numbers of ethylene oxide units per molecule is dispersible or soluble in water, soluble in alcohol and in methylene chloride.

It solidifies at 32 °C to 52 °C.

IDENTIFICATION

- A. It complies with the test for hydroxyl value (see Tests).
- B. It complies with the test for iodine value (see Tests).
- C. It complies with the test for saponification value (see Tests).
- D. Examine by thin-layer chromatography (2.2.27), using a suitable silica gel as the coating substance.

Test solution. Dissolve the amount of substance to be examined (given in Table 1123.-1) in a mixture of 1 volume of *water R* and 9 volumes of *methanol R* and dilute to 75 ml with the same mixture of solvents.

Table 1123.-1

Ethylene oxide units per molecule	Amount to be dissolved
2 - 6	5.0 g
10 - 22	10.0 g
25 - 33	15.0 g

Add 60 ml of *hexane R* and shake for 3 min. The formation of foam can be reduced by the addition of some drops of *alcohol R*. Pass the hexane layer through a filter with *anhydrous sodium sulphate R*, wash the filter with 3 quantities, each of 10 ml, of *hexane R* and evaporate the combined filtrates to dryness. Dissolve 0.05 g of the residue in 10 ml of *methanol R* (sometimes the solution is opalescent).

Reference solution. Dissolve 25 mg of *stearyl alcohol CRS* in *methanol R* and dilute to 25 ml with the same solvent.

Apply separately to the plate 20 µl of each solution. Develop over a path of 15 cm using *ethyl acetate R*. Dry and spray with vanillin-sulphuric acid reagent prepared as follows: dissolve 0.50 g of *vanillin R* in 50.0 ml of *alcohol R* and dilute to 100.0 ml with *sulphuric acid R*. Allow the plate to dry in air. Heat the plate at about 130 °C for 15 min and allow to cool in air. The chromatogram obtained with the test solution shows several spots; one of these spots corresponds to the principal spot in the chromatogram obtained with the reference solution.

- E. Dissolve or disperse 0.1 g in 5 ml of *alcohol R*, add 2 ml of *water R*, 10 ml of *dilute hydrochloric acid R*, 10 ml of *barium chloride solution R1* and 10 ml of a 100 g/l solution of *phosphomolybdic acid R*. A precipitate is formed.

TESTS

Appearance of solution. Dissolve 5.0 g in *alcohol R* and dilute to 50 ml with the same solvent. The solution is not more intensely coloured than reference solution BY₅ (2.2.2, *Method II*).

Alkalinity. Dissolve 2.0 g in a hot mixture of 10 ml of *water R* and 10 ml of *alcohol R*. Add 0.1 ml of *bromothymol blue solution R1*. Not more than 0.5 ml of 0.1 M *hydrochloric acid* is required to change the colour of the indicator to yellow.

Acid value (2.5.1). Not more than 1.0, determined on 5.0 g.

Hydroxyl value (2.5.3, *Method A*).

Ethylene oxide units per molecule (nominal value)	Hydroxyl value
2	150 - 180
3	135 - 155
5 - 6	100 - 134
10	75 - 90
12	67 - 77
15	58 - 67
20 - 22	40 - 55
25	36 - 46
30 - 33	32 - 40

Iodine value (2.5.4). Not more than 2.0.

Saponification value (2.5.6). Not more than 3.0, determined on 10.0 g.

Ethylene oxide and dioxan (2.4.25). Not more than 1 ppm of ethylene oxide and not more than 10 ppm of dioxan.

Water (2.5.12). Not more than 3.0 per cent, determined on 2.00 g by the semi-micro determination of water.

Total ash (2.4.16). Not more than 0.2 per cent, determined on 2.0 g.

STORAGE

Store in an airtight container.

LABELLING

The label states the amount of ethylene oxide reacted with cetostearyl alcohol (nominal value).

01/2005:1124

MACROGOL LAURYL ETHER**Macrogoli aether laurilicum****DEFINITION**

Mixture of ethers of mixed macrogols with fatty alcohols, mainly C₁₂H₂₆O. It contains a variable amount of free C₁₂H₂₆O and it may contain free macrogols. The number of moles of ethylene oxide reacted per mole of C₁₂H₂₆O is 3 to 23 (nominal value).

CHARACTERS

- Macrogol lauryl ether with 3 to 5 units of ethylene oxide per molecule.

Appearance: colourless liquid.

Solubility: practically insoluble in water, soluble or dispersible in alcohol, practically insoluble in light petroleum.

- Macrogol lauryl ether with 9 to 23 units of ethylene oxide per molecule.

Appearance: white, waxy mass.

Solubility: soluble or dispersible in water, soluble in alcohol, practically insoluble in light petroleum.

IDENTIFICATION

- It complies with the test for hydroxyl value (see Tests).
- It complies with the test for iodine value (see Tests).
- It complies with the test for saponification value (see Tests).
- Dissolve or disperse 0.1 g in 5 ml of *alcohol R*, add 10 ml of *dilute hydrochloric acid R*, 10 ml of *barium chloride solution R1* and 10 ml of a 100 g/l solution of *phosphomolybdic acid R*. A precipitate is formed.

TESTS

Appearance of solution. The solution is not more intensely coloured than reference solution BY₅ (2.2.2, *Method II*).

Dissolve 5.0 g in *alcohol R* and dilute to 50 ml with the same solvent.

Alkalinity. Dissolve 2.0 g in a hot mixture of 10 ml of *water R* and 10 ml of *alcohol R*. Add 0.1 ml of *bromothymol blue solution R1*. Not more than 0.5 ml of 0.1 M *hydrochloric acid* is required to change the colour of the indicator to yellow.

Acid value (2.5.1): maximum 1.0, determined on 5.0 g.

Hydroxyl value (2.5.3, *Method A*).

Ethylene oxide units per molecule (nominal value)	Hydroxyl value
3	165 - 180
4	145 - 165
5	130 - 140
9	90 - 100
10	85 - 95
12	73 - 83
15	64 - 74
20 - 23	40 - 60

Iodine value (2.5.4): maximum 2.0.

Saponification value (2.5.6): maximum 3.0, determined on 10.0 g.

Ethylene oxide and dioxan (2.4.25): maximum 1 ppm of ethylene oxide and maximum 10 ppm of dioxan.

Water (2.5.12): maximum 3.0 per cent, determined on 2.00 g.

Total ash (2.4.16): maximum 0.2 per cent, determined on 2.0 g.

STORAGE

In an airtight container.

LABELLING

The label states the number of moles of ethylene oxide reacted per mole of C₁₂H₂₆O (nominal value).

01/2005:1618

MACROGOL OLEATE**Macrogoli oleas****DEFINITION**

A mixture of monoesters and diesters of mainly oleic acid and macrogols. It may be obtained by ethoxylation of *Oleic acid* (0799) or by esterification of macrogols with oleic acid of animal or vegetable origin. It may contain free macrogols. The average polymer length is equivalent to 5-6 or 10 ethylene oxide units per molecule (nominal value). A suitable antioxidant may be added.

CHARACTERS

Appearance: slightly yellowish, viscous liquid.

Solubility: dispersible in water, soluble in alcohol and in 2-propanol, dispersible in oils, miscible with fatty oils and with waxes.

Refractive index: about 1.466.

IDENTIFICATION

First identification: A, C.

Second identification: A, B.

- It complies with the test for saponification value (see Tests).

- Thin-layer chromatography (2.2.27).

Test solution. To 20 mg add 10 ml of *methylene chloride R* and mix.

Plate: TLC silica gel F₂₅₄ plate R.

Mobile phase: 25 per cent V/V solution of *concentrated ammonia R*, 2-propanol R (20:80 V/V).

Application: 10 µl.

Development: over a path of 15 cm.

Drying: in air.

Detection: spray with *potassium iodobismuthate solution R4*. Examine the plate about 10 min later.

Results: the chromatogram obtained shows 3 principal spots, corresponding, in order of increasing R_f value, to free macrogol, macrogol mono-oleate and macrogol dioleate.

- It complies with the test for composition of fatty acids (see Tests).

TESTS

Alkalinity. Dissolve 2.0 g in *alcohol R* and dilute to 20 ml with the same solvent. To 2 ml of this solution add 0.05 ml of *phenol red solution R*. The solution is not red.

Acid value (2.5.1): maximum 1.0, determined on 10.0 g.

Hydroxyl value (2.5.3, *Method A*): see Table 1618-1.

Iodine value (2.5.4): see Table 1618-1.

Peroxide value (2.5.5): maximum 12.0.

Saponification value (2.5.6): see Table 1618-1.

Table 1618-1

	5-6 ethylene oxide units	10 ethylene oxide units
Hydroxyl value	50 - 70	65 - 90
Iodine value	50 - 60	27 - 34
Saponification value	105 - 120	68 - 85

Composition of fatty acids. Gas chromatography (2.4.22, Method A).

Composition:

- *myristic acid*: maximum 5.0 per cent,
- *stearic acid*: maximum 6.0 per cent,
- *palmitic acid*: maximum 16.0 per cent,
- *palmitoleic acid*: maximum 8.0 per cent,
- *oleic acid*: 65.0 per cent to 88.0 per cent,
- *linoleic acid*: maximum 18.0 per cent,
- *linolenic acid*: maximum 4.0 per cent,
- *fatty acids with a chain length greater than C₁₈*: maximum 4.0 per cent.

Residual ethylene oxide and dioxan (2.4.25): maximum 1 ppm of residual ethylene oxide and 10 ppm of residual dioxan.

Water (2.5.12): maximum 2.0 per cent, determined on 1.00 g using *anhydrous methanol R* as the solvent.

Total ash (2.4.16): maximum 0.3 per cent, determined on 1.0 g.

STORAGE

In an airtight container.

LABELLING

The label states:

- the number of ethylene oxide units per molecule (nominal value),
- the name and concentration of any added antioxidant.

01/2005:1125

MACROGOL OLEYL ETHER

Macrogoli aether oleicum

DEFINITION

Mixture of ethers of mixed macrogols with linear fatty alcohols, mainly oleyl alcohol. It contains a variable amount of free oleyl alcohol and it may contain free macrogols. The number of moles of ethylene oxide reacted per mole of oleyl alcohol is 2 to 20 (nominal value). A suitable antioxidant may be added.

CHARACTERS

- Macrogol oleyl ether with 2 to 5 units of ethylene oxide per molecule.

Appearance: yellow liquid.

Solubility: practically insoluble in water, soluble in alcohol, practically insoluble in light petroleum.

- Macrogol oleyl ether with 10 to 20 units of ethylene oxide per molecule.

Appearance: yellowish-white waxy mass.

Solubility: dispersible or soluble in water, soluble in alcohol, practically insoluble in light petroleum.

IDENTIFICATION

- It complies with the test for hydroxyl value (see Tests).
- It complies with the test for iodine value (see Tests).
- It complies with the test for saponification value (see Tests).
- Dissolve or disperse 0.1 g in 5 ml of *alcohol R*, add 2 ml of *water R*, 10 ml of *dilute hydrochloric acid R*, 10 ml of *barium chloride solution R1* and 10 ml of a 100 g/l solution of *phosphomolybdic acid R*. A precipitate is formed.

TESTS

Appearance of solution. The solution is not more intensely coloured than reference solution BY₅ (2.2.2, Method II).

Dissolve 5.0 g in *alcohol R* and dilute to 50 ml with the same solvent.

Alkalinity. Dissolve 2.0 g in a hot mixture of 10 ml of *water R* and 10 ml of *alcohol R*. Add 0.1 ml of *bromothymol blue solution R1*. Not more than 0.5 ml of 0.1 M *hydrochloric acid* is required to change the colour of the indicator to yellow.

Acid value (2.5.1): maximum 1.0, determined on 5.0 g.

Hydroxyl value (2.5.3, Method A). See Table 1125-1.

Iodine value (2.5.4). See Table 1125-1.

Table 1125-1

Ethylene oxide units per molecule (nominal value)	Hydroxyl value	Iodine value
2	158 - 178	48 - 74*
5	110 - 125	48 - 56
10	75 - 95	24 - 38
20	40 - 65	14 - 24

* This broad range is needed since 2 different grades of oleyl alcohol may be used for the synthesis. The iodine value does not differ by more than 5 units from the nominal iodine value and is within the limits stated in the table.

Peroxide value (2.5.5): maximum 10.0.

Saponification value (2.5.6): maximum 3.0.

Ethylene oxide and dioxan (2.4.25): maximum 1 ppm of ethylene oxide and 10 ppm of dioxan.

Water (2.5.12): maximum 3.0 per cent, determined on 2.00 g.

Total ash (2.4.16): maximum 0.2 per cent, determined on 2.0 g.

STORAGE

In an airtight container, protected from light.

LABELLING

The label states:

- the number of moles of ethylene oxide reacted per mole of oleyl alcohol (nominal value),
- the name and concentration of any added antioxidant,
- the nominal iodine value for the type with 2 units of ethylene oxide per molecule.

01/2005:1234 **Composition of fatty acids.** Gas chromatography (2.4.22, Method C).

MACROGOL STEARATE

Macrogoli stearas

DEFINITION

Mixture of monoesters and diesters of mainly stearic acid and/or palmitic acid and macrogols. It may be obtained by ethoxylation or by esterification of macrogols with stearic acid 50 (type I) or stearic acid 95 (type II) (see *Stearic acid* (1474)). It may contain free macrogols. The average polymer length is equivalent to 6 to 100 ethylene oxide units per molecule (nominal value).

CHARACTERS

Appearance: white or slightly yellowish waxy mass.

Solubility: soluble in alcohol and in 2-propanol. Macrogol stearate corresponding to a product with 6 to 9 units of ethylene oxide per molecule is practically insoluble, but freely dispersible in water and miscible with fatty oils and with waxes. Macrogol stearate corresponding to a product with 20 to 100 units of ethylene oxide per molecule is soluble in water and practically insoluble in fatty oils and in waxes.

IDENTIFICATION

- A. It complies with the test for saponification value (see Tests).
- B. It complies with the test for composition of fatty acids (see Tests).

TESTS

Alkalinity. Dissolve 2.0 g in *alcohol R* and dilute to 20 ml with the same solvent. To 2 ml of this solution add 0.05 ml of *phenol red solution R*. The solution is not red.

Melting point (2.2.15). See Table 1234.-1.

Melt about 10 g at 80-90 °C. Introduce into the tube by capillary action, a sufficient amount of the substance, to form in the tube a column of the prescribed height. Allow to stand at 0 °C for 2 h.

Acid value (2.5.1): maximum 2.0, determined on 2.0 g.

Hydroxyl value (2.5.3, Method A). See Table 1234.-1.

Iodine value (2.5.4): maximum 2.0.

Saponification value (2.5.6). See Table 1234.-1.

Table 1234.-1

Ethylene oxide units per molecule (nominal value)	Melting point (°C)	Hydroxyl value	Saponification value
6		90 - 110	85 - 105
8 - 9	26 - 35	80 - 105	88 - 100
20	33 - 40	50 - 62	46 - 56
40 - 50	38 - 52	23 - 40	20 - 35
100	48 - 60	15 - 30	5 - 20

Reducing substances. Dissolve or disperse 2.0 g in *water R* and dilute to 20 ml with the same solvent. Mix 1.0 ml of the solution with 9 ml of 0.1 M *sodium hydroxide* and 0.5 ml of *triphenyltetrazolium chloride solution R*. Heat in a water-bath at 70 °C. After 5 min, the solution is not more intensely coloured than a mixture of 0.15 ml of yellow primary solution, 0.9 ml of red primary solution and 8.95 ml of a 10 g/l solution of *hydrochloric acid R* (2.2.2, Method II).

Composition of the fatty acid fraction of the substance:

	Type of fatty acid used	Composition of fatty acids
Macrogol stearate type I	Stearic acid 50	<i>Stearic acid</i> : 40.0 per cent to 60.0 per cent, <i>Sum of the contents of palmitic and stearic acids</i> : not less than 90.0 per cent.
Macrogol stearate type II	Stearic acid 95	<i>Stearic acid</i> : 90.0 per cent to 99.0 per cent, <i>Sum of the contents of palmitic and stearic acids</i> : not less than 96.0 per cent.

Ethylene oxide and dioxan (2.4.25): maximum 1 ppm of ethylene oxide and 10 ppm of dioxan.

Heavy metals (2.4.8): maximum 10 ppm.

2.0 g complies with limit test C. Prepare the standard using 2 ml of *lead standard solution* (10 ppm Pb) R.

Water (2.5.12): maximum 3.0 per cent, determined on 0.50 g. Use as the solvent a mixture of equal volumes of *anhydrous methanol R* and *methylene chloride R*.

Total ash (2.4.16): maximum 0.3 per cent, determined on 1.0 g.

STORAGE

In an airtight container.

LABELLING

The label states:

- the number of ethylene oxide units per molecule (nominal value),
- the type of macrogol stearate.

01/2005:1340

MACROGOL STEARYL ETHER

Macrogoli aether stearylicus

DEFINITION

Macrogol stearyl ether is a mixture of ethers obtained by ethoxylation of stearyl alcohol. It may contain some free macrogols and various amounts of free stearyl alcohol. The amount of ethylene oxide reacted with stearyl alcohol is 2 to 20 units per molecule (nominal value).

CHARACTERS

A white to yellowish-white, waxy, unctuous mass, pellets, microbeads or flakes.

Macrogol stearyl ether with 2 units of ethylene oxide per molecule is practically insoluble in water, soluble in alcohol with heating and in methylene chloride. Macrogol stearyl ether with 10 units of ethylene oxide per molecule is soluble in water and in alcohol. Macrogol stearyl ether with 20 units of ethylene oxide per molecule is soluble in water, in alcohol and in methylene chloride.

After melting, it solidifies at about 45 °C.

IDENTIFICATION

- A. It complies with the test for hydroxyl value (see Tests).
- B. It complies with the test for iodine value (see Tests).
- C. It complies with the test for saponification value (see Tests).

01/2005:1122

D. Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel plate R*.

Test solution. Dissolve 10.0 g in a mixture of 1 volume of *water R* and 9 volumes of *methanol R* and dilute to 75 ml with the same mixture of solvents. Add 60 ml of *heptane R* and shake for 3 min. The formation of foam can be reduced by the addition of a few drops of *alcohol R*. Filter the upper layer through *anhydrous sodium sulphate R*, wash the filter with 3 quantities, each of 10 ml, of *heptane R* and evaporate the combined filtrates to dryness. Dissolve 50 mg of the residue in 10 ml of *methanol R* (the solution may be opalescent).

Reference solution. Dissolve 25 mg of *stearyl alcohol CRS* in *methanol R* and dilute to 25 ml with the same solvent.

Apply to the plate 20 µl of each solution. Develop over a path of 15 cm using *ethyl acetate R*. Dry and spray with vanillin-sulphuric acid reagent prepared as follows: dissolve 0.5 g of *vanillin R* in 50 ml of *alcohol R* and dilute to 100 ml with *sulphuric acid R*. Allow the plate to dry in air. Heat the plate at about 130 °C for 15 min and allow to cool in air. The chromatogram obtained in the test solution shows several spots; one of these spots corresponds to the principal spot in the chromatogram obtained with the reference solution.

E. Dissolve or disperse 0.1 g in 5 ml of *alcohol R*, add 2 ml of *water R*, 10 ml of *dilute hydrochloric acid R*, 10 ml of *barium chloride solution R1* and 10 ml of a 100 g/l solution of *phosphomolybdic acid R*. A precipitate is formed.

TESTS

Appearance of solution. Dissolve 5.0 g in *alcohol R* and dilute to 50 ml with the same solvent. The solution is not more intensely coloured than reference solution BY₅ (2.2.2, *Method II*).

Alkalinity. Dissolve 2.0 g in a hot mixture of 10 ml of *alcohol R* and 10 ml of *water R*. Add 0.1 ml of *bromothymol blue solution R1*. Not more than 0.5 ml of 0.1 M *hydrochloric acid* is required to change the colour of the indicator to yellow.

Acid value (2.5.1). Not more than 1.0, determined on 5.0 g.

Hydroxyl value (2.5.3, *Method A*). See Table 1340.-1.

Table 1340.-1

Ethylene oxide units per molecule (nominal value)	Hydroxyl value
2	150 to 180
10	75 to 90
20	40 to 60

Iodine value (2.5.4). Not more than 2.0.

Saponification value (2.5.6). Not more than 3.0, determined on 10.0 g.

Ethylene oxide and dioxan (2.4.25). Not more than 1 ppm of ethylene oxide and not more than 10 ppm of dioxan.

Water (2.5.12). Not more than 3.0 per cent, determined on 1.00 g by the semi-micro determination of water.

STORAGE

Store in an airtight container.

LABELLING

The label states the number of ethylene oxide units reacted with stearyl alcohol (nominal value).

MACROGOLGLYCEROL COCOATES

Macroglyceroli cocoates

DEFINITION

Macrogol glycerol cocoates are mixtures of mono-, di- and triesters of ethoxylated glycerol with fatty acids of vegetable origin having a composition corresponding to the fatty acid composition of the oil extracted from the hard, dried fraction of the endosperm of *Cocos nucifera* L. The average content of ethylene oxide is either 7 units per molecule or 23 units per molecule (nominal value).

CHARACTERS

A clear, yellowish, oily liquid, soluble in water and in alcohol and practically insoluble in light petroleum (50 °C to 70 °C) for macrogol 7 glycerol cocoate; soluble in water and in alcohol and practically insoluble in light petroleum (50 °C to 70 °C) for macrogol 23 glycerol cocoate. Macrogol 7 glycerol cocoate has a relative density of about 1.05 and macrogol 23 glycerol cocoate has a relative density of about 1.09.

IDENTIFICATION

A. Dissolve 1.0 g of macrogol 7 glycerol cocoate in 99 g of a mixture of 10 volumes of *2-propanol R* and 90 volumes of *water R*. Heat the solution to about 65 °C. A turbidity is produced. Allow to cool until the turbidity disappears. The cloud point is between 35 °C and 54 °C.

Heat a 10 g/l solution of macrogol 23 glycerol cocoate in a 100 g/l solution of *sodium chloride R* to about 90 °C. A turbidity is produced. Allow to cool until the turbidity disappears. The cloud point is between 65 °C and 85 °C.

B. They comply with the test for iodine value (see Tests).

C. They comply with the test for saponification value (see Tests).

TESTS

Appearance. The substance to be examined is clear (2.2.1) and not more intensely coloured than reference solution Y₂ (2.2.2, *Method I*).

Alkalinity. Dissolve 2.0 g in a hot mixture of 10 ml of *water R* and 10 ml of *alcohol R*. Add 0.1 ml of *bromothymol blue solution R1*. Not more than 0.5 ml of 0.1 M *hydrochloric acid* is required to change the colour of the indicator to yellow.

Acid value (2.5.1). Not more than 5.0, determined on 5.0 g.

Hydroxyl value (2.5.3, *Method A*). See Table 1122.-1.

Saponification value (2.5.6). See Table 1122.-1.

Table 1122.-1

Number of units of ethylene oxide (nominal value)	Hydroxyl value	Saponification value (determined on 2.0 g)
7	170 to 210	85 to 105
23	80 to 100	40 to 50

Iodine value (2.5.4). Not more than 5.0.

Composition of fatty acids (2.4.22, *Method A*). The fatty acid fraction has the following composition:

- *caproic acid*: not more than 1.0 per cent,
- *caprylic acid*: 5.0 per cent to 10.0 per cent,
- *capric acid*: 4.0 per cent to 10.0 per cent,
- *lauric acid*: 40.0 per cent to 55.0 per cent,
- *myristic acid*: 14.0 per cent to 23.0 per cent,

- *palmitic acid*: 8.0 per cent to 12.0 per cent,
- *stearic acid*: 1.0 per cent to 5.0 per cent,
- *oleic acid*: 5.0 per cent to 10.0 per cent,
- *linoleic acid*: not more than 3.0 per cent.

Ethylene oxide and dioxan (2.4.25). Not more than 1 ppm of ethylene oxide and not more than 10 ppm of dioxan.

Water (2.5.12). Not more than 1.0 per cent, determined on 1.0 g by the semi-micro determination of water.

Total ash (2.4.16). Not more than 0.3 per cent, determined on 1.0 g.

LABELLING

The label states the number of ethylene oxide units per molecule (nominal value).

01/2005:1083

MACROGOLGLYCEROL HYDROXYSTEARATE

Macroglglyceroli hydroxystearas

DEFINITION

Contains mainly trihydroxystearyl glycerol ethoxylated with 7 to 60 molecules of ethylene oxide (nominal value), with small amounts of macrogol hydroxystearate and of the corresponding free glycols. It results from the reaction of hydrogenated castor oil with ethylene oxide.

CHARACTERS

Appearance: if less than 10 units of ethylene oxide per molecule: yellowish, turbid, viscous liquid; if more than 20 units of ethylene oxide per molecule: white or yellowish semi-liquid or pasty mass.

Solubility: if less than 10 units of ethylene oxide per molecule: practically insoluble in water, soluble in acetone, dispersible in alcohol; if more than 20 units of ethylene oxide per molecule: freely soluble in water, in acetone and in alcohol, practically insoluble in light petroleum.

IDENTIFICATION

- It complies with the test for iodine value (see Tests).
- It complies with the test for saponification value (see Tests).

- Thin-layer chromatography (2.2.27).

Test solution. To 1 g of the substance to be examined, add 100 ml of a 100 g/l solution of *potassium hydroxide R* and boil under a reflux condenser for 30 min. Allow to cool. Acidify the solution with 20 ml of *hydrochloric acid R*. Shake the mixture with 50 ml of *ether R* and allow to stand until separation of the layers is obtained. Transfer the clear upper layer to a suitable tube, add 5 g of *anhydrous sodium sulphate R*, close the tube and allow to stand for 30 min. Filter and evaporate the filtrate to dryness on a water-bath. Dissolve 50 mg of the residue in 25 ml of *ether R*.

Reference solution. Dissolve 50 mg of *12-hydroxystearic acid R* in *methylene chloride R* and dilute to 25 ml with the same solvent.

Plate: TLC octadecylsilyl silica gel plate *R*.

Mobile phase: *methylene chloride R*, *glacial acetic acid R*, *acetone R* (10:40:50 V/V/V).

Application: 2 µl.

Development: over a path of 8 cm.

Drying: in a current of cold air.

Detection: spray with a 80 g/l solution of *phosphomolybdic acid R* in *2-propanol R* and heat at 120 °C for about 1-2 min.

Results: the principal spot in the chromatogram obtained with the test solution is similar in position and colour to the principal spot in the chromatogram obtained with the reference solution.

- Place about 2 g in a test-tube and add 0.2 ml of *sulphuric acid R*. Close the tube using a stopper fitted with a glass tube bent twice at right angles. Heat the tube until white fumes appear. Collect the fumes in 1 ml of *mercuric chloride solution R*. A white precipitate is formed and the fumes turn a filter paper impregnated with *alkaline potassium tetraiodomercurate solution R* black.

TESTS

Solution S. Dissolve 5.0 g of macroglglycerol hydroxystearate with less than 10 units of ethylene oxide per molecule in a mixture of 50 volumes of *acetone R* and 50 volumes of *ethanol R* and dilute to 50 ml with the same mixture of solvents.

Dissolve 5.0 g of macroglglycerol hydroxystearate with more than 20 units of ethylene oxide per molecule in *carbon dioxide-free water R* and dilute to 50 ml with the same solvent.

Appearance of solution. Solution S is not more opalescent than reference suspension III (2.2.1) and not more intensely coloured than reference solution BY₆ (2.2.2, *Method II*).

Alkalinity. To 2 ml of solution S add 0.5 ml of *bromothymol blue solution R1*. The solution is not blue.

Acid value (2.5.1): maximum 2.0, determined on 5.0 g.

Hydroxyl value (2.5.3, *Method A*). See Table 1083.-1.

Iodine value (2.5.4): maximum 5.0.

Saponification value (2.5.6). See Table 1083.-1.

Table 1083.-1

Ethylene oxide units per molecule (nominal value)	Hydroxyl value	Saponification value
7	115 - 135	125 - 140
25	70 - 90	70 - 90
40	60 - 80	45 - 69
60	45 - 67	40 - 51

Residual ethylene oxide and dioxan (2.4.25): maximum 1 ppm of residual ethylene oxide and 10 ppm of residual dioxan.

Heavy metals (2.4.8).

Substances soluble in acetone/ethanol: maximum 10 ppm. 12 ml of solution S complies with limit test B. Prepare the standard using lead standard solution (1 ppm Pb) obtained by diluting *lead standard solution (100 ppm Pb) R* with a mixture of equal volumes of *acetone R* and *ethanol R*.

Substances soluble in water: maximum 10 ppm.

12 ml of solution S complies with limit test A. Prepare the standard using *lead standard solution (1 ppm Pb) R*.

Water (2.5.12): maximum 3.0 per cent, determined on 2.000 g.

Total ash (2.4.16): maximum 0.3 per cent, determined on 2.0 g.

LABELLING

The label states the number of ethylene oxide units per molecule (nominal value).

01/2005:1082 TESTS

MACROGOLGLYCEROL RICINOLEATE

Macrogolglyceroli ricinoleas

DEFINITION

Contains mainly ricinoleyl glycerol ethoxylated with 30 to 50 molecules of ethylene oxide (nominal value), with small amounts of macrogol ricinoleate and of the corresponding free glycols. It results from the reaction of castor oil with ethylene oxide.

CHARACTERS

Appearance: clear, yellow viscous liquid or semi-solid.

Solubility: freely soluble in water, very soluble in methylene chloride, freely soluble in alcohol.

Relative density: about 1.05.

Viscosity: 500 mPas to 800 mPas at 25 °C.

IDENTIFICATION

- A. It complies with the test for iodine value (see Tests).
- B. It complies with the test for saponification value (see Tests).
- C. Thin-layer chromatography (2.2.27).

Test solution. To 1 g of the substance to be examined add 100 ml of a 100 g/l solution of *potassium hydroxide R* and boil under a reflux condenser for 30 min. Allow to cool. Acidify the solution with 20 ml of *hydrochloric acid R*. Shake the mixture with 50 ml of *ether R* and allow to stand until separation of the layers is obtained. Transfer the clear upper layer to a suitable tube, add 5 g of *anhydrous sodium sulphate R*, close the tube and allow to stand for 30 min. Filter and evaporate the filtrate to dryness on a water-bath. Dissolve 50 mg of the residue in 25 ml of *ether R*.

Reference solution. Dissolve 50 mg of *ricinoleic acid R* in *methylene chloride R* and dilute to 25 ml with the same solvent.

Plate: TLC octadecylsilyl silica gel plate *R*.

Mobile phase: *methylene chloride R*, *glacial acetic acid R*, *acetone R* (10:40:50 V/V/V).

Application: 2 µl.

Development: over a path of 8 cm.

Drying: in a current of cold air.

Detection: spray with an 80 g/l solution of *phosphomolybdic acid R* in *2-propanol R* and heat at 120 °C for 1-2 min.

Results: the principal spot in the chromatogram obtained with the test solution is similar in position and colour to the principal spot in the chromatogram obtained with the reference solution.

- D. Place about 2 g of the substance to be examined in a test-tube and add 0.2 ml of *sulphuric acid R*. Close the tube using a stopper fitted with a glass tube bent twice at right angles. Heat the tube until white fumes appear. Collect the fumes in 1 ml of *mercuric chloride solution R*. A white precipitate is formed and the fumes turn a filter paper impregnated with *alkaline potassium tetraiodomercurate solution R* black.

Solution S. Dissolve 5.0 g in *carbon dioxide-free water R* and dilute to 50 ml with the same solvent.

Appearance of solution. Solution S is not more opalescent than reference suspension III (2.2.1) and not more intensely coloured than reference solution BY₆ (2.2.2, *Method II*).

Alkalinity. Dissolve 2.0 g in a hot mixture of 10 ml of *water R* and 10 ml of *alcohol R*. Add 0.1 ml of *bromothymol blue solution R1*. Not more than 0.5 ml of 0.1 M *hydrochloric acid* is required to change the colour of the indicator to yellow.

Acid value (2.5.1): maximum 2.0, determined on 5.0 g.

Hydroxyl value (2.5.3, *Method A*). See Table 1082.-1.

Iodine value (2.5.4): 25 to 35.

Saponification value (2.5.6). See Table 1082.-1.

Table 1082.-1

Ethylene oxide units per molecule (nominal value)	Hydroxyl value	Saponification value
30 - 35	65 - 82	60 - 75
50	48 - 68	38 - 52

Residual ethylene oxide and dioxan (2.4.25): maximum 1 ppm of residual ethylene oxide and 10 ppm of residual dioxan.

Heavy metals (2.4.8): maximum 10 ppm.

12 ml of solution S, filtered if necessary, complies with limit test A. Prepare the standard using *lead standard solution (1 ppm Pb) R*.

Water (2.5.12): maximum 3.0 per cent, determined on 2.000 g.

Total ash (2.4.16): maximum 0.3 per cent, determined on 2.0 g.

STORAGE

Store protected from light.

LABELLING

The label states the amount of ethylene oxide reacted with castor oil (nominal value).

01/2005:1444

MACROGOLS

Macrogola

DEFINITION

Mixtures of polymers with the general formula $H(OCH_2CH_2)_nOH$ where n represents the average number of oxyethylene groups. The type of macrogol is defined by a number that indicates the average relative molecular mass. A suitable stabiliser may be added.

CHARACTERS

Type of macrogol	Appearance	Solubility
300 400 600	clear, viscous, colourless or almost colourless hygroscopic liquid	miscible with water, very soluble in acetone, in alcohol, and in methylene chloride, practically insoluble in fatty oils and in mineral oils
1000	white or almost white, hygroscopic solid with a waxy or paraffin-like appearance	very soluble in water, freely soluble in alcohol and in methylene chloride, practically insoluble in fatty oils and in mineral oils
1500	white or almost white solid with a waxy or paraffin-like appearance	very soluble in water and in methylene chloride, freely soluble in alcohol, practically insoluble in fatty oils and in mineral oils
3000 3350	white or almost white solid with a waxy or paraffin-like appearance	very soluble in water and in methylene chloride, very slightly soluble in alcohol, practically insoluble in fatty oils and in mineral oils
4000 6000 8000	white or almost white solid with a waxy or paraffin-like appearance	very soluble in water and in methylene chloride, practically insoluble in alcohol and in fatty oils and in mineral oils
20 000 35 000	white or almost white solid with a waxy or paraffin-like appearance	very soluble in water, soluble in methylene chloride, practically insoluble in alcohol and in fatty oils and in mineral oils

IDENTIFICATION

- A. It complies with the test for viscosity (see Tests).
- B. To 1 g in a test-tube add 0.5 ml of *sulphuric acid R*, close the test-tube with a stopper fitted with a bent delivery tube and heat until white fumes are evolved. Collect the fumes via the delivery tube into 1 ml of *mercuric chloride solution R*. An abundant white, crystalline precipitate is formed.
- C. To 0.1 g add 0.1 g of *potassium thiocyanate R* and 0.1 g of *cobalt nitrate R* and mix thoroughly with a glass rod. Add 5 ml of *methylene chloride R* and shake. The liquid phase becomes blue.

TESTS

Appearance of solution. The solution is clear (2.2.1) and not more intensely coloured than reference solution BY₆ (2.2.2, Method II).

Dissolve 12.5 g in *water R* and dilute to 50 ml with the same solvent.

Acidity or alkalinity. Dissolve 5.0 g in 50 ml of *carbon dioxide-free water R* and add 0.15 ml of *bromothymol blue solution R1*. The solution is yellow or green. Not more than 0.1 ml of 0.1 M *sodium hydroxide* is required to change the colour of the indicator to blue.

Viscosity (2.2.9). The viscosity is calculated using a density given in Table 1444-1.

Table 1444-1

Type of macrogol	Kinematic viscosity (mm ² ·s ⁻¹)	Dynamic viscosity (mPa·s)	Density* (g/ml)
300	71 - 94	80 - 105	1.120
400	94 - 116	105 - 130	1.120
600	13.9 - 18.5	15 - 20	1.080
1000	20.4 - 27.7	22 - 30	1.080
1500	31 - 46	34 - 50	1.080
3000	69 - 93	75 - 100	1.080
3350	76 - 110	83 - 120	1.080
4000	102 - 158	110 - 170	1.080
6000	185 - 250	200 - 270	1.080
8000	240 - 472	260 - 510	1.080
20 000	2500 - 3200	2700 - 3500	1.080
35 000	10 000 - 13 000	11 000 - 14 000	1.080

*Density of the substance for macrogols 300 and 400. Density of the 50 per cent *m/m* solution for the other macrogols.

For macrogols having a relative molecular mass greater than 400, determine the viscosity on a 50 per cent *m/m* solution of the substance to be examined.

Freezing point (2.2.18). See Table 1444-2.

Table 1444-2

Type of macrogol	Freezing point (°C)
600	15 - 25
1000	35 - 40
1500	42 - 48
3000	50 - 56
3350	53 - 57
4000	53 - 59
6000	55 - 61
8000	55 - 62
20 000	minimum 57
35 000	minimum 57

Hydroxyl value. Introduce m g (see Table 1444-3) into a dry conical flask fitted with a reflux condenser. Add 25.0 ml of *phthalic anhydride solution R*, swirl to dissolve and boil under a reflux condenser on a hot plate for 60 min. Allow to cool. Rinse the condenser first with 25 ml of *pyridine R* and then with 25 ml of *water R*, add 1.5 ml of *phenolphthalein solution R* and titrate with 1 M *sodium hydroxide* until a faint pink colour is obtained (n_1 ml). Carry out a blank test (n_2 ml). Calculate the hydroxyl value using the expression:

$$\frac{56.1 \times (n_2 - n_1)}{m}$$

Table 1444.3

Type of macrogol	Hydroxyl value	m (g)
300	340 - 394	1.5
400	264 - 300	1.9
600	178 - 197	3.5
1000	107 - 118	5.0
1500	70 - 80	7.0
3000	34 - 42	12.0
3350	30 - 38	12.0
4000	25 - 32	14.0
6000	16 - 22	18.0
8000	12 - 16	24.0
20 000	-	-
35 000	-	-

For macrogols having a relative molecular mass greater than 1000, if the water content is more than 0.5 per cent, dry a sample of suitable mass at 100-105 °C for 2 h and carry out the determination of the hydroxyl value on the dried sample.

Reducing substances. Dissolve 1 g in 1 ml of a 10 g/l solution of *resorcinol R* and warm gently if necessary. Add 2 ml of *hydrochloric acid R*. After 5 min the solution is not more intensely coloured than reference solution R₃ (2.2.2, *Method I*).

Formaldehyde: maximum 30 ppm.

Test solution. To 1.00 g add 0.25 ml of *chromotropic acid, sodium salt solution R*, cool in iced water and add 5.0 ml of *sulphuric acid R*. Allow to stand for 15 min and complete slowly to 10 ml with *water R*.

Reference solution. Dilute 0.860 g of *formaldehyde solution R* to 100 ml with *water R*. Dilute 1.0 ml of this solution to 100 ml with *water R*. In a 10 ml flask, mix 1.00 ml of this solution with 0.25 ml of *chromotropic acid, sodium salt solution R*, cool in iced water and add 5.0 ml of *sulphuric acid R*. Allow to stand for 15 min and complete slowly to 10 ml with *water R*.

Blank solution. In a 10 ml flask mix 1.00 ml of *water R* with 0.25 ml of *chromotropic acid, sodium salt solution R*, cool in iced water and add 5.0 ml of *sulphuric acid R*. Complete slowly to 10 ml with *water R*.

Determine the absorbance (2.2.25) of the test solution at 567 nm, against the blank solution. It is not higher than that of the reference solution.

If the use of macrogols with a higher content of formaldehyde may have adverse effects, the competent authority may impose a limit of not more than 15 ppm.

Ethylene glycol and diethylene glycol: carry out this test only if the macrogol has a relative molecular mass below 1000.

Gas chromatography (2.2.28).

Test solution. Dissolve 5.00 g of the substance to be examined in *acetone R* and dilute to 100.0 ml with the same solvent.

Reference solution. Dissolve 0.10 g of *ethylene glycol R* and 0.50 g of *diethylene glycol R* in *acetone R* and dilute to 100.0 ml with the same solvent. Dilute 1.0 ml of this solution to 10.0 ml with *acetone R*.

Column:

- **material:** glass,
- **size:** $l = 1.8 \text{ m}$, $\varnothing = 2 \text{ mm}$,

- **stationary phase:** silanised diatomaceous earth for gas chromatography *R*, impregnated with 5 per cent *m/m* of macrogol 20 000 *R*,

Carrier gas: nitrogen for chromatography *R*.

Flow rate: 30 ml/min.

Temperature:

- **column:** if necessary, precondition the column by heating at 200 °C for about 15 h; adjust the initial temperature of the column to obtain a retention time of 14-16 min for diethylene glycol; raise the temperature of the column by about 30 °C at a rate of 2 °C/min but without exceeding 170 °C;

- **injection port and detector:** 250 °C.

Detection: flame ionisation.

Injection: 2 µl.

Carry out 5 replicate injections to check the repeatability of the response.

Limit: maximum 0.4 per cent, calculated as the sum of the contents of ethylene glycol and diethylene glycol.

Ethylene oxide and dioxan (2.4.25): maximum 1 ppm of ethylene oxide and 10 ppm of dioxan.

Heavy metals (2.4.8): maximum 20 ppm.

Dissolve 2.0 g in *water R* and dilute to 20 ml with the same solvent. 12 ml of the solution complies with limit test A. Prepare the standard using *lead standard solution (2 ppm Pb) R*.

Water (2.5.12): maximum 2.0 per cent for macrogol with a relative molecular mass not greater than 1000 and maximum 1.0 per cent for macrogol with a relative molecular mass greater than 1000, determined on 2.00 g.

Sulphated ash (2.4.14): maximum 0.2 per cent, determined on 1.0 g.

STORAGE

In an airtight container.

LABELLING

The label states:

- the type of macrogol,
- the name and the concentration of any added stabiliser,
- the content of formaldehyde.

01/2005:1539
corrected

MAGALDRATE

Magaldratum

$\text{Al}_5\text{Mg}_{10}(\text{OH})_{31}(\text{SO}_4)_2 \cdot x\text{H}_2\text{O}$ M_r 1097 (anhydrous substance)

DEFINITION

Magaldrate is composed of aluminium and magnesium hydroxides and sulphates. Its composition corresponds approximately to the formula $\text{Al}_5\text{Mg}_{10}(\text{OH})_{31}(\text{SO}_4)_2 \cdot x\text{H}_2\text{O}$. It contains not less than 90.0 per cent and not more than the equivalent of 105.0 per cent of $\text{Al}_5\text{Mg}_{10}(\text{OH})_{31}(\text{SO}_4)_2$, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, crystalline powder, practically insoluble in water and in alcohol, soluble in dilute mineral acids.

IDENTIFICATION

- A. Dissolve 0.6 g in 20 ml of 3 *M* hydrochloric acid, add about 30 ml of water *R* and heat to boiling. Adjust to pH 6.2 with dilute ammonia *R1*, continue boiling for a further 2 min, filter and retain the precipitate and the filtrate. To 2 ml of the filtrate add 2 ml of ammonium chloride solution *R* and neutralise with a solution prepared by dissolving 2 g of ammonium carbonate *R* and 2 ml of dilute ammonia *R1* in 20 ml of water *R*; no precipitate is produced. Add disodium hydrogen phosphate solution *R*; a white, crystalline precipitate is produced which does not dissolve in dilute ammonia *R1*.
- B. The precipitate retained in identification test A gives the reaction of aluminium (2.3.1).
- C. The filtrate retained in identification test A gives reaction (a) of sulphates (2.3.1).

TESTS

Soluble chlorides. Disperse 1 g in 50 ml of water *R*, boil for 5 min, cool, dilute again to 50.0 ml, mix and filter. To 25.0 ml of the filtrate add 0.2 ml of potassium chromate solution *R* and titrate with 0.1 *M* silver nitrate until a persistent violet-red colour is obtained. Not more than 5.0 ml of 0.1 *M* silver nitrate is required (3.5 per cent).

Soluble sulphates. To 2.5 ml of the filtrate obtained in the test for soluble chlorides, add 30 ml of water *R*, neutralise to blue litmus paper *R* with hydrochloric acid *R*, add 3 ml of 1 *M* hydrochloric acid, 3 ml of a 120 g/l solution of barium chloride *R* and dilute to 50 ml with water *R*. Mix and allow to stand for 10 min. Any opalescence in the test solution is not more intense than that of a standard prepared at the same time in the same manner using 1 ml of 0.01 *M* sulphuric acid instead of 2.5 ml of filtrate (1.9 per cent).

Sulphates. 16.0 per cent to 21.0 per cent, calculated with reference to the dried substance. Dissolve 0.875 g in a mixture of 5 ml of glacial acetic acid *R* and 10 ml of water *R* and dilute to 25 ml with water *R*. Prepare a chromatographic column of 1 cm in internal diameter containing 15 ml of cation exchange resin *R* (150 µm to 300 µm), previously washed with 30 ml of water *R*. Transfer 5.0 ml of the solution to be examined to the column and elute with 15 ml of water *R*. To the eluate add 5 ml of a 53.6 g/l solution of magnesium acetate *R*, 32 ml of methanol *R* and 0.2 ml of alizarin *S* solution *R*. Add from a burette about 4.0 ml of 0.05 *M* barium chloride, add a further 0.2 ml of alizarin *S* solution *R* and slowly complete the titration until the yellow colour disappears and a violet-red tinge is visible.

1 ml of 0.05 *M* barium chloride is equivalent to 4.803 mg of SO₄.

Aluminium hydroxide: 32.1 per cent to 45.9 per cent, calculated with reference to the dried substance. Dissolve 0.800 g in 10 ml of dilute hydrochloric acid *R1*, heating on a water-bath. Cool and dilute to 50.0 ml with water *R*. To 10.0 ml of the solution, add dilute ammonia *R1* until a precipitate begins to appear. Add the smallest quantity of dilute hydrochloric acid *R* needed to dissolve the precipitate and dilute to 20 ml with water *R*. Carry out the complexometric titration of aluminium (2.5.11).

1 ml of 0.1 *M* sodium edetate is equivalent to 7.80 mg of Al(OH)₃.

Magnesium hydroxide: 49.2 per cent to 66.6 per cent, calculated with reference to the dried substance. Dissolve 0.100 g in 2 ml of dilute hydrochloric acid *R* and carry out the complexometric titration of magnesium (2.5.11).

1 ml of 0.1 *M* sodium edetate is equivalent to 5.832 mg of Mg(OH)₂.

Sodium. Not more than 0.10 per cent of Na, determined by atomic absorption spectrometry (2.2.23, Method I).

Test solution. Weigh 2.00 g of the substance to be examined into a 100 ml volumetric flask, place in an ice-bath, add 5 ml of nitric acid *R* and swirl to mix. Allow to warm to room temperature and dilute to 100 ml with water *R*. Filter, if necessary, to obtain a clear solution. Dilute 10.0 ml of the filtrate to 100.0 ml with water *R*.

Reference solutions. Prepare the reference solutions using sodium standard solution (200 ppm Na) *R*, diluted as necessary with dilute nitric acid *R*.

Measure the absorbance at 589 nm using a sodium hollow-cathode lamp as a source of radiation and an air-acetylene flame.

Heavy metals (2.4.8). Dissolve 2.0 g in 30 ml of hydrochloric acid *R1* and shake with 50 ml of methyl isobutyl ketone *R* for 2 min. Allow to stand, separate the aqueous layer and evaporate to dryness. Dissolve the residue in 30 ml of water *R*. 12 ml of the solution complies with limit test A for heavy metals (30 ppm). Prepare the standard using lead standard solution (2 ppm Pb) *R*.

Loss on drying (2.2.32): 10.0 per cent to 20.0 per cent, determined on 1.000 g by drying in an oven at 200 °C for 4 h.

ASSAY

To 1.500 g add 50.0 ml of 1 *M* hydrochloric acid. Titrate the excess hydrochloric acid with 1 *M* sodium hydroxide to pH 3.0, determining the end-point potentiometrically (2.2.20). Carry out a blank titration.

1 ml of 1 *M* hydrochloric acid is equivalent to 35.40 mg of Al₅Mg₁₀(OH)₃₁(SO₄)₂.

01/2005:2035

MAGNESIUM ACETATE
TETRAHYDRATE

Magnesii acetat tetrahydricus

Mg(CH₃COO)₂·4H₂OM_r 214.5

DEFINITION

Content: 98.0 per cent to 101.0 per cent of magnesium acetate (anhydrous substance).

CHARACTERS

Appearance: colourless crystals or white, crystalline powder.

Solubility: freely soluble in water and in alcohol.

IDENTIFICATION

- A. Dissolve about 100 mg in 2 ml of water *R*. Add 1 ml of dilute ammonia *R1* and heat. A white precipitate is formed that dissolves slowly on addition of 5 ml of ammonium chloride solution *R*. Add 1 ml of disodium hydrogen phosphate solution *R*. A white crystalline precipitate is formed.
- B. It gives reaction (b) of acetates (2.3.1).

TESTS

pH (2.2.3): 7.5 to 8.5.

Dissolve 2.5 g in carbon dioxide-free water *R* and dilute to 50 ml with the same solvent.

Chlorides (2.4.4): maximum 330 ppm.

Dissolve 1.0 g in water *R* and dilute to 100 ml with the same solvent. 15 ml of the solution complies with the limit test for chlorides.

Nitrates: maximum 3 ppm.

Dissolve 1.0 g in *distilled water R* and dilute to 10 ml with the same solvent, add 5 mg of *sodium chloride R*, 0.05 ml of *indigo carmine solution R* and while stirring, 10 ml of *nitrogen-free sulphuric acid R*. A blue colour is produced which persists for at least 10 min.

Sulphates (2.4.13): maximum 600 ppm.

Dissolve 0.25 g in *distilled water R* and dilute to 15 ml with the same solvent.

Aluminium (2.4.17): maximum 1 ppm.

Prescribed solution. Dissolve 4.0 g in *water R* and dilute to 100 ml with the same solvent. Add 10 ml of *acetate buffer solution pH 6.0 R*.

Reference solution. Mix 2 ml of *aluminium standard solution (2 ppm Al) R*, 10 ml of *acetate buffer solution pH 6.0 R* and 98 ml of *water R*.

Blank solution. Mix 10 ml of *acetate buffer solution pH 6.0 R* and 100 ml of *water R*.

Calcium (2.4.3): maximum 100 ppm.

Dissolve 1.0 g in *distilled water R* and dilute to 15 ml with the same solvent.

Potassium: maximum 0.1 per cent.

Atomic emission spectrometry (2.2.22, *Method II*).

Test solution. Dissolve 0.5 g in *water R* and dilute to 100 ml with the same solvent.

Reference solutions. Prepare the reference solutions using *potassium standard solution (600 ppm K) R*, diluted as necessary with *water R*.

Wavelength: 766.5 nm.

Sodium: maximum 0.5 per cent.

Atomic emission spectrometry (2.2.22, *Method II*).

Test solution. Dissolve 1.0 g in *water R* and dilute to 100 ml with the same solvent.

Reference solutions. Prepare the reference solutions using *sodium standard solution (200 ppm Na) R*, diluted as necessary with *water R*.

Wavelength: 589.0 nm.

Heavy metals (2.4.8): maximum 40 ppm.

Dissolve 1.0 g in *water R* and dilute to 20 ml with the same solvent. 12 ml of the solution complies with limit test A. Prepare the standard using *lead standard solution (2 ppm Pb) R*.

Readily oxidisable substances. Dissolve 2.0 g in 100 ml of boiling *water R*, add 6 ml of a 150 g/l solution of *sulphuric acid R* and 0.3 ml of 0.02 M *potassium permanganate*. Mix and boil gently for 5 min. The pink colour is not completely discharged.

Water (2.5.12): 33.0 per cent to 35.0 per cent, determined on 0.100 g.

ASSAY

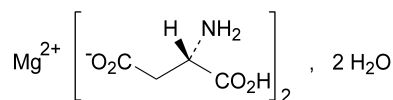
Dissolve 0.150 g in 300 ml of *water R*. Carry out the complexometric titration of magnesium (2.5.11).

1 ml of 0.1 M *sodium edetate* is equivalent to 14.24 mg of $C_8H_{12}MgN_2O_8 \cdot 2H_2O$.

01/2005:1445

MAGNESIUM ASPARTATE DIHYDRATE

Magnesii aspartas dihydricus


 $C_8H_{12}MgN_2O_8 \cdot 2H_2O$
 M_r 324.5

DEFINITION

Magnesium aspartate dihydrate contains not less than 98.0 per cent and not more than the equivalent of 102.0 per cent of magnesium di[(S)-2-aminohydrogenobutane-1,4-dioate], calculated with reference to the anhydrous substance.

CHARACTERS

A white, crystalline powder or colourless crystals, freely soluble in water.

IDENTIFICATION

- It complies with the test for specific optical rotation (see Tests).
- Examine the chromatograms obtained in the test for ninhydrin-positive substances. The principal spot in the chromatogram obtained with test solution (b) is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).
- Ignite about 15 mg until a white residue is obtained. Dissolve the residue in 1 ml of *dilute hydrochloric acid R*, neutralise to *red litmus paper R* by the addition of *dilute sodium hydroxide solution R* and filter if necessary. The solution gives the reaction of magnesium (2.3.1).

TESTS

Solution S. Dissolve 2.5 g in *carbon dioxide-free water R* prepared from *distilled water R* and dilute to 100 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and colourless (2.2.2, *Method II*).

pH (2.2.3). The pH of solution S is 6.0 to 8.0.

Specific optical rotation (2.2.7). Dissolve 0.50 g in a 515 g/l solution of *hydrochloric acid R* and dilute to 25.0 ml with the same acid. The specific optical rotation is + 20.5 to + 23.0, calculated with reference to the anhydrous substance.

Ninhydrin-positive substances. Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel plate R*.

Test solution (a). Dissolve 0.10 g of the substance to be examined in *water R* and dilute to 10 ml with the same solvent.

Test solution (b). Dilute 1 ml of test solution (a) to 50 ml with *water R*.

Reference solution (a). Dissolve 10 mg of *magnesium aspartate dihydrate CRS* in *water R* and dilute to 50 ml with the same solvent.

Reference solution (b). Dilute 5 ml of test solution (b) to 20 ml with *water R*.

Reference solution (c). Dissolve 10 mg of *magnesium aspartate dihydrate CRS* and 10 mg of *glutamic acid CRS* in 2 ml of *water R* and dilute to 25 ml with the same solvent.

Apply to the plate 5 µl of each solution. Allow the plate to dry in air. Develop over a path of 15 cm using a mixture of 20 volumes of *glacial acetic acid R*, 20 volumes of *water R*

and 60 volumes of *butanol R*. Allow the plate to dry in air and spray with *ninhydrin solution R*. Heat at 100 °C to 105 °C for 15 min. Any spot in the chromatogram obtained with test solution (a), apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.5 per cent). The test is not valid unless the chromatogram obtained with reference solution (c) shows two clearly separated principal spots.

Chlorides (2.4.4). Dilute 10 ml of solution S to 15 ml with *water R*. The solution complies with the limit test for chlorides (200 ppm).

Sulphates (2.4.13). Dilute 12 ml of solution S to 15 ml with *distilled water R*. The solution complies with the limit test for sulphates (500 ppm). Carry out the evaluation of the test after 30 min.

Ammonium (2.4.1). 50 mg complies with limit test B for ammonium (200 ppm). Prepare the standard using 0.1 ml of *ammonium standard solution (100 ppm NH₄) R*.

Iron (2.4.9). In a separating funnel, dissolve 0.20 g in 10 ml of *dilute hydrochloric acid R*. Shake with three quantities, each of 10 ml, of *methyl isobutyl ketone R1*, shaking for 3 min each time. To the combined organic layers add 10 ml of *water R* and shake for 3 min. The aqueous layer complies with the limit test for iron (50 ppm).

Heavy metals (2.4.8). Dissolve 2.0 g with gentle heating in 20 ml of *water R*. 12 ml of the solution complies with limit test A for heavy metals (10 ppm). Prepare the standard using *lead standard solution (1 ppm Pb) R*.

Water (2.5.12). 10.0 per cent to 14.0 per cent, determined on 0.100 g by the semi-micro determination of water. Dissolve the substance in 10 ml of *formamide R1* at 50 °C protected from moisture, add 10 ml of *anhydrous methanol R* and allow to cool. Carry out a blank determination.

ASSAY

Dissolve 0.260 g in 10 ml of *water R* and carry out the complexometric titration of magnesium (2.5.11).

1 ml of 0.1 M *sodium edetate* is equivalent to 28.85 mg of C₈H₁₂MgN₂O₈.

IMPURITIES

A. aspartic acid.

01/2005:0043

MAGNESIUM CARBONATE, HEAVY

Magnesii subcarbonas ponderosus

DEFINITION

Hydrated basic magnesium carbonate.

Content: 40.0 per cent to 45.0 per cent, calculated as MgO (*M_r* 40.30).

CHARACTERS

Appearance: white powder.

Solubility: practically insoluble in water. It dissolves in dilute acids with strong effervescence.

IDENTIFICATION

- 15 g has an apparent volume (2.9.15) before setting about 30 ml.
- It gives the reaction of carbonates (2.3.1).
- Dissolve about 15 mg in 2 ml of *dilute nitric acid R* and neutralise with *dilute sodium hydroxide solution R*. The solution gives the reaction of magnesium (2.3.1).

TESTS

Solution S. Dissolve 5.0 g in 100 ml of *dilute acetic acid R*. When the effervescence has ceased, boil for 2 min, cool and dilute to 100 ml with the same acid. Filter, if necessary, through a previously ignited and tared porcelain or silica filter crucible of suitable porosity to give a clear filtrate.

Appearance of solution. Solution S is not more intensely coloured than reference solution B₄ (2.2.2, *Method II*).

Soluble substances: maximum 1.0 per cent.

Mix 2.00 g with 100 ml of *water R* and boil for 5 min. Filter whilst hot through a sintered-glass filter (40), allow to cool and dilute to 100 ml with *water R*. Evaporate 50 ml of the filtrate to dryness and dry at 100-105 °C. The residue weighs not more than 10 mg.

Substances insoluble in acetic acid: maximum 0.05 per cent.

Any residue obtained during the preparation of solution S, washed, dried, and ignited at 600 °C, weighs not more than 2.5 mg.

Chlorides (2.4.4): maximum 0.07 per cent.

1.5 ml of solution S diluted to 15 ml with *water R* complies with the limit test for chlorides.

Sulphates (2.4.13): maximum 0.6 per cent.

0.5 ml of solution S diluted to 15 ml with *distilled water R* complies with the limit test for sulphates.

Arsenic (2.4.2): maximum 2 ppm.

10 ml of solution S complies with limit test A.

Calcium (2.4.3): maximum 0.75 per cent.

Dilute 2.6 ml of solution S to 150 ml with *distilled water R*. 15 ml of the solution complies with the limit test for calcium.

Iron (2.4.9): maximum 400 ppm.

Dissolve 0.1 g in 3 ml of *dilute hydrochloric acid R* and dilute to 10 ml with *water R*. 2.5 ml of the solution diluted to 10 ml with *water R* complies with the limit test for iron.

Heavy metals (2.4.8): maximum 20 ppm.

To 20 ml of solution S add 15 ml of *hydrochloric acid R1* and shake with 25 ml of *methyl isobutyl ketone R* for 2 min. Allow to stand, separate the aqueous layer and evaporate to dryness. Dissolve the residue in 1 ml of *acetic acid R* and dilute to 20 ml with *water R*. 12 ml of the solution complies with limit test A. Prepare the standard using *lead standard solution (1 ppm Pb) R*.

ASSAY

Dissolve 0.150 g in a mixture of 20 ml of *water R* and 2 ml of *dilute hydrochloric acid R*. Carry out the complexometric titration of magnesium (2.5.11).

1 ml of 0.1 M *sodium edetate* is equivalent to 4.030 mg of MgO.

01/2005:0042

MAGNESIUM CARBONATE, LIGHT

Magnesii subcarbonas levis

DEFINITION

Hydrated basic magnesium carbonate.

Content: 40.0 per cent to 45.0 per cent, calculated as MgO (*M_r* 40.30).

CHARACTERS

Appearance: white powder.

Solubility: practically insoluble in water. It dissolves in dilute acids with strong effervescence.

01/2005:1341

IDENTIFICATION

- A. 15 g has an apparent volume (2.9.15) before settling of about 180 ml.
- B. It gives the reaction of carbonates (2.3.1).
- C. Dissolve about 15 mg in 2 ml of *dilute nitric acid R* and neutralise with *dilute sodium hydroxide solution R*. The solution gives the reaction of magnesium (2.3.1).

TESTS

Solution S. Dissolve 5.0 g in 100 ml of *dilute acetic acid R*. When the effervescence has ceased, boil for 2 min, cool and dilute to 100 ml with the same acid. Filter, if necessary, through a previously ignited and tared porcelain or silica filter crucible of suitable porosity to give a clear filtrate.

Appearance of solution. Solution S is not more intensely coloured than reference solution B₄ (2.2.2, *Method II*).

Soluble substances: maximum 1.0 per cent.

Mix 2.00 g with 100 ml of *water R* and boil for 5 min. Filter whilst hot through a sintered-glass filter (40), allow to cool and dilute to 100 ml with *water R*. Evaporate 50 ml of the filtrate to dryness and dry at 100-105 °C. The residue weighs not more than 10 mg.

Substances insoluble in acetic acid: maximum 0.05 per cent.

Any residue obtained during the preparation of solution S, washed, dried and ignited at 600 °C, weighs not more than 2.5 mg.

Chlorides (2.4.4): maximum 0.07 per cent.

1.5 ml of solution S diluted to 15 ml with *water R* complies with the limit test for chlorides.

Sulphates (2.4.13): maximum 0.3 per cent.

1 ml of solution S diluted to 15 ml with *distilled water R* complies with the limit test for sulphates.

Arsenic (2.4.2): maximum 2 ppm.

10 ml of solution S complies with limit test A.

Calcium (2.4.3): maximum 0.75 per cent.

Dilute 2.6 ml of solution S to 150 ml with *distilled water R*. 15 ml of the solution complies with the limit test for calcium.

Iron (2.4.9): maximum 400 ppm.

Dissolve 0.1 g in 3 ml of *dilute hydrochloric acid R* and dilute to 10 ml with *water R*. 2.5 ml of the solution diluted to 10 ml with *water R* complies with the limit test for iron.

Heavy metals (2.4.8): maximum 20 ppm.

To 20 ml of solution S add 15 ml of *hydrochloric acid R1* and shake with 25 ml of *methyl isobutyl ketone R* for 2 min. Allow to stand, separate the aqueous layer and evaporate to dryness. Dissolve the residue in 1 ml of *acetic acid R* and dilute to 20 ml with *water R*. 12 ml of the solution complies with limit test A. Prepare the standard using *lead standard solution (1 ppm Pb) R*.

ASSAY

Dissolve 0.150 g in a mixture of 20 ml of *water R* and 2 ml of *dilute hydrochloric acid R*. Carry out the complexometric titration of magnesium (2.5.11).

1 ml of 0.1 M *sodium edetate* is equivalent to 4.030 mg of MgO.

MAGNESIUM CHLORIDE 4.5-HYDRATE

Magnesii chloridum 4.5-hydricum

MgCl₂·xH₂O with x≈4. 5 *M_r* 95.21 (anhydrous substance)

DEFINITION

Magnesium chloride 4.5 - hydrate contains not less than 98.5 per cent and not more than the equivalent of 101.0 per cent of MgCl₂, calculated with reference to the anhydrous substance.

CHARACTERS

A white or almost white granular powder, hygroscopic, very soluble in water, freely soluble in alcohol.

IDENTIFICATION

- A. It complies with the test for water (see Tests).
- B. It gives reaction (a) of chlorides (2.3.1).
- C. It gives the reaction of magnesium (2.3.1).

TESTS

Solution S. Dissolve 10.0 g in *carbon dioxide-free water R* prepared from *distilled water R* and dilute to 100.0 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and colourless (2.2.2, *Method II*).

Acidity or alkalinity. To 5 ml of solution S add 0.05 ml of *phenol red solution R*. Not more than 0.3 ml of 0.01 M *hydrochloric acid* or 0.01 M *sodium hydroxide* is required to change the colour of the indicator.

Bromides. Dilute 2.0 ml of solution S to 10.0 ml with *water R*. To 1.0 ml of the solution add 4.0 ml of *water R*, 2.0 ml of *phenol red solution R3* and 1.0 ml of *chloramine solution R2* and mix immediately. After exactly 2 min add 0.30 ml of 0.1 M *sodium thiosulphate*, mix and dilute to 10.0 ml with *water R*. The absorbance (2.2.25) of the solution measured at 590 nm, using *water R* as the compensation liquid, is not greater than that of a standard prepared at the same time and in the same manner using 5.0 ml of a 3 mg/l solution of *potassium bromide R* (500 ppm).

Sulphates (2.4.13). 15 ml of solution S complies with the limit test for sulphates (100 ppm).

Aluminium (2.4.17). If intended for use in the manufacture of peritoneal dialysis solutions, haemodialysis solutions, or haemofiltration solutions it complies with the test for aluminium. Dissolve 4 g in 100 ml of *water R* and add 10 ml of *acetate buffer solution pH 6.0 R*. The solution complies with the limit test for aluminium (1 ppm). Use as the reference solution a mixture of 2 ml of *aluminium standard solution (2 ppm Al) R*, 10 ml of *acetate buffer solution pH 6.0 R* and 98 ml of *water R*. To prepare the blank use a mixture of 10 ml of *acetate buffer solution pH 6.0 R* and 100 ml of *water R*.

Arsenic (2.4.2). 0.5 g complies with limit test A for arsenic (2 ppm).

Calcium (2.4.3). Dilute 1 ml of solution S to 15 ml with *distilled water R*. The solution complies with the limit test for calcium (0.1 per cent).

Heavy metals (2.4.8). 12 ml of solution S complies with limit test A for heavy metals (10 ppm). Prepare the standard using *lead standard solution (1 ppm Pb) R*.

Iron (2.4.9). 10 ml of solution S complies with the limit test for iron (10 ppm).

Potassium. If intended for use in the manufacture of parenteral dosage forms, not more than 500 ppm of K, determined by atomic emission spectrometry (2.2.22, *Method I*).

Test solution. Dissolve 1.00 g of the substance to be examined in *water R* and dilute to 100.0 ml with the same solvent.

Reference solution. Dissolve 1.144 g of *potassium chloride R* in *water R*, previously dried at 100 °C to 105 °C for 3 h, and dilute to 1000.0 ml with the same solvent (600 µg of K per millilitre). Dilute as required.

Measure the emission intensity at 766.5 nm.

Water (2.5.12): 44.0 per cent to 48.0 per cent, determined on 50.0 mg by the semi-micro determination of water.

ASSAY

Dissolve 0.250 g in 50 ml of *water R*. Carry out the complexometric titration of magnesium (2.5.11).

1 ml of 0.1 M sodium edetate is equivalent to 9.521 mg of MgCl₂.

STORAGE

Store in an airtight container.

LABELLING

The label states:

- where applicable, that the substance is suitable for use in the manufacture of peritoneal dialysis solutions, haemodialysis solutions or haemofiltration solutions,
- where applicable, that the substance is suitable for use in the manufacture of parenteral dosage forms.

01/2005:0402

MAGNESIUM CHLORIDE HEXAHYDRATE

Magnesii chloridum hexahydricum

MgCl₂·6H₂O

M_r 203.3

DEFINITION

Magnesium chloride hexahydrate contains not less than 98.0 per cent and not more than the equivalent of 101.0 per cent of MgCl₂·6H₂O.

CHARACTERS

Colourless crystals, hygroscopic, very soluble in water, freely soluble in alcohol.

IDENTIFICATION

- It complies with the test for water (see Tests).
- It gives reaction (a) of chlorides (2.3.1).
- It gives the reaction of magnesium (2.3.1).

TESTS

Solution S. Dissolve 10.0 g in *carbon dioxide-free water R* prepared from *distilled water R* and dilute to 100.0 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and colourless (2.2.2, *Method II*).

Acidity or alkalinity. To 5 ml of solution S add 0.05 ml of *phenol red solution R*. Not more than 0.3 ml of 0.01 M *hydrochloric acid* or 0.01 M *sodium hydroxide* is required to change the colour of the indicator.

Bromides. Dilute 2.0 ml of solution S to 10.0 ml with *water R*. To 1.0 ml of the solution add 4.0 ml of *water R*, 2.0 ml of *phenol red solution R3* and 1.0 ml of *chloramine solution R2* and mix immediately. After exactly 2 min, add 0.30 ml of 0.1 M *sodium thiosulphate*, mix and dilute to 10.0 ml with *water R*. The absorbance (2.2.25) of the solution measured at 590 nm, using *water R* as the compensation liquid, is not greater than that of a standard prepared at the same time and in the same manner using 5.0 ml of a 3 mg/l solution of *potassium bromide R* (500 ppm).

Sulphates (2.4.13). 15 ml of solution S complies with the limit test for sulphates (100 ppm).

Aluminium (2.4.17). If intended for use in the manufacture of peritoneal dialysis solutions, haemodialysis solutions, or haemofiltration solutions, it complies with the test for aluminium. Dissolve 4 g in 100 ml of *water R* and add 10 ml of *acetate buffer solution pH 6.0 R*. The solution complies with the limit test for aluminium (1 ppm). Use as the reference solution a mixture of 2 ml of *aluminium standard solution (2 ppm Al) R*, 10 ml of *acetate buffer solution pH 6.0 R* and 98 ml of *water R*. To prepare the blank use a mixture of 10 ml of *acetate buffer solution pH 6.0 R* and 100 ml of *water R*.

Arsenic (2.4.2). 0.5 g complies with limit test A for arsenic (2 ppm).

Calcium (2.4.3). Dilute 1 ml of solution S to 15 ml with *distilled water R*. The solution complies with the limit test for calcium (0.1 per cent).

Heavy metals (2.4.8). 12 ml of solution S complies with limit test A for heavy metals (10 ppm). Prepare the standard using *lead standard solution (1 ppm Pb) R*.

Iron (2.4.9). 10 ml of solution S complies with the limit test for iron (10 ppm).

Potassium. If intended for use in the manufacture of parenteral dosage forms, not more than 500 ppm of K, determined by atomic emission spectrometry (2.2.22, *Method I*).

Test solution. Dissolve 1.00 g of the substance to be examined in *water R* and dilute to 100.0 ml with the same solvent.

Reference solution. Dissolve 1.144 g of *potassium chloride R*, previously dried at 100 °C to 105 °C for 3 h in *water R* and dilute to 1000.0 ml with the same solvent (600 µg of K per millilitre). Dilute as required.

Measure the emission intensity at 766.5 nm.

Water (2.5.12). 51.0 per cent to 55.0 per cent, determined on 50.0 mg by the semi-micro determination of water.

ASSAY

Dissolve 0.300 g in 50 ml of *water R*. Carry out the complexometric titration of magnesium (2.5.11).

1 ml of 0.1 M *sodium edetate* is equivalent to 20.33 mg of MgCl₂·6H₂O.

STORAGE

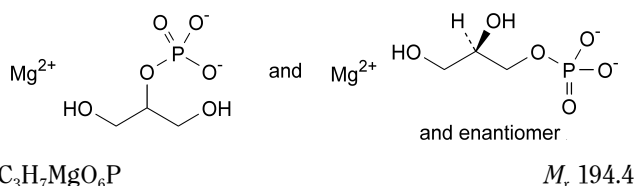
Store in an airtight container.

LABELLING

The label states:

- where applicable, that the substance is suitable for use in the manufacture of peritoneal dialysis solutions, haemodialysis solutions or haemofiltration solutions,
- where applicable, that the substance is suitable for use in the manufacture of parenteral dosage forms.

01/2005:1446

MAGNESIUM GLYCEROPHOSPHATE**Magnesii glycerophosphas****DEFINITION**

Magnesium glycerophosphate is a mixture, in variable proportions, of magnesium (*RS*)-2,3-dihydroxypropyl phosphate, and of magnesium 2-hydroxy-1-(hydroxymethyl)ethyl phosphate, which may be hydrated. Magnesium glycerophosphate contains not less than 11.0 per cent and not more than 12.5 per cent of Mg, calculated with reference to the dried substance.

CHARACTERS

A white powder, hygroscopic, practically insoluble in alcohol. It dissolves in dilute solutions of acids.

IDENTIFICATION

- A. Mix 1 g with 1 g of *potassium hydrogen sulphate R* in a test tube fitted with a glass tube. Heat strongly and direct the white vapour towards a piece of filter paper impregnated with a freshly prepared 10 g/l solution of *sodium nitroprusside R*. The filter paper develops a blue colour in contact with *piperidine R*.
- B. Ignite 0.1 g in a crucible. Take up the residue with 5 ml of *nitric acid R* and heat on a water-bath for 1 min. Filter. The filtrate gives reaction (b) of phosphates (2.3.1).
- C. It gives the reaction of magnesium (2.3.1).

TESTS

Solution S. Dissolve 2.5 g in *carbon dioxide-free water R* prepared from *distilled water R*, and dilute to 50 ml with the same solvent.

Appearance of solution. Solution S is not more opalescent than reference suspension III (2.2.1).

Acidity. Dissolve 1.0 g in 100 ml of *carbon dioxide-free water R*. Add 0.1 ml of *phenolphthalein solution R*. Not more than 1.5 ml of 0.1 M *sodium hydroxide* is required to change the colour of the indicator.

Glycerol and alcohol-soluble substances. Shake 1.0 g with 25 ml of *alcohol R* for 2 min. Filter and wash the residue with 5 ml of *alcohol R*. Combine the filtrate and the washings, evaporate to dryness on a water-bath and dry the residue at 70 °C for 1 h. The residue weighs not more than 15 mg (1.5 per cent).

Chlorides (2.4.4). Dissolve 1.0 g in *water R* and dilute to 100 ml with the same solvent. Dilute 3.5 ml of the solution to 15 ml with *water R*. The solution complies with the limit test for chlorides (0.15 per cent).

Phosphates (2.4.11). Dilute 4 ml of solution S to 100 ml with *water R*. Dilute 1 ml of the solution to 100 ml with *water R*. The solution complies with the limit test for phosphates (0.5 per cent).

Sulphates (2.4.13). Dilute 3 ml of solution S to 15 ml with *distilled water R*. The solution complies with the limit test for sulphates (0.1 per cent).

Iron (2.4.9). Dissolve 67 mg in *water R* and dilute to 10 ml with the same solvent. The solution complies with the limit test for iron (150 ppm).

Heavy metals (2.4.8). To 20 ml of solution S add 15 ml of *hydrochloric acid R* and shake with 25 ml of *methyl isobutyl ketone R* for 2 min. Allow to stand, separate the aqueous layer and evaporate to dryness. Dissolve the residue in 2.5 ml of *acetic acid R* and dilute to 20 ml with *water R*. 12 ml of the solution complies with limit test A for heavy metals (20 ppm). Prepare the standard using *lead standard solution (1 ppm Pb) R*.

Loss on drying (2.2.32). Not more than 12.0 per cent, determined on 1.000 g by drying in an oven at 150 °C for 4 h.

ASSAY

Dissolve 0.200 g in 40 ml of *water R*. Carry out the complexometric titration of magnesium (2.5.11).

1 ml of 0.1 M *sodium edetate* is equivalent to 2.431 mg of Mg.

STORAGE

Store in an airtight container.

01/2005:0039

MAGNESIUM HYDROXIDE**Magnesii hydroxidum****DEFINITION**

Content: 95.0 per cent to 100.5 per cent of $Mg(OH)_2$.

CHARACTERS

Appearance: white, fine, amorphous powder.

Solubility: practically insoluble in water. It dissolves in dilute acids.

IDENTIFICATION

- A. Dissolve about 15 mg in 2 ml of *dilute nitric acid R* and neutralise with *dilute sodium hydroxide solution R*. The solution gives the reaction of magnesium (2.3.1).
- B. It complies with the test for loss on ignition (see Tests).

TESTS

Solution S. Dissolve 5.0 g in a mixture of 50 ml of *acetic acid R* and 50 ml of *distilled water R*. Not more than slight effervescence is produced. Boil for 2 min, cool and dilute to 100 ml with *dilute acetic acid R*. Filter, if necessary, through a previously ignited and tared porcelain or silica filter crucible of suitable porosity to give a clear filtrate.

Appearance of solution. Solution S is not more intensely coloured than reference solution B₃ (2.2.2, *Method II*).

Soluble substances: maximum 2.0 per cent.

Mix 2.00 g with 100 ml of *water R* and boil for 5 min. Filter whilst hot through a sintered-glass filter (40), allow to cool and dilute to 100 ml with *water R*. Evaporate 50 ml of the filtrate to dryness and dry at 100-105 °C. The residue weighs not more than 20 mg.

Substances insoluble in acetic acid: maximum 0.1 per cent. Any residue obtained during the preparation of solution S, washed, dried, and ignited at 600 °C, weighs not more than 5 mg.

Chlorides (2.4.4): maximum 0.1 per cent.

1 ml of solution S diluted to 15 ml with *water R* complies with the limit test for chlorides.

Sulphates (2.4.13): maximum 0.5 per cent.

0.6 ml of solution S diluted to 15 ml with *distilled water R* complies with the limit test for sulphates.

Arsenic (2.4.2): maximum 4 ppm.

5 ml of solution S complies with limit test A.

Calcium (2.4.3): maximum 1.5 per cent.

Dilute 1.3 ml of solution S to 150 ml with *distilled water R*. 15 ml of the solution complies with the limit test for calcium.

Iron (2.4.9): maximum 0.07 per cent.

Dissolve 0.15 g in 5 ml of *dilute hydrochloric acid R* and dilute to 10 ml with *water R*. 1 ml of this solution diluted to 10 ml with *water R* complies with the limit test for iron.

Heavy metals (2.4.8): maximum 30 ppm.

Dissolve 2.0 g in 20 ml of *hydrochloric acid R1* and shake with 25 ml of *methyl isobutyl ketone R* for 2 min. Allow to stand, separate the aqueous layer and evaporate to dryness. Dissolve the residue in 30 ml of *water R*. 12 ml of the solution complies with limit test A. Prepare the standard using *lead standard solution (2 ppm Pb) R*.

Loss on ignition: 29.0 per cent to 32.5 per cent.

Heat 0.5 g gradually to 900 °C and ignite to constant mass.

ASSAY

Dissolve 0.100 g in a mixture of 20 ml of *water R* and 2 ml of *dilute hydrochloric acid R* and carry out the complexometric titration of magnesium (2.5.11).

1 ml of 0.1 M *sodium edetate* is equivalent to 5.832 mg of $\text{Mg}(\text{OH})_2$.

01/2005:0041

MAGNESIUM OXIDE, HEAVY

Magnesii oxidum ponderosum

MgO

M_r 40.30

DEFINITION

Content: from 98.0 per cent to 100.5 per cent of MgO (ignited substance).

CHARACTERS

Appearance: fine, white powder.

Solubility: practically insoluble in water. It dissolves in dilute acids with at most slight effervescence.

IDENTIFICATION

- 15 g has an apparent volume (2.9.15) before settling of about 30 ml.
- Dissolve about 15 mg in 2 ml of *dilute nitric acid R* and neutralise with *dilute sodium hydroxide solution R*. The solution gives the reaction of magnesium (2.3.1).
- It complies with the test for loss on ignition (see Tests).

TESTS

Solution S. Dissolve 5.0 g in a mixture of 30 ml of *distilled water R* and 70 ml of *acetic acid R*, boil for 2 min, cool and dilute to 100 ml with *dilute acetic acid R*. Filter, if necessary, through a previously ignited and tared porcelain or silica filter crucible of suitable porosity to give a clear solution.

Appearance of solution. Solution S is not more intensely coloured than reference solution B3 (2.2.2, *Method II*).

Soluble substances: maximum 2.0 per cent.

Mix 2.00 g with 100 ml of *water R* and boil for 5 min. Filter whilst hot through a sintered-glass filter (40), allow to cool and dilute to 100 ml with *water R*. Evaporate 50 ml of the filtrate to dryness and dry at 100-105 °C. The residue weighs not more than 20 mg.

Substances insoluble in acetic acid: maximum 0.1 per cent.

Any residue obtained during the preparation of solution S, washed, dried, and ignited at 600 °C, weighs not more than 5 mg.

Chlorides (2.4.4): maximum 0.1 per cent.

1 ml of solution S diluted to 15 ml with *water R* complies with the limit test for chlorides.

Sulphates (2.4.13): maximum 1.0 per cent.

0.3 ml of solution S diluted to 15 ml with *distilled water R* complies with the limit test for sulphates.

Arsenic (2.4.2): maximum 4 ppm.

5 ml of solution S complies with limit test A.

Calcium (2.4.3): maximum 1.5 per cent.

Dilute 1.3 ml of solution S to 150 ml with *distilled water R*. 15 ml of the solution complies with the limit test for calcium.

Iron (2.4.9): maximum 0.07 per cent.

Dissolve 0.15 g in 5 ml of *dilute hydrochloric acid R* and dilute to 10 ml with *water R*. 1 ml of the solution diluted to 10 ml with *water R* complies with the limit test for iron.

Heavy metals (2.4.8): maximum 30 ppm.

To 20 ml of solution S add 15 ml of *hydrochloric acid R1* and shake with 25 ml of *methyl isobutyl ketone R* for 2 min. Separate the layers, evaporate the aqueous layer to dryness, dissolve the residue in 1 ml of *acetic acid R* and dilute to 30 ml with *water R*. 12 ml of the solution complies with limit test A. Prepare the standard using *lead standard solution (1 ppm Pb) R*.

Loss on ignition: maximum 8.0 per cent, determined on 1.00 g at 900 °C.

ASSAY

Dissolve 0.320 g in 20 ml of *dilute hydrochloric acid R* and dilute to 100.0 ml with *water R*. Using 20.0 ml of the solution, carry out the complexometric titration of magnesium (2.5.11).

1 ml of 0.1 M *sodium edetate* is equivalent to 4.030 mg of MgO.

01/2005:0040

MAGNESIUM OXIDE, LIGHT

Magnesii oxidum leve

MgO

M_r 40.30

DEFINITION

Content: 98.0 per cent to 100.5 per cent of MgO (ignited substance).

CHARACTERS

Appearance: fine, white, amorphous powder.

Solubility: practically insoluble in water. It dissolves in dilute acids with at most slight effervescence.

IDENTIFICATION

- 15 g has an apparent volume (2.9.15) before settling of about 150 ml.

B. Dissolve about 15 mg in 2 ml of *dilute nitric acid R* and neutralise with *dilute sodium hydroxide solution R*. The solution gives the reaction of magnesium (2.3.1).

C. It complies with the test for loss on ignition (see Tests).

TESTS

Solution S. Dissolve 5.0 g in a mixture of 30 ml of *distilled water R* and 70 ml of *acetic acid R*, boil for 2 min, cool and dilute to 100 ml with *dilute acetic acid R*. Filter if necessary through a previously ignited and tared porcelain or silica filter crucible of a suitable porosity to give a clear filtrate.

Appearance of solution. Solution S is not more intensely coloured than reference solution B₂ (2.2.2, *Method II*).

Soluble substances: maximum 2.0 per cent.

To 2.00 g add 100 ml of *water R* and heat to boiling for 5 min. Filter whilst hot through a sintered-glass filter (40), allow to cool and dilute to 100 ml with *water R*. Evaporate 50 ml of the filtrate to dryness and dry at 100–105 °C. The residue weighs not more than 20 mg.

Substances insoluble in acetic acid: maximum 0.1 per cent.

Any residue obtained during the preparation of solution S, washed, dried, and ignited at 600 °C, weighs not more than 5 mg.

Chlorides (2.4.4): maximum 0.15 per cent.

0.7 ml of solution S diluted to 15 ml with *water R* complies with the limit test for chlorides.

Sulphates (2.4.13): maximum 1.0 per cent.

0.3 ml of solution S diluted to 15 ml with *distilled water R* complies with the limit test for sulphates.

Arsenic (2.4.2): maximum 4 ppm.

5 ml of solution S complies with limit test A.

Calcium (2.4.3): maximum 1.5 per cent.

Dilute 1.3 ml of solution S to 150 ml with *distilled water R*. 15 ml of the solution complies with the limit test for calcium.

Iron (2.4.9): maximum 0.1 per cent.

Dissolve 50 mg in 5 ml of *dilute hydrochloric acid R* and dilute to 10 ml with *water R*. 2 ml of this solution diluted to 10 ml with *water R* complies with the limit test for iron.

Heavy metals (2.4.8): maximum 30 ppm.

To 20 ml of solution S add 15 ml of *hydrochloric acid R1* and shake with 25 ml of *methyl isobutyl ketone R* for 2 min. Allow to stand, separate the aqueous layer and evaporate to dryness. Dissolve the residue in 1.5 ml of *acetic acid R* and dilute to 30 ml with *water R*. 12 ml of the solution complies with limit test A. Prepare the standard using *lead standard solution* (1 ppm Pb) *R*.

Loss on ignition: maximum 8.0 per cent, determined on 1.00 g at 900 °C.

ASSAY

Dissolve 0.320 g in 20 ml of *dilute hydrochloric acid R* and dilute to 100.0 ml with *water R*. Using 20.0 ml of the solution, carry out the complexometric titration of magnesium (2.5.11).

1 ml of 0.1 M *sodium edetate* is equivalent to 4.030 mg of MgO.

01/2005:1540

MAGNESIUM PEROXIDE

Magnesii peroxidum

DEFINITION

Magnesium peroxide is a mixture of magnesium peroxide and magnesium oxide. It contains not less than 22.0 per cent and not more than 28.0 per cent of MgO₂ (*M_r* 56.30).

CHARACTERS

A white or slightly yellow, amorphous, light powder, practically insoluble in water and in alcohol. It dissolves in dilute mineral acids.

IDENTIFICATION

A. Dissolve about 15 mg in 2 ml of *dilute nitric acid R* and neutralise with *dilute sodium hydroxide solution R*. The solution gives the reaction of magnesium (2.3.1).

B. Dissolve 0.1 g in 2 ml of *dilute sulphuric acid R* and dilute to 10 ml with *water R*. Shake 1 ml of the solution with 5 ml of *ether R* and 0.5 ml of *potassium dichromate solution R1*. The ether layer is blue.

TESTS

Solution S1. Dissolve cautiously 5.0 g in 40 ml of *hydrochloric acid R1*. Cautiously evaporate the solution to a volume of 10 ml and dilute to 100 ml with a mixture of equal volumes of *acetic acid R* and *distilled water R*. Filter, if necessary, through a previously ignited and tared porcelain or silica filter crucible of suitable porosity to give a clear filtrate. Keep the residue for the test for acid insoluble substances.

Solution S2. Dilute 5 ml of solution S1 to 25 ml with *distilled water R*.

Appearance of solution. Solution S1 is not more intensely coloured than reference solution B₄ (2.2.2, *Method II*).

Acid insoluble substances. Any residue obtained during the preparation of solution S1, washed, dried and ignited at 600 °C, weighs not more than 5 mg (0.1 per cent).

Acidity or alkalinity. To 2.0 g add 100 ml of *carbon dioxide-free water R* and heat to boiling for 5 min. Filter whilst hot through a sintered-glass filter (40), allow to cool and dilute to 100 ml with *carbon dioxide-free water R*. To 15 ml of the filtrate, add 0.1 ml of *phenolphthalein solution R*. The solution is red. Not more than 0.2 ml of 0.1 M *hydrochloric acid* is necessary to change the colour of the indicator. Keep the filtrate for the test for soluble substances.

Soluble substances. Take 50 ml of the filtrate obtained in the test for acidity or alkalinity, evaporate to dryness and dry at 100 °C to 105 °C. The residue weighs not more than 15 mg (1.5 per cent).

Chlorides (2.4.4). Dissolve 50 mg in 5 ml of *dilute nitric acid R* and dilute to 15 ml with *water R*. The solution complies with the limit test for chlorides (0.1 per cent).

Sulphates (2.4.13). 3 ml of solution S2 diluted to 15 ml with *distilled water R* complies with the limit test for sulphates (0.5 per cent).

Arsenic (2.4.2). 5 ml of solution S1 complies with limit test A for arsenic (4 ppm).

Calcium (2.4.3). 1 ml of solution S2 diluted to 15 ml with *distilled water R* complies with the limit test for calcium (1.0 per cent).

Iron (2.4.9). 2 ml of solution S2 diluted to 10 ml with *water R* complies with the limit test for iron (500 ppm).

Heavy metals (2.4.8). To 20 ml of solution S1 add 15 ml of *hydrochloric acid R1* and shake with 25 ml of *methyl isobutyl ketone R* for 2 min. Allow to stand, separate the aqueous layer and evaporate to dryness. Dissolve the residue in 1.5 ml of *acetic acid R* and dilute to 30 ml with *water R*. 12 ml of the solution complies with limit test A for heavy metals (30 ppm). Prepare the standard using *lead standard solution (1 ppm Pb) R*.

ASSAY

Dissolve 80.0 mg, shaking cautiously, in a mixture, previously cooled to 20 °C, of 10 ml of *sulphuric acid R* and 90 ml of *water R*. Titrate with 0.02 M *potassium permanganate* until a pink colour is obtained.

1 ml of 0.02 M *potassium permanganate* is equivalent to 2.815 mg of MgO₂.

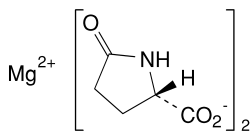
STORAGE

Store protected from light.

01/2005:1619

MAGNESIUM PIDOLATE

Magnesii pidolas



C₁₀H₁₂N₂O₆Mg

M_r 280.5

DEFINITION

Magnesium bis[(2S)-5-oxopyrrolidine-2-carboxylate].

Content: 8.49 per cent to 8.84 per cent of Mg (A_r = 24.31) (anhydrous substance).

CHARACTERS

Appearance: amorphous, white or almost white powder, hygroscopic.

Solubility: very soluble in water, soluble in methanol, practically insoluble in methylene chloride.

IDENTIFICATION

A. Thin-layer chromatography (2.2.27).

Test solution. Dissolve 60 mg in 2 ml of *water R* and dilute to 10 ml with *methanol R*.

Reference solution. Dissolve 55 mg of *pidolic acid CRS* in 2 ml of *water R* and dilute to 10 ml with *methanol R*.

Plate: TLC silica gel plate R.

Mobile phase: *methanol R*, *glacial acetic acid R*, *methylene chloride R* (15:20:65 V/V/V).

Application: 1 µl.

Development: over 2/3 of the plate.

Drying: at 100-105 °C for 15 min.

Detection: spray with *concentrated sodium hypochlorite solution R*. Allow to stand for 10 min and spray abundantly with *glacial acetic acid R*. Allow to stand again for 10 min and dry the plate at 100-105 °C for 2 min. Spray with *potassium iodide and starch solution R* until spots appear.

Results: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution. The chromatogram obtained with the test solution may show 2 faint secondary spots.

B. To 0.15 ml of solution S (see Tests) add 1.8 ml of *water R*. The solution gives the reaction of magnesium (2.3.1).

TESTS

Solution S. Dissolve 5.00 g in *carbon dioxide-free water R* prepared from *distilled water R* and dilute to 50.0 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and not more intensely coloured than reference solution B₈ (2.2.2, Method I).

pH (2.2.3): 5.5 to 7.0 for solution S.

Specific optical rotation (2.2.7): -23.3 to -26.5 (anhydrous substance), determined on solution S.

Related substances. Liquid chromatography (2.2.29).

Test solution. Dissolve 0.500 g of the substance to be examined in the mobile phase and dilute to 100.0 ml with the mobile phase.

Reference solution (a). Dilute 1.0 ml of the test solution to 100.0 ml with the mobile phase.

Reference solution (b). Dissolve 50.0 mg *pidolate impurity B CRS* in the mobile phase and dilute to 100.0 ml with the mobile phase. Dilute 5.0 ml of the solution to 50.0 ml with the mobile phase.

Reference solution (c). Dilute 10.0 ml of reference solution (b) to 100.0 ml with the mobile phase.

Reference solution (d). Dilute 1.0 ml of *nitrate standard solution (100 ppm NO₃) R* to 100.0 ml with the mobile phase.

Reference solution (e). Dilute 6.0 ml of reference solution (a) to 10.0 ml with reference solution (b).

Column:

- **size:** *l* = 0.25 m, Ø = 4.6 mm,
- **stationary phase:** *octadecylsilyl silica gel for chromatography R* (5 µm).

Mobile phase: dissolve 1.56 g of *sodium dihydrogen phosphate R* in 1000 ml of *water R* and adjust to pH 2.5 with a 10 per cent V/V solution of *phosphoric acid R*.

Flow rate: 1.5 ml/min.

Detection: spectrophotometer at 210 nm.

Injection: 10 µl loop injector; inject the test solution and reference solutions (b), (c), (d) and (e).

Run time: 4 times the retention time of pidolic acid.

Retention times: pidolic acid = about 4.5 min; impurity B = about 7.5 min.

System suitability: reference solution (e):

- **resolution:** minimum 10 between the peaks due to pidolic acid and to impurity B.

Limits:

- **impurity B:** not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (1.0 per cent),
- **total of other impurities:** not more than half of the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent),
- **disregard limit:** not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.05 per cent); disregard any peak corresponding to the nitrate ion (NO₃⁻).

Impurity A. Thin-layer chromatography (2.2.27).

Test solution. Dissolve 0.250 g of the substance to be examined in 4 ml of *water R* and dilute to 50.0 ml with *methanol R*.

Reference solution (a). Dissolve 60.0 mg of *glutamic acid R* in 50 ml of *water R* and dilute to 100.0 ml with *methanol R*. Dilute 1.0 ml of the solution to 20.0 ml with *methanol R*.

Reference solution (b). Dissolve 10 mg of *glutamic acid R* and 10 mg of *aspartic acid R* in *water R* and dilute to 25 ml with the same solvent. Dilute 1 ml of the solution to 10 ml with *water R*.

Plate: TLC silica gel plate *R*.

Mobile phase: *glacial acetic acid R*, *water R*, *butanol R* (20:20:60 V/V/V).

Application: 5 µl.

Development: over 2/3 of the plate.

Drying: in air.

Detection: spray with *ninhydrin solution R* and heat at 100–105 °C for 15 min.

System suitability: the test is not valid unless the chromatogram obtained with reference solution (b) shows 2 clearly separated spots.

Limit:

- **impurity A:** any spot corresponding to impurity A in the chromatogram obtained with the test solution is not more intense than the spot in the chromatogram obtained with reference solution (a) (0.6 per cent).

Chlorides (2.4.4): maximum 500 ppm.

Dilute 1.0 ml of solution S to 15.0 ml of *water R*. The solution complies with the limit test for chlorides.

Nitrates. Examine the chromatogram obtained with the test solution in the test for related substances.

Limit:

- **nitrates:** not more than the area of the principal peak in the chromatogram obtained with reference solution (d) (200 ppm).

Sulphates (2.4.13): maximum 0.1 per cent.

Dilute 1.5 ml of solution S to 15.0 ml with *distilled water R*. The solution complies with the limit test for sulphates.

Arsenic (2.4.2): maximum 2 ppm.

5.0 ml of solution S complies with limit test A.

Iron (2.4.9): maximum 200 ppm.

Dilute 0.5 ml of solution S to 10 ml of *water R*. The solution complies with the limit test for iron.

Heavy metals (2.4.8): maximum 20 ppm.

12 ml of solution S complies with limit test A. Prepare the standard using *lead standard solution (2 ppm Pb) R*.

Water (2.5.12): maximum 8.0 per cent, determined on 0.200 g.

ASSAY

Dissolve 0.300 g in 50 ml of *water R*. Carry out the complexometric titration of magnesium (2.5.11).

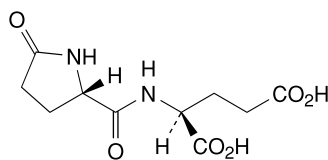
1 ml of 0.1 M *sodium edetate* is equivalent to 2.431 mg of Mg.

STORAGE

In an airtight container.

IMPURITIES

A. glutamic acid,



B. (2S)-2-[[[(2S)-5-oxopyrrolidin-2-yl]carbonyl]amino]pentanedioic acid.

01/2005:0229

MAGNESIUM STEARATE

Magnesii stearas

DEFINITION

Magnesium stearate is a mixture of magnesium salts of different fatty acids consisting mainly of stearic acid [(C₁₇H₃₅COO)₂Mg; *M_r* 591.3] and palmitic acid [(C₁₅H₃₁COO)₂Mg; *M_r* 535.1] with minor proportions of other fatty acids. It contains not less than 4.0 per cent and not more than 5.0 per cent of Mg (*A_r* 24.30), calculated with reference to the dried substance. The fatty acid fraction contains not less than 40.0 per cent of stearic acid and the sum of stearic acid and palmitic acid is not less than 90.0 per cent.

CHARACTERS

A white, very fine, light powder, greasy to the touch, practically insoluble in water and in ethanol.

IDENTIFICATION

First identification: C, D.

Second identification: A, B, D.

- The residue obtained in the preparation of solution S (see Tests) has a freezing point (2.2.18) not lower than 53 °C.
- The acid value of the fatty acids (2.5.1) is 195 to 210, determined on 0.200 g of the residue obtained in the preparation of solution S dissolved in 25 ml of the prescribed mixture of solvents.
- Examine the chromatograms obtained in the test for fatty acid composition. The retention times of the principal peaks in the chromatogram obtained with the test solution are approximately the same as those of the principal peaks in the chromatogram obtained with the reference solution.
- 1 ml of solution S gives the reaction of magnesium (2.3.1).

TESTS

Solution S. To 5.0 g add 50 ml of *peroxide-free ether R*, 20 ml of *dilute nitric acid R* and 20 ml of *distilled water R* and heat under a reflux condenser until dissolution is complete. Allow to cool. In a separating funnel, separate the aqueous layer and shake the ether layer with 2 quantities, each of 4 ml, of *distilled water R*. Combine the aqueous layers, wash with 15 ml of *peroxide-free ether R* and dilute to 50 ml with *distilled water R* (solution S). Evaporate the organic layer to dryness and dry the residue at 100–105 °C. Keep the residue for identification tests A and B.

Acidity or alkalinity. To 1.0 g add 20 ml of *carbon dioxide-free water R* and boil for 1 min with continuous stirring. Cool and filter. To 10 ml of the filtrate add 0.05 ml of *bromothymol blue solution R1*. Not more than 0.5 ml of 0.01 M *hydrochloric acid* or 0.01 M *sodium hydroxide* is required to change the colour of the indicator.

Chlorides (2.4.4). 0.5 ml of solution S diluted to 15 ml with *water R* complies with the limit test for chlorides (0.1 per cent).

Sulphates (2.4.13). 0.3 ml of solution S diluted to 15 ml with *distilled water R* complies with the limit test for sulphates (0.5 per cent).

Cadmium. Not more than 3 ppm of Cd, determined by atomic absorption spectrometry (2.2.23, *Method II*).

Test solution. Place 50.0 mg of the substance to be examined in a polytetrafluoroethylene digestion bomb and add 0.5 ml of a mixture of 1 volume of *hydrochloric acid R* and 5 volumes of *cadmium- and lead-free nitric acid R*. Allow to digest at 170 °C for 5 h. Allow to cool. Dissolve the residue in *water R* and dilute to 5.0 ml with the same solvent.

Reference solutions. Prepare the reference solutions using *cadmium standard solution (10 ppm Cd) R*, diluted as necessary with a 1 per cent V/V solution of *hydrochloric acid R*.

Measure the absorbance at 228.8 nm, using a cadmium hollow-cathode lamp as a source of radiation and a graphite furnace as atomic generator.

Lead. Not more than 10 ppm of Pb, determined by atomic absorption spectrometry (2.2.23, *Method II*).

Test solution. Use the solution described in the test for cadmium.

Reference solutions. Prepare the reference solutions using *lead standard solution (10 ppm Pb) R*, diluted as necessary with *water R*.

Measure the absorbance at 283.3 nm, using a lead hollow-cathode lamp as a source of radiation and a graphite furnace as atomic generator, depending on the apparatus the line at 217.0 nm may be used.

Nickel. Not more than 5 ppm of Ni, determined by atomic absorption spectrometry (2.2.23, *Method II*).

Test solution. Use the solution described in the test for cadmium.

Reference solutions. Prepare the reference solutions using *nickel standard solution (10 ppm Ni) R*, diluted as necessary with *water R*.

Measure the absorbance at 232.0 nm, using a nickel hollow-cathode lamp as a source of radiation and a graphite furnace as atomic generator.

Loss on drying (2.2.32). Not more than 6.0 per cent, determined on 1.000 g by drying in an oven at 100-105 °C.

Microbial contamination. Total viable aerobic count (2.6.12) not more than 10³ micro-organisms per gram, determined by plate count. It complies with the test for *Escherichia coli* (2.6.13).

ASSAY

Magnesium. To 0.500 g in a 250 ml conical flask add 50 ml of a mixture of equal volumes of *butanol R* and *ethanol R*, 5 ml of *concentrated ammonia R*, 3 ml of *ammonium chloride buffer solution pH 10.0 R*, 30.0 ml of 0.1 M *sodium edetate* and 15 mg of *mordant black 11 triturate R*. Heat to 45-50 °C until the solution is clear and titrate with 0.1 M *zinc sulphate* until the colour changes from blue to violet. Carry out a blank titration.

1 ml of 0.1 M *sodium edetate* is equivalent to 2.431 mg of Mg.

Fatty acid composition. Examine by gas chromatography (2.2.28).

Test solution. In a conical flask fitted with a reflux condenser, dissolve 0.10 g of the substance to be examined in 5 ml of *boron trifluoride-methanol solution R*. Boil under a reflux condenser for 10 min. Add 4 ml of *heptane R* through the condenser and boil again under a reflux condenser for 10 min. Allow to cool. Add 20 ml of a *saturated sodium*

chloride solution R. Shake and allow the layers to separate. Remove about 2 ml of the organic layer and dry over 0.2 g of *anhydrous sodium sulphate R*. Dilute 1.0 ml of the solution to 10.0 ml with *heptane R*.

Reference solution. Prepare the reference solution in the same manner as the test solution using 50.0 mg of *palmitic acid CRS* and 50.0 mg of *stearic acid CRS* instead of magnesium stearate.

The chromatographic procedure may be carried out using:

- a fused-silica column 30 m long and 0.32 mm in internal diameter coated with *macrogol 20 000 R* (film thickness 0.5 µm),
- *helium for chromatography R* as the carrier gas at a flow rate of 2.4 ml/min,
- a flame-ionisation detector,

with the following temperature programme:

	Time (min)	Temperature (°C)	Rate (°C/min)	Comment
Column	0 - 2	70	–	isothermal
	2 - 36	70 → 240	5	linear gradient
	36 - 41	240	–	isothermal
Injection port		220		
Detector		260		

Inject 1 µl of the reference solution. When the chromatogram is recorded in the prescribed conditions, the relative retention of methyl palmitate to that of methyl stearate is about 0.88. The test is not valid unless, in the chromatogram obtained with the reference solution, the resolution between the peaks corresponding to methyl stearate and methyl palmitate is at least 5.0.

Inject 1 µl of the test solution. Calculate the percentage content of stearic acid and palmitic acid from the areas of the peaks in the chromatogram obtained with the test solution by the normalisation procedure, disregarding the peak due to the solvent.

FUNCTIONALITY-RELATED CHARACTERISTICS

The following test is not a mandatory requirement but in view of its known importance for achieving consistency in manufacture, quality and performance of medicinal products, it is recommended that suppliers should verify this characteristic and provide information on the result and the analytical method applied to users. The method indicated below has been found suitable but other methods may be used.

The following characteristic is relevant for magnesium stearate used as a lubricant in solid dosage forms (compressed and powder).

Specific surface area (2.9.26, *Method I*). Determine the specific surface area in the P/P₀ range of 0.05 to 0.15.

Sample outgassing: 2 h at 40 °C.

01/2005:0044

MAGNESIUM SULPHATE HEPTAHYDRATE

Magnesii sulfas heptahydricus

MgSO₄·7H₂O

M_r 246.5

DEFINITION

Magnesium sulphate heptahydrate contains not less than 99.0 per cent and not more than the equivalent of 100.5 per cent of MgSO_4 , calculated with reference to the dried substance.

CHARACTERS

A white, crystalline powder or brilliant, colourless crystals, freely soluble in water, very soluble in boiling water, practically insoluble in alcohol.

IDENTIFICATION

- A. It gives the reactions of sulphates (2.3.1).
- B. It gives the reaction of magnesium (2.3.1).

TESTS

Solution S. Dissolve 5.0 g in *water R* and dilute to 50 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and colourless (2.2.2, *Method II*).

Acidity or alkalinity. To 10 ml of solution S add 0.05 ml of *phenol red solution R*. Not more than 0.2 ml of 0.01 M *hydrochloric acid* or 0.01 M *sodium hydroxide* is required to change the colour of the indicator.

Chlorides (2.4.4). 1.7 ml of solution S diluted to 15 ml with *water R* complies with the limit test for chlorides (300 ppm).

Arsenic (2.4.2). 0.5 g complies with limit test A for arsenic (2 ppm).

Iron (2.4.9). 5 ml of solution S diluted to 10 ml with *water R* complies with the limit test for iron (20 ppm).

Heavy metals (2.4.8). 12 ml of solution S complies with limit test A for heavy metals (10 ppm). Prepare the standard using *lead standard solution* (1 ppm Pb) *R*.

Loss on drying (2.2.32). 48.0 per cent to 52.0 per cent, determined on 0.500 g by drying in an oven at 110 °C to 120 °C for 1 h and then at 400 °C to constant mass.

ASSAY

Dissolve 0.450 g in 100 ml of *water R* and carry out the complexometric titration of magnesium (2.5.11).

1 ml of 0.1 M *sodium edetate* is equivalent to 12.04 mg of MgSO_4 .

01/2005:0403

MAGNESIUM TRISILICATE

Magnesii trisilicas

DEFINITION

Magnesium trisilicate has a variable composition corresponding approximately to $\text{Mg}_2\text{Si}_3\text{O}_8 \cdot x\text{H}_2\text{O}$ and contains not less than the equivalent of 29.0 per cent of magnesium oxide (MgO ; M_r 40.30) and not less than the equivalent of 65.0 per cent of silicon dioxide (SiO_2 ; M_r 60.1), both calculated with reference to the ignited substance.

CHARACTERS

A white powder, practically insoluble in water and in alcohol.

IDENTIFICATION

- A. 0.25 g gives the reaction of silicates (2.3.1).
- B. 1 ml of solution S (see Tests) neutralised with *dilute sodium hydroxide solution R* gives the reaction of magnesium (2.3.1).

TESTS

Solution S. To 2.0 g add a mixture of 4 ml of *nitric acid R* and 4 ml of *distilled water R*. Heat to boiling with frequent shaking. Add 12 ml of *distilled water R* and allow to cool. Filter or centrifuge to obtain a clear solution and dilute to 20 ml with *distilled water R*.

Alkalinity. To 10.0 g in a 200 ml conical flask, add 100.0 g of *water R* and heat on a water-bath for 30 min. Allow to cool and make up to the initial mass with *water R*. Allow to stand and filter or centrifuge until a clear liquid is obtained. To 10 ml of the liquid add 0.1 ml of *phenolphthalein solution R*. Not more than 1.0 ml of 0.1 M *hydrochloric acid* is required to change the colour of the indicator.

Water-soluble salts. In a platinum dish, evaporate to dryness on a water-bath 20.0 ml of the liquid obtained in the test for alkalinity. The residue, ignited to constant mass at 900 °C, weighs not more than 30 mg (1.5 per cent).

Chlorides (2.4.4). 0.5 ml of solution S diluted to 15 ml with *water R* complies with the limit test for chlorides (500 ppm). Prepare the standard using a mixture of 5 ml of *chloride standard solution* (5 ppm Cl) *R* and 10 ml of *water R*.

Sulphates (2.4.13). 0.3 ml of solution S diluted to 15 ml with *distilled water R* complies with the limit test for sulphates (0.5 per cent).

Arsenic (2.4.2). 2.5 ml of solution S complies with limit test A for arsenic (4 ppm).

Heavy metals (2.4.8). Neutralise 10 ml of solution S with *dilute ammonia R1*, using *metanil yellow solution R* as an external indicator. Dilute to 20 ml with *water R* and filter if necessary. 12 ml of the solution complies with limit test A for heavy metals (40 ppm). Prepare the standard using *lead standard solution* (2 ppm Pb) *R*.

Loss on ignition: 17 per cent to 34 per cent, determined on 0.5 g ignited to constant mass at 900 °C in a platinum crucible.

Acid-absorbing capacity. Suspend 0.25 g in 0.1 M *hydrochloric acid*, dilute to 100.0 ml with the same acid and allow to stand for 2 h in a water-bath at 37 ± 0.5 °C, with frequent shaking. Allow to cool. To 20.0 ml of the supernatant solution add 0.1 ml of *bromophenol blue solution R* and titrate with 0.1 M *sodium hydroxide* until a blue colour is obtained. The acid-absorbing capacity is not less than 100.0 ml of 0.1 M *hydrochloric acid* per gram.

ASSAY

Magnesium oxide. To 1.000 g in a 200 ml conical flask, add 35 ml of *hydrochloric acid R* and 60 ml of *water R* and heat in a water-bath for 15 min. Allow to cool, filter, wash the conical flask and the residue with *water R* and dilute the combined filtrate and washings to 250.0 ml with *water R*. Neutralise 50.0 ml of the solution with *strong sodium hydroxide solution R* (about 8 ml). Carry out the complexometric titration of magnesium (2.5.11).

1 ml of 0.1 M *sodium edetate* is equivalent to 4.030 mg of MgO .

Silicon dioxide. To 0.700 g add 10 ml of *dilute sulphuric acid R* and 10 ml of *water R*. Heat for 90 min on a water-bath with frequent shaking, replacing the evaporated water. Allow to cool and decant onto an ashless filter paper (diameter 7 cm). Wash the precipitate by decantation with three quantities, each of 5 ml, of hot *water R*, transfer it to the filter and wash it with hot *water R* until 1 ml of the filtrate remains clear after the addition of 0.05 ml of *dilute hydrochloric*

acid R and 2 ml of *barium chloride solution RI*. Incinerate the filter and its contents in a platinum crucible, then ignite the residue (SiO_2) at 900 °C to constant mass.

01/2005:1342

MAIZE OIL, REFINED

Maydis oleum raffinatum

DEFINITION

Refined maize oil is the fatty oil obtained from the seeds of *Zea mays* L. by expression or by extraction, then refined.

CHARACTERS

A clear, light yellow or yellow oil, practically insoluble in water and in alcohol, miscible with light petroleum (bp: 40 °C to 60 °C) and with methylene chloride.

It has a relative density of about 0.920 and a refractive index of about 1.474.

IDENTIFICATION

- A. Carry out the identification of fatty oils by thin-layer chromatography (2.3.2). The chromatogram obtained with the test solution is similar to that obtained with the reference solution.
- B. It complies with the test for composition of fatty acids (see Tests).

TESTS

Acid value (2.5.1). Not more than 0.5, determined on 10.0 g. If intended for use in the manufacture of parenteral dosage forms, not more than 0.3.

Peroxide value (2.5.5). Not more than 10.0. If intended for use in the manufacture of parenteral dosage forms, not more than 5.0.

Unsaponifiable matter (2.5.7). Not more than 2.8 per cent, determined on 5.0 g.

Alkaline impurities (2.4.19). It complies with the test for alkaline impurities in fatty oils.

Composition of fatty acids (2.4.22, Method A). The fatty-acid fraction of the oil has the following composition:

- *fatty acids of chain length less than C_{16}* : not more than 0.6 per cent,
- *palmitic acid*: 8.6 per cent to 16.5 per cent,
- *stearic acid*: not more than 3.3 per cent,
- *oleic acid*: 20.0 per cent to 42.2 per cent (equivalent chain length on polyethyleneglycol adipate 18.9),
- *linoleic acid*: 39.4 per cent to 65.6 per cent (equivalent chain length on polyethyleneglycol adipate 18.9),
- *linolenic acid*: 0.5 per cent to 1.5 per cent (equivalent chain length on polyethyleneglycol adipate 19.7),
- *arachidic acid*: not more than 0.8 per cent,
- *eicosenoic acid*: not more than 0.5 per cent (equivalent chain length on polyethyleneglycol adipate 20.3),
- *behenic acid*: not more than 0.5 per cent,
- *other fatty acids*: not more than 0.5 per cent.

Sterols. Determined by gas chromatography (2.4.23), the sterol fraction of the oil contains not more than 0.3 per cent of brassicasterol.

Water (2.5.32). If intended for use in the manufacture of parenteral dosage forms, not more than 0.1 per cent, determined on 5.00 g by the micro-determination of water. Use a mixture of equal volumes of *decanol R* and *anhydrous methanol R* as the solvent.

STORAGE

Store protected from light, at a temperature not exceeding 25 °C.

LABELLING

The label states:

- where applicable, that the substance is suitable for use in the manufacture of parenteral dosage forms,
- whether the oil is obtained by mechanical expression or by extraction.

01/2005:0344

MAIZE STARCH

Maydis amylum

DEFINITION

Maize starch is obtained from the caryopsis of *Zea mays* L.

CHARACTERS

Appearance: matt, white to slightly yellowish, very fine powder which creaks when pressed between the fingers.

Solubility: practically insoluble in cold water and in alcohol.

The presence of granules with cracks or irregularities on the edge is exceptional.

It is tasteless.

IDENTIFICATION

- A. Examined under a microscope, using not less than 20 × magnification and using equal volumes of *glycerol R* and *water R*, it appears as either angular polyhedral granules of irregular sizes with diameters ranging from about 2 µm to about 23 µm or as rounded or spheroidal granules of irregular sizes with diameters ranging from about 25 µm to about 35 µm. The central hilum consists of a distinct cavity or two-to five-rayed cleft and there are no concentric striations. Between crossed nicol prisms, the starch granules show a distinct black cross intersecting at the hilum.
- B. Suspend 1 g in 50 ml of *water R*, boil for 1 min and cool. A thin, cloudy mucilage is formed.
- C. To 10 ml of the mucilage obtained in identification test B add 0.04 ml of *iodine solution RI*. An orange-red to dark blue colour is produced which disappears on heating.

TESTS

pH (2.2.3): 4.0 to 7.0.

Shake 5.0 g with 25.0 ml of *carbon dioxide-free water R* for 60 s. Allow to stand for 15 min.

Foreign matter. Examined under a microscope using a mixture of equal volumes of *glycerol R* and *water R*, not more than traces of matter other than starch granules are present. No starch grains of any other origin are present.

Oxidising substances (2.5.30): maximum 20 ppm, calculated as H_2O_2 .

Sulphur dioxide (2.5.29): maximum 50 ppm.

Iron (2.4.9): maximum 10 ppm.

Shake 1.5 g with 15 ml of *dilute hydrochloric acid R*. Filter. The filtrate complies with the limit test for iron.

Loss on drying (2.2.32): maximum 15.0 per cent, determined on 1.000 g by drying in an oven at 130 °C for 90 min.

Sulphated ash (2.4.14): maximum 0.6 per cent, determined on 1.0 g.

Microbial contamination. Total viable aerobic count (2.6.12) not more than 10^3 bacteria and 10^2 fungi per gram, determined by plate count. It complies with the test for *Escherichia coli* (2.6.13).

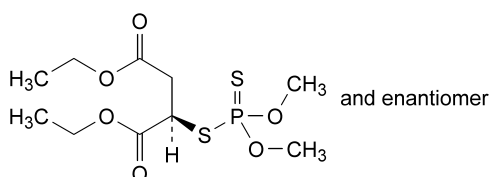
STORAGE

In an airtight container.

01/2005:1343

MALATHION

Malathionum



$C_{10}H_{19}O_6PS_2$

M_r 330.4

DEFINITION

Malathion contains not less than 98.0 per cent and not more than the equivalent of 102.0 per cent of diethyl (2RS)-2-(dimethoxyphosphinodithioyl)butanedioate, calculated with reference to the anhydrous substance.

CHARACTERS

A clear, colourless or slightly yellowish liquid, slightly soluble in water, miscible with alcohol and with acetone, with cyclohexane and with vegetable oils.

It freezes at about 3 °C.

IDENTIFICATION

Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *malathion CRS*.

TESTS

Relative density (2.2.5): 1.220 to 1.240.

Optical rotation (2.2.7). Dissolve 2.50 g in *alcohol R* and dilute to 25.0 ml with the same solvent. The angle of optical rotation is -0.1° to $+0.1^\circ$.

Related substances. Examine by liquid chromatography (2.2.29) as prescribed under Assay.

Inject 20 µl of test solution (a) and 20 µl of reference solution (d). In the chromatogram obtained with test solution (a): the area of any peak due to impurity A is not greater than three times the area of the corresponding peak in the chromatogram obtained with reference solution (d) (0.3 per cent); the area of any peak due to impurity B is not greater than the area of the corresponding peak in the chromatogram obtained with reference solution (d) (0.1 per cent); the sum of the areas of all the peaks apart from the principal peak and any peak due to impurity A or impurity B is not greater than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (1 per cent). Disregard any peak with an area less than 0.1 times that of the area of the principal peak in the chromatogram obtained with reference solution (b).

Water (2.5.12). Not more than 0.1 per cent, determined on 2.000 g by the semi-micro determination of water.

ASSAY

Examine by liquid chromatography (2.2.29).

Test solution (a). Dissolve 0.10 g of the substance to be examined in a mixture of 1 volume of *water R* and 3 volumes of *acetonitrile R* and dilute to 5.0 ml with the same mixture of solvents.

Test solution (b). Take 1.0 ml of test solution (a) and dilute to 10.0 ml with a mixture of 1 volume of *water R* and 3 volumes of *acetonitrile R*.

Reference solution (a). Dissolve 0.100 g of *malathion CRS* in a mixture of 1 volume of *water R* and 3 volumes of *acetonitrile R* and dilute to 50.0 ml with the same mixture of solvents.

Reference solution (b). Take 0.5 ml of test solution (a) and dilute to 100.0 ml with a mixture of 1 volume of *water R* and 3 volumes of *acetonitrile R*.

Reference solution (c). Dissolve 5.0 mg of *malathion impurity A CRS* and 5.0 mg of *malathion impurity B CRS* in a mixture of 1 volume of *water R* and 3 volumes of *acetonitrile R* and dilute to 50.0 ml with the same mixture of solvents.

Reference solution (d). Take 2.0 ml of reference solution (c) and dilute to 10.0 ml with a mixture of 1 volume of *water R* and 3 volumes of *acetonitrile R*.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.15 m long and 4.6 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (10 µm),
- as mobile phase at a flow rate of 1 ml/min a mixture of 45 volumes of *acetonitrile R* and 55 volumes of *water R*,
- as detector a spectrophotometer set at 210 nm, maintaining the temperature of the column at 35 °C.

Inject 20 µl of reference solution (b) and 20 µl of reference solution (c).

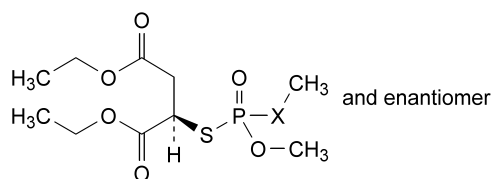
When the chromatograms are recorded in the prescribed conditions, the retention times are: impurity B about 3.5 min; impurity A about 5 min and malathion about 16 min. Adjust the sensitivity of the system so that the height of the principal peak in the chromatogram obtained with reference solution (b) is at least 50 per cent of the full scale of the recorder. The test is not valid unless in the chromatogram obtained with reference solution (c) the resolution between the peaks corresponding to impurity A and impurity B is at least 2.0. Inject reference solution (a) six times. The assay is not valid unless the relative standard deviation of the peak area for malathion is at most 1.0 per cent.

Inject alternately test solution (b) and reference solution (a). Calculate the percentage content of malathion.

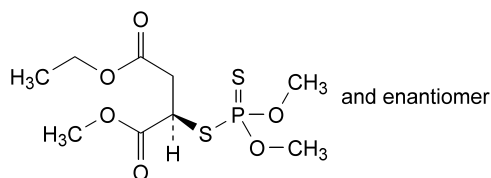
STORAGE

Store in an airtight container, protected from light.

IMPURITIES



- X = S: diethyl (2RS)-2-[(methoxy)(methylsulphonyl)-S-phosphinodithioyl]butanedioate (isomalathion),
- X = O: diethyl (2RS)-2-(dimethoxy-S-phosphinodithioyl)butanedioate (maloxon),

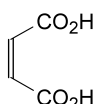


C. ethyl and methyl (2*RS*)-2-(dimethoxyphosphinodithiyl)butanedioate (methyl analogue).

01/2005:0365

MALEIC ACID

Acidum maleicum



$C_4H_4O_4$

M_r 116.1

DEFINITION

Maleic acid contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of (*Z*)-butenedioic acid, calculated with reference to the anhydrous substance.

CHARACTERS

A white, crystalline powder, freely soluble in water and in alcohol.

IDENTIFICATION

- Dilute 5 ml of solution S (see Tests) to 10 ml with *water R*. The pH of the dilution is less than 2.
- Examine the chromatograms obtained in the test for fumaric acid. The principal spot in the chromatogram obtained with test solution (b) is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a).
- Dissolve 0.1 g in 10 ml of *water R* (solution a). To 0.3 ml of solution (a) add a solution of 10 mg of *resorcinol R* in 3 ml of *sulphuric acid R*. Heat on a water-bath for 15 min; no colour develops. To 3 ml of solution (a) add 1 ml of *bromine water R*. Heat on a water-bath to remove the bromine (15 min), heat to boiling and cool. To 0.2 ml of this solution add a solution of 10 mg of *resorcinol R* in 3 ml of *sulphuric acid R*. Heat on a water-bath for 15 min. A violet-pink colour develops.

TESTS

Solution S. Dissolve 5.0 g in *water R* and dilute to 50 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and not more intensely coloured than reference solution Y_7 (2.2.2, Method II).

Fumaric acid. Examine by thin-layer chromatography (2.2.27), using *silica gel GF₂₅₄ R* as the coating substance.

Test solution (a). Dissolve 0.5 g of the substance to be examined in *acetone R* and dilute to 5 ml with the same solvent.

Test solution (b). Dilute 1 ml of test solution (a) to 50 ml with *acetone R*.

Reference solution (a). Dissolve 20 mg of *maleic acid CRS* in *acetone R* and dilute to 10 ml with the same solvent.

Reference solution (b). Dissolve 15 mg of *fumaric acid CRS* in *acetone R* and dilute to 10 ml with the same solvent.

Reference solution (c). Mix 5 ml of reference solution (a) and 5 ml of reference solution (b).

Apply separately to the plate 5 µl of test solutions (a) and (b), 5 µl of reference solutions (a) and (b) and 10 µl of reference solution (c). Develop in a non-saturated tank over a path of 10 cm using a mixture of 12 volumes of *anhydrous formic acid R*, 16 volumes of *chloroform R*, 32 volumes of *butanol R* and 44 volumes of *heptane R*. Dry the plate at 100 °C for 15 min and examine in ultraviolet light at 254 nm. Any spot corresponding to fumaric acid in the chromatogram obtained with test solution (a) is not more intense than the spot in the chromatogram obtained with reference solution (b) (1.5 per cent). The test is not valid unless the chromatogram obtained with reference solution (c) shows two clearly separated spots.

Iron. To 10 ml of solution S add 2 ml of *dilute hydrochloric acid R* and 0.05 ml of *bromine water R*. After 5 min, remove the excess of bromine by passing a current of air and add 3 ml of *potassium thiocyanate solution R*. Shake. Prepare a standard at the same time and in the same manner, using a mixture of 5 ml of *iron standard solution (1 ppm Fe) R*, 1 ml of *dilute hydrochloric acid R*, 6 ml of *water R* and 0.05 ml of *bromine water R*. Allow both solutions to stand for 5 min. Any red colour in the test solution is not more intense than that in the standard (5 ppm).

Heavy metals (2.4.8). 1.0 g complies with limit test D for heavy metals (10 ppm). Prepare the standard using 1 ml of *lead standard solution (10 ppm Pb) R*.

Water (2.5.12). Not more than 2.0 per cent, determined on 1.00 g by the semi-micro determination of water.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.500 g in 50 ml of *water R*. Titrate with 1 *M sodium hydroxide* using 0.5 ml of *phenolphthalein solution R* as indicator.

1 ml of 1 *M sodium hydroxide* is equivalent to 58.04 mg of $C_4H_4O_4$.

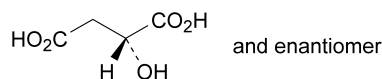
STORAGE

Store in a glass container, protected from light.

01/2005:2080

MALIC ACID

Acidum malicum



$C_4H_6O_5$

M_r 134.1

DEFINITION

(2*RS*)-2-Hydroxybutanedioic acid.

Content: 99.0 per cent to 101.0 per cent (anhydrous substance).

CHARACTERS

Appearance: white, crystalline powder.

Solubility: freely soluble in water and in alcohol, sparingly soluble in acetone.

IDENTIFICATION

A. Melting point (2.2.14): 128 °C to 132 °C.

B. Infrared absorption spectrophotometry (2.2.24).

Comparison: *Ph. Eur. reference spectrum of malic acid.*

TESTS

Solution S. Dissolve 5.00 g in *water R* and dilute to 25 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and colourless (2.2.2, *Method II*).

Optical rotation (2.2.7): -0.10° to $+0.10^{\circ}$, determined on solution S.

Water-insoluble substances: maximum 0.1 per cent.

Dissolve 25.0 g in 100 ml of *water R*, filter the solution through a tared sintered-glass filter crucible (16), wash the filter with hot *water R* and dry at 100–105 °C to constant weight. The residue weighs a maximum of 25 mg.

Related substances. Liquid chromatography (2.2.29).

Test solution. Dissolve 100.0 mg of the substance to be examined in the mobile phase and dilute to 100.0 ml with the mobile phase.

Reference solution (a). Dissolve 10.0 mg of *fumaric acid R* and 4.0 mg of *maleic acid R* in 25 ml of the mobile phase and dilute to 50.0 ml with the mobile phase.

Reference solution (b). Dilute 2.5 ml of reference solution (a) to 100.0 ml with the mobile phase.

Reference solution (c). Dissolve 20.0 mg of the substance to be examined in the mobile phase, add 1.0 ml of reference solution (a) and dilute to 20.0 ml with the mobile phase.

Column:

- **size:** $l = 0.30$ m, $\varnothing = 7.8$ mm,
- **stationary phase:** ion-exclusion resin for chromatography *R* (9 μ m),
- **temperature:** 37 °C.

Mobile phase: 0.005 *M* sulphuric acid.

Flow rate: 0.6 ml/min.

Detection: spectrophotometer at 210 nm.

Injection: 20 μ l.

Run time: twice the retention time of the principal peak in the chromatogram obtained with the test solution.

Relative retention with reference to malic acid (retention time = about 10 min): impurity B = about 0.8; impurity A = about 1.5.

System suitability: reference solution (c):

- **resolution:** minimum 2.5 between the peaks due to impurity B and malic acid.

Limits:

- **impurity A:** not more than twice the area of the corresponding peak in the chromatogram obtained with reference solution (b) (1.0 per cent),
- **impurity B:** not more than 0.25 times the area of the corresponding peak in the chromatogram obtained with reference solution (b) (0.05 per cent),
- **any other impurity:** for each impurity, not more than 0.5 times the area of the peak due to impurity B in the chromatogram obtained with reference solution (b) (0.1 per cent),
- **total of other impurities:** not more than 2.5 times the area of the peak due to impurity B in the chromatogram obtained with reference solution (b) (0.5 per cent),
- **disregard limit:** 0.1 times the area of the peak due to impurity B in the chromatogram obtained with reference solution (b) (0.02 per cent).

Heavy metals (2.4.8): maximum 20 ppm.

1.0 g complies with limit test F. Prepare the standard using 2 ml of *lead standard solution* (10 ppm *Pb*) *R*.

Water (2.5.12): maximum 2.0 per cent, determined on 1.00 g.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

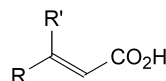
ASSAY

Dissolve 0.500 g in 50 ml of *carbon dioxide-free water R*. Titrate with 1 *M* sodium hydroxide determining the end-point potentiometrically (2.2.20).

1 ml of 1 *M* sodium hydroxide is equivalent to 67.05 mg of $C_4H_6O_5$.

IMPURITIES

Specified impurities: A, B.



A. $R = CO_2H$, $R' = H$: (*E*)-butenedioic acid (fumaric acid),

B. $R = H$, $R' = CO_2H$: (*Z*)-butenedioic acid (maleic acid).

01/2005:1541

MALLOW FLOWER

Malvae sylvestris flos

DEFINITION

Mallow flower consists of the whole or fragmented dried flower of *Malva sylvestris* L. or its cultivated varieties.

CHARACTERS

It has the macroscopic and microscopic characters described under identification tests A and B.

IDENTIFICATION

- A. The flower consists of an epicalyx with three oblong or elliptical-lanceolate parts that are shorter than those of the calyx and situated immediately below it; a calyx with five pubescent triangular lobes, gamosepalous at the base; a corolla three to four times longer than the calyx with five wedge-shaped, notched petals fused to the staminal tube at their base; numerous stamens, the filaments of which fuse into a staminal tube covered by small star-shaped trichomes and occasional simple trichomes visible using a lens; numerous wrinkled carpels, glabrous or sometimes pubescent, enclosed in the staminal tube and arranged into a circle around a central style ending with numerous filiform stigmas. In cultivated varieties, the epicalyx is 3 to 7 partite, the calyx 5 to 8 partite and the corolla 5 to 10 partite.
- B. Reduce to a powder (355). The powder is bluish-grey. Examine under a microscope using *chloral hydrate solution R*. The powder shows unicellular, thick-walled, stiff trichomes, up to 2 mm in length; small unicellular covering trichomes, somewhat curved, either isolated or in small star-shaped groups of 2 to 6; capitate glandular trichomes with multicellular heads; mesophyll fragments with vessels accompanied by cluster crystals of calcium oxalate; spherical pollen grains, about 150 μ m in diameter, with a roughly spiny exine.

When mounted with *alcohol R*, numerous elongated cells containing mucilage are seen in the petal fragments.

- C. Examine by thin-layer chromatography (2.2.27) using a *TLC silica gel plate R*.

Test solution. To 1 g of the powdered drug (355) add 10 ml of *alcohol* (60 per cent *V/V*) *R*. Stir for 15 min and filter.

Reference solution. A 0.5 g/l solution of *quinaldine red R* in *alcohol R*.

Apply to the plate as bands 10 µl of the test solution and 5 µl of the reference solution.

Develop over a path of 10 cm using a mixture of 15 volumes of *acetic acid R*, 30 volumes of *water R* and 60 volumes of *butanol R*. Allow the plate to dry in air. Examine the chromatograms in daylight. The chromatogram obtained with the reference solution shows an orange-red zone in the upper part of the middle third. The chromatogram obtained with the test solution shows, below the zone in the chromatogram obtained with the reference solution, two violet zones in the middle third; the principal zone (6''-malonyl malvin) is situated just below the other violet zone (malvin).

TESTS

Foreign matter (2.8.2). It complies with the test for foreign matter.

Swelling index (2.8.4). Not less than 15, determined on 0.2 g of the powdered drug (710) humidified with 0.5 ml of *ethanol R*.

Loss on drying (2.2.32). Not more than 12.0 per cent, determined on 1.000 g of the powdered drug by drying in an oven at 100 °C to 105 °C.

Total ash (2.4.16). Not more than 14.0 per cent.

Ash insoluble in hydrochloric acid (2.8.1). Not more than 2.0 per cent.

STORAGE

Store protected from light.

B. Melting point (2.2.14): 148 °C to 151 °C.

C. Dissolve 5.00 g in *water R* and dilute to 100.0 ml with the same solvent. The specific optical rotation (2.2.7) is + 105.5 to + 108.5, calculated with reference to the anhydrous substance.

D. Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel G plate R*.

Test solution. Dissolve 25 mg of the substance to be examined in *water R* and dilute to 10 ml with the same solvent.

Reference solution (a). Dissolve 25 mg of *maltitol CRS* in *water R* and dilute to 10 ml with the same solvent.

Reference solution (b). Dissolve 25 mg of *maltitol CRS* and 25 mg of *sorbitol CRS* in *water R* and dilute to 10 ml with the same solvent.

Apply to the plate 2 µl of each solution. Develop over a path of 17 cm using a mixture of 10 volumes of *water R*, 20 volumes of *ethyl acetate R* and 70 volumes of *propanol R*. Allow the plate to dry in air and spray with *4-aminobenzoic acid solution R*. Dry the plate in a current of cold air until the acetone is removed. Heat the plate at 100–105 °C for 15 min. Allow to cool and spray with a 2 g/l solution of *sodium periodate R*. Dry the plate in a current of cold air. Heat the plate at 100 °C for 15 min. The principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution (a). The test is not valid unless the chromatogram obtained with reference solution (b) shows two clearly separated spots.

TESTS

Appearance of solution. Dissolve 5.0 g in *water R* and dilute to 50 ml with the same solvent. The solution is clear (2.2.1) and colourless (2.2.2, *Method II*).

Conductivity (2.2.38). Not more than 20 µS cm⁻¹.

Dissolve 20.0 g in *carbon dioxide-free water R* prepared from *distilled water R* and dilute to 100.0 ml with the same solvent. Measure the conductivity of the solution at a temperature of 20 °C, while gently stirring with a magnetic stirrer.

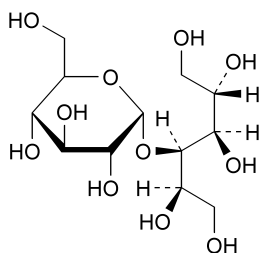
Reducing sugars. Dissolve 5.0 g in 6 ml of *water R* with the aid of gentle heat. Cool and add 20 ml of *cupri-citric solution R* and a few glass beads. Heat so that boiling begins after 4 min and maintain boiling for 3 min. Cool rapidly and add 100 ml of a 2.4 per cent V/V solution of *glacial acetic acid R* and 20.0 ml of 0.025 M *iodine*. With continuous shaking, add 25 ml of a mixture of 6 volumes of *hydrochloric acid R* and 94 volumes of *water R* and, when the precipitate has dissolved, titrate the excess of iodine with 0.05 M *sodium thiosulphate* using 1 ml of *starch solution R*, added towards the end of the titration, as indicator. Not less than 12.8 ml of 0.05 M *sodium thiosulphate* is required (0.2 per cent, calculated as glucose equivalent).

Related substances. Examine by liquid chromatography (2.2.29) as described under Assay. Inject 20 µl of reference solution (b). Adjust the sensitivity of the system so that the height of the peak due to maltitol is at least 50 per cent of the full scale of the recorder. Inject 20 µl of the test solution and continue the chromatogram for three times the retention time of maltitol. In the chromatogram obtained with the test solution: the area of any peak, apart from the principal peak, is not greater than the area of the principal peak in the chromatogram obtained with reference solution (b) (1 per cent); the sum of the areas of all the peaks, apart from the principal peak, is not greater than twice the area of the principal peak in the chromatogram obtained with reference

01/2005:1235

MALTITOL

Maltitolum



C₁₂H₂₄O₁₁

M_r 344.3

DEFINITION

Maltitol contains not less than 98.0 per cent and not more than the equivalent of 102.0 per cent of 4-O-α-D-glucopyranosyl-D-glucitol (D-maltitol), calculated with reference to the anhydrous substance.

CHARACTERS

A white, crystalline powder, very soluble in water, practically insoluble in ethanol.

IDENTIFICATION

First identification: A.

Second identification: B, C, D.

A. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *maltitol CRS*. Examine the substances prepared as discs.

solution (b) (2 per cent). Disregard any peak with an area less than the area of the principal peak in the chromatogram obtained with reference solution (c) (0.1 per cent).

Lead (2.4.10). It complies with the limit test for lead in sugars (0.5 ppm).

Nickel (2.4.15). It complies with the limit test for nickel in polyols (1 ppm).

Water (2.5.12). Not more than 1.0 per cent, determined on 1.00 g by the semi-micro determination of water.

Microbial contamination. If intended for use in the manufacture of parenteral dosage forms the total viable aerobic count (2.6.12) is not more than 10^2 bacteria and 10^2 fungi per gram, determined by plate count; it complies with the tests for *Escherichia coli* and *Salmonella* (2.6.13).

Bacterial endotoxins (2.6.14): if intended for use in the manufacture of parenteral dosage forms without a further appropriate procedure for the removal of bacterial endotoxins, less than 4 IU/g for parenteral dosage forms having a concentration of less than 100 g/l of maltitol, and less than 2.5 IU/g for parenteral dosage forms having a concentration of 100 g/l or more of maltitol.

ASSAY

Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 5.0 g of the substance to be examined in 20 ml of *water R* and dilute to 100.0 ml with the same solvent.

Reference solution (a). Dissolve 0.50 g of *maltitol CRS* in 2.0 ml of *water R* and dilute to 10.0 ml with the same solvent.

Reference solution (b). Dilute 1.0 ml of the test solution to 100.0 ml with *water R*.

Reference solution (c). Dilute 10.0 ml of reference solution (b) to 100.0 ml with *water R*.

Reference solution (d). Dissolve 0.5 g of *maltitol R* and 0.5 g of *sorbitol R* in 5 ml of *water R* and dilute to 10.0 ml with the same solvent.

The chromatography may be carried out using:

- a stainless steel column 0.3 m long and 7.8 mm in internal diameter packed with *strong cation exchange resin (calcium form) R* (9 µm) and maintained at 85 ± 1 °C,
- as mobile phase at a flow rate of 0.5 ml/min, degassed *water R*,
- as detector a refractometer maintained at a constant temperature.

Inject 20 µl of reference solution (d). Continue the chromatography for three times the retention time of maltitol.

When the chromatograms are recorded in the prescribed conditions, the retention time of maltitol is about 16 min and the relative retentions with reference to maltitol are: sorbitol about 1.8 and maltotriitol about 0.8. The test is not valid unless the resolution between the peaks due to maltitol and to sorbitol is at least 2 in the chromatogram obtained with reference solution (d).

Inject 20 µl of the test solution and 20 µl of reference solution (a). Continue the chromatography for three times the retention time of maltitol.

Calculate the percentage content of D-maltitol from the areas of the peaks and the declared content of *maltitol CRS*.

LABELLING

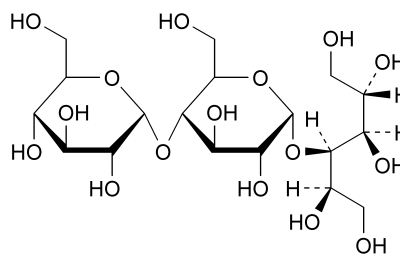
The label states:

- where applicable, the maximum concentration of bacterial endotoxins,

- where applicable, that the substance is suitable for use in the manufacture of parenteral dosage forms.

IMPURITIES

A. sorbitol,



B. *O*-α-D-glucopyranosyl-(1→4)-*O*-α-D-glucopyranosyl-(1→4)-D-glucitol (maltotriitol).

01/2005:1236
corrected

MALTITOL, LIQUID

Maltitolum liquidum

DEFINITION

Liquid maltitol is an aqueous solution of a hydrogenated, partly hydrolysed starch. It contains not less than 68.0 per cent *m/m* and not more than 85.0 per cent *m/m* of anhydrous substance composed of a mixture of mainly 4-*O*-α-D-glucopyranosyl-D-glucitol (D-maltitol) with D-glucitol (D-sorbitol) and hydrogenated oligo- and polysaccharides. It contains not less than 50.0 per cent *m/m* of D-maltitol ($C_{12}H_{24}O_{11}$) and not more than 8.0 per cent *m/m* of D-sorbitol ($C_6H_{14}O_6$), both calculated with reference to the anhydrous substance. It contains not less than 95.0 per cent and not more than 105.0 per cent of the content of D-maltitol stated on the label.

CHARACTERS

A clear, colourless, syrupy liquid, miscible with water and with glycerol.

IDENTIFICATION

First identification: A.

Second identification: B, C.

- Examine the chromatograms obtained in the assay. The principal peak in the chromatogram obtained with the test solution is similar in retention time to the principal peak in the chromatogram obtained with reference solution (a).
- To 3 ml of a freshly prepared 100 g/l solution of *pyrocatechol R*, add 6 ml of *sulphuric acid R* while cooling in iced water. To 3 ml of the cooled mixture, add 0.3 ml of solution S (see Tests). Heat gently over a naked-flame for about 30 s. A pink colour develops.
- Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel G plate R*.

Test solution. Dilute 0.35 g of the substance to be examined to 100 ml with *water R*.

Reference solution (a). Dissolve 20 mg of *maltitol CRS* in *water R* and dilute to 10 ml with the same solvent.

Reference solution (b). Dissolve 20 mg of *maltitol CRS* and 20 mg of *sorbitol CRS* in *water R* and dilute to 10 ml with the same solvent.

Apply to the plate 2 µl of each solution. Develop over a path of 17 cm using a mixture of 10 volumes of *water R*, 20 volumes of *ethyl acetate R* and 70 volumes

of *propanol R*. Allow the plate to dry in air and spray with *4-aminobenzoic acid solution R*. Dry the plate in a current of cold air until the acetone is removed. Heat the plate at 100 °C to 105 °C for 15 min. Allow to cool and spray with a 2 g/l solution of *sodium periodate R*. Dry the plate in a current of cold air. Heat the plate at 100 °C for 15 min. The principal spot in the chromatogram obtained with the test solution is similar in position and colour to the principal spot in the chromatogram obtained with the reference solution (a). The test is not valid unless the chromatogram obtained with reference solution (b) shows two clearly separated spots.

TESTS

Solution S. Dilute 7.0 g to 50 ml with *water R*.

Appearance of solution. Solution S is clear (2.2.1) and colourless (2.2.2, *Method II*).

Conductivity (2.2.38). Not more than 10 µS cm⁻¹ measured on the undiluted liquid maltitol while gently stirring with a magnetic stirrer.

Reducing sugars. To 5.0 g add 6 ml of *water R*, 20 ml of *cupri-citric solution R* and a few glass beads. Heat so that boiling begins after 4 min and maintain boiling for 3 min. Cool rapidly and add 100 ml of a 2.4 per cent V/V solution of *glacial acetic acid R* and 20.0 ml of 0.025 M *iodine*. With continuous shaking, add 25 ml of a mixture of 6 volumes of *hydrochloric acid R* and 94 volumes of *water R* and, when the precipitate has dissolved, titrate the excess of iodine with 0.05 M *sodium thiosulphate* using 1 ml of *starch solution R*, added towards the end of the titration, as indicator. Not less than 12.8 ml of 0.05 M *sodium thiosulphate* is required (0.2 per cent calculated as glucose equivalent).

Lead (2.4.10). It complies with the limit test for lead in sugars (0.5 ppm).

Nickel (2.4.15). It complies with the limit test for nickel in polyols (1 ppm).

Water (2.5.12). Not less than 15.0 per cent and not more than 32.0 per cent *m/m* determined on 0.100 g by the semi-micro determination of water.

ASSAY

Examine by liquid chromatography (2.2.29).

Test solution. Mix 1.00 g of the solution to be examined with 20 ml of *water R* and dilute to 50.0 ml with the same solvent.

Reference solution (a). Dissolve 50.0 mg of *maltitol CRS* in 2 ml of *water R* and dilute to 5.0 ml with the same solvent.

Reference solution (b). Dissolve 8.0 mg of *sorbitol CRS* in 2 ml of *water R* and dilute to 5.0 ml with the same solvent.

Reference solution (c). Dissolve 50 mg of *maltitol R* and 50 mg of *sorbitol R* in 2 ml of *water R* and dilute to 5.0 ml with the same solvent.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.3 m long and 7.8 mm in internal diameter packed with a *strong cation exchange resin (calcium form) R* (9 µm) and maintained at 85 °C ± 2 °C,
- as mobile phase at a flow rate of 0.5 ml/min degassed *water R*,
- as detector a refractometer maintained at a constant temperature.

Inject 20 µl of reference solution (c). Continue the chromatography for three times the retention time of maltitol.

When the chromatogram is recorded in the prescribed conditions, the retention time of maltitol is about 16 min and the relative retention of sorbitol is about 1.8. The test is not

valid unless the resolution between the peaks due to sorbitol and to maltitol is at least 2 in the chromatogram obtained with reference solution (c).

Inject 20 µl of the test solution, 20 µl of reference solution (a) and 20 µl of reference solution (b). Continue the chromatography for three times the retention time of maltitol.

Calculate the percentage contents of D-maltitol and D-sorbitol from the areas of the peaks and the declared contents of *maltitol CRS* and *sorbitol CRS*.

LABELLING

The label states the content of D-maltitol.

01/2005:1542

MALTODEXTRIN

Maltodextrinum

DEFINITION

Maltodextrin is a mixture of glucose, disaccharides and polysaccharides, obtained by the partial hydrolysis of starch. The degree of hydrolysis, expressed as dextrose equivalent (DE), is not more than 20 (nominal value).

CHARACTERS

A white or almost white, slightly hygroscopic powder or granules, freely soluble in water.

IDENTIFICATION

- Dissolve 0.1 g in 2.5 ml of *water R* and heat with 2.5 ml of *cupri-tartaric solution R*. A red precipitate is formed.
- Dip, for 1 s, a suitable stick with a reactive pad containing glucose-oxidase, peroxidase and a hydrogen-donating substance, such as tetramethylbenzidine, in a 100 g/l solution of the substance to be examined. Observe the colour of the reactive pad; within 60 s the colour changes from yellow to green or blue.
- It is a powder or granules.
- It complies with the test for dextrose equivalent (see Tests).

TESTS

Solution S. Dissolve 12.5 g in *carbon dioxide-free water R* and dilute to 50.0 ml with the same solvent.

pH (2.2.3). The pH of a mixture of 30 ml of solution S and 1 ml of a 223.6 g/l solution of *potassium chloride R* is 4.0 to 7.0.

Sulphur dioxide (2.5.29). Not more than 20 ppm.

Heavy metals (2.4.8). Dilute 4 ml of solution S to 30 ml with *water R*. The solution complies with limit test E for heavy metals (10 ppm). Prepare the standard using 10 ml of *lead standard solution (1 ppm Pb) R*.

Loss on drying (2.2.32). Not more than 6.0 per cent, determined on 10.00 g by drying in an oven at 100-105 °C.

Sulphated ash (2.4.14). Not more than 0.5 per cent, determined on 1.0 g.

Dextrose equivalent. Weigh an amount of the sample to be examined equivalent to 2.85-3.15 g of reducing carbohydrates, calculated as dextrose equivalent, into a 500 ml volumetric flask. Dissolve in *water R* and dilute to 500.0 ml with the same solvent. Transfer the solution to a 50 ml burette.

Pipette 25.0 ml of *cupri-tartaric solution R* into a 250 ml flask and add 18.5 ml of the sample solution from the

burette, mix and add boiling chips. Place the flask on a hot plate, previously adjusted so that the solution begins to boil within 2 min ± 15 s. Allow to boil for exactly 120 s, add 1 ml of a 1 g/l solution of *methylene blue R* and titrate with the sample solution (V_1) until the blue colour disappears. Maintain the solution at boiling throughout the titration. Standardise the cupri-tartaric solution using a 6.00 g/l solution of *glucose R* (V_0).

Calculate the dextrose equivalent (DE) from the equation:

$$DE = \frac{300 \times V_0 \times 100}{V_1 \times M \times D}$$

V_0 = total volume of glucose standard solution, in millilitres,

V_1 = total volume of sample solution, in millilitres,

M = sample weight, in grams,

D = percentage content of dry matter in the substance.

The dextrose equivalent (DE) is within 2 DE units of the nominal value.

Microbial contamination. Total viable aerobic count (2.6.12) not more than 10^3 bacteria and 10^2 fungi per gram, determined by plate count. It complies with the test for *Escherichia coli* and *Salmonella* (2.6.13).

LABELLING

The label states the dextrose equivalent (DE) (= nominal value).

01/2005:1543

MANGANESE SULPHATE MONOHYDRATE

Mangani sulfas monohydricus

$MnSO_4 \cdot H_2O$

M_r 169.0

DEFINITION

Manganese sulphate monohydrate contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of $MnSO_4$, calculated with reference to the ignited substance.

CHARACTERS

A pale pink crystalline powder, slightly hygroscopic, freely soluble in water, practically insoluble in alcohol.

IDENTIFICATION

- Solution S (see Tests) gives reaction (a) of sulphates (2.3.1).
- Dissolve 50 mg in 5 ml of *water R*. Add 0.5 ml of *ammonium sulphide solution R*. A pale pink precipitate is formed which dissolves on the addition of 1 ml of *anhydrous acetic acid R*.
- It complies with the test for loss on ignition (see Tests).

TESTS

Solution S. Dissolve 10.0 g in *distilled water R* and dilute to 100 ml with the same solvent.

Appearance of solution. Solution S is not more opalescent than reference suspension II (2.2.1).

Chlorides (2.4.4). Dilute 5 ml of solution S to 15 ml with *water R*. The solution complies with the limit test for chlorides (100 ppm).

Iron (2.4.9). 10 ml of solution S complies with the limit test for iron (10 ppm).

Zinc. To 10 ml of solution S add 1 ml of *sulphuric acid R* and 0.1 ml of *potassium ferrocyanide solution R*. After 30 s, any opalescence in the solution is not more intense than that in a mixture of 5 ml of *zinc standard solution (10 ppm Zn) R*, 5 ml of *water R*, 1 ml of *sulphuric acid R* and 0.1 ml of *potassium ferrocyanide solution R* (50 ppm).

Heavy metals (2.4.8). 12 ml of solution S complies with limit test A for heavy metals (20 ppm). Prepare the standard using *lead standard solution (2 ppm Pb) R*.

Loss on ignition: 10.0 per cent to 12.0 per cent, determined on 1.00 g at 500 °C.

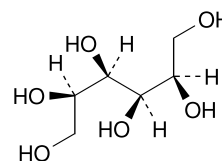
ASSAY

Dissolve 0.150 g in 50 ml of *water R*. Add 10 mg of *ascorbic acid R*, 20 ml of *ammonium chloride buffer solution pH 10.0 R* and 0.2 ml of a 2 g/l solution of *mordant black 11 R* in *triethanolamine R*. Titrate with 0.1 M *sodium edetate* until the colour changes from violet to pure blue. 1 ml of 0.1 M *sodium edetate* is equivalent to 15.10 mg of $MnSO_4$.

01/2005:0559

MANNITOL

Mannitolum



$C_6H_{14}O_6$

M_r 182.2

DEFINITION

Mannitol contains not less than 98.0 per cent and not more than the equivalent of 102.0 per cent of D-mannitol, calculated with reference to the anhydrous substance.

CHARACTERS

White or almost white, crystalline powder or free-flowing granules, freely soluble in water, very slightly soluble in alcohol.

It shows polymorphism.

IDENTIFICATION

First identification: A.

Second identification: B, C, D.

- Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *mannitol CRS*. Examine the substances prepared as discs. If the spectra obtained show differences, dissolve the substance to be examined and the reference substance separately in *water R*, evaporate to dryness and record new spectra using the residues.

- Melting point (2.2.14): 165 °C to 170 °C.

- Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel G plate R*.

Test solution. Dissolve 25 mg of the substance to be examined in *water R* and dilute to 10 ml with the same solvent.

Reference solution (a). Dissolve 25 mg of *mannitol CRS* in *water R* and dilute to 10 ml with the same solvent.

Reference solution (b). Dissolve 25 mg of mannitol CRS and 25 mg of sorbitol CRS in water R and dilute to 10 ml with the same solvent.

Apply to the plate 2 µl of each solution. Develop over a path of 17 cm using a mixture of 10 volumes of water R, 20 volumes of ethyl acetate R and 70 volumes of propanol R. Allow the plate to dry in air and spray with 4-aminobenzoic acid solution R. Dry the plate in a current of cold air until the acetone is removed. Heat the plate at 100 °C for 15 min. Allow to cool and spray with a 2 g/l solution of sodium periodate R. Dry the plate in a current of cold air. Heat the plate at 100 °C for 15 min. The principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a). The test is not valid unless the chromatogram obtained with reference solution (b) shows 2 clearly separated spots.

- D. Dissolve 2.00 g of the substance to be examined and 2.6 g of disodium tetraborate R in about 20 ml of water R at a temperature of 30 °C; shake continuously for 15-30 min without further heating. Dilute the resulting clear solution to 25.0 ml with water R. The specific optical rotation (2.2.7) is + 23 to + 25, calculated with reference to the anhydrous substance.

TESTS

Appearance of solution. Dissolve 5.0 g in water R and dilute to 50 ml with the same solvent. The solution is clear (2.2.1) and colourless (2.2.2, Method II).

Conductivity (2.2.38). Not more than 20 µS·cm⁻¹.

Dissolve 20.0 g in carbon dioxide-free water R prepared from distilled water R and dilute to 100.0 ml with the same solvent. Measure the conductivity of the solution while gently stirring with a magnetic stirrer.

Reducing sugars. Dissolve 5.0 g in 25 ml of water R with the aid of gentle heat. Cool and add 20 ml of cupri-citric solution R and a few glass beads. Heat so that boiling begins after 4 min and maintain boiling for 3 min. Cool rapidly and add 100 ml of a 2.4 per cent V/V solution of glacial acetic acid R and 20.0 ml of 0.025 M iodine. With continuous shaking, add 25 ml of a mixture of 6 volumes of hydrochloric acid R and 94 volumes of water R and, when the precipitate has dissolved, titrate the excess of iodine with 0.05 M sodium thiosulphate using 1 ml of starch solution R, added towards the end of the titration, as indicator. Not less than 12.8 ml of 0.05 M sodium thiosulphate is required (0.2 per cent, calculated as glucose equivalent).

Related substances. Examine by liquid chromatography (2.2.29) as described under Assay. Inject 20 µl of reference solution (b). Adjust the sensitivity of the system so that the height of the peak due to mannitol is at least 50 per cent of the full scale of the recorder. Inject 20 µl of the test solution and of reference solution (c) and continue the chromatography for twice the retention time of mannitol. In the chromatogram obtained with the test solution: the area of any peak, apart from the principal peak, is not greater than the area of the principal peak in the chromatogram obtained with reference solution (b) (2 per cent); the sum of the areas of all the peaks, apart from the principal peak, is not greater than the area of the principal peak in the chromatogram obtained with reference solution (b) (2 per cent). Disregard any peak with an area less than the area of the principal peak in the chromatogram obtained with reference solution (c) (0.1 per cent).

Lead (2.4.10). It complies with the limit test for lead in sugars (0.5 ppm). Dissolve the substance to be examined in 150.0 ml of the prescribed mixture of solvents.

Nickel (2.4.15). It complies with the limit test for nickel in polyols (1 ppm). Dissolve the substance to be examined in 150.0 ml of the prescribed mixture of solvents.

Water (2.5.12). Not more than 0.5 per cent, determined on 1.00 g by the semi-micro determination of water.

Microbial contamination. If intended for use in the manufacture of parenteral dosage forms, the total viable aerobic count (2.6.12) is not more than 10² bacteria and 10² fungi per gram, determined by plate count. It complies with the tests for *Escherichia coli* and *Salmonella* (2.6.13).

Bacterial endotoxins (2.6.14): if intended for use in the manufacture of parenteral dosage forms without a further appropriate procedure for the removal of bacterial endotoxins, less than 4 IU/g for parenteral dosage forms having a concentration of 100 g/l or less of mannitol, and less than 2.5 IU/g for parenteral dosage forms having a concentration of more than 100 g/l of mannitol.

ASSAY

Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 5.0 g of the substance to be examined in 25 ml of water R and dilute to 100.0 ml with the same solvent.

Reference solution (a). Dissolve 0.50 g of mannitol CRS in 2.5 ml of water R and dilute to 10.0 ml with the same solvent.

Reference solution (b). Dilute 2.0 ml of the test solution to 100.0 ml with water R.

Reference solution (c). Dilute 5.0 ml of reference solution (b) to 100.0 ml with water R.

Reference solution (d). Dissolve 0.5 g of mannitol R and 0.5 g of sorbitol R in 5 ml of water R and dilute to 10.0 ml with the same solvent.

The chromatography may be carried out using:

- a stainless steel column 0.3 m long and 7.8 mm in internal diameter packed with strong cation exchange resin (calcium form) R (9 µm) and maintained at 85 ± 1 °C,
- as mobile phase at a flow rate of 0.5 ml/min, degassed water R,
- as detector a refractometer maintained at a constant temperature.

Inject 20 µl of reference solution (d). Continue the chromatography for 3 times the retention time of mannitol.

When the chromatograms are recorded in the prescribed conditions, the retention time of mannitol is about 22 min and the relative retention of sorbitol with reference to mannitol is about 1.25. The test is not valid unless the resolution between the peaks due to mannitol and to sorbitol is at least 2 in the chromatogram obtained with reference solution (d).

Inject 20 µl of the test solution and 20 µl of reference solution (a). Continue the chromatography for twice the retention time of mannitol.

Calculate the percentage content of D-mannitol from the areas of the peaks and the declared content of mannitol CRS.

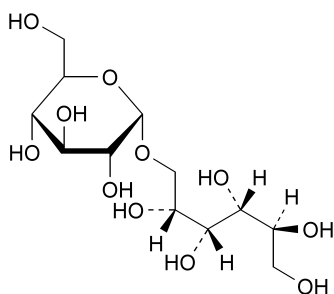
LABELLING

The label states:

- where applicable, the maximum concentration of bacterial endotoxins,
- where applicable, that the substance is suitable for use in the manufacture of parenteral dosage forms.

IMPURITIES

- A. sorbitol,
B. maltitol,

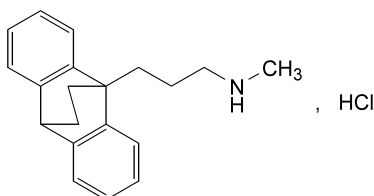


- C. 6-O-α-D-glucopyranosyl-D-glucitol (isomaltitol).

01/2005:1237

MAPROTILINE HYDROCHLORIDE

Maprotilini hydrochloridum

 $C_{20}H_{24}ClN$ M_r 313.9

DEFINITION

Maprotiline hydrochloride contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of 3-(9,10-ethanoanthracen-9(10H)-yl)-N-methylpropan-1-amine hydrochloride, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, crystalline powder, slightly soluble in water, freely soluble in methanol, soluble in alcohol, sparingly soluble in methylene chloride, very slightly soluble in acetone.

It shows polymorphism.

IDENTIFICATION

First identification: B, D.

Second identification: A, C, D.

- Dissolve 10 mg in 1 M hydrochloric acid and dilute to 100 ml with the same acid. Examined between 250 nm and 300 nm (2.2.25), the solution shows two absorption maxima, at 265 nm and 272 nm, and an absorption minimum at 268 nm. The ratio of the absorbance measured at the maximum at 272 nm to that measured at the maximum at 265 nm is 1.1 to 1.3.
- Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *maprotiline hydrochloride CRS*. Examine the substances prepared as discs. If the spectra obtained show differences, dissolve the substance to be examined and the reference substance separately in *methanol R*, evaporate to dryness and record new spectra using the residues.
- Examine by thin-layer chromatography (2.2.27), using as the coating substance a suitable silica gel with a fluorescent indicator having an optimal intensity at 254 nm.

Test solution. Dissolve 25 mg of the substance to be examined in *methanol R* and dilute to 5 ml with the same solvent.

Reference solution (a). Dissolve 25 mg of *maprotiline hydrochloride CRS* in *methanol R* and dilute to 5 ml with the same solvent.

Reference solution (b). Dissolve 10 mg of *maprotiline impurity D CRS* in reference solution (a) and dilute to 2 ml with the same solution.

Apply to the plate 5 µl of each solution. Develop over a path of 10 cm using a mixture of 4 volumes of *ethyl acetate R*, 5 volumes of *dilute ammonia R1* and 14 volumes of *2-butanol R*. Dry the plate in a current of warm air and examine at 254 nm. The principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a). The test is not valid unless the chromatogram obtained with reference solution (b) shows two clearly separated principal spots.

- Dilute 0.5 ml of solution S (see Tests) to 2 ml with *methanol R*. The solution gives reaction (a) of chlorides (2.3.1).

TESTS

Solution S. Dissolve 1.0 g in *methanol R* and dilute to 20 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and not more intensely coloured than reference solution BY₆ (2.2.2, Method II).

Related substances. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 0.10 g of the substance to be examined in the mobile phase and dilute to 100.0 ml with the mobile phase.

Reference solution (a). Dilute 1.0 ml of the test solution to 10.0 ml with the mobile phase. Dilute 2.0 ml of the solution to 100.0 ml with the mobile phase.

Reference solution (b). Dissolve 1.0 mg of *maprotiline impurity D CRS* in the test solution and dilute to 10.0 ml with the same solution.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4.6 mm in internal diameter packed with *silica gel for chromatography R* (5 µm),
- as mobile phase a flow rate of 1 ml/min a mixture as prepared as follows: dissolve about 0.580 g of *ammonium acetate R* in 200 ml of *water R* and add 2 ml of a 70 g/l solution of *concentrated ammonia R*; add 150 ml of *2-propanol R* and 650 ml of *methanol R*; the resulting apparent pH value is between 8.2 and 8.4,
- as detector a spectrophotometer set at 272 nm.

When the chromatograms are recorded in the prescribed conditions, the retention time of maprotiline is about 10.3 min and retention times relative to maprotiline are: impurity A about 0.3, impurity B about 0.47, impurity C about 0.74, impurity D about 0.81, impurity E about 1.26. Inject 20 µl of reference solution (b). Adjust the sensitivity of the system so that the height of the principal peak (maprotiline) in the chromatogram is at least 50 per cent of the full scale of the recorder. The test is not valid unless, in the chromatogram obtained, the resolution between the peaks corresponding to impurity D and maprotiline is between 1.8 and 3.2. If necessary adjust the mobile phase by adding a 50 per cent V/V solution of *acetic acid R* if the resolution is less than 1.8, or by adding a 70 g/l solution

of *concentrated ammonia R* if the resolution is greater than 3.2, to adjust the pH in steps of 0.1 pH units. Inject 20 µl of the test solution and 20 µl of reference solution (a). Continue the chromatography of the test solution for 1.5 times the retention time of the principal peak. In the chromatogram obtained with the test solution: the area of any peak, apart from the principal peak, is not greater than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent); the sum of the areas of all the peaks, apart from the principal peak, is not greater than five times the area of the principal peak in the chromatogram obtained with reference solution (a) (1 per cent). Disregard any peak with an area less than 0.1 times that of the principal peak in the chromatogram obtained with reference solution (a).

Loss on drying (2.2.32). Not more than 1.0 per cent, determined on 1.000 g by drying in an oven at 80 °C at a pressure not exceeding 2.5 kPa for 6 h.

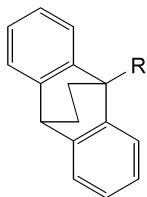
Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

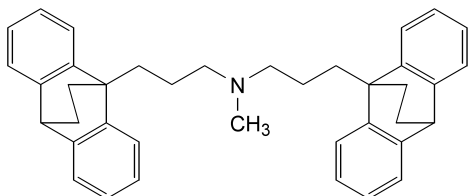
Dissolve 0.250 g in a mixture of 5 ml of 0.1 M *hydrochloric acid* and 50 ml of *alcohol R*. Carry out a potentiometric titration (2.2.20), using 0.1 M *sodium hydroxide*. Read the volume added between the two points of inflexion.

1 ml of 0.1 M *sodium hydroxide* is equivalent to 31.39 mg of C₂₀H₂₄ClN.

IMPURITIES



- A. R = CH=CH-CH=O: 3-(9,10-ethanoanthracen-9(10H)-yl)prop-2-enal,
 C. R = CH₂-CH₂-CH₂-NH₂: 3-(9,10-ethanoanthracen-9(10H)-yl)propan-1-amine,
 D. R = CH=CH-CH₂-NH-CH₃: 3-(9,10-ethanoanthracen-9(10H)-yl)-N-methylprop-2-en-1-amine (dehydromaprotiline),
 E. R = CH₂-CH₂-CH₂-N(CH₃)₂: 3-(9,10-ethanoanthracen-9(10H)-yl)-N,N-dimethylpropan-1-amine,



- B. 3-(9,10-ethanoanthracen-9(10H)-yl)-N-[3-(9,10-ethanoanthracen-9(10H)-yl)propyl]-N-methylpropan-1-amine.

01/2005:1856

MARSHMALLOW LEAF

Althaeae folium

DEFINITION

Whole or cut dried leaf of *Althaea officinalis* L.

CHARACTERS

Macroscopic and microscopic characters described under identification tests A and B.

IDENTIFICATION

- A. The leaves have long petioles and are about 7 cm to 10 cm long; the lamina is cordate to ovate with 3 to 5 shallow lobes and crenate to dentate margins; the venation is palmate. The petioles and both surfaces of the lamina are greyish-green and densely pubescent. Rarely, fragments of the inflorescence or immature fruits may be present.
 B. Reduce to a powder (355). The powder is greyish-green. Examine under a microscope using *chloral hydrate solution R*. The powder shows numerous long, rigid, unicellular covering trichomes with thick walls, pointed at the apex, angular and pitted at the base where they are sometimes still united to form stellate structures with up to 8 components; few secretory trichomes with unicellular stalks and globular, multicellular heads; fragments of the leaf epidermis with anomocytic or paracytic stomata (2.8.3); cluster crystals of calcium oxalate, isolated or in the parenchyma of the mesophyll; fragments of veins with small, spiral or annular vessels. Examine under a microscope using *ruthenium red solution R*. The powder shows groups of parenchyma containing mucilage which stains orange-red.
 C. Thin-layer chromatography (2.2.27).

Test solution. To 1 g of the powdered drug (355) add 10 ml of *methanol R*. Heat in a water-bath under a reflux condenser for 5 min. Cool and filter. Distil the filtrate under reduced pressure until the total volume is about 2 ml.

Reference solution. Dissolve 2.5 mg of *quercitrin R* and 2.5 mg of *chlorogenic acid R* in 10 ml of *methanol R*.

Plate: TLC silica gel plate *R*.

Mobile phase: anhydrous formic acid *R*, glacial acetic acid *R*, water *R*, ethyl acetate *R* (11:11:27:100 V/V/V/V).

Application: 10 µl, as bands.

Development: over a path of 15 cm.

Drying: at 100–105 °C.

Detection: spray with a 10 g/l solution of *diphenylboric acid aminoethyl ester R* in *methanol R*, then with a 50 g/l solution of *macrogol 400 R* in *methanol R*. Allow the plate to dry in air for 30 min. Examine in ultraviolet light at 365 nm.

Results: see below the sequence of the zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other fluorescent zones may be present in the chromatogram obtained with the test solution.

Top of the plate	
Quercitrin: an orange zone	A blue fluorescent zone
	A yellow fluorescent zone
	An orange fluorescent zone
	An orange fluorescent zone
Chlorogenic acid: a blue fluorescent zone	A blue fluorescent zone
	An orange fluorescent zone
	An intense yellow fluorescent zone
Reference solution	Test solution

TESTS

Foreign matter (2.8.2): maximum 4 per cent of leaves infected by *Puccinia malvacearum*, showing red spots and maximum 2 per cent of other foreign matter.

Loss on drying (2.2.32): maximum 10.0 per cent, determined on 1.000 g of the powdered drug (355) by drying in an oven at 100-105 °C for 2 h.

Total ash (2.4.16): maximum 18.0 per cent.

Swelling index (2.8.4): minimum 12, determined on 0.2 g of the powdered drug (355).

Ash insoluble in hydrochloric acid (2.8.1): maximum 2.0 per cent.

01/2005:1126

MARSHMALLOW ROOT

Althaeae radix

DEFINITION

Marshmallow root consists of the peeled or unpeeled, whole or cut, dried root of *Althaea officinalis* L.

CHARACTERS

It has the macroscopic and microscopic characters described under identification tests A and B.

IDENTIFICATION

- A. The unpeeled, non-fragmented drug consists of cylindrical, slightly twisted roots, up to 2 cm thick, with deep longitudinal furrows. The outer surface is greyish-brown and bears numerous rootlet scars. The fracture is fibrous externally, rugged and granular internally. The section shows a more or less thick, whitish bark with brownish periderm, separated by the well-marked, brownish cambium from a white xylem. The stratified structure of the bark and the radiate structure of xylem become more distinct when moistened.
- The peeled drug has a greyish-white finely fibrous outer surface. Cork and external cortical parenchyma are absent.
- B. Reduce to a powder (355). The powder is brownish-grey (unpeeled root) or whitish (peeled root). Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters: fragments of colourless, mainly unligified, thick-walled fibres with pointed or split ends; fragments of bordered, pitted or scalariformly thickened vessels; cluster crystals of calcium oxalate about 20 µm to 35 µm, mostly 25 µm to 30 µm in size; parenchymatous cells containing mucilage; fragments of cork with thin-walled, tabular cells in the unpeeled root. Examine under a microscope using *water R*. The powder shows numerous starch granules about 3 µm to 25 µm in size occasionally with a longitudinal hilum. The starch grains are mostly simple, a few being two to four compound.

TESTS

Foreign matter (2.8.2). Not more than 2 per cent of brown deteriorated drug. Not more than 2 per cent of cork in the peeled root.

Swelling index (2.8.4). Not less than 10, determined on the powdered drug (710).

Loss on drying (2.2.32). Not more than 12.0 per cent, determined on 1.000 g of powdered drug (710) by drying in an oven at 100 °C to 105 °C for 2 h.

Total ash (2.4.16). Not more than 6.0 per cent for peeled root and not more than 8.0 per cent for the unpeeled root.

STORAGE

Store protected from light.

01/2005:1876

MASTIC

Mastix

DEFINITION

Dried resinous exudate obtained from stems and branches of *Pistacia lentiscus* L. var. *latifolius* Coss.

Content: minimum 10 ml/kg of essential oil (anhydrous drug).

CHARACTERS

Macroscopic characters described under identification test A.

IDENTIFICATION

- A. Small light yellow to greenish-yellow, non-uniform, spherical or pyriform, clear or opaque, hard glassy fragments.
- B. Thin-layer chromatography (2.2.27).

Test solution. Dissolve 1 g of the substance to be examined in 10 ml of *methylene chloride R* and filter after 1-2 min.

Reference solution. Dissolve 25 mg of *eugenol R* and 25 mg of *borneol R* in 3 ml of *methylene chloride R*.

Plate: TLC silica gel plate R.

Mobile phase: *light petroleum R*, *toluene R* (5:95 V/V).

Application: 1 µl, as bands.

Development: over a path of 10 cm.

Drying: in air.

Detection: spray with *vanillin reagent R* and heat at 100-105 °C for 5 min.

Results: see below the sequence of the zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other zones of various colours may be present in the chromatogram obtained with the test solution.

Top of the plate	
	A violet zone
	A pale violet zone
	A very pale violet zone
Eugenol: a brown zone	A blue zone
Borneol: a greenish-blue zone	A bluish-violet zone
	A dark violet zone
Reference solution	Test solution

TESTS

Acid value (2.5.1): 50 to 70, determined on 1.0 g.

Foreign matter (2.8.2): maximum 2 per cent *m/m*.

Water (2.2.13): maximum 10 ml/kg, determined on 25.0 g of the drug reduced to a coarse powder (1400).

Total ash (2.4.16): maximum 0.5 per cent.

ASSAY

Essential oil (2.8.12). Use a 500 ml round-bottomed flask and 200 ml of *water R* as the distillation liquid. Reduce the drug to a coarse powder (1400) and immediately use 20.0 g for the determination. Introduce 0.50 ml of *xylene R* in the graduated tube. Distil at a rate of 2-3 ml/min for 2 h.

STORAGE

Do not powder.

01/2005:0404

MATRICARIA FLOWER

Matricariae flos

DEFINITION

Dried capitula of *Matricaria recutita* L. (*Chamomilla recutita* (L.) Rauschert).

Content:

- blue essential oil: minimum 4 ml/kg (dried drug),
- total apigenin 7-glucoside ($C_{21}H_{20}O_{10}$): minimum 0.25 per cent (dried drug).

CHARACTERS

Macroscopic and microscopic characters described under identification tests A and B.

IDENTIFICATION

- A. Capitula, when spread out, consisting of an involucre made up of many bracts arranged in 1 to 3 rows; an elongated-conical receptacle, occasionally hemispherical (young capitula); 12 to 20 marginal ligulate florets with a white ligule; several dozen yellow central tubular florets. The involucre bracts are ovate to lanceolate, with a brownish-grey scarious margin. The receptacle is hollow, without paleae. The corolla of the ligulate florets has a brownish-yellow tube at the base extending to form a white, elongated-oval ligule. The inferior ovary is dark brown, ovoid to spherical, and has a long style and bifid stigma. The tubular florets are yellow and have a five-toothed corolla tube, 5 syngenesious, epipetalous stamens and a gynoeceum similar to that of the ligulate florets.
- B. Separate the capitulum into its different parts. Examine under a microscope using *chloral hydrate solution R*. The bracts have a margin composed of thin-walled cells and a central region composed of elongated sclereids with occasional stomata. The inner epidermis of the corolla of the ligulate florets, in surface view, consisting of thin-walled, polygonal cells, slightly papillose, those of the outer epidermis markedly sinuous and strongly striated; corolla of the tubular florets with longitudinally elongated epidermal cells, and with small groups of papillae near the apex of the lobes. Glandular trichomes each consisting of a short stalk and a head of 2 to 3 tiers of 2 cells each occur on the outer surfaces of the bracts and on the corollas of both types of florets. The ovaries have a sclerous ring at the base and the wall is composed of vertical bands of thin-walled, longitudinally elongated cells with numerous glandular trichomes, alternating with fusiform groups of small, radially elongated cells containing mucilage. The cells at the apex of the stigmas are extended to form rounded papillae. Numerous small, cluster crystals of calcium oxalate occur in the inner tissues of the ovaries and the anther lobes. Pollen grains spherical to triangular, about 30 µm in diameter with 3 pores and a spiny exine.

C. Thin-layer chromatography (2.2.27).

Test solution. Dilute 50 µl of essential oil obtained in the assay of essential oil in 1 ml of *xylene R*.

Reference solution. Dissolve 2 µl of *chamazulene R*, 5 µl of *levomenol R* and 10 mg of *bornyl acetate R* in 5 ml of *toluene R*.

Plate: TLC silica gel plate *R*.

Mobile phase: *ethyl acetate R*, *toluene R* (5:95 V/V).

Application: 10 µl, as bands.

Development: over a path of 10 cm.

Drying: in air.

Detection: spray with *anisaldehyde solution R* and heat at 100-105 °C for 5-10 min. Examine immediately in daylight.

Results: see below the sequence of the zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other zones are present in the chromatogram obtained with the test solution.

Top of the plate	
Chamazulene: a red to reddish-violet zone	1 or 2 blue to bluish-violet zones A red to reddish-violet zone (chamazulene)
Bornyl acetate: a yellowish-brown zone	A brown zone (en-yne-dicycloether)
Levomenol: a reddish-violet to bluish-violet zone	A reddish-violet to bluish-violet zone (levomenol)
Reference solution	Test solution

TESTS

Broken drug: maximum 25 per cent, determined on 20.0 g, passes through a sieve (710).

Foreign matter (2.8.2): maximum 2 per cent *m/m*.

Loss on drying (2.2.32): maximum 12.0 per cent, determined on 1.000 g of the powdered drug (355) by drying in an oven at 100-105 °C for 2 h.

Total ash (2.4.16): maximum 13.0 per cent.

ASSAY

Essential oil (2.8.12). Use 30 g of whole drug, a 1000 ml flask, 300 ml of *water R* as distillation liquid and 0.50 ml of *xylene R* in the graduated tube. Distil at a rate of 3-4 ml/min for 4 h. Towards the end of this period, stop the flow of water to the condenser assembly but continue distilling until the blue, steam-volatile components have reached the lower end of the condenser. Immediately re-start the flow of water to the condenser assembly to avoid warming the separation space. Stop the distillation after a further 10 min.

Total apigenin 7-glucoside. Liquid chromatography (2.2.29).

Test solution. Reduce 40 g of the drug to a powder (500). Place 2.00 g of the powdered drug in a 500 ml round-bottomed flask. Add 200 ml of *alcohol R*. Heat the mixture under a reflux condenser on a water-bath for 15 min. Cool and filter. Rinse the filter and the residue with a few millilitres of *alcohol R*. To the filtrate add 10 ml of freshly prepared *dilute sodium hydroxide solution R* and heat the mixture under a reflux condenser on a water-bath for about 1 h. Cool. Dilute to 250.0 ml with *alcohol R*. To 50.0 ml of the solution add 0.5 g of *citric acid R*. Shake for 5 min and

01/2005:1544

filter. Dilute 5.0 ml to 10.0 ml with the mobile phase (initial mixture).

Reference solution (a). Dissolve 10.0 mg of *apigenin 7-glucoside R* in 100.0 ml of *methanol R*. Dilute 25.0 ml of this solution to 200 ml with the mobile phase (initial mixture).

Reference solution (b). Dissolve 10.0 mg of *5,7-dihydroxy-4-methylcoumarin R* in 100.0 ml of *methanol R*. Dilute 25.0 ml of this solution to 100 ml of the mobile phase (initial mixture). To 4.0 ml of this solution add 4.0 ml of reference solution (a) and dilute to 10.0 ml with the mobile phase (initial mixture).

Precolumn:

- size: $l = 8$ mm, $\emptyset = 4.6$ mm,
- stationary phase: octadecylsilyl silica gel for chromatography *R* (5 μ m).

Column:

- size: $l = 0.25$ m, $\emptyset = 4.6$ mm,
- stationary phase: octadecylsilyl silica gel for chromatography *R* (5 μ m).

Mobile phase:

- mobile phase A: phosphoric acid *R*, water *R* (0.5:99.5 V/V).
- mobile phase B: phosphoric acid *R*, acetonitrile *R* (0.5:99.5 V/V).

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)
0 - 9	75	25
9 - 19	75 $\rightarrow$ 25	25 $\rightarrow$ 75
19 - 24	25	75
24 - 29	25 $\rightarrow$ 75	75 $\rightarrow$ 25
29 - 30	75 $\rightarrow$ 90	25 $\rightarrow$ 10

Flow rate: 1 ml/min.

Detection: spectrophotometer at 340 nm.

Injection: 20 μ l.

System suitability: reference solution (b):

- resolution: minimum 1.8 between the peaks due to apigenin 7-glucoside and 5,7-dihydroxy-4-methylcoumarin.

Calculate the percentage content of total apigenin 7-glucoside from the expression:

$$\frac{A_1 \times m_2}{A_2 \times m_1} \times P \times 0.625$$

- A_1 = area of the peak due to apigenin 7-glucoside in the chromatogram obtained with the test solution,
- A_2 = area of the peak due to apigenin 7-glucoside in the chromatogram obtained with the reference solution,
- m_1 = mass of the drug in the test solution, in grams,
- m_2 = mass of *apigenin 7-glucoside R* in reference solution (a), in grams,
- P = percentage content of apigenin 7-glucoside in the reagent.

MATRICARIA LIQUID EXTRACT

Matricariae extractum fluidum

DEFINITION

Matricaria liquid extract is produced from *Matricaria flower (0404)*. It contains not less than 0.30 per cent of blue residual oil.

PRODUCTION

The extract is produced from the drug and a mixture of 2.5 parts of a 10 per cent *m/m* solution of ammonia (NH₃), 47.5 parts of water and 50 parts of alcohol with an appropriate procedure for liquid extracts.

CHARACTERS

A brownish, clear liquid with an intense characteristic odour and characteristic bitter taste; miscible with water and with alcohol with development of turbidity, soluble in alcohol (50 per cent V/V).

IDENTIFICATION

- A. Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel F₂₅₄ plate R*.

Test solution. Place 10 ml of the extract in a separating funnel and shake with 2 quantities, each of 10 ml, of *pentane R*. Combine the pentane layers, dry over 2 g of *anhydrous sodium sulphate R* and filter. Evaporate the filtrate to dryness on a water-bath and dissolve the residue in 0.5 ml of *toluene R*.

Reference solution. Dissolve 4 mg of *guaiazulene R*, 20 mg of *levomenol R* and 20 mg of *bornyl acetate R* in 10 ml of *toluene R*.

Apply to the plate as bands 10 μ l of each solution. Develop over a path of 10 cm using a mixture of 5 volumes of *ethyl acetate R* and 95 volumes of *toluene R*. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. The chromatogram obtained with the test solution shows several quenching zones, of which 2 main zones are in the middle third (en-yne-dicycloether). Examine in ultraviolet light at 365 nm. The chromatogram obtained with the test solution shows in the middle part an intense blue fluorescent zone (herniarin). Spray the plate with *anisaldehyde solution R*. Examine in daylight while heating at 100-105 °C for 5-10 min. The chromatogram obtained with the reference solution shows in the lower third a reddish-violet to bluish-violet zone (levomenol), in the middle third a yellowish-brown to greyish-green zone (bornyl acetate) and in the upper third a red to reddish-violet zone (guaiazulene). The chromatogram obtained with the test solution shows in the lower third yellowish-brown to greenish-yellow and violet zones and a reddish-violet to bluish-violet zone corresponding to levomenol in the chromatogram obtained with the reference solution; a brownish zone (en-yne-dicycloether) similar in position to bornyl acetate in the chromatogram obtained with the reference solution; a red or reddish-violet zone (chamazulene) corresponding to guaiazulene in the chromatogram obtained with the reference solution and immediately above it 1 or 2 blue to bluish-violet zones; further weak zones may be present in the chromatogram obtained with the test solution.

- B. Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel plate R*.

Test solution. Use the extract.

Reference solution. Dissolve 1.0 mg of *chlorogenic acid R*, 2.5 mg of *hyperoside R* and 2.5 mg of *rutin R* in 10 ml of *methanol R*.

Apply to the plate as bands 10 µl of each solution. Develop over a path of 15 cm using a mixture of 7.5 volumes of *anhydrous formic acid R*, 7.5 volumes of *glacial acetic acid R*, 18 volumes of *water R*, and 67 volumes of *ethyl acetate R*. Dry the plate at 100-105 °C and spray the warm plate with a 10 g/l solution of *diphenylboric acid aminoethyl ester R* in *methanol R*. Subsequently spray the plate with a 50 g/l solution of *macrogol 400 R* in *methanol R*. Allow the plate to dry in air for about 30 min and examine in ultraviolet light at 365 nm. The chromatogram obtained with the reference solution shows in the middle part a light blue fluorescent zone (chlorogenic acid), below it a yellowish-brown fluorescent zone (rutin) and above it a yellowish-brown fluorescent zone (hyperoside). The chromatogram obtained with the test solution shows a yellowish-brown fluorescent zone corresponding to the zone of rutin in the chromatogram obtained with the reference solution, a light blue fluorescent zone corresponding to the zone of chlorogenic acid in the chromatogram obtained with the reference solution, a yellowish-brown fluorescent zone similar in position to the zone of hyperoside in the chromatogram obtained with the reference solution; it also shows above the yellowish-brown fluorescent zone a green fluorescent zone, then several bluish or greenish fluorescent zones and near the solvent front a yellowish fluorescent zone.

TESTS

Ethanol (2.9.10): 38 per cent V/V to 53 per cent V/V.

Dry residue (2.8.16): minimum 12.0 per cent.

ASSAY

Place 20.0 g in a 1000 ml round-bottomed flask, add 300 ml of *water R* and distil until 200 ml has been collected in a flask. Transfer the distillate into a separating funnel. Dissolve 65 g of *sodium chloride R* in the distillate and shake with 3 quantities, each of 30 ml, of *pentane R* previously used to rinse the reflux condenser and the flask. Combine the pentane layers, dry over 2 g of *anhydrous sodium sulphate R* and filter into a tared 100 ml round-bottomed flask which has been dried in a desiccator for 3 h. Rinse the anhydrous sodium sulphate and the filter with 2 quantities, each of 20 ml, of *pentane R*. Evaporate the pentane in a water-bath at 45 °C. The residue of pentane is eliminated in a current of air for 3 min. Dry the flask in a desiccator for 3 h and weigh. The residual oil is blue (chamazulene).

01/2005:1836

MATRICARIA OIL

Matricariae aetheroleum

DEFINITION

Blue essential oil obtained by steam distillation from the fresh or dried flower-heads or flowering tops of *Matricaria recutita* L. (*Chamomilla recutita* L. Rauschert). There are 2 types of matricaria oil which are characterised as rich in bisabolol oxides, or rich in levomenol.

CHARACTERS

Appearance: clear, intensely blue, viscous liquid. It has an intense characteristic odour.

IDENTIFICATION

First identification: B.

Second identification: A.

A. Thin-layer chromatography (2.2.27).

Test solution. Dissolve 20 µl of the substance to be examined in 1.0 ml of *toluene R*.

Reference solution. Dissolve 2 mg of *guaiazulene R*, 5 µl of *levomenol R* and 10 mg of *bornyl acetate R* in 5.0 ml of *toluene R*.

Plate: TLC silica gel plate R.

Mobile phase: *ethyl acetate R*, *toluene R* (5:95 V/V).

Application: 10 µl, as bands.

Development: over a path of 10 cm.

Drying: in air.

Detection A: examine in daylight.

Results A: see below for the sequence of the zones present in the chromatograms obtained with the reference solution and the test solution.

Top of the plate	
Guaiazulene: a blue zone _____	A blue zone (chamazulene) _____
Reference solution	Test solution

Detection B: spray with *anisaldehyde solution R* and heat at 100-105 °C for 5-10 min. Examine immediately in daylight.

Results B: see below for the sequence of the zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, yellowish-brown to greenish-yellow zones (lower third), violet zones (lower third) and further weak zones may be present in the chromatogram obtained with the test solution.

Top of the plate	
Guaiazulene: a red to reddish-violet zone _____	1 or 2 blue to bluish-violet zones A red to reddish-violet zone (chamazulene) _____
Bornyl acetate: a yellowish-brown to greyish-green zone _____	A brown zone (en-yne-dicycloether) _____
Levomenol: a reddish-violet to bluish-violet zone	A reddish-violet to bluish-violet zone (levomenol) A brownish zone
Reference solution	Test solution

B. Examine the chromatograms obtained in the test for chromatographic profile.

Results: the characteristic peaks corresponding to levomenol and to chamazulene in the chromatogram obtained with the test solution are similar in retention time to those in the chromatogram obtained with the reference solution.

TESTS

Chromatographic profile. Gas chromatography (2.2.28): use the normalisation procedure.

Test solution. Dissolve 20 µl of the oil to be examined in *cyclohexane R* and dilute to 5.0 ml with the same solvent.

Reference solution. Dissolve 20 µl of *levomenol R*, 5 mg of *chamazulene R* and 6 mg of *guaiazulene R* in *cyclohexane R* and dilute to 5.0 ml with the same solvent.

Column:

- **material:** fused silica,
- **size:** $l = 30$ m (a film thickness of 1 µm may be used) to 60 m (a film thickness of 0.2 µm may be used), $\varnothing = 0.25$ – 0.53 mm, when using a column longer than 30 m, an adjustment of the temperature programme may be necessary,
- **stationary phase:** *macrogol 20 000 R*.

Carrier gas: *helium for chromatography R*.

Flow rate: 1–2 ml/min.

Split ratio: 1:100.

Temperature:

	Time (min)	Temperature (°C)
Column	0 – 40	70 → 230
	40 – 50	230
Injection port		250
Detector		250

Detection: flame ionisation.

Injection: 1.0 µl.

Elution order: order indicated in the composition of the reference solution. Record the retention times of these substances.

Relative retention with reference to chamazulene (retention time = about 34.4 min): β -farnesene = about 0.5; bisabolol oxide B = about 0.8; bisabolone = about 0.87; levomenol = about 0.9; bisabolol oxide A = about 1.02.

System suitability: reference solution:

- **resolution:** minimum of 1.5 between the peaks due to chamazulene and to guaiazulene.

Using the retention times determined from the chromatogram obtained with the reference solution, locate levomenol and chamazulene in the chromatogram obtained with the test solution; locate bisabolol oxides (bisabolol oxide B, bisabolone and bisabolol oxide A) using Figures 1836-1 and 1836-2 (disregard the peak due to cyclohexane). The chromatogram obtained with the test solution does not show a peak with the retention time of guaiazulene.

Determine the percentage content of the components. The limits are within the following ranges.

	Matricaria oil rich in bisabolol oxides (per cent)	Matricaria oil rich in levomenol (per cent)
Bisabolol oxides	29 – 81	
Levomenol		10 – 65
Chamazulene	≥ 1.0	≥ 1.0
Total of bisabolol oxides and levomenol		≥ 20

STORAGE

In a well-filled, airtight container, protected from light at a temperature not exceeding 25 °C.

LABELLING

The label states the type of matricaria oil (rich in bisabolol oxides or rich in levomenol).

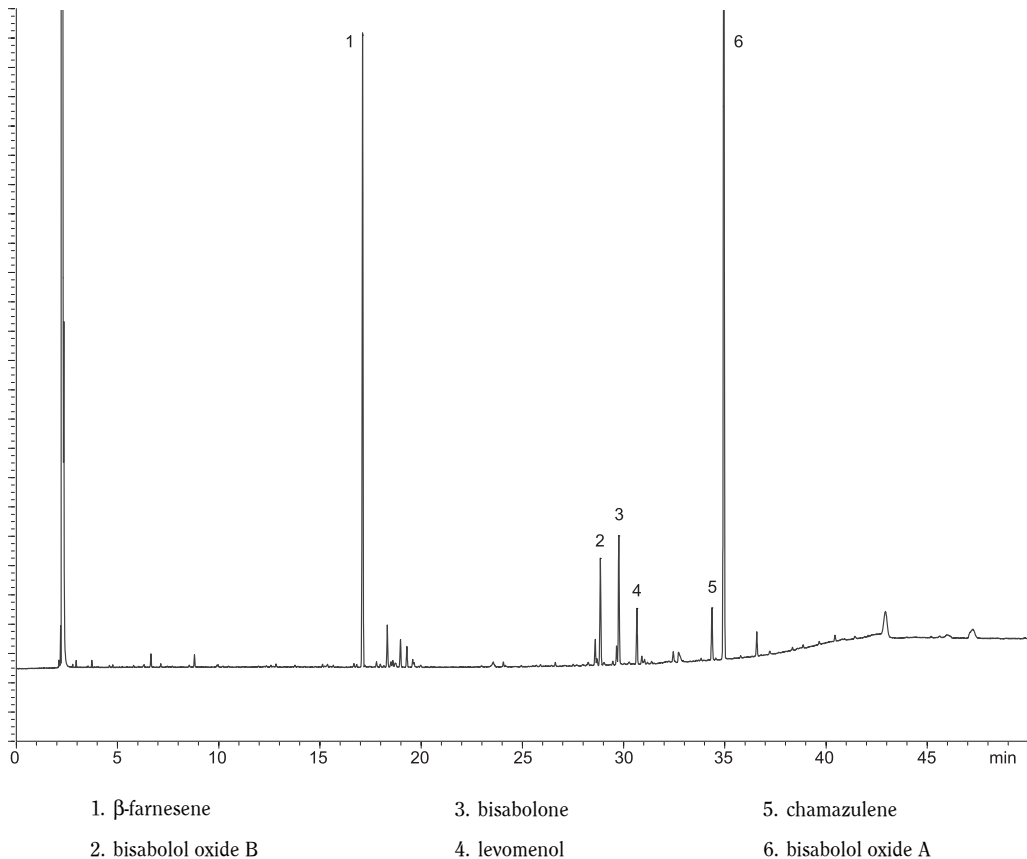


Figure 1836-1. – Chromatogram of matricaria oil rich in bisabolol oxides

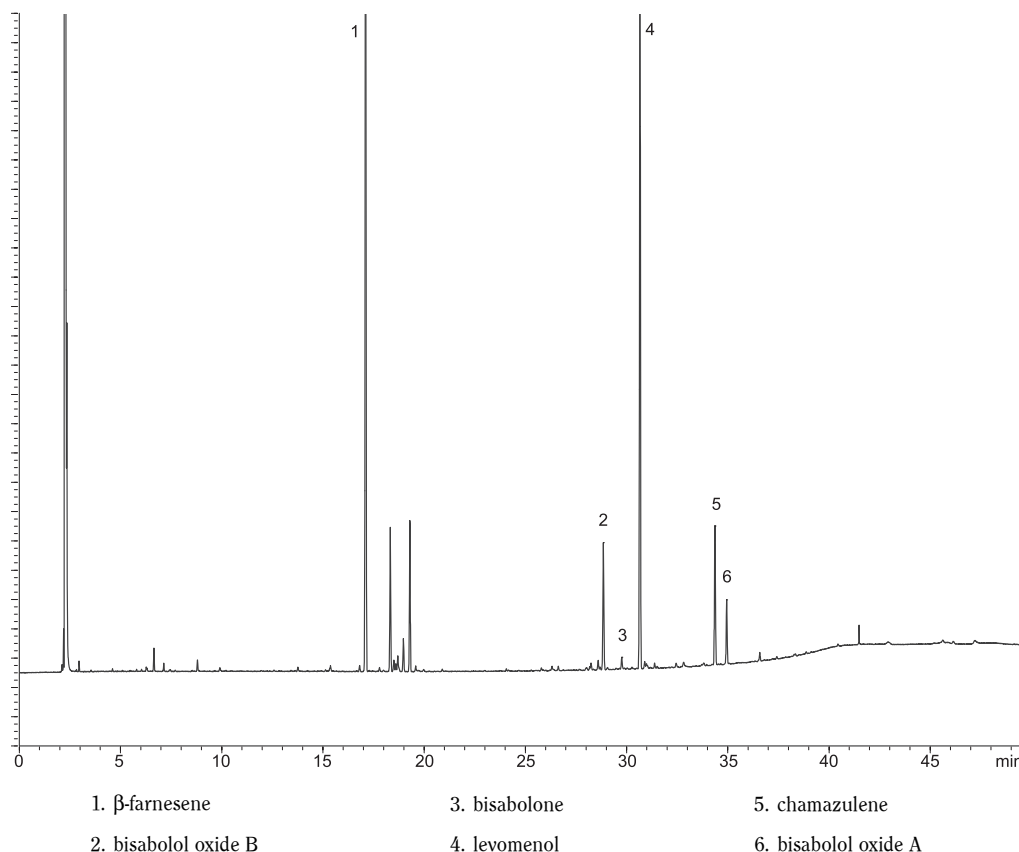


Figure 1836.-2. – Chromatogram of matricaria oil rich in levomenol

01/2005:1868

MEADOWSWEET**Filipendulae ulmariae herba****DEFINITION**

Whole or cut, dried flowering tops of *Filipendula ulmaria* (L.) Maxim. (= *Spiraea ulmaria* L.).

Content: minimum 1 ml/kg of steam-volatile substances (dried drug).

CHARACTERS

Aromatic odour of methyl salicylate, after crushing.

Macroscopic and microscopic characters described under identification tests A and B.

IDENTIFICATION

A. The stem, up to 5 mm in diameter, is greenish-brown, stiff, angular, hollow except at the apex, and has regular, straight, longitudinal furrows. The petiolate leaf, compound imparipinnate, has 2 reddish-brown angular stipules. It consists of 3 to 9 pairs of leaflets, unevenly dentate, some of which are small and fan-shaped. The leaflets are dark green and glabrous on the upper surface, tomentose and lighter, sometimes silvery on the lower surface. The terminal leaflet, the largest, is divided into 3 segments. The veins are prominent and brown on the lower surface. The inflorescence is complex and composed of very numerous flowers arranged in irregular cymose panicles. The flowers are creamish-white and about 3 mm to 6 mm in diameter; the calyx consists of 5 dark green, reflexed and hairy sepals fused at the base to a concave receptacle; the 5 free petals, which are readily detached, are pale yellow, obovate and distinctly narrowed at the base; the stamens are numerous with rounded anthers and they extend beyond the petals;

the gynoecium consists of about 4 to 6 carpels, each with a short style and a globular stigma; the carpels become twisted together spirally to form yellowish-brown fruits with a helicoidal twist. Unopened flower buds are frequently present. If the fruit is present, it has a helicoidal twist and contains brownish seeds.

- B. Reduce to a powder (355). The powder is yellowish-green. Examine under a microscope using *chloral hydrate solution R*. The powder shows unicellular covering trichomes, some thin-walled, very long and flexuous, with pointed ends, others shorter, thick-walled, conical and thickened at the base; occasional clavate glandular trichomes with a 1- to 3-celled, uniseriate stalk and a multicellular head containing dense brown contents; fragments of the leaves and sepals with sinuous to wavy epidermal cells, anomocytic stomata (2.8.3) on the lower surface only and cluster crystals of calcium oxalate in the mesophyll; thin-walled epidermal cells of the petals, some showing rounded papillae; numerous spherical pollen grains with 3 pores and a faintly pitted exine; fragments of the fibrous layer of the anthers with stellate thickenings; groups of small-celled parenchyma from the ovaries containing prism crystals of calcium oxalate; fragments of vascular tissue with spiral and annular vessels from the leaves and stems.
- C. Thin-layer chromatography (2.2.27).

Test solution. Xylene solution obtained in the assay.

Reference solution. Dissolve 0.1 ml of *methyl salicylate R* and 0.1 ml of *salicylaldehyde R* in *xylene R* and dilute to 5 ml with the same solvent.

Plate: TLC silica gel plate *R*.

Mobile phase: hexane *R*, toluene *R* (50:50 V/V).

Application: 10 µl, as bands.

Development: over a path of 10 cm.

Drying: in air.

Detection: spray the plate with 3 ml of *ferric chloride solution R3* and examine in daylight.

Results: see below the sequence of the zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other zones are present in the chromatogram obtained with the test solution.

Top of the plate	
Methyl salicylate: a violet-brown zone	A violet-brown zone (methyl salicylate)
Salicylaldehyde: a violet-brown zone	A violet-brown zone (salicylaldehyde)
Reference solution	Test solution

TESTS

Foreign matter (2.8.2): maximum 5.0 per cent of stems with a diameter greater than 5 mm and maximum 2.0 per cent of foreign matter.

Loss on drying (2.2.32): maximum 12.0 per cent, determined on 1.000 g of the powdered drug (355) by drying in an oven at 100-105 °C for 2 h.

Total ash (2.4.16): maximum 7.0 per cent.

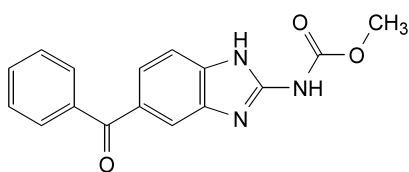
ASSAY

Examine according to the method described for the determination of essential oils in vegetable drugs (2.8.12). Use 50.0 g, a 1000 ml flask and 300 ml of *dilute hydrochloric acid R* as distillation liquid and 0.5 ml of *xylene R* in the graduated tube. Distil at a rate of 2-3 ml/min for 2 h.

01/2005:0845

MEBENDAZOLE

Mebendazolum

C₁₆H₁₃N₃O₃M_r 295.3

DEFINITION

Methyl (5-benzoyl-1*H*-benzimidazol-2-yl)carbamate.

Content: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

Appearance: white or almost white powder.

Solubility: practically insoluble in water, in alcohol and in methylene chloride.

It shows polymorphism.

IDENTIFICATION

Infrared absorption spectrophotometry (2.2.24).

Comparison: Ph. Eur. reference spectrum of mebendazole.

TESTS

Related substances. Liquid chromatography (2.2.29).

Test solution. Dissolve 25.0 mg of the substance to be examined in *dimethylformamide R* and dilute to 25.0 ml with the same solvent.

Reference solution (a). Dissolve 5.0 mg of *mebendazole for system suitability CRS* in *dimethylformamide R* and dilute to 5.0 ml with the same solvent.

Reference solution (b). Dilute 1.0 ml of the test solution to 100.0 ml with *dimethylformamide R*. Dilute 5.0 ml of this solution to 20.0 ml with *dimethylformamide R*.

Column:

- **size:** *l* = 0.10 m, Ø = 4.6 mm,
- **stationary phase:** base-deactivated octadecylsilyl silica gel for chromatography R (3 µm),
- **temperature:** 40 °C.

Mobile phase:

- **mobile phase A:** 7.5 g/l solution of *ammonium acetate R*,
- **mobile phase B:** *acetonitrile R*,

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)
0 - 15	80 → 70	20 → 30
15 - 20	70 → 10	30 → 90
20 - 25	10	90
25 - 26	10 → 80	90 → 20
26 - 30	80	20

Flow rate: 1.2 ml/min.

Detection: spectrophotometer at 250 nm.

Injection: 10 µl.

System suitability: reference solution (a):

- the chromatogram obtained is similar to the chromatogram supplied with *mebendazole for system suitability CRS*.

Limits:

- **correction factor:** for the calculation of content, multiply the peak area of impurity G by 1.4,
- **impurity G:** not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent),
- **any other impurity:** not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.25 per cent),
- **total:** not more than 4 times the area of the principal peak in the chromatogram obtained with reference solution (b) (1.0 per cent),
- **disregard limit:** 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

Loss on drying (2.2.32): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 100-105 °C for 4 h.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

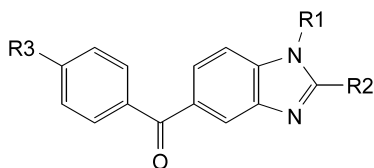
Dissolve 0.250 g in 3 ml of *anhydrous formic acid R* and add 50 ml of a mixture of 1 volume of *anhydrous acetic acid R* and 7 volumes of *methyl ethyl ketone R*. Titrate with 0.1 M *perchloric acid*, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *perchloric acid* is equivalent to 29.53 mg of C₁₆H₁₃N₃O₃.

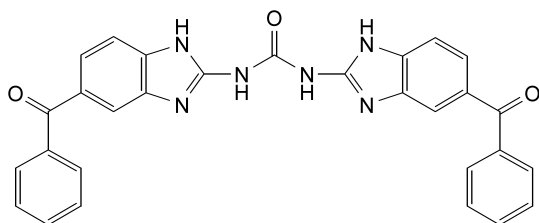
STORAGE

Protected from light.

IMPURITIES



- A. R1 = R3 = H, R2 = NH₂: (2-amino-1*H*-benzimidazol-5-yl)phenylmethanone,
 B. R1 = R3 = H, R2 = OH: (2-hydroxy-1*H*-benzimidazol-5-yl)phenylmethanone,
 C. R1 = CH₃, R2 = NH₂, R3 = H: (2-amino-1-methyl-1*H*-benzimidazol-5-yl)phenylmethanone,
 D. R1 = CH₃, R2 = NH-CO-OCH₃, R3 = H: methyl (5-benzoyl-1-methyl-1*H*-benzimidazol-2-yl)carbamate,
 E. R1 = R3 = H, R2 = NH-CO-OC₂H₅: ethyl (5-benzoyl-1*H*-benzimidazol-2-yl)carbamate,
 F. R1 = H, R2 = NH-CO-OCH₃, R3 = CH₃: methyl [5-(4-methylbenzoyl)-1*H*-benzimidazol-2-yl]carbamate,

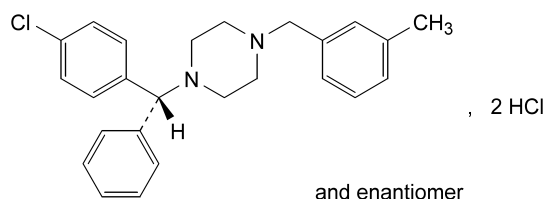


G. *N,N'*-bis(5-benzoyl-1*H*-benzimidazol-2-yl)urea.

01/2005:0622

MECLOZINE HYDROCHLORIDE

Meclozini hydrochloridum



C₂₅H₂₉Cl₃N₂

M_r 463.9

DEFINITION

Meclozine hydrochloride contains not less than 98.0 per cent and not more than the equivalent of 102.0 per cent of 1-[(*RS*)-(4-chlorophenyl)phenylmethyl]-4-(3-methylbenzyl)piperazine dihydrochloride, calculated with reference to the anhydrous substance.

CHARACTERS

A yellow or yellowish-white, crystalline powder, slightly soluble in water, soluble in alcohol and in methylene chloride.

IDENTIFICATION

First identification: B, D.

Second identification: A, C, D.

- A. Dissolve 15.0 mg in 0.1 *M* hydrochloric acid and dilute to 100.0 ml with the same acid. Dilute 10.0 ml of this solution to 100.0 ml with 0.1 *M* hydrochloric acid. Examined between 220 nm and 350 nm (2.2.25), the solution shows an absorption maximum at 232 nm and weak absorbance without a defined maximum between 260 nm and 300 nm. The specific absorbance at the maximum is 345 to 380, calculated with reference to the anhydrous substance.
 B. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *meclozine hydrochloride CRS*. Examine the substances prepared as discs using *potassium chloride R*.
 C. Examine the chromatograms obtained in the test for related substances. The principal spot in the chromatogram obtained with test solution (b) is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).
 D. Dissolve about 15 mg in 2 ml of *alcohol R*. The solution gives reaction (a) of chlorides (2.3.1).

TESTS

Appearance of solution. Dissolve 0.50 g in *alcohol R* and dilute to 25 ml with the same solvent. The solution is clear (2.2.1) and not more intensely coloured than reference solution Y₆ (2.2.2, *Method II*).

Acidity or alkalinity. Calculate the acidity or alkalinity from the titration volumes noted in the Assay using the equation:

$$A = V_2 - 2V_1$$

*V*₁ = volume of 0.1 *M* sodium hydroxide added at the first point of inflexion,

*V*₂ = volume of 0.1 *M* sodium hydroxide added at the second point of inflexion.

A is -0.3 ml to 0.3 ml for 0.3500 g of the substance to be examined.

Related substances. Examine by thin-layer chromatography (2.2.27), using *silica gel G R* as the coating substance.

Test solution (a). Dissolve 0.50 g of the substance to be examined in a mixture of equal volumes of *methanol R* and *methylene chloride R* and dilute to 10 ml with the same mixture of solvents.

Test solution (b). Dilute 1 ml of test solution (a) to 10 ml with a mixture of equal volumes of *methanol R* and *methylene chloride R*.

Reference solution (a). Dissolve 50 mg of *meclozine hydrochloride CRS* in a mixture of equal volumes of *methanol R* and *methylene chloride R* and dilute to 10 ml with the same mixture of solvents.

Reference solution (b). Dilute 0.5 ml of test solution (b) to 10 ml with a mixture of equal volumes of *methanol R* and *methylene chloride R*.

Apply separately to the plate 10 µl of each solution. Develop over a path of 15 cm using a mixture of 0.5 volumes of *concentrated ammonia R*, 5 volumes of *methanol R*, 30 volumes of *toluene R* and 60 volumes of *methylene chloride R*. Allow the plate to dry in air and spray with *dilute potassium iodobismuthate solution R*. Any spot in the chromatogram obtained with test solution (a), apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.5 per cent). Disregard any yellowish-white spot at the starting point.

Water (2.5.12). Not more than 5.0 per cent, determined on 0.200 g by the semi-micro determination of water.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.3500 g in 50 ml of *alcohol R*. Carry out a potentiometric titration (2.2.20), using 0.1 M *sodium hydroxide*. Read the volume added between the two points of inflexion.

1 ml of 0.1 M *sodium hydroxide* is equivalent to 46.39 mg of $C_{25}H_{29}Cl_3N_2$.

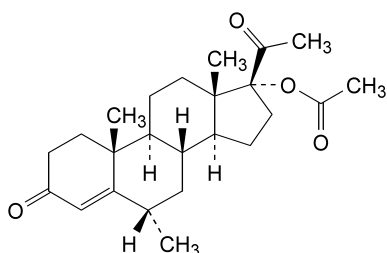
STORAGE

Store in an airtight container.

01/2005:0673

MEDROXYPROGESTERONE ACETATE

Medroxyprogesteroni acetat


 $C_{24}H_{34}O_4$
 M_r 386.5

DEFINITION

Medroxyprogesterone acetate contains not less than 97.0 per cent and not more than the equivalent of 103.0 per cent of 6 α -methyl-3,20-dioxopregn-4-en-17-yl acetate, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, crystalline powder, practically insoluble in water, freely soluble in methylene chloride, soluble in acetone and in dioxan, sparingly soluble in alcohol.

IDENTIFICATION

First identification: A, B.

Second identification: A, C.

- Melting point (2.2.14): 205 °C to 209 °C.
- Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *medroxyprogesterone acetate CRS*.
- Examine the chromatogram obtained in the test for impurity F after spraying at 365 nm. The principal spot in the chromatogram obtained with test solution (b) is similar in position, fluorescence in ultraviolet light at 365 nm and size to the principal spot in the chromatogram obtained with reference solution (b).

TESTS

Specific optical rotation (2.2.7). Dissolve 0.250 g in *dioxan R* and dilute to 25.0 ml with the same solvent. The specific optical rotation is + 45 to + 51, calculated with reference to the dried substance.

Impurity F (6 α -methyl-3,20-dioxo-5 β -pregnan-17-yl acetate). Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel plate R*.

Test solution (a). Dissolve 0.200 g of the substance to be examined in *methylene chloride R* and dilute to 10.0 ml with the same solvent.

Test solution (b). Dilute 1 ml of test solution (a) to 20 ml with *methylene chloride R*.

Reference solution (a). Add to 20.0 mg of *medroxyprogesterone acetate for performance test CRS* (containing 0.5 per cent of impurity F) 1.0 ml of *methylene chloride R* and dissolve.

Reference solution (b). Dissolve 10 mg of *medroxyprogesterone acetate CRS* in *methylene chloride R* and dilute to 10 ml with the same solvent.

Apply separately to the plate 10 μ l of each solution. Develop over a path of 10 cm using a mixture of 10 volumes of *tetrahydrofuran R*, 45 volumes of *1,1-dimethylethyl methyl ether R* and 45 volumes of *hexane R*. Allow the plate to dry in air and carry out a second development in the same direction over a path of 10 cm with the same mixture. Allow the plate to dry at 120 °C for 10 min. Spray with a 200 g/l solution of *toluenesulphonic acid R* in *alcohol R*. Heat at 120 °C for 10 min. Allow to cool. Examine the chromatograms in ultraviolet light at 365 nm. In the chromatogram obtained with test solution (a) any blue fluorescent spot with an R_f value higher than the principal spot due to medroxyprogesterone acetate is not more intense than the corresponding blue fluorescent spot of impurity F in the chromatogram obtained with reference solution (a) (0.5 per cent). The test is not valid unless the chromatogram obtained with reference solution (a) shows two clearly separated spots.

Related substances. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 25.0 mg of the substance to be examined in 1 ml of *tetrahydrofuran R* and dilute to 10.0 ml with the mobile phase.

Reference solution (a). Dissolve 2 mg of *medroxyprogesterone acetate CRS* and 5 mg of *megestrol acetate CRS* in the mobile phase and dilute to 100.0 ml with the mobile phase.

Reference solution (b). Dilute 1.0 ml of the test solution to 100.0 ml with the mobile phase.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4.6 mm in internal diameter packed with *base-deactivated end-capped octadecylsilyl silica gel for chromatography R* (5 μ m),
- as mobile phase at a flow rate of 2 ml/min a mixture prepared as follows: in a 1000 ml volumetric flask mix 100 ml of *tetrahydrofuran R* with 350 ml of *acetonitrile R* and 500 ml of *water R*; allow to equilibrate and adjust the volume to 1000 ml with *water R* and mix again,
- as detector a spectrophotometer set at 254 nm, maintaining the temperature of the column at 40 °C.

Equilibrate the column with the mobile phase at a flow rate of 2 ml/min for about 30 min.

Adjust the sensitivity of the system so that the height of the principal peak in the chromatogram obtained with 20 μ l of reference solution (b) is at least 50 per cent of the full scale of the recorder.

Inject 20 μ l of reference solution (a). When the chromatograms are recorded in the prescribed conditions, the retention times are: megestrol acetate about 14.5 min and medroxyprogesterone acetate about 16.5 min. The test is not valid unless the resolution between the peaks corresponding to megestrol acetate and medroxyprogesterone acetate is at least 3.3. If necessary, adjust the concentration of acetonitrile and/or tetrahydrofuran in the mobile phase. Inject 20 μ l of the solvent mixture of the test solution as a blank, 20 μ l of the test solution and 20 μ l of reference solution (b). Continue the chromatography of the test

solution for 1.5 times the retention time of the principal peak. In the chromatogram obtained with the test solution: the area of any peak, apart from the principal peak, is not greater than the area of the principal peak in the chromatogram obtained with reference solution (b) (1 per cent); the sum of the areas of all the peaks, apart from the principal peak, is not greater than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (1.5 per cent). Disregard any peak due to the blank and any peak with an area less than 0.05 times the area of the principal peak in the chromatogram obtained with reference solution (b).

Loss on drying (2.2.32). Not more than 1.0 per cent, determined on 0.500 g by drying in an oven at 100 °C to 105 °C for 3 h.

ASSAY

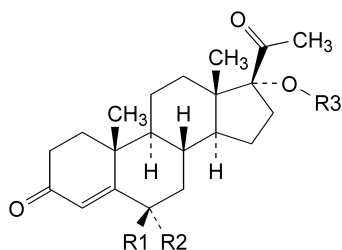
Dissolve 50.0 mg in *alcohol R* and dilute to 50.0 ml with the same solvent. Dilute 2.0 ml of the solution to 100.0 ml with *alcohol R*. Measure the absorbance (2.2.25) at the maximum at 241 nm.

Calculate the content of $C_{24}H_{34}O_4$ taking the specific absorbance to be 420.

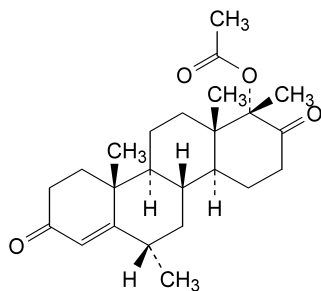
STORAGE

Store protected from light.

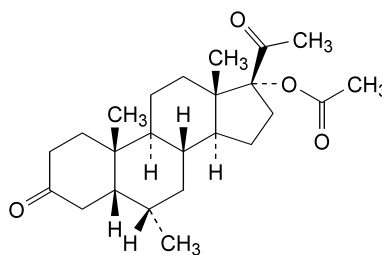
IMPURITIES



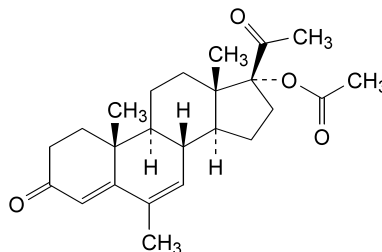
- A. $R_1 = OH$, $R_2 = CH_3$, $R_3 = CO-CH_3$: 6 β -hydroxy-6-methyl-3,20-dioxopregn-4-en-17-yl acetate (6 β -hydroxymedroxyprogesterone acetate),
- B. $R_1 = H$, $R_2 = CH_3$, $R_3 = H$: 17-hydroxy-6 α -methylpregn-4-ene-3,20-dione (medroxyprogesterone),
- D. $R_1 = CH_3$, $R_2 = H$, $R_3 = CO-CH_3$: 6 β -methyl-3,20-dioxopregn-4-en-17-yl acetate (6-epimedroxyprogesterone acetate),
- E. $R_1 + R_2 = CH_2$, $R_3 = CO-CH_3$: 6-methylene-3,20-dioxopregn-4-en-17-yl acetate (6-methylenedihydroxyprogesterone acetate),



- C. 6 α ,17 α -dimethyl-3,17-dioxo-*D*-homoandrost-4-en-17 α -yl acetate,



- F. 6 α -methyl-3,20-dioxo-5 β -pregnan-17-yl acetate (4,5 β -dihydromedroxyprogesterone acetate),

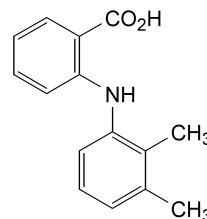


- G. 6-methyl-3,20-dioxopregna-4,6-dien-17-yl acetate.

01/2005:1240

MEFENAMIC ACID

Acidum mefenamicum



$C_{15}H_{15}NO_2$

M_r 241.3

DEFINITION

Mefenamic acid contains not less than 99.0 per cent and not more than the equivalent of 100.5 per cent of 2-[(2,3-dimethylphenyl)amino]benzoic acid, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, microcrystalline powder, practically insoluble in water, slightly soluble in alcohol and in methylene chloride. It dissolves in dilute solutions of alkali hydroxides.

It shows polymorphism.

IDENTIFICATION

First identification: B.

Second identification: A, C, D.

- A. Dissolve 20 mg in a mixture of 1 volume of 1 *M* hydrochloric acid and 99 volumes of *methanol R* and dilute to 100 ml with the same mixture of solvents. Dilute 5 ml of the solution to 50 ml with a mixture of 1 volume of 1 *M* hydrochloric acid and 99 volumes of *methanol R*. Examined between 250 nm and 380 nm (2.2.25), the solution shows two absorption maxima, at 279 nm and 350 nm. The ratio of the absorbance measured at the maximum at 279 nm to that measured at 350 nm is 1.1 to 1.3.

- B. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *mefenamic acid CRS*. Examine the substances prepared as discs. If the spectra obtained show differences, dissolve the substance to be examined and the reference substance separately in *alcohol R*, evaporate to dryness and record new spectra using the residues.
- C. Dissolve about 25 mg in 15 ml of *methylene chloride R* and examine in ultraviolet light at 365 nm; the solution exhibits a strong greenish-yellow fluorescence. Carefully add 0.5 ml of a saturated solution of *trichloroacetic acid R* dropwise and examine in ultraviolet light at 365 nm. The solution does not exhibit fluorescence.
- D. Dissolve about 5 mg in 2 ml of *sulphuric acid R* and add 0.05 ml of *potassium dichromate solution R1*. An intense blue colour is produced, turning rapidly to brownish-green.

TESTS

Related substances. Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel GF₂₅₄ plate R*.

Test solution. Dissolve 0.125 g of the substance to be examined in a mixture of 1 volume of *methanol R* and 3 volumes of *methylene chloride R* and dilute to 5 ml with the same mixture of solvents.

Reference solution (a). Dilute 1 ml of the test solution to 50 ml with a mixture of 1 volume of *methanol R* and 3 volumes of *methylene chloride R*. Dilute 1 ml of the solution to 10 ml with a mixture of 1 volume of *methanol R* and 3 volumes of *methylene chloride R*.

Reference solution (b). Dissolve 5 mg of *flufenamic acid R* and 5 mg of *mefenamic acid CRS* in a mixture of 1 volume of *methanol R* and 3 volumes of *methylene chloride R* and dilute to 10 ml with the same mixture of solvents.

Apply to the plate 20 µl of each solution. Develop over a path of 15 cm using a mixture of 1 volume of *glacial acetic acid R*, 25 volumes of *dioxan R* and 90 volumes of *toluene R*. Dry the plate in a current of warm air. Expose the plate to iodine vapour for 5 min and examine in ultraviolet light at 254 nm. Any spot in the chromatogram obtained with the test solution, apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (a) (0.2 per cent). The test is not valid unless the chromatogram obtained with reference solution (b) shows two clearly separated spots.

2,3-Dimethylaniline

Solution (a). Dissolve 0.250 g of the substance to be examined in a mixture of 1 volume of *methanol R* and 3 volumes of *methylene chloride R* and dilute to 10 ml with the same mixture of solvents. This solution is used to prepare the test solution.

Solution (b). Dissolve 50 mg of *2,3-dimethylaniline R* in a mixture of 1 volume of *methanol R* and 3 volumes of *methylene chloride R* and dilute to 100 ml with the same mixture of solvents. Dilute 1 ml of the solution to 100 ml with a mixture of 1 volume of *methanol R* and 3 volumes of *methylene chloride R*. This solution is used to prepare the standard.

Using three flat-bottomed tubes, place in the first 2 ml of solution (a), in the second 1 ml of solution (b) and 1 ml of a mixture of 1 volume of *methanol R* and 3 volumes

of *methylene chloride R* and in the third 2 ml of a mixture of 1 volume of *methanol R* and 3 volumes of *methylene chloride R* (used to prepare a blank). To each tube add 1 ml of a freshly prepared 10 g/l solution of *dimethylaminobenzaldehyde R* in *methanol R* and 2 ml of *glacial acetic acid R* and allow to stand at room temperature for 10 min. The intensity of the yellow colour of the test solution is between that of the blank and that of the standard (100 ppm).

Copper. Not more than 10 ppm of Cu, determined by atomic absorption spectrometry (2.2.23, *Method I*).

Test solution. Place 1.00 g of the substance to be examined in a silica crucible, moisten with *sulphuric acid R*, heat cautiously on a flame for 30 min and then progressively to 650 °C. Continue ignition until all black particles have disappeared. Allow to cool, dissolve the residue in 0.1 M *hydrochloric acid* and dilute to 25.0 ml with the same acid.

Reference solutions. Prepare the reference solutions using *copper standard solution (0.1 per cent Cu) R*, diluted as necessary with 0.1 M *nitric acid*.

Measure the absorbance at 324.8 nm using a copper hollow-cathode lamp as a source of radiation and an air-acetylene flame.

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying at 100 °C to 105 °C.

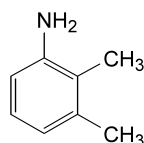
Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

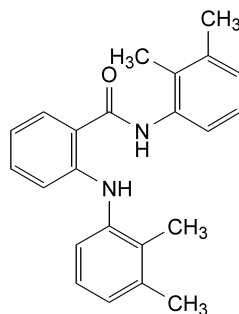
Dissolve with the aid of ultrasound 0.200 g in 100 ml of warm *ethanol R*, previously neutralised to *phenol red solution R*. Add 0.1 ml of *phenol red solution R* and titrate with 0.1 M *sodium hydroxide*.

1 ml of 0.1 M *sodium hydroxide* is equivalent to 24.13 mg of C₁₅H₁₅NO₂.

IMPURITIES



A. 2,3-dimethylaniline,

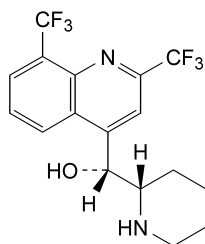


B. *N*-(2,3-dimethylphenyl)-2-[(2,3-dimethylphenyl)amino]benzamide.

01/2005:1241

MEFLOQUINE HYDROCHLORIDE

Mefloquini hydrochloridum



and enantiomer, HCl

 $C_{17}H_{17}ClF_6N_2O$ M_r 414.8

DEFINITION

Mefloquine hydrochloride contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of (RS)-[2,8-bis(trifluoromethyl)quinolin-4-yl][(2SR)-piperidin-2-yl]methanol hydrochloride, calculated with reference to the anhydrous substance.

CHARACTERS

A white or slightly yellow, crystalline powder, very slightly soluble in water, freely soluble in methanol, soluble in alcohol.

It melts at about 260 °C, with decomposition.

It shows polymorphism.

IDENTIFICATION

First identification: A, E.

Second identification: B, C, D, E.

- A. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *mefloquine hydrochloride CRS*. If the spectra obtained show differences, dissolve the substance to be examined and the reference substance separately in *methanol R*, evaporate to dryness and record new spectra using the residues.
- B. Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel F₂₅₄ plate R*. Predevelop the plate before use with a mixture of 20 volumes of *methanol R* and 80 volumes of *methylene chloride R*, and dry at 100 °C to 105 °C for 15 min before use.

Test solution. Dissolve 8 mg of the substance to be examined in *methanol R* and dilute to 5 ml with the same solvent.

Reference solution (a). Dissolve 8 mg of *mefloquine hydrochloride CRS* in *methanol R* and dilute to 5 ml with the same solvent.

Reference solution (b). Dilute 2.5 ml of the test solution to 100 ml with *methanol R*.

Reference solution (c). To 1 ml of reference solution (b) add 1 ml of a 0.016 g/l solution of *quinidine sulphate R* in *methanol R*.

Apply to the plate 20 µl of each solution. Develop over a path of 10 cm using a mixture of 10 volumes of *anhydrous acetic acid R*, 10 volumes of *methanol R* and 80 volumes of *methylene chloride R*. Dry the plate in a current of warm air for 15 min and examine in ultraviolet light at 254 nm. Lightly spray with a mixture prepared immediately before use of 1 volume of *sulphuric acid R* and 40 volumes of *iodoplatinate reagent R*. Spray with

strong hydrogen peroxide solution R. The principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a). The test is not valid unless the chromatogram obtained with reference solution (c) shows two clearly separated spots.

- C. Mix about 10 mg with 45 mg of *heavy magnesium oxide R* and ignite in a crucible until a practically white residue is obtained. Allow to cool, add 2 ml of *water R*, 0.05 ml of *phenolphthalein solution R1* and about 1 ml of *dilute hydrochloric acid R* to make the solution colourless. Filter. To the filtrate add a freshly prepared mixture of 0.1 ml of *alizarin S solution R* and 0.1 ml of *zirconyl nitrate solution R*. Mix, allow to stand for 5 min and compare the colour of the solution with a blank prepared in the same manner. The colour of the test solution is yellow and that of the blank is red.
- D. To about 20 mg add 0.2 ml of *sulphuric acid R*. Blue fluorescence appears in ultraviolet light at 365 nm.
- E. It gives reaction (b) of chlorides (2.3.1).

TESTS

Solution S. Dissolve 2.50 g in *methanol R* and dilute to 50.0 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and not more intensely coloured than reference solution BY₇ (2.2.2, *Method I*).

Optical rotation (2.2.7): −0.2° to +0.2°, determined on solution S.

Related substances. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 0.10 g of the substance to be examined in the mobile phase and dilute to 25.0 ml with the mobile phase.

Reference solution (a). Dilute 1.0 ml of the test solution to 50.0 ml with the mobile phase. Dilute 1.0 ml of the solution to 20.0 ml the mobile phase.

Reference solution (b). Dissolve 8 mg of *mefloquine hydrochloride CRS* and 8 mg of *quinidine sulphate R* in the mobile phase and dilute to 50.0 ml with the mobile phase. Dilute 5.0 ml of the solution to 100.0 ml with the mobile phase.

The chromatographic procedure may be carried out using:

- a stainless steel precolumn 0.025 m long and 4 mm in internal diameter and a stainless steel column 0.25 m long and 4 mm in internal diameter, both packed with *end-capped octadecylsilyl silica gel for chromatography R* (5 µm),
- as mobile phase at a flow rate of 0.8 ml/min a mixture prepared as follows: dissolve 1 g of *tetraheptylammonium bromide R* in a mixture of 200 volumes of *methanol R*, 400 volumes of a 1.5 g/l solution of *sodium hydrogen sulphate R* and 400 volumes of *acetonitrile R*,
- as detector a spectrophotometer set at 280 nm.

Equilibrate the column with the mobile phase at a flow rate of 2 ml/min for about 30 min.

Inject 20 µl of reference solution (a). Adjust the sensitivity of the system so that the height of the principal peak is at least 50 per cent of the full scale of the recorder.

Inject 20 µl of reference solution (b). When the chromatograms are recorded in the prescribed conditions, the retention times are: quinidine about 2 min, mefloquine about 4 min, impurity B about 15 min, impurity A about 36 min.

The test is not valid unless, in the chromatogram obtained with reference solution (b), the resolution between quinidine and mefloquine is at least 8.5.

Inject 20 µl of the test solution and 20 µl of reference solution (a). Continue the chromatography for 10 times the retention time of the principal peak. In the chromatogram obtained with the test solution: the area of any peak with a relative retention with reference to mefloquine of about 0.7 is not greater than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent); the area of any peaks apart from the principal peak is not greater than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent) and the sum of the areas of any such peaks is not greater than five times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent). Disregard any peak with an area less than 0.2 times that of the principal peak in the chromatogram obtained with reference solution (a) (0.02 per cent).

Heavy metals (2.4.8). 1.0 g complies with limit test C for heavy metals (20 ppm). Prepare the standard using 2 ml of lead standard solution (10 ppm Pb) R.

Water (2.5.12). Not more than 3.0 per cent, determined on 1.000 g by the semi-micro determination of water.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

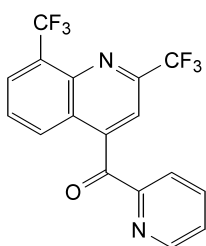
Dissolve 0.350 g in 15 ml of *anhydrous formic acid* R and add 40 ml of *acetic anhydride* R. Titrate with 0.1 M *perchloric acid*, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *perchloric acid* is equivalent to 41.48 mg of $C_{17}H_{17}ClF_6N_2O$.

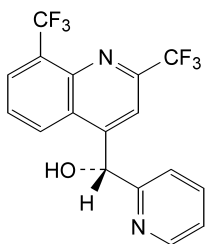
STORAGE

Store protected from light.

IMPURITIES

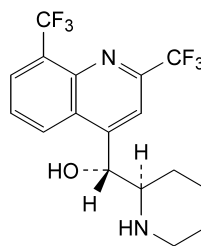


A. [2,8-bis(trifluoromethyl)quinolin-4-yl](pyridin-2-yl)methanone,



and enantiomer

B. (RS)-[2,8-bis(trifluoromethyl)quinolin-4-yl](pyridin-2-yl)methanol,



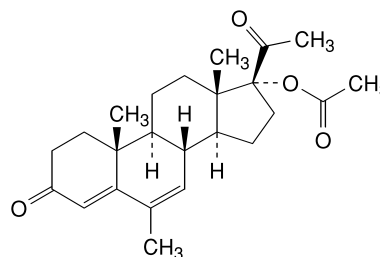
and enantiomer

C. (RS)-[2,8-bis(trifluoromethyl)quinolin-4-yl]-(2RS)-piperidin-2-yl)methanol.

01/2005:1593

MEGESTROL ACETATE

Megestrol acetate



$C_{24}H_{32}O_4$

M_r 384.5

DEFINITION

6-Methyl-3,20-dioxopregna-4,6-dien-17-yl acetate.

Content: 97.0 per cent to 103.0 per cent (dried substance).

CHARACTERS

Appearance: a white or almost white, crystalline powder.

Solubility: practically insoluble in water, soluble in acetone, sparingly soluble in alcohol.

mp: about 217 °C.

IDENTIFICATION

Infrared absorption spectrophotometry (2.2.24).

Comparison: Ph. Eur. reference spectrum of megestrol acetate.

TESTS

Specific optical rotation (2.2.7): + 14.0 to + 17.0 (dried substance).

Dissolve 2.50 g in *methylene chloride* R and dilute to 25.0 ml with the same solvent.

Related substances. Liquid chromatography (2.2.29).

Test solution. Dissolve 25.0 mg of the substance to be examined in a mixture of 145 volumes of *tetrahydrofuran* R and 255 volumes of *acetonitrile* R, dilute to 20 ml with the same mixture of solvents and then dilute to 50.0 ml with *water* R.

Reference solution (a). Dissolve 25.0 mg of *medroxyprogesterone acetate* CRS in a mixture of 145 volumes of *tetrahydrofuran* R and 255 volumes of *acetonitrile* R, dilute to 20 ml with the same mixture of solvents and then dilute to 50.0 ml with *water* R.

Reference solution (b). Dilute 1.0 ml of reference solution (a) to 200.0 ml with the mobile phase.

Reference solution (c). To 3 ml of the test solution, add 1 ml of reference solution (a) and dilute to 50 ml with the mobile phase.

Column:

- size: $l = 0.25$ m, $\varnothing = 4.6$ mm,
- stationary phase: octadecylsilyl silica gel for chromatography R (5 μ m).

Mobile phase: tetrahydrofuran R , acetonitrile R , water R (145:255:600 V/V/V).

Flow rate: 1.5 ml/min.

Detection: spectrophotometer at 254 nm.

Injection: 20 μ l.

Sensitivity: reference solution (b).

Run time: 1.5 times the retention time of megestrol acetate.

System suitability: reference solution (c):

- resolution: minimum 4.0 between the peaks corresponding to impurity A and megestrol acetate.

Limits:

- impurity A: not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent),
- total of other impurities: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (1.0 per cent),
- disregard limit: 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

Loss on drying (2.2.32): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 100–105 °C.

ASSAY

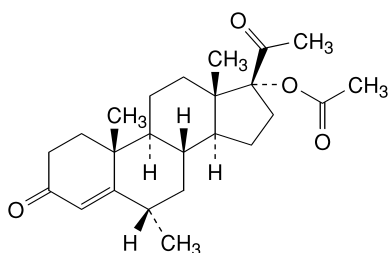
Dissolve 15.0 mg in *ethanol R* and dilute to 100.0 ml with the same solvent. Dilute 5.0 ml of the solution to 100.0 ml with *ethanol R*. Measure the absorbance (2.2.25) at the maximum at 287 nm.

Calculate the content of $C_{24}H_{32}O_4$ taking the specific absorbance to be 640.

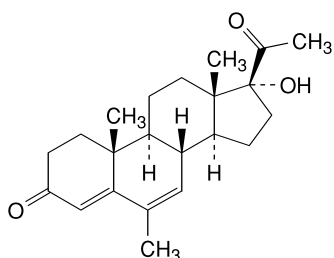
STORAGE

Protected from light.

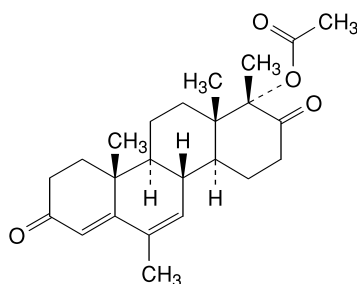
IMPURITIES



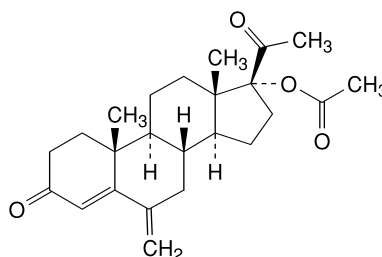
A. 6 α -methyl-3,20-dioxopregn-4-en-17-yl acetate (medroxyprogesterone acetate),



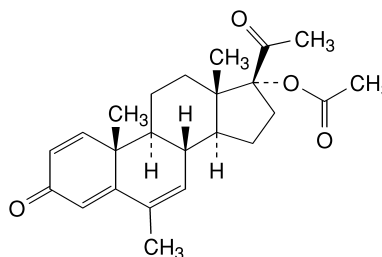
B. 6-methyl-17-hydroxypregna-4,6-diene-3,20-dione (megestrol),



C. 6,17a-dimethyl-3,17-dioxo-*D*-homoandrosta-4,6-dien-17 α -yl acetate (*D*-homo megestrol acetate),



D. 6-methylene-3,20-dioxopregn-4-en-17-yl acetate (6-methylene hydroxyprogesterone acetate),

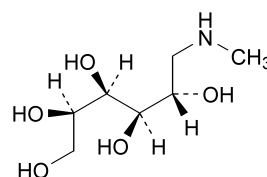


E. 6-methyl-3,20-dioxopregna-1,4,6-trien-17-yl acetate.

01/2005:2055

MEGLUMINE

Megluminum



$C_7H_{17}NO_5$

M_r 195.2

DEFINITION

1-Deoxy-1-(methylamino)-D-glucitol.

Content: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

Appearance: white or almost white, crystalline powder.

Solubility: freely soluble in water, sparingly soluble in alcohol, practically insoluble in methylene chloride.

mp: about 128 °C.

IDENTIFICATION

First identification: A.

Second identification: B, C, D.

A. Infrared absorption spectrophotometry (2.2.24).

Comparison: Ph. Eur. reference spectrum of meglumine.

- B. To 5 ml of *water R*, add 0.5 ml of *paraldehyde R* and 1 ml of *dilute sulphuric acid R*. Shake vigorously and heat on a water-bath until opalescence appears. Allow to stand for 15 min. To 1 ml of this solution add 0.2 ml of a freshly prepared 100 g/l solution of *sodium nitroprusside R*, 50 mg of the substance to be examined and 2 ml of a 50 g/l solution of *disodium tetraborate R*. A blue colour develops.
- C. Dissolve 0.1 g in 2 ml of *nitric acid R*. Add 5 ml of a 6.4 g/l solution of *sodium periodate R*, then add 5 ml of *nitric acid R* and 25 ml of a 17 g/l solution of *silver nitrate R*. Allow to stand in the dark for 10 min. A white precipitate is formed.
- D. It complies with the limits of the assay.

TESTS

Solution S. Dissolve 20.0 g in *distilled water R* and dilute to 100.0 ml with the same solvent.

Appearance of solution. The solution is clear (2.2.1) and its absorbance (2.2.25) at 420 nm is maximum 0.03.

Dissolve the residue obtained in the test for loss on drying in *water R* and dilute to 10 ml with the same solvent.

Specific optical rotation (2.2.7): -16.0 to -17.0 (dried substance).

Dilute 12.5 ml of solution S to 25.0 ml with *water R*.

Reducing substances: maximum 0.2 per cent, expressed as glucose.

Dilute 1.25 ml of solution S to 2.5 ml with *water R*, add 2 ml of *cupri-tartaric solution R*. Heat on a water-bath for 10 min. Cool under running water for 1 min and then sonicate the tube for 20 s. Immediately filter through a filter 25 mm in diameter and 0.5 μm in pore size. Rinse with 10 ml of *water R*. Prepare a standard in the same manner using 2.5 ml of a solution obtained by dissolving 20 mg of *glucose R* in *water R* and diluting to 100 ml with the same solvent.

Any precipitate on the membrane filter obtained with the test solution is not more intensely coloured than the precipitate obtained with the standard.

Chlorides (2.4.4): maximum 100 ppm.

To 2.5 ml of solution S add 12.5 ml of *water R*.

Sulphates (2.4.13): maximum 150 ppm.

To 5 ml of solution S add 10 ml of *distilled water R*.

Iron: maximum 10 ppm.

To 10 ml of solution S, adjusted to pH 1 by adding 0.8 ml of *hydrochloric acid R*, add 0.05 ml of *bromine water R*. Allow to stand for 5 min, evaporate the excess of bromine in a current of air and add 3 ml of *potassium thiocyanate solution R*. Prepare a reference solution at the same time and in the same manner using 10 ml of *iron standard solution* (2 ppm Fe) *R*, to which 2 ml of *hydrochloric acid R* has been added.

After 5 min, any red colour in the test solution is not more intense than that in the reference solution.

Heavy metals (2.4.8): maximum 10 ppm.

Adjust 10 ml of solution S to pH 3-4 with *dilute acetic acid R*. Dilute to 20 ml with *water R*. 12 ml of the solution complies with limit test A. Prepare the standard using *lead standard solution* (1 ppm Pb) *R*.

Loss on drying (2.2.32): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 100-105 °C for 3 h.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

Bacterial endotoxins (2.6.14): less than 1.5 IU/g.

ASSAY

Dissolve 0.180 g in 30 ml of *water R*. Titrate with 0.05 M *sulphuric acid*, determining the end-point potentiometrically (2.2.20).

1 ml of 0.05 M *sulphuric acid* is equivalent to 19.52 mg of $\text{C}_7\text{H}_{17}\text{NO}_5$.

01/2005:1447

MELISSA LEAF

Melissae folium

DEFINITION

Melissa leaf consists of the dried leaf of *Melissa officinalis* L. It contains not less than 4.0 per cent of total hydroxycinnamic derivatives expressed as rosmarinic acid ($\text{C}_{18}\text{H}_{16}\text{O}_8$; M_r 360.3), calculated with reference to the dried drug.

CHARACTERS

Melissa leaf has an odour reminiscent of lemon.

It has the macroscopic and microscopic characters described under identification tests A and B.

IDENTIFICATION

- A. Melissa leaf has a petiole varying length and is oval, cordate and up to about 8 cm long and 5 cm wide. The lamina is thin and the under surface has a conspicuous, raised, reticulate venation; the margins are roughly dentate or crenate. The upper surface is bright green and the lower surface is lighter in colour.
- B. Reduce to a powder (355). The powder is greenish. Examine under a microscope using *chloral hydrate solution R*. The powder shows fragments of the leaf epidermis with sinuous walls; short, straight, unicellular, conical covering trichomes with a finely striated cuticle; multicellular, uniseriate covering trichomes with pointed ends and thick, warty cuticles; eight-celled secretory trichomes of lamiaceous type; secretory trichomes with unicellular to tricellular stalks and unicellular or, more rarely bicellular, heads; diacytic stomata (2.8.3), on the lower surface only.
- C. Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel plate R*.

Test solution. Place 2.0 g of the powdered drug (355) in a 250 ml round-bottomed flask and add 100 ml of *water R*. Distil for 1 h using the apparatus for the determination of essential oils in vegetable drugs (2.8.12) and 0.5 ml of *xylene R* in the graduated tube. After distillation transfer the organic phase to a 1 ml volumetric flask rinsing the graduated tube of the apparatus with the aid of a small portion of *xylene R* and dilute to 1.0 ml with the same solvent.

Reference solution. Dissolve 1.0 μl of *citronellal R* and 10.0 μl of *citral R* in 25 ml of *xylene R*.

Apply to the plate as bands 10 μl of the reference solution and 20 μl of the test solution. Develop over a path of 15 cm using a mixture of 10 volumes of *ethyl acetate R* and 90 volumes of *hexane R*. Allow the plate to dry in air. Spray the plate with *anisaldehyde solution R*. Examine in daylight while heating at 100 °C to 105 °C for 10 min to 15 min. The chromatogram obtained with the reference solution shows in the lower third a greyish-violet to bluish-violet double zone (citral) and above it a grey to greyish-violet zone (citronellal). The chromatogram obtained with the test solution shows zones similar in position and colour to the zones in the chromatogram

obtained with the reference solution and between these zones a reddish-violet zone (epoxycaryophyllene). Further zones may be present.

TESTS

Foreign matter (2.8.2). Not more than 10 per cent of stems having a diameter greater than 1 mm and not more than 2 per cent of other foreign matters, determined on 20 g.

Loss on drying (2.2.32). Not more than 10.0 per cent, determined on 1.000 g of the powdered drug (355) by drying in an oven at 100 °C to 105 °C for 2 h.

Total ash (2.4.16). Not more than 12.0 per cent.

ASSAY

Stock solution. To 0.200 g of the powdered drug (355) add 190 ml of *alcohol* (50 per cent V/V) R. Boil in a water-bath under a reflux condenser for 30 min. Allow to cool and filter. Rinse the filter with 10 ml of *alcohol* (50 per cent V/V) R. Combine the filtrate and the rinsings in a volumetric flask and dilute to 200.0 ml with *alcohol* (50 per cent V/V) R.

Test solution. To 1.0 ml of the stock solution in a test-tube add 2 ml of 0.5 M *hydrochloric acid*, 2 ml of a solution prepared by dissolving 10 g of *sodium nitrite* R and 10 g of *sodium molybdate* R in 100 ml of *water* R and then add 2 ml of *dilute sodium hydroxide solution* R and dilute to 10.0 ml with *water* R and mix.

Compensation solution. In another test-tube place 1.0 ml of the stock solution, 2 ml of 0.5 M *hydrochloric acid*, 2 ml of *dilute sodium hydroxide solution* R and dilute to 10.0 ml with *water* R.

Immediately measure the absorbance (2.2.25) of the test solution at 505 nm by comparison with the compensation solution.

Calculate the percentage content of total hydroxycinnamic derivatives, expressed as rosmarinic acid, from the expression:

$$\frac{A \times 5}{m}$$

i.e. taking the specific absorbance to be 400 for rosmarinic acid at 505 nm.

A = absorbance of the test solution at 505 nm,

m = mass of the substance to be examined, in grams.

STORAGE

Store protected from light.

DEFINITION

Menadione contains not less than 98.5 per cent and not more than the equivalent of 101.0 per cent of 2-methylnaphthalene-1,4-dione, calculated with reference to the dried substance.

CHARACTERS

A pale-yellow, crystalline powder, practically insoluble in water, freely soluble in toluene, sparingly soluble in alcohol and in methanol. It is unstable in light.

IDENTIFICATION

First identification: A, B.

Second identification: A, C, D.

A. Melting point (2.2.14): 105 °C to 108 °C.

B. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *menadione CRS*.

C. Dissolve about 1 mg in 5 ml of *alcohol* R, add 2 ml of *ammonia* R and 0.2 ml of *ethyl cyanoacetate* R. An intense bluish-violet colour develops. Add 2 ml of *hydrochloric acid* R. The colour disappears.

D. Dissolve about 10 mg in 1 ml of *alcohol* R, add 1 ml of *hydrochloric acid* R and heat in a water-bath. A red colour develops.

TESTS

Related substances. Carry out the test protected from bright light. Examine by thin-layer chromatography (2.2.27), using *silica gel GF₂₅₄* R as the coating substance.

Test solution. Dissolve 0.2 g of the substance to be examined in *acetone* R and dilute to 10 ml with the same solvent.

Reference solution. Dilute 0.5 ml of the test solution to 100 ml with *acetone* R.

Apply separately to the plate 5 µl of each solution. Develop over a path of 15 cm using a mixture of 1 volume of *nitromethane* R, 2 volumes of *acetone* R, 5 volumes of *ethylene chloride* R and 90 volumes of *cyclohexane* R. Dry the plate in a current of hot air. Repeat the development and drying a further two times. Examine in ultraviolet light at 254 nm. Any spot in the chromatogram obtained with the test solution, apart from the principal spot, is not more intense than the spot in the chromatogram obtained with the reference solution (0.5 per cent).

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying over *diphosphorus pentoxide* R at a pressure of 2 kPa to 3 kPa for 4 h.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

01/2005:0507 ASSAY

Dissolve 0.150 g in 15 ml of *glacial acetic acid* R in a flask with a stopper fitted with a valve. Add 15 ml of *dilute hydrochloric acid* R and 1 g of *zinc powder* R. Close the flask. Allow the mixture to stand for 60 min, protected from light, with occasional shaking. Filter the solution over a cotton wad, wash with three quantities, each of 10 ml, of *carbon dioxide-free water* R. Add 0.1 ml of *ferroin* R and immediately titrate the combined filtrate and washings with 0.1 M *ammonium and cerium nitrate*.

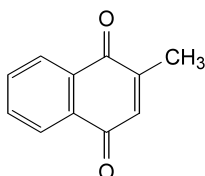
1 ml of 0.1 M *ammonium and cerium nitrate* is equivalent to 8.61 mg of C₁₁H₈O₂.

STORAGE

Store protected from light.

MENADIONE

Menadionum



C₁₁H₈O₂

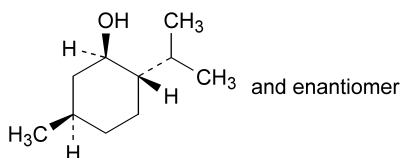
M_r 172.2

01/2005:0623

with iced *acetone R* and dry at 75 °C at a pressure not exceeding 2.7 kPa for 30 min. The crystals melt (2.2.14) at 130 °C to 131 °C.

MENTHOL, RACEMIC

Mentholum racemicum

C₁₀H₂₀OM_r 156.3

DEFINITION

Racemic menthol is a mixture of equal parts of (1*RS*,2*SR*,5*RS*)-5-methyl-2-(1-methylethyl)cyclohexanol.

CHARACTERS

A free-flowing or agglomerated, crystalline powder or prismatic or acicular, colourless, shiny crystals, practically insoluble in water, very soluble in alcohol and in light petroleum, freely soluble in fatty oils and in liquid paraffin, very slightly soluble in glycerol.

It melts at about 34 °C.

IDENTIFICATION

First identification: A, C.

Second identification: B, D.

- A. It complies with the test for angle of optical rotation (see Tests).
- B. Examine by thin-layer chromatography (2.2.27), using *silica gel G R* as the coating substance.
Test solution. Dissolve 25 mg of the substance to be examined in *methanol R* and dilute to 5 ml with the same solvent.
Reference solution. Dissolve 25 mg of *menthol CRS* in *methanol R* and dilute to 5 ml with the same solvent.
Apply separately to the plate 2 µl of each solution. Develop over a path of 15 cm using a mixture of 5 volumes of *ethyl acetate R* and 95 volumes of *toluene R*. Allow the plate to dry in air until the solvents have evaporated and spray with *anisaldehyde solution R*. Heat at 100 °C to 105 °C for 5 min to 10 min. The principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.
- C. Examine the chromatograms obtained in the test for related substances. The principal peak in the chromatogram obtained with test solution (b) is similar in position and approximate dimensions to the principal peak in the chromatogram obtained with reference solution (c).
- D. Dissolve 0.20 g in 0.5 ml of *anhydrous pyridine R*. Add 3 ml of a 150 g/l solution of *dinitrobenzoyl chloride R* in *anhydrous pyridine R*. Heat on a water-bath for 10 min. Add 7.0 ml of *water R* in small quantities with stirring and allow to stand in iced water for 30 min. A precipitate is formed. Allow to stand and decant the supernatant liquid. Wash the precipitate with two quantities, each of 5 ml, of iced *water R*, recrystallise from 10 ml of *acetone R*, wash

TESTS

Solution S. Dissolve 2.50 g in 10 ml of *alcohol R* and dilute to 25.0 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and colourless (2.2.2, Method II).

Acidity or alkalinity. Dissolve 1.0 g in *alcohol R* and dilute to 10 ml with the same solvent. Add 0.1 ml of *phenolphthalein solution R*; the solution is colourless. Not more than 0.5 ml of 0.01 M *sodium hydroxide* is required to change the colour of the indicator to pink.

Optical rotation (2.2.7). The angle of optical rotation of solution S is + 0.2° to − 0.2°.

Related substances. Examine by gas chromatography (2.2.28).

Test solution (a). Dissolve 0.20 g of the substance to be examined in *methylene chloride R* and dilute to 50.0 ml with the same solvent.

Test solution (b). Dilute 1.0 ml of test solution (a) to 10.0 ml with *methylene chloride R*.

Reference solution (a). Dissolve 40.0 mg of the substance to be examined and 40.0 mg of *isomenthol R* in *methylene chloride R* and dilute to 100.0 ml with the same solvent.

Reference solution (b). Dilute 0.10 ml of test solution (a) to 100.0 ml with *methylene chloride R*.

Reference solution (c). Dissolve 40.0 mg of *menthol CRS* in *methylene chloride R* and dilute to 100.0 ml with the same solvent.

The chromatographic procedure may be carried out using:

- a glass column 2.0 m long and 2 mm in internal diameter packed with *diatomaceous earth for gas chromatography R* impregnated with 15 per cent *m/m* of *macrogol 1500 R*,
- *nitrogen for chromatography R* as carrier gas at a flow rate of 30 ml/min,
- a flame-ionisation detector,

maintaining the temperature of the column at 120 °C, that of the injection port at 150 °C and that of the detector at 200 °C.

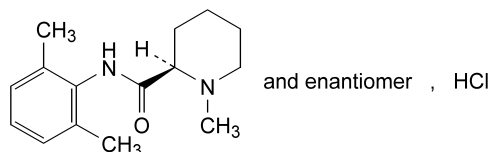
Inject separately 1 µl of each solution. Record the chromatograms for twice the retention time of the peak corresponding to menthol. In the chromatogram obtained with test solution (a), the sum of the areas of the peaks, apart from the principal peak, is not greater than 1 per cent of the area of the principal peak. Disregard any peak due to the solvent and any peak whose area is less than 0.05 per cent of the area of the principal peak. The test is not valid unless: in the chromatogram obtained with reference solution (a) the resolution between the peaks corresponding to menthol and isomenthol is at least 1.4 and the principal peak in the chromatogram obtained with reference solution (b) has a signal-to-noise ratio of at least 5.

Residue on evaporation. Evaporate 2.00 g on a water-bath and heat in an oven at 100 °C to 105 °C for 1 h. The residue weighs not more than 1.0 mg (0.05 per cent).

01/2005:1242 D. It gives reaction (a) of chlorides (2.3.1).

MEPIVACAINE HYDROCHLORIDE

Mepivacaini hydrochloridum

 $C_{15}H_{23}ClN_2O$ M_r 282.8

DEFINITION

Mepivacaine hydrochloride contains not less than 98.5 per cent and not more than the equivalent of 101.0 per cent of (*RS*)-*N*-(2,6-dimethylphenyl)-1-methylpiperidine-2-carboxamide hydrochloride, calculated with reference to the dried substance.

CHARACTERS

A white, crystalline powder, freely soluble in water and in alcohol, very slightly soluble in methylene chloride.

It melts at about 260 °C with decomposition.

IDENTIFICATION

First identification: A, B, D.

Second identification: B, C, D.

A. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *mepivacaine hydrochloride CRS*. Examine the substances prepared as discs.

B. Examine by thin-layer chromatography (2.2.27), using as the coating substance a suitable silica gel with a fluorescent indicator having an optimal intensity at 254 nm.

Test solution. Dissolve 20 mg of the substance to be examined in *alcohol R* and dilute to 5 ml with the same solvent.

Reference solution (a). Dissolve 20 mg of *mepivacaine hydrochloride CRS* in *alcohol R* and dilute to 5 ml with the same solvent.

Reference solution (b). Dissolve 20 mg of *mepivacaine hydrochloride CRS* and 20 mg of *lidocaine hydrochloride CRS* in *alcohol R* and dilute to 5 ml with the same solvent.

Apply to the plate 10 µl of each solution. Develop over a path of 12 cm using a mixture of 1 volume of *concentrated ammonia R*, 5 volumes of *methanol R* and 100 volumes of *ether R*. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. The principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a). The test is not valid unless the chromatogram obtained with reference solution (b) shows two clearly separated principal spots.

C. To 5 ml of solution S (see Tests) add 1 ml of *dilute sodium hydroxide solution R* and shake with two quantities, each of 10 ml, of *ether R*. Dry the combined upper layers over *anhydrous sodium sulphate R*. Filter and evaporate the ether on a water-bath. Dry the residue at 100–105 °C for 2 h. The melting point (2.2.14) is 151 °C to 155 °C.

TESTS

Solution S. Dissolve 1.5 g in *carbon dioxide-free water R* and dilute to 30 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and not more intensely coloured than reference solution B₇ (2.2.2, *Method II*).

pH (2.2.3). Dilute 2 ml of solution S to 5 ml with *carbon dioxide-free water R*. The pH of the solution is 4.0 to 5.0.

Optical rotation (2.2.7): −0.10° to +0.10°, determined on solution S.

Related substances. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 20.0 mg of the substance to be examined in the mobile phase and dilute to 10.0 ml with the mobile phase.

Reference solution (a). Dissolve 20.0 mg of the substance to be examined and 30.0 mg of *mepivacaine impurity B CRS* in the mobile phase and dilute to 100.0 ml with the mobile phase. Dilute 1.0 ml of the solution to 100.0 ml with the mobile phase.

Reference solution (b). Dilute 1.0 ml of the test solution to 100.0 ml with the mobile phase. Dilute 1.0 ml of the solution to 10.0 ml with the mobile phase.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.125 m long and 4.6 mm in internal diameter packed with *base-deactivated octadecylsilyl silica gel for chromatography R* (5 µm),
- as mobile phase at a flow rate of 1 ml/min a mixture of 35 volumes of *acetonitrile R* and 65 volumes of a 2.25 g/l solution of *phosphoric acid R* (adjusted to pH 7.6 with *strong sodium hydroxide solution R*),
- as detector a spectrophotometer set at 220 nm.

Inject 20 µl of reference solution (a). Adjust the sensitivity of the system so that the heights of the two principal peaks in the chromatogram obtained are at least 20 per cent of the full scale of the recorder. The test is not valid unless the resolution between the peaks corresponding to mepivacaine impurity B and mepivacaine is at least 2.5.

Inject 20 µl of the test solution and 20 µl of reference solution (b). Continue the chromatography of the test solution for three times the retention time of mepivacaine. In the chromatogram obtained with the test solution: the area of any peak, apart from the principal peak, is not greater than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent) and the area of not more than one of these peaks is greater than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent); the sum of the areas of all the peaks, apart from the principal peak, is not greater than five times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent). Disregard any peak with an area less than 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (b).

2,6-Dimethylaniline. Dissolve 0.50 g in *methanol R* and dilute to 10 ml with the same solvent. To 2 ml of the solution add 1 ml of a freshly prepared 10 g/l solution of *dimethylaminobenzaldehyde R* in *methanol R* and 2 ml of *glacial acetic acid R* and allow to stand for 10 min. Any yellow colour in the solution is not more intense than that in a standard prepared at the same time and in the same manner using 2 ml of a 5 mg/l solution of *2,6-dimethylaniline R* in *methanol R* (100 ppm).

Heavy metals (2.4.8). Dissolve 1.0 g in *water R* and dilute to 25 ml with the same solvent. Carry out the prefiltration. 10 ml of the filtrate complies with limit test E for heavy metals (5 ppm). Prepare the standard using 2 ml of *lead standard solution* (1 ppm Pb) *R*.

Loss on drying (2.2.32). Not more than 1.0 per cent, determined on 1.000 g by drying in an oven at 100–105 °C.

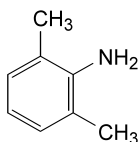
Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

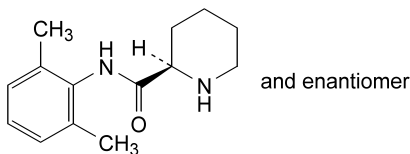
Dissolve 0.250 g in a mixture of 5.0 ml of 0.01 *M* hydrochloric acid and 50 ml of *alcohol R*. Carry out a potentiometric titration (2.2.20), using 0.1 *M* sodium hydroxide. Read the volume added between the two points of inflexion.

1 ml of 0.1 *M* sodium hydroxide is equivalent to 28.28 mg of $C_{15}H_{23}ClN_2O$.

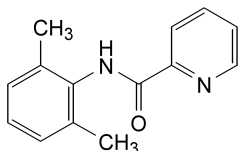
IMPURITIES



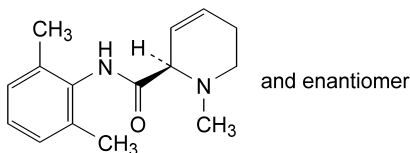
A. 2,6-dimethylaniline,



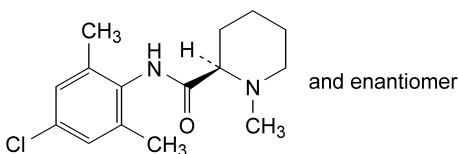
B. (*RS*)-*N*-(2,6-dimethylphenyl)piperidine-2-carboxamide,



C. *N*-(2,6-dimethylphenyl)pyridine-2-carboxamide,



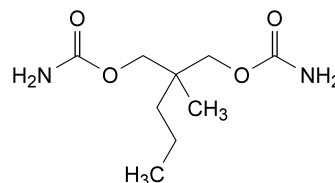
D. (*RS*)-*N*-(2,6-dimethylphenyl)-1-methyl-1,2,5,6-tetrahydropyridine-2-carboxamide,



E. (*RS*)-*N*-(4-chloro-2,6-dimethylphenyl)-1-methylpiperidine-2-carboxamide.

MEPROBAMATE

Meprobamatum



$C_9H_{18}N_2O_4$

M_r 218.3

DEFINITION

Meprobamate contains not less than 97.0 per cent and not more than the equivalent of 101.0 per cent of 2-methyl-2-propylpropane-1,3-diyl dicarbamate, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, amorphous or crystalline powder, slightly soluble in water, freely soluble in alcohol.

IDENTIFICATION

First identification: A, B.

Second identification: A, C, D.

A. Melting point (2.2.14): 104 °C to 108 °C.

B. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *meprobamate CRS*.

C. To 0.5 g add 1 ml of *acetic anhydride R* and 0.05 ml of *sulphuric acid R*, mix and allow to stand for 30 min, shaking frequently. Pour the solution dropwise into 50 ml of *water R*, mix and allow to stand. Initiate crystallisation by scratching the wall of the tube with a glass rod. Collect the precipitate by filtration, wash and dry at 60 °C. The precipitate melts (2.2.14) at 124 °C to 128 °C.

D. Dissolve 0.2 g in 15 ml of 0.5 *M* *alcoholic potassium hydroxide* and boil under a reflux condenser for 15 min. Add 0.5 ml of *glacial acetic acid R* and 1 ml of a 50 g/l solution of *cobalt nitrate R* in *ethanol R*. A deep-blue colour develops.

TESTS

Appearance of solution. Dissolve 1.0 g in 20 ml of *ethanol R*. The solution is clear (2.2.1) and colourless (2.2.2, *Method II*).

Related substances. Examine by thin-layer chromatography (2.2.27), using *silica gel G R* as the coating substance.

Test solution. Dissolve 0.20 g of the substance to be examined in *alcohol R* and dilute to 10 ml with the same solvent.

Reference solution. Dilute 0.1 ml of the test solution to 10 ml with *alcohol R*.

Apply separately to the plate 5 µl of each solution. Develop over a path of 15 cm using a mixture of 10 volumes of *pyridine R*, 30 volumes of *acetone R* and 70 volumes of *hexane R*. Dry the plate at 120 °C for 30 min, allow to cool and spray with a solution of 0.25 g of *vanillin R* in a cooled mixture of 10 ml of *alcohol R* and 40 ml of *sulphuric acid R* and heat at 100 °C to 105 °C for 30 min. Any spot in the chromatogram obtained with the test solution, apart from the principal spot, is not more intense than the spot in the chromatogram obtained with the reference solution (1.0 per cent).

Heavy metals (2.4.8). Dissolve 2.0 g in a mixture of 15 volumes of *water R* and 85 volumes of *acetone R* and dilute to 20 ml with the same mixture of solvents. 12 ml of the solution complies with limit test B for heavy metals (10 ppm). Prepare the standard using lead standard solution (1 ppm Pb) obtained by diluting *lead standard solution* (100 ppm Pb) *R* with the mixture of *water R* and *acetone R*.

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying *in vacuo* at 60 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

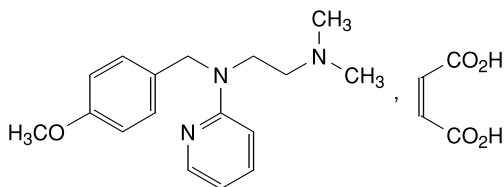
Dissolve 0.1000 g in 15 ml of a 25 per cent *V/V* solution of *sulphuric acid R* and boil under a reflux condenser for 3 h. Cool, dissolve by cautiously adding 30 ml of *water R*, cool again and place in a steam-distillation apparatus. Add 40 ml of *strong sodium hydroxide solution R* and distil immediately by passing steam through the mixture. Collect the distillate into 40 ml of a 40 g/l solution of *boric acid R* until the total volume in the receiver reaches about 200 ml. Add 0.25 ml of *methyl red mixed solution R*. Titrate with 0.1 *M hydrochloric acid* until the colour changes from green to violet. Carry out a blank titration.

1 ml of 0.1 *M hydrochloric acid* is equivalent to 10.91 mg of $C_{21}H_{27}N_3O_5$.

01/2005:0278

MEPYRAMINE MALEATE

Mepyramini maleas



$C_{21}H_{27}N_3O_5$

M_r 401.5

DEFINITION

Mepyramine maleate contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of *N*-(4-methoxybenzyl)-*N,N*-dimethyl-*N*-(pyridin-2-yl)ethane-1,2-diamine (*Z*)-butenedioate, calculated with reference to the dried substance.

CHARACTERS

A white or slightly yellowish, crystalline powder, very soluble in water, freely soluble in alcohol.

IDENTIFICATION

First identification: A, B.

Second identification: A, C, D, E.

A. Melting point (2.2.14): 99 °C to 103 °C.

B. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *mepyramine maleate CRS*. Examine the substances as 50 g/l solutions in *methylene chloride R* using a 0.1 mm cell.

C. Dissolve 0.100 g in 0.01 *M hydrochloric acid* and dilute to 100.0 ml with the same acid. Dilute 1.0 ml of this solution to 100.0 ml with 0.01 *M hydrochloric acid*.

Examined between 220 nm and 350 nm (2.2.25), the solution shows two absorption maxima, at 239 nm and 316 nm. The specific absorbances at the maxima are 431 to 477 and 196 to 220, respectively.

- D. Examine the chromatograms obtained in the test for related substances. The principal spot in the chromatogram obtained with test solution (b) is similar in position and size to the principal spot in the chromatogram obtained with reference solution (b).
- E. Triturate 0.1 g with 3 ml of *water R* and 1 ml of *strong sodium hydroxide solution R*. Shake with three quantities, each of 5 ml, of *ether R*. To 0.1 ml of the aqueous layer add a solution of 10 mg of *resorcinol R* in 3 ml of *sulphuric acid R*. Heat on a water-bath for 15 min; no colour develops. To the rest of the aqueous layer add 1 ml of *bromine water R*. Heat on a water-bath for 15 min and then heat to boiling and cool. To 0.2 ml of this solution add a solution of 10 mg of *resorcinol R* in 3 ml of *sulphuric acid R*. Heat on a water-bath for 15 min. A violet-pink colour develops.

TESTS

Solution S. Dissolve 5.0 g in *carbon dioxide-free water R* and dilute to 25 ml with the same solvent.

Appearance of solution. Dilute 5 ml of solution S to 25 ml with *carbon dioxide-free water R*. The solution is clear (2.2.1) and not more intensely coloured than reference solution Y_6 (2.2.2, *Method II*).

pH (2.2.3). Dilute 1 ml of solution S to 10 ml with *carbon dioxide-free water R*. The pH of this solution is 4.9 to 5.2.

Related substances. Examine by thin-layer chromatography (2.2.27), using *silica gel GF₂₅₄ R* as the coating substance.

Test solution (a). Dissolve 0.4 g of the substance to be examined in *chloroform R* and dilute to 10 ml with the same solvent. Prepare immediately before use.

Test solution (b). Dilute 1 ml of test solution (a) to 10 ml with *chloroform R*.

Reference solution (a). Dissolve 0.4 g of *mepyramine maleate CRS* in *chloroform R* and dilute to 10 ml with the same solvent. Prepare immediately before use.

Reference solution (b). Dilute 1 ml of reference solution (a) to 10 ml with *chloroform R*.

Reference solution (c). Dilute 0.1 ml of reference solution (a) to 50 ml with *chloroform R*.

Reference solution (d). To 2 ml of reference solution (c) add 2 ml of *chloroform R*.

Apply separately to the plate 5 µl of each solution. Develop over a path of 15 cm using a mixture of 2 volumes of *diethylamine R* and 100 volumes of *ethyl acetate R*. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. Any spot in the chromatogram obtained with test solution (a), apart from the principal spot, does not show more intense fluorescence quenching than the spot in the chromatogram obtained with reference solution (c) (0.2 per cent). The test is not valid unless the principal spots in the chromatograms obtained with test solution (a) and with reference solution (a) have R_f values not less than 0.2 and the spot in the chromatogram obtained with reference solution (d) is clearly visible. During evaluation, ignore the spot remaining at the starting point (maleic acid).

Chlorides (2.4.4). 2.5 ml of solution S diluted to 15 ml with *water R* complies with the limit test for chlorides (100 ppm).

Heavy metals (2.4.8). 1.0 g complies with limit test D for heavy metals (20 ppm). Prepare the standard using 2 ml of *lead standard solution* (10 ppm Pb) *R*.

Loss on drying (2.2.32). Not more than 0.25 per cent, determined on 1.00 g by drying in an oven at 80 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on the residue obtained in the test for loss on drying.

ASSAY

Dissolve 0.150 g in 40 ml of *anhydrous acetic acid R*. Titrate with 0.1 M *perchloric acid* determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *perchloric acid* is equivalent to 20.07 mg of $C_{21}H_{27}N_3O_5$.

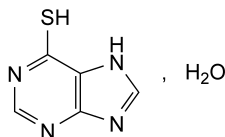
STORAGE

Store protected from light.

01/2005:0096

MERCAPTOPURINE

Mercaptopurinum


 $C_5H_4N_4S \cdot H_2O$
 M_r 170.2

DEFINITION

Mercaptopurine contains not less than 98.5 per cent and not more than the equivalent of 101.0 per cent of 7*H*-purine-6-thiol, calculated with reference to the anhydrous substance.

CHARACTERS

A yellow, crystalline powder, practically insoluble in water, slightly soluble in alcohol. It dissolves in solutions of alkali hydroxides.

IDENTIFICATION

- Dissolve 20 mg in 5 ml of *dimethyl sulphoxide R* and dilute to 100 ml with 0.1 M *hydrochloric acid*. Dilute 5 ml of the solution to 200 ml with 0.1 M *hydrochloric acid*. Examined between 230 nm and 350 nm (2.2.25), the solution shows only one absorption maximum, at 325 nm.
- Dissolve about 20 mg in 20 ml of *alcohol R* heated to 60 °C and add 1 ml of a saturated solution of *mercuric acetate R* in *alcohol R*. A white precipitate is formed.
- Dissolve about 20 mg in 20 ml of *alcohol R* heated to 60 °C and add 1 ml of a 10 g/l solution of *lead acetate R* in *alcohol R*. A yellow precipitate is formed.

TESTS

Hypoxanthine. Examine by thin-layer chromatography (2.2.27), using *silica gel GF₂₅₄ R* as the coating substance.

Test solution. Dissolve 50 mg of the substance to be examined in 1 ml of *dimethyl sulphoxide R* and dilute to 10 ml with *methanol R*.

Reference solution. Dissolve 10 mg of *hypoxanthine R* in 10 ml of *dimethyl sulphoxide R* and dilute to 100 ml with *methanol R*.

Apply separately to the plate 5 µl of each solution. Develop over a path of 10 cm using a mixture of 3 volumes of *concentrated ammonia R*, 7 volumes of *water R* and 90 volumes of *acetone R*. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. Any spot corresponding to hypoxanthine in the chromatogram

obtained with the test solution is not more intense than the spot in the chromatogram obtained with the reference solution (2.0 per cent).

Water (2.5.12). 10.0 per cent to 12.0 per cent, determined on 0.250 g by the semi-micro determination of water.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.100 g in 50 ml of *dimethylformamide R*. Titrate with 0.1 M *tetrabutylammonium hydroxide*, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *tetrabutylammonium hydroxide* is equivalent to 15.22 mg of $C_5H_4N_4S$.

STORAGE

Store protected from light.

01/2005:0120

MERCURIC CHLORIDE

Hydrargyri dichloridum

 $HgCl_2$
 M_r 271.5

DEFINITION

Mercuric chloride contains not less than 99.5 per cent and not more than the equivalent of 100.5 per cent of $HgCl_2$, calculated with reference to the dried substance.

CHARACTERS

A white, crystalline powder or colourless or white crystals or heavy crystalline masses, soluble in water and in glycerol, freely soluble in alcohol.

IDENTIFICATION

- It gives the reactions of chlorides (2.3.1).
- Solution S (see Tests) gives the reactions of mercury (2.3.1).

TESTS

Solution S. Dissolve 1.0 g in *carbon dioxide-free water R* and dilute to 20 ml with the same solvent.

Appearance of solution. Solution S is not more opalescent than reference suspension II (2.2.1) and is colourless (2.2.2, *Method II*).

Acidity or alkalinity. To 10 ml of solution S add 0.1 ml of *methyl red solution R*. The solution is red. Add 0.5 g of *sodium chloride R*. The solution becomes yellow. Not more than 0.5 ml of 0.01 M *hydrochloric acid* is required to change the colour to red.

Mercurous chloride. Dissolve 1.0 g in 30 ml of *ether R*. The solution shows no opalescence.

Loss on drying (2.2.32). Not more than 1.0 per cent, determined on 2.00 g by drying *in vacuo* for 24 h.

ASSAY

Dissolve 0.500 g in 100 ml of *water R*. Add 20.0 ml of 0.1 M *sodium edetate* and 5 ml of *buffer solution pH 10.9 R*.

Allow to stand for 15 min. Add 0.1 g of *mordant black 11 triturate R* and titrate with 0.1 M *zinc sulphate* until the colour changes to purple. Add 3 g of *potassium iodide R*, allow to stand for 2 min, add a further 0.1 g of *mordant black 11 triturate R* and titrate with 0.1 M *zinc sulphate*.

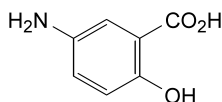
1 ml of 0.1 M *zinc sulphate* used in the second titration is equivalent to 27.15 mg of $HgCl_2$.

STORAGE

Store protected from light.

MESALAZINE

Mesalazinum



$C_7H_7NO_3$

M_r 153.1

DEFINITION

5-Amino-2-hydroxybenzoic acid.

Content: 98.5 per cent to 101.0 per cent (dried substance).

CHARACTERS

Appearance: almost white or light grey or light pink powder or crystals.

Solubility: very slightly soluble in water, practically insoluble in alcohol. It dissolves in dilute solutions of alkali hydroxides and in dilute hydrochloric acid.

IDENTIFICATION

First identification: B.

Second identification: A, C.

A. Dissolve 50.0 mg in 10 ml of a 10.3 g/l solution of *hydrochloric acid R* and dilute to 100.0 ml with the same acid. Dilute 5.0 ml to 200.0 ml with a 10.3 g/l solution of *hydrochloric acid R*. Examined between 210 nm and 250 nm (2.2.25), the solution shows an absorption maximum at about 230 nm. The specific absorbance at the maximum is 430 to 450.

B. Infrared absorption spectrophotometry (2.2.24).

Preparation: discs.

Comparison: mesalazine CRS.

C. Thin-layer chromatography (2.2.27).

Test solution. Dissolve 50 mg of the substance to be examined in 10 ml of a mixture of equal volumes of *glacial acetic acid R* and *water R* and dilute to 20.0 ml with *methanol R*.

Reference solution. Dissolve 50 mg of mesalazine CRS in 10 ml of a mixture of equal volumes of *glacial acetic acid R* and *water R* and dilute to 20.0 ml with *methanol R*.

Plate: a suitable silica gel as the coating substance.

Mobile phase: *glacial acetic acid R*, *methanol R*, *methyl isobutyl ketone R* (10:40:50 V/V/V).

Application: 5 µl.

Development: over a path of 10 cm.

Drying: in air.

Detection: examine in ultraviolet light at 365 nm.

Results: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

TESTS

Appearance of solution. Maintain the solutions at 40 °C during preparation and measurements. Dissolve 0.5 g in 1 M *hydrochloric acid* and dilute to 20 ml with the same

acid. The solution is clear (2.2.1). Immediately measure the absorbance (2.2.25) of the solution at 440 nm and 650 nm. The absorbance is not greater than 0.15 at 440 nm and 0.10 at 650 nm.

Reducing substances. Dissolve 0.10 g in *dilute hydrochloric acid R* and dilute to 25 ml with the same acid. Add 0.2 ml of *starch solution R* and 0.25 ml of 0.01 M *iodine*. Allow to stand for 2 min. The solution is blue or violet-brown.

Related substances. Liquid chromatography (2.2.29). Use freshly prepared solutions and mobile phases.

Test solution. Dissolve 50.0 mg of the substance to be examined in mobile phase A and dilute to 50.0 ml with mobile phase A.

Reference solution (a). Dilute 1.0 ml of the test solution to 100.0 ml with mobile phase A.

Reference solution (b). Dissolve 5.0 mg of 3-aminobenzoic acid *R* in mobile phase A and dilute to 100.0 ml with mobile phase A. Dilute 1.0 ml to 25.0 ml with the test solution.

Reference solution (c). Dissolve 5.0 mg of 3-aminobenzoic acid *R* in mobile phase A and dilute to 100.0 ml with mobile phase A. Dilute 1.0 ml to 50.0 ml with mobile phase A.

Reference solution (d). Dissolve 10.0 mg of 3-aminophenol *R* in mobile phase A and dilute to 100.0 ml with mobile phase A. Dilute 1.0 ml to 50.0 ml with mobile phase A.

Reference solution (e). Dissolve 5.0 mg of 2,5-dihydroxybenzoic acid *R* in mobile phase A and dilute to 100.0 ml with mobile phase A. Dilute 1.0 ml to 50.0 ml with mobile phase A.

Reference solution (f). Dissolve 15.0 mg of salicylic acid *R* in mobile phase A and dilute to 100.0 ml with mobile phase A. Dilute 1.0 ml to 50.0 ml with mobile phase A.

Blank solution. Mobile phase A.

Column:

- size: $l = 0.25$ m, $\varnothing = 4.6$ mm,
- stationary phase: spherical base-deactivated octylsilyl silica gel for chromatography *R* (5 µm).

Mobile phase:

- mobile phase A: dissolve 2.2 g of *perchloric acid R* and 1.0 g of *phosphoric acid R* in *water R* and dilute to 1000.0 ml with the same solvent,
- mobile phase B: dissolve 1.7 g of *perchloric acid R* and 1.0 g of *phosphoric acid R* in *acetonitrile R* and dilute to 1000.0 ml with the same solvent,

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)
0 - 7	100	0
7 - 25	100 → 40	0 → 60
25 - 30	40 → 100	60 → 0
30 - 40	100	0

Flow rate: 1.25 ml/min.

Detection: spectrophotometer at 220 nm.

Injection: 10 µl.

Relative retention with reference to mesalazine (retention time = about 5 min): impurity B = about 0.8; impurity D = about 1.2; impurity G = about 3.1; impurity H = about 3.9.

System suitability: reference solution (b):

- peak-to-valley ratio: minimum 1.5, where H_p = height above the baseline of the peak due to impurity D and H_v = height above the baseline of the lowest point of the curve separating this peak from the peak due to mesalazine.

Limits:

- **impurity B:** not more than the area of the principal peak in the chromatogram obtained with reference solution (d) (0.2 per cent),
- **impurity D:** not more than the area of the principal peak in the chromatogram obtained with reference solution (c) (0.1 per cent),
- **impurity G:** not more than the area of the principal peak in the chromatogram obtained with reference solution (e) (0.1 per cent),
- **impurity H:** not more than the area of the principal peak in the chromatogram obtained with reference solution (f) (0.3 per cent),
- **any other impurity:** not more than 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent),
- **total:** not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (1.0 per cent),
- **disregard limit:** 0.05 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent). Disregard any peaks obtained with the blank solution.

Impurity A and impurity C. Liquid chromatography (2.2.29). Use freshly prepared mobile phases.

Test solution. Dissolve 50.0 mg of the substance to be examined in mobile phase A and dilute to 50.0 ml with mobile phase A.

Reference solution (a). Dissolve 5.0 mg of 2-aminophenol R in mobile phase A and dilute to 100.0 ml with mobile phase A. Dilute 10.0 ml to 100.0 ml with mobile phase A.

Reference solution (b). Dissolve 5.0 mg of 4-aminophenol R in mobile phase A and dilute to 250.0 ml with mobile phase A. To 1.0 ml of this solution, add 1.0 ml of reference solution (a) and dilute to 100.0 ml with mobile phase A.

Reference solution (c). Dilute 1.0 ml of the test solution to 200.0 ml with mobile phase A. To 5.0 ml of this solution add 5.0 ml of reference solution (a).

Column:

- **size:** $l = 0.25$ m, $\varnothing = 4.6$ mm,
- **stationary phase:** spherical end-capped octadecylsilyl silica gel for chromatography R (3 μ m).

Mobile phase:

- **mobile phase A:** dissolve 2.2 g of perchloric acid R and 1.0 g of phosphoric acid R in water R and dilute to 1000.0 ml with the same solvent,
- **mobile phase B:** dissolve 1.7 g of perchloric acid R and 1.0 g of phosphoric acid R in acetonitrile R and dilute to 1000.0 ml with the same solvent,

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)
0 - 8	100	0
8 - 25	100 $\rightarrow$ 40	0 $\rightarrow$ 60
25 - 30	40 $\rightarrow$ 100	60 $\rightarrow$ 0
30 - 40	100	0

Flow rate: 1.0 ml/min.

Detection: spectrophotometer at 220 nm.

Injection: 20 μ l; inject the test solution and reference solutions (b) and (c).

Relative retention with reference to mesalazine (retention time = about 9 min): impurity A = about 0.5; impurity C = about 0.9.

System suitability: reference solution (c):

- **resolution:** minimum 3 between the peaks due to impurity C and mesalazine.

Limits:

- **impurity A:** not more than the area of the corresponding peak in the chromatogram obtained with reference solution (b) (200 ppm),
- **impurity C:** not more than 4 times the area of the corresponding peak in the chromatogram obtained with reference solution (b) (200 ppm).

Impurity K. Liquid chromatography (2.2.29).

Test solution. Dissolve 40.0 mg of the substance to be examined in the mobile phase and dilute to 20.0 ml with the mobile phase.

Reference solution. Dissolve 27.8 mg of aniline hydrochloride R in the mobile phase and dilute to 100.0 ml with the mobile phase. Dilute 0.20 ml of this solution to 20.0 ml with the mobile phase. Dilute 0.20 ml of this solution to 20.0 ml with the mobile phase.

Column:

- **size:** $l = 0.25$ m, $\varnothing = 4$ mm,
- **stationary phase:** spherical octadecylsilyl silica gel for chromatography R (5 μ m),
- **temperature:** 40 °C.

Mobile phase: mix 15 volumes of methanol R and 85 volumes of a solution containing 1.41 g/l of potassium dihydrogen phosphate R and 0.47 g/l of disodium hydrogen phosphate dihydrate R previously adjusted to pH 8.0 with a 42 g/l solution of sodium hydroxide R.

Flow rate: 1.0 ml/min.

Detection: spectrophotometer at 205 nm.

Injection: 50 μ l.

Retention time: impurity K = about 15 min.

System suitability: reference solution:

- **signal-to-noise ratio:** minimum 10 for the principal peak.

Limit:

- **impurity K:** not more than the area of the corresponding peak in the chromatogram obtained with the reference solution (10 ppm).

Chlorides: maximum 0.1 per cent.

Dissolve 1.50 g in 50 ml of anhydrous formic acid R. Add 100 ml of water R and 5 ml of 2 M nitric acid. Titrate with 0.005 M silver nitrate determining the end-point potentiometrically (2.2.20).

1 ml of 0.005 M silver nitrate is equivalent to 0.1773 mg of Cl.

Sulphates (2.4.13): maximum 200 ppm.

Shake 1.0 g with 20 ml of distilled water R for 1 min and filter. 15 ml of the filtrate complies with the limit test for sulphates.

Heavy metals (2.4.8): maximum 20 ppm.

1.0 g complies with limit test F. Prepare the standard using 2 ml of lead standard solution (10 ppm Pb) R.

Loss on drying (2.2.32): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 100-105 °C.

Sulphated ash (2.4.14): maximum 0.2 per cent, determined on 1.0 g.

ASSAY

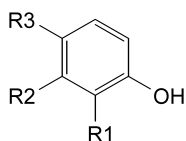
Dissolve 50.0 mg in 100 ml of boiling water R. Cool rapidly to room temperature and titrate with 0.1 M sodium hydroxide, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M sodium hydroxide is equivalent to 15.31 mg of $C_7H_7NO_3$.

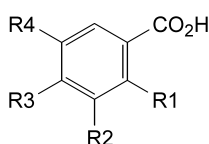
STORAGE

In an airtight container, protected from light.

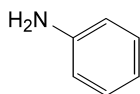
IMPURITIES



- A. $R_1 = R_2 = H, R_3 = NH_2$: 4-aminophenol,
 B. $R_1 = R_3 = H, R_2 = NH_2$: 3-aminophenol,
 C. $R_1 = NH_2, R_2 = R_3 = H$: 2-aminophenol,



- D. $R_1 = R_3 = R_4 = H, R_2 = NH_2$: 3-aminobenzoic acid,
 E. $R_1 = OH, R_2 = R_4 = H, R_3 = NH_2$: 4-amino-2-hydroxybenzoic acid (4-aminosalicylic acid),
 F. $R_1 = OH, R_2 = NH_2, R_3 = R_4 = H$: 3-amino-2-hydroxybenzoic acid (3-aminosalicylic acid),
 G. $R_1 = R_4 = OH, R_2 = R_3 = H$: 2,5-dihydroxybenzoic acid,
 H. $R_1 = OH, R_2 = R_3 = R_4 = H$: 2-hydroxybenzoic acid (salicylic acid),
 I. $R_1 = OH, R_2 = R_3 = H, R_4 = N=N-C_6H_5$: 2-hydroxy-5-(phenyldiazenyl)benzoic acid (phenylazosalicic acid),
 J. $R_1 = OH, R_2 = R_4 = NH_2, R_3 = H$: 3,5-diamino-2-hydroxybenzoic acid (diaminosalicic acid),
 L. $R_1 = Cl, R_2 = R_3 = R_4 = H$: 2-chlorobenzoic acid,
 M. $R_1 = Cl, R_2 = R_3 = H, R_4 = NO_2$: 2-chloro-5-nitrobenzoic acid,
 N. $R_1 = OH, R_2 = R_3 = H, R_4 = NO_2$: 2-hydroxy-5-nitrobenzoic acid (5-nitrosalicic acid),



- K. aniline.

Solubility: freely soluble in water, slightly soluble in alcohol, practically insoluble in cyclohexane.

IDENTIFICATION

A. Infrared absorption spectrophotometry (2.2.24).

Comparison: Ph. Eur. reference spectrum of mesna.

B. It gives reaction (a) of sodium (2.3.1).

TESTS

Solution S. Dissolve 10.0 g in carbon dioxide-free water R prepared from distilled water R and dilute to 50 ml with the same solvent.

Appearance of solution. Solution S is not more opalescent than reference suspension II (2.2.1) and not more intensely coloured than reference solution Y₇ (2.2.2, Method II).

pH (2.2.3): 4.5 to 6.0.

Dilute 10 ml of solution S to 20 ml with carbon dioxide-free water R.

Related substances. Liquid chromatography (2.2.29).

Test solution. Dissolve 0.10 g of the substance to be examined in the mobile phase and dilute to 25.0 ml with the mobile phase.

Reference solution (a). Dissolve 4.0 mg of mesna impurity C CRS in the mobile phase and dilute to 50.0 ml with the mobile phase. Dilute 2.0 ml of the solution to 20.0 ml with the mobile phase.

Reference solution (b). Dissolve 6.0 mg of mesna impurity D CRS in the mobile phase and dilute to 5.0 ml with the mobile phase. Dilute 1.0 ml of the solution to 10.0 ml with the mobile phase.

Reference solution (c). Dilute 3.0 ml of the test solution to 10.0 ml with the mobile phase. Dilute 1.0 ml of the solution to 100.0 ml with the mobile phase.

Reference solution (d). Dilute 6.0 ml of reference solution (c) to 20.0 ml with the mobile phase. To 10 ml of the solution, add 10 ml of reference solution (a).

Column:

- size: $l = 0.25$ m, $\varnothing = 4.6$ mm,
- stationary phase: octadecylsilyl silica gel for chromatography R (10 μ m).

Mobile phase: dissolve 2.94 g of potassium dihydrogen phosphate R, 2.94 g of dipotassium hydrogen phosphate R and 2.6 g of tetrabutylammonium hydrogen sulphate R in about 600 ml of water R. Adjust to pH 2.3 with phosphoric acid R, add 335 ml of methanol R and dilute to 1000 ml with water R.

Flow rate: 1 ml/min.

Detection: spectrophotometer at 235 nm.

Injection: 20 μ l.

Run time: 4 times the retention time of mesna.

Relative retention with reference to mesna (retention time = about 4.8): impurities A and B = about 0.6; impurity E = about 0.8; impurity C = about 1.4; impurity D = about 2.3.

System suitability: reference solution (d):

- resolution: minimum 3.0 between the peaks corresponding to mesna and to impurity C.

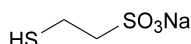
Limits:

- correction factor: for the calculation of contents, multiply the peak areas of impurities A, B and E by 0.01,
- impurity C: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (a) (0.2 per cent),

01/2005:1674

MESNA

Mesnum



$C_2H_5NaO_3S_2$

M_r 164.2

DEFINITION

Sodium 2-sulphanylethanesulphonate.

Content: 96.0 per cent to 102.0 per cent (dried substance).

CHARACTERS

Appearance: white or slightly yellow, crystalline powder, hygroscopic.

- **impurity D**: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (b) (3.0 per cent),
- **impurities A, B, E**: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (c) (0.3 per cent),
- **any other impurity**: for each impurity, not more than one third the area of the principal peak in the chromatogram obtained with reference solution (c) (0.1 per cent),
- **total of other impurities**: not more than the area of the principal peak in the chromatogram obtained with reference solution (c) (0.3 per cent),
- **disregard limit**: 0.15 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.045 per cent).

Chlorides (2.4.4): maximum 250 ppm.

Dilute 1 ml of solution S to 15 ml with *water R*.

Sulphates (2.4.13): maximum 300 ppm.

Dilute 5 ml of solution S to 30 ml with *distilled water R*. 15 ml of the solution complies with the limit test for sulphates.

Disodium edetate: maximum 500 ppm.

Dissolve 4.000 g in 90 ml of *water R* and adjust to pH 4.5 using *0.1 M hydrochloric acid*. Add 10 ml of *acetate buffer solution pH 4.5 R* and 50 ml of *2-propanol R*. Add 2 ml of a 0.25 g/l solution of *dithizone R* in *2-propanol R*. Titrate with *0.01 M zinc sulphate* until the colour changes from bluish-grey to pink.

1 ml of *0.01 M zinc sulphate* is equivalent to 3.72 mg of $\text{C}_{10}\text{H}_{14}\text{N}_2\text{Na}_2\text{O}_8 \cdot 2\text{H}_2\text{O}$.

Heavy metals (2.4.8): maximum 10 ppm.

Dilute 10 ml of solution S to 20 ml with *water R*. 12 ml of the solution complies with limit test A. Prepare the standard using *lead standard solution (1 ppm Pb R)*.

Loss on drying (2.2.32): maximum 1.0 per cent, determined on 1.000 g under high vacuum at 60 °C for 2 h.

ASSAY

Dissolve 0.120 g in 10 ml of *water R*. Add 10 ml of *1 M sulphuric acid* and 10.0 ml of *0.1 M iodine*. Titrate with *0.1 M sodium thiosulphate* adding 1 ml of *starch solution R* near the endpoint. Carry out a blank titration.

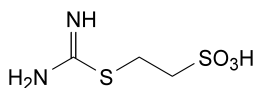
1 ml of *0.1 M sodium thiosulphate* is equivalent to 16.42 mg of $\text{C}_2\text{H}_5\text{NaO}_3\text{S}_2$.

STORAGE

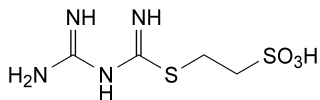
In an airtight container.

IMPURITIES

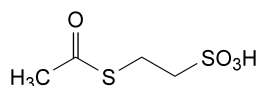
Specified impurities: A, B, C, D, E.



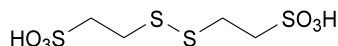
A. 2-(carbamimidoylsulphonyl)ethanesulphonic acid,



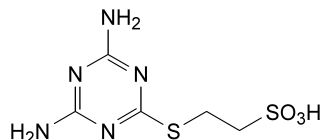
B. 2-[[[guanidino](imino)methyl]sulphonyl]ethanesulphonic acid,



C. 2-(acetylsulphonyl)ethanesulphonic acid,



D. 2,2'-(disulphanediyl)bis(ethanesulphonic acid),

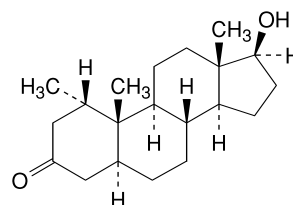


E. 2-(4,6-diamino-1,3,5-triazin-2-yl)sulphonyl ethanesulphonic acid.

01/2005:1730

MESTEROLONE

Mesterolonom



$\text{C}_{20}\text{H}_{32}\text{O}_2$

M_r 304.5

DEFINITION

17 β -Hydroxy-1 α -methyl-5 α -androstan-3-one.

Content: 98.0 per cent to 102.0 per cent (dried substance).

CHARACTERS

Appearance: white or yellowish crystalline powder.

Solubility: practically insoluble in water, sparingly soluble in acetone, in ethyl acetate and in methanol.

IDENTIFICATION

A. Melting point (2.2.14): 206 °C to 211 °C.

B. Infrared absorption spectrophotometry (2.2.24).

Preparation: discs.

Comparison: mesterolone CRS.

TESTS

Specific optical rotation (2.2.7): + 20 to + 24 (dried substance).

Dissolve 0.200 g in *methylene chloride R* and dilute to 10.0 ml with the same solvent.

Impurity B: maximum 0.5 per cent.

Thin-layer chromatography (2.2.27).

Test solution. Dissolve 100.0 mg of the substance to be examined in a mixture of equal volumes of *methanol R* and *methylene chloride R* and dilute to 10.0 ml with the same mixture of solvents.

Reference solution (a). Dilute 1.0 ml of the test solution to 200.0 ml with a mixture of equal volumes of *methanol R* and *methylene chloride R*.

Reference solution (b). Dissolve 5.0 mg of *mesterolone impurity A CRS* in reference solution (a) and dilute to 100.0 ml with the same solution.

Plate: TLC silica gel plate R.

Mobile phase: methanol *R*, acetone *R*, toluene *R* (2:15:85 V/V/V).

Application: 10 µl.

Development: over 2/3 of the plate.

Drying: in air.

Detection: examine in ultraviolet light at 366 nm; spray with a 200 g/l solution of *toluenesulphonic acid R* in alcohol *R* and heat the plate for 10 min at 120 °C.

System suitability: the chromatogram obtained with reference solution (b) shows 2 clearly separated spots (blue spot due to mesterolone and yellow spot due to impurity A).

Limit:

- **impurity B:** any blue spot, apart from the main spot, is not more intense than the spot in the chromatogram obtained with reference solution (a) (0.5 per cent).

Related substances. Liquid chromatography (2.2.29).

Test solution. Dissolve 50.0 mg of the substance to be examined in a mixture of 20 volumes of water *R* and 80 volumes of acetonitrile *R* and dilute to 25.0 ml with the same mixture of solvents.

Reference solution (a). Dissolve 50.0 mg of mesterolone CRS in a mixture of 20 volumes of water *R* and 80 volumes of acetonitrile *R* and dilute to 25.0 ml with the same mixture of solvents.

Reference solution (b). Dissolve 10.0 mg of mesterolone impurity A CRS in a mixture of 20 volumes of water *R* and 80 volumes of acetonitrile *R* and dilute to 5.0 ml with the same mixture of solvents.

Reference solution (c). Dilute 0.5 ml of reference solution (a) and 0.5 ml of reference solution (b) to 100.0 ml with a mixture of 20 volumes of water *R* and 80 volumes of acetonitrile *R*.

Column:

- **size:** $l = 0.25$ m, $\varnothing = 4.6$ mm,
- **stationary phase:** octadecylsilyl silica gel for chromatography *R* (3 µm).

Mobile phase: acetonitrile *R*, water *R*, methanol *R* (20:40:60 V/V/V).

Flow rate: 0.9 ml/min.

Detection: spectrophotometer at 200 nm.

Injection: 50 µl; inject the test solution and reference solution (c).

Run time: 3 times the retention time of mesterolone.

Relative retention with reference to mesterolone (retention time = about 22 min): impurity A = about 0.7.

System suitability: reference solution (c):

- **resolution:** minimum 6.0 between the peaks due to impurity A and to mesterolone.

Limits:

- **impurity A:** not more than the area of the peak due to impurity A in the chromatogram obtained with reference solution (c) (0.5 per cent),
- **any other impurity:** not more than half the area of the peak due to mesterolone in the chromatogram obtained with reference solution (c) (0.25 per cent),
- **total:** not more than 1.5 times the area of the peak due to mesterolone in the chromatogram obtained with reference solution (c) (0.75 per cent),
- **disregard limit:** 0.1 times the area of the peak due to mesterolone in the chromatogram obtained with reference solution (c) (0.05 per cent).

Loss on drying (2.2.32): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 100–105 °C.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

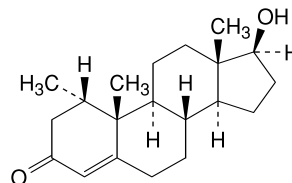
ASSAY

Liquid chromatography (2.2.29) as described in the test for related substances.

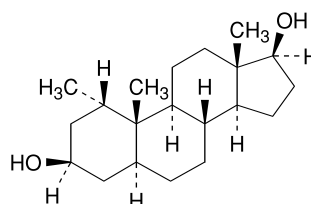
Injection: 10 µl; inject the test solution and reference solution (a).

Calculate the percentage content of $C_{20}H_{32}O_2$.

IMPURITIES



- A. 17β-hydroxy-1α-methylandrosta-4-en-3-one,

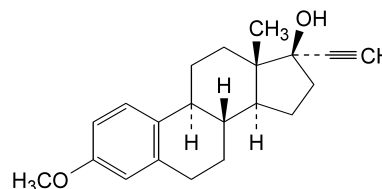


- B. 1α-methyl-5α-androstane-3β,17β-diol.

01/2005:0509

MESTRANOL

Mestranolum



$C_{21}H_{26}O_2$

M_r 310.4

DEFINITION

Mestranol contains not less than 98.0 per cent and not more than the equivalent of 102.0 per cent of 3-methoxy-19-nor-17α-pregna-1,3,5(10)-trien-20-yn-17-ol, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, crystalline powder, practically insoluble in water, sparingly soluble in alcohol.

IDENTIFICATION

First identification: B.

Second identification: A, C, D.

A. Melting point (2.2.14): 150 °C to 154 °C.

B. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with mestranol CRS.

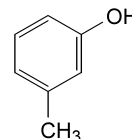
C. Examine the chromatograms obtained in the test for related substances in daylight and in ultraviolet light at 365 nm. The principal spot in the chromatogram obtained with test solution (b) is similar in position, colour, fluorescence and size to the principal spot in the chromatogram obtained with reference solution (a).

D. Dissolve about 5 mg in 1 ml of *sulphuric acid R*. A red colour develops with a greenish-yellow fluorescence in ultraviolet light at 365 nm. Add the solution to 10 ml of *water R* and mix. The solution becomes pink and a pink to violet precipitate is formed on standing.

01/2005:2077

METACRESOL

Metacresololum

C₇H₈OM_r 108.1

DEFINITION

3-Methylphenol.

CHARACTERS

Appearance: colourless or yellowish liquid.*Solubility*: sparingly soluble in water, miscible with alcohol and with methylene chloride.*Relative density*: about 1.03.

mp: about 11 °C.

bp: about 202 °C.

IDENTIFICATION

Infrared absorption spectrophotometry (2.2.24).

Comparison: *Ph. Eur. reference spectrum of metacresol*.

TESTS

Solution S. Dissolve 1.5 g in *carbon dioxide-free water R* and dilute to 100 ml with the same solvent.**Appearance of solution.** Freshly prepared solution S is not more opalescent than reference suspension III (2.2.1) and not more intensely coloured than reference solution BY₇ (2.2.2, *Method II*).**Acidity.** To 25 ml of solution S, add 0.15 ml of *methyl red solution R*. The solution is red. Not more than 0.5 ml of 0.01 M *sodium hydroxide* is required to change the colour of the indicator to yellow.**Related substances.** Gas chromatography (2.2.28): use the normalisation procedure.*Test solution.* Dissolve 0.250 g of the substance to be examined in *methanol R* and dilute to 100.0 ml with the same solvent.*Reference solution (a).* Dissolve 25 mg of *cresol R*, 25 mg of *p-cresol R* and 25 mg of the substance to be examined in *methanol R* and dilute to 20 ml with the same solvent.*Reference solution (b).* Dilute 1.0 ml of the test solution to 100.0 ml with *methanol R*. Dilute 1.0 ml to 20.0 ml with *methanol R*.*Column*:

- *material*: fused silica,
- *size*: *l* = 25 m, Ø = 0.25 mm,
- *stationary phase*: poly[(cyanopropyl)(methyl)][(phenyl)(methyl)]siloxane *R* (0.2 µm).

Carrier gas: *helium for chromatography R*.*Flow rate*: 1.5 ml/min.*Split ratio*: 1:50.

TESTS

Specific optical rotation (2.2.7). Dissolve 0.100 g in *anhydrous pyridine R* and dilute to 10.0 ml with the same solvent. The specific optical rotation is –20 to –24, calculated with reference to the dried substance.**Absorbance** (2.2.25). Dissolve 25.0 mg in *alcohol R* and dilute to 25.0 ml with the same solvent. Dilute 10.0 ml of this solution to 100.0 ml with *alcohol R*. Examined between 260 nm and 310 nm, the solution shows two absorption maxima, at 279 nm and 288 nm, and a minimum at 286 nm. The specific absorbances at the maxima are 62 to 68 and 59 to 64, respectively.**Related substances.** Examine by thin-layer chromatography (2.2.27), using *silica gel G R* as the coating substance.*Test solution (a).* Dissolve 0.10 g of the substance to be examined in *chloroform R* and dilute to 10 ml with the same solvent.*Test solution (b).* Dilute 1 ml of test solution (a) to 10 ml with *chloroform R*.*Reference solution (a).* Dissolve 10 mg of *mestranol CRS* in *chloroform R* and dilute to 10 ml with the same solvent.*Reference solution (b).* Dilute 1 ml of test solution (b) to 10 ml with *chloroform R*.*Reference solution (c).* Dilute 5 ml of reference solution (b) to 10 ml with *chloroform R*.

Apply separately to the plate 5 µl of each solution. Develop over a path of 15 cm using a mixture of 10 volumes of *alcohol R* and 90 volumes of *toluene R*. Allow the plate to dry in air until the solvent has evaporated. Heat at 110 °C for 10 min. Spray the hot plate with *alcoholic solution of sulphuric acid R*. Heat again at 110 °C for 10 min. Examine in daylight and in ultraviolet light at 365 nm. Any spot in the chromatogram obtained with test solution (a), apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (b) (1.0 per cent) and at most one such spot is more intense than the spot in the chromatogram obtained with reference solution (c) (0.5 per cent).

Loss on drying (2.2.32). Not more than 1.0 per cent, determined on 0.500 g by drying in an oven at 100 °C to 105 °C for 3 h.

ASSAY

Dissolve 0.200 g in 40 ml of *tetrahydrofuran R* and add 5 ml of a 100 g/l solution of *silver nitrate R*. Titrate with 0.1 M *sodium hydroxide*, determining the end-point potentiometrically (2.2.20). Carry out a blank titration.

1 ml of 0.1 M *sodium hydroxide* is equivalent to 31.04 mg of C₂₁H₂₆O₂.

STORAGE

Store protected from light.

Temperature:

	Time (min)	Temperature (°C)
Column	0 - 35	100
	35 - 40	100 → 150
	40 - 50	150
Injection port		200
Detector		200

Detection: flame ionisation.

Injection: 0.5 µl.

Relative retention with reference to metacresol (retention time = about 28 min): impurity B = about 0.75; impurity C = about 0.98.

System suitability: reference solution (a):

- **resolution:** minimum 1.4 between the peaks due to impurity C and metacresol.

Limits:

- **impurities B, C:** for each impurity, not more than 0.5 per cent,
- **any other impurity:** for each impurity, not more than 0.1 per cent,
- **total:** not more than 1.0 per cent.
- **disregard limit:** the area of the peak due to metacresol in the chromatogram obtained with reference solution (b) (0.05 per cent).

Residue on evaporation: maximum 0.1 per cent.

Evaporate 2.0 g to dryness on a water-bath under a fume hood and dry at 100-105 °C for 1 h. The residue weighs a maximum of 2 mg.

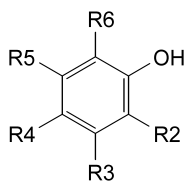
STORAGE

In an airtight container, protected from light.

IMPURITIES

Specified impurities: B, C.

Other detectable impurities: A, D, E, F, G, H, I, J, K, L, M.



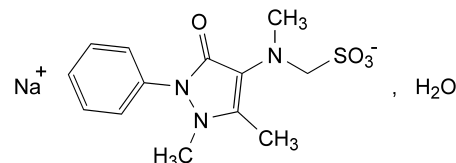
- A. R2 = R3 = R4 = R5 = R6 = H: phenol,
- B. R2 = CH₃, R3 = R4 = R5 = R6 = H: 2-methylphenol (*o*-cresol, cresol),
- C. R2 = R3 = R5 = R6 = H, R4 = CH₃: 4-methylphenol (*p*-cresol),
- D. R2 = R6 = CH₃, R3 = R4 = R5 = H: 2,6-dimethylphenol (2,6-xyleneol),
- E. R2 = C₂H₅, R3 = R4 = R5 = R6 = H: 2-ethylphenol (*o*-ethylphenol),
- F. R2 = R4 = CH₃, R3 = R5 = R6 = H: 2,4-dimethylphenol (2,4-xyleneol),
- G. R2 = R5 = CH₃, R3 = R4 = R6 = H: 2,5-dimethylphenol (2,5-xyleneol),
- H. R2 = CH(CH₃)₂, R3 = R4 = R5 = R6 = H: 2-(1-methylethyl)phenol,
- I. R2 = R3 = CH₃, R4 = R5 = R6 = H: 2,3-dimethylphenol (2,3-xyleneol),

- J. R2 = R4 = R6 = H, R3 = R5 = CH₃: 3,5-dimethylphenol (3,5-xyleneol),
- K. R2 = R3 = R5 = R6 = H, R4 = C₂H₅: 4-ethylphenol (*p*-ethylphenol),
- L. R2 = R5 = R6 = H, R3 = R4 = CH₃: 3,4-dimethylphenol (3,4-xyleneol),
- M. R2 = R3 = R5 = CH₃, R4 = R6 = H: 2,3,5-trimethylphenol.

01/2005:1346

METAMIZOLE SODIUM

Metamizolum natriicum

C₁₃H₁₆N₃NaO₄S·H₂OM_r 351.4

DEFINITION

Metamizole sodium contains not less than 99.0 and not more than the equivalent of 100.5 per cent of sodium [(1,5-dimethyl-3-oxo-2-phenyl-2,3-dihydro-1*H*-pyrazol-4-yl)-*N*-methylamino]methanesulphonate, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, crystalline powder, very soluble in water, soluble in alcohol.

IDENTIFICATION

First identification: A, D.

Second identification: B, C, D.

- A. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *metamizole sodium CRS*.
- B. Dissolve 50 mg in 1 ml of *strong hydrogen peroxide solution R*. A blue colour is produced which fades rapidly and turns to intense red in a few minutes.
- C. Place 0.10 g in a test tube, add some beads of glass and dissolve the substance in 1.5 ml of *water R*. Add 1.5 ml of *dilute hydrochloric acid R* and place a filter paper wetted with a solution of 20 mg of *potassium iodate R* in 2 ml of *starch solution R* at the open end of the test tube. Heat gently, the evolving vapour of sulphur dioxide colours the filter paper blue. After heating gently for 1 min take a glass rod with a drop of a 10 g/l solution of *chromotropic acid, sodium salt R* in *sulphuric acid R* and place in the opening of the tube. Within 10 min, a blue-violet colour develops in the drop of the reagent.
- D. 0.5 ml of solution S (see Tests) gives reaction (a) of sodium (2.3.1).

TESTS

Solution S. Dissolve 2.0 g in *carbon dioxide-free water R* and dilute to 40 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and immediately after preparation, not more intensely coloured than reference solution BY₆ (2.2.2, *Method I*).

Acidity or alkalinity. To 5 ml of solution S, add 0.1 ml of *phenolphthalein solution R1*. The solution is colourless. Not more than 0.1 ml of 0.02 M *sodium hydroxide* is required to change the colour of the indicator to pink.

Related substances. Examine by liquid chromatography (2.2.29).

Prepare the solutions immediately before use.

Test solution. Dissolve 50.0 mg of the substance to be examined in *methanol R* and dilute to 10.0 ml with the same solvent.

Reference solution (a). Dissolve 10.0 mg of *metamizole impurity A CRS* in *methanol R* and dilute to 20.0 ml with the same solvent.

Reference solution (b). Dilute 1.0 ml of reference solution (a) to 20.0 ml with *methanol R*.

Reference solution (c). Dissolve 40 mg of *metamizole sodium CRS* in *methanol R* and dilute to 20.0 ml with the same solvent.

Reference solution (d). Take 10 ml of reference solution (c) and boil under a reflux condenser for 10 min. Allow to cool to room temperature and dilute to 20.0 ml with *methanol R*.

Reference solution (e). To 6 ml of reference solution (a) add 1 ml of reference solution (c).

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4.6 mm in internal diameter packed with *base-deactivated octadecylsilyl silica gel for chromatography R* (5 µm),
- as mobile phase at a flow rate of 1.0 ml/min a mixture of 28 volumes of *methanol R* and 72 volumes of a buffer solution prepared by adjusting a mixture of 1000 volumes of a 6.0 g/l solution of *sodium dihydrogen phosphate R* and 1 volume of *triethylamine R* to pH 7.0 with *strong sodium hydroxide solution R*,
- as detector a spectrophotometer set at 254 nm.

When the chromatograms are recorded in the prescribed conditions, the substances elute in the following order: impurity A, metamizole, impurity B, impurity C and impurity D. Inject 10 µl of reference solution (b). Adjust the sensitivity of the system so that the height of the principal peak in the chromatogram obtained is at least 50 per cent of the full scale of the recorder.

Inject 10 µl of reference solution (d). The chromatogram shows two principal peaks due to metamizole and impurity C.

Inject 10 µl of reference solution (e). The test is not valid unless in the chromatogram obtained the resolution between the peaks corresponding to impurity A and metamizole is at least 2.5.

Inject 10 µl of the test solution and 10 µl of reference solution (b) and continue the chromatography for 3.5 times the retention time of metamizole. In the chromatogram obtained with the test solution: the area of any peak corresponding to impurity C is not greater than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent), the area of any peaks, apart from the principal peak and the peak due to impurity C is not greater than 0.4 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent). The sum of the areas of all the peaks, apart from the principal peak, is not greater than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent). Disregard any peak with an area less than 0.05 times that of the principal peak in the chromatogram obtained with the reference solution (b).

Sulphates (2.4.13). Dissolve 0.150 g in *distilled water R* and dilute to 15 ml with the same solvent. The solution complies with the limit test for sulphates (0.1 per cent).

Heavy metals (2.4.8). Dissolve 2.0 g in *water R* and dilute to 20 ml with the same solvent. 12 ml of the freshly prepared solution complies with limit test A for heavy metals (20 ppm). Prepare the standard using *lead standard solution (2 ppm Pb) R*.

Loss on drying (2.2.32): 4.9 per cent to 5.3 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C.

ASSAY

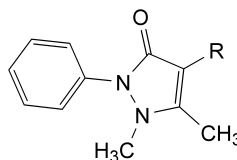
Dissolve 0.200 g in 10 ml of 0.01 *M hydrochloric acid* previously cooled in iced water and titrate immediately, dropwise, with 0.05 *M iodine*. Before each addition of 0.05 *M iodine* dissolve the precipitate by swirling. At the end of the titration add 2 ml of *starch solution R* and titrate until the blue colour of the solution persists for at least 2 min. The temperature of the solution during the titration must not exceed 10 °C.

1 ml of 0.05 *M iodine* is equivalent to 16.67 mg of $C_{13}H_{16}N_3NaO_4S$.

STORAGE

Store protected from light.

IMPURITIES

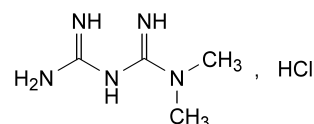


- A. R = NHCHO: 4-formylamino-1,5-dimethyl-2-phenyl-1,2-dihydro-3H-pyrazol-3-one,
- B. R = NH₂: 4-amino-1,5-dimethyl-2-phenyl-1,2-dihydro-3H-pyrazol-3-one,
- C. R = NHCH₃: 4-methylamino-1,5-dimethyl-2-phenyl-1,2-dihydro-3H-pyrazol-3-one,
- D. R = N(CH₃)₂: 4-dimethylamino-1,5-dimethyl-2-phenyl-1,2-dihydro-3H-pyrazol-3-one.

01/2005:0931

METFORMIN HYDROCHLORIDE

Metformini hydrochloridum



$C_4H_{12}ClN_5$

M_r 165.6

DEFINITION

1,1-Dimethylbiguanide hydrochloride.

Content: 98.5 per cent to 101.0 per cent (dried substance).

CHARACTERS

Appearance: white crystals.

Solubility: freely soluble in water, slightly soluble in alcohol, practically insoluble in acetone and in methylene chloride.

IDENTIFICATION

First identification: B, E.

Second identification: A, C, D, E.

A. Melting point (2.2.14): 222 °C to 226 °C.

B. Infrared absorption spectrophotometry (2.2.24).

Preparation: discs of *potassium chloride R*.

Comparison: *metformin hydrochloride CRS*.

C. Thin-layer chromatography (2.2.27).

Test solution. Dissolve 20 mg of the substance to be examined in *water R* and dilute to 5 ml with the same solvent.

Reference solution. Dissolve 20 mg of *metformin hydrochloride CRS* in *water R* and dilute to 5 ml with the same solvent.

Plate: *TLC silica gel G plate R*.

Mobile phase: upper layer of a mixture of 10 volumes of *glacial acetic acid R*, 40 volumes of *butanol R* and 50 volumes of *water R*.

Application: 5 µl.

Development: over a path of 15 cm.

Drying: at 100-105 °C for 15 min.

Detection: spray with a mixture of equal volumes of a 100 g/l solution of *sodium nitroprusside R*, a 100 g/l solution of *potassium ferricyanide R* and a 100 g/l solution of *sodium hydroxide R*, prepared 20 min before use.

Results: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

D. Dissolve about 5 mg in *water R* and dilute to 100 ml with the same solvent. To 2 ml of the solution add 0.25 ml of *strong sodium hydroxide solution R* and 0.10 ml of *α-naphthol solution R*. Mix and allow to stand in iced water for 15 min. Add 0.5 ml of *sodium hypobromite solution R* and mix. A pink colour develops.

E. It gives reaction (a) of chlorides (2.3.1).

TESTS

Solution S. Dissolve 2.0 g in *water R* and dilute to 20 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and colourless (2.2.2, *Method II*).

Related substances. Liquid chromatography (2.2.29).

Test solution. Dissolve 0.50 g of the substance to be examined in the mobile phase and dilute to 100.0 ml with the mobile phase.

Reference solution (a). Dissolve 20.0 mg of *cyanoguanidine R* in *water R* and dilute to 100.0 ml with the same solvent. Dilute 1.0 ml to 200.0 ml with the mobile phase.

Reference solution (b). Dilute 1.0 ml of the test solution to 50.0 ml with the mobile phase. Dilute 1.0 ml of this solution to 20.0 ml with the mobile phase.

Reference solution (c). Dissolve 10.0 mg of *melamine R* in about 90 ml of *water R*. Add 5.0 ml of the test solution and dilute to 100.0 ml with *water R*. Dilute 1.0 ml of this solution to 50.0 ml with the mobile phase.

Column:

- *size:* $l = 0.25$ m, $\varnothing = 4.6$ mm,
- *stationary phase:* irregular, porous silica gel to which benzenesulphonic acid groups have been chemically bonded (10 µm),

or

- *size:* $l = 0.11$ m, $\varnothing = 4.7$ mm,

- *stationary phase:* regular, porous silica gel to which benzenesulphonic acid groups have been chemically bonded (5 µm).

Mobile phase: 17 g/l solution of *ammonium dihydrogen phosphate R* adjusted to pH 3.0 with *phosphoric acid R*.

Flow rate: 1 ml/min.

Detection: spectrophotometer at 218 nm.

Injection: 20 µl.

Run time: twice the retention time of metformin hydrochloride.

System suitability: reference solution (c):

- *resolution:* minimum of 10 between the peaks due to melamine and to metformin hydrochloride.

Limits:

- *impurity A:* not more than the area of the corresponding peak in the chromatogram obtained with reference solution (a) (0.02 per cent),
- *any other impurity:* not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent).

Heavy metals (2.4.8): maximum 10 ppm.

12 ml of solution S complies with limit test A. Prepare the standard using *lead standard solution (1 ppm Pb) R*.

Loss on drying (2.2.32): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 100-105 °C for 5 h.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

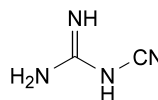
Dissolve 0.100 g in 4 ml of *anhydrous formic acid R*. Add 80 ml of *acetonitrile R*. Carry out the titration immediately. Titrate with 0.1 M *perchloric acid*, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *perchloric acid* is equivalent to 16.56 mg of $C_4H_{12}ClN_5$.

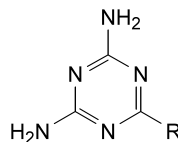
IMPURITIES

Specified impurities: A.

Other detectable impurities: B, C, D, E, F.



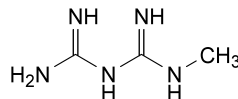
A. cyanoguanidine,



B. $R = \text{NH}-\text{C}(=\text{NH})-\text{NH}_2$: (4,6-diamino-1,3,5-triazin-2-yl)guanidine,

C. $R = \text{N}(\text{CH}_3)_2$: *N,N*-dimethyl-1,3,5-triazine-2,4,6-triamine,

D. $R = \text{NH}_2$: 1,3,5-triazine-2,4,6-triamine (melamine),



E. 1-methylbiguanide,

F. $\text{CH}_3-\text{NH}-\text{CH}_3$: *N*-methylmethanamine.

01/2005:1128

METHACRYLIC ACID - ETHYL ACRYLATE COPOLYMER (1:1)

Acidum methacrylicum et ethylis acrylas polymerisatum 1:1

DEFINITION

Methacrylic acid - ethyl acrylate copolymer (1:1) is a copolymer of methacrylic acid and ethyl acrylate having a mean relative molecular mass of about 250 000. The ratio of carboxylic groups to ester groups is about 1:1. It may contain suitable surface-active agents such as sodium dodecyl sulphate and polysorbate 80. It contains not less than 46.0 per cent *m/m* and not more than 50.6 per cent *m/m* of methacrylic acid units, calculated with reference to the dried substance.

CHARACTERS

A white, free-flowing powder, practically insoluble in water, freely soluble in ethanol and in 2-propanol, practically insoluble in ethyl acetate. It is freely soluble in a 40 g/l solution of sodium hydroxide.

IDENTIFICATION

- A. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the *Ph. Eur. reference spectrum of methacrylic acid - ethyl acrylate copolymer (1:1)*.
- B. It complies with the limits of the assay.

TESTS

Apparent viscosity. Dissolve a quantity of substance to be examined corresponding to 37.5 g of the dried substance in a mixture of 7.9 g of *water R* and 254.6 g of *2-propanol R*. Determine the viscosity (2.2.10) using a rotating viscometer at 20 °C. At a shear rate of 10 s⁻¹, the apparent viscosity is not less than 100 mPa.s and not more than 200 mPa.s.

Appearance of a film. Place 1 ml of the solution prepared for the apparent viscosity test on a glass plate and allow to dry. A clear brittle film is formed.

Ethyl acrylate and methacrylic acid. Total content: not more than 0.1 per cent, determined by liquid chromatography (2.2.29).

Blank solution. To 50.0 ml of *methanol R* add 25.0 ml of mobile phase.

Test solution. Dissolve 40 mg of the substance to be examined in 50.0 ml of *methanol R* and add 25.0 ml of mobile phase.

Reference solution. Dissolve 10 mg each of *ethyl acrylate R* and *methacrylic acid R* in *methanol R* and dilute to 50.0 ml with the same solvent. Dilute 0.1 ml of this solution to 50.0 ml with *methanol R* and add 25.0 ml of mobile phase.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.10 m long and 4 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (5 µm),
- as mobile phase at a flow rate of 2.5 ml/min a mixture of 30 volumes of *methanol R* and 70 volumes of *phosphate buffer solution pH 2.0 R*,
- as detector a spectrophotometer set at 202 nm.

Inject 50 µl of each solution. The test is not valid unless the resolution between the peaks corresponding to ethyl acrylate and methacrylic acid in the chromatogram obtained with the reference solution is at least 2.0. The test is not

valid if the chromatogram obtained with the blank solution shows peaks with the same retention times as ethyl acrylate or methacrylic acid.

Calculate the percentage content of monomers from the area of the peaks in the chromatograms obtained with the test solution and the reference solution and from the content of monomers in the reference solution.

Loss on drying (2.2.32). Not more than 5.0 per cent, determined on 1.000 g by drying at 100 °C to 105 °C for 6 h.

Sulphated ash (2.4.14). Not more than 0.4 per cent, determined on 1.0 g.

ASSAY

Dissolve 1.000 g in a mixture of 40 ml of *water R* and 60 ml of *2-propanol R*. Titrate slowly while stirring with 0.5 M *sodium hydroxide*, using *phenolphthalein solution R* as indicator.

1 ml of 0.5 M *sodium hydroxide* is equivalent to 43.05 mg of C₄H₆O₂ (methacrylic acid units).

LABELLING

The label states, where applicable, the name and concentration of any surface-active agents.

01/2005:1129

METHACRYLIC ACID - ETHYL ACRYLATE COPOLYMER (1:1) DISPERSION 30 PER CENT

Acidum methacrylicum et ethylis acrylas polymerisatum 1:1 dispersio 30 per centum

DEFINITION

Methacrylic acid - ethyl acrylate copolymer (1:1) dispersion 30 per cent is a dispersion in water of a copolymer of methacrylic acid and ethyl acrylate having a mean relative molecular mass of about 250 000. The ratio of carboxylic groups to ester groups is about 1:1. It may contain suitable surface-active agents such as sodium dodecyl sulphate and polysorbate 80. It contains not less than 46.0 per cent *m/m* and not more than 50.6 per cent *m/m* of methacrylic acid units, calculated with reference to the residue on evaporation.

CHARACTERS

An opaque, white, slightly viscous liquid, miscible with water. On addition of solvents such as acetone, ethanol or 2-propanol, a precipitate is formed which dissolves on addition of excess solvent. It is miscible with a 40 g/l solution of sodium hydroxide.

It is sensitive to spoilage by microbial contaminants.

IDENTIFICATION

- A. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the *Ph. Eur. reference spectrum of methacrylic acid - ethyl acrylate copolymer (1:1) dispersion 30 per cent*.
- B. It complies with the limits of the assay.

TESTS

Apparent viscosity. Determine the viscosity (2.2.10) using a rotating viscometer at 20 °C. At a shear rate of 50 s⁻¹, the apparent viscosity is not more than 15 mPa.s.

Appearance of a film. Place 1 ml on a glass plate and allow to dry. A clear, brittle film is formed.

01/2005:1127

Particulate matter. Filter 100.0 g through a tared stainless steel sieve (90). Rinse with *water R* until a clear filtrate is obtained and dry at 100 °C to 105 °C. The mass of the residue is not more than 1.00 g.

Ethyl acrylate and methacrylic acid. Total content not more than 0.1 per cent, determined by liquid chromatography (2.2.29) and calculated with reference to the dried substance.

Blank solution. To 50.0 ml of *methanol R* add 25.0 ml of mobile phase.

Test solution. Dissolve 40 mg of the substance to be examined in 50.0 ml of *methanol R* and add 25.0 ml of mobile phase.

Reference solution. Dissolve 10 mg each of *ethyl acrylate R* and *methacrylic acid R* in *methanol R* and dilute to 50.0 ml with the same solvent. Dilute 0.1 ml of this solution to 50.0 ml with *methanol R* and add 25.0 ml of mobile phase.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.10 m long and 4 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (5 µm),
- as mobile phase at a flow rate of 2.5 ml/min a mixture of 30 volumes of *methanol R* and 70 volumes of *phosphate buffer solution pH 2.0 R*,

as detector a spectrophotometer set at 202 nm.

Inject 50 µl of each solution. The test is not valid unless the resolution between the peaks corresponding to ethyl acrylate and methacrylic acid in the chromatogram obtained with the reference solution is at least 2.0. The test is not valid if the chromatogram obtained with the blank solution shows peaks with the same retention times as ethyl acrylate or methacrylic acid.

Calculate the percentage content of monomers from the area of the peaks in the chromatograms obtained with the test solution and the reference solution and from the content of monomers in the reference solution.

Residue on evaporation. Dry 1.000 g at 110 °C for 5 h. The residue weighs not less than 0.285 g and not more than 0.315 g.

Sulphated ash (2.4.14). Not more than 0.2 per cent, determined on 1.0 g.

Microbial contamination. Total viable aerobic count (2.6.12) not more than 10³ micro-organisms per gram, determined by plate count.

ASSAY

Dissolve 1.500 g in a mixture of 40 ml of *water R* and 60 ml of *2-propanol R*. Titrate slowly while stirring with 0.5 M *sodium hydroxide*, using *phenolphthalein solution R* as indicator.

1 ml of 0.5 M *sodium hydroxide* is equivalent to 43.05 mg of C₄H₆O₂ (methacrylic acid units).

STORAGE

Store protected from freezing. Handle the substance so as to minimise microbial contamination.

LABELLING

The label states, where applicable, the name and concentration of any surface-active agents.

METHACRYLIC ACID - METHYL METHACRYLATE COPOLYMER (1:1)

Acidum methacrylicum et methylis methacrylas polymerisatum 1:1

DEFINITION

Methacrylic acid - methyl methacrylate copolymer (1:1) is a copolymer of methacrylic acid and methyl methacrylate having a mean relative molecular mass of about 135 000. The ratio of carboxylic groups to ester groups is about 1:1. It contains not less than 46.0 per cent *m/m* and not more than 50.6 per cent of methacrylic acid units, calculated with reference to the dried substance.

CHARACTERS

A white, free-flowing powder, practically insoluble in water, freely soluble in ethanol and in 2-propanol, practically insoluble in ethyl acetate. It is freely soluble in a 40 g/l solution of sodium hydroxide.

IDENTIFICATION

- Examine by infrared absorption spectrophotometry (2.2.24), comparing with the *Ph. Eur. reference spectrum of methacrylic acid - methyl methacrylate copolymer (1:1)*.
- It complies with the limits of the assay.

TESTS

Apparent viscosity. Dissolve a quantity of substance to be examined corresponding to 37.5 g of the dried substance in a mixture of 7.9 g of *water R* and 254.6 g of *2-propanol R*. Determine the viscosity (2.2.10) using a rotating viscometer at 20 °C. At a shear rate of 10 s⁻¹, the apparent viscosity is not less than 50 mPa·s and not more than 200 mPa·s.

Appearance of a film. Place 1 ml of the solution prepared for the viscosity test on a glass plate and allow to dry. A clear brittle film is formed.

Methyl methacrylate and methacrylic acid. Total content: not more than 0.1 per cent, determined by liquid chromatography (2.2.29).

Blank solution. To 50.0 ml of *methanol R* add 25.0 ml of mobile phase.

Test solution. Dissolve 40 mg of the substance to be examined in 50.0 ml of *methanol R* and add 25.0 ml of mobile phase.

Reference solution. Dissolve 10 mg each of *methyl methacrylate R* and *methacrylic acid R* in *methanol R* and dilute to 50.0 ml with the same solvent. Dilute 0.1 ml of this solution to 50.0 ml with *methanol R* and add 25.0 ml of mobile phase.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.10 m long and 4 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (5 µm),
- as mobile phase at a flow rate of 2.5 ml/min a mixture of 30 volumes of *methanol R* and 70 volumes of *phosphate buffer solution pH 2.0 R*,
- as detector a spectrophotometer set at 202 nm.

Inject 50 µl of each solution. The test is not valid unless the resolution between the peaks corresponding to methyl methacrylate and methacrylic acid in the chromatogram obtained with the reference solution is at least 2.0. The test

is not valid if the chromatogram obtained with the blank solution shows peaks with the same retention times as methyl methacrylate or methacrylic acid.

Calculate the percentage content of monomers from the area of the peaks in the chromatograms obtained with the test solution and the reference solution and from the content of monomers in the reference solution.

Loss on drying (2.2.32). Not more than 5.0 per cent, determined on 1.000 g by drying at 100 °C to 105 °C for 6 h.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 1.000 g in a mixture of 40 ml of *water R* and 60 ml of *2-propanol R*. Titrate slowly while stirring with 0.5 M *sodium hydroxide*, using *phenolphthalein solution R* as indicator.

1 ml of 0.5 M *sodium hydroxide* is equivalent to 43.05 mg of $C_4H_6O_2$ (methacrylic acid units).

01/2005:1130

METHACRYLIC ACID - METHYL METHACRYLATE COPOLYMER (1:2)

Acidum methacrylicum et methylis methacrylas polymerisatum 1:2

DEFINITION

Methacrylic acid - methyl methacrylate copolymer (1:2) is a copolymer of methacrylic acid and methyl methacrylate having a mean relative molecular mass of about 135 000. The ratio of carboxylic groups to ester groups is about 1:2. It contains not less than 27.6 per cent *m/m* and not more than 30.7 per cent *m/m* of methacrylic acid units, calculated with reference to the dried substance.

CHARACTERS

A white, free-flowing powder, practically insoluble in water, freely soluble in ethanol and in 2-propanol, practically insoluble in ethyl acetate. It is freely soluble in a 40 g/l solution of sodium hydroxide.

IDENTIFICATION

A. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the *Ph. Eur. reference spectrum of methacrylic acid - methyl methacrylate copolymer (1:2)*.

B. It complies with the limits of the assay.

TESTS

Apparent viscosity. Dissolve a quantity of substance to be examined corresponding to 37.5 g of the dried substance in a mixture of 7.9 g of *water R* and 254.6 g of *2-propanol R*. Determine the viscosity (2.2.10) using a rotating viscometer at 20 °C. At a shear rate of 10 s⁻¹, the apparent viscosity is not less than 50 mPa·s and not more than 200 mPa·s.

Appearance of a film. Place 1 ml of the solution prepared for the viscosity test on a glass plate and allow to dry. A clear brittle film is formed.

Methyl methacrylate and methacrylic acid. Total content: not more than 0.1 per cent, determined by liquid chromatography (2.2.29).

Blank solution. To 50.0 ml of *methanol R* add 25.0 ml of mobile phase.

Test solution. Dissolve 40 mg of the substance to be examined in 50.0 ml of *methanol R* and add 25.0 ml of mobile phase.

Reference solution. Dissolve 10 mg each of *methyl methacrylate R* and *methacrylic acid R* in *methanol R* and dilute to 50.0 ml with the same solvent. Dilute 0.1 ml of this solution to 50.0 ml with *methanol R* and add 25.0 ml of mobile phase.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.10 m long and 4 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (5 µm),
- as mobile phase at a flow rate of 2.5 ml/min a mixture of 30 volumes of *methanol R* and 70 volumes of *phosphate buffer solution pH 2.0 R*,
- as detector a spectrophotometer set at 202 nm.

Inject 50 µl of each solution. The test is not valid unless the resolution between the peaks corresponding to methyl methacrylate and methacrylic acid in the chromatogram obtained with the reference solution is at least 2.0. The test is not valid if the chromatogram obtained with the blank solution shows peaks with the same retention times as methyl methacrylate or methacrylic acid.

Calculate the percentage content of monomers from the area of the peaks in the chromatograms obtained with the test solution and the reference solution and from the content of monomers in the reference solution.

Loss on drying (2.2.32). Not more than 5.0 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C for 6 h.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

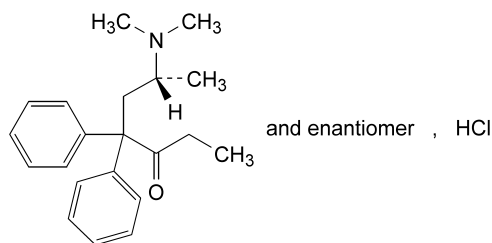
Dissolve 1.000 g in a mixture of 40 ml of *water R* and 60 ml of *2-propanol R*. Titrate slowly while stirring with 0.5 M *sodium hydroxide*, using *phenolphthalein solution R* as indicator.

1 ml of 0.5 M *sodium hydroxide* is equivalent to 43.05 mg of $C_4H_6O_2$ (methacrylic acid units).

01/2005:0408

METHADONE HYDROCHLORIDE

Methadoni hydrochloridum

C₂₁H₂₈ClNOM_r 345.9

DEFINITION

Methadone hydrochloride contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of (6*RS*)-6-(dimethylamino)-4,4-diphenylheptan-3-one hydrochloride, calculated with reference to the dried substance.

CHARACTERS

A white, crystalline powder, soluble in water, freely soluble in alcohol.

IDENTIFICATION

01/2005:0510

*First identification: A, C, E.**Second identification: A, B, D, E.*

- A. The optical rotation (2.2.7) of solution S (see Tests) measured in a 2 dm tube is -0.05° to $+0.05^{\circ}$.
- B. Melting point (2.2.14): 233°C to 236°C .
- C. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the *Ph. Eur. reference spectrum of methadone hydrochloride*.
- D. To 2.0 ml of solution S add 1 ml of 0.1 M hydrochloric acid and 6 ml of ammonium thiocyanate solution R. A white precipitate is formed which becomes crystalline on stirring for a few minutes. The crystalline precipitate, dried at 100°C to 105°C , melts (2.2.14) at 143°C to 148°C .
- E. Dilute 1 ml of solution S to 5 ml with water R and add 1 ml of dilute ammonia R1. Mix, allow to stand for 5 min and filter. The filtrate gives reaction (a) of chlorides (2.3.1).

TESTS

Solution S. Dissolve 2.50 g in carbon dioxide-free water R and dilute to 50.0 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and colourless (2.2.2, Method II).

Acidity or alkalinity. Dilute 10 ml of solution S to 25 ml with carbon dioxide-free water R. To 10 ml of the solution add 0.2 ml of methyl red solution R and 0.2 ml of 0.01 M sodium hydroxide. The solution is yellow. Add 0.4 ml of 0.01 M hydrochloric acid. The solution is red.

Related substances. Examine by thin-layer chromatography (2.2.27), using silica gel G R as the coating substance.

Test solution. Dissolve 0.5 g of the substance to be examined in alcohol R and dilute to 10 ml with the same solvent.

Reference solution. Dilute 1 ml of the test solution to 10 ml with alcohol R. Dilute 1 ml of this solution to 100 ml with alcohol R.

Apply to the plate 10 μl of each solution. Develop over a path of 15 cm using a mixture of 10 volumes of water R, 30 volumes of glacial acetic acid R and 60 volumes of alcohol R. Allow the plate to dry in air and spray with dilute potassium iodobismuthate solution R. Any spot in the chromatogram obtained with the test solution, apart from the principal spot, is not more intense than the spot in the chromatogram obtained with the reference solution (0.1 per cent).

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.00 g by drying in an oven at 100°C to 105°C .

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.300 g in 50 ml of anhydrous acetic acid R. Add 5 ml of mercuric acetate solution R. Using 0.1 ml of crystal violet solution R as indicator, titrate with 0.1 M perchloric acid until the colour changes from violet-blue to green.

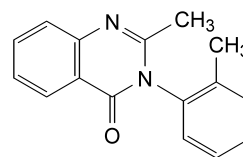
1 ml of 0.1 M perchloric acid is equivalent to 34.59 mg of $\text{C}_{16}\text{H}_{14}\text{N}_2\text{O}$.

STORAGE

Store protected from light.

METHAQUALONE

Methaqualonum

 $\text{C}_{16}\text{H}_{14}\text{N}_2\text{O}$ M_r 250.3

DEFINITION

Methaqualone contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of 2-methyl-3-(2-methylphenyl)quinazolin-4(3H)-one, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, crystalline powder, very slightly soluble in water, soluble in alcohol. It dissolves in dilute sulphuric acid.

IDENTIFICATION

*First identification: A, C.**Second identification: A, B, D.*

- A. Melting point (2.2.14): 114°C to 117°C .
- B. Dissolve 50.0 mg in 0.1 M hydrochloric acid, warming if necessary, and dilute to 100.0 ml with the same acid. Dilute 1.0 ml of this solution to 100.0 ml with 0.1 M hydrochloric acid. Examined between 220 nm and 300 nm (2.2.25), the solution shows two absorption maxima, at 235 nm and 270 nm. The specific absorbances at the maxima are 1270 to 1390 and 315 to 345, respectively.
- C. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the *Ph. Eur. reference spectrum of methaqualone*.
- D. Dissolve about 10 mg in 2 ml of alcohol R. Add 1 ml of dimethylaminobenzaldehyde solution R1 and heat on a water-bath for 5 min. A reddish-orange colour develops.

TESTS

Appearance of solution. Dissolve 1.0 g in methanol R and dilute to 25 ml with the same solvent. The solution is clear (2.2.1) and not more intensely coloured than reference solution BY₇ (2.2.2, Method II).

Acidity. Shake 0.6 g with 30 ml of carbon dioxide-free water R for 5 min and filter. To 10 ml of the filtrate add 0.1 ml of phenolphthalein solution R. Not more than 0.2 ml of 0.01 M sodium hydroxide is required to change the colour of the indicator.

2-Aminobenzoic acid. Dissolve 1.00 g in a mixture of 1 volume of water R and 3 volumes of alcohol R and dilute to 50.0 ml with the same mixture of solvents (test solution). Prepare a reference solution as follows: dissolve 40 mg of 2-aminobenzoic acid R in a mixture of 1 volume of water R and 3 volumes of alcohol R and dilute to 100.0 ml with the same mixture of solvents; dilute 10.0 ml of this solution to 100.0 ml with a mixture of 1 volume of water R and 3 volumes of alcohol R; dilute 0.5 ml of this solution to 50.0 ml with a mixture of 1 volume of water R and 3 volumes of alcohol R (reference solution). To the test solution and to the reference solution add 5 ml of dilute hydrochloric acid R and cool in iced water for 5 min. Add 0.1 ml of

sodium nitrite solution R and cool again in iced water for 5 min. Add 1.5 ml of a freshly prepared 10 g/l solution of *sulphamic acid R* and shake until the colour of the solution is discharged. Add 2.5 ml of a freshly prepared 2 g/l solution of *naphthylethylenediamine dihydrochloride R* and allow to stand for 2 h 30 min. Examine the solutions as described for 2.2.2, *Method II*. The test solution is not more intensely coloured than the reference solution (20 ppm).

***o*-Toluidine.** Dissolve 0.50 g in *acetone R* and dilute to 10.0 ml with the same solvent (test solution). Prepare a reference solution as follows: dissolve 50 mg of freshly distilled *o*-toluidine *R* in 5 ml of *acetone R* and dilute to 100.0 ml with *dilute hydrochloric acid R*; dilute 10.0 ml of the solution to 100.0 ml with *dilute hydrochloric acid R*; dilute 0.1 ml of this solution to 10.0 ml with *acetone R* (reference solution). To the test solution and to the reference solution add 5 ml of *water R* and cool in iced water for 5 min. Add 0.1 ml of *sodium nitrite solution R* and cool again in iced water for 5 min. Add 10 ml of a freshly prepared 2 g/l solution of *β*-naphthol *R* in *dilute sodium hydroxide solution R*. Examine the solutions as described for 2.2.2, *Method II*. The test solution is not more intensely coloured than the reference solution (10 ppm).

Heavy metals (2.4.8). 1.0 g complies with limit test C for heavy metals (20 ppm). Prepare the standard using 2 ml of *lead standard solution (10 ppm Pb) R*.

Loss on drying (2.2.32). Not more than 1.0 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.200 g in 30 ml of *anhydrous acetic acid R*. Titrate with 0.1 M *perchloric acid*, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *perchloric acid* is equivalent to 25.03 mg of C₁₆H₁₄N₂O.

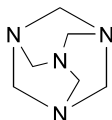
STORAGE

Store protected from light.

01/2005:1545

METHENAMINE

Methenaminum



C₆H₁₂N₄

M_r 140.2

DEFINITION

Methenamine contains not less than 99.0 per cent and not more than the equivalent of 100.5 per cent of 1,3,5,7-tetra-azatricyclo[3.3.1.1.1]decane, calculated with reference to the dried substance.

CHARACTERS

A white, crystalline powder or colourless crystals, freely soluble in water, soluble in alcohol and in methylene chloride.

IDENTIFICATION

First identification: A.

Second identification: B, C, D.

- Examine by infrared absorption spectrophotometry (2.2.24), comparing with the *Ph. Eur. reference spectrum of methenamine*.
- To 1 ml of solution S (see Tests) add 1 ml of *sulphuric acid R* and immediately heat to boiling. Allow to cool. To 1 ml of the solution add 4 ml of *water R* and 5 ml of *acetylacetone reagent R1*. Heat on a water-bath for 5 min. An intense yellow colour develops.
- To 1 ml of solution S add 1 ml of *dilute sulphuric acid R* and immediately heat to boiling. The solution gives the reaction of ammonium salts and salts of volatile bases (2.3.1).
- Dissolve 10 mg in 5 ml of *water R* and acidify with *dilute hydrochloric acid R*. Add 1 ml of *potassium iodobismuthate solution R*. An orange precipitate is formed immediately.

TESTS

Solution S. Dissolve 10.0 g in *carbon dioxide-free water R* prepared from *distilled water R* and dilute to 100 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and colourless (2.2.2, *Method II*).

Acidity or alkalinity. To 5 ml of solution S add 0.1 ml of *phenolphthalein solution R*. Not more than 0.2 ml of 0.1 M *hydrochloric acid* or 0.1 M *sodium hydroxide* is required to change the colour of the indicator.

Free formaldehyde. Dissolve 0.8 g in *water R* and dilute to 8 ml with the same solvent. Add 2 ml of *ammoniacal silver nitrate solution R*. After 5 min, any grey colour in the solution is not more intense than that in a standard prepared at the same time and in the same manner with a mixture of 8 ml of freshly prepared *formaldehyde standard solution (5 ppm CH₂O) R* and 2 ml of *ammoniacal silver nitrate solution R* (50 ppm).

Chlorides (2.4.4). Dilute 5 ml of solution S to 15 ml with *water R*. The solution complies with the limit test for chlorides (100 ppm).

Sulphates (2.4.13). 15 ml of solution S complies with the limit test for sulphates (100 ppm).

Ammonium (2.4.1). Dilute 2 ml of freshly prepared solution S to 13 ml with *water R*. Add 2 ml of *dilute sodium hydroxide solution R*. The solution complies with the limit test for ammonium (50 ppm).

Heavy metals (2.4.8). 12 ml of solution S complies with limit test A for heavy metals (20 ppm). Prepare the standard using *lead standard solution (2 ppm Pb) R*.

Loss on drying (2.2.32). Not more than 2.0 per cent, determined on 1.000 g by drying in a desiccator.

ASSAY

Dissolve 0.100 g in 30 ml of *methanol R*. Titrate with 0.1 M *perchloric acid*, determining the end-point potentiometrically (2.2.20).

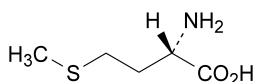
1 ml of 0.1 M *perchloric acid* is equivalent to 14.02 mg of C₆H₁₂N₄.

STORAGE

Store protected from light.

METHIONINE

Methioninum

C₅H₁₁NO₂SM_r 149.2

DEFINITION

Methionine contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of (2S)-2-amino-4-(methylsulphanyl)butanoic acid, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, crystalline powder or colourless crystals, soluble in water, very slightly soluble in alcohol.

IDENTIFICATION

First identification: A, B.

Second identification: A, C, D.

- It complies with the test for specific optical rotation (see Tests).
- Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *methionine CRS*. Examine the substances prepared as discs.
- Examine the chromatograms obtained in the test for ninhydrin-positive substances. The principal spot in the chromatogram obtained with test solution (b) is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).
- Dissolve 0.1 g of the substance to be examined and 0.1 g of *glycine R* in 4.5 ml of *dilute sodium hydroxide solution R*. Add 1 ml of a 25 g/l solution of *sodium nitroprusside R*. Heat to 40 °C for 10 min. Allow to cool and add 2 ml of a mixture of 1 volume of *phosphoric acid R* and 9 volumes of *hydrochloric acid R*. A dark red colour develops.

TESTS

Solution S. Dissolve 2.5 g in *carbon dioxide-free water R* and dilute to 100 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and colourless (2.2.2, *Method II*).

pH (2.2.3). The pH of solution S is 5.5 to 6.5.

Specific optical rotation (2.2.7). Dissolve 1.00 g in *hydrochloric acid R1* and dilute to 50.0 ml with the same acid. The specific optical rotation is + 22.5 to + 24.0, calculated with reference to the dried substance.

Ninhydrin-positive substances. Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel plate R*.

Test solution (a). Dissolve 0.10 g of the substance to be examined in *dilute hydrochloric acid R* and dilute to 10 ml with the same acid.

Test solution (b). Dilute 1 ml of test solution (a) to 50 ml with *water R*.

Reference solution (a). Dissolve 10 mg of *methionine CRS* in a 10 g/l solution of *hydrochloric acid R* and dilute to 50 ml with the same acid solution.

Reference solution (b). Dilute 5 ml of test solution (b) to 20 ml with *water R*.

01/2005:1027 Reference solution (c). Dissolve 10 mg of *methionine CRS* and 10 mg of *serine CRS* in a 10 g/l solution of *hydrochloric acid R* and dilute to 25 ml with the same acid solution.

Apply separately to the plate 5 µl of each solution. Develop over a path of 15 cm using a mixture of 20 volumes of *glacial acetic acid R*, 20 volumes of *water R* and 60 volumes of *butanol R*. Allow the plate to dry in air, spray with *ninhydrin solution R* and heat at 100 °C to 105 °C for 15 min. Any spot in the chromatogram obtained with test solution (a), apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.5 per cent). The test is not valid unless the chromatogram obtained with reference solution (c) shows two clearly separated spots.

Chlorides. To 10 ml of solution S add 25 ml of *water R*, 5 ml of *dilute nitric acid R* and 10 ml of *silver nitrate solution R2*. Allow to stand protected from light for 5 min. Any opalescence in the solution is not more intense than that in a standard prepared at the same time and in the same manner using 10 ml of *chloride standard solution (5 ppm Cl) R* (200 ppm). Examine the tubes laterally against a black background.

Sulphates (2.4.13). Dissolve 0.5 g in 3 ml of *dilute hydrochloric acid R* and dilute to 15 ml with *distilled water R*. The solution complies with the limit test for sulphates (300 ppm).

Ammonium (2.4.1). 0.10 g complies with limit test B for ammonium (200 ppm). Prepare the standard using 0.2 ml of *ammonium standard solution (100 ppm NH₄) R*.

Iron (2.4.9). In a separating funnel, dissolve 1.0 g in 10 ml of *dilute hydrochloric acid R*. Shake with three quantities, each of 10 ml, of *methyl isobutyl ketone R1*, shaking for 3 min each time. To the combined upper layers add 10 ml of *water R* and shake for 3 min. The lower layer complies with the limit test for iron (10 ppm).

Heavy metals (2.4.8). 2.0 g complies with limit test C for heavy metals (10 ppm). Prepare the standard using 2 ml of *lead standard solution (10 ppm Pb) R*.

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.125 g in 5 ml of *anhydrous formic acid R*. Add 30 ml of *anhydrous acetic acid R*. Titrate with 0.1 M *perchloric acid*, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *perchloric acid* is equivalent to 14.92 mg of C₅H₁₁NO₂S.

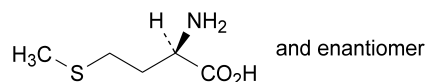
STORAGE

Store protected from light.

01/2005:0624

DL-METHIONINE

DL-Methioninum

C₅H₁₁NO₂SM_r 149.2

DEFINITION

DL-Methionine contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of (2*RS*)-2-amino-4-(methylsulphonyl)butanoic acid, calculated with reference to the dried substance.

CHARACTERS

Almost white, crystalline powder or small flakes, sparingly soluble in water, very slightly soluble in alcohol. It dissolves in dilute acids and in dilute solutions of the alkali hydroxides. It melts at about 270 °C (instantaneous method).

IDENTIFICATION

First identification: A, C.

Second identification: B, C, D.

- Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *DL-methionine CRS*. Dry the substances at 105 °C.
- Examine the chromatograms obtained in the test for related substances. The principal spot in the chromatogram obtained with test solution (b) is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).
- Dissolve 2.50 g in 1 *M* hydrochloric acid and dilute to 50.0 ml with the same acid. The angle of optical rotation (2.2.7) is -0.05° to $+0.05^{\circ}$.
- Dissolve 0.1 g of the substance to be examined and 0.1 g of *glycine R* in 4.5 ml of dilute sodium hydroxide solution *R*. Add 1 ml of a 25 g/l solution of sodium nitroprusside *R*. Heat to 40 °C for 10 min. Allow to cool and add 2 ml of a mixture of 1 volume of phosphoric acid *R* and 9 volumes of hydrochloric acid *R*. A deep-red colour develops.

TESTS

Solution S. Dissolve 1.0 g in carbon dioxide-free water *R* and dilute to 50 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and colourless (2.2.2, Method II).

pH (2.2.3). The pH of solution S is 5.4 to 6.1.

Related substances. Examine by thin-layer chromatography (2.2.27), using silica gel *G R* as the coating substance.

Test solution (a). Dissolve 0.2 g in water *R* and dilute to 10 ml with the same solvent.

Test solution (b). Dilute 1 ml of test solution (a) to 50 ml with water *R*.

Reference solution (a). Dissolve 20 mg of *DL-methionine CRS* in water *R* and dilute to 50 ml with the same solvent.

Reference solution (b). Dilute 1 ml of reference solution (a) to 10 ml with water *R*.

Apply separately to the plate 5 µl of each solution. Develop over a path of 10 cm using a mixture of 20 volumes of glacial acetic acid *R*, 20 volumes of water *R* and 60 volumes of butanol *R*. Allow the plate to dry in air and spray with ninhydrin solution *R*. Heat the plate at 100 °C to 105 °C for 15 min. Any spot in the chromatogram obtained with test solution (a), apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.2 per cent).

Chlorides. Dissolve 0.25 g in 35 ml of water *R*. Add 5 ml of dilute nitric acid *R* and 10 ml of silver nitrate solution *R2*. Allow to stand protected from light for 5 min. Any opalescence in the solution is not more intense than that in a standard prepared at the same time in the same manner

using a mixture of 10 ml of chloride standard solution (5 ppm *Cl*) *R* and 25 ml of water *R* (200 ppm). Examine the tubes laterally against a black background.

Sulphates (2.4.13). Dissolve 1.0 g in 20 ml of distilled water *R*, heating to 60 °C. Cool to 10 °C and filter. 15 ml of the solution complies with the limit test for sulphates (200 ppm).

Heavy metals (2.4.8). 1.0 g complies with limit test D for heavy metals (20 ppm). Prepare the standard using 2 ml of lead standard solution (10 ppm *Pb*) *R*.

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.140 g in 3 ml of anhydrous formic acid *R*. Add 30 ml of anhydrous acetic acid *R*. Immediately after dissolution, titrate with 0.1 *M* perchloric acid, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 *M* perchloric acid is equivalent to 14.92 mg of $C_5H_{11}NO_2S$.

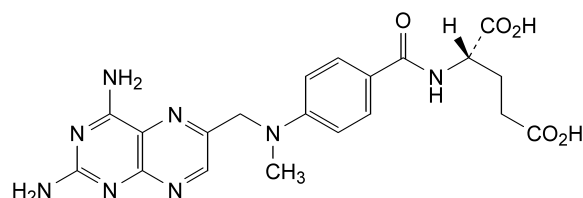
STORAGE

Store protected from light.

01/2005:0560

METHOTREXATE

Methotrexatum


 $C_{20}H_{22}N_8O_5$
 M_r 454.4

DEFINITION

Methotrexate contains not less than 98.0 per cent and not more than the equivalent of 102.0 per cent of (2*S*)-2-[[4-[(2,4-diaminopteridin-6-yl)methyl]methylamino]benzoyl]amino]pentanedioic acid, calculated with reference to the anhydrous substance.

CHARACTERS

A yellow or orange, crystalline, hygroscopic powder, practically insoluble in water, in alcohol and in methylene chloride. It dissolves in dilute solutions of mineral acids and in dilute solutions of alkali hydroxides and carbonates.

IDENTIFICATION

- Dissolve 0.250 g in a 14 g/l solution of sodium carbonate *R* and dilute to 25.0 ml with the same solution. The specific optical rotation (2.2.7) is $+19$ to $+24$ calculated with reference to the anhydrous substance.
- Dissolve 10 mg in a 4 g/l solution of sodium hydroxide *R* and dilute to 100 ml with the same solvent. Dilute 10 ml of this solution to 100 ml with a 4 g/l solution of sodium hydroxide *R*. Examined between 230 nm and 380 nm (2.2.25), the solution shows three absorption maxima, at

258 nm, 302 nm and 371 nm, respectively. The ratio of the absorbance measured at the maximum at 302 nm to that measured at the maximum at 371 nm is 2.8 to 3.3.

- C. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *methotrexate CRS*.

TESTS

Related substances. Examine by liquid chromatography (2.2.29), as prescribed under Assay but setting the spectrophotometer at 265 nm.

Inject 20 µl of reference solution (c). The test is not valid unless in the chromatogram obtained, the resolution between the peaks corresponding to impurity D and methotrexate respectively is at least 2.0.

Inject 20 µl of the test solution and 20 µl of reference solution (b). Continue the chromatography for three times the retention time of methotrexate.

When the chromatograms are recorded in the prescribed conditions, the approximate relative retention times are the following: impurity A = 0.2; impurity B = 0.3; impurity C = 0.4; impurity D = 0.8; impurity E = 2.3.

In the chromatogram obtained with the test solution, the area of at most five peaks, apart from the principal peak, is greater than 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent) and no such peak has an area greater than that of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent). The sum of the areas of all the peaks, apart from the principal peak, is not greater than 2.6 times the area of the principal peak in the chromatogram obtained with reference solution (b) (1.3 per cent). Disregard any peak with an area less than 0.05 times that of the principal peak in the chromatogram obtained with reference solution (b).

(R)-Methotrexate. Not more than 3.0 per cent. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 20.0 mg of the substance to be examined in the mobile phase and dilute to 100.0 ml with the mobile phase.

Reference solution (a). Dilute 1.0 ml of the test solution to 100.0 ml with the mobile phase.

Reference solution (b). Dissolve 4.0 mg of *(RS)-methotrexate R* in the mobile phase and dilute to 100.0 ml with the mobile phase.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.15 m long and 4.0 mm in internal diameter packed with *bovine albumin R* bound to *silica gel for chromatography R* (film thickness 7 µm; pore size 30 nm),
- as mobile phase at a flow rate of 1.5 ml/min a solution prepared as follows: to 500 ml of a 7.1 g/l solution of *anhydrous disodium hydrogen phosphate R* add 600 ml of a 6.9 g/l solution of *sodium dihydrogen phosphate monohydrate R* and mix. Adjust to pH 6.9 with *dilute sodium hydroxide solution R*. To 920 ml of this mixture add 80 ml of *propanol R*,
- as detector a spectrophotometer set at 302 nm.

Inject 20 µl of each solution. The test is not valid unless in the chromatogram obtained with reference solution (b); the resolution between the peaks corresponding to *(S)-methotrexate* and *(R)-methotrexate* respectively is at least 3.0.

In the chromatogram obtained with the test solution the area of any peak corresponding to *(R)-methotrexate* is not greater than three times the area of the principal peak in the chromatogram obtained with reference solution (a) (3.0 per cent).

Heavy metals (2.4.8). 1.0 g complies with limit test C for heavy metals (50 ppm). Prepare the standard using 5 ml of *lead standard solution (10 ppm Pb) R*.

Water (2.5.12). Not more than 13.0 per cent, determined on 0.10 g by the semi-micro determination of water.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 25.0 mg of the substance to be examined in the mobile phase and dilute to 250.0 ml with the mobile phase.

Reference solution (a). Dissolve 25.0 mg of *methotrexate CRS* in the mobile phase and dilute to 250.0 ml with the mobile phase.

Reference solution (b). Dilute 0.5 ml of reference solution (a) to 100.0 ml with the mobile phase.

Reference solution (c). Dissolve 25.0 mg of the substance to be examined and 25.0 mg of *methotrexate impurity D CRS* in the mobile phase and dilute to 250.0 ml with the mobile phase.

The chromatographic procedure may be carried out using:

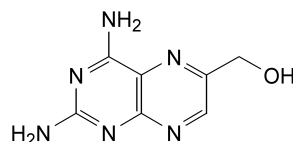
- a stainless steel column 0.25 m long and 4.6 mm in internal diameter packed with *base-deactivated octadecylsilyl silica gel for chromatography R* (5 µm),
- as mobile phase at a flow rate of 1.2 ml/min a mixture of 7 volumes of *acetonitrile R* and 93 volumes of a solution obtained as follows: dissolve 7.8 g of *citric acid R* and 17.9 g of *anhydrous disodium hydrogen phosphate R* in *water R* and dilute to 1000 ml with the same solvent,
- as detector a spectrophotometer set at 302 nm.

Inject reference solution (a) six times. The assay is not valid unless the relative standard deviation of the area of the methotrexate peak is not greater than 2.0 per cent. Inject 20 µl of the test solution and 20 µl of reference solution (a). Calculate the percentage content of methotrexate using the chromatograms obtained with the test solution and reference solution (a)

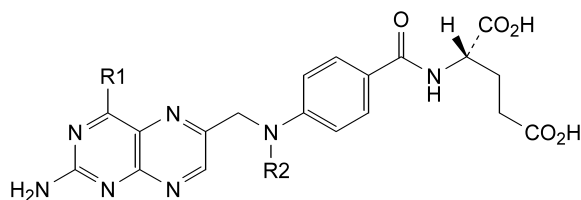
STORAGE

Store in an airtight container, protected from light.

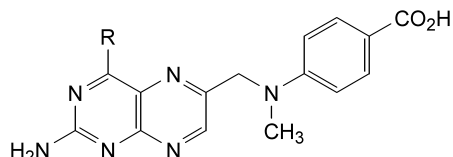
IMPURITIES



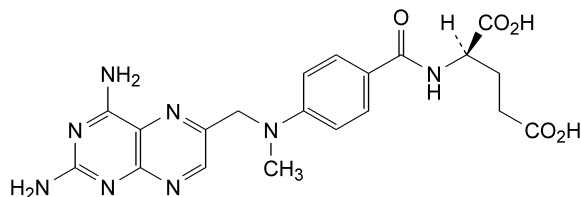
A. (2,4-diaminopteridin-6-yl)methanol,



- B. R1 = NH₂, R2 = H: (2S)-2-[[4-[(2,4-diaminopteridin-6-yl)methyl]amino]benzoyl]amino]pentanedioic acid (4-aminofolic acid, aminopteridine),
- C. R1 = OH, R2 = CH₃: (2S)-2-[[4-[(2-amino-4-hydroxypteridin-6-yl)methyl]methylamino]benzoyl]amino]pentanedioic acid (N-methylfolic acid, methopteridine),



- D. R = OH: 4-[[[(2-amino-4-hydroxypteridin-6-yl)methyl]methylamino]benzoic acid (N¹⁰-methylpteroic acid),
- E. R = NH₂: 4-[[[(2,4-diaminopteridin-6-yl)methyl]methylamino]benzoic acid (2,4-diamino-N¹⁰-methylpteroic acid, APA),

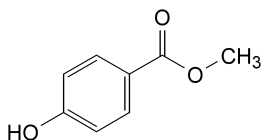


- F. (2R)-2-[[4-[[[(2,4-diaminopteridin-6-yl)methyl]methylamino]benzoyl]amino]pentanedioic acid ((R)-methotrexate).

01/2005:0409

METHYL PARAHYDROXYBENZOATE

Methylis parahydroxybenzoas

C₈H₈O₃M_r 152.1

DEFINITION

Methyl 4-hydroxybenzoate.

Content: 98.0 per cent to 102.0 per cent.

CHARACTERS

Appearance: white, crystalline powder or colourless crystals.*Solubility*: very slightly soluble in water, freely soluble in alcohol and in methanol.

IDENTIFICATION

First identification: A, B.*Second identification*: A, C, D.

A. Melting point (2.2.14): 125 °C to 128 °C.

B. Infrared absorption spectrophotometry (2.2.24).

Comparison: methyl parahydroxybenzoate CRS.

C. Examine the chromatograms obtained in the test for related substances.

Results: the principal spot in the chromatogram obtained with test solution (b) is similar in position and size to the principal spot in the chromatogram obtained with reference solution (b).

D. To about 10 mg in a test-tube add 1 ml of *sodium carbonate solution R*, boil for 30 s and cool (solution A). To a further 10 mg in a similar test-tube add 1 ml of *sodium carbonate solution R*; the substance partly dissolves (solution B). Add at the same time to solution A and solution B 5 ml of *aminopyrazolone solution R* and 1 ml of *potassium ferricyanide solution R* and mix. Solution B is yellow to orange-brown. Solution A is orange to red, the colour being clearly more intense than any similar colour which may be obtained with solution B.

TESTS

Solution S. Dissolve 1.0 g in *alcohol R* and dilute to 10 ml with the same solvent.**Appearance of solution.** Solution S is clear (2.2.1) and not more intensely coloured than reference solution BY₆ (2.2.2, Method II).**Acidity.** To 2 ml of solution S add 3 ml of *alcohol R*, 5 ml of *carbon dioxide-free water R* and 0.1 ml of *bromocresol green solution R*. Not more than 0.1 ml of 0.1 M *sodium hydroxide* is required to change the colour of the indicator to blue.**Related substances.** Thin-layer chromatography (2.2.27).*Test solution (a).* Dissolve 0.10 g of the substance to be examined in *acetone R* and dilute to 10 ml with the same solvent.*Test solution (b).* Dilute 1 ml of test solution (a) to 10 ml with *acetone R*.*Reference solution (a).* Dilute 0.5 ml of test solution (a) to 100 ml with *acetone R*.*Reference solution (b).* Dissolve 10 mg of *methyl parahydroxybenzoate CRS* in *acetone R* and dilute to 10 ml with the same solvent.*Reference solution (c).* Dissolve 10 mg of *ethyl parahydroxybenzoate CRS* in 1 ml of test solution (a) and dilute to 10 ml with *acetone R*.*Plate*: suitable octadecylsilyl silica gel with a fluorescent indicator having an optimal intensity at 254 nm as the coating substance.*Mobile phase*: glacial acetic acid R, water R, methanol R (1:30:70 V/V/V).*Application*: 2 µl.*Development*: over a path of 15 cm.*Drying*: in air.*Detection*: examine in ultraviolet light at 254 nm.*System suitability*: the chromatogram obtained with reference solution (c) shows 2 clearly separated principal spots.*Limits*:

— *any impurity*: any spot in the chromatogram obtained with test solution (a), apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (a) (0.5 per cent).

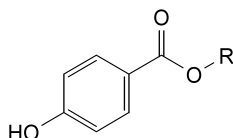
Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

To 1.000 g add 20.0 ml of 1 M sodium hydroxide. Heat at about 70 °C for 1 h. Cool rapidly in an ice bath. Prepare a blank in the same manner. Carry out the titration on the solutions at room temperature. Titrate the excess sodium hydroxide with 0.5 M sulphuric acid, continuing the titration until the second point of inflexion and determining the end-point potentiometrically (2.2.20).

1 ml of 1 M sodium hydroxide is equivalent to 152.1 mg of C₈H₈O₃.

IMPURITIES

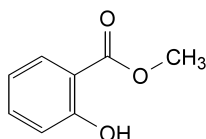


- A. R = H: 4-hydroxybenzoic acid,
 B. R = CH₂-CH₃: ethyl 4-hydroxybenzoate,
 C. R = CH₂-CH₂-CH₃: propyl 4-hydroxybenzoate,
 D. R = CH₂-CH₂-CH₂-CH₃: butyl 4-hydroxybenzoate.

01/2005:0230

METHYL SALICYLATE

Methylis salicylas

C₈H₈O₃*M*_r 152.1

DEFINITION

Methyl salicylate contains not less than 99.0 per cent *m/m* and not more than the equivalent of 100.5 per cent *m/m* of methyl 2-hydroxybenzoate.

CHARACTERS

A colourless or slightly yellow liquid, very slightly soluble in water, miscible with alcohol and with fatty and essential oils.

IDENTIFICATION

- A. Heat 0.25 ml with 2 ml of *dilute sodium hydroxide solution R* on a water-bath for 5 min. Add 3 ml of *dilute sulphuric acid R*. A crystalline precipitate is formed. Filter. The precipitate, washed with *water R* and dried at 100 °C to 105 °C, melts (2.2.14) at 156 °C to 161 °C.
 B. To 10 ml of a saturated solution add 0.05 ml of *ferric chloride solution R1*. A violet colour develops.

TESTS

Appearance of solution. To 2 ml add 10 ml of *alcohol R*. The solution is clear (2.2.1) and not more intensely coloured than reference solution Y₇ (2.2.2, *Method II*).

Acidity. Dissolve 5.0 g in a mixture of 0.2 ml of *bromocresol green solution R* and 50 ml of *alcohol R* previously neutralised to a blue colour by addition of 0.1 M sodium hydroxide. Not more than 0.4 ml of 0.1 M sodium hydroxide is required to restore the blue colour.

Refractive index (2.2.6): 1.535 to 1.538.

Relative density (2.2.5): 1.180 to 1.186.

ASSAY

Dissolve 0.500 g in 25 ml of *alcohol R*. Add 0.05 ml of *phenol red solution R* and neutralise with 0.1 M sodium hydroxide. To the neutralised solution add 50.0 ml of 0.1 M sodium hydroxide and heat under a reflux condenser on a water-bath for 30 min. Cool and titrate with 0.1 M hydrochloric acid. Calculate the volume of 0.1 M sodium hydroxide used in the saponification. Carry out a blank titration.

1 ml of 0.1 M sodium hydroxide is equivalent to 15.21 mg of C₈H₈O₃.

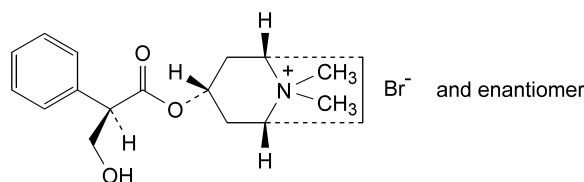
STORAGE

Store protected from light.

01/2005:0511

METHYLATROPINE BROMIDE

Methylatropini bromidum

C₁₈H₂₆BrNO₃*M*_r 384.3

DEFINITION

Methylatropine bromide contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of (1*R*,3*r*,5*S*)-3-[(2*RS*)-3-hydroxy-2-phenylpropanoyl]oxy]-8,8-dimethyl-8-azoniabicyclo[3.2.1]octane bromide, calculated with reference to the dried substance.

CHARACTERS

A white, crystalline powder or colourless crystals, freely soluble in water, sparingly soluble in alcohol.

It melts at about 219 °C, with decomposition.

IDENTIFICATION

First identification: B, E.

Second identification: A, C, D, E.

- A. It complies with the test for optical rotation (see Tests).
 B. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *methylatropine bromide CRS*.
 C. To 5 ml of solution S (see Tests) add 2 ml of *dilute sodium hydroxide solution R*. No precipitate is formed.
 D. To about 1 mg add 0.2 ml of *fuming nitric acid R* and evaporate to dryness on a water-bath. Dissolve the residue in 2 ml of *acetone R* and add 0.1 ml of a 30 g/l solution of *potassium hydroxide R* in *methanol R*. A violet colour develops.
 E. It gives reaction (a) of bromides (2.3.1).

TESTS

Solution S. Dissolve 1.25 g in *carbon dioxide-free water R* and dilute to 25 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and not more intensely coloured than reference solution B₉ (2.2.2, *Method II*).

Acidity or alkalinity. To 10 ml of solution S add 0.1 ml of *phenolphthalein solution R*; the solution is colourless. Add 0.5 ml of 0.01 M sodium hydroxide; the solution is red.

Optical rotation (2.2.7). Dissolve 2.50 g in *water R* and dilute to 25.0 ml with the same solvent. The angle of optical rotation, measured in a 2 dm tube, is -0.25° to $+0.05^{\circ}$.

Related substances. Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel G plate R*.

Test solution. Dissolve 0.2 g of the substance to be examined in a mixture of 1 volume of *water R* and 9 volumes of *methanol R* and dilute to 5 ml with the same mixture of solvents.

Reference solution. Dilute 0.5 ml of the test solution to 100 ml with a mixture of 1 volume of *water R* and 9 volumes of *methanol R*.

Apply to the plate 5 μ l of each solution. Develop over a path of 15 cm using a mixture of 10 volumes of *methanol R*, 15 volumes of *anhydrous formic acid R*, 15 volumes of *water R* and 60 volumes of *ethyl acetate R*. Dry the plate at 100°C to 105°C until the solvent has evaporated, allow to cool and spray with *dilute potassium iodobismuthate solution R* until the spots appear. Any spot in the chromatogram obtained with the test solution, apart from the principal spot, is not more intense than the spot in the chromatogram obtained with the reference solution (0.5 per cent).

Apomethylatropine. Dissolve 0.10 g in *0.01 M hydrochloric acid* and dilute to 100.0 ml with the same acid. Measure the absorbances (2.2.25) at the maxima at 252 nm and 257 nm. The ratio of the absorbance at 257 nm to that at 252 nm is at least 1.19.

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 0.500 g by drying in an oven at 100°C to 105°C .

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on the residue obtained in the test for loss on drying.

ASSAY

Dissolve 0.300 g in 50 ml of *anhydrous acetic acid R*, warming slightly if necessary. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (2.2.20). 1 ml of *0.1 M perchloric acid* is equivalent to 38.43 mg of $\text{C}_{18}\text{H}_{26}\text{BrNO}_3$.

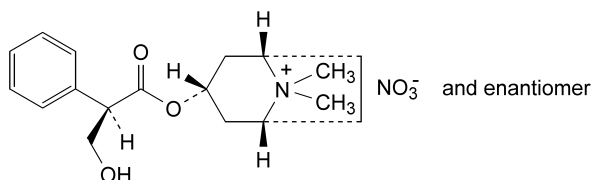
STORAGE

Store protected from light.

01/2005:0512

METHYLATROPINE NITRATE

Methylatropini nitras



$\text{C}_{18}\text{H}_{26}\text{N}_2\text{O}_6$

M_r 366.4

DEFINITION

Methylatropine nitrate contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of (1*R*,3*r*,5*S*)-3-[[[(2*RS*)-3-hydroxy-2-phenylpropanoyl]oxy]-8,8-dimethyl-8-azoniabicyclo[3.2.1]octane nitrate, calculated with reference to the dried substance.

CHARACTERS

A white, crystalline powder or colourless crystals, freely soluble in water, soluble in alcohol.

It melts at about 167°C .

IDENTIFICATION

First identification: B, E.

Second identification: A, C, D, E.

- It complies with the test for optical rotation (see Tests).
- Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *methylatropine nitrate CRS*.
- To a mixture of 2.5 ml of solution S (see Tests) and 2.5 ml of *water R* add 2 ml of *dilute sodium hydroxide solution R*. No precipitate is formed.
- To about 1 mg add 0.2 ml of *fuming nitric acid R* and evaporate to dryness on a water-bath. Dissolve the residue in 2 ml of *acetone R* and add 0.25 ml of a 30 g/l solution of *potassium hydroxide R* in *methanol R*. A violet colour develops.
- To 0.05 ml of *diphenylamine solution R* add 0.05 ml of a 1 in 10 dilution of solution S. An intense blue colour is produced.

TESTS

Solution S. Dissolve 1.25 g in *carbon dioxide-free water R* and dilute to 25 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and not more intensely coloured than reference solution B₉ (2.2.2, Method II).

Acidity or alkalinity. To 10 ml of solution S add 0.1 ml of *phenolphthalein solution R*; the solution is colourless. Add 0.5 ml of *0.01 M sodium hydroxide*; the solution is red.

Optical rotation (2.2.7). Dissolve 2.50 g in *water R* and dilute to 25.0 ml with the same solvent. The angle of optical rotation, measured in a 2 dm tube, is -0.25° to $+0.05^{\circ}$.

Related substances. Examine by thin-layer chromatography (2.2.27), using *silica gel G R* as the coating substance.

Test solution. Dissolve 0.2 g of the substance to be examined in a mixture of 1 volume of *water R* and 9 volumes of *methanol R* and dilute to 5 ml with the same mixture of solvents.

Reference solution. Dilute 0.5 ml of the test solution to 100 ml with a mixture of 1 volume of *water R* and 9 volumes of *methanol R*.

Apply to the plate 5 μ l of each solution. Develop over a path of 15 cm using a mixture of 10 volumes of *methanol R*, 15 volumes of *anhydrous formic acid R*, 15 volumes of *water R* and 60 volumes of *ethyl acetate R*. Dry the plate at 100°C to 105°C until the solvent has evaporated, allow to cool and spray with *dilute potassium iodobismuthate solution R* until the spots appear. Any spot in the chromatogram obtained with the test solution, apart from the principal spot, is not more intense than the spot in the chromatogram obtained with the reference solution (0.5 per cent).

Apomethylatropine. Dissolve 0.10 g in *0.01 M hydrochloric acid* and dilute to 100.0 ml with the same acid. Measure the absorbances (2.2.25) at the maxima at 252 nm and 257 nm. The ratio of the absorbance at 257 nm to that at 252 nm is not less than 1.17.

Halides. 15 ml of solution S complies with the limit test for chlorides (2.4.4). Prepare the standard using 1.5 ml of *chloride standard solution (5 ppm Cl) R* (10 ppm).

Silver. Dissolve 1.0 g in 10 ml of *water R* and add 0.1 ml of *sodium sulphide solution R*. Allow to stand for 2 min. The solution is not more intensely coloured than reference solution B₈ (2.2.2, *Method II*) (10 ppm).

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 0.500 g by drying in an oven at 100 °C to 105 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on the residue obtained in the test for loss on drying.

ASSAY

Dissolve 0.300 g in 50 ml of *anhydrous acetic acid R*. Titrate with 0.1 M *perchloric acid* determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *perchloric acid* is equivalent to 36.64 mg of C₁₈H₂₆N₂O₆.

STORAGE

Store protected from light.

01/2005:0345

METHYLCELLULOSE

Methylcellulosum

DEFINITION

Methylcellulose is a partly *O*-methylated cellulose.

CHARACTERS

A white, yellowish-white or greyish-white powder or granules, hygroscopic after drying, practically insoluble in hot water, in acetone, in ethanol and in toluene. It dissolves in cold water giving a colloidal solution.

IDENTIFICATION

- Heat 10 ml of solution S (see Tests) in a water-bath while stirring. At a temperature above 50 °C the solution becomes cloudy or a flocculent precipitate is formed. The solution becomes clear again on cooling.
- To 10 ml of solution S add 0.3 ml of *dilute acetic acid R* and 2.5 ml of a 100 g/l solution of *tannic acid R*. A yellowish-white, flocculent precipitate is formed which dissolves in *dilute ammonia RI*.
- In a test-tube about 160 mm long, thoroughly mix 1 g with 2 g of finely powdered *manganese sulphate R*. Introduce to a depth of 2 cm into the upper part of the tube a strip of filter paper impregnated with a freshly prepared mixture of 1 volume of a 20 per cent *V/V* solution of *diethanolamine R* and 11 volumes of a 50 g/l solution of *sodium nitroprusside R*, adjusted to about pH 9.8 with 1 M *hydrochloric acid*. Insert the tube 8 cm into a silicone-oil bath at 190 °C to 200 °C. The filter paper does not become blue within 10 min. Carry out a blank test.
- Dissolve 0.2 g completely, without heating, in 15 ml of a 70 per cent *m/m* solution of *sulphuric acid R*. Pour the solution with stirring into 100 ml of iced *water R* and dilute to 250 ml with iced *water R*. In a test-tube, mix thoroughly while cooling in iced water 1 ml of the solution with 8 ml of *sulphuric acid R*, added dropwise. Heat in a water-bath for exactly 3 min and immediately cool in iced water. While the mixture is cold, carefully add 0.6 ml of *ninhydrin solution R2* and mix well. Allow to stand at 25 °C. A pink colour is produced immediately and does not become violet within 100 min.

E. Place 1 ml of solution S on a glass plate. After evaporation of the water a thin film is formed.

F. 0.2 g does not dissolve in 10 ml of *toluene R* nor in 10 ml of *ethanol R*.

TESTS

Solution S. While stirring, introduce a quantity of the substance to be examined equivalent to 1.0 g of the dried substance into 50 g of *carbon dioxide-free water R* heated to 90 °C. Allow to cool, adjust the mass of the solution to 100 g with *carbon dioxide-free water R* and stir until dissolution is complete. Allow to stand at 2 °C to 8 °C for 1 h before carrying out the test for appearance of solution.

Appearance of solution. Solution S is not more opalescent than reference suspension III (2.2.1) and not more intensely coloured than reference solution Y₆ (2.2.2, *Method II*).

pH (2.2.3). The pH of solution S is 5.5 to 8.0.

Apparent viscosity. While stirring, introduce a quantity of the substance to be examined equivalent to 6.00 g of the dried substance into 150 g of *water R* heated to 90 °C. Stir with a propeller-type stirrer for 10 min, place the flask in a bath of iced water, continue the stirring, and allow to remain in the bath of iced water for 40 min to ensure that dissolution is complete. Adjust the mass of the solution to 300 g, and centrifuge the solution to expel any entrapped air. Adjust the temperature of the solution to 20 ± 0.1 °C. Determine the viscosity (2.2.10) with a rotating viscometer at 20 °C and a shear rate of 10 s⁻¹. The apparent viscosity is not less than 75 per cent and not more than 140 per cent of the value stated on the label.

Chlorides (2.4.4). 1 ml of solution S diluted to 15 ml with *water R* complies with the limit test for chlorides (0.5 per cent).

Heavy metals (2.4.8). 1.0 g complies with limit test C for heavy metals (20 ppm). Prepare the standard using 2 ml of *lead standard solution (10 ppm Pb) R*.

Loss on drying (2.2.32). Not more than 10.0 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C.

Sulphated ash (2.4.14). Not more than 1.0 per cent, determined on 1.000 g.

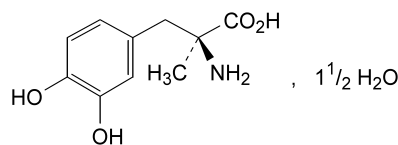
LABELLING

The label states the apparent viscosity in millipascal seconds for a 2 per cent *m/m* solution.

01/2005:0045

METHYLDOPA

Methyldopum



C₁₀H₁₃NO₄ · 1½H₂O

M_r 238.2

DEFINITION

Methyldopa contains not less than 98.5 per cent and not more than the equivalent of 101.0 per cent of (2*S*)-2-amino-3-(3,4-dihydroxyphenyl)-2-methylpropanoic acid, calculated with reference to the anhydrous substance.

CHARACTERS

A white or yellowish white, crystalline powder or colourless or almost colourless crystals, slightly soluble in water, very slightly soluble in alcohol. It is freely soluble in dilute mineral acids.

IDENTIFICATION

First identification: A.

Second identification: B, C, D.

- A. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *methyldopa CRS*.
- B. Dissolve about 2 mg in 2 ml of *water R* and add 0.2 ml of *feric chloride solution R2*. A green colour develops which changes to bluish-violet on the addition of 0.1 g of *hexamethylenetetramine R*.
- C. Dissolve about 5 mg in a mixture of 5 ml of 1 M *hydrochloric acid* and 5 ml of *water R*. Add 0.1 ml of *sodium nitrite solution R* containing 100 g/l of *ammonium molybdate R*. A yellow colour is produced which becomes brownish-red on the addition of *strong sodium hydroxide solution R*.
- D. To about 5 mg add 1 ml of *water R*, 1 ml of *pyridine R* and about 5 mg of *nitrobenzoyl chloride R* and heat to boiling. Add, while shaking, 0.2 ml of *sodium carbonate solution R*. An orange or amber colour develops.

TESTS

Appearance of solution. Dissolve 1.0 g in 1 M *hydrochloric acid* and dilute to 25 ml with the same solvent. The solution is not more intensely coloured than reference solution BY₆ or B₆ (2.2.2, *Method II*).

Acidity. Dissolve 1.0 g with heating in 100 ml of *carbon dioxide-free water R*. Add 0.1 ml of *methyl red solution R*. Not more than 0.5 ml of 0.1 M *sodium hydroxide* is required to produce the pure yellow colour of the indicator.

Optical rotation (2.2.7). Dissolve a quantity equivalent to 2.20 g of the anhydrous substance in *aluminium chloride solution R* and dilute to 50.0 ml with the same solution. The angle of optical rotation is -1.10° to -1.23° .

Absorbance (2.2.25). Dissolve 40.0 mg in 0.1 M *hydrochloric acid* and dilute to 100.0 ml with the same acid. Dilute 10.0 ml of this solution to 100.0 ml with 0.1 M *hydrochloric acid*. Examined between 230 nm and 350 nm, the solution shows a single absorption maximum, at 280 nm. The specific absorbance at this maximum is 122 to 137, calculated with reference to the anhydrous substance.

Methoxymethyldopa and related substances. Examine by thin-layer chromatography (2.2.27), using *cellulose for chromatography R* as the coating substance.

Test solution. Dissolve 0.1 g of the substance to be examined in a mixture of 0.4 ml of *hydrochloric acid R1* and 9.6 ml of *methanol R*.

Reference solution (a). Dissolve 5 mg of *3-methoxymethyldopa CRS* in 100 ml of *methanol R*.

Reference solution (b). To 1 ml of the test solution add 1 ml of reference solution (a).

Apply separately to the plate 10 µl of the test solution, 10 µl of reference solution (a) and 20 µl of reference solution (b). Develop over a path of 10 cm using a mixture of 15 volumes of *glacial acetic acid R*, 25 volumes of *water R* and 65 volumes of *butanol R*. Dry the plate immediately in a current of warm air and spray with a mixture of 5 volumes of a 50 g/l solution of *sodium nitrite R* and 45 volumes of a 3 g/l solution of *nitroaniline R* in a mixture of 20 volumes

of *water R* and 80 volumes of *hydrochloric acid R*. Dry the plate immediately in a current of warm air and spray with a 200 g/l solution of *sodium carbonate R*. Examine the chromatograms immediately. Any spot in the chromatogram obtained with the test solution, apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (a) (0.5 per cent). The test is not valid unless the chromatogram obtained with reference solution (b) shows 2 clearly separated spots.

Heavy metals (2.4.8). 1.0 g complies with limit test C for heavy metals (20 ppm). Prepare the standard using 2 ml of *lead standard solution (10 ppm Pb) R*.

Water (2.5.12). 10.0 per cent to 13.0 per cent, determined on 0.20 g by the semi-micro determination of water.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.200 g in a mixture of 15 ml of *anhydrous formic acid R* 30 ml of *anhydrous acetic acid R* and 30 ml of *dioxan R*. Titrate with 0.1 M *perchloric acid* until a green colour is obtained using 0.1 ml of *crystal violet solution R* as indicator.

1 ml of 0.1 M *perchloric acid* is equivalent to 21.12 mg of C₁₀H₁₃NO₄.

STORAGE

Store protected from light.

01/2005:0932

METHYLENE CHLORIDE

Methyleni chloridum

CH₂Cl₂M_r 84.9

DEFINITION

Methylene chloride is dichloromethane. It may contain not more than 2.0 per cent V/V of ethanol and/or not more than 0.03 per cent V/V of 2-methylbut-2-ene as stabiliser.

CHARACTERS

A clear, colourless, volatile liquid, sparingly soluble in water, miscible with alcohol.

IDENTIFICATION

First identification: B, C.

Second identification: A, D, E.

- A. It complies with the test for relative density (see Tests).
- B. It complies with the test for refractive index (see Tests).
- C. Examine the chromatograms obtained in the test for ethanol, 2-methylbut-2-ene and other related substances. The retention time and size of the principal peak in the chromatogram obtained with test solution (b) are approximately the same as those of the principal peak in the chromatogram obtained with reference solution (a).
- D. Heat 2 ml with 2 g of *potassium hydroxide R* and 20 ml of *alcohol R* under a reflux condenser for 30 min. Allow to cool. Add 15 ml of *dilute sulphuric acid R* and filter. To 1 ml of the filtrate add 1 ml of a 15 g/l solution of *chromotropic acid, sodium salt R*, 2 ml of *water R* and 8 ml of *sulphuric acid R*. A violet colour is produced.
- E. 2 ml of the filtrate obtained in identification test D gives reaction (a) of chlorides (2.3.1).

TESTS

Appearance. It is clear (2.2.1) and colourless (2.2.2, *Method II*).

Acidity. To 50 ml of *methanol R* previously neutralised to 0.1 ml of *bromothymol blue solution R1*, add 50 g of the substance to be examined. Not more than 0.15 ml of 0.1 *M sodium hydroxide* is required to change the colour of the indicator to blue.

Relative density (2.2.5): 1.320 to 1.332.

Refractive index (2.2.6): 1.423 to 1.425.

Ethanol, 2-methylbut-2-ene and other related substances. Examine by gas chromatography (2.2.28).

Test solution (a). The substance to be examined.

Test solution (b). Dilute 0.5 ml of test solution (a) to 100.0 ml with *water R*.

Reference solution (a). Dilute 0.5 ml of *methylene chloride CRS* to 100.0 ml with *water R*.

Reference solution (b). Dilute 2.0 ml of test solution (b) to 10.0 ml with *water R*.

Reference solution (c). To 20.0 ml of *ethanol R*, add 0.3 ml of *2-methylbut-2-ene R* and dilute to 100.0 ml with test solution (a). Dilute 1.0 ml of the solution to 10.0 ml with test solution (a).

Reference solution (d). Dilute 0.1 ml of *methanol R* and 0.1 ml of *methylene chloride CRS* to 100.0 ml with *water R*.

The chromatographic procedure may be carried out using:

- a glass column 2 m long and 2 mm in internal diameter, packed with *ethylvinylbenzene-divinylbenzene copolymer R* (136 µm to 173 µm),
- *nitrogen for chromatography R* as the carrier gas at a flow rate of 30 ml/min,
- a flame-ionisation detector,

maintaining the temperature of the column at 90 °C until injection, then raising the temperature at a rate of 4 °C per minute to 190 °C and maintaining at 190 °C for 15 min and maintaining the temperature of the injection port and the detector at 240 °C.

Inject 1 µl of reference solution (d). If a recorder is used, adjust the sensitivity of the detector such that the height of the peak due to methanol is not less than 25 per cent of the full scale of the recorder. The test is not valid unless: in the chromatogram obtained with reference solution (d), the resolution between the peaks corresponding to methanol and methylene chloride is at least 3.0.

Inject twice 2 µl of reference solution (c). If the peaks obtained show an area difference greater than 1.0 per cent, verify the repeatability by making four separate injections of reference solution (c); the test is not valid unless the relative standard deviation of the peak area is not more than 5.0 per cent.

Inject 2 µl of test solution (a), 2 µl of reference solution (b) and 2 µl of reference solution (c). In the chromatogram obtained with test solution (a): the areas of any peaks corresponding to ethanol and 2-methylbut-2-ene respectively are not greater than the difference between the areas of the peaks due to ethanol and 2-methylbut-2-ene in the chromatogram obtained with reference solution (c) and those of the peaks due to ethanol and 2-methylbut-2-ene in the chromatogram obtained with test solution (a) (2.0 per cent and 0.03 per cent, respectively).

In the chromatogram obtained with test solution (a), the sum of the areas of any peaks apart from the principal peak and any peaks due to ethanol and 2-methylbut-2-ene,

is not greater than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent).

Free chlorine. Place 5 ml in a ground-glass-stoppered tube. Add 5 ml of a 100 g/l solution of *potassium iodide R* and 0.2 g of *soluble starch R*. Shake for 30 s and allow to stand for 5 min. No blue colour develops.

Heavy metals (2.4.8). Evaporate 25.0 g to dryness on a water-bath. Allow to cool. Add 1 ml of *hydrochloric acid R* and evaporate again. Dissolve the residue in 1 ml of *acetic acid R* and dilute to 25 ml with *water R*. 12 ml of the solution complies with limit test A for heavy metals (1 ppm). Prepare the standard using 10 ml of *lead standard solution (1 ppm Pb) R*.

Residue on evaporation. Evaporate 50.0 g to dryness on a water-bath and dry at 100 °C to 105 °C for 30 min. The residue weighs not more than 1 mg (20 ppm).

Water (2.5.12). Not more than 0.05 per cent *m/m*, determined on 10.00 g by the semi-micro determination of water.

STORAGE

Store in an airtight container, protected from light.

LABELLING

The label states the name and the concentration of any stabilisers.

IMPURITIES

- A. carbon tetrachloride,
- B. chloroform,
- C. ethanol,
- D. methanol,
- E. 2-methylbut-2-ene.

01/2005:0346

METHYLHYDROXYETHYLCELLULOSE

Methylhydroxyethylcellulosum

DEFINITION

Methylhydroxyethylcellulose is a partly *O*-methylated and *O*-(2-hydroxyethylated) cellulose.

CHARACTERS

A white, yellowish-white or greyish-white powder or granules, hygroscopic after drying, practically insoluble in hot water, in acetone, in ethanol and in toluene. It dissolves in cold water giving a colloidal solution.

IDENTIFICATION

- A. Heat 10 ml of solution S (see Tests) in a water-bath while stirring. At a temperature above 50 °C, the solution becomes cloudy or a flocculent precipitate is formed. The solution becomes clear again on cooling.
- B. To 10 ml of solution S add 0.3 ml of *dilute acetic acid R* and 2.5 ml of a 100 g/l solution of *tannic acid R*. A yellowish-white, flocculent precipitate is formed which dissolves in *dilute ammonia R1*.
- C. In a test-tube about 160 mm long, thoroughly mix 1 g with 2 g of finely powdered *manganese sulphate R*. Introduce to a depth of 2 cm into the upper part of the tube a strip of filter paper impregnated with a freshly prepared mixture of 1 volume of a 20 per cent *V/V* solution of *diethanolamine R* and 11 volumes of a 50 g/l solution

01/2005:0189

of *sodium nitroprusside R*, adjusted to about pH 9.8 with *1 M hydrochloric acid*. Insert the tube 8 cm into a silicone-oil bath at 190 °C to 200 °C. The filter paper becomes blue within 10 min. Carry out a blank test.

D. Dissolve 0.2 g completely, without heating, in 15 ml of a 70 per cent *m/m* solution of *sulphuric acid R*. Pour the solution with stirring into 100 ml of iced *water R* and dilute to 250 ml with iced *water R*. In a test-tube, mix thoroughly, while cooling in iced water, 1 ml of the solution with 8 ml of *sulphuric acid R*, added dropwise. Heat in a water-bath for exactly 3 min and immediately cool in iced water. While the mixture is cold, carefully add 0.6 ml of *ninhydrin solution R2* and mix well. Allow to stand at 25 °C. A pink colour is produced immediately and does not become violet within 100 min.

E. Place 1 ml of solution S on a glass plate. After evaporation of the water a thin film is formed.

TESTS

Solution S. While stirring, introduce a quantity of the substance to be examined equivalent to 1.0 g of the dried substance into 50 g of *carbon dioxide-free water R* heated to 90 °C. Allow to cool, adjust the mass of the solution to 100 g with *carbon dioxide-free water R* and stir until dissolution is complete.

Appearance of solution. Solution S is not more opalescent than reference suspension III (2.2.1) and not more intensely coloured than reference solution Y₆ (2.2.2, *Method II*).

pH (2.2.3). The pH of solution S is 5.5 to 8.0.

Apparent viscosity. While stirring, introduce a quantity of the substance to be examined equivalent to 6.00 g of the dried substance into 150 g of *water R* heated to 90 °C. Stir with a propeller-type stirrer for 10 min, place the flask in a bath of iced water, continue the stirring and allow to remain in the bath of iced water for 40 min to ensure that dissolution is complete. Adjust the mass of the solution to 300 g and centrifuge the solution to expel any entrapped air. Adjust the temperature of the solution to 20 ± 0.1 °C. Determine the viscosity (2.2.10) with a rotating viscometer at 20 °C and a shear rate of 10 s⁻¹. The apparent viscosity is not less than 75 per cent and not more than 140 per cent of the value stated on the label.

Chlorides (2.4.4). 1 ml of solution S diluted to 15 ml with *water R* complies with the limit test for chlorides (0.5 per cent).

Heavy metals (2.4.8). 1.0 g complies with limit test C for heavy metals (20 ppm). Prepare the standard using 2 ml of *lead standard solution* (10 ppm Pb) *R*.

Loss on drying (2.2.32). Not more than 10.0 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C.

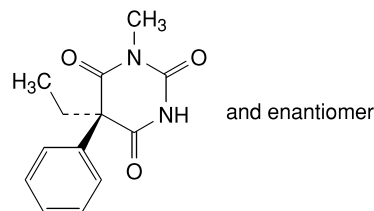
Sulphated ash (2.4.14). Not more than 1.0 per cent, determined on 1.000 g.

LABELLING

The label states the apparent viscosity in millipascal seconds for a 2 per cent *m/m* solution.

METHYLPHENOBARBITAL

Methylphenobarbitalum

C₁₃H₁₄N₂O₃M_r 246.3

DEFINITION

Methylphenobarbital contains not less than 99.0 per cent and not more than the equivalent of 102.0 per cent of (5*RS*)-5-ethyl-1-methyl-5-phenylpyrimidine-2,4,6-(1*H*,3*H*,5*H*)-trione, calculated with reference to the dried substance.

CHARACTERS

A white, crystalline powder or colourless crystals, practically insoluble in water, very slightly soluble in ethanol. It forms water-soluble compounds with alkali hydroxides and carbonates and with ammonia.

IDENTIFICATION

First identification: A, B.

Second identification: A, C, D.

- Determine the melting point (2.2.14) of the substance to be examined. Mix equal parts of the substance to be examined and *methylphenobarbital CRS* and determine the melting point of the mixture. The difference between the two melting points (which are about 178 °C) is not greater than 2 °C.
- Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *methylphenobarbital CRS*.
- Examine by thin-layer chromatography (2.2.27), using *silica gel GF₂₅₄ R* as the coating substance.

Test solution. Dissolve 0.1 g of the substance to be examined in *chloroform R* and dilute to 100 ml with the same solvent.

Reference solution. Dissolve 0.1 g of *methylphenobarbital CRS* in *chloroform R* and dilute to 100 ml with the same solvent.

Apply separately to the plate 10 µl of each solution. Develop over a path of 18 cm using the lower layer of a mixture of 5 volumes of *concentrated ammonia R*, 15 volumes of *alcohol R* and 80 volumes of *chloroform R*. Examine immediately in ultraviolet light at 254 nm. The principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with the reference solution.

- To about 10 mg add 0.2 ml of *sulphuric acid R* and 0.1 ml of *nitric acid R*. Heat on a water-bath for 10 min. Cool in iced water and add 5 ml of *water R* and 5 ml of *strong sodium hydroxide solution R*. Add 5 ml of *acetone R*, shake and allow to stand. A dark-red colour develops in the upper layer.

TESTS

Appearance of solution. Dissolve 1.0 g, with gentle heating, in a mixture of 4 ml of *dilute sodium hydroxide solution R* and 6 ml of *water R*. The solution is clear (2.2.1) and not more intensely coloured than reference solution Y₆ (2.2.2, *Method II*).

Acidity. Boil 1.0 g with 50 ml of *water R* for 2 min, allow to cool and filter. To 10 ml of the filtrate add 0.15 ml of *methyl red solution R*. The solution is orange-yellow. Not more than 0.1 ml of 0.1 M *sodium hydroxide* is required to produce a pure yellow colour.

Related substances. Examine by thin-layer chromatography (2.2.27), using *silica gel F₂₅₄ R* as the coating substance.

Test solution. Dissolve 1.0 g of the substance to be examined in *chloroform R* and dilute to 100 ml with the same solvent.

Reference solution. Dissolve 0.1 g of *phenobarbital CRS* in *chloroform R* and dilute to 100 ml with the same solvent. Dilute 10 ml of the solution to 100 ml with *chloroform R*.

Apply separately to the plate 20 µl of each solution. Develop over a path of 15 cm using the lower layer of a mixture of 5 volumes of *concentrated ammonia R*, 15 volumes of *alcohol R* and 80 volumes of *chloroform R*. Examine immediately in ultraviolet light at 254 nm. Spray with *diphenylcarbazone mercuric reagent R*. Allow the plate to dry in air and spray with freshly prepared *alcoholic potassium hydroxide solution R* diluted 1 in 5 with *aldehyde-free alcohol R*. Heat at 100 °C to 105 °C for 5 min and examine immediately. When examined in ultraviolet light and after spraying, any spot in the chromatogram obtained with the test solution, apart from the principal spot, is not more intense than the spot in the chromatogram obtained with the reference solution (1.0 per cent).

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.00 g by drying in an oven at 100 °C to 105 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

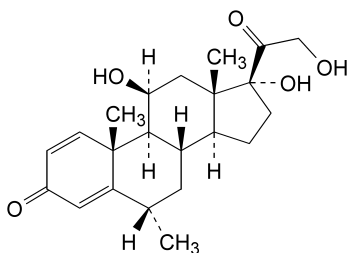
Dissolve 0.200 g in 5 ml of *pyridine R*. Add 0.5 ml of *thymolphthalein solution R* and 10 ml of *silver nitrate solution in pyridine R*. Titrate with 0.1 M *ethanolic sodium hydroxide* until a pure blue colour is obtained. Carry out a blank titration.

1 ml of 0.1 M *ethanolic sodium hydroxide* is equivalent to 24.63 mg of C₂₂H₃₀N₂O₅.

01/2005:0561

METHYLPREDNISOLONE

Methylprednisolonum

C₂₂H₃₀O₅M_r 374.5

DEFINITION

Methylprednisolone contains not less than 97.0 per cent and not more than the equivalent of 103.0 per cent of 11β,17,21-trihydroxy-6α-methylpregna-1,4-diene-3,20-dione, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, crystalline powder, practically insoluble in water, sparingly soluble in alcohol, slightly soluble in acetone and in methylene chloride.

It shows polymorphism.

IDENTIFICATION

First identification: A, B.

Second identification: C, D.

A. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *methylprednisolone CRS*. If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in the minimum volume of *acetone R*, evaporate to dryness on a water-bath and record new spectra using the residues.

B. Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel F₂₅₄ plate R*.

Test solution. Dissolve 10 mg of the substance to be examined in a mixture of 1 volume of *methanol R* and 9 volumes of *methylene chloride R* and dilute to 10 ml with the same mixture of solvents.

Reference solution (a). Dissolve 20 mg of *methylprednisolone CRS* in a mixture of 1 volume of *methanol R* and 9 volumes of *methylene chloride R* and dilute to 20 ml with the same mixture of solvents.

Reference solution (b). Dissolve 10 mg of *hydrocortisone CRS* in reference solution (a) and dilute to 10 ml with reference solution (a).

Apply to the plate 5 µl of each solution. Prepare the mobile phase by adding a mixture of 1.2 volumes of *water R* and 8 volumes of *methanol R* to a mixture of 15 volumes of *ether R* and 77 volumes of *methylene chloride R*. Develop over a path of 15 cm. Carry out a second development over a path of 15 cm using a mixture of 5 volumes of *butanol R* saturated with *water R*, 15 volumes of *toluene R* and 80 volumes of *ether R*. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. The principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a). Spray with *alcoholic solution of sulphuric acid R*. Heat at 120 °C for 10 min or until the spots appear. Allow to cool. Examine the chromatograms in daylight and in ultraviolet light at 365 nm. The principal spot in the chromatogram obtained with the test solution is similar in position, colour in daylight, fluorescence in ultraviolet light at 365 nm and size to the principal spot in the chromatogram obtained with reference solution (a). The test is not valid unless the chromatogram obtained with reference solution (b) shows two clearly separated spots.

C. Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel F₂₅₄ plate R*.

Test solution (a). Dissolve 25 mg of the substance to be examined in *methanol R* and dilute to 5 ml with the same solvent. This solution is also used to prepare test solution (b). Dilute 2 ml of the solution to 10 ml with *methylene chloride R*.

Test solution (b). Transfer 0.4 ml of the solution obtained during the preparation of test solution (a) to a glass tube 100 mm long and 20 mm in diameter and fitted with a ground-glass stopper or a polytetrafluoroethylene cap and evaporate the solvent with gentle heating under a stream of *nitrogen R*. Add 2 ml of a 15 per cent *V/V* solution of *glacial acetic acid R* and 50 mg of *sodium bismuthate R*. Stopper the tube and shake the suspension in a mechanical shaker protected from light for 1 h. Add 2 ml of a 15 per cent *V/V* solution of *glacial acetic acid R* and filter into a 50 ml separating funnel, washing the filter with two quantities, each of 5 ml, of *water R*. Shake the clear filtrate with 10 ml of *methylene chloride R*. Wash the organic layer with 5 ml of 1 *M sodium hydroxide* and two quantities, each of 5 ml, of *water R*. Dry over *anhydrous sodium sulphate R*.

Reference solution (a). Dissolve 25 mg of *methylprednisolone CRS* in *methanol R* and dilute to 5 ml with the same solvent. This solution is also used to prepare reference solution (b). Dilute 2 ml of the solution to 10 ml with *methylene chloride R*.

Reference solution (b). Transfer 0.4 ml of the solution obtained during preparation of reference solution (a) to a glass tube 100 mm long and 20 mm in diameter and fitted with a ground-glass stopper or a polytetrafluoroethylene cap and evaporate the solvent with gentle heating under a stream of *nitrogen R*. Add 2 ml of a 15 per cent *V/V* solution of *glacial acetic acid R* and 50 mg of *sodium bismuthate R*. Stopper the tube and shake the suspension in a mechanical shaker protected from light for 1 h. Add 2 ml of a 15 per cent *V/V* solution of *glacial acetic acid R* and filter into a 50 ml separating funnel, washing the filter with two quantities, each of 5 ml, of *water R*. Shake the clear filtrate with 10 ml of *methylene chloride R*. Wash the organic layer with 5 ml of 1 *M sodium hydroxide* and two quantities, each of 5 ml, of *water R*. Dry over *anhydrous sodium sulphate R*.

Apply to the plate 5 µl of test solution (a), 5 µl of reference solution (a), 10 µl of test solution (b) and 10 µl of reference solution (b), applying the latter two in small quantities in order to obtain small spots. Develop over a path of 15 cm using a mixture of 5 volumes of *butanol R* saturated with *water R*, 10 volumes of *toluene R* and 85 volumes of *ether R*. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. The principal spot in each of the chromatograms obtained with the test solutions is similar in position and size to the principal spot in the chromatogram obtained with the corresponding reference solution. Spray with *alcoholic solution of sulphuric acid R* and heat at 120 °C for 15 min. Allow to cool. Examine the plate in daylight and in ultraviolet light at 365 nm. The principal spot in each of the chromatograms obtained with the test solutions is similar in position, colour in daylight, fluorescence in ultraviolet light at 365 nm and size to the principal spot in the chromatogram obtained with the corresponding reference solution. The principal spots in the chromatograms obtained with test solution (b) and reference solution (b) have an R_f value distinctly higher than that of the principal spots in the chromatograms obtained with test solution (a) and reference solution (a).

- D. Add about 2 mg to 2 ml of *sulphuric acid R* and shake to dissolve. Within 5 min, an intense red colour develops. When examined in ultraviolet light at 365 nm, brownish-red fluorescence is seen. Add the solution to 10 ml of *water R* and mix. The colour fades and there is a yellowish-green fluorescence in ultraviolet light at 365 nm.

TESTS

Specific optical rotation (2.2.7). Dissolve 0.250 g in *dioxan R* and dilute to 25.0 ml with the same solvent. The specific optical rotation is + 79.0 to + 86.0, calculated with reference to the dried substance.

Related substances. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 25.0 mg of the substance to be examined in a mixture of equal volumes of *acetonitrile R* and *methanol R* and dilute to 10.0 ml with the same mixture of solvents.

Reference solution (a). Dissolve 2 mg of *methylprednisolone CRS* and 2 mg of *betamethasone CRS* in mobile phase A and dilute to 100.0 ml with the same mobile phase.

Reference solution (b). Dilute 1.0 ml of the test solution to 100.0 ml with mobile phase A.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4.6 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (5 µm),
- as mobile phase at a flow rate of 2.5 ml/min a linear-gradient programme using the following conditions:

Mobile phase A. In a 1000 ml volumetric flask mix 250 ml of *acetonitrile R* with 700 ml of *water R* and allow to equilibrate; adjust the volume to 1000 ml with *water R* and mix again,

Mobile phase B. *Acetonitrile R*,

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)	Comment
0	100	0	isocratic
15	100	0	begin linear gradient
40	0	100	end chromatogram, return to 100 A
41	100	0	begin equilibration with A
46 = 0	100	0	end equilibration, begin next chromatogram

- as detector a spectrophotometer set at 254 nm, maintaining the temperature of the column at 45 °C.

Equilibrate the column with mobile phase B at a flow rate of 2.5 ml/min for at least 30 min and then with mobile phase A for 5 min. For subsequent chromatograms, use the conditions described from 40 min to 46 min. Adjust the sensitivity of the system so that the height of the principal peak in the chromatogram obtained with 20 µl of reference solution (b) is at least 50 per cent of the full scale of the recorder.

Inject 20 µl of reference solution (a). When the chromatograms are recorded in the conditions described above, the retention times are: *methylprednisolone* about 11.5 min and *betamethasone* about 12.5 min. The test is not valid unless the resolution between the peaks corresponding to *methylprednisolone* and *betamethasone* is at least 1.5; if necessary, adjust the concentration of *acetonitrile* in mobile phase A.

Inject separately 20 µl of a mixture of equal volumes of *acetonitrile R* and *methanol R* as a blank, 20 µl of the test solution and 20 µl of reference solution (b). In the chromatogram obtained with the test solution: the area of any peak, apart from the principal peak, is not greater than half the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent); the sum of the areas of all the peaks, apart from the principal peak, is

not greater than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (2 per cent). Disregard any peak due to the blank and any peak with an area less than 0.05 times the area of the principal peak in the chromatogram obtained with reference solution (b).

Loss on drying (2.2.32). Not more than 1.0 per cent, determined on 0.500 g by drying in an oven at 100 °C to 105 °C.

ASSAY

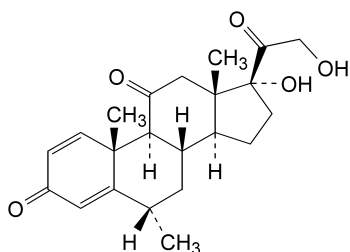
Dissolve 0.100 g in *alcohol R* and dilute to 100.0 ml with the same solvent. Dilute 2.0 ml of the solution to 100.0 ml with *alcohol R*. Measure the absorbance (2.2.25) at the maximum at 243 nm.

Calculate the content of $C_{22}H_{30}O_5$ taking the specific absorbance to be 395.

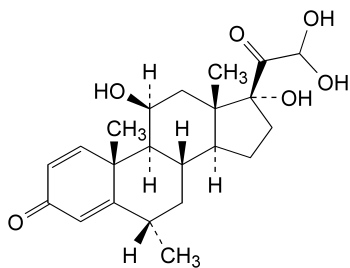
STORAGE

Store protected from light.

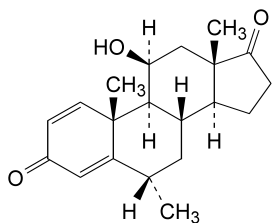
IMPURITIES



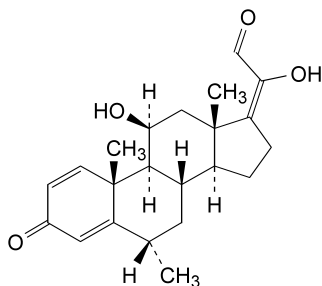
A. 17,21-dihydroxy-6α-methylpregna-1,4-diene-3,11,20-trione,



B. 11β,17,21,21-tetrahydroxy-6α-methylpregna-1,4-diene-3,20-dione,



C. 11β-hydroxy-6α-methylandrosta-1,4-diene-3,17-dione,



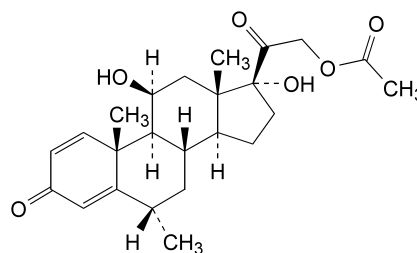
and (Z)-isomer

D. (E)- and (Z)-11β,20-dihydroxy-6α-methylpregna-1,4,17(20)-triene-3,21-dione.

01/2005:0933

METHYLPREDNISOLONE ACETATE

Methylprednisoloni acetat



$C_{24}H_{32}O_6$

M_r 416.5

DEFINITION

Methylprednisolone acetate contains not less than 97.0 per cent and not more than the equivalent of 103.0 per cent of 11β,17-dihydroxy-6α-methyl-3,20-dioxopregna-1,4-dien-21-yl acetate, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, crystalline powder, practically insoluble in water, sparingly soluble in acetone and in alcohol.

IDENTIFICATION

First identification: A, B.

Second identification: C, D, E.

A. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *methylprednisolone acetate CRS*. If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in the minimum volume of *acetone R*, evaporate to dryness and record new spectra using the residues.

B. Examine by thin-layer chromatography (2.2.27), using *silica gel GF₂₅₄ R* as the coating substance.

Test solution. Dissolve 10 mg of the substance to be examined in a mixture of 1 volume of *methanol R* and 9 volumes of *methylene chloride R* and dilute to 10 ml with the same mixture of solvents.

Reference solution (a). Dissolve 10 mg of *methylprednisolone acetate CRS* in a mixture of 1 volume of *methanol R* and 9 volumes of *methylene chloride R* and dilute to 10 ml with the same mixture of solvents.

Reference solution (b). Dissolve 10 mg of *prednisolone acetate CRS* and 10 mg of *methylprednisolone acetate CRS* in a mixture of 1 volume of *methanol R* and 9 volumes of *methylene chloride R* and dilute to 10 ml with the same mixture of solvents.

Apply separately to the plate 5 µl of each solution. Develop over a path of 15 cm using a mixture of 5 volumes of *butanol R*, 10 volumes of *toluene R* and 85 volumes of *ether R*. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. The principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a). Spray the plate with *alcoholic solution of sulphuric acid R*. Heat at 120 °C for 10 min or until the spots appear. Allow to cool. Examine in daylight and in ultraviolet light at 365 nm. The principal spot in the chromatogram obtained with the test solution is similar in position, colour in

daylight, fluorescence in ultraviolet light at 365 nm and size to the principal spot in the chromatogram obtained with reference solution (a). The test is not valid unless the chromatogram obtained with reference solution (b) shows two spots which, when examined in ultraviolet light at 365 nm, may not be completely separated.

- C. Examine by thin-layer chromatography (2.2.27), using *silica gel GF₂₅₄* R as the coating substance.

Test solution (a). Dissolve 25 mg of the substance to be examined in *methanol* R and dilute to 5 ml with the same solvent. This solution is also used to prepare test solution (b). Dilute 2 ml of the solution to 10 ml with *methylene chloride* R.

Test solution (b). Transfer 2 ml of the solution obtained during preparation of test solution (a) to a 15 ml glass tube with a ground-glass stopper or a polytetrafluoroethylene cap. Add 10 ml of *saturated methanolic potassium hydrogen carbonate solution* R and immediately pass a current of *nitrogen* R through the solution for 5 min. Stopper the tube. Heat in a water-bath at 45 °C protected from light for 1 h. Allow to cool.

Reference solution (a). Dissolve 25 mg of *methylprednisolone acetate* CRS in *methanol* R and dilute to 5 ml with the same solvent. This solution is also used to prepare reference solution (b). Dilute 2 ml of the solution to 10 ml with *methylene chloride* R.

Reference solution (b). Transfer 2 ml of the solution obtained during preparation of reference solution (a) to a 15 ml glass tube with a ground-glass stopper or a polytetrafluoroethylene cap. Add 10 ml of *saturated methanolic potassium hydrogen carbonate solution* R and immediately pass a current of *nitrogen* R through the solution for 5 min. Stopper the tube. Heat in a water-bath at 45 °C protected from light for 1 h. Allow to cool.

Apply separately to the plate 5 µl of each solution. Prepare the mobile phase by adding a mixture of 1.2 volumes of *water* R and 8 volumes of *methanol* R to a mixture of 15 volumes of *ether* R and 77 volumes of *methylene chloride* R. Develop over a path of 15 cm. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. The principal spot in each of the chromatograms obtained with the test solutions is similar in position and size to the principal spot in the chromatogram obtained with the corresponding reference solution. Spray with *alcoholic solution of sulphuric acid* R. Heat at 120 °C for 10 min or until the spots appear. Allow to cool. Examine in daylight and in ultraviolet light at 365 nm. The principal spot in each of the chromatograms obtained with the test solutions is similar in position, colour in daylight, fluorescence in ultraviolet light at 365 nm and size to the principal spot in the chromatogram obtained with the corresponding reference solution. The principal spots in the chromatograms obtained with test solution (b) and reference solution (b) have an *R_f* value distinctly lower than that of the principal spots in the chromatograms obtained respectively with test solution (a) and reference solution (a).

- D. Add about 2 mg to 2 ml of *sulphuric acid* R and shake to dissolve. Within 5 min, an intense red colour develops. When examined in ultraviolet light at 365 nm, a reddish-brown fluorescence is seen. Add the solution to 10 ml of *water* R and mix. The colour fades and there is a greenish-yellow fluorescence in ultraviolet light at 365 nm.
- E. About 10 mg gives the reaction of acetyl (2.3.1).

TESTS

Specific optical rotation (2.2.7). Dissolve 0.250 g in *dioxan* R and dilute to 25.0 ml with the same solvent. The specific optical rotation is + 97 to + 105, calculated with reference to the dried substance.

Related substances. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 20.0 mg of the substance to be examined in 5 ml of *tetrahydrofuran* R and dilute to 10.0 ml with *water* R.

Reference solution (a). Dissolve 4 mg of *methylprednisolone acetate* CRS and 4 mg of *dexamethasone acetate* CRS in the mobile phase and dilute to 20.0 ml with the mobile phase. Dilute 2.0 ml of this solution to 10.0 ml with the mobile phase.

Reference solution (b). Dilute 1.0 ml of the test solution to 50.0 ml with the mobile phase.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4.6 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography* R (5 µm),
- as mobile phase at a flow rate of 1 ml/min a mixture prepared as follows: in a 1000 ml volumetric flask mix 260 ml *tetrahydrofuran* R and 700 ml of *water* R and leave to equilibrate; adjust the volume to 1000 ml with *water* R and mix again,
- as detector a spectrophotometer set at 254 nm.

Equilibrate the column with the mobile phase at a flow rate of 1 ml/min for about 45 min.

Adjust the sensitivity of the system so that the height of the principal peak in the chromatogram obtained with 20 µl of reference solution (b) is at least 50 per cent of the full scale of the recorder.

Inject 20 µl of reference solution (a). When the chromatograms are recorded in the prescribed conditions, the retention times are: methylprednisolone acetate, about 43 min; dexamethasone acetate, about 57 min. The test is not valid unless the resolution between the peaks corresponding to methylprednisolone acetate and dexamethasone acetate is not less than 6.5. If necessary, adjust the concentration of *water* R in the mobile phase.

Inject separately 20 µl of the test solution and 20 µl of reference solution (b). Continue the chromatography for 1.5 times the retention time of the principal peak. In the chromatogram obtained with the test solution, the sum of the areas of all the peaks, apart from the principal peak, is not greater than half the area of the principal peak in the chromatogram obtained with reference solution (b) (1.0 per cent). Disregard any peak due to the solvent and any peak with an area less than 0.025 times the area of the principal peak in the chromatogram obtained with reference solution (b).

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C.

ASSAY

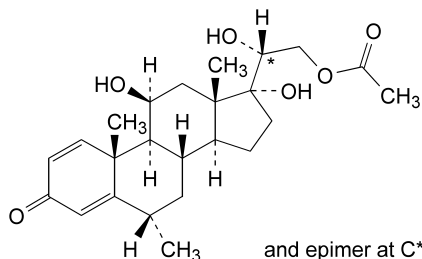
Dissolve 0.100 g in *alcohol* R and dilute to 100.0 ml with the same solvent. Dilute 1.0 ml of the solution to 100.0 ml with *alcohol* R. Measure the absorbance (2.2.25) at the maximum at 243 nm.

Calculate the content of C₂₄H₃₂O₆, taking the specific absorbance to be 355.

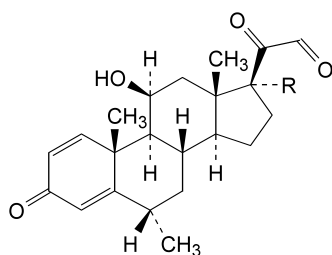
STORAGE

Store protected from light.

IMPURITIES

01/2005:1131
correctedA. (20*RS*)-11β,17,20-trihydroxy-6α-methyl-3-oxopregna-1,4-dien-21-yl acetate,

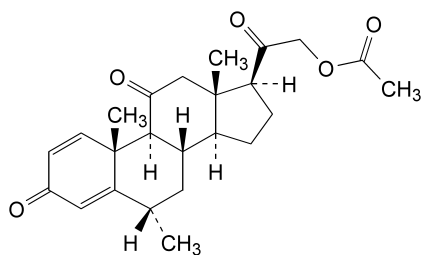
B. methylprednisolone,



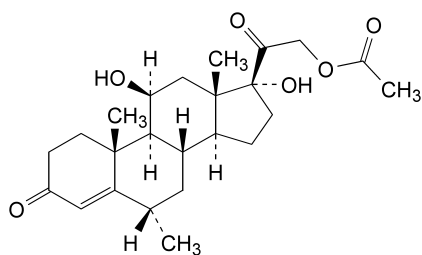
C. R = OH: 11β,17-dihydroxy-6α-methylpregna-1,4-diene-3,20,21-trione,

D. R = H: 11β-hydroxy-6α-methylpregna-1,4-diene-3,20,21-trione,

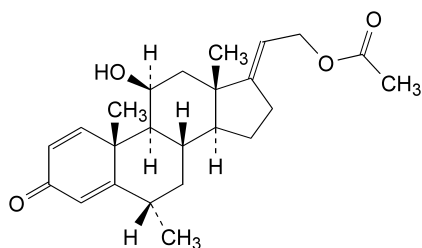
E. prednisolone acetate,



F. 6α-methyl-3,11,20-trioxopregna-1,4-dien-21-yl acetate,



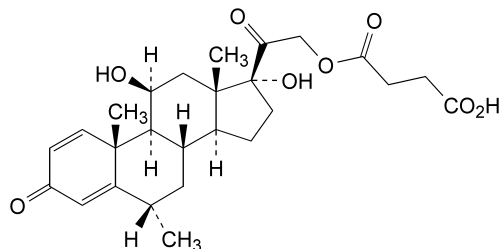
G. 11β,17-dihydroxy-6α-methyl-3,20-dioxopregn-4-en-21-yl acetate,



H. 11β-hydroxy-6α-methyl-3-oxopregna-1,4,17(20)-trien-21-yl acetate.

METHYLPREDNISOLONE HYDROGEN SUCCINATE

Methylprednisoloni hydrogenosuccinas

 $C_{26}H_{34}O_8$ M_r 474.6

DEFINITION

Methylprednisolone hydrogen succinate contains not less than 97.0 per cent and not more than the equivalent of 103.0 per cent of 4-[(11β,17-dihydroxy-6α-methyl-3,20-dioxopregna-1,4-dien-21-yl)oxy]-4-oxobutanoic acid, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, hygroscopic powder, practically insoluble in water, slightly soluble in acetone and in ethanol. It dissolves in dilute solutions of alkali hydroxides.

IDENTIFICATION

First identification: A, B.

Second identification: C, D.

A. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *methylprednisolone hydrogen succinate CRS*.

B. Examine by thin layer chromatography (2.2.27), using a *TLC silica gel F₂₅₄ plate R*.

Test solution. Dissolve 10 mg of the substance to be examined in a mixture of 1 volume of *methanol R* and 9 volumes of *methylene chloride R* and dilute to 10 ml with the same mixture of solvents.

Reference solution (a). Dissolve 20 mg of *methylprednisolone hydrogen succinate CRS* in a mixture of 1 volume of *methanol R* and 9 volumes of *methylene chloride R* and dilute to 20 ml with the same mixture of solvents.

Reference solution (b). Dissolve 10 mg of *hydrocortisone hydrogen succinate CRS* in reference solution (a) and dilute to 10 ml with the same reference solution.

Apply to the plate 10 µl of each solution. Develop over a path of 15 cm using a mixture of 0.1 volumes of *anhydrous formic acid R*, 1 volume of *ethanol R* and 15 volumes of *methylene chloride R*. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. The principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a). Spray the plate with *alcoholic solution of sulphuric acid R*. Heat at 120 °C for 10 min or until the spots appear. Allow to cool. Examine in daylight and in ultraviolet light at 365 nm. The principal spot in the chromatogram obtained with the test solution is similar in position, colour in daylight, fluorescence in ultraviolet light at 365 nm and size to the principal spot in the chromatogram obtained with reference solution (a).

The test is not valid unless the chromatogram obtained with reference solution (b) shows two spots which may, however, not be completely separated.

- C. Examine by thin layer chromatography (2.2.27), using a *TLC silica gel F₂₅₄ plate R*.

Test solution (a). Dissolve 25 mg of the substance to be examined in *methanol R* with gentle heating and dilute to 5 ml with the same solvent. This solution is also used to prepare test solution (b). Dilute 2 ml of the solution to 10 ml with *methylene chloride R*.

Test solution (b). Transfer 2 ml of the solution obtained during the preparation of test solution (a) to a 15 ml glass tube with a ground-glass stopper or a polytetrafluoroethylene cap. Add 10 ml of a 0.8 g/l solution of *sodium hydroxide R* in *methanol R* and immediately pass a stream of *nitrogen R* through the solution for 5 min. Stopper the tube. Heat in a water-bath at 45 °C, protected from light, for 30 min. Allow to cool.

Reference solution (a). Dissolve 25 mg of *methylprednisolone hydrogen succinate CRS* in *methanol R* with gentle heating and dilute to 5 ml with the same solvent. This solution is also used to prepare reference solution (b). Dilute 2 ml of the solution to 10 ml with *methylene chloride R*.

Reference solution (b). Transfer 2 ml of the solution obtained during preparation of reference solution (a) to a 15 ml glass tube with a ground-glass stopper or a polytetrafluoroethylene cap. Add 10 ml of a 0.8 g/l solution of *sodium hydroxide R* in *methanol R* and immediately pass a stream of *nitrogen R* through the solution for 5 min. Stopper the tube. Heat in a water-bath at 45 °C, protected from light, for 30 min. Allow to cool.

Apply to the plate 5 µl of each solution. Prepare the mobile phase by adding a mixture of 1.2 volumes of *water R* and 8 volumes of *methanol R* to a mixture of 15 volumes of *ether R* and 77 volumes of *methylene chloride R*. Develop over a path of 15 cm. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. The principal spot in each of the chromatograms obtained with the test solutions is similar in position and size to the principal spot in the chromatogram obtained with the corresponding reference solution.

Spray the plate with *alcoholic solution of sulphuric acid R*. Heat at 120 °C for 10 min or until the spots appear. Allow to cool. Examine in daylight and in ultraviolet light at 365 nm. The principal spot in each of the chromatograms obtained with the test solutions is similar in position, colour in daylight, fluorescence in ultraviolet light at 365 nm and size to the principal spot in the chromatogram obtained with the corresponding reference solution. The principal spot in each of the chromatograms obtained with test solution (b) and reference solution (b) has an *R_f* value distinctly higher than that of the principal spot in each of the chromatograms obtained with test solution (a) and reference solution (a).

- D. Add about 2 mg to 2 ml of *sulphuric acid R* and shake to dissolve. Within 5 min a reddish-brown colour develops. Add the solution to 10 ml of *water R* and mix. The colour fades and a precipitate is formed.

TESTS

Appearance of solution. Dissolve 0.100 g in 5 ml of *sodium hydrogen carbonate solution R*. The solution is clear (2.2.1).

Specific optical rotation (2.2.7). Dissolve 0.250 g in *dioxan R* and dilute to 25.0 ml with the same solvent. The specific optical rotation is + 87 to + 95, calculated with reference to the dried substance.

Related substances. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 25.0 mg of the substance to be examined in the mobile phase and dilute to 10.0 ml with the mobile phase.

Reference solution (a). Dissolve 25 mg of *methylprednisolone hydrogen succinate for performance test CRS* in the mobile phase and dilute to 10.0 ml with the mobile phase.

Reference solution (b). Dilute 1.0 ml of the test solution to 100.0 ml with the mobile phase.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4.0 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (5 µm),
- as mobile phase at a flow rate of 1 ml/min a mixture of 33 volumes of *acetonitrile R* and 67 volumes of a 3 per cent *V/V* solution of *glacial acetic acid R*,
- as detector a spectrophotometer set at 254 nm.

Equilibrate the column with the mobile phase at a flow rate of 1 ml/min for about 30 min.

Adjust the sensitivity of the system so that the height of the principal peak in the chromatogram obtained with 20 µl of reference solution (b) is at least 50 per cent of the full scale of the recorder.

Inject 20 µl of reference solution (a). When the chromatograms are recorded in the prescribed conditions, the retention times are: methylprednisolone hydrogen succinate about 22 min and methylhydrocortisone 21-(hydrogen succinate) (the impurity eluting immediately after the main peak and appearing as a shoulder) about 24 min. Measure the height (*A*) above the base-line of the peak due to methylhydrocortisone 21-(hydrogen succinate) and the height (*B*) above the base-line of the lowest point of the curve separating this peak from the peak due to methylprednisolone hydrogen succinate. The test is not valid unless *A* is greater than four times *B*. If necessary, adjust the concentration of acetonitrile in the mobile phase.

Inject separately 20 µl of the test solution and 20 µl of reference solution (b). Continue the chromatography for twice the retention time of the principal peak. In the chromatogram obtained with the test solution: the area of any peak, apart from the principal peak, is not greater than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent); the sum of the areas of all the peaks, apart from the principal peak, is not greater than the area of the principal peak in the chromatogram obtained with reference solution (b) (1 per cent). Disregard any peak due to the solvent and any peak with an area less than 0.05 times the area of the principal peak in the chromatogram obtained with reference solution (b).

Loss on drying (2.2.32). Not more than 1.0 per cent, determined on 1.000 g by drying in an oven at 100-105 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 50.0 mg in *alcohol R* and dilute to 100.0 ml with the same solvent. Dilute 2.0 ml of the solution to 50.0 ml with *alcohol R*. Measure the absorbance (2.2.25) at the maximum at 243 nm.

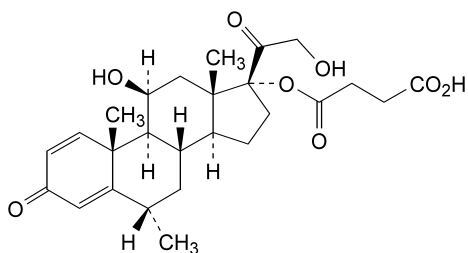
Calculate the content of $C_{26}H_{34}O_8$ taking the specific absorbance to be 316.

STORAGE

Store in an airtight container, protected from light.

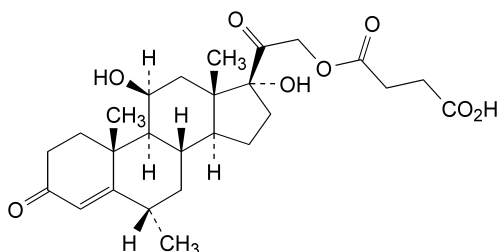
IMPURITIES

A. methylprednisolone,



B. 4-[(11β,21-dihydroxy-6α-methyl-3,20-dioxopregna-1,4-dien-17-yl)oxy]-4-oxobutanoic acid (methylprednisolone 17-(hydrogen succinate)),

C. methylprednisolone acetate,

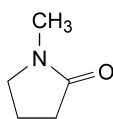


D. 4-[(11β,17-dihydroxy-6α-methyl-3,20-dioxopregn-4-en-21-yl)oxy]-4-oxobutanoic acid (methylhydrocortisone 21-(hydrogen succinate)).

01/2005:1675

N-METHYLPYRROLIDONE

N-Methylpyrrolidonum



C_5H_9NO

M_r 99.1

DEFINITION

1-Methylpyrrolidin-2-one.

CHARACTERS

Appearance: clear, colourless liquid.

Solubility: miscible with water and with alcohol.

bp: about 204 °C.

Relative density: about 1.034.

Refractive index: about 1.469.

IDENTIFICATION

Infrared absorption spectrophotometry (2.2.24).

Preparation: films.

Comparison: Ph. Eur. reference spectrum of N-methylpyrrolidone.

TESTS

Appearance. The substance to be examined is clear (2.2.1) and colourless (2.2.2, *Method II*).

Alkalinity. Dissolve 50 ml of the substance to be examined in 50 ml of *water R* previously adjusted with 0.02 M *potassium hydroxide* or 0.02 M *hydrochloric acid* until a yellow colour is obtained using 0.5 ml of *bromothymol blue solution RI* as indicator. Titrate with 0.02 M *hydrochloric acid* to the initial coloration. Not more than 8.0 ml of 0.02 M *hydrochloric acid* is required.

Related substances. Gas chromatography (2.2.28): use the normalisation procedure.

Test solution. The substance to be examined.

Reference solution. To 1 ml of the substance to be examined, add 1 ml of 2-pyrrolidone *R* and dilute to 20 ml with *methylene chloride R*.

Column:

- *material*: fused silica,
- *size*: $l = 30$ m, $\varnothing = 0.32$ mm,
- *stationary phase*: *poly(dimethyl)siloxane R* (5 µm).

Carrier gas: nitrogen for chromatography *R*.

Linear velocity: 20 cm/s.

Split ratio: 1:100.

Temperature:

	Time (min)	Temperature (°C)
Column	0	100
	0 - 23.3	100 → 170
	23.3 - 53	170
Injection port		280
Detector		280

Detection: flame ionisation.

Injection: 1 µl.

System suitability: reference solution:

- *resolution*: minimum 2.0 between the peaks due to N-methylpyrrolidone and impurity G.

Limits:

- *any impurity*: maximum 0.1 per cent,
- *total*: maximum 0.3 per cent,
- *disregard limit*: 0.02 per cent.

Heavy metals (2.4.8): maximum 10 ppm.

Dissolve 4.0 g in *water R* and dilute to 20.0 ml with the same solvent. 12 ml of the solution complies with limit test A. Prepare the standard using *lead standard solution (2 ppm Pb) R*.

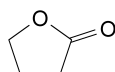
Water (2.5.32): maximum 0.1 per cent, determined on 1.000 g.

STORAGE

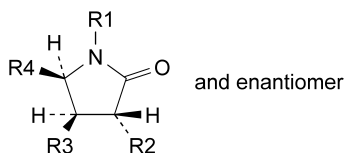
Protected from light.

IMPURITIES

A. H_3C-NH_2 : methanamine (methylaniline),



B. dihydrofuran-2(3H)-one (γ-butyrolactone),



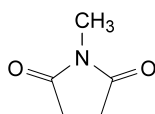
C. R1 = R2 = CH₃, R3 = R4 = H: (3*RS*)-1,3-dimethylpyrrolidin-2-one,

D. R1 = R3 = CH₃, R2 = R4 = H: (4*RS*)-1,4-dimethylpyrrolidin-2-one,

E. R1 = R4 = CH₃, R2 = R3 = H: (5*RS*)-1,5-dimethylpyrrolidin-2-one,

G. R1 = R2 = R3 = R4 = H: pyrrolidin-2-one (2-pyrrolidone),

F. HO[CH₂]₄-OH: butane-1,4-diol,



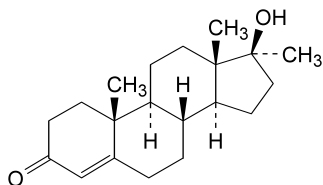
H. 1-methylpyrrolidine-2,5-dione (*N*-methylsuccinimide),

I. propylene glycol.

01/2005:0410

METHYLTESTOSTERONE

Methyltestosteronum



C₂₀H₃₀O₂

*M*_r 302.5

DEFINITION

Methyltestosterone contains not less than 97.0 per cent and not more than the equivalent of 103.0 per cent of 17β-hydroxy-17α-methylandroster-4-en-3-one, calculated with reference to the dried substance.

CHARACTERS

A white or slightly yellowish-white, crystalline powder, practically insoluble in water, freely soluble in alcohol.

IDENTIFICATION

First identification: B.

Second identification: A, C.

A. Melting point (2.2.14): 162 °C to 168 °C.

B. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *methyltestosterone CRS*.

C. After examination of the chromatograms obtained in the test for related substances, spray the plate with a saturated solution of *potassium dichromate R* in a

mixture of 30 volumes of *water R* and 70 volumes of *sulphuric acid R* and examine immediately in daylight. The principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).

TESTS

Specific optical rotation (2.2.7). Dissolve 0.250 g in *alcohol R* and dilute to 25.0 ml with the same solvent. The specific optical rotation is + 79 to + 85, calculated with reference to the dried substance.

Related substances. Examine by thin-layer chromatography (2.2.27), using as the coating substance a suitable silica gel containing a fluorescent indicator having an optimal intensity at 254 nm.

Test solution. Dissolve 0.2 g of the substance to be examined in a mixture of 1 volume of *methanol R* and 9 volumes of *chloroform R* and dilute to 10 ml with the same mixture of solvents.

Reference solution (a). Dissolve 20 mg of *methyltestosterone CRS* in 1 ml of a mixture of 1 volume of *methanol R* and 9 volumes of *chloroform R*.

Reference solution (b). Dilute 1 ml of the test solution to 100 ml with a mixture of 1 volume of *methanol R* and 9 volumes of *chloroform R*.

Reference solution (c). Dilute 5 ml of reference solution (b) to 10 ml with a mixture of 1 volume of *methanol R* and 9 volumes of *chloroform R*.

Reference solution (d). Dissolve 10 mg of *testosterone CRS* in 0.5 ml of reference solution (a) and dilute to 10 ml with a mixture of 1 volume of *methanol R* and 9 volumes of *chloroform R*.

Apply separately to the plate 5 µl of each solution. Develop over a path of 15 cm using a mixture of 1 volume of *anhydrous acetic acid R*, 30 volumes of *light petroleum R* and 70 volumes of *butyl acetate R*. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. Any spot in the chromatogram obtained with the test solution, apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (b) (1.0 per cent) and at most one such spot is more intense than the spot in the chromatogram obtained with reference solution (c) (0.5 per cent). The test is not valid unless the chromatogram obtained with reference solution (d) shows two clearly separated spots.

Loss on drying (2.2.32). Not more than 2.0 per cent, determined on 0.50 g by drying in an oven at 100 °C to 105 °C for 2 h.

ASSAY

Dissolve 50.0 mg in *alcohol R* and dilute to 50.0 ml with the same solvent. Dilute 10.0 ml of the solution to 100.0 ml with *alcohol R*. Dilute 10.0 ml of this solution to 100.0 ml with *alcohol R*. Measure the absorbance (2.2.25) at the maximum at 241 nm.

Calculate the content of C₂₀H₃₀O₂ taking the specific absorbance to be 540.

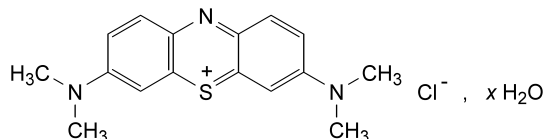
STORAGE

Store protected from light.

01/2005:1132
corrected

METHYLTHIONINIUM CHLORIDE

Methylthioninii chloridum

 $C_{16}H_{18}ClN_3S \cdot xH_2O$ M_r 319.9 (anhydrous substance)

DEFINITION

Methylthioninium chloride (methylene blue) contains not less than 95.0 per cent and not more than the equivalent of 101.0 per cent of 3,7-bis(dimethylamino)phenothiazin-5-ylum chloride, calculated with reference to the dried substance.

CHARACTERS

A dark blue, crystalline powder with a copper-coloured sheen, or green crystals with a bronze-coloured sheen, soluble in water, slightly soluble in alcohol.

IDENTIFICATION

- A. Dissolve 10 mg in *dilute hydrochloric acid R* and dilute to 100 ml with the same acid. Dilute 5 ml of the solution to 100 ml with *dilute hydrochloric acid R*. Examined between 240 nm and 800 nm (2.2.25), the solution shows four absorption maxima, at 255 nm to 260 nm, 285 nm to 290 nm, 675 nm to 685 nm and 740 nm to 750 nm.
- B. Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel plate R*.

Test solution. Dissolve 10 mg of the substance to be examined in *methanol R* and dilute to 10 ml with the same solvent. Dilute 1 ml to 10 ml with *methanol R*.

Reference solution. Dissolve 10 mg of *methylthioninium chloride CRS* in *methanol R* and dilute to 10 ml with the same solvent. Dilute 1 ml to 10 ml with *methanol R*.

Apply to the plate 2 µl of each solution. Develop over a path of 8 cm using a mixture of 20 volumes of *anhydrous formic acid R* and 80 volumes of *propanol R*. Allow the plate to dry in air protected from light. Examine in daylight. The principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with the reference solution. A secondary spot may appear above the principal spot in both chromatograms.

- C. Dissolve about 1 mg in 10 ml of *water R*. Add 1 ml of *glacial acetic acid R* and 0.1 g of *zinc powder R*. Heat to boiling. The solution becomes colourless. Filter and shake the filtrate. It becomes blue in contact with air.
- D. Ignite 50 mg with 0.5 g of *anhydrous sodium carbonate R*. Cool and dissolve the residue in 10 ml of *dilute nitric acid R*. Filter. The filtrate, without further addition of *dilute nitric acid R*, gives reaction (a) of chlorides (2.3.1).

TESTS

Methanol-insoluble substances. To 1.0 g add 20 ml of *methanol R* and boil under a reflux condenser for 5 min. Filter through a tared sintered-glass filter (40) and wash the filter with *methanol R* until a colourless filtrate is obtained. Dry the filter at 100 °C and weigh. The residue weighs not more than 10.0 mg (1.0 per cent).

Related substances. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 15.0 mg of the substance to be examined in the mobile phase and dilute to 100.0 ml with the mobile phase.

Reference solution (a). Dissolve 15.0 mg of *methylthioninium impurity A CRS* in the mobile phase and dilute to 100.0 ml with the mobile phase. To 1.0 ml of this solution, add 1.0 ml of the test solution and dilute to 10.0 ml with the mobile phase.

Reference solution (b). Dilute 1.0 ml of the test solution to 100.0 ml with the mobile phase.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (7 µm),
- as mobile phase at a flow rate of 1 ml/min a mixture of 27 volumes of *acetonitrile R* and 73 volumes of a mixture of 3.4 ml of *phosphoric acid R* and 1000 ml of *water R*,
- as detector a spectrophotometer set at 246 nm.

Inject 20 µl of each solution. Adjust the sensitivity of the detector so that the height of the peak due to the substance to be examined (retention time about 11 min) in the chromatogram obtained with reference solution (a) is at least 80 per cent of the full scale of the recorder. The test is not valid unless in the chromatogram obtained with reference solution (a), the resolution between the peaks due to impurity A and methylthioninium is at least 1.5. If necessary, adjust the concentration of acetonitrile in the mobile phase. Continue the chromatography of the test solution for twice the retention time of the principal peak. In the chromatogram obtained with the test solution the area of any peak corresponding to impurity A is not greater than five times the area of the principal peak in the chromatogram obtained with reference solution (b) (5.0 per cent); the area of any peak, apart from the principal peak and the peak due to impurity A, is not greater than half the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent) and the sum of the areas of any such peaks is not greater than the area of the principal peak in the chromatogram obtained with reference solution (b) (1.0 per cent). Disregard any peak with an area less than 0.1 times that of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent).

Metals. Examine by atomic emission spectrometry (2.2.22) in argon plasma, using as detector a conventional optical system or a mass spectrometer; in the case of a mass spectrometer, use indium as internal standard.

Test solution. In a 10 ml volumetric flask, dissolve with stirring 100 mg in 9 ml of *water R*, add 100.0 µl of a 10 µg/ml solution of indium prepared from indium *elementary standard solution for atomic spectrometry (1.000 g/l) R* in *nitric acid R* which has been diluted fifty-fold with *water R*. Dilute to 10.0 ml with *water R*.

Reference solutions. Into a 100 ml volumetric flask, introduce 10.0 ml of a standard solution containing 1.00 µg/ml of each of the metals to be determined and prepared by dilution, with *water R*, of each *elementary standard solution for atomic spectrometry (1.000 g/l) R* for the corresponding elements. Add 1.00 ml of a 10 µg/ml solution of indium prepared from indium *elementary standard solution for atomic spectrometry (1.000 g/l) R* in *nitric acid R* which has been diluted fifty-fold with *water R*. Dilute to 100.0 ml with *water R*.

Blank solution. Dilute one hundred-fold with *water R* the 10 µg/ml solution of indium used for the test and reference solutions.

Element	Optical detection			Mass detection
	Signal (nm)	Background 1 (nm)	Background 2 (nm)	Isotope
Aluminium	396.15	396.05	396.25	27
Cadmium	214.44	214.37	214.51	114
Chromium	283.56	283.49	283.64	*
Copper	327.40	327.31	327.48	65
Tin	190.00**	189.90	190.10	118
Iron	238.20	238.27	238.14	*
Manganese	260.57	260.50	260.64	55
Mercury	253.70***	253.60	253.80	200
Molybdenum	202.03	202.02	202.04	95
Nickel	231.60	231.54	231.66	60
Lead	217.00**	216.90	217.10	208
Zinc	213.86	213.80	213.91	66
Indium				115

*Element difficult, if not impossible, to be determined with a mass spectrometer as detector.

**Borderline sensitivity with conventional optical spectrometry.

***Mercury is often impossible to determine using conventional optical spectrometry; it may be quantified using a device for the determination of hydrides.

Element	Maximum content in ppm
Aluminium	100
Cadmium	1
Chromium	10
Copper	100
Tin	10
Iron	100
Manganese	10
Mercury	1
Molybdenum	10
Nickel	10
Lead	10
Zinc	100

Loss on drying (2.2.32): 8.0 per cent to 22.0 per cent, determined on 1.000 g by drying in an oven at 100-105 °C.

Sulphated ash (2.4.14). Not more than 0.25 per cent, determined on 1.0 g.

ASSAY

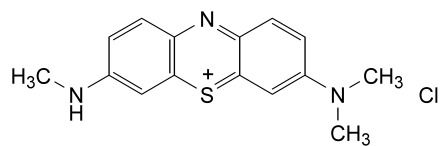
Dissolve 0.300 g in 30 ml of *water R* with heating. Cool, add 50.0 ml of *potassium dichromate solution R1* and dilute to 100.0 ml with *water R*. Allow to stand for 10 min. Filter and discard the first 20 ml of filtrate. Introduce 50.0 ml of the filtrate into a flask with a ground-glass neck, add 50 ml of *dilute sulphuric acid R* and 8.0 ml of *potassium iodide solution R*. Allow to stand protected from light for 5 min, then add 80 ml of *water R*. Titrate with 0.1 M *sodium thiosulphate* using 2 ml of *starch solution R*, added towards the end of the titration, as indicator. Carry out a blank titration.

1 ml of 0.1 M *sodium thiosulphate* is equivalent to 10.66 mg of C₁₆H₁₈ClN₃S.

STORAGE

Store in an airtight container, protected from light.

IMPURITIES

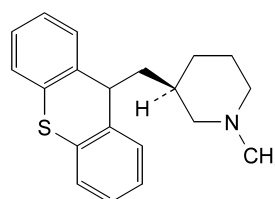


A. 3-(dimethylamino)-7-(methylamino)phenothiazin-5-ylum chloride.

01/2005:1347

METIXENE HYDROCHLORIDE

Metixeni hydrochloridum



and enantiomer, HCl, H₂O

C₂₀H₂₄ClN₃S·H₂O

M_r 363.9

DEFINITION

Metixene hydrochloride contains not less than 98.0 per cent and not more than the equivalent of 102.0 per cent of (*RS*)-1-methyl-3-[(9*H*-thioxanthen-9-yl)methyl]piperidine hydrochloride, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, crystalline or fine crystalline powder, soluble in water, soluble in alcohol and in methylene chloride.

IDENTIFICATION

- Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *metixene hydrochloride CRS*.
- It gives reaction (a) of chlorides (2.3.1).

TESTS

Appearance of solution. Dissolve 0.40 g in *methanol R* and dilute to 20.0 ml with the same solvent. The solution is clear (2.2.1) and not more intensely coloured than reference solution Y₆ (2.2.2, *Method I*).

pH (2.2.3). Dissolve 0.18 g in *carbon dioxide-free water R* heating if necessary at about 50 °C, cool and dilute to 10.0 ml with the same solvent. The pH of the solution, measured immediately, is 4.4 to 5.8.

Related substances. Examine by thin-layer chromatography (2.2.27), using a TLC silica gel plate *R*. Carry out the test rapidly and protected from light.

Test solution. Dissolve 50 mg of the substance to be examined in *methylene chloride R* and dilute to 5.0 ml with the same solvent.

Reference solution (a). Dissolve 5 mg of *metixene hydrochloride CRS* in *methylene chloride R* and dilute to 100.0 ml with the same solvent.

Reference solution (b). Dissolve 20 mg of *thioxanthene CRS* in 50 ml of *methylene chloride R*. Dilute 1.0 ml of the solution to 20.0 ml with *methylene chloride R*.

01/2005:1348

Reference solution (c). Dissolve 5 mg of *thioxanthone CRS* in 50 ml of *methylene chloride R*. Dilute 1.0 ml of the solution to 20.0 ml with *methylene chloride R*.

Reference solution (d). Dilute 4 ml of reference solution (a) to 10.0 ml with *methylene chloride R*.

Apply to the plate as narrow bands 5 µl of each solution. Develop over a path of 10 cm using a mixture of 10 volumes of *glacial acetic acid R*, 10 volumes of *methanol R* and 80 volumes of *methylene chloride R*. Dry the plate in a stream of cold air. Spray with a mixture of 1 volume of *sulphuric acid R* and 9 volumes of *alcohol R* and heat at 100 °C for 10 min. Allow the plate to cool and examine in ultraviolet light at 365 nm. Thioxanthene shows orange fluorescence and thioxanthone shows greenish-blue fluorescence. Any band corresponding to thioxanthene in the chromatogram obtained with the test solution is not more intense than the band in the chromatogram obtained with reference solution (b) (0.2 per cent); any band corresponding to thioxanthone in the chromatogram obtained with the test solution is not more intense than the band in the chromatogram obtained with reference solution (c) (0.05 per cent); any band, apart from the principal band and the bands corresponding to thioxanthene and thioxanthone, is not more intense than the band in the chromatogram obtained with reference solution (a) (0.5 per cent) and at most one such band is more intense than the band in the chromatogram obtained with reference solution (d) (0.2 per cent). The test is not valid unless the bands in the chromatograms obtained with reference solutions (b) and (c) are clearly visible and differentiated.

Loss on drying (2.2.32). Not less than 4.0 per cent and not more than 6.0 per cent, determined on 0.500 g by drying in an oven at 138–142 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

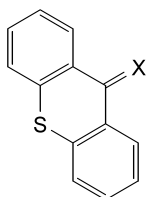
Dissolve 0.250 g in a mixture of 5.0 ml of 0.01 M *hydrochloric acid* and 50 ml of *alcohol R*. Carry out a potentiometric titration (2.2.20), using 0.1 M *sodium hydroxide*. Read the volume added between the 2 points of inflexion.

1 ml of 0.1 M *sodium hydroxide* is equivalent to 34.59 mg of C₁₄H₂₂ClN₃O₂.

STORAGE

Store protected from light.

IMPURITIES

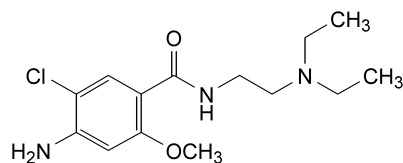


A. X = H₂: 9*H*-thioxanthene,

B. X = O: 9*H*-thioxanthene-9-one (thioxanthone).

METOCLOPRAMIDE

Metoclopramidum

C₁₄H₂₂ClN₃O₂M_r 299.8

DEFINITION

Metoclopramide contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of 4-amino-5-chloro-*N*-[2-(diethylamino)ethyl]-2-methoxybenzamide, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, fine powder, practically insoluble in water, sparingly soluble in *methylene chloride*, sparingly soluble to slightly soluble in *alcohol*.

It shows polymorphism.

IDENTIFICATION

First identification: A, B.

Second identification: A, C.

- Melting point (2.2.14): 145 °C to 149 °C.
- Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *metoclopramide CRS*. Examine the substances prepared as discs.
- Examine the chromatograms obtained in test A for related substances (see Tests) in ultraviolet light at 254 nm before spraying with *dimethylaminobenzaldehyde solution RI*. The principal spot in the chromatogram obtained with test solution (a) is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a).

TESTS

Appearance of solution. Dissolve 2.5 g in 25 ml of 1 M *hydrochloric acid*. The freshly prepared solution is clear (2.2.1) and not more intensely coloured than reference solution Y₆ (2.2.2, *Method II*).

Related substances

- Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel F₂₅₄ plate R*.
Test solution (a). Dissolve 40 mg of the substance to be examined in *methanol R* and dilute to 10 ml with the same solvent.
Test solution (b). Dissolve 0.160 g of the substance to be examined in *methanol R* and dilute to 10 ml with the same solvent.
Reference solution (a). Dissolve 20 mg of *metoclopramide CRS* and 10 mg of *sulpiride CRS* in *methanol R* and dilute to 5 ml with the same solvent.
Reference solution (b). Dissolve 20 mg of *N,N*-diethylethane-1,2-diamine *R* in *methanol R* and dilute to 50 ml with the same solvent. Dilute 2 ml of the solution to 25 ml with *methanol R*.
Apply to the plate 10 µl of each solution. Develop over a path of 12 cm using a mixture of 2 volumes of *concentrated ammonia R*, 10 volumes of *dioxan R*,

14 volumes of *methanol R* and 90 volumes of *methylene chloride R*. Allow the plate to dry in air. Examine in ultraviolet light at 254 nm (identification C). Spray with *dimethylaminobenzaldehyde solution R1*. Allow the plate to dry in air. Any spot corresponding to impurity E (not visualised in ultraviolet light at 254 nm) in the chromatogram obtained with test solution (b) is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.2 per cent). The test is not valid unless the chromatogram obtained with reference solution (a) shows two clearly separated spots.

B. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 10.0 mg of the substance to be examined in the mobile phase and dilute to 10.0 ml with the mobile phase.

Reference solution (a). Dilute 0.2 ml of the test solution to 100.0 ml with the mobile phase.

Reference solution (b). Dissolve 10.0 mg of *metoclopramide impurity A CRS* in the mobile phase and dilute to 100.0 ml with the mobile phase. Mix 1.0 ml of this solution with 0.1 ml of the test solution and dilute to 10.0 ml with the mobile phase.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4.6 mm in internal diameter packed with *octylsilyl silica gel for chromatography R* (5 µm),
- as mobile phase at a flow rate of 1.5 ml/min a mixture prepared as follows: dissolve 6.8 g of *potassium dihydrogen phosphate R* in 700 ml of *water R*; add 0.2 ml of *N,N*-dimethyloctylamine *R* and adjust to pH 4.0 with *dilute phosphoric acid R*; dilute to 1000 ml with *water R*, add 250 ml of *acetonitrile R* and mix,
- as detector a spectrophotometer set at 240 nm.

Inject 10 µl of each solution. Adjust the sensitivity of the system so that the heights of the principal peaks in the chromatogram obtained with reference solution (b) are at least 50 per cent of the full scale of the recorder. The test is not valid unless, in the chromatogram obtained with reference solution (b), the resolution between the two principal peaks is at least 2.0. Continue the chromatography of the test solution for eight times the retention time of metoclopramide. In the chromatogram obtained with the test solution: the area of any peak, apart from the principal peak, is not greater than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent) and the sum of the areas of any such peaks is not greater than three times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.6 per cent). Disregard any peak with an area less than 0.1 times that of the principal peak in the chromatogram obtained with reference solution (a).

Heavy metals (2.4.8). 1.0 g complies with limit test C for heavy metals (20 ppm). Prepare the standard using 2 ml of *lead standard solution (10 ppm Pb) R*.

Loss on drying (2.2.32). Not more than 1.0 per cent, determined on 1.000 g by drying in an oven at 100–105 °C.

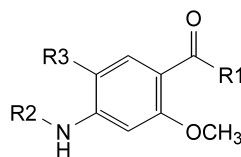
Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

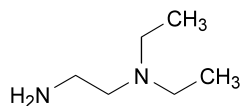
Dissolve 0.250 g in 50 ml of *anhydrous acetic acid R* and add 5 ml of *acetic anhydride R*. Titrate with 0.1 M *perchloric acid*, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *perchloric acid* is equivalent to 29.98 mg of $C_{14}H_{22}ClN_3O_2$.

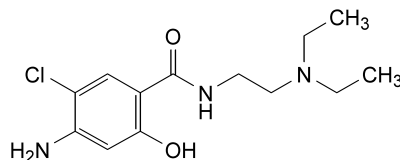
IMPURITIES



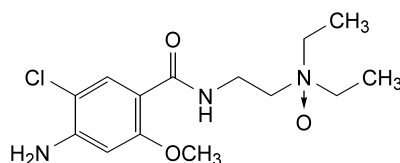
- A. R1 = NH-CH₂-CH₂-N(C₂H₅)₂, R2 = CO-CH₃, R3 = Cl: 4-(acetylamino)-5-chloro-*N*-[2-(diethylamino)ethyl]-2-methoxybenzamide,
- B. R1 = OCH₃, R2 = CO-CH₃, R3 = Cl: methyl 4-(acetylamino)-5-chloro-2-methoxybenzoate,
- C. R1 = OH, R2 = H, R3 = Cl: 4-amino-5-chloro-2-methoxybenzoic acid,
- D. R1 = OCH₃, R2 = CO-CH₃, R3 = H: methyl 4-(acetylamino)-2-methoxybenzoate,



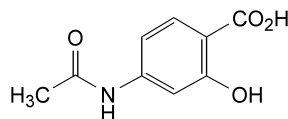
- E. *N,N*-diethylethane-1,2-diamine,



- F. 4-amino-5-chloro-*N*-[2-(diethylamino)ethyl]-2-hydroxybenzamide,



- G. *N'*-(4-amino-5-chloro-2-methoxybenzoyl)-*N,N*-diethylethane-1,2-diamine *N*-oxide,

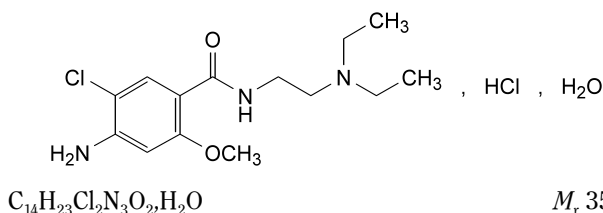


- H. 4-(acetylamino)-2-hydroxybenzoic acid.

01/2005:0674

**METOCLOPRAMIDE
HYDROCHLORIDE**

Metoclopramidi hydrochloridum



*M*_r 354.3

DEFINITION

Metoclopramide hydrochloride contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of 4-amino-5-chloro-*N*-[2-(diethylamino)ethyl]-2-methoxybenzamide hydrochloride, calculated with reference to the anhydrous substance.

CHARACTERS

White or almost white, crystalline powder or crystals, very soluble in water, freely soluble in alcohol, sparingly soluble in methylene chloride.

It melts at about 183 °C with decomposition.

IDENTIFICATION

First identification: A, B, D.

Second identification: A, C, D, E.

- The pH (2.2.3) of solution S (see Tests) is 4.5 to 6.0.
- Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *metoclopramide hydrochloride CRS*. Examine the substances as discs prepared using *potassium chloride R*.
- Examine the chromatograms obtained in the test for related substances in ultraviolet light before spraying with *dimethylaminobenzaldehyde solution R1*. The principal spot in the chromatogram obtained with test solution (b) is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a).
- Dilute 1 ml of solution S to 2 ml with *water R*. The solution gives reaction (a) of chlorides (2.3.1).
- Dissolve about 2 mg in 2 ml of *water R*. The solution gives the reaction of primary aromatic amines (2.3.1).

TESTS

Solution S. Dissolve 2.5 g in *carbon dioxide-free water R* and dilute to 25 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and colourless (2.2.2, *Method II*).

Related substances. Examine by thin-layer chromatography (2.2.27), using *silica gel HF₂₅₄ R* as the coating substance.

Test solution (a). Dissolve 0.40 g of the substance to be examined in *methanol R* and dilute to 10 ml with the same solvent.

Test solution (b). Dilute 1 ml of test solution (a) to 10 ml with *methanol R*.

Reference solution (a). Dissolve 20 mg of *metoclopramide hydrochloride CRS* in *methanol R* and dilute to 5 ml with the same solvent.

Reference solution (b). Dilute 5 ml of test solution (a) to 100 ml with *methanol R*. Dilute 1 ml of this solution to 10 ml with *methanol R*.

Reference solution (c). Dissolve 10 mg of *N,N*-diethylethylenediamine *R* in *methanol R* and dilute to 50 ml with the same solvent.

Apply separately to the plate 5 µl of each solution. Develop over a path of 12 cm using a mixture of 2 volumes of *concentrated ammonia R*, 10 volumes of *dioxan R*, 14 volumes of *methanol R* and 90 volumes of *methylene chloride R*. Allow the plate to dry in air. Examine in ultraviolet light at 254 nm. Any spot in the chromatogram obtained with test solution (a), apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.5 per cent). Spray with *dimethylaminobenzaldehyde solution R1*. Allow the plate to dry in air. Any spot in the chromatogram obtained with test solution (a) that has not been visualised in

ultraviolet light at 254 nm is not more intense than the spot in the chromatogram obtained with reference solution (c) (0.5 per cent).

Heavy metals (2.4.8). 12 ml of solution S complies with limit test A for heavy metals (20 ppm). Prepare the standard using *lead standard solution (2 ppm Pb) R*.

Water (2.5.12): 4.5 per cent to 5.5 per cent, determined on 0.500 g by the semi-micro determination of water.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.2500 g in a mixture of 5.0 ml of 0.01 *M* *hydrochloric acid* and 50 ml of *alcohol R*. Carry out a potentiometric titration (2.2.20), using 0.1 *M* *sodium hydroxide*. Read the volume of 0.1 *M* *sodium hydroxide* added between the two points of inflexion.

1 ml of 0.1 *M* *sodium hydroxide* is equivalent to 33.63 mg of C₁₄H₂₃Cl₂N₃O₂.

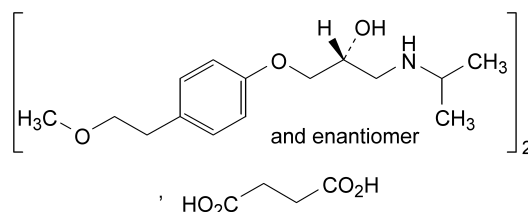
STORAGE

Store protected from light.

01/2005:1448

METOPROLOL SUCCINATE

Metoprololi succinas

C₃₄H₅₆N₂O₁₀M_r 653

DEFINITION

Bis[(2*RS*)-1-[4-(2-methoxyethyl)phenoxy]-3-[(1-methylethyl)amino]propan-2-ol] butanedioate.

Content: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

Appearance: white, crystalline powder.

Solubility: freely soluble in water, soluble in methanol, slightly soluble in alcohol, very slightly soluble in ethyl acetate.

IDENTIFICATION

Infrared absorption spectrophotometry (2.2.24).

Comparison: *Ph. Eur. reference spectrum of metoprolol succinate*.

TESTS

Solution S. Dissolve 0.500 g in *carbon dioxide-free water R* and dilute to 25.0 ml with the same solvent.

Appearance of solution. Solution S is not more opalescent than reference suspension II (2.2.1) and it is colourless (2.2.2, *Method II*).

pH (2.2.3): 7.0 to 7.6 for solution S.

Related substances

- Thin-layer chromatography (2.2.27).

Test solution. Dissolve 0.50 g of the substance to be examined in *methanol R* and dilute to 10 ml with the same solvent.

Reference solution. Dilute 1 ml of the test solution to 50 ml with *methanol R*. Dilute 5 ml of this solution to 50 ml with *methanol R*.

Plate: TLC silica gel plate *R*.

Mobile phase: place 2 beakers each containing 30 volumes of *concentrated ammonia R* at the bottom of a chromatographic tank containing a mixture of 20 volumes of *methanol R* and 80 volumes of *ethyl acetate R*.

Application: 10 µl.

Development: over a path of 12 cm in a tank saturated for at least 1 h.

Drying: in air for at least 3 h.

Detection: expose the plate to iodine vapour for at least 15 h.

Limits:

- **any impurity:** any spot, apart from the principal spot, is not more intense than the spot in the chromatogram obtained with the reference solution (0.2 per cent),
- **disregard** any spot on the starting line.

B. Liquid chromatography (2.2.29).

Test solution. Dissolve 20.0 mg of the substance to be examined in the mobile phase and dilute to 10.0 ml with the mobile phase.

Reference solution (a). Dissolve 5.0 mg of the substance to be examined and 3.0 mg of *metoprolol impurity A CRS* in the mobile phase and dilute to 100.0 ml with the mobile phase.

Reference solution (b). Dilute 1.0 ml of the test solution to 100.0 ml with the mobile phase. Dilute 1.0 ml of the solution to 10.0 ml with the mobile phase.

Reference solution (c). If this solution is required (see below), it is to be prepared in a fume cupboard. This solution is used only to identify the peak due to impurity C. Dissolve 10 mg of the substance to be examined in 10 ml of 0.1 M *hydrochloric acid*. Transfer this solution to an evaporating dish 10 cm in diameter. Place the dish so that the surface of the solution is 5 cm from a lamp emitting ultraviolet light (2.1.3) at 254 nm for 6 h. Dilute 0.5 ml of this solution to 25 ml with the mobile phase.

Column:

- **size:** $l = 0.15$ m, $\varnothing = 3.9$ mm,
- **stationary phase:** octadecylsilyl silica gel for chromatography *R1* (5 µm) with a pore size of 10 nm and a carbon loading of 19 per cent.

Mobile phase: dissolve 3.9 g of *ammonium acetate R* in 810 ml of *water R*, add 2.0 ml of *triethylamine R*, 10.0 ml of *glacial acetic acid R*, 3.0 ml of *phosphoric acid R* and 146 ml of *acetonitrile R* and mix.

Flow rate: 1 ml/min.

Detection: spectrophotometer at 280 nm.

Injection: 20 µl; inject the test solution and reference solutions (a) and (b).

Run time: 3 times the retention time of metoprolol.

Relative retention with reference to metoprolol (retention time = about 7 min): impurity C = about 0.3; impurity A = about 0.7.

System suitability: reference solution (a):

- **resolution:** minimum of 6.0 between the peaks due to impurity A and to metoprolol.

Limits:

- **any impurity:** not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent),
- **total:** not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent),
- **disregard limit:** 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent); disregard any peak due to succinic acid.

If a peak occurs with a retention time of about 2.3 min (impurity C) which has an area greater than the area of the principal peak in the chromatogram obtained with reference solution (b), prepare and inject reference solution (c). For the chromatogram obtained with the test solution, multiply the peak area of impurity C by a correction factor of 0.1.

Heavy metals (2.4.8): maximum 10 ppm.

Dissolve 2.0 g in 20 ml of *water R*. 12 ml of the solution complies with limit test A. Prepare the standard using *lead standard solution (1 ppm Pb) R*.

Loss on drying (2.2.32): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 100–105 °C.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.250 g in 40 ml of *anhydrous acetic acid R*. Titrate with 0.1 M *perchloric acid*, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *perchloric acid* is equivalent to 32.64 mg of $C_{34}H_{56}N_2O_{10}$.

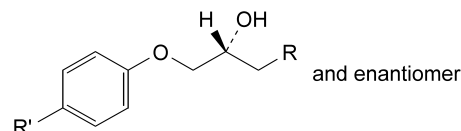
STORAGE

Protected from light.

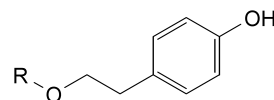
IMPURITIES

By liquid chromatography: A, B, C, D, E, F, G, H, J.

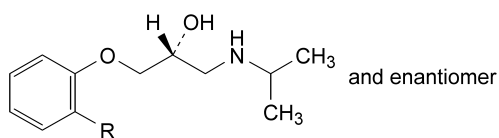
By thin-layer chromatography: M, N, O.



- A. $R = \text{NH-CH}_2\text{-CH}_3$, $R' = \text{CH}_2\text{-CH}_2\text{-OCH}_3$: (2*RS*)-1-(ethylamino)-3-[4-(2-methoxyethyl)phenoxy]propan-2-ol,
- C. $R = \text{NH-CH(CH}_3)_2$, $R' = \text{CHO}$: 4-[(2*RS*)-2-hydroxy-3-[(1-methylethyl)amino]propoxy]benzaldehyde,
- D. $R = \text{OH}$, $R' = \text{CH}_2\text{-CH}_2\text{-OCH}_3$: (2*RS*)-3-[4-(2-methoxyethyl)phenoxy]propane-1,2-diol,
- H. $R = \text{NH-CH(CH}_3)_2$, $R' = \text{CH}_2\text{-CH}_2\text{-OH}$: (2*RS*)-1-[4-(2-hydroxyethyl)phenoxy]-3-[(1-methylethyl)amino]propan-2-ol,
- J. $R = \text{O-CH}_2\text{-CHOH-CH}_2\text{-NH-CH(CH}_3)_2$, $R' = \text{CH}_2\text{-CH}_2\text{-OCH}_3$: 1-[2-hydroxy-3-[(1-methylethyl)amino]propoxy]-3-[4-(2-methoxyethyl)phenoxy]propan-2-ol,

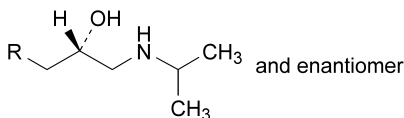


- B. $R = \text{CH}_3$: 4-(2-methoxyethyl)phenol,
- G. $R = \text{H}$: 2-(4-hydroxyphenyl)ethanol,



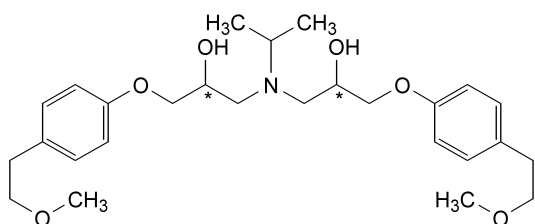
E. R = CH₂-CH₂-OCH₃: (2*RS*)-1-[2-(2-methoxyethyl)phenoxy]-3-[(1-methylethyl)amino]propan-2-ol,

F. R = H: (2*RS*)-1-[(1-methylethyl)amino]-3-phenoxypropan-2-ol,



M. R = NH-CH(CH₃)₂: 1,3-bis[(1-methylethyl)amino]propan-2-ol,

N. R = OH: (2*RS*)-3-[(1-methylethyl)amino]propane-1,2-diol,

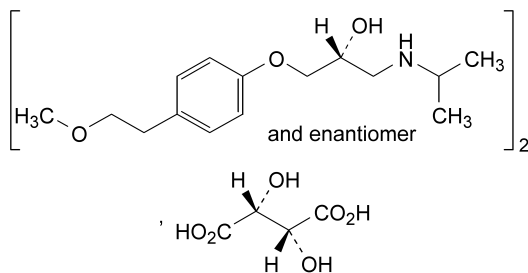


O. 1,1'-(1-methylethyl)imino]bis[3-[4-(2-methoxyethyl)phenoxy]propan-2-ol].

01/2005:1028

METOPROLOL TARTRATE

Metoprololi tartras



C₃₄H₅₆N₂O₁₂

M_r 685

DEFINITION

Bis[(2*RS*)-1-[4-(2-methoxyethyl)phenoxy]-3-[(1-methylethyl)amino]propan-2-ol] (2*R*,3*R*)-2,3-dihydroxybutanedioate.

Content: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

Appearance: white, crystalline powder or colourless crystals.

Solubility: very soluble in water, freely soluble in alcohol.

It shows polymorphism.

IDENTIFICATION

A. Specific optical rotation (see Tests).

B. Infrared absorption spectrophotometry (2.2.24).

Comparison: metoprolol tartrate CRS.

If the spectra obtained in the solid state show differences, record further spectra using discs prepared by placing 25 µl of a 100 g/l solution in *methylene chloride R* on a disc of *potassium bromide R* and evaporating the solvent. Examine immediately.

TESTS

Solution S. Dissolve 0.500 g in *carbon dioxide-free water R* and dilute to 25.0 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and not more intensely coloured than reference solution B₈ (2.2.2, *Method II*).

pH (2.2.3): 6.0 to 7.0 for solution S.

Specific optical rotation (2.2.7): + 7.0 to + 10.0 (dried substance), determined on solution S.

Related substances

A. Thin-layer chromatography (2.2.27).

Test solution. Dissolve 0.50 g of the substance to be examined in *methanol R* and dilute to 10 ml with the same solvent.

Reference solution (a). Dilute 1 ml of the test solution to 20 ml with *methanol R*. Dilute 5 ml of this solution to 50 ml with *methanol R*.

Reference solution (b). Dilute 4 ml of reference solution (a) to 10 ml with *methanol R*.

Plate: TLC silica gel plate *R*.

Mobile phase: place 2 beakers each containing 30 volumes of *concentrated ammonia R* at the bottom of a chromatographic tank containing a mixture of 20 volumes of *methanol R* and 80 volumes of *ethyl acetate R*.

Application: 5 µl.

Development: over a path of 12 cm in a tank saturated for at least 1 h.

Drying: in air for at least 3 h.

Detection: expose the plate to iodine vapour for at least 15 h.

Limits:

- *any impurity*: any spot, apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (a) (0.5 per cent) and at most 1 such spot is more intense than the spot in the chromatogram obtained with reference solution (b) (0.2 per cent),
- *disregard* any spot on the starting-line.

B. Liquid chromatography (2.2.29).

Test solution. Dissolve 20.0 mg of the substance to be examined in the mobile phase and dilute to 10.0 ml with the mobile phase.

Reference solution (a). Dissolve 5.0 mg of *metoprolol tartrate CRS* and 3.0 mg of *metoprolol impurity A CRS* in the mobile phase and dilute to 100.0 ml with the mobile phase.

Reference solution (b). Dilute 1.0 ml of the test solution to 20.0 ml with the mobile phase. Dilute 3.0 ml of the solution to 50.0 ml with the mobile phase.

Reference solution (c). If this solution is required (see below), it is to be prepared in a fume cupboard. This solution is used only to identify the peak due to impurity C. Dissolve 10 mg of *metoprolol tartrate CRS* in 10 ml of 0.1 M *hydrochloric acid*. Transfer this solution to an evaporating dish 10 cm in diameter. Place the dish

so that the surface of the solution is 5 cm from a lamp emitting ultraviolet light (2.1.3) at 254 nm for 6 h. Dilute 0.5 ml of this solution to 25 ml with the mobile phase.

Column:

- **size:** $l = 0.15$ m, $\varnothing = 3.9$ mm,
- **stationary phase:** octadecylsilyl silica gel for chromatography R1 (5 μ m) with a pore size of 10 nm and a carbon loading of 19 per cent.

Mobile phase: dissolve 3.9 g of ammonium acetate R in 810 ml of water R, add 2.0 ml of triethylamine R, 10.0 ml of glacial acetic acid R, 3.0 ml of phosphoric acid R and 146 ml of acetonitrile R and mix.

Flow rate: 1 ml/min.

Detection: spectrophotometer at 280 nm.

Injection: 20 μ l; inject the test solution and reference solutions (a) and (b).

Run time: 3 times the retention time of metoprolol.

Relative retention with reference to metoprolol (retention time = about 7 min): impurity C = about 0.3; impurity A = about 0.7.

System suitability: reference solution (a):

- **resolution:** minimum of 6.0 between the peaks due to impurity A and to metoprolol.

Limits:

- **any impurity (A to J):** not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.3 per cent),
- **total:** not more than 1.7 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent),
- **disregard limit:** 0.17 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent); disregard any peak due to tartaric acid.

If a peak occurs with a retention time of about 2.3 min (impurity C) which has an area greater than the area of the principal peak in the chromatogram obtained with reference solution (b), prepare and inject reference solution (c). For the chromatogram obtained with the test solution, multiply the peak area of impurity C by a correction factor of 0.1.

Heavy metals (2.4.8): maximum 10 ppm.

Dissolve 2.0 g in 20 ml of water R. 12 ml of the solution complies with limit test A. Prepare the standard using lead standard solution (1 ppm Pb) R.

Loss on drying (2.2.32): maximum 0.5 per cent, determined on 1.000 g by drying *in vacuo* over anhydrous calcium chloride R for 4 h.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.250 g in 30 ml of anhydrous acetic acid R. Titrate with 0.1 M perchloric acid, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M perchloric acid is equivalent to 34.24 mg of $C_{34}H_{56}N_2O_{12}$.

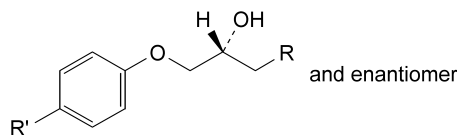
STORAGE

Protected from light.

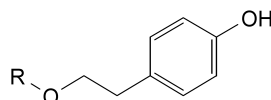
IMPURITIES

By liquid chromatography: A, B, C, D, E, F, G, H, J.

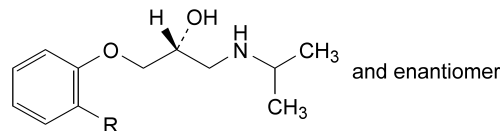
By thin-layer chromatography: M, N, O.



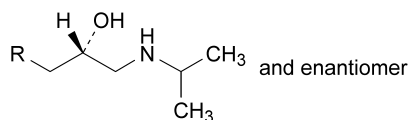
- A. R = NH-CH₂-CH₃, R' = CH₂-CH₂-OCH₃: (2*RS*)-1-(ethylamino)-3-[4-(2-methoxyethyl)phenoxy]propan-2-ol,
- C. R = NH-CH(CH₃)₂, R' = CHO: 4-[(2*RS*)-2-hydroxy-3-[(1-methylethyl)amino]propoxy]benzaldehyde,
- D. R = OH, R' = CH₂-CH₂-OCH₃: (2*RS*)-3-[4-(2-methoxyethyl)phenoxy]propane-1,2-diol,
- H. R = NH-CH(CH₃)₂, R' = CH₂-CH₂-OH: (2*RS*)-1-[4-(2-hydroxyethyl)phenoxy]-3-[(1-methylethyl)amino]propan-2-ol,
- J. R = O-CH₂-CHOH-CH₂-NH-CH(CH₃)₂, R' = CH₂-CH₂-OCH₃: 1-[2-hydroxy-3-[(1-methylethyl)amino]propoxy]-3-[4-(2-methoxyethyl)phenoxy]propan-2-ol,



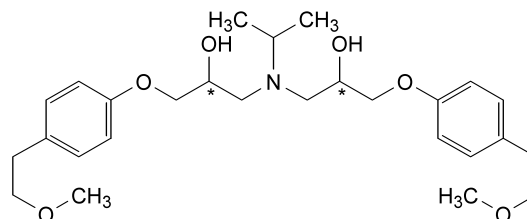
- B. R = CH₃: 4-(2-methoxyethyl)phenol,
- G. R = H: 2-(4-hydroxyphenyl)ethanol,



- E. R = CH₂-CH₂-OCH₃: (2*RS*)-1-[2-(2-methoxyethyl)phenoxy]-3-[(1-methylethyl)amino]propan-2-ol,
- F. R = H: (2*RS*)-1-[(1-methylethyl)amino]-3-phenoxypropan-2-ol,



- M. R = NH-CH(CH₃)₂: 1,3-bis[(1-methylethyl)amino]propan-2-ol,
- N. R = OH: (2*RS*)-3-[(1-methylethyl)amino]propane-1,2-diol,

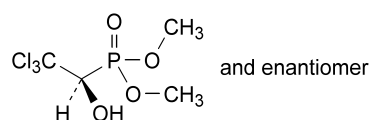


- O. 1,1'-[(1-methylethyl)imino]bis[3-[4-(2-methoxyethyl)phenoxy]propan-2-ol].

01/2005:1133

METRIFONATE

Metrifonatum



$C_4H_8Cl_3O_4P$

M_r 257.4

DEFINITION

Metrifonate contains not less than 98.0 per cent and not more than the equivalent of 100.5 per cent of dimethyl (RS)-(2,2,2-trichloro-1-hydroxyethyl)phosphonate, calculated with reference to the anhydrous substance.

CHARACTERS

A white, crystalline powder, freely soluble in water, very soluble in methylene chloride, freely soluble in acetone and in alcohol.

It melts between 76 °C and 81 °C.

IDENTIFICATION

First identification: A, B.

Second identification: B, C, D.

A. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *metrifonate CRS*. Examine the substances prepared as discs.

B. Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel plate R*.

Test solution. Dissolve 10 mg of the substance to be examined in *methanol R* and dilute to 10 ml with the same solvent.

Reference solution. Dissolve 10 mg of *metrifonate CRS* in *methanol R* and dilute to 10 ml with the same solvent.

Apply to the plate 10 µl of each solution. Develop in an unsaturated tank over a path of 15 cm using a mixture of 5 volumes of *glacial acetic acid R*, 25 volumes of *dioxan R* and 70 volumes of *toluene R*. Allow the plate to dry in air. Spray with a 50 g/l solution of 4-(4-nitrobenzyl)pyridine *R* in *acetone R* and heat at 120 °C for 15 min. Before the plate cools, spray with a 100 g/l solution of *tetraethylene pentamine R* in *acetone R*. Examine immediately. The principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

C. Dissolve about 20 mg in 1 ml of *dilute sodium hydroxide solution R*. Add 1 ml of *pyridine R*. Shake and heat on a water-bath for 2 min. A red colour develops in the upper layer.

D. To 0.1 g add 0.5 ml of *nitric acid R*, 0.5 ml of a 500 g/l solution of *ammonium nitrate R1* and 0.1 ml of *strong hydrogen peroxide solution R*. Heat on a water-bath for 10 min. Heat to boiling and add 1 ml of *ammonium molybdate solution R*. A yellow colour is produced or a yellow precipitate is formed.

TESTS

Appearance of solution. Dissolve 5.0 g in 20 ml of *methanol R*. The solution is clear (2.2.1) and not more intensely coloured than reference solution Y₇ (2.2.2, *Method II*).

Acidity. Dissolve 2.5 g in *carbon dioxide-free water R* and dilute to 50 ml with the same solvent. Add 0.1 ml of *methyl red solution R*. Not more than 1.0 ml of 0.1 M *sodium hydroxide* is required to change the colour of the indicator to yellow.

Optical rotation (2.2.7). Dissolve 0.1 g in *alcohol R* and dilute to 10.0 ml with the same solvent. The angle of optical rotation is –0.10° to +0.10°.

Related substances. Examine by liquid chromatography (2.2.29).

Solvent mixture. Prepare a mixture of 10 volumes of mobile phase B and 90 volumes of mobile phase A.

Test solution. Dissolve 0.20 g of the substance to be examined in the solvent mixture and dilute to 10.0 ml with the solvent mixture.

Reference solution (a). Use a freshly prepared solution. Dissolve 10.0 mg of *desmethylnmetrifonate CRS* in the solvent mixture and dilute to 20.0 ml with the solvent mixture. Dilute 1.0 ml of this solution to 5.0 ml with the solvent mixture.

Reference solution (b). Dissolve 0.10 g of *dichlorvos R* in the solvent mixture and dilute to 50.0 ml with the solvent mixture. Dilute 1.0 ml of this solution to 50.0 ml with the solvent mixture.

Reference solution (c). Dilute 1.0 ml of the test solution to 10.0 ml with the solvent mixture. Dilute 5.0 ml of this solution to 100.0 ml with the solvent mixture.

Reference solution (d). Use a freshly prepared solution. Mix 1.0 ml of reference solution (a), 1.0 ml of reference solution (b) and 0.025 ml of the test solution.

Reference solution (e). Dilute 4.0 ml of the test solution to 100.0 ml with the solvent mixture. Dilute 1.0 ml of this solution to 10.0 ml with the solvent mixture.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4.6 mm in internal diameter packed with a suitable octadecylsilyl silica gel for chromatography (10 µm),
- as mobile phase at a flow rate of 1 ml/min:

Mobile phase A. A 1.36 g/l solution of *potassium dihydrogen phosphate R*, previously adjusted to pH 2.9 with *phosphoric acid R*,

Mobile phase B. *Acetonitrile R*,

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)
0 - 5	90	10
5 - 25	90 → 85	10 → 15
25 - end	85 → 45	15 → 55

– as detector a spectrophotometer set at 210 nm, equilibrating the column for 5 min with the same mixture of mobile phases used for the first 5 min and maintaining the temperature of the column at 40 °C.

When the chromatograms are recorded in the prescribed conditions the peaks elute in the following order: desmethylnmetrifonate, metrifonate and dichlorvos. Inject 10 µl of reference solution (e). Adjust the sensitivity of the system so that the height of the principal peak in the chromatogram obtained is 50 per cent to 70 per cent of the full scale of the recorder. Inject 50 µl of reference solution (d). The test is not valid unless the resolution between the peaks corresponding to desmethylnmetrifonate and metrifonate is at least 3.0 and the resolution between the peaks corresponding to metrifonate and dichlorvos is at least 4.5.

Inject 50 µl of the test solution and 50 µl each of reference solutions (a), (b) and (c). Continue the chromatography of the test solution for three times the retention time of metrifonate. In the chromatogram obtained with the test solution: the area of any peak corresponding to desmethylnmetrifonate is not greater than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent); the area of any peak corresponding to dichlorvos is not greater than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent); the area of any other peak, apart from the principal peak and the peaks corresponding to desmethylnmetrifonate and dichlorvos respectively, is not greater than the area

of the principal peak in the chromatogram obtained with reference solution (c) (0.5 per cent) and the sum of the areas of all these peaks is not greater than twice the area of the principal peak in the chromatogram obtained with reference solution (c) (1 per cent). Disregard any peak with an area less than 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (e).

Chlorides. Not more than 500 ppm. Dissolve 5.00 g in 30 ml of *alcohol R* and add a mixture of 15 ml of *nitric acid R* and 100 ml of *water R*. Using a silver electrode, titrate with 0.01 M *silver nitrate*, determining the end-point potentiometrically (2.2.20).

1 ml of 0.01 M *silver nitrate* is equivalent to 0.3546 mg of Cl.

Heavy metals (2.4.8). Dissolve 2.0 g in 20 ml of *water R*. 12 ml of the solution complies with limit test A for heavy metals (10 ppm). Prepare the standard using *lead standard solution* (1 ppm Pb) *R*.

Water (2.5.12). Not more than 0.3 per cent, determined on 3.000 g by the semi-micro determination of water.

ASSAY

Dissolve 0.300 g in 30 ml of *alcohol R*. Add 10 ml of *ethanolamine R* and allow to stand for 1 h at 20–22 °C. Add a chilled mixture of 15 ml of *nitric acid R* and 100 ml of *water R* maintaining the temperature of the mixture at 20–22 °C. Maintain at that temperature and titrate with 0.1 M *silver nitrate*, using a silver electrode and determining the end-point potentiometrically (2.2.20).

Calculate the percentage content of $C_6H_9N_3O_3$, taking into account the content of chloride and using the following expression:

$$\left[\frac{V_P}{M_P} - \frac{V_{Cl} \times 0.1}{M_{Cl}} \right] \times 25.74 \times 0.1$$

V_P = volume of silver nitrate used in the assay, in millilitres,

M_P = mass of substance used in the assay, in grams,

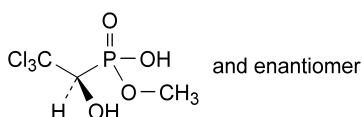
V_{Cl} = volume of silver nitrate used in the test for chlorides in millilitres,

M_{Cl} = mass of substance used in the test for chlorides, in grams.

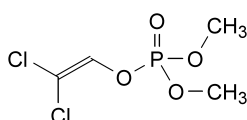
STORAGE

Store protected from light.

IMPURITIES



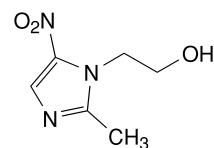
A. methyl (RS)-(2,2,2-trichloro-1-hydroxyethyl)phosphonate acid (desmethylnetrifonate),



B. 2,2-dichloroethenyl dimethyl phosphate (dichlorvos).

METRONIDAZOLE

Metronidazolium



$C_6H_9N_3O_3$

M_r 171.2

DEFINITION

2-(2-Methyl-5-nitro-1H-imidazol-1-yl)ethanol.

Content: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

Appearance: white or yellowish, crystalline powder.

Solubility: slightly soluble in water, in acetone, in alcohol and in methylene chloride.

IDENTIFICATION

First identification: C.

Second identification: A, B, D.

A. Melting point (2.2.14): 159 °C to 163 °C.

B. Dissolve 40.0 mg in 0.1 M *hydrochloric acid* and dilute to 100.0 ml with the same acid. Dilute 5.0 ml of the solution to 100.0 ml with 0.1 M *hydrochloric acid*. Examined between 230 nm and 350 nm (2.2.25), the solution shows an absorption maximum at 277 nm and a minimum at 240 nm. The specific absorbance at the maximum is 365 to 395.

C. Infrared absorption spectrophotometry (2.2.24).

Preparation: discs.

Comparison: metronidazole CRS.

D. To about 10 mg add about 10 mg of *zinc powder R*, 1 ml of *water R* and 0.25 ml of *dilute hydrochloric acid R*. Heat on a water-bath for 5 min. Cool. The solution gives the reaction of primary aromatic amines (2.3.1).

TESTS

Appearance of solution. The solution is not more opalescent than reference suspension II (2.2.1) and not more intensely coloured than reference solution GY₆ (2.2.2, *Method II*).

Dissolve 1.0 g in 1 M *hydrochloric acid* and dilute to 20 ml with the same acid.

Related substances. Liquid chromatography (2.2.29).

Prepare the solutions protected from light.

Test solution. Dissolve 0.05 g of the substance to be examined in the mobile phase and dilute to 100.0 ml with the mobile phase.

Reference solution (a). Dilute 1.0 ml of the test solution to 100.0 ml with the mobile phase and dilute 1.0 ml of this solution to 10.0 ml with the mobile phase.

Reference solution (b). Dissolve 5.0 mg of *metronidazole impurity A CRS* in the mobile phase, add 10.0 ml of the test solution and dilute to 100.0 ml with the mobile phase. Dilute 1.0 ml to 100.0 ml with the mobile phase.

Column:

– *size*: $l = 0.25$ m, $\varnothing = 4.6$ mm,

– *stationary phase*: octadecylsilyl silica gel for chromatography R (5 μ m).

01/2005:0934

Mobile phase: mix 30 volumes of *methanol R* and 70 volumes of a 1.36 g/l solution of *potassium dihydrogen phosphate R*,

Flow rate: 1 ml/min.

Detection: spectrophotometer at 315 nm.

Injection: 10 µl.

Run time: 3 times the retention time of metronidazole.

Relative retention with reference to metronidazole (retention time = about 7 min): impurity A = about 0.7.

System suitability: reference solution (b):

- **resolution:** minimum of 2.0 between the peaks due to metronidazole and to impurity A.

Limits:

- **any impurity:** not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent),
- **total:** not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent),
- **disregard limit:** 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.01 per cent).

Heavy metals (2.4.8): maximum 20 ppm.

1.0 g complies with limit test C. Prepare the standard using 2 ml of *lead standard solution (10 ppm Pb) R*.

Loss on drying (2.2.32): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 100–105 °C for 3 h.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

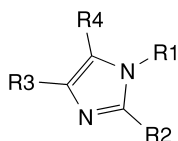
Dissolve 0.150 g in 50 ml of *anhydrous acetic acid R*. Titrate with 0.1 M *perchloric acid*, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *perchloric acid* is equivalent to 17.12 mg of C₆H₉N₃O₃.

STORAGE

Protected from light.

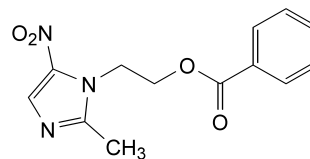
IMPURITIES



- R1 = R4 = H, R2 = CH₃, R3 = NO₂: 2-methyl-4-nitroimidazole,
- R1 = R2 = R4 = H, R3 = NO₂: 4-nitroimidazole,
- R1 = CH₂-CH₂-OH, R2 = R4 = H, R3 = NO₂: 2-(4-nitro-1*H*-imidazol-1-yl)ethanol,
- R1 = CH₂-CH₂-OH, R2 = R3 = H, R4 = NO₂: 2-(5-nitro-1*H*-imidazol-1-yl)ethanol,
- R1 = CH₂-CH₂-OH, R2 = CH₃, R3 = NO₂, R4 = H: 2-(2-methyl-4-nitro-1*H*-imidazol-1-yl)ethanol,
- R1 = CH₂-CH₂-O-CH₂-CH₂-OH, R2 = CH₃, R3 = H, R4 = NO₂: 2-[2-(2-methyl-5-nitro-1*H*-imidazol-1-yl)ethoxy]ethanol,
- R1 = CH₂-CO₂H, R2 = CH₃, R3 = H, R4 = NO₂: 2-(2-methyl-5-nitro-1*H*-imidazol-1-yl)acetic acid.

METRONIDAZOLE BENZOATE

Metronidazoli benzoas

C₁₃H₁₃N₃O₄M_r 275.3

DEFINITION

2-(2-Methyl-5-nitro-1*H*-imidazol-1-yl)ethyl benzoate.

Content: 98.5 per cent to 101.0 per cent (dried substance).

CHARACTERS

Appearance: white or slightly yellowish, crystalline powder or flakes.

Solubility: practically insoluble in water, freely soluble in methylene chloride, soluble in acetone, slightly soluble in alcohol.

IDENTIFICATION

First identification: C.

Second identification: A, B, D.

- Melting point (2.2.14): 99 °C to 102 °C.
- Dissolve 0.100 g in a 103 g/l solution of *hydrochloric acid R* and dilute to 100.0 ml with the same acid. Dilute 1.0 ml of the solution to 100.0 ml with a 103 g/l solution of *hydrochloric acid R*. Examined between 220 nm and 350 nm (2.2.25), the solution shows 2 absorption maxima, at 232 nm and 275 nm. The specific absorbance at the absorption maximum at 232 nm is 525 to 575.
- Infrared absorption spectrophotometry (2.2.24).
Comparison: Ph. Eur. reference spectrum of *metronidazole benzoate*.
- To about 10 mg add about 10 mg of *zinc powder R*, 1 ml of *water R* and 0.3 ml of *hydrochloric acid R*. Heat on a water-bath for 5 min and cool. The solution gives the reaction of primary aromatic amines (2.3.1).

TESTS

Appearance of solution. The solution is not more opalescent than reference suspension II (2.2.1) and not more intensely coloured than reference solution GY₃ (2.2.2, *Method II*).

Dissolve 1.0 g in *dimethylformamide R* and dilute to 10 ml with the same solvent.

Acidity. Dissolve 2.0 g in a mixture of 20 ml of *dimethylformamide R* and 20 ml of *water R*, previously neutralised with 0.02 M *hydrochloric acid* or 0.02 M *sodium hydroxide* using 0.2 ml of *methyl red solution R*. Not more than 0.25 ml of 0.02 M *sodium hydroxide* is required to change the colour of the indicator.

Related substances. Liquid chromatography (2.2.29).

Solvent mixture. Mix 45 volumes of mobile phase B and 55 volumes of mobile phase A.

Test solution. Dissolve 0.100 g of the substance to be examined in the solvent mixture and dilute to 10.0 ml with the solvent mixture.

Reference solution (a). Dilute 1.0 ml of the test solution to 100.0 ml with the solvent mixture. Dilute 1.0 ml of the solution to 10.0 ml with the solvent mixture.

Reference solution (b). Dissolve 5.0 mg of metronidazole CRS, 5.0 mg of 2-methyl-5-nitroimidazole R and 5.0 mg of benzoic acid R in the solvent mixture and dilute to 50.0 ml with the solvent mixture. Dilute 1.0 ml of the solution to 10.0 ml with the solvent mixture.

Column:

- **size:** $l = 0.25$ m, $\varnothing = 4.6$ mm,
- **stationary phase:** spherical di-isobutyloctadecylsilyl silica gel for chromatography R (5 μ m) with a specific surface area of 180 m²/g, a pore size of 8 nm and a carbon loading of 10 per cent.

Mobile phase:

- **mobile phase A:** 1.5 g/l solution of potassium dihydrogen phosphate R adjusted to pH 3.2 with phosphoric acid R,
- **mobile phase B:** acetonitrile R,

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)
0 - 5	80	20
5 - 15	80 → 55	20 → 45
15 - 40	55	45
40 - 41	55 → 80	45 → 20
41 - 45	80	20

Flow rate: 1 ml/min.

Detection: spectrophotometer at 235 nm.

Injection: 10 μ l.

Relative retention with reference to metronidazole benzoate (retention time = about 20 min): impurity B = about 0.17; impurity A = about 0.20; impurity C = about 0.7.

System suitability: reference solution (b):

- **resolution:** minimum 2.0 between the peaks due to impurity A and impurity B.

Limits:

- **impurities A, B, C:** for each impurity, not more than the area of the corresponding peak in the chromatogram obtained with reference solution (b) (0.1 per cent),
- **any other impurity:** for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent),
- **total:** not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent),
- **disregard limit:** 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.01 per cent).

Heavy metals (2.4.8): maximum 20 ppm.

1.0 g complies with limit test C. Prepare the standard using 2 ml of lead standard solution (10 ppm Pb) R.

Loss on drying (2.2.32): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 80 °C for 3 h.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.250 g in 50 ml of anhydrous acetic acid R. Titrate with 0.1 M perchloric acid, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M perchloric acid is equivalent to 27.53 mg of C₁₃H₁₈N₂O₄.

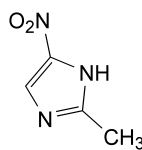
STORAGE

Protected from light.

IMPURITIES

Specified impurities: A, B, C.

A. metronidazole,



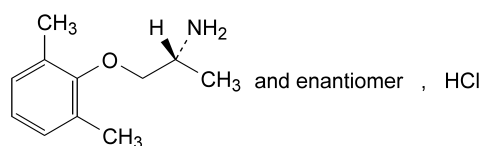
B. 2-methyl-5-nitroimidazole,

C. benzoic acid.

01/2005:1029

MEXILETINE HYDROCHLORIDE

Mexiletini hydrochloridum



C₁₁H₁₈ClNO

M_r 215.7

DEFINITION

(2RS)-1-(2,6-Dimethylphenoxy)propan-2-amine hydrochloride.

Content: 99.0 per cent to 101.0 per cent (anhydrous substance).

CHARACTERS

Appearance: white or almost white, crystalline powder.

Solubility: freely soluble in water and in methanol, sparingly soluble in methylene chloride.

It shows polymorphism.

IDENTIFICATION

A. Infrared absorption spectrophotometry (2.2.24).

Comparison: mexiletine hydrochloride CRS.

If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in methanol R, evaporate to dryness and record new spectra using the residues.

B. Dilute 1.5 ml of solution S (see Tests) to 15 ml with water R. The solution gives reaction (a) of chlorides (2.3.1).

TESTS

Solution S. Dissolve 2.0 g in carbon dioxide-free water R and dilute to 20 ml with the same solvent.

Appearance of solution. The solution is clear (2.2.1) and colourless (2.2.2, Method II).

Dilute 5 ml of solution S to 10 ml with water R.

pH (2.2.3): 4.0 to 5.5 for solution S.

Impurity D. Thin-layer chromatography (2.2.27).

Test solution. Dissolve 0.500 g of the substance to be examined in methanol R and dilute to 5.0 ml with the same solvent.

Reference solution (a). Dissolve the content of a vial of mexiletine impurity D CRS in 4.0 ml of methanol R.

Reference solution (b). Dilute 1.0 ml of the test solution to 20.0 ml with methanol R.

Reference solution (c). Dilute 1.0 ml of reference solution (a) to 5.0 ml with *methanol R*.

Reference solution (d). Dilute 1.0 ml of reference solution (a) to 5.0 ml with reference solution (b).

Plate: TLC silica gel plate *R*.

Mobile phase: concentrated ammonia *R*, alcohol *R*, acetone *R*, toluene *R* (3:7:45:45 V/V/V/V).

Application: 5 µl of the test solution and reference solutions (c) and (d).

Development: over a path of 10 cm.

Drying: in air.

Detection: spray with *ninhydrin solution R3* and heat at 100-105 °C for 15 min or until the spots appear.

System suitability: the chromatogram obtained with reference solution (b) shows 2 clearly separated spots.

Limit:

- **impurity D:** any spot corresponding to impurity D in the chromatogram obtained with the test solution is not more intense than the spot in the chromatogram obtained with reference solution (c) (0.1 per cent).

Related substances. Liquid chromatography (2.2.29).

Test solution. Dissolve 0.200 g of the substance to be examined in the mobile phase and dilute to 10.0 ml with the mobile phase.

Reference solution (a). Dilute 1.0 ml of the test solution to 10.0 ml with the mobile phase.

Reference solution (b). Dissolve the content of a vial of *mexiletine impurity C CRS* in the mobile phase and transfer the solution quantitatively to a volumetric flask containing 16.0 mg of *2,6-dimethylphenol R*. Dilute to 20.0 ml with the mobile phase. Mix 1.0 ml of this solution with 2.0 ml of reference solution (a) and dilute the mixture to 100.0 ml with the mobile phase.

Column:

- **size:** $l = 0.25$ m, $\varnothing = 4.6$ mm,
- **stationary phase:** end-capped octadecylsilyl silica gel for chromatography *R* (5 µm).

Mobile phase: mix 65 volumes of *methanol R2* and 35 volumes of a solution prepared as follows: dissolve 11.5 g of *anhydrous sodium acetate R* in 500 ml of *water R*, add 3.2 ml of *glacial acetic acid R*, mix and allow to cool; adjust to pH 4.8 with *glacial acetic acid R* and dilute to 1000 ml with *water R*.

Flow rate: 1.0 ml/min.

Detection: spectrophotometer at 262 nm.

Injection: 20 µl.

Run time: 5.5 times the retention time of mexiletine.

Relative retention with reference to mexiletine (retention time = about 4 min): impurity C = about 0.7; impurity A = about 1.8.

System suitability: reference solution (b):

- **resolution:** minimum 5.0 between the peaks due to mexiletine and impurity C.

Limits:

- **impurity A:** not more than 2.5 times the area of the corresponding peak in the chromatogram obtained with reference solution (b) (0.1 per cent),

- **impurity C:** not more than 20 times the area of the corresponding peak in the chromatogram obtained with reference solution (b) (0.1 per cent),
- **any other impurity:** for each impurity, not more than 0.5 times the area of the peak due to mexiletine in the chromatogram obtained with reference solution (b) (0.1 per cent),
- **total:** not more than 2.5 times the area of the peak due to mexiletine in the chromatogram obtained with reference solution (b) (0.5 per cent),
- **disregard limit:** 0.25 times the area of the peak due to mexiletine in the chromatogram obtained with reference solution (b) (0.05 per cent).

Heavy metals (2.4.8): maximum 10 ppm.

2.0 g complies with limit test C. Prepare the standard using 2 ml of *lead standard solution (10 ppm Pb) R*.

Water (2.5.12): maximum 0.5 per cent, determined on 1.00 g.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

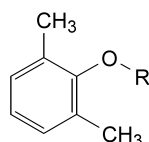
Dissolve 0.150 g in 50 ml of a mixture of equal volumes of *anhydrous acetic acid R* and *acetic anhydride R*. Titrate immediately with 0.1 M *perchloric acid*, determining the end-point potentiometrically (2.2.20) and completing the titration within 2 min.

1 ml of 0.1 M *perchloric acid* is equivalent to 21.57 mg of $C_{11}H_{18}ClNO$.

IMPURITIES

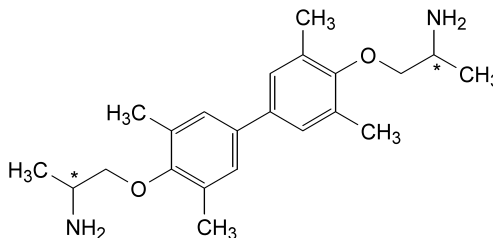
Specified impurities: A, C, D.

Other detectable impurities: B.

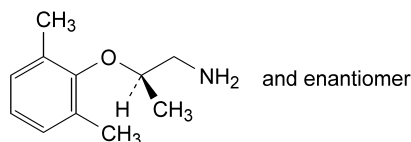


A. R = H: 2,6-dimethylphenol,

B. R = CH_2COCH_3 : 1-(2,6-dimethylphenoxy)propan-2-one,



C. 1,1'-(3,3',5,5'-tetramethylbiphenyl-4,4'-diyl)bisoxydipropyl-2-amine,

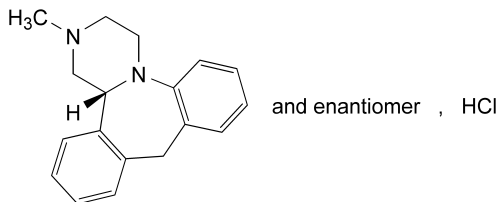


D. (2*RS*)-2-(2,6-dimethylphenoxy)propan-1-amine.

01/2005:0846

MIANSERIN HYDROCHLORIDE

Mianserini hydrochloridum

 $C_{18}H_{21}ClN_2$ M_r 300.8

DEFINITION

Mianserin hydrochloride contains not less than 98.5 per cent and not more than the equivalent of 101.0 per cent of (RS)-2-methyl-1,2,3,4,10,14b-hexahydrodibenzo[*c,f*]pyrazino[1,2-*a*]azepine hydrochloride, calculated with reference to the dried substance.

CHARACTERS

White or almost white, crystalline powder or crystals, sparingly soluble in water, soluble in methylene chloride, slightly soluble in alcohol.

IDENTIFICATION

First identification: B, D.

Second identification: A, C, D.

- A. Dissolve 50.0 mg in *water R* and dilute to 50.0 ml with the same solvent. Dilute 5.0 ml of the solution to 50.0 ml with *water R*. Examined between 230 nm and 350 nm (2.2.25), the solution shows an absorption maximum at 279 nm. The specific absorption at the maximum is 64 to 72.
- B. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *mianserin hydrochloride CRS*. Examine the substances as discs prepared using *potassium chloride R*. If the spectra obtained show differences, dissolve the substance to be examined and the reference substance separately in *methanol R*, evaporate to dryness and record new spectra using the residues.
- C. Examine by thin-layer chromatography (2.2.27), using *silica gel GF₂₅₄ R* as the coating substance.

Test solution. Dissolve 10 mg of the substance to be examined in *methylene chloride R* and dilute to 5 ml with the same solvent.

Reference solution (a). Dissolve 10 mg of *mianserin hydrochloride CRS* in *methylene chloride R* and dilute to 5 ml with the same solvent.

Reference solution (b). Dissolve 10 mg of *mianserin hydrochloride CRS* and 10 mg of *cyproheptadine hydrochloride CRS* in *methylene chloride R* and dilute to 5 ml with the same solvent.

Apply to the plate 2 µl of each solution. Develop over a path of 15 cm using a mixture of 5 volumes of *diethylamine R*, 20 volumes of *ether R* and 75 volumes of *cyclohexane R*. Examine in ultraviolet light at 254 nm. The principal spot in the chromatogram obtained with the test solution is similar in position and size to the

principal spot in the chromatogram obtained with reference solution (a). The test is not valid unless the chromatogram obtained with reference solution (b) shows two clearly separated principal spots.

D. It gives reaction (a) of chlorides (2.3.1).

TESTS

pH (2.2.3). Dissolve 0.10 g in *carbon dioxide-free water R* and dilute to 10 ml with the same solvent. The pH of the solution is 4.0 to 5.5.

Related substances. Examine by thin-layer chromatography (2.2.27), using *silica gel G R* as the coating substance.

Test solution. Dissolve 0.20 g of the substance to be examined in a mixture of 1 volume of *ammonia R* and 4 volumes of *methanol R* and dilute to 10 ml with the same mixture of solvents.

Reference solution (a). Dilute 1 ml of the test solution to 200 ml with a mixture of 1 volume of *ammonia R* and 4 volumes of *methanol R*.

Reference solution (b). Dilute 5 ml of reference solution (a) to 25 ml with a mixture of 1 volume of *ammonia R* and 4 volumes of *methanol R*.

Apply to the plate 5 µl of each solution. Develop over a path of 15 cm using a mixture of 10 volumes of *methanol R* and 90 volumes of *methylene chloride R*. Dry the plate in a current of cold air. Expose the plate to iodine vapour for 20 min. Any spot in the chromatogram obtained with the test solution, apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (a) (0.5 per cent) and at most one such spot is more intense than the spot in the chromatogram obtained with reference solution (b) (0.1 per cent).

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying over *diphosphorus pentoxide R* at 65 °C at a pressure not exceeding 700 Pa for 3 h.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

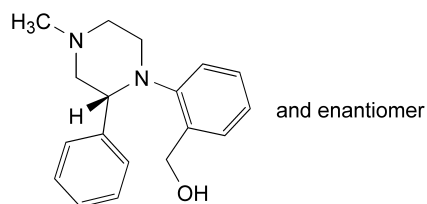
Dissolve 0.200 g in a mixture of 5.0 ml of 0.01 M *hydrochloric acid* and 50 ml of *alcohol R*. Carry out a potentiometric titration (2.2.20), using 0.1 M *sodium hydroxide*. Read the volume added between the two points of inflexion.

1 ml of 0.1 M *sodium hydroxide* is equivalent to 30.08 mg of $C_{18}H_{21}ClN_2$.

STORAGE

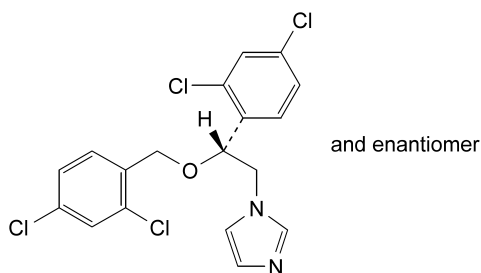
Store protected from light.

IMPURITIES



- A. [2-[(2RS)-4-methyl-2-phenylpiperazin-1-yl]phenyl]methanol.

01/2005:0935 TESTS

MICONAZOLE**Miconazolum** $C_{18}H_{14}Cl_4N_2O$ M_r 416.1**DEFINITION**

Miconazole contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of 1-[(2*RS*)-2-[(2,4-dichlorobenzyl)oxy]-2-(2,4-dichlorophenyl)ethyl]-1*H*-imidazole, calculated with reference to the dried substance.

CHARACTERS

A white or almost white powder, very slightly soluble in water, freely soluble in methanol, soluble in alcohol.

It shows polymorphism.

IDENTIFICATION

First identification: A, B.

Second identification: A, C, D.

- A. Melting point (2.2.14): 83 °C to 87 °C.
- B. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *miconazole CRS*. Examine the substances as discs prepared using *potassium bromide R*.
- C. Examine by thin-layer chromatography (2.2.27), using a suitable octadecylsilyl silica gel as the coating substance.
- Test solution.* Dissolve 30 mg of the substance to be examined in the mobile phase and dilute to 5 ml with the mobile phase.
- Reference solution (a).* Dissolve 30 mg of *miconazole CRS* in the mobile phase and dilute to 5 ml with the mobile phase.
- Reference solution (b).* Dissolve 30 mg of *miconazole CRS* and 30 mg of *econazole nitrate CRS* in the mobile phase and dilute to 5 ml with the mobile phase. Apply separately to the plate 5 µl of each solution. Develop over a path of 15 cm using a mixture of 20 volumes of *ammonium acetate solution R*, 40 volumes of *dioxan R* and 40 volumes of *methanol R*. Dry the plate in a current of warm air for 15 min and expose it to iodine vapour until the spots appear. Examine in daylight. The principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a). The test is not valid unless the chromatogram obtained with reference solution (b) shows 2 clearly separated spots.
- D. To 30 mg in a porcelain crucible add 0.3 g of *anhydrous sodium carbonate R*. Heat over an open flame for 10 min. Allow to cool. Take up the residue with 5 ml of *dilute nitric acid R* and filter. To 1 ml of the filtrate add 1 ml of *water R*. The solution gives reaction (a) of chlorides (2.3.1).

Solution S. Dissolve 0.1 g in *methanol R* and dilute to 10 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and is not more intensely coloured than reference solution Y_6 (2.2.2, *Method II*).

Optical rotation (2.2.7). The angle of optical rotation of solution S is -0.10° to $+0.10^\circ$.

Related substances. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 0.100 g of the substance to be examined in the mobile phase and dilute to 10.0 ml with the mobile phase.

Reference solution (a). Dissolve 2.5 mg of *miconazole CRS* and 2.5 mg of *econazole nitrate CRS* in the mobile phase and dilute to 100.0 ml with the mobile phase.

Reference solution (b). Dilute 1.0 ml of the test solution to 100.0 ml with the mobile phase. Dilute 5.0 ml of this solution to 20.0 ml with the mobile phase.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.10 m long and 4.6 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (3 µm),
- as mobile phase at a flow rate of 2 ml/min a solution of 6.0 g of *ammonium acetate R* in a mixture of 300 ml of *acetonitrile R*, 320 ml of *methanol R* and 380 ml of *water R*,
- as detector a spectrophotometer set at 235 nm.

Equilibrate the column with the mobile phase at a flow rate of 2 ml/min for about 30 min.

Adjust the sensitivity of the system so that the height of the principal peak in the chromatogram obtained with 10 µl of reference solution (b) is not less than 50 per cent of the full scale of the recorder.

Inject 10 µl of reference solution (a). When the chromatograms are recorded in the prescribed conditions, the retention times are: *econazole nitrate*, about 10 min; *miconazole*, about 20 min. The test is not valid unless the resolution between the peaks corresponding to *econazole nitrate* and *miconazole* is not less than 10; if necessary, adjust the composition of the mobile phase.

Inject separately 10 µl of the test solution and 10 µl of reference solution (b). Continue the chromatography for 1.2 times the retention time of the principal peak. In the chromatogram obtained with the test solution: the area of any peak, apart from the principal peak, is not greater than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.25 per cent); the sum of the areas of all the peaks, apart from the principal peak, is not greater than twice that of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent). Disregard any peak due to the solvent and any peak with an area less than 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (b).

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying *in vacuo* at 60 °C for 4 h.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.300 g in 50 ml of a mixture of 1 volume of *anhydrous acetic acid R* and 7 volumes of *methyl ethyl ketone R*. Using 0.2 ml of *naphtholbenzein solution R* as indicator, titrate with 0.1 *M perchloric acid* until the colour changes from orange-yellow to green.

1 ml of 0.1 M perchloric acid is equivalent to 41.61 mg of $C_{18}H_{14}Cl_4N_2O$.

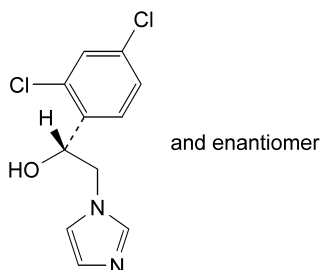
STORAGE

Store protected from light.

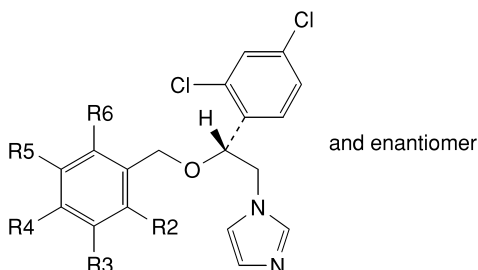
IMPURITIES

Specified impurities: A, B, C, D, E, F, G.

Other detectable impurities: H, I.



A. (1*RS*)-1-(2,4-dichlorophenyl)-2-(1*H*-imidazol-1-yl)ethanol,



B. R2 = R3 = R5 = R6 = H, R4 = Cl: 1-[(2*RS*)-2-[(4-chlorobenzyl)oxy]-2-(2,4-dichlorophenyl)ethyl]-1*H*-imidazole,

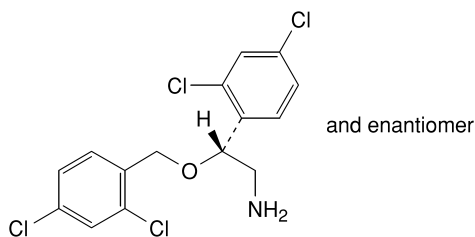
D. R2 = R6 = Cl, R3 = R4 = R5 = H: 1-[(2*RS*)-2-[(2,6-dichlorobenzyl)oxy]-2-(2,4-dichlorophenyl)ethyl]-1*H*-imidazole,

F. R2 = R5 = R6 = H, R3 = R4 = Cl: 1-[(2*RS*)-2-[(3,4-dichlorobenzyl)oxy]-2-(2,4-dichlorophenyl)ethyl]-1*H*-imidazole,

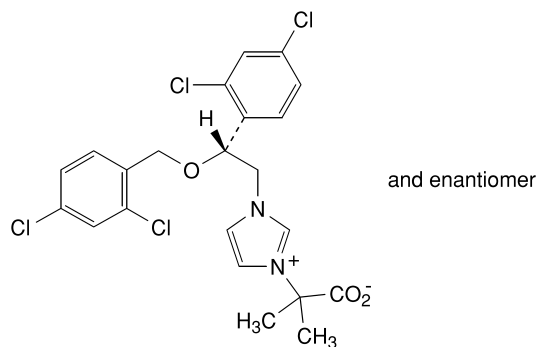
G. R2 = R5 = Cl, R3 = R4 = R6 = H: 1-[(2*RS*)-2-[(2,5-dichlorobenzyl)oxy]-2-(2,4-dichlorophenyl)ethyl]-1*H*-imidazole,

H. R2 = R3 = R4 = R5 = R6 = H: 1-[(2*RS*)-2-benzyloxy-2-(2,4-dichlorophenyl)ethyl]-1*H*-imidazole,

I. R2 = Cl, R3 = R4 = R5 = R6 = H: 1-[(2*RS*)-2-[(2-chlorobenzyl)oxy]-2-(2,4-dichlorophenyl)ethyl]-1*H*-imidazole,



C. (2*RS*)-2-[(2,4-dichlorobenzyl)oxy]-2-(2,4-dichlorophenyl)ethanamine,

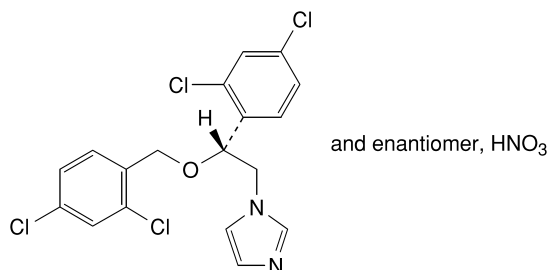


E. 2-[1-[(2*RS*)-2-[(2,4-dichlorobenzyl)oxy]-2-(2,4-dichlorophenyl)ethyl]-1*H*-imidazol-3-yl]-2-methylpropanoate.

01/2005:0513

MICONAZOLE NITRATE

Miconazoli nitras



$C_{18}H_{15}Cl_4N_3O_4$

M_r 479.1

DEFINITION

Miconazole nitrate contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of 1-[(2*RS*)-2-[(2,4-dichlorobenzyl)oxy]-2-(2,4-dichlorophenyl)ethyl]-1*H*-imidazole nitrate, calculated with reference to the dried substance.

CHARACTERS

A white or almost white powder, very slightly soluble in water, sparingly soluble in methanol, slightly soluble in alcohol.

IDENTIFICATION

First identification: A, B.

Second identification: A, C, D.

A. Melting point (2.2.14): 178 °C to 184 °C.

B. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *miconazole nitrate CRS*. Examine the substances prepared as discs using *potassium bromide R*.

C. Examine by thin-layer chromatography (2.2.27), using a suitable octadecylsilyl silica gel as the coating substance.

Test solution. Dissolve 30 mg of the substance to be examined in the mobile phase and dilute to 5 ml with the mobile phase.

Reference solution (a). Dissolve 30 mg of *miconazole nitrate CRS* in the mobile phase and dilute to 5 ml with the mobile phase.

Reference solution (b). Dissolve 30 mg of *miconazole nitrate CRS* and 30 mg of *econazole nitrate CRS* in the mobile phase and dilute to 5 ml with the mobile phase.

Apply separately to the plate 5 µl of each solution. Develop over a path of 15 cm using a mixture of 20 volumes of *ammonium acetate solution R*, 40 volumes of *dioxan R* and 40 volumes of *methanol R*. Dry the plate in a current of warm air for 15 min and expose it to iodine vapour until the spots appear. Examine in daylight. The principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a). The test is not valid unless the chromatogram obtained with reference solution (b) shows two clearly separated spots.

D. It gives the reaction of nitrates (2.3.1).

TESTS

Solution S. Dissolve 0.1 g in *methanol R* and dilute to 10 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and is not more intensely coloured than reference solution Y₇ (2.2.2, *Method II*).

Optical rotation (2.2.7). The angle of optical rotation of solution S is -0.10° to $+0.10^{\circ}$.

Related substances. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 0.100 g of the substance to be examined in the mobile phase and dilute to 10.0 ml with the mobile phase.

Reference solution (a). Dissolve 2.5 mg of *miconazole nitrate CRS* and 2.5 mg of *econazole nitrate CRS* in the mobile phase and dilute to 100.0 ml with the mobile phase.

Reference solution (b). Dilute 1.0 ml of the test solution to 100.0 ml with the mobile phase. Dilute 5.0 ml of this solution to 20.0 ml with the mobile phase.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.10 m long and 4.6 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (3 µm),
- as mobile phase at a flow rate of 2 ml/min a solution of 6.0 g of *ammonium acetate R* in a mixture of 300 ml of *acetonitrile R*, 320 ml of *methanol R* and 380 ml of *water R*,
- as detector a spectrophotometer set at 235 nm.

Equilibrate the column with the mobile phase at a flow rate of 2 ml/min for about 30 min.

Adjust the sensitivity of the system so that the height of the principal peak in the chromatogram obtained with 10 µl of reference solution (b) is not less than 50 per cent of the full scale of the recorder.

Inject 10 µl of reference solution (a). When the chromatogram is recorded in the prescribed conditions, the retention times are: econazole nitrate, about 10 min; miconazole nitrate, about 20 min. The test is not valid unless the resolution between the peaks corresponding to econazole nitrate and miconazole nitrate is at least 10; if necessary, adjust the composition of the mobile phase.

Inject separately 10 µl of the test solution and 10 µl of reference solution (b). Continue the chromatography for 1.2 times the retention time of the principal peak. In the chromatogram obtained with the test solution: the area of any peak apart from the principal peak is not greater than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.25 per cent); the sum of the areas of the peaks apart from the principal peak is not greater than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per

cent). Disregard any peak due to the nitrate ion and any peak with an area less than 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (b).

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C for 2 h.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.350 g in 75 ml of *anhydrous acetic acid R*, with slight heating if necessary. Titrate with 0.1 M *perchloric acid* determining the end-point potentiometrically (2.2.20). Carry out a blank titration.

1 ml of 0.1 M *perchloric acid* is equivalent to 47.91 mg of C₁₈H₁₅Cl₄N₃O₄.

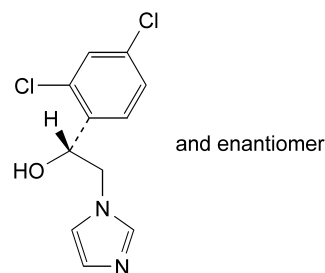
STORAGE

Store protected from light.

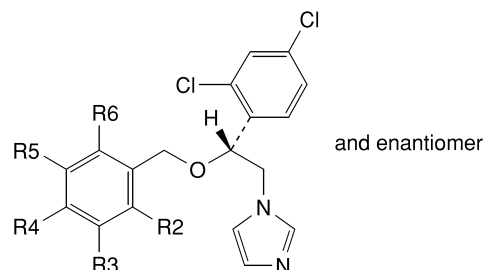
IMPURITIES

Specified impurities: A, B, C, D, E, F, G.

Other detectable impurities: H, I.



A. (1*RS*)-1-(2,4-dichlorophenyl)-2-(1*H*-imidazol-1-yl)ethanol,



B. R₂ = R₃ = R₅ = R₆ = H, R₄ = Cl: 1-[(2*RS*)-2-[(4-chlorobenzyl)oxy]-2-(2,4-dichlorophenyl)ethyl]-1*H*-imidazole,

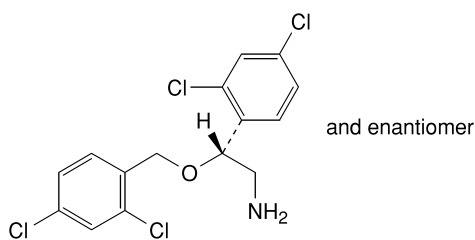
D. R₂ = R₆ = Cl, R₃ = R₄ = R₅ = H: 1-[(2*RS*)-2-[(2,6-dichlorobenzyl)oxy]-2-(2,4-dichlorophenyl)ethyl]-1*H*-imidazole,

F. R₂ = R₅ = R₆ = H, R₃ = R₄ = Cl: 1-[(2*RS*)-2-[(3,4-dichlorobenzyl)oxy]-2-(2,4-dichlorophenyl)ethyl]-1*H*-imidazole,

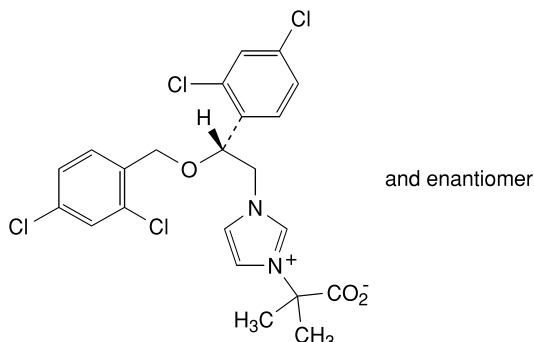
G. R₂ = R₅ = Cl, R₃ = R₄ = R₆ = H: 1-[(2*RS*)-2-[(2,5-dichlorobenzyl)oxy]-2-(2,4-dichlorophenyl)ethyl]-1*H*-imidazole,

H. R₂ = R₃ = R₄ = R₅ = R₆ = H: 1-[(2*RS*)-2-benzoyloxy-2-(2,4-dichlorophenyl)ethyl]-1*H*-imidazole,

I. R₂ = Cl, R₃ = R₄ = R₅ = R₆ = H: 1-[(2*RS*)-2-[(2-chlorobenzyl)oxy]-2-(2,4-dichlorophenyl)ethyl]-1*H*-imidazole,



- C. (2*RS*)-2-[(2,4-dichlorobenzyl)oxy]-2-(2,4-dichlorophenyl)ethanamine,

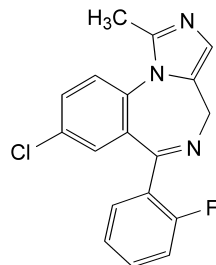


- E. 2-[1-[(2*RS*)-2-[(2,4-dichlorobenzyl)oxy]-2-(2,4-dichlorophenyl)ethyl]-1*H*-imidazol-3-yl]-2-methylpropanoate.

01/2005:0936

MIDAZOLAM

Midazolamum



$C_{18}H_{13}ClFN_3$

M_r 325.8

DEFINITION

Midazolam contains not less than 98.5 per cent and not more than the equivalent of 101.5 per cent of 8-chloro-6-(2-fluorophenyl)-1-methyl-4*H*-imidazo[1,5-*a*][1,4]benzodiazepine, calculated with reference to the dried substance.

CHARACTERS

A white or yellowish, crystalline powder, practically insoluble in water, freely soluble in acetone and in alcohol, soluble in methanol.

IDENTIFICATION

First identification: B.

Second identification: A, C, D, E.

- Melting point (2.2.14): 161 °C to 164 °C.
- Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *midazolam CRS*.
- Examine the chromatograms obtained in the test for related substances in ultraviolet light at 254 nm. The principal spot in the chromatogram obtained with test

solution (b) is similar in position and size to the principal spot in the chromatogram obtained with reference solution (b).

- Mix 90 mg with 0.30 g of *anhydrous sodium carbonate R* and ignite in a crucible until an almost white residue is obtained (normally in less than 5 min). Allow to cool and dissolve the residue in 5 ml of *dilute nitric acid R*. Filter (the filtrate is also used in identification test E). Add 1.0 ml of the filtrate to a freshly prepared mixture of 0.1 ml of *alizarin S solution R* and 0.1 ml of *zirconyl nitrate solution R*. Mix, allow to stand for 5 min and compare the colour of the solution with that of a blank prepared in the same manner. The colour of the test solution is yellow and that of the blank is red.
- To 1 ml of the filtrate obtained in identification test D add 1 ml of *water R*. The solution gives reaction (a) of chlorides (2.3.1).

TESTS

Appearance of solution. Dissolve 0.1 g in 0.1 *M* *hydrochloric acid* and dilute to 10 ml with the same acid. The solution is clear (2.2.1) and not more intensely coloured than reference solution Y_6 (2.2.2, *Method II*).

Related substances. Examine by thin-layer chromatography (2.2.27), using *silica gel GF₂₅₄ R* as the coating substance.

Test solution (a). Dissolve 0.2 g of the substance to be examined in *alcohol R* and dilute to 5 ml with the same solvent.

Test solution (b). Dilute 1 ml of test solution (a) to 50 ml with *alcohol R*.

Reference solution (a). Dilute 1 ml of test solution (a) to 10 ml with *alcohol R*. Dilute 2 ml of the solution to 100 ml with *alcohol R*.

Reference solution (b). Dissolve 8 mg of *midazolam CRS* in *alcohol R* and dilute to 10 ml with the same solvent.

Reference solution (c). Dissolve 8 mg of *midazolam CRS* and 8 mg of *chlordiazepoxide CRS* in *alcohol R* and dilute to 10 ml with the same solvent.

Apply separately to the plate 5 µl of each solution. Develop over a path of 12 cm using a mixture of 2 volumes of *glacial acetic acid R*, 15 volumes of *water R*, 20 volumes of *methanol R* and 80 volumes of *ethyl acetate R*. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. Any spot in the chromatogram obtained with test solution (a), apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (a) (0.2 per cent). The test is not valid unless the chromatogram obtained with reference solution (c) shows two clearly separated spots.

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C for 2 h.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g in a platinum crucible.

ASSAY

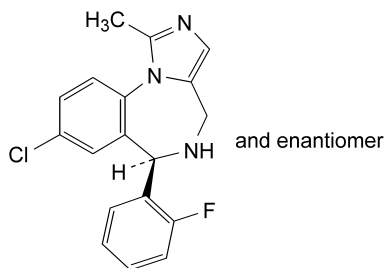
Dissolve 0.120 g in 30 ml of *anhydrous acetic acid R* and add 20 ml of *acetic anhydride R*. Titrate with 0.1 *M* *perchloric acid* determining the end-point potentiometrically (2.2.20). Titrate to the second point of inflexion.

1 ml of 0.1 *M* *perchloric acid* is equivalent to 16.29 mg of $C_{18}H_{13}ClFN_3$.

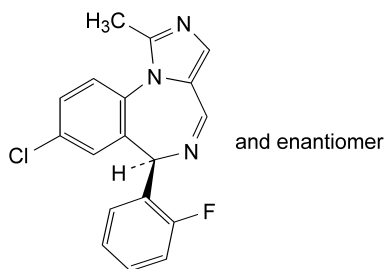
STORAGE

Store protected from light.

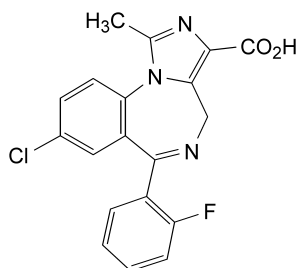
IMPURITIES



- A. (6*RS*)-8-chloro-6-(2-fluorophenyl)-1-methyl-5,6-dihydro-4*H*-imidazo[1,5-*a*][1,4]benzodiazepine,



- B. (6*RS*)-8-chloro-6-(2-fluorophenyl)-1-methyl-6*H*-imidazo[1,5-*a*][1,4]benzodiazepine,



- C. 8-chloro-6-(2-fluorophenyl)-1-methyl-4*H*-imidazo[1,5-*a*][1,4]benzodiazepine-3-carboxylic acid.

01/2005:1860

MILK-THISTLE FRUIT

Silybi mariani fructus

DEFINITION

Mature fruit, devoid of the pappus, of *Silybum marianum* L. Gaertner.

Content: minimum 1.5 per cent of silymarin expressed as silibinin (C₂₅H₂₂O₁₀; *M_r* 482.4) (dried drug).

CHARACTERS

No rancid odour.

Macroscopic and microscopic characters described under identification tests A and B.

IDENTIFICATION

- A. Strongly compressed, elongate-obovate achenes, about 6 mm to 8 mm long, 3 mm broad and 1.5 mm thick; outer surface smooth and shiny with a grey to pale brown ground colour variably streaked dark brown longitudinally to give an overall pale greyish to brown colour; tapering at the base and crowned at the apex with a glistening, pale yellow extension forming a collar about 1 mm high surrounding the remains of the style. Cut transversely, the fruit shows a narrow, brown outer area and 2 large, dense, white oily cotyledons.

- B. Reduce to a powder (355). The powder is brownish-yellow with darker specks. Examine under a microscope using *chloral hydrate solution R*. The powder shows: fragments of the epicarp composed of colourless cells, polygonal in surface view, the lumen appearing fairly large or as a small slit, depending on the orientation; groups of parenchymatous cells from the pigment layer, some of them containing colouring matter which appears bright red; very abundant groups of large sclereids from the testa with bright yellow pitted walls and a narrow lumen; occasionally fragments of small-celled parenchyma with pitted and beaded walls; abundant thin-walled parenchymatous cells from the cotyledons containing oil globules and scattered cluster crystals of calcium oxalate; a few larger, prismatic crystals of calcium oxalate.

- C. Thin-layer chromatography (2.2.27).

Test solution. To 1.0 g of powdered drug (500) add 10 ml of *methanol R*. Heat under reflux in a water-bath at 70 °C for 5 min. Cool and filter. Evaporate the filtrate to dryness and dissolve the residue in 1.0 ml of *methanol R*.

Reference solution. Dissolve 2 mg of *silibinin R* and 5 mg of *taxifolin R* in 10 ml of *methanol R*.

Plate: TLC silica gel plate *R*.

Mobile phase: *anhydrous formic acid R*, *acetone R*, *methylene chloride R* (8.5:16.5:75 V/V/V).

Application: 30 µl of the test solution and 10 µl of the reference solution, as bands.

Development: over a path of 10 cm.

Drying: at 100-105 °C.

Detection: spray the warm plate with a 10 g/l solution of *diphenylboric acid aminoethyl ester R* in *methanol R* and subsequently spray with a 50 g/l solution of *macrogol 400 R* in *methanol R*. Allow the plate to dry for 30 min and examine in ultraviolet light at 365 nm.

Results: see below the sequence of the zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other orange and yellowish-green fluorescent zones are present between the zones of silibinin and taxifolin in the chromatogram obtained with the test solution.

Top of the plate	
Silibinin: a yellowish-green fluorescent zone	A yellowish-green fluorescent zone (silibinin)
Taxifolin: an orange fluorescent zone	An orange fluorescent zone (taxifolin)
	A yellowish-green fluorescent zone (silicristin)
	A light blue fluorescent zone (starting line)
Reference solution	Test solution

TESTS

Foreign matter (2.8.2): maximum 2 per cent.

Loss on drying (2.2.32): maximum 8.0 per cent, determined on 1.000 g of the powdered drug (500) by drying in an oven at 100-105 °C for 2 h.

Total ash (2.4.16): maximum 8.0 per cent.

ASSAY

Liquid chromatography (2.2.29).

Test solution. Place 5.00 g of the powdered drug (500) in a continuous-extraction apparatus. Add 100 ml of *light petroleum R* and heat in a water-bath for 8 h. Allow the defatted drug to dry at room temperature. In a continuous-extraction apparatus, extract the latter with 100 ml of *methanol R* in a water-bath for 5 h. Evaporate the methanolic extract *in vacuo* to a volume of about 30 ml. Filter into a 50 ml volumetric flask, rinsing the extraction flask and the filter, and diluting to 50.0 ml with *methanol R*. Dilute 5.0 ml of this solution to 50.0 ml with *methanol R*.

Reference solution (a). Dissolve 5.0 mg of *silibinin R*, dried *in vacuo*, in *methanol R* and dilute to 50.0 ml with the same solvent.

Reference solution (b). Dissolve 1.0 mg of *silicristin R* in *methanol R* and dilute to 10.0 ml with the same solvent.

Reference solution (c). Dissolve 1.0 mg of *silidianin R* in *methanol R* and dilute to 10.0 ml with the same solvent.

Reference solution (d). Dissolve 1.0 mg of *isosilibinin R* in *methanol R* and dilute to 10.0 ml with the same solvent.

Column:

- size: $l = 0.125$ m, $\varnothing = 4$ mm,
- stationary phase: octadecylsilyl silica gel for chromatography *R* (5 μ m).

Mobile phase:

- mobile phase A: phosphoric acid *R*, *methanol R*, water *R* (0.5:35:65 V/V/V),
- mobile phase B: phosphoric acid *R*, *methanol R*, water *R* (0.5:50:50 V/V/V),

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)
0 - 28	100 → 0	0 → 100
28 - 35	0	100
35 - 36	0 → 100	100 → 0
36 - 51	100	0

Flow rate: 0.8 ml/min.

Detection: spectrophotometer at 288 nm.

Injection: 10 μ l.

Retention time: silibinin B = about 30 min. If necessary, adjust the time periods of the gradient.

System suitability: reference solution:

- resolution: minimum 1.8 between the peaks due to silibinin A and silibinin B.

Using the retention times determined from the chromatogram obtained with the reference solutions, locate the peaks of silicristin, silidianin, silibinin A, silibinin B, isosilibinin A and isosilibinin B in the chromatogram obtained with the test solution. The peak due to silidianin may vary in size, be absent or be present as the major peak. Determine the area of the peaks due to silicristin, silidianin, silibinin A, silibinin B, isosilibinin A and isosilibinin B.

Calculate the percentage content of silymarin, calculated as silibinin, from the expression:

$$\frac{(A1 + A2 + A3 + A4 + A5 + A6) \times m_1 \times p \times 1000}{(A7 + A8) \times m_2 \times (100 - d)}$$

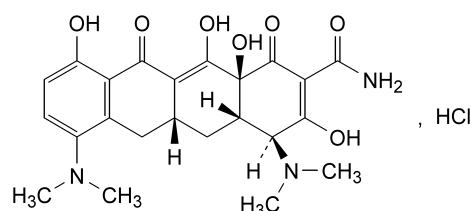
- A1 = area of the peak due to silicristin in the chromatogram obtained with the test solution,
- A2 = area of the peak due to silidianin in the chromatogram obtained with the test solution,

- A3 = area of the peak due to silibinin A in the chromatogram obtained with the test solution,
- A4 = area of the peak due to silibinin B in the chromatogram obtained with the test solution,
- A5 = area of the peak due to isosilibinin A in the chromatogram obtained with the test solution,
- A6 = area of the peak due to isosilibinin B in the chromatogram obtained with the test solution,
- A7 = area of the peak due to silibinin A in the chromatogram obtained with reference solution (a),
- A8 = area of the peak due to silibinin B in the chromatogram obtained with reference solution (a),
- m_1 = mass of *silibinin R* in the reference solution, in grams,
- m_2 = mass of the drug to be examined, in grams,
- p = combined percentage content of silibinin A and silibinin B in *silibinin R*,
- d = percentage loss on drying of the drug.

01/2005:1030

MINOCYCLINE HYDROCHLORIDE

Minocyclini hydrochloridum



$C_{23}H_{28}ClN_3O_7$

M_r 493.9

DEFINITION

(4*S*,4*aS*,5*aR*,12*aS*)-4,7-Bis(dimethylamino)-3,10,12,12*a*-tetrahydroxy-1,11-dioxo-1,4,4*a*,5,5*a*,6,11,12*a*-octahydrotetracene-2-carboxamide hydrochloride.

Content: 96.0 per cent to 102.5 per cent (anhydrous substance).

CHARACTERS

Appearance: yellow, crystalline powder, hygroscopic.

Solubility: sparingly soluble in water, slightly soluble in alcohol. It dissolves in solutions of alkali hydroxides and carbonates.

IDENTIFICATION

A. Thin-layer chromatography (2.2.27).

Test solution. Dissolve 5 mg of the substance to be examined in *methanol R* and dilute to 10 ml with the same solvent.

Reference solution (a). Dissolve 5 mg of *minocycline hydrochloride CRS* in *methanol R* and dilute to 10 ml with the same solvent.

Reference solution (b). Dissolve 5 mg of *minocycline hydrochloride CRS* and 5 mg of *oxytetracycline hydrochloride CRS* in *methanol R* and dilute to 10 ml with the same solvent.

Plate: TLC octadecylsilyl silica gel F_{254} plate *R*.

Mobile phase: mix 20 volumes of *acetonitrile R*, 20 volumes of *methanol R* and 60 volumes of a 63 g/l solution of *oxalic acid R* previously adjusted to pH 2 with *concentrated ammonia R*.

Application: 1 µl

Development: over 3/4 of the plate.

Drying: in air.

Detection: examine in ultraviolet light at 254 nm.

System suitability: reference solution (b):

- the chromatogram shows 2 clearly separated spots.

Results: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a).

B. To about 2 mg add 5 ml of *sulphuric acid R*. A bright yellow colour develops. Add 2.5 ml of *water R* to the solution. The solution becomes pale yellow.

C. It gives reaction (a) of chlorides (2.3.1).

TESTS

Solution S. Dissolve 0.200 g in *carbon dioxide-free water R* and dilute to 20.0 ml with the same solvent.

Appearance of solution. The solution is clear (2.2.1) and its absorbance (2.2.25) at 450 nm using a 1 cm cell is not greater than 0.23.

Dilute 1.0 ml of solution S to 10.0 ml with *water R*.

pH (2.2.3): 3.5 to 4.5 for solution S.

Light-absorbing impurities. Carry out the measurement within 1 h of preparing solution S.

The absorbance (2.2.25) of solution S measured at 560 nm is not greater than 0.06.

Related substances. Liquid chromatography (2.2.29).

Carry out the test protected from bright light. Store the solutions at a temperature of 2–8 °C and use them within 3 h of preparation.

Test solution (a). Dissolve 25.0 mg of the substance to be examined in the mobile phase and dilute to 100.0 ml with the mobile phase.

Test solution (b). Dilute 10.0 ml of test solution (a) to 20.0 ml with the mobile phase.

Reference solution (a). Dissolve 12.5 mg of *minocycline hydrochloride CRS* in the mobile phase and dilute to 100.0 ml with the mobile phase.

Reference solution (b). Dilute 2.0 ml of test solution (a) to 100.0 ml with the mobile phase.

Reference solution (c). Dilute 1.2 ml of test solution (a) to 100.0 ml with the mobile phase.

Reference solution (d). Dissolve 10 mg of *minocycline hydrochloride CRS* in 1 ml of *water R*. Boil the solution on a water-bath for 20 min. Dilute to 25 ml with the mobile phase.

Column:

- size: $l = 0.20$ m, $\varnothing = 4.6$ mm,
- stationary phase: octylsilyl silica gel for chromatography *R* (5 µm).

Mobile phase: mix 25 volumes of a 4 g/l solution of *sodium edetate R*, 27 volumes of *dimethylformamide R* and 50 volumes of a 28 g/l solution of *ammonium oxalate R*, adjust to pH 7.0 using *tetrabutylammonium hydroxide solution (104 g/l) R*.

Flow rate: 1 ml/min.

Detection: spectrophotometer at 280 nm.

Injection: 20 µl; inject test solution (a) and reference solutions (a), (b), (c) and (d).

Run time: 1.5 times the retention time of minocycline.

System suitability:

- resolution: minimum 2.0 between the peaks due to impurity A and minocycline in the chromatogram obtained with reference solution (d),
- number of theoretical plates: minimum 15 000, calculated for the peak due to minocycline in the chromatogram obtained with reference solution (a).

Limits:

- impurity A: not more than the area of the principal peak in the chromatogram obtained with reference solution (c) (1.2 per cent),
- any other impurity: not more than the area of the principal peak in the chromatogram obtained with reference solution (c) (1.2 per cent),
- total of other impurities: not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (2.0 per cent).

Heavy metals (2.4.8): maximum 50 ppm.

0.5 g complies with limit test C. Prepare the standard using 2.5 ml of *lead standard solution (10 ppm Pb) R*.

Water (2.5.12): 5.0 per cent to 8.0 per cent, determined on 0.500 g.

Sulphated ash (2.4.14): maximum 0.5 per cent, determined on 1.0 g.

Bacterial endotoxins (2.6.14): less than 1.25 IU/mg, if intended for use in the manufacture of parenteral dosage forms without a further appropriate procedure for the removal of bacterial endotoxins.

ASSAY

Liquid chromatography (2.2.29) as described in the test for related substances with the following modifications.

Injection: test solution (b) and reference solution (a).

System suitability:

- repeatability: maximum relative standard deviation of the peak area for minocycline of 1.5 per cent after 6 injections of reference solution (a).

Calculate the percentage content of $C_{23}H_{28}ClN_3O_7$.

STORAGE

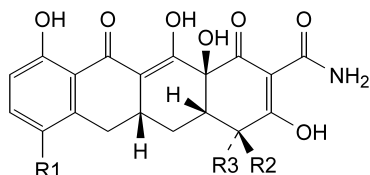
In an airtight container, protected from light. If the substance is sterile, store in a sterile, airtight, tamper-proof container.

LABELLING

The label states, where applicable, that the substance is free from bacterial endotoxins.

IMPURITIES

Specified impurities: A, B, C, D.

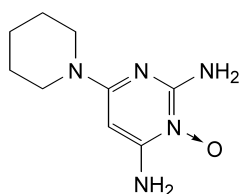


- A. $R_1 = R_3 = N(CH_3)_2$, $R_2 = H$: (4*R*,4*aS*,5*aR*,12*aS*)-4,7-bis(dimethylamino)-3,10,12,12*a*-tetrahydroxy-1,11-dioxo-1,4,4*a*,5,5*a*,6,11,12*a*-octahydrotetracene-2-carboxamide (4-epiminocycline),
- B. $R_1 = R_3 = H$, $R_2 = N(CH_3)_2$: (4*S*,4*aS*,5*aR*,12*aS*)-4-(dimethylamino)-3,10,12,12*a*-tetrahydroxy-1,11-dioxo-1,4,4*a*,5,5*a*,6,11,12*a*-octahydrotetracene-2-carboxamide (sancycline),
- C. $R_1 = NH-CH_3$, $R_2 = N(CH_3)_2$, $R_3 = H$: (4*S*,4*aS*,5*aR*,12*aS*)-4-(dimethylamino)-3,10,12,12*a*-tetrahydroxy-7-(methylamino)-1,11-dioxo-1,4,4*a*,5,5*a*,6,11,12*a*-octahydrotetracene-2-carboxamide (7-monodemethylminocycline),
- D. $R_1 = NH_2$, $R_2 = N(CH_3)_2$, $R_3 = H$: (4*S*,4*aS*,5*aR*,12*aS*)-7-amino-4-(dimethylamino)-3,10,12,12*a*-tetrahydroxy-1,11-dioxo-1,4,4*a*,5,5*a*,6,11,12*a*-octahydrotetracene-2-carboxamide (7-aminosancycline).

01/2005:0937
corrected

MINOXIDIL

Minoxidilum



$C_9H_{15}N_5O$

M_r 209.3

DEFINITION

Minoxidil contains not less than 98.5 per cent and not more than the equivalent of 101.0 per cent of 6-(piperidin-1-yl)pyrimidine-2,4-diamine 3-oxide, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, crystalline powder, slightly soluble in water, soluble in methanol and in propylene glycol.

IDENTIFICATION

First identification: A, B.

Second identification: A, C, D.

- A. Dissolve 20.0 mg in 0.1 *M* hydrochloric acid and dilute to 100.0 ml with the same solvent (solution a). Dilute 2.0 ml of solution (a) to 100.0 ml with 0.1 *M* hydrochloric acid (solution b) and dilute 2.0 ml of solution (a) to 100.0 ml with 0.1 *M* sodium hydroxide (solution c). Examine solutions (b) and (c) between 200 nm and 350 nm (2.2.25). Solution (b) shows two absorption maxima, at 230 nm and 281 nm. The specific absorbance at the maximum at 230 nm is 1015 to 1120 and that at the maximum at 281 nm is 1060 to 1170. Solution (c) shows three

absorption maxima, at 230 nm, 262 nm and 288 nm. The specific absorbance at the maximum at 230 nm is 1525 to 1685, that at the maximum at 262 nm is 485 to 535 and that at the maximum at 288 nm is 555 to 605.

- B. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with minoxidil CRS.

- C. Examine by thin-layer chromatography (2.2.27), using silica gel GF_{254} R as the coating substance.

Test solution. Dissolve 10 mg of the substance to be examined in methanol R and dilute to 10 ml with the same solvent.

Reference solution. Dissolve 10 mg of minoxidil CRS in methanol R and dilute to 10 ml with the same solvent.

Apply to the plate 2 µl of each solution. Develop over a path of 10 cm using a mixture of 1.5 volumes of concentrated ammonia R and 100 volumes of methanol R. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. The principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with the reference solution.

- D. Dissolve about 10 mg in 1 ml of methanol R. Add 0.1 ml of copper sulphate solution R. A green colour develops. The solution becomes greenish-yellow on the addition of 0.1 ml of dilute hydrochloric acid R.

TESTS

Appearance of solution. Dissolve 0.5 g in 12.5 ml of methanol R and dilute to 25 ml with water R. The solution is clear (2.2.1) and not more intensely coloured than reference solution Y₆ (2.2.2, Method II).

Related substances. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 25.0 mg of the substance to be examined in the mobile phase and dilute to 100.0 ml with the mobile phase.

Reference solution (a). Dilute 1.0 ml of the test solution to 100.0 ml with the mobile phase.

Reference solution (b). Dissolve 5 mg of deoxyminoxidil CRS (minoxidil impurity E) in the mobile phase and dilute to 20 ml with the mobile phase. To 2 ml of this solution, add 2 ml of the test solution and dilute to 10 ml with the mobile phase.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.10 m long and 3 mm in internal diameter packed with octadecylsilyl silica gel for chromatography R (5 µm),
- as mobile phase at a flow rate of 1 ml/min a mixture prepared as follows: dissolve 3.0 g of docusate sodium R in a mixture of 10 ml of glacial acetic acid R and 300 ml of water R, adjust to pH 3.0 with perchloric acid R and add 700 ml of methanol R,
- as detector a spectrophotometer set at 240 nm,
- a 10 µl loop injector.

Inject 10 µl of each solution and continue the chromatography for twice the retention time of the principal peak. In the chromatogram obtained with the test solution, the sum of the areas of any peaks, apart from the principal peak, is not greater than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (1.5 per cent). The test is not valid unless, in the chromatogram obtained with reference solution (b), the resolution between the peaks corresponding to minoxidil and deoxyminoxidil is at least 2.0. Disregard any peak with an area less than 0.1 times of that of the peak in the chromatogram obtained with reference solution (a).

01/2005:1838

Heavy metals (2.4.8). 1.0 g complies with the limit test C for heavy metals (20 ppm). Prepare the standard using 2 ml of *lead standard solution* (10 ppm Pb) R.

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 100-105 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

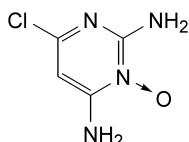
Dissolve 0.150 g in 50 ml of *anhydrous acetic acid* R. Titrate with 0.1 M *perchloric acid*, determining the end-point potentiometrically (2.2.20). Carry out a blank titration.

1 ml of 0.1 M *perchloric acid* is equivalent to 20.93 mg of C₉H₁₅N₅O.

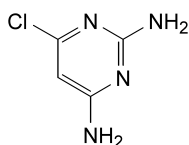
STORAGE

Store protected from light.

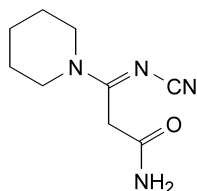
IMPURITIES



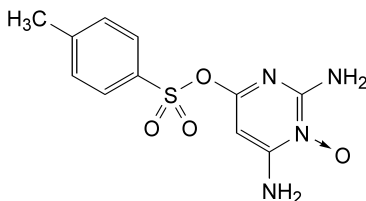
A. 6-chloropyrimidine-2,4-diamine 3-oxide,



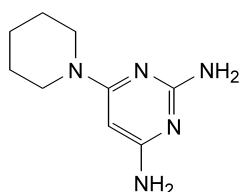
B. 6-chloropyrimidine-2,4-diamine,



C. 3-(cyanoimino)-3-(piperidin-1-yl)propanamide,



D. 6-[[[(4-methylphenyl)sulphonyl]oxy]pyrimidine-2,4-diamine 3-oxide,



E. 6-(piperidin-1-yl)pyrimidine-2,4-diamine (desoxyminoxidil).

MINT OIL, PARTLY DEMENTHOLISED

Menthae arvensis aetheroleum partim mentholi privum

DEFINITION

Essential oil obtained by steam distillation from the fresh, flowering aerial parts, recently gathered from *Mentha canadensis* L. (syn. *M. arvensis* L. var. *glabrata* (Benth) Fern., *M. arvensis* var. *piperascens* Malinv. ex Holmes), followed by partial separation of menthol by crystallisation.

CHARACTERS

Appearance: colourless or pale yellow to greenish-yellow liquid with a characteristic odour.

IDENTIFICATION

First identification: B.

Second identification: A.

A. Thin-layer chromatography (2.2.27).

Test solution. Dissolve 0.1 ml of the substance to be examined in 1.0 ml of *toluene* R.

Reference solution. Dissolve 4 µl of *carvone* R, 4 µl of *pulegone* R, 10 µl of *menthyl acetate* R, 20 µl of *cineole* R and 50 mg of *menthol* R in 5 ml of *toluene* R.

Plate: TLC silica gel F₂₅₄ plate R.

Mobile phase: *ethyl acetate* R, *toluene* R (5:95 V/V).

Application: 10 µl, as bands.

Development: over a path of 15 cm.

Drying: in air.

Detection A: examine in ultraviolet light at 254 nm.

Results A: see below the sequence of the zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, a quenching zone may be present in the upper third of the chromatogram obtained with the test solution.

Top of the plate	
Carvone and pulegone: a quenching zone	A quenching zone
	A quenching zone
Reference solution	Test solution

Detection B: spray with *anisaldehyde solution* R and heat at 100-105 °C for 5-10 min. Examine immediately in daylight.

Results B: see below the sequence of the zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, the zone due to cineole in the reference solution is absent in the chromatogram obtained with the test solution. No yellowish-brown zone below the intense reddish-violet zone is present in the chromatogram obtained with the test solution.

Top of the plate	
Menthyl acetate: a bluish-violet zone	An intense reddish-violet zone (near the solvent front) A bluish-violet zone (menthyl acetate) A strongly greenish zone A greenish zone
Carvone and pulegone: a reddish zone	A reddish zone
Cineole: a violet zone	A distinctly violet zone
Menthol: an intense blue zone	A very intense blue zone (menthol)
Reference solution	Test solution

B. Examine the chromatograms obtained in the test for chromatographic profile.

Results: the characteristic peaks in the chromatogram obtained with the test solution are approximately similar in retention time to those in the chromatogram obtained with the reference solution. Carvone may be absent from the chromatogram obtained with the test solution.

TESTS

Relative density (2.2.5): 0.888 to 0.910.

Refractive index (2.2.6): 1.456 to 1.470.

Optical rotation (2.2.7): -16.0° to -34.0° .

Acid value (2.5.1): maximum 1.0, determined on 5.00 g of the substance to be examined dissolved in 50 ml of the prescribed mixture of solvents.

Chromatographic profile. Gas chromatography (2.2.28): use the normalisation procedure.

Test solution. Dissolve 0.20 g of the substance to be examined in *hexane R* and dilute to 10.0 ml with the same solvent.

Reference solution. Dissolve 10 mg of *limonene R*, 20 mg of *cineole R*, 40 mg of *menthone R*, 10 mg of *isomenthone R*, 40 mg of *menthyl acetate R*, 20 mg of *isopulegol R*, 60 mg of *menthol R*, 20 mg of *pulegone R* and 10 mg of *carvone R* in *hexane R* and dilute to 10.0 ml with the same solvent.

Column:

- **material:** fused silica,
- **size:** $l = 30$ m (a film thickness of 1 μm may be used) to 60 m (a film thickness of 0.2 μm may be used), $\varnothing = 0.25$ –0.53 mm,
- **stationary phase:** *macrogol 20 000 R*.

Carrier gas: *helium for chromatography R*.

Flow rate: 1.5 ml/min.

Split ratio: 1:100.

Temperature:

	Time (min)	Temperature ($^{\circ}\text{C}$)
Column	0 - 10	60
	10 - 70	60 $\rightarrow$ 180
	70 - 75	180
Injection port		200
Detector		220

Detection: flame ionisation.

Injection: 1.0 μl .

Elution order: order indicated in the composition of the reference solution. Record the retention times of these substances.

System suitability: reference solution:

- **resolution:** minimum 1.5 between the peaks due to limonene and cineole.

Using the retention times determined from the chromatogram obtained with the reference solution, locate the components of the reference solution in the chromatogram obtained with the test solution.

Determine the percentage content of these components. The percentages are within the following ranges:

- **limonene:** 1.5 per cent to 7.0 per cent,
- **cineole:** maximum 1.5 per cent,
- **menthone:** 17.0 per cent to 35.0 per cent,
- **isomenthone:** 5.0 per cent to 13.0 per cent,
- **menthyl acetate:** 1.5 per cent to 7.0 per cent,
- **isopulegol:** 1.0 per cent to 3.0 per cent,
- **menthol:** 30.0 per cent to 50.0 per cent,
- **pulegone:** maximum 2.0 per cent,
- **carvone:** maximum 2.0 per cent.

The ratio of cineole content to limonene content is less than 1.

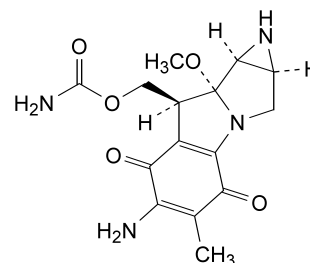
STORAGE

In a well-filled, airtight container, protected from light, at a temperature not exceeding 25 $^{\circ}\text{C}$.

01/2005:1655

MITOMYCIN

Mitomycinum



$\text{C}_{15}\text{H}_{18}\text{N}_4\text{O}_5$

M_r 334.3

DEFINITION

[(1aS,8S,8aR,8bS)-6-Amino-8a-methoxy-5-methyl-4,7-dioxo-1,1a,2,4,7,8,8a,8b-octahydroazirino[2',3':3,4]pyrrolo[1,2-a]-indol-8-yl]methyl carbamate (mitomycin C).

Substance produced by a strain of *Streptomyces caespitosus*.

Content: 97.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

Appearance: blue-violet crystals or crystalline powder.

Solubility: slightly soluble in water, freely soluble in dimethylacetamide, sparingly soluble in methanol, slightly soluble in acetone.

IDENTIFICATION

A. Infrared absorption spectrophotometry (2.2.24).

Comparison: *mitomycin CRS*.

B. Examine the chromatograms obtained in the assay.

Results: the principal peak in the chromatogram obtained with the test solution is similar in retention time and size to the principal peak in the chromatogram obtained with reference solution (a).

TESTS

pH (2.2.3): 5.5 to 7.5.

Dissolve 10 mg in 10 ml of *carbon dioxide-free water R*.

Related substances. Liquid chromatography (2.2.29). Prepare the solutions immediately before use.

Test solution. Dissolve 50.0 mg of the substance to be examined in *methanol R* and dilute to 10.0 ml with the same solvent.

Reference solution (a). Dilute 1.0 ml of the test solution to 100.0 ml with *methanol R*. Dilute 5.0 ml of this solution to 10.0 ml with *methanol R*.

Reference solution (b). Dissolve 10 mg of *cinnamamide R* in *methanol R* and dilute to 10 ml with the same solvent. Mix 2 ml of this solution and 1 ml of the test solution and dilute to 10 ml with *methanol R*.

Column:

- **size:** $l = 0.25$ m, $\varnothing = 4.6$ mm,
- **stationary phase:** spherical base-deactivated end-capped octadecylsilyl silica gel for chromatography *R* (5 μ m),
- **temperature:** 30 °C.

Mobile phase:

- **mobile phase A:** *methanol R*, 0.77 g/l solution of ammonium acetate *R* (20:80 V/V);
- **mobile phase B:** *methanol R*, 0.77 g/l solution of ammonium acetate *R* (50:50 V/V);

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)
0 - 10	100	0
10 - 30	100 → 0	0 → 100
30 - 45	0	100
45 - 50	0 → 100	100 → 0

Flow rate: 1.0 ml/min.

Detection: spectrophotometer at 254 nm.

Injection: 10 μ l.

Relative retention with reference to mitomycin (retention time = about 21 min): impurity D = about 0.6; impurity C = about 1.2; impurity A = about 1.3; impurity B = about 1.6.

System suitability: reference solution (b):

- **resolution:** minimum 15.0 between the peaks due to mitomycin and impurity A.

Limits:

- **correction factor:** for the calculation of content, multiply the peak area of impurity A by 0.35,
- **impurities A, B, C, D:** for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent),
- **any other impurity:** for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent),
- **total:** not more than 4 times the area of the principal peak in the chromatogram obtained with reference solution (a) (2.0 per cent),

- **disregard limit:** 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

Water (2.5.12): maximum 2.5 per cent, determined on 0.30 g.

Bacterial endotoxins (2.6.14, *Method B*): less than 10 IU/ mg, if intended for use in the manufacture of parenteral dosage forms without a further appropriate procedure for the removal of bacterial endotoxins.

ASSAY

Liquid chromatography (2.2.29).

Test solution. Dissolve 50.0 mg of the substance to be examined in *dimethylacetamide R* and dilute to 100.0 ml with the same solvent.

Reference solution (a). Dissolve 50.0 mg of *mitomycin CRS* in *dimethylacetamide R* and dilute to 100.0 ml with the same solvent.

Reference solution (b). Dissolve 10 mg of *cinnamamide R* in *methanol R* and dilute to 20 ml with the same solvent. Mix 2 ml of this solution with 2 ml of reference solution (a).

Column:

- **size:** $l = 0.30$ m, $\varnothing = 3.9$ mm,
- **stationary phase:** end-capped phenylsilyl silica gel for chromatography *R* (10 μ m) with a specific surface area of 330 m²/g, a carbon loading of 8 per cent and a pore size of 12.5 nm.

Mobile phase: mix 23 volumes of *methanol R*, 77 volumes of a solution containing 2.05 g/l of ammonium acetate *R* and 2.8 ml/l of dilute acetic acid *R*.

Flow rate: 2.0 ml/min.

Detection: variable wavelength spectrophotometer capable of operating at 365 nm and 254 nm.

Injection: 20 μ l.

Run time: twice the retention time of mitomycin.

Relative retention with reference to mitomycin (retention time = about 8 min): impurity A = about 1.2.

System suitability:

- **resolution:** minimum 1.8 between the peaks due to mitomycin and impurity A in the chromatogram obtained with reference solution (b) at 254 nm,
- **symmetry factor:** maximum 1.3 for the principal peak in the chromatogram obtained with reference solution (a) at 365 nm.

Calculate the percentage content of C₁₅H₁₈N₄O₅, from the chromatograms obtained at 365 nm and the declared content of *mitomycin CRS*.

STORAGE

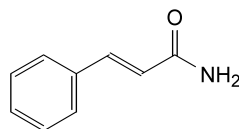
Protected from light.

LABELLING

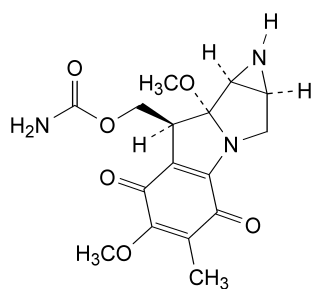
The label states where applicable that the substance is free from bacterial endotoxins.

IMPURITIES

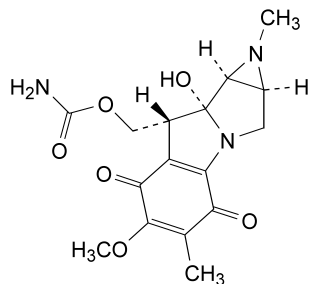
Specified impurities: A, B, C, D.



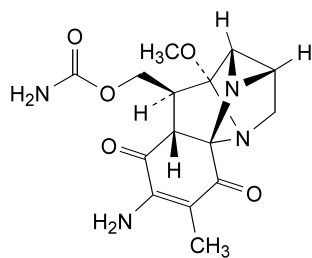
A. (*E*)-3-phenylprop-2-enamide (cinnamamide),



- B. [(1a*S*,8*S*,8a*R*,8b*S*)-6,8a-dimethoxy-5-methyl-4,7-dioxo-1,1a,2,4,7,8,8a,8b-octahydroazirino[2',3':3,4]pyrrolo[1,2-*a*]indol-8-yl)methyl carbamate (mitomycin A),



- C. [(1a*S*,8*R*,8a*R*,8b*S*)-8a-hydroxy-6-methoxy-1,5-dimethyl-4,7-dioxo-1,1a,2,4,7,8,8a,8b-octahydroazirino[2',3':3,4]pyrrolo[1,2-*a*]indol-8-yl)methyl carbamate (mitomycin B),

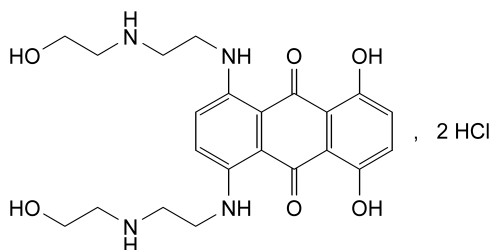


- D. [(1*S*,2*S*,4*S*,5*R*,6*S*,6a*R*,10a*S*,11*S*)-8-amino-5-methoxy-9-methyl-7,10-dioxo-2,3,6,6a,7,10-hexahydro-1,2,5-metheno-1*H*,5*H*-imidazo[2,1-*i*]indol-6-yl)methyl carbamate (albomitomycin C).

01/2005:1243

MITOXANTRONE HYDROCHLORIDE

Mitoxantroni hydrochloridum

C₂₂H₃₀Cl₂N₄O₆M_r 517.4

DEFINITION

Mitoxantrone hydrochloride contains not less than 97.0 per cent and not more than the equivalent of 102.0 per cent of 1,4-dihydroxy-5,8-bis[[2-[(2-hydroxyethyl)amino]ethyl]amino]anthracene-9,10-dione dihydrochloride, calculated with reference to the anhydrous, ethanol-free substance.

CHARACTERS

A dark blue, electrostatic powder, hygroscopic, sparingly soluble in water, slightly soluble in methanol, practically insoluble in acetone.

CAUTION: Mitoxantrone hydrochloride and impurity A are electrostatic. The use of an antistatic gun or other suitable method to discharge the solids before weighing or transfer is recommended.

IDENTIFICATION

- A. Dissolve 2 mg to 3 mg in 1 ml of *methanol R* by warming in a water-bath at 40-50 °C. Evaporate the solution to dryness under a stream of dry nitrogen, warming gently if necessary. Examine the residue by infrared absorption spectrophotometry (2.2.24), comparing with the *Ph. Eur.* reference spectrum of mitoxantrone hydrochloride.

- B. It gives reaction (b) of chlorides (2.3.1).

TESTS

Ethanol. Not more than 1.6 per cent *m/m* of ethanol, determined by gas chromatography (2.2.28), using *propanol R* as the internal standard.

Internal standard solution. Dilute 2.0 ml of *propanol R* to 100 ml with *water R*. Dilute 5.0 ml of this solution to 100 ml with *water R*.

Test solution. Mix 0.100 g of the substance to be examined with 2.0 ml of the internal standard solution and dilute to 5.0 ml with *water R*. Place the flask in a sonic bath for 2 min then shake the flask for 2 min. If necessary repeat the sonication and shaking until dissolution is complete.

Reference solution. Dilute 2.0 ml of *ethanol R* to 100.0 ml with *water R*. Dilute 5.0 ml of this solution to 100.0 ml with *water R*. Dilute 10.0 ml of this solution and 10.0 ml of the internal standard solution to 25.0 ml with *water R*.

The chromatographic procedure may be carried out using:

- a column 2 m long and 3 mm in internal diameter packed with *ethylvinylbenzene-divinylbenzene copolymer R*,
- *helium for chromatography R* as the carrier gas at a flow rate of 19 ml/min,
- a flame-ionisation detector,

maintaining the column at a temperature of 120 °C, the temperature of the injector at 175 °C and the temperature of the detector at 210 °C.

Inject 1 µl of the test solution and 1 µl of the reference solution. The retention times are about 1 min for ethanol and about 2 min for propanol. The test is not valid unless the resolution between the peaks due to ethanol and propanol in the chromatogram obtained with the reference solution is at least six. Calculate the content of ethanol taking its density (2.2.5) to be 0.790 g/ml at 20 °C.

Related substances. Examine by liquid chromatography (2.2.29) as prescribed under Assay. In the chromatogram obtained with the test solution, the area of any peak apart from the principal peak is not greater than the area of the peak in the chromatogram obtained with reference solution (b) (1 per cent) and the sum of the areas of all the peaks apart from the principal peak is not greater than twice the area of the peak in the chromatogram obtained with reference solution (b) (2 per cent). Disregard any peak with an area less than that of the principal peak in the chromatogram obtained with reference solution (d).

Water (2.5.12). Not more than 6.0 per cent, determined on 0.300 g by the semi-micro determination of water.

ASSAY

Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 20.0 mg of the substance to be examined in about 40 ml of the mobile phase, if necessary with the aid of ultrasonication, and dilute to 50.0 ml with the mobile phase.

Reference solution (a). Dissolve 20.0 mg of *mitoxantrone hydrochloride CRS* in about 40 ml of the mobile phase, if necessary with the aid of ultrasonication, and dilute to 50.0 ml with the mobile phase.

Reference solution (b). Dilute 1 ml of the test solution to 100 ml with the mobile phase.

Reference solution (c). Dissolve 2.0 mg of *mitoxantrone impurity A CRS* in 1.0 ml of reference solution (a).

Reference solution (d). Dilute 1 ml of reference solution (b) to 10 ml with the mobile phase.

The chromatographic procedure may be carried out using:

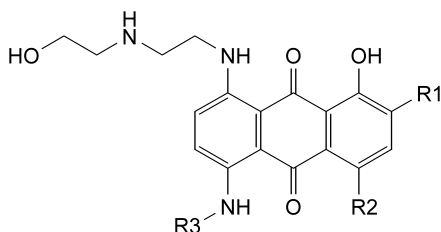
- a column 0.30 m long and 3.0 mm in internal diameter packed with *phenylsilyl silica gel for chromatography R* (10 µm),
- as mobile phase at a flow rate of 3 ml/min a mixture of 750 volumes of *water R*, 250 volumes of *acetonitrile R* and 25 volumes of a solution prepared as follows: dissolve 22.0 g of *sodium heptanesulphonate R* in about 150 ml of *water R* and filter through a 0.45 µm filter; wash the filter with *water R* and combine the filtrate and washings; add 32.0 ml of *glacial acetic acid R* and dilute to 250 ml with *water R*,
- as detector a spectrophotometer set at 254 nm,
- a loop injector.

Inject separately 50 µl of each solution. The test is not valid unless the resolution between the two principal peaks in the chromatogram obtained with reference solution (c) is at least 3.0. Record the chromatogram obtained with the test solution for three times the retention time of the principal peak. Calculate the content of $C_{22}H_{30}Cl_2N_4O_6$ from the peak areas in the chromatograms obtained with the test solution and reference solution (a) and the declared content of $C_{22}H_{30}Cl_2N_4O_6$ in *mitoxantrone hydrochloride CRS*.

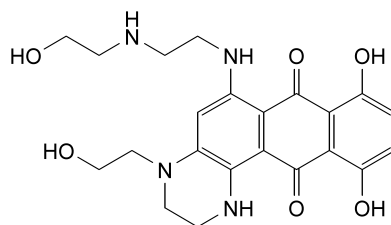
STORAGE

Store in an airtight container.

IMPURITIES



- A. R1 = R3 = H, R2 = OH: 1-amino-5,8-dihydroxy-4-[[2-[(2-hydroxyethyl)amino]ethyl]amino]anthracene-9,10-dione,
- B. R1 = R2 = H, R3 = $CH_2-CH_2-NH-CH_2-CH_2-OH$: 5-hydroxy-1,4-bis[[2-[(2-hydroxyethyl)amino]ethyl]amino]anthracene-9,10-dione,
- C. R1 = Cl, R2 = OH, R3 = $CH_2-CH_2-NH-CH_2-CH_2-OH$: 2-chloro-1,4-dihydroxy-5,8-bis[[2-[(2-hydroxyethyl)amino]ethyl]amino]anthracene-9,10-dione,



- D. 8,11-dihydroxy-4-(2-hydroxyethyl)-6-[[2-[(2-hydroxyethyl)amino]ethyl]amino]-1,2,3,4-tetrahydronaphtho[2,3-f]quinoxaline-7,12-dione.

01/2005:1641

MOLGRAMOSTIM CONCENTRATED SOLUTION

Molgramostimi solutio concentrata

APARSPSPST	QPWEHVNAIQ	EARRLLNLSR
DTAAEMNETV	EVISEMFDLQ	EPTCLQTRLE
LYKQGLRGSL	TKLKGPLTMM	ASHYKQHCPP
TPETSCATQI	ITFESFKENL	KDFLLVIPFD
CWEPVQE		

$C_{639}H_{1007}N_{171}O_{196}S_8$

M_r 14 477

DEFINITION

Solution of a protein having the structure of the granulocyte macrophage colony stimulating factor which is produced and secreted by various human blood cell types. The protein stimulates the differentiation and proliferation of leucocyte stem cells into mature granulocytes and macrophages.

Content: minimum 2.0 mg of protein per millilitre.

Potency: minimum 0.7×10^7 IU per milligram of protein.

PRODUCTION

Molgramostim concentrated solution is produced by a method based on recombinant DNA (rDNA) technology, using bacteria as host cells. It is produced under conditions designed to minimise microbial contamination of the product.

Prior to release, the following tests are carried out on each batch of the final bulk product, unless exemption has been granted by the competent authority.

Host-cell derived proteins: the limit is approved by the competent authority.

Host-cell or vector derived DNA: the limit is approved by the competent authority.

CHARACTERS

Appearance: clear, colourless liquid.

IDENTIFICATION

A. It shows the expected biological activity (see Assay).

B. Isoelectric focusing (2.2.54).

Test solution. Dilute the preparation to be examined with *water R* to obtain a concentration of 0.25 mg/ml.

Reference solution (a). Dilute *molgramostim CRS* with *water R* to obtain a concentration of 0.25 mg/ml.

Reference solution (b). Use an isoelectric point (pI) calibration solution, in the pI range of 2.5-6.5, prepared according to the manufacturer's instructions.

Focusing:

- *pH gradient*: 4.0-6.5,
- *catholyte*: 8.91 g/l (0.1 M) solution of *βalanine R*,
- *anolyte*: 14.7 g/l (0.1 M) solution of *glutamic acid R* in a 50 per cent V/V solution of *dilute phosphoric acid R* (0.5 M),
- *application*: 20 µl.

Detection: immerse the gel in a suitable volume of a solution containing 115 g/l of *trichloroacetic acid R* and 34.5 g/l of *sulphosalicylic acid R* and shake the container gently for 30 min. Transfer the gel to a mixture of 32 volumes of *glacial acetic acid R*, 100 volumes of *ethanol R* and 268 volumes of *water R* (mixture A) and rinse for 5 min. Immerse the gel for 10 min in a staining solution prewarmed to 60 °C and prepared by adding *brilliant blue R* at a concentration of 1.2 g/l to mixture A. Wash the gel in several containers with mixture A and keep the gel in this mixture until the background is clear (12-24 h). After adequate destaining, soak the gel for 1 h in a 10 per cent V/V solution of *glycerol R* in mixture A.

System suitability:

- in the electropherogram obtained with reference solution (b), the relevant isoelectric point markers are distributed along the entire length of the gel,
- in the electropherogram obtained with reference solution (a), the pI of the principal band is 4.9 to 5.4.

Results: the principal band in the electropherogram obtained with the test solution corresponds in position to the principal band in the electropherogram obtained with reference solution (a). Plot the migration distances of the relevant pI markers versus their pI and determine the isoelectric points of the principal component of each of the test solution and reference solution (a). They do not differ by more than 0.2 pI units.

- C. Examine the electropherograms obtained under reducing conditions in the test for impurities with molecular masses differing from that of molgramostim. The principal band in the electropherogram obtained with test solution (a) is similar in position to the principal band in the electropherogram obtained with reference solution (a).
- D. Peptide mapping (2.2.55).

Test solution. Introduce 50 µl of *tris-hydrochloride buffer solution pH 8.0 R* and 50 µl of the preparation to be examined at a concentration of 2 mg/ml into a polypropylene tube of 0.5 ml capacity. Add 4 µl of a 1 mg/ml solution of *trypsin for peptide mapping R* in a 0.01 per cent V/V solution of *trifluoroacetic acid R*, cap tightly and mix well. Incubate at about 37 °C for 18 h. Add 125 µl of a 764 g/l (8 M) solution of *guanidine hydrochloride R* and mix well. Add 10 µl of a 154.2 g/l (1 M) solution of *dithiothreitol R* and mix well. Place the capped tube in boiling water for 1 min. Cool to room temperature.

Reference solution. Prepare at the same time and in the same manner as for the test solution but use *molgramostim CRS* instead of the preparation to be examined.

Examine the 2 tryptic digests by liquid chromatography (2.2.29).

Column:

- *size*: $l = 0.10$ m, $\varnothing = 4.6$ mm,
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm) with a pore size of 30 nm.

Mobile phase:

- *mobile phase A*: dilute 1 ml of *trifluoroacetic acid R* in 1000 ml of *water R*;
- *mobile phase B*: dilute 1 ml of *trifluoroacetic acid R* in 100 ml of *water R*; add 900 ml of *acetonitrile for chromatography R* and mix;

Time (min)	Mobile Phase A (per cent V/V)	Mobile Phase B (per cent V/V)
0 - 35.0	100 → 65	0 → 35
35.0 - 105.0	65 → 35	35 → 65
105.0 - 107.5	35 → 100	65 → 0
107.5 - 120.0	100	0

Flow rate: 1.0 ml/min.

Detection: spectrophotometer at 214 nm.

Equilibration: at initial conditions for at least 12 min.

Injection: 200 µl.

System suitability: the chromatograms obtained with the reference solution and the test solution are qualitatively similar to the *Ph. Eur. reference chromatogram of molgramostim digest*.

Results: the profile of the chromatogram obtained with the test solution corresponds to that of the chromatogram obtained with the reference solution.

E. N-Terminal sequence analysis.

Perform the Edman degradation using an automated solid-phase sequencer, operated in accordance with the manufacturer's instructions.

Load about 1 nmol of the test preparation to a sequencing cartridge using the protocol provided by the manufacturer. Run 16 sequencing cycles, noting, if appropriate, the presence of proline at positions 2, 6, 8 and 12.

Identify the phenylthiohydantoin (PTH)-amino acids released at each sequencing cycle by reverse-phase liquid chromatography. The procedure may be carried out using the column and reagents recommended by the manufacturer of the sequencing equipment for the separation of PTH-amino acids.

The separation procedure is calibrated using:

- the mixture of PTH-amino acids provided by the manufacturer of the sequencer, with the gradient conditions adjusted as indicated to achieve optimum resolution of all amino acids,
- a sample obtained from a blank sequencing cycle obtained as recommended by the equipment manufacturer.

Results: the first 16 amino acids are: Ala-Pro-Ala-Arg-Ser-Pro-Ser-Pro-Ser-Thr-Gln-Pro-Trp-Glu-His-Val.

TESTS

Impurities with molecular masses differing from that of molgramostim. Polyacrylamide gel electrophoresis (2.2.31) under both reducing and non-reducing conditions.

Gel dimensions: 0.75 mm thick.

Resolving gel: 14 per cent acrylamide.

Sample buffer A. Mix equal volumes of *water R* and *concentrated SDS-PAGE sample buffer R*.

Sample buffer B (reducing conditions). Mix equal volumes of *water R* and *concentrated SDS-PAGE sample buffer for reducing conditions R*.

Test solution (a). Dilute the preparation to be examined in *water R* to obtain a concentration of 1.0 mg/ml. To 1 volume of this solution add 1 volume of *concentrated SDS-PAGE sample buffer R*.

Test solution (b) (2 per cent control). Dilute 0.020 ml of test solution (a) to 1.0 ml with sample buffer A.

Test solution (c) (1 per cent control). To 0.20 ml of test solution (b) add 0.20 ml of sample buffer A.

Test solution (d) (0.5 per cent control). To 0.20 ml of test solution (c) add 0.20 ml of sample buffer A.

Test solution (e) (0.25 per cent control). To 0.20 ml of test solution (d) add 0.20 ml of sample buffer A.

Test solution (f) (0.1 per cent control). To 0.20 ml of test solution (e) add 0.30 ml of sample buffer A.

Test solution (g) (0.05 per cent control). To 0.20 ml of test solution (f) add 0.20 ml of sample buffer A.

Test solution (h) (0.025 per cent control). To 0.20 ml of test solution (g) add 0.20 ml of sample buffer A.

Test solution (i). Prepare as for test solution (a), but using *concentrated SDS-PAGE sample buffer for reducing conditions R*.

Test solutions (j)-(p). Prepare as for test solutions (b)-(h), but using sample buffer B.

Reference solution (a). Dilute *molgramostim CRS* in *water R* to obtain a concentration of 0.02 mg/ml. Mix 1 volume of this solution with 1 volume of *concentrated SDS-PAGE sample buffer R*.

Reference solution (b). Prepare as for reference solution (a), but using *concentrated SDS-PAGE sample buffer for reducing conditions R*.

Reference solution (c). Use a solution of molecular mass markers suitable for calibrating SDS-PAGE gels in the range of 14 400-94 000. Dissolve in sample buffer or sample buffer (reducing conditions), as appropriate.

Sample treatment: boil for 3 min.

Application: 50 µl; apply reduced and non-reduced solutions to separate gels.

Detection: silver staining as described below.

Immerse the gel overnight in a mixture of 10 volumes of *acetic acid R*, 40 volumes of *water R* and 50 volumes of *methanol R*. Transfer the gel to a 100 g/l solution of *glutaraldehyde R* and shake for about 30 min. Replace the glutaraldehyde solution with *water R*, and keep the gel in *water R* for 20 min. Repeat this washing-step twice. Transfer the gel to a mixture containing 0.75 g/l of *sodium hydroxide R*, 14 g/l of *concentrated ammonia R* and 8 g/l of *silver nitrate R*. This solution is prepared immediately before use. Place the gel on a shaker in the dark for 5 min. Wash the gel for 30 s in each of 3 containers with *water R* and shake the gel in a mixture consisting of 0.05 g/l of *citric acid R*, 0.05 per cent V/V of *formaldehyde R* and 0.005 per cent V/V of *methanol R* in *water R*. Protein bands become visible during this step. Keep the gel in the solution until sufficiently stained and then rinse the gel repeatedly with *water R* in a shaking water bath. Soak gels in a solution consisting of 10 per cent V/V of *acetic acid R* and 1 per cent V/V of *glycerol R*.

System suitability:

- the validation criteria are met (2.2.31),
- a band is seen in the electropherogram obtained with test solution (h),
- a gradation of intensity of staining is seen in the electropherograms obtained with test solutions (a)-(h) and (i)-(p),

- the molecular mass of the principal band in the electropherogram obtained with reference solution (a) or (b) is within the range of 15 100 to 17 100.

Limits: compare the staining intensity of each non-molgramostim band observed in the electropherogram obtained with test solution (a) to the staining intensity of the principal band in the electropherograms obtained with test solutions (b)-(h). Proceed similarly with the electropherograms obtained with test solutions (i)-(p). The impurity level is estimated as the dilution, in percentage, of the solution giving the electropherogram with the closest intensity of staining.

Reducing conditions:

- *impurity with an apparent molecular mass of 20 000:* maximum 1 per cent,
- *impurity with an apparent molecular mass of 25 000:* maximum 0.1 per cent,
- *impurity with an apparent molecular mass of 30 000:* maximum 0.3 per cent,
- *total:* maximum 2 per cent.

Non-reducing conditions:

- *total of all impurities of molecular masses higher than 30 000:* maximum 1 per cent.

Related proteins. Liquid chromatography (2.2.29): use the normalisation procedure.

Test solution (a). Dilute the preparation to be examined with 0.05 M phosphate buffer solution pH 7.0 R to obtain a concentration of 0.5 mg/ml.

Test solution (b). Mix 1 volume of test solution (a) with 4 volumes of a 0.125 mg/ml solution of *human albumin R* or *bovine albumin R* in 0.05 M phosphate buffer solution pH 7.0 R.

Reference solution (a). Dilute *molgramostim CRS* with 0.05 M phosphate buffer solution pH 7.0 R to obtain a concentration of 0.5 mg/ml.

Reference solution (b). Mix 1 volume of reference solution (a) with 4 volumes of a 0.125 mg/ml solution of *human albumin R* or *bovine albumin R* in 0.05 M phosphate buffer solution pH 7.0 R.

Column:

- *size:* $l = 0.15$ m, $\varnothing = 4.6$ mm,
- *stationary phase:* butylsilyl silica gel for chromatography R (5 µm) with a pore size of 30 nm.

Mobile phase:

- *mobile phase A:* to about 800 ml of *water R* add 1.0 ml of *trifluoroacetic acid R* and dilute to 1000 ml with *water R*;
- *mobile phase B:* to 100 ml of *water R* add 1.0 ml of *trifluoroacetic acid R* and 900 ml of *acetonitrile for chromatography R*;

Time (min)	Mobile Phase A (per cent V/V)	Mobile Phase B (per cent V/V)
0 - 30	64 → 44	36 → 56
30 - 35	44 → 0	56 → 100
35 - 45	0	100
45 - 50	0 → 64	100 → 36
50 - 60	64	36

Flow rate: 1.2 ml/min.

Detection: spectrophotometer at 214 nm.

Injection: 100 µl of test solution (a), reference solutions (a) and (b).

System suitability: reference solution (b):

- **retention time:** molgramostin = about 22 min,
- **repeatability:** maximum relative standard deviation of 5.0 per cent after 4 injections,
- **resolution:** minimum 2 between the peaks due to albumin and molgramostin.

Limits:

- **any impurity:** for each impurity, maximum 1.5 per cent,
- **total of impurities eluting between 5 min and 30 min:** maximum 4 per cent.

Bacterial endotoxins (2.6.14): less than 5 IU in the volume that contains 1.0 mg of protein.

ASSAY

Protein. Liquid chromatography (2.2.29) as described in the test for related proteins.

Injection: 150 µl of test solution (b) and reference solution (b).

Calculate the content of molgramostin using the declared content of molgramostin in *molgramostin CRS*.

Potency. Determination of the biological activity of molgramostin concentrated solution based on the stimulation of proliferation of TF-1 cells by molgramostin.

The following method uses the conversion of tetrazolium bromide (MTT) as a staining method. Validated alternative stains such as Almar blue have also been found suitable.

TF-1 cells are incubated with varying dilutions of test and reference preparations of molgramostin. They are then incubated with a solution of MTT. This cytochemical stain is converted by cellular dehydrogenases to a purple formazan product. The formazan is then measured spectrophotometrically. The potency of the preparation to be examined is determined by comparison of the dilutions of the test preparation with the dilutions of the appropriate International Standard of molgramostin or with a reference preparation calibrated in International Units, which yield the same response (50 per cent maximal stimulation).

The International Unit is the activity contained in a stated amount of the appropriate International Standard. The equivalence in International Units of the International Standard is stated by the World Health Organisation.

Add 50 µl of dilution medium to all wells of a 96-well microtitre plate. Add an additional 50 µl of this solution to the wells designed for the blanks. Add 50 µl of each solution to be tested in triplicate (test preparation and reference preparation at a concentration of about 65 IU/ml, plus a series of 10 twofold dilutions to obtain a standard curve). Then add to each well 50 µl of a TF-1 cell suspension containing 3×10^5 cells per millilitre, maintaining the cells in a uniform suspension during addition.

Incubate the plate at 36.0–38.0 °C for a minimum of 24 h in a humidified incubator using 6 ± 1 per cent CO₂. Add 25 µl of a 5.0 g/l sterile solution of *tetrazolium bromide R* to each well. Reincubate for 5 h. Remove the plates from the incubator and add to each well 100 µl of a 240 g/l solution of *sodium dodecyl sulphate R* previously adjusted to pH 2.7 with hydrochloric acid. Reincubate overnight.

Determine the relative quantity of purple formazan product formed in each well by measuring the absorbance (2.2.25) using a 96-well microtitre plate reader. Read each plate at 570 nm and at 690 nm. Subtract the reading at 690 nm from the reading at 570 nm. Analyse the data by fitting a sigmoidal dose-response curve to the data obtained and by using a suitable statistical method, for example the 4-parameter model (see 5.3).

The estimated potency is not less than 80 per cent and not more than 125 per cent of the stated potency. The confidence limits ($P = 0.95$) of the estimated potency are not less than 74 per cent and not more than 136 per cent of the stated potency.

STORAGE

In an airtight container, protected from light, at a temperature below –65 °C.

LABELLING

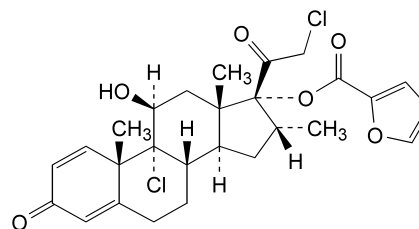
The label states:

- the content, in milligrams of protein per millilitre,
- the potency, in International Units per milligram of protein.

01/2005:1449
corrected

MOMETASONE FUROATE

Mometasoni furoas



C₂₇H₃₀Cl₂O₆

M_r 521.4

DEFINITION

Mometasone furoate contains not less than 97.0 per cent and not more than the equivalent of 103.0 per cent of 9,21-dichloro-11β-hydroxy-16α-methyl-3,20-dioxopregna-1,4-dien-17-yl furan-2-carboxylate, calculated with reference to the dried substance.

CHARACTERS

A white or almost white powder, practically insoluble in water, soluble in acetone and in methylene chloride, slightly soluble in alcohol.

It melts at about 220 °C, with decomposition.

IDENTIFICATION

First identification: A, B.

Second identification: B, C, D.

- Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *mometasone furoate CRS*. Examine the substances prepared as discs.
- Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel F₂₅₄ plate R*.
Test solution. Dissolve 10 mg of the substance to be examined in *methylene chloride R* and dilute to 10 ml with the same solvent.
Reference solution (a). Dissolve 20 mg of *mometasone furoate CRS* in *methylene chloride R* and dilute to 20 ml with the same solvent.
Reference solution (b). Dissolve 10 mg of *beclometasone dipropionate CRS* in reference solution (a) and dilute to 10 ml with the same solution.
Apply separately to the plate 5 µl of each solution. Prepare the mobile phase by adding a mixture of 1.2 volumes of *water R* and 8 volumes of *methanol R* to a mixture

of 15 volumes of *ether R* and 77 volumes of *methylene chloride R*. Develop over a path of 15 cm. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. The principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a). Spray with *alcoholic solution of sulphuric acid R*. Heat at 120 °C for 10 min or until the spots appear. Allow to cool. Examine the chromatograms in daylight and in ultraviolet light at 365 nm. The principal spot in the chromatogram obtained with the test solution is similar in position, colour in daylight, fluorescence in ultraviolet light at 365 nm and size to the principal spot in the chromatogram obtained with reference solution (a). The test is not valid unless the chromatogram obtained with reference solution (b) shows two spots which, when examined in ultraviolet light at 365 nm, may not be completely separated.

- C. Add about 2 mg to 2 ml of *sulphuric acid R* and shake to dissolve. Within 15 min a light yellow colour develops. When examined in ultraviolet light at 365 nm, no fluorescence is seen. Add the solution to 10 ml of *water R* and mix. The colour fades and there is no fluorescence.
- D. Mix 80 mg with 0.30 g of *anhydrous sodium carbonate R* and ignite in a crucible until an almost white residue is obtained. Allow to cool and dissolve the residue in 5 ml of *dilute nitric acid R*. Filter. To 1 ml of the filtrate add 1 ml of *water R*. The solution gives reaction (a) of chlorides (2.3.1).

TESTS

Specific optical rotation (2.2.7). Dissolve 50.0 mg in *alcohol R* and dilute to 10.0 ml with the same solvent. The specific optical rotation is + 50 to + 55, calculated with reference to the dried substance.

Related substances. Examine by liquid chromatography (2.2.29). Prepare the solutions immediately before use.

Solvent solution. Prepare a mixture of equal volumes of *acetonitrile R* and *water R* to which is added 0.1 volumes of *acetic acid R*.

Test solution. Dissolve 20.0 mg of the substance to be examined in 4.0 ml of *acetonitrile R* and dilute to 20.0 ml with the solvent solution.

Reference solution (a). Dissolve 2 mg of *mometasone furoate CRS* and 6 mg of *beclometasone dipropionate CRS* in the solvent solution and dilute to 10.0 ml with the same solvent. Dilute 0.25 ml of the solution to 10.0 ml with the solvent solution.

Reference solution (b). Dilute 1.0 ml of the test solution to 20.0 ml with the solvent solution. Dilute 1.0 ml of the solution to 10.0 ml with the solvent solution.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4.6 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (5 µm),
- as mobile phase at a flow rate of 1 ml/min a mixture of equal volumes of *acetonitrile R* and *water R*,
- as detector a spectrophotometer set at 254 nm.

Adjust the sensitivity of the system so that the height of the principal peak in the chromatogram obtained with 20 µl of reference solution (b) is at least 50 per cent of the full scale of the recorder.

Inject 20 µl of reference solution (a). When the chromatograms are recorded in the prescribed conditions, the retention times are: mometasone furoate about 17 min and beclometasone dipropionate about 22 min.

The test is not valid unless the resolution between the peaks corresponding to mometasone furoate and beclometasone dipropionate is at least 6. If necessary adjust the concentration of acetonitrile in the mobile phase.

Inject separately 20 µl of the test solution and 20 µl of reference solution (b). Continue the chromatography for twice the retention time of the principal peak in the chromatogram obtained with the test solution. In the chromatogram obtained with the test solution: the area of any peak apart from the principal peak, is not greater than 0.6 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.3 per cent); the sum of the areas of all peaks apart from the principal peak, is not greater than 1.2 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.6 per cent). Disregard any peak due to the solvent and any peak with an area less than 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (b).

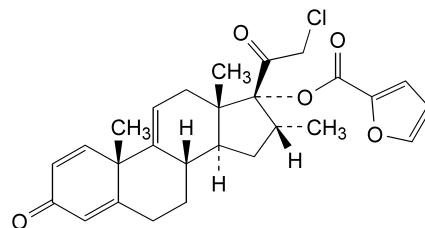
Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C.

ASSAY

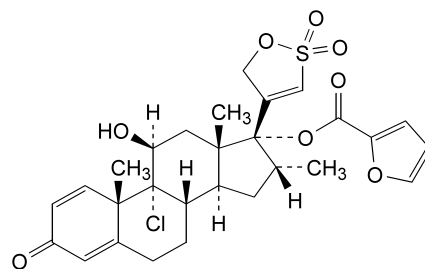
Dissolve 50.0 mg in *alcohol R* and dilute to 100.0 ml with the same solvent. Dilute 2.0 ml of this solution to 100.0 ml with *alcohol R*. Measure the absorbance (2.2.25) at the maximum at 249 nm.

Calculate the content of $C_{27}H_{30}Cl_2O_6$ taking the specific absorbance to be 481.

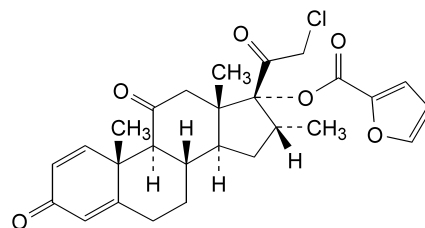
IMPURITIES



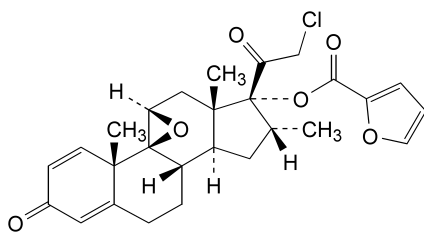
A. 21-chloro-16α-methyl-3,20-dioxopregna-1,4,9(11)-trien-17-yl furan-2-carboxylate,



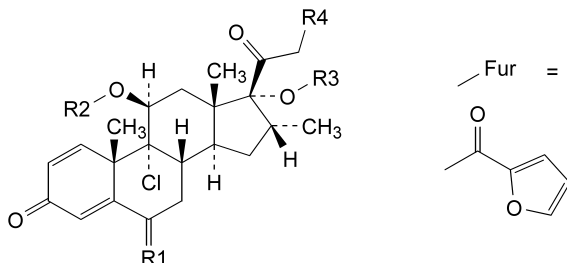
B. 4-[9-chloro-17-[(furan-2-ylcarbonyl)oxy]-11β-hydroxy-16α-methyl-3-oxoandrosta-1,4-dien-17β-yl]-5H-1,2-oxathiole 2,2-dioxide,



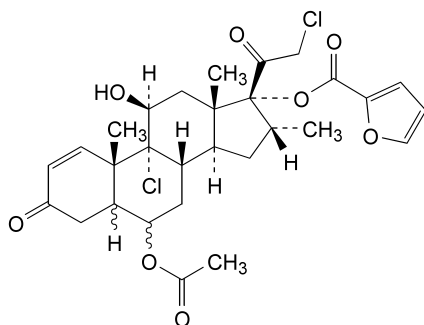
C. 21-chloro-16α-methyl-3,11,20-trioxopregna-1,4-dien-17-yl furan-2-carboxylate,



D. 21-chloro-9,11β-epoxy-16α-methyl-3,20-dioxo-9β-pregna-1,4-dien-17-yl furan-2-carboxylate,



- E. R1 = H₂, R2 = R3 = Fur, R4 = Cl: 9,21-dichloro-16α-methyl-3,20-dioxopregna-1,4-diene-11β,17-diyl bis(furan-2-carboxylate),
- F. R1 = O, R2 = H, R3 = Fur, R4 = Cl: 9,21-dichloro-11β-hydroxy-16α-methyl-3,6,20-trioxopregna-1,4-dien-17-yl furan-2-carboxylate,
- G. R1 = H₂, R2 = R3 = H, R4 = Cl: 9,21-dichloro-11β,17-dihydroxy-16α-methylpregna-1,4-diene-3,20-dione (mometasone),
- H. R1 = H₂, R2 = H, R3 = Fur, R4 = OH: 9-chloro-11β,21-dihydroxy-16α-methyl-3,20-dioxopregna-1,4-dien-17-yl furan-2-carboxylate,

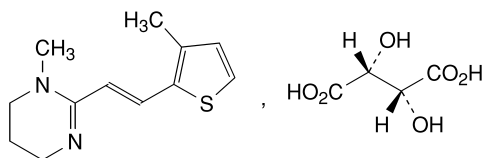


I. 9,21-dichloro-11β-hydroxy-16α-methyl-3,20-dioxo-5ξ-pregn-1-ene-6ξ,17-diyl 6-acetate 17-(furan-2-carboxylate).

01/2005:1546

MORANTEL HYDROGEN TARTRATE FOR VETERINARY USE

Moranteli hydrogenotartras
ad usum veterinarium



C₁₆H₂₂N₂O₆S

M_r 370.4

DEFINITION

1-Methyl-2-[(*E*)-2-(3-methylthiophen-2-yl)ethenyl]-1,4,5,6-tetrahydropyrimidine hydrogen tartrate.

Content: 98.5 per cent to 101.5 per cent (dried substance).

CHARACTERS

Appearance: white or pale yellow, crystalline powder.

Solubility: very soluble in water and in alcohol, practically insoluble in ethyl acetate.

IDENTIFICATION

First identification: B.

Second identification: A, C, D.

A. Melting point (2.2.14): 167 °C to 172 °C.

B. Infrared absorption spectrophotometry (2.2.24).

Comparison: morantel hydrogen tartrate CRS.

C. Dissolve about 10 mg in 1 ml of a 5 g/l solution of ammonium vanadate R. Evaporate to dryness. Add 0.1 ml of sulphuric acid R. A purple colour is produced.

D. Dissolve about 10 mg in 1 ml of 0.1 M sodium hydroxide. Transfer to a separating funnel and shake with 5 ml of methylene chloride R. Discard the organic layer. Neutralise the aqueous layer with a few drops of dilute hydrochloric acid R. The solution gives reaction (b) of tartrates (2.3.1).

TESTS

Solution S. Dissolve 0.25 g in carbon dioxide-free water R and dilute to 25.0 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and not more intensely coloured than reference solution GY₆ or Y₆ (2.2.2, Method II).

pH (2.2.3): 2.8 to 3.2 for solution S.

Related substances. Liquid chromatography (2.2.29).

Carry out the test protected from light.

Test solution. Dissolve 50.0 mg of the substance to be examined in the mobile phase and dilute to 100.0 ml with the mobile phase.

Reference solution (a). Dilute 1.0 ml of the test solution to 100.0 ml with the mobile phase.

Reference solution (b). Dilute 2.0 ml of reference solution (a) to 100.0 ml with the mobile phase.

Reference solution (c). Expose 10 ml of reference solution (a) to daylight for 15 min before injection.

Reference solution (d). Dissolve 15.0 mg of tartaric acid R in the mobile phase and dilute to 100.0 ml with the mobile phase.

Column:

— *size*: *l* = 0.25 m, Ø = 4.6 mm,

— *stationary phase*: base-deactivated end-capped octadecylsilyl silica gel for chromatography R (5 µm).

Mobile phase: to a mixture of 0.35 volumes of triethylamine R and 85 volumes of water R adjusted to pH 2.5 with phosphoric acid R, add 5 volumes of tetrahydrofuran R and 10 volumes of methanol R.

Flow rate: 0.75 ml/min.

Detection: spectrophotometer at 226 nm.

Injection: 20 µl.

Run time: twice the retention time of morantel.

System suitability: reference solution (c):

— *resolution*: minimum of 2 between the principal peak and the preceding peak ((*Z*)-isomer).

Limits:

- *any impurity apart from the peak due to tartaric acid*: not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent),
- *total*: not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (1 per cent),
- *disregard limit*: the area of the principal peak in the chromatogram obtained with reference solution (b) (0.02 per cent).

Heavy metals (2.4.8): maximum 20 ppm.

1.0 g complies with limit test C. Prepare the standard using 2 ml of *lead standard solution* (10 ppm Pb) R.

Loss on drying (2.2.32): maximum 1.5 per cent, determined on 1.000 g by drying in an oven at 100–105 °C.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

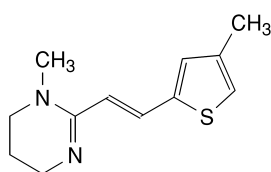
ASSAY

Dissolve 0.280 g in 40 ml of *anhydrous acetic acid* R. Titrate with 0.1 M *perchloric acid*, determining the end-point potentiometrically (2.2.20).

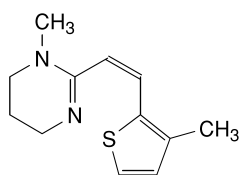
1 ml of 0.1 M *perchloric acid* is equivalent to 37.04 mg of $C_{16}H_{22}N_2O_6S$.

STORAGE

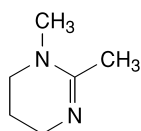
Protected from light.

IMPURITIES

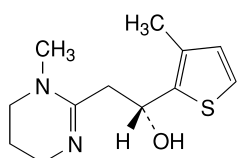
- A. 1-methyl-2-[(*E*)-2-(4-methylthiophen-2-yl)ethenyl]-1,4,5,6-tetrahydropyrimidine,



- B. 1-methyl-2-[(*Z*)-2-(3-methylthiophen-2-yl)ethenyl]-1,4,5,6-tetrahydropyrimidine,

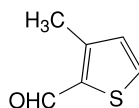


- C. 1,2-dimethyl-1,4,5,6-tetrahydropyrimidine,



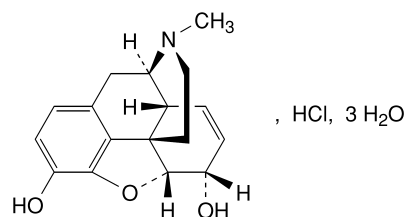
and enantiomer

- D. (1*R*)-2-(1-methyl-1,4,5,6-tetrahydropyrimidin-2-yl)-1-(3-methylthiophen-2-yl)ethanol,



- E. 3-methylthiophene-2-carbaldehyde.

01/2005:0097

MORPHINE HYDROCHLORIDE**Morphini hydrochloridum**

$C_{17}H_{20}ClNO_3 \cdot 3H_2O$

M_r 375.8

DEFINITION

Morphine hydrochloride contains not less than 98.0 per cent and not more than the equivalent of 101.0 per cent of 7,8-didehydro-4,5 α -epoxy-17-methylmorphinan-3,6 α -diol hydrochloride, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, crystalline powder or colourless, silky needles or cubical masses, efflorescent in a dry atmosphere, soluble in water and in glycerol, slightly soluble in alcohol.

IDENTIFICATION

- Dissolve 10 mg in *water* R and dilute to 100.0 ml with the same solvent. Examined between 250 nm and 350 nm (2.2.25), the solution shows a single absorption maximum, at 285 nm. The specific absorbance at the maximum is about 41.
- Dissolve 10 mg in 0.1 M *sodium hydroxide* and dilute to 100.0 ml with the same solvent. Examined between 265 nm and 350 nm (2.2.25), the solution shows a single absorption maximum, at 298 nm. The specific absorbance at the maximum is about 70.
- To about 1 mg of powdered substance in a porcelain dish add 0.5 ml of *sulphuric acid-formaldehyde reagent* R. A purple colour develops and becomes violet.
- Dissolve about 5 mg in 5 ml of *water* R and add 0.15 ml of a freshly prepared 10 g/l solution of *potassium ferricyanide* R and 0.05 ml of *ferric chloride solution* R1. A blue colour is produced immediately.
- Dissolve about 5 mg in 5 ml of *water* R and add 1 ml of *dilute hydrogen peroxide solution* R, 1 ml of *dilute ammonia* R1 and 0.05 ml of a 40 g/l solution of *copper sulphate* R. A red colour develops.
- It gives reaction (a) of chlorides (2.3.1).
- It gives the reaction of alkaloids (2.3.1).

TESTS

Solution S. Dissolve 0.50 g in *water* R and dilute to 25.0 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and not more intensely coloured than reference solution Y_6 or BY_6 (2.2.2, *Method II*).

Acidity or alkalinity. To 10 ml of solution S add 0.05 ml of *methyl red solution R*. Not more than 0.2 ml of 0.02 M sodium hydroxide or 0.02 M hydrochloric acid is required to change the colour of the indicator.

Specific optical rotation (2.2.7). –110 to –115, determined on solution S and calculated with reference to the dried substance.

Related substances. Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel G plate R*.

Test solution. Dissolve 0.10 g of the substance to be examined in a mixture of equal volumes of *alcohol R* and *water R* and dilute to 10 ml with the same mixture of solvents.

Reference solution. Dissolve 50 mg of *codeine phosphate R* in 5 ml of the test solution. Dilute 0.1 ml of the solution to 10 ml with a mixture of equal volumes of *alcohol R* and *water R*.

Apply separately to the plate 10 µl of each solution. Develop over a path of 15 cm using a freshly prepared mixture of 2.5 volumes of *concentrated ammonia R*, 32.5 volumes of *acetone R*, 24.5 volumes of *ethanol R*, 10.5 volumes of *water R* and 35 volumes of *toluene R*, prepared in the order given. Dry the plate in a current of air. Spray with *potassium iodobismuthate solution R* and dry for 15 min in a current of air. Spray with *dilute hydrogen peroxide solution R*. Any spot corresponding to codeine in the chromatogram obtained with the test solution is not more intense than the corresponding spot in the chromatogram obtained with the reference solution (1 per cent); any spot apart from the principal spot and the spot corresponding to codeine, is not more intense than the spot corresponding to morphine in the chromatogram obtained with the reference solution (1 per cent). The test is not valid unless the chromatogram obtained with the reference solution shows two clearly separated spots.

Meconate. To 10 ml of solution S add 1 ml of *hydrochloric acid R* and 0.1 ml of *ferric chloride solution R1*. The absorbance (2.2.25) measured at 480 nm is not greater than 0.05 (0.2 per cent). Use as the compensation liquid a blank solution prepared at the same time and in the same manner using 10 ml of *water R*.

Loss on drying (2.2.32). 12.0 per cent to 15.0 per cent, determined on 0.500 g by drying in an oven at 130 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on the residue from the test for loss on drying.

ASSAY

Dissolve 0.350 g, heating if necessary, in 30 ml of *anhydrous acetic acid R*. Cool and add 6 ml of *mercuric acetate solution R*. Titrate with 0.1 M *perchloric acid*, using 0.1 ml of *crystal violet solution R* as indicator.

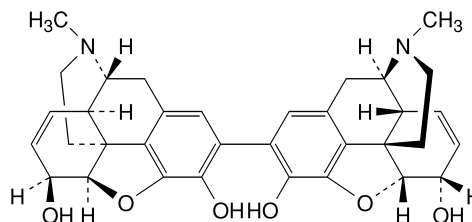
1 ml of 0.1 M *perchloric acid* is equivalent to 32.18 mg of C₁₇H₂₀ClNO₃.

STORAGE

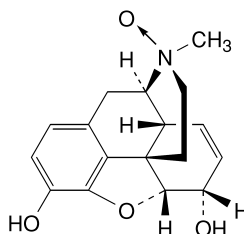
Store protected from light.

IMPURITIES

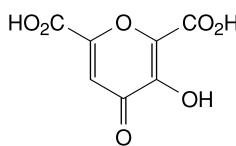
A. codeine,



B. 2,2'-bimorphine (pseudomorphine),



C. morphine *N*-oxide,

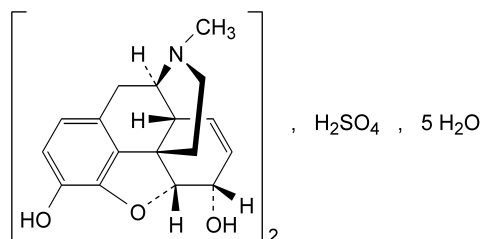


D. 3-hydroxy-4-oxo-4*H*-pyran-2,6-dicarboxylic acid (meconic acid).

01/2005:1244

MORPHINE SULPHATE

Morphini sulfas



C₃₄H₄₀N₂O₁₀S·5H₂O

M_r 759

DEFINITION

Morphine sulphate contains not less than 98.0 per cent and not more than the equivalent of 102.0 per cent of di(7,8-didehydro-4,5α-epoxy-17-methylmorphinan-3,6α-diol) sulphate, calculated with reference to the anhydrous, ethanol-free substance.

CHARACTERS

A white or almost white, crystalline powder, soluble in water, very slightly soluble in alcohol, practically insoluble in toluene.

IDENTIFICATION

First identification: A, E.

Second identification: B, C, D, E.

A. Examine by infrared absorption spectrophotometry (2.2.24), after drying the substance to be examined at 145 °C for 1 h, comparing with the *Ph. Eur. reference spectrum of morphine sulphate*.

B. Dissolve 0.100 g in *water R* and dilute to 100.0 ml with the same solvent (solution A). Dilute 10.0 ml to 100.0 ml with *water R*. Examined between 250 nm and 300 nm

(2.2.25), the solution shows a single absorption maximum, at 285 nm. The specific absorbance is in the range 37 to 43. Dilute 10.0 ml of solution A to 100.0 ml with 0.1 M sodium hydroxide. Examined between 250 nm and 350 nm (2.2.25), the solution shows a single absorption maximum, at 298 nm. The specific absorbance is in the range 64 to 72.

C. To about 1 mg of powdered substance in a porcelain dish add 0.5 ml of *sulphuric acid-formaldehyde reagent R*. A purple colour develops and becomes violet.

D. It gives the reaction of alkaloids (2.3.1).

E. It gives the reactions of sulphates (2.3.1).

TESTS

Solution S. Dissolve 0.500 g in *water R* and dilute to 25.0 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and not more intensely coloured than reference solution Y₆ or BY₅ (2.2.2, *Method II*).

Acidity or alkalinity. To 10 ml of solution S add 0.05 ml of *methyl red solution R*. Not more than 0.2 ml of 0.02 M sodium hydroxide or 0.02 M hydrochloric acid is required to change the colour of the indicator.

Specific optical rotation (2.2.7): –107 to –110, determined on solution S and calculated with reference to the anhydrous, ethanol-free substance.

Related substances. Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel G plate R*.

Test solution. Dissolve 0.20 g of the substance to be examined in a mixture of equal volumes of *alcohol R* and *water R* and dilute to 10 ml with the same mixture of solvents.

Reference solution (a). Dissolve 25 mg of *codeine phosphate R* in 5 ml of the test solution. Dilute 0.2 ml of the solution to 10 ml with a mixture of equal volumes of *alcohol R* and *water R*.

Reference solution (b). Dilute 0.1 ml of the test solution to 20 ml with a mixture of equal volumes of *alcohol R* and *water R*.

Reference solution (c). Dilute 2.0 ml of reference solution (b) to 5.0 ml with a mixture of equal volumes of *alcohol R* and *water R*.

Reference solution (d). Dilute 2.0 ml of reference solution (b) to 10.0 ml with a mixture of equal volumes of *alcohol R* and *water R*.

Apply to the plate 10 µl of each solution. Develop over a path of 10 cm using a freshly prepared mixture of 2.5 volumes of *concentrated ammonia R*, 32.5 volumes of *acetone R*, 24.5 volumes of *ethanol R*, 10.5 volumes of *water R* and 35 volumes of *toluene R*, prepared in the order given. Dry the plate in a current of air. Spray with *potassium iodobismuthate solution R* and dry for 15 min in a current of air. Spray with *dilute hydrogen peroxide solution R*. Any spot corresponding to impurity A in the chromatogram

obtained with the test solution is not more intense than the corresponding spot in the chromatogram obtained with reference solution (a) (0.5 per cent). Any spot in the chromatogram obtained with the test solution, apart from the principal spot and any spot corresponding to impurity A is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.5 per cent) and not more than two such spots are more intense than the spot in the chromatogram obtained with reference solution (c) (0.2 per cent). The test is not valid unless the chromatogram obtained with reference solution (a) shows two such clearly separated spots and the spot in the chromatogram obtained with reference solution (d) is clearly visible.

Ethanol (2.4.24). Not more than 0.5 per cent.

Iron (2.4.9). Dissolve the residue from the test for sulphated ash in *water R* and dilute to 10.0 ml with the same solvent. The solution complies with the limit test for iron (5 ppm).

Water (2.5.12): 10.4 per cent to 13.4 per cent, determined on 0.200 g by the semi-micro determination of water.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 2.0 g.

ASSAY

Dissolve 0.500 g in 120 ml of *anhydrous acetic acid R*. Titrate with 0.1 M *perchloric acid*, determining the end-point potentiometrically (2.2.20).

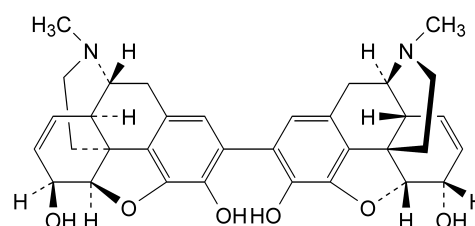
1 ml of 0.1 M *perchloric acid* is equivalent to 66.88 mg of C₃₄H₄₀N₂O₁₀S.

STORAGE

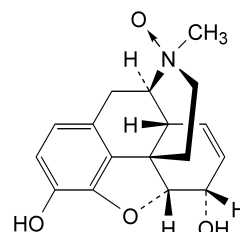
Store protected from light.

IMPURITIES

A. codeine,



B. 2,2'-bimorphine (pseudomorphine),



C. morphine N-oxide.

01/2005:1833

MOTHERWORT**Leonuri cardiaca herba****DEFINITION**

Whole or cut, dried flowering aerial parts of *Leonurus cardiaca* L.

Content: minimum 0.2 per cent of flavonoids, expressed as hyperoside ($C_{21}H_{20}O_{12}$; M_r 464.4) (dried drug).

CHARACTERS

Macroscopic and microscopic characters described under identification tests A and B.

IDENTIFICATION

A. The stem pieces are hairy, longitudinally striated, quadrangular, hollow, and up to about 10 mm wide; they bear opposite and decussate, petiolate leaves and, in the axils of the upper leaves, about 6 to 12 small flowers, arranged in sessile whorls forming a long leafy spike. The lower leaves are ovate-orbicular, palmately 3 to 5-lobed, rarely 7-lobed, the lobes irregularly dentate. The upper leaves are entire or slightly trifid, lanceolate with a serrate margin and cuneate at the base. The upper surface of the leaves is green with scattered hairs, the lower surface is paler green, densely pubescent and shows a prominent palmate and reticulate venation. The flowers have a funnel-shaped calyx, 3 mm to 5 mm long with 5 stiff, recurved teeth; the corolla is 2-lipped, the upper lip pink and pubescent on the outer surface, the lower lip white with purplish spots; stamens 4, densely pubescent.

B. Reduce to a powder (355). The powder is green. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters: fragments of the leaf lamina with a one-layered palisade mesophyll reaching almost to the centre and a loosely arranged spongy parenchyma; fragments of leaf epidermis; upper epidermal cells with straight anticlinal walls and a striated cuticle; lower epidermal cells with sinuous anticlinal walls; stomata of the diacytic type (2.8.3) more numerous on the lower surface; glandular trichomes with a short unicellular stalk and a globular head composed of 8, sometimes up to 16 cells or with a unicellular head; covering trichomes conical, uniseriate, up to about 300 µm long, occasionally up to 1500 µm, composed of from 2 to 8 cells with slight swellings at the junctions and a warty or striated cuticle; fragments of the calyx containing small, cluster crystals of calcium oxalate; spherical pollen grains, about 25 µm to 30 µm in diameter, with 3 pores and 3 furrows and a smooth exine; thick-walled, lignified fibres and spirally and annularly thickened vessels from the stem; occasional brown fragments of pericarp with single crystals of calcium oxalate.

C. Thin-layer chromatography (2.2.27).

Test solution. To 0.5 g of the powdered drug (355) add 5 ml of *methanol R*. Heat in a water-bath at 65 °C for 5 min with shaking. Cool and filter.

Reference solution. Dissolve 5 mg of *naphthol yellow S R* and 2.0 mg of *catalpol R* in 5.0 ml of *methanol R*.

Plate: TLC silica gel plate *R*.

Mobile phase: *glacial acetic acid R*, *water R*, *ethyl acetate R* (20:20:60 V/V/V).

Application: 20 µl as bands.

Development: over a path of 10 cm.

Drying: in air.

Detection: spray with *dimethylaminobenzaldehyde solution R2*, using about 5 ml for a plate 200 mm square; heat at 100-105 °C for 10 min until the spots appear; examine in daylight.

Results: see below the sequence of the zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, further weak greyish-blue zones may be present in the chromatogram obtained with the test solution.

Top of the plate	
	A wide white zone
	A greyish-blue zone (iridoid)
Naphthol yellow S: an intense yellow zone	1 or 2 greyish-blue zones (iridoid)
Catalpol: a greyish-blue zone	
Reference solution	Test solution

TESTS

Foreign matter (2.8.2): maximum 2 per cent of brown or yellow leaves and maximum 2 per cent of other foreign matter.

Loss on drying (2.2.32): maximum 12.0 per cent, determined on 1.000 g of the powdered drug (355) by drying in an oven at 100-105 °C for 2 h.

Total ash (2.4.16): maximum 12.0 per cent.

ASSAY

Stock solution. In a 100 ml round-bottomed flask, place 1.00 g of the powdered drug (355), add 1 ml of a 5 g/l solution of *hexamethylenetetramine R*, 20 ml of *acetone R* and 2 ml of *hydrochloric acid R1*. Boil the mixture under a reflux condenser for 30 min. Filter the liquid through a plug of absorbent cotton into a flask. Add the absorbent cotton to the residue in the round-bottomed flask and extract with 2 quantities, each of 20 ml, of *acetone R*, each time boiling under a reflux condenser for 10 min. Allow to cool, filter each extract through the plug of absorbent cotton into the flask. After cooling, filter the combined acetone extracts through a paper filter into a volumetric flask, dilute to 100.0 ml with *acetone R* by rinsing the flask and the paper filter. Introduce 20.0 ml of the solution into a separating funnel, add 20 ml of *water R* and shake the mixture with 1 quantity of 15 ml and then with 3 quantities, each of 10 ml, of *ethyl acetate R*. Combine the ethyl acetate extracts in a separating funnel, wash with 2 quantities, each of 50 ml, of *water R*, and filter the extracts over 10 g of *anhydrous sodium sulphate R* into a volumetric flask and dilute to 50.0 ml with *ethyl acetate R*.

Test solution. To 10.0 ml of the stock solution add 1 ml of *aluminium chloride reagent R* and dilute to 25.0 ml with a 5 per cent V/V solution of *glacial acetic acid R* in *methanol R*.

Compensation liquid. Dilute 10.0 ml of the stock solution to 25.0 ml with a 5 per cent V/V solution of *glacial acetic acid R* in *methanol R*.

Measure the absorbance (2.2.25) of the test solution after 30 min, by comparison with the compensation liquid at 425 nm. Calculate the percentage content of flavonoids, calculated as hyperoside, from the expression:

$$\frac{A \times 1.25}{m}$$

i.e. taking the specific absorbance of hyperoside to be 500.

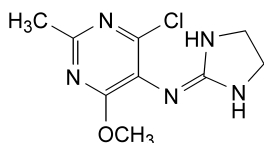
A = absorbance at 425 nm,

m = mass of the substance to be examined, in grams.

01/2005:1758

MOXONIDINE

Moxonidinum



$C_9H_{12}ClN_5O$

M_r 241.7

DEFINITION

4-Chloro-*N*-(imidazolidin-2-ylidene)-6-methoxy-2-methylpyrimidin-5-amine.

Content: 97.5 per cent to 102.0 per cent (dried substance).

CHARACTERS

Appearance: white or almost white powder.

Solubility: very slightly soluble in water, sparingly soluble in methanol, slightly soluble in methylene chloride, very slightly soluble in acetonitrile.

IDENTIFICATION

Infrared absorption spectrophotometry (2.2.24).

Preparation: discs.

Comparison: moxonidine CRS.

TESTS

Related substances. Liquid chromatography (2.2.29).

Test solution. Dissolve 0.100 g of the substance to be examined in a mixture of equal volumes of *methanol R* and *water R* and dilute to 100.0 ml with the same mixture of solvents.

Reference solution (a). Dissolve 10.0 mg of *moxonidine CRS* in a mixture of equal volumes of *methanol R* and *water R* and dilute to 10.0 ml with the same mixture of solvents.

Reference solution (b). Dilute 1.0 ml of reference solution (a) to 100.0 ml with a mixture of equal volumes of *methanol R* and *water R*. Dilute 2.0 ml of this solution to 20.0 ml with a mixture of equal volumes of *methanol R* and *water R*.

Reference solution (c). Dissolve 5.0 mg of *moxonidine impurity A CRS* in a mixture of equal volumes of *methanol R* and *water R* and dilute to 100.0 ml with the same mixture of solvents.

Reference solution (d). Dilute 6.0 ml of reference solution (c) to 100.0 ml with a mixture of equal volumes of *methanol R* and *water R*.

Reference solution (e). Dilute 2.5 ml of reference solution (a) to 50.0 ml with reference solution (c).

Column:

– *size*: $l = 0.25$ m, $\varnothing = 4$ mm,

– *stationary phase*: base-deactivated octylsilyl silica gel for chromatography *R* (5 μ m),

– *temperature*: 40 °C.

Mobile phase: mix 136 volumes of *acetonitrile R* with 1000 volumes of a 3.48 g/l solution of *sodium pentanesulphonate R* previously adjusted to pH 3.5 with *dilute sulphuric acid R*.

Flow rate: 1.2 ml/min.

Detection: spectrophotometer at 230 nm.

Injection: 20 μ l; inject a blank, the test solution and reference solutions (b), (d) and (e).

Run time: twice the retention time of moxonidine.

Relative retentions with reference to moxonidine (retention time = about 11.6 min): impurity A = about 0.9; impurity B = about 1.7.

System suitability: reference solution (e):

– *resolution*: minimum of 2 between the peaks due to impurity A and moxonidine.

Limits:

– *impurity A*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (d) (0.3 per cent),

– *impurity B*: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.3 per cent),

– *any other impurity*: not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent),

– *total*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent),

– *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent). Disregard any peak observed with the blank run.

Loss on drying (2.2.32): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 100–105 °C for 3 h.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

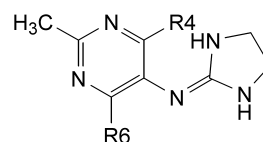
ASSAY

Liquid chromatography (2.2.29) as described in the test for related substances with the following modification.

Injection: test solution and reference solution (a).

Calculate the percentage content of $C_9H_{12}ClN_5O$ from the areas of the peaks and the declared content of *moxonidine CRS*.

IMPURITIES



A. $R_4 = R_6 = Cl$: 4,6-dichloro-*N*-(imidazolidin-2-ylidene)-2-methylpyrimidin-5-amine (6-chloromoxonidine),

B. $R_4 = R_6 = OCH_3$: *N*-(imidazolidin-2-ylidene)-4,6-dimethoxy-2-methylpyrimidin-5-amine (4-methoxymoxonidine),

C. $R_4 = OH$, $R_6 = OCH_3$: 5-[(imidazolidin-2-ylidene)amino]-6-methoxy-2-methylpyrimidin-4-ol (4-hydroxymoxonidine),

D. $R_4 = OH$, $R_6 = Cl$: 6-chloro-5-[(imidazolidin-2-ylidene)amino]-2-methylpyrimidin-4-ol (6-desmethyloxonidine).

01/2005:1853

MULLEIN FLOWER

Verbasci flos

DEFINITION

Dried flower, reduced to the corolla and the androecium, of *Verbascum thapsus* L., *V. densiflorum* Bertol. (*V. thapsiforme* Schrad), and *V. phlomoides* L.

CHARACTERS

Macroscopic and microscopic characters described under identification tests A and B.

IDENTIFICATION

- A. The corolla of *V. thapsus* is about 20 mm in diameter, pale yellow, yellow to brown, funnel-shaped with 5 slightly unequal and spreading lobes. The corolla lobes are densely hairy on the outer surface, glabrous on the inner surface, with a fine network of light brown veins. There are 5 stamens, alternating with the petal lobes, 2 of these are long, with glabrous filaments, the other 3 shorter, with densely tormentose filaments. The anthers are attached transversely. In *V. phlomoides* the corolla is up to about 30 mm in diameter, bright yellow to orange, and the anthers are obliquely attached to the filaments. The corolla of *V. densiflorum*, about 30 mm in diameter, is almost flat and deeply divided into 5 slightly unequal lobes, with rounded apices.
- B. Reduce to a powder (355). The powder is yellow or yellowish-brown. Examine under a microscope using *chloral hydrate solution R*. The powder shows many covering trichomes from the corolla, whole and fragmented; there are pluricellular, of the candelabra type with a central uniseriate axis from which whorls of branch cells arise at the position of the cross walls and at the apex. The covering trichomes from the stamen filaments are unicellular, long, thin-walled and tubular, sometimes with a club-shaped tip; they have a distinctly granular or striated surface. Numerous pollen grains, ovoid with a finely granular exine with 3 pores. Fragments of the fibrous layer of the anther with thickened walls giving a characteristic star-shaped appearance. Yellow fragments of the petals in the surface view, the epidermal cells polygonal and isodiametric; fragments of the mesophyll consisting of irregular parenchymatous cells and scattered spiral vessels.

- C. Thin-layer chromatography (2.2.27).

Test solution. Heat 1.0 g of the powdered drug (355) in 10 ml of *methanol R* in a water-bath at 60 °C for 5 min, with stirring. Cool and filter.

Reference solution. Dissolve 1 mg of *caffeic acid R*, 2.5 mg of *hyperoside R* and 2.5 mg of *rutin R* in *methanol R* and dilute to 10 ml with the same solvent.

Plate: TLC silica gel plate *R*.

Mobile phase: *water R*, *anhydrous formic acid R*, *methyl ethyl ketone R*, *ethyl acetate R* (10:10:30:50 V/V/V/V).

Application: 10 µl of the reference solution and 30 µl of the test solution, as bands.

Development: over a path of 15 cm.

Drying: at 100-105 °C.

Detection: spray the warm plate with a 10 g/l solution of *diphenylboric acid aminoethyl ester R* in *methanol R*. Subsequently spray with a 50 g/l solution of *macrogol 400 R* in *methanol R*. Allow the plate to dry in air for 30 min and examine in ultraviolet light at 365 nm.

Results: see below the sequence of the zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other faint zones may be present in the chromatogram obtained with the test solution.

Top of the plate	
Caffeic acid: a greenish-blue fluorescent zone.	A yellow to yellowish-green fluorescent zone.
	A bluish fluorescent zone.
	A greenish fluorescent zone.
	A yellowish-green fluorescent zone.
	A bluish fluorescent zone.
Hyperoside: a yellowish-brown fluorescent zone.	A greenish fluorescent zone.
Rutin: a yellowish-brown fluorescent zone.	
Reference solution	Test solution

- D. Boil 1.0 g of the powdered drug (355) with 15 ml of *water R* for 1 min. Filter. Add 1 ml of *hydrochloric acid R* and boil for 1 min. A greenish-blue colour develops and, after a few minutes, cloudiness appears and then a blackish precipitate (iridoids).

TESTS

Foreign matter (2.8.2): maximum 5 per cent of brown petals and maximum 2 per cent of fragments of the calyx and other foreign matter, determined on 20 g.

Swelling index (2.8.4): minimum 9, determined on the powdered drug (710), humidified with 2 ml of *alcohol R*.

Loss on drying (2.2.32): maximum 12.0 per cent, determined on 1.000 g of the powdered drug (710) by drying in an oven at 100-105 °C for 2 h.

Total ash (2.4.16): maximum 6.0 per cent.

Ash insoluble in hydrochloric acid (2.8.1): maximum 2.0 per cent.

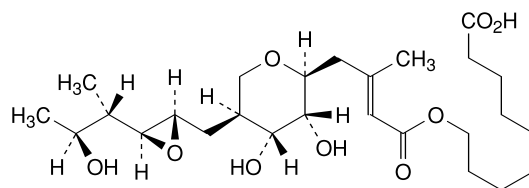
STORAGE

In an airtight container.

01/2005:1450

MUPIROCIN

Mupirocinum

C₂₆H₄₄O₉M_r 500.6

DEFINITION

Mupirocin contains not less than 93.0 per cent and not more than the equivalent of 100.5 per cent of 9-[(2*E*)-4-[(2*S*,3*R*,4*R*,5*S*)-5-[(2*S*,3*S*,4*S*,5*S*)-2,3-epoxy-5-

hydroxy-4-methylhexyl]-3,4-dihydroxy-3,4,5,6-tetrahydro-2H-pyran-2-yl]-3-methylbut-2-enoyl]oxy]nonanoic acid, calculated with reference to the anhydrous substance.

CHARACTERS

A white or almost white powder, slightly soluble in water, freely soluble in acetone, in ethanol and in methylene chloride.

It shows polymorphism.

IDENTIFICATION

Examine by infrared absorption spectrophotometry (2.2.24), comparing with the *Ph. Eur. reference spectrum of mupirocin*.

TESTS

pH (2.2.3). The pH of a freshly prepared saturated solution (about 10 g/l) in *carbon dioxide-free water R* is 3.5 to 4.0.

Specific optical rotation (2.2.7). Dissolve 0.50 g in *methanol R* and dilute to 10.0 ml with the same solvent. The specific optical rotation is -17 to -21 , calculated with reference to the anhydrous substance.

Related substances. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 50.0 mg of the substance to be examined in a mixture of equal volumes of *methanol R* and of a 13.6 g/l solution of *sodium acetate R*, adjusted to pH 4.0 with *acetic acid R* and dilute to 10.0 ml with the same mixture of solvents.

Reference solution (a). Dilute 1.0 ml of the test solution to 50.0 ml with a mixture of equal volumes of *methanol R* and of a 13.6 g/l solution of *sodium acetate R*, adjusted to pH 4.0 with *acetic acid R*.

Reference solution (b). Adjust 10 ml of reference solution (a) to pH 2.0 using *hydrochloric acid R* and allow to stand for 20 h.

Reference solution (c). Dissolve 25 mg of *mupirocin lithium CRS* in a mixture of equal volumes of *methanol R* and of a 13.6 g/l solution of *sodium acetate R*, adjusted to pH 4.0 with *acetic acid R* and dilute to 200.0 ml with the same mixture of solvents.

The chromatographic procedure may be carried out using:

- a column 0.25 m long and 4.6 mm in internal diameter packed with *octylsilyl silica gel for chromatography R* (5 μ m),
- as mobile phase at a flow rate of 1 ml/min a mixture of 20 volumes of *water R*, 30 volumes of *tetrahydrofuran R* and 50 volumes of a 10.5 g/l solution of *ammonium acetate R* previously adjusted to pH 5.7 with *acetic acid R*,
- as detector a spectrophotometer set at 240 nm.

Inject 20 μ l of reference solution (b). The test is not valid unless in the chromatogram obtained, the resolution between the second of the 2 peaks corresponding to hydrolysis products and the peak corresponding to mupirocin is at least 7.0. Inject 20 μ l of reference solution (c). When the chromatogram is recorded in the prescribed conditions, the retention time relative to mupirocin is about 0.75 for impurity C. Inject 20 μ l of the test solution and 20 μ l of reference solution (a). Continue the chromatography of the test solution for 3.5 times the retention time of mupirocin. In the chromatogram obtained with the test solution: the area of any peak corresponding to impurity C is not greater than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (4 per cent); the area of any peak, apart from the principal peak and any peak corresponding to impurity C, is not greater than half the area of the principal peak in the chromatogram obtained with

reference solution (a) (1 per cent); the sum of the areas of all the peaks, apart from the principal peak, is not greater than 3 times the area of the principal peak in the chromatogram obtained with reference solution (a) (6 per cent). Disregard any peak with an area less than 0.05 times the area of the principal peak in the chromatogram obtained with reference solution (a).

Water (2.5.12). Not more than 1.0 per cent, determined on 0.500 g by the semi-micro determination of water.

ASSAY

Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 25.0 mg of the substance to be examined in 5 ml of *methanol R* and dilute to 200.0 ml with a 7.5 g/l solution of *ammonium acetate R* adjusted to pH 5.7 with *acetic acid R*.

Reference solution (a). Dissolve 25.0 mg of *mupirocin lithium CRS* in 5 ml of *methanol R* and dilute to 200.0 ml with a 7.5 g/l solution of *ammonium acetate R* adjusted to pH 5.7 with *acetic acid R*.

Reference solution (b). Adjust 10 ml of the test solution to pH 2.0 using *hydrochloric acid R* and allow to stand for 20 h.

The chromatographic procedure may be carried out using:

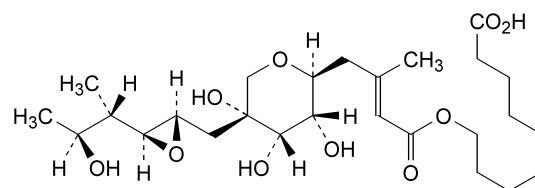
- a column 0.25 m long and 4.6 mm in internal diameter packed with *octylsilyl silica gel for chromatography R* (5 μ m),
- as mobile phase at a flow rate of 1 ml/min a mixture of 19 volumes of *water R*, 32 volumes of *tetrahydrofuran R* and 49 volumes of a 10.5 g/l solution of *ammonium acetate R* adjusted to pH 5.7 with *acetic acid R*,
- as detector a spectrophotometer set at 230 nm.

Inject 20 μ l of reference solution (b). The test is not valid unless in the chromatogram obtained, the resolution between the second of the 2 peaks corresponding to hydrolysis products and the peak corresponding to mupirocin is at least 7.0. Inject reference solution (a) 6 times. The test is not valid unless the relative standard deviation of the peak area of mupirocin is at most 1.0 per cent. Inject the test solution and reference solution (a).

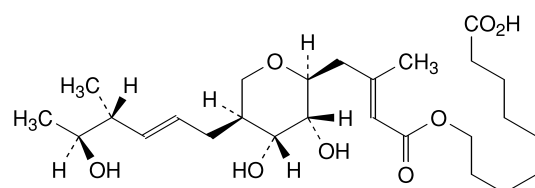
STORAGE

Protected from light.

IMPURITIES



A. 9-[(2E)-4-[(2S,3R,4R,5S)-5-[(2S,3S,4S,5S)-2,3-epoxy-5-hydroxy-4-methylhexyl]-3,4,5-trihydroxy-3,4,5,6-tetrahydro-2H-pyran-2-yl]-3-methylbut-2-enoyl]oxy]nonanoic acid (pseudomonic acid B),

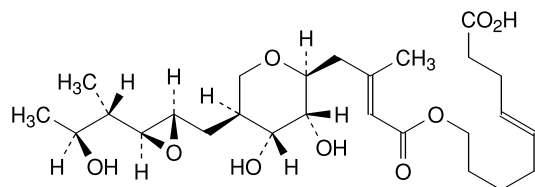


B. 9-[(2E)-4-[(2S,3R,4R,5S)-3,4-dihydroxy-5-[(2E,4R,5S)-5-hydroxy-4-methylhex-2-enyl]-3,4,5,6-tetrahydro-2H-pyran-2-yl]-3-methylbut-2-enoyl]oxy]nonanoic acid (pseudomonic acid C),

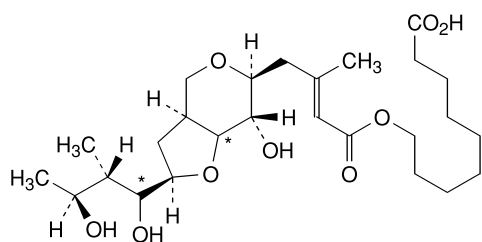
01/2005:1451

MUPIROCIN CALCIUM

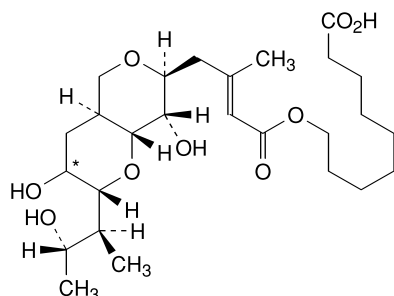
Mupirocinum calcicum



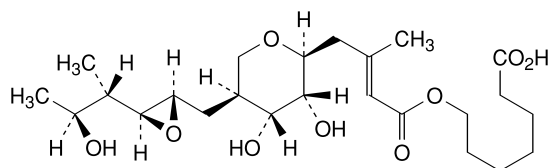
C. (4*E*)-9-[[[(2*E*)-4-[(2*S*,3*R*,4*R*,5*S*)-5-[(2*S*,3*S*,4*S*,5*S*)-2,3-epoxy-5-hydroxy-4-methylhexyl]-3,4-dihydroxy-3,4,5,6-tetrahydro-2*H*-pyran-2-yl]-3-methylbut-2-enoyl]oxy]non-4-enoic acid (pseudomonic acid D),



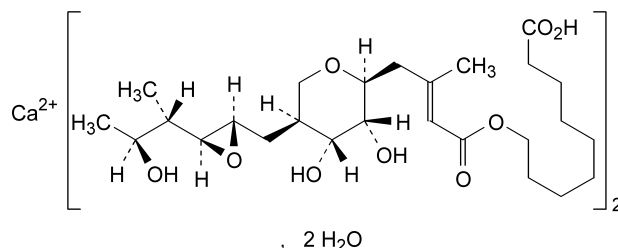
D. 9-[[[(2*E*)-4-[(2*R*,3*aS*,6*S*,7*S*)-2-[(2*S*,3*S*)-1,3-dihydroxy-2-methylbutyl]-7-hydroxy-2,3,3*a*,6,7,7*a*-hexahydro-4*H*-furo[3,2-*c*]pyran-6-yl]-3-methylbut-2-enoyl]oxy]nonanoic acid,



E. 9-[[[(2*E*)-4-[(2*R*,3*RS*,4*aS*,7*S*,8*S*,8*aR*)-3,8-dihydroxy-2-[(1*S*,2*S*)-2-hydroxy-1-methylpropyl]-3,4,4*a*,7,8,8*a*-hexahydro-2*H*,5*H*-pyrano[4,3-*b*]pyran-7-yl]-3-methylbut-2-enoyl]oxy]nonanoic acid,



F. 7-[[[(2*E*)-4-[(2*S*,3*R*,4*R*,5*S*)-5-[(2*S*,3*S*,4*S*,5*S*)-2,3-epoxy-5-hydroxy-4-methylhexyl]-3,4-dihydroxy-3,4,5,6-tetrahydro-2*H*-pyran-2-yl]-3-methylbut-2-enoyl]oxy]heptanoic acid.

C₅₂H₈₆O₁₈Ca, 2H₂OM_r 1075

DEFINITION

Mupirocin calcium contains not less than 93.0 per cent and not more than the equivalent of 100.5 per cent of calcium bis[9-[[[(2*E*)-4-[(2*S*,3*R*,4*R*,5*S*)-3,4-dihydroxy-5-[(2*S*,3*S*)-3-[(1*S*,2*S*)-2-hydroxy-1-methylpropyl]oxiranyl]methyl]tetrahydro-2*H*-pyran-2-yl]-3-methylbut-2-enoyl]oxy]nonanoate], calculated with reference to the anhydrous substance.

CHARACTERS

A white or almost white powder, very slightly soluble in water, sparingly soluble in ethanol and in methylene chloride.

IDENTIFICATION

- A. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the *Ph. Eur. reference spectrum of mupirocin calcium*.
- B. It gives reaction (a) of calcium (2.3.1).

TESTS

Specific optical rotation (2.2.7). Dissolve 0.50 g in *methanol R* and dilute to 10.0 ml with the same solvent. The specific optical rotation is -16 to -20 , calculated with reference to the anhydrous substance.

Related substances. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 50.0 mg of the substance to be examined in a mixture of equal volumes of a 13.6 g/l solution of *sodium acetate R*, adjusted to pH 4.0 with *acetic acid R*, and *methanol R*. Dilute to 10.0 ml with the same mixture of solvents.

Reference solution (a). Dilute 1.0 ml of the test solution to 50.0 ml with a mixture of equal volumes of a 13.6 g/l solution of *sodium acetate R*, adjusted to pH 4.0 with *acetic acid R* and *methanol R*.

Reference solution (b). Adjust 10 ml of reference solution (a) to pH 2.0 using *hydrochloric acid R* and allow to stand for 20 h.

Reference solution (c). Dissolve 25 mg of *mupirocin lithium CRS* in a mixture of equal volumes of a 13.6 g/l solution of *sodium acetate R*, adjusted to pH 4.0 with *acetic acid R*, and *methanol R*. Dilute to 200.0 ml with the same mixture of solvents.

The chromatographic procedure may be carried out using:

- a column 0.25 m long and 4.6 mm in internal diameter packed with *octylsilyl silica gel for chromatography R* (5 μ m),

- as mobile phase at a flow rate of 1 ml/min a mixture of 20 volumes of *water R*, 30 volumes of *tetrahydrofuran R* and 50 volumes of a 10.5 g/l solution of *ammonium acetate R* previously adjusted to pH 5.7 with *acetic acid R*,
- as detector a spectrophotometer set at 240 nm.

Inject 20 µl of reference solution (b). The test is not valid unless in the chromatogram obtained, the resolution between the second of the two peaks corresponding to hydrolysis products and the peak corresponding to mupirocin is at least 7.0. Inject 20 µl of reference solution (c). When the chromatogram is recorded in the prescribed conditions, the retention time relative to mupirocin is about 0.75 for impurity C. Inject 20 µl of the test solution and 20 µl of reference solution (a). Continue the chromatography of the test solution for 3.5 times the retention time of mupirocin. In the chromatogram obtained with the test solution: the area of any peak corresponding to impurity C is not greater than 1.25 times the area of the principal peak in the chromatogram obtained with reference solution (a) (2.5 per cent); the area of any peak, apart from the principal peak and any peak corresponding to impurity C, is not greater than half the area of the principal peak in the chromatogram obtained with reference solution (a) (1 per cent); the sum of the areas of all the peaks, apart from the principal peak, is not greater than 2.25 times the area of the principal peak in the chromatogram obtained with reference solution (a) (4.5 per cent). Disregard any peak with an area less than 0.05 times the area of the principal peak in the chromatogram obtained with reference solution (a).

Chlorides (2.4.4). Dissolve 10.0 mg in a mixture of 1 ml of *dilute nitric acid R* and 15 ml of *methanol R*. The solution complies with the limit test for chlorides (0.5 per cent).

Water (2.5.12). 3.0 per cent to 4.5 per cent, determined on 0.500 g by the semi-micro determination of water.

ASSAY

Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 25.0 mg of the substance to be examined in 5 ml of *methanol R* and dilute to 200.0 ml with a 7.5 g/l solution of *ammonium acetate R*, adjusted to pH 5.7 with *acetic acid R*.

Reference solution (a). Dissolve 25.0 mg of *mupirocin lithium CRS* in 5 ml of *methanol R* and dilute to 200.0 ml with a 7.5 g/l solution of *ammonium acetate R*, adjusted to pH 5.7 with *acetic acid R*.

Reference solution (b). Adjust 10 ml of the test solution to pH 2.0 using *hydrochloric acid R* and allow to stand for 20 h.

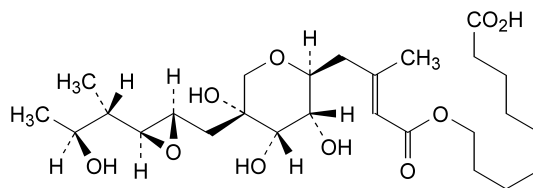
The chromatographic procedure may be carried out using:

- a column 0.25 m long and 4.6 mm in internal diameter packed with *octylsilyl silica gel for chromatography R* (5 µm),
- as mobile phase at a flow rate of 1 ml/min a mixture of 19 volumes of *water R*, 32 volumes of *tetrahydrofuran R* and 49 volumes of a 10.5 g/l solution of *ammonium acetate R* adjusted to pH 5.7 with *acetic acid R*,
- as detector a spectrophotometer set at 230 nm.

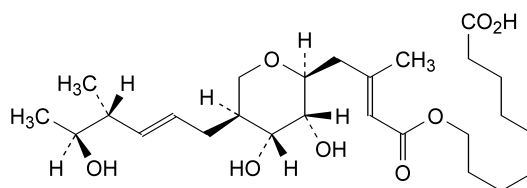
Inject 20 µl of the reference solution (b). The test is not valid unless, in the chromatogram obtained, the resolution between the second of the two peaks corresponding to hydrolysis products and the peak corresponding to mupirocin is at least 7.0. Inject reference solution (a) six times. The test is not valid unless the relative standard deviation of the peak area of mupirocin is at most 1.0 per cent. Inject the test solution and the reference solution (a).

Calculate the percentage content of mupirocin calcium by multiplying the percentage content of mupirocin lithium by 1.038.

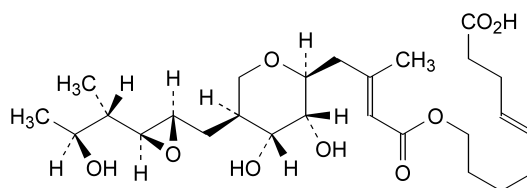
IMPURITIES



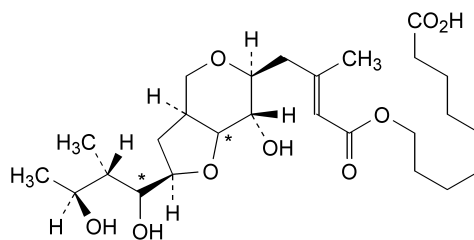
- A. 9-[[[(2E)-4-[(2S,3R,4R,5R)-3,4,5-trihydroxy-5-[[[(2S,3S)-3-[(1S,2S)-2-hydroxy-1-methylpropyl]oxiranyl]methyl]tetrahydro-2H-pyran-2-yl]-3-methylbut-2-enyl]oxy]nonanoic acid (pseudomonic acid B),



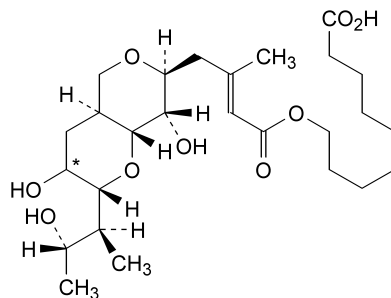
- B. 9-[[[(2E)-4-[(2S,3R,4R,5S)-3,4-dihydroxy-5-[(2E,4R,5S)-5-hydroxy-4-methylhex-2-enyl]tetrahydro-2H-pyran-2-yl]-3-methylbut-2-enyl]oxy]nonanoic acid (pseudomonic acid C),



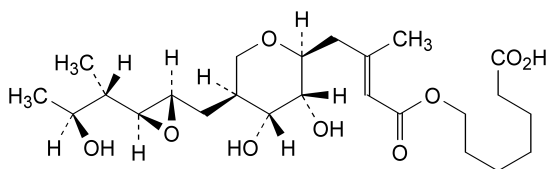
- C. (4E)-9-[[[(2E)-4-[(2S,3R,4R,5S)-3,4-dihydroxy-5-[[[(2S,3S)-3-[(1S,2S)-2-hydroxy-1-methylpropyl]oxiranyl]methyl]tetrahydro-2H-pyran-2-yl]-3-methylbut-2-enyl]oxy]non-4-enoic acid (pseudomonic acid D),



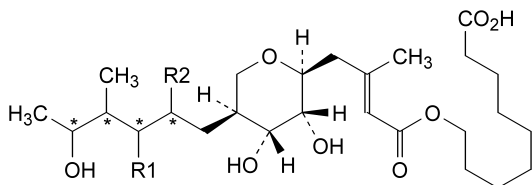
- D. 9-[[[(2E)-4-[(2R,3aS,6S,7S)-2-[(2S,3S)-1,3-dihydroxy-2-methylbutyl]-7-hydroxyhexahydro-4H-furo[3,2-c]pyran-6-yl]-3-methylbut-2-enyl]oxy]nonanoic acid,



- E. 9-[[[(2E)-4-[(2R,3RS,4aS,7S,8S,8aR)-3,8-dihydroxy-2-[(1S,2S)-2-hydroxy-1-methylpropyl]hexahydro-2H,5H-pyrano[4,3-b]pyran-7-yl]-3-methylbut-2-enyl]oxy]nonanoic acid,

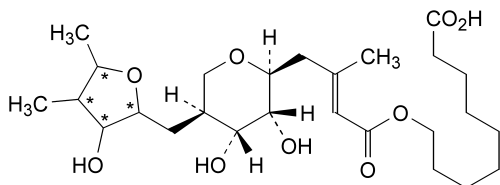


- F. 7-[[[(2E)-4-[(2S,3R,4R,5S)-3,4-dihydroxy-5-[[[(2S,3S)-3-[(1S,2S)-2-hydroxy-1-methylpropyl]oxiranyl]methyl]tetrahydro-2H-pyran-2-yl]-3-methylbut-2-enoyl]oxy]heptanoic acid,



- G. R1 = OH, R2 = Cl: 9-[[[(2E)-4-[(2S,3R,4R,5S)-5-(2-chloro-3,5-dihydroxy-4-methylhexyl)-3,4-dihydroxytetrahydro-2H-pyran-2-yl]-3-methylbut-2-enoyl]oxy]nonanoic acid,

- H. R1 = Cl, R2 = OH: 9-[[[(2E)-4-[(2S,3R,4R,5S)-5-(3-chloro-2,5-dihydroxy-4-methylhexyl)-3,4-dihydroxytetrahydro-2H-pyran-2-yl]-3-methylbut-2-enoyl]oxy]nonanoic acid,



- I. 9-[[[(2E)-4-[(2S,3R,4R,5S)-3,4-dihydroxy-5-[(3-hydroxy-4,5-dimethyltetrahydrofuran-2-yl)methyl]tetrahydro-2H-pyran-2-yl]-3-methylbut-2-enoyl]oxy]nonanoic acid.

01/2005:1349

MYRRH

Myrrha

DEFINITION

Myrrh consists of a gum-resin, hardened in air, obtained by incision or produced by spontaneous exudation from the stem and branches of *Commiphora molmol* Engler and/or other species of *Commiphora*.

CHARACTERS

Myrrh has a bitter taste.

It has the macroscopic and microscopic characters described under identification tests A and B.

IDENTIFICATION

- The light or dark orange-brown, irregular or roundish grains or pieces of different size show components of various colours. Their surface is mostly covered with grey to yellowish-brown dust.
- Reduce to a powder (355). The powder is brownish-yellow to reddish-brown. Examine under a microscope, using *chloral hydrate solution R*. The powder shows only a few tissue fragments from the original plants including the following: reddish-brown cork fragments; single or grouped polyhedral to elongated stone cells with partly strongly thickened, pitted and lignified walls with a brownish content; fragments of thin-walled parenchyma and sclerenchymatous fibres; irregular prismatic to polyhedral crystals of calcium oxalate, about 10 µm to 25 µm in size.

- Examine the chromatograms obtained in the test for *Commiphora mukul*. Spray the plate with *anisaldehyde solution R*, and examine in daylight while heating at 100 °C to 105 °C for 10 min. The chromatogram obtained with the reference solution shows in the lower third an orange-red zone (thymol) and in the middle third a violet zone (anethole).

The chromatogram obtained with the test solution shows an intense violet zone (furanoeudesma-1,3-diene), exceeding the other zones in size and intensity, above the zone of anethole in the chromatogram obtained with the reference solution; a violet zone similar in position to the zone of anethole in the chromatogram obtained with the reference solution; two intense violet zones similar in position to the zone of thymol in the chromatogram obtained with the reference solution, the upper one corresponding to curzerenone and the lower one to 2-methoxyfuranodiene. Further mostly violet zones are present in the chromatogram obtained with the test solution.

TESTS

Foreign matter (2.8.2). It complies with the test for foreign matter.

Commiphora mukul. Examine by thin-layer chromatography (2.2.27) using a *TLC silica gel plate R*.

Test solution. To 0.5 g of the powdered drug (355) add 5.0 ml of *alcohol R* and warm the mixture on a water-bath for 2 min to 3 min. Cool and filter.

Reference solution. Dissolve 10 mg of *thymol R* and 40 µl of *anethole R* in 10 ml of *alcohol R*.

Apply to the plate as bands 10 µl of each solution. Develop over a path of 15 cm using a mixture of 2 volumes of *ethyl acetate R* and 98 volumes of *toluene R*. Allow the plate to dry in air. Examine in ultraviolet light at 365 nm, the chromatogram obtained with the test solution shows no blue to violet fluorescent zones in the lower third of the chromatogram.

Matter insoluble in alcohol. Not more than 70 per cent. Place 1.00 g of the powdered drug (250) in a flask. Add 30 ml of *alcohol R* and shake vigorously for 10 min. Filter the supernatant through a tared sintered-glass filter (16) avoiding the transfer of sediment from the flask. Repeat the extraction with two quantities, each of 20 ml, of *alcohol R*. Quantitatively transfer the sediment to the filter by rinsing the flask with *alcohol R*. Dry the filter and the residue in an oven at 100 °C to 105 °C and weigh.

Loss on drying (2.2.32). Not more than 15.0 per cent, determined on 1.000 g of powdered drug (355) by drying in an oven at 100 °C to 105 °C for 2 h.

Total ash (2.4.16). Not more than 7.0 per cent.

STORAGE

Store protected from light.

01/2005:1877

MYRRH TINCTURE

Myrrhae tinctura

DEFINITION

Tincture produced from *Myrrh* (1349).

PRODUCTION

The tincture is produced from 1 part of the drug and 5 parts of ethanol (90 per cent V/V) by a suitable procedure.

CHARACTERS

Clear yellowish-brown or orange-brown liquid.

IDENTIFICATION

Thin-layer chromatography (2.2.27).

Test solution. Dilute 5 ml of the tincture to be examined to 10 ml with *alcohol R*.

Reference solution. Dissolve 10 mg of *thymol R* and 40 µl of *anethole R* in 10 ml of *ether R*.

Plate: TLC silica gel plate *R*.

Mobile phase: *ethyl acetate R*, *toluene R* (2:98 *V/V*).

Application: 10 µl, as bands.

Development: over a path of 15 cm.

Drying: in air.

Detection: spray with *anisaldehyde solution R* and examine in daylight whilst heating at 100-105 °C for 10 min.

Results: see below the sequence of the zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other zones mostly violet, are present in the chromatogram obtained with the test solution.

Top of the plate	
Anethole: a violet zone	An intense violet zone exceeding the others in size and intensity (furanoeudesma-1,3-diene) A violet zone
Thymol: an orange-red zone	Two intense violet zones (curzerenone and below 2-methoxyfuranodiene)
Reference solution	Test solution

TESTS

Ethanol content (2.9.10): 82 per cent *V/V* to 88 per cent *V/V*.

Methanol and 2-propanol (2.9.11): maximum 0.05 per cent *V/V* of methanol and maximum 0.05 per cent *V/V* of 2-propanol.

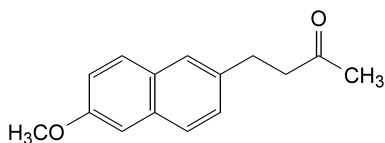
Dry residue (2.8.16): minimum 4.0 per cent *m/m*.

STORAGE

Plastic containers are not recommended.

NABUMETONE

Nabumetonum

C₁₅H₁₆O₂M_r 228.3

DEFINITION

Nabumetone contains not less than 97.0 per cent and not more than the equivalent of 102.0 per cent of 4-(6-methoxynaphthalen-2-yl)butan-2-one, calculated with reference to the anhydrous substance.

CHARACTERS

A white or almost white, crystalline powder, practically insoluble in water, freely soluble in acetone, slightly soluble in methanol.

IDENTIFICATION

Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *nabumetone CRS*.

TESTS

Related substances. Examine by liquid chromatography (2.2.29) as described under Assay.

Inject 20 µl of test solution (a), 20 µl of reference solution (b) and 20 µl of reference solution (c). In the chromatogram obtained with test solution (a): the area of any peak due to impurity F, is not greater than the area of the principal peak in the chromatogram obtained with reference solution (c) (0.3 per cent); the sum of the areas of any other peak, apart from the principal peak and a peak due to impurity F, is not greater than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent). Disregard any peak with an area less than 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (b).

Heavy metals (2.4.8). 2.0 g complies with limit test C for heavy metals (10 ppm). Prepare the standard using 2 ml of *lead standard solution* (10 ppm Pb) R.

Water (2.5.12). Not more than 0.2 per cent, determined on 1.000 g by the semi-micro determination of water.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Examine by liquid chromatography (2.2.29).

Test solution (a). Dissolve 50.0 mg of the substance to be examined in *acetonitrile R* and dilute to 10.0 ml with the same solvent.

Test solution (b). Dilute 1.0 ml of test solution (a) to 25.0 ml with *acetonitrile R*. Dilute 1.0 ml of the solution to 5.0 ml with *acetonitrile R*.

Reference solution (a). Dissolve 20.0 mg of *nabumetone CRS* in *acetonitrile R* and dilute to 10.0 ml with the same solvent. Dilute 1.0 ml of the solution to 50.0 ml with *acetonitrile R*.

Reference solution (b). Dilute 0.5 ml of test solution (a) to 100.0 ml with *acetonitrile R*.

01/2005:1350 Reference solution (c). Dissolve 1.5 mg of *nabumetone impurity F CRS* in *acetonitrile R* and dilute to 100.0 ml with the same solvent.

Reference solution (d). Dissolve 4 mg of *nabumetone impurity D CRS* in *acetonitrile R* and dilute to 100 ml with the same solvent. To 5 ml of the solution, add 5 ml of test solution (b).

The chromatographic procedure may be carried out using:

- a stainless steel column 0.15 m long and 4.6 mm in internal diameter packed with *base-deactivated octadecylsilyl silica gel for chromatography R* (4 µm),
- as mobile phase at a flow rate of 1 ml/min:

Mobile phase A. A mixture of 12 volumes of *tetrahydrofuran R*, 28 volumes of *acetonitrile for chromatography R* and 60 volumes of a 0.1 per cent V/V solution of *glacial acetic acid R* in *carbon dioxide-free water R* prepared from *distilled water R*,

Mobile phase B. A mixture of 24 volumes of *tetrahydrofuran R*, 56 volumes of *acetonitrile for chromatography R* and 20 volumes of a 0.1 per cent V/V solution of *glacial acetic acid R* in *carbon dioxide-free water R* prepared from *distilled water R*,

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)	Comment
0 - 12	100	0	isocratic
12 - 28	100 → 0	0 → 100	linear gradient
28 - 33	0	100	isocratic
33 - 34	0 → 100	100 → 0	linear gradient
34 - 35	100	0	isocratic

- as detector a spectrophotometer set at 254 nm, maintaining the temperature of the column at 40 °C.

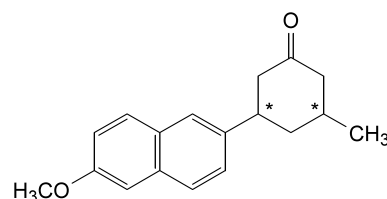
Inject 20 µl of reference solution (b) and 20 µl of reference solution (d). Adjust the sensitivity of the system so that the height of the principal peak in the chromatogram obtained with reference solution (b) is at least 70 per cent of the full scale of the recorder. When the chromatograms are recorded under the prescribed conditions the retention time for nabumetone is about 11 min. The test is not valid unless, in the chromatogram obtained with reference solution (d), the resolution between the peaks corresponding to nabumetone and impurity D is at least 1.5. Inject reference solution (a) six times. The test is not valid unless the relative standard deviation of the peak area for nabumetone is at most 1.0 per cent. Inject alternately test solution (b) and reference solution (a).

Calculate the percentage content of nabumetone using the chromatogram obtained with reference solution (a).

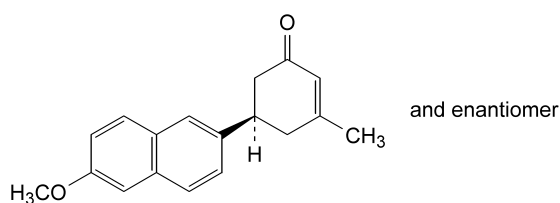
STORAGE

Store protected from light.

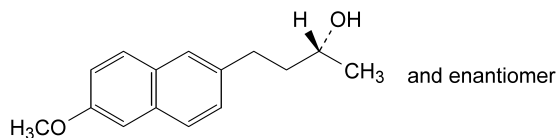
IMPURITIES



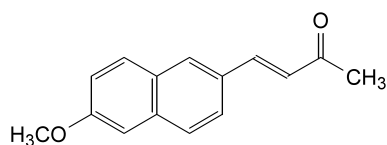
A. 3-(6-methoxynaphthalen-2-yl)-5-methylcyclohexanone,



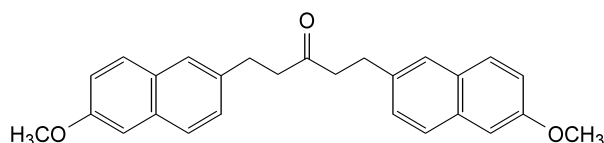
B. (5*RS*)-5-(6-methoxynaphthalen-2-yl)-3-methylcyclohex-2-enone,



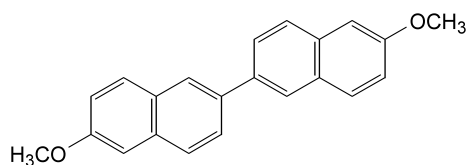
C. (2*RS*)-4-(6-methoxynaphthalen-2-yl)butan-2-ol,



D. (*E*)-4-(6-methoxynaphthalen-2-yl)but-3-en-2-one,



E. 1,5-bis(6-methoxynaphthalen-2-yl)pentan-3-one,

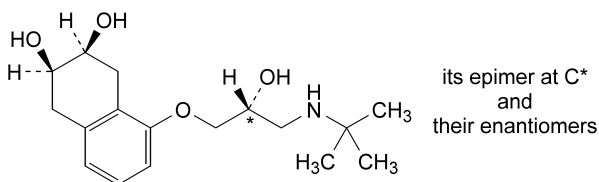


F. 6,6'-dimethoxy-2,2'-binaphthalenyl.

01/2005:1789

NADOLOL

Nadololum



$C_{17}H_{27}NO_4$

M_r 309.4

DEFINITION

cis-5-[3-[(1,1-Dimethylethyl)amino]-2-hydroxypropoxy]-1,2,3,4-tetrahydronaphthalene-2,3-diol.

It consists of 2 pairs of enantiomers which are present as 2 racemic compounds: racemate A and racemate B.

Content: 98.5 per cent to 101.0 per cent (dried substance).

CHARACTERS

Appearance: white or almost white, crystalline powder.

Solubility: slightly soluble in water, freely soluble in alcohol, practically insoluble in acetone.

IDENTIFICATION

Infrared absorption spectrophotometry (2.2.24).

Comparison: nadolol CRS.

TESTS

Racemate content. Infrared absorption spectrophotometry (2.2.24).

Prepare a mull in *liquid paraffin R* of the substance to be examined (dried substance), adjusting the thickness of the mull to give an absorbance reading of 0.6 ± 0.1 at 1587 cm^{-1} . Record the spectrum from 1667 to 1111 cm^{-1} , using *liquid paraffin R* as reference. Measure the absorbance A_a , corresponding to racemate A, at the maximum at 1266 cm^{-1} and the absorbance A_b , corresponding to racemate B, at the maximum at 1250 cm^{-1} . The ratio A_a/A_b is 0.72 to 1.08 (corresponding to racemate A content of between 40 per cent and 60 per cent).

Related substances. Liquid chromatography (2.2.29).

Test solution. Dissolve 0.100 g of the substance to be examined in 4.0 ml of *methanol R* and dilute to 100.0 ml with a mixture of 20 volumes of *acetonitrile R* and 80 volumes of *water R*.

Reference solution (a). Dilute 1.0 ml of the test solution to 50.0 ml with a mixture of 20 volumes of *acetonitrile R* and 80 volumes of *water R*. Dilute 5.0 ml of the solution to 50.0 ml with a mixture of 20 volumes of *acetonitrile R* and 80 volumes of *water R*.

Reference solution (b). Dissolve 20 mg of *pindolol CRS* and 20 mg of the substance to be examined in 20 ml of *methanol R* and dilute to 100.0 ml with a mixture of 20 volumes of *acetonitrile R* and 80 volumes of *water R*. Dilute 1.0 ml of the solution to 100.0 ml with a mixture of 20 volumes of *acetonitrile R* and 80 volumes of *water R*.

Reference solution (c). Dilute 5.0 ml of reference solution (a) to 20.0 ml with a mixture of 20 volumes of *acetonitrile R* and 80 volumes of *water R*.

Column:

- *size*: $l = 0.30\text{ m}$, $\varnothing = 3.9\text{ mm}$,
- *stationary phase*: spherical *end-capped octadecylsilyl silica gel for chromatography R* ($5\text{ }\mu\text{m}$), with a specific surface area of $700\text{ m}^2/\text{g}$, a pore size of 0.9 nm and a carbon loading of 12 per cent.
- *temperature*: $40\text{ }^\circ\text{C}$.

Mobile phase:

- *mobile phase A*: 5.6 g/l solution of *sodium octanesulphonate R* adjusted to pH 3.5 with a 300 g/l solution of *phosphoric acid R*,
- *mobile phase B*: *acetonitrile R*,

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)
0 - 7	77	23
7 - 30	77 → 65	23 → 35
30 - 45	65	35
45 - 50	65 → 77	35 → 23
50 - 60	77	23

Flow rate: 1 ml/min.

Detection: spectrophotometer at 206 nm.

Injection: 20 μl .

Relative retention with reference to nadolol (retention time = about 18 min): impurity A = 0.25; impurity B = 0.4; impurity C (doublet) = 0.6 and 0.7; impurity D = 1.4; impurity E = 1.6; impurity F = 2.2; impurity G = 2.6.

System suitability: reference solution (b):

- **resolution:** minimum of 8.0 between the peaks due to nadolol and to pindolol.

Limits:

- **correction factors:** for the calculation of contents, multiply the peak areas of the following impurities by the corresponding correction factor: impurity A = 1.7; impurity B = 1.7; impurity C = 1.7 (multiply the sum of the areas of the 2 peaks);
- **impurity A, B, C, D, E, F, G:** for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent);
- **any other impurity:** not more than half the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent);
- **total:** not more than 2.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent);
- **disregard limit:** area of the principal peak in the chromatogram obtained with reference solution (c) (0.05 per cent).

Heavy metals (2.4.8): maximum 30 ppm.

1.0 g complies with limit test D. Prepare the standard using 3 ml of *lead standard solution* (10 ppm Pb) R.

Loss on drying (2.2.32): maximum 2.0 per cent, determined on 1.000 g by drying *in vacuo* at 60 °C for 3 h.

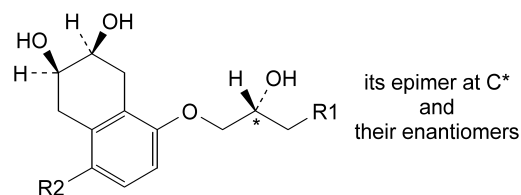
Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

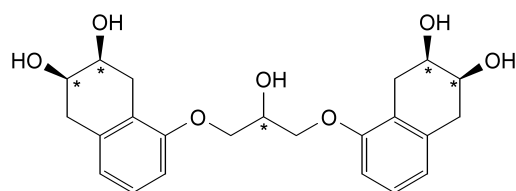
Dissolve 0.250 g in 100 ml of *anhydrous acetic acid* R. Titrate with 0.1 M *perchloric acid*, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *perchloric acid* is equivalent to 30.94 mg of C₁₇H₂₇NO₄.

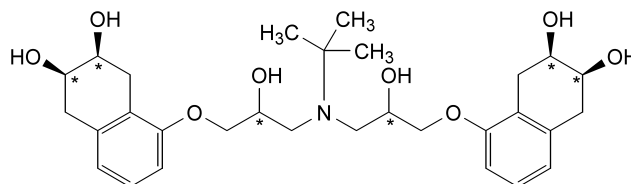
IMPURITIES



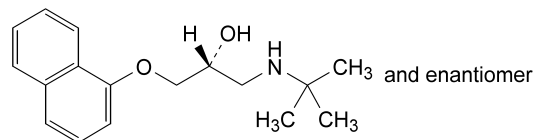
- A. R1 = OH, R2 = H: *cis*-5-[(2*RS*)-2,3-dihydroxypropoxy]-1,2,3,4-tetrahydronaphthalene-2,3-diol (tetraol),
- B. R1 = OCH₃, R2 = H: *cis*-5-[(2*RS*)-2-hydroxy-3-methoxypropoxy]-1,2,3,4-tetrahydronaphthalene-2,3-diol,
- E. R1 = NH-C(CH₃)₃, R2 = I: *cis*-5-[(2*RS*)-3-[(1,1-dimethylethyl)amino]-2-hydroxypropoxy]-8-iodo-1,2,3,4-tetrahydronaphthalene-2,3-diol,



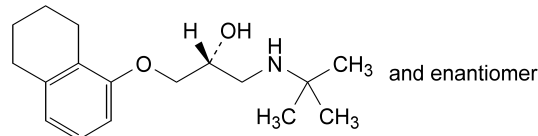
- C. 5,5'-[(2-hydroxypropane-1,3-diyl)bis(oxy)]bis(*cis*-1,2,3,4-tetrahydronaphthalene-2,3-diol),



- D. 5,5'-[[[(1,1-dimethylethyl)imino]bis[(2-hydroxypropane-1,3-diyl)oxy]]bis(*cis*-1,2,3,4-tetrahydronaphthalene-2,3-diol),



- F. (2*RS*)-1-[(1,1-dimethylethyl)amino]-3-(naphthalen-1-yloxy)propan-2-ol,

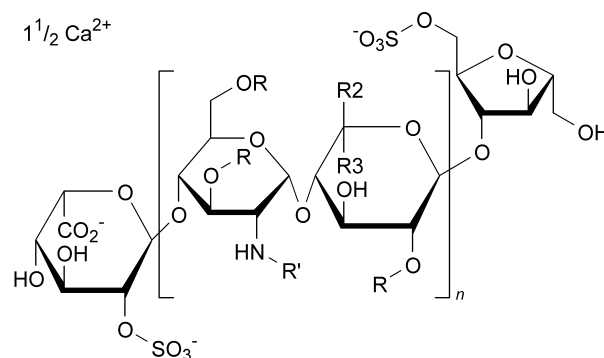


- G. (2*RS*)-1-[(1,1-dimethylethyl)amino]-3-[(5,6,7,8-tetrahydronaphthalen-1-yl)oxy]propan-2-ol.

01/2005:1134

NADROPARIN CALCIUM

Nadroparinum calcicum



R = H or SO₃($1/2 \text{ Ca}$), R' = H or SO₃($1/2 \text{ Ca}$) or CO-CH₃
R2 = H and R3 = CO₂($1/2 \text{ Ca}$) or R2 = CO₂($1/2 \text{ Ca}$) and R3 = H

DEFINITION

Nadroparin calcium is the calcium salt of low-molecular-mass heparin obtained by nitrous acid depolymerisation of heparin from pork intestinal mucosa, followed by fractionation to eliminate selectively most of the chains with a molecular mass lower than 2000. The majority of the components have a 2-*O*-sulpho-α-*L*-idopyranosuronic acid structure at the non-reducing end and a 6-*O*-sulpho-2,5-anhydro-*D*-mannitol structure at the reducing end of their chain.

Nadroparin calcium complies with the monograph on *Heparins, low-molecular-mass (0828)* with the modifications and additional requirements below.

The mass-average molecular mass ranges between 3600 and 5000 with a characteristic value of about 4300.

The degree of sulphatation is about 2 per disaccharide unit. The potency is not less than 95 IU and not more than 130 IU of anti-factor Xa activity per milligram, calculated with reference to the dried substance. The ratio of anti-factor Xa activity to anti-factor IIa activity is between 2.5 and 4.0.

IDENTIFICATION

Carry out identification test C as described in the monograph on *Heparins, low-molecular-mass (0828)*. The following requirements apply.

The mass-average molecular mass ranges between 3600 and 5000. The mass percentage of chains lower than 2000 is not more than 15 per cent. The mass percentage of chains between 2000 and 8000 ranges between 75 per cent and 95 per cent. The mass percentage of chains between 2000 and 4000 ranges between 35 per cent and 55 per cent.

TESTS

Appearance of solution. Dissolve 0.5 g of the substance to be examined in 10 ml of *water R*. The solution is not more intensely opalescent than the reference suspension II (2.2.1) and not more intensely coloured than the reference solution Y₅ (2.2.2, *Method II*).

Ethanol. Not more than 1.0 per cent *m/m*, determined by head-space gas chromatography (2.2.28), using *2-propanol R* as the internal standard.

Internal standard solution. Dilute 1.0 ml of *2-propanol R* to 100.0 ml with *water R*. Dilute 1.0 ml of this solution to 50.0 ml with *water R*.

Reference solution. Dilute 1.0 ml of *ethanol R* to 100.0 ml with *water R*. Dilute 0.5 ml of this solution to 20.0 ml with *water R*.

Vial filling. Place the following into four separate vials which can be crimp-sealed and which are compatible with the injection system:

- 1.0 ml of *water R* (blank vial),
- 0.50 ml of the reference solution and 0.50 ml of the internal standard solution (reference vial),
- 10.0 mg of the substance to be examined. Add 1.0 ml of *water R* (test vial A),
- 10.0 mg of the substance to be examined. Add 0.50 ml of *water R* and 0.50 ml of the internal standard solution (test vial B).

The chromatographic procedure may be carried out using:

- a nickel column 1.5 m long and 2 mm in internal diameter packed with *ethylvinylbenzene divinylbenzene copolymer R* (150 µm to 180 µm),
- *helium for chromatography R* or *nitrogen for chromatography R* as the carrier gas at a flow rate of 30 ml/min,
- a flame-ionisation detector.

Maintain the temperature of the column at 150 °C and that of the injection port and that of the detector at 250 °C.

Equilibrate each vial in the head-space system at 90 °C for 15 min. The pre-injection pressurisation time is 1 min.

The chromatogram obtained with the reference vial shows two peaks which correspond in order of increasing retention time to ethanol and 2-propanol (with retention times of approximately 2.5 min and 4 min). Calculate the content of ethanol (*m/m*) taking its density at 20 °C to be 0.792 g/ml.

N-NO groups. Not more than 0.25 ppm, determined by cleavage of the N-NO bond with hydrobromic acid in ethyl acetate under a reflux condenser and detection of the released NO by chemiluminescence.

Description of the apparatus (Figure 1134-1). Use a 500 ml borosilicate glass round-bottomed flask, above which is attached a condenser which is equipped with:

- on one side, a torion joint through which a stream of *argon R* can be introduced via a cannula,

- on the other side, a screw joint with a piston equipped with a septum through which the reference solution and test solution will be injected.

The round-bottomed flask is connected in series to three bubble traps which are themselves connected to two cold traps, which are in turn connected to a chemiluminescence detector. Suitable tubing ensures the junctions are leak-free.

Preparation of the chemiluminescence detector. Switch on the chemiluminescence detector 48 h before use and start the vacuum pump. The vacuum must be less than 0.5 mm Hg. 1 h before use, open the oxygen valve at a pressure of 0.2 MPa and a flow rate of 9.4 ml/min.

Preparation of the bubble trap. In each bubble trap, place 30 ml of a 300 g/l solution of *sodium hydroxide R* in *water R*.

Preparation of the cold traps.

- Trap at –120 °C: Slowly add liquid nitrogen to an isothermic flask containing 250 ml of *ethanol R* whilst stirring with a wooden spatula until a paste is obtained. Place the cold trap in the isothermic flask prepared as described.
- Trap at –160 °C: Slowly add liquid nitrogen to an isothermic flask containing 250 ml of *2-methylbutane R* whilst stirring with a wooden spatula until a paste is obtained. Place the cold trap in the isothermic flask prepared as described.

Drying of the 500 ml borosilicate-glass round-bottomed flask and condenser. Boil 50 ml of *ethyl acetate R* under reflux for 1 h under *argon R* without connecting the system to the chemiluminescence detector.

Test solution. Dry the substance to be examined for 12 h over *diphosphorus pentoxide R* at 60 °C under vacuum. Dissolve 0.10 g of the treated substance to be examined in 1.0 ml of *treated formamide R*. Shake the solution obtained for 30 min.

Reference solution. Dilute 0.1 ml of *nitrosodipropylamine solution R* in 6.0 ml of *ethanol R*. Dilute 0.1 ml of the solution obtained in 1.0 ml of *treated formamide R*. (This solution is equivalent to 0.05 ppm of N-NO groups).

Place 50 ml of *treated ethyl acetate R* in the dry 500 ml borosilicate glass round-bottomed flask equipped with a septum. Connect the round-bottomed flask to the condenser which has been previously cooled to –15 °C for 2 h.

Connect the *argon R* cannula and adjust the flow rate to 0.1 litre/min. Check that the system is leak-free. Only the connector to the chemiluminescence detector remains open in order to avoid excess pressure.

Heat the *treated ethyl acetate R* to boiling.

Evacuate the system by slowly turning the valve of the chemiluminescence detector. At the same time tighten the inlet on the chemiluminescence detector.

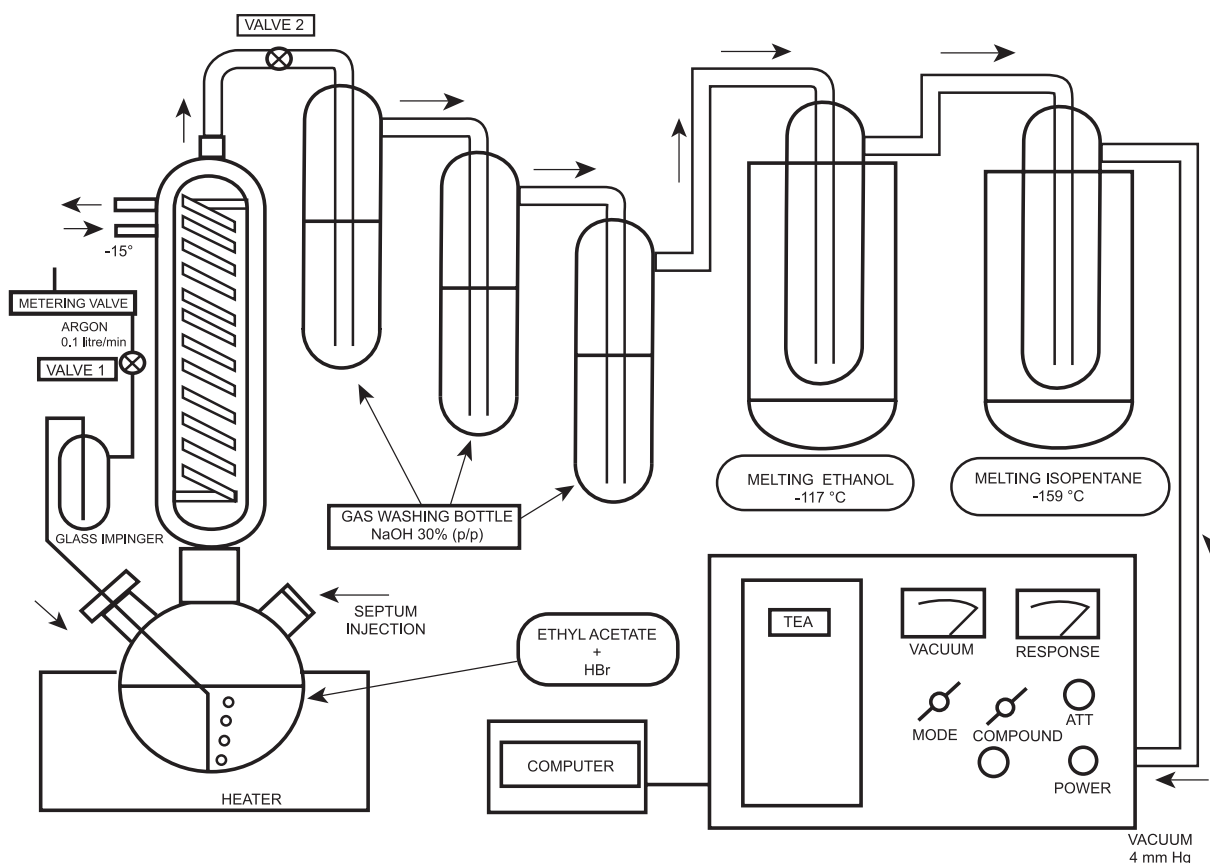
When the system is equilibrated, the vacuum reaches 4 mm Hg.

The signal of the zero adjuster on the chemiluminescence detector is set to 10 per cent of the full scale of the recorder.

Through the septum of the 500 ml borosilicate glass round-bottomed flask, sequentially inject 0.5 ml of *water R*, 2.0 ml of *dilute hydrobromic acid R* and then another 2.0 ml of *dilute hydrobromic acid R*, making sure that the recorder pen has returned to the baseline between each injection.

Inject 50.0 µl of the reference solution, then 50.0 µl of the test solution after the recorder pen has returned to the baseline.

Calculate the content of N-NO groups of the substance to be examined.



Bubble traps. Height: 24 cm, internal diameter: 2.5 cm, internal tubing 23 cm in length by 0.5 cm internal diameter. Centrally positioned Rotulex mounting. Equipped with torion joints on the inlet and outlet.

Chemiluminescence detector.

Cold trap. Height: 16.5 cm, internal diameter: 4 cm, internal tubing 14 cm in length and internal diameter 1.3 cm. Equipped with torion joints on the inlet and outlet.

Condenser. Height: 21 cm, internal diameter: 3 cm. Lower rodavis joint and upper torion joint.

Flask. Round-bottomed borosilicate glass flask equipped with a central rodavis joint, a torion joint on the left neck and a 15 cm screw joint on the right neck.

Isothermic flask. Internal depth: 22 cm, internal diameter: 8 cm.

Septum. Silicone material, diameter: 14 mm; thickness: 3.5 mm.

Torion joint.

Tubing. Polytetrafluoroethylene FEP material, internal diameter: 3.2 mm, thickness: 0.8 mm.

Figure 1134.-1. – Apparatus used for the assay of N-NO groups

Free sulphates. Not more than 0.5 per cent, determined by liquid chromatography (2.2.29) using an instrument equipped with a conductivity detector.

Test solution. Dissolve 30.0 mg of the substance to be examined in *water R* and dilute to 10.0 ml with the same solvent.

Reference solution. Dissolve 1.4787 g of *anhydrous sodium sulphate R* in *water R* and dilute to 1000.0 ml with the same solvent. Dilute 1.0 ml of this solution to 200.0 ml with *distilled water R* (5 ppm of sulphate ions).

The chromatographic procedure may be carried out using:

- an anion separation column 50 mm long and 4.6 mm in internal diameter,
- a chemical neutralisation system: neutralisation micromembrane in line with the mobile phase for anion detection,

- elute with a 1.91 g solution of *disodium tetraborate R* in 1000 ml of *water R* as mobile phase for 15 min. Change to 100 per cent of 0.1 M *sodium hydroxide* for a period of 0.5 min. Elute with this solution for 10 min. Return to the initial conditions for a period of 0.5 min. The flow rate is 1.0 ml/min,
- a detector with a sensitivity of 30 µS.

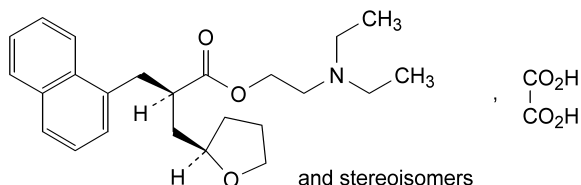
Continuously pump the chemical neutralisation system in counter-flow with a 2.45 g/l solution of *sulphuric acid R*, at a flow rate of 4 ml/min.

Inject 50 µl of each solution. The chromatogram obtained with the reference solution shows a principal peak which corresponds to the sulphate ion (retention time of about 7.5 min). Change the composition of the mobile phase, if necessary, to obtain the prescribed retention time. Calculate the sulphate content of the substance to be examined.

01/2005:1594

NAFTIDROFURYL HYDROGEN OXALATE

Naftidrofuryli hydrogenooxalas

 $C_{26}H_{35}NO_7$ M_r 473.6

DEFINITION

Mixture of 4 stereoisomers of 2-(diethylamino)ethyl 2-[(naphthalen-1-yl)methyl]-3-(tetrahydrofuran-2-yl)propanoate hydrogen oxalate.

Content: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

Appearance: white or almost white powder.

Solubility: freely soluble in water, freely soluble or soluble in alcohol, slightly or sparingly soluble in acetone.

IDENTIFICATION

A. Infrared absorption spectrophotometry (2.2.24).

Preparation: dissolve 1.0 g in *water R* and dilute to 50 ml with the same solvent. Add 2 ml of *concentrated ammonia R* and shake with 3 quantities, each of 10 ml, of *methylene chloride R*. To the combined lower layers, add *anhydrous sodium sulphate R*, shake, filter and evaporate the filtrate at a temperature not exceeding 30 °C, using a rotary evaporator. Use the residue obtained.

Comparison: *Ph. Eur.* reference spectrum of *naftidrofuryl*.

B. Dissolve 0.5 g in *water R* and dilute to 10 ml with the same solvent. Add 2.0 ml of *calcium chloride solution R*. A white precipitate is formed. The precipitate dissolves after the addition of 3.0 ml of *hydrochloric acid R*.

TESTS

Absorbance (2.2.25): maximum 0.1 at 430 nm.

Dissolve 1.5 g in *water R* and dilute to 10 ml with the same solvent. If necessary use an ultrasonic bath.

Related substances

A. Liquid chromatography (2.2.29).

Test solution. Dissolve 80.0 mg of the substance to be examined in the mobile phase and dilute to 20.0 ml with the mobile phase. Treat in an ultrasonic bath for 10 s. A precipitate is formed. Filter through a 0.45 µm membrane filter, discarding the first 5 millilitres. *Use a freshly prepared solution.*

Reference solution (a). Dissolve 5.0 mg of *naftidrofuryl impurity A CRS* in *acetonitrile R* and dilute to 25.0 ml with the same solvent. Dilute 1.0 ml of this solution to 50.0 ml with the mobile phase.

Reference solution (b). Dissolve 5 mg of *naftidrofuryl impurity B CRS* and 5 mg of the substance to be examined in *acetonitrile R* and dilute to 50 ml with the same solvent. Dilute 1 ml of this solution to 50 ml with the mobile phase.

Column:

- **size:** $l = 0.25$ m, $\varnothing = 4.6$ mm,
- **stationary phase:** spherical *end-capped octadecylsilyl silica gel for chromatography R* (5 µm) with a specific surface area of 350 m²/g, a pore size of 10 nm and a carbon loading of 14 per cent.

Mobile phase: mix 60 ml of *methanol R* with 150 ml of *tetrabutylammonium buffer solution pH 7.0 R* and dilute to 1000 ml with *acetonitrile R*.

Flow rate: 1 ml/min.

Detection: spectrophotometer at 283 nm.

Injection: 20 µl.

Run time: 2.3 times the retention time of *naftidrofuryl*.

Relative retention with reference to *naftidrofuryl* (retention time = about 7 min): *impurity A* = about 0.5; *impurity B* = about 0.8; *impurity C* = about 1.8.

System suitability: reference solution (b):

- **resolution:** minimum 3.0 between the peaks due to *naftidrofuryl* and *impurity B*.

Limits:

- **impurities A, B, C:** for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent),
- **any other impurity:** for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent),
- **total:** not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.3 per cent),
- **disregard limit:** 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.02 per cent).

B. Gas chromatography (2.2.28).

Test solution (a). Dissolve 1.0 g of the substance to be examined in *water R* and dilute to 50 ml with the same solvent. Add 2 ml of *concentrated ammonia R* and shake with 3 quantities, each of 10 ml, of *methylene chloride R*. To the combined lower layers, add *anhydrous sodium sulphate R*, shake, filter and evaporate the filtrate at a temperature not exceeding 30 °C, using a rotary evaporator. Take up the residue with *methylene chloride R* and dilute to 20.0 ml with the same solvent.

Test solution (b). Dilute 1.0 ml of test solution (a) to 10.0 ml with *methylene chloride R*.

Reference solution. Dissolve 5 mg of *naftidrofuryl impurity F CRS* in *methylene chloride R* and dilute to 50 ml with the same solvent.

Column:

- **material:** fused silica,
- **size:** $l = 25$ m, $\varnothing = 0.32$ mm,
- **stationary phase:** *poly(dimethyl)(diphenyl)siloxane R* (film thickness 0.45 µm).

Carrier gas: *helium for chromatography R*.

Splitter flow rate: 25 ml/min.

Flow rate: 2.9 ml/min.

Temperature:

	Time (min)	Temperature (°C)
Column	0 - 4	210
	4 - 8	210 → 230
	8 - 18	230 → 260
	18 - 30	260
Injection port		290
Detector		290

Detection: flame ionisation.

Injection: 1 µl.

Relative retention with reference to the second eluting peak of naftidrofuryl: impurity D = about 0.14; impurity B = about 0.55 (for the second eluting peak); impurity E = about 0.86; impurity F = about 1.04 (for the second eluting peak).

System suitability: test solution (b):

- *resolution:* minimum 1.0 between the 2 peaks due to the diastereoisomers of naftidrofuryl.

Limits: test solution (a):

- *impurity F:* for the sum of the areas of the 2 peaks, maximum 0.20 per cent of the sum of the areas of the 2 peaks due to naftidrofuryl (0.20 per cent),
- *impurity E:* maximum 0.20 per cent of the sum of the areas of the 2 peaks due to naftidrofuryl (0.20 per cent),
- *impurity D:* maximum 0.10 per cent of the sum of the areas of the 2 peaks due to naftidrofuryl (0.10 per cent),
- *any other impurity:* for each impurity, maximum 0.10 per cent of the sum of the areas of the 2 peaks due to naftidrofuryl (0.10 per cent),
- *total:* maximum 0.50 per cent of the sum of the areas of the 2 peaks due to naftidrofuryl (0.50 per cent),
- *disregard limit:* 0.02 per cent of the sum of the areas of the 2 peaks due to naftidrofuryl (0.02 per cent); disregard any peaks due to impurity B.

Diastereoisomer ratio. Gas chromatography (2.2.28) as described in test B for related substances.

Limits: test solution (b):

- *first eluting naftidrofuryl diastereoisomer:* minimum 30 per cent of the sum of the areas of the 2 peaks due to naftidrofuryl.

Heavy metals (2.4.8): maximum 10 ppm.

In a silica crucible, mix thoroughly 1.0 g of the substance to be examined with 0.5 g of *magnesium oxide R1*. Ignite to dull redness until a homogeneous white or greyish-white mass is obtained. If after 30 min of ignition the mixture remains coloured, allow to cool, mix using a fine glass rod and repeat the ignition. If necessary repeat the operation. Heat at 800 °C for about 1 h. Take up the residue with 2 quantities, each of 5 ml, of a mixture of equal volumes of *hydrochloric acid R1* and *water R*. Add 0.1 ml of *phenolphthalein solution R* and then *concentrated ammonia R* until a pink colour is obtained. Cool, add *glacial acetic acid R* until the solution is decolorised and add 0.5 ml in excess. Filter if necessary and wash the filter. Dilute to 20 ml with *water R*. The solution complies with limit test E. Prepare the standard using 10 ml of *lead standard solution (1 ppm Pb) R*.

Loss on drying (2.2.32): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 100–105 °C.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

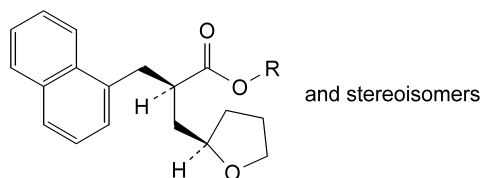
ASSAY

Dissolve 0.350 g in 50 ml of *anhydrous acetic acid R*. Titrate with 0.1 M *perchloric acid*, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *perchloric acid* is equivalent to 47.36 mg of C₂₆H₃₅NO₇.

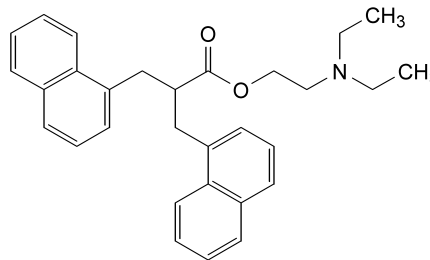
IMPURITIES

Specified impurities: A, B, C, D, E, F.

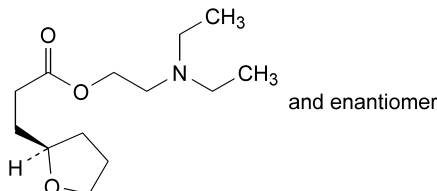


A. R = H: 2-[(naphthalen-1-yl)methyl]-3-(tetrahydrofuran-2-yl)propanoic acid,

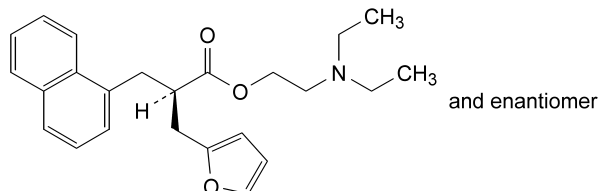
B. R = C₂H₅: ethyl 2-[(naphthalen-1-yl)methyl]-3-(tetrahydrofuran-2-yl)propanoate,



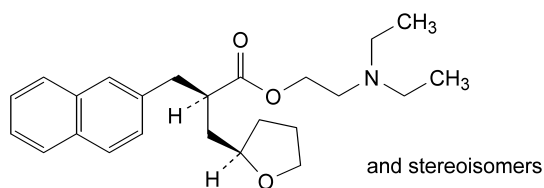
C. 2-(diethylamino)ethyl 3-(naphthalen-1-yl)-2-[(naphthalen-1-yl)methyl]propanoate,



D. 2-(diethylamino)ethyl 3-[(2*RS*)-tetrahydrofuran-2-yl]propanoate,



E. 2-(diethylamino)ethyl (2*RS*)-2-[(furan-2-yl)methyl]-3-(naphthalen-1-yl)propanoate,

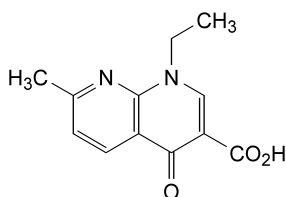


F. 2-(diethylamino)ethyl 2-[(naphthalen-2-yl)methyl]-3-(tetrahydrofuran-2-yl)propanoate.

01/2005:0701 *Reference solution (b).* Dilute 2 ml of test solution (b) to 10 ml with *methylene chloride R*.

NALIDIXIC ACID

Acidum nalidixicum



$C_{12}H_{12}N_2O_3$

M_r 232.2

DEFINITION

Nalidixic acid contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of 1-ethyl-7-methyl-4-oxo-1,4-dihydro-1,8-naphthyridine-3-carboxylic acid, calculated with reference to the dried substance.

CHARACTERS

An almost white or pale yellow, crystalline powder, practically insoluble in water, soluble in methylene chloride, slightly soluble in acetone and in alcohol. It dissolves in dilute solutions of alkali hydroxides.

It melts at about 230 °C.

IDENTIFICATION

First identification: B.

Second identification: A, C, D.

- Dissolve 12.5 mg in 0.1 M sodium hydroxide and dilute to 50.0 ml with the same solvent. Dilute 2.0 ml of this solution to 100.0 ml with 0.1 M sodium hydroxide. Examined between 230 nm and 350 nm (2.2.25), the solution shows two absorption maxima, at 258 nm and 334 nm. The ratio of the absorbance measured at 258 nm to that measured at 334 nm is 2.2 to 2.4.
- Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *nalidixic acid CRS*. Examine the substances prepared as discs.
- Examine the chromatograms obtained in the test for related substances. The principal spot in the chromatogram obtained with the test solution (b) is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a).
- Dissolve 0.1 g in 2 ml of *hydrochloric acid R*. Add 0.5 ml of a 100 g/l solution of β -naphthol *R* in *alcohol R*. An orange-red colour develops.

TESTS

Absorbance. Dissolve 1.50 g in *methylene chloride R* and dilute to 50.0 ml with the same solvent. The absorbance (2.2.25) measured at 420 nm is not greater than 0.10.

Related substances. Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel F₂₅₄ plate R*.

Test solution (a). Dissolve 0.20 g of the substance to be examined in *methylene chloride R* and dilute to 10 ml with the same solvent.

Test solution (b). Dilute 1 ml of test solution (a) to 20 ml with *methylene chloride R*.

Reference solution (a). Dissolve 20 mg of *nalidixic acid CRS* in *methylene chloride R* and dilute to 20 ml with the same solvent.

Reference solution (c). Dilute 1 ml of reference solution (b) to 10 ml with *methylene chloride R*.

Reference solution (d). Dilute 1 ml of reference solution (b) to 25 ml with *methylene chloride R*.

Apply to the plate 10 µl of each solution. Develop over a path of 15 cm using a mixture of 10 volumes of *dilute ammonia R1*, 20 volumes of *methylene chloride R* and 70 volumes of *alcohol R*. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. Any spot in the chromatogram obtained with the test solution (a), apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (c) (0.1 per cent) and not more than one such spot is more intense than the spot in the chromatogram obtained with reference solution (d).

Heavy metals (2.4.8). 1.0 g complies with limit test D for heavy metals (20 ppm). Prepare the standard using 2 ml of *lead standard solution (10 ppm Pb) R*.

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.150 g in 10 ml of *methylene chloride R* and add 30 ml of *2-propanol R* and 10 ml of *carbon dioxide-free water R*. Keep the titration vessel covered and pass *nitrogen R* through the solution throughout the titration. Keep the temperature of the solution between 15 °C and 20 °C. Titrate with 0.1 M *ethanolic sodium hydroxide*, determining the end-point potentiometrically (2.2.20) using a silver-silver chloride comparison electrode with a sleeve diaphragm or a capillary tip, filled with a saturated solution of *lithium chloride R* in *ethanol R*, and a glass electrode as indicator electrode.

1 ml of 0.1 M *ethanolic sodium hydroxide* is equivalent to 23.22 mg of $C_{12}H_{12}N_2O_3$.

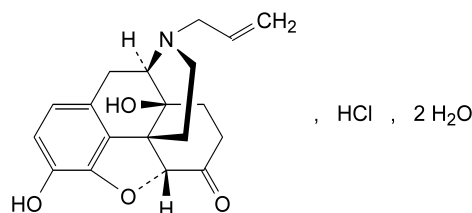
STORAGE

Store in an airtight container, protected from light.

01/2005:0729

NALOXONE HYDROCHLORIDE DIHYDRATE

Naloxoni hydrochloridum dihydricum



$C_{19}H_{22}ClNO_4 \cdot 2H_2O$

M_r 399.9

DEFINITION

Naloxone hydrochloride dihydrate contains not less than 98.0 per cent and not more than the equivalent of 102.0 per cent of 4,5 α -epoxy-3,14-dihydroxy-17-(prop-2-enyl)morphinan-6-one hydrochloride, calculated with reference to the anhydrous substance.

CHARACTERS

A white or almost white, crystalline powder, hygroscopic, freely soluble in water, soluble in alcohol, practically insoluble in toluene.

IDENTIFICATION

First identification: A, C.

Second identification: B, C.

- A. Examine by infrared absorption spectrophotometry (2.2.24) comparing with the spectrum obtained with *naloxone hydrochloride dihydrate CRS*.
- B. Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel G plate R*.

Test solution. Dissolve 8 mg of the substance to be examined in 0.5 ml of *water R* and dilute to 1 ml with *methanol R*.

Reference solution. Dissolve 8 mg of *naloxone hydrochloride dihydrate CRS* in 0.5 ml of *water R* and dilute to 1 ml with *methanol R*.

Apply to the plate 5 µl of each solution. Develop protected from light over a path of 10 cm using a mixture of 5 volumes of *methanol R* and 95 volumes of the upper layer from a mixture of 60 ml of *dilute ammonia R2* and 100 ml of *butanol R*. Dry the plate in a current of air. Spray with a freshly prepared 5 g/l solution of *potassium ferricyanide R* in *ferric chloride solution R1*. Examine in daylight. The principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

- C. It gives reaction (a) of chlorides (2.3.1).

TESTS

Solution S. Dissolve 0.50 g in *carbon dioxide-free water R* and dilute to 25.0 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and colourless (2.2.2, *Method II*).

Acidity or alkalinity. To 10.0 ml of solution S add 0.05 ml of *methyl red solution R*. Not more than 0.2 ml of 0.02 M *sodium hydroxide* or 0.02 M *hydrochloric acid* is required to change the colour of the indicator.

Specific optical rotation (2.2.7): –170 to –181, determined on solution S and calculated with reference to the anhydrous substance.

Related substances. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 0.125 g of the substance to be examined in 0.1 M *hydrochloric acid* and dilute to 25.0 ml with the same acid.

Reference solution (a). Dissolve 10.0 mg of *naloxone hydrochloride dihydrate CRS* and 10.0 mg of *naloxone impurity A CRS* in 0.1 M *hydrochloric acid* and dilute to 10.0 ml with the same acid. Dilute 1.0 ml to 100.0 ml with 0.1 M *hydrochloric acid*.

Reference solution (b). Dilute 1.0 ml of the test solution to 20.0 ml with 0.1 M *hydrochloric acid*. Dilute 1.0 ml to 10.0 ml with 0.1 M *hydrochloric acid*.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.125 m long and 4.0 mm in internal diameter packed with *end-capped octylsilyl silica gel for chromatography R* (5 µm),
- as mobile phase at a flow rate of 1.5 ml/min a linear gradient programme using the following conditions:
Mobile phase A. Mix 20 ml of *acetonitrile R*, 40 ml of *tetrahydrofuran R* and 940 ml of a solution prepared as follows: dissolve 1.17 g of *sodium octanesulphonate R* in 1000 ml of *water R*, adjust the pH to 2.0 with a 50 per cent V/V solution of *phosphoric acid R* and filter (octanesulphonic acid solution).
Mobile phase B. Mix 170 ml of *acetonitrile R*, 40 ml of *tetrahydrofuran R* and 790 ml of the octanesulphonic acid solution.

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)	Comment
0 - 40	100 → 0	0 → 100	linear gradient
40 - 50	0	100	isocratic

- as detector a spectrophotometer set at 230 nm, maintaining the temperature of the column at 40 °C.

Inject separately 20 µl of each solution.

The test is not valid unless: the peak corresponding to naloxone in the chromatogram obtained with reference solution (a) has a signal-to-noise ratio of at least ten; in the chromatogram obtained with reference solution (a), the resolution between the peaks corresponding to impurity A and naloxone is not less than four. If necessary, adjust the chromatographic conditions.

When the chromatograms are recorded in the prescribed conditions, the retention time of naloxone is about 11 min and the relative retention time of impurity A is about 0.8 with respect to naloxone.

In the chromatogram obtained with the test solution: the area of any peak, apart from the principal peak, is not greater than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent); the sum of the areas of all the peaks, apart from the principal peak, is not greater than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (1 per cent). Disregard any peak with an area less than 0.1 times that of the principal peak in the chromatogram obtained with reference solution (b).

Water (2.5.12): 7.5 per cent to 11.0 per cent, determined on 0.200 g by the semi-micro determination of water.

Sulphated ash (2.4.14). Not more than 0.2 per cent, determined on 0.50 g.

ASSAY

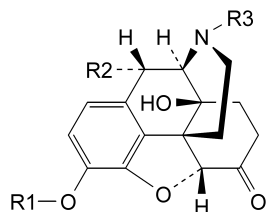
Dissolve 0.300 g in 50 ml of *alcohol R* and add 5.0 ml of 0.01 M *hydrochloric acid*. Carry out a potentiometric titration (2.2.20), using 0.1 M *ethanolic sodium hydroxide*. Read the volume added between the two points of inflexion.

1 ml of 0.1 M *ethanolic sodium hydroxide* is equivalent to 36.38 mg of C₁₉H₂₂ClNO₄.

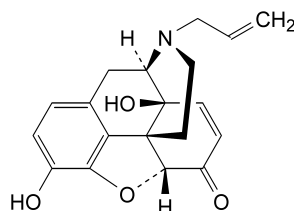
STORAGE

Store in an airtight container, protected from light.

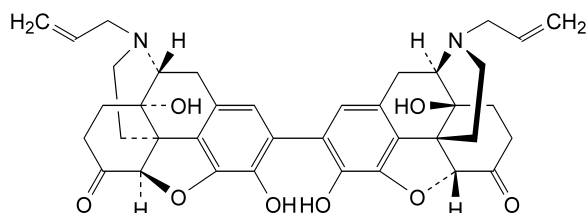
IMPURITIES



- A. R1 = R2 = R3 = H: 4,5 α -epoxy-3,14-dihydroxymorphinan-6-one (noroxymorphone),
 B. R1 = R3 = CH₂-CH=CH₂, R2 = H: 4,5 α -epoxy-14-hydroxy-17-(prop-2-enyl)-3-(prop-2-enyloxy)morphinan-6-one (3-*O*-allylnaloxone),
 C. R1 = H, R2 = OH, R3 = CH₂-CH=CH₂: 4,5 α -epoxy-3,10 α ,14-trihydroxy-17-(prop-2-enyl)morphinan-6-one (10 α -hydroxynaloxone),



- D. 7,8-didehydro-4,5 α -epoxy-3,14-dihydroxy-17-(prop-2-enyl)morphinan-6-one (7,8-didehydronaloxone),

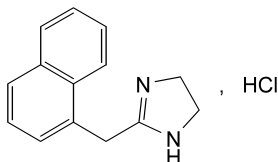


- E. 4,5 α :4',5' α -diepoxy-3,3',14,14'-tetrahydroxy-17,17'-bis(prop-2-enyl)-2,2'-bimorphinan-6,6'-dione (2,2'-bisnaloxone).

01/2005:0730

NAPHAZOLINE HYDROCHLORIDE

Naphazolini hydrochloridum

C₁₄H₁₅ClN₂M_r 246.7

DEFINITION

2-(Naphthalen-1-ylmethyl)-4,5-dihydro-1H-imidazole hydrochloride.

Content: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

Appearance: white or almost white, crystalline powder.

Solubility: freely soluble in water, soluble in alcohol.

mp: about 259 °C, with decomposition.

IDENTIFICATION

First identification: B.

Second identification: A, C.

- A. Dissolve 50.0 mg in 0.01 M hydrochloric acid and dilute to 250.0 ml with the same acid. Dilute 25.0 ml of the solution to 100.0 ml with 0.01 M hydrochloric acid. Examined between 230 nm and 350 nm (2.2.25), the solution shows 4 absorption maxima, at 270 nm, 280 nm, 287 nm and 291 nm. The ratios of the absorbances measured at the maxima at 270 nm, 287 nm and 291 nm to that measured at the maximum at 280 nm are 0.82 to 0.86, 0.67 to 0.70 and 0.65 to 0.69, respectively.
 B. Infrared absorption spectrophotometry (2.2.24).
Comparison: Ph. Eur. reference spectrum of naphazoline hydrochloride.
 C. It gives reaction (a) of chlorides (2.3.1).

TESTS

Solution S. Dissolve 0.5 g in carbon dioxide-free water R and dilute to 50 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and colourless (2.2.2, Method II).

Acidity or alkalinity. To 20 ml of solution S add 0.2 ml of 0.01 M sodium hydroxide and 0.1 ml of methyl red solution R. The solution is yellow. Not more than 0.6 ml of 0.01 M hydrochloric acid is required to change the colour of the solution to red.

Related substances. Liquid chromatography (2.2.29).

Test solution. Dissolve 50.0 mg of the substance to be examined in the mobile phase and dilute to 100.0 ml with the mobile phase.

Reference solution (a). Dissolve 5 mg of 1-naphthylacetic acid R in the mobile phase, add 5 ml of the test solution and dilute to 100 ml with the mobile phase.

Reference solution (b). Dissolve 5.0 mg of naphazoline impurity A CRS in the mobile phase and dilute to 100.0 ml with the mobile phase. Dilute 1.0 ml to 100.0 ml with the mobile phase.

Reference solution (c). Dilute 1.0 ml of the test solution to 10.0 ml with the mobile phase. Dilute 1.0 ml of this solution to 100.0 ml with the mobile phase.

Column:

- size: $l = 0.25$ m, $\varnothing = 4.0$ mm,
- stationary phase: base-deactivated end-capped octylsilyl silica gel for chromatography R (4 μ m) with a pore size of 6 nm.

Mobile phase: dissolve 1.1 g of sodium octanesulphonate R in a mixture of 5 ml of glacial acetic acid R, 300 ml of acetonitrile R and 700 ml of water R.

Flow rate: 1 ml/min.

Detection: spectrophotometer at 280 nm.

Injection: 20 μ l.

Run time: 3 times the retention time of naphazoline.

Retention time: naphazoline = about 14 min.

System suitability: reference solution (a):

- resolution: minimum 5.0 between the peaks due to naphazoline and to impurity B.

Limits:

- impurity A: not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent),
- any other impurity: not more than the area of the principal peak in the chromatogram obtained with reference solution (c) (0.1 per cent),

- *total*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.5 per cent),
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.05 per cent).

Loss on drying (2.2.32): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 100–105 °C.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.200 g in a mixture of 5.0 ml of 0.01 M hydrochloric acid and 50 ml of alcohol R. Carry out a potentiometric titration (2.2.20), using 0.1 M sodium hydroxide. Read the volume added between the 2 points of inflexion.

1 ml of 0.1 M sodium hydroxide is equivalent to 24.67 mg of C₁₄H₁₅CIN₂.

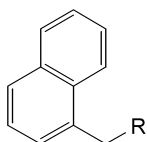
STORAGE

Protected from light.

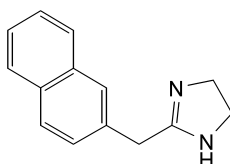
IMPURITIES

Specified impurities: A.

Other detectable impurities: B, C, D.



- A. R = CO-NH-[CH₂]₂-NH₂: N-(2-aminoethyl)-2-(naphthalen-1-yl)acetamide (naphthylacetylenediamine),
- B. R = CO₂H: (naphthalen-1-yl)acetic acid (1-naphthylacetic acid),
- C. R = CN: (naphthalen-1-yl)acetonitrile (1-naphthylacetonitrile),

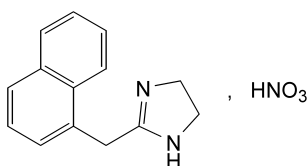


- D. 2-(naphthalen-2-ylmethyl)-4,5-dihydro-1H-imidazole (β-naphazoline).

01/2005:0147

NAPHAZOLINE NITRATE

Naphazolini nitras



C₁₄H₁₅N₃O₃

M_r 273.3

DEFINITION

2-(Naphthalen-1-ylmethyl)-4,5-dihydro-1H-imidazole nitrate.

Content: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

Appearance: white or almost white, crystalline powder.

Solubility: sparingly soluble in water, soluble in alcohol.

IDENTIFICATION

First identification: C.

Second identification: A, B, D.

A. Melting point (2.2.14): 167 °C to 170 °C.

B. Dissolve 50.0 mg in 0.01 M hydrochloric acid and dilute to 250.0 ml with the same acid. Dilute 25.0 ml of the solution to 100.0 ml with 0.01 M hydrochloric acid. Examined between 230 nm and 350 nm (2.2.25), the solution shows 4 absorption maxima, at 270 nm, 280 nm, 287 nm and 291 nm. The ratios of the absorbances measured at the maxima at 270 nm, 287 nm and 291 nm to that measured at the maximum at 280 nm are 0.82 to 0.86, 0.67 to 0.70 and 0.65 to 0.69, respectively.

C. Infrared absorption spectrophotometry (2.2.24).

Comparison: Ph. Eur. reference spectrum of naphazoline nitrate.

D. Dissolve 45 mg of the substance to be examined in 2 ml of water R. Add 1 ml of sulphuric acid R. Shake carefully and allow to cool. Add 1 ml of ferrous sulphate solution R2 dropwise along the walls of the container. At the junction of the 2 liquids, a brown colour develops.

TESTS

Solution S. Dissolve 0.5 g in carbon dioxide-free water R, warming gently, and dilute to 50 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and colourless (2.2.2, Method II).

pH (2.2.3): 5.0 to 6.5 for solution S.

Related substances. Liquid chromatography (2.2.29).

Test solution. Dissolve 50.0 mg of the substance to be examined in the mobile phase and dilute to 100.0 ml with the mobile phase.

Reference solution (a). Dissolve 5 mg of 1-naphthylacetic acid R in the mobile phase, add 5 ml of the test solution and dilute to 100 ml with the mobile phase.

Reference solution (b). Dissolve 5.0 mg of naphazoline impurity A CRS in the mobile phase and dilute to 100.0 ml with the same solvent. Dilute 5.0 ml to 100.0 ml with the mobile phase.

Reference solution (c). Dilute 2.0 ml of the test solution to 10.0 ml with the mobile phase. Dilute 1.0 ml of this solution to 100.0 ml with the mobile phase.

Column:

- *size:* *l* = 0.25 m, Ø = 4.0 mm,
- *stationary phase:* end-capped base-deactivated octylsilyl silica gel for chromatography R (4 µm) with a pore size of 6 nm.

Mobile phase: dissolve 1.1 g of sodium octanesulphonate R in a mixture of 5 ml of glacial acetic acid R, 300 ml of acetonitrile R and 700 ml of water R.

Flow rate: 1 ml/min.

Detection: spectrophotometer at 280 nm.

Injection: 20 µl.

Run time: 3 times the retention time of naphazoline.

Retention time: naphazoline = about 14 min.

System suitability: reference solution (a):

- *resolution:* minimum 5.0 between the peaks due to naphazoline and impurity B.

Limits:

- *impurity A*: not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent),
- *any other impurity*: not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.1 per cent),
- *total*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (c) (1.0 per cent),
- *disregard limit*: 0.25 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.05 per cent); disregard any peak due to the nitrate ion.

Chlorides (2.4.4): maximum 330 ppm.

15 ml of solution S complies with the limit test for chlorides.

Loss on drying (2.2.32): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 100–105 °C.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.200 g in 30 ml of *anhydrous acetic acid R*. Titrate with 0.1 M *perchloric acid*, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *perchloric acid* is equivalent to 27.33 mg of $C_{14}H_{15}N_3O_3$.

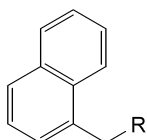
STORAGE

Protected from light.

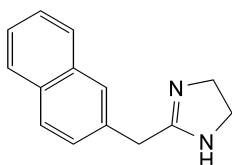
IMPURITIES

Specified impurities: A.

Other detectable impurities: B, C, D.



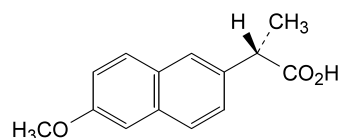
- A. R = CO-NH-[CH₂]₂-NH₂: *N*-(2-aminoethyl)-2-(naphthalen-1-yl)acetamide (naphthylacetylenediamine),
- B. R = CO₂H: (naphthalen-1-yl)acetic acid (1-naphthylacetic acid),
- C. R = CN: (naphthalen-1-yl)acetonitrile (1-naphthylacetonitrile),



- D. 2-(naphthalen-2-ylmethyl)-4,5-dihydro-1H-imidazole (β-naphazoline).

NAPROXEN

Naproxenum



$C_{14}H_{14}O_3$

M_r 230.3

DEFINITION

Naproxen contains not less than 98.5 per cent and not more than the equivalent of 100.5 per cent of (2*S*)-2-(6-methoxynaphthalen-2-yl)propanoic acid, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, crystalline powder, practically insoluble in water, soluble in alcohol and in methanol.

IDENTIFICATION

First identification: A, B, D.

Second identification: A, B, C, E.

- A. It complies with the test for specific optical rotation (see Tests).
- B. Melting point (2.2.14): 154 °C to 158 °C.
- C. Dissolve 40.0 mg in *methanol R* and dilute to 100.0 ml with the same solvent. Dilute 10.0 ml of the solution to 100.0 ml with *methanol R*. Examined between 230 nm and 350 nm (2.2.25), the solution shows four absorption maxima, at 262 nm, 271 nm, 316 nm and 331 nm. The specific absorbances at the maxima are 216 to 238, 219 to 241, 61 to 69 and 79 to 87, respectively.
- D. Examine by infrared absorption spectrophotometry (2.2.24) comparing with the spectrum obtained with *naproxen CRS*. Examine the substances prepared as discs, using *potassium bromide R*.
- E. Dissolve about 2 mg in 2 ml of *sulphuric acid R*. The solution is yellow. Add 50 mg of *chloral hydrate R* and shake to dissolve. The solution becomes orange and then orange-red.

TESTS

Appearance of solution. Dissolve 1.25 g in *methanol R* and dilute to 25 ml with the same solvent. The solution is clear (2.2.1) and not more intensely coloured than reference solution BY₇ (2.2.2, *Method II*).

Specific optical rotation (2.2.7). Dissolve 0.500 g in *chloroform R* and dilute to 25.0 ml with the same solvent. The specific optical rotation is + 63 to + 68.5, calculated with reference to the dried substance.

Related substances. Examine by thin-layer chromatography (2.2.27), using *silica gel GF₂₅₄ R* as the coating substance.

Test solution. Dissolve 0.25 g of the substance to be examined in *methanol R* and dilute to 5 ml with the same solvent.

Reference solution. Dilute 0.5 ml of the test solution to 100 ml with *methanol R*.

Apply separately to the plate 10 µl of each solution. Develop over a path of 15 cm using a mixture of 3 volumes of *glacial acetic acid R*, 9 volumes of *tetrahydrofuran R* and 90 volumes of *toluene R*. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. Any spot in the

chromatogram obtained with the test solution, apart from the principal spot, is not more intense than the spot in the chromatogram obtained with the reference solution (0.5 per cent).

Heavy metals (2.4.8). 1.0 g complies with limit test C for heavy metals (20 ppm). Prepare the standard using 2 ml of *lead standard solution* (10 ppm Pb) R.

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 100–105 °C for 3 h.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.200 g in a mixture of 25 ml of *water* R and 75 ml of *methanol* R. Titrate with 0.1 M *sodium hydroxide*, using 1 ml of *phenolphthalein solution* R as indicator.

1 ml of 0.1 M *sodium hydroxide* is equivalent to 23.03 mg of $C_{28}H_{36}O_{15}$.

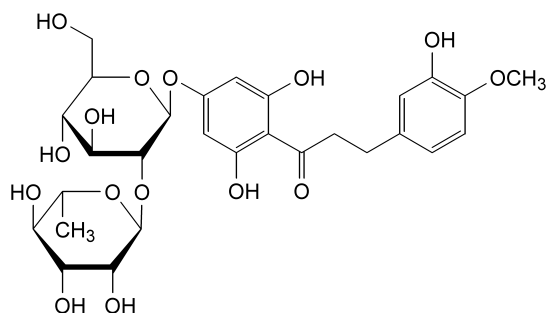
STORAGE

Store protected from light.

01/2005:1547

NEOHESPERIDIN-DIHYDROCHALCONE

Neohesperidin-dihydrochalconum



$C_{28}H_{36}O_{15}$

M_r 613

DEFINITION

1-[4-[[2-O-(6-Deoxy- α -L-mannopyranosyl)- β -D-glucopyranosyl]oxy]-2,6-dihydroxyphenyl]-3-(3-hydroxy-4-methoxyphenyl)propan-1-one.

Content: 96.0 per cent to 101.0 per cent (anhydrous substance).

CHARACTERS

Appearance: white or yellowish-white powder.

Solubility: practically insoluble in water, freely soluble in dimethyl sulphoxide, soluble in methanol, practically insoluble in methylene chloride.

IDENTIFICATION

A. Infrared absorption spectrophotometry (2.2.24).

Comparison: *neohesperidin-dihydrochalcone* CRS.

B. Examine the chromatograms obtained in the assay.

Results: the principal peak in the chromatogram obtained with test solution (b) is similar in retention time and size to the principal peak in the chromatogram obtained with reference solution (a).

TESTS

Appearance of solution. The solution is clear (2.2.1) and not more intensely coloured than reference solution Y_4 (2.2.2, *Method II*).

Dissolve 0.25 g in *methanol* R and dilute to 25 ml with the same solvent.

Related substances. Liquid chromatography (2.2.29).

Test solution (a). Dissolve 0.10 g of the substance to be examined in *dimethyl sulphoxide* R and dilute to 50.0 ml with the same solvent.

Test solution (b). Dilute 10.0 ml of test solution (a) to 20.0 ml with *dimethyl sulphoxide* R.

Reference solution (a). Dissolve 50.0 mg of *neohesperidin-dihydrochalcone* CRS in *dimethyl sulphoxide* R and dilute to 50.0 ml with the same solvent.

Reference solution (b). Dissolve 4.0 mg of *neohesperidin-dihydrochalcone impurity B* CRS in *dimethyl sulphoxide* R and dilute to 100.0 ml with the same solvent.

Reference solution (c). Dilute 1.0 ml of test solution (a) to 100.0 ml with *dimethyl sulphoxide* R.

Reference solution (d). In order to prepare *in situ* impurity F and impurity G, suspend 0.10 g of the substance to be examined in 10.0 ml of a 100 g/l solution of *sulphuric acid* R. Heat the sample for 5 min on a water-bath. Dilute immediately 1.0 ml of the resulting solution to 50.0 ml with *dimethyl sulphoxide* R.

Column:

- **size:** $l = 0.15$ m, $\varnothing = 3.9$ mm,
- **stationary phase:** spherical *octadecylsilyl silica gel for chromatography* R (4 μ m) with a carbon loading of 7 per cent,
- **temperature:** 30 °C.

Mobile phase: mix 20 volumes of *acetonitrile* R and 80 volumes of a solution prepared by adding 5.0 ml of *glacial acetic acid* R to 1000.0 ml of *water* R.

Flow rate: 1.0 ml/min.

Detection: spectrophotometer at 282 nm.

Injection: 10 μ l; inject test solution (a) and reference solutions (a), (b), (c) and (d).

Run time: 5 times the retention time of *neohesperidin-dihydrochalcone* which is about 10 min.

Relative retention with reference to *neohesperidin-dihydrochalcone*: impurity B = about 0.4; impurity D = about 0.7; impurity F = about 1.2; impurity G = about 3.7.

System suitability:

- **resolution:** minimum of 2.5 between the first peak (*neohesperidin-dihydrochalcone*) and the second peak (impurity F) in the chromatogram obtained with reference solution (d),
- chromatogram obtained with reference solution (a) is similar to the chromatogram provided with *neohesperidin-dihydrochalcone* CRS.

Limits:

- **impurity B:** not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (2 per cent),
- **impurity D:** not more than twice the area of the principal peak in the chromatogram obtained with reference solution (c) (2 per cent),
- **any other impurity:** not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.5 per cent),

- *total of all impurities apart from impurity B*: not more than 2.5 times the area of the principal peak in the chromatogram obtained with reference solution (c) (2.5 per cent),
- *disregard limit*: 0.05 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.05 per cent).

Heavy metals (2.4.8): maximum 10 ppm.

2.0 g complies with limit test D. Prepare the standard using 2 ml of *lead standard solution* (10 ppm Pb) R.

Water (2.5.12): maximum 12.0 per cent, determined on 0.200 g.

Sulphated ash (2.4.14): maximum 0.2 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (2.2.29) as described in the test for related substances.

Injection: 10 µl; inject test solution (b) and reference solutions (a) and (d).

System suitability:

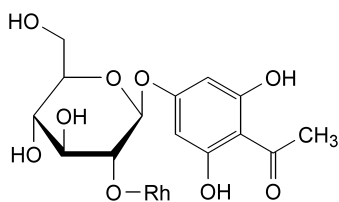
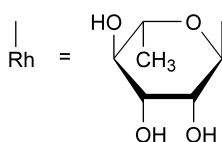
- *resolution*: minimum of 2.5 between the first peak (neohesperidin-dihydrochalcone) and the second peak (impurity F) in the chromatogram obtained with reference solution (d),
- *repeatability*: reference solution (a).

Calculate the percentage content of $C_{28}H_{36}O_{15}$ using the chromatogram obtained with reference solution (a) and the stated content of $C_{28}H_{36}O_{15}$ in *neohesperidin-dihydrochalcone CRS*, correcting for the water content of the substance to be examined.

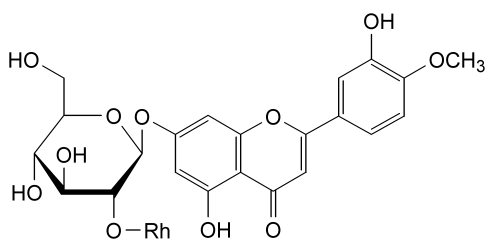
STORAGE

Protected from light.

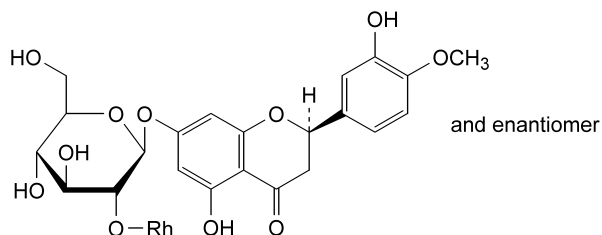
IMPURITIES



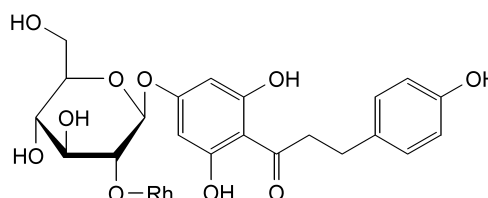
- A. 1-[4-[[2-*O*-(6-deoxy- α -L-mannopyranosyl)- β -D-glucopyranosyl]oxy]-2,6-dihydroxyphenyl]ethanone (phloroacetophenone neohesperidoside),



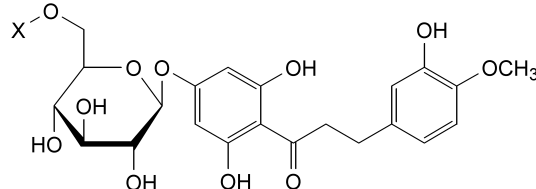
- B. 7-[[2-*O*-(6-deoxy- α -L-mannopyranosyl)- β -D-glucopyranosyl]oxy]-5-hydroxy-2-(3-hydroxy-4-methoxyphenyl)-4*H*-1-benzopyran-4-one (neodiosmin),



- C. (2*RS*)-7-[[2-*O*-(6-deoxy- α -L-mannopyranosyl)- β -D-glucopyranosyl]oxy]-5-hydroxy-2-(3-hydroxy-4-methoxyphenyl)-2,3-dihydro-4*H*-1-benzopyran-4-one (neohesperidin),

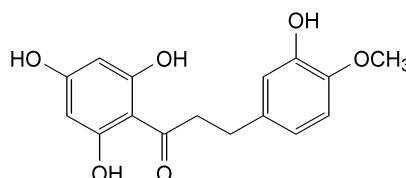


- D. 1-[4-[[2-*O*-(6-deoxy- α -L-mannopyranosyl)- β -D-glucopyranosyl]oxy]-2,6-dihydroxyphenyl]-3-(4-hydroxyphenyl)propan-1-one (naringin-dihydrochalcone),



- E. X = Rh: 1-[4-[[6-*O*-(6-deoxy- α -L-mannopyranosyl)- β -D-glucopyranosyl]oxy]-2,6-dihydroxyphenyl]-3-(3-hydroxy-4-methoxyphenyl)propan-1-one (hesperidin-dihydrochalcone),

- F. X = H: 1-[4-[(β -D-glucopyranosyloxy)-2,6-dihydroxyphenyl]-3-(3-hydroxy-4-methoxyphenyl)propan-1-one (hesperetin-dihydrochalcone 7'-glucoside),

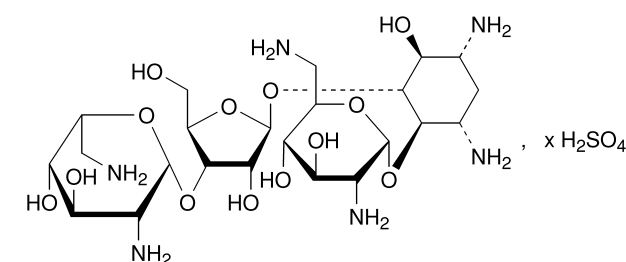


- G. 3-(3-hydroxy-4-methoxyphenyl)-1-(2,4,6-trihydroxyphenyl)propan-1-one (hesperetin-dihydrochalcone).

01/2005:0197

NEOMYCIN SULPHATE

Neomycini sulfas



M_r 615 (base)

DEFINITION

Mixture of sulphates of substances produced by the growth of certain selected strains of *Streptomyces fradiae*, the main component being the sulphate of 2-deoxy-4-*O*-(2,6-diamino-2,6-dideoxy- α -D-glucopyranosyl)-5-*O*-[3-*O*-(2,6-diamino-2,6-dideoxy- β -L-idopyranosyl)- β -D-ribofuranosyl]-D-streptamine (neomycin B).

Content: minimum of 680 IU/mg (dried substance).

CHARACTERS

Appearance: white or yellowish-white powder, hygroscopic.

Solubility: very soluble in water, very slightly soluble in alcohol, practically insoluble in acetone.

IDENTIFICATION

A. Examine the chromatograms obtained in the test for related substances.

Results:

- the retention time of the principal peak in the chromatogram obtained with the test solution is approximately the same as that of the principal peak in the chromatogram obtained with reference solution (e),
- it complies with the limits given for impurity C.

B. It gives reaction (a) of sulphates (2.3.1).

TESTS

pH (2.2.3): 5.0 to 7.5.

Dissolve 0.1 g in *carbon dioxide-free water R* and dilute to 10 ml with the same solvent.

Specific optical rotation (2.2.7): + 53.5 to + 59.0 (dried substance).

Dissolve 1.00 g in *water R* and dilute to 10.0 ml with the same solvent.

Related substances. Liquid chromatography (2.2.29).

Test solution. Dissolve 25.0 mg of the substance to be examined in the mobile phase and dilute to 50.0 ml with the mobile phase.

Reference solution (a). Dissolve 25.0 mg of *framycetin sulphate CRS* in the mobile phase and dilute to 50.0 ml with the mobile phase.

Reference solution (b). Dilute 5.0 ml of reference solution (a) to 100.0 ml with the mobile phase.

Reference solution (c). Dilute 1.0 ml of reference solution (a) to 100.0 ml with the mobile phase.

Reference solution (d). Dissolve the contents of a vial of *neamine CRS* (corresponding to 0.5 mg) in the mobile phase and dilute to 50.0 ml with the mobile phase.

Reference solution (e). Dissolve 10 mg of *neomycin sulphate CRS* in the mobile phase and dilute to 100.0 ml with the mobile phase.

Column:

- **size:** $l = 0.25$ m, $\varnothing = 4.6$ mm,
- **stationary phase:** base-deactivated octadecylsilyl silica gel for chromatography R (5 μ m),
- **temperature:** 25 °C.

Mobile phase: mix 20.0 ml of *trifluoroacetic acid R*, 6.0 ml of *carbonate-free sodium hydroxide solution R* and 500 ml of *water R*, allow to equilibrate, dilute to 1000 ml with *water R* and degas.

Flow rate: 0.7 ml/min.

Post-column solution: carbonate-free sodium hydroxide solution *R* diluted 1 in 25 previously degassed, which is added pulse-less to the column effluent using a 375 μ l polymeric mixing coil.

Flow rate: 0.5 ml/min.

Detection: pulsed amperometric detector with a gold indicator electrode, a silver-silver chloride reference electrode and a stainless steel auxiliary electrode which is the cell body, held at respectively 0.00 V detection, + 0.80 V oxidation and – 0.60 V reduction potentials, with pulse durations according to the instrument used.

Injection: 10 μ l; inject the test solution and the reference solutions (b), (c), (d) and (e).

Run time: 1.5 times the retention time of neomycin B.

Relative retention with reference to neomycin B (retention time = about 10 min): impurity A = about 0.65; impurity C = about 0.9; impurity G = about 1.1.

System suitability:

- **resolution:** minimum of 2.0 between the peaks due to impurity C and to neomycin B in the chromatogram obtained with reference solution (e); if necessary, adjust the volume of the carbonate-free sodium hydroxide solution in the mobile phase,
- **signal-to-noise ratio:** minimum 10 for the principal peak in the chromatogram obtained with reference solution (c).

Limits:

- **impurity A:** not more than the area of the principal peak in the chromatogram obtained with reference solution (d) (2.0 per cent),
- **impurity C:** not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (b) (15.0 per cent) and not less than 0.6 times the area of the principal peak in the chromatogram obtained with reference solution (b) (3.0 per cent),
- **any other impurity:** not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (5.0 per cent),
- **total of other impurities:** not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (b) (15.0 per cent),
- **disregard limit:** area of the principal peak in the chromatogram obtained with reference solution (c) (1.0 per cent).

Sulphate: 27.0 per cent to 31.0 per cent (dried substance).

Dissolve 0.250 g in 100 ml of *water R* and adjust the solution to pH 11 using *concentrated ammonia R*. Add 10.0 ml of 0.1 *M barium chloride* and about 0.5 mg of *phthalein purple R*. Titrate with 0.1 *M sodium edetate* adding 50 ml of *alcohol R* when the colour of the solution begins to change, continuing the titration until the violet-blue colour disappears.

1 ml of 0.1 *M barium chloride* is equivalent to 9.606 mg of SO_4 .

Loss on drying (2.2.32): maximum 8.0 per cent, determined on 1.000 g by drying at 60 °C over *diphosphorus pentoxide R* at a pressure not exceeding 0.7 kPa for 3 h.

Sulphated ash (2.4.14): maximum 1.0 per cent, determined on 1.0 g.

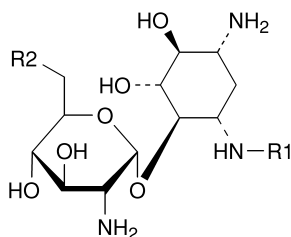
ASSAY

Carry out the microbiological assay of antibiotics (2.7.2). Use *neomycin sulphate for microbiological assay CRS* as the reference substance.

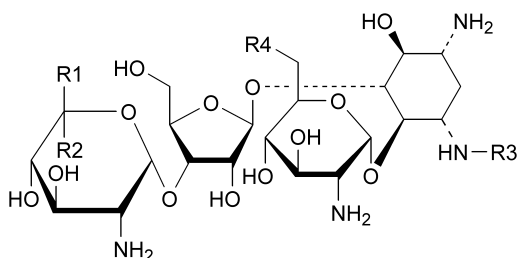
STORAGE

In an airtight container, protected from light.

IMPURITIES



- A. R1 = H, R2 = NH₂: 2-deoxy-4-*O*-(2,6-diamino-2,6-dideoxy-α-D-glucopyranosyl)-D-streptamine (neamine or neomycin A-LP),
- B. R1 = CO-CH₃, R2 = NH₂: 3-*N*-acetyl-2-deoxy-4-*O*-(2,6-diamino-2,6-dideoxy-α-D-glucopyranosyl)-D-streptamine (3-acetylneamine),
- D. R1 = H, R2 = OH: 4-*O*-(2-amino-2-deoxy-α-D-glucopyranosyl)-2-deoxy-D-streptamine (paromamine or neomycin D),

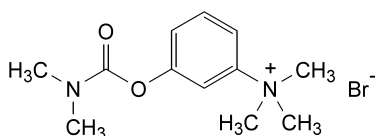


- C. R1 = CH₂-NH₂, R2 = R3 = H, R4 = NH₂: 2-deoxy-4-*O*-(2,6-diamino-2,6-dideoxy-α-D-glucopyranosyl)-5-*O*-[3-*O*-(2,6-diamino-2,6-dideoxy-α-D-glucopyranosyl)-β-D-ribofuranosyl]-D-streptamine (neomycin C),
- E. R1 = R3 = H, R2 = CH₂-NH₂, R4 = OH: 4-*O*-(2-amino-2-deoxy-α-D-glucopyranosyl)-2-deoxy-5-*O*-[3-*O*-(2,6-diamino-2,6-dideoxy-β-L-idopyranosyl)-β-D-ribofuranosyl]-D-streptamine (paromomycin I or neomycin E),
- F. R1 = CH₂-NH₂, R2 = R3 = H, R4 = OH: 4-*O*-(2-amino-2-deoxy-α-D-glucopyranosyl)-2-deoxy-5-*O*-[3-*O*-(2,6-diamino-2,6-dideoxy-α-D-glucopyranosyl)-β-D-ribofuranosyl]-D-streptamine (paromomycin II or neomycin F),
- G. R1 = H, R2 = CH₂-NH₂, R3 = CO-CH₃, R4 = NH₂: 3-*N*-acetyl-2-deoxy-4-*O*-(2,6-diamino-2,6-dideoxy-α-D-glucopyranosyl)-5-*O*-[3-*O*-(2,6-diamino-2,6-dideoxy-β-L-idopyranosyl)-β-D-ribofuranosyl]-D-streptamine (neomycin B-LP).

01/2005:0046

NEOSTIGMINE BROMIDE

Neostigmini bromidum

C₁₂H₁₉BrN₂O₂M_r 303.2

DEFINITION

Neostigmine bromide contains not less than 98.5 per cent and not more than the equivalent of 101.0 per cent of 3-[(dimethylcarbamoyl)oxy]-*N,N,N*-trimethylanilinium bromide, calculated with reference to the dried substance.

CHARACTERS

A white, crystalline powder or colourless crystals, hygroscopic, very soluble in water, freely soluble in alcohol.

IDENTIFICATION

First identification: B, D.

Second identification: A, C, D.

- A. Dissolve 20 mg in 0.5 *M* sulphuric acid and dilute to 100 ml with the same acid. Examined between 230 nm and 350 nm, the solution shows 2 absorption maxima (2.2.25), at 260 nm and 266 nm. The specific absorbances at the maxima are about 16 and about 14, respectively.
- B. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *neostigmine bromide CRS*.
- C. Heat about 50 mg with a mixture of 0.4 g of *potassium hydroxide R* and 2 ml of *alcohol R* on a water-bath for 3 min, replacing the evaporated alcohol. Cool and add 2 ml of *water R* and 2 ml of *diazobenzenesulphonic acid solution R1*. An orange-red colour develops.
- D. It gives the reactions of bromides (2.3.1).

TESTS

Solution S. Dissolve 2.5 g in *distilled water R* and dilute to 50 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and colourless (2.2.2, *Method II*).

(3-Hydroxyphenyl)trimethylammonium bromide. Dissolve 50 mg in a mixture of 1 ml of *sodium carbonate solution R* and 9 ml of *water R*. The absorbance (2.2.25) measured immediately at 294 nm is not greater than 0.25.

Sulphates (2.4.13). 15 ml of solution S complies with the limit test for sulphates (200 ppm).

Loss on drying (2.2.32). Not more than 1.0 per cent, determined on 1.00 g by drying in an oven at 100 °C to 105 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

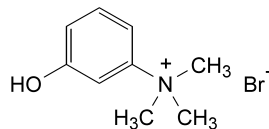
ASSAY

Dissolve 0.225 g in 2 ml of *anhydrous formic acid R*. Add 50 ml of *acetic anhydride R*. Titrate with 0.1 *M* perchloric acid, determining the end-point potentiometrically (2.2.20). 1 ml of 0.1 *M* perchloric acid is equivalent to 30.32 mg of C₁₂H₁₉BrN₂O₂.

STORAGE

Store protected from light.

IMPURITIES

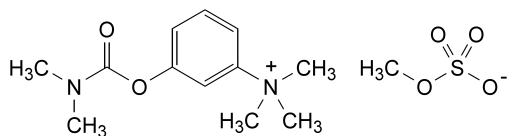


- A. 3-hydroxy-*N,N,N*-trimethylanilinium bromide.

01/2005:0626

NEOSTIGMINE METILSULFATE

Neostigmini metilsulfas

 $C_{13}H_{22}N_2O_6S$ M_r 334.4**DEFINITION**

Neostigmine metilsulfate contains not less than 98.5 per cent and not more than the equivalent of 101.0 per cent of 3-[(dimethylcarbamoyl)oxy]-*N,N,N*-trimethylanilinium methyl sulphate, calculated with reference to the dried substance.

CHARACTERS

A white, crystalline powder or colourless crystals, hygroscopic, very soluble in water, freely soluble in alcohol.

IDENTIFICATION

First identification: A, C.

Second identification: A, B, D, E.

A. Melting point (2.2.14): 144 °C to 149 °C.

B. Dissolve 50 mg in 0.5 M sulphuric acid and dilute to 100.0 ml with the same acid. Examined between 230 nm and 350 nm (2.2.25), the solution shows two absorption maxima, at 261 nm and 267 nm. The ratio of the absorbance at the maximum at 267 nm to that at the maximum at 261 nm is 0.84 to 0.87. The identification is not valid unless in the test for resolution (2.2.25) the ratio of the absorbances is not less than 1.9.

C. Examine by infrared absorption spectrophotometry (2.2.24) comparing with the spectrum obtained with *neostigmine metilsulfate CRS*. Examine the substances prepared as discs.

D. To 50 mg add 0.4 g of *potassium hydroxide R* and 2 ml of *alcohol R* and heat on a water-bath for 3 min, replacing the evaporated alcohol. Cool and add 2 ml of *water R* and 2 ml of *diazobenzenesulphonic acid solution R1*. An orange-red colour develops.

E. Dissolve 0.1 g in 5 ml of *distilled water R* and add 1 ml of *barium chloride solution R1*. No precipitate is formed. Add 2 ml of *hydrochloric acid R* and heat in a water-bath for 10 min. A fine, white precipitate is formed.

TESTS

Solution S. Dissolve 2.5 g in *distilled water R* and dilute to 50 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and colourless (2.2.2, *Method II*).

Acidity or alkalinity. To 4.0 ml of solution S add 6.0 ml of *water R* and 0.1 ml of *phenolphthalein solution R1*. The solution is colourless. Add 0.3 ml of 0.01 M *sodium hydroxide*; the solution becomes red. Add 0.4 ml of 0.01 M *hydrochloric acid*; the solution becomes colourless. Add 0.1 ml of *methyl red solution R*; the solution becomes red or yellowish-red.

(3-Hydroxyphenyl)trimethylammonium methyl sulphate. Dissolve 50 mg in a mixture of 1 ml of *sodium carbonate solution R* and 9 ml of *water R*. The absorbance (2.2.25) measured immediately at 294 nm is not greater than 0.20.

Sulphates (2.4.13). 15 ml of solution S complies with the limit test for sulphates (200 ppm).

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.300 g in 150 ml of *water R* and add 100 ml of *dilute sodium hydroxide solution R*. Distil collecting the distillate in 40 ml of a 40 g/l solution of *boric acid R* until the total volume in the collecting vessel is about 250 ml. Titrate the solution in the collecting vessel with 0.1 M *hydrochloric acid*, using 0.25 ml of *methyl red mixed solution R* as indicator. Carry out a blank test.

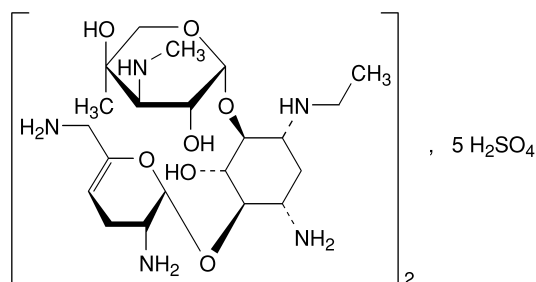
1 ml of 0.1 M *hydrochloric acid* is equivalent to 33.44 mg of $C_{13}H_{22}N_2O_6S$.

STORAGE

Store in a airtight container, protected from light.

01/2005:1351
corrected**NETILMICIN SULPHATE**

Netilmicini sulfas

 $C_{42}H_{92}N_{10}O_{34}S_5$ M_r 1442**DEFINITION**

2-Deoxy-6-*O*-[3-deoxy-4-*C*-methyl-3-(methylamino)-β-*L*-arabinopyranosyl]-4-*O*-(2,6-diamino-2,3,4,6-tetra-deoxy-α-*D*-glycero-hex-4-enopyranosyl)-1-*N*-ethyl-*D*-streptamine sulphate.

Substance obtained by synthesis from sisomicin.

Content: minimum 650 IU/mg (dried substance).

CHARACTERS

Appearance: white or yellowish-white powder, very hygroscopic.

Solubility: very soluble in water, practically insoluble in acetone and in alcohol.

IDENTIFICATION

A. Examine the chromatograms obtained in the test for related substances.

Results: the retention time and size of the principal peak in the chromatogram obtained with test solution (a) are approximately the same as those of the principal peak in the chromatogram obtained with reference solution (a).

B. It gives reaction (a) of sulphates (2.3.1).

TESTS

Solution S. Dissolve 0.80 g in *carbon dioxide-free water R* and dilute to 20.0 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and its absorbance at 400 nm (2.2.25) has a maximum of 0.08.

pH (2.2.3): 3.5 to 5.5 for solution S.

Specific optical rotation (2.2.7): + 88.0 to + 96.0 (dried substance).

Dissolve 0.50 g in *water R* and dilute to 10.0 ml with the same solvent.

Related substances. Liquid chromatography (2.2.29).

Test solution (a). Dissolve 25.0 mg of the substance to be examined in the mobile phase and dilute to 25.0 ml with the mobile phase.

Test solution (b). Dilute 1.0 ml of test solution (a) to 100.0 ml with the mobile phase. Dilute 1.0 ml of this solution to 10.0 ml with the mobile phase.

Reference solution (a). Dissolve 25.0 mg of *netilmicin sulphate CRS* in the mobile phase and dilute to 25.0 ml with the mobile phase.

Reference solution (b). Dissolve 25.0 mg of *sisomicin sulphate CRS* in the mobile phase and dilute to 25.0 ml with the mobile phase.

Reference solution (c). Dissolve 20.5 mg of *1-N-ethylgaramine sulphate CRS* in the mobile phase and dilute to 25.0 ml with the mobile phase.

Reference solution (d). Dilute 1.0 ml of reference solution (a), 1.0 ml of reference solution (b) and 1.0 ml of reference solution (c) to 100.0 ml with the mobile phase.

Column:

- **size:** $l = 0.25$ m, $\varnothing = 4.6$ mm,
- **stationary phase:** *styrene-divinylbenzene copolymer R* (8 μ m) with a pore size of 100 nm,
- **temperature:** 50 °C.

Mobile phase: prepare a solution in *carbon dioxide-free water R* containing 35 g/l of *anhydrous sodium sulphate R*, 0.5 g/l of *sodium octanesulphonate R*, 10 ml/l of *tetrahydrofuran R*, 50 ml/l of 0.2 M *potassium dihydrogenphosphate R* previously adjusted to pH 3.0 with a 22.5 g/l solution of *phosphoric acid R* and degassed.

Flow rate: 1.0 ml/min.

Post-column solution: 20 g/l carbonate-free solution of *sodium hydroxide R* previously degassed, which is added pulse-less to the column effluent using a 375 μ l polymeric mixing coil.

Flow rate: 0.3 ml/min.

Detection: pulsed amperometric detector with a gold indicator electrode, a silver-silver chloride reference electrode and a stainless steel auxiliary electrode which is the cell body, held at respectively + 0.05 V detection, + 0.75 V oxidation and – 0.15 V reduction potentials, with pulse durations according to the instrument used.

Injection: 20 μ l; inject test solutions (a) and (b) and reference solution (d).

Run time: 3 times the retention time of netilmicin.

Retention time: netilmicin = about 12 min.

System suitability:

- **resolution:** minimum of 2.0 between the peaks due to impurity B (first peak) and to impurity A (second peak); minimum of 3.0 between the peaks due to impurity A (second peak) and to netilmicin (third peak) in the chromatogram obtained with reference solution (d). If necessary, adjust the concentration of sodium octanesulphonate in the mobile phase.
- **signal-to-noise ratio:** minimum of 10 for the principal peak in the chromatogram obtained with test solution (b).

Limits:

- **impurity A:** not more than the area of the second peak in the chromatogram obtained with reference solution (d) and taking into account the declared content of *sisomicin sulphate CRS* (1 per cent),
- **impurity B:** not more than the area of the first peak in the chromatogram obtained with reference solution (d) and taking into account the declared content of *1-N-ethylgaramine sulphate CRS* (1 per cent),
- **any other impurity:** not more than the area of the third peak in the chromatogram obtained with reference solution (d) (1 per cent),
- **total of other impurities:** not more than twice the area of the third peak in the chromatogram obtained with reference solution (d) (2 per cent),
- **disregard limit:** any peak with an area less than that of the principal peak in the chromatogram obtained with test solution (b) (0.1 per cent).

Sulphate: 31.5 per cent to 35.0 per cent (dried substance).

Dissolve 0.12 g in 100 ml of *water R* and adjust the solution to pH 11 using *concentrated ammonia R*. Add 30.0 ml of 0.1 M *barium chloride* and about 0.5 mg of *phthalein purple R*. Titrate with 0.1 M *sodium edetate* adding 50 ml of *alcohol R* when the colour of the solution begins to change and continue the titration until the violet-blue colour disappears.

1 ml of 0.1 M *barium chloride* is equivalent to 9.606 mg of SO_4 .

Loss on drying (2.2.32): maximum 15.0 per cent, determined on 0.500 g by drying at 110 °C under high vacuum for 3 h.

Sulphated ash (2.4.14): maximum 1.0 per cent, determined on 0.5 g.

Bacterial endotoxins (2.6.14): less than 1.25 IU/mg, if intended for use in the manufacture of parenteral dosage forms without a further appropriate procedure for the removal of bacterial endotoxins.

ASSAY

Carry out the microbiological assay of antibiotics (2.7.2), using the diffusion method.

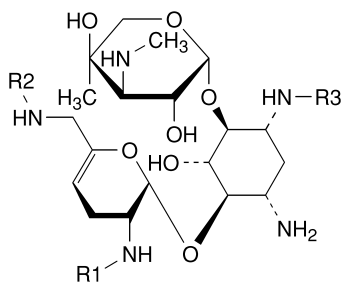
STORAGE

In an airtight container, protected from light. If the substance is sterile, store in a sterile, airtight, tamper-proof container.

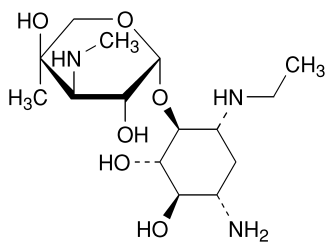
LABELLING

The label states, where applicable, that the substance is free from bacterial endotoxins.

IMPURITIES



- A. R1 = R2 = R3 = H: 2-deoxy-4-*O*-[3-deoxy-4-*C*-methyl-3-(methylamino)- β -L-arabinopyranosyl]-6-*O*-(2,6-diamino-2,3,4,6-tetra-deoxy- α -D-*glycero*-hex-4-enopyranosyl)-L-streptamine (sisomicin),
- C. R1 = R3 = C₂H₅, R2 = H: 4-*O*-[6-amino-2,3,4,6-tetra-deoxy-2-(ethylamino)- α -D-*glycero*-hex-4-enopyranosyl]-2-deoxy-6-*O*-[3-deoxy-4-*C*-methyl-3-(methylamino)- β -L-arabinopyranosyl]-1-*N*-ethyl-D-streptamine (2'-*N*-ethylnetilmicin),
- D. R1 = H, R2 = R3 = C₂H₅: 4-*O*-[2-amino-2,3,4,6-tetra-deoxy-6-(ethylamino)- α -D-*glycero*-hex-4-enopyranosyl]-2-deoxy-6-*O*-[3-deoxy-4-*C*-methyl-3-(methylamino)- β -L-arabinopyranosyl]-1-*N*-ethyl-D-streptamine (6'-*N*-ethylnetilmicin),

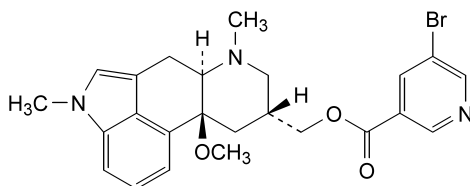


- B. 2-deoxy-6-*O*-[3-deoxy-4-*C*-methyl-3-(methylamino)- β -L-arabinopyranosyl]-1-*N*-ethyl-D-streptamine (1-*N*-ethylgaramine).

01/2005:1998

NICERGOLINE

Nicergolinum

C₂₄H₂₆BrN₃O₃M_r 484.4

DEFINITION

[(6*aR*,9*R*,10*aS*)-10*a*-Methoxy-4,7-dimethyl-4,6,6*a*,7,8,9,10,10*a*-octahydroindolo[4,3-*fg*]quinolin-9-yl]methyl 5-bromopyridine-3-carboxylate.

Content: 99.0 per cent to 101.0 per cent (anhydrous and solvent-free substance).

CHARACTERS

Appearance: fine to granular, white or yellowish powder.

Solubility: practically insoluble in water, freely soluble in methylene chloride, soluble in alcohol.

It shows polymorphism.

IDENTIFICATION

First identification: A, C.

Second identification: A, B, D.

A. Specific optical rotation (2.2.7): + 4.8 to + 5.8 (anhydrous substance).

Dissolve 0.50 g in *alcohol R* and dilute to 10.0 ml with the same solvent.

B. Dissolve 50.0 mg in *alcohol R* and dilute to 100.0 ml with the same solvent. Dilute 5.0 ml to 50.0 ml with *alcohol R*. Examined between 220 nm and 350 nm (2.2.25), the solution shows an absorption maximum at 288 nm and an absorption minimum at 251 nm. The specific absorbance at the maximum at 288 nm is 175 to 185 (anhydrous substance).

C. Infrared absorption spectrophotometry (2.2.24).

Preparation: discs.

Comparison: *Ph. Eur. reference spectrum of nicergoline.*

If the spectra obtained show differences, dissolve the substance to be examined in *alcohol R*, evaporate to dryness and record a further spectrum using the residue.

D. Dissolve 2 mg in 2 ml of *sulphuric acid R*. A blue colour develops.

TESTS

Appearance of solution. The solution is not more opalescent than reference suspension II (2.2.1) and not more intensely coloured than intensity 5 of the range of reference solutions of the most appropriate colour (2.2.2, *Method II*).

Dissolve 0.5 g in *alcohol R* and dilute to 10 ml with the same solvent.

Related substances. Liquid chromatography (2.2.29).

Test solution. Dissolve 25.0 mg in *acetonitrile R* and dilute to 25.0 ml with the same solvent.

Reference solution (a). Dilute 1.0 ml of the test solution to 100.0 ml with *acetonitrile R*. Dilute 10.0 ml of this solution to 50.0 ml with *acetonitrile R*.

Reference solution (b). Dissolve 25.0 mg of the substance to be examined and 10.0 mg of *nicergoline impurity A CRS* in *acetonitrile R* and dilute to 25.0 ml with the same solvent. Dilute 1.0 ml to 100.0 ml with *acetonitrile R*.

Column:

— **size:** *l* = 0.25 m, Ø = 4.6 mm,

— **stationary phase:** octadecylsilyl silica gel for chromatography *R* (5 µm).

Mobile phase: mix 30 volumes of *acetonitrile R*, 35 volumes of *methanol R* and 35 volumes of a freshly prepared solution of 6.8 g/l *potassium dihydrogen phosphate R* previously adjusted to pH 7.0 with *triethylamine R*.

Flow rate: 1.0 ml/min.

Detection: spectrophotometer at 288 nm.

Injection: 20 µl.

Run time: twice the retention time of nicergoline.

Relative retention with reference to nicergoline (retention time = about 25 min): impurity B = 0.5.

System suitability: reference solution (b):

— **resolution:** minimum 1.5 between the peaks due to nicergoline and impurity A.

Limits:

— **impurity B:** not more than 4 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.8 per cent),

- *any other impurity*: not more than 2.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent) and not more than 2 such peaks have an area greater than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent),
- *total*: not more than 7.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (1.5 per cent),
- *disregard limit*: 0.25 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

Water (2.5.32): maximum 0.5 per cent, determined on 0.100 g.

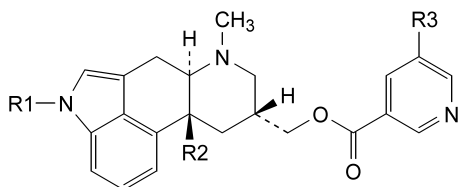
Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

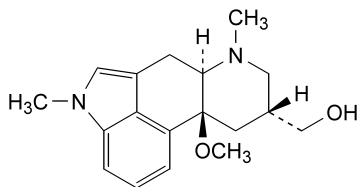
Dissolve 0.400 g in 50 ml of *acetone R*. Titrate with 0.1 M *perchloric acid*, determining the end-point potentiometrically (2.2.20). Titrate to the 1st point of inflexion.

1 ml of 0.1 M *perchloric acid* is equivalent to 48.44 mg of C₂₄H₂₆BrN₃O₃.

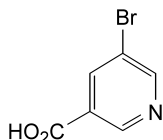
IMPURITIES



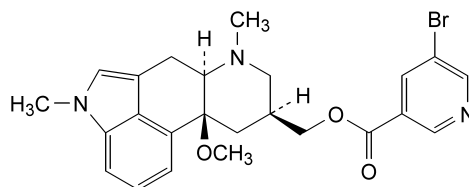
- A. R1 = CH₃, R2 = OCH₃, R3 = Cl: [(6a*R*,9*R*,10a*S*)-10a-methoxy-4,7-dimethyl-4,6,6a,7,8,9,10,10a-octahydroindolo[4,3-*fg*]quinolin-9-yl]methyl 5-chloropyridine-3-carboxylate,
- B. R1 = H, R2 = OCH₃, R3 = Br: [(6a*R*,9*R*,10a*S*)-10a-methoxy-7-methyl-4,6,6a,7,8,9,10,10a-octahydroindolo[4,3-*fg*]quinolin-9-yl]methyl 5-bromopyridine-3-carboxylate,
- E. R1 = CH₃, R2 = OH, R3 = Br: [(6a*R*,9*R*,10a*S*)-10a-hydroxy-4,7-dimethyl-4,6,6a,7,8,9,10,10a-octahydroindolo[4,3-*fg*]quinolin-9-yl]methyl 5-bromopyridine-3-carboxylate,
- G. R1 = CH₃, R2 = H, R3 = Br: [(6a*R*,9*R*,10a*R*)-4,7-dimethyl-4,6,6a,7,8,9,10,10a-octahydroindolo[4,3-*fg*]quinolin-9-yl]methyl 5-bromopyridine-3-carboxylate



- C. [(6a*R*,9*R*,10a*S*)-10a-methoxy-4,7-dimethyl-4,6,6a,7,8,9,10,10a-octahydroindolo[4,3-*fg*]quinolin-9-yl]methanol,



- D. 5-bromopyridine-3-carboxylic acid,

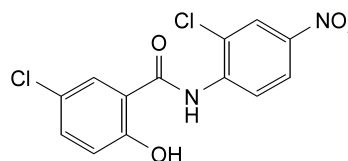


- F. [(6a*R*,9*S*,10a*S*)-10a-methoxy-4,7-dimethyl-4,6,6a,7,8,9,10,10a-octahydroindolo[4,3-*fg*]quinolin-9-yl]methyl 5-bromopyridine-3-carboxylate.

01/2005:0679

NICLOSAMIDE, ANHYDROUS

Niclosamidum anhydricum



C₁₃H₈Cl₂N₂O₄

M_r 327.1

DEFINITION

Anhydrous niclosamide contains not less than 98.0 per cent and not more than the equivalent of 101.0 per cent of 5-chloro-*N*-(2-chloro-4-nitrophenyl)-2-hydroxybenzamide, calculated with reference to the dried substance.

CHARACTERS

Yellowish-white or yellowish, fine crystals, practically insoluble in water, sparingly soluble in acetone, slightly soluble in ethanol.

IDENTIFICATION

First identification: B, E.

Second identification: A, C, D, E.

- A. Melting point (2.2.14): 227 °C to 232 °C.
- B. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *anhydrous niclosamide CRS*. Examine as discs prepared using about 0.5 mg of substance and 0.3 g of *potassium bromide R*.
- C. To 50 mg add 5 ml of 1 M *hydrochloric acid* and 0.1 g of *zinc powder R*, heat in a water-bath for 10 min, cool and filter. To the filtrate add 1 ml of a 5 g/l solution of *sodium nitrite R* and allow to stand for 3 min; add 2 ml of a 20 g/l solution of *ammonium sulphamate R*, shake, allow to stand for 3 min and add 2 ml of a 5 g/l solution of *naphthylethylenediamine dihydrochloride R*. A violet colour is produced.
- D. Heat the substance on a copper wire in a non-luminous flame. The flame becomes green.
- E. It complies with the test for loss on drying (see Tests).

TESTS

Related substances. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 50 mg of the substance to be examined in *methanol R*, heating gently, cool and dilute to 50.0 ml with the same solvent.

Reference solution. Dilute 1.0 ml of the test solution to 100.0 ml with *acetonitrile R*. Dilute 1.0 ml of this solution to 20.0 ml with *acetonitrile R*.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.125 m long and 4 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (5 µm),
- as mobile phase at a flow-rate of 1.0 ml/min, a mixture of equal volumes of *acetonitrile R* and a solution containing 2 g/l of *potassium dihydrogen phosphate R*, 1 g/l of *disodium hydrogen phosphate R* and 2 g/l of *tetrabutylammonium hydrogen sulphate R*,
- as detector a spectrophotometer set at 230 nm.

Adjust the sensitivity so that the height of the peak corresponding to niclosamide in the chromatogram obtained with the reference solution is not less than 20 per cent of the full scale of the recorder. Inject 20 µl of each solution and record the chromatogram for twice the retention time of niclosamide. In the chromatogram obtained with the test solution, the sum of the areas of the peaks, apart from the peak corresponding to niclosamide and the peak due to the solvent, is not greater than four times the area of the principal peak in the chromatogram obtained with the reference solution (0.2 per cent). Disregard any peak with an area less than 10 per cent of the area of the peak corresponding to niclosamide in the chromatogram obtained with the reference solution.

5-Chlorosalicylic acid

Test solution. To 1.0 g of the substance to be examined add 15 ml of *water R*, boil for 2 min, cool, filter through a membrane filter (nominal pore size: 0.45 µm), wash the filter and dilute the combined filtrate and washings to 20.0 ml with *water R*.

Reference solution. Dissolve 30 mg of *5-chlorosalicylic acid R* in 20 ml of *methanol R* and dilute to 100.0 ml with *water R*. Dilute 1.0 ml of the solution to 100.0 ml with *water R*.

To 10.0 ml of the test solution and to 10.0 ml of the reference solution add separately 0.1 ml of *ferric chloride solution R2*. Any violet colour in the test solution is not more intense than that in the reference solution (60 ppm).

2-Chloro-4-nitroaniline

Test solution. To 0.250 g of the substance to be examined add 5 ml of *methanol R*, heat to boiling, cool, add 45 ml of *1 M hydrochloric acid*, heat again to boiling, cool, filter and dilute the filtrate to 50.0 ml with *1 M hydrochloric acid*.

Reference solution. Dissolve 50 mg of *2-chloro-4-nitroaniline R* in *methanol R* and dilute to 100.0 ml with the same solvent. Dilute 1.0 ml of the solution to 100.0 ml with *methanol R*. Dilute 2.0 ml of this solution to 20.0 ml with *1 M hydrochloric acid*.

To 10.0 ml of the test solution and to 10.0 ml of the reference solution add separately 0.5 ml of a 5 g/l solution of *sodium nitrite R* and allow to stand for 3 min. Add 1 ml of a 20 g/l solution of *ammonium sulphamate R*, shake, allow to stand for 3 min and add 1 ml of a 5 g/l solution of *naphthylethylenediamine dihydrochloride R*. Any pinkish-violet colour in the test solution is not more intense than that in the reference solution (100 ppm).

Chlorides (2.4.4). To 2 g add a mixture of 1.2 ml of *acetic acid R* and 40 ml of *water R*, boil for 2 min, cool and filter. 2 ml of the filtrate diluted to 15 ml with *water R* complies with the limit test for chlorides (500 ppm).

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C for 4 h.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.3000 g in 80 ml of a mixture of equal volumes of *acetone R* and *methanol R*. Titrate with 0.1 M *tetrabutylammonium hydroxide*, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *tetrabutylammonium hydroxide* is equivalent to 32.71 mg of C₁₃H₈Cl₂N₂O₄.

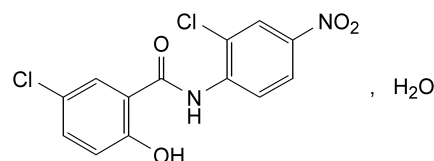
STORAGE

Store in an airtight container, protected from light.

01/2005:0680

NICLOSAMIDE MONOHYDRATE

Niclosamidum monohydricum



C₁₃H₈Cl₂N₂O₄·H₂O

*M*_r 345.1

DEFINITION

Niclosamide monohydrate contains not less than 98.0 per cent and not more than the equivalent of 101.0 per cent of 5-chloro-*N*-(2-chloro-4-nitrophenyl)-2-hydroxybenzamide, calculated with reference to the dried substance.

CHARACTERS

Yellowish, fine crystals, practically insoluble in water, sparingly soluble in acetone, slightly soluble in ethanol.

IDENTIFICATION

First identification: B, E.

Second identification: A, C, D, E.

- Melting point (2.2.14): 227 °C to 232 °C, determined after drying at 100 °C to 105 °C for 4 h.
- Dry the substance to be examined at 100 °C to 105 °C for 4 h. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *anhydrous niclosamide CRS*. Examine as discs prepared using about 0.5 mg of substance and 0.3 g of *potassium bromide R*.
- To 50 mg add 5 ml of *1 M hydrochloric acid* and 0.1 g of *zinc powder R*, heat in a water-bath for 10 min, cool and filter. To the filtrate add 1 ml of a 5 g/l solution of *sodium nitrite R* and allow to stand for 3 min; add 2 ml of a 20 g/l solution of *ammonium sulphamate R*, shake, allow to stand for 3 min and add 2 ml of a 5 g/l solution of *naphthylethylenediamine dihydrochloride R*. A violet colour is produced.
- Heat the substance on a copper wire in a non-luminous flame. The flame becomes green.
- It complies with the test for loss on drying (see Tests).

TESTS

Related substances. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 50 mg of the substance to be examined in *methanol R*, heating gently, cool and dilute to 50.0 ml with the same solvent.

Reference solution. Dilute 1.0 ml of the test solution to 100.0 ml with *acetonitrile R*. Dilute 1.0 ml of this solution to 20.0 ml with *acetonitrile R*.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.125 m long and 4 mm in internal diameter, packed with *octadecylsilyl silica gel for chromatography R* (5 µm),
- as mobile phase at a flow-rate of 1.0 ml/min, a mixture of equal volumes of *acetonitrile R* and a solution containing 2 g/l of *potassium dihydrogen phosphate R*, 1 g/l of *disodium hydrogen phosphate R* and 2 g/l of *tetrabutylammonium hydrogen sulphate R*,
- as detector a spectrophotometer set at 230 nm.

Adjust the sensitivity so that the height of the peak corresponding to niclosamide in the chromatogram obtained with the reference solution is not less than 20 per cent of full-scale of the recorder. Inject 20 µl of each solution and record the chromatogram for twice the retention time of niclosamide. In the chromatogram obtained with the test solution, the sum of the areas of the peaks, apart from the peak corresponding to niclosamide and the peak due to the solvent, is not greater than four times the area of the principal peak in the chromatogram obtained with the reference solution (0.2 per cent). Disregard any peak with an area less than 10 per cent of the area of the peak corresponding to niclosamide in the chromatogram obtained with the reference solution.

5-Chlorosalicylic acid

Test solution. To 1.0 g of the substance to be examined add 15 ml of *water R*, boil for 2 min, cool, filter through a membrane filter (nominal pore size: 0.45 µm), wash the filter and dilute the combined filtrate and washings to 20.0 ml with *water R*.

Reference solution. Dissolve 30 mg of *5-chlorosalicylic acid R* in 20 ml of *methanol R* and dilute to 100.0 ml with *water R*. Dilute 1.0 ml of the solution to 100.0 ml with *water R*.

To 10.0 ml of the test solution and to 10.0 ml of the reference solution add separately 0.1 ml of *ferric chloride solution R2*. Any violet colour produced in the test solution is not more intense than that in the reference solution (60 ppm).

2-Chloro-4-nitroaniline

Test solution. To 0.250 g of the substance to be examined add 5 ml of *methanol R*, heat to boiling, cool, add 45 ml of *1 M hydrochloric acid*, heat again to boiling, cool, filter and dilute the filtrate to 50.0 ml with *1 M hydrochloric acid*.

Reference solution. Dissolve 50 mg of *2-chloro-4-nitroaniline R* in *methanol R* and dilute to 100.0 ml with the same solvent. Dilute 1.0 ml of the solution to 100.0 ml with *methanol R*. Dilute 2.0 ml of this solution to 20.0 ml with *1 M hydrochloric acid*.

To 10.0 ml of the test solution and to 10.0 ml of the reference solution add separately 0.5 ml of a 5 g/l solution of *sodium nitrite R* and allow to stand for 3 min. Add 1 ml of a 20 g/l solution of *ammonium sulphamate R*, shake, allow to stand for 3 min and add 1 ml of a 5 g/l solution of *naphthylethylenediamine dihydrochloride R*. Any pinkish-violet colour produced in the test solution is not more intense than that in the reference solution (100 ppm).

Chlorides (2.4.4). To 2 g add a mixture of 1.2 ml of *acetic acid R* and 40 ml of *water R*, boil for 2 min, cool and filter. 2 ml of the filtrate diluted to 15 ml with *water R* complies with the limit test for chlorides (500 ppm).

Loss on drying (2.2.32): 4.5 per cent to 6.0 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C for 4 h.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.3000 g in 80 ml of a mixture of equal volumes of *acetone R* and *methanol R*. Titrate with *0.1 M tetrabutylammonium hydroxide*, determining the end-point potentiometrically (2.2.20).

1 ml of *0.1 M tetrabutylammonium hydroxide* is equivalent to 32.71 mg of $C_{13}H_{18}Cl_2N_2O_4$.

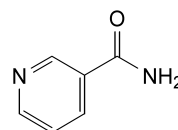
STORAGE

Store protected from light.

01/2005:0047

NICOTINAMIDE

Nicotinamidum



$C_6H_6N_2O$

M_r 122.1

DEFINITION

Nicotinamide contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of pyridine-3-carboxamide, calculated with reference to the dried substance.

CHARACTERS

A white, crystalline powder or colourless crystals, freely soluble in water and in ethanol.

IDENTIFICATION

First identification: A, B.

Second identification: A, C, D.

A. Melting point (2.2.14): 128 °C to 131 °C.

B. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *nicotinamide CRS*.

C. Boil 0.1 g with 1 ml of *dilute sodium hydroxide solution R*. Ammonia is evolved which is recognisable by its odour.

D. Dilute 2 ml of solution S (see Tests) to 100 ml with *water R*. To 2 ml of the solution, add 2 ml of *cyanogen bromide solution R* and 3 ml of a 25 g/l solution of *aniline R* and shake. A yellow colour develops.

TESTS

Solution S. Dissolve 2.5 g in *carbon dioxide-free water R* and dilute to 50 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and not more intensely coloured than reference solution BY₇ (2.2.2, *Method II*).

pH (2.2.3). The pH of solution S is 6.0 to 7.5.

Related substances. Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel GF₂₅₄ plate R*.

Test solution. Dissolve 0.4 g of the substance to be examined in a mixture of equal volumes of *alcohol R* and *water R* and dilute to 5.0 ml with the same mixture of solvents.

Reference solution. Dilute 0.5 ml of the test solution to 200 ml with a mixture of equal volumes of *alcohol R* and *water R*.

Apply to the plate 5 µl of each solution. Develop over a path of 10 cm using a mixture of 4 volumes of *water R*, 45 volumes of *ethanol R* and 48 volumes of *chloroform R*. Allow the plate to dry and examine in ultraviolet light at 254 nm. Any spot in the chromatogram obtained with the test solution, apart from the principal spot, is not more intense than the spot in the chromatogram obtained with the reference solution (0.25 per cent).

Heavy metals (2.4.8). Dilute 12 ml of solution S to 18 ml with *water R*. 12 ml of the solution complies with limit test A for heavy metals (30 ppm). Prepare the standard using *lead standard solution (1 ppm Pb) R*.

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.00 g by drying *in vacuo* for 18 h.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

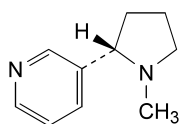
Dissolve 0.250 g in 20 ml of *anhydrous acetic acid R*, heating slightly if necessary, and add 5 ml of *acetic anhydride R*. Titrate with 0.1 M *perchloric acid*, using *crystal violet solution R* as indicator until the colour changes to greenish-blue.

1 ml of 0.1 M *perchloric acid* is equivalent to 12.21 mg of C₆H₆N₂O.

01/2005:1452

NICOTINE

Nicotinum

C₁₀H₁₄N₂*M*_r 162.2

DEFINITION

Nicotine contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of 3-[(2*S*)-1-methylpyrrolidin-2-yl]pyridine, calculated with reference to the anhydrous substance.

CHARACTERS

A colourless or brownish viscous liquid, volatile, hygroscopic, soluble in water, miscible with ethanol.

IDENTIFICATION

- It complies with the test for specific optical rotation (see Tests).
- Examine by infrared absorption spectrophotometry (2.2.24), comparing with the *Ph. Eur. reference spectrum of nicotine*.

TESTS

Appearance of solution. Dissolve 1.0 g in *water R* and dilute to 10 ml with the same solvent. The solution is clear (2.2.1) and not more intensely coloured than reference solution Y₅, BY₅ or R₅ (2.2.2, *Method II*).

Specific optical rotation (2.2.7). Dissolve 1.00 g in *ethanol R* and dilute to 50.0 ml with the same solvent. The specific optical rotation is –140 to –152.

Related substances. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 20.0 mg of the substance to be examined in the mobile phase and dilute to 25.0 ml with the mobile phase.

Reference solution (a). Dissolve 4 mg of *nicotine ditartrate CRS* and 2 mg of *myosmine R* in the mobile phase and dilute to 50.0 ml with the mobile phase.

Reference solution (b). Dilute 0.4 ml of the test solution to 100.0 ml with the mobile phase.

The chromatography may be carried out using:

- a stainless steel column 0.10 m long and 8 mm in internal diameter, packed with *octadecylsilyl silica gel for chromatography R* (4 µm),
- as mobile phase at a flow rate of 1.5 ml/min a solution prepared as follows: dissolve 2.31 g of *sodium dodecyl sulphate R* in a mixture of 250 ml of *acetonitrile R* and 750 ml of a 13.6 g/l solution of *potassium dihydrogen phosphate R*, adjusted to pH 4.5 with *sodium hydroxide R* or *phosphoric acid R*,
- as detector a spectrophotometer set at 254 nm.

Inject 25 µl of reference solution (a). When the chromatograms are recorded in the prescribed conditions the retention times are: nicotine about 13 min and impurity D about 11 min. The test is not valid unless the resolution between the impurity D peak eluting closest to the nicotine peak and the peak due to nicotine is at least 1.5; if necessary, adjust the concentration of acetonitrile in the mobile phase.

Adjust the sensitivity of the system so that the height of the principal peak in the chromatogram obtained with 25 µl of reference solution (b) is at least 50 per cent of the full scale of the recorder.

Inject 25 µl of the test solution and 25 µl of reference solution (b). Continue the chromatography for twice the retention time of the principal peak. In the chromatogram obtained with the test solution: the area of any peak, apart from the principal peak, is not greater than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.4 per cent); the sum of the areas of all the peaks, apart from the principal peak, is not greater than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (0.8 per cent). Disregard any peak with an area less than 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (b).

Water (2.5.12). Not more than 0.5 per cent, determined on 1.00 g by the semi-micro determination of water.

ASSAY

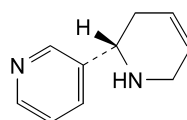
Dissolve 60.0 mg in 30 ml of *anhydrous acetic acid R*. Titrate with 0.1 M *perchloric acid* determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *perchloric acid* is equivalent to 8.11 mg of C₁₀H₁₄N₂.

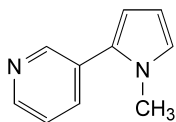
STORAGE

Store, under nitrogen, in an airtight container, protected from light.

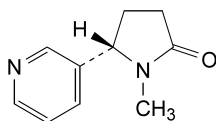
IMPURITIES



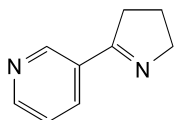
- (2*S*)-1,2,3,6-tetrahydro-2,3'-bipyridyl (anatabine),



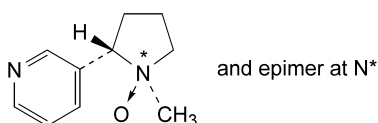
B. 3-(1-methyl-1*H*-pyrrol-2-yl)pyridine (β-nicotyrine),



C. (5*S*)-1-methyl-5-(pyridin-3-yl)pyrrolidin-2-one (cotinine),



D. 3-(4,5-dihydro-3*H*-pyrrol-2-yl)pyridine (myosmine),



E. (1*R*,2*S*)-1-methyl-2-(pyridin-3-yl)pyrrolidine 1-oxide (nicotine *N*-oxide).

01/2005:1792

NICOTINE RESINATE

Nicotini resinas

DEFINITION

Complex of nicotine (3-[(2*S*)-1-methylpyrrolidin-2-yl]pyridine) with a weak cationic exchange resin.

Content: 95.0 per cent to 115.0 per cent of the declared content of nicotine stated on the label (anhydrous substance). It may contain glycerol.

CHARACTERS

Appearance: white or slightly yellowish powder.

Solubility: practically insoluble in water.

IDENTIFICATION

A. Infrared absorption spectrophotometry (2.2.24).

Preparation: shake a quantity of the substance to be examined equivalent to 100 mg of nicotine with a mixture of 10 ml of *dilute ammonia R2*, 10 ml of *water R*, 5 ml of *strong sodium hydroxide solution R* and 20 ml of *hexane R* for 5 min. Transfer the upper layer to a beaker and evaporate to produce an oily residue. Record the spectrum of the oily residue as a thin film between *sodium chloride R* plates.

Comparison: *Ph. Eur. reference spectrum of nicotine*.

B. It complies with the test for nicotine release (see Tests).

TESTS

Nicotine release: minimum 70 per cent of the content determined under Assay in 10 min.

Transfer an accurately weighed quantity of the substance to be examined equivalent to about 4 mg of nicotine, to a glass-stoppered test-tube, add 10.0 ml of a 9 g/l solution of *sodium chloride R* previously heated to 37 °C and shake vigorously for 10 min. Immediately filter the liquid through a dry filter paper discarding the first millilitre of filtrate.

Transfer 1.0 ml of the filtrate to a 20 ml volumetric flask, dilute to volume with 0.1 *M hydrochloric acid* and mix. Determine the absorbance (2.2.25) at the minima at about 236 nm and 282 nm and at the maximum at 259 nm using 1.0 ml of a 9 g/l solution of *sodium chloride R* diluted to 20 ml with 0.1 *M hydrochloric acid* as compensation liquid. Calculate the percentage of nicotine release from the expression:

$$\frac{20 \times 10^6 \times (A_{259} - 0.5A_{236} - 0.5A_{282})}{323 \times C \times m}$$

323 = specific absorbance of nicotine at 259 nm,

C = percentage of nicotine in the substance to be examined on the basis of the amount determined in the assay,

m = mass of the substance to be examined, in milligrams,

$A_{259}, A_{236}, A_{282}$ = absorbances of the solution at the wavelength indicated by the subscript.

Related substances. Liquid chromatography (2.2.29).

Test solution. Accurately weigh a quantity of the substance to be examined equivalent to 20 mg of nicotine into a glass-stoppered test-tube, add 5.0 ml of *dilute ammonia R2*, 5.0 ml of *water R* and shake vigorously for 10 min. Centrifuge at about 2000 r/min for 10 min and dilute 3.0 ml of the clear solution to 10.0 ml with a 39.5 g/l solution of *phosphoric acid R*.

Reference solution (a). Weigh 60.0 mg of *nicotine ditartrate CRS* into a glass-stoppered test-tube, add 5.0 ml of *dilute ammonia R2*, 5.0 ml of *water R* and shake until dissolution is complete. Dilute 3.0 ml of the clear solution to 10.0 ml with a 39.5 g/l solution of *phosphoric acid R*.

Reference solution (b). Dissolve 5 mg of *myosmine R* in *acetonitrile R* and dilute to 5.0 ml with the same solvent. To 2.0 ml add 1.0 ml of the clear solution obtained during preparation of reference solution (a) and dilute to 10.0 ml with a 39.5 g/l solution of *phosphoric acid R*.

Reference solution (c). Dilute 1.0 ml of the test solution to 10.0 ml with a 39.5 g/l solution of *phosphoric acid R*. Dilute 1.0 ml to 20.0 ml with the mobile phase.

Column:

- size: *l* = 0.10 m, Ø = 8 mm,
- stationary phase: octadecylsilyl silica gel for chromatography *R* (4 µm).

Mobile phase: dissolve 2.31 g of *sodium dodecyl sulphate R* in a mixture of 250 ml of *acetonitrile R* and 750 ml of a 13.6 g/l solution of *potassium dihydrogen phosphate R* adjusted to pH 4.5 with *dilute sodium hydroxide R* or *dilute phosphoric acid R*.

Flow rate: 1.5 ml/min.

Detection: spectrophotometer at 254 nm.

Injection: 50 µl.

Run time: twice the retention time of nicotine.

System suitability: reference solution (b):

- resolution: minimum 1.5 between the peaks due to impurity D and nicotine.

Limits:

- any impurity: not more than the area of the principal peak in the chromatogram obtained with reference solution (c) (0.5 per cent),

- *total*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (c) (1.0 per cent),
- *disregard limit*: 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.05 per cent).

Water (2.5.12): maximum 5.0 per cent.

Suspend 1.0 g in 20.0 ml of *methanol R*, shake for 30 min and allow to stand for 30 min. Use 10 ml of the methanol layer for the titration. Carry out a blank titration.

ASSAY

Liquid chromatography (2.2.29) as described in the test for related substances.

Calculate the percentage content of nicotine using the chromatograms obtained with the test solution and reference solution (a) taking into account the declared content of *nicotine ditartrate CRS*.

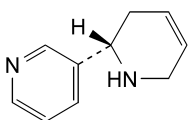
STORAGE

In an airtight container, protected from light.

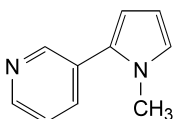
LABELLING

The label states the content of nicotine.

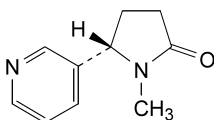
IMPURITIES



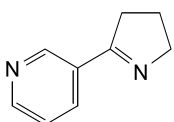
- A. (2*S*)-1,2,3,6-tetrahydro-2,3'-bipyridyl (anatabine),



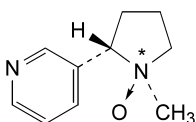
- B. 3-(1-methyl-1*H*-pyrrol-2-yl)pyridine (β-nicotyrine),



- C. (5*S*)-1-methyl-5-(pyridin-3-yl)pyrrolidin-2-one (cotinine),



- D. 3-(4,5-dihydro-3*H*-pyrrol-2-yl)pyridine (myosmine),

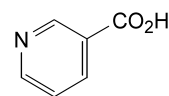


and epimer at N*

- E. (1*RS*,2*S*)-1-methyl-2-(pyridin-3-yl)pyrrolidine 1-oxide (nicotine *N*-oxide).

NICOTINIC ACID

Acidum nicotinicum



$C_6H_5NO_2$

M_r 123.1

DEFINITION

Nicotinic acid contains not less than 99.5 per cent and not more than the equivalent of 100.5 per cent of pyridine-3-carboxylic acid, calculated with reference to the dried substance.

CHARACTERS

A white, crystalline powder, soluble in boiling water and in boiling alcohol, sparingly soluble in water. It dissolves in dilute solutions of the alkali hydroxides and carbonates.

IDENTIFICATION

First identification: A, B.

Second identification: A, C.

A. Melting point (2.2.14): 234 °C to 240 °C.

B. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *nicotinic acid CRS*.

C. Dissolve about 10 mg in 10 ml of *water R*. To 2 ml of the solution add 2 ml of *cyanogen bromide solution R* and 3 ml of a 25 g/l solution of *aniline R* and shake. A yellow colour develops.

TESTS

Related substances. Examine by thin-layer chromatography (2.2.27), using *silica gel GF₂₅₄ R* as the coating substance.

Test solution. Dissolve 0.5 g of the substance to be examined in *water R*, warming slightly if necessary, and dilute to 25 ml with the same solvent.

Reference solution. Dilute 0.5 ml of the test solution to 100 ml with *water R*.

Apply separately to the plate 5 µl of each solution. Develop over a path of 15 cm using a mixture of 5 volumes of *water R*, 10 volumes of *anhydrous formic acid R* and 85 volumes of *propanol R*. Dry the plate at 100 °C to 105 °C for 10 min and examine in ultraviolet light at 254 nm. Any spot in the chromatogram obtained with the test solution, apart from the principal spot, is not more intense than the spot in the chromatogram obtained with the reference solution (0.5 per cent).

Chlorides (2.4.4). Dissolve 0.25 g in *water R*, heating on a water-bath, and dilute to 15 ml with the same solvent. The solution complies with the limit test for chlorides (200 ppm).

Heavy metals (2.4.8). 1.0 g complies with limit test C for heavy metals (20 ppm). Prepare the standard using 2 ml of *lead standard solution (10 ppm Pb) R*.

Loss on drying (2.2.32). Not more than 1.0 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C for 1 h.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.250 g in 50 ml of *water R*. Titrate with 0.1 M *sodium hydroxide*, using 0.25 ml of *phenolphthalein solution R* as indicator, until a pink colour is obtained. Carry out a blank titration.

1 ml of 0.1 M *sodium hydroxide* is equivalent to 12.31 mg of $C_{17}H_{18}N_2O_6$.

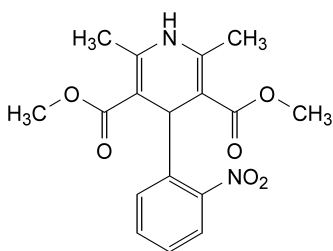
STORAGE

Store protected from light.

01/2005:0627
corrected

NIFEDIPINE

Nifedipinum



$C_{17}H_{18}N_2O_6$

M_r 346.3

DEFINITION

Dimethyl 2,6-dimethyl-4-(2-nitrophenyl)-1,4-dihydropyridine-3,5-dicarboxylate.

Content: 98.0 per cent to 102.0 per cent (dried substance).

CHARACTERS

Appearance: yellow, crystalline powder.

Solubility: practically insoluble in water, freely soluble in acetone, sparingly soluble in ethanol.

When exposed to daylight and to artificial light of certain wavelengths, it readily converts to a nitrosophenylpyridine derivative. Exposure to ultraviolet light leads to the formation of a nitrophenylpyridine derivative.

Prepare solutions immediately before use in the dark or under long-wavelength light (> 420 nm) and protect them from light.

IDENTIFICATION

First identification: B.

Second identification: A, C, D.

A. Melting point (2.2.14): 171 °C to 175 °C.

B. Infrared absorption spectrophotometry (2.2.24).

Comparison: *nifedipine CRS*.

C. Thin-layer chromatography (2.2.27).

Test solution. Dissolve 10 mg of the substance to be examined in *methanol R* and dilute to 10 ml with the same solvent.

Reference solution. Dissolve 10 mg of *nifedipine CRS* in *methanol R* and dilute to 10 ml with the same solvent.

Plate: TLC silica gel F_{254} plate *R*.

Mobile phase: *ethyl acetate R*, *cyclohexane R* (40:60 V/V).

Application: 5 µl.

Development: over 3/4 of the plate.

Drying: in air.

Detection: examine in ultraviolet light at 254 nm.

Results: the principal spot in the chromatogram obtained with the test solution is similar in position, appearance at 254 nm and size to the principal spot in the chromatogram obtained with the reference solution.

D. To 25 mg in a test tube, add 10 ml of a mixture of 1.5 volumes of *hydrochloric acid R*, 3.5 volumes of *water R* and 5 volumes of *alcohol R* and dissolve with gentle heating. Add 0.5 g of *zinc R* in granules and allow to stand for 5 min with occasional swirling. Filter into a second test tube, add 5 ml of a 10 g/l solution of *sodium nitrite R* to the filtrate and allow to stand for 2 min. Add 2 ml of a 50 g/l solution of *ammonium sulphamate R*, shake vigorously with care and add 2 ml of a 5 g/l solution of *naphthylethylenediamine dihydrochloride R*. An intense red colour develops which persists for not less than 5 min.

TESTS

Impurity D and other basic impurities. Transfer 4 g to a 250 ml conical flask and dissolve in 160 ml of *glacial acetic acid R* using an ultrasonic bath. Titrate with 0.1 M *perchloric acid* using 0.25 ml of *naphtholbenzein solution R* as indicator until the colour changes from brownish-yellow to green. Not more than 0.48 ml of 0.1 M *perchloric acid* is required (0.14 per cent).

Related substances. Liquid chromatography (2.2.29).

Test solution. Dissolve 0.200 g of the substance to be examined in 20 ml of *methanol R* and dilute to 50.0 ml with the mobile phase.

Reference solution (a). Dissolve 10 mg of *nifedipine impurity A CRS* in *methanol R* and dilute to 25.0 ml with the same solvent.

Reference solution (b). Dissolve 10 mg of *nifedipine impurity B CRS* in *methanol R* and dilute to 25.0 ml with the same solvent.

Reference solution (c). Mix 1.0 ml of reference solution (a), 1.0 ml of reference solution (b) and 0.1 ml of the test solution and dilute to 20.0 ml with the mobile phase. Dilute 2.0 ml of this solution to 10.0 ml with the mobile phase.

Column:

- *size*: $l = 0.15$ m, $\varnothing = 4.6$ mm,
- *stationary phase*: octadecylsilyl silica gel for chromatography *R* (5 µm).

Mobile phase: *acetonitrile R*, *methanol R*, *water R* (9:36:55 V/V/V).

Flow rate: 1.0 ml/min.

Detection: spectrophotometer at 235 nm.

Injection: 20 µl; inject the test solution and reference solution (c).

Run time: twice the retention time of nifedipine.

Elution order: impurity A, impurity B, nifedipine.

Retention time: nifedipine = about 15.5 min.

System suitability: reference solution (c):

- *resolution*: minimum 1.5 between the peaks due to impurity A and impurity B and minimum 1.5 between the peaks due to impurity B and nifedipine.

Limits:

- *impurity A*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (c) (0.1 per cent),
- *impurity B*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (c) (0.1 per cent),

- *any other impurity*: not more than the area of the peak due to nifedipine in the chromatogram obtained with reference solution (c) (0.1 per cent),
- *total*: not more than 0.3 per cent,
- *disregard limit*: 0.1 times the area of the peak due to nifedipine in the chromatogram obtained with reference solution (c) (0.01 per cent).

Loss on drying (2.2.32): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 100–105 °C for 2 h.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.1300 g in a mixture of 25 ml of *2-methyl-2-propanol R* and 25 ml of *perchloric acid solution R*. Titrate with 0.1 M *cerium sulphate* using 0.1 ml of *ferroin R* as indicator, until the pink colour disappears. Titrate slowly towards the end of the titration. Carry out a blank titration.

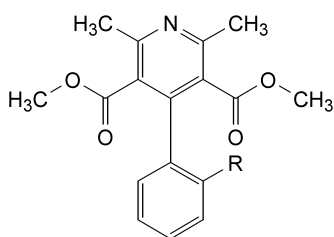
1 ml of 0.1 M *cerium sulphate* is equivalent to 17.32 mg of $C_{17}H_{18}N_2O_6$.

STORAGE

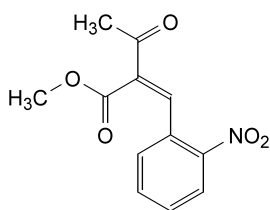
Protected from light.

IMPURITIES

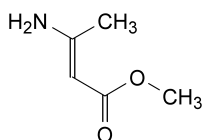
Specified impurities: A, B, C, D.



- A. R = NO₂: dimethyl 2,6-dimethyl-4-(2-nitrophenyl)pyridine-3,5-dicarboxylate (nitrophenylpyridine analogue),
- B. R = NO: dimethyl 2,6-dimethyl-4-(2-nitrosophenyl)pyridine-3,5-dicarboxylate (nitrosophenylpyridine analogue),



- C. methyl 2-(2-nitrobenzylidene)-3-oxobutanoate,

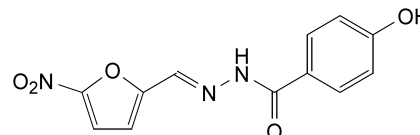


- D. methyl 3-aminobut-2-enoate.

01/2005:1999
corrected

NIFUROXAZIDE

Nifuroxazidum



$C_{12}H_9N_3O_5$

M_r 275.2

DEFINITION

1-(4-Hydroxybenzoyl)-2-[(5-nitrofuran-2-yl)methylene]-diazane.

Content: 98.5 per cent to 101.5 per cent (dried substance).

CHARACTERS

Appearance: bright yellow, crystalline powder.

Solubility: practically insoluble in water, slightly soluble in alcohol, practically insoluble in methylene chloride.

IDENTIFICATION

Infrared absorption spectrophotometry (2.2.24).

Comparison: Ph. Eur. reference spectrum of nifuroxazide.

TESTS

Specific absorbance (2.2.25): 940 to 1000 at the absorption maximum at 367 nm.

Protected from light, dissolve 10.0 mg in 10 ml of *ethylene glycol monomethyl ether R* and dilute to 100.0 ml with *methanol R*. Dilute 5.0 ml of this solution to 100.0 ml with *methanol R*.

Impurity A: maximum 0.05 per cent.

Test solution (a). Dissolve 1.0 g of the substance to be examined in *dimethyl sulphoxide R* and dilute to 10.0 ml with the same solvent.

Test solution (b). To 5.5 ml of test solution (a) add 50.0 ml of *water R* while stirring. Allow to stand for 15 min and filter.

Reference solution. To 0.5 ml of test solution (a) add 5.0 ml of a 50 mg/l solution of *4-hydroxybenzohydrazide R* in *dimethyl sulphoxide R*. Add 50.0 ml of *water R* while stirring. Allow to stand for 15 min and filter.

Add 0.5 ml of *phosphomolybdotungstic reagent R* and 10.0 ml of *sodium carbonate solution R* separately to 10.0 ml of test solution (b) and to 10.0 ml of the reference solution. Allow to stand for 1 h. Examine the 2 solutions at 750 nm. The absorbance (2.2.25) of the solution obtained with test solution (b) is not greater than that obtained with the reference solution.

Related substances. Liquid chromatography (2.2.29). *Use freshly prepared solutions, protected from light.*

Test solution. Dissolve 0.100 g of the substance to be examined in 15.0 ml of *dimethylformamide R* and dilute to 100.0 ml with the mobile phase. If precipitation occurs, use the supernatant liquid.

Reference solution (a). Dissolve 10.0 mg of *methyl parahydroxybenzoate R* (impurity B) in 2.0 ml of *dimethylformamide R* and dilute to 20.0 ml with the mobile phase. Dilute 1.0 ml of this solution to 100.0 ml with the mobile phase.

Reference solution (b). Dissolve 5 mg of the substance to be examined and 10 mg of *methyl parahydroxybenzoate R* in 2 ml of *dimethylformamide R* and dilute to 20 ml with the mobile phase. Dilute 1 ml of this solution to 100 ml with the mobile phase.

Column:

- **size:** $l = 0.25$ m, $\varnothing = 4.6$ mm,
- **stationary phase:** spherical octadecylsilyl silica gel for chromatography *R* (5 μ m) with a specific surface area of 340 m²/g, a pore size of 10 nm and a carbon loading of 19 per cent.

Mobile phase: acetonitrile *R*, water *R* (35:65 V/V).

Flow rate: 1 ml/min.

Detection: spectrophotometer at 280 nm.

Injection: 20 μ l.

Run time: 6 times the retention time of nifuroxazide.

Relative retention with reference to nifuroxazide (retention time = about 6.5 min): impurity A = about 0.4; impurity B = about 1.2; impurity C = about 2.8; impurity D = about 5.2.

System suitability: reference solution (b):

- **resolution:** minimum 3.0 between the peaks due to nifuroxazide and impurity B.

Limits:

- **any impurity:** not more than 0.6 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.3 per cent), and not more than 1 such peak has an area greater than 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent),
- **total:** not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent),
- **disregard limit:** 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

Heavy metals (2.4.8): maximum 20 ppm.

1.0 g complies with limit test D. Prepare the standard using 2 ml of *lead standard solution* (10 ppm Pb) *R*.

Loss on drying (2.2.32): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 100–105 °C for 3 h.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.200 g, if necessary with heating, in 30 ml of *dimethylformamide R* and add 20 ml of *water R*. Titrate with 0.1 *M sodium hydroxide*, determining the end-point potentiometrically (2.2.20).

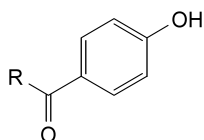
1 ml of 0.1 *M sodium hydroxide* is equivalent to 27.52 mg of C₁₂H₉N₃O₅.

STORAGE

Protected from light.

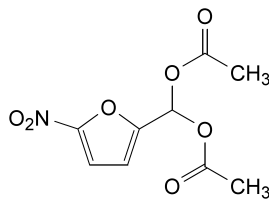
IMPURITIES

Specified impurities: A, B, C, D.

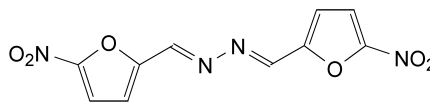


A. R = NH-NH₂: (4-hydroxybenzoyl)diazane (*p*-hydroxybenzohydrazide),

B. R = OCH₃: methyl 4-hydroxybenzoate,



C. (5-nitrofuran-2-yl)methylene diacetate,

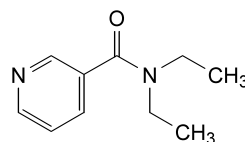


D. 1,2-bis[(5-nitrofuran-2-yl)methylene]diazane (5-nitrofurfural azine).

01/2005:0233

NIKETHAMIDE

Nicethamidum



C₁₀H₁₄N₂O

*M*_r 178.2

DEFINITION

Nikethamide contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of *N,N*-diethylpyridine-3-carboxamide, calculated with reference to the anhydrous substance.

CHARACTERS

An oily liquid or a crystalline mass, colourless or slightly yellowish, miscible with water and with alcohol.

IDENTIFICATION

First identification: A, B.

Second identification: A, C, D.

A. Dissolve 0.15 g in 0.01 *M hydrochloric acid* and dilute to 100.0 ml with the same acid. Dilute 1.0 ml of this solution to 100.0 ml with 0.01 *M hydrochloric acid*. Examined between 230 nm and 350 nm (2.2.25) in a 2 cm cell, the solution shows a single absorption maximum, at 263 nm. The specific absorbance at the maximum is about 285.

B. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *nikethamide CRS*.

C. Heat 0.1 g with 1 ml of *dilute sodium hydroxide solution R*. Diethylamine is evolved progressively and is recognisable by its characteristic odour and by its turning *red litmus paper R* blue.

D. Dilute 1 ml of solution S (see Tests) to 250 ml with *water R*. To 2 ml of this solution add 2 ml of *cyanogen bromide solution R*. Add 3 ml of a 25 g/l solution of *aniline R* and shake. A yellow colour develops.

TESTS

Solution S. Dissolve 2.5 g in *carbon dioxide-free water R* and dilute to 10 ml with the same solvent.

Appearance. The substance to be examined, in liquid form or liquefied by slight heating, is clear (2.2.1) and not more intensely coloured than reference solution Y₅ (2.2.2, Method II).

pH (2.2.3). The pH of solution S is 6.0 to 7.8.

Refractive index (2.2.6). 1.524 to 1.526.

Related substances. Examine by thin-layer chromatography (2.2.27), using *silica gel GF₂₅₄ R* as the coating substance.

Test solution. Dissolve 0.4 g of the substance to be examined in *methanol R* and dilute to 10 ml with the same solvent.

Reference solution (a). Dissolve 40 mg of *ethylnicotinamide CRS* in *methanol R* and dilute to 100 ml with the same solvent.

Reference solution (b). Dilute 1 ml of reference solution (a) to 10 ml with *methanol R*.

Apply separately to the plate 10 µl of each solution. Develop over a path of 15 cm using a mixture of 25 volumes of *propanol R* and 75 volumes of *chloroform R*. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. In the chromatogram obtained with the test solution, any spot corresponding to ethylnicotinamide is not more intense than the spot in the chromatogram obtained with reference solution (a) (1.0 per cent) and any spot, apart from the principal spot and the spot corresponding to ethylnicotinamide, is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.1 per cent).

Heavy metals (2.4.8). Dilute 10 ml of solution S to 25 ml with *water R*. 12 ml of this solution complies with limit test A for heavy metals (10 ppm). Prepare the standard using *lead standard solution (1 ppm Pb) R*.

Water (2.5.12). Not more than 0.3 per cent, determined on 2.00 g by the semi-micro determination of water.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

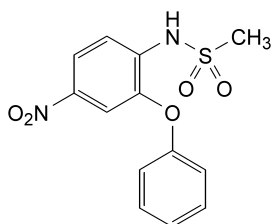
Dissolve 0.150 g in a mixture of 5 ml of *acetic anhydride R* and 20 ml of *anhydrous acetic acid R*. Titrate with 0.1 M *perchloric acid*, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *perchloric acid* is equivalent to 17.82 mg of C₁₃H₁₂N₂O₅S.

01/2005:1548

NIMESULIDE

Nimesulidum



C₁₃H₁₂N₂O₅S

*M*_r 308.3

DEFINITION

N-(4-Nitro-2-phenoxyphenyl)methanesulphonamide.

Content: 98.5 per cent to 101.5 per cent (dried substance).

CHARACTERS

Appearance: yellowish crystalline powder.

Solubility: practically insoluble in water, freely soluble in acetone, slightly soluble in ethanol.

mp: about 149 °C.

It shows polymorphism.

IDENTIFICATION

Infrared absorption spectrophotometry (2.2.24).

Preparation: discs.

Comparison: *nimesulide CRS*.

If the spectra obtained show differences, dissolve the substance to be examined and the reference substance separately in *acetone R*, evaporate to dryness and record new spectra using the residues.

TESTS

Absorbance (2.2.25): maximum 0.50 at 450 nm.

Dissolve 1.0 g in *acetone R* and dilute to 10.0 ml with the same solvent.

Related substances. Liquid chromatography (2.2.29).

Test solution. Dissolve 20 mg of the substance to be examined in 8 ml of *acetonitrile R* and dilute to 20.0 ml with *water R*.

Reference solution (a). Dissolve 10 mg of *nimesulide impurity C CRS* and 10 mg of *nimesulide impurity D CRS* in 20 ml of *acetonitrile R* and dilute to 50.0 ml with *water R*. Dilute 1.0 ml of the solution to 50.0 ml with the mobile phase.

Reference solution (b). Dilute 1.0 ml of the test solution to 10.0 ml with the mobile phase. Dilute 1.0 ml of the solution to 100.0 ml with the mobile phase.

Column:

- *dimensions:* *l* = 0.125 m, Ø = 4.0 mm,
- *stationary phase:* octadecylsilyl silica gel for chromatography *R*.

Mobile phase: a mixture of 35 volumes of *acetonitrile R* and 65 volumes of a 1.15 g/l solution of *ammonium dihydrogen phosphate R* adjusted to pH 7.0 with *ammonia R*.

Flow rate: 1.3 ml/min.

Detection: spectrophotometer at 230 nm.

Injection: 20 µl.

Run time: 7 times the retention time of nimesulide.

System suitability:

- *resolution:* minimum of 2.0 between the 2 principal peaks in the chromatogram obtained with reference solution (a).

Limits:

- *any impurity:* not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent),
- *total:* not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent),
- *disregard limit:* 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.01 per cent).

Heavy metals (2.4.8): maximum 20 ppm.

1.0 g complies with limit test D. Prepare the standard using 2 ml of *lead standard solution (10 ppm Pb) R*.

Loss on drying (2.2.32): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 100-105 °C for 4 h.

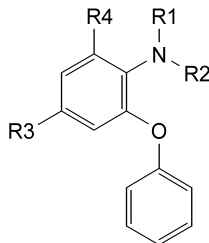
Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

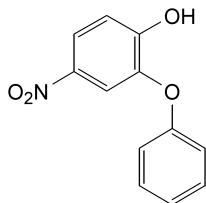
Dissolve 0.240 g in 30 ml of previously neutralised *acetone R* and add 20 ml of *water R*. Titrate with 0.1 M sodium hydroxide, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M sodium hydroxide is equivalent to 30.83 mg of $C_{13}H_{12}N_2O_5S$.

IMPURITIES



- A. $R_1 = SO_2CH_3$, $R_2 = H$, $R_3 = R_4 = NO_2$: *N*-(2,4-dinitro-6-phenoxyphenyl)methanesulphonamide,
 B. $R_1 = SO_2CH_3$, $R_2 = R_3 = R_4 = H$: *N*-(2-phenoxyphenyl)methanesulphonamide,
 C. $R_1 = R_2 = R_3 = R_4 = H$: 2-phenoxyaniline,
 D. $R_1 = R_2 = R_4 = H$, $R_3 = NO_2$: 4-nitro-2-phenoxyaniline,
 E. $R_1 = R_2 = SO_2CH_3$, $R_3 = R_4 = H$: *N,N*-bis(methylsulphonyl)-2-phenoxyaniline,
 F. $R_1 = R_2 = SO_2CH_3$, $R_3 = NO_2$, $R_4 = H$: *N,N*-bis(methylsulphonyl)-4-nitro-2-phenoxyaniline,

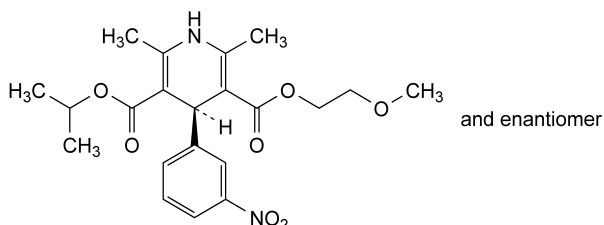


- G. 4-nitro-2-phenoxyphenol.

01/2005:1245

NIMODIPINE

Nimodipinum

 $C_{21}H_{26}N_2O_7$ M_r 418.4

DEFINITION

Nimodipine contains not less than 98.5 per cent and not more than the equivalent of 101.5 per cent of 2-methoxyethyl 1-methylethyl (4*RS*)-2,6-dimethyl-4-(3-nitrophenyl)-1,4-dihydropyridine-3,5-dicarboxylate, calculated with reference to the dried substance.

CHARACTERS

A light yellow or yellow, crystalline powder, practically insoluble in water, freely soluble in ethyl acetate, sparingly soluble in ethanol.

It shows polymorphism.

Exposure to ultraviolet light leads to the formation of a nitrophenylpyridine derivative.

Prepare solutions immediately before use either protected from light or under long-wavelength light (> 420 nm).

IDENTIFICATION

Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *nimodipine CRS*. If the spectra obtained in the solid state show differences, record further spectra using 20 g/l solutions in *methylene chloride R* and a 0.2 mm cell.

TESTS

Solution S. Dissolve 1.0 g in *acetone R* and dilute to 20.0 ml with *acetone R*.

Appearance of solution. Solution S is clear (2.2.1).

Optical rotation (2.2.7). The angle of optical rotation, determined on solution S, is -0.10° to $+0.10^\circ$.

Related substances. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 40.0 mg of the substance to be examined in 2.5 ml of *tetrahydrofuran R* and dilute to 25.0 ml with the mobile phase.

Reference solution (a). Dilute 1.0 ml of the test solution to 100.0 ml with the mobile phase. Dilute 2.0 ml of the solution to 10.0 ml with the mobile phase.

Reference solution (b). Dissolve 20.0 mg of *nimodipine impurity A CRS* in 2.5 ml of *tetrahydrofuran R* and dilute to 25.0 ml with the mobile phase. Dilute 1.0 ml of the solution to 20.0 ml with the mobile phase.

Reference solution (c). Dilute 0.5 ml of the test solution to 20.0 ml with the mobile phase.

Reference solution (d). Mix 1.0 ml of reference solution (b) and 1.0 ml of reference solution (c) and dilute to 25.0 ml with the mobile phase.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.125 m long and 4.6 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (5 μ m),
- as mobile phase at a flow rate of 2.0 ml/min a mixture of 20 volumes of *methanol R*, 20 volumes of *tetrahydrofuran R* and 60 volumes of *water R*,
- as detector a spectrophotometer set at 235 nm, maintaining the temperature of the column at 40 $^\circ$ C.

Adjust the sensitivity of the system so that the height of the peak corresponding to nimodipine in the chromatogram obtained with 20 μ l of reference solution (d) is at least 50 per cent of the full scale of the recorder.

Inject 20 μ l of reference solution (d). When the chromatograms are recorded in the prescribed conditions, the retention times are: impurity A about 7 min and nimodipine about 8 min. The test is not valid unless the resolution between the peaks corresponding to impurity A and nimodipine is at least 1.5.

Inject separately 20 μ l of the test solution and 20 μ l of reference solution (a). Record the chromatogram of the test solution for four times the retention time of nimodipine. In the chromatogram obtained with the test solution: the area of the peak due to impurity A is not greater than the corresponding peak in the chromatogram obtained with reference solution (d) (0.1 per cent); none of the peaks, apart from the principal peak and the peak due to impurity A, has an area greater than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per

cent); the sum of the areas of all the peaks, apart from the principal peak, is not greater than 2.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent). Disregard any peak due to the solvent and any peak with an area less than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (d).

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 100–105 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

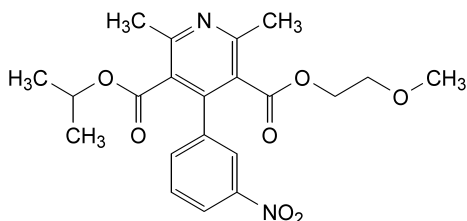
Dissolve 0.180 g with gentle heating in a mixture of 25 ml of 2-methyl-2-propanol *R* and 25 ml of perchloric acid solution *R*. Add 0.1 ml of ferroin *R*. Titrate with 0.1 *M* cerium sulphate. Titrate slowly towards the end of the titration. Carry out a blank titration.

1 ml of 0.1 *M* cerium sulphate is equivalent to 20.92 mg of C₂₁H₂₆N₂O₇.

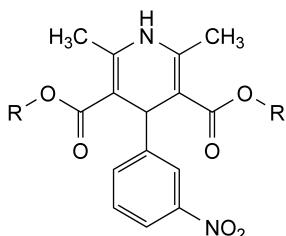
STORAGE

Store protected from light.

IMPURITIES



A. 2-methoxyethyl 1-methylethyl 2,6-dimethyl-4-(3-nitrophenyl)pyridine-3,5-dicarboxylate,



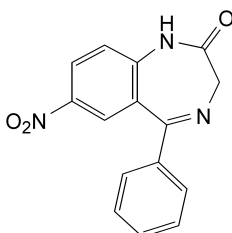
B. R = CH(CH₃)₂: bis(1-methylethyl) 2,6-dimethyl-4-(3-nitrophenyl)-1,4-dihydropyridine-3,5-dicarboxylate,

C. R = CH₂CH₂OCH₃: bis(2-methoxyethyl) 2,6-dimethyl-4-(3-nitrophenyl)-1,4-dihydropyridine-3,5-dicarboxylate.

01/2005:0415

NITRAZEPAM

Nitrazepamum



C₁₅H₁₁N₃O₃

*M*_r 281.3

DEFINITION

Nitrazepam contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of 7-nitro-5-phenyl-1,3-dihydro-2*H*-1,4-benzodiazepin-2-one, calculated with reference to the dried substance.

CHARACTERS

A yellow, crystalline powder, practically insoluble in water, slightly soluble in alcohol.

IDENTIFICATION

First identification: A, C.

Second identification: A, B, D, E.

A. Melting point (2.2.14): 226 °C to 230 °C.

B. *Protect the solutions from light and measure the absorbances immediately.* Dissolve 25.0 mg in a 5 g/l solution of sulphuric acid *R* in methanol *R* and dilute to 250.0 ml with the same solvent. Dilute 5.0 ml of the solution to 100.0 ml with a 5 g/l solution of sulphuric acid *R* in methanol *R*. Examined between 230 nm and 350 nm (2.2.25), the solution shows an absorption maximum at 280 nm. The specific absorbance at the maximum is 890 to 950.

C. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with nitrazepam CRS.

D. Dissolve about 20 mg in a mixture of 5 ml of hydrochloric acid *R* and 10 ml of water *R*. Boil for 5 min, cool and add 2 ml of a 1 g/l solution of sodium nitrite *R*. Allow to stand for 1 min and add 1 ml of a 5 g/l solution of sulphamic acid *R* and mix. Allow to stand for 1 min and add 1 ml of a 1 g/l solution of naphthylethylenediamine dihydrochloride *R*. A red colour is produced.

E. Dissolve about 10 mg in 1 ml of methanol *R*, warming if necessary, and add 0.05 ml of dilute sodium hydroxide solution *R*. An intense yellow colour is produced.

TESTS

Related substances. Carry out the test protected from light. Examine by thin-layer chromatography (2.2.27), using silica gel GF₂₅₄ *R* as the coating substance.

Test solution. Dissolve 0.2 g of the substance to be examined in acetone *R* and dilute to 10 ml with the same solvent. Prepare the solution immediately before use.

Reference solution (a). Dissolve 10 mg of aminonitrobenzophenone *R* in acetone *R* and dilute to 100 ml with the same solvent. Dilute 10 ml of the solution to 50 ml with acetone *R*.

Reference solution (b). Dissolve 10 mg of nitrazepam impurity A CRS in acetone *R* and dilute to 100 ml with the same solvent. Dilute 10 ml of the solution to 50 ml with acetone *R*.

Reference solution (c). Dilute 1 ml of the test solution to 20 ml with acetone *R*. Dilute 1 ml of this solution to 50 ml with acetone *R*.

Apply separately to the plate 10 µl of each solution. Develop over a path of 12 cm using a mixture of 15 volumes of ethyl acetate *R* and 85 volumes of nitromethane *R*. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. In the chromatogram obtained with the test solution: any spot corresponding to aminonitrobenzophenone is not more intense than the spot in the chromatogram obtained with reference solution (a) (0.1 per cent); any spot corresponding to nitrazepam impurity A is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.1 per cent); any spot apart from the principal spot and the spots corresponding to aminonitrobenzophenone and

nitrazepam impurity A is not more intense than the spot in the chromatogram obtained with reference solution (c) (0.1 per cent).

Heavy metals (2.4.8). 1.0 g complies with limit test D for heavy metals (20 ppm). Prepare the standard using 2 ml of *lead standard solution* (10 ppm Pb) *R*.

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.00 g by drying in an oven at 100 °C to 105 °C for 4 h.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

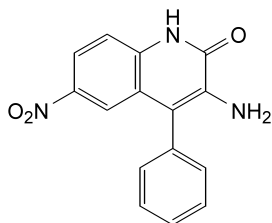
Dissolve 0.250 g in 25 ml of *acetic anhydride R*. Titrate with 0.1 M *perchloric acid*, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *perchloric acid* is equivalent to 28.13 mg of $C_{15}H_{11}N_3O_3$.

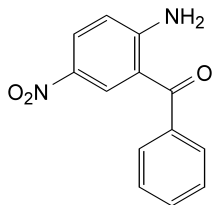
STORAGE

Store protected from light.

IMPURITIES



A. 3-amino-6-nitro-4-phenylquinolin-2(1H)-one,

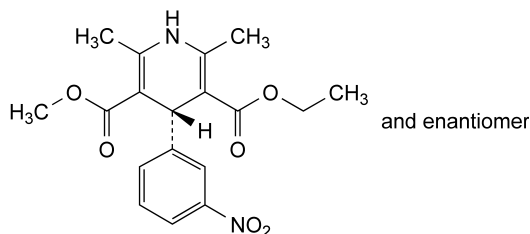


B. (2-amino-5-nitrophenyl)phenylmethanone.

01/2005:1246

NITRENDIPINE

Nitrendipinum



$C_{18}H_{20}N_2O_6$

M_r 360.4

DEFINITION

Nitrendipine contains not less than 98.5 per cent and not more than the equivalent of 101.5 per cent of ethyl methyl (4*RS*)-2,6-dimethyl-4-(3-nitrophenyl)-1,4-dihydropyridine-3,5-dicarboxylate, calculated with reference to the dried substance.

CHARACTERS

A yellow, crystalline powder, practically insoluble in water, freely soluble in ethyl acetate, sparingly soluble in ethanol and in methanol.

It shows polymorphism.

Exposure to ultraviolet light leads to the formation of a nitrophenylpyridine derivative

Prepare solutions immediately before use either protected from light or under long-wavelength light (> 420 nm).

IDENTIFICATION

Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *nitrendipine CRS*. If the spectra obtained in the solid state show differences, record further spectra using 20 g/l solutions in *methylene chloride R* and a 0.2 mm cell.

TESTS

Optical rotation (2.2.7). Dissolve 0.2 g in *acetone R* and dilute to 10.0 ml with the same solvent. The angle of optical rotation is -0.10° to $+0.10^\circ$.

Related substances. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 40.0 mg of the substance to be examined in 2.5 ml of *tetrahydrofuran R* and dilute to 25.0 ml with the mobile phase.

Reference solution (a). Dilute 2.0 ml of the test solution to 10.0 ml with the mobile phase. Dilute 1.0 ml of this solution to 25.0 ml with the mobile phase.

Reference solution (b). Dissolve 20.0 mg of *nitrendipine impurity A CRS* in 2.5 ml of *tetrahydrofuran R* and dilute to 25.0 ml with the mobile phase. Dilute 1.0 ml of this solution to 20.0 ml with the mobile phase.

Reference solution (c). Dilute 0.5 ml of the test solution to 20.0 ml with the mobile phase.

Reference solution (d). Mix 1.0 ml of reference solution (b) and 1.0 ml of reference solution (c) and dilute to 25.0 ml with the mobile phase.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.125 m long and 4 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (5 μ m),
- as mobile phase at a flow rate of 1 ml/min a mixture of 14 volumes of *acetonitrile R*, 22 volumes of *tetrahydrofuran R* and 64 volumes of *water R*,
- as detector a spectrophotometer set at 235 nm, maintaining the temperature of the column at 40 °C.

Adjust the sensitivity of the system so that the height of the nitrendipine peak in the chromatogram obtained with 20 μ l of reference solution (d) is at least 50 per cent of the full scale of the recorder.

Inject 20 μ l of reference solution (d). When the chromatogram is recorded in the prescribed conditions, the retention times are: nitrendipine impurity A about 6 min and nitrendipine about 8 min. The test is not valid unless the resolution between the peaks corresponding to nitrendipine impurity A and nitrendipine is at least 2.0.

Inject separately 20 μ l of the test solution and 20 μ l of reference solution (a). Record the chromatogram of the test solution for five times the retention time of the principal peak. In the chromatogram obtained with the test solution: the area of the peak corresponding to nitrendipine impurity A is not greater than the area of the corresponding peak in the chromatogram obtained with reference solution (d) (0.1 per cent); none of the peaks, apart from the principal peak and the peaks corresponding to nitrendipine impurity A

has an area greater than that of the peak corresponding to nitrendipine in the chromatogram obtained with reference solution (a) (0.8 per cent); the sum of the areas of all the peaks, apart from the principal peak, is not greater than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (1.2 per cent). Disregard any peak with an area less than 0.5 times the area of the nitrendipine peak in the chromatogram obtained with reference solution (d).

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 100–105 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

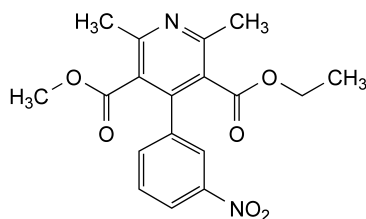
Dissolve 0.160 g with gentle heating if necessary in a mixture of 25 ml of *2-methyl-2-propanol R* and 25 ml of *perchloric acid solution R*. Titrate with *0.1 M cerium sulphate*, using *0.1 ml of ferroin R* as indicator. Titrate slowly towards the end of the titration. Carry out a blank titration.

1 ml of *0.1 M cerium sulphate* is equivalent to 18.02 mg of $C_{18}H_{20}N_2O_6$.

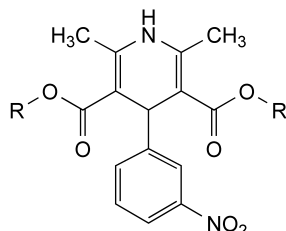
STORAGE

Store protected from light.

IMPURITIES



A. ethyl methyl 2,6-dimethyl-4-(3-nitrophenyl)pyridine-3,5-dicarboxylate,



B. $R = CH_3$: dimethyl 2,6-dimethyl-4-(3-nitrophenyl)-1,4-dihydropyridine-3,5-dicarboxylate,

C. $R = CH_2CH_3$: diethyl 2,6-dimethyl-4-(3-nitrophenyl)-1,4-dihydropyridine-3,5-dicarboxylate.

01/2005:1549

NITRIC ACID

Acidum nitricum

 HNO_3
 M_r 63.0

DEFINITION

Nitric acid contains not less than 68.0 per cent *m/m* and not more than 70.0 per cent *m/m* of HNO_3 .

CHARACTERS

A clear, colourless to almost colourless liquid, miscible with water.

It has a relative density of about 1.41.

IDENTIFICATION

- Dilute 1 ml to 100 ml with *water R*. The solution is strongly acid (2.2.4).
- 0.2 ml of the solution obtained in identification test A gives the reaction of nitrates (2.3.1).

TESTS

Appearance of solution. Dilute 2 ml to 10 ml with *water R*. The solution is clear (2.2.1) and not more intensely coloured than reference solution Y_6 (2.2.2, *Method II*).

Chlorides (2.4.4). To 5 g add 10 ml of *water R* and 0.3 ml of *silver nitrate solution R2* and allow to stand for 2 min protected from light. Any opalescence is not more intense than that of a standard prepared in the same manner using 13 ml of *water R*, 0.5 ml of *nitric acid R*, 0.5 ml of *chloride standard solution (5 ppm Cl) R* and 0.3 ml of *silver nitrate solution R2* (0.5 ppm).

Sulphates (2.4.13). To 15 g add 0.2 g of *sodium carbonate R*. After carbon dioxide has evolved, evaporate to dryness. Dissolve the residue in 15 ml of *distilled water R*. The solution complies with the limit test for sulphates (10 ppm).

Iron (2.4.9). Dissolve the residue obtained in the test for sulphated ash in 1 ml of *dilute hydrochloric acid R* and dilute to 20 ml with *water R*. Dilute 1 ml to 10 ml with *water R*. The solution complies with the limit test for iron (10 ppm).

Heavy metals (2.4.8). Carefully evaporate 10.0 g to dryness on a water-bath. Moisten the residue with a few drops of *dilute hydrochloric acid R* and dilute to 20 ml with *water R*. 12 ml of the solution complies with limit test A for heavy metals (2 ppm). Prepare the standard using *lead standard solution (2 ppm Pb) R*.

Sulphated ash. Carefully evaporate 20.00 g to dryness. Moisten the residue with a few drops of *sulphuric acid R* and ignite to dull red. The residue does not exceed 0.01 per cent.

ASSAY

To 0.750 g add 50 ml of *water R* and titrate with *1 M sodium hydroxide*, determining the end-point potentiometrically (2.2.20).

1 ml of *1 M sodium hydroxide* is equivalent to 63.0 mg of HNO_3 .

STORAGE

Store protected from light.

01/2005:1550

NITRIC OXIDE

Nitrogenii oxidum

 NO
 M_r 30.01

DEFINITION

Nitric oxide contains not less than 99.0 per cent *V/V* of NO . This monograph applies to nitric oxide for medicinal use.

CHARACTERS

A colourless gas which turns brown when exposed to air. At 20 °C and at a pressure of 101 kPa, 1 volume dissolves in about 21 volumes of water.

PRODUCTION

Carbon dioxide. Not more than 3000 ppm *V/V*, determined by gas chromatography (2.2.28).

Gas to be examined. The substance to be examined.

Reference gas. Use a mixture containing 3000 ppm V/V of carbon dioxide R1 in nitrogen R.

The chromatography may be carried out using:

- a stainless steel column 3.5 m long and 2 mm in internal diameter packed with *ethylvinylbenzene-divinylbenzene copolymer R*,
- *helium for chromatography R* as the carrier gas at a flow rate of 15 ml/min,
- a thermal conductivity detector,
- a loop injector,

maintaining the temperature of the column at 50 °C.

Inject the gas to be examined and the reference gas. The test is not valid unless the chromatograms obtained show a clear separation of carbon dioxide from nitric oxide.

Calculate the carbon dioxide content in the gas to be examined from the area of the carbon dioxide peak in the chromatogram obtained with the reference gas.

Nitrogen. Not more than 3000 ppm V/V, determined by gas chromatography (2.2.28).

Gas to be examined. The substance to be examined.

Reference gas. Use a mixture containing 3000 ppm V/V of nitrogen R in *helium for chromatography R*.

The chromatography may be carried out using:

- a stainless steel column 3.5 m long and 2 mm in internal diameter packed with *molecular sieve for chromatography R* (0.5 nm),
- *helium for chromatography R* as the carrier gas at a flow rate of 15 ml/min,
- a thermal conductivity detector,
- a loop injector,

maintaining the temperature of the column at 50 °C.

Inject the gas to be examined and the reference gas. The test is not valid unless the chromatograms obtained show a clear separation of nitrogen from nitric oxide.

Calculate the nitrogen content in the gas to be examined from the area of the peak due to nitrogen in the chromatogram obtained with the reference gas.

Nitrogen dioxide. Not more than 400 ppm V/V, determined using an ultraviolet absorption spectrophotometry analyser.

Gas to be examined. The substance to be examined.

Reference gas (a). Use *nitrogen R1*.

Reference gas (b). Use a mixture containing 400 ppm V/V of *nitrogen dioxide R* in *nitrogen R*.

The apparatus consists of the following:

- an ultraviolet-visible light source (analytical wavelength about 400 nm),
- a sample gas cell through which the feed gas flows,
- a closed reference gas cell containing *nitrogen R1* in parallel with the sample gas cell,
- a rotating chopper which feeds light alternately through the reference gas cell and the sample gas cell,
- a semiconductor detector which generates a frequency modulated output whose amplitude is a measure of the difference of absorption of the sample gas and the reference gas.

Carry out the analysis in the following way:

- set the zero of the instrument using reference gas (a) through the sample gas cell at a flow rate of 1 litre/min,
- adjust the span while feeding reference gas (b) through the sample gas cell at a flow rate of 1 litre/min,

- feed the gas to be examined through the sample gas cell at a flow rate of 1 litre/min, read the value from the instrument output and calculate if necessary the concentration of nitrogen dioxide.

Nitrous oxide. Not more than 3000 ppm V/V, determined by gas chromatography (2.2.28).

Gas to be examined. The substance to be examined.

Reference gas. Use a mixture containing 3000 ppm V/V of *nitrous oxide R* in *nitrogen R*.

The chromatography may be carried out using:

- a stainless steel column 3.5 m long and 2 mm in internal diameter packed with *ethylvinylbenzene-divinylbenzene copolymer R*,
- *helium for chromatography R* as the carrier gas at a flow rate of 15 ml/min,
- a thermal conductivity detector,
- a loop injector,

maintaining the temperature of the column at 50 °C.

Inject the gas to be examined and the reference gas. The test is not valid unless the chromatograms obtained show a clear separation of nitrous oxide from nitric oxide.

Calculate the nitrous oxide content in the gas to be examined from the area of the peak due to nitrous oxide in the chromatogram obtained with the reference gas.

Water. Not more than 100 ppm V/V, determined using an electrolytic hygrometer (2.5.28).

Assay. Determine the content of nitric oxide by difference using the mass balance equation after determining the sum of the impurities described under Production.

IDENTIFICATION

Examine by infrared absorption spectrophotometry (2.2.24), comparing with the *Ph. Eur. reference spectrum of nitric oxide*.

STORAGE

Store compressed at a pressure not exceeding 2.5 MPa (25 bars) measured at 15 °C, in suitable containers complying with the legal regulations.

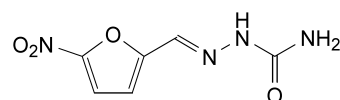
IMPURITIES

- carbon dioxide,
- nitrogen,
- nitrogen dioxide,
- nitrous oxide,
- water.

01/2005:1135

NITROFURAL

Nitrofuralum



$C_6H_6N_4O_4$

M_r 198.1

DEFINITION

Nitrofural contains not less than 97.0 per cent and not more than the equivalent of 103.0 per cent of 2-[(5-nitrofuran-2-yl)methylene]diazanecarboxamide, calculated with reference to the dried substance.

CHARACTERS

A yellow or brownish-yellow, crystalline powder, very slightly soluble in water, slightly soluble in alcohol.

IDENTIFICATION

First identification: B.

Second identification: A, C, D.

- Carry out the test protected from bright light.* Use the solution prepared for the assay. Examined between 220 nm and 400 nm (2.2.25), the solution shows two absorption maxima, at 260 nm and 375 nm. The ratio of the absorbance measured at the maximum at 375 nm to that measured at the maximum at 260 nm is 1.15 to 1.30.
- Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *nitrofural CRS*. Examine the substances prepared as discs.
- Examine by thin-layer chromatography (2.2.27), using *silica gel G R* as the coating substance.
Test solution. Dissolve 10 mg of the substance to be examined in *methanol R* and dilute to 10 ml with the same solvent.
Reference solution. Dissolve 10 mg of *nitrofural CRS* in *methanol R* and dilute to 10 ml with the same solvent. Apply to the plate 5 µl of each solution. Develop over a path of 15 cm using a mixture of 10 volumes of *methanol R* and 90 volumes of *nitromethane R*. Allow the plate to dry in air and spray with *phenylhydrazine hydrochloride solution R*. The principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.
- Dissolve about 1 mg in 1 ml of *dimethylformamide R* and add 0.1 ml of *alcoholic potassium hydroxide solution R*. A violet-red colour is produced.

TESTS

pH (2.2.3). To 1.0 g add 100 ml of *carbon dioxide-free water R*. Shake and filter. The pH of the filtrate is 5.0 to 7.0.

Related substances. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 0.10 g of the substance to be examined in the mobile phase and dilute to 100.0 ml with the mobile phase.

Reference solution (a). Dissolve 10.0 mg of (5-nitro-2-furyl)methylene diacetate *R* in the mobile phase and dilute to 20.0 ml with the mobile phase. Dilute 1.0 ml of this solution to 100.0 ml with the mobile phase.

Reference solution (b). Dissolve 10 mg of *nitrofural CRS* and 10 mg of *nitrofurantoin R* in the mobile phase and dilute to 100 ml with the mobile phase. Dilute 5 ml of this solution to 100 ml with the mobile phase.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4.6 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (5 µm),
- as mobile phase at a flow rate of 1 ml per minute a mixture of 40 volumes of *acetonitrile R* and 60 volumes of *water R*,
- as detector a spectrophotometer set at 310 nm.

Inject 20 µl of reference solution (a). Adjust the sensitivity of the system so that the height of the principal peak in the chromatogram obtained is not less than 50 per cent of the full scale of the recorder. Inject 20 µl of reference solution (b). The test is not valid unless, in the chromatogram obtained,

the resolution between the peaks due to nitrofurantoin and nitrofural is at least 2.0. Inject 20 µl of the test solution and 20 µl of reference solution (a). Continue the chromatography for ten times the retention time of nitrofural, which is about 3 min. In the chromatogram obtained with the test solution: the area of any peak, apart from the principal peak, is not greater than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent); the sum of the areas of all the peaks, apart from the principal peak, is not greater than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (1.0 per cent). Disregard any peak with an area less than 0.05 times that of the principal peak in the chromatogram obtained with reference solution (a).

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 100–105 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

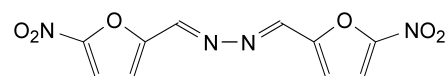
ASSAY

Carry out the assay protected from bright light. Dissolve 60.0 mg in 20 ml of *dimethylformamide R* and dilute to 500.0 ml with *water R*. Dilute 5.0 ml to 100.0 ml with *water R*. Prepare a reference solution in the same manner using 60.0 mg of *nitrofural CRS*. Measure the absorbances (2.2.25) of the two solutions at the maximum at 375 nm. Calculate the content of C₈H₆N₄O₅ from the absorbances measured and the concentrations of the solutions.

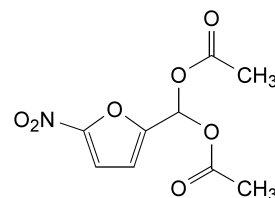
STORAGE

Store protected from light.

IMPURITIES



A. bis[(5-nitrofuran-2-yl)methylene]diazane,

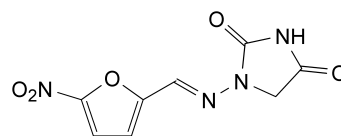


B. (5-nitrofuran-2-yl)methylene diacetate.

01/2005:0101

NITROFURANTOIN

Nitrofurantoinum

C₈H₆N₄O₅M_r 238.2

DEFINITION

Nitrofurantoin contains not less than 98.0 per cent and not more than the equivalent of 102.0 per cent of 1-[(5-nitrofuran-2-yl)methylene]aminolimidazolidine-2,4-dione, calculated with reference to the dried substance.

CHARACTERS

A yellow, crystalline powder or yellow crystals, odourless or almost odourless, very slightly soluble in water and in alcohol, soluble in dimethylformamide.

IDENTIFICATION

- A. *Carry out the test protected from bright light.* Use the solution prepared for the assay. Examined between 220 nm and 400 nm (2.2.25), the solution shows two absorption maxima, at 266 nm and 367 nm. The ratio of the absorbance at the maximum at 367 nm to that at the maximum at 266 nm is 1.36 to 1.42.
- B. Dissolve about 10 mg in 10 ml of *dimethylformamide R*. To 1 ml of the solution add 0.1 ml of 0.5 M alcoholic *potassium hydroxide*. A brown colour develops.

TESTS

Related substances. Examine by thin-layer chromatography (2.2.27), using *silica gel HF₂₅₄ R* as the coating substance.

Test solution. Dissolve 0.25 g of the substance to be examined in a minimum of *dimethylformamide R* and dilute to 10 ml with *acetone R*.

Reference solution. Dilute 1 ml of the test solution to 100 ml with *acetone R*.

Apply separately to the plate 10 µl of each solution. Develop over a path of 15 cm using a mixture of 10 volumes of *methanol R* and 90 volumes of *nitromethane R*. Allow the plate to dry in air and heat at 100 °C to 105 °C for 5 min. Examine in ultraviolet light at 254 nm. Spray with *phenylhydrazine hydrochloride solution R*. Heat the plate at 100 °C to 105 °C for a further 10 min. When examined in ultraviolet light and after spraying, any spot in the chromatogram obtained with the test solution, apart from the principal spot, is not more intense than the spot in the chromatogram obtained with the reference solution (1.0 per cent).

Loss on drying (2.2.32). Not more than 1.0 per cent, determined on 1.00 g by drying in an oven at 100 °C to 105 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Carry out the assay protected from bright light. Dissolve 0.120 g in 50 ml of *dimethylformamide R* and dilute to 1000.0 ml with *water R*. Dilute 5.0 ml of the solution to 100.0 ml with a solution containing 18 g/l of *sodium acetate R* and 0.14 per cent V/V of *glacial acetic acid R*. Measure the absorbance (2.2.25) at the absorption maximum at 367 nm, using the sodium acetate solution described above as compensation liquid.

Calculate the content of C₈H₆N₄O₅, taking the specific absorbance to be 765.

STORAGE

Store protected from light, at a temperature below 25 °C.

DEFINITION

Nitrogen contains not less than 99.5 per cent V/V of N₂. This monograph applies to nitrogen for medicinal use.

CHARACTERS

A colourless, odourless gas. At 20 °C and at a pressure of 101 kPa, 1 volume dissolves in about 62 volumes of water and about 10 volumes of alcohol.

PRODUCTION

Carbon dioxide. Not more than 300 ppm V/V, determined using an infrared analyser (2.5.24).

Gas to be examined. The substance to be examined. It must be filtered to avoid stray light phenomena.

Reference gas (a). Use *nitrogen R1*.

Reference gas (b). Use a mixture containing 300 ppm V/V of *carbon dioxide R1* in *nitrogen R1*.

Calibrate the apparatus and set the sensitivity using reference gases (a) and (b). Measure the content of carbon dioxide in the gas to be examined.

Carbon monoxide. Not more than 5 ppm V/V, determined using an infrared analyser (2.5.25).

Gas to be examined. The substance to be examined. It must be filtered to avoid stray light phenomena.

Reference gas (a). Use *nitrogen R1*.

Reference gas (b). Use a mixture containing 5 ppm V/V of *carbon monoxide R* in *nitrogen R1*.

Calibrate the apparatus and set the sensitivity using reference gases (a) and (b). Measure the content of carbon monoxide in the gas to be examined.

Oxygen. Not more than 50 ppm V/V, determined using an oxygen analyser with a detector scale ranging from 0 ppm V/V to 100 ppm V/V and equipped with an electrochemical cell.

The gas to be examined passes through a detection cell containing an aqueous solution of an electrolyte, generally potassium hydroxide. The presence of oxygen in the gas to be examined produces variation in the electric signal recorded at the outlet of the cell that is proportional to the oxygen content.

Calibrate the analyser according to the instructions of the manufacturer. Pass the gas to be examined through the analyser using a suitable pressure regulator and airtight metal tubes and operating at the prescribed flow-rates until constant readings are obtained.

Water. Not more than 67 ppm V/V, determined using an electrolytic hygrometer (2.5.28).

Assay. Examine by gas chromatography (2.2.28).

Gas to be examined. The substance to be examined.

Reference gas (a). Use ambient air.

Reference gas (b). Use *nitrogen R1*.

The chromatographic procedure may be carried out using:

- a stainless steel column 2 m long and 2 mm in internal diameter packed with an appropriate molecular sieve for chromatography (0.5 nm),
- *helium for chromatography R* as the carrier gas at a flow rate of 40 ml/min,
- a thermal conductivity detector,
- a loop injector,

maintaining the temperature of the column at 50 °C and that of the detector at 130 °C.

Inject reference gas (a). Adjust the injected volumes and operating conditions so that the height of the peak due to nitrogen in the chromatogram obtained with the reference

01/2005:1247

NITROGEN

Nitrogenium

N₂M_r 28.01

gas is at least 35 per cent of the full scale of the recorder. The assay is not valid unless the chromatograms obtained show a clear separation of oxygen and nitrogen.

Inject the gas to be examined and reference gas (b). Calculate the content of N₂ in the gas to be examined.

IDENTIFICATION

First identification: A.

Second identification: B, C.

- A. Examine the chromatograms obtained in the Assay. The retention time of the principal peak in the chromatogram obtained with the substance to be examined is approximately the same as that of the principal peak in the chromatogram obtained with reference gas (b).
- B. In a 250 ml conical flask replace the air by the substance to be examined. Place a burning or glowing splinter of wood in the flask. The splinter is extinguished.
- C. In a suitable test tube, place 0.1 g of *magnesium R* in turnings. Close the tube with a two-hole stopper fitted with a glass tube reaching about 1 cm above the turnings. Pass the substance to be examined through the glass tube for 1 min without heating, then for 15 min while heating the test tube to a red glow. After cooling, add 5 ml of *dilute sodium hydroxide solution R*. The evolving vapours change the colour of moistened *red litmus paper R* blue.

TESTS

Carbon dioxide. Not more than 300 ppm V/V, determined using a carbon dioxide detector tube (2.1.6).

Carbon monoxide. Not more than 5 ppm V/V, determined using a carbon monoxide detector tube (2.1.6).

Water vapour. Not more than 67 ppm V/V, determined using a water vapour detector tube (2.1.6).

STORAGE

Store as a compressed gas or a liquid in appropriate containers complying with the legal regulations.

IMPURITIES

- A. carbon dioxide,
- B. carbon monoxide,
- C. oxygen,
- D. water.

01/2005:1685

NITROGEN, LOW-OXYGEN

Nitrogenium oxygenio depletum

N₂

M_r 28.01

DEFINITION

This monograph applies to nitrogen which is used for inerting finished medicinal products which are particularly sensitive to degradation by oxygen. It does not necessarily apply to nitrogen used in earlier production steps.

Content: minimum 99.5 per cent V/V of N₂, calculated by deduction of the sum of impurities found when performing the test for impurities.

CHARACTERS

Colourless and odourless gas.

Solubility: at 20 °C and at a pressure of 101 kPa, 1 volume dissolves in about 62 volumes of water and about 10 volumes of alcohol.

PRODUCTION

Oxygen: maximum 5 ppm V/V, determined using an oxygen analyser with a detector scale ranging from 0 ppm V/V to 100 ppm V/V and equipped with an electrochemical cell.

The gas to be examined passes through a detection cell containing an aqueous solution of an electrolyte, generally potassium hydroxide. The presence of oxygen in the gas to be examined produces variation in the electric signal recorded at the outlet of the cell that is proportional to the oxygen content.

Calibrate the analyser according to the manufacturer's instructions. Pass the gas to be examined through the analyser using a suitable pressure regulator and airtight metal tubes and operating at the prescribed flow rates until constant readings are obtained.

Impurities. Gas chromatography (2.2.28).

Gas to be examined. The substance to be examined.

Reference gas (a). Use ambient air.

Reference gas (b). Use *nitrogen R1*.

Column:

- *material:* stainless steel,
- *size:* *l* = 2 m, Ø = 2 mm,
- *stationary phase:* appropriate molecular sieve for chromatography (0.5 nm).

Carrier gas: *helium for chromatography R*.

Flow rate: 40 ml/min.

Temperature:

- *column:* 50 °C,
- *detector:* 130 °C.

Detection: thermal conductivity.

System suitability: reference gas (a): adjust the injected volumes and operating conditions so that the height of the peak due to nitrogen in the chromatogram obtained is at least 35 per cent of the full scale of the recorder:

- the chromatogram obtained shows a clear separation of oxygen and nitrogen.

Limit:

- *total:* not more than 0.5 per cent of the sum of the areas of all the peaks (0.5 per cent V/V).

IDENTIFICATION

First identification: A.

Second identification: B, C.

- A. Examine the chromatograms obtained in the test for impurities (see Production).

Results: the principal peak in the chromatogram obtained with the gas to be examined is similar in retention time to the principal peak in the chromatogram obtained with reference gas (b).

- B. In a 250 ml conical flask replace the air by the gas to be examined. Place a burning or glowing splinter of wood in the flask. The splinter is extinguished.
- C. In a suitable test tube, place 0.1 g of *magnesium R* in turnings. Close the tube with a two-hole stopper fitted with a glass tube reaching about 1 cm above the turnings. Pass the gas to be examined through the glass tube for 1 min without heating, then for 15 min while heating the test tube to a red glow. After cooling, add 5 ml of *dilute sodium hydroxide solution R*. The evolving vapours turn the colour of moistened *red litmus paper R* blue.

STORAGE

Where the gas has to be stored, store as a compressed gas or a liquid in appropriate containers complying with the legal regulations.

IMPURITIES

- A. oxygen,
- B. argon.

01/2005:0416

NITROUS OXIDE

Dinitrogenii oxidum

N₂OM_r 44.01

DEFINITION

Content: minimum 98.0 per cent V/V of N₂O in the gaseous phase, when sampled at 15 °C.

This monograph applies to nitrous oxide for medicinal use.

CHARACTERS

Appearance: colourless gas.

Solubility: at 20 °C and at a pressure of 101 kPa, 1 volume dissolves in about 1.5 volumes of water.

PRODUCTION

Nitrous oxide is produced from ammonium nitrate by thermic decomposition.

Examine the gaseous phase.

If the test is performed on a cylinder, keep the cylinder at room temperature for at least 6 h before carrying out the tests. Keep the cylinder in the vertical position with the outlet valve uppermost.

Carbon dioxide. Gas chromatography (2.2.28).

Gas to be examined. The substance to be examined.

Reference gas. A mixture containing 300 ppm V/V of carbon dioxide R1 in nitrous oxide R.

Column:

- **material:** stainless steel,
- **size:** $l = 3.5$ m, $\varnothing = 2$ mm,
- **stationary phase:** ethylvinylbenzene-divinylbenzene copolymer R.

Carrier gas: helium for chromatography R.

Flow rate: 15 ml/min.

Temperature:

- **column:** 40 °C,
- **detector:** 90 °C.

Detection: thermal conductivity.

Injection: loop injector.

Adjust the injected volumes and operating conditions so that the height of the peak due to carbon dioxide in the chromatogram obtained with the reference gas is at least 35 per cent of the full scale of the recorder. The test is not valid unless the chromatograms obtained show a clear separation of carbon dioxide from nitrous oxide.

Limit:

- **carbon dioxide:** not more than the area of the corresponding peak in the chromatogram obtained with the reference gas (300 ppm V/V).

Carbon monoxide. Gas chromatography (2.2.28). *When the test is carried out on a cylinder, use the first portion of gas to be withdrawn.*

Gas to be examined. The substance to be examined.

Reference gas. A mixture containing 5 ppm V/V of carbon monoxide R in nitrous oxide R.

Column:

- **material:** stainless steel,
- **size:** $l = 2$ m, $\varnothing = 4$ mm,
- **stationary phase:** suitable molecular sieve for chromatography (0.5 nm).

Carrier gas: helium for chromatography R.

Flow rate: 60 ml/min.

Temperature:

- **column:** 50 °C,
- **injection port and detector:** 130 °C.

Detection: flame ionisation with methaniser.

Injection: loop injector.

Adjust the injected volumes and the operating conditions so that the height of the peak due to carbon monoxide in the chromatogram obtained with the reference gas is at least 35 per cent of the full scale of the recorder.

Limit:

- **carbon monoxide:** not more than the area of the corresponding peak in the chromatogram obtained with the reference gas (5 ppm V/V).

Nitrogen monoxide and nitrogen dioxide: maximum

2 ppm V/V in total in the gaseous and liquid phases, determined using a chemiluminescence analyser (2.5.26).

Gas to be examined. The substance to be examined.

Reference gas (a). Nitrous oxide R.

Reference gas (b). A mixture containing 2 ppm V/V of nitrogen monoxide R in nitrogen R1.

Calibrate the apparatus and set the sensitivity using reference gases (a) and (b). Measure the content of nitrogen monoxide and nitrogen dioxide, separately examining the samples collected from the gaseous phase and the liquid phase of the gas to be examined.

Multiply the result obtained by the quenching correction factor in order to correct the quenching effect on the analyser response caused by the nitrous oxide matrix effect.

The quenching correction factor is determined by applying a known reference mixture of nitrogen monoxide in nitrous oxide and comparing the actual content with the content indicated by the analyser which has been calibrated with a NO/N₂ reference mixture.

$$\text{Quenching correction factor} = \frac{\text{actual nitrogen monoxide content}}{\text{indicated nitrogen monoxide content}}$$

Water: maximum 67 ppm V/V, determined using an electrolytic hygrometer (2.5.28).

Assay. Gas chromatography (2.2.28).

Gas to be examined. The substance to be examined.

Reference gas. Nitrous oxide R.

Column:

- **material:** stainless steel,
- **size:** $l = 2$ m, $\varnothing = 2$ mm,
- **stationary phase:** silica gel for chromatography R (250-355 μm).

Carrier gas: helium for chromatography R.

Flow rate: 50 ml/min.

Temperature:

- **column and injection port:** 60 °C,

– detector: 130 °C.

Detection: thermal conductivity.

Injection: loop injector.

Adjust the injected volumes and the operating conditions so that the height of the peak due to nitrous oxide in the chromatogram obtained with the reference gas is at least 35 per cent of the full scale of the recorder.

The area of the peak due to nitrous oxide in the chromatogram obtained with the gas to be examined is at least 98.0 per cent of the area of the peak due to nitrous oxide in the chromatogram obtained with the reference gas.

IDENTIFICATION

First identification: A.

Second identification: B, C.

A. Infrared absorption spectrophotometry (2.2.24).

Comparison: Ph. Eur. reference spectrum of nitrous oxide.

B. Place a glowing splinter of wood in the substance to be examined. The splinter bursts into flame.

C. Introduce the substance to be examined into *alkaline pyrogallol solution R*. A brown colour does not develop.

TESTS

Examine the gaseous phase.

If the test is performed on a cylinder, keep the cylinder of the substance to be examined at room temperature for at least 6 h before carrying out the tests. Keep the cylinder in the vertical position with the outlet valve uppermost.

Carbon dioxide: maximum 300 ppm V/V, determined using a carbon dioxide detector tube (2.1.6).

Carbon monoxide: maximum 5 ppm V/V, determined using a carbon monoxide detector tube (2.1.6). When the test is carried out on a cylinder, use the first portion of the gas to be withdrawn.

Nitrogen monoxide and nitrogen dioxide: maximum 2 ppm V/V, determined using a nitrogen monoxide and nitrogen dioxide detector tube (2.1.6).

Water vapour: maximum 67 ppm V/V, determined using a water vapour detector tube (2.1.6).

STORAGE

Store liquefied under pressure in suitable containers complying with the legal regulations. The taps and valves are not greased or oiled.

IMPURITIES

A. carbon dioxide,

B. carbon monoxide,

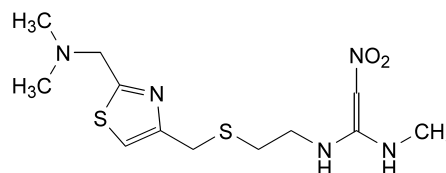
C. nitrogen monoxide,

D. nitrogen dioxide,

E. water.

NIZATIDINE

Nizatidinum



$C_{12}H_{21}N_5O_2S_2$

M_r 331.5

DEFINITION

Nizatidine contains not less than 97.0 per cent and not more than the equivalent of 101.0 per cent of (EZ)-N-[2-[[[2-[(dimethylamino)methyl]thiazol-4-yl]methyl]sulphanyl]ethyl]-N'-methyl-2-nitroethene-1,1-diamine, calculated with reference to the dried substance.

CHARACTERS

An almost white or slightly brownish, crystalline powder, sparingly soluble in water, soluble in methanol.

IDENTIFICATION

First identification: C.

Second identification: A, B, D.

A. Melting point (2.2.14): 131 °C to 134 °C.

B. Dissolve 0.10 g of the substance to be examined in *methanol R* and dilute to 100.0 ml with the same solvent. Dilute 2.0 ml of the solution to 100.0 ml with *methanol R*. Examined between 220 nm and 350 nm (2.2.25), the solution shows two absorption maxima, at 242 nm and 325 nm. The ratio of the absorbance measured at the maximum at 325 nm to that measured at the maximum at 242 nm is 2.2 to 2.5.

C. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *nizatidine CRS*. Examine the substance prepared as discs.

D. Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel plate R*.

Test solution. Dissolve 50 mg of the substance to be examined in *methanol R* and dilute to 10 ml with the same solvent.

Reference solution (a). Dissolve 50 mg of *nizatidine CRS* in *methanol R* and dilute to 10 ml with the same solvent.

Reference solution (b). Dissolve 50 mg of *nizatidine CRS* and 50 mg of *ranitidine hydrochloride CRS* in *methanol R* and dilute to 10 ml with the same solvent.

Apply to the plate 5 µl of each solution. Develop over a path corresponding to two thirds of the height of the plate using a mixture of 2 volumes of *water R*, 4 volumes of *concentrated ammonia R1*, 15 volumes of *2-propanol R* and 25 volumes of *ethyl acetate R*. Allow the plate to dry in air and expose to iodine vapour until the spots are clearly visible. Examine in daylight. The principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a). The test is not valid unless the chromatogram obtained with reference solution (b) shows two clearly separated spots.

TESTS

Appearance of solution. Dissolve 0.2 g of the substance to be examined in a 10 g/l solution of *hydrochloric acid R* and dilute to 20 ml with the same acid solution. The solution is clear (2.2.1) and not more intensely coloured than reference solution Y₅ (2.2.2, *Method II*).

pH (2.2.3). Dissolve 0.2 g of the substance to be examined in *carbon dioxide-free water R* and dilute to 20 ml with the same solvent. The pH of the solution is 8.5 to 10.0.

Related substances. Examine by liquid chromatography (2.2.29) as described under Assay, replacing the mixture of mobile phases by the following elution programme:

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)	Comment
0 - 3	76	24	isocratic
3 - 20	76 → 50	24 → 50	linear gradient
20 - 45	50	50	isocratic
45 - 50	50 → 76	50 → 24	linear gradient
50 - 60	76	24	re-equilibration

Inject 20 µl of reference solution (a). Adjust the sensitivity of the system so that the height of the principal peak in the chromatogram obtained with the reference solution is at least 50 per cent of the full scale of the recorder. The test is not valid unless the retention time of nizatidine is between 10 min and 20 min and the symmetry factor of the peak due to nizatidine is not greater than 2.0. Inject 20 µl of reference solution (c). The test is not valid unless the resolution between the peak due to nizatidine (first peak) and impurity F (second peak) is at least 2.0.

Inject 20 µl of test solution (a). In the chromatogram obtained, the area of any peak apart from the principal peak, is not greater than 0.3 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.3 per cent) and the sum of the areas of all these peaks is not greater than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (1.5 per cent). Disregard any peak with an area less than 0.03 times the area of the principal peak in the chromatogram obtained with reference solution (a).

Heavy metals (2.4.8). 1.0 g complies with limit test C for heavy metals (20 ppm). Prepare the standard using 2 ml of *lead standard solution* (10 ppm Pb) *R*.

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Examine by liquid chromatography (2.2.29).

Test solution (a). Dissolve 50.0 mg of the substance to be examined in a mixture of 24 volumes of mobile phase B and 76 volumes of mobile phase A and dilute to 10.0 ml with the same mixture of mobile phases.

Test solution (b). Dissolve 15.0 mg of the substance to be examined in a mixture of 24 volumes of mobile phase B and 76 volumes of mobile phase A and dilute to 50.0 ml with the same mixture of mobile phases.

Reference solution (a). Dilute 1.0 ml of test solution (a) to 100.0 ml with a mixture of 24 volumes of mobile phase B and 76 volumes of mobile phase A.

Reference solution (b). Dissolve 15.0 mg of *nizatidine CRS* in a mixture of 24 volumes of mobile phase B and 76 volumes of mobile phase A and dilute to 50.0 ml with the same mixture of mobile phases.

Reference solution (c). Dissolve 5 mg of *nizatidine CRS* and 0.5 mg of *nizatidine impurity F CRS* in a mixture of 24 volumes of mobile phase B and 76 volumes of mobile phase A and dilute to 100.0 ml with the same mixture of mobile phases.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4.6 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (5 µm),
- as mobile phase at a flow rate of 1.0 ml/min a mixture of 35 volumes of mobile phase B and 65 volumes of mobile phase A:

Mobile phase A. Dissolve 5.9 g of *ammonium acetate R* in 760 ml of *water R*, add 1 ml of *diethylamine R*, and adjust the pH to 7.5 with *acetic acid R*,

Mobile phase B. Methanol R,

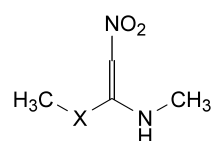
- as detector a spectrophotometer set at 254 nm.

Inject 20 µl of reference solution (b). The test is not valid unless the retention time of nizatidine is between 8 min and 10 min and the symmetry of the peak due to nizatidine is not greater than 2.0.

Inject reference solution (b) six times. The test is not valid unless the relative standard deviation of the peak area for nizatidine is at most 2.0 per cent.

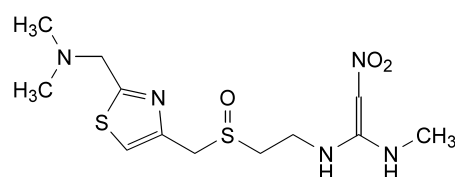
Inject 20 µl of test solution (b) and 20 µl of reference solution (b). Calculate the percentage content of nizatidine from the areas of the peaks and the declared content of *nizatidine CRS*.

IMPURITIES

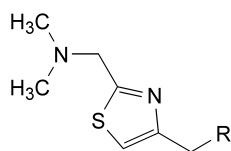


A. X = NH: *N,N'*-dimethyl-2-nitroethene-1,1-diamine,

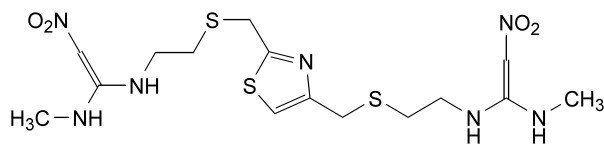
B. X = S: (*EZ*)-*N*-methyl-1-(methylsulphonyl)-2-nitroethen-1-amine,



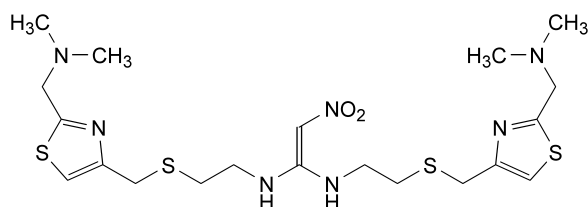
C. (*EZ*)-*N*-[2-[[2-[(dimethylamino)methyl]thiazol-4-yl]methyl]sulphonyl]ethyl]-*N'*-methyl-2-nitroethene-1,1-diamine,



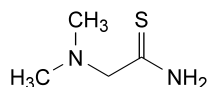
- D. $R = S-CH_2-CH_2-NH_2$: 2-[[2-[(dimethylamino)methyl]thiazol-4-yl]methyl]sulphanyl]ethanamine,
- E. $R = S-CH_2-CH_2-NH-CO-CH_2-NO_2$: *N*-[2-[[2-[(dimethylamino)methyl]thiazol-4-yl]methyl]sulphanyl]ethyl]-2-nitroacetamide,
- I. $R = S-CH_2-CH_2-NH-CO-NH-CH_3$: *N*-[2-[[2-[(dimethylamino)methyl]thiazol-4-yl]methyl]sulphanyl]ethyl]-*N'*-methylurea,
- J. $R = OH$: [2-[(dimethylamino)methyl]thiazol-4-yl]methanol,



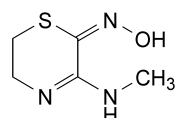
- F. (*EZ*)-*N*¹,*N*^{1'}-[thiazole-2,4-diylbis(methylenesulphanediyl-ethylene)]bis(*N'*-methyl-2-nitroethene-1,1-diamine),



- G. *N,N'*-bis[2-[[2-[(dimethylamino)methyl]thiazol-4-yl]methyl]sulphanyl]ethyl]-2-nitroethene-1,1-diamine,



- H. 2-(dimethylamino)thioacetamide,



- K. 3-(methylamino)-5,6-dihydro-2*H*-1,4-thiazin-2-one oxime.

DEFINITION

Nomegestrol acetate contains not less than 97.0 per cent and not more than the equivalent of 103.0 per cent of 6-methyl-3,20-dioxo-19-norpregna-4,6-dien-17-yl acetate, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, crystalline powder, practically insoluble in water, freely soluble in acetone, soluble in alcohol.

IDENTIFICATION

Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *nomegestrol acetate CRS*.

TESTS

Appearance of solution. Dissolve 1.0 g in *methylene chloride R* and dilute to 10 ml with the same solvent. The solution is clear (2.2.1) and not more intensely coloured than reference solution Y₅ (2.2.2, *Method II*).

Specific optical rotation (2.2.7). Dissolve 0.500 g in *ethanol R* and dilute to 25.0 ml with the same solvent. The specific optical rotation is -60.0 to -64.0 , calculated with reference to the dried substance.

Related substances. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 25.0 mg of the substance to be examined in *methanol R* and dilute to 50.0 ml with the same solvent.

Reference solution (a). Dilute 1.0 ml of the test solution to 200.0 ml with the mobile phase.

Reference solution (b). Dissolve 25.0 mg of *nomegestrol acetate impurity A CRS* in *methanol R* and dilute to 50.0 ml with the same solvent.

Reference solution (c). Dissolve 25.0 mg of *nomegestrol acetate CRS* in 20 ml of *methanol R*, add 0.25 ml of reference solution (b) and dilute to 50.0 ml with the mobile phase.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4.6 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (5 μ m),
- as mobile phase at a flow rate of 1.3 ml/min a mixture of 24 volumes of *acetonitrile R*, 38 volumes of *methanol R* and 38 volumes of *water R*,
- as detector, a variable wavelength spectrophotometer capable of operating at 245 nm and at 290 nm.

Inject 10 μ l of reference solution (c) and record the chromatogram with the detector set at 245 nm.

When the chromatogram is recorded in the prescribed conditions, the retention times are: nomegestrol acetate about 17 min and impurity A about 18.5 min. Adjust the sensitivity of the system at 245 nm so that the height of the peak due to impurity A in the chromatogram obtained with reference solution (c) is at least 50 per cent of the full scale of the recorder.

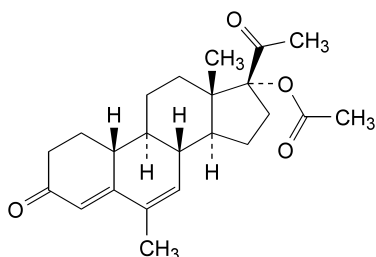
Measure the height H_p above the baseline of the peak due to impurity A and the height H_r above the baseline of the lowest point of the curve separating this peak from the peak due to nomegestrol acetate. The test is not valid unless H_p is greater than 5 times H_r .

Inject 10 μ l of reference solution (a) and record the chromatogram with the detector set at 290 nm. Adjust the sensitivity of the system at 290 nm so that the height of the

01/2005:1551

NOMEGESTROL ACETATE

Nomegestroli acetat

C₂₃H₃₀O₄M_r 370.5

principal peak in the chromatogram obtained with reference solution (a) is at least 50 per cent of the full scale of the recorder.

Inject 10 µl of the test solution and record the chromatograms at 245 nm and 290 nm for 1.5 times the retention time of the principal peak.

In the chromatogram obtained with the test solution at 290 nm: the area of any peak, apart from the principal peak, is not greater than 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent). Disregard any peak with an area less than 0.04 times that of the principal peak in the chromatogram obtained with reference solution (a) (0.02 per cent). In the chromatogram obtained with the test solution at 245 nm: the area of any peak corresponding to impurity A is not greater than 0.4 times the area of the peak due to impurity A in the chromatogram obtained with reference solution (c) (0.2 per cent); the area of any peak, apart from the principal peak and any peak corresponding to impurity A, is not greater than 0.2 times the area of the peak due to impurity A in the chromatogram obtained with reference solution (c) (0.1 per cent). Disregard any peak with an area less than 0.1 times that of the peak due to impurity A in the chromatogram obtained with reference solution (c) (0.05 per cent).

In the chromatograms obtained at 290 nm and 245 nm, the sum of the related substances apart from impurity A is not greater than 0.3 per cent.

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 100-105 °C.

ASSAY

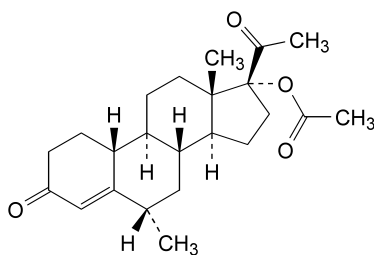
Dissolve 50.0 mg in *ethanol R* and dilute to 100.0 ml with the same solvent. Dilute 2.0 ml of the solution to 100.0 ml with *ethanol R*. Measure the absorbance (2.2.25) at the maximum at 287 nm.

Calculate the content of $C_{23}H_{30}O_4$ taking the specific absorbance to be 685.

STORAGE

Store protected from light.

IMPURITIES



A. 6α-methyl-3,20-dioxo-19-norpregn-4-en-17-yl acetate.

01/2005:1454

NONOXINOL 9

Nonoxinolum 9

DEFINITION

α-(4-Nonylphenyl)-ω-hydroxynona(oxyethylene).

Mixture consisting mainly of monononylphenyl ethers of macrogols corresponding to the formula: $C_9H_{19}C_6H_4-[OCH_2CH_2]_n-OH$ where the average value of n is 9. It may contain free macrogols.

CHARACTERS

Appearance: clear, colourless or light yellow, viscous liquid.

Solubility: miscible with water, with alcohol and with vegetable oils.

IDENTIFICATION

A. Infrared absorption spectrophotometry (2.2.24).

Comparison: *Ph. Eur. reference spectrum of nonoxinol 9.*

Preparation: film between *sodium chloride R* plates.

B. It complies with the test for cloud point (see Tests).

TESTS

Acidity or alkalinity. Boil 1.0 g with 20 ml of *carbon dioxide-free water R* for 1 min, with constant stirring. Cool and filter. To 10 ml of the filtrate, add 0.05 ml of *bromothymol blue solution R1*. Not more than 0.5 ml of 0.01 M *hydrochloric acid* or 0.01 M *sodium hydroxide* is required to change the colour of the indicator.

Hydroxyl value (2.5.3, *Method A*): 84 to 94.

Cloud point: 52 °C to 58 °C.

Dissolve 1.0 g in 99 g of *water R*. Transfer about 30 ml of this solution into a test-tube, heat on a water-bath and stir continuously until the solution becomes cloudy. Remove the test-tube from the water-bath (ensuring that the temperature does not increase more than 2 °C) and continue to stir. The cloud point is the temperature at which the solution becomes sufficiently clear that the entire thermometer bulb is plainly seen.

Ethylene oxide and dioxan (2.4.25): maximum 1 ppm of ethylene oxide and maximum 10 ppm of dioxan.

Heavy metals (2.4.8): maximum 10 ppm.

Dissolve 2.0 g in *distilled water R* and dilute to 20.0 ml with the same solvent. 12 ml of this solution complies with limit test A. Prepare the standard using *lead standard solution (1 ppm Pb) R*.

Water (2.5.12): maximum 0.5 per cent, determined on 2.00 g.

Total ash (2.4.16): maximum 0.4 per cent, determined on 1.0 g.

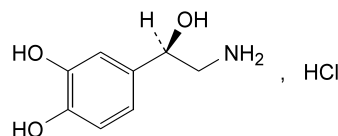
STORAGE

In an airtight container.

01/2005:0732

NORADRENALINE HYDROCHLORIDE

Noradrenalini hydrochloridum



$C_8H_{12}ClNO_3$

M_r 205.6

DEFINITION

(*R*)-2-Amino-1-(3,4-dihydroxyphenyl)ethanol hydrochloride.

Content: 98.5 per cent to 101.0 per cent (anhydrous substance).

CHARACTERS

Appearance: white or brownish-white, crystalline powder.

Solubility: very soluble in water, slightly soluble in alcohol. It becomes coloured on exposure to air and light.

IDENTIFICATION

- A. Specific optical rotation (see Tests).
 B. Infrared absorption spectrophotometry (2.2.24).

Dissolve 2 g in 20 ml of a 5 g/l solution of *sodium metabisulphite R* and make alkaline by addition of *ammonia R*. Keep in iced water for 1 h and filter. Wash the precipitate with 3 quantities, each of 2 ml, of *water R*, with 5 ml of *alcohol R* and finally with 5 ml of *ether R* and dry *in vacuo* for 3 h. Examine the noradrenaline base thus prepared, comparing with the spectrum obtained with noradrenaline base prepared by the same method from a suitable amount of *noradrenaline tartrate CRS*. Examine the substances prepared as discs.

- C. 0.2 ml of solution S (see Tests) gives reaction (a) of chlorides (2.3.1).

TESTS

Solution S. Dissolve 0.500 g in *carbon dioxide-free water R* and dilute to 25.0 ml with the same solvent.

Appearance of solution. The solution is clear (2.2.1) and not more intensely coloured than a mixture of 0.2 ml of blue primary solution, 0.4 ml of yellow primary solution, 0.4 ml of red primary solution and 9 ml of a 13.7 per cent V/V solution of *dilute hydrochloric acid R* (2.2.2, Method II).

Dissolve 0.2 g in *carbon dioxide-free water R* and dilute to 10 ml with the same solvent. Examine the solution immediately.

pH (2.2.3): 3.5 to 4.5 for solution S.

Specific optical rotation (2.2.7): –37 to –41, determined on solution S (anhydrous substance).

Noradrenalone: maximum 0.12 per cent.

Dissolve 30.0 mg in 0.01 M *hydrochloric acid* and dilute to 25.0 ml with the same acid. The absorbance (2.2.25) of the solution measured at 310 nm is not greater than 0.20.

Adrenaline. Thin-layer chromatography (2.2.27).

Test solution. Dissolve 0.15 g of the substance to be examined in *water R* and dilute to 10 ml with the same solvent. Prepare immediately before use.

Reference solution (a). Dissolve 12.5 mg of *adrenaline tartrate CRS* in *water R* and dilute to 10 ml with the same solvent. Prepare immediately before use.

Reference solution (b). Dilute 2 ml of reference solution (a) to 10 ml with *water R*.

Reference solution (c). Mix 2 ml of the test solution and 2 ml of reference solution (b).

Plate: TLC silica gel G plate R.

Mobile phase: *anhydrous formic acid R*, *acetone R*, *methylene chloride R* (0.5:50:50 V/V/V).

Application: apply as bands 20 mm by 2 mm, 6 µl of the test solution, 6 µl of reference solution (a), 6 µl of reference solution (b) and 12 µl of reference solution (c). Allow to dry in air and spray the bands with a saturated solution of *sodium hydrogen carbonate R*. Allow the plate to dry in air and spray the bands twice with *acetic anhydride R*, drying between the 2 sprayings. Heat the plate at 50 °C for 90 min.

Development: over a path of 15 cm.

Drying: in air.

Detection: spray with a solution freshly prepared by mixing 2 volumes of *ethylenediamine R* and 8 volumes of *methanol R* and adding 2 volumes of a 5 g/l solution of *potassium ferricyanide R*. Dry the plate at 60 °C for 10 min and examine in ultraviolet light at 254 nm and 365 nm.

System suitability: the chromatogram obtained with reference solution (c) shows above the most intense zone a clearly separated zone corresponding to the most intense zone in the chromatogram obtained with reference solution (a).

Limits: any zone situated immediately above the most intense zone is not more intense than the corresponding zone in the chromatogram obtained with reference solution (b) (1.0 per cent).

Water (2.5.12): maximum 0.5 per cent, determined on 1.000 g.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 0.50 g.

ASSAY

Dissolve 0.180 g in 50 ml of *acetic anhydride R* and add 10 ml of *anhydrous formic acid R*. Titrate with 0.1 M *perchloric acid*, determining the end-point potentiometrically (2.2.20). 1 ml of 0.1 M *perchloric acid* is equivalent to 20.56 mg of C₈H₁₂ClNO₃.

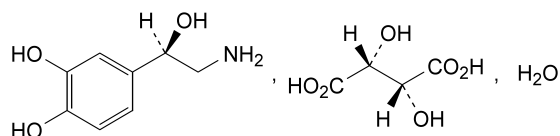
STORAGE

Store in an airtight container, or preferably in a sealed tube under vacuum or under an inert gas, protected from light.

01/2005:0285

NORADRENALINE TARTRATE

Noradrenalini tartras

C₁₂H₁₇NO₉·H₂OM_r 337.3

DEFINITION

(1R)-2-Amino-1-(3,4-dihydroxyphenyl)ethanol hydrogen (2R,3R)-2,3-dihydroxybutanedioate monohydrate.

Content: 98.5 per cent to 101.0 per cent (anhydrous substance).

CHARACTERS

Appearance: white or almost white, crystalline powder.

Solubility: freely soluble in water, slightly soluble in alcohol.

IDENTIFICATION

- A. Dissolve 2 g in 20 ml of a 5 g/l solution of *sodium metabisulphite R* and make alkaline by addition of *ammonia R*. Keep in iced water for 1 h and filter. Reserve the filtrate for identification test C. Wash the precipitate with 3 quantities, each of 2 ml, of *water R*, with 5 ml of *alcohol R* and finally with 5 ml of *ether R* and dry *in vacuo* for 3 h. The specific optical rotation (2.2.7) of the precipitate (noradrenaline base) is –44 to –48, determined using a 20.0 g/l solution in 0.5 M *hydrochloric acid*.
- B. Infrared absorption spectrophotometry (2.2.24).
 Use noradrenaline base prepared as described under identification test A and compare with the spectrum obtained with noradrenaline base prepared by the same method from a suitable amount of *noradrenaline tartrate CRS*. Examine the substances prepared as discs.
- C. 0.2 ml of the filtrate obtained in identification test A gives reaction (b) of tartrates (2.3.1).

TESTS

01/2005:0234

Appearance of solution. The solution is clear (2.2.1) and not more intensely coloured than reference solution BY₅ (2.2.2, Method II).

Dissolve 0.2 g in *water R* and dilute to 10 ml with the same solvent. Examine the solution immediately.

Noradrenalone: the absorbance (2.2.25) of the solution measured at 310 nm is not greater than 0.20.

Dissolve 50.0 mg in 0.01 M hydrochloric acid and dilute to 25.0 ml with the same acid.

Adrenaline. Thin-layer chromatography (2.2.27).

Test solution. Dissolve 0.25 g of the substance to be examined in *water R* and dilute to 10 ml with the same solvent. Prepare immediately before use.

Reference solution (a). Dissolve 12.5 mg of *adrenaline tartrate CRS* in *water R* and dilute to 10 ml with the same solvent. Prepare immediately before use.

Reference solution (b). Dilute 2 ml of reference solution (a) to 10 ml with *water R*.

Reference solution (c). Mix 2 ml of the test solution with 2 ml of reference solution (b).

Plate: TLC silica gel G plate *R*.

Mobile phase: anhydrous formic acid *R*, acetone *R*, methylene chloride *R* (0.5:50:50 V/V/V).

Application: apply as bands 20 mm by 2 mm, 6 µl of the test solution, 6 µl of reference solution (a), 6 µl of reference solution (b) and 12 µl of reference solution (c). Allow to dry in air and spray the bands with a saturated solution of sodium hydrogen carbonate *R*. Allow the plate to dry in air and spray the bands twice with acetic anhydride *R*, drying between the 2 sprayings. Heat the plate at 50 °C for 90 min.

Development: over a path of 15 cm.

Drying: in air.

Detection: spray with a solution freshly prepared by mixing 2 volumes of ethylenediamine *R* and 8 volumes of methanol *R* and adding 2 volumes of a 5 g/l solution of potassium ferricyanide *R*. Dry the plate at 60 °C for 10 min and examine in ultraviolet light at 254 nm and 365 nm.

System suitability: the chromatogram obtained with reference solution (c) shows above the most intense zone a clearly separated zone corresponding to the most intense zone in the chromatogram obtained with reference solution (a).

Limit:

- *adrenaline:* any zone situated immediately above the most intense zone is not more intense than the corresponding zone in the chromatogram obtained with reference solution (b) (1.0 per cent).

Water (2.5.12): 4.5 per cent to 5.8 per cent, determined on 0.500 g.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 0.5 g.

ASSAY

Dissolve 0.300 g in 50 ml of anhydrous acetic acid *R*, heating gently if necessary. Titrate with 0.1 M perchloric acid using 0.1 ml of crystal violet solution *R* as indicator, until a bluish-green colour is obtained.

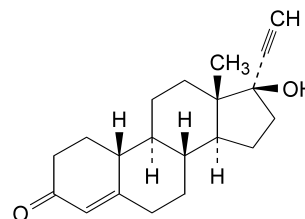
1 ml of 0.1 M perchloric acid is equivalent to 31.93 mg of C₁₂H₁₇NO₉.

STORAGE

In an airtight container, or preferably in a sealed tube under vacuum or under an inert gas, protected from light.

NORETHISTERONE

Norethisteronum

C₂₀H₂₆O₂M_r 298.4

DEFINITION

Norethisterone contains not less than 98.0 per cent and not more than the equivalent of 102.0 per cent of 17-hydroxy-19-nor-17α-pregn-4-en-20-yn-3-one, calculated with reference to the dried substance.

CHARACTERS

A white or yellowish-white, crystalline powder, practically insoluble in water, slightly soluble in alcohol.

It melts at about 206 °C, with decomposition.

IDENTIFICATION

First identification: A, B, E.

Second identification: B, C, D, E.

- Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *norethisterone CRS*. Examine the substances prepared in the form of discs. If the spectra obtained with the substance to be examined and the reference substance show differences, dissolve the substances in *chloroform R*, evaporate to dryness on a water-bath and then record the spectra.
- Examine by thin-layer chromatography (2.2.27), using *kieselguhr G R* as the coating substance. Impregnate the plate by placing it in a tank containing the necessary quantity of a mixture of 10 volumes of *formamide R* and 90 volumes of *acetone R* so that the plate dips about 5 mm into the liquid. When the front of the impregnation mixture has risen at least 1 cm above the level prescribed for the mobile phase, remove the plate and allow it to stand at room temperature until the solvent has completely evaporated (about 2 min to 5 min). Use the impregnated plate within 2 h and carry out the chromatography in the same direction as the impregnation.

Test solution. Dissolve 10 mg of the substance to be examined in *chloroform R* and dilute to 10 ml with the same solvent.

Reference solution. Dissolve 10 mg of *norethisterone CRS* in *chloroform R* and dilute to 10 ml with the same solvent.

Apply separately to the plate 2 µl of each solution. Develop over a path of 15 cm using a mixture of 20 volumes of *dioxan R* and 80 volumes of *hexane R*. Heat the plate at 120 °C for 15 min and spray with *alcoholic solution of sulphuric acid R*. Heat at 120 °C for 10 min to 15 min or until the spots appear. Allow to cool and examine in daylight and in ultraviolet light at 365 nm. The principal spot in the chromatogram obtained with the test solution is similar in position, colour, fluorescence and size to the principal spot in the chromatogram obtained with the reference solution.

- C. Dissolve about 2 mg in 2 ml of *alcohol R*, add 1 ml of *ammoniacal silver nitrate solution R* and heat on a water-bath. The solution becomes turbid and a white precipitate is formed which becomes grey when heated. A silver mirror is deposited on the walls of the tube.
- D. Dissolve about 2 mg in a cooled mixture of 2 ml of *ethanol R* and 2 ml of *sulphuric acid R* and heat to 70 °C. The resulting solution is dichroic, appearing blue-violet in transmitted light and red in reflected light. The solution shows a bright-red fluorescence in ultraviolet light at 365 nm.
- E. Dissolve about 2 mg in 2 ml of *alcohol R*, add 1 ml of a 10 g/l solution of *butylhydroxytoluene R* in *alcohol R* and 2 ml of 1 M *sodium hydroxide*. Heat in a water-bath at 80 °C for 30 min and cool to room temperature. A yellowish-pink colour is produced.

TESTS

Solution S. Dissolve 0.200 g in *dioxan R* and dilute to 10.0 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and not more intensely coloured than reference solution Y₆ (2.2.2, Method I).

Specific optical rotation (2.2.7). Dilute 5.0 ml of solution S to 10.0 ml with *dioxan R*. The specific optical rotation is –33 to –37, calculated with reference to the dried substance.

Absorbance (2.2.25). Dissolve 10.0 mg in *alcohol R* and dilute to 100.0 ml with the same solvent. Dilute 10.0 ml of this solution to 100.0 ml with *alcohol R*. The solution shows an absorption maximum at 240 nm. The specific absorbance at the maximum is 550 to 590, calculated with reference to the dried substance.

Related substances. Examine by thin-layer chromatography (2.2.27), using a suitable silica gel as the coating substance.

Test solution. Dissolve 50 mg of the substance to be examined in a mixture of 1 volume of *methanol R* and 9 volumes of *chloroform R* and dilute to 10 ml with the same mixture of solvents.

Reference solution (a). Dilute 1.0 ml of the test solution to 200 ml with a mixture of 1 volume of *methanol R* and 9 volumes of *chloroform R*.

Reference solution (b). Dissolve 25 mg of *ethisterone CRS* in a mixture of 1 volume of *methanol R* and 9 volumes of *chloroform R*, add 5 ml of the test solution and dilute to 100 ml with the same mixture of solvents.

Apply separately to the plate, as two applications of 5 µl, 10 µl of each solution. Develop over a path of 15 cm using a mixture of 10 volumes of *acetone R* and 90 volumes of *chloroform R*. Allow the plate to dry in air, spray with *alcoholic solution of sulphuric acid R* and heat at 100 °C to 105 °C for 5 min. Examine in ultraviolet light at 365 nm.

Any spot in the chromatogram obtained with the test solution, apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (a) (0.5 per cent). The test is not valid unless the chromatogram obtained with reference solution (b) shows two clearly separated spots of approximately equal intensity.

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.00 g by drying in an oven at 100 °C to 105 °C for 3 h.

ASSAY

Dissolve 0.200 g in 40 ml of *tetrahydrofuran R*. Add 10 ml of a 100 g/l solution of *silver nitrate R*. Using 2 ml of *bromocresol green solution R* as indicator, titrate with 0.1 M *sodium hydroxide* until a violet colour is obtained. Carry out a blank titration.

1 ml of 0.1 M *sodium hydroxide* is equivalent to 29.84 mg of C₂₀H₂₆O₂.

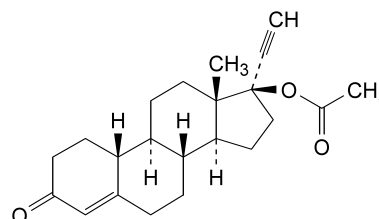
STORAGE

Store protected from light.

01/2005:0850

NORETHISTERONE ACETATE

Norethisteroni acetat

C₂₂H₂₈O₃M_r 340.5

DEFINITION

3-Oxo-19-nor-17α-pregn-4-en-20-yn-17-yl acetate.

Content: 98.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

Appearance: white or yellowish-white, crystalline powder.

Solubility: practically insoluble in water, freely soluble in methylene chloride, soluble in alcohol.

It shows polymorphism.

IDENTIFICATION

Infrared absorption spectrophotometry (2.2.24).

Preparation: discs.

Comparison: *norethisterone acetate CRS*.

If the spectra show differences, dissolve the substance to be examined and the reference substance separately in *methylene chloride R*, evaporate to dryness on a water-bath and record new spectra using the residues.

TESTS

Specific optical rotation (2.2.7) –30 to –35 (dried substance).

Dissolve 0.500 g in *ethanol R* and dilute to 25.0 ml with the same solvent.

Related substances. Liquid chromatography (2.2.29).

Test solution. Dissolve 25.0 mg of the substance to be examined in the mobile phase and dilute to 10.0 ml with the mobile phase.

Reference solution (a). Dissolve 2 mg of *desoxycortone acetate CRS* and 2 mg of *norethisterone acetate CRS* in the mobile phase and dilute to 50.0 ml with the mobile phase.

Reference solution (b). Dilute 1.0 ml of the test solution to 100.0 ml with the mobile phase.

Column:

- size: *l* = 0.25 m, Ø = 4.6 mm,
- stationary phase: octadecylsilyl silica gel for chromatography R (5 µm).

Mobile phase: acetonitrile *R*, water *R* (60:40 *V/V*).

Flow rate: 1.0 ml/min.

Detection: variable wavelength spectrophotometer capable of operating at 254 nm and at 210 nm.

Injection: 20 µl.

Run time: 3 times the retention time of norethisterone acetate.

Relative retention with reference to norethisterone acetate (retention time = about 10 min): impurity A = about 0.48; impurity D = about 0.65; impurity E = about 0.83; impurity C = about 1.35; impurity B = about 1.40.

System suitability: reference solution (a) at 254 nm:

- resolution: minimum of 3.5 between the peaks due to norethisterone acetate and to desoxycortone acetate.

Limits: spectrophotometer at 254 nm:

- any impurity: not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent),
- total: not more than 0.75 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.75 per cent),
- disregard limit: 0.05 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

Limits: spectrophotometer at 210 nm:

- any impurity with a relative retention between 1.0 and 1.6, with reference to norethisterone acetate (retention time = about 10 min): not more than 0.3 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.3 per cent),
- total of these impurities: not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent),
- disregard limit: 0.05 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

Loss on drying (2.2.32): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 100–105 °C.

ASSAY

Dissolve 0.200 g in 40 ml of *tetrahydrofuran R*. Add 10 ml of a 100 g/l solution of *silver nitrate R* and titrate with 0.1 *M* sodium hydroxide, determining the end-point potentiometrically (2.2.20). Carry out a blank titration.

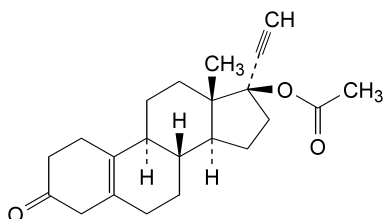
1 ml of 0.1 *M* sodium hydroxide is equivalent to 34.05 mg of $C_{22}H_{28}O_3$.

IMPURITIES

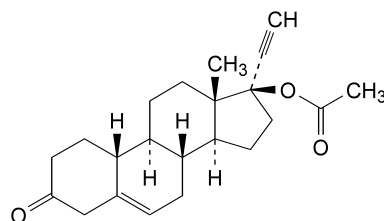
Specified impurities: A, B, C, D, E.

Other detectable impurities: F, G.

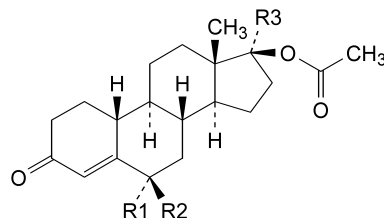
A. norethisterone,



B. 3-oxo-19-nor-17 α -pregn-5(10)-en-20-yn-17-yl acetate,



C. 3-oxo-19-nor-17 α -pregn-5-en-20-yn-17-yl acetate,



D. R1 = H, R2 = CO-CH₃, R3 = C $\equiv$ CH: 6 β -acetyl-3-oxo-19-nor-17 α -pregn-4-en-20-yn-17-yl acetate,

E. R1 = R2 = H, R3 = CO-CH₃: 3,20-dioxo-19-nor-17 α -pregn-4-en-17-yl acetate,

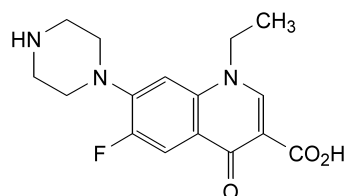
F. R1 = H, R2 = OH, R3 = C $\equiv$ CH: 6 β -hydroxy-3-oxo-19-nor-17 α -pregn-4-en-20-yn-17-yl acetate,

G. R1 + R2 = O, R3 = C $\equiv$ CH: 3,6-dioxo-19-nor-17 α -pregn-4-en-20-yn-17-yl acetate.

01/2005:1248

NORFLOXACIN

Norfloxacinum



$C_{16}H_{18}FN_3O_3$

M_r 319.3

DEFINITION

Norfloxacin contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of 1-ethyl-6-fluoro-4-oxo-7-(piperazin-1-yl)-1,4-dihydroquinoline-3-carboxylic acid, calculated with reference to the dried substance.

CHARACTERS

A white or pale-yellow, hygroscopic, photosensitive, crystalline powder, very slightly soluble in water, slightly soluble in acetone and in alcohol.

IDENTIFICATION

Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *norfloxacin CRS*. Examine the substances prepared as discs.

TESTS

Appearance of solution. Dissolve 0.5 g in a previously filtered 4 g/l solution of *sodium hydroxide R* in *methanol R* and dilute to 50 ml with the same solution. The solution is not more opalescent than reference suspension II (2.2.1) and not more intensely coloured than reference solution B₇ (2.2.2, *Method II*).

01/2005:0940

Related substances. Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel GF₂₅₄* plate *R*, previously washed with *methanol R* and dried in air.

Test solution (a). Dissolve 40 mg of the substance to be examined in a mixture of equal volumes of *methanol R* and *methylene chloride R* and dilute to 5 ml with the same mixture of solvents.

Test solution (b). Dilute 1 ml of test solution (a) to 10 ml with a mixture of equal volumes of *methanol R* and *methylene chloride R*.

Reference solution (a). Dilute 1 ml of test solution (b) to 50 ml with a mixture of equal volumes of *methanol R* and *methylene chloride R*.

Reference solution (b). Dissolve 4.0 mg of *norfloxacin impurity A CRS* in a mixture of equal volumes of *methanol R* and *methylene chloride R* and dilute to 5 ml with the same mixture of solvents. Dilute 1 ml of this solution to 2 ml with test solution (b).

Apply to the plate 5 µl of test solution (a) and 5 µl of each reference solution. Develop over a path of 18 cm using a mixture of 8 volumes of *water R*, 14 volumes of *diethylamine R*, 20 volumes of *toluene R*, 40 volumes of *chloroform R* and 40 volumes of *methanol R*. Dry the plate in a current of air and examine in ultraviolet light at 254 nm and then 365 nm. Any spot in the chromatogram obtained with test solution (a), apart from the principal spot, is not more intense than the principal spot in the chromatogram obtained with reference solution (a) (0.2 per cent) and there are no more than three such spots. The test is not valid unless, in the chromatogram obtained with reference solution (b), the ratio of the *R_f* value of impurity A to the *R_f* value of norfloxacin is at least 1.2.

Heavy metals (2.4.8). 2.0 g complies with limit test D for heavy metals (15 ppm). Prepare the standard using 3 ml of *lead standard solution (10 ppm Pb) R*.

Loss on drying (2.2.32). Not more than 1.0 per cent, determined on 1.000 g by drying in an oven at 100-105 °C under high vacuum for 2 h.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g in a platinum crucible.

ASSAY

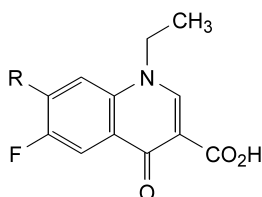
Dissolve 0.240 g in 80 ml of *anhydrous acetic acid R*. Titrate with 0.1 M *perchloric acid*, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *perchloric acid* is equivalent to 31.93 mg of C₁₆H₁₈FN₃O₃.

STORAGE

Store in an airtight container, protected from light.

IMPURITIES

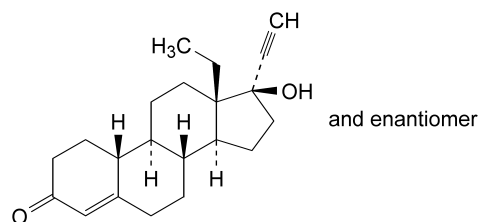


A. R = Cl: 7-chloro-1-ethyl-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylic acid,

B. R = NH-CH₂-CH₂-NH₂: 7-[(2-aminoethyl)amino]-1-ethyl-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylic acid.

NORGESTREL

Norgestrelum

C₂₁H₂₈O₂*M_r* 312.5

DEFINITION

Norgestrel contains not less than 98.0 per cent and not more than the equivalent of 102.0 per cent of *rac*-13-ethyl-17-hydroxy-18,19-dinor-17α-pregn-4-en-20-yn-3-one, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, crystalline powder, practically insoluble in water, sparingly soluble in methylene chloride, slightly soluble in alcohol.

IDENTIFICATION

- Dissolve 0.5 g in *methylene chloride R* and dilute to 10.0 ml with the same solvent. The angle of optical rotation (2.2.7) is + 0.05° to - 0.05°.
- Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *norgestrel CRS*.

TESTS

Related substances. Examine by thin-layer chromatography (2.2.27), using *silica gel G R* as the coating substance.

Test solution. Dissolve 0.2 g of the substance to be examined in *methylene chloride R* and dilute to 10 ml with the same solvent.

Reference solution (a). Dilute 1 ml of the test solution to 10 ml with *methylene chloride R*. Dilute 1 ml of this solution to 20 ml with *methylene chloride R*.

Reference solution (b). Dilute 4 ml of reference solution (a) to 10 ml with *methylene chloride R*.

Reference solution (c). Dissolve 5 mg of *norgestrel CRS* and 5 mg of *ethinylestradiol CRS* in *methylene chloride R* and dilute to 50 ml with the same solvent.

Apply to the plate 10 µl of each solution. Develop over a path of 15 cm using a mixture of 20 volumes of *ethyl acetate R* and 80 volumes of *methylene chloride R*. Allow the plate to dry in air, spray with a 100 g/l solution of *phosphomolybdic acid R* in *alcohol R*, heat at 100-105 °C for 15 min and examine immediately. Any spot in the chromatogram obtained with the test solution, apart from the principal spot, is not more intense than the principal spot in the chromatogram obtained with reference solution (a) (0.5 per cent) and at most two such spots are more intense than the spot in the chromatogram obtained with reference solution (b) (0.2 per cent). The test is not valid unless the chromatogram obtained with reference solution (c) shows two clearly separated spots.

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 100-105 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.200 g in 45 ml of *tetrahydrofuran R*. Add 10 ml of a 100 g/l solution of *silver nitrate R*. After 1 min, titrate with 0.1 M *sodium hydroxide* determining the end-point potentiometrically (2.2.20). Carry out a blank titration. 1 ml of 0.1 M *sodium hydroxide* is equivalent to 31.25 mg of $C_{21}H_{28}O_2$.

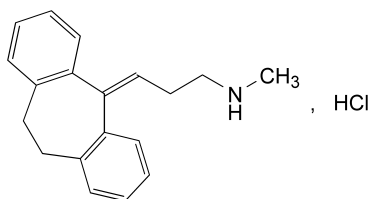
STORAGE

Store protected from light.

01/2005:0941

NORTRIPTYLINE HYDROCHLORIDE

Nortriptylini hydrochloridum

 $C_{19}H_{22}ClN$ M_r 299.8

DEFINITION

Nortriptyline hydrochloride contains not less than 98.0 per cent and not more than the equivalent of 101.0 per cent of 3-(10,11-dihydro-5H-dibenzo[a,d][7]annulen-5-ylidene)-N-methylpropan-1-amine hydrochloride, calculated with reference to the dried substance.

CHARACTERS

A white or almost white powder, sparingly soluble in water, soluble in alcohol and in methylene chloride.

IDENTIFICATION

First identification: C, E.

Second identification: A, B, D, E.

- Melting point (2.2.14): 216 °C to 220 °C.
- Dissolve 20.0 mg in *methanol R* and dilute to 100.0 ml with the same solvent. Dilute 5.0 ml of this solution to 100.0 ml with *methanol R*. Examined between 230 nm and 350 nm (2.2.25), the solution shows an absorption maximum at 239 nm. The specific absorbance at the maximum is 465 to 495.
- Examine by infrared absorption spectrophotometry (2.2.24), comparing with the *Ph. Eur. reference spectrum of nortriptyline hydrochloride*.
- Dissolve 50 mg in 3 ml of warm *water R*, cool and add 0.05 ml of a 25 g/l solution of *quinhydrone R* in *methanol R*. A red colour develops slowly.
- 50 mg gives reaction (b) of chlorides (2.3.1).

TESTS

Appearance of solution. Dissolve 0.5 g in *water R* with gentle heating and dilute to 25 ml with the same solvent. The solution is clear (2.2.1) and not more intensely coloured than reference solution B₇ (2.2.2, *Method II*).

Acidity or alkalinity. Dissolve 0.2 g with gentle heating in *carbon dioxide-free water R* and dilute to 10 ml with the same solvent. Add 0.1 ml of *methyl red solution R* and 0.2 ml of 0.01 M *sodium hydroxide*. The solution is yellow. Add 0.4 ml of 0.01 M *hydrochloric acid*. The solution is red.

Related substances. Prepare the solutions in subdued light and develop the chromatograms protected from light. Examine by thin-layer chromatography (2.2.27), using a TLC silica gel plate R.

Test solution (a). Dissolve 0.20 g of the substance to be examined in *alcohol R* and dilute to 10 ml with the same solvent.

Test solution (b). Dilute 1 ml of test solution (a) to 2 ml with *alcohol R*.

Reference solution (a). Dissolve 10 mg of *dibenzosuberone CRS* in *alcohol R* and dilute to 10 ml with the same solvent. Dilute 1 ml of the solution to 100 ml with *alcohol R*.

Reference solution (b). Dissolve 10 mg of *norcyclobenzaprine CRS* in *alcohol R* and dilute to 10 ml with the same solvent. Dilute 1 ml of the solution to 100 ml with *alcohol R*.

Reference solution (c). To 0.1 ml of test solution (b) add 10 ml of reference solution (b).

Apply to the plate 5 µl of each solution. Develop over a path of 15 cm in an unsaturated tank using a mixture of 3 volumes of *diethylamine R*, 15 volumes of *ethyl acetate R* and 85 volumes of *cyclohexane R*. Allow the plate to dry in air and spray with a freshly prepared mixture of 4 volumes of *formaldehyde solution R* and 96 volumes of *sulphuric acid R*. Examine immediately in ultraviolet light at 365 nm and then at 254 nm. In the chromatogram obtained with test solution (a): any spot corresponding to dibenzosuberone, is not more intense than the spot in the chromatogram obtained with reference solution (a) (0.05 per cent); and any spot in the chromatogram obtained with test solution (b), apart from the principal spot and any spot corresponding to dibenzosuberone, is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.1 per cent). The test is not valid unless the chromatogram obtained with reference solution (c) shows two clearly separated spots.

Heavy metals (2.4.8). 1.0 g complies with limit test C for heavy metals (20 ppm). Prepare the standard using 2 ml of *lead standard solution (10 ppm Pb) R*.

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 100-105 °C for 2 h.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

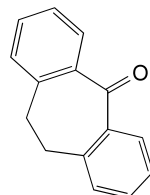
Dissolve 0.250 g in 30 ml of *alcohol R*. Add 1.0 ml of 0.1 M *hydrochloric acid*. Carry out a potentiometric titration (2.2.20), using 0.1 M *sodium hydroxide*. Read the volume added between the two points of inflexion.

1 ml of 0.1 M *sodium hydroxide* is equivalent to 29.98 mg of $C_{19}H_{22}ClN$.

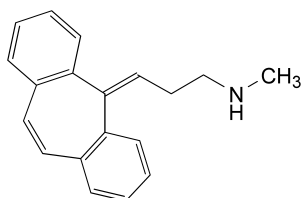
STORAGE

Store protected from light.

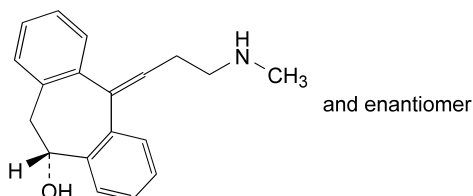
IMPURITIES



- A. 10,11-dihydro-5H-dibenzo[a,d][7]annulen-5-one (dibenzosuberone),



- B. 3-(5*H*-dibenzo[*a,d*][7]annulen-5-ylidene)-*N*-methylpropan-1-amine (norcyclobenzaprine),

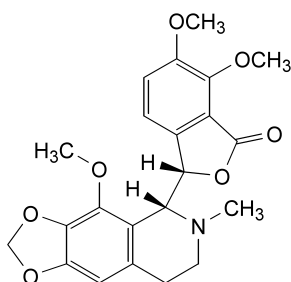


- C. 10,11-dihydro-5-[3-(methylamino)propylidene]-5*H*-dibenzo[*a,d*][7]annulen-10-ol.

01/2005:0516

NOSCAPINE

Noscapinum

C₂₂H₂₃NO₇M_r 413.4

DEFINITION

(3*S*)-6,7-Dimethoxy-3-[(5*R*)-4-methoxy-6-methyl-5,6,7,8-tetrahydro-1,3-dioxolo[4,5-*g*]isoquinolin-5-yl]isobenzofuran-1(3*H*)-one.

Content: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

Appearance: white, crystalline powder or colourless crystals.

Solubility: practically insoluble in water, soluble in acetone, slightly soluble in alcohol. It dissolves in strong acids; on dilution of the solution with water, the base may be precipitated.

IDENTIFICATION

First identification: C, E.

Second identification: A, B, D, E.

- A. It complies with the test for specific optical rotation (see Tests).

- B. Melting point (2.2.14): 174 °C to 177 °C.

- C. Infrared absorption spectrophotometry (2.2.24).

Comparison: noscapine CRS.

- D. Thin-layer chromatography (2.2.27).

Test solution. Dissolve 25 mg of the substance to be examined in *acetone R* and dilute to 100 ml with the same solvent.

Reference solution. Dissolve 25 mg of *noscapine CRS* in *acetone R* and dilute to 100 ml with the same solvent.

Plate: TLC silica gel plate *R*.

Mobile phase: concentrated ammonia *R*, alcohol *R*, acetone *R*, toluene *R* (1:3:20:20 V/V/V/V).

Application: 10 µl.

Development: over 2/3 of the plate.

Drying: in air.

Detection: spray with dilute potassium iodobismuthate solution *R*.

Results: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

- E. To 20 mg add 10 ml of *water R* and shake. It does not dissolve.

TESTS

Appearance of solution. The solution is clear (2.2.1) and not more intensely coloured than reference solution Y₆ (2.2.2, Method II).

Dissolve 0.2 g in *acetone R* and dilute to 10 ml with the same solvent. Examine immediately after preparation.

Specific optical rotation (2.2.7): + 42 to + 48 (dried substance).

Dissolve 0.500 g in 0.1 *M* hydrochloric acid and dilute to 25.0 ml with the same acid.

Related substances. Liquid chromatography (2.2.29).

Test solution. Dissolve 20.0 mg of the substance to be examined with gentle heating in 14 ml of *methanol R*, cool the solution and dilute to 20.0 ml with *phosphate buffer solution pH 6.0 R1*.

Reference solution (a). Dilute 1.0 ml of the test solution to 20.0 ml with the mobile phase. Dilute 1.0 ml of the solution to 10.0 ml with the mobile phase.

Reference solution (b). Dissolve 5 mg of *papaverine hydrochloride R* in the mobile phase and dilute to 50.0 ml with the mobile phase. Dilute 1.0 ml of the solution to 20.0 ml with the mobile phase.

Reference solution (c). Dissolve 1.5 mg of *papaverine hydrochloride R* in 10 ml of the test solution and dilute to 50 ml with the mobile phase.

Column:

– *size*: *l* = 0.125 m, Ø = 4.6 mm,

– *stationary phase*: nitrile silica gel for chromatography *R* (5 µm).

Mobile phase: *methanol R*, *phosphate buffer solution pH 6.0 R1* (350:650 V/V).

Flow rate: 1 ml/min.

Detection: spectrophotometer at 240 nm.

Injection: 20 µl.

Run time: 2.5 times the retention time of noscapine.

Relative retention with reference to noscapine (retention time = about 10 min): impurity A = about 1.3.

System suitability: reference solution (c):

– *resolution*: minimum 2 between the peaks due to noscapine and to impurity A.

Limits:

– *impurity A*: not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent),

– *any other impurity*: not more than 0.4 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent),

- *total of other impurities*: not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent),
- *disregard limit*: 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

Loss on drying (2.2.32): maximum 1.0 per cent, determined on 0.500 g by drying in an oven at 100–105 °C.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.350 g in 40 ml of *anhydrous acetic acid R*, warming gently. Titrate with 0.1 M *perchloric acid*, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *perchloric acid* is equivalent to 41.34 mg of $C_{22}H_{23}NO_7$.

STORAGE

Protected from light.

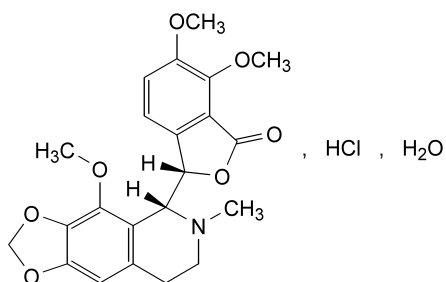
IMPURITIES

A. papaverine.

01/2005:0515

NOSCAPINE HYDROCHLORIDE

Noscapini hydrochloridum



$C_{22}H_{24}ClNO_7 \cdot H_2O$

M_r 467.9

DEFINITION

(3*S*)-6,7-Dimethoxy-3-[(5*R*)-4-methoxy-6-methyl-5,6,7,8-tetrahydro-1,3-dioxolo[4,5-*g*]isoquinolin-5-yl]isobenzofuran-1(3*H*)-one hydrochloride monohydrate.

Content: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

Appearance: white, crystalline powder or colourless crystals, hygroscopic.

Solubility: freely soluble in water and in alcohol. Aqueous solutions are faintly acid; the base may be precipitated when the solutions are allowed to stand.

mp: about 200 °C, with decomposition.

IDENTIFICATION

First identification: C, E.

Second identification: A, B, D, E.

A. It complies with the test for specific optical rotation (see Tests).

B. Melting point (2.2.14) of the precipitate obtained in identification test E: 174 °C to 177 °C.

C. Infrared absorption spectrophotometry (2.2.24).

Preparation: examine the precipitate obtained in identification test E.

Comparison: noscapine CRS.

D. Thin-layer chromatography (2.2.27).

Test solution. Dissolve 25 mg of the substance to be examined in *alcohol R* and dilute to 100 ml with the same solvent.

Reference solution. Dissolve 22 mg of *noscapine CRS* in *acetone R* and dilute to 100 ml with the same solvent.

Plate: TLC silica gel plate *R*.

Mobile phase: concentrated ammonia *R*, alcohol *R*, acetone *R*, toluene *R* (1:3:20:20 V/V/V/V).

Application: 10 µl.

Development: over 2/3 of the plate.

Drying: in air.

Detection: spray with dilute potassium iodobismuthate solution *R*.

Results: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

E. Dissolve about 40 mg in a mixture of 2 ml of *water R* and 3 ml of *alcohol R* and add 1 ml of dilute ammonia *R*2. Heat until dissolution is complete. Allow to cool, scratching the wall of the tube with a glass rod. Filter. The filtrate gives reaction (a) of chlorides (2.3.1). Wash the precipitate with *water R*, dry at 100–105 °C and reserve for identification tests B and C.

TESTS

Appearance of solution. The solution is clear (2.2.1) and not more intensely coloured than reference solution Y₆ or BY₆ (2.2.2, Method II).

Dissolve 0.5 g in *water R*, add 0.3 ml of 0.1 M *hydrochloric acid* and dilute to 25 ml with *water R*.

pH (2.2.3): minimum 3.0.

Dissolve 0.2 g in 10 ml of carbon dioxide-free *water R*.

Specific optical rotation (2.2.7): + 38.5 to + 44.0 (dried substance).

Dissolve 0.500 g in 0.01 M *hydrochloric acid* and dilute to 25.0 ml with the same acid.

Related substances. Liquid chromatography (2.2.29).

Test solution. Dissolve 20.0 mg of the substance to be examined with gentle heating in 14 ml of *methanol R*, cool the solution and dilute to 20.0 ml with *phosphate buffer solution pH 6.0 RI*.

Reference solution (a). Dilute 1.0 ml of the test solution to 20.0 ml with the mobile phase. Dilute 1.0 ml of the solution to 10.0 ml with the mobile phase.

Reference solution (b). Dissolve 5 mg of *papaverine hydrochloride R* in the mobile phase and dilute to 50.0 ml with the mobile phase. Dilute 1.0 ml of the solution to 20.0 ml with the mobile phase.

Reference solution (c). Dissolve 1.5 mg of *papaverine hydrochloride R* in 10 ml of the test solution and dilute to 50 ml with the mobile phase.

Column:

– *size*: $l = 0.125$ m, $\varnothing = 4.6$ mm,

– *stationary phase*: nitrile silica gel for chromatography *R* (5 µm).

Mobile phase: *methanol R*, *phosphate buffer solution pH 6.0 RI* (350:650 V/V).

Flow rate: 1 ml/min.

Detection: spectrophotometer at 240 nm.

Injection: 20 µl.

Run time: 2.5 times the retention time of noscapine.

Relative retention with reference to noscapine (retention time = about 10 min): impurity A = about 1.3.

System suitability: reference solution (c):

- **resolution:** minimum 2 between the peaks due to noscapine and to impurity A.

Limits:

- **impurity A:** not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent),
- **any other impurity:** not more than 0.4 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent),
- **total of other impurities:** not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent),
- **disregard limit:** 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

Loss on drying (2.2.32): 2.5 per cent to 6.5 per cent, determined on 0.200 g by drying in an oven at 100–105 °C.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.400 g in a mixture of 5.0 ml of 0.01 M hydrochloric acid and 50 ml of alcohol R. Carry out a potentiometric titration (2.2.20), using 0.1 M sodium hydroxide. Read the volume added between the 2 points of inflexion.

1 ml of 0.1 M sodium hydroxide is equivalent to 44.99 mg of C₂₂H₂₄ClNO₇.

STORAGE

In an airtight container, protected from light.

IMPURITIES

A. papaverine.

01/2005:1552

NUTMEG OIL

Myristicae fragrantis aetheroleum

DEFINITION

Nutmeg oil is obtained by steam distillation of the dried and crushed kernels of *Myristica fragrans* Houtt.

CHARACTERS

A colourless or pale yellow liquid, with a spicy odour.

IDENTIFICATION

First identification: B.

Second identification: A.

A. Examine by thin-layer chromatography (2.2.27), using a TLC silica gel plate R.

Test solution. Dissolve 1 ml of the substance to be examined in toluene R and dilute to 10 ml with the same solvent.

Reference solution. Dissolve 20 µl of myristicine R in 10 ml of toluene R.

Apply to the plate as bands 10 µl of each solution. Develop over a path of 15 cm using a mixture of 5 volumes of ethyl acetate R and 95 volumes of toluene R. Allow the plate to dry in air and spray with vanillin reagent R. Heat the plate at 100 °C to 105 °C for 10 min. Examine in daylight.

The chromatogram obtained with the reference solution shows in the upper third a pink to reddish-brown zone (myristicine). The chromatogram obtained with the test solution shows a series of zones of which one is similar in position and colour to the zone in the chromatogram obtained with the reference solution. Above this zone a brownish zone (safrole) and a violet zone (hydrocarbons) are present. Below the myristicine zone, 5 blue zones of variable intensity are present.

B. Examine the chromatograms obtained in the test for chromatographic profile. The retention times of the principal peaks in the chromatogram obtained with the test solution are similar to those in the chromatogram obtained with the reference solution.

TESTS

Relative density (2.2.5): 0.885 to 0.905.

Refractive index (2.2.6): 1.475 to 1.485.

Optical rotation (2.2.7): + 8° to + 18°.

Chromatographic profile. Examine by gas chromatography (2.2.28).

Test solution. The substance to be examined.

Reference solution. Dissolve 15 µl of α-pinene R, 15 µl of β-pinene R, 15 µl of sabinene R, 5 µl of car-3-ene R, 5 µl of limonene R, 5 µl of γ-terpinene R, 5 µl of terpinen-4-ol R, 5 µl of safrole R and 10 µl of myristicine R in 1 ml of hexane R.

The chromatographic procedure may be carried out using:

- a fused-silica column 25 m to 60 m long and about 0.3 mm in internal diameter, coated with macrogol 20 000 R as the bonded phase,
- helium for chromatography R as the carrier gas at a flow rate of 1.5 ml/min,
- a flame-ionisation detector,
- a split ratio of 1:100.
- with the following temperature programme:

	Time (min)	Temperature (°C)	Rate (°C/min)	Comment
Column	0 - 10	50	–	isothermal
	10 - 75	50 → 180	2	linear gradient
	75 - 130	180	–	isothermal
Injection port		200 - 220		
Detector		240 - 250		

Inject 0.2 µl of the reference solution. When the chromatogram is recorded in the prescribed conditions, the components elute in the order indicated in the composition of the reference solution. Record the retention times of these substances.

The test is not valid unless the resolution between the peaks corresponding to β-pinene and sabinene is at least 1.5.

Inject 0.2 µl of the test solution. Using the retention times determined from the chromatogram obtained with the reference solution, locate the components of the reference solution in the chromatogram obtained with the test solution. Determine the percentage content of each of these components by the normalisation procedure.

The percentages are within the following ranges:

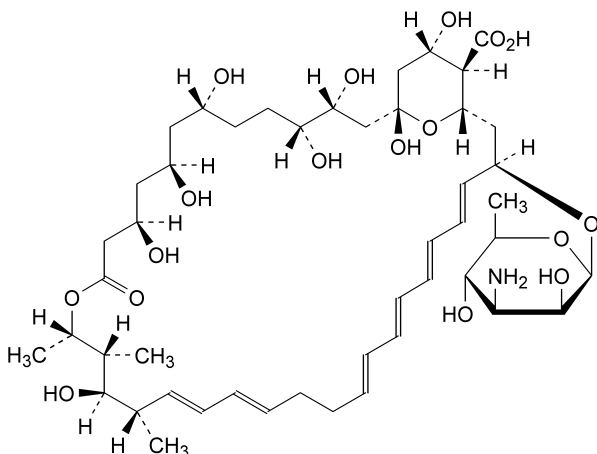
- α-pinene: 15 per cent to 28 per cent,
- β-pinene: 13 per cent to 18 per cent,
- sabinene: 14 per cent to 29 per cent,
- car-3-ene: 0.5 per cent to 2.0 per cent,
- limonene: 2.0 per cent to 7.0 per cent,
- γ-terpinene: 2.0 per cent to 6.0 per cent,

- *terpinen-4-ol*: 2.0 per cent to 6.0 per cent,
- *safrole*: less than 2.5 per cent,
- *myristicine*: 5.0 per cent to 12.0 per cent.

STORAGE

Store in a well-filled, airtight container, protected from light and heat.

01/2005:0517

NYSTATIN**Nystatinum**C₄₇H₇₅NO₁₇M_r 926**DEFINITION**

Antifungal substance obtained by fermentation using certain strains of *Streptomyces noursei* as the production micro-organism. It contains mainly tetraenes, the principal component being (1*S*,3*R*,4*R*,7*R*,9*R*,11*R*,15*S*,16*R*,17*R*,18*S*,19*E*,21*E*,25*E*,27*E*,29*E*,31*E*,33*R*,35*S*,36*R*,37*S*)-33-[(3-amino-3,6-dideoxy-β-D-mannopyranosyl)oxy]-1,3,4,7,9,11,17,37-octahydroxy-15,16,18-trimethyl-13-oxo-14,39-dioxabicyclo[33.3.1]nonatriaconta-19,21,25,27,29,31-hexaene-36-carboxylic acid (nystatin A1).

Content: minimum 4400 IU/mg (dried substance) and minimum 5000 IU/mg (dried substance) if intended for oral administration.

PRODUCTION

If nystatin is not intended for cutaneous administration, the method of manufacture is validated to demonstrate that the product, if tested, would comply with the following test.

Abnormal toxicity (2.6.9). Inject intraperitoneally into each mouse a quantity equivalent to not less than 600 IU suspended in 0.5 ml of a 5 g/l solution of *acacia R*.

CHARACTERS

Appearance: yellow or slightly brownish powder, hygroscopic.

Solubility: practically insoluble in water, freely soluble in dimethylformamide and in dimethyl sulphoxide, slightly soluble in methanol, practically insoluble in alcohol.

IDENTIFICATION

First identification: B, E.

Second identification: A, C, D.

A. Examine the solution prepared in the test for absorbance between 220 nm and 350 nm (2.2.25). The solution shows 4 absorption maxima at 230 nm, 291 nm, 305 nm

and 319 nm, and a shoulder at 280 nm. The ratios of the absorbances at the absorption maxima at 291 nm and 319 nm to the absorbance at the absorption maximum at 305 nm are 0.61 to 0.73 and 0.83 to 0.96, respectively. The ratio of the absorbance measured at the absorption maximum at 230 nm to that measured at the shoulder at 280 nm is 0.83 to 1.25.

B. Infrared absorption spectrophotometry (2.2.24).

Comparison: *nystatin CRS*.

C. To about 2 mg add 0.1 ml of *hydrochloric acid R*. A brown colour develops.

D. To about 2 mg add 0.1 ml of *sulphuric acid R*. A brown colour develops that becomes violet on standing.

E. Examine the chromatograms obtained in the test for composition.

Results: the principal peak in the chromatogram obtained with the test solution is similar in retention time to the principal peak in the chromatogram obtained with reference solution (a).

TESTS

Absorbance (2.2.25). Dissolve 0.10 g in a mixture of 5.0 ml of *glacial acetic acid R* and 50 ml of *methanol R* and dilute to 100.0 ml with *methanol R*. Dilute 1.0 ml of the solution to 100.0 ml with *methanol R*. Determined at the maximum at 305 nm within 30 min of preparation of the solution, the absorbance is not less than 0.60.

Composition. Liquid chromatography (2.2.29): use the normalisation procedure. Carry out the test protected from light.

Test solution. Dissolve 20 mg of the substance to be examined in *dimethyl sulphoxide R* and dilute to 50 ml with the same solvent.

Reference solution (a). Dissolve 20 mg of *nystatin CRS* in *dimethyl sulphoxide R* and dilute to 50 ml with the same solvent.

Reference solution (b). Dissolve 20 mg of the substance to be examined in 25 ml of *methanol R* and dilute to 50 ml with *water R*. To 10.0 ml of the solution add 2.0 ml of *dilute hydrochloric acid R*. Allow to stand at room temperature for 1 h.

Reference solution (c). Dilute 1.0 ml of reference solution (a) to 100.0 ml with *dimethyl sulphoxide R*. Dilute 1.0 ml of this solution to 10.0 ml with *dimethyl sulphoxide R*.

Column:

- size: $l = 0.15$ m, $\varnothing = 4.6$ mm,
- stationary phase: base-deactivated end-capped octadecylsilyl silica gel for chromatography *R* (5 μ m),
- temperature: 30 °C.

Mobile phase:

- mobile phase A: *acetonitrile R*, 3.85 g/l solution of *ammonium acetate R* (29:71 V/V),
- mobile phase B: 3.85 g/l solution of *ammonium acetate R*, *acetonitrile R* (40:60 V/V),

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)
0 - 25	100	0
25 - 35	100 → 0	0 → 100
35 - 45	0	100
45 - 50	0 → 100	100 → 0
50 - 55	100	0

Flow rate: 1.0 ml/min.

Detection: spectrophotometer at 305 nm.

Injection: 20 µl

Retention time: nystatin A1 = about 14 min.

System suitability: reference solution (b):

- **resolution:** minimum 3.5 between the 2 principal peaks (retention time = about 13 min and 19 min).

Composition:

- **nystatin A1:** minimum 85.0 per cent,
- **any other compound:** maximum 4.0 per cent,
- **disregard limit:** the area of the principal peak in the chromatogram obtained with reference solution (c); disregard any peak with a retention time of less than 2 min.

Heavy metals (2.4.8): maximum 20 ppm.

1.0 g complies with limit test C. Prepare the standard using 2 ml of *lead standard solution (10 ppm Pb) R*.

Loss on drying (2.2.32): maximum 5.0 per cent, determined on 1.000 g by drying at 60 °C over *diphosphorus pentoxide R* at a pressure not exceeding 0.1 kPa for 3 h.

Sulphated ash (2.4.14): maximum 3.5 per cent, determined on 1.0 g.

ASSAY

Carry out the microbiological assay of antibiotics (2.7.2).

Protect the solutions from light throughout the assay.

Dissolve the substance to be examined and *nystatin CRS* separately in *dimethylformamide R* and dilute with a mixture of 5 volumes of *dimethylformamide R* and 95 volumes of buffer solution pH 6.0.

STORAGE

In an airtight container, protected from light.

LABELLING

The label states where applicable, that the substance is only for cutaneous use.

OAK BARK

Quercus cortex

DEFINITION

Cut and dried bark from the fresh young branches of *Quercus robur* L., *Q. petraea* (Matt.) Liebl. and *Q. pubescens* Willd.

Content: minimum 3.0 per cent of tannins, expressed as pyrogallol ($C_6H_6O_3$; M_r 126.1) (dried drug).

CHARACTERS

Macroscopic and microscopic characters described under identification tests A and B.

IDENTIFICATION

- A. The bark occurs in channelled or quilled pieces, not more than 3 mm thick. The outer surface is light grey or greenish-grey, rather smooth, with occasional lenticels. The inner surface is dull brown or reddish-brown and has slightly raised longitudinal striations about 0.5 mm to 1 mm wide. The fracture is splintery and fibrous.
- B. Reduce to a powder (355). The powder is light brown to reddish-brown and fibrous. Examine under a microscope using *chloral hydrate solution R*. The powder shows groups of thick-walled fibres surrounded by a moderately thickened parenchymatous sheath containing prism crystals of calcium oxalate; fragments of cork composed of thin-walled tabular cells filled with brownish or reddish contents; abundant sclereids, isolated and in groups, some large with thick, stratified walls and branching pits, others smaller and thinner-walled with simple pits, often with dense brown contents; fragments of parenchyma containing cluster crystals of calcium oxalate; occasional fragments of sieve tissue, thin-walled, some showing sieve areas on the oblique end-walls.
- C. To 1 g of the powdered drug (710) add 10 ml of *alcohol* (30 per cent V/V) *R* and heat the mixture under a reflux condenser on a water-bath for 30 min. Cool and filter. To 1 ml of the solution add 2 ml of a 10 g/l solution of *vanillin R* in *hydrochloric acid R*. A red colour develops.

TESTS

Foreign matter (2.8.2): maximum 2 per cent.

Loss on drying (2.2.32): maximum 10.0 per cent, determined on 1.000 g of the powdered drug (710) by drying in an oven at 100–105 °C for 2 h.

Total ash (2.4.16): maximum 8.0 per cent.

ASSAY

Carry out the determination of tannins in herbal drugs (2.8.14). Use 0.700 g of the powdered drug (710).

01/2005:1887 CHARACTERS

Appearance: clear, colourless or light yellow, viscous liquid.

Solubility: miscible with water, with ethanol and with vegetable oils.

IDENTIFICATION

A. Infrared absorption spectrophotometry (2.2.24).

Comparison: *Ph. Eur. reference spectrum of octoxinol 10*.

Preparation: film between *sodium chloride R* plates.

B. It complies with the test for cloud point (see Tests).

TESTS

Acidity or alkalinity. Boil 1.0 g with 20 ml of *carbon dioxide-free water R* for 1 min, with constant stirring. Cool and filter. To 10 ml of the filtrate, add 0.05 ml of *bromothymol blue solution R1*. Not more than 0.5 ml of 0.01 M *hydrochloric acid* or 0.01 M *sodium hydroxide* is required to change the colour of the indicator.

Hydroxyl value (2.5.3, *Method A*): 85 to 101.

Cloud point: 63 °C to 70 °C.

Dissolve 1.0 g in 99 g of *water R*. Transfer about 30 ml of this solution to a test-tube, heat on a water-bath and stir continuously until the solution becomes cloudy. Remove the test-tube from the water-bath (ensuring that the temperature does not increase more than 2 °C), and continue to stir. The cloud point is the temperature at which the solution becomes sufficiently clear that the entire thermometer bulb is plainly seen.

Ethylene oxide and dioxan (2.4.25): maximum 1 ppm of ethylene oxide and maximum 10 ppm of dioxan.

Heavy metals (2.4.8): maximum 10 ppm.

Dissolve 2.0 g in *distilled water R* and dilute to 20.0 ml with the same solvent. 12 ml of this solution complies with limit test A. Prepare the standard using *lead standard solution* (1 ppm Pb) *R*.

Water (2.5.12): maximum 0.5 per cent, determined on 2.00 g.

Total ash (2.4.16): maximum 0.4 per cent, determined on 1.0 g.

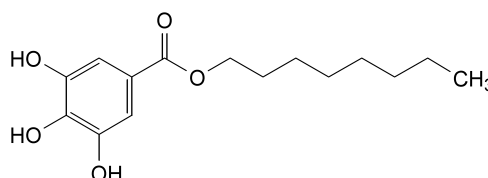
STORAGE

In an airtight container.

01/2005:2057

OCTYL GALLATE

Octylis gallas


 $C_{15}H_{22}O_5$
 M_r 282.3

DEFINITION

Octyl 3,4,5-trihydroxybenzoate.

Content: 97.0 per cent to 103.0 per cent (dried substance).

CHARACTERS

Appearance: white or almost white, crystalline powder.

OCTOXINOL 10

Octoxinolum 10

DEFINITION

α -[4-(1,1,3,3-Tetramethylbutyl)phenyl]- ω -hydroxydeca(oxyethylene).

Mixture consisting mainly of mono-octylphenyl ethers of macrogols corresponding to the formula $C_8H_{17}C_6H_4-[OCH_2CH_2]_n-OH$ where the average value of n is 10. It may contain free macrogols.

01/2005:1553

Solubility: practically insoluble in water, freely soluble in ethanol (96 per cent), practically insoluble in methylene chloride.

IDENTIFICATION

A. Melting point (2.2.14).

Determine the melting point of the substance to be examined. Mix equal parts of the substance to be examined and *octyl gallate CRS* and determine the melting point of the mixture. The difference between the melting points (which are about 101 °C) is not greater than 2 °C.

B. Examine the chromatograms obtained in the test for impurity A.

Results: the principal spot in the chromatogram obtained with test solution (b) is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).

TESTS

Impurity A. Thin-layer chromatography (2.2.27).

Test solution (a). Dissolve 0.20 g of the substance to be examined in *acetone R* and dilute to 10 ml with the same solvent.

Test solution (b). Dilute 1.0 ml of test solution (a) to 20 ml with *acetone R*.

Reference solution (a). Dissolve 10 mg of *octyl gallate CRS* in *acetone R* and dilute to 10 ml with the same solvent.

Reference solution (b). Dissolve 20 mg of *gallic acid R* in *acetone R* and dilute to 20 ml with the same solvent.

Reference solution (c). Dilute 1.0 ml of reference solution (b) to 10 ml with *acetone R*.

Reference solution (d). Dilute 1.0 ml of reference solution (b) to 5 ml with test solution (a).

Plate: *TLC silica gel plate R*.

Mobile phase: *anhydrous formic acid R, ethyl formate R, toluene R* (10:40:50 V/V/V).

Application: 5 µl of test solutions (a) and (b) and reference solutions (a), (c) and (d).

Development: over 2/3 of the plate.

Drying: in air for 10 min.

Detection: spray with a mixture of 1 volume of *ferric chloride solution R1* and 9 volumes of *ethanol (96 per cent) R*.

System suitability: reference solution (d):

- the chromatogram shows 2 clearly separated principal spots.

Limit: test solution (a):

- **impurity A:** any spot due to impurity A is not more intense than the spot in the chromatogram obtained with reference solution (c) (0.5 per cent).

Chlorides (2.4.4): maximum 100 ppm.

To 1.65 g add 50 ml of *water R*. Shake for 5 min. Filter. 15 ml of the filtrate complies with the test.

Heavy metals (2.4.8): maximum 10 ppm.

2.0 g complies with limit test C. Prepare the reference solution using 2 ml of *lead standard solution (10 ppm Pb) R*.

Loss on drying (2.2.32): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 70 °C.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.100 g in *methanol R* and dilute to 250.0 ml with the same solvent. Dilute 5.0 ml of the solution to 200.0 ml with *methanol R*. Measure the absorbance (2.2.25) at the absorption maximum at 275 nm.

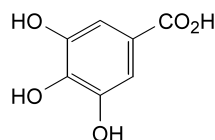
Calculate the content of $C_{15}H_{32}O_2$ taking the specific absorbance to be 387.

STORAGE

In a non-metallic container, protected from light.

IMPURITIES

Specified impurities: A.

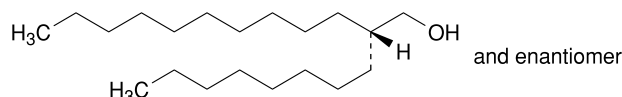


A. 3,4,5-trihydroxybenzoic acid (gallic acid).

01/2005:1136

OCTYLDODECANOL

Octyldodecanolum



DEFINITION

Condensation product of saturated liquid fatty alcohols.

Content: not less than 90 per cent of (2*RS*)-2-octyldodecan-1-ol ($C_{20}H_{42}O$; M_r 298.6), the remainder consisting mainly of related alcohols.

CHARACTERS

Appearance: clear, colourless or yellowish, oily liquid.

Solubility: practically insoluble in water, miscible with alcohol.

Relative density: about 0.840.

Refractive index: about 1.455.

IDENTIFICATION

A. It complies with the test for hydroxyl value (see Tests).

B. Thin-layer chromatography (2.2.27).

Test solution. Dissolve 0.20 g of the substance to be examined in *toluene R* and dilute to 20 ml with the same solvent.

Reference solution. Dissolve 0.20 g of *octyldodecanol CRS* in *toluene R* and dilute to 20 ml with the same solvent.

Plate: suitable silica gel plate.

Mobile phase: *ethyl acetate R, toluene R* (5:95 V/V).

Application: 2 µl.

Development: over a path of 12 cm.

Drying: in air.

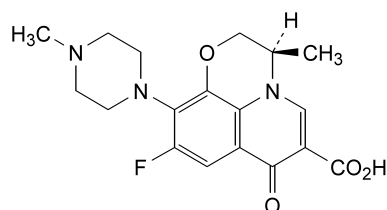
Detection: spray with about 7 ml of a mixture of 1 volume of a 25 g/l solution of *vanillin R* in *alcohol R* and 4 volumes of *sulphuric acid R* and heat at 130 °C for 5-10 min.

Results: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

01/2005:1455

OFLOXACIN

Ofloxacinum

 $C_{18}H_{20}FN_3O_4$ M_r 361.4

DEFINITION

Ofloxacin contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of (RS)-9-fluoro-3-methyl-10-(4-methylpiperazin-1-yl)-7-oxo-2,3-dihydro-7H-pyrido[1,2,3-de]-1,4-benzoxazine-6-carboxylic acid, calculated with reference to the dried substance.

CHARACTERS

A pale yellow or bright yellow, crystalline powder, slightly soluble in water, soluble in glacial acetic acid, slightly soluble to soluble in methylene chloride, slightly soluble in methanol.

IDENTIFICATION

Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *ofloxacin CRS*. Examine the substances prepared as discs.

TESTS

Absorbance (2.2.25). Dissolve 0.5 g in 0.1 M hydrochloric acid and dilute to 100 ml with the same solvent. The absorbance of the solution measured at 440 nm is not greater than 0.25.

Optical rotation (2.2.7). Dissolve 0.300 g in a mixture of 10 volumes of *methanol R* and 40 volumes of *methylene chloride R* and dilute to 10 ml with the same mixture of solvents. The angle of optical rotation is -0.10° to $+0.10^\circ$.

Impurity A. Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel GF₂₅₄ plate R* (2 µm to 10 µm).

Test solution. Dissolve 0.250 g of the substance to be examined in a mixture of 10 volumes of *methanol R* and 40 volumes of *methylene chloride R* and dilute to 5.0 ml with the same mixture of solvents.

Reference solution. Dissolve 10 mg of *ofloxacin impurity A CRS* in a mixture of 10 volumes of *methanol R* and 40 volumes of *methylene chloride R* and dilute to 100.0 ml with the same mixture of solvents.

Apply to the plate 10 µl of each solution. Develop over a path of 10 cm using a mixture of 1 volume of *glacial acetic acid R*, 1 volume of *water R* and 2 volumes of *ethyl acetate R*. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. Any spot due to impurity A in the chromatogram obtained with the test solution is not more intense than the spot in the chromatogram obtained with the reference solution (0.2 per cent).

Related substances. Examine by liquid chromatography (2.2.29). Prepare the solutions immediately before use.

Test solution. Dissolve 10.0 mg of the substance to be examined in a mixture of 10 volumes of *acetonitrile R* and 60 volumes of *water R* and dilute to 50.0 ml with the same mixture of solvents.

TESTS

Acidity or alkalinity. Mix 5.0 g thoroughly for 1 min with a mixture of 0.1 ml of *bromothymol blue solution R1*, 2 ml of *heptane R* and 10 ml of *water R*. If the aqueous layer is blue, not more than 0.15 ml of 0.01 M hydrochloric acid is required to change the colour of the indicator to yellow. If the aqueous layer is yellow, add 0.45 ml of 0.1 M sodium hydroxide and shake vigorously. After standing to ensure complete separation, the aqueous layer is blue.

Optical rotation (2.2.7): -0.10° to $+0.10^\circ$.

Dissolve 2.50 g in *alcohol R* and dilute to 25 ml with the same solvent.

Hydroxyl value (2.5.3, Method A): 175 to 190.

Iodine value (2.5.4): maximum 8.0.

Peroxide value (2.5.5): maximum 5.0.

Saponification value (2.5.6): maximum 5.0.

Heavy metals (2.4.8): maximum 10 ppm.

2.0 g complies with limit test C. Prepare the standard using 2 ml of *lead standard solution (10 ppm Pb) R*.

Water (2.5.12): maximum 0.5 per cent, determined on 2.00 g.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Gas chromatography (2.2.28).

Internal standard solution. Dissolve 0.4 g of *tetradecane R* in *hexane R* and dilute to 100.0 ml with the same solvent.

Test solution. Dissolve 0.100 g of the substance to be examined in the internal standard solution and dilute to 10.0 ml with the same solution.

Reference solution. Dissolve 0.100 g of *octyldodecanol CRS* in the internal standard solution and dilute to 10.0 ml with the same solution.

Column:

- **material:** stainless steel,
- **size:** $l = 60$ m, $\varnothing = 0.25$ mm,
- **stationary phase:** *poly(dimethyl)(diphenyl)(divinyl)siloxane R* (film thickness 0.25 µm).

Carrier gas: *helium for chromatography R*.

Flow rate: 0.68 ml/min.

Temperature:

	Time (min)	Temperature (°C)
	0 - 2	180
Column	2 - 22	180 → 280
	22 - 52	280
Injection port		290
Detector		300

Detection: flame ionisation.

Injection: 1 µl.

Calculate the content of $C_{20}H_{42}O$ in the substance to be examined.

STORAGE

Protected from light.

Reference solution (a). Dilute 1.0 ml of the test solution to 50.0 ml with a mixture of 10 volumes of *acetonitrile R* and 60 volumes of *water R*. Dilute 1.0 ml of this solution to 10.0 ml with a mixture of 10 volumes of *acetonitrile R* and 60 volumes of *water R*.

Reference solution (b). Dissolve 10.0 mg of *ofloxacin impurity E CRS* in a mixture of 10 volumes of *acetonitrile R* and 60 volumes of *water R* and dilute to 100.0 ml with the same mixture of solvents. Mix 10.0 ml of this solution with 5.0 ml of the test solution. Dilute to 50.0 ml with a mixture of 10 volumes of *acetonitrile R* and 60 volumes of *water R*. Dilute 1.0 ml of this solution to 50.0 ml with a mixture of 10 volumes of *acetonitrile R* and 60 volumes of *water R*.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.15 m long and 4.6 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (5 µm),
- as mobile phase a mixture prepared as follows: dissolve 4.0 g of *ammonium acetate R* and 7.0 g of *sodium perchlorate R* in 1300 ml of *water R*. Adjust to pH 2.2 with *phosphoric acid R*. Add 240 ml of *acetonitrile R*. Adjust the flow rate of the mobile phase so that a retention time of about 20 min is obtained for ofloxacin,
- as detector a spectrophotometer set at 294 nm, maintaining the temperature of the column at 45 °C.

Inject 10 µl of reference solution (b). Adjust the sensitivity of the system so that the heights of the two principal peaks in the chromatogram obtained are at least 50 per cent of the full scale of the recorder. The test is not valid unless: in the chromatogram obtained, the resolution between the peaks corresponding to impurity E and ofloxacin is at least 2.0. Inject 10 µl of the test solution and 10 µl of reference solution (a). Continue the chromatography for 2.5 times the retention time of the principal peak. In the chromatogram obtained with the test solution, the area of any peak, apart from the principal peak, is not greater than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent); the sum of the areas of all the peaks is not greater than 2.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent). Disregard any peak with an area less than 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (a).

Heavy metals (2.4.8). 2.0 g complies with limit test C for heavy metals (10 ppm). Prepare the standard using 2 ml of *lead standard solution (10 ppm Pb) R*.

Loss on drying (2.2.32). Not more than 0.2 per cent, determined on 1.000 g by drying at 100 °C to 105 °C for 4 h.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

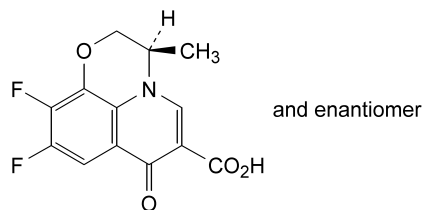
Dissolve 0.300 g in 100 ml of *anhydrous acetic acid R*. Titrate with 0.1 M *perchloric acid* determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *perchloric acid* is equivalent to 36.14 mg of C₁₈H₂₀FN₃O₄.

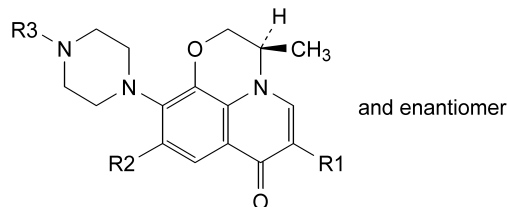
STORAGE

Store in an airtight container, protected from light.

IMPURITIES



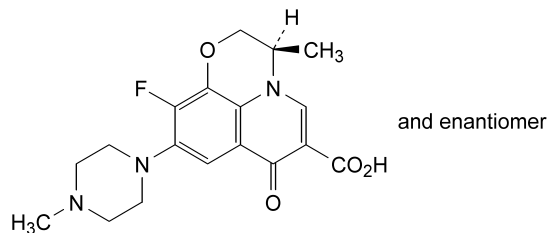
A. (RS)-9,10-difluoro-3-methyl-7-oxo-2,3-dihydro-7H-pyrido[1,2,3-de]-1,4-benzoxazine-6-carboxylic acid (FPA),



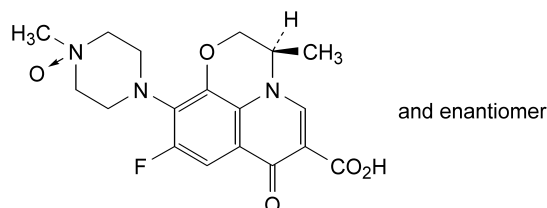
B. R1 = H, R2 = F, R3 = CH₃: (RS)-9-fluoro-3-methyl-10-(4-methylpiperazin-1-yl)-2,3-dihydro-7H-pyrido[1,2,3-de]-1,4-benzoxazine-7-one,

C. R1 = CO₂H, R2 = H, R3 = CH₃: (RS)-3-methyl-10-(4-methylpiperazin-1-yl)-7-oxo-2,3-dihydro-7H-pyrido[1,2,3-de]-1,4-benzoxazine-6-carboxylic acid,

E. R1 = CO₂H, R2 = F, R3 = H: (RS)-9-fluoro-3-methyl-7-oxo-10-(piperazin-1-yl)-2,3-dihydro-7H-pyrido[1,2,3-de]-1,4-benzoxazine-6-carboxylic acid,



D. (RS)-10-fluoro-3-methyl-9-(4-methylpiperazin-1-yl)-7-oxo-2,3-dihydro-7H-pyrido[1,2,3-de]-1,4-benzoxazine-6-carboxylic acid,



F. 4-[(RS)-6-carboxy-9-fluoro-3-methyl-7-oxo-2,3-dihydro-7H-pyrido[1,2,3-de]-1,4-benzoxazine-10-yl]-1-methylpiperazine 1-oxide.

01/2005:0799

OLEIC ACID

Acidum oleicum

DEFINITION

(Z)-Octadec-9-enoic acid (C₁₈H₃₄O₂; M_r 282.5), together with varying amounts of saturated and other unsaturated fatty acids. A suitable antioxidant may be added.

Content: 65.0 per cent to 88.0 per cent of C₁₈H₃₄O₂.

CHARACTERS

Appearance: clear, yellowish or brownish, oily liquid.

Solubility: practically insoluble in water, miscible with alcohol and with methylene chloride.

Relative density: about 0.892.

IDENTIFICATION

- A. It complies with the test for acid value (see Tests).
- B. It complies with the test for iodine value (see Tests).
- C. It complies with the test for composition of fatty acids (see Tests).

Margaric acid: maximum 0.2 per cent for oleic acid of vegetable origin and maximum 4.0 per cent for oleic acid of animal origin.

TESTS

Appearance. The substance to be examined is not more intensely coloured than reference solution Y₁ or BY₁ (2.2.2, Method I).

Acid value (2.5.1): 195 to 204, determined on 0.5 g.

Iodine value (2.5.4): 89 to 105.

Peroxide value (2.5.5): maximum 10.0.

Composition of fatty acids. Gas chromatography (2.4.22, Method C).

Test solution. Prepare as described in the method but omitting the initial hydrolysis.

Composition of the fatty acid fraction of the substance:

- *myristic acid*: maximum 5.0 per cent,
- *palmitic acid*: maximum 16.0 per cent,
- *palmitoleic acid*: maximum 8.0 per cent,
- *stearic acid*: maximum 6.0 per cent,
- *oleic acid*: 65.0 per cent to 88.0 per cent,
- *linoleic acid*: maximum 18.0 per cent,
- *linolenic acid*: maximum 4.0 per cent,
- *fatty acids of chain length greater than C₁₈*: maximum 4.0 per cent.

Total ash (2.4.16): maximum 0.1 per cent, determined on 2.00 g.

STORAGE

In an airtight, well-filled container, protected from light.

LABELLING

The label states:

- the name and concentration of any added antioxidant,
- the origin of oleic acid (animal or vegetable).

01/2005:1249

OLEOYL MACROGOLGLYCERIDES

Macrogolglyceridorum oleates

DEFINITION

Oleoyl macrogolglycerides are mixtures of monoesters, diesters and triesters of glycerol and monoesters and diesters of macrogols. They are obtained by partial alcoholysis of an unsaturated oil mainly containing triglycerides of oleic acid using macrogol with a mean relative molecular mass between 300 and 400 or by esterification of glycerol and macrogol with unsaturated fatty acids or by mixing glycerol esters and condensates of ethylene oxide with the fatty acids of this unsaturated oil.

CHARACTERS

Amber oily liquids, which may give rise to a deposit after prolonged periods at 20 °C, practically insoluble but dispersible in water, freely soluble in methylene chloride.

The viscosity at 40 °C is about 35 mPas, the relative density at 20 °C is about 0.95 and the refractive index at 20 °C is about 1.47.

IDENTIFICATION

- A. Examine by thin-layer chromatography (2.2.27), using a suitable silica gel as the coating substance.

Test solution. Dissolve 1.0 g of the substance to be examined in *methylene chloride R* and dilute to 20 ml with the same solvent.

Apply to the plate 10 µl of the test solution. Develop over a path of 15 cm using a mixture of 30 volumes of *hexane R* and 70 volumes of *ether R*. Allow the plate to dry in air. Spray with a 0.1 g/l solution of *rhodamine B R* in *alcohol R* and examine in ultraviolet light at 365 nm. The chromatogram shows a spot corresponding to triglycerides with an *R_f* value of about 0.9 (*R_{st}* 1) and spots corresponding to 1,3-diglycerides (*R_f* 0.7), to 1,2-diglycerides (*R_f* 0.6), to monoglycerides (*R_f* 0.1) and to esters of macrogol (*R_f* 0).

- B. They comply with the test for hydroxyl value (see Tests).
- C. They comply with the test for fatty acid composition (see Tests).
- D. They comply with the test for saponification value (see Tests).

TESTS

Acid value (2.5.1). Not more than 2.0, determined on 2.0 g.

Hydroxyl value (2.5.3, Method A): 45 to 65, determined on 1.0 g.

Iodine value (2.5.4): 75 to 95.

Peroxide value (2.5.5). Not more than 12.0, determined on 2.0 g.

Saponification value (2.5.6): 150 to 170, determined on 2.0 g.

Alkaline impurities. Introduce 5.0 g into a test-tube and carefully add a mixture, neutralised if necessary with 0.01 *M* hydrochloric acid or with 0.01 *M* sodium hydroxide, of 0.05 ml of a 0.4 g/l solution of *bromophenol blue R* in *alcohol R*, 0.3 ml of *water R* and 10 ml of *alcohol R*. Shake and allow to stand. Not more than 1.0 ml of 0.01 *M* hydrochloric acid is required to change the colour of the upper layer to yellow.

Free glycerol. Not more than 3.0 per cent. Dissolve 1.20 g in 25.0 ml of *methylene chloride R*. Heat if necessary. After cooling, add 100 ml of *water R*. Shake and add 25.0 ml of a 6 g/l solution of *periodic acid R*. Shake and allow to stand for 30 min. Add 40 ml of a 75 g/l solution of *potassium iodide R*. Allow to stand for 1 min. Add 1 ml of *starch solution R*. Titrate the iodine with 0.1 *M* sodium thiosulphate. Carry out a blank titration.

1 ml of 0.1 *M* sodium thiosulphate is equivalent to 2.3 mg of glycerol.

Composition of fatty acids (2.4.22, Method A). The fatty-acid fraction has the following composition:

- *palmitic acid*: 4.0 per cent to 9.0 per cent,
- *stearic acid*: not more than 6.0 per cent,
- *oleic acid*: 58.0 per cent to 80.0 per cent,
- *linoleic acid*: 15.0 per cent to 35.0 per cent,
- *linolenic acid*: not more than 2.0 per cent,

- *arachidic acid*: not more than 2.0 per cent,
- *eicosenoic acid*: not more than 2.0 per cent.

Ethylene oxide and dioxan (2.4.25). Not more than 1 ppm of ethylene oxide and 10 ppm of dioxan.

Heavy metals (2.4.8). 2.0 g complies with limit test C for heavy metals (10 ppm). Prepare the standard using 2 ml of *lead standard solution* (10 ppm Pb) R.

Water (2.5.12). Not more than 1.0 per cent, determined on 1.0 g by the semi-micro determination of water. Use a mixture of 30 volumes of *anhydrous methanol* R and 70 volumes of *methylene chloride* R as solvent.

Total ash (2.4.16). Not more than 0.1 per cent, determined on 1.0 g.

STORAGE

Store protected from light and at room temperature.

LABELLING

The label states the type of macrogol used (mean relative molecular mass) or the number of units of ethylene oxide per molecule (nominal value).

01/2005:2073

OLEYL ALCOHOL

Alcohol oleicus

DEFINITION

Mixture of unsaturated and saturated long-chain fatty alcohols consisting mainly of octadec-9-enol (oleyl alcohol and elaidyl alcohol; $C_{18}H_{36}O$; M_r 268.5). It may be of vegetable or animal origin.

CHARACTERS

Appearance: colourless or light yellow liquid.

IDENTIFICATION

- It complies with the test for hydroxyl value (see Tests).
- It complies with the test for composition of fatty alcohols (see Tests).

TESTS

Appearance. The substance to be examined is clear (2.2.1) and not more intensely coloured than reference solution B₆ (2.2.2, *Method II*).

Refractive index (2.2.6): 1.458 to 1.460, determined at 25 °C.

Cloud point: maximum 10 °C.

Introduce about 60 g into a cylindrical flat-bottomed container, 30-33.5 mm internal diameter and 115-125 mm high. Heat to 30 °C, cool, and immerse the container in iced water with the surfaces of the water and the sample at the same level. Insert a thermometer and, using it as a stirring rod begin stirring rapidly and steadily when the temperature falls below 20 °C. Keep the thermometer immersed throughout the test, remove and examine the container at regular intervals. The cloud point is the temperature at which the immersed portion of the thermometer, positioned vertically in the centre of the container, is no longer visible when viewed horizontally through the container and sample.

Acid value (2.5.1): maximum 1.0, determined on 5.0 g.

Hydroxyl value (2.5.3, *Method A*): 205 to 215.

Saponification value (2.5.6): maximum 2.0.

Composition of fatty alcohols. Gas chromatography (2.2.28): use the normalisation procedure.

Test solution. Mix 25 mg of the substance to be examined with 1.0 ml of *methylene chloride* R.

Reference solution (a). Dissolve 25 mg of each of *arachidyl alcohol* R, *linolenyl alcohol* R, *linoleyl alcohol* R, *oleyl alcohol* R, *palmityl alcohol* R and *stearyl alcohol* R in *methylene chloride* R and dilute to 5 ml with the same solvent. Dilute 1 ml of this solution to 5 ml with *methylene chloride* R.

Reference solution (b). Dissolve 10 mg of *linoleyl alcohol* R and 1 g of *oleyl alcohol* R in *methylene chloride* R and dilute to 40 ml with the same solvent.

Column:

- **size**: $l = 30$ m, $\varnothing = 0.32$ mm,
- **stationary phase**: *poly(dimethyl)siloxane* R (film thickness 1 μ m).

Carrier gas: *helium for chromatography* R.

Flow rate: 1 ml/min.

Split ratio: 1:11.

Temperature:

	Time (min)	Temperature (°C)
Column	0 - 1	170
	1 - 9	170 → 210
	9 - 65	210
Injection port		270
Detector		280

Detection: flame ionisation.

Injection: 1 μ l.

Identify the peaks using the chromatogram obtained with reference solution (a).

Relative retention with reference to oleyl alcohol (retention time = about 30 min): *palmityl alcohol* = about 0.6; *linolenyl alcohol* = about 0.8; *linoleyl alcohol* = about 0.9; *stearyl alcohol* = about 1.1; *arachidyl alcohol* = about 1.9 (*elaidyl alcohol* co-elutes with *oleyl alcohol*).

System suitability: reference solution (b):

- **peak-to-valley ratio**: minimum 1.2 between the peaks due to *linoleyl alcohol* and *oleyl alcohol*.

Limits:

- *palmityl alcohol*: maximum 8.0 per cent,
- *stearyl alcohol*: maximum 5.0 per cent,
- *oleyl alcohol* (sum of *oleyl* and *elaidyl alcohols*): minimum 80.0 per cent,
- *linoleyl alcohol*: maximum 3.0 per cent,
- *linolenyl alcohol*: maximum 0.5 per cent,
- *arachidyl alcohol*: maximum 0.3 per cent.

01/2005:1878

OLIVE LEAF

Oleae folium

DEFINITION

Dried leaf of *Olea europaea* L.

Content: minimum 5.0 per cent of oleuropein ($C_{25}H_{32}O_{13}$; M_r 540.5) (dried drug).

CHARACTERS

Macroscopic and microscopic characters described under identification tests A and B.

IDENTIFICATION

- A. The leaf is simple, thick and coriaceous, lanceolate to obovate, 30 mm to 50 mm long and 10 mm to 15 mm wide, with a mucronate apex and tapering at the base to a short petiole; the margins are entire and reflexed abaxially. The upper surface is greyish-green, smooth and shiny, the lower surface paler and pubescent, particularly along the midrib and main lateral veins.
- B. Reduce to a powder (355). The powder is yellowish-green. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters: fragments of the epidermis in surface view with small, thick-walled polygonal cells and, in the lower epidermis only, small anomocytic stomata (2.8.3); fragments of the lamina in sectional view showing a thick cuticle, a palisade composed of 3 layers of cells and a small-celled spongy parenchyma; numerous sclereids, very thick-walled and mostly fibre-like with blunt or, occasionally, forked ends, isolated or associated with the parenchyma of the mesophyll; abundant, very large peltate trichomes, with a central unicellular stalk from which radiate some 10 to 30 thin-walled cells which become free from the adjoining cells at the margin of the shield, given an uneven, jagged appearance.
- C. Thin-layer chromatography (2.2.27).

Test solution. To 1.0 g of the powdered drug (355) add 10 ml of *methanol R*. Boil under a reflux condenser for 15 min. Cool and filter.

Reference solution. Dissolve 10 mg of *oleuropein R* and 1 mg of *rutin R* in 1 ml of *methanol R*.

Plate: TLC silica gel plate *R*.

Mobile phase: *water R*, *methanol R*, *methylene chloride R* (1.5:15:85 V/V/V).

Application: 10 µl as bands.

Development: over a path of 10 cm.

Drying: in air.

Detection: spray with *vanillin reagent R* and heat at 100-105 °C for 5 min; examine in daylight.

Results: see below the sequence of the zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other faint zones may be present in the chromatogram obtained with the test solution.

Top of the plate	
	A dark violet-blue zone (solvent front)
	A dark violet-blue zone
Oleuropein: a brownish-green zone	A brownish-green zone (oleuropein)
Rutin: a brownish-yellow zone	
Reference solution	Test solution

TESTS

Foreign matter (2.8.2): maximum 2 per cent.

Loss on drying (2.2.32): maximum 10.0 per cent, determined on 1.000 g of the powdered drug (355) by drying in an oven at 100-105 °C for 2 h.

Total ash (2.4.16): maximum 9.0 per cent.

ASSAY

Liquid chromatography (2.2.29).

Test solution. In a flask, place 1.000 g of the powdered drug (355) and add 50 ml of *methanol R*. Heat in a water-bath at 60 °C for 30 min with shaking. Allow to cool and filter into a 100 ml volumetric flask. Rinse the flask and the filter with *methanol R* and dilute to 100.0 ml with the same solvent. Dilute 2.0 ml of the solution to 20.0 ml with *water R*.

Reference solution. Dissolve 5.0 mg of *oleuropein R* in 5.0 ml of *methanol R*. Dilute 1.0 ml of the solution to 25.0 ml with *water R*.

Column:

- size: $l = 0.15$ m, $\varnothing = 3.9$ mm,
- stationary phase: octadecylsilyl silica gel for chromatography *R* (5 µm),
- temperature: 25 °C.

Mobile phase:

- mobile phase A: dilute 1.0 ml of *glacial acetic acid R* to 100 ml with *water R*,
- mobile phase B: *methanol R*,

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)
0 - 5	85 → 40	15 → 60
5 - 12	40 → 20	60 → 80
12 - 15	20 → 85	80 → 15

Flow rate: 1 ml/min.

Detection: spectrophotometer at 254 nm.

Injection: 20 µl.

Retention time: oleuropein = about 9 min.

Calculate the percentage content of oleuropein from the expression.

$$\frac{A_1 \times m_2 \times p \times 8}{A_2 \times m_1}$$

A_1 = area of the peak due to oleuropein in the chromatogram obtained with the test solution,

A_2 = area of the peak due to oleuropein in the chromatogram obtained with the reference solution,

m_1 = mass of the drug to be examined, in grams,

m_2 = mass of *oleuropein R* in the reference solution, in grams,

p = percentage content of oleuropein in *oleuropein R*.

01/2005:1456

OLIVE OIL, REFINED

Olivae oleum raffinatum

DEFINITION

Refined olive oil is the fatty oil obtained by refining of crude olive oil, obtained by cold expression or other suitable mechanical means from the ripe drupes of *Olea europaea* L. A suitable antioxidant may be added.

CHARACTERS

A clear, colourless or greenish-yellow, transparent liquid, practically insoluble in alcohol, miscible with light petroleum (50 °C to 70 °C).

When cooled, it begins to become cloudy at 10 °C and becomes a butter-like mass at about 0 °C. It has a relative density of about 0.913.

IDENTIFICATION

- A. It complies with the test for absorbance (see Tests).
 B. Carry out the test for identification of fatty oils by thin-layer chromatography (2.3.2). The chromatogram obtained shows spots corresponding to those in the typical chromatogram for olive oil. For certain types of refined olive oil, the difference in the size of spots E and F is less pronounced than in the typical chromatogram.

TESTS

Acid value (2.5.1). Not more than 0.5, determined on 10.0 g.

Peroxide value (2.5.5, Method A). Not more than 10.0. If intended for use in the manufacture of parenteral dosage forms, not more than 5.0.

Unsaponifiable matter. Not more than 1.5 per cent. Place 5.0 g (*m* g) in a 150 ml flask fitted with a reflux condenser. Add 50 ml of 2 *M* alcoholic potassium hydroxide *R* and heat on a water-bath for 1 h, shaking frequently. Add 50 ml of water *R* through the top of the condenser, shake, allow to cool and transfer the contents of the flask to a separating funnel. Rinse the flask with several portions to a total of 50 ml of light petroleum *R1* and add the rinsings to the separating funnel. Shake vigorously for 1 min. Allow to separate and transfer the aqueous layer to a second separating funnel. If an emulsion forms, add small quantities of alcohol *R* or a concentrated solution of potassium hydroxide *R*. Shake the aqueous layer with 2 quantities, each of 50 ml, of light petroleum *R1*. Combine the light petroleum layers in a third separating funnel and wash with 3 quantities, each of 50 ml, of alcohol (50 per cent *V/V*) *R*. Transfer the light petroleum layer to a tared 250 ml flask. Rinse the separating funnel with small quantities of light petroleum *R1* and add to the flask. Evaporate the light petroleum on a water-bath and dry the residue at 100 °C to 105 °C for 15 min, keeping the flask horizontal. Allow to cool in a desiccator and weigh (*a* g). Repeat the drying for successive periods of 15 min until the loss of mass between 2 successive weighings does not exceed 0.1 per cent. Dissolve the residue in 20 ml of alcohol *R*, previously neutralised to 0.1 ml of bromophenol blue solution *R*. If necessary, titrate with 0.1 *M* hydrochloric acid (*b* ml).

Calculate the percentage content of unsaponifiable matter from the expression:

$$\frac{100(a - 0.032b)}{m}$$

If 0.032*b* is greater than 5 per cent of *a*, the test is invalid and must be repeated.

Alkaline impurities (2.4.19). It complies with the test for alkaline impurities in fatty oils.

Absorbance (2.2.25). Dissolve 1.00 g in cyclohexane *R* and dilute to 100.0 ml with the same solvent. The absorbance measured at 270 nm is 0.20 to 1.20.

Composition of fatty acids (2.4.22, Method A). The fatty acid fraction of the oil has the following composition:

- saturated fatty acids of chain length less than C_{16} : not more than 0.1 per cent,
- palmitic acid: 7.5 per cent to 20.0 per cent,
- palmitoleic acid (equivalent chain length on polyethyleneglycol adipate 16.3): not more than 3.5 per cent,
- stearic acid: 0.5 per cent to 5.0 per cent,

- oleic acid (equivalent chain length on polyethyleneglycol adipate 18.3): 56.0 per cent to 85.0 per cent,
- linoleic acid (equivalent chain length on polyethyleneglycol adipate 18.9): 3.5 per cent to 20.0 per cent,
- linolenic acid (equivalent chain length on polyethyleneglycol adipate 19.7): not more than 1.2 per cent,
- arachidic acid: not more than 0.7 per cent,
- eicosenoic acid (equivalent chain length on polyethyleneglycol adipate 20.3): not more than 0.4 per cent,
- behenic acid: not more than 0.2 per cent,
- lignoceric acid: not more than 0.2 per cent.

Sterols (2.4.23). The sterol fraction of the oil has the following composition:

- sum of contents of β -sitosterol, $\Delta 5,23$ -stigmastadienol, clerosterol, sitostanol, $\Delta 5$ -avenasterol and $\Delta 5,24$ -stigmastadienol: not less than 93.0 per cent,
- cholesterol: not more than 0.5 per cent,
- $\Delta 7$ -stigmasterol: not more than 0.5 per cent,
- campesterol: not more than 4.0 per cent,

and the content of stigmasterol is not more than that of campesterol.

Sesame oil. In a ground-glass-stoppered cylinder shake 10 ml for about 1 min with a mixture of 0.5 ml of a 0.35 per cent *V/V* solution of furfural *R* in acetic anhydride *R* and 4.5 ml of acetic anhydride *R*. Filter through a filter paper impregnated with acetic anhydride *R*. To the filtrate add 0.2 ml of sulphuric acid *R*. No bluish-green colour develops.

Water (2.5.32). If intended for use in the manufacture of parenteral dosage forms, not more than 0.1 per cent, determined on 5.0 g by the coulometric method. Use a mixture of equal volumes of decanol *R* and anhydrous methanol *R* as solvent.

STORAGE

Store in a well-filled container, protected from light, at a temperature not exceeding 25 °C. If intended for use in the manufacture of parenteral dosage forms, store under an inert gas.

LABELLING

The label states:

- where applicable, that the substance is suitable for use in the manufacture of parenteral dosage forms,
- the name and concentration of any added antioxidant,
- the name of the inert gas.

01/2005:0518

OLIVE OIL, VIRGIN

Olivae oleum virginale

DEFINITION

Virgin olive oil is the fatty oil obtained by cold expression or other suitable mechanical means from the ripe drupes of *Olea europaea* L.

CHARACTERS

A clear, yellow or greenish-yellow, transparent liquid with a characteristic odour, practically insoluble in alcohol, miscible with light petroleum (50 °C to 70 °C).

When cooled, it begins to become cloudy at 10 °C and becomes a butter-like mass at about 0 °C. It has a relative density of about 0.913.

IDENTIFICATION

Carry out the test for identification of fatty oils by thin-layer chromatography (2.3.2). The chromatogram obtained shows spots corresponding to those in the typical chromatogram for olive oil. For certain types of olive oil, the difference in the size of spots E and F is less pronounced than in the typical chromatogram.

TESTS

Acid value (2.5.1). Not more than 2.0, determined on 5.0 g.

Peroxide value (2.5.5, Method A). Not more than 20.0.

Unsaponifiable matter. Not more than 1.5 per cent. Place 5.0 g (*m* g) in a 150 ml flask fitted with a reflux condenser. Add 50 ml of 2 *M* alcoholic potassium hydroxide *R* and heat on a water-bath for 1 h, shaking frequently. Add 50 ml of water *R* through the top of the condenser, shake, allow to cool and transfer the contents of the flask to a separating funnel. Rinse the flask with several portions to a total of 50 ml of light petroleum *R1* and add the rinsings to the separating funnel. Shake vigorously for 1 min. Allow to separate and transfer the aqueous layer to a second separating funnel. If an emulsion forms, add small quantities of alcohol *R* or a concentrated solution of potassium hydroxide *R*. Shake the aqueous layer with 2 quantities, each of 50 ml, of light petroleum *R1*. Combine the light petroleum layers in a third separating funnel and wash with 3 quantities, each of 50 ml, of alcohol (50 per cent *V/V*) *R*. Transfer the light petroleum layer to a tared 250 ml flask. Rinse the separating funnel with small quantities of light petroleum *R1* and add to the flask. Evaporate the light petroleum on a water-bath and dry the residue at 100 °C to 105 °C for 15 min, keeping the flask horizontal. Allow to cool in a desiccator and weigh (*a* g). Repeat the drying for successive periods of 15 min until the loss of mass between 2 successive weighings does not exceed 0.1 per cent. Dissolve the residue in 20 ml of alcohol *R*, previously neutralised to 0.1 ml of bromophenol blue solution *R*. If necessary, titrate with 0.1 *M* hydrochloric acid (*b* ml).

Calculate the percentage content of unsaponifiable matter from the expression:

$$\frac{100(a - 0.032b)}{m}$$

If 0.032*b* is greater than 5 per cent of *a*, the test is invalid and must be repeated.

Absorbance (2.2.25). Dissolve 1.00 g in cyclohexane *R* and dilute to 100.0 ml with the same solvent. The absorbance measured at 270 nm is not greater than 0.20. The ratio of the absorbance at 232 nm to that at 270 nm is greater than 8.

Composition of fatty acids (2.4.22, Method A). The fatty acid fraction of the oil has the following composition:

- saturated fatty acids of chain length less than *C*₁₆: not more than 0.1 per cent,
- palmitic acid: 7.5 per cent to 20.0 per cent,
- palmitoleic acid (equivalent chain length on polyethyleneglycol adipate 16.3): not more than 3.5 per cent,
- stearic acid: 0.5 per cent to 5.0 per cent,
- oleic acid (equivalent chain length on polyethyleneglycol adipate 18.3): 56.0 per cent to 85.0 per cent,

- linoleic acid (equivalent chain length on polyethyleneglycol adipate 18.9): 3.5 per cent to 20.0 per cent,
- linolenic acid (equivalent chain length on polyethyleneglycol adipate 19.7): not more than 1.2 per cent,
- arachidic acid: not more than 0.7 per cent,
- eicosenoic acid (equivalent chain length on polyethyleneglycol adipate 20.3): not more than 0.4 per cent,
- behenic acid: not more than 0.2 per cent,
- lignoceric acid: not more than 0.2 per cent.

Sterols (2.4.23). The sterol fraction of the oil has the following composition:

- sum of contents of β -sitosterol, $\Delta^5,23$ -stigmastadienol, clerosterol, sitostanol, Δ^5 -avenasterol and $\Delta^5,24$ -stigmastadienol: not less than 93.0 per cent,
 - cholesterol: not more than 0.5 per cent,
 - Δ^7 -stigmasterol: not more than 0.5 per cent,
 - campesterol: not more than 4.0 per cent,
- and the content of stigmasterol is not more than that of campesterol.

Sesame oil. In a ground-glass-stoppered cylinder shake 10 ml for about 1 min with a mixture of 0.5 ml of a 0.35 per cent *V/V* solution of furfural *R* in acetic anhydride *R* and 4.5 ml of acetic anhydride *R*. Filter through a filter paper impregnated with acetic anhydride *R*. To the filtrate add 0.2 ml of sulphuric acid *R*. No bluish-green colour develops.

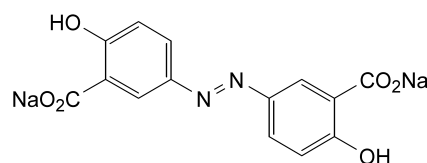
STORAGE

Store in a well-filled container, protected from light, at a temperature not exceeding 25 °C.

01/2005:1457

OLSALAZINE SODIUM

Olsalazinum natricum



$C_{14}H_8N_2Na_2O_6$

M_r 346.2

DEFINITION

Olsalazine sodium contains not less than 98.0 per cent and not more than the equivalent of 102.0 per cent of disodium 3,3'-diazenediylbis(6-hydroxybenzoate), calculated with reference to the dried and acetate-free substance.

CHARACTERS

A yellow, fine, crystalline powder, sparingly soluble in water, soluble in dimethyl sulphoxide, very slightly soluble in methanol.

It shows polymorphism.

IDENTIFICATION

First identification: B, D.

Second identification: A, C, D.

A. Dissolve 40.0 mg in 5 ml of 0.1 *M* sodium hydroxide and dilute to 100.0 ml with a 7.8 g/l solution of sodium dihydrogen phosphate *R* adjusted to pH 7.2 with strong sodium hydroxide solution *R* (buffer solution). Dilute

2.0 ml of the solution to 100.0 ml with the buffer solution. Examined between 240 nm and 400 nm (2.2.25), the solution shows absorption maxima at 255 nm and 362 nm. The ratio of the absorbance measured at the maximum at 255 nm to that measured at the maximum at 362 nm is 0.53 to 0.56.

B. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *olsalazine sodium CRS*. If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in *methanol R*, evaporate to dryness and record new spectra using the residues.

C. Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel F₂₅₄ plate R*.

Test solution. Dissolve 10 mg of the substance to be examined in a mixture of 1 volume of *dilute ammonia R2* and 4 volumes of *alcohol R* and dilute to 10 ml with the same mixture of solvents.

Reference solution (a). Dissolve 10 mg of *olsalazine sodium CRS* in a mixture of 1 volume of *dilute ammonia R2* and 4 volumes of *alcohol R* and dilute to 10 ml with the same mixture of solvents.

Reference solution (b). Dissolve 5 mg of *sulfasalazine CRS* in reference solution (a) and dilute to 5 ml with reference solution (a).

Apply to the plate 10 µl of each solution. Develop over a path of 15 cm using a mixture of 5 volumes of *anhydrous formic acid R*, 50 volumes of *acetone R* and 60 volumes of *methylene chloride R*. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. The principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a). The test is not valid unless the chromatogram obtained with reference solution (b) shows two separated spots.

D. To 0.5 g of the substance to be examined add 2 ml of *sulphuric acid R*. Progressively heat to ignition and continue heating until an almost white, or at most greyish, residue is obtained. Carry out the ignition at a temperature up to 800 °C. Dissolve the residue in 10 ml of boiling *water R* and filter. 2 ml of the filtrate gives reaction (a) of sodium (2.3.1).

TESTS

Acetate. Not more than 1.0 per cent, determined by liquid chromatography (2.2.29).

Test solution. Dissolve 0.125 g of the substance to be examined in 25.0 ml of *water R* and add 1.0 ml of *dilute hydrochloric acid R*. Centrifuge and then filter the solution through a 0.45 µm filter and also through an appropriate filter for removal of chlorides.

Reference solution (a). Dissolve 0.140 g of *sodium acetate R*, 0.150 g of *sodium formate R* and 0.180 g of *potassium sulphate R* in 100.0 ml of *water R*. Dilute 1.0 ml of this solution to 100.0 ml with *water R*.

Reference solution (b). Use suitable amounts of *sodium acetate R* to prepare not fewer than five reference solutions containing 10 µg/ml to 50 µg/ml of acetate.

The ion-exclusion chromatographic procedure may be carried out using:

- a separation column 0.25 m long and 6 mm in internal diameter packed with *ion-exclusion resin for chromatography R* with a capacity of about 27 meq/column.

- a suppressor column,
- as mobile phase at a flow rate of 0.9 ml/min 0.0001 M *hydrochloric acid*,
- a conductivity detector set at 10 µS·cm⁻¹.

Inject 0.1 ml of reference solution (a). The chromatogram shows three separated peaks. Inject 0.1 ml of the test solution and 0.1 ml of reference solution (b). Prepare a calibration curve from the average of the readings obtained with the reference solutions and determine the concentration of acetate in the test solution from the curve obtained. Measure the peak area for acetate. Calculate the percentage content of acetate content from the following expression:

$$\frac{2.6 \ c}{m}$$

c = concentration of acetate in the test solution (µg/ml), determined by linear interpolation of the standard curve for reference solution (b),

m = mass of sample (mg).

Methanesulphonic acid. Liquid chromatography (2.2.29).

Test solution. Dissolve 0.25 g of the substance to be examined in 20 ml of *water R*, add 1.0 ml of *dilute hydrochloric acid R* and dilute to 25.0 ml with *water R*. Centrifuge and then filter the solution through a 0.45 µm filter and also through an appropriate filter for removal of chloride.

Reference solution (a). Dissolve 0.25 g of *methanesulphonic acid R* in 50 ml of *water R*. Add 0.58 g of *sodium acetate R* and 0.08 g of *sodium chloride R* and dilute to 100.0 ml with *water R*. Dilute 1.0 ml of the solution to 100.0 ml with *water R*.

Reference solution (b). Dissolve 0.10 g of *methanesulphonic acid R* in *water R* and dilute to 100.0 ml with *water R*. Dilute 3.0 ml of the solution to 100.0 ml with *water R*.

The reversed-phase ion chromatographic procedure may be carried out using:

- a pre-column 0.035 m long and 4 mm in internal diameter packed with *reversed-phase ion resin for chromatography R* (10 µm),
- a separation column 0.25 m long and 4 mm in internal diameter packed with *reversed-phase ion resin for chromatography R* (10 µm),
- as mobile phase at a flow rate of 1.0 ml/min, a mixture of 10 volumes of *acetonitrile for chromatography R* and 990 volumes of a solution containing 1.6 g/l of *tetrabutylammonium hydroxide R* and 0.053 g/l of *anhydrous sodium carbonate R*,
- a conductivity detector set at 50 µS·cm⁻¹.

Inject 100 µl of reference solution (a). The test is not valid unless the chromatogram shows 3 separated peaks. Inject 100 µl each of the test solution and reference solution (b). In the chromatogram obtained with the test solution, the area of the peak corresponding to methanesulphonic acid is not greater than the area of the corresponding peak in the chromatogram obtained with reference solution (b) (0.3 per cent).

Related substances. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 20.0 mg of the substance to be examined in mobile phase A and dilute to 25.0 ml with mobile phase A.

Reference solution (a). Dilute 0.5 ml of the test solution to 100.0 ml with the mobile phase A.

Reference solution (b). Dissolve 20.0 mg of *olsalazine sodium for performance test CRS* in mobile phase A and dilute to 25.0 ml with mobile phase A.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.125 m long and 4.0 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (5 µm),
- as mobile phase at a flow rate of 1 ml/min, the following linear gradient programme:

Mobile phase A. Dissolve 2.38 g of *tetrabutylammonium hydrogen sulphate R* and 3.6 g of *disodium hydrogen phosphate dihydrate R* in 900 ml of *water R*. Adjust to pH 7.6 with *dilute sodium hydroxide solution R*. Dilute to 1000.0 ml with *water R*. Mix 700 ml of this buffer solution with 300 ml of *methanol R*,

Mobile phase B. Dissolve 4.75 g of *tetrabutylammonium hydrogen sulphate R* and 3.6 g of *disodium hydrogen phosphate dihydrate R* in 900 ml of *water R*. Adjust to pH 7.6 with *dilute sodium hydroxide solution R*. Dilute to 1000.0 ml with *water R*. Mix 350 ml of this buffer solution with 650 ml of *methanol R*,

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)	Comment
0 - 15	55	45	isocratic
15 - 45	55 → 0	45 → 100	linear gradient
45 - 50	0 → 55	100 → 45	return to initial composition
50 - 65	55	45	equilibration

- as detector a spectrophotometer set at 360 nm,

maintaining the temperature of the column at 30 °C.

Inject 20 µl of reference solution (a). Adjust the sensitivity of the system so that the height of the principal peak in the chromatogram obtained is at least 50 per cent of the full scale of the recorder.

Inject 20 µl of reference solution (b). The test is not valid unless the chromatogram obtained is similar to the chromatogram obtained with *olsalazine sodium for performance test CRS*. If necessary, adjust the proportion of mobile phase A in the mobile phase (increasing the proportion of mobile phase A increases the retention time).

Inject 20 µl of the test solution. In the chromatogram obtained with the test solution: the area of any peak, apart from the principal peak, is not greater than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (1 per cent), and not more than one such peak has an area greater than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent); the sum of the areas of all the peaks, apart from the principal peak, is not greater than four times the area of the principal peak in the chromatogram obtained with reference solution (a) (2 per cent). Disregard any peak with an area less than 0.05 times that of the principal peak in the chromatogram obtained with reference solution (a) (0.025 per cent).

Heavy metals (2.4.8). 1.0 g complies with limit test D for heavy metals (20 ppm). Prepare the standard using 2 ml of *lead standard solution (10 ppm Pb) R*.

Loss on drying (2.2.32). Not more than 2.0 per cent, determined on 1.000 g by drying in an oven at 150 °C.

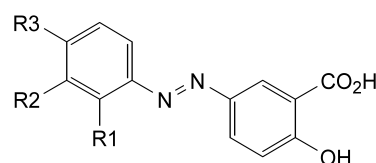
ASSAY

Dissolve 0.100 g in 15 ml of *ethylene glycol R*. Add 40 ml of *dioxan R* and 0.2 ml of a 224 g/l solution of *potassium chloride R*. Titrate with 0.1 M *hydrochloric acid*, determining the end-point potentiometrically (2.2.20). Carry out a blank titration.

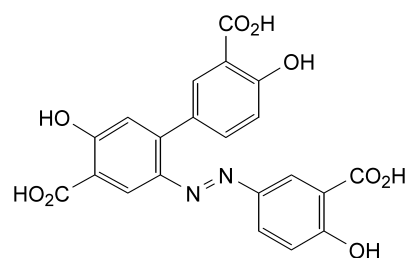
Correct the volume consumed for the content of acetate, taking the molecular mass of acetate to be 59.0.

1 ml of 0.1 M *hydrochloric acid* is equivalent to 17.31 mg of C₁₄H₈N₂Na₂O₆.

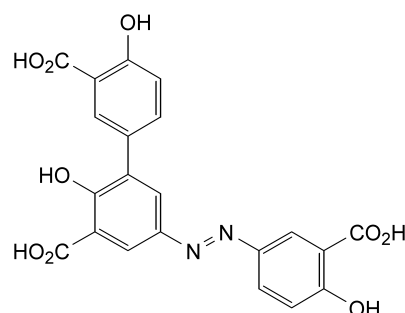
IMPURITIES



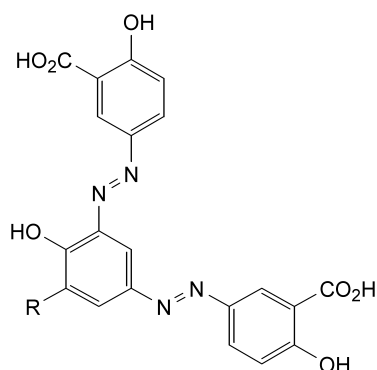
- A. R1 = H, R2 = CO₂H, R3 = OCH₃: 6-hydroxy-6'-methoxy-3,3'-diazenediylidibenzoic acid,
- B. R1 = OH, R2 = CO₂H, R3 = H: 2,6'-dihydroxy-3,3'-diazenediylidibenzoic acid,
- C. R1 = R2 = H, R3 = OH: 2-hydroxy-5-[(4-hydroxyphenyl)diazenyl]benzoic acid,
- D. R1 = H, R2 = CO₂H, R3 = Cl: 6-chloro-6'-hydroxy-3,3'-diazenediylidibenzoic acid,
- E. R1 = H, R2 = CO-CH₂-SO₃H, R3 = OH: 2-hydroxy-5-[[4-hydroxy-3-(sulphoacetyl)phenyl]diazenyl]benzoic acid,



- F. 2'-[(3-carboxy-4-hydroxyphenyl)diazenyl]-4,5'-dihydroxybiphenyl-3,4'-dicarboxylic acid,



- G. 5-[(3-carboxy-4-hydroxyphenyl)diazenyl]-2,4'-dihydroxybiphenyl-3,3'-dicarboxylic acid,



- H. R = CO₂H: 3,3'-[5-carboxy-4-hydroxy-1,3-phenylenebis(diazenediyl)]bis(6-hydroxybenzoic) acid,
- I. R = H: 3,3'-[4-hydroxy-1,3-phenylenebis(diazenediyl)]bis(6-hydroxybenzoic) acid.

01/2005:2063
corrected

OMEGA-3-ACID ETHYL ESTERS 60

Omega-3 acidorum esteri ethylici 60

DEFINITION

Ethyl esters of *alpha*-linolenic acid (C18:3 n-3), moroctic acid (C18:4 n-3), eicosatetraenoic acid (C20:4 n-3), timnodonic (eicosapentaenoic) acid (C20:5 n-3; EPA), heneicosapentaenoic acid (C21:5 n-3), clupanodonic acid (C22:5 n-3) and cervonic (docosahexaenoic) acid (C22:6 n-3; DHA). Omega-3-acid ethyl esters 60 are obtained by transesterification of the body oil of fat fish species coming from families like *Engraulidae*, *Carangidae*, *Clupeidae*, *Osmeridae*, *Salmonidae* and *Scombridae* and subsequent physico-chemical purification processes, including molecular distillation. The minimum content of total omega-3-acid ethyl esters and the minimum content of the omega-3-acids EPA and DHA ethyl esters are indicated in Table 2063-1.

Table 2063-1

Total omega-3-acid ethyl esters	EPA and DHA ethyl esters	EPA ethyl esters	DHA ethyl esters
Minimum content (per cent)			
65	50	25	20
60	50	–	40
55	50	40	–

Tocopherol may be added as an antioxidant.

CHARACTERS

Appearance: light yellow liquid.

It has a slight fish-like odour.

Solubility: practically insoluble in water, very soluble in acetone, in ethanol, in heptane and in methanol.

IDENTIFICATION

- A. Examine the chromatograms obtained in the assay for EPA and DHA ethyl esters.

Results: the peaks due to eicosapentaenoic acid ethyl ester and to docosahexaenoic acid ethyl ester in the chromatogram obtained with the test solution are similar in retention time to the corresponding peaks in the chromatogram obtained with the reference solution.

- B. The substance to be examined complies with the assay for Total omega-3-acid ethyl esters.

TESTS

Absorbance (2.2.25): maximum 0.60 at 233 nm.

Dilute 0.300 g of the substance to be examined to 50.0 ml with *trimethylpentane R*. Dilute 2.0 ml of this solution to 50.0 ml with *trimethylpentane R*.

Acid value (2.5.1): maximum 2.0, determined on 10 g in 50 ml of the prescribed mixture of solvents.

Peroxide value (2.5.5, Method A): maximum 10.0.

Anisidine value: maximum 20.0.

The anisidine value is defined as 100 times the absorbance measured in a 1 cm cell of a solution containing 1 g of the substance to be examined in 100 ml of a mixture of solvents and reagents according to the method described below.

Carry out the operations as rapidly as possible, avoiding exposure to actinic light.

Test solution (a). Dilute 0.500 g of the substance to be examined to 25.0 ml with *trimethylpentane R*.

Test solution (b). To 5.0 ml of test solution (a) add 1.0 ml of a 2.5 g/l solution of *p*-anisidine *R* in *glacial acetic acid R*, shake and protect from light.

Reference solution. To 5.0 ml of *trimethylpentane R* add 1.0 ml of a 2.5 g/l solution of *p*-anisidine *R* in *glacial acetic acid R*, shake and protect from light.

Measure the absorbance (2.2.25) of test solution (a) at 350 nm using *trimethylpentane R1* as the compensation liquid. Measure the absorbance of test solution (b) at 350 nm exactly 10 min after its preparation, using the reference solution as the compensation liquid.

Calculate the anisidine value from the expression:

$$\frac{25 \times (1.2 A_s - A_b)}{m}$$

A_s = absorbance of test solution (b) at 350 nm,

A_b = absorbance of test solution (a) at 350 nm,

m = mass of the substance to be examined in test solution (a), in grams.

Oligomers and partial glycerides. Size exclusion chromatography (2.2.30).

Test solution. Dilute 10.0 mg of the substance to be examined to 10.0 ml with *tetrahydrofuran R*.

Reference solution. In a 100 ml volumetric flask dissolve 50 mg of *monodocosahexaenoin R*, 30 mg of *didocosahexaenoin R* and 20 mg of *tridocosahexaenoin R* in *tetrahydrofuran R* and dilute to 100.0 ml with the same solvent.

Column 1:

– **dimensions:** $l = 0.3$ m, $\varnothing = 7.8$ mm,

– **stationary phase:** *styrene-divinylbenzene copolymer R* (7 μ m), with a pore size of 10 nm.

Columns 2 and 3 placed closest to the injector:

– **dimensions:** $l = 0.3$ m, $\varnothing = 7.8$ mm,

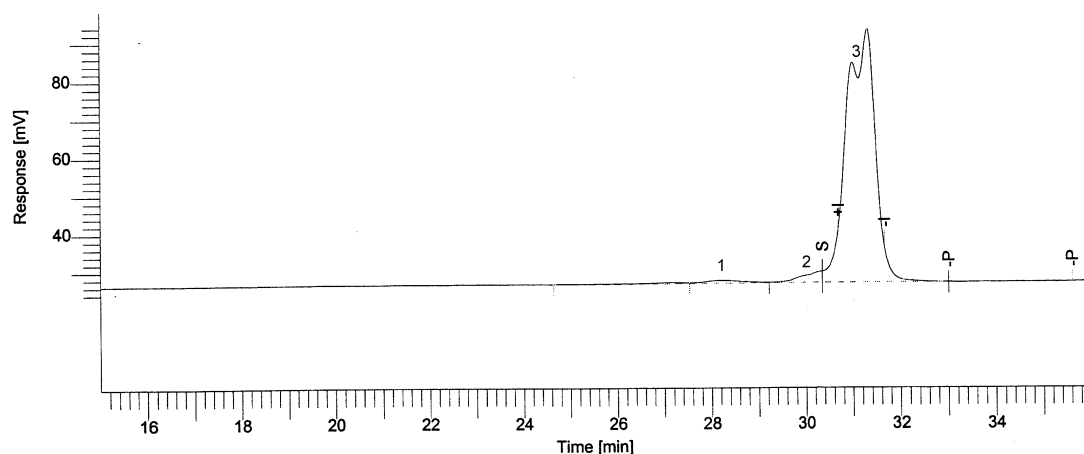
– **stationary phase:** *styrene-divinylbenzene copolymer R* (7 μ m), with a pore size of 50 nm.

Mobile phase: *tetrahydrofuran R*.

Flow rate: 0.8 ml/min.

Detection: differential refractometer.

Injection: 40 μ l.

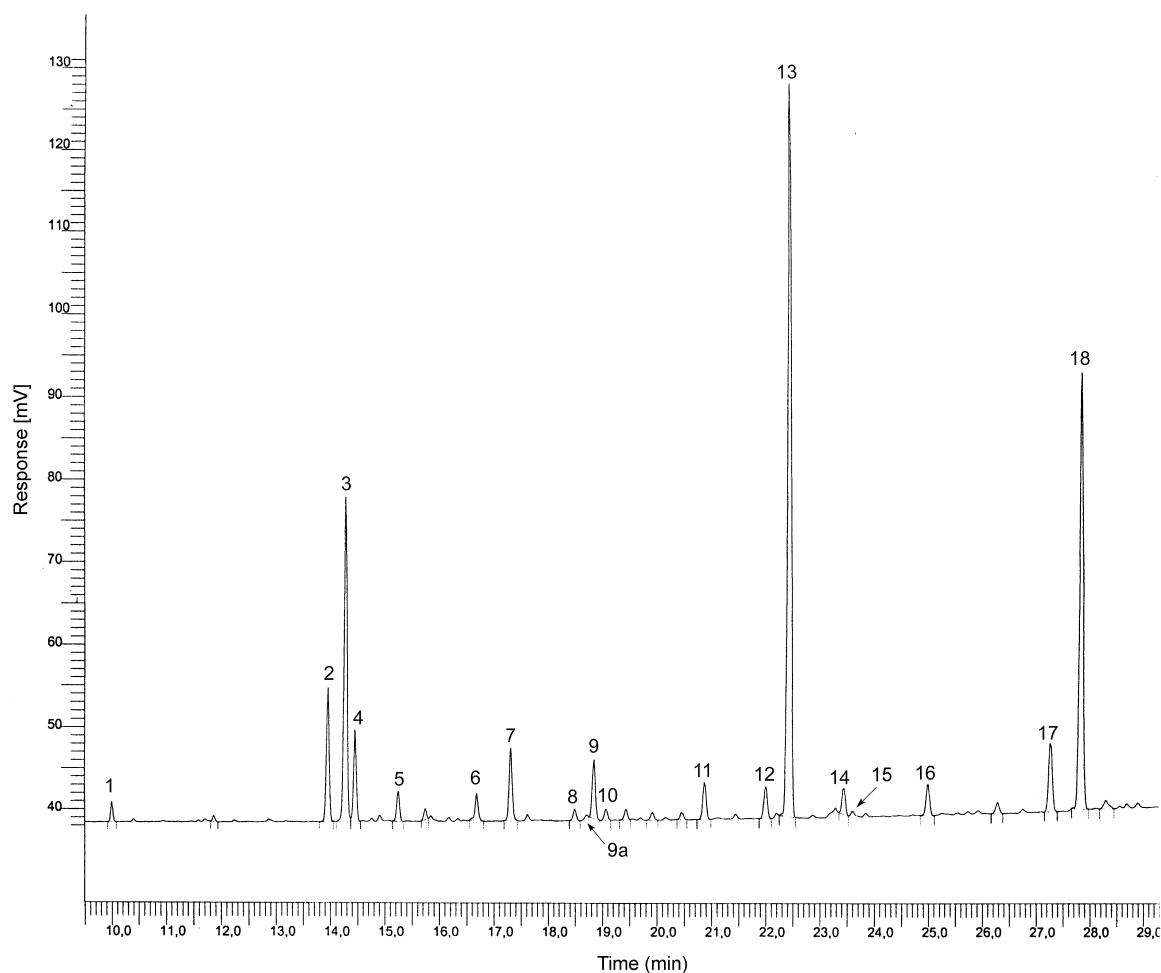


1. oligomers

2. monoglycerides

3. fatty acid ethyl esters

Figure 2063.1. — Chromatogram for the test for oligomers and partial glycerides in omega-3-acid ethyl esters 60



- | | | | | |
|--------------|--------------|----------------|----------------|---------------|
| 1. C16:0 | 5. C18:2 n-6 | 9. C20:1 n-9 | 12. C20:4 n-6 | 16. C21:5 n-3 |
| 2. C18:0 | 6. C18:3 n-3 | 9a. C20:1 n-11 | 13. C20:5 n-3 | 17. C22:5 n-6 |
| 3. C18:1 n-9 | 7. C18:4 n-3 | 10. C20:1 n-7 | 14. C22:1 n-11 | 18. C22:6 n-3 |
| 4. C18:1 n-7 | 8. C20:0 | 11. C20:4 n-3 | 15. C22:1 n-9 | |

Figure 2063.2. — Chromatogram for the assay of omega-3-acid ethyl ester in omega-3-acid ethyl esters

System suitability:

01/2005:1250

- *elution order* in the chromatogram obtained with the reference solution: tridocosahexaenoin, didocosahexaenoin and monodocosahexaenoin;
- *resolution* in the chromatogram obtained with the reference solution: minimum of 2.0 between the peaks due to monodocosahexaenoin and to didocosahexaenoin; minimum of 1.0 between the peaks due to didocosahexaenoin and tridocosahexaenoin,
- if the method of standard addition to the test solution is used, the recovery for the added *eicosapentaenoic acid ethyl ester CRS* or *docosahexaenoic acid ethyl ester CRS* is minimum 95 per cent.

Calculate the percentage content of oligomers plus partial glycerides using the following expression:

$$\frac{B}{A} \times 100$$

- A* = sum of areas of all the peaks in the chromatogram,
B = sum of the areas of the peaks with a retention time smaller than those of the ethyl ester peaks.

The ethyl ester peaks, which may be present in the form of an unresolved double peak, are identified as the major peaks in the chromatogram (Figure 2063.-1).

Limit:

- *oligomers + partial glycerides*: maximum 7.0 per cent.

ASSAY

EPA and DHA ethyl esters (2.4.29). See Figure 2063.-2.

Total omega-3-acids ethyl esters (2.4.29). See Figure 2063.-2.

STORAGE

Under an inert gas, in an airtight container, protected from light.

LABELLING

The label states:

- the content of total omega-3-acid ethyl esters,
- the content of EPA ethyl ester and DHA ethyl ester,
- the concentration of any added tocopherol.

OMEGA-3-ACID ETHYL ESTERS 90

Omega-3 acidorum esteri ethylici 90

DEFINITION

Ethyl esters of *alpha*-linolenic acid (C18:3 n-3), morotic acid (C18:4 n-3), eicosatetraenoic acid (C20:4 n-3), timnodonic (eicosapentaenoic) acid (C20:5 n-3; EPA), heneicosapentaenoic acid (C21:5 n-3), clupanodonic acid (C22:5 n-3) and cervonic (docosahexaenoic) acid (C22:6 n-3; DHA). Omega-3-acid ethyl esters are obtained by transesterification of the body oil of fat fish species coming from families such as *Engraulidae*, *Carangidae*, *Clupeidae*, *Osmeridae*, *Salmonidae* and *Scombridae* and subsequent physico-chemical purification processes, including urea fractionation followed by molecular distillation.

Content:

- EPA and DHA ethyl esters: minimum 80 per cent, with minimum 40 per cent of EPA ethyl esters and minimum 34 per cent of DHA ethyl esters,
 - total omega-3-acid ethyl esters: minimum 90 per cent.
- Tocopherol may be added as an antioxidant.

CHARACTERS

Appearance: light yellow liquid.

It has a slight fish-like odour.

Solubility: practically insoluble in water, very soluble in acetone, in ethanol, in heptane and in methanol.

IDENTIFICATION

Examine the chromatograms obtained in the assay for EPA and DHA ethyl esters.

Results: the peaks due to eicosapentaenoic acid ethyl ester and to docosahexaenoic acid ethyl ester in the chromatogram obtained with the test solution are similar in retention time and size to the corresponding peaks in the chromatogram obtained with the reference solution.

TESTS

Absorbance (2.2.25): maximum 0.55 at 233 nm.

Dilute 0.300 g of the substance to be examined to 50.0 ml with *trimethylpentane R*. Dilute 2.0 ml of this solution to 50.0 ml with *trimethylpentane R*.

Acid value (2.5.1): maximum 2.0, determined on 10 g in 50 ml of the prescribed mixture of solvents.

Anisidine value: maximum 20.0.

The anisidine value is defined as 100 times the absorbance measured in a 1 cm cell of a solution containing 1 g of the substance to be examined in 100 ml of a mixture of solvents and reagents according to the method described below.

Carry out the operations as rapidly as possible, avoiding exposure to actinic light.

Test solution (a). Dilute 0.500 g of the substance to be examined to 25.0 ml with *trimethylpentane R*.

Test solution (b). To 5.0 ml of test solution (a) add 1.0 ml of a 2.5 g/l solution of *p-anisidine R* in *glacial acetic acid R*, shake and protect from light.

Reference solution. To 5.0 ml of *trimethylpentane R* add 1.0 ml of a 2.5 g/l solution of *p-anisidine R* in *glacial acetic acid R*, shake and protect from light.

Measure the absorbance (2.2.25) of test solution (a) at 350 nm using *trimethylpentane R1* as the compensation liquid. Measure the absorbance of test solution (b) at 350 nm exactly 10 min after its preparation, using the reference solution as the compensation liquid.

Calculate the anisidine value from the expression:

$$\frac{25 \times (1.2A_s - A_b)}{m}$$

- A_s = absorbance of test solution (b) at 350 nm,
 A_b = absorbance of test solution (a) at 350 nm,
 m = mass of the substance to be examined in test solution (a), in grams.

Peroxide value (2.5.5, *Method A*): maximum 10.0.

Oligomers. Size-exclusion chromatography (2.2.30).

Test solution. Dilute 10.0 mg of the substance to be examined to 10.0 ml with *tetrahydrofuran R*.

Reference solution. Into a 100 ml volumetric flask dissolve 50 mg of *monodocosahexaenoin R*, 30 mg of *didocosahexaenoin R* and 20 mg of *tridocosahexaenoin R* in *tetrahydrofuran R* and dilute to 100.0 ml with the same solvent.

Column 1:

- size: $l = 0.3$ m, $\varnothing = 7.8$ mm,
- stationary phase: *styrene-divinylbenzene copolymer R* (7 μ m) with a pore size of 10 nm.

Columns 2 and 3 placed closest to the injector:

- size: $l = 0.3$ m, $\varnothing = 7.8$ mm,
- stationary phase: *styrene-divinylbenzene copolymer R* (7 μ m) with a pore size of 50 nm.

Mobile phase: *tetrahydrofuran R*.

Flow rate: 0.8 ml/min.

Detection: differential refractometer.

Injection: 40 μ l.

System suitability:

- **elution order** in the chromatogram obtained with the reference solution: tridocosahexaenoin, didocosahexaenoin, monodocosahexaenoin,
- **resolution** in the chromatogram obtained with the reference solution: minimum of 2.0 between the peaks due to monodocosahexaenoin and to didocosahexaenoin and minimum of 1.0 between the peaks due to didocosahexaenoin and to tridocosahexaenoin,
- if the method of standard addition to the test solution is used, the recovery for the added *eicosapentaenoic acid ethyl ester CRS* or *docosahexaenoic acid ethyl ester CRS* is minimum 95 per cent.

Calculate the percentage content of oligomers using the following expression:

$$\frac{B}{A} \times 100$$

- A = sum of areas of all the peaks in the chromatogram,
 B = sum of the areas of the peaks with a retention time smaller than those of the ethyl ester peaks.

The ethyl ester peaks, which may be present in the form of an unresolved double peak, are identified as the major peaks in the chromatogram (Figure 1250-1).

Limit:

- **oligomers:** maximum 1.0 per cent.

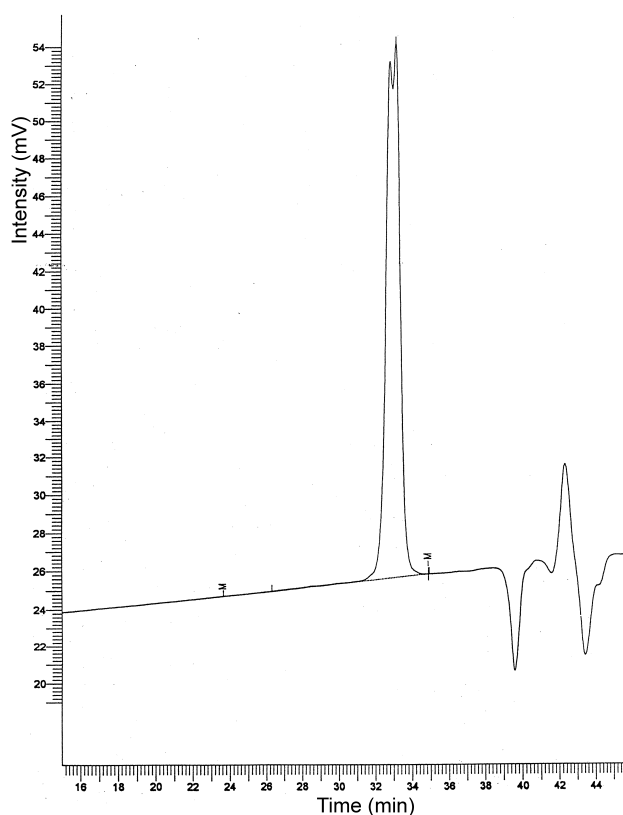


Figure 1250-1. – Chromatogram of the test for oligomers in omega-3-acid ethyl esters 90

ASSAY

EPA and DHA ethyl esters (2.4.29). See Figure 1250-2.

Total omega-3-acid ethyl esters (2.4.29). See Figure 1250-2.

STORAGE

Under an inert gas, in an airtight container, protected from light.

LABELLING

The label states the concentration of any added tocopherol.

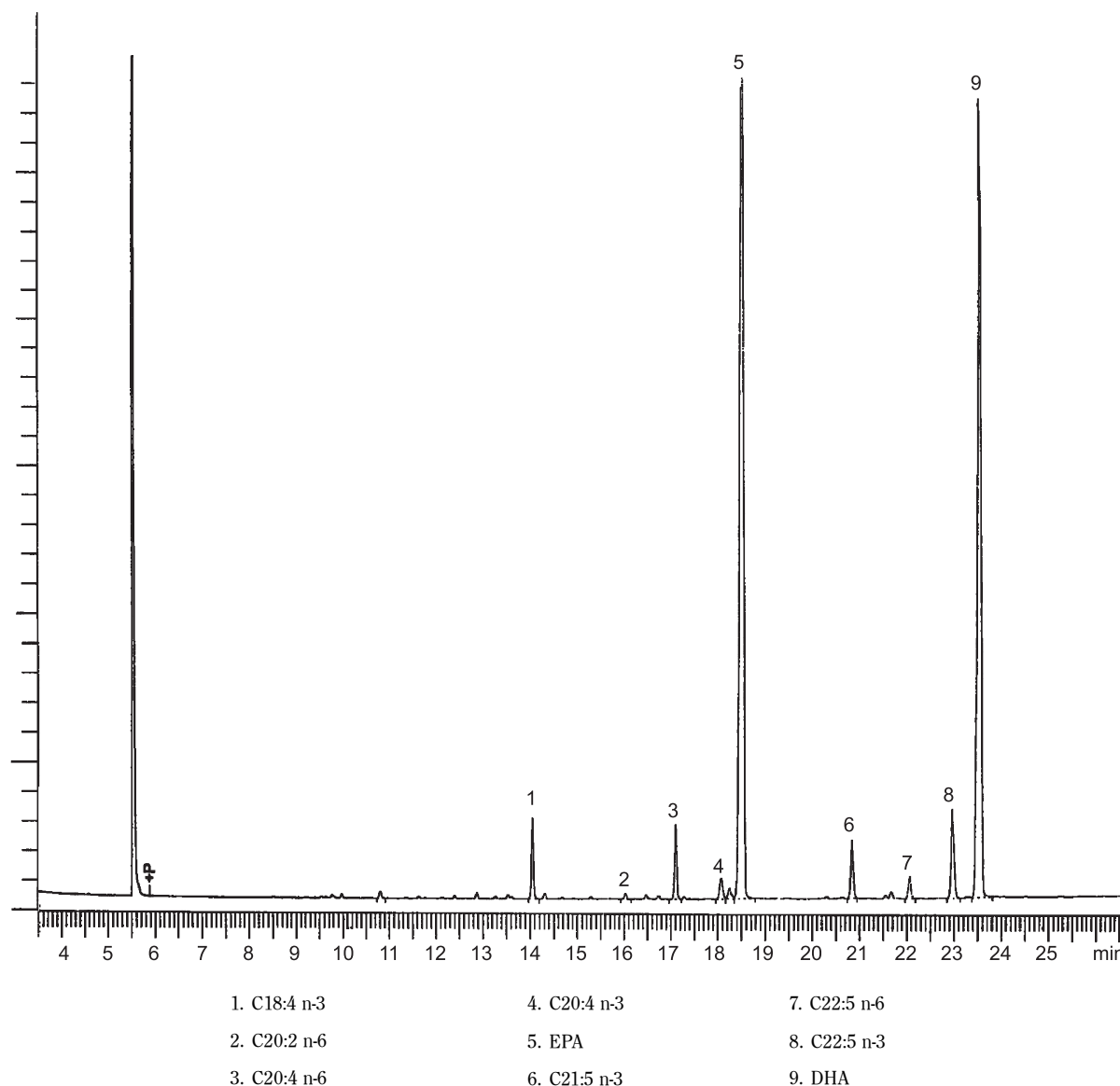


Figure 1250.-2. - Chromatogram for the assay of total omega-3-acid ethyl esters in omega-3-acid ethyl esters 90

01/2005:1352 — total omega-3 acids, expressed as triglycerides: minimum 60.0 per cent.

Tocopherol may be added as an antioxidant.

OMEGA-3-ACID TRIGLYCERIDES

Omega-3 acidorum triglycerida

DEFINITION

Mixture of mono-, di- and triesters of omega-3 acids with glycerol containing mainly triesters and obtained either by esterification of concentrated and purified omega-3 acids with glycerol or by transesterification of the omega-3 acid ethyl esters with glycerol. The origin of the omega-3 acids is the body oil from fatty fish species coming from families like *Engraulidae*, *Carangidae*, *Clupeidae*, *Osmeridae*, *Salmonidae* and *Scombridae*. The omega-3 acids are identified as the following acids: alpha-linolenic acid (C18:3 n-3), moroctic acid (C18:4 n-3), eicosatetraenoic acid (C20:4 n-3), timnodonic (eicosapentaenoic) acid (C20:5 n-3; EPA), heneicosapentaenoic acid (C21:5 n-3), clupanodonic acid (C22:5 n-3) and cervonic (docosahexaenoic) acid (C22:6 n-3; DHA).

Content:

- sum of the contents of the omega-3 acids EPA and DHA, expressed as triglycerides: minimum 45.0 per cent,

CHARACTERS

Appearance: pale yellow liquid.

Solubility: practically insoluble in water, very soluble in acetone and in heptane, slightly soluble in ethanol.

IDENTIFICATION

Examine the chromatograms obtained in the assay for EPA and DHA.

Results: the peaks due to eicosapentaenoic acid methyl ester and to docosahexaenoic acid methyl ester in the chromatogram obtained with test solution (b) are similar in retention time and size to the corresponding peaks in the chromatogram obtained with reference solution (a).

TESTS

Absorbance (2.2.25): maximum 0.73 at 233 nm.

Dilute 0.300 g of the substance to be examined to 50.0 ml with *trimethylpentane R*. Dilute 2.0 ml of this solution to 50.0 ml with *trimethylpentane R*.

Acid value (2.5.1): maximum 3.0, determined on 10.0 g in 50 ml of the prescribed mixture of solvents.

Anisidine value: maximum 30.0.

The anisidine value is defined as 100 times the absorbance measured in a 1 cm cell filled with a solution containing 1 g of the substance to be examined in 100 ml of a mixture of solvents and reagents according to the method described below.

Carry out the operations as rapidly as possible, avoiding exposure to actinic light.

Test solution (a). Dilute 0.500 g of the substance to be examined to 25.0 ml with *trimethylpentane R*.

Test solution (b). To 5.0 ml of test solution (a) add 1.0 ml of a 2.5 g/l solution of *p-anisidine R* in *glacial acetic acid R*, shake and store protected from light.

Reference solution. To 5.0 ml of *trimethylpentane R* add 1.0 ml of a 2.5 g/l solution of *p-anisidine R* in *glacial acetic acid R*, shake and store protected from light.

Measure the absorbance (2.2.25) of test solution (a) at 350 nm using *trimethylpentane R* as the compensation liquid. Measure the absorbance of test solution (b) at 350 nm exactly 10 min after its preparation, using the reference solution as the compensation liquid.

Calculate the anisidine value from the expression:

$$\frac{25 \times (1.2A_s - A_b)}{m}$$

A_s = absorbance of test solution (b),

A_b = absorbance of test solution (a),

m = mass of the substance to be examined in test solution (a), in grams.

Peroxide value (2.5.5, Method A): maximum 10.0.

Oligomers and partial glycerides. Size-exclusion chromatography (2.2.30).

Test solution. Dilute 10.0 mg of the substance to be examined to 10.0 ml with *tetrahydrofuran R*.

Reference solution. In a 100 ml volumetric flask dissolve 50 mg of *monodocosahexaenoin R*, 30 mg of *didocosahexaenoin R* and 20 mg of *tridocosahexaenoin R* in *tetrahydrofuran R* and dilute to 100.0 ml with the same solvent.

Column 1:

- **dimensions:** $l = 0.3$ m, $\varnothing = 7.8$ mm,
- **stationary phase:** *styrene-divinylbenzene copolymer R* (7 μ m) with a pore size of 10 nm.

Columns 2 and 3 placed closest to the injector:

- **dimensions:** $l = 0.3$ m, $\varnothing = 7.8$ mm,
- **stationary phase:** *styrene-divinylbenzene copolymer R* (7 μ m) with a pore size of 50 nm.

Mobile phase: *tetrahydrofuran R*.

Flow rate: 0.8 ml/min.

Detection: differential refractometer.

Injection: 40 μ l.

System suitability: reference solution:

- **elution order:** tridocosahexaenoin, didocosahexaenoin, monodocosahexaenoin,
- **resolution:** minimum of 2.0 between the peaks due to monodocosahexaenoin and to didocosahexaenoin and minimum of 1.0 between the peaks due to didocosahexaenoin and to tridocosahexaenoin.

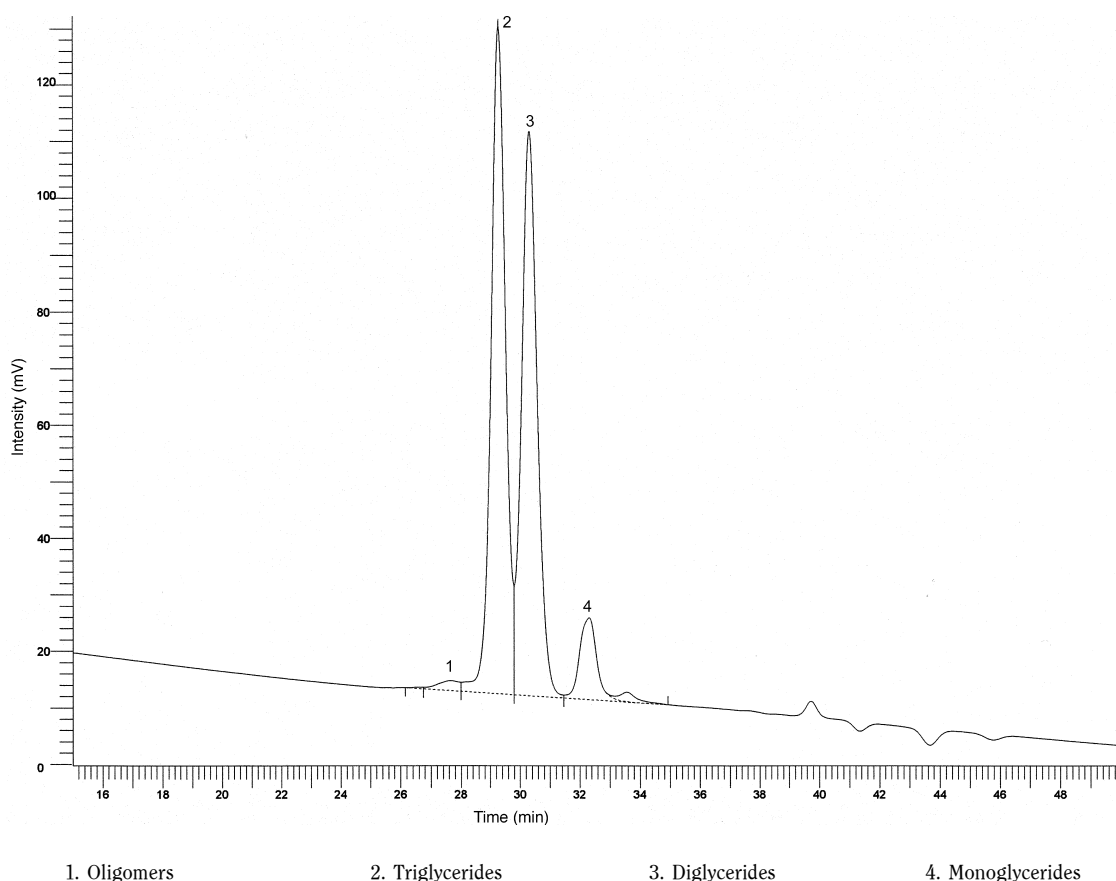


Figure 1352-1. – Chromatogram of the test for oligomers and partial glycerides in omega-3-acid triglycerides

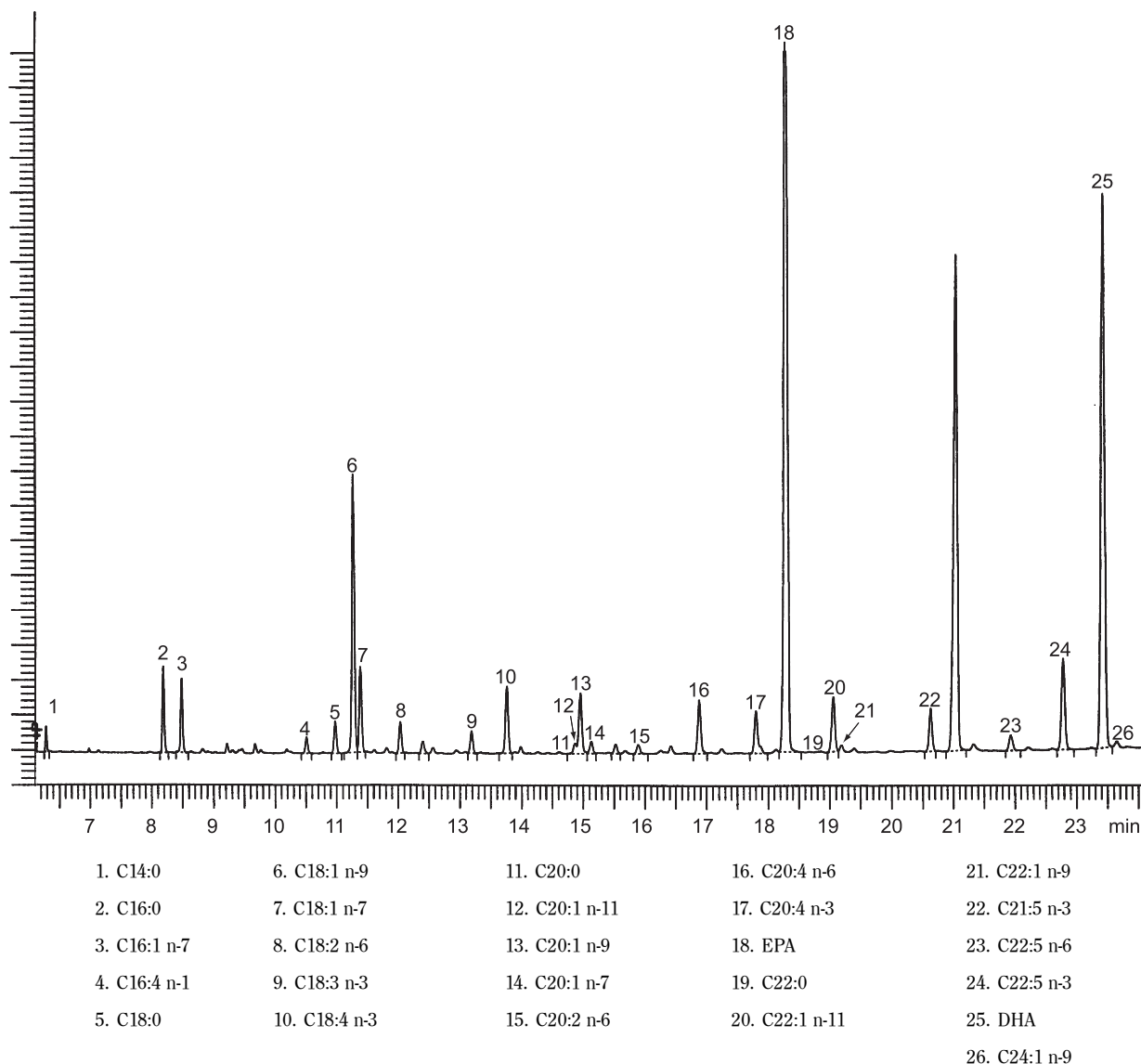


Figure 1352.-2. – Chromatogram for the assay of total omega-3 acids in omega-3-acid triglycerides

Identify the peaks from the chromatogram (Figure 1352.-1). Calculate the percentage content of oligomers using the following expression:

$$\frac{B}{A} \times 100$$

- A = sum of the areas of all the peaks in the chromatogram,
 B = area of the peak with a retention time smaller than the retention time of the triglyceride peak.

Calculate the percentage content of partial glycerides using the following expression:

$$\frac{C}{A} \times 100$$

- C = (sum of the) area(s) of the peak(s) due to the mono- and diglycerides.

Limits:

- *oligomers*: maximum 3.0 per cent,
- *partial glycerides*: maximum 50.0 per cent.

ASSAY

EPA and DHA (2.4.29). See Figure 1352.-2.

Total omega-3-acids (2.4.29). See Figure 1352.-2.

STORAGE

In an airtight, well-filled container, protected from light, under an inert gas.

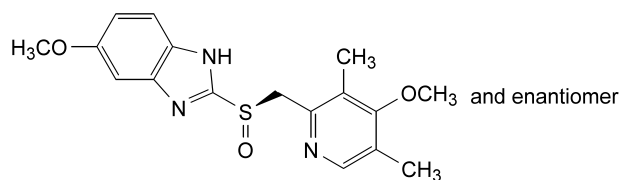
LABELLING

The label states the concentration of any added tocopherol.

01/2005:0942

OMEPRAZOLE

Omeprazolium



$C_{17}H_{19}N_3O_3S$

M_r 345.4

DEFINITION

Omeprazole contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of 5-methoxy-2-[(*RS*)-[(4-methoxy-3,5-dimethylpyridin-2-yl)methyl]sulphonyl]-1*H*-benzimidazole, calculated with reference to the dried substance.

CHARACTERS

A white or almost white powder, very slightly soluble in water, soluble in methylene chloride, sparingly soluble in alcohol and in methanol. It dissolves in dilute solutions of alkali hydroxides.

It shows polymorphism.

IDENTIFICATION

First identification: B.

Second identification: A, C.

- A. Dissolve 2.0 mg in 0.1 *M* sodium hydroxide and dilute to 100.0 ml with the same solvent. Examined between 230 nm and 350 nm (2.2.25), the solution shows 2 absorption maxima at 276 nm and 305 nm. The ratio of the absorbance measured at the absorption maximum at 305 nm to that measured at the absorption maximum at 276 nm is 1.6 to 1.8.
- B. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with omeprazole CRS. If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in methanol *R*, evaporate to dryness and record new spectra using the residues.
- C. Examine the chromatograms obtained in the test for impurity C. The principal spot in the chromatogram obtained with test solution (b) is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a). Place the plate in a tank saturated with vapour from acetic acid *R*. The spots rapidly turn brown.

TESTS

Solution S. Dissolve 0.50 g in methylene chloride *R* and dilute to 25 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1).

Absorbance (2.2.25). The absorbance of solution S measured at 440 nm is not more than 0.10 (this limit corresponds to 0.035 per cent of impurity F or impurity G).

Impurity C. Examine by thin-layer chromatography (2.2.27), using a TLC silica gel F_{254} plate *R*.

Test solution (a). Dissolve 0.10 g of the substance to be examined in 2.0 ml of a mixture of equal volumes of methanol *R* and methylene chloride *R*.

Test solution (b). Dilute 1.0 ml of test solution (a) to 10 ml with methanol *R*.

Reference solution (a). Dissolve 10 mg of omeprazole CRS in 2.0 ml of methanol *R*.

Reference solution (b). Dilute 1 ml of test solution (a) to 10 ml with a mixture of equal volumes of methanol *R* and methylene chloride *R*. Dilute 1 ml of this solution to 100 ml with a mixture of equal volumes of methanol *R* and methylene chloride *R*.

Apply to the plate 10 µl of each solution. Develop over a path of 15 cm using a mixture of 20 volumes of 2-propanol *R*, 40 volumes of methylene chloride *R* previously shaken with concentrated ammonia *R* (shake 100 ml of methylene chloride *R* with 30 ml of concentrated ammonia *R* in a separating funnel; allow the layers to separate and use

the lower layer) and 40 volumes of methylene chloride *R*. Allow the plate to dry in air. Examine in ultraviolet light at 254 nm. Any spot in the chromatogram obtained with test solution (a) with a higher R_f value than that of the spot due to omeprazole is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.1 per cent).

Related substances. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 3.0 mg of the substance to be examined in the mobile phase and dilute to 25.0 ml with the mobile phase.

Reference solution (a). Dissolve 1.0 mg of omeprazole CRS and 1.0 mg of omeprazole impurity D CRS in the mobile phase and dilute to 10.0 ml with the mobile phase.

Reference solution (b). Dilute 1.0 ml of the test solution to 100.0 ml with the mobile phase. Dilute 1.0 ml of this solution to 10.0 ml with the mobile phase.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.15 m long and 4 mm in internal diameter packed with octylsilyl silica gel for chromatography *R* (5 µm),
- as mobile phase at a flow rate of 1 ml/min a mixture of 27 volumes of acetonitrile *R* and 73 volumes of a 1.4 g/l solution of disodium hydrogen phosphate *R* previously adjusted to pH 7.6 with phosphoric acid *R*,
- as detector a spectrophotometer set at 280 nm.

When the chromatograms are recorded under the prescribed conditions, the retention time of omeprazole is about 9 min and the relative retention time of impurity D is about 0.8. Inject separately 40 µl of each solution and continue the chromatography for 3 times the retention time of omeprazole. Adjust the sensitivity of the system so that the height of the principal peak in the chromatogram obtained with reference solution (b) is at least 15 per cent of the full scale of the recorder. The test is not valid unless in the chromatogram obtained with reference solution (a), the resolution between the peaks due to impurity D and omeprazole is greater than 3. If necessary, adjust the pH of the mobile phase or the concentration of acetonitrile *R*; an increase in the pH will improve the resolution. The area of any peak, apart from the principal peak, in the chromatogram obtained with the test solution is not greater than the area of the peak in the chromatogram obtained with reference solution (b) (0.1 per cent).

Residual solvents. Examine by head-space gas chromatography (2.2.28), using the standard additions method. The content of chloroform is not more than 50 ppm, and the content of methylene chloride is not more than 100 ppm.

The chromatographic procedure may be carried out using:

- a fused-silica column 30 m long and 0.32 mm in internal diameter coated with a 1.8 µm film of cross-linked poly[(cyanopropyl)(phenyl)][dimethyl]siloxane *R*,
- nitrogen for chromatography *R* as the carrier gas,
- a flame-ionisation detector,
- a suitable head-space sampler.

Place 0.50 g of the substance to be examined in a 10 ml vial. Add 4.0 ml of dimethylacetamide *R* and stopper the vial. Equilibrate the vial at 80 °C for 1 h.

Loss on drying (2.2.32): maximum 0.2 per cent, determined on 1.000 g by drying under high vacuum at 60 °C for 4 h.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

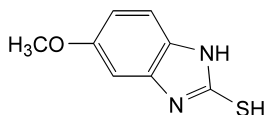
Dissolve 1.100 g in a mixture of 10 ml of *water R* and 40 ml of *alcohol R*. Titrate with 0.5 M sodium hydroxide, determining the end-point potentiometrically (2.2.20).

1 ml of 0.5 M sodium hydroxide is equivalent to 0.1727 g of $C_{17}H_{19}N_3O_3S$.

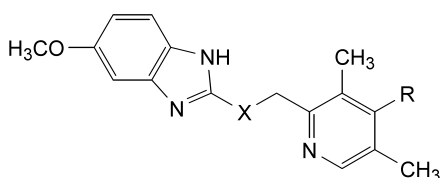
STORAGE

In an airtight container, protected from light, at a temperature between 2 °C and 8 °C.

IMPURITIES



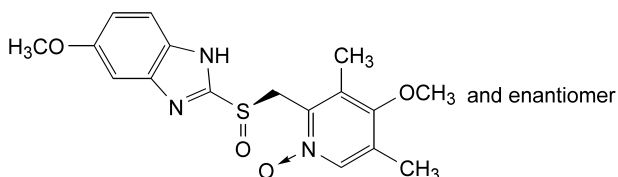
A. 5-methoxy-1*H*-benzimidazole-2-thiol,



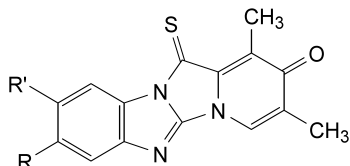
B. R = H, X = SO: 2-[(*RS*)-[(3,5-dimethylpyridin-2-yl)methyl]sulphinyl]-5-methoxy-1*H*-benzimidazole,

C. R = OCH₃, X = S: 5-methoxy-2-[(4-methoxy-3,5-dimethylpyridin-2-yl)methyl]sulphonyl]-1*H*-benzimidazole (ufiprazole),

D. R = OCH₃, X = SO₂: 5-methoxy-2-[(4-methoxy-3,5-dimethylpyridin-2-yl)methyl]sulphonyl]-1*H*-benzimidazole (omeprazole sulphone),



E. 4-methoxy-2-[(*RS*)-(5-methoxy-1*H*-benzimidazol-2-yl)sulphinyl]methyl]-3,5-dimethylpyridine 1-oxide,

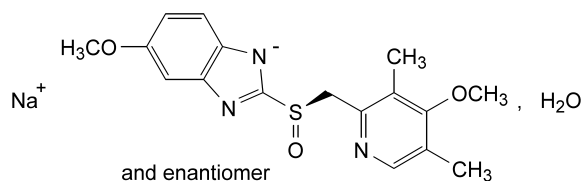


F. R = OCH₃, R' = H: 1,3-dimethyl-8-methoxy-12-thioxopyrido[1',2':3,4]imidazo[1,2-*a*]benzimidazol-2(12*H*)-one,

G. R = H, R' = OCH₃: 1,3-dimethyl-9-methoxy-12-thioxopyrido[1',2':3,4]imidazo[1,2-*a*]benzimidazol-2(12*H*)-one.

OMEPRAZOLE SODIUM

Omeprazolum natricum



$C_{17}H_{18}N_3NaO_3S \cdot H_2O$

M_r 385.4

DEFINITION

Omeprazole sodium contains not less than 98.0 per cent and not more than the equivalent of 101.0 per cent of sodium 5-methoxy-2-[(*RS*)-[(4-methoxy-3,5-dimethylpyridin-2-yl)methyl]sulphinyl]-1*H*-benzimidazole, calculated with reference to the anhydrous substance.

CHARACTERS

A white or almost white powder, hygroscopic, freely soluble in water and in alcohol, soluble in propylene glycol, very slightly soluble in methylene chloride.

IDENTIFICATION

- Dissolve 2.0 mg in 0.1 M sodium hydroxide and dilute to 100.0 ml with the same solvent. Examined between 230 nm and 350 nm (2.2.25), the solution shows two absorption maxima, at 276 nm and 305 nm. The ratio of the absorbance measured at the maximum at 305 nm to that measured at the maximum at 276 nm is 1.6 to 1.8.
- Examine the chromatograms obtained in the test for omeprazole impurity C. The principal spot in the chromatogram obtained with test solution (b) is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a). Place the plate in a tank saturated with vapour of *acetic acid R*. The spots rapidly turn brown.
- Ignite 1 g and cool. Add 1 ml of *water R* to the residue and neutralise with *hydrochloric acid R*. Filter and dilute the filtrate to 4 ml with *water R*. 0.1 ml of the solution gives reaction (b) of sodium (2.3.1).

TESTS

Solution S. Dissolve 0.50 g in *carbon dioxide-free water R* and dilute to 25 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and not more intensely coloured than reference solution B₆ (2.2.2, *Method II*).

pH (2.2.3). The pH of solution S is 10.3 to 11.3.

Omeprazole impurity C. Examine by thin-layer chromatography (2.2.27), using *silica gel HF*₂₅₄ *R* as the coating substance.

Test solution (a). Dissolve 0.10 g of the substance to be examined in 2.0 ml of *methanol R*.

Test solution (b). Dilute 1.0 ml of test solution (a) to 10 ml with *methanol R*.

Reference solution (a). Dissolve 9 mg of *omeprazole CRS* in 2.0 ml of *methanol R*.

Reference solution (b). Dilute 1.0 ml of test solution (b) to 100 ml with *methanol R*.

Apply separately to the plate 10 µl of each solution. Develop over a path of 15 cm using a mixture of 20 volumes of 2-propanol *R*, 40 volumes of *methylene chloride R*

previously shaken with *concentrated ammonia R* (shake 100 ml of *methylene chloride R* with 30 ml of *concentrated ammonia R* in a separating funnel, allow the layers to separate and use the lower layer) and 40 volumes of *methylene chloride R*. Allow the plate to dry in air. Examine in ultraviolet light at 254 nm. Any spot in the chromatogram obtained with test solution (a) with a higher R_f value than that of the spot corresponding to omeprazole is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.1 per cent).

Related substances. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 3.0 mg of the substance to be examined in the mobile phase and dilute to 25.0 ml with the mobile phase.

Reference solution (a). Dissolve 1.0 mg of *omeprazole CRS* and 1.0 mg of *omeprazole impurity D CRS* in the mobile phase and dilute to 10.0 ml with the mobile phase.

Reference solution (b). Dilute 1.0 ml of the test solution to 100.0 ml with the mobile phase. Dilute 1.0 ml of this solution to 10.0 ml with the mobile phase.

The chromatography may be carried out using:

- a stainless steel column 0.15 m long and 4 mm in internal diameter packed with *octylsilyl silica gel for chromatography R* (5 µm),
- as mobile phase at a flow rate of 1 ml/min a mixture of 27 volumes of *acetonitrile R* and 73 volumes of a 1.4 g/l solution of *disodium hydrogen phosphate R*, previously adjusted to pH 7.6 with *phosphoric acid R*,
- as detector a spectrophotometer set at 280 nm.

When the chromatograms are recorded in the prescribed conditions, the retention time of omeprazole is about 9 min and the relative retention time of omeprazole impurity D is about 0.8. Inject separately 40 µl of each solution and continue the chromatography for three times the retention time of omeprazole. Adjust the sensitivity of the detector so that the height of the principal peak in the chromatogram obtained with reference solution (b) is not less than 15 per cent of the full scale of the recorder. The test is not valid unless in the chromatogram obtained with reference solution (a), the resolution between the peaks corresponding to omeprazole impurity D and omeprazole is greater than 3. If necessary adjust the pH of the mobile phase or the concentration of *acetonitrile R*, an increase in the pH will improve the resolution. The area of any peak apart from the principal peak in the chromatogram obtained with the test solution is not greater than the area of the peak in the chromatogram obtained with reference solution (b) (0.1 per cent).

Heavy metals (2.4.8). 1.0 g complies with limit test C for heavy metals (20 ppm). Prepare the standard using 2 ml of *lead standard solution (10 ppm Pb) R*.

Water (2.5.12): 4.5 per cent to 10.0 per cent, determined on 0.300 g by the semi-micro determination of water.

ASSAY

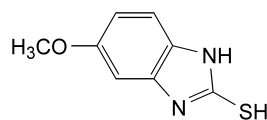
Dissolve 0.300 g in 50 ml of *water R*. Titrate with 0.1 M *hydrochloric acid*, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *hydrochloric acid* corresponds to 36.74 mg of $C_{17}H_{18}N_3NaO_3S$.

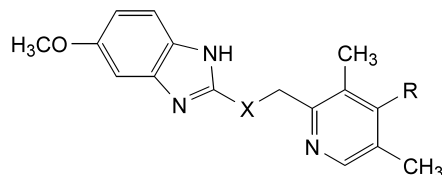
STORAGE

Store in an airtight container, protected from light.

IMPURITIES



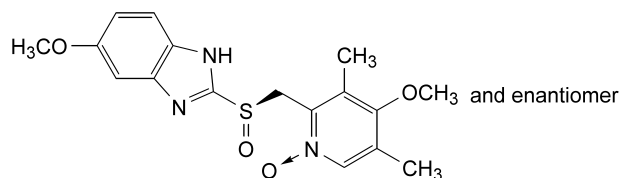
A. 5-methoxy-1*H*-benzimidazole-2-thiol,



B. R = H, X = SO: 2-[(*RS*)-[(3,5-dimethylpyridin-2-yl)methyl]sulphinyl]-5-methoxy-1*H*-benzimidazole,

C. R = OCH₃, X = S: 5-methoxy-2-[[4-methoxy-3,5-dimethylpyridin-2-yl)methyl]thio]-1*H*-benzimidazole (ufiprazole),

D. R = OCH₃, X = SO₂: 5-methoxy-2-[[4-methoxy-3,5-dimethylpyridin-2-yl)methyl]sulfonyl]-1*H*-benzimidazole (omeprazole-sulphone),

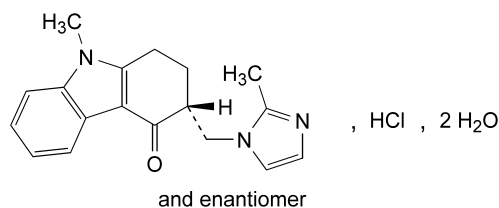


E. 4-methoxy-2-[[(*RS*)-(5-methoxy-1*H*-benzimidazol-2-yl)sulphinyl]methyl]-3,5-dimethylpyridine 1-oxide,

01/2005:2016

ONDANSETRON HYDROCHLORIDE DIHYDRATE

Ondansetroni hydrochloridum dihydricum



$C_{18}H_{20}ClN_3O \cdot 2H_2O$

M_r 365.9

DEFINITION

(3*RS*)-9-Methyl-3-[(2-methyl-1*H*-imidazol-1-yl)methyl]-1,2,3,9-tetrahydro-4*H*-carbazol-4-one hydrochloride dihydrate.

Content: 97.5 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

Appearance: white or almost white powder.

Solubility: sparingly soluble in water and in alcohol, soluble in methanol, slightly soluble in methylene chloride.

IDENTIFICATION

A. Infrared absorption spectrophotometry (2.2.24).

Comparison: *ondansetron hydrochloride dihydrate CRS*.

B. It gives reaction (a) of chlorides (2.3.1).

TESTS

Impurity B. Thin-layer chromatography (2.2.27).

Test solution. Dissolve 0.125 g of the substance to be examined in a mixture of 0.5 volumes of *concentrated ammonia R*, 100 volumes of *alcohol R* and 100 volumes of *methanol R*, and dilute to 10.0 ml with the same mixture of solvents.

Reference solution (a). Dissolve 12.5 mg of *ondansetron for TLC system suitability CRS* in a mixture of 0.5 volumes of *concentrated ammonia R*, 100 volumes of *alcohol R* and 100 volumes of *methanol R*, and dilute to 1.0 ml with the same mixture of solvents.

Reference solution (b). Dilute 1 ml of the test solution to 100 ml with a mixture of 0.5 volumes of *concentrated ammonia R*, 100 volumes of *alcohol R* and 100 volumes of *methanol R*. Dilute 4.0 ml to 10.0 ml with a mixture of 0.5 volumes of *concentrated ammonia R*, 100 volumes of *alcohol R* and 100 volumes of *methanol R*.

Plate: TLC silica gel F_{254} plate *R*.

Mobile phase: *concentrated ammonia R*, *methanol R*, *ethyl acetate R*, *methylene chloride R* (2:40:50:90 V/V/V/V).

Application: 20 µl.

Development: over 3/4 of the plate.

Drying: in air.

Detection: examine in ultraviolet light at 254 nm.

Order of elution: ondansetron, impurity B, impurity A.

System suitability: the chromatogram obtained with reference solution (a) shows 3 clearly separated spots.

Limit:

- **impurity B:** any spot corresponding to impurity B in the chromatogram obtained with the test solution is not more intense than the principal spot in the chromatogram obtained with reference solution (b) (0.4 per cent).

Related substances. Liquid chromatography (2.2.29).

Test solution (a). Dissolve 50.0 mg of the substance to be examined in the mobile phase and dilute to 100.0 ml with the mobile phase.

Test solution (b). Dissolve 90.0 mg of the substance to be examined in the mobile phase and dilute to 100.0 ml with the mobile phase. Dilute 10.0 ml to 100.0 ml with the mobile phase.

Reference solution (a). Dilute 2.0 ml of test solution (a) to 100.0 ml with the mobile phase. Dilute 10.0 ml to 100.0 ml with the mobile phase.

Reference solution (b). Dissolve 10.0 mg of *imidazole R* and 10.0 mg of *2-methylimidazole R* in the mobile phase and dilute to 100.0 ml with the mobile phase. Dilute 1.0 ml to 100.0 ml with the mobile phase.

Reference solution (c). Dissolve 5.0 mg of *ondansetron for LC system suitability CRS* in the mobile phase and dilute to 10.0 ml with the mobile phase.

Reference solution (d). Dissolve 5.0 mg of *ondansetron impurity D CRS* in the mobile phase and dilute to 100.0 ml with the mobile phase. Dilute 1.0 ml to 100.0 ml with the mobile phase.

Reference solution (e). Dissolve 90.0 mg of *ondansetron hydrochloride dihydrate CRS* in the mobile phase and dilute to 100.0 ml with the mobile phase. Dilute 10.0 ml to 100.0 ml with the mobile phase.

Column:

- **size:** $l = 0.25$ m, $\varnothing = 4.6$ mm,

- **stationary phase:** spherical *nitrile silica gel for chromatography R* (5 µm) with a specific surface area of 220 m²/g and a pore size of 8 nm.

Mobile phase: mix 20 volumes of *acetonitrile R* and 80 volumes of a 2.8 g/l solution of *sodium dihydrogen phosphate monohydrate R* previously adjusted to pH 5.4 with a 40 g/l solution of *sodium hydroxide R*.

Flow rate: 1.5 ml/min.

Detection: spectrophotometer at 216 nm.

Injection: 20 µl; inject test solution (a) and reference solutions (a), (b), (c) and (d).

Run time: 1.5 times the retention time of ondansetron.

Relative retentions with reference to ondansetron (retention time = about 18 min): impurity E = about 0.1; impurity F = about 0.2; impurity C = about 0.4; impurity D = about 0.5; impurity H = about 0.7; impurity A = about 0.8; impurity G = about 0.9.

System suitability:

- **resolution:** minimum of 1.3 between the peak due to impurity E (first peak) and the peak due to impurity F (second peak) in the chromatogram obtained with reference solution (b) and minimum of 2.5 between the peak due to impurity C (first peak) and the peak due to impurity D (second peak) in the chromatogram obtained with reference solution (c).

Limits:

- **correction factor:** for the calculation of contents, multiply the peak area of impurity C by 0.6,
- **impurity C:** not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent),
- **impurity D:** not more than the area of the principal peak in the chromatogram obtained with reference solution (d) (0.1 per cent),
- **impurity E:** not more than the area of the corresponding peak in the chromatogram obtained with reference solution (b) (0.2 per cent),
- **impurity F:** not more than the area of the corresponding peak in the chromatogram obtained with reference solution (b) (0.2 per cent),
- **any other impurity:** not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent),
- **total:** not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.4 per cent),
- **disregard limit:** 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.04 per cent).

Water (2.5.12): 9.0 per cent to 10.5 per cent, determined on 0.200 g.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (2.2.29) as described in the test for related substances with the following modification.

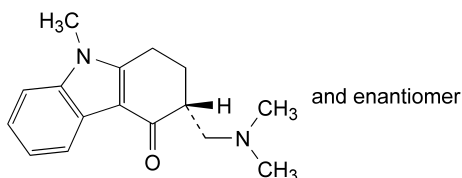
Injection: test solution (b) and reference solution (e).

Calculate the percentage content of $C_{18}H_{20}ClN_3O$.

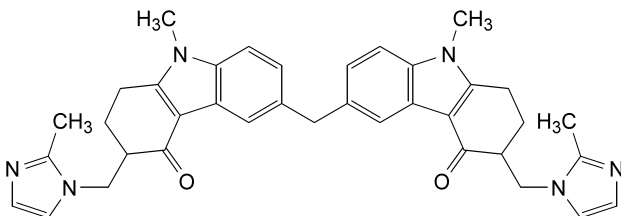
STORAGE

Protected from light.

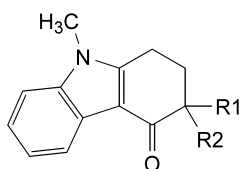
IMPURITIES



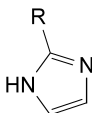
- A. (3RS)-3-[(dimethylamino)methyl]-9-methyl-1,2,3,9-tetrahydro-4H-carbazol-4-one,



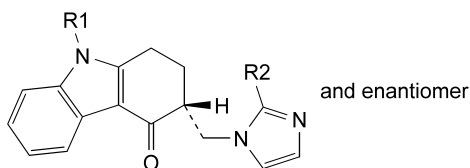
- B. 6,6'-methylenebis[(3RS)-9-methyl-3-[(2-methyl-1H-imidazol-1-yl)methyl]-1,2,3,9-tetrahydro-4H-carbazol-4-one],



- C. R1 = R2 = H: 9-methyl-1,2,3,9-tetrahydro-4H-carbazol-4-one,
D. R1 + R2 = CH₂: 9-methyl-3-methylene-1,2,3,9-tetrahydro-4H-carbazol-4-one,



- E. R = H: 1H-imidazole,
F. R = CH₃: 2-methyl-1H-imidazole,



- G. R1 = CH₃, R2 = H: (3RS)-3-[(1H-imidazol-1-yl)methyl]-9-methyl-1,2,3,9-tetrahydro-4H-carbazol-4-one (C-demethylondansetron),
H. R1 = H, R2 = CH₃: (3RS)-3-[(2-methyl-1H-imidazol-1-yl)methyl]-1,2,3,9-tetrahydro-4H-carbazol-4-one (N-demethylondansetron).

Content:

- morphine (C₁₇H₁₉NO₃; M_r 285.3): 9.8 per cent to 10.2 per cent (drug dried at 100-105 °C for 4 h),
- codeine (C₁₈H₂₁NO₃; M_r 299.4): minimum 1.0 per cent (drug dried at 100-105 °C for 4 h).

Content adjusted if necessary by adding a suitable excipient or raw opium powder.

CHARACTERS

Appearance: yellowish-brown or dark brown powder.

IDENTIFICATION

- A. Examine under a microscope using a 20 g/l solution of *potassium hydroxide R*. It is seen to consist of granules of latex agglomerated in irregular masses, and of light brown elongated filaments. Some fragments of vessels and rather elongated, refringent crystals are also visible, as well as a smaller number of round pollen grains and fragments of elongated fibres. Hairs of various lengths with sharp points and fragments of epicarp consisting of polygonal cells with thick walls defining a stellate lumen may be present. Examine under a microscope using *glycerol (85 per cent) R*. Particles of excipient and a few grains of starch introduced during the handling of the latex may be seen.

- B. Thin-layer chromatography (2.2.27).

Test solution. Triturate 0.10 g of the drug to be examined with 5 ml of *alcohol (70 per cent V/V) R*, rinse with 3 ml of *alcohol (70 per cent V/V) R*, transfer to a 25 ml conical flask. Heat in a water-bath at 50-60 °C with stirring for 30 min. Cool, filter, wash the filter with *alcohol (70 per cent V/V) R* and dilute the filtrate to 10 ml with the same solvent.

Reference solution. Dissolve 2.0 mg of *papaverine hydrochloride R*, 12.0 mg of *codeine phosphate R*, 12.0 mg of *noscaphine hydrochloride R* and 25.0 mg of *morphine hydrochloride R* in *alcohol (70 per cent V/V) R* and dilute to 25.0 ml with the same solvent.

Plate: TLC silica gel G plate R.

Mobile phase: concentrated ammonia R, alcohol R, acetone R, toluene R (2:6:40:40 V/V/V/V).

Application: 20 µl, as bands of 20 mm by 3 mm.

Development: over a path of 15 cm.

Drying: at 100-105 °C for 15 min.

Detection: allow to cool and spray with *potassium iodobismuthate solution R2* and then with a 4 g/l solution of *sulphuric acid R*, examine in daylight.

Results: see below the sequence of the zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, a dark red zone (thebaine) situated between the codeine zone and the papaverine zone may be present in the chromatogram obtained with the test solution.

Top of the plate	
Noscaphine: an orange-red or red zone	An orange-red or red zone (noscaphine)
Papaverine: an orange-red or red zone	An orange-red or red zone (papaverine)
Codeine: an orange-red or red zone	An orange-red or red zone (codeine)
Morphine: an orange-red or red zone	An orange-red or red zone (morphine)
Reference solution	Test solution

01/2005:1840

OPIUM, PREPARED

Opii pulvis normatus

DEFINITION

Raw opium powdered (180), and dried at a temperature not exceeding 70 °C.

C. To 1.0 g of the drug to be examined add 5 ml of *water R*, shake for 5 min and filter. To the filtrate add 0.25 ml of *ferric chloride solution R2*. A red colour develops which does not disappear on the addition of 0.5 ml of *dilute hydrochloric acid R*.

TESTS

Thebaine. Liquid chromatography (2.2.29).

Test solution. Suspend 1.00 g of the drug to be examined in 50 ml of *alcohol (50 per cent V/V) R*, mix using sonication for 1 h, allow to cool and dilute to 100.0 ml with the same solvent. Allow to stand. To 10.0 ml of the supernatant liquid, add 5 ml of *ammonium chloride buffer solution pH 9.5 R*, dilute to 25.0 ml with *water R* and mix. Transfer 20.0 ml of the solution to a chromatography column about 0.15 m long and about 30 mm in internal diameter containing 15 g of *kieselguhr for chromatography R*. Allow to stand for 15 min. Elute with 2 quantities, each of 40 ml, of a mixture of 15 volumes of *2-propanol R* and 85 volumes of *methylene chloride R*. Evaporate the eluate to dryness *in vacuo* at 40 °C. Transfer the residue to a volumetric flask with the aid of the mobile phase and dilute to 25.0 ml with the mobile phase.

Reference solution. Dissolve 25.0 mg of *thebaine R* in the mobile phase and dilute to 25.0 ml with the mobile phase. Dilute 10.0 ml of the solution to 100.0 ml with the mobile phase.

Precolumn:

- **size:** $l = 40$ mm, $\varnothing = 4.6$ mm,
- **stationary phase:** *octylsilyl silica gel for chromatography R* (5 μ m).

Column:

- **size:** $l = 0.25$ m, $\varnothing = 4.6$ mm,
- **stationary phase:** *octylsilyl silica gel for chromatography R* (5 μ m).

Mobile phase: dissolve 1.0 g of *sodium heptanesulphonate monohydrate R* in 420 ml of *water R*, adjust to pH 3.2 with phosphoric acid (4.9 g/l H_3PO_4) (about 5 ml) and add 180 ml of *acetonitrile R*.

Flow rate: 1.5 ml/min.

Detection: spectrophotometer at 280 nm.

Injection: a suitable volume with a loop injector.

System suitability: reference solution:

- **mass distribution ratio:** minimum 3.0 for the peak due to thebaine.

Calculate the percentage content of alkaloid from the expression:

$$\frac{m_1 \times A_2 \times 125}{m_2 \times A_1} \times \frac{100}{100 - h}$$

- m_1 = mass of the alkaloid in the reference solution, in grams,
- m_2 = mass of the substance to be examined in the test solution, in grams,
- A_1 = area of the peak due to the alkaloid in the chromatogram obtained with the reference solution,
- A_2 = area of the peak due to the alkaloid in the chromatogram obtained with the test solution,
- h = percentage loss on drying.

Limit:

- **thebaine:** maximum 3.0 per cent (dried drug).

Loss on drying (2.2.32): maximum 8.0 per cent, determined on 1.000 g by drying in an oven at 100–105 °C for 4 h.

Total ash (2.4.16): maximum 6.0 per cent.

ASSAY

Liquid chromatography (2.2.29) as described in the test for thebaine with the following modifications.

Reference solution. Dissolve 0.100 g of *morphine hydrochloride R* and 25.0 mg of *codeine R* in the mobile phase and dilute to 25.0 ml with the mobile phase. Dilute 10.0 ml of the solution to 100.0 ml with the mobile phase.

System suitability: reference solution:

- **resolution:** minimum 2.5 between the peaks due to morphine and codeine; if necessary, adjust the volume of acetonitrile in the mobile phase,
- **repeatability:** maximum relative standard deviation of 1.0 per cent for the peak area due to morphine, determined on 6 replicate injections.

Calculate the percentage content of morphine and codeine from the expression given in the test for thebaine. For the calculation, 1 mg of *morphine hydrochloride R* is taken to be equivalent to 0.759 mg of morphine and 1 mg of *codeine R* is taken to be equivalent to 0.943 mg of codeine.

LABELLING

The label states the name of any excipient used.

01/2005:0777

OPIUM, RAW

Opium crudum

DEFINITION

Raw opium is intended only as starting material for the manufacture of galenical preparations. It is not dispensed as such.

Raw opium is the air-dried latex obtained by incision from the unripe capsules of *Papaver somniferum* L. It contains not less than 10.0 per cent of morphine ($\text{C}_{17}\text{H}_{19}\text{NO}_3$; M_r 285.3) and not less than 2.0 per cent of codeine ($\text{C}_{18}\text{H}_{21}\text{NO}_3$; M_r 299.4), both calculated with reference to the drug dried at 100 °C to 105 °C.

CHARACTERS

Raw opium has a characteristic odour and a blackish-brown colour. It has the microscopic characters described in identification test A. It consists of masses of various sizes, which tend to be soft and shiny and, after drying, become hard and brittle.

IDENTIFICATION

Strip off any covering, cut the substance to be examined into thin slices, if necessary, dry at about 60 °C for 48 h and reduce to a powder (500).

A. Examined under a microscope, a suspension of raw opium in a 20 g/l solution of *potassium hydroxide R* is seen to consist of granules of latex agglomerated in irregular masses, and of light-brown elongated filaments. Some fragments of vessels and rather elongated, refringent crystals are also visible, as well as a smaller number of round pollen grains and fragments of elongated fibres. Hairs of various lengths with sharp points and a few grains of starch introduced during the handling of the latex may be present. Fragments of epicarp consisting of polygonal cells with thick walls defining a stellate lumen may also be present.

- B. Examine by thin-layer chromatography (2.2.27), using *silica gel G R* as the coating substance.

Test solution. Triturate 0.10 g of the powdered drug with 5 ml of *alcohol (70 per cent V/V) R*, add 3 ml of *alcohol (70 per cent V/V) R*, transfer to a 25 ml conical flask and heat in a water-bath at 50 °C to 60 °C with stirring for 30 min. Cool, filter, wash the filter with *alcohol (70 per cent V/V) R* and dilute the filtrate to 10 ml with the same solvent.

Reference solution. Dissolve 2.0 mg of *papaverine hydrochloride R*, 12.0 mg of *codeine phosphate R*, 12.0 mg of *noscipine hydrochloride R* and 25.0 mg of *morphine hydrochloride R* in *alcohol (70 per cent V/V) R* and dilute to 25.0 ml with the same solvent.

Apply separately to the plate as bands 20 mm by 3 mm 20 µl of each solution. Develop over a path of 15 cm using a freshly prepared mixture of 2 volumes of *concentrated ammonia R*, 6 volumes of *alcohol R*, 40 volumes of *acetone R* and 40 volumes of *toluene R*. Dry the plate at 100 °C to 105 °C for 15 min, allow to cool and spray with *potassium iodobismuthate solution R2* and then with a 4 g/l solution of *sulphuric acid R*. The chromatogram obtained with the reference solution shows in the lower part an orange-red or red zone (morphine), above it a similarly coloured zone (codeine) and in the upper part an orange-red or red zone (papaverine) and above it a similarly coloured zone (noscipine). The chromatogram obtained with the test solution shows orange-red or red zones corresponding to those in the chromatogram obtained with the reference solution. The chromatogram obtained with the test solution may also show a dark red zone (thebaine) situated between those due to codeine and to papaverine.

- C. To 1.0 g of the powdered drug add 5 ml of *water R*, shake for 5 min and filter. To the filtrate add 0.25 ml of *ferric chloride solution R2*. A red colour develops which does not disappear on the addition of 0.5 ml of *dilute hydrochloric acid R*.

TESTS

Thebaine. Not more than 3.0 per cent, determined by liquid chromatography (2.2.29) and calculated with reference to the dried drug.

Test solution. Prepare the test solution as described in the assay.

Reference solution. Dissolve 25.0 mg of *thebaine R* in the mobile phase and dilute to 25.0 ml with the mobile phase. Dilute 10.0 ml of the solution to 100.0 ml with the mobile phase.

The chromatographic procedure is carried out as described in the assay. The test is not valid unless the mass distribution ratio for thebaine is at least 3.0 and the number of theoretical plates is at least 3000. Calculate the percentage content of thebaine from the expression given in the assay.

Loss on drying (2.2.32). Not more than 15.0 per cent, determined on 1.000 g of raw opium cut into thin slices, by drying in an oven at 100 °C to 105 °C for 4 h.

Total ash (2.4.16). Not more than 6.0 per cent.

ASSAY

Examine by liquid chromatography (2.2.29).

Test solution. Suspend 1.00 g of raw opium, cut into thin slices, in 50 ml of *alcohol (50 per cent V/V) R*, mix with the aid of ultrasound for 1 h, allow to cool and dilute to 100.0 ml with the same solvent. Allow to stand. To 10.0 ml of the supernatant liquid add 5 ml of *ammonium chloride buffer solution pH 9.5 R*, dilute to 25.0 ml with *water R* and mix. Transfer 20.0 ml of the solution to a chromatography column about 0.15 m long and about 30 mm in internal diameter containing 15 g of *kieselguhr for chromatography R*. Allow to stand for 15 min. Elute with two quantities, each of 40 ml, of a mixture of 15 volumes of *2-propanol R* and 85 volumes of *methylene chloride R*. Evaporate the eluate to dryness *in vacuo* at 40 °C. Transfer the residue to a volumetric flask with the aid of the mobile phase and dilute to 25.0 ml with the mobile phase.

Reference solution. Dissolve 0.100 g of *morphine hydrochloride R* and 25.0 mg of *codeine R* in the mobile phase and dilute to 25.0 ml with the mobile phase. Dilute 10.0 ml of the solution to 100.0 ml with the mobile phase.

The chromatographic procedure may be carried out using:

- a column 0.25 m long and 4.6 mm in internal diameter packed with *octylsilyl silica gel for chromatography R* (5 µm), equipped with a guard column 40 mm long and 4.6 mm in internal diameter packed with *octylsilyl silica gel for chromatography R* (5 µm),
- as mobile phase at a flow rate of 1.5 ml/min a solution prepared as follows: dissolve 1.0 g of *sodium heptanesulphonate monohydrate R* in 420 ml of *water R*, adjust to pH 3.2 by addition of phosphoric acid (4.9 g/l H_3PO_4) (about 5 ml) and add 180 ml of *acetonitrile R*,
- as detector a spectrophotometer set at 280 nm,
- an loop injector.

Inject suitable volumes of each solution.

The assay is not valid unless the resolution between the peaks corresponding to morphine and codeine is at least 2.5. If necessary, adjust the volume of acetonitrile in the mobile phase. Inject the reference solution six times. The assay is not valid unless the relative standard deviation of the peak area for morphine is at most 1.0 per cent. Inject alternately the test solution and the reference solution.

Calculate the percentage content of each alkaloid from the expression:

$$\frac{m_1 \times A_2 \times 625}{m_2 \times A_1 \times 5} \times \frac{100}{100 - h}$$

- m_1 = mass in grams of the alkaloid used to prepare the reference solution,
 m_2 = mass in grams of the substance to be examined used to prepare the test solution,
 A_1 = area of the peak corresponding to the alkaloid in the chromatogram obtained with the reference solution,
 A_2 = area of the peak corresponding to the alkaloid in the chromatogram obtained with the test solution,
 h = percentage loss on drying.

For the calculation, 1 mg of *morphine hydrochloride R* is taken to be equivalent to 0.759 mg of morphine and 1 mg of *codeine R* is taken to be equivalent to 0.943 mg of codeine.

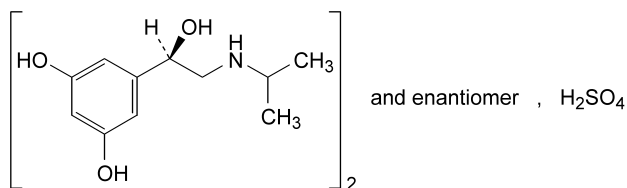
STORAGE

Store protected from light.

01/2005:1033

ORCIPRENALINE SULPHATE

Orciprenalini sulfas

C₂₂H₃₆N₂O₁₀SM_r 520.6

DEFINITION

Orciprenaline sulphate contains not less than 98.0 per cent and not more than the equivalent of 102.0 per cent of di[(RS)-1-(3,5-dihydroxyphenyl)-2-[(1-methylethyl)amino]ethanol] sulphate, calculated with reference to the anhydrous, solvent-free substance.

CHARACTERS

A white, crystalline powder, slightly hygroscopic, freely soluble in water and in alcohol, practically insoluble in methylene chloride.

IDENTIFICATION

First identification: B, E.

Second identification: A, C, D, E.

A. Dissolve 50.0 mg in a 0.04 per cent V/V solution of *hydrochloric acid R* and dilute to 50.0 ml with the same acid solution. Dilute 5.0 ml of the solution to 50.0 ml with a 0.04 per cent V/V solution of *hydrochloric acid R*. Examined between 240 nm and 350 nm (2.2.25), the solution shows an absorption maximum at 278 nm. The specific absorbance at the maximum is 68.5 to 76.0, calculated with reference to the anhydrous and solvent-free substance.

B. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *orciprenaline sulphate CRS*. Examine the substances prepared as discs. If the spectra obtained show differences, dissolve 50 mg of the substance to be examined and the reference substance separately in the minimum volume of *water R* with heating. Add 10 ml of *acetone R* and centrifuge. Dry the precipitate at 40 °C under reduced pressure for 3 h and record new spectra using the residues.

C. Examine by thin-layer chromatography (2.2.27), using *silica gel G R* as the coating substance.

Test solution. Dissolve 10 mg of the substance to be examined in *alcohol R* and dilute to 10 ml with the same solvent.

Reference solution (a). Dissolve 10 mg of *orciprenaline sulphate CRS* in *alcohol R* and dilute to 10 ml with the same solvent.

Reference solution (b). Dissolve 10 mg of *orciprenaline sulphate CRS* and 10 mg of *salbutamol CRS* in *alcohol R* and dilute to 10 ml with the same solvent.

Apply separately to the plate 2 µl of each solution. Develop over a path of 15 cm using a mixture of 1.5 volumes of *ammonia R*, 10 volumes of *water R* and 90 volumes of *aldehyde-free methanol R*. Allow the plate to dry in air and spray with a 10 g/l solution of *potassium*

permanganate R. The principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a). The test is not valid unless the chromatogram obtained with reference solution (b) shows two clearly separated principal spots.

D. Dissolve about 20 mg in 2 ml of *alcohol R*. Add 2 ml of a 1 g/l solution of *dichloroquinonechlorimide R* in *alcohol R* and 1 ml of *sodium carbonate solution R*. A violet colour is produced, turning to brown.

E. It gives reaction (a) of sulphates (2.3.1).

TESTS

Solution S. Dissolve 2.0 g in *carbon dioxide-free water R* and dilute to 20 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and colourless (2.2.2, *Method II*).

pH (2.2.3). The pH of solution S is 4.0 to 5.5.

Related substances. Examine by thin-layer chromatography (2.2.27), using *silica gel G R* as the coating substance.

Test solution. Dissolve 0.20 g of the substance to be examined in a mixture of 1 volume of *water R* and 5 volumes of *methanol R* and dilute to 10 ml with the same mixture of solvents.

Reference solution (a). Dilute 1 ml of the test solution to 100 ml with a mixture of 1 volume of *water R* and 5 volumes of *methanol R*.

Reference solution (b). Dilute 5 ml of reference solution (a) to 10 ml with a mixture of 1 volume of *water R* and 5 volumes of *methanol R*.

Reference solution (c). Dilute 5 ml of reference solution (a) to 20 ml with a mixture of 1 volume of *water R* and 5 volumes of *methanol R*.

Apply separately to the plate 10 µl of each solution. Develop over a path of 15 cm using a mixture of 4 volumes of *concentrated ammonia R*, 16 volumes of *water R*, 30 volumes of *2-propanol R* and 50 volumes of *ethyl acetate R*. Allow the plate to dry in air. Expose the plate to iodine vapour. Any spot in the chromatogram obtained with the test solution, apart from the principal spot, is not more intense than the principal spot in the chromatogram obtained with reference solution (a) (1.0 per cent) and at most one such spot is more intense than the principal spot in the chromatogram obtained with reference solution (b) (0.5 per cent). The test is not valid unless the spot in the chromatogram obtained with reference solution (c) is clearly visible.

Methanol and 2-propanol. Not more than 0.1 per cent *m/m* of methanol and 0.3 per cent *m/m* of 2-propanol, determined by gas chromatography (2.2.28), using *ethanol R1* as internal standard.

Internal standard solution. Dilute 2 ml of *ethanol R1* to 100 ml with *water R*. Dilute 1 ml of the solution to 10 ml with *water R*.

Test solution (a). Dissolve 1.0 g of the substance to be examined in *water R* and dilute to 10.0 ml with the same solvent.

Test solution (b). Dissolve 1.0 g of the substance to be examined in *water R*, add 1.0 ml of the internal standard solution and dilute to 10.0 ml with *water R*.

Reference solution. Dilute a mixture of 1.0 ml of *methanol R* and 3.0 ml of *2-propanol R* to 100.0 ml with *water R*. Dilute 1.0 ml of the solution to 10.0 ml with *water R*. To 1.0 ml of this solution, add 1.0 ml of the internal standard solution and dilute to 10.0 ml with *water R*.

The chromatographic procedure may be carried out using:

- a glass column 2 m long and 2 mm in internal diameter packed with *ethylvinylbenzene-divinylbenzene copolymer R* (150 µm to 180 µm),
- *nitrogen for chromatography R* as the carrier gas at a flow rate of 30 ml/min,
- a flame-ionisation detector,

maintaining the temperature of the column at 140 °C and that of the injection port and of the detector at 180 °C.

Inject 1 µl of each solution. In the chromatogram obtained with test solution (a), verify that there is no peak with the same retention time as the internal standard. Calculate the content of methanol and 2-propanol taking their density at 20 °C to be 0.792 g/ml and 0.785 g/ml, respectively.

Phenone. Dissolve 0.50 g in a 0.04 per cent V/V solution of *hydrochloric acid R* and dilute to 25.0 ml with the same acid solution. The absorbance (2.2.25) of the solution measured at 328 nm is not greater than 0.16 (0.1 per cent).

Iron (2.4.9). The residue obtained in the test for sulphated ash complies with the limit test for iron (20 ppm). Prepare the standard using *iron standard solution (2 ppm Fe) R*.

Heavy metals. 1.0 g complies with limit test C for heavy metals (20 ppm). Prepare the standard using 2 ml of *lead standard solution (10 ppm Pb) R*.

Water (2.5.12). Not more than 2.0 per cent, determined on 1.00 g by the semi-micro determination of water.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

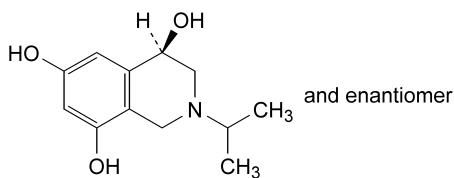
Dissolve 0.400 g in 30 ml of *anhydrous acetic acid R*. Titrate with 0.1 M *perchloric acid* using 0.1 ml of *crystal violet solution R* as indicator.

1 ml of 0.1 M *perchloric acid* is equivalent to 52.06 mg of C₂₂H₃₆N₂O₁₀S.

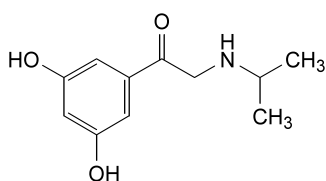
STORAGE

Store protected from light.

IMPURITIES



- A. 2-(1-methylethyl)-1,2,3,4-tetrahydroisoquinoline-4,6,8-triol,



- B. 1-(3,5-dihydroxyphenyl)-2-[(1-methylethyl)amino]ethanone.

OREGANO

Origani herba

DEFINITION

Dried leaves and flowers separated from the stems of *Origanum onites* L. or *Origanum vulgare* L. subsp. *hirtum* (Link) Ietsw., or a mixture of both species.

Content: minimum 25 ml/kg of essential oil and minimum 1.5 per cent of carvacrol and thymol (both C₁₀H₁₄O; *M_r* 150.2) (anhydrous drug).

CHARACTERS

Macroscopic and microscopic characters described under identification tests A and B.

IDENTIFICATION

A. *O. onites*. The leaf is yellowish-green, usually 4 mm to 22 mm long and 3 mm to 14 mm wide. It has long or short petiole or is sessile. The lamina is ovate, elliptic or ovate-lanceolate. Margins are entire or serrate, apex is acute or obtuse. The veins are yellowish and conspicuous on the adaxial surface. Flowers are solitary or seen as broken parts of the corymb. The calyx is bract-like and inconspicuous. The corolla is white, on top of inflorescences or single flowers, or inconspicuous. The bract is imbricate and green like the leaves. The drug consists yellowish or yellowish-brown stem parts.

O. vulgare (subsp. *hirtum*). The leaf is green and usually 3 mm to 28 mm long and 2.5 mm to 19 mm wide. It is petiolate or sessile. The lamina is ovate or ovate-elliptic. The margins are entire or serrate, the apex is acute or obtuse. Flowers are rare, found as broken parts of the corymbs. Bracts are greenish-yellow and imbricate. Calyx is corolla-like and inconspicuous. Corolla is white on top of inflorescences, slightly conspicuous or inconspicuous.

B. Reduce to a powder (355). The powder is green (*O. vulgare*) to yellowish-green (*O. onites*). Examine under a microscope using *chloral hydrate solution R*. The covering trichomes are of lamiate type or short, unicellular and rarely conical; conical trichomes shaped as pointed teeth are more abundant in *O. vulgare*. Covering trichomes are thick-walled in *O. vulgare*. Covering trichomes contain prismatic crystals in *O. onites*, minute needles in *O. vulgare*. Cuticle on covering trichomes is smooth; warty in *O. vulgare*. The epidermises of the leaves have cells with walls which are sinuous and the stomata are of diacytic type (2.8.3); in *O. vulgare* cells of the upper epidermis are beaded; secretory trichomes with 8-16 cells (12 in *O. vulgare*); glandular trichomes are numerous in *O. onites*; rare in *O. vulgare*. They have unicellular head and unicellular, bicellular or tricellular (bicellular or tricellular in *O. vulgare*) stalk; pollen grains are smooth, spherical and more abundant in *O. onites*.

C. Thin-layer chromatography (2.2.27).

Test solution. To 1.0 g of the powdered drug (355) add 5 ml of *methylene chloride R* and shake for 3 min, filter through about 2 g of *anhydrous sodium sulphate R*.

Reference solution. Dissolve 1 mg of *thymol R* and 10 µl of *carvacrol R* in 10 ml of *methylene chloride R*.

Plate: TLC silica gel plate *R*.

Mobile phase: *methylene chloride R*.

Application: 20 µl, as bands.

Development: over a path of 15 cm.

Drying: in air.

combined upper layers add *anhydrous sodium sulphate R*, shake, filter and evaporate the filtrate, at a temperature not exceeding 50 °C, using a rotary evaporator. Take up the residue with *toluene R* and dilute to 20.0 ml with the same solvent.

Reference solution. Dissolve 30 mg of *orphenadrine citrate CRS* and 30 mg of *orphenadrine impurity E CRS* in 20 ml of *water R*. Add 1 ml of *concentrated ammonia R* and shake with 3 quantities, each of 5 ml, of *toluene R*. To the combined upper layers add *anhydrous sodium sulphate R*, shake, filter and evaporate the filtrate, at a temperature not exceeding 50 °C, using a rotary evaporator. Take up the residue with *toluene R* and dilute to 20.0 ml with the same solvent.

Column:

- *size*: $l = 60$ m, $\varnothing = 0.32$ mm,
- *stationary phase*: *poly(dimethyl)(diphenyl)siloxane R* (film thickness 1.0 μ m).

Carrier gas: *helium for chromatography R*.

Flow rate: 1 ml/min.

Split ratio: 1:25.

Temperature:

- *column*: 240 °C,
- *injection port and detector*: 290 °C.

Detection: flame ionisation.

Injection: 2 μ l.

Run time: 1.3 times the retention time of orphenadrine.

System suitability: reference solution:

- *resolution*: minimum of 1.5 between the peaks due to orphenadrine and to impurity E.

Limits:

- *any impurity*: maximum 0.3 per cent,
- *total*: maximum 1.0 per cent,
- *disregard limit*: 0.02 per cent.

Heavy metals (2.4.8): maximum 10 ppm.

2.0 g complies with limit test C. Prepare the standard using 2 ml of *lead standard solution (10 ppm Pb) R*.

Loss on drying (2.2.32): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 100–105 °C for 3 h.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

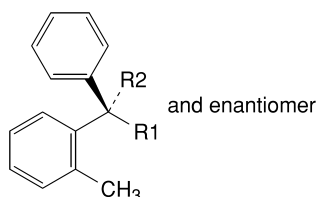
Dissolve 0.350 g in 50 ml of *anhydrous acetic acid R*. Titrate with 0.1 M *perchloric acid*, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *perchloric acid* is equivalent to 46.15 mg of $C_{24}H_{31}NO_8$.

STORAGE

Protected from light. If the substance is sterile, store in a sterile, airtight, tamper-proof container, protected from light.

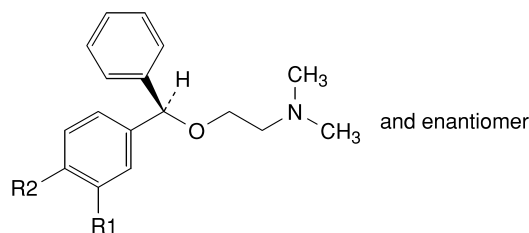
IMPURITIES



- A. $R_1 = OH$, $R_2 = H$: (*RS*)-(2-methylphenyl)phenylmethanol (2-methylbenzhydrol),

- B. $R_1 + R_2 = O$: (2-methylphenyl)phenylmethanone (2-methylbenzophenone),

- C. $R_1 = O-CH_2-CH_2-NH_2$, $R_2 = H$: (*RS*)-2-[(2-methylphenyl)phenylmethoxy]ethanamine,



- D. $R_1 = R_2 = H$: diphenhydramine,

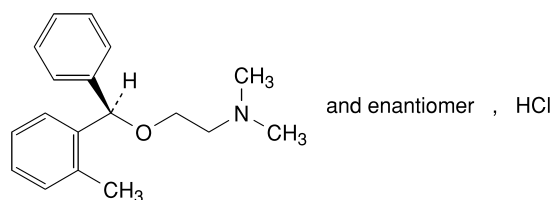
- E. $R_1 = CH_3$, $R_2 = H$: (*RS*)-*N,N*-dimethyl-2-[(3-methylphenyl)phenylmethoxy]ethanamine (*meta*-methylbenzyl isomer),

- F. $R_1 = H$, $R_2 = CH_3$: (*RS*)-*N,N*-dimethyl-2-[(4-methylphenyl)phenylmethoxy]ethanamine (*para*-methylbenzyl isomer).

01/2005:1760

ORPHENADRINE HYDROCHLORIDE

Orphenadrini hydrochloridum



$C_{18}H_{24}ClNO$

M_r 305.9

DEFINITION

(*RS*)-*N,N*-Dimethyl-2-[(2-methylphenyl)phenylmethoxy]ethanamine hydrochloride.

Content: 98.5 per cent to 101.0 per cent (dried substance).

CHARACTERS

Appearance: white or almost white, crystalline powder.

Solubility: freely soluble in water and in alcohol.

mp: about 160 °C.

IDENTIFICATION

- A. Infrared absorption spectrophotometry (2.2.24).

Preparation: discs.

Comparison: *orphenadrine hydrochloride CRS*.

- B. It gives reaction (a) of chlorides (2.3.1).

TESTS

Appearance of solution. The solution is clear (2.2.1) and its absorbance (2.2.25) at 436 nm has a maximum of 0.050.

Dissolve 0.70 g in *alcohol R* and dilute to 10.0 ml with the same solvent.

Related substances. Gas chromatography (2.2.28): use the normalisation procedure.

Test solution. Dissolve 0.300 g of the substance to be examined in *water R* and dilute to 50 ml with the same solvent. Add 2 ml of *concentrated ammonia R* and shake with 3 quantities, each of 10 ml, of *toluene R*. To the combined upper layers add *anhydrous sodium sulphate R*, shake, filter and evaporate the filtrate, at a temperature not

exceeding 50 °C, using a rotary evaporator. Take up the residue with *toluene R* and dilute to 20.0 ml with the same solvent.

Reference solution. Dissolve 20 mg of *orphenadrine hydrochloride CRS* and 20 mg of *orphenadrine impurity E CRS* in 20 ml of *water R*. Add 1 ml of *concentrated ammonia R* and shake with 3 quantities, each of 5 ml, of *toluene R*. To the combined upper layers add *anhydrous sodium sulphate R*, shake, filter and evaporate the filtrate, at a temperature not exceeding 50 °C, using a rotary evaporator. Take up the residue with *toluene R* and dilute to 20.0 ml with the same solvent.

Column:

- *size*: $l = 60$ m, $\varnothing = 0.32$ mm,
- *stationary phase*: *poly(dimethyl)(diphenyl)siloxane R* (film thickness 1.0 μ m).

Carrier gas: *helium for chromatography R*.

Flow rate: 1 ml/min.

Split ratio: 1:25.

Temperature:

- *column*: 240 °C,
- *injection port and detector*: 290 °C.

Detection: flame ionisation.

Injection: 2 μ l.

Run time: 1.3 times the retention time of orphenadrine.

System suitability: reference solution:

- *resolution*: minimum of 1.5 between the peaks due to orphenadrine and to impurity E.

Limits:

- *any impurity*: maximum 0.3 per cent,
- *total*: maximum 1.0 per cent,
- *disregard limit*: 0.02 per cent.

Heavy metals (2.4.8): maximum 10 ppm.

2.0 g complies with limit test C. Prepare the standard using 2 ml of *lead standard solution (10 ppm Pb) R*.

Loss on drying (2.2.32): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 100–105 °C for 3 h.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

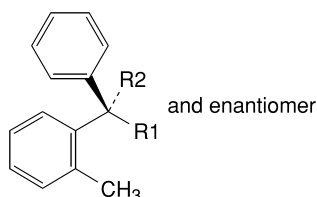
Dissolve 0.250 g in 50 ml of *acetic anhydride R*. Titrate with 0.1 M *perchloric acid*, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *perchloric acid* is equivalent to 30.59 mg of $C_{18}H_{24}ClNO$.

STORAGE

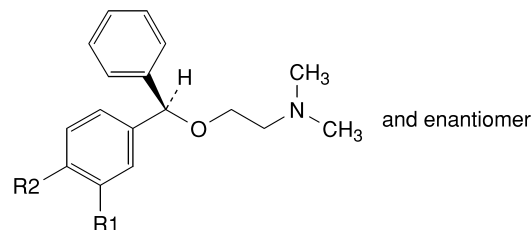
Protected from light. If the substance is sterile, store in a sterile, airtight, tamper-proof container, protected from light.

IMPURITIES



- A. $R_1 = OH$, $R_2 = H$: (*RS*)-(2-methylphenyl)phenylmethanol (2-methylbenzhydrol),
- B. $R_1 + R_2 = O$: (2-methylphenyl)phenylmethanone (2-methylbenzophenone),

- C. $R_1 = O-CH_2-CH_2-NH_2$, $R_2 = H$: (*RS*)-2-[(2-methylphenyl)phenylmethoxy]ethanamine,

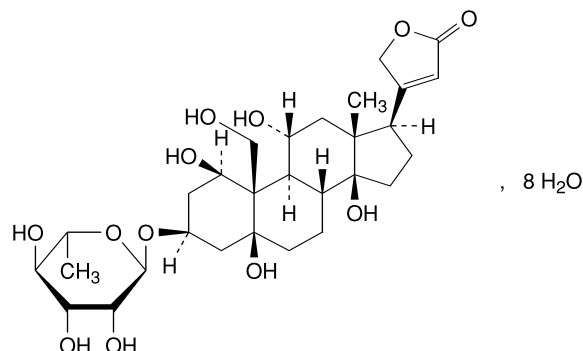


- D. $R_1 = R_2 = H$: diphenhydramine,
- E. $R_1 = CH_3$, $R_2 = H$: (*RS*)-*N,N*-dimethyl-2-[(3-methylphenyl)phenylmethoxy]ethanamine (*meta*-methylbenzyl isomer),
- F. $R_1 = H$, $R_2 = CH_3$: (*RS*)-*N,N*-dimethyl-2-[(4-methylphenyl)phenylmethoxy]ethanamine (*para*-methylbenzyl isomer).

01/2005:0048

OUABAIN

Ouabainum



$C_{29}H_{44}O_{12} \cdot 8H_2O$

M_r 729

DEFINITION

Ouabain contains not less than 96.0 per cent and not more than the equivalent of 104.0 per cent of 3 β -[(6-deoxy- α -L-mannopyranosyl)oxy]-1 β ,5,11 α ,14,19-pentahydroxy-5 β ,14 β -card-20(22)-enolide, calculated with reference to the anhydrous substance.

CHARACTERS

A white, crystalline powder or colourless crystals, sparingly soluble in water and in ethanol, practically insoluble in ethyl acetate.

IDENTIFICATION

- A. Examine the chromatograms obtained in the test for related substances. The principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the spot in the chromatogram obtained with reference solution (a).
- B. Dissolve 2 mg to 3 mg in 2 ml of *sulphuric acid R*; a pink colour develops which quickly changes to red. The solution shows green fluorescence in ultraviolet light.
- C. Dissolve about 1 mg in 1 ml of *dinitrobenzene solution R* and add 0.2 ml of *dilute sodium hydroxide solution R*. An intense blue colour develops.
- D. Dissolve 0.1 g in 5 ml of a 150 g/l solution of *sulphuric acid R* and boil for a few minutes. The solution becomes yellow and turbid. Filter and add to the filtrate 5 ml

of a 120 g/l solution of *sodium hydroxide R* and 3 ml of *cupri-tartaric solution R*. Heat. A red precipitate is formed.

TESTS

Solution S. Dissolve 0.20 g in 15 ml of *water R*, heating on a water-bath. Allow to cool and dilute to 20.0 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and colourless (2.2.2, *Method II*).

Specific optical rotation (2.2.7). –30 to –33, determined on solution S and calculated with reference to the anhydrous substance.

Related substances. Examine by thin-layer chromatography (2.2.27), using *silica gel G R* as the coating substance.

Test solution. Dissolve a quantity of the substance to be examined corresponding to 20 mg of the anhydrous substance in 1.0 ml of a mixture of 32 volumes of *water R*, 100 volumes of *chloroform R* and 100 volumes of *methanol R*.

Reference solution (a). Dissolve a quantity of *ouabain CRS* corresponding to 20 mg of the anhydrous substance in 1.0 ml of a mixture of 32 volumes of *water R*, 100 volumes of *chloroform R* and 100 volumes of *methanol R*.

Reference solution (b). Dissolve a quantity of *ouabain CRS* corresponding to 10 mg of the anhydrous substance in a mixture of 32 volumes of *water R*, 100 volumes of *chloroform R*, 100 volumes of *methanol R* and dilute to 25 ml with the same mixture of solvents.

Reference solution (c). Dilute 2.5 ml of reference solution (b) to 10 ml with a mixture of 32 volumes of *water R*, 100 volumes of *chloroform R* and 100 volumes of *methanol R*.

Apply separately to the plate 5 µl of each solution. Develop over a path of 13 cm using a homogeneous mixture of 4 volumes of *water R*, 15 volumes of *methanol R*, 15 volumes of *dimethyl sulphoxide R* and 70 volumes of *chloroform R*. Dry the plate immediately at 140 °C for 30 min in a ventilated drying oven. Allow to cool, spray with *alcoholic sulphuric acid solution R* and heat at 140 °C for 15 min. Any spot in the chromatogram obtained with the test solution, apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (b) (2.0 per cent). The test is not valid unless the principal spot in the chromatogram obtained with reference solution (a) and the principal spot in the chromatogram obtained with the test solution migrate over a distance sufficient to give unequivocal separation of the secondary spots and the spot in the chromatogram obtained with reference solution (c) is clearly visible.

Alkaloids and strophanthin-K. To 5.0 ml of solution S add 0.5 ml of a 100 g/l solution of *tannic acid R*. No precipitate is formed.

Water (2.5.12). 18.0 per cent to 22.0 per cent, determined on 0.100 g by the semi-micro determination of water.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 40.0 mg in *alcohol R* and dilute to 50.0 ml with the same solvent. Dilute 5.0 ml of the solution to 100.0 ml with *alcohol R*. Prepare a reference solution in the same manner using 40.0 mg of *ouabain CRS*. To 5.0 ml of each solution add 3.0 ml of *alkaline sodium picrate solution R*, allow to stand protected from bright light for 30 min and measure the absorbance (2.2.25) of each solution at the maximum

at 495 nm using as the compensation liquid a mixture of 5.0 ml of *alcohol R* and 3.0 ml of *alkaline sodium picrate solution R* prepared at the same time.

Calculate the content of $C_{29}H_{44}O_{12}$ from the absorbances measured and the concentrations of the solutions.

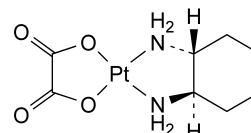
STORAGE

Store protected from light.

01/2005:2017

OXALIPLATIN

Oxaliplatinum



$C_8H_{14}N_2O_4Pt$

M_r 397.3

DEFINITION

(*SP-4-2*)-[(1*R*,2*R*)-Cyclohexane-1,2-diamine-κ*N*,κ*N'*][ethanedioato(2-)-κ*O*¹,κ*O*²]platinum.

Content: 98.0 per cent to 102.0 per cent (dried substance).

CHARACTERS

Appearance: white or almost white, crystalline powder.

Solubility: slightly soluble in water, very slightly soluble in methanol, practically insoluble in ethanol.

IDENTIFICATION

A. Infrared absorption spectrophotometry (2.2.24).

Comparison: *oxaliplatin CRS*.

B. It complies with the test for specific optical rotation (see Tests).

TESTS

Appearance of solution. The solution is clear (2.2.1) and colourless (2.2.2, *Method II*).

Dissolve 0.10 g in *water R* and dilute to 50 ml with the same solvent.

Acidity. Dissolve 0.10 g in *carbon dioxide-free water R*, dilute to 50 ml with the same solvent and add 0.5 ml of *phenolphthalein solution R1*. The solution is colourless. Not more than 0.60 ml of 0.01 *M sodium hydroxide* is required to change the colour of the indicator to pink.

Specific optical rotation (2.2.7): + 74.5 to + 78.0 (dried substance).

Dissolve 0.250 g in *water R* and dilute to 50.0 ml with the same solvent.

Related substances

A. Impurity A. Liquid chromatography (2.2.29). *Use vigorous shaking and very brief sonication to dissolve the substance to be examined. Inject the test solution within 20 min of preparation.*

Test solution. Dissolve 0.100 g of the substance to be examined in *water R* and dilute to 50.0 ml with the same solvent.

Reference solution (a). Dissolve 14.0 mg of *oxalic acid R* (impurity A) in *water R* and dilute to 250.0 ml with the same solvent.

Reference solution (b). Dilute 5.0 ml of reference solution (a) to 200.0 ml with *water R*.

Reference solution (c). Dissolve 12.5 mg of *sodium nitrate R* in *water R* and dilute to 250.0 ml with the same solvent. Dilute a mixture of 2.0 ml of this solution and 25.0 ml of reference solution (a) to 100.0 ml with *water R*.

Column:

- *size:* $l = 25$ cm, $\emptyset = 4.6$ mm,
- *stationary phase:* base-deactivated octadecylsilyl silica gel for chromatography *R* (5 μ m).

Temperature: 40 °C.

Mobile phase: mix 20 volumes of *acetonitrile R* with 80 volumes of a solution prepared as follows: to 10 ml of a 320 g/l solution of *tetrabutylammonium hydroxide R* add 1.36 g of *potassium dihydrogen phosphate R* and dilute to 1000 ml with *water R*; adjust this solution to pH 6.0 with *phosphoric acid R*.

Flow rate: 2 ml/min.

Detection: spectrophotometer at 205 nm.

Injection: 20 μ l; inject the test solution and reference solutions (b) and (c).

Run time: twice the retention time of impurity A.

Retention times: nitrate = about 2.7 min; impurity A = about 4.7 min.

System suitability:

- *resolution:* minimum 9 between the peaks due to nitrate and impurity A in the chromatogram obtained with reference solution (c),
- *signal-to-noise ratio:* minimum of 10 for the peak due to impurity A in the chromatogram obtained with reference solution (b).

Limits:

- *impurity A:* not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent).

- B. Impurity B. Liquid chromatography (2.2.29). Use vigorous shaking and very brief sonication to dissolve the substance to be examined. Inject the test solution within 20 min of preparation. Use suitable polypropylene containers for the preparation and injection of all solutions. Glass pipettes may be used for diluting solutions.

Test solution. Dissolve 0.100 g of the substance to be examined in *water R* and dilute to 50.0 ml with the same solvent.

Reference solution (a). Dissolve 12.5 mg of *oxaliplatin impurity B CRS* in 63 ml of *methanol R* and dilute to 250.0 ml with *water R*. Dilute 3.0 ml to 200.0 ml with *water R*.

Reference solution (b). In order to prepare *in situ* the degradation compound (impurity E) dissolve 12.5 mg of *oxaliplatin impurity B CRS* in 63 ml of *methanol R* and dilute to 250 ml with *water R*. Adjust to pH 6.0 with a 0.2 g/l solution of *sodium hydroxide R*. Heat for 4 h at 70 °C and allow to cool.

Column:

- *size:* $l = 25$ cm, $\emptyset = 4.6$ mm,
- *stationary phase:* base-deactivated octadecylsilyl silica gel for chromatography *R* (5 μ m).

Temperature: 40 °C.

Mobile phase: mix 20 volumes of *acetonitrile R* with 80 volumes of a solution prepared as follows: dissolve 1.36 g of *potassium dihydrogen phosphate R* and 1 g of *sodium heptanesulphonate R* in 1000 ml of *water R*; adjust this solution to pH 3.0 ± 0.05 with *phosphoric acid R*.

Flow rate: 2.0 ml/min.

Detection: spectrophotometer at 215 nm.

Injection: 20 μ l.

Run time: 2.5 times the retention time of impurity B.

Retention times: impurity B = about 4.3 min; impurity E = about 6.4 min.

System suitability:

- *resolution:* minimum 7 between the peaks due to impurity B and impurity E in the chromatogram obtained with reference solution (b),
- *signal-to-noise ratio:* minimum of 10 for the peak due to impurity B in the chromatogram obtained with reference solution (a).

Limits:

- *impurity B:* not more than 3.3 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent).

- C. Impurity C and other related substances. Liquid chromatography (2.2.29). Use vigorous shaking and very brief sonication to dissolve the substance to be examined. Inject the test solution within 20 min of preparation.

Test solution (a). Dissolve 0.100 g of the substance to be examined in *water R* and dilute to 50.0 ml with the same solvent.

Test solution (b). Dissolve 50.0 mg of the substance to be examined in *water R* and dilute to 500.0 ml with the same solvent.

Reference solution (a). Dissolve 10 mg of *oxaliplatin impurity C CRS* and 10 mg of *oxaliplatin CRS* in *water R* and dilute to 100.0 ml with the same solvent.

Reference solution (b). Dilute 1.0 ml of reference solution (a) to 100.0 ml with *water R*.

Reference solution (c). Dissolve 5 mg of *dichlorodiaminocyclohexaneplatinum CRS* in *methanol R* and dilute to 50.0 ml with the same solvent. To 10.0 ml of this solution add 10.0 ml of reference solution (a) and dilute to 100.0 ml with *water R*.

Reference solution (d). Dissolve 50.0 mg of *oxaliplatin CRS* in *water R* and dilute to 500.0 ml with the same solvent.

Reference solution (e). Dissolve 5.0 mg of *dichlorodiaminocyclohexaneplatinum CRS* in reference solution (d) and dilute to 50.0 ml with the same solvent.

Reference solution (f). To 0.100 g of the substance to be examined add 1.0 ml of reference solution (a) and dilute to 50.0 ml with *water R*.

Column:

- *size:* $l = 25$ cm, $\emptyset = 4.6$ mm,
- *stationary phase:* octadecylsilyl silica gel for chromatography *R* (5 μ m).

Temperature: 40 °C.

Mobile phase: mixture of solutions A and B (99:1 V/V).

- *solution A:* dilute 0.6 ml of *dilute phosphoric acid R* in 1000 ml of *water R* and adjust to pH 3.0 with either *sodium hydroxide solution R* or *phosphoric acid R*,
- *solution B:* *acetonitrile R*.

Flow rate: 1.2 ml/min.

Detection: spectrophotometer at 210 nm.

Injection: 10 μ l; inject test solution (a) and reference solutions (b), (c) and (f).

Run time: 3 times the retention time of oxaliplatin.

Retention times: impurity C = about 4.4 min;
dichlorodiaminocyclohexaneplatinum = about 6.9 min;
oxaliplatin = about 8.0 min.

System suitability:

- **resolution:** minimum 2.0 between the peaks due to dichlorodiaminocyclohexaneplatinum and oxaliplatin in the chromatogram obtained with reference solution (c),
- **signal-to-noise ratio:** minimum 50 for the peak due to impurity C and minimum 10 for the peak due to oxaliplatin in the chromatogram obtained with reference solution (b).

Limits:

- **impurity C:** not more than half the area of the peak due to impurity C in the chromatogram obtained with reference solution (f) (0.1 per cent),
- **any other impurity:** not more than twice the area of the peak due to oxaliplatin in the chromatogram obtained with reference solution (b) (0.1 per cent),
- **total of other impurities:** not more than twice the area of the peak due to oxaliplatin in the chromatogram obtained with reference solution (b) (0.1 per cent),
- **disregard limit:** the area of the peak due to oxaliplatin in the chromatogram obtained with reference solution (b) (0.05 per cent); disregard any peak with a retention time less than 2 min.

D. Total of impurities: the sum of impurities A, B, C and other related impurities is not greater than 0.30 per cent.

Impurity D. Liquid chromatography (2.2.29).

Test solution. Dissolve 30 mg of the substance to be examined in *methanol R* and dilute to 50.0 ml with the same solvent.

Reference solution (a). Dissolve 5 mg of *oxaliplatin impurity D CRS* in *methanol R* and dilute to 100.0 ml with the same solvent.

Reference solution (b). Dilute 15.0 ml of reference solution (a) to 50.0 ml with *methanol R*.

Reference solution (c). Dissolve 150.0 mg of *oxaliplatin CRS* in *methanol R* and dilute to 200.0 ml with the same solvent.

Reference solution (d). Dilute 5.0 ml of reference solution (c) to 100.0 ml with *methanol R*.

Reference solution (e). To 40 ml of reference solution (c) add 1.0 ml of reference solution (b) and dilute to 50.0 ml with *methanol R*.

Reference solution (f). Mix 4.0 ml of reference solution (a) and 5.0 ml of reference solution (d) and dilute to 50.0 ml with *methanol R*.

Column:

- **size:** $l = 25$ cm, $\varnothing = 4.6$ mm,
- **stationary phase:** *silica gel OC for chiral separations R*.

Temperature: 40 °C.

Mobile phase: *ethanol R*, *methanol R* (3:7 V/V).

Flow rate: 0.3 ml/min.

Detection: spectrophotometer at 254 nm.

Injection: 20 µl; inject the test solution and reference solutions (e) and (f).

Run time: twice the retention time of oxaliplatin.

Retention times: oxaliplatin = about 14 min;
impurity D = about 16 min.

System suitability:

- **resolution:** minimum 1.5 between the peaks due to oxaliplatin and impurity D in the chromatogram obtained with reference solution (f),

- **signal-to-noise ratio:** minimum 10 for the peak due to impurity D in the chromatogram obtained with reference solution (e).

Limits:

- **impurity D:** not more than twice the peak height of the corresponding peak in the chromatogram obtained with reference solution (e) (0.1 per cent).

Silver: maximum 5 ppm.

Atomic absorption spectrometry (2.2.23, *Method II*).

Test solution. Dissolve 0.1000 g of the substance to be examined in *water R* and dilute to 50.0 ml with the same solvent. Dilute 20 µl of this solution to 40 µl with 0.5 M *nitric acid*.

Reference solution (a). Dilute a solution of *silver nitrate R* containing 1000 ppm of silver in 0.5 M *nitric acid* with 0.5 M *nitric acid* to obtain a solution which contains 10 ppb of silver.

Reference solution (b). Mix 20 µl of the test solution and 8 µl of reference solution (a) and dilute to 40 µl with 0.5 M *nitric acid*.

Reference solution (c). Mix 20 µl of the test solution and 16 µl of reference solution (a) and dilute to 40 µl with 0.5 M *nitric acid*.

Source: silver hollow-cathode lamp.

Wavelength: 328.1 nm.

Atomisation device: furnace.

Measure the absorbance of the test solution and reference solutions (b) and (c).

Loss on drying (2.2.32): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 100–105 °C for 2 h.

Bacterial endotoxins (2.6.14): less than 1.0 IU/mg, if intended for use in the manufacture of parenteral dosage forms without a further appropriate procedure for the removal of bacterial endotoxins.

ASSAY

Liquid chromatography (2.2.29) as described in the test for impurity C and other related substances with the following modifications.

Injection: 20 µl; inject test solution (b) and reference solutions (d) and (e).

System suitability:

- **resolution:** minimum 2.0 between the peaks due to dichlorodiaminocyclohexaneplatinum and oxaliplatin in the chromatogram obtained with reference solution (e),
- **repeatability:** reference solution (d).

Calculate the percentage content of oxaliplatin using the chromatogram obtained with reference solution (d).

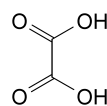
LABELLING

The label states where applicable, that the substance is free from bacterial endotoxins.

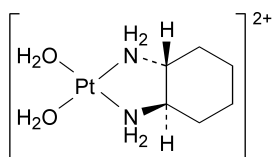
IMPURITIES

Specified impurities: A, B, C, D.

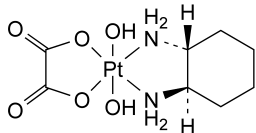
Other detectable impurities: E.



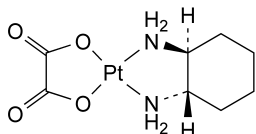
A. ethanedioic acid (oxalic acid),



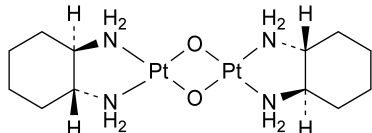
- B. (SP-4-2)-diaqua[(1R,2R)-cyclohexane-1,2-diamine-κN, κN']platinum (diaquodiaminocyclohexaneplatinum),



- C. (OC-6-33)-[(1R,2R)-cyclohexane-1,2-diamine-κN, κN']ethanedioato(2-)-κO', κO'' dihydroxyplatinum,



- D. (SP-4-2)-[(1S,2S)-cyclohexane-1,2-diamine-κN, κN']ethanedioato(2-)-κO', κO'' platinum (S,S-enantiomer of oxaliplatin),

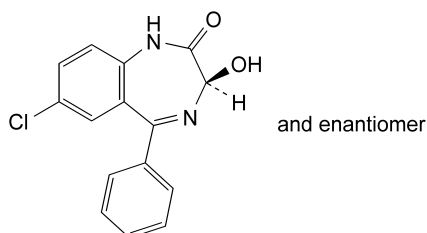


- E. (SP-4-2)-di-μ-oxobis[(1R,2R)-cyclohexane-1,2-diamine-κN, κN']diplatinum (diaquodiaminocyclohexaneplatinum dimer).

01/2005:0778

OXAZEPAM

Oxazepamum

C₁₅H₁₁ClN₂O₂M_r 286.7

DEFINITION

Oxazepam contains not less than 98.5 per cent and not more than the equivalent of 101.0 per cent of (3RS)-7-chloro-3-hydroxy-5-phenyl-1,3-dihydro-2H-1,4-benzodiazepin-2-one, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, crystalline powder, practically insoluble in water, slightly soluble in alcohol and in methylene chloride.

IDENTIFICATION

First identification: B, C.

Second identification: A, C, D.

A. Prepare the solutions immediately before use, protected from light. Dissolve 20.0 mg in alcohol R and dilute to 100.0 ml with the same solvent. Dilute 10.0 ml of

the solution to 50.0 ml with alcohol R (solution A). Dilute 10.0 ml of solution A to 100.0 ml with alcohol R (solution B). Examined between 300 nm and 350 nm (2.2.25), solution A shows an absorption maximum at 316 nm. Examined between 220 nm and 250 nm, solution B shows an absorption maximum at 229 nm. The specific absorbance at the maximum at 229 nm is 1220 to 1300.

- B. Examine by infrared absorption spectrophotometry (2.2.24) comparing with the spectrum obtained with oxazepam CRS. Examine the substances prepared as discs.
- C. Examine the chromatograms obtained in the test for related substances in ultraviolet light at 254 nm. The principal spot in the chromatogram obtained with test solution (b) is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a).
- D. Dissolve about 20 mg in a mixture of 5 ml of hydrochloric acid R and 10 ml of water R. Heat to boiling for 5 min and cool. Add 2 ml of a 1 g/l solution of sodium nitrite R and allow to stand for 1 min. Add 1 ml of a 5 g/l solution of sulphamic acid R, mix and allow to stand for 1 min. Add 1 ml of a 1 g/l solution of naphthylethylenediamine dihydrochloride R. A red colour develops.

TESTS

Related substances. Carry out the test protected from light. Examine by thin-layer chromatography (2.2.27), using as the coating substance a suitable silica gel with a fluorescent indicator having an optimal intensity at 254 nm. Before use, wash the plate with methanol R until the solvent front has migrated at least 17 cm. Allow the plate to dry in air and heat at 100 °C to 105 °C for 30 min.

Test solution (a). Dissolve 50 mg of the substance to be examined in acetone R and dilute to 10 ml with the same solvent.

Test solution (b). Dilute 2 ml of test solution (a) to 10 ml with acetone R.

Reference solution (a). Dissolve 10 mg of oxazepam CRS in acetone R and dilute to 10 ml with the same solvent.

Reference solution (b). Dissolve 10 mg of oxazepam CRS and 10 mg of bromazepam CRS in acetone R and dilute to 10 ml with the same solvent.

Reference solution (c). Dilute 1 ml of test solution (b) to 100 ml with acetone R.

Reference solution (d). Dilute 5 ml of reference solution (c) to 10 ml with acetone R.

Apply separately to the plate 20 µl of each solution. Develop over a path of 15 cm, in the same direction as the washing with methanol R, using a mixture of 10 volumes of methanol R and 100 volumes of methylene chloride R. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. Any spot in the chromatogram obtained with test solution (a), apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (c) (0.2 per cent) and at most one such spot is more intense than the spot in the chromatogram obtained with reference solution (d) (0.1 per cent). The test is not valid unless the chromatogram obtained with reference solution (b) shows two clearly separated spots.

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C at a pressure not exceeding 0.7 kPa.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.250 g in a mixture of 10 ml of *anhydrous acetic acid R* and 90 ml of *acetic anhydride R*. Titrate with 0.1 M *perchloric acid* determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *perchloric acid* is equivalent to 28.67 mg of $C_{15}H_{11}ClN_2O_2$.

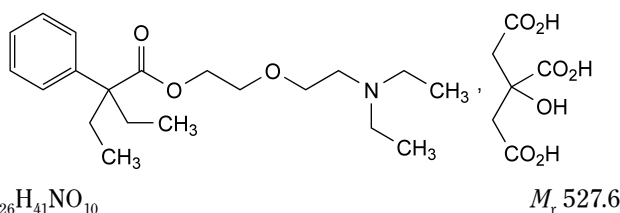
STORAGE

Store protected from light.

01/2005:1761

OXELADIN HYDROGEN CITRATE

Oxeladini hydrogenocitras



DEFINITION

2-[2-(Diethylamino)ethoxy]ethyl 2-ethyl-2-phenylbutanoate dihydrogen 2-hydroxypropane-1,2,3-tricarboxylate.

Content: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

Appearance: white or almost white, crystalline powder.

Solubility: freely soluble in water, slightly to very slightly soluble in ethyl acetate.

It shows polymorphism.

IDENTIFICATION

Infrared absorption spectrophotometry (2.2.24).

Comparison: oxeladin hydrogen citrate CRS.

If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in *anhydrous ethanol R*, evaporate to dryness and record new spectra using the residues.

TESTS

Appearance of solution. The solution is clear (2.2.1) and not more intensely coloured than reference solution Y₆ (2.2.2, Method II).

Dissolve 2.0 g in *water R* and dilute to 10.0 ml with the same solvent.

Related substances. Gas chromatography (2.2.28): use the normalisation procedure. *Prepare the solutions immediately before use.*

Test solution. Dissolve 0.500 g of the substance to be examined in *water R* and dilute to 50 ml with the same solvent. Add 1 ml of a 10.3 g/l solution of *hydrochloric acid R* and shake with 3 quantities, each of 10 ml, of *methylene chloride R*. Combine the lower layers. Add 5 ml of *concentrated ammonia R* to the aqueous layer and shake with 3 quantities, each of 10 ml, of *methylene chloride R*. Combine the lower layers obtained to the lower layers obtained previously, add *anhydrous sodium sulphate R*, shake, filter and evaporate the filtrate, at a temperature not exceeding 30 °C, using a rotary evaporator. Take up the residue with *methylene chloride R* and dilute to 20.0 ml with the same solvent.

Reference solution (a). Dissolve 5 mg of *oxeladin impurity D CRS* in 10 ml of *water R*, add 0.5 ml of *concentrated ammonia R* and shake with 3 quantities, each of 2 ml, of *methylene chloride R*. To the combined lower layers, add 0.2 ml of the test solution and dilute to 10.0 ml with *methylene chloride R*.

Reference solution (b). Dilute 1.0 ml of the test solution to 100.0 ml with *methylene chloride R*. Dilute 1.0 ml of this solution to 20.0 ml with *methylene chloride R*.

Reference solution (c). Dissolve 5 mg of *oxeladin impurity C CRS* in 10 ml of *water R*, add 0.5 ml of *concentrated ammonia R* and shake with 3 quantities, each of 2 ml, of *methylene chloride R*. Combine the lower layers and dilute to 10 ml with *methylene chloride R*.

Column:

- *material*: fused silica,
- *size*: $l = 25$ m, $\varnothing = 0.32$ mm,
- *stationary phase*: poly(dimethyl)(diphenyl)siloxane *R* (film thickness 0.4 µm).

Carrier gas: helium for chromatography *R*.

Flow rate: 1.0 ml/min. Adjust the flow rate if necessary to obtain a retention time of about 13 min for oxeladin.

Split ratio: 1:15.

Temperature:

	Time (min)	Temperature (°C)
Column	0 - 4	160
	4 - 12	160 → 240
	12 - 21	240
	21 - 30	240 → 160
Injection port		280
Detector		280

Detection: flame ionisation.

Injection: 1 µl.

Relative retention with reference to oxeladin (retention time = about 13 min): impurity A = about 0.2; impurity B = about 0.4; impurity C = about 0.8; impurity D = about 0.9.

System suitability: reference solution (a):

- *resolution*: minimum 10 between the peaks due to impurity D and oxeladin.

Limits:

- *impurity C*: maximum 0.2 per cent,
- *impurity D*: maximum 0.3 per cent,
- *any other impurity*: for each impurity, maximum 0.1 per cent,
- *total*: maximum 1.0 per cent,
- *disregard limit*: the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

Loss on drying (2.2.32): maximum 0.5 per cent, determined on 1.000 g by drying *in vacuo* at 60 °C for 3 h.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

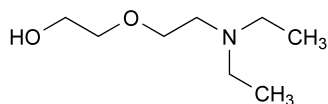
Dissolve 0.400 g in 50 ml of *anhydrous acetic acid R*. Titrate with 0.1 M *perchloric acid*, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *perchloric acid* is equivalent to 52.76 mg of $C_{26}H_{41}NO_{10}$.

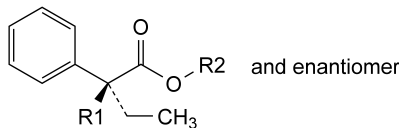
IMPURITIES

Specified impurities: C, D.

Other detectable impurities: A, B.



A. 2-[2-(diethylamino)ethoxy]ethanol,



B. R1 = C₂H₅, R2 = H: 2-ethyl-2-phenylbutanoic acid,

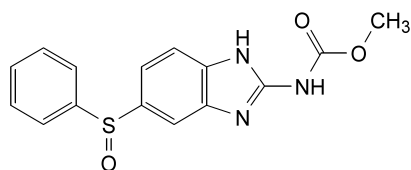
C. R1 = C₂H₅, R2 = [CH₂]₂-N(C₂H₅)₂: 2-(diethylamino)ethyl 2-ethyl-2-phenylbutanoate,

D. R1 = H, R2 = [CH₂]₂-O-[CH₂]₂-N(C₂H₅)₂: 2-[2-(diethylamino)ethoxy]ethyl (2*RS*)-2-phenylbutanoate.

01/2005:1458

OXFENDAZOLE FOR VETERINARY USE

Oxfendazolum ad usum veterinarium



C₁₅H₁₃N₃O₃S

*M*_r 315.4

DEFINITION

Methyl [5-(phenylsulphonyl)-1*H*-benzimidazol-2-yl]carbamate.

Content: 97.5 per cent to 100.5 per cent (dried substance).

CHARACTERS

Appearance: white or almost white powder.

Solubility: practically insoluble in water, slightly soluble in alcohol and in methylene chloride.

It shows polymorphism.

IDENTIFICATION

Infrared absorption spectrophotometry (2.2.24).

Comparison: oxfendazole CRS.

If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in *alcohol R*, evaporate to dryness and record new spectra using the residues.

TESTS

Related substances. Liquid chromatography (2.2.29).

Test solution. Dissolve 25.0 mg of the substance to be examined in the mobile phase and dilute to 100.0 ml with the mobile phase.

Reference solution (a). Dilute 1.0 ml of the test solution to 100.0 ml with the mobile phase.

Reference solution (b). To 10 ml of the test solution, add 0.25 ml of *strong hydrogen peroxide solution R* and dilute to 25 ml with the mobile phase.

Reference solution (c). Dissolve 5.0 mg of *fenbendazole CRS* and 10.0 mg of *oxfendazole impurity B CRS* in the mobile phase and dilute to 100.0 ml with the mobile phase. Dilute 1.0 ml to 20.0 ml with the mobile phase.

Reference solution (d). Dissolve 5 mg of *oxfendazole with impurity D CRS* in the mobile phase and dilute to 20 ml with the mobile phase (solution used for identification of impurity D).

Column:

- *size*: *l* = 0.25 m, Ø = 4.6 mm,
- *stationary phase*: spherical *end-capped octadecylsilyl silica gel for chromatography R* (5 µm) with a specific surface area of 350 m²/g, a pore size of 10 nm and a carbon loading of 14 per cent.

Mobile phase: mix 36 volumes of *acetonitrile R* and 64 volumes of a 2 g/l solution of *sodium pentanesulphonate R* adjusted to pH 2.7 with a 2.8 per cent V/V solution of *sulphuric acid R*.

Flow rate: 1 ml/min.

Detection: spectrophotometer at 254 nm.

Injection: 20 µl.

Run time: 4 times the retention time of oxfendazole.

Retention time: oxfendazole = about 6.5 min.

System suitability: reference solution (b):

- *resolution*: minimum 4.0 between the 2 principal peaks corresponding to impurity C (1st peak) and oxfendazole (2nd peak).

Limits:

- *impurity A*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (c) (1.0 per cent),
- *impurity B*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (c) (2.0 per cent),
- *impurity C or D*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (1.0 per cent),
- *any other impurity*: not more than 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent),
- *total*: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (a) (3.0 per cent),
- *disregard limit*: 0.05 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

Loss on drying (2.2.32): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 100-105 °C at a pressure not exceeding 0.7 kPa for 2 h.

Sulphated ash (2.4.14): maximum 0.2 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.250 g in 3 ml of *anhydrous formic acid R*. Add 40 ml of *anhydrous acetic acid R*. Titrate with 0.1 *M perchloric acid*, determining the end-point potentiometrically (2.2.20).

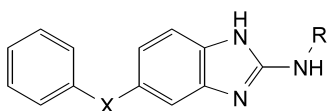
1 ml of 0.1 *M perchloric acid* is equivalent to 31.54 mg of C₁₅H₁₃N₃O₃S.

STORAGE

Protected from light.

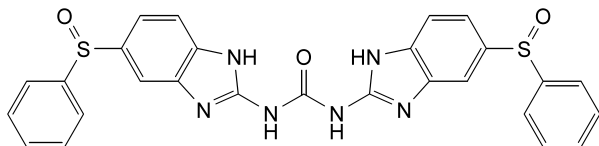
IMPURITIES

A. fenbendazole,



B. X = SO₂, R = CO₂-CH₃: methyl [5-(phenylsulphonyl)-1H-benzimidazol-2-yl]carbamate,

C. X = SO, R = H: 5-(phenylsulphinyl)-1H-benzimidazol-2-amine,

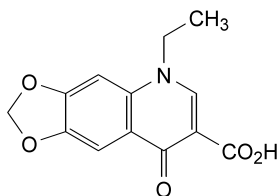


D. N,N'-bis[5-(phenylsulphonyl)-1H-benzimidazol-2-yl]urea.

01/2005:1353

OXOLINIC ACID

Acidum oxolinicum

C₁₃H₁₁NO₅M_r 261.2

DEFINITION

Oxolinic acid contains not less than 98.0 per cent and not more than the equivalent of 102.0 per cent of 5-ethyl-8-oxo-5,8-dihydro-1,3-dioxolo[4,5-g]quinoline-7-carboxylic acid, calculated with reference to the dried substance.

CHARACTERS

An almost white or pale yellow, crystalline powder, practically insoluble in water, very slightly soluble in methylene chloride, practically insoluble in alcohol. It dissolves in dilute solutions of alkali hydroxides.

IDENTIFICATION

First identification: B.

Second identification: A, C.

A. Dissolve 25.0 mg in 5 ml of 0.1 M sodium hydroxide, heating on a water-bath. Allow to cool and dilute to 100.0 ml with methanol R. Dilute 2.0 ml of the solution to 100.0 ml with 0.1 M hydrochloric acid. Examined between 220 nm and 350 nm (2.2.25), the solution shows three absorption maxima, at 260 nm, 322 nm and 336 nm respectively. The ratio of the absorbance measured at the maximum at 260 nm to that measured at the maximum at 336 nm is 4.9 to 5.2.

B. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with oxolinic acid CRS. Examine the substances prepared as discs.

C. Examine by thin-layer chromatography (2.2.27), using a suitable silica gel as the coating substance.

Test solution. Dissolve 10 mg of the substance to be examined in 3 ml of dilute sodium hydroxide solution R and dilute to 20 ml with alcohol R.

Reference solution (a). Dissolve 10 mg of oxolinic acid CRS in 3 ml of dilute sodium hydroxide solution R and dilute to 20 ml with alcohol R.

Reference solution (b). Dissolve 5 mg of ciprofloxacin hydrochloride CRS in methanol R and dilute to 10 ml with the same solvent. Dilute 1 ml of the solution to 2 ml with reference solution (a).

Apply separately to the plate 10 µl of each solution. At the bottom of a chromatographic tank, place an evaporating disk containing 50 ml of concentrated ammonia R. Close the tank and expose the plate to the ammonia vapour for 15 min. Withdraw the plate and transfer to a chromatographic tank and develop over a path of 15 cm using a mixture of 10 volumes of acetonitrile R, 20 volumes of concentrated ammonia R, 40 volumes of methanol R and 40 volumes of methylene chloride R. Allow the plate to dry in air. Examine in ultraviolet light at 254 nm. The principal spot in the chromatogram obtained with the test solution is similar in position, fluorescence and size to the principal spot in the chromatogram obtained with reference solution (a). The identification is not valid unless the chromatogram obtained with reference solution (b) shows two clearly separated spots.

TESTS

Solution S. Dissolve 0.6 g in 20 ml of a 40 g/l solution of sodium hydroxide R.

Appearance of solution. Solution S is clear (2.2.1) and not more intensely coloured than reference solution B₇ (2.2.2, Method II).

Related substances. Examine by thin-layer chromatography (2.2.27), using as the coating substance a suitable cellulose with a particle size of narrow distribution.

Test solution. Dissolve 50 mg in 3 ml of dilute sodium hydroxide solution R and dilute to 10 ml with alcohol R.

Reference solution (a). Dilute 1 ml of the test solution to 50.0 ml with alcohol R. Dilute 1.0 ml of the solution to 5.0 ml with alcohol R.

Reference solution (b). Dissolve 2 mg of oxolinic acid impurity B CRS in alcohol R and dilute to 10 ml with the same solvent. Dilute 0.5 ml of the solution to 10 ml with alcohol R.

Reference solution (c). Dissolve 5 mg of the substance to be examined and 5 mg of oxolinic acid impurity A CRS in 2 ml of dilute sodium hydroxide solution R and dilute to 40 ml with alcohol R.

Apply separately to the plate 5 µl of each solution, in sufficiently small portions to obtain small spots. Develop over a path of 6 cm (corresponding to two thirds of the plate height) with a mixture of 15 volumes of ammonia R, 30 volumes of water R and 55 volumes of propanol R. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. In the chromatogram obtained with the test solution: any spot corresponding to oxolinic acid impurity B is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.2 per cent); any spot apart from the principal spot and any spot corresponding to oxolinic acid impurity B is not more intense than the spot in the chromatogram obtained with reference solution (a) (0.4 per cent). The test is not valid unless the chromatogram obtained with reference solution (c) shows two clearly separated principal spots.

Heavy metals (2.4.8). 2.0 g complies with limit test D for heavy metals (10 ppm). Prepare the standard using 2 ml of lead standard solution (10 ppm Pb) R.

Loss on drying (2.2.32). Not more than 0.5 per cent determined on 1.000 g by heating in an oven at 100 °C to 105 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent determined on 1.0 g.

ASSAY

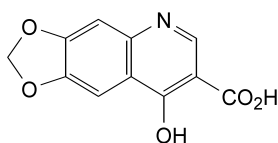
Dissolve 0.200 g in 150 ml of *dimethylformamide R*. Titrate with 0.1 M *tetrabutylammonium hydroxide*, determining the end-point potentiometrically (2.2.20). Use a glass indicator electrode and a calomel reference electrode containing, as the electrolyte, a saturated solution of *potassium chloride R* in *methanol R*. Carry out a blank titration.

1 ml of 0.1 M *tetrabutylammonium hydroxide* is equivalent to 26.12 mg of $C_{13}H_{11}NO_5$.

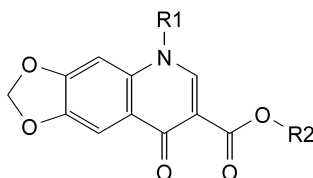
STORAGE

Store protected from light.

IMPURITIES



A. 8-hydroxy-1,3-dioxolo[4,5-*g*]quinoline-7-carboxylic acid,



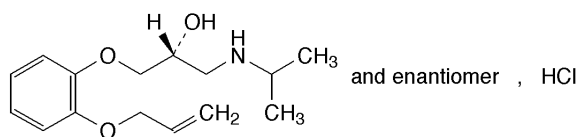
B. R1 = R2 = C_2H_5 : ethyl 5-ethyl-8-oxo-5,8-dihydro-1,3-dioxolo[4,5-*g*]quinoline-7-carboxylate,

C. R1 = CH_3 , R2 = H: 5-methyl-8-oxo-5,8-dihydro-1,3-dioxolo[4,5-*g*]quinoline-7-carboxylic acid.

01/2005:0628

OPXPENOLOL HYDROCHLORIDE

Oxprenololi hydrochloridum



$C_{15}H_{24}ClNO_3$

M_r 301.8

DEFINITION

(2*RS*)-1-[(1-methylethyl)amino]-3-[2-(prop-2-enyloxy)phenoxy]propan-2-ol hydrochloride.

Content: 98.5 per cent to 101.5 per cent (dried substance).

CHARACTERS

Appearance: white or almost white, crystalline powder.

Solubility: very soluble in water, freely soluble in alcohol.

IDENTIFICATION

First identification: B, D.

Second identification: A, C, D.

A. Melting point (2.2.14): 107 °C to 110 °C.

B. Infrared absorption spectrophotometry (2.2.24).

Comparison: *oxprenolol hydrochloride CRS*.

If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in *ethyl acetate R*, evaporate to dryness and record new spectra using the residues.

C. Examine the chromatograms obtained in the test for related substances.

Results: the principal spot in the chromatogram obtained with test solution (b) is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).

D. It gives reaction (a) of chlorides (2.3.1).

TESTS

Solution S. Dissolve 2.0 g in *carbon dioxide-free water R* and dilute to 20 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and not more intensely coloured than reference solution GY₆ (2.2.2, *Method II*).

pH (2.2.3): 4.5 to 6.0 for freshly prepared solution S.

Related substances. Thin-layer chromatography (2.2.27).

Test solution (a). Dissolve 0.10 g of the substance to be examined in 2 ml of a mixture of 1 volume of *methanol R* and 9 volumes of *methylene chloride R*.

Test solution (b). Dilute 1 ml of test solution (a) to 10 ml with a mixture of 1 volume of *methanol R* and 9 volumes of *methylene chloride R*.

Reference solution (a). Dissolve 10 mg of *oxprenolol hydrochloride CRS* in 2 ml of a mixture of 1 volume of *methanol R* and 9 volumes of *methylene chloride R*.

Reference solution (b). Dilute 0.4 ml of test solution (a) to 100 ml with a mixture of 1 volume of *methanol R* and 9 volumes of *methylene chloride R*.

Reference solution (c). Dilute 5 ml of reference solution (b) to 10 ml with a mixture of 1 volume of *methanol R* and 9 volumes of *methylene chloride R*.

Reference solution (d). Dissolve 5 mg of *alprenolol hydrochloride CRS* in 1 ml of reference solution (a).

Plate: TLC silica gel G plate R.

Mobile phase: concentrated ammonia R, *methanol R*, *methylene chloride R* (2:12:88 V/V/V).

Application: 2 µl; allow the spots to dry in air for 15 min.

Development: over a path of 13 cm.

Drying: in a current of warm air for 10 min.

Detection: allow to cool and spray with *anisaldehyde solution R*. Heat at 100-105 °C for 5-10 min. Examine in daylight.

System suitability: the test is not valid unless the chromatogram obtained with reference solution (d) shows 2 clearly separated spots.

Limits: in the chromatogram obtained with test solution (a):

— **any impurity:** any spot, apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.4 per cent); not more than 1 such spot is more intense than the spot in the chromatogram obtained with reference solution (c) (0.2 per cent).

Lead: maximum 5 ppm.

Atomic absorption spectrometry (2.2.23, *Method II*).

Test solution. Dissolve 1.00 g of the substance to be examined in *water R* and dilute to 25.0 ml with the same solvent.

Reference solutions. Prepare the reference solutions using 0.5 ml and 1.0 ml respectively of *lead standard solution* (10 ppm Pb) R diluted to 25.0 ml with *water R*.

Source: lead hollow-cathode lamp.

Wavelength: 217.0 nm.

Loss on drying (2.2.32): maximum 0.5 per cent, determined on 1.000 g by drying *in vacuo* at 60 °C for 6 h.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.250 g in a mixture of 5.0 ml of 0.01 M hydrochloric acid and 50 ml of alcohol R. Carry out a potentiometric titration (2.2.20), using 0.1 M sodium hydroxide. Read the volume added between the 2 points of inflexion.

1 ml of 0.1 M sodium hydroxide is equivalent to 30.18 mg of $C_{17}H_{29}ClN_2O_3$.

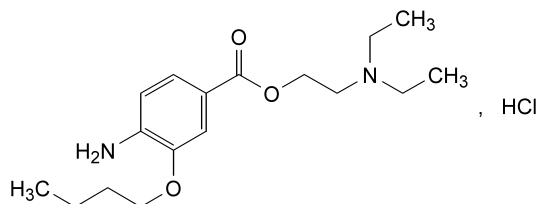
STORAGE

Protected from light.

01/2005:1251

OXYBUPROCAINE HYDROCHLORIDE

Oxybuprocaini hydrochloridum



$C_{17}H_{29}ClN_2O_3$

M_r 344.9

DEFINITION

Oxybuprocaine hydrochloride contains not less than 98.5 per cent and not more than the equivalent of 101.5 per cent of 2-(diethylamino)ethyl 4-amino-3-butoxybenzoate hydrochloride, calculated with reference to the dried substance.

CHARACTERS

A white, crystalline powder or colourless crystals, very soluble in water, freely soluble in alcohol.

It shows polymorphism.

IDENTIFICATION

First identification: B, D.

Second identification: A, C, D.

A. Melting point (2.2.14): 158 °C to 162 °C.

B. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with oxybuprocaine hydrochloride CRS. Examine the substances prepared as discs. If the spectra obtained show differences, dissolve the substance to be examined and the reference substance separately in methanol R, evaporate to dryness and record the spectra again using the residues.

C. Examine by thin-layer chromatography (2.2.27), using as the coating substance a suitable silica gel with a fluorescent indicator having an optimal intensity at 254 nm.

Test solution. Dissolve 40 mg of the substance to be examined in methanol R and dilute to 10 ml with the same solvent.

Reference solution (a). Dissolve 40 mg of oxybuprocaine hydrochloride CRS in methanol R and dilute to 10 ml with the same solvent.

Reference solution (b). Dissolve 20 mg of procaine hydrochloride R in reference solution (a) and dilute to 5 ml with the same solution.

Apply separately to the plate 5 µl of each solution. Develop over a path of 10 cm using a mixture of 10 volumes of anhydrous formic acid R, 15 volumes of methanol R, 15 volumes of water R and 60 volumes of ethyl acetate R. Dry the plate in a current of hot air for 10 min and examine in ultraviolet light at 254 nm. Spray with dimethylaminobenzaldehyde solution R7. The principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a). The test is not valid unless the chromatogram obtained with reference solution (b) shows two clearly separated spots.

D. Dilute 0.2 ml of solution S (see Tests) to 2 ml with water R. The solution gives reaction (a) of chlorides (2.3.1).

TESTS

Solution S. Dissolve 5.0 g in carbon dioxide-free water R and dilute to 50 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and not more intensely coloured than reference solution Y₅ (2.2.2, Method II).

pH (2.2.3). The pH of solution S is 4.5 to 6.0.

Related substances. Examine by liquid chromatography (2.2.29).

Buffer solution pH 2.5. Add 6 ml of perchloric acid solution R and 12 ml of dilute phosphoric acid R to 950 ml of water R. Adjust the pH to 2.5 with a 40 g/l solution of sodium hydroxide R and dilute to 1000.0 ml with water R.

Test solution. Dissolve 10.0 mg of the substance to be examined in the mobile phase and dilute to 25.0 ml with the mobile phase.

Reference solution (a). Dilute 1.0 ml of the test solution to 20.0 ml with the mobile phase and dilute 5.0 ml of this solution to 100.0 ml with the mobile phase.

Reference solution (b). Mix 1.0 ml of the test solution with 1 ml of a 40 g/l solution of sodium hydroxide R and allow to stand for 20 min. Add 1 ml of dilute phosphoric acid R and dilute to 100.0 ml with the mobile phase. Dilute 25 ml to 100.0 ml with the mobile phase.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.15 m long and 3.9 mm in internal diameter packed with octadecylsilyl silica gel for chromatography R1 (5 µm) with a pore size of 10 nm and a carbon loading of 19 per cent,
- as mobile phase at a flow rate of 1 ml/min a mixture of 25 volumes of acetonitrile R and 75 volumes of buffer solution pH 2.5,
- as detector a spectrophotometer set at 309 nm, maintaining the temperature of the column at 35 °C.

When the chromatograms are recorded in the prescribed conditions the retention time is about 9 min for oxybuprocaine hydrochloride. Adjust the sensitivity of the system so that the height of the principal peak in the chromatogram obtained with reference solution (a) is at least 50 per cent of the full scale of the recorder.

Inject 20 µl of reference solution (b). The test is not valid unless: in the chromatogram obtained with reference solution (b), the resolution between the main peaks corresponding to oxybuprocaine and oxybuprocaine impurity B (hydrolysis product) is at least twelve.

Inject 20 µl of the test solution and 20 µl of reference solution (a). Continue the chromatography for four times the retention time of the principal peak. In the chromatogram obtained with the test solution: the area of any peak, apart from the principal peak, is not greater than 0.4 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent); the sum of the areas of all the peaks apart from the principal peak is not greater than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.25 per cent). Disregard any peak with an area less than 0.05 times that of the peak due to oxybuprocaine hydrochloride in the chromatogram obtained with reference solution (a).

Heavy metals (2.4.8). 12 ml of solution S complies with limit test A for heavy metals (10 ppm). Prepare the standard using *lead standard solution* (1 ppm Pb) R.

Loss on drying (2.2.32). Not more than 0.5 per cent determined on 1.000 g by drying in an oven at 100–105 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

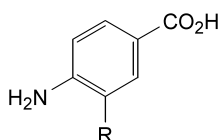
Dissolve 0.300 g in a mixture of 20 ml of *anhydrous acetic acid* R and 20 ml of *acetic anhydride* R. Titrate with 0.1 M *perchloric acid*, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *perchloric acid* is equivalent to 34.49 mg of C₁₇H₂₉ClN₂O₃.

STORAGE

Store protected from light.

IMPURITIES

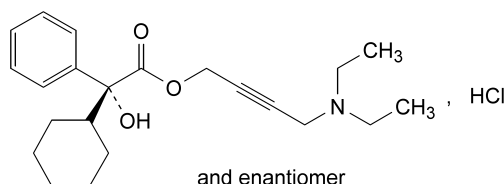


- A. R = H: 4-aminobenzoic acid,
 B. R = O-CH₂-CH₂-CH₂-CH₃: 4-amino-3-butoxybenzoic acid,
 C. R = OH: 4-amino-3-hydroxybenzoic acid.

01/2005:1354

OXYBUTYNIN HYDROCHLORIDE

Oxybutynini hydrochloridum



C₂₂H₃₂ClNO₃

M_r 394.0

DEFINITION

Oxybutynin hydrochloride contains not less than 99.0 per cent and not more than the equivalent of 102.0 per cent of 4-(diethylamino)but-2-ynyl (RS)-2-cyclohexyl-2-hydroxy-2-phenylacetate hydrochloride, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, crystalline powder, freely soluble in water and in alcohol, soluble in acetone, slightly soluble in cyclohexane.

IDENTIFICATION

First identification: B, D.

Second identification: A, C, D.

A. Melting point (2.2.14): 124 °C to 129 °C.

B. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *oxybutynin hydrochloride CRS*. Examine the substances prepared as discs.

C. Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel plate R*.

Test solution. Dissolve 50 mg of the substance to be examined in *alcohol R* and dilute to 10 ml with the same solvent.

Reference solution. Dissolve 10 mg of *oxybutynin hydrochloride CRS* in *alcohol R* and dilute to 2 ml with the same solvent.

Apply to the plate 5 µl of each solution. Develop over a path of 15 cm, using *methanol R*. Allow the plate to dry in air, and expose the plate to iodine vapour for 30 min. The principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

D. It gives reaction (a) of chlorides (2.3.1).

TESTS

Solution S. Dissolve 2.00 g in *water R* and dilute to 20.0 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and not more intensely coloured than reference solution BY₅ (2.2.2, *Method II*).

Optical rotation (2.2.7). The angle of optical rotation, determined on solution S, is –0.10° to +0.10°.

Related substances. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 50.0 mg of the substance to be examined in the mobile phase and dilute to 10.0 ml with the mobile phase.

Reference solution (a). Dissolve 50.0 mg of *oxybutynin hydrochloride CRS* and 50.0 mg of *oxybutynin impurity A CRS* in the mobile phase and dilute to 100.0 ml with the mobile phase. Dilute 10.0 ml of the solution to 100.0 ml with the mobile phase.

Reference solution (b). Dilute 1.0 ml of the test solution to 200.0 ml with the mobile phase.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.15 m long and 3.9 mm in internal diameter packed with *octylsilyl silica gel for chromatography R2* (5 µm),
- as mobile phase at a flow rate of 1 ml/min a mixture of 49 volumes of a solution containing 3.4 g/l of *potassium dihydrogen phosphate R* and 4.36 g/l of *dipotassium hydrogen phosphate R* and 51 volumes of *acetonitrile R*,

– as detector a spectrophotometer set at 210 nm.

Inject 10 µl of reference solution (a). When the chromatograms are recorded in the prescribed conditions the retention times are: oxybutynin hydrochloride about 15 min and impurity A about 24 min. Adjust the sensitivity of the system so that the heights of the peaks in the chromatogram obtained are about 20 per cent of the full scale of the recorder. The test is not valid unless the resolution between the peaks due to oxybutynin hydrochloride and to impurity A is at least 11.0.

Inject 10 µl of the test solution, 10 µl of reference solution (a) and 10 µl of reference solution (b). Continue the chromatography for about twice the retention time of the principal peak. In the chromatogram obtained with the test solution: the area of any peak due to impurity A is not greater than 1.5 times the area of the peak due to impurity A in the chromatogram obtained with reference solution (a) (1.5 per cent); the sum of the areas of all the peaks, apart from the principal peak and the peak due to impurity A, is not greater than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent). Disregard any peak with an area less than 0.05 times the area of the principal peak in the chromatogram obtained with reference solution (b).

Heavy metals (2.4.8). 12 ml of solution S complies with limit test A for heavy metals (20 ppm). Prepare the standard using 2 ml of *lead standard solution* (10 ppm Pb) *R*.

Loss on drying (2.2.32). Not more than 3.0 per cent determined on 1.000 g by drying in an oven at 100–105 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

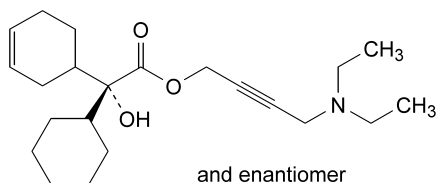
Dissolve 0.300 g in a mixture of 5.0 ml of 0.01 *M* hydrochloric acid and 50 ml of alcohol *R*. Carry out a potentiometric titration (2.2.20), using 0.1 *M* sodium hydroxide. Read the volume added between the two points of inflexion.

1 ml of 0.1 *M* sodium hydroxide is equivalent to 39.4 mg of $C_{22}H_{32}ClNO_3$.

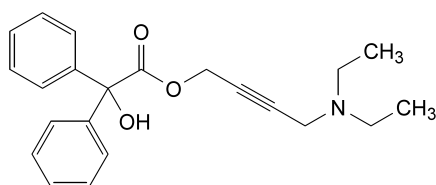
STORAGE

Store protected from light.

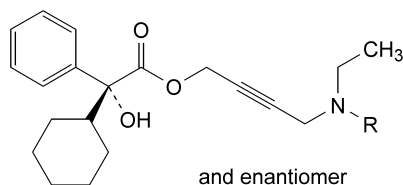
IMPURITIES



- A. 4-(diethylamino)but-2-ynyl (*RS*)-2-(cyclohex-3-en-1-yl)-2-cyclohexyl-2-hydroxyacetate,

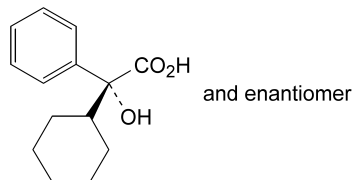


- B. 4-(diethylamino)but-2-ynyl 2-hydroxy-2,2-diphenylacetate (diphenyl analogue of oxybutynin),



- C. $R = CH_3$: 4-(ethylmethylamino)but-2-ynyl (*RS*)-2-cyclohexyl-2-hydroxy-2-phenylacetate (methylethyl analogue of oxybutynin),

- E. $R = CH_2-CH_2-CH_3$: 4-(ethylpropylamino)but-2-ynyl (*RS*)-2-cyclohexyl-2-hydroxy-2-phenylacetate (ethylpropyl analogue of oxybutynin),



- D. (*RS*)-2-cyclohexyl-2-hydroxy-2-phenylacetic acid (phenylcyclohexylglycolic acid).

01/2005:0417

OXYGEN

Oxygenium

O_2

M_r 32.00

DEFINITION

Oxygen contains not less than 99.5 per cent *V/V* of O_2 .

This monograph applies to oxygen for medicinal use.

CHARACTERS

A colourless, odourless gas. At 20 °C and at a pressure of 101 kPa, 1 volume dissolves in about 32 volumes of water.

PRODUCTION

Carbon dioxide. Not more than 300 ppm *V/V*, determined using an infrared analyser (2.5.24).

Gas to be examined. The substance to be examined. It must be filtered to avoid stray light phenomena.

Reference gas (a). Use oxygen *R*.

Reference gas (b). Use a mixture containing 300 ppm *V/V* of carbon dioxide *R1* in nitrogen *R1*.

Calibrate the apparatus and set the sensitivity using reference gases (a) and (b). Measure the content of carbon dioxide in the gas to be examined.

Carbon monoxide. Not more than 5 ppm *V/V*, determined using an infrared analyser (2.5.25).

Gas to be examined. The substance to be examined. It must be filtered to avoid stray light phenomena.

Reference gas (a). Use oxygen *R*.

Reference gas (b). Use a mixture containing 5 ppm *V/V* of carbon monoxide *R* in nitrogen *R1*.

Calibrate the apparatus and set the sensitivity using reference gases (a) and (b). Measure the content of carbon monoxide in the gas to be examined.

Water. Not more than 67 ppm *V/V*, determined using an electrolytic hygrometer (2.5.28).

Assay. Determine the concentration of oxygen using a paramagnetic analyser (2.5.27).

IDENTIFICATION

First identification: C.

Second identification: A, B.

- A. Place a glowing splinter of wood in the substance to be examined. The splinter bursts into flame.
- B. Shake with *alkaline pyrogallol solution R*. The substance to be examined is absorbed and the solution becomes dark brown.
- C. It complies with the limits of the assay.

TESTS

Carbon dioxide. Not more than 300 ppm V/V, determined using a carbon dioxide detector tube (2.1.6).

Carbon monoxide. Not more than 5 ppm V/V, determined using a carbon monoxide detector tube (2.1.6).

Water vapour. Not more than 67 ppm V/V, determined using a water vapour detector tube (2.1.6).

STORAGE

Store as a compressed gas or liquid in appropriate containers, complying with the legal regulations. Taps and valves are not to be greased or oiled.

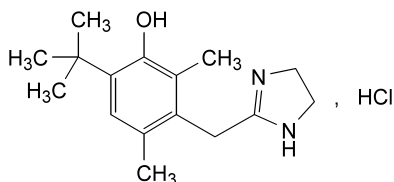
IMPURITIES

- A. carbon dioxide,
- B. carbon monoxide,
- C. water.

01/2005:0943

OXYMETAZOLINE HYDROCHLORIDE

Oxymetazolini hydrochloridum

C₁₆H₂₅ClN₂OM_r 296.8

DEFINITION

Oxymetazoline hydrochloride contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of 3-[(4,5-dihydro-1H-imidazol-2-yl)methyl]-6-(1,1-dimethylethyl)-2,4-dimethylphenol hydrochloride, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, crystalline powder, freely soluble in water and in alcohol.

IDENTIFICATION

First identification: A, D.

Second identification: B, C, D.

- A. Examine by infrared absorption spectrophotometry (2.2.24) comparing with the spectrum obtained with *oxymetazoline hydrochloride CRS*.
- B. Examine the chromatograms obtained in the test for related substances. The principal spot in the chromatogram obtained with test solution (b) is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).

- C. To a solution of about 2 mg in 1 ml of *water R* add 0.2 ml of a 50 g/l solution of *sodium nitroprusside R* and 0.2 ml of *dilute sodium hydroxide solution R*. Allow to stand for 10 min. Add 2 ml of *sodium hydrogen carbonate solution R*. A violet colour develops.

- D. It gives reaction (a) of chlorides (2.3.1).

TESTS

Solution S. Dissolve 2.5 g in *water R* and dilute to 50 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and not more intensely coloured than reference solution BY₇ (2.2.2, *Method II*)

Acidity or alkalinity. Dissolve 0.25 g in *water R* and dilute to 25 ml with the same solvent. Add 0.1 ml of *methyl red solution R* and 0.2 ml of 0.01 M *hydrochloric acid*. The solution is red. Not more than 0.4 ml of 0.01 M *sodium hydroxide* is required to change the colour of the indicator to yellow.

Related substances. Examine by thin-layer chromatography (2.2.27), using *silica gel G R* as the coating substance.

Test solution (a). Dissolve 0.40 g of the substance to be examined in a mixture of equal volumes of *ethyl acetate R* and *methanol R* and dilute to 10 ml with the same mixture of solvents.

Test solution (b). Dilute 1 ml of test solution (a) to 10 ml with a mixture of equal volumes of *ethyl acetate R* and *methanol R*.

Reference solution (a). Dissolve 40 mg of *oxymetazoline hydrochloride CRS* in a mixture of equal volumes of *ethyl acetate R* and *methanol R* and dilute to 10 ml with the same mixture of solvents.

Reference solution (b). Dilute 1 ml of test solution (b) to 20 ml with a mixture of equal volumes of *ethyl acetate R* and *methanol R*.

Reference solution (c). Dilute 1 ml of test solution (b) to 40 ml with a mixture of equal volumes of *ethyl acetate R* and *methanol R*.

Apply separately to the plate 5 µl of each solution. Develop over a path of 15 cm using a mixture of 6 volumes of *diethylamine R*, 15 volumes of *cyclohexane R* and 79 volumes of *ethanol R*. Dry the plate in a current of warm air for 5 min. Allow to cool and spray with a freshly prepared 5.0 g/l solution of *potassium ferricyanide R* in *ferric chloride solution R2*. Examine in daylight. Any spot in the chromatogram obtained with test solution (a), apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.5 per cent) and at most one such spot is more intense than the spot in the chromatogram obtained with reference solution (c) (0.25 per cent).

Loss on drying (2.2.32). Not more than 1.0 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C.

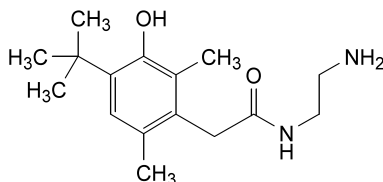
Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.200 g in a mixture of 20 ml of *anhydrous acetic acid R* and 20 ml of *acetic anhydride R*. Titrate with 0.1 M *perchloric acid* determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *perchloric acid* is equivalent to 29.68 mg of C₁₆H₂₅ClN₂O.

IMPURITIES

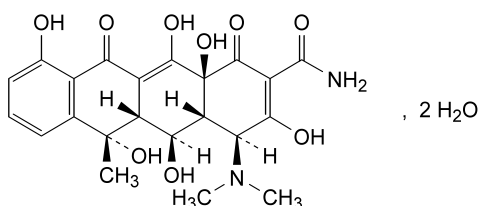


- A. *N*-(2-aminoethyl)-2-[4-(1,1-dimethylethyl)-3-hydroxy-2,6-dimethylphenyl]acetamide.

01/2005:0199

OXYTETRACYCLINE DIHYDRATE

Oxytetracyclinum dihydricum

 $C_{22}H_{24}N_2O_9 \cdot 2 H_2O$ M_r 496.4

DEFINITION

(4*S*,4*aR*,5*S*,5*aR*,6*S*,12*aS*)-4-(Dimethylamino)-3,5,6,10,12,12*a*-hexahydroxy-6-methyl-1,11-dioxo-1,4,4*a*,5,5*a*,6,11,12*a*-octahydrotetracene-2-carboxamide dihydrate.

Substance produced by the growth of certain strains of *Streptomyces rimosus* or obtained by any other means.

Content: 95.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

Appearance: yellow, crystalline powder.

Solubility: very slightly soluble in water. It dissolves in dilute acid and alkaline solutions.

IDENTIFICATION

- A. Thin-layer chromatography (2.2.27).

Test solution. Dissolve 5 mg of the substance to be examined in *methanol R* and dilute to 10 ml with the same solvent.

Reference solution (a). Dissolve 5 mg of *oxytetracycline CRS* in *methanol R* and dilute to 10 ml with the same solvent.

Reference solution (b). Dissolve 5 mg of *oxytetracycline CRS*, 5 mg of *tetracycline hydrochloride R* and 5 mg of *minocycline hydrochloride R* in *methanol R* and dilute to 10 ml with the same solvent.

Plate: TLC octadecylsilyl silica gel F_{254} plate *R*.

Mobile phase: mix 20 volumes of *acetonitrile R*, 20 volumes of *methanol R* and 60 volumes of a 63 g/l solution of *oxalic acid R* previously adjusted to pH 2 with *concentrated ammonia R*.

Application: 1 μ l.

Development: over 3/4 of the plate.

Drying: in air.

Detection: examine in ultraviolet light at 254 nm.

System suitability: the chromatogram obtained with reference solution (b) shows 3 clearly separated spots.

Results: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a).

- B. To about 2 mg add 5 ml of *sulphuric acid R*. A deep red colour develops. Add the solution to 2.5 ml of *water R*. The colour becomes yellow.
- C. Dissolve about 10 mg in a mixture of 1 ml of *dilute nitric acid R* and 5 ml of *water R*. Shake and add 1 ml of *silver nitrate solution R2*. Any opalescence in the solution is not more intense than that in a mixture of 1 ml of *dilute nitric acid R*, 5 ml of a 0.021 g/l solution of *potassium chloride R* and 1 ml of *silver nitrate solution R2*.

TESTS

pH (2.2.3): 4.5 to 7.5.

Suspend 0.1 g in 10 ml of *carbon dioxide-free water R*.

Specific optical rotation (2.2.7): -203 to -216 (anhydrous substance).

Dissolve 0.250 g in 0.1 *M hydrochloric acid* and dilute to 25.0 ml with the same acid.

Specific absorbance (2.2.25): 290 to 310 determined at 353 nm (anhydrous substance).

Dissolve 20.0 mg in *buffer solution pH 2.0 R* and dilute to 100.0 ml with the same buffer solution. Dilute 10.0 ml of the solution to 100.0 ml with *buffer solution pH 2.0 R*.

Light-absorbing impurities. Carry out the measurements within 1 h of preparing the solutions.

Dissolve 20.0 mg in a mixture of 1 volume of 1 *M hydrochloric acid* and 99 volumes of *methanol R* and dilute to 10.0 ml with the same mixture of solvents. The absorbance (2.2.25), determined at 430 nm has a maximum of 0.25 (anhydrous substance).

Dissolve 0.100 g in a mixture of 1 volume of 1 *M hydrochloric acid* and 99 volumes of *methanol R* and dilute to 10.0 ml with the same mixture of solvents. The absorbance (2.2.25) determined at 490 nm has a maximum of 0.20 (anhydrous substance).

Related substances. Liquid chromatography (2.2.29).

Test solution. Dissolve 20.0 mg of the substance to be examined in 0.01 *M hydrochloric acid* and dilute to 25.0 ml with the same acid.

Reference solution (a). Dissolve 20.0 mg of *oxytetracycline CRS* in 0.01 *M hydrochloric acid* and dilute to 25.0 ml with the same acid.

Reference solution (b). Dissolve 20.0 mg of 4-epioxytetracycline *CRS* in 0.01 *M hydrochloric acid* and dilute to 25.0 ml with the same acid.

Reference solution (c). Dissolve 20.0 mg of *tetracycline hydrochloride CRS* in 0.01 *M hydrochloric acid* and dilute to 25.0 ml with the same acid.

Reference solution (d). Mix 1.5 ml of reference solution (a), 1.0 ml of reference solution (b) and 3.0 ml of reference solution (c) and dilute to 25.0 ml with 0.01 *M hydrochloric acid*.

Reference solution (e). Mix 1.0 ml of reference solution (b) and 4.0 ml of reference solution (c) and dilute to 200.0 ml with 0.01 *M hydrochloric acid*.

Column:

- size: $l = 0.25$ m, $\varnothing = 4.6$ mm,
- stationary phase: styrene-divinylbenzene copolymer *R* (8 μ m),
- temperature: 60 °C.

Mobile phase: weigh 60.0 g of 2-methyl-2-propanol *R* and transfer to a 1000 ml volumetric flask with the aid of 200 ml of water *R*; add 60 ml of 0.33 *M* phosphate buffer solution pH 7.5 *R*, 50 ml of a 10 g/l solution of tetrabutylammonium hydrogen sulphate *R* adjusted to pH 7.5 with dilute sodium hydroxide solution *R* and 10 ml of a 0.4 g/l solution of sodium edetate *R* adjusted to pH 7.5 with dilute sodium hydroxide solution *R*; dilute to 1000 ml with water *R*.

Flow rate: 1.0 ml/min.

Detection: spectrophotometer at 254 nm.

Injection: 20 µl; inject the test solution and reference solutions (d) and (e).

System suitability: reference solution (d):

- **resolution:** minimum 4.0 between the peaks due to impurity A (1st peak) and oxytetracycline (2nd peak) and minimum 5.0 between the peaks and due to oxytetracycline and impurity B (3rd peak); adjust the 2-methyl-2-propanol content in the mobile phase if necessary,
- **symmetry factor:** maximum 1.25 for the peak due to oxytetracycline.

Limits:

- **impurity A:** not more than the area of the corresponding peak in the chromatogram obtained with reference solution (e) (0.5 per cent),
- **impurity B:** not more than the area of the corresponding peak in the chromatogram obtained with reference solution (e) (2.0 per cent),
- **impurity C (eluting on the tail of the principal peak):** not more than 4 times the area of the peak due to impurity A in the chromatogram obtained with reference solution (e) (2.0 per cent),
- **disregard limit:** 0.02 times the area of the peak due to oxytetracycline in the chromatogram obtained with reference solution (d) (0.1 per cent).

Heavy metals (2.4.8): maximum 50 ppm.

0.5 g complies with limit test F. Prepare the standard using 2.5 ml of lead standard solution (10 ppm Pb) *R*.

Water (2.5.12): 6.0 per cent to 9.0 per cent, determined on 0.250 g.

Sulphated ash (2.4.14): maximum 0.5 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (2.2.29) as described in the test for related substances with the following modification.

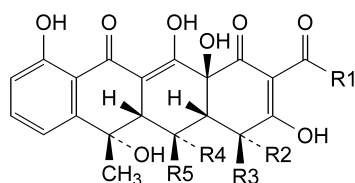
Injection: test solution and reference solution (a).

Calculate the percentage content of C₂₂H₂₄N₂O₉.

STORAGE

In an airtight container, protected from light.

IMPURITIES



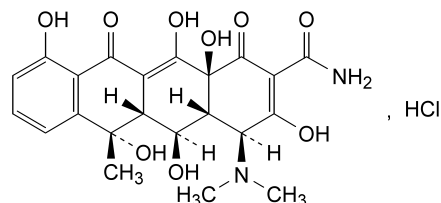
- A. R1 = NH₂, R2 = N(CH₃)₂, R3 = R4 = H, R5 = OH:
(4*R*,4*aR*,5*S*,5*aR*,6*S*,12*aS*)-4-(dimethylamino)-3,5,6,10,12,12*a*-hexahydroxy-6-methyl-1,11-dioxo-1,4,4*a*,5,5*a*,6,11,12*a*-octahydrotetracene-2-carboxamide (4-epioxytetracycline),
- B. R1 = NH₂, R2 = R4 = R5 = H, R3 = N(CH₃)₂: tetracycline,

- C. R1 = CH₃, R2 = R4 = H, R3 = N(CH₃)₂, R5 = OH:
(4*S*,4*aR*,5*S*,5*aR*,6*S*,12*aS*)-2-acetyl-4-(dimethylamino)-3,5,6,10,12,12*a*-hexahydroxy-6-methyl-4*a*,5*a*,6,12*a*-tetrahydrotetracene-1,11(4*H*,5*H*)-dione
(2-acetyl-2-decarbamoxyloxytetracycline).

01/2005:0198

OXYTETRACYCLINE HYDROCHLORIDE

Oxytetracyclini hydrochloridum



C₂₂H₂₅ClN₂O₉

M_r 496.9

DEFINITION

(4*S*,4*aR*,5*S*,5*aR*,6*S*,12*aS*)-4-(Dimethylamino)-3,5,6,10,12,12*a*-hexahydroxy-6-methyl-1,11-dioxo-1,4,4*a*,5,5*a*,6,11,12*a*-octahydrotetracene-2-carboxamide hydrochloride.

Substance produced by the growth of certain strains of *Streptomyces rimosus* or obtained by any other means.

Content: 95.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

Appearance: yellow, crystalline powder, hygroscopic.

Solubility: freely soluble in water, sparingly soluble in alcohol. Solutions in water become turbid on standing, owing to the precipitation of oxytetracycline.

IDENTIFICATION

A. Thin-layer chromatography (2.2.27).

Test solution. Dissolve 5 mg of the substance to be examined in methanol *R* and dilute to 10 ml with the same solvent.

Reference solution (a). Dissolve 5 mg of oxytetracycline hydrochloride CRS in methanol *R* and dilute to 10 ml with the same solvent.

Reference solution (b). Dissolve 5 mg of oxytetracycline hydrochloride CRS, 5 mg of tetracycline hydrochloride *R* and 5 mg of minocycline hydrochloride *R* in methanol *R* and dilute to 10 ml with the same solvent.

Plate: TLC octadecylsilyl silica gel F₂₅₄ plate *R*.

Mobile phase: mix 20 volumes of acetonitrile *R*, 20 volumes of methanol *R* and 60 volumes of a 63 g/l solution of oxalic acid *R* previously adjusted to pH 2 with concentrated ammonia *R*.

Application: 1 µl.

Development: over 3/4 of the plate.

Drying: in air.

Detection: examine in ultraviolet light at 254 nm.

System suitability: the chromatogram obtained with reference solution (b) shows 3 clearly separated spots.

Results: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a).

- B. To about 2 mg add 5 ml of *sulphuric acid R*. A deep red colour develops. Add the solution to 2.5 ml of *water R*. The colour becomes yellow.
- C. It gives reaction (a) of chlorides (2.3.1).

TESTS

pH (2.2.3): 2.3 to 2.9.

Dissolve 0.1 g in 10 ml of *carbon dioxide-free water R*.

Specific optical rotation (2.2.7): –188 to –200 (anhydrous substance).

Dissolve 0.250 g in 0.1 M *hydrochloric acid* and dilute to 25.0 ml with the same acid.

Specific absorbance (2.2.25): 270 to 290 determined at 353 nm (anhydrous substance).

Dissolve 20.0 mg in *buffer solution pH 2.0 R* and dilute to 100.0 ml with the same buffer solution. Dilute 10.0 ml of the solution to 100.0 ml with *buffer solution pH 2.0 R*.

Light-absorbing impurities. Carry out the measurements within 1 h of preparing the solutions.

Dissolve 20.0 mg in a mixture of 1 volume of 1 M *hydrochloric acid* and 99 volumes of *methanol R* and dilute to 10.0 ml with the same mixture of solvents. The absorbance (2.2.25) determined at 430 nm has a maximum of 0.50 (anhydrous substance).

Dissolve 0.100 g in a mixture of 1 volume of 1 M *hydrochloric acid* and 99 volumes of *methanol R* and dilute to 10.0 ml with the same mixture of solvents. The absorbance (2.2.25) determined at 490 nm has a maximum of 0.20 (anhydrous substance).

Related substances. Liquid chromatography (2.2.29).

Test solution. Dissolve 20.0 mg of the substance to be examined in 0.01 M *hydrochloric acid* and dilute to 25.0 ml with the same acid.

Reference solution (a). Dissolve 20.0 mg of *oxytetracycline CRS* in 0.01 M *hydrochloric acid* and dilute to 25.0 ml with the same acid.

Reference solution (b). Dissolve 20.0 mg of 4-epioxytetracycline *CRS* in 0.01 M *hydrochloric acid* and dilute to 25.0 ml with the same acid.

Reference solution (c). Dissolve 20.0 mg of *tetracycline hydrochloride CRS* in 0.01 M *hydrochloric acid* and dilute to 25.0 ml with the same acid.

Reference solution (d). Dissolve 8.0 mg of α -apo-oxytetracycline *CRS* in 5 ml of 0.01 M *sodium hydroxide* and dilute to 100.0 ml with 0.01 M *hydrochloric acid*.

Reference solution (e). Dissolve 8.0 mg of β -apo-oxytetracycline *CRS* in 5 ml of 0.01 M *sodium hydroxide* and dilute to 100.0 ml with 0.01 M *hydrochloric acid*.

Reference solution (f). Mix 1.5 ml of reference solution (a), 1.0 ml of reference solution (b), 3.0 ml of reference solution (c), 3.0 ml of reference solution (d) and 3.0 ml of reference solution (e) and dilute to 25.0 ml with 0.01 M *hydrochloric acid*.

Reference solution (g). Mix 1.0 ml of reference solution (b), 4.0 ml of reference solution (c) and 40.0 ml of reference solution (e) and dilute to 200.0 ml with 0.01 M *hydrochloric acid*.

Column:

- size: $l = 0.25$ m, $\varnothing = 4.6$ mm,
- stationary phase: *styrene-divinylbenzene copolymer R* (8 μ m),
- temperature: 60 °C.

Mobile phase: weigh 30.0 g (for mobile phase A) and 100.0 g (for mobile phase B) of 2-methyl-2-propanol *R* and transfer separately to 1000 ml volumetric flasks with the aid of 200 ml of *water R*; to each flask add 60 ml of 0.33 M *phosphate buffer solution pH 7.5 R*, 50 ml of a 10 g/l solution of *tetrabutylammonium hydrogen sulphate R* adjusted to pH 7.5 with *dilute sodium hydroxide solution R* and 10 ml of a 0.4 g/l solution of *sodium edetate R* adjusted to pH 7.5 with *dilute sodium hydroxide solution R*; dilute each solution to 1000 ml with *water R*,

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)
0 - 15	70	30
15 - 30	30	70
30 - 45	70	30

Flow rate: 1 ml/min.

Detection: spectrophotometer at 254 nm.

Injection: 20 μ l; inject the test solution and reference solutions (f) and (g).

System suitability: reference solution (f):

- resolution: minimum 4.0 between the peaks due to impurity A (1st peak) and oxytetracycline (2nd peak), minimum 5.0 between the peaks due to oxytetracycline and impurity B (3rd peak) and minimum 3.5 between the peaks due to impurity D (4th peak) and impurity E (5th peak); if necessary, adapt the ratio mobile phase A: mobile phase B and/or adjust the time programme used to produce the one-step gradient elution,
- symmetry factor: maximum 1.25 for the peak due to oxytetracycline.

Limits:

- impurity A: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (g) (0.5 per cent),
- impurity B: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (g) (2.0 per cent),
- impurity C (eluting on the tail of the main peak): not more than 4 times the area of the peak due to impurity A in the chromatogram obtained with reference solution (g) (2.0 per cent),
- total of impurities D, E and F (eluting between the latter two): not more than the area of the peak due to impurity E in the chromatogram obtained with reference solution (g) (2.0 per cent),
- disregard limit: 0.02 times the area of the peak due to oxytetracycline in the chromatogram obtained with reference solution (f) (0.1 per cent).

Heavy metals (2.4.8): maximum 50 ppm.

0.5 g complies with limit test F. Prepare the standard using 2.5 ml of *lead standard solution (10 ppm Pb) R*.

Water (2.5.12): maximum 2.0 per cent, determined on 0.500 g.

Sulphated ash (2.4.14): maximum 0.5 per cent, determined on 1.0 g.

Bacterial endotoxins (2.6.14): less than 0.4 IU/mg, if intended for use in the manufacture of parenteral dosage forms without a further appropriate procedure for the removal of bacterial endotoxins.

ASSAY

Liquid chromatography (2.2.29) as described in the test for related substances with the following modification.

Injection: test solution and reference solution (a).

01/2005:0780

Calculate the percentage content of $C_{22}H_{25}ClN_2O_9$ taking 1 mg of oxytetracycline as equivalent to 1.079 mg of oxytetracycline hydrochloride.

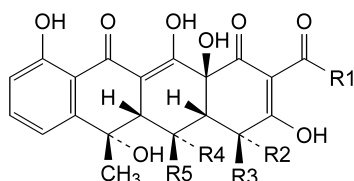
STORAGE

In an airtight container, protected from light. If the substance is sterile, store in a sterile, airtight, tamper-proof container.

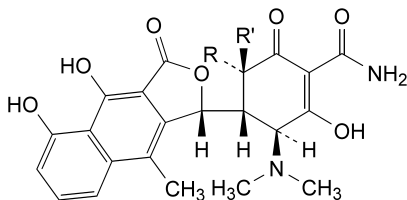
LABELLING

The label states, where applicable, that the substance is free from bacterial endotoxins.

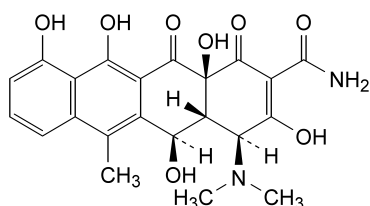
IMPURITIES



- A. $R_1 = NH_2$, $R_2 = N(CH_3)_2$, $R_3 = R_4 = H$, $R_5 = OH$: (4*R*,4*aR*,5*S*,5*aR*,6*S*,12*aS*)-4-(dimethylamino)-3,5,6,10,12,12*a*-hexahydroxy-6-methyl-1,11-dioxo-1,4,4*a*,5,5*a*,6,11,12*a*-octahydrotetracene-2-carboxamide (4-epioxytetracycline),
- B. $R_1 = NH_2$, $R_2 = R_4 = R_5 = H$, $R_3 = N(CH_3)_2$: tetracycline,
- C. $R_1 = CH_3$, $R_2 = R_4 = H$, $R_3 = N(CH_3)_2$, $R_5 = OH$: (4*S*,4*aR*,5*S*,5*aR*,6*S*,12*aS*)-2-acetyl-4-(dimethylamino)-3,5,6,10,12,12*a*-hexahydroxy-6-methyl-4*a*,5*a*,6,12*a*-tetrahydrotetracene-1,11-(4*H*,5*H*)-dione (2-acetyl-2-decarbamoxyloxytetracycline),



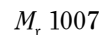
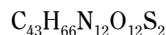
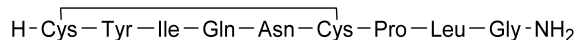
- D. $R = OH$, $R' = H$: (3*S*,4*S*,5*S*)-4-[(1*R*)-4,5-dihydroxy-9-methyl-3-oxo-1,3-dihydronaphtho[2,3-*c*]furan-1-yl]-3-(dimethylamino)-2,5-dihydroxy-6-oxocyclohex-1-enecarboxamide (α-apo-oxytetracycline),
- E. $R = H$, $R' = OH$: (3*S*,4*S*,5*R*)-4-[(1*R*)-4,5-dihydroxy-9-methyl-3-oxo-1,3-dihydronaphtho[2,3-*c*]furan-1-yl]-3-(dimethylamino)-2,5-dihydroxy-6-oxocyclohex-1-enecarboxamide (β-apo-oxytetracycline),



- F. (4*S*,4*aR*,5*R*,12*aS*)-4-(dimethylamino)-3,5,10,11,12*a*-pentahydroxy-6-methyl-1,12-dioxo-1,4,4*a*,5,12,12*a*-hexahydrotetracene-2-carboxamide (anhydro-oxytetracycline).

OXYTOCIN

Oxytocinum



DEFINITION

Oxytocin is a cyclic nonapeptide having the structure of the hormone produced by the posterior lobe of the pituitary gland that stimulates contraction of the uterus and milk ejection in receptive mammals. It is obtained by chemical synthesis and is available in the freeze-dried form as an acetate. It contains not less than 93.0 per cent and not more than the equivalent of 102.0 per cent of the peptide $C_{43}H_{66}N_{12}O_{12}S_2$, calculated with reference to the anhydrous, acetic acid-free substance.

By convention, for the purpose of labelling oxytocin preparations, 1 mg of oxytocin peptide ($C_{43}H_{66}N_{12}O_{12}S_2$) is equivalent to 600 IU of biological activity.

CHARACTERS

A white or almost white powder, hygroscopic, very soluble in water and in dilute solutions of acetic acid and of ethanol.

IDENTIFICATION

Examine the chromatograms obtained in the assay. The retention time of the principal peak in the chromatogram obtained with the test solution is approximately the same as that of the principal peak in the chromatogram obtained with the reference solution.

TESTS

pH (2.2.3). Dissolve 0.200 g in *carbon dioxide-free water R* and dilute to 10.0 ml with the same solvent. The pH of the solution is 3.0 to 6.0.

Amino acids. Examine by means of an amino-acid analyser. Standardise the apparatus with a mixture containing equimolar amounts of ammonia, glycine and the L-form of the following amino acids:

lysine	threonine	alanine	leucine
histidine	serine	valine	tyrosine
arginine	glutamic acid	methionine	phenylalanine
aspartic acid	proline	isoleucine	

together with half the equimolar amount of L-cystine. For the validation of the method, an appropriate internal standard, such as *DL-norleucine R*, is used.

Test solution. Place 1.0 mg of the substance to be examined in a rigorously cleaned hard-glass tube 100 mm long and 6 mm in internal diameter. Add a suitable amount of a 50 per cent *V/V* solution of *hydrochloric acid R*. Immerse the tube in a freezing mixture at $-5^\circ C$, reduce the pressure to below 133 Pa and seal. Heat at $110^\circ C$ to $115^\circ C$ for 16 h. Cool, open the tube, transfer the contents to a 10 ml flask with the aid of five quantities, each of 0.2 ml, of *water R* and evaporate to dryness over *potassium hydroxide R* under reduced pressure. Take up the residue in *water R* and evaporate to dryness over *potassium hydroxide R* under reduced pressure; repeat these operations once. Take up the residue in a buffer solution suitable for the amino-acid analyser used and dilute to a suitable volume with the same buffer solution. Apply a suitable volume to the amino-acid analyser.

Express the content of each amino acid in moles. Calculate the relative proportions of the amino acids, taking one-sixth of the sum of the number of moles of aspartic acid, glutamic acid, proline, glycine, isoleucine and leucine as equal to one. The values fall within the following limits: aspartic acid 0.95 to 1.05; glutamic acid 0.95 to 1.05; proline 0.95 to 1.05; glycine 0.95 to 1.05; leucine 0.90 to 1.10; isoleucine 0.90 to 1.10; tyrosine 0.7 to 1.05; half-cystine 1.4 to 2.1; not more than traces of other amino acids are present.

Related peptides. Examine by liquid chromatography (2.2.29) as described under Assay.

Inject 50 µl of the test solution. In the chromatogram obtained the area of any peak, apart from the principal peak, is not greater than 1.5 per cent of the total area of the peaks; the sum of the areas of all the peaks, apart from the principal peak, is not greater than 5 per cent of the total area of the peaks. Disregard any peak due to the solvent and any peak with an area less than 0.1 per cent of that of the principal peak.

Acetic acid (2.5.34): 6.0 per cent to 10.0 per cent.

Test solution. Dissolve 15.0 mg of the substance to be examined in a mixture of 5 volumes of mobile phase B and 95 volumes of mobile phase A and dilute to 10.0 ml with the same mixture of solvents.

Water (2.5.12). Not more than 5.0 per cent, determined on at least 50 mg by the semi-micro determination of water.

Bacterial endotoxins (2.6.14): less than 300 IU/mg, if intended for use in the manufacture of parenteral dosage forms without a further appropriate procedure for removal of bacterial endotoxins.

ASSAY

Examine by liquid chromatography (2.2.29).

Test solution. Prepare a 0.25 mg/ml solution of the substance to be examined in a 15.6 g/l solution of *sodium dihydrogen phosphate R*.

Reference solution. Dissolve the contents of a vial of *oxytocin CRS* in a 15.6 g/l solution of *sodium dihydrogen phosphate R* to obtain a concentration of 0.25 mg/ml.

Resolution solution. Dissolve the contents of a vial of *oxytocin/desmopressin validation mixture CRS* in 500 µl of a 15.6 g/l solution of *sodium dihydrogen phosphate R*.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.125 m long and 4.6 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (5 µm),

- as mobile phase at a flow rate of 1 ml/min:

Mobile phase A. A 15.6 g/l solution of *sodium dihydrogen phosphate R*,

Mobile phase B. Mix 1 volume of *acetonitrile for chromatography R* with 1 volume of *water R*,

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)	Comment
0 - 30	70 → 40	30 → 60	linear gradient
30 - 30.1	40 → 70	60 → 30	switch to initial eluent composition
30.1 - 45	70	30	re-equilibration

- as detector a spectrophotometer set at 220 nm.

Equilibrate the column with a mixture of 30 volumes of mobile phase B and 70 volumes of mobile phase A.

Inject 25 µl of the resolution solution. When the chromatograms are recorded in the prescribed conditions, the retention times are: oxytocin about 7.5 min and

desmopressin about 10 min. The test is not valid unless the resolution between the peaks corresponding to desmopressin and oxytocin is at least 5.0.

Inject 25 µl of the test solution and 25 µl of the reference solution.

Calculate the content of oxytocin ($C_{43}H_{66}N_{12}O_{12}S_2$) from the peak areas in the chromatograms obtained with the test solution and the reference solution and the declared content of $C_{43}H_{66}N_{12}O_{12}S_2$ in *oxytocin CRS*.

STORAGE

Store in an airtight container, protected from light, at a temperature of 2 °C to 8 °C. If the substance is sterile, store in a sterile, airtight, tamper-proof container.

LABELLING

The label states:

- the oxytocin peptide content ($C_{43}H_{66}N_{12}O_{12}S_2$),
- where applicable, that the substance is free from bacterial endotoxins.

01/2005:0779

OXYTOCIN BULK SOLUTION

Oxytocini solutio

DEFINITION

Oxytocin bulk solution is a solution of oxytocin, a cyclic nonapeptide having the structure of the hormone produced by the posterior lobe of the pituitary gland that stimulates contraction of the uterus and milk ejection in receptive mammals. It is obtained by chemical synthesis. It is available as a bulk solution with a stated concentration of not less than 0.25 mg of oxytocin per millilitre, in a solvent that may contain an appropriate antimicrobial preservative. It contains not less than 95.0 per cent and not more than 105.0 per cent of the amount of the peptide $C_{43}H_{66}N_{12}O_{12}S_2$ stated per millilitre.

By convention, for the purpose of labelling oxytocin preparations, 1 mg of oxytocin peptide ($C_{43}H_{66}N_{12}O_{12}S_2$) is equivalent to 600 IU of biological activity.

CHARACTERS

A clear, colourless liquid.

IDENTIFICATION

Examine the chromatograms obtained under Assay. The retention time of the principal peak in the chromatogram obtained with the test solution is similar to that of the principal peak in the chromatogram obtained with the reference solution.

TESTS

pH (2.2.3). The pH of the preparation to be examined is 3.0 to 5.0.

Amino acids. Examine by means of an amino-acid analyser. Standardise the apparatus with a mixture containing equimolar amounts of ammonia, glycine and the L-form of the following amino acids:

lysine	threonine	alanine	leucine
histidine	serine	valine	tyrosine
arginine	glutamic acid	methionine	phenylalanine
aspartic acid	proline	isoleucine	

together with half the equimolar amount of L-cystine. For the validation of the method, an appropriate internal standard, such as *DL-norleucine R*, is used.

Test solution. Place a volume of the preparation to be examined containing 0.25 mg of peptide in a rigorously cleaned hard-glass tube 100 mm long and 6 mm in internal diameter. Evaporate to dryness. Add a suitable amount of a 50 per cent *V/V* solution of *hydrochloric acid R*. Immerse the tube in a freezing mixture at -5°C , reduce the pressure to below 133 Pa and seal. Heat at 110°C to 115°C for 16 h. Cool, open the tube, transfer the contents to a 10 ml flask with the aid of five quantities, each of 0.2 ml, of *water R* and evaporate to dryness over *potassium hydroxide R* under reduced pressure. Take up the residue in *water R* and evaporate to dryness over *potassium hydroxide R* under reduced pressure; repeat these operations once. Take up the residue in a buffer solution suitable for the amino-acid analyser used and dilute to a suitable volume with the same buffer solution.

Apply to the amino-acid analyser a suitable, accurately measured volume of the test solution such that the peak given by the amino acid present in the largest amount occupies most of the available chart height of the recorder.

Express the content of each amino acid in moles. Calculate the relative proportions of the amino acids taking one sixth of the sum of the number of moles of aspartic acid, glutamic acid, proline, glycine, isoleucine and leucine as equal to 1. The values fall within the following limits: aspartic acid 0.95 to 1.05; glutamic acid 0.95 to 1.05; proline 0.95 to 1.05; glycine 0.95 to 1.05; leucine 0.90 to 1.10; isoleucine 0.90 to 1.10; tyrosine 0.7 to 1.05; half cystine 1.4 to 2.1; not more than traces of other amino acids are present.

Related peptides. Examine by liquid chromatography (2.2.29) as described under Assay.

Inject 50 μl of the test solution. In the chromatogram obtained, the area of any peak apart from the principal peak is not greater than 1.5 per cent of the total area of the peaks; the sum of the areas of all peaks, apart from the principal peak, is not greater than 5 per cent of the total area of the peaks. Disregard any peak due to the solvent or to the antimicrobial preservative and any peak with an area less than 0.1 per cent of that of the principal peak.

Bacterial endotoxins (2.6.14): less than 300 IU in the volume that contains 1 mg of oxytocin, if intended for use in the manufacture of parenteral dosage forms without a further appropriate procedure for the removal of bacterial endotoxins.

ASSAY

Examine by liquid chromatography (2.2.29).

Test solution. Use the preparation to be examined.

Reference solution. Dissolve the contents of a vial of *oxytocin CRS* in a 15.6 g/l solution of *sodium dihydrogen phosphate R* to obtain a concentration of 0.25 mg/ml.

Resolution solution. Dissolve the contents of a vial of *oxytocin/desmopressin validation mixture CRS* with 500 μl of a 15.6 g/l solution of *sodium dihydrogen phosphate R*.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.125 m long and 4.6 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (5 μm),

- as mobile phases at a flow rate of 1 ml/min:

Mobile phase A. A 15.6 g/l solution of *sodium dihydrogen phosphate R*,

Mobile phase B. Mix 1 volume of *acetonitrile for chromatography R* with 1 volume of *water R*,

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)	Comment
0 - 30	70 $\rightarrow$ 40	30 $\rightarrow$ 60	linear gradient
30 - 30.1	40 $\rightarrow$ 70	60 $\rightarrow$ 30	return to initial conditions
30.1 - 45	70	30	re-equilibration

- as detector a spectrophotometer set at 220 nm.

Equilibrate the column with a mixture of 30 volumes of mobile phase B and 70 volumes of mobile phase A.

Inject 25 μl of the resolution solution. When the chromatograms are recorded in the prescribed conditions, the retention times are: oxytocin about 7.5 min and desmopressin about 10 min. The test is not valid unless the resolution between the desmopressin peak and the oxytocin peak is at least 5.0.

Inject 25 μl of the reference solution and 25 μl of the test solution. Calculate the content of oxytocin ($\text{C}_{43}\text{H}_{66}\text{N}_{12}\text{O}_{12}\text{S}_2$) from the peak areas in the chromatograms obtained with the test solution and the reference solution and the declared content of $\text{C}_{43}\text{H}_{66}\text{N}_{12}\text{O}_{12}\text{S}_2$ in *oxytocin CRS*.

STORAGE

Store at a temperature between 2°C and 8°C , protected from light. If the substance is sterile, store in a sterile, airtight, tamper-proof container.

LABELLING

The label states:

- the oxytocin peptide content in milligrams of $\text{C}_{43}\text{H}_{66}\text{N}_{12}\text{O}_{12}\text{S}_2$ per millilitre,
- where applicable, that the preparation is free from bacterial endotoxins,
- the name of any added antimicrobial preservative.

01/2005:1904

PALMITIC ACID**Acidum palmiticum****DEFINITION**

Hexadecanoic acid ($C_{16}H_{32}O_2$; M_r 256.4), obtained from fats or oils of vegetable or animal origin.

Content: minimum 92.0 per cent.

CHARACTERS

Appearance: white, waxy solid.

Solubility: practically insoluble in water, soluble in alcohol.

IDENTIFICATION

- It complies with the test for freezing point (see Tests).
- Acid value (2.5.1): 216 to 220, determined on 0.1 g.
- Examine the chromatograms obtained in the assay.

Results: the principal peak in the chromatogram obtained with the test solution is similar in retention time to the principal peak in the chromatogram obtained with the reference solution.

TESTS

Appearance. Heat the substance to be examined to about 75 °C. The resulting liquid is not more intensely coloured than reference solution Y_7 or BY_7 (2.2.2, Method I).

Acidity. Melt 5.0 g, stir for 2 min in 10 ml of hot *carbon dioxide-free water R*, cool slowly and filter. To the filtrate add 0.05 ml of *methyl orange solution R*. No red colour develops.

Freezing point (2.2.18): 60 °C to 66 °C.

Iodine value (2.5.4): maximum 1.

Stearic acid: maximum 6.0 per cent, determined as prescribed in the assay.

Nickel (2.4.27): maximum 1 ppm.

ASSAY

Gas chromatography (2.4.22, Method C). Prepare the solutions as described in the method but omitting the initial hydrolysis.

Reference solution. Prepare the reference solution in the same manner as the test solution using a mixture of 50 mg of *palmitic acid R* and 50 mg of *stearic acid R* instead of the substance to be examined.

Relative retention with reference to methyl stearate: methyl palmitate = about 0.9.

System suitability:

- *resolution*: minimum 5.0 between the peaks due to methyl stearate and methyl palmitate.

01/2005:0350

PANCREAS POWDER**Pancreatis pulvis****DEFINITION**

Pancreas powder is prepared from the fresh or frozen pancreases of mammals. It contains various enzymes having proteolytic, lipolytic and amylolytic activities.

1 milligram of pancreas powder contains not less than 1.0 European Pharmacopoeia Unit (Ph. Eur. U.) of total proteolytic activity, 15 European Pharmacopoeia Units of lipolytic activity and 12 European Pharmacopoeia Units of amylolytic activity.

Pancreas powder is prepared in conditions designed to minimise the degree of microbial contamination.

CHARACTERS

A slightly brown, amorphous powder, partly soluble in water, practically insoluble in alcohol.

IDENTIFICATION

- Triturate 0.5 g with 10 ml of *water R* and adjust to pH 8 by the addition of 0.1 M *sodium hydroxide*, using 0.1 ml of *cresol red solution R* as indicator. Divide the suspension into two equal parts (suspension (a) and suspension (b)). Boil suspension (a). To each suspension add 10 mg of *fibrin congo red R*, heat to 38 °C to 40 °C and maintain at this temperature for 1 h. Suspension (a) is colourless or slightly pink and suspension (b) is distinctly more red.

- Triturate 0.25 g with 10 ml of *water R* and adjust to pH 8 by the addition of 0.1 M *sodium hydroxide*, using 0.1 ml of *cresol red solution R* as indicator. Divide the suspension into two equal parts (suspension (a) and suspension (b)). Boil suspension (a). Dissolve 0.1 g of *soluble starch R* in 100 ml of boiling *water R*, boil for 2 min, cool and dilute to 150 ml with *water R*. To 75 ml of the starch solution add suspension (a) and to the remaining 75 ml add suspension (b). Heat each mixture to 38 °C to 40 °C and maintain at this temperature for 5 min.

To 1 ml of each mixture add 10 ml of *iodine solution R2*. The mixture obtained with suspension (a) has an intense blue-violet colour; the mixture obtained with suspension (b) has the colour of the iodine solution.

TESTS

Fat content. In an extraction apparatus, treat 1.0 g with *light petroleum R1* for 3 h. Evaporate the solvent and dry the residue at 100 °C to 105 °C for 2 h. The residue weighs not more than 50 mg (5.0 per cent).

Loss on drying (2.2.32). Not more than 5.0 per cent, determined on 0.50 g by drying at 60 °C at a pressure not exceeding 670 Pa for 4 h.

Microbial contamination. Total viable aerobic count (2.6.12) not more than 10^4 micro-organisms per gram, determined by plate count. It complies with the tests for *Escherichia coli* and *Salmonella* (2.6.13).

ASSAY

Total proteolytic activity. The total proteolytic activity of pancreas powder is determined by comparing the quantity of peptides non-precipitable by a 50 g/l solution of *trichloroacetic acid R* released per minute from a substrate of casein solution with the quantity of such peptides released by *pancreas powder (protease) BRP* from the same substrate in the same conditions.

Casein solution. Suspend a quantity of *casein BRP* equivalent to 1.25 g of dried substance in 5 ml of *water R*, add 10 ml of 0.1 M *sodium hydroxide* and stir for 1 min. (Determine the water content of *casein BRP* prior to the test by heating at 60 °C *in vacuo* for 4 h.) Add 60 ml of *water R* and stir with a magnetic stirrer until the solution is practically clear. Adjust to pH 8.0 with 0.1 M *sodium hydroxide* or 0.1 M *hydrochloric acid*. Dilute to 100.0 ml with *water R*. Use the solution on the day of preparation.

Enterokinase solution. Dissolve 50 mg of *enterokinase BRP* in 0.02 M calcium chloride solution R and dilute to 50.0 ml with the same solvent. Use the solution on the day of preparation.

For the test suspension and the reference suspension, prepare the suspension and carry out the dilution at 0 °C to 4 °C.

Test suspension. Triturate 0.100 g of the substance to be examined for 5 min adding gradually 25 ml of 0.02 M calcium chloride solution R. Transfer completely to a volumetric flask and dilute to 100.0 ml with 0.02 M calcium chloride solution R. To 10.0 ml of this suspension add 10.0 ml of enterokinase solution and heat on a water-bath at 35 ± 0.5 °C for 15 min. Cool and dilute with borate buffer solution pH 7.5 R at 5 ± 3 °C to a final concentration of about 0.065 Ph. Eur. U. of total proteolytic activity per millilitre calculated on the basis of the stated activity.

Reference suspension. Prepare a suspension of *pancreas powder (protease) BRP* as described for the test suspension but without the addition of enterokinase so as to obtain a known final concentration of about 0.065 Ph. Eur. U. per millilitre calculated on the basis of the stated activity.

Designate tubes in duplicate T, T_b, S₁, S_{1b}, S₂, S_{2b}, S₃, S_{3b}; designate a tube B.

Add borate buffer solution pH 7.5 R to the tubes as follows:

B: 3.0 ml,

S₁ and S_{1b}: 2.0 ml,

S₂, S_{2b}, T and T_b: 1.0 ml.

Add the reference suspension to the tubes as follows:

S₁ and S_{1b}: 1.0 ml,

S₂ and S_{2b}: 2.0 ml,

S₃ and S_{3b}: 3.0 ml.

Add 2.0 ml of the test suspension to tubes T and T_b.

Add 5.0 ml of a 50 g/l solution of *trichloroacetic acid R* to tubes B, S_{1b}, S_{2b}, S_{3b} and T_b. Mix by shaking.

Place the tubes and the casein solution in a water-bath at 35 ± 0.5 °C. Place a glass rod in each tube. When temperature equilibrium is reached, add 2.0 ml of the casein solution to tubes B, S_{1b}, S_{2b}, S_{3b} and T_b. Mix. At time zero, add 2.0 ml of casein solution successively and at intervals of 30 s to tubes S₁, S₂, S₃ and T. Mix immediately after each addition. Exactly 30 min after addition of the casein solution, taking into account the regular interval adopted, add 5.0 ml of a 50 g/l solution of *trichloroacetic acid R* to tubes S₁, S₂, S₃ and T. Mix. Withdraw the tubes from the water-bath and allow to stand at room temperature for 20 min.

Filter the contents of each tube twice through the same suitable filter paper previously washed with a 50 g/l solution of *trichloroacetic acid R*, then with *water R* and dried.

A suitable filter paper complies with the following test: filter 5 ml of a 50 g/l of *trichloroacetic acid R* on a 7 cm disc of white filter paper; the absorbance (2.2.25) of the filtrate, measured at 275 nm using unfiltered trichloroacetic acid solution as the compensation liquid, is less than 0.04.

A schematic presentation of the above operations is shown in Table 0350.-1.

Table 0350.-1

	Tubes								
	S ₁	S _{1b}	S ₂	S _{2b}	S ₃	S _{3b}	T	T _b	B
Buffer solution	2	2	1	1			1	1	3
Reference solution	1	1	2	2	3	3			
Suspension test							2	2	
Trichloroacetic acid solution		5		5		5		5	5
Mix		+		+		+		+	+
Water-bath 35 °C	+	+	+	+	+	+	+	+	+
Casein solution		2		2		2		2	2
Mix		+		+		+		+	+
Casein solution	2		2		2		2		
Mix	+		+		+		+		
Water-bath 35 °C 30 min	+	+	+	+	+	+	+	+	+
Trichloroacetic solution	5		5		5		5		
Mix	+		+		+		+		
Room temperature 20 min	+	+	+	+	+	+	+	+	+
Filter	+	+	+	+	+	+	+	+	+

Measure the absorbance (2.2.25) of the filtrates at 275 nm using the filtrate obtained from tube B as the compensation liquid.

Correct the average absorbance values for the filtrates obtained from tubes S₁, S₂ and S₃ by subtracting the average values obtained for the filtrates from tubes S_{1b}, S_{2b} and S_{3b} respectively. Draw a calibration curve of the corrected values against volume of reference suspension used.

Determine the activity of the substance to be examined using the corrected absorbance for the test suspension (T – T_b) and the calibration curve and taking into account the dilution factors.

The test is not valid unless the corrected absorbance values are between 0.15 and 0.60.

Lipolytic activity. The lipolytic activity is determined by comparing the rate at which a suspension of pancreas powder hydrolyses a substrate of olive oil emulsion with the rate at which a suspension of *pancreas powder (amylase and lipase) BRP* hydrolyses the same substrate under the same conditions. *The test is carried out under nitrogen.*

Olive oil stock emulsion. In an 800 ml beaker 9 cm in diameter, place 40 ml of *olive oil R*, 330 ml of *acacia solution R* and 30 ml of *water R*. Place an electric mixer at the bottom of the beaker. Place the beaker in a vessel containing *alcohol R* and a sufficient quantity of ice as a cooling mixture. Emulsify using the mixer at an average speed of 1000 r/min to 2000 r/min. Cool to 5 °C to 10 °C. Increase the mixing speed to 8000 r/min. Mix for 30 min keeping the temperature below 25 °C by the continuous addition of crushed ice into the cooling mixture. (A mixture of calcium chloride and crushed ice is also suitable). Store the stock emulsion in a refrigerator and use within 14 days. The emulsion must not separate into two distinct layers. Check the diameter of the globules of the emulsion under a microscope. At least 90 per cent have a diameter below 3 µm and none has a diameter greater than 10 µm. Shake the emulsion thoroughly before preparing the emulsion substrate.

Olive oil emulsion. For ten determinations, mix the following solutions in the order indicated: 100 ml of stock emulsion, 80 ml of *tris(hydroxymethyl)aminomethane*

solution R1, 20 ml of a freshly prepared 80 g/l of *sodium taurocholate BRP* and 95 ml of *water R*. Use on the day of preparation.

Apparatus. Use a reaction vessel of about 50 ml capacity provided with:

- a device that will maintain a temperature of 37 ± 0.5 °C,
- a magnetic stirrer,
- a lid with holes for the insertion of electrodes, the tip of a burette, a tube for the admission of nitrogen and the introduction of reagents.

An automatic or manual titration apparatus may be used. In the latter case, the burette is graduated in 0.005 ml and the pH-meter is provided with a wide reading scale and glass-calomel electrodes. After each test the reaction vessel is evacuated by suction and washed several times with *water R*, the washings being removed each time by suction.

Test suspension. In a small mortar cooled to 0 °C to 4 °C, triturate carefully a quantity of the substance to be examined equivalent to about 2500 Ph. Eur. U. of lipolytic activity with 1 ml of cooled *maleate buffer solution pH 7.0 R* (lipase solvent) until a very fine suspension is obtained. Dilute the suspension with cold *maleate buffer solution pH 7.0 R*, transfer quantitatively to a volumetric flask and dilute to 100.0 ml with the cold buffer solution. Keep the flask containing the test suspension in iced water during the titration.

Reference suspension. To avoid absorption of water formed by condensation, allow the reference preparation to reach room temperature before opening the container. Prepare a suspension of *pancreas powder (amylase and lipase) BRP* as described for the test suspension using a quantity equivalent to about 2500 Ph. Eur. U.

Carry out the titrations immediately after preparation of the test suspension and the reference suspension. Place 29.5 ml of olive oil emulsion in the reaction vessel equilibrated at 37 ± 0.5 °C. Fit the vessel with the electrodes, a stirrer and the burette (the tip being immersed in the olive oil emulsion).

Put the lid in place and switch on the apparatus. Carefully add 0.1 M sodium hydroxide with stirring. Adjust to pH 9.2. Using a rapid-flow graduated pipette transfer about 0.5 ml of the previously homogenised reference suspension, start the chronometer and add continuously 0.1 M sodium hydroxide to maintain the pH at 9.0. After exactly 1 min, note the volume of 0.1 M sodium hydroxide used. Carry out the measurement a further four times. Discard the first reading and determine the average of the four others (S_1). Make two further determinations (S_2 and S_3). Calculate the average of the values S_1 , S_2 and S_3 . The average volume of 0.1 M sodium hydroxide used should be about 0.12 ml/min with limits of 0.08 ml to 0.16 ml.

Carry out three determinations in the same manner for the test suspension (T_1 , T_2 and T_3). If the quantity of 0.1 M sodium hydroxide used is outside the limits 0.08 ml to 0.16 ml per minute, the assay is repeated with a quantity of test suspension which is more suitable but situated between 0.4 ml and 0.6 ml. Otherwise the quantity of the substance to be examined is adjusted to comply with the conditions of the test. Calculate the average of the values T_1 , T_2 and T_3 .

Calculate the activity in Ph. Eur. Units per milligram from the expression:

$$\frac{n \times m_1}{n_1 \times m} \times A$$

- n = average volume of 0.1 M sodium hydroxide used per minute during the titration of the test suspension,
- n_1 = average volume of 0.1 M sodium hydroxide used per minute during the titration of the reference suspension,
- m = mass in milligrams of the substance to be examined,
- m_1 = mass in milligrams of the reference preparation,
- A = activity of *pancreas powder (amylase and lipase) BRP* in Ph. Eur. Units per milligram.

Amylolytic activity. The amylolytic activity is determined by comparing the rate at which a suspension of pancreas powder hydrolyses a substrate of starch solution with the rate at which a suspension of *pancreas powder (amylase and lipase) BRP* hydrolyses the same substrate under the same conditions.

Starch solution. To a quantity of *starch BRP* equivalent to 2.0 g of the dried substance add 10 ml of *water R* and mix. (Determine the water content of *starch BRP* prior to the test by heating at 120 °C for 4 h). Add this suspension, whilst stirring continuously, to 160 ml of boiling *water R*. Wash the container several times with successive quantities, each of 10 ml, of *water R* and add the washings to the hot starch solution. Heat to boiling, stirring continuously. Cool to room temperature and dilute to 200 ml with *water R*. Use the solution on the day of preparation.

For the test suspension and the reference suspension, prepare the suspension and carry out the dilution at 0 °C to 4 °C.

Test suspension. Triturate a quantity of the substance to be examined equivalent to about 1500 Ph. Eur. U. of amylolytic activity with 60 ml of *phosphate buffer solution pH 6.8 R1* for 15 min. Transfer quantitatively to a volumetric flask and dilute to 100.0 ml with *phosphate buffer solution pH 6.8 R1*.

Reference suspension. Prepare a suspension of *pancreas powder (amylase and lipase) BRP* as described for the test suspension, using a quantity equivalent to about 1500 Ph. Eur. U.

In a test tube 200 mm long and 22 mm in diameter, fitted with a ground-glass stopper, place 25.0 ml of starch solution, 10.0 ml of *phosphate buffer solution pH 6.8 R1* and 1.0 ml of a 11.7 g/l solution of *sodium chloride R*. Close the tube, shake and place in a water-bath at 25.0 ± 0.1 °C. When the temperature equilibrium has been reached, add 1.0 ml of the test suspension and start the chronometer. Mix and place the tube in the water-bath. After exactly 10 min, add 2 ml of 1 M hydrochloric acid. Transfer the mixture quantitatively to a 300 ml conical flask fitted with a ground-glass stopper. Whilst shaking continuously, add 10.0 ml of 0.05 M iodine and immediately, 45 ml of 0.1 M sodium hydroxide. Allow to stand in the dark at a temperature between 15 °C and 25 °C for 15 min. Add 4 ml of a mixture of 1 volume of sulphuric acid R and 4 volumes of *water R*. Titrate the excess of iodine with 0.1 M sodium thiosulphate using a microburette. Carry out a blank titration adding the 2 ml of 1 M hydrochloric acid before introducing the test suspension. Carry out the titration of the reference suspension in the same manner.

Calculate the amylolytic activity in Ph. Eur. Units per milligram from the expression:

$$\frac{(n' - n) m_1}{(n'_1 - n_1) m} \times A$$

- n = number of millilitres of 0.1 M sodium thiosulphate used in the titration of the test suspension,
 n_1 = number of millilitres of 0.1 M sodium thiosulphate used in the titration of the reference suspension,
 n' = number of millilitres of 0.1 M sodium thiosulphate used in the blank titration of the test suspension,
 n'_1 = number of millilitres of 0.1 M sodium thiosulphate used in the blank titration of the reference suspension,
 m = mass in milligrams of the substance to be examined,
 m_1 = mass in milligrams of the reference preparation,
 A = activity of pancreas powder (amylase and lipase) BRP in Ph. Eur. Units per milligram.

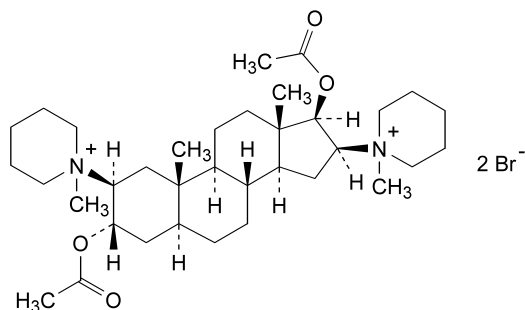
STORAGE

Store in an airtight container, at a temperature of 2 °C to 8 °C.

01/2005:0681

PANCURONIUM BROMIDE

Pancuronii bromidum



$C_{35}H_{60}Br_2N_2O_4$

M_r 733

DEFINITION

1,1'-[3 α ,17 β -Bis(acetyloxy)-5 α -androstane-2 β ,16 β -diyl]bis(1-methylpiperidinium) dibromide.

Content: 98.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

Appearance: white or almost white, crystalline powder, hygroscopic.

Solubility: very soluble to freely soluble in water, very soluble in methylene chloride, freely soluble in alcohol.

IDENTIFICATION

A. Infrared absorption spectrophotometry (2.2.24).

Preparation: discs.

Comparison: pancuronium bromide CRS.

B. Examine the chromatograms obtained in the test for related substances.

Results: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).

C. It gives reaction (a) of bromides (2.3.1).

TESTS

Appearance of solution. The solution is clear (2.2.1) and colourless (2.2.2, Method II).

Dissolve 50 mg in water R and dilute to 25 ml with the same solvent.

Specific optical rotation (2.2.7): + 38 to + 42 (anhydrous substance).

Dissolve 0.75 g in water R and dilute to 25.0 ml with the same solvent.

Related substances. Thin-layer chromatography (2.2.27). Prepare the solutions immediately before use.

Test solution. Dissolve 50 mg of the substance to be examined in methylene chloride R and dilute to 5 ml with the same solvent.

Reference solution (a). Dissolve 50 mg of pancuronium bromide CRS in methylene chloride R and dilute to 5 ml with the same solvent.

Reference solution (b). Dilute 0.1 ml of the test solution to 20 ml with methylene chloride R.

Reference solution (c). Dissolve 5 mg of pancuronium impurity A CRS in methylene chloride R and dilute to 50 ml with the same solvent.

Reference solution (d). Dissolve 5 mg of pancuronium bromide CRS in 1 ml of reference solution (c).

Plate: TLC silica gel plate R.

Mobile phase: 400 g/l solution of sodium iodide R, acetonitrile R, 2-propanol R (5:10:85 V/V/V).

Application: 2 μ l.

Development: in an unlined and unsaturated tank over a path of 12 cm.

Drying: in a current of cold air.

Detection: expose the plate to iodine vapour for about 10 min and cover the plate with a glass plate.

System suitability:

- the chromatogram obtained with reference solution (d) shows 2 distinct spots and the ratio of the R_f values of impurity A and of pancuronium bromide is at least 1.2,
- a spot is clearly visible in the chromatogram obtained with reference solution (b).

Limits:

- **impurity A:** any spot corresponding to impurity A is not more intense than the principal spot in the chromatogram obtained with reference solution (c) (1.0 per cent),
- **any other impurity:** any spot, apart from the principal spot and any spot corresponding to impurity A, is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.5 per cent).

Water (2.5.12): maximum 8.0 per cent, determined on 0.300 g.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

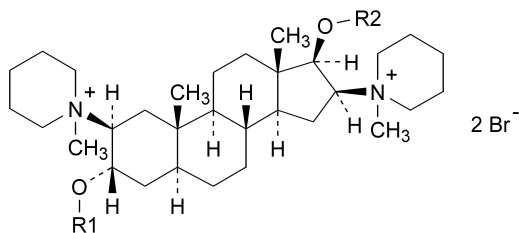
Dissolve 0.2000 g in 50 ml of acetic anhydride R, heating if necessary. Titrate with 0.1 M perchloric acid, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M perchloric acid is equivalent to 36.63 mg of $C_{35}H_{60}Br_2N_2O_4$.

STORAGE

In an airtight container, protected from light.

IMPURITIES

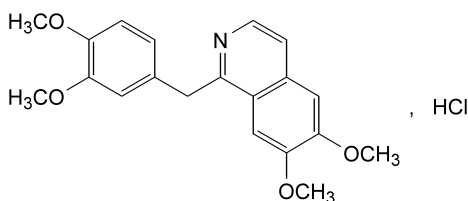


- A. R1 = CO-CH₃, R2 = H: 1,1'-[3 α -(acetyloxy)-17 β -hydroxy-5 α -androstane-2 β ,16 β -diyl]bis(1-methylpiperidinium) dibromide (dacuronium bromide),
- B. R1 = H, R2 = CO-CH₃: 1,1'-[17 β -(acetyloxy)-3 α -hydroxy-5 α -androstane-2 β ,16 β -diyl]bis(1-methylpiperidinium) dibromide,
- C. R1 = R2 = H: 1,1'-(3 α ,17 β -dihydroxy-5 α -androstane-2 β ,16 β -diyl)bis(1-methylpiperidinium) dibromide.

01/2005:0102
corrected

PAPAVERINE HYDROCHLORIDE

Papaverini hydrochloridum



C₂₀H₂₂ClNO₄

M_r 375.9

DEFINITION

1-(3,4-Dimethoxybenzyl)-6,7-dimethoxyisoquinoline hydrochloride.

Content: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

Appearance: white or almost white, crystalline powder or white or almost white crystals.

Solubility: sparingly soluble in water, slightly soluble in alcohol.

IDENTIFICATION

First identification: A, D.

Second identification: B, C, D.

A. Infrared absorption spectrophotometry (2.2.24).

Comparison: papaverine hydrochloride CRS.

B. Thin-layer chromatography (2.2.27).

Test solution. Dissolve 5 mg of the substance to be examined in methanol R and dilute to 10 ml with the same solvent.

Reference solution. Dissolve 5 mg of papaverine hydrochloride CRS in methanol R and dilute to 10 ml with the same solvent.

Plate: TLC silica gel GF₂₅₄ plate R.

Mobile phase: diethylamine R, ethyl acetate R, toluene R (10:20:70 V/V/V).

Application: 10 μ l.

Development: over 2/3 of the plate.

Drying: at 100-105 °C for 2 h.

Detection: examine in ultraviolet light at 254 nm.

Results: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with the reference solution.

C. To 10 ml of solution S (see Tests) add 5 ml of ammonia R dropwise and allow to stand for 10 min. The precipitate, washed and dried, melts (2.2.14) at 146 °C to 149 °C.

D. It gives reaction (a) of chlorides (2.3.1).

TESTS

Solution S. Dissolve 0.4 g in carbon dioxide-free water R, heating gently if necessary, and dilute to 20 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and not more intensely coloured than reference solution BY₆ (2.2.2, Method II).

pH (2.2.3): 3.0 to 4.0 for solution S.

Related substances. Liquid chromatography (2.2.29).

Solvent mixture: acetonitrile R, mobile phase A (20:80 V/V).

Test solution. Dissolve 20.0 mg of the substance to be examined in the solvent mixture and dilute to 10.0 ml with the solvent mixture.

Reference solution (a). Dilute 1.0 ml of the test solution to 100.0 ml with the solvent mixture. Dilute 1.0 ml of this solution to 10.0 ml with the solvent mixture.

Reference solution (b). Dissolve 12 mg of noscapine CRS in 1.0 ml of the test solution and dilute to 100.0 ml with the solvent mixture.

Column:

– size: l = 0.25 m, $\varnothing$ = 4.0 mm,

– stationary phase: base-deactivated octylsilyl silica gel for chromatography R (5 μ m).

Mobile phase:

– mobile phase A: 3.4 g/l solution of potassium dihydrogen phosphate R adjusted to pH 3.0 with dilute phosphoric acid R,

– mobile phase B: acetonitrile R,

– mobile phase C: methanol R,

Time (min)	Mobile phase A (per cent V/V/V)	Mobile phase B (per cent V/V/V)	Mobile phase C (per cent V/V/V)
0 - 5	85	5	10
5 - 12	85 $\rightarrow$ 60	5	10 $\rightarrow$ 35
12 - 20	60	5	35
20 - 24	60 $\rightarrow$ 40	5 $\rightarrow$ 20	35 $\rightarrow$ 40
24 - 27	40	20	40
27 - 32	40 $\rightarrow$ 85	20 $\rightarrow$ 5	40 $\rightarrow$ 10
32 - 40	85	5	10

Flow rate: 1 ml/min.

Detection: spectrophotometer at 238 nm.

Injection: 10 μ l.

Relative retention with reference to papaverine (retention time = about 23.4 min): impurity E = about 0.7; impurity C = about 0.75; impurity B = about 0.8; impurity A = about 0.9; impurity F = about 1.1; impurity D = about 1.2.

System suitability: reference solution (b):

- *resolution:* minimum 1.5 between the peaks due to impurity A and papaverine.

Limits:

- *correction factors:* for the calculation of contents, multiply the peak areas of the following impurities by the corresponding correction factor: impurity C = 2.7; impurity D = 0.5; impurity A = 6.2;
- *any impurity:* not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent);
- *total:* not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent);
- *disregard limit:* 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

Loss on drying (2.2.32): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 100–105 °C.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on the residue from the test for loss on drying.

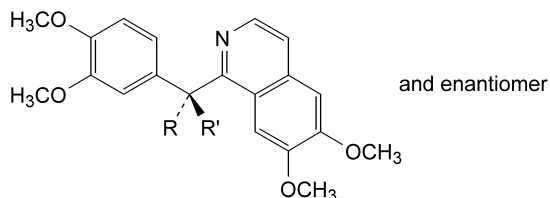
ASSAY

Dissolve 0.300 g in a mixture of 5.0 ml of 0.01 M hydrochloric acid and 50 ml of alcohol R. Carry out a potentiometric titration (2.2.20), using 0.1 M sodium hydroxide. Read the volume added between the 2 points of inflexion.

1 ml of 0.1 M sodium hydroxide is equivalent to 37.59 mg of $C_{20}H_{22}ClNO_4$.

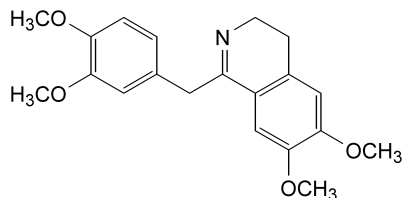
IMPURITIES

A. noscapine,

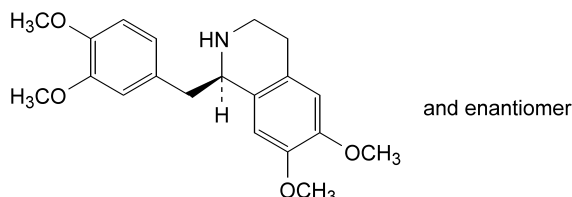


B. R = OH, R' = H: (RS)-(3,4-dimethoxyphenyl)(6,7-dimethoxyisoquinolin-1-yl)methanol (papaverinol),

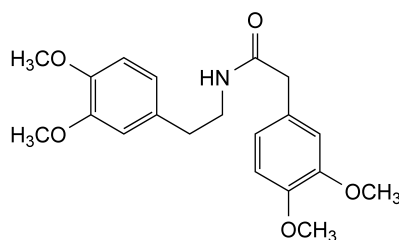
D. R + R' = O: (3,4-dimethoxyphenyl)(6,7-dimethoxyisoquinolin-1-yl)methanone (papaveraldine),



C. 1-(3,4-dimethoxybenzyl)-6,7-dimethoxy-3,4-dihydroisoquinoline (dihydropapaverine),



E. (1RS)-1-(3,4-dimethoxybenzyl)-6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline (tetrahydropapaverine),

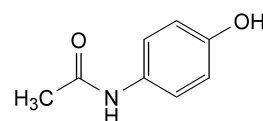


F. 2-(3,4-dimethoxyphenyl)-N-[2-(3,4-dimethoxyphenyl)ethyl]acetamide.

01/2005:0049

PARACETAMOL

Paracetamolum



$C_8H_9NO_2$

M_r 151.2

DEFINITION

N-(4-Hydroxyphenyl)acetamide.

Content: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

Appearance: white, crystalline powder.

Solubility: sparingly soluble in water, freely soluble in alcohol, very slightly soluble in methylene chloride.

IDENTIFICATION

First identification: A, C.

Second identification: A, B, D, E.

A. Melting point (2.2.14): 168 °C to 172 °C.

B. Dissolve 0.1 g in methanol R and dilute to 100.0 ml with the same solvent. To 1.0 ml of the solution add 0.5 ml of a 10.3 g/l solution of hydrochloric acid R and dilute to 100.0 ml with methanol R. Protect the solution from bright light and immediately measure the absorbance (2.2.25) at the absorption maximum at 249 nm. The specific absorbance at the maximum is 860 to 980.

C. Infrared absorption spectrophotometry (2.2.24).

Preparation: discs.

Comparison: paracetamol CRS.

D. To 0.1 g add 1 ml of hydrochloric acid R, heat to boiling for 3 min, add 1 ml of water R and cool in an ice bath. No precipitate is formed. Add 0.05 ml of a 4.9 g/l solution of potassium dichromate R. A violet colour develops which does not change to red.

E. It gives the reaction of acetyl (2.3.1). Heat over a naked flame.

TESTS

Related substances. Liquid chromatography (2.2.29).

Prepare the solutions immediately before use.

Test solution. Dissolve 0.200 g of the substance to be examined in 2.5 ml of methanol R containing 4.6 g/l of a 400 g/l solution of tetrabutylammonium hydroxide R and dilute to 10.0 ml with a mixture of equal volumes of a 17.9 g/l solution of disodium hydrogen phosphate R and of a 7.8 g/l solution of sodium dihydrogen phosphate R.

Reference solution (a). Dilute 1.0 ml of the test solution to 50.0 ml with the mobile phase. Dilute 5.0 ml of this solution to 100.0 ml with the mobile phase.

Reference solution (b). Dilute 1.0 ml of reference solution (a) to 10.0 ml with the mobile phase.

Reference solution (c). Dissolve 5.0 mg of 4-aminophenol *R*, 5 mg of paracetamol *CRS* and 5.0 mg of chloroacetanilide *R* in methanol *R* and dilute to 20.0 ml with the same solvent. Dilute 1.0 ml to 250.0 ml with the mobile phase.

Reference solution (d). Dissolve 20.0 mg of 4-nitrophenol *R* in methanol *R* and dilute to 50.0 ml with the same solvent. Dilute 1.0 ml to 20.0 ml with the mobile phase.

Column:

- **size:** $l = 0.25$ m, $\varnothing = 4.6$ mm,
- **stationary phase:** octylsilyl silica gel for chromatography *R* (5 μ m),
- **temperature:** 35 °C.

Mobile phase: mix 375 volumes of a 17.9 g/l solution of disodium hydrogen phosphate *R*, 375 volumes of a 7.8 g/l solution of sodium dihydrogen phosphate *R* and 250 volumes of methanol *R* containing 4.6 g/l of a 400 g/l solution of tetrabutylammonium hydroxide *R*.

Flow rate: 1.5 ml/min.

Detection: spectrophotometer at 245 nm.

Injection: 20 μ l.

Run time: 12 times the retention time of paracetamol.

Relative retentions with reference to paracetamol (retention time = about 4 min): impurity K = about 0.8; impurity F = about 3; impurity J = about 7.

System suitability: reference solution (c):

- **resolution:** minimum 4.0 between the peaks due to impurity K and to paracetamol,
- **signal-to-noise ratio:** minimum 50 for the peak due to impurity J.

Limits:

- **impurity J:** not more than 0.2 times the area of the corresponding peak in the chromatogram obtained with reference solution (c) (10 ppm),
- **impurity K:** not more than the area of the corresponding peak in the chromatogram obtained with reference solution (c) (50 ppm),
- **impurity F:** not more than half the area of the corresponding peak in the chromatogram obtained with reference solution (d) (0.05 per cent),
- **any other impurity:** not more than half the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent),
- **total of other impurities:** not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent),
- **disregard limit** for the calculation of the total of other impurities: the area of the principal peak in the chromatogram obtained with reference solution (b) (0.01 per cent).

Heavy metals (2.4.8): maximum 20 ppm.

Dissolve 1.0 g in a mixture of 15 volumes of water *R* and 85 volumes of acetone *R* and dilute to 20 ml with the same mixture of solvents. 12 ml of the solution complies with limit test B. Prepare the standard using lead standard solution (1 ppm Pb) obtained by diluting lead standard solution (100 ppm Pb) *R* with a mixture of 15 volumes of water *R* and 85 volumes of acetone *R*.

Loss on drying (2.2.32): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 100–105 °C.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

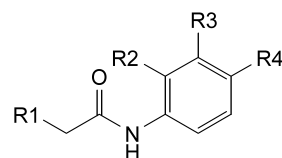
Dissolve 0.300 g in a mixture of 10 ml of water *R* and 30 ml of dilute sulphuric acid *R*. Boil under a reflux condenser for 1 h, cool and dilute to 100.0 ml with water *R*. To 20.0 ml of the solution add 40 ml of water *R*, 40 g of ice, 15 ml of dilute hydrochloric acid *R* and 0.1 ml of ferroin *R*. Titrate with 0.1 M cerium sulphate until a greenish-yellow colour is obtained. Carry out a blank titration.

1 ml of 0.1 M cerium sulphate is equivalent to 7.56 mg of $C_8H_9NO_2$.

STORAGE

Protected from light.

IMPURITIES



A. $R_1 = R_3 = R_4 = H$, $R_2 = OH$: *N*-(2-hydroxyphenyl)acetamide,

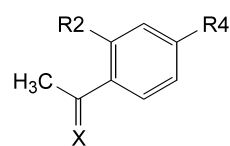
B. $R_1 = CH_3$, $R_2 = R_3 = H$, $R_4 = OH$: *N*-(4-hydroxyphenyl)propanamide,

C. $R_1 = R_2 = H$, $R_3 = Cl$, $R_4 = OH$: *N*-(3-chloro-4-hydroxyphenyl)acetamide,

D. $R_1 = R_2 = R_3 = R_4 = H$: *N*-phenylacetamide,

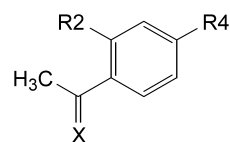
H. $R_1 = R_2 = R_3 = H$, $R_4 = O-CO-CH_3$: 4-(acetylamino)phenyl acetate,

J. $R_1 = R_2 = R_3 = H$, $R_4 = Cl$: *N*-(4-chlorophenyl)acetamide (chloroacetanilide),

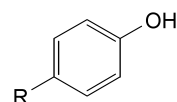


E. $X = O$, $R_2 = H$, $R_4 = OH$: 1-(4-hydroxyphenyl)ethanone,

G. $X = N-OH$, $R_2 = H$, $R_4 = OH$: 1-(4-hydroxyphenyl)ethanone oxime,



I. $X = O$, $R_2 = OH$, $R_4 = H$: 1-(2-hydroxyphenyl)ethanone,



F. $R = NO_2$: 4-nitrophenol,

K. $R = NH_2$: 4-aminophenol.

01/2005:1034

01/2005:0240

PARAFFIN, HARD**Paraffinum solidum****DEFINITION**

Hard paraffin is a purified mixture of solid saturated hydrocarbons generally obtained from petroleum. It may contain a suitable antioxidant.

CHARACTERS

A colourless or white mass, practically insoluble in water, freely soluble in methylene chloride, practically insoluble in alcohol. The melted substance is free from fluorescence in daylight.

IDENTIFICATION

First identification: A, C.

Second identification: B, C.

- A. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the *Ph. Eur. reference spectrum of hard paraffin*.
- B. It complies with the test for acidity or alkalinity (see Tests).
- C. Melting point (2.2.16): 50 °C to 61 °C.

TESTS

Acidity or alkalinity. To 15 g add 30 ml of boiling *water R* and shake vigorously for 1 min. Allow to cool and to separate. To 10 ml of the aqueous layer add 0.1 ml of *phenolphthalein solution R*. The solution is colourless. Not more than 1.0 ml of 0.01 M *sodium hydroxide* is required to change the colour of the indicator to red. To a further 10 ml of the aqueous layer add 0.1 ml of *methyl red solution R*. The solution is yellow. Not more than 0.5 ml of 0.01 M *hydrochloric acid* is required to change the colour of the indicator to red.

Polycyclic aromatic hydrocarbons. *Use reagents for ultraviolet absorption spectrophotometry.* Dissolve 0.50 g in 25 ml of *heptane R* and place in a 125 ml separating funnel with unlubricated ground-glass parts (stopper, stopcock). Add 5.0 ml of *dimethyl sulphoxide R*. Shake vigorously for 1 min and allow to stand until two clear layers are formed. Transfer the lower layer to a second separating funnel, add 2 ml of *heptane R* and shake the mixture vigorously. Allow to stand until two clear layers are formed. Separate the lower layer and measure its absorbance (2.2.25) between 265 nm and 420 nm using as the compensation liquid the clear lower layer obtained by vigorously shaking 5.0 ml of *dimethyl sulphoxide R* with 25 ml of *heptane R* for 1 min. Prepare a 7.0 mg/l reference solution of *naphthalene R* in *dimethyl sulphoxide R* and measure the absorbance of this solution at the maximum at 278 nm using *dimethyl sulphoxide R* as the compensation liquid. At wavelengths from 265 nm to 420 nm, the absorbance of the test solution is not greater than one-third that of the reference solution at 278 nm.

Sulphates (2.4.13). Introduce 2.0 g of the melted substance to be examined into a 50 ml ground-glass-stoppered separating funnel. Add 30 ml of boiling *distilled water R* and shake vigorously for 1 min. Filter. 15 ml of the filtrate complies with the limit test for sulphates (150 ppm).

STORAGE

Store protected from light.

LABELLING

The label states, where applicable, the name and concentration of any added antioxidant.

PARAFFIN, LIGHT LIQUID**Paraffinum perliquidum****DEFINITION**

Light liquid paraffin is a purified mixture of liquid saturated hydrocarbons obtained from petroleum.

CHARACTERS

A colourless, transparent, oily liquid, free from fluorescence in daylight. Practically insoluble in water, slightly soluble in ethanol (96 per cent), miscible with hydrocarbons.

IDENTIFICATION

First identification: A, C.

Second identification: B, C.

- A. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the *Ph. Eur. reference spectrum of liquid paraffin*.
- B. In a test tube cautiously boil 1 ml with 1 ml of 0.1 M *sodium hydroxide*, with continuous shaking, for about 30 s. On cooling to room temperature, 2 phases separate. To the aqueous phase add 0.1 ml of *phenolphthalein solution R*. The colour turns to red.
- C. It complies with the test for viscosity (see Tests).

TESTS

Acidity or alkalinity. To 10 ml add 20 ml of boiling *water R* and shake vigorously for 1 min. Separate the aqueous layer and filter. To 10 ml of the filtrate, add 0.1 ml of *phenolphthalein solution R*. The solution is colourless. Not more than 0.1 ml of 0.1 M *sodium hydroxide* is required to change the colour of the indicator to pink.

Relative density (2.2.5): 0.810 to 0.875.

Viscosity (2.2.9): 25 mPa·s to 80 mPa·s.

Polycyclic aromatic hydrocarbons. *Use reagents for ultraviolet spectrophotometry.*

Introduce 25.0 ml into a 125 ml separating funnel with unlubricated ground-glass parts (stopper, stopcock). Add 25 ml of *hexane R* which has been previously shaken twice with one-fifth its volume of *dimethyl sulphoxide R*. Mix and add 5.0 ml of *dimethyl sulphoxide R*. Shake vigorously for 1 min and allow to stand until 2 clear layers are formed. Transfer the lower layer to a second separating funnel, add 2 ml of *hexane R* and shake the mixture vigorously. Allow to stand until 2 clear layers are formed. Separate the lower layer and measure its absorbance (2.2.25) between 260 nm and 420 nm, using as the compensation liquid the clear lower layer obtained by vigorously shaking 5.0 ml of *dimethyl sulphoxide R* with 25 ml of *hexane R* for 1 min. Prepare a 7.0 mg/l reference solution of *naphthalene R* in *trimethylpentane R* and measure the absorbance of the solution at the maximum at 275 nm, using *trimethylpentane R* as the compensation liquid. At no wavelength between 260 nm and 420 nm does the absorbance of the test solution exceed one-third that of the reference solution at 275 nm.

Readily carbonisable substances. Use a ground-glass-stoppered tube about 125 mm long and 18 mm in internal diameter, graduated at 5 ml and 10 ml; wash with hot *water R* (temperature at least 60 °C), *acetone R*, *heptane R* and finally with *acetone R*, dry at 100–110 °C. Cool in a desiccator. Introduce 5 ml of the substance to be examined and add 5 ml of *nitrogen-free sulphuric acid R1*. Insert the stopper and shake as vigorously as possible, in the

longitudinal direction of the tube, for 5 s. Loosen the stopper, immediately place the tube in a water-bath, avoiding contact of the tube with the bottom or side of the bath, and heat for 10 min. After 2 min, 4 min, 6 min and 8 min, remove the tube from the bath and shake as vigorously as possible, in the longitudinal direction of the tube for 5 s. At the end of 10 min of heating, remove the tube from the water-bath and allow to stand for 10 min. Centrifuge at 2000 *g* for 5 min. The lower layer is not more intensely coloured (2.2.2, *Method I*) than a mixture of 0.5 ml of blue primary solution, 1.5 ml of red primary solution, 3.0 ml of yellow primary solution and 2 ml of a 10 g/l solution of *hydrochloric acid R*.

Solid paraffins. Dry a suitable quantity of the substance to be examined by heating at 100 °C for 2 h and cool in a desiccator over *sulphuric acid R*. Place in a glass tube with an internal diameter of about 25 mm, close the tube and immerse in a bath of iced water. After 4 h, the liquid is sufficiently clear for a black line, 0.5 mm wide, to be easily seen against a white background held vertically behind the tube.

STORAGE

Protected from light.

01/2005:0239

PARAFFIN, LIQUID

Paraffinum liquidum

DEFINITION

Liquid paraffin is a purified mixture of liquid saturated hydrocarbons obtained from petroleum.

CHARACTERS

A colourless, transparent, oily liquid, free from fluorescence in daylight. Practically insoluble in water, slightly soluble in ethanol (96 per cent), miscible with hydrocarbons.

IDENTIFICATION

First identification: A, C.

Second identification: B, C.

- A. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the *Ph. Eur. reference spectrum of liquid paraffin*.
- B. In a test tube cautiously boil 1 ml with 1 ml of 0.1 *M* sodium hydroxide, with continuous shaking, for about 30 s. On cooling to room temperature, 2 phases separate. To the aqueous phase add 0.1 ml of *phenolphthalein solution R*. The colour turns to red.
- C. It complies with the test for viscosity (see Tests).

TESTS

Acidity or alkalinity. To 10 ml add 20 ml of boiling *water R* and shake vigorously for 1 min. Separate the aqueous layer and filter. To 10 ml of the filtrate, add 0.1 ml of *phenolphthalein solution R*. The solution is colourless. Not more than 0.1 ml of 0.1 *M* sodium hydroxide is required to change the colour of the indicator to pink.

Relative density (2.2.5): 0.827 to 0.890.

Viscosity (2.2.9): 110 mPas to 230 mPas.

Polycyclic aromatic hydrocarbons. Use reagents for ultraviolet spectrophotometry.

Introduce 25.0 ml into a 125 ml separating funnel with unlubricated ground-glass parts (stopper, stopcock). Add

25 ml of *hexane R* which has been previously shaken twice with one-fifth its volume of *dimethyl sulphoxide R*. Mix and add 5.0 ml of *dimethyl sulphoxide R*. Shake vigorously for 1 min and allow to stand until 2 clear layers are formed. Transfer the lower layer to a second separating funnel, add 2 ml of *hexane R* and shake the mixture vigorously. Allow to stand until 2 clear layers are formed. Separate the lower layer and measure its absorbance (2.2.25) between 260 nm and 420 nm, using as the compensation liquid the clear lower layer obtained by vigorously shaking 5.0 ml of *dimethyl sulphoxide R* with 25 ml of *hexane R* for 1 min. Prepare a 7.0 mg/l reference solution of *naphthalene R* in *trimethylpentane R* and measure the absorbance of the solution at the maximum at 275 nm, using *trimethylpentane R* as the compensation liquid. At no wavelength between 260 nm and 420 nm does the absorbance of the test solution exceed one-third that of the reference solution at 275 nm.

Readily carbonisable substances. Use a ground-glass-stoppered tube about 125 mm long and 18 mm in internal diameter, graduated at 5 ml and 10 ml; wash with hot *water R* (temperature at least 60 °C), *acetone R*, *heptane R* and finally with *acetone R*, dry at 100–110 °C. Cool in a desiccator. Introduce 5 ml of the substance to be examined and add 5 ml of *nitrogen-free sulphuric acid R1*. Insert the stopper and shake as vigorously as possible, in the longitudinal direction of the tube, for 5 s. Loosen the stopper, immediately place the tube in a water-bath, avoiding contact of the tube with the bottom or side of the bath, and heat for 10 min. After 2 min, 4 min, 6 min and 8 min, remove the tube from the bath and shake as vigorously as possible, in the longitudinal direction of the tube for 5 s. At the end of 10 min of heating, remove the tube from the water-bath and allow to stand for 10 min. Centrifuge at 2000 *g* for 5 min. The lower layer is not more intensely coloured (2.2.2, *Method I*) than a mixture of 0.5 ml of blue primary solution, 1.5 ml of red primary solution, 3.0 ml of yellow primary solution and 2 ml of a 10 g/l solution of *hydrochloric acid R*.

Solid paraffins. Dry a suitable quantity of the substance to be examined by heating at 100 °C for 2 h and cool in a desiccator over *sulphuric acid R*. Place in a glass tube with an internal diameter of about 25 mm, close the tube and immerse in a bath of iced water. After 4 h, the liquid is sufficiently clear for a black line, 0.5 mm wide, to be easily seen against a white background held vertically behind the tube.

STORAGE

Protected from light.

01/2005:1799

PARAFFIN, WHITE SOFT

Vaselinum album

DEFINITION

Purified and wholly or nearly decolorised mixture of semi-solid hydrocarbons, obtained from petroleum. It may contain a suitable antioxidant. White soft paraffin described in this monograph is not suitable for oral use.

CHARACTERS

Appearance: white or almost white, translucent, soft unctuous mass, slightly fluorescent in daylight when melted.
Solubility: practically insoluble in water, soluble in methylene chloride, practically insoluble in alcohol and in glycerol.

IDENTIFICATION

First identification: A, B, D.

Second identification: A, C, D.

A. The drop point is between 35 °C and 70 °C and does not differ by more than 5 °C from the value stated on the label, according to method (2.2.17) with the following modification to fill the cup: heat the substance to be examined at a temperature not exceeding 80 °C, with stirring to ensure uniformity. Warm the metal cup at a temperature not exceeding 80 °C in an oven, remove it from the oven, place on a clean plate or ceramic tile and pour a sufficient quantity of the melted sample into the cup to fill it completely. Allow the filled cup to cool for 30 min on the plate or the ceramic tile and place it in a water bath at 24-26 °C for 30-40 min. Level the surface of the sample with a single stroke of a knife or razor blade, avoiding compression of the sample.

B. Infrared absorption spectrophotometry (2.2.24).

Comparison: Ph. Eur. reference spectrum of white soft paraffin.

C. Melt 2 g and when a homogeneous phase is obtained, add 2 ml of *water R* and 0.2 ml of 0.05 M *iodine*. Shake. Allow to cool. The solid upper layer is violet-pink.

D. It complies with the test for appearance (see Tests).

TESTS

Appearance. The substance is white. Melt 12 g on a water-bath. The melted mass is not more intensely coloured than a mixture of 1 volume of yellow primary solution and 9 volumes of a 1 per cent *m/V* solution of *hydrochloric acid R* (2.2.2, *Method II*).

Acidity or alkalinity. To 10 g add 20 ml of boiling *water R* and shake vigorously for 1 min. Allow to cool and decant. To 10 ml of the aqueous layer add 0.1 ml of *phenolphthalein solution R*. The solution is colourless. Not more than 0.5 ml of 0.01 M *sodium hydroxide* is required to change the colour of the indicator to red.

Consistency (2.9.9): 60 to 300.

Polycyclic aromatic hydrocarbons: maximum 300 ppm.

Use reagents for ultraviolet spectrophotometry. Dissolve 1.0 g in 50 ml of *hexane R* which has been previously shaken twice with 10 ml of *dimethyl sulphoxide R*. Transfer the solution to a 125 ml separating funnel with unlubricated ground-glass parts (stopper, stopcock). Add 20 ml of *dimethyl sulphoxide R*. Shake vigorously for 1 min and allow to stand until 2 clear layers are formed. Transfer the lower layer to a second separating funnel. Repeat the extraction with a further 20 ml of *dimethyl sulphoxide R*. Shake vigorously the combined lower layers with 20 ml of *hexane R* for 1 min. Allow to stand until 2 clear layers are formed. Separate the lower layer and dilute to 50.0 ml with *dimethyl sulphoxide R*. Measure the absorbance (2.2.25) over the range 260 nm to 420 nm using a path length of 4 cm and as compensation liquid the clear lower layer obtained by vigorously shaking 10 ml of *dimethyl sulphoxide R* with 25 ml of *hexane R* for 1 min. Prepare a reference solution in *dimethyl sulphoxide R* containing 6.0 mg of *naphthalene R* per litre and measure the absorbance of the solution at the maximum at 278 nm using a path length of 4 cm and *dimethyl sulphoxide R* as compensation liquid. At no wavelength in the range 260 nm to 420 nm does the absorbance of the test solution exceed that of the reference solution at 278 nm.

Sulphated ash (2.4.14): maximum 0.05 per cent, determined on 2.0 g.

STORAGE

Protected from light.

LABELLING

The label states:

- the nominal drop point,
- where applicable, the name and concentration of any added antioxidant.

01/2005:1554

PARAFFIN, YELLOW SOFT

Vaselinum flavum

DEFINITION

Yellow soft paraffin is a purified mixture of semi-solid hydrocarbons, obtained from petroleum. It may contain a suitable antioxidant.

CHARACTERS

A yellow, translucent, unctuous mass, slightly fluorescent in daylight when melted, practically insoluble in water, soluble in methylene chloride, practically insoluble in alcohol and in glycerol.

IDENTIFICATION

First identification: A, B, D.

Second identification: A, C, D.

A. The drop point (2.2.17) is 40 °C to 60 °C and does not differ by more than 5 °C from the value stated on the label, with the following modification to fill the cup: heat the substance to be examined at 118 °C to 122 °C, with stirring to ensure uniformity, then cool to 100 °C to 107 °C. Warm the metal cup at 103 °C to 107 °C in an oven, remove it from the oven, place on a clean plate or ceramic tile and pour a sufficient quantity of the melted sample into the cup to fill it completely. Allow the filled cup to cool for 30 min on the ceramic tile and place it in a water-bath at 24 °C to 26 °C for a further 30 min to 40 min. Level the surface of the sample with a single stroke of a knife or razor blade, avoiding compression of the sample.

B. Examine by infrared absorption spectrophotometry (2.2.24) comparing with the *Ph. Eur. reference spectrum of yellow soft paraffin*. The spectrum obtained shows major peaks similar in position and intensity to the *Ph. Eur. reference spectrum*.

C. Melt 2 g and when a homogenous phase is obtained, add 2 ml of *water R* and 0.2 ml of 0.05 M *iodine*. Shake. Allow to cool. The solid upper layer is violet-pink.

D. It complies with the test for appearance (see Tests).

TESTS

Appearance. The substance is yellow. Melt 12 g on a water-bath. The melted mass is not more intensely coloured than a mixture of 7.6 volumes of yellow primary solution and 2.4 volumes of red primary solution (2.2.2, *Method II*).

Acidity or alkalinity. To 10 g add 20 ml of boiling *water R* and shake vigorously for 1 min. Allow to cool and decant. To 10 ml of the aqueous layer add 0.1 ml of *phenolphthalein solution R*. The solution is colourless. Not more than 0.5 ml of 0.01 M *sodium hydroxide* is required to change the colour of the indicator to red.

Consistency (2.9.9). The consistency is 100 to 300.

Polycyclic aromatic hydrocarbons. Use reagents for ultraviolet absorption spectrophotometry. Dissolve 1.0 g in 50 ml of *hexane R* which has been previously shaken twice with one-fifth its volume of *dimethyl sulphoxide R*. Transfer the solution to a 125 ml separating funnel with unlubricated ground-glass parts (stopper, stopcock). Add 20 ml of *dimethyl sulphoxide R*. Shake vigorously for 1 min and allow to stand until two clear layers are formed. Transfer the lower layer to a second separating funnel. Repeat the extraction with a further 20 ml of *dimethyl sulphoxide R*. Shake vigorously the combined lower layers with 20 ml of *hexane R* for 1 min. Allow to stand until two clear layers are formed. Separate the lower layer and dilute to 50.0 ml with *dimethyl sulphoxide R*. Measure the absorbance (2.2.25) between 260 nm and 420 nm using a path length of 4 cm and using as the compensation liquid the clear lower layer obtained by vigorously shaking 10 ml of *dimethyl sulphoxide R* with 25 ml of *hexane R* for 1 min. Prepare a 9.0 mg/l reference solution of *naphthalene R* in *dimethyl sulphoxide R* and measure the absorbance of this solution at the maximum at 278 nm using a path length of 4 cm and using *dimethyl sulphoxide R* as the compensation liquid. At no wavelength in the range of 260 nm to 420 nm does the absorbance of the test solution exceed that of the reference solution at 278 nm.

Sulphated ash (2.4.14). Not more than 0.05 per cent, determined on 2.0 g.

STORAGE

Store protected from light.

LABELLING

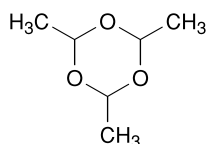
The label states:

- the nominal drop point,
- where applicable, the name and concentration of any added antioxidant.

01/2005:0351

PARALDEHYDE

Paraldehydum


 $C_6H_{12}O_3$
 M_r 132.2

DEFINITION

Paraldehyde is 2,4,6-trimethyl-1,3,5-trioxane, the cyclic trimer of acetaldehyde. It may contain a suitable quantity of an antioxidant.

CHARACTERS

A colourless or slightly yellow, transparent liquid. It solidifies on cooling to form a crystalline mass. Soluble in water, but less soluble in boiling water, miscible with alcohol and with essential oils.

IDENTIFICATION

- Solution S (see Tests) is clear (2.2.1) but becomes turbid on warming.
- To 5 ml add 0.1 ml of *dilute sulphuric acid R* and heat. Acetaldehyde, recognisable by its odour, is evolved.

- To 5 ml of solution S in a test-tube add 5 ml of *ammoniacal silver nitrate solution R* and heat in a water-bath. Silver is deposited as a mirror on the wall of the tube.

TESTS

Solution S. Dissolve 20.0 ml in *carbon dioxide-free water R* and dilute to 200.0 ml with the same solvent.

Acidity. To 50.0 ml of solution S add 0.05 ml of *phenolphthalein solution R*. Not more than 1.5 ml of 0.1 M *sodium hydroxide* is required to change the colour of the indicator.

Refractive index (2.2.6): 1.403 to 1.406.

Relative density (2.2.5): 0.991 to 0.996.

Distillation range (2.2.11). Not more than 10 per cent distils below 123 °C and not less than 95 per cent distils below 126 °C.

Freezing point (2.2.18): 10 °C to 13 °C.

Acetaldehyde. To 5.0 ml add a mixture of 0.2 ml of *methyl orange solution R*, 5 ml of *alcohol (60 per cent V/V) R* and 5 ml of *alcoholic hydroxylamine solution R* and shake. Not more than 0.8 ml of 0.5 M *sodium hydroxide* is required to change the colour of the indicator to pure yellow.

Peroxides. Place 50.0 ml of solution S in a ground-glass-stoppered flask, add 5 ml of *dilute sulphuric acid R* and 10 ml of *potassium iodide solution R*, close the flask and allow to stand protected from light for 15 min. Titrate with 0.1 M *sodium thiosulphate* using 1 ml of *starch solution R* as indicator. Allow to stand for 5 min and, if necessary complete the titration. Not more than 2.0 ml of 0.1 M *sodium thiosulphate* is required.

Non-volatile residue. Heat 5.0 ml in a tared evaporating dish on a water-bath and dry at 105 °C for 1 h. The residue weighs not more than 3 mg (0.6 g/l).

STORAGE

Store in a small, well-filled, airtight container, protected from light. If the substance has solidified the whole contents of the container must be liquefied before use.

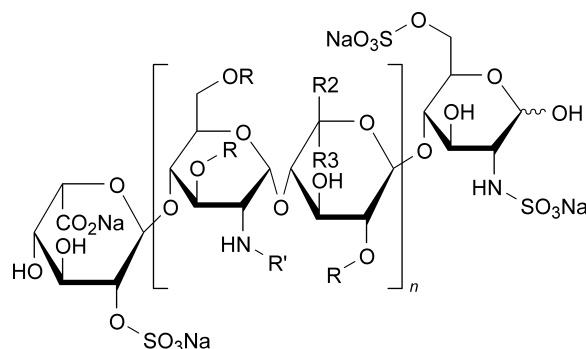
LABELLING

The label states the name and quantity of any added antioxidant.

01/2005:1252

PARNAPARIN SODIUM

Parnaparinum natricum



$n = 1$ to 21, $R = H$ or SO_3Na , $R' = SO_3Na$ or $CO-CH_3$
 $R_2 = H$ and $R_3 = CO_2Na$ or $R_2 = CO_2Na$ and $R_3 = H$

DEFINITION

Sodium salt of a low-molecular-mass heparin that is obtained by radical-catalysed depolymerisation, with hydrogen peroxide and with a cupric salt, of heparin from bovine or porcine intestinal mucosa. The majority of the components have a 2-*O*-sulpho- α -L-idopyranosuronic acid structure at the non-reducing end and a 2-*N*,6-*O*-disulpho-D-glucosamine structure at the reducing end of their chain.

Parnaparin sodium complies with the monograph on *Low-molecular-mass heparins* (0828), with the modifications and additional requirements below.

The mass-average molecular mass ranges between 4000 and 6000 with a characteristic value of about 5000.

The degree of sulphatation is 2.0 to 2.6 per disaccharide unit.

The potency is not less than 75 IU and not more than 110 IU of anti-factor Xa activity per milligram calculated with reference to the dried substance. The ratio of anti-factor Xa activity to anti-factor IIa activity is between 1.5 and 3.0.

IDENTIFICATION

Carry out identification test C as described in the monograph on *Low-molecular-mass heparins* (0828). In order to verify the suitability of the system in the lower molecular mass ranges (for example M_r 2000), a suitable reference preparation is used. The following requirements apply.

The mass-average molecular mass ranges between 4000 and 6000. The mass percentage of chains lower than 3000 is not more than 30 per cent. The mass percentage of chains between 3000 and 8000 ranges between 50 per cent and 60 per cent.

TESTS

Appearance of solution. The solution is clear (2.2.1) and not more intensely coloured than reference solution Y₅ (2.2.2, Method II).

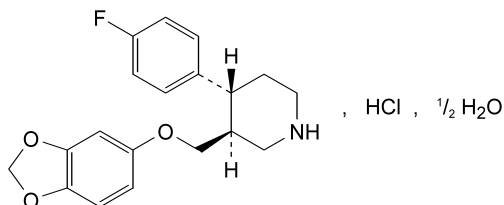
Dissolve 1.5 g in 10 ml of *water R*.

Copper: maximum 10 ppm, determined by atomic absorption spectrometry (2.2.23, Method I) and calculated with reference to the dried substance.

01/2005:2018

PAROXETINE HYDROCHLORIDE
HEMIHYDRATE

Paroxetini hydrochloridum hemihydricum

C₁₉H₂₁ClFNO₃ · 1/2 H₂O M_r 374.8

DEFINITION

(3*S*,4*R*)-3-[(1,3-Benzodioxol-5-yloxy)methyl]-4-(4-fluorophenyl)piperidine hydrochloride hemihydrate.

Content: 97.5 per cent to 102.0 per cent (anhydrous substance).

PRODUCTION

Impurity G: not more than 1 ppm, determined by liquid chromatography, coupled with tandem mass spectrometry using a suitable, validated method.

CHARACTERS

Appearance: white or almost white, crystalline powder.

Solubility: slightly soluble in water, freely soluble in methanol, sparingly soluble in alcohol and in methylene chloride.

It shows pseudopolymorphism.

IDENTIFICATION

A. Infrared absorption spectrophotometry (2.2.24).

Comparison: *paroxetine hydrochloride hemihydrate CRS*.

If the spectra obtained show differences, dissolve 1 part of the substance to be examined and 1 part of the reference substance separately in 10 parts of a mixture of 1 volume of *water R* and 9 volumes of *2-propanol R*, heat to 70 °C to dissolve. Recrystallise and record new spectra using the residues.

B. Examine the chromatograms obtained in the test for impurity D.

Injection: test solution and reference solution (c).

Results: the principal peak in the chromatogram obtained with the test solution is similar in retention time and size to the principal peak in the chromatogram obtained with reference solution (c).

C. It complies with the test for water (see Tests).

D. It gives reaction (b) of chlorides (2.3.1).

TESTS

Impurity D. Liquid chromatography (2.2.29).

Test solution. Dissolve 0.1000 g of the substance to be examined in 20 ml of *methanol R* and dilute to 100.0 ml with the mobile phase.

Reference solution (a). Dilute 1.0 ml of the test solution to 100.0 ml with the mobile phase. Dilute 1.0 ml to 10.0 ml with the mobile phase.

Reference solution (b). Dissolve 5 mg of *paroxetine impurity D CRS* and 5 mg of *paroxetine hydrochloride hemihydrate CRS* in 2 ml of *methanol R* and dilute to 100.0 ml with the mobile phase.

Reference solution (c). Dissolve 10 mg of *paroxetine hydrochloride hemihydrate CRS* in 2 ml of *methanol R* and dilute to 10.0 ml with the mobile phase.

Column:

- size: $l = 0.10$ m, $\varnothing = 4.0$ mm,
- stationary phase: *silica gel AGP for chiral chromatography R* (5 μ m).

Mobile phase: mix 2 volumes of *methanol R* and 8 volumes of a 5.8 g/l solution of *sodium chloride R*.

Flow rate: 0.5 ml/min.

Detection: spectrophotometer at 295 nm.

Injection: 10 μ l; inject the test solution and reference solutions (a) and (b).

Run time: 2.5 times the retention time of paroxetine.

Retention time: paroxetine = about 30 min.

System suitability: reference solution (b):

- resolution: minimum 2.2 between the peaks due to impurity D and paroxetine.

Limit:

- **impurity D:** not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent).

Related substances. Liquid chromatography (2.2.29).

Solvent mixture. Mix 1 volume of *tetrahydrofuran R* and 9 volumes of *water R*.

Test solution. Dissolve 50.0 mg of the substance to be examined in the solvent mixture and dilute to 50.0 ml with the same solvent mixture.

Reference solution (a). Dilute 5.0 ml of the test solution to 50.0 ml with the solvent mixture.

Reference solution (b). Dissolve 2 mg of *paroxetine impurity C CRS* in the solvent mixture and dilute to 20.0 ml with the same solvent mixture.

Reference solution (c). To 2.0 ml of reference solution (a) add 2.0 ml of reference solution (b) and dilute to 20.0 ml with the solvent mixture.

Reference solution (d). Dilute 2.0 ml of reference solution (a) to 200.0 ml with the solvent mixture.

Reference solution (e). Dissolve 2 mg of *paroxetine impurity A CRS* in the solvent mixture and dilute to 20 ml with the same solvent mixture.

Column:

- size: $l = 0.25$ m, $\varnothing = 4.6$ mm,
- stationary phase: end-capped octylsilyl silica gel for chromatography *R* (5 μ m),
- temperature: 40 °C.

Mobile phase:

- mobile phase A: trifluoroacetic acid *R*, tetrahydrofuran *R*, water *R* (5:100:900 V/V/V),
- mobile phase B: trifluoroacetic acid *R*, tetrahydrofuran *R*, acetonitrile *R* (5:100:900 V/V/V),

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)
0 - 30	80	20
30 - 50	80 → 20	20 → 80
50 - 60	20	80
60 - 65	20 → 80	80 → 20
65 - 70	80	20

Flow rate: 1 ml/min.

Detection: spectrophotometer at 295 nm.

Injection: 20 μ l; inject the test solution and reference solutions (c), (d) and (e).

Relative retention with reference to paroxetine: impurity A = about 0.8.

System suitability: reference solution (c):

- resolution: minimum 3.5 between the peaks due to impurity C and paroxetine.

Limits:

- **impurity A:** not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (d) (0.3 per cent),
- **any other impurity:** not more than the area of the principal peak in the chromatogram obtained with reference solution (d) (0.1 per cent),
- **total:** not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (d) (0.5 per cent),
- **disregard limit:** 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (d) (0.05 per cent).

Heavy metals (2.4.8): maximum 20 ppm.

1.0 g complies with limit test C. Use a platinum crucible. Prepare the standard using 2 ml of *lead standard solution* (10 ppm Pb) *R*.

Water (2.5.12): 2.2 per cent to 2.7 per cent, determined on 0.300 g.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g in a platinum crucible.

ASSAY

Liquid chromatography (2.2.29).

Test solution. Dissolve 50.0 mg of the substance to be examined in *water R* and dilute to 100.0 ml with the same solvent.

Reference solution (a). Dissolve 50.0 mg of *paroxetine hydrochloride hemihydrate CRS* in *water R* and dilute to 100.0 ml with the same solvent.

Reference solution (b). Dissolve 5.0 mg of *paroxetine hydrochloride hemihydrate CRS* and 5 mg of *paroxetine impurity A CRS* in *water R* and dilute to 10.0 ml with the same solvent.

Column:

- size: $l = 0.25$ m, $\varnothing = 4.6$ mm,
- stationary phase: trimethylsilyl silica gel for chromatography *R* (5 μ m).

Mobile phase: dissolve 3.85 g of ammonium acetate *R* in *water R*, adjust to pH 5.5 with *anhydrous acetic acid R* and dilute to 600 ml with the same solvent; add 400 ml of *acetonitrile R*; slowly add with stirring, 10 ml of *triethylamine R* and readjust to pH 5.5 with *anhydrous acetic acid R*.

Flow rate: 1 ml/min.

Detection: spectrophotometer at 295 nm.

Injection: 10 μ l.

Run time: twice the retention time of paroxetine.

System suitability: reference solution (b):

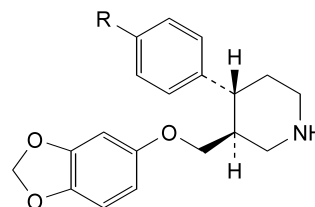
- resolution: minimum 2 between the peaks due to paroxetine and impurity A.

Calculate the percentage content of paroxetine hydrochloride using the chromatogram obtained with reference solution (a).

STORAGE

Protected from light.

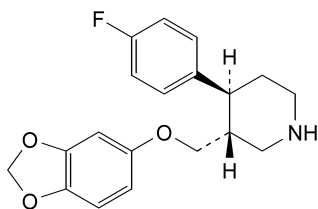
IMPURITIES



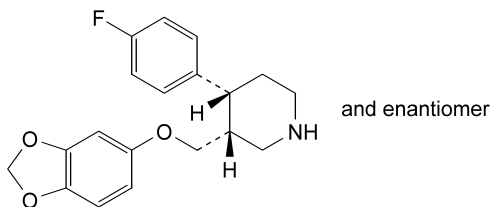
A. R = H: (3*S*,4*R*)-3-[(1,3-benzodioxol-5-yloxy)methyl]-4-phenylpiperidine (desfluoroparoxetine),

B. R = OCH₃: (3*S*,4*R*)-3-[(1,3-benzodioxol-5-yloxy)methyl]-4-(4-methoxyphenyl)piperidine,

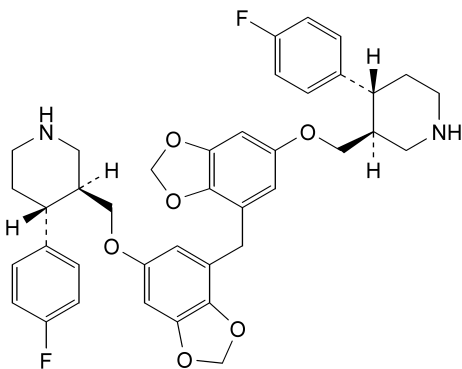
C. R = OC₂H₅: (3*S*,4*R*)-3-[(1,3-benzodioxol-5-yloxy)methyl]-4-(4-ethoxyphenyl)piperidine,



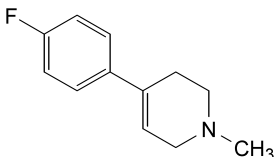
D. (3*R*,4*S*)-3-[(1,3-benzodioxol-5-yloxy)methyl]-4-(4-fluorophenyl)piperidine ((+)-*trans*-paroxetine),



E. (3*RS*,4*RS*)-3-[(1,3-benzodioxol-5-yloxy)methyl]-4-(4-fluorophenyl)piperidine (*cis*-paroxetine),



F. 3,3'-[methylenebis(1,3-benzodioxole-6,4-diylloxymethylene)]bis[4-(4-fluorophenyl)piperidine],



G. 4-(4-fluorophenyl)-1-methyl-1,2,3,6-tetrahydropyridine.

01/2005:1459

PASSION FLOWER

Passiflorae herba

DEFINITION

Passion flower consists of the fragmented or cut, dried aerial parts of *Passiflora incarnata* L. It may also contain flowers and/or fruits. It contains not less than 1.5 per cent of total flavonoids expressed as vitexin (C₂₁H₂₀O₁₀; *M_r* 432.4), calculated with reference to the dried drug.

CHARACTERS

It has the macroscopic and microscopic characters described under Identification tests A and B.

IDENTIFICATION

A. The green to greenish-grey or brownish stem is ligneous, hollow, longitudinally striated, glabrous or very slightly pubescent, with a diameter that is generally less than

8 mm. The green or greenish-brown leaves are alternate, finely dentate and pubescent, deeply divided into three acute lobes of which the central lobe is the largest. The midrib is much more prominent on the lower surface. The petiole is pubescent and bears two dark-coloured nectaries near the lamina. The tendrils are very numerous and grow from the axils of the leaves; they are fine, smooth, round and terminated in cylindrical spirals. The radiate flowers, if present, have three small bracts and a corolla consisting of five white, elongated petals with several rows of filiform, petaloid appendices. If present, the greenish to brownish fruit is flattened and oval; it contains several flattened, brownish-yellow, pitted seeds.

B. Reduce to a powder (355). The powder is light green. Examine under a microscope using *chloral hydrate solution R*. The powder shows fragments of the leaf epidermis with sinuous walls and anomocytic stomata (2.8.3); numerous cluster crystals of calcium oxalate isolated or aligned along the veins; many isolated or grouped fibres from the stems associated with pitted vessels and tracheids; uniseriate trichomes with one to three thin-walled cells, straight or slightly curved, ending in a point or sometimes a hook. In addition the powder shows, if flowers are present, papillose epidermises of the petals and appendages and pollen grains with a reticulate exine; and if mature fruits are present, scattered brown tannin cells and brownish-yellow, pitted fragments of the testa.

C. Examine the chromatograms obtained in the test for other species of *Passiflora*.

The chromatogram obtained with the test solution shows below the zone due to rutin in the chromatogram obtained with the reference solution a zone of intense yellow fluorescence, above it a zone of green fluorescence (diglycosylflavone), below the zone due to hyperoside in the chromatogram obtained with the reference solution a zone of yellow fluorescence (iso-orientin) and above a zone of green fluorescence (isovitexin), above the zone due to hyperoside in the chromatogram obtained with the reference solution a zone of brownish-yellow fluorescence (orientin) and above it a zone of green fluorescence (vitexin). These latter two zones may be absent. Further zones may be present.

TESTS

Foreign matter (2.8.2). It complies with the test for foreign matter.

Other species of *Passiflora*. Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel plate R*.

Test solution. To 1.0 g of the powdered drug (355) add 5 ml of *methanol R*. Heat to boiling under a reflux condenser for 10 min. Cool and filter.

Reference solution. Dissolve with heating 2.0 mg of *rutin R* and 2.0 mg of *hyperoside R* in 10 ml of *methanol R*.

Apply separately to the plate as bands 10 µl of each solution. Develop over a path of 15 cm using a mixture of 10 volumes of *anhydrous formic acid R*, 10 volumes of *water R*, 30 volumes of *methyl ethyl ketone R* and 50 volumes of *ethyl acetate R*. Allow the plate to dry in air. Spray with a 10 g/l solution of *diphenylboric acid aminoethyl ester R* in *methanol R* and then with a 50 g/l solution of *macrogol 400 R* in *methanol R*. Allow the plate to dry in air for 30 min. Examine the plate in ultraviolet light at 365 nm. The chromatogram obtained with the reference solution shows in the lower third a zone of yellowish-brown fluorescence due to rutin and in the middle third a zone of yellowish-brown fluorescence due to hyperoside.

The chromatogram obtained with the test solution shows no intense zones of greenish-yellow or orange-yellow fluorescence between the zone due to diglycosylflavones and that due to iso-orientin (*P. coerulea* and *P. edulis*).

Total ash (2.4.16). Not more than 13.0 per cent.

Loss on drying (2.2.32). Not more than 10.0 per cent, determined on 1.000 g of the powdered drug (355) by drying in an oven at 100 °C to 105 °C for 2 h.

ASSAY

Stock solution. In a 100 ml round-bottomed flask, introduce 0.200 g of the powdered drug (250) and add 40 ml of *alcohol* (60 per cent V/V) *R*. Heat in a water-bath at 60 °C under a reflux condenser for 30 min while shaking frequently. Allow to cool and filter the mixture through a plug of absorbent cotton in a 100 ml flask. Transfer the absorbent cotton with the drug residue into the round-bottomed flask. Add 40 ml of *alcohol* (60 per cent V/V) *R* and heat again in a water-bath at 60 °C under reflux for 10 min. Allow to cool and filter the mixture and the first filtrate from the 100 ml flask through a paper filter in the 100 ml volumetric flask. Dilute to 100 ml with the same solvent, while rinsing the flask, round-bottomed flask and filter.

Test solution. Introduce 5.0 ml of stock solution into a flask. Evaporate to dryness under reduced pressure and take up the residue with 10 ml of a mixture of 10 volumes of *methanol R* and 100 volumes of *glacial acetic acid R*. Add 10 ml of a solution consisting of 25 g/l of *boric acid R* and 20 g/l of oxalic acid in *anhydrous formic acid R* and dilute to 25.0 ml with *anhydrous acetic acid R*.

Compensation solution. Into a second flask introduce 5.0 ml of the stock solution. Evaporate to dryness under reduced pressure and take up the residue with 10 ml of a mixture of 10 volumes of *methanol R* and 100 volumes of *glacial acetic acid R*. Add 10 ml of *anhydrous formic acid R* and dilute to 25.0 ml with *anhydrous acetic acid R*.

Measure the absorbance (2.2.25) of the test solution after 30 min by comparison with the compensation solution at 401 nm.

Calculate the percentage content of total flavonoids, expressed as vitexin, from the expression:

$$\frac{A \times 0.8}{m}$$

taking the value of the specific absorbance to be 628.

A = absorbance of the test solution at 401 nm,

m = mass of the substance to be examined, in grams.

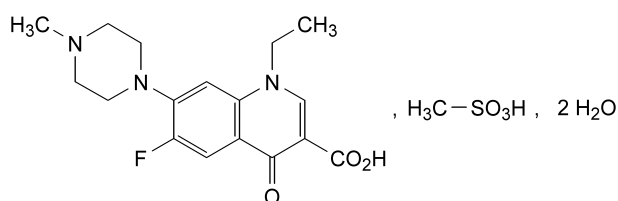
STORAGE

Store protected from light.

01/2005:1460
corrected

PEFLOXACIN MESILATE DIHYDRATE

Pefloxacin mesilas dihydricus



$C_{18}H_{24}FN_3O_6S \cdot 2H_2O$

M_r 465.5

DEFINITION

Pefloxacin mesilate dihydrate contains not less than 98.5 per cent and not more than the equivalent of 101.5 per cent of 1-ethyl-6-fluoro-7-(4-methylpiperazin-1-yl)-4-oxo-1,4-dihydroquinoline-3-carboxylic acid methanesulphonate, calculated with reference to the anhydrous substance.

PRODUCTION

The production method must be evaluated to determine the potential for formation of alkyl mesilates, which is particularly likely to occur if the reaction medium contains lower alcohols. Where necessary, the production method is validated to demonstrate that alkyl mesilates are not detectable in the final product.

CHARACTERS

A fine, white or almost white powder, freely soluble in water, slightly soluble in alcohol, very slightly soluble in methylene chloride.

IDENTIFICATION

- A. Dissolve separately 0.1 g of the substance to be examined and 0.1 g of *pefloxacin mesilate dihydrate CRS* in 10 ml of *water R*. Add 5 ml of 1 *M sodium hydroxide*. Adjust the pH of the solution to 7.4 ± 0.1 with *phosphoric acid R* and shake with 2 quantities, each of 30 ml, of *methylene chloride R*. Combine the organic layers and dry over *anhydrous sodium sulphate R*. Evaporate to dryness. Examine the residues by infrared absorption spectrophotometry (2.2.24), comparing the spectra obtained. Examine the residues as discs prepared using *potassium bromide R*.
- B. Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel plate R*.

Test solution. Dissolve 40 mg in *water R* and dilute to 1 ml with the same solvent.

Reference solution. Dissolve 60 mg of *methanesulphonic acid R* in *water R* and dilute to 10 ml with the same solvent.

Apply to the plate 10 µl of each solution. Develop over a path of 15 cm using a mixture of 5 volumes of *water R*, 10 volumes of *ammonia R*, 20 volumes of *butanol R* and 65 volumes of *acetone R*. Allow the plate to dry in air. Spray with a 0.4 g/l solution of *bromocresol purple R* in *alcohol* (50 per cent V/V) *R*, adjusted to pH 10 using 1 *M sodium hydroxide*. The spot in the chromatogram obtained with the test solution is similar in position, colour and size to the spot in the chromatogram obtained with the reference solution.

TESTS

Solution S. Dissolve 1.0 g in *carbon dioxide-free water R* and dilute to 10.0 ml with the same solvent.

Appearance of solution. Examined within 1 h after its preparation, solution S is not more opalescent than reference suspension II (2.2.1) and not more intensely coloured than intensity 3 of the range of reference solutions of the most appropriate colour (2.2.2, *Method II*).

pH (2.2.3). Dilute 1 ml of solution S to 10 ml with *carbon dioxide-free water R*. The pH of the solution is 3.5 to 4.5.

Related substances. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 20.0 mg of the substance to be examined in the mobile phase and dilute to 100.0 ml with the mobile phase.

Reference solution (a). Dissolve 5.0 mg of *pefloxacin impurity B CRS* in the mobile phase and dilute to 50.0 ml with the mobile phase. Dilute 1.0 ml of this solution to 100.0 ml with the mobile phase. In 2.0 ml of the solution obtained, dissolve the contents of a vial of *pefloxacin impurity C CRS*.

Reference solution (b). Dissolve 10.0 mg of *norfloxacin impurity A CRS* (pefloxacin impurity F) in the mobile phase and dilute to 100.0 ml with the mobile phase. Dilute 1.0 ml of this solution to 100.0 ml with the mobile phase.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.15 m long and 6 mm in internal diameter packed with *octadecylsilyl vinyl polymer for chromatography R* (5 µm),
- as mobile phase at a flow rate of 1 ml/min a mixture prepared as follows: 30 volumes of *acetonitrile R*, 70 volumes of a solution containing 2.70 g/l of *cetyltrimethylammonium bromide R* and 6.18 g/l of *boric acid R* (exactly adjusted to pH 8.30 with 1 M *sodium hydroxide*), and 0.2 volumes of *thiodiethylene glycol R*,
- as detector a spectrophotometer set at 258 nm and 273 nm.

Inject 20 µl of reference solution (a). Record the chromatogram at 273 nm. The test is not valid unless the resolution between the peaks corresponding to impurity B and impurity C is at least 1.5. Inject 20 µl of the test solution and 20 µl of reference solution (b). Record the chromatograms of the test solution at 258 nm and 273 nm for 4 times the retention time of pefloxacin (about 60 min). Record the chromatogram of reference solution (b) at 258 nm. When the chromatograms are recorded in the prescribed conditions, the relative retentions are indicated in the following table.

	Approximate relative retention	Correction factor
Impurity E	0.2	–
Impurity D	0.3	–
Impurity A	0.5	–
Impurity G	0.8	1.4
Pefloxacin	1	–
Impurity C	1.7	2.4
Impurity B	1.8	–
Impurity H	2.4	1.8
Impurity F	3.5	–

From the chromatogram obtained at 258 nm with the test solution, calculate the percentage content of impurity C, F, G and H using the area of the principal peak in the chromatogram obtained at 258 nm with reference solution (b) (external standardisation) taking into account the correction factors indicated in the table.

From the chromatogram obtained at 273 nm with the test solution, calculate the percentage content of impurity A, B, D and E and of any unknown impurity from the areas of the peaks in the chromatogram obtained with the test solution by the normalisation procedure, disregarding the peaks with an area less than 0.0005 times that of the principal peak in the chromatogram obtained at 273 nm with the test solution.

None of the impurities has a content of more than 0.5 per cent and not more than 3 impurities have a content between 0.2 per cent and 0.5 per cent. The sum of the contents of the impurities is not more than 1.0 per cent.

Heavy metals (2.4.8). 1.0 g complies with limit test E for heavy metals (10 ppm). Prepare the standard using 10.0 ml of *lead standard solution (1 ppm Pb) R*.

Water (2.5.12): 7.0 per cent to 8.5 per cent, determined on 50.0 mg by the semi-micro determination of water using a mixture of 10 volumes of *methanol R* and 50 volumes of *methylene chloride R*.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

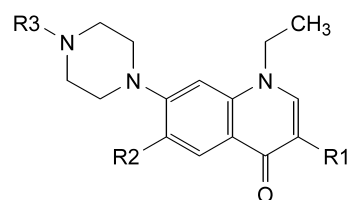
Dissolve 0.200 g in 15.0 ml of *anhydrous acetic acid R* and add 75.0 ml of *acetic anhydride R*. Titrate with 0.1 M *perchloric acid*, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *perchloric acid* is equivalent to 21.48 mg of C₁₈H₂₄FN₃O₆S.

STORAGE

Store in an airtight container, protected from light.

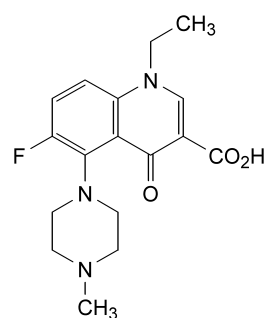
IMPURITIES



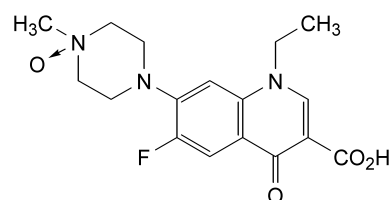
A. R1 = CO₂H, R2 = F, R3 = H: 1-ethyl-6-fluoro-4-oxo-7-(piperazin-1-yl)-1,4-dihydroquinoline-3-carboxylic acid (demethylated pefloxacin or norfloxacin),

B. R1 = CO₂H, R2 = Cl, R3 = CH₃: 6-chloro-1-ethyl-7-(4-methylpiperazin-1-yl)-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (chlorinated homologue of pefloxacin),

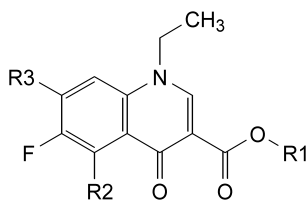
E. R1 = H, R2 = F, R3 = CH₃: 1-ethyl-6-fluoro-7-(4-methylpiperazin-1-yl)quinoline-4(1H)-one (decarboxylated pefloxacin),



C. 1-ethyl-6-fluoro-5-(4-methylpiperazin-1-yl)-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (isopefloxacin),



D. 4-(3-carboxy-1-ethyl-6-fluoro-4-oxo-1,4-dihydroquinolin-7-yl)-1-methylpiperazine 1-oxide (*N*-oxide of pefloxacin),

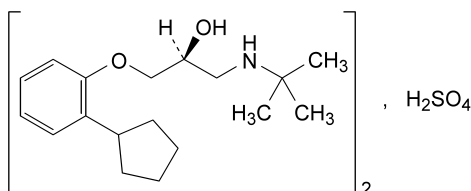


- F. R₁ = R₂ = H, R₃ = Cl: 7-chloro-1-ethyl-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (*N*-ethyl acid) (norfloxacin impurity A),
- G. R₁ = C₂H₅, R₂ = H, R₃ = Cl: ethyl 7-chloro-1-ethyl-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylate (*N*-ethyl ester),
- H. R₁ = R₃ = H, R₂ = Cl: 5-chloro-1-ethyl-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (iso-*N*-ethyl acid).

01/2005:1461

PENBUTOLOL SULPHATE

Penbutololi sulfas

C₃₆H₆₀N₂O₈SM_r 681

DEFINITION

Penbutolol sulphate contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of di[(2*S*)-1-(2-cyclopentylphenoxy)-3-[(1,1-dimethylethyl)amino]propan-2-ol] sulphate, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, crystalline powder, slightly soluble in water, soluble in methanol, practically insoluble in cyclohexane.

IDENTIFICATION

First identification: A, C, D.

Second identification: B, C, D.

- A. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *penbutolol sulphate CRS*.
- B. Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel F₂₅₄ plate R*.
Test solution. Dissolve 40 mg of the substance to be examined in 1 ml of *methanol R*.
Reference solution. Dissolve 40 mg of *penbutolol sulphate CRS* in 1 ml of *methanol R*.
 Apply to the plate 5 µl of each solution. Develop over a path of 15 cm using a mixture of 10 volumes of *glacial acetic acid R*, 20 volumes of *water R*, 35 volumes of *butanol R* and 35 volumes of *ethyl acetate R*. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. The principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with the reference solution.
- C. Dissolve 50 mg in a mixture of 5 ml of *water R* and 1 ml of 0.1 M *hydrochloric acid*. The solution gives reaction (a) of sulphates (2.3.1).

D. It complies with the test for specific optical rotation (see Tests).

TESTS

Solution S. Dissolve 1.00 g in *methanol R* and dilute to 20.0 ml with the same solvent.

Acidity or alkalinity. To 4 ml of solution S add 4 ml of *carbon dioxide-free water R*. Add 0.1 ml of *methyl red solution R* and 0.2 ml of 0.01 M *sodium hydroxide*. The solution is yellow. Add 0.4 ml of 0.01 M *hydrochloric acid*. The solution is red.

Specific optical rotation (2.2.7): −23 to −25 determined on solution S, calculated with reference to the dried substance.

Related substances. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 40.0 mg of the substance to be examined in a mixture of 40 volumes of mobile phase B and 60 volumes of mobile phase A and dilute to 10.0 ml with the same mixture of solvents.

Reference solution (a). Dissolve 4.0 mg of the substance to be examined and 1.0 mg of *penbutolol impurity A CRS* in 5.0 ml of a mixture of 40 volumes of mobile phase B and 60 volumes of mobile phase A.

Reference solution (b). Dilute 1.0 ml of the test solution to 200.0 ml with a mixture of 40 volumes of mobile phase B and 60 volumes of mobile phase A.

Reference solution (c). Dilute 1.0 ml of reference solution (b) to 10.0 ml with a mixture of 40 volumes of mobile phase B and 60 volumes of mobile phase A.

Reference solution (d). Dissolve 5.0 mg of *penbutolol impurity A CRS* in a mixture of 40 volumes of mobile phase B and 60 volumes of mobile phase A and dilute to 50.0 ml with the same mixture of solvents. Dilute 2.0 ml to 10.0 ml with the same mixture of solvents.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4.6 mm in internal diameter, packed with *octadecylsilyl silica gel for chromatography R* (5 µm),
- as mobile phase at a flow rate of 1.0 ml/min:
Mobile phase A. Mix 39 volumes of *acetonitrile for chromatography R* and 61 volumes of *methanol R*,
Mobile phase B. Dissolve 11 g of *sodium heptanesulphonate R* in 1000 ml of *water R*, add 5.0 ml of *triethylamine R* and adjust to pH 2.7 with *phosphoric acid R*,

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)	Comment
0 - 15	60	40	isocratic
15 - 35	60 → 80	40 → 20	linear gradient
35 - 36	80 → 60	20 → 40	linear gradient

— as detector a spectrophotometer set at 270 nm.

Inject 10 µl of reference solution (b) and adjust the sensitivity of the system so that the height of the second peak (penbutolol) is at least 20 per cent of the full scale of the recorder. The resolution between the 2 principal peaks must be at least 3.0.

Inject 10 µl of each of the other solutions. In the chromatogram obtained with the test solution, the area of any peak corresponding to impurity A is not greater than the area of the principal peak in the chromatogram obtained with reference solution (d) (0.5 per cent), the area of any peak, apart from the principal peak and that of impurity A, is not greater than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per

cent) and the sum of the areas of all the peaks, apart from the principal peak and the peak corresponding to impurity A, is not greater than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (1 per cent). Disregard any peak with an area less than that of the principal peak in the chromatogram obtained with reference solution (c).

Heavy metals (2.4.8). 1.0 g complies with limit test F for heavy metals (10 ppm). Prepare the reference solution using 1 ml of *lead standard solution* (10 ppm Pb) R.

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 100–105 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.500 g in 40 ml of *anhydrous acetic acid* R. Titrate with 0.1 M *perchloric acid* determining the end-point potentiometrically (2.2.20).

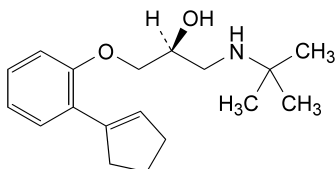
1 ml of 0.1 M *perchloric acid* is equivalent to 68.10 mg of C₃₆H₆₀N₂O₈S.

STORAGE

Store protected from light.

IMPURITIES

Specified impurities: A.

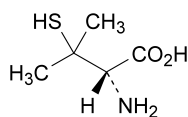


A. (2S)-1-[2-(cyclopent-1-enyl)phenoxy]-3-[(1,1-dimethylethyl)amino]propan-2-ol.

01/2005:0566

PENICILLAMINE

Penicillaminum



C₅H₁₁NO₂S

M_r 149.2

DEFINITION

Penicillamine contains not less than 98.0 per cent and not more than the equivalent of 101.0 per cent of (2S)-2-amino-3-methyl-3-sulphanylbutoanoic acid, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, crystalline powder, freely soluble in water, slightly soluble in alcohol.

IDENTIFICATION

First identification: A, B, D.

Second identification: A, C, D.

A. Dissolve 0.5 g in a mixture of 0.5 ml of *hydrochloric acid* R and 4 ml of warm *acetone* R, cool in iced water and initiate crystallisation by scratching the wall of the tube with a glass rod. A white precipitate is formed.

Filter with the aid of vacuum, wash with *acetone* R and dry with suction. A 10 g/l solution of the precipitate is dextrorotatory.

B. Examine the chromatograms obtained in the test for penicillamine disulphide. The principal peak in the chromatogram obtained with the test solution is similar in position and approximate size to the principal peak in the chromatogram obtained with reference solution (a).

C. Examine by thin-layer chromatography (2.2.27), using *silica gel G* R as the coating substance.

Test solution. Dissolve 10 mg of the substance to be examined in 4 ml of *water* R.

Reference solution. Dissolve 10 mg of *penicillamine CRS* in 4 ml of *water* R.

Apply separately to the plate 2 µl of each solution.

Develop over a path of 10 cm using a mixture of 18 volumes of *glacial acetic acid* R, 18 volumes of *water* R and 72 volumes of *butanol* R. Dry the plate at 100 °C to 105 °C for 5 min to 10 min and expose to iodine vapour for 5 min to 10 min. The principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

D. Dissolve 40 mg in 4 ml of *water* R and add 2 ml of *phosphotungstic acid solution* R. Allow to stand for 5 min. A blue colour develops.

TESTS

Solution S. Dissolve 2.5 g in *carbon dioxide-free water* R and dilute to 25 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and not more intensely coloured than intensity 6 of the range of reference solutions of the most appropriate colour (2.2.2, *Method II*).

pH (2.2.3). Dilute 1 ml of solution S to 10 ml with *carbon dioxide-free water* R. The pH of this solution is 4.5 to 5.5.

Specific optical rotation (2.2.7). Dissolve 0.500 g in 1 M *sodium hydroxide* and dilute to 10.0 ml with the same solvent. The specific optical rotation is –61.0 to –65.0, calculated with reference to the dried substance.

Penicillamine disulphide. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 40 mg of the substance to be examined in the mobile phase and dilute to 10.0 ml with the mobile phase.

Reference solution (a). Dissolve 40 mg of *penicillamine CRS* in the mobile phase and dilute to 10.0 ml with the mobile phase.

Reference solution (b). Dissolve 20 mg of *penicillamine disulphide CRS* in the mobile phase and dilute to 50.0 ml with the mobile phase. Dilute 1.0 ml of this solution to 10.0 ml with the mobile phase.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 5 mm in internal diameter packed with *octylsilyl silica gel for chromatography* R (10 µm),
- as mobile phase at a flow rate of 2.0 ml/min a solution containing 0.1 g/l of *sodium edetate* R and 2 g/l of *methanesulphonic acid* R,
- as detector a spectrophotometer set at 220 nm.

In the chromatogram obtained with the test solution the area of any peak due to penicillamine disulphide is not greater than the area of the corresponding peak in the chromatogram obtained with reference solution (b) (1 per cent).

Ultraviolet-absorbing substances. Dissolve 0.100 g in *water R* and dilute to 50.0 ml with the same solvent. The absorbance (2.2.25) of the solution at 268 nm is not greater than 0.07 (about 0.5 per cent of penilloic acid).

Heavy metals (2.4.8). 12 ml of solution S complies with limit test A for heavy metals (20 ppm). Prepare the standard using *lead standard solution* (2 ppm Pb) *R*.

Mercury. Not more than 10 ppm of Hg, determined by atomic absorption spectrometry (2.2.23, *Method I*).

Test solution. To 1.00 g of the substance to be examined add 10 ml of *water R* and 0.15 ml of *perchloric acid R* and swirl until dissolution is complete. Add 1.0 ml of a 10 g/l solution of *ammonium pyrrolidinedithiocarbamate R* which has been washed immediately before use three times, each time with an equal volume of *methyl isobutyl ketone R*. Mix and add 2.0 ml of *methyl isobutyl ketone R* and shake for 1 min. Dilute to 25.0 ml with *water R* and allow the layers to separate; use the methyl isobutyl ketone layer.

Reference solutions. Dissolve a quantity of *mercuric oxide R* equivalent to 0.108 g of HgO in the smallest necessary volume of *dilute hydrochloric acid R* and dilute to 1000.0 ml with *water R* (100 ppm Hg). Prepare the reference solutions in the same manner as the test solution but using instead of the substance to be examined suitable volumes of the solution containing 100 ppm of Hg.

Measure the absorbance at 254 nm using a mercury hollow-cathode lamp as source of radiation and an air-acetylene flame. Set the zero of the instrument using a methyl isobutyl ketone layer obtained as described for the test solution but omitting the substance to be examined.

Penicillin. Carry out all the operations in a penicillin-free atmosphere and with equipment reserved for this test. Sterilise the equipment at 180 °C for 3 h and the buffer solutions at 121 °C for 20 min before use.

Test solution (a). Dissolve 1.000 g of the substance to be examined in 8 ml of *buffer solution pH 2.5 R* and add 8 ml of *ether R*. Shake vigorously for 1 min. Repeat the extraction and combine the ether layers. Add 8 ml of *buffer solution pH 2.5 R*. Shake for 1 min, allow to settle and quantitatively separate the upper layer, taking care to eliminate the aqueous phase completely (*penicillin is unstable at pH 2.5; carry out operations at this pH within 6 min to 7 min*). Add 8 ml of *phosphate buffer solution pH 6.0 R2*; shake for 5 min, allow to settle, separate the aqueous layer and check that the pH is 6.0.

Test solution (b). To 2 ml of test solution (a) add 20 µl of *penicillinase solution R* and incubate at 37 °C for 1 h.

Reference solution (a). Dissolve 5 mg of *benzylpenicillin sodium R* in 500 ml of *phosphate buffer solution pH 6.0 R2*. Dilute 0.25 ml of this solution to 200.0 ml with *buffer solution pH 2.5 R*. Carry out the extraction using 8 ml of this solution as described for test solution (a).

Reference solution (b). To 2 ml of reference solution (a), add 20 µl of *penicillinase solution R* and incubate at 37 °C for 1 h.

Reference solution (c). Prepare the solution as described for test solution (a) but omitting the substance to be examined (blank).

Liquefy a suitable nutrient medium such as that described below and inoculate it at a suitable temperature with a culture of *Micrococcus flavus* (ATCC 9341) to give 5×10^4

micro-organisms per millilitre or a different quantity if necessary to obtain the required sensitivity and formation of clearly defined inhibition zones of suitable diameter. Immediately pour the inoculated medium into five Petri dishes 10 cm in diameter to give uniform layers 2 mm to 5 mm deep. The medium may alternatively consist of two layers, only the upper layer being inoculated. Store the dishes so that no appreciable growth or death of the micro-organisms occurs before use and so that the surface of the medium is dry at the time of use. In each dish, place five stainless steel hollow cylinders 6 mm in diameter on the surface of the agar evenly spaced on a circle with a radius of about 25 mm and concentric with the dish. For each dish, place in separate cylinders 0.15 ml of test solutions (a) and (b) and reference solutions (a), (b) and (c). Maintain at 30 °C for at least 24 h. Measure the diameters of the inhibition zones to at least 0.1 mm. The test is valid if reference solution (a) gives a clear inhibition zone and if reference solutions (b) and (c) give no inhibition zone. If test solution (a) gives an inhibition zone, this is caused by penicillin if test solution (b) gives no inhibition zone. If this is so, the average diameter of the inhibition zones given by test solution (a) for the five Petri dishes is less than the average diameter of the inhibition zones given by reference solution (a) (0.1 ppm).

Nutrient medium (pH 6.0)

Peptone	5 g
Yeast extract	1.5 g
Meat extract	1.5 g
Sodium chloride	3.5 g
Agar	15 g
<i>Distilled water R</i>	1000 ml

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying over *diphosphorus pentoxide R* at 60 °C at a pressure not exceeding 670 Pa.

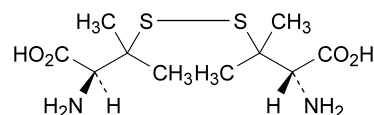
Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

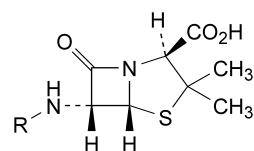
Dissolve 0.1000 g in 30 ml of *anhydrous acetic acid R*. Titrate with 0.1 M *perchloric acid*, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *perchloric acid* is equivalent to 14.92 mg of $C_5H_{11}NO_2S$.

IMPURITIES



A. 3,3'-(disulphanediyl)bis[(2S)-2-amino-3-methylbutanoic] acid (penicillamine disulphide),

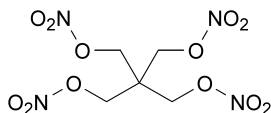


B. penicillin.

01/2005:1355

PENTAERYTHRITYL TETRANITRATE, DILUTED

Pentaerythrityli tetranitras dilutus

 $C_5H_8N_4O_{12}$ M_r 316.1

DEFINITION

Diluted pentaerythrityl tetranitrate is a dry mixture of pentaerythrityl tetranitrate and *Lactose monohydrate* (0187) or *Mannitol* (0559). It contains not less than 95.0 per cent *m/m* and not more than 105.0 per cent *m/m* of the declared content of 2,2-bis(hydroxymethyl)propane-1,3-diol tetranitrate.

CHARACTERS

Undiluted pentaerythrityl tetranitrate is a white or slightly yellowish powder, practically insoluble in water, soluble in acetone, slightly soluble in alcohol.

The solubility of the diluted product depends on the diluent and its concentration.

IDENTIFICATION

First identification: A, B, D.

Second identification: A, C, D.

- The melting point (2.2.14) of the residue obtained with the substance to be examined in identification test B is 138 °C to 142 °C.
- Shake a quantity of the substance to be examined and a quantity of *diluted pentaerythrityl tetranitrate CRS*, each corresponding to 25 mg of pentaerythrityl tetranitrate with 10 ml of *acetone R* for 5 min. Filter, evaporate to dryness at a temperature below 40 °C and dry the residue over *diphosphorus pentoxide R* at a pressure of 0.7 kPa for 16 h. Examine the residue obtained with the substance to be examined by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum of the residue obtained with *diluted pentaerythrityl tetranitrate CRS*. Examine the residues prepared as discs.
- Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel G plate R*.

Test solution. Shake a quantity of the substance to be examined corresponding to 10 mg of pentaerythrityl tetranitrate with 10 ml of *alcohol R* for 5 min and filter.

Reference solution. Shake a quantity of *diluted pentaerythrityl tetranitrate CRS* corresponding to 10 mg of pentaerythrityl tetranitrate with 10 ml of *alcohol R* for 5 min and filter.

Apply separately to the plate 10 µl of each solution. Develop over a path of 15 cm using a mixture of 20 volumes of *ethyl acetate R* and 80 volumes of *toluene R*. Dry the plate in a current of air. Spray with freshly prepared *potassium iodide and starch solution R*. Expose to ultraviolet light at 254 nm for 15 min. Examine in daylight. The principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

- Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel G plate R*.

Test solution. Shake a quantity of the substance to be examined corresponding to 0.10 g of lactose or mannitol with 10 ml of *water R*. Filter if necessary.

Reference solution (a) Dissolve 0.10 g of *lactose R* in *water R* and dilute to 10 ml with the same solvent.

Reference solution (b). Dissolve 0.10 g of *mannitol R* in *water R* and dilute to 10 ml with the same solvent.

Reference solution (c). Mix equal volumes of reference solutions (a) and (b).

Apply separately to the plate 1 µl of each solution and thoroughly dry the starting points. Develop over a path of 15 cm using a mixture of 10 volumes of *water R*, 15 volumes of *methanol R*, 25 volumes of *anhydrous acetic acid R* and 50 volumes of *ethylene chloride R*, measured accurately since a slight excess of water produces cloudiness. Dry the plate in a current of warm air. Repeat immediately the development after renewing the mobile phase. Dry the plate in a current of warm air. Spray with *4-aminobenzoic acid solution R*. Dry the plate in a current of cold air until the acetone is removed. Heat the plate at 100 °C for 15 min. Allow to cool and spray with a 2 g/l solution of *sodium periodate R*. Dry the plate in a current of cold air. Heat the plate at 100 °C for 15 min. The principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a) for lactose or to the principal spot in the chromatogram obtained with reference solution (b) for mannitol. The test is not valid unless the chromatogram obtained with reference solution (c) shows two clearly separated spots.

TESTS

Inorganic nitrates. Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel plate R*.

Test solution. Shake a quantity of the substance to be examined corresponding to 0.10 g of pentaerythrityl tetranitrate with 5 ml of *alcohol R* and filter.

Reference solution. Dissolve 10 mg of *potassium nitrate R* in 1 ml of *water R* and dilute to 100 ml with *alcohol R*.

Apply separately to the plate 10 µl of each solution. Develop over a path of 15 cm using a mixture of 15 volumes of *glacial acetic acid R*, 30 volumes of *acetone R* and 60 volumes of *toluene R*. Dry the plate thoroughly in a current of air until the acetic acid is completely removed. Spray copiously with freshly prepared *potassium iodide and starch solution R*. Expose the plate to ultraviolet light at 254 nm for 15 min. Examine in daylight. Any spot corresponding to nitrate ion in the chromatogram obtained with the test solution is not more intense than the spot in the chromatogram obtained with the reference solution (0.5 per cent, calculated as potassium nitrate).

Related substances. Examine by liquid chromatography (2.2.29) as described under Assay.

Adjust the sensitivity of the system so that the height of the principal peak in the chromatogram obtained with reference solution (c) is at least 20 per cent of the full scale of the recorder.

The test is not valid unless in the chromatogram obtained with reference solution (e), the resolution between the peaks corresponding to glyceryl trinitrate and to pentaerythrityl tetranitrate is at least 2.0.

Inject 20 µl of test solution (a) and 20 µl of reference solution (c) and record the chromatogram of test solution (a) for at least five times the retention time of pentaerythrityl tetranitrate. In the chromatogram obtained with test solution (a); the area of any peak, apart from the principal

peak is not greater than the area of the principal peak in the chromatogram obtained with reference solution (c) (0.3 per cent); the sum of the areas of all the peaks, apart from the principal peak, is not greater than twice the area of the principal peak in the chromatogram obtained with reference solution (c) (0.6 per cent). Disregard any peak with an area less than 0.2 times that of the principal peak in the chromatogram obtained with reference solution (c).

ASSAY

Examine by liquid chromatography (2.2.29).

Test solution (a). Sonicate for 15 min a quantity of the substance to be examined corresponding to 25.0 mg of pentaerythrityl tetranitrate in 20 ml of *methanol R* and dilute to 25.0 ml with the mobile phase. Filter through a suitable membrane filter.

Test solution (b). Dilute 1.0 ml of test solution (a) to 10.0 ml with the mobile phase.

Reference solution (a). Sonicate for 15 min a quantity of *diluted pentaerythrityl tetranitrate CRS* corresponding to 25.0 mg of pentaerythrityl tetranitrate in 20 ml of *methanol R* and dilute to 25.0 ml with the mobile phase. Filter through a suitable membrane filter.

Reference solution (b). Dilute 1.0 ml of reference solution (a) to 10.0 ml with the mobile phase.

Reference solution (c). Dilute 0.3 ml of reference solution (b) to 10.0 ml with the mobile phase.

Reference solution (d). Sonicate for 15 min a quantity of *glyceryl trinitrate solution CRS* corresponding to 20.0 mg of glyceryl trinitrate in 20 ml of *methanol R* and dilute to 25.0 ml with the mobile phase. Filter through a suitable membrane filter. Dilute 1.0 ml of the filtrate to 10.0 ml with the mobile phase.

Reference solution (e). To 1 ml of reference solution (b), add 1 ml of reference solution (d) and dilute to 10 ml with the mobile phase.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4.6 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (10 µm),
- as mobile phase at a flow rate of 2 ml/min a mixture of 40 volumes of *water R* and 60 volumes of *methanol R*,
- as detector a spectrophotometer set at 230 nm.

When the chromatograms are recorded in the prescribed conditions the retention time of pentaerythrityl tetranitrate is about 8 min. Inject 20 µl of reference solution (b). Adjust the sensitivity of the system so that the height of the principal peak in the chromatogram obtained is at least 50 per cent of the full scale of the recorder. Inject reference solution (b) six times. The assay is not valid unless the relative standard deviation for the area of the principal peak is at most 2.0 per cent. Inject test solution (b) and reference solution (b) alternately.

STORAGE

Store protected from light and heat.

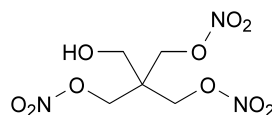
LABELLING

The label states:

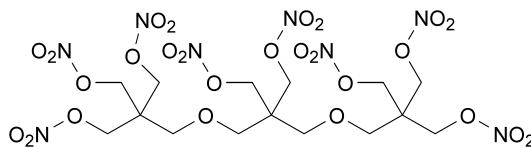
- the content of pentaerythrityl tetranitrate as a percentage,
- the diluent used.

IMPURITIES

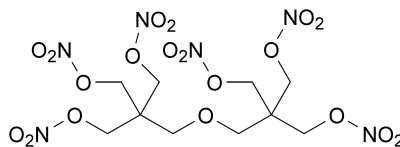
A. inorganic nitrates,



B. pentaerythrityl trinitrate,



C. tripentaerythrityl octanitrate,

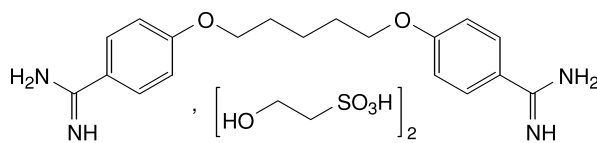


D. dipentaerythrityl hexanitrate.

01/2005:1137

PENTAMIDINE DISETIONATE

Pentamidini diisetonas



$C_{23}H_{36}N_4O_{10}S_2$

M_r 592.7

DEFINITION

4,4'-[Pentane-1,5-diylbis(oxy)]dibenzamidine di(2-hydroxyethanesulphonate).

Content: 98.5 per cent to 101.5 per cent (dried substance).

CHARACTERS

Appearance: white or almost white powder or colourless crystals, hygroscopic.

Solubility: freely soluble in water, sparingly soluble in alcohol, practically insoluble in methylene chloride.

IDENTIFICATION

A. Infrared absorption spectrophotometry (2.2.24).

Preparation: discs.

Comparison: *pentamidine diisetonate CRS*.

B. Dissolve about 40 mg in 5 ml of *water R* and add dropwise with shaking 1 ml of a 10 g/l solution of *sodium chloride R*. Allow to stand for 5 min. The mixture remains clear.

C. Treat 0.15 g by the oxygen-flask method (2.5.10). Use 10 ml of *dilute hydrogen peroxide solution R* to absorb the combustion products. The solution gives reaction (a) of sulphates (2.3.1).

TESTS

Appearance of solution. The solution is not more opalescent than reference suspension II (2.2.1) and not more intensely coloured than intensity 6 of the range of reference solutions of the most appropriate colour (2.2.2, *Method II*).

Dissolve 2.0 g in *water R* and dilute to 20 ml with the same solvent.

pH (2.2.3): 4.5 to 6.5.

Dissolve 0.5 g in *carbon dioxide-free water R* and dilute to 10 ml with the same solvent.

Related substances. Liquid chromatography (2.2.29).

Test solution. Dissolve 0.100 g of the substance to be examined in the mobile phase and dilute to 100.0 ml with the mobile phase.

Reference solution (a). Dilute 2.0 ml of the test solution to 100.0 ml with the mobile phase. Dilute 1.0 ml of this solution to 10.0 ml with the mobile phase.

Reference solution (b). To 0.1 g in a conical flask, add 40 ml of *water R* and glass beads. Adjust to pH 10.5 with *dilute sodium hydroxide solution R* and boil under reflux for 20 min. Cool and dilute to 50 ml with *water R*. Dilute 1 ml of the solution to 50 ml with the mobile phase.

Column:

- size: $l = 0.25$ m, $\varnothing = 4.6$ mm,
- stationary phase: octadecylsilyl silica gel for chromatography *R* (5 μ m).

Mobile phase: mix 65 volumes of *methanol R* and 35 volumes of a 30 g/l solution of *ammonium acetate R* previously adjusted to pH 7.5 using *triethylamine R*.

Flow rate: 1 ml/min.

Detection: spectrophotometer at 265 nm.

Injection: 10 μ l.

Run time: 3.5 times the retention time of pentamidine.

System suitability: reference solution (b):

- the chromatogram obtained shows 2 principal peaks,
- resolution: minimum of 2.0 between the 2 principal peaks.

Limits:

- **any impurity:** not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent),
- **total:** not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.4 per cent),
- **disregard limit:** 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.02 per cent).

Heavy metals (2.4.8): maximum 20 ppm.

1.0 g complies with limit test C. Prepare the standard using 2 ml of *lead standard solution* (10 ppm *Pb*) *R*.

Loss on drying (2.2.32): maximum 4.0 per cent, determined on 1.000 g by drying in an oven at 100–105 °C.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

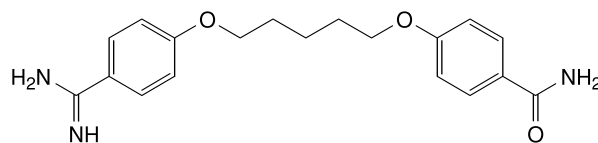
Dissolve 0.250 g in 50 ml of *dimethylformamide R*. Add 0.25 ml of *thymol blue solution R*. Titrate with 0.1 *M tetrabutylammonium hydroxide*, under a current of *nitrogen R*, until the colour of the indicator changes to blue. Carry out a blank titration.

1 ml of 0.1 *M tetrabutylammonium hydroxide* is equivalent to 29.63 mg of $C_{23}H_{36}N_4O_{10}S_2$.

STORAGE

In an airtight container.

IMPURITIES

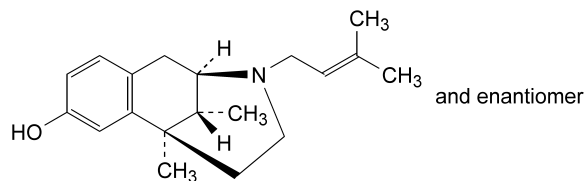


A. 4-[[5-(4-aminidinophenoxy)pentyl]oxy]benzenecarboxamide.

01/2005:1462

PENTAZOCINE

Pentazocinum



$C_{19}H_{27}NO$

M_r 285.4

DEFINITION

Pentazocine contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of (2*RS*,6*RS*,11*RS*)-6,11-dimethyl-3-(3-methylbut-2-enyl)-1,2,3,4,5,6-hexahydro-2,6-methano-3-benzazocin-8-ol, calculated with reference to the dried substance.

CHARACTERS

A white or almost white powder, practically insoluble in water, freely soluble in methylene chloride and soluble in alcohol.

It shows polymorphism.

IDENTIFICATION

Examine by infrared absorption spectrophotometry (2.2.24), comparing with the *Ph. Eur. reference spectrum for pentazocine (form A)*.

TESTS

Absorbance (2.2.25). Dissolve 0.100 g in a mixture of 20 ml of *water R* and 1 ml of 1 *M hydrochloric acid*, and dilute to 100.0 ml with *water R*. To 10.0 ml add 1 ml of 1 *M hydrochloric acid* and dilute to 100.0 ml with *water R*. The specific absorbance at the maximum at 278 nm is 0.67 to 0.71, calculated with reference to the dried substance.

Related substances. Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel F₂₅₄ plate R*.

Test solution. Dissolve 0.20 g of the substance to be examined in *methylene chloride R* and dilute to 10 ml with the same solvent.

Reference solution (a). Dilute 1 ml of the test solution to 100 ml with *methylene chloride R*.

Reference solution (b). Dilute 5 ml of reference solution (a) to 10 ml with *methylene chloride R*.

Reference solution (c). Dilute 5 ml of reference solution (a) to 20 ml with *methylene chloride R*.

Apply to the plate 10 μ l of each solution. Develop over a path corresponding to two thirds of the plate height using a mixture of 3 volumes of *isopropylamine R*, 3 volumes of *methanol R* and 94 volumes of *methylene chloride R*. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. Heat the plate at 100 °C to 105 °C for 15 min, allow to cool, expose to iodine vapour and re-examine under ultraviolet light at 254 nm. By each method of

visualisation: any spot in the chromatogram obtained with the test solution, apart from the principal spot, is not more intense than the spot obtained with reference solution (a) (1 per cent); not more than one such spot is more intense than the spot in the chromatogram obtained with reference solution (b) (0.5 per cent) and not more than four such spots are more intense than the spot in the chromatogram obtained with reference solution (c) (0.25 per cent).

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying at 60 °C at a pressure not exceeding 0.7 kPa for 4 h.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.200 g in 50 ml of *anhydrous acetic acid R*. Titrate with 0.1 M *perchloric acid*, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *perchloric acid* is equivalent to 28.54 mg of $C_{19}H_{27}NO$.

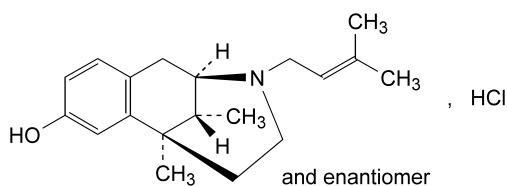
STORAGE

Store protected from light.

01/2005:1463

PENTAZOCINE HYDROCHLORIDE

Pentazocini hydrochloridum


 $C_{19}H_{28}ClNO$
 M_r 321.9

DEFINITION

Pentazocine hydrochloride contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of (2*RS*,6*RS*,11*RS*)-6,11-dimethyl-3-(3-methylbut-2-enyl)-1,2,3,4,5,6-hexahydro-2,6-methano-3-benzazocin-8-ol hydrochloride, calculated with reference to the dried substance.

CHARACTERS

A white or almost white powder, sparingly soluble in water, soluble in alcohol and sparingly soluble in methylene chloride.

It shows polymorphism.

IDENTIFICATION

- Examine by infrared absorption spectrophotometry (2.2.24), comparing with the *Ph. Eur. reference spectrum of pentazocine hydrochloride*.
- It gives reaction (a) of chlorides (2.3.1).

TESTS

pH (2.2.3). Dissolve 0.1 g in 10 ml of *carbon dioxide-free water R*. The pH of the solution is 4.0 to 6.0.

Absorbance (2.2.25). Dissolve 0.100 g in a mixture of 20 ml of *water R* and 1 ml of 1 M *hydrochloric acid*, and dilute to 100.0 ml with *water R*. To 10.0 ml add 1 ml of 1 M

hydrochloric acid and dilute to 100.0 ml with *water R*. The specific absorbance at the maximum at 278 nm is 0.59 to 0.63, calculated with reference to the dried substance.

Related substances. Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel F₂₅₄ plate R*.

Test solution. Dissolve 0.20 g in 3 ml of *methanol R* and dilute to 10 ml with *methylene chloride R*.

Reference solution (a). Dilute 1 ml of the test solution to 100 ml with *methylene chloride R*.

Reference solution (b). Dilute 5 ml of reference solution (a) to 10 ml with *methylene chloride R*.

Reference solution (c). Dilute 5 ml of reference solution (a) to 20 ml with *methylene chloride R*.

Apply to the plate 10 µl of each solution. Develop over a path corresponding to two-thirds of the plate height using a mixture of 3 volumes of *isopropylamine R*, 3 volumes of *methanol R* and 94 volumes of *methylene chloride R*. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. Heat the plate at 100 °C to 105 °C for 15 min, allow to cool, expose to iodine vapour and re-examine under ultraviolet light at 254 nm. By each method of visualisation: any spot in the chromatogram obtained with the test solution, apart from the principal spot, is not more intense than the spot obtained with reference solution (a) (1 per cent); not more than one such spot is more intense than the spot in the chromatogram obtained with reference solution (b) (0.5 per cent); and not more than four such spots are more intense than the spot in the chromatogram obtained with reference solution (c) (0.25 per cent).

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying at 60 °C at a pressure not exceeding 0.7 kPa for 4 h.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.250 g in 50 ml of *alcohol R*. Add 5 ml of 0.01 M *hydrochloric acid*. Carry out a potentiometric titration (2.2.20), using 0.1 M *sodium hydroxide*. Read the volume added between the two points of inflection.

1 ml of 0.1 M *sodium hydroxide* is equivalent to 32.19 mg of $C_{19}H_{28}ClNO$.

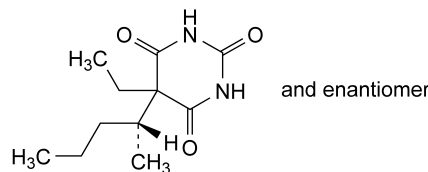
STORAGE

Store protected from light.

01/2005:0200

PENTOBARBITAL

Pentobarbitalum


 $C_{11}H_{18}N_2O_3$
 M_r 226.3

DEFINITION

Pentobarbital contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of 5-ethyl-5-[(1*RS*)-1-methylbutyl]pyrimidine-2,4,6(1*H*,3*H*,5*H*)-trione, calculated with reference to the dried substance.

CHARACTERS

A white, crystalline powder or colourless crystals, very slightly soluble in water, freely soluble in ethanol. It forms water-soluble compounds with alkali hydroxides and carbonates and with ammonia.

IDENTIFICATION

A. Determine the melting point (2.2.14) of the substance to be examined. Mix equal parts of the substance to be examined and *pentobarbital CRS* and determine the melting point of the mixture. The difference between the melting points (which are about 133 °C) is not greater than 2 °C.

B. Examine by thin-layer chromatography (2.2.27), using *silica gel GF₂₅₄ R* as the coating substance.

Test solution. Dissolve 0.1 g of the substance to be examined in *alcohol R* and dilute to 100 ml with the same solvent.

Reference solution. Dissolve 0.1 g of *pentobarbital CRS* in *alcohol R* and dilute to 100 ml with the same solvent.

Apply to the plate 10 µl of each solution. Develop over a path of 18 cm using the lower layer of a mixture of 5 volumes of *concentrated ammonia R*, 15 volumes of *alcohol R* and 80 volumes of *chloroform R*. Examine immediately in ultraviolet light at 254 nm. The principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with the reference solution.

C. To about 10 mg add about 10 mg of *vanillin R* and 2 ml of *sulphuric acid R*. Mix and heat on a water-bath for 2 min. A reddish-brown colour develops. Cool and add cautiously 5 ml of *ethanol R*. The colour becomes violet and then blue.

TESTS

Appearance of solution. Dissolve 1.0 g in a mixture of 4 ml of *dilute sodium hydroxide solution R* and 6 ml of *water R*. The solution is clear (2.2.1) and not more intensely coloured than reference solution Y₆ (2.2.2, *Method II*).

Acidity. Boil 1.0 g with 50 ml of *water R* for 2 min, allow to cool and filter. To 10 ml of the filtrate add 0.15 ml of *methyl red solution R*. The solution is orange-yellow. Not more than 0.1 ml of 0.1 M *sodium hydroxide* is required to produce a pure yellow colour.

Related substances. Examine by thin-layer chromatography (2.2.27), using *silica gel GF₂₅₄ R* as the coating substance.

Test solution. Dissolve 1.0 g of the substance to be examined in *alcohol R* and dilute to 100 ml with the same solvent.

Reference solution. Dilute 0.5 ml of the test solution to 100 ml with *alcohol R*.

Apply to the plate 20 µl of each solution. Develop over a path of 15 cm using the lower layer of a mixture of 5 volumes of *concentrated ammonia R*, 15 volumes of *alcohol R* and 80 volumes of *chloroform R*. Examine immediately in ultraviolet light at 254 nm. Spray with *diphenylcarbazone mercuric reagent R*. Allow the plate to dry in air and spray with freshly prepared *alcoholic potassium hydroxide solution R* diluted 1 in 5 with *aldehyde-free alcohol R*. Heat at 100 °C to 105 °C for 5 min and examine immediately. When examined in ultraviolet light and after spraying, any spot in the chromatogram obtained with the test solution, apart from the principal spot, is not more intense than the spot in the chromatogram obtained with the reference solution (0.5 per cent).

Isomer. Dissolve 0.3 g in 5 ml of a 50 g/l solution of *anhydrous sodium carbonate R*, heating slightly if necessary. Add a solution of 0.3 g of *nitrobenzyl chloride R* in 10 ml of *alcohol R* and heat under a reflux condenser for 30 min. Cool to 25 °C, filter and wash the precipitate with five quantities, each of 5 ml, of *water R*. In a small flask, heat the precipitate with 25 ml of *alcohol R* under a reflux condenser until dissolved (about 10 min). Cool to 25 °C, if necessary scratching the wall of the flask with a glass rod to induce crystallisation, and filter. The precipitate, washed with two quantities, each of 5 ml, of *water R* and dried at 100 °C to 105 °C for 30 min, melts (2.2.14) at 136 °C to 148 °C.

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

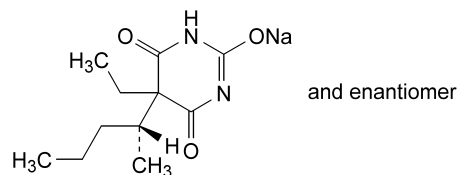
Dissolve 0.100 g in 5 ml of *pyridine R*. Add 0.5 ml of *thymolphthalein solution R* and 10 ml of *silver nitrate solution in pyridine R*. Titrate with 0.1 M *ethanolic sodium hydroxide* until a pure blue colour is obtained. Carry out a blank titration.

1 ml of 0.1 M *ethanolic sodium hydroxide* is equivalent to 11.31 mg of C₁₁H₁₈N₂O₃.

01/2005:0419

PENTOBARBITAL SODIUM

Pentobarbitalum natricum

C₁₁H₁₇N₂NaO₃M_r 248.3

DEFINITION

Pentobarbital sodium contains not less than 99.0 per cent and not more than the equivalent of 101.5 per cent of the sodium derivative of 5-ethyl-5-[(1*RS*)-1-methylbutyl]pyrimidine-2,4,6-(1*H*,3*H*,5*H*)-trione, calculated with reference to the dried substance.

CHARACTERS

A white, crystalline powder, hygroscopic, very soluble in water.

IDENTIFICATION

A. Dissolve 1 g in 10 ml of *water R* and add 5 ml of *dilute acetic acid R*. A white, crystalline precipitate is formed. Filter, wash the precipitate with *water R* and dry at 100 °C to 105 °C. Determine the melting point (2.2.14) of the precipitate. Mix equal parts of the precipitate and *pentobarbital CRS* and determine the melting point of the mixture. The difference between the melting points (which are about 131 °C) is not greater than 2 °C.

B. Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel GF₂₅₄ plate R*.

Test solution. Dissolve 25 mg of the precipitate obtained in identification test A in *alcohol R* and dilute to 25 ml with the same solvent.

Reference solution. Dissolve 25 mg of *pentobarbital CRS* in *alcohol R* and dilute to 25 ml with the same solvent.

Apply to the plate 10 µl of each solution. Develop over a path of 18 cm using the lower layer from a mixture of 5 volumes of *concentrated ammonia R*, 15 volumes of *alcohol R* and 80 volumes of *chloroform R*. Examine immediately in ultraviolet light at 254 nm. The principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with the reference solution.

C. To about 10 mg add about 10 mg of *vanillin R* and 2 ml of *sulphuric acid R*. Mix and heat on a water-bath for 2 min. A reddish-brown colour develops. Cool and add cautiously 5 ml of *ethanol R*. The colour becomes violet and then blue.

D. Ignite 1 g. The residue gives reaction (a) of sodium (2.3.1).

TESTS

pH (2.2.3). Dissolve 1.0 g in *carbon dioxide-free water R* and dilute to 10 ml with the same solvent. The pH measured immediately after preparation of the solution is 9.6 to 11.0.

Related substances. Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel GF₂₅₄ plate R*.

Test solution. Dissolve 0.2 g of the substance to be examined in *alcohol R* and dilute to 10 ml with the same solvent.

Reference solution. Dilute 0.5 ml of the test solution to 100 ml with *alcohol R*.

Apply to the plate 10 µl of each solution. Develop over a path of 15 cm using the lower layer from a mixture of 5 volumes of *concentrated ammonia R*, 15 volumes of *alcohol R* and 80 volumes of *chloroform R*. Examine immediately in ultraviolet light at 254 nm. Any spot in the chromatogram obtained with the test solution, apart from the principal spot, is not more intense than the spot in the chromatogram obtained with the reference solution (0.5 per cent). Spray with *diphenylcarbazone mercuric reagent R*. Allow the plate to dry in air and spray with freshly prepared *alcoholic potassium hydroxide solution R* diluted 1 in 5 with *aldehyde-free alcohol R*. Heat at 100 °C to 105 °C for 5 min and examine immediately in daylight. Any spot in the chromatogram obtained with the test solution, apart from the principal spot, is not more intense than the spot in the chromatogram obtained with the reference solution (0.5 per cent).

Free pentobarbital. Not more than 3.5 per cent. Dissolve 2.00 g in 75 ml of *dimethylformamide R*, heating gently if necessary. Titrate with 0.1 M *sodium methoxide* until the colour changes from olive-green to blue, using 0.25 ml of a 10 g/l solution of *thymol blue R* in *dimethylformamide R* as indicator. Carry out a blank titration.

1 ml of 0.1 M *sodium methoxide* is equivalent to 22.63 mg of pentobarbital.

Isomer. Dissolve 0.3 g in 5 ml of a 50 g/l solution of *anhydrous sodium carbonate R*. Add a solution of 0.3 g of *nitrobenzyl chloride R* in 10 ml of *alcohol R* and heat under a reflux condenser for 30 min. Cool to 25 °C, if necessary scratching the wall of the container with a glass rod to induce crystallisation. Filter and wash the precipitate with five quantities, each of 5 ml, of *water R*. In a small flask, heat the precipitate with 25 ml of *alcohol R* under a reflux condenser until dissolved (about 10 min). Cool to 25 °C, if necessary scratching the wall of the flask with a glass rod to induce crystallisation, and filter. The precipitate, washed with two quantities, each of 5 ml, of *water R* and dried at 100 °C to 105 °C for 30 min, melts (2.2.14) at 136 °C to 148 °C.

Heavy metals (2.4.8). Dissolve 1.0 g in *water R* and dilute to 10.0 ml with the same solvent. To 9 ml of the solution, add 3 ml of *dilute acetic acid R* and 3 ml of *buffer solution pH 3.5 R* and filter. Dilute the filtrate to 18 ml with *water R*. 12 ml of the solution complies with limit test A for heavy metals (20 ppm). In preparing the test solution, replace the buffer solution with *water R*. Prepare the standard using *lead standard solution (1 ppm Pb) R*.

Loss on drying (2.2.32). Not more than 3.0 per cent, determined on 1.00 g by drying in an oven at 100 °C to 105 °C.

ASSAY

Dissolve 0.200 g in 15 ml of a 127.5 g/l solution of *silver nitrate R* in *pyridine R*. Titrate with 0.1 M *ethanolic sodium hydroxide* until a pure blue colour is obtained, using 0.5 ml of *thymolphthalein solution R* as indicator. Carry out a blank titration.

1 ml of 0.1 M *ethanolic sodium hydroxide* is equivalent to 24.83 mg of C₁₁H₁₇N₂NaO₃.

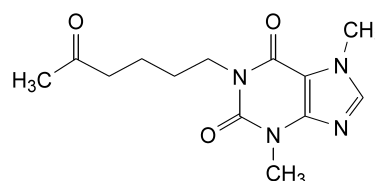
STORAGE

Store in an airtight container.

01/2005:0851

PENTOXIFYLLINE

Pentoxifyllinum



C₁₃H₁₈N₄O₃

*M*_r 278.3

DEFINITION

Pentoxifylline contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of 3,7-dimethyl-1-(5-oxohexyl)-3,7-dihydro-1*H*-purine-2,6-dione, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, crystalline powder, soluble in water, freely soluble in methylene chloride, sparingly soluble in alcohol.

IDENTIFICATION

First identification: A, B.

Second identification: A, C, D.

A. Melting point (2.2.14): 103 °C to 107 °C.

B. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *pentoxifylline CRS*. Examine the substances prepared as discs.

C. Examine the chromatograms obtained in the test for related substances in ultraviolet light at 254 nm. The principal spot in the chromatogram obtained with test solution (b) is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a).

D. It gives the reaction of xanthines (2.3.1).

TESTS

01/2005:1621

Solution S. Dissolve 2.5 g in *carbon dioxide-free water R* prepared from *distilled water R* and dilute to 50 ml with the same solvent.

Appearance of solution. Dilute 4 ml of solution S to 10 ml with *water R*. The solution is clear (2.2.1) and not more intensely coloured than reference solution Y₇ (2.2.2, *Method II*).

Acidity. To 8 ml of solution S add 12 ml of *water R* and 0.05 ml of *bromothymol blue solution R1*. The solution is green or yellow. Not more than 0.2 ml of 0.01 M *sodium hydroxide* is required to change the colour of the indicator to blue.

Related substances. Examine by thin-layer chromatography (2.2.27), using *silica gel GF₂₅₄ R* as the coating substance.

Test solution (a). Dissolve 0.10 g of the substance to be examined in *methanol R* and dilute to 5 ml with the same solvent.

Test solution (b). Dilute 1 ml of test solution (a) to 10 ml with *methanol R*.

Reference solution (a). Dissolve 20 mg of *pentoxifylline CRS* in *methanol R* and dilute to 10 ml with the same solvent.

Reference solution (b). Dilute 1 ml of test solution (b) to 50 ml with *methanol R*.

Reference solution (c). Dissolve 20 mg of *pentoxifylline CRS* and 20 mg of *theophylline CRS* in *methanol R* and dilute to 10 ml with the same solvent.

Apply separately to the plate 20 µl of each solution. Develop over a path of 10 cm using a mixture of 15 volumes of *methanol R* and 85 volumes of *ethyl acetate R*. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. Any spot in the chromatogram obtained with test solution (a), apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.2 per cent). The test is not valid unless the chromatogram obtained with reference solution (c) shows two clearly separated principal spots.

Chlorides (2.4.4). Place 20 ml of solution S in a separating funnel and shake with two quantities, each of 20 ml, of *2-methylpropan-1-ol R*. Dilute 10 ml of the aqueous layer to 15 ml with *water R*. The solution complies with the limit test for chlorides (100 ppm).

Sulphates (2.4.13). 15 ml of solution S complies with the limit test for sulphates (200 ppm).

Heavy metals (2.4.8). 2.0 g complies with the limit test C for heavy metals (10 ppm). Prepare the standard using 2 ml of *lead standard solution (10 ppm Pb) R*.

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying over *diphosphorus pentoxide R* at 60 °C at a pressure not exceeding 700 Pa.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.200 g in 5 ml of *anhydrous acetic acid R*. Add 20 ml of *acetic anhydride R*. Titrate with 0.1 M *perchloric acid* determining the end-point potentiometrically (2.2.20). 1 ml of 0.1 M *perchloric acid* is equivalent to 27.83 mg of C₁₃H₁₈N₄O₃.

STORAGE

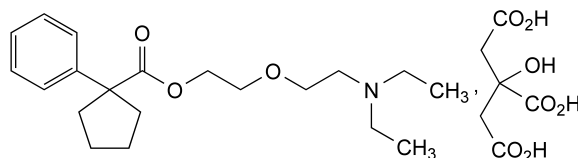
Store protected from light.

IMPURITIES

A. theobromine.

PENTOXYVERINE HYDROGEN CITRATE

Pentoxoxyverini hydrogenocitras

C₂₆H₃₉N₂O₁₀M_r 525.6

DEFINITION

2-[2-(Diethylamino)ethoxy]ethyl 1-phenylcyclopentanecarboxylate dihydrogen 2-hydroxypropane-1,2,3-tricarboxylate.

Content: 98.5 per cent to 101.0 per cent (dried substance).

CHARACTERS

Appearance: white or almost white, crystalline powder.

Solubility: freely soluble in water, very soluble in glacial acetic acid, freely soluble in methanol, soluble in alcohol and in methylene chloride.

mp: about 93 °C.

IDENTIFICATION

A. Infrared absorption spectrophotometry (2.2.24).

Comparison: *Ph. Eur. reference spectrum of pentoxoxyverine hydrogen citrate.*

B. Dissolve 0.25 g in 5 ml of *water R*. The solution gives the reaction of citrates (2.3.1).

TESTS

Solution S. Dissolve 5.0 g in *carbon dioxide-free water R* and dilute to 50 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and not more intensely coloured than reference solution Y₆ (2.2.2, *Method II*).

pH (2.2.3): 3.3 to 3.7 for solution S.

Related substances. Liquid chromatography (2.2.29).

Test solution. Dissolve 25.0 mg of the substance to be examined in the mobile phase and dilute to 25.0 ml with the mobile phase.

Reference solution. Introduce 5.0 mg of *pentoxoxyverine impurity A CRS* and 5.0 mg of *pentoxoxyverine impurity B CRS* in a conical flask, add 5.0 ml of the test solution and dilute to 100.0 ml with the mobile phase. Dilute 3.0 ml of the solution to 50.0 ml with the mobile phase.

Column:

- **size:** *l* = 0.15 m, Ø = 3.9 mm,
- **stationary phase:** *end-capped octylsilyl silica gel for chromatography R* (5 µm) with a pore size of 10 nm and a carbon loading of 12 per cent,
- **temperature:** 50 °C.

Mobile phase: mix 35 volumes of *acetonitrile R* and 65 volumes of a 0.15 per cent (*m/V*) solution of *sodium heptanesulphonate R* adjusted to pH 3.0 with *dilute sulphuric acid R*.

Flow rate: 1.0 ml/min.

Detection: spectrophotometer at 205 nm.

Injection: 20 µl.

Run time: 3 times the retention time of pentoxoxyverine.

Relative retention with reference to pentoxyverine (retention time = about 6 min): impurity B = about 0.8; impurity A = about 1.5.

System suitability: reference solution:

- **resolution:** minimum of 5.0 between the peaks due to pentoxyverine and to impurity A,
- **signal-to-noise ratio:** minimum of 100 for the peak due to pentoxyverine,
- **symmetry factor:** maximum of 2.0 for the peak due to pentoxyverine.

Limits:

- **impurity A:** not more than the area of the corresponding peak in the chromatogram obtained with the reference solution (0.3 per cent),
- **impurity B:** not more than the area of the corresponding peak in the chromatogram obtained with the reference solution (0.3 per cent),
- **any other impurity:** not more than one-third of the area of the peak due to pentoxyverine in the chromatogram obtained with the reference solution (0.1 per cent),
- **total of any other impurity:** not more than the area of the peak due to pentoxyverine in the chromatogram obtained with the reference solution (0.3 per cent),
- **disregard limit:** 0.1 times the area of the peak due to pentoxyverine in the chromatogram obtained with the reference solution (0.03 per cent); disregard any peak with a retention time less than or equal to 2.5 min.

Loss on drying (2.2.32): maximum 0.5 per cent, determined on 1.000 g by drying *in vacuo* at 60 °C for 4 h.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

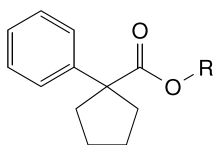
Dissolve 0.400 g in 70 ml of *anhydrous acetic acid R*. Titrate with 0.1 M *perchloric acid*, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *perchloric acid* is equivalent to 52.56 mg of $C_{26}H_{39}NO_{10}$.

STORAGE

Protected from light.

IMPURITIES



- A. R = H: 1-phenylcyclopentanecarboxylic acid,
 B. R = $CH_2CH_2N(CH_2CH_3)_2$: 2-(diethylamino)ethyl 1-phenylcyclopentanecarboxylate (caramiphen).

01/2005:0406

PEPPERMINT LEAF

Menthae piperitae folium

DEFINITION

Peppermint leaf consists of the whole or cut dried leaves of *Mentha × piperita* L. The whole drug contains not less than 12 ml/kg of essential oil. The cut drug contains not less than 9 ml/kg of essential oil.

CHARACTERS

Peppermint leaf has a characteristic and penetrating odour and a characteristic aromatic taste; it is green to brownish-green, with brownish-violet veins in some varieties. The petioles are green to brownish-violet.

It has the macroscopic and microscopic characters described under Identification tests A and B.

IDENTIFICATION

- A. The leaf is entire, broken or cut, thin, fragile; the entire leaf is 3 cm to 9 cm long and 1 cm to 3 cm wide and often crumpled. The lamina is oval or lanceolate, the apex acuminate, the margin sharply dentate and the base asymmetrical. Venation is pinnate, prominent on the lower surface, with lateral veins leaving the midrib at about 45°. The lower surface is slightly pubescent and secretory trichomes are visible under a lens (6×) as bright yellowish points. The petiole is grooved, usually up to 1 mm in diameter and 0.5 cm to 1 cm long.
- B. Reduce to a powder (355). The powder is brownish-green. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characteristics: leaf-tissue fragments with cells of the epidermis having sinuous-wavy walls and the cuticle striated over the veins and diacytic stomata predominantly present on the lower epidermis; epidermis fragments from near the leaf margin with isodiametric cells straighter-walled showing distinct beading and pitting in anticlinal walls; covering trichomes short, conical, unicellular or bicellular, or elongated, uniseriate with three to eight cells with striated cuticle; glandular trichomes of two types: (a) unicellular base with small, rounded unicellular head 15 µm to 25 µm in diameter; (b) unicellular base with enlarged oval head 55 µm to 70 µm in diameter composed of eight radiating cells; dorsiventral mesophyll fragment with a single palisade layer and four to six layers of spongy parenchyma; yellowish crystals of menthol under the cuticle of secretory cells. Calcium oxalate crystals are absent.

- C. Examine by thin-layer chromatography (2.2.27), using *silica gel GF₂₅₄ R* as the coating substance.
- Test solution.** To 0.2 g of the recently powdered drug add 2 ml of *methylene chloride R*, shake for a few minutes and filter. Evaporate the filtrate to dryness at about 40 °C and dissolve the residue in 0.1 ml of *toluene R*.

Reference solution. Dissolve 50 mg of *menthol R*, 20 µl of *cineole R*, 10 mg of *thymol R* and 10 µl of *menthyl acetate R* in *toluene R* and dilute to 10 ml with the same solvent.

Apply separately to the plate as bands 10 µl of the reference solution and 20 µl of the test solution. Develop over a path of 15 cm using a mixture of 5 volumes of *ethyl acetate R* and 95 volumes of *toluene R*. Allow the plate to dry in air until the solvent has evaporated and examine in ultraviolet light at 254 nm. The chromatogram obtained with the test solution may show a light quenching zone situated just below the level of the zone (thymol) in the chromatogram obtained with the reference solution (carvone, pulegone). Spray with *anisaldehyde solution R* and examine in daylight while heating for 5 min to 10 min at 100 °C to 105 °C. The chromatogram obtained with the reference solution shows, in order of increasing R_f value: in the lower third a deep-blue to violet zone (menthol); a violet-blue to brown zone (cineole); a pink zone (thymol); and a bluish-violet zone (menthyl acetate). The chromatogram obtained with the test solution shows: a zone due to menthol (the most intense); a faint zone due to cineole; at R_f values between those of the

cineole and thymol zones in the chromatogram obtained with the reference solution, it may show light pink, or bluish-grey or greyish-green zones (carvone, pulegone, isomenthone); in the middle of the chromatogram, a bluish-violet zone (menthyl acetate) and just below it a greenish-blue zone (menthone); an intense reddish-violet zone (hydrocarbons) appears near the solvent front; other less intensely coloured zones also appear.

TESTS

Foreign matter (2.8.2). Carry out the determinations using 10 g of the drug.

Foreign organs. Not more than 5 per cent of stems; the diameter of the stems is not greater than 1.5 mm.

Foreign elements. Not more than 2 per cent. Not more than 8 per cent of the leaves show brown stains due to *Puccinia menthae*.

Water (2.2.13). Not more than 110 ml/kg, determined on 20.0 g by distillation.

Total ash (2.4.16). Not more than 15.0 per cent.

Ash insoluble in hydrochloric acid (2.8.1). Not more than 1.5 per cent.

ASSAY

Carry out the determination of essential oils in vegetable drugs (2.8.12). Use 20.0 g of crushed drug, a 500 ml flask, 200 ml of *water R* as the distillation liquid and 0.50 ml of *xylene R* in the graduated tube. Distil at a rate of 3-4 ml/min for 2 h.

STORAGE

Store protected from light.

01/2005:0405

PEPPERMINT OIL

Menthae piperitae aetheroleum

DEFINITION

Essential oil obtained by steam distillation from the fresh aerial parts of the flowering plant of *Mentha × piperita* L.

CHARACTERS

Appearance: a colourless, pale yellow or pale greenish-yellow liquid.

It has a characteristic odour and taste followed by a sensation of cold.

Solubility: miscible with alcohol and with methylene chloride.

IDENTIFICATION

First identification: B.

Second identification: A.

A. Examine the chromatograms obtained in the test for mint oil.

Results A: see below the sequence of the zones present in the chromatograms obtained with the reference solution and the test solution.

Top of the plate	
Thymol: a quenching zone	Quenching zones may be present (carvone, pulegone)
Reference solution	Test solution

Results B: see below the sequence of the zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other less intensely coloured zones may be present in the chromatogram obtained with the test solution.

Top of the plate	
Menthyl acetate: a violet-blue zone	An intense violet-red zone (near the solvent front) (hydrocarbons) A brownish-yellow zone (menthofuran) A violet-blue zone (menthyl acetate) A greenish-blue zone (menthone)
Thymol: a pink zone	Light pink or greyish-blue or greyish-green zones may be present (carvone, pulegone, isomenthone)
Cineole: a violet-blue to brown zone	A faint violet-blue to brown zone (cineole)
Menthol: an intense blue to violet zone	An intense blue to violet zone (menthol)
Reference solution	Test solution

B. Examine the chromatograms obtained in the test for chromatographic profile.

Results: the characteristic peaks in the chromatogram obtained with the test solution are similar in retention time to those in the chromatogram obtained with the reference solution. Carvone and pulegone may be present in the chromatogram obtained with the test solution.

TESTS

Relative density (2.2.5): 0.900 to 0.916.

Refractive index (2.2.6): 1.457 to 1.467.

Optical rotation (2.2.7): -10° to -30° .

Acid value (2.5.1): maximum 1.4, determined on 5.0 g diluted in 50 ml of the prescribed mixture of solvents.

Fatty oils and resinified essential oils (2.8.7). It complies with the test for fatty oils and resinified essential oils.

Mint oil

A. Thin-layer chromatography (2.2.27).

Test solution. Mix 0.1 g of the substance to be examined with *toluene R* and dilute to 10 ml with the same solvent.

Reference solution. Dissolve 50 mg of *menthol R*, 20 µl of *cineole R*, 10 mg of *thymol R* and 10 µl of *menthyl acetate R* in *toluene R* and dilute to 10 ml with the same solvent.

Plate: TLC silica gel F_{254} plate *R*.

Mobile phase: *ethyl acetate R*, *toluene R* (5:95 V/V).

Application: 10 µl of the reference solution and 20 µl of the test solution, as bands.

Development: over a path of 15 cm.

Drying: in air.

Detection A: examine in ultraviolet light at 254 nm.

Detection B: spray with *anisaldehyde solution R* and heat at 100-105 °C for 5-10 min. Examine immediately in daylight.

Result B: the chromatogram obtained with the test solution shows no blue zone between the zones due to cineole and menthol.

- B. Examine the chromatograms obtained in the test for chromatographic profile.

Results: the chromatogram obtained with the test solution does not show a peak with the retention time of isopulegol that has an area of more than 0.2 per cent of the total area.

Chromatographic profile. Gas chromatography (2.2.28): use the normalisation procedure.

Test solution. Mix 0.20 g of the substance to be examined with *hexane R* and dilute to 10.0 ml with the same solvent.

Reference solution (a). Dissolve 10 µl of *limonene R*, 20 µl of *cineole R*, 40 µl of *menthone R*, 10 µl of *menthofuran R*, 10 µl of *isomenthone R*, 40 µl of *menthyl acetate R*, 20 µl of *isopulegol R*, 60 mg of *menthol R*, 20 µl of *pulegone R*, 10 µl of *piperitone R* and 10 µl of *carvone R* in *hexane R* and dilute to 10.0 ml with the same solvent.

Reference solution (b). Dissolve 5 µl of *isopulegol R* in *hexane R* and dilute to 10 ml with the same solvent. Dilute 0.1 ml to 5 ml with *hexane R*.

Column:

- *material*: fused silica,
- *size*: $l = 60$ m, $\varnothing = 0.25$ mm,
- *stationary phase*: *macrogol 20 000 R* (film thickness 0.25 µm).

Carrier gas: *helium for chromatography R*.

Flow rate: 1.5 ml/min.

Split ratio: 1:50.

Temperature:

	Time (min)	Temperature (°C)
Column	0 - 10	60
	10 - 70	60 - 180
	70 - 75	180
Injection port		200
Detector		220

Detection: flame ionisation.

Injection: 1 µl.

Elution order: order indicated in the composition of reference solution (a); record the retention times of these substances.

System suitability: reference solution (a):

- *resolution*: minimum 1.5 between the peaks due to limonene and cineole and minimum 1.5 between the peaks due to piperitone and carvone.

Using the retention times determined from the chromatogram obtained with reference solution (a), locate the components of the reference solution in the chromatogram obtained with the test solution (disregard the peak due to hexane).

Determine the percentage content of the components. The limits are within the following ranges:

- *limonene*: 1.0 per cent to 5.0 per cent,
- *cineole*: 3.5 per cent to 14.0 per cent,

- *menthone*: 14.0 per cent to 32.0 per cent,
- *menthofuran*: 1.0 per cent to 9.0 per cent,
- *isomenthone*: 1.5 per cent to 10.0 per cent,
- *menthyl acetate*: 2.8 per cent to 10.0 per cent,
- *isopulegol*: maximum 0.2 per cent,
- *menthol*: 30.0 per cent to 55.0 per cent,
- *pulegone*: maximum 4.0 per cent,
- *carvone*: maximum 1.0 per cent,
- *disregard limit*: peak area obtained with reference solution (b) (0.05 per cent).

The ratio of cineole content to limonene content is minimum 2.

STORAGE

In a well-filled, airtight container, protected from light, at a temperature not exceeding 25 °C.

01/2005:0682
corrected

PEPSIN POWDER

Pepsini pulvis

DEFINITION

Pepsin powder is prepared from the gastric mucosa of pigs, cattle or sheep. It contains gastric proteinases, active in acid medium (pH 1 to 5). It has an activity not less than 0.5 Ph. Eur. U./mg, calculated with reference to the dried substance.

PRODUCTION

The animals from which pepsin powder is derived must fulfil the requirements for the health of animals suitable for human consumption to the satisfaction of the competent authority.

It must have been shown to what extent the method of production allows inactivation or removal of any contamination by viruses or other infectious agents.

CHARACTERS

A white or slightly yellow, crystalline or amorphous powder, hygroscopic, soluble in water, practically insoluble in alcohol. The solution in water may be slightly opalescent with a weak acidic reaction.

IDENTIFICATION

In a mortar, pound 30 mg of *fibrin blue R*. Suspend in 20 ml of *dilute hydrochloric acid R2*. Filter the suspension on a filter paper and wash with *dilute hydrochloric acid R2* until a colourless filtrate is obtained. Perforate the filter paper and wash the *fibrin blue R* through it into a conical flask using 20 ml of *dilute hydrochloric acid R2*. Shake before use. Dissolve a quantity of the substance to be examined, equivalent to not less than 20 Ph. Eur. U., in 2 ml of *dilute hydrochloric acid R2* and adjust to pH 1.6 ± 0.1 . Add 1 ml of this solution to a test-tube containing 4 ml of the fibrin blue suspension, mix and place in a water-bath at 25 °C with gentle shaking. Prepare a blank solution at the same time and in the same manner using 1 ml of *water R*. After 15 min of incubation the blank solution is colourless and the test solution is blue.

TESTS

Loss on drying (2.2.32). Not more than 5.0 per cent, determined on 0.500 g by drying at 60 °C over *diphosphorus pentoxide R* at a pressure not exceeding 670 Pa for 4 h.

Microbial contamination. Total viable aerobic count (2.6.12) not more than 10^4 micro-organisms per gram, determined by plate-count. It complies with the tests for *Escherichia coli* and *Salmonella* (2.6.13).

ASSAY

The activity of pepsin powder is determined by comparing the quantity of peptides, non-precipitable by *trichloroacetic acid solution R* and assayed using the *phosphomolybdotungstic reagent R*, which are released per minute from a substrate of *haemoglobin solution R*, with the quantity of such peptides released by *pepsin powder BRP* from the same substrate in the same conditions.

For the test solution and the reference solution, prepare the solution and carry out the dilution at 0 °C to 4 °C.

Avoid shaking and foaming during preparation of the test and reference solutions.

Test solution. Immediately before use, prepare a solution of the substance to be examined expected to contain 0.5 Ph. Eur. U./ml in *dilute hydrochloric acid R2*; before dilution to volume, adjust to pH 1.6 ± 0.1 , if necessary, using *1 M hydrochloric acid*.

Reference solution. Less than 15 min before use, prepare a solution of *pepsin powder BRP* containing 0.5 Ph. Eur. U./ml in *dilute hydrochloric acid R2*; before dilution to volume, adjust to pH 1.6 ± 0.1 , if necessary, using *1 M hydrochloric acid*.

Designate tubes in duplicate T, T_b, S₁, S_{1b}, S₂, S_{2b}, S₃, S_{3b}; designate a tube B.

Add *dilute hydrochloric acid R2* to the tubes as follows:

B: 1.0 ml

S₁ and S_{1b}: 0.5 ml

S₂, S_{2b} and T and T_b: 0.25 ml

Add the reference solution to the tubes as follows:

S₁ and S_{1b}: 0.5 ml

S₂ and S_{2b}: 0.75 ml

S₃ and S_{3b}: 1.0 ml

Add 0.75 ml of the test solution to tubes T and T_b.

Add 10.0 ml of *trichloroacetic acid solution R* to tubes S_{1b}, S_{2b}, S_{3b}, T_b and B. Mix by shaking.

Place the tubes and *haemoglobin solution R* in a water bath at 25 ± 0.1 °C. When temperature equilibrium is reached, add 5.0 ml of *haemoglobin solution R* to tubes B, S_{1b}, S_{2b}, S_{3b} and T_b. Mix.

At time zero add 5.0 ml of *haemoglobin solution R* successively and at intervals of 30 s to tubes S₁, S₂, S₃ and T. Mix immediately after each addition.

Exactly 10 min after adding the *haemoglobin solution R*, stop the reaction by adding, at intervals of 30 s, 10.0 ml of *trichloroacetic acid solution R* to tubes S₁, S₂, S₃ and T (the use of a fast-flowing or blow-out pipette is recommended) and mix.

Filter the contents of each tube (samples and blanks) twice through the same suitable filter paper previously washed with a 50 g/l solution of *trichloroacetic acid R*, then with *water R* and dried. Discard the first 5 ml of filtrate. Place 3.0 ml of each filtrate separately in a tube containing 20 ml of *water R*. Mix.

A suitable filter paper complies with the following test: filter 5 ml of a 50 g/l solution of *trichloroacetic acid R* through a 7 cm disc of white filter paper: the absorbance (2.2.25) of the filtrate, measured at 275 nm using unfiltered *trichloroacetic acid R* solution as the compensation liquid, is less than 0.04.

Add to each tube 1.0 ml of *sodium hydroxide solution R* and 1.0 ml of *phosphomolybdotungstic reagent R*, beginning with the blanks and then the samples of each set, in a known order.

A schematic presentation of the above operations is shown in Table 0682.-1.

After 15 min measure the absorbance (2.2.25) of solutions S₁, S₂, S₃, S_{1b}, S_{2b}, S_{3b} and T at 540 nm using the filtrate obtained from tube B as the compensation liquid. Correct the average absorbance values for the filtrates obtained from tubes S₁, S₂ and S₃ by subtracting the average values obtained for the filtrates from tubes S_{1b}, S_{2b}, S_{3b} respectively.

Draw a calibration curve of the corrected values against volume of reference solution used. Determine the activity of the substance to be examined using the corrected absorbance for the test solution (T – T_b) together with the calibration curve and taking into account the dilution factors.

Table 0682.-1

	Tubes								
	S ₁	S _{1b}	S ₂	S _{2b}	S ₃	S _{3b}	T	T _b	B
<i>Dilute hydrochloric acid R2</i> (ml)	0.5	0.5	0.25	0.25			0.25	0.25	1.0
Reference solution (ml)	0.5	0.5	0.75	0.75	1.0	1.0			
Test solution (ml)							0.75	0.75	
<i>Trichloroacetic acid solution R</i> (ml)		10.0		10.0		10.0		10.0	10.0
Mix		+		+		+		+	+
Water bath at 25 °C	+	+	+	+	+	+	+	+	+
<i>Haemoglobin solution R</i> (ml)		5.0		5.0		5.0		5.0	5.0
Mix		+		+		+		+	+
<i>Haemoglobin solution R</i> (ml)	5.0		5.0		5.0		5.0		
Mix	+		+		+		+		
Water bath at 25 °C, 10 min	+	+	+	+	+	+	+	+	+
<i>Trichloroacetic acid solution R</i> (ml)	10.0		10.0		10.0		10.0		
Mix	+		+		+		+		
Filter	+	+	+	+	+	+	+	+	+

STORAGE

Store in an airtight container, protected from light, at a temperature of 2 °C to 8 °C.

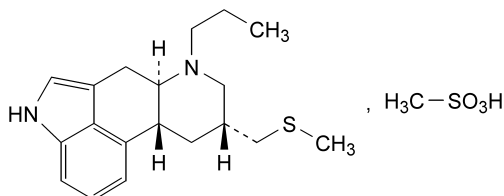
LABELLING

The label states the activity in European Pharmacopoeia Units per milligram.

01/2005:1555

PERGOLIDE MESILATE

Pergolidi mesilas

C₂₀H₃₀N₂O₃S₂M_r 410.6

DEFINITION

Pergolide mesilate contains not less than 97.5 per cent and not more than 102.0 per cent of (6a*R*,9*R*,10a*R*)-9-[(methylsulphonyl)methyl]-7-propyl-4,6,6a,7,8,9,10,10a-octahydroindolo[4,3-*fg*]quinoline monomethanesulphonate, calculated with reference to the dried substance.

PRODUCTION

The production method must be evaluated to determine the potential for formation of alkyl mesilates, which is particularly likely to occur if the reaction medium contains lower alcohols. Where necessary, the production method is validated to demonstrate that alkyl mesilates are not detectable in the final product.

CHARACTERS

A white or almost white, crystalline powder, slightly soluble in water, sparingly soluble in methanol, slightly soluble in alcohol and in methylene chloride, very slightly soluble in acetone.

IDENTIFICATION

- The specific optical rotation (2.2.7) is –17 to –23, calculated with reference to the dried substance and determined on a solution prepared as follows: dissolve 0.25 g in *dimethylformamide R* and dilute to 25.0 ml with the same solvent.
- Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *pergolide mesilate CRS*. Examine the substances prepared as discs.

TESTS

Related substances. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 30.0 mg of the substance to be examined in *methanol R* and dilute to 10.0 ml with the same solvent.

Reference solution (a). Dilute 1.0 ml of the test solution to 100.0 ml with *methanol R*. Dilute 1.0 ml of this solution to 10.0 ml with *methanol R*.

Reference solution (b). Dissolve 10 mg of 4,4'-dimethoxybenzophenone *R* in *methanol R* and dilute to 10 ml with the same solvent. To 1 ml of this

solution add 2 ml of the test solution and dilute to 100 ml with *methanol R*. Dilute 1 ml of this solution to 10 ml with *methanol R*.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4.6 mm in internal diameter packed with *base-deactivated octadecylsilyl silica gel for chromatography R* (5 µm),
- as mobile phase at a flow rate of 1 ml/min:
 - Mobile phase A.** Mix 5.0 ml of *morpholine for chromatography R* with 995 ml of *water R* and adjust to pH 7.0 with *phosphoric acid R*. Use within 24 h,
 - Mobile phase B.** Mix equal volumes of *acetonitrile R*, *methanol R* and *tetrahydrofuran R*,

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)	Comment
0 - 35	70 → 0	30 → 100	linear gradient
35 - 40	0 → 70	100 → 30	return to initial conditions
40 - 50	70	30	re-equilibration

- as detector a spectrophotometer set at 280 nm, maintaining the temperature of the column at 40 °C.

Adjust the sensitivity of the system so that the height of the principal peak in the chromatogram obtained with 20 µl of reference solution (a) is at least 90 per cent of the full scale of the recorder.

Inject 20 µl of reference solution (b). The test is not valid unless, in the chromatogram obtained, the resolution between the peaks corresponding to 4,4'-dimethoxybenzophenone (first peak) and pergolide (second peak) is at least 2.0.

Inject 20 µl of the test solution and 20 µl of reference solution (a).

In the chromatogram obtained with the test solution: the area of any peak, apart from the principal peak, is not greater than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent); the sum of the areas of all the peaks, apart from the principal peak, is not greater than 5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent). Disregard any peak with an area less than 0.2 times that of the principal peak in the chromatogram obtained with reference solution (a) (0.02 per cent).

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying *in vacuo* at 100-105 °C for 1 h.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Examine by liquid chromatography (2.2.29).

Solution A. Dissolve 5.0 mg of *DL-methionine R* in 500 ml of 0.01 M hydrochloric acid. Add 500 ml of *methanol R* and mix.

Test solution. Dissolve 65.0 mg of the substance to be examined in solution A and dilute to 100.0 ml with the same solution. Dilute 10.0 ml to 100.0 ml with solution A.

Reference solution. Dissolve 65.0 mg of *pergolide mesilate CRS* in solution A and dilute to 100.0 ml with the same solution. Dilute 10.0 ml to 100.0 ml with solution A.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4.6 mm in internal diameter packed with *base-deactivated octylsilyl silica gel for chromatography R* (5 µm),

– as mobile phase at a flow rate of 1 ml/min a mixture of 1 volume of *acetonitrile R*, 1 volume of *methanol R* and 2 volumes of a mixture prepared as follows: dissolve 2.0 g of *sodium octanesulphonate R* in *water R*, add 1.0 ml of *anhydrous acetic acid R* and dilute to 1000 ml with *water R*,

– as detector a spectrophotometer set at 280 nm, maintaining the temperature of the column at 40 °C.

Inject 20 µl of the reference solution. When the chromatogram is recorded in the prescribed conditions, the retention time of pergolide is about 9 min. Adjust the sensitivity of the system so that the height of the principal peak in the chromatogram obtained with the reference solution is at least 50 per cent of the full scale of the recorder. The assay is not valid unless the symmetry factor of the peak due to pergolide is at most 1.5.

Inject 20 µl of the test solution.

Calculate the percentage content of $C_{20}H_{30}N_2O_3S_2$ from the areas of the peaks and the declared content of *pergolide mesilate CRS*.

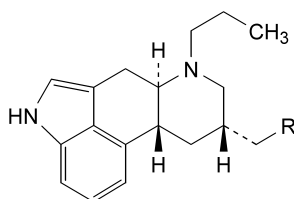
STORAGE

Store protected from light.

IMPURITIES

Specified impurities: A.

Other detectable impurities: B.

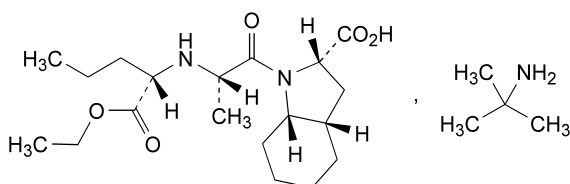


- A. $R = SO-CH_3$: (6a*R*,9*R*,10a*R*)-9-[(methylsulphinyl)methyl]-7-propyl-4,6,6a,7,8,9,10,10a-octahydroindolo[4,3-*fg*]quinoline (pergolide sulfoxide),
- B. $R = SO_2-CH_3$: (6a*R*,9*R*,10a*R*)-9-[(methylsulphonyl)methyl]-7-propyl-4,6,6a,7,8,9,10,10a-octahydroindolo[4,3-*fg*]quinoline (pergolide sulphone).

01/2005:2019

PERINDOPRIL *tert*-BUTYLAMINE

tert-Butylamini perindoprilum



$C_{23}H_{43}N_3O_5$

M_r 441.6

DEFINITION

2-Methylpropan-2-amine (2*S*,3a*S*,7a*S*)-1-[(2*S*)-2-[(1*S*)-1-(ethoxycarbonyl)butyl]amino]propanoyl]octahydro-1*H*-indole-2-carboxylate.

Content: 99.0 per cent to 101.0 per cent (anhydrous substance).

CHARACTERS

Appearance: white or almost white, crystalline powder, slightly hygroscopic.

Solubility: freely soluble in water and in alcohol, sparingly soluble in methylene chloride.

It shows polymorphism.

IDENTIFICATION

A. Specific optical rotation (2.2.7): -66 to -69 (anhydrous substance).

Dissolve 0.250 g in *alcohol R* and dilute to 25.0 ml with the same solvent.

B. Infrared absorption spectrophotometry (2.2.24).

Preparation: discs.

Comparison: *perindopril tert-butylamine CRS*.

If the spectra obtained show differences, dissolve the substance to be examined and the reference substance separately in *methylene chloride R*, evaporate to dryness and record new spectra using the residues.

C. Examine the chromatograms obtained in the test for impurity A.

Results: in the chromatogram obtained with the test solution a spot is observed with the same R_f as the spot with the higher R_f in the chromatogram with reference solution (c) (*tert*-butylamine).

TESTS

Impurity A. Thin-layer chromatography (2.2.27).

Test solution. Dissolve 0.20 g of the substance to be examined in *methanol R* and dilute to 10 ml with the same solvent.

Reference solution (a). Dissolve 5 mg of *perindopril impurity A CRS* in *methanol R* and dilute to 25.0 ml with the same solvent.

Reference solution (b). Dilute 5 ml of reference solution (a) to 20 ml with *methanol R*.

Reference solution (c). To 5 ml of reference solution (a) add 5 ml of a 20 g/l solution of *1,1-dimethylethylamine R* in *methanol R*.

Plate: TLC silica gel plate *R*.

Mobile phase: *glacial acetic acid R*, *toluene R*, *methanol R* (1:40:60 V/V/V).

Application: 10 µl; apply the test solution and reference solutions (b) and (c).

Development: in a saturated tank, over 2/3 of the plate.

Drying: in a current of warm air.

Detection: expose to iodine vapour for at least 20 h.

System suitability: the chromatogram obtained with reference solution (c) shows 2 clearly separated spots.

Limit:

- *impurity A*: any spot due to impurity A is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.25 per cent).

Stereochemical purity. Liquid chromatography (2.2.29).

Test solution. Dissolve 20 mg of the substance to be examined in *alcohol R* and dilute to 10.0 ml with the same solvent.

Reference solution (a). Dilute 1.0 ml of the test solution to 200.0 ml with *alcohol R*.

Reference solution (b). Dissolve 10 mg of *perindopril for stereochemical purity CRS* in *alcohol R* and dilute to 5.0 ml with the same solvent.

Reference solution (c). Dilute 10.0 ml of reference solution (a) to 50.0 ml with *alcohol R*.

Column:

- *size*: $l = 0.25$ m, $\varnothing = 4.6$ mm,

- *stationary phase*: spherical *octadecylsilyl silica gel for chromatography R* (5 µm) with a specific surface area of 450 m²/g and a pore size of 10 nm,
- *temperature*: 50 °C for the column and at least 30 cm of the tubing preceding the column.

Mobile phase: mix, in the following order, 21.7 volumes of *acetonitrile R*, 0.3 volumes of *pentanol R* and 78 volumes of a 1.50 g/l solution of *sodium heptanesulphonate R*, previously adjusted to pH 2.0 with a mixture of equal volumes of *perchloric acid R* and *water R*.

Flow rate: 0.8 ml/min.

Detection: spectrophotometer at 215 nm.

Equilibration: minimum 4 h.

Injection: 10 µl.

Run time: 1.5 times the retention time of perindopril.

Retention time: perindopril = about 100 min.

System suitability:

- *signal-to-noise ratio*: minimum 3 for the principal peak in the chromatogram obtained with reference solution (c),
- *peak-to-valley ratio*: minimum 3, where H_p = height above the baseline of the peak due to impurity I and H_v = height above the baseline of the lowest point of the curve separating this peak from the peak due to perindopril in the chromatogram obtained with reference solution (b),
- the chromatogram obtained with reference solution (b) is similar to the chromatogram provided with *perindopril for stereochemical purity CRS*.

Limits:

- *any impurity*: not more than 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent); disregard any peak with a retention time less than 0.6 times the retention time of perindopril and any peak with a retention time greater than 1.4 times the retention time of perindopril.

Related substances. Liquid chromatography (2.2.29).

Test solution. Dissolve 60 mg of the substance to be examined in mobile phase A and dilute to 20.0 ml with mobile phase A.

Reference solution (a). Dissolve 15 mg of *perindopril for system suitability CRS* in mobile phase A and dilute to 5.0 ml with mobile phase A.

Reference solution (b). Dilute 1.0 ml of the test solution to 200.0 ml with mobile phase A.

Column:

- *size*: $l = 0.25$ m, $\varnothing = 4$ mm,
- *stationary phase*: spherical *octylsilyl silica gel for chromatography R* (4 µm) with a pore size of 6 nm,
- *temperature*: 70 °C.

Mobile phase:

- *mobile phase A*: dissolve 0.92 g of *sodium heptanesulphonate R* in 1000 ml of *water R*, add 1 ml of *triethylamine R* and adjust to pH 2.0 with a mixture of equal volumes of *perchloric acid R* and *water R*,
- *mobile phase B*: *acetonitrile R1*,

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)
0 - 1	70	30
1 - 20	70 → 40	30 → 60
20 - 25	40	60
25 - 35	40 → 20	60 → 80
35 - 40	20 → 0	80 → 100
40 - 45	0 → 70	100 → 30

Flow rate: 1.5 ml/min.

Detection: spectrophotometer at 215 nm.

Injection: 20 µl.

Relative retention with reference to perindopril (retention time = about 8 min): impurity B = about 0.4; impurity C = about 0.8; impurity D = about 0.9; impurity E = about 1.4; impurity F = about 1.7; impurity G = about 2.2 and 2.3; impurity H = about 3.6 and 3.7.

System suitability: reference solution (a):

- *peak-to-valley ratio*: minimum 10, where H_p = height above the baseline of the peak due to impurity D and H_v = height above the baseline of the lowest point of the curve separating this peak from the peak due to perindopril.

Limits:

- *impurity B*: not more than 0.6 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.3 per cent),
- *impurity E*: not more than 0.8 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.4 per cent),
- *impurities F, H*: for each impurity, not more than 0.4 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent),
- *any other impurity*: not more than 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent),
- *total*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (1 per cent),
- *disregard limit*: 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

Water (2.5.12): maximum 1.0 per cent, determined on 0.50 g.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.160 g in 50 ml of *anhydrous acetic acid R*. Titrate with 0.1 M *perchloric acid*, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *perchloric acid* is equivalent to 22.08 mg of C₂₃H₄₃N₃O₅.

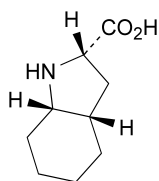
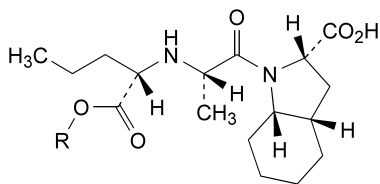
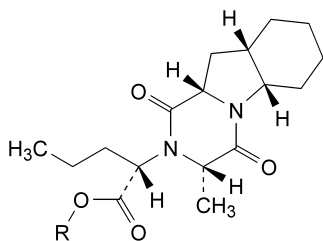
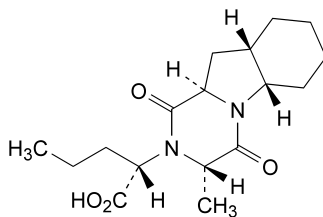
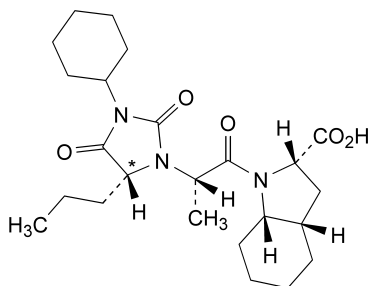
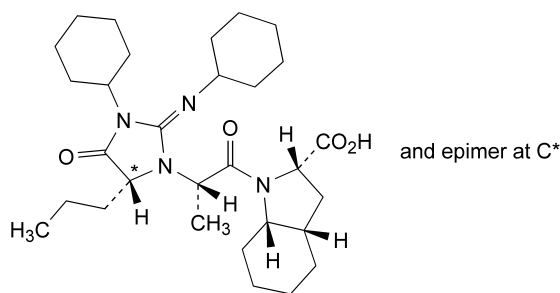
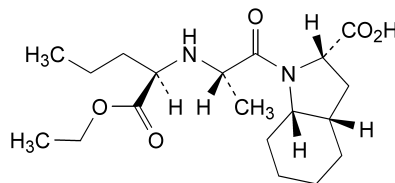
STORAGE

In an airtight container.

IMPURITIES

Specified impurities: A, B, E, F, H, I.

Other detectable impurities: C, D, G.

A. (2*S*,3*aS*,7*aS*)-octahydro-1*H*-indole-2-carboxylic acid,B. R = H: (2*S*,3*aS*,7*aS*)-1-[(2*S*)-2-[(1*S*)-1-carboxybutyl]amino]propanoyl]octahydro-1*H*-indole-2-carboxylic acid,E. R = CH(CH₃)₂: (2*S*,3*aS*,7*aS*)-1-[(2*S*)-2-[(1*S*)-1-[(1-methylethoxy)carbonyl]butyl]amino]propanoyl]octahydro-1*H*-indole-2-carboxylic acid,C. R = H: (2*S*)-2-[(3*S*,5*aS*,9*aS*,10*aS*)-3-methyl-1,4-dioxodecahydropyrazino[1,2-*a*]indol-2(1*H*)-yl]pentanoic acid,F. R = C₂H₅: ethyl (2*S*)-2-[(3*S*,5*aS*,9*aS*,10*aS*)-3-methyl-1,4-dioxodecahydropyrazino[1,2-*a*]indol-2(1*H*)-yl]pentanoate,D. (2*S*)-2-[(3*S*,5*aS*,9*aS*,10*aR*)-3-methyl-1,4-dioxodecahydropyrazino[1,2-*a*]indol-2(1*H*)-yl]pentanoic acid,G. (2*S*,3*aS*,7*aS*)-1-[(2*S*)-2-[(5*RS*)-3-cyclohexyl-2,4-dioxo-5-propylimidazolidin-1-yl]propanoyl]octahydro-1*H*-indole-2-carboxylic acid,H. (2*S*,3*aS*,7*aS*)-1-[(2*S*)-2-[(5*RS*)-3-cyclohexyl-2-(cyclohexylimino)-4-oxo-5-propylimidazolidin-1-yl]propanoyl]octahydro-1*H*-indole-2-carboxylic acid,I. (2*S*,3*aS*,7*aS*)-1-[(2*S*)-2-[(1*R*)-1-(ethoxycarbonyl)butyl]amino]propanoyl]octahydro-1*H*-indole-2-carboxylic acid.

01/2005:0862

PERITONEAL DIALYSIS, SOLUTIONS FOR

Solutiones ad peritonealem dialysim

DEFINITION

Solutions for peritoneal dialysis are preparations for intraperitoneal use containing electrolytes with a concentration close to the electrolytic composition of plasma. They contain glucose in varying concentrations or other suitable osmotic agents.

Solutions for peritoneal dialysis are supplied in:

- rigid or semi-rigid plastic containers,
- flexible plastic containers fitted with a special connecting device; these are generally filled to a volume below their nominal capacity and presented in closed protective envelopes,
- glass containers.

The containers and closures comply with the requirements for containers for preparations for parenteral use (3.2.1 and 3.2.2).

Several formulations are used. The concentrations of the components per litre of solution are usually in the following range:

Table 0862.-1

	Expression in mmol	Expression in mEq
Sodium	125 - 150	125 - 150
Potassium	0 - 4.5	0 - 4.5
Calcium	0 - 2.5	0 - 5.0
Magnesium	0.25 - 1.5	0.50 - 3.0
Acetate and/or lactate and/or hydrogen carbonate	30 - 60	30 - 60
Chloride	90 - 120	90 - 120
Glucose	25 - 250	

When hydrogen carbonate is present, the solution of sodium hydrogen carbonate is supplied in a container or a separate compartment and is added to the electrolyte solution immediately before use.

Unless otherwise justified and authorised, antioxidants such as metabisulphite salts are not added to the solutions.

IDENTIFICATION

According to the stated composition, the solution to be examined gives the following identification reactions (2.3.1):

- potassium: reaction (b);
- calcium: reaction (a);
- sodium: reaction (b);
- chlorides: reaction (a);
- acetates: to 5 ml of the solution to be examined add 1 ml of *hydrochloric acid R* in a test-tube fitted with a stopper and a bent tube, heat and collect a few millilitres of distillate; carry out reaction (b) of acetates on the distillate;
- lactates, hydrogen carbonates; the identification is carried out together with the assay;
- magnesium: to 0.1 ml of *titan yellow solution R* add 10 ml of *water R*, 2 ml of the solution to be examined and 1 ml of 1 M *sodium hydroxide*; a pink colour is produced;
- glucose: to 5 ml of the solution to be examined, add 2 ml of *dilute sodium hydroxide solution R* and 0.05 ml of *copper sulphate solution R*; the solution is blue and clear; heat to boiling; an abundant red precipitate is formed.

TESTS

Appearance of solution. The solution is clear (2.2.1) and not more intensely coloured than reference solution Y₄ (2.2.2, *Method I*).

pH (2.2.3). The pH of the solution is 5.0 to 6.5. If the solution contains hydrogen carbonate, the pH is 6.5 to 8.0.

Hydroxymethylfurfural. To a volume of the solution containing the equivalent of 25 mg of glucose, add 5.0 ml of a 100 g/l solution of *p-toluidine R* in 2-propanol *R* containing 10 per cent V/V of *glacial acetic acid R* and 1.0 ml of a 5 g/l solution of *barbituric acid R*. The absorbance (2.2.25) determined at 550 nm after allowing the mixture to stand for 2 min to 3 min is not greater than that of a standard prepared at the same time in the same manner using a solution containing 10 µg of *hydroxymethylfurfural R* in the same volume as the solution to be examined. If the solution contains hydrogen carbonate, use as the standard a solution containing 20 µg of *hydroxymethylfurfural R*.

Aluminium (2.4.17). Take 400 ml, adjust to pH 6.0 and add 10 ml of *acetate buffer solution pH 6.0 R*. The solution complies with the limit test for aluminium (15 µg/l). Use as the reference solution a mixture of 3 ml of *aluminium standard solution (2 ppm Al) R*, 10 ml of *acetate buffer solution pH 6.0 R* and 9 ml of *water R*. To prepare the blank use a mixture of 10 ml of *acetate buffer solution pH 6.0 R* and 10 ml of *water R*.

Particulate contamination. Carry out the test for sub-visible particles (2.9.19) using 50 ml of solution.

Table 0862-2

Particles larger than	10 µm	25 µm
Maximum number of particles per millilitre	25	3

Extractable volume (2.9.17). The solution complies with the test prescribed for parenteral infusions.

Sterility (2.6.1). The solution complies with the test for sterility.

Bacterial endotoxins (2.6.14): less than 0.25 IU/ml.

Pyrogens (2.6.8). Solutions for which a validated test for bacterial endotoxins cannot be carried out comply with the test for pyrogens. Inject per kilogram of the rabbit's mass 10 ml of the solution.

ASSAY

Sodium: 97.5 per cent to 102.5 per cent of the content of sodium (Na) stated on the label, determined by atomic absorption spectrometry (2.2.23, *Method II*).

Test solution. If necessary, dilute the solution to be examined with *water R* to a concentration suitable for the instrument to be used.

Reference solutions. Prepare the reference solutions using *sodium standard solution (200 ppm Na) R*.

Measure the absorbance at 589.0 nm using a sodium hollow-cathode lamp as source of radiation and an air-propane or an air-acetylene flame.

Potassium. 95.0 per cent to 105.0 per cent of the content of potassium (K) stated on the label, determined by atomic absorption spectrometry (2.2.23, *Method I*).

Test solution. If necessary, dilute the solution to be examined with *water R* to a concentration suitable for the instrument to be used. To 100 ml of the solution add 10 ml of a 22 g/l solution of *sodium chloride R*.

Reference solutions. Prepare the reference solutions using *potassium standard solution (100 ppm K) R*. To 100 ml of each reference solution add 10 ml of a 22 g/l solution of *sodium chloride R*.

Measure the absorbance at 766.5 nm, using a potassium hollow-cathode lamp as source of radiation and an air-propane or an air-acetylene flame.

Calcium. 95.0 per cent to 105.0 per cent of the content of calcium (Ca) stated on the label, determined by atomic absorption spectrometry (2.2.23, *Method I*).

Test solution. If necessary, dilute the solution to be examined with *water R* to a concentration suitable for the instrument to be used.

Reference solutions. Prepare the reference solutions using *calcium standard solution (400 ppm Ca) R*.

Measure the absorbance at 422.7 nm using a calcium hollow-cathode lamp as source of radiation and an air-propane or an air-acetylene flame.

Magnesium: 95.0 per cent to 105.0 per cent of the content of magnesium (Mg) stated on the label, determined by atomic absorption spectrometry (2.2.23, *Method I*).

Test solution. If necessary, dilute the solution to be examined with *water R* to a concentration suitable for the instrument to be used.

Reference solutions. Prepare the reference solutions using *magnesium standard solution (100 ppm Mg) R*.

Measure the absorbance at 285.2 nm using a magnesium hollow-cathode lamp as source of radiation and an air-propane or an air-acetylene flame.

Total chloride. 95.0 per cent to 105.0 per cent of the content of chloride (Cl) stated on the label. Dilute to 50 ml with *water R* an accurately measured volume of the solution to be examined containing the equivalent of about 60 mg of chloride. Add 5 ml of *dilute nitric acid R*, 25.0 ml of 0.1 M *silver nitrate* and 2 ml of *dibutyl phthalate R*. Shake.

Using 2 ml of *ferric ammonium sulphate solution R2* as indicator, titrate with 0.1 M *ammonium thiocyanate* until a reddish-yellow colour is obtained.

1 ml of 0.1 M *silver nitrate* is equivalent to 3.545 mg of Cl.

Acetate: 95.0 per cent to 105.0 per cent of the content of acetate stated on the label. To a volume of the solution to be examined, corresponding to about 0.7 mmol of acetate, add 10.0 ml of 0.1 M *hydrochloric acid*. Carry out a potentiometric titration (2.2.20), using 0.1 M *sodium hydroxide*. Read the volume added between the two points of inflexion.

1 ml of 0.1 M *sodium hydroxide* is equivalent to 0.1 mmol of acetate.

Lactate: 95.0 per cent to 105.0 per cent of the content of lactate stated on the label. To a volume of the solution to be examined, corresponding to about 0.7 mmol of lactate, add 10.0 ml of 0.1 M *hydrochloric acid*. Then add 50 ml of *acetonitrile R*. Carry out a potentiometric titration (2.2.20), using 0.1 M *sodium hydroxide*. Read the volume added between the two points of inflexion.

1 ml of 0.1 M *sodium hydroxide* is equivalent to 0.1 mmol of lactate.

Sodium hydrogen carbonate: 95.0 per cent to 105.0 per cent of the content of sodium hydrogen carbonate stated on the label. Titrate with 0.1 M *hydrochloric acid*, a volume of the solution to be examined corresponding to about 0.1 g of sodium hydrogen carbonate, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *hydrochloric acid* is equivalent to 8.40 mg of NaHCO_3 .

Lactate and hydrogen carbonate: 95.0 per cent to 105.0 per cent of the content of lactates and/or hydrogen carbonates stated on the label. Examine by liquid chromatography (2.2.29).

Test solution. Solution to be examined.

Reference solution. Dissolve in 100 ml of *water for chromatography R* quantities of lactates and hydrogen carbonates, accurately weighed, in order to obtain solutions having concentrations representing about 90 per cent, 100 per cent and 110 per cent of the concentrations indicated on the label.

The chromatographic procedure may be carried out using:

- a column 0.30 m long and 7.8 mm in internal diameter packed with *cation exchange resin R* (9 µm),
 - as mobile phase at a flow rate of 0.6 ml/min 0.005 M *sulphuric acid* previously degassed with *helium R*,
 - a differential refractometer detector,
- maintaining the temperature of the column at 85 °C.

Inject in duplicate 20 µl of test solution and 20 µl of each reference solution. When the chromatograms are recorded in the prescribed conditions, the peaks elute in the following order: lactates then hydrogen carbonates.

Determine the concentration of lactates and hydrogen carbonates in the test solution by interpolating the peak area for lactate and the peak height for hydrogen carbonate from the linear regression curve obtained with the reference solutions.

Reducing sugars (expressed as anhydrous glucose). 95.0 per cent to 105.0 per cent of the content of glucose stated on the label. Transfer a volume of solution to be examined containing the equivalent of 25 mg of glucose to a 250 ml conical flask with a ground-glass neck and add 25.0 ml of *cupri-citric solution R*. Add a few grains of pumice, fit a reflux condenser, heat so that boiling occurs within 2 min and boil for exactly 10 min. Cool and add 3 g of *potassium*

iodide R dissolved in 3 ml of *water R*. Carefully add, in small amounts, 25 ml of a 25 per cent *m/m* solution of *sulphuric acid R*. Titrate with 0.1 M *sodium thiosulphate* using *starch solution R*, added towards the end of the titration, as indicator. Carry out a blank titration using 25.0 ml of *water R*.

Calculate the content of reducing sugars expressed as anhydrous glucose ($\text{C}_6\text{H}_{12}\text{O}_6$), by means of Table 0862-3:

Table 0862-3

Volume of 0.1 M sodium thiosulphate (ml)	Anhydrous glucose (mg)
8	19.8
9	22.4
10	25.0
11	27.6
12	30.3
13	33.0
14	35.7
15	38.5
16	41.3

STORAGE

Store at a temperature not below 4 °C.

LABELLING

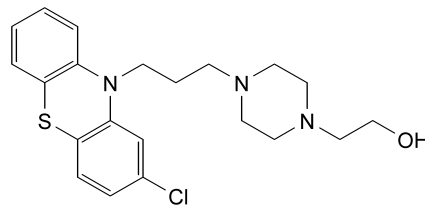
The label states:

- the formula of the solution for peritoneal dialysis, expressed in grams per litre and in millimoles per litre,
- the calculated osmolarity, expressed in milliosmoles per litre,
- the nominal volume of the solution for peritoneal dialysis in the container,
- that the solution is free from bacterial endotoxins, or where applicable, that it is apyrogenic,
- the storage conditions,
- that the solution is not to be used for intravenous infusion,
- that any unused portion of solution is to be discarded.

01/2005:0629

PERPHENAZINE

Perphenazinum



$\text{C}_{21}\text{H}_{26}\text{ClN}_3\text{OS}$

M_r 404.0

DEFINITION

Perphenazine contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of 2-[4-[3-(2-chlorophenothiazin-10-yl)propyl]piperazin-1-yl]ethanol, calculated with reference to the dried substance.

CHARACTERS

A white or yellowish-white, crystalline powder, practically insoluble in water, freely soluble in methylene chloride, soluble in alcohol. It dissolves in dilute solutions of hydrochloric acid.

IDENTIFICATION

First identification: A, C

Second identification: A, B, D.

- A. Melting point (2.2.14): 96 °C to 100 °C.
- B. Dissolve 10 mg in *methanol R* and dilute to 100 ml with the same solvent. Dilute 10 ml of the solution to 100 ml with *methanol R*. Examined between 230 nm and 350 nm (2.2.25), the solution shows two absorption maxima, at 257 nm and 313 nm. The ratio of the absorbance measured at the maximum at 313 nm to that measured at the maximum at 257 nm is 0.120 to 0.128.
- C. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *perphenazine CRS*. Examine the substances prepared as discs.
- D. Examine by thin-layer chromatography (2.2.27), using *kieselguhr G R* as the coating substance. Impregnate the plate by placing it in a closed tank containing the necessary quantity of the impregnation mixture containing 2.5 per cent V/V of *phenoxyethanol R* and 7.5 per cent V/V of *formamide R* in *acetone R* so that the plate dips about 5 mm beneath the surface of the liquid. When the impregnation mixture has risen at least 17 cm from the lower edge of the plate, remove the plate and use immediately for chromatography. Carry out the chromatography in the same direction as the impregnation.

Test solution. Dissolve 20 mg of the substance to be examined in *chloroform R* and dilute to 10 ml with the same solvent.

Reference solution. Dissolve 20 mg of *perphenazine CRS* in *chloroform R* and dilute to 10 ml with the same solvent. Apply separately to the plate 2 µl of each solution. Develop in the dark over a path of 15 cm using a mixture of 2 volumes of *diethylamine R* and 100 volumes of *light petroleum R*, saturated with phenoxyethanol (add *phenoxyethanol R* - 6 volumes to 8 volumes - to the above mixture of solvents until there is a persistent cloudiness after shaking, decant, and use the supernatant liquid, even if it is cloudy). Expose the plate to ultraviolet light at 365 nm and examine after a few minutes. The principal spot in the chromatogram obtained with the test solution is similar in position, fluorescence and size to the principal spot in the chromatogram obtained with the reference solution. Dry the plate at 120 °C for 20 min, allow to cool and spray with a 10 per cent V/V solution of *sulphuric acid R* in *alcohol R*. The principal spot in the chromatogram obtained with the test solution is of the same colour as the principal spot in the chromatogram obtained with the reference solution.

TESTS

Appearance of solution. Dissolve 0.20 g in 10 ml of *methanol R*. The solution is clear (2.2.1).

Related substances. Examine by thin-layer chromatography (2.2.27), using *silica gel GF₂₅₄ R* as the coating substance. Prepare the solutions immediately before use.

Test solution. Dissolve 0.1 g of the substance to be examined in *methanol R* and dilute to 10 ml with the same solvent.

Reference solution. Dilute 0.5 ml of the test solution to 100 ml with *methanol R*.

Apply separately to the plate 10 µl of each solution.

Develop over a path of 15 cm using a mixture of 1 volume of *concentrated ammonia R*, 14 volumes of *water R* and 85 volumes of *butanol R*. Allow the plate to dry in air. Examine in ultraviolet light at 254 nm. Any spot in the chromatogram obtained with the test solution, apart from the principal spot, is not more intense than the spot in the chromatogram obtained with the reference solution (0.5 per cent).

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying *in vacuo* at 65 °C for 4 h.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.1500 g in 25 ml of *anhydrous acetic acid R*. Titrate with 0.1 M *perchloric acid* determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *perchloric acid* is equivalent to 20.20 mg of C₂₁H₂₆ClN₃OS.

STORAGE

Store protected from light.

01/2005:0754

PERU BALSAM

Balsamum peruvianum

DEFINITION

Peru balsam is the balsam obtained from the scorched and wounded trunk of *Myroxylon balsamum* (L.) Harms var. *pereirae* (Royle) Harms. It contains not less than 45.0 per cent *m/m* and not more than 70.0 per cent *m/m* of esters, mainly benzyl benzoate and benzyl cinnamate.

CHARACTERS

A dark brown, viscous liquid which is transparent and yellowish-brown when viewed in a thin layer; the liquid is not sticky, it is non-drying and does not form threads; practically insoluble in water, freely soluble in ethanol, not miscible with fatty oils, except for castor oil.

IDENTIFICATION

- A. Dissolve 0.20 g in 10 ml of *alcohol R*. Add 0.2 ml of *ferric chloride solution R1*. A green to olive-green colour develops.
- B. Examine by thin-layer chromatography (2.2.27), using *silica gel GF₂₅₄ R* as the coating substance.
- Test solution.* Dissolve 0.5 g of the substance to be examined in 10 ml of *ethyl acetate R*.
- Reference solution.* Dissolve 4 mg of *thymol R*, 30 mg of *benzyl cinnamate R* and 80 µl of *benzyl benzoate R* in 5 ml of *ethyl acetate R*.

Apply separately to the plate as bands 20 mm by 3 mm 10 µl of each solution. Develop twice over a path of 10 cm using a mixture of 0.5 volumes of *glacial acetic acid R*, 10 volumes of *ethyl acetate R* and 90 volumes of *hexane R*. Allow the plate to dry in air, examine in ultraviolet light at 254 nm and mark the quenching zones. The chromatogram obtained with the reference solution shows in the upper third two quenching zones, the higher one corresponding to benzyl benzoate and the lower one to benzyl cinnamate. The chromatogram obtained with the test solution shows two quenching zones at the same levels and of approximately the same size. Spray the plate with a freshly prepared 200 g/l

solution of *phosphomolybdic acid R* in *alcohol R*, using 10 ml for a plate 200 mm square and examine the plate in daylight while heating at 100 °C to 105 °C for 5 min to 10 min. The zones due to benzyl benzoate and benzyl cinnamate are coloured blue against a yellow background. The chromatogram obtained with the reference solution shows at about the middle a violet-grey zone (thymol). In the chromatogram obtained with the test solution, a blue zone (nerolidol) is seen just below the level of the zone due to thymol in the chromatogram obtained with the reference solution. Just below the zone due to nerolidol, no blue zone is seen corresponding to a quenching zone seen when examined in ultraviolet light at 254 nm (colophony). In the upper and lower part of the chromatogram obtained with the test solution, other faint blue zones may be seen.

TESTS

Relative density (2.2.5): 1.14 to 1.17.

Saponification value (2.5.6): 230 to 255, determined on the residue obtained in the assay.

Artificial balsams. Shake 0.20 g with 6 ml of *light petroleum R1*. The light petroleum solution is clear and colourless and the whole of the insoluble parts of the balsam stick to the wall of the test-tube.

Fatty oils. Shake 1 g with 3 ml of a 1000 g/l solution of *chloral hydrate R*. The resulting solution is as clear as the 1000 g/l solution of *chloral hydrate R*.

Turpentine. Evaporate to dryness 4 ml of the solution obtained in the test for artificial balsams. The residue has no odour of turpentine.

ASSAY

To 2.50 g in a separating funnel add 7.5 ml of *dilute sodium hydroxide solution R* and 40 ml of *peroxide-free ether R* and shake vigorously for 10 min. Separate the lower layer and shake it with three quantities, each of 15 ml, of *peroxide-free ether R*. Combine the ether layers, dry over 10 g of *anhydrous sodium sulphate R* and filter. Wash the sodium sulphate with two quantities, each of 10 ml, of *peroxide-free ether R*. Combine the ether layers and evaporate to dryness. Dry the residue (esters) at 100 °C to 105 °C for 30 min and weigh.

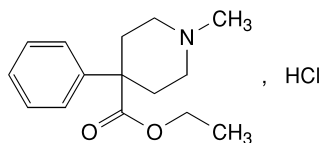
STORAGE

Store protected from light.

01/2005:0420

PETHIDINE HYDROCHLORIDE

Pethidini hydrochloridum

C₁₅H₂₂ClNO₂M_r 283.8

DEFINITION

Ethyl 1-methyl-4-phenylpiperidine-4-carboxylate hydrochloride.

Content: 99.0 per cent to 101.0 per cent (dried substance).

PRODUCTION

If intended for use in the manufacture of parenteral dosage forms, the manufacturing process is validated to show that the content of impurity B is not more than 0.1 ppm.

CHARACTERS

Appearance: white, crystalline powder.

Solubility: very soluble in water, freely soluble in alcohol.

IDENTIFICATION

First identification: B, D.

Second identification: A, C, D.

A. Melting point (2.2.14): 187 °C to 190 °C.

B. Infrared absorption spectrophotometry (2.2.24).

Comparison: Ph. Eur. reference spectrum of pethidine hydrochloride.

C. Dissolve 0.1 g in 10 ml of *ethanol R* and add 10 ml of *picric acid solution R*. A crystalline precipitate is formed which, when washed with *water R* and dried at 100–105 °C, melts (2.2.14) at 186 °C to 193 °C. Mix equal quantities of the precipitate and the substance to be examined and determine the melting point of the mixture. The melting point is at least 20 °C lower than that of the precipitate.

D. To 5 ml of solution S (see Tests) add 5 ml of *water R*. The solution gives reaction (a) of chlorides (2.3.1).

TESTS

Solution S. Dissolve 0.5 g in *carbon dioxide-free water R* and dilute to 25 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and colourless (2.2.2, Method II).

Acidity or alkalinity. To 10 ml of solution S add 0.2 ml of *methyl red solution R* and 0.2 ml of 0.01 M *sodium hydroxide*. The solution is yellow. Add 0.3 ml of 0.01 M *hydrochloric acid*. The solution is red.

Impurity B. Liquid chromatography (2.2.29).

Test solution (a). Dissolve 0.100 g of the substance to be examined in a mixture of 20 volumes of *acetonitrile R* and 80 volumes of *water R* and dilute to 25.0 ml with the same mixture of solvents.

Test solution (b). Dissolve 0.125 g of the substance to be examined in a mixture of 20 volumes of *acetonitrile R* and 80 volumes of *water R* and dilute to 10.0 ml with the same mixture of solvents.

Reference solution (a). Dilute 0.5 ml of test solution (a) to 100.0 ml with a mixture of 20 volumes of *acetonitrile R* and 80 volumes of *water R*.

Reference solution (b). Dissolve 10.0 mg of *pethidine impurity A CRS* in a mixture of 20 volumes of *acetonitrile R* and 80 volumes of *water R* and dilute to 100.0 ml with the same mixture of solvents.

Reference solution (c). Dissolve 12.5 mg of *1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine R* in a mixture of 20 volumes of *acetonitrile R* and 80 volumes of *water R* and dilute to 10.0 ml with the same mixture of solvents. Dilute 1.0 ml of the solution to 100.0 ml with a mixture of 20 volumes of *acetonitrile R* and 80 volumes of *water R*.

Reference solution (d). Dilute 5.0 ml of reference solution (b) and 1.0 ml of reference solution (c) to 100.0 ml with a mixture of 20 volumes of *acetonitrile R* and 80 volumes of *water R*.

Column:

— size: *l* = 0.25 m, Ø = 4.0 mm,

- *stationary phase*: spherical *end-capped octadecylsilyl silica gel for chromatography R* (5 µm) with a specific surface area of 340 m²/g, a pore size of 10 nm and a carbon loading of 19 per cent.

Mobile phase:

- *mobile phase A*: mix equal volumes of a 42.0 g/l solution of *sodium perchlorate R* and of a 11.6 g/l solution of *phosphoric acid R*, adjust to pH 2.0 with *triethylamine R*,
- *mobile phase B*: *acetonitrile R*,

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)
0 - 15	80 → 75	20 → 25
15 - 31	75 → 55	25 → 45
31 - 40	55	45
40 - 41	55 → 80	45 → 20
41 - 50	80	20

Flow rate: 1.0 ml/min.

Detection: spectrophotometer at 210 nm.

Injection: 50 µl; inject test solution (b) and reference solution (d).

Relative retention with reference to pethidine (retention time = about 24 min): impurity B = about 0.66; impurity A = about 0.68.

System suitability: reference solution (d):

- *signal-to-noise ratio*: minimum 10 for the first peak,
- *peak-to-valley ratio*: minimum 4, where H_p = height above the baseline of the peak due to impurity B, and H_v = height above the baseline of the lowest point of the curve separating this peak from the peak due to impurity A.

Limit:

- *impurity B*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (d) (10 ppm) if intended for non-parenteral use.

Related substances. Liquid chromatography (2.2.29) as described in the test for impurity B with the following modifications.

Injection: 20 µl; inject test solution (a) and reference solution (a).

Limits:

- *any impurity*: not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent),
- *total*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (1.0 per cent),
- *disregard limit*: 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

Loss on drying (2.2.32): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 100-105 °C.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.220 g in 50 ml of *alcohol R*. Add 5.0 ml of 0.01 M *hydrochloric acid*. Titrate with 0.1 M *sodium hydroxide* determining the end-point potentiometrically (2.2.20). Read the volume added between the 2 points of inflexion.

1 ml of 0.1 M *sodium hydroxide* is equivalent to 28.38 mg of C₁₅H₁₂N₂O.

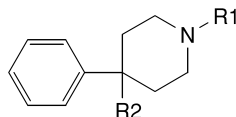
STORAGE

In an airtight container, protected from light.

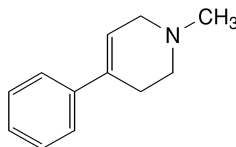
LABELLING

The label states, where applicable, that the substance is suitable for use in the manufacture of parenteral dosage forms.

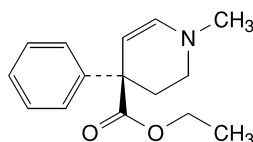
IMPURITIES



- A. R1 = CH₃, R2 = H: 1-methyl-4-phenylpiperidine (MPP),
- C. R1 = CH₃, R2 = CO₂H: 1-methyl-4-phenylpiperidine-4-carboxylic acid,
- D. R1 = CH₃, R2 = CO₂-CH₃: methyl 1-methyl-4-phenylpiperidine-4-carboxylate,
- E. R1 = H, R2 = CO₂-CH₂-CH₃: ethyl 4-phenylpiperidine-4-carboxylate,
- F. R1 = CH₂-C₆H₅, R2 = CO₂H: 1-benzyl-4-phenylpiperidine-4-carboxylic acid,
- G. R1 = CH₃, R2 = CO₂-CH(CH₃)₂: 1-methylethyl 1-methyl-4-phenylpiperidine-4-carboxylate,
- H. R1 = CH₂-C₆H₅, R2 = CO₂-CH₂-CH₃: ethyl 1-benzyl-4-phenylpiperidine-4-carboxylate,
- J. R1 = CH₂-CH₃, R2 = CO₂-CH₂-CH₃: ethyl 1-ethyl-4-phenylpiperidine-4-carboxylate,



- B. 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP),



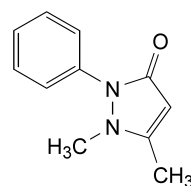
and enantiomer

- I. ethyl (4RS)-1-methyl-4-phenyl-1,2,3,4-tetrahydropyridine-4-carboxylate.

01/2005:0421

PHENAZONE

Phenazonum



C₁₁H₁₂N₂O

M_r 188.2

DEFINITION

Phenazone contains not less than 99.0 per cent and not more than the equivalent of 100.5 per cent of 1,5-dimethyl-2-phenyl-1,2-dihydro-3H-pyrazol-3-one, calculated with reference to the dried substance.

CHARACTERS

01/2005:1357

A white or almost white, crystalline powder or colourless crystals, very soluble in water, in alcohol and in methylene chloride.

IDENTIFICATION

First identification: A, B.

Second identification: A, C, D.

- A. Melting point (2.2.14): 109 °C to 113 °C.
- B. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *phenazone CRS*. Examine the substances prepared as discs using *potassium bromide R*.
- C. To 1 ml of solution S (see Tests) add 4 ml of *water R* and 0.25 ml of *dilute sulphuric acid R*. Add 1 ml of *sodium nitrite solution R*. A green colour develops.
- D. To 1 ml of solution S add 4 ml of *water R* and 0.5 ml of *ferric chloride solution R2*. A red colour develops which is discharged on the addition of *dilute sulphuric acid R*.

TESTS

Solution S. Dissolve 2.5 g in *carbon dioxide-free water R* and dilute to 50 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and colourless (2.2.2, *Method II*).

Acidity or alkalinity. To 10 ml of solution S add 0.1 ml of *phenolphthalein solution R*. The solution is colourless. Add 0.2 ml of 0.01 M *sodium hydroxide*. The solution is red. Add 0.25 ml of *methyl red solution R* and 0.4 ml of 0.01 M *hydrochloric acid*. The solution is red or yellowish-red.

Chlorides (2.4.4). 10 ml of solution S diluted to 15 ml with *water R* complies with the limit test for chlorides (100 ppm).

Sulphates (2.4.13). Dissolve 1.5 g in *distilled water R* and dilute to 15 ml with the same solvent. The solution complies with the limit test for sulphates (100 ppm).

Heavy metals (2.4.8). 12 ml of solution S complies with limit test A for heavy metals (20 ppm). Prepare the standard using *lead standard solution (1 ppm Pb R)*.

Loss on drying (2.2.32). Not more than 1.0 per cent, determined on 1.000 g by drying *in vacuo* at 60 °C for 6 h.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.150 g in 20 ml of *water R*. Add 2 g of *sodium acetate R* and 25.0 ml of 0.05 M *iodine*. Allow to stand protected from light for 30 min. Add 25 ml of *methylene chloride R* and shake until the precipitate dissolves. Titrate with 0.1 M *sodium thiosulphate*, using 1 ml of *starch solution R*, added towards the end of the titration, as indicator. Carry out a blank titration.

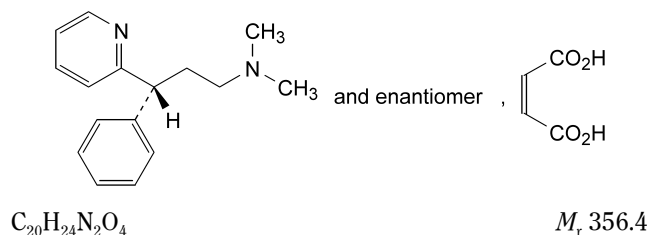
1 ml of 0.05 M *iodine* is equivalent to 9.41 mg of $C_{20}H_{24}N_2O_4$.

STORAGE

Store protected from light.

PHENIRAMINE MALEATE

Pheniramine maleate



DEFINITION

Pheniramine maleate contains not less than 98.0 per cent and not more than the equivalent of 102.0 per cent of (3*RS*)-*N,N*-dimethyl-3-phenyl-3-(pyridin-2-yl)propan-1-amine (*Z*)-butenedioate, calculated with reference to the dried substance.

CHARACTERS

A white, crystalline powder, very soluble in water, freely soluble in alcohol, in methanol and in methylene chloride.

IDENTIFICATION

First identification: C, D.

Second identification: A, B, D.

- A. Melting point (2.2.14): 106 °C to 109 °C.
- B. Dissolve 40.0 mg in 0.1 M *hydrochloric acid* and dilute to 100.0 ml with the same acid. Dilute 5.0 ml of this solution to 50.0 ml with 0.1 M *hydrochloric acid*. Examined between 220 nm and 320 nm (2.2.25), the solution shows a shoulder at 261 nm and an absorption maximum at 265 nm. The specific absorbance at the maximum is 200 to 220.
- C. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *pheniramine maleate CRS*. Examine the substances prepared as discs.
- D. Examine by thin-layer chromatography (2.2.27), using as the coating substance a suitable silica gel with a fluorescent indicator having an optimal intensity at 254 nm.

Test solution. Dissolve 0.10 g of the substance to be examined in *methanol R* and dilute to 5.0 ml with the same solvent.

Reference solution (a). Dissolve 65 mg of *maleic acid R* in *methanol R* and dilute to 10 ml with the same solvent.

Reference solution (b). Dissolve 0.10 g of *pheniramine maleate CRS* in *methanol R* and dilute to 5.0 ml with the same solvent.

Apply to the plate 5 µl of each solution. Develop over a path of 12 cm using a mixture of 3 volumes of *water R*, 7 volumes of *anhydrous formic acid R*, 20 volumes of *methanol R* and 70 volumes of *di-isopropyl ether R*. Examine in ultraviolet light at 254 nm. The chromatogram obtained with the test solution shows two clearly separated spots. The upper spot is similar in position and size to the spot in the chromatogram obtained with reference solution (a). The lower spot is similar in position and size to the spot in the chromatogram obtained with reference solution (b).

TESTS

Solution S. Dissolve 2.0 g in *water R* and dilute to 20 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and not more intensely coloured than reference solution BY₆ (2.2.2, *Method II*).

pH (2.2.3). Dissolve 0.20 g in 20.0 ml of *carbon dioxide-free water R*. The pH of the solution is 4.5 to 5.5.

Optical rotation (2.2.7). The angle of optical rotation, determined on solution S, is -0.10° to $+0.10^{\circ}$.

Related substances. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 20.0 mg of the substance to be examined in a mixture of 1 volume of *acetonitrile R* and 9 volumes of mobile phase A and dilute to 20.0 ml with the same mixture of solvents.

Reference solution (a). Dissolve 10.0 mg of *2-benzylpyridine R* in 10.0 ml of the test solution. Dilute to 100.0 ml with a mixture of 1 volume of *acetonitrile R* and 9 volumes of mobile phase A.

Reference solution (b). Dilute 2.0 ml of the test solution to 100.0 ml with a mixture of 1 volume of *acetonitrile R* and 9 volumes of mobile phase A. Dilute 1.0 ml of this solution to 10.0 ml with a mixture of 1 volume of *acetonitrile R* and 9 volumes of mobile phase A.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.30 m long and 3.9 mm in internal diameter packed with *dimethyloctadecylsilyl silica gel for chromatography R* (10 μ m),
- as mobile phase at a flow rate of 1 ml/min:

Mobile phase A. A 5.056 g/l solution of *sodium heptanesulphonate R* adjusted to pH 2.5 with *phosphoric acid R*,

Mobile phase B. *Acetonitrile R*,

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)	Comment
0	90	10	equilibration
0 - 35	90 $\rightarrow$ 62	10 $\rightarrow$ 38	linear gradient
35 - 37	62 $\rightarrow$ 90	38 $\rightarrow$ 10	linear gradient

- as detector a spectrophotometer set at 264 nm.

Inject 20 μ l of each solution. Adjust the sensitivity of the system so that the height of the principal peak in the chromatogram obtained with reference solution (b) is at least 50 per cent of the full scale of the recorder. The test is not valid unless: the chromatogram obtained with reference solution (a) shows three principal peaks (maleic acid, 2-benzylpyridine and pheniramine in order of elution); the resolution between the peaks corresponding to 2-benzylpyridine and pheniramine is at least 8. In the chromatogram obtained with the test solution: the area of any peak, apart from the principal peak and the peak due to maleic acid, is not greater than the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent); the sum of the areas of all the peaks, apart from the principal peak and the peak due to maleic acid, is not greater than five times the area of the principal peak in the chromatogram obtained with reference solution (b) (1 per cent). Disregard any peak with an area less than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b).

Heavy metals (2.4.8). 1.0 g complies with limit test C for heavy metals (20 ppm). Prepare the standard using 2 ml of *lead standard solution* (10 ppm Pb) *R*.

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 60 $^{\circ}$ C *in vacuo* for 3 h.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

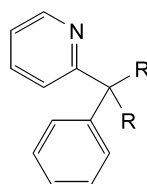
Dissolve 0.260 g in 50 ml of *anhydrous acetic acid R*. Titrate with 0.1 M *perchloric acid*, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *perchloric acid* is equivalent to 17.82 mg of C₂₀H₂₄N₂O₄.

STORAGE

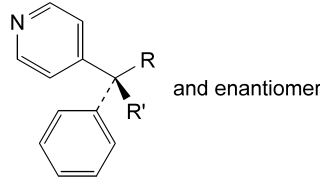
Store protected from light.

IMPURITIES



A. R = H: 2-benzylpyridine,

D. R = CH₂-CH₂-N(CH₃)₂: *N,N,N',N'*-tetramethyl-3-phenyl-3-(pyridin-2-yl)pentane-1,5-diamine.



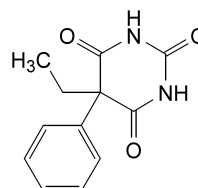
B. R = R' = H: 4-benzylpyridine,

C. R = CH₂-CH₂-N(CH₃)₂, R' = H: (3*RS*)-*N,N*-dimethyl-3-phenyl-3-(pyridin-4-yl)propan-1-amine.

01/2005:0201

PHENOBARBITAL

Phenobarbitalum

C₁₂H₁₂N₂O₃*M*_r 232.2

DEFINITION

Phenobarbital contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of 5-ethyl-5-phenylpyrimidine-2,4,6(1*H*,3*H*,5*H*)-trione, calculated with reference to the dried substance.

CHARACTERS

A white, crystalline powder or colourless crystals, very slightly soluble in water, freely soluble in alcohol. It forms water-soluble compounds with alkali hydroxides and carbonates and with ammonia.

IDENTIFICATION

First identification: A, B.

Second identification: A, C, D.

- A. Determine the melting point (2.2.14) of the substance to be examined. Mix equal parts of the substance to be examined and *phenobarbital CRS* and determine the melting point of the mixture. The difference between the melting points (which are about 176 °C) is not greater than 2 °C.
- B. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *phenobarbital CRS*.
- C. Examine by thin-layer chromatography (2.2.27), using *silica gel GF₂₅₄ R* as the coating substance.

Test solution. Dissolve 0.1 g of the substance to be examined in *alcohol R* and dilute to 100 ml with the same solvent.

Reference solution. Dissolve 0.1 g of *phenobarbital CRS* in *alcohol R* and dilute to 100 ml with the same solvent.

Apply separately to the plate 10 µl of each solution. Develop over a path of 18 cm using the lower layer of a mixture of 5 volumes of *concentrated ammonia R*, 15 volumes of *alcohol R* and 80 volumes of *chloroform R*. Examine immediately in ultraviolet light at 254 nm. The principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with the reference solution.

- D. It gives the reaction of non-nitrogen substituted barbiturates (2.3.1).

TESTS

Appearance of solution. Dissolve 1.0 g in a mixture of 4 ml of *dilute sodium hydroxide solution R* and 6 ml of *water R*. The solution is clear (2.2.1) and not more intensely coloured than reference solution Y₆ (2.2.2, *Method II*).

Acidity. Boil 1.0 g with 50 ml of *water R* for 2 min, allow to cool and filter. To 10 ml of the filtrate add 0.15 ml of *methyl red solution R*. The solution is orange-yellow. Not more than 0.1 ml of 0.1 M *sodium hydroxide* is required to produce a pure yellow colour.

Related substances. Examine by thin-layer chromatography (2.2.27), using *silica gel GF₂₅₄ R* as the coating substance.

Test solution. Dissolve 1.0 g of the substance to be examined in *alcohol R* and dilute to 100 ml with the same solvent.

Reference solution. Dilute 0.5 ml of the test solution to 100 ml with *alcohol R*.

Apply separately to the plate 20 µl of each solution. Develop over a path of 15 cm using the lower layer of a mixture of 5 volumes of *concentrated ammonia R*, 15 volumes of *alcohol R* and 80 volumes of *chloroform R*. Examine immediately in ultraviolet light at 254 nm. Spray with *diphenylcarbazone mercuric reagent R*. Allow the plate to dry in air and spray with freshly prepared *alcoholic potassium hydroxide solution R* diluted 1 in 5 with *aldehyde-free alcohol R*. Heat at 100 °C to 105 °C for 5 min and examine immediately. When examined in ultraviolet light and after spraying, any spot in the chromatogram obtained with the test solution, apart from the principal spot, is not more intense than the spot in the chromatogram obtained with the reference solution (0.5 per cent).

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.00 g by drying in an oven at 100 °C to 105 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

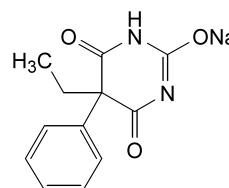
Dissolve 0.100 g in 5 ml of *pyridine R*. Add 0.5 ml of *thymolphthalein solution R* and 10 ml of *silver nitrate solution in pyridine R*. Titrate with 0.1 M *ethanolic sodium hydroxide* until a pure blue colour is obtained. Carry out a blank titration.

1 ml of 0.1 M *ethanolic sodium hydroxide* is equivalent to 11.61 mg of C₁₂H₁₁N₂O₃.

01/2005:0630

PHENOBARBITAL SODIUM

Phenobarbitalum natricum



C₁₂H₁₁N₂NaO₃

M_r 254.2

DEFINITION

Phenobarbital sodium contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of the sodium derivative of 5-ethyl-5-phenylpyrimidine-2,4,6(1*H*,3*H*,5*H*)-trione, calculated with reference to the dried substance.

CHARACTERS

A white, crystalline powder, hygroscopic, freely soluble in carbon dioxide-free water (a small fraction may be insoluble), soluble in alcohol, practically insoluble in methylene chloride.

IDENTIFICATION

First identification: A, B, E.

Second identification: A, C, D, E.

- A. Acidify 10 ml of solution S (see Tests) with *dilute hydrochloric acid R* and shake with 20 ml of *ether R*. Separate the ether layer, wash with 10 ml of *water R*, dry over *anhydrous sodium sulphate R* and filter. Evaporate the filtrate to dryness and dry the residue at 100 °C to 105 °C. Determine the melting point (2.2.14) of the test residue. Mix equal parts of the residue and of *phenobarbital CRS* and determine the melting point of the mixture. The difference between the two melting points (which are about 176 °C) is not greater than 2 °C.
- B. Examine by infrared absorption spectrophotometry (2.2.24), comparing the residue obtained during identification test A with the spectrum obtained with *phenobarbital CRS*. If the spectra obtained in the solid state show differences, dissolve the test residue and the reference substance separately in *ethanol R*, evaporate to dryness and record the spectra again.
- C. Examine by thin-layer chromatography (2.2.27), using *silica gel GF₂₅₄ R* as the coating substance.
Test solution. Dissolve 0.10 g of the substance to be examined in *alcohol (50 per cent V/V) R* and dilute to 100 ml with the same solvent.
Reference solution. Dissolve 90 mg of *phenobarbital CRS* in *alcohol (50 per cent V/V) R* and dilute to 100 ml with the same solvent.

01/2005:0631

Apply separately to the plate 10 µl of each solution. Develop over a path of 18 cm using the lower layer from a mixture of 5 volumes of *concentrated ammonia R*, 15 volumes of *alcohol R* and 80 volumes of *chloroform R*. Examine immediately in ultraviolet light at 254 nm. The principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with the reference solution.

D. It gives the reaction of non-nitrogen substituted barbiturates (2.3.1).

E. It gives reaction (a) of sodium (2.3.1).

TESTS

Solution S. Dissolve 5.0 g in *alcohol (50 per cent V/V) R* and dilute to 50 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and not more intensely coloured than reference solution Y₇ (2.2.2, *Method II*).

pH (2.2.3). Dissolve 5.0 g as completely as possible in *carbon dioxide-free water R* and dilute to 50 ml with the same solvent. The pH of the solution is not greater than 10.2.

Related substances. Examine by thin-layer chromatography (2.2.27), using *silica gel GF₂₅₄ R* as the coating substance.

Test solution. Dissolve 1.0 g of the substance to be examined in *alcohol (50 per cent V/V) R* and dilute to 100 ml with the same solvent.

Reference solution. Dilute 0.5 ml of the test solution to 100 ml with *alcohol (50 per cent V/V) R*.

Apply separately to the plate 20 µl of each solution. Develop over a path of 15 cm using the lower layer from a mixture of 5 volumes of *concentrated ammonia R*, 15 volumes of *alcohol R* and 80 volumes of *chloroform R*. Examine immediately in ultraviolet light at 254 nm. Spray with *diphenylcarbazone mercuric reagent R*. Allow the plate to dry in air and spray with freshly prepared *alcoholic potassium hydroxide solution R* diluted 1 in 5 with *aldehyde-free alcohol R*. Heat at 100 °C to 105 °C for 5 min and examine immediately. When examined in ultraviolet light and after spraying, any spot in the chromatogram obtained with the test solution, apart from the principal spot, is not more intense than the spot in the chromatogram obtained with the reference solution (0.5 per cent). Disregard any spot at the starting-point.

Loss on drying (2.2.32). Not more than 7.0 per cent, determined on 0.500 g by drying in an oven at 150 °C for 4 h.

ASSAY

Dissolve 0.150 g in 2 ml of *water R* and add 8 ml of 0.05 M *sulphuric acid*. Heat to boiling and cool. Add 30 ml of *methanol R* and shake until dissolution is complete. Carry out a potentiometric titration (2.2.20), using 0.1 M *sodium hydroxide*. After the first point of inflexion, interrupt the addition of sodium hydroxide, add 10 ml of *pyridine R*, mix and continue the titration. Read the volume added between the two points of inflexion.

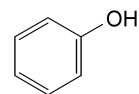
1 ml of 0.1 M *sodium hydroxide* is equivalent to 25.42 mg of C₁₂H₁₁N₂NaO₃.

STORAGE

Store in an airtight container.

PHENOL

Phenolum

C₆H₆OM_r 94.1

DEFINITION

Phenol contains not less than 99.0 per cent and not more than the equivalent of 100.5 per cent of C₆H₆O.

CHARACTERS

Colourless or faintly pink or faintly yellowish crystals or crystalline masses, deliquescent, soluble in water, very soluble in alcohol, in glycerol and in methylene chloride.

IDENTIFICATION

- Dissolve 0.5 g in 2 ml of *concentrated ammonia R*. The substance dissolves completely. Dilute to about 100 ml with *water R*. To 2 ml of the dilute solution add 0.05 ml of *strong sodium hypochlorite solution R*. A blue colour develops and becomes progressively more intense.
- To 1 ml of solution S (see Tests) add 10 ml of *water R* and 0.1 ml of *ferric chloride solution R1*. A violet colour is produced which disappears on addition of 5 ml of *2-propanol R*.
- To 1 ml of solution S add 10 ml of *water R* and 1 ml of *bromine water R*. A pale-yellow precipitate is formed.

TESTS

Solution S. Dissolve 1.0 g in *water R* and dilute to 15 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and not more intensely coloured than reference solution B₆ (2.2.2, *Method II*).

Acidity. To 2 ml of solution S add 0.05 ml of *methyl orange solution R*. The solution is yellow.

Freezing point (2.2.18). Not less than 39.5 °C.

Residue on evaporation. Not more than 0.05 per cent, determined by evaporating 5.000 g to dryness on a water-bath and drying the residue at 100 °C to 105 °C for 1 h.

ASSAY

Dissolve 2.000 g in *water R* and dilute to 1000.0 ml with the same solvent. Transfer 25.0 ml of the solution to a ground-glass-stoppered flask and add 50.0 ml of 0.0167 M *bromide-bromate* and 5 ml of *hydrochloric acid R*, close the flask, allow to stand with occasional swirling for 30 min and then allow to stand for a further 15 min. Add 5 ml of a 200 g/l solution of *potassium iodide R*, shake and titrate with 0.1 M *sodium thiosulphate* until a faint yellow colour remains. Add 0.5 ml of *starch solution R* and 10 ml of *chloroform R* and continue the titration with vigorous shaking. Carry out a blank titration.

1 ml of 0.0167 M *bromide-bromate* is equivalent to 1.569 mg of C₆H₆O.

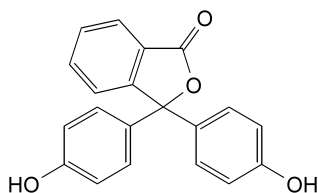
STORAGE

Store in an airtight container, protected from light.

01/2005:1584

PHENOLPHTHALEIN

Phenolphthaleinum

 $C_{20}H_{14}O_4$ M_r 318.3

DEFINITION

Phenolphthalein contains not less than 98.0 per cent and not more than the equivalent of 101.0 per cent of 3,3-bis(4-hydroxyphenyl)isobenzofuran-1(3*H*)-one, calculated with reference to the dried substance.

CHARACTERS

A white or almost white powder, practically insoluble in water, soluble in alcohol.

It melts at about 260 °C.

IDENTIFICATION

- A. Dissolve 25.0 mg in *alcohol R* and dilute to 100.0 ml with the same solvent (solution A). To 2.0 ml of solution A add 5.0 ml of 1 *M* hydrochloric acid and dilute to 50.0 ml with *alcohol R* (solution A₁). To 10.0 ml of solution A add 5.0 ml of 1 *M* hydrochloric acid and dilute to 50.0 ml with *alcohol R* (solution A₂). To 2.0 ml of solution A add 5.0 ml of 1 *M* sodium hydroxide and dilute to 50.0 ml with *alcohol R* (solution B). Examined between 220 nm and 250 nm (2.2.25), solution A₁ shows an absorption maximum at 229 nm. The specific absorbance at the maximum at 229 nm is 922 to 1018. Examined between 250 nm and 300 nm, solution A₂ shows an absorption maximum at 276 nm. The specific absorbance at the maximum at 276 nm is 142 to 158. Examined between 230 nm and 270 nm, solution B shows an absorption maximum at 249 nm. The specific absorbance at the maximum at 249 nm is 744 to 822.
- B. Dissolve about 10 mg in *alcohol R*. Add 1 ml of dilute sodium hydroxide solution *R*. The solution is red. Add 5 ml of dilute sulphuric acid *R*. The colour disappears.

TESTS

Solution S. To 2.0 g add 40 ml of distilled water *R* and heat to boiling. Cool and filter.

Appearance of solution. Dissolve 0.20 g in 5 ml of *alcohol R*. The solution is clear (2.2.1) and not more intensely coloured than reference solution Y₇ (2.2.2, Method II).

Acidity or alkalinity. To 10 ml of solution S add 0.15 ml of bromothymol blue solution *R1*. Add 0.05 ml of 0.01 *M* hydrochloric acid, the solution is yellow. Add 0.10 ml of 0.01 *M* sodium hydroxide, the solution is blue.

Related substances. Examine by thin-layer chromatography (2.2.27), using a TLC silica gel F₂₅₄ plate *R*.

Test solution. Dissolve 0.5 g of the substance to be examined in *alcohol R* and dilute to 10 ml with the same solvent.

Reference solution (a). Dilute 1 ml of the test solution to 10 ml with *alcohol R*. Dilute 5 ml of this solution to 100 ml with *alcohol R*.

Reference solution (b). Dissolve 25 mg of fluorene *R* in *alcohol R*, add 0.5 ml of the test solution and dilute to 10 ml with *alcohol R*.

Apply to the plate 5 µl of the test solution and 5 µl of each of the reference solutions. Develop over a path corresponding to two-thirds of the plate height using a mixture of 50 volumes of *acetone R* and 50 volumes of *methylene chloride R*. Allow the plate to dry in air. Examine in ultraviolet light at 254 nm and re-examine after exposure to ammonia vapour. Any spot in the chromatogram obtained with the test solution, apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (a) (0.5 per cent). The test is not valid unless the chromatogram obtained with reference solution (b) shows 2 clearly separated spots.

Chlorides (2.4.4). Dilute 10 ml of solution S to 15 ml with *water R*. The solution complies with the limit test for chlorides (100 ppm).

Sulphates (2.4.13). 15 ml of solution S complies with the limit test for sulphates (200 ppm).

Heavy metals (2.4.8). Heat 3 g with 50 ml of dilute hydrochloric acid *R* on a water-bath for 5 min and filter. Evaporate the filtrate almost to dryness and dissolve the residue in 30 ml of *water R*. 12 ml of this solution complies with limit test A for heavy metals (10 ppm). Prepare the standard using 10 ml of lead standard solution (1 ppm Pb) *R*.

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.100 g in 5 ml of dimethylformamide *R*. Add 5 ml of sodium carbonate solution *R*, 10 ml of sodium hydrogen carbonate solution *R*, 35 ml of *water R* and 50.0 ml of 0.05 *M* iodine. Add 10 ml of *methylene chloride R* and 20 ml of dilute sulphuric acid *R*. Titrate the excess of iodine with 0.1 *M* sodium thiosulphate, using 0.3 ml of starch solution *R* added towards the end of the titration, as indicator. Carry out a blank titration.

1 ml of 0.05 *M* iodine is equivalent to 3.979 mg of C₂₀H₁₄O₄.

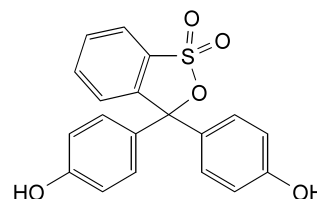
STORAGE

Store protected from light.

01/2005:0242

PHENOLSULFONPHTHALEIN

Phenolsulfonphthaleinum

 $C_{19}H_{14}O_5S$ M_r 354.4

DEFINITION

Phenolsulfonphthalein (phenol red) contains not less than 98.0 per cent and not more than the equivalent of 102.0 per cent of 3,3-bis(4-hydroxyphenyl)-3*H*-2,1-benzoxathiole 1,1-dioxide, calculated with reference to the dried substance.

CHARACTERS

A bright-red to dark-red, crystalline powder, very slightly soluble in water, slightly soluble in alcohol.

IDENTIFICATION

- A. Dissolve 10 mg in a 10 g/l solution of *sodium carbonate R* and dilute to 200.0 ml with the sodium carbonate solution. Dilute 5.0 ml of the solution to 100.0 ml with a 10 g/l solution of *sodium carbonate R*. Examined between 400 nm and 630 nm (2.2.25), the solution shows an absorption maximum at 558 nm. The specific absorbance at the maximum is 1900 to 2100.
- B. Dissolve about 10 mg in 1 ml of *dilute sodium hydroxide solution R* and add 9 ml of *water R*. The solution is deep red. To 5 ml of the solution add a slight excess of *dilute sulphuric acid R*. The colour becomes orange.
- C. To 5 ml of the solution prepared for identification test B add 1 ml of 0.0167 M bromide-bromate and 1 ml of *dilute hydrochloric acid R*, shake and allow to stand for 15 min. Make alkaline with *dilute sodium hydroxide solution R*. An intense violet-blue colour is produced.

TESTS

Related substances. Examine by thin-layer chromatography (2.2.27), using *silica gel GF₂₅₄ R* as the coating substance.

Test solution. Dissolve 0.1 g of the substance to be examined in 0.1 M *sodium hydroxide* and dilute to 5 ml with the same solvent.

Reference solution. Dilute 0.5 ml of the test solution to 100 ml with 0.1 M *sodium hydroxide*.

Apply separately to the plate 10 µl of each solution. Develop over a path of 15 cm using a mixture of 25 volumes of *glacial acetic acid R*, 25 volumes of *water R* and 100 volumes of *tert-pentyl alcohol R*. Allow the plate to dry in air until the solvent has evaporated and expose the plate to the vapour from *concentrated ammonia R*. Examine in ultraviolet light at 254 nm. Not more than one spot, apart from the principal spot, appears in the chromatogram obtained with the test solution and this spot is not more intense than the spot in the chromatogram obtained with the reference solution (0.5 per cent).

Insoluble matter. To 1.0 g of the finely powdered substance to be examined add 12 ml of *sodium hydrogen carbonate solution R*. Allow to stand for 1 h, shaking frequently. Dilute to 100 ml with *water R* and allow to stand for 15 h. Centrifuge at 2000 g to 3000 g, for 30 min, decant the supernatant liquid and wash the residue with 25 ml of a 10 g/l solution of *sodium hydrogen carbonate R* and then 25 ml of *water R*. Dry at 100 °C to 105 °C. The residue weighs not more than 5 mg (0.5 per cent).

Loss on drying (2.2.32). Not more than 1.0 per cent, determined on 1.00 g of the powdered substance to be examined by drying in an oven at 100 °C to 105 °C.

Sulphated ash (2.4.14). Not more than 0.2 per cent, determined on 0.5 g.

ASSAY

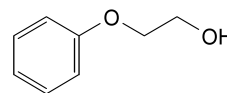
Dissolve 0.900 g in 15 ml of 1 M *sodium hydroxide* and dilute to 250.0 ml with *water R*. To 10.0 ml of the solution in a glass-stoppered flask add 25 ml of *glacial acetic acid R*, 20.0 ml of 0.0167 M *potassium bromate*, 5 ml of a 100 g/l solution of *potassium bromide R* and 5 ml of *hydrochloric acid R*. Allow to stand protected from light for 15 min, add 10 ml of a 100 g/l solution of *potassium iodide R* and titrate immediately with 0.1 M *sodium thiosulphate*, using 0.1 ml of *starch solution R* as indicator.

1 ml of 0.0167 M *potassium bromate* is equivalent to 4.43 mg of C₈H₁₀O₂S.

01/2005:0781

PHENOXYETHANOL

Phenoxyethanolum

C₈H₁₀O₂M_r 138.2

DEFINITION

Phenoxyethanol contains not less than 99.0 per cent and not more than the equivalent of 100.5 per cent *m/m* of 2-phenoxyethanol.

CHARACTERS

A colourless, slightly viscous liquid, slightly soluble in water, miscible with acetone, with alcohol and with glycerol, slightly soluble in arachis oil and in olive oil.

IDENTIFICATION

First identification: C.

Second identification: A, B, D.

- A. Refractive index (2.2.6): 1.537 to 1.539.
- B. Dissolve 80.0 mg in *water R* and dilute to 100.0 ml with the same solvent. Dilute 10.0 ml of the solution to 100.0 ml with *water R*. Examined between 240 nm and 350 nm (2.2.25), the solution shows two absorption maxima, at 269 nm and at 275 nm. The specific absorbances at the maxima are 95 to 105 and 75 to 85 respectively.
- C. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *phenoxyethanol CRS*.
- D. Shake 2 ml with a mixture of 4 g of *potassium permanganate R*, 5.4 g of *sodium carbonate R* and 75 ml of *water R* for 30 min. Add 25 g of *sodium chloride R* and stir continuously for 60 min, filter and acidify with *hydrochloric acid R* to about pH 1.7. The melting point of the precipitate, after recrystallisation from *water R*, is 96 °C to 99 °C (2.2.14).

TESTS

Relative density (2.2.5): 1.105 to 1.110.

Related substances. Examine by gas chromatography (2.2.28), using *methyl laurate R* as internal standard.

Internal standard solution. Dissolve 1.25 g of *methyl laurate R* in *methylene chloride R* and dilute to 25 ml with the same solvent.

Test solution (a). Dissolve 5.0 g of the substance to be examined in *methylene chloride R* and dilute to 10.0 ml with the same solvent.

Test solution (b). Dissolve 5.0 g of the substance to be examined in *methylene chloride R*, add 1.0 ml of the internal standard solution and dilute to 10.0 ml with *methylene chloride R*.

Reference solution. To 1.0 ml of test solution (a), add 10.0 ml of the internal standard solution and dilute to 100.0 ml with *methylene chloride R*.

The chromatographic procedure may be carried out using:

01/2005:0148
corrected

- a glass column 1.5 m long and 4 mm in internal diameter packed with *silanised diatomaceous earth for gas chromatography R* (150 µm to 180 µm) impregnated with 3 per cent *m/m* of *polymethylphenylsiloxane R*,
- *nitrogen for chromatography R* as the carrier gas at a flow rate of 30 ml/min,
- a flame-ionisation detector,

maintaining the temperature of the column at 130 °C and that of the injection port and of the detector at 200 °C.

Inject 1 µl of the reference solution and adjust the sensitivity of the detector so that the heights of the two peaks, apart from the solvent peak, are not less than 70 per cent of the full scale of the recorder. The substances elute in the following order: phenoxyethanol and methyl laurate. The test is not valid unless, in the chromatogram obtained with the reference solution, the resolution between the peaks corresponding to phenoxyethanol and methyl laurate is not less than twelve.

Inject 1 µl of test solution (a). In the chromatogram obtained, verify that there is no peak with the same retention time as the internal standard.

Inject separately 1 µl of test solution (b) and the reference solution. Continue the chromatography for five times the retention time of phenoxyethanol (which is about 5 min). From the chromatogram obtained with the reference solution, calculate the ratio (*R*) of the area of the peak due to phenoxyethanol to the area of the peak due to the internal standard. From the chromatogram obtained with test solution (b), calculate the ratio of the sum of the areas of any peaks, apart from the principal peak, the peak due to the internal standard and the peak due to the solvent, to the area of the peak due to the internal standard: this ratio is not greater than *R* (1.0 per cent).

Phenol. Dissolve 1.00 g in 50 ml of *methylene chloride R*, add 1 ml of *dilute sodium hydroxide solution R* and 10 ml of *water R*. Shake. Wash the upper layer with two quantities, each of 20 ml, of *methylene chloride R* and dilute to 100.0 ml with *water R*. The absorbance (2.2.25) of the resulting solution at the maximum at 287 nm is not more than 0.27 (0.1 per cent).

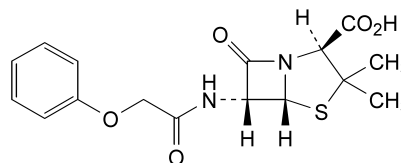
ASSAY

To 2.000 g in an acetylation flask fitted with an air condenser, add 10.0 ml of freshly prepared *acetic anhydride solution R1* and heat with frequent shaking in a water-bath for 45 min. Cool and carefully add 10 ml of *water R*. Heat for a further 2 min. Cool, add 10 ml of *butanol R*, shake vigorously and titrate the excess of acetic acid with 1 M *sodium hydroxide* using 0.2 ml of *phenolphthalein solution R* as indicator. Repeat the procedure without the substance to be examined. The difference between the volumes used in the titrations represents the amount of acetic anhydride required for the acetylation of the substance to be examined.

1 ml of 1 M *sodium hydroxide* is equivalent to 0.1382 g of C₈H₁₀O₂.

PHENOXYMETHYLPENICILLIN

Phenoxyethylpenicillinum



C₁₆H₁₈N₂O₅S

M_r 350.4

DEFINITION

Phenoxyethylpenicillin is (2*S*,5*R*,6*R*)-3,3-dimethyl-7-oxo-6-[(phenoxyacetyl)amino]-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid, a substance produced by the growth of certain strains of *Penicillium notatum* or related organisms on a culture medium containing an appropriate precursor, or obtained by any other means. The sum of the percentage contents of phenoxyethylpenicillin and 4-hydroxyphenoxyethylpenicillin is not less than 95.0 per cent and not more than the equivalent of 100.5 per cent, calculated with reference to the anhydrous substance.

CHARACTERS

A white or almost white, crystalline powder, slightly hygroscopic, very slightly soluble in water, soluble in alcohol.

IDENTIFICATION

First identification: B.

Second identification: A, C, D.

A. It complies with the test for pH (see Tests).

B. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *phenoxyethylpenicillin CRS*.

C. Examine by thin-layer chromatography (2.2.27), using *silanised silica gel H R* as the coating substance.

Test solution. Dissolve 25 mg of the substance to be examined in 5 ml of *acetone R*.

Reference solution (a). Dissolve 25 mg of *phenoxyethylpenicillin CRS* in 5 ml of *acetone R*.

Reference solution (b). Dissolve 25 mg of *benzylpenicillin potassium CRS* and 25 mg of *phenoxyethylpenicillin potassium CRS* in 5 ml of *water R*.

Apply to the plate 1 µl of each solution. Develop over a path of 15 cm using a mixture of 30 volumes of *acetone R* and 70 volumes of a 154 g/l solution of *ammonium acetate R*, adjusted to pH 5.0 with *glacial acetic acid R*. Allow the plate to dry in air and expose it to iodine vapour until the spots appear. Examine in daylight. The principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a). The test is not valid unless the chromatogram obtained with reference solution (b) shows 2 clearly separated spots.

D. Place about 2 mg in a test-tube about 150 mm long and 15 mm in diameter. Moisten with 0.05 ml of *water R* and add 2 ml of *sulphuric acid-formaldehyde reagent R*. Mix the contents of the tube by swirling; the solution is reddish-brown. Place the test-tube on a water-bath for 1 min; a dark reddish-brown colour develops.

TESTS

pH (2.2.3). Suspend 50 mg in 10 ml of *carbon dioxide-free water R*. The pH of the suspension is 2.4 to 4.0.

Specific optical rotation (2.2.7). Dissolve 0.250 g in *butanol R* and dilute to 25.0 ml with the same solvent. The specific optical rotation is + 186 to + 200, calculated with reference to the anhydrous substance.

Related substances. Examine by liquid chromatography (2.2.29) as described under Assay. Inject 20 µl of reference solution (d) and elute isocratically with the chosen mobile phase until elution of the phenoxymethylpenicillin peak. Adjust the sensitivity of the system to obtain a peak with a signal-to-noise ratio of at least 3. Inject 20 µl of reference solution (e). Inject 20 µl of test solution (b) and start the elution isocratically. Immediately after elution of the phenoxymethylpenicillin peak start the following linear gradient.

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)	Comment
0 - 20	60 → 0	40 → 100	linear gradient
20 - 35	0	100	isocratic
35 - 50	0 → 60	100 → 40	re-equilibration

Inject the dissolution mixture and use the same elution pattern to obtain a blank. In the chromatogram obtained with test solution (b), the area of any peak, apart from the principal peak and any peak corresponding to 4-hydroxyphenoxymethylpenicillin, is not greater than the area of the principal peak in the chromatogram obtained with reference solution (e) (1 per cent).

4-Hydroxyphenoxymethylpenicillin. Not more than 4.0 per cent, calculated with reference to the anhydrous substance and determined by liquid chromatography (2.2.29), as described under Assay.

Water (2.5.12). Not more than 0.5 per cent, determined on 1.000 g by the semi-micro determination of water.

ASSAY

Examine by liquid chromatography (2.2.29).

Dissolution mixture. To 250 ml of 0.2 M *potassium dihydrogen phosphate R* add 500 ml of *water R* and adjust to pH 6.5 with an 8.4 g/l solution of *sodium hydroxide R*. Dilute to 1000 ml with *water R*.

Test solution (a). Dissolve 50.0 mg of the substance to be examined in the dissolution mixture and dilute to 50.0 ml with the same mixture.

Test solution (b). Prepare immediately before use. Dissolve 80.0 mg of the substance to be examined in the dissolution mixture and dilute to 20.0 ml with the same mixture.

Reference solution (a). Dissolve 55.0 mg of *phenoxymethylpenicillin potassium CRS* in the dissolution mixture and dilute to 50.0 ml with the same mixture.

Reference solution (b). Dissolve 10.0 mg of *4-hydroxyphenoxymethylpenicillin CRS* in the dissolution mixture and dilute to 25.0 ml with the same mixture. Dilute 5.0 ml of the solution to 100.0 ml with the dissolution mixture.

Reference solution (c). Dissolve 10 mg of *phenoxymethylpenicillin potassium CRS* and 10 mg of *benzylpenicillin sodium CRS* in the dissolution mixture and dilute to 50 ml with the same mixture.

Reference solution (d). Dilute 1.0 ml of reference solution (a) to 20 ml with the dissolution mixture. Dilute 1.0 ml of the solution to 50 ml with the dissolution mixture.

Reference solution (e). Dilute 1.0 ml of reference solution (a) to 25.0 ml with the dissolution mixture.

The chromatographic procedure may be carried out using:

- a column 0.25 m long and 4.6 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (5 µm),

- as mobile phase at a flow rate of 1.0 ml/min:

Mobile phase A. Mix 10 volumes of *phosphate buffer solution pH 3.5 R*, 30 volumes of *methanol R* and 60 volumes of *water R*,

Mobile phase B. Mix 10 volumes of *phosphate buffer solution pH 3.5 R*, 35 volumes of *water R* and 55 volumes of *methanol R*,

- as detector a spectrophotometer set at 254 nm.

Equilibrate the column with a mobile phase ratio A:B of 60:40. Inject 20 µl of reference solution (c). The test is not valid unless, in the chromatogram obtained, the resolution between the 2 principal peaks is at least 6.0 (if necessary, adjust the ratio A:B of the mobile phase) and the mass distribution ratio for the second peak (phenoxymethylpenicillin) is 5.0 to 7.0. Inject reference solution (a) 6 times. The test is not valid unless the relative standard deviation for the area of the principal peak is at most 1.0 per cent. Inject alternately test solution (a) and reference solutions (a) and (b).

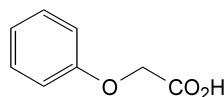
Calculate the percentage content of phenoxymethylpenicillin by multiplying the percentage content of phenoxymethylpenicillin potassium by 0.902. Calculate the percentage content of 4-hydroxyphenoxymethylpenicillin by multiplying if necessary by the correction factor supplied with the CRS.

STORAGE

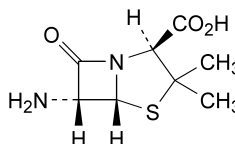
Store in an airtight container.

IMPURITIES

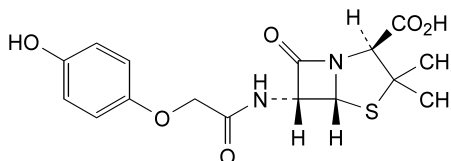
A. benzylpenicillin,



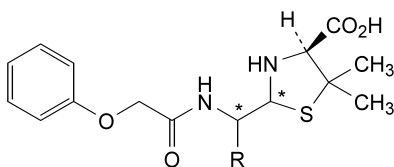
B. phenoxyacetic acid,



C. (2*S*,5*R*,6*R*)-6-amino-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid (6-aminopenicillanic acid),



D. (2*S*,5*R*,6*R*)-3,3-dimethyl-7-oxo-6-[[2-(4-hydroxyphenoxy)acetyl]amino]-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid (4-hydroxyphenoxymethylpenicillin),

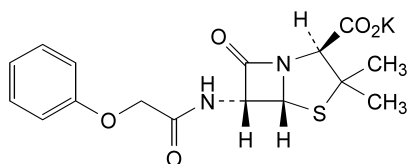


- E. R = CO₂H: (4*S*)-2-[carboxy[(phenoxyacetyl)amino]methyl]-5,5-dimethylthiazolidine-4-carboxylic acid (penicilloic acids of phenoxymethylpenicillin),
- F. R = H: (2*RS*,4*S*)-5,5-dimethyl-2-[(phenoxyacetyl)amino]methylthiazolidine-4-carboxylic acid (penilloic acids of phenoxymethylpenicillin).

01/2005:0149
corrected

PHENOXYMETHYLPENICILLIN POTASSIUM

Phenoxymethylpenicillinum kalicum



C₁₆H₁₇KN₂O₅S

*M*_r 388.5

DEFINITION

Phenoxymethylpenicillin potassium is the potassium salt of (2*S*,5*R*,6*R*)-3,3-dimethyl-7-oxo-6-[(phenoxyacetyl)amino]-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid, a substance produced by the growth of certain strains of *Penicillium notatum* or related organisms on a culture medium containing an appropriate precursor, or obtained by any other means. The sum of the percentage contents of phenoxymethylpenicillin potassium and 4-hydroxyphenoxymethylpenicillin potassium is not less than 95.0 per cent and not more than the equivalent of 100.5 per cent, calculated with reference to the anhydrous substance.

CHARACTERS

A white or almost white, crystalline powder, freely soluble in water, practically insoluble in alcohol.

IDENTIFICATION

First identification: A, D.

Second identification: B, C, D.

- A. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *phenoxymethylpenicillin potassium CRS*.
- B. Examine by thin-layer chromatography (2.2.27), using *silanised silica gel H R* as the coating substance.
Test solution. Dissolve 25 mg of the substance to be examined in 5 ml of *water R*.
Reference solution (a). Dissolve 25 mg of *phenoxymethylpenicillin potassium CRS* in 5 ml of *water R*.

Reference solution (b). Dissolve 25 mg of *benzylpenicillin potassium CRS* and 25 mg of *phenoxymethylpenicillin potassium CRS* in 5 ml of *water R*.

Apply to the plate 1 µl of each solution. Develop over a path of 15 cm using a mixture of 30 volumes of *acetone R* and 70 volumes of a 154 g/l solution of *ammonium*

acetate R, adjusted to pH 5.0 with *glacial acetic acid R*. Allow the plate to dry in air and expose it to iodine vapour until the spots appear. Examine in daylight. The principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a). The test is not valid unless the chromatogram obtained with reference solution (b) shows 2 clearly separated spots.

- C. Place about 2 mg in a test-tube about 150 mm long and 15 mm in diameter. Moisten with 0.05 ml of *water R* and add 2 ml of *sulphuric acid-formaldehyde reagent R*. Mix the contents of the tube by swirling; the solution is reddish-brown. Place the test-tube in a water-bath for 1 min; a dark reddish-brown colour develops.
- D. It gives reaction (a) of potassium (2.3.1).

TESTS

pH (2.2.3). Dissolve 50 mg in *carbon dioxide-free water R* and dilute to 10 ml with the same solvent. The pH of the solution is 5.5 to 7.5.

Specific optical rotation (2.2.7). Dissolve 0.250 g in *carbon dioxide-free water R* and dilute to 25.0 ml with the same solvent. The specific optical rotation is + 215 to + 230, calculated with reference to the anhydrous substance.

Related substances. Examine by liquid chromatography (2.2.29) as described under Assay. Inject 20 µl of reference solution (d) and elute isocratically with the chosen mobile phase until elution of the phenoxymethylpenicillin peak. Adjust the sensitivity of the system to obtain a peak with a signal-to-noise ratio of at least 3. Inject 20 µl of reference solution (e). Inject 20 µl of test solution (b) and start the elution isocratically. Immediately after elution of the phenoxymethylpenicillin peak start the following linear gradient.

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)	Comment
0 - 20	60 → 0	40 → 100	linear gradient
20 - 35	0	100	isocratic
35 - 50	0 → 60	100 → 40	re-equilibration

Inject the dissolution mixture and use the same elution pattern to obtain a blank. In the chromatogram obtained with test solution (b), the area of any peak, apart from the principal peak and any peak corresponding to 4-hydroxyphenoxymethylpenicillin, is not greater than the area of the principal peak in the chromatogram obtained with reference solution (e) (1 per cent).

4-Hydroxyphenoxymethylpenicillin potassium. Not more than 4.0 per cent, calculated with reference to the anhydrous substance and determined by liquid chromatography (2.2.29), as described under Assay.

Water (2.5.12). Not more than 1.0 per cent, determined on 1.000 g by the semi-micro determination of water.

ASSAY

Examine by liquid chromatography (2.2.29).

Dissolution mixture. To 250 ml of 0.2 M *potassium dihydrogen phosphate R* add 500 ml of *water R* and adjust to pH 6.5 with an 8.4 g/l solution of *sodium hydroxide R*. Dilute to 1000 ml with *water R*.

Test solution (a). Dissolve 50.0 mg of the substance to be examined in the dissolution mixture and dilute to 50.0 ml with the same mixture.

Test solution (b). Prepare immediately before use. Dissolve 80.0 mg of the substance to be examined in the dissolution mixture and dilute to 20.0 ml with the same mixture.

Reference solution (a). Dissolve 50.0 mg of *phenoxymethylpenicillin potassium CRS* in the dissolution mixture and dilute to 50.0 ml with the same mixture.

Reference solution (b). Dissolve 10.0 mg of *4-hydroxyphenoxymethylpenicillin CRS* in the dissolution mixture and dilute to 25.0 ml with the same mixture. Dilute 5.0 ml of the solution to 100.0 ml with the dissolution mixture.

Reference solution (c). Dissolve 10 mg of *phenoxymethylpenicillin potassium CRS* and 10 mg of *benzylpenicillin sodium CRS* in the dissolution mixture and dilute to 50 ml with the same mixture.

Reference solution (d). Dilute 1.0 ml of reference solution (a) to 20 ml with the dissolution mixture. Dilute 1.0 ml of the solution to 50 ml with the dissolution mixture.

Reference solution (e). Dilute 1.0 ml of reference solution (a) to 25.0 ml with the dissolution mixture.

The chromatographic procedure may be carried out using:

- a column 0.25 m long and 4.6 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (5 µm),

- as mobile phase at a flow rate of 1.0 ml/min:

Mobile phase A. Mix 10 volumes of *phosphate buffer solution pH 3.5 R*, 30 volumes of *methanol R* and 60 volumes of *water R*,

Mobile phase B. Mix 10 volumes of *phosphate buffer solution pH 3.5 R*, 35 volumes of *water R* and 55 volumes of *methanol R*,

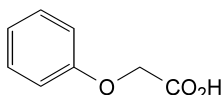
- as detector a spectrophotometer set at 254 nm.

Equilibrate the column with a mobile phase ratio A:B of 60:40. Inject 20 µl of reference solution (c). The test is not valid unless, in the chromatogram obtained, the resolution between the 2 principal peaks is at least 6.0 (if necessary, adjust the ratio A:B of the mobile phase) and the mass distribution ratio for the second peak (phenoxymethylpenicillin) is 5.0 to 7.0. Inject reference solution (a) 6 times. The test is not valid unless the relative standard deviation for the area of the principal peak is at most 1.0 per cent. Inject alternately test solution (a) and reference solutions (a) and (b).

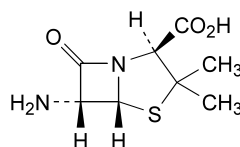
Calculate the percentage content of phenoxymethylpenicillin potassium. Calculate the percentage content of 4-hydroxyphenoxymethylpenicillin potassium by multiplying the percentage content of 4-hydroxyphenoxymethylpenicillin if necessary by the correction factor supplied with the CRS.

IMPURITIES

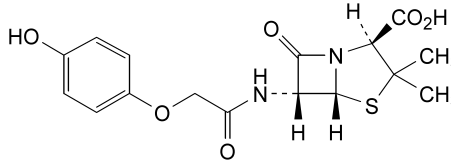
A. benzylpenicillin,



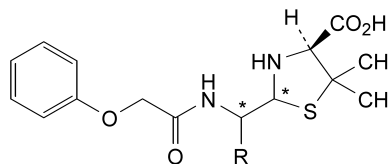
B. phenoxyacetic acid,



C. (2*S*,5*R*,6*R*)-6-amino-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid (6-aminopenicillanic acid),



D. (2*S*,5*R*,6*R*)-3,3-dimethyl-7-oxo-6-[[2-(4-hydroxyphenoxy)acetyl]amino]-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid (4-hydroxyphenoxymethylpenicillin),



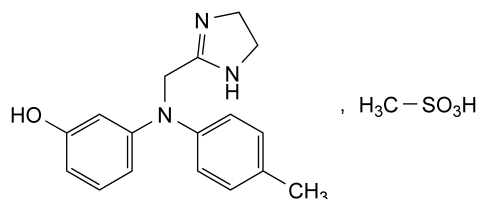
E. R = CO₂H: (4*S*)-2-[carboxy[(phenoxyacetyl)amino]methyl]-5,5-dimethylthiazolidine-4-carboxylic acid (penicilloic acids of phenoxymethylpenicillin),

F. R = H: (2*R**S*,4*S*)-5,5-dimethyl-2-[[2-(phenoxyacetyl)amino]methyl]thiazolidine-4-carboxylic acid (penilloic acids of phenoxymethylpenicillin).

01/2005:1138

PHENTOLAMINE MESILATE

Phentolamini mesilas



C₁₈H₂₃N₃O₄S

M_r 377.5

DEFINITION

Phentolamine mesilate contains not less than 98.0 per cent and not more than the equivalent of 100.5 per cent of 3-[[4-(4,5-dihydro-1*H*-imidazol-2-yl)methyl](4-methylphenyl)amino]phenol methanesulphonate, calculated with reference to the dried substance.

PRODUCTION

The production method must be evaluated to determine the potential for formation of alkyl mesilates, which is particularly likely to occur if the reaction medium contains lower alcohols. Where necessary, the production method is validated to demonstrate that alkyl mesilates are not detectable in the final product.

CHARACTERS

A white, crystalline powder, slightly hygroscopic, freely soluble in water and in alcohol, practically insoluble in methylene chloride.

IDENTIFICATION

First identification: C, E

Second identification: A, B, D, E.

- A. Melting point (2.2.14): 178 °C to 182 °C.
- B. Dissolve 60.0 mg in *water R* and dilute to 100.0 ml with the same solvent. Dilute 5.0 ml of the solution to 100.0 ml with *water R*. Examined between 230 nm and 350 nm (2.2.25), the solution shows an absorption maximum at 278 nm. The specific absorbance at the maximum is 220 to 245.
- C. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the *Ph. Eur. reference spectrum of phentolamine mesilate*.
- D. Dissolve 0.5 g in a mixture of 5 ml of *alcohol R* and 5 ml of a 10 g/l solution of *hydrochloric acid R* and add 0.5 ml of a 5 g/l solution of *ammonium vanadate R*. A light green precipitate is produced.
- E. Mix 50 mg with 0.2 g of *sodium hydroxide R*, heat to fusion and continue heating for a few seconds. Allow to cool and add 0.5 ml of warm *water R*. Acidify with *dilute hydrochloric acid R* and heat. Sulphur dioxide is evolved, which turns moistened *starch iodate paper R* blue.

TESTS

Acidity. Dissolve 0.1 g in *carbon dioxide-free water R* and dilute to 10 ml with the same solvent. Add 0.1 ml of *methyl red solution R*. If the solution is red, not more than 0.05 ml of 0.1 M *sodium hydroxide* is required to change the colour of the indicator to yellow.

Related substances. Examine by thin-layer chromatography (2.2.27), using *silica gel G R* as the coating substance.

Test solution. Dissolve 0.10 g of the substance to be examined in *alcohol R* and dilute to 5 ml with the same solvent.

Reference solution (a). Dilute 0.5 ml of the test solution to 100 ml with *alcohol R*.

Reference solution (b). Dilute 5 ml of reference solution (a) to 10 ml with *alcohol R*.

Apply to the plate 10 µl of each solution. Develop over a path of 15 cm using a mixture of 5 volumes of *concentrated ammonia R*, 15 volumes of *acetone R* and 85 volumes of *methyl ethyl ketone R*. Allow the plate to dry in air and spray with *dilute potassium iodobismuthate solution R*. Any spot in the chromatogram obtained with the test solution, apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (a) (0.5 per cent) and at most one such spot is more intense than the spot in the chromatogram obtained with reference solution (b) (0.25 per cent).

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 100–105 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

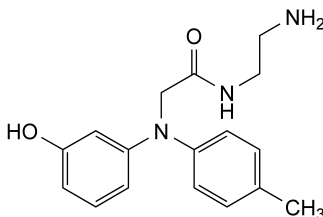
Dissolve 0.300 g in 100 ml of *2-propanol R1*. Titrate under a current of nitrogen with 0.1 M *tetrabutylammonium hydroxide in 2-propanol*. Determine the end-point potentiometrically (2.2.20), using a glass electrode as indicator electrode and a calomel electrode containing a saturated solution of *tetramethylammonium chloride R* in *2-propanol R1* as the comparison electrode. Carry out a blank titration.

1 ml of 0.1 M *tetrabutylammonium hydroxide in 2-propanol* is equivalent to 37.75 mg of $C_{18}H_{23}N_3O_4S$.

STORAGE

In an airtight container, protected from light.

IMPURITIES

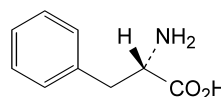


A. *N*-(2-aminoethyl)-2-[(3-hydroxyphenyl)(4-methylphenyl)amino]acetamide.

01/2005:0782

PHENYLALANINE

Phenylalaninum



$C_9H_{11}NO_2$

M_r 165.2

DEFINITION

Phenylalanine contains not less than 98.5 per cent and not more than the equivalent of 101.0 per cent of (S)-2-amino-3-phenylpropanoic acid, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, crystalline powder, or shiny, white flakes, sparingly soluble in water, very slightly soluble in alcohol. It dissolves in dilute mineral acids and in dilute solutions of alkali hydroxides.

IDENTIFICATION

First identification: A, B.

Second identification: A, C, D.

- A. It complies with the test for specific optical rotation (see Tests).
- B. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *phenylalanine CRS*. Examine the substances prepared as discs.
- C. Examine the chromatograms obtained in the test for ninhydrin-positive substances. The principal spot in the chromatogram obtained with test solution (b) is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).
- D. To about 10 mg add 0.5 g of *potassium nitrate R* and 2 ml of *sulphuric acid R*. Heat on a water-bath for 20 min. Allow to cool. Add 5 ml of a 50 g/l solution of *hydroxylamine hydrochloride R* and allow to stand in iced water for 10 min. Add 9 ml of *strong sodium hydroxide solution R*. A violet-red to violet-brown colour develops.

TESTS

Appearance of solution. Dissolve 0.5 g in 1 M *hydrochloric acid* and dilute to 10 ml with the same acid. The solution is clear (2.2.1) and not more intensely coloured than reference solution BY₆ (2.2.2, *Method II*).

Specific optical rotation (2.2.7). Dissolve 0.50 g in *water R* and dilute to 25.0 ml with the same solvent. The specific optical rotation is -33.0 to -35.5 , calculated with reference to the dried substance.

Ninhydrin-positive substances. Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel plate R*.

Test solution (a). Dissolve 0.10 g of the substance to be examined in a mixture of equal volumes of *glacial acetic acid R* and *water R* and dilute to 10 ml with the same mixture of solvents.

Test solution (b). Dilute 1 ml of test solution (a) to 50 ml with a mixture of equal volumes of *glacial acetic acid R* and *water R*.

Reference solution (a). Dissolve 10 mg of *phenylalanine CRS* in a mixture of equal volumes of *glacial acetic acid R* and *water R* and dilute to 50 ml with the same mixture of solvents.

Reference solution (b). Dilute 5 ml of test solution (b) to 20 ml with a mixture of equal volumes of *glacial acetic acid R* and *water R*.

Reference solution (c). Dissolve 10 mg of *phenylalanine CRS* and 10 mg of *tyrosine CRS* in a mixture of equal volumes of *glacial acetic acid R* and *water R* and dilute to 25 ml with the same mixture of solvents.

Apply separately to the plate 5 µl of each solution. Develop over a path of 15 cm using a mixture of 20 volumes of *glacial acetic acid R*, 20 volumes of *water R* and 60 volumes of *butanol R*. Allow the plate to dry in air, spray with *ninhydrin solution R* and heat at 100 °C to 105 °C for 15 min. Any spot in the chromatogram obtained with test solution (a), apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.5 per cent). The test is not valid unless the chromatogram obtained with reference solution (c) shows two clearly separated spots.

Chlorides (2.4.4). Dissolve 0.25 g in 3 ml of *dilute nitric acid R* and dilute to 15 ml with *water R*. The solution complies with the limit test for chlorides, without any further addition of nitric acid (200 ppm).

Sulphates (2.4.13). Dissolve 0.5 g in a mixture of 5 volumes of *dilute hydrochloric acid R* and 25 volumes of *distilled water R* and dilute to 15 ml with the same mixture of solvents. The solution complies with the limit test for sulphates (300 ppm).

Ammonium (2.4.1). 50 mg complies with limit test B for ammonium (200 ppm). Prepare the standard using 0.1 ml of *ammonium standard solution* (100 ppm NH_4) *R*.

Iron (2.4.9). In a separating funnel, dissolve 1.0 g in 10 ml of *dilute hydrochloric acid R*. Shake with three quantities, each of 10 ml, of *methyl isobutyl ketone RI*, shaking for 3 min each time. To the combined organic layers add 10 ml of *water R* and shake for 3 min. The aqueous layer complies with the limit test for iron (10 ppm).

Heavy metals (2.4.8). 2.0 g complies with limit test D for heavy metals (10 ppm). Prepare the standard using 2 ml of *lead standard solution* (10 ppm *Pb*) *R*.

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.100 g in 3 ml of *anhydrous formic acid R*. Add 30 ml of *anhydrous acetic acid R*. Titrate with 0.1 *M perchloric acid* using 0.1 ml of *naphtholbenzein solution R* as indicator, until the colour changes from yellow to green. 1 ml of 0.1 *M perchloric acid* is equivalent to 16.52 mg of $\text{C}_{19}\text{H}_{20}\text{N}_2\text{O}_2$.

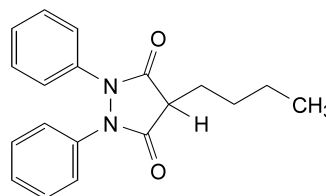
STORAGE

Store protected from light.

01/2005:0422

PHENYLBUTAZONE

Phenylbutazonum



$\text{C}_{19}\text{H}_{20}\text{N}_2\text{O}_2$

M_r 308.4

DEFINITION

4-Butyl-1,2-diphenylpyrazolidine-3,5-dione.

Content: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

Appearance: white or almost white, crystalline powder.

Solubility: practically insoluble in water, sparingly soluble in alcohol. It dissolves in alkaline solutions.

IDENTIFICATION

First identification: A, C.

Second identification: A, B, D.

A. Melting point (2.2.14): 104 °C to 107 °C.

B. Thin-layer chromatography (2.2.27).

Test solution. Dissolve 25 mg of the substance to be examined in a mixture of equal volumes of *ethanol R* and *methylene chloride R* and dilute to 25 ml with the same mixture of solvents.

Reference solution. Dissolve 25 mg of *phenylbutazone CRS* in a mixture of equal volumes of *ethanol R* and *methylene chloride R* and dilute to 25 ml with the same mixture of solvents.

Plate: *TLC silica gel GF₂₅₄ plate R*.

Mobile phase: *acetone R*, *methylene chloride R* (20:80 V/V).

Application: 5 µl.

Development: over a path of 10 cm.

Drying: in air.

Detection: examine in ultraviolet light at 254 nm.

Results: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with the reference solution.

C. Infrared absorption spectrophotometry (2.2.24).

Comparison: *phenylbutazone CRS*.

D. To 0.1 g add 1 ml of *glacial acetic acid R* and 2 ml of *hydrochloric acid R* and heat the mixture under a reflux condenser for 30 min. Cool, add 10 ml of *water R* and filter. To the filtrate add 3 ml of a 7 g/l solution of

sodium nitrite R. A yellow colour is produced. To 1 ml of the solution add a solution of 10 mg of *β-naphthol R* in 5 ml of *sodium carbonate solution R*. A brownish-red to violet-red precipitate is formed.

TESTS

Solution S. Dissolve 1.0 g with shaking in 20 ml of *dilute sodium hydroxide solution R* and maintain the solution at 25 °C for 3 h.

Appearance of solution. Solution S is clear (2.2.1).

Acidity or alkalinity. Heat to boiling 1.0 g in 50 ml of *water R*, cool with shaking in a closed flask and filter. To 25 ml of the filtrate add 0.5 ml of *phenolphthalein solution R*. The solution is colourless. Not more than 0.5 ml of 0.01 M *sodium hydroxide* is required to change the colour of the indicator. Add 0.6 ml of 0.01 M *hydrochloric acid* and 0.1 ml of *methyl red solution R*; the solution is red or orange.

Absorbance (2.2.25): maximum 0.20 for solution S at 420 nm in a 4 cm cell.

Related substances. Liquid chromatography (2.2.29). Prepare the solutions immediately before use.

Test solution. Dissolve 100.0 mg of the substance to be examined in *acetonitrile R* and dilute to 10.0 ml with the same solvent.

Reference solution (a). Dilute 1.0 ml of the test solution to 100.0 ml with *acetonitrile R*. Dilute 1.0 ml to 10.0 ml with *acetonitrile R*.

Reference solution (b). Dissolve 5 mg of *phenylbutazone impurity B CRS* and 5 mg of *1,2-diphenylhydrazine R* in *acetonitrile R*, add 0.5 ml of the test solution and dilute to 50 ml with *acetonitrile R*. Dilute 2.5 ml to 10 ml with *acetonitrile R*.

Reference solution (c). Dissolve 1.0 mg of *benzidine R* in *acetonitrile R* and dilute to 100.0 ml with the same solvent. Dilute 1.0 ml to 100.0 ml with *acetonitrile R*. Dilute 5.0 ml to 10.0 ml with *acetonitrile R*.

Column:

- **size:** $l = 0.125$ m, $\varnothing = 4.0$ mm,
- **stationary phase:** *octadecylsilyl silica gel for chromatography R* (5 μ m),
- **temperature:** 30 °C.

Mobile phase:

- **mobile phase A:** dissolve 1.36 g of *sodium acetate R* in *water R*, adjust to pH 5.2 with a 52.5 g/l solution of *citric acid R* and dilute to 1000 ml with *water R*,
- **mobile phase B:** *acetonitrile R*,

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)
0 - 10	70	30
10 - 20	70 → 40	30 → 60
20 - 35	40	60
35 - 40	40 → 70	60 → 30

Flow rate: 1.5 ml/min.

Detection: spectrophotometer at 240 nm.

Injection: 20 μ l; inject the test solution and reference solutions (a) and (b).

Relative retentions with reference to phenylbutazone (retention time = about 13 min): impurity E = about 0.2; impurity A = about 0.5; impurity B = about 1.2; impurity C = about 1.3; impurity D = about 1.7.

System suitability: reference solution (b):

- **resolution:** minimum 2.0 between the peaks due to phenylbutazone and to impurity B.

Limits:

- **correction factor:** for the calculation of content, multiply the peak area of impurity C by 0.55,
- **impurities A, B:** for each impurity, not more than 2.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.25 per cent),
- **impurity C:** not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.20 per cent),
- **any other impurity:** not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent),
- **total:** not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent),
- **disregard limit:** 0.25 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.025 per cent); disregard any peak due to impurity E.

Impurity E. Liquid chromatography (2.2.29) as described in the test for related substances with the following modifications.

Detection: spectrophotometer at 280 nm.

Injection: test solution and reference solution (c).

System suitability: reference solution (c):

- **signal-to-noise ratio:** minimum 10 for the principal peak.

Limit:

- **impurity E:** not more than the area of the principal peak in the chromatogram obtained with reference solution (c) (5 ppm).

Heavy metals (2.4.8): maximum 20 ppm.

1.0 g complies with limit test C. Prepare the standard using 2 ml of *lead standard solution (10 ppm Pb) R*.

Loss on drying (2.2.32): maximum 0.2 per cent, determined on 1.000 g by drying *in vacuo* at 80 °C for 4 h.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

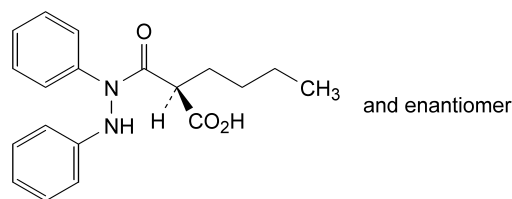
Dissolve 0.250 g in 25 ml of *acetone R* and add 0.5 ml of *bromothymol blue solution R1*. Titrate with 0.1 M *sodium hydroxide* until a blue colour is obtained which persists for 15 s. Carry out a blank titration.

1 ml of 0.1 M *sodium hydroxide* is equivalent to 30.84 mg of $C_{19}H_{20}N_2O_2$.

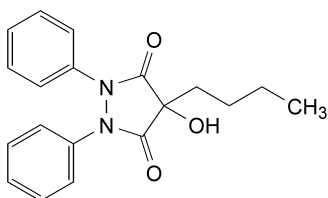
STORAGE

Protected from light.

IMPURITIES



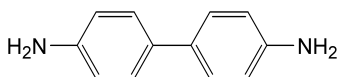
A. (2*RS*)-2-[(1,2-diphenyldiazanyl)carbonyl]hexanoic acid,



B. 4-butyl-4-hydroxy-1,2-diphenylpyrazolidine-3,5-dione,

C. $\text{C}_6\text{H}_5\text{-NH-NH-C}_6\text{H}_5$: 1,2-diphenyldiazane,
(1,2-diphenylhydrazine),

D. $\text{C}_6\text{H}_5\text{-N=N-C}_6\text{H}_5$: 1,2-diphenyldiazene,

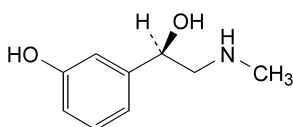


E. biphenyl-4,4'-diamine (benzidine).

01/2005:1035

PHENYLEPHRINE

Phenylephrinum



$\text{C}_9\text{H}_{13}\text{NO}_2$

M_r 167.2

DEFINITION

Phenylephrine contains not less than 99.0 per cent and not more than the equivalent of 100.5 per cent of (1*R*)-1-(3-hydroxyphenyl)-2-(methylamino)ethanol, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, crystalline powder, slightly soluble in water, sparingly soluble in methanol, slightly soluble in alcohol. It dissolves in dilute mineral acids and in dilute solutions of alkali hydroxides.

It melts at about 174 °C.

IDENTIFICATION

First identification: A, B.

Second identification: A, C, D.

- It complies with the test for specific optical rotation (see Tests).
- Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *phenylephrine CRS*.
- Examine the chromatograms obtained in the test for related substances. The principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).

D. Dissolve about 10 mg in 1 ml of 1 *M* hydrochloric acid, add 0.05 ml of copper sulphate solution *R* and 1 ml of a 200 g/l solution of sodium hydroxide *R*. A violet colour develops. Add 1 ml of ether *R* and shake. The upper layer remains colourless.

TESTS

Appearance of solution. Dissolve 1 g in 1 *M* hydrochloric acid and dilute to 10 ml with the same acid. The solution is clear (2.2.1) and not more intensely coloured than reference solution *Y*₇ (2.2.2, *Method II*).

Specific optical rotation (2.2.7). Dissolve 1.250 g in 1 *M* hydrochloric acid and dilute to 25.0 ml with the same acid. The specific optical rotation is −53 to −57, calculated with reference to the dried substance.

Absorbance. Dissolve 0.50 g in 0.1 *M* hydrochloric acid and dilute to 200 ml with the same solvent. Measure the absorbance (2.2.25) at 315 nm using 0.1 *M* hydrochloric acid as the compensation liquid. The absorbance is not more than 0.15 (0.4 per cent, calculated as impurity C).

Related substances. Examine by thin-layer chromatography (2.2.27), using silica gel *HF*₂₅₄ *R* as the coating substance.

Solvent mixture. Prepare a mixture of equal volumes of methylene chloride *R* and methanolic hydrochloric acid hydrochloric acid *R* diluted to 10 volumes with methanol *R*.

Test solution. Dissolve 0.1 g of the substance to be examined in the solvent mixture and dilute to 5 ml with the same solvent mixture.

Reference solution (a). Dissolve 20 mg of *phenylephrine CRS* in the solvent mixture and dilute to 1 ml with the same solvent mixture.

Reference solution (b). Dilute 0.1 ml of the test solution to 20 ml with the solvent mixture.

Reference solution (c). Dilute 0.1 ml of the test solution to 50 ml with the solvent mixture.

Apply separately to the plate 10 µl of each solution and develop over a path of 15 cm using a mixture of 0.5 volumes of concentrated ammonia *R*, 25 volumes of methanol *R* and 70 volumes of methylene chloride *R*. Dry the plate in a current of cold air. Examine in ultraviolet light at 254 nm. Spray with a 1 g/l solution of fast red B salt *R* in a 50 g/l solution of sodium carbonate *R* and examine in daylight. Any spot in the chromatogram obtained with the test solution, apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.5 per cent) and at most two such spots are more intense than the spot in the chromatogram obtained with reference solution (c) (0.2 per cent).

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

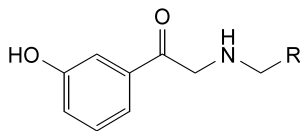
Dissolve 0.150 g in 60 ml of anhydrous acetic acid *R*. Titrate with 0.1 *M* perchloric acid determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 *M* perchloric acid is equivalent to 16.72 mg of $\text{C}_9\text{H}_{13}\text{NO}_2$.

STORAGE

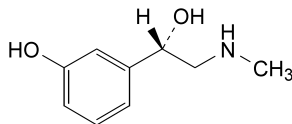
Store in an airtight container, protected from light.

IMPURITIES



A. R = C₆H₅: 2-(benzylamino)-1-(3-hydroxyphenyl)ethanone,

C. R = H: 1-(3-hydroxyphenyl)-2-(methylamino)ethanone,

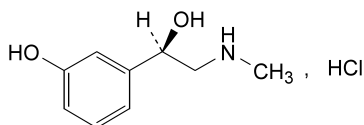


B. (1S)-1-(3-hydroxyphenyl)-2-(methylamino)ethanol.

01/2005:0632

PHENYLEPHRINE HYDROCHLORIDE

Phenylephrini hydrochloridum



C₉H₁₄ClNO₂

M_r 203.7

DEFINITION

Phenylephrine hydrochloride contains not less than 98.5 per cent and not more than the equivalent of 101.0 per cent of (1R)-1-(3-hydroxyphenyl)-2-(methylamino)ethanol hydrochloride, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, crystalline powder, freely soluble in water and in alcohol.

It melts at about 143 °C.

IDENTIFICATION

First identification: A, B, E.

Second identification: A, C, D, E.

- It complies with the test for specific optical rotation (see Tests).
- Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *phenylephrine hydrochloride CRS*. Examine the substances prepared as discs.
- Dissolve 0.3 g in 3 ml of *water R*, add 1 ml of *dilute ammonia R1* and initiate crystallisation by scratching the wall of the tube with a glass rod. The crystals, washed with iced *water R* and dried at 105 °C for 2 h, melt (2.2.14) at 171 °C to 176 °C.
- Dissolve about 10 mg in 1 ml of *water R* and add 0.05 ml of a 125 g/l solution of *copper sulphate R* and 1 ml of a 200 g/l solution of *sodium hydroxide R*. A violet colour is produced. Add 1 ml of *ether R* and shake; the ether layer remains colourless.
- It gives reaction (a) of chlorides (2.3.1).

TESTS

Solution S. Dissolve 2.00 g in *carbon dioxide-free water R* prepared from *distilled water R* and dilute to 100.0 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and colourless (2.2.2, *Method II*).

Acidity or alkalinity. To 10 ml of solution S add 0.1 ml of *methyl red solution R* and 0.2 ml of 0.01 M *sodium hydroxide*. The solution is yellow. Not more than 0.4 ml of 0.01 M *hydrochloric acid* is required to change the colour of the indicator to red.

Specific optical rotation (2.2.7): −43 to −47, determined on solution S and calculated with reference to the dried substance.

Related substances. Examine by thin-layer chromatography (2.2.27), using *silica gel H R* as the coating substance.

Test solution. Dissolve 0.10 g of the substance to be examined in *methanol R* and dilute to 5 ml with the same solvent.

Reference solution (a). Dilute 1 ml of the test solution to 200 ml with *methanol R*.

Reference solution (b). Dilute 2 ml of reference solution (a) to 5 ml with *methanol R*.

Apply separately to the plate 5 µl of each solution. Develop over a path of 15 cm using a mixture of 5 volumes of *chloroform R*, 15 volumes of *ammonia R* and 80 volumes of *2-propanol R*. Dry the plate in a current of cold air, spray with *ninhydrin solution R* and heat at 100 °C to 105 °C for 5 min to 10 min. Examine in daylight. Any spot in the chromatogram obtained with the test solution, apart from the principal spot, is not more intense than the spot in the chromatogram obtained with the reference solution (a) (0.5 per cent) and at most two such spots are more intense than the spot in the chromatogram obtained with reference solution (b) (0.2 per cent).

Ketones. Dilute 10.0 ml of solution S to 50.0 ml with 0.01 M *hydrochloric acid*. The absorbance (2.2.25), measured at 310 nm using 0.01 M *hydrochloric acid* as the compensation liquid, is not greater than 0.20.

Sulphates (2.4.13). 15 ml of solution S complies with the limit test for sulphates (500 ppm).

Loss on drying (2.2.32). Not more than 1.0 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

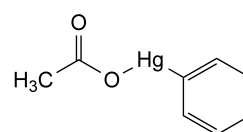
ASSAY

Dissolve 0.1500 g in a mixture of 0.5 ml of 0.1 M *hydrochloric acid* and 80 ml of *alcohol R*. Carry out a potentiometric titration (2.2.20) using 0.1 M *ethanolic sodium hydroxide*. Read the volume added between the two points of inflexion. 1 ml of 0.1 M *ethanolic sodium hydroxide* is equivalent to 20.37 mg of C₉H₁₄ClNO₂.

01/2005:2042

PHENYLMERCURIC ACETATE

Phenylhydrargyri acetat



C₈H₈HgO₂

M_r 336.7

DEFINITION

Content: 98.0 per cent to 100.5 per cent (dried substance).

CHARACTERS

Appearance: white or yellowish, crystalline powder or small, colourless crystals.

Solubility: slightly soluble in water, soluble in acetone and in alcohol.

IDENTIFICATION

First identification: A.

Second identification: B, C.

A. Infrared absorption spectrophotometry (2.2.24).

Comparison: Ph. Eur. reference spectrum of phenylmercuric acetate.

B. To 5 ml of solution S (see Tests) add 5 ml of *water R* and 0.1 ml of *sodium sulphide solution R*. A white precipitate is formed that darkens slowly on heating.

C. To 10 ml of solution S add 2 ml of *potassium iodide solution R* and shake vigorously. Filter. The filtrate gives reaction (b) of acetates (2.3.1).

TESTS

Solution S. Dissolve 0.250 g in 40 ml of *water R* by heating to boiling. Allow to cool and dilute to 50 ml with *water R*. Prepare the solution immediately before use.

Appearance of solution. Solution S is not more opalescent than reference suspension II (2.2.1) and is colourless (2.2.2, Method II).

Ionised mercury: maximum 0.2 per cent.

To 2 ml of solution S add 8 ml of *water R*, 2 ml of *potassium iodide solution R* and 3 ml of *dilute hydrochloric acid R*. Filter. The filtrate is not more coloured than the potassium iodide solution used. Wash the precipitate with 3 ml of *water R*. Combine the filtrate and the washings, add 2 ml of *dilute sodium hydroxide solution R* and dilute to 20 ml with *water R*. 12 ml of this solution complies with limit test A for heavy metals (2.4.8). Prepare the standard using *lead standard solution (1 ppm Pb) R*.

Polymercuric benzene compounds: maximum 1.5 per cent. Shake 0.2 g with 10 ml of *acetone R*. Filter. Wash the residue twice with 5 ml of *acetone R*. Dry the residue at 105 °C for 1 h. The residue weighs a maximum of 3 mg.

Loss on drying (2.2.32): maximum 0.5 per cent, determined on 0.500 g by drying in an oven at 45 °C for 15 h.

ASSAY

Dissolve with heating 0.300 g in 100 ml of *water R*. Cool and add 3 ml of *nitric acid R*. Titrate with 0.1 M *ammonium thiocyanate* using 2 ml of *ferric ammonium sulphate solution R2* as indicator, until a persistent reddish-yellow colour is obtained.

1 ml of 0.1 M *ammonium thiocyanate* is equivalent to 33.67 mg of phenylmercuric acetate.

STORAGE

Protected from light.

01/2005:0103

PHENYLMERCURIC BORATE

Phenylhydrargyri boras

DEFINITION

Phenylmercuric borate is a compound consisting of equimolecular proportions of phenylmercuric orthoborate and phenylmercuric hydroxide ($C_{12}H_{13}BHg_2O_4$; M_r 633) or of

the dehydrated form (metaborate, $C_{12}H_{11}BHg_2O_3$; M_r 615) or a mixture of the two compounds. It contains not less than 64.5 per cent and not more than 66.0 per cent of Hg (A_r 200.6) and not less than the equivalent of 9.8 per cent and not more than the equivalent of 10.3 per cent of borates, expressed as H_3BO_3 , both calculated with reference to the dried substance.

CHARACTERS

A white or slightly yellowish, crystalline powder or colourless, shiny crystals, slightly soluble in water and in alcohol.

IDENTIFICATION

A. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the Ph. Eur. reference spectrum of phenylmercuric borate. Examine the substance as a disc.

B. To 2 ml of solution S (see Tests) add 8 ml of *water R* and 0.1 ml of *sodium sulphide solution R*. A white precipitate is formed that darkens slowly on heating.

C. Dissolve about 20 mg in 2 ml of *methanol R*. The solution is clear and colourless. Ignite; the solution burns with a green-edged flame.

TESTS

Solution S. Dissolve 0.25 g by sprinkling it on the surface of 25 ml of boiling *water R*, cool and dilute to 25 ml with *water R*.

Appearance of solution. Solution S is clear (2.2.1) and colourless (2.2.2, Method II).

Ionised mercury. To 10 ml of solution S add 2 ml of *potassium iodide solution R* and 3 ml of *dilute hydrochloric acid R*. Filter. The filtrate is colourless. Wash the precipitate with 3 ml of *water R*. Combine the filtrate and the washings, add 2 ml of *dilute sodium hydroxide solution R* and dilute to 20 ml with *water R*. 12 ml of this solution complies with limit test A for heavy metals (2.4.8). Prepare the standard using a mixture of 2.5 ml of *lead standard solution (2 ppm Pb) R* and 7.5 ml of *water R*.

Loss on drying (2.2.32). Not more than 3.5 per cent, determined on 0.50 g by drying in an oven at 45 °C for 15 h ($\pm$ 30 min).

ASSAY

Mercury. Dissolve 0.300 g in 100 ml of *water R* and add 3 ml of *nitric acid R*. Titrate with 0.1 M *ammonium thiocyanate*, using 2 ml of *ferric ammonium sulphate solution R2* as indicator, until a persistent reddish-yellow colour is obtained.

1 ml of 0.1 M *ammonium thiocyanate* is equivalent to 20.06 mg of Hg.

Borates. Dissolve 0.600 g with heating in 25 ml of *water R*. Dissolve 10 g of *sorbitol R* in the hot solution and cool. Titrate with 0.1 M *sodium hydroxide*, using 0.5 ml of *phenolphthalein solution R* as indicator, until a persistent pink colour is obtained. Carry out a blank titration.

1 ml of 0.1 M *sodium hydroxide* is equivalent to 6.18 mg of H_3BO_3 .

STORAGE

Store protected from light.

01/2005:0783

01/2005:0683

PHENYLMERCURIC NITRATE

Phenylhydrargyri nitras

DEFINITION

Phenylmercuric nitrate is a mixture of phenylmercuric nitrate ($\text{C}_6\text{H}_5\text{HgNO}_3$; M_r 339.7) and phenylmercuric hydroxide ($\text{C}_6\text{H}_5\text{HgOH}$; M_r 294.7). It contains not less than 62.5 per cent and not more than 64.0 per cent of Hg (A_r 200.6), calculated with reference to the dried substance.

CHARACTERS

A white or pale yellow powder, very slightly soluble in water and in alcohol, slightly soluble in hot water. It dissolves in glycerol and in fatty oils.

IDENTIFICATION

- To 5 ml of solution S (see Tests) add 8 ml of *water R* and 0.1 ml of *sodium sulphide solution R*. A white precipitate is formed that darkens slowly on heating.
- To 1 ml of a saturated solution of the substance to be examined add 1 ml of *dilute hydrochloric acid R*. A white, flocculent precipitate is formed.
- To 5 ml of solution S add 1 ml of *dilute hydrochloric acid R*, 2 ml of *methylene chloride R* and 0.2 ml of *dithizone solution R*. Shake. The lower layer is orange-yellow.
- About 10 mg gives the reaction of nitrates (2.3.1).

TESTS

Solution S. To 0.1 g add 45 ml of *water R* and heat to boiling with shaking. Cool, filter and dilute to 50 ml with *water R*.

Appearance of solution. Solution S is colourless (2.2.2, *Method II*).

Inorganic mercuric compounds. To 10 ml of solution S add 2 ml of *potassium iodide solution R* and 3 ml of *dilute hydrochloric acid R*. Filter. The filtrate is colourless. Wash the precipitate with 2 ml of *water R*. Combine the filtrate and washings, add 2 ml of *dilute sodium hydroxide solution R* and dilute to 20 ml with *water R*. 12 ml of the solution complies with limit test A for heavy metals (0.1 per cent) (2.4.8). Prepare the standard using *lead standard solution (1 ppm Pb) R*.

Loss on drying (2.2.32). Not more than 1.0 per cent, determined on 1.000 g by drying *in vacuo* for 24 h.

ASSAY

Dissolve 0.150 g in a mixture of 10 ml of *dilute nitric acid R* and 90 ml of *water R*, heating to boiling. Cool to 15 °C to 20 °C. Titrate with 0.1 M *ammonium thiocyanate* using 2 ml of *ferric ammonium sulphate solution R2* as indicator, until a persistent reddish-yellow colour is obtained. Carry out a blank titration.

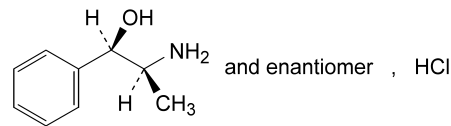
1 ml of 0.1 M *ammonium thiocyanate* is equivalent to 20.06 mg of Hg.

STORAGE

Store protected from light.

PHENYLPROPANOLAMINE HYDROCHLORIDE

Phenylpropanolamini hydrochloridum

 $\text{C}_9\text{H}_{14}\text{ClNO}$ M_r 187.7**DEFINITION**

Phenylpropanolamine hydrochloride contains not less than 99.0 per cent and not more than the equivalent of 101.5 per cent of (1*RS*,2*SR*)-2-amino-1-phenylpropan-1-ol hydrochloride, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, crystalline powder, freely soluble in water and in alcohol, practically insoluble in methylene chloride.

IDENTIFICATION

First identification: B, E.

Second identification: A, C, D, E.

- Melting point (2.2.14): 194 °C to 197 °C.
- Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *phenylpropanolamine hydrochloride CRS*. Examine the substances prepared as discs without recrystallisation.
- Examine the chromatograms obtained in the test for related substances. The principal spot in the chromatogram obtained with test solution (b) is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).
- Dissolve 50 mg in 5 ml of *water R*, add 0.2 ml of *copper sulphate solution R* and 0.3 ml of *dilute sodium hydroxide solution R*. A violet colour develops. Add 2 ml of *ether R* and shake. A violet precipitate is formed between the two layers.
- It gives reaction (a) of chlorides (2.3.1).

TESTS

Solution S. Dissolve 1.25 g in *water R* and dilute to 25 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and colourless (2.2.2, *Method II*).

Acidity or alkalinity. To 10 ml of solution S add 0.1 ml of *methyl red solution R* and 0.2 ml of 0.01 M *sodium hydroxide*. The solution is yellow. Add 0.4 ml of 0.01 M *hydrochloric acid*. The solution is red.

Related substances. Examine by thin-layer chromatography (2.2.27), using *silica gel H R* as the coating substance.

Test solution (a). Dissolve 0.20 g of the substance to be examined in *alcohol R* and dilute to 10 ml with the same solvent.

Test solution (b). Dilute 1 ml of test solution (a) to 10 ml with *alcohol R*.

Reference solution (a). Dissolve 20 mg of *phenylpropanolamine hydrochloride CRS* in *alcohol R* and dilute to 10 ml with the same solvent.

Reference solution (b). Dilute 1 ml of reference solution (a) to 10 ml with *alcohol R*.

Reference solution (c). Dissolve 20 mg of *norpseudoephedrine hydrochloride CRS* in *alcohol R*, add 1 ml of test solution (a) and dilute to 10 ml with *alcohol R*.

Reference solution (d). Dissolve 60 mg of *ammonium chloride R* in *methanol R* and dilute to 10 ml with the same solvent.

Before applying the solutions, spray the plate with a 20 g/l solution of *disodium tetraborate R*, using 8 ml for a plate 100 mm by 200 mm and dry in a stream of cold air for 30 min. Apply separately to the plate as bands about 10 mm by 3 mm 10 µl of each solution. Develop over a path of 10 cm using a mixture of 6 volumes of *concentrated ammonia R*, 24 volumes of *alcohol R* and 70 volumes of *butanol R*. Dry the plate in a current of warm air until the solvents have evaporated, allow to cool, spray with a 2 g/l solution of *ninhydrin R* in *alcohol R* and heat at 110 °C for 15 min. Any spot in the chromatogram obtained with test solution (a) apart from the principal spot and the spot corresponding to ammonium chloride is not more intense than the spot in the chromatogram obtained with reference solution (b) (1.0 per cent). The test is not valid unless the chromatogram obtained with reference solution (c) shows two clearly separated spots.

Phenylpropanonamine. Dissolve 1.0 g in 0.01 M *hydrochloric acid* and dilute to 50.0 ml with the same acid. The absorbance (2.2.25) of the solution measured at 283 nm is not greater than 0.10.

Heavy metals (2.4.8). 12 ml of solution S complies with limit test A for heavy metals (20 ppm). Prepare the standard using *lead standard solution (1 ppm Pb) R*.

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

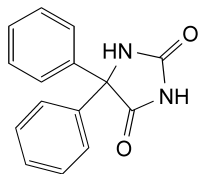
Dissolve 0.1500 g in a mixture of 5 ml of 0.01 M *hydrochloric acid* and 50 ml of *alcohol R*. Carry out a potentiometric titration (2.2.20), using 0.1 M *sodium hydroxide*. Read the volume added between the two points of inflexion.

1 ml of 0.1 M *sodium hydroxide* is equivalent to 18.77 mg of $C_{15}H_{12}N_2O_2$.

01/2005:1253

PHENYTOIN

Phenytoinum



$C_{15}H_{12}N_2O_2$

M_r 252.3

DEFINITION

Phenytoin contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of 5,5-diphenylimidazolidine-2,4-dione, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, crystalline powder, practically insoluble in water, sparingly soluble in alcohol, very slightly soluble in methylene chloride. It dissolves in dilute solutions of alkali hydroxides.

IDENTIFICATION

First identification: A.

Second identification: B, C, D.

- Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *phenytoin CRS*.
- Examine the chromatograms obtained in the test for related substances. The principal spot in the chromatogram obtained with test solution (b) is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a).
- To about 10 mg add 1 ml of *water R* and 0.05 ml of *ammonia R*. Heat until boiling begins. Add 0.05 ml of a 50 g/l solution of *copper sulphate R* in *dilute ammonia R2* and shake. A pink, crystalline precipitate is formed.
- It complies with the test for sulphated ash (see Tests).

TESTS

Appearance of solution. Dissolve 1.0 g in a mixture of 5 ml of 1 M *sodium hydroxide* and 20 ml of *water R*. The solution is clear (2.2.1) and not more intensely coloured than reference solution BY₆ (2.2.2, *Method II*).

Acidity or alkalinity. To 1.0 g add 45 ml of *water R* and boil for 2 min. Allow to cool and filter. Wash the filter with *carbon dioxide-free water R* and dilute the combined filtrate and washings to 50 ml with the same solvent. To 10 ml of the solution add 0.15 ml of *methyl red solution R*. Not more than 0.5 ml of 0.01 M *hydrochloric acid* is required to change the colour of the indicator to red. To 10 ml of the solution add 0.15 ml of *bromothymol blue solution R1*. Not more than 0.5 ml of 0.01 M *sodium hydroxide* is required to change the colour of the indicator to blue.

Related substances. Examine by thin-layer chromatography (2.2.27), using as the coating substance a suitable silica gel with a fluorescent indicator having an optimal intensity at 254 nm. Before use, wash the plate with a mixture of 30 volumes of *dioxan R* and 75 volumes of *hexane R*. Allow the plate to dry in air.

Test solution (a). Dissolve 0.40 g of the substance to be examined in a mixture of equal volumes of *acetone R* and *methanol R* and dilute to 10 ml with the same mixture of solvents.

Test solution (b). Dilute 1 ml of test solution (a) to 20 ml with a mixture of equal volumes of *acetone R* and *methanol R*.

Reference solution (a). Dissolve 20 mg of *phenytoin CRS* in a mixture of equal volumes of *acetone R* and *methanol R* and dilute to 10 ml with the same mixture of solvents.

Reference solution (b). Dissolve 8 mg of *benzophenone R* in a mixture of equal volumes of *acetone R* and *methanol R* and dilute to 100 ml with the same mixture of solvents.

Reference solution (c). Dissolve 8 mg of *benzil R* in a mixture of equal volumes of *acetone R* and *methanol R* and dilute to 100 ml with the same mixture of solvents.

Reference solution (d). Dilute 1 ml of test solution (a) to 100 ml with a mixture of equal volumes of *acetone R* and *methanol R*.

Reference solution (e). Mix 1 ml of reference solution (b) and 1 ml of reference solution (c).

Apply separately to the plate 10 µl of each solution and dry the plate in a stream of cold air for 2 min. Develop over a path of 15 cm using a mixture of 30 volumes of *dioxan R* and 75 volumes of *hexane R*. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. In the chromatogram obtained with test solution (a): any spot corresponding to benzophenone is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.2 per cent); any spot corresponding to benzil is not more intense than the spot in the chromatogram obtained with reference solution (c) (0.2 per cent) and any spot, apart from the principal spot and any spot corresponding to benzo-phenone and benzil, is not more intense than the spot in the chromatogram obtained with reference solution (d) (1 per cent). The test is not valid unless the chromatogram obtained with reference solution (e) shows two clearly separated principal spots.

Heavy metals (2.4.8). 2.0 g complies with limit test C for heavy metals (10 ppm). Prepare the standard using 2 ml of *lead standard solution (10 ppm Pb) R*.

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C.

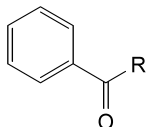
Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.200 g in 50 ml of *dimethylformamide R*. Titrate with 0.1 M *sodium methoxide*, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *sodium methoxide* is equivalent to 25.23 mg of $C_{15}H_{12}N_2O_2$.

IMPURITIES



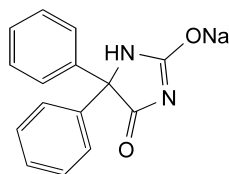
A. R = C_6H_5 : diphenylmethanone (benzophenone),

B. R = $CO-C_6H_5$: diphenylethanedione (benzil).

01/2005:0521

PHENYTOIN SODIUM

Phenytoinum natricum



$C_{15}H_{11}N_2NaO_2$

M_r 274.3

DEFINITION

Phenytoin sodium contains not less than 98.5 per cent and not more than the equivalent of 100.5 per cent of sodium 4-oxo-5,5-diphenyl-4,5-dihydro-1H-imidazol-2-olate, calculated with reference to the anhydrous substance.

CHARACTERS

A white, crystalline powder, slightly hygroscopic, soluble in water and in alcohol, practically insoluble in methylene chloride.

IDENTIFICATION

First identification: A, C.

Second identification: B, C.

- Dissolve 0.1 g in 20 ml of *water R*. Acidify with *dilute hydrochloric acid R* and shake with three quantities, each of 30 ml, of *chloroform R*. Wash the combined chloroform layers with *water R*, evaporate to dryness and dry the residue at 100 °C to 105 °C (test residue). Repeat the operations using 0.1 g of *phenytoin sodium CRS* (reference residue). Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *phenytoin sodium CRS*. Examine as discs prepared using *potassium bromide R*.
- To about 10 mg add 1 ml of *water R* and 0.05 ml of *ammonia R*. Heat until boiling begins. Add 0.05 ml of a 50 g/l solution of *copper sulphate R* in *dilute ammonia R2* and shake. A pink, crystalline precipitate is formed.
- Ignite 1 g and cool. Add 2 ml of *water R* to the residue and neutralise the solution with *hydrochloric acid R*. Filter and dilute the filtrate to 4 ml with *water R*. 0.1 ml of the solution gives reaction (b) of sodium (2.3.1).

TESTS

Appearance of solution. Suspend 1.0 g in 5 ml of *water R* and dilute to 20 ml with 0.1 M *sodium hydroxide*. The solution is clear (2.2.1) and not more intensely coloured than reference solution BY₆ (2.2.2, *Method II*).

Related substances. Examine by thin-layer chromatography (2.2.27), using as the coating substance a suitable silica gel containing a fluorescent indicator having an optimal intensity at 254 nm.

Test solution. Dissolve 0.4 g of the substance to be examined in *methanol R* and dilute to 10 ml with the same solvent.

Reference solution (a). Dilute 1 ml of the test solution to 100 ml with *methanol R*.

Reference solution (b). Dissolve 20 mg of *benzophenone R* in *methanol R* and dilute to 100 ml with the same solvent.

Apply separately to the plate 10 µl of each solution and dry the plate in a stream of cold air for 2 min. Develop over a path of 15 cm using a mixture of 10 volumes of *concentrated ammonia R*, 45 volumes of *chloroform R* and 45 volumes of *2-propanol R*. Dry the plate in an oven at 80 °C for 5 min and examine in ultraviolet light at 254 nm. In the chromatogram obtained with the test solution, any spot corresponding to benzophenone is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.5 per cent) and any spot, apart from the principal spot and the spot corresponding to benzophenone, is not more intense than the spot in the chromatogram obtained with reference solution (a) (1.0 per cent).

Free phenytoin. Dissolve 0.30 g in 10 ml of a mixture of equal volumes of *pyridine R* and *water R*. Add 0.5 ml of *phenolphthalein solution R* and 3 ml of *silver nitrate solution in pyridine R*. Not more than 1.0 ml of 0.1 M *sodium hydroxide* is required to change the colour of the indicator to pink.

Heavy metals (2.4.8). 2.0 g complies with limit test C for heavy metals (10 ppm). Prepare the standard using 2 ml of *lead standard solution (10 ppm Pb) R*.

Water (2.5.12). Not more than 3.0 per cent, determined on 1.000 g by the semi-micro determination of water.

ASSAY

Suspend 0.180 g in 2 ml of *water R*. Add 8.0 ml of 0.05 M *sulphuric acid* and heat gently for 1 min. Add 30 ml of *methanol R* and cool. Carry out a potentiometric titration (2.2.20), using 0.1 M *sodium hydroxide*. After reaching the first point of inflexion, interrupt the addition of 0.1 M *sodium hydroxide*, add 5 ml of *silver nitrate solution in pyridine R*, mix and continue the titration. Read the volume of 0.1 M *sodium hydroxide* added between the two points of inflexion.

1 ml of 0.1 M *sodium hydroxide* is equivalent to 27.43 mg of $C_{15}H_{11}N_2NaO_2$.

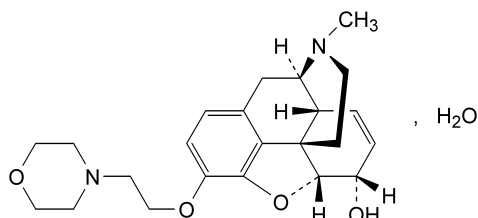
STORAGE

Store in an airtight container.

01/2005:0522

PHOLCODINE

Pholcodinum


 $C_{23}H_{30}N_2O_4 \cdot H_2O$
 M_r 416.5

DEFINITION

Pholcodine contains not less than 98.5 per cent and not more than the equivalent of 100.5 per cent of 7,8-didehydro-4,5 α -epoxy-17-methyl-3-[2-(morpholin-4-yl)ethoxy]morphinan-6 α -ol, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, crystalline powder or colourless crystals, sparingly soluble in water, freely soluble in acetone and in alcohol. It dissolves in dilute mineral acids.

IDENTIFICATION

First identification: A.

Second identification: B, C.

- Examine by infrared absorption spectrophotometry (2.2.24), comparing with the *Ph. Eur. reference spectrum of pholcodine*.
- Dissolve 0.100 g in *water R* and dilute to 100.0 ml with the same solvent. To 10.0 ml of this solution add 75 ml of *water R* and 10 ml of 1 M *sodium hydroxide* and dilute to 100.0 ml with *water R*. Examined between 230 nm and 350 nm (2.2.25), the solution shows an absorption maximum at 284 nm. The specific absorbance at the maximum is 36 to 38.
- Dissolve 50 mg in 1 ml of *sulphuric acid R* and add 0.05 ml of *ammonium molybdate solution R*. A pale-blue colour is produced which becomes deep blue on gentle warming. Add 0.05 ml of *dilute nitric acid R*. The colour becomes brownish-red.

TESTS

Specific optical rotation (2.2.7). Dissolve 1.000 g in *alcohol R* and dilute to 50.0 ml with the same solvent. The specific optical rotation is -94 to -98 , calculated with reference to the dried substance.

Related substances. Examine by thin-layer chromatography (2.2.27), using *silica gel G R* as the coating substance.

Test solution. Dissolve 0.25 g of the substance to be examined in *chloroform R* and dilute to 10 ml with the same solvent.

Reference solution (a). Dilute 0.5 ml of the test solution to 50 ml with *chloroform R*.

Reference solution (b). Dilute 5 ml of reference solution (a) to 10 ml with *chloroform R*.

Apply separately to the plate 10 μ l of each solution. Develop over a path of 15 cm using a mixture of 2.5 volumes of *concentrated ammonia R*, 32.5 volumes of *acetone R*, 35 volumes of *alcohol R* and 35 volumes of *toluene R*. Dry the plate in a current of air and spray with *dilute potassium iodobismuthate solution R*. In the chromatogram obtained with the test solution, any spot apart from the principal spot is not more intense than the spot in the chromatogram obtained with reference solution (a) (1.0 per cent) and not more than one such spot situated above the principal spot is more intense than the spot in the chromatogram obtained with reference solution (b) (0.5 per cent).

Morphine. Dissolve 0.10 g in 0.1 M *hydrochloric acid* and dilute to 5 ml with the same acid. Add 2 ml of a 10 g/l solution of *sodium nitrite R*, allow to stand for 15 min and add 3 ml of *dilute ammonia R1*. The solution is not more intensely coloured than reference solution B₄ (2.2.2, *Method II*) (about 0.13 per cent of morphine).

Loss on drying (2.2.32): 3.9 per cent to 4.5 per cent, determined on 0.500 g by drying in an oven at 100 °C to 105 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.180 g in 50 ml of *anhydrous acetic acid R*, warming gently. Titrate with 0.1 M *perchloric acid* determining the end-point potentiometrically (2.2.20) at the second point of inflexion.

1 ml of 0.1 M *perchloric acid* is equivalent to 19.93 mg of $C_{23}H_{30}N_2O_4$.

01/2005:0004

PHOSPHORIC ACID, CONCENTRATED

Acidum phosphoricum concentratum

 H_3PO_4
 M_r 98.0

DEFINITION

Concentrated phosphoric acid contains not less than 84.0 per cent *m/m* and not more than 90.0 per cent *m/m* of H_3PO_4 .

CHARACTERS

A clear, colourless, syrupy liquid, corrosive, miscible with water and with alcohol. When stored at a low temperature it may solidify into a mass of colourless crystals which do not melt at a temperature below 28 °C.

It has a relative density of about 1.7.

IDENTIFICATION

- Dilute with *water R*. The solution is strongly acid (2.2.4).
- Solution S (see Tests) neutralised with *dilute sodium hydroxide solution R* gives the reactions of phosphates (2.3.1).

TESTS

Solution S. Dilute 10.0 g to 150 ml with *water R*.

Appearance of solution. Solution S is clear (2.2.1) and colourless (2.2.2, *Method II*).

Substances precipitated with ammonia. To 10 ml of solution S add 8 ml of *dilute ammonia R1*. Any opalescence in the solution is not more intense than that in a mixture of 10 ml of solution S and 8 ml of *water R*.

Hypophosphorous acid and phosphorous acid. To 5 ml of solution S add 2 ml of *silver nitrate solution R2* and heat on a water-bath for 5 min. The solution shows no change in appearance.

Chlorides (2.4.4). 15 ml of solution S complies with the limit test for chlorides (50 ppm).

Sulphates (2.4.13). 1.5 g diluted to 15 ml with *distilled water R* complies with the limit test for sulphates (100 ppm).

Arsenic (2.4.2). 7.5 ml of solution S complies with limit test A for arsenic (2 ppm).

Iron (2.4.9). 3 ml of solution S diluted to 10 ml with *water R* complies with the limit test for iron (50 ppm).

Heavy metals (2.4.8). To 2.5 g add 4 ml of *dilute ammonia R1* and dilute to 25 ml with *water R*. 12 ml of the solution complies with limit test A for heavy metals (10 ppm). Prepare the standard using *lead standard solution (1 ppm Pb) R*.

ASSAY

To 1.000 g add a solution of 10 g of *sodium chloride R* in 30 ml of *water R*. Titrate with 1 M *sodium hydroxide*, using *phenolphthalein solution R* as indicator.

1 ml of 1 M *sodium hydroxide* is equivalent to 49.00 mg of H_3PO_4 .

STORAGE

Store in a glass container.

01/2005:0005

PHOSPHORIC ACID, DILUTE

Acidum phosphoricum dilutum

DEFINITION

Dilute phosphoric acid contains 9.5 per cent *m/m* to 10.5 per cent *m/m* of H_3PO_4 (M_r 98.0).

PREPARATION

To 115 g of concentrated phosphoric acid add 885 g of *water R* and mix.

IDENTIFICATION

- A. It is strongly acid (2.2.4).
- B. Solution S (see Tests), neutralised with *dilute sodium hydroxide solution R*, gives the reactions of phosphates (2.3.1).

TESTS

Solution S. Dilute 86 g to 150 ml with *water R*.

Appearance of solution. Solution S is clear (2.2.1) and colourless (2.2.2, *Method II*).

Substances precipitated with ammonia. To 10 ml of solution S add 8 ml of *dilute ammonia R1*. Any opalescence in the solution is not more intense than that in a mixture of 10 ml of solution S and 8 ml of *water R*.

Hypophosphorous acid and phosphorous acid. To 5 ml of solution S add 2 ml of *silver nitrate solution R2* and heat on a water-bath for 5 min. The solution shows no change in appearance.

Chlorides (2.4.4). 15 ml of solution S complies with the limit test for chlorides (6 ppm).

Sulphates (2.4.13). 15 ml of the substance to be examined complies with the limit test for sulphates (10 ppm).

Arsenic (2.4.2). 7.5 ml of solution S complies with limit test A for arsenic (0.2 ppm).

Iron (2.4.9). 3 ml of solution S diluted to 10 ml with *water R* complies with the limit test for iron (6 ppm).

Heavy metals (2.4.8). To 20 g of the substance to be examined add 4 ml of *dilute ammonia R1* and dilute to 25 ml with *water R*. 12 ml of the solution complies with limit test A for heavy metals (1 ppm). Prepare the standard using a mixture of 8 ml of *lead standard solution (1 ppm Pb) R* and 2 ml of *water R*.

ASSAY

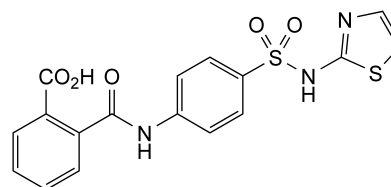
To 8.60 g add a solution of 10 g of *sodium chloride R* in 30 ml of *water R*. Titrate with 1 M *sodium hydroxide*, using *phenolphthalein solution R* as indicator.

1 ml of 1 M *sodium hydroxide* is equivalent to 49.00 mg of H_3PO_4 .

01/2005:0352

PHTHALYLSULFATHIAZOLE

Phthalylsulfathiazolum

 $\text{C}_{17}\text{H}_{13}\text{N}_3\text{O}_5\text{S}_2$ M_r 403.4

DEFINITION

Phthalylsulfathiazole contains not less than 98.5 per cent and not more than the equivalent of 101.5 per cent of 2-[[4-(thiazol-2-ylsulphamoyl)phenyl]carbamoyl]benzoic acid, calculated with reference to the dried substance.

CHARACTERS

A white or yellowish-white, crystalline powder, practically insoluble in water, freely soluble in dimethylformamide, slightly soluble in acetone and in alcohol.

IDENTIFICATION

First identification: A, B, E.

Second identification: B, C, D, E.

- A. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *phthalylsulfathiazole CRS*.
- B. To 1 g add 8.5 ml of *dilute sodium hydroxide solution R* and boil under a reflux condenser for 30 min. Cool and add 17.5 ml of *dilute hydrochloric acid R*. Shake vigorously and filter. Neutralise the filtrate with *dilute sodium hydroxide solution R*. Filter, wash the precipitate with *water R*, recrystallise from *water R* and dry the crystals at 100 °C to 105 °C. The crystals melt (2.2.14) at 200 °C to 203 °C.

01/2005:0286

- C. To 0.1 g in a test-tube add 3 ml of *dilute sulphuric acid R* and 0.5 g of *zinc powder R*. Fumes are evolved which produce a black stain on *lead acetate paper R*.
- D. To 0.1 g add 0.5 g of *resorcinol R* and 0.3 ml of *sulphuric acid R* and heat on a water-bath until a homogeneous mixture is obtained. Allow to cool. Add 5 ml of *dilute sodium hydroxide solution R*. Dilute 0.1 ml of this brownish-red mixture to 25 ml with *water R*. An intense green fluorescence appears which disappears on acidification.
- E. Dissolve about 10 mg of the crystals obtained in identification test B in 200 ml of *0.1 M hydrochloric acid*. 2 ml of the solution gives the reaction of primary aromatic amines (2.3.1) with formation of an orange precipitate.

TESTS

Appearance of solution. Dissolve 1.0 g in *1 M sodium hydroxide* and dilute to 20 ml with the same solvent. The solution is clear (2.2.1) and not more intensely coloured than reference solution BY₅ (2.2.2, Method II).

Acidity. To 2.0 g add 20 ml of *water R*, shake continuously for 30 min and filter. To 10 ml of the filtrate add 0.1 ml of *phenolphthalein solution R*. Not more than 0.2 ml of *0.1 M sodium hydroxide* is required to change the colour of the indicator.

Sulfathiazole and other primary aromatic amines. Dissolve 5 mg in a mixture of 3.5 ml of *water R*, 6 ml of *dilute hydrochloric acid R* and 25 ml of *alcohol R*, previously cooled to 15 °C. Place immediately in iced water and add 1 ml of a 2.5 g/l solution of *sodium nitrite R*. Allow to stand for 3 min, add 2.5 ml of a 40 g/l solution of *sulphamic acid R* and allow to stand for 5 min. Add 1 ml of a 4 g/l solution of *naphthylethylenediamine dihydrochloride R* and dilute to 50 ml with *water R*. Measured at 550 nm, the absorbance (2.2.25) is not greater than that of a standard prepared at the same time and in the same manner using a mixture of 1 ml of a solution containing in 100 ml; 10 mg of *sulfathiazole R* and 0.5 ml of *hydrochloric acid R*, 2.5 ml of *water R*, 6 ml of *dilute hydrochloric acid R* and 25 ml of *alcohol R*.

Heavy metals (2.4.8). 1.0 g complies with limit test C for heavy metals (20 ppm). Prepare the standard using 2 ml of *lead standard solution (10 ppm Pb) R*.

Loss on drying (2.2.32). Not more than 2 per cent, determined on 1.00 g by drying in an oven at 100 °C to 105 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.300 g in 40 ml of *dimethylformamide R*. Titrate with *0.1 M sodium hydroxide* until the colour becomes blue, using 0.2 ml of *thymolphthalein solution R* as indicator. Carry out a blank titration.

1 ml of *0.1 M sodium hydroxide* is equivalent to 20.17 mg of C₂₂H₂₇N₃O₅S₂.

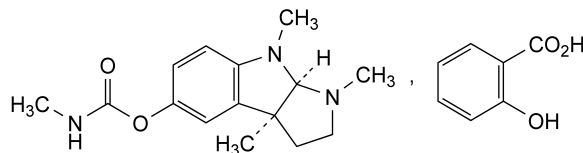
STORAGE

Store protected from light.

PHYSOSTIGMINE SALICYLATE

Physostigmini salicylas

Eserini salicylas

C₂₂H₂₇N₃O₅M_r 413.5

DEFINITION

Physostigmine salicylate contains not less than 98.5 per cent and not more than the equivalent of 101.0 per cent of (3a*S*,8a*R*)-1,2,3,3a,8,8a-hexahydro-1,3a,8-trimethylpyrrolo[2,3-*b*]indol-5-yl methylcarbamate salicylate, calculated with reference to the dried substance.

CHARACTERS

Colourless or almost colourless crystals, sparingly soluble in water, soluble in alcohol. The crystals gradually become red when exposed to air and light; the colour develops more quickly when the crystals are also exposed to moisture. Aqueous solutions are unstable.

It melts at about 182 °C, with decomposition.

IDENTIFICATION

First identification: A, B.

Second identification: B, C, D.

- Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *physostigmine salicylate CRS*.
- Examine the chromatograms obtained in the test for related substances. The principal spot in the chromatogram obtained with test solution (b) is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).
- Heat about 10 mg in a porcelain dish with a few drops of *dilute ammonia R1*. An orange colour develops. Evaporate the solution to dryness. The residue dissolves in *alcohol R* giving a blue solution. Add 0.1 ml of *glacial acetic acid R*. The colour becomes violet. Dilute with *water R*. An intense red fluorescence appears.
- Solution S (see Tests) gives reaction (a) of salicylates (2.3.1).

TESTS

Solution S. Dissolve 0.900 g, without heating, in 95 ml of *carbon dioxide-free water R* prepared from *distilled water R* and dilute to 100.0 ml with the same solvent. Prepare immediately before use.

Appearance of solution. Solution S is clear (2.2.1) and colourless (2.2.2, Method II).

pH (2.2.3). The pH of solution S is 5.1 to 5.9.

Specific optical rotation (2.2.7): −90 to −94, determined on solution S and calculated with reference to the dried substance.

Related substances. Examine by thin-layer chromatography (2.2.27), using *silica gel G R* as the coating substance.

Test solution (a). Dissolve 0.2 g of the substance to be examined in *alcohol R* and dilute to 10 ml with the same solvent.

Test solution (b). Dilute 2.5 ml of test solution (a) to 50 ml with *alcohol R*.

Reference solution (a). Dissolve 10 mg of *physostigmine salicylate CRS* in *alcohol R* and dilute to 10 ml with the same solvent.

Reference solution (b). Dilute 2 ml of reference solution (a) to 20 ml with *alcohol R*.

Apply to the plate 20 µl of each solution. Develop over a path of 15 cm using a mixture of 2 volumes of *concentrated ammonia R*, 23 volumes of *2-propanol R* and 100 volumes of *cyclohexane R*. Dry the plate in a current of cold air and carry out a second development in the same direction. Allow the plate to dry in air and spray with freshly prepared *potassium iodobismuthate solution R* and then with *dilute hydrogen peroxide solution R*. Examine the plate within 2 min. Any spot in the chromatogram obtained with test solution (a), apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.5 per cent).

Eseridine. To 5 ml of solution S add a few crystals of *potassium iodate R*, 0.05 ml of *dilute hydrochloric acid R* and 2 ml of *chloroform R*. Shake. No violet colour develops in the chloroform layer within 1 min.

Sulphates (2.4.13). 15 ml of solution S complies with the limit test for sulphates (0.1 per cent).

Loss on drying (2.2.32). Not more than 1.0 per cent, determined on 1.00 g by drying in an oven at 100 °C to 105 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on the residue obtained in the test for loss on drying.

ASSAY

Dissolve 0.350 g in 50 ml of a mixture of equal volumes of *anhydrous acetic acid R* and *chloroform R*. Titrate with 0.1 M *perchloric acid* determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *perchloric acid* is equivalent to 41.35 mg of $C_{30}H_{44}N_6O_8$.

STORAGE

Store in an airtight container, protected from light.

DEFINITION

Physostigmine sulphate contains not less than 97.0 per cent and not more than the equivalent of 101.0 per cent of di[(3aS,8aR)-1,2,3,3a,8,8a-hexahydro-1,3a,8-trimethylpyrrolo[2,3-b]indol-5-yl methylcarbamate] sulphate, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, crystalline powder, hygroscopic, very soluble in water, freely soluble in alcohol. It gradually becomes red when exposed to air and light; the colour develops more quickly when the substance is also exposed to moisture. Aqueous solutions are unstable.

It melts at about 145 °C, with decomposition.

IDENTIFICATION

First identification: A, D.

Second identification: B, C, D.

- Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *physostigmine sulphate CRS*. Examine the substances prepared as discs using *potassium bromide R*.
- Examine the chromatograms obtained in the test for related substances. The principal spot in the chromatogram obtained with test solution (b) is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).
- Heat about 10 mg in a porcelain dish with 0.5 ml of *dilute ammonia R1*. An orange colour develops. Evaporate the solution to dryness. The residue dissolves in *alcohol R* giving a blue solution. Add 0.1 ml of *glacial acetic acid R*. The colour becomes violet. Dilute with water. An intense red fluorescence appears.
- It gives reaction (a) of sulphates (2.3.1).

TESTS

Solution S. Dissolve 0.500 g without heating in *carbon dioxide-free water R* and dilute to 50.0 ml with the same solvent. Prepare immediately before use.

Appearance of solution. Solution S is clear (2.2.1) and colourless (2.2.2, *Method II*).

pH (2.2.3). The pH of solution S is 3.5 to 5.5.

Specific optical rotation (2.2.7): –116 to –120, determined on solution S and calculated with reference to the dried substance.

Related substances. Examine by thin-layer chromatography (2.2.27), using *silica gel G R* as the coating substance.

Test solution (a). Dissolve 0.15 g of the substance to be examined in *alcohol R* and dilute to 5 ml with the same solvent.

Test solution (b). Dilute 1 ml of test solution (a) to 10 ml with *alcohol R*.

Reference solution (a). Dissolve 30 mg of *physostigmine sulphate CRS* in *alcohol R* and dilute to 10 ml with the same solvent.

Reference solution (b). Dilute 5 ml of test solution (b) to 100 ml with *alcohol R*.

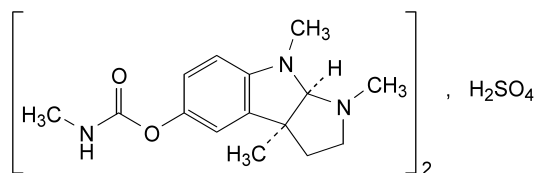
Apply separately to the plate 10 µl of each solution. Develop over a path of 15 cm using a mixture of 2 volumes of *concentrated ammonia R*, 23 volumes of *2-propanol R* and 100 volumes of *cyclohexane R*. Dry the plate in a current of cold air and carry out a second development in the same direction. Allow the plate to dry in air and spray with freshly prepared *potassium iodo-bismuthate solution R* and then with *dilute hydrogen peroxide solution R*. Examine the plate within 2 min. Any spot in the chromatogram obtained

01/2005:0684

PHYSOSTIGMINE SULPHATE

Physostigmini sulfas

Eserini sulfas



$C_{30}H_{44}N_6O_8S$

M_r 649

with test solution (a), apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.5 per cent).

Eseridine. To 5 ml of solution S add a few crystals of *potassium iodate R*, 0.05 ml of *dilute hydrochloric acid R* and 2 ml of *chloroform R* and shake. After 1 min, the chloroform layer is not more intensely coloured than a reference solution prepared at the same time in the same manner using 5 ml of *water R* instead of solution S.

Loss on drying (2.2.32). Not more than 1.0 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on the residue obtained in the test for loss on drying.

ASSAY

Dissolve 0.5000 g in a mixture of 20 ml of *anhydrous acetic acid R* and 40 ml of *acetic anhydride R*. Titrate with 0.1 M *perchloric acid* determining the end-point potentiometrically (2.2.20) at the first inflexion point.

1 ml of 0.1 M *perchloric acid* is equivalent to 64.88 mg of $C_{30}H_{44}N_6O_8S$.

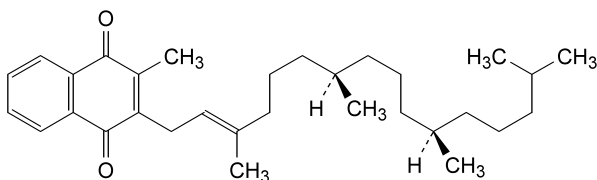
STORAGE

Store in a well-filled, airtight glass container, protected from light.

01/2005:1036

PHYTOMENADIONE

Phytomenadionum



$C_{31}H_{46}O_2$

M_r 450.7

DEFINITION

Phytomenadione is a mixture of 2-methyl-3-[(2E)-(7R,11R)-3,7,11,15-tetramethylhexadec-2-enyl]naphthalene-1,4-dione (*trans*-phytomenadione), 2-methyl-3-[(2Z)-(7R,11R)-3,7,11,15-tetramethylhexadec-2-enyl]naphthalene-1,4-dione (*cis*-phytomenadione) and 2,3-epoxy-2-methyl-3-[(2E)-(7R,11R)-3,7,11,15-tetramethylhexadec-2-enyl]-2,3-dihydronaphthalene-1,4-dione (*trans*-epoxyphytomenadione). It contains not more than 4.0 per cent of *trans*-epoxyphytomenadione and not less than 75.0 per cent of *trans*-phytomenadione. The total of the three components is not less than 97.0 per cent and not more than the equivalent of 103.0 per cent.

CHARACTERS

A clear, intense yellow, viscous, oily liquid, practically insoluble in water, sparingly soluble in alcohol, miscible with fatty oils.

It decomposes on exposure to actinic light.

The refractive index is about 1.526.

IDENTIFICATION

Carry out all operations as rapidly as possible avoiding exposure to actinic light.

- Dissolve 10.0 mg in *trimethylpentane R* and dilute to 100.0 ml with the same solvent. Examined between 275 nm and 340 nm (2.2.25), the solution shows an absorption maximum at 327 nm and an absorption minimum at 285 nm. The specific absorbance at the maximum is 67 to 73. Dilute 10.0 ml of the solution to 50.0 ml with *trimethylpentane R*. Examined between 230 nm and 280 nm, the solution shows four absorbance maxima, at 243 nm, 249 nm, 261 nm and 270 nm respectively.
- Examine the chromatograms obtained in the test for menadione and other related substances. The principal spot in the chromatogram obtained with test solution (b) is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).
- Dissolve 50 mg in 10 ml of *methanol R* and add 1 ml of a 200 g/l solution of *potassium hydroxide R* in *methanol R*. A green colour is produced which becomes violet-red on heating in a water-bath at 40 °C and then reddish-brown on standing.

TESTS

Appearance of solution. Dissolve 2.5 g in *trimethylpentane R* and dilute to 25 ml with the same solvent. The solution is clear (2.2.1).

Acid value (2.5.1). Not more than 2.0, determined on 2.00 g.

Menadione and other related substances. Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel F₂₅₄ plate R*.

Test solution (a). Dissolve 0.40 g of the substance to be examined in *cyclohexane R* and dilute to 10 ml with the same solvent.

Test solution (b). Dilute 1 ml of test solution (a) to 10 ml with *cyclohexane R*.

Reference solution (a). Dissolve 40 mg of *phytomenadione CRS* in *cyclohexane R* and dilute to 10 ml with the same solvent.

Reference solution (b). Dilute 1 ml of test solution (b) to 20 ml with *cyclohexane R*.

Reference solution (c). Dissolve 4.0 mg of *menadione R* in *cyclohexane R* and dilute to 50 ml with the same solvent.

Apply to the plate 10 µl of each solution. Develop over a path of 15 cm using a mixture of 20 volumes of *cyclohexane R* and 80 volumes of *toluene R*. Allow the plate to dry in air for 5 min. Examine in ultraviolet light at 254 nm and spray with a 100 g/l solution of *phosphomolybdic acid R* in *ethanol R*. Heat at 120 °C for 5 min. Examine in daylight. In the chromatogram obtained with test solution (a): any spot corresponding to menadione is not more intense than the spot in the chromatogram obtained with reference solution (c) (0.2 per cent); any spot, apart from the principal spot and any spot corresponding to menadione, is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.5 per cent). Disregard any spot below the principal spot, which may not be completely separated from the principal spot.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 15.0 mg of the substance to be examined in the mobile phase and dilute to 10.0 ml with the mobile phase.

Reference solution (a). Dissolve 15.0 mg of *phytomenadione CRS* in the mobile phase and dilute to 10.0 ml with the mobile phase.

Reference solution (b). Dissolve 15.0 mg of *phytomenadione CRS* and 4.0 mg of *trans-epoxyphytomenadione CRS* in the mobile phase and dilute to 10.0 ml with the mobile phase.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4.6 mm in internal diameter packed with spherical *silica gel for chromatography R* (5 µm) with a porosity of 8 nm,
- as mobile phase at a flow rate of 0.4 ml/min a mixture of 0.67 volumes of *octanol R*, 3.3 volumes of *di-isopropyl ether R* and 1000 volumes of *heptane R*,
- as detector a spectrophotometer set at 254 nm,
- a 20 µl loop injector.

Inject reference solution (b). When using a recorder, adjust the sensitivity of the system so that the height of the principal peak is at least 50 per cent of the full scale of the recorder. The test is not valid unless the order of elution of the peaks is: *trans-epoxyphytomenadione*, *cis-phytomenadione* and *trans-phytomenadione*. Carry out six replicate injections of reference solution (a). The assay is not valid unless the relative standard deviation of the peak area of the *trans*-isomer is less than 1.0 per cent and the resolution between the peaks corresponding to *trans-phytomenadione* and *cis-phytomenadione* is at least 2.5. Inject the test solution and reference solution (a) and calculate the percentage contents of *trans-phytomenadione*, *cis-phytomenadione* and *trans-epoxyphytomenadione* using the following expressions:

$$\text{trans-phytomenadione} = \frac{m' \times A'_{\text{trans}} \times S_{\text{trans}}}{m \times S'_{\text{trans}}}$$

$$\text{cis-phytomenadione} = \frac{m' \times A'_{\text{cis}} \times S_{\text{cis}}}{m \times S'_{\text{cis}}}$$

$$\text{trans-epoxyphytomenadione} = \frac{m' \times A'_{\text{epoxy}} \times S_{\text{epoxy}}}{m \times S'_{\text{epoxy}}}$$

- m' = mass of the reference substance in reference solution (a), in milligrams,
- m = mass of the substance to be examined in the test solution, in milligrams,
- A'_{trans} = percentage content of *trans-phytomenadione* in *phytomenadione CRS*,
- A'_{cis} = percentage content of *cis-phytomenadione* in *phytomenadione CRS*,
- A'_{epoxy} = percentage content of *trans-epoxyphytomenadione* in *phytomenadione CRS*,
- S_{trans} = area of the peak corresponding to the *trans*-isomer in the chromatogram obtained with the test solution,
- S_{cis} = area of the peak corresponding to the *cis*-isomer in the chromatogram obtained with the test solution,
- S_{epoxy} = area of the peak corresponding to *trans-epoxyphytomenadione* in the chromatogram obtained with the test solution,

S'_{trans} = area of the peak corresponding to the *trans*-isomer in the chromatogram obtained with reference solution (a),

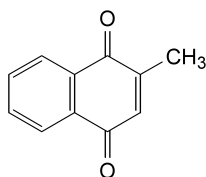
S'_{cis} = area of the peak corresponding to the *cis*-isomer in the chromatogram obtained with reference solution (a),

S'_{epoxy} = area of the peak corresponding to *trans-epoxyphytomenadione* in the chromatogram obtained with reference solution (a).

STORAGE

Store protected from light.

IMPURITIES



A. 2-methylnaphthalene-1,4-dione (menadione).

01/2005:1911

PHYTOSTEROL

Phytosterolum

DEFINITION

Natural mixture of sterols obtained from plants of the genera *Hypoxis*, *Pinus* and *Picea*.

Content: minimum 70.0 per cent of β -sitosterol (dried substance).

CHARACTERS

Appearance: white or almost white powder.

Solubility: practically insoluble in water, soluble in tetrahydrofuran, sparingly soluble in ethyl acetate.

IDENTIFICATION

- A. Mix 1 ml of *acetic anhydride R* with 0.5 ml of solution S (see Tests). After the addition of 0.1 ml of *sulphuric acid R* a red colour is produced which changes rapidly to violet then to blue and finally to green.
- B. Examine the chromatograms obtained in the assay.
- Results:** the principal peak in the chromatogram obtained with the test solution is similar in retention time to the peak in the chromatogram obtained with reference solution (b).

TESTS

Solution S. Dissolve 1.0 g in *tetrahydrofuran R* and dilute to 20 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and not more intensely coloured than reference solution Y₆ (2.2.2, Method II).

Acidity or alkalinity. Shake 0.20 g with a mixture of 4.0 ml of *ethyl acetate R* and 10.0 ml of *carbon dioxide-free water R* for 3 min. Allow the layers to separate. To the aqueous layer add 0.1 ml of *bromothymol blue solution R1*. If the solution is yellow not more than 0.5 ml of 0.01 M *sodium hydroxide* is required to change the colour to blue. If the solution is blue not more than 0.5 ml of 0.01 M *hydrochloric acid* is required to change the colour to yellow.

Specific optical rotation (2.2.7): -15 to -28 (dried substance).

Dissolve 0.500 g in *ethyl acetate R* and dilute to 10.0 ml with the same solvent.

Acid value (2.5.1): maximum 1.0, determined on 2.0 g.

Peroxide value (2.5.5): maximum 10.0.

Saponification value (2.5.6): maximum 3.

Other sterols. Examine the chromatogram obtained with the test solution in the assay (Figure 1911-1).

Composition of the other sterols:

- *cholesterol*: maximum 0.5 per cent,
- *brassicasterol*: maximum 0.5 per cent,
- *campesterol*: maximum 15.0 per cent,
- *campestanol*: maximum 5.0 per cent,
- *stigmasterol*: maximum 5.0 per cent,
- *sitostanol*: maximum 15.0 per cent,
- Δ^7 -*stigmasterol*: maximum 5.0 per cent.

Loss on drying (2.2.32): maximum 4.0 per cent, determined on 0.250 g by drying in an oven at 100–105 °C for 2 h.

Total ash (2.4.16): maximum 0.5 per cent, determined on 1.0 g.

ASSAY

Gas chromatography (2.2.28): use the normalisation procedure.

Test solution. Dissolve 0.100 g in *tetrahydrofuran R* and dilute to 10.0 ml with the same solvent. Introduce 100 µl of this solution into a 3 ml flask and evaporate to dryness under *nitrogen R*. Add 100 µl of a freshly prepared mixture of 50 µl of *1-methylimidazole R* and 1.0 ml of *heptafluoro-N-methyl-N-(trimethylsilyl)butanamide R*, close the flask tightly and heat to 100 °C for 15 min. Allow to cool.

Reference solution (a). Dissolve 25 mg of β -sitosterol *R* and 25 mg of sitostanol *R* in *tetrahydrofuran R* and dilute to 10.0 ml with the same solvent. Introduce 100 µl of this solution into a 3 ml flask and evaporate to dryness under *nitrogen R*. Add 100 µl of a freshly prepared mixture of 50 µl of *1-methylimidazole* and 1.0 ml of *heptafluoro-N-methyl-N-(trimethylsilyl)butanamide R*. Close the flask tightly and heat to 100 °C for 15 min. Allow to cool.

Reference solution (b). Dissolve 0.100 g of β -sitosterol *R* in *tetrahydrofuran R* and dilute to 10.0 ml with the same solvent. Introduce 100 µl of this solution into a 3 ml flask and evaporate to dryness under *nitrogen R*. Add 100 µl of a freshly prepared mixture of 50 µl of *1-methylimidazole R* and 1.0 ml of *heptafluoro-N-methyl-N-(trimethylsilyl)butanamide R*. Close the flask tightly and heat to 100 °C for 15 min. Allow to cool.

Column:

- *material*: quartz,
- *size*: $l = 25$ m, $\varnothing = 0.3$ mm,
- *stationary phase*: poly(dimethyl)(diphenyl)(divinyl)siloxane *R* (1 µm).

Carrier gas: hydrogen for chromatography *R*.

Flow rate: 2 ml/min.

Split ratio: 1:20.

Temperature:

- *column*: 280 °C,
- *injection port and detector*: 300 °C.

Detection: flame ionisation.

Injection: 1 µl.

Relative retentions with reference to β -sitosterol (retention time = about 16 min): cholesterol = about 0.7; brassicasterol = about 0.77; campesterol = about 0.84; campestanol = about 0.86; stigmasterol = about 0.9; sitostanol = about 1.02; Δ^7 -stigmasterol = about 1.1.

System suitability: reference solution (a):

- *resolution*: minimum of 1.0 between the peaks due to β -sitosterol and sitostanol.

STORAGE

In an airtight container, protected from light.

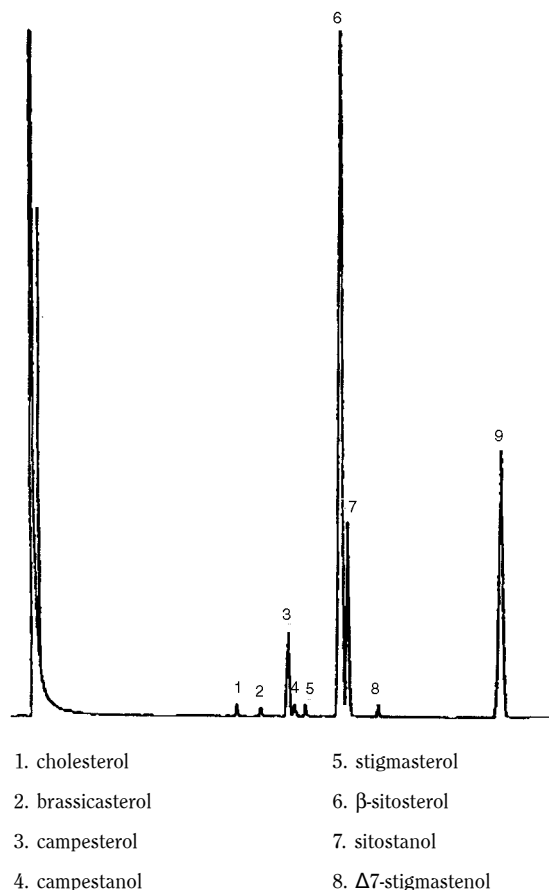
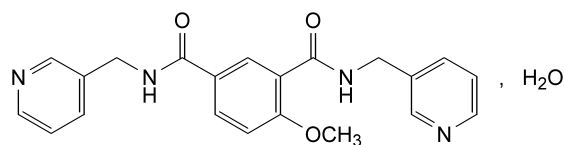


Figure 1911-1. – Assay of phytosterol (trimethylsilane derivatives)

01/2005:1358

PICOTAMIDE MONOHYDRATE

Picotamidum monohydricum



$C_{21}H_{20}N_4O_3 \cdot H_2O$

M_r 394.4

DEFINITION

Picotamide monohydrate contains not less than 98.0 per cent and not more than 101.0 per cent of 4-methoxy-*N,N'*-bis(pyridin-3-ylmethyl)benzene-1,3-dicarboxamide, calculated with reference to the anhydrous substance.

CHARACTERS

A white or almost white, crystalline powder, slightly soluble in water, soluble in ethanol and in methylene chloride. It dissolves in dilute mineral acids.

It shows polymorphism.

IDENTIFICATION

Examine by infrared spectrophotometry (2.2.24), comparing with the spectrum obtained with *picotamide monohydrate CRS*. If the spectra obtained in the solid state shows differences, dissolve the substance to be examined and the reference substance separately in *acetone R*, evaporate to dryness and record new spectra using the residues.

TESTS

Appearance of solution. Dissolve 2.5 g in *methanol R* and dilute to 50 ml with the same solvent. The solution is clear (2.2.1) and not more intensely coloured than reference solution Y₆ (2.2.2, *Method II*).

Related substances. Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel F₂₅₄ plate R*.

Test solution. Dissolve 0.5 g of the substance to be examined in *methanol R* and dilute to 10 ml with the same solvent.

Reference solution (a). Dilute 1 ml of the test solution to 10 ml with *methanol R*. Dilute 1 ml of the solution to 20 ml with *methanol R*.

Reference solution (b). Dilute 5 ml of reference solution (a) to 10 ml with *methanol R*.

Reference solution (c). Dissolve 0.5 g of the substance to be examined and 5 mg of *picotamide impurity A CRS* in *methanol R* and dilute to 10 ml with the same solvent.

Apply to the plate 5 µl of each solution. Develop over a path of 15 cm using a mixture 0.8 volumes of *glacial acetic acid R*, 1 volume of *water R*, 2.5 volumes of *methanol R* and 8 volumes of *butanol R*. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. Any spot in the chromatogram obtained with the test solution, apart from the principal spot, is not more intense than the principal spot in the chromatogram obtained with reference solution (a) (0.5 per cent) and not more than one such spot is more intense than the spot in the chromatogram obtained with reference solution (b) (0.25 per cent). The test is not valid unless the chromatogram obtained with reference solution (c) shows two clearly separated principal spots.

Chlorides (2.4.4). Dissolve 0.25 g in a mixture of 2.5 ml of *dilute nitric acid R* and 12.5 ml of *water R*. The solution complies with the limit test for chlorides (200 ppm).

Heavy metals (2.4.8). Dissolve 1.0 g by gently heating in a mixture of 15 volumes of *water R* and 85 volumes of *methanol R* and dilute to 20 ml with the same mixture of solvents. 12 ml of the solution complies with limit test B for heavy metals (20 ppm). Prepare the standard, using lead standard solution (1 ppm Pb) obtained by diluting *lead standard solution (100 ppm Pb) R* with a mixture of 15 volumes of *water R* and 85 volumes of *methanol R*.

Water (2.5.12): 4.5 per cent to 5.0 per cent, determined on 0.300 g by the semi-micro determination of water.

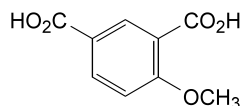
Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

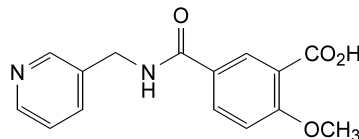
Dissolve 0.150 g in a mixture of 20 ml of *anhydrous acetic acid R* and 20 ml of *acetic anhydride R*. Titrate with 0.1 M *perchloric acid*, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *perchloric acid* is equivalent to 18.82 mg of C₂₁H₂₀N₄O₃.

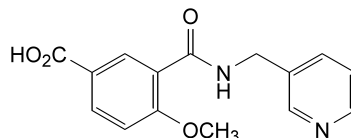
IMPURITIES



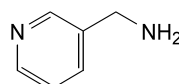
A. 4-methoxybenzene-1,3-dicarboxylic acid,



B. 2-methoxy-5-[(pyridin-3-ylmethyl)amino]carbonylbenzoic acid,



C. 4-methoxy-3-[(pyridin-3-ylmethyl)amino]carbonylbenzoic acid,

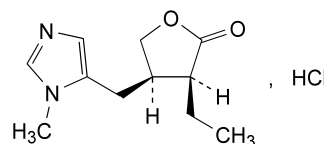


D. (pyridin-3-yl)methanamine.

01/2005:0633

PILOCARPINE HYDROCHLORIDE

Pilocarpini hydrochloridum



C₁₁H₁₇ClN₂O₂

M_r 244.7

DEFINITION

Pilocarpine hydrochloride contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of (3*S*,4*R*)-3-ethyl-4-[(1-methyl-1*H*-imidazol-5-yl)methyl]dihydrofuran-2(3*H*)-one hydrochloride, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, crystalline powder or colourless crystals, hygroscopic, very soluble in water and in alcohol. It melts at about 203 °C.

IDENTIFICATION

First identification: A, B, E.

Second identification: A, C, D, E.

A. It complies with the test for specific optical rotation (see Tests).

B. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *pilocarpine hydrochloride CRS*. If the substances are examined as discs, prepare using *potassium chloride R*.

- C. Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel G plate R*.

Test solution. Dissolve 10 mg in *methanol R* and dilute to 2 ml with the same solvent.

Reference solution. Dissolve 10 mg of *pilocarpine hydrochloride CRS* in *methanol R* and dilute to 2 ml with the same solvent.

Apply to the plate 2 µl of each solution. Develop over a path of 15 cm using a mixture of 1 volume of *concentrated ammonia R*, 14 volumes of *methanol R* and 85 volumes of *methylene chloride R*. Dry the plate at 100–105 °C for 10 min, allow to cool and spray with *dilute potassium iodobismuthate solution R*. The principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

- D. Dilute 0.2 ml of solution S (see Tests) to 2 ml with *water R*. Add 0.05 ml of a 50 g/l solution of *potassium dichromate R*, 1 ml of *dilute hydrogen peroxide solution R* and 2 ml of *methylene chloride R* and shake. A violet colour develops in the organic layer.

- E. It gives reaction (a) of chlorides (2.3.1).

TESTS

Solution S. Dissolve 2.50 g in *carbon dioxide-free water R* and dilute to 50.0 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and not more intensely coloured than reference solution Y₇ (2.2.2, *Method II*).

pH (2.2.3). The pH of solution S is 3.5 to 4.5.

Specific optical rotation (2.2.7): + 89 to + 93, determined on solution S and calculated with reference to the dried substance.

Related substances. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 0.100 g of the substance to be examined in *water R* and dilute to 100.0 ml with the same solvent.

Reference solution (a). Dilute 5.0 ml of the test solution to 100.0 ml with *water R*. Dilute 2.0 ml of the solution to 20.0 ml with *water R*.

Reference solution (b). Dissolve 5.0 mg of *pilocarpine nitrate for system suitability CRS* in *water R* and dilute to 50.0 ml with the same solvent.

Reference solution (c). To 5 ml of the test solution, add 0.1 ml of *ammonia R* and heat the solution on a water-bath for 30 min, cool and dilute to 25 ml with *water R*. Take 3 ml of this solution and dilute to 25 ml with *water R*. Mainly pilocarpic acid is formed.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.15 m long and 4.6 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R1* (5 µm) with a pore size of 10 nm and a carbon loading of 19 per cent,
- as mobile phase at a flow rate of 1.2 ml/min a solution prepared as follows: mix 55 volumes of *methanol R*, 60 volumes of *acetonitrile R* and 885 volumes of a 0.679 g/l solution of *tetrabutylammonium dihydrogen phosphate R*, previously adjusted to pH 7.7 with *dilute ammonia R2*,
- as detector a spectrophotometer set at 220 nm.

Inject 20 µl of each solution. Continue the chromatography for twice the retention time of the principal component (about 40 min). When the chromatograms are recorded in the prescribed conditions, the substances are eluted in the following sequence: pilocarpic acid, isopilocarpic acid, isopilocarpine and pilocarpine. The test is not valid unless the resolution between the peaks corresponding to isopilocarpine and pilocarpine in the chromatogram obtained with reference solution (b) is at least 1.6. In the chromatogram obtained with the test solution: the area of the peak corresponding to isopilocarpine is not greater than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (1 per cent); the sum of the areas of the peaks corresponding to isopilocarpine and pilocarpic acid is not greater than 3 times the area of the principal peak in the chromatogram obtained with reference solution (a) (1.5 per cent); and the sum of the areas of any peaks apart from the principal peak and the peaks corresponding to isopilocarpine and pilocarpic acid is not greater than the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent). Disregard any peak with an area less than 0.4 times the area of the principal peak in the chromatogram obtained with reference solution (a).

Iron (2.4.9). 10 ml of solution S complies with the limit test for iron (10 ppm). Prepare the standard using 5 ml of *iron standard solution (1 ppm Fe) R* and 5 ml of *water R*.

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 100–105 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

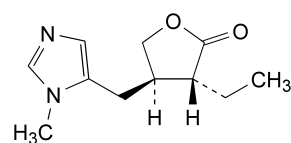
Dissolve 0.200 g in 50 ml of *alcohol R* and add 5 ml of 0.01 M *hydrochloric acid*. Titrate with 0.1 M *sodium hydroxide*, determining the end-point potentiometrically (2.2.20). Read the volume added between the two points of inflexion.

1 ml of 0.1 M *sodium hydroxide* is equivalent to 24.47 mg of C₁₁H₁₇ClN₂O₂.

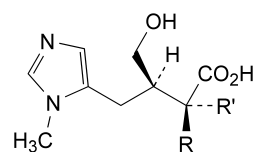
STORAGE

Store in an airtight container, protected from light.

IMPURITIES



- A. (3*R*,4*R*)-3-ethyl-4-[(1-methyl-1*H*-imidazol-5-yl)methyl]dihydrofuran-2(3*H*)-one (isopilocarpine),

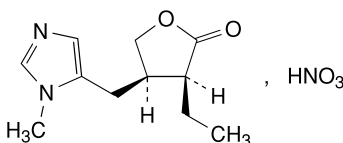


- B. R = C₂H₅, R' = H: (2*S*,3*R*)-2-ethyl-3-(hydroxymethyl)-4-(1-methyl-1*H*-imidazol-5-yl)butanoic acid (pilocarpic acid),
- C. R = H, R' = C₂H₅: (2*R*,3*R*)-2-ethyl-3-(hydroxymethyl)-4-(1-methyl-1*H*-imidazol-5-yl)butanoic acid (isopilocarpic acid).

01/2005:0104

PILOCARPINE NITRATE

Pilocarpini nitras

 $C_{11}H_{17}N_3O_5$ M_r 271.3

DEFINITION

Pilocarpine nitrate contains not less than 98.5 per cent and not more than the equivalent of 101.0 per cent of (3*S*,4*R*)-3-ethyl-4-[(1-methyl-1*H*-imidazol-5-yl)methyl]dihydrofuran-2(3*H*)-one nitrate, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, crystalline powder or colourless crystals, sensitive to light, freely soluble in water, sparingly soluble in alcohol.

It melts at about 174 °C with decomposition.

IDENTIFICATION

First identification: A, B, E.

Second identification: A, C, D, E.

- A. It complies with the test for specific optical rotation (see Tests).
- B. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *pilocarpine nitrate CRS*.
- C. Examine thin-layer chromatography (2.2.27), using a *TLC silica gel G plate R*.

Test solution. Dissolve 10 mg of the substance to be examined in *water R* and dilute to 10 ml with the same solvent.

Reference solution. Dissolve 10 mg of *pilocarpine nitrate CRS* in *water R* and dilute to 10 ml with the same solvent.

Apply to the plate 10 µl of each solution. Develop over a path of 15 cm using a mixture of 1 volume of *concentrated ammonia R*, 14 volumes of *methanol R* and 85 volumes of *methylene chloride R*. Dry the plate at 100-105 °C for 10 min, allow to cool and spray with *potassium iodobismuthate solution R*. The principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

- D. Dilute 0.2 ml of solution S (see Tests) to 2 ml with *water R*. Add 0.05 ml of a 50 g/l solution of *potassium dichromate R*, 1 ml of *dilute hydrogen peroxide solution R* and 2 ml of *methylene chloride R* and shake. A violet colour develops in the organic layer.
- E. It gives the reaction of nitrates (2.3.1).

TESTS

Solution S. Dissolve 2.50 g in *carbon dioxide-free water R* and dilute to 50.0 ml with the same solvent. Prepare immediately before use.

Appearance of solution. Solution S is clear (2.2.1) and not more intensely coloured than reference solution Y₆ (2.2.2, Method II).

pH (2.2.3). The pH of solution S is 3.5 to 4.5.

Specific optical rotation (2.2.7). + 80 to + 83, determined on solution S and calculated with reference to the dried substance.

Related substances. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 0.100 g of the substance to be examined in *water R* and dilute to 100.0 ml with the same solvent.

Reference solution (a). Dissolve 5.0 ml of the test solution to 100.0 ml with *water R*. Dilute 2.0 ml of this solution to 20.0 ml with *water R*.

Reference solution (b). Dissolve 5.0 mg of *pilocarpine nitrate for system suitability CRS* in *water R* and dilute to 50.0 ml with the same solvent.

Reference solution (c). To 5 ml of the test solution, add 0.1 ml of *ammonia R* and heat the solution on a water-bath for 30 min, cool and dilute to 25 ml with *water R*. Take 3 ml of this solution and dilute to 25 ml with *water R*. Mainly pilocarpic acid is formed.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.15 m long and 4.6 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R1* (5 µm) with a pore size of 10 nm and a carbon loading of 19 per cent,
- as mobile phase at a flow rate of 1.2 ml/min a solution prepared as follows: mix 55 volumes of *methanol R*, 60 volumes of *acetonitrile R* and 885 volumes of a 0.679 g/l solution of *tetrabutylammonium dihydrogen phosphate R*, previously adjusted to pH 7.7 with *diluted ammonia R2*,
- as detector a spectrophotometer set at 220 nm.

Inject 20 µl of each solution. Continue the chromatography for twice the retention time of the principal peak (about 40 min). When the chromatograms are recorded in the prescribed conditions, the substances are eluted in the following sequence: pilocarpic acid, isopilocarpic acid, isopilocarpine and pilocarpine. The test is not valid unless the resolution between the peaks corresponding to isopilocarpine and pilocarpine in the chromatogram obtained with reference solution (b) is at least 1.6. In the chromatogram obtained with the test solution: the area of the peak corresponding to isopilocarpine is not greater than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (1 per cent); the sum of the area of the peaks corresponding to isopilocarpine and pilocarpic acid is less than 3 times the area of the principal peak in the chromatogram obtained with reference solution (a) (1.5 per cent); and the sum of the areas of any peaks apart from the principal peak and the peaks corresponding to isopilocarpine and pilocarpic acid is not greater than the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent). Disregard any peak with an area less than 0.4 times the area of the principal peak in the chromatogram obtained with reference solution (a) and any peak due to the nitrate ion with a relative retention time relative to pilocarpine of about 0.3.

Chlorides (2.4.4). 15 ml of solution S complies with the limit test for chlorides (70 ppm).

Iron (2.4.9). 10 ml of solution S complies with the limit test for iron (10 ppm). Prepare the standard using 5 ml of *iron standard solution* (1 ppm Fe) *R* and 5 ml of *water R*.

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 100-105 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

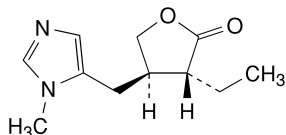
Dissolve 0.250 g in 30 ml of *anhydrous acetic acid R*. Titrate with 0.1 M *perchloric acid* determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *perchloric acid* is equivalent to 27.13 mg of $C_{11}H_{17}N_3O_5$.

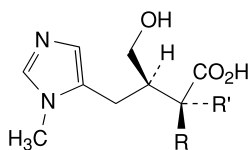
STORAGE

Store protected from light.

IMPURITIES



- A. (3*R*,4*R*)-3-ethyl-4-[(1-methyl-1*H*-imidazol-5-yl)methyl]dihydrofuran-2(3*H*)-one (isopilocarpine),

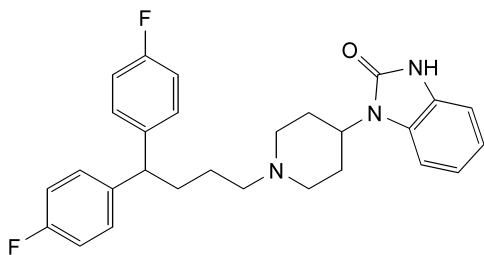


- B. $R = C_2H_5$, $R' = H$: (2*S*,3*R*)-2-ethyl-3-(hydroxymethyl)-4-(1-methyl-1*H*-imidazol-5-yl)butanoic acid (pilocarpic acid),
 C. $R = H$, $R' = C_2H_5$: (2*R*,3*R*)-2-ethyl-3-(hydroxymethyl)-4-(1-methyl-1*H*-imidazol-5-yl)butanoic acid (isopilocarpic acid).

01/2005:1254

PIMOZIDE

Pimozidum


 $C_{28}H_{29}F_2N_3O$
 M_r 461.6

DEFINITION

Pimozide contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of 1-[1-[4,4-bis(4-fluorophenyl)butyl]piperidin-4-yl]-1,3-dihydro-2*H*-benzimidazol-2-one, calculated with reference to the dried substance.

CHARACTERS

A white or almost white powder, practically insoluble in water, soluble in methylene chloride, sparingly soluble in methanol, slightly soluble in alcohol.

IDENTIFICATION

First identification: B.

Second identification: A, C, D.

- A. Melting point (2.2.14): 216 °C to 220 °C.

- B. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *pimozide CRS*. Examine the substances prepared as discs.

- C. Examine by thin-layer chromatography (2.2.27), using a suitable silica gel as the coating substance.

Test solution. Dissolve 30 mg of the substance to be examined in a mixture of 1 volume of *acetone R* and 9 volumes of *methanol R* and dilute to 10 ml with the same mixture of solvents.

Reference solution (a). Dissolve 30 mg of *pimozide CRS* in a mixture of 1 volume of *acetone R* and 9 volumes of *methanol R* and dilute to 10 ml with the same mixture of solvents.

Reference solution (b). Dissolve 30 mg of *pimozide CRS* and 30 mg of *benperidol CRS* in a mixture of 1 volume of *acetone R* and 9 volumes of *methanol R* and dilute to 10 ml with the same mixture of solvents.

Apply separately to the plate 10 µl of each solution.

Develop over a path of 15 cm using a mixture of 1 volume of *acetone R* and 9 volumes of *methanol R*. Allow the plate to dry in a current of warm air for 15 min and expose it to iodine vapour until the spots appear. The principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a). The test is not valid unless the chromatogram obtained with reference solution (b) shows two clearly separated spots.

- D. Mix about 5 mg with 45 mg of *heavy magnesium oxide R* and ignite in a crucible until an almost white residue is obtained (usually less than 5 min). Allow to cool, add 1 ml of *water R*, 0.05 ml of *phenolphthalein solution R1* and about 1 ml of *dilute hydrochloric acid R* to render the solution colourless. Filter. To a freshly prepared mixture of 0.1 ml of *alizarin S solution R* and 0.1 ml of *zirconyl nitrate solution R*, add 1.0 ml of the filtrate. Mix, allow to stand for 5 min and compare the colour of the solution with that of a blank prepared in the same manner. The test solution is yellow and the blank is red.

TESTS

Appearance of solution. Dissolve 0.2 g in *methanol R* and dilute to 20 ml with the same solvent. The solution is clear (2.2.1) and not more intensely coloured than reference solution Y₇ (2.2.2, *Method II*).

Related substances. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 0.10 g of the substance to be examined in *methanol R* and dilute to 10.0 ml with the same solvent.

Reference solution (a). Dissolve 5.0 mg of *pimozide CRS* and 2.0 mg of *mebendazole CRS* in *methanol R* and dilute to 100.0 ml with the same solvent.

Reference solution (b). Dilute 5.0 ml of the test solution to 100.0 ml with *methanol R*. Dilute 1.0 ml of this solution to 10.0 ml with *methanol R*.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.1 m long and 4.6 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (3 µm),

- as mobile phase at a flow rate of 2.0 ml/min:

Mobile phase A. A solution containing 2.5 g/l of *ammonium acetate R* and 8.5 g/l of *tetrabutylammonium hydrogen sulphate R*,

Mobile phase B. *Acetonitrile R*,

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)	Comment
0 - 10	80 → 70	20 → 30	linear gradient
10 - 15	70	30	isocratic elution
15 - 20	80	20	switch to initial eluent composition
20 = 0	80	20	restart gradient

— as detector a spectrophotometer set at 280 nm.

Equilibrate the column for at least 10 min at the initial elution composition.

Adjust the sensitivity of the system so that the height of the principal peak in the chromatogram obtained with 10 µl of reference solution (b) is at least 50 per cent of the full scale of the recorder.

Inject 10 µl of reference solution (a). When the chromatogram is recorded in the prescribed conditions, the retention times are: mebendazole, about 7 min and pimozone, about 8 min. The test is not valid unless the resolution between the peaks due to mebendazole and pimozone is at least 5.0. If necessary, adjust the concentration of acetonitrile in the mobile phase or adjust the time programme for the linear-gradient elution.

Inject separately 10 µl of *methanol R* as a blank, 10 µl of the test solution and 10 µl of reference solution (b). In the chromatogram obtained with the test solution: the area of any peak, apart from the principal peak, is not greater than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent); the sum of the areas of all peaks, apart from the principal peak, is not greater than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.75 per cent). Disregard any peak in the chromatogram obtained with the blank run and any peak with an area less than 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (b).

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 100-105 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g in a platinum crucible.

ASSAY

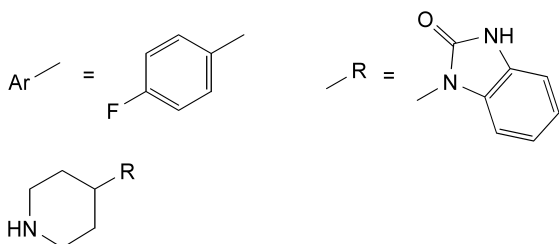
Dissolve 0.300 g in 50 ml of a mixture of 1 volume of *anhydrous acetic acid R* and 7 volumes of *methyl ethyl ketone R* and titrate with 0.1 M *perchloric acid*, using 0.2 ml of *naphtholbenzein solution R* as indicator.

1 ml 0.1 M *perchloric acid* is equivalent to 46.16 mg of C₂₈H₂₉F₂N₃O.

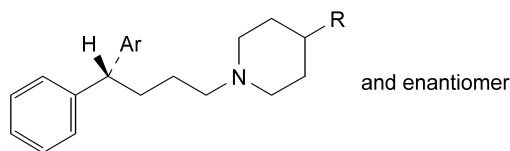
STORAGE

Store protected from light.

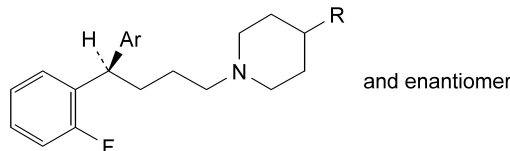
IMPURITIES



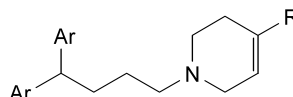
A. 1-(piperidin-4-yl)-1,3-dihydro-2H-benzimidazol-2-one,



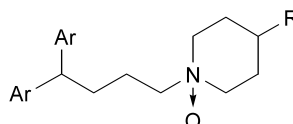
B. 1-[1-[(4RS)-4-(4-fluorophenyl)-4-phenylbutyl]piperidin-4-yl]-1,3-dihydro-2H-benzimidazol-2-one,



C. 1-[1-[(4RS)-4-(2-fluorophenyl)-4-(4-fluorophenyl)butyl]piperidin-4-yl]-1,3-dihydro-2H-benzimidazol-2-one,



D. 1-[1-[4,4-bis(4-fluorophenyl)butyl]-1,2,3,6-tetrahydropyridin-4-yl]-1,3-dihydro-2H-benzimidazol-2-one,

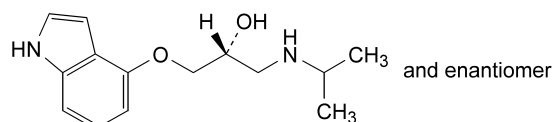


E. 1-[1-[4,4-bis(4-fluorophenyl)butyl]piperidin-4-yl]-1-oxide]-1,3-dihydro-2H-benzimidazol-2-one.

01/2005:0634

PINDOLOL

Pindololum



C₁₄H₂₀N₂O₂

M_r 248.3

DEFINITION

Pindolol contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of (2RS)-1-(1H-indol-4-yloxy)-3-[(1-methylethyl)amino]propan-2-ol, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, crystalline powder, practically insoluble in water, slightly soluble in methanol. It dissolves in dilute mineral acids.

IDENTIFICATION

First identification: A, C.

Second identification: A, B, D.

A. Melting point (2.2.14): 169 °C to 174 °C.

B. Dissolve 20.0 mg in a 0.085 per cent V/V solution of *hydrochloric acid R* in *methanol R* and dilute to 100.0 ml with the same solution. Dilute 10.0 ml of the solution to 100.0 ml with a 0.085 per cent V/V solution of *hydrochloric acid R* in *methanol R*. Examined between 230 nm and 320 nm (2.2.25), the solution shows two

absorption maxima, at 264 nm and at 287 nm, and a shoulder at 275 nm. The specific absorbance at the maxima are 330 to 350 and 170 to 190, respectively.

- C. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *pindolol CRS*.
- D. Examine in daylight the chromatograms on plate A obtained in the test for related substances. The principal spot in the chromatogram obtained with test solution (b) is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).

TESTS

Appearance of solution. Dissolve 0.5 g in *dilute acetic acid R* and dilute to 10 ml with the same acid. The solution is clear (2.2.1) and not more intensely coloured than reference solution BY₅ or B₅ (2.2.2, *Method II*).

Related substances. Examine by thin-layer chromatography (2.2.27), using *silica gel GF₂₅₄ R* as the coating substance. Carry out all operations as rapidly as possible, protected from light.

Test solution (a). Dissolve 0.10 g of the substance to be examined in a mixture of 1 volume of *anhydrous acetic acid R* and 99 volumes of *methanol R* and dilute to 5 ml with the same mixture of solvents. Prepare immediately before use and apply this solution to the plate last.

Test solution (b). Dilute 1 ml of test solution (a) to 10 ml with a mixture of 1 volume of *anhydrous acetic acid R* and 99 volumes of *methanol R*.

Reference solution (a). Dissolve 20 mg of *pindolol CRS* in a mixture of 1 volume of *anhydrous acetic acid R* and 99 volumes of *methanol R* and dilute to 10 ml with the same mixture of solvents.

Reference solution (b). Dilute 1.5 ml of reference solution (a) to 50 ml with a mixture of 1 volume of *anhydrous acetic acid R* and 99 volumes of *methanol R*.

- A. Apply separately 5 µl of each solution. Develop the plate without delay over a path of 10 cm using a freshly prepared mixture of 4 volumes of *concentrated ammonia R*, 50 volumes of *ethyl acetate R* and 50 volumes of *methanol R*. Dry the plate briefly in a current of cold air. Spray the plate without delay with *dimethylaminobenzaldehyde solution R7* and heat to 50 °C for 20 min. Any spot in the chromatogram obtained with test solution (a), apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.3 per cent).
- B. Apply separately 10 µl of each solution. Develop the plate without delay over a path of 10 cm using a freshly prepared mixture of 4 volumes of *concentrated ammonia R*, 50 volumes of *ethyl acetate R* and 50 volumes of *methanol R*. Dry the plate briefly in a current of cold air. Examine the plate without delay in ultraviolet light at 254 nm. Any spot in the chromatogram obtained with test solution (a), apart from the principal spot and the spots detected on plate A, is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.3 per cent).

Heavy metals (2.4.8). 1.0 g complies with limit test C for heavy metals (20 ppm). Prepare the standard using 2 ml of *lead standard solution (10 ppm Pb) R*.

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

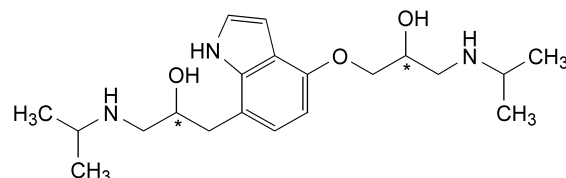
Dissolve 0.200 g in 80 ml of *methanol R*. Titrate with 0.1 M *hydrochloric acid*, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *hydrochloric acid* is equivalent to 24.83 mg of C₁₄H₂₀N₂O₂.

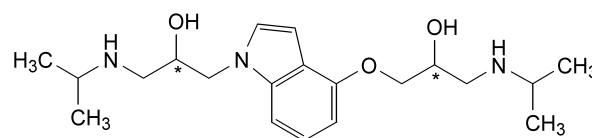
STORAGE

Store protected from light.

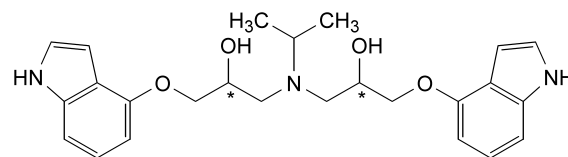
IMPURITIES



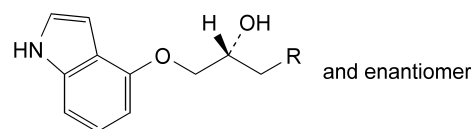
- A. 1-[[7-[2-hydroxy-3-[(1-methylethyl)amino]propyl]-1H-indol-4-yl]oxy]-3-[(1-methylethyl)amino]propan-2-ol,



- B. 1-[4-[2-hydroxy-3-[(1-methylethyl)amino]propoxy]-1H-indol-1-yl]-3-[(1-methylethyl)amino]propan-2-ol,

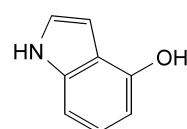


- C. 1,1'-(1-methylethyl)imino]bis[3-(1H-indol-4-yloxy)propan-2-ol],



- D. R = OH: (2RS)-3-(1H-indol-4-yloxy)propane-1,2-diol,

- F. R = Cl: (2RS)-1-chloro-3-(1H-indol-4-yloxy)propan-2-ol,

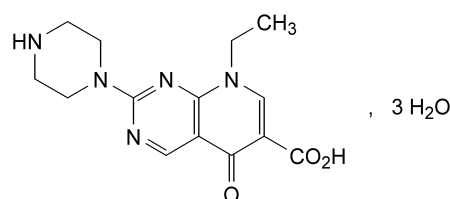


- E. 1H-indol-4-ol.

01/2005:1743

PIPEMIDIC ACID TRIHYDRATE

Acidum pipemidicum trihydricum



C₁₄H₁₇N₅O₃·3H₂O

M_r 357.4

DEFINITION

8-Ethyl-5-oxo-2-(piperazin-1-yl)-5,8-dihydropyrido[2,3-*d*]pyrimidine-6-carboxylic acid trihydrate.

Content: 98.5 per cent to 101.0 per cent (dried substance).

CHARACTERS

Appearance: pale yellow or yellow, crystalline powder.

Solubility: very slightly soluble in water. It dissolves in dilute solutions of acids and of alkali hydroxides.

IDENTIFICATION

Infrared absorption spectrophotometry (2.2.24).

Comparison: Ph. Eur. reference spectrum of pipemidic acid trihydrate.

TESTS

Related substances. Liquid chromatography (2.2.29).

Test solution. Dissolve 20 mg of the substance to be examined in 10 ml of the mobile phase and dilute to 20.0 ml with the mobile phase. Dilute 1.0 ml of the solution to 10.0 ml with the mobile phase.

Reference solution (a). Dilute 2.0 ml of the test solution to 10.0 ml with the mobile phase. Dilute 1.0 ml of the solution to 100.0 ml with the mobile phase.

Reference solution (b). Dissolve 10.0 mg of *ethyl parahydroxybenzoate R* in 2.0 ml of the test solution and dilute to 20.0 ml with the mobile phase.

Column:

- *size*: $l = 0.15$ m, $\varnothing = 4.6$ mm,
- *stationary phase*: octadecylsilyl silica gel for chromatography R1 (5 μ m) with a pore size of 18 nm and a carbon loading of 13 per cent.

Mobile phase: mix 20 volumes of *acetonitrile R*, 20 volumes of *methanol R* and 60 volumes of a solution containing 5.7 g/l of *citric acid R* and 1.7 g/l of *sodium decanesulphonate R*.

Flow rate: 0.8 ml/min.

Detection: spectrophotometer at 275 nm.

Injection: 20 μ l.

Run time: 2.5 times the retention time of pipemidic acid.

System suitability: reference solution (b):

- *resolution*: minimum 4.0 between the peaks due to pipemidic acid and to ethyl parahydroxybenzoate.

Limits:

- *any impurity*: not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent),
- *total*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (1.0 per cent),
- *disregard limit*: 0.25 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

Heavy metals (2.4.8): maximum 20 ppm.

1.0 g complies with limit test C. Prepare the standard using 2.0 ml of *lead standard solution (10 ppm Pb) R*.

Loss on drying (2.2.32): 14.0 per cent to 16.0 per cent, determined on 1.000 g by drying in an oven at 100–105 °C.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

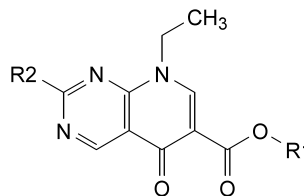
Dissolve 0.240 g in 50 ml of *anhydrous acetic acid R*. Titrate with 0.1 M *perchloric acid*, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *perchloric acid* is equivalent to 30.33 mg of $C_{14}H_{17}N_5O_3$.

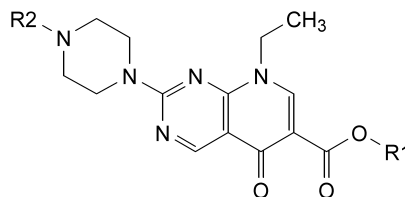
STORAGE

Protected from light.

IMPURITIES



- A. R1 = H, R2 = OH: 8-ethyl-2-hydroxy-5-oxo-5,8-dihydropyrido[2,3-*d*]pyrimidine-6-carboxylic acid,
 B. R1 = H, R2 = OCH₃: 8-ethyl-2-methoxy-5-oxo-5,8-dihydropyrido[2,3-*d*]pyrimidine-6-carboxylic acid,
 C. R1 = H, R2 = OC₂H₅: 2-ethoxy-8-ethyl-5-oxo-5,8-dihydropyrido[2,3-*d*]pyrimidine-6-carboxylic acid,
 D. R1 = C₂H₅, R2 = Cl: ethyl 2-chloro-8-ethyl-5-oxo-5,8-dihydropyrido[2,3-*d*]pyrimidine-6-carboxylate,

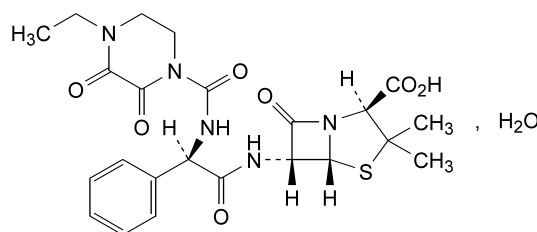


- E. R1 = C₂H₅, R2 = H: ethyl 8-ethyl-5-oxo-2-(piperazin-1-yl)-5,8-dihydropyrido[2,3-*d*]pyrimidine-6-carboxylate,
 F. R1 = H, R2 = CO-CH₃: 2-(4-acetyl)piperazin-1-yl)-8-ethyl-5-oxo-5,8-dihydropyrido[2,3-*d*]pyrimidine-6-carboxylic acid (acetyl pipemidic acid).

01/2005:1169

PIPERACILLIN

Piperacillinum



$C_{23}H_{27}N_5O_7S \cdot H_2O$

M_r 535.6

DEFINITION

Piperacillin contains not less than 96.0 per cent and not more than the equivalent of 101.0 per cent of (2*S*,5*R*,6*R*)-6-[[[(2*R*)-2-[[[(4-ethyl-2,3-dioxopiperazin-1-yl)carbonyl]amino]-2-phenylacetyl]amino]-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid, calculated with reference to the anhydrous substance.

CHARACTERS

A white or almost white powder, slightly soluble in water, freely soluble in methanol, slightly soluble in ethyl acetate.

IDENTIFICATION

Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *piperacillin CRS*.

TESTS

Solution S. Dissolve 2.50 g in *sodium carbonate solution R* and dilute to 25 ml with the same solvent.

Appearance of solution. Solution S is not more opalescent than reference suspension II (2.2.1). The absorbance of solution S measured at 430 nm (2.2.25) is not greater than 0.10.

Specific optical rotation (2.2.7). Dissolve 0.250 g in *methanol R* and dilute to 25.0 ml with the same solvent. The specific optical rotation is + 165 to + 175, calculated with reference to the anhydrous substance.

Related substances. Examine by liquid chromatography (2.2.29) as prescribed under Assay. Inject 20 µl of reference solution (b) and elute isocratically with the chosen mobile phase. Inject 20 µl of test solution (b). Start the elution isocratically. Immediately after elution of the piperacillin peak start the following linear gradient.

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)	Comment
0 - 30	88 → 0	12 → 100	linear gradient
30 - 45	0 → 88	100 → 12	re-equilibration

In the chromatogram obtained with test solution (b), the area of any peak, apart from the principal peak, is not greater than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (2 per cent). Disregard any peak due to the solvent.

N,N-Dimethylaniline (2.4.26, *Method A*). Not more than 20 ppm.

Heavy metals (2.4.8). 1.0 g complies with limit test C for heavy metals (20 ppm). Prepare the standard using 2 ml of *lead standard solution* (10 ppm Pb) *R*.

Water (2.5.12): 2.0 per cent to 4.0 per cent, determined on 0.500 g by the semi-micro determination of water.

ASSAY

Examine by liquid chromatography (2.2.29).

Solvent mixture. Mix 25 volumes of *acetonitrile R* and 75 volumes of a 31.2 g/l solution of *sodium dihydrogen phosphate R*.

Test solution (a). Dissolve 25.0 mg of the substance to be examined in the solvent mixture and dilute to 50.0 ml with the solvent mixture.

Test solution (b). Prepare the solution immediately before use. Dissolve 40.0 mg of the substance to be examined in the solvent mixture and dilute to 20.0 ml with the solvent mixture.

Reference solution (a). Dissolve 25.0 mg of *piperacillin CRS* in the solvent mixture and dilute to 50.0 ml with the solvent mixture.

Reference solution (b). Dilute 1.0 ml of reference solution (a) to 25.0 ml with the solvent mixture.

Reference solution (c). Dissolve 10.0 mg of *piperacillin CRS* and 10.0 mg of *anhydrous ampicillin CRS* in the solvent mixture and dilute to 50.0 ml with the solvent mixture.

Reference solution (d). Dilute 1.0 ml of reference solution (a) to 100.0 ml with the solvent mixture. Dilute 1.0 ml of the solution to 50.0 ml with the solvent mixture.

The chromatographic procedure may be carried out using:

- a column 0.25 m long and 4.6 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (5 µm),
- as mobile phase at a flow rate of 1.0 ml/min a mixture of 88 volumes of mobile phase A and 12 volumes of mobile phase B:

Mobile phase A. Mix 576 ml of *water R*, 200 ml of a 31.2 g/l solution of *sodium dihydrogen phosphate R* and 24 ml of an 80 g/l solution of *tetrabutylammonium hydroxide R*. If necessary, adjust to pH 5.5 with *dilute phosphoric acid R* or *dilute sodium hydroxide solution R*; then add 200 ml of *acetonitrile R*,

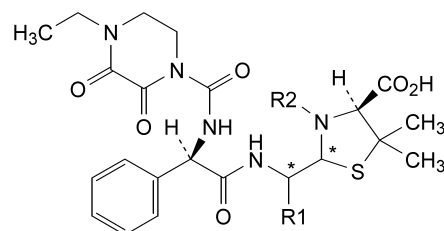
Mobile phase B. Mix 126 ml of *water R*, 200 ml of a 31.2 g/l solution of *sodium dihydrogen phosphate R* and 24 ml of an 80 g/l solution of *tetrabutylammonium hydroxide R*. If necessary, adjust to pH 5.5 with *dilute phosphoric acid R* or *dilute sodium hydroxide solution R*; then add 650 ml of *acetonitrile R*,

- as detector a spectrophotometer set at 220 nm.

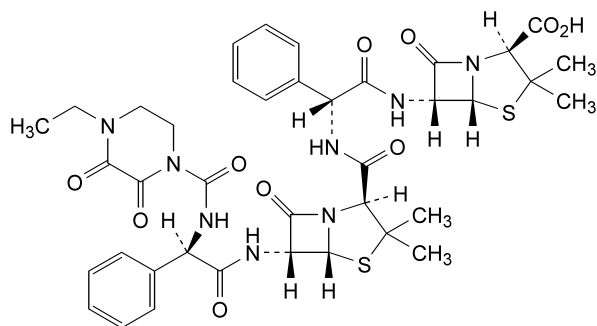
Inject 20 µl of reference solution (c). The test is not valid unless in the chromatogram obtained, the resolution between the peaks corresponding to ampicillin and to piperacillin is at least 10 (if necessary, adjust the ratio A:B of the mobile phase) and the mass distribution ratio for the second peak (piperacillin) is 2.0 to 3.0. Inject 20 µl of reference solution (d). Adjust the sensitivity of the system to obtain a peak with a signal-to-noise ratio of at least 3. Inject reference solution (a) 6 times. The test is not valid unless the relative standard deviation of the peak area of piperacillin is at most 1.0 per cent. Inject alternately test solution (a) and reference solution (a).

IMPURITIES

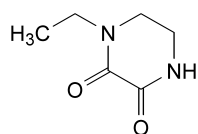
A. ampicillin,



- B. R1 = CO₂H, R2 = H: (4S)-2-[carboxy[[[(2R)-2-[(4-ethyl-2,3-dioxopiperazin-1-yl)carbonyl]amino]-2-phenylacetyl]amino]methyl]-5,5-dimethylthiazolidine-4-carboxylic acid (penicilloic acids of piperacillin),
- C. R1 = R2 = H: (2RS,4S)-2-[[[(2R)-2-[(4-ethyl-2,3-dioxopiperazin-1-yl)carbonyl]amino]-2-phenylacetyl]amino]methyl]-5,5-dimethylthiazolidine-4-carboxylic acid (penilloic acids of piperacillin),
- F. R1 = CO₂H, R2 = CO-CH₃: (4S)-3-acetyl-2-[carboxy[[[(2R)-2-[(4-ethyl-2,3-dioxopiperazin-1-yl)carbonyl]amino]-2-phenylacetyl]amino]methyl]-5,5-dimethylthiazolidine-4-carboxylic acid (acetylated penicilloic acids of piperacillin),



D. (2*S*,5*R*,6*R*)-6-[[[(2*R*)-2-[[[(2*S*,5*R*,6*R*)-6-[(2*R*)-2-[(4-ethyl-2,3-dioxopiperazin-1-yl)carbonyl]amino]-2-phenylacetyl]amino]-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]hept-2-yl]carbonyl]amino]-2-phenylacetyl]amino]-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid (piperacillinylampicillin),

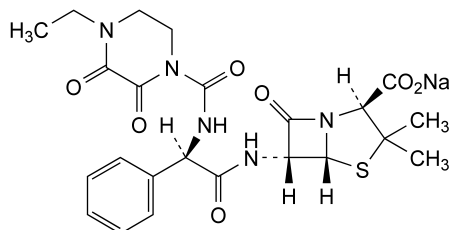


E. 1-ethylpiperazine-2,3-dione.

01/2005:1168

PIPERACILLIN SODIUM

Piperacillinum natricum



$C_{23}H_{26}N_5NaO_7S$

M_r 539.5

DEFINITION

Piperacillin sodium contains not less than 95.0 per cent and not more than the equivalent of 101.0 per cent of sodium (2*S*,5*R*,6*R*)-6-[[[(2*R*)-2-[(4-ethyl-2,3-dioxopiperazin-1-yl)carbonyl]amino]-2-phenylacetyl]amino]-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylate, calculated with reference to the anhydrous substance.

CHARACTERS

A white or almost white powder, hygroscopic, freely soluble in water and in methanol, practically insoluble in ethyl acetate.

IDENTIFICATION

A. Dissolve 0.250 g in *water R*, add 0.5 ml of *dilute hydrochloric acid R* and 5 ml of *ethyl acetate R*; stir and allow to stand for 10 min in iced water. Filter the crystals through a small sintered-glass filter (40), applying suction. Wash with 5 ml of *water R* and 5 ml of *ethyl acetate R* and dry in an oven at 60 °C for 60 min. Examine the crystals by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *piperacillin CRS*.

B. It gives reaction (a) of sodium (2.3.1).

TESTS

Solution S. Dissolve 2.50 g in *carbon dioxide-free water R* and dilute to 25 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1). The absorbance of solution S measured at 430 nm (2.2.25) is not greater than 0.10.

pH (2.2.3). The pH of solution S is 5.0 to 7.0.

Specific optical rotation (2.2.7). Dissolve 0.250 g in *water R* and dilute to 25.0 ml with the same solvent. The specific optical rotation is + 175 to + 190, calculated with reference to the anhydrous substance.

Related substances. Examine by liquid chromatography (2.2.29) as prescribed under Assay. Inject 20 µl of reference solution (b) and elute isocratically with the chosen mobile phase. Inject 20 µl of test solution (b). Start the elution isocratically. Immediately after elution of the piperacillin peak start the following linear gradient.

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)	Comment
0 - 30	88 → 0	12 → 100	linear gradient
30 - 45	0 → 88	100 → 12	re-equilibration

In the chromatogram obtained with test solution (b), the area of any peak, apart from the principal peak, is not greater than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (2 per cent). Disregard any peak due to the solvent.

***N,N*-Dimethylaniline** (2.4.26, *Method A*). Not more than 20 ppm.

Heavy metals (2.4.8). 1.0 g complies with the limit test C for heavy metals (20 ppm). Prepare the standard using 2 ml of *lead standard solution* (10 ppm Pb) *R*.

Water (2.5.12). Not more than 2.0 per cent, determined on 0.500 g by the semi-micro determination of water.

Bacterial endotoxins (2.6.14): less than 0.07 IU/mg, if intended for use in the manufacture of parenteral dosage forms without a further appropriate procedure for the removal of bacterial endotoxins.

ASSAY

Examine by liquid chromatography (2.2.29).

Solvent mixture. Mix 25 volumes of *acetonitrile R* and 75 volumes of a 31.2 g/l solution of *sodium dihydrogen phosphate R*.

Test solution (a). Dissolve 25.0 mg of the substance to be examined in the solvent mixture and dilute to 50.0 ml with the solvent mixture.

Test solution (b). Prepare the solution immediately before use. Dissolve 40.0 mg of the substance to be examined in the solvent mixture and dilute to 20.0 ml with the solvent mixture.

Reference solution (a). Dissolve 25.0 mg of *piperacillin CRS* in the solvent mixture and dilute to 50.0 ml with the solvent mixture.

Reference solution (b). Dilute 1.0 ml of reference solution (a) to 25.0 ml with the solvent mixture.

Reference solution (c). Dissolve 10.0 mg of *piperacillin CRS* and 10.0 mg of *anhydrous ampicillin CRS* in the solvent mixture and dilute to 50.0 ml with the solvent mixture.

Reference solution (d). Dilute 1.0 ml of reference solution (a) to 100.0 ml with the solvent mixture. Dilute 1.0 ml of the solution to 50.0 ml with the solvent mixture.

The chromatographic procedure may be carried out using:

- a column 0.25 m long and 4.6 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (5 µm),
- as mobile phase at a flow rate of 1.0 ml/min a mixture of 88 volumes of mobile phase A and 12 volumes of mobile phase B:

Mobile phase A. Mix 576 ml of *water R*, 200 ml of a 31.2 g/l solution of *sodium dihydrogen phosphate R* and 24 ml of a 80 g/l solution of *tetrabutylammonium hydroxide R*. If necessary, adjust to pH 5.5 with *dilute phosphoric acid R* or *dilute sodium hydroxide solution R*; then add 200 ml of *acetonitrile R*,

Mobile phase B. Mix 126 ml of *water R*, 200 ml of a 31.2 g/l solution of *sodium dihydrogen phosphate R* and 24 ml of a 80 g/l solution of *tetrabutylammonium hydroxide R*. If necessary, adjust to pH 5.5 with *dilute phosphoric acid R* or *dilute sodium hydroxide solution R*; then add 650 ml of *acetonitrile R*,

- as detector a spectrophotometer set at 220 nm.

Inject 20 µl of reference solution (c). The test is not valid unless in the chromatogram obtained, the resolution between the peaks corresponding to ampicillin and to piperacillin is at least ten (if necessary, adjust the A:B ratio of the mobile phase) and the mass distribution ratio for the second peak (piperacillin) is 2.0 to 3.0. Inject 20 µl of reference solution (d). Adjust the sensitivity of the system to obtain a peak with a signal-to-noise ratio of at least 3. Inject reference solution (a) six times. The test is not valid unless the relative standard deviation of the peak area of piperacillin is at most 1.0 per cent. Inject alternately test solution (a) and reference solution (a).

Calculate the percentage content of piperacillin sodium multiplying the result by 1.042.

STORAGE

Store in an airtight container. If the substance is sterile, store in a sterile, airtight, tamper-proof container.

LABELLING

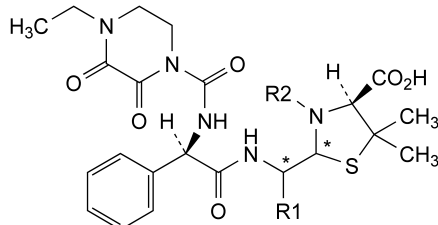
The label states, where applicable, that the substance is free from bacterial endotoxins.

IMPURITIES

Specified impurities: A, B, C, D, E, F, G.

Other detectable impurities: H.

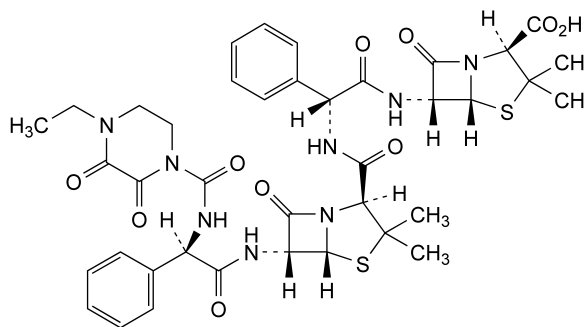
A. ampicillin,



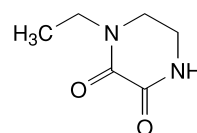
B. R1 = CO₂H, R2 = H: (4S)-2-[carboxy[[[(2R)-2-[(4-ethyl-2,3-dioxopiperazin-1-yl)carbonyl]amino]-2-phenylacetyl]amino]methyl]-5,5-dimethylthiazolidine-4-carboxylic acid (penicilloic acids of piperacillin),

C. R1 = R2 = H: (2RS,4S)-2-[[[(2R)-2-[(4-ethyl-2,3-dioxopiperazin-1-yl)carbonyl]amino]-2-phenylacetyl]amino]methyl]-5,5-dimethylthiazolidine-4-carboxylic acid (penilloic acids of piperacillin),

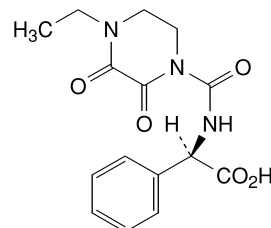
F. R1 = CO₂H, R2 = CO-CH₃: (4S)-3-acetyl-2-[carboxy[[[(2R)-2-[(4-ethyl-2,3-dioxopiperazin-1-yl)carbonyl]amino]-2-phenylacetyl]amino]methyl]-5,5-dimethylthiazolidine-4-carboxylic acid (acetylated penicilloic acids of piperacillin).



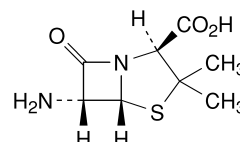
D. (2S,5R,6R)-6-[[[(2R)-2-[[[(2S,5R,6R)-6-[[[(2R)-2-[(4-ethyl-2,3-dioxopiperazin-1-yl)carbonyl]amino]-2-phenylacetyl]amino]-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]hept-2-yl]carbonyl]amino]-2-phenylacetyl]amino]-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid (piperacillinylampicillin),



E. 1-ethylpiperazine-2,3-dione,



G. (2R)-2-[[[(4-ethyl-2,3-dioxopiperazin-1-yl)carbonyl]amino]-2-phenylacetic acid,

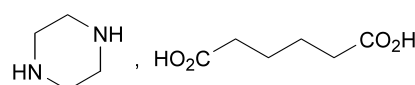


H. (2S,5R,6R)-6-amino-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid (6-aminopenicillanic acid).

01/2005:0423

PIPERAZINE ADIPATE

Piperazini adipas



C₁₀H₂₀N₂O₄

M_r 232.3

DEFINITION

Piperazine adipate contains not less than 98.0 per cent and not more than the equivalent of 101.0 per cent of piperazine hexanedioate, calculated with reference to the anhydrous substance.

CHARACTERS

A white crystalline powder, soluble in water, practically insoluble in alcohol. It melts at about 250 °C, with decomposition.

IDENTIFICATION

First identification: A.

Second identification: B, C.

- A. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *piperazine adipate CRS*. Examine the substances prepared as discs.
- B. Examine the chromatograms obtained in the test for related substances after spraying with the ninhydrin solutions. The principal spot in the chromatogram obtained with test solution (b) is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).
- C. To 10 ml of solution S (see Tests) add 5 ml of *hydrochloric acid R* and shake with three quantities, each of 10 ml, of *ether R*. Evaporate the combined ether layers to dryness. The residue, washed with 5 ml of *water R* and dried at 100 °C to 105 °C, melts (2.2.14) at 150 °C to 154 °C.

TESTS

Solution S. Dissolve 2.5 g in *water R* and dilute to 50 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and not more intensely coloured than reference solution B₈ (2.2.2, *Method II*).

Related substances. Examine by thin-layer chromatography (2.2.27), using a suitable silica gel as the coating substance.

Test solution (a). Dissolve 1.0 g of the substance to be examined in 6 ml of *concentrated ammonia R* and dilute to 10 ml with *ethanol R*.

Test solution (b). Dilute 1 ml of test solution (a) to 10 ml with a mixture of 2 volumes of *ethanol R* and 3 volumes of *concentrated ammonia R*.

Reference solution (a). Dissolve 0.1 g of *piperazine adipate CRS* in a mixture of 2 volumes of *ethanol R* and 3 volumes of *concentrated ammonia R* and dilute to 10 ml with the same mixture of solvents.

Reference solution (b). Dissolve 25 mg of *ethylenediamine R* in a mixture of 2 volumes of *ethanol R* and 3 volumes of *concentrated ammonia R* and dilute to 100 ml with the same mixture of solvents.

Reference solution (c). Dissolve 25 mg of *triethylenediamine R* in a mixture of 2 volumes of *ethanol R* and 3 volumes of *concentrated ammonia R* and dilute to 100 ml with the same mixture of solvents.

Reference solution (d). Dissolve 12.5 mg of *triethylenediamine R* in 5.0 ml of test solution (a) and dilute to 50 ml with a mixture of 2 volumes of *ethanol R* and 3 volumes of *concentrated ammonia R*.

Apply separately to the plate 5 µl of each solution. Develop over a path of 15 cm using a freshly prepared mixture of 20 volumes of *concentrated ammonia R* and 80 volumes of *acetone R*. Dry the plate at 105 °C and spray successively with a 3 g/l solution of *ninhydrin R* in a mixture of 3 volumes of *anhydrous acetic acid R* and 100 volumes of *butanol R* and a 1.5 g/l solution of *ninhydrin R* in *ethanol R*. Dry the plate at 105 °C for 10 min. Any spot in the chromatogram obtained with test solution (a), apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.25 per cent). Spray the plate with 0.05 M *iodine* and allow to stand for about

10 min. Any spot corresponding to triethylenediamine in the chromatogram obtained with test solution (a) is not more intense than the spot in the chromatogram obtained with reference solution (c) (0.25 per cent). The test is not valid unless the chromatogram obtained with reference solution (d) shows two clearly separated spots. Disregard any spots remaining on the starting line.

Heavy metals (2.4.8). 12 ml of solution S complies with limit test A for heavy metals (20 ppm). Prepare the standard using *lead standard solution (1 ppm Pb) R*.

Water (2.5.12). Not more than 0.5 per cent, determined on 1.00 g by the semi-micro determination of water.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

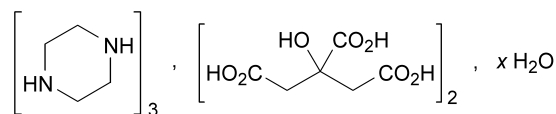
Dissolve 0.100 g in 10 ml of *anhydrous acetic acid R* with gentle heating and dilute to 70 ml with the same acid. Titrate with 0.1 M *perchloric acid* using 0.25 ml of *naphtholbenzein solution R* as indicator until the colour changes from brownish-yellow to green.

1 ml of 0.1 M *perchloric acid* is equivalent to 11.61 mg of C₁₀H₂₀N₂O₄.

01/2005:0424

PIPERAZINE CITRATE

Piperazini citras

C₂₄H₄₆N₆O₁₄·xH₂OM_r 643 (anhydrous substance)

DEFINITION

Piperazine citrate contains not less than 98.0 per cent and not more than the equivalent of 101.0 per cent of tripiperazine bis(2-hydroxy-propane-1,2,3-tricarboxylate), calculated with reference to the anhydrous substance. It contains a variable quantity of water.

CHARACTERS

A white granular powder, freely soluble in water, practically insoluble in alcohol.

After drying at 100 °C to 105 °C, it melts at about 190 °C.

IDENTIFICATION

First identification: A.

Second identification: B, C.

- A. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *piperazine citrate CRS*. Dry the substance to be examined and the reference substance at 120 °C for 5 h, powder the substances avoiding uptake of water, prepare discs and record the spectra without delay.
- B. Examine the chromatograms obtained in the test for related substances after spraying with the ninhydrin solutions. The principal spot in the chromatogram obtained with test solution (b) is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).
- C. Dissolve 0.5 g in *water R* and dilute to 5 ml with the same solvent. The solution gives the reaction of citrates (2.3.1).

TESTS

01/2005:0425

Solution S. Dissolve 1.25 g in *water R* and dilute to 25 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and not more intensely coloured than reference solution B₈ (2.2.2, Method II).

Related substances. Examine by thin-layer chromatography (2.2.27), using a suitable silica gel as the coating substance.

Test solution (a). Dissolve 1.0 g of the substance to be examined in 6 ml of *concentrated ammonia R* and dilute to 10 ml with *ethanol R*.

Test solution (b). Dilute 1 ml of test solution (a) to 10 ml with a mixture of 2 volumes of *ethanol R* and 3 volumes of *concentrated ammonia R*.

Reference solution (a). Dissolve 0.1 g of *piperazine citrate CRS* in a mixture of 2 volumes of *ethanol R* and 3 volumes of *concentrated ammonia R* and dilute to 10 ml with the same mixture of solvents.

Reference solution (b). Dissolve 25 mg of *ethylenediamine R* in a mixture of 2 volumes of *ethanol R* and 3 volumes of *concentrated ammonia R* and dilute to 100 ml with the same mixture of solvents.

Reference solution (c). Dissolve 25 mg of *triethylenediamine R* in a mixture of 2 volumes of *ethanol R* and 3 volumes of *concentrated ammonia R* and dilute to 100 ml with the same mixture of solvents.

Reference solution (d). Dissolve 12.5 mg of *triethylenediamine R* in 5.0 ml of test solution (a) and dilute to 50 ml with a mixture of 2 volumes of *ethanol R* and 3 volumes of *concentrated ammonia R*.

Apply separately to the plate 5 µl of each solution. Develop over a path of 15 cm using a freshly prepared mixture of 20 volumes of *concentrated ammonia R* and 80 volumes of *acetone R*. Dry the plate at 105 °C and spray successively with a 3 g/l solution of *ninhydrin R* in a mixture of 3 volumes of *anhydrous acetic acid R* and 100 volumes of *butanol R* and a 1.5 g/l solution of *ninhydrin R* in *ethanol R*. Dry the plate at 105 °C for 10 min. Any spot in the chromatogram obtained with test solution (a), apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.25 per cent). Spray the plate with 0.05 M *iodine* and allow to stand for about 10 min. Any spot corresponding to triethylenediamine in the chromatogram obtained with test solution (a) is not more intense than the spot in the chromatogram obtained with reference solution (c) (0.25 per cent). The test is not valid unless the chromatogram obtained with reference solution (d) shows two clearly separated spots. Disregard any spots remaining on the starting line.

Heavy metals (2.4.8). 12 ml of solution S complies with limit test A for heavy metals (20 ppm). Prepare the standard using *lead standard solution* (1 ppm Pb) R.

Water (2.5.12). 10.0 per cent to 14.0 per cent, determined on 0.300 g by the semi-micro determination of water.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

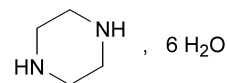
ASSAY

Dissolve 0.100 g in 10 ml of *anhydrous acetic acid R* with gentle heating and dilute to 70 ml with the same acid. Titrate with 0.1 M *perchloric acid* using 0.25 ml of *naphtholbenzein solution R* as indicator until the colour changes from brownish-yellow to green.

1 ml of 0.1 M *perchloric acid* is equivalent to 10.71 mg of C₂₄H₄₆N₆O₁₄.

PIPERAZINE HYDRATE

Piperazinum hydricum

C₄H₁₀N₂·6H₂OM_r 194.2

DEFINITION

Piperazine hydrate contains not less than 98.0 per cent and not more than the equivalent of 101.0 per cent of piperazine hexahydrate.

CHARACTERS

Colourless, deliquescent crystals, freely soluble in water and in alcohol.

It melts at about 43 °C.

IDENTIFICATION

First identification: A.

Second identification: B, C.

- A. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *piperazine hydrate CRS*. Dry the substance to be examined and the reference substance over *diphosphorus pentoxide R in vacuo* for 48 h, powder the substances avoiding uptake of water, prepare discs and record the spectra without delay.
- B. Examine the chromatograms obtained in the test for related substances after spraying with the ninhydrin solutions. The principal spot in the chromatogram obtained with test solution (b) is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).
- C. Dissolve 0.5 g in 5 ml of *dilute sodium hydroxide solution R*. Add 0.2 ml of *benzoyl chloride R* and mix. Continue to add *benzoyl chloride R* in portions of 0.2 ml until no further precipitate is formed. Filter and wash the precipitate with a total of 10 ml of *water R* added in small portions. Dissolve the precipitate in 2 ml of hot *alcohol R* and pour the solution into 5 ml of *water R*. Allow to stand for 4 h, filter, wash the crystals with *water R* and dry at 100 °C to 105 °C. The crystals melt (2.2.14) at 191 °C to 196 °C.

TESTS

Solution S. Dissolve 1.0 g in *carbon dioxide-free water R* and dilute to 20 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and not more intensely coloured than reference solution B₈ (2.2.2, Method II).

pH (2.2.3). The pH of solution S is 10.5 to 12.0.

Related substances. Examine by thin-layer chromatography (2.2.27), using a suitable silica gel as the coating substance.

Test solution (a). Dissolve 1.0 g of the substance to be examined in 6 ml of *concentrated ammonia R* and dilute to 10 ml with *ethanol R*.

Test solution (b). Dilute 1 ml of test solution (a) to 10 ml with a mixture of 2 volumes of *ethanol R* and 3 volumes of *concentrated ammonia R*.

Reference solution (a). Dissolve 0.1 g of *piperazine hydrate CRS* in a mixture of 2 volumes of *ethanol R* and 3 volumes of *concentrated ammonia R* and dilute to 10 ml with the same mixture of solvents.

Reference solution (b). Dissolve 25 mg of *ethylenediamine R* in a mixture of 2 volumes of *ethanol R* and 3 volumes of *concentrated ammonia R* and dilute to 100 ml with the same mixture of solvents.

Reference solution (c). Dissolve 25 mg of *triethylenediamine R* in a mixture of 2 volumes of *ethanol R* and 3 volumes of *concentrated ammonia R* and dilute to 100 ml with the same mixture of solvents.

Reference solution (d). Dissolve 12.5 mg of *triethylenediamine R* in 5.0 ml of test solution (a) and dilute to 50 ml with a mixture of 2 volumes of *ethanol R* and 3 volumes of *concentrated ammonia R*.

Apply separately to the plate 5 µl of each solution. Develop over a path of 15 cm using a freshly prepared mixture of 20 volumes of *concentrated ammonia R* and 80 volumes of *acetone R*. Dry the plate at 105 °C and spray successively with a 3 g/l solution of *ninhydrin R* in a mixture of 3 volumes of *anhydrous acetic acid R* and 100 volumes of *butanol R* and a 1.5 g/l solution of *ninhydrin R* in *ethanol R*. Dry the plate at 105 °C for 10 min. Any spot in the chromatogram obtained with test solution (a), apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.25 per cent). Spray the plate with 0.05 M *iodine* and allow to stand for about 10 min. Any spot corresponding to triethylenediamine in the chromatogram obtained with test solution (a) is not more intense than the spot in the chromatogram obtained with reference solution (c) (0.25 per cent). The test is not valid unless the chromatogram obtained with reference solution (d) shows two clearly separated spots.

Heavy metals (2.4.8). 12 ml of solution S complies with limit test A for heavy metals (20 ppm). Prepare the standard using *lead standard solution (1 ppm Pb) R*.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 80.0 mg in 10 ml of *anhydrous acetic acid R* with gentle heating and dilute to 70 ml with the same acid. Titrate with 0.1 M *perchloric acid* using 0.25 ml of *naphtholbenzein solution R* as indicator until the colour changes from brownish-yellow to green.

1 ml of 0.1 M *perchloric acid* is equivalent to 9.705 mg of C₆H₁₀N₂O₂.

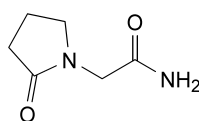
STORAGE

Store in an airtight container, protected from light.

01/2005:1733

PIRACETAM

Piracetamum



C₆H₁₀N₂O₂

*M*_r 142.2

DEFINITION

2-(2-Oxopyrrolidin-1-yl)acetamide.

Content: 98.0 per cent to 102.0 per cent (dried substance).

CHARACTERS

Appearance: white or almost white powder.

Solubility: freely soluble in water, soluble in alcohol.

It shows polymorphism.

IDENTIFICATION

Infrared absorption spectrophotometry (2.2.24).

Comparison: *piracetam CRS*.

If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in *alcohol R*, evaporate to dryness on a water-bath and record new spectra using the residues.

TESTS

Appearance of solution. The solution is clear (2.2.1) and colourless (2.2.2, *Method II*).

Dissolve 2.0 g in *water R* and dilute to 10 ml with the same solvent.

Related substances. Liquid chromatography (2.2.29).

Test solution. Dissolve 50.0 mg of the substance to be examined in a mixture of 10 volumes of *acetonitrile R* and 90 volumes of *water R* and dilute to 100.0 ml with the same mixture of solvents.

Reference solution (a). Dissolve 5 mg of the substance to be examined and 10 mg of *2-pyrrolidone R* in a mixture of 10 volumes of *acetonitrile R* and 90 volumes of *water R* and dilute to 100.0 ml with the same mixture of solvents.

Reference solution (b). Dilute 1.0 ml of the test solution to 100.0 ml with a mixture of 10 volumes of *acetonitrile R* and 90 volumes of *water R*. Dilute 5.0 ml to 50.0 ml with a mixture of 10 volumes of *acetonitrile R* and 90 volumes of *water R*.

Column:

- **size:** *l* = 0.25 m, Ø = 4.6 mm,
- **stationary phase:** end-capped octadecylsilyl silica gel for chromatography R (5 µm).

Mobile phase: mix 10 volumes of *acetonitrile R* and 90 volumes of a 1.0 g/l solution of *dipotassium hydrogen phosphate R*, adjust to pH 6.0 with *dilute phosphoric acid R*.

Flow rate: 1.0 ml/min.

Detection: spectrophotometer at 205 nm.

Injection: 20 µl.

Run time: 8 times the retention time of piracetam.

Retention time: piracetam = about 4 min.

System suitability: reference solution (a):

- **resolution:** minimum 3.0 between the peaks due to piracetam and impurity A.
- **symmetry factor:** maximum 2.0 for the peak due to piracetam.

Limits:

- **any impurity:** not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent),
- **total:** not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.3 per cent),
- **disregard limit:** half the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

Heavy metals (2.4.8): maximum 10 ppm.

Dissolve 2 g in 20 ml of *water R*. 12 ml of the solution complies with limit test A. Prepare the standard using *lead standard solution (1 ppm Pb) R*.

Loss on drying (2.2.32): maximum 1.0 per cent, determined on 1.000 g by drying in an oven at 100–105 °C.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

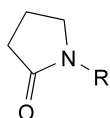
Dissolve 0.750 g in 50 ml of *carbon dioxide-free water R*. Add 20.0 ml of 1 M *sodium hydroxide* and heat to boiling for 15 min. Allow to cool and add 25.0 ml of 1 M *hydrochloric acid*. Heat to boiling for 2 min and allow to cool. Add 0.1 ml of *phenolphthalein solution R1*. Titrate with 1 M *sodium hydroxide* until a pink colour is obtained.

1 ml of 1 M *sodium hydroxide* is equivalent to 142.2 mg of $C_{19}H_{23}Cl_2N_5O_2 \cdot H_2O$.

STORAGE

Protected from light.

IMPURITIES

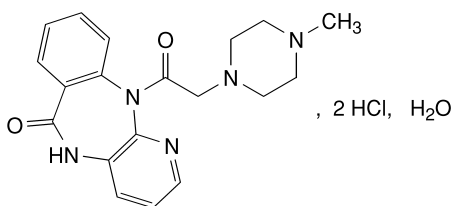


- A. R = H: pyrrolidin-2-one (2-pyrrolidone),
 B. R = CH_2COOCH_3 : methyl (2-oxopyrrolidin-1-yl)acetate,
 C. R = $CH_2COOC_2H_5$: ethyl (2-oxopyrrolidin-1-yl)acetate,
 D. R = CH_2CO_2H : (2-oxopyrrolidin-1-yl)acetic acid.

01/2005:2001

PIRENZEPINE DIHYDROCHLORIDE MONOHYDRATE

Pirenzepini dihydrochloridum monohydricum



$C_{19}H_{23}Cl_2N_5O_2 \cdot H_2O$

M_r 442.3

DEFINITION

11-[(4-Methylpiperazin-1-yl)acetyl]-5,11-dihydro-6H-pyrido[2,3-b][1,4]benzodiazepin-6-one dihydrochloride monohydrate.

Content: 98.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

Appearance: white or yellowish, crystalline powder.

Solubility: freely soluble in water, slightly soluble in methanol, very slightly soluble in ethanol, practically insoluble in methylene chloride.

IDENTIFICATION

First identification: B, D.

Second identification: A, C, D.

- A. Dissolve 30.0 mg in *methanol R* and dilute to 100.0 ml with the same solvent. Dilute 10.0 ml of the solution to 100.0 ml with *methanol R*. Examined between 240 nm

and 360 nm (2.2.25), the solution shows an absorption maximum at 283 nm. The specific absorbance at the maximum is 190 to 205 (anhydrous substance).

- B. Infrared absorption spectrophotometry (2.2.24).

Comparison: *pirenzepine dihydrochloride monohydrate CRS*.

- C. Examine the chromatograms obtained in the test for impurity D.

Results: the principal band obtained in the chromatogram obtained with test solution (b) is similar in position, colour and size to the principal band in the chromatogram obtained with reference solution (d).

- D. To 0.2 ml of solution S (see Tests) add 1.8 ml of *water R*. The solution gives reaction (a) of chlorides (2.3.1).

TESTS

Solution S. Dissolve 2.5 g in *carbon dioxide-free water R* and dilute to 25 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and not more intensely coloured than reference solution GY₅ (2.2.2, *Method II*).

pH (2.2.3): 1.0 to 2.0 for solution S.

Impurity D. Thin-layer chromatography (2.2.27).

Test solution (a). To 0.10 g add 0.1 ml of *concentrated ammonia R* and dilute to 10 ml with *methanol R*.

Test solution (b). Dilute 1 ml of test solution (a) to 10 ml with *methanol R*.

Reference solution (a). To 0.1 g of *pirenzepine dihydrochloride monohydrate CRS* add 0.1 ml of *concentrated ammonia R* and dilute to 10 ml with *methanol R*.

Reference solution (b). Dissolve 25 mg of *methylpiperazine R* in *methanol R* and dilute to 25 ml with the same solvent. Dilute 2.0 ml of the solution to 100 ml with *methanol R*.

Reference solution (c). Dilute 5 ml of test solution (a) to 100 ml with *methanol R*. Dilute 4 ml of this solution to 100 ml with *methanol R*. Mix 1 ml with 1 ml of reference solution (b).

Reference solution (d). Dilute 1 ml of reference solution (a) to 10 ml with *methanol R*.

Plate: TLC silica gel plate R.

Mobile phase: *concentrated ammonia R*, *methanol R*, *ethyl acetate R*, *toluene R* (7:25:28:40 V/V/V/V).

Application: 20 µl as bands of 20 mm by 2 mm.

Development: over 2/3 of the plate.

Drying: in air.

Detection: expose the plate to iodine vapour until the band in the chromatogram obtained with reference solution (b) is clearly visible (at most 60 min).

System suitability: the test is not valid unless the chromatogram obtained with reference solution (c) shows 2 clearly separated bands.

Limit:

- **impurity D:** any band corresponding to impurity D in the chromatogram obtained with test solution (a) is not more intense than the band in the chromatogram obtained with reference solution (b) (0.2 per cent).

Related substances. Liquid chromatography (2.2.29).

Test solution. Dissolve 0.30 g of the substance to be examined in *water R* and dilute to 10.0 ml with the same solvent. To 1.0 ml of the solution add 5 ml of *methanol R* and dilute to 10.0 ml with mobile phase A.

Reference solution (a). Dilute 2.0 ml of the test solution to 100.0 ml with mobile phase A. Dilute 1.0 ml of this solution to 10.0 ml with mobile phase A.

Reference solution (b). Dissolve 0.1 g of 1-phenylpiperazine *R* in methanol *R* and dilute to 10 ml with the same solvent. Mix 1 ml of the solution with 1 ml of the test solution, add 5 ml of methanol *R* and dilute to 10 ml with mobile phase A.

Column:

- **size:** $l = 0.125$ m, $\varnothing = 4.6$ mm,
- **stationary phase:** octadecylsilyl silica gel for chromatography *R* (5 μ m).

Mobile phase:

- **mobile phase A:** dissolve 2.0 g of sodium dodecyl sulphate *R* in water *R*, adjust to pH 3.2 with acetic acid *R* and dilute to 1000 ml with water *R*,
- **mobile phase B:** methanol *R*,
- **mobile phase C:** acetonitrile *R*,

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)	Mobile phase C (per cent V/V)
0 - 15	55 → 25	30	15 → 45
15 - 18	25 → 20	30 → 0	45 → 80
18 - 20	20 → 55	0 → 30	80 → 15
20 - 25	55	30	15

Flow rate: 1 ml/min.

Detection: spectrophotometer at 283 nm.

Injection: 10 μ l.

System suitability: reference solution (b):

- **resolution:** minimum of 5.0 between the peaks corresponding to pirenzepine and to 1-phenylpiperazine.

Limits:

- **any impurity:** not more than the peak area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent),
- **total:** not more than 2.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent),
- **disregard limit:** 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.04 per cent).

Water (2.5.12): 3.5 per cent to 5.0 per cent, determined on 0.250 g.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

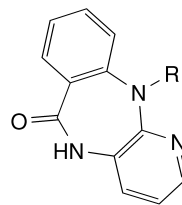
Dissolve 0.300 g in 50 ml of water *R*. Carry out a potentiometric titration (2.2.20), using 0.1 *M* sodium hydroxide. Read the volume at the first point of inflection.

1 ml of 0.1 *M* sodium hydroxide is equivalent to 42.43 mg of $C_{19}H_{23}Cl_2N_5O_2$.

STORAGE

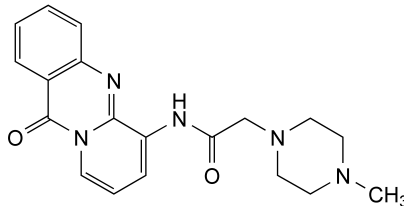
Protected from light.

IMPURITIES

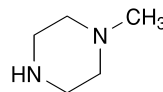


A. R = CO-CH₂-Cl: 11-(chloroacetyl)-5,11-dihydro-6*H*-pyrido[2,3-*b*][1,4]benzodiazepin-6-one,

B. R = H: 5,11-dihydro-6*H*-pyrido[2,3-*b*][1,4]benzodiazepin-6-one,



C. 6-[[[(4-methylpiperazin-1-yl)acetyl]amino]-11*H*-pyrido[2,1-*b*]quinazolin-11-one,

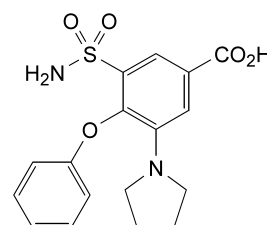


D. 1-methylpiperazine.

01/2005:1556

PIRETANIDE

Piretanidum



$C_{17}H_{18}N_2O_5S$

M_r 362.4

DEFINITION

Piretanide contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of 4-phenoxy-3-(pyrrolidin-1-yl)-5-sulphamoylbenzoic acid, calculated with reference to the dried substance.

CHARACTERS

A yellowish-white to yellowish powder, very slightly soluble in water, sparingly soluble in ethanol.

It shows polymorphism.

IDENTIFICATION

Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *piretanide CRS*. Examine the substances prepared as discs. If the spectra obtained show differences, dissolve the substance to be examined and the reference substance separately in acetone *R*, evaporate to dryness and record new spectra using the residues.

TESTS

Appearance of solution. Dissolve 0.1 g in *methanol R* and dilute to 10 ml with the same solvent. The solution is clear (2.2.1) and not more intensely coloured than reference solution GY₄ (2.2.2, *Method II*).

Related substances. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 20 mg of the substance to be examined in a mixture of 10 volumes of *ethanol R*, 45 volumes of *acetonitrile R* and 45 volumes of *water R* and dilute to 20.0 ml with the same mixture of solvents.

Reference solution (a). Dissolve 10 mg of *piretanide CRS* and 3 mg of *piretanide impurity A CRS* in a mixture of 10 volumes of *ethanol R*, 45 volumes of *acetonitrile R* and 45 volumes of *water R* and dilute to 10.0 ml with the same mixture of solvents.

Reference solution (b). Dilute 0.3 ml of the test solution to 100.0 ml with a mixture of 10 volumes of *ethanol R*, 45 volumes of *acetonitrile R* and 45 volumes of *water R*.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.125 m long and 4 mm in internal diameter packed with *octylsilyl silica gel for chromatography R* (5 µm),
- as mobile phase at a flow rate of 1 ml/min a mixture of 35 volumes of *acetonitrile R* and 65 volumes of a solution prepared as follows: add 1 ml of *trifluoroacetic acid R* to 500 ml of *water R*, add 1 ml of *triethylamine R* and dilute to 1000 ml with *water R*,
- as detector a spectrophotometer set at 232 nm.

Inject 10 µl of reference solution (a). When the chromatograms are recorded in the conditions described the relative retention of impurity A is about 0.9. The test is not valid unless the resolution between the peaks corresponding to impurity A and piroxicam is not less than 2.

Inject 10 µl of the test solution and 10 µl of reference solution (b). Continue the chromatography of the test solution for 5 times the retention time of the principal peak. In the chromatogram obtained with the test solution: the area of any peak, apart from the principal peak, is not greater than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.3 per cent); the sum of the areas of all the peaks, apart from the principal peak, is not greater than 3.33 times the area of the principal peak in the chromatogram obtained with reference solution (b) (1.0 per cent). Disregard any peak with an area less than 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (b).

Heavy metals (2.4.8). 2.0 g complies with limit test C for heavy metals (10 ppm). Prepare the standard using 2 ml of *lead standard solution (10 ppm Pb) R*.

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C for 4 h.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

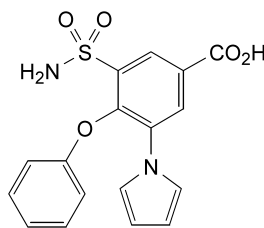
Dissolve 0.300 g in 25 ml of *anhydrous acetic acid R*. Titrate with 0.1 M *perchloric acid* determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *perchloric acid* is equivalent to 36.24 mg of C₁₇H₁₈N₃O₅S.

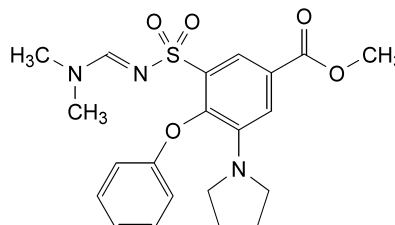
STORAGE

Store protected from light.

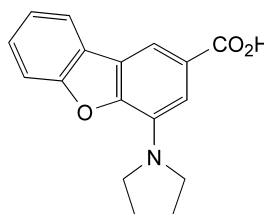
IMPURITIES



A. 4-phenoxy-3-(1*H*-pyrrol-1-yl)-5-sulphamoylbenzoic acid,



B. methyl-3-[[[(dimethylamino)methylene]sulphamoyl]-4-phenoxy-5-(pyrrolidin-1-yl)benzoate,

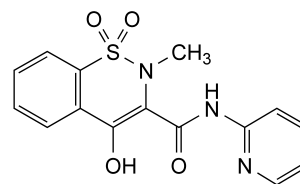


C. 4-(pyrrolidin-1-yl)dibenzo[*b,d*]furan-2-carboxylic acid.

01/2005:0944
corrected

PIROXICAM

Piroxicamum



C₁₅H₁₃N₃O₄S

*M*_r 331.4

DEFINITION

Piroxicam contains not less than 98.5 per cent and not more than the equivalent of 101.0 per cent of 4-hydroxy-2-methyl-*N*-(pyridin-2-yl)-2*H*-1,2-benzothiazine-3-carboxamide 1,1-dioxide, calculated with reference to the dried substance.

CHARACTERS

A white or slightly yellow, crystalline powder, practically insoluble in water, soluble in methylene chloride, slightly soluble in ethanol.

It shows polymorphism.

IDENTIFICATION

Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *piroxicam CRS*. Examine the substances as discs prepared using *potassium bromide R*. If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the

reference substance separately in the minimum volume of *methylene chloride R*, evaporate to dryness on a water-bath and record new spectra using the residues.

TESTS

Related substances. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 75 mg of the substance to be examined in *acetonitrile R* warming slightly if necessary and dilute to 50.0 ml with the same solvent.

Reference solution (a). Dissolve 10 mg of *piroxicam for system suitability CRS* in *acetonitrile R* and dilute to 50.0 ml with the same solvent.

Reference solution (b). Dilute 1.0 ml of the test solution to 10.0 ml with *acetonitrile R*. Dilute 1.0 ml of the solution to 50.0 ml with *acetonitrile R*.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4.6 mm in internal diameter packed with *base-deactivated octadecylsilyl silica gel for chromatography R* (5 µm),
- as mobile phase at a flow rate of 1 ml/min, a mixture of 40 volumes of *acetonitrile R* and 60 volumes of a 6.81 g/l solution of *potassium dihydrogen phosphate R* adjusted to pH 3.0 with *phosphoric acid R*,
- as detector a spectrophotometer set at 230 nm, maintaining the temperature of the column at 40 °C.

Inject 20 µl of each solution and continue the chromatography for 5 times the retention time of piroxicam. The test is not valid unless the chromatogram obtained with reference solution (a) has a similar profile to the chromatogram supplied with *piroxicam for system suitability CRS* and shows a peak due to impurity B with a relative retention of about 0.85 and a symmetry factor of at most 1.5.

In the chromatogram obtained with the test solution: the area of any peak, apart from the principal peak, is not greater than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent) and the sum of the areas of such peaks is not greater than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (0.4 per cent). Disregard any peak with an area less than 0.1 times that of the principal peak in the chromatogram obtained with reference solution (b).

Heavy metals (2.4.8). 1.0 g complies with limit test C (20 ppm). Prepare the standard using 2 ml of *lead standard solution (10 ppm Pb) R*.

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying *in vacuo* at 100–105 °C for 4 h.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

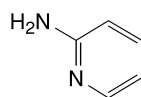
Dissolve 0.250 g in 60 ml of a mixture of equal volumes of *acetic anhydride R* and *anhydrous acetic acid R*. Titrate with 0.1 M *perchloric acid*, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *perchloric acid* is equivalent to 33.14 mg of C₁₅H₁₃N₃O₄S.

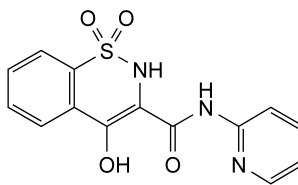
STORAGE

In an airtight container, protected from light.

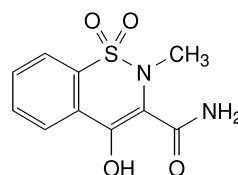
IMPURITIES



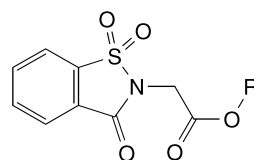
A. pyridin-2-amine,



B. 4-hydroxy-N-(pyridin-2-yl)-2H-1,2-benzothiazine-3-carboxamide 1,1-dioxide,



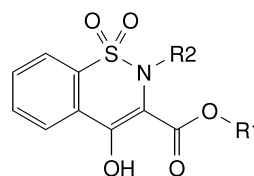
C. 4-hydroxy-2-methyl-2H-1,2-benzothiazine-3-carboxamide 1,1-dioxide,



D. R = CH₃: methyl (1,1-dioxido-3-oxo-1,2-benzisothiazol-2(3H)-yl)acetate,

E. R = C₂H₅: ethyl (1,1-dioxido-3-oxo-1,2-benzisothiazol-2(3H)-yl)acetate,

F. R = CH(CH₃)₂: 1-methylethyl (1,1-dioxido-3-oxo-1,2-benzisothiazol-2(3H)-yl)acetate,



G. R₁ = CH₃, R₂ = H: methyl 4-hydroxy-2H-1,2-benzothiazine-3-carboxylate 1,1-dioxide,

H. R₁ = C₂H₅, R₂ = H: ethyl 4-hydroxy-2H-1,2-benzothiazine-3-carboxylate 1,1-dioxide,

I. R₁ = CH(CH₃)₂, R₂ = H: 1-methylethyl 4-hydroxy-2H-1,2-benzothiazine-3-carboxylate 1,1-dioxide,

J. R₁ = R₂ = CH₃: methyl 4-hydroxy-2-methyl-2H-1,2-benzothiazine-3-carboxylate 1,1-dioxide,

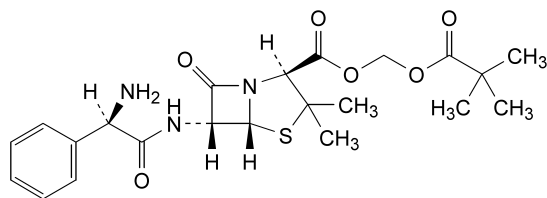
K. R₁ = C₂H₅, R₂ = CH₃: ethyl 4-hydroxy-2-methyl-2H-1,2-benzothiazine-3-carboxylate 1,1-dioxide,

L. R₁ = CH(CH₃)₂, R₂ = CH₃: 1-methylethyl 4-hydroxy-2-methyl-2H-1,2-benzothiazine-3-carboxylate 1,1-dioxide.

01/2005:0852 TESTS

PIVAMPICILLIN

Pivampicillinum

 $C_{22}H_{29}N_3O_6S$ M_r 463.6

DEFINITION

Pivampicillin contains not less than 95.0 per cent and not more than the equivalent of 101.0 per cent of methylene (2*S*, 5*R*, 6*R*)-6-[[*(2R)*-2-amino-2-phenylacetyl]amino]-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylate 2,2-dimethylpropanoate, calculated with reference to the anhydrous substance.

CHARACTERS

A white or almost white, crystalline powder, practically insoluble in water, freely soluble in methanol, soluble in ethanol. It dissolves in dilute acids.

IDENTIFICATION

First identification: A.

Second identification: B, C.

- A. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *pivampicillin CRS*.
- B. Examine by thin-layer chromatography (2.2.27), using *silanised silica gel H R* as the coating substance.

Test solution. Dissolve 10 mg of the substance to be examined in 2 ml of *methanol R*.

Reference solution (a). Dissolve 10 mg of *pivampicillin CRS* in 2 ml of *methanol R*.

Reference solution (b). Dissolve 10 mg of *bacampicillin hydrochloride CRS*, 10 mg of *pivampicillin CRS* and 10 mg of *talampicillin hydrochloride CRS* in 2 ml of *methanol R*.

Apply to the plate 1 µl of each solution. Develop over a path of 15 cm using a mixture of 10 volumes of a 272 g/l solution of *sodium acetate R*, adjusted to pH 5.0 with *glacial acetic acid R*, 40 volumes of *water R* and 50 volumes of *alcohol R*. Dry the plate in a current of warm air, spray with *ninhydrin solution R1* and heat the plate at 60 °C for 10 min. The principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a). The test is not valid unless the chromatogram obtained with reference solution (b) shows 3 clearly separated spots.

- C. Place about 2 mg in a test-tube about 150 mm long and 15 mm in diameter. Moisten with 0.05 ml of *water R* and add 2 ml of *sulphuric acid-formaldehyde reagent R*. Mix the contents of the tube by swirling; the solution is almost colourless. Place the test-tube in a water-bath for 1 min; a dark yellow colour develops.

Appearance of solution. Dissolve 50 mg in 12 ml of 0.1 *M hydrochloric acid*. The solution is not more opalescent than reference suspension II (2.2.1) and not more intensely coloured than reference solution B₇ (2.2.2, *Method I*).

Specific optical rotation (2.2.7). Dissolve 0.100 g in 5.0 ml of *alcohol R* and dilute to 10.0 ml with 0.1 *M hydrochloric acid*. The specific optical rotation is + 208 to + 222, calculated with reference to the anhydrous substance.

Related substances. Examine by liquid chromatography (2.2.29). Prepare the solutions immediately before use.

Test solution. Dissolve 50.0 mg of the substance to be examined in 10.0 ml of *acetonitrile R* and dilute to 20 ml with a 1 g/l solution of *phosphoric acid R*.

Reference solution. Mix 2.0 ml of the test solution with 9.0 ml of *acetonitrile R* and 9.0 ml of a 1 g/l solution of *phosphoric acid R*.

The chromatographic procedure may be carried out using:

- a column 0.125 m long and 4 mm in internal diameter packed with *end-capped octylsilyl silica gel for chromatography R*,

- as mobile phase at a flow rate of 1.5 ml/min:

Mobile phase A. A mixture of 50 volumes of a 1.32 g/l solution of *ammonium phosphate R*, adjusted to pH 2.5 with a 100 g/l solution of *phosphoric acid R*, and 50 volumes of *acetonitrile R*,

Mobile phase B. A mixture of 15 volumes of a 1.32 g/l solution of *ammonium phosphate R*, adjusted to pH 2.5 with a 100 g/l solution of *phosphoric acid R*, and 85 volumes of *acetonitrile R*,

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)	Comment
0 - 10	100	0	isocratic
10 - 12	0	100	isocratic
12 - 17	100	0	re-equilibration

- as detector a spectrophotometer set at 220 nm.

Inject 50 µl of the test solution and 50 µl of the reference solution. The test is not valid unless the ratio of the mass distribution ratio of pivampicillin dimer (which has a retention time of about 5 min) to that of pivampicillin (principal peak) is at least 12. In the chromatogram obtained with the test solution, the sum of the areas of all the peaks, apart from the principal peak, is not greater than 0.3 times the area of the principal peak in the chromatogram obtained with the reference solution (3 per cent). Disregard any peak due to the solvent and any peak with an area less than 0.01 times the area of the principal peak in the chromatogram obtained with the reference solution.

***N,N*-Dimethylaniline** (2.4.26, *Method B*). Not more than 20 ppm.

Test solution. To 1.00 g of the substance to be examined in a ground-glass-stoppered tube add 10 ml of 0.5 *M sulphuric acid*. Heat the tube for 10 min in a water-bath, cool and add 15 ml of 1 *M sodium hydroxide* and 1.0 ml of the internal standard solution. Stopper the tube and shake vigorously for 1 min. Centrifuge if necessary and use the upper layer.

Triethanolamine. Examine by thin-layer chromatography (2.2.27), using *silica gel H R* as the coating substance.

Test solution. Dissolve 0.100 g of the substance to be examined in 1.0 ml of a mixture of 1 volume of *water R* and 9 volumes of *acetonitrile R*.

Reference solution. Dissolve 5.0 mg of triethanolamine *R* in a mixture of 1 volume of water *R* and 9 volumes of acetonitrile *R* and dilute to 100 ml with the same mixture of solvents.

Apply to the plate 10 µl of each solution. Develop over a path of 12 cm using a mixture of 5 volumes of methanol *R*, 15 volumes of butanol *R*, 24 volumes of phosphate buffer solution pH 5.8 *R*, 40 volumes of glacial acetic acid *R* and 80 volumes of butyl acetate *R*. Dry the plate at 110 °C for 10 min and allow to cool. Place in a chromatographic tank an evaporating dish containing a mixture of 1 volume of hydrochloric acid *R1*, 1 volume of water *R* and 2 volumes of a 15 g/l solution of potassium permanganate *R*. Close the tank and allow to stand for 15 min. Place the dried plate in the tank and close the tank. Leave the plate in contact with the chlorine vapour in the tank for 15-20 min. Remove the plate, allow it to stand in air for 2-3 min and spray with tetramethyldiaminodiphenylmethane reagent *R*. Any spot corresponding to triethanolamine in the chromatogram obtained with the test solution is not more intense than the spot in the chromatogram obtained with the reference solution (0.05 per cent).

Water (2.5.12). Not more than 1.0 per cent, determined on 0.30 g by the semi-micro determination of water.

Sulphated ash (2.4.14). Not more than 0.5 per cent, determined on 1.0 g.

ASSAY

Examine by liquid chromatography (2.2.29). Use the solutions within 2 h of preparation.

Test solution. Dissolve 50.0 mg of the substance to be examined in the mobile phase and dilute to 50.0 ml with the mobile phase. Dilute 10.0 ml of the solution to 50.0 ml with the mobile phase.

Reference solution (a). Dissolve 50.0 mg of pivampicillin CRS in the mobile phase and dilute to 50.0 ml with the mobile phase. Dilute 10.0 ml of the solution to 50.0 ml with the mobile phase.

Reference solution (b). Dissolve 25.0 mg of propyl parahydroxybenzoate CRS in the mobile phase and dilute to 50.0 ml with the mobile phase. Dilute 10.0 ml of the solution to 50.0 ml with the mobile phase. Mix 5.0 ml of this solution with 5.0 ml of reference solution (a).

The chromatographic procedure may be carried out using:

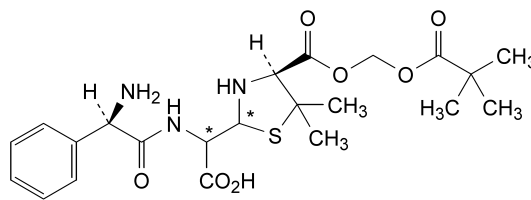
- a stainless steel column 0.125 m long and 4 mm in internal diameter packed with octadecylsilyl silica gel for chromatography *R* (5 µm),
- as mobile phase at a flow rate of 1.5 ml/min a mixture of 40 volumes of acetonitrile *R* and 60 volumes of a 2.22 g/l solution of phosphoric acid *R* adjusted to pH 2.5 with triethylamine *R*,
- as detector a spectrophotometer set at 220 nm.

Inject 20 µl of reference solution (b). Adjust the sensitivity of the system so that the height of each of the 2 principal peaks in the chromatogram obtained is at least 50 per cent of the full scale of the recorder. The test is not valid unless the resolution between the first peak (pivampicillin) and the second peak (propyl parahydroxybenzoate) is at least 5.0 and the symmetry factor for the pivampicillin peak is at most 2.0. Inject 20 µl of reference solution (a) 6 times. The test is not valid unless the relative standard deviation for the area of the principal peak is at most 1.0 per cent. Inject alternately the test solution and reference solution (a).

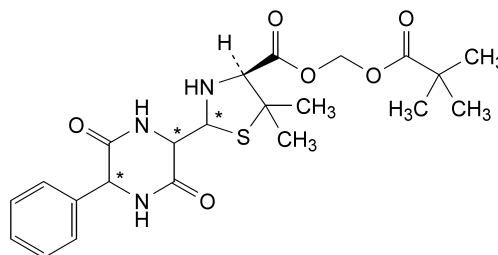
STORAGE

Store in an airtight container.

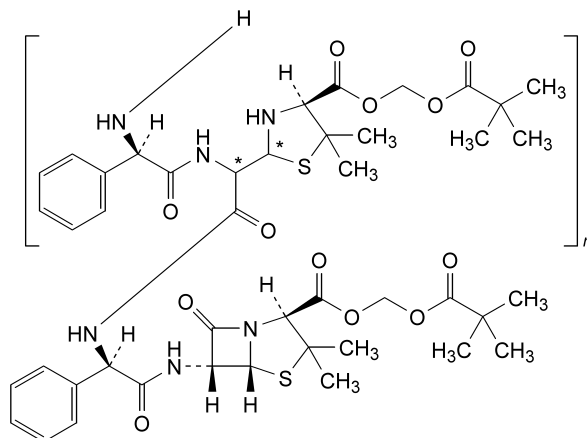
IMPURITIES



- A. 2-[[[(2*R*)-2-amino-2-phenylacetyl]amino]-2-[(4*S*)-4-[[[(2,2-dimethylpropanoyl)oxy]methoxy]carbonyl]-5,5-dimethylthiazolidin-2-yl]acetic acid (penicilloic acids of pivampicillin),



- B. methylene (4*S*)-5,5-dimethyl-2-(3,6-dioxo-5-phenylpiperazin-2-yl)thiazolidine-4-carboxylate 2,2-dimethylpropanoate (diketopiperazines of pivampicillin),

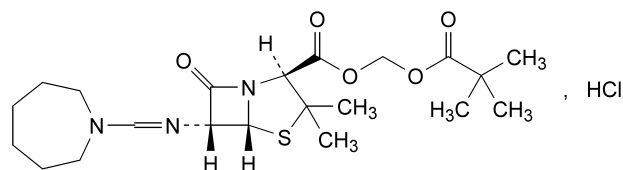


- C. co-oligomers of pivampicillin and of penicilloic acids of pivampicillin.

01/2005:1359

PIVMECILLINAM HYDROCHLORIDE

Pivmecillinami hydrochloridum



C₂₁H₃₄ClN₃O₅S

M_r 476.0

DEFINITION

Pivmecillinam hydrochloride contains not less than 97.0 per cent and not more than the equivalent of 101.5 per cent of methylene 2,2-dimethylpropanoate (2*S*,5*R*,6*R*)-6-[[[hexahydro-1*H*-azepin-1-yl]methylene]amino]-3,3-dimethyl-

7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylate hydrochloride, calculated with reference to the anhydrous substance.

CHARACTERS

A white or almost white, crystalline powder, freely soluble in water, in ethanol and in methanol, slightly soluble in acetone.

IDENTIFICATION

- A. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *pivmecillinam hydrochloride CRS*. Examine the substances prepared as discs.
- B. It gives reaction (a) of chlorides (2.3.1).

TESTS

Appearance of solution. Dissolve 0.5 g in *water R* and dilute to 10 ml with the same solvent. The solution is not more opalescent than reference suspension II (2.2.1) and not more intensely coloured than reference solution B₈ (2.2.2, *Method I*).

pH (2.2.3). Dissolve 1.0 g in *carbon dioxide-free water R* and dilute to 10 ml with the same solvent. The pH of the solution is 2.8 to 3.8.

Related substances. Examine by liquid chromatography (2.2.29) as prescribed under Assay. Inject 20 µl of reference solution (b). Adjust the sensitivity of the system so that the height of the principal peak in the chromatogram obtained is at least 50 per cent of the full-scale of the recorder. Inject 20 µl of test solution (b) and continue the chromatography for 3 times the retention time of the principal peak. In the chromatogram obtained with test solution (b): the area of any peak, apart from the principal peak, is not greater than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (1.5 per cent); the sum of the areas of all the peaks, apart from the principal peak, is not greater than 3 times the area of the principal peak in the chromatogram obtained with reference solution (b) (3 per cent). Disregard any peak with an area less than 0.1 times that of the principal peak in the chromatogram obtained with reference solution (b).

***N,N*-Dimethylaniline** (2.4.26, *Method A*): maximum 20 ppm.

Test solution. Prepare as described in the general method but heat at about 27 °C after the addition of *strong sodium hydroxide solution R*, to dissolve the precipitate formed, then add the *trimethylpentane R*.

Heavy metals (2.4.8). Dissolve 1.0 g in *water R* and dilute to 20 ml with the same solvent. 12 ml of the solution complies with limit test A for heavy metals (20 ppm). Prepare the standard using *lead standard solution (1 ppm Pb) R*.

Water (2.5.12). Not more than 0.5 per cent, determined on 1.00 g by the semi-micro determination of water.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Examine by liquid chromatography (2.2.29). *Prepare the test and reference solutions immediately before use.*

Solvent mixture. To 45 volumes of *acetonitrile R* add 55 volumes of a 13.5 g/l solution of *potassium dihydrogen phosphate R* previously adjusted to pH 3.0 with *dilute phosphoric acid R*.

Test solution (a). Dissolve 20.0 mg of the substance to be examined in the solvent mixture and dilute to 200.0 ml with the solvent mixture.

Test solution (b). Dissolve 25.0 mg of the substance to be examined in the solvent mixture and dilute to 25.0 ml with the solvent mixture.

Reference solution (a). Dissolve 20.0 mg of *pivmecillinam hydrochloride CRS* in the solvent mixture and dilute to 200.0 ml with the solvent mixture.

Reference solution (b). Dilute 5.0 ml of reference solution (a) to 50.0 ml with the solvent mixture.

Reference solution (c). Dissolve 5 mg of *pivmecillinam hydrochloride CRS* and 5 mg of *pivmecillinam impurity C CRS* in the solvent mixture and dilute to 50 ml with the solvent mixture.

The chromatographic procedure may be carried out using:

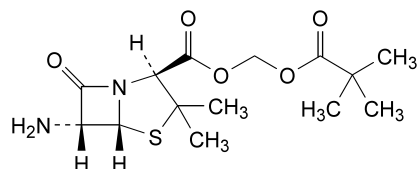
- a stainless steel column 0.25 m long and 4.0 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (5 µm),
- as mobile phase at a flow rate of 1.0 ml/min a mixture prepared as follows: dissolve 0.55 g of *tetraethylammonium hydrogen sulphate R* and 1.0 g of *tetramethylammonium hydrogen sulphate R* in the solvent mixture and dilute to 1000 ml with the solvent mixture,
- as detector a spectrophotometer set at 220 nm.

Inject 20 µl of reference solution (c). Adjust the sensitivity of the system so that the heights of the 2 principal peaks in the chromatogram obtained are at least 50 per cent of the full scale of the recorder. The test is not valid unless, in the chromatogram obtained, the resolution between the first peak (*pivmecillinam*) and the second peak (*impurity C*) is at least 3.5. Inject reference solution (a) 6 times. The test is not valid unless the relative standard deviation of the peak area for *pivmecillinam* is at most 1.0 per cent. Inject alternately test solution (a) and reference solution (a).

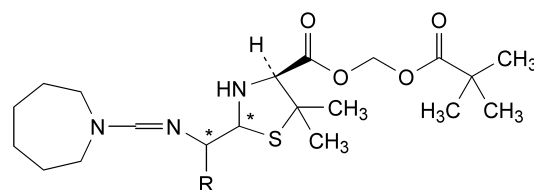
STORAGE

Store protected from light, at a temperature of 2 °C to 8 °C.

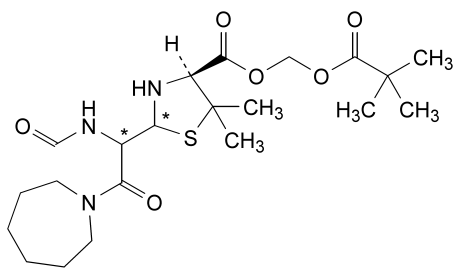
IMPURITIES



- A. methylene (2*S*,5*R*,6*R*)-6-amino-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylate 2,2-dimethylpropanoate (pivaloyloxymethyl 6-aminopenicillanate),



- B. R = CO₂H: 2-[(hexahydro-1*H*-azepin-1-yl)methylene]amino-2-[(4*S*)-4-[[[(2,2-dimethylpropanoyl)oxy]methoxy]carbonyl]-5,5-dimethylthiazolidin-2-yl]acetic acid (penicilloic acids of pivmecillinam),
- C. R = H: methylene 2,2-dimethylpropanoate (2*RS*,4*S*)-2-[[[(hexahydro-1*H*-azepin-1-yl)methylene]amino]methyl]-5,5-dimethylthiazolidin-4-carboxylate,



D. methylene 2,2-dimethylpropanoate (4S)-2-[1-(formylamino)-2-(hexahydro-1H-azepin-1-yl)-2-oxoethyl]-5,5-dimethylthiazolidin-4-carboxylate.

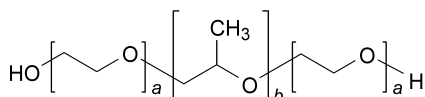
01/2005:1464

POLOXAMERS

Poloxamera

DEFINITION

Synthetic block copolymer of ethylene oxide and propylene oxide, represented by the following general formula:



Poloxamer type	Ethylene oxide units (a)	Propylene oxide units (b)	Content of oxyethylene (per cent)	Average molecular mass
124	10 - 15	18 - 23	44.8 - 48.6	2090 - 2360
188	75 - 85	25 - 30	79.9 - 83.7	7680 - 9510
237	60 - 68	35 - 40	70.5 - 74.3	6840 - 8830
338	137 - 146	42 - 47	81.4 - 84.9	12 700 - 17 400
407	95 - 105	54 - 60	71.5 - 74.9	9840 - 14 600

A suitable antioxidant may be added.

CHARACTERS

Appearance: colourless or almost colourless liquid (poloxamer 124); white or almost white, waxy powder, microbeads or flakes.

Solubility: very soluble in water and in alcohol, practically insoluble in light petroleum (50-70 °C).

mp: about 50 °C for poloxamers 188, 237, 338 and 407.

IDENTIFICATION

First identification: A, B.

Second identification: B, C.

A. Infrared absorption spectrophotometry (2.2.24).

Comparison: chemical reference substance of the Ph. Eur. corresponding to the type of poloxamer to be examined.

B. It complies with the test for average molecular mass (see Tests).

C. It complies with the test for oxypropylene:oxyethylene ratio (see Tests).

TESTS

Solution S. Dissolve 10.0 g in carbon dioxide-free water R and dilute to 100 ml with the same solvent.

Appearance of solution. Solution S is not more intensely coloured than reference solution BY₇ (2.2.2, Method II).

pH (2.2.3): 5.0 to 7.5 for solution S.

Ethylene oxide, propylene oxide and dioxan. Head-space gas chromatography (2.2.28).

Ethylene oxide stock solution. Introduce 0.5 g of ethylene oxide solution R5 in a vial and dilute to 50.0 ml with dimethyl sulphoxide R1. Mix carefully.

Ethylene oxide solution. Dilute 1.0 ml of ethylene oxide stock solution to 250 ml with dimethyl sulphoxide R1.

Propylene oxide stock solution. Introduce about 7 ml of methylene chloride R in a volumetric flask and add 0.500 g (m) of propylene oxide R. Dilute to 10.0 ml with methylene chloride R. Dilute 0.5 ml of this solution to 50.0 ml with dimethyl sulphoxide R1. Mix carefully. Calculate the exact concentration of propylene oxide in mg/ml using the following expression:

$$\frac{m \times 1000 \times 0.5}{10 \times 50}$$

Propylene oxide solution. Dilute 1.0 ml of propylene oxide stock solution to 50.0 ml with dimethyl sulphoxide R1.

Calculate the exact concentration of propylene oxide in µg/ml using the following expression:

$$\frac{C \times 1000 \times 1}{50}$$

C = concentration of the propylene oxide stock solution in mg/ml.

Dioxan solution. Introduce 0.100 g (m) of dioxan R in a flask and dilute to 50.0 ml with dimethyl sulphoxide R1. Dilute 2.50 ml of this solution to 100.0 ml with dimethyl sulphoxide R1.

Calculate the exact concentration of dioxan in µg/ml using the following expression:

$$\frac{m \times 2.50 \times 1000 \times 1000}{50 \times 100}$$

Mixture solution. Dilute a mixture of 6.0 ml of ethylene oxide solution, 6.0 ml of propylene oxide solution and 2.5 ml of dioxan solution to 25.0 ml with dimethyl sulphoxide R1.

Test solution. To 1.000 g of the substance to be examined in a head-space vial, add 4.0 ml of dimethyl sulphoxide R1 and close the vial immediately.

Reference solution. To 1.000 g of the substance to be examined in a head-space vial, add 2.0 ml of dimethyl sulphoxide R1 and 2.0 ml of the mixture solution. Close the vial immediately.

Column:

- **material:** fused silica,
- **size:** l = 50 m, Ø = 0.32 mm,
- **stationary phase:** poly(dimethyl)(diphenyl)siloxane R (film thickness 5 µm).

Carrier gas: helium for chromatography R.

Flow rate: 1.4 ml/min.

Static head-space conditions:

- **equilibrium temperature:** 110 °C,
- **equilibration time:** 30 min,
- **transfer-line temperature:** 140 °C,
- **pressurisation time:** 1 min,
- **injection time:** 0.05 min.

Temperature:

	Time (min)	Temperature (°C)
	0 - 10	70
Column	10 - 27	70 → 240
Injection port		250
Detector		250

Detection: flame ionisation.

Injection: inject a suitable volume of the gaseous phase, for example 1 ml.

Relative retention with reference to ethylene oxide (retention time = about 6 min): propylene oxide = about 1.3; methylene chloride = about 1.6; dioxan = about 3.0; dimethyl sulphoxide = about 3.7.

Limits:

- **ethylene oxide:** not more than half the area of the corresponding peak in the chromatogram obtained with the reference solution (1 ppm),
- **propylene oxide:** not more than half the area of the corresponding peak in the chromatogram obtained with the reference solution (5 ppm),
- **dioxan:** not more than half the area of the corresponding peak in the chromatogram obtained with the reference solution (10 ppm).

Average molecular mass. Weigh 15 g (*m*) of the substance to be examined into a 250 ml ground-glass-stoppered flask, add 25.0 ml of *phthalic anhydride solution R* and a few glass beads and swirl to dissolve. Boil gently under a reflux condenser for 1 h, allow to cool and add 2 quantities, each of 10 ml, of *pyridine R*, through the condenser. Add 10 ml of *water R*, mix and allow to stand for 10 min. Add 40.0 ml of 0.5 M sodium hydroxide and 0.5 ml of a 10 g/l solution of *phenolphthalein R* in *pyridine R*. Titrate with 0.5 M sodium hydroxide to a light pink endpoint that persists for 15 s and record the volume of sodium hydroxide used (*S*). Prepare a blank in the same manner but omitting the substance to be examined. Record the volume of sodium hydroxide used (*B*). Calculate the average molecular mass using the expression:

$$\frac{4000m}{B - S}$$

Oxypropylene:oxyethylene ratio. Nuclear magnetic resonance spectrometry (2.2.33).

Use a 100 g/l solution of the substance to be examined in *deuterated chloroform R*. Record the average area of the doublet appearing at about 1.08 ppm due to the methyl groups of the oxypropylene units (*A*₁) and the average area of the composite band from 3.2 ppm to 3.8 ppm due to CH₂O groups of both the oxyethylene and oxypropylene units and the CHO groups of the oxypropylene units (*A*₂) with reference to the internal standard.

Calculate the percentage of oxyethylene, by weight, in the sample being examined using the following expression:

$$\frac{3300\alpha}{33\alpha + 58}$$

where $\alpha = \frac{A_2}{A_1} - 1$

Water (2.5.12): maximum 1.0 per cent, determined on 1.000 g.

Total ash (2.4.16): maximum 0.4 per cent, determined on 1.0 g.

STORAGE

In an airtight container.

LABELLING

The label states:

- the type of poloxamer,
- the name and concentration of any added antioxidant.

01/2005:0733

POLYACRYLATE DISPERSION 30 PER CENT

Polyacrylatis dispersio 30 per centum

DEFINITION

Polyacrylate dispersion 30 per cent is a dispersion in water of a copolymer of ethyl acrylate and methyl methacrylate having a mean relative molecular mass of about 800 000. It may contain a suitable emulsifier. The residue on evaporation is not less than 28.5 per cent *m/m* and not more than 31.5 per cent *m/m*.

CHARACTERS

An opaque, white, slightly viscous liquid, miscible with water, soluble in acetone, in ethanol and in 2-propanol.

IDENTIFICATION

First identification: A.

Second identification: B, C, D, E.

- Examine by infrared absorption spectrophotometry (2.2.24), comparing with the *Ph. Eur. reference spectrum of polyacrylate*.
- To 1 g add 5 ml of *water R* and mix; the mixture remains opaque. Take three 1 g portions and mix separately with 5 g each of *ethanol R*, *acetone R* and *2-propanol R*. Transparent solutions are obtained.
- To 1 g add 10 ml of 0.1 M sodium hydroxide. The mixture remains opaque.
- It complies with the test for appearance of a film (see Tests).
- Dry 4 g in a Petri dish at 60 °C in an oven for 4 h and transfer the resulting clear film to a small test-tube (100 mm × 12 mm). Heat over a flame and collect the fumes that evolve in a second test-tube held over the mouth of the first tube. The condensate gives the reaction of esters (2.3.1).

TESTS

Relative density (2.2.5): 1.037 to 1.047.

Apparent viscosity. Determine the viscosity (2.2.10) using a rotating viscometer at 20 °C. At a shear rate of 10 s⁻¹, the apparent viscosity is not more than 50 mPa.s.

Appearance of a film. Pour 1 ml on a glass plate and allow to dry. A clear elastic film is formed.

Particulate matter. Filter 100.0 g through a tared stainless steel sieve (90). Rinse with *water R* until a clear filtrate is obtained and dry at 80 °C to constant mass. The mass of the residue is not more than 0.500 g.

Residual monomers. Not more than 100 ppm, determined by liquid chromatography (2.2.29).

Test solution. Dissolve 1.00 g of the substance to be examined in *tetrahydrofuran R* and dilute to 50.0 ml with the same solvent. To 5.0 ml of a 35 g/l solution of *sodium perchlorate R* add 10.0 ml of the solution dropwise

01/2005:0203
corrected

whilst stirring continuously. Centrifuge and filter the clear supernatant liquid. Dilute 5.0 ml to 10.0 ml with *water R*.

Reference solution. Dissolve 10 mg each of *ethyl acrylate R* and *methyl methacrylate R* in *tetrahydrofuran R* and dilute to 50.0 ml with the same solvent. Dilute 1.0 ml of this solution to 100.0 ml with *tetrahydrofuran R*. To 10.0 ml of the final solution add 5.0 ml of a 35 g/l solution of *sodium perchlorate R* and mix. Dilute 5.0 ml of the mixture to 10.0 ml with *water R*.

The chromatographic procedure may be carried out using:

- a column 0.12 m long and 4.6 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (5 µm to 10 µm),
- as mobile phase at a flow rate of 2 ml/min a mixture of 15 volumes of *acetonitrile R* and 85 volumes of *water R*,
- as detector a spectrophotometer set at 205 nm.

Inject separately equal volumes (about 50 µl) of each solution.

Calculate the percentage content of monomers from the area of the peaks in the chromatograms obtained with the test solution and the reference solution and from the content of monomers in the reference solution.

Heavy metals (2.4.8). 1.0 g complies with limit test C for heavy metals (20 ppm). Prepare the standard using 2 ml of *lead standard solution (10 ppm Pb) R*.

Sulphated ash (2.4.14). Not more than 0.4 per cent, determined on 1.0 g.

Microbial contamination. Total viable aerobic count (2.6.12) not more than 10³ micro-organisms per gram, determined by plate count.

ASSAY

Dry 1.000 g at 110 °C for 3 h and weigh the mass of the residue.

STORAGE

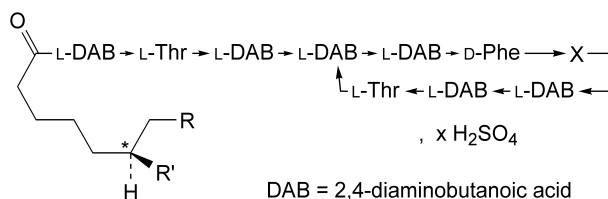
Store at a temperature of 5 °C to 25 °C. Protect from freezing. Handle the substance so as to minimise microbial contamination.

LABELLING

The label states, where applicable, the name and concentration of any added emulsifier.

POLYMYXIN B SULPHATE

Polymyxini B sulfas



polymyxin	R	R'	X	Molecular formula	<i>M_r</i>
B1	CH ₃	CH ₃	L-Leu	C ₅₆ H ₉₈ N ₁₆ O ₁₃	1204
B2	H	CH ₃	L-Leu	C ₅₅ H ₉₆ N ₁₆ O ₁₃	1190
B3	CH ₃	H	L-Leu	C ₅₅ H ₉₆ N ₁₆ O ₁₃	1190
B1-I	CH ₃	CH ₃	L-Ile	C ₅₆ H ₉₈ N ₁₆ O ₁₃	1204

DEFINITION

Mixture of the sulphates of polypeptides produced by the growth of certain strains of *Paenibacillus polymyxa*, or obtained by any other means, the main component being polymyxin B1.

Content:

- sum of polymyxins B1, B2, B3 and B1-I: minimum 80.0 per cent (dried substance),
- polymyxin B3: maximum 6.0 per cent (dried substance),
- polymyxin B1-I: maximum 15.0 per cent (dried substance).

CHARACTERS

Appearance: white or almost white powder, hygroscopic.

Solubility: soluble in water, slightly soluble in alcohol.

IDENTIFICATION

First identification: B, D.

Second identification: A, C, D.

A. Thin-layer chromatography (2.2.27).

Test solution. Dissolve 5 mg of the substance to be examined in 1 ml of a mixture of equal volumes of *hydrochloric acid R* and *water R*. Heat at 135 °C in a sealed tube for 5 h. Evaporate to dryness on a water-bath and continue the heating until the hydrochloric acid has evaporated. Dissolve the residue in 0.5 ml of *water R*.

Reference solution (a). Dissolve 20 mg of *leucine R* in *water R* and dilute to 10 ml with the same solvent.

Reference solution (b). Dissolve 20 mg of *threonine R* in *water R* and dilute to 10 ml with the same solvent.

Reference solution (c). Dissolve 20 mg of *phenylalanine R* in *water R* and dilute to 10 ml with the same solvent.

Reference solution (d). Dissolve 20 mg of *serine R* in *water R* and dilute to 10 ml with the same solvent.

Plate: TLC silica gel G plate R.

Mobile phase: *water R*, *phenol R* (25:75 V/V).

Carry out the following procedures protected from light.

Application: 5 µl, as bands of 10 mm.

Place the plate in the chromatographic tank, so that it is not in contact with the mobile phase, and allow to become impregnated with the vapour of the solvent for at least 12 h.

Development: over a path of 12 cm using the same mobile phase.

Drying: at 100-105 °C.

Detection: spray with *ninhydrin solution R1* and heat at 110 °C for 5 min.

Results: the chromatogram obtained with the test solution shows bands corresponding to those in the chromatograms obtained with reference solutions (a), (b) and (c), but shows no band corresponding to that in the chromatogram obtained with reference solution (d). The chromatogram obtained with the test solution also shows a band with a very low R_f value (2,4-diaminobutyric acid).

B. Examine the chromatograms obtained in the assay.

Results: the peaks due to polymyxins B1, B2, B3 and B1-I in the chromatogram obtained with the test solution are similar in retention time to the corresponding peaks in the chromatogram obtained with reference solution (a).

C. Dissolve about 2 mg in 5 ml of *water R* and add 5 ml of a 100 g/l solution of *sodium hydroxide R*. Shake and add dropwise 0.25 ml of a 10 g/l solution of *copper sulphate R*, shaking after each addition. A reddish-violet colour develops.

D. It gives reaction (a) of sulphates (2.3.1).

TESTS

pH (2.2.3): 5.0 to 7.0.

Dissolve 0.2 g in *carbon dioxide-free water R* and dilute to 10 ml with the same solvent.

Specific optical rotation (2.2.7): –78 to –90 (dried substance).

Dissolve 0.50 g in *water R* and dilute to 25.0 ml with the same solvent.

Related substances. Liquid chromatography (2.2.29): use the normalisation procedure.

Test solution. Dissolve 50.0 mg of the substance to be examined in a mixture of 20 volumes of *acetonitrile R* and 80 volumes of *water R* and dilute to 100.0 ml with the same mixture of solvents.

Reference solution (a). Dissolve 50.0 mg of *polymyxin B sulphate CRS* in a mixture of 20 volumes of *acetonitrile R* and 80 volumes of *water R* and dilute to 100.0 ml with the same mixture of solvents.

Reference solution (b). Dilute 1.0 ml of reference solution (a) to 100.0 ml with a mixture of 20 volumes of *acetonitrile R* and 80 volumes of *water R*.

Column:

- **size:** $l = 0.25$ m, $\varnothing = 4.6$ mm,
- **stationary phase:** base-deactivated end-capped octadecylsilyl silica gel for chromatography *R* (5 μ m),
- **temperature:** 30 °C.

Mobile phase: mix 20 volumes of *acetonitrile R* and 80 volumes of a solution prepared as follows: dissolve 4.46 g of *anhydrous sodium sulphate R* in 900 ml of *water R*, adjust to pH 2.3 using *dilute phosphoric acid R* and dilute to 1000 ml with *water R*.

Flow rate: 1.0 ml/min.

Detection: spectrophotometer at 215 nm.

Injection: 20 μ l.

Run time: 1.4 times the retention time of polymyxin B1.

Relative retention with reference to polymyxin B1 (retention time = about 35 min): polymyxin B2 = about 0.5; polymyxin B3 = about 0.6; polymyxin B1-I = about 0.8.

System suitability: reference solution (a):

- **resolution:** minimum of 3.0 between the peaks due to polymyxin B2 and polymyxin B3.

Limits:

- **any impurity:** maximum 3.0 per cent,
- **total:** maximum 17.0 per cent,
- **disregard limit:** 0.7 times the area of the principal peak in the chromatogram obtained with reference solution (b).

Sulphate: 15.5 per cent to 17.5 per cent (dried substance).

Dissolve 0.250 g in 100 ml of *water R* and adjust the solution to pH 11 using *concentrated ammonia R*. Add 10.0 ml of 0.1 M *barium chloride* and about 0.5 mg of *phthalein purple R*. Titrate with 0.1 M *sodium edetate*, adding 50 ml of *alcohol R* when the colour of the solution begins to change and continuing the titration until the violet-blue colour disappears.

1 ml of 0.1 M *barium chloride* is equivalent to 9.606 mg of SO_4 .

Loss on drying (2.2.32): maximum 6.0 per cent, determined on 1.000 g by drying at 60 °C over *diphosphorus pentoxide R* at a pressure not exceeding 670 Pa for 3 h.

Sulphated ash (2.4.14): maximum 0.75 per cent, determined on 1.0 g.

Pyrogens (2.6.8). If intended for use in the manufacture of parenteral dosage forms without a further appropriate procedure for the removal of pyrogens, it complies with the test for pyrogens. Inject, per kilogram of the rabbit's mass, 1 ml of a solution in *water for injections R* containing 1.5 mg of the substance to be examined per millilitre.

ASSAY

Liquid chromatography (2.2.29) as described in the test for related substances with the following modification.

Injection: test solution and reference solution (a).

Calculate the percentage content of polymyxin B3 and B1-I, and the sum of polymyxins B1, B2, B3 and B1-I using the corresponding declared contents of *polymyxin sulphate CRS*.

STORAGE

In an airtight container, protected from light. If the substance is sterile, store in a sterile, airtight, tamper-proof container.

LABELLING

The label states, where applicable, that the substance is apyrogenic.

01/2005:0426
corrected

POLYSORBATE 20

Polysorbatum 20

DEFINITION

Mixture of partial esters of fatty acids, mainly lauric acid, with sorbitol and its anhydrides ethoxylated with approximately 20 moles of ethylene oxide for each mole of sorbitol and sorbitol anhydrides.

CHARACTERS

Appearance: oily, yellow to brownish-yellow, clear or slightly opalescent liquid.

Solubility: soluble in water, in ethanol, in ethyl acetate and in methanol, practically insoluble in fatty oils and in liquid paraffin.

Relative density: about 1.10.

Viscosity: about 400 mPas at 25 °C.

IDENTIFICATION

First identification: A, D.

Second identification: B, C, D, E.

A. Infrared absorption spectrophotometry (2.2.24).

Comparison: Ph. Eur. reference spectrum of polysorbate 20.

B. It complies with the test for hydroxyl value (see Tests).

C. It complies with the test for saponification value (see Tests).

D. It complies with the test for composition of fatty acids (see Tests).

E. Dissolve 0.1 g in 5 ml of *methylene chloride R*. Add 0.1 g of *potassium thiocyanate R* and 0.1 g of *cobalt nitrate R*. Stir with a glass rod. The solution becomes blue.

TESTS

Acid value (2.5.1): maximum 2.0.

Dissolve 5.0 g in 50 ml of the prescribed mixture of solvent.

Hydroxyl value (2.5.3, Method A): 96 to 108.

Peroxide value: maximum 10.0.

Introduce 10.0 g into a 100 ml beaker, dissolve with *glacial acetic acid R* and dilute to 20 ml with the same solvent. Add 1 ml of *saturated potassium iodide solution R* and allow to stand for 1 min. Add 50 ml of *carbon dioxide-free water R* and a magnetic stirring bar. Titrate with 0.01 M *sodium thiosulphate*, determining the end-point potentiometrically (2.2.20). Carry out a blank titration.

Determine the peroxide value using the following expression:

$$\frac{(n_1 - n_2) \times M \times 1000}{m}$$

n_1 = volume of 0.01 M *sodium thiosulphate* required for the substance to be examined, in millilitres,

n_2 = volume of 0.01 M *sodium thiosulphate* required for the blank, in millilitres,

M = molarity of the sodium thiosulphate solution, in moles per litre,

m = mass of substance to be examined, in grams.

Saponification value (2.5.6): 40 to 50, determined on 4.0 g.

Use 15.0 ml of 0.5 M *alcoholic potassium hydroxide* and dilute with 50 ml of *alcohol R* before carrying out the titration. Heat under reflux for 60 min.

Composition of fatty acids (2.4.22, Method C). Prepare reference solution (a) as indicated in Table 2.4.22-2.

Column:

- *material:* fused silica,
- *dimensions:* $l = 30$ m, $\varnothing = 0.32$ mm,
- *stationary phase:* *macrogol 20 000 R* (film thickness 0.5 µm).

Carrier gas: *helium for chromatography R*.

Linear velocity: 50 cm/s.

Temperature:

	Time (min)	Temperature (°C)
Column	0 - 14	80 → 220
	14 - 54	220
Injection port		250
Detector		250

Detection: flame ionisation.

Injection: 1 µl.

Composition of the fatty acid fraction of the substance:

- *caproic acid:* maximum 1.0 per cent,
- *caprylic acid:* maximum 10.0 per cent,
- *capric acid:* maximum 10.0 per cent,
- *lauric acid:* 40.0 per cent to 60.0 per cent,
- *myristic acid:* 14.0 per cent to 25.0 per cent,
- *palmitic acid:* 7.0 per cent to 15.0 per cent,
- *stearic acid:* maximum 7.0 per cent,
- *oleic acid:* maximum 11.0 per cent,
- *linoleic acid:* maximum 3.0 per cent.

Ethylene oxide and dioxan (2.4.25, Method A): maximum 1 ppm of ethylene oxide and 10 ppm of dioxan.

Heavy metals (2.4.8): maximum 10 ppm.

2.0 g complies with limit test C. Prepare the standard using 2 ml of *lead standard solution (10 ppm Pb) R*.

Water (2.5.12): maximum 3.0 per cent, determined on 1.00 g.

Total ash (2.4.16): maximum 0.25 per cent, determined on 2.0 g.

STORAGE

In an airtight container, protected from light.

01/2005:1914

POLYSORBATE 40

Polysorbatum 40

DEFINITION

Mixture of partial esters of fatty acids, mainly *Palmitic acid (1904)*, with sorbitol and its anhydrides ethoxylated with approximately 20 moles of ethylene oxide for each mole of sorbitol and sorbitol anhydrides.

CHARACTERS

Appearance: oily, viscous, yellowish or brownish-yellow liquid.

Solubility: miscible with water, with ethanol, with ethyl acetate and with methanol, practically insoluble in fatty oils and in liquid paraffin.

Relative density: about 1.10.

Viscosity: about 400 mPas at 30 °C.

IDENTIFICATION

First identification: A, D.

Second identification: B, C, D, E.

A. Infrared absorption spectrophotometry (2.2.24).

Comparison: Ph. Eur. reference spectrum of polysorbate 40.

B. It complies with the test for hydroxyl value (see Tests).

C. It complies with the test for saponification value (see Tests).

- D. It complies with the test for composition of fatty acids (see Tests).
- E. Dissolve 0.1 g in 5 ml of *methylene chloride R*. Add 0.1 g of *potassium thiocyanate R* and 0.1 g of *cobalt nitrate R*. Stir with a glass rod. The solution becomes blue.

TESTS

Acid value (2.5.1): maximum 2.0.

Dissolve 5.0 g in 50 ml of the prescribed mixture of solvents.

Hydroxyl value (2.5.3, *Method A*): 89 to 105.

Peroxide value: maximum 10.0.

Introduce 10.0 g into a 100 ml beaker, dissolve with *glacial acetic acid R* and dilute to 20 ml with the same solvent. Add 1 ml of *saturated potassium iodide solution R* and allow to stand for 1 min. Add 50 ml of *carbon dioxide-free water R* and a magnetic stirring bar. Titrate with *0.01 M sodium thiosulphate*, determining the end-point potentiometrically (2.2.20). Carry out a blank titration.

Determine the peroxide value using the following expression:

$$\frac{(n_1 - n_2) \times M \times 1000}{m}$$

- n_1 = volume of *0.01 M sodium thiosulphate* required for the substance to be examined, in millilitres,
- n_2 = volume of *0.01 M sodium thiosulphate* required for the blank, in millilitres,
- M = molarity of the sodium thiosulphate solution, in moles per litre,
- m = mass of substance to be examined, in grams.

Saponification value (2.5.6): 41 to 52, determined on 4.0 g.

Use 15.0 ml of *0.5 M alcoholic potassium hydroxide* and dilute with 50 ml of *alcohol R* before carrying out the titration. Heat under reflux for 60 min.

Composition of fatty acids (2.4.22, *Method C*). Prepare reference solution (a) as indicated in Table 2.4.22-1.

Column:

- **material**: fused silica,
- **size**: $l = 30$ m, $\varnothing = 0.32$ mm,
- **stationary phase**: *macrogol 20 000 R* (film thickness 0.5 μ m).

Carrier gas: *helium for chromatography R*.

Linear velocity: 50 cm/s.

Temperature:

	Time (min)	Temperature (°C)
Column	0 - 14	80 → 220
	14 - 54	220
Injection port		250
Detector		250

Detection: flame ionisation.

Injection: 1 μ l.

Composition of the fatty acid fraction of the substance:

- **palmitic acid**: minimum 92.0 per cent.

Ethylene oxide and dioxan (2.4.25, *Method A*): maximum 1 ppm of ethylene oxide and maximum 10 ppm of dioxan.

Heavy metals (2.4.8): maximum 10 ppm.

2.0 g complies with limit test C. Prepare the standard using 2 ml of *lead standard solution (10 ppm Pb) R*.

Water (2.5.12): maximum 3.0 per cent, determined on 1.00 g.

Total ash (2.4.16): maximum 0.25 per cent, determined on 2.0 g.

STORAGE

In an airtight container, protected from light.

01/2005:0427

POLYSORBATE 60

Polysorbatum 60

DEFINITION

Mixture of partial esters of fatty acids, mainly *Stearic acid 50 (1474)*, with sorbitol and its anhydrides ethoxylated with approximately 20 moles of ethylene oxide for each mole of sorbitol and sorbitol anhydrides.

CHARACTERS

Appearance: yellowish-brown gelatinous mass which becomes a clear liquid at temperatures above 25 °C.

Solubility: soluble in water, in ethanol, in ethyl acetate and in methanol, practically insoluble in fatty oils and in liquid paraffin.

Relative density: about 1.10.

Viscosity: about 400 mPa·s at 30 °C.

IDENTIFICATION

First identification: A, D.

Second identification: B, C, D, E.

A. Infrared absorption spectrophotometry (2.2.24).

Comparison: *Ph. Eur. reference spectrum of polysorbate 60*.

B. It complies with the test for hydroxyl value (see Tests).

C. It complies with the test for saponification value (see Tests).

D. It complies with the test for composition of fatty acids (see Tests).

E. Dissolve 0.1 g in 5 ml of *methylene chloride R*. Add 0.1 g of *potassium thiocyanate R* and 0.1 g of *cobalt nitrate R*. Stir with a glass rod. The solution becomes blue.

TESTS

Acid value (2.5.1): maximum 2.0.

Dissolve 5.0 g in 50 ml of the prescribed mixture of solvents.

Hydroxyl value (2.5.3, *Method A*): 81 to 96.

Peroxide value: maximum 10.0.

Introduce 10.0 g into a 100 ml beaker, dissolve with *glacial acetic acid R* and dilute to 20 ml with the same solvent. Add 1 ml of *saturated potassium iodide solution R* and allow to stand for 1 min. Add 50 ml of *carbon dioxide-free water R* and a magnetic stirring bar. Titrate with *0.01 M sodium thiosulphate*, determining the end-point potentiometrically (2.2.20). Carry out a blank titration.

Determine the peroxide value using the following expression:

$$\frac{(n_1 - n_2) \times M \times 1000}{m}$$

- n_1 = volume of *0.01 M sodium thiosulphate* required for the substance to be examined, in millilitres,
- n_2 = volume of *0.01 M sodium thiosulphate* required for the blank, in millilitres,

M = molarity of the sodium thiosulphate solution, in moles per litre,

m = mass of substance to be examined, in grams.

Saponification value (2.5.6): 45 to 55, determined on 4.0 g. Use 15.0 ml of 0.5 *M* alcoholic potassium hydroxide and dilute with 50 ml of alcohol *R* before carrying out the titration. Heat under reflux for 60 min.

Composition of fatty acids (2.4.22, Method C). Prepare reference solution (a) as indicated in Table 2.4.22-1.

Column:

- **material:** fused silica,
- **size:** $l = 30$ m, $\varnothing = 0.32$ mm,
- **stationary phase:** macrogol 20 000 *R* (film thickness 0.5 μ m).

Carrier gas: helium for chromatography *R*.

Linear velocity: 50 cm/s.

Temperature:

	Time (min)	Temperature (°C)
Column	0 - 14	80 → 220
	14 - 54	220
Injection port		250
Detector		250

Detection: flame ionisation.

Injection: 1 μ l.

Composition of the fatty acid fraction of the substance:

- **stearic acid:** 40.0 per cent to 60.0 per cent,
- **sum of the contents of palmitic and stearic acids:** minimum 90.0 per cent.

Ethylene oxide and dioxan (2.4.25, Method A): maximum 1 ppm of ethylene oxide and maximum 10 ppm of dioxan.

Heavy metals (2.4.8): maximum 10 ppm.

2.0 g complies with limit test C. Prepare the standard using 2 ml of lead standard solution (10 ppm Pb) *R*.

Water (2.5.12): maximum 3.0 per cent, determined on 1.00 g.

Total ash (2.4.16): maximum 0.25 per cent, determined on 2.0 g.

STORAGE

In an airtight container, protected from light.

01/2005:0428

POLYSORBATE 80

Polysorbatum 80

DEFINITION

Mixture of partial esters of fatty acids, mainly *Oleic acid* (0799), with sorbitol and its anhydrides ethoxylated with approximately 20 moles of ethylene oxide for each mole of sorbitol and sorbitol anhydrides.

CHARACTERS

Appearance: oily, yellowish or brownish-yellow, clear liquid.

Solubility: miscible with water, with ethanol, with ethyl acetate and with methanol, practically insoluble in fatty oils and in liquid paraffin.

Relative density: about 1.10.

Viscosity: about 400 mPa·s at 25 °C.

IDENTIFICATION

First identification: A, D.

Second identification: B, C, D, E.

A. Infrared absorption spectrophotometry (2.2.24).

Comparison: Ph. Eur. reference spectrum of polysorbate 80.

B. It complies with the test for hydroxyl value (see Tests).

C. It complies with the test for saponification value (see Tests).

D. It complies with the test for composition of fatty acids (see Tests).

E. Dissolve 0.1 g in 5 ml of methylene chloride *R*. Add 0.1 g of potassium thiocyanate *R* and 0.1 g of cobalt nitrate *R*. Stir with a glass rod. The solution becomes blue.

TESTS

Acid value (2.5.1): maximum 2.0.

Dissolve 5.0 g in 50 ml of the prescribed mixture of solvents.

Hydroxyl value (2.5.3, Method A): 65 to 80.

Peroxide value: maximum 10.0.

Introduce 10.0 g into a 100 ml beaker, dissolve with glacial acetic acid *R* and dilute to 20 ml with the same solvent. Add 1 ml of saturated potassium iodide solution *R* and allow to stand for 1 min. Add 50 ml of carbon dioxide-free water *R* and a magnetic stirring bar. Titrate with 0.01 *M* sodium thiosulphate, determining the end-point potentiometrically (2.2.20). Carry out a blank titration.

Determine the peroxide value using the following expression:

$$\frac{(n_1 - n_2) \times M \times 1000}{m}$$

n_1 = volume of 0.01 *M* sodium thiosulphate required for the substance to be examined, in millilitres,

n_2 = volume of 0.01 *M* sodium thiosulphate required for the blank, in millilitres,

M = molarity of the sodium thiosulphate solution, in moles per litre,

m = mass of substance to be examined, in grams.

Saponification value (2.5.6): 45 to 55, determined on 4.0 g. Use 15.0 ml of 0.5 *M* alcoholic potassium hydroxide and dilute with 50 ml of alcohol *R* before carrying out the titration. Heat under reflux for 60 min.

Composition of fatty acids (2.4.22, Method C). Prepare reference solution (a) as indicated in Table 2.4.22-3.

Column:

- **material:** fused silica,
- **size:** $l = 30$ m, $\varnothing = 0.32$ mm,
- **stationary phase:** macrogol 20 000 *R* (film thickness 0.5 μ m).

Carrier gas: helium for chromatography *R*.

Linear velocity: 50 cm/s.

Temperature:

	Time (min)	Temperature (°C)
Column	0 - 14	80 → 220
	14 - 54	220
Injection port		250
Detector		250

Detection: flame ionisation.

Injection: 1 μ l.

Composition of the fatty acid fraction of the substance:

- *myristic acid*: maximum 5.0 per cent,
- *palmitic acid*: maximum 16.0 per cent,
- *palmitoleic acid*: maximum 8.0 per cent,
- *stearic acid*: maximum 6.0 per cent,
- *oleic acid*: 58.0 per cent to 85.0 per cent,
- *linoleic acid*: maximum 18.0 per cent,
- *linolenic acid*: maximum 4.0 per cent,

Ethylene oxide and dioxan (2.4.25, *Method A*): maximum 1 ppm of ethylene oxide and maximum 10 ppm of dioxan.

Heavy metals (2.4.8): maximum 10 ppm.

2.0 g complies with limit test C. Prepare the standard using 2 ml of *lead standard solution* (10 ppm Pb) R.

Water (2.5.12): maximum 3.0 per cent, determined on 1.00 g.

Total ash (2.4.16): maximum 0.25 per cent, determined on 2.0 g.

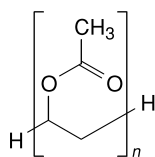
STORAGE

In an airtight container, protected from light.

01/2005:1962

POLY(VINYL ACETATE)

Poly(vinylis acetat)

**DEFINITION**

Poly(vinyl acetate) is a thermoplastic polymer obtained by polymerisation of vinyl acetate using a suitable starter, without solvent or with water or 2-propanol. The vast majority of the acetate moieties are attached to non-neighbouring carbon atoms of the chain.

The index *n* is about 100 - 17 000. The relative molecular mass lies between 10 000 and 1500 000. The viscosity is 4 to 250 mPas. The ester value, which characterises the degree of hydrolysis, is 615 to 675.

CHARACTERS

Appearance: white powder or colourless granules or beads.

Solubility: practically insoluble in water, freely soluble in ethyl acetate, soluble in alcohol. It is hygroscopic and swells in water.

It softens at temperatures above 40-50 °C.

IDENTIFICATION**A. Infrared absorption spectrophotometry** (2.2.24).

Preparation: discs prepared as follows: dissolve about 200 mg in 5 ml of *acetone* R, place 2 drops on a *potassium bromide* R disc and dry to evaporate the solvent.

The spectrum obtained shows absorption maxima corresponding to poly(vinyl acetate) at 1750 cm⁻¹, 1400 cm⁻¹ and 1020 cm⁻¹.

B. It complies with the test for viscosity (see Tests).**C. Saponify** 0.500 g in a mixture of 25.0 ml of 0.5 M *alcoholic potassium hydroxide* R and 25.0 ml of *water* R (2.5.6). The solution obtained gives the reaction of acetates (2.3.1).**TESTS**

Solution S. Suspend 50.0 g in 100 ml of *ethyl acetate* R in a borosilicate glass flask with a ground-glass neck. Heat under a reflux condenser with constant stirring for 30 min. Allow to cool. Filter through a tared sintered glass filter (No. 16) and wash the residue with 50.0 ml of *ethyl acetate* R, pour the filtrate into a 250 ml graduated flask. Dilute to 250 ml with *ethyl acetate* R.

Appearance of solution. Solution S is clear (2.2.1) and colourless (2.2.2, *Method I*).

Viscosity (2.2.49): 85 per cent to 115 per cent of the value stated on the label.

Determine the viscosity immediately after preparation of solution S at 20 ± 0.1 °C by using a rolling ball viscosimeter.

Acid value (2.5.1): maximum 2.0, determined on 5.0 g dissolved in 50 ml of *alcohol* R by shaking for 3 h.

Ester value (2.5.2): 615 to 675.

Saponify 0.500 g in a mixture of 25.0 ml of 0.5 M *alcoholic potassium hydroxide* and 25.0 ml of *water* R (2.5.6).

Residual peroxides: maximum 100 ppm, calculated as hydrogen peroxide.

Place 0.85 g in a borosilicate glass flask with a ground-glass neck. Add 10 ml of *ethyl acetate* R and heat under a reflux condenser with constant agitation. Allow to cool. Replace the air in the container with *oxygen-free nitrogen* R and add a solution of 1 ml of *glacial acetic acid* R and 0.5 g of *sodium iodide* R in 40 ml of *water* R. Shake thoroughly and allow to stand protected from light for 20 min. Titrate with 0.005 M *sodium thiosulphate* R until the yellow colour is discharged. Carry out a blank titration. The difference between the titration volumes is not greater than 1.0 ml.

Vinyl acetate. Head-space gas chromatography (2.2.28).

Reference solution. Place 15 ml of *dimethylformamide* R in a 20 ml vial, add 45 µl of *vinyl acetate* R and 50.0 µl of *butanal* R and dilute to volume with *dimethylformamide* R. Dilute 1 ml of the solution to 10 ml with *dimethylformamide* R.

Test solution (a). Place 0.2000 g of the substance to be examined in a 20 ml vial and add 1.00 ml of *dimethylformamide* R. Close the vial and secure the stopper. Shake, avoiding contact between the stopper and the liquid.

Test solution (b). Place 0.2000 g of the substance to be examined in a 20 ml vial and add 1.00 ml of the reference solution. Close the vial and secure the stopper. Shake, avoiding contact between the stopper and the liquid.

Column:

- **material:** fused silica,
- **size:** *l* = 25 m, Ø = 0.32 mm,
- **stationary phase:** poly(dimethyl)(diphenyl)(divinyl)siloxane R (film thickness: 0.32 µm),

Carrier gas: *nitrogen for chromatography* R.

Flow rate: 20 ml/min.

Static head-space conditions which may be used:

- **equilibration temperature:** 60 °C,
- **equilibration time:** 20 min,
- **transfer-line temperature:** 120 °C,
- **carrier gas:** *nitrogen for chromatography* R.

Temperature:

- **column:** 155 °C,
- **injection port:** 120 °C,
- **detector:** 180 °C.

Detection: flame ionisation.

Sulphated ash (2.4.14): maximum 1.0 per cent, determined on 1.0 g.

The following test concerning the pharmacotechnological properties may be carried out depending on the intended formulation. It is not a mandatory requirement.

Infrared absorption spectrum (2.2.24). The determination of the spectrum and comparison with a suitable sample is useful for ensuring suitable functionality-related properties. The intensities of the absorption maxima at 1720 cm^{-1} and 1260 cm^{-1} are inversely proportional to the degree of hydrolysis.

LABELLING

The label states:

- the viscosity for a 40 g/l solution,
- the ester value.

01/2005:1139
corrected

POTASSIUM ACETATE

Kalii acetas

$\text{C}_2\text{H}_3\text{KO}_2$

M_r 98.1

DEFINITION

Potassium acetate contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of $\text{C}_2\text{H}_3\text{KO}_2$, calculated with reference to the dried substance.

CHARACTERS

A white, crystalline powder or colourless crystals, deliquescent, very soluble in water, freely soluble in alcohol.

IDENTIFICATION

- It gives reaction (a) of acetates (2.3.1).
- It gives reaction (a) of potassium (2.3.1).

TESTS

Solution S. Dissolve 10.0 g in *distilled water R* and dilute to 100 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and colourless (2.2.2, *Method II*).

pH (2.2.3). Dissolve 1.0 g in *carbon dioxide-free water R* and dilute to 20 ml with the same solvent. The pH of the solution is 7.5 to 9.0.

Reducing substances. Dilute 10 ml of solution S to 100 ml with *water R*. Add 5 ml of *dilute sulphuric acid R* and 0.5 ml of a 0.32 g/l solution of *potassium permanganate R*. Mix and boil gently for 5 min. The solution remains pink.

Chlorides (2.4.4). Dilute 2.5 ml of solution S to 15 ml with *water R*. The solution complies with the limit test for chlorides (200 ppm).

Sulphates (2.4.13). Dilute 7.5 ml of solution S to 15 ml with *distilled water R*. The solution complies with the limit test for sulphates (200 ppm).

Aluminium (2.4.17). If intended for use in the manufacture of peritoneal dialysis solutions, haemofiltration solutions or haemodialysis solutions, it complies with the test for aluminium. Dissolve 2.0 g in 50 ml of *water R* and add 5 ml of *acetate buffer solution pH 6.0 R*. The solution complies with the limit test for aluminium (1 ppm). Use as a reference

solution a mixture of 1 ml of *aluminium standard solution (2 ppm Al) R*, 5 ml of *acetate buffer solution pH 6.0 R* and 49 ml of *water R*. To prepare the blank use a mixture of 5 ml of *acetate buffer solution pH 6.0 R* and 50 ml of *water R*.

Iron (2.4.9). Dilute 5 ml of solution S to 10 ml with *water R*. The solution complies with the limit test for iron (20 ppm).

Heavy metals (2.4.8). Dissolve 5.0 g in *water R* and dilute to 20 ml with the same solvent. 12 ml of the solution complies with limit test A for heavy metals (4 ppm). Prepare the standard using *lead standard solution (1 ppm Pb) R*.

Sodium. Not more than 0.5 per cent of Na, determined by atomic emission spectrometry (2.2.22, *Method II*).

Test solution. Dissolve 1.00 g in *water R* and dilute to 100.0 ml with the same solvent.

Reference solutions. Prepare the reference solutions using *sodium standard solution (200 ppm Na) R*, diluted as necessary with *water R*.

Measure the emission intensity at 589 nm.

Loss on drying (2.2.32). Not more than 3.0 per cent, determined on 1.000 g by drying in an oven at $100\text{ }^\circ\text{C}$ to $105\text{ }^\circ\text{C}$.

ASSAY

Dissolve 80.0 mg in 20 ml of *anhydrous acetic acid R*. Add 0.2 ml of *naphtholbenzein solution R*. Titrate with 0.1 M *perchloric acid*. Carry out a blank titration.

1 ml of 0.1 M *perchloric acid* is equivalent to 9.81 mg of $\text{C}_2\text{H}_3\text{KO}_2$.

STORAGE

Store in an airtight container.

01/2005:0184

POTASSIUM BROMIDE

Kalii bromidum

KBr

M_r 119.0

DEFINITION

Content: 98.0 per cent to 100.5 per cent (dried substance).

CHARACTERS

Appearance: white, crystalline powder or colourless crystals.

Solubility: freely soluble in water and in glycerol, slightly soluble in alcohol.

IDENTIFICATION

- It gives reaction (a) of bromides (2.3.1).
- Solution S (see Tests) gives the reactions of potassium (2.3.1).

TESTS

Solution S. Dissolve 10.0 g in *carbon dioxide-free water R* prepared from *distilled water R* and dilute to 100 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and colourless (2.2.2, *Method II*).

Acidity or alkalinity. To 10 ml of solution S add 0.1 ml of *bromothymol blue solution R1*. Not more than 0.5 ml of 0.01 M *hydrochloric acid* or 0.01 M *sodium hydroxide* is required to change the colour of the indicator.

01/2005:1557

POTASSIUM CARBONATE

Kalii carbonas

 M_r 138.2

DEFINITION

Potassium carbonate contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of K_2CO_3 , calculated with reference to the dried substance.

CHARACTERS

A white granular powder, hygroscopic, freely soluble in water, practically insoluble in alcohol.

IDENTIFICATION

- Dissolve 1 g in 10 ml of *water R*. The solution is strongly alkaline (2.2.4).
- 2 ml of the solution prepared for identification test A gives the reaction of carbonates and bicarbonates (2.3.1).
- 1 ml of the solution prepared for identification test A gives reaction (b) of potassium (2.3.1).

TESTS

Solution S. Dissolve 10.0 g in 25 ml of *distilled water R*. Slowly add 14 ml of *hydrochloric acid R*. When the effervescence has ceased, boil for a few minutes. Allow to cool and dilute to 50 ml with *distilled water R*.

Appearance of solution. Solution S is not more opalescent than reference suspension II (2.2.1) and not more intensely coloured than reference solution Y_6 (2.2.2, *Method II*).

Chlorides (2.4.4). Dissolve 0.50 g in 10 ml of *water R*. Carefully add dropwise 1 ml of *nitric acid R*. Boil. Cool, add 5 ml of *dilute nitric acid R* and dilute to 15 ml with *water R*. The solution complies with the limit test for chlorides (100 ppm).

Sulphates (2.4.13). Dilute 7.50 ml of solution S to 15 ml with *distilled water R*. The solution complies with the limit test for sulphates (100 ppm).

Calcium (2.4.3). To 5 ml of solution S, add 1 ml of *concentrated ammonia R*. Boil. Cool. Dilute to 15 ml with *distilled water R*. The solution complies with the limit test for calcium (100 ppm).

Iron (2.4.9). 5 ml of solution S diluted to 10 ml with *water R* complies with the limit test for iron (10 ppm).

Heavy metals (2.4.8). Dilute 10 ml of solution S to 20 ml with *water R*. 12 ml of the solution complies with limit test A for heavy metals (20 ppm). Prepare the standard using *lead standard solution* (2 ppm Pb) *R*.

Loss on drying (2.2.32). Not more than 5.0 per cent, determined on 0.300 g by drying in an oven at 120 °C to 125 °C for 5 h.

ASSAY

Dissolve 0.500 g in 50 ml of *carbon dioxide-free water R*. Carry out a potentiometric titration (2.2.20), using 1 M *hydrochloric acid*. Read the volume added at the second point of inflexion.

1 ml of 1 M *hydrochloric acid* is equivalent to 69.1 mg of K_2CO_3 .

STORAGE

Store in an airtight container.

Bromates. To 10 ml of solution S add 1 ml of *starch solution R*, 0.1 ml of a 100 g/l solution of *potassium iodide R* and 0.25 ml of 0.5 M *sulphuric acid* and allow to stand protected from light for 5 min. No blue or violet colour develops.

Chlorides: maximum 0.6 per cent.

In a conical flask, dissolve 1.000 g in 20 ml of *dilute nitric acid R*. Add 5 ml of *strong hydrogen peroxide solution R* and heat on a water-bath until the solution is completely decolorised. Wash down the sides of the flask with a little *water R* and heat on a water-bath for 15 min. Allow to cool, dilute to 50 ml with *water R* and add 5.0 ml of 0.1 M *silver nitrate* and 1 ml of *dibutyl phthalate R*. Shake and titrate with 0.1 M *ammonium thiocyanate*, using 5 ml of *ferric ammonium sulphate solution R2* as indicator. Not more than 1.7 ml of 0.1 M *silver nitrate* is used. Note the volume of 0.1 M *silver nitrate* used (see Assay). Carry out a blank test.

Iodides. To 5 ml of solution S add 0.15 ml of *ferric chloride solution R1* and 2 ml of *methylene chloride R*. Shake and allow to separate. The lower layer is colourless (2.2.2, *Method I*).

Sulphates (2.4.13): maximum 100 ppm.

15 ml of solution S complies with the limit test for sulphates.

Iron (2.4.9): maximum 20 ppm.

5 ml of solution S diluted to 10 ml with *water R* complies with the limit test for iron.

Magnesium and alkaline-earth metals (2.4.7): maximum 200 ppm, calculated as Ca.

10.0 g complies with the limit test for magnesium and alkaline-earth metals. The volume of 0.01 M *sodium edetate* used does not exceed 5.0 ml.

Heavy metals (2.4.8): maximum 10 ppm.

12 ml of solution S complies with limit test A. Prepare the standard using *lead standard solution* (1 ppm Pb) *R*.

Loss on drying (2.2.32): maximum 1.0 per cent, determined on 1.000 g by drying in an oven at 100–105 °C for 3 h.

ASSAY

Dissolve 2.000 g in *water R* and dilute to 100.0 ml with the same solvent. To 10.0 ml of the solution add 50 ml of *water R*, 5 ml of *dilute nitric acid R*, 25.0 ml of 0.1 M *silver nitrate* and 2 ml of *dibutyl phthalate R*. Shake. Titrate with 0.1 M *ammonium thiocyanate*, using 2 ml of *ferric ammonium sulphate solution R2* as indicator and shaking vigorously towards the end-point.

1 ml of 0.1 M *silver nitrate* is equivalent to 11.90 mg of KBr.

Calculate the percentage content of KBr from the expression:

$$a - 3.357 b$$

a = percentage content of KBr and KCl obtained in the assay and calculated as KBr,

b = percentage content of Cl in the test for chlorides.

01/2005:0185

POTASSIUM CHLORIDE

Kalii chloridum

KCl

 M_r 74.6

DEFINITION

Potassium chloride contains not less than 99.0 per cent and not more than the equivalent of 100.5 per cent of KCl, calculated with reference to the dried substance.

CHARACTERS

A white, crystalline powder or colourless crystals, freely soluble in water, practically insoluble in ethanol.

IDENTIFICATION

- A. It gives the reactions of chlorides (2.3.1).
B. Solution S (see Tests) gives the reactions of potassium (2.3.1).

TESTS

Solution S. Dissolve 10.0 g in *carbon dioxide-free water R* prepared from *distilled water R* and dilute to 100 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and colourless (2.2.2, *Method II*).

Acidity or alkalinity. To 50 ml of solution S add 0.1 ml of *bromothymol blue solution R1*. Not more than 0.5 ml of 0.01 M *hydrochloric acid* or 0.01 M *sodium hydroxide* is required to change the colour of the indicator.

Bromides. Dilute 1.0 ml of solution S to 50 ml with *water R*. To 5.0 ml of the solution add 2.0 ml of *phenol red solution R2* and 1.0 ml of *chloramine solution R1* and mix immediately. After exactly 2 min add 0.15 ml of 0.1 M *sodium thiosulphate*, mix and dilute to 10.0 ml with *water R*. The absorbance (2.2.25) of the solution measured at 590 nm, using *water R* as the compensation liquid, is not greater than that of a standard prepared at the same time and in the same manner using 5 ml of a 3.0 mg/l solution of *potassium bromide R* (0.1 per cent).

Iodides. Moisten 5 g by the dropwise addition of a freshly prepared mixture of 0.15 ml of *sodium nitrite solution R*, 2 ml of 0.5 M *sulphuric acid*, 25 ml of *iodide-free starch solution R* and 25 ml of *water R*. After 5 min, examine in daylight. The substance shows no blue colour.

Sulphates (2.4.13). 5 ml of solution S diluted to 15 ml with *distilled water R* complies with the limit test for sulphates (300 ppm).

Barium. To 5 ml of solution S add 5 ml of *distilled water R* and 1 ml of *dilute sulphuric acid R*. After 15 min, any opalescence in the solution is not more intense than that in a mixture of 5 ml of solution S and 6 ml of *distilled water R*.

Heavy metals (2.4.8). 12 ml of solution S complies with limit test A for heavy metals (10 ppm). Prepare the standard using *lead standard solution (1 ppm Pb R)*.

Iron (2.4.9). 5 ml of solution S diluted to 10 ml with *water R* complies with the limit test for iron (20 ppm).

Magnesium and alkaline-earth metals (2.4.7). 10.0 g complies with the limit test for magnesium and alkaline-earth metals. The volume of 0.01 M *sodium edetate* used does not exceed 5.0 ml (200 ppm, calculated as Ca).

Sodium. If intended for use in the manufacture of parenteral dosage forms or haemodialysis solutions, it complies with the test for sodium. Not more than 0.1 per cent of Na, determined by atomic emission spectrometry (2.2.22).

Test solution. Dissolve 1.00 g of the substance to be examined in *water R* and dilute to 100.0 ml with the same solvent.

Reference solutions. Dissolve in *water R* 0.5084 g of *sodium chloride R*, previously dried at 100 °C to 105 °C for 3 h, and dilute to 1000.0 ml with the same solvent (200 µg of Na per millilitre). Dilute as required.

Measure the emission intensity at 589 nm.

Aluminium (2.4.17). If intended for use in the manufacture of haemodialysis solutions, it complies with the test for aluminium. Dissolve 4 g in 100 ml of *water R* and add 10 ml of *acetate buffer solution pH 6.0 R*. The solution complies with the limit test for aluminium (1 ppm). Use as the reference solution a mixture of 2 ml of *aluminium standard solution (2 ppm Al) R*, 10 ml of *acetate buffer solution pH 6.0 R* and 98 ml of *water R*. To prepare the blank use a mixture of 10 ml of *acetate buffer solution pH 6.0 R* and 100 ml of *water R*.

Loss on drying (2.2.32). Not more than 1.0 per cent, determined on 1.000 g by drying in an oven at 100-105 °C for 3 h.

ASSAY

Dissolve 1.300 g in *water R* and dilute to 100.0 ml with the same solvent. To 10.0 ml of the solution add 50 ml of *water R*, 5 ml of *dilute nitric acid R*, 25.0 ml of 0.1 M *silver nitrate* and 2 ml of *dibutyl phthalate R*. Shake. Titrate with 0.1 M *ammonium thiocyanate*, using 2 ml of *ferric ammonium sulphate solution R2* as indicator and shaking vigorously towards the end-point.

1 ml of 0.1 M *silver nitrate* is equivalent to 7.46 mg of KCl.

LABELLING

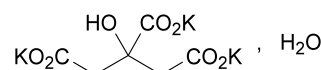
The label states:

- where applicable, that the substance is suitable for use in the manufacture of parenteral dosage forms,
- where applicable, that the substance is suitable for use in the manufacture of haemodialysis solutions.

01/2005:0400

POTASSIUM CITRATE

Kalii citras

 $\text{C}_6\text{H}_5\text{K}_3\text{O}_7 \cdot \text{H}_2\text{O}$ M_r 324.4

DEFINITION

Potassium citrate contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of tripotassium 2-hydroxypropane-1,2,3-tricarboxylate, calculated with reference to the anhydrous substance.

CHARACTERS

A white, granular powder or transparent crystals, hygroscopic, very soluble in water, practically insoluble in alcohol.

IDENTIFICATION

- A. To 1 ml of solution S (see Tests) add 4 ml of *water R*. The solution gives the reaction of citrates (2.3.1).

B. 0.5 ml of solution S gives reaction (b) of potassium (2.3.1).

01/2005:1140

TESTS

Solution S. Dissolve 10.0 g in *carbon dioxide-free water R* prepared from *distilled water R* and dilute to 100 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and colourless (2.2.2, *Method II*).

Acidity or alkalinity. To 10 ml of solution S add 0.1 ml of *phenolphthalein solution R*. Not more than 0.2 ml of 0.1 M *hydrochloric acid* or 0.1 M *sodium hydroxide* is required to change the colour of the indicator.

Readily carbonisable substances. To 0.20 g of the powdered substance to be examined add 10 ml of *sulphuric acid R* and heat in a water-bath at 90 ± 1 °C for 60 min. Cool rapidly. The solution is not more intensely coloured than reference solution Y₂ or GY₂ (2.2.2, *Method II*).

Chlorides (2.4.4). Dilute 10 ml of solution S to 15 ml with *water R*. The solution complies with the limit test for chlorides (50 ppm).

Oxalates. Dissolve 0.50 g in 4 ml of *water R*, add 3 ml of *hydrochloric acid R* and 1 g of granulated *zinc R* and heat on a water-bath for 1 min. Allow to stand for 2 min, decant the liquid into a test-tube containing 0.25 ml of a 10 g/l solution of *phenylhydrazine hydrochloride R* and heat to boiling. Cool rapidly, transfer to a graduated cylinder and add an equal volume of *hydrochloric acid R* and 0.25 ml of *potassium ferricyanide solution R*. Shake and allow to stand for 30 min. Any pink colour in the solution is not more intense than that in a standard prepared at the same time and in the same manner using 4 ml of a 0.05 g/l solution of *oxalic acid R* (300 ppm).

Sulphates (2.4.13). To 10 ml of solution S add 2 ml of *hydrochloric acid R1* and dilute to 15 ml with *distilled water R*. The solution complies with the limit test for sulphates (150 ppm).

Heavy metals (2.4.8). 12 ml of solution S complies with limit test A for heavy metals (10 ppm). Prepare the standard using *lead standard solution (1 ppm Pb) R*.

Sodium. Not more than 0.3 per cent of Na, determined by atomic emission spectrometry (2.2.22, *Method II*).

Test solution. To 10 ml of solution S add 1 ml of *dilute hydrochloric acid R* and dilute to 100 ml with *distilled water R*.

Reference solutions. Dilute as necessary with *distilled water R* a solution of *sodium chloride R* containing 1 mg of Na per millilitre.

Measure the emission intensity at 589 nm.

Water (2.5.12): 4.0 per cent to 7.0 per cent, determined on 0.500 g by the semi-micro determination of water. After adding the substance to be examined, stir for 15 min before titrating.

ASSAY

Dissolve 0.150 g in 20 ml of *anhydrous acetic acid R*, heating to about 50 °C. Allow to cool. Titrate with 0.1 M *perchloric acid* using 0.25 ml of *naphtholbenzein solution R* as indicator until a green colour is obtained.

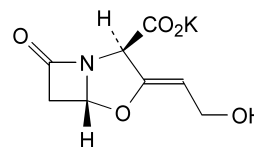
1 ml of 0.1 M *perchloric acid* is equivalent to 10.21 mg of C₈H₈K₃O₇.

STORAGE

Store in an airtight container.

POTASSIUM CLAVULANATE

Kalii clavulanas



C₈H₈KNO₅

M_r 237.3

DEFINITION

Potassium (2R,3Z,5R)-3-(2-hydroxyethylidene)-7-oxo-4-oxa-1-azabicyclo[3.2.0]heptane-2-carboxylate, the potassium salt of a substance produced by the growth of certain strains of *Streptomyces clavuligerus* or obtained by any other means. *Content*: 96.5 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

Appearance: white or almost white, crystalline powder, hygroscopic.

Solubility: freely soluble in water, slightly soluble in alcohol, very slightly soluble in acetone.

PRODUCTION

The method of production, extraction and purification are such that clavam-2-carboxylate is eliminated or present at a level not exceeding 0.01 per cent.

IDENTIFICATION

A. Infrared absorption spectrophotometry (2.2.24).

Comparison: *Ph. Eur. reference spectrum of potassium clavulanate*.

B. It gives reaction (b) of potassium (2.3.1).

TESTS

Solution S. Dissolve 0.400 g in *carbon dioxide-free water R* and dilute to 20.0 ml with the same solvent.

pH (2.2.3): 5.5 to 8.0.

Dilute 5 ml of solution S to 10 ml with *carbon dioxide-free water R*.

Specific optical rotation (2.2.7): + 53 to + 63 (anhydrous substance), determined on solution S.

Absorbance (2.2.25): maximum 0.40 at 278 nm.

Dissolve 50.0 mg in 0.1 M *phosphate buffer solution pH 7.0 R* and dilute to 50.0 ml with the same solution. Measure immediately the absorbance of this solution.

Related substances. Liquid chromatography (2.2.29). *Prepare the solutions immediately before use.*

Test solution. Dissolve 0.250 g of the substance to be examined in mobile phase A and dilute to 25.0 ml with mobile phase A.

Reference solution (a). Dilute 1.0 ml of the test solution to 100.0 ml with mobile phase A.

Reference solution (b). Dissolve 10 mg of *lithium clavulanate CRS* and 10 mg of *amoxicillin trihydrate CRS* in mobile phase A and dilute to 100 ml with mobile phase A.

Column:

- *size*: $l = 0.10$ m, $\varnothing = 4.6$ mm,
- *stationary phase*: octadecylsilyl silica gel for chromatography R (5 μ m),
- *temperature*: 40 °C.

Mobile phase:

- **mobile phase A:** a 7.8 g/l solution of *sodium dihydrogen phosphate R* adjusted to pH 4.0 with *phosphoric acid R* and filtered through a 0.5 µm filter,
- **mobile phase B:** a mixture of equal volumes of mobile phase A and *methanol R*,

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)
0 - 4	100	0
4 - 15	100 → 50	0 → 50
15 - 18	50	50
18 - 24	50 → 100	50 → 0
24 - 39	100	0

Flow rate: 1 ml/min.

Detection: spectrophotometer at 230 nm.

Injection: 20 µl.

System suitability: reference solution (b):

- **resolution:** minimum 13 between the first peak (clavulanate) and the second peak (amoxicillin).

Limits:

- **any impurity:** not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (1.0 per cent),
- **total:** not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (2.0 per cent),
- **disregard limit:** 0.05 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

Aliphatic amines. Gas chromatography (2.2.28).

The method shown below can be used to determine the following aliphatic amines: 1,1-dimethylethylamine; diethylamine; *N,N,N',N'*-tetramethylethylenediamine; 1,1,3,3-tetramethylbutylamine; *N,N'*-diisopropylethylenediamine; 2,2'-oxydi(*N,N*)dimethylethylamine.

Internal standard solution: dissolve 50 µl of 3-methylpentan-2-one *R* in *water R* and dilute to 100.0 ml with the same solvent.

Test solution. Weigh 1.00 g of the substance to be examined into a centrifuge tube. Add 5.0 ml of the internal standard solution, 5.0 ml of *dilute sodium hydroxide solution R*, 10.0 ml of *water R*, 5.0 ml of 2-methylpropanol *R* and 5 g of *sodium chloride R*. Shake vigorously for 1 min. Centrifuge to separate the layers.

Reference solution. Dissolve 80.0 mg of each of the following amines 1,1-dimethylethylamine *R*; diethylamine *R*; tetramethylethylenediamine *R*; 1,1,3,3-tetramethylbutylamine *R*; *N,N'*-diisopropylethylenediamine *R* and 2,2'-oxybis(*N,N*-dimethylethylamine) *R* in *dilute hydrochloric acid R* and dilute to 200.0 ml with the same acid. Introduce 5.0 ml of this solution into a centrifuge tube. Add 5.0 ml of the internal standard solution, 10.0 ml of *dilute sodium hydroxide solution R*, 5.0 ml of 2-methylpropanol *R* and 5 g of *sodium chloride R*. Shake vigorously for 1 min. Centrifuge to separate the layers.

Column:

- **material:** fused silica,
- **size:** *l* = 50 m, Ø = 0.53 mm,
- **stationary phase:** poly(dimethyl)(diphenyl)siloxane *R* (film thickness 5 µm).

Carrier gas: helium for chromatography *R*.

Flow rate: 8 ml/min.

Split ratio: 1:10.

Temperature:

	Time (min)	Temperature (°C)
Column	0 - 7	35
	7 - 10.8	35 → 150
	10.8 - 25.8	150
Injection port		200
Detector		250

Detection: flame ionisation.

Injection: 1 µl of the upper layers obtained from test solution and reference solution.

Relative retention with reference to 3-methylpentan-2-one (retention time = about 11.4 min): impurity H = about 0.55; impurity I = about 0.76; impurity J = about 1.07; impurity K = about 1.13; impurity L = about 1.33; impurity M = about 1.57.

Limit:

- **aliphatic amines:** maximum 0.2 per cent.

2-Ethylhexanoic acid (2.4.28): maximum 0.8 per cent.

Water (2.5.12): maximum 0.5 per cent, determined on 1.00 g.

Bacterial endotoxins (2.6.14): less than 0.03 IU/mg if intended for use in the manufacture of parenteral dosage forms without a further appropriate procedure for the removal of bacterial endotoxins.

ASSAY

Liquid chromatography (2.2.29). *Prepare the solutions immediately before use.*

Test solution. Dissolve 50.0 mg of the substance to be examined in a 4.1 g/l solution of *sodium acetate R* previously adjusted to pH 6.0 with *glacial acetic acid R*, and dilute to 50.0 ml with the same solution.

Reference solution (a). Dissolve 50.0 mg of *lithium clavulanate CRS* in a 4.1 g/l solution of *sodium acetate R* previously adjusted to pH 6.0 with *glacial acetic acid R* and dilute to 50.0 ml with the same solution.

Reference solution (b). Dissolve 50.0 mg of *lithium clavulanate CRS* and 50.0 mg of *amoxicillin trihydrate CRS* in a 4.1 g/l solution of *sodium acetate R* previously adjusted to pH 6.0 with *glacial acetic acid R* and dilute to 50.0 ml with the same solution.

Column:

- **size:** *l* = 0.3 m, Ø = 4.6 mm,
- **stationary phase:** octadecylsilyl silica gel for chromatography *R* (5 µm).

Mobile phase: mix 5 volumes of *methanol R1* and 95 volumes of a 15 g/l solution of *sodium dihydrogen phosphate R* previously adjusted to pH 4.0 with *dilute phosphoric acid R*.

Flow rate: 1 ml/min.

Detection: spectrophotometer at 230 nm.

Injection: 10 µl.

System suitability: reference solution (b):

- **resolution:** minimum 3.5 between the first peak (clavulanate) and the second peak (amoxicillin).

1 mg of clavulanate (C₈H₉NO₅) is equivalent to 1.191 mg of C₈H₈KNO₅.

STORAGE

In an airtight container, at a temperature of 2 °C to 8 °C. If the substance is sterile, store in a sterile, airtight, tamper-proof container.

LABELLING

The label states, where applicable, that the substance is free from bacterial endotoxins.

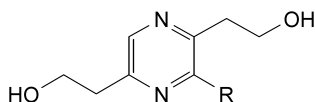
IMPURITIES

Specified impurities: A, B, C, D, G, H, I, J, K, L, M.

Other detectable impurities: E, F.

By liquid chromatography: A, B, C, D, E, F, G.

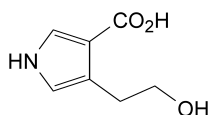
By gas chromatography: H, I, J, K, L, M.



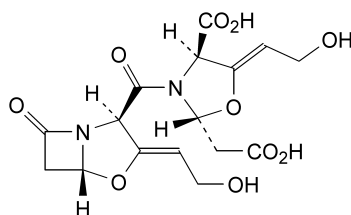
A. R = H: 2,2'-(pyrazine-2,5-diyl)diethanol,

B. R = CH₂-CH₂-CO₂H: 3-[3,6-bis(2-hydroxyethyl)pyrazin-2-yl]propanoic acid,

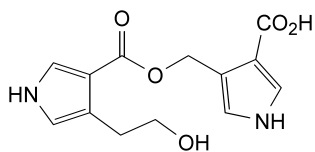
C. R = CH₂-CH₃: 2,2'-(3-ethylpyrazine-2,5-diyl)diethanol,



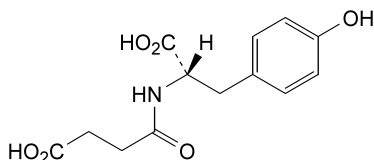
D. 4-(2-hydroxyethyl)pyrrole-3-carboxylic acid,



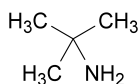
E. (2*R*,4*R*,5*Z*)-2-(carboxymethyl)-5-(2-hydroxyethylidene)-3-[[[(2*R*,3*Z*,5*R*)-3-(2-hydroxyethylidene)-7-oxo-4-oxa-1-azabicyclo[3.2.0]hept-2-yl]carbonyl]oxazolidine-4-carboxylic acid,



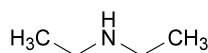
F. 4-[[[4-(2-hydroxyethyl)-1*H*-pyrrol-3-yl]carbonyl]oxy]methyl]-1*H*-pyrrole-3-carboxylic acid,



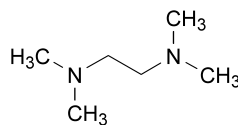
G. 4-[[[(1*S*)-1-carboxy-2-(4-hydroxyphenyl)ethyl]amino]-4-oxobutanoic acid (*N*-succinyltyrosine),



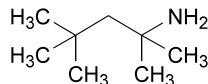
H. 2-amino-2-methylpropane (1,1-dimethylethylamine),



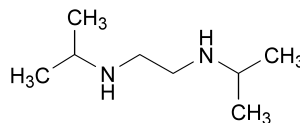
I. diethylamine,



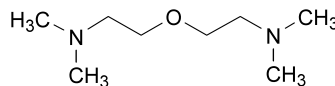
J. 1,2-bis(dimethylamino)ethane (*N,N,N',N'*-tetramethylethylenediamine),



K. 2-amino-2,4,4-trimethylpentane (1,1,3,3-tetramethylbutylamine),



L. *N,N'*-bis(1-methylethyl)-1,2-ethanediamine (*N,N'*-diisopropylethylenediamine),

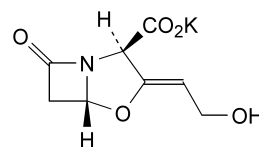


M. bis(2-dimethylamino)ethyl ether [2,2'-oxybis(*N,N*-dimethylethylamine)].

01/2005:1653
corrected

POTASSIUM CLAVULANATE, DILUTED

Kalii clavulanate dilutus



C₈H₈KNO₅

M_r 237.3

DEFINITION

Dry mixture of *Potassium clavulanate* (1140) and *Cellulose, microcrystalline* (0316) or *Silica, colloidal anhydrous* (0434) or *Silica, colloidal hydrated* (0738).

Content: 91.2 per cent to 107.1 per cent of the content of potassium clavulanate stated on the label.

CHARACTERS

Appearance of diluted potassium clavulanate: white or almost white powder, hygroscopic.

Solubility of potassium clavulanate: freely soluble in water, slightly soluble in alcohol, very slightly soluble in acetone.

The solubility of the diluted product depends on the diluent and its concentration.

IDENTIFICATION

A. Examine the chromatograms obtained in the assay.

Results: the principal peak in the chromatogram obtained with the test solution is similar in retention time to the principal peak in the chromatogram obtained with reference solution (a).

- B. It gives reaction (b) of potassium (2.3.1).
- C. Depending on the diluent used, carry out the corresponding identification test (a) or (b).
- (a) A quantity of the substance to be examined, corresponding to 20 mg of cellulose, when placed on a watch-glass and dispersed in 4 ml of *iodinated zinc chloride solution R*, becomes violet-blue.
- (b) It gives the reaction of silicates (2.3.1).

TESTS

pH (2.2.3): 4.8 to 8.0.

Suspend a quantity of the substance to be examined corresponding to 0.200 g of potassium clavulanate in 20 ml of *carbon dioxide-free water R*.

Absorbance (2.2.25): maximum 0.40 measured immediately at 278 nm.

Disperse a quantity of the substance to be examined corresponding to 50.0 mg of potassium clavulanate in 10 ml of *0.1 M phosphate buffer solution pH 7.0 R*, dilute to 50.0 ml with the same buffer solution and filter.

Related substances. Liquid chromatography (2.2.29). *Prepare the solutions immediately before use.*

Test solution. Disperse a quantity of the substance to be examined corresponding to 0.250 g of potassium clavulanate in 5 ml of mobile phase A, dilute to 25.0 ml with mobile phase A and filter.

Reference solution (a). Dilute 1.0 ml of the test solution to 100.0 ml with mobile phase A.

Reference solution (b). Dissolve 10 mg of *amoxicillin trihydrate CRS* in 1 ml of the test solution and dilute to 100 ml with mobile phase A.

Column:

- *size*: $l = 0.10$ m, $\varnothing = 4.6$ mm,
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 μ m),
- *temperature*: 40 °C.

Mobile phase:

- *mobile phase A*: 7.8 g/l solution of *sodium dihydrogen phosphate R* adjusted to pH 4.0 with *dilute phosphoric acid R*,
- *mobile phase B*: mixture of equal volumes of mobile phase A and *methanol R*,

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)
0 - 4	100	0
4 - 15	100 → 50	0 → 50
15 - 18	50	50
18 - 24	50 → 100	50 → 0
24 - 39	100	0

Flow rate: 1 ml/min.

Detection: spectrophotometer at 230 nm.

Injection: 20 μ l.

System suitability: reference solution (b):

- *resolution*: minimum of 13 between the peak due to clavulanate (1st peak) and the peak due to amoxicillin (2nd peak).

Limits:

- *any impurity*: not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (1.0 per cent),

- *total*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (2.0 per cent),
- *disregard limit*: 0.05 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

Water (2.5.12): maximum 2.5 per cent, determined on 1.000 g.

ASSAY

Liquid chromatography (2.2.29). *Prepare the solutions immediately before use.*

Test solution. Disperse a quantity of the substance to be examined corresponding to 50.0 mg of potassium clavulanate in a 4.1 g/l solution of *sodium acetate R* previously adjusted to pH 6.0 with *glacial acetic acid R*, dilute to 50.0 ml with the same solution and filter.

Reference solution (a). Dissolve 50.0 mg of *lithium clavulanate CRS* in a 4.1 g/l solution of *sodium acetate R* previously adjusted to pH 6.0 with *glacial acetic acid R* and dilute to 50.0 ml with the same solution.

Reference solution (b). Dissolve 10 mg of *amoxicillin trihydrate CRS* in 10 ml of reference solution (a).

Column:

- *size*: $l = 0.3$ m, $\varnothing = 4.6$ mm,
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (10 μ m).

Mobile phase: mix 5 volumes of *methanol R1* and 95 volumes of a 15 g/l solution of *sodium dihydrogen phosphate R* previously adjusted to pH 4.0 with *dilute phosphoric acid R*.

Flow rate: 1 ml/min.

Detection: spectrophotometer at 230 nm.

Injection: 10 μ l.

System suitability: reference solution (b):

- *resolution*: minimum 3.5 between the peak due to clavulanate (1st peak) and the peak due to amoxicillin (2nd peak).

1 mg of $C_8H_9NO_5$ is equivalent to 1.191 mg of $C_8H_8KNO_5$.

STORAGE

In an airtight container.

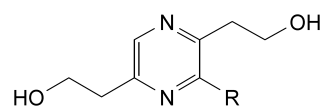
LABELLING

The label states the *m/m* percentage content of potassium clavulanate and the diluent used to prepare the mixture.

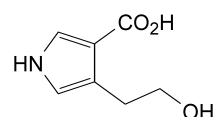
IMPURITIES

Specified impurities: A, B, C, D, G.

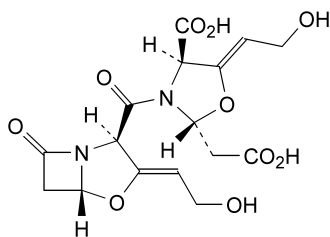
Other detectable impurities: E, F.



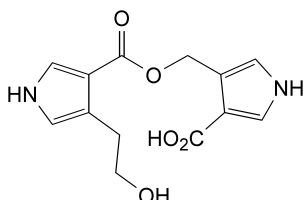
- A. R = H: 2,2'-(pyrazine-2,5-diyl)diethanol,
- B. R = $CH_2CH_2CO_2H$: 3-[3,6-bis(2-hydroxyethyl)pyrazin-2-yl]propanoic acid,
- C. R = CH_2CH_3 : 2,2'-(3-ethylpyrazine-2,5-diyl)diethanol,



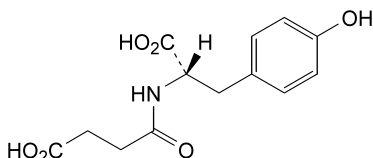
- D. 4-(2-hydroxyethyl)-1H-pyrrole-3-carboxylic acid,



- E. (2*R*,4*R*,5*Z*)-2-(carboxymethyl)-5-(2-hydroxyethylidene)-3-[[[(2*R*,3*Z*,5*R*)-3-(2-hydroxyethylidene)-7-oxo-4-oxa-1-azabicyclo[3.2.0]hept-2-yl]carbonyl]oxazolidine-4-carboxylic acid,



- F. 4-[[[4-(2-hydroxyethyl)-1*H*-pyrrol-3-yl]carbonyl]oxy]methyl]-1*H*-pyrrole-3-carboxylic acid,



- G. 4-[[[(1*S*)-1-carboxy-2-(4-hydroxyphenyl)ethyl]amino]-4-oxobutanoic acid (*N*-succinyltyrosine).

01/2005:0920

POTASSIUM DIHYDROGEN PHOSPHATE

Kalii dihydrogenophosphas

KH₂PO₄*M*_r 136.1

DEFINITION

Potassium dihydrogen phosphate contains not less than 98.0 per cent and not more than the equivalent of 100.5 per cent of KH₂PO₄, calculated with reference to the dried substance.

CHARACTERS

A white, crystalline powder or colourless crystals, freely soluble in water, practically insoluble in alcohol.

IDENTIFICATION

- A. Solution S (see Tests) is faintly acid (2.2.4).
B. Solution S gives reaction (b) of phosphates (2.3.1).
C. 0.5 ml of solution S gives reaction (b) of potassium (2.3.1).

TESTS

Solution S. Dissolve 10.0 g in *carbon dioxide-free water R* prepared from *distilled water R* and dilute to 100 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and colourless (2.2.2, *Method II*).

pH (2.2.3). To 5 ml of solution S add 5 ml of *carbon dioxide-free water R*. The pH of the solution is 4.2 to 4.5.

Reducing substances. To 5 ml of solution S add 5 ml of *dilute sulphuric acid R* and 0.25 ml of 0.02 *M* *potassium permanganate*. Heat on a water-bath for 5 min. The colour of the permanganate is not completely discharged.

Chlorides (2.4.4). Dilute 2.5 ml of solution S to 15 ml with *water R*. The solution complies with the limit test for chlorides (200 ppm).

Sulphates (2.4.13). To 5 ml of solution S add 0.5 ml of *hydrochloric acid R* and dilute to 15 ml with *distilled water R*. The solution complies with the limit test for sulphates (300 ppm).

Arsenic (2.4.2). 0.5 g complies with limit test A for arsenic (2 ppm).

Iron (2.4.9). 10 ml of solution S complies with the limit test for iron (10 ppm).

Sodium. If intended for use in the manufacture of parenteral dosage forms, it complies with the test for sodium. Not more than 0.1 per cent of Na, determined by atomic emission spectrometry (2.2.22, *Method I*).

Test solution. Dissolve 1.00 g of the substance to be examined in *water R* and dilute to 100.0 ml with the same solvent.

Reference solution. Dissolve in *water R* 0.5084 g of *sodium chloride R*, previously dried at 100 °C to 105 °C for 3 h, and dilute to 1000.0 ml with the same solvent (200 µg of Na per millilitre). Dilute as necessary.

Measure the emission intensity at 589 nm.

Heavy metals (2.4.8). 12 ml of solution S complies with limit test A for heavy metals (10 ppm). Prepare the standard using *lead standard solution* (1 ppm Pb) *R*.

Loss on drying (2.2.32). Not more than 2.0 per cent, determined on 1.000 g by drying in an oven at 125 °C to 130 °C.

ASSAY

Dissolve 1.000 g in 50 ml of *carbon dioxide-free water R*. Titrate with carbonate-free 1 *M* *sodium hydroxide*, determining the end-point potentiometrically (2.2.20).

1 ml of 1 *M* *sodium hydroxide* is equivalent to 0.1361 g of KH₂PO₄.

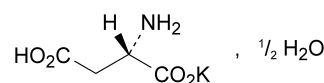
LABELLING

The label states, where applicable, that the substance is suitable for use in the manufacture of parenteral dosage forms.

01/2005:2076

POTASSIUM HYDROGEN ASPARTATE HEMIHYDRATE

Kalii hydrogenoaspartas hemihydricus

C₄H₆KNO₄ · 1/2 H₂O*M*_r 180.2

DEFINITION

Potassium hydrogen (2*S*)-2-aminobutanedioate hemihydrate.

Content: 99.0 per cent to 101.0 per cent (anhydrous substance).

CHARACTERS

Appearance: white powder, or white crystalline powder or colourless crystals.

Solubility: very soluble in water, practically insoluble in alcohol and in methylene chloride.

IDENTIFICATION

A. It complies with the test for specific optical rotation (see Tests).

B. Examine the chromatograms obtained in the test for ninhydrin-positive substances.

Results: the principal spot in the chromatogram obtained with test solution (b) is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).

C. It gives reaction (b) of potassium (2.3.1).

TESTS

Solution S. Dissolve 2.5 g in *carbon dioxide-free water R* prepared from *distilled water R* and dilute to 100 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and colourless (2.2.2, *Method II*).

pH (2.2.3): 6.0 to 7.5 for solution S.

Specific optical rotation (2.2.7): + 18.0 to + 20.5 (anhydrous substance).

Dissolve 0.50 g in a mixture of equal volumes of *hydrochloric acid R* and *water R* and dilute to 25.0 ml with the same mixture of solvents.

Ninhydrin-positive substances. Thin-layer chromatography (2.2.27).

Test solution (a). Solution S.

Test solution (b). Dilute 1.0 ml of solution S to 10.0 ml with *water R*.

Reference solution (a). Dissolve 25 mg of *potassium hydrogen aspartate hemihydrate CRS* in *water R* and dilute to 10 ml with the same solvent.

Reference solution (b). Dilute 1.0 ml of test solution (b) to 20.0 ml with *water R*.

Reference solution (c). Dissolve 10 mg of *glutamic acid CRS* and 10 mg of the substance to be examined in *water R* and dilute to 25 ml with the same solvent.

Plate: *TLC silica gel plate R*.

Mobile phase: *glacial acetic acid R*, *water R*, *butanol R* (20:20:60 V/V/V).

Application: 5 µl.

Development: over 2/3 of the plate.

Drying: in air.

Detection: spray with *ninhydrin solution R* and heat at 100-105 °C for 15 min.

System suitability: reference solution (c):

- the chromatogram shows 2 clearly separated principal spots.

Limits: test solution (a):

- *any impurity:* any spot, apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.5 per cent).

Chlorides (2.4.4): maximum 200 ppm.

To 10 ml of solution S add 5 ml of *water R*.

Sulphates (2.4.13): maximum 500 ppm.

To 12 ml of solution S add 3 ml of *distilled water R*.

Ammonium (2.4.1, *Method B*): maximum 200 ppm, determined on 50 mg.

Prepare the standard using 0.1 ml of *ammonium standard solution* (100 ppm NH_4) *R*.

Iron (2.4.9): maximum 30 ppm.

In a separating funnel, dissolve 0.33 g in 10 ml of *dilute hydrochloric acid R*. Shake with 3 quantities, each of 10 ml, of *methyl isobutyl ketone R1*, shaking for 3 min each time. To the combined organic layers add 10 ml of *water R* and shake for 3 min. The aqueous layer complies with the limit test for iron.

Heavy metals (2.4.8): maximum 10 ppm.

Dissolve 2.0 g in *water R* and dilute to 20 ml with the same solvent. 12 ml of the solution complies with limit test A. Prepare the standard using *lead standard solution* (1 ppm Pb) *R*.

Water (2.5.12): 4.0 per cent to 6.0 per cent, determined on 0.200 g.

Dissolve the substance to be examined in 10 ml of *formamide R1* and add 10 ml of *anhydrous methanol R*.

ASSAY

Dissolve 70.0 mg in 5 ml of *anhydrous formic acid R*, add 50 ml of *anhydrous acetic acid R*. Titrate with 0.1 M *perchloric acid*, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *perchloric acid* is equivalent to 8.56 mg of $\text{C}_4\text{H}_6\text{KNO}_4$.

01/2005:1141

POTASSIUM HYDROGEN CARBONATE

Kalii hydrogenocarbonas

 KHCO_3
 M_r 100.1

DEFINITION

Potassium hydrogen carbonate contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of KHCO_3 .

CHARACTERS

A white, crystalline powder or colourless crystals, freely soluble in water, practically insoluble in alcohol. When heated in the dry state or in solution, it is gradually converted to potassium carbonate.

IDENTIFICATION

A. To 5 ml of solution S (see Tests) add 0.1 ml of *phenolphthalein solution R*. A pale pink colour is produced. Heat; gas is evolved and the colour becomes red.

B. It gives the reaction of carbonates and bicarbonates (2.3.1).

C. 1 ml of solution S gives reaction (b) of potassium (2.3.1).

TESTS

Solution S. Dissolve 5.0 g in 90 ml of *carbon dioxide-free water R* prepared from *distilled water R* and dilute to 100 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and colourless (2.2.2, *Method II*).

Carbonates. The pH (2.2.3) of freshly prepared solution S is not more than 8.6.

Chlorides (2.4.4). Dilute 7 ml of solution S to 15 ml with *dilute nitric acid R*. The solution complies with the limit test for chlorides (150 ppm).

Sulphates (2.4.13). Dilute 10 ml of solution S to 15 ml with *acetic acid R*. The solution complies with the limit test for sulphates (150 ppm). Prepare the standard using a mixture of 7.5 ml of *sulphate standard solution (10 ppm SO₄) R* and 7.5 ml of *distilled water R*.

Ammonium (2.4.1). 10 ml of solution S diluted to 15 ml with *water R* complies with the limit test for ammonium (20 ppm). Prepare the standard using a mixture of 5 ml of *water R* and 10 ml of *ammonium standard solution (1 ppm NH₄) R*.

Calcium (2.4.3). Dilute 10 ml of solution S to 15 ml with *acetic acid R*. The solution complies with the limit test for calcium (100 ppm). Prepare the standard using 5 ml of *calcium standard solution (10 ppm Ca) R* and 10 ml of *distilled water R*.

Heavy metals (2.4.8). Dissolve 2.0 g in a mixture of 2 ml of *hydrochloric acid R* and 18 ml of *water R*. 12 ml of the solution complies with limit test A for heavy metals (10 ppm). Prepare the standard using *lead standard solution (1 ppm Pb) R*.

Iron (2.4.9). 10 ml of solution S complies with the limit test for iron (20 ppm).

Sodium. Not more than 0.5 per cent of Na, determined by atomic emission spectrometry (2.2.22, *Method II*).

Test solution. Dissolve 1.00 g in *water R* and dilute to 100.0 ml with the same solvent.

Reference solutions. Prepare the reference solutions using *sodium standard solution (200 ppm Na) R*, diluted as necessary with *water R*.

Measure the emission intensity at 589 nm.

ASSAY

Dissolve 0.800 g in 50 ml of *carbon dioxide-free water R*. Add 0.1 ml of *methyl orange solution R*. Titrate with 1 M *hydrochloric acid* until the yellow colour begins to change to yellowish-pink. Heat cautiously and boil for at least 2 min. The solution becomes yellow. Cool and titrate until a yellowish-red colour is obtained.

1 ml of 1 M *hydrochloric acid* is equivalent to 0.1001 g of KHCO₃.

CHARACTERS

Appearance: white, crystalline powder or colourless crystals.

Solubility: slightly soluble in water, practically insoluble in alcohol. It dissolves in dilute solutions of mineral acids and alkali hydroxides.

IDENTIFICATION

- It complies with the test for specific optical rotation (see Tests).
- Suspend 0.5 g in 50 ml of *water R* and boil until dissolution is complete. Allow to cool (solution A). To 5 ml of solution A, add 0.1 ml of *methyl red solution R*. The solution is red.
- Solution A gives reaction (a) of tartrates (2.3.1).
- Solution A gives reaction (b) of potassium (2.3.1).

TESTS

Specific optical rotation (2.2.7): + 8.0 to + 9.2 (dried substance).

Dissolve 2.50 g in 20 ml of 1 M *hydrochloric acid* with heating. Allow to cool. Dilute to 25.0 ml with *water R*.

Oxalic acid: maximum 500 ppm.

Dissolve 0.43 g in 4 ml of *water R*. Add 3 ml of *hydrochloric acid R* and 1 g of *zinc R* in granules and boil for 1 min. Allow to stand for 2 min. Collect the liquid in a test-tube containing 0.25 ml of a 10 g/l solution of *phenylhydrazine hydrochloride R* and heat to boiling. Cool rapidly, transfer to a graduated cylinder and add an equal volume of *hydrochloric acid R* and 0.25 ml of a 50 g/l solution of *potassium ferricyanide R*. Shake and allow to stand for 30 min. Any pink colour in the solution is not more intense than that in a standard prepared at the same time in the same manner using a mixture of 1 ml of *water R* and 3 ml of a 0.1 g/l solution of *oxalic acid R*.

Chlorides (2.4.4): maximum 500 ppm.

Dissolve 1.0 g with heating in a mixture of 3 ml of *dilute nitric acid R* and 50 ml of *water R*. Dilute to 100 ml with *water R*. Dilute 10 ml of the solution to 15 ml with *water R*. The solution complies with the limit test for chlorides.

Sulphates (2.4.13): maximum 500 ppm.

Suspend 0.30 g in 3.0 ml of *dilute hydrochloric acid R* and dilute to 15 ml with *distilled water R*. Heat until dissolution is complete. The solution complies with the limit test for sulphates.

Barium. Suspend 0.50 g in a mixture of 1.5 ml of *dilute hydrochloric acid R* and 8.5 ml of *water R*. Heat until dissolution is complete. Allow to cool. Add 1 ml of *dilute sulphuric acid R*. The solution remains clear (2.2.1) on standing for 15 min.

Heavy metals (2.4.8): maximum 10 ppm.

2.0 g complies with limit test C. Prepare the standard using 2 ml of *lead standard solution (10 ppm Pb) R*.

Loss on drying (2.2.32): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 100-105 °C.

ASSAY

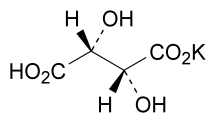
Dissolve 0.170 g in 100 ml of *water R* at 100 °C. Titrate the hot solution with 0.1 M *sodium hydroxide*, using 0.3 ml of *phenolphthalein solution R* as indicator.

1 ml of 0.1 M *sodium hydroxide* is equivalent to 18.82 mg of C₄H₅KO₆.

01/2005:1984

POTASSIUM HYDROGEN TARTRATE

Kalii hydrogenotartras



C₄H₅KO₆

M_r 188.2

DEFINITION

Potassium hydrogen (2*R*,3*R*)-2,3-dihydroxybutane-1,4-dioate.

Content: 99.5 per cent to 100.5 per cent (dried substance).

01/2005:0840

POTASSIUM HYDROXIDE

Kalii hydroxidum

KOH

 M_r 56.11

DEFINITION

Potassium hydroxide contains not less than 85.0 per cent and not more than the equivalent of 100.5 per cent of total alkali, calculated as KOH.

CHARACTERS

White, crystalline, hard masses, supplied as sticks, pellets or irregularly shaped pieces, deliquescent in air, hygroscopic, absorbing carbon dioxide, very soluble in water, freely soluble in alcohol.

IDENTIFICATION

- A. Dissolve 0.1 g in 10 ml of *water R* (this solution is used for identification test B). Dilute 1 ml of the solution to 100 ml with *water R*. The pH (2.2.3) of this solution is not less than 10.5.
- B. 1 ml of the initial solution prepared in identification test A gives reaction (b) of potassium (2.3.1).

TESTS

Solution S1. Dissolve 2.5 g in 10 ml of *water R*. Carefully add 2 ml of *nitric acid R*, with cooling, and dilute to 25 ml with *dilute nitric acid R*.

Solution S2. Dissolve 10 g in 15 ml of *distilled water R*. Carefully add 12 ml of *hydrochloric acid R*, with cooling, and dilute to 50 ml with *dilute hydrochloric acid R*.

Appearance of solution. Dissolve 5 g in *carbon dioxide-free water R* and dilute to 50 ml with the same solvent. The solution is clear (2.2.1) and colourless (2.2.2, *Method II*).

Carbonates. Not more than 2.0 per cent, calculated as K_2CO_3 , as determined in the assay.

Chlorides (2.4.4). Dilute 10 ml of solution S1 to 15 ml with *water R*. The solution complies with the limit test for chlorides (50 ppm).

Phosphates (2.4.11). Dilute 5 ml of solution S1 to 100 ml with *water R*. The solution complies with the limit test for phosphates (20 ppm).

Sulphates (2.4.13). 15 ml of solution S2 complies with the limit test for sulphates (50 ppm).

Aluminium (2.4.17). If intended for use in the manufacture of haemodialysis solutions, it complies with the test for aluminium. Dissolve 20 g in 100 ml of *water R* and add 10 ml of *acetate buffer solution pH 6.0 R*. The solution complies with the limit test for aluminium (0.2 ppm). Use as the reference solution a mixture of 2 ml of *aluminium standard solution (2 ppm Al) R*, 10 ml of *acetate buffer solution pH 6.0 R* and 98 ml of *water R*. To prepare the blank use a mixture of 10 ml of *acetate buffer solution pH 6.0 R* and 100 ml of *water R*.

Iron (2.4.9). Dilute 5 ml of solution S2 to 10 ml with *water R*. The solution complies with the limit test for iron (10 ppm).

Sodium. Not more than 1.0 per cent of Na, determined by atomic absorption spectrometry (2.2.23, *Method II*).

Test solution. Dissolve 1.00 g in 50 ml of *water R*, add 5 ml of *sulphuric acid R* and dilute to 100.0 ml with *water R*. Dilute 1.0 ml of the solution to 10.0 ml with *water R*.

Reference solutions. Prepare the reference solutions using *sodium standard solution (200 ppm Na) R*, diluted as necessary with *water R*.

Measure the absorbance at 589 nm using a sodium hollow-cathode lamp as a source of radiation and an air-acetylene flame.

Heavy metals (2.4.8). Dilute 10 ml of solution S2 to 20 ml with *water R*. 12 ml of the solution complies with limit test A for heavy metals (10 ppm). Prepare the standard using *lead standard solution (1 ppm Pb) R*.

ASSAY

Dissolve 2.000 g in 25 ml of *carbon-dioxide free water R*. Add 25 ml of freshly prepared *barium chloride solution R1* and 0.3 ml of *phenolphthalein solution R*. Add slowly with shaking 25.0 ml of *1 M hydrochloric acid* and continue the titration with *1 M hydrochloric acid* until the colour changes from pink to colourless. Add 0.3 ml of *bromophenol blue solution R* and continue the titration with *1 M hydrochloric acid* until the colour changes from violet-blue to yellow.

1 ml of *1 M hydrochloric acid* used in the second part of the titration is equivalent to 69.11 mg of K_2CO_3 .

1 ml of *1 M hydrochloric acid* used in the combined titrations is equivalent to 56.11 mg of total alkali, calculated as KOH.

STORAGE

Store in an airtight, non-metallic container.

LABELLING

The label states, where applicable, that the substance is suitable for use in the manufacture of haemodialysis solutions.

01/2005:0186

POTASSIUM IODIDE

Kalii iodidum

KI

166.0

DEFINITION

Potassium iodide contains not less than 99.0 per cent and not more than the equivalent of 100.5 per cent of KI, calculated with reference to the dried substance.

CHARACTERS

A white powder or colourless crystals, very soluble in water, freely soluble in glycerol, soluble in alcohol.

IDENTIFICATION

- A. Solution S (see Tests) gives the reactions of iodides (2.3.1).
- B. Solution S gives the reactions of potassium (2.3.1).

TESTS

Solution S. Dissolve 10.0 g in *carbon dioxide-free water R* prepared from *distilled water R* and dilute to 100 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and colourless (2.2.2, *Method II*).

Alkalinity. To 12.5 ml of solution S add 0.1 ml of *bromothymol blue solution R1*. Not more than 0.5 ml of *0.01 M hydrochloric acid* is required to change the colour of the indicator.

Iodates. To 10 ml of solution S add 0.25 ml of *iodide-free starch solution R* and 0.2 ml of *dilute sulphuric acid R* and allow to stand protected from light for 2 min. No blue colour develops.

Sulphates (2.4.13). 10 ml of solution S diluted to 15 ml with *distilled water R* complies with the limit test for sulphates (150 ppm).

Thiosulphates. To 10 ml of solution S add 0.1 ml of *starch solution R* and 0.1 ml of 0.005 M *iodine*. A blue colour is produced.

Heavy metals (2.4.8). 12 ml of solution S complies with limit test A for heavy metals (10 ppm). Prepare the standard using *lead standard solution (1 ppm Pb) R*.

Iron (2.4.9). 5 ml of solution S diluted to 10 ml with *water R* complies with the limit test for iron (20 ppm).

Loss on drying (2.2.32). Not more than 1.0 per cent, determined on 1.00 g of previously powdered substance by drying in an oven at 100-105 °C for 3 h.

ASSAY

Dissolve 1.500 g in *water R* and dilute to 100.0 ml with the same solvent. To 20.0 ml of the solution add 40 ml of *hydrochloric acid R* and titrate with 0.05 M *potassium iodate* until the colour changes from red to yellow. Add 5 ml of *chloroform R* and continue the titration, shaking vigorously, until the chloroform layer is decolourised. 1 ml of 0.05 M *potassium iodate* is equivalent to 16.60 mg of KI.

STORAGE

Store protected from light.

01/2005:2075

POTASSIUM METABISULPHITE

Kalii metabisulfis

K₂S₂O₅

*M*_r 222.3

DEFINITION

Potassium metabisulphite (potassium disulphite).

Content: 95.0 per cent to 101.0 per cent.

CHARACTERS

Appearance: white powder or colourless crystals.

Solubility: freely soluble in water, slightly soluble in alcohol.

IDENTIFICATION

- It complies with the test for pH (see Tests).
- To 5 ml of solution S (see Tests), add 0.5 ml of 0.05 M *iodine*. The mixture is colourless and gives reaction (a) of sulphates (2.3.1).
- Solution S gives reaction (a) of potassium (2.3.1).

TESTS

Solution S. Dissolve 5.0 g in *carbon dioxide-free water R* and dilute to 100 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and colourless (2.2.2, *Method I*).

pH (2.2.3): 3.0 to 4.5 for solution S.

Thiosulphates. To 2.00 g add 25 ml of a 42.5 g/l solution of *sodium hydroxide R* and 75 ml of *water R*. Shake until dissolved and add 10 ml of *formaldehyde R* and 10 ml of

acetic acid R. After 5 min, titrate with 0.03 M *iodine* using 1 ml of *starch solution R*. Carry out a blank titration. The difference between the volumes consumed in the 2 titrations is maximum 0.15 ml.

Iron: maximum 10 ppm.

Atomic absorption spectrometry (2.2.23, *Method I*).

Test solution. Dilute 20 ml of solution S to 50 ml with *water R*.

Reference solutions. Prepare the reference solutions using *iron standard solution (20 ppm Fe) R*, diluted as necessary with *water R*.

Source: iron hollow-cathode lamp.

Wavelength: 248.3 nm.

Atomisation device: air-acetylene flame.

Selenium: maximum 10 ppm.

To 3.0 g add 10 ml of *formaldehyde R*. Carefully add 2 ml of *hydrochloric acid R* in small portions. Heat on a water-bath for 20 min. Any pink colour in the solution is not more intense than that of a reference solution prepared at the same time in the same manner using 1.0 g of the substance to be examined to which 0.2 ml of *selenium standard solution (100 ppm Se) R* has been added.

Zinc: maximum 25 ppm.

Atomic absorption spectrometry (2.2.23, *Method I*).

Test solution. Dilute 20 ml of solution S to 50 ml with *water R*.

Reference solutions. Prepare the reference solutions using *zinc standard solution (100 ppm Zn) R*, diluted as necessary with *water R*.

Source: zinc hollow-cathode lamp.

Wavelength: 213.9 nm.

Atomisation device: air-acetylene flame.

Heavy metals (2.4.8): maximum 10 ppm.

Introduce 40 ml of solution S into a silica crucible, add 10 ml of *hydrochloric acid R* and evaporate to dryness. Dissolve the residue in 19 ml of *water R* and add 1 ml of a 40 g/l solution of *sodium fluoride R*. The solution complies with limit test E. Prepare the standard using 20 ml of *lead standard solution (1 ppm Pb) R*.

ASSAY

In a 500 ml conical flask containing 50.0 ml of 0.05 M *iodine* introduce 0.150 g and add 5 ml of *hydrochloric acid R*. Titrate the excess of iodine with 0.1 M *sodium thiosulphate* using 0.1 ml of *starch solution R*.

1 ml of 0.05 M *iodine* is equivalent to 5.558 mg of K₂S₂O₅.

STORAGE

In an airtight container, protected from light.

01/2005:1465

POTASSIUM NITRATE

Kalii nitras

KNO₃

*M*_r 101.1

DEFINITION

Potassium nitrate contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of KNO₃, calculated with reference to the dried substance.

CHARACTERS

A white, crystalline powder or colourless crystals, freely soluble in water, very soluble in boiling water, practically insoluble in alcohol.

IDENTIFICATION

- A. It gives the reaction of nitrates (2.3.1).
B. Solution S (see Tests) gives the reactions of potassium (2.3.1).

TESTS

Solution S. Dissolve 10.0 g in *carbon dioxide-free water R* prepared from *distilled water R* and dilute to 100 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and colourless (2.2.2, *Method II*).

Acidity or alkalinity. To 10 ml of solution S add 0.05 ml of *bromothymol blue solution R1*. Not more than 0.5 ml of 0.01 M *hydrochloric acid* or 0.01 M *sodium hydroxide* is required to change the colour of the indicator.

Reducible substances. To 10 ml of solution S, add 0.5 ml of *dilute sulphuric acid R* and 2 ml of *zinc iodide and starch solution R*. The solution does not become blue within 2 min.

Chlorides (2.4.4). If intended for ophthalmic use, it complies with the test for chlorides. Dissolve 2.5 g in *water R* and dilute to 15 ml with the same solvent. The solution complies with the limit test for chlorides (20 ppm).

Sulphates (2.4.13). Dilute 10 ml of solution S to 15 ml with *distilled water R*. The solution complies with the limit test for sulphates (150 ppm).

Ammonium (2.4.1). 1 ml of solution S complies with the limit test (A) for ammonium (100 ppm). If intended for ophthalmic use, not more than 50 ppm of ammonium.

Calcium (2.4.3). Dilute 10 ml of solution S to 15 ml with *distilled water R*. The solution complies with the limit test for calcium (100 ppm). If intended for ophthalmic use, not more than 50 ppm of calcium.

Iron (2.4.9). Dilute 5 ml of solution S to 10 ml with *water R*. The solution complies with the limit test for iron (20 ppm). If intended for ophthalmic use, not more than 10 ppm of iron.

Sodium. Not more than 0.1 per cent of Na, determined by atomic emission spectrometry (2.2.22, *Method II*).

Test solution. Dissolve 1.00 g in *water R* and dilute to 100.0 ml with the same solvent.

Reference solutions. Prepare the reference solutions using *sodium standard solution* (200 ppm Na) *R*, diluted as necessary with *water R*.

Measure the emission intensity at 589 nm.

Heavy metals (2.4.8). 12 ml of solution S complies with limit test A for heavy metals (10 ppm). Prepare the standard using *lead standard solution* (1 ppm Pb) *R*.

Loss on drying (2.2.32). Not more than 0.5 per cent determined on 1.000 g by drying in an oven at 100 °C to 105 °C.

ASSAY

Prepare a chromatography column 0.3 m long and 10 mm in internal diameter and filled with 10 g of *strongly acidic cation-exchange resin R* covered with *carbon dioxide-free water R*. Maintain a 1 cm layer of liquid above the resin at all times. Allow 100 ml of *dilute hydrochloric acid R* to run through the column at a flow rate of about 5 ml/min. Wash the column (with the tap completely open) with *carbon dioxide-free water R* until neutral to *blue litmus paper R*. Dissolve 0.200 g of the substance to be examined in 2 ml

of *carbon dioxide-free water R* in a beaker and transfer it to the column reservoir, allow the solution to run through the column at a flow rate of about 3 ml/min and collect the eluate. Wash the beaker with 10 ml of *carbon dioxide-free water R* and transfer this solution at the same flow rate to the column before it runs dry. Finally wash the column with 200 ml of *carbon dioxide-free water R* (with the tap completely open) until neutral to *blue litmus paper R*. Titrate the combined eluate and washings with 0.1 M *sodium hydroxide*, using 1 ml of *phenolphthalein solution R* as indicator.

1 ml of 0.1 M *sodium hydroxide* is equivalent to 10.11 mg of KNO₃.

LABELLING

The label states, where applicable, that the substance is suitable for ophthalmic use.

01/2005:1987

POTASSIUM PERCHLORATE

Kalii perchloras

KClO₄ *M_r* 138.6

DEFINITION

Content: 99.0 per cent to 102.0 per cent.

CHARACTERS

Appearance: white, crystalline powder or colourless crystals.
Solubility: sparingly soluble in water, practically insoluble in alcohol.

IDENTIFICATION

- A. Dissolve 0.1 g in 5 ml of *water R*. Add 5 ml of *indigo carmine solution R* and heat to boiling. The colour of the solution does not disappear.
B. It complies with the test for chlorates and chlorides (see Tests).
C. Heat 10 mg over a flame for 2 min. Dissolve the residue in 2 ml of *water R*. The solution gives reaction (a) of chlorides (2.3.1).
D. Dissolve 50 mg with heating in 5 ml of *water R*. Allow to cool to room temperature. The solution gives reaction (a) of potassium (2.3.1).

TESTS

Solution S. Suspend 5.0 g in 90 ml of *distilled water R* and heat to boiling. Allow to cool. Filter. Dilute the filtrate to 100 ml with *carbon dioxide-free water R*.

Appearance of solution. The solution is clear (2.2.1) and colourless (2.2.2, *Method II*).

Dissolve 0.20 g in *water R* and dilute to 20 ml with the same solvent.

Acidity or alkalinity. To 5 ml of solution S add 5 ml of *water R* and 0.1 ml of *phenolphthalein solution R*. Not more than 0.25 ml of 0.01 M *sodium hydroxide* is required to change the colour of the indicator. To 5 ml of solution S, add 5 ml of *water R* and 0.1 ml of *bromocresol green solution R*. Not more than 0.25 ml of 0.01 M *hydrochloric acid* is required to change the colour of the indicator.

Chlorates and chlorides (2.4.4): maximum 100 ppm (calculated as chlorides).

To 5 ml of solution S, add 5 ml of *water R* and heat to boiling. Add 1 ml of *nitric acid R* and 0.1 g of *sodium nitrite R*. Allow to cool to room temperature. Dilute to 15 ml

with *water R*. The solution complies with the limit test for chlorides. Prepare the standard using 5 ml of *chloride standard solution (5 ppm Cl) R* and 10 ml of *water R*, and adding only 1 ml of *dilute nitric acid R*.

Sulphates (2.4.13): maximum 100 ppm.

15 ml of solution S complies with the limit test for sulphates. Prepare the standard using a mixture of 7.5 ml of *sulphate standard solution (10 ppm SO₄) R* and 7.5 ml of *water R*.

Calcium (2.4.3): maximum 100 ppm.

15 ml of solution S complies with the limit test for calcium. Prepare the standard using a mixture of 7.5 ml of *calcium standard solution (10 ppm Ca) R*, 1 ml of *dilute acetic acid R* and 7.5 ml of *distilled water R*.

Heavy metals (2.4.8): maximum 20 ppm.

12 ml of solution S complies with limit test A. Prepare the standard using *lead standard solution (1 ppm Pb) R*.

ASSAY

Prepare a chromatography column 0.3 m long and 10 mm in internal diameter and filled with 10 g of *strongly acidic ion-exchange resin R* covered with *carbon dioxide-free water R*. Maintain a 1 cm layer of liquid above the resin throughout the determination. Allow 100 ml of *dilute hydrochloric acid R* to run through the column at a flow rate of about 5 ml/min. Wash the column (with the tap completely open) with *carbon dioxide-free water R* until the eluate is neutral to *blue litmus paper R*. Dissolve 0.100 g of the substance to be examined in 10 ml of *carbon dioxide-free water R* in a beaker and transfer it to the column reservoir, allow the solution to run through the column at a flow rate of about 3 ml/min and collect the eluate. Wash the beaker 3 times with 10 ml of *carbon dioxide-free water R* and transfer this solution at the same flow rate to the column before it runs dry. Finally, wash the column with 200 ml of *carbon dioxide-free water R* (with the tap completely open) until the eluate is neutral to *blue litmus paper R*. Titrate the combined eluate and washings with 0.1 M *sodium hydroxide*, using 1 ml of *phenolphthalein solution R* as indicator.

1 ml of 0.1 M *sodium hydroxide* is equivalent to 13.86 mg of KClO₄.

01/2005:0121

POTASSIUM PERMANGANATE

Kalii permanganas

KMnO₄

M_r 158.0

DEFINITION

Potassium permanganate contains not less than 99.0 per cent and not more than the equivalent of 100.5 per cent of KMnO₄.

CHARACTERS

A dark purple or brownish-black, granular powder or dark purple or almost black crystals, usually having a metallic lustre, soluble in cold water, freely soluble in boiling water. It decomposes on contact with certain organic substances.

IDENTIFICATION

- Dissolve about 50 mg in 5 ml of *water R* and add 1 ml of *alcohol R* and 0.3 ml of *dilute sodium hydroxide solution R*. A green colour develops. Heat to boiling. A dark brown precipitate is formed.
- Filter the mixture obtained in identification test A. The filtrate gives reaction (b) of potassium (2.3.1).

TESTS

Solution S. Dissolve 0.75 g in 25 ml of *distilled water R*, add 3 ml of *alcohol R* and boil for 2 min to 3 min. Cool, dilute to 30 ml with *distilled water R* and filter.

Appearance of solution. Solution S is colourless (2.2.2, *Method II*).

Substances insoluble in water. Dissolve 0.5 g in 50 ml of *water R* and heat to boiling. Filter through a tared sintered-glass filter (16). Wash with *water R* until the filtrate is colourless and collect the residue on the filter. The residue, dried in an oven at 100 °C to 105 °C, weighs not more than 5 mg (1.0 per cent).

Chlorides (2.4.4). 10 ml of solution S diluted to 15 ml with *water R* complies with the limit test for chlorides (200 ppm).

Sulphates (2.4.13). 12 ml of solution S diluted to 15 ml with *distilled water R* complies with the limit test for sulphates (500 ppm).

ASSAY

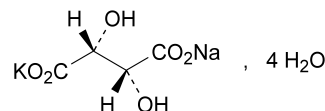
Dissolve 0.300 g in *water R* and dilute to 100.0 ml with the same solvent. To 20.0 ml of the solution add 20 ml of *water R*, 1 g of *potassium iodide R* and 10 ml of *dilute hydrochloric acid R*. Titrate the liberated iodine with 0.1 M *sodium thiosulphate*, using 1 ml of *starch solution R* as indicator.

1 ml of 0.1 M *sodium thiosulphate* is equivalent to 3.160 mg of KMnO₄.

01/2005:1986

POTASSIUM SODIUM TARTRATE TETRAHYDRATE

Kalii natrii tartras tetrahydricus



C₄H₄KNaO₆·4H₂O

M_r 282.2

DEFINITION

Potassium sodium (+)-(2*R*,3*R*)-2,3-dihydroxybutanedioate tetrahydrate.

Content: 98.0 per cent to 101.0 per cent (anhydrous substance).

CHARACTERS

Appearance: white, crystalline powder or colourless, transparent crystals.

Solubility: very soluble in water, practically insoluble in alcohol.

IDENTIFICATION

- It complies with the test for specific optical rotation (see Tests).
- It gives reaction (b) of tartrates (2.3.1).
- It gives reaction (b) of potassium (2.3.1).
- It gives reaction (a) of sodium (2.3.1).

TESTS

Solution S. Dissolve 5.000 g in *carbon dioxide-free water R*, prepared from *distilled water R*, and dilute to 100.0 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and colourless (2.2.2, *Method II*).

Acidity or alkalinity. To 5 ml of solution S, add 0.1 ml of *phenolphthalein solution R*. Not more than 0.5 ml of 0.01 M *hydrochloric acid* or 0.01 M *sodium hydroxide* is required to change the colour of the indicator.

Specific optical rotation (2.2.7): + 28.0 to + 30.0 (anhydrous substance), determined on solution S.

Chlorides (2.4.4): maximum 100 ppm.

Dilute 10 ml of solution S to 15 ml with *water R*. The solution complies with the limit test for chlorides.

Sulphates (2.4.13): maximum 50 ppm.

Dissolve 1.0 g in *distilled water R* and dilute to 15 ml with the same solvent. The solution complies with the limit test for sulphates. Prepare the reference solution with a mixture of 5 ml of *sulphate standard solution (10 ppm SO₄) R* and 10 ml of *distilled water R*.

Ammonium (2.4.1): maximum 40 ppm.

5 ml of solution S complies with the limit test for ammonium.

Barium and oxalates. To 5 ml of solution S, add 3 ml of *calcium sulphate solution R*. Allow to stand for 5 min. Any opalescence in the solution is not more intense than that in a mixture of 3 ml of *calcium sulphate solution R* and 5 ml of *distilled water R*.

Calcium (2.4.3): maximum 200 ppm.

Dilute 10 ml of solution S to 15 ml with *distilled water R*. The solution complies with the limit test for calcium.

Heavy metals (2.4.8): maximum 10 ppm.

Dissolve 2.0 g in *water R* and dilute to 20 ml with the same solvent. 12 ml of the solution complies with limit test A. Prepare the standard using *lead standard solution (1 ppm Pb) R*.

Water (2.5.12): 24.0 per cent to 26.5 per cent, determined on 50.0 mg. Use 50 ml of *anhydrous methanol R*. Titrate slowly.

ASSAY

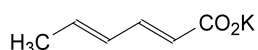
To 0.100 g of finely powdered substance add 40 ml of *anhydrous acetic acid R* and 20 ml of *acetic anhydride R*. Titrate slowly with 0.1 M *perchloric acid*, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *perchloric acid* is equivalent to 10.51 mg of C₆H₇KNaO₆.

01/2005:0618

POTASSIUM SORBATE

Kalii sorbas



C₆H₇KO₂

M_r 150.2

DEFINITION

Potassium sorbate contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of potassium (*E,E*)-hexa-2,4-dienoate, calculated with reference to the dried substance.

CHARACTERS

White or almost white powder or granules, very soluble in water, slightly soluble in alcohol.

IDENTIFICATION

First identification: B, D.

Second identification: A, C, D.

- Dissolve 50.0 mg in *water R* and dilute to 250.0 ml with the same solvent. Dilute 2.0 ml of this solution to 200.0 ml with 0.1 M *hydrochloric acid*. Examined between 230 nm and 350 nm (2.2.25), the solution shows a maximum at 264 nm. The specific absorbance at the maximum is 1650 to 1900.
- Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *potassium sorbate CRS*.
- Dissolve 1.0 g in 50 ml of *water R*, add 10 ml of *dilute hydrochloric acid R* and shake. Filter the crystalline precipitate, wash with *water R* and dry in a vacuum over *sulphuric acid R* for 4 h. The residue obtained melts (2.2.14) at 132 °C to 136 °C.
- Dissolve 0.2 g in 2 ml of *water R* and add 2 ml of *dilute acetic acid R*. Filter. The solution gives reaction (b) of potassium (2.3.1).

TESTS

Solution S. Dissolve 2.5 g in *carbon dioxide-free water R* and dilute to 50 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and not more intensely coloured than reference solution Y₅ (2.2.2, *Method II*).

Acidity or alkalinity. To 20 ml of solution S add 0.1 ml of *phenolphthalein solution R*. Not more than 0.25 ml of 0.1 M *sodium hydroxide* or 0.1 M *hydrochloric acid* is required to change the colour of the indicator.

Aldehydes. Dissolve 1.0 g in a mixture of 30 ml of *water R* and 50 ml of 2-propanol *R*, adjust the solution to pH 4 with 1 M *hydrochloric acid* and dilute to 100 ml with *water R*. To 10 ml of the solution add 1 ml of *decolorised fuchsin solution R* and allow to stand for 30 min. Any colour in the solution is not more intense than that in a standard prepared at the same time by adding 1 ml of *decolorised fuchsin solution R* to a mixture of 1.5 ml of *acetaldehyde standard solution (100 ppm C₂H₄O) R*, 4 ml of 2-propanol *R* and 4.5 ml of *water R* (0.15 per cent, calculated as C₂H₄O).

Heavy metals (2.4.8). 2.0 g complies with limit test D for heavy metals (10 ppm). Prepare the standard using 2 ml of *lead standard solution (10 ppm Pb) R*.

Loss on drying (2.2.32). Not more than 1.0 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C for 3 h.

ASSAY

Dissolve 0.120 g in 20 ml of *anhydrous acetic acid R*. Titrate with 0.1 M *perchloric acid* using 0.1 ml of *crystal violet solution R* as indicator until the colour changes from violet to bluish-green.

1 ml of 0.1 M *perchloric acid* is equivalent to 15.02 mg of C₆H₇KO₂.

STORAGE

Store protected from light.

01/2005:1622 ASSAY

POTASSIUM SULPHATE**Kalii sulfas** K_2SO_4 M_r 174.3**DEFINITION**

Content: 98.5 per cent to 101.0 per cent of K_2SO_4 (dried substance).

CHARACTERS

Appearance: white, crystalline powder or colourless crystals.

Solubility: soluble in water, practically insoluble in ethanol.

IDENTIFICATION

A. It gives the reactions of sulphates (2.3.1).

B. It gives the reactions of potassium (2.3.1).

TESTS

Solution S. Dissolve 10.0 g in 90 ml of *carbon dioxide-free water R* prepared from *distilled water R*, heating gently. Allow to cool and dilute to 100 ml with *carbon dioxide-free water R* prepared from *distilled water R*.

Appearance of solution. Solution S is clear (2.2.1) and colourless (2.2.2, *Method II*).

Acidity or alkalinity. To 10 ml of solution S add 0.1 ml of *bromothymol blue solution R1*. Not more than 0.5 ml of 0.01 M *hydrochloric acid* or 0.01 M *sodium hydroxide* is required to change the colour of the indicator.

Chlorides (2.4.4): maximum 40 ppm.

Dilute 12.5 ml of solution S to 15 ml with *water R*.

Calcium (2.4.3): maximum 200 ppm.

Dilute 5 ml of solution S to 15 ml with *distilled water R*.

Iron (2.4.9): maximum 10 ppm, determined on 10 ml of solution S.

Magnesium: maximum 20 ppm.

To 5 ml of solution S add 5 ml of *water R*, 1 ml of *glycerol* (85 per cent) *R*, 0.15 ml of *titan yellow solution R*, 0.25 ml of *ammonium oxalate solution R* and 5 ml of *dilute sodium hydroxide solution R* and shake. Any pink colour in the test solution is not more intense than that in a standard prepared at the same time and in the same manner using a mixture of 1 ml of *magnesium standard solution* (10 ppm Mg) *R* and 9 ml of *water R*.

Sodium: maximum 0.10 per cent.

Atomic emission spectrometry (2.2.22, *Method I*).

Test solution. Dissolve 1.00 g of the substance to be examined in *water R* and dilute to 100.0 ml with the same solvent.

Reference solutions. Dissolve in *water R* 0.50 g of *sodium chloride R*, previously dried at 100–105 °C for 3 h, and dilute to 1000.0 ml with the same solvent (200 µg of Na per millilitre). Dilute as required.

Wavelength: 589 nm.

Heavy metals (2.4.8): maximum 20 ppm.

12 ml of solution S complies with limit test A. Prepare the standard using *lead standard solution* (2 ppm Pb) *R*.

Loss on drying (2.2.32): maximum 1.0 per cent, determined on 1.000 g by drying in an oven at 130 °C for 4 h.

Dissolve 0.150 g in 40 ml of *water R*. Add 0.2 ml of 0.1 M *hydrochloric acid* and 80 ml of *methanol R*. Carry out a potentiometric titration (2.2.20), using 0.1 M *lead nitrate* and as indicator electrode a lead-selective electrode and as reference electrode a silver-silver chloride electrode.

1 ml of 0.1 M *lead nitrate* is equivalent to 17.43 mg of K_2SO_4 .

01/2005:0355

POTATO STARCH**Solani amyllum****DEFINITION**

Potato starch is obtained from the tuber of *Solanum tuberosum* L.

CHARACTERS

Appearance: very fine, white powder which creaks when pressed between the fingers.

Solubility: practically insoluble in cold water and in alcohol.

Potato starch does not contain starch grains of any other origin. It may contain a minute quantity, if any, of tissue fragments of the original plant.

IDENTIFICATION

- Examined under a microscope using a mixture of equal volumes of *glycerol R* and *water R*, it presents granules, either irregularly shaped, ovoid or pear-shaped, usually 30 µm to 100 µm in size but occasionally exceeding 100 µm, or rounded, 10 µm to 35 µm in size. There are occasional compound granules having 2 to 4 components. The ovoid and pear-shaped granules have an eccentric hilum and the rounded granules acentric or slightly eccentric hilum. All granules show clearly visible concentric striations. Between crossed nicol prisms, the granules show a distinct black cross intersecting at the hilum.
- Suspend 1 g in 50 ml of *water R*, boil for 1 min and cool. A thick, opalescent mucilage is formed.
- To 1 ml of the mucilage obtained in identification test B, add 0.05 ml of *iodine solution R1*. An orange-red to dark blue colour is produced which disappears on heating.

TESTS

pH (2.2.3): 5.0 to 8.0.

Shake 5.0 g with 25.0 ml of *carbon dioxide-free water R* for 60 s. Allow to stand for 15 min.

Foreign matter. Examined under a microscope using a mixture of equal volumes of *glycerol R* and *water R*, not more than traces of matter other than starch granules are present. No starch grains of any other origin are present.

Oxidising substances (2.5.30): maximum 20 ppm, calculated as H_2O_2 .

Sulphur dioxide (2.5.29): maximum 50 ppm.

Iron (2.4.9): maximum 10 ppm.

Shake 1.5 g with 15 ml of *dilute hydrochloric acid R*. Filter. The filtrate complies with the limit test for iron.

Loss on drying (2.2.32): maximum 20.0 per cent, determined on 1.000 g by drying in an oven at 130 °C for 90 min.

Sulphated ash (2.4.14): maximum 0.6 per cent, determined on 1.0 g.

Microbial contamination. Total viable aerobic count (2.6.12) not more than 10^3 bacteria and not more than 10^2 fungi per gram, determined by plate count. It complies with the test for *Escherichia coli* (2.6.13).

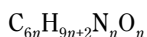
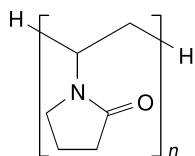
D. To 0.1 ml of solution S1 add 5 ml of *water R* and 0.2 ml of 0.05 M iodine. A red colour is produced.

E. It is freely soluble in *water R*.

01/2005:0685
corrected

POVIDONE

Povidonum



DEFINITION

α -Hydro- ω -hydropoly[1-(2-oxopyrrolidin-1-yl)ethylene]. It consists of linear polymers of 1-ethenylpyrrolidin-2-one.

Content: 11.5 per cent to 12.8 per cent of nitrogen (N; A_r 14.01) (anhydrous substance).

The different types of povidone are characterised by their viscosity in solution, expressed as a *K*-value.

The *K*-value of povidone having a stated *K*-value of 15 or less is 85.0 per cent to 115.0 per cent of the stated value.

The *K*-value of povidone having a stated *K*-value or a stated *K*-value range with an average of more than 15 is 90.0 per cent to 108.0 per cent of the stated value or of the average of the stated range.

CHARACTERS

Appearance: white or yellowish-white powder or flakes, hygroscopic.

Solubility: freely soluble in water, in alcohol and in methanol, slightly soluble in acetone.

IDENTIFICATION

First identification: A, E.

Second identification: B, C, D, E.

A. Infrared absorption spectrophotometry (2.2.24).

Preparation: dry the substances previously at 105 °C for 6 h. Record the spectra using 4 mg of substance.

Comparison: povidone CRS.

B. To 0.4 ml of solution S1 (see Tests) add 10 ml of *water R*, 5 ml of *dilute hydrochloric acid R* and 2 ml of *potassium dichromate solution R*. An orange-yellow precipitate is formed.

C. To 1 ml of solution S1 add 0.2 ml of *dimethylaminobenzaldehyde solution R1* and 0.1 ml of *sulphuric acid R*. A pink colour is produced.

TESTS

Solution S. Dissolve 1.0 g in *carbon dioxide-free water R* and dilute to 20 ml with the same solvent. Add the substance to be examined to the water in small portions with magnetic stirring.

Solution S1. Dissolve 2.5 g in *carbon dioxide-free water R* and dilute to 25 ml with the same solvent. Add the substance to be examined to the water in small portions with magnetic stirring.

Appearance of solution. Solution S is clear (2.2.1) and not more intensely coloured than reference solution B₆, BY₆ or R₆ (2.2.2, *Method II*).

pH (2.2.3): 3.0 to 5.0 for solution S, for povidone having a stated *K*-value of at most 30; 4.0 to 7.0 for solution S, for povidone having a stated *K*-value of more than 30.

Viscosity, expressed as *K*-value. For povidone having a stated value of 18 or less, use a 50 g/l solution. For povidone having a declared value of more than 18, use a 10 g/l solution. For povidone having a declared value of more than 95, use a 1.0 g/l solution. Allow to stand for 1 h and determine the viscosity (2.2.9) of the solution at 25 °C, using viscometer No.1 with a minimum flow time of 100 s. Calculate the *K*-value from the expression:

$$\frac{1.5 \log \eta - 1}{0.15 + 0.003c} + \frac{\sqrt{300c \log \eta + (c + 1.5c \log \eta)^2}}{0.15c + 0.003c^2}$$

c = concentration of the substance to be examined, calculated with reference to the anhydrous substance, in grams per 100 ml,

η = viscosity of the solution relative to that of *water R*.

Aldehydes: maximum 500 ppm, expressed as acetaldehyde.

Test solution. Dissolve 1.0 g of the substance to be examined in *phosphate buffer solution pH 9.0 R* and dilute to 100.0 ml with the same solvent. Stopper the flask and heat at 60 °C for 1 h. Allow to cool.

Reference solution. Dissolve 0.140 g of *acetaldehyde ammonia trimer trihydrate R* in *water R* and dilute to 200.0 ml with the same solvent. Dilute 1.0 ml of this solution to 100.0 ml with *phosphate buffer solution pH 9.0 R*.

Into 3 identical spectrophotometric cells with a path length of 1 cm, introduce separately 0.5 ml of the test solution, 0.5 ml of the reference solution and 0.5 ml of *water R* (blank). To each cell, add 2.5 ml of *phosphate buffer solution pH 9.0 R* and 0.2 ml of *nicotinamide-adenine dinucleotide solution R*. Mix and stopper tightly. Allow to stand at 22 ± 2 °C for 2-3 min and measure the absorbance (2.2.25) of each solution at 340 nm, using *water R* as the compensation liquid. To each cell, add 0.05 ml of *aldehyde dehydrogenase solution R*, mix and stopper tightly. Allow to stand at 22 ± 2 °C for 5 min. Measure the absorbance of each solution at 340 nm using *water R* as the compensation liquid.

Determine the content of aldehydes using the expression:

$$\frac{(A_{t2} - A_{t1}) - (A_{b2} - A_{b1})}{(A_{s2} - A_{s1}) - (A_{b2} - A_{b1})} \times \frac{100\,000 \times C}{m}$$

- A_{t1} = absorbance of the test solution before the addition of aldehyde dehydrogenase,
 A_{t2} = absorbance of the test solution after the addition of aldehyde dehydrogenase,
 A_{s1} = absorbance of the reference solution before the addition of aldehyde dehydrogenase,
 A_{s2} = absorbance of the reference solution after the addition of aldehyde dehydrogenase,
 A_{b1} = absorbance of the blank before the addition of aldehyde dehydrogenase,
 A_{b2} = absorbance of the blank after the addition of aldehyde dehydrogenase,
 m = mass of povidone calculated with reference to the anhydrous substance, in grams,
 C = concentration of acetaldehyde in the reference solution, calculated from the weight of the acetaldehyde ammonia trimer trihydrate with the factor 0.72, in milligrams per millilitre.

Peroxides: maximum 400 ppm, expressed as H_2O_2 .

Dissolve 2.0 g in 50 ml of *water R*. To 25 ml of this solution, add 2 ml of *titanium trichloride-sulphuric acid reagent R*. Allow to stand for 30 min. The absorbance (2.2.25) of the solution, measured at 405 nm using a mixture of 25 ml of a 40 g/l solution of the substance to be examined and 2 ml of a 13 per cent V/V solution of *sulphuric acid R* as the compensation liquid, is not greater than 0.35.

Hydrazine. Thin-layer chromatography (2.2.27). Use freshly prepared solutions.

Test solution. Dissolve 2.5 g of the substance to be examined in 25 ml of *water R*. Add 0.5 ml of a 50 g/l solution of *salicylaldehyde R* in *methanol R*, mix and heat in a water-bath at 60 °C for 15 min. Allow to cool, add 2.0 ml of *toluene R*, shake for 2 min and centrifuge. Use the clear supernatant layer.

Reference solution. Dissolve 9 mg of *salicylaldehydeazine R* in *toluene R* and dilute to 100 ml with the same solvent. Dilute 1 ml of the solution to 10 ml with *toluene R*.

Plate: TLC silanised silica gel plate *R*.

Mobile phase: *water R*, *methanol R* (1:2 V/V).

Application: 10 µl.

Development: over a path of 15 cm.

Drying: in air.

Detection: examine in ultraviolet light at 365 nm.

Limit:

- *hydrazine*: any spot corresponding to *salicylaldehydeazine* in the chromatogram obtained with the test solution is not more intense than the spot in the chromatogram obtained with the reference solution (1 ppm).

Impurity A. Liquid chromatography (2.2.29).

Test solution. Dissolve 0.25 g of the substance to be examined in the mobile phase and dilute to 10.0 ml with the mobile phase.

Reference solution (a). Dissolve 50 mg of *1-vinylpyrrolidin-2-one R* in *methanol R* and dilute to 100.0 ml with the same solvent. Dilute 1.0 ml of the solution to 100.0 ml with *methanol R*. Dilute 5.0 ml of this solution to 100.0 ml with the mobile phase.

Reference solution (b). Dissolve 10 mg of *1-vinylpyrrolidin-2-one R* and 0.5 g of *vinyl acetate R* in *methanol R* and dilute to 100.0 ml with the same solvent. Dilute 1.0 ml of the solution to 100.0 ml with the mobile phase.

Precolumn:

- *size*: $l = 0.025$ m, $\varnothing = 4$ mm,
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

Column:

- *size*: $l = 0.25$ m, $\varnothing = 4$ mm,
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm),
- *temperature*: 40 °C.

Mobile phase: *acetonitrile R*, *water R* (10:90 V/V).

Flow rate: adjusted so that the retention time of the peak corresponding to impurity A is about 10 min.

Detection: spectrophotometer at 235 nm.

Injection: 50 µl. After injection of the test solution, wait for about 2 min and wash the precolumn by passing the mobile phase backward, at the same flow rate applied in the test, for 30 min.

System suitability:

- *resolution*: minimum 2.0 between the peaks due to impurity A and to vinyl acetate in the chromatogram obtained with reference solution (b),
- *repeatability*: maximum relative standard deviation of 2.0 per cent after 5 injections of reference solution (a).

Limit:

- *impurity A*: not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (10 ppm).

Impurity B. Liquid chromatography (2.2.29).

Test solution. Dissolve 100 mg of the substance to be examined in *water R* and dilute to 50.0 ml with the same solvent.

Reference solution. Dissolve 100 mg of *2-pyrrolidone R* in *water R* and dilute to 100 ml with the same solvent. Dilute 3.0 ml to 50.0 ml with *water R*.

Precolumn:

- *size*: $l = 0.025$ m, $\varnothing = 4$ mm,
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

Column:

- *size*: $l = 0.25$ m, $\varnothing = 4$ mm,
- *stationary phase*: *spherical aminohexadecylsilyl silica gel for chromatography R* (5 µm),
- *temperature*: 30 °C.

Mobile phase: *water R*, adjusted to pH 2.4 with *phosphoric acid R*.

Flow rate: 1 ml/min.

Detection: spectrophotometer at 205 nm. A detector is placed between the precolumn and the analytical column. A second detector is placed after the analytical column.

Injection: 10 µl. When impurity B has left the precolumn (after about 1.2 min) switch the flow directly from the pump to the analytical column. Before the next chromatogram is run, wash the precolumn by reversed flow.

System suitability:

- *symmetry factor*: maximum 2.0 for the peak due to impurity B.

Limit:

- **impurity B:** not more than the area of the principal peak in the chromatogram obtained with the reference solution (3.0 per cent).

Heavy metals (2.4.8): maximum 10 ppm.

2.0 g complies with limit test D. Prepare the standard using 2.0 ml of *lead standard solution* (10 ppm Pb) R.

Water (2.5.12): maximum 5.0 per cent, determined on 0.500 g.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Place 100.0 mg of the substance to be examined (*m* mg) in a combustion flask, add 5 g of a mixture of 1 g of *copper sulphate R*, 1 g of *titanium dioxide R* and 33 g of *dipotassium sulphate R*, and 3 glass beads. Wash any adhering particles from the neck into the flask with a small quantity of *water R*. Add 7 ml of *sulphuric acid R*, allowing it to run down the sides of the flask, and mix the contents by rotation. Close the mouth of the flask loosely, for example by means of a glass bulb with a short stem, to avoid excessive loss of sulphuric acid. Heat gradually at first, then increase the temperature until there is vigorous boiling with condensation of sulphuric acid in the neck of the flask; precautions are to be taken to prevent the upper part of the flask from becoming overheated. Continue the heating for 45 min. Cool, dissolve the solid material by cautiously adding to the mixture 20 ml of *water R*, cool again and place in a steam-distillation apparatus. Add 30 ml of *strong sodium hydroxide solution R* through the funnel, rinse cautiously the funnel with 10 ml of *water R* and distil immediately by passing steam through the mixture. Collect about 80–100 ml of distillate in a mixture of 30 ml of a 40 g/l solution of *boric acid R* and 3 drops of *bromocresol green-methyl red solution R* and enough *water R* to cover the tip of the condenser. Towards the end of the distillation lower the receiver so that the tip of the condenser is above the surface of the acid solution and rinse the end part of the condenser with a small quantity of *water R*. Titrate the distillate with 0.025 M *sulphuric acid* until the colour of the solution changes from green through pale greyish-blue to pale greyish-red-purple (n_1 ml of 0.025 M *sulphuric acid*).

Repeat the test using about 100.0 mg of *glucose R* in place of the substance to be examined (n_2 ml of 0.025 M *sulphuric acid*).

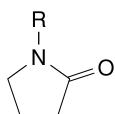
$$\text{percentage content of nitrogen} = \frac{0.7004 (n_1 - n_2)}{m} \times 100$$

STORAGE

In an airtight container.

LABELLING

The label indicates the nominal *K*-value.

IMPURITIES

A. R = CH=CH₂: 1-ethenylpyrrolidin-2-one (1-vinylpyrrolidin-2-one),

B. R = H: pyrrolidin-2-one (2-pyrrolidone).

POVIDONE, IODINATED**Povidonum iodinatum****DEFINITION**

Complex of iodine and povidone.

Content: 9.0 per cent to 12.0 per cent of available iodine (dried substance).

PRODUCTION

It is produced using povidone that complies with the monograph on *Povidone* (0685), except that the povidone used may contain not more than 2.0 per cent of formic acid and not more than 8.0 per cent of water.

CHARACTERS

Appearance: yellowish-brown or reddish-brown, amorphous powder.

Solubility: soluble in water and in ethanol (96 per cent), practically insoluble in acetone.

IDENTIFICATION

A. Infrared absorption spectrophotometry (2.2.24).

Comparison: iodinated povidone CRS.

B. Dissolve 10 mg in 10 ml of *water R* and add 1 ml of *starch solution R*. An intense blue colour is produced.

C. Dissolve 0.1 g in 5 ml of *water R* and add a 10 g/l solution of *sodium sulphite R* dropwise, until the solution becomes colourless. Add 2 ml of *potassium dichromate solution R* and 1 ml of *hydrochloric acid R*. A light brown precipitate is formed.

TESTS

pH (2.2.3): 1.5 to 5.0.

Dissolve 1.0 g in 10 ml of *carbon dioxide-free water R*.

Iodide: maximum 6.0 per cent (dried substance).

Dissolve 0.500 g in 100 ml of *water R*. Add *sodium metabisulphite R* until the colour of the iodine has disappeared. Add 25.0 ml of 0.1 M *silver nitrate*, 10 ml of *nitric acid R* and 5 ml of *ferric ammonium sulphate solution R2*. Titrate with 0.1 M *ammonium thiocyanate*. Carry out a blank titration.

1 ml of 0.1 M *silver nitrate* is equivalent to 12.69 mg of total iodine. From the percentage of total iodine, calculated with reference to the dried substance, subtract the percentage of available iodine as determined in the assay to obtain the percentage of iodide.

Loss on drying (2.2.32): maximum 8.0 per cent, determined on 0.500 g by drying in an oven at 100–105 °C for 3 h.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Transfer 1.000 g into a ground-glass-stoppered flask containing 150 ml of *water R* and stir for 1 h. Add 0.1 ml of *dilute acetic acid R* and titrate with 0.1 M *sodium thiosulphate* using *starch solution R* as indicator.

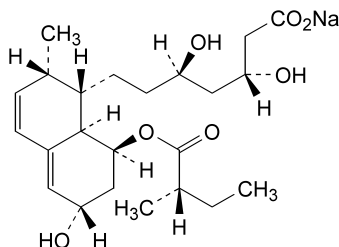
1 ml of 0.1 M *sodium thiosulphate* is equivalent to 12.69 mg of available iodine.

STORAGE

Protected from light.

01/2005:2059
corrected**PRAVASTATIN SODIUM**

Pravastatinum natricum

 $C_{23}H_{35}NaO_7$ M_r 446.5**DEFINITION**

Sodium (3*R*,5*R*)-3,5-dihydroxy-7-[(1*S*,2*S*,6*S*,8*S*,8*aR*)-6-hydroxy-2-methyl-8-[[[(2*S*)-2-methylbutanoyl]oxy]-1,2,6,7,8,8*a*-hexahydronaphthalen-1-yl]heptanoate.

Content: 97.0 per cent to 102.0 per cent (anhydrous and ethanol-free substance).

CHARACTERS

Appearance: white to yellowish white powder or crystalline powder, hygroscopic.

Solubility: freely soluble in water and in methanol, soluble in ethanol.

IDENTIFICATION

A. Specific optical rotation (see Tests).

B. Infrared absorption spectrophotometry (2.2.24).

Comparison: Ph. Eur. reference spectrum of pravastatin sodium.

C. 1 ml of solution S (see Tests) gives reaction (a) of sodium (2.3.1).

TESTS

Solution S. Dissolve 1.00 g in carbon dioxide free water *R* and dilute to 20.0 ml with the same solvent.

Appearance of solution. The solution is clear (2.2.1) and not more intensely coloured than reference solution BY₆ (2.2.2, Method II).

Dilute 2.0 ml of solution S to 10.0 ml with water *R*.

pH (2.2.3): 7.2 to 9.0 for solution S.

Specific optical rotation (2.2.7): + 153 to + 159 (anhydrous and ethanol-free substance).

Dilute 2.0 ml of solution S to 20.0 ml with water *R*.

Related substances. Liquid chromatography (2.2.29).

Solvent mixture. Mix 9 volumes of methanol *R* with 11 volumes of water *R*.

Test solution (a). Dissolve 0.1000 g of the substance to be examined in the solvent mixture and dilute to 100.0 ml with the solvent mixture.

Test solution (b). Dilute 10.0 ml of test solution (a) to 100.0 ml with the solvent mixture.

Reference solution (a). Dissolve 5.0 mg of the substance to be examined and 5.0 mg of pravastatin impurity A CRS in the solvent mixture and dilute to 50.0 ml with the solvent mixture.

Reference solution (b). Dilute 2.0 ml of test solution (a) to 100.0 ml with the solvent mixture. Dilute 1.0 ml to 10.0 ml with the solvent mixture.

Reference solution (c). Dissolve 12.4 mg of pravastatin 1,1,3,3-tetramethylbutylamine CRS in the solvent mixture and dilute to 100.0 ml with the solvent mixture.

Column:

- *size*: $l = 0.15$ m, $\varnothing = 4.6$ mm,
- *stationary phase*: octadecylsilyl silica gel for chromatography *R* (5 μ m),
- *temperature*: 25 °C.

Mobile phase: glacial acetic acid *R*, triethylamine *R*, methanol *R*, water *R* (1:1:450:550 V/V/V/V).

Flow rate: 1.3 ml/min.

Detection: spectrophotometer at 238 nm.

Injection: 10 μ l; inject test solution (a) and reference solutions (a) and (b).

Run time: 2.5 times the retention time of pravastatin.

Relative retention with reference to pravastatin (retention time = about 21 min): impurity B = about 0.2; impurity A = about 0.6; impurity C = about 2.1.

System suitability: reference solution (a):

- *resolution*: minimum 7.0 between the peaks due to impurity A and to pravastatin.

Limits:

- *impurity A*: not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.3 per cent),
- *any impurity*: not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent),
- *total*: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.6 per cent),
- *disregard limit*: 0.25 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

Ethanol (2.4.24, System A): maximum 3.0 per cent *m/m*.

Heavy metals (2.4.8): maximum 20 ppm.

Dissolve 2.0 g of substance to be examined in a mixture of 15 volumes of water *R* and 85 volumes of methanol *R* and dilute to 20 ml with the same mixture of solvents. 12 ml of this solution complies with limit test B. Prepare the standard using lead standard solution (2 ppm Pb) prepared by diluting lead standard solution (100 ppm Pb) *R* with a mixture of 15 volumes of water *R* and 85 volumes of methanol *R*.

Water (2.5.12): maximum 4.0 per cent, determined on 0.500 g.

ASSAY

Liquid chromatography (2.2.29) as described in the test for related substances.

Injection: test solution (b) and reference solution (c).

Calculate the percentage content of $C_{23}H_{35}NaO_7$ using the chromatogram obtained with reference solution (c) and the declared content of pravastatin in pravastatin 1,1,3,3-tetramethylbutylamine CRS.

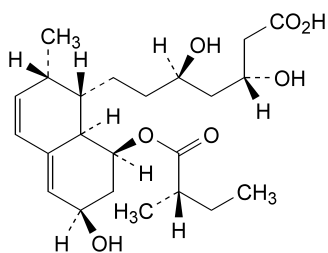
1 mg of pravastatin is equivalent to 1.052 mg of pravastatin sodium.

STORAGE

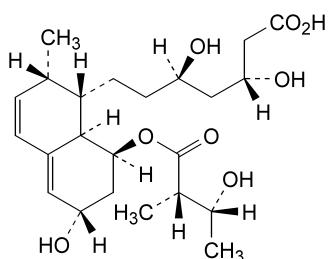
In an airtight container.

IMPURITIES

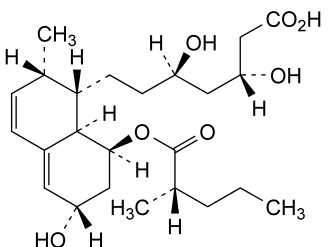
01/2005:1466



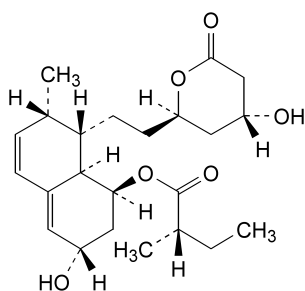
- A. (3*R*,5*R*)-3,5-dihydroxy-7-[(1*S*,2*S*,6*R*,8*S*,8*aR*)-6-hydroxy-2-methyl-8-[(2*S*)-2-methylbutanoyl]oxy]-1,2,6,7,8,8*a*-hexahydronaphthalen-1-yl]heptanoic acid (6'-epipravastatin),



- B. (3*R*,5*R*)-3,5-dihydroxy-7-[(1*S*,2*S*,6*S*,8*S*,8*aR*)-6-hydroxy-8-[(2*S*,3*R*)-3-hydroxy-2-methylbutanoyl]oxy]-2-methyl-1,2,6,7,8,8*a*-hexahydronaphthalen-1-yl]heptanoic acid (3''-hydroxypravastatin),



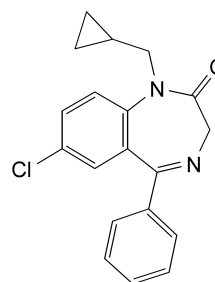
- C. (3*R*,5*R*)-3,5-dihydroxy-7-[(1*S*,2*S*,6*S*,8*S*,8*aR*)-6-hydroxy-2-methyl-8-[(2*S*)-2-methylpentanoyl]oxy]-1,2,6,7,8,8*a*-hexahydronaphthalen-1-yl]heptanoic acid,



- D. (1*S*,3*S*,7*S*,8*S*,8*aR*)-3-hydroxy-8-[2-[(2*R*,4*R*)-4-hydroxy-6-oxotetrahydro-2*H*-pyran-2-yl]ethyl]-7-methyl-1,2,3,7,8,8*a*-hexahydronaphthalen-1-yl (2*S*)-2-methylbutanoate (pravastatin lactone).

PRAZEPAM

Prazepamum

 $C_{19}H_{17}ClN_2O$ M_r 324.8

DEFINITION

Prazepam contains not less than 98.5 per cent and not more than the equivalent of 101.0 per cent of 7-chloro-1-(cyclopropylmethyl)-5-phenyl-1,3-dihydro-2*H*-1,4-benzodiazepin-2-one, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, crystalline powder, practically insoluble in water, freely soluble in methylene chloride, sparingly soluble in ethanol.

It melts at about 145 °C.

IDENTIFICATION

First identification: B.

Second identification: A, C.

- A. Dissolve 30.0 mg in *alcohol R* and dilute to 100.0 ml with the same solvent. Dilute 20.0 ml of the solution to 100.0 ml (solution A) and 2.0 ml of the solution to 100.0 ml (solution B) with the same solvent. Examined between 300 nm and 350 nm (2.2.25), solution A shows an absorption maximum at 312 nm. Examined between 210 nm and 300 nm, solution B shows an absorption maximum at 228 nm and a point of inflexion at about 252 nm. The specific absorbance at the maximum at 228 nm is 900 to 940. The specific absorbance at the maximum at 312 nm is 59 to 63.
- B. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *prazepam CRS*.
- C. Examine the chromatograms obtained in the test for related substances. The principal spot in the chromatogram obtained with test solution (b) is similar in position, fluorescence at 365 nm and size to the principal spot in the chromatogram obtained with reference solution (b).

TESTS

Appearance of solution. Dissolve 0.25 g in *alcohol R* and dilute to 10 ml with the same solvent. The solution is clear (2.2.1) and colourless (2.2.2, *Method II*).

Related substances. Examine by thin-layer chromatography (2.2.27), using as the coating substance a suitable silica gel with a fluorescent indicator having an optimal intensity at 254 nm.

Test solution (a). Dissolve 0.50 g of the substance to be examined in *acetone R* and dilute to 5 ml with the same solvent.

Test solution (b). Dilute 1 ml of test solution (a) to 100 ml with *acetone R*.

Reference solution (a). Dilute 1 ml of test solution (b) to 10 ml with *acetone R*.

Reference solution (b). Dissolve 10 mg of *prazepam CRS* in *acetone R* and dilute to 10 ml with the same solvent.

Reference solution (c). Dissolve 15 mg of *nordazepam CRS* in *acetone R* and dilute to 50 ml with the same solvent.

Reference solution (d). To 1 ml of reference solution (a) add 1 ml of reference solution (c) and mix.

Apply to the plate 5 µl of each solution. Develop over a path of 10 cm using a freshly prepared mixture of 50 volumes of *ethyl acetate R* and 50 volumes of *heptane R*. Dry the plate in air and examine in ultraviolet light at 254 nm and 365 nm. In the chromatogram obtained with test solution (a): any spot corresponding to nordazepam is not more intense than the spot in the chromatogram obtained with reference solution (c) (0.3 per cent); not more than four additional spots are present, none of which are more intense than the spot in the chromatogram obtained with reference solution (a) (0.1 per cent). The test is not valid unless the chromatogram obtained with reference solution (d) shows two clearly separated spots.

Heavy metals (2.4.8). 1.0 g complies with limit test C for heavy metals (20 ppm). Prepare the standard using 2 ml of *lead standard solution (10 ppm Pb) R*.

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

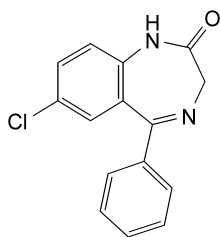
Dissolve 0.250 g in 25 ml of *anhydrous acetic acid R*. Titrate with 0.1 M *perchloric acid*, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *perchloric acid* is equivalent to 32.48 mg of $C_{19}H_{17}ClN_2O$.

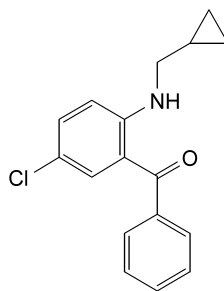
STORAGE

Store protected from light.

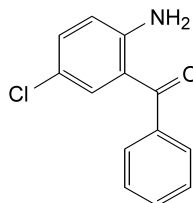
IMPURITIES



A. 7-chloro-5-phenyl-1,3-dihydro-2H-1,4-benzodiazepin-2-one (nordazepam),



B. [5-chloro-2-[(cyclopropylmethyl)amino]phenyl]phenylmethanone,

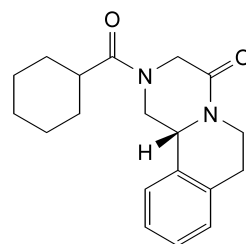


C. (2-amino-5-chlorophenyl)phenylmethanone.

01/2005:0855

PRAZIQUANTEL

Praziquantelum



and enantiomer

$C_{19}H_{24}N_2O_2$

M_r 312.4

DEFINITION

(11bRS)-2-(Cyclohexylcarbonyl)-1,2,3,6,7,11b-hexahydro-4H-pyrazino[2,1-a]isoquinolin-4-one.

Content: 97.5 per cent to 102.0 per cent (dried substance).

CHARACTERS

Appearance: white or almost white, crystalline powder.

Solubility: very slightly soluble in water, freely soluble in alcohol and in methylene chloride.

It shows polymorphism.

IDENTIFICATION

Infrared absorption spectrophotometry (2.2.24).

Comparison: *praziquantel CRS*.

If the spectra obtained show differences, dissolve 50 mg of the substance to be examined and 50 mg of the reference substance separately in 2 ml of *methanol R*. Evaporate and dry the residue at 60 °C at a pressure not exceeding 0.7 kPa. Record new spectra using the residues.

TESTS

Related substances. Liquid chromatography (2.2.29).

Test solution (a). Dissolve 40.0 mg of the substance to be examined in the mobile phase and dilute to 10.0 ml with the mobile phase.

Test solution (b). Dilute 1.0 ml of test solution (a) to 20.0 ml with the mobile phase.

Reference solution (a). Dissolve 40.0 mg of praziquantel CRS in the mobile phase and dilute to 10.0 ml with the mobile phase. Dilute 1.0 ml to 20.0 ml with the mobile phase.

Reference solution (b). Dissolve 5 mg of praziquantel impurity A CRS in reference solution (a) and dilute to 25.0 ml with reference solution (a). Dilute 2.0 ml of this solution to 20.0 ml with the mobile phase.

Reference solution (c). Dilute 1.0 ml of test solution (a) to 20.0 ml with the mobile phase. Dilute 5.0 ml of this solution to 50.0 ml with the mobile phase.

Column:

- **size:** $l = 0.25$ m, $\varnothing = 4.0$ mm,
- **stationary phase:** octadecylsilyl silica gel for chromatography R (5 μ m).

Mobile phase: acetonitrile R, water R (45:55 V/V).

Flow rate: 1 ml/min.

Detection: spectrophotometer at 210 nm.

Injection: 20 μ l; inject test solution (a) and reference solutions (b) and (c).

Run time: 5 times the retention time of praziquantel (retention time = about 9 min).

System suitability: reference solution (b):

- **resolution:** minimum 3.0 between the peaks corresponding to impurity A and praziquantel.

Limits:

- **any impurity:** not more than the area of the principal peak in the chromatogram obtained with reference solution (c) (0.5 per cent), not more than 1 such peak having an area greater than 0.4 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.2 per cent),
- **total:** not more than the area of the principal peak in the chromatogram obtained with reference solution (c) (0.5 per cent),
- **disregard limit:** 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.05 per cent).

Heavy metals (2.4.8): maximum 20 ppm.

1.0 g complies with limit test C. Prepare the standard using 2 ml of lead standard solution (10 ppm Pb) R.

Loss on drying (2.2.32): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 50 °C over diphosphorus pentoxide R at a pressure not exceeding 0.7 kPa for 2 h.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (2.2.29) as described in the test for related substances with the following modification.

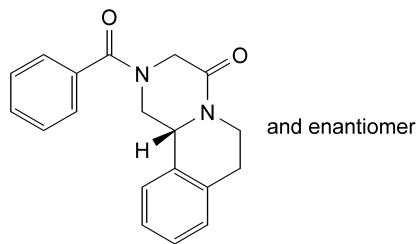
Injection: test solution (b) and reference solution (a).

Calculate the percentage content of $C_{19}H_{24}N_2O_2$.

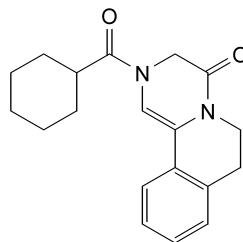
STORAGE

Protected from light.

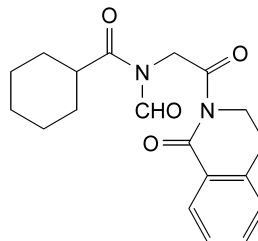
IMPURITIES



A. (11bRS)-2-benzoyl-1,2,3,6,7,11b-hexahydro-4H-pyrazino[2,1-a]isoquinolin-4-one,



B. 2-(cyclohexylcarbonyl)-2,3,6,7-tetrahydro-4H-pyrazino[2,1-a]isoquinolin-4-one,

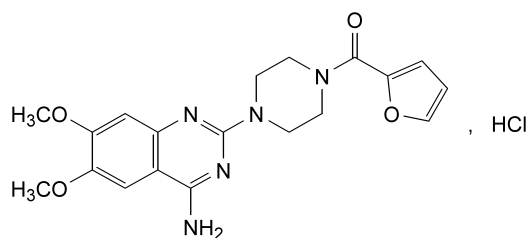


C. N-formyl-N-[2-oxo-2-(1-oxo-3,4-dihydroisoquinolin-2(1H-yl)ethyl)cyclohexanecarboxamide.

01/2005:0856

PRAZOSIN HYDROCHLORIDE

Prazosini hydrochloridum



$C_{19}H_{22}ClN_5O_4$

M_r 419.9

DEFINITION

1-(4-Amino-6,7-dimethoxyquinazolin-2-yl)-4-(furan-2-ylcarbonyl)piperazine hydrochloride.

Content: 98.5 per cent to 101.0 per cent (anhydrous substance).

CHARACTERS

Appearance: white or almost white powder.

Solubility: very slightly soluble in water, slightly soluble in alcohol and in methanol, practically insoluble in acetone.

IDENTIFICATION

First identification: B, D.

Second identification: A, C, D.

A. Dissolve 50.0 mg in a 0.1 per cent V/V solution of *hydrochloric acid R* in *methanol R* and dilute to 100.0 ml with the same acid solution. Dilute separately 1.0 ml and 5.0 ml of this solution to 100.0 ml with a 0.1 per cent V/V solution of *hydrochloric acid R* in *methanol R* (solution A and solution B, respectively). Examined between 220 nm and 280 nm (2.2.25), solution A shows an absorption maximum at 247 nm. The specific absorbance at the maximum is 1320 to 1400. Examined between 280 nm and 400 nm, solution B shows 2 absorption maxima, at 330 nm and 343 nm. The specific absorbances at the maxima are 260 to 280 and 240 to 265, respectively.

B. Infrared absorption spectrophotometry (2.2.24).

Preparation: discs of *potassium chloride R*.

Comparison: *prazosin hydrochloride CRS*.

C. Thin-layer chromatography (2.2.27).

Test solution. Dissolve 10 mg of the substance to be examined in a mixture of 1 volume of *diethylamine R*, 10 volumes of *methanol R* and 10 volumes of *methylene chloride R* and dilute to 10 ml with the same mixture of solvents.

Reference solution. Dissolve 10 mg of *prazosin hydrochloride CRS* in a mixture of 1 volume of *diethylamine R*, 10 volumes of *methanol R* and 10 volumes of *methylene chloride R* and dilute to 10 ml with the same mixture of solvents.

Plate: TLC silica gel GF₂₅₄ plate *R*.

Mobile phase: *diethylamine R*, *ethyl acetate R* (5:95 V/V).

Application: 10 µl.

Development: over 2/3 of the plate.

Drying: in a current of warm air.

Detection: examine in ultraviolet light at 254 nm.

Results: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with the reference solution.

D. Dissolve about 2 mg in 2 ml of *water R*. The solution gives reaction (a) of chlorides (2.3.1).

TESTS

Related substances. Liquid chromatography (2.2.29).

Test solution. Dissolve 50.0 mg of the substance to be examined in the mobile phase and dilute to 50.0 ml with the mobile phase.

Reference solution (a). Dilute 1.0 ml of the test solution to 100.0 ml with the mobile phase. Dilute 1.0 ml of the solution to 10.0 ml with the mobile phase.

Reference solution (b). Dissolve 8 mg of *metoclopramide hydrochloride CRS* in 1 ml of the test solution and dilute to 25.0 ml with the mobile phase. Dilute 1.0 ml of the solution to 10.0 ml with the mobile phase.

Column:

- *size*: $l = 0.25$ m, $\varnothing = 4.6$ mm,
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm),

Mobile phase: mix 50 volumes of *methanol R* and 50 volumes of a solution containing 3.484 g/l of *sodium pentanesulphonate R* and 3.64 g/l of *tetramethylammonium hydroxide R* adjusted to pH 5.0 with *glacial acetic acid R*.

Flow rate: 1 ml/min.

Detection: spectrophotometer at 254 nm.

Injection: 20 µl.

Run time: 6 times the retention time of prazosin.

Retention times: prazosin = about 9 min;
metoclopramide = about 5 min.

System suitability: reference solution (b):

- *resolution*: minimum 8 between the peaks due to metoclopramide and to prazosin.

Limits:

- *any impurity*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent),
- *total*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent),
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

Iron: maximum 100 ppm.

Atomic absorption spectrometry (2.2.23, *Method I*).

Test solution. To 1.0 g add dropwise about 1.5 ml of *nitric acid R*. After fuming has subsided, evaporate on a water-bath and ignite by gradually raising the temperature from 150 °C to 1000 °C, maintaining the final temperature for 1 h. Cool, dissolve the residue in 20 ml of *dilute hydrochloric acid R*, evaporate to about 5 ml and dilute to 25.0 ml with *dilute hydrochloric acid R*.

Reference solutions. Prepare the reference solutions using *iron standard solution (8 ppm Fe) R*, diluted as necessary with *water R*.

Source: iron hollow-cathode lamp.

Wavelength: 248 nm.

Flame: air-acetylene.

Nickel: maximum 50 ppm.

Atomic absorption spectrometry (2.2.23, *Method I*).

Test solution. Use the test solution prepared in the test for iron.

Reference solutions. Prepare the reference solutions using *nickel standard solution (10 ppm Ni) R*, diluted as necessary with *water R*.

Source: nickel hollow-cathode lamp.

Wavelength: 232 nm.

Flame: air-acetylene.

Water (2.5.12): maximum 0.5 per cent, determined on 1.000 g. Use a mixture of equal volumes of *methanol R* and *methylene chloride R* as the solvent.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

In order to avoid overheating in the reaction medium, mix thoroughly throughout and stop the titration immediately after the end-point has been reached.

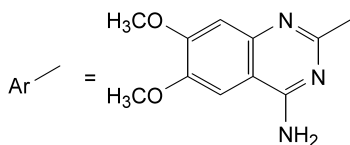
Dissolve 0.350 g in a mixture of 20 ml of *anhydrous formic acid R* and 30 ml of *acetic anhydride R*. Titrate quickly with 0.1 M *perchloric acid*, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *perchloric acid* is equivalent to 41.99 mg of $C_{19}H_{22}ClN_5O_4$.

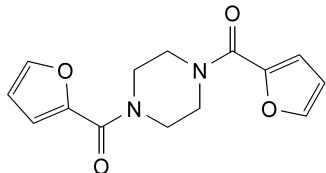
STORAGE

Protected from light.

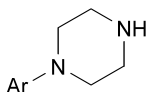
IMPURITIES



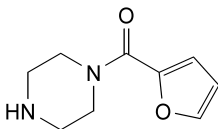
A. Ar-Cl: 2-chloro-6,7-dimethoxyquinazolin-4-amine,



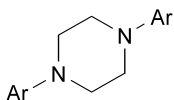
B. 1,4-bis(furan-2-ylcarbonyl)piperazine,



C. 6,7-dimethoxy-2-(piperazin-1-yl)quinazolin-4-amine,



D. 1-(furan-2-ylcarbonyl)piperazine,

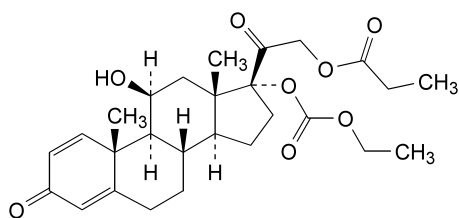


E. 2,2'-(piperazin-1,4-diyl)bis(6,7-dimethoxyquinazolin-4-amine).

01/2005:1467

PREDNICARBATE

Prednicarbatum



$C_{27}H_{36}O_8$

M_r 488.6

DEFINITION

Prednicarbate contains not less than 97.0 per cent and not more than the equivalent of 102.0 per cent of 11 β -hydroxy-3,20-dioxopregna-1,4-diene-17,21-diyl 17-ethylcarbonate 21-propanoate, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, crystalline powder, practically insoluble in water, freely soluble in alcohol and in acetone, sparingly soluble in propylene glycol.

It shows polymorphism.

IDENTIFICATION

A. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *prednicarbate CRS*. If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in the minimum volume of *alcohol R*, evaporate to dryness on a water-bath and record the spectra again using the residues.

B. Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel F₂₅₄ plate R*.

Test solution. Dissolve 10 mg of the substance to be examined in a mixture of 1 volume of *methanol R* and 9 volumes of *methylene chloride R* and dilute to 10 ml with the same mixture of solvents.

Reference solution (a). Dissolve 10 mg of *prednicarbate CRS* in a mixture of 1 volume of *methanol R* and 9 volumes of *methylene chloride R* and dilute to 10 ml with the same mixture of solvents.

Reference solution (b). Dissolve 5 mg of *prednisolone acetate CRS* in 5 ml of reference solution (a).

Apply to the plate 5 μ l of each solution. Prepare the mobile phase by adding a mixture of 1.2 volumes of *water R* and 8 volumes of *methanol R* to a mixture of 15 volumes of *ether R* and 77 volumes of *methylene chloride R*. Develop over a path of 15 cm. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. The principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a). Spray the plate with *alcoholic solution of sulphuric acid R*. Heat at 120 °C for 10 min or until the spots appear. Allow to cool. Examine in daylight and in ultraviolet light at 365 nm. The principal spot in the chromatogram obtained with the test solution is similar in position, colour in daylight, fluorescence in ultraviolet light at 365 nm and size to the principal spot in the chromatogram obtained with reference solution (a). The test is not valid unless the chromatogram obtained with reference solution (b) shows two clearly separated spots.

TESTS

Specific optical rotation (2.2.7). Dissolve 0.250 g in *alcohol R* and dilute to 25.0 ml with the same solvent. The specific optical rotation is + 60 to + 66, calculated with reference to the dried substance.

Related substances. Examine by liquid chromatography (2.2.29) as described under Assay.

Inject 20 μ l of reference solution (a). Adjust the sensitivity of the system so that the heights of the two principal peaks in the chromatogram obtained are about 50 per cent of the full scale of the recorder. When the chromatograms are recorded in the conditions described above, the retention times are: prednicarbate about 17 min and impurity F about 19 min. The test is not valid unless the resolution between the peaks corresponding to prednicarbate and impurity F is at least 3.0; if this resolution is not achieved, adjust the composition of the mobile phase.

Inject 20 μ l of the test solution and 20 μ l of reference solution (b). Continue the chromatography for twice the retention time of the principal peak. In the chromatogram obtained with the test solution: the area of any peak corresponding to impurity F is not greater than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (1 per cent); the area of any peak, apart from the principal peak and a peak corresponding to impurity F, is not greater than the area of the principal peak

in the chromatogram obtained with reference solution (b) (0.5 per cent); the sum of the areas of all the peaks, apart from the principal peak, is not greater than four times the area of the principal peak in the chromatogram obtained with reference solution (b) (2 per cent). Disregard any peak with an area less than 0.025 times the area of the principal peak in the chromatogram obtained with reference solution (b).

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C.

ASSAY

Examine by liquid chromatography (2.2.29). *Prepare the solutions immediately before use.*

Test solution. Dissolve 30.0 mg of the substance to be examined in the mobile phase and dilute to 50.0 ml with the mobile phase.

Reference solution (a). Dissolve 3 mg of *prednicarbate impurity F CRS* in the mobile phase, add 5.0 ml of the test solution and dilute to 100.0 ml with the mobile phase. Dilute 1.0 ml to 10.0 ml with the mobile phase.

Reference solution (b). Dilute 1.0 ml of the test solution to 200.0 ml with the mobile phase.

Reference solution (c). Dissolve 30.0 mg of *prednicarbate CRS* in the mobile phase and dilute to 50.0 ml with the mobile phase.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.125 m long and 4 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (5 µm),
- as mobile phase at a flow rate of 0.7 ml/min a mixture of 5 volumes of *acetonitrile R* and 6 volumes of *water R*,
- as detector a spectrophotometer set at 243 nm.

Inject 20 µl of reference solution (a). When the chromatograms are recorded in the prescribed conditions the retention times are: prednicarbate about 17 min and impurity F about 19 min. The test is not valid unless the resolution between the peaks corresponding to prednicarbate and impurity F is not less than 3.0; if this resolution is not achieved, adjust the composition of the mobile phase.

Inject 20 µl of reference solution (c). Adjust the sensitivity of the system so that the height of the principal peak in the chromatogram obtained is at least 50 per cent of the full scale of the recorder.

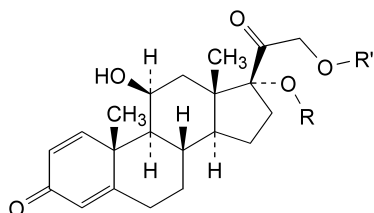
Inject 20 µl of the test solution.

Calculate the percentage content of prednicarbate.

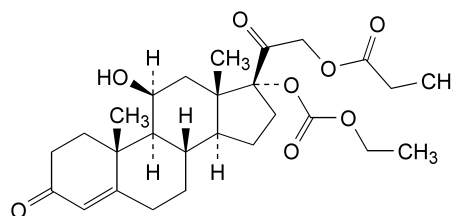
STORAGE

Store protected from light.

IMPURITIES



- A. R = R' = H: prednisolone,
 B. R = CO-O-C₂H₅, R' = H: prednisolone 17-ethylcarbonate,
 C. R = H, R' = CO-C₂H₅: prednisolone 21-propanoate,
 D. R = H, R' = CO-O-C₂H₅: prednisolone 21-ethylcarbonate,
 E. R = CO-O-C₂H₅, R' = CO-CH₃: prednisolone 21-acetate 17-ethylcarbonate,

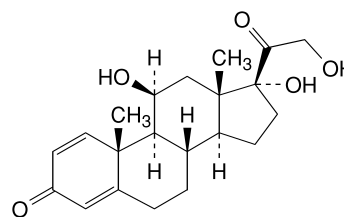


F. 11β-hydroxy-3,20-dioxopregn-4-ene-17,21-diyl 17-ethylcarbonate 21-propanoate(1,2-dihydroprednicarbate).

01/2005:0353

PREDNISOLONE

Prednisolonum



C₂₁H₂₈O₅

M_r 360.4

DEFINITION

Prednisolone contains not less than 97.0 per cent and not more than the equivalent of 103.0 per cent of 11β,17,21-trihydroxypregna-1,4-diene-3,20-dione, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, crystalline powder, hygroscopic, very slightly soluble in water, soluble in alcohol and in methanol, sparingly soluble in acetone, slightly soluble in methylene chloride.

It shows polymorphism.

IDENTIFICATION

- A. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *prednisolone CRS*. If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in the minimum volume of *acetone R* and evaporate to dryness on a water-bath. Record new spectra using the residues.
- B. Examine by thin-layer chromatography (2.2.27), using as the coating substance a suitable silica gel with a fluorescent indicator having an optimal intensity at 254 nm.

Test solution. Dissolve 10 mg of the substance to be examined in a mixture of 1 volume of *methanol R* and 9 volumes of *methylene chloride R* and dilute to 10 ml with the same mixture of solvents.

Reference solution (a). Dissolve 20 mg of *prednisolone CRS* in a mixture of 1 volume of *methanol R* and 9 volumes of *methylene chloride R* and dilute to 20 ml with the same mixture of solvents.

Reference solution (b). Dissolve 10 mg of *hydrocortisone CRS* in reference solution (a) and dilute to 10 ml with reference solution (a).

Apply to the plate 5 µl of each solution. Develop over a path of 15 cm using a mixture of 10 volumes of *methanol R* and 90 volumes of *methylene chloride R*. Allow the plate to dry in air and examine in ultraviolet

light at 254 nm. The principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a). Spray with *alcoholic solution of sulphuric acid R*. Heat at 120 °C for 10 min or until the spots appear. Allow to cool. Examine the chromatograms in daylight and in ultraviolet light at 365 nm. The principal spot in the chromatogram obtained with the test solution is similar in position, colour in daylight, fluorescence in ultraviolet light at 365 nm and size to the principal spot in the chromatogram obtained with reference solution (a). The test is not valid unless the chromatogram obtained with reference solution (b) shows 2 clearly separated spots.

TESTS

Specific optical rotation (2.2.7). Dissolve 0.250 g in *dioxan R* and dilute to 25.0 ml with the same solvent. The specific optical rotation is + 96 to + 102, calculated with reference to the dried substance.

Related substances. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 25.0 mg of the substance to be examined in 2 ml of *tetrahydrofuran R* and dilute to 10.0 ml with *water R*.

Reference solution (a). Dissolve 2 mg of *prednisolone CRS* and 2 mg of *hydrocortisone CRS* in the mobile phase and dilute to 100.0 ml with the mobile phase.

Reference solution (b). Dilute 1.0 ml of the test solution to 100.0 ml with the mobile phase.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4.6 mm in internal diameter packed with *base-deactivated end-capped octadecylsilyl silica gel for chromatography R* (5 µm),
- as mobile phase at a flow rate of 1 ml/min a mixture prepared as follows: in a 1000 ml volumetric flask mix 220 ml of *tetrahydrofuran R* with 700 ml of *water R* and allow to equilibrate; dilute to 1000 ml with *water R* and mix again,
- as detector a spectrophotometer set at 254 nm, maintaining the temperature of the column at 45 °C.

Equilibrate the column with the mobile phase at a flow rate of 1 ml/min for about 30 min.

Adjust the sensitivity of the system so that the height of the principal peak in the chromatogram obtained with 20 µl of reference solution (b) is at least 50 per cent of the full scale of the recorder.

Inject 20 µl of reference solution (a). When the chromatograms are recorded in the prescribed conditions, the retention times are: prednisolone about 14 min and hydrocortisone about 15.5 min. The test is not valid unless in the chromatogram obtained with reference solution (a) the resolution between the peaks due to prednisolone and hydrocortisone is at least 2.2. If necessary, adjust the concentration of *tetrahydrofuran R* in the mobile phase.

Inject separately 20 µl of the solvent mixture of the test solution as a blank, 20 µl of the test solution and 20 µl of reference solution (b). Continue the chromatography of the test solution for 4.5 times the retention time of the principal peak. In the chromatogram obtained with the test solution: the area of any peak, apart from the principal peak, is not greater than the area of the principal peak in the chromatogram obtained with reference solution (b) (1 per cent) and not more than one such peak has an area greater than half the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent); the sum

of the areas of all the peaks, apart from the principal peak, is not greater than 2.0 times the area of the principal peak in the chromatogram obtained with reference solution (b) (2 per cent). Disregard any peak obtained with the blank run and any peak with an area less than 0.05 times the area of the principal peak in the chromatogram obtained with reference solution (b).

Loss on drying (2.2.32). Not more than 1.0 per cent, determined on 0.500 g by drying in an oven at 100-105 °C.

ASSAY

Dissolve 0.100 g in *alcohol R* and dilute to 100.0 ml with the same solvent. Dilute 2.0 ml of the solution to 100.0 ml with *alcohol R*. Measure the absorbance (2.2.25) at the maximum at 243.5 nm.

Calculate the content of C₂₁H₂₈O₅ taking the specific absorbance to be 415.

STORAGE

Store in an airtight container, protected from light.

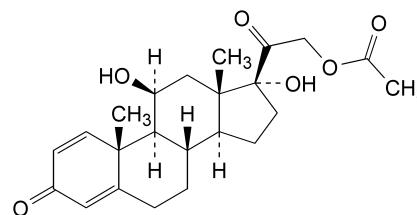
IMPURITIES

A. hydrocortisone.

01/2005:0734

PREDNISOLONE ACETATE

Prednisoloni acetat



C₂₃H₃₀O₆

M_r 402.5

DEFINITION

Prednisolone acetate contains not less than 97.0 per cent and not more than the equivalent of 103.0 per cent of 11β,17-dihydroxy-3,20-dioxopregna-1,4-dien-21-yl acetate, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, crystalline powder, practically insoluble in water, slightly soluble in alcohol and in methylene chloride.

It melts at about 230 °C, with decomposition.

IDENTIFICATION

First identification: A, B.

Second identification: C, D, E.

A. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *prednisolone acetate CRS*. Examine the substances prepared as discs.

B. Examine by thin-layer chromatography (2.2.27), using as the coating substance a suitable silica gel with a fluorescent indicator having an optimal intensity at 254 nm.

Test solution. Dissolve 10 mg of the substance to be examined in a mixture of 1 volume of *methanol R* and 9 volumes of *methylene chloride R* and dilute to 10 ml with the same mixture of solvents.

Reference solution (a). Dissolve 20 mg of *prednisolone acetate CRS* in a mixture of 1 volume of *methanol R* and 9 volumes of *methylene chloride R* and dilute to 20 ml with the same mixture of solvents.

Reference solution (b). Dissolve 10 mg of *prednisolone pivalate CRS* in reference solution (a) and dilute to 10 ml with the same solution.

Apply separately to the plate 5 µl of each solution. Prepare the mobile phase by adding a mixture of 1.2 volumes of *water R* and 8 volumes of *methanol R* to a mixture of 15 volumes of *ether R* and 77 volumes of *methylene chloride R*. Develop over a path of 15 cm. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. The principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a). Spray the plate with *alcoholic solution of sulphuric acid R*. Heat at 120 °C for 10 min or until the spots appear. Allow to cool. Examine in daylight and in ultraviolet light at 365 nm. The principal spot in the chromatogram obtained with the test solution is similar in position, colour in daylight, fluorescence in ultraviolet light at 365 nm and size to the principal spot in the chromatogram obtained with reference solution (a). The test is not valid unless the chromatogram obtained with reference solution (b) shows two clearly separated spots.

- C. Examine by thin-layer chromatography (2.2.27), using as the coating substance a suitable silica gel with a fluorescent indicator having an optimal intensity at 254 nm.

Test solution (a). Dissolve 25 mg of the substance to be examined in *methanol R* with gentle heating and dilute to 5 ml with the same solvent. This solution is also used to prepare test solution (b). Dilute 2 ml of the solution to 10 ml with *methylene chloride R*.

Test solution (b). Transfer 2 ml of the solution obtained during preparation of test solution (a) to a 15 ml glass tube with a ground-glass stopper or a polytetrafluoroethylene cap. Add 10 ml of *saturated methanolic potassium hydrogen carbonate solution R* and immediately pass a stream of *nitrogen R* briskly through the solution for 5 min. Stopper the tube. Heat in a water-bath at 45 °C, protected from light, for 2 h 30 min. Allow to cool.

Reference solution (a). Dissolve 25 mg of *prednisolone acetate CRS* in *methanol R* with gentle heating and dilute to 5 ml with the same solvent. This solution is also used to prepare reference solution (b). Dilute 2 ml of the solution to 10 ml with *methylene chloride R*.

Reference solution (b). Transfer 2 ml of the solution obtained during preparation of reference solution (a) to a 15 ml glass tube with a ground-glass stopper or a polytetrafluoroethylene cap. Add 10 ml of *saturated methanolic potassium hydrogen carbonate solution R* and immediately pass a stream of *nitrogen R* briskly through the solution for 5 min. Stopper the tube. Heat in a water-bath at 45 °C, protected from light, for 2 h 30 min. Allow to cool.

Apply separately to the plate 5 µl of each solution. Prepare the mobile phase by adding a mixture of 1.2 volumes of *water R* and 8 volumes of *methanol R* to a mixture of 15 volumes of *ether R* and 77 volumes of *methylene chloride R*. Develop over a path of 15 cm. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. The principal spot in each of the chromatograms obtained with the test solutions is similar in position and size to the principal spot in the chromatogram obtained with the corresponding reference solution. Spray the plate

with *alcoholic solution of sulphuric acid R*. Heat at 120 °C for 10 min or until the spots appear. Allow to cool. Examine in daylight and in ultraviolet light at 365 nm. The principal spot in each of the chromatograms obtained with the test solutions is similar in position, colour in daylight, fluorescence in ultraviolet light at 365 nm and size to the principal spot in the chromatogram obtained with the corresponding reference solution. The principal spot in each of the chromatograms obtained with test solution (b) and reference solution (b) has an R_f value distinctly lower than that of the principal spots in each of the chromatograms obtained with test solution (a) and reference solution (a).

- D. Add about 2 mg to 2 ml of *sulphuric acid R* and shake to dissolve. Within 5 min, an intense red colour develops. When examined in ultraviolet light at 365 nm, a reddish-brown fluorescence is seen. Add the solution to 10 ml of *water R* and mix. The colour fades and there is greenish-yellow fluorescence in ultraviolet light at 365 nm.

- E. About 10 mg gives the reaction of acetyl (2.3.1).

TESTS

Specific optical rotation (2.2.7). Dissolve 0.250 g in *dioxan R* and dilute to 25.0 ml with the same solvent. The specific optical rotation is + 112 to + 119, calculated with reference to the dried substance.

Related substances. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 25.0 mg of the substance to be examined in *methanol R* and dilute to 10.0 ml with the same solvent.

Reference solution (a). Dissolve 2 mg of *prednisolone acetate CRS* and 2 mg of *hydrocortisone acetate CRS* in the mobile phase and dilute to 100.0 ml with the mobile phase.

Reference solution (b). Dilute 1.0 ml of the test solution to 100.0 ml with the mobile phase.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4.6 mm in internal diameter, packed with *base-deactivated end-capped octadecylsilyl silica gel for chromatography R* (5 µm),
- as mobile phase at a flow rate of 1 ml/min a mixture prepared as follows: in a 1000 ml volumetric flask mix 350 ml of *acetonitrile R* with 600 ml of *water R* and allow to equilibrate; adjust the volume to 1000 ml with *water R* and mix again,
- as detector a spectrophotometer set at 254 nm.

Equilibrate the column with the mobile phase at a flow rate of 1 ml/min for about 30 min.

Adjust the sensitivity of the system so that the height of the principal peak in the chromatogram obtained with 20 µl of reference solution (b) is at least 50 per cent of the full scale of the recorder.

Inject 20 µl of reference solution (a). When the chromatograms are recorded in the prescribed conditions, the retention times are: prednisolone acetate, about 24 min and hydrocortisone acetate, about 26 min. The test is not valid unless the resolution between the peaks corresponding to prednisolone acetate and hydrocortisone acetate is at least 2.5; if necessary, adjust the concentration of acetonitrile in the mobile phase.

Inject separately 20 µl of the test solution and 20 µl of reference solution (b). Continue the chromatography for 2.5 times the retention time of the principal peak in the chromatogram obtained with the test solution. In the chromatogram obtained with the test solution: the area of

any peak, apart from the principal peak, is not greater than the area of the principal peak in the chromatogram obtained with reference solution (b) (1 per cent) and not more than one such peak has an area greater than half the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent); the sum of the areas of all the peaks, apart from the principal peak, is not greater than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (2 per cent). Disregard any peak due to the solvent and any peak with an area less than 0.05 times the area of the principal peak in the chromatogram obtained with reference solution (b).

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C.

ASSAY

Dissolve 0.100 g in *alcohol R* and dilute to 100.0 ml with the same solvent. Dilute 2.0 ml of the solution to 100.0 ml with *alcohol R*. Measure the absorbance (2.2.25) at the maximum at 243 nm.

Calculate the content of $C_{26}H_{30}O_6$ taking the specific absorbance to be 370.

STORAGE

Store protected from light.

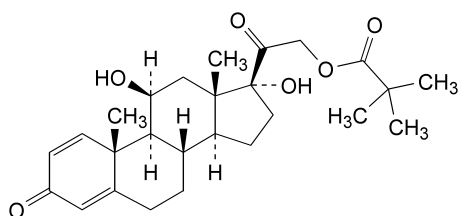
IMPURITIES

- A. hydrocortisone acetate,
- B. prednisolone.

01/2005:0736

PREDNISOLONE PIVALATE

Prednisoloni pivalas



$C_{26}H_{36}O_6$

M_r 444.6

DEFINITION

Prednisolone pivalate contains not less than 97.0 per cent and not more than the equivalent of 103.0 per cent of 11 β ,17-dihydroxy-3,20-dioxopregna-1,4-dien-21-yl 2,2-dimethylpropanoate, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, crystalline powder, practically insoluble in water, slightly soluble in alcohol, soluble in methylene chloride.

It melts at about 229 °C, with decomposition.

IDENTIFICATION

First identification: B, C.

Second identification: A, C, D.

- A. Dissolve 10.0 mg in *ethanol R* and dilute to 100.0 ml with the same solvent. Place 2.0 ml of this solution in a ground-glass-stoppered tube, add 10.0 ml of

phenylhydrazine-sulphuric acid solution R, mix and heat in a water-bath at 60 °C for 20 min. Cool immediately. The absorbance (2.2.25) of the solution at the maximum at 415 nm is 0.20 to 0.30.

- B. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *prednisolone pivalate CRS*. If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in the minimum volume of *alcohol R*, evaporate to dryness on a water-bath and record the spectra again using the residues.
- C. Examine by thin-layer chromatography (2.2.27), using as the coating substance a suitable silica gel with a fluorescent indicator having an optimal intensity at 254 nm.

Test solution. Dissolve 10 mg of the substance to be examined in a mixture of 1 volume of *methanol R* and 9 volumes of *methylene chloride R* and dilute to 10 ml with the same mixture of solvents.

Reference solution (a). Dissolve 10 mg of *prednisolone pivalate CRS* in a mixture of 1 volume of *methanol R* and 9 volumes of *methylene chloride R* and dilute to 10 ml with the same mixture of solvents.

Reference solution (b). Dissolve 10 mg of *prednisolone acetate CRS* in a mixture of 1 volume of *methanol R* and 9 volumes of *methylene chloride R* and dilute to 10 ml with the same mixture of solvents. Dilute 5 ml of this solution to 10 ml with reference solution (a).

Apply separately to the plate 5 μ l of each solution. Prepare the mobile phase by adding a mixture of 1.2 volumes of *water R* and 8 volumes of *methanol R* to a mixture of 15 volumes of *ether R* and 77 volumes of *methylene chloride R*. Develop over a path of 15 cm. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. The principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a). Spray the plate with *alcoholic solution of sulphuric acid R*. Heat at 120 °C for 10 min or until the spots appear. Allow to cool. Examine in daylight and in ultraviolet light at 365 nm. The principal spot in the chromatogram obtained with the test solution is similar in position, colour in daylight, fluorescence in ultraviolet light at 365 nm and size to the principal spot in the chromatogram obtained with reference solution (a). The test is not valid unless the chromatogram obtained with reference solution (b) shows two clearly separated spots.

- D. Add about 2 mg to 2 ml of *sulphuric acid R* and shake to dissolve. Within 5 min, an intense red colour develops. When examined in ultraviolet light at 365 nm, a reddish-brown fluorescence is seen. Add the solution to 10 ml of *water R* and mix. The colour fades and there is greenish-yellow fluorescence in ultraviolet light at 365 nm.

TESTS

Specific optical rotation (2.2.7). Dissolve 0.250 g in *dioxan R* and dilute to 25.0 ml with the same solvent. The specific optical rotation is + 104 to + 112, calculated with reference to the dried substance.

Related substances. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 62.5 mg of the substance to be examined in 2 ml of a mixture of 1 volume of *water R* and 4 volumes of *tetrahydrofuran R* and dilute to 25.0 ml with the mobile phase.

01/2005:0735

Reference solution (a). Dissolve 25 mg of *prednisolone acetate CRS*, 25 mg of *cortisone acetate CRS* and 25 mg of *prednisolone pivalate CRS* in 2 ml of a mixture of 1 volume of *water R* and 4 volumes of *tetrahydrofuran R* and dilute to 25.0 ml with the mobile phase. Dilute 1.0 ml of this solution to 25.0 ml with the mobile phase.

Reference solution (b). Dilute 1.0 ml of the test solution to 50.0 ml with the mobile phase.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.15 m long and 4.6 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (5 µm),
- as mobile phase at a flow rate of 1 ml/min a mixture prepared as follows: mix 19 ml of *butyl acetate R1* carefully with 37 ml of *tetrahydrofuran R* and 213 ml of *ethylene glycol monomethyl ether R* and then with 231 ml of *water R*, leave to equilibrate for 1 h and filter through a 0.45 µm filter,
- as detector a spectrophotometer set at 254 nm.

Adjust the sensitivity so that the height of the principal peak in the chromatogram obtained with reference solution (b) is 70 per cent to 90 per cent of the full scale.

Equilibrate the column with the mobile phase at a flow rate of 1 ml/min for about 30 min.

Inject 20 µl of reference solution (a). When the chromatograms are recorded in the conditions described above the retention times are: prednisolone acetate about 3.5 min, cortisone acetate about 4.5 min and prednisolone pivalate about 13 min. The test is not valid unless the resolution between the peaks corresponding to prednisolone acetate and cortisone acetate is at least 2.5; if this resolution is not achieved, adjust the concentration of water in the mobile phase.

Inject separately 20 µl of the test solution and 20 µl of reference solution (b). Continue the chromatography for 1.5 times the retention time of the principal peak. In the chromatogram obtained with the test solution: the area of any peak apart from the principal peak is not greater than the area of the principal peak in the chromatogram obtained with reference solution (b) (2.0 per cent) and not more than one such peak has an area greater than half the area of the principal peak in the chromatogram obtained with reference solution (b) (1.0 per cent); the sum of the areas of all the peaks apart from the principal peak is not greater than 1.25 times the area of the principal peak in the chromatogram obtained with reference solution (b) (2.5 per cent). Disregard any peak due to the solvent and any peak with an area less than 0.025 times the area of the principal peak in the chromatogram obtained with reference solution (b).

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C.

ASSAY

Dissolve 0.100 g in *alcohol R* and dilute to 100.0 ml with the same solvent. Dilute 5.0 ml of the solution to 250.0 ml with *alcohol R*. Measure the absorbance (2.2.25) at the maximum at 243 nm.

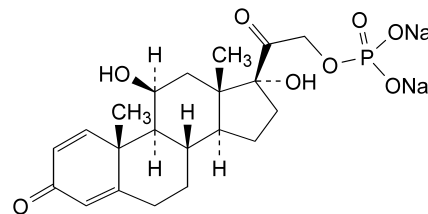
Calculate the content of $C_{21}H_{27}O_6$ taking the specific absorbance to be 337.

STORAGE

Store protected from light.

PREDNISOLONE SODIUM PHOSPHATE

Prednisoloni natrii phosphas


 $C_{21}H_{27}Na_2O_8P$
 M_r 484.4

DEFINITION

Prednisolone sodium phosphate contains not less than 96.0 per cent and not more than the equivalent of 103.0 per cent of 11β,17-dihydroxy-3,20-dioxpregna-1,4-dien-21-yl disodium phosphate, calculated with reference to the anhydrous substance.

CHARACTERS

A white or almost white, crystalline powder, hygroscopic, freely soluble in water, very slightly soluble in alcohol.

IDENTIFICATION

First identification: B, C.

Second identification: A, C, D, E.

- Dissolve 10.0 mg in 5 ml of *water R* and dilute to 100.0 ml with *ethanol R*. Place 2.0 ml of this solution in a ground-glass-stoppered tube, add 10.0 ml of *phenylhydrazine-sulphuric acid solution R*, mix and heat in a water-bath at 60 °C for 20 min. Cool immediately. The absorbance (2.2.25) of the solution at the maximum at 415 nm is 0.10 to 0.20.
- Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *prednisolone sodium phosphate CRS*. If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in the minimum volume of *alcohol R*, evaporate to dryness on a water-bath and record the spectra again using the residues.
- Examine by thin-layer chromatography (2.2.27), using as the coating substance a suitable silica gel with a fluorescent indicator having an optimal intensity at 254 nm.

Test solution. Dissolve 10 mg of the substance to be examined in *methanol R* and dilute to 10 ml with the same solvent.

Reference solution (a). Dissolve 10 mg of *prednisolone sodium phosphate CRS* in *methanol R* and dilute to 10 ml with the same solvent.

Reference solution (b). Dissolve 10 mg of *dexamethasone sodium phosphate CRS* in *methanol R* and dilute to 10 ml with the same solvent. Dilute 5 ml of this solution to 10 ml with reference solution (a).

Apply separately to the plate 5 µl of each solution. Develop over a path of 15 cm using a mixture of 20 volumes of *glacial acetic acid R*, 20 volumes of *water R* and 60 volumes of *butanol R*. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. The principal spot in the chromatogram obtained with the test solution is similar in position and size to the

principal spot in the chromatogram obtained with reference solution (a). Spray the plate with *alcoholic solution of sulphuric acid R*. Heat at 120 °C for 10 min or until the spots appear. Allow to cool. Examine in daylight and in ultraviolet light at 365 nm. The principal spot in the chromatogram obtained with the test solution is similar in position, colour in daylight, fluorescence in ultraviolet light at 365 nm and size to the principal spot in the chromatogram obtained with reference solution (a). The test is not valid unless the chromatogram obtained with reference solution (b) shows two spots which may however not be completely separated.

- D. Add about 2 mg to 2 ml of *sulphuric acid R* and shake to dissolve. Within 5 min, an intense red colour develops. When examined in ultraviolet light at 365 nm, a reddish-brown fluorescence is seen. Add the solution to 10 ml of *water R* and mix. The colour fades and there is greenish-yellow fluorescence in ultraviolet light at 365 nm.
- E. To about 40 mg add 2 ml of *sulphuric acid R* and heat gently until white fumes are evolved. Add *nitric acid R* dropwise, continue the heating until the solution is almost colourless and cool. Add 2 ml of *water R*, heat until white fumes are again evolved, cool, add 10 ml of *water R* and neutralise to *red litmus paper R* with *dilute ammonia R1*. The solution gives reaction (a) of sodium (2.3.1) and reaction (b) of phosphates (2.3.1).

TESTS

Solution S. Dissolve 1.0 g in *carbon dioxide-free water R* and dilute to 20 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and not more intensely coloured than reference solution B₇ (2.2.2, Method II).

pH (2.2.3). The pH of solution S is 7.5 to 9.0.

Specific optical rotation (2.2.7). Dissolve 0.250 g in *water R* and dilute to 25.0 ml with the same solvent. The specific optical rotation is + 94 to + 100, calculated with reference to the anhydrous substance.

Related substances. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 62.5 mg of the substance to be examined in the mobile phase and dilute to 25.0 ml with the mobile phase.

Reference solution (a). Dissolve 25 mg of *prednisolone sodium phosphate CRS* and 25 mg of *prednisolone CRS* in the mobile phase and dilute to 25.0 ml with the same solvent. Dilute 1.0 ml of this solution to 25.0 ml with the mobile phase.

Reference solution (b). Dilute 1.0 ml of the test solution to 50.0 ml with the mobile phase.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.15 m long and 4.6 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (5 µm),
- as mobile phase at a flow rate of 1 ml/min a mixture prepared as follows: in a 250 ml conical flask weigh 1.360 g of *potassium dihydrogen phosphate R* and 0.600 g of *hexylamine R*, mix and allow to stand for 10 min and then dissolve in 185 ml of *water R*; add 65 ml of *acetonitrile R*, mix and filter through a 0.45 µm filter,
- as detector a spectrophotometer set at 254 nm.

Inject 20 µl of reference solution (b). Adjust the sensitivity of the system so that the height of the principal peak in the chromatogram obtained with reference solution (b) is 70 per cent to 90 per cent of the full scale of the recorder.

Equilibrate the column with the mobile phase at a flow rate of 1 ml/min for about 30 min.

Inject 20 µl of reference solution (a). When the chromatograms are recorded in the conditions described above the retention times are: *prednisolone sodium phosphate*, about 6.5 min and *prednisolone*, about 8.5 min. The test is not valid unless the resolution between the peaks corresponding to *prednisolone sodium phosphate* and *prednisolone* is at least 4.5; if this resolution is not achieved, increase the concentration of *acetonitrile R* or increase the concentration of *water R* in the mobile phase.

Inject separately 20 µl of the test solution and 20 µl of reference solution (b). Continue the chromatography for three times the retention time of the principal peak. In the chromatogram obtained with the test solution: the area of any peak apart from the principal peak is not greater than the area of the principal peak in the chromatogram obtained with reference solution (b) (2 per cent) and not more than one such peak has an area greater than half the area of the principal peak in the chromatogram obtained with reference solution (b) (1 per cent); the sum of the areas of all the peaks apart from the principal peak is not greater than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (3 per cent). Disregard any peak due to the solvent and any peak with an area less than 0.025 times the area of the principal peak in the chromatogram obtained with reference solution (b).

Inorganic phosphate. Dissolve 50 mg in *water R* and dilute to 100 ml with the same solvent. To 10 ml of this solution add 5 ml of *molybdovanadic reagent R*, mix and allow to stand for 5 min. Any yellow colour in the solution is not more intense than that in a standard prepared at the same time in the same manner using 10 ml of *phosphate standard solution* (5 ppm PO₄) *R* (1 per cent).

Water (2.5.12). Not more than 8.0 per cent, determined on 0.200 g by the semi-micro determination of water.

ASSAY

Dissolve 0.100 g in *water R* and dilute to 100.0 ml with the same solvent. Dilute 5.0 ml of the solution to 250.0 ml with *water R*. Measure the absorbance (2.2.25) at the maximum at 247 nm.

Calculate the content of C₂₁H₂₇Na₂O₈P taking the specific absorbance to be 312.

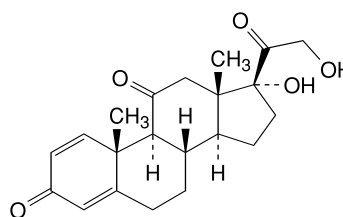
STORAGE

Store protected from light.

01/2005:0354

PREDNISONE

Prednisonum



C₂₁H₂₆O₅

M_r 358.4

DEFINITION

Prednisone contains not less than 97.0 per cent and not more than the equivalent of 103.0 per cent of 17,21-dihydroxypregna-1,4-diene-3,11,20-trione, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, crystalline powder, practically insoluble in water, slightly soluble in alcohol and in methylene chloride.

It shows polymorphism.

IDENTIFICATION

First identification: A, B.

Second identification: C, D.

- A. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *prednisone CRS*. If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in the minimum volume of *acetone R*, evaporate to dryness on a water-bath and record new spectra using the residues.
- B. Examine by thin-layer chromatography (2.2.27), using as the coating substance a suitable silica gel with a fluorescent indicator having an optimal intensity at 254 nm.

Test solution. Dissolve 10 mg of the substance to be examined in a mixture of 1 volume of *methanol R* and 9 volumes of *methylene chloride R* and dilute to 10 ml with the same mixture of solvents.

Reference solution (a). Dissolve 20 mg of *prednisone CRS* in a mixture of 1 volume of *methanol R* and 9 volumes of *methylene chloride R* and dilute to 20 ml with the same mixture of solvents.

Reference solution (b). Dissolve 10 mg of *betamethasone CRS* in reference solution (a) and dilute to 10 ml with the same solution.

Apply to the plate 5 µl of each solution. Prepare the mobile phase by adding a mixture of 1.2 volumes of *water R* and 8 volumes of *methanol R* to a mixture of 15 volumes of *ether R* and 77 volumes of *methylene chloride R*. Develop over a path of 15 cm. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. The principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a). Spray with *alcoholic solution of sulphuric acid R*. Heat at 120 °C for 10 min or until the spots appear. Allow to cool. Examine the plate in daylight and in ultraviolet light at 365 nm. The principal spot in the chromatogram obtained with the test solution is similar in position, colour in daylight, fluorescence in ultraviolet light at 365 nm and size to the principal spot in the chromatogram obtained with reference solution (a). The test is not valid unless the chromatogram obtained with reference solution (b) shows two clearly separated spots.

- C. Examine by thin-layer chromatography (2.2.27), using as the coating substance a suitable silica gel with a fluorescent indicator having an optimal intensity at 254 nm.

Test solution (a). Dissolve 25 mg of the substance to be examined in *methanol R* and dilute to 5 ml with the same solvent. This solution is also used to prepare test solution (b). Dilute 2 ml of the solution to 10 ml with *methylene chloride R*.

Test solution (b). Transfer 0.4 ml of the solution obtained during preparation of test solution (a) to a glass tube 100 mm long and 20 mm in diameter and fitted with a ground-glass stopper or a polytetrafluoroethylene cap and evaporate the solvent with gentle heating under a stream of *nitrogen R*. Add 2 ml of a 15 per cent V/V solution of *glacial acetic acid R* and 50 mg of *sodium bismuthate R*. Stopper the tube and shake the suspension in a mechanical shaker, protected from light, for 1 h. Add 2 ml of a 15 per cent V/V solution of *glacial acetic acid R* and filter into a 50 ml separating funnel, washing the filter with two quantities, each of 5 ml, of *water R*. Shake the clear filtrate with 10 ml of *methylene chloride R*. Wash the organic layer with 5 ml of 1 M *sodium hydroxide* and two quantities, each of 5 ml, of *water R*. Dry over *anhydrous sodium sulphate R*.

Reference solution (a). Dissolve 25 mg of *prednisone CRS* in *methanol R* and dilute to 5 ml with the same solvent. This solution is also used to prepare reference solution (b). Dilute 2 ml of the solution to 10 ml with *methylene chloride R*.

Reference solution (b). Transfer 0.4 ml of the solution obtained during preparation of reference solution (a) to a glass tube 100 mm long and 20 mm in diameter and fitted with a ground-glass stopper or a polytetrafluoroethylene cap and evaporate the solvent with gentle heating under a stream of *nitrogen R*. Add 2 ml of a 15 per cent V/V solution of *glacial acetic acid R* and 50 mg of *sodium bismuthate R*. Stopper the tube and shake the suspension in a mechanical shaker, protected from light, for 1 h. Add 2 ml of a 15 per cent V/V solution of *glacial acetic acid R* and filter into a 50 ml separating funnel, washing the filter with two quantities, each of 5 ml, of *water R*. Shake the clear filtrate with 10 ml of *methylene chloride R*. Wash the organic layer with 5 ml of 1 M *sodium hydroxide* and two quantities, each of 5 ml, of *water R*. Dry over *anhydrous sodium sulphate R*.

Apply to the plate 5 µl of test solution (a) and reference solution (a) and 50 µl of test solution (b) and reference solution (b), applying the latter two in small quantities in order to obtain small spots. Prepare the mobile phase by adding a mixture of 1.2 volumes of *water R* and 8 volumes of *methanol R* to a mixture of 15 volumes of *ether R* and 77 volumes of *methylene chloride R*. Develop over a path of 15 cm. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. The principal spot in each of the chromatograms obtained with the test solutions is similar in position and size to the principal spot in the chromatogram obtained with the corresponding reference solution. Spray with *alcoholic solution of sulphuric acid R*. Heat at 120 °C for 10 min or until the spots appear. Allow to cool. Examine the chromatograms in daylight and in ultraviolet light at 365 nm. The principal spot in each of the chromatograms obtained with the test solutions is similar in position, colour in daylight, fluorescence in ultraviolet light at 365 nm and size to the principal spot in the chromatogram obtained with the corresponding reference solution. The principal spots in the chromatograms obtained with test solution (b) and reference solution (b) have an R_f value distinctly higher than that of the principal spots in the chromatograms obtained with test solution (a) and reference solution (a).

- D. Add about 2 mg to 2 ml of *sulphuric acid R* and shake to dissolve. Within 5 min, a yellow colour develops with a blue fluorescence in ultraviolet light at 365 nm. Add the solution to 10 ml of *water R* and mix. The colour fades but the blue fluorescence in ultraviolet light does not disappear.

TESTS

Specific optical rotation (2.2.7). Dissolve 0.125 g in *dioxan R* and dilute to 25.0 ml with the same solvent. The specific optical rotation is + 167 to + 175, calculated with reference to the dried substance.

Related substances. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 25.0 mg of the substance to be examined in *methanol R* and dilute to 10.0 ml with the same solvent.

Reference solution (a). Dissolve 2 mg of *prednisone CRS* and 2 mg of *prednisolone CRS* in *methanol R* and dilute to 100.0 ml with the same solvent.

Reference solution (b). Dilute 1.0 ml of the test solution to 100.0 ml with *methanol R*.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4.6 mm in internal diameter, packed with *octadecylsilyl silica gel for chromatography R* (5 µm),
- as mobile phase at a flow rate of 2.5 ml/min a linear gradient programme using the following conditions:
Mobile phase A. In a 1000 ml volumetric flask mix 100 ml of *acetonitrile R* with 200 ml of *methanol R* and 650 ml of *water R* and allow to equilibrate; adjust the volume to 1000 ml with *water R* and mix again,
Mobile phase B. *Acetonitrile R*,

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)	Comment
0	100	0	isocratic
25	100	0	begin linear gradient
40	40	60	end chromatogram, change to 100B
41	0	100	begin treatment with B
46	0	100	end treatment, return to 100A
47	100	0	begin equilibration with A
52 = 0	100	0	end equilibration, begin next chromatogram

- as detector a spectrophotometer set at 254 nm, maintaining the temperature of the column at 45 °C.
- Equilibrate the column for at least 30 min with mobile phase B at a flow rate of 2.5 ml/min and then with the mobile phase A for 5 minutes. For subsequent chromatograms, use the conditions described from 40.0 to 52.0 min. Adjust the sensitivity of the system so that the height of the principal peak in the chromatogram obtained with 20 µl of reference solution (b) is not less than 50 per cent of the full scale of the recorder.

Inject 20 µl of reference solution (a). When the chromatograms are recorded in the prescribed conditions, the retention times are: prednisone, about 19 minutes and prednisolone, about 23 minutes. The test is not valid unless the resolution between the peaks corresponding to prednisone and prednisolone is at least 2.7; if necessary, adjust the concentration of acetonitrile in mobile phase A. Inject separately 20 µl of *methanol R* as a blank, 20 µl of the test solution and 20 µl of reference solution (b). In the chromatogram obtained with the test solution: the area of any peak apart from the principal peak is not greater than 0.25 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.25 per cent); the sum of the areas of all the peaks, apart from the principal peak, is not greater than 0.75 times the area of the

principal peak in the chromatogram obtained with reference solution (b) (0.75 per cent). Disregard any peak due to the blank run and any peak with an area less than 0.05 times the area of the principal peak in the chromatogram obtained with reference solution (b).

Loss on drying (2.2.32). Not more than 1.0 per cent, determined on 0.500 g by drying in an oven at 100 °C to 105 °C.

ASSAY

Dissolve 0.100 g in *alcohol R* and dilute to 100.0 ml with the same solvent. Dilute 2.0 ml of the solution to 100.0 ml with *alcohol R*. Measure the absorbance (2.2.25) at the maximum at 238 nm.

Calculate the content of C₂₁H₂₆O₅ taking the specific absorbance to be 425.

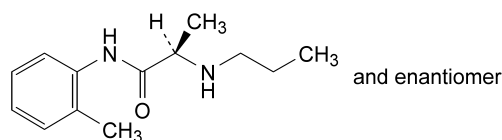
STORAGE

Store protected from light.

01/2005:1362

PRILOCAINE

Prilocainum

C₁₃H₂₀N₂OM_r 220.3

DEFINITION

Prilocaine contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of (RS)-N-(2-methylphenyl)-2-(propylamino)propanamide, calculated with reference to the anhydrous substance.

CHARACTERS

A white or almost white, crystalline powder, slightly soluble in water, very soluble in acetone and in alcohol.

IDENTIFICATION

First identification: B.

Second identification: A, C.

- Melting point (2.2.14): 36 °C to 39 °C, determined without previous drying.
- Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *prilocaine CRS*. Examine the substances by applying 50 µl of a 30 g/l solution in *ether R* to *potassium bromide R* discs and evaporating the solvent.
- Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel GF₂₅₄ plate R*.

Test solution. Dissolve 20.0 mg of the substance to be examined in *alcohol R* and dilute to 5 ml with the same solvent.

Reference solution (a). Dissolve 20.0 mg of *prilocaine CRS* in *alcohol R* and dilute to 5 ml with the same solvent.

Reference solution (b). Dissolve 20.0 mg of *prilocaine CRS* and 20.0 mg of *lidocaine CRS* in *alcohol R* and dilute to 5 ml with the same solvent.

Apply to the plate 10 µl of each solution. Develop over a path of 12 cm, using a mixture of 1 volume of *concentrated ammonia R*, 5 volumes of *methanol R* and

100 volumes of *ether R*. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. The principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a). The test is not valid unless the chromatogram obtained with reference solution (b) shows two clearly separated spots.

TESTS

Solution S. Dissolve 2.50 g in 15 ml of *dilute hydrochloric acid R* and dilute to 50.0 ml with *water R*.

Appearance of solution. Solution S is clear (2.2.1) and colourless (2.2.2, *Method II*).

Optical rotation (2.2.7). The angle of optical rotation, determined on solution S, is -0.10° to $+0.10^\circ$.

Related substances. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 25.0 mg of the substance to be examined in the mobile phase and dilute to 10.0 ml with the mobile phase.

Reference solution (a). Dissolve 2.5 mg of the substance to be examined and 3.0 mg of *prilocaine impurity E CRS* in the mobile phase and dilute to 100.0 ml with the mobile phase. Dilute 1.0 ml to 10.0 ml with the mobile phase.

Reference solution (b). Dilute 1.0 ml of the test solution to 100.0 ml with the mobile phase. Dilute 1.0 ml to 10.0 ml with the mobile phase.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.125 m long and 4.6 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (5 μ m),
- as mobile phase at a flow rate of 1 ml/min a mixture of 40 volumes of *acetonitrile R* and 60 volumes of a solution prepared as follows: dissolve 0.180 g of *sodium dihydrogen phosphate monohydrate R* and 2.89 g of *disodium hydrogen phosphate dihydrate R* in 1000 ml of *water R* (pH 8.0),
- as detector a spectrophotometer set at 240 nm.

Inject 20 μ l of reference solution (a). Adjust the sensitivity of the system so that the heights of the two principal peaks in the chromatogram obtained are at least 20 per cent of the full scale of the recorder. The test is not valid unless the resolution between the peaks corresponding to impurity E and prilocaine is at least 3.0. Inject 20 μ l of the test solution and 20 μ l of reference solution (b). Continue the chromatography of the test solution for twice the retention time of prilocaine, which is about 7 min. In the chromatogram obtained with the test solution: the area of any peak, apart from the principal peak, is not greater than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent) and not more than one such peak has an area greater than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent); the sum of the areas of all the peaks, apart from the principal peak, is not greater than five times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent). Disregard any peak with an area less than 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (b).

***o*-Toluidine.** Use freshly prepared solutions. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 0.100 g of the substance to be examined in the mobile phase and dilute to 10.0 ml with the mobile phase.

Reference solution. Dissolve 10.0 mg of *o*-toluidine hydrochloride *R* in the mobile phase and dilute to 100.0 ml with the mobile phase. Dilute 1.0 ml of this solution to 100.0 ml with the mobile phase.

Carry out the chromatography using the same system as described under Related substances. Inject 20 μ l of the reference solution. Adjust the sensitivity of the system so that the height of the peak in the chromatogram obtained is at least 50 per cent of the full scale of the recorder. Inject 20 μ l of the test solution. Continue the chromatography of the test solution for twice the retention time of prilocaine. In the chromatogram obtained with the test solution, the area of any peak corresponding to *o*-toluidine is not greater than the area of the principal peak in the chromatogram obtained with the reference solution (100 ppm).

Heavy metals (2.4.8). 1.0 g complies with limit test C for heavy metals (20 ppm). Prepare the standard using 2 ml of *lead standard solution (10 ppm Pb) R*.

Water (2.5.12). Not more than 0.5 per cent, determined on 1.00 g by the semi-micro determination of water.

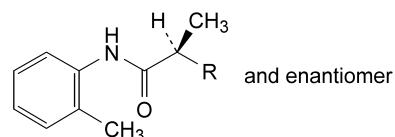
Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.400 g in 20 ml of *anhydrous acetic acid R*. Titrate with 0.1 M *perchloric acid*, determining the end-point potentiometrically (2.2.20).

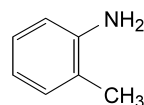
1 ml of 0.1 M *perchloric acid* is equivalent to 22.03 mg of $C_{13}H_{20}N_2O$.

IMPURITIES

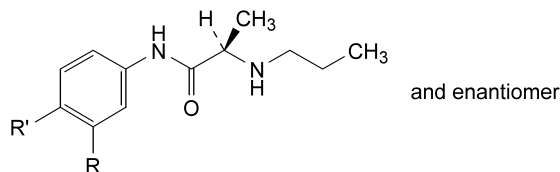


A. R = Cl: (*RS*)-2-chloro-*N*-(2-methylphenyl)propanamide,

C. R = $NH-C_2H_5$: (*RS*)-2-(ethylamino)-*N*-(2-methylphenyl)propanamide,



B. 2-methylbenzenamine (*o*-toluidine),



D. R = CH_3 , R' = H: (*RS*)-*N*-(3-methylphenyl)-2-(propylamino)propanamide,

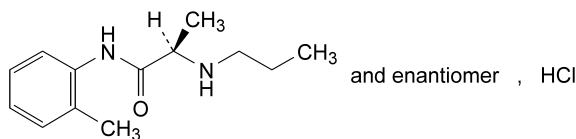
E. R = H, R' = CH_3 : (*RS*)-*N*-(4-methylphenyl)-2-(propylamino)propanamide,

F. R = R' = H: (*RS*)-*N*-phenyl-2-(propylamino)propanamide.

01/2005:1363

PRILOCAINE HYDROCHLORIDE

Prilocaini hydrochloridum

 $C_{13}H_{21}ClN_2O$ M_r 256.8

DEFINITION

Prilocaine hydrochloride contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of (*RS*)-*N*-(2-methylphenyl)-2-(propylamino)propanamide hydrochloride, calculated with reference to the dried substance.

CHARACTERS

A white, crystalline powder or colourless crystals, freely soluble in water and in alcohol, very slightly soluble in acetone.

IDENTIFICATION

First identification: B, D.

Second identification: A, C, D.

- A. Melting point (2.2.14): 168 °C to 171 °C.
- B. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *prilocaine hydrochloride CRS*. Examine the substances prepared as discs.
- C. Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel GF₂₅₄ plate R*.

Test solution. Dissolve 20.0 mg of the substance to be examined in *alcohol R* and dilute to 5 ml with the same solvent.

Reference solution (a). Dissolve 20.0 mg of *prilocaine hydrochloride CRS* in *alcohol R* and dilute to 5 ml with the same solvent.

Reference solution (b). Dissolve 20.0 mg of *prilocaine hydrochloride CRS* and 20.0 mg of *lidocaine hydrochloride CRS* in *alcohol R* and dilute to 5 ml with the same solvent.

Apply to the plate 10 µl of each solution. Develop over a path of 12 cm, using a mixture of 1 volume of *concentrated ammonia R*, 5 volumes of *methanol R* and 100 volumes of *ether R*. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. The principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a). The test is not valid unless the chromatogram obtained with reference solution (b) shows two clearly separated spots.

- D. It gives reaction (a) of chlorides (2.3.1).

TESTS

Solution S. Dissolve 2.50 g in *carbon dioxide-free water R* and dilute to 50.0 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and colourless (2.2.2, *Method II*).

Optical rotation (2.2.7). The angle of optical rotation, determined on solution S, is -0.10° to $+0.10^\circ$.

Acidity or alkalinity. Dilute 4 ml of solution S to 10 ml with *carbon dioxide-free water R*. Add 0.1 ml of *bromocresol green solution R* and 0.40 ml of 0.01 M *sodium hydroxide*. The solution is blue. Add 0.80 ml of 0.01 M *hydrochloric acid*. The solution is yellow.

Related substances. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 30.0 mg of the substance to be examined in the mobile phase and dilute to 10.0 ml with the mobile phase.

Reference solution (a). Dissolve 3.0 mg of the substance to be examined and 3.0 mg of *prilocaine impurity E CRS* in the mobile phase and dilute to 100.0 ml with the mobile phase. Dilute 1.0 ml of this solution to 10.0 ml with the mobile phase.

Reference solution (b). Dilute 1.0 ml of the test solution to 100.0 ml with the mobile phase. Dilute 1.0 ml of this solution to 10.0 ml with the mobile phase.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.125 m long and 4.6 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (5 µm),
- as mobile phase at a flow rate of 1 ml/min a mixture of 40 volumes of *acetonitrile R* and 60 volumes of a solution prepared as follows: dissolve 0.180 g of *sodium dihydrogen phosphate monohydrate R* and 2.89 g of *disodium hydrogen phosphate dihydrate R* in 1000 ml of *water R* (pH 8.0),
- as detector a spectrophotometer set at 240 nm.

Inject 20 µl of reference solution (a). Adjust the sensitivity of the system so that the heights of the two principal peaks in the chromatogram obtained are at least 20 per cent of the full scale of the recorder. The test is not valid unless the resolution between the peaks corresponding to impurity E and prilocaine is at least 3.0. Inject 20 µl of the test solution and 20 µl of reference solution (b). Continue the chromatography of the test solution for twice the retention time of prilocaine, which is about 7 min. In the chromatogram obtained with the test solution: the area of any peak, apart from the principal peak, is not greater than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent) and not more than one such peak has an area greater than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent); the sum of the areas of all the peaks, apart from the principal peak, is not greater than five times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent). Disregard any peak with an area less than 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (b).

***o*-Toluidine.** Use *freshly prepared solutions*. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 0.100 g of the substance to be examined in the mobile phase and dilute to 10.0 ml with the mobile phase.

Reference solution. Dissolve 10.0 mg of *o*-toluidine hydrochloride *R* in the mobile phase and dilute to 100.0 ml with the mobile phase. Dilute 1.0 ml of this solution to 100.0 ml with the mobile phase.

Carry out the chromatography using the same system as described under Related substances. Inject 20 µl of the reference solution. Adjust the sensitivity of the system so that the height of the peak in the chromatogram obtained is at least 50 per cent of the full scale of the recorder. Inject 20 µl of the test solution. Continue the chromatography of

the test solution for twice the retention time of prilocaine. In the chromatogram obtained with the test solution, the area of any peak corresponding to *o*-toluidine is not greater than the area of the principal peak in the chromatogram obtained with the reference solution (100 ppm).

Heavy metals (2.4.8). 1.0 g complies with limit test C for heavy metals (20 ppm). Prepare the standard using 2 ml of *lead standard solution* (10 ppm Pb) R.

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C.

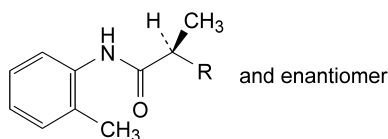
Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.400 g in a mixture of 5.0 ml of 0.01 M *hydrochloric acid* and 50 ml of *alcohol* R. Carry out a potentiometric titration (2.2.20), using 0.1 M *sodium hydroxide*. Read the volume added between the two points of inflexion.

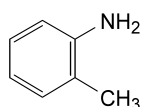
1 ml of 0.1 M *sodium hydroxide* is equivalent to 25.68 mg of C₁₃H₂₁ClN₂O.

IMPURITIES

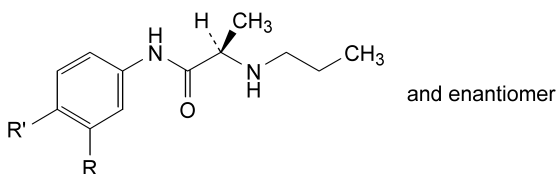


A. R = Cl: (RS)-2-chloro-*N*-(2-methylphenyl)propanamide,

C. R = NH-C₂H₅: (RS)-2-ethylamino-*N*-(2-methylphenyl)propanamide,



B. 2-methylbenzenamine (*o*-toluidine),



D. R = CH₃, R' = H: (RS)-*N*-(3-methylphenyl)-2-(propylamino)propanamide,

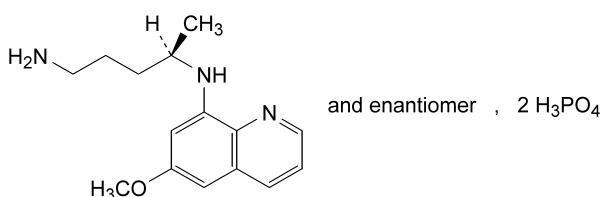
E. R = H, R' = CH₃: (RS)-*N*-(4-methylphenyl)-2-(propylamino)propanamide,

F. R = R' = H: (RS)-*N*-phenyl-2-(propylamino)propanamide.

01/2005:0635
corrected

PRIMAQUINE DIPHOSPHATE

Primaquini diphosphas



C₁₅H₂₇N₃O₉P₂

M_r 455.3

DEFINITION

Primaquine diphosphate contains not less than 98.5 per cent and not more than the equivalent of 101.5 per cent of (4*RS*)-*N*-(6-methoxyquinolin-8-yl)pentane-1,4-diamine bisphosphate, calculated with reference to the dried substance.

CHARACTERS

An orange, crystalline powder, soluble in water, practically insoluble in alcohol.

It melts at about 200 °C, with decomposition.

IDENTIFICATION

First identification: B, D.

Second identification: A, C, D.

A. Dissolve 15 mg in 0.01 M *hydrochloric acid* and dilute to 100.0 ml with the same acid. Examined between 310 nm and 450 nm (2.2.25), the solution shows two absorption maxima, at 332 nm and 415 nm. The specific absorbances at the maxima are 45 to 52 and 27 to 35, respectively. Dilute 5.0 ml of the solution to 50.0 ml with 0.01 M *hydrochloric acid*. Examined between 215 nm and 310 nm, the solution shows three absorption maxima, at 225 nm, 265 nm and 282 nm. The specific absorbances at the maxima are 495 to 515, 335 to 350 and 330 to 345, respectively.

B. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *primaquine diphosphate CRS*. Examine the substances as discs prepared as follows: dissolve separately 0.1 g of the substance to be examined and 0.1 g of the reference substance in 5 ml of *water* R, add 2 ml of *dilute ammonia R2* and 5 ml of *methylene chloride R* and shake; dry the methylene chloride layer over 0.5 g of *anhydrous sodium sulphate R*; prepare a blank disc using about 0.3 g of *potassium bromide R*; apply dropwise to the disc 0.1 ml of the methylene chloride layer, allowing the methylene chloride to evaporate between applications; dry the disc at 50 °C for 2 min.

C. Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel GF₂₅₄ plate R*.

Carry out all operations as rapidly as possible, protected from light. Prepare the test and reference solutions immediately before use.

Test solution. Dissolve 0.20 g of the substance to be examined in 5 ml of *water* R and dilute to 10 ml with *methanol* R. Dilute 1 ml of the solution to 10 ml with a mixture of equal volumes of *methanol* R and *water* R.

Reference solution. Dissolve 20 mg of *primaquine diphosphate CRS* in 5 ml of *water* R and dilute to 10 ml with *methanol* R.

Carry out pre-washing of the plate with a mixture of 1 volume of *concentrated ammonia R*, 40 volumes of *methanol* R and 60 volumes of *methylene chloride R*. Allow the plate to dry in air. Apply to the plate 5 µl of each solution. Develop over a path of 15 cm using the mixture of solvents prescribed for pre-washing. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. The principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with the reference solution.

D. Dissolve 50 mg in 5 ml of *water* R. Add 2 ml of *dilute sodium hydroxide solution R* and shake with two quantities, each of 5 ml, of *methylene chloride R*. The aqueous layer, acidified by addition of *nitric acid R*, gives reaction (b) of phosphates (2.3.1).

TESTS

01/2005:0584

Related substances. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 50 mg of the substance to be examined in *water R* and dilute to 5.0 ml with the same solvent. To 1.0 ml of the solution add 0.2 ml of *concentrated ammonia R* and shake with 10.0 ml of the mobile phase. Use the clear lower layer.

Reference solution (a). Dissolve 50 mg of *primaquine diphosphate CRS* in *water R* and dilute to 5.0 ml with the same solvent. To 1.0 ml of the solution add 0.2 ml of *concentrated ammonia R* and shake with 10.0 ml of the mobile phase. Use the clear lower layer.

Reference solution (b). Dilute 3.0 ml of the test solution to 100.0 ml with the mobile phase.

Reference solution (c). Dilute 1.0 ml of the test solution to 10.0 ml with the mobile phase. Dilute 1.0 ml of this solution to 50.0 ml with the mobile phase.

The chromatographic procedure may be carried out using:

- a column 0.2 m long and 4.6 mm in internal diameter packed with *silica gel for chromatography R* (10 µm),
- as mobile phase at a flow rate of 3.0 ml/min a mixture of 0.1 volumes of *concentrated ammonia R*, 10 volumes of *methanol R*, 45 volumes of *methylene chloride R* and 45 volumes of *hexane R*,
- as detector a spectrophotometer set at 261 nm,
- a loop injector.

Inject 20 µl of each solution and continue the chromatography for at least twice the retention time of primaquine. The test is not valid unless in the chromatogram obtained with reference solution (a), just before the principal peak there is a peak whose area is about 6 per cent of that of the principal peak and the resolution between these peaks is not less than 2.0; in the chromatogram obtained with reference solution (c) the signal-to-noise ratio of the principal peak is not less than five. In the chromatogram obtained with the test solution, the sum of the areas of any peaks, apart from the principal peak, is not greater than the area of the principal peak in the chromatogram obtained with reference solution (b) (3.0 per cent). Disregard the peak due to the solvent and any peak whose area is less than that of the principal peak in the chromatogram obtained with reference solution (c).

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C.

ASSAY

Dissolve 0.2000 g in 40 ml of *anhydrous acetic acid R*, heating gently. Allow to cool and titrate with 0.1 M *perchloric acid*, determining the end-point potentiometrically (2.2.20).

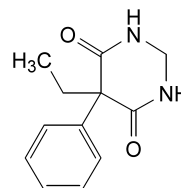
1 ml of 0.1 M *perchloric acid* is equivalent to 22.77 mg of $C_{15}H_{27}N_3O_9P_2$.

STORAGE

Store protected from light.

PRIMIDONE

Primidonum


 $C_{12}H_{14}N_2O_2$
 M_r 218.3

DEFINITION

5-Ethyl-5-phenyldihydropyrimidine-4,6(1*H*,5*H*)-dione.

Content: 98.0 per cent to 102.0 per cent (dried substance).

CHARACTERS

Appearance: white or almost white, crystalline powder.

Solubility: very slightly soluble in water, slightly soluble in ethanol (96 per cent). It dissolves in alkaline solutions.

IDENTIFICATION

First identification: B.

Second identification: A, C, D.

- Use the solution prescribed for the assay. Examined between 240 nm and 300 nm (2.2.25), the solution shows 3 absorption maxima, at 252 nm, 257 nm and 264 nm, and 2 absorption minima, at 254 nm and 261 nm. The ratio of the absorbance measured at the absorption maximum at 257 nm to that measured at the absorption minimum at 261 nm is 2.00 to 2.20. The identification is valid if, in the test for resolution (2.2.25), the ratio of the absorbances is not less than 2.0.
- Infrared absorption spectrophotometry (2.2.24).
Preparation: discs of *potassium bromide R*.
Comparison: *primidone CRS*.
- Dissolve 0.1 g in 5 ml of a 5 g/l solution of *chromotropic acid, sodium salt R* in a mixture of 4 volumes of *water R* and 9 volumes of *sulphuric acid R*. A pinkish-blue colour develops on heating.
- Mix 0.2 g and 0.2 g of *anhydrous sodium carbonate R*. Heat until the mixture melts. Ammonia is evolved which is detectable by its alkaline reaction (2.2.4).

TESTS

Related substances. Liquid chromatography (2.2.29).

Test solution. Dissolve 50.0 mg of the substance to be examined in *methanol R1* and dilute to 50.0 ml with the same solvent.

Reference solution (a). Dilute 1.0 ml of the test solution to 100.0 ml with *methanol R1*. Dilute 1.0 ml of this solution to 10.0 ml with *methanol R1*.

Reference solution (b). Dissolve 5 mg of *primidone for peak identification CRS* (containing impurities A, B, C, D, E and F) in *methanol R1* and dilute to 5 ml with the same solvent.

Column:

- **size:** $l = 0.10$ m, $\varnothing = 4.6$ mm,
- **stationary phase:** *monolithic octadecylsilyl silica gel for chromatography R*.

Mobile phase:

- **mobile phase A:** 1.36 g/l solution of *potassium dihydrogen phosphate R*,

– mobile phase B: methanol R1,

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)
0 - 1	75	25
1 - 6	75 → 40	25 → 60
6 - 8	40	60
8 - 8.5	40 → 75	60 → 25
8.5 - 10	75	25

Flow rate: 3.2 ml/min.

Detection: spectrophotometer at 215 nm.

Injection: 10 µl.

Identification of impurities: use the chromatogram supplied with *primidone* for peak identification CRS and the chromatogram obtained with reference solution (b) to identify the peaks.

Relative retention with reference to primidone (retention time = about 2.2 min): impurity A = about 0.5; impurity B = about 1.4; impurity C = about 1.6; impurity D = about 1.75; impurity E = about 2.0; impurity F = about 2.8.

System suitability: reference solution (b):

- resolution: minimum 2.5 between the peaks due to impurity B and impurity C.

Limits:

- correction factors: for the calculation of contents, multiply the peak areas of the following impurities by the corresponding correction factor: impurity A = 1.5; impurity C = 1.5; impurity D = 1.4; impurity E = 1.3;
- impurity F: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.3 per cent);
- impurities A, B, C, D, E: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent);
- any other impurity: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent);
- total: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent);
- disregard limit: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

Heavy metals (2.4.8): maximum 10 ppm.

2.0 g complies with limit test D. Prepare the reference solution using 2 ml of *lead standard solution* (10 ppm Pb) R.

Loss on drying (2.2.32): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 100–105 °C for 2 h.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

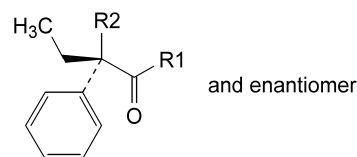
ASSAY

Dissolve 60.0 mg with heating in 70 ml of *ethanol* (96 per cent) R, cool and dilute to 100.0 ml with the same solvent. Prepare a reference solution in the same manner using 60.0 mg of *primidone* CRS. Measure the absorbance (2.2.25) of the 2 solutions at the absorption maximum at 257 nm.

Calculate the content of C₁₂H₁₄N₂O₂ from the absorbances measured and the concentrations of the solutions.

IMPURITIES

Specified impurities: A, B, C, D, E, F.



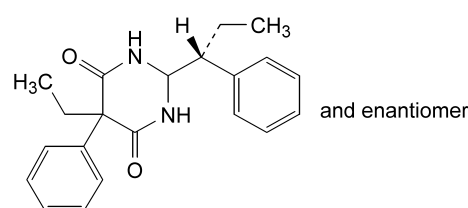
A. R1 = NH₂, R2 = CO-NH₂: 2-ethyl-2-phenylpropanediamide (ethylphenylmalonamide),

C. R1 = NH₂, R2 = H: (2*RS*)-2-phenylbutanamide,

D. R1 = NH₂, R2 = CN: (2*RS*)-2-cyano-2-phenylbutanamide,

E. R1 = OH, R2 = H: (2*RS*)-2-phenylbutanoic acid,

B. phenobarbital,



F. 5-ethyl-5-phenyl-2-[(1*RS*)-1-phenylpropyl]dihydropyrimidine-4,6(1*H*,5*H*)-dione.

01/2005:1364

PRIMULA ROOT

Primulae radix

DEFINITION

Primula root consists of the whole or cut, dried rhizome and root of *Primula veris* L. or *P. elatior* (L.) Hill.

CHARACTERS

Primula root has a bitter taste.

It has the macroscopic and microscopic characters described under identification tests A and B.

IDENTIFICATION

- The coarsely torose, greyish-brown rhizome is straight or slightly curved, about 1 cm to 5 cm long and about 2 mm to 4 mm thick. The rhizome crown often bears the remains of stems and leaves. Attached to the rhizome are numerous brittle roots, about 1 mm thick and usually 6 cm to 8 cm long. The root of *P. elatior* is light brown to reddish-brown, that of *P. veris* light yellow to yellowish-white. The fracture is smooth.
- Reduce to a powder (355). The powder is greyish-brown. Examine under a microscope, using *chloral hydrate solution* R. The powder shows fragments of parenchyma from the root bark, medulla and bark of the rhizome consisting of rounded cells with thickened and pitted walls; brownish fragments from the surface tissue showing root hairs; vessels with reticulate thickening. Yellowish-green groups of strongly pitted stone cells are characteristic of the presence of *P. elatior*. Examine under a microscope using a 50 per cent V/V solution of *glycerol* R. The powder shows single or compound starch grains of various size and shape.
- Use the chromatograms obtained in the test for *Vincetoxicum hirundinaria medicus* root. Spray the plate with *anisaldehyde solution* R. Heat at 100 °C to 105 °C for 5 min to 10 min. Examine in daylight. The main zone (aescin) in the chromatogram obtained with

the reference solution is bluish-violet and is situated near the boundary between the lower and middle thirds. The chromatogram obtained with the test solution shows one or two strong dark violet zones a little below the aescin zone in the chromatogram obtained with the reference solution; further pale violet, yellowish or brownish-green zones may be visible.

TESTS

Foreign matter (2.8.2). It complies with the test for foreign matter.

Vincetoxicum hirundinaria medicus root. Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel F₂₅₄* plate R.

Test solution. To 1.0 g of the powdered drug (500) add 10 ml of *alcohol* (70 per cent V/V) R and heat under a reflux condenser for 15 min. Cool and filter.

Reference solution. Dissolve 10 mg of *aescin* R in 1.0 ml of *alcohol* (70 per cent V/V) R.

Apply to the plate as bands 20 µl of each solution. Develop over a path of 12 cm using the upper phase of a mixture of 10 volumes of *glacial acetic acid* R, 40 volumes of *water* R and 50 volumes of *butanol* R. Allow the plate to dry in an oven at 100 °C to 105 °C. Examine in ultraviolet light at 254 nm. The chromatograms obtained with the reference solution and the test solution show a quenching zone (*aescin*) near the boundary between the lower and the middle thirds. Mark this zone. Examine in ultraviolet light at 365 nm. In the chromatogram obtained with the test solution no zones of light-blue or greenish fluorescence occur below the main zone of *aescin* in the chromatogram obtained with the reference solution.

Loss on drying (2.2.32). Not more than 10.0 per cent, determined on 1.000 g of powdered drug (355) by drying in an oven at 100 °C to 105 °C for 2 h.

Total ash (2.4.16). Not more than 9.0 per cent.

Ash insoluble in hydrochloric acid (2.8.1). Not more than 3.0 per cent.

STORAGE

Store protected from light.

IDENTIFICATION

First identification: A, C.

Second identification: A, B, D.

A. Melting point (2.2.14): 197 °C to 202 °C.

B. Dissolve 20 mg in a mixture of 1 volume of 0.1 M *hydrochloric acid* and 9 volumes of *alcohol* R and dilute to 100.0 ml with the same mixture of solvents. Dilute 5.0 ml of the solution to 100.0 ml with a mixture of 1 volume of 0.1 M *hydrochloric acid* and 9 volumes of *alcohol* R. Examined between 220 nm and 350 nm (2.2.25), the solution shows two absorption maxima, at 223 nm and 248 nm. The specific absorbance at the maximum at 248 nm is 310 to 350.

C. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *probenecid CRS*.

D. Dissolve 0.2 g in the smallest necessary quantity of *dilute ammonia* R2 (about 0.6 ml). Add 3 ml of *silver nitrate solution* R2. A white precipitate is formed which dissolves in an excess of ammonia.

TESTS

Appearance of solution. Dissolve 1.0 g in 1 M *sodium hydroxide* and dilute to 10 ml with the same solvent. The solution is clear (2.2.1) and not more intensely coloured than reference solution Y₆ (2.2.2, *Method II*).

Acidity. To 2.0 g add 100 ml of *water* R and heat on a water-bath for 30 min. Make up to the original volume with *water* R, allow to cool to room temperature and filter. To 50 ml of the filtrate add 0.1 ml of *phenolphthalein solution* R. Not more than 0.5 ml of 0.1 M *sodium hydroxide* is required to change the colour of the indicator.

Related substances. Examine by thin-layer chromatography (2.2.27), using *silica gel GF₂₅₄* R as the coating substance.

Test solution. Dissolve 0.1 g of the substance to be examined in *acetone* R and dilute to 10 ml with the same solvent.

Reference solution. Dilute 0.5 ml of the test solution to 100 ml with *acetone* R.

Apply separately to the plate 20 µl of each solution. Develop over a path of 15 cm using a mixture of 10 volumes of *glacial acetic acid* R, 15 volumes of *chloroform* R, 20 volumes of *di-isopropyl ether* R and 55 volumes of *toluene* R. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. Any spot in the chromatogram obtained with the test solution, apart from the principal spot, is not more intense than the spot in the chromatogram obtained with the reference solution (0.5 per cent).

Heavy metals (2.4.8). 1.0 g complies with limit test C for heavy metals (20 ppm). Prepare the standard using 2 ml of *lead standard solution* (10 ppm Pb) R.

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.00 g by drying in an oven at 100 °C to 105 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

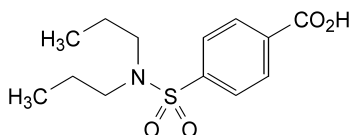
Dissolve 0.250 g in 50 ml of *alcohol* R, shaking and heating slightly if necessary. Titrate with 0.1 M *sodium hydroxide*, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *sodium hydroxide* is equivalent to 28.54 mg of C₁₃H₁₉NO₄S.

01/2005:0243

PROBENECID

Probenecidum

C₁₃H₁₉NO₄SM_r 285.4

DEFINITION

Probenecid contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of 4-(dipropylsulfamoyl)benzoic acid, calculated with reference to the dried substance.

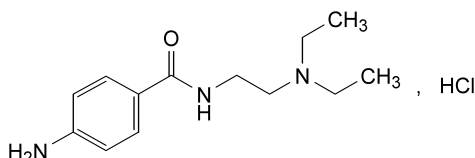
CHARACTERS

A white or almost white, crystalline powder or small crystals, practically insoluble in water, soluble in acetone, sparingly soluble in ethanol.

01/2005:0567

PROCAINAMIDE HYDROCHLORIDE

Procainamidi hydrochloridum

 $C_{13}H_{22}ClN_3O$ M_r 271.8**DEFINITION**

Procainamide hydrochloride contains not less than 98.0 per cent and not more than the equivalent of 101.0 per cent of 4-amino-*N*-[2-(diethylamino)ethyl]benzamide hydrochloride, calculated with reference to the dried substance.

CHARACTERS

A white or very slightly yellow, crystalline powder, hygroscopic, very soluble in water, freely soluble in alcohol, slightly soluble in acetone.

IDENTIFICATION

First identification: C, D.

Second identification: A, B, D, E.

- A. Melting point (2.2.14): 166 °C to 170 °C.
- B. Dissolve 10.0 mg in 0.1 *M* sodium hydroxide and dilute to 100.0 ml with the same solvent. Dilute 10.0 ml of the solution to 100.0 ml with 0.1 *M* sodium hydroxide. Examined between 220 nm and 350 nm (2.2.25), the solution shows an absorption maximum at 273 nm. The specific absorbance at the maximum is 580 to 610.
- C. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with procainamide hydrochloride CRS.
- D. Dilute 1 ml of solution S to 5 ml with water *R*. The solution gives reaction (a) of chlorides (2.3.1).
- E. Dilute 1 ml of solution S (see Tests) to 2 ml with water *R*. 1 ml of this solution gives the reaction of primary aromatic amines (2.3.1).

TESTS

Solution S. Dissolve 2.5 g in carbon dioxide-free water *R* and dilute to 25 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and not more intensely coloured than reference solution B₆ (2.2.2, Method II).

pH (2.2.3). The pH of solution S is 5.6 to 6.3.

Related substances. Examine by thin-layer chromatography (2.2.27), using silica gel GF₂₅₄ *R* as the coating substance.

Test solution. Dissolve 0.10 g of the substance to be examined in alcohol *R* and dilute to 10 ml with the same solvent.

Reference solution. Dilute 1 ml of the test solution to 200 ml with alcohol *R*.

Apply to the plate 5 µl of each solution. Develop over a path of 12 cm using a mixture of 15 volumes of glacial acetic acid *R*, 30 volumes of water *R* and 60 volumes of butanol *R*. Place the plate in a stream of cold air until the plate appears dry. Examine in ultraviolet light at 254 nm. Any spot in the chromatogram obtained with the test solution, apart from

the principal spot, is not more intense than the spot in the chromatogram obtained with the reference solution (0.5 per cent).

Heavy metals (2.4.8). 1.0 g complies with limit test C for heavy metals (20 ppm). Prepare the standard using 2 ml of lead standard solution (10 ppm Pb) *R*.

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.2500 g in 50 ml of dilute hydrochloric acid *R*. Carry out the determination of primary aromatic amino-nitrogen (2.5.8).

1 ml of 0.1 *M* sodium nitrite is equivalent to 27.18 mg of $C_{13}H_{22}ClN_3O$.

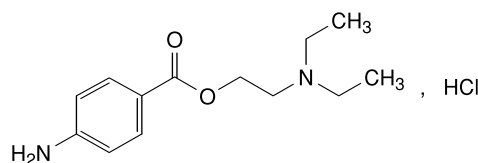
STORAGE

Store in an airtight container, protected from light.

01/2005:0050

PROCAINE HYDROCHLORIDE

Procaini hydrochloridum

 $C_{13}H_{21}ClN_2O_2$ M_r 272.8**DEFINITION**

Procaine hydrochloride contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of 2-(diethylamino)ethyl 4-aminobenzoate hydrochloride, calculated with reference to the dried substance.

CHARACTERS

A white, crystalline powder or colourless crystals, very soluble in water, soluble in alcohol.

IDENTIFICATION

First identification: A, B, E.

Second identification: A, C, D, E, F.

A. Melting point (2.2.14): 154 °C to 158 °C.

B. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with procaine hydrochloride CRS.

C. To about 5 mg add 0.5 ml of fuming nitric acid *R*. Evaporate to dryness on a water-bath, allow to cool and dissolve the residue in 5 ml of acetone *R*. Add 1 ml of 0.1 *M* alcoholic potassium hydroxide. Only a brownish-red colour develops.

D. To 0.2 ml of solution S (see Tests) add 2 ml of water *R* and 0.5 ml of dilute sulphuric acid *R* and shake. Add 1 ml of a 1 g/l solution of potassium permanganate *R*. The colour is immediately discharged.

E. It gives reaction (a) of chlorides (2.3.1).

F. Dilute 1 ml of solution S to 100 ml with water *R*. 2 ml of this solution gives the reaction of primary aromatic amines (2.3.1).

TESTS

Solution S. Dissolve 2.5 g in *carbon dioxide-free water R* and dilute to 50 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and colourless (2.2.2, *Method II*).

pH (2.2.3). Dilute 4 ml of solution S to 10 ml with *carbon dioxide-free water R*. The pH of the solution is 5.0 to 6.5.

Related substances. Examine by thin-layer chromatography (2.2.27), using *silica gel GF₂₅₄ R* as the coating substance.

Test solution. Dissolve 1.0 g of the substance to be examined in *water R* and dilute to 10 ml with the same solvent.

Reference solution. Dissolve 50 mg of 4-aminobenzoic acid *R* in *water R* and dilute to 100 ml with the same solvent. Dilute 1 ml of the solution to 10 ml with *water R*.

Apply separately to the plate 5 µl of each solution. Develop over a path of 10 cm using a mixture of 4 volumes of *glacial acetic acid R*, 16 volumes of *hexane R* and 80 volumes of *dibutyl ether R*. Dry the plate at 100 °C to 105 °C for 10 min and examine in ultraviolet light at 254 nm. Any spot in the chromatogram obtained with the test solution, apart from the principal spot, is not more intense than the spot in the chromatogram obtained with the reference solution (0.05 per cent). The principal spot in the chromatogram obtained with the test solution remains on the starting point.

Heavy metals (2.4.8). Dissolve 1.0 g in *water R* and dilute to 25.0 ml with the same solvent. Carry out the prefiltration. 10 ml of the prefiltrate complies with limit test E for heavy metals (5 ppm). Prepare the standard using 2 ml of *lead standard solution (1 ppm Pb) R*.

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.00 g by drying in an oven at 100 °C to 105 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.400 g in 50 ml of *dilute hydrochloric acid R*. Carry out the determination of primary aromatic amino nitrogen (2.5.8).

1 ml of 0.1 M *sodium nitrite* is equivalent to 27.28 mg of C₁₃H₂₁ClN₃O₂.

STORAGE

Store protected from light.

DEFINITION

Prochlorperazine maleate contains not less than 98.0 per cent and not more than the equivalent of 101.0 per cent of 2-chloro-10-[3-(4-methylpiperazin-1-yl)propyl]-10*H*-phenothiazine bis[hydrogen (*Z*)-butenedioate], calculated with reference to the dried substance.

CHARACTERS

A white or pale-yellow, crystalline powder, very slightly soluble in water and in alcohol.

IDENTIFICATION

First identification: B, C, D.

Second identification: A, C, D.

- A. Carry out the test protected from light. Dissolve 50 mg in 0.1 M *hydrochloric acid* and dilute to 500.0 ml with the same acid. Examined immediately between 280 nm and 350 nm (2.2.25), the solution shows an absorption maximum at 305 nm. Dilute 10.0 ml of the solution to 100.0 ml with 0.1 M *hydrochloric acid*. Examined immediately between 230 nm and 280 nm, the solution shows an absorption maximum at 255 nm. The specific absorbance at the maximum is 525 to 575.
- B. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *prochlorperazine maleate CRS*.
- C. It complies with the identification of phenothiazines by thin-layer chromatography (2.3.3), with the following modifications:
Test solution. Dissolve 20 mg of the substance to be examined in a mixture of equal volumes of *chloroform R* and *methanol R* and dilute to 20 ml with the same mixture of solvents.
Reference solution. Dissolve 20 mg of *prochlorperazine maleate CRS* in a mixture of equal volumes of *chloroform R* and *methanol R* and dilute to 20 ml with the same mixture of solvents.
 Apply separately to the plate 4 µl of each solution.
- D. Triturate 0.2 g with a mixture of 1 ml of *strong sodium hydroxide solution R* and 3 ml of *water R*. Shake with three quantities, each of 5 ml, of *ether R*. To 0.1 ml of the aqueous layer add a solution of 10 mg of *resorcinol R* in 3 ml of *sulphuric acid R*. Heat in a water-bath for 15 min. No colour develops. To the remainder of the aqueous layer add 2 ml of *bromine solution R*. Heat in a water-bath for 15 min and then heat to boiling. Cool. To 0.1 ml of the solution add a solution of 10 mg of *resorcinol R* in 3 ml of *sulphuric acid R*. Heat in a water-bath for 15 min. A blue colour develops.

TESTS

pH (2.2.3). The pH of a freshly prepared saturated solution in *carbon dioxide-free water R* is 3.0 to 4.0.

Related substances. Carry out the test protected from light. Examine by thin-layer chromatography (2.2.27), using *silica gel GF₂₅₄ R* as the coating substance.

Test solution. Dissolve 0.2 g of the substance to be examined in a mixture of 5 volumes of *diethylamine R* and 95 volumes of *methanol R* and dilute to 10 ml with the same mixture of solvents. Prepare immediately before use.

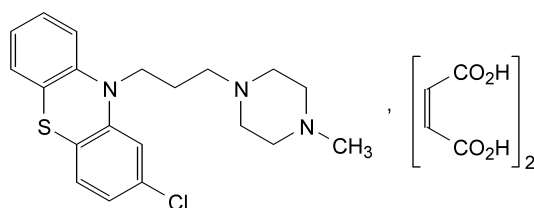
Reference solution. Dilute 1 ml of the test solution to 200 ml with a mixture of 5 volumes of *diethylamine R* and 95 volumes of *methanol R*.

Apply separately to the plate 10 µl of each solution. Develop over a path of 12 cm using a mixture of 10 volumes of *acetone R*, 10 volumes of *diethylamine R* and 80 volumes of *cyclohexane R*. Allow the plate to dry in air and examine in

01/2005:0244

PROCHLORPERAZINE MALEATE

Prochlorperazini maleas

C₂₈H₃₂ClN₃O₈SM_r 606

ultraviolet light at 254 nm. Any spot in the chromatogram obtained with the test solution, apart from the principal spot, is not more intense than the spot in the chromatogram obtained with the reference solution (0.5 per cent). Disregard any spots remaining at the starting-points.

Loss on drying (2.2.32). Not more than 1.0 per cent, determined on 1.00 g by drying in an oven at 100 °C to 105 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.200 g of the powdered substance to be examined in 50 ml of *anhydrous acetic acid R*, warming on a water-bath. Allow to cool to room temperature. Titrate with 0.1 M *perchloric acid* determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *perchloric acid* is equivalent to 30.31 mg of $C_{21}H_{30}O_2$.

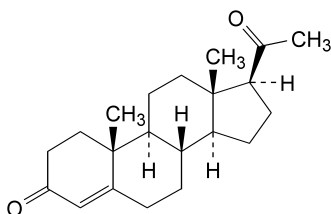
STORAGE

Store protected from light.

01/2005:0429

PROGESTERONE

Progesteronum



$C_{21}H_{30}O_2$

M_r 314.5

DEFINITION

Pregn-4-ene-3,20-dione.

Content: 97.0 per cent to 103.0 per cent (dried substance).

CHARACTERS

Appearance: white or almost white, crystalline powder or colourless crystals.

Solubility: practically insoluble in water, freely soluble in ethanol, sparingly soluble in acetone and in fatty oils.

It shows polymorphism.

IDENTIFICATION

A. Infrared absorption spectrophotometry (2.2.24).

Comparison: *progesterone CRS*.

If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in *ethanol R*, evaporate to dryness and record new spectra using the residues.

B. Thin-layer chromatography (2.2.27)

Test solution. Dissolve 10 mg of the substance to be examined in a mixture of 1 volume of *methanol R* and 9 volumes of *methylene chloride R* and dilute to 10 ml with the same mixture of solvents.

Reference solution. Dissolve 10 mg of *progesterone CRS* in a mixture of 1 volume of *methanol R* and 9 volumes of *methylene chloride R* and dilute to 10 ml with the same mixture of solvents.

Plate: *TLC silica gel F₂₅₄ plate R*.

Mobile phase: *ethyl acetate R, methylene chloride R* (33:66 V/V).

Application: 5 µl.

Development: over 3/4 of the plate.

Drying: in air.

Detection A: examine in ultraviolet light at 254 nm.

Detection B: spray with *alcoholic solution of sulphuric acid R*, heat at 120 °C for 15 min and allow to cool. Examine in daylight and in ultraviolet light at 365 nm.

Results A: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

Results B: the principal spot in the chromatogram obtained with the test solution is similar in position, fluorescence in ultraviolet light at 365 nm and size to the principal spot in the chromatogram obtained with the reference solution.

TESTS

Specific optical rotation (2.2.7): + 186 to + 194 (dried substance).

Dissolve 0.250 g in *ethanol R* and dilute to 25.0 ml with the same solvent.

Related substances. Liquid chromatography (2.2.29).

Test solution. Dissolve 20.0 mg of the substance to be examined in *methanol R* and dilute to 50.0 ml with the same solvent.

Reference solution (a). Dissolve 2.0 mg of *progesterone CRS* and 2.0 mg of *progesterone impurity C CRS* in *methanol R* and dilute to 50.0 ml with the same solvent.

Reference solution (b). Dilute 1.0 ml of the test solution to 100.0 ml with *methanol R*.

Column:

- **size:** $l = 0.15$ m, $\varnothing = 4.6$ mm,
- **stationary phase:** spherical *end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

Mobile phase:

- **mobile phase A:** *water R*,
- **mobile phase B:** *acetonitrile R*.

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)
0 - 20	50	50
20 - 27	50 → 20	50 → 80
27 - 45	20	80
45 - 50	50	50

Flow rate: 0.8 ml/min.

Detection: spectrophotometer at 241 nm.

Injection: 10 µl.

System suitability: reference solution (a):

- **resolution:** minimum 1.5 between the peaks due to impurity C and to progesterone.

Limits:

- **any impurity:** not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent),
- **total:** not more than 0.8 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.8 per cent),

- *disregard limit*: 0.05 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

Loss on drying (2.2.32): maximum 0.5 per cent, determined on 0.500 g by drying in an oven at 100–105 °C for 2 h.

ASSAY

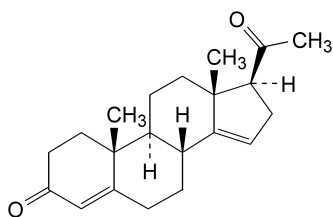
Dissolve 25.0 mg in *alcohol R* and dilute to 250.0 ml with the same solvent. Dilute 5.0 ml of the solution to 50.0 ml with *alcohol R*. Measure the absorbance (2.2.25) at the maximum at 241 nm.

Calculate the content of $C_{21}H_{30}O_2$ taking the specific absorbance to be 535.

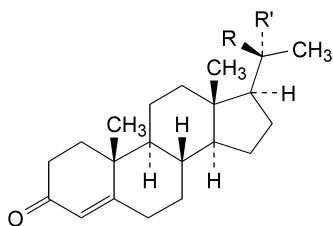
STORAGE

Protected from light.

IMPURITIES



A. pregna-4,14-diene-3,20-dione,

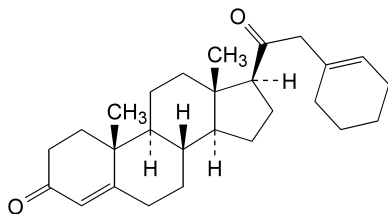


B. R = OH, R' = H: (20S)-20-hydroxypregn-4-en-3-one,

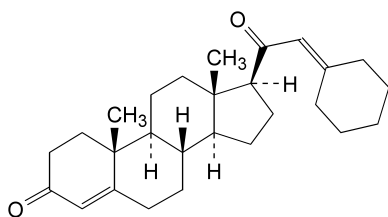
C. R = H, R' = OH: (20R)-20-hydroxypregn-4-en-3-one,

D. R = O-CO-CH₃, R' = H: (20S)-3-oxopregn-4-en-20-yl acetate,

E. R = H, R' = O-CO-CH₃: (20R)-3-oxopregn-4-en-20-yl acetate,



F. 21-(cyclohex-1-en-1-yl)pregn-4-ene-3,20-dione,

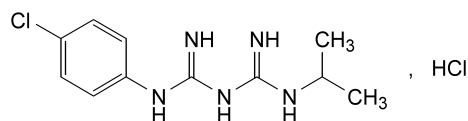


G. 21-(cyclohexylidene)pregn-4-ene-3,20-dione.

01/2005:2002

PROGUANIL HYDROCHLORIDE

Proguanili hydrochloridum



$C_{11}H_{17}Cl_2N_5$

M_r 290.2

DEFINITION

1-(4-Chlorophenyl)-5-(1-methylethyl)biguanide hydrochloride.

Content: 98.5 per cent to 101.0 per cent (dried substance).

CHARACTERS

Appearance: white, crystalline powder.

Solubility: slightly soluble in water, sparingly soluble in ethanol, practically insoluble in methylene chloride.

IDENTIFICATION

First identification: A, D.

Second identification: B, C, D.

A. Infrared absorption spectrophotometry (2.2.24).

Comparison: Ph. Eur. reference spectrum of proguanil hydrochloride.

B. Dissolve 0.4 g in 50 ml of *water R* (solution A). To 15 ml of solution A add 2 ml of *dilute sodium hydroxide solution R*. Extract with 20 ml of *ethyl acetate R*. Wash the organic layer with *water R*, evaporate to dryness and dry at 105 °C. The melting point (2.2.14) of the residue is 130 °C to 133 °C.

C. To 10 ml of solution A, add 1 drop of *copper sulphate solution R* and 2 ml of *dilute ammonia R1*. Add 5 ml of *toluene R* and stir. Allow to stand until separation of the layers is obtained. The upper layer is violet-red.

D. It gives reaction (a) of chlorides (2.3.1).

TESTS

Acidity or alkalinity. To 35 ml of *water R* maintained at 60–65 °C, add 0.2 ml of *methyl red mixed solution R*. Neutralise to a grey colour with either 0.01 M *sodium hydroxide* or 0.01 M *hydrochloric acid*. Add 0.4 g of the substance to be examined and stir until completely dissolved. The solution is grey or green. Not more than 0.2 ml of 0.01 M *hydrochloric acid* is required to change the colour of the solution to reddish-violet.

Chloroaniline: maximum 250 ppm.

Dissolve 0.10 g in 1 ml of 2 M *hydrochloric acid* and dilute to 20 ml with *water R*. Cool to 5 °C. Add 1 ml of a 3.45 g/l solution of *sodium nitrite R* and allow to stand at 5 °C for 5 min. Add 2 ml of a 50 g/l solution of *ammonium sulphamate R* and allow to stand for 10 min. Add 2 ml of *naphthylethylenediamine dihydrochloride solution R*, dilute to 50 ml with *water R* and allow to stand for 30 min. Any red colour produced is not more intense than that of a standard prepared at the same time and in the same manner, using 20 ml of a 1.25 mg/l solution of *chloroaniline R*.

Related substances. Liquid chromatography (2.2.29).

Test solution. Dissolve 10.0 mg of the substance to be examined in the mobile phase and dilute to 100.0 ml with the mobile phase.

Reference solution (a). Dilute 1.0 ml of the test solution to 50.0 ml with the mobile phase. Dilute 1.0 ml of this solution to 10.0 ml with the mobile phase.

Reference solution (b). Dissolve 5 mg of *proguanil impurity C CRS* in the mobile phase and dilute to 100 ml with the mobile phase. Dilute 0.1 ml to 10 ml with the mobile phase.

Reference solution (c). Dissolve 5 mg of *proguanil impurity D CRS* in the mobile phase and dilute to 100 ml with the mobile phase. Dilute 0.1 ml to 10 ml with the mobile phase.

Reference solution (d). Dilute 1 ml of the test solution to 200 ml with the mobile phase. To 1 ml add 1 ml of reference solution (c) and mix.

Column:

- **size:** $l = 0.125$ m, $\varnothing = 4.6$ mm,
- **stationary phase:** octadecylsilyl silica gel for chromatography *R* (5 μ m).

Mobile phase: dissolve 3.78 g of sodium hexanesulphonate *R* in a mixture of 10 volumes of *glacial acetic acid R*, 800 volumes of *water R* and 1200 volumes of *methanol R*.

Flow rate: 1 ml/min.

Detection: spectrophotometer at 230 nm and 254 nm.

Injection: 20 μ l.

Run time: 5 times the retention time of proguanil.

Retention time: proguanil = about 6 min.

System suitability: reference solution (d) at 230 nm:

- **resolution:** minimum 5 between the peaks due to impurity D and proguanil.

Limits:

- **impurity C:** not more than 3.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) at 230 nm (0.7 per cent),
- **impurity D:** not more than the area of the principal peak in the chromatogram obtained with reference solution (a) at 230 nm (0.2 per cent),
- **any other impurity:** not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) at 230 nm and at 254 nm (0.1 per cent),
- **total:** the sum of the calculated percentage contents of known and unknown impurities is not greater than 1 per cent, considering each peak at the wavelength at which the peak shows the higher value,
- **disregard limit:** 0.25 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

Loss on drying (2.2.32): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 100–105 °C.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

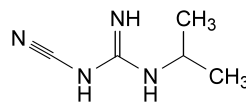
Suspend 0.100 g in 20 ml of *anhydrous acetic acid R*, shake and heat at 50 °C for 5 min. Cool to room temperature and add 40 ml of *acetic anhydride R*. Titrate with 0.1 M *perchloric acid*, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *perchloric acid* is equivalent to 14.51 mg of $C_{11}H_{17}Cl_2N_5$.

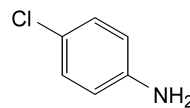
STORAGE

Protected from light.

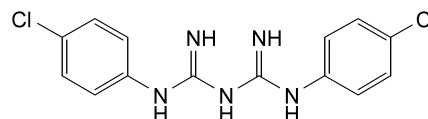
IMPURITIES



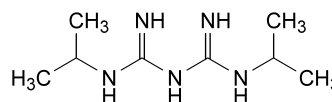
A. 1-cyano-3-(1-methylethyl)guanidine,



B. 4-chloroaniline,



C. 1,5-bis(4-chlorophenyl)biguanide,

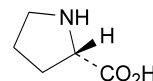


D. 1,5-bis(1-methylethyl)biguanide.

01/2005:0785

PROLINE

Prolinum



$C_5H_9NO_2$

M_r 115.1

DEFINITION

Proline contains not less than 98.5 per cent and not more than the equivalent of 101.0 per cent of (*S*)-pyrrolidine-2-carboxylic acid, calculated with reference to the dried substance.

CHARACTERS

White or almost white, crystalline powder or colourless crystals, very soluble in water, freely soluble in alcohol.

IDENTIFICATION

First identification: A, B.

Second identification: A, C.

- It complies with the test for specific optical rotation (see Tests).
- Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *proline CRS*. Examine the substances prepared as discs.
- Examine the chromatograms obtained in the test for ninhydrin-positive substances. The principal spot in the chromatogram obtained with test solution (b) is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).

TESTS

Solution S. Dissolve 2.5 g in *distilled water R* and dilute to 50 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and colourless (2.2.2, *Method II*).

Specific optical rotation (2.2.7). Dissolve 1.00 g in *water R* and dilute to 25.0 ml with the same solvent. The specific optical rotation is -84.0 to -86.0 , calculated with reference to the dried substance.

Ninhydrin-positive substances. Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel plate R*.

Test solution (a). Dissolve 0.10 g of the substance to be examined in *0.1 M hydrochloric acid* and dilute to 10 ml with the same acid.

Test solution (b). Dilute 1 ml of test solution (a) to 50 ml with *water R*.

Reference solution (a). Dissolve 10 mg of *proline CRS* in *0.1 M hydrochloric acid* and dilute to 50 ml with the same acid.

Reference solution (b). Dilute 5 ml of test solution (b) to 20 ml with *water R*.

Reference solution (c). Dissolve 10 mg of *proline CRS* and 10 mg of *threonine CRS* in *0.1 M hydrochloric acid* and dilute to 25 ml with the same acid.

Apply to the plate 5 μ l of each solution. Allow the plate to dry in air. Develop over a path of 15 cm using a mixture of 20 volumes of *glacial acetic acid R*, 20 volumes of *water R* and 60 volumes of *butanol R*. Allow the plate to dry in air, spray with *ninhydrin solution R* and heat at $100\text{ }^{\circ}\text{C}$ to $105\text{ }^{\circ}\text{C}$ for 15 min. Any spot in the chromatogram obtained with test solution (a), apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.5 per cent). The test is not valid unless the chromatogram obtained with reference solution (c) shows two clearly separated spots.

Chlorides (2.4.4). Dilute 5 ml of solution S to 15 ml with *water R*. The solution complies with the limit test for chlorides (200 ppm).

Sulphates (2.4.13). Dilute 10 ml of solution S to 15 ml with *distilled water R*. The solution complies with the limit test for sulphates (300 ppm).

Ammonium (2.4.1). 50 mg complies with limit test B for ammonium (200 ppm). Prepare the standard using 0.1 ml of *ammonium standard solution (100 ppm NH_4) R*.

Iron (2.4.9). In a separating funnel, dissolve 1.0 g in 10 ml of *dilute hydrochloric acid R*. Shake with three quantities, each of 10 ml, of *methyl isobutyl ketone R1*, shaking for 3 min each time. To the combined organic layers add 10 ml of *water R* and shake for 3 min. The aqueous layer complies with the limit test for iron (10 ppm).

Heavy metals (2.4.8). Dissolve 2.0 g in *water R* and dilute to 20 ml with the same solvent. 12 ml of the solution complies with limit test A for heavy metals (10 ppm). Prepare the standard using the *lead solution (1 ppm Pb) R*.

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at $100\text{ }^{\circ}\text{C}$ to $105\text{ }^{\circ}\text{C}$.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.100 g in 3 ml of *anhydrous formic acid R*. Add 30 ml of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid* using 0.1 ml of *naphtholbenzein solution R* as indicator, until the colour changes from brownish-yellow to green.

1 ml of *0.1 M perchloric acid* is equivalent to 11.51 mg of $\text{C}_{17}\text{H}_{21}\text{ClN}_2\text{S}$.

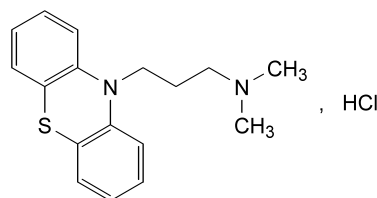
STORAGE

Store protected from light.

01/2005:1365

PROMAZINE HYDROCHLORIDE

Promazini hydrochloridum



$\text{C}_{17}\text{H}_{21}\text{ClN}_2\text{S}$

M_r 320.9

DEFINITION

Promazine hydrochloride contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of 3-(10*H*-phenothiazin-10-yl)-*N,N*-dimethylpropan-1-amine hydrochloride, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, crystalline powder, slightly hygroscopic, very soluble in water, in alcohol and in methylene chloride.

It melts at about $179\text{ }^{\circ}\text{C}$.

IDENTIFICATION

First identification: A, B, D.

Second identification: B, C, D.

- Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *promazine hydrochloride CRS*.
- It complies with the identification test for phenothiazines by thin-layer chromatography (2.3.3). Use *promazine hydrochloride CRS* to prepare the reference solution.
- Dissolve about 5 mg in 2 ml of *sulphuric acid R* and allow to stand for 5 min. An orange colour is produced.
- It gives reaction (b) of chlorides (2.3.1).

TESTS

pH (2.2.3). Dissolve 0.5 g in *carbon dioxide-free water R* and dilute to 10 ml with the same solvent. The pH of the freshly prepared solution is 4.2 to 5.2.

Related substances. Carry out the test protected from bright light. Prepare the solutions immediately before use.

Examine by thin layer chromatography (2.2.27), using a *TLC silica gel F₂₅₄ plate R*.

Test solution. Dissolve 0.10 g of the substance to be examined in a mixture of 5 volumes of *diethylamine R* and 95 volumes of *methanol R* and dilute to 10 ml with the same mixture of solvents.

Reference solution (a). Dilute 1 ml of the test solution to 200 ml with a mixture of 5 volumes of *diethylamine R* and 95 volumes of *methanol R*.

Reference solution (b). Dissolve 10 mg of *chlorprothixene hydrochloride CRS* in a mixture of 5 volumes of *diethylamine R* and 95 volumes of *methanol R*, add 1 ml of the test solution and dilute to 10 ml with the same mixture of solvents.

Apply to the plate 10 µl of each solution. Develop over a path of 15 cm using a mixture of 10 volumes of *acetone R*, 10 volumes of *diethylamine R* and 80 volumes of *cyclohexane R*. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. Any spot in the chromatogram obtained with the test solution, apart from the principal spot, is not more intense than the spot in the chromatogram obtained with the reference solution (a) (0.5 per cent). Disregard any spot at the starting point. The test is not valid unless the chromatogram obtained with reference solution (b) shows two clearly separated principal spots.

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

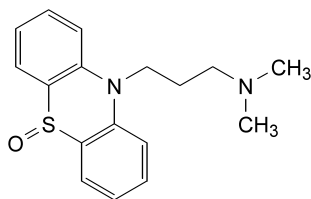
Dissolve 0.250 g in a mixture of 5.0 ml of 0.01 M *hydrochloric acid* and 50 ml of *alcohol R*. Carry out a potentiometric titration (2.2.20), using 0.1 M *sodium hydroxide*. Read the volume added between the two points of inflexion.

1 ml of 0.1 M *sodium hydroxide* is equivalent to 32.09 mg of C₁₇H₂₁ClN₂S.

STORAGE

Store protected from light.

IMPURITIES

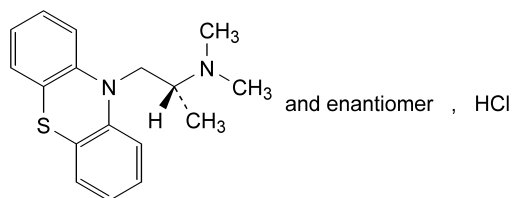


- A. 3-(10H-phenothiazin-10-yl)-N,N-dimethylpropan-1-amine S-oxide (promazine sulphoxide).

01/2005:0524

PROMETHAZINE HYDROCHLORIDE

Promethazini hydrochloridum



C₁₇H₂₁ClN₂S

M_r 320.9

DEFINITION

Promethazine hydrochloride contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of (2*RS*)-N,N-dimethyl-1-(10H-phenothiazin-10-yl)propan-2-amine hydrochloride, calculated with reference to the dried substance.

CHARACTERS

A white or faintly yellowish, crystalline powder, very soluble in water, freely soluble in alcohol and in methylene chloride.

It melts at about 222 °C, with decomposition.

IDENTIFICATION

First identification: A, B, D.

Second identification: B, C, D.

- Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *promethazine hydrochloride CRS*.
- It complies with the identification test for phenothiazines by thin-layer chromatography (2.3.3).
- Dissolve 0.1 g in 3 ml of *water R*. Add dropwise 1 ml of *nitric acid R*. A precipitate is formed which rapidly dissolves to give a red solution, becoming orange and then yellow. Heat to boiling. The solution becomes orange and an orange-red precipitate is formed.
- It gives reaction (b) of chlorides (2.3.1).

TESTS

pH (2.2.3). Dissolve 1.0 g in *carbon dioxide-free water R* and dilute to 10 ml with the same solvent. The pH of the solution measured immediately after preparation is 4.0 to 5.0.

Related substances. Carry out the test protected from bright light. Prepare the solutions immediately before use. Examine by thin-layer chromatography (2.2.27), using a suitable silica gel as the coating substance.

Test solution. Dissolve 0.20 g of the substance to be examined in a mixture of 5 volumes of *diethylamine R* and 95 volumes of *methanol R* and dilute to 10 ml with the same mixture of solvents.

Reference solution (a). Dissolve 20 mg of *isopromethazine hydrochloride CRS* in a mixture of 5 volumes of *diethylamine R* and 95 volumes of *methanol R* and dilute to 10 ml with the same mixture of solvents.

Reference solution (b). Dilute 0.5 ml of the test solution to 100 ml with a mixture of 5 volumes of *diethylamine R* and 95 volumes of *methanol R*.

Reference solution (c). Dilute 0.2 ml of the test solution to 100 ml with a mixture of 5 volumes of *diethylamine R* and 95 volumes of *methanol R*.

Apply separately to the plate 10 µl of the test solution and 10 µl of each reference solution. Develop in an unsaturated tank over a path of 12 cm using a mixture of 5 volumes of *diethylamine R*, 10 volumes of *acetone R* and 85 volumes of *cyclohexane R*. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. Disregard any spot at the starting point.

In the chromatogram obtained with the test solution: any spot corresponding to isopromethazine hydrochloride is not more intense than the spot in the chromatogram obtained with reference solution (a) (1 per cent); any spot, apart from the principal spot and the spot corresponding to isopromethazine hydrochloride, is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.5 per cent) and at most three such spots are more intense than the spot in the chromatogram obtained with reference solution (c) (0.2 per cent).

Heavy metals (2.4.8). Dissolve 1.0 g in 5 ml of *water R*, add 5 ml of *acetone R* and 5 ml of *buffer solution pH 3.5 R*. Carry out the prefiltration. The prefiltrate complies with limit test E for heavy metals (10 ppm). Prepare the standard using 5 ml of *lead standard solution (2 ppm Pb) R*.

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

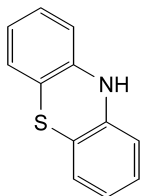
Dissolve 0.250 g in a mixture of 5.0 ml of 0.01 M hydrochloric acid and 50 ml of alcohol R. Carry out a potentiometric titration (2.2.20), using 0.1 M sodium hydroxide. Read the volume added between the two points of inflexion.

1 ml of 0.1 M sodium hydroxide is equivalent to 32.09 mg of $C_{17}H_{21}ClN_2S$.

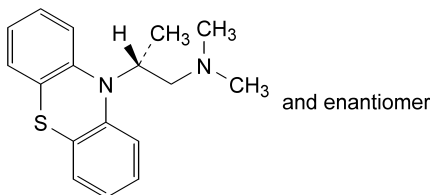
STORAGE

Store protected from light.

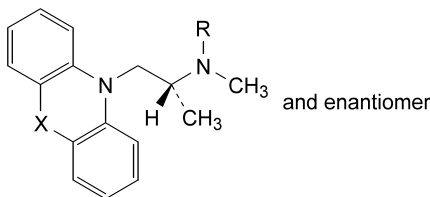
IMPURITIES



A. phenothiazine,



B. (2RS)-N,N-dimethyl-2-(10H-phenothiazin-10-yl)propan-1-amine (isopromethazine),



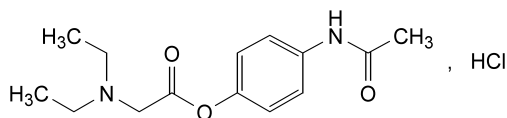
C. R = H, X = S: (2RS)-N-methyl-1-(10H-phenothiazin-10-yl)propan-2-amine,

D. R = CH₃, X = SO: (2RS)-N,N-dimethyl-1-(10H-phenothiazin-10-yl)propan-2-amine S-oxide.

01/2005:1366

PROPACETAMOL HYDROCHLORIDE

Propacetamoli hydrochloridum



$C_{14}H_{21}ClN_2O_3$

M_r 300.8

DEFINITION

Propacetamol hydrochloride contains not less than 98.0 per cent and not more than the equivalent of 102.0 per cent of 4-(acetlamino)phenyl (diethylamino)acetate hydrochloride, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, crystalline powder, freely soluble in water, slightly soluble in ethanol, practically insoluble in acetone.

IDENTIFICATION

A. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the Ph. Eur. reference spectrum of propacetamol hydrochloride.

B. It gives reaction (a) of chlorides (2.3.1).

TESTS

Solution S. Prepare the solution immediately before use. Dissolve 1.75 g in water R and dilute to 10.0 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and not more intensely coloured than reference solution Y₆ or BY₆ (2.2.2, Method II).

Absorbance. The absorbance (2.2.25) of solution S measured at 390 nm is not greater than 0.05.

Related substances. Examine by liquid chromatography (2.2.29).

Solution A. Dissolve 2.16 g of sodium octanesulphonate R in 900 ml of water R and dilute to 1000 ml with the same solvent. Adjust to pH 3.0 with acetic acid R.

Test solution. Suspend 1.00 g of the substance to be examined in 10.0 ml of acetonitrile R. Shake for 10 min. Allow to stand. Take 3.0 ml of the supernatant solution and dilute to 10.0 ml with solution A. Inject this solution immediately after preparation.

Reference solution (a). Dissolve 50 mg of paracetamol R in acetonitrile R and dilute to 50.0 ml with the same solvent. Dilute 1.0 ml of the solution to 50.0 ml with acetonitrile R. Dilute 3.0 ml of the latter solution to 10.0 ml with solution A.

Reference solution (b). Dissolve 10 mg of paracetamol R and 0.100 g of 4-aminophenol R in acetonitrile R and dilute to 50.0 ml with the same solvent. Dilute 1.0 ml of the solution to 50.0 ml with acetonitrile R. Dilute 3.0 ml of the latter solution to 10.0 ml with solution A.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4.6 mm in internal diameter packed with octadecylsilyl silica gel for chromatography R (5 µm),
- as mobile phase at a flow rate of 1 ml/min a mixture of 30 volumes of acetonitrile R and 70 volumes of solution A,
- as detector a spectrophotometer set at 246 nm.

Inject 20 µl of reference solution (b). The chromatogram obtained shows a peak corresponding to paracetamol (first peak) and a peak corresponding to 4-aminophenol (second peak) with a retention time relative to paracetamol of about 1.6. Adjust the sensitivity of the system so that the height of the two principal peaks is at least 20 per cent of the full scale of the recorder.

Inject 20 µl of the test solution and 20 µl of reference solution (a). Continue the chromatography of the test solution for twice the retention time of the principal peak. In the chromatogram obtained with the test solution: the area of any peak corresponding to paracetamol is not greater than that of the peak due to paracetamol in the chromatogram obtained with reference solution (a) (200 ppm); the area of any peak, apart from the principal peak and the peak due to paracetamol, is not greater than 3.2 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent taking into account the response factor of paracetamol of 1.6); the sum of the areas of any peak, apart from the principal peak, is not greater than 6.4 times the area of the paracetamol peak in the chromatogram obtained with reference solution (a) (0.2 per cent taking into account the relative response factor

of paracetamol of 1.6). Disregard any peak with an area less than 0.01 times the area of the principal peak in the chromatogram obtained with reference solution (a).

4-Aminophenol. Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel F₂₅₄ plate R*.

Test solution. Suspend 4.00 g of the substance to be examined in 8 ml of *acetonitrile R*. Shake for 30 min and filter. Dilute to 10 ml with *acetonitrile R*.

Reference solution (a). Dissolve 25 mg of *4-aminophenol R* in *acetonitrile R* and dilute to 50 ml with the same solvent. Dilute 10 ml of the solution to 50 ml with *acetonitrile R*.

Reference solution (b). Dilute 5 ml of reference solution (a) to 50 ml with *acetonitrile R*.

Reference solution (c). Dilute 0.2 ml of reference solution (a) to 5 ml with the test solution.

Apply to the plate 50 µl of the test solution and 50 µl each of reference solutions (b) and (c). Develop over a path of 15 cm using a mixture of 3 volumes of *anhydrous formic acid R*, 4 volumes of *water R*, 30 volumes of *methanol R* and 64 volumes of *methylene chloride R*. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. Spray with a 10 g/l solution of *dimethylaminobenzaldehyde R* in *alcohol R*. Reference solution (c) shows two spots: one visible in ultraviolet light and corresponding to propacetamol hydrochloride and the other one yellow, visible after spraying and corresponding to 4-aminophenol. An additional spot may appear in ultraviolet light and corresponds to paracetamol. In the chromatogram obtained with the test solution, any yellow spot corresponding to 4-aminophenol and not visible in ultraviolet light is not more intense than the spot in the chromatogram obtained with reference solution (b) (25 ppm). The test is not valid unless the chromatogram obtained with reference solution (c) shows two clearly separated spots.

Methanol. Examine by gas chromatography (2.2.28), using *propanol R* as the internal standard.

Internal standard solution. Dilute 2.0 ml of *propanol R* to 20.0 ml with *water R*. Dilute 1.0 ml of the solution to 25.0 ml with *water R*. Dilute 1.0 ml of this solution to 25.0 ml with *water R*.

Test solution. Dissolve 2.00 g of the substance to be examined in *water R*, add 2.0 ml of the internal standard solution and dilute to 10.0 ml with *water R*.

Reference solution. Dilute 0.8 ml of *methanol R* to 50.0 ml with *water R*. Dilute 1.0 ml of the solution to 25.0 ml with *water R*. To 2.0 ml of the solution, add 2.0 of the internal standard solution and dilute to 10.0 ml with *water R*.

The chromatographic procedure may be carried out using:

- a glass column 2 m long and 2 mm in internal diameter packed with carbon molecular sieve impregnated with 0.2 per cent of macrogol 1500,
- *nitrogen for chromatography R* as the carrier gas,
- a flame-ionisation detector,

with the following temperature programme:

	Time (min)	Temperature (°C)	Rate (°C/min)	Comment
Column	0 - 1.5	60	–	isothermal
	1.5 - 5.5	60 → 80	5	linear gradient
	5.5 - 15.5	80	–	isothermal
Injection port		170		
Detector		220		

Inject 2 µl of the test solution and 2 µl of the reference solution.

Calculate the ratio of the area of the peak due to methanol to the area of the peak due to propanol for both chromatograms. The ratio for the test solution is not greater than that for the reference solution (500 ppm).

Heavy metals (2.4.8). Dissolve 2.0 g in *water R* and dilute to 20 ml with the same solvent. 12 ml of the solution complies with limit test A for heavy metals (10 ppm). Prepare the standard using *lead standard solution 1 ppm (Pb) R*.

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 100-105 °C for 3 h.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.250 g in a mixture of 25 ml of *anhydrous acetic acid R* and 25 ml of *acetic anhydride R*. Titrate with 0.1 M *perchloric acid*, determining the end-point potentiometrically (2.2.20).

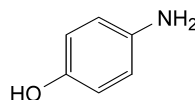
1 ml of 0.1 M *perchloric acid* is equivalent to 30.08 mg of C₁₄H₂₁ClN₂O₃.

STORAGE

Store protected from humidity.

IMPURITIES

A. paracetamol,

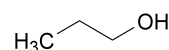


B. 4-aminophenol.

01/2005:2036
corrected

PROPANOL

Propanolum



C₃H₈O

M_r 60.1

DEFINITION

Propan-1-ol.

CHARACTERS

Appearance: clear, colourless liquid.

Solubility: miscible with water and with ethanol.

IDENTIFICATION

First identification: C, B.

Second identification: A, B, D.

A. Refractive index (2.2.6): 1.384 to 1.387.

B. Boiling point (2.2.12): 96 °C to 98 °C.

C. Infrared absorption spectrophotometry (2.2.24).

Comparison: *Ph. Eur. reference spectrum of propanol.*

D. To 1.0 ml add 0.10 g of *dinitrobenzoyl chloride R* and 0.05 ml of *sulphuric acid R*. Boil under reflux for 30 min. Evaporate until the excess of propanol is removed, add 5 ml of *heptane R* to the residue and heat to boiling. Filter the hot solution. Wash the crystals formed on cooling with *heptane R* and dry in vacuum (2 kPa, at room temperature for 24 h). The small, colourless, shiny plates melt (2.2.14) between 71 °C and 74 °C.

TESTS

Solution S. Dissolve the residue obtained in the test for non-volatile matter in 1 ml of 1 M hydrochloric acid and dilute to 50.0 ml with water R.

Appearance. The substance to be examined is clear (2.2.1) and colourless (2.2.2, Method II). Dilute 2 ml to 10 ml with water R. After 5 min, the solution is clear (2.2.1).

Acidity or alkalinity. To 10.0 ml of carbon dioxide-free water R add 0.1 ml of phenolphthalein solution R and 0.01 M sodium hydroxide until the solution becomes pale pink. After addition of 5.0 ml of the substance to be examined the colour of the solution does not become more intense. If the colour fades, add 0.2 ml of 0.01 M sodium hydroxide. The solution is pink.

Absorbance (2.2.25). Measure the absorbance between 230 nm and 310 nm using water R as the compensation liquid. The absorbance *A* is not greater than the following values.

Wavelength (nm)	Absorbance <i>A</i>
230	0.300
250	0.100
270	0.030
290	0.020
310	0.010

The absorption curve does not show any peaks.

Reducing substances. Place 10.0 ml in a test tube of about 20 mm in diameter in a water bath at 20 °C. Keep protected from actinic light and add 1.0 ml of a freshly prepared 0.16 g/l solution of potassium permanganate R. The mixture, maintained at 20 °C, slowly changes its colour from violet to red. After 30 min, the test solution is not less intensely coloured (2.2.2, Method II) than 10.0 ml of a reference solution prepared as follows: to 5.5 ml of primary solution yellow, add 13.0 ml of primary solution red and dilute to 100.0 ml with water R.

Related substances. Gas chromatography (2.2.28).

Test solution. The substance to be examined.

Reference solution (a). Dilute 1.0 ml of the test solution to 100.0 ml with heptane R. Dilute 1.0 ml of the solution to 10.0 ml with heptane R.

Reference solution (b). Mix 0.1 ml of acetone R with 0.1 ml of 2-propanol R and dilute to 100 ml with the test solution.

Column:

- **material:** fused silica,
- **size:** *l* = 30 m, Ø = 0.25 mm,
- **stationary phase:** poly[(cyanopropyl)(phenyl)][dimethylsiloxane R (film thickness 1.4 µm).

Carrier gas: helium for chromatography R.

Linear velocity: 25 cm/s.

Split ratio: 1:200.

Temperature:

	Time (min)	Temperature (°C)
Column	0 - 12	40
	12 - 28	40 → 200
	28 - 38	200
Injection port		240
Detector		240

Detection: flame ionisation.

Injection: 1 µl.

System suitability: reference solution (b):

- **resolution:** minimum 2.0 between the peaks due to impurity D and impurity E.

Limits:

- **any impurity:** not more than the area of the peak due to propanol in the chromatogram obtained with reference solution (a) (0.1 per cent),
- **total:** not more than 3 times the area of the peak due to propanol in the chromatogram obtained with reference solution (a) (0.3 per cent),
- **disregard limit:** 0.1 times the area of the peak due to propanol in the chromatogram obtained with reference solution (a) (0.01 per cent).

Non-volatile matter: maximum 0.004 per cent.

Evaporate 50 ml of the substance to be examined to dryness at 100 °C and dry the residue in an oven at 100-105 °C to constant mass. The residue weighs a maximum of 2 mg. The residue is used for the preparation of solution S.

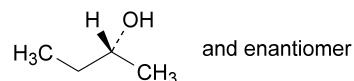
Water (2.5.12): maximum 0.2 per cent, determined on 10 g.

STORAGE

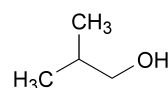
Protected from light.

IMPURITIES

- A. CH₃-OH: methanol,
 B. ethanol,
 C. CH₃-CH₂-CHO: propanal,
 D. acetone,
 E. isopropyl alcohol (2-propanol),



- F. butan-2-ol (sec-butanol),

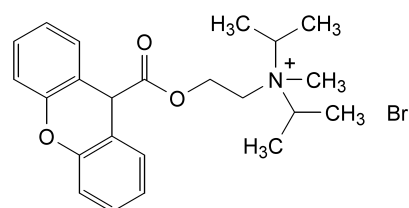


- G. 2-methylpropan-1-ol (isobutanol),
 H. CH₃-[CH₂]₃-OH: butan-1-ol (*n*-butanol),
 I. CH₃-[CH₂]₄-OH: pentan-1-ol (*n*-pentanol),
 J. CH₃-[CH₂]₅-OH: hexan-1-ol (*n*-hexanol).

01/2005:0857

PROPANTHELINE BROMIDE

Propanthelini bromidum



C₂₃H₃₀BrNO₃

*M*_r 448.4

DEFINITION

Propantheline bromide contains not less than 98.5 per cent and not more than the equivalent of 101.0 per cent of *N*-methyl-*N*,*N*-bis(1-methylethyl)-2-[(9*H*-xanthen-9-ylcarbonyl)oxy]ethanaminium bromide, calculated with reference to the dried substance.

CHARACTERS

A white or yellowish-white powder, slightly hygroscopic, very soluble in water, in alcohol and in methylene chloride.

IDENTIFICATION

- A. Dissolve 60 mg in *methanol R* and dilute to 100.0 ml with the same solvent. Dilute 10.0 ml of the solution to 100.0 ml with *methanol R*. Examined between 230 nm and 350 nm (2.2.25), the solution shows two absorption maxima, at 246 nm and 282 nm. The specific absorbances at the maxima are 115 to 125 and 57 to 63, respectively.
- B. Dissolve 0.2 g in 15 ml of *water R* and add 1 ml of *strong sodium hydroxide solution R*. Boil for 2 min and cool slightly. Add 7.5 ml of *dilute hydrochloric acid R* and filter. Wash the residue with *water R* and recrystallise from *alcohol (50 per cent V/V) R*. Dry at 100 °C to 105 °C for 1 h. Dissolve about 10 mg of the residue in 5 ml of *sulphuric acid R*. The solution has an intense yellow colour and shows an intense yellowish-green fluorescence when examined in ultraviolet light at 365 nm.
- C. Dissolve 50 mg in 0.1 ml of *water R* in a 25 ml flask and add 1 ml of a saturated solution of *potassium permanganate R*. Attach a fractionating column and a condenser, with the end of the delivery tube immersed in 1 ml of *water R* in a test-tube placed in a bath of iced water. Distil fairly vigorously and continue heating for 1 min after a dry residue has been obtained in the flask. Prepare a blank by introducing into an identical test-tube a volume of *water R* equal to that of the distillate. Place the tubes in a bath of iced water. To each tube, add 0.5 ml of a 20 per cent *V/V* solution of *morpholine R* and 0.5 ml of a freshly prepared 50 g/l solution of *sodium nitroprusside R*. Mix and allow to stand at 0 °C for 5 min and then at room temperature for 3 min. No blue colour develops in either tube. Add 1 g of *ammonium sulphate R*, mix and allow to stand for 15 min. A stable, intense pink colour develops in the test solution. A brownish-yellow colour develops in the blank.
- D. It gives reaction (a) of bromides (2.3.1).

TESTS

Appearance of solution. Dissolve 0.6 g in *water R* and dilute to 20 ml with the same solvent. The solution is clear (2.2.1).

Related substance. Examine by liquid chromatography (2.2.29).

Test solution (a). Dissolve 6 mg of the substance to be examined in a mixture of 40 volumes of *acetonitrile R* and 60 volumes of *water R* and dilute to 50 ml with the same mixture of solvents.

Test solution (b). Dissolve 6 mg of the substance to be examined in 30 ml of a mixture of 40 volumes of *acetonitrile R* and 60 volumes of *water R*. Add 5 ml of reference solution (b) and dilute to 50 ml with a mixture of 40 volumes of *acetonitrile R* and 60 volumes of *water R*.

Test solution (c). Dissolve 6 mg of *xanthidrol R1* and 6 mg of the substance to be examined in a mixture of 40 volumes of *acetonitrile R* and 60 volumes of *water R* and dilute to 50 ml with the same mixture of solvents.

Reference solution (a). Dissolve 6 mg of *xanthidrol R1* in a mixture of 40 volumes of *acetonitrile R* and 60 volumes of *water R* and dilute to 50 ml with the same mixture of solvents.

Reference solution (b). Dilute 5 ml of reference solution (a) to 50 ml with a mixture of 40 volumes of *acetonitrile R* and 60 volumes of *water R*.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4.6 mm in internal diameter, packed with *octadecylsilyl silica gel for chromatography R* (5 µm),
- as mobile phase at a flow rate of 1 ml/min a mixture of equal volumes of *acetonitrile R* and a solution containing 28 g/l of *sodium perchlorate R* and 11 g/l of *phosphoric acid R*, adjusted to pH 3.8 with *strong sodium hydroxide solution R* and then with 0.1 *M* *sodium hydroxide*,
- as detector a spectrophotometer set at 206 nm,
- a loop injector.

Inject 20 µl of test solution (c). The test is not valid unless the resolution between the peaks corresponding to propantheline and xanthidrol is at least 8.0. Inject 20 µl of test solution (a), 20 µl of reference solution (a) and 20 µl of test solution (b). Continue the chromatography for twice the retention time of the propantheline peak. In the chromatogram obtained with test solution (a), there is no peak corresponding to the principal peak in the chromatogram obtained with reference solution (a). In the chromatogram obtained with test solution (b), the area of any peak, apart from the principal peak and the xanthidrol peak, is not greater than the area of the xanthidrol peak (1.0 per cent) and not more than one such peak has an area greater than or equal to half the area of the xanthidrol peak (0.5 per cent). Disregard any peak with a retention time relative to that of the propantheline peak, less than 0.2 (bromide).

Loss on drying (2.2.32). Not more than 1.0 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.400 g in 50 ml of *acetic anhydride R*. Titrate with 0.1 *M* *perchloric acid*, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 *M* *perchloric acid* corresponds to 44.84 mg of $C_{23}H_{30}BrNO_3$.

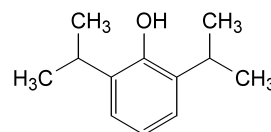
STORAGE

Store in an airtight container.

01/2005:1558

PROPOFOL

Propofolum



$C_{12}H_{18}O$

M_r 178.3

DEFINITION

Propofol contains not less than 98.0 per cent and not more than the equivalent of 102.0 per cent of 2,6-bis(1-methylethyl)phenol.

CHARACTERS

A colourless or very light yellow, clear liquid, very slightly soluble in water, miscible with hexane and with methanol.

IDENTIFICATION

Examine by infrared absorption spectrophotometry (2.2.24) as a thin film between potassium bromide plates, comparing with the spectrum obtained with the *propofol CRS*.

TESTS

Refractive index (2.2.6). The refractive index is not less than 1.5125 and not more than 1.5145.

Impurity J. Examine by liquid chromatography (2.2.29).

Prepare the solutions immediately before use and protect from light.

Test solution. Dissolve 0.5 g of the substance to be examined in *hexane R* and dilute to 10.0 ml with the same solvent.

Reference solution. Dissolve 5 µl of *propofol impurity J CRS* (corresponding to 5 mg) in *hexane R* and dilute to 50.0 ml with the same solvent. Dilute 5.0 ml of this solution to 100.0 ml with *hexane R*.

The chromatographic procedure used in the test for related substances may be used, by monitoring the eluate of the column at 254 nm in place of 275 nm.

Inject 20 µl of the test solution and 20 µl of the reference solution and continue the chromatography for 6 times the retention time of the propofol peak. In the chromatogram obtained with the test solution the area of any peak due to impurity J is not greater than 5 times the area of the peak due to impurity J in the chromatogram obtained with the reference solution (0.05 per cent).

Related substances. Examine by liquid chromatography (2.2.29) as described under Assay.

Inject 10 µl of reference solution (a) and continue the chromatography for 15 min. The resolution of the 2 principal peaks observed (impurity J, approximate retention time 2.5 min and propofol, approximate retention time 3 min) is at least 4.0.

Inject 10 µl of reference solution (b) and locate the peaks due to impurity G (relative retention about 0.5) and impurity E (relative retention about 5), both with respect to propofol.

Inject 10 µl of reference solution (c) and adjust the sensitivity of the system so that the height of the peak due to propofol is at least 50 per cent of the full scale of the recorder.

Inject separately 10 µl of test solution (a) and reference solution (c) and record the chromatogram of test solution (a) for 6 times the retention time of the peak due to propofol. In the chromatogram obtained with test solution (a): the area of any peak due to impurity G is not greater than 0.4 times the area of the peak due to propofol in the chromatogram obtained with reference solution (c) (0.2 per cent taking into account a response factor of 0.2); and the area of any peak due to impurity E is not greater than 0.4 times the area of the peak due to propofol in the chromatogram obtained with reference solution (c) (0.01 per cent taking into account a response factor of 4.0). The area of any other peak apart from that due to propofol and the 2 impurities is not greater than 0.5 times the area of the peak due to propofol in the chromatogram obtained with reference solution (c) (0.05 per cent). The sum of all impurities including impurity G and impurity E is not greater than 0.3 per cent. Disregard any peak with an area less than 0.25 times the area of the peak due to propofol in the chromatogram obtained with reference solution (c) (0.025 per cent).

ASSAY

Examine by liquid chromatography (2.2.29).

Test solution (a). Dissolve 1.00 g of the substance to be examined in *hexane R* and dilute to 10.0 ml with the same solvent.

Test solution (b). Dissolve 0.240 g of the substance to be examined in *hexane R* and dilute to 100.0 ml with the same solvent.

Reference solution (a). Dissolve 5 µl of the substance to be examined and 15 µl of *propofol impurity J CRS* in *hexane R* and dilute to 50.0 ml with the same solvent.

Reference solution (b). Dilute 0.1 ml of *propofol for system suitability CRS* to 1.0 ml with *hexane R*.

Reference solution (c). Dilute 1.0 ml of test solution (a) to 100.0 ml with *hexane R*. Dilute 1.0 ml of this solution to 10.0 ml with *hexane R*.

Reference solution (d). Dissolve 0.240 g of *propofol CRS* in *hexane R* and dilute to 100.0 ml with the same solvent.

The chromatographic procedure may be carried out using:

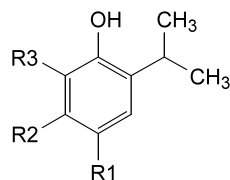
- a stainless steel column 0.20 m long and 5 mm in internal diameter packed with *silica gel for chromatography R* (5 µm),
- as mobile phase at a flow rate of 2.0 ml/min a mixture of 1.0 volumes of *ethanol R*, 7.5 volumes of *acetonitrile R* and 990 volumes of *hexane R*,
- as detector a spectrophotometer set at 275 nm.

Inject 10 µl of test solution (b) and 10 µl of reference solution (d). Continue the chromatography for 5 times the retention time of the peak due to propofol.

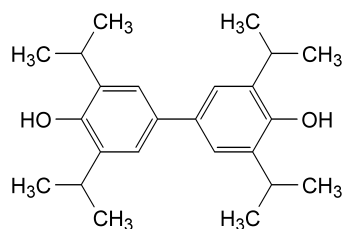
STORAGE

Store protected from light under an inert atmosphere of gas.

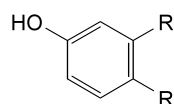
IMPURITIES



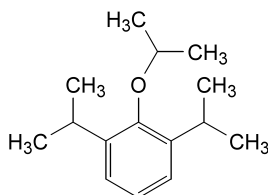
- A. R1 = CH(CH₃)₂, R2 = R3 = H: 2,4-bis(1-methylethyl)phenol,
 B. R1 = R2 = H, R3 = CCH₃=CH₂: 2-(1-methylethenyl)-6-(1-methylethyl)phenol,
 C. R1 = R2 = R3 = H: 2-(1-methylethyl)phenol,
 D. R1 = R3 = H, R2 = CH(CH₃)₂: 2,5-bis(1-methylethyl)phenol,



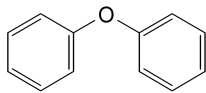
- E. 3,3',5,5'-tetrakis(1-methylethyl)biphenyl-4,4'-diol,



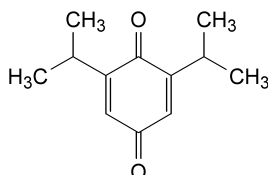
- F. R = CH(CH₃)₂, R' = H: 3-(1-methylethyl)phenol,
 H. R = H, R' = CH(CH₃)₂: 4-(1-methylethyl)phenol,



G. 2-(1-methylethoxy)-1,3-bis(1-methylethyl)benzene,



I. oxydibenzene,

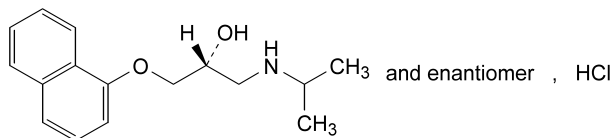


J. 2,6-bis(1-methylethyl)-1,4-benzoquinone.

01/2005:0568
corrected

PROPRANOLOL HYDROCHLORIDE

Propranololi hydrochloridum



$C_{16}H_{22}ClNO_2$

M_r 295.8

DEFINITION

Propranolol hydrochloride contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of (2*RS*)-1-[(1-methylethyl)amino]-3-(naphthalen-1-yloxy)propan-2-ol hydrochloride, calculated with reference to the dried substance.

CHARACTERS

A white or almost white powder, soluble in water and in alcohol.

IDENTIFICATION

First identification: B, D.

Second identification: A, C, D.

- Melting point (2.2.14): 163 °C to 166 °C.
- Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *propranolol hydrochloride CRS*.
- Examine by thin-layer chromatography (2.2.27), using *silica gel G R* as the coating substance.

Test solution. Dissolve 10 mg of the substance to be examined in 1 ml of *methanol R*.

Reference solution. Dissolve 10 mg of *propranolol hydrochloride CRS* in 1 ml of *methanol R*.

Apply separately to the plate 10 µl of each solution. Develop over a path of 15 cm using a mixture of 1 volume of *concentrated ammonia RI* and 99 volumes of *methanol R*. Dry the plate at 100 °C to 105 °C and spray

with *anisaldehyde solution R*. Heat the plate at 100 °C to 105 °C until the colour of the spots reaches maximum intensity (10 min to 15 min). The principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

D. It gives reaction (a) of chlorides (2.3.1).

TESTS

Appearance of solution. Dissolve 2.0 g in *methanol R* and dilute to 20 ml with the same solvent. The solution is clear (2.2.1) and not more intensely coloured than intensity 6 of the range of reference solutions of the most appropriate colour (2.2.2, *Method II*).

Acidity or alkalinity. Dissolve 0.20 g in *carbon dioxide-free water R* and dilute to 20 ml with the same solvent. Add 0.2 ml of *methyl red solution R* and 0.2 ml of 0.01 *M hydrochloric acid*. The solution is red. Add 0.4 ml of 0.01 *M sodium hydroxide*. The solution is yellow.

Related substances. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 20.0 mg of the substance to be examined in the mobile phase and dilute to 10.0 ml with the mobile phase.

Reference solution (a). Dissolve 10.0 mg of *propranolol hydrochloride for performance test CRS* in the mobile phase and dilute to 10.0 ml with the mobile phase.

Reference solution (b). Dilute 2.0 ml of the test solution to 100.0 ml with the mobile phase. Dilute 1.0 ml of the solution to 10.0 ml with the mobile phase.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4.6 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (5 µm),
- as mobile phase at a flow rate of 1.8 ml/min a mixture prepared as follows: mix 1.6 g of *sodium laurilsulfate R* and 0.31 g of *tetrabutylammonium dihydrogen phosphate R* in a mixture of 1 ml of *sulphuric acid R*, 450 ml of *water R* and 550 ml of *acetonitrile R*; adjust to pH 3.3 using *dilute sodium hydroxide solution R*,
- as detector a spectrophotometer set at 292 nm.

Equilibrate the column for at least 30 min.

Inject 20 µl of reference solution (a). As a system suitability requirement, baseline separation is obtained between the peaks due to impurity A and propranolol; use the chromatogram supplied with *propranolol hydrochloride for performance test CRS* to identify the peak due to impurity A. Inject 20 µl of reference solution (b). Adjust the sensitivity of the system so that the height of the principal peak in the chromatogram obtained is at least 50 per cent of the full scale of the recorder.

Inject 20 µl of the test solution. Continue the chromatography for five times the retention time of the principal peak. In the chromatogram obtained with the test solution, the area of any peak apart from the principal peak, is not greater than half the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent) and the sum of the area of all peaks, apart from the principal peak, is not greater than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (0.4 per cent).

Heavy metals (2.4.8). Dissolve 1.0 g in a mixture of 15 volumes of *water R* and 85 volumes of *methanol R* and dilute to 20 ml with the same mixture of solvents. 12 ml of the solution complies with limit test B for heavy metals (20 ppm). Prepare the standard using lead standard solution

(1 ppm Pb) prepared by diluting *lead standard solution* (100 ppm Pb) *R* with a mixture of 15 volumes of *water R* and 85 volumes of *methanol R*.

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C.

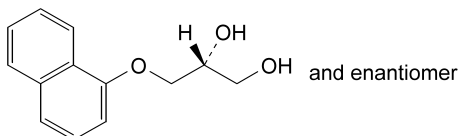
Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

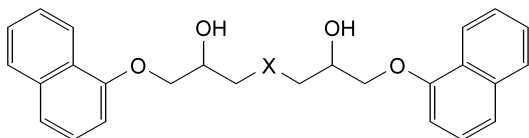
Dissolve 0.250 g in 25 ml of *alcohol R*. Titrate with 0.1 M *sodium hydroxide*, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *sodium hydroxide* is equivalent to 29.58 mg of $C_{16}H_{22}ClNO_2$.

IMPURITIES



A. 3-(naphthalen-1-yloxy)propane-1,2-diol (diol derivative),



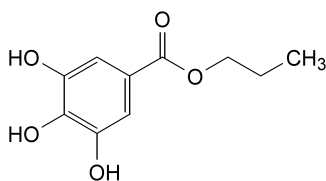
B. X = N-CH(CH₃)₂: 1,1'-[(1-methylethyl)imino]bis[3-(naphthalen-1-yloxy)propan-2-ol] (tertiary amine derivative),

C. X = O: 1,1'-oxybis[3-(naphthalen-1-yloxy)propan-2-ol] (bis-ether derivative).

01/2005:1039

PROPYL GALLATE

Propylis gallas



$C_{10}H_{12}O_5$

M_r 212.2

DEFINITION

Propyl gallate contains not less than 97.0 per cent and not more than the equivalent of 103.0 per cent of propyl 3,4,5-trihydroxybenzoate, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, crystalline powder, very slightly soluble in water, freely soluble in ethanol (96 per cent). It dissolves in dilute solutions of alkali hydroxides.

IDENTIFICATION

First identification: B.

Second identification: A, C, D.

A. Melting point (2.2.14): 148 °C to 151 °C.

B. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *propyl gallate CRS*.

C. Examine the chromatograms obtained in the test for gallic acid. The principal spot in the chromatogram obtained with test solution (b) is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).

D. Dissolve about 10 mg in 10 ml of *water R* by heating to about 70 °C. Cool and add 1 ml of *bismuth subnitrate solution R*. A bright yellow precipitate is formed.

TESTS

Appearance of solution. Dissolve 1.0 g in *ethanol* (96 per cent) *R* and dilute to 20 ml with the same solvent. The solution is clear (2.2.1) and not more intensely coloured than reference solution BY₅ (2.2.2, *Method II*).

Gallic acid. Examine by thin-layer chromatography (2.2.27), using *silica gel G R* as the coating substance.

Test solution (a). Dissolve 0.20 g of the substance to be examined in *acetone R* and dilute to 10 ml with the same solvent.

Test solution (b). Dilute 1 ml of test solution (a) to 20 ml with *acetone R*.

Reference solution (a). Dissolve 10 mg of *propyl gallate CRS* in *acetone R* and dilute to 10 ml with the same solvent.

Reference solution (b). Dissolve 20 mg of *gallic acid R* in *acetone R* and dilute to 20 ml with the same solvent. Dilute 1 ml of the solution to 10 ml with *acetone R*.

Reference solution (c). Dilute 0.5 ml of test solution (b) to 5 ml with reference solution (b).

Apply separately to the plate 5 µl of each solution. Develop over a path of 8 cm using a mixture of 10 volumes of *anhydrous formic acid R*, 40 volumes of *ethyl formate R* and 50 volumes of *toluene R*. Allow the plate to dry in air for 10 min and spray with a mixture of 1 volume of *ferric chloride solution R1* and 9 volumes of *ethanol* (96 per cent) *R*. Any spot due to gallic acid in the chromatogram obtained with test solution (a) is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.5 per cent). The test is not valid unless the chromatogram obtained with reference solution (c) shows 2 clearly separated principal spots.

Total chlorine. Mix 0.5 g with 2 g of *calcium carbonate R1*. Dry and ignite at 700 °C. Take up the residue with 20 ml of *dilute nitric acid R* and dilute to 30 ml with *water R*. 15 ml of the solution, without further addition of *dilute nitric acid R*, complies with the limit test for chlorides (2.4.4) (200 ppm).

Chlorides (2.4.4). To 1.65 g add 50 ml of *water R*. Shake for 5 min. Filter. 15 ml of the filtrate complies with the limit test for chlorides (100 ppm).

Zinc. Not more than 25 ppm of Zn, determined by atomic absorption spectrometry (2.2.23, *Method II*).

Test solution. To 2.5 ml of the solution obtained in the test for heavy metals, add 2.5 ml of *water R*.

Reference solutions. Prepare the reference solutions using *zinc standard solution* (10 ppm Zn) *R*, diluted as necessary with *water R*.

Measure the absorbance at 213.9 nm using a zinc hollow-cathode lamp as the source of radiation and an air-acetylene flame.

Heavy metals (2.4.8). 2.0 g complies with limit test C for heavy metals (10 ppm). Prepare the reference solution using 2 ml of *lead standard solution* (10 ppm Pb) *R*.

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 100–105 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.100 g in *methanol R* and dilute to 250.0 ml with the same solvent. Dilute 5.0 ml of the solution to 200.0 ml with *methanol R*. Measure the absorbance (2.2.25) at the absorption maximum at 275 nm.

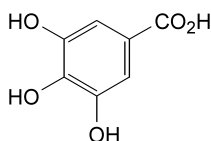
Calculate the content of $C_{10}H_{12}O_3$ taking the specific absorbance to be 503.

STORAGE

Protected from light.

IMPURITIES

Specified impurities: A.

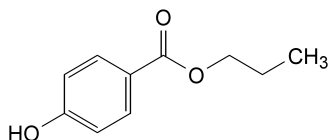


A. 3,4,5-trihydroxybenzoic acid (gallic acid).

01/2005:0431

PROPYL PARAHYDROXYBENZOATE

Propylis parahydroxybenzoas



$C_{10}H_{12}O_3$

M_r 180.2

DEFINITION

Propyl 4-hydroxybenzoate.

Content: 98.0 per cent to 102.0 per cent.

CHARACTERS

Appearance: white, crystalline powder.

Solubility: very slightly soluble in water, freely soluble in alcohol and in methanol.

IDENTIFICATION

First identification: A, B.

Second identification: A, C, D.

A. Melting point (2.2.14): 96 °C to 99 °C.

B. Infrared absorption spectrophotometry (2.2.24).

Comparison: propyl parahydroxybenzoate CRS.

C. Examine the chromatograms obtained in the test for related substances.

Results: the principal spot in the chromatogram obtained with test solution (b) is similar in position and size to the principal spot in the chromatogram obtained with reference solution (b).

D. To about 10 mg in a test-tube add 1 ml of *sodium carbonate solution R*, boil for 30 s and cool (solution A). To a further 10 mg in a similar test-tube add 1 ml of

sodium carbonate solution R; the substance partly dissolves (solution B). Add at the same time to solution A and solution B 5 ml of *aminopyrazolone solution R* and 1 ml of *potassium ferricyanide solution R* and mix. Solution B is yellow to orange-brown. Solution A is orange to red, the colour being clearly more intense than any similar colour which may be obtained with solution B.

TESTS

Solution S. Dissolve 1.0 g in *alcohol R* and dilute to 10 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and not more intensely coloured than reference solution BY₆ (2.2.2, Method II).

Acidity. To 2 ml of solution S add 3 ml of *alcohol R*, 5 ml of *carbon dioxide-free water R* and 0.1 ml of *bromocresol green solution R*. Not more than 0.1 ml of 0.1 M *sodium hydroxide* is required to change the colour of the indicator to blue.

Related substances. Thin-layer chromatography (2.2.27).

Test solution (a). Dissolve 0.10 g of the substance to be examined in *acetone R* and dilute to 10 ml with the same solvent.

Test solution (b). Dilute 1 ml of test solution (a) to 10 ml with *acetone R*.

Reference solution (a). Dilute 0.5 ml of test solution (a) to 100 ml with *acetone R*.

Reference solution (b). Dissolve 10 mg of *propyl parahydroxybenzoate CRS* in *acetone R* and dilute to 10 ml with the same solvent.

Reference solution (c). Dissolve 10 mg of *ethyl parahydroxybenzoate CRS* in 1 ml of test solution (a) and dilute to 10 ml with *acetone R*.

Plate: suitable octadecylsilyl silica gel with a fluorescent indicator having an optimal intensity at 254 nm as the coating substance.

Mobile phase: *glacial acetic acid R*, *water R*, *methanol R* (1:30:70 V/V/V).

Application: 2 µl.

Development: over a path of 15 cm.

Drying: in air.

Detection: examine in ultraviolet light at 254 nm.

System suitability: the chromatogram obtained with reference solution (c) shows 2 clearly separated principal spots.

Limits:

- *any impurity:* any spot in the chromatogram obtained with test solution (a), apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (a) (0.5 per cent).

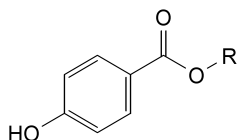
Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

To 1.000 g add 20.0 ml of 1 M *sodium hydroxide*. Heat at about 70 °C for 1 h. Cool rapidly in an ice bath. Prepare a blank in the same manner. Carry out the titration on the solutions at room temperature. Titrate the excess sodium hydroxide with 0.5 M *sulphuric acid*, continuing the titration until the second point of inflexion and determining the end-point potentiometrically (2.2.20).

1 ml of 1 M *sodium hydroxide* is equivalent to 180.2 mg of $C_{10}H_{12}O_3$.

IMPURITIES

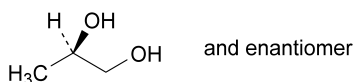


- A. R = H: 4-hydroxybenzoic acid,
 B. R = CH₃: methyl 4-hydroxybenzoate,
 C. R = CH₂-CH₃: ethyl 4-hydroxybenzoate,
 D. R = CH₂-CH₂-CH₂-CH₃: butyl 4-hydroxybenzoate.

01/2005:0430

PROPYLENE GLYCOL

Propylenglycolum

C₃H₈O₂M_r 76.1

DEFINITION

Propylene glycol is (*RS*)-propane-1,2-diol.

CHARACTERS

A viscous, clear, colourless, hygroscopic liquid, miscible with water and with ethanol (96 per cent).

IDENTIFICATION

- A. It complies with the test for relative density (see Tests).
 B. It complies with the test for refractive index (see Tests).
 C. Boiling point (2.2.12): 184 °C to 189 °C.
 D. To 0.5 ml add 5 ml of *pyridine R* and 2 g of finely ground *nitrobenzoyl chloride R*. Boil for 1 min and pour into 15 ml of cold *water R* with shaking. Filter, wash the precipitate with 20 ml of a saturated solution of *sodium hydrogen carbonate R* and then with *water R* and dry. Dissolve in boiling *ethanol (80 per cent V/V) R* and filter the hot solution. On cooling, crystals are formed which, after drying at 100-105 °C, melt (2.2.14) at 121 °C to 128 °C.

TESTS

Appearance. It is clear (2.2.1) and colourless (2.2.2, *Method II*).

Relative density (2.2.5): 1.035 to 1.040.

Refractive index (2.2.6): 1.431 to 1.433.

Acidity. To 10 ml add 40 ml of *water R* and 0.1 ml of *bromothymol blue solution R1*. The solution is greenish-yellow. Not more than 0.05 ml of 0.1 M *sodium hydroxide* is required to change the colour of the indicator to blue.

Oxidising substances. To 10 ml add 5 ml of *water R*, 2 ml of *potassium iodide solution R* and 2 ml of *dilute sulphuric acid R* and allow to stand in a ground-glass-stoppered flask protected from light for 15 min. Titrate with 0.05 M *sodium thiosulphate*, using 1 ml of *starch solution R* as indicator. Not more than 0.2 ml of 0.05 M *sodium thiosulphate* is required.

Reducing substances. To 1 ml add 1 ml of *dilute ammonia R1* and heat in a water-bath at 60 °C for 5 min. The solution is not yellow. Immediately add 0.15 ml of 0.1 M *silver nitrate* and allow to stand for 5 min. The solution does not change its appearance.

Heavy metals (2.4.8). Mix 4 ml with 16 ml of *water R*. 12 ml of the solution complies with limit test A for heavy metals (5 ppm *m/V*). Prepare the reference solution using *lead standard solution (1 ppm Pb) R*.

Water (2.5.12). Not more than 0.2 per cent, determined on 5.00 g by the semi-micro determination of water.

Sulphated ash (2.4.14). Heat 50 g until it burns and ignite. Allow to cool. Moisten the residue with *sulphuric acid R* and ignite; repeat the operations. The residue weighs not more than 5 mg (0.01 per cent).

STORAGE

Store in an airtight container.

01/2005:2122

PROPYLENE GLYCOL
DICAPRYLOCAPRATE

Propylenglycoli dicaprylocapras

DEFINITION

Propylene glycol diesters of saturated fatty acids, mainly caprylic acid (octanoic acid; C₈H₁₆O₂) and capric acid (decanoic acid; C₁₀H₂₀O₂), of vegetable origin.

CHARACTERS

Appearance: almost colourless to light yellow, oily liquid.

Solubility: practically insoluble in water, soluble in fatty oils and in light petroleum, slightly soluble in anhydrous ethanol.

IDENTIFICATION

- A. Refractive index (2.2.6): 1.439 to 1.442.
 B. Relative density (2.2.5): 0.910 to 0.930.
 C. Viscosity (2.2.9): 9 mPas to 12 mPas.
 D. It complies with the test for composition of fatty acids (see Tests).

TESTS

Appearance. The substance to be examined is clear (2.2.1) and not more intensely coloured than reference solution BY₆ (2.2.2, *Method II*).

Acid value (2.5.1): maximum 0.2.

Hydroxyl value (2.5.3, *Method A*): maximum 10.

Iodine value (2.5.4): maximum 1.0.

Peroxide value (2.5.5, *Method A*): maximum 1.0.

Saponification value (2.5.6): 320 to 340.

Unsaponifiable matter (2.5.7): maximum 0.3 per cent, determined on 5.0 g.

Alkaline impurities. Dissolve 2.00 g of the substance to be examined in a mixture of 1.5 ml of *ethanol (96 per cent) R* and 3.0 ml of *ether R*. Add 0.05 ml of *bromophenol blue solution R*. Not more than 0.15 ml of 0.01 M *hydrochloric acid* is required to change the colour of the indicator to yellow.

Composition of fatty acids. Gas chromatography (2.4.22, Method C). Prepare reference solution (a) as indicated in Table 2.4.22.-2.

Column:

- **material:** fused silica,
- **size:** $l = 30$ m, $\varnothing = 0.32$ mm,
- **stationary phase:** macrogol 20 000 R (film thickness 0.5 μ m),

Carrier gas: helium for chromatography R.

Flow rate: 1.3 ml/min.

Split ratio: 1:100.

Temperature:

	Time (min)	Temperature (°C)
Column	0 - 1	70
	1 - 35	70 $\rightarrow$ 240
	35 - 50	240
Injection port		250
Detector		250

Detection: flame ionisation.

Composition of the fatty acid fraction of the substance to be examined:

- **caproic acid:** maximum 2.0 per cent,
- **caprylic acid:** 50.0 per cent to 80.0 per cent,
- **capric acid:** 20.0 per cent to 50.0 per cent,
- **lauric acid:** maximum 3.0 per cent,
- **myristic acid:** maximum 1.0 per cent.

Water (2.5.12): maximum 0.1 per cent, determined on 5.00 g.

Total ash (2.4.16): maximum 0.1 per cent, determined on 2.0 g.

STORAGE

Protected from light.

Reference solution. Dissolve 0.1 g of *propylene glycol dilaurate CRS* in *methylene chloride R* and dilute to 2 ml with the same solvent.

Plate: TLC silica gel plate R.

Mobile phase: hexane R, ether R (30:70 V/V).

Application: 10 μ l.

Development: over a path of 15 cm.

Drying: in air.

Detection: spray with a 0.1 g/l solution of *rhodamine 6 G R* in *alcohol R*. Examine in ultraviolet light at 365 nm.

Results: the spots in the chromatogram obtained with the test solution are similar in position to those in the chromatogram obtained with the reference solution.

B. It complies with the test for composition of fatty acids (see Tests).

C. It complies with the assay (content of diesters).

TESTS

Acid value (2.5.1): maximum 4.0, determined on 5.00 g.

Iodine value (2.5.4, Method A): maximum 1.0.

Saponification value (2.5.6): 230 to 250.

Composition of fatty acids. Gas chromatography (2.4.22, Method C). Use the mixture of calibrating substances in Table 2.4.22.-2.

Composition of the fatty acid fraction of the substance:

- **caprylic acid:** maximum 0.5 per cent,
- **capric acid:** maximum 2.0 per cent,
- **lauric acid:** minimum 95.0 per cent,
- **myristic acid:** maximum 3.0 per cent,
- **palmitic acid:** maximum 1.0 per cent.

Free propylene glycol: maximum 2.0 per cent, determined as prescribed under Assay.

Water (2.5.12): maximum 1.0 per cent, determined on 1.00 g.

Total ash (2.4.16): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Size-exclusion chromatography (2.2.30).

Stock solution. Introduce 0.100 g of *propylene glycol R* into a flask and dilute to 25.0 ml with *tetrahydrofuran R*.

Test solution. In a 15 ml flask, weigh 0.200 g (*m*). Add 5.0 ml of *tetrahydrofuran R* and shake to dissolve. Reweigh the flask and calculate the total mass of solvent and substance (*M*).

Reference solutions. Into four 15 ml flasks, introduce respectively 0.25 ml, 0.5 ml, 1.0 ml and 2.5 ml of stock solution and add 5.0 ml of *tetrahydrofuran R*. Weigh each flask and calculate the concentration of propylene glycol in milligrams per gram for each reference solution.

Column:

- **size:** $l = 0.6$ m, $\varnothing = 7$ mm,
- **stationary phase:** styrene-divinylbenzene copolymer R (5 μ m) with a pore size of 10 nm.

Mobile phase: *tetrahydrofuran R*.

Flow rate: 1 ml/min.

Detection: differential refractometer.

Injection: 40 μ l.

Relative retention with reference to propylene glycol: diesters = about 0.85; monoesters = about 0.90.

01/2005:2087
corrected

PROPYLENE GLYCOL DILAURATE

Propylenglycoli dilauras

DEFINITION

Mixture of propylene glycol mono- and diesters of lauric acid.

Content: minimum 70.0 per cent of diesters and maximum 30.0 per cent of monoesters.

CHARACTERS

Appearance: clear, oily liquid at 20 °C, colourless or slightly yellow.

Solubility: practically insoluble in water, very soluble in alcohol, in methanol and in methylene chloride.

IDENTIFICATION

A. Thin-layer chromatography (2.2.27).

Test solution. Dissolve 0.1 g of the substance to be examined in *methylene chloride R* and dilute to 2 ml with the same solvent.

Calculations:

- **free propylene glycol:** from the calibration curve obtained with the reference solutions, determine the concentration (*C*) in milligrams per gram in the test solution and calculate the percentage content in the substance to be examined using the following expression:

$$\frac{C \times M}{m \times 10}$$

- **monoesters:** calculate the percentage content of monoesters using the following expression:

$$\frac{A}{A + B} \times (100 - D)$$

A = area of the peak due to the monoesters,

B = area of the peak due to the diesters,

D = percentage content of free propylene glycol + percentage content of free fatty acids.

Calculate the percentage content of free fatty acids using the expression:

$$\frac{I_A \times 200}{561.1}$$

I_A = acid value.

- **diesters:** calculate the percentage content of diesters using the following expression:

$$\frac{B}{A + B} \times (100 - D)$$

STORAGE

Protected from moisture.

01/2005:1915
corrected

PROPYLENE GLYCOL MONOLAUROATE**Propylenglycoli monolauras****DEFINITION**

Mixture of propylene glycol mono- and diesters of lauric acid.

Content:

- propylene glycol monolaurate (type I): 45.0 per cent to 70.0 per cent of monoesters and 30.0 per cent to 55.0 per cent of diesters,
- propylene glycol monolaurate (type II): minimum 90.0 per cent of monoesters and maximum 10.0 per cent of diesters.

CHARACTERS

Appearance: clear, oily liquid at 20 °C, colourless or slightly yellow.

Solubility: practically insoluble in water, very soluble in alcohol, in methanol and in methylene chloride.

IDENTIFICATION**A. Thin-layer chromatography (2.2.27).**

Test solution. Dissolve 0.1 g of the substance to be examined in *methylene chloride R* and dilute to 2 ml with the same solvent.

Reference solution. Dissolve 0.1 g of *propylene glycol monolaurate CRS* in *methylene chloride R* and dilute to 2 ml with the same solvent.

Plate: *TLC silica gel plate R.*

Mobile phase: *hexane R, ether R (30:70 V/V).*

Application: 10 µl.

Development: over a path of 15 cm.

Drying: in air.

Detection: spray with a 0.1 g/l solution of *rhodamine 6 G R* in *alcohol R*. Examine in ultraviolet light at 365 nm.

Results: the spots in the chromatogram obtained with the test solution are similar in position to those in the chromatogram obtained with the reference solution.

B. It complies with the test for composition of fatty acids (see Tests).

C. It complies with the assay (content of monoesters).

TESTS

Acid value (2.5.1): maximum 4.0, determined on 5.00 g.

Iodine value (2.5.4, Method A): maximum 1.0.

Saponification value (2.5.6): 210 to 245 for propylene glycol monolaurate (type I) and 200 to 230 for propylene glycol monolaurate (type II).

Composition of fatty acids. Gas chromatography (2.4.22, Method C). Use the mixture of calibrating substances in Table 2.4.22.-2.

Composition of the fatty acid fraction of the substance:

- *caprylic acid:* maximum 0.5 per cent,
- *capric acid:* maximum 2.0 per cent,
- *lauric acid:* minimum 95.0 per cent,
- *myristic acid:* maximum 3.0 per cent,
- *palmitic acid:* maximum 1.0 per cent.

Free propylene glycol: maximum 5.0 per cent for propylene glycol monolaurate (type I) and maximum 1.0 per cent for propylene glycol monolaurate (type II), determined as prescribed under Assay.

Water (2.5.12): maximum 1.0 per cent, determined on 1.00 g.

Total ash (2.4.16): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Size-exclusion chromatography (2.2.30).

Stock solution. Introduce 0.100 g of *propylene glycol R* into a vial and dilute to 25.0 ml with *tetrahydrofuran R*.

Test solution. In a 15 ml flask, weigh 0.200 g (*m*). Add 5.0 ml of *tetrahydrofuran R* and shake to dissolve. Reweigh the flask and calculate the total mass of solvent and substance (*M*).

Reference solutions. Into four 15 ml flasks, introduce respectively 0.25 ml, 0.5 ml, 1.0 ml and 2.5 ml of stock solution and add 5.0 ml of *tetrahydrofuran R*. In a fifth 15 ml flask, introduce 5.0 ml of stock solution. Weigh each flask and calculate the concentration of propylene glycol in milligrams per gram for each reference solution.

Column:

- **size:** *l* = 0.6 m, Ø = 7 mm,
- **stationary phase:** *styrene-divinylbenzene copolymer R* (5 µm) with a pore size of 10 nm.

Mobile phase: *tetrahydrofuran R.*

Flow rate: 1 ml/min.

Detection: differential refractometer.

Injection: 40 µl.

Relative retention with reference to propylene glycol: diesters = about 0.85; monoesters = about 0.90.

Calculations:

- *free propylene glycol*: from the calibration curve obtained with the reference solutions, determine the concentration (*C*) in milligrams per gram in the test solution and calculate the percentage content in the substance to be examined using the following expression:

$$\frac{C \times M}{m \times 10}$$

- *monoesters*: calculate the percentage content of monoesters using the following expression:

$$\frac{A}{A + B} \times (100 - D)$$

- A* = area of the peak due to the monoesters,
B = area of the peak due to the diesters,
D = percentage content of free propylene glycol + percentage content of free fatty acids.

Calculate the percentage content of free fatty acids using the expression:

$$\frac{I_A \times 200}{561.1}$$

I_A = acid value.

- *diesters*: calculate the percentage content of diesters using the following expression:

$$\frac{B}{A + B} \times (100 - D)$$

STORAGE

Protected from moisture.

LABELLING

The label states the type of propylene glycol monolaurate (type I or type II).

01/2005:1469

PROPYLENE GLYCOL MONOPALMITOSTEARATE

Propylenglycoli monopalmitostearas

DEFINITION

Mixture of propylene glycol mono- and diesters and of stearic and palmitic acids, produced by the condensation of propylene glycol and stearic acid 50 of vegetable or animal origin (see *Stearic acid* (1474)).

Content: minimum of 50.0 per cent of monoesters.

CHARACTERS

Appearance: white or almost white, waxy solid.

Solubility: practically insoluble in water, soluble in acetone and in hot alcohol.

IDENTIFICATION

- A. It complies with the test for melting point (see Tests).
- B. It complies for the test for composition of fatty acids (see Tests).
- C. It complies with the assay (monoesters content).

TESTS

Melting point (2.2.15): 33 °C to 40 °C.

Acid value (2.5.1): maximum 4.0, determined on 10.0 g.

Iodine value (2.5.4): maximum 3.0.

Saponification value (2.5.6): 170 to 185, determined on 2.0 g.

Composition of fatty acids (2.4.22, Method A). The fatty acid fraction has the following composition:

- *stearic acid*: 40.0 per cent to 60.0 per cent,
- *sum of contents of palmitic acid and stearic acid*: minimum 90.0 per cent.

Free propylene glycol: maximum 5.0 per cent, determined as prescribed under Assay.

Total ash (2.4.16): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Size-exclusion chromatography (2.2.30).

Test solution. In a 15 ml flask, weigh about 0.2 g (*m*), to the nearest 0.1 mg. Add 5.0 ml of *tetrahydrofuran R* and shake to dissolve. Heat gently, if necessary. Reweigh the flask and calculate the total mass of solvent and substance (*M*).

Reference solutions. In four 15 ml flasks, weigh, to the nearest 0.1 mg, about 2.5 mg, 5.0 mg, 10.0 mg and 20.0 mg of *propylene glycol R*. Add 5.0 ml of *tetrahydrofuran R* and shake to dissolve. Weigh the flasks again and calculate the concentration of propylene glycol in milligrams per gram for each reference solution.

Column:

- *size*: *l* = 0.6 m, Ø = 7 mm,
- *stationary phase*: *styrene-divinylbenzene copolymer R* (particle diameter 5 µm, pore size 10 nm).

Mobile phase: *tetrahydrofuran R*.

Flow rate: 1 ml/min.

Detection: differential refractometer.

Injection: 40 µl.

Relative retention with reference to propylene glycol: diesters = about 0.78, monoesters = about 0.84.

Limits:

- *free propylene glycol*: from the calibration curve obtained with the reference solutions, determine the concentration (*C*) in milligrams per gram in the test solution and calculate the percentage content in the substance to be examined using the following expression:

$$\frac{C \times M}{m \times 10}$$

- *monoesters*: calculate the percentage content of monoesters using the following expression:

$$\frac{A}{A + B} \times (100 - D)$$

- A = area of the peak due to the monoesters,
 B = area of the peak due to the diesters,
 D = percentage content of free propylene glycol + percentage content of free fatty acids which is determined using the following expression:

$$\frac{I_A \times 270}{561.1}$$

I_A = acid value.

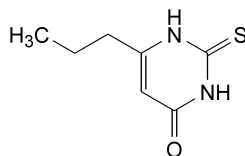
STORAGE

Protected from light.

01/2005:0525

PROPYLTHIOURACIL

Propylthiouracilum



$C_7H_{10}N_2OS$

M_r 170.2

DEFINITION

Propylthiouracil contains not less than 98.0 per cent and not more than the equivalent of 100.5 per cent of 2,3-dihydro-6-propyl-2-thioxypyrimidin-4(1H)-one, calculated with reference to the dried substance.

CHARACTERS

White or almost white, crystalline powder or crystals, very slightly soluble in water, sparingly soluble in alcohol. It dissolves in solutions of alkali hydroxides.

IDENTIFICATION

First identification: A, B.

Second identification: A, C, D.

- A. Melting point (2.2.14): 217 °C to 221 °C.
- B. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *propylthiouracil CRS*. Examine as discs prepared using 1 mg of substance and 0.3 g of *potassium bromide R*.
- C. Examine the chromatograms obtained in the test for impurity A and related substances in ultraviolet light at 254 nm before exposure of the plate to iodine vapour. The principal spot in the chromatogram obtained with test solution (b) is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a).
- D. To about 20 mg add 8 ml of *bromine water R* and shake for a few minutes. Boil until the mixture is decolourised, allow to cool and filter. To the filtrate add 2 ml of *barium*

chloride solution R1. A white precipitate is formed whose colour does not become violet on the addition of 5 ml of *dilute sodium hydroxide solution R*.

TESTS

Impurity A and related substances. Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel GF₂₅₄ plate R*.

Test solution (a). Dissolve 0.1 g of the substance to be examined in *methanol R* and dilute to 10 ml with the same solvent.

Test solution (b). Dilute 1 ml of test solution (a) to 10 ml with *methanol R*.

Reference solution (a). Dissolve 10 mg of *propylthiouracil CRS* in *methanol R* and dilute to 10 ml with the same solvent.

Reference solution (b). Dissolve 50 mg of *thiourea R* in *methanol R* and dilute to 100 ml with the same solvent. Dilute 1 ml of this solution to 100 ml with *methanol R*.

Reference solution (c). Dilute 1 ml of test solution (a) to 100 ml with *methanol R*.

Apply separately to the plate 10 µl of each solution. Develop over a path of 15 cm using a mixture of 0.1 volumes of *glacial acetic acid R*, 6 volumes of *2-propanol R* and 50 volumes of *chloroform R*. Allow the plate to dry in air. Examine in ultraviolet light at 254 nm. Expose the plate to iodine vapour for 10 min. In the chromatogram obtained with test solution (a), any spot corresponding to impurity A is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.05 per cent) and any spot apart from the principal spot and any spot corresponding to impurity A is not more intense than the spot in the chromatogram obtained with reference solution (c) (1.0 per cent).

Heavy metals (2.4.8). 1.0 g complies with limit test F for heavy metals (20 ppm). Prepare the standard using 2 ml of *lead standard solution (10 ppm Pb) R*.

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

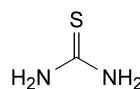
To 0.300 g add 30 ml of *water R* and 30.0 ml of 0.1 M *sodium hydroxide*. Boil and shake until dissolution is complete. Add 50 ml of 0.1 M *silver nitrate* while stirring, boil gently for 5 min and cool. Titrate with 0.1 M *sodium hydroxide*, determining the end-point potentiometrically (2.2.20). The volume of 0.1 M *sodium hydroxide* used is equal to the sum of the volume added initially and the volume used in the final titration.

1 ml of 0.1 M *sodium hydroxide* is equivalent to 8.511 mg of $C_7H_{10}N_2OS$.

STORAGE

Store protected from light.

IMPURITIES

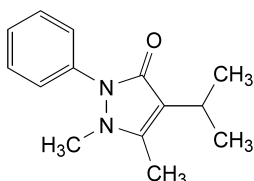


A. thiourea.

01/2005:0636

PROPYPHENAZONE

Propyphenazonum

 $C_{14}H_{18}N_2O$ M_r 230.3

DEFINITION

Propyphenazone contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of 1,5-dimethyl-4-(1-methylethyl)-2-phenyl-1,2-dihydro-3H-pyrazol-3-one, calculated with reference to the dried substance.

CHARACTERS

A white or slightly yellowish, crystalline powder, slightly soluble in water, freely soluble in alcohol and in methylene chloride.

IDENTIFICATION

First identification: A, B.

Second identification: A, C, D.

- Melting point (2.2.14): 102 °C to 106 °C.
- Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *propyphenazone* CRS. Examine the substances prepared as discs.
- Examine the chromatograms obtained in the test for related substances. The principal spot in the chromatogram obtained with test solution (b) is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).
- To 1 ml of solution S (see Tests) add 0.1 ml of *ferric chloride solution R1*. A brownish-red colour appears which becomes yellow on addition of 1 ml of *dilute hydrochloric acid R*.

TESTS

Solution S. Dissolve 2 g in a mixture of equal volumes of *alcohol R* and *carbon dioxide-free water R* and dilute to 50 ml with the same mixture of solvents.

Appearance of solution. Solution S is clear (2.2.1) and colourless (2.2.2, *Method II*).

Acidity or alkalinity. To 10 ml of solution S add 0.1 ml of *phenolphthalein solution R*. The solution is colourless. Add 0.2 ml of 0.01 M *sodium hydroxide*; the solution becomes pink. Add 0.4 ml of 0.01 M *hydrochloric acid*; the solution becomes colourless. Add 0.2 ml of *methyl red solution R*. The solution becomes orange or red.

Related substances. Examine by thin-layer chromatography (2.2.27), using *silica gel HF₂₅₄ R* as the coating substance.

Test solution (a). Dissolve 0.40 g of the substance to be examined in *methanol R* and dilute to 5 ml with the same solvent.

Test solution (b). Dilute 1 ml of test solution (a) to 5 ml with *methanol R*.

Reference solution (a). Dissolve 80 mg of *propyphenazone* CRS in *methanol R* and dilute to 5 ml with the same solvent.

Reference solution (b). Dilute 1 ml of test solution (b) to 100 ml with *methanol R*.

Apply to the plate 5 µl of each solution. Develop over a path of 15 cm using a mixture of 10 volumes of *butanol R*, 45 volumes of *cyclohexane R* and 45 volumes of *ethyl acetate R*. Dry the plate in a current of hot air for 15 min and examine in ultraviolet light at 254 nm. Any spot in the chromatogram obtained with test solution (a), apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.2 per cent). Spray with a mixture of equal volumes of *potassium ferricyanide solution R* and *ferric chloride solution R1*. Any spot in the chromatogram obtained with test solution (a), apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.2 per cent).

Heavy metals (2.4.8). 1.0 g complies with limit test C (10 ppm). Prepare the standard using 1 ml of *lead standard solution (10 ppm Pb) R*.

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying *in vacuo* at 60 °C for 4 h.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 0.5 g.

ASSAY

Dissolve 0.2000 g in 10 ml of *anhydrous acetic acid R* and add 75 ml of *ethylene chloride R*. Titrate with 0.1 M *perchloric acid*, determining the end-point potentiometrically (2.2.20).

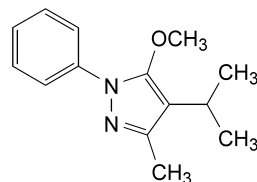
1 ml of 0.1 M *perchloric acid* is equivalent to 23.03 mg of $C_{14}H_{18}N_2O$.

STORAGE

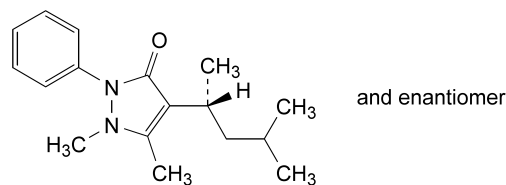
Store protected from light.

IMPURITIES

A. phenazone,



B. 5-methoxy-3-methyl-4-(1-methylethyl)-1-phenyl-1H-pyrazole,



C. 4-[(1*RS*)-1,3-dimethylbutyl]-1,5-dimethyl-2-phenyl-1,2-dihydro-3H-pyrazol-3-one.

01/2005:0686

PROTAMINE HYDROCHLORIDE

Protamini hydrochloridum

DEFINITION

Protamine hydrochloride consists of the hydrochlorides of basic peptides extracted from the sperm or roe of fish, usually species of *Salmonidae* and *Clupeidae*. It binds with

heparin in solution, inhibiting its anticoagulant activity; in the conditions of the assay this binding gives rise to a precipitate. Calculated with reference to the dried substance, 1 mg of protamine hydrochloride precipitates not less than 100 IU of heparin.

PRODUCTION

Protamine hydrochloride is prepared in conditions designed to minimise the risk of microbial contamination.

The method of manufacture is validated to demonstrate that the product, if tested, would comply with the following test:

Abnormal toxicity (2.6.9). Inject into each mouse 0.5 mg dissolved in 0.5 ml of *water for injections R*.

CHARACTERS

Appearance: white or almost white powder, hygroscopic.

Solubility: soluble in water, practically insoluble in alcohol.

IDENTIFICATION

A. Specific optical rotation (2.2.7): -40 to -60 (dried substance).

Dissolve 1.000 g in 0.1 M *hydrochloric acid* and dilute to 100.0 ml with the same solvent.

B. In the conditions of the assay, protamine hydrochloride forms a precipitate.

C. To 0.5 ml of solution S (see Tests) add 4.5 ml of *water R*, 1.0 ml of a 100 g/l solution of *sodium hydroxide R* and 1.0 ml of a 0.2 g/l solution of α -*naphthol R* and mix. Cool the mixture to 5 °C. Add 0.5 ml of *sodium hypobromite solution R*. An intense red colour is produced.

D. Heat 2 ml of solution S in a water-bath at 60 °C and add 0.1 ml of *mercuric sulphate solution R*. Mix. No precipitate is formed. Cool the mixture in iced water. A precipitate is formed.

E. It gives reaction (a) of chlorides (2.3.1).

TESTS

Solution S. Dissolve 0.50 g in *water R* and dilute to 25.0 ml with the same solvent.

Appearance of solution. The solution is not more opalescent than reference suspension II (2.2.1) and not more intensely coloured than reference solution BY₆ or Y₆ (2.2.2, *Method II*). To 2.5 ml of solution S add 7.5 ml of *water R*.

Absorbance (2.2.25): maximum 0.1 between wavelengths of 260 nm to 280 nm.

Dilute 2.5 ml of solution S to 5.0 ml with *water R*.

Chloride: 12.3 per cent to 19.0 per cent (dried substance).

Dissolve 0.400 g in 50 ml of *water R*. Add 5 ml of *dilute nitric acid R*, 25.0 ml of 0.1 M *silver nitrate* and 2 ml of *dibutyl phthalate R* and shake. Titrate with 0.1 M *ammonium thiocyanate* using 2 ml of *ferric ammonium sulphate solution R2* as indicator; shake vigorously when approaching the end-point.

1 ml of 0.1 M *silver nitrate* is equivalent to 3.545 mg of chloride (Cl).

Sulphates: maximum 4.0 per cent (dried substance).

Dissolve 0.500 g in 200 ml of *distilled water R*, add 5.0 ml of *dilute hydrochloric acid R* and heat to boiling. Add dropwise 10 ml of a hot 100 g/l solution of *barium chloride R* while stirring with a glass rod, cover the beaker with a watch glass and allow to stand on a water-bath for 2 h to obtain a coarse granular precipitate. Add 0.1 ml of a 100 g/l solution of *barium chloride R* to the clear supernatant liquid. If a turbidity develops, repeat the precipitation procedure. Transfer the precipitate quantitatively to a previously ignited

and tared porcelain crucible and wash with hot *distilled water R* until the addition of *silver nitrate solution R1* to the washings produces no opalescence. Ignite the precipitate at 600 °C for 1 h. Allow to cool in a desiccator and weigh.

1 mg of residue is equivalent to 0.412 mg of sulphate (SO₄).

Barium: maximum 10 ppm.

Atomic absorption spectrometry (2.2.23, *Method I*).

Test solution. Dissolve 1.0 g of the substance to be examined in *distilled water R*, add 1 ml of a 250 g/l solution of *caesium chloride R* and 0.2 ml of *hydrochloric acid R* and dilute to 20.0 ml with *distilled water R*.

Reference solution. To 1.0 ml of *barium standard solution* (50 ppm Ba) *R* add 5 ml of a 250 g/l solution of *caesium chloride R* and 1 ml of *hydrochloric acid R* and dilute to 100.0 ml with *distilled water R*.

Source: hollow-cathode lamp.

Wavelength: 553.3 nm.

Flame: air-acetylene-nitrous oxide flame of suitable composition.

Iron (2.4.9): maximum 10 ppm.

Dissolve 1.0 g with heating in *water R* and dilute to 10 ml with the same solvent.

Mercury: maximum 10 ppm.

Introduce 2.0 g of the substance to be examined into a ground-glass-stoppered 250 ml conical flask and add 20 ml of a mixture of equal volumes of *nitric acid R* and *sulphuric acid R*. Boil under a reflux condenser for 1 h, cool and cautiously dilute with *water R*. Boil until nitrous fumes are no longer seen. Cool the solution, cautiously dilute to 200.0 ml with *water R*, mix and filter. Transfer 50.0 ml of the filtrate to a separating funnel. Shake with successive small portions of *chloroform R* until the chloroform layer remains colourless. Discard the chloroform layers. To the aqueous layer add 25 ml of *dilute sulphuric acid R*, 115 ml of *water R* and 10 ml of a 200 g/l solution of *hydroxylamine hydrochloride R*. Titrate with *dithizone solution R2*; after each addition, shake the mixture 20 times and towards the end of the titration allow to separate and discard the chloroform layer. Titrate until a bluish-green colour is obtained. Calculate the content of mercury using the equivalent in micrograms of mercury per millilitre of titrant determined in the standardisation of the *dithizone solution R2*.

Nitrogen: 23.0 per cent to 27.0 per cent (dried substance).

Carry out the determination of nitrogen by sulphuric acid digestion (2.5.9), using 10.0 mg and heating for 3-4 h.

Heavy metals (2.4.8): maximum 20 ppm.

1.0 g complies with limit test D. Prepare the standard using 2 ml of *lead standard solution* (10 ppm Pb) *R*.

Loss on drying (2.2.32): maximum 5.0 per cent, determined on 1.000 g by drying in an oven at 100-105 °C for 3 h.

Bacterial endotoxins (2.6.14): less than 7.0 IU/mg, if intended for use in the manufacture of parenteral dosage forms without a further appropriate procedure for the removal of bacterial endotoxins.

ASSAY

Test solution (a). Dissolve 15.0 mg of the substance to be examined in *water R* and dilute to 100.0 ml with the same solvent.

Test solution (b). Dilute 2.0 ml of test solution (a) to 3.0 ml with *water R*.

Test solution (c). Dilute 1.0 ml of test solution (a) to 3.0 ml with *water R*.

Use as titrant a 1 in 6 dilution of *heparin sodium BRP* in *water R* (for example, 1.7 ml diluted to 10.0 ml with *water R*). Titrate each test solution in duplicate as follows: introduce an accurately measured volume of the solution to be titrated, for example, 1.5 ml, into the cell of a suitable colorimeter, set the apparatus for measurement at a suitable wavelength (none is critical) in the visible range, add the titrant in small volumes until there is a sharp increase in the absorbance and note the volume of titrant added.

Carry out 3 independent assays. For each individual titration, calculate the number of International Units of heparin in the volume of titrant added at the end-point per milligram of the substance to be examined. Calculate the potency of the substance as the average of the 18 values. Test the linearity of the response by the usual statistical methods. Calculate the 3 standard deviations for the results obtained with each of the 3 test solutions. Calculate the three standard deviations for the results obtained with each of the 3 independent assays. The assay is not valid unless each of the 6 standard deviations is less than 5 per cent of the average result.

STORAGE

In an airtight, tamper-proof container. If the substance is sterile, store in a sterile, airtight, tamper-proof container.

LABELLING

The label states, where applicable, that the substance is free from bacterial endotoxins.

01/2005:0569

PROTAMINE SULPHATE

Protamini sulfas

DEFINITION

Protamine sulphate consists of the sulphates of basic peptides extracted from the sperm or roe of fish, usually species of *Salmonidae* and *Clupeidae*. It binds with heparin in solution, inhibiting its anticoagulant activity; in the conditions of the assay this binding gives rise to a precipitate. Calculated with reference to the dried substance, 1 mg of protamine sulphate precipitates not less than 100 IU of heparin.

PRODUCTION

Protamine sulphate is prepared in conditions designed to minimise the risk of microbial contamination.

The method of manufacture is validated to demonstrate that the product, if tested, would comply with the following test:

Abnormal toxicity (2.6.9). Inject into each mouse 0.5 mg dissolved in 0.5 ml of *water for injections R*.

CHARACTERS

Appearance: white or almost white powder, hygroscopic.

Solubility: sparingly soluble in water, practically insoluble in alcohol.

IDENTIFICATION

A. Specific optical rotation (2.2.7): –65 to –85 (dried substance).

Dissolve 1.000 g in 0.1 M *hydrochloric acid* and dilute to 100.0 ml with the same solvent.

B. In the conditions of the assay, protamine sulphate forms a precipitate.

C. To 0.5 ml of solution S (see Tests) add 4.5 ml of *water R*, 1.0 ml of a 100 g/l solution of *sodium hydroxide R* and 1.0 ml of a 0.2 g/l solution of α -*naphthol R* and mix. Cool the mixture to 5 °C. Add 0.5 ml of *sodium hypobromite solution R*. An intense red colour is produced.

D. Heat 2 ml of solution S in a water-bath at 60 °C and add 0.1 ml of *mercuric sulphate solution R*. Mix. No precipitate is formed. Cool the mixture in iced water. A precipitate is formed.

E. It gives reaction (a) of sulphates (2.3.1).

TESTS

Solution S. Dissolve 0.20 g in *water R* and dilute to 10.0 ml with the same solvent.

Appearance of solution. The solution is not more opalescent than reference suspension II (2.2.1) and not more intensely coloured than reference solution BY₆ or Y₆ (2.2.2, *Method II*). To 2.5 ml of solution S add 7.5 ml of *water R*.

Absorbance (2.2.25): maximum 0.1 between wavelengths of 260 nm to 280 nm.

Dilute 2.5 ml of solution S to 5.0 ml with *water R*.

Sulphate: 16 per cent to 24 per cent (dried substance).

Dissolve 0.150 g in 15 ml of *distilled water R* in a beaker. Add 5 ml of *dilute hydrochloric acid R*. Heat to boiling and slowly add to the boiling solution 10 ml of a 100 g/l solution of *barium chloride R*. Cover the beaker and heat on a water-bath for 1 h. Filter. Wash the precipitate several times with small quantities of hot *water R*. Dry and ignite the residue at 600 °C to constant mass.

1.0 g of residue is equivalent to 0.4117 g of sulphate (SO₄).

Iron (2.4.9): maximum 10 ppm.

Dissolve 1.0 g with heating in *water R* and dilute to 10 ml with the same solvent.

Mercury: maximum 10 ppm.

Introduce 2.0 g of the substance to be examined into a 250 ml ground-glass-stoppered conical flask and add 20 ml of a mixture of equal volumes of *nitric acid R* and *sulphuric acid R*. Boil under a reflux condenser for 1 h, cool and cautiously dilute with *water R*. Boil until nitrous fumes are no longer seen. Cool the solution, cautiously dilute to 200.0 ml with *water R*, mix and filter. Transfer 50.0 ml of the filtrate to a separating funnel. Shake with successive small portions of *chloroform R* until the chloroform layer remains colourless. Discard the chloroform layers. To the aqueous layer add 25 ml of *dilute sulphuric acid R*, 115 ml of *water R* and 10 ml of a 200 g/l solution of *hydroxylamine hydrochloride R*. Titrate with *dithizone solution R2*; after each addition, shake the mixture 20 times and towards the end of the titration allow to separate and discard the chloroform layer. Titrate until a bluish-green colour is obtained. Calculate the content of mercury using the equivalent in micrograms of mercury per millilitre of titrant, determined in the standardisation of the *dithizone solution R2*.

Nitrogen: 21.0 per cent to 26.0 per cent (dried substance).

Carry out the determination of nitrogen by sulphuric acid digestion (2.5.9), using 10.0 mg and heating for 3–4 h.

Heavy metals (2.4.8): maximum 20 ppm.

1.0 g complies with limit test D. Prepare the standard using 2 ml of *lead standard solution (10 ppm Pb) R*.

Loss on drying (2.2.32): maximum 5.0 per cent, determined on 1.000 g by drying in an oven at 100–105 °C for 3 h.

Bacterial endotoxins (2.6.14): less than 7.0 IU/mg, if intended for use in the manufacture of parenteral dosage forms without a further appropriate procedure for the removal of bacterial endotoxins.

ASSAY

Test solution (a). Dissolve 15.0 mg of the substance to be examined in *water R* and dilute to 100.0 ml with the same solvent.

Test solution (b). Dilute 2.0 ml of test solution (a) to 3.0 ml with *water R*.

Test solution (c). Dilute 1.0 ml of test solution (a) to 3.0 ml with *water R*.

Use as titrant a 1 to 6 dilution of *heparin sodium BRP* in *water R* (for example, 1.7 ml diluted to 10.0 ml with *water R*). Titrate each test solution in duplicate as follows: introduce an accurately measured volume of the solution to be titrated, for example 1.5 ml, into the cell of a suitable colorimeter and set the apparatus for measurement at a suitable wavelength (none is critical) in the visible range. Add the titrant in small volumes until there is a sharp increase in the absorbance and note the volume of titrant added.

Carry out 3 independent assays. For each individual titration, calculate the number of International Units of heparin in the volume of titrant added at the end-point per milligram of the substance to be examined. Calculate the potency of the substance as the average of the 18 values. Test the linearity of the response by the usual statistical methods. Calculate the 3 standard deviations for the results obtained with each of the 3 test solutions. Calculate the 3 standard deviations for the results obtained with each of the 3 independent assays. The assay is not valid unless each of the 6 standard deviations is less than 5 per cent of the average result.

STORAGE

In an airtight, tamper-proof container. If the substance is sterile, store in a sterile, airtight, tamper-proof container.

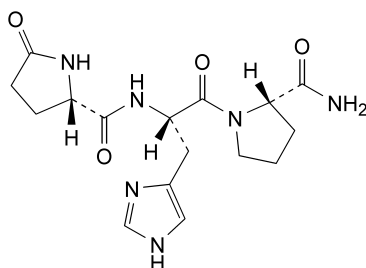
LABELLING

The label states, where applicable, that the substance is free from bacterial endotoxins.

01/2005:1144

PROTIRELIN

Protirelinum



$C_{16}H_{22}N_6O_4$

M_r 362.4

DEFINITION

5-Oxo-L-prolyl-L-histidyl-L-prolinamide.

Synthetic tripeptide with the same sequence of amino acids as the natural hypothalamic neurohormone, which stimulates the release and synthesis of thyrotropin.

Content: 97.0 per cent to 102.0 per cent (anhydrous and acetic acid-free substance).

CHARACTERS

Appearance: white or yellowish-white powder, hygroscopic.

Solubility: very soluble in water, freely soluble in methanol.

IDENTIFICATION

A. Infrared absorption spectrophotometry (2.2.24).

Comparison: *protirelin CRS*.

B. Examine the chromatograms obtained in the assay.

Results: the principal peak in the chromatogram obtained with the test solution is similar in retention time and size to the principal peak in the chromatogram obtained with the reference solution.

TESTS

Appearance of solution. A 10 g/l solution is clear (2.2.1) and not more intensely coloured than reference solution Y_5 (2.2.2, *Method II*).

Specific optical rotation (2.2.7): -62 to -70 (anhydrous and acetic acid-free substance).

Dissolve 10 mg in 1.0 ml of *water R*.

Related substances. Liquid chromatography (2.2.29).

Test solution. Dissolve 5.0 mg of the substance to be examined in mobile phase A and dilute to 5.0 ml with mobile phase A.

Reference solution (a). Dissolve the contents of a vial of *D-His-protirelin CRS* in an appropriate volume of mobile phase A to obtain a concentration of 1 mg/ml. Mix equal volumes of this solution and the test solution.

Reference solution (b). Dilute 0.2 ml of the test solution to 10.0 ml with mobile phase A.

Column:

- **size:** $l = 0.25$ m, $\varnothing = 4.0$ mm,
- **stationary phase:** spherical *octadecylsilyl silica gel for chromatography R* (5 μ m) with a pore size of 12 nm.

Mobile phase:

- **mobile phase A:** a mixture of 100 ml of *acetonitrile for chromatography R*, 1900 ml of *water R* and 2.0 g of *sodium octanesulphonate R*, containing 2.5 ml/l of *tetraethylammonium hydroxide solution R*; adjust to pH 3.5 with *phosphoric acid R*,
- **mobile phase B:** a mixture of 300 ml of *acetonitrile for chromatography R*, 1700 ml of *water R* and 2.0 g of *sodium octanesulphonate R*, containing 2.5 ml/l of *tetraethylammonium hydroxide solution R*; adjust to pH 3.5 with *phosphoric acid R*,

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)
0 - 30	74 $\rightarrow$ 41	26 $\rightarrow$ 59
30 - 35	41 $\rightarrow$ 74	59 $\rightarrow$ 26
35 - 50	74	26

Flow rate: 1 ml/min.

Detection: spectrophotometer at 210 nm.

Injection: 10 μ l.

Relative retention with reference to protirelin (retention time = about 18 min): impurity C = about 0.2; impurity D = about 0.68; impurity A = about 0.91; impurity B = about 0.95; impurity E = about 1.08.

System suitability: reference solution (a):

- **resolution:** minimum 2.5 between the peaks due to impurity A and protirelin,
- **symmetry factor:** 0.9 to 1.2 for the peak due to protirelin.

Limits:

- **any impurity:** for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (2 per cent),
- **total:** not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (3 per cent),
- **disregard limit:** 0.05 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent).

Acetic acid (2.5.34): maximum 2.0 per cent.

Test solution. Dissolve 40.0 mg of the substance to be examined in a mixture of 5 volumes of mobile phase B and 95 volumes of mobile phase A and dilute to 10.0 ml with the same mixture of solvents.

Water (2.5.12): maximum 7.0 per cent, determined on 0.200 g.

Bacterial endotoxins (2.6.14): less than 0.7 IU/mg, if intended for use in the manufacture of parenteral dosage forms without a further appropriate procedure for the removal of bacterial endotoxins.

ASSAY

Liquid chromatography (2.2.29) as described in the test for related substances with the following modification.

Reference solution. Dissolve the contents of a vial of *protirelin CRS* in an appropriate volume of mobile phase A to obtain a concentration of 1.0 mg/ml.

Calculate the content of protirelin ($C_{16}H_{22}N_6O_4$) using the peak areas of the chromatograms obtained with the test solution and the reference solution and the declared content of $C_{16}H_{22}N_6O_4$ in *protirelin CRS*.

STORAGE

In an airtight container, protected from light at a temperature of 2 °C to 8 °C. If the substance is sterile, store in a sterile, airtight, tamper-proof container.

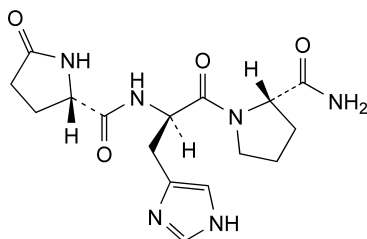
LABELLING

The label states:

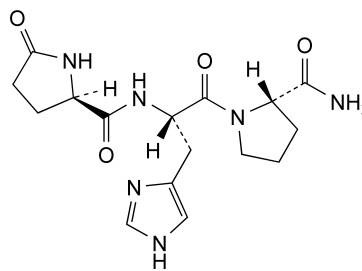
- the mass of peptide in the container,
- where applicable, that the substance is free from bacterial endotoxins.

IMPURITIES

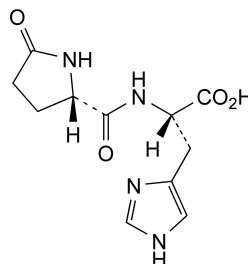
Specified impurities: A, B, C, D, E.



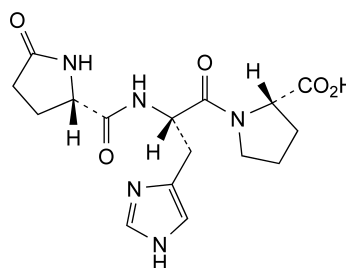
A. 5-oxo-L-prolyl-D-histidyl-L-prolinamide,



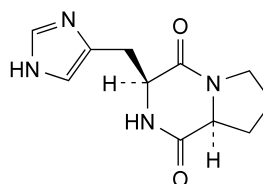
B. 5-oxo-D-prolyl-L-histidyl-L-prolinamide,



C. 5-oxo-L-prolyl-L-histidine,

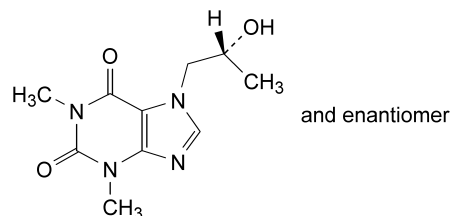


D. 5-oxo-L-prolyl-L-histidyl-L-proline,



E. (3*S*,8*aS*)-3-(1*H*-imidazol-4-ylmethyl)hexahydropyrrolo[1,2-*a*]pyrazine-1,4-dione (cyclo-(*L*-histidyl-*L*-prolyl-)).

01/2005:0526

PROXYPHYLLINE**Proxiphyllinum**

$C_{10}H_{14}N_4O_3$

M_r 238.2

DEFINITION

Proxiphylline contains not less than 98.5 per cent and not more than the equivalent of 101.0 per cent of 7-[(2*RS*)-2-hydroxypropyl]-1,3-dimethyl-3,7-dihydro-1*H*-purine-2,6-dione, calculated with reference to the dried substance.

CHARACTERS

A white, crystalline powder, very soluble in water, soluble in alcohol.

IDENTIFICATION

First identification: B, C.

Second identification: A, C, D.

- A. Melting point (2.2.14): 134 °C to 136 °C.
- B. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *proxiphylline CRS*. Examine the substances as discs prepared using 0.5 mg to 1 mg of the substance to be examined in 0.3 g of *potassium bromide R*.
- C. Dissolve 1 g in 5 ml of *acetic anhydride R* and boil under a reflux condenser for 15 min. Allow to cool and add 100 ml of a mixture of 20 volumes of *ether R* and 80 volumes of *light petroleum R*. Cool in iced water for at least 20 min, shaking from time to time. Filter, wash the precipitate with a mixture of 20 volumes of *ether R* and 80 volumes of *light petroleum R*, recrystallise from *alcohol R* and dry *in vacuo*. The crystals melt (2.2.14) at 87 °C to 92 °C.
- D. It gives the reaction of xanthines (2.3.1).

TESTS

Solution S. Dissolve 2.5 g in *carbon dioxide-free water R* and dilute to 50 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and colourless (2.2.2, *Method II*).

Acidity or alkalinity. To 10 ml of solution S add 0.25 ml of *bromothymol blue solution R1*. The solution is yellow or green. Not more than 0.4 ml of 0.01 M *sodium hydroxide* is required to change the colour of the indicator to blue.

Related substances. Examine by thin-layer chromatography (2.2.27), using *silica gel HF₂₅₄ R* as the coating substance.

Test solution. Dissolve 0.3 g of the substance to be examined in a mixture of 20 volumes of *water R* and 30 volumes of *methanol R* and dilute to 10 ml with the same mixture of solvents. Prepare immediately before use.

Reference solution (a). Dilute 1 ml of the test solution to 100 ml with *methanol R*.

Reference solution (b). Dilute 0.2 ml of the test solution to 100 ml with *methanol R*.

Reference solution (c). Dissolve 10 mg of *theophylline R* in *methanol R*, add 0.3 ml of the test solution and dilute to 10 ml with *methanol R*.

Apply separately to the plate 10 µl of each solution. Develop over a path of 15 cm using a mixture of 1 volume of *concentrated ammonia R*, 10 volumes of *ethanol R* and 90 volumes of *chloroform R*. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. Any spot in the chromatogram obtained with the test solution, apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (a) (1 per cent) and at most one such spot is more intense than the spot in the chromatogram obtained with reference solution (b) (0.2 per cent). The test is not valid unless the chromatogram obtained with reference solution (c) shows two clearly separated spots.

Chlorides (2.4.4). Dilute 2.5 ml of solution S to 15 ml with *water R*. The solution complies with the limit test for chlorides (400 ppm).

Heavy metals (2.4.8). 12 ml of solution S complies with limit test A for heavy metals (20 ppm). Prepare the standard using *lead standard solution (1 ppm Pb) R*.

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

In order to avoid overheating in the reaction medium, mix thoroughly throughout and stop the titration immediately after the end-point has been reached.

Dissolve 0.200 g in 3.0 ml of *anhydrous formic acid R* and add 50.0 ml of *acetic anhydride R*. Titrate with 0.1 M *perchloric acid* determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *perchloric acid* is equivalent to 23.82 mg of C₁₀H₁₄N₄O₃.

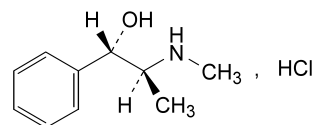
STORAGE

Store protected from light.

01/2005:1367
corrected

PSEUDOEPHEDRINE
HYDROCHLORIDE

Pseudoephedrini hydrochloridum



C₁₀H₁₆ClNO

M_r 201.7

DEFINITION

Pseudoephedrine hydrochloride contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of (1S,2S)-2-(methylamino)-1-phenylpropan-1-ol hydrochloride, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, crystalline powder or colourless crystals, freely soluble in water and in alcohol, sparingly soluble in methylene chloride.

It melts at about 184 °C.

IDENTIFICATION

First identification: A, B, D.

Second identification: A, C, D.

A. It complies with the test for specific optical rotation (see Tests).

B. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *pseudoephedrine hydrochloride CRS*. Examine the substances prepared as discs.

C. Examine by thin-layer chromatography (2.2.27), using a suitable silica gel as the coating substance.

Test solution. Dissolve 20 mg of the substance to be examined in *methanol R* and dilute to 10 ml with the same solvent.

Reference solution (a). Dissolve 20 mg of *pseudoephedrine hydrochloride CRS* in *methanol R* and dilute to 10 ml with the same solvent.

Reference solution (b). Dissolve 10 mg of *ephedrine hydrochloride CRS* in reference solution (a) and dilute to 5 ml with the same solution.

Apply to the plate 10 µl of each solution. Develop over a path of 15 cm using a mixture of 5 volumes of *methylene chloride R*, 15 volumes of *concentrated ammonia R* and 80 volumes of *2-propanol R*. Allow the plate to dry in air and spray with *ninhydrin solution R*. Heat at 110 °C for 5 min. The principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a). The test is not valid unless the chromatogram obtained with reference solution (b) shows two clearly separated spots.

D. Solution S (see Tests) gives reaction (a) of chlorides (2.3.1).

TESTS

Solution S. Dissolve 1.25 g in *carbon dioxide-free water R* and dilute to 25.0 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and colourless (2.2.2, *Method II*).

Acidity or alkalinity. Dilute 2 ml of solution S to 10 ml with *carbon dioxide-free water R*. Add 0.1 ml of *methyl red solution R* and 0.1 ml of 0.01 M *sodium hydroxide*; the solution is yellow. Add 0.2 ml of 0.01 M *hydrochloric acid*; the solution is red.

Specific optical rotation (2.2.7): + 61.0 to + 62.5, determined on solution S and calculated with reference to the dried substance.

Related substances. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 50.0 mg of the substance to be examined in the mobile phase and dilute to 25.0 ml with the mobile phase.

Reference solution (a). Dissolve 20.0 mg of *ephedrine hydrochloride CRS* in the mobile phase and dilute to 20.0 ml with the mobile phase. Dilute 1.0 ml of the solution to 50.0 ml with the mobile phase.

Reference solution (b). Dilute 1.0 ml of the test solution to 200.0 ml with the mobile phase.

Reference solution (c). Dissolve 10 mg of *ephedrine hydrochloride CRS* in 5 ml of the test solution and dilute to 100 ml with the mobile phase.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4.6 mm in internal diameter packed with *phenylsilyl silica gel for chromatography R* (5 µm),
- as mobile phase at a flow rate of 1 ml/min a mixture of 6 volumes of *methanol R* and 94 volumes of a 11.6 g/l solution of *ammonium acetate R* adjusted to pH 4.0 with *glacial acetic acid R*,
- as detector a spectrophotometer set at 257 nm.

Inject 20 µl of reference solution (c). Adjust the sensitivity of the system so that the heights of the two peaks in the chromatogram obtained are at least 50 per cent of the full scale of the recorder. The test is not valid unless the resolution between the peaks corresponding to ephedrine and pseudoephedrine is at least 2.0. If necessary reduce the content of methanol in the mobile phase. Inject 20 µl of the test solution, 20 µl of reference solution (a) and 20 µl of reference solution (b). Continue the chromatography of the test solution for 1.5 times the retention time of pseudoephedrine. In the chromatogram obtained with the test solution: the area of any peak due to ephedrine is not greater than the area of the principal peak in the chromatogram obtained with reference solution (a) (1 per cent); the area of any peak, apart from the principal peak and any peak due to ephedrine, is not greater than the

area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent) and the sum of the areas of such peaks is not greater than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (1 per cent). Disregard any peak with an area less than 0.1 times that of the principal peak in the chromatogram obtained with reference solution (b).

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 100–105 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.170 g in 30 ml of *alcohol R*. Add 5.0 ml of 0.01 M *hydrochloric acid*. Carry out a potentiometric titration (2.2.20), using 0.1 M *sodium hydroxide*. Read the volume added between the two points of inflexion.

1 ml of 0.1 M *sodium hydroxide* is equivalent to 20.17 mg of C₁₀H₁₆ClNO.

STORAGE

Store protected from light.

IMPURITIES

A. ephedrine.

01/2005:0858

PSYLLIUM SEED

Psyllii semen

DEFINITION

Psyllium seed consists of the ripe, whole, dry seeds of *Plantago afra* L. (*Plantago psyllium* L.) or *Plantago indica* L. (*Plantago arenaria* Waldstein and Kitaibel).

CHARACTERS

Psyllium seed has a sweet taste.

It has the macroscopic characters described under Identification.

IDENTIFICATION

P. afra seeds are light brown to very dark brown but never black, smooth and shiny having an elliptical oblong shape. They are 2 mm to 3 mm long and 0.8 mm to 1.0 mm wide, one end being wider than the other. Towards the middle of the dorsal surface there is a fairly marked transverse constriction of light colour. On the ventral surface, there is a linear lighter-coloured groove in the middle of which is a clear spot corresponding to the hilum and bounded by swollen edges.

P. indica seeds are almost identical to the seeds of *P. afra*, but a little less shiny; they are 2 mm to 3 mm long and have a maximum diameter of 1.5 mm.

TESTS

Swelling index (2.8.4). Not less than 10.

Foreign matter (2.8.2). Not more than 1.0 per cent, determined on 10.0 g of the drug, including greenish unripe seeds. Psyllium seed does not contain seeds having a dark central spot on the groove (*Plantago lanceolata* L. and *P. major* L.) or seeds with brownish-grey or pinkish outer coats (*P. ovata* Forssk. and *P. sempervirens* Crantz).

Loss on drying (2.2.32). Not more than 14.0 per cent, determined on 1.000 g of drug by drying in an oven at 100 °C to 105 °C for 2 h.

Total ash (2.4.16). Not more than 4.0 per cent.

STORAGE

Store protected from light and moisture.

01/2005:1886

PYGEUM AFRICANUM BARK

Pruni africanae cortex

DEFINITION

Whole or cut, dried bark of the stems and branches of *Prunus africana* (Hook f.) Kalkm. (syn. *Pygeum africanum* Hook f.).

CHARACTERS

Macroscopic and microscopic characters described under identification tests A and B.

IDENTIFICATION

- A. The dark brown to reddish-brown bark occurs in curved, hard, irregular pieces. The outer surface has a wrinkled dark reddish-brown cork with areas of adhering lichen. The reddish-brown to dark brown inner surface bears longitudinal striations. It may also occur in rolled fragments with a fibrous fracture.
- B. Reduce to a powder (355). The powder is reddish-brown. Examine under a microscope using *chloral hydrate solution R*. The powdered drug shows thick-walled sclereids, solitary or in groups; calcium oxalate cluster crystals of different size; numerous lignified fibres, thick-walled and with narrow lumen, some of them solitary and most in groups with forked ends; fragments of pigmented polygonal cells of reddish-brown colour; fragments of cork. Examine under a microscope using a 50 per cent *V/V* solution of *glycerol R*, the powder shows some isolated small starch grains that stain bluish-black against *iodine solution R1*.
- C. Thin-layer chromatography (2.2.27).

Test solution. Extract 15.0 g of powdered drug (250) with *methylene chloride R* for 30 min in a continuous extraction apparatus (Soxhlet type). Filter. Evaporate the solvent to dryness under reduced pressure. Dissolve the residue in 1 ml of *methylene chloride R*.

Reference solution. Dissolve 20 mg of β -sitosterol *R* and 20 mg of ursolic acid *R* in 10 ml of a mixture of equal volumes of *methanol R* and *methylene chloride R*.

Plate: TLC silica gel plate *R*.

Mobile phase: *methanol R*, *methylene chloride R* (10:90 *V/V*).

Application: 10 μ l, as 1 cm bands.

Development: over a path of 15 cm.

Drying: in air.

Detection: spray with *vanillin reagent R*. Heat the plate at 100-105 °C for 10 min and allow to cool; examine in daylight.

Results: see below the sequence of the zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other zones may be present in the chromatogram obtained with the test solution.

Top of the plate	
	A violet zone Several weak violet, blue or grey zones
β -Sitosterol: a violet zone Ursolic acid: a blue zone	A violet zone (β -sitosterol) A blue zone (ursolic acid) Several weak violet, blue or grey zones
	A violet zone (β -sitosterol glucoside)
Reference solution	Test solution

TESTS

Foreign matter (2.8.2): maximum 3.0 per cent.

Loss on drying (2.2.32): maximum 12.0 per cent, determined on 1.000 g of powdered drug (355) by drying in an oven at 100-105 °C for 2 h.

Total ash (2.4.16): maximum 10.0 per cent.

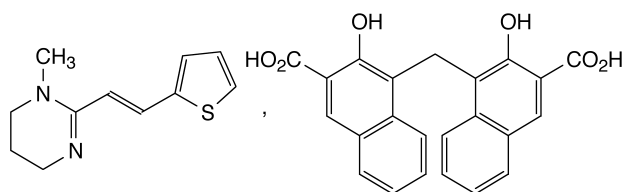
Extractable matter: minimum 0.5 per cent.

Extract 20.0 g of the powdered drug (250) with *methylene chloride R* for 4 h in a continuous extraction apparatus (Soxhlet type). Evaporate the solution to dryness on a water-bath *in vacuo* and then dry the residue at 80 °C for 2 h. The residue weighs a minimum of 0.10 g.

01/2005:1680

PYRANTEL EMBONATE

Pyranteli embonas

C₃₄H₃₀N₂O₆SM_r 594.7

DEFINITION

1-Methyl-2-[(*E*)-2-(thiophen-2-yl)ethenyl]-1,4,5,6-tetrahydropyrimidine hydrogen 4,4'-methylenebis(3-hydroxynaphthalene-2-carboxylate).

Content: 98.0 per cent to 102.0 per cent (dried substance).

CHARACTERS

Appearance: pale yellow or yellow powder.

Solubility: practically insoluble in water, soluble in dimethyl sulphoxide, practically insoluble in methanol.

IDENTIFICATION

Infrared absorption spectrophotometry (2.2.24).

Comparison: *pyrantel embonate CRS*.

TESTS

Related substances. Liquid chromatography (2.2.29).

Prepare the solutions immediately before use and strictly protect from light at all stages.

Solvent mixture. Mix 5 volumes of *glacial acetic R* with 5 volumes of *water R* and add 2 volumes of *diethylamine R* with cooling.

Test solution. Dissolve 80 mg in 7 ml of the solvent mixture and dilute to 100.0 ml with *acetonitrile R*.

Reference solution (a). Dissolve 10.0 mg of *pyrantel impurity A CRS* in the solvent mixture, add 2.5 ml of the test solution and dilute to 50.0 ml with the solvent mixture. Dilute 2.0 ml of this solution to 100.0 ml with the solvent mixture.

Reference solution (b). Dilute 1.0 ml of the test solution to 200.0 ml with the mobile phase.

Column:

- **size:** $l = 0.25$ m, $\varnothing = 4.6$ mm,
- **stationary phase:** *silica gel for chromatography R* (5 μ m).

Mobile phase: solvent mixture, *acetonitrile for chromatography R* (72:928 V/V).

Flow rate: 1 ml/min.

Detection: spectrophotometer at 288 nm.

Injection: 20 μ l.

Run time: 4 times the retention time of pyrantel.

Relative retention with reference to pyrantel (retention time = about 11 min): embonic acid = about 0.5; impurity A = about 1.3; impurity B = about 1.8 (impurity A also gives rise to an embonate peak).

System suitability: reference solution (a):

- **resolution:** minimum 4.0 between the peaks due to pyrantel and impurity A.

Limits:

- **correction factor:** for the calculation of content, multiply the peak area of impurity B by 0.4,
- **impurity A:** not more than the area of the corresponding peak in the chromatogram obtained with reference solution (a) (0.5 per cent),
- **impurity B:** not more than 0.4 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent),
- **any other impurity:** for each impurity, not more than 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent),
- **sum of impurities other than A and B:** not more than 0.6 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.3 per cent),
- **disregard limit:** 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

Chlorides (2.4.4): maximum 360 ppm.

To 0.46 g add 10 ml of *dilute nitric acid R* and 30 ml of *water R*. Heat on a water-bath for 5 min. Cool, dilute to 50 ml with *water R*, mix well and filter. 15 ml complies with the limit test for chlorides.

Sulphates (2.4.13): maximum 0.1 per cent.

To 0.50 g add 2.5 ml of *dilute nitric acid R* and dilute to 50 ml with *distilled water R*. Heat on a water-bath for 5 min, shake for 2 min, cool and filter. 15 ml complies with the limit test for sulphates.

Iron (2.4.9): maximum 75 ppm.

Ignite 0.66 g at 800 °C for 2 h. Dissolve the residue in 2.5 ml of *dilute hydrochloric acid R* with gentle heating for 10 min. Cool and dilute to 50 ml with *water R*. 10 ml complies with the limit test for iron.

Heavy metals (2.4.8): maximum 20 ppm.

1.0 g complies with limit test D. Prepare the reference solution using 2.0 ml of *lead standard solution (10 ppm Pb) R*.

Loss on drying (2.2.32): maximum 1.0 per cent, determined on 1.000 g by drying *in vacuo* at 60 °C for 3 h.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

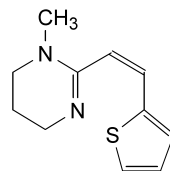
To 0.450 g add 10 ml of *acetic anhydride R* and 50 ml *glacial acetic acid R*, heat at 50 °C and stir for 10 min. Allow to cool (a clear solution is not obtained). Titrate with 0.1 M *perchloric acid*, determining the end-point potentiometrically (2.2.20). Carry out a blank titration. 1 ml of 0.1 M *perchloric acid* is equivalent to 59.47 mg of $C_{34}H_{30}N_2O_6S$.

STORAGE

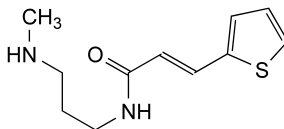
Protected from light.

IMPURITIES

Specified impurities: A, B.



A. 1-methyl-2-[(Z)-2-(thiophen-2-yl)ethenyl]-1,4,5,6-tetrahydropyrimidine,

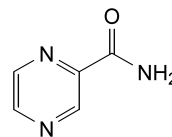


B. (E)-N-[3-(methylamino)propyl]-3-(thiophen-2-yl)prop-2-enamide.

01/2005:0859

PYRAZINAMIDE

Pyrazinamidum



$C_5H_5N_3O$

M_r 123.1

DEFINITION

Pyrazinamide contains not less than 99.0 per cent and not more than the equivalent of 100.5 per cent of pyrazine-2-carboxamide, calculated with reference to the anhydrous substance.

CHARACTERS

A white, crystalline powder, sparingly soluble in water, slightly soluble in alcohol and in methylene chloride.

IDENTIFICATION

First identification: C.

Second identification: A, B, D.

A. Melting point (2.2.14): 188 °C to 191 °C.

- B. Dissolve 50.0 mg in *water R* and dilute to 100.0 ml with the same solvent (solution (a)). Dilute 1.0 ml of solution (a) to 10.0 ml with *water R*. Examined between 290 nm and 350 nm (2.2.25), the solution shows an absorption maximum at 310 nm. Dilute 2.0 ml of solution (a) to 100.0 ml with *water R*. Examined between 230 nm and 290 nm, the solution shows an absorption maximum at 268 nm. The specific absorbance at the maximum is 640 to 680.
- C. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *pyrazinamide CRS*. Examine the substances prepared as discs. If the spectra obtained show differences, dissolve the substance to be examined and the reference substance separately in *alcohol R*, evaporate to dryness and record new spectra using the residues.
- D. Dissolve 0.1 g in 5 ml of *water R*. Add 1 ml of *ferrous sulphate solution R2*. The solution becomes orange. Add 1 ml of *dilute sodium hydroxide solution R*. The solution becomes dark blue.

TESTS

Solution S. Dissolve 0.5 g in *carbon dioxide-free water R* and dilute to 50 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and colourless (2.2.2, *Method II*).

Acidity or alkalinity. To 25 ml of solution S add 0.05 ml of *phenolphthalein solution R1* and 0.2 ml of *0.01 M sodium hydroxide*. The solution is red. Add 1.0 ml of *0.01 M hydrochloric acid*. The solution is colourless. Add 0.15 ml of *methyl red solution R*. The solution is red.

Related substances. Examine by thin-layer chromatography (2.2.27), using *silica gel GF₂₅₄ R* as the coating substance.

Test solution. Dissolve 0.1 g of the substance to be examined in a mixture of 1 volume of *methanol R* and 9 volumes of *methylene chloride R* and dilute to 10 ml with the same mixture of solvents.

Reference solution (a). Dilute 1 ml of the test solution to 50 ml with a mixture of 1 volume of *methanol R* and 9 volumes of *methylene chloride R*. Dilute 1 ml of the solution to 10 ml with a mixture of 1 volume of *methanol R* and 9 volumes of *methylene chloride R*.

Reference solution (b). Dissolve 10 mg of *nicotinic acid CRS* in a mixture of 1 volume of *methanol R* and 9 volumes of *methylene chloride R*, add 1 ml of the test solution and dilute to 10 ml with the same mixture of solvents.

Apply separately to the plate 20 µl of each solution. Develop over a path of 10 cm using a mixture of 20 volumes of *glacial acetic acid R*, 20 volumes of *water R* and 60 volumes of *butanol R*. Allow the plate to dry in air and examine immediately in ultraviolet light at 254 nm. Any spot in the chromatogram obtained with the test solution, apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (a) (0.2 per cent). The test is not valid unless the chromatogram obtained with reference solution (b) shows two clearly separated principal spots.

Heavy metals (2.4.8). 1.0 g complies with limit test C for heavy metals (10 ppm). Prepare the standard using 1 ml of *lead standard solution (10 ppm Pb) R*.

Water (2.5.12). Not more than 0.5 per cent, determined on 2.00 g by the semi-micro determination of water.

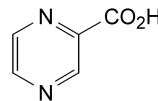
Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.100 g in 50 ml of *acetic anhydride R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (2.2.20).

1 ml of *0.1 M perchloric acid* is equivalent to 12.31 mg of $C_9H_{13}BrN_2O_2$.

IMPURITIES

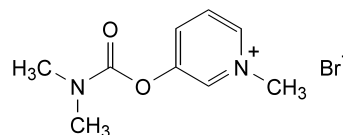


A. pyrazine-2-carboxylic acid.

01/2005:1255

PYRIDOSTIGMINE BROMIDE

Pyridostigmini bromidum

 $C_9H_{13}BrN_2O_2$ M_r 261.1

DEFINITION

Pyridostigmine bromide contains not less than 98.5 per cent and not more than the equivalent of 101.0 per cent of 3-(dimethylcarbamoyloxy)-1-methylpyridinium bromide, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, crystalline, deliquescent powder, very soluble in water and in alcohol.

IDENTIFICATION

- A. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *pyridostigmine bromide CRS*.
- B. It gives reaction (a) of bromides (2.3.1).

TESTS

Solution S. Dissolve 1.0 g in *carbon dioxide-free water R* and dilute to 100 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and colourless (2.2.2, *Method II*).

Acidity or alkalinity. To 40 ml of solution S add a few drops of *methyl red solution R*. To 20 ml of this solution add 0.2 ml of *0.02 M sodium hydroxide*. The solution is yellow. To the other 20 ml add 0.2 ml of *0.02 M hydrochloric acid*. The solution is red.

Related substances. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 50 mg of the substance to be examined in the mobile phase at about 40 °C. Allow to cool and dilute to 50.0 ml with the same solvent.

Reference solution (a). Dissolve 4 mg of *pyridostigmine impurity A CRS* and 4 mg of *pyridostigmine bromide CRS* in the mobile phase and dilute to 100.0 ml with the same solvent. Dilute 5.0 ml to 100.0 ml with the mobile phase.

Reference solution (b). Dilute 1.0 ml of the test solution to 100.0 ml with the mobile phase. Dilute 10.0 ml to 50.0 ml with the mobile phase.

Reference solution (c). Dilute 5.0 ml of reference solution (b) to 20.0 ml with the mobile phase.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4.0 mm in internal diameter packed with *base-deactivated octadecylsilyl silica gel for chromatography R* (5 µm to 10 µm),
- as mobile phase at a flow rate of 1.1 ml/min a mixture of 30 volumes of *acetonitrile R* and 70 volumes of a 4.33 g/l solution of *sodium dodecyl sulphate R*, previously adjusted to pH 2.0 with *phosphoric acid R*,
- as detector a spectrophotometer set at 220 nm.

Inject 20 µl of reference solution (b). Adjust the sensitivity of the system so that the height of the principal peak in the chromatogram obtained is at least 50 per cent of the full scale of the recorder. Inject 20 µl of reference solution (a). The test is not valid unless in the chromatogram obtained, the resolution between the peaks due to pyridostigmine and pyridostigmine impurity A is at least 1.5. Inject separately 20 µl of the test solution, 20 µl of reference solution (b) and 20 µl of reference solution (c). Continue the chromatography for twice the retention time of pyridostigmine. In the chromatogram obtained with the test solution: the area of any peak apart from the principal peak is not greater than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (0.4 per cent), at most one such peak has an area greater than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent) and at most one further peak has an area greater than half of the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent); the sum of the area of all of the peaks apart from the principal peak is not greater than 2.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent). Disregard any peak due to the solvent and any peak with an area less than the area of the principal peak in the chromatogram obtained with reference solution (c).

Heavy metals (2.4.8). 1.0 g complies with limit test C for heavy metals (20 ppm). Prepare the standard using 2 ml of *lead standard solution (10 ppm Pb) R*.

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

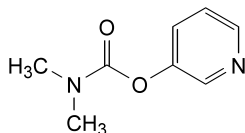
ASSAY

Dissolve 0.230 g in 10 ml of *anhydrous acetic acid R*. Add 40 ml of *acetic anhydride R*. Titrate with 0.1 M *perchloric acid*, determining the end-point potentiometrically (2.2.20). 1 ml of 0.1 M *perchloric acid* is equivalent to 26.11 mg of C₉H₁₃BrN₂O₂.

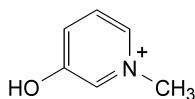
STORAGE

Store in an airtight container, protected from light. If the substance is sterile, store in a sterile, airtight, tamper-proof container, protected from light.

IMPURITIES



A. pyridin-3-yl dimethylcarbamate,

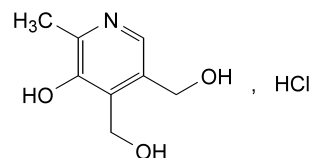


B. 3-hydroxy-1-methylpyridinium.

01/2005:0245

PYRIDOXINE HYDROCHLORIDE

Pyridoxini hydrochloridum



C₈H₁₂ClNO₃

M_r 205.6

DEFINITION

Pyridoxine hydrochloride contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of (5-hydroxy-6-methylpyridine-3,4-diyl)dimethanol hydrochloride, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, crystalline powder, freely soluble in water, slightly soluble in alcohol.

It melts at about 205 °C, with decomposition.

IDENTIFICATION

First identification: B, D.

Second identification: A, C, D.

- Dilute 1.0 ml of solution S (see Tests) to 50.0 ml with 0.1 M *hydrochloric acid* (solution A). Dilute 1.0 ml of solution A to 100.0 ml with 0.1 M *hydrochloric acid*. Examined between 250 nm and 350 nm (2.2.25), the solution shows an absorption maximum at 288 nm to 296 nm. The specific absorbance at the maximum is 425 to 445. Dilute 1.0 ml of solution A to 100.0 ml with a mixture of equal volumes of 0.025 M *potassium dihydrogen phosphate solution* and 0.025 M *disodium hydrogen phosphate solution* (2.2.3). Examined between 220 nm and 350 nm, the solution shows 2 absorption maxima, at 248 nm to 256 nm and at 320 nm to 327 nm. The specific absorbances at the maxima are 175 to 195 and 345 to 365, respectively.
- Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *pyridoxine hydrochloride CRS*.
- Examine the chromatograms obtained in the test for related substances. The principal spot in the chromatogram obtained with test solution (b) is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).
- Solution S gives reaction (a) of chlorides (2.3.1).

TESTS

Solution S. Dissolve 2.50 g in *carbon dioxide-free water R* and dilute to 50.0 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and not more intensely coloured than reference solution Y₇ (2.2.2, *Method II*).

pH (2.2.3). The pH of solution S is 2.4 to 3.0.

Related substances. Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel G plate R*.

Test solution (a). Dissolve 1.0 g of the substance to be examined in *water R* and dilute to 10 ml with the same solvent.

Test solution (b). Dilute 1 ml of test solution (a) to 10 ml with *water R*.

Reference solution (a). Dissolve 0.10 g of *pyridoxine hydrochloride CRS* in *water R* and dilute to 10 ml with the same solvent.

Reference solution (b). Dilute 2.5 ml of test solution (a) to 100 ml with *water R*. Dilute 1 ml of this solution to 10 ml with *water R*.

Apply to the plate 2 µl of each solution. Develop in an unsaturated tank over a path of 15 cm using a mixture of 9 volumes of *concentrated ammonia R*, 13 volumes of *methylene chloride R*, 13 volumes of *tetrahydrofuran R* and 65 volumes of *acetone R*. Allow the plate to dry in air and spray with a 50 g/l solution of *sodium carbonate R* in a mixture of 30 volumes of *alcohol R* and 70 volumes of *water R*. Dry the plate in a current of air, spray with a 1 g/l solution of *dichloroquinonechlorimide R* in *alcohol R* and examine the chromatograms immediately. Any spot in the chromatogram obtained with test solution (a), apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.25 per cent). Disregard any spots remaining on the starting line.

Heavy metals (2.4.8). 12 ml of solution S complies with limit test A (20 ppm). Prepare the standard using *lead standard solution (1 ppm Pb) R*.

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 100–105 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

In order to avoid overheating in the reaction medium, mix thoroughly throughout and stop the titration immediately after the end-point has been reached.

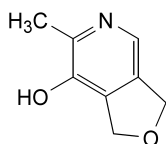
Dissolve 0.150 g in 5 ml of *anhydrous formic acid R*. Add 50 ml of *acetic anhydride R*. Titrate with 0.1 M *perchloric acid*, determining the end-point potentiometrically (2.2.20). Carry out a blank titration.

1 ml of 0.1 M *perchloric acid* is equivalent to 20.56 mg of C₈H₁₂ClNO₃.

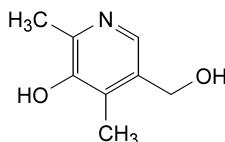
STORAGE

Store protected from light.

IMPURITIES



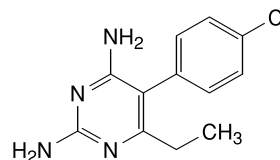
A. 6-methyl-1,3-dihydrofuro[3,4-c]pyridin-7-ol,



B. 5-(hydroxymethyl)-2,4-dimethylpyridin-3-ol.

PYRIMETHAMINE

Pyrimethaminum



C₁₂H₁₃ClN₄

M_r 248.7

DEFINITION

Pyrimethamine contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of 5-(4-chlorophenyl)-6-ethylpyrimidine-2,4-diamine, calculated with reference to the dried substance.

CHARACTERS

An almost white, crystalline powder or colourless crystals, practically insoluble in water, slightly soluble in alcohol.

IDENTIFICATION

First identification: C.

Second identification: A, B, D.

A. Melting point (2.2.14): 239 °C to 243 °C.

B. Dissolve 0.14 g in *ethanol R* and dilute to 100.0 ml with the same solvent. Dilute 10.0 ml of this solution to 100.0 ml with 0.1 M *hydrochloric acid*. Dilute 10.0 ml of this solution to 100.0 ml with 0.1 M *hydrochloric acid*. Examined between 250 nm and 300 nm (2.2.25), the solution shows an absorption maximum at 272 nm and an absorption minimum at 261 nm. The specific absorbance at the maximum is 310 to 330.

C. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *pyrimethamine CRS*.

D. Examine the chromatograms obtained in the test for related substances in ultraviolet light at 254 nm. The principal spot in the chromatogram obtained with test solution (b) is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a).

TESTS

Solution S. Shake 1.0 g with 50 ml of *distilled water R* for 2 min and filter.

Appearance of solution. Prepare the solution immediately before use.

Dissolve 0.25 g in a mixture of 1 volume of *methanol R* and 3 volumes of *methylene chloride R* and dilute to 10 ml with the same mixture of solvents. The solution is clear (2.2.1) and not more intensely coloured than reference solution BY₆ (2.2.2, Method II).

Acidity or alkalinity. To 10 ml of solution S add 0.05 ml of *phenolphthalein solution R1*. The solution is colourless. Not more than 0.2 ml of 0.01 M *sodium hydroxide* is required to change the colour of the indicator to pink. Add 0.4 ml of 0.01 M *hydrochloric acid* and 0.05 ml of *methyl red solution R*. The solution is red or orange.

Related substances. Examine by thin-layer chromatography (2.2.27), using *silica gel GF₂₅₄ R* as the coating substance. Prepare the solutions immediately before use.

Test solution (a). Dissolve 0.25 g of the substance to be examined in a mixture of 1 volume of *methanol R* and 9 volumes of *chloroform R* and dilute to 25 ml with the same mixture of solvents.

Test solution (b). Dilute 1 ml of test solution (a) to 10 ml with a mixture of 1 volume of *methanol R* and 9 volumes of *chloroform R*.

Reference solution (a). Dissolve 0.1 g of *pyrimethamine CRS* in a mixture of 1 volume of *methanol R* and 9 volumes of *chloroform R* and dilute to 100 ml with the same mixture of solvents.

Reference solution (b). Dilute 2.5 ml of test solution (a) to 100 ml with a mixture of 1 volume of *methanol R* and 9 volumes of *chloroform R*. Dilute 1 ml of the solution to 10 ml with a mixture of 1 volume of *methanol R* and 9 volumes of *chloroform R*.

Apply to the plate 20 µl of each solution. Develop over a path of 10 cm using a mixture of 4 volumes of *chloroform R*, 8 volumes of *propanol R*, 12 volumes of *glacial acetic acid R* and 76 volumes of *toluene R*. Allow the plate to dry in air. Examine in ultraviolet light at 254 nm. Any spot in the

chromatogram obtained with test solution (a), apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.25 per cent).

Sulphates (2.4.13). 15 ml of solution S complies with the limit test for sulphates (80 ppm). Prepare the standard using a mixture of 2.5 ml of *sulphate standard solution (10 ppm SO₄) R* and 12.5 ml of *distilled water R*.

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 0.50 g by drying in an oven at 100 °C to 105 °C for 4 h.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.200 g in 25 ml of *anhydrous acetic acid R*, heating gently. Cool. Titrate with 0.1 M *perchloric acid* determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *perchloric acid* is equivalent to 24.87 mg of C₁₂H₁₃ClN₄.

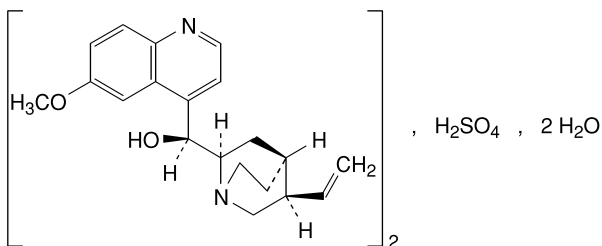
STORAGE

Store protected from light.

01/2005:0017 **Appearance of solution.** Solution S is clear (2.2.1) and not more intensely coloured than reference solution GY₆ (2.2.2, Method II).

QUINIDINE SULPHATE

Chinidini sulfas



$C_{40}H_{50}N_4O_8S \cdot 2H_2O$

M_r 783

DEFINITION

Quinidine sulphate contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of alkaloid monosulphates, calculated as bis[(S)-[(2R,4S,5R)-5-ethenyl-1-azabicyclo[2.2.2]oct-2-yl](6-methoxyquinolin-4-yl)methanol] sulphate, with reference to the dried substance.

CHARACTERS

A white or almost white, crystalline powder or silky, colourless needles, slightly soluble in water, soluble in boiling water and in alcohol, practically insoluble in acetone.

IDENTIFICATION

- A. Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel G plate R*.

Test solution. Dissolve 0.10 g of the substance to be examined in *methanol R* and dilute to 10 ml with the same solvent.

Reference solution. Dissolve 0.10 g of *quinidine sulphate CRS* in *methanol R* and dilute to 10 ml with the same solvent.

Apply to the plate 5 µl of each solution. Develop over a path of 15 cm using a mixture of 10 volumes of *diethylamine R*, 24 volumes of *ether R* and 40 volumes of *toluene R*. Dry the plate in a current of air for 15 min and repeat the development. Dry the plate at 105 °C for 30 min, allow to cool and spray with *iodoplatinate reagent R*. The principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

- B. Dissolve about 5 mg in 5 ml of *water R*. Add 0.2 ml of *bromine water R* and 1 ml of *dilute ammonia R2*. A green colour develops.
- C. Dissolve 0.1 g in 3 ml of *dilute sulphuric acid R* and dilute to 100 ml with *water R*. When examined in ultraviolet light at 366 nm, an intense blue fluorescence appears which disappears almost completely on addition of 1 ml of *hydrochloric acid R*.
- D. Dissolve about 50 mg in 5 ml of hot *water R*, cool, add 1 ml of *silver nitrate solution R1* and stir with a glass rod. After a few minutes, a white precipitate is formed that dissolves on the addition of *dilute nitric acid R*.
- E. It gives reaction (a) of sulphates (2.3.1).
- F. It complies with the test for pH (see Tests).

TESTS

Solution S. Dissolve 0.500 g in 0.1 M *hydrochloric acid* and dilute to 25.0 ml with the same acid.

pH (2.2.3). Dissolve 0.10 g in *carbon dioxide-free water R* and dilute to 10 ml with the same solvent. The pH of the solution is 6.0 to 6.8.

Specific optical rotation (2.2.7): + 275 to + 290, determined on solution S and calculated with reference to the dried substance.

Other cinchona alkaloids. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 20 mg of the substance to be examined, with gentle heating if necessary, in 5 ml of the mobile phase and dilute to 10 ml with the mobile phase.

Reference solution (a). Dissolve 20 mg of *quinine sulphate CRS*, with gentle heating if necessary, in 5 ml of the mobile phase and dilute to 10 ml with the mobile phase.

Reference solution (b). Dissolve 20 mg of *quinidine sulphate CRS*, with gentle heating if necessary, in 5 ml of the mobile phase and dilute to 10 ml with the mobile phase.

Reference solution (c). To 1 ml of reference solution (a) add 1 ml of reference solution (b).

Reference solution (d). Dilute 1.0 ml of reference solution (a) to 10.0 ml with the mobile phase. Dilute 1.0 ml of this solution to 50.0 ml with the mobile phase.

Reference solution (e). Dissolve 10 mg of *thiourea R* in the mobile phase and dilute to 10 ml with the mobile phase.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.15 m to 0.25 m long and 4.6 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (5 µm or 10 µm),
- as mobile phase at a flow rate of 1.5 ml/min a mixture prepared as follows: dissolve 6.8 g of *potassium dihydrogen phosphate R* and 3.0 g of *hexylamine R* in 700 ml of *water R*, adjust to pH 2.8 with *dilute phosphoric acid R*, add 60 ml of *acetonitrile R* and dilute to 1000 ml with *water R*,
- as detector a spectrophotometer set at 250 nm for recording the chromatogram obtained with reference solution (e) and at 316 nm for the other solutions.

Inject 10 µl of reference solution (b) and 10 µl of reference solution (e). If necessary, adjust the concentration of acetonitrile in the mobile phase so that, in the chromatogram obtained with reference solution (b), the mass distribution factor of the peak corresponding to quinidine is 3.5 to 4.5, t_R being calculated from the peak corresponding to thiourea in the chromatogram obtained with reference solution (e).

Inject 10 µl each of reference solutions (a), (b), (c) and (d). The chromatogram obtained with reference solution (a) shows a principal peak corresponding to quinine and a peak corresponding to dihydroquinine, with a retention time relative to quinine of about 1.4. The chromatogram obtained with reference solution (b) shows a principal peak corresponding to quinidine and a peak corresponding to dihydroquinidine, with a retention time relative to quinidine of about 1.5. The chromatogram obtained with reference solution (c) shows 4 peaks corresponding to quinidine, quinine, dihydroquinidine and dihydroquinine which are identified by comparison of their retention times with those of the corresponding peaks in the chromatograms obtained with reference solutions (a) and (b).

The test is not valid unless: in the chromatogram obtained with reference solution (c), the resolution between the peaks corresponding to quinine and quinidine is at least 3.0 and the resolution between the peaks corresponding

01/2005:0018

to dihydroquinidine and quinine is at least 2.0; and the chromatogram obtained with reference solution (d) shows a principal peak with a signal-to-noise ratio of at least 4.

Inject 10 µl of the test solution and record the chromatogram for 2.5 times the retention time of the principal peak. Calculate the percentage content of related substances from the areas of the peaks in the chromatogram obtained with the test solution by the normalisation procedure, disregarding any peaks with an area less than that of the peak in the chromatogram obtained with reference solution (d). The content of dihydroquinidine is not greater than 15 per cent, the content of any related substance eluted before quinidine is not greater than 5 per cent and the content of any other related substance is not greater than 2.5 per cent.

Boron. Avoid where possible the use of glassware.

Test solution. Dissolve 1.00 g in a mixture of 0.5 ml of *hydrochloric acid R* and 4.0 ml of *water R*.

Reference solution. Dissolve 0.572 g of *boric acid R* in *water R* and dilute to 1000.0 ml with the same solvent. Dilute 5.0 ml to 100.0 ml with *water R*. To 1.0 ml of this solution add 3.0 ml of *water R* and 0.5 ml of *hydrochloric acid R*.

Blank solution. Add 0.5 ml of *hydrochloric acid R* to 4.0 ml of *water R*.

Add 3.0 ml of a 100 g/l solution of *2-ethylhexane-1,3-diol R* in *methylene chloride R* to the test solution, to the reference solution and to the blank solution and shake for 1 min. Allow to stand for 6 min. To 1.0 ml of the lower layer, add 2.0 ml of a 3.75 g/l solution of *curcumin R* in *anhydrous acetic acid R* and 0.3 ml of *sulphuric acid R*. Mix and after 20 min add 25.0 ml of *alcohol R*. Mix. The colour of the blank solution is yellow. Any red colour in the test solution is not more intense than that in the reference solution (5 ppm of B).

Loss on drying (2.2.32). 3.0 per cent to 5.0 per cent, determined on 1.000 g by drying in an oven at 130 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.200 g in 20 ml of *acetic anhydride R*. Titrate with 0.1 M *perchloric acid*, using 0.15 ml of *naphtholbenzein solution R* as indicator.

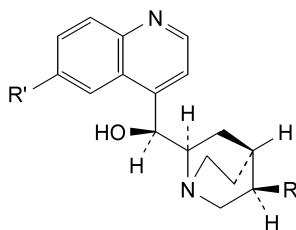
1 ml of 0.1 M *perchloric acid* is equivalent to 24.90 mg of C₂₀H₂₅ClN₂O₂·2H₂O.

STORAGE

Store protected from light.

IMPURITIES

A. quinine,

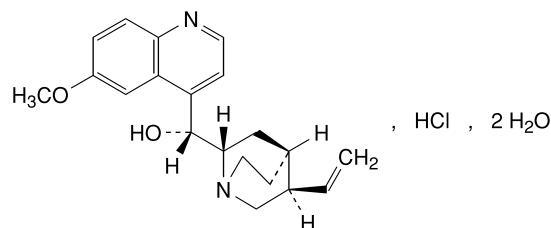


B. R = CH=CH₂, R' = H: (S)-[(2R,4S,5R)-5-ethenyl-1-azabicyclo[2.2.2]oct-2-yl](quinolin-4-yl)methanol (cinchonine),

C. R = C₂H₅, R' = OCH₃: (S)-[(2R,4S,5R)-5-ethyl-1-azabicyclo[2.2.2]oct-2-yl](6-methoxyquinolin-4-yl)methanol (dihydroquinidine).

QUININE HYDROCHLORIDE

Chinini hydrochloridum



C₂₀H₂₅ClN₂O₂·2H₂O

M_r 396.9

DEFINITION

Quinine hydrochloride contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of alkaloid monohydrochlorides, calculated as (R)-[(2S,4S,5R)-5-ethenyl-1-azabicyclo[2.2.2]oct-2-yl](6-methoxyquinolin-4-yl)methanol hydrochloride, with reference to the dried substance.

CHARACTERS

Fine, silky needles, often in clusters, colourless, soluble in water, freely soluble in alcohol.

IDENTIFICATION

A. Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel G plate R*.

Test solution. Dissolve 0.10 g of the substance to be examined in *methanol R* and dilute to 10 ml with the same solvent.

Reference solution. Dissolve 0.10 g of *quinine sulphate CRS* in *methanol R* and dilute to 10 ml with the same solvent.

Apply separately to the plate 5 µl of each solution. Develop over a path of 15 cm using a mixture of 10 volumes of *diethylamine R*, 24 volumes of *ether R* and 40 volumes of *toluene R*. Dry the plate in a current of air for 15 min and repeat the development. Dry the plate at 105 °C for 30 min, allow to cool and spray with *iodoplatinate reagent R*. The principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

B. Dissolve about 10 mg in *water R* and dilute to 10 ml with the same solvent. To 5 ml of the solution add 0.2 ml of *bromine water R* and 1 ml of *dilute ammonia R2*. A green colour develops.

C. Dissolve 0.1 g in 3 ml of *dilute sulphuric acid R* and dilute to 100 ml with *water R*. When examined in ultraviolet light at 366 nm, an intense blue fluorescence appears which disappears almost completely on the addition of 1 ml of *hydrochloric acid R*.

D. It gives the reactions of chlorides (2.3.1).

E. It complies with the test for pH (see Tests).

TESTS

Solution S. Dissolve 1.0 g in *carbon dioxide-free water R* prepared from *distilled water R* and dilute to 50 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and not more intensely coloured than reference solution Y₆ (2.2.2, Method II).

pH (2.2.3). Dilute 10 ml of solution S to 20 ml with *carbon dioxide-free water R*. The pH of the solution is 6.0 to 6.8.

Specific optical rotation (2.2.7). Dissolve 0.500 g in 0.1 M *hydrochloric acid* and dilute to 25.0 ml with the same acid. The specific optical rotation is -245 to -258 , calculated with reference to the dried substance.

Other cinchona alkaloids. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 20 mg of the substance to be examined, with gentle heating if necessary, in 5 ml of the mobile phase and dilute to 10 ml with the mobile phase.

Reference solution (a). Dissolve 20 mg of *quinine sulphate CRS*, with gentle heating if necessary, in 5 ml of the mobile phase and dilute to 10 ml with the mobile phase.

Reference solution (b). Dissolve 20 mg of *quinidine sulphate CRS*, with gentle heating if necessary, in 5 ml of the mobile phase and dilute to 10 ml with the mobile phase.

Reference solution (c). To 1 ml of reference solution (a) add 1 ml of reference solution (b).

Reference solution (d). Dilute 1.0 ml of reference solution (a) to 10.0 ml with the mobile phase. Dilute 1.0 ml of this solution to 50.0 ml with the mobile phase.

Reference solution (e). Dissolve 10 mg of *thiourea R* in the mobile phase and dilute to 10 ml with the mobile phase.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.15 m to 0.25 m long and 4.6 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (5 μ m or 10 μ m),
- as mobile phase at a flow rate of 1.5 ml/min a mixture prepared as follows: dissolve 6.8 g of *potassium dihydrogen phosphate R* and 3.0 g of *hexylamine R* in 700 ml of *water R*, adjust to pH 2.8 with *dilute phosphoric acid R*, add 60 ml of *acetonitrile R* and dilute to 1000 ml with *water R*,
- as detector a spectrophotometer set at 250 nm for recording the chromatogram obtained with reference solution (e) and at 316 nm for the other solutions.

Inject 10 μ l of reference solution (b) and 10 μ l of reference solution (e). If necessary, adjust the concentration of acetonitrile in the mobile phase so that, in the chromatogram obtained with reference solution (b), the mass distribution factor of the peak corresponding to quinidine is 3.5 to 4.5, t_R being calculated from the peak corresponding to thiourea in the chromatogram obtained with reference solution (e).

Inject 10 μ l each of reference solutions (a), (b), (c) and (d). The chromatogram obtained with reference solution (a) shows a principal peak corresponding to quinine and a peak corresponding to dihydroquinine, with a retention time relative to quinine of about 1.4. The chromatogram obtained with reference solution (b) shows a principal peak corresponding to quinidine and a peak corresponding to dihydroquinidine with a retention time relative to quinidine of about 1.5. The chromatogram obtained with reference solution (c) shows 4 peaks corresponding to quinidine, quinine, dihydroquinidine and dihydroquinine, which are identified by comparison of their retention times with those of the corresponding peaks in the chromatograms obtained with reference solutions (a) and (b).

The chromatographic system is not satisfactory unless in the chromatogram obtained with reference solution (c), the resolution between the peaks corresponding to quinine and quinidine is at least 3.0 and the resolution between the peaks corresponding to dihydroquinidine and quinine is at least 2.0 and the chromatogram obtained with reference solution (d) shows a principal peak with a signal-to-noise ratio of at least 4.

Inject 10 μ l of the test solution. Record the chromatograms for 2.5 times the retention time of the principal peak. Calculate the percentage content of related substances from the areas of the peaks in the chromatogram obtained with the test solution by the normalisation procedure, ignoring any peaks with an area less than that of the peak in the chromatogram obtained with reference solution (d). The content of dihydroquinine is not greater than 10 per cent, the content of any related substance eluted before quinine is not greater than 5 per cent and the content of any other related substance is not greater than 2.5 per cent.

Sulphates (2.4.13). 15 ml of solution S complies with the limit test for sulphate (500 ppm).

Barium. To 15 ml of solution S add 1 ml of *dilute sulphuric acid R*. After at least 15 min, any opalescence in the solution is not more intense than that in a mixture of 15 ml of solution S and 1 ml of *distilled water R*.

Loss on drying (2.2.32). 6.0 per cent to 10.0 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.250 g in 50 ml of *alcohol R* and add 5.0 ml of 0.01 M *hydrochloric acid*. Titrate with 0.1 M *sodium hydroxide*, determining the end-point potentiometrically (2.2.20). Read the volume added between the 2 inflexion points.

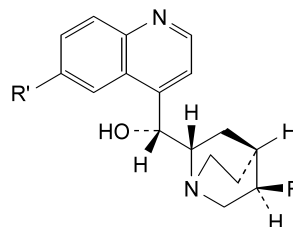
1 ml of 0.1 M *sodium hydroxide* is equivalent to 36.09 mg of $C_{20}H_{25}ClN_2O_2$

STORAGE

Store protected from light.

IMPURITIES

A. quinidine,



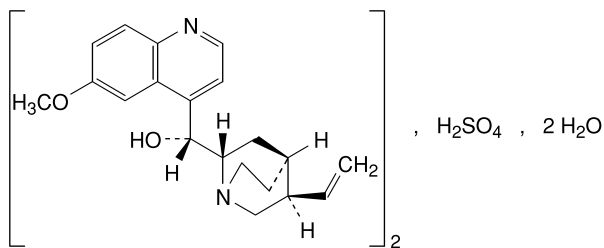
B. $R = CH=CH_2$, $R' = H$: (*R*)-[(2*S*,4*S*,5*R*)-5-ethenyl-1-azabicyclo[2.2.2]oct-2-yl](quinolin-4-yl)methanol (cinchonidine),

C. $R = C_2H_5$, $R' = OCH_3$: (*R*)-[(2*S*,4*S*,5*R*)-5-ethyl-1-azabicyclo[2.2.2]oct-2-yl](6-methoxyquinolin-4-yl)methanol (dihydroquinine).

01/2005:0019 pH (2.2.3). The pH of a 10 g/l suspension in *water R* is 5.7 to 6.6.

QUININE SULPHATE

Chinini sulfas



$C_{40}H_{50}N_4O_8S \cdot 2H_2O$

M_r 783

DEFINITION

Quinine sulphate contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of alkaloid monosulphates, calculated as bis[(*R*)-[(2*S*,4*S*,5*R*)-5-ethenyl-1-azabicyclo[2.2.2]oct-2-yl](6-methoxyquinolin-4-yl)methanol] sulphate, with reference to the dried substance.

CHARACTERS

A white or almost white, crystalline powder or fine, colourless needles, slightly soluble in water, sparingly soluble in boiling water and in alcohol.

IDENTIFICATION

- A. Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel G plate R*.

Test solution. Dissolve 0.10 g of the substance to be examined in *methanol R* and dilute to 10 ml with the same solvent.

Reference solution. Dissolve 0.10 g of *quinine sulphate CRS* in *methanol R* and dilute to 10 ml with the same solvent.

Apply to the plate 5 µl of each solution. Develop over a path of 15 cm using a mixture of 10 volumes of *diethylamine R*, 24 volumes of *ether R* and 40 volumes of *toluene R*. Dry the plate in a current of air for 15 min and repeat the development. Dry the plate at 105 °C for 30 min, allow to cool and spray with *iodoplatinate reagent R*. The principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

- B. Dissolve about 5 mg in 5 ml of *water R*. Add 0.2 ml of *bromine water R* and 1 ml of *dilute ammonia R2*. A green colour develops.
- C. Dissolve 0.1 g in 3 ml of *dilute sulphuric acid R* and dilute to 100 ml with *water R*. When examined in ultraviolet light at 366 nm, an intense blue fluorescence appears which disappears almost completely on the addition of 1 ml of *hydrochloric acid R*.
- D. Dissolve about 45 mg in 5 ml of *dilute hydrochloric acid R*. The solution gives reaction (a) of sulphates (2.3.1).
- E. It complies with the test for pH (see Tests).

TESTS

Solution S. Dissolve 0.500 g in 0.1 *M* *hydrochloric acid* and dilute to 25.0 ml with the same acid.

Appearance of solution. Solution S is clear (2.2.1) and not more intensely coloured than reference solution GY₆ (2.2.2, *Method II*).

Specific optical rotation (2.2.7). –237 to –245, determined on solution S and calculated with reference to the dried substance.

Other cinchona alkaloids. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 20 mg of the substance to be examined, with gentle heating if necessary, in 5 ml of the mobile phase and dilute to 10 ml with the mobile phase.

Reference solution (a). Dissolve 20 mg of *quinine sulphate CRS*, with gentle heating if necessary, in 5 ml of the mobile phase and dilute to 10 ml with the mobile phase.

Reference solution (b). Dissolve 20 mg of *quinidine sulphate CRS*, with gentle heating if necessary, in 5 ml of the mobile phase and dilute to 10 ml with the mobile phase.

Reference solution (c). To 1 ml of reference solution (a) add 1 ml of reference solution (b).

Reference solution (d). Dilute 1.0 ml of reference solution (a) to 10.0 ml with the mobile phase. Dilute 1.0 ml of this solution to 50.0 ml with the mobile phase.

Reference solution (e). Dissolve 10 mg of *thiourea R* in the mobile phase and dilute to 10 ml with the mobile phase.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.15 m to 0.25 m long and 4.6 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (5 µm or 10 µm),
- as mobile phase at a flow rate of 1.5 ml/min a mixture prepared as follows: dissolve 6.8 g of *potassium dihydrogen phosphate R* and 3.0 g of *hexylamine R* in 700 ml of *water R*, adjust to pH 2.8 with *dilute phosphoric acid R*, add 60 ml of *acetonitrile R* and dilute to 1000 ml with *water R*,
- as detector a spectrophotometer set at 250 nm for recording the chromatogram obtained with reference solution (e) and at 316 nm for the other solutions.

Inject 10 µl of reference solution (b) and 10 µl of reference solution (e). If necessary, adjust the concentration of acetonitrile in the mobile phase so that, in the chromatogram obtained with reference solution (b), the mass distribution factor of the peak corresponding to quinidine is 3.5 to 4.5, t_R being calculated from the peak corresponding to thiourea in the chromatogram obtained with reference solution (e).

Inject 10 µl each of reference solutions (a), (b), (c) and (d). The chromatogram obtained with reference solution (a) shows a principal peak corresponding to quinine and a peak, corresponding to dihydroquinine, with a retention time relative to quinine of about 1.4. The chromatogram obtained with reference solution (b) shows a principal peak corresponding to quinidine and a peak, corresponding to dihydroquinidine, with a retention time relative to quinidine of about 1.5. The chromatogram obtained with reference solution (c) shows 4 peaks corresponding to quinidine, quinine, dihydroquinidine and dihydroquinine which are identified by comparison of their retention times with those of the corresponding peaks in the chromatograms obtained with reference solutions (a) and (b).

The test is not valid unless: in the chromatogram obtained with reference solution (c), the resolution between the peaks corresponding to quinine and quinidine is at least 3.0 and the resolution between the peaks corresponding to dihydroquinidine and quinine is at least 2.0; and the chromatogram obtained with reference solution (d) shows a principal peak with a signal-to-noise ratio of at least 4.

Inject 10 µl of the test solution. Record the chromatograms for 2.5 times the retention time of the principal peak. Calculate the percentage content of related substances from the areas of the peaks in the chromatogram obtained with the test solution by the normalisation procedure, disregarding any peaks with an area less than that of the peak in the chromatogram obtained with reference solution (d). The content of dihydroquinine is not greater than 10 per cent, the content of any related substance eluted before quinine is not greater than 5 per cent and the content of any other related substance is not greater than 2.5 per cent.

Loss on drying (2.2.32): 3.0 per cent to 5.0 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

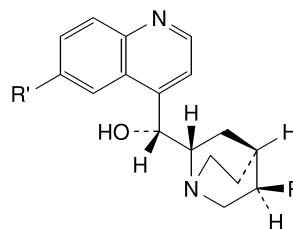
Dissolve 0.300 g in a mixture of 10 ml of *chloroform R* and 20 ml of *acetic anhydride R*. Titrate with 0.1 M *perchloric acid*, determining the end-point potentiometrically (2.2.20). 1 ml of 0.1 M *perchloric acid* is equivalent to 24.90 mg of C₄₀H₅₀N₄O₈S.

STORAGE

Store protected from light.

IMPURITIES

A. quinidine,



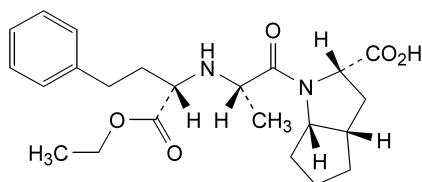
B. R = CH=CH₂, R' = H: (*R*)-[(2*S*,4*S*,5*R*)-5-ethenyl-1-azabicyclo[2.2.2]oct-2-yl](quinolin-4-yl)methanol (cinchonidine),

C. R = C₂H₅, R' = OCH₃: (*R*)-[(2*S*,4*S*,5*R*)-5-ethyl-1-azabicyclo[2.2.2]oct-2-yl](6-methoxyquinolin-4-yl)methanol (dihydroquinine).

01/2005:1368

RAMIPRIL

Ramiprilum

 $C_{23}H_{32}N_2O_5$ M_r 416.5

DEFINITION

Ramipril contains not less than 98.0 per cent and not more than the equivalent of 101.0 per cent of (2S,3aS,6aS)-1-[(S)-2-[(S)-1-(ethoxycarbonyl)-3-phenylpropyl]amino]propanoyl]octahydrocyclopenta[b]pyrrole-2-carboxylic acid, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, crystalline powder, sparingly soluble in water, freely soluble in methanol.

IDENTIFICATION

- It complies with the test for specific optical rotation (see Tests).
- Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *ramipril CRS*.

TESTS

Appearance of solution. Dissolve 0.1 g in *methanol R* and dilute to 10 ml with the same solvent. The solution is clear (2.2.1) and colourless (2.2.2, *Method II*).

Specific optical rotation (2.2.7). Dissolve 0.250 g in a mixture of 14 volumes of *hydrochloric acid R1* and 86 volumes of *methanol R* and dilute to 25.0 ml with the same mixture of solvents. The specific optical rotation is + 32.0 to + 38.0, calculated with reference to the dried substance.

Related substances. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 20.0 mg of the substance to be examined in mobile phase A and dilute to 20.0 ml with the same mobile phase.

Reference solution (a). Dissolve 5 mg each of *ramipril impurity A CRS*, *ramipril impurity B CRS*, *ramipril impurity C CRS* and *ramipril impurity D CRS* in 5 ml of the test solution and dilute to 10 ml with mobile phase B.

Reference solution (b). Dilute 5.0 ml of the test solution to 100.0 ml with mobile phase B. Dilute 5.0 ml of the solution to 50.0 ml with mobile phase B.

Reference solution (c). Dilute 1.0 ml of reference solution (b) to 10.0 ml with mobile phase B.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4.0 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (3 µm),
- as mobile phase at a flow rate of 1.0 ml/min:

Mobile phase A. Dissolve 2.0 g of *sodium perchlorate R* in a mixture of 0.5 ml of *triethylamine R* and 800 ml of *water R*; adjust to pH 3.6 with *phosphoric acid R* and add 200 ml of *acetonitrile R*,

Mobile phase B. Dissolve 2.0 g of *sodium perchlorate R* in a mixture of 0.5 ml of *triethylamine R* and 300 ml of *water R*; adjust to pH 2.6 with *phosphoric acid R* and add 700 ml of *acetonitrile R*,

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)	Comment
0 - 6	90	10	isocratic
6 - 7	90 → 75	10 → 25	linear gradient
7 - 20	75 → 65	25 → 35	linear gradient
20 - 30	65 → 25	35 → 75	linear gradient
30 - 40	25	75	isocratic
40 - 45	25 → 90	75 → 10	linear gradient
45 - 55	90	10	re-equilibration

– as detector a spectrophotometer set at 210 nm,

maintaining the temperature of the column at 65 °C.

Equilibrate the column with a mixture of 90 per cent mobile phase A and 10 per cent mobile phase B for at least 35 min. If a suitable baseline cannot be obtained, use another grade of triethylamine.

Inject 10 µl of reference solution (c). Adjust the sensitivity of the system so that the chromatogram obtained shows a visible peak. Inject 10 µl of reference solution (a), 10 µl of reference solution (b) and 10 µl of the test solution. The test is not valid unless: in the chromatogram obtained with reference solution (a) the resolution between the peaks corresponding to ramipril impurity A and ramipril is at least 3.0; in the chromatogram obtained with reference solution (c) the principal peak has a signal-to-noise ratio of at least three; and in the chromatogram obtained with the test solution, the symmetry factor of the principal peak is 0.8 to 2.0.

When the chromatograms are recorded in the prescribed conditions, the retention times are: ramipril impurity A about 14 min, ramipril about 18 min, ramipril impurity B about 22 min, toluene about 24 min, ramipril impurity C about 26 min and ramipril impurity D about 28 min.

In the chromatogram obtained with the test solution, multiply the area of any peak corresponding to ramipril impurity C by a correction factor of 2.4.

In the chromatogram obtained with the test solution: the area of each peak corresponding to ramipril impurity A, ramipril impurity B, ramipril impurity C and ramipril impurity D is not greater than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent); the area of any peak, apart from the principal peak and any peak corresponding to the impurities A, B, C and D, is not greater than 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent); the sum of the areas of all peaks, apart from the principal peak, is not greater than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (1 per cent). Disregard any peak with an area less than that of the principal peak in the chromatogram obtained with reference solution (c).

Palladium. Not more than 20 ppm of Pd, determined by atomic absorption spectrometry (2.2.23, *Method I*).

Test solution. Dissolve 0.200 g of the substance to be examined in a mixture of 0.3 volumes of *nitric acid R* and 99.7 volumes of *water R* and dilute to 100.0 ml with the same mixture of solvents.

Reference solutions. Use solutions containing 0.02 µg, 0.03 µg and 0.05 µg of palladium per millilitre, freshly prepared by dilution of *palladium standard solution* (0.5 ppm Pd) *R* with a mixture of 0.3 volumes of *nitric acid R* and 99.7 volumes of *water R*.

Modifier solution. Dissolve 0.150 g of *magnesium nitrate R* in a mixture of 0.3 volumes of *nitric acid R* and 99.7 volumes of *water R* and dilute to 100.0 ml with the same mixture of solvents.

Inject separately 20 µl of the test solution and 20 µl of the reference solution, and 10 µl of the modifier solution. Measure the absorbance at 247.6 nm using a palladium hollow-cathode lamp as a source of radiation, a transmission band of preferably 1 nm and a graphite tube.

Loss on drying (2.2.32). Not more than 0.2 per cent, determined on 1.000 g by drying in an oven under high vacuum at 60 °C for 4 h.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.300 g in 25 ml of *methanol R* and add 25 ml of *water R*. Titrate with 0.1 M *sodium hydroxide*, determining the end-point potentiometrically (2.2.20). Carry out a blank titration.

1 ml of 0.1 M *sodium hydroxide* is equivalent to 41.65 mg of C₂₃H₃₂N₂O₅.

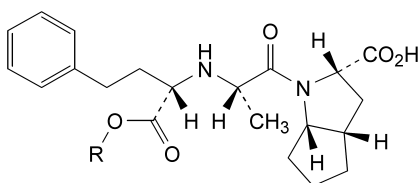
STORAGE

Store protected from light.

IMPURITIES

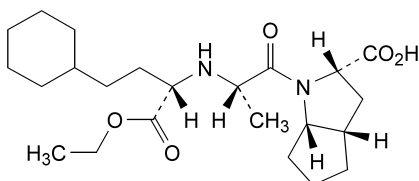
Specified impurities: A, B, C, D.

Other detectable impurities: E, F, G, H, I, J, K, L, M, N.

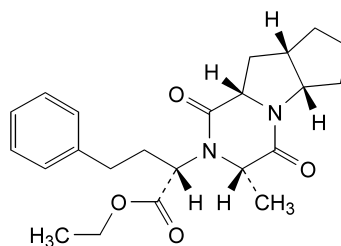


A. R = CH₃: (2*S*,3*aS*,6*aS*)-1-[(*S*)-2-[(*S*)-1-(methoxycarbonyl)-3-phenylpropyl]amino]propanoyl]octahydrocyclopenta[*b*]pyrrole-2-carboxylic acid (ramipril methyl ester),

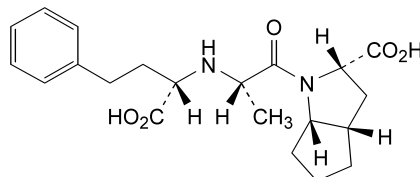
B. R = CH(CH₃)₂: (2*S*,3*aS*,6*aS*)-1-[(*S*)-2-[(*S*)-1-[(1-methylethoxy)carbonyl]-3-phenylpropyl]amino]propanoyl]octahydrocyclopenta[*b*]pyrrole-2-carboxylic acid (ramipril isopropyl ester),



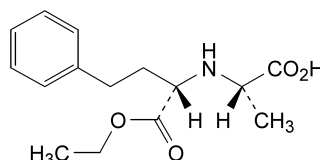
C. (2*S*,3*aS*,6*aS*)-1-[(*S*)-2-[(*S*)-3-cyclohexyl-1-(ethoxycarbonyl)propyl]amino]propanoyl]octahydrocyclopenta[*b*]pyrrole-2-carboxylic acid (hexahydroramipril),



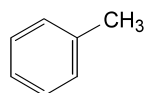
D. ethyl (2*S*)-2-[(3*S*,5*aS*,8*aS*,9*aS*)-3-methyl-1,4-dioxodecahydro-2*H*-cyclopenta[4,5]pyrrolo[1,2-*a*]pyrazin-2-yl]-4-phenylbutanoate (ramipril diketopiperazine),



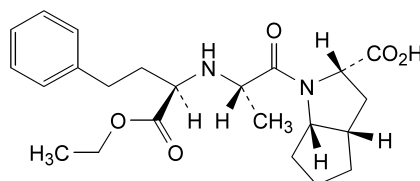
E. (2*S*,3*aS*,6*aS*)-1-[(*S*)-2-[(*S*)-1-carboxy-3-phenylpropyl]amino]propanoyl]octahydrocyclopenta[*b*]pyrrole-2-carboxylic acid (ramipril diacid),



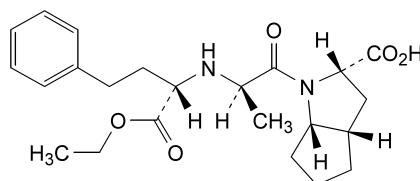
F. (*S*)-2-[(*S*)-1-(ethoxycarbonyl)-3-phenylpropyl]amino]propanoic acid,



G. toluene,

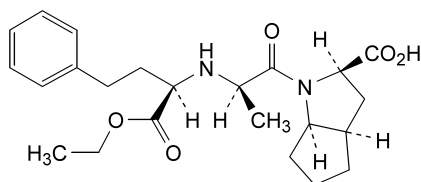


H. (2*S*,3*aS*,6*aS*)-1-[(*R*)-2-[(*S*)-1-(ethoxycarbonyl)-3-phenylpropyl]amino]propanoyl]octahydrocyclopenta[*b*]pyrrole-2-carboxylic acid ((*R,S,S,S*) isomer of ramipril),

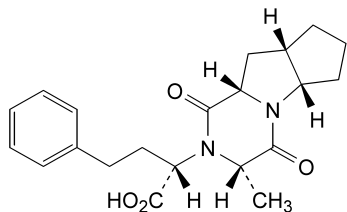


I. (2*S*,3*aS*,6*aS*)-1-[(*S*)-2-[(*R*)-1-(ethoxycarbonyl)-3-phenylpropyl]amino]propanoyl]octahydrocyclopenta[*b*]pyrrole-2-carboxylic acid ((*S,R,S,S*) isomer of ramipril),

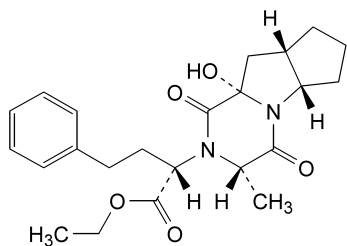
01/2005:0946



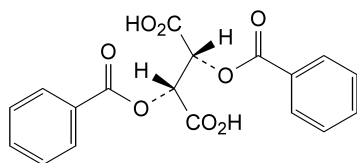
- J. (2*R*,3*aR*,6*aR*)-1-[(*R*)-2-[(*R*)-1-(ethoxycarbonyl)-3-phenylpropyl]amino]propanoyl]octahydrocyclopenta[*b*]pyrrole-2-carboxylic acid ((*R*,*R*,*R*,*R*) isomer of ramipril),



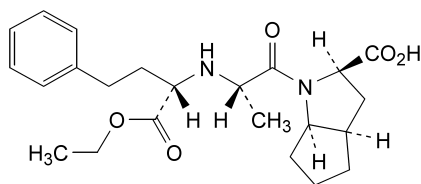
- K. (2*S*)-2-[(3*S*,5*aS*,8*aS*,9*aS*)-3-methyl-1,4-dioxodecahydro-2*H*-cyclopenta[4,5]pyrrolo[1,2-*a*]pyrazin-2-yl]-4-phenylbutanoic acid (ramipril diketopiperazine acid),



- L. ethyl (2*S*)-2-[(3*S*,5*aS*,8*aS*,9*aS*)-9*a*-hydroxy-3-methyl-1,4-dioxodecahydro-2*H*-cyclopenta[4,5]pyrrolo[1,2-*a*]pyrazin-2-yl]-4-phenylbutanoate (ramipril hydroxydiketopiperazine),



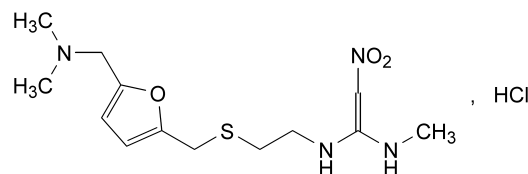
- M. (2*R*,3*R*)-2,3-di(benzoyloxy)butanedioic acid (dibenzoyltartric acid),



- N. (2*R*,3*aR*,6*aR*)-1-[(*S*)-2-[(*S*)-1-(ethoxycarbonyl)-3-phenylpropyl]amino]propanoyl]octahydrocyclopenta[*b*]pyrrole-2-carboxylic acid ((*S*,*S*-*R*,*R*,*R*) isomer of ramipril).

RANITIDINE HYDROCHLORIDE

Ranitidini hydrochloridum

C₁₃H₂₃ClN₄O₃S*M*_r 350.9

DEFINITION

Ranitidine hydrochloride contains not less than 98.5 per cent and not more than the equivalent of 101.0 per cent of *N*-[2-[[[5-[(dimethylamino)methyl]furan-2-yl]methyl]sulphonyl]ethyl]-*N'*-methyl-2-nitroethene-1,1-diamine hydrochloride, calculated with reference to the dried substance.

CHARACTERS

A white or pale yellow, crystalline powder, freely soluble in water and in methanol, sparingly soluble in ethanol, very slightly soluble in methylene chloride.

It shows polymorphism.

IDENTIFICATION

First identification: B, D.

Second identification: A, C, D.

- Dissolve 10 mg in *water R* and dilute to 100.0 ml with the same solvent. Dilute 5.0 ml of the solution to 50.0 ml with *water R*. Examined between 220 nm and 360 nm (2.2.25), the solution shows two absorption maxima, at 229 nm and 315 nm. The ratio of the absorbance measured at the maximum at 229 nm to that measured at the maximum at 315 nm is 1.01 to 1.07.
- Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *ranitidine hydrochloride CRS*. Examine the substances as mulls in *liquid paraffin R*. If the spectra show differences, dissolve 20 mg of the substance to be examined and 20 mg of the reference substance separately in 5 ml of *methanol R*. Evaporate to dryness in a water-bath at 40 °C under reduced pressure and with constant stirring. Dry the residues under high vacuum at 60 °C for 1 h and record new spectra using the residues.
- Examine the chromatograms obtained in the test for related substances (see Tests). The principal spot in the chromatogram obtained with test solution (b) is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).
- It gives reaction (a) of chlorides (2.3.1).

TESTS

Solution S. Dissolve 1.0 g in *carbon dioxide-free water R* and dilute to 100.0 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and not more intensely coloured than reference solution BY₅ (2.2.2, *Method II*).

pH (2.2.3). The pH of solution S is 4.5 to 6.0.

Related substances. Examine by thin-layer chromatography (2.2.27), using *silica gel G R* as the coating substance.

Test solution (a). Dissolve 0.50 g of the substance to be examined in *methanol R* and dilute to 25 ml with the same solvent.

Test solution (b). Dilute 1 ml of test solution (a) to 10 ml with *methanol R*.

Reference solution (a). Dissolve 20 mg of *ranitidine hydrochloride CRS* in *methanol R* and dilute to 10 ml with the same solvent.

Reference solution (b). Dilute 3.0 ml of test solution (b) to 100 ml with *methanol R*.

Reference solution (c). Dilute 2.0 ml of test solution (b) to 100 ml with *methanol R*.

Reference solution (d). Dilute 1.0 ml of test solution (b) to 100 ml with *methanol R*.

Reference solution (e). Dilute 0.5 ml of test solution (b) to 100 ml with *methanol R*.

Reference solution (f). Dissolve 10 mg of *ranitidine impurity A CRS* in *methanol R* and dilute to 100 ml with the same solvent.

Reference solution (g). Dissolve 10 mg of *ranitidine impurity B CRS* in *methanol R* and dilute to 10 ml with the same solvent.

Reference solution (h). Dissolve 10 mg of *ranitidine impurity B CRS* in test solution (a) and dilute to 10 ml with the same solution.

Apply to the plate 10 µl of each solution. Develop over a path of 15 cm using a mixture of 2 volumes of *water R*, 4 volumes of *concentrated ammonia R1*, 15 volumes of *2-propanol R* and 25 volumes of *ethyl acetate R*. Allow the plate to dry in air and expose to iodine vapour until the spots are clearly visible. Examine in daylight. In the chromatogram obtained with test solution (a): any spot corresponding to ranitidine impurity A is not more intense than the spot in the chromatogram obtained with reference solution (f) (0.5 per cent); any spot apart from the principal spot and any spot corresponding to ranitidine impurity A is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.3 per cent); at most three such spots are more intense than the spot in the chromatogram obtained with reference solution (d) (0.1 per cent) and at most one of these is more intense than the spot in the chromatogram obtained with reference solution (c) (0.2 per cent). The test is not valid unless the chromatogram obtained with reference solution (h) shows two clearly separated spots, corresponding to ranitidine impurity B (whose R_f value is obtained from the chromatogram obtained with reference solution (g)) and ranitidine, and the chromatogram obtained with reference solution (e) shows a clearly visible spot.

Heavy metals (2.4.8). 1.0 g complies with limit test C for heavy metals (20 ppm). Prepare the standard using 2 ml of *lead standard solution (10 ppm Pb) R*.

Loss on drying (2.2.32). Not more than 0.75 per cent, determined on 1.000 g by drying under high vacuum at 60 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

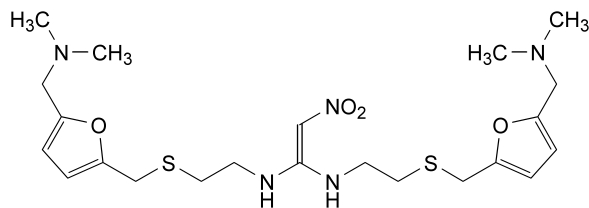
Dissolve 0.280 g in 35 ml of *water R*. Titrate with 0.1 M *sodium hydroxide*, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *sodium hydroxide* is equivalent to 35.09 mg of $C_{13}H_{23}ClN_4O_3S$.

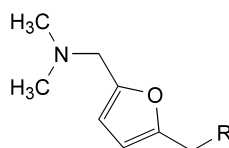
STORAGE

Store in an airtight container, protected from light.

IMPURITIES



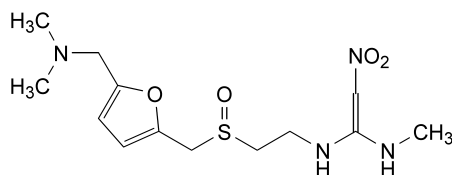
A. *N,N'*-bis[2-[[[5-[(dimethylamino)methyl]furan-2-yl]methyl]sulphonyl]ethyl]-2-nitroethene-1,1-diamine,



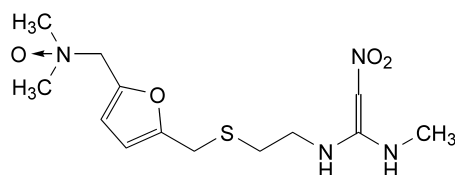
B. $R = S-CH_2-CH_2-NH_2$: 2-[[[5-[(dimethylamino)methyl]furan-2-yl]methyl]sulphonyl]ethanamine,

D. $R = S-CH_2-CH_2-NH-CO-CH_2-NO_2$: *N*-[2-[[[5-[(dimethylamino)methyl]furan-2-yl]methyl]sulphonyl]ethyl]-2-nitroacetamide,

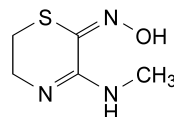
F. $R = OH$: [5-[(dimethylamino)methyl]furan-2-yl]methanol,



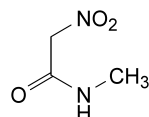
C. *N*-[2-[[[5-[(dimethylamino)methyl]furan-2-yl]methyl]sulphanyl]ethyl]-*N'*-methyl-2-nitroethene-1,1-diamine,



E. *N,N*-dimethyl[5-[[[2-[1-(methylamino)-2-nitroethenyl]amino]ethyl]sulphonyl]methyl]furan-2-yl]methanamine *N*-oxide,



G. 3-(methylamino)-5,6-dihydro-2H-1,4-thiazin-2-one oxime,



H. *N*-methyl-2-nitroacetamide.

01/2005:1369 IDENTIFICATION

RAPESEED OIL, REFINED

Rapae oleum raffinatum

DEFINITION

Refined rapeseed oil is the fatty oil obtained from the seeds of *Brassica napus* L. and *Brassica campestris* L. by mechanical expression or by extraction. It is then refined. A suitable antioxidant may be added.

CHARACTERS

A clear, light yellow liquid, practically insoluble in water and in alcohol, miscible with light petroleum (bp: 40 °C to 60 °C).
It has a relative density of about 0.917 and a refractive index of about 1.473.

IDENTIFICATION

Carry out the identification of fatty oils by thin-layer chromatography (2.3.2). The chromatogram obtained is similar to the typical chromatogram for rapeseed oil.

TESTS

- Acid value** (2.5.1). Not more than 0.5, determined on 10.0 g.
- Peroxide value** (2.5.5). Not more than 10.0.
- Unsaponifiable matter** (2.5.7). Not more than 1.5 per cent, determined on 5.0 g.
- Alkaline impurities** (2.4.19). It complies with the test for alkaline impurities in fatty oils.
- Composition of fatty acids** (2.4.22, Method A). The fatty acid fraction of the oil has the following composition:
- *palmitic acid*: 2.5 per cent to 6.0 per cent,
 - *stearic acid*: not more than 3.0 per cent,
 - *oleic acid*: 50.0 per cent to 67.0 per cent,
 - *linoleic acid*: 16.0 per cent to 30.0 per cent,
 - *linolenic acid*: 6.0 per cent to 14.0 per cent,
 - *eicosenoic acid*: not more than 5.0 per cent,
 - *erucic acid*: not more than 2.0 per cent.

STORAGE

Store in an airtight, well-filled container, protected from light.

LABELLING

- The label states:
- the name and concentration of any added antioxidant,
 - whether the oil is obtained by mechanical expression or by extraction.

- A. The petal is dark red to dark violet-brown, very thin, floppy, wrinkled, often crumpled into a ball and velvety to the touch. It is broadly ovate with an entire margin, about 6 cm long and 4 cm to 6 cm wide, narrowing at the base where there is a black spot. The vascular bundles radiate from the base and they anastomose in a continuous arc, all at the same short distance from the margin.
- B. Reduce to a powder (355). Examine under a microscope using *chloral hydrate solution R*. The powder is an intense reddish-pink and shows fragments of epidermis composed of elongated, sinuous-walled cells with small, rounded, anomocytic stomata (2.8.3), numerous vascular bundles with spiral vessels embedded in the parenchyma; occasional fragments of the fibrous layer of the anthers; rounded pollen grains, about 30 µm in diameter, with 3 pores and a finely verrucose exine.
- C. Thin-layer chromatography (2.2.27).
Test solution. To 1.0 g of the powdered drug (355) add 10 ml of *alcohol (60 per cent V/V) R*. Stir for 15 min. Filter through a filter paper.
Reference solution. Dissolve 1 mg of *quinaldine red R* and 1 mg of *sulphan blue R* in 2 ml of *methanol R*.
Plate: TLC silica gel plate R.
Mobile phase: *anhydrous formic acid R, water R, butanol R* (10:12:40 V/V/V).
Application: 10 µl, as bands.
Development: over a path of 10 cm.
Drying: in air.
Detection: examine in daylight.
Results: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other bands may be present in the chromatogram obtained with the test solution.

Top of the plate	
Quinaldine red: an orange-red zone	2 yellow zones
Sulphan blue: a blue zone	A violet principal zone A violet zone A yellow zone
	A compact group of violet zones
Reference solution	Test solution

TESTS

- Foreign matter** (2.8.2): maximum 2.0 per cent of capsules and maximum 1.0 per cent of other foreign matter.
- Loss on drying** (2.2.32): maximum 12.0 per cent, determined on 1.000 g of the powdered drug (355) by drying in an oven at 100-105 °C for 2 h.
- Total ash** (2.4.16): maximum 11.0 per cent.
- Colouring capacity.** Place 1.0 g of the powdered drug (355) in a 250 ml flask and add 100 ml of *ethanol (30 per cent V/V) R*. Allow to macerate for 4 h with frequent stirring. Filter and discard the first 10 ml. Transfer 10.0 ml of the filtrate to a 100 ml volumetric flask and add 2 ml of *hydrochloric acid R*. Dilute to 100.0 ml with *ethanol (30 per*

01/2005:1881

RED POPPY PETALS

Papaveris rhoeados flos

DEFINITION

Dried, whole or fragmented petals of *Papaver rhoeas* L.

CHARACTERS

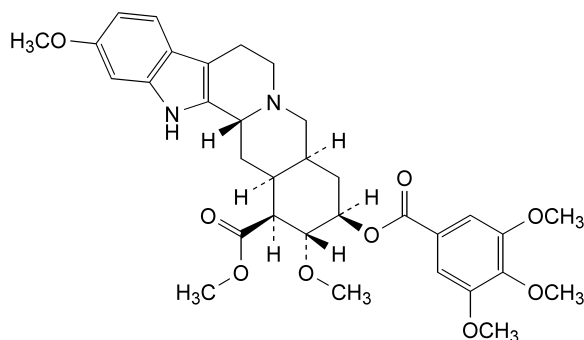
Macroscopic and microscopic characters described under identification tests A and B.

cent V/V) *R*. Allow to stand for 10 min. The absorbance (2.2.25) measured at 523 nm using *ethanol* (30 per cent V/V) *R* as compensation liquid is not less than 0.6.

01/2005:0528

RESERPINE

Reserpinum

C₃₃H₄₀N₂O₉M_r 609

DEFINITION

Reserpine contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of total alkaloids and not less than 98.0 per cent and not more than the equivalent of 102.0 per cent of methyl 11,17-α-dimethoxy-18β-[(3,4,5-trimethoxybenzoyl)oxy]-3β,20α-yohimban-16β-carboxylate, both calculated with reference to the dried substance.

CHARACTERS

A crystalline powder or small, white to slightly yellow crystals, darkening slowly on exposure to light, practically insoluble in water, very slightly soluble in alcohol.

IDENTIFICATION

First identification: B.

Second identification: A, C, D, E.

- Dissolve 20.0 mg in *chloroform R* and dilute to 10.0 ml with the same solvent. Dilute 1.0 ml of this solution to 100.0 ml with *alcohol R*. Examined immediately between 230 nm and 350 nm (2.2.25), the solution shows a maximum at 268 nm. The specific absorbance at the maximum is 265 to 285. Over the range 288 nm to 295 nm, the curve shows a slight absorption minimum followed by a shoulder or a slight absorption maximum; over this range, the specific absorbance is about 170.
- Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *reserpine CRS*. Examine the substances prepared as discs.
- To about 1 mg add 0.1 ml of a 1 g/l solution of *sodium molybdate R* in *sulphuric acid R*. A yellow colour is produced which becomes blue within 2 min.
- To about 1 mg add 0.2 ml of a freshly prepared 10 g/l solution of *vanillin R* in *hydrochloric acid R*. A pink colour develops within 2 min.
- Mix about 0.5 mg with 5 mg of *dimethylaminobenzaldehyde R* and 0.2 ml of *glacial acetic acid R* and add 0.2 ml of *sulphuric acid R*. A green colour is produced. Add 1 ml of *glacial acetic acid R*. The colour becomes red.

TESTS

Specific optical rotation (2.2.7). Carry out the determination immediately after preparing the solution. Dissolve 0.250 g in *chloroform R* and dilute to 25.0 ml with the same solvent. The specific optical rotation is –116 to –128, calculated with reference to the dried substance.

Oxidation products. Dissolve 20 mg in *glacial acetic acid R* and dilute to 100.0 ml with the same acid. The absorbance (2.2.25) measured immediately at 388 nm is not greater than 0.10.

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 0.500 g by drying at 60 °C over *diphosphorus pentoxide R* at a pressure not exceeding 667 Pa for 3 h.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 0.5 g.

ASSAY

Total alkaloids. Dissolve 0.500 g in a mixture of 6 ml of *acetic anhydride R* and 40 ml of *anhydrous acetic acid R*. Titrate with 0.1 M *perchloric acid*, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *perchloric acid* is equivalent to 60.9 mg of total alkaloids.

Reserpine. Protect the solutions from light. Moisten 25.0 mg with 2 ml of *alcohol R*, add 2 ml of 0.25 M *sulphuric acid* and 10 ml of *alcohol R*, and warm gently to effect solution. Cool and dilute to 100.0 ml with *alcohol R*. Dilute 5.0 ml of this solution to 50.0 ml with *alcohol R*. Prepare a reference solution in the same manner using 25.0 mg of *reserpine CRS*. Place 10.0 ml of each solution separately in two boiling-tubes, add 2.0 ml of 0.25 M *sulphuric acid* and 2.0 ml of a freshly prepared 3 g/l solution of *sodium nitrite R*. Mix and heat in a water-bath at 55 °C for 35 min. Cool, add 1.0 ml of a freshly prepared 50 g/l solution of *sulphamic acid R* and dilute to 25.0 ml with *alcohol R*. Measure the absorbance (2.2.25) of each solution at the maximum at 388 nm, using as the compensation liquid 10.0 ml of the same solution treated at the same time in the same manner, but omitting the sodium nitrite.

Calculate the content of C₃₃H₄₀N₂O₉ from the absorbances measured and the concentrations of the solutions.

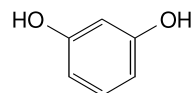
STORAGE

Store protected from light.

01/2005:0290

RESORCINOL

Resorcinolum

C₆H₆O₂M_r 110.1

DEFINITION

Resorcinol contains not less than 98.5 per cent and not more than the equivalent of 101.0 per cent of benzene-1,3-diol, calculated with reference to the dried substance.

CHARACTERS

A colourless or slightly pinkish-grey, crystalline powder or crystals, turning red on exposure to light and air, very soluble in water and in alcohol.

IDENTIFICATION

01/2005:1879

- A. Melting point (2.2.14): 109 °C to 112 °C.
- B. Dissolve 0.1 g in 1 ml of *water R*, add 1 ml of *strong sodium hydroxide solution R* and 0.1 ml of *chloroform R*, heat and allow to cool. An intense, deep-red colour develops which becomes pale yellow on the addition of a slight excess of *hydrochloric acid R*.
- C. Thoroughly mix about 10 mg with about 10 mg of *potassium hydrogen phthalate R*, both finely powdered. Heat over a naked flame until an orange-yellow colour is obtained. Cool and add 1 ml of *dilute sodium hydroxide solution R* and 10 ml of *water R* and shake to dissolve. The solution shows an intense green fluorescence.

TESTS

Solution S. Dissolve 2.5 g in *carbon dioxide-free water R* and dilute to 25 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and not more intensely coloured than reference solution B₅ or R₅ (2.2.2, *Method II*) and remains so when heated in a water-bath for 5 min.

Acidity or alkalinity. To 10 ml of solution S add 0.05 ml of *bromophenol blue solution R2*. Not more than 0.05 ml of 0.1 M *hydrochloric acid* or 0.1 M *sodium hydroxide* is required to change the colour of the indicator.

Related substances. Examine by thin-layer chromatography (2.2.27), using *silica gel G R* as the coating substance.

Test solution. Dissolve 0.5 g of the substance to be examined in *methanol R* and dilute to 10 ml with the same solvent.

Reference solution. Dilute 0.1 ml of the test solution to 20 ml with *methanol R*.

Apply separately to the plate 2 µl of each solution. Develop over a path of 15 cm using a mixture of 40 volumes of *ethyl acetate R* and 60 volumes of *hexane R*. Allow the plate to dry in air for 15 min and expose it to iodine vapour. Any spot in the chromatogram obtained with the test solution, apart from the principal spot, is not more intense than the spot in the chromatogram obtained with the reference solution (0.5 per cent).

Pyrocatechol. To 2 ml of solution S add 1 ml of *ammonium molybdate solution R2* and mix. Any yellow colour in the solution is not more intense than that in a standard prepared at the same time in the same manner using 2 ml of a 0.1 g/l solution of *pyrocatechol R*.

Loss on drying (2.2.32). Not more than 1.0 per cent, determined on 1.00 g of powdered substance by drying in a desiccator for 4 h.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.500 g in *water R* and dilute to 250.0 ml with the same solvent. To 25.0 ml of the solution in a ground-glass-stoppered flask add 1.0 g of *potassium bromide R*, 50.0 ml of 0.0167 M *potassium bromate*, 15 ml of *chloroform R* and 15.0 ml of *hydrochloric acid R1*. Stopper the flask, shake and allow to stand in the dark for 15 min, shaking occasionally. Add 10 ml of a 100 g/l solution of *potassium iodide R*, shake thoroughly, allow to stand for 5 min and titrate with 0.1 M *sodium thiosulphate*, using 1 ml of *starch solution R* as indicator.

1 ml of 0.0167 M *potassium bromate* is equivalent to 1.835 mg of C₆H₆O₂.

STORAGE

Store protected from light.

RESTHARROW ROOT

Ononidis radix

DEFINITION

Whole or cut, dried root of *Ononis spinosa* L.

CHARACTERS

Macroscopic and microscopic characters described under identification tests A and B.

IDENTIFICATION

A. The root is more or less flattened, twisted and branched, deeply wrinkled, brown in colour and grooved longitudinally. The transversely cut surface shows a thin bark and a xylem cylinder with a conspicuously radiate structure. The fracture of the root is short and fibrous.

B. Reduce to a powder (355). The powder is light brown or brown. Examine under a microscope using *chloral hydrate solution R*. The powder shows brown fragments of cork composed of thin-walled polygonal cells; groups of thick-walled narrow fibres, often accompanied by a parenchymatous crystal sheath containing prisms of calcium oxalate; fragments of vessels with numerous small bordered pits; parenchymatous cells with single prisms of calcium oxalate. Examine under a microscope using a mixture of equal volumes of *glycerol R* and *water R*. The powder shows numerous simple, round starch granules, 5 µm to 10 µm in diameter.

C. Thin-layer chromatography (2.2.27).

Test solution. To 1.0 g of the powdered drug (180) add 15.0 ml of *methanol R* and boil under a reflux condenser for 30 min. Cool and filter.

Reference solution. Dissolve 10 mg of *resorcinol R* and 50 mg of *vanillin R* in 10 ml of *methanol R*.

Plate: TLC silica gel F₂₅₄ plate R.

Mobile phase: *alcohol R*, *methylene chloride R*, *toluene R* (10:45:45 V/V/V).

Application: 20 µl, as bands.

Development: over a path of 15 cm.

Drying: in air.

Detection A: examine in ultraviolet light at 254 nm and 365 nm.

Results A: see below the sequence of the zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other fluorescent zones are present in the middle third of the chromatogram obtained with the test solution.

Top of the plate	
Vanillin: a zone visible at 254 nm	
Resorcinol: a zone visible at 254 nm	An intense blue fluorescent zone visible at 365 nm
Reference solution	Test solution

Detection B: spray with *anisaldehyde solution R*. Heat at 100-105 °C for 5-10 min. Examine in daylight.

Results B: see below the sequence of the zones present in the chromatograms obtained with the reference solution and the test solution.

Top of the plate	
Vanillin: a greyish-violet zone	A violet zone (onocol)
Resorcinol: a red zone	
Reference solution	Test solution

TESTS

Extractable matter: minimum 15.0 per cent.

To 2.00 g of the powdered drug (250) add a mixture of 8 g of *water R* and 12 g of *alcohol R* and allow to macerate for 2 h, shaking frequently. Filter, evaporate 5 g of the filtrate to dryness on a water-bath and dry at 100-105 °C for 2 h. The residue weighs a minimum of 75 mg.

Foreign matter (2.8.2). It complies with the test for foreign matter.

Loss on drying (2.2.32): maximum 10.0 per cent, determined on 1.000 g of the powdered drug (355) by drying in an oven at 100-105 °C for 2 h.

Total ash (2.4.16): maximum 8.0 per cent.

01/2005:0289

RHATANY ROOT

Ratanhiae radix

DEFINITION

Rhatany root, known as Peruvian rhatany, consists of the dried, usually fragmented, underground organs of *Krameria triandra* Ruiz and Pavon. It contains not less than 5.0 per cent of tannins, expressed as pyrogallol ($C_6H_6O_3$; M_r 126.1), calculated with reference to the dried drug.

CHARACTERS

It has the macroscopic and microscopic characters described under identification tests A and B.

IDENTIFICATION

A. The taproot is dark red-brown and has a thick, knotty crown. The secondary roots are the same colour and nearly straight or somewhat tortuous. The bark is rugged to scaly in the older pieces and smooth with sharp, transverse fissures in the younger pieces; it separates readily from the wood. The fracture is fibrous in the bark and splintery in the wood. The smooth, transversely cut surface shows a dark brown-red bark about one third of the radius in thickness; a dense, pale red-brown and finely porous wood is present with numerous fine medullary rays; the central heartwood is often darker.

B. Reduce to a powder (355). The powder is brown-red. Examine under a microscope using *chloral hydrate solution R*. The powder shows cork cells containing dark brown phlobaphenes; fragments of unlignified phloem fibres, usually 12 µm to 30 µm in diameter with moderately thick walls; phloem parenchyma cells in files containing prisms and microcrystals of calcium oxalate; fragments of vessels usually 20 µm to 60 µm in diameter with bordered pits; fragments of tracheids up to 20 µm wide with slit-shaped pits. Examine under a microscope using a 50 per cent *V/V* solution of *glycerol R*. The powder shows rounded starch granules, simple or two-

four-compound, an individual granule measuring up to 30 µm in diameter and some granules being found in the cells of the medullary rays and in the parenchyma.

C. Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel plate R*.

Test solution. To 1.0 g of the powdered drug (355) add 10 ml of a mixture of 3 volumes of *water R* and 7 volumes of *alcohol R*, shake for 10 min and filter. To the filtrate add 10 ml of *light petroleum R* and shake. Separate the light petroleum layer, add 2 g of *anhydrous sodium sulphate R*, shake and filter. Evaporate the filtrate to dryness. Dissolve the residue in 0.5 ml of *methanol R*.

Reference solution. Dissolve 5.0 mg of *Sudan red G R* in 10 ml of *methanol R*.

Apply to the plate as bands 10 µl of each solution. Develop over a path of 15 cm using a mixture of 2 volumes of *ethyl acetate R* and 98 volumes of *toluene R*. Allow the plate to dry in air and spray the plate with a 5 g/l solution of *fast blue B salt R*. Allow the plate to dry in air and spray the plate with 0.1 *M* *ethanolic sodium hydroxide*. Examine in daylight. The chromatogram obtained with the reference solution shows in the lower third a red zone due to Sudan red G. The chromatogram obtained with the test solution shows a violet zone due to rhatany phenol I similar in position to the zone of Sudan red G in the chromatogram obtained with the reference solution, below it the brownish zone due to rhatany phenol II and below it the bluish-grey zone due to rhatany phenol III. Further zones may be present.

TESTS

Foreign matter (2.8.2). Not more than 2 per cent of foreign matter and not more than 5 per cent of fragments of crown or root exceeding 25 mm in diameter. Root without bark may be present in very small quantities.

Loss on drying (2.2.32). Not more than 12.0 per cent, determined on 1.000 g of the powdered drug (355) by drying in an oven at 100 °C to 105 °C.

Total ash (2.4.16). Not more than 5.5 per cent.

ASSAY

Carry out the determination of tannins in herbal drugs (2.8.14). Use 0.750 g of powdered drug (180).

STORAGE

Store protected from light.

01/2005:1888

RHATANY TINCTURE

Ratanhiae tinctura

DEFINITION

Tincture produced from *Rhatany root* (0289).

Content: minimum 1.0 per cent *m/m* of tannins, expressed as pyrogallol ($C_6H_6O_3$; M_r 126.1).

PRODUCTION

The tincture is produced from 1 part of the drug and 5 parts of ethanol (70 per cent *V/V*) by a suitable procedure.

CHARACTERS

Appearance: reddish-brown liquid.

IDENTIFICATION

Thin-layer chromatography (2.2.27).

Test solution. To 5 ml of the tincture to be examined, add 10 ml of *light petroleum R* and shake. Separate the light petroleum layer, add 2 g of *anhydrous sodium sulphate R*, shake and filter. Evaporate the filtrate to dryness. Dissolve the residue in 0.5 ml of *methylene chloride R*.

Reference solution. Dissolve 5 mg of *thymol R* and 10 mg of *dichlorophenolindophenol, sodium salt R* in 10 ml of *alcohol (60 per cent V/V) R*.

Plate: *TLC silica gel plate R*.

Mobile phase: *methylene chloride R*.

Application: 10 µl, as bands.

Development: over a path of 10 cm.

Drying: in air.

Detection: spray with a 5 g/l solution of *fast blue B salt R*; allow the plate to dry in air and spray with 0.1 M *ethanolic sodium hydroxide*; examine in daylight.

Results: see below the sequence of the zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other zones may be present in the chromatogram obtained with the test solution.

Top of the plate	
Thymol: an orange brownish-yellow zone	A violet zone
	A greenish-grey zone
	A bluish-grey zone
	A yellowish-brown zone
Dichlorophenolindophenol: a greyish-blue zone	A violet zone
Reference solution	Test solution

TESTS

Ethanol (2.9.10): 63 per cent V/V to 67 per cent V/V.

Methanol and 2-propanol (2.9.11): maximum 0.05 per cent V/V of methanol and maximum 0.05 per cent V/V of 2-propanol.

ASSAY

Carry out the determination of tannins in herbal drugs (2.8.14) using 2.500 g of the tincture to be examined.

01/2005:0291

RHUBARB

Rhei radix

DEFINITION

Rhubarb consists of the whole or cut, dried underground parts of *Rheum palmatum* L. or of *Rheum officinale* Baillon or of hybrids of these two species or of a mixture. The underground parts are often divided; the stem and most of the bark with the rootlets are removed. It contains not less than 2.2 per cent of hydroxyanthracene derivatives, expressed as rhein (C₁₅H₈O₆, M_r 284.2), calculated with reference to the dried drug.

CHARACTERS

Rhubarb has a characteristic, aromatic odour.

It has the macroscopic and microscopic characters described under identification tests A and B.

IDENTIFICATION

A. The appearance is variable: disc-shaped pieces up to 10 cm in diameter and 1 cm to 5 cm in thickness; cylindrical pieces; oval or planoconvex pieces. The surface has a pinkish tinge and is usually covered with a layer of brownish-yellow powder. It shows, especially after moistening, a reticulum of darker lines. This structure causes the marbled appearance of the drug. The fracture is granular. The transverse section of the rhizome shows a narrow outer zone of radiating brownish-red lines. These medullary rays are crossed perpendicularly by a dark cambial ring. Inside this zone is a ring of small star-spot formations of anomalous vascular bundles. The root shows a more radiate structure.

B. Reduce to a powder (355). The powder is orange to brownish-yellow. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters: large calcium oxalate cluster crystals, which may measure more than 100 µm, and their fragments; reticulately thickened non-lignified vessels measuring up to 175 µm. Numerous groups of rounded or polygonal, thin-walled parenchyma cells. Sclereids and fibres are absent. Examine under a microscope using a 50 per cent V/V solution of *glycerol R*. The powder shows simple, rounded or compound (2 to 4) starch granules with a star-shaped hilum.

C. Examine by thin-layer chromatography (2.2.27), using a suitable silica gel as the coating substance.

Test solution. Heat 50 mg of the powdered drug (180) in a water-bath for 15 min with a mixture of 1 ml of *hydrochloric acid R* and 30 ml of *water R*. Allow to cool and shake the liquid with 25 ml of *ether R*. Dry the ether layer over *anhydrous sodium sulphate R* and filter. Evaporate the ether layer to dryness and dissolve the residue in 0.5 ml of *ether R*.

Reference solution. Dissolve 5 mg of *emodin R* in 5 ml of *ether R*.

Apply separately to the plate as bands 20 µl of each solution. Develop over a path of 10 cm using a mixture of 1 volume of *anhydrous formic acid R*, 25 volumes of *ethyl acetate R* and 75 volumes of *light petroleum R*. Allow the plate to dry in air and examine in ultraviolet light at 365 nm. The chromatogram obtained with the reference solution shows in its central part a zone of orange fluorescence (emodin). The chromatogram obtained with the test solution shows: a zone due to emodin; above the emodin zone, two zones of similar fluorescence (physcione and chrysophanol, in order of increasing R_f value); below the emodin zone, also two zones of similar fluorescence (rhein and aloe-emodin, in order of decreasing R_f value). Spray with a 100 g/l solution of *potassium hydroxide R* in *methanol R*. All the zones become red to violet.

D. To about 50 mg of the powdered drug (180) add 25 ml of *dilute hydrochloric acid R* and heat the mixture on a water-bath for 15 min. Allow to cool, shake with 20 ml of *ether R* and discard the aqueous layer. Shake the ether layer with 10 ml of *dilute ammonia R1*. The aqueous layer becomes red to violet.

TESTS

Rheum rhaponticum. Examine by thin-layer chromatography (2.2.27), using *silica gel G R* as the coating substance.

Test solution. To 0.2 g of the powdered drug (180) add 2 ml of *methanol R* and boil for 5 min under a reflux condenser. Allow to cool and filter. Use the filtrate as the test solution.

Reference solution. Dissolve 10 mg of *rhaponticin R* in 10 ml of *methanol R*.

01/2005:2109

Apply separately to the plate, as bands not more than 20 mm by 3 mm, 20 µl of each solution. Develop over a path of 12 cm using a mixture of 20 volumes of *methanol R* and 80 volumes of *methylene chloride R*. Allow the plate to dry in air and spray with *phosphomolybdic acid solution R*. The chromatogram obtained with the test solution does not show a blue zone near the starting-line (rhaponticin) corresponding to the zone in the chromatogram obtained with the reference solution.

Foreign matter (2.8.2). It complies with the test for foreign matter.

Loss on drying (2.2.32). Not more than 12.0 per cent, determined on 1.000 g of the powdered drug (180) by drying in an oven at 100 °C to 105 °C.

Total ash (2.4.16). Not more than 12.0 per cent.

Ash insoluble in hydrochloric acid (2.8.1). Not more than 2.0 per cent.

ASSAY

Carry out the assay protected from bright light.

Introduce 0.100 g of the powdered drug (180) into a 100 ml flask. Add 30.0 ml of *water R*, mix and weigh. Heat in a water-bath under a reflux condenser for 15 min. Allow to cool, add 50 mg of *sodium hydrogen carbonate R*, weigh and adjust to the original mass with *water R*. Centrifuge and transfer 10.0 ml of the liquid to a 100 ml round-bottomed flask with a ground-glass neck. Add 20 ml of *ferric chloride solution RI* and mix. Heat under a reflux condenser on a water-bath for 20 min, add 1 ml of *hydrochloric acid R* and heat for a further 20 min, shaking frequently. Cool, transfer to a separating funnel and shake with three quantities, each of 25 ml, of *ether R* previously used to rinse the flask. Combine the ether extracts and wash with two quantities, each of 15 ml, of *water R*. Filter the ether extracts through a plug of absorbent cotton into a volumetric flask and dilute to 100.0 ml with *ether R*. Evaporate 10.0 ml carefully to dryness on a water-bath and dissolve the residue in 10.0 ml of a 5 g/l solution of *magnesium acetate R* in *methanol R*. Measure the absorbance (2.2.25) at 515 nm, using *methanol R* as the compensation liquid.

Calculate the percentage content of rhein from the expression:

$$\frac{A \times 0.64}{m}$$

i.e. taking the specific absorbance of rhein to be 468, calculated on the basis of the specific absorbance of barbaloin.

A = absorbance at 515 nm,

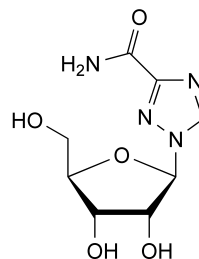
m = mass of the drug used, in grams.

STORAGE

Store protected from light.

RIBAVIRIN

Ribavirinum


 $C_8H_{12}N_4O_5$
 M_r 244.2

DEFINITION

1-β-D-Ribofuranosyl-1H-1,2,4-triazole-3-carboxamide.

Content: 98.0 per cent to 102.0 per cent (dried substance).

CHARACTERS

Appearance: white or almost white, crystalline powder.

Solubility: freely soluble in water, slightly soluble in ethanol (96 per cent), slightly soluble or very slightly soluble in methylene chloride.

It shows polymorphism.

IDENTIFICATION

Infrared absorption spectrophotometry (2.2.24).

Comparison: *ribavirin CRS*.

If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in *methylene chloride R*, evaporate to dryness and record new spectra using the residues.

TESTS

pH (2.2.3): 4.0 to 6.5.

Dissolve 0.200 g in *carbon dioxide-free water R* and dilute to 10.0 ml with the same solvent.

Specific optical rotation (2.2.7): –33 to –37 (dried substance).

Dissolve 0.250 g in *water R* and dilute to 25.0 ml with the same solvent. Determine the specific optical rotation within 10 min of preparing the solution.

Related substances. Liquid chromatography (2.2.29).

Test solution. Dissolve 25.0 mg of the substance to be examined in the mobile phase and dilute to 100.0 ml with the mobile phase. Dilute 10.0 ml of the solution to 100.0 ml with the mobile phase.

Reference solution (a). Dilute 1.0 ml of the test solution to 100.0 ml with the mobile phase. Dilute 1.0 ml of this solution to 10.0 ml with the mobile phase.

Reference solution (b). Dissolve the contents of a vial of *ribavirin for system suitability CRS* in 2.0 ml of the mobile phase.

Reference solution (c). Dissolve 25.0 mg of *ribavirin CRS* in the mobile phase and dilute to 100.0 ml with the mobile phase. Dilute 10.0 ml of the solution to 100.0 ml with the mobile phase.

Column:

- **size:** $l = 0.10$ m, $\varnothing = 7.8$ mm,
- **stationary phase:** *strong cation-exchange resin R* (9 µm),
- **temperature:** 40 °C.

Mobile phase: water R adjusted to pH 2.5 with sulphuric acid R.

Flow rate: 1 ml/min.

Detection: spectrophotometer at 207 nm.

Injection: 10 µl of the test solution and reference solutions (a) and (b).

Run time: 11 times the retention time of ribavirin.

Relative retention with reference to ribavirin (retention time = about 4 min): impurity F = about 1.2.

System suitability: reference solution (b):

- **peak-to-valley ratio:** minimum 1.2, where H_p = height above the baseline of the peak due to impurity F and H_v = height above the baseline of the lowest point of the curve separating this peak from the peak due to ribavirin.

Limits:

- **impurity F:** not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent),
- **any other impurity:** for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent),
- **total:** not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent),
- **disregard limit:** 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

Heavy metals (2.4.8): maximum 10 ppm.

Dissolve 4.0 g in 20 ml of water R. 12 ml of the solution complies with limit test A. Prepare the reference solution using 10 ml of lead standard solution (2 ppm Pb) R.

Loss on drying (2.2.32): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 100–105 °C for 5 h.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (2.2.29) as described in the test for related substances with the following modification.

Injection: test solution and reference solution (c).

Calculate the percentage content of $C_8H_{12}N_4O_5$ using the declared content of $C_8H_{12}N_4O_5$ in ribavirin CRS.

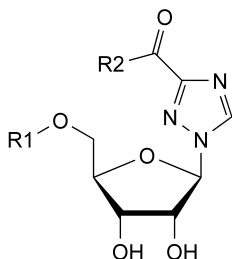
STORAGE

Protected from light.

IMPURITIES

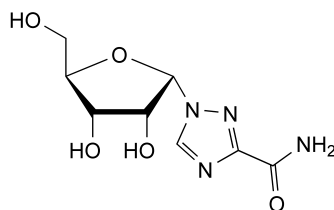
Specified impurities: F.

Other detectable impurities: A, B, C, D, E, G.

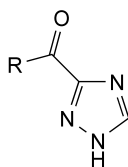


- A. R1 = H, R2 = OH: 1-β-D-ribofuranosyl-1H-1,2,4-triazole-3-carboxylic acid,
- E. R1 = CO-C₆H₅, R2 = NH₂: 1-(5-O-benzoyl-β-D-ribofuranosyl)-1H-1,2,4-triazole-3-carboxamide (5'-O-benzoylribavirin),

- F. R1 = CO-CH₃, R2 = NH₂: 1-(5-O-acetyl-β-D-ribofuranosyl)-1H-1,2,4-triazole-3-carboxamide (5'-O-acetylribavirin),

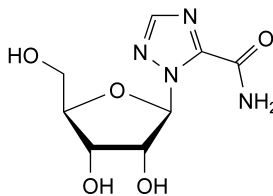


- B. 1-α-D-ribofuranosyl-1H-1,2,4-triazole-3-carboxamide (anomer),



- C. R = OH: 1H-1,2,4-triazole-3-carboxylic acid,

- D. R = NH₂: 1H-1,2,4-triazole-3-carboxamide,

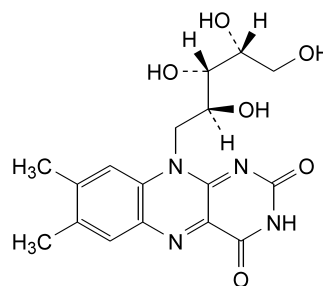


- G. 1-β-D-ribofuranosyl-1H-1,2,4-triazole-5-carboxamide (N-isomer).

01/2005:0292

RIBOFLAVIN

Riboflavinum



$C_{17}H_{20}N_4O_6$

M_r 376.4

DEFINITION

7,8-Dimethyl-10-[(2S,3S,4R)-2,3,4,5-tetrahydroxypentyl]benzo[g]pteridine-2,4(3H,10H)-dione.

Contents: 97.0 per cent to 103.0 per cent (dried substance).

CHARACTERS

Appearance: yellow or orange-yellow, crystalline powder.

Solubility: very slightly soluble in water, practically insoluble in alcohol.

Solutions deteriorate on exposure to light, especially in the presence of alkali.

It shows polymorphism.

IDENTIFICATION

- A. It complies with the test for specific optical rotation (see Tests).

- B. Examine the chromatogram obtained in the test for lumiflavin.

Results: the principal spot in the chromatogram obtained with test solution (a) is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a).

- C. Dissolve about 1 mg in 100 ml of *water R*. The solution has, by transmitted light, a pale greenish-yellow colour, and, by reflected light, an intense yellowish-green fluorescence which disappears on the addition of mineral acids or alkalis.

TESTS

Specific optical rotation (2.2.7): –115 to –135 (dried substance).

Dissolve 50.0 mg in 0.05 M *sodium hydroxide* free from carbonate and dilute to 10.0 ml with the same alkaline solution. Measure the optical rotation within 30 min of dissolution.

Absorbance (2.2.25). Dilute the final solution prepared for the assay with an equal volume of *water R*. The solution shows 4 maxima, at 223 nm, 267 nm, 373 nm and 444 nm. The ratio of the absorbance at the maximum at 373 nm to that at the maximum at 267 nm is 0.31 to 0.33, and the ratio of the absorbance at the maximum at 444 nm to that at the maximum at 267 nm is 0.36 to 0.39.

Lumiflavin. Thin-layer chromatography (2.2.27).

Test solution (a). Suspend 25 mg in 10 ml of *water R*; shake for 5 min and filter the suspension to remove the undissolved material.

Test solution (b). Shake 25 mg of the substance to be examined with 10.0 ml of *methylene chloride R* and filter the suspension to remove the undissolved material.

Reference solution (a). Suspend 25 mg of *riboflavin CRS* in 10 ml of *water R*; shake for 5 min and filter the suspension to remove the undissolved material.

Reference solution (b). Dissolve 25 mg of *lumiflavin R* in *methylene chloride R* and dilute to 50.0 ml with the same solvent. Dilute 1.0 ml of the solution to 20.0 ml with *methylene chloride R*.

Reference solution (c). Dilute 2.5 ml of reference solution (b) to 100.0 ml with *methylene chloride R*.

Plate: *TLC silica gel plate R* (2–10 µm).

Mobile phase: *water R*.

Application: as follows, dry in a current of cold air after each individual application:

- 1st application: 2 µl of *methylene chloride R* followed by 2 µl of test solution (a),
- 2nd application: 2 µl of *methylene chloride R* followed by 2 µl of reference solution (a),
- 3rd application: 2 µl of reference solution (b) followed by 2 µl of reference solution (a),
- 4th application: 10 µl of test solution (b),
- 5th application: 10 µl of reference solution (c).

Development: over a path of 6 cm.

Drying: in a current of cold air.

Detection: examine in ultraviolet light at 365 nm.

System suitability: the chromatogram obtained with the 3rd application shows 2 clearly separated spots.

Limit: in the chromatogram obtained with test solution (b):

- *lumiflavin*: any spot corresponding to lumiflavin is not more intense than the principal spot in the chromatogram obtained with reference solution (c) (0.025 per cent).

Loss on drying (2.2.32): maximum 1.5 per cent, determined on 1.000 g by drying in an oven at 100–105 °C.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on the residue obtained in the test for loss on drying.

ASSAY

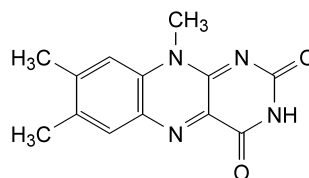
Carry out the assay protected from light.

In a brown-glass 500 ml volumetric flask, suspend 65.0 mg in 5 ml of *water R* ensuring that it is completely wetted and dissolve in 5 ml of *dilute sodium hydroxide solution R*. As soon as dissolution is complete, add 100 ml of *water R* and 2.5 ml of *glacial acetic acid R* and dilute to 500.0 ml with *water R*. Place 20.0 ml of this solution in a 200 ml brown-glass volumetric flask, add 3.5 ml of a 14 g/l solution of *sodium acetate R* and dilute to 200.0 ml with *water R*. Measure the absorbance (2.2.25) at the maximum at 444 nm. Calculate the content of C₁₇H₂₀N₄O₆ taking the specific absorbance to be 328.

STORAGE

In an airtight container, protected from light.

IMPURITIES

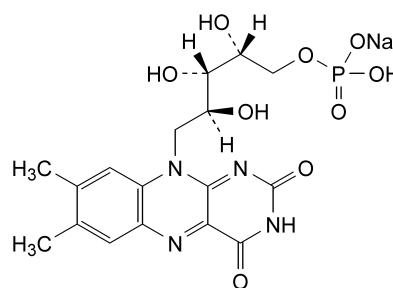


- A. 7,8,10-trimethylbenzo[*g*]pteridine-2,4(3*H*,10*H*)-dione (lumiflavin).

01/2005:0786

RIBOFLAVIN SODIUM PHOSPHATE

Riboflavini natrii phosphas



C₁₇H₂₀N₄NaO₉P

*M*_r 478.3

DEFINITION

Riboflavin sodium phosphate is a mixture containing riboflavin 5'-(sodium hydrogen phosphate) as the main component and other riboflavin sodium monophosphates. It contains not less than 73.0 per cent and not more than 79.0 per cent of riboflavin (C₁₇H₂₀N₄O₆; *M*_r 376.4), calculated with reference to the dried substance. It contains a variable amount of water.

CHARACTERS

A yellow or orange-yellow, crystalline powder, hygroscopic, soluble in water, very slightly soluble in alcohol.

IDENTIFICATION

- A. Dissolve 50.0 mg in *phosphate buffer solution pH 7.0 R* and dilute to 100.0 ml with the same buffer solution. Dilute 2.0 ml of the solution to 100.0 ml with *phosphate*

buffer solution pH 7.0 R. Examined between 230 nm and 350 nm (2.2.25), the solution shows an absorption maximum at 266 nm. The specific absorbance at the maximum is 580 to 640.

- B. Examine the chromatograms obtained in the test for related substances. The principal peak in the chromatogram obtained with the test solution is similar in position and approximate size to the principal peak in the chromatogram obtained with reference solution (b).
- C. Dissolve about 10 mg in *dilute sodium hydroxide solution R* and dilute to 100 ml with the same solution. Expose 1 ml to ultraviolet light at 254 nm for 5 min, add sufficient *acetic acid R* to make the solution acidic to *blue litmus paper R* and shake with 2 ml of *methylene chloride R*. The lower layer shows yellow fluorescence.
- D. To 0.5 g add 10 ml of *nitric acid R*, evaporate the mixture to dryness on a water-bath. Ignite the residue until it becomes white, dissolve the residue in 5 ml of *water R* and filter. The filtrate gives reaction (a) of sodium and reaction (b) of phosphates (2.3.1).

TESTS

pH (2.2.3). Dissolve 0.5 g in *carbon dioxide-free water R* and dilute to 50 ml with the same solvent. The pH of the solution is 5.0 to 6.5.

Specific optical rotation (2.2.7). Dissolve 0.300 g in 18.2 ml of *hydrochloric acid R1* and dilute to 25.0 ml with *water R*. The specific optical rotation is + 38.0 to + 43.0, calculated with reference to the dried substance.

Lumiflavin. To about 35 mg add 10 ml of *methylene chloride R*, shake for 5 min and filter. The filtrate is not more intensely coloured than reference solution BY₆ (2.2.2, Method II).

Related substances. Examine by liquid chromatography (2.2.29). Carry out the test protected from actinic light.

Test solution. Dissolve 0.100 g of the substance to be examined in 50 ml of *water R* and dilute to 100.0 ml with the mobile phase. Dilute 8.0 ml of this solution to 50.0 ml with the mobile phase.

Reference solution (a). Dissolve 60 mg of *riboflavin CRS* in 1 ml of *hydrochloric acid R* and dilute to 250.0 ml with *water R*. Dilute 4.0 ml to 100.0 ml with the mobile phase.

Reference solution (b). Dissolve 0.100 g of *riboflavin sodium phosphate CRS* in 50 ml of *water R* and dilute to 100.0 ml with the mobile phase. Dilute 8.0 ml of this solution to 50.0 ml with the mobile phase.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4.6 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (5 µm),
- as mobile phase at a flow rate of 2 ml/min a mixture of 150 volumes of *methanol R* and 850 volumes of a 7.35 g/l solution of *potassium dihydrogen phosphate R*,
- as detector a spectrophotometer set at 266 nm.

When the chromatograms are recorded in the prescribed conditions, the retention time of riboflavin 5'-monophosphate is about 20 min and the relative retention times are: riboflavin 3',4'-diphosphate about 0.2, riboflavin 3',5'-diphosphate about 0.3, riboflavin 4',5'-diphosphate about 0.5, riboflavin 3'-monophosphate about 0.7, riboflavin 4'-mono-phosphate about 0.9, riboflavin 5'-monophosphate 1, riboflavin about 2.

Inject 100 µl of reference solution (a). Adjust the sensitivity of the system so that the height of the principal peak in the chromatogram obtained is at least 50 per cent of the full scale of the recorder. Inject 100 µl of reference solution (b). Continue the chromatography until the peak corresponding to riboflavin can be clearly evaluated. The test is not valid unless in the chromatogram obtained with reference solution (b), the resolution between the peaks corresponding to riboflavin 4'-monophosphate and riboflavin 5'-monophosphate is at least 1.5.

Inject 100 µl of the test solution, 100 µl of reference solution (a) and 100 µl of reference solution (b). Calculate the percentage content of free riboflavin and of riboflavin in the form of the diphosphates of riboflavin from the areas of the peaks in the chromatogram obtained with the test solution and the amount of free riboflavin in reference solution (a). The content of free riboflavin is not greater than 6.0 per cent and the content of riboflavin in the form of the diphosphates of riboflavin is not greater than 6.0 per cent, both calculated with reference to dried material.

Inorganic phosphate. Dissolve 0.10 g in *water R* and dilute to 100 ml with the same solvent. To 5 ml of the solution, add 10 ml of *water R*, 5 ml of *buffered copper sulphate solution pH 4.0 R*, 2 ml of a 30 g/l solution of *ammonium molybdate R*, 1 ml of a freshly prepared solution containing 20 g/l of *4-methylaminophenol sulphate R* and 50 g/l of *sodium metabisulphite R* and 1 ml of a 3 per cent V/V solution of *perchloric acid R*. Dilute to 25.0 ml with *water R* and measure, within 15 min of its preparation, the absorbance of the solution at 800 nm (2.2.25) using as the compensation liquid a solution prepared in the same manner but without the substance to be examined. The absorbance is not greater than that of a solution prepared as follows: to 15 ml of *phosphate standard solution (5 ppm PO₄) R*, add 5 ml of *buffered copper sulphate solution pH 4.0 R*, 2 ml of a 30 g/l solution of *ammonium molybdate R*, 1 ml of a freshly prepared solution containing 20 g/l of *4-methylaminophenol sulphate R* and 50 g/l of *sodium metabisulphite R* and 1 ml of a 3 per cent V/V solution of *perchloric acid R*; dilute to 25.0 ml with *water R* (1.5 per cent).

Heavy metals (2.4.8). To 2.0 g in a silica crucible add 2 ml of *nitric acid R*, dropwise, followed by 0.25 ml of *sulphuric acid R*. Heat cautiously until white fumes are evolved and ignite. Extract the cooled residue with two quantities, each of 2 ml, of *hydrochloric acid R* and evaporate the extracts to dryness. Dissolve the residue in 2 ml of *dilute acetic acid R* and dilute to 20 ml with *water R*. 12 ml of the solution complies with limit test A for heavy metals (10 ppm). Prepare the standard using 10 ml of *lead standard solution (1 ppm Pb) R*.

Loss on drying (2.2.32). Not more than 8.0 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C at a pressure not exceeding 0.7 kPa for 5 h.

ASSAY

Carry out the assay protected from light.

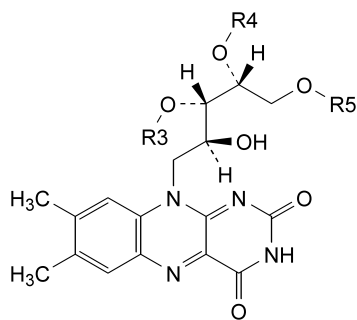
Dissolve 0.100 g in 150 ml of *water R*, add 2 ml of *glacial acetic acid R* and dilute to 1000.0 ml with *water R*. To 10.0 ml add 3.5 ml of a 14 g/l solution of *sodium acetate R* and dilute to 50.0 ml with *water R*. Measure the absorbance (2.2.25) at the maximum at 444 nm.

Calculate the content of C₁₇H₂₀N₄O₆ taking the specific absorbance to be 328.

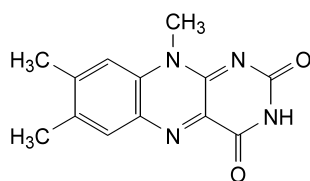
STORAGE

Store in an airtight container, protected from light.

IMPURITIES



- A. R3 = R4 = PO₃H₂, R5 = H: riboflavin 3',4'-diphosphate,
 B. R3 = R5 = PO₃H₂, R4 = H: riboflavin 3',5'-diphosphate,
 C. R3 = H, R4 = R5 = PO₃H₂: riboflavin 4',5'-diphosphate,
 D. R3 = R4 = R5 = H: riboflavin,



- E. 7,8,10-trimethylbenzo[*g*]pteridine-2,4(3*H*,10*H*)-dione (lumiflavin).

01/2005:1884

RIBWORT PLANTAIN

Plantaginis lanceolatae folium

DEFINITION

Whole or fragmented, dried leaf and scape of *Plantago lanceolata* L. s. l.

Content: minimum 1.5 per cent of total *ortho*-dihydroxycinnamic acid derivatives expressed as acteoside (C₂₉H₃₆O₁₅; *M_r* 624.6) (dried drug).

CHARACTERS

Macroscopic and microscopic characters described under identification tests A and B.

IDENTIFICATION

- A. The leaf is up to 30 cm long and 4 cm wide, yellowish-green to brownish-green, with a prominent, whitish-green, almost parallel venation on the abaxial surface. It consists of a lanceolate lamina narrowing at the base into a channelled petiole. The margin is indistinctly dentate and often undulate. It has 3, 5 or 7 primary veins, nearly equal in length and running almost parallel. Hairs may be almost absent, sparsely scattered or sometimes abundant, especially on the lower surface and over the veins. The scape is brownish-green, longer than the leaves, 3 mm to 4 mm in diameter and is deeply grooved longitudinally, with 5 to 7 conspicuous ribs. The surface is usually covered with fine hairs.
- B. Reduce to a powder (355). The powder is yellowish-green. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters: fragments of epidermis, composed of cells with irregularly sinuous anticlinal walls, the fragments from the scape with thickened outer walls and a coarsely

ridged cuticle; stomata mostly of the diacytic type (2.8.3) and sometimes anomocytic; the multicellular, uniseriate, conical covering trichomes are highly characteristic, with a basal cell larger than the other epidermal cells followed by a short cell supporting 2 or more elongated cells with the lumen narrow and variable, occluded at intervals corresponding to slight swellings in the trichome and giving a jointed appearance; the terminal cell has an acute apex and a filiform lumen; the glandular trichomes have a unicellular cylindrical stalk and a multicellular, elongated, conical head consisting of several rows of small cells and a single terminal cell; dense groups of lignified fibro-vascular tissue with narrow, spirally and annularly thickened vessels and slender, moderately thickened fibres.

- C. Examine the chromatograms obtained for *Digitalis lanata* leaves.

Results A: see below the sequence of the zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other zones may be present in the chromatograms obtained with the test solution.

Top of the plate	
Acteoside: a yellow zone	A yellow zone (acteoside)
Aucubin: a blue zone	A blue zone (aucubin)
Reference solution	Test solution

TESTS

***Digitalis lanata* leaves.** Thin layer chromatography (2.2.27).

Test solution. Use a freshly prepared solution. To 1 g of the powdered drug (355) in a 25 ml flask, add 10 ml of a mixture of 30 volumes of *water R* and 70 volumes of *methanol R* and shake for 30 min. Filter, rinse the flask and filter with 2 quantities, each of 5 ml, with the mixture of 30 volumes of *water R* and 70 volumes of *methanol R*. Dilute to 25 ml with a mixture of 30 volumes of *water R* and 70 volumes of *methanol R*.

Reference solution. Dissolve 1 mg of *acteoside R* and 1 mg of *aucubin R* in 1 ml of a mixture of 30 volumes of *water R* and 70 volumes of *methanol R*.

Plate: TLC silica gel *F₂₅₄* plate *R*.

Mobile phase: *acetic acid R*, *anhydrous formic acid R*, *water R*, *ethyl acetate R* (11:11:27:100 V/V/V/V).

Application: 10 µl as bands.

Development: over a path of 8 cm; heat immediately after development at about 120 °C for 5-10 min.

Detection A: examine in daylight.

Detection B: examine in ultraviolet light at 365 nm.

Results B: the chromatogram obtained with the test solution shows no bright blue fluorescent zone just below the reddish-brown fluorescent zone corresponding to aucubin in the chromatogram obtained with the reference solution.

Foreign matter (2.8.2): maximum 5 per cent of leaves of different colour and maximum 2 per cent of other foreign matter.

Loss on drying (2.2.32): maximum 10.0 per cent, determined on 1.000 g of the powdered drug (355) by drying in an oven at 100-105 °C for 2 h.

Total ash (2.4.16): maximum 14.0 per cent.

ASSAY

Stock solution. In a flask, place 1.000 g of the powdered drug (355) and add 90 ml of *alcohol* (50 per cent V/V) *R*. Boil in a water-bath under a reflux condenser for 30 min. Allow to cool and filter into a 100 ml volumetric flask. Rinse the flask and the filter with 10 ml of *alcohol* (50 per cent V/V) *R*. Combine the filtrate and the rinsings and dilute to 100.0 ml with *alcohol* (50 per cent V/V) *R*.

Test solution. To a 10 ml volumetric flask add, mixing after each addition, 1.0 ml of stock solution, 2 ml of 0.5 *M* hydrochloric acid, 2 ml of a solution prepared by dissolving 10 g of sodium nitrite *R* and 10 g of sodium molybdate *R* in 100 ml of water *R* and then add 2 ml of dilute sodium hydroxide solution *R*. Dilute to 10.0 ml with water *R*.

Immediately measure the absorbance (2.2.25) of the test solution at 525 nm using as compensation liquid a solution prepared as follows: to a 10 ml volumetric flask, add 1.0 ml of stock solution, 2 ml of 0.5 *M* hydrochloric acid, 2 ml of dilute sodium hydroxide solution *R* and dilute to 10.0 ml with water *R*.

Calculate the percentage content of total *ortho*-dihydroxycinnamic acid derivatives, expressed as acteoside, from the expression:

$$\frac{A \times 1000}{185 \times m}$$

i.e. taking the specific absorbance to be 185 for acteoside at 525 nm.

A = absorbance of the test solution at 525 nm,

m = mass of the substance to be examined, in grams.

01/2005:0349

RICE STARCH

Oryzae amyllum

DEFINITION

Rice starch is obtained from the caryopsis of *Oryza sativa* L.

CHARACTERS

A very fine, white powder which creaks when pressed between the fingers, tasteless, practically insoluble in cold water and in alcohol. The presence of granules with cracks or irregularities on the edge is exceptional.

DESCRIPTION

Examined under a microscope it presents polyhedral granules 2 µm to 5 µm in size, either isolated or aggregated in ovoid masses of 10 µm to 20 µm in size. The granules have a poorly visible central hilum and there are no concentric striations. Between crossed nicol prisms, the starch granules show a distinct black cross intersecting at the hilum.

IDENTIFICATION

- Suspend 1 g in 50 ml of water *R*, boil for 1 min and cool. A thin, cloudy mucilage is formed.
- To 1 ml of the mucilage obtained in identification test A add 0.05 ml of iodine solution *R1*. A dark-blue colour is produced which disappears on heating.

TESTS

Acidity. Add 10 g to 100 ml of *alcohol* (70 per cent V/V) *R* previously neutralised to 0.5 ml of phenolphthalein solution *R* and shake for 1 h. Filter and take 50 ml of the filtrate. Not more than 2.0 ml of 0.1 *M* sodium hydroxide is required to change the colour of the indicator.

Foreign matter. Not more than traces of cell membranes and protoplasm are present.

Loss on drying (2.2.32). Not more than 15.0 per cent, determined on 1.00 g by drying in an oven at 100-105 °C.

Sulphated ash (2.4.14). Not more than 1.0 per cent, determined on 1.0 g.

Microbial contamination. Total viable aerobic count (2.6.12) not more than 10³ bacteria and 10² fungi per gram, determined by plate count. It complies with the test for *Escherichia coli* (2.6.13).

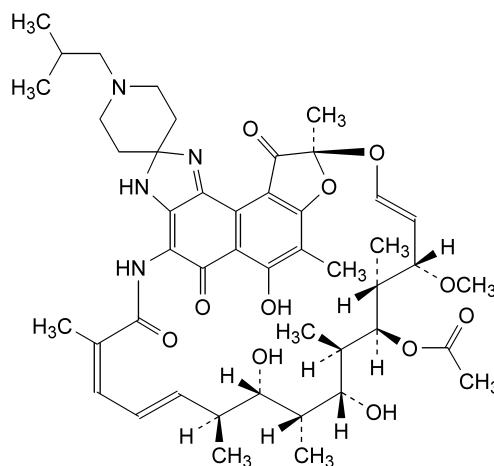
STORAGE

In an airtight container.

01/2005:1657
corrected

RIFABUTIN

Rifabutinum

C₄₆H₆₂N₄O₁₁*M*_r 847

DEFINITION

(9*S*,12*E*,14*S*,15*R*,16*S*,17*R*,18*R*,19*R*,20*S*,21*S*,22*E*,24*Z*)-6,18,20-trihydroxy-14-methoxy-7,9,15,17,19,21,25-heptamethyl-1'-(2-methylpropyl)-5,10,26-trioxo-3,5,9,10-tetrahydrospiro[9,4-(epoxypentadeca[1,11,13]trienimino)-2*H*-furo[2',3':7,8]naphtho[1,2-*d*]imidazole-2,4'-piperidine]-16-yl acetate.

Content: 96.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

Appearance: reddish-violet amorphous powder.

Solubility: slightly soluble in water, soluble in methanol, slightly soluble in alcohol.

IDENTIFICATION

- Infrared absorption spectrophotometry (2.2.24).

Preparation: discs.

Comparison: rifabutin CRS.

- Examine the chromatograms obtained in the test for related substances.

Results: the principal peak in the chromatogram obtained with the test solution is similar in retention time and size to the principal peak in the chromatogram obtained with reference solution (a).

TESTS

Impurity A. Thin-layer chromatography (2.2.27).

Test solution. Dissolve 0.100 g of the substance to be examined in a mixture of equal volumes of *methanol R* and *methylene chloride R* and dilute to 10 ml with the same mixture of solvents.

Reference solution. Dissolve 10 mg of *rifabutin impurity A CRS* in a mixture of equal volumes of *methanol R* and *methylene chloride R* and dilute to 10 ml with the same mixture of solvents. Dilute 3 ml of the solution to 100 ml with a mixture of equal volumes of *methanol R* and *methylene chloride R*.

Plate: TLC silica gel F_{254} plate *R*.

Mobile phase: *acetone R*, *light petroleum R* (23:77 V/V).

Application: 10 µl.

Development: over 2/3 of the plate.

Drying: in air.

Detection: expose the plate to iodine vapour for about 5 min, then spray with *potassium iodide and starch solution R* and allow to stand for 5 min.

Limit:

- **impurity A:** any spot corresponding to impurity A is not more intense than the spot in the chromatogram obtained with the reference solution (0.3 per cent).

Related substances. Liquid chromatography (2.2.29).

Test solution. Dissolve 50.0 mg of the substance to be examined in the mobile phase and dilute to 50.0 ml with the mobile phase.

Reference solution (a). Dissolve 50.0 mg of *rifabutin CRS* in the mobile phase and dilute to 50.0 ml with the mobile phase.

Reference solution (b). Dilute 1.0 ml of reference solution (a) to 100.0 ml with the mobile phase.

Reference solution (c). Dissolve about 10 mg of *rifabutin CRS* in 2 ml of *methanol R*, add 1 ml of *dilute sodium hydroxide solution R* and allow to stand for about 4 min. Add 1 ml of *dilute hydrochloric acid R* and dilute to 50 ml with the mobile phase.

Column:

- **size:** $l = 0.110$ m, $\varnothing = 4.6$ mm,
- **stationary phase:** *octylsilyl silica gel for chromatography R* (5 µm).

Mobile phase: mix equal volumes of *acetonitrile R* and a 13.6 g/l solution of *potassium dihydrogen phosphate R* adjusted to pH 6.5 with *dilute sodium hydroxide solution R*.

Flow rate: 1 ml/min.

Detection: spectrophotometer at 254 nm.

Injection: 20 µl.

Run time: 2.5 times the retention time of rifabutin.

Relative retention with reference to rifabutin (retention time = about 9 min): impurity E = about 0.5; impurity B = about 0.6; impurity D = about 0.9; impurity C = about 1.3.

System suitability: reference solution (c):

- **resolution:** minimum 2.0 between the second peak of the 3 peaks due to degradation products and the peak due to rifabutin.

Limits:

- **any impurity:** not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (1.0 per cent); not more than 1 such peak has an area greater than half the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent),
- **total:** not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (b) (3.0 per cent),
- **disregard limit:** 0.05 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

Water (2.5.12): maximum 2.5 per cent, determined on 0.200 g.

Sulphated ash (2.4.14): maximum 0.3 per cent, determined on 1.0 g.

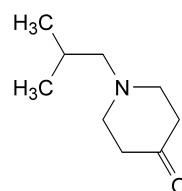
ASSAY

Liquid chromatography (2.2.29) as described in the test for related substances with the following modification.

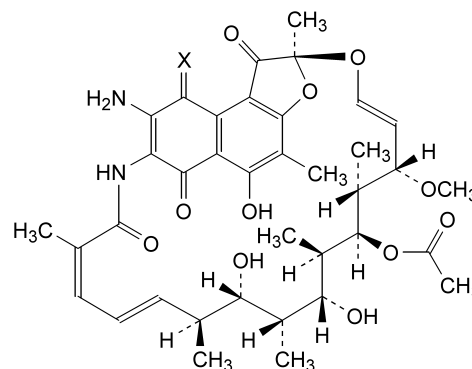
Injection: test solution and reference solution (a).

Calculate the percentage content of rifabutin.

IMPURITIES

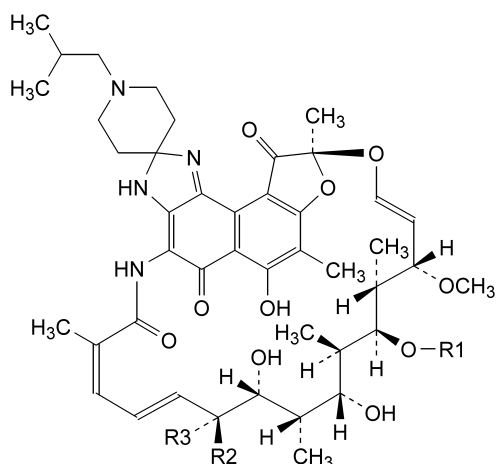


A. 1-(2-methylpropyl)piperidin-4-one,



B. X = O: 3-aminorifamycin S,

D. X = NH: 3-amino-4-imidorifamycin S,

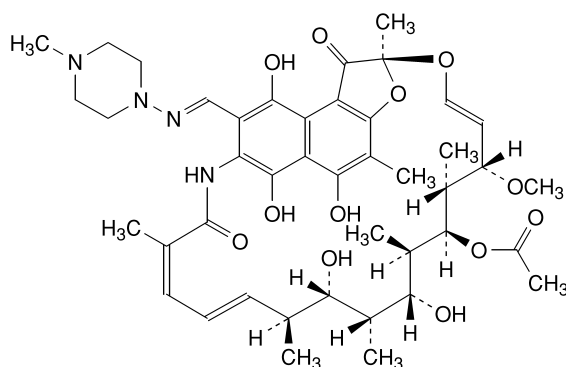


- C. R1 = CO-CH₃, R2 + R3 = CH₂: 21,31-didehydrorifabutin,
 E. R1 = R3 = H, R2 = CH₃: 16-deacetyl rifabutin.

01/2005:0052

RIFAMPICIN

Rifampicinum

C₄₃H₅₈N₄O₁₂M_r 823

DEFINITION

Rifampicin is (2*S*,12*Z*,14*E*,16*S*,17*S*,18*R*,19*R*,20*R*,21*S*,22*R*,23*S*,24*E*)-5,6,9,17,19-pentahydroxy-23-methoxy-2,4,12,16,18,20,22-heptamethyl-8-[[[4-methylpiperazin-1-yl]imino]methyl]-1,11-dioxo-1,2-dihydro-2,7-(epoxypentadeca[1,11,13]trienimino)naphto[2,1-*b*]furan-21-yl acetate, a semisynthetic antibiotic obtained from rifamycin SV. It contains not less than 97.0 per cent and not more than the equivalent of 102.0 per cent of C₄₃H₅₈N₄O₁₂, calculated with reference to the dried substance.

CHARACTERS

A reddish-brown or brownish-red, crystalline powder, slightly soluble in water, soluble in methanol, slightly soluble in acetone and in alcohol.

IDENTIFICATION

- A. Dissolve 50 mg in 50 ml of *methanol R*. Dilute 1 ml of the solution to 50 ml with *phosphate buffer solution pH 7.4 R*. Examined between 220 nm and 500 nm, the solution shows 4 absorption maxima (2.2.25), at 237 nm, 254 nm, 334 nm and 475 nm. The ratio of the absorbance at the maximum at 334 nm to that at 475 nm is about 1.75.
- B. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *rifampicin CRS*. Examine the substances prepared as mulls in *liquid paraffin R*.

- C. Suspend about 25 mg in 25 ml of *water R*, shake for 5 min and filter. To 5 ml of the filtrate add 1 ml of a 100 g/l solution of *ammonium persulphate R* in *phosphate buffer solution pH 7.4 R* and shake for a few minutes. The colour changes from orange-yellow to violet-red and no precipitate is formed.

TESTS

pH (2.2.3). The pH of a 10 g/l suspension in *carbon dioxide-free water R* is 4.5 to 6.5.

Related substances. Examine by liquid chromatography (2.2.29). Prepare the test solution and the reference solution immediately before use.

Solvent mixture. To 10 volumes of a 210.1 g/l solution of *citric acid R* add 23 volumes of a 136.1 g/l solution of *potassium dihydrogen phosphate R*, 77 volumes of a 174.2 g/l solution of *dipotassium hydrogen phosphate R*, 250 volumes of *acetonitrile R* and 640 volumes of *water R*.

Test solution. Dissolve 20.0 mg of the substance to be examined in *acetonitrile R* and dilute to 10.0 ml with the same solvent. Dilute 5.0 ml to 50.0 ml with the solvent mixture.

Reference solution. Dissolve 20.0 mg of *rifampicin quinone CRS* in *acetonitrile R* and dilute to 100.0 ml with the same solvent. To 1.0 ml of the solution add 1.0 ml of the test solution and dilute to 100.0 ml with the solvent mixture.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.12 m long and 4.6 mm in internal diameter packed with *octylsilyl silica gel for chromatography R* (5 µm),
- as mobile phase at a flow rate of 1.5 ml/min a mixture of 35 volumes of *acetonitrile R* and 65 volumes of a solution containing 0.1 per cent V/V of *phosphoric acid R*, 1.9 g/l of *sodium perchlorate R*, 5.9 g/l of *citric acid R* and 20.9 g/l of *potassium dihydrogen phosphate R*,
- as detector a spectrophotometer set at 254 nm,
- a 20 µl loop injector.

Inject the reference solution. Adjust the sensitivity of the detector so that the height of the 2 principal peaks is not less than half the full scale of the recorder. The test is not valid unless the resolution between the 2 principal peaks is at least 4.0. Adjust the concentration of acetonitrile in the mobile phase, if necessary. Inject the test solution and continue the chromatography for at least twice the retention time of rifampicin: the area of any peak corresponding to rifampicin quinone is not greater than 1.5 times the area of the corresponding peak in the chromatogram obtained with the reference solution (1.5 per cent); the area of any peak, apart from the principal peak and the peak corresponding to rifampicin quinone, is not greater than the area of the rifampicin peak in the chromatogram obtained with the reference solution (1.0 per cent) and the sum of the areas of any such peaks is not greater than 3.5 times the area of the rifampicin peak in the chromatogram obtained with the reference solution (3.5 per cent). Disregard any peak due to the solvent and any peak with an area less than 0.05 times that of the peak corresponding to rifampicin in the chromatogram obtained with the reference solution.

Heavy metals (2.4.8). 1.0 g complies with limit test C for heavy metals (20 ppm). Prepare the standard using 2 ml of *lead standard solution (10 ppm Pb) R*.

Loss on drying (2.2.32). Not more than 1.0 per cent, determined on 1.000 g by drying at 80 °C at a pressure not exceeding 670 Pa for 4 h.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 2.0 g.

ASSAY

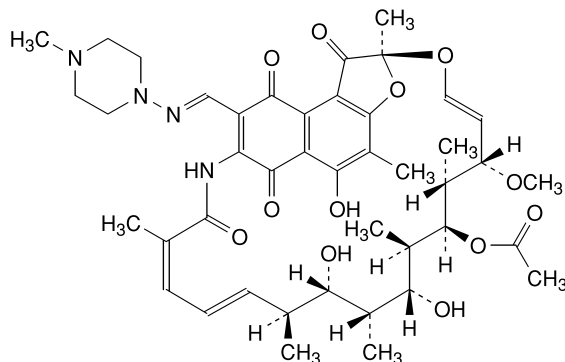
Dissolve 0.100 g in *methanol R* and dilute to 100.0 ml with the same solvent. Dilute 2.0 ml of the solution to 100.0 ml with *phosphate buffer solution pH 7.4 R*. Measure the absorbance (2.2.25) at the maximum at 475 nm, using *phosphate buffer solution pH 7.4 R* as the compensation liquid.

Calculate the content of $C_{43}H_{58}N_4O_{12}$, taking the specific absorbance to be 187.

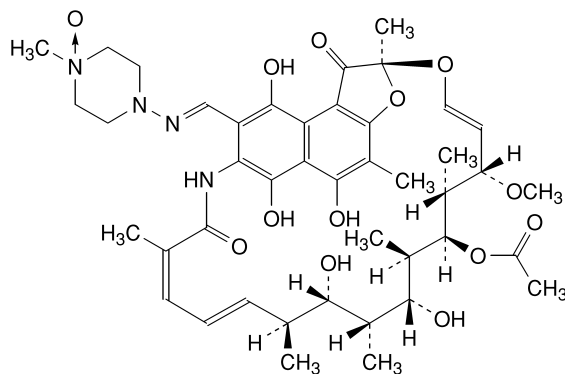
STORAGE

Store under nitrogen in an airtight container, protected from light at a temperature not exceeding 25 °C.

IMPURITIES



A. rifampicin quinone,

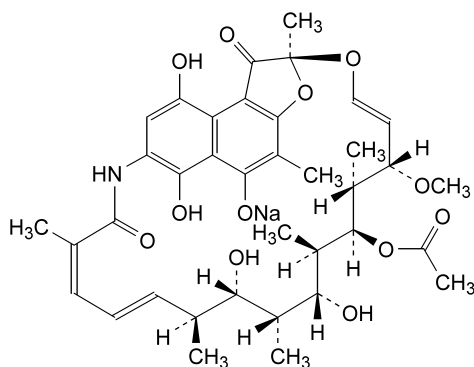


B. rifampicin N-oxide.

01/2005:0432

RIFAMYCIN SODIUM

Rifamycinum natricum

 $C_{37}H_{46}NNaO_{12}$ M_r 720

DEFINITION

Rifamycin sodium is sodium (2*S*,12*Z*,14*E*,16*S*,17*S*,18*R*,19*R*,20*R*,21*S*,22*R*,23*S*,24*E*)-21-(acetyloxy)-6,9,17,19-tetrahydroxy-23-methoxy-2,4,12,16,18,20,22-heptamethyl-1,11-dioxo-1,2-dihydro-2,7-(epoxypentadeca[1,11,13]trienimino)naphtho[2,1-*b*]furan-5-olate, the monosodium salt of rifamycin SV, a substance obtained by chemical transformation of rifamycin B, which is produced during the growth of certain strains of *Amycolatopsis mediterranei*. Rifamycin SV may also be obtained directly from certain *A. mediterranei* mutants. The potency is not less than 900 IU/mg, calculated with reference to the anhydrous substance.

PRODUCTION

It is produced by methods of manufacture designed to minimise or eliminate substances lowering blood pressure. The method of manufacture is validated to demonstrate that the product if tested would comply with the following test.

Abnormal toxicity (2.6.9). Inject into each mouse 4 mg dissolved in 0.5 ml of *water for injections R*.

CHARACTERS

A fine or slightly granular, red powder, soluble in water, freely soluble in ethanol.

IDENTIFICATION

- Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *rifamycin sodium CRS*. Examine the substances as discs prepared using *potassium bromide R*.
- It gives reaction (a) of sodium (2.3.1).

TESTS

pH (2.2.3). Dissolve 0.5 g in *carbon dioxide-free water R* and dilute to 10 ml with the same solvent. The pH is 6.5 to 8.0.

Absorbance (2.2.25). Dissolve 20.0 mg in 5 ml of *methanol R* and dilute to 100.0 ml with freshly prepared *phosphate buffer solution pH 7.0 R1* to which 1 g/l of *ascorbic acid R* has been added immediately before use. Dilute 5.0 ml of the solution to 50.0 ml with the same phosphate buffer solution containing ascorbic acid. Allow to stand for 30 min. The solution shows an absorption maximum at 445 nm. The specific absorbance at the maximum is 190 to 210, calculated with reference to the anhydrous substance.

Rifamycin B, rifamycin S and other related substances.

Examine by liquid chromatography (2.2.29). *Prepare the solutions immediately before use.*

Test solution. Dissolve 50.0 mg of the substance to be examined in a mixture of equal volumes of a 3.9 g/l solution of *sodium dihydrogen phosphate R*, adjusted to pH 3.0 with *phosphoric acid R*, and *acetonitrile R* and dilute to 50.0 ml with the same mixture of solvents.

Reference solution (a). Dissolve 10.0 mg of *rifamycin B CRS* and 40.0 mg of *rifamycin S CRS* in a mixture of equal volumes of a 3.9 g/l solution of *sodium dihydrogen phosphate R*, adjusted to pH 3.0 with *phosphoric acid R*, and *acetonitrile R* and dilute to 200.0 ml with the same mixture of solvents. Dilute 5.0 ml of the solution to 50.0 ml with the same mixture of solvents.

Reference solution (b). Dissolve 25 mg of the substance to be examined and 8 mg of *rifamycin S CRS* in a mixture of equal volumes of a 3.9 g/l solution of *sodium dihydrogen phosphate R*, adjusted to pH 3.0 with *phosphoric acid R*, and *acetonitrile R* and dilute to 250.0 ml with the same mixture of solvents.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4.6 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (5 µm),
- as mobile phase at a flow rate of 1 ml/min the following solutions, prepared and maintained at a temperature not lower than 20 °C:

Mobile phase A. Mix 10 volumes of *acetonitrile R* and 90 volumes of a 3.9 g/l solution of *sodium dihydrogen phosphate R*, adjusted to pH 7.5 with *dilute sodium hydroxide solution R*,

Mobile phase B. Mix 70 volumes of *acetonitrile R* and 30 volumes of a 3.9 g/l solution of *sodium dihydrogen phosphate R*, adjusted to pH 7.5 with *dilute sodium hydroxide solution R*,

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)	Comment
0 - 40	80 → 20	20 → 80	linear gradient
40 - 45	20	80	isocratic
45 - 47	20 → 80	80 → 20	linear gradient
47 - 55	80	20	re-equilibration

- as detector a spectrophotometer set at 254 nm,
- a 20 µl loop injector.

Inject reference solution (a). When the chromatogram is recorded in the prescribed conditions, the substances elute in the following order: rifamycin B, rifamycin SV, rifamycin S. Inject reference solution (b). Adjust the sensitivity of the system so that the height of the peak corresponding to rifamycin S in the chromatogram obtained is at least 50 per cent of the full scale of the recorder. The test is not valid unless the resolution between the peaks corresponding to rifamycin SV and rifamycin S is at least 5.0. Inject the test solution and reference solution (a). In the chromatogram obtained with the test solution: the area of any peak corresponding to rifamycin B is not greater than that of the peak due to rifamycin B in the chromatogram obtained with reference solution (a) (0.5 per cent); the area of any peak corresponding to rifamycin S is not greater than that of the peak due to rifamycin S in the chromatogram obtained with reference solution (a) (2 per cent); the sum of the areas of all the peaks, apart from the principal peak and any peaks corresponding to rifamycin B and rifamycin S, is not greater than the area of the peak corresponding to rifamycin S in the chromatogram obtained with reference solution (a) (2 per cent). Disregard any peak with an area less than 0.05 times that of the area of the peak corresponding to rifamycin S in the chromatogram obtained with reference solution (a).

Heavy metals (2.4.8). 2.0 g complies with limit test C for heavy metals (10 ppm). Prepare the standard using 2 ml of *lead standard solution (10 ppm Pb) R*.

Water (2.5.12): 12.0 per cent to 17.0 per cent, determined on 0.200 g by the semi-micro determination of water.

Bacterial endotoxins (2.6.14): less than 0.50 IU/mg, if intended for use in the manufacture of parenteral dosage forms without a further appropriate procedure for removal of bacterial endotoxins.

ASSAY

Carry out the microbiological assay of antibiotics (2.7.2).

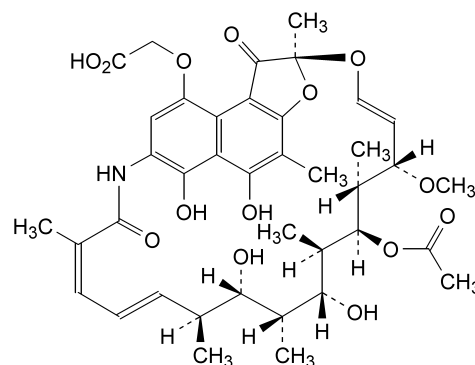
STORAGE

Store in an airtight container, protected from light at a temperature of 2 °C to 8 °C. If the substance is sterile, store in a sterile, airtight, tamper-proof container.

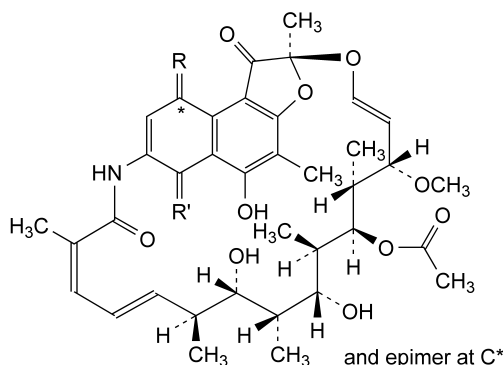
LABELLING

The label states, where applicable, that the substance is free from bacterial endotoxins.

IMPURITIES



A. rifamycin B,



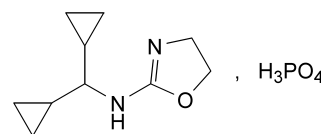
B. R = R' = =O: rifamycin S,

C. -R- = -O-CO-CH₂-O-, R' = =O: rifamycin O.

01/2005:2020

RILMENIDINE DIHYDROGEN PHOSPHATE

Rilmenidini dihydrogenophosphas



C₁₀H₁₉N₂O₅P

M_r 278.2

DEFINITION

N-(Dicyclopropylmethyl)-4,5-dihydro-oxazol-2-amine dihydrogen phosphate.

Content: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

Appearance: white or almost white powder.

Solubility: freely soluble in water, slightly soluble in alcohol, practically insoluble in methylene chloride.

IDENTIFICATION

A. Infrared absorption spectrophotometry (2.2.24).

Comparison: *Ph. Eur. reference spectrum of rilmenidine dihydrogen phosphate.*

- B. Dissolve 10 mg in *water R* and dilute to 1 ml with the same solvent. The solution gives reaction (b) of phosphates (2.3.1).

TESTS

Related substances. Liquid chromatography (2.2.29).

Test solution. Dissolve 60.0 mg of the substance to be examined in *water R* and dilute to 20.0 ml with the same solvent.

Reference solution (a). Dilute 1.0 ml of the test solution to 100.0 ml with *water R* and dilute 10.0 ml of this solution to 50.0 ml with the same solvent.

Reference solution (b). Dilute 5.0 ml of reference solution (a) to 20.0 ml with *water R*.

Reference solution (c). Dissolve 15.0 mg of *rilmenidine for system suitability CRS* in *water R* and dilute to 5.0 ml with the same solvent.

Column:

- **size:** $l = 0.15$ m, $\varnothing = 3$ mm,
- **stationary phase:** base-deactivated octadecylsilyl silica gel for chromatography *R* (5 μ m) with a pore size of 10 nm and a carbon loading of 25 per cent,
- **temperature:** 40 °C.

Mobile phase:

- **mobile phase A:** dissolve 3 g of *sodium heptanesulphonate R* in *water R* and dilute to 860 ml with the same solvent; add 130 ml of *methanol R2*, 10 ml of *tetrahydrofuran for chromatography R* and 1.0 ml of *phosphoric acid R*,
- **mobile phase B:** dissolve 3 g of *sodium heptanesulphonate R* in *water R* and dilute to 600 ml with the same solvent; add 350 ml of *acetonitrile for chromatography R*, 50 ml of *tetrahydrofuran for chromatography R* and 1.0 ml of *phosphoric acid R*,

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)
0 - 14	100 → 0	0 → 100
14 - 15	0 → 100	100 → 0
15 - 30	100	0

Flow rate: 1 ml/min.

Detection: spectrophotometer at 205 nm.

Injection: 20 μ l.

Relative retention with reference to *rilmenidine* (retention time = about 13 min): impurity A = about 0.6; impurity B = about 0.9; impurity C = about 1.4.

With these conditions the inflexion of the baseline, corresponding to the beginning of the gradient, appears on the recorder after a minimum time t of 5 min. If this is not the case ($t < 5$ min) modify the chromatographic sequence by adding an isocratic elution with 100 per cent of mobile phase A for a time corresponding to (5- t) min before the linear gradient.

System suitability: reference solution (c):

- **peak-to-valley ratio:** minimum 3, where H_p = height above the baseline of the peak due to impurity B and H_v = height above the baseline of the lowest point of the curve separating this peak from the peak due to *rilmenidine*.

Limits:

- **any impurity:** not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent),

- **total:** not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent),
- **disregard limit:** area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

Loss on drying (2.2.32): maximum 0.5 per cent, determined on 1.000 g by drying in an oven *in vacuo* at 50 °C over *diphosphorus pentoxide R* for 2 h.

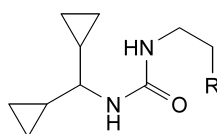
ASSAY

Dissolve 0.200 g in 50 ml of *anhydrous acetic acid R*. Titrate with 0.1 *M perchloric acid*, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 *M perchloric acid* is equivalent to 27.82 mg of $C_{10}H_{19}N_2O_5P$.

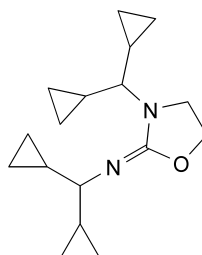
IMPURITIES

Specified impurities: A, B, C.



A. R = OH: 1-(dicyclopropylmethyl)-3-(2-hydroxyethyl)urea,

B. R = Cl: 1-(2-chloroethyl)-3-(dicyclopropylmethyl)urea,

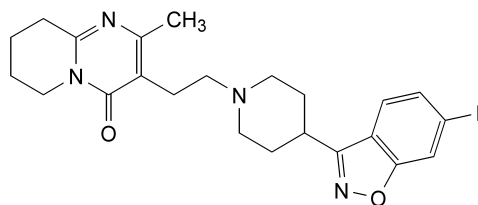


C. *N*,3-bis(dicyclopropylmethyl)oxazolidin-2-imine.

01/2005:1559

RISPERIDONE

Risperidonum



$C_{23}H_{27}FN_4O_2$

M_r 410.5

DEFINITION

3-[2-[4-(6-Fluoro-1,2-benzisoxazol-3-yl)piperidin-1-yl]ethyl]-2-methyl-6,7,8,9-tetrahydro-4*H*-pyrido[1,2-*a*]pyrimidin-4-one.

Content: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

Appearance: white or almost white powder.

Solubility: practically insoluble in water, freely soluble in methylene chloride, sparingly soluble in ethanol (96 per cent). It dissolves in dilute acid solutions.

It shows polymorphism.

IDENTIFICATION

Infrared absorption spectrophotometry (2.2.24).

Preparation: discs.

Comparison: risperidone CRS.

If the spectra obtained show differences, dissolve the substance to be examined and the reference substance separately in *acetone R*, evaporate to dryness and record new spectra using the residues.

TESTS

Appearance of solution. The solution is clear (2.2.1) and colourless (2.2.2, *Method II*).

Dissolve 0.1 g in a 7.5 g/l solution of *tartaric acid R* and dilute to 100 ml with the same acid solution.

Related substances. Liquid chromatography (2.2.29).

Test solution. Dissolve 0.100 g of the substance to be examined in *methanol R* and dilute to 10.0 ml with the same solvent.

Reference solution (a). Dissolve 10 mg of *risperidone for system suitability CRS* (containing impurities A, B, C, D and E) in *methanol R* and dilute to 1.0 ml with the same solvent.

Reference solution (b). Dilute 1.0 ml of the test solution to 100.0 ml with *methanol R*. Dilute 5.0 ml of this solution to 25.0 ml with *methanol R*.

Column:

- size: $l = 0.10$ m, $\varnothing = 4.6$ mm,
- stationary phase: base-deactivated octadecylsilyl silica gel for chromatography *R* (3 μ m).

Mobile phase:

- mobile phase A: 5 g/l solution of ammonium acetate *R*,
- mobile phase B: *methanol R*,

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)
0 - 2	70	30
2 - 17	70 $\rightarrow$ 30	30 $\rightarrow$ 70
17 - 22	30	70
22 - 23	30 $\rightarrow$ 70	70 $\rightarrow$ 30
23 - 27	70	30

Flow rate: 1.5 ml/min.

Detection: spectrophotometer at 260 nm.

Injection: 10 μ l.

Relative retention with reference to risperidone (retention time = about 12 min): impurity A = about 0.69; impurity B = about 0.75; impurity C = about 0.81; impurity D = about 0.94; impurity H = about 0.96; impurity G = about 1.04; impurity E = about 1.12; impurity F = about 1.32; impurity I = about 1.60.

System suitability: reference solution (a):

- peak-to-valley ratio: minimum 1.5, where H_p = height above the baseline of the peak due to impurity D and H_v = height above the baseline of the lowest point of the curve separating this peak from the peak due to risperidone,

- the chromatogram obtained is similar to the chromatogram supplied with *risperidone for system suitability CRS*.

Limits:

- impurities A, B, C, D, E: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent),
- any other impurity: for each impurity, not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent),
- total: not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.3 per cent),
- disregard limit: 0.25 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

Loss on drying (2.2.32): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 100-105 °C for 4 h.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g in a platinum crucible.

ASSAY

Dissolve 0.160 g in 70 ml of a mixture of 1 volume of *anhydrous acetic acid R* and 7 volumes of *methyl ethyl ketone R* and titrate with 0.1 M *perchloric acid*. Determine the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *perchloric acid* is equivalent to 20.53 mg of $C_{23}H_{27}FN_4O_2$.

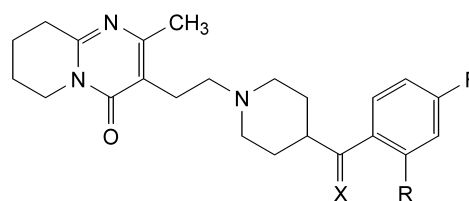
STORAGE

Protected from light.

IMPURITIES

Specified impurities: A, B, C, D, E.

Other detectable impurities: F, G, H, I.

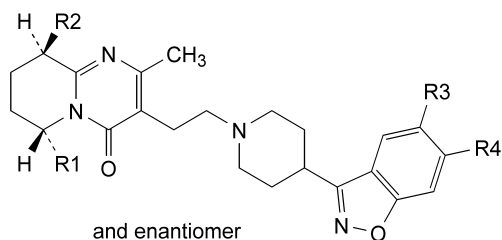


A. R = F, X = N-OH: 3-[2-[4-(*E*)-(2,4-difluorophenyl)(hydroxyimino)methyl]piperidin-1-yl]ethyl]-2-methyl-6,7,8,9-tetrahydro-4*H*-pyrido[1,2-*a*]pyrimidin-4-one,

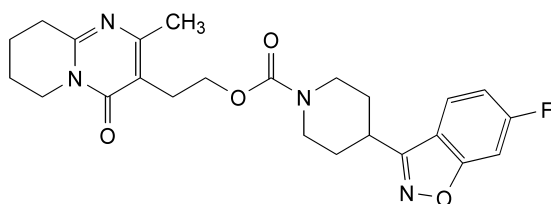
B. R = F, X = N-OH: 3-[2-[4-(*Z*)-(2,4-difluorophenyl)(hydroxyimino)methyl]piperidin-1-yl]ethyl]-2-methyl-6,7,8,9-tetrahydro-4*H*-pyrido[1,2-*a*]pyrimidin-4-one,

G. R = OH, X = O: 3-[2-[4-(4-fluoro-2-hydroxybenzoyl)piperidin-1-yl]ethyl]-2-methyl-6,7,8,9-tetrahydro-4*H*-pyrido[1,2-*a*]pyrimidin-4-one,

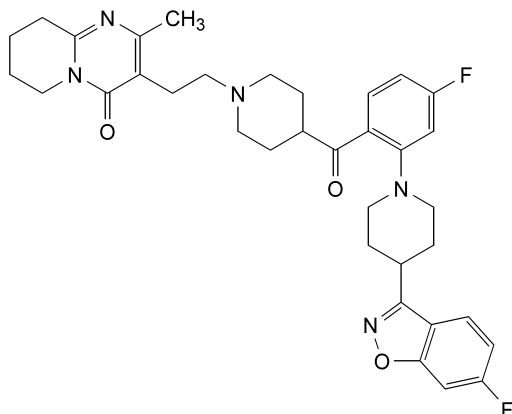
H. R = F, X = O: 3-[2-[4-(2,4-difluorobenzoyl)piperidin-1-yl]ethyl]-2-methyl-6,7,8,9-tetrahydro-4*H*-pyrido[1,2-*a*]pyrimidin-4-one,



- C. R1 = R3 = H, R2 = OH, R4 = F: (9*RS*)-3-[2-[4-(6-fluoro-1,2-benzisoxazol-3-yl)piperidin-1-yl]ethyl]-9-hydroxy-2-methyl-6,7,8,9-tetrahydro-4*H*-pyrido[1,2-*a*]pyrimidin-4-one,
- D. R1 = R2 = R4 = H, R3 = F: 3-[2-[4-(5-fluoro-1,2-benzisoxazol-3-yl)piperidin-1-yl]ethyl]-2-methyl-6,7,8,9-tetrahydro-4*H*-pyrido[1,2-*a*]pyrimidin-4-one,
- E. R1 = CH₃, R2 = R3 = H, R4 = F: (6*RS*)-3-[2-[4-(6-fluoro-1,2-benzisoxazol-3-yl)piperidin-1-yl]ethyl]-2,6-dimethyl-6,7,8,9-tetrahydro-4*H*-pyrido[1,2-*a*]pyrimidin-4-one,



- F. 2-[2-methyl-4-oxo-6,7,8,9-tetrahydro-4*H*-pyrido[1,2-*a*]pyrimidin-3-yl]ethyl 4-(6-fluoro-1,2-benzisoxazol-3-yl)piperidin-1-carboxylate,



- I. 3-[2-[4-[4-fluoro-2-[4-(6-fluoro-1,2-benzisoxazol-3-yl)piperidin-1-yl]benzoyl]piperidin-1-yl]ethyl]-2-methyl-6,7,8,9-tetrahydro-4*H*-pyrido[1,2-*a*]pyrimidin-4-one.

IDENTIFICATION

- A. The calyx is joined in the lower half to form an urceolate structure, the upper half dividing to form 5 long acuminate recurved tips. The tips have a prominent, slightly protruding midrib and a large, thick nectary gland about 1 mm in diameter. The epicalyx consist of 8 to 12 small, obovate leaflets which are adnate to the base of the calyx. The calyx and epicalyx are fleshy, dry, easily fragmented and coloured bright-red to deep-purple, somewhat lighter at the base of the inner side.
- B. Reduce to a powder (355). The powder is red to purplish-red. Examine under a microscope using *chloral hydrate solution R*. The powder shows predominantly red coloured fragments of the parenchyme containing numerous crystal clusters of calcium oxalate and, sporadically, mucilage filled cavities, sometimes associated with polygonal epidermal cells and anisocytic stomata (2.8.3); numerous fragments of vascular bundles with spiral and reticulate vessels; sclerenchymatous fibres with a wide lumen; rarely, rectangular, pitted parenchymatous cells; fragments of unicellular, smooth, bent covering trichomes and occasional glandular trichomes; rounded pollen grains with spiny exine.
- C. Thin-layer chromatography (2.2.27).

Test solution. To 1.0 g of the powdered drug (355) add 10 ml of *alcohol (60 per cent) V/V R*. Shake for 15 min and filter.

Reference solution. Dissolve 2.5 mg of *quinaldine red R* in 10 ml of *alcohol R*.

Plate: *TLC silica gel plate R*.

Mobile phase: *acetic acid R, water R, butanol R* (15:30:60 *V/V/V*).

Application: 20 µl, as bands.

Development: over a path of 10 cm.

Drying: in air.

Detection: examine in daylight.

Results: see below the sequence of the zones present in the chromatograms obtained with the reference and test solutions.

Top of the plate	
Quinaldine red: an orange red zone	A pale violet zone A violet-blue zone A violet-blue zone A violet-blue zone
Reference solution	Test solution

01/2005:1623

ROSELLE

Hibisci sabdariffae flos

DEFINITION

Whole or cut dried calyces and epicalyces of *Hibiscus sabdariffa* L. collected during fruiting.

Content: minimum 13.5 per cent of acids, expressed as citric acid (C₆H₈O₇; *M_r* 192.1) (dried drug).

CHARACTERS

Acidic taste.

Macroscopic and microscopic characters described under identification tests A and B.

TESTS

Foreign matter (2.8.2): maximum 2 per cent of fragments of fruits: red funicles and parts of the 5 caverned capsule with yellowish-grey pericarp, whose thin walls consist of several layers of differently directed fibres; flattened, reniform seeds with a dotted surface.

Loss on drying (2.2.32): maximum 11.0 per cent, determined on 1.000 g of the powdered drug (355) by drying in an oven at 100-105 °C for 2 h.

Total ash (2.4.16): maximum 10.0 per cent.

Colouring power: reduce the drug to a coarse powder (1400) and mix 100 g of drug. Reduce about 10 g of this mixture to a powder (355). To 1.0 g of the powdered drug (355) add 25 ml of boiling *water R* in a 100 ml flask and heat for 15 min on a water-bath with frequent shaking. Filter the

hot mixture into a 50 ml graduated flask; rinse successively the 100 ml flask and the filter 3 times with 5 ml of warm *water R*. After cooling dilute to 50 ml with *water R*.

Dilute 5 ml of this solution to 50 ml with *water R*. Measure the absorbance (2.2.25) at 520 nm using *water R* as the compensation liquid. The absorbance is not less than 0.350 for the whole drug and not less than 0.250 for the cut drug.

ASSAY

Shake 1.000 g of the powdered drug (355) with 100 ml of *carbon dioxide free water R* for 15 min. Filter. To 50.0 ml of the filtrate add 100 ml of *carbon dioxide free water R*. Titrate with 0.1 M *sodium hydroxide* until pH 7.0, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *sodium hydroxide* is equivalent to 6.4 mg of citric acid.

01/2005:1560

ROSEMARY LEAF

Rosmarini folium

DEFINITION

Whole, dried leaf of *Rosmarinus officinalis* L.

Content:

- minimum 12 ml/kg of essential oil (anhydrous drug),
- minimum 3 per cent of total hydroxycinnamic derivatives, expressed as rosmarinic acid ($C_{18}H_{16}O_8$; M_r 360) (anhydrous drug).

CHARACTERS

Strongly aromatic odour.

Macroscopic and microscopic characters described under identification tests A and B.

IDENTIFICATION

- The leaves are sessile, tough, linear to linear-lanceolate, 1 cm to 4 cm long and 2 mm to 4 mm wide, with recurved edges. The upper surface is dark green, glabrous and grainy, the lower surface is greyish-green and densely tomentose with a prominent midrib.
- Reduce to a powder (355). The powder is greyish-green to yellowish-green. Examine under a microscope using *chloral hydrate solution R*. The powder shows fragments of lower epidermis with straight to sinuous-walled cells and numerous diacytic stomata (2.8.3); fragments of the upper epidermis with straight-walled cells, slightly thickened and pitted, and an underlying hypodermis composed of large, irregular cells with thickened and beaded anticlinal walls; fragments in sectional view showing the hypodermal cells extending across the lamina at intervals, separating the one or two-layered palisade into large, crescent-shaped areas; numerous multicellular, extensively branched, covering trichomes of the lower epidermis and rare conical covering trichomes of the upper epidermis; glandular trichomes of 2 types, the majority with a short, unicellular stalk and a radiate head composed of 8 cells, others, less abundant, with a unicellular stalk and a spherical, unicellular or bicellular head.

C. Thin-layer chromatography (2.2.27).

Test solution. Dissolve 20 µl of the oil obtained in the assay in 1 ml of *hexane R*.

Reference solution. Dissolve 5 mg of *borneol R*, 5 mg of *bornyl acetate R* and 10 µl of *cineole R* in 1 ml of *hexane R*.

Plate: TLC silica gel G plate R.

Mobile phase: *ethyl acetate R*, *toluene R* (5:95 V/V).

Application: 10 µl, as bands.

Development: over a path of 15 cm.

Drying: in air.

Detection: spray with *anisaldehyde solution R*. Heat at 100-105 °C for 10 min. Examine in daylight.

Results: see below the sequence of the zones present in the chromatograms obtained with the reference solution and the test solution.

Top of the plate	
Bornyl acetate: a yellowish-brown zone	A red zone A yellowish-brown zone of low intensity A coloured zone of low intensity
Cineole: a violet zone	A violet zone Coloured zones of low intensity
Borneol: a violet-brown zone	A violet-brown zone A coloured zone of low intensity
Reference solution	Test solution

D. Thin-layer chromatography (2.2.27).

Test solution. Grind 1.0 g of the drug in 10 ml of *methanol R* and filter.

Reference solution. Dissolve 5.0 mg of *rosmarinic acid R* and 1.0 mg of *caffeic acid R* in 10 ml of *methanol R*.

Plate: TLC silica gel plate R.

Mobile phase: *anhydrous formic acid R*, *acetone R*, *methylene chloride R* (8.5:25:85 V/V/V).

Application: 10 µl of the test solution and 20 µl of the reference solution, as bands.

Development: over a path of 8 cm.

Drying: in air.

Detection: examine in ultraviolet light at 365 nm.

Results: see below the sequence of the zones present in the chromatograms obtained with the reference solution and the test solution.

Top of the plate	
Caffeic acid: a light blue fluorescent zone Rosmarinic acid: a light blue fluorescent zone	A pink fluorescent zone A blue fluorescent zone of low intensity An intense light blue fluorescent zone
Reference solution	Test solution

TESTS

Foreign matter (2.8.2): maximum 5 per cent of stems and maximum 2 per cent of other foreign matter.

Water (2.2.13): maximum 100 ml/kg, determined on 20.0 g of the powdered drug (355).

Total ash (2.4.16): maximum 9.0 per cent.

ASSAY

Total hydroxycinnamic derivatives

Stock solution. To 0.200 g of the powdered drug (355) add 80 ml of *alcohol (50 per cent V/V) R*. Boil in a water-bath under a reflux condenser for 30 min. Allow to cool and filter. Rinse the filter with 10 ml of *alcohol (50 per cent V/V) R*. Combine the filtrate and the rinsings in a volumetric flask and dilute to 100.0 ml with *alcohol (50 per cent V/V) R*.

Test solution. To 1.0 ml of the stock solution add 2 ml of 0.5 M hydrochloric acid, 2 ml of a solution prepared by dissolving 10 g of sodium nitrite R and 10 g of sodium molybdate R in 100 ml of water R and then add 2 ml of dilute sodium hydroxide solution R and dilute to 10.0 ml with water R; mix.

Compensation solution. Dilute 1.0 ml of the stock solution to 10.0 ml with water R.

Measure immediately the absorbance (2.2.25) of the test solution at 505 nm.

Calculate the percentage content of total hydroxycinnamic derivatives, expressed as rosmarinic acid, from the expression:

$$\frac{A \times 2.5}{m}$$

i.e. taking the specific absorbance of rosmarinic acid to be 400.

A = absorbance of the test solution at 505 nm,

m = mass of the substance to be examined, in grams.

Essential oil (2.8.12). Use 25.0 g of the crushed drug, a 1000 ml flask and 300 ml of water R as the distillation liquid. Distil at a rate of 2-3 ml/min for 3 h.

01/2005:1846

ROSEMARY OIL

Rosmarini aetheroleum

DEFINITION

Essential oil obtained by steam distillation from the flowering aerial parts of *Rosmarinus officinalis* L.

CHARACTERS

Appearance: clear, mobile, colourless to pale yellow liquid with a characteristic odour.

IDENTIFICATION

First identification: B.

Second identification: A.

A. Thin-layer chromatography (2.2.27).

Test solution. Dissolve 0.5 ml of the substance to be examined in toluene R and dilute to 10 ml with the same solvent.

Reference solution. Dissolve 50 mg of borneol R, 50 mg of bornyl acetate R and 100 µl of cineole R in toluene R and dilute to 10 ml with the same solvent.

Plate: TLC silica gel plate R.

Mobile phase: ethyl acetate R, toluene R (5:95 V/V).

Application: 10 µl, as bands.

Development: over a path of 15 cm.

Drying: in air.

Detection: spray the plate with vanillin reagent R and heat the plate at 100-105 °C for 10 min. Examine immediately in daylight.

Results: see below the sequence of the zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, several violet-blue to violet-grey zones of medium intensity (terpene alcohols) are present in the lower third of the chromatogram obtained with the test solution.

Top of the plate	
	An intense violet zone
	A violet-grey zone
Bornyl acetate: a bluish-grey zone of low intensity	A bluish-grey zone of low intensity (bornyl acetate)
	A violet-pink zone
Cineole: an intense blue zone	An intense blue zone (cineole)
Borneol: a violet-blue zone of medium intensity	A violet-blue zone of medium intensity (borneol)
Reference solution	Test solution

B. Examine the chromatograms obtained in the test for chromatographic profile.

Results: the characteristic peaks in the chromatogram obtained with the test solution are similar in retention time to those in the chromatogram obtained with the reference solution.

TESTS

Relative density (2.2.5): 0.895 to 0.920.

Refractive index (2.2.6): 1.464 to 1.473.

Optical rotation (2.2.7): −5° to +8°.

Acid value (2.5.1): maximum 1.0.

Chromatographic profile. Gas chromatography (2.2.28): use the normalisation procedure.

Test solution. Dissolve 0.20 ml of the substance to be examined in hexane R and dilute to 10.0 ml with the same solvent.

Reference solution. Dissolve 20 µl of α-pinene R, 10 mg of camphene R, 20 µl of β-pinene R, 10 µl of β-myrcene R, 20 µl of limonene R, 50 µl of cineole R, 10 µl of p-cymene R, 50 mg of camphor R, 30 mg of bornyl acetate R, 10 mg of α-terpineol R, 10 mg of borneol R and 10 µl of verbenone R in hexane R and dilute to 10.0 ml with the same solvent.

Column:

- **material:** fused silica,
- **size:** $l = 30$ m (a film thickness of 1 µm may be used) to 60 m (a film thickness of 0.2 µm may be used), $\varnothing = 0.25$ -0.53 mm,
- **stationary phase:** macrogol 20 000 R.

Carrier gas: helium for chromatography R.

Flow rate: 1 ml/min.

Split ratio: 1:50.

Temperature:

	Time (min)	Temperature (°C)
Column	0 - 10	50
	10 - 85	50 → 200
	85 - 110	200
Injection port		200
Detector		250

Detection: flame ionisation.

Injection: 1 µl.

Elution order: order indicated in the composition of the reference solution. Record the retention times of these substances.

System suitability: reference solution:

01/2005:1146

- *resolution*: minimum 1.5 between the peaks due to limonene and cineole and minimum 1.5 between the peaks due to α -terpineol and borneol.

Using the retention times determined from the chromatogram obtained with the reference solution, locate the components of the reference solution in the chromatogram obtained with the test solution.

Determine the percentage content of these components.

For rosemary oil, Spanish type, the percentages are within the following ranges:

- *α -pinene*: 18 per cent to 26 per cent,
- *camphene*: 8.0 per cent to 12.0 per cent,
- *β -pinene*: 2.0 per cent to 6.0 per cent,
- *β -myrcene*: 1.5 per cent to 5.0 per cent,
- *limonene*: 2.5 per cent to 5.0 per cent,
- *cineole*: 16.0 per cent to 25.0 per cent,
- *p-cymene*: 1.0 per cent to 2.2 per cent,
- *camphor*: 13.0 per cent to 21.0 per cent,
- *bornyl acetate*: 0.5 per cent to 2.5 per cent,
- *α -terpineol*: 1.0 per cent to 3.5 per cent,
- *borneol*: 2.0 per cent to 4.5 per cent,
- *verbenone*: 0.7 per cent to 2.5 per cent.

For rosemary oil, Moroccan and Tunisian type, the percentages are within the following ranges:

- *α -pinene*: 9.0 per cent to 14.0 per cent,
- *camphene*: 2.5 per cent to 6.0 per cent,
- *β -pinene*: 4.0 per cent to 9.0 per cent,
- *β -myrcene*: 1.0 per cent to 2.0 per cent,
- *limonene*: 1.5 per cent to 4.0 per cent,
- *cineole*: 38.0 per cent to 55.0 per cent,
- *p-cymene*: 0.8 per cent to 2.5 per cent,
- *camphor*: 5.0 per cent to 15.0 per cent,
- *bornyl acetate*: 0.1 per cent to 1.5 per cent,
- *α -terpineol*: 1.0 per cent to 2.6 per cent,
- *borneol*: 1.5 per cent to 5.0 per cent,
- *verbenone*: maximum 0.4 per cent.

STORAGE

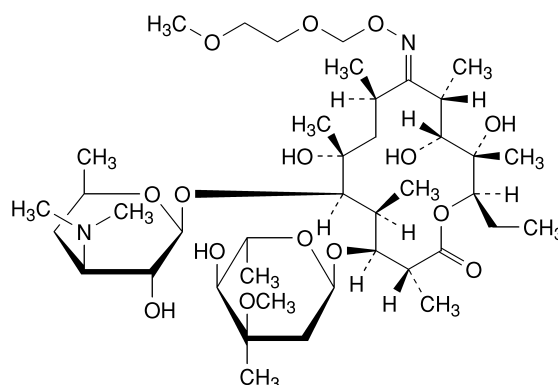
In a well-filled, airtight container, protected from light, at a temperature not exceeding 25 °C.

LABELLING

The label states that the content is Spanish type or Moroccan and Tunisian type.

ROXITHROMYCIN

Roxithromycinum



C₄₁H₇₆N₂O₁₅

M_r 837

DEFINITION

(3*R*,4*S*,5*S*,6*R*,7*R*,9*R*,11*S*,12*R*,13*S*,14*R*)-4-[(2,6-Dideoxy-3-C-methyl-3-*O*-methyl- α -L-ribo-hexopyranosyl)oxy]-14-ethyl-7,12,13-trihydroxy-10-[(*E*)-[(2-methoxyethoxy)methoxy]imino]-3,5,7,9,11,13-hexamethyl-6-[[3,4,6-trideoxy-3-(dimethylamino)- β -D-xyllo-hexopyranosyl]oxy]oxacyclotetradecan-2-one (erythromycin 9-(*E*)-[O-[(2-methoxyethoxy)methyl]oxime]).

Content: 96.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

Appearance: white, crystalline powder.

Solubility: very slightly soluble in water, freely soluble in acetone, in alcohol and in methylene chloride. It is slightly soluble in dilute hydrochloric acid.

It shows polymorphism.

IDENTIFICATION

A. Infrared absorption spectrophotometry (2.2.24).

Comparison: roxithromycin CRS.

If the spectra obtained shows differences, prepare further spectra using 90 g/l solutions in *methylene chloride R*.

B. Examine the chromatograms obtained in the assay.

Results: the principal peak in the chromatogram obtained with the test solution is similar in retention time and size to the principal peak in the chromatogram obtained with reference solution (a).

TESTS

Appearance of solution. The solution is clear (2.2.1) and colourless (2.2.2, *Method II*).

Dissolve 0.2 g in *methanol R* and dilute to 20 ml with the same solvent.

Specific optical rotation (2.2.7): –93 to –96 (anhydrous substance).

Dissolve 0.500 g in *acetone R* and dilute to 50.0 ml with the same solvent.

Related substances. Liquid chromatography (2.2.29).

Solution A. Mix 30 volumes of *acetonitrile R* and 70 volumes of a 48.6 g/l solution of *ammonium dihydrogen phosphate R*, adjusted to pH 5.3 with *dilute sodium hydroxide solution R*.

Test solution. Dissolve 50.0 mg of the substance to be examined in solution A and dilute to 25.0 ml with solution A.

Reference solution (a). Dissolve 50.0 mg of *roxithromycin CRS* in solution A and dilute to 25.0 ml with solution A.

Reference solution (b). Dilute 1.0 ml of reference solution (a) to 100.0 ml with solution A.

Reference solution (c). Dissolve 2.0 mg of *roxithromycin for system suitability CRS* in solution A and dilute to 1.0 ml with solution A.

Reference solution (d). Dilute 1.0 ml of *toluene R* to 100.0 ml with *acetonitrile R*. Dilute 0.2 ml of this solution to 200.0 ml with solution A.

Column:

- **size:** $l = 0.15$ m, $\varnothing = 4.6$ mm,
- **stationary phase:** spherical *end-capped octadecylsilyl silica gel for chromatography R* (5 μ m) with a 10 nm pore size and a carbon loading of about 19 per cent,
- **temperature:** 15 °C.

Mobile phase:

- **mobile phase A:** mix 26 volumes of *acetonitrile R* and 74 volumes of a 59.7 g/l solution of *ammonium dihydrogen phosphate R*, adjusted to pH 4.3 with *dilute sodium hydroxide solution R*,
- **mobile phase B:** *water R*, *acetonitrile R* (30:70 V/V),

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)
0 - 50	100	0
50 - 51	100 → 90	0 → 10
51 - 80	90	10
80 - 81	90 → 100	10 → 0
81 - 100	100	0

Flow rate: 1.1 ml/min.

Detection: spectrophotometer at 205 nm.

Injection: 20 μ l, using an injector maintained at 8 °C, of the test solution and reference solutions (b), (c) and (d).

Relative retention with reference to roxithromycin (retention time = about 22 min): impurity A = about 0.28; impurity B = about 0.31; impurity C = about 0.33; impurity D = about 0.62; impurity E = about 0.67; impurity F = about 0.83; impurity G = about 1.15; impurity K = about 1.7; impurity H = about 1.85; impurity J = about 2.65; impurity I = about 3.1.

System suitability: reference solution (c):

- **peak-to-valley ratio:** minimum 2.0, where H_p = height above the baseline of the peak due to impurity G and H_v = height above the baseline of the lowest point of the curve separating this peak from the peak due to roxithromycin.

Limits:

- **impurity G:** not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (1.0 per cent),
- **impurities A, B, C, D, E, F, H, I, J:** for each impurity, not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent),
- **total:** not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (b) (3.0 per cent),

- **disregard limit:** 0.05 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent). Disregard any peak due to toluene (use reference solution (d) to identify it).

Heavy metals (2.4.8): maximum 10 ppm.

Dissolve 2.0 g in a mixture of 15 volumes of *water R* and 85 volumes of *acetone R* and dilute to 20 ml with the same mixture of solvents. 12 ml of the solution complies with the limit test B. Prepare the standard using lead standard solution (1 ppm Pb) obtained by diluting *lead standard solution (100 ppm Pb) R* with a mixture of 15 volumes of *water R* and 85 volumes of *acetone R*.

Water (2.5.12): maximum 3.0 per cent, determined on 0.200 g.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (2.2.29) as described in the test for related substances, with the following modifications.

Column:

- **size:** $l = 0.25$ m.

Mobile phase: mix 307 volumes of *acetonitrile R* and 693 volumes of a 49.1 g/l solution of *ammonium dihydrogen phosphate R* adjusted to pH 5.3 with *dilute sodium hydroxide solution R*.

Flow rate: 1.5 ml/min.

Injection: test solution and reference solutions (a) and (c).

Retention time: roxithromycin = about 12 min.

System suitability: reference solution (c):

- **peak-to-valley ratio:** minimum 1.5, where H_p = height above the baseline of the peak due to impurity G and H_v = height above the baseline of the lowest point of the curve separating this peak from the peak due to roxithromycin.

STORAGE

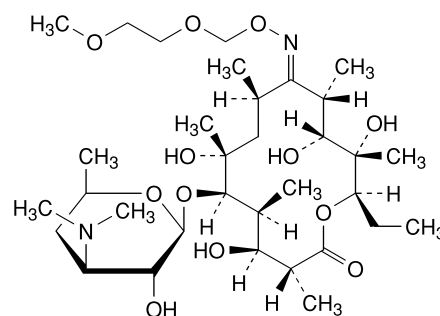
In an airtight container.

IMPURITIES

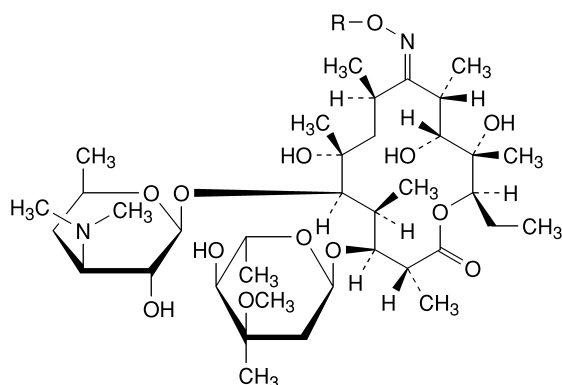
Specified impurities: A, B, C, D, E, F, G, H, I, J.

Other detectable impurities: K.

- A. (3*R*,4*S*,5*S*,6*R*,7*R*,9*R*,11*R*,12*R*,13*S*,14*R*)-4-[(2,6-dideoxy-3-*C*-methyl-3-*O*-methyl- α -L-*ribo*-hexopyranosyl)oxy]-14-ethyl-7,12,13-trihydroxy-3,5,7,9,11,13-hexamethyl-6-[[3,4,6-trideoxy-3-(dimethylamino)- β -D-*xylo*-hexopyranosyl]oxy]oxacyclotetradecane-2,10-dione (erythromycin A),



- B. 3-*O*-de(2,6-dideoxy-3-*C*-methyl-3-*O*-methyl- α -L-*ribo*-hexopyranosyl)erythromycin 9-(*E*)-[*O*-(2-methoxyethoxy)methyl]oxime],

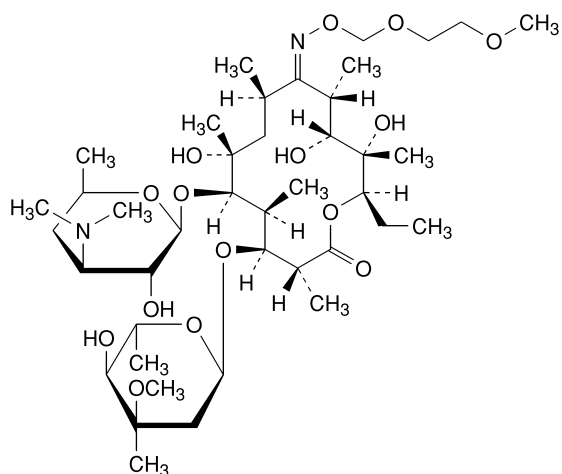


C. R = H: erythromycin 9-(*E*)-oxime,

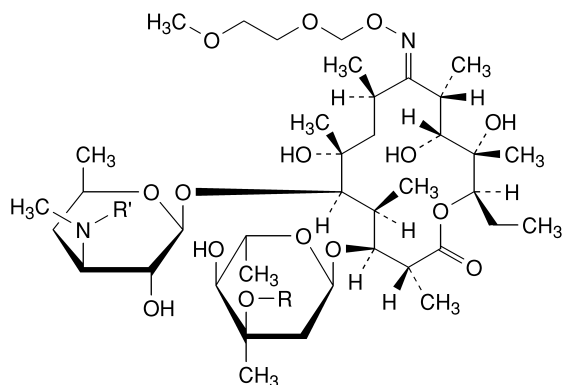
G. R = CH₂-O-CH₂-O-CH₂-CH₂-OCH₃: erythromycin
9-(*E*)-[O-[(2-methoxyethoxy)methoxy]methyl]oxime],

J. R = CH₂-O-CH₂-CH₂Cl: erythromycin 9-(*E*)-[O-(2-chloroethoxy)methyl]oxime],

K. R = CH₂-O-CH₂-CH₂-O-CH₂OH: erythromycin
9-(*E*)-[O-[2-(hydroxymethoxy)ethoxy]methyl]oxime].

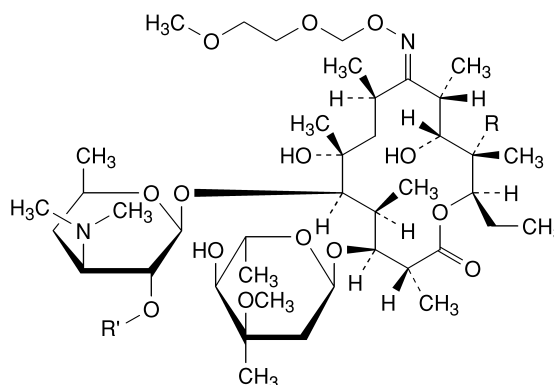


D. erythromycin 9-(Z)-[O-(2-methoxyethoxy)methyl]oxime],



E. R = H, R' = CH₃: 3''-O-demethylerythromycin
9-(*E*)-[O-[(2-methoxyethoxy)methyl]oxime],

F. R = CH₃, R' = H: 3'-*N*-demethylethromycin
9-(*E*)-[O-[(2-methoxyethoxy)methyl]oxime],



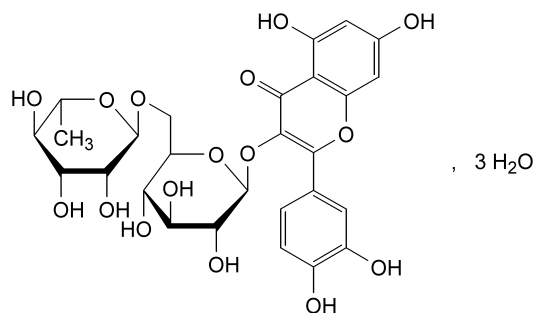
H. R = R' = H: 12-deoxyerythromycin 9-(*E*)-[O-[(2-methoxyethoxy)methyl]oxime].

I. R = OH, R' = CH₂-O-CH₂-CH₂-OCH₃: 2'-O-[(2-methoxyethoxy)methyl]erythromycin 9-(E)-[O-[(2-methoxyethoxy)methyl]oxime].

01/2005:1795

RUTOSIDE TRIHYDRATE

Rutosidum trihydricum


$$\text{C}_{27}\text{H}_{30}\text{O}_{16}, 3\text{H}_2\text{O}$$

M. 665

DEFINITION

3-[[6-*O*-(6-Deoxy- α -L-mannopyranosyl)- β -D-glucopyranosyl]oxy]-2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-4*H*-1-benzopyran-4-one.

Content: 95.0 per cent to 101.0 per cent (anhydrous substance).

CHARACTERS

Appearance: yellow or greenish-yellow, crystalline powder.

Solubility: practically insoluble in water, soluble in methanol, sparingly soluble in ethanol, practically insoluble in methylene chloride. It dissolves in solutions of alkali hydroxides.

IDENTIFICATION

First identification: B.

Second identification: A, C, D.

A. Dissolve 50.0 mg in *methanol R*, dilute to 250.0 ml with the same solvent and filter if necessary. Dilute 5.0 ml of the solution to 50.0 ml with *methanol R*. Examined between 210 nm and 450 nm (2.2.25), the solution shows 2 absorption maxima, at 257 nm and 358 nm. The specific absorbance at the maximum at 358 nm is 305 to 330, calculated with reference to the anhydrous substance.

B. Infrared absorption spectrophotometry (2.2.24).

Comparison: rutoside trihydrate CRS.

C. Thin-layer chromatography (2.2.27).

Test solution. Dissolve 25 mg of the substance to be examined in *methanol R* and dilute to 10.0 ml with the same solvent.

Reference solution. Dissolve 25 mg of *rutoside trihydrate CRS* in *methanol R* and dilute to 10.0 ml with the same solvent.

Plate: TLC silica gel G plate *R*.

Mobile phase: *butanol R*, *anhydrous acetic acid R*, *water R*, *methyl ethyl ketone R*, *ethyl acetate R* (5:10:10:30:50 *V/V/V/V/V*).

Application: 10 µl.

Development: over a path of 10 cm.

Drying: in air.

Detection: spray with a mixture of 7.5 ml of a 10 g/l solution of *potassium ferricyanide R* and 2.5 ml of *ferric chloride solution R1* and examine for 10 min.

Results: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

- D. Dissolve 10 mg in 5 ml of *alcohol R*, add 1 g of *zinc R* and 2 ml of *hydrochloric acid R1*. A red colour develops.

TESTS

Light absorbing impurities (2.2.25): maximum 0.10 at wavelengths between 450 nm and 800 nm.

Dissolve 0.200 g in 40 ml of *2-propanol R*. Stir for 15 min, dilute to 50.0 ml with *2-propanol R* and filter.

Substances insoluble in methanol: maximum 3 per cent.

Shake 2.5 g for 15 min in 50 ml of *methanol R* at 20–25 °C. Filter under reduced pressure through a sintered-glass filter (1.6) previously dried for 15 min at 100–105 °C, allowed to cool in a desiccator and tared. Wash the filter 3 times with 20 ml of *methanol R*. Dry the filter for 30 min at 100–105 °C. Allow to cool and weigh. The residue weighs a maximum of 75 mg.

Related substances. Liquid chromatography (2.2.29).

Test solution. Dissolve 0.10 g of the substance to be examined in 20 ml of *methanol R* and dilute to 100.0 ml with mobile phase B.

Reference solution (a). Dissolve 10.0 mg of *rutoside trihydrate CRS* in 10.0 ml of *methanol R*.

Reference solution (b). Dilute 1.0 ml of reference solution (a) to 50.0 ml with mobile phase B.

Column:

- **size:** $l = 0.25$ m, $\varnothing = 4.0$ mm,
- **stationary phase:** *octylsilyl silica gel for chromatography R* (5 µm),
- **temperature:** 30 °C.

Mobile phase:

- **mobile phase A:** mix 5 volumes of *tetrahydrofuran R* with 95 volumes of a 15.6 g/l solution of *sodium dihydrogen phosphate R* adjusted to pH 3.0 with *phosphoric acid R*,
- **mobile phase B:** mix 40 volumes of *tetrahydrofuran R* with 60 volumes of a 15.6 g/l solution of *sodium dihydrogen phosphate R* adjusted to pH 3.0 with *phosphoric acid R*,

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)
0 - 10	50 → 0	50 → 100
10 - 15	0	100
15 - 16	0 → 50	100 → 50
16 - 20	50	50

Flow rate: 1 ml/min.

Detection: spectrophotometer at 280 nm.

Injection: 20 µl.

Relative retention with reference to rutoside (retention time = about 7 min): impurity B = about 1.1; impurity A = about 1.2; impurity C = about 2.5.

System suitability: reference solution (a):

- **peak-to-valley ratio:** minimum 10, where H_p = height above the baseline of the peak due to impurity B and H_v = height above the baseline of the lowest point of the curve separating this peak from the peak due to rutoside.

Limits: locate the impurities by comparison with the chromatogram provided with *rutoside trihydrate CRS*.

- **correction factors:** for the calculation of contents, multiply the peak areas of the following impurities by the corresponding correction factor: impurity A = 0.8; impurity C = 0.5,
- **impurity A:** not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (2.0 per cent),
- **impurity B:** not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (2.0 per cent),
- **impurity C:** not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (2.0 per cent),
- **total:** not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (4.0 per cent),
- **disregard limit:** 0.05 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent).

Water (2.5.12): 7.5 per cent to 9.5 per cent, determined on 0.100 g.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

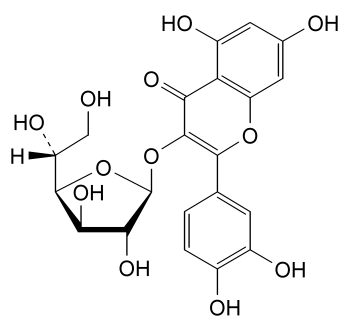
Dissolve 0.200 g in 20 ml of *dimethylformamide R*. Titrate with 0.1 M *tetrabutylammonium hydroxide*, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *tetrabutylammonium hydroxide* is equivalent to 30.53 mg of $C_{27}H_{30}O_{16}$.

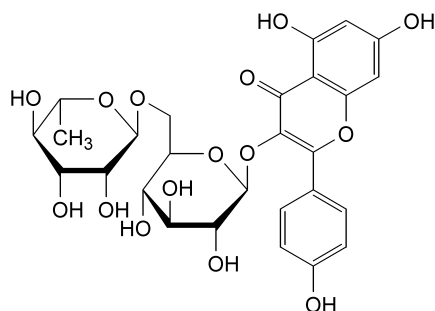
STORAGE

Protected from light.

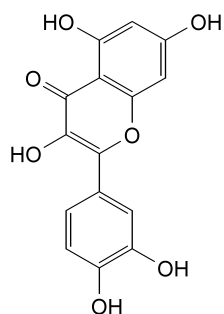
IMPURITIES



A. 2-(3,4-dihydroxyphenyl)-3-(β -D-glucofuranosyloxy)-5,7-dihydroxy-4*H*-1-benzopyran-4-one (isoquercitrin),



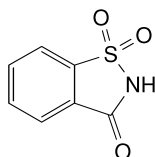
B. 3-[[6-*O*-(6-deoxy- α -L-mannopyranosyl)- β -D-glucopyranosyl]oxy]-5,7-dihydroxy-2-(4-hydroxyphenyl)-4*H*-1-benzopyran-4-one (kaempferol 3-rutinoside),



C. 2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4*H*-1-benzopyran-4-one (quercetin).

SACCHARIN

Saccharinum

C₇H₅NO₃SM_r 183.2

DEFINITION

1,2-Benzisothiazol-3(2H)-one 1,1-dioxide.

Content: 98.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

Appearance: white, crystalline powder or colourless crystals.*Solubility*: sparingly soluble in boiling water and in alcohol, slightly soluble in cold water. It dissolves in dilute solutions of alkali hydroxides and carbonates.

IDENTIFICATION

First identification: C.*Second identification*: A, B, D, E.A. A saturated solution, prepared without heating, turns *blue litmus paper R* red.

B. Melting point (2.2.14): 226 °C to 230 °C.

C. Infrared absorption spectrophotometry (2.2.24).

Preparation: discs.*Comparison*: saccharin CRS.D. Mix about 10 mg with about 10 mg of *resorcinol R*, add 0.25 ml of *sulphuric acid R* and carefully heat the mixture over a naked flame until a dark green colour is produced. Allow to cool, add 10 ml of *water R* and *dilute sodium hydroxide solution R* until an alkaline reaction is produced. An intense green fluorescence develops.E. To 0.2 g add 1.5 ml of *dilute sodium hydroxide solution R*, evaporate to dryness and heat the residue carefully until it melts, avoiding carbonisation. Allow to cool, dissolve the mass in about 5 ml of *water R*, add *dilute hydrochloric acid R* until a weak acid reaction is produced and filter, if necessary. To the filtrate add 0.2 ml of *ferric chloride solution R2*. A violet colour develops.

TESTS

Solution S. Dissolve 5.0 g in 20 ml of a 200 g/l solution of *sodium acetate R* and dilute to 25 ml with the same solution.**Appearance of solution.** Solution S is clear (2.2.1) and colourless (2.2.2, *Method II*).***o*- and *p*-Toluenesulphonamide.** Gas chromatography (2.2.28).*Internal standard solution.* Dissolve 25 mg of *caffeine R* in *methylene chloride R* and dilute to 100 ml with the same solvent.*Test solution.* Suspend 10.0 g of the substance to be examined in 20 ml of *water R* and dissolve using 5 ml to 6 ml of *strong sodium hydroxide solution R*. If necessary adjust the solution to pH 7-8 with 1 M *sodium hydroxide* or 1 M *hydrochloric acid* and dilute to 50 ml with *water R*. Shake the solution with 4 quantities, each of 50 ml, of *methylene**chloride R*. Combine the lower layers, dry over *anhydrous sodium sulphate R* and filter. Wash the filter and the sodium sulphate with 10 ml of *methylene chloride R*. Combine the solution and the washings and evaporate almost to dryness in a water-bath at a temperature not exceeding 40 °C. Using a small quantity of *methylene chloride R*, quantitatively transfer the residue into a suitable 10 ml tube, evaporate to dryness in a current of nitrogen and dissolve the residue in 1.0 ml of the internal standard solution.*Blank solution.* Evaporate 200 ml of *methylene chloride R* to dryness in a water-bath at a temperature not exceeding 40 °C. Dissolve the residue in 1 ml of *methylene chloride R*.*Reference solution.* Dissolve 20.0 mg of *o*-toluenesulphonamide *R* and 20.0 mg of *p*-toluenesulphonamide *R* in *methylene chloride R* and dilute to 100.0 ml with the same solvent. Dilute 5.0 ml of the solution to 50.0 ml with *methylene chloride R*. Evaporate 5.0 ml of the final solution to dryness in a current of nitrogen. Dissolve the residue in 1.0 ml of the internal standard solution.*Column*:

- *material*: fused silica,
- *size*: *l* = 10 m, Ø = 0.53 mm,
- *stationary phase*: *polymethylphenylsiloxane R* (film thickness 2 µm).

Carrier gas: nitrogen for chromatography *R*.*Flow rate*: 10 ml/min.*Split ratio*: 1:2.*Temperature*:

- *column*: 180 °C,
- *injection port and detector*: 250 °C.

Detection: flame ionisation.*Injection*: 1 µl.*Order of elution*: *o*-toluenesulphonamide, *p*-toluenesulphonamide, caffeine.*System suitability*: reference solution:

- *resolution*: minimum of 1.5 between the peaks due to *o*-toluenesulphonamide and *p*-toluenesulphonamide.

Limits:

- *o*-toluenesulphonamide: the ratio of its area to that of the internal standard is not greater than the corresponding ratio in the chromatogram obtained with the reference solution (10 ppm),
- *p*-toluenesulphonamide: the ratio of its area to that of the internal standard is not greater than the corresponding ratio in the chromatogram obtained with the reference solution (10 ppm).

Heavy metals (2.4.8): maximum 20 ppm.Dilute 10 ml of solution S to 20 ml with *water R*. 12 ml of the solution complies with limit test A. Prepare the standard using *lead standard solution* (2 ppm Pb) *R*.**Loss on drying** (2.2.32): maximum 1.0 per cent, determined on 1.000 g by drying in an oven at 100-105 °C for 4 h.**Sulphated ash** (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

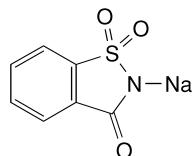
ASSAY

Dissolve 0.150 g in 25 ml of *alcohol R*, with slight heating if necessary. Add 25 ml of *water R* and 0.25 ml of *phenolphthalein solution R1*. Titrate with 0.1 M *sodium hydroxide*. Carry out a blank titration.1 ml of 0.1 M *sodium hydroxide* is equivalent to 18.32 mg of C₇H₅NO₃S.

01/2005:0787 ***o*- and *p*-Toluenesulphonamide**. Gas chromatography (2.2.28).

SACCHARIN SODIUM

Saccharinum natricum



$C_7H_4NNaO_3S$

M_r 205.2

DEFINITION

2-Sodio-1,2-benzisothiazol-3(2*H*)-one 1,1-dioxide.

Content: 99.0 per cent to 101.0 per cent (anhydrous substance). It may contain a variable quantity of water.

CHARACTERS

Appearance: white, crystalline powder or colourless crystals, efflorescent in dry air.

Solubility: freely soluble in water, sparingly soluble in alcohol.

IDENTIFICATION

First identification: B, E.

Second identification: A, C, D, E.

A. To 5 ml of solution S (see Tests) add 3 ml of *dilute hydrochloric acid R*. A white precipitate is formed. Filter and wash with *water R*. Dry the precipitate at 100–105 °C. The melting point (2.2.14) is 226 °C to 230 °C.

B. Infrared absorption spectrophotometry (2.2.24).

Preparation: discs; dry the substances at 100–105 °C before use.

Comparison: *saccharin sodium CRS*.

C. Mix about 10 mg with about 10 mg of *resorcinol R*, add 0.25 ml of *sulphuric acid R* and carefully heat the mixture over a naked flame until a dark green colour is produced. Allow to cool, add 10 ml of *water R* and *dilute sodium hydroxide solution R* until an alkaline reaction is produced. An intense green fluorescence develops.

D. To 0.2 g add 1.5 ml of *dilute sodium hydroxide solution R*, evaporate to dryness and heat the residue carefully until it melts, avoiding carbonisation. Allow to cool, dissolve the mass in about 5 ml of *water R*, add *dilute hydrochloric acid R* until a weak acid reaction is produced and filter, if necessary. To the filtrate add 0.2 ml of *ferric chloride solution R2*. A violet colour develops.

E. 0.5 ml of solution S gives reaction (a) of sodium (2.3.1).

TESTS

Solution S. Dissolve 5.0 g in *carbon dioxide-free water R* and dilute to 50.0 ml with the same solvent.

Appearance of solution. The solution is clear (2.2.1) and colourless (2.2.2, *Method II*).

Dissolve 5.0 g in 25 ml of *carbon dioxide-free water R*.

Acidity or alkalinity. To 10.0 ml of solution S add 5.0 ml of 0.005 *M sulphuric acid*. Heat to boiling and cool. Add 0.1 ml of *phenolphthalein solution R*. Not less than 4.5 ml and not more than 5.5 ml of 0.01 *M sodium hydroxide* is required to change the colour of the indicator to pink.

Internal standard solution. Dissolve 25 mg of *caffeine R* in *methylene chloride R* and dilute to 100 ml with the same solvent.

Test solution. Dissolve 10.0 g of the substance to be examined in 50 ml of *water R*. If necessary adjust the solution to pH 7–8 by addition of 1 *M sodium hydroxide* or 1 *M hydrochloric acid*. Shake the solution with 4 quantities, each of 50 ml, of *methylene chloride R*. Combine the lower layers, dry over *anhydrous sodium sulphate R* and filter. Wash the filter and the sodium sulphate with 10 ml of *methylene chloride R*. Combine the solution and the washings and evaporate almost to dryness in a water-bath at a temperature not exceeding 40 °C. Using a small quantity of *methylene chloride R*, quantitatively transfer the residue into a suitable 10 ml tube, evaporate to dryness in a current of *nitrogen R* and add 1.0 ml of the internal standard solution.

Blank solution. Evaporate 200 ml of *methylene chloride R* to dryness in a water-bath at a temperature not exceeding 40 °C. Dissolve the residue in 1 ml of *methylene chloride R*.

Reference solution. Dissolve 20.0 mg of *o*-toluenesulphonamide *R* and 20.0 mg of *p*-toluenesulphonamide *R* in *methylene chloride R* and dilute to 100.0 ml with the same solvent. Dilute 5.0 ml of the solution to 50.0 ml with *methylene chloride R*. Evaporate 5.0 ml of the final solution to dryness in a current of *nitrogen R*. Take up the residue using 1.0 ml of the internal standard solution.

Column:

- **material:** fused silica,
- **size:** $l = 10$ m, $\varnothing = 0.53$ mm,
- **stationary phase:** *polymethylphenylsiloxane R* (film thickness 2 µm).

Carrier gas: *nitrogen for chromatography R*.

Flow rate: 10 ml/min.

Split ratio: 1:2.

Temperature:

- **column:** 180 °C,
- **injection port and detector:** 250 °C.

Detection: flame ionisation.

Injection: 1 µl.

Elution order: *o*-toluenesulphonamide, *p*-toluenesulphonamide, *caffeine*.

System suitability: reference solution:

- **resolution:** minimum of 1.5 between the peaks due to *o*-toluenesulphonamide and *p*-toluenesulphonamide.

Limits:

- ***o*-toluenesulphonamide:** the ratio of its area to that of the internal standard is not greater than the corresponding ratio in the chromatogram obtained with the reference solution (10 ppm),
- ***p*-toluenesulphonamide:** the ratio of its area to that of the internal standard is not greater than the corresponding ratio in the chromatogram obtained with the reference solution (10 ppm).

Heavy metals (2.4.8): maximum 20 ppm.

12 ml of solution S complies with limit test A. Prepare the standard using *lead standard solution* (2 ppm Pb) *R*.

Water (2.5.12): maximum 15.0 per cent, determined on 0.200 g.

ASSAY

Dissolve 0.150 g in 50 ml of *anhydrous acetic acid R*, with slight heating if necessary. Titrate with 0.1 M *perchloric acid*, determining the end-point potentiometrically (2.2.20). 1 ml of 0.1 M *perchloric acid* is equivalent to 20.52 mg of $C_7H_4NNaO_3S$.

STORAGE

In an airtight container.

01/2005:2088

SAFFLOWER OIL, REFINED

Carthami oleum raffinatum

DEFINITION

Fatty oil obtained from seeds of *Carthamus tinctorius* L. (type I) or from seeds of hybrids of *Carthamus tinctorius* L. (type II), by expression and/or extraction followed by refining. Type II refined safflower oil is rich in oleic acid. It may contain a suitable antioxidant.

CHARACTERS

Appearance: clear, viscous, yellow to pale yellow liquid.

Solubility: miscible with light petroleum (bp: 40-60 °C), practically insoluble in alcohol.

	Refined safflower oil (type I)	Refined safflower oil (type II)
Relative density	about 0.922	about 0.914
Refractive index	about 1.476	about 1.472

IDENTIFICATION

First identification: B.

Second identification: A.

- A. Carry out the identification of fatty oils by thin-layer chromatography (2.3.2). The chromatogram obtained is comparable with the type chromatogram for type I or type II refined safflower oil.
- B. It complies with the test for composition of fatty acids (see Tests).

TESTS

Acid value (2.5.1): maximum 0.5.

Peroxide value (2.5.5): maximum 10.0. Maximum 5.0, if intended for the manufacture of parenteral dosage forms.

Unsaponifiable matter (2.5.7): maximum 1.5 per cent, determined on 5.0 g.

Alkaline impurities (2.4.19). It complies with the test for alkaline impurities in fatty oils.

Composition of fatty acids (2.4.22, *Method A*). Use the mixture of calibrating substances in Table 2.4.22-3.

Composition of the fatty acid fraction of type I refined safflower oil:

- *saturated fatty acids of chain length less than C14*: maximum 0.2 per cent,
- *myristic acid*: maximum 0.2 per cent,
- *palmitic acid*: 4.0 per cent to 10.0 per cent,
- *stearic acid*: 1.0 per cent to 5.0 per cent,
- *oleic acid* (equivalent chain length on polyethyleneglycol adipate 18.3): 8.0 per cent to 21.0 per cent,
- *linoleic acid* (equivalent chain length on polyethyleneglycol adipate 18.9): 68.0 per cent to 83.0 per cent,

- *linolenic acid* (equivalent chain length on polyethyleneglycol adipate 19.7): maximum 0.5 per cent,
- *arachidic acid*: maximum 0.5 per cent,
- *eicosenoic acid* (equivalent chain length on polyethyleneglycol adipate 20.3): maximum 0.5 per cent,
- *behenic acid*: maximum 1.0 per cent.

Composition of the fatty acid fraction of type II refined safflower oil:

- *saturated fatty acids of chain length less than C14*: maximum 0.2 per cent,
- *myristic acid*: maximum 0.2 per cent,
- *palmitic acid*: 3.6 per cent to 6.0 per cent,
- *stearic acid*: 1.0 per cent to 5.0 per cent,
- *oleic acid* (equivalent chain length on polyethyleneglycol adipate 18.3): 70.0 per cent to 84.0 per cent,
- *linoleic acid* (equivalent chain length on polyethyleneglycol adipate 18.9): 7.0 per cent to 23.0 per cent,
- *linolenic acid* (equivalent chain length on polyethyleneglycol adipate 19.7): maximum 0.5 per cent,
- *arachidic acid*: maximum 1.0 per cent,
- *eicosenoic acid* (equivalent chain length on polyethyleneglycol adipate 20.3): maximum 1.0 per cent,
- *behenic acid*: maximum 1.2 per cent.

Brassicasterol (2.4.23): maximum 0.3 per cent of brassicasterol in the sterol fraction of the oil.

Water (2.5.32): maximum 0.1 per cent, determined on 5.00 g, if intended for use in the manufacture of parenteral dosage forms.

STORAGE

In a well-filled, airtight container, protected from light.

LABELLING

The label states:

- where applicable, that the substance is suitable for use in the manufacture of parenteral dosage forms,
- the name and concentration of any added antioxidant,
- the type of oil (type I or type II).

01/2005:1370

SAGE LEAF (*SALVIA OFFICINALIS*)*Salviae officinalis folium*

DEFINITION

Whole or cut dried leaves of *Salvia officinalis* L.

Content: minimum 15 ml/kg of essential oil for the whole drug and minimum 10 ml/kg of essential oil for the cut drug (anhydrous drug).

CHARACTERS

Sage leaf (*Salvia officinalis*) oil is rich in thujone.

Macroscopic and microscopic characters described under identification tests A and B.

IDENTIFICATION

- A. The lamina of whole sage leaf (*Salvia officinalis*) is about 2 cm to 10 cm long and 1 cm to 2 cm wide, oblong-ovate, elliptical. The margin is finely crenate to smooth. The apex is rounded or subacute and the base is shrunk at the petiole and rounded or cordate. The upper surface

01/2005:1561

is greenish-grey and finely granular; the lower surface is white and pubescent and shows a dense network of raised veinlets.

- B. Reduce to a powder (355). The powder is light grey to brownish-green. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters: very numerous articulated and bent trichomes with narrow elongated cells and a very thick cell at the base as well as fragments of these trichomes; fragments of the upper epidermis with pitted, somewhat polygonal cells; fragments of the lower epidermis with sinuous cells and numerous diacytic stomata (2.8.3); rare single glandular trichomes with a uni- or bicellular head and a stalk consisting of 1 to 4 cells; abundant glandular trichomes with a unicellular stalk and a head composed of 8 radiating cells with a raised common cuticle.

- C. Thin-layer chromatography (2.2.27).

Test solution. Shake 0.5 g of the freshly powdered drug (355) with 5 ml of *ethanol R* for 5 min.

Reference solution. Dissolve 20 µl of *thujone R* and 25 µl of *cineole R* in 20 ml of *ethanol R*.

Plate: *TLC silica gel plate R*.

Mobile phase: *ethyl acetate R*, *toluene R* (5:95 V/V).

Application: 20 µl, as bands.

Development: over a path of 15 cm.

Drying: in air.

Detection: spray the plate with a 200 g/l solution of *phosphomolybdic acid R* in *ethanol R* and heat at 100-105 °C for 10 min. Examine in daylight.

Results: see below the sequence of the zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other zones are present in the chromatogram obtained with the test solution.

Top of the plate	
_____	A blue zone (near the solvent front)
α-Thujone and β-thujone: 2 pinkish-violet zones	2 pinkish-violet zones (α-thujone and β-thujone)
Cineole: a blue zone	A blue zone (cineole)
_____	Blue zones
Reference solution	Test solution

TESTS

Foreign matter (2.8.2): maximum 3 per cent of stems and maximum 2 per cent of other foreign matter.

Water (2.2.13): maximum 100 ml/kg, determined on 20.0 g.

Total ash (2.4.16): maximum 10.0 per cent.

ASSAY

Carry out the determination of essential oils in vegetable drugs (2.8.12). Use 20.0 g of the substance to be examined, cut, if necessary, immediately before the assay, a 500 ml flask, 250 ml of *water R* as the distillation liquid and 0.5 ml of *xylene R* in the graduated tube. Distil at a rate of 2-3 ml/min for 2 h.

SAGE LEAF, THREE-LOBED

Salviae trilobae folium

DEFINITION

Three-lobed sage leaf consists of the whole or cut, dried leaves of *Salvia fruticosa* Mill. (*S. triloba* L. fil). The whole drug contains not less than 18 ml/kg of essential oil, and the cut drug not less than 12 ml/kg of essential oil, both calculated with reference to the anhydrous drug.

CHARACTERS

Three-lobed sage leaf has a spicy odour when ground, similar to eucalyptus oil.

It has the macroscopic and microscopic characters described under identification tests A and B.

IDENTIFICATION

- A. The lamina of the whole three-lobed sage leaf is about 8 mm to 50 mm long and about 4 mm to 20 mm wide, and oblong-ovate to lanceolate. The margin is finely crenate and undulate but indistinct owing to the dense hairy covering on both surfaces. The base is obtuse and sometimes bears one or two more or less developed lobes. The upper surface is grey-tomentose pubescent, the lower surface is densely white-tomentose pubescent; the venation is indistinct. The densely white-tomentose pubescent petiole is about 1 mm in diameter.
- B. Reduce to a powder (355). The powder is greyish-green and tomentose. Examine under a microscope using *chloral hydrate solution R*. The powder shows very numerous, whole and fragmented, covering and glandular trichomes, scattered and attached to fragments of the epidermises; covering trichomes articulated, uniseriate, thick-walled and bluntly tapering, those on the upper epidermis straight, those on the lower epidermis longer, tortuous and more densely packed; glandular trichomes, some with a unicellular or bicellular head and a stalk consisting of from one to four cells, the majority having a short, unicellular stalk and a head composed of eight radiating cells with a raised common cuticle; the upper epidermis with pitted and beaded cells, somewhat polygonal, with a few diacytic stomata (2.8.3); the lower epidermis with sinuous- to wavy-walled cells and numerous diacytic stomata.
- C. Examine the chromatogram obtained in the test for thujone. The chromatogram obtained with the test solution shows a blue zone corresponding to cineole, equal or greater in size and intensity to the zone in the chromatogram obtained with the reference solution. Further zones are present.

TESTS

Thujone. Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel plate R*.

Test solution. Shake 0.3 g of the freshly powdered drug (355) with 5.0 ml of *ethanol R* for 5 min.

Reference solution. Dissolve 20 µl of *thujone R* and 25 µl of *cineole R* in 20 ml of *ethanol R*.

Apply to the plate as bands 20 µl of each solution. Develop over a path of 15 cm using a mixture of 5 volumes of *ethyl acetate R* and 95 volumes of *toluene R*. Allow the plate to dry in air. Spray with a 200 g/l solution of *phosphomolybdic acid R* in *ethanol R* and heat at 100 °C to 105 °C for 10 min. Examine in daylight. The chromatogram obtained with the reference solution shows in the middle part a blue zone

(cineole) and in the upper part a pink-blue zone (thujone). The chromatogram obtained with the test solution shows no zone or a very faint pink-blue zone corresponding to thujone.

Foreign matter (2.8.2). Not more than 8 per cent of stems and not more than 2 per cent of other foreign matter.

Water (2.2.13). Not more than 100 ml/kg, determined on 20.0 g by distillation.

Total ash (2.4.16). Not more than 10.0 per cent.

ASSAY

Carry out the determination of essential oils in vegetable drugs (2.8.12). Use 20.0 g of drug, if necessary cut immediately before the assay, a 500 ml flask, 250 ml of *water R* as the distillation liquid and 0.50 ml of *xylene R* in the graduated tube. Distil at a rate of 2 ml/min to 3 ml/min for 2 h.

STORAGE

Store protected from light.

01/2005:1889

SAGE TINCTURE

Salviae tinctura

DEFINITION

Tincture produced from *Sage leaf* (*Salvia officinalis*) (1370). *Content*: minimum 0.1 per cent *m/m* of essential oil.

PRODUCTION

The tincture is produced from 1 part of comminuted drug and 10 parts of ethanol (70 per cent *V/V*) by a suitable procedure.

CHARACTERS

Appearance: brownish liquid with a characteristic odour.

IDENTIFICATION

Thin-layer chromatography (2.2.27).

Test solution. The tincture to be examined.

Reference solution. Dissolve 20 µl of *thujone R* and 25 µl of *cineole R* in 20 ml of *ethanol R*.

Plate: TLC silica gel plate *R*.

Mobile phase: *ethyl acetate R*, *toluene R* (5:95 *V/V*).

Application: 20 µl, as bands.

Development: over a path of 15 cm.

Drying: in air.

Detection: spray with a 200 g/l solution of *phosphomolybdic acid R* in *ethanol R* and heat at 100-105 °C for 10 min.

Examine in daylight.

Results: see below the sequence of the zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other zones are present in the chromatogram obtained with the test solution.

Top of the plate	
	A blue zone (near the solvent front)
_____	_____
α-Thujone and β-thujone: 2 pinkish-violet zones	2 pinkish-violet zones (α-thujone and β-thujone)
Cineole: a blue zone	A blue zone (cineole)
_____	_____
	Blue zones
Reference solution	Test solution

TESTS

Ethanol content (2.9.10): 64 per cent *V/V* to 69 per cent *V/V*.

Methanol and 2-propanol (2.9.11): maximum 0.05 per cent *V/V* of methanol and maximum 0.05 per cent of 2-propanol.

Dry residue (2.8.16): minimum 2.0 per cent *m/m*, determined on 3.00 g.

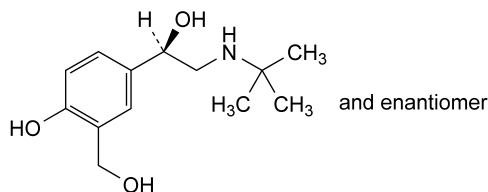
ASSAY

In a 500 ml round-bottomed flask, place 30.0 g of the tincture and add 100 ml of *water R*. Distil, using a descending condenser, into a separating funnel which has been marked beforehand at 50 ml. Stop the distillation process as soon as the distillate reaches the 50 ml mark. Rinse the condenser with 10 ml of *pentane R*. Dissolve in the distillate sufficient *sodium chloride R* to produce a saturated solution. Shake with 3 quantities, each of 20 ml, of *pentane R*. Dry the combined pentane layers, including the pentane from rinsing the condenser, over *anhydrous sodium sulphate R* and filter through a plug of absorbent cotton into a weighed 100 ml round-bottomed flask. Wash the sodium sulphate several times with small quantities of *pentane R*. Remove the pentane carefully at a temperature not exceeding 40 °C. Dry the residue in a desiccator over *diphosphorus pentoxide R* and hard paraffin at atmospheric pressure and at room temperature for 2 h. Weigh the residue (essential oil).

01/2005:0529

SALBUTAMOL

Salbutamolum



$C_{13}H_{21}NO_3$

M_r 239.3

DEFINITION

(1*R,S*)-2-[(1,1-Dimethylethyl)amino]-1-[4-hydroxy-3-(hydroxymethyl)phenyl]ethanol.

Content: 98.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

Appearance: white or almost white, crystalline powder.

Solubility: sparingly soluble in water, soluble in alcohol.

mp: about 155 °C, with decomposition.

IDENTIFICATION

First identification: B.

Second identification: A, C, D.

A. Dissolve 80.0 mg in a 10 g/l solution of *hydrochloric acid R* and dilute to 100.0 ml with the same acid. Dilute 10.0 ml of the solution to 100.0 ml with a 10 g/l solution of *hydrochloric acid R*. Examined between 230 nm and 350 nm (2.2.25), the solution shows an absorption maximum at 276 nm. The specific absorbance at the maximum is 66 to 75.

B. Infrared absorption spectrophotometry (2.2.24).

Comparison: *salbutamol CRS*.

C. Thin-layer chromatography (2.2.27).

Test solution. Dissolve 10 mg of the substance to be examined in *methanol R* and dilute to 50 ml with the same solvent.

Reference solution. Dissolve 10 mg of *salbutamol CRS* in *methanol R* and dilute to 50 ml with the same solvent.

Plate: *TLC silica gel plate R*.

Mobile phase: concentrated ammonia *R*, water *R*, ethyl acetate *R*, 2-propanol *R*, methyl isobutyl ketone *R* (3:18:35:45:50 V/V/V/V/V).

Application: 5 µl.

Development: over a path of 18 cm.

Drying: in air.

Detection: spray with a 1 g/l solution of methylbenzothiazolone hydrazone hydrochloride *R* in a 90 per cent V/V solution of *methanol R*, followed by a 20 g/l solution of potassium ferricyanide *R* in a mixture of 1 volume of concentrated ammonia *R1* and 3 volumes of water *R*, followed by a further spraying with a 1 g/l solution of methylbenzothiazolone hydrazone hydrochloride *R* in a 90 per cent V/V solution of *methanol R*.

Results: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

- D. Dissolve about 10 mg in 50 ml of a 20 g/l solution of disodium tetraborate *R*. Add 1 ml of a 30 g/l solution of aminopyrazolone *R*, 10 ml of methylene chloride *R* and 10 ml of a 20 g/l solution of potassium ferricyanide *R*. Shake and allow to separate. An orange-red colour develops in the methylene chloride layer.

TESTS

Solution S. Dissolve 0.50 g in *methanol R* and dilute to 25.0 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and not more intensely coloured than reference solution BY₅ (2.2.2, Method II).

Optical rotation (2.2.7): -0.10° to $+0.10^{\circ}$ determined on solution S.

Related substances. Liquid chromatography (2.2.29).

Test solution. Dissolve 0.100 g of the substance to be examined in the mobile phase and dilute to 50.0 ml with the mobile phase.

Reference solution. Dissolve 2.0 mg of *salbutamol CRS*, 2.0 mg of *salbutamol impurity B CRS*, 3.0 mg of *salbutamol impurity D CRS*, 3.0 mg of *salbutamol impurity F CRS*, 3.0 mg of *salbutamol impurity G CRS* and 3.0 mg of *salbutamol impurity I CRS* in the mobile phase and dilute to 10.0 ml with the mobile phase. Dilute 2.0 ml of the solution to 100.0 ml with the mobile phase.

Column:

- size: $l = 0.15$ m, $\varnothing = 3.9$ mm,
- stationary phase: spherical end-capped octylsilyl silica gel for chromatography *R* (5 µm) with a specific surface of 335 m²/g, a pore size of 10 nm and a carbon loading of 11.7 per cent.

Mobile phase: mix 22 volumes of acetonitrile *R* and 78 volumes of a solution containing 2.87 g/l of sodium heptanesulphonate *R* and 2.5 g/l of potassium dihydrogen phosphate *R* adjusted to pH 3.65 with dilute phosphoric acid *R*.

Flow rate: 1 ml/min.

Detection: spectrophotometer at 220 nm.

Injection: 20 µl.

Run time: 25 times the retention time of salbutamol.

Relative retention with reference to salbutamol (retention time = about 1.9 min): impurity B = about 1.3, impurity A = about 1.7, impurity C = about 2.0, impurity D = about 2.7, impurity H = about 3.0, impurity E = about 3.1, impurity G = about 4.1, impurity F = about 6.2, impurity I = about 23.2.

System suitability: reference solution:

- resolution: minimum of 3.0 between the peaks due to salbutamol and to impurity B.

Limits:

- impurity D: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (0.3 per cent),
- impurity F: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (0.3 per cent),
- impurity G: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (0.3 per cent),
- impurity I: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (0.3 per cent),
- any other impurity: not more than 1.5 times the area of the peak due to salbutamol in the chromatogram obtained with reference solution (0.3 per cent),
- total: maximum 1.0 per cent,
- disregard limit: 0.05 per cent.

Impurity J: maximum 0.2 per cent.

Dissolve 50.0 mg in a 1 g/l solution of hydrochloric acid *R* and dilute to 25.0 ml with the same solvent. The absorbance (2.2.25) of the solution measured at 310 nm is not greater than 0.10.

Boron: maximum 50 ppm.

Test solution. To 50 mg of the substance to be examined add 5 ml of a solution containing 13 g/l of anhydrous sodium carbonate *R* and 17 g/l of potassium carbonate *R*. Evaporate to dryness on a water-bath and dry at 120 °C. Ignite the residue rapidly until the organic matter has been destroyed, allow to cool and add 0.5 ml of water *R* and 3.0 ml of a freshly prepared 1.25 g/l solution of curcumin *R* in glacial acetic acid *R*. Warm gently to effect solution, allow to cool and add 3.0 ml of a mixture prepared by adding 5 ml of sulphuric acid *R*, slowly and with stirring, to 5 ml of glacial acetic acid *R*. Mix and allow to stand for 30 min. Dilute to 100.0 ml with alcohol *R*, filter and use the filtrate.

Reference solution. Dissolve 0.572 g of boric acid *R* in 1000.0 ml of water *R*. Dilute 1.0 ml to 100.0 ml with water *R*. To 2.5 ml of the solution add 5 ml of a solution containing 13 g/l of anhydrous sodium carbonate *R* and 17 g/l of potassium carbonate *R*, and treat this mixture in the same manner as the test solution.

Measure the absorbance (2.2.25) of the test solution and of the reference solution at the maximum at about 555 nm. The absorbance of the test solution is not greater than that of the reference solution.

Loss on drying (2.2.32): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 100–105 °C.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

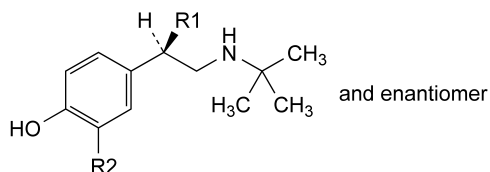
Dissolve 0.200 g in 30 ml of anhydrous acetic acid *R*. Titrate with 0.1 M perchloric acid, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M perchloric acid is equivalent to 23.93 mg of $C_{13}H_{21}NO_3$.

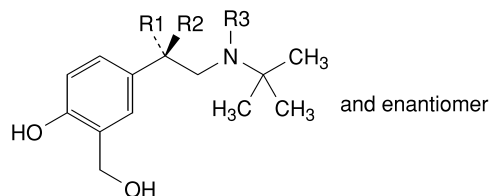
STORAGE

Protected from light.

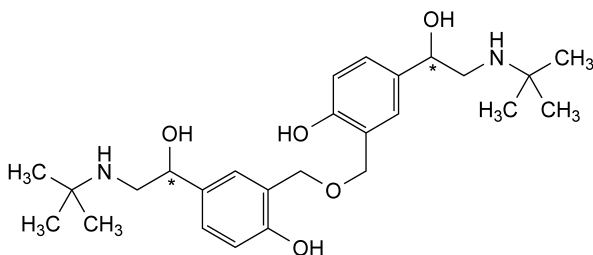
IMPURITIES



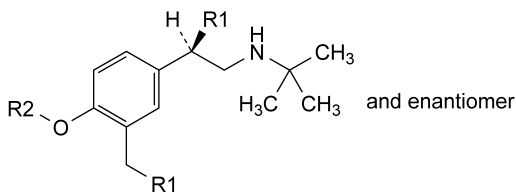
- A. R1 = OCH_3 , R2 = CH_2OH : [5-[(1*RS*)-2-[(1,1-dimethylethyl)amino]-1-methoxyethyl]-2-hydroxyphenyl]methanol,
- B. R1 = OH, R2 = H: (1*RS*)-2-[(1,1-dimethylethyl)amino]-1-(4-hydroxyphenyl)ethanol,
- C. R1 = OH, R2 = CH_3 : (1*RS*)-2-[(1,1-dimethylethyl)amino]-1-(4-hydroxy-3-methylphenyl)ethanol,
- D. R1 = OH, R2 = CHO: 5-[(1*RS*)-2-[(1,1-dimethylethyl)amino]-1-hydroxyethyl]-2-hydroxybenzaldehyde,



- E. R1 = H, R2 = OH, R3 = $CH_2-C_6H_5$: (1*RS*)-2-[benzyl(1,1-dimethylethyl)amino]-1-[4-hydroxy-3-(hydroxymethyl)phenyl]ethanol,
- G. R1+R2 = O, R3 = $CH_2-C_6H_5$: 2-[benzyl(1,1-dimethylethyl)amino]-1-[4-hydroxy-3-(hydroxymethyl)phenyl]ethanone,
- J. R1+R2 = O, R3 = H: 2-[(1,1-dimethylethyl)amino]-1-[4-hydroxy-3-(hydroxymethyl)phenyl]ethanone (salbutamone),



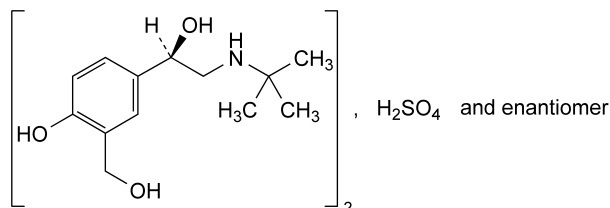
- F. 1,1'-[oxybis[methylene(4-hydroxy-1,3-phenylene)]]bis[2-[(1,1-dimethylethyl)amino]ethanol],



- H. R1 = R2 = H: 4-[2-[(1,1-dimethylethyl)amino]ethyl]-2-methylphenol,
- I. R1 = OH, R2 = $CH_2-C_6H_5$: (1*RS*)-2-[(1,1-dimethylethyl)amino]-1-[3-(hydroxymethyl)-4-benzyloxyphenyl]ethanol.

SALBUTAMOL SULPHATE

Salbutamoli sulfas



$C_{26}H_{44}N_2O_{10}S$

M_r 576.7

DEFINITION

Bis[(1*RS*)-2-[(1,1-dimethylethyl)amino]-1-[4-hydroxy-3-(hydroxymethyl)phenyl]ethanol] sulphate.

Content: 98.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

Appearance: white or almost white, crystalline powder.

Solubility: freely soluble in water, practically insoluble or very slightly soluble in alcohol and in methylene chloride.

IDENTIFICATION

First identification: B, E.

Second identification: A, C, D, E.

- A. Dissolve 80.0 mg in a 10 g/l solution of hydrochloric acid R and dilute to 100.0 ml with the same acid. Dilute 10.0 ml of the solution to 100.0 ml with a 10 g/l solution of hydrochloric acid R. Examined between 230 nm and 350 nm (2.2.25), the solution shows an absorption maximum at 276 nm. The specific absorbance at the maximum is 55 to 64.

- B. Infrared absorption spectrophotometry (2.2.24).

Preparation: discs of potassium bromide R.

Comparison: salbutamol sulphate CRS.

- C. Thin-layer chromatography (2.2.27).

Test solution. Dissolve 12 mg of the substance to be examined in water R and dilute to 10 ml with the same solvent.

Reference solution. Dissolve 12 mg of salbutamol sulphate CRS in water R and dilute to 10 ml with the same solvent.

Plate: TLC silica gel plate R.

Mobile phase: concentrated ammonia R, water R, ethyl acetate R, 2-propanol R, methyl isobutyl ketone R (3:18:35:45:50 V/V/V/V/V).

Application: 1 µl.

Development: over a path of 18 cm.

Drying: in air.

Detection: spray with a 1 g/l solution of methylbenzothiazolone hydrazone hydrochloride R in a 90 per cent V/V solution of methanol R, followed by a 20 g/l solution of potassium ferricyanide R in a mixture of 1 volume of concentrated ammonia R1 and 3 volumes of water R, followed by a further spraying with a 1 g/l solution of methylbenzothiazolone hydrazone hydrochloride R in a 90 per cent V/V solution of methanol R.

Results: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

D. Dissolve about 10 mg in 50 ml of a 20 g/l solution of *disodium tetraborate R*. Add 1 ml of a 30 g/l solution of *aminopyrazolone R*, 10 ml of *methylene chloride R* and 10 ml of a 20 g/l solution of *potassium ferricyanide R*. Shake and allow to separate. An orange-red colour develops in the methylene chloride layer.

E. It gives reaction (a) of sulphates (2.3.1).

TESTS

Solution S. Dissolve 0.250 g in *carbon dioxide-free water R* and dilute to 25.0 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and not more intensely coloured than reference solution BY₆ (2.2.2, Method II).

Optical rotation (2.2.7): -0.10° to $+0.10^{\circ}$, determined on solution S.

Acidity or alkalinity. To 10 ml of solution S add 0.15 ml of *methyl red solution R* and 0.2 ml of 0.01 M *sodium hydroxide*. The solution is yellow. Not more than 0.4 ml of 0.01 M *hydrochloric acid* is required to change the colour of the indicator to red.

Related substances. Liquid chromatography (2.2.29).

Test solution. Dissolve 0.100 g of the substance to be examined in the mobile phase and dilute to 50.0 ml with the mobile phase.

Reference solution. Dissolve 2.4 mg of *salbutamol sulphate CRS*, 2.0 mg of *salbutamol impurity B CRS*, 3.0 mg of *salbutamol impurity D CRS*, 3.0 mg of *salbutamol impurity F CRS*, 3.0 mg of *salbutamol impurity G CRS* and 3.0 mg of *salbutamol impurity I CRS* in the mobile phase and dilute to 10.0 ml with the mobile phase. Dilute 2.0 ml of the solution to 100.0 ml with the mobile phase.

Column:

- **size:** $l = 0.15$ m, $\varnothing = 3.9$ mm,
- **stationary phase:** spherical *end-capped octylsilyl silica gel for chromatography R* (5 μ m) with a specific surface area of 335 m²/g, a pore size of 10 nm and a carbon loading of 11.7 per cent.

Mobile phase: mix 22 volumes of *acetonitrile R* and 78 volumes of a solution containing 2.87 g/l of *sodium heptanesulphonate R* and 2.5 g/l of *potassium dihydrogen phosphate R* adjusted to pH 3.65 with *dilute phosphoric acid R*.

Flow rate: 1 ml/min.

Detection: spectrophotometer at 220 nm.

Injection: 20 μ l.

Run time: 25 times the retention time of salbutamol.

Relative retention with reference to salbutamol (retention time = about 1.9 min): impurity B = about 1.3, impurity A = about 1.7, impurity C = about 2.0, impurity D = about 2.7, impurity H = about 3.0, impurity E = about 3.1, impurity G = about 4.1, impurity F = about 6.2, impurity I = about 23.2.

System suitability: reference solution:

- **resolution:** minimum of 3.0 between the peaks due to salbutamol and to impurity B.

Limits:

- **impurity D:** not more than the area of the corresponding peak in the chromatogram obtained with the reference solution (0.3 per cent),
- **impurity F:** not more than the area of the corresponding peak in the chromatogram obtained with the reference solution (0.3 per cent),
- **impurity G:** not more than the area of the corresponding peak in the chromatogram obtained with the reference solution (0.3 per cent),
- **impurity I:** not more than the area of the corresponding peak in the chromatogram obtained with the reference solution (0.3 per cent),
- **any other impurity:** not more than 1.5 times the area of the peak due to salbutamol in the chromatogram obtained with the reference solution (0.3 per cent),
- **total:** maximum 1.0 per cent,
- **disregard limit:** 0.05 per cent.

Impurity J: maximum 0.2 per cent.

Dissolve 60.0 mg in a 1 g/l solution of *hydrochloric acid R* and dilute to 25.0 ml with the same solvent. The absorbance (2.2.25) of the solution measured at 310 nm is not greater than 0.10.

Boron: maximum 50 ppm.

Test solution. To 50 mg of the substance to be examined add 5 ml of a solution containing 13 g/l of *anhydrous sodium carbonate R* and 17 g/l of *potassium carbonate R*. Evaporate to dryness on a water-bath and dry at 120 °C. Ignite the residue rapidly until the organic matter has been destroyed, allow to cool and add 0.5 ml of *water R* and 3.0 ml of a freshly prepared 1.25 g/l solution of *curcumin R* in *glacial acetic acid R*. Warm gently to effect solution, allow to cool and add 3.0 ml of a mixture prepared by adding 5 ml of *sulphuric acid R*, slowly and with stirring, to 5 ml of *glacial acetic acid R*. Mix and allow to stand for 30 min. Dilute to 100.0 ml with *alcohol R*, filter and use the filtrate.

Reference solution. Dissolve 0.572 g of *boric acid R* in 1000.0 ml of *water R*. Dilute 1.0 ml to 100.0 ml with *water R*. To 2.5 ml of the solution add 5 ml of a solution containing 13 g/l of *anhydrous sodium carbonate R* and 17 g/l of *potassium carbonate R*, and treat this mixture in the same manner as the test solution.

Measure the absorbance (2.2.25) of the test solution and of the reference solution at the maximum at about 555 nm. The absorbance of the test solution is not greater than that of the reference solution.

Loss on drying (2.2.32): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 100–105 °C.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.400 g in 5 ml of *anhydrous formic acid R* and add 35 ml of *anhydrous acetic acid R*. Titrate with 0.1 M *perchloric acid*, determining the end-point potentiometrically (2.2.20).

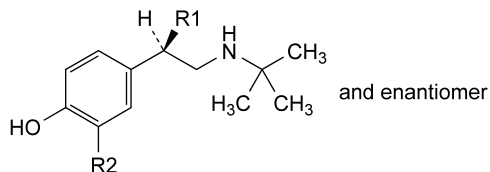
1 ml of 0.1 M *perchloric acid* is equivalent to 57.67 mg of C₂₆H₄₄N₂O₁₀S.

STORAGE

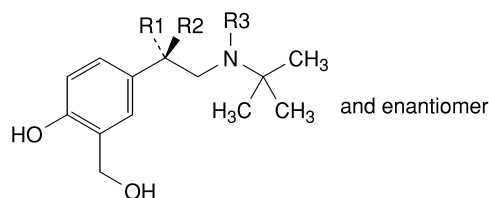
Protected from light.

IMPURITIES

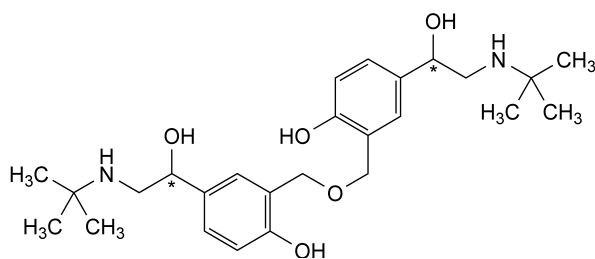
01/2005:0366



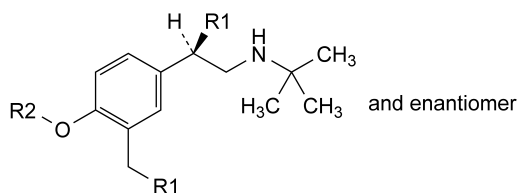
- A. R1 = OCH₃, R2 = CH₂OH: [5-[(1RS)-2-[(1,1-dimethylethyl)amino]-1-methoxyethyl]-2-hydroxyphenyl]methanol,
- B. R1 = OH, R2 = H: (1RS)-2-[(1,1-dimethylethyl)amino]-1-(4-hydroxyphenyl)ethanol,
- C. R1 = OH, R2 = CH₃: (1RS)-2-[(1,1-dimethylethyl)amino]-1-(4-hydroxy-3-methylphenyl)ethanol,
- D. R1 = OH, R2 = CHO: 5-[(1RS)-2-[(1,1-dimethylethyl)amino]-1-hydroxyethyl]-2-hydroxybenzaldehyde,



- E. R1 = H, R2 = OH, R3 = CH₂-C₆H₅: (1RS)-2-[benzyl(1,1-dimethylethyl)amino]-1-[4-hydroxy-3-(hydroxymethyl)phenyl]ethanol,
- G. R1+R2 = O, R3 = CH₂-C₆H₅: 2-[benzyl(1,1-dimethylethyl)amino]-1-[4-hydroxy-3-(hydroxymethyl)phenyl]ethanone,
- J. R1+R2 = O, R3 = H: 2-[(1,1-dimethylethyl)amino]-1-[4-hydroxy-3-(hydroxymethyl)phenyl]ethanone (salbutamone),



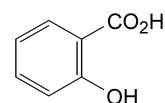
- F. 1,1'-[oxybis[methylene(4-hydroxy-1,3-phenylene)]]bis[2-[(1,1-dimethylethyl)amino]ethanol],



- H. R1 = R2 = H: 4-[2-[(1,1-dimethylethyl)amino]ethyl]-2-methylphenol,
- I. R1 = OH, R2 = CH₂-C₆H₅: (1RS)-2-[(1,1-dimethylethyl)amino]-1-[3-(hydroxymethyl)-4-benzyloxyphenyl]ethanol.

SALICYLIC ACID

Acidum salicylicum

C₇H₆O₃M_r 138.1

DEFINITION

Salicylic acid contains not less than 99.0 per cent and not more than the equivalent of 100.5 per cent of 2-hydroxybenzenecarboxylic acid, calculated with reference to the dried substance.

CHARACTERS

A white, crystalline powder or white or colourless, acicular crystals, slightly soluble in water, freely soluble in alcohol, sparingly soluble in methylene chloride.

IDENTIFICATION

First identification: A, B.

Second identification: A, C.

- A. Melting point (2.2.14): 158 °C to 161 °C.
- B. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *salicylic acid CRS*.
- C. Dissolve about 30 mg in 5 ml of 0.05 M sodium hydroxide, neutralise if necessary and dilute to 20 ml with *water R*. 1 ml of the solution gives reaction (a) of salicylates (2.3.1).

TESTS

Solution S. Dissolve 2.5 g in 50 ml of boiling *distilled water R*, cool and filter.

Appearance of solution. Dissolve 1 g in 10 ml of *alcohol R*. The solution is clear (2.2.1) and colourless (2.2.2, *Method II*).

Related substances. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 0.50 g of the substance to be examined in the mobile phase and dilute to 100.0 ml with the mobile phase.

Reference solution (a). Dissolve 10 mg of *phenol R* in the mobile phase and dilute to 100.0 ml with the mobile phase.

Reference solution (b). Dissolve 25 mg of 4-hydroxyisophthalic acid *R* in the mobile phase and dilute to 100.0 ml with the mobile phase.

Reference solution (c). Dissolve 50 mg of 4-hydroxybenzoic acid *R* in the mobile phase and dilute to 100.0 ml with the mobile phase.

Reference solution (d). Dilute 1.0 ml of reference solution (a) to 10.0 ml with the mobile phase.

Reference solution (e). Dilute a mixture of 1.0 ml of each of reference solutions (a), (b) and (c) to 10.0 ml with the mobile phase.

Reference solution (f). Dilute a mixture of 0.1 ml of each of reference solutions (a), (b) and (c) to 10.0 ml with the mobile phase.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.15 m long and 4.6 mm in internal diameter packed with non-deactivated octadecylsilyl silica gel for chromatography *R* (5 µm),

- as mobile phase at a flow rate of 0.5 ml/min a mixture of 1 volume of *glacial acetic acid R*, 40 volumes of *methanol R* and 60 volumes of *water R*,
- as detector a spectrophotometer set at 270 nm.

Inject 10 µl of reference solutions (d) and (e). When the chromatograms are recorded in the prescribed conditions, the retention times relative to phenol are: 4-hydroxybenzoic acid about 0.70 and 4-hydroxyisophthalic acid about 0.90. Adjust the sensitivity of the system so that the height of the principal peak in the chromatogram obtained with reference solution (f) is at least 70 per cent of the full scale of the recorder. The test is not valid unless: in the chromatogram obtained with reference solution (e), the third peak corresponds to the phenol peak in the chromatogram obtained with reference solution (d) and the resolution between the peaks corresponding to 4-hydroxyisophthalic acid and to phenol is at least 1.0. If this resolution is not obtained adjust the quantity of acetic acid in the mobile phase.

Inject 10 µl of the test solution and 10 µl of reference solution (f). In the chromatogram obtained with the test solution: the areas of the peaks due to 4-hydroxybenzoic acid, 4-hydroxyisophthalic acid and phenol are not greater than the areas of the corresponding peaks in the chromatogram obtained with reference solution (f) (0.1 per cent for 4-hydroxybenzoic acid; 0.05 per cent for 4-hydroxyisophthalic acid and 0.02 per cent for phenol).

In the chromatogram obtained with the test solution: the area of any peak, apart from the principal peak and the peaks due to 4-hydroxybenzoic acid, 4-hydroxyisophthalic acid and phenol, is not greater than that of the peak due to 4-hydroxyisophthalic acid in the chromatogram obtained with reference solution (f) (0.05 per cent); the sum of the areas of all the peaks, apart from the principal peak, is not greater than twice the area of the peak due to 4-hydroxybenzoic acid in the chromatogram obtained with reference solution (f) (0.2 per cent). Disregard any peak with an area less than 0.01 times that of the principal peak in the chromatogram obtained with reference solution (f).

Chlorides (2.4.4). 10 ml of solution S diluted to 15 ml with *water R* complies with the limit test for chlorides (100 ppm).

Sulphates. Not more than 200 ppm. Dissolve 1.0 g in 5 ml of *dimethylformamide R* and add 4 ml of *water R*. Mix thoroughly. Add 0.2 ml of *dilute hydrochloric acid R* and 0.5 ml of a 25 per cent *m/m* solution of *barium chloride R*. After 15 min any opalescence in the solution is not more intense than that in a standard prepared as follows: to 2 ml of *sulphate standard solution (100 ppm SO₄) R* add 0.2 ml of *dilute hydrochloric acid R*, 0.5 ml of a 25 per cent *m/m* solution of *barium chloride R*, 3 ml of *water R* and 5 ml of *dimethylformamide R*.

Heavy metals (2.4.8). Dissolve 2.0 g in 15 ml of *alcohol R* and add 5 ml of *water R*. 12 ml of the solution complies with limit test B for heavy metals (20 ppm). Prepare the standard using lead standard solution (2 ppm Pb) prepared by diluting *lead standard solution (100 ppm Pb) R* with a mixture of 5 volumes of *water R* and 15 volumes of *alcohol R*.

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in a desiccator.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 2.0 g.

ASSAY

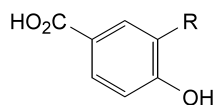
Dissolve 0.120 g in 30 ml of *alcohol R* and add 20 ml of *water R*. Titrate with 0.1 M *sodium hydroxide*, using 0.1 ml of *phenol red solution R* as indicator.

1 ml of 0.1 M *sodium hydroxide* is equivalent to 13.81 mg of C₇H₆O₃.

STORAGE

Store protected from light.

IMPURITIES



- A. R = H: 4-hydroxybenzoic acid,
- B. R = CO₂H: 4-hydroxyisophthalic acid,
- C. phenol.

01/2005:1910

SALMON OIL, FARMED

Salmonis domestici oleum

DEFINITION

Purified fatty oil obtained from fresh farmed *Salmo salar*. The positional distribution (β(2)-acyl) is 60-70 per cent for cervonic (docosahexaenoic) acid (C22:6 n-3; DHA), 25-35 per cent for timnodonic (eicosapentaenoic) acid (C20:5 n-3; EPA) and 40-55 per cent for moroctic acid (C18:4 n-3).

Content:

- sum of the contents of EPA and DHA (expressed as triglycerides): 10.0 per cent to 28.0 per cent.

Authorised antioxidants in concentrations not exceeding the levels specified by the competent authority may be added.

PRODUCTION

The fish shall only be given feed with a composition that is in accordance with the relevant EU or other applicable regulations.

The oil is produced by mechanical expression of fresh raw materials, either from the whole fish, or fish where the fillets have been removed, at a temperature not exceeding 100 °C, and without using solvents. After centrifugation, solid substances may be removed from the oil by cooling and filtering (winterisation).

CHARACTERS

Appearance: pale pink liquid.

Solubility: practically insoluble in water, very soluble in acetone and in heptane, slightly soluble in anhydrous ethanol.

IDENTIFICATION

Examine the ¹³C NMR spectra obtained in the assay for positional distribution (β(2)-acyl) of fatty acids. The spectra contain peaks between 172 ppm and 173 ppm with shifts similar to those in the type spectrum (Figure 1910-2). The oil to be examined complies with the limits of this assay.

TESTS

Absorbance (2.2.25): minimum 0.10, measured at the maximum between 470 nm and 480 nm.

Dissolve 5.0 ml in 5.0 ml of *trimethylpentane R*.

Acid value (2.5.1): maximum 2.0.

Anisidine value (2.5.36): maximum 10.0.

Peroxide value (2.5.5, Method A): maximum 5.0.

Unsaponifiable matter (2.5.7): maximum 1.5 per cent, determined on 5.0 g.

Linoleic acid (2.4.29): maximum 11.0 per cent.

Identify the peak due to linoleic acid using the chromatogram in Figure 1910.-1. Determine the percentage content by normalisation.

ASSAY

Positional distribution (β (2)-acyl) of fatty acids (2.2.33)

Use a high resolution FT-NMR spectrometer operating at minimum 300 MHz.

Test solution. Dissolve 190-210 mg of fresh salmon oil in 500 μ l of deuterated chloroform *R*. Prepare at least 3 samples and examine within 3 days.

Acquisition of ^{13}C NMR spectra. The following parameters may be used.

- *sweep width*: 200 ppm (–5 to 195 ppm),
- *irradiation frequency offset*: 95 ppm,

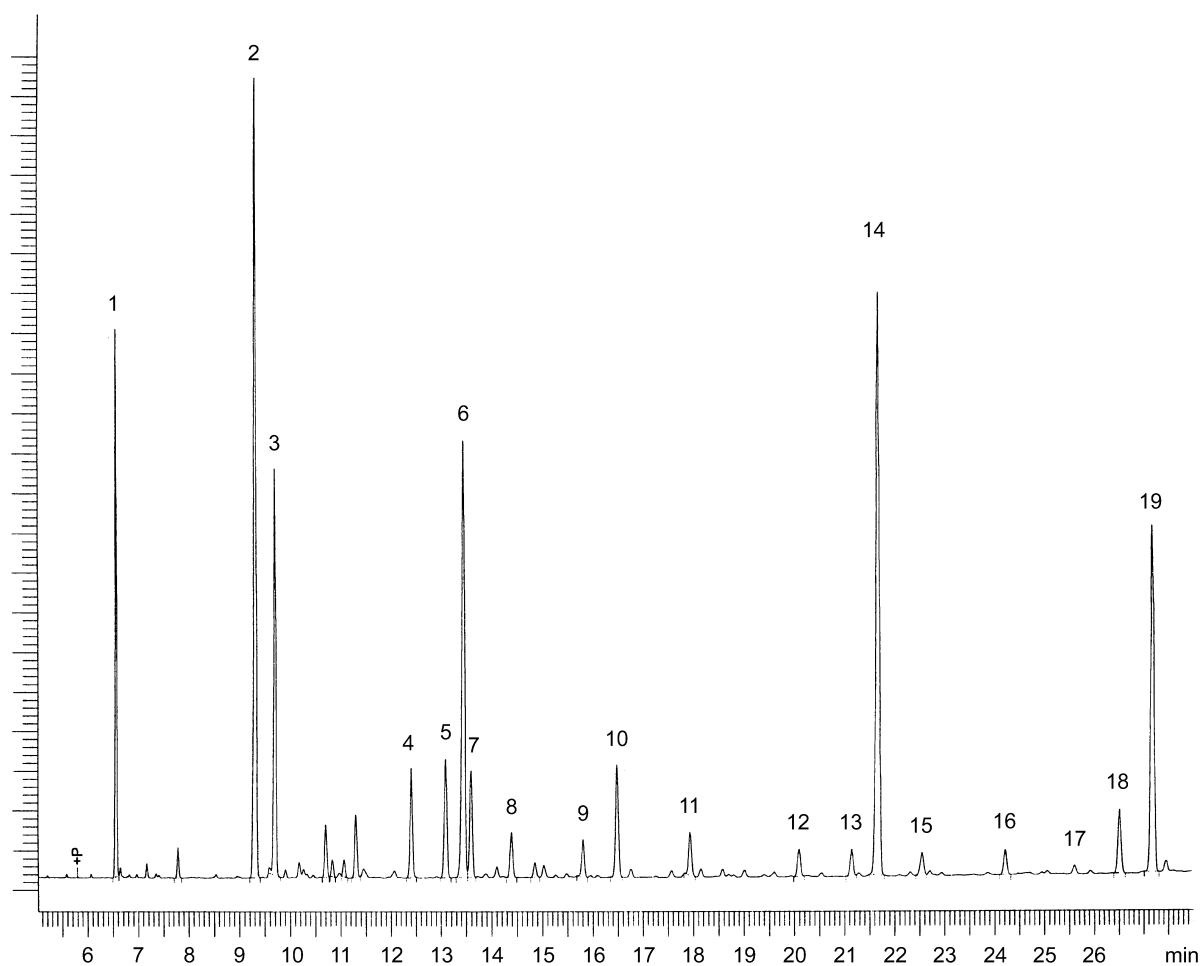
- *time domain*: 64 K,
- *pulse delay*: 2 s,
- *pulse program*: zgig 30 (inverse gated, 30° excitation pulse),
- *dummy scans*: 4,
- *number of scans*: 4096.

Processing and plotting. The following parameters may be used:

- *size*: 64 K (zero-filling),
- *window multiplication*: exponential,
- *Lorentzian broadening factor*: 0.2 Hz.

Use the CDCl_3 signal for shift referencing. The shift of the central peak of the 1:1:1 triplet is set to 77.16 ppm.

Plot the spectral region δ 171.5-173.5 ppm. Compare the spectrum with the reference spectrum in Figure 1910.-2. The shift values lie within the ranges given in Table 1910.-1.



1. C14:0	6. C18:1 n-9	11. C20:1 n-9	16. C21:5 n-3
2. C16:0	7. C18:1 n-7	12. C20:4 n-6	17. C22:5 n-6
3. C16:1 n-7	8. C18:2 n-6	13. C20:4 n-3	18. C22:5 n-3
4. C16:4 n-1	9. C18:3 n-3	14. EPA	19. DHA
5. C18:0	10. C18:4 n-3	15. C22:1 n-11	

Figure 1910.-1. – Chromatogram for the composition of fatty acids in farmed salmon oil

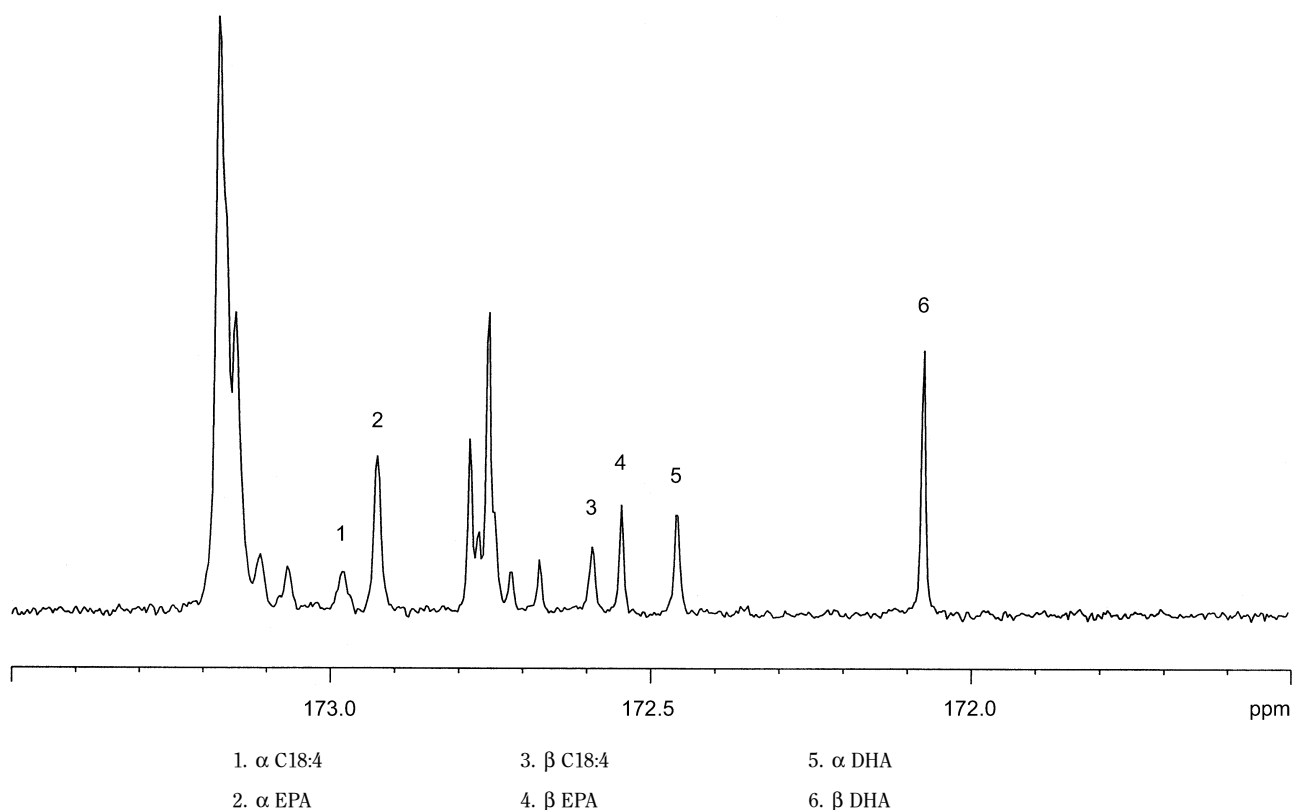
Figure 1910.-2. – ^{13}C NMR spectrum carbonyl region of farmed salmon oil

Table 1910.-1 – Shift values

Signal	Shift range (ppm)
β DHA	172.05 - 172.09
α DHA	172.43 - 172.47
β EPA	172.52 - 172.56
α EPA	172.90 - 172.94
β C18:4	172.56 - 172.60
α C18:4	172.95 - 172.99

System suitability: calculate the signal-to-noise ratio for the smallest relevant peak corresponding to α C18:4 signal (in the range δ 172.95-172.99 ppm). Measure the peak width at half-height for the central CDCl_3 signal (at δ 77.16 ppm). The system suitability criteria in Table 1910.-2 are fulfilled.

Table 1910.-2 – System suitability criteria

S/N smallest peak	Peak width (ppm)
minimum 5	maximum 0.02

Calculation of positional distribution: calculate the positional distribution ($\beta(2)$ -acyl) using the expression:

$$\frac{\beta}{\alpha + \beta} \times 100$$

- α = peak area of the corresponding α -carbonyl peak,
 β = peak area of β -carbonyl peak from C22:6 n-3, C20:5 n-3 or C18:4 n-3, respectively.

Limits:

	Positional distribution (mole %)		
	β DHA	β EPA	β C18:4
Limits	60 - 70	25 - 35	40 - 55

EPA and DHA (2.4.29). See Figure 1910.-1.

STORAGE

In an airtight, well-filled container, protected from light, under inert gas.

LABELLING

The label states the name and concentration of any added antioxidant.

01/2005:1848

SAW PALMETTO FRUIT

Sabal serrulatae fructus

DEFINITION

Dried ripe fruit of *Serenoa repens* (Bartram) Small. (*Sabal serrulata* (Michaux) Nichols).

Content: minimum 11.0 per cent of total fatty acids (dried drug).

CHARACTERS

Characteristic, strong, unpleasant but not rancid odour.

Macroscopic and microscopic characters described under identification tests A and B.

IDENTIFICATION

First identification: A, B, D.

Second identification: A, B, C.

- A. The fruit is an ovoid to subspherical drupe, with a dark brown to blackish, roughly wrinkled surface and more or less coppery sheen, up to 2.5 cm long and 1.5 cm in diameter. The apex sometimes bears the remains of the style and tubular calyx, with 3 teeth, and the base bears a small depression with the scar of the stalk. The epicarp and underlying mesocarp form a thin fragile layer, which partially peels off, revealing the thin, hard, pale brown endocarp, which is fibrous and easily separable. The

seed is irregularly spherical to ovoid, up to 12 mm long and 8 mm in diameter, with a hard, smooth or finely pitted surface which is reddish-brown with a paler, raised and membranous area over the raphe and micropyle; cut transversely, the seed has a thin testa, narrow perisperm and a large area of dense, horny, greyish-white endosperm, with the embryo positioned to one side.

- B. Reduce to a powder (710). The powder is reddish or blackish-brown and oily. Examine under a microscope using *chloral hydrate solution R*. The powder shows fragments of epicarp composed of several layers of thin-walled, reddish-brown, pigmented, polyhedral cells (10 µm to 40 µm) which are strongly cuticularised; those of the outer layers are much smaller than those of the inner layers. Parenchyma cells of the mesocarp may be large and filled with oil droplets, or smaller and containing nodules of silica. Groups of xylem tissue of the mesocarp show small lignified, annular or spirally thickened vessels. Stone cells of the mesocarp (20 µm to 200 µm) may be found scattered, usually singly but sometimes in small groups, the walls are moderately thickened, distinctly striated and finely pitted. Fragments of endocarp contain groups of elongated sclereids about 300 µm long, with strongly thickened walls and numerous pits. The seed testa consists of small, thin-walled cells with brownish contents and underlying sclereids; albumen cells are thick-walled with large conspicuous pits and contain aleurone grains and fixed oil.

C. Thin-layer chromatography (2.2.27).

Test solution. To 1.5 g of the powdered drug (710), add 20 ml of *alcohol R* and stir for 15 min. Filter.

Reference solution. Dissolve 4 mg of *β-amyryn R* and 10 mg of *β-sitosterol R* in 10 ml of *alcohol R*.

Plate: TLC silica gel plate *R* (2-10 µm).

Mobile phase: *acetic acid R*, *ethyl acetate R*, *toluene R* (1:30:70 V/V/V).

Application: 8 µl of the test solution and 2 µl of the reference solution, as bands.

Development: over a path of 10 cm.

Drying: in air.

Detection: spray with *anisaldehyde solution R*; dry the plate at 100-105 °C for 5-10 min; examine in daylight.

Results: see below the sequence of the zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other faint zones are present, especially in the lower third, in the chromatogram obtained with the test solution.

Top of the plate	
	A strong blue zone
	A faint blue zone
	A faint blue zone
	A strong bluish-violet zone
	A faint blue zone
β-Amyryn: a blue zone	
β-Sitosterol: a blue zone	
	A faint blue zone
Reference solution	Test solution

- D. Examine the chromatograms obtained in the assay of total fatty acids.

Results: the characteristic peaks in the chromatogram obtained with the test solution are similar in retention time to the characteristic peaks in the chromatogram obtained with the reference solution. The principal peak is due to lauric acid.

TESTS

Foreign matter (2.8.2): maximum 2.0 per cent.

Loss on drying (2.2.32): maximum 12.0 per cent, determined on 1.000 g of the powdered drug (710) by drying in an oven at 100-105 °C for 2 h.

Total ash (2.4.16): maximum 5.0 per cent.

ASSAY

Total fatty acids. Gas chromatography (2.2.28).

Internal standard solution. Dissolve 0.47 g of *methyl pelargonate R* and 0.47 g of *methyl margarate R* in 20.0 ml of *dimethylformamide R* and dilute to 100.0 ml with the same solvent.

Test solution. Reduce 50 g of the drug to a powder (200). Place 4.0 g of the powdered drug in a 100 ml volumetric flask. Add 60.0 ml of *dimethylformamide R*. Mix using sonication for 15 min and then shake for 30 min. Dilute to 100.0 ml with *dimethylformamide R*. Allow to stand for a few minutes and filter. To 20.0 ml of this solution add 4.0 ml of the internal standard solution and dilute to 25.0 ml with *dimethylformamide R*. To 0.4 ml of this solution add 0.6 ml of an 18.84 g/l solution of *trimethylsulphonium hydroxide R* in *methanol R* and mix.

Reference solution. Dissolve 32.0 mg of *caproic acid R*, 62.0 mg of *caprylic acid R*, 68.0 mg of *capric acid R*, 0.699 g of *lauric acid R*, 0.267 g of *myristic acid R*, 10.0 mg of *palmitoleic acid R*, 0.217 g of *palmitic acid R*, 0.115 g of *linoleic acid R*, 18.0 mg of *linolenic acid R*, 0.870 g of *oleic acid R* and 49.0 mg of *stearic acid R* in *dimethylformamide R* and dilute to 10.0 ml with the same solvent. To 1.0 ml of the solution add 4.0 ml of the internal standard solution and dilute to 25.0 ml with *dimethylformamide R*. To 0.4 ml of this solution add 0.6 ml of an 18.84 g/l solution of *trimethylsulphonium hydroxide R* in *methanol R* and mix.

Column:

- **material:** fused silica,
- **size:** *l* = 25 m (a film thickness of 1 µm may be used) to 60 m (a film thickness of 0.2 µm may be used), Ø = 0.20-0.53 mm,
- **stationary phase:** *poly(dimethyl)siloxane R*.

Carrier gas: *helium for chromatography R*.

Flow rate: 0.5 ml/min.

Split ratio: 1:40.

Temperature:

	Time (min)	Temperature (°C)
Column	0 - 2	150
	2 - 7	150 → 190
	7 - 22	190 → 220
Injection port		300
Detector		300

Detection: flame ionisation.

Injection: 1.0 µl.

Using the retention times determined from the chromatogram obtained with the reference solution, locate the components of the reference solution on the chromatogram obtained with the test solution.

Use the expression below to determine the percentage content of the different fatty acids. Determine the content of caproic acid, caprylic acid, capric acid and lauric acid using methyl pelargonate as the internal standard. Determine the content of myristic acid, palmitoleic acid, palmitic acid, linoleic acid, linolenic acid, oleic acid and stearic acid using methyl margarate as the internal standard. The peak area of lauric acid is not less than 20 per cent of the total area of the peaks.

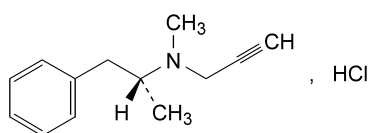
$$\frac{A_1}{A_3} \times \frac{A_2}{A_4} \times \frac{m_2}{m_1} \times p \times 0.5$$

- A_1 = area of the peak due to the considered derivatised fatty acid in the chromatogram obtained with the test solution,
- A_2 = area of the peak due to methyl pelargonate or methyl margarate in the chromatogram obtained with the reference solution,
- A_3 = area of the peak due to methyl pelargonate or methyl margarate in the chromatogram obtained with the test solution,
- A_4 = area of the peak due to the considered derivatised fatty acid in the chromatogram obtained with the reference solution,
- m_1 = mass of the test sample, in grams,
- m_2 = mass of the considered fatty acid in the reference solution, in grams,
- p = percentage purity of the considered fatty acid in the reference solution.

01/2005:1260

SELEGILINE HYDROCHLORIDE

Selegilini hydrochloridum

 $C_{13}H_{18}ClN$ M_r 223.7

DEFINITION

Selegiline hydrochloride contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of *N*-methyl-*N*[(1*R*)-1-methyl-2-phenylethyl]prop-2-yn-1-amine hydrochloride, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, crystalline powder, freely soluble in water and in methanol, slightly soluble in acetone.

It melts at about 143 °C.

IDENTIFICATION

- A. Dissolve 2.000 g in *carbon dioxide-free water R* and dilute to 20.0 ml with the same solvent. The specific optical rotation (2.2.7) is -10.0 to -12.0 , calculated with reference to the dried substance.
- B. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *selegiline hydrochloride CRS*. Examine the substances as discs prepared using *potassium chloride R*.

C. It gives reaction (a) of chlorides (2.3.1).

TESTS

pH (2.2.3). Dissolve 0.20 g in *carbon dioxide-free water R* and dilute to 10 ml with the same solvent. The pH of the solution is 3.5 to 4.5.

Related substances. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 20 mg of the substance to be examined in the mobile phase and dilute to 10.0 ml with the mobile phase.

Reference solution (a). Dissolve 50.0 mg of *selegiline hydrochloride CRS* and 10.0 mg of *butyl parahydroxybenzoate R* in the mobile phase and dilute to 50.0 ml with the mobile phase. Dilute 1.0 ml to 20.0 ml with the mobile phase.

Reference solution (b). Dilute 1.0 ml of the test solution to 10.0 ml with the mobile phase. Dilute 1.0 ml to 50.0 ml with the mobile phase.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4.6 mm in internal diameter packed with *octylsilyl silica gel for chromatography R* (5 µm),
- as mobile phase at a flow rate of 1 ml/min a mixture prepared as follows: dilute 500 ml of *acetonitrile R* to 1000.0 ml with a butylammonium acetate buffer solution pH 6.5 prepared by dissolving 4 ml of *butylamine R* in 900 ml of *water R* and adjusting to pH 6.5 with *acetic acid R* before diluting to 1000.0 ml with *water R*,
- as detector a spectrophotometer set at 215 nm.

Inject 20 µl of reference solution (a). The test is not valid unless, in the chromatogram obtained with reference solution (a), the resolution between the peaks corresponding to selegiline and butyl parahydroxybenzoate is greater than three. Inject 20 µl of the test solution and 20 µl of reference solution (b). Continue the chromatography for 1.7 times the retention time of selegiline. In the chromatogram obtained with the test solution: the area of any peak apart from the principal peak is not greater than that of the peak in the chromatogram obtained with reference solution (b) (0.2 per cent); and the sum of the areas of such peaks is not greater than 2.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent). Disregard any peak due to chlorides and any peak with an area less than 0.1 times that of the principal peak in the chromatogram obtained with reference solution (b) (0.02 per cent).

(S)-Selegiline. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 20.0 mg of the substance to be examined in a mixture of 1 ml of *2-propanol R* and 10 µl of *butylamine R* and dilute to 10.0 ml with the mobile phase.

Reference solution (a). Dissolve 8.0 mg of *(RS)*-selegiline hydrochloride CRS in a mixture of 10 µl of *butylamine R* and 1 ml of *2-propanol R* and dilute to 10.0 ml with the mobile phase.

Reference solution (b). Dilute 0.5 ml of reference solution (a) to 20.0 ml with the mobile phase.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4.6 mm in internal diameter packed with *silica gel OD for chiral separation R*,
- as the mobile phase at a flow rate of 1 ml/min a mixture of 0.2 volumes of *2-propanol R* and 99.8 volumes of *cyclohexane R*,
- as detector a spectrophotometer set at 220 nm.

Inject 20 µl of each solution. When the chromatograms are recorded in the prescribed conditions, the retention time of (S)-selegiline is about 10 min. Adjust the sensitivity of the system so that the height of the peaks in the chromatogram obtained with reference solution (b) is about 10 per cent of the full scale of the recorder. The test is not valid unless in the chromatogram obtained with reference solution (a) the resolution between the peaks corresponding to (S)-selegiline and (R)-selegiline is at least 1.5. If necessary, adjust the concentration of 2-propanol in the mobile phase. In the chromatogram obtained with the test solution, any peak due to (S)-selegiline is not greater than the area of the corresponding peak in the chromatogram obtained with reference solution (b) (0.5 per cent).

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying at 60 °C at a pressure not exceeding 0.5 kPa.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

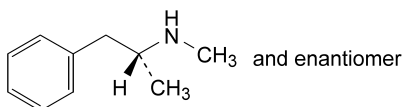
Dissolve 0.180 g in 50 ml of *acetic anhydride R*. Titrate with 0.1 M *perchloric acid*, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *perchloric acid* is equivalent to 22.37 mg of C₁₃H₁₈ClN.

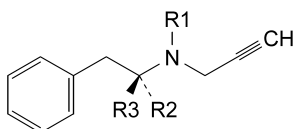
STORAGE

Store protected from light.

IMPURITIES



- A. (2*RS*)-*N*-methyl-1-phenylpropan-2-amine [(*RS*)-metamphetamine],
- B. (2*R*)-1-phenylpropan-2-amine (amphetamine),
- C. (1*RS*,2*SR*)-2-amino-1-phenylpropan-1-ol (phenylpropanolamine),



- D. R₁ = R₃ = H, R₂ = CH₃: *N*-[(1*R*)-1-methyl-2-phenylethyl]prop-2-yn-1-amine (demethylselegiline),
- E. R₁ = R₃ = CH₃, R₂ = H: *N*-methyl-*N*-[(1*S*)-1-methyl-2-phenylethyl]prop-2-yn-1-amine [(*S*)-selegiline].

01/2005:1147

SELENIUM DISULPHIDE

Selenii disulfidum

SeS₂

*M*_r 143.1

DEFINITION

Selenium disulphide contains not less than 52.0 per cent and not more than 55.5 per cent of Se.

CHARACTERS

A bright-orange or reddish-brown powder, practically insoluble in water.

IDENTIFICATION

- A. Gently boil about 50 mg with 5 ml of *nitric acid R* for 30 min. Dilute to 50 ml with *water R* and filter. To 5 ml of the filtrate add 10 ml of *water R* and 5 g of *urea R*. Heat to boiling, cool and add 1.5 ml of *potassium iodide solution R*. A yellow to orange colour is produced which darkens rapidly on standing. This solution is used in identification test B.
- B. Allow the coloured solution obtained under identification A to stand for 10 min and filter through *kieselguhr for chromatography R*. 5 ml of the filtrate give reaction (a) of sulphates (2.3.1).

TESTS

Soluble selenium compounds. To 10 g add 100 ml of *water R*, mix well, allow to stand for 1 h with frequent shaking and filter. To 10 ml of the filtrate add 2 ml of a 115 g/l solution of *anhydrous formic acid R*, dilute to 50 ml with *water R* and adjust to pH 2.0 to 3.0 with an 115 g/l solution of *anhydrous formic acid R*. Add 2 ml of a 5 g/l solution of 3,3'-*diaminobenzidine tetrahydrochloride R*. Allow to stand for 45 min and then adjust to pH 6.0 to 7.0 with *dilute ammonia R1*. Shake the solution for 1 min with 10 ml of *toluene R* and allow the phases to separate. The absorbance (2.2.25) of the upper layer measured at 420 nm is not greater than that of a standard prepared at the same time and in the same manner beginning at the words "add 2 ml of an 115 g/l solution of *anhydrous formic acid R*" and using 5 ml of *selenium standard solution (1 ppm Se) R* (5 ppm, calculated as Se).

ASSAY

To 0.100 g add 25 ml of *fuming nitric acid R* and heat on a water-bath for 1 h; a small insoluble residue may remain. Cool and dilute to 100.0 ml with *water R*. To 25.0 ml add 50 ml of *water R* and 5 g of *urea R* and heat to boiling. Cool, add 7 ml of *potassium iodide solution R*, 3 ml of *starch solution R* and titrate immediately with 0.1 M *sodium thiosulphate*. Carry out a blank titration.

1 ml of 0.1 M *sodium thiosulphate* is equivalent to 1.974 mg of Se.

01/2005:0202

SENEGA ROOT

Polygalae radix

DEFINITION

Senega root consists of the dried and usually fragmented root and root crown of *Polygala senega* L. or of certain other closely related species or of a mixture of these *Polygala* species.

CHARACTERS

Senega root has a faint, sweet odour, slightly rancid or reminiscent of methyl salicylate.

Reduced to a powder, it is irritant and sternutatory. Shaken with water, the powder produces a copious froth.

It has the macroscopic and microscopic characters described under identification tests A, B and C.

IDENTIFICATION

- A. The root crown is greyish-brown and wider than the root; it forms an irregular head consisting of numerous remains of stems and tightly packed purplish-brown buds. The taproot is brown to yellow, occasionally branched, sometimes flexuous, usually tortuous without secondary

roots, except in the Japanese varieties and species, which contain numerous fibrous rootlets. The diameter is usually 1 mm to 8 mm at the crown, gradually tapering to the tip; the surface is transversely and longitudinally striated and often shows a more or less distinct decurrent, elongated spiral keel. The fracture is short and shows a yellowish cortex of varying thickness surrounding a paler central woody area somewhat circular or irregular in shape depending on the species.

- B. Examine under a microscope using *chloral hydrate solution R*. The transverse section of the root shows cork formed from several layers of thin-walled cells, phelloderm of slightly collenchymatous cells containing droplets of oil; the phloem and xylem arrangement is usually normal, especially near the crown but where a keel is present this is formed by increased development of phloem; other anomalous secondary development sometimes occurs, resulting in the formation of one or two large wedge-shaped rays in the phloem and xylem, the parenchymatous cells of which contain droplets of oil. The xylem is usually central and consists of vessels up to 60 µm in diameter associated with numerous thin-walled tracheids and a few small lignified parenchymatous cells.
- C. Reduce to a powder (355). The powder is light brown. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters: longitudinal fragments of lignified tissue made up of pitted tracheids and somewhat larger vessels with numerous bordered pits or with reticulate thickening; yellowish parenchyma and collenchymatous cells containing droplets of oil; occasional fragments of cork, and of epidermal tissue with stomata and unicellular trichomes from the bud scales. Crystals and stone cells are absent.
- D. Examine by thin-layer chromatography (2.2.27), using *silica gel G R* as the coating substance.

Test solution. To 1.0 g of the powdered drug (355) add 10 ml of *alcohol (70 per cent V/V) R*, boil under a reflux condenser for 15 min, filter and allow to cool.

Reference solution. Dissolve 10 mg of *aescin R* in *alcohol (70 per cent V/V) R* and dilute to 10 ml with the same solvent.

Apply to the plate as bands 20 mm by 3 mm 10 µl of the test solution and 10 µl and 40 µl of the reference solution. Develop over a path of 12 cm using the upper layer of a mixture of 10 volumes of *glacial acetic acid R*, 40 volumes of *water R* and 50 volumes of *butanol R*. Dry the plate at 100-105 °C, spray with about 10 ml of *anisaldehyde solution R* for a plate 200 mm square and heat again at 100-105 °C, observing the chromatogram obtained with the test solution until red zones (corresponding to saponosides) appear. In the chromatogram obtained with the test solution, three to five red zones appear in the lower and middle parts, similar in position to the grey-violet zones corresponding to aescin in the chromatogram obtained with the reference solution. Spray the plate with about 10 ml of a 200 g/l solution of *phosphomolybdic acid R* in *ethanol R* and heat at 100-105 °C, observing the chromatograms, until the zones corresponding to saponosides become blue. The intensity and size of the zones in the chromatogram obtained with the test solution are between those of the two bands corresponding to aescin in the chromatograms obtained respectively with 10 µl and 40 µl of the reference solution.

TESTS

Foreign matter (2.8.2). It complies with the test for foreign matter.

Total ash (2.4.16). Not more than 6.0 per cent.

Ash insoluble in hydrochloric acid (2.8.1). Not more than 3.0 per cent.

STORAGE

Store protected from light and humidity.

01/2005:0206

SENNA LEAF

Sennae folium

DEFINITION

Senna leaf consists of the dried leaflets of *Cassia senna* L. (*C. acutifolia* Delile), known as Alexandrian or Khartoum senna, or *Cassia angustifolia* Vahl, known as Tinnevely senna, or a mixture of the two species. It contains not less than 2.5 per cent of hydroxyanthracene glycosides, calculated as sennoside B ($C_{42}H_{38}O_{20}$; M_r 863) with reference to the dried drug.

CHARACTERS

Senna leaf has a slight characteristic odour.

It has the macroscopic and microscopic characters described under identification tests A and B.

IDENTIFICATION

- A. *C. senna* occurs as greyish-green to brownish-green, thin, fragile leaflets, lanceolate, mucronate, asymmetrical at the base, usually 15 mm to 40 mm long and 5 mm to 15 mm wide, the maximum width being at a point slightly below the centre; the lamina is slightly undulant with both surfaces covered with fine, short trichomes. Pinnate venation is visible mainly on the lower surface, with lateral veins leaving the midrib at an angle of about 60° and anastomosing to form a ridge near the margin. **Stomatal index (2.8.3):** 10-12.5-15.

C. angustifolia occurs as yellowish-green to brownish-green leaflets, elongated and lanceolate, slightly asymmetrical at the base, usually 20 mm to 50 mm long and 7 mm to 20 mm wide at the centre. Both surfaces are smooth with a very small number of short trichomes and are frequently marked with transverse or oblique lines. **Stomatal index (2.8.3):** 14-17.5-20

- B. Reduce to a powder (355). The powder is light green to greenish-yellow. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters: polygonal epidermal cells showing paracytic stomata (2.8.3); unicellular trichomes, conical in shape, with warted walls, isolated or attached to fragments of epidermis; fragments of vascular bundles with a crystal sheath of prismatic crystals of calcium oxalate; cluster crystals isolated or in fragments of parenchyma.
- C. Examine by thin-layer chromatography (2.2.27), using *silica gel G R* as the coating substance.
- Test solution.** To 0.5 g of the powdered drug (180) add 5 ml of a mixture of equal volumes of *alcohol R* and *water R* and heat to boiling. Centrifuge and use the supernatant liquid.
- Reference solution.** Dissolve 10 mg of *senna extract CRS* in 1 ml of a mixture of equal volumes of *alcohol R* and *water R* (a slight residue remains).

Apply to the plate as bands 20 mm by 2 mm 10 µl of each solution. Develop over a path of 10 cm using a mixture of 1 volume of *glacial acetic acid R*, 30 volumes of *water R*, 40 volumes of *ethyl acetate R* and 40 volumes of *propanol R*. Allow the plate to dry in air, spray with a 20 per cent V/V solution of *nitric acid R* and heat at 120 °C for 10 min. Allow to cool and spray with a 50 g/l solution of *potassium hydroxide R* in *alcohol (50 per cent V/V) R* until the zones appear. The principal zones in the chromatogram obtained with the test solution are similar in position (sennosides B, A, D and C in the order of increasing R_f value), colour and size to the principal zones in the chromatogram obtained with the reference solution. Between the zones corresponding to sennosides D and C a red zone corresponding to rhein-8-glucoside may be visible.

- D. Place about 25 mg of the powdered drug (180) in a conical flask and add 50 ml of *water R* and 2 ml of *hydrochloric acid R*. Heat in a water-bath for 15 min, cool and shake with 40 ml of *ether R*. Separate the ether, dry over *anhydrous sodium sulphate R*, evaporate 5 ml to dryness and to the cooled residue add 5 ml of *dilute ammonia R1*. A yellow or orange colour develops. Heat on a water-bath for 2 min. A reddish-violet colour develops.

TESTS

Foreign matter (2.8.2). Not more than 3 per cent of foreign organs and not more than 1 per cent of foreign elements.

Loss on drying (2.2.32). Not more than 12.0 per cent, determined on 1.000 g of the powdered drug (355) by drying in an oven at 100-105 °C for 2 h.

Total ash (2.4.16). Not more than 12.0 per cent.

Ash insoluble in hydrochloric acid (2.8.1). Not more than 2.5 per cent.

ASSAY

Carry out the assay protected from bright light.

Place 0.150 g of the powdered drug (180) in a 100 ml flask. Add 30.0 ml of *water R*, mix, weigh and place in a water-bath. Heat under a reflux condenser for 15 min. Allow to cool, weigh and adjust to the original mass with *water R*. Centrifuge and transfer 20.0 ml of the supernatant liquid to a 150 ml separating funnel. Add 0.1 ml of *dilute hydrochloric acid R* and shake with three quantities, each of 15 ml, of *chloroform R*. Allow to separate and discard the chloroform layer. Add 0.10 g of *sodium hydrogen carbonate R* and shake for 3 min. Centrifuge and transfer 10.0 ml of the supernatant liquid to a 100 ml round-bottomed flask with a ground-glass neck. Add 20 ml of *ferric chloride solution R1* and mix. Heat for 20 min under a reflux condenser in a water-bath with the water level above that of the liquid in the flask; add 1 ml of *hydrochloric acid R* and heat for a further 20 min, with frequent shaking, to dissolve the precipitate. Cool, transfer the mixture to a separating funnel and shake with three quantities, each of 25 ml, of *ether R* previously used to rinse the flask. Combine the ether layers and wash with two quantities, each of 15 ml, of *water R*. Transfer the ether layers to a volumetric flask and dilute to 100.0 ml with *ether R*. Evaporate 10.0 ml carefully to dryness and dissolve the residue in 10.0 ml of a 5 g/l solution of *magnesium acetate R* in *methanol R*. Measure the absorbance (2.2.25) at 515 nm, using *methanol R* as the compensation liquid.

Calculate the percentage content of sennoside B from the expression:

$$\frac{A \times 1.25}{m}$$

taking the specific absorbance to be 240.

A = absorbance at 515 nm,

m = mass of the substance to be examined, in grams.

STORAGE

Store protected from light and moisture.

01/2005:1261

SENNA LEAF DRY EXTRACT, STANDARDISED

Sennae folii extractum siccum normatum

DEFINITION

Standardised senna leaf dry extract is produced from *Senna leaf (0206)*. It contains not less than 5.5 per cent and not more than 8.0 per cent of hydroxyanthracene glycosides, calculated as sennoside B ($C_{42}H_{38}O_{20}$; M_r 863) with reference to the dried extract.

The measured content does not deviate from the value stated on the label by more than ± 10 per cent.

PRODUCTION

The extract is produced from the drug and ethanol 50 to 80 per cent V/V with an appropriate procedure.

CHARACTERS

Brownish or brown powder.

IDENTIFICATION

- A. Examine by thin-layer chromatography (2.2.27), using a suitable silica gel as the coating substance.

Test solution. To 0.1 g of the extract add 5 ml of a mixture of equal volumes of *alcohol R* and *water R* and heat to boiling. Cool and centrifuge. Use the supernatant liquid.

Reference solution. Dissolve 10 mg of *senna extract CRS* in 1 ml of a mixture of equal volumes of *alcohol R* and *water R* (a slight residue remains).

Apply separately to the plate, as bands, 10 µl of each solution. Develop over a path of 10 cm using a mixture of 1 volume of *glacial acetic acid R*, 30 volumes of *water R*, 40 volumes of *ethyl acetate R* and 40 volumes of *1-propanol R*. Allow the plate to dry in air, spray with a 20 per cent V/V solution of *nitric acid R* and heat at 120 °C for 10 min. Allow to cool and spray with a 50 g/l solution of *potassium hydroxide R* in *alcohol (50 per cent V/V) R* until the zones appear. The principal zones in the chromatogram obtained with the test solution are similar in position, colour and size to the principal zones in the chromatogram obtained with the reference solution. The chromatograms show in the lower third a prominent brown zone corresponding to sennoside B and above it a yellow zone followed by another prominent brown zone corresponding to sennoside A. In the upper half of the chromatograms are visible, in increasing R_f value, a prominent reddish-brown band and an orange-brown zone followed by a faint pink band and two yellow bands. Close to the solvent front a dark pink zone appears, which may be followed by several faint bands.

- B. Place about 25 mg of the extract in a conical flask and add 50 ml of *water R* and 2 ml of *hydrochloric acid R*. Heat in a water-bath for 15 min, cool and shake with 40 ml of *ether R*. Separate the ether, dry over *anhydrous sodium sulphate R*, evaporate 5 ml to dryness and to

the cooled residue add 5 ml of *dilute ammonia R1*. A yellow or orange colour develops. Heat on a water-bath for 2 min. A reddish-violet colour develops.

TESTS

Loss on drying (2.8.17): maximum 5.0 per cent.

Microbial contamination. Total viable aerobic count (2.6.12) not more than 10^4 micro-organisms per gram, of which not more than 10^2 fungi per gram, determined by plate-count. It complies with the tests for *Escherichia coli* and for *Salmonella* (2.6.13).

ASSAY

Carry out the assay protected from bright light.

Place 0.150 g of the extract in a 100 ml flask, dissolve in *water R* and dilute to 100.0 ml with the same solvent. Filter the solution, discard the first 10 ml of the filtrate. Transfer 20.0 ml of the filtrate to a 150 ml separating funnel. Add 0.1 ml of *dilute hydrochloric acid R* and shake with three quantities, each of 15 ml, of *ether R*. Allow the layers to separate and discard the ether layer. Add 0.10 g of *sodium hydrogen carbonate R* to the aqueous layer and shake for 3 min. Centrifuge and transfer 10.0 ml of the supernatant liquid to a 100 ml round-bottomed flask with a ground-glass neck. Add 20 ml of *ferric chloride solution R1* and mix. Heat for 20 min under a reflux condenser in a water-bath with the water level above that of the liquid in the flask; add 3 ml of *hydrochloric acid R* and heat for a further 30 min with frequent shaking to dissolve the precipitate. Cool, transfer the mixture to a separating funnel and shake with three quantities, each of 25 ml, of *ether R* previously used to rinse the flask. Combine the ether layers and wash with two quantities, each of 15 ml, of *water R*. Transfer the ether layers to a volumetric flask and dilute to 100.0 ml with *ether R*. Evaporate 10.0 ml carefully to dryness and dissolve the residue in 10.0 ml of a 5.0 g/l solution of *magnesium acetate R* in *methanol R*. Measure the absorbance (2.2.25) at 515 nm using *methanol R* as the compensation liquid.

Calculate the percentage of hydroxyanthracene glycosides expressed as sennoside B from the expression:

$$\frac{A \times 4.167}{m}$$

i.e. taking the specific absorbance of sennoside B to be 240.

A = absorbance at 515 nm,

m = mass of the substance to be examined, in grams.

STORAGE

Store in an airtight container, protected from light.

LABELLING

The label states the content of hydroxyanthracene glycosides.

01/2005:0207

SENNA PODS, ALEXANDRIAN

Sennae fructus acutifoliae

DEFINITION

Alexandrian senna pods consist of the dried fruit of *Cassia senna* L. (*C. acutifolia* Delile). They contain not less than 3.4 per cent of hydroxyanthracene glycosides, calculated as sennoside B ($C_{42}H_{38}O_{20}$; M_r 863) with reference to the dried drug.

CHARACTERS

Alexandrian senna pods have a slight odour.

They have the macroscopic and microscopic characters described under identification tests A and B.

IDENTIFICATION

A. They occur as flattened reniform pods, green to greenish-brown with brown patches at the positions corresponding to the seeds, usually 40 mm to 50 mm long and at least 20 mm wide. At one end is a styler point and at the other a short stalk. The pods contain six or seven flattened and obovate seeds, green to pale brown, with a continuous network of prominent ridges on the testa.

B. Reduce to a powder (355). The powder is brown. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters: epicarp with polygonal cells and a small number of conical warty trichomes and occasional anomocytic or paracytic stomata (2.8.3); fibres in two crossed layers accompanied by a crystal sheath of calcium oxalate prisms; characteristic palisade cells in the seed and stratified cells in the endosperm; clusters and prisms of calcium oxalate.

C. Examine by thin-layer chromatography (2.2.27), using *silica gel G R* as the coating substance.

Test solution. To 0.5 g of the powdered drug (180) add 5 ml of a mixture of equal volumes of *alcohol R* and *water R* and heat to boiling. Centrifuge and use the supernatant liquid.

Reference solution. Dissolve 10 mg of *senna extract CRS* in 1 ml of a mixture of equal volumes of *alcohol R* and *water R* (a slight residue remains).

Apply to the plate as bands 20 mm by 2 mm, 10 µl of each solution. Develop over a path of 10 cm using a mixture of 1 volume of *glacial acetic acid R*, 30 volumes of *water R*, 40 volumes of *ethyl acetate R* and 40 volumes of *propanol R*. Allow the plate to dry in air, spray with a 20 per cent V/V solution of *nitric acid R* and heat at 120 °C for 10 min. Allow to cool and spray with a 50 g/l solution of *potassium hydroxide R* in *alcohol (50 per cent V/V) R* until the zones appear. The principal zones in the chromatogram obtained with the test solution are similar in position (sennosides B, A, D and C in the order of increasing R_f value), colour and size to the principal zones in the chromatogram obtained with the reference solution. Between the zones corresponding to sennosides D and C, a red zone corresponding to rhein-8-glucoside may be visible. The zones corresponding to sennosides D and C are faint in the chromatogram obtained with the test solution.

D. Place about 25 mg of the powdered drug (180) in a conical flask and add 50 ml of *water R* and 2 ml of *hydrochloric acid R*. Heat in a water-bath for 15 min, cool and shake with 40 ml of *ether R*. Separate the ether, dry over *anhydrous sodium sulphate R*, evaporate 5 ml to dryness and to the cooled residue add 5 ml of *dilute ammonia R1*. A yellow or orange colour develops. Heat on a water-bath for 2 min. A reddish-violet colour develops.

TESTS

Foreign matter (2.8.2). Not more than 1 per cent.

Loss on drying (2.2.32). Not more than 12.0 per cent, determined on 1.000 g of the powdered drug (355) by drying in an oven at 100-105 °C for 2 h.

Total ash (2.4.16). Not more than 9.0 per cent.

Ash insoluble in hydrochloric acid (2.8.1). Not more than 2.0 per cent.

ASSAY

Carry out the assay protected from bright light.

Place 0.150 g of the powdered drug (180) in a 100 ml flask. Add 30.0 ml of *water R*, mix, weigh and place in a water-bath. Heat under a reflux condenser for 15 min. Allow to cool, weigh and adjust to the original mass with *water R*. Centrifuge and transfer 20.0 ml of the supernatant liquid to a 150 ml separating funnel. Add 0.1 ml of *dilute hydrochloric acid R* and shake with three quantities, each of 15 ml, of *chloroform R*. Allow to separate and discard the chloroform layer. Add 0.10 g of *sodium hydrogen carbonate R* and shake for 3 min. Centrifuge and transfer 10.0 ml of the supernatant liquid to a 100 ml round-bottomed flask with a ground-glass neck. Add 20 ml of *ferric chloride solution R1* and mix. Heat for 20 min under a reflux condenser in a water-bath with the water level above that of the liquid in the flask; add 1 ml of *hydrochloric acid R* and heat for a further 20 min, with frequent shaking, to dissolve the precipitate. Cool, transfer the mixture to a separating funnel and shake with three quantities, each of 25 ml, of *ether R* previously used to rinse the flask. Combine the ether layers and wash with two quantities, each of 15 ml, of *water R*. Transfer the ether layers to a volumetric flask and dilute to 100.0 ml with *ether R*. Evaporate 10.0 ml carefully to dryness and dissolve the residue in 10.0 ml of a 5 g/l solution of *magnesium acetate R* in *methanol R*. Measure the absorbance (2.2.25) at 515 nm using *methanol R* as the compensation liquid. Calculate the percentage content of sennoside B from the expression:

$$\frac{A \times 1.25}{m}$$

i.e. taking the specific absorbance to be 240.

A = absorbance at 515 nm,

m = mass of the substance to be examined, in grams.

STORAGE

Store protected from light and moisture.

01/2005:0208

SENNA PODS, TINNEVELLY

Sennae fructus angustifoliae

DEFINITION

Tinnevelly senna pods consist of the dried fruit of *Cassia angustifolia* Vahl. They contain not less than 2.2 per cent of hydroxyanthracene glycosides, calculated as sennoside B ($C_{42}H_{38}O_{20}$; M_r 863) with reference to the dried drug.

CHARACTERS

Tinnevelly senna pods have a slight odour.

They have the macroscopic and microscopic characters described under identification tests A and B.

IDENTIFICATION

A. They occur as flattened, slightly reniform pods, yellowish-brown to brown with dark brown patches at the positions corresponding to the seeds, usually 35 mm to 60 mm long and 14 mm to 18 mm wide. At one end is a stylar point and at the other a short stalk. The pods contain five to eight flattened and obovate seeds, green to pale brown, with incomplete, wavy, transverse ridges on the testa.

B. Reduce to a powder (355). The powder is brown. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters: epicarp with polygonal cells and a small number of conical warty trichomes and occasional anomocytic or paracytic stomata (2.8.3); fibres in two crossed layers accompanied by a crystal sheath of calcium oxalate prisms; characteristic palisade cells in the seed and stratified cells in the endosperm; clusters and prisms of calcium oxalate.

C. Examine by thin-layer chromatography (2.2.27), using *silica gel g R* as the coating substance.

Test solution. To 0.5 g of the powdered drug (180) add 5 ml of a mixture of equal volumes of *alcohol R* and *water R* and heat to boiling. Centrifuge and use the supernatant liquid.

Reference solution. Dissolve 10 mg of *senna extract CRS* in 1 ml of a mixture of equal volumes of *alcohol R* and *water R* (a slight residue remains).

Apply to the plate as bands 20 mm by 2 mm 10 µl of each solution. Develop over a path of 10 cm using a mixture of 1 volume of *glacial acetic acid R*, 30 volumes of *water R*, 40 volumes of *ethyl acetate R* and 40 volumes of *propanol R*. Allow the plate to dry in air, spray with a 20 per cent V/V solution of *nitric acid R* and heat at 120 °C for 10 min. Allow to cool and spray with a 50 g/l solution of *potassium hydroxide R* in *alcohol* (50 per cent V/V) *R* until the zones appear. The principal zones in the chromatogram obtained with the test solution are similar in position (sennosides B, A, D and C in the order of increasing R_f value), colour and size to the principal zones in the chromatogram obtained with the reference solution. Between the zones corresponding to sennosides D and C a red zone corresponding to rhein-8-glucoside may be visible. The zones corresponding to sennosides D and C are faint in the chromatogram obtained with the test solution.

D. Place about 25 mg of the powdered drug (180) in a conical flask and add 50 ml of *water R* and 2 ml of *hydrochloric acid R*. Heat in a water-bath for 15 min, cool and shake with 40 ml of *ether R*. Separate the ether, dry over *anhydrous sodium sulphate R*, evaporate 5 ml to dryness and to the cooled residue add 5 ml of *dilute ammonia R1*. A yellow or orange colour develops. Heat on a water-bath for 2 min. A reddish-violet colour develops.

TESTS

Foreign matter (2.8.2). Not more than 1 per cent.

Loss on drying (2.2.32). Not more than 12.0 per cent, determined on 1.000 g of the powdered drug (355) by drying in an oven at 100-105 °C for 2 h.

Total ash (2.4.16). Not more than 9.0 per cent.

Ash insoluble in hydrochloric acid (2.8.1). Not more than 2.0 per cent.

ASSAY

Carry out the assay protected from bright light.

Place 0.150 g of the powdered drug (180) in a 100 ml flask. Add 30.0 ml of *water R*, mix, weigh and place in a water-bath. Heat under a reflux condenser for 15 min. Allow to cool, weigh and adjust to the original mass with *water R*. Centrifuge and transfer 20.0 ml of the supernatant liquid to a 150 ml separating funnel. Add 0.1 ml of *dilute hydrochloric acid R* and shake with three quantities, each of 15 ml, of *chloroform R*. Allow to separate and discard the chloroform layer. Add 0.10 g of *sodium hydrogen carbonate R* and shake for 3 min. Centrifuge and transfer 10.0 ml of the

supernatant liquid to a 100 ml round-bottomed flask with a ground-glass neck. Add 20 ml of *ferric chloride solution R1* and mix. Heat for 20 min under a reflux condenser in a water-bath with the water level above that of the liquid in the flask; add 1 ml of *hydrochloric acid R* and heat for a further 20 min, with frequent shaking, to dissolve the precipitate. Cool, transfer the mixture to a separating funnel and shake with three quantities, each of 25 ml, of *ether R* previously used to rinse the flask. Combine the ether layers and wash with two quantities, each of 15 ml, of *water R*. Transfer the ether layers to a volumetric flask and dilute to 100.0 ml with *ether R*. Evaporate 10.0 ml carefully to dryness and dissolve the residue in 10.0 ml of a 5 g/l solution of *magnesium acetate R* in *methanol R*. Measure the absorbance (2.2.25) at 515 nm using *methanol R* as the compensation liquid. Calculate the percentage content of sennoside B from the expression:

$$\frac{A \times 1.25}{m}$$

i.e. taking the specific absorbance to be 240.

A = absorbance at 515 nm,

m = mass of the substance to be examined, in grams.

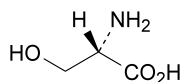
STORAGE

Store protected from light and moisture.

01/2005:0788

SERINE

Serinum



$C_3H_7NO_3$

M_r 105.1

DEFINITION

Serine contains not less than 98.5 per cent and not more than the equivalent of 101.0 per cent of (S)-2-amino-3-hydroxypropanoic acid, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, crystalline powder or colourless crystals, freely soluble in water, practically insoluble in alcohol.

IDENTIFICATION

First identification: A, B.

Second identification: A, C, D.

- It complies with the test for specific optical rotation (see Tests).
- Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *serine CRS*. Examine the substances prepared as discs.
- Examine the chromatograms obtained in the test for ninhydrin-positive substances. The principal spot in the chromatogram obtained with test solution (b) is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).
- To 1 ml of a 10 g/l solution of the substance to be examined in a test-tube, add 5 ml of a 20 g/l solution of *sodium periodate R*. Heat on a water-bath and collect the vapour on glass wool moistened with *water R* and

inserted in the opening of the test tube. After heating for 5 min, transfer the glass wool to a test-tube containing 1 ml of a 15 g/l solution of *chromotropic acid, sodium salt R* and 3 ml of *sulphuric acid R*. Heat on a water-bath for 10 min. A violet-red colour is produced.

TESTS

Solution S. Dissolve 2.5 g in *distilled water R* and dilute to 50 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and not more intensely coloured than reference solution BY₆ (2.2.2, Method II).

Specific optical rotation (2.2.7). Dissolve 2.50 g in *dilute hydrochloric acid R* and dilute to 25.0 ml with the same acid. The specific optical rotation is + 14.0 to + 16.0, calculated with reference to the dried substance.

Ninhydrin-positive substances. Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel plate R*.

Test solution (a). Dissolve 0.10 g of the substance to be examined in 0.1 M *hydrochloric acid* and dilute to 10 ml with the same acid.

Test solution (b). Dilute 1 ml of test solution (a) to 50 ml with *water R*.

Reference solution (a). Dissolve 10 mg of *serine CRS* in 0.1 M *hydrochloric acid* and dilute to 50 ml with the same acid.

Reference solution (b). Dilute 5 ml of test solution (b) to 20 ml with *water R*.

Reference solution (c). Dissolve 10 mg of *methionine CRS* and 10 mg of *serine CRS* in 0.1 M *hydrochloric acid* and dilute to 25 ml with the same acid.

Apply to the plate 5 µl of each solution. Develop over a path of 15 cm using a mixture of 20 volumes of *glacial acetic acid R*, 20 volumes of *water R* and 60 volumes of *butanol R*. Allow the plate to dry in air, spray with *ninhydrin solution R* and heat at 100 °C to 105 °C for 15 min. Any spot in the chromatogram obtained with test solution (a), apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.5 per cent). The test is not valid unless the chromatogram obtained with reference solution (c) shows two clearly separated spots.

Chlorides (2.4.4). Dilute 5 ml of solution S to 15 ml with *water R*. The solution complies with the limit test for chlorides (200 ppm).

Sulphates (2.4.13). Dilute 10 ml of solution S to 15 ml with *distilled water R*. The solution complies with the limit test for sulphates (300 ppm).

Ammonium (2.4.1). 50 mg complies with limit test B for ammonium (200 ppm). Prepare the standard using 0.1 ml of *ammonium standard solution (100 ppm NH₄) R*.

Iron (2.4.9). In a separating funnel, dissolve 1.0 g in 10 ml of *dilute hydrochloric acid R*. Shake with three quantities, each of 10 ml, of *methyl isobutyl ketone R1*, shaking for 3 min each time. To the combined organic layers add 10 ml of *water R* and shake for 3 min. The aqueous layer complies with the limit test for iron (10 ppm).

Heavy metals (2.4.8). Dissolve 2.0 g in *water R* and dilute to 20 ml with the same solvent. 12 ml of the solution complies with limit test A for heavy metals (10 ppm). Prepare the standard using *lead standard solution (1 ppm Pb) R*.

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.100 g in 3 ml of *anhydrous formic acid R*. Add 30 ml of *anhydrous acetic acid R*. Using 0.1 ml of *naphtholbenzein solution R* as indicator, titrate with 0.1 M *perchloric acid* until the colour changes from brownish-yellow to green.

1 ml of 0.1 M *perchloric acid* is equivalent to 10.51 mg of $C_{20}H_{16}Cl_3N_3O_4S$.

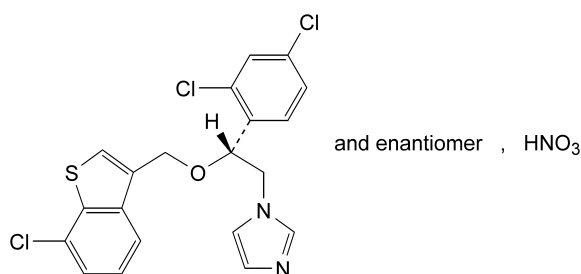
STORAGE

Store protected from light.

01/2005:1148

SERTACONAZOLE NITRATE

Sertaconazoli nitras


 $C_{20}H_{16}Cl_3N_3O_4S$
 M_r 500.8

DEFINITION

Sertaconazole nitrate contains not less than 98.5 per cent and not more than the equivalent of 101.0 per cent of (RS)-1-[2-[(7-chloro-1-benzothiophen-3-yl)methoxy]-2-(2,4-dichlorophenyl)ethyl]-1H-imidazole nitrate, calculated with reference to the anhydrous substance.

CHARACTERS

A white or almost white powder, practically insoluble in water, soluble in methanol, sparingly soluble in alcohol and in methylene chloride.

IDENTIFICATION

First identification: A, C.

Second identification: A, B, D, E.

- Melting point (2.2.14): 156 °C to 161 °C.
- Dissolve 0.1 g in *methanol R* and dilute to 100 ml with the same solvent. Dilute 10 ml of the solution to 100 ml with *methanol R*. Examined between 240 nm and 320 nm (2.2.25), the solutions shows three absorption maxima, at 260 nm, 293 nm and 302 nm. The ratio of the absorbance measured at the maximum at 302 nm to that measured at the maximum at 293 nm is 1.16 to 1.28.
- Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *sertaconazole nitrate CRS*. Dry the substances at 100-105 °C for 2 h and examine as discs prepared using *potassium bromide R*.
- Examine by thin-layer chromatography (2.2.27), using *silica gel G R* as the coating substance.
Test solution. Dissolve 40 mg of the substance to be examined in a mixture of 1 volume of *concentrated ammonia R* and 9 volumes of *methanol R* and dilute to 10 ml with the same mixture of solvents.

Reference solution (a). Dissolve 40 mg of *sertaconazole nitrate CRS* in a mixture of 1 volume of *concentrated ammonia R* and 9 volumes of *methanol R* and dilute to 10 ml with the same mixture of solvents.

Reference solution (b). Dissolve 20 mg of *miconazole nitrate CRS* in reference solution (a) and dilute to 5 ml with the same solution.

Apply to the plate 5 µl of each solution. Develop over a path of 15 cm using a mixture of 1 volume of *concentrated ammonia R*, 40 volumes of *toluene R* and 60 volumes of *dioxan R*. Dry the plate in a current of air for 15 min and expose the plate to iodine vapour for 30 min. The principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the spot in the chromatogram obtained with reference solution (a). The test is not valid unless the chromatogram obtained with reference solution (b) shows two clearly separated spots.

E. About 1 mg gives the reaction of nitrates (2.3.1).

TESTS

Appearance of solution. Dissolve 0.1 g in *alcohol R* and dilute to 10 ml with the same solvent. The solution is clear (2.2.1) and not more intensely coloured than reference solution Y₅ (2.2.2, *Method II*).

Related substances. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 10.0 mg of the substance to be examined in the mobile phase and dilute to 10.0 ml with the mobile phase.

Reference solution (a). Dilute 5.0 ml of the test solution to 100.0 ml with the mobile phase. Dilute 1.0 ml of the solution to 20.0 ml with the mobile phase.

Reference solution (b). Dissolve 5.0 mg of *sertaconazole nitrate CRS* and 5.0 mg of *miconazole nitrate CRS* in the mobile phase and dilute to 20.0 ml with the mobile phase. Dilute 1.0 ml of the solution to 50.0 ml with the mobile phase.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4.0 mm in internal diameter packed with *nitrile silica gel for chromatography R1* (10 µm),
- as mobile phase at a flow rate of 1.6 ml/min a mixture of 37 volumes of *acetonitrile R* and 63 volumes of a 1.5 g/l solution of *sodium dihydrogen phosphate R*,
- as detector a spectrophotometer set at 220 nm.

Inject 20 µl of reference solution (a) and 20 µl of reference solution (b). When the chromatograms are recorded in the prescribed conditions, the retention times are: nitrate ion, about 1 min; miconazole, about 17 min; sertaconazole, about 19 min. When using a recorder, adjust the sensitivity of the system so that the height of the principal peak in the chromatogram obtained with reference solution (a) is not less than 25 per cent of the full scale of the recorder. The test is not valid unless in the chromatogram obtained with reference solution (b), the resolution between the peaks due to miconazole and sertaconazole is at least 2.0.

Inject separately 20 µl of the test solution and 20 µl of reference solution (a). Continue the chromatography for 1.3 times the retention time of the principal peak. In the chromatogram obtained with the test solution: the area of any peak, apart from the principal peak and the peak due to nitrate ion, is not greater than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.25 per cent) and the sum of the areas of such peaks, is not greater than twice the area of the principal peak in

the chromatogram obtained with the reference solution (a) (0.5 per cent). Disregard any peak with an area less than 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (a).

Water (2.5.12). Not more than 1.0 per cent, determined on 0.50 g by the semi-micro determination of water.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

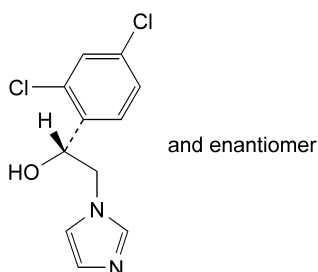
Dissolve 0.400 g in 50 ml of a mixture of equal volumes of *anhydrous acetic acid R* and *methyl ethyl ketone R*. Titrate with 0.1 M perchloric acid, determining the end-point potentiometrically (2.2.20). Carry out a blank titration.

1 ml of 0.1 M perchloric acid is equivalent to 50.08 mg of $C_{20}H_{16}Cl_3N_3O_4S$.

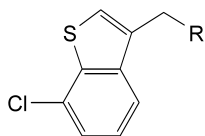
STORAGE

Store protected from light.

IMPURITIES



A. (1*RS*)-1-(2,4-dichlorophenyl)-2-(1*H*-imidazol-1-yl)ethanol,



B. R = Br: 3-(bromomethyl)-7-chloro-1-benzothiophen,

C. R = OH: (7-chloro-1-benzothiophen-3-yl)methanol.

01/2005:0433

SESAME OIL, REFINED

Sesami oleum raffinatum

DEFINITION

Refined sesame oil is the fatty oil obtained from the ripe seeds of *Sesamum indicum* L. by expression or extraction, then refined. Improved colour and odour may be obtained by further refining. It may contain a suitable antioxidant.

CHARACTERS

A clear, light yellow liquid, almost colourless, practically insoluble in alcohol, miscible with light petroleum.

It has a relative density of about 0.919.

It solidifies to a soft mass at about $-4\text{ }^{\circ}\text{C}$.

IDENTIFICATION

First identification: C.

Second identification: A, B.

A. It complies with the test for refractive index (see Tests).

B. Carry out the identification of fatty oils by thin-layer chromatography (2.3.2). The chromatogram obtained is similar to the type chromatogram for sesame oil.

C. It complies with the test for composition of triglycerides (see Tests).

TESTS

Refractive index (2.2.6): 1.470 to 1.476.

Acid value (2.5.1). Not more than 0.6, determined on 10.0 g. If intended for use in the manufacture of parenteral dosage forms, not more than 0.3.

Peroxide value (2.5.5). Not more than 10.0. If intended for use in the manufacture of parenteral dosage forms, not more than 5.0.

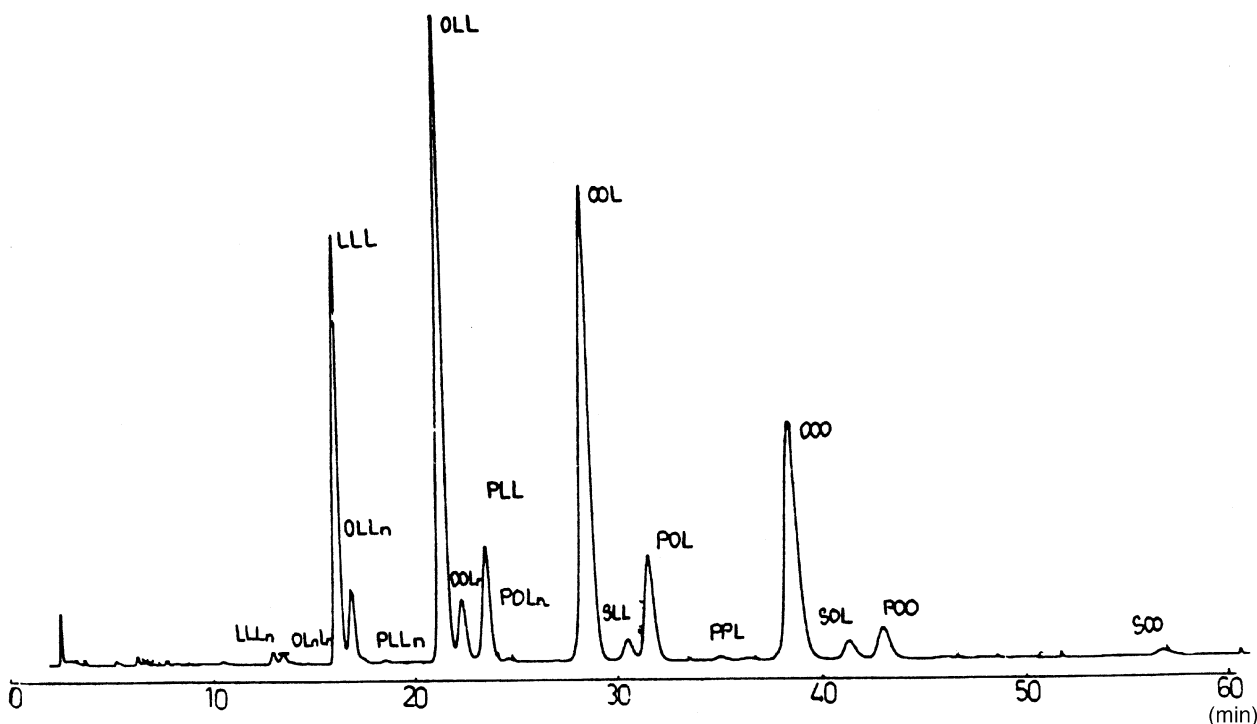


Figure 0433-1. – Chromatogram for the composition of triglycerides in sesame oil

01/2005:1149

Unsaponifiable matter (2.5.7). Not more than 2.0 per cent, determined on 5.0 g.

Alkaline impurities (2.4.19). It complies with the test for alkaline impurities in fatty oils.

Cottonseed oil. Mix 5 ml in a test-tube with 5 ml of a mixture of equal volumes of *pentanol R* and a 10 g/l solution of *sulphur R* in *carbon disulphide R*. Warm the mixture carefully until the carbon disulphide is expelled, and immerse the tube to one-third of its depth in boiling *saturated sodium chloride solution R*. No reddish colour develops within 15 min.

Composition of triglycerides. Examine by liquid chromatography (2.2.29).

Test solution. Into a 10 ml volumetric flask, weigh 0.200 g and dilute to 10.0 ml with the mobile phase.

The chromatographic procedure may be carried out using:

- two stainless steel columns 0.25 m long and 4.6 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (5 µm) in series,
- as mobile phase at a flow rate of 1.0 ml/min a mixture of 1 volume of *methylene chloride R* and 2 volumes of *acetonitrile R*,
- as detector a refractometer.

Inject 20 µl of the test solution. Identify the peaks from the type chromatogram (Figure 0433.-1). The fatty acid radicals are designated as linolenic (Ln), linoleic (L), oleic (O), palmitic (P) and stearic (S).

Calculate the percentage content of triglycerides from the areas of the peaks in the chromatogram obtained with the test solution by the normalisation procedure.

The composition of triglycerides is the following:

- LLL: 7.0 per cent to 19.0 per cent,
- OLL: 13.0 per cent to 30.0 per cent,
- PLL: 5.0 per cent to 9.0 per cent,
- OOL: 14.0 per cent to 25.0 per cent,
- POL: 8.0 per cent to 16.0 per cent,
- OOO: 5.0 per cent to 14.0 per cent,
- SOL: 2.0 per cent to 8.0 per cent,
- POO: 2.0 per cent to 10.0 per cent.

Water (2.5.12). If intended for use in the manufacture of parenteral dosage forms, not more than 0.05 per cent, determined on 5.0 g by the semi-micro determination of water.

STORAGE

Store in an airtight, well-filled container, protected from light.

Refined sesame oil intended for use in the manufacture of parenteral dosage forms is stored under an inert gas in an airtight container.

When the container has been opened, its contents are to be used as soon as possible. Any part of the contents not used at once is protected by an atmosphere of an inert gas.

LABELLING

The label states:

- whether the oil is obtained by expression or extraction,
- where applicable, the name and amount of any added antioxidant,
- where applicable, that the substance is suitable for use in the manufacture of parenteral dosage forms,
- where applicable, the name of the inert gas used.

SHELLAC

Lacca

DEFINITION

Shellac is a purified material obtained from the resinous secretion of the female insect *Kerria lacca* (Kerr) Lindinger (*Laccifer lacca* Kerr). There are four types of shellac depending on the nature of the treatment of crude secretion (seedlac): wax-containing shellac, bleached shellac, dewaxed shellac and bleached, dewaxed shellac.

Wax-containing shellac is obtained from seedlac; it is purified by filtration of the molten substance and/or by hot extraction using a suitable solvent.

Bleached shellac is obtained from seedlac by treatment with sodium hypochlorite after dissolution in a suitable alkaline solution, precipitation by dilute acid and drying.

Dewaxed shellac is obtained from wax-containing shellac or seedlac by treatment with a suitable solvent and removal of the insoluble wax by filtering.

Bleached, dewaxed shellac is obtained from wax-containing shellac or seedlac by treatment with sodium hypochlorite after dissolution in a suitable alkaline solution; the insoluble wax is removed by filtration. It is precipitated by dilute acid and dried.

CHARACTERS

Shellac occurs as brownish-orange or yellow, shining, translucent, hard or brittle, more or less thin flakes (wax-containing shellac and dewaxed shellac), or a creamy white or brownish-yellow powder (bleached shellac and bleached, dewaxed shellac).

Shellac is practically insoluble in water. With ethanol, it gives a more or less opalescent solution (wax containing shellac and bleached shellac) or a clear solution (dewaxed shellac and bleached, dewaxed shellac). When warmed, it is sparingly soluble or soluble in alkaline solutions.

IDENTIFICATION

- A. Examine by thin-layer chromatography (2.2.27), using as the coating substance a suitable silica gel with a fluorescent indicator having an optimal intensity at 254 nm.

Test solution. Heat 0.25 g of the powdered substance (500) on a water-bath with 2 ml of *dilute sodium hydroxide solution R* for 5 min. Cool, add 5 ml of *ethyl acetate R* and slowly, with stirring, 2 ml of *dilute acetic acid R*. Shake and filter the upper layer through *anhydrous sodium sulphate R*.

Reference solution. Dissolve 6.0 mg of *aleuritic acid R* in 1.0 ml of *methanol R*, heating slightly if necessary.

Apply to the plate as bands 10 µl of each solution. Develop twice over a path of 15 cm using a mixture of 1 volume of *acetic acid R*, 8 volumes of *methanol R*, 32 volumes of *methylene chloride R* and 60 volumes of *ethyl acetate R*. Allow the plate to dry in air. Spray the plate with *anisaldehyde solution R*. Heat the plate at 100° C to 105° C for 5 min to 10 min and examine in daylight. The chromatogram obtained with the test solution shows several coloured zones, one of which is similar in position and colour to the zone in the chromatogram obtained with the reference solution. Above this zone the chromatogram obtained with the test solution shows a pink zone and below it several violet zones. Below the zone corresponding to aleuritic acid,

there is a light blue zone (shellolic acid) accompanied by zones of the same colour but of lower intensity. Other faint grey and violet zones may be visible.

- B. Examine the chromatograms obtained in the test for colophony. For wax-containing shellac, in the chromatogram obtained with the test solution, a more or less strong bluish-grey zone is visible, just above the zone due to thymolphthalein in the chromatogram obtained with the reference solution. For dewaxed shellac, no such zone is visible just above the zone due to thymolphthalein in the chromatogram obtained with the reference solution.

TESTS

Colophony. Examine by thin-layer chromatography (2.2.27), as described under identification test A with the following modifications.

Test solution. Dissolve 50 mg of the powdered substance (500), while heating, in a mixture of 0.5 ml of *methylene chloride R* and 0.5 ml of *methanol R*.

Reference solution. Dissolve 2.0 mg of *thymolphthalein R* in 1.0 ml of *methanol R*.

Examine the plate in ultraviolet light at 254 nm. Mark the quenching zones in the chromatogram obtained with the test solution that have similar R_f values to that of the quenching zone of thymolphthalein in the chromatogram obtained with the reference solution. Spray with *anisaldehyde solution R*, heat the plate at 100 °C to 105 °C for 5 min to 10 min and examine in daylight. The chromatogram obtained with the reference solution shows a principal zone with a reddish-violet colour (thymolphthalein). None of the quenching zones in the chromatogram obtained with the test solution that have an R_f value similar to the zone due to thymolphthalein in the reference solution show a more or less strong violet or brownish colour (colophony). Disregard any faint violet zone at this level that does not show quenching before spraying and heating.

Acid value (2.5.1): 65 to 95, calculated with reference to the dried substance. Examine 1.00 g of the coarsely ground substance. Determine the end-point by potentiometry (2.2.20).

Arsenic (2.4.2). Introduce 0.33 g of the substance to be examined and 5 ml of *sulphuric acid R* into a combustion flask. Carefully add a few millilitres of *strong hydrogen peroxide solution R* and heat to boiling until a clear, colourless solution is obtained. Continue heating to eliminate the water and as much sulphuric acid as possible and dilute to 25 ml with *water R*. The solution complies with limit test A for arsenic (3 ppm).

Heavy metals (2.4.8). 2.0 g complies with limit test D for heavy metals (10 ppm). Prepare the standard using 2 ml of *lead standard solution (10 ppm Pb) R*.

Loss on drying (2.2.32). Not more than 2.0 per cent for unbleached shellac and not more than 6.0 per cent for bleached shellac, determined on 1.000 g of the powdered substance (500) by drying in an oven at 40 °C to 45 °C for 24 h.

STORAGE

Store protected from light. Store bleached shellac and bleached, dewaxed shellac at a temperature not exceeding 15 °C.

LABELLING

The label indicates the type of shellac.

01/2005:0434

SILICA, COLLOIDAL ANHYDROUS

Silica colloidalis anhydrica

SiO₂ M_r 60.1

DEFINITION

Colloidal anhydrous silica contains not less than 99.0 per cent and not more than the equivalent of 100.5 per cent of SiO₂, determined on the ignited substance.

CHARACTERS

A light, fine, white, amorphous powder, with a particle size of about 15 nm, practically insoluble in water and in mineral acids except hydrofluoric acid. It dissolves in hot solutions of alkali hydroxides.

IDENTIFICATION

About 20 mg gives the reaction of silicates (2.3.1).

TESTS

pH (2.2.3). Shake 1.0 g with 30 ml of *carbon dioxide-free water R*. The pH of the suspension is 3.5 to 5.5.

Chlorides (2.4.4). To 1.0 g add a mixture of 20 ml of *dilute nitric acid R* and 30 ml of *water R* and heat on a water-bath for 15 min, shaking frequently. Dilute to 50 ml with *water R* if necessary, filter and cool. 10 ml of the filtrate diluted to 15 ml with *water R* complies with the limit test for chlorides (250 ppm).

Heavy metals (2.4.8). Suspend 2.5 g in sufficient *water R* to produce a semi-fluid slurry. Dry at 140 °C. When the dried substance is white, break up the mass with a glass rod. Add 25 ml of 1 M *hydrochloric acid* and boil gently for 5 min, stirring frequently with the glass rod. Centrifuge for 20 min and filter the supernatant liquid through a membrane filter. To the residue in the centrifuge tube add 3 ml of *dilute hydrochloric acid R* and 9 ml of *water R* and boil. Centrifuge for 20 min and filter the supernatant liquid through the same membrane filter. Wash the residue with small quantities of *water R*, combine the filtrates and washings and dilute to 50 ml with *water R*. To 20 ml of the solution add 50 mg of *ascorbic acid R* and 1 ml of *concentrated ammonia R*. Neutralise with *dilute ammonia R2*. Dilute to 25 ml with *water R*. 12 ml of the solution complies with limit test A for heavy metals (25 ppm). Prepare the standard using *lead standard solution (1 ppm Pb) R*.

Loss on ignition. Not more than 5.0 per cent, determined on 0.200 g by ignition in a platinum crucible at 900 °C for 2 h. Allow to cool in a desiccator before weighing.

ASSAY

To the residue obtained in the test for loss on ignition add 0.2 ml of *sulphuric acid R* and sufficient *alcohol R* to moisten the residue completely. Add 6 ml of *hydrofluoric acid R* and evaporate to dryness on a hot-plate at 95 °C to 105 °C, taking care to avoid loss from sputtering. Wash down the sides of the dish with 6 ml of *hydrofluoric acid R* and evaporate to dryness. Ignite at 900 °C, allow to cool in a desiccator and weigh.

The difference between the mass of the final residue and the mass of the residue obtained in the test for loss on ignition gives the amount of SiO₂ in the quantity of the substance to be examined used.

01/2005:0738 ASSAY

SILICA, COLLOIDAL HYDRATED**Silica colloidalis hydrica****DEFINITION**

Colloidal hydrated silica contains not less than 98.0 per cent and not more than the equivalent of 100.5 per cent of SiO_2 (M_r 60.1), determined on the ignited substance.

CHARACTERS

A white or almost white, light, fine, amorphous powder, practically insoluble in water and in mineral acids, with the exception of hydrofluoric acid. It dissolves in hot solutions of alkali hydroxides.

IDENTIFICATION

- A. About 20 mg gives the reaction of silicates (2.3.1).
B. When heated in an oven at 100 °C to 105 °C for 2 h, it shows a loss of mass greater than 3 per cent.

TESTS

Solution S. To 2.5 g add 50 ml of *hydrochloric acid R* and mix. Heat on a water-bath for 30 min, stirring from time to time. Maintain the original volume by adding *dilute hydrochloric acid R*. Evaporate to dryness. Add to the residue a mixture of 8 ml of *dilute hydrochloric acid R* and 24 ml of *water R*. Heat to boiling and filter under reduced pressure through a sintered-glass filter (16). Wash the residue on the filter with a hot mixture of 3 ml of *dilute hydrochloric acid R* and 9 ml of *water R*. Wash with small quantities of *water R*, combine the filtrate and washings and dilute to 50 ml with *water R*.

pH (2.2.3). Suspend 1.0 g in 30 ml of a 75 g/l solution of *potassium chloride R*. The pH of the suspension is 4.0 to 7.0.

Water-absorption capacity. In a mortar, triturate 5 g with 5 ml of *water R*, added drop by drop. The mixture remains powdery.

Substances soluble in hydrochloric acid. In a platinum dish, evaporate to dryness 10.0 ml of solution S and dry to constant mass at 100 °C to 105 °C. The mass of the residue is not more than 10 mg (2.0 per cent).

Chlorides (2.4.4). Heat 0.5 g with 50 ml of *water R* on a water-bath for 15 min. Dilute to 100 ml with *water R* and centrifuge at 1500 *g* for 5 min. 10 ml of the supernatant solution diluted to 15 ml with *water R* complies with the limit test for chlorides (0.1 per cent).

Sulphates (2.4.13). Dilute 2 ml of solution S to 100 ml with *distilled water R*. 15 ml of the solution complies with the limit test for sulphates (1 per cent).

Iron (2.4.9). To 2 ml of solution S add 28 ml of *water R*. 10 ml of the solution complies with the limit test for iron (300 ppm).

Heavy metals (2.4.8). To 20 ml of solution S add 50 mg of *hydroxylamine hydrochloride R* and 1 ml of *concentrated ammonia R*. Adjust to pH 3.5 by adding *dilute ammonia R2*, monitoring the pH potentiometrically. Dilute to 25 ml with *water R*. 12 ml of the solution complies with limit test A for heavy metals (25 ppm). Prepare the standard using *lead standard solution* (1 ppm Pb) *R*.

Loss on ignition. Not more than 20.0 per cent, determined on 0.200 g in a platinum crucible by heating at 100 °C to 105 °C for 1 h and then at 900 °C for 2 h.

ASSAY

To the residue obtained in the test for loss on ignition add 0.2 ml of *sulphuric acid R* and a quantity of *alcohol R* sufficient to moisten the residue completely. Add 6 ml of *hydrofluoric acid R* and evaporate to dryness at 95 °C to 105 °C, taking care to avoid loss from sputtering. Wash the inside of the dish with 6 ml of *hydrofluoric acid R* and evaporate to dryness again. Ignite at 900 °C, allow to cool in a desiccator and weigh. The difference between the mass of the final residue and that of the mass obtained in the test for loss on ignition corresponds to the mass of SiO_2 in the test sample.

01/2005:1562

SILICA, DENTAL TYPE**Silica ad usum dentalem****DEFINITION**

Dental type silica is an amorphous silica (precipitated, gel or obtained by flame hydrolysis). It contains not less than 94.0 per cent and not more than 100.5 per cent of SiO_2 determined on the ignited substance.

CHARACTERS

A white or almost white, light, fine amorphous powder, practically insoluble in water and in mineral acids. It dissolves in hydrofluoric acid and hot solutions of alkali hydroxides.

IDENTIFICATION

About 20 mg gives the reaction of silicates (2.3.1).

TESTS

Solution S. To 2.5 g add 50 ml of *hydrochloric acid R* and mix. Heat on a water-bath for 30 min, stirring from time to time. Evaporate to dryness. Add to the residue a mixture of 8 ml of *dilute hydrochloric acid R* and 24 ml of *water R*. Heat to boiling and filter under reduced pressure through a sintered-glass filter (16). Wash the residue on the filter with a hot mixture of 3 ml of *dilute hydrochloric acid R* and 9 ml of *water R*. Wash with small quantities of *water R*, combine the washings and the filtrate and dilute to 50 ml with *water R*.

pH (2.2.3). Suspend 5 g in a mixture of 5 ml of a 7.46 g/l solution of *potassium chloride R* and 90 ml of *carbon dioxide-free water R*. The pH of the suspension is 3.2 to 8.9.

Chlorides. Examine by liquid chromatography (2.2.29) as prescribed under sulphates.

Inject 25 µl of the test solution and 25 µl of the reference solution. In the chromatogram obtained with the test solution the area of any peak corresponding to chloride is not greater than that of the corresponding peak in the chromatogram obtained with the reference solution (0.3 per cent).

Sulphates. Examine by liquid chromatography (2.2.29).

Test solution. To 0.625 g add 30 ml of *water R* and boil for 2 h. Allow to cool and quantitatively transfer to a 50 ml graduated flask. Dilute to 50.0 ml with *water R*. Dilute 5.0 ml of the supernatant to 50.0 ml with *water R* and filter through a membrane filter (nominal pore size: 0.45 µm).

Reference solution. Dissolve 0.50 g of *anhydrous sodium sulphate R* and 0.062 g of *sodium chloride R* in *water R* and dilute to 1000.0 ml with *water R*. Dilute 5.0 ml of this solution to 50.0 ml with *water R*.

The chromatographic procedure may be carried out using:

- a non-metallic column 0.25 m long and 4.6 mm in internal diameter packed with a suitable anion-exchange resin (30 µm to 50 µm),
- as mobile phase at a flow rate of 1.2 ml/min a solution prepared as follows: dissolve 0.508 g of *sodium carbonate R* and 0.05 g of *sodium hydrogen carbonate R* in *water R* and dilute to 1000 ml with the same solvent,
- a conductivity detector,
- a loop injector.

Inject 25 µl of the test solution and 25 µl of the reference solution. When the chromatograms are recorded in the prescribed conditions, the retention times are: sulphate about 8 min and chloride about 4 min.

In the chromatogram obtained with the test solution the area of any peak corresponding to sulphate is not greater than that of the corresponding peak in the chromatogram obtained with the reference solution (4.0 per cent calculated as sodium sulphate).

Iron (2.4.9). Dilute 2 ml of solution S to 40 ml with *water R*. 10 ml of the solution complies with the limit test for iron (400 ppm).

Heavy metals (2.4.8). To 20 ml of solution S, add 50 mg of *hydroxylamine hydrochloride R* and 1 ml of *concentrated ammonia R*. Adjust to pH 3.5 by adding *dilute ammonia R2*, monitoring the pH potentiometrically. Dilute to 25 ml with *water R*. 12 ml of the solution complies with limit test A for heavy metals (25 ppm). Prepare the standard using *lead standard solution (1 ppm Pb) R*.

Loss on ignition. Not more than 25.0 per cent, determined on 0.200 g in a platinum crucible by heating at 100 °C to 105 °C for 1 h and then at 1000 °C for 2 h.

ASSAY

To the residue obtained in the test for loss on ignition add 0.2 ml of *sulphuric acid R* and a quantity of *alcohol R* sufficient to moisten the residue completely. Add 6 ml of *hydrofluoric acid R* and evaporate to dryness at 95 °C to 105 °C, taking care to avoid loss from sputtering. Wash the inside of the crucible with 6 ml of *hydrofluoric acid R* and evaporate to dryness again. Ignite at 900 °C, allow to cool in a desiccator and weigh. The difference between the mass of the final residue and that of the mass obtained in the test for loss on ignition corresponds to the mass of SiO₂ in the test sample.

01/2005:0009

SILVER NITRATE

Argenti nitras

AgNO₃

M_r 169.9

DEFINITION

Silver nitrate contains not less than 99.0 per cent and not more than the equivalent of 100.5 per cent of AgNO₃.

CHARACTERS

A white, crystalline powder or transparent, colourless crystals, very soluble in water, soluble in alcohol.

IDENTIFICATION

- 10 mg gives the reaction of nitrates (2.3.1).
- 10 mg gives the reaction of silver (2.3.1).

TESTS

Solution S. Dissolve 2.0 g in *water R* and dilute to 50 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and colourless (2.2.2, *Method II*).

Acidity or alkalinity. To 2 ml of solution S add 0.1 ml of *bromocresol green solution R*. The solution is blue. To 2 ml of solution S add 0.1 ml of *phenol red solution R*. The solution is yellow.

Foreign salts. To 30 ml of solution S, add 7.5 ml of *dilute hydrochloric acid R*, shake vigorously, heat for 5 min on a water-bath and filter. Evaporate 20 ml of the filtrate to dryness on a water-bath and dry at 100-105 °C. The residue weighs not more than 2 mg (0.3 per cent).

Aluminium, lead, copper and bismuth. Dissolve 1.0 g in a mixture of 4 ml of *concentrated ammonia R* and 6 ml of *water R*. The solution is clear (2.2.1) and colourless (2.2.2, *Method II*).

ASSAY

Dissolve 0.300 g in 50 ml of *water R* and add 2 ml of *dilute nitric acid R* and 2 ml of *ferric ammonium sulphate solution R2*. Titrate with 0.1 M *ammonium thiocyanate* until a reddish-yellow colour is obtained.

1 ml of 0.1 M *ammonium thiocyanate* is equivalent to 16.99 mg of AgNO₃.

STORAGE

Store in a non-metallic container, protected from light.

01/2005:1470

SIMETICONE

Simeticonum

DEFINITION

Simeticone is prepared by incorporation of 4 per cent to 7 per cent silica into poly(dimethylsiloxane) with a degree of polymerisation between 20 and 400. Simeticone contains 90.5 per cent to 99.0 per cent of poly(dimethylsiloxane).

PRODUCTION

Poly(dimethylsiloxane) is obtained by hydrolysis and polycondensation of dichlorodimethylsilane and chlorotrimethylsilane and the silica is modified at the surface by incorporation of methylsilyl groups.

CHARACTERS

A viscous, greyish-white, opalescent liquid, practically insoluble in water, very slightly soluble to practically insoluble in ethanol, practically insoluble in methanol, partly miscible with ethyl acetate, with methylene chloride, with methyl ethyl ketone and with toluene.

IDENTIFICATION

- Examine by infrared absorption spectrophotometry (2.2.24). Absorption maxima are observed at 2964 cm⁻¹, 2905 cm⁻¹, 1412 cm⁻¹, 1260 cm⁻¹ and 1020 cm⁻¹. Examine the substances as thin films between *sodium chloride R* plates.
- Heat 0.5 g in a test-tube over a small flame until white fumes begin to appear. Invert the tube over a second tube containing 1 ml of a 1 g/l solution of *chromotropic acid, sodium salt R* in *sulphuric acid R* so that the fumes reach the solution. Shake the second tube for about 10 s and heat on a water-bath for 5 min. The solution is violet.

C. The residue obtained in the test for silica under Assay, gives the reaction of silicates (2.3.1).

TESTS

Acidity. To 2.0 g add 25 ml of a mixture of equal volumes of *ethanol R* and *ether R* previously neutralised to 0.2 ml of *bromothymol blue solution R1*, and shake. Not more than 3.0 ml of 0.01 M *sodium hydroxide* is required to change the colour of the solution to blue.

Defoaming activity

Foaming solution. Dissolve 5.0 g of *docusate sodium R* in 1 litre of *water R* (warm to 50 °C if necessary).

Defoaming solution. To 50 ml of *methyl ethyl ketone R* add 0.250 g of simeticone, warm to not more than 50 °C with shaking.

Into a 250 ml cylindrical tube about 5 cm in diameter introduce 100 ml of foaming solution and 1 ml of defoaming solution. Close tightly and fix the tube on a suitable oscillating shaker that complies with the following conditions:

- 250 to 300 oscillations per minute,
- angle of oscillation of about 10°,
- oscillation radius of about 10 cm.

Shake for 10 s and record the time between the end of the shaking and the instant the first portion of foam-free liquid surface appears.

This duration does not exceed 15 s.

Mineral oils. Place 2.0 g in a test-tube and examine in ultraviolet light at 365 nm. The fluorescence is not more intense than that of a solution containing 0.1 ppm of *quinine sulphate R* in 0.005 M *sulphuric acid* examined in the same conditions.

Phenylated compounds. Dissolve 5.0 g with shaking in 10.0 ml of *cyclohexane R*. Determine the absorbance of the solution between 200 nm and 350 nm (2.2.25) using *cyclohexane R* as the compensation liquid. The corrected absorbance (absorbance measured at the maximum between 250 nm and 270 nm minus the absorbance measured at 300 nm) is not greater than 0.2.

Heavy metals. Mix 1.0 g with *methylene chloride R* and dilute to 20 ml with the same solvent. Add 1.0 ml of a freshly prepared 0.02 g/l solution of *dithizone R* in *methylene chloride R*, 0.5 ml of *water R* and 0.5 ml of a mixture of 1 volume of *dilute ammonia R2* and 9 volumes of a 2 g/l solution of *hydroxylamine hydrochloride R*. At the same time, prepare a standard as follows: to 20 ml of *methylene chloride R* add 1.0 ml of a freshly prepared 0.02 g/l solution of *dithizone R* in *methylene chloride R*, 0.5 ml of *lead standard solution* (10 ppm Pb) *R* and 0.5 ml of a mixture of 1 volume of *dilute ammonia R2* and 9 volumes of a 2 g/l solution of *hydroxylamine hydrochloride R*. Immediately shake each solution vigorously for 1 min. Any red colour in the test solution is not more intense than that in the standard (5 ppm).

Volatile matter. Not more than 1.0 per cent, determined on 1.00 g by heating in an oven at 150 °C for 2 h. Carry out the test using a dish 60 mm in diameter and 10 mm deep.

ASSAY

Silica. Not more than 7 per cent, determined on not less than 20.0 mg. Heat the substance to be examined to 800 °C increasing the temperature by 20 °C/min under a current of *nitrogen R* at a flow rate of 200 ml/min and weigh the residue (silica).

Dimeticone

Test solution. Place about 50 mg (*E*) in a screw-capped 125 ml cylindrical tube, add 25.0 ml of *toluene R*, swirl manually to disperse and add 50 ml of *dilute hydrochloric acid R*, close the tube and place on a vortex mixer; shake for 5 min. Transfer the contents of the tube to a separating funnel, allow to settle and transfer 5 ml of the upper layer to a screw-capped test tube containing 0.5 g of *anhydrous sodium sulphate R*. Cap and shake vigorously manually. Centrifuge to obtain a clear test solution.

Reference solution. Introduce about 0.20 g of *dimeticone CRS* in 100.0 ml of *toluene R*. Prepare the reference solution in the same way as for the test solution, using 25.0 ml of the dimeticone solution obtained above. Prepare a blank by shaking 10 ml of *toluene R* with 1 g of *anhydrous sodium sulphate R*. Centrifuge the resulting suspension.

Record the infrared absorption spectra for the test solution and the reference solution in 0.5 mm cells, from 1330 cm⁻¹ to 1180 cm⁻¹, and determine the absorbance of the band at 1260 cm⁻¹ (2.2.24).

Calculate the percentage content of dimeticone using the expression:

$$\frac{25C \times A_M \times 100}{A_E \times E}$$

A_M = absorbance of the test solution,

A_E = absorbance of the reference solution,

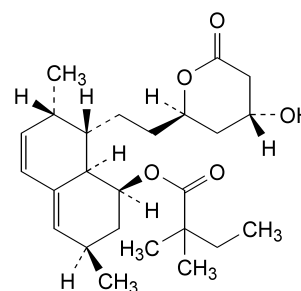
C = concentration of the reference solution, in milligrams per millilitre,

E = mass of the substance to be examined, in milligrams.

01/2005:1563

SIMVASTATIN

Simvastatinum



C₂₅H₃₈O₅

M_r 418.6

DEFINITION

Simvastatin contains not less than 97.0 per cent and not more than the equivalent of 102.0 per cent of (1*S*,3*R*,7*S*,8*S*,8*aR*)-8-[2-[(2*R*,4*R*)-4-hydroxy-6-oxotetrahydro-2*H*-pyran-2-yl]ethyl]-3,7-dimethyl-1,2,3,7,8,8*a*-hexahydronaphthalen-1-yl 2,2-dimethylbutanoate, calculated with reference to the dried substance. A suitable antioxidant may be added.

CHARACTERS

A white or almost white, crystalline powder, practically insoluble in water, very soluble in methylene chloride, freely soluble in alcohol.

IDENTIFICATION

- A. It complies with the test for specific optical rotation (see Tests).
- B. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *simvastatin CRS*. Examine the substances prepared as discs.

TESTS

Appearance of solution. Dissolve 0.200 g in *methanol R* and dilute to 20 ml with the same solvent. The solution is clear (2.2.1) and not more intensely coloured than reference solution BY₇ (2.2.2, *Method II*).

Specific optical rotation (2.2.7). Dissolve 0.125 g in *acetonitrile R* and dilute to 25.0 ml with the same solvent. The specific optical rotation is + 285 to + 300, calculated with reference to the dried substance.

Related substances. Examine by liquid chromatography (2.2.29) as prescribed under Assay.

Inject 5 µl of reference solution (b). Adjust the sensitivity of the system so that the height of the principal peak in the chromatogram obtained is at least 20 per cent of the full scale of the recorder. Inject 5 µl of test solution (a) and continue the chromatography for five times the retention time of simvastatin. When the chromatograms are recorded under the prescribed conditions the relative retentions are: impurity A about 0.45, lovastatin (impurity E) and epilovastatin (impurity F) about 0.60, impurity G about 0.80, impurity B about 2.38, impurity C about 2.42 and impurity D about 3.80 (retention time of simvastatin: about 2.6 min). In the chromatogram obtained with test solution (a): the area of the peak due to lovastatin and epilovastatin is not greater than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (1.0 per cent); the area of any peak apart from the principal peak and the peak due to lovastatin and epilovastatin is not greater than 0.8 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.4 per cent); the sum of the areas of all peaks, apart from the principal peak and the peak due to lovastatin and epilovastatin, is not greater than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (1.0 per cent). Disregard any peak with an area less than 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

Heavy metals (2.4.8). 1.0 g complies with limit test C for heavy metals (20 ppm). Prepare the standard using 2 ml of *lead standard solution (10 ppm Pb) R*.

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in a desiccator under high vacuum at 60 °C for 3 h.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Examine by liquid chromatography (2.2.29). *Prepare the solutions immediately before use.*

Solvent mixture. Prepare a mixture of 40 volumes of a solution of a 1.4 g/l solution of *potassium dihydrogen phosphate R*, adjusted to pH 4.0 with *phosphoric acid R*, and 60 volumes of *acetonitrile R*. Filter.

Test solution (a). Dissolve 75.0 mg of the substance to be examined in the solvent mixture and dilute to 50.0 ml with the solvent mixture.

Test solution (b). Dissolve 40.0 mg of the substance to be examined in the solvent mixture and dilute to 50.0 ml with the solvent mixture.

Reference solution (a). Dissolve 1.0 mg of *simvastatin CRS* and 1.0 mg of *lovastatin CRS* in the solvent mixture and dilute to 50.0 ml with the solvent mixture.

Reference solution (b). Dilute 0.5 ml of test solution (a) to 100.0 ml with the solvent mixture.

Reference solution (c). Dissolve 40.0 mg of *simvastatin CRS* in the solvent mixture and dilute to 50.0 ml with the solvent mixture.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.033 m long and 4.6 mm in internal diameter packed with *end-capped octadecylsilyl silica gel for chromatography R* (3 µm),
- as mobile phase at a flow rate of 3.0 ml/min:

Mobile phase A. Mix 50 volumes of *acetonitrile R* and 50 volumes of a 0.1 per cent V/V solution of *phosphoric acid R*,

Mobile phase B. A 0.1 per cent V/V solution of *phosphoric acid R* in *acetonitrile R*,

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)	Comment
0 - 4.5	100	0	isocratic
4.5 - 4.6	100 → 95	0 → 5	linear gradient
4.6 - 8.0	95 → 25	5 → 75	linear gradient
8.0 - 11.5	25	75	isocratic
11.5 - 11.6	25 → 100	75 → 0	linear gradient
11.6 - 13	100	0	re-equilibration

- as detector a spectrophotometer set at 238 nm.

Inject 5 µl of reference solution (a). The test and the assay are not valid unless, in the chromatogram obtained, the resolution between the peak corresponding to lovastatin and epilovastatin and the peak corresponding to simvastatin is at least 5.0. When the chromatograms are recorded under the prescribed conditions the retention times are: lovastatin and epilovastatin about 1.6 min and simvastatin about 2.6 min. Inject 5 µl of reference solution (c). Adjust the sensitivity of the system so that the height of the principal peak is at least 50 per cent of the full scale of the recorder. Inject 5 µl of test solution (b).

Calculate the content of simvastatin from the peak areas in the chromatograms obtained with test solution (b) and reference solution (c) and the declared content of *simvastatin CRS*.

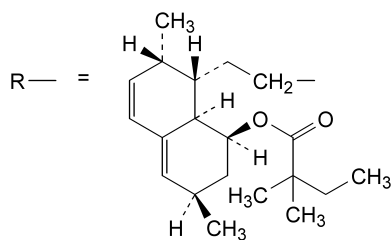
STORAGE

Store under nitrogen, in an airtight container, protected from light.

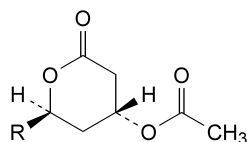
LABELLING

The label states the name and concentration of any added antioxidant.

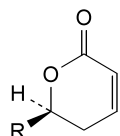
IMPURITIES



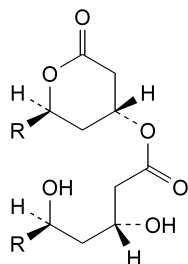
- A. (3*R*,5*R*)-7-[(1*S*,2*S*,6*R*,8*S*,8*aR*)-8-[(2,2-dimethylbutanoyl)oxy]-2,6-dimethyl-1,2,6,7,8,8*a*-hexahydronaphthalen-1-yl]-3,5-dihydroxyheptanoic acid (hydroxy acid),



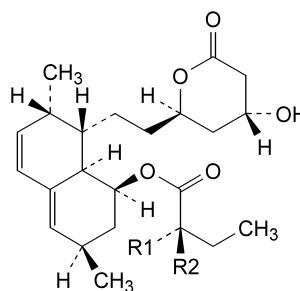
- B. (1*S*,3*R*,7*S*,8*S*,8*aR*)-8-[2-[(2*R*,4*R*)-4-(acetyloxy)-6-oxotetrahydro-2*H*-pyran-2-yl]ethyl]-3,7-dimethyl-1,2,3,7,8,8*a*-hexahydronaphthalen-1-yl 2,2-dimethylbutanoate (acetate ester),



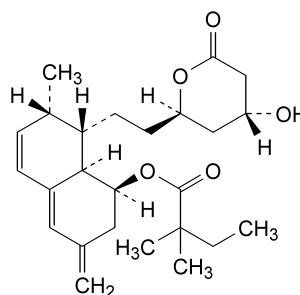
- C. (1*S*,3*R*,7*S*,8*S*,8*aR*)-3,7-dimethyl-8-[2-[(2*R*)-6-oxo-3,6-dihydro-2*H*-pyran-2-yl]ethyl]-1,2,3,7,8,8*a*-hexahydronaphthalen-1-yl 2,2-dimethylbutanoate (anhydrosimvastatin),



- D. (2*R*,4*R*)-2-[(1*S*,2*S*,6*R*,8*S*,8*aR*)-8-[(2,2-dimethylbutanoyl)oxy]-2,6-dimethyl-1,2,6,7,8,8*a*-hexahydronaphthalen-1-yl]ethyl]-6-oxotetrahydro-2*H*-pyran-4-yl (3*R*,5*R*)-7-[(1*S*,2*S*,6*R*,8*S*,8*aR*)-8-[(2,2-dimethylbutanoyl)oxy]-2,6-dimethyl-1,2,6,7,8,8*a*-hexahydronaphthalen-1-yl]-3,5-dihydroxyheptanoate (dimer),



- E. R1 = CH₃, R2 = H: (1*S*,3*R*,7*S*,8*S*,8*aR*)-8-[2-[(2*R*,4*R*)-4-hydroxy-6-oxotetrahydro-2*H*-pyran-2-yl]ethyl]-3,7-dimethyl-1,2,3,7,8,8*a*-hexahydronaphthalen-1-yl (2*S*)-2-methylbutanoate (lovastatin),
- F. R1 = H, R2 = CH₃: (1*S*,3*R*,7*S*,8*S*,8*aR*)-8-[2-[(2*R*,4*R*)-4-hydroxy-6-oxotetrahydro-2*H*-pyran-2-yl]ethyl]-3,7-dimethyl-1,2,3,7,8,8*a*-hexahydronaphthalen-1-yl (2*R*)-2-methylbutanoate (epilovastatin),

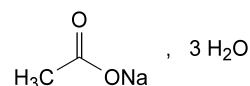


- G. (1*S*,7*S*,8*S*,8*aR*)-8-[2-[(2*R*,4*R*)-4-hydroxy-6-oxotetrahydro-2*H*-pyran-2-yl]ethyl]-7-methyl-3-methylene-1,2,3,7,8,8*a*-hexahydronaphthalen-1-yl 2,2-dimethylbutanoate.

01/2005:0411

SODIUM ACETATE TRIHYDRATE

Natrii acetat trihydricus

C₂H₃NaO₂·3H₂OM_r 136.1

DEFINITION

Sodium ethanoate trihydrate.

Content: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

Appearance: colourless crystals.*Solubility*: very soluble in water, soluble in alcohol.

IDENTIFICATION

- A. 1 ml of solution S (see Tests) gives reaction (b) of acetates (2.3.1).
- B. 1 ml of solution S gives reaction (a) of sodium (2.3.1).
- C. It complies with the test for loss on drying (see Tests).

TESTS

Solution S. Dissolve 10.0 g in carbon dioxide-free water *R* prepared from distilled water *R* and dilute to 100 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and colourless (2.2.2, Method II).

pH (2.2.3): 7.5 to 9.0.

Dilute 5 ml of solution S to 10 ml with *carbon dioxide-free water R*.

Reducing substances. Dissolve 1.0 g in 100 ml of boiling *water R*, add 5 ml of *dilute sulphuric acid R* and 0.5 ml of 0.002 M *potassium permanganate*, mix and boil gently for 5 min. The pink colour is not completely discharged.

Chlorides (2.4.4): maximum 200 ppm.

2.5 ml of solution S diluted to 15 ml with *water R* complies with the limit test for chlorides.

Sulphates (2.4.13): maximum 200 ppm.

7.5 ml of solution S diluted to 15 ml with *distilled water R* complies with the limit test for sulphates.

Aluminium (2.4.17): maximum 0.2 ppm, if intended for use in the manufacture of dialysis solutions.

Dissolve 20 g in 100 ml of *water R* and adjust to pH 6.0 by the addition of 1 M *hydrochloric acid* (about 10 ml). The solution complies with the limit test for aluminium. Use as the reference solution a mixture of 2 ml of *aluminium standard solution* (2 ppm Al) *R*, 10 ml of *acetate buffer solution pH 6.0 R* and 98 ml of *water R*. To prepare the blank, use a mixture of 10 ml of *acetate buffer solution pH 6.0 R* and 100 ml of *water R*.

Arsenic (2.4.2): maximum 2 ppm.

0.5 g complies with limit test A.

Calcium and magnesium: maximum 50 ppm, calculated as Ca.

To 200 ml of *water R* add 10 ml of *ammonium chloride buffer solution pH 10.0 R*, 0.1 g of *mordant black 11 triturate R*, 2.0 ml of 0.05 M *zinc chloride* and, dropwise, 0.02 M *sodium edetate* until the colour changes from violet to blue. Add to the solution 10.0 g of the substance to be examined and shake to dissolve. Titrate with 0.02 M *sodium edetate* until the blue colour is restored. Not more than 0.65 ml of 0.02 M *sodium edetate* is required.

Heavy metals (2.4.8): maximum 10 ppm.

12 ml of solution S complies with limit test A. Prepare the standard using *lead standard solution* (1 ppm Pb) *R*.

Iron (2.4.9): maximum 10 ppm.

10 ml of solution S complies with the limit test for iron.

Loss on drying (2.2.32): 39.0 per cent to 40.5 per cent, determined on 1.000 g by drying in an oven at 130 °C. Introduce the substance to be examined into the oven while the latter is cold.

ASSAY

Dissolve 0.250 g in 50 ml of *anhydrous acetic acid R*, add 5 ml of *acetic anhydride R*, mix and allow to stand for 30 min. Using 0.3 ml of *naphtholbenzein solution R* as indicator, titrate with 0.1 M *perchloric acid* until a green colour is obtained.

1 ml of 0.1 M *perchloric acid* is equivalent to 8.20 mg of $C_4H_{12}NNaO_7P_2 \cdot 3H_2O$.

STORAGE

In an airtight container.

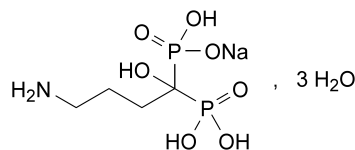
LABELLING

The label states, where applicable, that the substance is suitable for use in the manufacture of dialysis solutions.

01/2005:1564
corrected

SODIUM ALENDRONATE

Natrii alendronas



$C_4H_{12}NNaO_7P_2 \cdot 3H_2O$

M_r 325.1

DEFINITION

Sodium alendronate contains not less than 98.0 per cent and not more than the equivalent of 102.0 per cent of (4-amino-1-hydroxybutylidene)bisphosphonic acid monosodium salt, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, crystalline powder, soluble in water, very slightly soluble in methanol, practically insoluble in methylene chloride.

IDENTIFICATION

A. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *sodium alendronate CRS*. Examine the substances prepared as discs.

B. It gives reaction (a) of sodium (2.3.1).

TESTS

Solution S. Dissolve 0.5 g in *carbon dioxide-free water R* prepared from *distilled water R* and dilute to 50 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and not more intensely coloured than reference solution B₇ or BY₇ (2.2.2, *Method II*).

pH (2.2.3). The pH of solution S is 4.0 to 5.0.

4-aminobutanoic acid. Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel plate R*.

Test solution. Dissolve 0.10 g of the substance to be examined in *water R* and dilute to 10 ml with the same solvent.

Reference solution (a). Dissolve 0.10 g of 4-aminobutanoic acid *R* in *water R* and dilute to 200 ml with the same solvent.

Reference solution (b). Dilute 1 ml of reference solution (a) to 10 ml with *water R*.

Apply to the plate 5 µl of the test solution and 5 µl of reference solution (b). Allow the plate to dry in air. Develop over a path of 15 cm using a mixture of 20 volumes of *water R*, 20 volumes of *glacial acetic acid R* and 60 volumes of *butanol R*. Dry the plate in a current of warm air. Spray with *ninhydrin solution R* and heat at 100 °C to 105 °C for 15 min. Any spots corresponding to 4-aminobutanoic acid in the chromatogram obtained with the test solution are not more intense than the spot in the chromatogram obtained with reference solution (b) (0.5 per cent).

Phosphate and phosphite. Examine the chromatograms obtained in the assay. In the chromatogram obtained with the test solution: the area of any peak corresponding to phosphate is not greater than that of the peak due to phosphate in the chromatogram obtained with reference solution (d) (0.5 per cent); the area of any peak corresponding

to phosphite is not greater than that of the peak due to phosphite in the chromatogram obtained with reference solution (d) (0.5 per cent).

Heavy metals (2.4.8). 1.0 g complies with limit test F for heavy metals (20 ppm). Prepare the standard using 2 ml of *lead standard solution* (10 ppm Pb) R.

Loss on drying (2.2.32): 16.1 per cent to 17.1 per cent, determined on 1.000 g by drying in an oven at 140 °C to 145 °C.

ASSAY

Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 50.0 mg of the substance to be examined in *water R* and dilute to 25.0 ml with the same solvent.

Reference solution (a). Dissolve 50.0 mg of *sodium alendronate CRS* in *water R* and dilute to 25.0 ml with the same solvent.

Reference solution (b). Dissolve 3.0 g of *phosphoric acid R* in *water R* and dilute to 100.0 ml with the same solvent. Dilute 1.0 ml of the solution to 100.0 ml with *water R*.

Reference solution (c). Dissolve 2.5 g of *phosphorous acid R* in *water R* and dilute to 100.0 ml with the same solvent. Dilute 1.0 ml of the solution to 100.0 ml with *water R*.

Reference solution (d). Mix 2.0 ml of reference solution (b) and 2.0 ml of reference solution (c) and dilute to 50.0 ml with *water R*.

The chromatographic procedure may be carried out using:

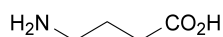
- a column 0.15 m long and 4.6 mm in internal diameter packed with *anion exchange resin R1* (7 µm),
- as mobile phase at a flow rate of 1.2 ml/min a solution of 0.2 ml of *anhydrous formic acid R* in 1000 ml of *water R*, adjusted to pH 3.5 with 2 M *sodium hydroxide solution*,
- as detector a refractometer,
- a 100 µl loop injector,

maintaining the temperature of the column at 35 °C.

Inject reference solution (a) six times. The assay is not valid unless the relative standard deviation of the peak area of sodium alendronate is at most 1.0 per cent. Inject the test solution, reference solution (a) and reference solution (d). The retention time of sodium alendronate is about 16 min and the relative retention times are: phosphate about 1.3 and phosphite about 1.6. Record the chromatograms for twice the retention time of the principal peak in the chromatogram obtained with the test solution.

Calculate the percentage content of C₄H₁₂NNaO₇P₂ from the peak areas and the declared content of *sodium alendronate CRS*.

IMPURITIES



- A. 4-aminobutanoic acid,
- B. phosphate,
- C. phosphite.

01/2005:0625

SODIUM ALGINATE

Natrii alginas

DEFINITION

Sodium alginate consists mainly of the sodium salt of alginic acid, which is a mixture of polyuronic acids [C₆H₈O₆]_n composed of residues of D-mannuronic acid and L-guluronic acid, and is obtained mainly from algae belonging to the Phaeophyceae.

CHARACTERS

A white or pale yellowish-brown powder, slowly soluble in water forming a viscous, colloidal solution, practically insoluble in alcohol.

IDENTIFICATION

- A. Dissolve 0.2 g with shaking in 20 ml of *water R*. To 5 ml of this solution add 1 ml of *calcium chloride solution R*. A voluminous gelatinous mass is formed.
- B. To 10 ml of the solution prepared in identification test A add 1 ml of *dilute sulphuric acid R*. A gelatinous mass is formed.
- C. To 5 mg add 5 ml of *water R*, 1 ml of a freshly prepared 10 g/l solution of *1,3-dihydroxynaphthalene R* in *alcohol R* and 5 ml of *hydrochloric acid R*. Boil for 3 min, cool, add 5 ml of *water R*, and shake with 15 ml of *di-isopropyl ether R*. Carry out a blank test. The upper layer obtained with the substance to be examined exhibits a deeper bluish-red colour than that obtained with the blank.
- D. It complies with the test for sulphated ash. The residue obtained, dissolved in 2 ml of *water R*, gives reaction (a) of sodium (2.3.1).

TESTS

Solution S. Dissolve 0.10 g in *water R*, with constant stirring, dilute to 30 ml with the same solvent and allow to stand for 1 h.

Appearance of solution. Dilute 1 ml of solution S to 10 ml with *water R*. The solution is not more opalescent than reference suspension II (2.2.1) and not more intensely coloured than intensity 6 of the range of reference solutions of the most appropriate colour (2.2.2, *Method II*).

Chlorides. Not more than 1.0 per cent. To 2.50 g add 50 ml of *dilute nitric acid R*, shake for 1 h and dilute to 100.0 ml with *dilute nitric acid R*. Filter. To 50.0 ml of the filtrate add 10.0 ml of 0.1 M *silver nitrate* and 5 ml of *toluene R*. Titrate with 0.1 M *ammonium thiocyanate*, using 2 ml of *ferric ammonium sulphate solution R2* as indicator and shaking vigorously towards the end point.

1 ml of 0.1 M *silver nitrate* is equivalent to 3.545 mg of Cl.

Calcium. Not more than 1.5 per cent of Ca, determined by atomic absorption spectrometry (2.2.23, *Method II*).

Test solution. Dissolve 0.10 g of the substance to be examined in 50 ml of *dilute ammonia R2*, heating on a water-bath. Allow to cool and dilute to 100.0 ml with *distilled water R* (solution (a)). Dilute 3.0 ml of solution (a) to 100.0 ml with *distilled water R*.

Reference solutions. Prepare three reference solutions in the same manner as the test solution but add 0.75 ml, 1.0 ml and 1.5 ml respectively of *calcium standard solution* (100 ppm Ca) R to the 3.0 ml of solution (a).

Set the zero of the instrument using a mixture of 1.5 volumes of *dilute ammonia R2* and 98.5 volumes of *distilled water R*. Measure the absorbance at 422.7 nm using a calcium hollow-cathode lamp as source of radiation and an air-acetylene flame.

Heavy metals (2.4.8). 1.0 g complies with the limit test F for heavy metals (20 ppm). Prepare the standard using 2 ml of *lead standard solution (10 ppm Pb) R*.

Loss on drying (2.2.32). Not more than 15.0 per cent, determined on 0.1000 g by drying in an oven at 100 °C to 105 °C for 4 h.

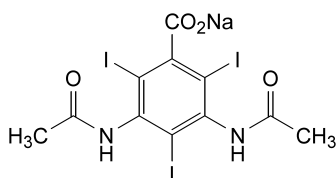
Sulphated ash (2.4.14): 30.0 per cent to 36.0 per cent, determined on 0.1000 g and calculated with reference to the dried substance.

Microbial contamination. Total viable aerobic count (2.6.12) not more than 10³ micro-organisms per gram, determined by plate-count. It complies with the tests for *Escherichia coli* and *Salmonella* (2.6.13).

01/2005:1150

SODIUM AMIDOTRIZOATE

Natrii amidotrizoas

C₁₁H₈I₃N₂NaO₄M_r 636

DEFINITION

Sodium amidotrizoate contains not less than 98.0 per cent and not more than the equivalent of 101.0 per cent of sodium 3,5-bis(acetylamino)-2,4,6-tri-iodobenzoate, calculated with reference to the anhydrous substance.

CHARACTERS

A white or almost white powder, freely soluble in water, slightly soluble in alcohol, practically insoluble in acetone. It melts at about 261 °C with decomposition.

IDENTIFICATION

First identification: A, D.

Second identification: B, C, D.

- Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *sodium amidotrizoate CRS*. Dry both the substance to be examined and the reference substance at 100 °C to 105 °C for 3 h.
- Examine the chromatograms obtained in the test for related substances. The principal spot in the chromatogram obtained with test solution (b) is similar in position and size to the principal spot in the chromatogram obtained with reference solution (b).
- Heat 50 mg gently in a small porcelain dish over a naked flame. Violet vapour is evolved.
- It gives reaction (a) of sodium (2.3.1).

TESTS

Solution S. Dissolve 10 g in *carbon dioxide-free water R* and dilute to 20 ml with the same solvent.

Appearance of solution. Dilute 1 ml of solution S to 10 ml with *water R*. The solution is clear (2.2.1) and colourless (2.2.2, *Method II*).

pH (2.2.3). The pH of solution S is 7.5 to 9.5.

Related substances. Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel GF₂₅₄ plate R*. Prepare the solutions in subdued light and develop the chromatograms protected from light.

Test solution (a). Dissolve 0.50 g of the substance to be examined in a 3 per cent V/V solution of *ammonia R* in *methanol R* and dilute to 10 ml with the same solution.

Test solution (b). Dilute 1 ml of test solution (a) to 10 ml with a 3 per cent V/V solution of *ammonia R* in *methanol R*.

Reference solution (a). Dilute 1 ml of test solution (b) to 50 ml with a 3 per cent V/V solution of *ammonia R* in *methanol R*.

Reference solution (b). Dissolve 50 mg of *sodium amidotrizoate CRS* in a 3 per cent V/V solution of *ammonia R* in *methanol R* and dilute to 10 ml with the same solution.

Apply separately to the plate 2 µl of each solution. Develop over a path of 15 cm using a mixture of 20 volumes of *anhydrous formic acid R*, 25 volumes of *methyl ethyl ketone R* and 60 volumes of *toluene R*. Allow the plate to dry until the solvents have evaporated and examine in ultraviolet light at 254 nm. Any spot in the chromatogram obtained with test solution (a), apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (a) (0.2 per cent).

Free aromatic amines. Maintain the solutions and reagents in iced water, protected from light. To 0.50 g in a 50 ml volumetric flask add 15 ml of *water R*. Shake and add 1 ml of *dilute sodium hydroxide solution R*. Cool in iced water, add 5 ml of a freshly prepared 5 g/l solution of *sodium nitrite R* and 12 ml of *dilute hydrochloric acid R*. Shake gently and allow to stand for exactly 2 min after adding the hydrochloric acid. Add 10 ml of a 20 g/l solution of *ammonium sulphamate R*. Allow to stand for 5 min, shaking frequently, and add 0.15 ml of a 100 g/l solution of *α-naphthol R* in *alcohol R*. Shake and allow to stand for 5 min. Add 3.5 ml of *buffer solution pH 10.9 R*, mix and dilute to 50.0 ml with *water R*. The absorbance (2.2.25), measured within 20 min at 485 nm using as the compensation liquid a solution prepared at the same time and in the same manner but omitting the substance to be examined, is not greater than 0.30.

Free iodine and iodides. Not more than 50 ppm. Dissolve 1.0 g in *distilled water R* and dilute to 10 ml with the same solvent. Add dropwise *dilute nitric acid R* until the precipitation is complete, then add 3 ml of *dilute nitric acid R*. Filter and wash the precipitate with 5 ml of *water R*. Collect the filtrate and washings. Add 1 ml of *strong hydrogen peroxide solution R* and 1 ml of *methylene chloride R*. Shake. The lower layer is not more intensely coloured than a reference solution prepared simultaneously and in the same manner, using a mixture of 5 ml of *iodide standard solution (10 ppm I) R*, 3 ml of *dilute nitric acid R* and 15 ml of *water R*.

Heavy metals (2.4.8). Dilute 4 ml of solution S to 20 ml with *water R*. 12 ml of this solution complies with limit test A for heavy metals (20 ppm). Prepare the standard using *lead standard solution (2 ppm Pb) R*.

Water (2.5.12). Not more than 11.0 per cent, determined on 0.400 g by the semi-micro determination of water.

ASSAY

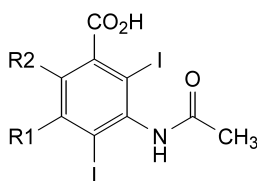
To 0.150 g in a 250 ml round-bottomed flask add 5 ml of *strong sodium hydroxide solution R*, 20 ml of *water R*, 1 g of *zinc powder R* and a few glass beads. Boil under a reflux condenser for 30 min. Allow to cool and rinse the condenser with 20 ml of *water R*, adding the rinsings to the flask. Filter through a sintered-glass filter and wash the filter with several quantities of *water R*. Collect the filtrate and washings. Add 40 ml of *dilute sulphuric acid R* and titrate immediately with 0.1 M *silver nitrate*. Determine the end-point potentiometrically (2.2.20), using a suitable electrode system such as silver-mercurous sulphate.

1 ml of 0.1 M *silver nitrate* is equivalent to 21.20 mg of $C_{11}H_8I_3N_2NaO_4$.

STORAGE

Store protected from light.

IMPURITIES

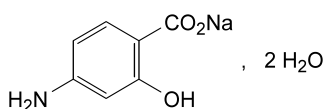


- A. $R_1 = NH_2$, $R_2 = I$: 3-acetylamino-5-amino-2,4,6-tri-iodobenzoic acid,
 B. $R_1 = NHCOCH_3$, $R_2 = H$: 3,5-bis(acetylamino)-2,4-di-iodobenzoic acid.

01/2005:1993

SODIUM AMINOSALICYLATE
DIHYDRATE

Natrii aminosalicylas dihydricus

 $C_7H_6NNaO_3 \cdot 2H_2O$ M_r 211.2

DEFINITION

Sodium 4-amino-2-hydroxybenzoate dihydrate.

Content: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

Appearance: white, crystalline powder or white or almost white crystals, slightly hygroscopic.

Solubility: freely soluble in water, sparingly soluble in alcohol, practically insoluble in methylene chloride.

IDENTIFICATION

First identification: A, E.

Second identification: B, C, D, E.

A. Infrared absorption spectrophotometry (2.2.24).

Comparison: Ph. Eur. reference spectrum of sodium aminosalicylate dihydrate.

B. Introduce 0.3 g into a porcelain crucible. Cautiously heat on a small flame until vapour is evolved. Cover the crucible with a watch glass and collect the white sublimate. The melting point (2.2.14) of the sublimate is 120 °C to 124 °C.

C. To 0.1 ml of solution S (see Tests) add 5 ml of *water R* and 0.1 ml of *ferric chloride solution R1*. A reddish-brown colour develops.

D. 2 ml of solution S gives the reaction of primary aromatic amines (2.3.1).

E. 0.5 ml of solution S gives reaction (a) of sodium (2.3.1).

TESTS

Solution S. Dissolve 0.50 g in *carbon dioxide-free water R* and dilute to 25 ml with the same solvent.

Appearance of solution. The freshly prepared solution is clear (2.2.1) and not more intensely coloured than reference solution B₅ (2.2.2, *Method II*).

Dissolve 2.5 g in *water R* and dilute to 25 ml with the same solvent.

pH (2.2.3): 6.5 to 8.5 for solution S.

Related substances. Liquid chromatography (2.2.29). Use freshly prepared solutions and mobile phases.

Test solution. Dissolve 50.0 mg of the substance to be examined in *water R* and dilute to 50.0 ml with the same solvent.

Reference solution (a). Dissolve 5.0 mg of 3-aminophenol R in *water R* and dilute to 100.0 ml with the same solvent.

Reference solution (b). Dissolve 5.0 mg of mesalazine CRS in *water R* and dilute to 100.0 ml with the same solvent. To 10.0 ml of this solution add 1.0 ml of reference solution (a) and dilute to 50.0 ml with *water R*.

Column:

- *size*: $l = 0.25$ m, $\varnothing = 4.6$ mm,
- *stationary phase*: spherical base-deactivated octylsilyl silica gel for chromatography R (5 μ m).

Mobile phase:

- *mobile phase A*: dissolve 2.2 g of *perchloric acid R* and 1.0 g of *phosphoric acid R* in *water R* and dilute to 1000.0 ml with the same solvent,
- *mobile phase B*: dissolve 1.7 g of *perchloric acid R* and 1.0 g of *phosphoric acid R* in *acetonitrile R* and dilute to 1000.0 ml with the same solvent,

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)
0 - 15	100	0
15 - 30	100 → 40	0 → 60
30 - 35	40 → 100	60 → 0
35 - 45	100	0

Flow rate: 1.25 ml/min.

Detection: spectrophotometer at 220 nm.

Injection: 10 μ l.

Relative retention with reference to 4-aminosalicylate (retention time = about 12 min): impurity A = about 0.30; impurity B = about 0.37.

System suitability: reference solution (b):

- *resolution*: minimum 4.0 between the peaks due to impurity A and impurity B.

Limits:

- *impurity A*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (b) (0.1 per cent),
- *impurity B*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (b) (1.0 per cent),

- *any other impurity*: for each impurity, not more than 0.1 times the area of the peak due to impurity B in the chromatogram obtained with reference solution (b) (0.1 per cent),
- *total*: not more than the area of the peak due to impurity B in the chromatogram obtained with reference solution (b) (1.0 per cent),
- *disregard limit*: 0.05 times the area of the peak due to impurity B in the chromatogram obtained with reference solution (b) (0.05 per cent).

Heavy metals (2.4.8): maximum 10 ppm.

2.0 g complies with limit test C. Prepare the standard using 2 ml of *lead standard solution* (10 ppm Pb) R.

Loss on drying (2.2.32): 16.0 per cent to 17.5 per cent, determined on 1.000 g by drying in an oven at 100–105 °C.

Pyrogens (2.6.8). If intended for use in the manufacture of parenteral dosage forms without a further appropriate procedure for the removal of pyrogens, it complies with the test for pyrogens. Inject per kilogram of the rabbit's mass, 10 ml of a 20 mg/ml solution of the substance to be examined in *water for injections* R.

ASSAY

Dissolve 0.150 g in 20 ml of *water* R. Add 10 ml of a 500 g/l solution of *sodium bromide* R and 25 ml of *glacial acetic acid* R. Add 5 ml of 0.1 M *sodium nitrite* rapidly and continue the titration with the same titrant, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *sodium nitrite* is equivalent to 17.52 mg of $C_7H_6NNaO_3$.

STORAGE

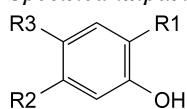
In an airtight container, protected from light. If the substance is sterile, store in a sterile, airtight, tamper-proof container.

LABELLING

The label states, where applicable, that the substance is apyrogenic.

IMPURITIES

Specified impurities: A, B.



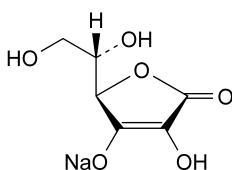
A. R1 = R3 = H, R2 = NH₂: 3-aminophenol,

B. R1 = CO₂H, R2 = H, R3 = NH₂: 5-amino-2-hydroxybenzoic acid (mesalazine).

01/2005:1791

SODIUM ASCORBATE

Natrii ascorbas



$C_6H_7NaO_6$

M_r 198.1

DEFINITION

Sodium (2*R*)-2-[(1*S*)-1,2-dihydroxyethyl]-4-hydroxy-5-oxo-2,5-dihydrofuran-3-olate.

Content: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

Appearance: white or yellowish, crystalline powder or crystals.

Solubility: freely soluble in water, sparingly soluble in alcohol, practically insoluble in methylene chloride.

IDENTIFICATION

First identification: B, D.

Second identification: A, C, D.

A. Specific optical rotation (2.2.7) (see Tests).

B. Infrared absorption spectrophotometry (2.2.24).

Preparation: discs.

Comparison: *sodium ascorbate CRS*.

C. To 1 ml of solution S (see Tests) add 0.2 ml of *dilute nitric acid* R and 0.2 ml of *silver nitrate solution* R2. A grey precipitate is formed.

D. 1 ml of solution S gives reaction (a) of sodium (2.3.1).

TESTS

Solution S. Dissolve 10.0 g in *carbon dioxide-free water* R prepared from *distilled water* R and dilute to 100.0 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and not more intensely coloured than reference solution Y₆ or BY₆ (2.2.2, *Method II*). Examine the colour immediately after preparation of the solution.

pH (2.2.3): 7.0 to 8.0 for solution S.

Specific optical rotation (2.2.7): + 103 to + 108 (dried substance), determined on freshly prepared solution S.

Oxalic acid: maximum 0.3 per cent.

Test solution. Dissolve 0.25 g in 5 ml of *water* R. Add 1 ml of *dilute acetic acid* R and 0.5 ml of *calcium chloride solution* R.

Reference solution. Dissolve 70 mg of *oxalic acid* R in *water* R and dilute to 500 ml with the same solvent; to 5 ml of the solution add 1 ml of *dilute acetic acid* R and 0.5 ml of *calcium chloride solution* R.

Allow the solutions to stand for 1 h. Any opalescence in the test solution is not more intense than that in the reference solution.

Benzene (2.4.24, *System A*): maximum 2 ppm.

Sulphates (2.4.13): maximum 150 ppm.

To 10 ml of solution S add 2 ml of *hydrochloric acid* R1 and dilute to 15 ml with *distilled water* R. The solution complies with the limit test for sulphates.

Copper: maximum 5 ppm.

Atomic absorption spectrometry (2.2.23, *Method I*).

Test solution. Dissolve 2.0 g in 0.1 M *nitric acid* and dilute to 25.0 ml with the same solvent.

Reference solutions. Prepare reference solutions (0.2 ppm, 0.4 ppm and 0.6 ppm) by diluting *copper standard solution* (10 ppm Cu) R with 0.1 M *nitric acid*.

Source: copper hollow-cathode lamp.

Wavelength: 324.8 nm.

Flame: air-acetylene.

Iron: maximum 2 ppm.

Atomic absorption spectrometry (2.2.23, *Method I*).

Test solution. Dissolve 5.0 g in 0.1 M *nitric acid* and dilute to 25.0 ml with the same solvent.

Reference solutions. Prepare reference solutions (0.2 ppm, 0.4 ppm and 0.6 ppm) by diluting *iron standard solution* (20 ppm Fe) *R* with 0.1 M nitric acid.

Source: iron hollow-cathode lamp.

Wavelength: 248.3 nm.

Flame: air-acetylene.

Nickel: maximum 1 ppm.

Atomic absorption spectrometry (2.2.23, *Method I*).

Test solution. Dissolve 10.0 g in 0.1 M nitric acid and dilute to 25.0 ml with the same solvent.

Reference solutions. Prepare reference solutions (0.2 ppm, 0.4 ppm and 0.6 ppm) by diluting *nickel standard solution* (10 ppm Ni) *R* with 0.1 M nitric acid.

Source: nickel hollow-cathode lamp.

Wavelength: 232.0 nm.

Flame: air-acetylene.

Heavy metals (2.4.8): maximum 10 ppm.

Dissolve 2.0 g in *water R* and dilute to 20 ml with the same solvent. 12 ml of the solution complies with limit test A.

Prepare the standard using *lead standard solution* (1 ppm Pb) *R*.

Loss on drying (2.2.32): maximum 0.25 per cent, determined on 1.000 g by drying in an oven at 100–105 °C.

ASSAY

Dissolve 80 mg in a mixture of 10 ml of *dilute sulphuric acid R* and 80 ml of *carbon dioxide-free water R*. Add 1 ml of *starch solution R*. Titrate with 0.05 M iodine until a persistent violet-blue colour is obtained.

1 ml of 0.05 M iodine is equivalent to 9.91 mg of C₆H₅NaO₂.

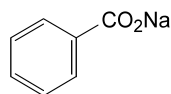
STORAGE

Non-metallic container, protected from light.

01/2005:0123

SODIUM BENZOATE

Natrii benzoas



C₇H₅NaO₂

*M*_r 144.1

DEFINITION

Sodium benzoate contains not less than 99.0 per cent and not more than the equivalent of 100.5 per cent of sodium benzenecarboxylate, calculated with reference to the dried substance.

CHARACTERS

A white, crystalline or granular powder or flakes, slightly hygroscopic, freely soluble in water, sparingly soluble in alcohol (90 per cent V/V).

IDENTIFICATION

- A. It gives reactions (b) and (c) of benzoates (2.3.1).
- B. It gives reaction (a) of sodium (2.3.1).

TESTS

Solution S. Dissolve 10.0 g in *carbon dioxide-free water R* and dilute to 100 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and not more intensely coloured than reference solution Y₆ (2.2.2, *Method II*).

Acidity or alkalinity. To 10 ml of solution S add 10 ml of *carbon dioxide-free water R* and 0.2 ml of *phenolphthalein solution R*. Not more than 0.2 ml of 0.1 M sodium hydroxide or 0.1 M hydrochloric acid is required to change the colour of the indicator.

Halogenated compounds. All glassware used must be chloride-free and may be prepared by soaking overnight in a 500 g/l solution of nitric acid *R*, rinsed with *water R* and stored full of *water R*. It is recommended that glassware be reserved exclusively for this test.

To 20.0 ml of solution S add 5 ml of *water R* and dilute to 50.0 ml with *alcohol R* (test solution).

Determination of ionised chlorine. In three 25 ml volumetric flasks, prepare the following solutions.

Solution (a). To 4.0 ml of the test solution add 3 ml of *dilute sodium hydroxide solution R* and 3 ml of *alcohol R*. This solution is used to prepare solution A.

Solution (b). To 3 ml of *dilute sodium hydroxide solution R* add 2 ml of *water R* and 5 ml of *alcohol R*. This solution is used to prepare solution B.

Solution (c). To 4.0 ml of *chloride standard solution* (8 ppm Cl) *R* add 6.0 ml of *water R*. This solution is used to prepare solution C.

In a fourth 25 ml volumetric flask, place 10 ml of *water R*. To each flask add 5 ml of *ferric ammonium sulphate solution R5*, mix and add dropwise and with swirling 2 ml of *nitric acid R* and 5 ml of *mercuric thiocyanate solution R*. Shake. Dilute the contents of each flask to 25.0 ml with *water R* and allow the solutions to stand in a water-bath at 20 °C for 15 min. Measure at 460 nm in a 2 cm cell the absorbance (2.2.25) of solution A using solution B as the compensation liquid, and the absorbance of solution C using the solution obtained with 10 ml of *water R* as the compensation liquid. The absorbance of solution A is not greater than that of solution C (200 ppm).

Determination of total chlorine

Solution (a). To 10.0 ml of the test solution add 7.5 ml of *dilute sodium hydroxide solution R* and 0.125 g of *nickel-aluminium alloy R* and heat on a water-bath for 10 min. Allow to cool to room temperature, filter into a 25 ml volumetric flask and wash the filter with 3 quantities, each of 2 ml, of *alcohol R* (a slight precipitate may form that disappears on acidification). Dilute the filtrate and washings to 25.0 ml with *water R*. This solution is used to prepare solution A.

Solution (b). In the same manner, prepare a similar solution replacing the test solution by a mixture of 5 ml of *alcohol R* and 5 ml of *water R*. This solution is used to prepare solution B.

Solution (c). To 6.0 ml of *chloride standard solution* (8 ppm Cl) *R* add 4.0 ml of *water R*. This solution is used to prepare solution C.

In four 25 ml volumetric flasks, place separately 10 ml of solution (a), 10 ml of solution (b), 10 ml of solution (c) and 10 ml of *water R*. To each flask add 5 ml of *ferric ammonium sulphate solution R5*, mix and add dropwise and with swirling 2 ml of *nitric acid R* and 5 ml of *mercuric thiocyanate solution R*. Shake. Dilute the contents of each flask to 25.0 ml with *water R* and allow the solutions to stand in a water-bath at 20 °C for 15 min. Measure at 460 nm in a 2 cm cell the absorbance (2.2.25) of solution A using solution B as the compensation liquid, and the absorbance of

solution C using the solution obtained with 10 ml of *water R* as the compensation liquid. The absorbance of solution A is not greater than that of solution C (300 ppm).

Heavy metals (2.4.8). 2.0 g complies with limit test C for heavy metals (10 ppm). Prepare the standard using 2 ml of *lead standard solution (10 ppm Pb) R*.

Loss on drying (2.2.32). Not more than 2.0 per cent, determined on 1.00 g by drying in an oven at 100 °C to 105 °C.

ASSAY

Dissolve 0.250 g in 20 ml of *anhydrous acetic acid R*, heating to 50 °C if necessary. Cool. Using 0.05 ml of *naphtholbenzein solution R* as indicator, titrate with 0.1 M *perchloric acid* until a green colour is obtained.

1 ml of 0.1 M *perchloric acid* is equivalent to 14.41 mg of $C_7H_5NaO_2$.

01/2005:0190

SODIUM BROMIDE

Natrii bromidum

NaBr

 M_r 102.9

DEFINITION

Content: 98.0 per cent to 100.5 per cent (dried substance).

CHARACTERS

Appearance: white, granular powder or small, colourless, transparent or opaque crystals, slightly hygroscopic.

Solubility: freely soluble in water, soluble in alcohol.

IDENTIFICATION

- It gives reaction (a) of bromides (2.3.1).
- Solution S (see Tests) gives the reactions of sodium (2.3.1).

TESTS

Solution S. Dissolve 10.0 g in *carbon dioxide-free water R* prepared from *distilled water R* and dilute to 100 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and colourless (2.2.2, *Method II*).

Acidity or alkalinity. To 10 ml of solution S add 0.1 ml of *bromothymol blue solution R1*. Not more than 0.5 ml of 0.01 M *hydrochloric acid* or 0.01 M *sodium hydroxide* is required to change the colour of the indicator.

Bromates. To 10 ml of solution S add 1 ml of *starch solution R*, 0.1 ml of a 100 g/l solution of *potassium iodide R* and 0.25 ml of 0.5 M *sulphuric acid* and allow to stand protected from light for 5 min. No blue or violet colour develops.

Chlorides: maximum 0.6 per cent.

In a conical flask, dissolve 1.000 g in 20 ml of *dilute nitric acid R*. Add 5 ml of *strong hydrogen peroxide solution R* and heat on a water-bath until the solution is completely decolourised. Wash down the sides of the flask with a little *water R* and heat on a water-bath for 15 min. Allow to cool, dilute to 50 ml with *water R* and add 5.0 ml of 0.1 M *silver nitrate* and 1 ml of *dibutyl phthalate R*. Shake and titrate with 0.1 M *ammonium thiocyanate*, using 5 ml of *ferric ammonium sulphate solution R2* as indicator. Not more than 1.7 ml of 0.1 M *silver nitrate* is used. Note the volume of 0.1 M *silver nitrate* used (see Assay). Carry out a blank test.

Iodides. To 5 ml of solution S add 0.15 ml of *ferric chloride solution R1* and 2 ml of *methylen chloride R*. Shake and allow to separate. The lower layer is colourless (2.2.2, *Method I*).

Sulphates (2.4.13): maximum 100 ppm.

15 ml of solution S complies with the limit test for sulphates.

Iron (2.4.9): maximum 20 ppm.

5 ml of solution S diluted to 10 ml with *water R* complies with the limit test for iron.

Magnesium and alkaline-earth metals (2.4.7): maximum 200 ppm, calculated as Ca.

10.0 g complies with the limit test for magnesium and alkaline-earth metals. The volume of 0.01 M *sodium edetate* used does not exceed 5.0 ml.

Heavy metals (2.4.8): maximum 10 ppm.

12 ml of solution S complies with limit test A. Prepare the standard using *lead standard solution (1 ppm Pb) R*.

Loss on drying (2.2.32): maximum 3.0 per cent, determined on 1.000 g by drying in an oven at 100-105 °C for 3 h.

ASSAY

Dissolve 2.000 g in *water R* and dilute to 100.0 ml with the same solvent. To 10.0 ml of the solution add 50 ml of *water R*, 5 ml of *dilute nitric acid R*, 25.0 ml of 0.1 M *silver nitrate* and 2 ml of *dibutyl phthalate R*. Shake. Titrate with 0.1 M *ammonium thiocyanate*, using 2 ml of *ferric ammonium sulphate solution R2* as indicator and shaking vigorously towards the end-point.

1 ml of 0.1 M *silver nitrate* is equivalent to 10.29 mg of NaBr. Calculate the percentage content of NaBr from the expression:

$$a - 2.902 b$$

a = percentage content of NaBr and NaCl obtained in the assay and calculated as NaBr,

b = percentage content of Cl in the test for chlorides.

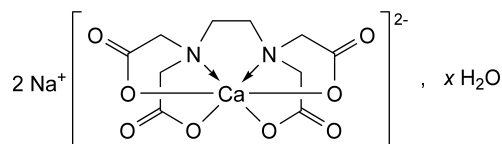
STORAGE

In an airtight container.

01/2005:0231

SODIUM CALCIUM EDETATE

Natrii calcii edetas



$C_{10}H_{12}CaN_2Na_2O_{16} \cdot xH_2O$ M_r 374.3 (anhydrous substance)

DEFINITION

Disodium [(ethylenedinitrilo)tetraacetato]calciate(2-).

Content: 98.0 per cent to 102.0 per cent (anhydrous substance).

It contains a variable amount of water of crystallisation.

CHARACTERS

Appearance: white or almost white powder, hygroscopic.

Solubility: freely soluble in water, practically insoluble in alcohol.

IDENTIFICATION

First identification: A, C, D.

Second identification: B, C, D.

A. Infrared absorption spectrophotometry (2.2.24).

Preparation: discs.

Comparison: sodium calcium edetate CRS.

B. Dissolve 2 g in 10 ml of *water R*, add 6 ml of *lead nitrate solution R*, shake and add 3 ml of *potassium iodide solution R*. No yellow precipitate is formed. Make alkaline to *red litmus paper R* by the addition of *dilute ammonia R2* and add 3 ml of *ammonium oxalate solution R*. A white precipitate is formed.

C. Ignite. The residue gives the reactions of calcium (2.3.1).

D. The residue obtained in identification test C gives the reactions of sodium (2.3.1).

TESTS

Solution S. Dissolve 5.0 g in *water R* and dilute to 100 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and colourless (2.2.2, *Method II*).

pH (2.2.3): 6.5 to 8.0.

Dissolve 5.0 g in *carbon dioxide-free water R* and dilute to 25 ml with the same solvent.

Impurity A. Liquid chromatography (2.2.29). Carry out the test protected from light.

Solvent mixture. Dissolve 10.0 g of *ferric sulphate pentahydrate R* in 20 ml of *0.5 M sulphuric acid* and add 780 ml of *water R*. Adjust to pH 2.0 with *1 M sodium hydroxide* and dilute to 1000 ml with *water R*.

Test solution. Dissolve 0.100 g of the substance to be examined in the solvent mixture and dilute to 25.0 ml with the solvent mixture.

Reference solution. Dissolve 40.0 mg of *nitrilotriacetic acid R* in the solvent mixture and dilute to 100.0 ml with the solvent mixture. To 1.0 ml of the solution add 0.1 ml of the test solution and dilute to 100.0 ml with the solvent mixture.

Column:

- size: $l = 0.10$ m, $\varnothing = 4.6$ mm,
- stationary phase: spherical graphitised carbon for chromatography R1 (5 μ m) with a specific surface area of 120 m²/g and a pore size of 25 nm.

Mobile phase: dissolve 50.0 mg of *ferric sulphate pentahydrate R* in 50 ml of *0.5 M sulphuric acid* and add 750 ml of *water R*. Adjust to pH 1.5 with *0.5 M sulphuric acid* or *1 M sodium hydroxide*, add 20 ml of *ethylene glycol R* and dilute to 1000 ml with *water R*.

Flow rate: 1 ml/min.

Detection: spectrophotometer at 273 nm.

Injection: 20 μ l; filter the solutions and inject immediately.

Run time: 4 times the retention time of the iron complex of impurity A.

Retention time: iron complex of impurity A = about 5 min; iron complex of edetic acid = about 10 min.

System suitability: reference solution:

- resolution: minimum 7 between the peaks due to the iron complex of impurity A and the iron complex of edetic acid,
- signal-to-noise ratio: minimum 50 for the peak due to impurity A.

Limit:

- impurity A: not more than the area of the corresponding peak in the chromatogram obtained with the reference solution (0.1 per cent).

Disodium edetate: maximum 1.0 per cent.

Dissolve 5.0 g in 250 ml of *water R*. Add 10 ml of *ammonium chloride buffer solution pH 10.0 R* and about 50 mg of *mordant black 11 triturate R*. Not more than 1.5 ml of *0.1 M magnesium chloride* is required to change the colour of the indicator to violet.

Chlorides (2.4.4): maximum 0.1 per cent.

To 20 ml of solution S add 30 ml of *dilute nitric acid R*, allow to stand for 30 min and filter. Dilute 2.5 ml of the filtrate to 15 ml with *water R*.

Iron (2.4.9): maximum 80 ppm.

Dilute 2.5 ml of solution S to 10 ml with *water R*. Add 0.25 g of *calcium chloride R* to the test solution and the standard before the addition of the *thioglycollic acid R*.

Heavy metals (2.4.8): maximum 20 ppm.

1.0 g complies with limit test D. Prepare the standard using 2 ml of *lead standard solution (10 ppm Pb) R*.

Water (2.5.12, *Method B*): 5.0 per cent to 13.0 per cent, determined on 0.100 g.

ASSAY

Dissolve 0.300 g in *water R* and dilute to 300 ml with the same solvent. Add 2 g of *hexamethylenetetramine R* and 2 ml of *dilute hydrochloric acid R*. Titrate with *0.1 M lead nitrate*, using about 50 mg of *xylene orange triturate R* as indicator.

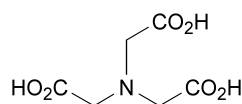
1 ml of *0.1 M lead nitrate* is equivalent to 37.43 mg of $C_{10}H_{12}CaN_2Na_2O_8$.

STORAGE

In an airtight container, protected from light.

IMPURITIES

Specified impurities: A.

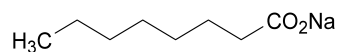


A. nitrilotriacetic acid.

01/2005:1471

SODIUM CAPRYLATE

Natrii caprylas



$C_8H_{15}NaO_2$

M_r 166.2

DEFINITION

Sodium caprylate contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of sodium octanoate, calculated with reference to the anhydrous substance.

CHARACTERS

A white, crystalline powder, very soluble or freely soluble in water, freely soluble in acetic acid, sparingly soluble in alcohol, practically insoluble in acetone.

IDENTIFICATION

A. Examine the chromatograms obtained in the test for related substances. The retention time and size of the principal peak in the chromatogram obtained with the

test solution are approximately the same as those of the principal peak in the chromatogram obtained with reference solution (a).

- B. To 0.2 ml of solution S (see Tests) add 0.3 ml of *water R*. The solution gives reaction (b) of sodium (2.3.1).

TESTS

Solution S. Dissolve 2.5 g in *carbon dioxide-free water R* and dilute to 25 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and colourless (2.2.2, *Method II*).

pH (2.2.3). The pH of solution S is 8.0 to 10.5.

Related substances. Examine by gas chromatography (2.2.28).

Test solution. Dissolve 0.116 g in *water R* and dilute to 5 ml with the same solvent. Add 1 ml of a 2.8 per cent V/V solution of *sulphuric acid R* and shake with 10 ml of *ethyl acetate R*. Separate the organic layer and dry over *anhydrous sodium sulphate R*.

Reference solution (a). Dissolve 0.10 g of *caprylic acid CRS* in *ethyl acetate R* and dilute to 10 ml with the same solvent.

Reference solution (b). Dilute 1 ml of the test solution to 100 ml with *ethyl acetate R*. Dilute 5 ml of the solution to 50 ml with *ethyl acetate R*.

The chromatographic procedure may be carried out using:

- a fused-silica column 30 m long and 0.25 mm in internal diameter coated with *macrogol 20 000 2-nitroterephthalate R* (film thickness 0.25 µm),
- *helium for chromatography R* as the carrier gas at a flow rate of 1.5 ml/min,
- a flame-ionisation detector,
- a split ratio of 1:100,

with the following temperature programme:

	Time (min)	Temperature (°C)	Rate (°C/min)	Comment
Column	0 - 1	100		isothermal
	1 - 25	100 → 220	5	linear gradient
	25 - 35	220		isothermal
Injection port		250		
Detector		250		

Inject 1 µl of reference solution (b). The test is not valid unless in the chromatogram obtained the principal peak has a signal-to-noise ratio of at least 5.

Inject 1 µl of the test solution and 1 µl of reference solution (a). Calculate the percentage of related substances from the areas of the peaks in the chromatogram obtained with the test solution by the normalisation procedure, disregarding any peaks with an area less than 0.5 times the area of the peak in the chromatogram obtained with reference solution (b) and any peak due to the solvent. The content of any related substance is not greater than 0.3 per cent and the sum of the related substances is not greater than 0.5 per cent.

Heavy metals (2.4.8). Dissolve 2.0 g in *glacial acetic acid R* and dilute to 10 ml with the same acid. Add 10 ml of *alcohol R*. 12 ml of the solution complies with limit test B for heavy metals (10 ppm). Prepare the standard using 1 ml of *lead standard solution (10 ppm Pb) R* and 9 ml of a mixture of equal volumes of *glacial acetic acid R* and *alcohol R*.

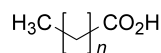
Water (2.5.12). Not more than 3.0 per cent, determined on 1.000 g by the semi-micro determination of water.

ASSAY

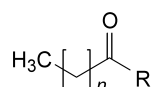
Dissolve 0.150 g in 50 ml of *anhydrous acetic acid R*. Titrate with 0.1 M *perchloric acid*, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M perchloric acid is equivalent to 16.62 mg of $C_8H_{15}NaO_9$.

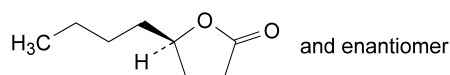
IMPURITIES



- A. $n = 4$: hexanoic acid,
B. $n = 5$: heptanoic acid,
C. $n = 7$: nonanoic acid,
D. $n = 8$: decanoic acid,
E. valproic acid.



- F. $R = \text{OCH}_3$, $n = 6$: methyl octanoate,
G. $R = \text{OC}_2\text{H}_5$, $n = 6$: ethyl octanoate,
H. $R = \text{OCH}_3$, $n = 8$: methyl decanoate,
I. $R = \text{CH}_2$, $n = 8$: undecan-2-one.



- J. (*RS*)-5-butyltetrahydrofuran-2-one (γ -hydroxyoctanoic acid lactone).

01/2005:0773

SODIUM CARBONATE, ANHYDROUS

Natrii carbonas anhydricus

 M_r 106.0

DEFINITION

Anhydrous sodium carbonate contains not less than 99.5 per cent and not more than the equivalent of 100.5 per cent of Na_2CO_3 , calculated with reference to the dried substance.

CHARACTERS

A white or almost white, slightly granular powder, hygroscopic, freely soluble in water, practically insoluble in alcohol.

IDENTIFICATION

- A. Dissolve 1 g in *water R* and dilute to 10 ml with the same solvent. The solution is strongly alkaline (2.2.4).
- B. The solution prepared for identification test A gives the reaction of carbonates (2.3.1).
- C. The solution prepared for identification test A gives reaction (a) of sodium (2.3.1).
- D. It complies with the test for loss on drying (see Tests).

TESTS

Solution S. Dissolve 2.0 g in portions in a mixture of 5 ml of *hydrochloric acid R* and 25 ml of *distilled water R*. Heat the solution to boiling and cool. Add *dilute sodium hydroxide solution R* until the solution is neutral and dilute to 50 ml with *distilled water R*.

Appearance of solution. Dissolve 2.0 g in 10 ml of *water R*. The solution is clear (2.2.1) and not more intensely coloured than reference solution Y₆ (2.2.2, *Method I*).

Alkali hydroxides and bicarbonates. Dissolve 0.4 g in 20 ml of *water R*. Add 20 ml of *barium chloride solution R1* and filter. To 10 ml of the filtrate add 0.1 ml of *phenolphthalein solution R*. The solution does not become red. Boil the remainder of the filtrate for 2 min. The solution remains clear (2.2.1).

Chlorides (2.4.4). Dissolve 0.4 g in *water R*, add 4 ml of *dilute nitric acid R* and dilute to 15 ml with *water R*. The solution complies with the limit test for chlorides (125 ppm).

Sulphates (2.4.13). 15 ml of solution S complies with the limit test for sulphates (250 ppm).

Arsenic (2.4.2). 5 ml of solution S complies with limit test A for arsenic (5 ppm).

Iron (2.4.9). Dilute 5 ml of solution S to 10 ml with *water R*. The solution complies with the limit test for iron (50 ppm).

Heavy metals (2.4.8). 12 ml of solution S complies with limit test A for heavy metals (50 ppm). Prepare the standard using *lead standard solution (2 ppm Pb) R*.

Loss on drying (2.2.32). Not more than 1.0 per cent, determined on 1.000 g by drying at 300 °C.

ASSAY

Dissolve 1.000 g in 25 ml of *water R*. Add 0.2 ml of *methyl orange solution R* as indicator. Titrate with 1 M *hydrochloric acid* until the colour changes from yellow to red.

1 ml of 1 M *hydrochloric acid* is equivalent to 52.99 mg of Na₂CO₃.

STORAGE

Store in an airtight container.

Appearance of solution. Dissolve 4.0 g in 10 ml of *water R*. The solution is clear (2.2.1) and not more intensely coloured than reference solution Y₆ (2.2.2, *Method I*).

Alkali hydroxides and bicarbonates. Dissolve 1.0 g in 20 ml of *water R* add 20 ml of *barium chloride solution R1* and filter. To 10 ml of the filtrate add 0.1 ml of *phenolphthalein solution R*. The solution does not become red. Heat the remainder of the filtrate to boiling for 2 min. The solution remains clear (2.2.1).

Chlorides (2.4.4). Dissolve 1.0 g in *water R*, add 4 ml of *dilute nitric acid R* and dilute to 15 ml with *water R*. The solution complies with the limit test for chlorides (50 ppm).

Sulphates (2.4.13). 15 ml of solution S complies with the limit test for sulphates (100 ppm).

Arsenic (2.4.2). 5 ml of solution S complies with limit test A for arsenic (2 ppm).

Heavy metals (2.4.8). 12 ml of solution S complies with limit test A for heavy metals (20 ppm). Prepare the standard using *lead standard solution (2 ppm Pb) R*.

Iron (2.4.9). 5 ml of solution S diluted to 10 ml with *water R* complies with the limit test for iron (20 ppm).

ASSAY

Dissolve 2.000 g in 25 ml of *water R*. Titrate with 1 M *hydrochloric acid*, using 0.2 ml of *methyl orange solution R* as indicator.

1 ml of 1 M *hydrochloric acid* is equivalent to 52.99 mg of Na₂CO₃.

STORAGE

Store in an airtight container.

01/2005:0192

01/2005:0191

SODIUM CARBONATE DECAHYDRATE

Natrii carbonas decahydricus

Na₂CO₃·10H₂O *M_r* 286.1

DEFINITION

Sodium carbonate decahydrate contains not less than 36.7 per cent and not more than the equivalent of 40.0 per cent of Na₂CO₃.

CHARACTERS

A white, crystalline powder or colourless, transparent crystals, efflorescent, freely soluble in water, practically insoluble in alcohol.

IDENTIFICATION

- Dissolve 1 g in *water R* and dilute to 10 ml with the same solvent. The solution is strongly alkaline (2.2.4).
- The solution prepared for identification test A gives the reaction of carbonates (2.3.1).
- The solution prepared for identification test A gives the reactions of sodium (2.3.1).

TESTS

Solution S. Dissolve 5.0 g in portions in a mixture of 5 ml of *hydrochloric acid R* and 25 ml of *distilled water R*. Heat the solution to boiling and cool. Add *dilute sodium hydroxide solution R* until the solution is neutral and dilute to 50 ml with *distilled water R*.

SODIUM CARBONATE MONOHYDRATE

Natrii carbonas monohydricus

Na₂CO₃·H₂O *M_r* 124.0

DEFINITION

Sodium carbonate monohydrate contains not less than 83.0 per cent and not more than the equivalent of 87.5 per cent of Na₂CO₃.

CHARACTERS

A white, crystalline powder or colourless crystals, freely soluble in water, practically insoluble in alcohol.

IDENTIFICATION

- Dissolve 1 g in *water R* and dilute to 10 ml with the same solvent. The solution is strongly alkaline (2.2.4).
- The solution prepared for identification test A gives the reaction of carbonates (2.3.1).
- The solution prepared for identification test A gives the reactions of sodium (2.3.1).

TESTS

Solution S. Dissolve 2.0 g in portions in a mixture of 5 ml of *hydrochloric acid R* and 25 ml of *distilled water R*. Heat the solution to boiling and cool. Add *dilute sodium hydroxide solution R* until the solution is neutral and dilute to 50 ml with *distilled water R*.

Appearance of solution. Dissolve 2.0 g in 10 ml of *water R*. The solution is clear (2.2.1) and not more intensely coloured than reference solution Y₆ (2.2.2, *Method I*).

Alkali hydroxides and bicarbonates. Dissolve 0.4 g in 20 ml of *water R*, add 20 ml of *barium chloride solution R1* and filter. To 10 ml of the filtrate add 0.1 ml of *phenolphthalein solution R*. The solution does not become red. Heat the remainder of the filtrate to boiling for 2 min. The solution remains clear (2.2.1).

Chlorides (2.4.4). Dissolve 0.4 g in *water R*, add 4 ml of *dilute nitric acid R* and dilute to 15 ml with *water R*. The solution complies with the limit test for chlorides (125 ppm).

Sulphates (2.4.13). 15 ml of solution S complies with the limit test for sulphates (250 ppm).

Arsenic (2.4.2). 5 ml of solution S complies with limit test A for arsenic (5 ppm).

Heavy metals (2.4.8). 12 ml of solution S complies with limit test A for heavy metals (50 ppm). Prepare the standard using *lead standard solution* (2 ppm Pb) *R*.

Iron (2.4.9). 5 ml of solution S diluted to 10 ml with *water R* complies with the limit test for iron (50 ppm).

ASSAY

Dissolve 1.000 g in 25 ml of *water R*. Titrate with 1 M *hydrochloric acid*, using 0.2 ml of *methyl orange solution R* as indicator.

1 ml of 1 M *hydrochloric acid* is equivalent to 52.99 mg of Na₂CO₃.

STORAGE

Store in an airtight container.

01/2005:0847

SODIUM CETOSTEARYL SULPHATE

Natrii cetylo- et stearylosulfas.

DEFINITION

Sodium cetostearyl sulphate is a mixture of sodium cetyl sulphate (C₁₆H₃₃NaO₄S; *M_r* 344.5) and sodium stearyl sulphate (C₁₈H₃₇NaO₄S; *M_r* 372.5). It contains not less than 90.0 per cent of sodium cetostearyl sulphate and not less than 40.0 per cent of sodium cetyl sulphate, both contents calculated with reference to the anhydrous substance. A suitable buffer may be added.

CHARACTERS

A white or pale yellow, amorphous or crystalline powder, soluble in hot water giving an opalescent solution, practically insoluble in cold water, partly soluble in alcohol.

IDENTIFICATION

First identification: B, D, F.

Second identification: A, C, D, E, F.

A. Examine by thin-layer chromatography (2.2.27), using a *TLC silanised silica gel plate R*.

Test solution. Dissolve 50 mg of the substance to be examined in 10 ml of *alcohol* (70 per cent V/V) *R*, heating on a water-bath.

Reference solution. Dissolve 50 mg of *sodium cetostearyl sulphate CRS* in 10 ml of *alcohol* (70 per cent V/V) *R*, heating on a water-bath.

Apply to the plate 2 µl of each solution. Develop over a path of 12 cm using a mixture of 20 volumes of *water R*, 40 volumes of *acetone R* and 40 volumes of *methanol R*.

Allow the plate to dry in air and spray with a 50 g/l solution of *phosphomolybdic acid R* in *alcohol R*. Heat at 120 °C until the appearance of the spots (about 3 h). The principal spots in the chromatogram obtained with the test solution are similar in position and colour to the principal spots in the chromatogram obtained with the reference solution.

- B. Examine the chromatograms obtained in the assay. The retention times of the two principal peaks in the chromatogram obtained with test solution (c) are similar to those of the two principal peaks in the chromatogram obtained with the reference solution.
- C. Dissolve 0.1 g in 10 ml of *water R* and shake. A foam is formed.
- D. It gives a yellow colour to a non-luminous flame.
- E. To 0.1 ml of the solution prepared for identification test C add 0.1 ml of a 1 g/l solution of *methylene blue R* and 2 ml of *dilute sulphuric acid R*. Add 2 ml of *methylene chloride R* and shake. The methylene chloride layer has an intense blue colour.
- F. Mix about 10 mg with 10 ml of *ethanol R*. Heat to boiling on a water-bath, shaking frequently. Filter immediately and evaporate to dryness. Dissolve the residue in 7 ml of *water R*, add 3 ml of *dilute hydrochloric acid R* and evaporate the solution to half its volume. Allow to cool. Filter. To the filtrate add 1 ml of *barium chloride solution R1*. A white, crystalline precipitate is formed.

TESTS

Acidity or alkalinity. Dissolve 0.5 g with heating in a mixture of 10 ml of *water R* and 15 ml of *alcohol* (90 per cent V/V) *R*. Add 0.1 ml of *phenolphthalein solution R1*. The solution is colourless. Add 0.1 ml of 0.1 M *sodium hydroxide*. The solution is red.

Sodium chloride and sodium sulphate. Not more than a total of 8.0 per cent.

Sodium chloride. Dissolve 5.00 g in 50 ml of *water R*, add *dilute nitric acid R* dropwise until the solution is neutral to *blue litmus paper R*. Add 2 ml of *potassium chromate solution R* and titrate with 0.1 M *silver nitrate*.

1 ml of 0.1 M *silver nitrate* is equivalent to 5.844 mg of NaCl.

Sodium sulphate. Dissolve 0.500 g in 20 ml of *water R*, warming gently if necessary, and add 1 ml of a 0.5 g/l solution of *dithizone R* in *acetone R*. If the solution is red, add 1 M *nitric acid*, dropwise, until a bluish-green colour is obtained. Add 2.0 ml of *dichloroacetic acid solution R* and 80 ml of *acetone R*. Titrate with 0.01 M *lead nitrate* until a persistent orange-red colour is obtained.

1 ml of 0.01 M *lead nitrate* is equivalent to 1.420 mg of Na₂SO₄.

Free cetostearyl alcohol. Not more than 4.0 per cent. Examine the chromatogram obtained with test solution (a) in the assay.

Calculate the percentage content of free cetostearyl alcohol in the substance to be examined, using the expression:

$$S \frac{100 \times m_H}{S_{Ha(corr)} \times m}$$

- S* = sum of the areas of the peaks corresponding to cetyl alcohol and stearyl alcohol in the chromatogram obtained with test solution (a),
- m_H* = mass of the internal standard added in the preparation of test solution (a), in milligrams,

$S_{Ha(corr)}$ = corrected area of the peak corresponding to the internal standard in the chromatogram obtained with test solution (a),

m = mass of sodium cetostearyl sulphate used for the preparation of test solution (a), in milligrams.

Water (2.5.12). Not more than 1.5 per cent, determined on 5.00 g by the semi-micro determination of water.

ASSAY

Examine by gas chromatography (2.2.28).

Internal standard solution. Dissolve 0.20 g of *heptadecanol CRS* in *ethanol R* and dilute to 50 ml with the same solvent.

Test solution (a). Dissolve 0.300 g of the substance to be examined in 50 ml of *ethanol R* and add 2 ml of the internal standard solution and 48 ml of *water R*. Shake with four quantities, each of 25 ml, of *pentane R*, adding *sodium chloride R*, if necessary, to facilitate the separation of the layers. Combine the organic layers. Reserve the hydro-alcoholic layer for the preparation of test solutions (c) and (d). Wash the organic layer with two quantities, each of 30 ml, of *water R*. Dry over *anhydrous sodium sulphate R* and filter.

Test solution (b). Dissolve 0.300 g of the substance to be examined in 50 ml of *ethanol R* and add 50 ml of *water R*. Shake with four quantities, each of 25 ml, of *pentane R*, adding *sodium chloride R*, if necessary, to facilitate the separation of the layers. Combine the organic layers, wash with two quantities, each of 30 ml, of *water R*. Dry over *anhydrous sodium sulphate R* and filter.

Test solution (c). Transfer 25 ml of the hydro-alcoholic solution obtained in the preparation of test solution (a) to a 200 ml flask that can be fitted with a reflux condenser. Add 20 ml of *hydrochloric acid R* and 10 ml of the internal standard solution. Boil under a reflux condenser for 2 h. Allow to cool and shake with four quantities, each of 20 ml, of *pentane R*. Combine the organic layers and wash with two quantities, each of 20 ml, of *water R*. Dry over *anhydrous sodium sulphate R* and filter.

Test solution (d). Transfer 25 ml of the hydro-alcoholic solution obtained in the preparation of test solution (a) to a 200 ml flask that can be fitted with a reflux condenser. Add 20 ml of *hydrochloric acid R* and 10 ml of *ethanol R*. Boil under a reflux condenser for 2 h. Allow to cool and shake with four quantities, each of 20 ml, of *pentane R*. Combine the organic layers and wash with two quantities, each of 20 ml, of *water R*. Dry over *anhydrous sodium sulphate R* and filter.

Reference solution. Dissolve 50 mg of *cetyl alcohol CRS* and 50 mg of *stearyl alcohol CRS* in *ethanol R* and dilute to 10 ml with the same solvent.

The chromatographic procedure may be carried out using:

- a fused-silica column 25 m long and 0.25 mm in internal diameter coated with *poly(dimethyl)siloxane R* or another suitable polar phase,
- *nitrogen for chromatography R* as the carrier gas at a flow rate of 1 ml/min,
- a flame-ionisation detector,
- a split ratio of 1:100,

with the following temperature programme:

	Time (min)	Temperature (°C)	Rate (°C/min)	Comment
Column	0 - 20	150 → 250	5	linear gradient
Injection port		250		
Detector		250		

The substances are eluted in the following order: cetyl alcohol, heptadecanol (internal standard) and stearyl alcohol.

Correction for interference. Inject 1 µl of test solution (a) and 1 µl of test solution (b). If the chromatogram obtained with test solution (b) shows a peak with the same retention time as the peak corresponding to the internal standard in the chromatogram obtained with test solution (a), calculate the ratio:

$$r = \frac{S_{ci}}{S_i}$$

S_{ci} = area of the peak corresponding to cetyl alcohol in the chromatogram obtained with test solution (b),

S_i = area of the peak with the same retention time as the peak corresponding to the internal standard in the chromatogram obtained with test solution (a).

If r is less than 300, calculate the corrected area $S_{Ha(corr)}$ of the peak corresponding to the internal standard in the chromatogram obtained with test solution (a):

$$S_{Ha(corr)} = S'_{Ha} - \frac{S_i \times S_c}{S_{ci}}$$

S'_{Ha} = area of the peak corresponding to the internal standard in the chromatogram obtained with test solution (a),

S_c = area of the peak corresponding to cetyl alcohol in the chromatogram obtained with test solution (a).

Inject 1 µl of test solution (c) and 1 µl of test solution (d). Carry out the correction for interference in the same manner as for test solution (a) and calculate the corrected area $S_{Hc(corr)}$ of the peak corresponding to the internal standard in the chromatogram obtained with test solution (c).

Inject equal volumes of the reference solution, test solution (c) and test solution (d). Identify the peaks in the chromatograms obtained with the test solutions by comparison of their retention times with those of the peaks in the chromatogram obtained with the reference solution. Determine the area of each peak.

Calculate the percentage content of sodium cetyl sulphate in the substance to be examined, using the expression:

$$\frac{(A \times 1.421) \times m'_H \times 100}{S_{Hc(corr)} \times m'}$$

A = area of the peak corresponding to cetyl alcohol in the chromatogram obtained with test solution (c),

m'_H = mass of the internal standard added in the preparation of test solution (c), in milligrams,

$S_{Hc(corr)}$ = corrected area of the peak corresponding to the internal standard in the chromatogram obtained with test solution (c),

m' = mass of the substance to be examined in test solution (c), in milligrams.

Calculate the percentage content of sodium stearyl sulphate in the substance to be examined, using the expression:

$$\frac{(B \times 1.377) \times m'_H \times 100}{S_{Hc(corr)} \times m'}$$

B = area of the peak corresponding to stearyl alcohol in the chromatogram obtained with test solution (c).

The percentage content of sodium cetostearyl sulphate corresponds to the sum of the percentage content of sodium cetyl sulphate and the percentage content of sodium stearyl sulphate.

LABELLING

The label states, where appropriate, the name and concentration of any added buffer.

01/2005:0193

SODIUM CHLORIDE

Natrii chloridum

NaCl

M_r 58.44

DEFINITION

Sodium chloride contains not less than 99.0 per cent and not more than the equivalent of 100.5 per cent of NaCl, calculated with reference to the dried substance.

CHARACTERS

A white, crystalline powder or colourless crystals or white pearls, freely soluble in water, practically insoluble in ethanol.

IDENTIFICATION

- A. It gives the reactions of chlorides (2.3.1).
- B. It gives the reactions of sodium (2.3.1).

TESTS

If the substance is in the form of pearls crush before use.

Solution S. Dissolve 20.0 g in carbon dioxide-free water *R* prepared from distilled water *R* and dilute to 100.0 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and colourless (2.2.2, Method II).

Acidity or alkalinity. To 20 ml of solution S add 0.1 ml of bromothymol blue solution *R1*. Not more than 0.5 ml of 0.01 *M* hydrochloric acid or 0.01 *M* sodium hydroxide is required to change the colour of the indicator.

Bromides. To 0.5 ml of solution S add 4.0 ml of water *R*, 2.0 ml of phenol red solution *R2* and 1.0 ml of a 0.1 g/l solution of chloramine *R* and mix immediately. After exactly 2 min, add 0.15 ml of 0.1 *M* sodium thiosulphate, mix and dilute to 10.0 ml with water *R*. The absorbance (2.2.25) of the solution measured at 590 nm, using water *R* as the compensation liquid, is not greater than that of a standard prepared at the same time and in the same manner, using 5.0 ml of a 3.0 mg/l solution of potassium bromide *R* (100 ppm).

Ferrocyanides. Dissolve 2.0 g in 6 ml of water *R*. Add 0.5 ml of a mixture of 5 ml of a 10 g/l solution of ferric ammonium sulphate *R* in a 2.5 g/l solution of sulphuric acid *R* and 95 ml of a 10 g/l solution of ferrous sulphate *R*. No blue colour develops within 10 min.

Iodides. Moisten 5 g by the dropwise addition of a freshly prepared mixture of 0.15 ml of sodium nitrite solution *R*, 2 ml of 0.5 *M* sulphuric acid, 25 ml of iodide-free starch solution *R* and 25 ml of water *R*. After 5 min, examine in daylight. The substance shows no blue colour.

Nitrites. To 10 ml of solution S add 10 ml of water *R*. Measure the absorbance (2.2.25) of the solution at 354 nm. The absorbance is not greater than 0.01.

Phosphates (2.4.11). Dilute 2 ml of solution S to 100 ml with water *R*. The solution complies with the limit test for phosphates (25 ppm).

Sulphates (2.4.13). Dilute 7.5 ml of solution S to 30 ml with distilled water *R*. 15 ml of the solution complies with the limit test for sulphates (200 ppm).

Aluminium (2.4.17). If intended for use in the manufacture of peritoneal dialysis solutions, haemodialysis solutions or haemofiltration solutions, it complies with the test for aluminium. Dissolve 20.0 g in 100 ml of water *R* and add 10 ml of acetate buffer solution pH 6.0 *R*. The solution complies with the limit test for aluminium (0.2 ppm). Use as the reference solution a mixture of 2 ml of aluminium standard solution (2 ppm Al) *R*, 10 ml of acetate buffer solution pH 6.0 *R* and 98 ml of water *R*. To prepare the blank, use a mixture of 10 ml of acetate buffer solution pH 6.0 *R* and 100 ml of water *R*.

Arsenic (2.4.2). 5 ml of solution S complies with limit test A for arsenic (1 ppm).

Barium. To 5 ml of solution S add 5 ml of distilled water *R* and 2 ml of dilute sulphuric acid *R*. After 2 h, any opalescence in the solution is not more intense than that in a mixture of 5 ml of solution S and 7 ml of distilled water *R*.

Iron (2.4.9). 10 ml of solution S complies with the limit test for iron (2 ppm). Prepare the standard using a mixture of 4 ml of iron standard solution (1 ppm Fe) *R* and 6 ml of water *R*.

Magnesium and alkaline-earth metals (2.4.7). 10.0 g complies with the limit test for magnesium and alkaline-earth metals (use 150 mg of mordant black 11 triturate *R*. The volume of 0.01 *M* sodium edetate used does not exceed 2.5 ml (100 ppm, calculated as Ca).

Potassium. If intended for use in the manufacture of parenteral dosage forms or haemodialysis, haemofiltration or peritoneal dialysis solutions, it contains not more than 500 ppm of K, determined by atomic emission spectrometry (2.2.22, Method I).

Test solution. Dissolve 1.00 g of the substance to be examined in water *R* and dilute to 100.0 ml with the same solvent.

Reference solutions. Dissolve 1.144 g of potassium chloride *R*, previously dried at 100-105 °C for 3 h, in water *R* and dilute to 1000.0 ml with the same solvent (600 µg of K per millilitre). Dilute as required.

Measure the emission intensity at 766.5 nm.

Heavy metals (2.4.8). 12 ml of solution S complies with limit test A for heavy metals (5 ppm). Prepare the standard using lead standard solution (1 ppm Pb) *R*.

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 100-105 °C for 2 h.

Bacterial endotoxins (2.6.14): less than 5 IU/g, if intended for use in the manufacture of parenteral dosage forms without a further appropriate procedure for removal of bacterial endotoxins.

ASSAY

Dissolve 50.0 mg in *water R* and dilute to 50 ml with the same solvent. Titrate with 0.1 M *silver nitrate* determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *silver nitrate* is equivalent to 5.844 mg of NaCl.

LABELLING

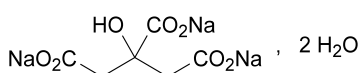
The label states:

- where applicable, that the substance is suitable for use in the manufacture of parenteral dosage forms,
- where applicable, that the substance is free from bacterial endotoxins,
- where applicable, that the substance is suitable for use in the manufacture of peritoneal dialysis solutions, haemodialysis solutions or haemofiltration solutions.

01/2005:0412

SODIUM CITRATE

Natrii citras


 $C_6H_5Na_3O_7 \cdot 2H_2O$
 M_r 294.1

DEFINITION

Sodium citrate contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of trisodium 2-hydroxypropane-1,2,3-tricarboxylate, calculated with reference to the anhydrous substance.

CHARACTERS

A white, crystalline powder or white, granular crystals, slightly deliquescent in moist air, freely soluble in water, practically insoluble in alcohol.

IDENTIFICATION

- A. To 1 ml of solution S (see Tests) add 4 ml of *water R*. The solution gives the reaction of citrates (2.3.1).
- B. 1 ml of solution S gives reaction (a) of sodium (2.3.1).

TESTS

Solution S. Dissolve 10.0 g in *carbon dioxide-free water R* prepared from *distilled water R* and dilute to 100 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and colourless (2.2.2, *Method II*).

Acidity or alkalinity. To 10 ml of solution S add 0.1 ml of *phenolphthalein solution R*. Not more than 0.2 ml of 0.1 M *hydrochloric acid* or 0.1 M *sodium hydroxide* is required to change the colour of the indicator.

Readily carbonisable substances. To 0.20 g of the powdered substance to be examined add 10 ml of *sulphuric acid R* and heat in a water-bath at $90 \pm 1^\circ C$ for 60 min. Cool rapidly. The solution is not more intensely coloured than reference solution Y₂ or GY₂ (2.2.2, *Method II*).

Chlorides (2.4.4). Dilute 10 ml of solution S to 15 ml with *water R*. The solution complies with the limit test for chlorides (50 ppm).

Oxalates. Dissolve 0.50 g in 4 ml of *water R*, add 3 ml of *hydrochloric acid R* and 1 g of granulated *zinc R* and heat on a water-bath for 1 min. Allow to stand for 2 min, decant the liquid into a test-tube containing 0.25 ml of a 10 g/l solution of *phenylhydrazine hydrochloride R* and heat to

boiling. Cool rapidly, transfer to a graduated cylinder and add an equal volume of *hydrochloric acid R* and 0.25 ml of *potassium ferricyanide solution R*. Shake and allow to stand for 30 min. Any pink colour in the solution is not more intense than that in a standard prepared at the same time in the same manner using 4 ml of a 50 mg/l solution of *oxalic acid R* (300 ppm).

Sulphates (2.4.13). To 10 ml of solution S add 2 ml of *hydrochloric acid R1* and dilute to 15 ml with *distilled water R*. The solution complies with the limit test for sulphates (150 ppm).

Heavy metals (2.4.8). 12 ml of solution S complies with limit test A for heavy metals (10 ppm). Prepare the standard using *lead standard solution* (1 ppm Pb) R.

Water (2.5.12): 11.0 per cent to 13.0 per cent, determined on 0.300 g by the semi-micro determination of water. After adding the substance to be examined, stir for 15 min before titrating.

Pyrogens (2.6.8). If intended for use in large-volume preparations for parenteral use, the competent authority may require that it comply with the test for pyrogens. Inject per kilogram of the rabbit's mass 10 ml of a freshly prepared solution in *water for injections R* containing per millilitre 10.0 mg of the substance to be examined and 7.5 mg of pyrogen-free *calcium chloride R*.

ASSAY

Dissolve 0.150 g in 20 ml of *anhydrous acetic acid R*, heating to about $50^\circ C$. Allow to cool. Using 0.25 ml of *naphtholbenzein solution R* as indicator, titrate with 0.1 M *perchloric acid* until a green colour is obtained.

1 ml of 0.1 M *perchloric acid* is equivalent to 8.602 mg of $C_6H_5Na_3O_7$.

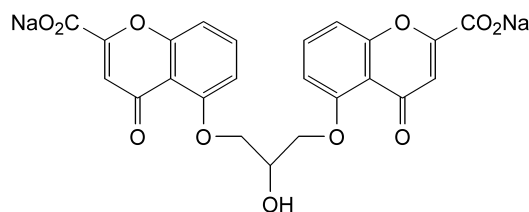
STORAGE

Store in an airtight container.

01/2005:0562

SODIUM CROMOGLICATE

Natrii cromoglicas


 $C_{23}H_{14}Na_2O_{11}$
 M_r 512.3

DEFINITION

Sodium cromoglicate contains not less than 98.0 per cent and not more than the equivalent of 101.0 per cent of disodium 5,5'-[(2-hydroxypropane-1,3-diyl)bis(oxy)]bis(4-oxo-4H-1-benzopyran-2-carboxylate, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, crystalline powder, hygroscopic, soluble in water, practically insoluble in alcohol.

IDENTIFICATION

First identification: B, D.

Second identification: A, C, D.

- A. Dissolve 10.0 mg in *phosphate buffer solution pH 7.4 R* and dilute to 100.0 ml with the same solvent. Dilute 10.0 ml of this solution to 100.0 ml with the same solvent. Examined between 230 nm and 350 nm (2.2.25), the solution shows two absorption maxima, at 239 nm and 327 nm. The ratio of the absorbance at the maximum at 327 nm to that at the maximum at 239 nm is 0.25 to 0.30.
- B. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *sodium cromoglicate CRS*. Examine the substances prepared as discs.
- C. Dissolve about 5 mg in 0.5 ml of *methanol R*. Add 3 ml of a solution in *methanol R* containing 5 g/l of *aminopyrazolone R* and 1 per cent V/V of *hydrochloric acid R*. Allow to stand for 5 min. The solution shows an intense yellow colour.
- D. It gives reaction (a) of sodium (2.3.1).

TESTS

Solution S. Dissolve 0.5 g in *carbon dioxide-free water R* and dilute to 25 ml with the same solvent.

Appearance of solution. Solution S is not more opalescent than reference suspension II (2.2.1) and not more intensely coloured than reference solution BY₅ (2.2.2, *Method II*).

Acidity or alkalinity. To 10 ml of solution S add 0.1 ml of *phenolphthalein solution R*. The solution is colourless. Add 0.2 ml of 0.01 M *sodium hydroxide*. The solution is pink. Add 0.4 ml of 0.01 M *hydrochloric acid*. The solution is colourless. Add 0.25 ml of *methyl red solution R*. The solution is red.

Related substances. Examine by thin-layer chromatography (2.2.27), using *silica gel GF₂₅₄ R* as the coating substance.

Test solution. Dissolve 0.2 g of the substance to be examined in a mixture of 1 volume of *acetone R*, 4 volumes of *tetrahydrofuran R* and 6 volumes of *water R* and dilute to 10 ml with the same mixture of solvents.

Reference solution. Dissolve 10 mg of *1,3-bis(2-acetyl-3-hydroxyphenoxy)-2-propanol CRS* in *chloroform R* and dilute to 100 ml with the same solvent.

Apply separately to the plate 5 µl of each solution. Develop over a path of 10 cm using a mixture of 5 volumes of *glacial acetic acid R*, 50 volumes of *ethyl acetate R* and 50 volumes of *toluene R*. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. Any spot in the chromatogram obtained with the test solution, apart from the principal spot (which remains at the starting point), is not more intense than the spot in the chromatogram obtained with the reference solution (0.5 per cent).

Oxalate. Dissolve 0.10 g in 20 ml of *water R*, add 5.0 ml of *iron salicylate solution R* and dilute to 50.0 ml with *water R*. Determine the absorbance (2.2.25) at 480 nm. The absorbance is not less than that of a standard prepared in the same manner using 0.35 mg of *oxalic acid R* instead of the substance to be examined.

Heavy metals (2.4.8). 1.0 g complies with limit test C for heavy metals (20 ppm). Prepare the standard using 2 ml of *lead standard solution (10 ppm Pb) R*.

Loss on drying (2.2.32). Not more than 10.0 per cent, determined on 1.000 g by drying over *diphosphorus pentoxide R* at 100 °C to 105 °C and at a pressure of 300 Pa to 600 Pa.

ASSAY

Dissolve 0.200 g with heating in a mixture of 5 ml of *2-propanol R* and 25 ml of *ethylene glycol R*. Cool and add 30 ml of *dioxan R*. Titrate with 0.1 M *perchloric acid*, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *perchloric acid* is equivalent to 25.62 mg of C₂₃H₁₄Na₂O₁₁.

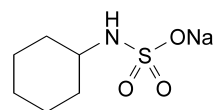
STORAGE

Store in an airtight container, protected from light.

01/2005:0774

SODIUM CYCLAMATE

Natrii cyclamas

C₆H₁₂NNaO₃SM_r 201.2

DEFINITION

Sodium cyclamate contains not less than 98.5 per cent and not more than the equivalent of 101.0 per cent of sodium *N*-cyclohexylsulphamate, calculated with reference to the dried substance.

CHARACTERS

A white, crystalline powder or colourless crystals, freely soluble in water, slightly soluble in alcohol.

IDENTIFICATION

First identification: A, E.

Second identification: B, C, D, E.

- A. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *sodium cyclamate CRS*.
- B. Examine the chromatograms obtained in the test for sulphamic acid. The principal spot in the chromatogram obtained with test solution (b) is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).
- C. To 1 ml of solution S (see Tests), add 1 ml of *water R* and 2 ml of *silver nitrate solution R1* and shake. A white, crystalline precipitate is formed.
- D. To 1 ml of solution S add 5 ml of *water R*, 2 ml of *dilute hydrochloric acid R* and 4 ml of *barium chloride solution R1* and mix. The solution is clear. Add 2 ml of *sodium nitrite solution R*. A voluminous white precipitate is formed and gas is given off.
- E. A mixture of 1 ml of solution S and 1 ml of *water R* gives reaction (a) of sodium (2.3.1).

TESTS

Solution S. Dissolve 5 g in *carbon dioxide-free water R* prepared from *distilled water R* and dilute to 50 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and colourless (2.2.2, *Method II*).

pH (2.2.3). The pH of solution S is 5.5 to 7.5.

Absorbance (2.2.25). The absorbance of solution S, measured at 270 nm, is not greater than 0.10.

Sulphamic acid. Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel G plate R*.

Test solution (a). Use solution S.

Test solution (b). Dilute 1 ml of test solution (a) to 10 ml with *water R*.

Reference solution (a). Dissolve 0.10 g of *sodium cyclamate CRS* in *water R* and dilute to 10 ml with the same solvent.

Reference solution (b). Dissolve 10 mg of *sulphamic acid R* in *water R* and dilute to 100 ml with the same solvent.

Apply to the plate 2 µl of each solution. Develop over a path of 12 cm using a mixture of 10 volumes of *concentrated ammonia R*, 10 volumes of *water R*, 20 volumes of *ethyl acetate R* and 70 volumes of *propanol R*. Dry the plate in a current of warm air, heat at 105 °C for 5 min and spray the hot plate with *strong sodium hypochlorite solution R* diluted to a concentration of 5 g/l of active chlorine. Place the plate in a current of cold air until an area of coating below the points of application gives at most a faint blue colour with a drop of *potassium iodide and starch solution R*; avoid prolonged exposure to cold air. Spray with *potassium iodide and starch solution R* and examine the chromatograms within 5 min. Any spot corresponding to sulphamic acid in the chromatogram obtained with test solution (a) is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.1 per cent).

Aniline, cyclohexylamine and dicyclohexylamine. Not more than 1 ppm of aniline, not more than 10 ppm of cyclohexylamine and not more than 1 ppm of dicyclohexylamine determined by gas chromatography (2.2.28) using *tetradecane R* as the internal standard.

Internal standard solution. Dissolve 2 µl of *tetradecane R* in *methylene chloride R* and dilute to 100 ml with the same solvent.

Test solution. Dissolve 2.00 g of the substance to be examined in 20 ml of *water R* and add 0.5 ml of *strong sodium hydroxide solution R* and shake with 30 ml of *toluene R*. Shake 20 ml of the upper layer with 4 ml of a mixture of equal volumes of *dilute acetic acid R* and *water R*. Separate the lower layer and add 0.5 ml of *strong sodium hydroxide solution R* and 0.5 ml of the internal standard solution. Shake and use the lower layer for chromatography immediately after separation.

Reference solution. Dissolve 10.0 mg (about 12 µl) of *cyclohexylamine R*, 1.0 mg (about 1.1 µl) of *dicyclohexylamine R* and 1.0 mg (about 1 µl) of *aniline R* in *water R* and dilute to 1000 ml with the same solvent. Dilute 10.0 ml of this solution to 100.0 ml with *water R* (solution A). To 20.0 ml of solution A, add 0.5 ml of *strong sodium hydroxide solution R* and extract with 30 ml of *toluene R*. Shake 20 ml of the upper layer with 4 ml of a mixture of equal volumes of *dilute acetic acid R* and *water R*. Separate the lower layer and add 0.5 ml of *strong sodium hydroxide solution R* and 0.5 ml of the internal standard solution. Shake and use the lower layer for chromatography immediately after separation.

The chromatographic procedure may be carried out using:

- a fused silica column 25 m long and 0.32 mm in internal diameter coated with *poly(dimethyl)(diphenyl)siloxane R* (0.51 µm),
 - *helium for chromatography R* as the carrier gas at a flow rate of 1.8 ml/min,
 - a flame-ionisation detector,
 - a split vent at a flow rate of 20 ml/min,
- with the following temperature programme:

	Time (min)	Temperature (°C)	Rate (°C/min)	Comment
Column	0 - 1	85		isothermal
	1 - 9	85 → 150	8	linear gradient
	9 - 13	150		isothermal
Injection port		250		
Detector		270		

Inject 1.5 µl of each solution. When the chromatograms are recorded in the conditions prescribed, the retention times relative to cyclohexylamine (about 2.3 min) are the following: aniline about 1.4, tetradecane about 4.3 and dicyclohexylamine about 4.5.

Sulphates (2.4.13). Dilute 1.5 ml of solution S to 15 ml with *distilled water R*. The solution complies with the limit test for sulphates (0.1 per cent).

Heavy metals (2.4.8). 12 ml of solution S complies with limit test A for heavy metals (10 ppm). Prepare the standard using *lead standard solution (1 ppm Pb) R*.

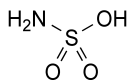
Loss on drying (2.2.32). Not more than 1.0 per cent, determined on 1.000 g in an oven at 100 °C to 105 °C for 4 h.

ASSAY

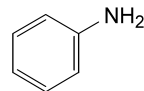
Dissolve without heating 0.150 g in 60 ml of *anhydrous acetic acid R*. Titrate with 0.1 M *perchloric acid*, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *perchloric acid* is equivalent to 20.12 mg of C₆H₁₂NNaO₃S.

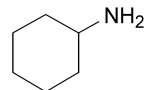
IMPURITIES



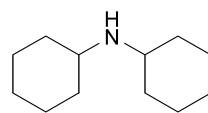
A. sulphamic acid,



B. aniline (phenylamine),



C. cyclohexylamine,



D. *N*-cyclohexylcyclohexylamine.

01/2005:0194

SODIUM DIHYDROGEN PHOSPHATE DIHYDRATE

Natrii dihydrogenophosphas dihydricus



M_r 156.0

DEFINITION

Sodium dihydrogen phosphate dihydrate contains not less than 98.0 per cent and not more than the equivalent of 100.5 per cent of NaH₂PO₄, calculated with reference to the dried substance.

CHARACTERS

A white powder or colourless crystals, very soluble in water, very slightly soluble in alcohol.

IDENTIFICATION

- A. Solution S (see Tests) is faintly acid (2.2.4).
 B. Solution S gives the reactions of phosphates (2.3.1).
 C. Solution S previously neutralised using a 100 g/l solution of *potassium hydroxide R* gives reaction (a) of sodium (2.3.1).

TESTS

Solution S. Dissolve 10.0 g in *carbon dioxide-free water R* prepared from *distilled water R* and dilute to 100 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and colourless (2.2.2, *Method II*).

pH (2.2.3). To 5 ml of solution S add 5 ml of *carbon dioxide-free water R*. The pH of the solution is 4.2 to 4.5.

Reducing substances. To 5 ml of solution S add 0.25 ml of 0.02 M *potassium permanganate* and 5 ml of *dilute sulphuric acid R* and heat in a water-bath for 5 min. The solution retains a slight red colour.

Chlorides (2.4.4). 2.5 ml of solution S diluted to 15 ml with *water R* complies with the limit test for chlorides (200 ppm).

Sulphates (2.4.13). To 5 ml of solution S add 0.5 ml of *hydrochloric acid R* and dilute to 15 ml with *distilled water R*. The solution complies with the limit test for sulphates (300 ppm).

Arsenic (2.4.2). 0.5 g complies with limit test A for arsenic (2 ppm).

Heavy metals (2.4.8). 12 ml of solution S complies with limit test A for heavy metals (10 ppm). Prepare the standard using *lead standard solution (1 ppm Pb) R*.

Iron (2.4.9). 10 ml of solution S complies with the limit test for iron (10 ppm).

Loss on drying (2.2.32). 21.5 per cent to 24.0 per cent, determined on 0.50 g by drying in an oven at 130 °C.

ASSAY

Dissolve 2.500 g in 40 ml of *water R*. Titrate with carbonate-free 1 M *sodium hydroxide*, determining the end-point potentiometrically (2.2.20).

1 ml of 1 M *sodium hydroxide* is equivalent to 0.120 g of NaH_2PO_4 .

DEFINITION

Sodium fluoride contains not less than 98.5 per cent and not more than the equivalent of 100.5 per cent of NaF, calculated with reference to the dried substance.

CHARACTERS

A white powder or colourless crystals, soluble in water, practically insoluble in alcohol.

IDENTIFICATION

- A. To 2 ml of solution S (see Tests) add 0.5 ml of *calcium chloride solution R*. A gelatinous white precipitate is formed which dissolves on adding 5 ml of *ferric chloride solution R1*.
 B. To about 4 mg add a mixture of 0.1 ml of *alizarin S solution R* and 0.1 ml of *zirconyl nitrate solution R* and mix. The colour changes from red to yellow.
 C. Solution S gives reaction (a) of sodium (2.3.1).

TESTS

Solution S. Dissolve 2.5 g in *carbon dioxide-free water R* without heating and dilute to 100 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and colourless (2.2.2, *Method II*).

Acidity or alkalinity. Dissolve 2.5 g of *potassium nitrate R* in 40 ml of solution S and dilute to 50 ml with *carbon dioxide-free water R*. Cool to 0 °C and add 0.2 ml of *phenolphthalein solution R*. If the solution is colourless, not more than 1.0 ml of 0.1 M *sodium hydroxide* is required to produce a red colour that persists for at least 15 s. If the solution is red, not more than 0.25 ml of 0.1 M *hydrochloric acid* is required to change the colour of the indicator.

Chlorides (2.4.4). Dilute 10 ml of solution S to 15 ml with *water R*. The solution complies with the limit test for chlorides (200 ppm).

Fluorosilicates. Heat to boiling the neutralised solution obtained in the test for acidity or alkalinity and titrate whilst hot. Not more than 0.75 ml of 0.1 M *sodium hydroxide* is required to change the colour of the indicator to red.

Sulphates (2.4.13). Dissolve 0.25 g in 10 ml of a saturated solution of *boric acid R* in *distilled water R*. Add 5 ml of *distilled water R* and 0.6 ml of *hydrochloric acid R1*. The solution complies with the limit test for sulphates (200 ppm). Prepare the standard by mixing 0.6 ml of *hydrochloric acid R1*, 5 ml of *sulphate standard solution (10 ppm SO₄) R* and 10 ml of a saturated solution of *boric acid R* in *distilled water R*.

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 130 °C for 3 h.

01/2005:0514

ASSAY

To 80.0 mg add a mixture of 5 ml of *acetic anhydride R* and 20 ml of *anhydrous acetic acid R* and heat to dissolve. Allow to cool and add 20 ml of *dioxan R*. Using 0.1 ml of *crystal violet solution R* as indicator, titrate with 0.1 M *perchloric acid* until a green colour is obtained. Carry out a blank titration.

1 ml of 0.1 M *perchloric acid* is equivalent to 4.199 mg of NaF.

SODIUM FLUORIDE

Natrii fluoridum

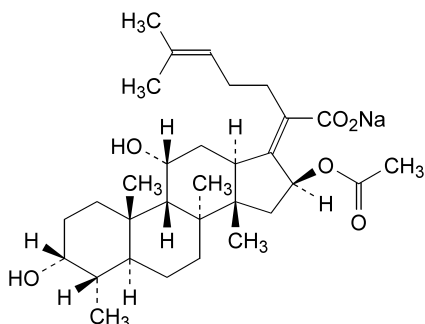
NaF

 M_r 41.99

01/2005:0848

SODIUM FUSIDATE

Natrii fusidas

 $C_{31}H_{47}NaO_6$ M_r 538.7

DEFINITION

Sodium fusidate is sodium sodium (*Z*)-*ent*-16 α -(acetyloxy)-3 β ,11 β -dihydroxy-4 β ,8,14-trimethyl-18-nor-5 β ,10 α -cholesta-17(20),24-dien-21-oate, an antimicrobial substance produced by the growth of certain strains of *Fusidium coccineum* or by any other means. It contains not less than 97.5 per cent and not more than the equivalent of 101.0 per cent of $C_{31}H_{47}NaO_6$, calculated with reference to the anhydrous substance.

CHARACTERS

A white or almost white, crystalline powder, slightly hygroscopic, freely soluble in water and in alcohol.

IDENTIFICATION

- A. Examine by infrared absorption spectrophotometry (2.2.24). Dissolve 0.1 g in 5 ml of *water R*, add 5 ml of *chloroform R* and 0.1 ml of *dilute phosphoric acid R*. Shake vigorously for 1 min, allow to separate and filter the lower layer through absorbent cotton covered with *anhydrous sodium sulphate R*. Repeat the extraction with two quantities, each of 5 ml, of *chloroform R* and evaporate the combined extracts to dryness under reduced pressure. Dry the residue over *diphosphorus pentoxide R* under reduced pressure for 2 h, dissolve in 1 ml of *chloroform R*. Compare the spectrum obtained with the *Ph. Eur. reference spectrum of fusidic acid*.
- B. Examine by thin-layer chromatography (2.2.27), using *silica gel HF₂₅₄ R* as the coating substance.

Test solution. Dissolve 20 mg of the substance to be examined in *methanol R* and dilute to 10 ml with the same solvent.

Reference solution. Dissolve 24 mg of *diethanolamine fusidate CRS* in *methanol R* and dilute to 10 ml with the same solvent.

Apply to the plate 10 μ l of each solution. Develop over a path of 15 cm using a mixture of 2.5 volumes of *methanol R*, 10 volumes of *glacial acetic acid R*, 10 volumes of *cyclohexane R* and 80 volumes of *chloroform R*. Dry the plate in a current of hot air. Examine in ultraviolet light at 254 nm. The principal spot

in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with the reference solution.

- C. Ignite 1 g. The residue gives reaction (a) of sodium (2.3.1).

TESTS

Appearance of solution. Dissolve 1.5 g in 10 ml of *water R*. The solution is not more intensely coloured than reference solution B₆ (2.2.2, *Method II*).

pH (2.2.3). Dissolve 0.125 g in 10 ml of *carbon dioxide-free water R*. The pH of the solution is 7.5 to 9.0.

Related substances. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 50 mg of the substance to be examined in the mobile phase and dilute to 10.0 ml with the mobile phase.

Reference solution (a). Dissolve 5 mg of *3-ketofusidic acid CRS* in 5 ml of the mobile phase. To 1.0 ml of this solution add 0.20 ml of the test solution and dilute to 20.0 ml with the mobile phase.

Reference solution (b). Dilute 20 μ l of the test solution to 100.0 ml with the mobile phase.

The chromatographic procedure may be carried out using:

- a steel column 0.125 m to 0.15 m long and 4 mm to 5 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (5 μ m),
- as mobile phase at a flow rate of 2 ml/min a mixture of 10 volumes of *methanol R*, 20 volumes of a 10 g/l solution of *phosphoric acid R*, 20 volumes of *water R* and 50 volumes of *acetonitrile R*,
- as detector a spectrophotometer set at 235 nm,
- a 20 μ l loop injector.

Continue the chromatography for at least 3.5 times the retention time of the principal peak. In the chromatogram obtained with the test solution, the sum of the areas of the peaks, apart from the principal peak and the solvent peak, is not greater than twice the area of the peak corresponding to sodium fusidate in the chromatogram obtained with reference solution (a) (2.0 per cent). Disregard any peak with an area less than that of the principal peak in the chromatogram obtained with reference solution (b). The test is not valid unless: the resolution between the peaks corresponding to 3-ketofusidic acid and sodium fusidate in the chromatogram obtained with reference solution (a) is at least 2.5; the principal peak in the chromatogram obtained with reference solution (b) has a signal-to-noise ratio of at least 3.

Water (2.5.12). Not more than 2.0 per cent, determined on 0.50 g by the semi-micro determination of water.

ASSAY

Dissolve 0.500 g in 30 ml of *water R* and add 40 ml of *alcohol R*. Titrate with 0.1 M *hydrochloric acid*, determining the end-point potentiometrically (2.2.20).

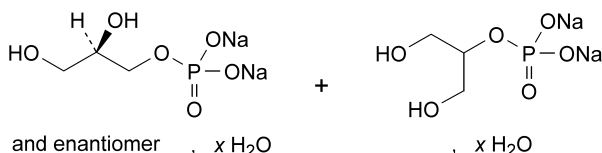
1 ml of 0.1 M *hydrochloric acid* is equivalent to 53.87 mg of $C_{31}H_{47}NaO_6$.

STORAGE

Store in an airtight container, protected from light, at a temperature of 2 °C to 8 °C.

01/2005:1995 **Heavy metals** (2.4.8): maximum 20 ppm.**SODIUM GLYCEROPHOSPHATE, HYDRATED**

Natrii glycerophosphas hydricus

C₃H₇Na₂O₆P, xH₂O M_r 216.0 (anhydrous substance)**DEFINITION**

Mixture of variable proportions of sodium (2*RS*)-2,3-dihydroxypropyl phosphate and sodium 2-hydroxy-1-(hydroxymethyl)ethyl phosphate. The degree of hydration is 4 to 6.

Content: 98.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

Appearance: white, crystalline powder or crystals.

Solubility: freely soluble in water, practically insoluble in acetone and in alcohol.

IDENTIFICATION

- A. Solution S (see Tests) gives reaction (a) of sodium (2.3.1).
- B. To 0.1 g add 5 ml of *dilute nitric acid R*. Heat to boiling and boil for 1 min. Cool. The solution gives reaction (b) of phosphates (2.3.1).
- C. In a test-tube fitted with a glass tube, mix 0.1 g with 5 g of *potassium hydrogen sulphate R*. Heat strongly and direct the white vapour into 5 ml of *decolorised fuchsin solution R*. A violet-red colour develops which becomes violet upon heating for 30 min on a water-bath.

TESTS

Solution S. Dissolve 10.0 g in *carbon dioxide-free water R* prepared from *distilled water R* and dilute to 100 ml with the same solvent.

Appearance of solution. Solution S is not more opalescent than reference suspension II (2.2.1) and not more intensely coloured than reference solution Y₆ (2.2.2, *Method II*).

Alkalinity. To 10 ml of solution S add 0.2 ml of *phenolphthalein solution R*. Not more than 1.0 ml of 0.1 *M hydrochloric acid* is required to change the colour of the indicator, (*n*₂).

Glycerol and alcohol-soluble substances: maximum 1.0 per cent.

Shake 1.000 g with 25 ml of *alcohol R* for 10 min. Filter. Evaporate the filtrate on a water-bath and dry the residue at 70 °C for 1 h. The residue weighs not more than 10 mg.

Chlorides (2.4.4): maximum 200 ppm.

Dilute 2.5 ml of solution S to 15 ml with *water R*.

Phosphates (2.4.11): maximum 0.1 per cent.

Dilute 1 ml of solution S to 10 ml with *water R*. Dilute 1 ml of this solution to 100 ml with *water R*.

Sulphates (2.4.13): maximum 500 ppm.

Dilute 3 ml of solution S to 15 ml with *water R*.

Iron (2.4.9): maximum 20 ppm.

Dilute 5 ml of solution S to 10 ml with *water R*.

Dilute 10 ml of solution S to 20 ml with *water R*. 12 ml of the solution complies with limit test A. Prepare the standard using 10 ml of *lead standard solution (1 ppm Pb) R*.

Water (2.5.12): 25.0 per cent to 35.0 per cent, determined on 0.100 g.

ASSAY

Dissolve 0.250 g in 30 ml of *water R*. Titrate with 0.05 *M sulphuric acid*, determining the end-point potentiometrically (2.2.20), (*n*₁).

Calculate the percentage content of sodium glycerophosphate (anhydrous substance) from the expression:

$$\frac{216.0 \left(n_1 - \frac{n_2}{4} \right)}{m (100 - a)}$$

a = percentage content of water,

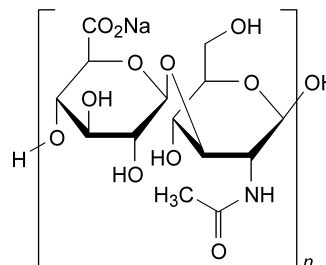
*n*₁ = volume of 0.05 *M sulphuric acid* used in the assay, in millilitres,

*n*₂ = volume of 0.1 *M hydrochloric acid* used in the test for alkalinity, in millilitres,

m = mass of the substance to be examined, in grams.

01/2005:1472
corrected**SODIUM HYALURONATE**

Natrii hyaluronas

(C₁₄H₂₀NNaO₁₁)_n**DEFINITION**

Sodium hyaluronate is the sodium salt of hyaluronic acid, a glycosaminoglycan consisting of D-glucuronic acid and N-acetyl-D-glucosamine disaccharide units. It contains not less than 95.0 per cent and not more than the equivalent of 105.0 per cent of sodium hyaluronate, calculated with reference to the dried substance. It has an intrinsic viscosity of not less than 90 per cent and not more than 120 per cent of the value stated on the label.

PRODUCTION

Sodium hyaluronate is extracted from cocks' combs or obtained by fermentation from *Streptococci*, Lancefield Groups A and C. It is produced by methods of manufacture designed to minimise or eliminate infectious agents. When produced by fermentation of gram-positive bacteria, the process must be shown to reduce or eliminate pyrogenic or inflammatory components of the cell wall.

CHARACTERS

A white or almost white, very hygroscopic powder or a fibrous aggregate, sparingly soluble to soluble in water, practically insoluble in acetone and in ethanol.

IDENTIFICATION

- A. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the *Ph. Eur. reference spectrum of sodium hyaluronate*.
- B. It gives reaction (a) of sodium (2.3.1).

TESTS

Solution S. Weigh a quantity of the substance to be examined equivalent to 0.10 g of the dried substance and add 30.0 ml of a 9 g/l solution of *sodium chloride R*. Mix gently on a shaker until dissolved (about 12 h).

Appearance of solution. Solution S is clear (2.2.1). The absorbance of solution S measured at 600 nm (2.2.25) is not greater than 0.01.

pH (2.2.3). Dissolve the substance to be examined in *carbon dioxide-free water R* to obtain a solution containing a quantity equivalent to 5 mg of the dried substance per millilitre. The pH of the solution is 5.0 to 8.5.

Intrinsic viscosity. *Sodium hyaluronate is very hygroscopic and must be protected from moisture during weighing.*

Buffer solution (0.15 M sodium chloride in 0.01 M phosphate buffer solution pH 7.0). Dissolve 0.78 g of *sodium dihydrogen phosphate R* and 4.50 g of *sodium chloride R* in *water R* and dilute to 500.0 ml with the same solvent (solution A). Dissolve 1.79 g of *disodium hydrogen phosphate R* and 4.50 g of *sodium chloride R* in *water R* and dilute to 500.0 ml with the same solvent (solution B). Mix solutions A and B until a pH of 7.0 is reached. Filter through a sintered-glass filter (4).

Test solution (a) (concentration c_1 of sodium hyaluronate). Weigh 0.200 g (m_{op}) (*NOTE: this value is only indicative and should be adjusted after an initial measurement of the viscosity of test solution (a)*) of the substance to be examined and dilute with 50.0 g (m_{os}) of buffer solution at 4 °C. Mix the solution by shaking at 4 °C during 24 h. Weigh 5.00 g (m_{1p}) of this solution and dilute with 100.0 g (m_{1s}) of buffer solution at 25 °C. Mix this solution by shaking for 20 min. Filter the solution through a sintered-glass filter (100), and discard the first 10 ml.

Test solution (b) (concentration c_2 of sodium hyaluronate). Weigh 30.0 g (m_{2p}) of test solution (a) and dilute with 10.0 g (m_{2s}) of buffer solution at 25 °C. Mix this solution by shaking for 20 min. Filter the solution through a sintered-glass filter (100) and discard the first 10 ml.

Test solution (c) (concentration c_3 of sodium hyaluronate). Weigh 20.0 g (m_{3p}) of test solution (a) and dilute with 20.0 g (m_{3s}) of buffer solution at 25 °C. Mix this solution by shaking for 20 min. Filter the solution through a sintered-glass filter (100) and discard the first 10 ml.

Test solution (d) (concentration c_4 of sodium hyaluronate). Weigh 10.0 g (m_{4p}) of test solution (a) and dilute with 30.0 g (m_{4s}) of buffer solution at 25 °C. Mix this solution by shaking for 20 min. Filter the solution through a sintered-glass filter (100) and discard the first 10 ml.

Determine the flow-time for the buffer solution (t_0) and the flow times for the four test solutions (t_1 , t_2 , t_3 and t_4), at 25.00 ± 0.03 °C (2.2.9). Use an appropriate suspended level viscometer (specifications: viscometer constant about $0.005 \text{ mm}^2/\text{s}^2$, kinematic viscosity range 1 to $5 \text{ mm}^2/\text{s}^2$, internal diameter of tube *R* 0.53 mm, volume of bulb *C* 5.6 ml, internal diameter of tube *N* 2.8 mm to 3.2 mm) with a funnel-shaped lower capillary end. Use the same viscometer for all measurements; measure all outflow times in triplicate. The test is not valid unless the results do not differ by more

than 0.35 per cent from the mean and if the flow time t_1 is not less than 1.6 and not more than 1.8 times t_0 . If this is not the case, adjust the value of m_{op} and repeat the procedure.

Calculation of the relative viscosities

Since the densities of the sodium hyaluronate solutions and of the solvent are almost equal, the relative viscosities η_{ri} (being η_{r1} , η_{r2} , η_{r3} and η_{r4}) can be calculated from the ratio of the flow times for the respective solutions t_i (being t_1 , t_2 , t_3 and t_4) to the flow time of the solvent t_0 , but taking into account the kinetic energy correction factor for the capillary ($B = 30\,800 \text{ s}^3$), as shown below:

$$\eta_{ri} = \frac{t_i - \frac{B}{t_i^2}}{t_0 - \frac{B}{t_0^2}}$$

Calculation of the concentrations

Calculation of the concentration c_1 (expressed in kg/m^3) of sodium hyaluronate in test solution (a)

$$c_1 = m_{op} \times \frac{x}{100} \times \frac{100 - h}{100} \times \frac{1}{m_{op} + m_{os}} \times \frac{m_{1p}}{m_{1p} + m_{1s}} \times \rho_{25}$$

x = percentage content of sodium hyaluronate as determined under Assay,

h = loss on drying as a percentage,

ρ_{25} = $1005 \text{ kg}/\text{m}^3$ (density of the test solution at 25 °C).

Calculation of the other concentrations

$$c_2 = c_1 \times \frac{m_{2p}}{m_{2s} + m_{2p}}$$

$$c_3 = c_1 \times \frac{m_{3p}}{m_{3s} + m_{3p}}$$

$$c_4 = c_1 \times \frac{m_{4p}}{m_{4s} + m_{4p}}$$

Calculation of the intrinsic viscosity

The intrinsic viscosity $[\eta]$ is calculated by linear least-squares regression analysis using the Martin equation:

$$\log \left(\frac{\eta_r - 1}{c} \right) = \log [\eta] + k [\eta] c$$

The decimal antilogarithm of the intercept is the intrinsic viscosity expressed in m^3/kg .

Sulphated glycosaminoglycans. *If the product is extracted from cocks' combs, it complies with the following requirement. Appropriate safety precautions are to be taken when handling perchloric acid at elevated temperature.*

Test solution. Introduce a quantity of the substance to be examined equivalent to 50.0 mg of the dried substance into a test-tube 150 mm long and 16 mm in internal diameter and dissolve in 1.0 ml of *perchloric acid R*.

Reference solution. Dissolve 0.149 g of *anhydrous sodium sulphate R* in *water R* and dilute to 100.0 ml with the same solvent. Dilute 10.0 ml to 100.0 ml with *water R*. Evaporate 1.0 ml in a test-tube 150 mm long and 16 mm in internal diameter in a heating block at 90 °C to 95 °C, and dissolve the residue in 1.0 ml of *perchloric acid R*.

Plug each test-tube with a piece of glass wool. Place the test-tubes in a heating block or a silicone oil bath maintained at 180 °C and heat until clear, colourless solutions are obtained (about 12 h). Remove the test-tubes and cool to room temperature. Add to each test-tube 3.0 ml of a 33.3 g/l solution of *barium chloride R*, cap and shake vigorously.

Allow the test-tubes to stand for 30 min. Shake each test-tube once again, and determine the absorbance (2.2.25) at 660 nm, using *water R* as a blank.

The absorbance obtained with the test solution is not greater than the absorbance obtained with the reference solution (1 per cent).

Nucleic acids. The absorbance (2.2.25) of solution S at 260 nm is not greater than 0.5.

Protein. Not more than 0.3 per cent. If intended for use in the manufacture of parenteral dosage forms, not more than 0.1 per cent.

Test solution (a). Dissolve the substance to be examined in *water R* to obtain a solution containing a quantity equivalent to about 10 mg of the dried substance per millilitre.

Test solution (b). Mix equal volumes of test solution (a) and *water R*.

Reference solutions. Prepare a 0.5 mg/ml stock solution of *bovine albumin R* in *water R*. Prepare five dilutions of the stock solution containing between 5 µg/ml and 50 µg/ml of *bovine albumin R*.

Add 2.5 ml of freshly prepared *cupri-tartaric solution R3* to test-tubes containing 2.5 ml of *water R* (blank), 2.5 ml of the test solutions (a) or (b) or 2.5 ml of the reference solutions. Mix after each addition. After about 10 min, add to each test-tube 0.50 ml of a mixture of equal volumes of *water R* and *phosphomolybdotungstic reagent R* prepared immediately before use. Mix after each addition. After 30 min, measure the absorbance (2.2.25) of each solution at 750 nm against the blank. From the calibration curve obtained with the five reference solutions determine the content of protein in the test solutions.

Chlorides (2.4.4). Dissolve 67 mg in 100 ml of *water R*. 15 ml of the solution complies with the limit test for chlorides (0.5 per cent).

Iron. Not more than 80 ppm of Fe, determined by atomic absorption spectrometry (2.2.23, *Method II*).

Test solution. Dissolve a quantity of the substance to be examined equivalent to 0.25 g of the dried substance in 1 ml of *nitric acid R* by heating on a water-bath. Cool and dilute to 10.0 ml with *water R*.

Reference solutions. Prepare two reference solutions in the same manner as the test solution, adding 1.0 ml and 2.0 ml respectively of *iron standard solution (10 ppm Fe) R* to the dissolved substance to be examined.

Measure the absorbance at 248.3 nm, using an iron hollow-cathode lamp as source of radiation, a transmission band of 0.2 nm and an air-acetylene flame.

Loss on drying (2.2.32). Not more than 20.0 per cent, determined on 0.500 g by drying at 100–110 °C over *diphosphorus pentoxide R* for 6 h.

Microbial contamination. Total aerobic viable count (2.6.12) not more than 10² micro-organisms per gram. Use 1 g of the substance to be examined.

Bacterial endotoxins (2.6.14): if intended for use in the manufacture of parenteral dosage forms without a further appropriate procedure for the removal of bacterial endotoxins, less than 0.5 IU/mg. If intended for use in the manufacture of intra-ocular preparations or intra-articular preparations without a further appropriate procedure for the removal of bacterial endotoxins, less than 0.05 IU/mg.

ASSAY

Determine the glucuronic acid content by reaction with carbazole as described below.

Reagent A. Dissolve 0.95 g of *disodium tetraborate R* in 100.0 ml of *sulphuric acid R*.

Reagent B. Dissolve 0.125 g of *carbazole R* in 100.0 ml of *ethanol R*.

Test solution. Prepare this solution in triplicate. Dissolve 0.170 g of the substance to be examined in *water R* and dilute to 100.0 g with the same solvent. Dilute 10.0 g of this solution to 200.0 g with *water R*.

Reference stock solution. Dissolve 0.100 g of *D-glucuronic acid R*, previously dried to constant mass in vacuum over *diphosphorus pentoxide R* (2.2.32), in *water R* and dilute to 100.0 g with the same solvent.

Reference solutions. Prepare five dilutions of the reference stock solution containing between 6.5 µg/g and 65 µg/g of *D-glucuronic acid R*.

Place 25 test-tubes, numbered 1 to 25, in iced water. Add 1.0 ml of the five reference solutions in triplicate to the test-tubes 1 to 15 (reference tubes), 1.0 ml of the three test solutions in triplicate to the test-tubes 16 to 24 (sample tubes), and 1.0 ml of *water R* to test-tube 25 (blank). Add 5.0 ml of freshly prepared reagent A to each test-tube. Tightly close the test-tubes with plastic caps, shake the contents, and place on a water bath for exactly 15 min. Cool in iced water, and add to each test tube 0.20 ml of reagent B. Recap the tubes, shake, and put them again on a water-bath for exactly 15 min. Cool to room temperature and measure the absorbance (2.2.25) of the solutions at 530 nm, against the blank.

From the calibration curve obtained with the mean absorbances read for each reference solution, determine the mean concentrations of *D-glucuronic acid* in the test solutions.

Calculate the percentage content of sodium hyaluronate from the expression:

$$\frac{c_g}{c_s} \times Z \times \frac{100}{100 - h} \times \frac{401.3}{194.1}$$

- c_g = mean of concentrations of *D-glucuronic acid* in the test solutions, in milligrams per gram,
 c_s = mean of concentrations of the substance to be examined in the test solutions, in milligrams per gram,
 Z = determined percentage content of $C_6H_{10}O_7$ in *D-glucuronic acid R*,
 h = loss on drying as a percentage,
 401.3 = relative molecular mass of the disaccharide fragment,
 194.1 = relative molecular mass of glucuronic acid.

STORAGE

Store in an airtight container, protected from light and humidity. If the substance is sterile, store in a sterile, airtight, tamper-proof container.

LABELLING

The label states:

- the intrinsic viscosity,
- the origin of the substance,
- the intended use of the substance,
- where applicable, that the substance is suitable for parenteral administration other than intra-articular administration,

- where applicable, that the substance is suitable for parenteral administration, including intra-articular administration,
- where applicable that the material is suitable for intra-ocular use.

01/2005:0195

SODIUM HYDROGEN CARBONATE**Natrii hydrogenocarbonas**NaHCO₃*M_r* 84.0**DEFINITION**

Sodium hydrogen carbonate contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of NaHCO₃.

CHARACTERS

A white, crystalline powder, soluble in water, practically insoluble in alcohol. When heated in the dry state or in solution, it gradually changes into sodium carbonate.

IDENTIFICATION

- To 5 ml of solution S (see Tests) add 0.1 ml of *phenolphthalein solution R*. A pale pink colour is produced. Heat; gas is evolved and the solution becomes red.
- It gives the reaction of carbonates and bicarbonates (2.3.1).
- Solution S gives reaction (a) of sodium (2.3.1).

TESTS

Solution S. Dissolve 5.0 g in 90 ml of *carbon dioxide-free water R* and dilute to 100.0 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and colourless (2.2.2, *Method II*).

Carbonates. The pH (2.2.3) of freshly prepared solution S is not more than 8.6.

Chlorides (2.4.4). To 7 ml of solution S add 2 ml of *nitric acid R* and dilute to 15 ml with *water R*. The solution complies with the limit test for chlorides (150 ppm).

Sulphates (2.4.13). To a suspension of 1.0 g in 10 ml of *distilled water R* add *hydrochloric acid R* until neutral and about 1 ml in excess. Dilute to 15 ml with *distilled water R*. The solution complies with the limit test for sulphates (150 ppm).

Ammonium (2.4.1). 10 ml of solution S diluted to 15 ml with *water R* complies with the limit test for ammonium (20 ppm). Prepare the standard using a mixture of 5 ml of *water R* and 10 ml of *ammonium standard solution* (1 ppm NH₄) *R*.

Arsenic (2.4.2). 0.5 g complies with limit test A for arsenic (2 ppm).

Calcium (2.4.3). To a suspension of 1.0 g in 10 ml of *distilled water R* add *hydrochloric acid R* until neutral and dilute to 15 ml with *distilled water R*. The solution complies with the limit test for calcium (100 ppm).

Iron (2.4.9). Dissolve 0.5 g in 5 ml of *dilute hydrochloric acid R* and dilute to 10 ml with *water R*. The solution complies with the limit test for iron (20 ppm).

Heavy metals (2.4.8). Dissolve 2.0 g in a mixture of 2 ml of *hydrochloric acid R* and 18 ml of *water R*. 12 ml of the solution complies with limit test A for heavy metals (10 ppm). Prepare the standard using *lead standard solution* (1 ppm Pb) *R*.

ASSAY

Dissolve 1.500 g in 50 ml of *carbon dioxide-free water R*. Titrate with 1 *M hydrochloric acid*, using 0.2 ml of *methyl orange solution R* as indicator.

1 ml of 1 *M hydrochloric acid* is equivalent to 84.0 mg of NaHCO₃.

01/2005:0677

SODIUM HYDROXIDE**Natrii hydroxidum**

NaOH

M_r 40.00**DEFINITION**

Sodium hydroxide contains not less than 97.0 per cent and not more than the equivalent of 100.5 per cent of total alkali, calculated as NaOH.

CHARACTERS

White, crystalline masses, supplied as pellets, sticks or slabs, deliquescent, readily absorbing carbon dioxide, very soluble in water, freely soluble in alcohol.

IDENTIFICATION

- Dissolve 0.1 g in 10 ml of *water R*. Dilute 1 ml of the solution to 100 ml with *water R*. The pH (2.2.3) of the final solution is not less than 11.0.
- 2 ml of solution S (see Tests) gives reaction (a) of sodium (2.3.1).

TESTS

Solution S. Carry out the procedure described below with caution. Dissolve 5.0 g in 12 ml of *distilled water R*. Add 17 ml of *hydrochloric acid R1*, adjust to pH 7 with 1 *M hydrochloric acid* and dilute to 50 ml with *distilled water R*.

Appearance of solution. Dissolve 1.0 g in 10 ml of *water R*. The solution is clear (2.2.1) and colourless (2.2.2, *Method II*).

Carbonates. Not more than 2.0 per cent, calculated as Na₂CO₃, as determined in the assay.

Chlorides (2.4.4). Dissolve 1.0 g in 5 ml of *water R*, acidify the solution with about 4 ml of *nitric acid R* and dilute to 15 ml with *water R*. The solution, without addition of *dilute nitric acid R*, complies with the limit test for chlorides (50 ppm).

Sulphates (2.4.13). Dissolve 3.0 g in 6 ml of *distilled water R*, adjust to pH 7 with *hydrochloric acid R* (about 7.5 ml) and dilute to 15 ml with *distilled water R*. The solution complies with the limit test for sulphates (50 ppm).

Iron (2.4.9). 10 ml of solution S complies with the limit test for iron (10 ppm).

Heavy metals (2.4.8). 12 ml of solution S complies with limit test A for heavy metals (20 ppm). Prepare the standard using *lead standard solution* (2 ppm Pb) *R*.

ASSAY

Dissolve 2.000 g in about 80 ml of *carbon dioxide-free water R*. Add 0.3 ml of *phenolphthalein solution R* and titrate with 1 *M hydrochloric acid*. Add 0.3 ml of *methyl orange solution R* and continue the titration with 1 *M hydrochloric acid*.

1 ml of 1 *M hydrochloric acid* used in the second part of the titration is equivalent to 0.1060 g of Na₂CO₃.

1 ml of 1 *M hydrochloric acid* used in the combined titrations is equivalent to 40.00 mg of total alkali, calculated as NaOH.

STORAGE

Store in an airtight, non-metallic container.

01/2005:1151

01/2005:0196

SODIUM IODIDE

Natrii iodidum

NaI

 M_r 149.9

DEFINITION

Sodium iodide contains not less than 99.0 per cent and not more than the equivalent of 100.5 per cent of NaI, calculated with reference to the dried substance.

CHARACTERS

A white, crystalline powder or colourless crystals, hygroscopic, very soluble in water, freely soluble in alcohol.

IDENTIFICATION

- A. Solution S (see Tests) gives the reactions of iodides (2.3.1).
B. Solution S gives the reactions of sodium (2.3.1).

TESTS

Solution S. Dissolve 10.0 g in *carbon dioxide-free water R* prepared from *distilled water R* and dilute to 100 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and colourless (2.2.2, *Method II*).

Alkalinity. To 12.5 ml of solution S add 0.1 ml of *bromothymol blue solution R1*. Not more than 0.7 ml of 0.01 M *hydrochloric acid* is required to change the colour of the indicator.

Iodates. To 10 ml of solution S add 0.25 ml of *iodide-free starch solution R* and 0.2 ml of *dilute sulphuric acid R* and allow to stand protected from light for 2 min. No blue colour develops.

Sulphates (2.4.13). 10 ml of solution S diluted to 15 ml with *distilled water R* complies with the limit test for sulphates (150 ppm).

Thiosulphates. To 10 ml of solution S add 0.1 ml of *starch solution R* and 0.1 ml of 0.005 M *iodine*. A blue colour is produced.

Heavy metals (2.4.8). 12 ml of solution S complies with limit test A for heavy metals (10 ppm). Prepare the standard using *lead standard solution* (1 ppm Pb) R.

Iron (2.4.9). 5 ml of solution S diluted to 10 ml with *water R* complies with the limit test for iron (20 ppm).

Loss on drying (2.2.32). Not more than 3.0 per cent, determined on 1.00 g by drying in an oven at 100 °C to 105 °C for 3 h.

ASSAY

Dissolve 1.300 g in *water R* and dilute to 100.0 ml with the same solvent. To 20.0 ml of the solution add 40 ml of *hydrochloric acid R* and titrate with 0.05 M *potassium iodate* until the colour changes from red to yellow. Add 5 ml of *chloroform R* and continue the titration, shaking vigorously, until the chloroform layer is decolorised.

1 ml of 0.05 M *potassium iodate* is equivalent to 14.99 mg of NaI.

STORAGE

Store protected from light.

SODIUM LACTATE SOLUTION

Natrii lactatis solutio

DEFINITION

Solution of a mixture of the enantiomers of sodium 2-hydroxypropionate in approximately equal proportions.

Content: minimum 50.0 per cent *m/m* of sodium 2-hydroxypropionate ($C_3H_5NaO_3$; M_r 112.1); 96.0 per cent to 104.0 per cent of the content of sodium lactate stated on the label.

CHARACTERS

Appearance: clear, colourless, slightly syrupy liquid.

Solubility: miscible with water and with alcohol.

IDENTIFICATION

- A. To 0.1 ml add 10 ml of *water R*. 5 ml of the solution gives the reaction of lactates (2.3.1).
B. It gives reaction (a) of sodium (2.3.1).

TESTS

Solution S. Dilute a quantity of the substance to be examined corresponding to 40.0 g of sodium lactate to 200 ml with *distilled water R*.

Appearance of solution. The substance to be examined is clear (2.2.1) and not more intensely coloured than reference solution BY₇ (2.2.2, *Method II*).

pH (2.2.3): 6.5 to 9.0 for the substance to be examined.

Reducing sugars and sucrose. To 5 ml of the substance to be examined add 2 ml of *dilute sodium hydroxide solution R* and 0.2 ml of *copper sulphate solution R*. The solution is clear and blue and remains so on boiling. Add to the hot solution 4 ml of *hydrochloric acid R*. Boil for 1 min. Add 6 ml of *strong sodium hydroxide solution R* and heat to boiling again. The solution is clear and blue.

Methanol. Gas chromatography (2.4.24).

Limit:

- *methanol*: maximum 50 ppm, calculated with reference to sodium lactate, if intended for use in the manufacture of parenteral dosage forms, dialysis, haemodialysis or haemofiltration solutions.

Chlorides (2.4.4): maximum 50 ppm calculated with reference to sodium lactate.

Dilute 5 ml of solution S to 15 ml with *water R*. The solution complies with the limit test for chlorides.

Oxalates and phosphates. To 1 ml of the substance to be examined add 15 ml of *alcohol R* and 2 ml of *calcium chloride solution R*. Heat at 75 °C for 5 min. Any opalescence in the solution is not more intense than that of a standard prepared at the same time and in the same manner using a mixture of 1 ml of the substance to be examined, 15 ml of *alcohol R* and 2 ml of *water R*.

Sulphates (2.4.13): maximum 100 ppm calculated with reference to sodium lactate.

To 7.5 ml of solution S, add 1.9 ml of *hydrochloric acid R1* and dilute to 15 ml with *distilled water R*. The solution complies with the limit test for sulphates without addition of 0.5 ml of *acetic acid R*. Acidify the standard solution with 0.05 ml of *hydrochloric acid R1* instead of 0.5 ml of *acetic acid R*.

01/2005:2033

Aluminium: maximum 0.1 ppm, if intended for use in the manufacture of parenteral dosage forms, dialysis, haemodialysis or haemofiltration solutions.

Atomic absorption spectrometry (2.2.23, *Method I*). For the preparation of the solutions, use equipment that is aluminium-free or that will not release aluminium under the conditions of use (glass, polyethylene, etc).

Modifier solution. Dissolve 100.0 g of *ammonium nitrate R* in a mixture of 50 ml of *water R* and 4 ml of *nitric acid R* and dilute to 200 ml with *water R*.

Blank solution. Dilute to 2.0 ml of the modifier solution to 25.0 ml with *water R*.

Test solution. To 1.25 g add 2.0 ml of the modifier solution and dilute to 25.0 ml with *water R*.

Reference solutions. Prepare the reference solutions immediately before use (0.010 ppm to 0.050 ppm of aluminium) using *aluminium standard solution (200 ppm Al) R*.

Source: aluminium hollow-cathode lamp.

Wavelength: 309.3 nm.

Atomisation device: a graphite furnace.

Carrier gas: argon *R*.

Conditions: the device is equipped with a non-specific absorption correction system. Heat the oven to 120 °C for as many seconds as there are microlitres of solution introduced into the apparatus, then heat at 1000 °C for 30 s and finally at 2700 °C for 6 s.

Barium. To 10 ml of solution S add 10 ml of *calcium sulphate solution R*. Allow to stand for 30 min. Any opalescence (2.2.1) in the solution is not more intense than that of a standard prepared at the same time and in the same manner using a mixture of 10 ml of solution S and 10 ml of *distilled water R*.

Iron (2.4.9): maximum 10 ppm calculated with reference to sodium lactate.

Dilute 5 ml of solution S to 10 ml with *water R*. The solution complies with the limit test for iron.

Heavy metals (2.4.8): maximum 10 ppm calculated with reference to sodium lactate.

12 ml of solution S complies with limit test A. Prepare the standard using *lead standard solution (2 ppm Pb) R*.

Bacterial endotoxins (2.6.14): less than 5 IU/g, if intended for use in the manufacture of parenteral dosage forms without a further appropriate procedure for the removal of bacterial endotoxins.

ASSAY

Dissolve a quantity of the substance to be examined corresponding to 75.0 mg of sodium lactate in a mixture of 10 ml of *glacial acetic acid R* and 20 ml of *acetic anhydride R*. Allow to stand for 15 min. Add 1 ml of *naphtholbenzein solution R* and titrate with 0.1 M *perchloric acid*.

1 ml of 0.1 M *perchloric acid* is equivalent to 11.21 mg of $C_3H_5NaO_3$.

LABELLING

The label states:

- where applicable, that the substance is suitable for use in the manufacture of dialysis, haemodialysis and haemofiltration solutions,
- where applicable, that the substance is suitable for use in the manufacture of parenteral dosage forms,
- the declared content of sodium lactate.

SODIUM (S)-LACTATE SOLUTION

Natrii (S)-lactatis solutio

DEFINITION

Content: minimum 50.0 per cent *m/m* of sodium (S)-2-hydroxypropanoate ($C_3H_5NaO_3$; M_r 112.1); 96.0 per cent to 104.0 per cent of the content of sodium lactate stated on the label, not less than 95.0 per cent of which is the (S)-enantiomer.

CHARACTERS

Appearance: clear, colourless, slightly syrupy liquid.

Solubility: miscible with water and with alcohol.

IDENTIFICATION

A. To 0.1 ml add 10 ml of *water R*. 5 ml of the solution gives the reaction of lactates (2.3.1).

B. It gives reaction (a) of sodium (2.3.1).

C. It complies with the limits of the assay.

TESTS

Solution S. Dilute a quantity of the substance to be examined corresponding to 40.0 g of sodium lactate to 200 ml with *distilled water R*.

Appearance of solution. The substance to be examined is clear (2.2.1) and not more intensely coloured than reference solution BY₇ (2.2.2, *Method II*).

pH (2.2.3): 6.5 to 9.0 for the substance to be examined.

Reducing sugars and sucrose. To 5 ml of the substance to be examined add 2 ml of *dilute sodium hydroxide solution R* and 0.2 ml of *copper sulphate solution R*. The solution is clear and blue and remains so on boiling. Add to the hot solution 4 ml of *hydrochloric acid R*. Boil for 1 min. Add 6 ml of *strong sodium hydroxide solution R* and heat to boiling again. The solution is clear and blue.

Methanol. Gas chromatography (2.4.24).

Limit:

- *methanol*: maximum 50 ppm, calculated with reference to sodium lactate, if intended for use in the manufacture of parenteral dosage forms, dialysis, haemodialysis or haemofiltration solutions.

Chlorides (2.4.4): maximum 50 ppm calculated with reference to sodium lactate.

Dilute 5 ml of solution S to 15 ml with *water R*. The solution complies with the limit test for chlorides.

Oxalates and phosphates. To 1 ml of the substance to be examined add 15 ml of *alcohol R* and 2 ml of *calcium chloride solution R*. Heat at 75 °C for 5 min. Any opalescence in the solution is not more intense than that of a standard prepared at the same time and in the same manner using a mixture of 1 ml of the substance to be examined, 15 ml of *alcohol R* and 2 ml of *water R*.

Sulphates (2.4.13): maximum 100 ppm calculated with reference to sodium lactate.

To 7.5 ml of solution S, add 1.9 ml of *hydrochloric acid RI* and dilute to 15 ml with *distilled water R*. The solution complies with the limit test for sulphates without addition of 0.5 ml of *acetic acid R*. Acidify the standard solution with 0.05 ml of *hydrochloric acid RI* instead of 0.5 ml of *acetic acid R*.

Aluminium: maximum 0.1 ppm, if intended for use in the manufacture of parenteral dosage forms, dialysis, haemodialysis or haemofiltration solutions.

Atomic absorption spectrometry (2.2.23, *Method I*). For the preparation of the solutions, use equipment that is aluminium-free or that will not release aluminium under the conditions of use (glass, polyethylene, etc).

Modifier solution. Dissolve 100.0 g of *ammonium nitrate R* in a mixture of 50 ml of *water R* and 4 ml of *nitric acid R* and dilute to 200 ml with *water R*.

Blank solution. Dilute 2.0 ml of the modifier solution to 25.0 ml with *water R*.

Test solution. To 1.25 g add 2.0 ml of the modifier solution and dilute to 25.0 ml with *water R*.

Reference solutions. Prepare the reference solutions immediately before use (0.010 ppm to 0.050 ppm of aluminium) using *aluminium standard solution (200 ppm Al) R*.

Source: aluminium hollow-cathode lamp.

Wavelength: 309.3 nm.

Atomisation device: a graphite furnace.

Carrier gas: argon *R*.

Conditions: the device is equipped with a non-specific absorption correction system. Heat the oven to 120 °C for as many seconds as there are microlitres of solution introduced into the apparatus, then heat at 1000 °C for 30 s and finally at 2700 °C for 6 s.

Barium. To 10 ml of solution S add 10 ml of *calcium sulphate solution R*. Allow to stand for 30 min. Any opalescence (2.2.1) in the solution is not more intense than that of a standard prepared at the same time and in the same manner using a mixture of 10 ml of solution S and 10 ml of *distilled water R*.

Iron (2.4.9): maximum 10 ppm calculated with reference to sodium lactate.

Dilute 5 ml of solution S to 10 ml with *water R*. The solution complies with the limit test for iron.

Heavy metals (2.4.8): maximum 10 ppm calculated with reference to sodium lactate.

12 ml of solution S complies with limit test A. Prepare the standard using *lead standard solution (2 ppm Pb) R*.

Bacterial endotoxins (2.6.14): less than 5 IU/g if intended for use in the manufacture of parenteral dosage forms without a further appropriate procedure for the removal of bacterial endotoxins.

ASSAY

Dissolve a quantity of the substance to be examined corresponding to 75.0 mg of sodium lactate in a mixture of 10 ml of *glacial acetic acid R* and 20 ml of *acetic anhydride R*. Allow to stand for 15 min. Add 1 ml of *naphtholbenzein solution R* and titrate with 0.1 M *perchloric acid*.

1 ml of 0.1 M *perchloric acid* is equivalent to 11.21 mg of $C_3H_5NaO_3$.

(S)-enantiomer. Transfer a quantity of the substance to be examined corresponding to 2.50 g of sodium lactate into a 50 ml volumetric flask, dilute with about 30 ml of *water R* and add 5.0 g of *ammonium molybdate R*. Dissolve and dilute with *water R* to 50.0 ml. Measure the angle of optical rotation (2.2.7). Calculate the percentage content of (S)-enantiomer using the expression:

$$50 + \left(24.04 \times \alpha \times \frac{5.0}{m} \times \frac{50}{c} \right)$$

α = angle of optical rotation (absolute value),

m = mass of the substance to be examined, in grams,

c = percentage content of $C_3H_5NaO_3$ in the substance to be examined.

The complex of sodium (S)-lactate formed under these test conditions is leavorotatory.

LABELLING

The label states:

- where applicable, that the substance is suitable for use in the manufacture of dialysis, haemodialysis and haemofiltration solutions,
- where applicable, that the substance is suitable for use in the manufacture of parenteral dosage forms,
- the declared content of sodium lactate.

01/2005:0098

SODIUM LAURILSULFATE

Natrii laurilsulfas

DEFINITION

Sodium laurilsulfate is a mixture of sodium alkyl sulphates consisting chiefly of sodium dodecyl sulphate, $C_{12}H_{25}NaO_4S$ (M_r 288.4). It contains not less than 85.0 per cent of sodium alkyl sulphates, calculated as $C_{12}H_{25}NaO_4S$.

CHARACTERS

A white or pale yellow powder or crystals, freely soluble in water giving an opalescent solution, partly soluble in alcohol.

IDENTIFICATION

- Dissolve 0.1 g in 10 ml of *water R* and shake. A copious foam is formed.
- To 0.1 ml of the solution prepared for identification test A, add 0.1 ml of a 1 g/l solution of *methylene blue R* and 2 ml of *dilute sulphuric acid R*. Add 2 ml of *methylene chloride R* and shake. An intense blue colour develops in the methylene chloride layer.
- Mix about 10 mg with 10 ml of *ethanol R*. Heat to boiling on a water-bath, shaking frequently. Filter immediately and evaporate the ethanol. Dissolve the residue in 8 ml of *water R*, add 3 ml of *dilute hydrochloric acid R*, evaporate the solution to half its volume and allow to cool. Separate the congealed fatty alcohols by filtration. To the filtrate add 1 ml of *barium chloride solution R1*. A white, crystalline precipitate is formed.
- Ignite 0.5 g. The residue gives reaction (a) of sodium (2.3.1).

TESTS

Alkalinity. Dissolve 1.0 g in 100 ml of *carbon dioxide-free water R* and add 0.1 ml of *phenol red solution R*. Not more than 0.5 ml of 0.1 M *hydrochloric acid* is required to change the colour of the indicator.

Non-esterified alcohols. Dissolve 10 g in 100 ml of *water R*, add 100 ml of *alcohol R* and shake the solution with 3 quantities, each of 50 ml, of *pentane R*, adding *sodium chloride R*, if necessary, to promote separation of the 2 layers. Wash the combined organic layers with 3 quantities, each of 50 ml, of *water R*, dry over *anhydrous sodium sulphate R*, filter and evaporate on a water-bath until the

solvent has evaporated. Heat the residue at 105 °C for 15 min and cool. The residue weighs not more than 0.4 g (4 per cent).

Sodium chloride and sodium sulphate. Not more than a total of 8.0 per cent of NaCl and Na₂SO₄.

Sodium chloride. Dissolve 5.00 g in 50 ml of *water R*, add *dilute nitric acid R* dropwise until the solution is neutral to *blue litmus paper R*, add 2 ml of *potassium chromate solution R* and titrate with 0.1 M *silver nitrate*.

1 ml of 0.1 M *silver nitrate* is equivalent to 5.844 mg of NaCl.

Sodium sulphate. Dissolve 0.500 g in 20 ml of *water R*, warming gently if necessary, and add 1 ml of a 0.5 g/l solution of *dithizone R1* in *acetone R*. If the solution is red, add 1 M *nitric acid*, dropwise, until the solution becomes bluish-green. Add 2.0 ml of *dichloroacetic acid solution R* and 80 ml of *acetone R*. Titrate with 0.01 M *lead nitrate* until a persistent violet-red or orange-red colour is obtained. Carry out a blank titration.

1 ml of 0.01 M *lead nitrate* is equivalent to 1.420 mg of Na₂SO₄.

ASSAY

Dissolve 1.15 g in *water R*, warming if necessary, and dilute to 1000.0 ml with the same solvent. To 20.0 ml of the solution add 15 ml of *chloroform R* and 10 ml of *dimidium bromide-sulphan blue mixed solution R*. Titrate with 0.004 M *benzethonium chloride*, shaking vigorously and allowing the layers to separate before each addition, until the pink colour of the chloroform layer is completely discharged and a greyish-blue colour is obtained.

1 ml of 0.004 M *benzethonium chloride* is equivalent to 1.154 mg of sodium alkyl sulphates, calculated as C₁₂H₂₅NaO₄S.

01/2005:0849

SODIUM METABISULPHITE

Natrii metabisulfis

Na₂S₂O₅*M*_r 190.1

DEFINITION

Sodium metabisulphite (sodium disulphite) contains not less than 95.0 per cent and not more than the equivalent of 100.5 per cent of Na₂S₂O₅.

CHARACTERS

A white or almost white, crystalline powder or colourless crystals, freely soluble in water, slightly soluble in alcohol.

IDENTIFICATION

- Solution S (see Tests) complies with the test for pH (see Tests).
- To 0.4 ml of *iodinated potassium iodide solution R* add 8 ml of *distilled water R* and 1 ml of a 1 to 10 dilution of solution S in *distilled water R*. The solution is colourless and gives reaction (a) of sulphates (2.3.1).
- Solution S gives reaction (a) of sodium (2.3.1).

TESTS

Solution S. Dissolve 5.0 g in *carbon dioxide-free water R* prepared from *distilled water R* and dilute to 100 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and colourless (2.2.2, *Method II*).

pH (2.2.3). The pH of solution S is 3.5 to 5.0.

Thiosulphates. To 5 ml of solution S add 5 ml of *dilute hydrochloric acid R*. The solution remains clear (2.2.1) for not less than 15 min.

Arsenic (2.4.2). Mix 0.20 g with 2 ml of *water R* in a dish. Add, drop by drop, 1.5 ml of *nitric acid R*. Evaporate the mixture to dryness on a water-bath. Heat over a flame until no more vapour is evolved. Take up the residue in 25 ml of *water R*. The solution complies with limit test A for arsenic (5 ppm).

Iron (2.4.9). 10 ml of solution S complies with the limit test for iron (20 ppm).

Heavy metals (2.4.8). To 40 ml of solution S in a silica crucible, add 10 ml of *hydrochloric acid R* and evaporate to dryness. Dissolve the residue in 19 ml of *water R* and add 1 ml of a 40 g/l solution of *sodium fluoride R*. 12 ml of the solution complies with limit test A for heavy metals (20 ppm). Prepare the standard using *lead standard solution* (2 ppm Pb) R.

ASSAY

Dissolve 0.200 g in 50.0 ml of 0.05 M *iodine*. Add 5 ml of *dilute hydrochloric acid R*. Titrate with 0.1 M *sodium thiosulphate* using 1 ml of *starch solution R*, added towards the end of the titration, as indicator.

1 ml of 0.05 M *iodine* is equivalent to 4.753 mg of Na₂S₂O₅.

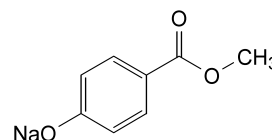
STORAGE

Store protected from light.

01/2005:1262

SODIUM METHYL PARAHYDROXYBENZOATE

Methylis parahydroxybenzoas natricum

C₈H₇NaO₃*M*_r 174.1

DEFINITION

Sodium methyl parahydroxybenzoate contains not less than 99.0 per cent and not more than the equivalent of 102.0 per cent of sodium 4-(methoxycarbonyl)phenolate, calculated with reference to the anhydrous substance.

CHARACTERS

A white, crystalline powder, freely soluble in water, sparingly soluble in alcohol, practically insoluble in methylene chloride.

IDENTIFICATION

First identification: A, B, E.

Second identification: A, C, D, E.

- Dissolve 0.5 g in 50 ml of *water R*. Immediately add 5 ml of *hydrochloric acid R1*. Filter and wash the precipitate with *water R*. Dry under vacuum at 80 °C for 2 h. The precipitate obtained melts (2.2.14) at 125 °C to 128 °C.
- Examine the precipitate obtained in identification test A by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *methyl parahydroxybenzoate CRS*.

- C. Examine the chromatograms obtained in the test for related substances. The principal spot in the chromatogram obtained with test solution (b) is similar in position and size to the principal spot in the chromatogram obtained with reference solution (c).
- D. To about 10 mg in a test-tube add 1 ml of *sodium carbonate solution R*, boil for 30 s and cool. Add 5 ml of *aminopyrazolone solution R* and 1 ml of *potassium ferricyanide solution R* and mix. An orange to red colour develops.
- E. To 1 ml of solution S (see Tests) add 1 ml of *water R*. The solution gives reaction (a) of sodium (2.3.1).

TESTS

Solution S. Dissolve 5.0 g in *carbon dioxide-free water R* prepared from *distilled water R* and dilute to 50 ml with the same solvent.

Appearance of solution. Solution S examined immediately after preparation is clear (2.2.1) and not more intensely coloured than reference solution BY₆ (2.2.2, Method I).

pH (2.2.3). Dilute 1 ml of solution S to 100 ml with *carbon dioxide-free water R*. The pH of the solution is 9.5 to 10.5.

Related substances. Examine by thin-layer chromatography (2.2.27), using as the coating substance a suitable octadecylsilyl silica gel with a fluorescent indicator having an optimal intensity at 254 nm.

Test solution (a). Dissolve 0.100 g in 10 ml of *water R*. Immediately add 2 ml of *hydrochloric acid R* and shake with 50 ml of *ether R*. Evaporate the upper layer to dryness and dissolve the residue to 10 ml with *acetone R*.

Test solution (b). Dilute 1 ml of test solution (a) to 10 ml with *acetone R*.

Reference solution (a). Dissolve 34.3 mg of *4-hydroxybenzoic acid R* in *acetone R* and dilute to 100 ml with the same solvent.

Reference solution (b). Dilute 0.5 ml of test solution (a) to 100 ml with *acetone R*.

Reference solution (c). Dissolve 10 mg of *methyl parahydroxybenzoate CRS* in *acetone R* and dilute to 10 ml with the same solvent.

Reference solution (d). Dissolve 10 mg of *ethyl parahydroxybenzoate CRS* in 1 ml of test solution (a) and dilute to 10 ml with *acetone R*.

Apply separately to the plate 5 µl of each solution. Develop over a path of 15 cm using a mixture of 1 volume of *glacial acetic acid R*, 30 volumes of *water R* and 70 volumes of *methanol R*. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. In the chromatogram obtained with test solution (a): any spot due to 4-hydroxybenzoic acid is not more intense than the spot in the chromatogram obtained with reference solution (a) (4 per cent) and any spot, apart from the principal spot and the spot due to 4-hydroxybenzoic acid, is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.5 per cent). The test is not valid unless the chromatogram obtained with reference solution (d) shows two clearly separated principal spots.

Chlorides (2.4.4). To 10 ml of solution S, add 30 ml of *water R* and 1 ml of *nitric acid R* and dilute to 50 ml with *water R*. Shake and filter. Dilute 10 ml of the filtrate to 15 ml with *water R*. The solution complies with the limit test for chlorides (350 ppm). Prepare the standard using 14 ml of *chloride standard solution (5 ppm Cl) R* to which 1 ml of *water R* has been added.

Sulphates (2.4.13). To 25 ml of solution S, add 5 ml of *distilled water R* and 10 ml of *hydrochloric acid R* and dilute to 50 ml with *distilled water R*. Shake and filter. Dilute 10 ml of the filtrate to 15 ml with *distilled water R*. The solution complies with the limit test for sulphates (300 ppm).

Heavy metals (2.4.8). 2.0 g complies with limit test C for heavy metals (10 ppm). Prepare the standard using 2 ml of *lead standard solution (10 ppm Pb) R*.

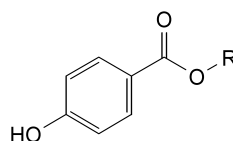
Water (2.5.12). Not more than 5.0 per cent, determined on 0.500 g by the semi-micro determination of water.

ASSAY

Dissolve 0.150 g in 50 ml of *anhydrous acetic acid R*. Titrate with 0.1 M *perchloric acid*, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *perchloric acid* is equivalent to 17.41 mg of C₈H₇NaO₃.

IMPURITIES



- A. R = H: 4-hydroxybenzoic acid,
 B. R = CH₂-CH₃: ethyl 4-hydroxybenzoate,
 C. R = CH₂-CH₂-CH₃: propyl 4-hydroxybenzoate,
 D. R = CH₂-CH₂-CH₂-CH₃: butyl 4-hydroxybenzoate.

01/2005:1565

SODIUM MOLYBDATE DIHYDRATE

Natrii molybdatas dihydricus

Na₂MoO₄·2H₂OM_r 241.9

DEFINITION

Sodium molybdate dihydrate contains not less than 98.0 per cent and not more than the equivalent of 100.5 per cent of Na₂MoO₄, calculated with reference to the dried substance.

CHARACTERS

A white powder or colourless crystals, freely soluble in water.

IDENTIFICATION

- A. It complies with the test for loss on drying (see Tests).
 B. Dissolve 0.2 g in 5 ml of a mixture of equal volumes of *nitric acid R* and *water R* and add 0.1 g of *ammonium chloride R*. Add 0.3 ml of *disodium hydrogen phosphate solution R* and heat slowly at 50 °C to 60 °C. A yellow precipitate is formed.
 C. Dissolve 0.15 g in 2 ml of *water R*, the solution gives reaction (a) of sodium (2.3.1).

TESTS

Solution S. Dissolve 10.0 g in *water R* and dilute to 50 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and colourless (2.2.2, Method II).

Chlorides. To 10 ml of a mixture of equal volumes of *nitric acid R* and *water R* add 10 ml of solution S with shaking. Add 1 ml of 0.1 M *silver nitrate*. Any opalescence in the solution is not more intense after 5 min than that of a

standard solution prepared at the same time in the same manner with 2 ml of *chloride standard solution* (50 ppm Cl) R (50 ppm).

Phosphates. Dissolve 2.0 g by heating in 13 ml of *water R*. In the still hot solution, dissolve 8.0 g of *ammonium nitrate R1*. Add this solution to 27 ml of a mixture of equal volumes of *nitric acid R* and *water R*. Any yellow colour or opalescence in the solution is not more intense within 3 h than that in a standard solution prepared at the same time as follows: dissolve 1.0 g in 12 ml of *water R* and add 1 ml of *phosphate standard solution* (200 ppm PO₄) R (200 ppm).

Ammonium (2.4.1). 0.10 g complies with limit test B for ammonium (10 ppm). Prepare the standard using 1 ml of *ammonium standard solution* (1 ppm NH₄) R.

Heavy metals. To 10 ml of solution S, add 2 ml of *water R*, 6 ml of a 168 g/l solution of *sodium hydroxide R* and 2 ml of *concentrated ammonia R* (solution A). To 0.5 ml of *thioacetamide reagent R* add a mixture of 15 ml of solution A and 5 ml of *water R*. Any coloration of the solution is not more intense after 2 min than that of a standard solution prepared at the same time as follows: to 0.5 ml of *thioacetamide reagent R* add a mixture of 5 ml of solution A, 1 ml of *lead standard solution* (10 ppm Pb) R and 14 ml of *water R* (10 ppm).

Loss on drying (2.2.32): 14.0 per cent to 16.0 per cent, determined on 1.000 g by drying in an oven at 140 °C for 3 h.

ASSAY

Dissolve 0.100 g in 30 ml of *water R*, add 0.5 g of *hexamethylenetetramine R* and 0.1 ml of a 250 g/l solution of *nitric acid R*. Heat to 60 °C. Titrate with 0.05 M *lead nitrate* using 4-(2-pyridylazo)resorcinol monosodium salt R as indicator.

1 ml of 0.05 M *lead nitrate* is equivalent to 10.30 mg of Na₂MoO₄.

01/2005:1996

SODIUM NITRITE

Natrii nitris

NaNO₂*M_r* 69.0

DEFINITION

Content: 98.5 per cent to 100.5 per cent (dried substance).

CHARACTERS

Appearance: colourless crystals or mass or yellowish rods, hygroscopic.

Solubility: freely soluble in water, soluble in alcohol.

IDENTIFICATION

- Dilute 1 ml of solution S1 (see Tests) to 25 ml with *water R*. To 0.1 ml of the solution add 1 ml of *sulphanilic acid solution R1*. Allow to stand for 2-3 min. Add 1 ml of *β-naphthol solution R* and 1 ml of *dilute sodium hydroxide solution R*. An intense red colour develops.
- To 1 ml of the solution prepared for identification test A add 3 ml of a 20 g/l solution of *phenazone R* and 0.4 ml of *dilute sulphuric acid R*. An intense green colour develops.
- To 0.15 ml of solution S1, add 0.35 ml of *water R*. The solution gives reaction (b) of sodium (2.3.1).

TESTS

Solution S1. Dissolve 2.5 g in *carbon dioxide-free water R* and dilute to 50 ml with the same solvent.

Solution S2. Dissolve 3 g in *distilled water R*. Cautiously add 10 ml of *nitric acid R* and evaporate to dryness. Dissolve the residue in 10 ml of *distilled water R*, neutralise with *dilute sodium hydroxide solution R* and dilute to 30 ml with *distilled water R*.

Appearance of solution. Solution S1 is clear (2.2.1) and not more intensely coloured than reference solution B₆ (2.2.2, Method II).

Acidity or alkalinity. To 10 ml of solution S1, add 0.05 ml of *phenol red solution R*. Add 0.1 ml of 0.01 M *sodium hydroxide*. The solution is red. Add 0.3 ml of 0.01 M *hydrochloric acid*. The solution is yellow.

Chlorides (2.4.4): maximum 50 ppm.

Dilute 10 ml of solution S2 to 15 ml with *water R*. The solution complies with the limit test for chlorides.

Sulphates (2.4.13): maximum 200 ppm.

Dilute 7.5 ml of solution S2 to 15 ml with *distilled water R*. The solution complies with the limit test for sulphates.

Heavy metals (2.4.8): maximum 20 ppm.

Dilute 10 ml of solution S2 to 20 ml with *water R*. 12 ml of the solution complies with limit test A. Prepare the standard using *lead standard solution* (1 ppm Pb) R.

Loss on drying (2.2.32): maximum 1.0 per cent, determined on 1.000 g by drying *in vacuo*.

ASSAY

Dissolve 0.400 g in 100.0 ml of *water R*. Introduce 20.0 ml of the solution, while stirring continuously and keeping the tip of the pipette below the surface of the liquid, into a conical flask containing 30.0 ml of 0.1 M *cerium sulphate*. Immediately stopper the flask and allow to stand for 2 min. Add 10 ml of a 200 g/l solution of *potassium iodide R* and 2 ml of *starch solution R*.

While stirring continuously, titrate with 0.1 M *sodium thiosulphate* until the blue colour disappears. Carry out a blank titration.

1 ml of 0.1 M *cerium sulphate* is equivalent to 3.45 mg of NaNO₂.

STORAGE

In an airtight container.

01/2005:0565

SODIUM NITROPRUSSIDE

Natrii nitroprussias

Na₂[Fe(CN)₅NO]·2H₂O*M_r* 298.0

DEFINITION

Sodium nitroprusside contains not less than 99.0 per cent and not more than the equivalent of 100.5 per cent of sodium pentacyanonitrosylferrate (III), calculated with reference to the anhydrous substance.

CHARACTERS

Reddish-brown powder or crystals, freely soluble in water, slightly soluble in alcohol.

IDENTIFICATION

- A. Dissolve 0.700 g in *water R* and dilute to 100.0 ml with the same solvent. Examined between 350 nm and 600 nm (2.2.25) immediately after preparation, the solution shows an absorption maximum at 395 nm, a shoulder at about 510 nm and a minimum at 370 nm. The specific absorbance at the maximum is 0.65 to 0.80.
- B. Dissolve about 20 mg in 2 ml of *water R* and add 0.1 ml of *sodium sulphide solution R*. A deep violet-red colour is produced.
- C. Dissolve 50 mg in 1 ml of *water R* and acidify the solution by the addition of *hydrochloric acid R*. Place a drop of the solution in an oxidising flame. A persistent yellow colour is produced.

TESTS

Insoluble matter. Dissolve 10 g without heating in 50 ml of *water R*. Allow to stand for 30 min and filter through a sintered-glass filter (16). Wash the filter with cold *water R* until the filtrate is colourless. Dry the residue on the filter at 105 °C. The residue weighs not more than 1 mg (100 ppm).

Chlorides (2.4.4). In a metallic crucible (nickel) mix 1.0 g of the substance to be examined with 8 ml of a 200 g/l solution of *sodium hydroxide R*. Heat slowly and evaporate carefully to dryness over a small flame, then heat to a dull red colour for 30 min. Allow to cool and transfer the solid residue with three successive portions, each of 8 ml of *dilute sulphuric acid R*. Filter the sulphuric acid extracts on a filter paper and collect the filtrates. Render the filtrate acid to *litmus paper R* by adding, if necessary, a few drops of *dilute sulphuric acid R*. Wash the crucible and the filter paper with three successive portions of 10 ml of *water R*, add the washings to the main sulphuric acid solution and dilute to 60 ml with *water R*. Mix. 15 ml of the solution complies with the limit test for chlorides (200 ppm).

Ferricyanides. Dissolve 1.25 g in *acetate buffer solution pH 4.6 R* and dilute to 50.0 ml with the same buffer solution. Use three 50 ml volumetric flasks (A, B, C). To flask B add 1.0 ml of *ferricyanide standard solution (50 ppm Fe(CN)₆ R*. To flasks A and B add 1 ml of a 5 g/l solution of *ferrous ammonium sulphate R*. To the three flasks add 10.0 ml of the solution of the substance to be examined. Dilute the contents of each flask to 50.0 ml with *water R*. Allow to stand for 30 min. The absorbance (2.2.25) of the solution in flask A measured at 720 nm using the solution in flask C as the compensation liquid is not greater than the absorbance of the solution in flask B measured at 720 nm using the solution in flask A as the compensation liquid (200 ppm).

Ferrocyanides. Dissolve 4.0 g in *water R* and dilute to 100.0 ml with the same solvent. Use three 50 ml volumetric flasks (A, B, C). To flask B add 2.0 ml of *ferrocyanide standard solution (100 ppm Fe(CN)₆ R*. To flasks A and B add 1 ml of *ferric chloride solution R2*. To the three flasks add 25.0 ml of the solution of the substance to be examined. Dilute the contents of each flask to 50.0 ml with *water R*. Allow to stand for 30 min. The absorbance (2.2.25) of the solution in flask A measured at 695 nm using the solution in flask C as the compensation liquid is not greater than the absorbance of the solution in flask B measured at 695 nm using the solution in flask A as the compensation liquid (200 ppm).

Sulphates. For the preparation and dilution of the solutions use *distilled water R*.

Test solution. Dissolve 3.6 g in 120 ml of water, add with mixing 4 ml of *sulphate standard solution (10 ppm SO₄ R* and 20 ml of a 250 g/l solution of *cupric chloride R* and

dilute to 150.0 ml with water. Allow to stand for 16 h and filter or centrifuge until a clear light-blue solution is obtained.

Reference solution. To 40 ml of *sulphate standard solution (10 ppm SO₄ R* add 80 ml of water and 12 ml to 13 ml of a 250 g/l solution of *cupric chloride R*. Dilute to 150.0 ml with water. The volume of cupric chloride solution added is such that the colour of the final solution matches that of the test solution.

Allow the solutions to stand. Filter both solutions, discarding the first 25 ml of filtrate. To 100 ml of each filtrate, add 0.5 ml of *acetic acid R*. Mix and add 2 ml of a 250 g/l solution of *barium chloride R* and mix again. The test solution is not more opalescent than the reference solution (100 ppm).

Water (2.5.12): 9.0 per cent to 15.0 per cent, determined on 0.250 g by the semi-micro determination of water.

ASSAY

Dissolve 0.250 g in 100 ml of *water R* and add 0.1 ml of *dilute sulphuric acid R*. Titrate with 0.1 M *silver nitrate*, determining the end-point potentiometrically (2.2.20) with a silver-mercurous sulphate electrode system.

1 ml of 0.1 M *silver nitrate* is equivalent to 13.10 mg of Na₂[Fe(CN)₅NO].

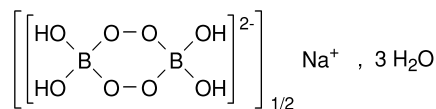
STORAGE

Store protected from light.

01/2005:1997

SODIUM PERBORATE, HYDRATED

Natrii perboras hydricus



NaBO₃·4H₂O or NaBO₂·H₂O₂·3H₂O

M_r 153.9

DEFINITION

Content: 96.0 per cent to 103.0 per cent.

CHARACTERS

Appearance: colourless, prismatic crystals or white powder, stable in the crystalline form.

Solubility: sparingly soluble in water, with slow decomposition. It dissolves in dilute mineral acids.

IDENTIFICATION

- A. Mix 1 ml of a saturated solution with a mixture of 1 ml of *dilute sulphuric acid R* and 0.2 ml of a 70 g/l solution of *potassium dichromate R*, shake with 2 ml of *ether R* and allow to stand. A blue colour is produced in the ether layer.
- B. The mixture obtained by treating about 100 mg with 0.1 ml of *sulphuric acid R* and 5 ml of *methanol R* burns with a greenish flame when ignited.
- C. It gives reaction (a) of sodium (2.3.1).

TESTS

Chlorides (2.4.4): maximum 330 ppm.

Dissolve 0.15 g in 15 ml of *water R*. The solution complies with the limit test for chlorides.

Sulphates (2.4.13): maximum 1.2 per cent.

Dissolve 0.13 g in 150 ml of *distilled water R*. 15 ml of the solution complies with the limit test for sulphates.

Iron (2.4.9): maximum 20 ppm.

Dissolve 2.5 g in 10 ml of *dilute hydrochloric acid R* with heating, evaporate to dryness, with stirring, and dissolve the residue in 25 ml of hot *water R*. Dilute 5 ml of the obtained solution to 10 ml with *water R*. The solution complies with the limit test for iron.

Heavy metals (2.4.8): maximum 10 ppm.

12 ml of the solution obtained in the test for iron complies with limit test A. Prepare the standard using *lead standard solution (1 ppm Pb) R*.

ASSAY

Dissolve 0.300 g in 50.0 ml of *water R*. Dilute 10.0 ml of the solution to 50 ml with *water R* and add 10 ml of *dilute sulphuric acid R*. Titrate with 0.02 M *potassium permanganate*.

1 ml of 0.02 M *potassium permanganate* is equivalent to 7.693 mg of NaH_8BO_7 .

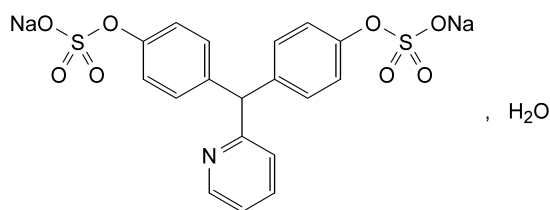
STORAGE

In an airtight container.

01/2005:1031

SODIUM PICOSULFATE

Natrii picosulfas



$\text{C}_{18}\text{H}_{13}\text{NNa}_2\text{O}_8\text{S}_2 \cdot \text{H}_2\text{O}$

M_r 499.4

DEFINITION

Sodium picosulfate contains not less than 98.5 per cent and not more than the equivalent of 100.5 per cent of 4,4'-(pyridin-2-ylmethylene)bisphenyl bis(sodium sulphate), calculated with reference to the anhydrous substance.

CHARACTERS

A white or almost white, crystalline powder, freely soluble in water, slightly soluble in alcohol.

IDENTIFICATION

First identification: A, E.

Second identification: B, C, D, E.

- Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *sodium picosulfate CRS*. Examine the substances prepared as discs.
- Examine the chromatograms obtained in the test for related substances in ultraviolet light at 254 nm. The principal spot in the chromatogram obtained with test solution (b) is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a).
- To 5 ml of solution S (see Tests) add 1 ml of *dilute hydrochloric acid R* and heat to boiling. Add 1 ml of *barium chloride solution R1*. A white precipitate is formed.

D. To about 10 mg add 3 ml of *sulphuric acid R* and 0.1 ml of *potassium dichromate solution R1*. A violet colour develops.

E. The solution S gives reaction (a) of sodium (2.3.1).

TESTS

Solution S. Dissolve 2.5 g in *distilled water R* and dilute to 50 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and not more intensely coloured than reference solution GY₇ (2.2.2, *Method II*).

Acidity or alkalinity. To 10 ml of solution S add 0.05 ml of *phenolphthalein solution R*. The solution is colourless. Not more than 0.25 ml of 0.01 M *sodium hydroxide* is required to change the colour of the indicator to pink.

Related substances. Examine by thin-layer chromatography (2.2.27), using *silica gel GF₂₅₄ R* as the coating substance.

Test solution (a). Dissolve 0.20 g of the substance to be examined in *methanol R* and dilute to 5 ml with the same solvent.

Test solution (b). Dilute 1 ml of test solution (a) to 10 ml with *methanol R*.

Reference solution (a). Dissolve 20 mg of *sodium picosulfate CRS* in *methanol R* and dilute to 5 ml with the same solvent.

Reference solution (b). Dilute 2 ml of test solution (b) to 100 ml with *methanol R*.

Reference solution (c). Dissolve 0.20 g of the substance to be examined in 2 ml of a 103 g/l solution of *hydrochloric acid R*. Heat rapidly to boiling and maintain boiling for 10 s. Cool in iced water and dilute to 10 ml with *methanol R*.

Apply to the plate 5 µl of each solution. Develop over a path of 10 cm using a mixture of 2.5 volumes of *anhydrous formic acid R*, 12.5 volumes of *water R*, 25 volumes of *methanol R* and 60 volumes of *ethyl acetate R*. Dry the plate in a current of hot air for 15 min and examine in ultraviolet light at 254 nm. Spray with a 200 g/l solution of *hydrochloric acid R* in *methanol R* and heat at 110 °C for 10 min. Spray the hot plate with a freshly prepared solution containing 50 g/l of *ferric chloride R* and 1 g/l of *potassium ferricyanide R*. Examine the wet plate. Any spot in the chromatogram obtained with test solution (a), apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.2 per cent). The test is not valid unless the chromatogram obtained with reference solution (c) shows three clearly separated spots. A fourth spot may be present on the starting-line.

Chlorides (2.4.4). Dilute 5 ml of solution S to 15 ml with *water R*. The solution complies with the limit test for chlorides (200 ppm).

Sulphates (2.4.13). Dilute 7.5 ml of solution S to 15 ml with *distilled water R*. The solution complies with the limit test for sulphates (400 ppm).

Heavy metals (2.4.8). 12 ml of solution S complies with limit test A for heavy metals (20 ppm). Prepare the standard using 10 ml of *lead standard solution (1 ppm Pb) R*.

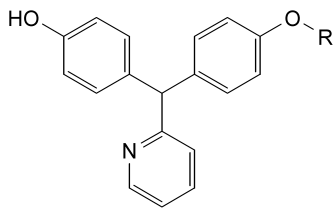
Water (2.5.12): 3.0 per cent to 5.0 per cent, determined on 0.500 g by the semi-micro determination of water.

ASSAY

Dissolve 0.400 g in 80 ml of *methanol R*. Titrate with 0.1 M *perchloric acid*, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *perchloric acid* is equivalent to 48.14 mg of $\text{C}_{18}\text{H}_{13}\text{NNa}_2\text{O}_8\text{S}_2$.

IMPURITIES



- A. R = SO₃Na: 4-[(pyridin-2-yl)(4-hydroxyphenyl)methyl]phenyl sodium sulphate,
 B. R = H: 4,4'-[(pyridin-2-yl)methylene]bisphenol.

01/2005:1909
corrected

SODIUM POLYSTYRENE SULPHONATE

Natrii polystyrenesulfonas

DEFINITION

Polystyrene sulphonate resin prepared in the sodium form.

Exchange capacity: 2.8 mmol to 3.4 mmol of potassium per gram (dried substance).

Content: 9.4 per cent to 11.0 per cent of Na (dried substance).

CHARACTERS

Appearance: almost white or light brown powder.

Solubility: practically insoluble in water, in alcohol and in methylene chloride.

IDENTIFICATION

- A. Infrared absorption spectrophotometry (2.2.24).

Preparation: discs using finely ground substance.

Comparison: Ph. Eur. reference spectrum of sodium polystyrene sulphonate.

- B. Suspend 0.1 g in *water R*, add 2 ml of a 150 g/l solution of *potassium carbonate R*, and heat to boiling. Allow to cool and filter. To the filtrate add 4 ml of *potassium pyroantimonate solution R* and heat to boiling. Allow to cool in iced water and if necessary rub the inside of the test-tube with a glass rod. A dense white precipitate is formed.

TESTS

Styrene. Liquid chromatography (2.2.29).

Test solution. Shake 10.0 g of the substance to be examined with 10 ml of *acetone R* for 30 min, centrifuge and use the supernatant liquid.

Reference solution. Dissolve 10 mg of *styrene R* in *acetone R* and dilute to 100 ml with the same solvent. Dilute 1 ml of this solution to 100 ml with *acetone R*.

Column:

- *size*: $l = 0.25$ m, $\varnothing = 4$ mm,
- *stationary phase*: octadecylsilyl silica gel for chromatography *R* (5 μ m).

Mobile phase: acetonitrile *R*, *water R* (1:1 V/V).

Flow rate: 2 ml/min.

Detection: spectrophotometer at 254 nm.

Injection: 20 μ l.

Limit:

- *styrene*: not more than the area of the principal peak in the chromatogram obtained with the reference solution (1 ppm).

Calcium: maximum 0.10 per cent.

Atomic emission spectrometry (2.2.22, *Method I*).

Test solution. To 1.10 g add 5 ml of *hydrochloric acid R*, heat to boiling, cool and add 10 ml of *water R*. Filter, wash the filter and residue with *water R* and dilute the filtrate and washing to 25.0 ml with *water R*.

Reference solutions. Prepare the reference solutions using *calcium standard solution (400 ppm Ca) R*, diluted as necessary with *water R*.

Wavelength: 422.7 nm.

Potassium: maximum 0.10 per cent.

Atomic emission spectrometry (2.2.22, *Method I*).

Test solution. To 1.10 g add 5 ml of *hydrochloric acid R*, heat to boiling, cool and add 10 ml of *water R*. Filter, wash the filter and residue with *water R* and dilute the filtrate and washings to 25.0 ml with *water R*.

Reference solutions. Prepare the reference solutions using *potassium standard solution (100 ppm K) R*, diluted as necessary with *water R*.

Wavelength: 766.5 nm.

Heavy metals (2.4.8): maximum 10 ppm.

Treat 1.0 g as described in limit test F. After the addition of the *buffer solution pH 3.5 R* and of the *thioacetamide reagent R*, dilute to 50 ml with *water R* and continue as described in limit test E, beginning at the words “mix and allow to stand for 10 min...”.

Prepare the standard using 10 ml of *lead standard solution (1 ppm Pb) R*.

Loss on drying (2.2.32): maximum 7.0 per cent, determined on 1.000 g by drying in an oven at 100–105 °C.

Microbial contamination (2.6.13): not more than 10² enterobacteria and certain other gram-negative bacteria per gram.

ASSAY

Sodium. Atomic emission spectrometry (2.2.22, *Method I*).

Test solution. In a platinum crucible moisten 0.90 g with a few drops of *sulphuric acid R*, ignite very gently and allow to cool. Moisten with a few drops of *sulphuric acid R* again, ignite at 800 °C until a carbon-free ash is obtained and allow to cool.

Add 20 ml of *water R* to the crucible, warm gently on a water-bath until dissolution, cool, transfer quantitatively to a 100 ml graduated flask and dilute to 100.0 ml with *water R*. Dilute 5 ml of this solution to 1000.0 ml with *water R*.

Reference solutions. Prepare the reference solutions using *sodium standard solution (200 ppm Na) R*, diluted as necessary with *water R*.

Wavelength: 589 nm.

Exchange capacity. Atomic emission spectrometry (2.2.22, *Method I*).

Solution A. 9.533 g/l solution of *potassium chloride R*.

Test solution. To 1.6 g of the substance to be examined in a dry 250 ml ground-glass-stoppered flask add 100 ml of solution A, stopper and shake for 15 min. Filter, discard the first 20 ml of the filtrate and dilute 4 ml of the filtrate to 1000 ml with *water R*.

Reference solutions. Prepare the reference solutions by diluting 0, 1, 2, 3 and 4 ml of solution A respectively and 4, 3, 2, 1 and 0 ml of a 7.63 g/l solution of *sodium chloride R* to 1000 ml with *water R*.

Wavelength: 766.5 nm.

Prepare a calibration curve using the reference solutions and calculate the potassium exchange capacity of the substance to be examined in millimoles per gram taking the concentration of potassium in solution A as 128 mmoles of K per litre.

STORAGE

In an airtight container.

IMPURITIES

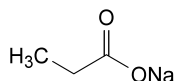
Specified impurities: A.

A. styrene.

01/2005:2041

SODIUM PROPIONATE

Natrii propionas



$C_3H_5NaO_2$

M_r 96.1

DEFINITION

Sodium propanoate.

Content: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

Appearance: colourless crystals or white powder, slightly hygroscopic.

Solubility: freely soluble in water, sparingly soluble in alcohol, practically insoluble in methylene chloride.

IDENTIFICATION

First identification: A, D.

Second identification: B, C, D.

A. Infrared absorption spectrophotometry (2.2.24).

Comparison: Ph. Eur. reference spectrum of sodium propionate.

B. Dissolve 0.1 g in a mixture of 2 ml of *copper sulphate solution R* and 2 ml of *methylene chloride R*. Shake vigorously and allow to stand. Both the upper and the lower layer show a blue colour.

C. To 5 ml of solution S (see Tests) add 2 ml of 0.1 M *silver nitrate*. A white precipitate is formed.

D. Solution S gives reaction (a) of sodium (2.3.1).

TESTS

Solution S. Dissolve 10 g in *carbon dioxide-free water R* prepared from *distilled water R* and dilute to 100 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and colourless (2.2.2, *Method II*).

pH (2.2.3): 7.8 to 9.2.

Dilute 1 ml of solution S to 5 ml with *water R*.

Related substances. Liquid chromatography (2.2.29).

Test solution. Dissolve 0.250 g of the substance to be examined in *water R* and dilute to 100 ml with the same solvent.

Reference solution (a). Dissolve 10 mg of the substance to be examined and 10 mg of *sodium acetate R* in *water R* and dilute to 100 ml with the same solvent.

Reference solution (b). Dilute 1.0 ml of the test solution to 100 ml with *water R*.

Column:

- size: $l = 0.25$ m, $\varnothing = 4.6$ mm,
- stationary phase: octadecylsilyl silica gel for chromatography R (5 μ m).

Mobile phase: dilute 1 ml of *phosphoric acid R* to 1000 ml with *water R*.

Flow rate: 1 ml/min.

Detection: spectrophotometer at 210 nm.

Injection: 20 μ l.

System suitability: reference solution (a):

- resolution: minimum 5 between the peaks due to sodium acetate and sodium propionate.

Limits:

- any impurity: not more than 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent),
- total: not more than half the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent),
- disregard limit: 0.05 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

Readily oxidisable substances. In a ground-glass-stoppered conical flask introduce 10 g of the substance to be examined. Add 100 ml of *water R* and stir to dissolve. Add 25 ml of *sodium hypobromite solution R* and 10 ml of a 200 g/l solution of *sodium acetate R*, stopper the flask and allow to stand for 15 min. Add 10 ml of *potassium iodide solution R* and 20 ml of *hydrochloric acid R* while cooling. Titrate with 0.2 M *sodium thiosulphate*, adding 2 ml of *starch solution R* towards the end of the titration. Carry out a blank titration. The difference between the volumes used in the 2 titrations is not greater than 2.2 ml.

Iron (2.4.9): maximum 10 ppm.

10 ml of solution S complies with the limit test for iron.

Heavy metals (2.4.8): maximum 10 ppm.

12 ml of solution S complies with limit test A. Prepare the standard using *lead standard solution* (1 ppm Pb) R.

Loss on drying (2.2.32): maximum 0.5 per cent, determined on 1.000 g by heating in an oven at 100–105 °C for 3 h.

ASSAY

Dissolve 80.0 mg in 30 ml of *anhydrous acetic acid R*. Titrate with 0.1 M *perchloric acid*, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *perchloric acid* is equivalent to 9.61 mg of $C_3H_5NaO_2$.

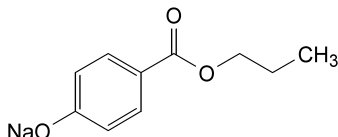
STORAGE

In an airtight container.

01/2005:1263

SODIUM PROPYL PARAHYDROXYBENZOATE

Propylis parahydroxybenzoas natricum

 $C_{10}H_{11}NaO_3$ M_r 202.2

DEFINITION

Sodium propyl parahydroxybenzoate contains not less than 99.0 per cent and not more than the equivalent of 104.0 per cent of sodium 4-(propoxycarbonyl)phenolate, calculated with reference to the anhydrous substance.

CHARACTERS

A white, crystalline powder, freely soluble in water, sparingly soluble in alcohol, practically insoluble in methylene chloride.

IDENTIFICATION

First identification: A, B, E.

Second identification: A, C, D, E.

- Dissolve 0.5 g in 50 ml of *water R*. Immediately add 5 ml of *hydrochloric acid R1*. Filter, and wash the precipitate with *water R*. Dry at 80 °C *in vacuo* for 2 h. The melting point (2.2.14) of the precipitate is 96 °C to 99 °C.
- Examine the precipitate obtained in identification test A by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *propyl parahydroxybenzoate CRS*.
- Examine the chromatograms obtained in the test for related substances. The principal spot in the chromatogram obtained with test solution (b) is similar in position and size to the principal spot in the chromatogram obtained with reference solution (c).
- To about 10 mg in a test-tube add 1 ml of *sodium carbonate solution R*, boil for 30 s and cool. Add 5 ml of *aminopyrazolone solution R* and 1 ml of *potassium ferricyanide solution R* and mix. An orange to red colour develops.
- To 1 ml of solution S (see Tests) add 1 ml of *water R*. The solution gives reaction (a) of sodium (2.3.1).

TESTS

Solution S. Dissolve 5.0 g in *carbon dioxide-free water R* prepared from *distilled water R*, and dilute to 50 ml with the same solvent.

Appearance of solution. Solution S, examined immediately after preparation, is clear (2.2.1) and not more intensely coloured than reference solution BY₆ (2.2.2, Method II).

pH (2.2.3). Dilute 1 ml of solution S to 100 ml with *carbon dioxide-free water R*. The pH of the solution is 9.5 to 10.5.

Related substances. Examine by thin-layer chromatography (2.2.27), using as the coating substance a suitable octadecylsilyl silica gel with a fluorescent indicator having an optimal intensity at 254 nm.

Test solution (a). Dissolve 0.100 g of the substance to be examined in 10 ml of *water R*. Immediately add 2 ml of *hydrochloric acid R* and shake with 50 ml of *ether R*.

Evaporate the upper layer to dryness and take up the residue with 10 ml of *acetone R*.

Test solution (b). Dilute 1 ml of test solution (a) to 10 ml with *acetone R*.

Reference solution (a). Dissolve 34.3 mg of *4-hydroxybenzoic acid R* in *acetone R* and dilute to 100 ml with the same solvent.

Reference solution (b). Dilute 0.5 ml of test solution (a) to 100 ml with *acetone R*.

Reference solution (c). Dissolve 10 mg of *propyl parahydroxybenzoate CRS* in *acetone R* and dilute to 10 ml with the same solvent.

Reference solution (d). Dissolve 10 mg of *ethyl parahydroxybenzoate CRS* in 1 ml of test solution (a) and dilute to 10 ml with *acetone R*.

Apply separately to the plate 5 µl of each solution. Develop over a path of 15 cm using a mixture of 1 volume of *glacial acetic acid R*, 30 volumes of *water R* and 70 volumes of *methanol R*. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. In the chromatogram obtained with test solution (a): any spot due to 4-hydroxybenzoic acid is not more intense than the spot in the chromatogram obtained with reference solution (a) (4 per cent); and any spot, apart from the principal spot and the spot due to 4-hydroxybenzoic acid, is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.5 per cent). The test is not valid unless the chromatogram obtained with reference solution (d) shows two clearly separated principal spots.

Chlorides (2.4.4). To 10 ml of solution S add 30 ml of *water R* and 1 ml of *nitric acid R* and dilute to 50 ml with *water R*. Shake and filter. Dilute 10 ml of the filtrate to 15 ml with *water R*. The solution complies with the limit test for chlorides (350 ppm). Prepare the standard using 14 ml of *chloride standard solution (5 ppm Cl) R* to which 1 ml of *water R* has been added.

Sulphates (2.4.13). To 25 ml of solution S add 5 ml of *distilled water R* and 10 ml of *hydrochloric acid R* and dilute to 50 ml with *distilled water R*. Shake and filter. Dilute 10 ml of the filtrate to 15 ml with *distilled water R*. The solution complies with the limit test for sulphates (300 ppm).

Heavy metals (2.4.8). 2.0 g complies with limit test C for heavy metals (10 ppm). Prepare the standard using 2 ml of *lead standard solution (10 ppm Pb) R*.

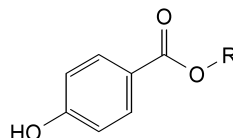
Water (2.5.12). Not more than 5.0 per cent, determined on 0.500 g by the semi-micro determination of water.

ASSAY

Dissolve 0.150 g in 50 ml of *anhydrous acetic acid R*. Titrate with 0.1 M *perchloric acid*, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *perchloric acid* is equivalent to 20.22 mg of $C_{10}H_{11}NaO_3$.

IMPURITIES

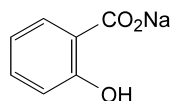


- R = H: 4-hydroxybenzoic acid,
- R = CH₃: methyl 4-hydroxybenzoate,
- R = CH₂-CH₃: ethyl 4-hydroxybenzoate,
- R = CH₂-CH₂-CH₂-CH₃: butyl 4-hydroxybenzoate.

01/2005:0413

SODIUM SALICYLATE

Natrii salicylas

 $C_7H_5NaO_3$ M_r 160.1**DEFINITION**

Sodium salicylate contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of sodium 2-hydroxybenzenecarboxylate, calculated with reference to the dried substance.

CHARACTERS

A white, crystalline powder or small, colourless crystals or shiny flakes, freely soluble in water, sparingly soluble in alcohol.

IDENTIFICATION

First identification: A, C.

Second identification: B, C.

- A. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *sodium salicylate CRS*.
- B. Solution S (see Tests) gives the reactions of salicylates (2.3.1).
- C. It gives reaction (b) of sodium (2.3.1).

TESTS

Solution S. Dissolve 5.0 g in *carbon dioxide-free water R* prepared from *distilled water R* and dilute to 50 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and not more intensely coloured than reference solution BY₆ (2.2.2, *Method II*).

Acidity. To 20 ml of solution S add 0.1 ml of *phenol red solution R*. The solution is yellow. Not more than 2.0 ml of 0.01 M *sodium hydroxide* is required to change the colour of the indicator to reddish-violet.

Chlorides (2.4.4). To 5 ml of solution S add 5 ml of *water R* and 10 ml of *dilute nitric acid R* and filter. 10 ml of the filtrate diluted to 15 ml with *water R* complies with the limit test for chlorides (200 ppm).

Sulphates (2.4.13). 2.5 ml of solution S diluted to 15 ml with *distilled water R* complies with the limit test for sulphates (600 ppm).

Heavy metals (2.4.8). Dissolve 1.6 g in 16 ml of a mixture of 5 volumes of *water R* and 10 volumes of *alcohol R*. 12 ml of the solution complies with limit test B for heavy metals (20 ppm). Prepare the standard using lead standard solution (2 ppm Pb) prepared by diluting *lead standard solution (100 ppm Pb) R* with a mixture of 5 volumes of *water R* and 10 volumes of *alcohol R*.

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.00 g by drying in an oven at 100 °C to 105 °C.

ASSAY

Dissolve 0.130 g in 30 ml of *anhydrous acetic acid R*. Titrate with 0.1 M *perchloric acid*, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *perchloric acid* is equivalent to 16.01 mg of $C_7H_5NaO_3$.

STORAGE

Store in an airtight container, protected from light.

01/2005:1677

SODIUM SELENITE PENTAHYDRATE

Natrii selenis pentahydricus

 $Na_2SeO_3 \cdot 5H_2O$ M_r 263.0**DEFINITION**

Content: 98.5 per cent to 101.5 per cent.

CHARACTERS

Appearance: white, crystalline powder, hygroscopic.

Solubility: freely soluble in water, practically insoluble in alcohol.

IDENTIFICATION

- A. Dissolve 50 mg in 5 ml of a mixture of equal volumes of *dilute hydrochloric acid R* and *water R* and heat to boiling. Add 0.05 g of *ascorbic acid R*; a red precipitate is formed which may become black.
- B. Dissolve 50 mg in a mixture of 1 ml of *dilute hydrochloric acid R* and 5 ml of *water R*. Add 1 ml of *barium chloride solution R1*; the solution remains clear.
- C. It gives reaction (a) of sodium (2.3.1).
- D. It complies with the limits of the assay.

TESTS

Solution S. Dissolve 5.0 g in *carbon dioxide-free water R* and dilute to 50.0 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and colourless (2.2.2, *Method II*).

Acidity or alkalinity. To 10 ml of solution S, add 0.1 ml of *thymolphthalein solution R*; the solution is blue. Not more than 0.3 ml of 1 M *hydrochloric acid* is required to change the colour of the indicator from blue to colourless.

Chlorides (2.4.4): maximum 50 ppm.

To 10 ml of solution S, add 2 ml of *nitric acid R* and dilute to 15 ml with *water R*.

Sulphates and selenates (2.4.13): maximum 300 ppm (determined as sulphates).

Dissolve 0.5 g in 10 ml of *distilled water R*. Add 0.5 ml of *hydrochloric acid R1* and dilute to 15 ml with *distilled water R*.

Iron: maximum 50 ppm.

To 2 ml of solution S, add 2 ml of a 200 g/l solution of *sulphosalicylic acid R*, 5 ml of *concentrated ammonia R* and dilute to 10 ml with *water R*. The solution is not more intensely coloured than a reference solution prepared in the same manner using 1 ml of *iron standard solution (10 ppm Fe) R*.

ASSAY

Dissolve 0.120 g in 50 ml of *water R*, add 7 ml of *glacial acetic acid R*, 25.0 ml of 0.1 M *sodium thiosulphate* and 0.5 g of *potassium iodide R*. Titrate immediately with 0.05 M *iodine solution* using *starch solution R* as indicator.

1 ml of 0.1 M *sodium thiosulphate* is equivalent to 6.575 mg of $Na_2SeO_3 \cdot 5H_2O$.

STORAGE

In an airtight container.

01/2005:0983

SODIUM STARCH GLYCOLATE (TYPE A)

Carboxymethylamylum natricum A

DEFINITION

Sodium starch glycolate (type A) is the sodium salt of a cross-linked partly *O*-carboxymethylated potato starch. It contains not less than 2.8 per cent and not more than 4.2 per cent of Na (*A*, 22.99), calculated with reference to the substance washed with alcohol (80 per cent *V/V*) and dried.

CHARACTERS

A white or almost white, fine, free-flowing powder, very hygroscopic, practically insoluble in methylene chloride. It gives a translucent suspension in water.

Examined under a microscope it is seen to consist of: granules, irregularly shaped, ovoid or pear-shaped, 30 µm to 100 µm in size, or rounded, 10 µm to 35 µm in size; compound granules consisting of two to four components occur occasionally; the granules have an eccentric hilum and clearly visible concentric striations; between crossed nicol prisms, the granules show a distinct black cross intersecting at the hilum; small crystals are visible at the surface of the granules. The granules show considerable swelling in contact with water.

IDENTIFICATION

- A. It complies with the test for pH (see Tests).
- B. Prepare with shaking and without heating a mixture of 4.0 g and 20 ml of *carbon dioxide-free water R*. The mixture has the appearance of a gel. Add 100 ml of *carbon dioxide-free water R* and shake. A suspension forms that settles after standing.
- C. To 5 ml of the suspension obtained in identification test B add 0.05 ml of *iodine solution R1*. A dark blue colour is produced.
- D. Solution S2 (see Tests) gives reaction (a) of sodium (2.3.1).

TESTS

Solution S1. Centrifuge the suspension obtained in identification test B at 2500 *g* for 10 min. Collect carefully the supernatant liquid.

Solution S2. Place 2.5 g in a silica or platinum crucible and add 2 ml of a 500 g/l solution of *sulphuric acid R*. Heat on a water-bath, then cautiously over a naked flame, raising the temperature progressively, and then incinerate in a muffle furnace at 600 ± 25 °C. Continue heating until all black particles have disappeared. Allow to cool, add a few drops of *dilute sulphuric acid R* and heat and incinerate as above. Allow to cool, add a few drops of *ammonium carbonate solution R*, evaporate to dryness and incinerate cautiously. Allow to cool and dissolve the residue in 50 ml of *water R*.

Appearance of solution S1. Solution S1 is clear (2.2.1) and colourless (2.2.2, *Method II*).

pH (2.2.3). The pH of solution S1 is 5.5 to 7.5.

Sodium glycolate. Carry out the test protected from light.

Test solution. Place 0.20 g of the substance to be examined in a beaker. Add 5 ml of *acetic acid R* and 5 ml of *water R*. Stir until dissolution is complete (about 10 min). Add 50 ml of *acetone R* and 1 g of *sodium chloride R*. Filter through a fast filter paper impregnated with *acetone R*, rinse the beaker and filter with *acetone R*. Combine the filtrate and washings and dilute to 100.0 ml with *acetone R*. Allow to stand for 24 h without shaking. Use the clear supernatant liquid.

Reference solution. Dissolve 0.310 g of *glycollic acid R*, previously dried *in vacuo* over *diphosphorus pentoxide R*, in *water R* and dilute to 500.0 ml with the same solvent. To 5.0 ml of this solution, add 5 ml of *acetic acid R* and allow to stand for about 30 min. Add 50 ml of *acetone R* and 1 g of *sodium chloride R* and dilute to 100.0 ml with *acetone R*.

Heat 2.0 ml of the test solution on a water-bath for 20 min. Cool to room temperature and add 20.0 ml of *2,7-dihydroxynaphthalene solution R*. Shake and heat in a water-bath for 20 min. Cool under running water, transfer to a volumetric flask and dilute to 25.0 ml with *sulphuric acid R*, maintaining the flask under running water. Within 10 min, measure the absorbance at 540 nm (2.2.25) using *water R* as the compensation liquid. The absorbance of the solution prepared with the test solution is not greater than that of a solution prepared at the same time and in the same manner with 2.0 ml of the reference solution (2.0 per cent).

Sodium chloride. Not more than 7.0 per cent. Shake 1.00 g with 20 ml of *alcohol (80 per cent V/V) R* for 10 min and filter. Repeat the operation four times. Dry the residue to constant mass at 100 °C and set aside for the assay. Combine the filtrates. Evaporate to dryness, take up the residue with *water R* and dilute to 25.0 ml with the same solvent. To 10.0 ml of the solution add 30 ml of *water R* and 5 ml of *dilute nitric acid R*. Titrate with 0.1 *M silver nitrate*, determining the end-point potentiometrically (2.2.20), using a silver indicator electrode.

1 ml of 0.1 *M silver nitrate* is equivalent to 5.844 mg of NaCl.

Iron (2.4.9). 10 ml of solution S2 complies with the limit test for iron (20 ppm).

Heavy metals (2.4.8). 1.0 g complies with limit test D for heavy metals (20 ppm). Prepare the standard using 2 ml of *lead standard solution (10 ppm Pb) R*.

Loss on drying (2.2.32). Not more than 10.0 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C for 4 h.

Microbial contamination. It complies with the test for *Escherichia coli* and *Salmonella* (2.6.13).

ASSAY

To 0.500 g of the dried and crushed residue obtained in the test for sodium chloride add 80 ml of *anhydrous acetic acid R* and heat under a reflux condenser for 2 h. Cool the solution to room temperature. Titrate with 0.1 *M perchloric acid*, determining the end-point potentiometrically (2.2.20). Carry out a blank test.

1 ml of 0.1 *M perchloric acid* is equivalent to 2.299 mg of Na.

STORAGE

Store in an airtight container, protected from light.

01/2005:0984

SODIUM STARCH GLYCOLATE (TYPE B)

Carboxymethylamylum natricum B

DEFINITION

Sodium starch glycolate (type B) is the sodium salt of a cross-linked partly *O*-carboxymethylated potato starch. It contains not less than 2.0 per cent and not more than 3.4 per cent of Na (*A*, 22.99), calculated with reference to the substance washed with alcohol (80 per cent *V/V*) and dried.

CHARACTERS

A white or almost white, fine, free-flowing powder, very hygroscopic, practically insoluble in methylene chloride. It gives a translucent suspension in water.

Examined under a microscope it is seen to consist of: granules, irregularly shaped, ovoid or pear shaped, 30 µm to 100 µm in size, or rounded, 10 µm to 35 µm in size; compound granules consisting of two to four components occur occasionally; the granules have an eccentric hilum and clearly visible concentric striations; between crossed nicol prisms, the granules show a distinct black cross intersecting at the hilum; small crystals are visible at the surface of the granules. The granules show considerable swelling in contact with water.

IDENTIFICATION

- A. It complies with the test for pH (see Tests).
- B. Prepare with shaking and without heating a mixture of 4.0 g and 20 ml of *carbon dioxide-free water R*. The mixture has the appearance of a gel. Add 100 ml of *carbon dioxide-free water R* and shake. A suspension forms that settles after standing.
- C. To 5 ml of the suspension obtained in identification test B add 0.05 ml of *iodine solution R1*. A dark blue colour is produced.
- D. Solution S2 (see Tests) gives reaction (a) of sodium (2.3.1).

TESTS

Solution S1. Centrifuge the suspension obtained in identification test B at 2500 *g* for 10 min. Collect carefully the supernatant liquid.

Solution S2. Place 2.5 g in a silica or platinum crucible and add 2 ml of a 500 g/l solution of *sulphuric acid R*. Heat on a water-bath, then cautiously over a naked flame, raising the temperature progressively, and then incinerate in a muffle furnace at 600 ± 25 °C. Continue heating until all black particles have disappeared. Allow to cool, add a few drops of *dilute sulphuric acid R* and heat and incinerate as above. Allow to cool, add a few drops of *ammonium carbonate solution R*, evaporate to dryness and incinerate cautiously. Allow to cool and dissolve the residue in 50 ml of *water R*.

Appearance of solution S1. Solution S1 is clear (2.2.1) and colourless (2.2.2, *Method II*).

pH (2.2.3). The pH of solution S1 is 3.0 to 5.0.

Sodium glycolate. Carry out the test protected from light.

Test solution. Place 0.20 g of the substance to be examined in a beaker. Add 5 ml of *acetic acid R* and 5 ml of *water R*. Stir until dissolution is complete (about 10 min). Add 50 ml of *acetone R* and 1 g of *sodium chloride R*. Filter through a fast filter paper impregnated with *acetone R*, rinse the beaker and filter with *acetone R*. Combine the filtrate and washings

and dilute to 100.0 ml with *acetone R*. Allow to stand for 24 h without shaking. Use the clear supernatant liquid.

Reference solution. Dissolve 0.310 g of *glycollic acid R*, previously dried *in vacuo* over *diphosphorus pentoxide R*, in *water R* and dilute to 500.0 ml with the same solvent. To 5.0 ml of this solution, add 5 ml of *acetic acid R* and allow to stand for about 30 min. Add 50 ml of *acetone R* and 1 g of *sodium chloride R* and dilute to 100.0 ml with *acetone R*.

Heat 2.0 ml of the test solution on a water-bath for 20 min. Cool to room temperature and add 20.0 ml of *2,7-dihydroxynaphthalene solution R*. Shake and heat in a water-bath for 20 min. Cool under running water, transfer quantitatively to a volumetric flask and dilute to 25.0 ml with *sulphuric acid R*, maintaining the flasks under running water. Within 10 min, measure the absorbance at 540 nm (2.2.25) using *water R* as the compensation liquid. The absorbance of the solution prepared with the test solution is not greater than that of a solution prepared at the same time and in the same manner with 2.0 ml of the reference solution (2.0 per cent).

Sodium chloride. Not more than 7.0 per cent. Shake 1.00 g with 20 ml of *alcohol (80 per cent V/V) R* for 10 min and filter. Repeat the operation four times. Dry the residue to constant mass at 100 °C and set aside for the assay. Combine the filtrates. Evaporate to dryness, take up the residue with *water R* and dilute to 25.0 ml with the same solvent. To 10.0 ml of the solution add 30 ml of *water R* and 5 ml of *dilute nitric acid R*. Titrate with 0.1 *M silver nitrate*, determining the end-point potentiometrically (2.2.20) using a silver indicator electrode.

1 ml of 0.1 *M silver nitrate* is equivalent to 5.844 mg of NaCl.

Iron (2.4.9). 10 ml of solution S2 complies with the limit test for iron (20 ppm).

Heavy metals (2.4.8). 1.0 g complies with limit test D for heavy metals (20 ppm). Prepare the standard using 2 ml of *lead standard solution (10 ppm Pb) R*.

Loss on drying (2.2.32). Not more than 10.0 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C for 4 h.

Microbial contamination. It complies with the test for *Escherichia coli* and *Salmonella* (2.6.13).

ASSAY

To 0.500 g of the dried and crushed residue obtained in the test for sodium chloride add 80 ml of *anhydrous acetic acid R* and heat under a reflux condenser for 2 h. Cool the solution to room temperature. Titrate with 0.1 *M perchloric acid*, determining the end-point potentiometrically (2.2.20). Carry out a blank test.

1 ml of 0.1 *M perchloric acid* is equivalent to 2.299 mg of Na.

STORAGE

Store in an airtight container, protected from light.

01/2005:1566

SODIUM STARCH GLYCOLATE (TYPE C)

Carboxymethylamylum natricum C

DEFINITION

Sodium starch glycolate (type C) is the sodium salt of a cross-linked by physical dehydration, partly *O*-carboxymethylated starch. It contains not less than

2.8 per cent and not more than 5.0 per cent of Na (A_r 22.99), calculated with reference to the substance washed with alcohol (80 per cent V/V) and dried.

CHARACTERS

A white or almost white, fine, free-flowing powder, very hygroscopic, soluble in water, practically insoluble in methylene chloride. It gives a translucent gel-like product in water.

Examined under a microscope it is seen to consist of granules, irregularly shaped, ovoid or pear-shaped, 30 µm to 100 µm in size, or rounded, 10 µm to 35 µm in size; compound granules consisting of two to four components occur occasionally; the granules have an eccentric hilum and clearly visible concentric striations; between crossed nicol prisms, the granules show a distinct black cross intersecting at the hilum; small crystals are visible at the surface of the granules. The granules show considerable swelling in contact with water.

IDENTIFICATION

- A. It complies with the test for pH (see Tests).
- B. Mix with shaking and without heating 4.0 g and 20 ml of *carbon dioxide-free water R*. The mixture has the appearance of a gel. Add 100 ml of *carbon dioxide-free water R* and shake: the gel remains stable (difference from types A and B). Keep the gel for the tests for appearance of gel and pH.
- C. To 5 ml of the gel obtained in identification test B add 0.05 ml of *iodine solution RI*. A dark blue colour is produced.
- D. Solution S (see Tests) gives reaction (a) of sodium (2.3.1).

TESTS

Solution S. Place 2.5 g in a silica or platinum crucible and add 2 ml of a 500 g/l solution of *sulphuric acid R*. Heat on a water-bath, then cautiously over a naked flame, raising the temperature progressively, and then incinerate in a muffle furnace at 600 ± 25 °C. Continue heating until all black particles have disappeared. Allow to cool, add a few drops of *sulphuric acid R* and heat and incinerate as described above. Allow to cool, add a few drops of *ammonium carbonate solution R*, evaporate to dryness and incinerate cautiously. Allow to cool and dissolve the residue in 50 ml of *water R*.

Appearance of gel. The gel prepared under identification test B is colourless (2.2.2, *Method II*).

pH (2.2.3). The pH of the gel prepared under identification test B is 5.5 to 7.5.

Sodium glycolate. Carry out the test protected from light.

Test solution. Place 0.20 g of the substance to be examined in a beaker. Add 5 ml of *acetic acid R* and 5 ml of *water R*. Stir until dissolution is complete (about 10 min). Add 50 ml of *acetone R* and 1 g of *sodium chloride R*. Filter through a fast filter paper impregnated with *acetone R*, rinse the beaker and filter with *acetone R*. Combine the filtrate and washings and dilute to 100.0 ml with *acetone R*. Allow to stand for 24 h without shaking. Use the clear supernatant liquid.

Reference solution. Dissolve 0.310 g of *glycollic acid R*, previously dried *in vacuo* over *diphosphorus pentoxide R*, in *water R* and dilute to 500.0 ml with the same solvent. To 5.0 ml of this solution, add 5 ml of *acetic acid R* and allow to stand for about 30 min. Add 50 ml of *acetone R* and 1 g of *sodium chloride R* and dilute to 100.0 ml with *acetone R*.

Heat 2.0 ml of the test solution on a water-bath for 20 min. Cool to room temperature and add 20.0 ml of *2,7-dihydroxynaphthalene solution R*. Shake and heat on a water-bath for 20 min. Cool under running water, transfer

to a volumetric flask and dilute to 25.0 ml with *sulphuric acid R*, maintaining the flask under running water. Within 10 min, measure the absorbance at 540 nm (2.2.25) using *water R* as the compensation liquid. The absorbance of the solution prepared with the test solution is not greater than that of a solution prepared at the same time and in the same manner with 2.0 ml of the reference solution (2.0 per cent).

Sodium chloride. Not more than 1 per cent. Shake 1.00 g with 20 ml of *alcohol (80 per cent V/V) R* for 10 min and filter. Repeat the operation four times. Dry the residue to constant mass at 100 °C and set aside for the assay. Combine the filtrates. Evaporate to dryness, take up the residue with *water R* and dilute to 25.0 ml with the same solvent. To 10.0 ml of the solution add 30 ml of *water R* and 5 ml of *dilute nitric acid R*. Titrate with 0.1 M *silver nitrate*, determining the end-point potentiometrically (2.2.20), using a silver indicator electrode.

1 ml of 0.1 M *silver nitrate* is equivalent to 5.844 mg of NaCl.

Iron (2.4.9). 10 ml of solution S complies with the limit test for iron (20 ppm).

Heavy metals (2.4.8). 1.0 g complies with limit test D for heavy metals (20 ppm). Prepare the standard using 2 ml of *lead standard solution (10 ppm Pb) R*.

Loss on drying (2.2.32). Not more than 7.0 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C for 4 h.

Microbial contamination. It complies with the test for *Escherichia coli* and *Salmonella* (2.6.13).

ASSAY

To 0.500 g of the dried and crushed residue obtained in the test for sodium chloride add 80 ml of *anhydrous acetic acid R* and heat under a reflux condenser for 2 h. Cool the solution to room temperature. Titrate with 0.1 M *perchloric acid*, determining the end-point potentiometrically (2.2.20). Carry out a blank test.

1 ml of 0.1 M *perchloric acid* is equivalent to 2.299 mg of Na.

STORAGE

Store in an airtight container, protected from light.

01/2005:2058

SODIUM STEARATE

Natrii stearas

DEFINITION

Mixture of sodium salts of different fatty acids consisting mainly of stearic acid [$C_{17}H_{35}COONa$; M_r 306.5] and palmitic acid [$C_{15}H_{31}COONa$; M_r 278.4].

Content:

- sodium: 7.4 per cent to 8.5 per cent (A_r 22.99) (dried substance),
- stearic acid in the fatty acid fraction: minimum 40 per cent,
- sum of stearic acid and palmitic acid in the fatty acid fraction: minimum 90 per cent.

CHARACTERS

Appearance: white or yellowish, fine powder, with a greasy touch.

Solubility: slightly soluble in water and in alcohol.

IDENTIFICATION

First identification: C, D.

Second identification: A, B, D.

- A. Freezing point (2.2.18): minimum 53 °C for the residue obtained in the preparation of solution S (see Tests).
- B. Acid value (2.5.1): 195 to 210, determined on 0.200 g of the residue obtained in the preparation of solution S dissolved in 25 ml of the prescribed mixture of solvents.
- C. Examine the chromatograms obtained in the assay of stearic acid and palmitic acid.

Results: the 2 principal peaks in the chromatogram obtained with the test solution are similar in retention time and size to the 2 principal peaks in the chromatogram obtained with the reference solution.

- D. Solution S gives reaction (b) of sodium (2.3.1).

TESTS

Solution S. To 10.0 g add 100 ml of *peroxide-free ether R* and 80 ml of *acetic acid R*. Boil under a reflux condenser until dissolution is complete. Allow to cool. In a separating funnel, separate the aqueous layer and shake the ether layer with 2 quantities, each of 8 ml, of *acetic acid R*. Combine the aqueous layers, wash with 30 ml of *peroxide-free ether R* and dilute to 100 ml with *distilled water R* (solution S). Evaporate the ether layers to dryness on a water-bath and dry the residue at 100-105 °C.

Acidity or alkalinity. Dissolve 2.0 g in 50 ml of previously neutralised *alcohol R* and add 3 drops of *phenolphthalein solution R*; the solution is colourless. Not less than 0.60 ml and not more than 0.85 ml of 0.1 M sodium hydroxide is required to change the colour of the indicator.

Chlorides (2.4.4): maximum 0.1 per cent.

Dilute 0.5 ml of solution S to 15 ml with *water R*.

Sulphates (2.4.13): maximum 0.3 per cent.

Dilute 0.5 ml of solution S to 15 ml with *distilled water R*.

Nickel: maximum 5 ppm.

Atomic absorption spectrometry (2.2.23, *Method II*).

Test solution. Place 50.0 mg of the substance to be examined in a polytetrafluoroethylene digestion flask and add 0.5 ml of a mixture of 1 volume of *heavy metal-free hydrochloric acid R* and 5 volumes of *heavy metal-free nitric acid R*. Allow to digest at 170 °C for 5 h. Allow to cool. Dissolve the residue in *water R* and dilute to 5.0 ml with the same solvent.

Reference solutions. Prepare the reference solutions using *nickel standard solution (10 ppm Ni) R*, diluted as necessary with *water R*.

Source: nickel hollow-cathode lamp.

Wavelength: 232.0 nm.

Atomisation device: air-acetylene flame.

Loss on drying (2.2.32): maximum 5.0 per cent.

In a weighing glass introduce 1.0 g of previously washed *sand R*, dry at 100-105 °C and weigh. Add 0.500 g of the substance to be examined and 10 ml of *alcohol R*. Evaporate the alcohol at 80 °C and dry the residue at 100-105 °C for 4 h.

Microbial contamination. Total viable aerobic count (2.6.12) not more than 10³ micro-organisms per gram, determined by plate count. It complies with the test for *Escherichia coli* (2.6.13).

ASSAY

Sodium. Dissolve 0.250 g with gentle heating in a mixture of 5 ml of *acetic anhydride R* and 20 ml of *anhydrous acetic acid R*. Cool and add 20 ml of *dioxan R*. Titrate with 0.1 M *perchloric acid*, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *perchloric acid* is equivalent to 2.299 mg of Na.

Stearic acid and palmitic acid. Gas chromatography (2.2.28): use the normalisation procedure.

Test solution. In a conical flask fitted with a reflux condenser, dissolve 0.10 g of the substance to be examined in 5 ml of *boron trifluoride-methanol solution R*. Boil under a reflux condenser for 10 min. Add 4 ml of *heptane R* through the condenser and boil again under a reflux condenser for 10 min. Allow to cool. Add 20 ml of *saturated sodium chloride solution R*. Shake and allow the layers to separate. Remove about 2 ml of the organic layer and dry over 0.2 g of *anhydrous sodium sulphate R*. Dilute 1.0 ml of the solution to 100.0 ml with *heptane R*.

Reference solution. Prepare the reference solution in the same manner as the test solution using 50.0 mg of *palmitic acid CRS* and 50.0 mg of *stearic acid CRS* instead of the substance to be examined.

Column:

- **material:** fused silica,
- **size:** *l* = 30 m, Ø = 0.32 mm,
- **stationary phase:** *macrogol 20 000 R* (film thickness 0.5 µm).

Carrier gas: *helium for chromatography R*.

Flow rate: 2.4 ml/min.

Temperature:

	Time (min)	Temperature (°C)
Column	0 - 2	70
	2 - 36	70 → 240
	36 - 41	240
Injection port		220
Detector		260

Detection: flame ionisation.

Injection: 1 µl.

Relative retention with reference to methyl stearate (retention time = about 40 min): methyl palmitate = about 0.88.

System suitability: reference solution:

- **resolution:** minimum 5.0 between the peaks due to methyl stearate and methyl palmitate.

Calculate the content of stearic acid and palmitic acid.

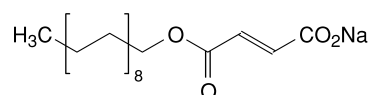
STORAGE

In an airtight container, protected from light.

01/2005:1567

SODIUM STEARYL FUMARATE

Natrii stearylis fumaras



C₂₂H₃₉NaO₄

*M*_r 390.5

DEFINITION

Sodium octadecyl (*E*)-butenedioate.

Content: 99.0 per cent to 101.5 per cent (anhydrous substance).

CHARACTERS

Appearance: white or almost white, fine powder with agglomerates of flat, circular particles.

Solubility: practically insoluble in water, slightly soluble in methanol, practically insoluble in acetone and in ethanol.

IDENTIFICATION

Infrared absorption spectrophotometry (2.2.24).

Comparison: sodium stearyl fumarate CRS.

TESTS

Related substances. Gas chromatography (2.2.28): use the normalisation procedure.

Silylation solution. To 2 ml of *N,O*-bis(trimethylsilyl)trifluoroacetamide *R* add 0.02 ml of chlorotrimethylsilane *R* and mix.

Test solution. Introduce 15.0 mg of the substance to be examined in a vial with a screw cap and add 1 ml of the silylation solution. Seal the vial and heat at about 70 °C for 1 h. After the reaction a precipitate remains in the vial; filter the solution through a nylon filter (pore size 0.45 µm).

Reference solution. Introduce 1.0 mg of sodium stearyl maleate CRS and 1.0 mg of sodium stearyl fumarate CRS into a vial with a screw cap and add 1 ml of the silylation solution. Seal the vial and heat at about 70 °C for 1 h.

Column:

- *material*: fused silica,
- *size*: *l* = 15 m, Ø = 0.53 mm,
- *stationary phase*: poly(dimethyl)siloxane *R* (film thickness 0.15 µm).

Carrier gas: helium for chromatography *R*.

Flow rate: 2 ml/min.

Split ratio: 1:25.

Temperature:

	Time (min)	Temperature (°C)
	0 - 1	180
Column	1 - 21	180 → 320
	21 - 26	320
Injection port		250
Detector		320

Detection: flame ionisation.

Injection: 2 µl.

Relative retention with reference to stearyl trimethylsilyl fumarate (retention time = about 9 min): stearyl alcohol = 0.30; stearyl trimethylsilyl ether = 0.35; palmityl trimethylsilyl fumarate = 0.80; heptadecyl trimethylsilyl fumarate = 0.85; stearyl trimethylsilyl maleate = 0.90; nonadecyl trimethylsilyl fumarate = 1.05; eicos-11-enyl trimethylsilyl fumarate = 1.15; distearyl fumarate = 2.25.

System suitability:

- *resolution*: minimum 1.5 between the peaks in the chromatogram obtained with the reference solution.

Limits:

- *any impurity*: maximum 0.5 per cent,
- *total*: maximum 5.0 per cent.

Water (2.5.12): maximum 5.0 per cent, determined on 0.250 g.

ASSAY

Dissolve 0.250 g, accurately weighed, in 10 ml of methylene chloride *R* and add 30 ml of anhydrous acetic acid *R*. Titrate with 0.1 *M* perchloric acid, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 *M* perchloric acid is equivalent to 39.05 mg of C₂₂H₃₉NaO₄.

01/2005:0099

SODIUM SULPHATE, ANHYDROUS

Natrii sulfas anhydricus

Na₂SO₄*M_r* 142.0

DEFINITION

Content: 98.5 per cent to 101.0 per cent (dried substance).

CHARACTERS

Appearance: white powder, hygroscopic.

Solubility: freely soluble in water.

IDENTIFICATION

- A. It gives the reactions of sulphates (2.3.1).
- B. It gives the reactions of sodium (2.3.1).
- C. It complies with the test for loss on drying (see Tests).

TESTS

Solution S. Dissolve 2.2 g in carbon dioxide-free water *R* prepared from distilled water *R* and dilute to 100 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and colourless (2.2.2, Method II).

Acidity or alkalinity. To 10 ml of solution S add 0.1 ml of bromothymol blue solution *R1*. Not more than 0.5 ml of 0.01 *M* hydrochloric acid or 0.01 *M* sodium hydroxide is required to change the colour of the indicator.

Chlorides (2.4.4): maximum 450 ppm.

5 ml of solution S diluted to 15 ml with water *R* complies with the limit test for chlorides.

Calcium (2.4.3): maximum 450 ppm, if intended for use in the manufacture of parenteral dosage forms.

10 ml of solution S diluted to 15 ml with distilled water *R* complies with the limit test for calcium.

Iron (2.4.9): maximum 90 ppm, if intended for use in the manufacture of parenteral dosage forms.

5 ml of solution S diluted to 10 ml with water *R* complies with the limit test for iron.

Magnesium: maximum 200 ppm, if intended for use in the manufacture of parenteral dosage forms.

To 10 ml of solution S add 1 ml of glycerol (85 per cent) *R*, 0.15 ml of titan yellow solution *R*, 0.25 ml of ammonium oxalate solution *R* and 5 ml of dilute sodium hydroxide solution *R* and shake. Any pink colour in the test solution is not more intense than that in a standard prepared at the same time in the same manner using a mixture of 5 ml of magnesium standard solution (10 ppm Mg) *R* and 5 ml of water *R*.

Heavy metals (2.4.8): maximum 45 ppm.

12 ml of solution S complies with limit test A. Prepare the standard using lead standard solution (1 ppm Pb) *R*.

Loss on drying (2.2.32): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 130 °C.

ASSAY

Dissolve 0.100 g in 40 ml of *water R*. Add a mixture of 0.2 ml of 0.1 M hydrochloric acid and 80 ml of *methanol R*. Carry out a potentiometric titration (2.2.20), using 0.1 M lead nitrate and as indicator electrode a lead-selective electrode and as reference electrode a silver-silver chloride electrode.

1 ml of 0.1 M lead nitrate is equivalent to 14.20 mg of Na₂SO₄.

STORAGE

Store in an airtight container.

LABELLING

The label states, where applicable, that the substance is suitable for use in the manufacture of parenteral dosage forms.

01/2005:0100

SODIUM SULPHATE DECAHYDRATE

Natrii sulfas decahydricus

Na₂SO₄·10H₂O

*M*_r 322.2

DEFINITION

Content: 98.5 per cent to 101.0 per cent (dried substance).

CHARACTERS

Appearance: white, crystalline powder or colourless, transparent crystals.

Solubility: freely soluble in water, practically insoluble in alcohol. It partly dissolves in its own water of crystallisation at about 33 °C.

IDENTIFICATION

- It gives the reactions of sulphates (2.3.1).
- It gives the reactions of sodium (2.3.1).
- It complies with the test for loss on drying (see Tests).

TESTS

Solution S. Dissolve 5.0 g in *carbon dioxide-free water R* prepared from *distilled water R* and dilute to 100 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and colourless (2.2.2, *Method II*).

Acidity or alkalinity. To 10 ml of solution S add 0.1 ml of *bromothymol blue solution R1*. Not more than 0.5 ml of 0.01 M hydrochloric acid or 0.01 M sodium hydroxide is required to change the colour of the indicator.

Chlorides (2.4.4): maximum 200 ppm.

5 ml of solution S diluted to 15 ml with *water R* complies with the limit test for chlorides.

Calcium (2.4.3): maximum 200 ppm, if intended for use in the manufacture of parenteral dosage forms.

10 ml of solution S diluted to 15 ml with *distilled water R* complies with the limit test for calcium.

Iron (2.4.9): maximum 40 ppm, if intended for use in the manufacture of parenteral dosage forms.

5 ml of solution S diluted to 10 ml with *water R* complies with the limit test for iron.

Magnesium: maximum 100 ppm, if intended for use in the manufacture of parenteral dosage forms.

To 10 ml of solution S add 1 ml of *glycerol (85 per cent) R*, 0.15 ml of *titan yellow solution R*, 0.25 ml of *ammonium oxalate solution R* and 5 ml of *dilute sodium hydroxide solution R* and shake. Any pink colour in the test solution is not more intense than that in a standard prepared at the same time in the same manner using a mixture of 5 ml of *magnesium standard solution (10 ppm Mg) R* and 5 ml of *water R*.

Heavy metals (2.4.8): maximum 20 ppm.

12 ml of solution S complies with limit test A. Prepare the standard using *lead standard solution (1 ppm Pb) R*.

Loss on drying (2.2.32): 52.0 per cent to 57.0 per cent, determined on 1.000 g by drying at 30 °C for 1 h, then at 130 °C.

ASSAY

Dissolve 0.250 g in 40 ml of *water R*. Add a mixture of 0.2 ml of 0.1 M hydrochloric acid and 80 ml of *methanol R*. Carry out a potentiometric titration (2.2.20), using 0.1 M lead nitrate and as indicator electrode a lead-selective electrode and as reference electrode a silver-silver chloride electrode.

1 ml of 0.1 M lead nitrate is equivalent to 14.20 mg of Na₂SO₄.

LABELLING

The label states, where applicable, that the substance is suitable for use in the manufacture of parenteral dosage forms.

01/2005:0775

SODIUM SULPHITE, ANHYDROUS

Natrii sulfis anhydricus

Na₂SO₃

*M*_r 126.0

DEFINITION

Anhydrous sodium sulphite contains not less than 95.0 per cent and not more than the equivalent of 100.5 per cent of Na₂SO₃.

CHARACTERS

A white powder, freely soluble in water, very slightly soluble in alcohol.

IDENTIFICATION

- Solution S (see Tests) is slightly alkaline (2.2.4).
- To 5 ml of solution S, add 0.5 ml of 0.05 M iodine. The solution is colourless and gives reaction (a) of sulphates (2.3.1).
- Solution S gives reaction (a) of sodium (2.3.1).
- It complies with the limits of the assay.

TESTS

Solution S. Dissolve 5 g in *water R* and dilute to 100 ml with the same solvent.

Solution S1. To 10.0 g add 25 ml of *water R*. Shake until mostly dissolved and carefully and progressively add 15 ml of *hydrochloric acid R*. Heat to boiling. Cool and dilute to 100.0 ml with *water R*.

Appearance of solution. Solution S is clear (2.2.1) and colourless (2.2.2, *Method I*).

Thiosulphates. To 2.00 g add 100 ml of *water R*. Shake and add 10 ml of *formaldehyde solution R* and 10 ml of *acetic acid R*. Allow to stand for 5 min. Add 0.5 ml of *starch solution R* and titrate with 0.05 M *iodine*. Carry out a blank titration. The difference between the volumes used in the titrations is not more than 0.15 ml (0.1 per cent).

Iron (2.4.9). 10 ml of solution S1 complies with the limit test for iron (10 ppm).

Selenium. To 3.0 g add 10 ml of *formaldehyde solution R* and carefully and progressively add 2 ml of *hydrochloric acid R*. Heat on a water-bath for 20 min. Any pink colour in the solution is not more intense than that of a standard prepared at the same time and in the same manner using 1.0 g of the substance to be examined to which has been added 0.2 ml of *selenium standard solution (100 ppm Se) R* (10 ppm).

Zinc. Not more than 25 ppm of Zn, determined by atomic absorption spectrometry (2.2.23, *Method I*).

Test solution. Dilute 2.0 ml of solution S1 to 10.0 ml with *water R*.

Reference solutions. Prepare the reference solutions using *zinc standard solution (100 ppm Zn) R*, diluted as necessary with *water R*.

Measure the absorbance at 213.9 nm using a zinc hollow-cathode lamp as source of radiation and an air-acetylene flame.

Heavy metals (2.4.8). 12 ml of solution S1 complies with limit test A for heavy metals (10 ppm). Prepare the standard using *lead standard solution (1 ppm Pb) R*.

ASSAY

Introduce 0.250 g into a 500 ml conical flask containing 50.0 ml of 0.05 M *iodine*. Shake until completely dissolved. Add 1 ml of *starch solution R* and titrate the excess of iodine with 0.1 M *sodium thiosulphate*. Carry out a blank titration. 1 ml of 0.05 M *iodine* is equivalent to 6.30 mg of Na₂SO₃.

STORAGE

Store in an airtight container.

01/2005:0776

SODIUM SULPHITE HEPTAHYDRATE

Natrii sulfis heptahydricus

Na₂SO₃·7H₂O

*M*_r 252.2

DEFINITION

Sodium sulphite heptahydrate contains not less than 48.0 per cent and not more than the equivalent of 52.5 per cent of Na₂SO₃.

CHARACTERS

Colourless crystals, freely soluble in water, very slightly soluble in alcohol.

IDENTIFICATION

- Solution S (see Tests) is slightly alkaline (2.2.4).
- To 5 ml of solution S, add 0.5 ml of 0.05 M *iodine*. The solution is colourless and gives reaction (a) of sulphates (2.3.1).
- Solution S gives reaction (a) of sodium (2.3.1).
- It complies with the limits of the assay.

TESTS

Solution S. Dissolve 10 g in *water R* and dilute to 100 ml with the same solvent.

Solution S1. To 20.0 g add 25 ml of *water R*. Shake until mostly dissolved and carefully and progressively add 15 ml of *hydrochloric acid R*. Heat to boiling. Cool and dilute to 100.0 ml with *water R*.

Appearance of solution. Solution S is clear (2.2.1) and colourless (2.2.2, *Method I*).

Thiosulphates. To 4.00 g add 100 ml of *water R*. Shake to dissolve and add 10 ml of *formaldehyde solution R* and 10 ml of *acetic acid R*. Allow to stand for 5 min. Add 0.5 ml of *starch solution R* and titrate with 0.05 M *iodine*. Carry out a blank titration. The difference between the volumes used in the titrations is not more than 0.15 ml (0.05 per cent).

Iron (2.4.9). 10 ml of solution S1 complies with the limit test for iron (5 ppm).

Selenium. To 6.0 g add 10 ml of *formaldehyde solution R* and carefully and progressively add 2 ml of *hydrochloric acid R*. Heat on a water-bath for 20 min. Any pink colour in the solution is not more intense than that of a standard prepared at the same time and in the same manner using 2.0 g of the substance to be examined to which has been added 0.2 ml of *selenium standard solution (100 ppm Se) R* (5 ppm).

Zinc. Not more than 12 ppm of Zn, determined by atomic absorption spectrometry (2.2.23, *Method I*).

Test solution. Dilute 2.0 ml of solution S1 to 10.0 ml with *water R*.

Reference solutions. Prepare the reference solutions using *zinc standard solution (100 ppm Zn) R*, diluted as necessary with *water R*.

Measure the absorbance at 213.9 nm using a zinc hollow-cathode lamp as source of radiation and an air-acetylene flame.

Heavy metals (2.4.8). 12 ml of solution S1 complies with limit test A for heavy metals (5 ppm). Prepare the standard using *lead standard solution (1 ppm Pb) R*.

ASSAY

Introduce 0.500 g into a 500 ml conical flask containing 50.0 ml of 0.05 M *iodine*. Shake until completely dissolved. Add 1 ml of *starch solution R* and titrate the excess of iodine with 0.1 M *sodium thiosulphate*. Carry out a blank titration. 1 ml of 0.05 M *iodine* is equivalent to 6.30 mg of Na₂SO₃.

01/2005:0414

SODIUM THIOSULPHATE

Natrii thiosulfas

Na₂S₂O₃·5H₂O

*M*_r 248.2

DEFINITION

Sodium thiosulphate contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of Na₂S₂O₃·5H₂O.

CHARACTERS

Transparent, colourless crystals, efflorescent in dry air, very soluble in water, practically insoluble in alcohol. It dissolves in its water of crystallisation at about 49 °C.

IDENTIFICATION

- It decolourises *iodinated potassium iodide solution R*.

- B. To 0.5 ml of solution S (see Tests) add 0.5 ml of *water R* and 2 ml of *silver nitrate solution R2*. A white precipitate is formed which rapidly becomes yellowish and then black.
- C. To 2.5 ml of solution S add 2.5 ml of *water R* and 1 ml of *hydrochloric acid R*. A precipitate of sulphur is formed and gas is evolved which gives a blue colour to *starch iodate paper R*.
- D. 1 ml of solution S gives reaction (a) of sodium (2.3.1).

TESTS

Solution S. Dissolve 10.0 g in *carbon dioxide-free water R* prepared from *distilled water R* and dilute to 100 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and colourless (2.2.2, *Method II*).

pH (2.2.3). The pH of solution S is 6.0 to 8.4.

Sulphates and sulphites. Dilute 2.5 ml of solution S to 10 ml with *distilled water R*. To 3 ml of this solution first add 2 ml of *iodinated potassium iodide solution R* and continue the addition dropwise until a very faint persistent yellow colour appears. Dilute to 15 ml with *distilled water R*. The solution complies with the limit test for sulphates (2.4.13) (0.2 per cent).

Sulphides. To 10 ml of solution S add 0.05 ml of a freshly prepared 50 g/l solution of *sodium nitroprusside R*. The solution does not become violet.

Heavy metals. To 10 ml of solution S add 0.05 ml of *sodium sulphide solution R*. After 2 min, any brown colour in the solution is not more intense than that in a standard prepared at the same time and in the same manner using 10 ml of *lead standard solution (1 ppm Pb) R* (10 ppm).

ASSAY

Dissolve 0.500 g in 20 ml of *water R* and titrate with 0.05 M *iodine*, using 1 ml of *starch solution R*, added towards the end of the titration, as indicator.

1 ml of 0.05 M *iodine* is equivalent to 24.82 mg of $\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$.

STORAGE

Store in an airtight container.

IDENTIFICATION

- A. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *sodium valproate CRS*. If the spectra obtained in the solid state for the substance to be examined and the reference substance show differences, record further spectra using discs prepared by placing 50 µl of a 100 g/l solution in *methanol R* on a disc of *potassium bromide R* and evaporating the solvent *in vacuo*. Use the discs immediately.
- B. Examine the chromatograms obtained in the test for related substances. The retention time of the principal peak in the chromatogram obtained with test solution (b) corresponds to that of the principal peak in the chromatogram obtained with reference solution (b).
- C. 2 ml of solution S (see Tests) gives reaction (a) of sodium (2.3.1).

TESTS

Solution S. Dissolve 1.25 g in 20 ml of *distilled water R* in a separating funnel, add 5 ml of *dilute nitric acid R* and shake. Allow the mixture to stand for 12 h. Use the lower layer.

Appearance of solution. Dissolve 2.0 g in *water R* and dilute to 10 ml with the same solvent. The solution is not more opalescent than reference suspension II (2.2.1) and not more intensely coloured than reference solution Y_6 (2.2.2, *Method II*).

Acidity or alkalinity. Dissolve 1.0 g in 10 ml of *water R*. Add 0.1 ml of *phenolphthalein solution R*. Not more than 0.75 ml of 0.1 M *hydrochloric acid* or 0.1 M *sodium hydroxide* is required to change the colour of the indicator.

Related substances. Examine by gas chromatography (2.2.28), using *butyric acid R* as the internal standard.

Internal standard solution. Dissolve 10 mg of *butyric acid R* in *heptane R* and dilute to 200 ml with the same solvent.

Test solution (a). Dissolve 0.500 g of the substance to be examined in 10 ml of *water R*. Add 5 ml of *dilute sulphuric acid R* and shake with three quantities, each of 20 ml, of *heptane R*. Add 10.0 ml of the internal standard solution to the combined upper layers, shake with *anhydrous sodium sulphate R*, filter and evaporate the filtrate, at a temperature not exceeding 30 °C, using a rotary evaporator. Take up the residue with *heptane R* and dilute to 10.0 ml with the same solvent. Dilute 1.0 ml of the solution to 10.0 ml with *heptane R*.

Test solution (b). Dissolve 40 mg of the substance to be examined in 100 ml of *water R*. To 10 ml of the solution add 0.5 ml of *dilute sulphuric acid R* and shake with three quantities, each of 5 ml, of *heptane R*. Shake with *anhydrous sodium sulphate R*, filter and evaporate the filtrate, at a temperature not exceeding 30 °C, to a volume of about 10 ml, using a rotary evaporator.

Reference solution (a). Dissolve 20 mg of 2-(1-methylethyl)pentanoic acid CRS in 5.0 ml of test solution (b) and dilute to 10 ml with *heptane R*. Dilute 1 ml of the solution to 10 ml with *heptane R*.

Reference solution (b). Prepare as prescribed for test solution (b), using *sodium valproate CRS* instead of the substance to be examined.

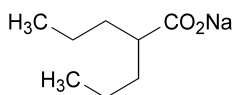
The chromatographic procedure may be carried out using:

- a wide-bore fused-silica column 30 m long and 0.53 mm in internal diameter coated with *macrogol 20 000 2-nitroterephthalate R* (film thickness 0.5 µm),
- *helium for chromatography R* as the carrier gas at a flow rate of 8 ml/min,
- a flame-ionisation detector,

01/2005:0678

SODIUM VALPROATE

Natrii valproas

 $\text{C}_8\text{H}_{15}\text{NaO}_2$ M_r 166.2

DEFINITION

Sodium valproate contains not less than 98.5 per cent and not more than the equivalent of 101.0 per cent of sodium 2-propylpentanoate, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, crystalline powder, hygroscopic, very soluble in water, slightly to freely soluble in alcohol.

with the following temperature programme:

	Time (min)	Temperature (°C)	Rate (°C/min)	Comment
Column	0 - 10	130	-	isothermal
	10 - 30	130 → 190	3	linear gradient
Injection port		220		
Detector		220		

Inject 1 µl of each solution. Adjust the sensitivity of the system so that the height of the peak due to the internal standard is at least 20 per cent of the full scale of the recorder. The test is not valid unless, in the chromatogram obtained with reference solution (a), the resolution between the peaks corresponding to 2-(1-methylethyl)pentanoic acid and valproic acid is at least 3.0. In the chromatogram obtained with test solution (a): the sum of the areas of the peaks, apart from the principal peak is not greater than three times the area of the peak due to the internal standard (0.3 per cent); none of the peaks, apart from the principal peak, has an area greater than that of the peak due to the internal standard (0.1 per cent). Disregard any peak with an area less than 0.1 times the area of the peak due to the internal standard.

Chlorides (2.4.4). To 5 ml of solution S add 10 ml of *water R*. The solution complies with the limit test for chlorides (200 ppm).

Sulphates (2.4.13). Solution S complies with the limit test for sulphates (200 ppm).

Heavy metals (2.4.8). 1.0 g complies with limit test C for heavy metals (20 ppm). Prepare the standard using 2 ml of *lead standard solution (10 ppm Pb) R*.

Loss on drying (2.2.32). Not more than 2.0 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C.

ASSAY

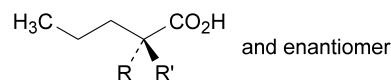
Dissolve 0.1500 g in 25 ml of *anhydrous acetic acid R*. Titrate with 0.1 M *perchloric acid* determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *perchloric acid*, is equivalent to 16.62 mg of C₈H₁₅NaO₂.

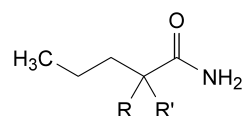
STORAGE

Store in an airtight container.

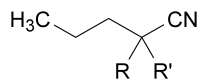
IMPURITIES



- A. R = R' = H: pentanoic acid (valeric acid),
 B. R = H, R' = CH₂-CH₃: (2*RS*)-2-ethylpentanoic acid,
 C. R = H, R' = CH(CH₃)₂: (2*RS*)-2-(1-methylethyl)pentanoic acid,
 D. R = R' = CH₂-CH₂-CH₃: 2,2-dipropylpentanoic acid,



- E. R = R' = H: pentanamide (valeramide),
 F. R = H, R' = CH₂-CH₂-CH₃: 2-propylpentanamide,
 G. R = R' = CH₂-CH₂-CH₃: 2,2-dipropylpentanamide,



- H. R = R' = H: pentanenitrile (valeronitrile),
 I. R = H, R' = CH₂-CH₂-CH₃: 2-propylpentanenitrile,
 J. R = R' = CH₂-CH₂-CH₃: 2,2-dipropylpentanenitrile.

01/2005:1264

SOLUTIONS FOR ORGAN PRESERVATION

Solutiones ad conservationem partium corporis

DEFINITION

Solutions for organ preservation are sterile, aqueous preparations, intended for storage, protection and/or perfusion of mammalian body organs that are in particular destined for transplantation.

They contain electrolytes that are typically at a concentration close to the intracellular electrolyte composition.

They may contain carbohydrates (such as glucose or mannitol), amino acids, calcium-complexing agents (such as citrate or phosphate), hydrocolloids (such as starch or gelatin derivatives) and other excipients, for example to make the preparation isotonic with blood, to adjust or buffer the pH, to prevent deterioration of the ingredients, but not to adversely affect the intended action of the preparation or, at the concentration used, to cause toxicity or undue local irritation. Solutions for organ preservation may also contain active substances or these may be added immediately before use.

Solutions for organ preservation, examined under suitable conditions of visibility, are clear and practically free from particles.

Solutions for organ preservation may also be presented as concentrated solutions. They are diluted to the prescribed volume with a prescribed liquid immediately before use. After dilution, they comply with the requirements for solutions for organ preservation.

Before use, the solutions for organ preservation are cooled below room temperature, typically to 2 °C to 6 °C, to reduce the temperature of the body organ and its metabolism.

Where applicable, the containers for solutions for organ preservation comply with the requirements for *Materials used for the manufacture of containers* (3.1 and subsections) and *Containers* (3.2 and subsections). Solutions for organ preservation are supplied in glass containers (3.2.1) or in other containers such as plastic containers (3.2.2 and 3.2.8). The tightness of the container is ensured by suitable means. Closures ensure a good seal, prevent the access of micro-organisms and other contaminants and usually permit the withdrawal of a part or the whole of the contents without removal of the closure. The plastic materials or elastomers of which the closure is composed are sufficiently firm and elastic to allow the passage of a needle with the least possible shedding of particles.

PRODUCTION

Solutions for organ preservation are prepared using materials and methods designed to ensure their sterility and to avoid the introduction of contaminants and the growth of micro-organisms; recommendations on this aspect are provided in the text on *Methods of preparation of sterile products* (5.1.1).

Unless otherwise justified and authorised, solutions for organ preservation are prepared from *water for injections R* and do not contain antimicrobial preservatives.

TESTS

pH (2.2.3). Carry out the test at room temperature. The pH of the solution is 5.0 to 8.0.

Osmolality (2.2.35). The osmolality of the solution is 250 mosmol/kg to 380 mosmol/kg.

Hydroxymethylfurfural. If the solution contains glucose, it complies with the following test: to a volume of the preparation to be examined containing the equivalent of 25 mg of glucose, add 5.0 ml of a 100 g/l solution of *p*-toluidine *R* in 2-propanol *R* containing 10 per cent *V/V* of glacial acetic acid *R* and 1.0 ml of a 5 g/l solution of barbituric acid *R*. The absorbance (2.2.25), determined at 550 nm after allowing the mixture to stand for 2 min to 3 min, is not greater than that of a standard prepared at the same time in the same manner using a solution containing 10 µg of hydroxymethylfurfural *R* in the same volume as the preparation to be examined.

Particulate contamination. Carry out the test for sub-visible particles (2.9.19) using 50 ml of preparation to be examined. The solution contains not more than 50 particles per millilitre larger than 10 µm and not more than 5 particles per millilitre larger than 25 µm.

Products for which the label states that the product is to be used with a final filter are exempted from these requirements.

Sterility (2.6.1). The solution complies with the test for sterility.

Bacterial endotoxins (2.6.14): less than 0.5 IU/ml.

Pyrogens (2.6.8). Solutions for which a validated test for bacterial endotoxins cannot be carried out comply with the test for pyrogens. Inject per kilogram of the rabbit's mass 10 ml of the solution, unless otherwise justified and authorised.

LABELLING

The label states:

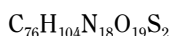
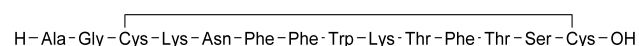
- that the solution is not to be used for injection,
- the formula of the solution for organ preservation expressed in grams per litre and in millimoles per litre,
- the nominal volume of the solution for organ preservation in the container,
- the osmolality, expressed in milliosmoles per kilogram,
- that any unused portion of the ready-to-use solution, of the concentrated solution or of the diluted solution must be discarded,
- the storage conditions,
- if applicable, that the solution is to be used in conjunction with a final filter.

In addition, for concentrated solutions the label states that the solution must be diluted with a suitable liquid immediately before use.

01/2005:0949

SOMATOSTATIN

Somatostatinum



DEFINITION

Somatostatin is a cyclic tetradecapeptide having the structure of the hypothalamic hormone that inhibits the release of human growth hormone. It is obtained by chemical synthesis. It contains a variable amount of acetic acid. It is available in the freeze-dried form.

Content: 95.0 per cent to 103.0 per cent of somatostatin (anhydrous and acetic acid-free substance).

CHARACTERS

Appearance: white, amorphous powder.

Solubility: freely soluble in water and in acetic acid, practically insoluble in methylene chloride.

IDENTIFICATION

A. Thin-layer chromatography (2.2.27).

Test solution. Dissolve 1.0 mg of the substance to be examined in 1.0 ml of *water R*.

Reference solution. Dissolve the contents of a vial of *somatostatin CRS* in *water R* and dilute with the same solvent to obtain a final concentration of 1 mg/ml.

Plate: TLC silica gel plate *R*.

Mobile phase: glacial acetic acid *R*, pyridine *R*, *water R*, butanol *R* (10:15:20:45 *V/V/V/V*).

Application: 20 µl.

Development: over a path of 15 cm.

Drying: in a current of warm air.

Detection: spray with a 1 g/l solution of *ninhydrin R* and heat the plate in an oven at 110 °C for about 5 min.

Results: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

B. Examine the chromatograms obtained in the assay.

Results: the principal peak in the chromatogram obtained with the test solution is similar in retention time to the principal peak in the chromatogram obtained with the reference solution.

TESTS

Specific optical rotation (2.2.7): –37 to –47 (anhydrous and acetic acid-free substance).

Dissolve 2.0 mg in 1.0 ml of a 1.0 per cent *V/V* solution of glacial acetic acid *R*.

Absorbance (2.2.25): maximum 0.20 at 280 nm (calculated with reference to the peptide content as determined in the assay).

Dissolve 5.0 mg in a 9 g/l solution of *sodium chloride R* and dilute to 100.0 ml with the same solvent.

Amino acids. Examine by means of an amino-acid analyser. Standardise the apparatus with a mixture containing equimolar amounts of ammonia, glycine and the L-form of the following amino acids:

Lysine	Threonine	Alanine	Leucine
Histidine	Serine	Valine	Tyrosine
Arginine	Glutamic acid	Methionine	Phenylalanine
Aspartic acid	Proline	Isoleucine	

together with half the equimolar amount of L-cystine. For the validation of the method, an appropriate internal standard, such as *DL-norleucine R*, is used.

Test solution. Place 1.0 mg of the substance to be examined in a rigorously cleaned hard-glass tube 100 mm long and 6 mm in internal diameter. Add a suitable amount of a

50 per cent *V/V* solution of *hydrochloric acid R*. Immerse the tube in a freezing mixture at -5°C , reduce the pressure to below 133 Pa and seal. Heat at $110\text{--}115^{\circ}\text{C}$ for 16 h. Cool, open the tube, transfer the contents to a 10 ml flask with the aid of 5 quantities, each of 0.2 ml, of *water R* and evaporate to dryness over *potassium hydroxide R* under reduced pressure. Take up the residue in *water R* and evaporate to dryness over *potassium hydroxide R* under reduced pressure; repeat these operations once. Take up the residue in a buffer solution suitable for the amino-acid analyser used and dilute to a suitable volume with the same buffer solution. Apply a suitable volume to the amino-acid analyser.

Express the content of each amino acid in moles. Calculate the relative proportions of the amino acids taking one eighth of the sum of the number of moles of aspartic acid, alanine, lysine, glycine and phenylalanine as equal to one. The values fall within the following limits: aspartic acid 0.95 to 1.05; glycine 0.95 to 1.05; alanine 0.95 to 1.05; phenylalanine 2.85 to 3.15; serine 0.7 to 1.05; threonine 1.4 to 2.1; half-cystine 1.4 to 2.1; lysine 1.9 to 2.1. Not more than traces of other amino acids are present.

Related peptides. Liquid chromatography (2.2.29): use the normalisation procedure.

Test solution. Dissolve 5.0 mg of the substance to be examined in *water R* and dilute to 10.0 ml with the same solvent.

Column:

- size: $l = 0.05\text{ m}$, $\varnothing = 4.6\text{ mm}$,
- stationary phase: octadecylsilyl silica gel for chromatography *R* (5 μm).

Mobile phase:

- mobile phase A: dilute 11 ml of *phosphoric acid R* with *water R*, adjust to pH 2.3 with *triethylamine R* and dilute to 1000 ml with *water R*,
- mobile phase B: acetonitrile for chromatography *R*,

Time (min)	Mobile phase A (per cent <i>V/V</i>)	Mobile phase B (per cent <i>V/V</i>)
0 - 18	79 → 60	21 → 40
18 - 20	60	40
20 - 21	60 → 79	40 → 21
21 - 26	79	21

Flow rate: 1.5 ml/min.

Detection: spectrophotometer at 215 nm.

Injection: 25 μl .

Limits:

- any impurity: maximum 1 per cent,
- total: maximum 2 per cent,
- disregard limit: 0.03 per cent.

Acetic acid (2.5.34): 3.0 per cent to 15.0 per cent.

Test solution. Dissolve 7.0 mg of the substance to be examined in a mixture of 5 volumes of mobile phase B and 95 volumes of mobile phase A and dilute to 10.0 ml with the same mixture of solvents.

Water (2.5.12): maximum 8.0 per cent, determined on 10.0 mg.

Bacterial endotoxins (2.6.14): less than 10 IU/mg, if intended for use in the manufacture of parenteral dosage forms without a further appropriate procedure for the removal of bacterial endotoxins.

ASSAY

Liquid chromatography (2.2.29).

Test solution. Dissolve 5.0 mg of the substance to be examined in *water R* and dilute to 10.0 ml with the same solvent.

Reference solution. Dissolve the contents of a vial of *somatostatin CRS* in *water R* and dilute with the same solvent to obtain a final concentration of 0.5 mg/ml.

Column:

- size: $l = 0.05\text{ m}$, $\varnothing = 4.6\text{ mm}$,
- stationary phase: octadecylsilyl silica gel for chromatography *R* (5 μm).

Mobile phase: mix 25 volumes of mobile phase B and 75 volumes of mobile phase A,

- mobile phase A: dilute 11 ml of *phosphoric acid R* with *water R*, adjust to pH 2.3 with *triethylamine R* and dilute to 1000 ml with *water R*,
- mobile phase B: acetonitrile for chromatography *R*.

Flow rate: 1.5 ml/min.

Detection: spectrophotometer at 215 nm.

Injection: 25 μl .

Run time: 15 min.

Calculate the content of somatostatin ($\text{C}_{76}\text{H}_{104}\text{N}_{18}\text{O}_{19}\text{S}_2$) using the chromatograms obtained with the test solution and the reference solution and the declared content of $\text{C}_{76}\text{H}_{104}\text{N}_{18}\text{O}_{19}\text{S}_2$ in *somatostatin CRS*.

STORAGE

In an airtight container protected from light, at a temperature of 2°C to 8°C . If the substance is sterile, store in a sterile, airtight, tamper-proof container.

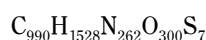
LABELLING

The label states, where applicable, that the substance is free from bacterial endotoxins.

01/2005:0951
corrected

SOMATROPIN

Somatropinum



M_r 22 125

DEFINITION

Somatropin is a protein having the structure (191 amino-acid residues) of the major component of growth hormone produced by the human pituitary. It contains not less than 91.0 per cent and not more than the equivalent of 105.0 per cent of somatropin⁽¹⁾ ($\text{C}_{990}\text{H}_{1528}\text{N}_{262}\text{O}_{300}\text{S}_7$), calculated with reference to the anhydrous substance.

PRODUCTION

Somatropin is produced by a method based on recombinant DNA (rDNA) technology. During the course of product development, it must be demonstrated that the manufacturing process produces a product having a biological activity of not less than 2.5 IU/mg, using a validated bioassay based on growth promotion and approved by the competent authority.

Somatropin complies with the following additional requirements.

(1) 1 mg of anhydrous somatropin ($\text{C}_{990}\text{H}_{1528}\text{N}_{262}\text{O}_{300}\text{S}_7$) is equivalent to 3.0 IU of biological activity.

Host-cell-derived proteins. The limit is approved by the competent authority.

Host-cell- and vector-derived DNA. The limit is approved by the competent authority.

CHARACTERS

A white or almost white powder.

IDENTIFICATION

- A. Examine the electropherograms obtained in the test for isoform distribution. In the electropherogram obtained with test solution (a), the principal band corresponds in position to that in the electropherogram obtained with reference solution (a).
- B. Examine the chromatograms obtained in the test for related proteins. The retention time of the principal peak in the chromatogram obtained with the test solution is similar to that of the principal peak in the chromatogram obtained with the reference solution.
- C. Examine by peptide mapping.

Test solution. Prepare a solution of the substance to be examined in 0.05 M tris-hydrochloride buffer solution pH 7.5 R to obtain a solution containing 2.0 mg/ml of somatropin and transfer about 1.0 ml to a tube made from suitable material such as polypropylene. Prepare a 1 mg/ml solution of *trypsin for peptide mapping R* in 0.05 M tris-hydrochloride buffer solution pH 7.5 R and add 30 µl to the solution of the substance to be examined. Cap the tube and place in a water-bath at 37 °C for 4 h. Remove from the water-bath and stop the reaction immediately, for example by freezing. If analysed immediately using an automatic injector, maintain the temperature at 2 °C to 8 °C.

Reference solution. Prepare at the same time and in the same manner as for the test solution but using *somatropin CRS* instead of the substance to be examined.

Examine by liquid chromatography (2.2.29).

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4.6 mm in internal diameter packed with *octylsilyl silica gel for chromatography R* (5 µm to 10 µm),
- as mobile phase at a flow rate of 1 ml/min:

Mobile phase A. Dilute 1 ml of *trifluoroacetic acid R* to 1000 ml with *water R*,

Mobile phase B. To 100 ml of *water R* add 1 ml of *trifluoroacetic acid R* and dilute to 1000 ml with *acetonitrile for chromatography R*,

following the elution conditions as described in the table below (if necessary, the gradient or the temperature of the column may be modified to improve separation of the digest):

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)
0 – 20	100 → 80	0 → 20
20 - 40	80 → 75	20 → 25
40 - 65	75 → 50	25 → 50
65 - 70	50 → 20	50 → 80
70 - 71	20 → 100	80 → 0
71 - 85	100	0

- as detector a spectrophotometer set at 214 nm,

maintaining the temperature of the column at 30 °C.

Equilibrate the column with mobile phase A for at least 15 min. Carry out a blank run using the above-mentioned gradient.

Inject 100 µl of the test solution and 100 µl of the reference solution. The test is not valid unless the chromatogram obtained with each solution is qualitatively similar to the chromatogram of somatropin digest supplied with *somatropin CRS*. The profile of the chromatogram obtained with the test solution corresponds to that of the chromatogram obtained with the reference solution.

- D. Examine the chromatograms obtained in the assay. The retention time of the principal peak in the chromatogram obtained with the test solution is similar to that of the principal peak in the chromatogram obtained with the reference solution.

TESTS

Related proteins. Examine by liquid chromatography (2.2.29).

Test solution. Prepare a solution of the substance to be examined in 0.05 M tris-hydrochloride buffer solution pH 7.5 R, containing 2.0 mg/ml of somatropin.

Reference solution. Prepare a solution of *somatropin CRS* in 0.05 M tris-hydrochloride buffer solution pH 7.5 R, containing 2.0 mg/ml of somatropin.

Resolution solution (somatropin/desamido-somatropin resolution mixture). Prepare a solution of *somatropin CRS* in 0.05 M tris-hydrochloride buffer solution pH 7.5 R to obtain a 2.0 mg/ml solution of somatropin. Either filter through a sterile filter or add *sodium azide R* to a concentration of 0.1 mg/ml and allow to stand at room temperature for 24 h.

Maintain the solutions at 2 °C to 8 °C and use within 24 h. If an automatic injector is used, maintain the temperature at 2 °C to 8 °C.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4.6 mm in internal diameter packed with a suitable singly end-capped butylsilyl silica gel, with a granulometry of 5 µm and a porosity of 30 nm. A silica saturation column is to be placed between the pump and the injector valve,
- as mobile phase at a flow rate of 0.5 ml/min a mixture of 29 volumes of *propanol R* and 71 volumes of 0.05 M tris-hydrochloride buffer solution pH 7.5 R,
- as detector a spectrophotometer set at 220 nm,

maintaining the temperature of the column at 45 °C.

Prior to use, rinse the column with 200 ml to 500 ml of a 0.1 per cent V/V solution of *trifluoroacetic acid R* in a 50 per cent V/V solution of *acetonitrile R*. Repeat as necessary, to improve column performance.

Inject 20 µl of the reference solution. If necessary, adjust the concentration of *propanol R* in the mobile phase so that the retention time of the principal peak is about 33 min.

Inject 20 µl of the resolution solution. Desamido-somatropin appears as a small peak at a retention time of about 0.85 relative to the principal peak. The test is not valid unless the resolution between the peaks corresponding to somatropin and desamido-somatropin is at least 1.0 and the symmetry factor of the somatropin peak is 0.9 to 1.8.

Inject 20 µl of the test solution. In the chromatogram obtained, the sum of the areas of all the peaks, apart from the principal peak, is not greater than 6.0 per cent of the total area of the peaks. Disregard any peak due to the solvent.

Dimer and related substances of higher molecular mass.

Examine by size-exclusion chromatography (2.2.30) as described under Assay.

Inject 20 µl of the test solution. In the chromatogram obtained with the test solution, the sum of the areas of any peak with a retention time less than that of the principal peak is not greater than 4.0 per cent of the total area of the peaks. Disregard any peak due to the solvent.

Isoform distribution. Examine by isoelectric focusing.

Test solution (a). Prepare a solution of the substance to be examined in 0.025 M phosphate buffer solution pH 7.0 R, containing 2.0 mg/ml of somatropin.

Test solution (b). Add 0.1 ml of test solution (a) to 1.9 ml of 0.025 M phosphate buffer solution pH 7.0 R.

Reference solution (a). Prepare a solution of somatropin CRS in 0.025 M phosphate buffer solution pH 7.0 R, containing 2.0 mg/ml of somatropin.

Reference solution (b). Use an isoelectric point calibration solution in the pH range of 2.5 to 6.5, prepared and used according to the manufacturer's instructions.

Operate the apparatus in accordance with the manufacturer's instructions. The isoelectric focusing procedure may be carried out using a pre-cast gel 245 mm × 110 mm × 1 mm, with a pH in the range 4.0 to 6.5. Apply to the gel 15 µl of each solution. Use as the anode solution a 14.7 g/l solution of glutamic acid R in phosphoric acid (50 g/l H₃PO₄) and as the cathode solution an 89.1 g/l solution of β-alanine R. Adjust the operating conditions to 2000 V and 25 mA. Allow focusing to take place for 2.5 h at a constant voltage and at a power of not more than 25 W. Immerse the gel for 30 min in a solution containing 115 g/l of trichloroacetic acid R and 34.5 g/l of sulphosalicylic acid R, and then for 5 min in a mixture of 8 volumes of acetic acid R, 25 volumes of ethanol R and 67 volumes of deionised water R (de-stain solution). Stain the gel by immersion in a 1.15 g/l solution of acid blue 83 R in de-stain solution at 60 °C for 10 min, and then place the gel in de-stain solution until excess stain is removed.

The test is not valid unless the distribution of bands in the electropherogram obtained with reference solution (b) corresponds to the manufacturer's indications. The electropherogram obtained with reference solution (a) contains a major band with an isoelectric point of approximately five, and a slightly more acidic minor band at approximately 4.8. In the electropherogram obtained with test solution (a), no band apart from the major band is more intense than the major band in the electropherogram obtained with test solution (b) (5 per cent).

Water (2.5.32). Not more than 10.0 per cent, determined by the micro determination of water.

Bacterial endotoxins (2.6.14): less than 5 IU/mg, if intended for use in the manufacture of parenteral dosage forms without a further appropriate procedure for removal of bacterial endotoxins.

ASSAY

Examine by size-exclusion chromatography (2.2.30).

Test solution. Prepare a solution of the substance to be examined in 0.025 M phosphate buffer solution pH 7.0 R, containing 1.0 mg/ml of somatropin.

Reference solution. Dissolve the contents of a vial of somatropin CRS in 0.025 M phosphate buffer solution pH 7.0 R and dilute with the same solvent to obtain a concentration of 1.0 mg/ml.

Resolution solution. Place one vial of somatropin CRS in an oven at 50 °C for a period (typically between 12 and 24 h) sufficient to generate 1 per cent to 2 per cent of dimer. Dissolve its contents in 0.025 M phosphate buffer solution pH 7.0 R and dilute with the same solvent to obtain a concentration of 1.0 mg/ml.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.30 m long and 7.8 mm in internal diameter packed with hydrophilic silica gel for chromatography R of a grade suitable for fractionation of globular proteins in the molecular mass range 5000 to 150 000,
- as mobile phase at a flow rate of 0.6 ml/min a mixture (filtered and degassed) consisting of 3 volumes of 2-propanol R and 97 volumes of 0.063 M phosphate buffer solution pH 7.0 R,
- as detector a spectrophotometer set at 214 nm.

Inject 20 µl of the resolution solution. In the chromatogram obtained, the main peak elutes at a retention time of approximately 12 min to 17 min and the peaks corresponding to the somatropin dimer and to the higher molecular weight proteins at relative retention times of 0.90 and 0.65 respectively, relative to the main peak. The resolution, defined by the ratio of the height above the baseline of the valley separating the monomer and dimer peaks to the height of the dimer peak, is not greater than 0.4.

Inject 20 µl of the test solution and 20 µl of the reference solution.

Calculate the content of somatropin (C₉₉₀H₁₅₂₈N₂₆₂O₃₀₀S₇) from the peak areas in the chromatograms obtained with the test solution and the reference solution and the declared content of (C₉₉₀H₁₅₂₈N₂₆₂O₃₀₀S₇) in somatropin CRS.

STORAGE

Store in an airtight container at a temperature of 2 °C to 8 °C. If the substance is sterile, store in a sterile, airtight, tamper-proof container.

LABELLING

The label states, where applicable, that the substance is free from bacterial endotoxins.

01/2005:0950
corrected

SOMATROPIN BULK SOLUTION**Somatropini solutio ad praeparationem****DEFINITION**

Somatropin bulk solution is a solution containing a protein having the structure (191 amino-acid residues) of the major component of growth hormone produced by the human pituitary. It may contain buffer salts and other auxiliary substances. It contains not less than 91.0 per cent and not more than 105.0 per cent of the amount of somatropin⁽²⁾ (C₉₉₀H₁₅₂₈N₂₆₂O₃₀₀S₇) stated on the label.

PRODUCTION

Somatropin bulk solution is produced by a method based on recombinant DNA (rDNA) technology. During the course of product development, it must be demonstrated that the manufacturing process produces a product having a biological activity of at least 2.5 IU/mg, using a validated bioassay based on growth promotion and approved by the competent authority.

(2) 1 mg of anhydrous somatropin (C₉₉₀H₁₅₂₈N₂₆₂O₃₀₀S₇) is equivalent to 3.0 IU of biological activity.

Somatropin bulk solution complies with the following additional requirements.

Host-cell-derived proteins. The limit is approved by the competent authority.

Host-cell- and vector-derived DNA. The limit is approved by the competent authority.

CHARACTERS

A clear or slightly turbid, colourless solution.

IDENTIFICATION

- Examine the electropherograms obtained in the test for isoform distribution. In the electropherogram obtained with test solution (a), the principal band corresponds in position to that in the electropherogram obtained with reference solution (a).
- Examine the chromatograms obtained in the test for related proteins. The retention time of the principal peak in the chromatogram obtained with the test solution is similar to that of the principal peak in the chromatogram obtained with the reference solution.
- Examine by peptide mapping.

Test solution. Dilute the solution to be examined with 0.05 M tris-hydrochloride buffer solution pH 7.5 R so that it contains 2.0 mg/ml of somatropin, and transfer about 1.0 ml to a tube made from suitable material such as polypropylene. Prepare a 1 mg/ml solution of *trypsin for peptide mapping R* in 0.05 M tris-hydrochloride buffer solution pH 7.5 R and add 30 µl to the solution of the substance to be examined. Cap the tube and place in a water-bath at 37 °C for 4 h. Remove from the water-bath and stop the reaction immediately, for example by freezing. If analysed immediately using an automatic injector, maintain the temperature at 2 °C to 8 °C.

Note: If a 2 mg/ml somatropin concentration is not obtainable, a similar digest relationship (µg of *trypsin* per mg of somatropin) may be used.

Reference solution. Prepare a solution of somatropin CRS in 0.05 M tris-hydrochloride buffer solution pH 7.5 R containing 2.0 mg/ml of somatropin and treat at the same time and in the same manner as for the test solution.

Examine by liquid chromatography (2.2.29).

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4.6 mm in internal diameter packed with *octylsilyl silica gel for chromatography R* (5 µm to 10 µm),
- as mobile phase at a flow rate of 1 ml/min:

Mobile phase A. Dilute 1 ml of *trifluoroacetic acid R* to 1000 ml with *water R*,

Mobile phase B. To 100 ml of *water R* add 1 ml of *trifluoroacetic acid R* and dilute to 1000 ml with *acetonitrile for chromatography R*,

following the elution conditions as described in the table below (if necessary, the gradient or the temperature of the column may be modified to improve separation of the digest):

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)
0 – 20	100 → 80	0 → 20
20 – 40	80 → 75	20 → 25
40 – 65	75 → 50	25 → 50
65 – 70	50 → 20	50 → 80
70 – 71	20 → 100	80 → 0
71 – 85	100	0

- as detector a spectrophotometer set at 214 nm, maintaining the temperature of the column at 30 °C.

Equilibrate the column with mobile phase A for at least 15 min. Carry out a blank run using the above-mentioned gradient.

Inject 100 µl of the test solution and 100 µl of the reference solution. The test is not valid unless the chromatogram obtained with each solution is qualitatively similar to the chromatogram of somatropin digest supplied with *somatropin CRS*. The profile of the chromatogram obtained with the test solution corresponds to that of the chromatogram obtained with the reference solution.

- Examine the chromatograms obtained in the assay. The retention time of the principal peak in the chromatogram obtained with the test solution is similar to that of the principal peak in the chromatogram obtained with the reference solution.

TESTS

Related proteins. Examine by liquid chromatography (2.2.29).

Test solution. Dilute the solution to be examined with 0.05 M tris-hydrochloride buffer solution pH 7.5 R so as to contain 2.0 mg/ml of somatropin or less, but then correct the injection volume accordingly.

Reference solution. Prepare a solution of *somatropin CRS* in 0.05 M tris-hydrochloride buffer solution pH 7.5 R, containing 2.0 mg/ml of somatropin.

Resolution solution (somatropin/desamido-somatropin resolution mixture). Prepare a solution of *somatropin CRS* in 0.05 M tris-hydrochloride buffer solution pH 7.5 R, containing 2.0 mg/ml of somatropin. Either filter through a sterile filter or add *sodium azide R* to a concentration of 0.1 mg/ml and allow to stand at room temperature for 24 h.

Maintain the solutions at 2 °C to 8 °C and use within 24 h. If an automatic injector is used, maintain the temperature at 2 °C to 8 °C.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4.6 mm in internal diameter packed with a suitable singly end-capped butylsilyl silica gel, with a granulometry of 5 µm and a porosity of 30 nm. A silica saturation column is to be placed between the pump and the injector valve,
- as mobile phase at a flow rate of 0.5 ml/min a mixture of 29 volumes of *propanol R* and 71 volumes of 0.05 M tris-hydrochloride buffer solution pH 7.5 R,
- as detector a spectrophotometer set at 220 nm, maintaining the temperature of the column at 45 °C.

Prior to use, rinse the column with 200 ml to 500 ml of a 0.1 per cent V/V solution of *trifluoroacetic acid R* in a 50 per cent V/V solution of *acetonitrile R*. Repeat as necessary, to improve column performance.

Inject 20 µl of the reference solution. If necessary, adjust the concentration of *propanol R* in the mobile phase so that the retention time of the principal peak is about 33 min.

Inject 20 µl of the resolution solution. Desamido-somatropin appears as a small peak at a retention time of about 0.85 relative to the principal peak. The test is not valid unless the resolution between the peaks corresponding to somatropin and desamido-somatropin is at least 1.0 and the symmetry factor of the somatropin peak is 0.9 to 1.8.

Inject 20 µl of the test solution. In the chromatogram obtained, the sum of the areas of all the peaks, apart from the principal peak, is not greater than 6.0 per cent of the total area of the peaks. Disregard any peak due to the solvent.

Dimer and related substances of higher molecular mass.

Examine by size-exclusion chromatography (2.2.30) as described under Assay.

Inject 20 µl of the test solution. In the chromatogram obtained with the test solution, the sum of the areas of any peak with a retention time less than that of the principal peak is not greater than 4.0 per cent of the total area of the peaks. Disregard any peak due to the solvent.

Isoform distribution. Examine by isoelectric focusing.

Test solution (a). Dilute the solution to be examined with 0.025 M phosphate buffer solution pH 7.0 R so as to contain 2.0 mg/ml of somatropin.

Test solution (b). Add 0.1 ml of test solution (a) to 1.9 ml of 0.025 M phosphate buffer solution pH 7.0 R.

Reference solution (a). Prepare a solution of somatropin CRS in 0.025 M phosphate buffer solution pH 7.0 R, containing 2.0 mg/ml of somatropin.

Reference solution (b). Use an isoelectric point calibration solution in the pH range of 2.5 to 6.5, prepared and used according to the manufacturer's instructions.

Operate the apparatus in accordance with the manufacturer's instructions. The isoelectric focusing procedure may be carried out using a pre-cast gel 245 mm × 110 mm × 1 mm, with a pH in the range 4.0 to 6.5. Apply to the gel 15 µl of each solution. Use as the anode solution a 14.7 g/l solution of glutamic acid R in phosphoric acid (50 g/l H₃PO₄) and as the cathode solution an 89.1 g/l solution of β-alanine R. Adjust the operating conditions to 2000 V and 25 mA. Allow focusing to take place for 2.5 h at a constant voltage and at a power of not more than 25 W. Immerse the gel for 30 min in a solution containing 115 g/l of trichloroacetic acid R and 34.5 g/l of sulphosalicylic acid R, and then for 5 min in a mixture of 8 volumes of acetic acid R, 25 volumes of ethanol R and 67 volumes of deionised water R (de-stain solution). Stain the gel by immersion in a 1.15 g/l solution of acid blue 83 R in de-stain solution at 60 °C for 10 min, and then place the gel in de-stain solution until excess stain is removed.

The test is not valid unless the distribution of bands in the electropherogram obtained with reference solution (b) corresponds to the manufacturer's indications. The electropherogram obtained with reference solution (a) contains a major band with an isoelectric point of approximately five, and a slightly more acidic minor band at approximately 4.8. In the electropherogram obtained with test solution (a), no band apart from the major band is more intense than the major band in the electropherogram obtained with test solution (b) (5 per cent).

Bacterial endotoxins (2.6.14): less than 5 IU in the volume that contains 1 mg of somatropin, if intended for use in the manufacture of parenteral dosage forms without a further appropriate procedure for removal of bacterial endotoxins.

ASSAY

Examine by size-exclusion chromatography (2.2.30).

Test solution. Dilute the solution to be examined with 0.025 M phosphate buffer solution pH 7.0 R so as to contain 1.0 mg/ml of somatropin.

Reference solution. Dissolve the contents of a vial of somatropin CRS in 0.025 M phosphate buffer solution pH 7.0 R and dilute with the same solvent to obtain a concentration of 1.0 mg/ml.

Resolution solution. Place one vial of somatropin CRS in an oven at 50 °C for a period (typically between 12 h and 24 h) sufficient to generate 1 per cent to 2 per cent of dimer. Dissolve its contents in 0.025 M phosphate buffer solution pH 7.0 R and dilute with the same solvent to obtain a concentration of 1.0 mg/ml.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.30 m long and 7.8 mm in internal diameter packed with hydrophilic silica gel for chromatography R of a grade suitable for fractionation of globular proteins in the molecular mass range 5000 to 150 000,
- as mobile phase at a flow rate of 0.6 ml/min a mixture (filtered and degassed) consisting of 3 volumes of 2-propanol R and 97 volumes of 0.063 M phosphate buffer solution pH 7.0 R,
- as detector a spectrophotometer set at 214 nm.

Inject 20 µl of the resolution solution. In the chromatogram obtained, the main peak elutes at a retention time of approximately 12 min to 17 min and the peaks corresponding to the somatropin dimer and to the higher molecular weight proteins at relative retention times of 0.90 and 0.65 respectively, relative to the main peak. The resolution, defined by the ratio of the height above the baseline of the valley separating the monomer and dimer peaks to the height of the dimer peak, is not greater than 0.4.

Inject 20 µl of the test solution and 20 µl of the reference solution.

Calculate the content of somatropin (C₉₉₀H₁₅₂₈N₂₆₂O₃₀₀S₇) from the peak areas in the chromatograms obtained with the test solution and the reference solution and the declared content of (C₉₉₀H₁₅₂₈N₂₆₂O₃₀₀S₇) in somatropin CRS.

STORAGE

Store in an airtight container at a temperature of –20 °C. Avoid repeated freezing and thawing. If the solution is sterile, store in a sterile, airtight, tamper-proof container.

LABELLING

The label states:

- the content of somatropin in milligrams per millilitre,
- the name and concentration of any auxiliary substance,
- where applicable, that the solution is free from bacterial endotoxins.

01/2005:0952
corrected

SOMATROPIN FOR INJECTION

Somatropinum ad iniectabilium

DEFINITION

Somatropin for injection is a freeze-dried, sterile preparation of a protein having the structure (191 amino-acid residues) of the major component of growth hormone produced by the human pituitary. It contains not less than 89.0 per cent and not more than 105.0 per cent of the amount of

somatropin⁽³⁾ (C₉₉₀H₁₅₂₈N₂₆₂O₃₀₀S₇) stated on the label. It complies with the requirements of the monographs on *Parenteral preparations* (0520).

PRODUCTION

Somatropin for injection is prepared either from *Somatropin* (0951) or from *Somatropin bulk solution* (0950), or by a method based on recombinant DNA (rDNA) technology in which the injectable preparation is produced without the isolation of an intermediate solid or liquid bulk. In the latter case, during the course of product development, it must be demonstrated that the manufacturing process produces a product having a biological activity of not less than 2.5 IU/mg, using a validated bioassay based on growth promotion and approved by the competent authority. The purified preparation, to which buffers and stabilisers may be added, is filtered through a bacteria-retentive filter, aseptically distributed in sterile containers of glass type I (3.2.1) and freeze-dried. The containers are immediately sealed so as to exclude microbial contamination and moisture.

Somatropin for injection complies with the following additional requirements.

Host-cell-derived proteins. The limit is approved by the competent authority.

Host-cell- and vector-derived DNA. The limit is approved by the competent authority.

Where somatropin for injection is prepared from *Somatropin* (0951) or from *Somatropin bulk solution* (0950), compliance with the requirements for host-cell-derived proteins, host-cell- and vector-derived DNA and with identification test C need not be reconfirmed by the manufacturer during subsequent production of somatropin for injection.

CHARACTERS

A white or almost white powder.

IDENTIFICATION

- A. Examine the electropherograms obtained in the test for isoform distribution. In the electropherogram obtained with test solution (a), the principal band corresponds in position to that in the electropherogram obtained with reference solution (a).
- B. Examine the chromatograms obtained in the test for related proteins. The retention time of the principal peak in the chromatogram obtained with the test solution is similar to that of the principal peak in the chromatogram obtained with the reference solution.

- C. Examine by peptide mapping.

Test solution. Prepare a solution of the substance to be examined in 0.05 M tris-hydrochloride buffer solution pH 7.5 R to obtain a solution containing 2.0 mg/ml of somatropin, and transfer about 1.0 ml to a tube made from suitable material such as polypropylene. Prepare a 1 mg/ml solution of *trypsin for peptide mapping R* in 0.05 M tris-hydrochloride buffer solution pH 7.5 R and add 30 µl to the solution of the substance to be examined. Cap the tube and place in a water-bath at 37 °C for 4 h. Remove from the water-bath and stop the reaction immediately, for example by freezing. If analysed immediately using an automatic injector, maintain the temperature at 2 °C to 8 °C.

Reference solution. Prepare at the same time and in the same manner as for the test solution but using *somatropin CRS* instead of the substance to be examined.

Examine by liquid chromatography (2.2.29).

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4.6 mm in internal diameter packed with *octylsilyl silica gel for chromatography R* (5 µm to 10 µm),
- as mobile phase at a flow rate of 1 ml/min:

Mobile phase A. Dilute 1 ml of *trifluoroacetic acid R* to 1000 ml with *water R*,

Mobile phase B. To 100 ml of *water R* add 1 ml of *trifluoroacetic acid R* and dilute to 1000 ml with *acetonitrile for chromatography R*,

following the elution conditions as described in the table below (if necessary, the gradient or the temperature of the column may be modified to improve separation of the digest):

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)
0 – 20	100 → 80	0 → 20
20 – 40	80 → 75	20 → 25
40 – 65	75 → 50	25 → 50
65 – 70	50 → 20	50 → 80
70 – 71	20 → 100	80 → 0
71 – 85	100	0

- as detector a spectrophotometer set at 214 nm, maintaining the temperature of the column at 30 °C.

Equilibrate the column with mobile phase A for at least 15 min. Carry out a blank run using the above-mentioned gradient.

Inject 100 µl of the test solution and 100 µl of the reference solution. The test is not valid unless the chromatogram obtained with each solution is qualitatively similar to the chromatogram of somatropin digest supplied with *somatropin CRS*. The profile of the chromatogram obtained with the test solution corresponds to that of the chromatogram obtained with the reference solution.

- D. Examine the chromatograms obtained in the assay. The retention time of the principal peak in the chromatogram obtained with the test solution is similar to that of the principal peak in the chromatogram obtained with the reference solution.

TESTS

Related proteins. Examine by liquid chromatography (2.2.29).

Test solution. Prepare a solution of the substance to be examined in 0.05 M tris-hydrochloride buffer solution pH 7.5 R, containing 2.0 mg/ml of somatropin.

Reference solution. Prepare a solution of *somatropin CRS* in 0.05 M tris-hydrochloride buffer solution pH 7.5 R, containing 2.0 mg/ml of somatropin.

Resolution solution (somatropin/desamido-somatropin resolution mixture). Prepare a solution of *somatropin CRS* in 0.05 M tris-hydrochloride buffer solution pH 7.5 R, containing 2.0 mg/ml of somatropin. Either filter through a sterile filter or add *sodium azide R* to a concentration of 0.1 mg/ml and allow to stand at room temperature for 24 h.

Maintain the solutions at 2 °C to 8 °C and use within 24 h. If an automatic injector is used, maintain the temperature at 2 °C to 8 °C.

(3) 1 mg of anhydrous somatropin (C₉₉₀H₁₅₂₈N₂₆₂O₃₀₀S₇) is equivalent to 3.0 IU of biological activity.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4.6 mm in internal diameter packed with a suitable singly end-capped butylsilyl silica gel, with a granulometry of 5 µm and a porosity of 30 nm. A silica saturation column is to be placed between the pump and the injector valve,
- as mobile phase at a flow rate of 0.5 ml/min a mixture of 29 volumes of *propanol R* and 71 volumes of 0.05 M *tris-hydrochloride buffer solution pH 7.5 R*,
- as detector a spectrophotometer set at 220 nm, maintaining the temperature of the column at 45 °C.

Prior to use, rinse the column with 200 ml to 500 ml of a 0.1 per cent V/V solution of *trifluoroacetic acid R* in a 50 per cent V/V solution of *acetonitrile R*. Repeat as necessary, to improve column performance.

Inject 20 µl of the reference solution. If necessary, adjust the concentration of *propanol R* in the mobile phase so that the retention time of the principal peak is about 33 min.

Inject 20 µl of the resolution solution. Desamido-somatropin appears as a small peak at a retention time of about 0.85 relative to the principal peak. The test is not valid unless the resolution between the peaks corresponding to somatropin and desamido-somatropin is at least 1.0 and the symmetry factor of the somatropin peak is 0.9 to 1.8.

Inject 20 µl of the test solution. In the chromatogram obtained, the sum of areas of all the peaks, apart from the principal peak, is not greater than 13 per cent of the total area of the peaks. Disregard any peak due to the solvent.

Dimer and related substances of higher molecular mass.

Examine by size-exclusion chromatography (2.2.30) as described under Assay.

Inject 20 µl of the test solution. In the chromatogram obtained with the test solution, the sum of the areas of any peak with a retention time less than that of the principal peak is not greater than 6.0 per cent of the total area of the peaks. Disregard any peak due to the solvent.

Isoform distribution. Examine by isoelectric focusing.

Test solution (a). Prepare a solution of the substance to be examined in 0.025 M *phosphate buffer solution pH 7.0 R*, containing 2.0 mg/ml of somatropin.

Test solution (b). Add 0.1 ml of test solution (a) to 1.5 ml of 0.025 M *phosphate buffer solution pH 7.0 R*.

Reference solution (a). Prepare a solution of *somatropin CRS* in 0.025 M *phosphate buffer solution pH 7.0 R*, containing 2.0 mg/ml of somatropin.

Reference solution (b). Use an isoelectric point calibration solution in the pH range of 2.5 to 6.5, prepared and used according to the manufacturer's instructions.

Operate the apparatus in accordance with the manufacturer's instructions. The isoelectric focusing procedure may be carried out using a pre-cast gel 245 mm × 110 mm × 1 mm, with a pH in the range 4.0 to 6.5. Apply to the gel 15 µl of each solution. Use as the anode solution a 14.7 g/l solution of *glutamic acid R* in phosphoric acid (50 g/l H_3PO_4) and as the cathode solution an 89.1 g/l solution of *β-alanine R*. Adjust the operating conditions to 2000 V and 25 mA. Allow focusing to take place for 2.5 h at a constant voltage and at a power of not more than 25 W. Immerse the gel for 30 min in a solution containing 115 g/l of *trichloroacetic acid R* and 34.5 g/l of *sulphosalicylic acid R*, and then for 5 min in a mixture of 8 volumes of *acetic acid R*, 25 volumes of *ethanol R* and 67 volumes of deionised *water R* (de-stain solution). Stain the gel by immersion in a 1.15 g/l solution of *acid blue 83 R* in de-stain solution at 60 °C for 10 min, and then place the gel in de-stain solution until excess stain is removed.

The test is not valid unless the distribution of bands in the electropherogram obtained with reference solution (b) corresponds to the manufacturer's indications. The electropherogram obtained with reference solution (a) contains a major band with an isoelectric point of approximately five, and a slightly more acidic minor band at approximately 4.8. In the electropherogram obtained with test solution (a), no band apart from the major band is more intense than the major band in the electropherogram obtained with test solution (b) (6.25 per cent).

Water (2.5.32). Not more than 3.0 per cent, unless otherwise justified and authorised, determined by the micro determination of water.

Bacterial endotoxins (2.6.14): less than 5 IU/mg.

ASSAY

Examine by size-exclusion chromatography (2.2.30).

Test solution. Prepare a solution of the substance to be examined in 0.025 M *phosphate buffer solution pH 7.0 R*, containing 1.0 mg/ml of somatropin.

Reference solution. Dissolve the contents of a vial of *somatropin CRS* in 0.025 M *phosphate buffer solution pH 7.0 R* and dilute with the same solvent to obtain a concentration of 1.0 mg/ml.

Resolution solution. Place one vial of *somatropin CRS* in an oven at 50 °C for a period (typically between 12 h and 24 h) sufficient to generate 1 per cent to 2 per cent of dimer. Dissolve its contents in 0.025 M *phosphate buffer solution pH 7.0 R* and dilute with the same solvent to obtain a concentration of 1.0 mg/ml.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.30 m long and 7.8 mm in internal diameter packed with *hydrophilic silica gel for chromatography R* of a grade suitable for fractionation of globular proteins in the molecular mass range 5000 to 150 000,
- as mobile phase at a flow rate of 0.6 ml/min a mixture (filtered and degassed) consisting of 3 volumes of 2-propanol *R* and 97 volumes of 0.063 M *phosphate buffer solution pH 7.0 R*,
- as detector a spectrophotometer set at 214 nm.

Inject 20 µl of the resolution solution. In the chromatogram obtained, the main peak elutes at a retention time of approximately 12 min to 17 min and the peaks corresponding to the somatropin dimer and to the higher molecular weight proteins at relative retention times of 0.90 and 0.65 respectively, relative to the main peak. The resolution defined by the ratio of the height above the baseline of the valley separating the monomer and dimer peaks, to the height of the dimer peak, is not greater than 0.4.

Inject 20 µl of the test solution and 20 µl of the reference solution.

Calculate the content of somatropin ($\text{C}_{990}\text{H}_{1528}\text{N}_{262}\text{O}_{300}\text{S}_7$) from the peak areas in the chromatograms obtained with the test solution and the reference solution and the declared content of ($\text{C}_{990}\text{H}_{1528}\text{N}_{262}\text{O}_{300}\text{S}_7$) in *somatropin CRS*.

STORAGE

Store in a sterile, airtight, tamper-proof container, at a temperature of 2 °C to 8 °C.

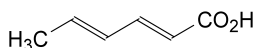
LABELLING

The label states:

- the content of somatropin in the container, in milligrams,
- the composition and volume of the liquid to be added for reconstitution,

- the time within which the reconstituted solution shall be used and the storage conditions during this period,
- the name and quantity of any added substance,
- the storage temperature,
- that the preparation shall not be shaken during reconstitution.

01/2005:0592

SORBIC ACID**Acidum sorbicum** $C_6H_8O_2$ M_r 112.1**DEFINITION**

Sorbic acid contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of (*E,E*)-hexa-2,4-dienoic acid, calculated with reference to the anhydrous substance.

CHARACTERS

A white or almost white, crystalline powder, slightly soluble in water, freely soluble in alcohol.

IDENTIFICATION

First identification: A, C.

Second identification: A, B, D.

- Melting point (2.2.14): 132 °C to 136 °C.
- Dissolve 50.0 mg in *water R* and dilute to 250.0 ml with the same solvent. Dilute 2.0 ml of this solution to 200.0 ml with 0.1 M *hydrochloric acid*. Examined between 230 nm and 350 nm (2.2.25), the solution shows a maximum at 264 nm. The specific absorbance at the maximum is 2150 to 2550.
- Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *sorbic acid CRS*.
- Dissolve 0.2 g in 2 ml of *alcohol R* and add 0.2 ml of *bromine water R*. The solution is decolorised.

TESTS

Solution S. Dissolve 1.25 g in *alcohol R* and dilute to 25 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and colourless (2.2.2, *Method II*).

Aldehydes. Dissolve 1.0 g in a mixture of 30 ml of *water R* and 50 ml of 2-propanol *R*, adjust the solution to pH 4 with 0.1 M *hydrochloric acid* or 0.1 M *sodium hydroxide* and dilute to 100 ml with *water R*. To 10 ml of the solution add 1 ml of *decolorised fuchsin solution R* and allow to stand for 30 min. Any colour in the solution is not more intense than that in a standard prepared at the same time by adding 1 ml of *decolorised fuchsin solution R* to a mixture of 1.5 ml of *acetaldehyde standard solution* (100 ppm C_2H_4O) *R*, 4 ml of 2-propanol *R* and 4.5 ml of *water R* (0.15 per cent, calculated as C_2H_4O).

Heavy metals (2.4.8). 12 ml of solution S complies with limit test B for heavy metals (10 ppm). Prepare the standard using 5 ml of *lead standard solution* (1 ppm Pb) *R* and 5 ml of *alcohol R*.

Water (2.5.12). Not more than 1.0 per cent, determined on 2.000 g by the semi-micro determination of water.

Sulphated ash (2.4.14). Not more than 0.2 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.1000 g in 20 ml of *alcohol R*. Using 0.2 ml of *phenolphthalein solution R* as indicator, titrate with 0.1 M *sodium hydroxide* until a pink colour is obtained.

1 ml of 0.1 M *sodium hydroxide* is equivalent to 11.21 mg of $C_6H_8O_2$.

STORAGE

Store protected from light.

01/2005:1040

SORBITAN LAURATE**Sorbitani lauras****DEFINITION**

Mixture usually obtained by partial esterification of sorbitol and its mono- and di-anhydrides with lauric acid.

CHARACTERS

Appearance: brownish-yellow, viscous liquid.

Solubility: practically insoluble, but dispersible in water, miscible with alcohol.

Relative density: about 0.98.

IDENTIFICATION

- It complies with the test for hydroxyl value (see Tests).
- It complies with the test for iodine value (see Tests).
- It complies with the test for composition of fatty acids (see Tests).

TESTS

Acid value (2.5.1): maximum 7.0, determined on 5.0 g.

Hydroxyl value (2.5.3, *Method A*): 330 to 358.

Iodine value (2.5.4): maximum 10.

Peroxide value (2.5.5): maximum 5.0.

Saponification value (2.5.6): 158 to 170.

Carry out the saponification for 1 h.

Composition of fatty acids. Gas chromatography (2.4.22, *Method C*).

Prepare reference solution (a) as indicated in tables 2.4.22-1 and 2.4.22-2.

Composition of the fatty acid fraction of the substance:

- *caproic acid*: maximum 1.0 per cent,
- *caprylic acid*: maximum 10.0 per cent,
- *capric acid*: maximum 10.0 per cent,
- *lauric acid*: 40.0 per cent to 60.0 per cent,
- *myristic acid*: 14.0 per cent to 25.0 per cent,
- *palmitic acid*: 7.0 per cent to 15.0 per cent,
- *stearic acid*: maximum 7.0 per cent,
- *oleic acid*: maximum 11.0 per cent,
- *linoleic acid*: maximum 3.0 per cent.

Heavy metals (2.4.8): maximum 10 ppm.

2.0 g complies with limit test D. Prepare the standard using 2 ml of *lead standard solution* (10 ppm Pb) *R*.

Water (2.5.12): maximum 1.5 per cent, determined on 1.00 g.

Total ash (2.4.16): maximum 0.5 per cent.

STORAGE

Protected from light.

01/2005:1041

01/2005:1042

SORBITAN OLEATE**Sorbitani oleas****DEFINITION**

Mixture usually obtained by esterification of 1 mole of sorbitol and its mono- and di-anhydrides per mole of oleic acid. A suitable antioxidant may be added.

CHARACTERS

Appearance: brownish-yellow, viscous liquid.

Solubility: practically insoluble but dispersible in water, soluble in fatty oils producing a hazy solution, miscible with alcohol.

Relative density: about 0.99.

IDENTIFICATION

- A. It complies with the test for hydroxyl value (see Tests).
- B. It complies with the test for iodine value (see Tests).
- C. It complies with the test for composition of fatty acids (see Tests).

Margaric acid: maximum 0.2 per cent for oleic acid of vegetable origin and maximum 4.0 per cent for oleic acid of animal origin.

TESTS

Acid value (2.5.1): maximum 8.0, determined on 5.0 g.

Hydroxyl value (2.5.3, *Method A*): 190 to 210.

Iodine value (2.5.4): 62 to 76.

Peroxide value (2.5.5): maximum 10.0.

Saponification value (2.5.6): 145 to 160.

Carry out the saponification for 1 h.

Composition of fatty acids. Gas chromatography (2.4.22, *Method C*).

Composition of the fatty acid fraction of the substance:

- *myristic acid*: maximum 5.0 per cent,
- *palmitic acid*: maximum 16.0 per cent,
- *palmitoleic acid*: maximum 8.0 per cent,
- *stearic acid*: maximum 6.0 per cent,
- *oleic acid*: 65.0 per cent to 88.0 per cent,
- *linoleic acid*: maximum 18.0 per cent,
- *linolenic acid*: maximum 4.0 per cent,
- *fatty acids with chain length greater than C₁₈*: maximum 4.0 per cent.

Heavy metals (2.4.8): maximum 10 ppm.

2.0 g complies with limit test D. Prepare the standard using 2 ml of *lead standard solution* (10 ppm Pb) R.

Water (2.5.12): maximum 1.5 per cent, determined on 1.000 g.

Total ash (2.4.16): maximum 0.5 per cent, determined on 1.5 g.

STORAGE

Protected from light.

LABELLING

The label states:

- the name and concentration of any added antioxidant,
- the origin of the oleic acid used (animal or vegetable).

SORBITAN PALMITATE**Sorbitani palmitas****DEFINITION**

Mixture usually obtained by partial esterification of sorbitol and its mono- and di-anhydrides with palmitic acid.

CHARACTERS

Appearance: yellow or yellowish powder, waxy flakes or hard masses.

Solubility: practically insoluble in water, soluble in fatty oils, slightly soluble in alcohol.

IDENTIFICATION

- A. Melting point (2.2.15): 44 °C to 51 °C.

Introduce the melted substance into the glass capillary tubes and allow to stand at a temperature below 10 °C for 24 h.

- B. It complies with the test for hydroxyl value (see Tests).
- C. It complies with the test for composition of fatty acids (see Tests).

TESTS

Acid value (2.5.1): maximum 8.0, determined on 5.0 g.

Hydroxyl value (2.5.3, *Method A*): 270 to 305.

Peroxide value (2.5.5): maximum 5.0.

Saponification value (2.5.6): 140 to 155.

Carry out the saponification for 1 h.

Composition of fatty acids. Gas chromatography (2.4.22, *Method C*).

Composition of the fatty acid fraction of the substance:

- *palmitic acid*: minimum 92.0 per cent,
- *stearic acid*: maximum 6.0 per cent.

Heavy metals (2.4.8): maximum 10 ppm.

2.0 g complies with limit test D. Prepare the standard using 2 ml of *lead standard solution* (10 ppm Pb) R.

Water (2.5.12): maximum 1.5 per cent, determined on 1.00 g.

Total ash (2.4.16): maximum 0.5 per cent.

STORAGE

Protected from light.

01/2005:1916

SORBITAN SESQUIOLEATE**Sorbitani sesquioleas****DEFINITION**

Mixture usually obtained by esterification of 2 moles of sorbitol and its mono- and di-anhydrides per 3 moles of oleic acid. A suitable antioxidant may be added.

CHARACTERS

Appearance: pale yellow or slightly brownish-yellow paste, which becomes a viscous, oily, brownish-yellow liquid at about 25 °C.

Solubility: dispersible in water, soluble in fatty oils, slightly soluble in ethanol.

Relative density: about 0.99.

IDENTIFICATION

- A. It complies with the test for hydroxyl value (see Tests).
 B. It complies with the test for iodine value (see Tests).
 C. It complies with the test for composition of fatty acids (see Tests).

Margaric acid: maximum 0.2 per cent for oleic acid of vegetable origin and maximum 4.0 per cent for oleic acid of animal origin.

TESTS

Acid value (2.5.1): maximum 16.0, determined on 5.0 g.

Hydroxyl value (2.5.3, *Method A*): 180 to 215.

Iodine value (2.5.4): 70 to 95.

Peroxide value (2.5.5): maximum 10.0.

Saponification value (2.5.6): 145 to 166.

Carry out the saponification for 1 h.

Composition of fatty acids. Gas chromatography (2.4.22, *Method C*).

Composition of the fatty acid fraction of the substance:

- *myristic acid*: maximum 5.0 per cent,
- *palmitic acid*: maximum 16.0 per cent,
- *palmitoleic acid*: maximum 8.0 per cent,
- *stearic acid*: maximum 6.0 per cent,
- *oleic acid*: 65.0 per cent to 88.0 per cent,
- *linoleic acid*: maximum 18.0 per cent,
- *linolenic acid*: maximum 4.0 per cent,
- *fatty acids with chain length greater than C₁₈*: maximum 4.0 per cent.

Heavy metals (2.4.8): maximum 10 ppm.

2.0 g complies with limit test D. Prepare the standard using 2 ml of *lead standard solution* (10 ppm Pb) R.

Water (2.5.12): maximum 1.5 per cent, determined on 1.000 g.

Total ash (2.4.16): maximum 0.5 per cent, determined on 1.5 g.

STORAGE

Protected from light.

LABELLING

The label states:

- the name and concentration of any added antioxidant,
- the origin of the oleic acid used (animal or vegetable).

01/2005:1043

SORBITAN STEARATE

Sorbitani stearas

DEFINITION

Mixture usually obtained by partial esterification of sorbitol and its mono- and di-anhydrides with *Stearic acid 50* (1474) or *Stearic acid 70* (1474).

CHARACTERS

Appearance: pale yellow, waxy solid.

Solubility: practically insoluble, but dispersible in water, slightly soluble in alcohol.

IDENTIFICATION

- A. Melting point (2.2.15): 50 °C to 60 °C.

Introduce the melted substance into the capillary tubes and allow to stand at a temperature below 10 °C for 24 h.

- B. It complies with the test for hydroxyl value (see Tests).
 C. It complies with the test for composition of fatty acids (see Tests).

TESTS

Acid value (2.5.1): maximum 10.0, determined on 5.0 g.

Hydroxyl value (2.5.3, *Method A*): 235 to 260.

Peroxide value (2.5.5): maximum 5.0.

Saponification value (2.5.6): 147 to 157.

Carry out the saponification for 1 h.

Composition of fatty acids. Gas chromatography (2.4.22, *Method C*).

Composition of the fatty acid fraction of the substance:

Type of fatty acid used		Composition of fatty acids
Sorbitan stearate (type I)	Stearic acid 50	<i>Stearic acid</i> : 40.0 per cent to 60.0 per cent, <i>Sum of the contents of palmitic and stearic acids</i> : minimum 90.0 per cent.
Sorbitan stearate (type II)	Stearic acid 70	<i>Stearic acid</i> : 60.0 per cent to 80.0 per cent, <i>Sum of the contents of palmitic and stearic acids</i> : minimum 90.0 per cent.

Heavy metals (2.4.8): maximum 10 ppm.

2.0 g complies with limit test D. Prepare the standard using 2 ml of *lead standard solution* (10 ppm Pb) R.

Water (2.5.12): maximum 1.5 per cent, determined on 1.00 g.

Total ash (2.4.16): maximum 0.5 per cent.

STORAGE

Protected from light.

LABELLING

The label states the type of sorbitan stearate.

01/2005:1044

SORBITAN TRIOLEATE

Sorbitani trioleas

DEFINITION

Mixture usually obtained by esterification of 1 mole of sorbitol and its mono-anhydride per 3 moles of oleic acid. A suitable antioxidant may be added.

CHARACTERS

Appearance: pale yellow, light yellowish or brown solid, which becomes a viscous, oily, brownish-yellow liquid at about 25 °C.

Solubility: practically insoluble but dispersible in water, soluble in fatty oils, slightly soluble in alcohol.

Relative density: about 0.98.

IDENTIFICATION

- A. It complies with the test for hydroxyl value (see Tests).
 B. It complies with the test for iodine value (see Tests).
 C. It complies with the test for composition of fatty acids (see Tests).

Margaric acid: maximum 0.2 per cent for oleic acid of vegetable origin and maximum 4.0 per cent for oleic acid of animal origin.

TESTS

Acid value (2.5.1): maximum 16.0, determined on 5.0 g.

Hydroxyl value (2.5.3, Method A): 55 to 75.

Iodine value (2.5.4): 76 to 90.

Peroxide value (2.5.5): maximum 10.0.

Saponification value (2.5.6): 170 to 190.

Carry out the saponification for 1 h.

Composition of fatty acids. Gas chromatography (2.4.22, Method C).

Composition of the fatty acid fraction of the substance:

- *myristic acid*: maximum 5.0 per cent,
- *palmitic acid*: maximum 16.0 per cent,
- *palmitoleic acid*: maximum 8.0 per cent,
- *stearic acid*: maximum 6.0 per cent,
- *oleic acid*: 65.0 per cent to 88.0 per cent,
- *linoleic acid*: maximum 18.0 per cent,
- *linolenic acid*: maximum 4.0 per cent,
- *fatty acids with chain length greater than C₁₈*: maximum 4.0 per cent.

Heavy metals (2.4.8): maximum 10 ppm.

2.0 g complies with limit test D. Prepare the standard using 2 ml of *lead standard solution* (10 ppm Pb) R.

Water (2.5.12): maximum 1.5 per cent, determined on 1.000 g.

Total ash (2.4.16): maximum 0.5 per cent, determined on 1.5 g.

STORAGE

Protected from light.

LABELLING

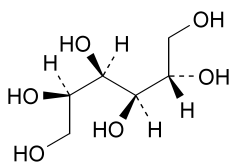
The label states:

- the name and concentration of any added antioxidant,
- the origin of the oleic acid used (animal or vegetable).

01/2005:0435

SORBITOL

Sorbitolum



C₆H₁₄O₆

M_r 182.2

DEFINITION

Sorbitol contains not less than 97.0 per cent and not more than the equivalent of 102.0 per cent of D-glucitol (D-sorbitol), calculated with reference to the anhydrous substance.

CHARACTERS

A white or almost white, crystalline powder, very soluble in water, practically insoluble in alcohol.

It shows polymorphism.

IDENTIFICATION

First identification: A.

Second identification: B, C, D.

A. Examine the chromatograms obtained in the assay.

Results: the principal peak in the chromatogram obtained with the test solution is similar in retention time and size to the principal peak in the chromatogram obtained with reference solution (a).

B. Dissolve 0.5 g with heating in a mixture of 0.5 ml of *pyridine R* and 5 ml of *acetic anhydride R*. After 10 min, pour the solution into 25 ml of *water R* and allow to stand in iced water for 2 h. The precipitate, recrystallised from a small volume of *alcohol R* and dried *in vacuo*, melts (2.2.14) at 98 °C to 104 °C.

C. Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel G plate R*.

Test solution. Dissolve 25 mg of the substance to be examined in *water R* and dilute to 10 ml with the same solvent.

Reference solution (a). Dissolve 25 mg of *sorbitol CRS* in *water R* and dilute to 10 ml with the same solvent.

Reference solution (b). Dissolve 25 mg of *mannitol CRS* and 25 mg of *sorbitol CRS* in *water R* and dilute to 10 ml with the same solvent.

Apply to the plate 2 µl of each solution. Develop over a path of 17 cm using a mixture of 10 volumes of *water R*, 20 volumes of *ethyl acetate R* and 70 volumes of *propanol R*. Allow the plate to dry in air and spray with *4-aminobenzoic acid solution R*. Dry the plate in a current of cold air until the acetone is removed. Heat the plate at 100 °C for 15 min. Allow to cool and spray with a 2 g/l solution of *sodium periodate R*. Dry the plate in a current of cold air. Heat the plate at 100 °C for 15 min. The principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a). The test is not valid unless the chromatogram obtained with reference solution (b) shows 2 clearly separated spots.

D. Dissolve 5.00 g of the substance to be examined and 6.4 g of *disodium tetraborate R* in 40 ml of *water R*. Allow to stand for 1 h, shaking occasionally, and dilute to 50.0 ml with *water R*. Filter if necessary. The specific optical rotation (2.2.7) is + 4.0 to + 7.0, calculated with reference to the anhydrous substance.

TESTS

Appearance of solution. Dissolve 5 g in *water R* and dilute to 50 ml with the same solvent. The solution is clear (2.2.1) and colourless (2.2.2, Method II).

Conductivity (2.2.38). Not more than 20 µS cm⁻¹.

Dissolve 20.0 g in *carbon dioxide-free water R* prepared from *distilled water R* and dilute to 100.0 ml with the same solvent. Measure the conductivity of the solution while gently stirring with a magnetic stirrer.

Reducing sugars. Dissolve 5.0 g in 6 ml of *water R* with the aid of gentle heat. Cool and add 20 ml of *cupri-citric solution R* and a few glass beads. Heat so that boiling begins after 4 min and maintain boiling for 3 min. Cool rapidly and add 100 ml of a 2.4 per cent V/V solution of *glacial acetic acid R* and 20.0 ml of 0.025 M *iodine*. With continuous shaking, add 25 ml of a mixture of 6 volumes of *hydrochloric acid R* and 94 volumes of *water R* and, when the precipitate has dissolved, titrate the excess of iodine with 0.05 M *sodium thiosulphate* using 1 ml of *starch solution R*, added towards the end of the titration, as indicator. Not less than 12.8 ml of 0.05 M *sodium thiosulphate* is required (0.2 per cent, calculated as glucose equivalent).

Related products. Examine by liquid chromatography (2.2.29) as described in the assay. Inject 20 µl of reference solution (b). Adjust the sensitivity of the system so that the height of the peak due to sorbitol is at least 50 per cent of the full scale of the recorder. Inject 20 µl of the test solution and 20 µl of reference solution (c) and continue the chromatography for twice the retention time of sorbitol. In the chromatogram obtained with the test solution: the area of any peak, apart from the principal peak, is not greater than the area of the principal peak in the chromatogram obtained with reference solution (b) (2 per cent); the sum of the areas of all the peaks, apart from the principal peak, is not greater than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (3 per cent). Disregard any peak with an area less than the area of the principal peak in the chromatogram obtained with reference solution (c) (0.1 per cent).

Lead (2.4.10). It complies with the limit test for lead in sugars (0.5 ppm).

Nickel (2.4.15). It complies with the limit test for nickel in polyols (1 ppm). Dissolve the substance to be examined in 150.0 ml of the prescribed mixture of solvents.

Water (2.5.12). Not more than 1.5 per cent, determined on 1.00 g by the semi-micro determination of water.

Microbial contamination. If intended for use in the manufacture of parenteral dosage forms the total viable aerobic count (2.6.12) is not more than 10² bacteria and 10² fungi per gram, determined by plate count. It complies with the tests for *Escherichia coli* and *Salmonella* (2.6.13).

Bacterial endotoxins (2.6.14): if intended for use in the manufacture of parenteral dosage forms without a further appropriate procedure for the removal of bacterial endotoxins, less than 4 IU/g for parenteral dosage forms having a concentration of less than 100 g/l of sorbitol, and less than 2.5 IU/g for parenteral dosage forms having a concentration of 100 g/l or more of sorbitol.

ASSAY

Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 5.0 g of the substance to be examined in 20 ml of *water R* and dilute to 100.0 ml with the same solvent.

Reference solution (a). Dissolve 0.50 g of *sorbitol CRS* in 2 ml of *water R* and dilute to 10.0 ml with the same solvent.

Reference solution (b). Dilute 2.0 ml of the test solution to 100.0 ml with *water R*.

Reference solution (c). Dilute 5.0 ml of reference solution (b) to 100.0 ml with *water R*.

Reference solution (d). Dissolve 0.5 g of *sorbitol R* and 0.5 g of *mannitol R* in 5 ml of *water R* and dilute to 10.0 ml with the same solvent.

The chromatography may be carried out using:

- a stainless steel column 0.3 m long and 7.8 mm in internal diameter packed with *strong cation exchange resin (calcium form) R* (9 µm) and maintained at a temperature of 85 ± 1 °C,
- as mobile phase at a flow rate of 0.5 ml/min, degassed *water R*,
- as detector, a refractometer maintained at a constant temperature.

Inject 20 µl of reference solution (d). Continue the chromatography for 3 times the retention time of sorbitol.

When the chromatograms are recorded in the prescribed conditions, the retention time of sorbitol is about 27 min and the relative retentions with reference to sorbitol are: maltitol

about 0.6, mannitol about 0.8 and iditol about 1.1. The test is not valid unless the resolution between the peaks due to sorbitol and to mannitol is at least 2 in the chromatogram obtained with reference solution (d).

Inject 20 µl of the test solution and 20 µl of reference solution (a). Continue the chromatography for twice the retention time of sorbitol.

Calculate the percentage content of D-sorbitol from the areas of the peaks and the declared content of *sorbitol CRS*.

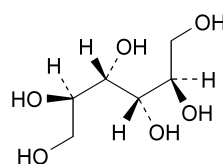
LABELLING

The label states:

- where applicable, the maximum concentration of bacterial endotoxins,
- where applicable, that the substance is suitable for use in the manufacture of parenteral dosage forms.

IMPURITIES

A. mannitol,



B. iditol,

C. maltitol.

01/2005:0436
corrected

SORBITOL, LIQUID (CRYSTALLISING)

Sorbitolum liquidum cristallisabile

DEFINITION

Aqueous solution of a hydrogenated, partly hydrolysed starch.

Content:

- anhydrous substance: 68.0 per cent *m/m* to 72.0 per cent *m/m*,
- D-glucitol (D-sorbitol, C₆H₁₄O₆): 92.0 per cent to 101.0 per cent (anhydrous substance).

CHARACTERS

Appearance: clear, colourless, syrupy liquid, miscible with water.

IDENTIFICATION

A. Examine the chromatograms obtained in the assay.

Results: the principal peak in the chromatogram obtained with the test solution is similar in retention time to the principal peak in the chromatogram obtained with reference solution (a).

B. To 7.0 g add 40 ml of *water R* and 6.4 g of *disodium tetraborate R*, allow to stand for 1 h, shaking occasionally, and dilute to 50.0 ml with *water R*. Filter if necessary. The angle of rotation (2.2.7) is 0° to + 1.5°.

C. It is a clear, syrupy liquid at a temperature of 25 °C.

TESTS

Appearance of solution. The solution is clear (2.2.1) and colourless (2.2.2, *Method II*).

Dilute 7.0 g to 50 ml with *water R*.

Conductivity (2.2.38): maximum $10 \mu\text{S cm}^{-1}$ measured on the undiluted liquid sorbitol (crystallising) while gently stirring with a magnetic stirrer.

Reducing sugars: maximum 0.2 per cent calculated as glucose equivalent.

To 5.0 g add 6 ml of *water R*, 20 ml of *cupri-citric solution R* and a few glass beads. Heat so that boiling begins after 4 min and maintain boiling for 3 min. Cool rapidly and add 100 ml of a 2.4 per cent *V/V* solution of *glacial acetic acid R* and 20.0 ml of 0.025 *M* *iodine*. With continuous shaking, add 25 ml of a mixture of 6 volumes of *hydrochloric acid R* and 94 volumes of *water R* and, when the precipitate has dissolved, titrate the excess of iodine with 0.05 *M* *sodium thiosulphate* using 1 ml of *starch solution R*, added towards the end of the titration, as indicator. Not less than 12.8 ml of 0.05 *M* *sodium thiosulphate* is required.

Lead (2.4.10): maximum 0.5 ppm.

Nickel (2.4.15): maximum 1 ppm.

Water (2.5.12): 28.0 per cent to 32.0 per cent *m/m*, determined on 0.1 g.

ASSAY

Liquid chromatography (2.2.29).

Test solution. Mix 1.00 g of the substance to be examined with 20 ml of *water R* and dilute to 50.0 ml with the same solvent.

Reference solution (a). Dissolve 65.0 mg of *sorbitol CRS* in 2 ml of *water R* and dilute to 5.0 ml with the same solvent.

Reference solution (b). Dissolve 65 mg of *mannitol R* and 65 mg of *sorbitol R* in 2 ml of *water R* and dilute to 5.0 ml with the same solvent.

Column:

- **size:** $l = 0.3 \text{ m}$, $\varnothing = 7.8 \text{ mm}$,
- **stationary phase:** strong cation exchange resin (calcium form) *R* (9 μm),
- **temperature:** $85 \pm 1^\circ\text{C}$.

Mobile phase: degassed *water R*.

Flow rate: 0.5 ml/min.

Detection: refractometer maintained at a constant temperature.

Injection: 20 μl .

Run time: 3 times the retention time of sorbitol.

Relative retention with reference to sorbitol (retention time = about 27 min): mannitol = about 0.8.

System suitability: reference solution (b):

- **resolution:** minimum 2 between the peaks due to mannitol and to sorbitol.

Calculate the percentage content of D-sorbitol from the areas of the peaks and the declared content of *sorbitol CRS*.

01/2005:0437
corrected

SORBITOL, LIQUID (NON-CRYSTALLISING)

Sorbitolum liquidum non cristallisabile

DEFINITION

Aqueous solution of a hydrogenated, partly hydrolysed starch.

Content:

- anhydrous substance: 68.0 per cent *m/m* to 72.0 per cent *m/m*,

- D-glucitol (D-sorbitol, $\text{C}_6\text{H}_{14}\text{O}_6$): 72.0 per cent to 92.0 per cent (anhydrous substance).

CHARACTERS

Appearance: clear, colourless, syrupy liquid, miscible with water.

IDENTIFICATION

A. Examine the chromatograms obtained in the assay.

Results: the principal peak in the chromatogram obtained with the test solution is similar in retention time to the principal peak in the chromatogram obtained with reference solution (a).

B. To 7.0 g add 40 ml of *water R* and 6.4 g of *disodium tetraborate R*. Allow to stand for 1 h, shaking occasionally, and dilute to 50.0 ml with *water R*. Filter if necessary. The angle of rotation (2.2.7) is $+1.5^\circ$ to $+3.5^\circ$.

C. It is a clear, syrupy liquid at 25°C .

TESTS

Appearance of solution. The solution is clear (2.2.1) and colourless (2.2.2, *Method II*).

Dilute 7.0 g to 50 ml with *water R*.

Conductivity (2.2.38): maximum $10 \mu\text{S cm}^{-1}$ measured on the undiluted liquid sorbitol (non crystallising) while gently stirring with a magnetic stirrer.

Reducing sugars: maximum 0.2 per cent calculated as glucose equivalent.

To 5.0 g add 6 ml of *water R*, 20 ml of *cupri-citric solution R* and a few glass beads. Heat so that boiling begins after 4 min and maintain boiling for 3 min. Cool rapidly and add 100 ml of a 2.4 per cent *V/V* solution of *glacial acetic acid R* and 20.0 ml of 0.025 *M* *iodine*. With continuous shaking, add 25 ml of a mixture of 6 volumes of *hydrochloric acid R* and 94 volumes of *water R* and, when the precipitate has dissolved, titrate the excess of iodine with 0.05 *M* *sodium thiosulphate* using 1 ml of *starch solution R*, added towards the end of the titration, as indicator. Not less than 12.8 ml of 0.05 *M* *sodium thiosulphate* is required.

Reducing sugars after hydrolysis: maximum 9.3 per cent calculated as glucose equivalent.

To 6.0 g add 35 ml of *water R*, 40 ml of 1 *M* *hydrochloric acid* and a few glass beads. Boil under a reflux condenser for 4 h. Cool and neutralise with *dilute sodium hydroxide solution R* using 0.2 ml of *bromothymol blue solution R1* as indicator. Cool and dilute to 100.0 ml with *water R*. To 3.0 ml of the solution add 5 ml of *water R*, 20 ml of *cupri-citric solution R* and a few glass beads. Heat so that boiling begins after 4 min and maintain boiling for 3 min. Cool rapidly and add 100 ml of a 2.4 per cent *V/V* solution of *glacial acetic acid R* and 20.0 ml of 0.025 *M* *iodine*. With continuous shaking, add 25 ml of a mixture of 6 volumes of *hydrochloric acid R* and 94 volumes of *water R*. When the precipitate has dissolved, titrate the excess of iodine with 0.05 *M* *sodium thiosulphate* using 1 ml of *starch solution R*, added towards the end of the titration, as indicator. Not less than 8.0 ml of 0.05 *M* *sodium thiosulphate* is required.

Lead (2.4.10): maximum 0.5 ppm.

Nickel (2.4.15): maximum 1 ppm.

Water (2.5.12): 28.0 per cent to 32.0 per cent *m/m*, determined on 0.1 g.

ASSAY

Liquid chromatography (2.2.29).

Test solution. Mix 1.00 g of the substance to be examined with 20 ml of *water R* and dilute to 50.0 ml with the same solvent.

Reference solution (a). Dissolve 55.0 mg of *sorbitol CRS* in 2 ml of *water R* and dilute to 5.0 ml with the same solvent.

Reference solution (b). Dissolve 55 mg of *mannitol R* and 55 mg of *sorbitol R* in 2 ml of *water R* and dilute to 5.0 ml with the same solvent.

Column:

- size: $l = 0.3$ m, $\varnothing = 7.8$ mm,
- stationary phase: strong cation exchange resin (calcium form) *R* (9 μ m),
- temperature: 85 ± 1 °C.

Mobile phase: degassed *water R*.

Flow rate: 0.5 ml/min.

Detection: refractometer maintained at a constant temperature.

Injection: 20 μ l.

Run time: 3 times the retention time of sorbitol.

Relative retention with reference to sorbitol (retention time = about 27 min): mannitol = about 0.8.

System suitability: reference solution (b):

- resolution: minimum 2 between the peaks due to mannitol and to sorbitol.

Calculate the percentage content of D-sorbitol from the areas of the peaks and the declared content of *sorbitol CRS*.

01/2005:2048

SORBITOL, LIQUID, PARTIALLY DEHYDRATED

Sorbitolum liquidum partim deshydricum

DEFINITION

Liquid sorbitol, partially dehydrated is obtained by acid-catalysed partial internal dehydration of liquid sorbitol. It contains not less than 68.0 per cent *m/m* and not more than 85.0 per cent *m/m* of anhydrous substances, composed of a mixture of mainly D-sorbitol and 1,4-sorbitan with mannitol, hydrogenated oligo- and disaccharides, and sorbitans.

Content (nominal value):

- 1,4-sorbitan ($C_6H_{12}O_5$): minimum 15.0 per cent (anhydrous substance),
- D-sorbitol ($C_6H_{14}O_6$): minimum 25.0 per cent (anhydrous substance).

The contents of 1,4-sorbitan and D-sorbitol are within 95.0 per cent to 105.0 per cent of the nominal values.

CHARACTERS

Appearance: clear, colourless, syrupy liquid.

Solubility: miscible with water, practically insoluble in mineral oils and vegetable oils.

IDENTIFICATION

Examine the chromatograms obtained in the assay.

Results: the 2 principal peaks in the chromatogram obtained with the test solution are similar in retention time and size to the peaks in the chromatogram obtained with reference solution (a).

TESTS

Solution S. Dilute the substance to be examined with *carbon dioxide-free water R* prepared from *distilled water R* to obtain a solution containing 50.0 per cent *m/m* of anhydrous substance.

Appearance of solution. Solution S is clear (2.2.1) and colourless (2.2.2, *Method II*).

Conductivity (2.2.38): maximum 20 μ S $\cdot$ cm⁻¹.

Measure the conductivity of solution S, while gently stirring with a magnetic stirrer.

Reducing sugars: maximum 0.3 per cent, calculated as glucose (anhydrous substance).

To an amount of the substance to be examined equivalent to 3.3 g of anhydrous substance, add 3 ml of *water R*, 20.0 ml of *cupri-citric solution R* and a few glass beads. Heat so that boiling begins after 4 min. Maintain boiling for 3 min. Cool rapidly and add 100 ml of a 2.4 per cent *V/V* solution of *glacial acetic acid R* and 20.0 ml of 0.025 *M* iodine. With continuous shaking, add 25 ml of a mixture of 6 ml of *hydrochloric acid R* and 94 ml of *water R*. When the precipitate has dissolved, titrate the excess of iodine with 0.05 *M* sodium thiosulphate using 2 ml of *starch solution R*, added towards the end of the titration, as indicator. Not less than 12.8 ml of 0.05 *M* sodium thiosulphate is required.

Nickel (2.4.15): maximum 1 ppm (anhydrous substance).

Water (2.5.12): 15.0 per cent to 32.0 per cent, determined on 0.10 g.

Microbial contamination. Total viable aerobic count (2.6.12) not more than 10³ micro-organisms per gram and not more than 10² fungi per gram, determined by plate count. It complies with the tests for *Escherichia coli* and *Salmonella* (2.6.13).

ASSAY

Liquid chromatography (2.2.29).

Test solution. Dissolve 0.400 g of the substance to be examined in *water R* and dilute to 20.0 ml with the same solvent.

Reference solution (a). Dissolve 50.0 mg of *sorbitol CRS* and 20.0 mg of 1,4-sorbitan *CRS* in *water R* and dilute to 5.0 ml with the same solvent.

Reference solution (b). Dissolve 0.100 g of *mannitol R* and 0.100 g of *sorbitol R* in *water R* and dilute to 10.0 ml with the same solvent.

Column:

- size: $l = 0.3$ m, $\varnothing = 7.8$ mm,
- stationary phase: strong cation exchange resin (calcium form) *R* (9 μ m),
- temperature: 80 ± 5 °C.

Mobile phase: degassed *water R*.

Flow rate: 0.5 ml/min.

Detection: refractometer maintained at a constant temperature of about 30-35 °C.

Injection: 40 μ l.

Relative retention with reference to D-sorbitol (retention time = about 25 min): 1,4-sorbitan = about 0.5; mannitol = about 0.8.

System suitability: reference solution (b):

- resolution: minimum 2.0 between the peaks due to mannitol and D-sorbitol.

Calculate the percentage contents of 1,4-sorbitan and D-sorbitol using the chromatogram obtained with reference solution (a) and the declared contents of 1,4-sorbitan CRS and of sorbitol CRS.

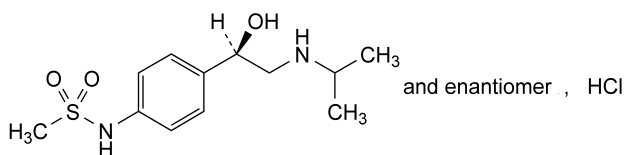
LABELLING

The label states the content of D-sorbitol and the content of 1,4-sorbitan (= nominal values).

01/2005:2004

SOTALOL HYDROCHLORIDE

Sotaloli hydrochloridum



$C_{12}H_{21}ClN_2O_3S$

M_r 308.8

DEFINITION

N-[4-[(1*S*)-1-hydroxy-2-[(1-methylethyl)amino]ethyl]phenyl]methanesulphonamide hydrochloride.

Content: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

Appearance: white or almost white powder.

Solubility: freely soluble in water, soluble in alcohol, practically insoluble in methylene chloride.

IDENTIFICATION

A. Infrared absorption spectrophotometry (2.2.24).

Comparison: sotalol hydrochloride CRS.

B. It gives reaction (a) of chlorides (2.3.1).

TESTS

Solution S. Dissolve 5.0 g in carbon dioxide-free water *R* and dilute to 50.0 ml with the same solvent.

Appearance of solution. Solution S is not more opalescent than reference suspension III (2.2.1) and not more intensely coloured than reference solution Y_6 (2.2.2, *Method II*).

pH (2.2.3): 4.0 to 5.0.

Dilute 5.0 ml of solution S to 10.0 ml with carbon dioxide-free water *R*.

Optical rotation (2.2.7): -0.10° to $+0.10^\circ$.

Dilute 25.0 ml of solution S to 50.0 ml with water *R*.

Related substances. Liquid chromatography (2.2.29).

Test solution. Dissolve 20.0 mg of the substance to be examined in the mobile phase and dilute to 10.0 ml with the mobile phase.

Reference solution (a). Dilute 1.0 ml of the test solution to 100.0 ml with the mobile phase. Dilute 3.0 ml of this solution to 10.0 ml with the mobile phase.

Reference solution (b). Dissolve 8.0 mg of sotalol impurity B CRS in the mobile phase and dilute to 10.0 ml with the mobile phase.

Reference solution (c). Dilute 1.0 ml of reference solution (b) to 100.0 ml with the mobile phase.

Reference solution (d). Dilute 1.5 ml of reference solution (b) to 100 ml with the mobile phase. To 1 ml of this solution add 1 ml of reference solution (a).

Column:

– *size*: $l = 0.25$ m, $\varnothing = 4.6$ mm,

– *stationary phase*: octadecylsilyl silica gel for chromatography *R* (5 μ m).

Mobile phase: dissolve 2 g of sodium octanesulphonate *R* in 790 ml of water *R*. Adjust to pH 3.0 with phosphoric acid *R* and add 210 ml of acetonitrile *R*.

Flow rate: 1 ml/min.

Detection: spectrophotometer at 228 nm.

Injection: 10 μ l; inject the test solution and reference solutions (a), (c) and (d).

Run time: 2.5 times the retention time of sotalol.

System suitability: reference solution (d):

– *resolution*: minimum 4.0 between the peaks due to sotalol and to impurity B.

Limits:

– *impurity B*: not more than 0.25 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.1 per cent),

– *any other impurity*: not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.3 per cent), and not more than 1 such peak has an area greater than 0.3 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent),

– *total of other impurities*: not more than 1.65 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent),

– *disregard limit*: 0.17 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

Palladium: maximum 0.5 ppm.

Atomic absorption spectrometry (2.2.23, *Method I*).

Test solution. Dissolve 1.00 g in a mixture of 0.25 volumes of nitric acid *R*, 0.75 volumes of hydrochloric acid *R* and 99.0 volumes of water *R* and dilute to 20.0 ml with the same mixture of solvents.

Reference solutions. Use solutions containing 0.02 μ g, 0.03 μ g and 0.05 μ g of palladium per millilitre, freshly prepared by dilution of palladium standard solution (0.5 ppm Pd) *R* with a mixture of 0.25 volumes of nitric acid *R*, 0.75 volumes of hydrochloric acid *R* and 99.0 volumes of water *R*.

Source: palladium hollow-cathode lamp.

Wavelength: 247.6 nm.

Use a graphite tube.

Heavy metals (2.4.8): maximum 20 ppm.

To 10 ml of solution S add 10 ml of water *R*. 12 ml of the solution complies with limit test A. Prepare the standard using lead standard solution (1 ppm Pb) *R*.

Loss on drying (2.2.32): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 100–105 $^\circ$ C.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

In order to avoid overheating during the titration, mix thoroughly throughout and stop the titration immediately after the end-point has been reached.

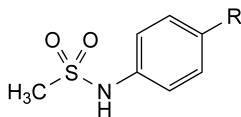
Dissolve 0.250 g in 10 ml of anhydrous formic acid *R*, if necessary with the aid of ultrasound. Add 40 ml of acetic anhydride *R* and titrate immediately with 0.1 M perchloric acid. Determine the end-point potentiometrically (2.2.20).

1 ml of 0.1 M perchloric acid is equivalent to 30.88 mg of $C_{12}H_{21}ClN_2O_3S$.

STORAGE

Protected from light.

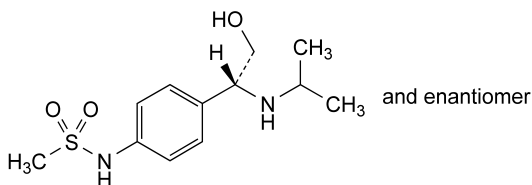
IMPURITIES



A. R = $CH_2CH_2NHCH(CH_3)_2$: N-[4-[2-[(1-methylethyl)amino]ethyl]phenyl]methanesulphonamide,

B. R = $COCH_2NHCH(CH_3)_2$: N-[4-[(1-methylethyl)amino]acetyl]phenyl]methanesulphonamide,

C. R = CHO: N-(4-formylphenyl)methanesulphonamide,



D. N-[4-[(1RS)-2-hydroxy-1-[(1-methylethyl)amino]ethyl]phenyl]methanesulphonamide.

01/2005:1265

SOYA-BEAN OIL, HYDROGENATED

Soiae oleum hydrogenatum

DEFINITION

Hydrogenated soya-bean oil is the product obtained by refining, bleaching, hydrogenation and deodorisation of oil obtained from seeds of *Glycine soja* Sieb. and Zucc. and *Glycine max* (L.) Merr. (*G. hispida* (Moench) Maxim.). The product consists mainly of triglycerides of palmitic and stearic acids.

CHARACTERS

A white mass or powder which melts to a clear, pale yellow liquid when heated, practically insoluble in water, freely soluble in methylene chloride, in light petroleum (bp: 65 °C to 70 °C) after heating and in toluene, very slightly soluble in alcohol.

IDENTIFICATION

- It complies with the test for melting point (see Tests).
- It complies with the test for foreign fatty oils (see Tests).

TESTS

Melting point (2.2.15): 66 °C to 72 °C.

Acid value (2.5.1). Not more than 0.5, determined on 10.0 g. Dissolve the substance to be examined in 50 ml of a hot mixture of equal volumes of *alcohol R* and *toluene R*, previously neutralised with 0.1 M potassium hydroxide using 0.5 ml of *phenolphthalein solution R1* as indicator. Titrate the solution immediately while still hot.

Peroxide value (2.5.5). Not more than 5.0.

Unsaponifiable matter (2.5.7). Not more than 1.0 per cent, determined on 5.0 g.

Alkaline impurities in fatty oils (2.4.19). Dissolve 2.0 g with gentle heating in a mixture of 1.5 ml of *alcohol R* and 3 ml of *toluene R*. Add 0.05 ml of a 0.4 g/l solution of *bromophenol blue R* in *alcohol R*. Not more than 0.4 ml of 0.01 M *hydrochloric acid* is required to change the colour to yellow.

Composition of fatty acids (2.4.22, Method A).

The chromatographic procedure may be carried out using:

- a fused-silica column 25 m long and 0.25 mm in internal diameter coated with *poly(cyanopropyl)siloxane R* (film thickness 0.2 µm),
- helium for chromatography R* as the carrier gas at a flow rate of 0.65 ml/min,
- a flame-ionisation detector,
- a split injector (1:100),

maintaining the temperature of the column at 180 °C for 20 min and that of the injection port and the detector at 250 °C.

The fatty acid fraction of the oil has the following composition:

- saturated fatty acids of chain length less than C_{14} : not more than 0.1 per cent,
- myristic acid: not more than 0.5 per cent,
- palmitic acid: 9.0 per cent to 16.0 per cent,
- stearic acid: 79.0 per cent to 89.0 per cent,
- oleic acid and isomers ($C_{18:1}$ equivalent chain length on *poly(cyanopropyl)siloxane* 18.5 to 18.8): not more than 4.0 per cent,
- linoleic acid and isomers ($C_{18:2}$ equivalent chain length on *poly(cyanopropyl)siloxane* 19.4 to 19.8): not more than 1.0 per cent,
- linolenic acid and isomers ($C_{18:3}$ equivalent chain length on *poly(cyanopropyl)siloxane* 20.3 to 20.7): not more than 0.2 per cent,
- arachidic acid: not more than 1.0 per cent,
- behenic acid: not more than 1.0 per cent.

Nickel. Not more than 1 ppm of Ni, determined by atomic absorption spectrometry (2.2.23, Method II).

Test solution. Introduce 5.0 g into a platinum or silica crucible, previously tared after calcination. Cautiously heat and introduce into the substance a wick formed from twisted ashless filter paper. Light the wick. When the substance is alight stop heating. After combustion, ignite in a muffle furnace at about 600 °C. Continue the ignition until white ash is obtained. After cooling, take up the residue with two quantities, each of 2 ml, of *dilute hydrochloric acid R* and transfer into a 25 ml graduated flask. Add 0.3 ml of *nitric acid R* and dilute to 25.0 ml with *water R*.

Reference solutions. Prepare three reference solutions by adding 1.0 ml, 2.0 ml and 4.0 ml of *nickel standard solution* (0.2 ppm Ni) *R* to 2.0 ml of the test solution and diluting to 10.0 ml with *water R*.

Measure the absorbance at 232 nm using a nickel hollow-cathode lamp as a source of radiation, a graphite furnace as an atomic generator and *argon R* as the carrier gas.

Water (2.5.12). Not more than 0.3 per cent, determined on 1.000 g by the semi-micro determination of water.

STORAGE

Store protected from light.

01/2005:1473 STORAGE

SOYA-BEAN OIL, REFINED**Soiae oleum raffinatum****DEFINITION**

Refined soya-bean oil is the fatty oil obtained from seeds of *Glycine soja* Sieb. and Zucc. and *Glycine max* (L.) Merr. (*G. hispida* (Moench) Maxim.) by extraction and subsequent refining. It may contain a suitable antioxidant.

CHARACTERS

A clear, pale yellow, liquid, miscible with light petroleum (50 °C to 70 °C), practically insoluble in alcohol.

It has a relative density of about 0.922 and a refractive index of about 1.475.

IDENTIFICATION

Carry out the identification of fatty oils by thin-layer chromatography (2.3.2). The chromatogram obtained is similar to the typical chromatogram for soya-bean oil.

TESTS

Acid value (2.5.1). Not more than 0.5, determined on 10.0 g.

Peroxide value (2.5.5, Method A). Not more than 10.0. If intended for use in the manufacture of parenteral dosage forms, the peroxide value is not more than 5.0.

Unsaponifiable matter (2.5.7). Not more than 1.5 per cent, determined on 5.0 g.

Alkaline impurities (2.4.19). It complies with the test for alkaline impurities in fatty oils.

Composition of fatty acids (2.4.22, Method A). The fatty acid fraction of the oil has the following composition:

- *saturated fatty acids of chain length less than C₁₄*: not more than 0.1 per cent,
- *myristic acid*: not more than 0.2 per cent,
- *palmitic acid*: 9.0 per cent to 13.0 per cent,
- *palmitoleic acid* (equivalent chain length on polyethyleneglycol adipate 16.3): not more than 0.3 per cent,
- *stearic acid*: 3.0 per cent to 5.0 per cent,
- *oleic acid* (equivalent chain length on polyethyleneglycol adipate 18.3): 17.0 per cent to 30.0 per cent,
- *linoleic acid* (equivalent chain length on polyethyleneglycol adipate 18.9): 48.0 per cent to 58.0 per cent,
- *linolenic acid* (equivalent chain length on polyethyleneglycol adipate 19.7): 5.0 per cent to 11.0 per cent,
- *arachidic acid*: not more than 1.0 per cent,
- *eicosenoic acid* (equivalent chain length on polyethyleneglycol adipate 20.3): not more than 1.0 per cent,
- *behenic acid*: not more than 1.0 per cent.

Brassicasterol (2.4.23). The sterol fraction of the oil contains not more than 0.3 per cent of brassicasterol.

Water (2.5.32). If intended for use in the manufacture of parenteral dosage forms, not more than 0.1 per cent, determined on 5.00 g by the coulometric determination of water. Use a mixture of equal volumes of *decanol R* and *anhydrous methanol R* as the solvent.

STORAGE

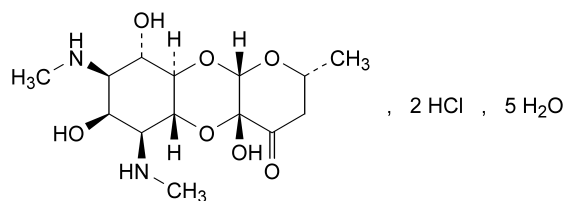
Store in a well-filled container, protected from light, at a temperature not exceeding 25 °C.

LABELLING

The label states:

- where applicable, that the substance is suitable for use in the manufacture of parenteral dosage forms,
- the name and concentration of any added antioxidant.

01/2005:1152

SPECTINOMYCIN HYDROCHLORIDE**Spectinomycini hydrochloridum**C₁₄H₂₆Cl₂N₂O₇·5H₂OM_r 495.4**DEFINITION**

Spectinomycin hydrochloride is the pentahydrate of the dihydrochloride of (2*R*,4*aR*,5*aR*,6*S*,7*S*,8*R*,9*S*,9*aR*,10*aS*)-4*a*,7,9-trihydroxy-2-methyl-6,8-bis(methylamino)decahydro-4*H*-pyrano[2,3-*b*][1,4]benzodioxin-4-one dihydrochloride, an antimicrobial substance produced by *Streptomyces spectabilis* or by any other means. It contains not less than 95.0 per cent and not more than the equivalent of 100.5 per cent of C₁₄H₂₆Cl₂N₂O₇ calculated with reference to the anhydrous substance.

CHARACTERS

A white or almost white powder, slightly hygroscopic, freely soluble in water, very slightly soluble in alcohol.

IDENTIFICATION

- A. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *spectinomycin hydrochloride CRS*.
- B. Dilute 1.0 ml of solution S (see Tests) to 10 ml with *water R*. The solution gives reaction (a) of chlorides (2.3.1).

TESTS

Solution S. Dissolve 2.0 g in *carbon dioxide-free water R* and dilute to 20.0 ml with the same solvent.

Appearance of solution. Dilute 2.0 ml of solution S to 20.0 ml with *water R*. The solution is clear (2.2.1) and colourless (2.2.2, Method II).

pH (2.2.3). The pH of solution S is 3.8 to 5.6.

Specific optical rotation (2.2.7): + 15 to + 21, determined on solution S within 20 min of preparation and calculated with reference to the anhydrous substance.

Related substances. Examine by thin-layer chromatography (2.2.27), using *silica gel G R* as the coating substance.

Test solution. Dilute 2.0 ml of solution S to 10 ml with *water R*.

Reference solution. Dilute 1.0 ml of the test solution to 100 ml with *water R*.

Apply separately to the plate 10 µl of each solution. Develop over a path of 12 cm using a mixture of 5 volumes of *glacial acetic acid R*, 5 volumes of *pyridine R*, 40 volumes of *water R* and 50 volumes of *propanol R*. Dry the plate in a current of warm air and spray with a 50 g/l solution of *potassium permanganate R*. Allow the plate to stand for 2 min to 3 min. Any spot in the chromatogram obtained with the test solution, apart from the principal spot, is not more intense than the spot in the chromatogram obtained with the reference solution (1.0 per cent).

Water (2.5.12): 16.0 per cent to 20.0 per cent, determined on 0.200 g by the semi-micro determination of water.

Sulphated ash (2.4.14). Not more than 1.0 per cent, determined on 1.000 g.

Bacterial endotoxins (2.6.14): less than 0.09 IU/mg, if intended for use in the manufacture of parenteral dosage forms without a further appropriate procedure for the removal of bacterial endotoxins. Prepare the solutions using a 0.42 per cent *m/m* solution of *sodium hydrogen carbonate R*.

ASSAY

Examine by gas chromatography (2.2.28), using *phenazone R* as the internal standard.

Internal standard solution. Dissolve 0.150 g of *phenazone R* in *dimethylformamide R* and dilute to 100 ml with the same solvent.

Allow the test solutions and the reference solution to stand for 1 h. Carry out the assay immediately.

Test solution (a). To 60.0 mg of the substance to be examined in a volumetric flask, add 10 ml of *dimethylformamide R* and 2.0 ml of *hexamethyldisilazane R*. Shake to dissolve and dilute to 20.0 ml with *dimethylformamide R*.

Test solution (b). To 60.0 mg of the substance to be examined in a volumetric flask add 10.0 ml of the internal standard solution and 2.0 ml of *hexamethyldisilazane R*. Shake to dissolve and dilute to 20.0 ml with *dimethylformamide R*.

Reference solution. To 60.0 mg of *spectinomycin hydrochloride CRS* in a volumetric flask add 10.0 ml of the internal standard solution and 2.0 ml of *hexamethyldisilazane R*. Shake to dissolve and dilute to 20.0 ml with *dimethylformamide R*.

The chromatographic procedure may be carried out using:

- a glass column 1.5 m long and 4 mm in internal diameter packed with *silanised diatomaceous earth for gas chromatography R* (125 µm to 150 µm) impregnated with 3 per cent *m/m* of a mixture of *polyphenylmethylsiloxane R*,
- *nitrogen for chromatography R* as the carrier gas at a flow rate of 45 ml/min,
- a flame-ionisation detector,
- an electronic integrator.

Maintain the temperature of the column at 200 °C and that of the injection port and of the detector between 200 °C and 230 °C. Inject the chosen volumes of the test solutions. The assay is not valid unless in the chromatogram obtained with test solution (b) the resolution between the main peak and the peak corresponding to the internal standard is at least 8.0. Inject alternately test solution (b) and the reference solution.

Calculate the percentage content of spectinomycin.

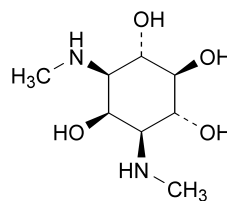
STORAGE

Store in an airtight container, at a temperature not exceeding 30 °C. If the substance is sterile, store in a sterile, airtight, tamper-proof container.

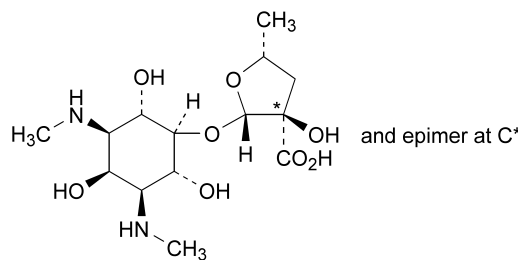
LABELLING

The label states, where applicable, that the substance is free from bacterial endotoxins.

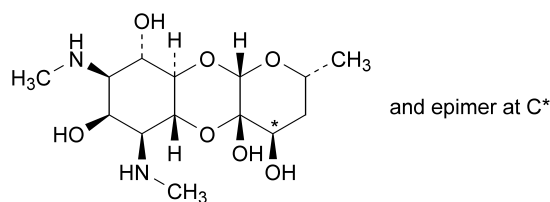
IMPURITIES



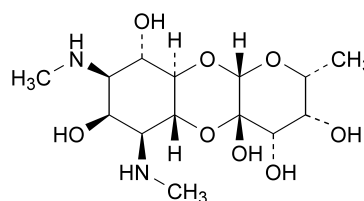
- A. 1,3-dideoxy-1,3-bis(methylamino)-*myo*-inositol (actinamine),



- B. (2*S*,3*RS*,5*R*)-3-hydroxy-5-methyl-2-[[[(1*r*,2*R*,3*S*,4*r*,5*R*,6*S*)-2,4,6-trihydroxy-3,5-bis(methylamino)cyclohexyl]oxy]tetrahydrofuran-3-carboxylic acid (actinospectinoic acid),



- C. (2*R*,4*RS*,4*aS*,5*aR*,6*S*,7*S*,8*R*,9*S*,9*aR*,10*aS*)-2-methyl-6,8-bis(methylamino)decahydro-2*H*-pyrano[2,3-*b*][1,4]benzodioxine-4,4*a*,7,9-tetrol (dihydrospectinomycins),

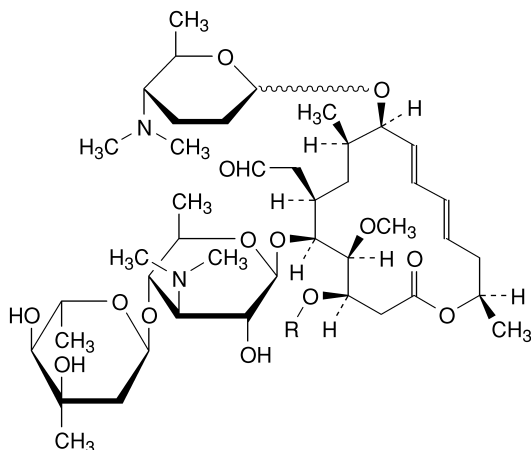


- D. (2*R*,3*R*,4*S*,4*aS*,5*aR*,6*S*,7*S*,8*R*,9*S*,9*aR*,10*aS*)-2-methyl-6,8-bis(methylamino)decahydro-2*H*-pyrano[2,3-*b*][1,4]benzodioxine-3,4,4*a*,7,9-pentol (dihydroxyspectinomycin).

01/2005:0293
corrected

SPIRAMYCIN

Spiramycinum



Spiramycin I	R = H	C ₄₃ H ₇₄ N ₂ O ₁₄
Spiramycin II	R = CO-CH ₃	C ₄₅ H ₇₆ N ₂ O ₁₅
Spiramycin III	R = CO-CH ₂ -CH ₃	C ₄₆ H ₇₈ N ₂ O ₁₅

C₄₃H₇₄N₂O₁₄

DEFINITION

Spiramycin is a macrolide antibiotic produced by the growth of certain strains of *Streptomyces ambofaciens* or obtained by any other means. The main component is (4*R*,5*S*,6*S*,7*R*,9*R*,10*R*,11*E*,13*E*,16*R*)-6-[[3,6-dideoxy-4-*O*-(2,6-dideoxy-3-*C*-methyl-α-*L*-ribohexopyranosyl)-3-(dimethylamino)-β-*D*-glucopyranosyl]oxy]-4-hydroxy-5-methoxy-9,16-dimethyl-7-(2-oxoethyl)-10-[[2,3,4,6-tetra-deoxy-4-(dimethylamino)-*D*-erythrohexopyranosyl]oxy]oxacyclohexadeca-11,13-dien-2-one (spiramycin I, *M_r* 843). Spiramycin II (4-*O*-acetylsiramycin I) and spiramycin III (4-*O*-propanoylsiramycin I) are also present. The potency is at least 4100 IU/mg, calculated with reference to the dried substance.

CHARACTERS

A white or slightly yellowish powder, slightly hygroscopic, slightly soluble in water, freely soluble in acetone, in alcohol and in methanol.

IDENTIFICATION

- A. Dissolve 0.10 g in *methanol R* and dilute to 100.0 ml with the same solvent. Dilute 1.0 ml of the solution to 100.0 ml with *methanol R*. Examined between 220 nm and 350 nm (2.2.25), the solution shows an absorption maximum at 232 nm. The specific absorbance at the maximum is about 340.
- B. Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel G plate R*.
- Test solution.* Dissolve 40 mg of the substance to be examined in *methanol R* and dilute to 10 ml with the same solvent.
- Reference solution (a).* Dissolve 40 mg of *spiramycin CRS* in *methanol R* and dilute to 10 ml with the same solvent.
- Reference solution (b).* Dissolve 40 mg of *erythromycin A CRS* in *methanol R* and dilute to 10 ml with the same solvent.

Apply to the plate 5 µl of each solution. Develop over a path of 15 cm using the upper layer of a mixture of 4 volumes of *2-propanol R*, 8 volumes of a 150 g/l solution of *ammonium acetate R* previously adjusted to pH 9.6 with *strong sodium hydroxide solution R*, and 9 volumes of *ethyl acetate R*. Allow the plate to dry in air, spray with *anisaldehyde solution R1* and heat at 110 °C for 5 min. The principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a). If in the chromatogram obtained with the test solution one or two spots occur with *R_f* values slightly higher than that of the principal spot, these spots are similar in position and colour to the secondary spots in the chromatogram obtained with reference solution (a) and differ from the spots in the chromatogram obtained with reference solution (b).

- C. Dissolve 0.5 g in 10 ml of 0.05 *M* sulphuric acid and add 25 ml of *water R*. Adjust to about pH 8 with 0.1 *M* sodium hydroxide and dilute to 50 ml with *water R*. To 5 ml of this solution add 2 ml of a mixture of 1 volume of *water R* and 2 volumes of sulphuric acid *R*. A brown colour develops.

TESTS

pH (2.2.3). Dissolve 0.5 g in 5 ml of *methanol R* and dilute to 100 ml with *carbon dioxide-free water R*. The pH of the solution is 8.5 to 10.5.

Specific optical rotation (2.2.7). Dissolve 1.00 g in a 10 per cent *V/V* solution of *dilute acetic acid R* and dilute to 50.0 ml with the same acid. The specific optical rotation is –80 to –85, calculated with reference to the dried substance.

Composition. Not less than 80.0 per cent of spiramycin I, not more than 5.0 per cent of spiramycin II and not more than 10.0 per cent of spiramycin III; the sum of the contents of spiramycin I, spiramycin II and spiramycin III is not less than 90.0 per cent, all contents are calculated with reference to the dried substance. Examine by liquid chromatography (2.2.29) as prescribed in the test for related substances.

Inject reference solution (a) 6 times. The test is not valid unless the relative standard deviation of the peak area for spiramycin I is at most 1.0 per cent. Inject alternately the test solution and reference solution (a). Continue the chromatography of the test solution for 3 times the retention time of spiramycin I. Calculate the percentage content of spiramycin I, spiramycin II and spiramycin III using the declared contents of *spiramycin CRS*.

Related substances. Examine by liquid chromatography (2.2.29). Prepare the solutions immediately before use.

Test solution. Dissolve 25.0 mg of the substance to be examined in a mixture of 3 volumes of *acetonitrile R* and 7 volumes of *water R* and dilute to 100.0 ml with the same mixture of solvents.

Reference solution (a). Dissolve 25.0 mg of *spiramycin CRS* in a mixture of 3 volumes of *acetonitrile R* and 7 volumes of *water R* and dilute to 100.0 ml with the same mixture of solvents.

Reference solution (b). Dilute 2.0 ml of reference solution (a) to 100.0 ml with a mixture of 3 volumes of *acetonitrile R* and 7 volumes of *water R*.

Reference solution (c). Dilute 5.0 ml of reference solution (a) to 100.0 ml with a mixture of 3 volumes of *acetonitrile R* and 7 volumes of *water R*.

Reference solution (d). Dissolve 5 mg of *spiramycin CRS* in 25 ml of the mobile phase, then heat in a water-bath at 60 °C for 30 min.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4.6 mm in internal diameter packed with spherical *octylsilyl silica gel for chromatography R* (5 µm) with a specific surface area of 350 m²/g and a pore size of 10 nm,
- as mobile phase at a flow rate of 0.8 ml/min a mixture of 30 volumes of *acetonitrile R* and 70 volumes of *buffer solution pH 2.2 R* containing 9.3 g/l of *sodium perchlorate R*,
- as detector a spectrophotometer set at 232 nm.

Inject 20 µl of reference solution (b). Adjust the sensitivity of the system so that the height of the principal peak in the chromatogram obtained is at least 50 per cent of the full scale of the recorder. Inject 20 µl of reference solution (c) and 20 µl of reference solution (d). The test is not valid unless: in the chromatogram obtained with reference solution (c), there is no significant peak with a retention time, relative to spiramycin I, of about 1.1; in the chromatogram obtained with reference solution (d), the resolution between impurity A (eluting first) and spiramycin I (eluting at 13 min to 17 min) is at least 6.3 (see Figure 0293.-1). If necessary, adjust the concentration of acetonitrile in the mobile phase (increasing the concentration to decrease the retention time or decreasing the concentration to increase the retention time).

Inject 20 µl of the test solution and 20 µl of reference solution (b). Continue the chromatography of the test solution for 3 times the retention time of spiramycin I. In the chromatogram obtained with the test solution: the area of any peak, apart from the peaks corresponding to spiramycin I, spiramycin II and spiramycin III, is not greater than the area of the principal peak in the chromatogram obtained with reference solution (b) (2 per cent). Disregard any peak with an area less than 0.05 times that of the principal peak in the chromatogram obtained with reference solution (b).

Heavy metals (2.4.8). 1.0 g complies with limit test C (20 ppm). Prepare the standard using 2 ml of *lead standard solution (10 ppm Pb) R*.

Loss on drying (2.2.32). Not more than 3.5 per cent, determined on 0.50 g by drying over *diphosphorus pentoxide R* at 80 °C at a pressure not exceeding 670 Pa for 6 h.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Carry out the microbiological assay of antibiotics (2.7.2).

STORAGE

Store in an airtight container.

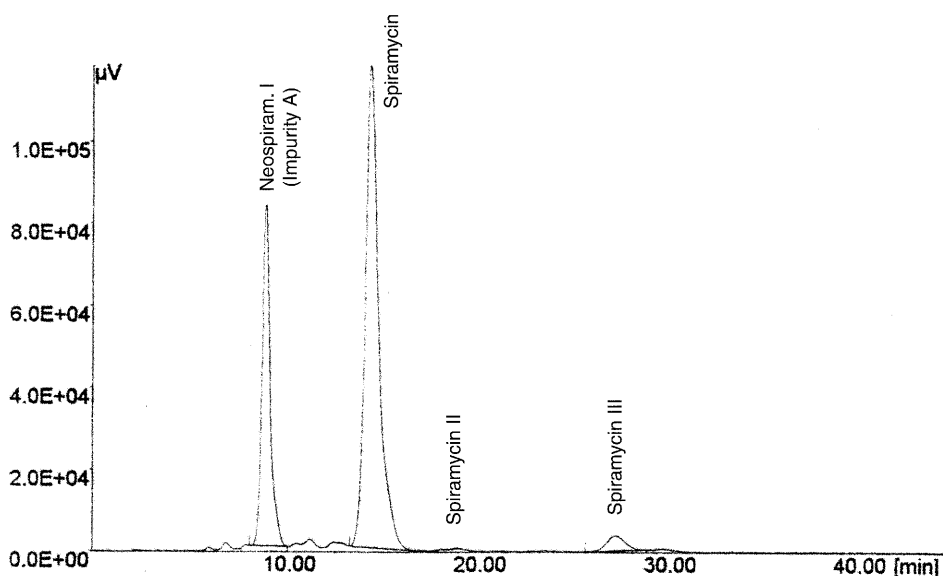
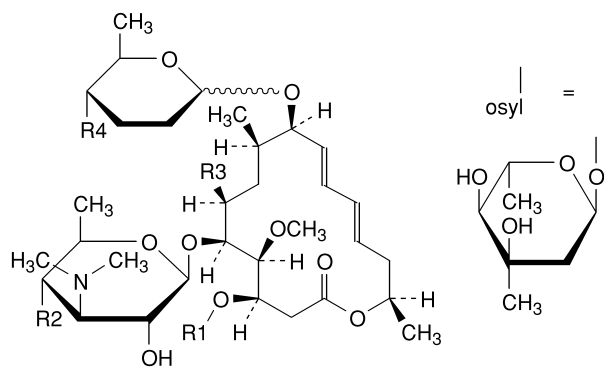


Figure 0293.-1.— Chromatogram for the test for related substances of spiramycin

IMPURITIES

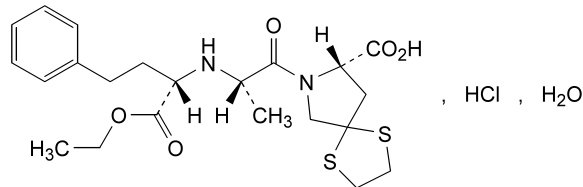
01/2005:1766



- A. $R_1 = H$, $R_2 = OH$, $R_3 = CH_2-CHO$, $R_4 = N(CH_3)_2$: (4*R*,5*S*,6*S*,7*R*,9*R*,10*R*,11*E*,13*E*,16*R*)-6-[[[3,6-dideoxy-3-(dimethylamino)-β-D-glucopyranosyl]oxy]-4-hydroxy-5-methoxy-9,16-dimethyl-7-(2-oxoethyl)-10-[[2,3,4,6-tetra-deoxy-4-(dimethylamino)-D-erythro-hexopyranosyl]oxy]oxacyclohexadeca-11,13-dien-2-one (neospiramycin I),
- B. $R_1 = H$, $R_2 = osyl$, $R_3 = CH_2-CH_2OH$, $R_4 = N(CH_3)_2$: (4*R*,5*S*,6*S*,7*R*,9*R*,10*R*,11*E*,13*E*,16*R*)-6-[[[3,6-dideoxy-4-O-(2,6-dideoxy-3-C-methyl-α-L-ribo-hexopyranosyl)-3-(dimethylamino)-β-D-glucopyranosyl]oxy]-4-hydroxy-7-(2-hydroxyethyl)-5-methoxy-9,16-dimethyl-10-[[2,3,4,6-tetra-deoxy-4-(dimethylamino)-D-erythro-hexopyranosyl]oxy]oxacyclohexadeca-11,13-dien-2-one,
- C. $R_1 = H$, $R_2 = osyl$, $R_3 = CH_2-CH_2-CHO$, $R_4 = N(CH_3)_2$: (4*R*,5*S*,6*S*,7*S*,9*R*,10*R*,11*E*,13*E*,16*R*)-6-[[[3,6-dideoxy-4-O-(2,6-dideoxy-3-C-methyl-α-L-ribo-hexopyranosyl)-3-(dimethylamino)-β-D-glucopyranosyl]oxy]-4-hydroxy-5-methoxy-9,16-dimethyl-7-(2-oxopropyl)-10-[[2,3,4,6-tetra-deoxy-4-(dimethylamino)-D-erythro-hexopyranosyl]oxy]oxacyclohexadeca-11,13-dien-2-one,
- D. $R_1 = H$, $R_2 = osyl$, $R_3 = CH_2-CHO$, $R_4 = OH$: (4*R*,5*S*,6*S*,7*R*,9*R*,10*R*,11*E*,13*E*,16*R*)-6-[[[3,6-dideoxy-4-O-(2,6-dideoxy-3-C-methyl-α-L-ribo-hexopyranosyl)-3-(dimethylamino)-β-D-glucopyranosyl]oxy]-4-hydroxy-5-methoxy-9,16-dimethyl-7-(2-oxoethyl)-10-[(2,3,6-trideoxy-D-erythro-hexopyranosyl)oxy]oxacyclohexadeca-11,13-dien-2-one,
- E. $R_1 = H$, $R_2 = osyl$, $R_3 = CH_2-CH_3$, $R_4 = N(CH_3)_2$: (4*R*,5*S*,6*S*,7*R*,9*R*,10*R*,11*E*,13*E*,16*R*)-6-[[[3,6-dideoxy-4-O-(2,6-dideoxy-3-C-methyl-α-L-ribo-hexopyranosyl)-3-(dimethylamino)-β-D-glucopyranosyl]oxy]-7-ethyl-4-hydroxy-5-methoxy-9,16-dimethyl-10-[[2,3,4,6-tetra-deoxy-4-(dimethylamino)-D-erythro-hexopyranosyl]oxy]oxacyclohexadeca-11,13-dien-2-one,
- G. $R_1 = CO-CH_3$, $R_2 = OH$, $R_3 = CH_2-CHO$, $R_4 = N(CH_3)_2$: (4*R*,5*S*,6*S*,7*R*,9*R*,10*R*,11*E*,13*E*,16*R*)-6-[[[3,6-dideoxy-3-(dimethylamino)-β-D-glucopyranosyl]oxy]-5-methoxy-9,16-dimethyl-2-oxo-7-(2-oxoethyl)-10-[[2,3,4,6-tetra-deoxy-4-(dimethylamino)-D-erythro-hexopyranosyl]oxy]oxacyclohexadeca-11,13-dien-4-yl acetate (neospiramycin II),
- H. $R_1 = CO-C_2H_5$, $R_2 = OH$, $R_3 = CH_2-CHO$, $R_4 = N(CH_3)_2$: (4*R*,5*S*,6*S*,7*R*,9*R*,10*R*,11*E*,13*E*,16*R*)-6-[[[3,6-dideoxy-3-(dimethylamino)-β-D-glucopyranosyl]oxy]-5-methoxy-9,16-dimethyl-2-oxo-7-(2-oxoethyl)-10-[[2,3,4,6-tetra-deoxy-4-(dimethylamino)-D-erythro-hexopyranosyl]oxy]oxacyclohexadeca-11,13-dien-4-yl propanoate (neospiramycin III),
- F. spiramycin dimer.

SPIRAPRIL HYDROCHLORIDE MONOHYDRATE

Spirapril hydrochloridum monohydricum

 $C_{22}H_{31}ClN_2O_5S_2 \cdot H_2O$ M_r 521.1

DEFINITION

(8*S*)-7-[(2*S*)-2-[[[(1*S*)-1-(Ethoxycarbonyl)-3-phenylpropyl]amino]propanoyl]-1,4-dithia-7-azaspiro[4.4]nonane-8-carboxylic acid hydrochloride monohydrate.

Content: 97.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

Appearance: white or almost white, fine crystalline powder.

Solubility: very slightly soluble in water, soluble in methanol, slightly soluble in acetonitrile, practically insoluble in methylene chloride.

IDENTIFICATION

A. Specific optical rotation (see Tests).

B. Infrared absorption spectrophotometry (2.2.24).

Preparation: discs of potassium bromide *R*.

Comparison: spirapril hydrochloride monohydrate CRS.

C. It gives the reactions of chlorides (2.3.1).

TESTS

Specific optical rotation (2.2.7): -11.0 to -13.0 (anhydrous substance).

Dissolve 0.200 g in dimethylformamide *R* and dilute to 20.0 ml with the same solvent.

Related substances. Liquid chromatography (2.2.29).

Solvent mixture: acetonitrile *R1*, water *R* (2:8 V/V).

Test solution. Dissolve 50.0 mg of the substance to be examined in the solvent mixture and dilute to 20.0 ml with the solvent mixture.

Reference solution (a). Dissolve 6.0 mg of spirapril for system suitability CRS (spirapril spiked with impurity B and impurity D) in the solvent mixture and dilute to 20 ml with the solvent mixture.

Reference solution (b). Dilute 5.0 ml of the test solution to 100.0 ml with the solvent mixture. Dilute 5.0 ml of this solution to 50.0 ml with the solvent mixture.

Reference solution (c). Dilute 1.0 ml of reference solution (b) to 10.0 ml with the solvent mixture.

Column:

- *size*: $l = 0.125$ m, $\varnothing = 4.6$ mm,
- *stationary phase*: octadecylsilyl silica gel for chromatography *R* (5 μ m),
- *temperature*: 70 °C.

Mobile phase:

- **mobile phase A:** dissolve 4.5 g of *tetramethylammonium hydroxide R* in 900 ml of *water R*, add 100 ml of *acetonitrile R1* and adjust to pH 2.2 with *phosphoric acid R*,
- **mobile phase B:** dissolve 4.5 g of *tetramethylammonium hydroxide R* in 400 ml of *water R*, add 600 ml of *acetonitrile R1* and adjust to pH 2.2 with *phosphoric acid R*,

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)
0 - 4	90	10
4 - 14	90 → 10	10 → 90
14 - 20	10	90
20 - 23	10 → 90	90 → 10
23 - 33	90	10

Flow rate: 2.0 ml/min.

Detection: spectrophotometer at 210 nm.

Injection: 20 µl.

Relative retention with reference to spirapril (retention time = about 10 min): impurity C = about 0.6; impurity B = about 0.7; impurity A = about 1.26; impurity D = about 1.38.

System suitability: reference solution (a):

- **resolution:** minimum 3.5 between the peaks due to impurity B and spirapril, and minimum 5.5 between the peaks due to spirapril and impurity D.

Limits:

- **impurities A, C:** for each impurity, not more than 0.4 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent),
- **impurity B:** not more than 0.6 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.3 per cent),
- **impurity D:** not more than 0.8 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.4 per cent),
- **any other impurity:** for each impurity, not more than 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent),
- **total:** not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (1.0 per cent),
- **disregard limit:** area of the principal peak in the chromatogram obtained with reference solution (c) (0.05 per cent); disregard any peak due to the blank (solvent mixture).

Water (2.5.12): 3.0 per cent to 4.0 per cent, determined on 0.200 g.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (2.2.29).

Solvent mixture. Mix equal volumes of *acetonitrile R1* and *water R*.

Test solution. Dissolve 20.0 mg of the substance to be examined in the solvent mixture and dilute to 100.0 ml with the solvent mixture.

Reference solution (a). Dissolve 20.0 mg of *spirapril hydrochloride monohydrate CRS* in the solvent mixture and dilute to 100.0 ml with the solvent mixture.

Reference solution (b). Dissolve 6.0 mg of *spirapril for system suitability CRS* (spirapril spiked with impurity B and impurity D) in a mixture of 2 volumes of *acetonitrile R* and 8 volumes of *water R* and dilute to 20 ml with the same mixture of solvents.

Solution A. Dissolve 4.5 g of *tetramethylammonium hydroxide R* in 900 ml of *water R*, adjust to pH 1.75 with *phosphoric acid R* and add 100 ml of *acetonitrile R1*.

Solution B. Dissolve 4.5 g of *tetramethylammonium hydroxide R* in 400 ml of *water R*, adjust to pH 1.75 with *phosphoric acid R* and add 600 ml of *acetonitrile R1*.

Column:

- **size:** $l = 0.125$ m, $\varnothing = 4.6$ mm,
- **stationary phase:** octadecylsilyl silica gel for chromatography R (5 µm),
- **temperature:** 70 °C.

Mobile phase: solution A, solution B (45:55 V/V).

Flow rate: 2.0 ml/min.

Detection: spectrophotometer at 210 nm.

Injection: 20 µl.

Retention time: spirapril = 1.6 min to 2.9 min; impurity D = about 13 min. Adjust the proportion of solution B in the mobile phase if necessary.

System suitability: reference solution (b):

- **resolution:** minimum 15 between the peaks due to spirapril and impurity D.

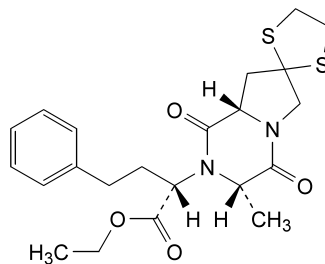
Calculate the percentage content of $C_{22}H_{31}ClN_2O_5S_2$ from the chromatograms obtained with the test solution and reference solution (a) and the declared content of *spirapril hydrochloride monohydrate CRS*.

STORAGE

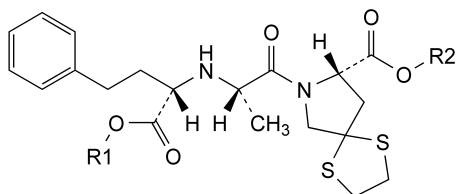
In an airtight container, protected from light.

IMPURITIES

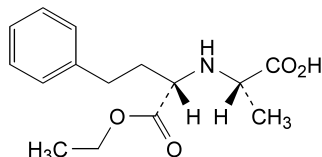
Specified impurities: A, B, C, D.



A. ethyl (2S)-2-[(3'S,8'aS)-3'-methyl-1',4'-dioxo-hexahydrospiro[1,3-dithiolane-2,7'-(6'H)-pyrrolo[1,2-a]pyrazin]-2'-yl]-4-phenylbutanoate,



- B. $R_1 = R_2 = H$: (8*S*)-7-[(2*S*)-2-[(1*S*)-1-carboxy-3-phenylpropyl]amino]propanoyl]-1,4-dithia-7-azaspiro[4.4]nonane-8-carboxylic acid (spiraprilat),
- D. $R_1 = C_2H_5$, $R_2 = CH(CH_3)_2$: 1-methylethyl (8*S*)-7-[(2*S*)-2-[(1*S*)-1-(ethoxycarbonyl)-3-phenylpropyl]amino]propanoyl]-1,4-dithia-7-azaspiro[4.4]nonane-8-carboxylate,

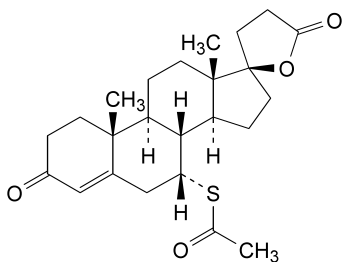


- C. (2*S*)-2-[(1*S*)-1-(ethoxycarbonyl)-3-phenylpropyl]amino]propanoic acid.

01/2005:0688

SPIRONOLACTONE

Spironolactonum

 $C_{24}H_{32}O_4S$ M_r 416.6

DEFINITION

Spironolactone contains not less than 97.0 per cent and not more than the equivalent of 102.0 per cent of 7 α -(acetylsulfanyl)-3',4'-dihydrospiro[androst-4-ene-17, 2'(5'*H*)-furan]-3,5'-dione, calculated with reference to the dried substance.

CHARACTERS

A white or yellowish-white powder, practically insoluble in water, soluble in alcohol.

It shows polymorphism.

IDENTIFICATION

First identification: A, C.

Second identification: B, C.

- A. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *spironolactone CRS*. Examine the substances as 50 g/l solutions in *chloroform R*.

- B. Examine by thin-layer chromatography (2.2.27), using *silica gel GF₂₅₄ R* as the coating substance.

Test solution. Dissolve 20 mg of the substance to be examined in *methylene chloride R* and dilute to 10 ml with the same solvent.

Reference solution. Dissolve 20 mg of *spironolactone CRS* in *methylene chloride R* and dilute to 10 ml with the same solvent.

Apply separately to the plate 5 μ l of each solution.

Develop over a path of 15 cm using a mixture of 1 volume of *water R*, 24 volumes of *cyclohexane R* and 75 volumes of *ethyl acetate R*. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. The principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with the reference solution.

- C. To about 10 mg add 2 ml of a 50 per cent V/V solution of *sulphuric acid R* and shake. An orange solution with an intense yellowish-green fluorescence is produced. Heat the solution gently; the colour becomes deep red and hydrogen sulphide, which blackens *lead acetate paper R*, is evolved. Add the solution to 10 ml of *water R*; a greenish-yellow solution is produced which shows opalescence or a precipitate.

TESTS

Specific optical rotation (2.2.7). Dissolve 0.100 g in *chloroform R* and dilute to 10.0 ml with the same solvent. The specific optical rotation is -33 to -37 , calculated with reference to the dried substance.

Related substances. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 62.5 mg of the substance to be examined in 2.5 ml of *tetrahydrofuran R* and dilute to 25.0 ml with the mobile phase.

Reference solution (a). Dilute 1.0 ml of the test solution to 100.0 ml with the mobile phase.

Reference solution (b). Dissolve 25.0 mg of *canrenone CRS* in 1 ml of *tetrahydrofuran R* and dilute to 10.0 ml with the mobile phase.

Reference solution (c). Dilute 1.0 ml of reference solution (b) to 100.0 ml with the mobile phase.

Reference solution (d). Mix 1 ml of the test solution with 1 ml of reference solution (b) and dilute to 100 ml with the mobile phase.

Reference solution (e). Dilute 0.50 ml of reference solution (a) to 10.0 ml with the mobile phase.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.15 m long and 4.6 mm in internal diameter packed with *octylsilyl silica gel for chromatography R* (5 μ m),
- a mixture of 8 volumes of *acetonitrile R*, 18 volumes of *tetrahydrofuran R* and 74 volumes of *water R* as the mobile phase at a flow rate of 1.8 ml/min,
- a variable wavelength spectrophotometer capable of operating at 254 nm and at 283 nm as detector.

Inject separately 20 μ l of the test solution and 20 μ l of reference solution (a), 20 μ l of reference solution (d) and 20 μ l of reference solution (e) and record the chromatograms with the detector set for recording at 254 nm. Continue the chromatography for twice the retention time of spironolactone. In the chromatogram obtained with the test solution, the sum of the areas of the peaks, except those corresponding to spironolactone and canrenone, is not greater than the area of the peak corresponding to spironolactone in the chromatogram obtained with reference solution (a) (1.0 per cent). Disregard any peak whose area is less than that of the principal peak in the chromatogram obtained with reference solution (e) (0.05 per cent). Inject 20 μ l of the test solution and 20 μ l of reference solution (c) and record the chromatogram with the detector

set for recording at 283 nm. In the chromatogram obtained with the test solution, the area of any peak corresponding to canrenone is not greater than the area of the peak corresponding to canrenone in the chromatogram obtained with reference solution (c) (1.0 per cent). Calculate the percentage content of canrenone found when recording at 283 nm and the percentage content of the other related substances found when recording at 254 nm. Add together these percentages. The sum is not greater than 1.0 per cent. The test is not valid unless: the chromatogram obtained with reference solution (d) shows peaks, corresponding to canrenone and spironolactone, with a resolution greater than 1.4; the principal peak in the chromatogram obtained with reference solution (e) has a signal-to-noise ratio of at least 6.

Free mercapto compounds. To 2.0 g add 20 ml of *water R*, shake for 1 min and filter. To 10 ml of the filtrate add 0.05 ml of 0.01 M iodine and 0.1 ml of *starch solution R* and mix. A blue colour develops.

Chromium. To 0.20 g in a platinum crucible add 1 g of *potassium carbonate R* and 0.3 g of *potassium nitrate R*. Heat gently until fused, and ignite at 600 °C to 650 °C until carbon is removed. Cool, dissolve the residue as completely as possible in 10 ml of *water R* with the aid of gentle heat, filter, and dilute to 20 ml with *water R*. To 10 ml of this solution add 0.5 g of *urea R*, and add a 14 per cent V/V solution of *sulphuric acid R* until the solution is just acid. When effervescence ceases, add a further 1 ml of the sulphuric acid, dilute to 20 ml with *water R* and add 0.5 ml of *diphenylcarbazide solution R*. The solution is not more intensely coloured than a standard prepared by adding 1 ml of a 14 per cent V/V solution of *sulphuric acid R* to 0.50 ml of a freshly prepared 28.3 mg/l solution of *potassium dichromate R*, diluting to 20 ml with *water R* and adding 0.5 ml of *diphenylcarbazide solution R* (50 ppm).

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C for 3 h.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 50.0 mg in *methanol R* and dilute to 250.0 ml with the same solvent. Dilute 5.0 ml of this solution to 100.0 ml with *methanol R*. Measure the absorbance (2.2.25) of the solution at the maximum at 238 nm.

Calculate the content of $C_{30}H_{62}O_4S$, taking the specific absorbance to be 470.

STORAGE

Store protected from light.

DEFINITION

2,6,10,15,19,23-Hexamethyltetracosane (perhydrosqualene). It may be of vegetable (unsaponifiable matter of olive oil) or animal (shark liver oil) origin.

Content: 96.0 per cent to 103.0 per cent.

CHARACTERS

Appearance: clear, colourless, oily liquid.

Solubility: practically insoluble in water, miscible with most fats and oils, freely soluble in acetone and in cyclohexane, practically insoluble in alcohol.

Relative density: about 0.815.

IDENTIFICATION

A. Infrared absorption spectrophotometry (2.2.24).

Comparison: *squalane CRS*.

B. It complies with the test for refractive index (see Tests).

C. Examine the chromatograms obtained in the assay.

Results: the principal peak in the chromatogram obtained with the test solution is similar in retention time and size to the principal peak in the chromatogram obtained with reference solution (a).

The chromatogram obtained with squalane of vegetable origin shows a peak corresponding to cyclosqualane (Figure 1630-1 and Figure 1630-2).

TESTS

Appearance. The substance to be examined is clear (2.2.1) and colourless (2.2.2, *Method II*).

Refractive index (2.2.6): 1.450 to 1.454.

Acid value (2.5.1): maximum 0.2.

Iodine value (2.5.4): maximum 4.0.

Saponification value (2.5.6): maximum 3.0.

Nickel (2.4.27): maximum 1 ppm.

Total ash (2.4.16): maximum 0.5 per cent, determined on 1.000 g.

ASSAY

Gas chromatography (2.2.28).

Internal standard solution. To 1.0 ml of *dimethylacetamide R*, add 100.0 ml of *heptane R*.

Test solution. Dissolve 0.100 g in the internal standard solution and dilute to 25.0 ml with the same solution.

Reference solution (a). Dissolve 0.100 g of *squalane CRS* in the internal standard solution and dilute to 25.0 ml with the same solution.

Reference solution (b). To 0.1 ml of *methyl erucate R* add 0.100 g of the substance to be examined, dissolve in the internal standard solution and dilute to 25.0 ml with the same solution.

Column:

- **material:** fused silica,
- **size:** $l = 30$ m, $\varnothing = 0.32$ mm,
- **stationary phase:** *poly(dimethyl)siloxane R* (film thickness 1 μ m).

Carrier gas: *helium for chromatography R*.

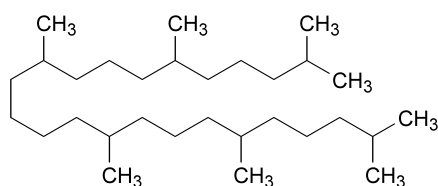
Flow rate: 1.7 ml/min.

Split ratio: 1:12.

01/2005:1630

SQUALANE

Squalanum



$C_{30}H_{62}$

M_r 422.8

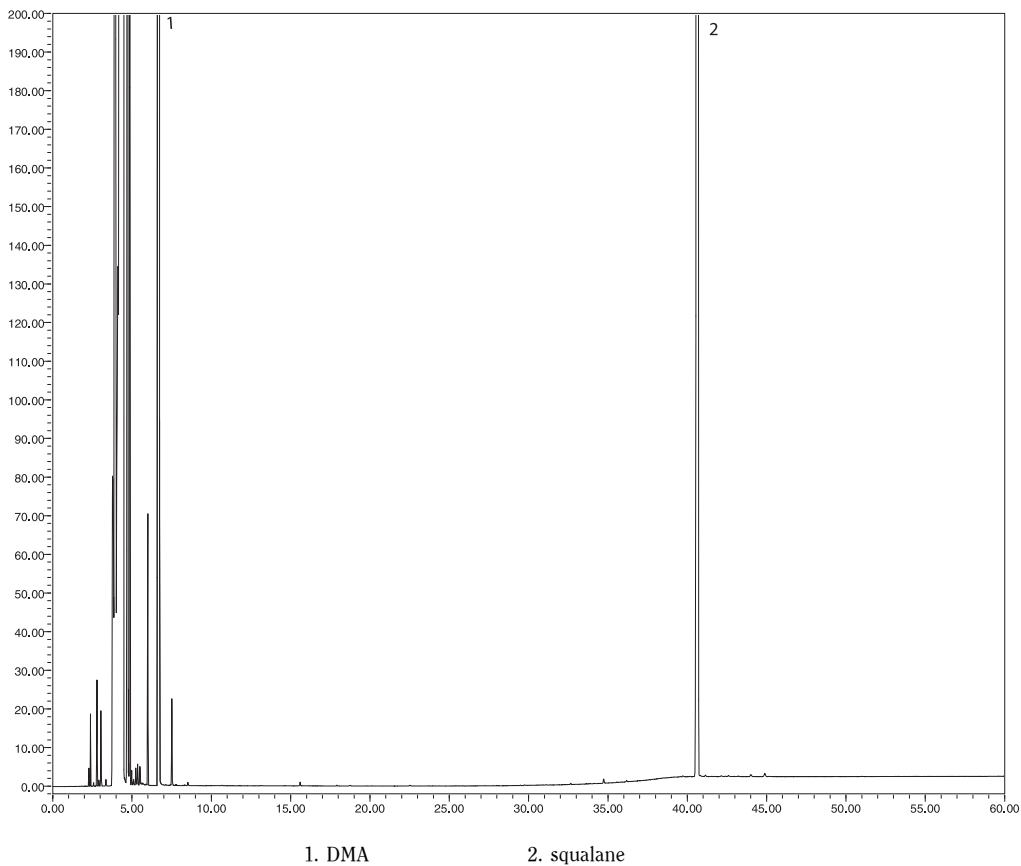


Figure 1630.-1. – Chromatogram of squalane of animal origin

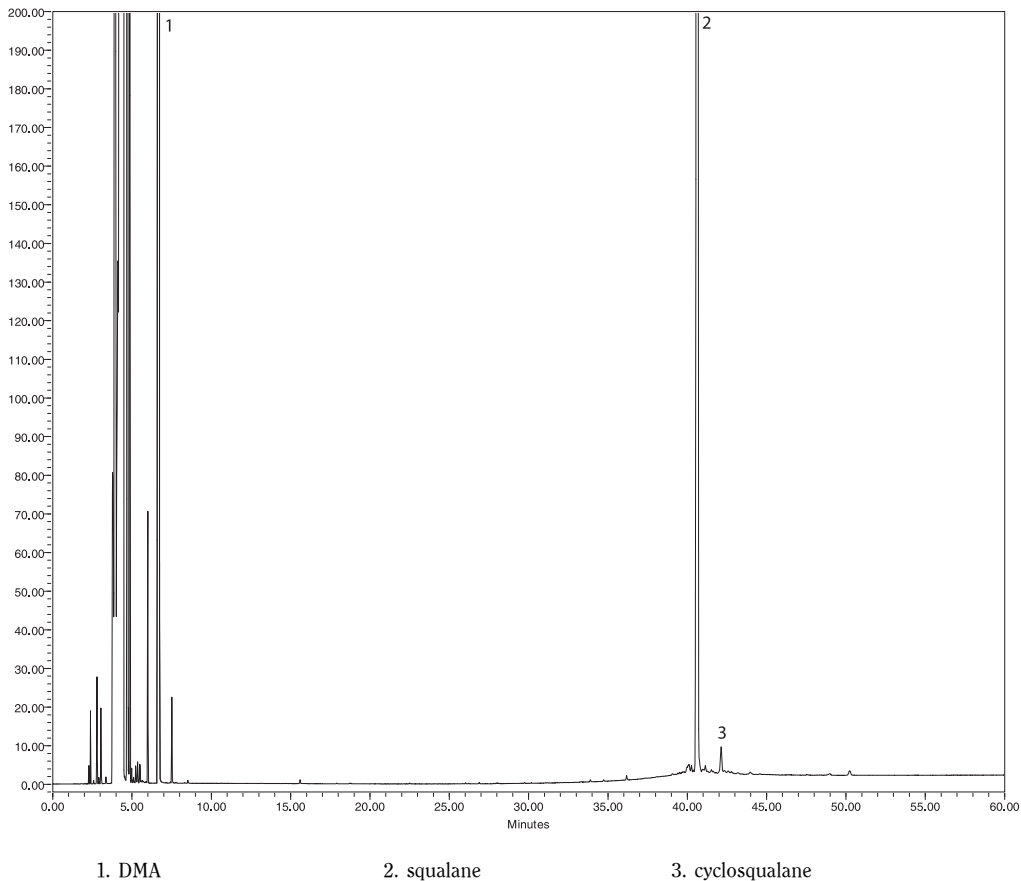


Figure 1630.-2. – Chromatogram of squalane of vegetable origin

Temperature:

	Time (min)	Temperature (°C)
Column	0 - 39	60 - 290
	39 - 50	290
Injection port		275
Detector		300

Detection: flame ionisation.

Injection: 1 µl.

Relative retentions with reference to squalane (retention time = about 41 min): internal standard = about 0.2; methyl erucate = about 0.9; cyclosqualane = 1.05.

System suitability: reference solution (b):

- *resolution:* minimum 5 between the peaks due to methyl erucate and squalane.

Calculate the percentage content of squalane from the areas of the peaks and the declared content of *squalane CRS*.

LABELLING

The label states the origin of squalane (vegetable or animal).

01/2005:1438

St. JOHN'S WORT

Hyperici herba

DEFINITION

St. John's wort consists of the whole or cut, dried flowering tops of *Hypericum perforatum* L., harvested during flowering time. It contains not less than 0.08 per cent of total hypericins expressed as hypericin ($C_{30}H_{16}O_8$; M_r 504.4), calculated with reference to the dried drug.

CHARACTERS

It has the macroscopic and microscopic characters described under identification tests A and B.

IDENTIFICATION

- The branched and bare stem shows 2 more-or-less prominent longitudinal ridges. The leaves are opposite, sessile, exstipulate, oblong-oval and 15 mm to 30 mm long; present on the leaf margins are glands which appear as black dots and over all the surface of the leaves many small, strongly translucent excretory glands which are visible in transmitted light. The flowers are regular and form corymbose clusters at the apex of the stem. They have 5 green, acute sepals, with black secretory glands on the margins; 5 orange-yellow petals, also with black secretory glands on the margins; 3 staminal blades, each divided into many orange-yellow stamens and 3 carpels surmounted by red styles.
- Reduce to a powder (355). The powder is greenish-yellow. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters: fragments of polygonal cells of the epidermis with thickened and beaded walls and paracytic or anomocytic stomata (2.8.3); fragments of the leaf and sepal with large oil glands and red pigment cells; thin-walled, elongated cells of the petal epidermis with straight or wavy anticlinal walls; tracheids and tracheidal

vessels with pitted walls and groups of thick-walled fibres; fragments of rectangular, lignified and pitted parenchyma; fibrous layer of the anther and elongated, thin-walled cells of the filament with a striated cuticle; numerous pollen grains with 3 pores and a smooth exine, occur singly or in dense groups, and calcium oxalate cluster crystals.

- Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel plate R*.

Test solution. Stir 0.5 g of the powdered drug (500) in 10 ml of *methanol R* in a water-bath at 60 °C for 10 min and filter.

Reference solution. Dissolve 5 mg of *rutin R* and 5 mg of *hyperoside R* in *methanol R* and dilute to 5 ml with the same solvent.

Apply to the plate as 10 mm bands 10 µl of the test solution and 5 µl of the reference solution. Develop over a path of 10 cm using a mixture of 6 volumes of *anhydrous formic acid R*, 9 volumes of *water R* and 90 volumes of *ethyl acetate R*. Allow the plate to dry at 100-105 °C for 10 min. Spray the plate with a 10 g/l solution of *diphenylboric acid aminoethyl ester R* in *methanol R* and then with a 50 g/l solution of *macrogol 400 R* in *methanol R*. Examine the plate after about 30 min in ultraviolet light at 365 nm. The chromatogram obtained with the reference solution shows in the lower third the zone due to rutin and above it the zone due to hyperoside, both with yellow-orange fluorescence. The chromatogram obtained with the test solution shows in the lower third the reddish-orange fluorescent zones of rutin and hyperoside and in the lower part of the upper third the zone of pseudohypericin and above it the zone of hypericin, both with red fluorescence. Other yellow or blue fluorescent zones are visible.

TESTS

Foreign matter (2.8.2). Not more than 3 per cent of stems with a diameter greater than 5 mm, and not more than 2 per cent of other foreign matter.

Loss on drying (2.2.32). Not more than 10.0 per cent, determined on 1.000 g of the powdered drug (500) by drying in an oven at 100-105 °C for 2 h.

Total ash (2.4.16). Not more than 7.0 per cent.

ASSAY

Test solution. In a 100 ml round-bottomed flask, introduce 0.800 g of the powdered drug (500), 60 ml of a mixture of 20 volumes of *water R* and 80 volumes of *tetrahydrofuran R* and a magnetic stirrer. Boil the mixture in a water-bath at 70 °C under a reflux condenser for 30 min. Centrifuge (2 min at 700 g) and decant the supernatant into a 250 ml flask. Take up the residue with 60 ml of a mixture of 20 volumes of *water R* and 80 volumes of *tetrahydrofuran R*. Heat again under a reflux condenser for 30 min. Centrifuge (2 min at 700 g) and decant the supernatant. Combine the extracts and evaporate to dryness. Take up the residue with 15 ml of *methanol R* with the help of ultrasound and transfer to a 25 ml measuring flask. Rinse the 250 ml flask with *methanol R* and dilute to 25.0 ml with the same solvent. Centrifuge again, filter 10 ml through a syringe filter (0.2 µm). Discard the first 2 millilitres of the filtrate. Introduce 5.0 ml of the filtrate into a measuring flask and dilute to 25.0 ml with *methanol R*.

Compensation liquid. *Methanol R*.

Measure the absorbance (2.2.25) of the test solution at 590 nm by comparison with the compensation liquid.

Calculate the percentage content of total hypericins, expressed as hypericin, from the expression:

$$\frac{A \times 125}{m \times 870}$$

i.e. taking the specific absorbance of hypericin to be 870.

A = absorbance at 590 nm,

m = mass of the drug to be examined, in grams.

STORAGE

Protected from light.

01/2005:1266

STANNOUS CHLORIDE DIHYDRATE

Stannosi chloridum dihydricum

$\text{SnCl}_2 \cdot 2\text{H}_2\text{O}$

M_r 225.6

DEFINITION

Stannous chloride dihydrate contains not less than 98.0 per cent and not more than the equivalent of 101.0 per cent of $\text{SnCl}_2 \cdot 2\text{H}_2\text{O}$.

CHARACTERS

A white, crystalline powder or colourless crystals, efflorescent in air, freely soluble in water (the solution becomes cloudy after standing or on dilution), freely soluble in alcohol. It dissolves in dilute hydrochloric acid.

IDENTIFICATION

- To 1 ml of solution S1 (see Tests) add a mixture of 5 ml of *water R* and 0.05 ml of *mercuric chloride solution R*. A blackish-grey precipitate forms.
- Dissolve 1.0 g in 3.0 ml of *water R*. Add 0.5 ml of *dilute sodium hydroxide solution R* to the cloudy solution; a yellowish flocculent precipitate is formed. Add 6.5 ml of *water R*. To 1.0 ml of the previously shaken suspension add 1.0 ml of *strong sodium hydroxide solution R*; the precipitate dissolves and the resulting solution is clear and colourless.
- Dissolve 10 mg in 2 ml of *dilute nitric acid R*. The solution gives reaction (a) of chlorides (2.3.1).

TESTS

Solution S1. To 0.40 g add 1 ml of *dilute hydrochloric acid R* and dilute to 20 ml with *distilled water R*.

Solution S2. Dissolve 1.0 g in *dilute hydrochloric acid R* and dilute to 30 ml with the same acid. Heat to boiling. Add 30 ml of *thioacetamide solution R* and boil for 15 min (solution A). Use 5 ml, filter and heat the filtrate to boiling. Add 5 ml of *thioacetamide solution R* and boil for 15 min. If a precipitate is formed, add the remainder of solution A (solution A') to the mixture. Add 10 ml of *thioacetamide solution R* and boil. Repeat the series of operations from "Use 5 ml,..." until a precipitate is no longer formed on addition of *thioacetamide solution R* to the filtrate obtained from the 5 ml of solution A (solution A', solution A'',..., respectively). If no precipitate is formed or if no more precipitate is formed combine the solution obtained with the remainder of solution A (solution A', solution A'',..., respectively), filter and wash the precipitate with 10 ml of *water R*. Heat the filtrate until the resulting vapour no longer turns a moistened piece of *lead acetate paper R* blackish-grey. Allow to cool and dilute to 50 ml with *water R*.

Appearance of solution. Dissolve 10.0 g in *dilute hydrochloric acid R* and dilute to 20 ml with the same acid. The solution is clear (2.2.1) and colourless (2.2.2, *Method II*).

Substances not precipitated by thioacetamide. Evaporate 25 ml of solution S2 to dryness and ignite at 600 °C. The residue weighs not more than 1 mg (0.2 per cent).

Sulphates (2.4.13). 15 ml of solution S1 complies with the limit test for sulphates (500 ppm).

Iron (2.4.9). Dilute 5 ml of solution S2 to 10 ml with *water R*. The solution complies with the limit test for iron (100 ppm).

Heavy metals. Dissolve 1.0 g in 2 ml of a mixture of 1 volume of *nitric acid R* and 3 volumes of *hydrochloric acid R*. Heat the solution on a water-bath until nitrous vapour is no longer evolved. Dissolve the residue in *water R* and dilute to 25 ml with the same solvent. To 5 ml of the solution add 3 ml of *strong sodium hydroxide solution R* and 2 ml of *water R*. Heat until a clear solution is obtained, then cool and add 0.5 ml of *thioacetamide reagent R*. After 2 min, any colour in the solution is not more intense than that of a mixture of 1.0 ml of *lead standard solution (10 ppm Pb) R*, 6 ml of *water R*, 3 ml of *strong sodium hydroxide solution R* and 0.5 ml of *thioacetamide reagent R* (50 ppm).

ASSAY

Dissolve 0.100 g in 1.5 ml of *hydrochloric acid R1* and dilute to 50 ml with *water R*. Add 5 g of *sodium potassium tartrate R*, 10 g of *sodium hydrogen carbonate R* and 1 ml of *starch solution R*. Titrate immediately with 0.05 M *iodine*. Carry out a blank titration.

1 ml of 0.05 M *iodine* is equivalent to 11.28 mg of $\text{SnCl}_2 \cdot 2\text{H}_2\text{O}$.

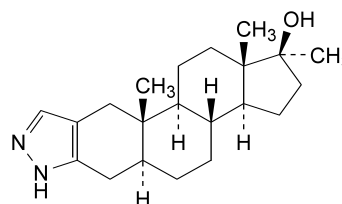
STORAGE

Store in an airtight container.

01/2005:1568

STANOZOLOL

Stanozololum



$\text{C}_{21}\text{H}_{32}\text{N}_2\text{O}$

M_r 328.5

DEFINITION

Stanozolol contains not less than 98.5 per cent and not more than the equivalent of 101.0 per cent of 17-methyl-2'H-5α-androst-2-eno[3,2-c]pyrazol-17β-ol, calculated with reference to the dried substance.

CHARACTERS

A white or almost white crystalline powder, hygroscopic, practically insoluble in water, soluble in dimethylformamide, slightly soluble in alcohol, very slightly soluble in methylene chloride.

It shows polymorphism.

IDENTIFICATION

- Examine by infrared spectrophotometry (2.2.24), comparing with the spectrum obtained with *stanozolol CRS*. If the spectra obtained in the solid

state show differences, dissolve the substance to be examined and the reference substance separately in the minimum volume of methylene chloride, evaporate to dryness at room temperature under an air-stream and record new spectra using the residues.

- B. Examine the chromatograms obtained in the test for related substances. The principal spot in the chromatogram obtained with test solution (b) is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (c).

TESTS

Specific optical rotation (2.2.7). Dissolve 0.10 g in *chloroform R* and dilute to 10.0 ml with the same solvent. The specific optical rotation is + 34 to + 40, calculated with reference to the dried substance.

Related substances. Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel F₂₅₄ plate R*.

Test solution (a). Dissolve 0.10 g of the substance to be examined in a mixture of 1 volume of *methanol R* and 9 volumes of *methylene chloride R* and dilute to 5 ml with the same mixture of solvents.

Test solution (b). Dilute 1 ml of test solution (a) to 10 ml with a mixture of 1 volume of *methanol R* and 9 volumes of *methylene chloride R*.

Reference solution (a). Dilute 1.0 ml of test solution (a) to 200 ml with a mixture of 1 volume of *methanol R* and 9 volumes of *methylene chloride R*.

Reference solution (b). Dissolve 5 mg of *stanazolol impurity A CRS* in reference solution (a) and dilute to 50 ml with the same solution.

Reference solution (c). Dissolve 10 mg of *stanazolol CRS* in a mixture of 1 volume of *methanol R* and 9 volumes of *methylene chloride R* and dilute to 5 ml with the same mixture of solvents.

Apply to the plate 5 µl of each solution. Develop over a path corresponding to two thirds of the plate height using a mixture of 10 volumes of *methanol R* and 90 volumes of *methylene chloride R*. Allow the plate to dry in air, spray with *alcoholic solution of sulphuric acid R*, heat at 105 °C for 15 min and examine under ultraviolet light at 365 nm. Any secondary spot in the chromatogram obtained with test solution (a) is not more intense than the spot in the chromatogram obtained with reference solution (a) (0.5 per cent). The test is not valid unless the chromatogram obtained with reference solution (b) shows two clearly separated spots.

Loss on drying (2.2.32). Not more than 1.0 per cent, determined on 1.000 g by drying at 100 °C at a pressure not exceeding 0.7 kPa.

ASSAY

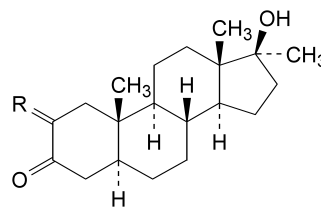
Dissolve 0.250 g in 50 ml of *anhydrous acetic acid R*. Titrate with 0.1 M *perchloric acid*, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *perchloric acid* is equivalent to 32.85 mg of C₂₁H₃₂N₂O.

STORAGE

Store in an airtight container, protected from light.

IMPURITIES



- A. R = H₂: 17β-hydroxy-17-methyl-5α-androstan-3-one (mestanolone),
 B. R = CH-OH: 17β-hydroxy-2-(hydroxymethylene)-17-methyl-5α-androstan-3-one (oxymetholone).

01/2005:1153

STAR ANISE

Anisi stellati fructus

DEFINITION

Dried composite fruit of *Illicium verum* Hooker fil.

Content: minimum 70 ml/kg of essential oil (anhydrous drug).

CHARACTERS

The fruit carpels are brown.

Odour of anethole.

Macroscopic and microscopic characters described under identification tests A and B.

IDENTIFICATION

- A. The fruit consists of 6 to 11 (usually 8), often unequally developed, boat-shaped follicles, each 12 mm to 20 mm long and 6 mm to 11 mm thick, radially arranged around a short, central, blunt-ending columella. The follicles are bluntly peaked at the apex, flat at the base and the outer surface is greyish-brown, roughly wrinkled. The ventral suture is frequently open exposing a single, hard, ovoid, laterally compressed, shiny, reddish-brown seed about 8 mm long. The pedicel, if present, is distinctly curved and thickened at the apex. Separated follicles and seeds may be present.
- B. Reduce to a powder (355). The powder is reddish-brown. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters: brown epicarpal cells, polygonal in surface view, with strongly striated cuticles and occasional anomocytic stomata (2.8.3); fragments of the endocarp with palisade-like cells up to about 600 µm long; fragments of the mesocarp with large parenchymatous cells, vessels, oil-containing cells and groups of large very elongated stone cells with thickened and pitted walls; fragments of the testa with palisade-like, yellow stone cells up to 200 µm long, with strongly pitted walls; fragments of the endosperm containing droplets of oil and aleurone grains; fragments of the columella and the fruit stalk with strongly thickened irregular stone cells up to 400 µm long and about 150 µm wide, with pointed, star-shaped projections (astrosclereids); rhomboidal or rectangular crystals of calcium oxalate.

- C. Thin-layer chromatography (2.2.27).

Test solution. Shake 0.20 g of the freshly powdered drug (710) with 2.0 ml of *toluene R* for 15 min and filter.

Reference solution. Dissolve 3 µl of *anethole R* and 20 µl of *olive oil R* in 1 ml of *toluene R*.

Plate: *TLC silica gel plate R*.

Mobile phase: toluene R.

Application: 20 µl, as bands.

Development: over a path of 10 cm.

Drying: in air.

Detection: spray with *anisaldehyde solution R*. Heat at 120 °C for 5 min and examine in daylight.

Results: see below the sequence of the zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other weaker zones may be present in the chromatogram obtained with the test solution.

Top of the plate	
_____	_____
Anethole: a reddish-violet zone	A reddish-violet zone (anethole)
_____	_____
Triglycerides of olive oil: a bluish-violet zone	A bluish-violet zone
Reference solution	Test solution

TESTS

***Illicium anisatum* (= *I. religiosum*).** The fruit must not contain follicles with an upwardly turned beak nor straight fruit stalks that are not curved at the distal end. Fragments of the endocarp with palisade-like cells less than about 360 µm long and fragments of the columella, previously separated from the fruit, with strongly thickened rounded stone cells without star-shaped projections must be absent.

Foreign matter (2.8.2): maximum 2 per cent.

Water (2.2.13): maximum 100 ml/kg, determined by distillation on 20.0 g of the powdered drug (710).

Total ash (2.4.16): maximum 4.0 per cent.

ASSAY

Carry out the determination of essential oils in vegetable drugs (2.8.12). Use a 250 ml round-bottomed flask and 100 ml of *water R* as the distillation liquid. Immediately before the determination, reduce 50.0 g of the drug to a coarse powder (1400) and mix. Further reduce about 10.0 g of this mixture to a finer powder (710). Use 2.50 g of the powder for the determination. Introduce 0.50 ml of *xylene R* into the graduated tube. Distil at a rate of 2-3 ml/min for 2 h.

01/2005:2108
corrected

STAR ANISE OIL

Anisi stellati aetheroleum

DEFINITION

Essential oil obtained by steam distillation from the dry ripe fruits of *Illicium verum* Hook. fil.

CHARACTERS

Appearance: clear, colourless or pale yellow liquid.

IDENTIFICATION

First identification: B.

Second identification: A.

A. Thin-layer chromatography (2.2.27).

Test solution. Dissolve 1 g of the substance to be examined in *toluene R* and dilute to 10 ml with the same solvent.

Reference solution. Dissolve 10 µl of *linalol R*, 30 µl of *anisaldehyde R* and 200 µl of *anethole R* and in *toluene R* and dilute to 15 ml with the same solvent. Dilute 1 ml of this solution to 5 ml with *toluene R*.

Plate: TLC silica gel F₂₅₄ plate R.

Mobile phase: ethyl acetate R, toluene R (7:93 V/V).

Application: 5 µl as bands of 10 mm (for normal TLC plates) or 2 µl as bands of 10 mm (for fine particle TLC plates).

Development: over a path of 15 cm (for normal TLC plates) or over a path of 6 cm (for fine particle size plates).

Drying: in air.

Detection A: examine in ultraviolet light at 254 nm.

Results A: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other zones may be present in the chromatogram obtained with the test solution.

Top of the plate	
_____	_____
Anethole: a quenching zone	A quenching zone, partly separated
_____	_____
Anisaldehyde: a quenching zone	A very strong quenching zone (anethole)
_____	_____
	A quenching zone (anisaldehyde)
Reference solution	Test solution

Detection B: spray with *methyl 4-acetylbenzoate reagent R* and heat at 100-105 °C for 10 min; examine the still hot plate in daylight within 10 min.

Results B: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other zones may be present in the chromatogram obtained with the test solution.

Top of the plate	
_____	_____
Anethole: a brown zone	A violet-brown zone, not fully separated
_____	_____
Anisaldehyde: a yellow zone	A very strong brown zone (anethole)
_____	_____
Linalol: a grey zone	A yellow zone (anisaldehyde)

	A grey zone (linalol)
Reference solution	Test solution

B. Examine the chromatograms obtained in the test for chromatographic profile.

Results: the characteristic peaks in the chromatogram obtained with the test solution are similar in retention time to those in the chromatogram obtained with the reference solution.

TESTS

Relative density (2.2.5): 0.979 to 0.985.

Refractive index (2.2.6): 1.553 to 1.556.

Freezing point (2.2.18): 15 °C to 19 °C.

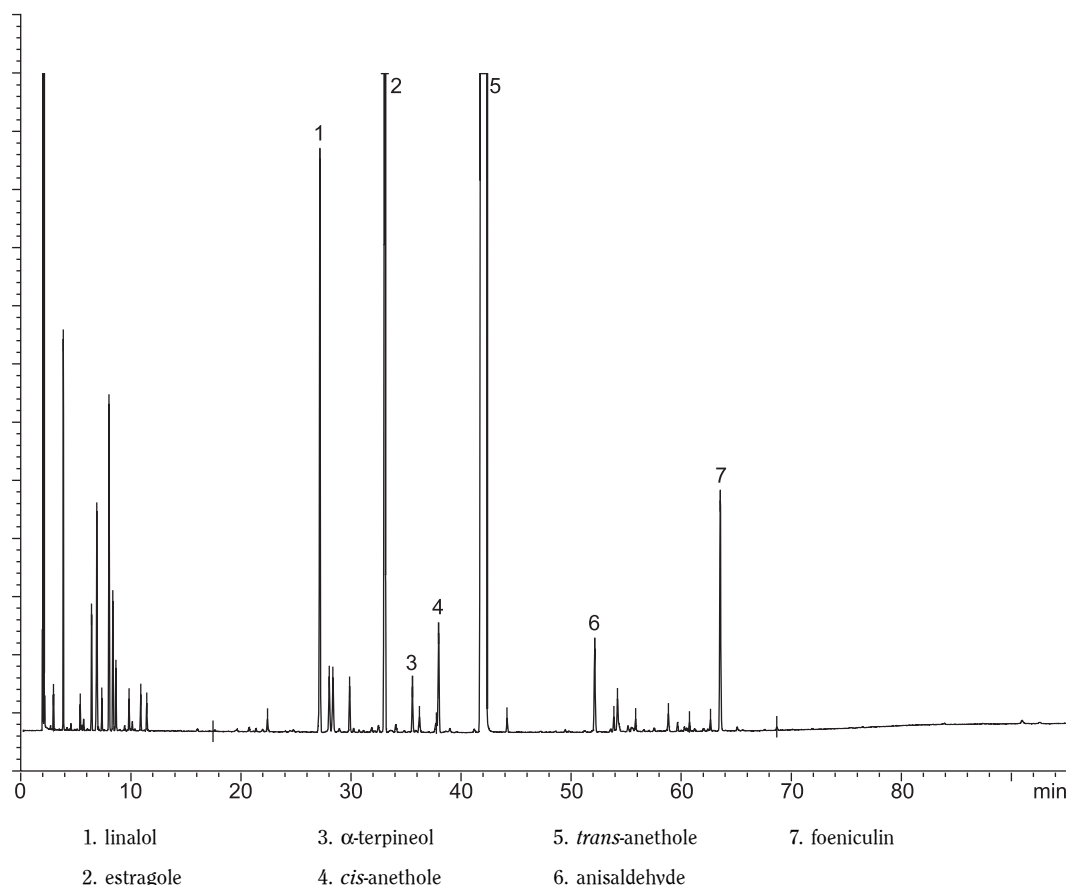


Figure 2108.-1. – Chromatogram for the test for chromatographic profile of star anise oil

Fenchone. Gas chromatography (2.2.28) as described in the test for chromatographic profile with the following modifications.

Test solution. Dissolve 400 µl of the substance to be examined in 2.0 ml of *hexane R*.

Reference solution (a). Dilute 10 µl of *fenchone R* to 1.2 g with *hexane R*.

Reference solution (b). Dilute 100 µl of reference solution (a) to 100 ml with *hexane R*.

System suitability: reference solution (b):

– *signal-to-noise ratio*: minimum 10 for the principal peak.

Limit:

– *fenchone*: maximum 0.01 per cent.

Pseudoisoeugenyl 2-methylbutyrate. Gas chromatography (2.2.28) as described in the test for chromatographic profile with the following modifications.

Test solution. The substance to be examined.

Reference solution (a). Dilute 10 mg of the test solution to 1.000 g with *hexane R*. Dilute 0.5 ml of this solution to 100 ml with *hexane R*.

Reference solution (b). *Pseudoisoeugenyl 2-methylbutyrate for peak identification CRS*.

System suitability:

– the chromatogram obtained with reference solution (b) is similar to the chromatogram provided with *pseudoisoeugenyl 2-methylbutyrate for peak identification CRS*.

– *signal-to-noise ratio*: minimum 10 for the principal peak in the chromatogram obtained with reference solution (a).

Limit: locate the peak due to pseudoisoeugenyl 2-methylbutyrate by comparison with the chromatogram provided with *pseudoisoeugenyl 2-methylbutyrate for peak identification CRS*.

– *pseudoisoeugenyl 2-methylbutyrate*: maximum 0.01 per cent.

Fatty oils and resinified essential oils (2.8.7). It complies with the test for fatty oils and resinified essential oils.

Chromatographic profile. Gas chromatography (2.2.28): use the normalisation procedure.

Test solution. Dissolve 200 µl of the substance to be examined in 1.0 ml of *hexane R*.

Reference solution. To 1.0 ml of *hexane R*, add 20 µl of *linalol R*, 20 µl of *estragole R*, 20 µl of *α-terpineol R*, 60 µl of *anethole R* and 30 µl of *anisaldehyde R*.

Column:

– *material*: fused silica,

– *size*: $l = 30$ m, $\varnothing = 0.25$ mm,

– *stationary phase*: *macrogol 20 000 R* (film thickness 0.25 µm).

Carrier gas: *helium for chromatography R*.

Flow rate: 1.0 ml/min.

Split ratio: 1:100.

Temperature:

	Time (min)	Temperature (°C)
Column	0 - 5	60
	5 - 80	60 → 210
	80 - 95	210
Injection port		200
Detector		220

Detection: flame ionisation.

Injection: 0.2 µl.

Elution order: order indicated in the composition of the reference solution; record the retention times of these substances.

System suitability: reference solution:

- **resolution:** minimum 1.5 between the peaks due to estragole and α -terpineol.

Using the retention times determined from the chromatogram obtained with the reference solution, locate the components of the reference solution in the chromatogram obtained with the test solution and locate *cis*-anethole and foeniculin using the chromatogram shown in Figure 2108.-1 (disregard any peak due to hexane).

Determine the percentage content of these components. The percentages are within the following ranges:

- **linalol:** 0.2 per cent to 2.5 per cent,
- **estragole:** 0.5 per cent to 6.0 per cent,
- **α -terpineol:** less than 0.3 per cent,
- ***cis*-anethole:** 0.1 per cent to 0.5 per cent,
- ***trans*-anethole:** 86 per cent to 93 per cent,
- **anisaldehyde:** 0.1 per cent to 0.5 per cent,
- **foeniculin:** 0.1 per cent to 3.0 per cent.

STORAGE

In a well-filled, airtight container, protected from light and at a temperature not exceeding 25 °C.

01/2005:1267

STARCH, PREGELATINISED

Amylum pregelificatum

DEFINITION

Pregelatinised starch is prepared from *Maize starch* (0344), *Potato starch* (0355) or *Rice starch* (0349) by mechanical processing in the presence of water, with or without heat, to rupture all or part of the starch granules and subsequent drying. It contains no added substances but it may be modified to render it compressible and to improve its flow characteristics.

CHARACTERS

Appearance: white or yellowish-white powder.

It swells in cold water.

IDENTIFICATION

- Examined under a microscope using a mixture of equal volumes of *glycerol R* and *water R* it presents irregular, translucent, white or yellowish-white flakes or pieces with an uneven surface. Under polarised light (between crossed nicol prisms), starch granules with a distinct black cross intersecting at the hilum may be seen.
- Disperse 0.5 g in 2 ml of *water R* without heating and add 0.05 ml of *iodine solution R1*. A reddish-violet to blue colour is produced.

TESTS

pH (2.2.3): 4.5 to 7.0.

Progressively add 3.0 g to 100.0 ml of *carbon dioxide-free water R*, stirring continuously. Determine the pH when a homogeneous solution is obtained.

Oxidising substances (2.5.30). It complies with the test for oxidising substances. Use a mixture of equal volumes of *water R* and *methanol R* as solvent.

Sulphur dioxide (2.5.29): maximum 50 ppm.

Iron (2.4.9): maximum 20 ppm.

Dissolve the residue obtained in the test for sulphated ash in 20 ml of *dilute hydrochloric acid R*. Filter. 10 ml of the filtrate complies with the limit test for iron

Foreign matter. Examined under a microscope using a mixture of equal volumes of *glycerol R* and *water R*, not more than traces of matter other than starch granules are present.

Loss on drying (2.2.32): maximum 15.0 per cent, determined on 1.000 g by drying in an oven at 130 °C for 90 min.

Sulphated ash (2.4.14): maximum 0.6 per cent, determined on 1.0 g.

Microbial contamination. Total viable aerobic count (2.6.12) not more than 10³ bacteria and not more than 10² fungi per gram, determined by plate-count. It complies with the test for *Escherichia coli* (2.6.13).

LABELLING

The type of starch used as starting material is stated.

01/2005:1474

STEARIC ACID

Acidum stearicum

DEFINITION

Mixture consisting mainly of stearic acid (C₁₈H₃₆O₂; *M_r* 284.5) and palmitic acid (C₁₆H₃₂O₂; *M_r* 256.4) obtained from fats or oils of vegetable or animal origin.

Content:

Stearic acid 50	<i>Stearic acid:</i> 40.0 per cent to 60.0 per cent. <i>Sum of the contents of stearic and palmitic acids:</i> minimum 90.0 per cent.
Stearic acid 70	<i>Stearic acid:</i> 60.0 per cent to 80.0 per cent. <i>Sum of the contents of stearic and palmitic acids:</i> minimum 90.0 per cent.
Stearic acid 95	<i>Stearic acid:</i> minimum 90.0 per cent. <i>Sum of the contents of stearic and palmitic acids:</i> minimum 96.0 per cent.

CHARACTERS

Appearance: white, waxy, flaky crystals, white hard masses or white or yellowish-white powder.

Solubility: practically insoluble in water, soluble in alcohol and in light petroleum (50-70 °C).

IDENTIFICATION

- It complies with the test for freezing point (see Tests).
- Acid value (2.5.1): 194 to 212, determined on 0.1 g.
- Examine the chromatograms obtained in the assay.

Results: the retention times of the principal peaks in the chromatogram obtained with the test solution are approximately the same as those of the principal peaks in the chromatogram obtained with the reference solution.

TESTS

Appearance. Heat the substance to be examined to about 75 °C. The resulting liquid is not more intensely coloured than reference solution Y₇ or BY₇ (2.2.2, *Method I*).

Acidity. Melt 5.0 g, shake for 2 min with 10 ml of hot *carbon dioxide-free water R*, cool slowly and filter. To the filtrate add 0.05 ml of *methyl orange solution R*. No red colour develops.

Iodine value (2.5.4). See Table 1474.-1.

Freezing point (2.2.18). See Table 1474.-1.

01/2005:1268

Table 1474.-1.

Type	Iodine value	Freezing point (°C)
Stearic acid 50	maximum 4.0	53 - 59
Stearic acid 70	maximum 4.0	57 - 64
Stearic acid 95	maximum 1.5	64 - 69

Nickel (2.4.27): maximum 1 ppm.

ASSAY

Gas chromatography (2.2.28): use the normalisation procedure.

Test solution. In a conical flask fitted with a reflux condenser, dissolve 0.100 g of the substance to be examined in 5 ml of *boron trifluoride-methanol solution R*. Boil under reflux for 10 min. Add 4.0 ml of *heptane R* through the condenser and boil again under reflux for 10 min. Allow to cool. Add 20 ml of a saturated solution of *sodium chloride R*. Shake and allow the layers to separate. Remove about 2 ml of the organic layer and dry it over 0.2 g of *anhydrous sodium sulphate R*. Dilute 1.0 ml of this solution to 10.0 ml with *heptane R*.

Reference solution. Prepare the reference solution in the same manner as the test solution using 50 mg of *palmitic acid R* and 50 mg of *stearic acid R* instead of the substance to be examined.

Column:

- **material:** fused silica,
- **size:** $l = 30$ m, $\varnothing = 0.32$ mm,
- **stationary phase:** *macrogol 20 000 R* (film thickness 0.5 μ m).

Carrier gas: *helium for chromatography R*.

Flow rate: 2.4 ml/min.

Temperature:

	Time (min)	Temperature (°C)
Column	0 - 2	70
	2 - 36	70 → 240
	36 - 41	240
Injection port		220
Detector		260

Detection: flame ionisation.

Injection: 1 μ l.

Relative retention with reference to methyl stearate: methyl palmitate = about 0.88.

System suitability: reference solution:

- **resolution:** minimum 5.0 between the peaks due to methyl stearate and methyl palmitate.

LABELLING

The label states the type of stearic acid (50, 70, 95).

STEAROYL MACROGOLGLYCERIDES

Macrogolglyceridum stearates

DEFINITION

Stearoyl macrogolglycerides are mixtures of monoesters, diesters and triesters of glycerol and monoesters and diesters of macrogols with a mean relative molecular mass between 300 and 4000. They are obtained by partial alcoholysis of saturated oils containing mainly triglycerides of stearic acid using macrogol or by esterification of glycerol and macrogol with saturated fatty acids or by mixture of glycerol esters and condensates of ethylene oxide with the fatty acids of these hydrogenated oils. The hydroxyl value does not differ by more than 15 units from the nominal value. The saponification value does not differ by more than 10 units from the nominal value.

CHARACTERS

Pale yellow waxy solids, dispersible in warm water and in warm liquid paraffin, freely soluble in methylene chloride, soluble in warm ethanol.

IDENTIFICATION

A. Examine by thin-layer chromatography (2.2.27), using a suitable silica gel as the coating substance.

Test solution. Dissolve 1.0 g of the substance to be examined in *methylene chloride R* and dilute to 20 ml with the same solvent.

Apply to the plate 10 μ l of the test solution. Develop over a path of 15 cm using a mixture of 30 volumes of *hexane R* and 70 volumes of *ether R*. Allow the plate to dry in air. Spray with a 0.1 g/l solution of *rhodamine B R* in *alcohol R* and examine in ultraviolet light at 365 nm. The chromatogram shows a spot corresponding to triglycerides with an R_f value of about 0.9 (R_{st} 1) and spots corresponding to 1,3-diglycerides (R_{st} 0.7), to 1,2-diglycerides (R_{st} 0.6), to monoglycerides (R_{st} 0.1) and to esters of macrogol (R_{st} 0).

B. They comply with the test for hydroxyl value (see Tests).

C. They comply with the test for saponification value (see Tests).

D. They comply with the test for fatty acid composition (see Tests).

TESTS

Acid value (2.5.1). Not more than 2.0, determined on 2.0 g.

Hydroxyl value (2.5.3, Method A). Determined on 1.0 g, the hydroxyl value does not differ by more than 15 units from the nominal value.

Peroxide value (2.5.5). Not more than 6.0, determined on 2.0 g.

Saponification value (2.5.6). Determined on 2.0 g, the saponification value does not differ by more than 10 units from the nominal value.

Alkaline impurities. Into a test-tube introduce 5.0 g and carefully add a mixture, neutralised if necessary with 0.01 M *hydrochloric acid* or with 0.01 M *sodium hydroxide*, of 0.05 ml of a 0.4 g/l solution of *bromophenol blue R* in *alcohol R*, 0.3 ml of *water R* and 10 ml of *alcohol R*. Shake and allow to stand. Not more than 1.0 ml of 0.01 M *hydrochloric acid* is required to change the colour of the upper layer to yellow.

Free glycerol. Not more than 3.0 per cent. Dissolve 1.20 g in 25.0 ml of *methylene chloride R*. Heat if necessary. After cooling, add 100 ml of *water R*. Shake and add 25.0 ml of a 6 g/l solution of *periodic acid R*. Shake and allow to stand for 30 min. Add 40 ml of a 75 g/l solution of *potassium iodide R*. Allow to stand for 1 min. Add 1 ml of *starch solution R*. Titrate the iodine with 0.1 M *sodium thiosulphate*. Carry out a blank titration.

1 ml of 0.1 M *sodium thiosulphate* is equivalent to 2.3 mg of glycerol.

Composition of fatty acids (2.4.22, *Method A*). The constitutive fatty acid composition is the following:

- lauric acid: not more than 5.0 per cent,
- myristic acid: not more than 5.0 per cent,
- different nominal amounts of stearic acid and of palmitic acid. The sum of $C_{18}H_{36}O_2$ and $C_{16}H_{32}O_2$ is not less than 90.0 per cent.

Ethylene oxide and dioxan (2.4.25). Not more than 1 ppm of ethylene oxide and not more than 10 ppm of dioxan.

Heavy metals (2.4.8). 2.0 g complies with limit test C for heavy metals (10 ppm). Prepare the standard using 2 ml of *lead standard solution (10 ppm Pb) R*.

Water (2.5.12). Not more than 1.0 per cent, determined on 1.0 g by the semi-micro determination of water using a mixture of 30 volumes of *anhydrous methanol R* and 70 volumes of *methylene chloride R* as solvent.

Total ash (2.4.16). Not more than 0.2 per cent, determined on 1.0 g.

LABELLING

The label states the nominal hydroxyl value, the nominal saponification value and the type of the macrogol used (mean relative molecular mass) or the number of ethylene oxide units per molecule (nominal value).

Hydroxyl value (2.5.3, *Method A*): 197 to 217.

Iodine value (2.5.4). Not more than 2.0, determined on 2.00 g dissolved in 25 ml of *chloroform R*, warming if necessary.

Saponification value (2.5.6). Not more than 2.0.

ASSAY

Examine by gas chromatography (2.2.28).

Test solution. Dissolve 0.100 g of the substance to be examined in *ethanol R* and dilute to 10.0 ml with the same solvent.

Reference solution (a). Dissolve 0.100 g of *stearyl alcohol CRS* in *ethanol R* and dilute to 10.0 ml with the same solvent.

Reference solution (b). Dissolve 5 mg of *cetyl alcohol CRS* in 4.5 ml of reference solution (a) and dilute to 5.0 ml with *ethanol R*.

The chromatographic procedure may be carried out using:

- a stainless steel column 2 m long and 2 mm in internal diameter packed with *diatomaceous earth for gas chromatography R* impregnated with 10 per cent *m/m* of *poly(dimethyl)siloxane R*,
- *nitrogen for chromatography R* as the carrier gas at a flow rate of 30 ml/min,
- a flame-ionisation detector,

maintaining the temperature of the column at 220 °C, that of the injection port at 275 °C and that of the detector at 250 °C. Inject 2 µl of reference solution (b). The assay is not valid unless the resolution between the peaks corresponding to cetyl alcohol and stearyl alcohol is at least 4.0. Inject 2 µl of the test solution and 2 µl of reference solution (a). Calculate the percentage content of octadecanol using the normalisation procedure.

01/2005:0246

01/2005:0753

STEARYL ALCOHOL

Alcohol stearylicus

DEFINITION

Stearyl alcohol is a mixture of solid alcohols; it contains not less than 95.0 per cent of octadecanol ($C_{18}H_{38}O$; M_r 270.5).

CHARACTERS

White, unctuous flakes, granules or mass, practically insoluble in water, soluble in alcohol. When melted, it is miscible with fatty oils, with liquid paraffin and with melted wool fat.

IDENTIFICATION

Examine the chromatograms obtained in the Assay. The retention time and size of the principal peak in the chromatogram obtained with the test solution are approximately the same as those of the principal peak in the chromatogram obtained with reference solution (a).

TESTS

Appearance of solution. Dissolve 0.50 g in 20 ml of *alcohol R* by heating to boiling. Allow to cool. The solution is clear (2.2.1) and not more intensely coloured than reference solution B₆ (2.2.2, *Method II*).

Melting point (2.2.14): 57 °C to 60 °C.

Acid value (2.5.1). Not more than 1.0.

STRAMONIUM LEAF

Stramonii folium

DEFINITION

Stramonium leaf consists of the dried leaf or of the dried leaf, flowering tops and occasionally, fruit-bearing tops of *Datura stramonium* L. and its varieties. It contains not less than 0.25 per cent of total alkaloids, calculated as hyoscyamine ($C_{17}H_{23}NO_3$; M_r 289.4) with reference to the drug dried at 100 °C to 105 °C. The alkaloids consist mainly of hyoscyamine with varying proportions of hyoscyne (scopolamine).

CHARACTERS

Stramonium leaf has an unpleasant odour.

It has the macroscopic and microscopic characters described under identification tests A and B.

IDENTIFICATION

- A. The leaves are dark brownish-green to dark greyish-green, often much twisted and shrunken during drying, thin and brittle, ovate or triangular-ovate, dentately lobed with an acuminate apex and often unequal at the base. Young leaves are pubescent on the veins, older leaves are nearly glabrous. Stems are green or purplish-green, slender, curved and twisted, wrinkled longitudinally and sometimes wrinkled transversely, branched dichasially, with a single flower or an immature fruit in the fork. Flowers, on short pedicels, have a gamosepalous calyx with 5 lobes and trumpet-shaped brownish-white or

purplish corolla. The fruit is a capsule, usually covered with numerous short, stiff emergences; seeds are brown to black with a minutely pitted testa.

- B. Reduce to a powder (355). The powder is greyish-green. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters: fragments of leaf lamina showing epidermal cells with slightly wavy anticlinal walls and smooth cuticle; stomata are more frequent on the lower epidermis (anisocytic and anomocytic) (2.8.3); covering trichomes are conical, uniseriate with 3 to 5 cells and warty walls; glandular trichomes are short and clavate with heads formed by 2 to 7 cells; dorsiventral mesophyll, with a single layer of palisade cells and a spongy parenchyma containing cluster crystals of calcium oxalate; annularly and spirally thickened vessels. The powdered drug may also show the following: fibres and reticulately thickened vessels from the stem; subspherical pollen grains usually about 60 µm to 80 µm in diameter with 3 germinal pores and nearly smooth exine; fragments of the corolla with papillose epidermis; seed fragments containing yellowish-brown, sinuous, thick-walled sclereids of testa; occasional prisms and microsphenoidal crystals of calcium oxalate.
- C. Examine the chromatograms obtained in the chromatography test. The principal zones in the chromatogram obtained with the test solution are similar in position, colour and size to the principal zones in the chromatogram obtained with the same volume of the reference solution.
- D. Shake 1 g of the powdered drug (180) with 10 ml of 0.05 M *sulphuric acid* for 2 min. Filter and add to the filtrate 1 ml of *concentrated ammonia R* and 5 ml of *water R*. Shake cautiously with 15 ml of *peroxide-free ether R* to avoid the formation of an emulsion. Separate the ether layer and dry over *anhydrous sodium sulphate R*. Filter and evaporate the ether in a porcelain dish. Add 0.5 ml of *nitric acid R* and evaporate to dryness on a water-bath. Add 10 ml of *acetone R* and, dropwise, a 30 g/l solution of *potassium hydroxide R* in *alcohol R*. A deep violet colour develops.

TESTS

Chromatography. Examine by thin-layer chromatography (2.2.27), using *silica gel G R* as the coating substance.

Test solution. To 1.0 g of the powdered drug (180) add 10 ml of 0.05 M *sulphuric acid*, shake for 15 min and filter. Wash the filter with 0.05 M *sulphuric acid* until 25 ml of filtrate is obtained. To the filtrate add 1 ml of *concentrated ammonia R* and shake with 2 quantities, each of 10 ml, of *peroxide-free ether R*. If necessary, separate by centrifugation. Dry the combined ether layers over *anhydrous sodium sulphate R*, filter and evaporate to dryness on a water-bath. Dissolve the residue in 0.5 ml of *methanol R*.

Reference solution. Dissolve 50 mg of *hyoscyamine sulphate R* in 9 ml of *methanol R*. Dissolve 15 mg of *hyoscine hydrobromide R* in 10 ml of *methanol R*. Mix 3.8 ml of the hyoscyamine sulphate solution and 4.2 ml of the hyoscine hydrobromide solution and dilute to 10 ml with *methanol R*.

Apply separately to the plate as bands 20 mm by 3 mm 10 µl and 20 µl of each solution, leaving 1 cm between the bands. Develop over a path of 10 cm using a mixture of 3 volumes of *concentrated ammonia R*, 7 volumes of *water R* and 90 volumes of *acetone R*. Dry the plate

at 100-105 °C for 15 min, allow to cool and spray with *potassium iodobismuthate solution R2*, using about 10 ml for a plate 200 mm square, until the orange or brown zones become visible against a yellow background. The zones in the chromatograms obtained with the test solution are similar to those in the chromatograms obtained with the reference solution with respect to their position (hyoscyamine in the lower third, hyoscine in the upper third of the chromatogram) and their colour. The zones in the chromatograms obtained with the test solution are at least equal in size to the corresponding zones in the chromatogram obtained with the same volume of the reference solution. Faint secondary zones may appear, particularly in the middle of the chromatogram obtained with 20 µl of the test solution or near the starting point in the chromatogram obtained with 10 µl of the test solution. Spray the plate with *sodium nitrite solution R* until the coating is transparent. Examine after 15 min. The zones corresponding to hyoscyamine in the chromatograms obtained with the test solution and the reference solution change from brown to reddish-brown but not to greyish-blue (atropine) and any secondary zones disappear.

Foreign matter (2.8.2). Not more than 3 per cent of stem having a diameter exceeding 5 mm.

Total ash (2.4.16). Not more than 20.0 per cent.

Ash insoluble in hydrochloric acid (2.8.1). Not more than 4.0 per cent.

ASSAY

Take about 50 g of the drug and completely reduce it to a powder (180). Using the powder, determine the loss on drying and the total alkaloids.

a) Determine the loss on drying (2.2.32) on 2.000 g by drying in an oven at 100 °C to 105 °C.

b) Moisten 10.0 g of the powder with a mixture of 5 ml of *ammonia R*, 10 ml of *alcohol R* and 30 ml of *peroxide-free ether R* and mix thoroughly. Transfer the mixture to a suitable percolator, if necessary with the aid of the extracting mixture. Allow to macerate for 4 h and percolate with a mixture of 1 volume of *chloroform R* and 3 volumes of *peroxide-free ether R* until the alkaloids are completely extracted. Evaporate to dryness a few millilitres of the liquid flowing from the percolator, dissolve the residue in 0.25 M *sulphuric acid* and verify the absence of alkaloids using *potassium tetraiodomercurate solution R*. Concentrate the percolate to about 50 ml by distilling on a water-bath and transfer it to a separating funnel, rinsing with *peroxide-free ether R*. Add a quantity of *peroxide-free ether R* equal to at least 2.1 times the volume of the percolate to produce a liquid of a density well below that of water. Shake the solution with no fewer than 3 quantities, each of 20 ml, of 0.25 M *sulphuric acid*, separate the 2 layers by centrifugation if necessary and transfer the acid layers to a second separating funnel. Make the acid layer alkaline with *ammonia R* and shake with 3 quantities, each of 30 ml, of *chloroform R*. Combine the chloroform layers, add 4 g of *anhydrous sodium sulphate R* and allow to stand for 30 min with occasional shaking. Decant the chloroform and wash the sodium sulphate with 3 quantities, each of 10 ml, of *chloroform R*. Add the washings to the chloroform extract, evaporate to dryness on a water-bath and heat in an oven at 100-105 °C for 15 min. Dissolve the residue in a few millilitres of *chloroform R*, add 20.0 ml of 0.01 M *sulphuric acid* and remove the chloroform by evaporation on a water-bath. Titrate the excess of acid with 0.02 M *sodium hydroxide* using *methyl red mixed solution R* as indicator.

Calculate the percentage content of total alkaloids, expressed as hyoscyamine, from the expression:

$$\frac{57.88 (20 - n)}{(100 - d) m}$$

- d = loss on drying as a percentage,
 n = volume of 0.02 M sodium hydroxide used in millilitres,
 m = mass of drug used, in grams.

STORAGE

Protected from moisture.

01/2005:0247

STRAMONIUM, PREPARED

Stramonii pulvis normatus

DEFINITION

Prepared stramonium is stramonium leaf powder (180) adjusted, if necessary, by the addition of powdered lactose or stramonium leaf of lower alkaloidal content to contain 0.23 per cent to 0.27 per cent of total alkaloids, calculated as hyoscyamine ($C_{17}H_{23}NO_3$; M_r 289.4) with reference to the dried drug.

CHARACTERS

Greyish-green powder with an unpleasant odour.

IDENTIFICATION

- Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters: fragments of leaf lamina showing epidermal cells with slightly wavy anticlinal walls and smooth cuticle; stomata are more frequent on the lower epidermis (anisocytic and anomocytic) (2.8.3); covering trichomes are conical, uniseriate with three to five cells and warty walls; glandular trichomes are short and clavate with heads formed by 2 to 7 cells; dorsiventral mesophyll, with a single layer of palisade cells and a spongy parenchyma containing cluster crystals of calcium oxalate; annularly and spirally thickened vessels. The powdered drug may also show the following: fibres and reticulately thickened vessels from the stem; subspherical pollen grains usually about 60 µm to 80 µm in diameter with three germinal pores and nearly smooth exine; fragments of the corolla with papillose epidermis; seed fragments containing yellowish-brown, sinuous, thick-walled sclereids of testa; occasional prisms and microsphenoidal crystals of calcium oxalate. Examined in *glycerol (85 per cent) R*, it may be seen to contain lactose crystals.
- Examine the chromatograms obtained in the chromatography test. The principal zones in the chromatogram obtained with the test solution are similar in position, colour and size to the principal zones in the chromatogram obtained with the same volume of the reference solution.
- Shake 1 g with 10 ml of 0.05 M sulphuric acid for 2 min. Filter and add to the filtrate 1 ml of concentrated ammonia R and 5 ml of water R. Shake cautiously with 15 ml of peroxide-free ether R to avoid the formation of an emulsion. Separate the ether layer and dry over anhydrous sodium sulphate R. Filter and evaporate the ether in a porcelain dish. Add 0.5 ml of nitric acid R

and evaporate to dryness on a water-bath. Add 10 ml of acetone R and, dropwise, a 30 g/l solution of potassium hydroxide R in alcohol R. A deep violet colour develops.

TESTS

Chromatography. Examine by thin-layer chromatography (2.2.27), using silica gel G R as the coating substance.

Test solution. To 1.0 g add 10 ml of 0.05 M sulphuric acid, shake for 15 min and filter. Wash the filter with 0.05 M sulphuric acid until 25 ml of filtrate is obtained. To the filtrate add 1 ml of concentrated ammonia R and shake with two quantities, each of 10 ml, of peroxide-free ether R. If necessary, separate by centrifugation. Dry the combined ether layers over anhydrous sodium sulphate R, filter and evaporate to dryness on a water-bath. Dissolve the residue in 0.5 ml of methanol R.

Reference solution. Dissolve 50 mg of hyoscyamine sulphate R in 9 ml of methanol R. Dissolve 15 mg of hyoscine hydrobromide R in 10 ml of methanol R. Mix 3.8 ml of the hyoscyamine sulphate solution and 4.2 ml of the hyoscine hydrobromide solution and dilute to 10 ml with methanol R.

Apply separately to the plate as bands 20 mm by 3 mm 10 µl and 20 µl of each solution, leaving 1 cm between the bands. Develop over a path of 10 cm using a mixture of 3 volumes of concentrated ammonia R, 7 volumes of water R and 90 volumes of acetone R. Dry the plate at 100 °C to 105 °C for 15 min, allow to cool and spray with potassium iodobismuthate solution R2, using about 10 ml for a plate 200 mm square, until the orange or brown zones become visible against a yellow background. The zones in the chromatograms obtained with the test solution are similar to those in the chromatograms obtained with the reference solution with respect to their position (hyoscyamine in the lower third, hyoscine in the upper third of the chromatogram) and their colour. The zones in the chromatograms obtained with the test solution are at least equal in size to the corresponding zones in the chromatogram obtained with the same volume of the reference solution. Faint secondary zones may appear, particularly in the middle of the chromatogram obtained with 20 µl of the test solution or near the starting point in the chromatogram obtained with 10 µl of the test solution. Spray the plate with sodium nitrite solution R until the coating is transparent. Examine after 15 min. The zones corresponding to hyoscyamine in the chromatograms obtained with the test solution and the reference solution change from brown to reddish-brown but not to greyish-blue (atropine) and any secondary zones disappear.

Loss on drying (2.2.32). Not more than 5.0 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C.

Total ash (2.4.16). Not more than 20.0 per cent.

Ash insoluble in hydrochloric acid (2.8.1). Not more than 4.0 per cent.

ASSAY

- Determine the loss on drying (2.2.32) on 2.000 g by drying in an oven at 100 °C to 105 °C.
- Moisten 10.0 g with a mixture of 5 ml of ammonia R, 10 ml of alcohol R and 30 ml of peroxide-free ether R and mix thoroughly. Transfer the mixture to a suitable percolator, if necessary with the aid of the extracting mixture. Allow to macerate for 4 h and percolate with a mixture of 1 volume of chloroform R and 3 volumes of peroxide-free ether R until the alkaloids are completely extracted. Evaporate to dryness a few millilitres of the liquid flowing from the percolator,

dissolve the residue in 0.25 M sulphuric acid and verify the absence of alkaloids using *potassium tetraiodomercurate solution R*. Concentrate the percolate to about 50 ml by distilling on a water-bath and transfer it to a separating funnel, rinsing with *peroxide-free ether R*. Add a quantity of *peroxide-free ether R* equal to at least 2.1 times the volume of the percolate to produce a liquid of a density well below that of water. Shake the solution with no fewer than three quantities, each of 20 ml, of 0.25 M sulphuric acid, separate the two layers by centrifugation if necessary and transfer the acid layers to a second separating funnel. Make the acid layer alkaline with *ammonia R* and shake with three quantities, each of 30 ml, of *chloroform R*. Combine the chloroform layers, add 4 g of *anhydrous sodium sulphate R* and allow to stand for 30 min with occasional shaking. Decant the chloroform and wash the sodium sulphate with three quantities, each of 10 ml, of *chloroform R*. Add the washings to the chloroform extract, evaporate to dryness on a water-bath and heat in an oven at 100 °C to 105 °C for 15 min. Dissolve the residue in a few millilitres of *chloroform R*, add 20.0 ml of 0.01 M sulphuric acid and remove the chloroform by evaporation on a water-bath. Titrate the excess of acid with 0.02 M sodium hydroxide using *methyl red mixed solution R* as indicator.

Calculate the percentage content of total alkaloids, expressed as hyoscyamine, from the expression:

$$\frac{57.88 (20 - n)}{(100 - d) m}$$

d = loss on drying as a percentage,

n = volume of 0.02 M sodium hydroxide used in millilitres,

m = mass of drug used in grams.

STORAGE

Store in an airtight container, protected from light.

01/2005:0356

STREPTOKINASE BULK SOLUTION

Streptokinasi solutio ad praeparationem

DEFINITION

Streptokinase bulk solution is a preparation of a protein obtained from culture filtrates of certain strains of haemolytic *Streptococcus* group C; it has the property of combining with human plasminogen to form plasminogen activator. It may contain buffer salts and other auxiliary substances. The potency is not less than 96 000 IU per milligram of protein.

PRODUCTION

If intended for use in the manufacture of parenteral dosage forms, the method of manufacture is validated to demonstrate that the product, if tested, would comply with the following test.

Abnormal toxicity (2.6.9). Inject into each mouse a quantity of the preparation to be examined (if necessary, dilute with *water for injections R*) equivalent to 50 000 IU of streptokinase activity in 0.5 ml, the injection lasting 15-20 s.

CHARACTERS

Appearance: clear, colourless liquid.

IDENTIFICATION

- Place 0.5 ml of citrated human plasma in a haemolysis tube maintained in a water-bath at 37 °C. Add 0.1 ml of a dilution of the preparation to be examined containing 10 000 IU of streptokinase activity per millilitre in *phosphate buffer solution pH 7.2 R* and 0.1 ml of a solution of *human thrombin R* containing 20 IU/ml in *phosphate buffer solution pH 7.2 R*. Mix immediately. A clot forms and lyses within 30 min. Repeat the procedure using citrated bovine plasma. The clot does not lyse within 60 min.
- Dissolve 0.6 g of agar in 50.0 ml of *barbital buffer solution pH 8.6 RI*, heating until a clear solution is obtained. Use glass plates 50 mm square (transparency mounts) free from traces of grease. Using a pipette, apply to each plate 4 ml of the agar solution. Maintain the plates horizontal. Allow to cool. Pierce a cavity 6 mm in diameter in the centre of the agar and an appropriate number of cavities (not exceeding 6) at distances of 11 mm from the central cavity. Remove the residual agar from the cavities using a cannula connected to a vacuum pump. Using pipettes graduated in microlitres, place in the central cavity about 80 µl of goat or rabbit antistreptokinase serum containing 10 000 units of antistreptokinase activity per millilitre; place in each of the surrounding cavities about 80 µl of a dilution of the preparation to be examined containing 125 000 IU of streptokinase activity per millilitre. Allow the plates to stand in a humidified tank for 24 h. Only one precipitation arc appears and it is well defined and localised between the application point of the serum and each cavity containing the solution of the preparation to be examined.

TESTS

pH (2.2.3): 6.8 to 7.5.

Dilute the preparation to be examined in *carbon dioxide-free water R* to obtain a solution containing 5000 IU of streptokinase activity per millilitre.

Streptodornase: maximum 10 IU of streptodornase activity per 100 000 IU of streptokinase activity.

Test solution. Dilute the preparation to be examined in *imidazole buffer solution pH 6.5 R* to obtain a solution containing 150 000 IU of streptokinase activity per millilitre.

Reference solution. Dissolve in *imidazole buffer solution pH 6.5 R* a reference preparation of streptodornase, calibrated in International Units against the International Standard of streptodornase, to obtain a solution containing 20 IU of streptodornase activity per millilitre. The equivalence in International Units of the International Standard is stated by the World Health Organisation.

To each of 8 numbered centrifuge tubes, add 0.5 ml of a 1 g/l solution of *sodium deoxyribonucleate R* in *imidazole buffer solution pH 6.5 R*. To tube number 1 and tube number 2 add 0.25 ml of *imidazole buffer solution pH 6.5 R*, 0.25 ml of the test solution and, immediately, 3.0 ml of perchloric acid (25 g/l HClO₄). Mix, centrifuge at about 3000 *g* for 5 min and measure the absorbances (2.2.25) of the supernatant liquids at 260 nm, using as the compensation liquid a mixture of 1.0 ml of *imidazole buffer solution pH 6.5 R* and 3.0 ml of perchloric acid (25 g/l HClO₄) (absorbances A₁ and A₂). To the other 6 tubes (numbers 3 to 8) add 0.25 ml, 0.25 ml, 0.125 ml, 0.125 ml, 0 ml and 0 ml respectively of *imidazole buffer solution pH 6.5 R*; add to each tube 0.25 ml of the test solution and 0 ml, 0 ml, 0.125 ml, 0.125 ml, 0.25 ml and 0.25 ml respectively of the reference solution. Mix the contents of each tube and heat at 37 °C for 15 min. To each tube add 3.0 ml of perchloric acid (25 g/l HClO₄), mix and centrifuge. Measure the absorbances (2.2.25) of the

supernatant liquids at 260 nm using the compensation liquid described above (absorbances A_3 to A_8). The absorbances comply with the following requirement:

$$(A_3 + A_4) - (A_1 + A_2) < \frac{(A_5 + A_6 + A_7 + A_8)}{2} - (A_3 + A_4)$$

Streptolysin. In a haemolysis tube, use a quantity of the preparation to be examined equivalent to 500 000 IU of streptokinase activity and dilute to 0.5 ml with a mixture of 1 volume of *phosphate buffer solution pH 7.2 R* and 9 volumes of a 9 g/l solution of *sodium chloride R*. Add 0.4 ml of a 23 g/l solution of *sodium thioglycollate R*. Heat in a water-bath at 37 °C for 10 min. Add 0.1 ml of a solution of a reference preparation of human antistreptolysin O containing 5 IU/ml. Heat at 37 °C for 5 min. Add 1 ml of *rabbit erythrocyte suspension R*. Heat at 37 °C for 30 min. Centrifuge at about 1000 g. In the same manner, prepare a haemolysis tube in which the solution of the preparation to be examined has been replaced by 0.5 ml of a mixture of 1 volume of *phosphate buffer solution pH 7.2 R* and 9 volumes of a 9 g/l solution of *sodium chloride R*. Measure the absorbances (2.2.25) of the supernatant liquids at 550 nm. The absorbance of the test solution is not more than 50 per cent greater than that of the reference solution.

Bacterial endotoxins (2.6.14): less than 0.02 IU per 100 IU of streptokinase activity, if intended for use in the manufacture of parenteral dosage forms without a further appropriate procedure for the removal of bacterial endotoxins.

ASSAY

Protein. (2.5.33, *Method 7, Procedure A*). 1 mg of N is equivalent to 6.25 mg of protein.

Potency

The potency of streptokinase is determined by comparing its capacity to activate plasminogen to form plasmin with the same capacity of a reference preparation of streptokinase calibrated in International Units; the formation of plasmin is determined using a suitable chromogenic substrate.

The International Unit is the activity of a stated amount of the International Standard for streptokinase. The equivalence in International Units of the International Standard is stated by the World Health Organisation.

Reference and test solutions

Prepare 2 independent series of 4 dilutions of each of the substance to be examined and of the reference preparation of streptokinase in *tris(hydroxymethyl)aminomethane sodium chloride buffer solution pH 7.4 R1*, in the range of 0.5–4.0 IU/ml. Prepare and maintain all solutions at 37 °C.

Substrate solution

Mix 1.0 ml of *tris(hydroxymethyl)aminomethane buffer solution pH 7.4 R* with 1.0 ml of *chromophore substrate R3*. Add 5 µl of a 100 g/l solution of *polysorbate 20 R*. Keep at 37 °C in a water-bath. Immediately before commencing the activation assay, add 45 µl of a 1 mg/ml solution of *human plasminogen R*.

Method

Analyse each streptokinase dilution, maintained at 37 °C, in duplicate. Initiate the activation reaction by adding 60 µl of each dilution to 40 µl of substrate solution. For blank wells, use 60 µl of *tris(hydroxymethyl)aminomethane sodium chloride buffer solution pH 7.4 R1* instead of the reference and test solutions. Allow the reaction to proceed at 37 °C for 20 min and read the absorbance (2.2.25) at 405 nm. If a suitable thermostatted plate reader is available, this may be used to monitor the reaction. Alternatively, it may be necessary to stop the reaction after 20 min using

50 µl of a 50 per cent *V/V* solution of *glacial acetic acid R*. Best results are obtained when the absorbance for the highest streptokinase concentration is between 0.1 and 0.2 (after blank subtraction). If necessary, adjust the time of incubation in order to reach this range of absorbances.

Calculate the regression of the absorbance on log concentrations of the solutions of the substance to be examined and of the reference preparation of streptokinase and calculate the potency of the substance to be examined using the usual statistical methods for parallel-line assays.

The estimated potency is not less than 90 per cent and not more than 111 per cent of the stated potency. The confidence limits ($P = 0.95$) of the estimated potency are not less than 80 per cent and not more than 125 per cent of the stated potency.

STORAGE

In a sealed container, protected from light and at a temperature of –20 °C. If the substance is sterile, store in a sterile, airtight, tamper-proof container.

LABELLING

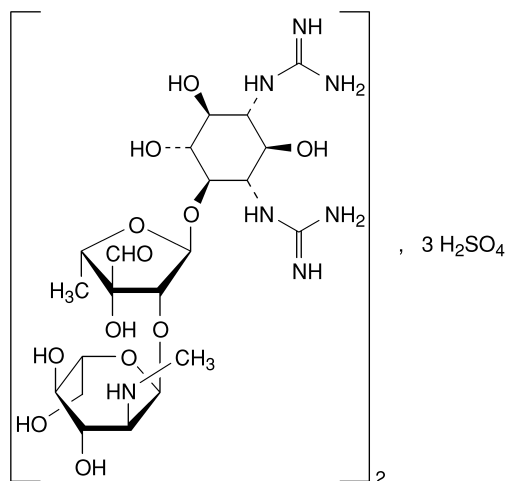
The label states:

- the number of International Units of streptokinase activity per milligram, calculated with reference to the dried preparation,
- the name and quantity of any added substance,
- where applicable, that the substance is free from bacterial endotoxins,
- where applicable, that the substance is suitable for use in the manufacture of parenteral dosage forms.

01/2005:0053

STREPTOMYCIN SULPHATE

Streptomycini sulfas



$C_{42}H_{84}N_{14}O_{36}S_3$

M_r 1457

DEFINITION

Streptomycin sulphate is bis[*N,N'*-bis(aminoiminomethyl)-4-*O*-[5-deoxy-2-*O*-[2-deoxy-2-(methylamino)-α-*L*-glucopyranosyl]-3-*C*-formyl-α-*L*-lyxofuranosyl]-D-streptamine] trisulphate, a substance produced by the growth of certain strains of *Streptomyces griseus* or obtained by any other means. Stabilisers may be added. The potency is not less than 720 IU/mg, calculated with reference to the dried substance.

PRODUCTION

It is produced by methods of manufacture designed to eliminate or minimise substances lowering blood pressure. The method of manufacture is validated to demonstrate that the product if tested would comply with the following test:

Abnormal toxicity (2.6.9). Inject into each mouse 1 mg of the substance to be examined dissolved in 0.5 ml of *water for injections R*.

CHARACTERS

A white or almost white powder, hygroscopic, very soluble in water, practically insoluble in ethanol.

IDENTIFICATION

- A. Examine by thin-layer chromatography (2.2.27), using a plate coated with a 0.75 mm layer of the following mixture: mix 0.3 g of *carbomer R* with 240 ml of *water R* and allow to stand, with moderate shaking, for 1 h; adjust to pH 7 by the gradual addition, with continuous shaking, of *dilute sodium hydroxide solution R* and add 30 g of *silica gel H R*.

Heat the plate at 110 °C for 1 h, allow to cool and use immediately.

Test solution. Dissolve 10 mg of the substance to be examined in *water R* and dilute to 10 ml with the same solvent.

Reference solution (a). Dissolve 10 mg of *streptomycin sulphate CRS* in *water R* and dilute to 10 ml with the same solvent.

Reference solution (b). Dissolve 10 mg of *kanamycin monosulphate CRS*, 10 mg of *neomycin sulphate CRS* and 10 mg of *streptomycin sulphate CRS* in *water R* and dilute to 10 ml with the same solvent.

Apply separately to the plate 10 µl of each solution. Develop over a path of 12 cm using a 70 g/l solution of *potassium dihydrogen phosphate R*. Dry the plate in a current of warm air, and spray with a mixture of equal volumes of a 2 g/l solution of *1,3-dihydroxynaphthalene R* in *alcohol R* and a 460 g/l solution of *sulphuric acid R*. Heat at 150 °C for 5 min to 10 min. The principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the spot in the chromatogram obtained with reference solution (a). The test is not valid unless the chromatogram obtained with reference solution (b) shows three clearly separated spots.

- B. Dissolve 5 mg to 10 mg in 4 ml of *water R* and add 1 ml of *1 M sodium hydroxide*. Heat in a water-bath for 4 min. Add a slight excess of *dilute hydrochloric acid R* and 0.1 ml of *ferric chloride solution R1*. A violet colour develops.
- C. Dissolve 0.1 g in 2 ml of *water R*, add 1 ml of α -*naphthol solution R* and 2 ml of a mixture of equal volumes of *strong sodium hypochlorite solution R* and *water R*. A red colour develops.
- D. Dissolve about 10 mg in 5 ml of *water R* and add 1 ml of *1 M hydrochloric acid*. Heat in a water-bath for 2 min. Add 2 ml of a 5 g/l solution of α -*naphthol R* in *1 M sodium hydroxide* and heat in a water-bath for 1 min. A faint yellow colour develops.
- E. It gives the reactions of sulphates (2.3.1).

TESTS

Solution S. Dissolve 2.5 g in *carbon dioxide-free water R* and dilute to 10 ml with the same solvent.

Appearance of solution. Solution S is not more intensely coloured than intensity 3 of the range of reference solutions of the most appropriate colour (2.2.2, *Method II*). Allow to stand protected from light, at a temperature of about 20 °C for 24 h. Solution S is not more opalescent than reference suspension II (2.2.1).

pH (2.2.3). The pH of solution S is 4.5 to 7.0.

Methanol. Examine by gas chromatography (2.2.28).

Test solution. Dissolve 1.00 g of the substance to be examined in *water R* and dilute to 25.0 ml with the same solvent.

Reference solution. Dilute 12.0 mg of *methanol R* to 100 ml with *water R*.

The chromatographic procedure may be carried out using:

- a column 1.5 m to 2.0 m long and 2 mm to 4 mm in internal diameter, packed with *ethylvinylbenzene-divinylbenzene copolymer R* (150 µm to 180 µm),
- *nitrogen for chromatography R* as the carrier gas at a constant flow rate of 30 ml to 40 ml per minute,
- a flame-ionisation detector.

Maintain the column at a constant temperature between 120 °C and 140 °C and the injection port and the detector at a temperature at least 50 °C higher than that of the column. Inject the test solution and the reference solution. The area of the peak corresponding to methanol in the chromatogram obtained with the test solution is not greater than the area of the peak in the chromatogram obtained with the reference solution (0.3 per cent).

Streptomycin B. Examine by thin-layer chromatography (2.2.27), using *silica gel G R* as the coating substance.

Test solution. Dissolve 0.2 g of the substance to be examined in a freshly prepared mixture of 3 volumes of *sulphuric acid R* and 97 volumes of *methanol R* and dilute to 5 ml with the same mixture of solvents. Heat under a reflux condenser for 1 h, cool, rinse the condenser with *methanol R* and dilute to 20 ml with the same solvent (10 g/l solution).

Reference solution. Dissolve 36 mg of *mannose R* in a freshly prepared mixture of 3 volumes of *sulphuric acid R* and 97 volumes of *methanol R* and dilute to 5 ml with the same mixture of solvents. Heat under a reflux condenser for 1 h, cool, rinse the condenser with *methanol R* and dilute to 50 ml with the same solvent. Dilute 5 ml of the solution to 50 ml with *methanol R* (0.3 g/l solution expressed as streptomycin B; 1 mg of *mannose R* is equivalent to 4.13 mg of streptomycin B).

Apply separately to the plate 10 µl of each solution. Develop over a path of 13 cm to 15 cm using a mixture of 25 volumes of *glacial acetic acid R*, 25 volumes of *methanol R* and 50 volumes of *toluene R*. Allow the plate to dry in air and spray with a freshly prepared mixture of equal volumes of a 2 g/l solution of *1,3-dihydroxynaphthalene R* in *alcohol R* and a 20 per cent V/V solution of *sulphuric acid R* and heat at 110 °C for 5 min. Any spot corresponding to streptomycin B in the chromatogram obtained with the test solution is not more intense than the spot in the chromatogram obtained with the reference solution (3.0 per cent).

Loss on drying (2.2.32). Not more than 7.0 per cent, determined on 1.000 g by drying at 60 °C over *diphosphorus pentoxide R* at a pressure not exceeding 0.1 kPa for 24 h.

Sulphated ash (2.4.14). Not more than 1.0 per cent, determined on 1.000 g.

Sulphate. 18.0 per cent to 21.5 per cent of sulphate (SO₄), calculated with reference to the dried substance. Dissolve 0.250 g in 100 ml of *water R* and adjust the solution to

pH 11 using *concentrated ammonia R*. Add 10.0 ml of 0.1 M *barium chloride* and about 0.5 mg of *phthalein purple R*. Titrate with 0.1 M *sodium edetate* adding 50 ml of *alcohol R* when the colour of the solution begins to change and continue the titration until the violet-blue colour disappears. 1 ml of 0.1 M *barium chloride* is equivalent to 9.606 mg of sulphate (SO_4).

Colorimetric test. Dry the substance to be examined and *streptomycin sulphate CRS* at 60 °C over *diphosphorus pentoxide R* at a pressure not exceeding 0.1 kPa for 24 h. Dissolve 0.100 g of the dried substance to be examined in *water R* and dilute to 100.0 ml with the same solvent. Prepare a reference solution in the same manner using 0.100 g of the dried *streptomycin sulphate CRS*. Place 5.0 ml of each solution separately in two volumetric flasks and in a third flask place 5 ml of *water R*. To each flask add 5.0 ml of 0.2 M *sodium hydroxide* and heat for exactly 10 min in a water-bath. Cool in ice for exactly 5 min, add 3 ml of a 15 g/l solution of *ferric ammonium sulphate R* in 0.5 M *sulphuric acid*, dilute to 25.0 ml with *water R* and mix. Exactly 20 min after the addition of the ferric ammonium sulphate solution measure the absorbance (2.2.25) of the test solution and the reference solution in a 2 cm cell at the maximum at 525 nm, using as compensation liquid the solution prepared from 5 ml of *water R*. The absorbance of the test solution is not less than 90.0 per cent of that of the reference solution.

Bacterial endotoxins (2.6.14): less than 0.25 IU/mg, if intended for use in the manufacture of parenteral dosage forms without a further appropriate procedure for removal of bacterial endotoxins.

ASSAY

Carry out the microbiological assay of antibiotics (2.7.2).

STORAGE

Store in an airtight container. If the substance is sterile, store in a sterile, airtight, tamper-proof container.

LABELLING

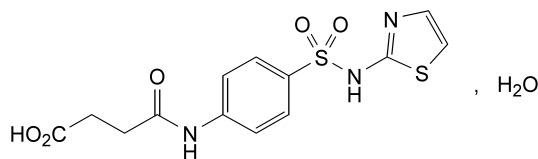
The label states:

- where applicable, the name and quantity of any added stabiliser,
- where applicable, that the substance is free from bacterial endotoxins.

01/2005:0357

SUCCINYLSULFATHIAZOLE

Succinylsulfathiazolum



$\text{C}_{13}\text{H}_{13}\text{N}_3\text{O}_5\text{S}_2 \cdot \text{H}_2\text{O}$

M_r 373.4

DEFINITION

Succinylsulfathiazole contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of 4-oxo-4-[[4-(thiazol-2-ylsulphamoyl)phenyl]amino]butanoic acid, calculated with reference to the dried substance.

CHARACTERS

A white or yellowish-white, crystalline powder, very slightly soluble in water, slightly soluble in acetone and in alcohol. It dissolves in solutions of alkali hydroxides and carbonates.

IDENTIFICATION

First identification: A, D.

Second identification: B, C, D, E.

- Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *succinylsulfathiazole CRS*. If the spectra obtained in the solid state with the substance to be examined and the reference substance show differences, dissolve in hot *water R*, allow to crystallise, dry the crystals carefully between two sheets of filter paper and prepare new discs.
- To 2 g add 10 ml of *water R* and 10 ml of *strong sodium hydroxide solution R* and boil for 10 min. Cool and adjust to pH 3.0 with *hydrochloric acid R1*. Cool, adjust to pH 7.0 with *sodium hydrogen carbonate solution R* and filter. The precipitate, washed with *water R* and dried at 100 °C to 105 °C, melts (2.2.14) at 196 °C to 204 °C. Introduce the capillary containing the precipitate into the bath at a temperature of 190 °C.
- Heat 0.1 g in a test-tube over a small flame. Fumes are evolved which blacken *lead acetate paper R*.
- Place 0.1 g in a borosilicate-glass tube of about 30 ml capacity and add 0.5 g of *hydroquinone R* and 1 ml of *sulphuric acid R*. Heat in a glycerin bath at 135 °C for 10 min, mixing at the beginning of heating to obtain a homogeneous liquid phase. Allow to cool and place in iced water. Add carefully and with shaking 15 ml of *water R*. Add 5 ml of *toluene R*, shake for 5 s to 10 s and allow to stand for 2 min; promote separation of the two layers using a stirrer. The upper (toluene) layer shows an intense pink colour.
- Dissolve about 10 mg of the precipitate obtained in identification test B in 200 ml of 0.1 M *hydrochloric acid*. 2 ml of the solution gives the reaction of primary aromatic amines (2.3.1) with formation of an orange precipitate.

TESTS

Appearance of solution. Dissolve 1.0 g in a mixture of 5 ml of *dilute sodium hydroxide solution R* and 15 ml of *water R*. The solution is clear (2.2.1) and not more intensely coloured than reference solution Y_4 or BY_4 (2.2.2, *Method II*).

Acidity. To 2.0 g add 20 ml of *water R*, shake continuously for 30 min and filter. To 10 ml of the filtrate add 0.1 ml of *phenolphthalein solution R*. Not more than 2 ml of 0.1 M *sodium hydroxide* is required to change the colour of the indicator.

Sulfathiazole and other primary aromatic amines. Dissolve 20 mg in a mixture of 3.5 ml of *water R*, 6 ml of *dilute hydrochloric acid R* and 25 ml of *alcohol R*, previously cooled to 15 °C. Place immediately in iced water and add 1 ml of a 2.5 g/l solution of *sodium nitrite R*. Allow to stand for 3 min, add 2.5 ml of a 40 g/l solution of *sulphamic acid R* and allow to stand for 5 min. Add 1 ml of a 4 g/l solution of *naphthylethylenediamine dihydrochloride R* and dilute to 50 ml with *water R*. Measured at 550 nm, the absorbance (2.2.25) is not greater than that of a standard prepared at the same time and in the same manner using a mixture of 1.5 ml of a solution containing 10 mg of *sulfathiazole R* and 0.5 ml of *hydrochloric acid R* per 100 ml; 2 ml of *water R*; 6 ml of *dilute hydrochloric acid R*; and 25 ml of *alcohol R*.

Heavy metals (2.4.8). 1.0 g complies with limit test D for heavy metals (20 ppm). Prepare the standard using 2 ml of *lead standard solution (10 ppm Pb) R*.

Loss on drying (2.2.32): 4.0 per cent to 5.5 per cent, determined on 1.00 g by drying in an oven at 100 °C to 105 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.300 g in 100 ml of a mixture of 1 volume of *hydrochloric acid R* and 2 volumes of *water R*. Heat under a reflux condenser for 1 h. Carry out the determination of primary aromatic amino-nitrogen (2.5.8), determining the end-point electrometrically.

1 ml of 0.1 M *sodium nitrite* is equivalent to 35.54 mg of $C_{12}H_{22}O_{11}$.

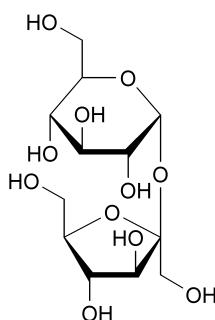
STORAGE

Store protected from light.

01/2005:0204
corrected

SUCROSE

Saccharum



$C_{12}H_{22}O_{11}$

M_r 342.3

DEFINITION

β -D-Fructofuranosyl α -D-glucopyranoside.

It contains no additives.

CHARACTERS

Appearance: white or almost white, crystalline powder, or shiny, colourless or white or almost white crystals.

Solubility: very soluble in water, slightly soluble in alcohol, practically insoluble in ethanol.

IDENTIFICATION

First identification: A.

Second identification: B, C.

A. Infrared absorption spectrophotometry (2.2.24).

Comparison: *sucrose CRS*.

B. Thin-layer chromatography (2.2.27).

Test solution. Dissolve 10 mg of the substance to be examined in a mixture of 2 volumes of *water R* and 3 volumes of *methanol R* and dilute to 20 ml with the same mixture of solvents.

Reference solution (a). Dissolve 10 mg of *sucrose CRS* in a mixture of 2 volumes of *water R* and 3 volumes of *methanol R* and dilute to 20 ml with the same mixture of solvents.

Reference solution (b). Dissolve 10 mg each of *fructose CRS*, *glucose CRS*, *lactose CRS* and *sucrose CRS* in a mixture of 2 volumes of *water R* and 3 volumes of *methanol R* and dilute to 20 ml with the same mixture of solvents.

Plate: TLC silica gel G plate R.

Mobile phase: cold saturated boric acid solution R, 60 per cent V/V solution of *glacial acetic acid R*, *ethanol R*, *acetone R*, *ethyl acetate R* (10:15:20:60:60 V/V/V/V/V).

Application: 2 μ l.

Development: in an unsaturated tank over a path of 15 cm.

Drying: in a current of warm air.

Detection: spray with a solution of 0.5 g of *thymol R* in a mixture of 5 ml of *sulphuric acid R* and 95 ml of *alcohol R*. Heat the plate at 130 °C for 10 min.

System suitability: the chromatogram obtained with reference solution (b) shows 4 clearly separated spots.

Results: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).

C. Dilute 1 ml of solution S (see Tests) to 100 ml with *water R*. To 5 ml of the solution add 0.15 ml of freshly prepared *copper sulphate solution R* and 2 ml of freshly prepared *dilute sodium hydroxide solution R*. The solution is blue and clear and remains so after boiling. To the hot solution add 4 ml of *dilute hydrochloric acid R* and boil for 1 min. Add 4 ml of *dilute sodium hydroxide solution R*. An orange precipitate is formed immediately.

TESTS

Solution S. Dissolve 50.0 g in *carbon dioxide-free water R* prepared from *distilled water R* and dilute to 100 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1).

Conductivity (2.2.38): maximum 35 μ S $\cdot$ cm $^{-1}$.

Dissolve 31.3 g in *carbon dioxide-free water R* prepared from *distilled water R* and dilute to 100 ml with the same solvent. Measure the conductivity of the solution (C_1), while gently stirring with a magnetic stirrer, and that of the water used for preparing the solution (C_2). The readings must be stable within 1 per cent over a period of 30 s. Calculate the conductivity of the solution of the substance to be examined from the following expression:

$$C_1 - 0.35 C_2$$

Specific optical rotation (2.2.7): + 66.3 to + 67.0.

Dissolve 26.0 g in *water R* and dilute to 100.0 ml with the same solvent.

Colour value: maximum 45.

Dissolve 50.0 g in 50.0 ml of *water R*. Mix, filter (diameter of pores 0.45 μ m) and degas. Measure the absorbance (2.2.25) at 420 nm, using a cell of at least 4 cm (a cell length of 10 cm or more is preferred).

Calculate the colour value using the following expression:

$$\frac{A \times 1000}{b \times c}$$

- A* = absorbance measured at 420 nm,
b = path length in centimetres,
c = concentration of the solution, in grams per millilitre, calculated from the refractive index (2.2.6) of the solution; use Table 0204.-1 and interpolate the values if necessary.

Table 0204.-1

n_D^{20}	<i>c</i> (g/ml)
1.4138	0.570
1.4159	0.585
1.4179	0.600
1.4200	0.615
1.4221	0.630
1.4243	0.645
1.4264	0.661

System suitability:

- **repeatability:** the absolute difference between 2 results is maximum 3.

Dextrins. If intended for use in the preparation of large-volume infusions, it complies with the test for dextrins. To 2 ml of solution S add 8 ml of *water R*, 0.05 ml of *dilute hydrochloric acid R* and 0.05 ml of *0.05 M iodine*. The solution remains yellow.

Reducing sugars. To 5 ml of solution S in a test-tube about 150 mm long and 16 mm in diameter add 5 ml of *water R*, 1.0 ml of *1 M sodium hydroxide* and 1.0 ml of a 1 g/l solution of *methylene blue R*. Mix and place in a water-bath. After exactly 2 min, take the tube out of the bath and examine the solution immediately. The blue colour does not disappear completely. Ignore any blue colour at the air/solution interface.

Sulphites: maximum 10 ppm, calculated as SO₂.

Determine the sulphites content by a suitable enzymatic method based on the following reactions. Sulphite is oxidised by sulphite oxidase to sulphate and hydrogen peroxide which in turn is reduced by nicotinamide-adenine dinucleotide-peroxidase in the presence of reduced nicotinamide-adenine dinucleotide (NADH). The amount of NADH oxidised is proportional to the amount of sulphite.

Test solution. Dissolve 4.0 g of the substance to be examined in freshly prepared *distilled water R* and dilute to 10.0 ml with the same solvent.

Reference solution. Dissolve 4.0 g of the substance to be examined in freshly prepared *distilled water R*, add 0.5 ml of *sulphite standard solution (80 ppm SO₂) R* and dilute to 10.0 ml with freshly prepared *distilled water R*.

Blank solution. Freshly prepared *distilled water R*.

Separately introduce 2.0 ml each of the test solution, the reference solution and the blank in 10 mm cuvettes and add the reagents as described in the instructions in the kit for sulphite determination. Measure the absorbance (2.2.25) at the absorption maximum at about 340 nm before and at the end of the reaction time and subtract the value obtained with the blank.

The absorbance difference of the test solution is not greater than half the absorbance difference of the reference solution.

Lead: maximum 0.5 ppm.

Atomic absorption spectrometry (2.2.23, *Method II*).

Test solution. Dissolve 1.5 g of the substance to be examined in 1.5 ml of *deionised distilled water R* in a digestion tube⁽⁴⁾. Add 0.75 ml of *lead-free nitric acid R1* and slowly warm to 90-95 °C, avoiding spattering. Heat for about 60 min, add again 0.75 ml of *lead-free nitric acid R1*. Heat until all brown vapours have dissipated and any reddish tint is gone (about 60 min). Cool. Add 0.5 ml of *strong hydrogen peroxide solution R* dropwise and heat at 90-95 °C for 15 min. Cool. Add again 0.5 ml of *strong hydrogen peroxide solution R* dropwise, heat at 90-95 °C for 60 min and cool. Repeat these operations until a clear, light yellow solution is obtained. Dilute to 10.0 ml with *deionised distilled water R*. Store in capped plastic vials.

Reference solutions. Prepare 3 reference solutions in the same manner as the test solution but adding 0.5 ml, 1.0 ml and 1.5 ml respectively of *lead standard solution (0.5 ppm Pb) R* in addition to the 1.5 g of the substance to be examined.

Blank solution. Prepare a blank in the same manner as for the test solution but without the substance to be examined.

Zero-setting solution. *Deionised distilled water R*.

Apparatus. Suitable graphite furnace atomic absorption spectrometer equipped with a background compensation system, an autosampler and pyrolytically-coated tubes or with pyrolytic graphite platforms.

Source: hollow-cathode lamp or electrodeless discharge lamp.

Gas:

- *argon R* as the purge gas,
- air as alternate gas during the charring step.

Flow rate: to be adapted according to the apparatus; usually between 200 ml/min and 300 ml/min.

Wavelength: 283.3 nm.

Method. Separately inject 20 µl each of the zero-setting solution, the blank solution, the test solution, the reference solutions, and add immediately 5 µl of *magnesium nitrate solution R1*, which is used as matrix modifier, to each of the solutions. Inject each solution in triplicate.

The ashing and atomisation temperatures and times vary according to the apparatus, the background compensation system, and the graphite tube etc. The following parameters are given for guidance and have to be adjusted.

Heat the furnace progressively to 200 °C and maintain the drying temperature at 200 °C for 30 s, the ashing temperature at 750 °C for 40 s after a 40 s temperature rise, and the atomisation temperature at 1800 °C for 10 s (0 s temperature rise). Clean out at 2600 °C for 7 s after a 1 s temperature rise. Use the zero-setting solution to set the instrument zero. Calculate the mean readings of the test solution and of the reference solutions subtracting the mean blank. If necessary, dilute with the zero-setting solution to obtain a reading within the linear range.

System suitability:

- the relative standard deviation of the 3 readings obtained for the triplicate injections of the test solution and the reference solutions to which the mean blank has been subtracted is not greater than 15 per cent.

Loss on drying (2.2.32): maximum 0.1 per cent, determined on 2.000 g by drying in an oven at 100-105 °C for 3 h.

(4) An acid-cleaned high density polyethylene tube, a polypropylene tube, a teflon tube or a quartz tube is suitable.

Bacterial endotoxins (2.6.14): less than 0.25 IU/mg, if intended for use in the preparation of large-volume infusions.

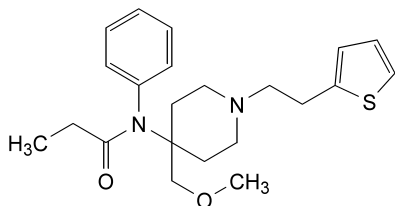
LABELLING

The label states, where applicable, that the substance is suitable for use in the manufacture of large-volume parenteral dosage forms.

01/2005:1569

SUFENTANIL

Sufentanilum

C₂₂H₃₀N₂O₂SM_r 386.6

DEFINITION

Sufentanil contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of *N*-[4-(methoxymethyl)-1-[2-(thiophen-2-yl)ethyl]piperidin-4-yl]-*N*-phenylpropanamide, calculated with reference to the dried substance.

CHARACTERS

A white or almost white powder, practically insoluble in water, freely soluble in alcohol and in methanol.

It melts at about 98 °C.

IDENTIFICATION

Examine by infrared absorption spectrophotometry (2.2.24), comparing with the *Ph. Eur. reference spectrum of sufentanil*.

TESTS

Appearance of solution. Dissolve 0.10 g in *methanol R* and dilute to 20 ml with the same solvent. The solution is clear (2.2.1) and colourless (2.2.2, *Method II*).

Related substances. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 0.100 g of the substance to be examined in *methanol R* and dilute to 10.0 ml with the same solvent.

Reference solution (a). In order to prepare *in situ* the degradation compound (sufentanil impurity E), dissolve 10 mg of the substance to be examined in 10.0 ml of *dilute hydrochloric acid R*. Heat on a water-bath under a reflux condenser for 4 h. Add 10.0 ml of *dilute sodium hydroxide solution R*. Evaporate to dryness on a water-bath. Cool and take up the residue in 10 ml of *methanol R*. Filter.

Reference solution (b). Dilute 1.0 ml of the test solution to 100.0 ml with *methanol R*. Dilute 5.0 ml of this solution to 20.0 ml with *methanol R*.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.1 m long and 4.6 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (3 µm),
- as mobile phase at a flow rate of 1.5 ml/min:

Mobile phase A. A 5 g/l solution of *ammonium carbonate R* in a mixture of 10 volumes of *tetrahydrofuran R* and 90 volumes of *water R*,

Mobile phase B. *Acetonitrile R*,

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)	Comment
0 - 15	90 → 40	10 → 60	linear gradient
15 - 20	40	60	isocratic
20 - 21	40 → 90	60 → 10	switch to initial eluent composition
21 - 25	90	10	re-equilibration

- as detector a spectrophotometer set at 220 nm.

Equilibrate the column for at least 30 min with *acetonitrile R* and then equilibrate with the initial eluent composition for at least 5 min.

Adjust the sensitivity of the system so that the height of the principal peak in the chromatogram obtained with 10 µl of reference solution (b) is at least 50 per cent of the full scale of the recorder.

Inject 10 µl of reference solution (a). When the chromatogram is recorded in the prescribed conditions, the retention times are: impurity E about 12 min and sufentanil about 13 min. The test is not valid unless the resolution between the peaks corresponding to sufentanil and impurity E is at least 4.0. If necessary, adjust the concentration of acetonitrile in the mobile phase or adjust the time programme for the linear gradient elution.

Inject 10 µl of *methanol R* as a blank, 10 µl of the test solution and 10 µl of reference solution (b). In the chromatogram obtained with the test solution: the area of any peak, apart from the principal peak, is not greater than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.25 per cent); the sum of the areas of all the peaks, apart from the principal peak, is not greater than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent). Disregard any peak due to the blank and any peak with an area less than 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying *in vacuo* at 60 °C for 2 h.

ASSAY

Dissolve 0.300 g in 50 ml of a mixture of 1 volume of *anhydrous acetic acid R* and 7 volumes of *methyl ethyl ketone R* and titrate with 0.1 M *perchloric acid*, using 0.2 ml of *naphtholbenzein solution R* as indicator.

1 ml of 0.1 M *perchloric acid* is equivalent to 38.66 mg of C₂₂H₃₀N₂O₂S.

STORAGE

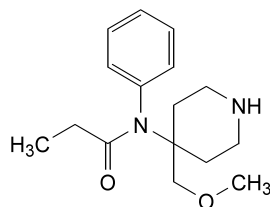
Store protected from light.

IMPURITIES

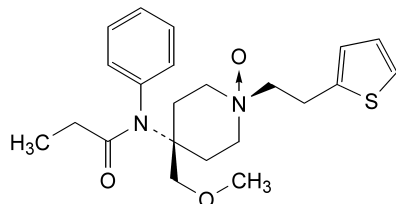
Specified impurities: D, F, H.

Other detectable impurities: A, B, C, E, G, I.

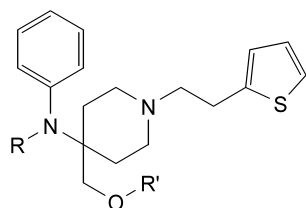
01/2005:1269



A. *N*-[4-(methoxymethyl)piperidin-4-yl]-*N*-phenylpropanamide,



B. *cis*-4-(methoxymethyl)-4-(phenylpropanoylamino)-1-[2-(thiophen-2-yl)ethyl]piperidine 1-oxide,



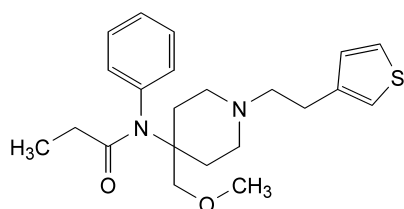
C. $R = R' = H$: [4-(phenylamino)-1-[2-(thiophen-2-yl)ethyl]piperidin-4-yl]methanol,

D. $R = CO-CH_3$, $R' = CH_3$: *N*-[4-(methoxymethyl)-1-[2-(thiophen-2-yl)ethyl]piperidin-4-yl]-*N*-phenylacetamide,

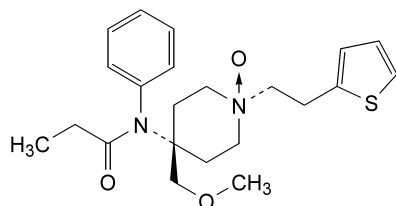
E. $R = H$, $R' = CH_3$: 4-(methoxymethyl)-*N*-phenyl-1-[2-(thiophen-2-yl)ethyl]piperidin-4-amine,

G. $R = R' = CO-CH_2-CH_3$: [4-(phenylpropanoylamino)-1-[2-(thiophen-2-yl)ethyl]piperidin-4-yl]methyl propanoate,

H. $R = CO-CH_2-CH_2-CH_3$, $R' = CH_3$: *N*-[4-(methoxymethyl)-1-[2-(thiophen-2-yl)ethyl]piperidin-4-yl]-*N*-phenylbutanamide,



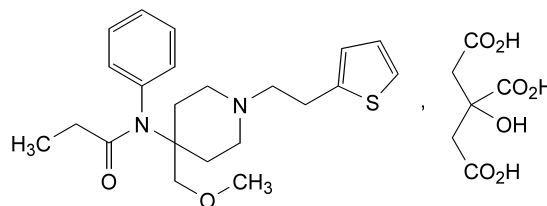
F. *N*-[4-(methoxymethyl)-1-[2-(thiophen-3-yl)ethyl]piperidin-4-yl]-*N*-phenylpropanamide,



I. *trans*-4-(methoxymethyl)-4-(phenylpropanoylamino)-1-[2-(thiophen-2-yl)ethyl]piperidine 1-oxide.

SUFENTANIL CITRATE

Sufentanili citras


 $C_{28}H_{38}N_2O_9S$
 M_r 578.7

DEFINITION

Sufentanil citrate contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of *N*-[4-(methoxymethyl)-1-[2-(thiophen-2-yl)ethyl]piperidin-4-yl]-*N*-phenylpropanamide citrate, calculated with reference to the dried substance.

CHARACTERS

A white or almost white powder, soluble in water and in alcohol, freely soluble in methanol.

It melts at about 140 °C, with decomposition.

IDENTIFICATION

Examine by infrared absorption spectrophotometry (2.2.24), comparing with the *Ph. Eur. reference spectrum of sufentanil citrate*.

TESTS

Appearance of solution. Dissolve 0.2 g of the substance to be examined in *water R* and dilute to 20 ml with the same solvent. The solution is clear (2.2.1) and colourless (2.2.2, *Method II*).

Related substances. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 0.100 g of the substance to be examined in *methanol R* and dilute to 10.0 ml with the same solvent.

Reference solution (a). In order to prepare *in situ* the degradation compound (sufentanil impurity E), dissolve 10 mg of the substance to be examined in 10.0 ml of *dilute hydrochloric acid R*. Heat on a water-bath under a reflux condenser for 4 h. Add 10.0 ml of *dilute sodium hydroxide solution R*. Evaporate to dryness on a water-bath. Cool and take up the residue in 10 ml of *methanol R*. Filter.

Reference solution (b). Dilute 5.0 ml of the test solution to 100.0 ml with *methanol R*. Dilute 1.0 ml of this solution to 10.0 ml with *methanol R*.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.1 m long and 4.6 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (3 µm),
- as mobile phase at a flow rate of 1.5 ml/min the following mixtures:

Mobile phase A. A 5 g/l solution of *ammonium carbonate R* in a mixture of 10 volumes of *tetrahydrofuran R* and 90 volumes of *water R*,

Mobile phase B. *Acetonitrile R*,

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)	Comment
0 - 15	90 → 40	10 → 60	linear gradient
15 - 20	40	60	isocratic elution
20 - 25	90	10	switch to initial eluent composition
25 = 0	90	10	restart gradient

— as detector a spectrophotometer set at 220 nm.

Equilibrate the column for at least 30 min with *acetonitrile R* and then equilibrate at the initial eluent composition for at least 5 min.

Adjust the sensitivity of the system so that the height of the principal peak in the chromatogram obtained with 10 µl of reference solution (b) is at least 50 per cent of the full scale of the recorder.

Inject 10 µl of reference solution (a). When the chromatograms are recorded in the prescribed conditions, the retention times are: sufentanil impurity E about 12 min and sufentanil about 13 min. The test is not valid unless the resolution between the peaks corresponding to sufentanil and sufentanil impurity E is at least 4.0. If necessary, adjust the concentration of acetonitrile in the mobile phase or adjust the time programme for the linear gradient elution.

Inject separately 10 µl of *methanol R* as a blank, 10 µl of the test solution and 10 µl of reference solution (b). In the chromatogram obtained with the test solution: the area of any peak, apart from the principal peak, is not greater than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent); the sum of the areas of all of the peaks, apart from the principal peak, is not greater than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (1 per cent). Disregard any peak obtained with the blank run, any peak with a retention time relative to the main compound of 0.05 or less, and any peak with an area less than 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (b).

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying *in vacuo* at 60 °C.

ASSAY

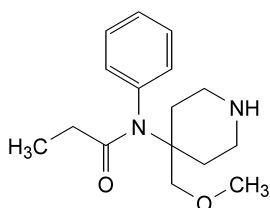
Dissolve 0.400 g in 50 ml of a mixture of 1 volume of *anhydrous acetic acid R* and 7 volumes of *methyl ethyl ketone R* and titrate with 0.1 M *perchloric acid*, using 0.2 ml of *naphtholbenzein solution R* as indicator.

1 ml of 0.1 M *perchloric acid* is equivalent to 57.87 mg of C₂₈H₃₈N₂O₉S.

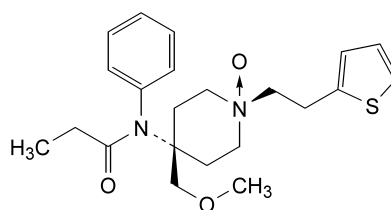
STORAGE

Store protected from light.

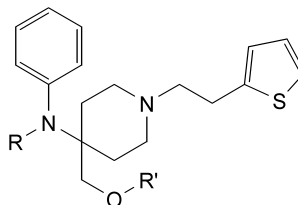
IMPURITIES



A. *N*-[4-(methoxymethyl)piperidin-4-yl]-*N*-phenylpropanamide,



B. *cis*-4-(methoxymethyl)-4-(phenylpropanoylamino)-1-[2-(thiophen-2-yl)ethyl]piperidine 1-oxide,



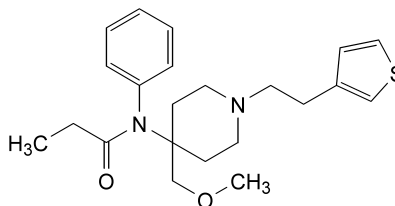
C. R = R' = H: [4-(phenylamino)-1-[2-(thiophen-2-yl)ethyl]piperidin-4-yl]methanol,

D. R = CO-CH₃, R' = CH₃: *N*-[4-(methoxymethyl)-1-[2-(thiophen-2-yl)ethyl]piperidin-4-yl]-*N*-phenylacetamide,

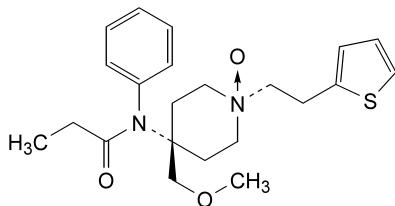
E. R = H, R' = CH₃: 4-(methoxymethyl)-*N*-phenyl-1-[2-(thiophen-2-yl)ethyl] piperidin-4-amine,

G. R = R' = CO-CH₂-CH₃: [4-(phenylpropanoylamino)-1-[2-(thiophen-2-yl)ethyl] piperidin-4-yl]methyl propanoate,

H. R = CO-CH₂-CH₂-CH₃, R' = CH₃: *N*-[4-(methoxymethyl)-1-[2-(thiophen-2-yl)ethyl]piperidin-4-yl]-*N*-phenylbutanamide,



F. *N*-[4-(methoxymethyl)-1-[2-(thiophen-3-yl)ethyl]piperidin-4-yl]-*N*-phenylpropanamide,



I. *trans*-4-(methoxymethyl)-4-(phenylpropanoylamino)-1-[2-(thiophen-2-yl)ethyl]piperidine 1-oxide.

01/2005:1570

SUGAR SPHERES

Sacchari spheri

DEFINITION

Sugar spheres contain not more than 92 per cent of sucrose, calculated on the dried basis. The remainder consists of maize starch and may also contain starch hydrolysates and colour additives. The diameter of sugar spheres varies usually from 200 µm to 2000 µm and the upper and lower limits of the size of the sugar spheres are stated on the label.

IDENTIFICATION

- A. Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel G plate R*.

Test solution. Mix 2 ml of solution S (see Tests) with 3 ml of *methanol R*. Dilute to 20 ml with a mixture of 2 volumes of *water R* and 3 volumes of *methanol R*.

Reference solution (a). Dissolve 10 mg of *sucrose CRS* in a mixture of 2 volumes of *water R* and 3 volumes of *methanol R* and dilute to 20 ml with the same mixture of solvents.

Reference solution (b). Dissolve 10 mg of *fructose CRS*, 10 mg of *glucose CRS*, 10 mg of *lactose CRS* and 10 mg of *sucrose CRS* in a mixture of 2 volumes of *water R* and 3 volumes of *methanol R* and dilute to 20 ml with the same mixture of solvents.

Apply to the plate 2 µl of each solution and thoroughly dry the starting points. Develop over a path of 15 cm using a mixture of 10 volumes of *water R*, 15 volumes of *methanol R*, 25 volumes of *anhydrous acetic acid R* and 50 volumes of *ethylene chloride R*, measured accurately as a slight excess of water causes cloudiness of the solution. Dry the plate in a current of warm air. Repeat the development immediately after renewing the mobile phase. Dry the plate in a current of warm air and spray evenly with a 5 g/l solution of *thymol R* in a mixture of 5 volumes of *sulphuric acid R* and 95 volumes of *alcohol R*. Heat at 130 °C for 10 min. The principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a). The test is not valid unless the chromatogram obtained with reference solution (b) shows 4 clearly separated spots.

- B. To water slurry of the insoluble portion obtained (see Assay), add 0.05 ml of *iodine solution R1*. A dark-blue is produced which disappears on heating.
- C. To 5 ml of solution S add 0.15 ml of freshly prepared *copper sulphate solution R* and 2 ml of freshly prepared *dilute sodium hydroxide solution R*. The solution is blue and clear and remains so after boiling. To the hot solution add 4 ml of *dilute hydrochloric acid R* and boil for 1 min. Add 4 ml of *dilute sodium hydroxide solution R*. An orange precipitate is formed immediately.

TESTS

Solution S. To 0.5 g in a 100 ml volumetric flask add 80 ml of *water R* and shake until the sucrose is dissolved. Dilute to 100.0 ml with *water R*. Filter under vacuum to obtain a clear solution.

Fineness (2.9.12). Minimum of 90 per cent (*m/m*) of the sugar spheres are between the lower and the upper limits of the size of the sugar spheres stated on the label.

Heavy metals (2.4.8). 2.0 g complies with limit test C for heavy metals (5 ppm). Prepare the standard using 1.0 ml of *lead standard solution (10 ppm Pb) R*.

Loss on drying (2.2.32). Not more than 5.0 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C for 4 h.

Sulphated ash (2.4.14). Not more than 0.2 per cent, determined on 2 g.

Microbial contamination. Total viable aerobic count (2.6.12) not more than 10³ bacteria and 10² fungi per gram, determined by plate count. It complies with the tests for *Escherichia coli* and *Salmonella* (2.6.13).

ASSAY

Sucrose content

Weigh 10.000 g of ground sugar spheres in a 100 ml graduated flask and complete to 100.0 ml with *water R*. Stir and decant. Filter under vacuum to obtain a clear solution (the insoluble portion is used for the identification test B). Measure the angle of optical rotation (2.2.7) and calculate the sucrose percentage content as follows:

$$\frac{10^6 \times \alpha}{66.5 \times l \times m \times (100 - H)}$$

α = angle of rotation,

l = length of the polarimeter tube, in decimetres,

m = exact weight of the granules sample, in grams,

H = loss on drying.

LABELLING

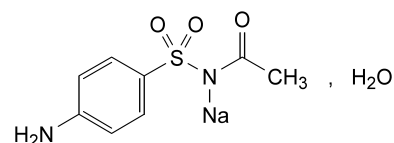
The label states:

- the upper and the lower limits of the size of the sugar spheres,
- any added colour additives,
- any added starch hydrolysates.

01/2005:0107

SULFACETAMIDE SODIUM

Sulfacetamidum natricum

C₈H₉N₂NaO₃S·H₂O*M*_r 254.2

DEFINITION

Sulfacetamide sodium contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of the sodium derivative of *N*-(4-aminophenyl)sulfonylacetamide, calculated with reference to the anhydrous substance.

CHARACTERS

A white or yellowish-white, crystalline powder, freely soluble in water, slightly soluble in ethanol.

IDENTIFICATION

First identification: B, F.

Second identification: A, C, D, E, F.

- A. Dissolve 0.1 g in *phosphate buffer solution pH 7.0 R* and dilute to 100.0 ml with the same buffer solution. Dilute 1.0 ml of the solution to 100.0 ml with *phosphate buffer solution pH 7.0 R*. Examined between 230 nm and 350 nm (2.2.25), the solution shows an absorption maximum at 255 nm. The specific absorbance at the maximum is 660 to 720, calculated with reference to the anhydrous substance.
- B. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *sulfacetamide sodium CRS*.
- C. Dissolve 1 g in 10 ml of *water R*, add 6 ml of *dilute acetic acid R* and filter. The precipitate, washed with a small quantity of *water R* and dried at 100 °C to 105 °C for 4 h, melts (2.2.14) at 181 °C to 185 °C.

01/2005:0294

- D. Dissolve 0.1 g of the precipitate obtained in identification test C in 5 ml of *alcohol R*. Add 0.2 ml of *sulphuric acid R* and heat. The odour of ethyl acetate is perceptible.
- E. Dissolve about 1 mg of the precipitate obtained in identification test C, with heating, in 1 ml of *water R*. The solution gives the reaction of primary aromatic amines (2.3.1) with formation of an orange-red precipitate.
- F. Solution S (see Tests) gives the reactions of sodium (2.3.1).

TESTS

Solution S. Dissolve 1.25 g in *carbon dioxide-free water R* and dilute to 25 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and not more intensely coloured than reference solution GY₄ (2.2.2, *Method II*).

pH (2.2.3). The pH of solution S is 8.0 to 9.5.

Related substances. Examine by thin-layer chromatography (2.2.27), using *silica gel HF₂₅₄ R* as the coating substance.

Test solution. Dissolve 1.5 g of the substance to be examined in *water R* and dilute to 15 ml with the same solvent.

Reference solution (a). Dissolve 5 mg of *sulfanilamide R* in *water R* and dilute to 10 ml with the same solvent.

Reference solution (b). Dilute 5 ml of reference solution (a) to 10 ml with *water R*.

Reference solution (c). Dissolve 5 mg of *sulfanilamide R* in 10 ml of the test solution.

Apply to the plate 5 µl of each solution. Develop over a path of 15 cm using a mixture of 10 volumes of *concentrated ammonia R*, 25 volumes of *ethanol R*, 25 volumes of *water R* and 50 volumes of *butanol R*. Allow the plate to dry in air and spray with *dimethylaminobenzaldehyde solution R2*. Any spot in the chromatogram obtained with the test solution, apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (a) (0.5 per cent), and not more than one such spot is more intense than the spot in the chromatogram obtained with reference solution (b) (0.25 per cent). The test is not valid unless the chromatogram obtained with reference solution (c) shows two clearly separated spots.

Sulphates (2.4.13). Dissolve 2.5 g in *distilled water R* and dilute to 25 ml with the same solvent. Add 25 ml of *dilute acetic acid R*, shake for 30 min and filter. 15 ml of the filtrate complies with the limit test for sulphates (200 ppm).

Heavy metals (2.4.8). 12 ml of the filtrate obtained in the test for sulphates complies with limit test A for heavy metals (20 ppm). Prepare the standard using *lead standard solution (1 ppm Pb) R*.

Water (2.5.12). 6.0 per cent to 8.0 per cent, determined on 0.200 g by the semi-micro determination of water.

ASSAY

Dissolve 0.500 g in a mixture of 50 ml of *water R* and 20 ml of *dilute hydrochloric acid R*. Cool the solution in iced water and carry out the determination of primary aromatic amino-nitrogen (2.5.8), determining the end-point electrometrically.

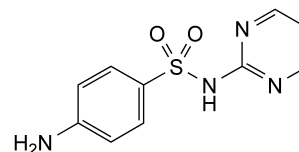
1 ml of 0.1 M *sodium nitrite* is equivalent to 23.62 mg of C₈H₉N₃NaO₃S.

STORAGE

Store protected from light.

SULFADIAZINE

Sulfadiazinum

C₁₀H₁₀N₄O₂SM_r 250.3

DEFINITION

Sulfadiazine contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of 4-amino-*N*-pyrimidin-2-ylbenzenesulphonamide, calculated with reference to the dried substance.

CHARACTERS

White, yellowish-white or pinkish-white, crystalline powder or crystals, practically insoluble in water, slightly soluble in acetone, very slightly soluble in alcohol. It dissolves in solutions of alkali hydroxides and in dilute mineral acids. It melts at about 255 °C, with decomposition.

IDENTIFICATION

First identification: A, B.

Second identification: B, C, D.

- Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *sulfadiazine CRS*. Examine the substances prepared as discs.
- Examine the chromatograms obtained in the test for related substances. The principal spot in the chromatogram obtained with test solution (a) corresponds in position and size to the principal spot in the chromatogram obtained with reference solution (a).
- Place 3 g in a dry tube. Immerse the lower part of the tube, inclined at 45°, in a silicone oil bath and heat to about 270 °C. The substance to be examined decomposes and a white or yellowish-white sublimate is formed which, after recrystallisation from *toluene R* and drying at 100 °C, melts (2.2.14) at 123 °C to 127 °C.
- Dissolve about 5 mg in 10 ml of 1 M *hydrochloric acid*. Dilute 1 ml of the solution to 10 ml with *water R*. The solution, without further acidification, gives the reaction of primary aromatic amines (2.3.1).

TESTS

Appearance of solution. Dissolve 0.8 g in a mixture of 5 ml of *dilute sodium hydroxide solution R* and 5 ml of *water R*. The solution is not more intensely coloured than reference solution Y₅, BY₅ or GY₅ (2.2.2, *Method II*).

Acidity. To 1.25 g, finely powdered, add 25 ml of *carbon dioxide-free water R*. Heat at about 70 °C for 5 min. Cool in iced water for about 15 min and filter. To 20 ml of the filtrate add 0.1 ml of *bromothymol blue solution R1*. Not more than 0.2 ml of 0.1 M *sodium hydroxide* is required to change the colour of the indicator.

Related substances. Examine by thin-layer chromatography (2.2.27), using *silica gel GF₂₅₄ R* as the coating substance.

Test solution (a). Dissolve 20 mg of the substance to be examined in 3 ml of a mixture of 2 volumes of *concentrated ammonia R* and 48 volumes of *methanol R* and dilute to 5.0 ml with the same mixture of solvents.

Test solution (b). Dissolve 0.10 g of the substance to be examined in 0.5 ml of *concentrated ammonia R* and dilute to 5.0 ml with *methanol R*. If the solution is not clear, heat gently until dissolution is complete.

Reference solution (a). Dissolve 20 mg of *sulfadiazine CRS* in 3 ml of a mixture of 2 volumes of *concentrated ammonia R* and 48 volumes of *methanol R* and dilute to 5.0 ml with the same mixture of solvents.

Reference solution (b). Dilute 1.25 ml of test solution (a) to 50 ml with a mixture of 2 volumes of *concentrated ammonia R* and 48 volumes of *methanol R*.

Apply to the plate 5 µl of each solution. Develop over a path of 15 cm using a mixture of 3 volumes of *dilute ammonia R1*, 5 volumes of *water R*, 40 volumes of *nitromethane R* and 50 volumes of *dioxan R*. Dry the plate at 100 °C to 105 °C and examine in ultraviolet light at 254 nm. Any spot in the chromatogram obtained with test solution (b), apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.5 per cent).

Heavy metals (2.4.8). 1.0 g complies with limit test D for heavy metals (20 ppm). Prepare the standard using 2 ml of *lead standard solution (10 ppm Pb) R*.

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.00 g by drying in an oven at 100 °C to 105 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.200 g in a mixture of 20 ml of *dilute hydrochloric acid R* and 50 ml of *water R*. Cool the solution in iced water. Carry out the determination of primary aromatic amino-nitrogen (2.5.8), determining the end-point electrometrically. 1 ml of 0.1 M *sodium nitrite* is equivalent to 25.03 mg of C₁₂H₁₄N₄O₂S.

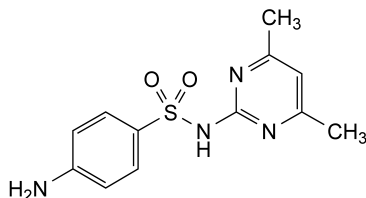
STORAGE

Store protected from light.

01/2005:0295

SULFADIMIDINE

Sulfadimidinum



C₁₂H₁₄N₄O₂S

M_r 278.3

DEFINITION

Sulfadimidine contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of 4-amino-*N*-(4,6-dimethylpyrimidin-2-yl)benzenesulphonamide, calculated with reference to the dried substance.

CHARACTERS

White or almost white powder or crystals, very slightly soluble in water, soluble in acetone, slightly soluble in alcohol. It dissolves in solutions of alkali hydroxides and in dilute mineral acids.

It melts at about 197 °C, with decomposition.

IDENTIFICATION

First identification: A, B.

Second identification: B, C, D.

- Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *sulfadimidine CRS*. Examine the substances prepared as discs.
- Examine the chromatograms obtained in the test for related substances. The principal spot in the chromatogram obtained with test solution (a) corresponds in position and size to the principal spot in the chromatogram obtained with reference solution (a).
- Place 3 g in a dry tube. Immerse the lower part of the tube, inclined at 45°, in a silicone oil bath and heat to about 270 °C. The substance to be examined decomposes and a white or yellowish-white sublimate is formed which, after recrystallisation from *toluene R* and drying at 100 °C, melts (2.2.14) at 150 °C to 154 °C.
- Dissolve about 5 mg in 10 ml of 1 M *hydrochloric acid*. Dilute 1 ml of the solution to 10 ml with *water R*. The solution, without further acidification, gives the reaction of primary aromatic amines (2.3.1).

TESTS

Appearance of solution. Dissolve 0.5 g in a mixture of 5 ml of *dilute sodium hydroxide solution R* and 5 ml of *water R*. The solution is not more intensely coloured than reference solution Y₅, BY₅ or GY₅ (2.2.2, *Method II*).

Acidity. To 1.25 g, finely powdered, add 25 ml of *carbon dioxide-free water R*. Heat at about 70 °C for 5 min. Cool in iced water for about 15 min and filter. To 20 ml of the filtrate add 0.1 ml of *bromothymol blue solution R1*. Not more than 0.2 ml of 0.1 M *sodium hydroxide* is required to change the colour of the indicator.

Related substances. Examine by thin-layer chromatography (2.2.27), using *silica gel GF₂₅₄ R* as the coating substance.

Test solution (a). Dissolve 20 mg of the substance to be examined in 3 ml of a mixture of 2 volumes of *concentrated ammonia R* and 48 volumes of *methanol R* and dilute to 5.0 ml with the same mixture of solvents.

Test solution (b). Dissolve 0.10 g of the substance to be examined in 0.5 ml of *concentrated ammonia R* and dilute to 5.0 ml with *methanol R*. If the solution is not clear, heat gently until dissolution is complete.

Reference solution (a). Dissolve 20 mg of *sulfadimidine CRS* in 3 ml of a mixture of 2 volumes of *concentrated ammonia R* and 48 volumes of *methanol R* and dilute to 5.0 ml with the same mixture of solvents.

Reference solution (b). Dilute 1.25 ml of test solution (a) to 50 ml with a mixture of 2 volumes of *concentrated ammonia R* and 48 volumes of *methanol R*.

Apply to the plate 5 µl of each solution. Develop over a path of 15 cm using a mixture of 3 volumes of *dilute ammonia R1*, 5 volumes of *water R*, 40 volumes of *nitromethane R* and 50 volumes of *dioxan R*. Dry the plate at 100 °C to 105 °C and examine in ultraviolet light at 254 nm. Any spot in the chromatogram obtained with test solution (b), apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.5 per cent).

Heavy metals (2.4.8). 1.0 g complies with limit test D for heavy metals (20 ppm). Prepare the standard using 2 ml of *lead standard solution (10 ppm Pb) R*.

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.00 g by drying in an oven at 100 °C to 105 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.250 g in a mixture of 20 ml of *dilute hydrochloric acid R* and 50 ml of *water R*. Cool the solution in iced water. Carry out the determination of primary aromatic amino-nitrogen (2.5.8), determining the end-point electrometrically.

1 ml of 0.1 M *sodium nitrite* is equivalent to 27.83 mg of $C_{12}H_{14}N_4O_4S$.

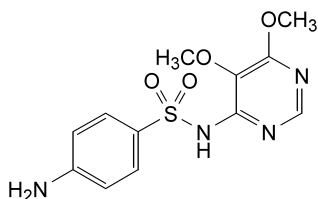
STORAGE

Store protected from light.

01/2005:0740

SULFADOXINE

Sulfadoxinum


 $C_{12}H_{14}N_4O_4S$
 M_r 310.3

DEFINITION

Sulfadoxine contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of 4-amino-*N*-(5,6-dimethoxypyrimidin-4-yl)benzenesulphonamide, calculated with reference to the dried substance.

CHARACTERS

White or yellowish-white crystalline powder or crystals, very slightly soluble in water, slightly soluble in alcohol and in methanol. It dissolves in solutions of alkali hydroxides and in dilute mineral acids.

It melts at about 198 °C, with decomposition.

IDENTIFICATION

First identification: A, C.

Second identification: B, C, D.

- Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *sulfadoxine CRS*. Examine the substances prepared as discs.
- Examine the chromatograms obtained in the test for related substances. The principal spot in the chromatogram obtained with test solution (b) is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a).
- Dissolve 0.5 g in 1 ml of a 40 per cent *V/V* solution of *sulphuric acid R*, heating gently. Continue heating until a crystalline precipitate appears (about 2 min). Allow to cool and add 10 ml of *dilute sodium hydroxide solution R*. Cool again. Add 25 ml of *ether R* and shake for 5 min. Separate the ether layer, dry over *anhydrous*

sodium sulphate R and filter. Evaporate the solvent by heating in a water-bath. The residue melts (2.2.14) at 80 °C to 82 °C or at 90 °C to 92 °C.

- Dissolve about 5 mg in 10 ml of 1 M *hydrochloric acid*. Dilute 1 ml of the solution to 10 ml with *water R*. The solution, without further acidification, gives the reaction of primary aromatic amines (2.3.1).

TESTS

Appearance of solution. Dissolve 1.0 g in a mixture of 5 ml of *dilute sodium hydroxide solution R* and 5 ml of *water R*. The solution is not more intensely coloured than reference solution Y_5 , BY_5 or GY_5 (2.2.2, *Method II*).

Acidity. To 1.25 g, finely powdered, add 25 ml of *carbon dioxide-free water R*. Heat at 70 °C for 5 min. Cool in a bath of iced water for about 15 min and filter. To 20 ml of the filtrate add 0.1 ml of *bromothymol blue solution R1*. Not more than 0.2 ml of 0.1 M *sodium hydroxide* is required to change the colour of the indicator.

Related substances. Examine by thin-layer chromatography (2.2.27), using *silica gel GF₂₅₄ R* as the coating substance.

Test solution (a). Dissolve 0.10 g of the substance to be examined in 3 ml of a mixture of 2 volumes of *concentrated ammonia R* and 48 volumes of *methanol R* and dilute to 5 ml with the same mixture of solvents.

Test solution (b). Dilute 1 ml of test solution (a) to 5 ml with a mixture of 2 volumes of *concentrated ammonia R* and 48 volumes of *methanol R*.

Reference solution (a). Dissolve 20 mg of *sulfadoxine CRS* in 3 ml of a mixture of 2 volumes of *concentrated ammonia R* and 48 volumes of *methanol R* and dilute to 5 ml with the same mixture of solvents.

Reference solution (b). Dilute 2.5 ml of test solution (b) to 100 ml with a mixture of 2 volumes of *concentrated ammonia R* and 48 volumes of *methanol R*.

Apply to the plate 5 µl of each solution. Develop over a path of 15 cm using a mixture of 3 volumes of *dilute ammonia R1*, 5 volumes of *water R*, 40 volumes of *nitromethane R* and 50 volumes of *dioxan R*. Dry the plate at 100 °C to 105 °C and examine in ultraviolet light at 254 nm. Any spot in the chromatogram obtained with test solution (a), apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.5 per cent).

Heavy metals (2.4.8). 1.0 g complies with limit test D for heavy metals (20 ppm). Prepare the standard using 2 ml of *lead standard solution (10 ppm Pb) R*.

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Carry out the determination of primary aromatic amino-nitrogen (2.5.8), using 0.250 g and determining the end-point electrometrically.

1 ml of 0.1 M *sodium nitrite* is equivalent to 31.03 mg of $C_{12}H_{14}N_4O_4S$.

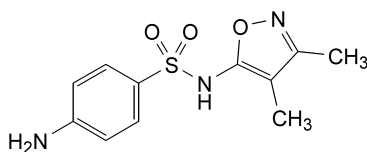
STORAGE

Store protected from light.

01/2005:0741 **Related substances.** Examine by thin-layer chromatography (2.2.27), using *silica gel GF₂₅₄ R* as the coating substance.

SULFAFURAZOLE

Sulfafurazolum



$C_{11}H_{13}N_3O_3S$

M_r 267.3

DEFINITION

Sulfafurazole contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of 4-amino-*N*-(3,4-dimethylisoxazol-5-yl)benzenesulphonamide, calculated with reference to the dried substance.

CHARACTERS

White or yellowish-white, crystalline powder or crystals, practically insoluble in water, sparingly soluble in alcohol, slightly soluble in methylene chloride. It dissolves in solutions of alkali hydroxides and in dilute mineral acids.

It melts at about 197 °C, with decomposition.

IDENTIFICATION

First identification: A, C.

Second identification: B, C, D.

- Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *sulfafurazole CRS*. Examine the substances prepared as discs.
- Examine the chromatograms obtained in the test for related substances. The principal spot in the chromatogram obtained with test solution (b) is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a).
- To 0.5 g add 1 ml of a 40 per cent *V/V* solution of *sulphuric acid R* and heat over a low flame to dissolve. Continue heating until a crystalline precipitate appears (about 2 min). Allow to cool and add 10 ml of *dilute sodium hydroxide solution R*. Cool. Shake the solution for 5 min with 25 ml of *ether R*. Separate the ether layer, dry over *anhydrous sodium sulphate R* and filter. Evaporate the solvent by heating on a water-bath. The residue melts (2.2.14) at 119 °C to 123 °C.
- Dissolve about 5 mg in 10 ml of *1 M hydrochloric acid*. Dilute 1 ml of the solution to 10 ml with *water R*. The solution, without further acidification, gives the reaction of primary aromatic amines (2.3.1).

TESTS

Appearance of solution. Dissolve 0.4 g in a mixture of 5 ml of *dilute sodium hydroxide solution R* and 5 ml of *water R*, with gently warming if necessary. The solution is not more intensely coloured than reference solution Y_6 , BY_6 or GY_6 (2.2.2, *Method II*).

Acidity. To 1.25 g, finely powdered, add 25 ml of *carbon dioxide-free water R*. Heat at 70 °C for 5 min. Cool in iced water for about 15 min and filter. To 20 ml of the filtrate add 0.1 ml of *bromothymol blue solution R1*. Not more than 0.2 ml of *0.1 M sodium hydroxide* is required to change the colour of the indicator.

Test solution (a). Dissolve 0.10 g of the substance to be examined in 3 ml of a mixture of 2 volumes of *concentrated ammonia R* and 48 volumes of *methanol R* and dilute to 5 ml with the same mixture of solvents.

Test solution (b). Dilute 1 ml of test solution (a) to 5 ml with a mixture of 2 volumes of *concentrated ammonia R* and 48 volumes of *methanol R*.

Reference solution (a). Dissolve 20 mg of *sulfafurazole CRS* in 3 ml of a mixture of 2 volumes of *concentrated ammonia R* and 48 volumes of *methanol R* and dilute to 5 ml with the same mixture of solvents.

Reference solution (b). Dilute 1.25 ml of test solution (b) to 50 ml with a mixture of 2 volumes of *concentrated ammonia R* and 48 volumes of *methanol R*.

Apply to the plate 5 µl of each solution. Develop over a path of 15 cm using a mixture of 1 volume of *concentrated ammonia R*, 25 volumes of *methanol R* and 75 volumes of *methylene chloride R*. Dry the plate at 100 °C to 105 °C and examine in ultraviolet light at 254 nm. Any spot in the chromatogram obtained with test solution (a), apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.5 per cent).

Heavy metals (2.4.8). 1.0 g complies with limit test D for heavy metals (20 ppm). Prepare the standard using 2 ml of *lead standard solution (10 ppm Pb) R*.

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.200 g in 50 ml of *acetone R*. Titrate with *0.1 M tetrabutylammonium hydroxide* using a 4 g/l solution of *thymol blue R* in *methanol R* as indicator.

1 ml of *0.1 M tetrabutylammonium hydroxide* is equivalent to 26.73 mg of $C_{11}H_{13}N_3O_3S$.

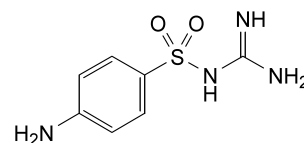
STORAGE

Store protected from light.

01/2005:1476

SULFAGUANIDINE

Sulfaguanidinum



$C_7H_{10}N_4O_2S$

M_r 214.3

DEFINITION

Sulfaguanidine contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of (4-aminophenylsulphonyl)guanidine, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, fine crystalline powder, very slightly soluble in water, slightly soluble in acetone, very slightly soluble in alcohol, practically insoluble in methylene chloride. It dissolves in dilute solutions of mineral acids.

IDENTIFICATION

First identification: A, B.

Second identification: A, C, D, E.

- A. Melting point (2.2.14): 189 °C to 193 °C, determined on the dried substance.
- B. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *sulfaguandine CRS*.
- C. Examine the chromatograms obtained in the test for related substances. The principal spot in the chromatogram obtained with test solution (b) is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a).
- D. Dissolve about 5 mg in 10 ml of 1 M hydrochloric acid. Dilute 1 ml of the solution to 10 ml with *water R*. The solution, without further acidification, gives the reaction of primary aromatic amines (2.3.1).
- E. Suspend 0.1 g in 2 ml of *water R*, add 1 ml of α -naphthol solution *R* and 2 ml of a mixture of equal volumes of *water R* and strong sodium hypochlorite solution *R*. A red colour develops.

TESTS

Solution S. To 2.5 g, add 40 ml of carbon dioxide-free *water R*. Heat at about 70 °C for 5 min. Cool while stirring in iced water for about 15 min, filter and dilute to 50 ml with carbon dioxide-free *water R*.

Acidity. To 20 ml of solution S, add 0.1 ml of bromothymol blue solution *R1*. Not more than 0.2 ml of 0.1 M sodium hydroxide is required to change the colour of the indicator.

Related substances. Examine by thin layer chromatography (2.2.27), using a TLC silica gel GF₂₅₄ plate *R*.

Test solution (a). Dissolve 50 mg of the substance to be examined in acetone *R* and dilute to 5 ml with the same solvent.

Test solution (b). Dilute 2 ml of test solution (a) to 10 ml with acetone *R*.

Reference solution (a). Dissolve 10 mg of sulfaguandine *CRS* in acetone *R* and dilute to 5 ml with the same solvent.

Reference solution (b). Dilute 5 ml of test solution (b) to 200 ml with acetone *R*.

Reference solution (c). Dilute 5 ml of reference solution (b) to 10 ml with acetone *R*.

Reference solution (d). Dissolve 10 mg of sulfanilamide *R* in test solution (b) and dilute to 5 ml with the same solution.

Apply to the plate 10 µl of each solution. Develop over a path of 15 cm using a mixture of 10 volumes of anhydrous formic acid *R*, 20 volumes of methanol *R* and 70 volumes of methylene chloride *R*. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. Any spot in the chromatogram obtained with test solution (a), apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.5 per cent) and at most one such spot is more intense than the spot in the chromatogram obtained with reference solution (c) (0.25 per cent). The test is not valid unless the chromatogram obtained with reference solution (d) shows two clearly separated principal spots.

Heavy metals (2.4.8). 12 ml of solution S complies with limit test F for heavy metals (20 ppm). Prepare the standard using of lead standard solution (1 ppm Pb) *R*.

Loss on drying (2.2.32). Not more than 8.0 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

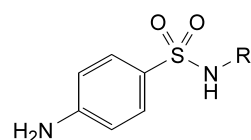
Dissolve 0.175 g in 50 ml of dilute hydrochloric acid *R*. Cool the solution in iced water. Carry out the determination of primary aromatic amino-nitrogen (2.5.8), determining the end-point electrometrically.

1 ml of 0.1 M sodium nitrite is equivalent to 21.42 mg of C₇H₁₀N₄O₂S.

STORAGE

Store protected from light.

IMPURITIES

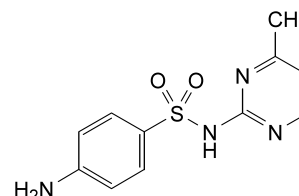


- A. R = H: 4-aminobenzenesulphonamide (sulphanilamide),
- B. R = CO-NH₂: N-[(4-aminophenyl)sulphonyl]urea (sulphacarbamide).

01/2005:0358

SULFAMERAZINE

Sulfamerazinum

C₁₁H₁₂N₄O₂SM_r 264.3

DEFINITION

Sulfamerazine contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of 4-amino-N-(4-methyl-2-pyrimidinyl)benzenesulphonamide, calculated with reference to the dried substance.

CHARACTERS

White, yellowish-white or pinkish-white, crystalline powder or crystals, very slightly soluble in water, sparingly soluble in acetone, slightly soluble in alcohol, very slightly soluble in methylene chloride. It dissolves in solutions of alkali hydroxides and in dilute mineral acids.

It melts at about 235 °C, with decomposition.

IDENTIFICATION

First identification: A, B.

Second identification: B, C, D.

- A. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *sulfamerazine CRS*. Examine the substances as discs.

- B. Examine the chromatograms obtained in the test for related substances. The principal spot in the chromatogram obtained with test solution (b) is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).
- C. Place 3 g in a dry tube. Incline the tube by about 45°, immerse the bottom of the tube in a silicone-oil bath and heat to about 270 °C. The substance decomposes, producing a white or yellowish-white sublimate which, after recrystallisation from *toluene R* and drying at 100 °C, melts (2.2.14) at 157 °C to 161 °C.
- D. Dissolve about 20 mg in 0.5 ml of *dilute hydrochloric acid R* and add 1 ml of *water R*. The solution gives, without further addition of acid, the identification reaction of primary aromatic amines (2.3.1).

TESTS

Appearance of solution. Dissolve 0.8 g in a mixture of 5 ml of *dilute sodium hydroxide solution R* and 5 ml of *water R*. The solution is not more intensely coloured than reference solution Y₄, BY₄ or GY₄ (2.2.2, *Method II*).

Acidity. To 1.25 g, finely powdered, add 40 ml of *carbon dioxide-free water R* and heat at about 70 °C for 5 min. Cool for about 15 min in iced water and filter. To 20 ml of the filtrate add 0.1 ml of *bromothymol blue solution R1*. Not more than 0.2 ml of 0.1 M *sodium hydroxide* is required to change the colour of the indicator.

Related substances. Examine by thin-layer chromatography (2.2.27) using *silica gel GF₂₅₄ R* as the coating substance.

Test solution (a). Dissolve 0.10 g of the substance to be examined in 3 ml of a mixture of 2 volumes of *concentrated ammonia R* and 48 volumes of *methanol R* and dilute to 5 ml with the same mixture of solvents.

Test solution (b). Dilute 1 ml of test solution (a) to 10 ml with a mixture of 2 volumes of *concentrated ammonia R* and 48 volumes of *methanol R*.

Reference solution (a). Dissolve 10 mg of *sulfamerazine CRS* in 3 ml of a mixture of 2 volumes of *concentrated ammonia R* and 48 volumes of *methanol R* and dilute to 5 ml with the same mixture of solvents.

Reference solution (b). Dilute 2.5 ml of test solution (b) to 50 ml with a mixture of 2 volumes of *concentrated ammonia R* and 48 volumes of *methanol R*.

Apply to the plate 5 µl of each solution. Develop over a path of 15 cm with a mixture of 3 volumes of *dilute ammonia R1*, 5 volumes of *water R*, 40 volumes of *nitromethane R* and 50 volumes of *dioxan R*. Dry the plate at 100 °C to 105 °C and examine in ultraviolet light at 254 nm. Any spot in the chromatogram obtained with test solution (a), apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.5 per cent).

Heavy metals (2.4.8). 1.0 g complies with limit test C for heavy metals (20 ppm). Prepare the standard using 2 ml of *lead standard solution (10 ppm Pb) R*.

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.2500 g in a mixture of 20 ml of *dilute hydrochloric acid R* and 50 ml of *water R*. Cool the solution in iced water. Carry out the determination of primary aromatic amino-nitrogen (2.5.8), determining the end-point electrometrically.

1 ml of 0.1 M *sodium nitrite* is equivalent to 26.43 mg of C₁₁H₁₂N₄O₂S.

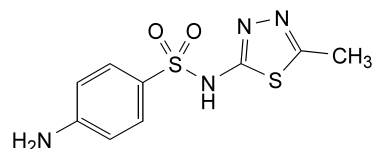
STORAGE

Store protected from light.

01/2005:0637

SULFAMETHIZOLE

Sulfamethizolum

C₉H₁₀N₄O₂S₂M_r 270.3

DEFINITION

Sulfamethizole contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of 4-amino-*N*-(5-methyl-1,3,4-thiadiazol-2-yl)benzenesulphonamide, calculated with reference to the dried substance.

CHARACTERS

White or yellowish-white crystalline powder or crystals, very slightly soluble in water, soluble in acetone, sparingly soluble in alcohol. It dissolves in dilute solutions of alkali hydroxides and in dilute mineral acids.

It melts at about 210 °C.

IDENTIFICATION

First identification: A, B.

Second identification: B, C, D.

- A. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *sulfamethizole CRS*. Examine the substances prepared as discs.
- B. Examine the chromatograms obtained in the test for related substances. The principal spot in the chromatogram obtained with test solution (b) is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a).
- C. Dissolve 50 mg in 4 ml of *methanol R* and add 0.2 ml of a 40 g/l solution of *copper acetate R*. A flocculent, yellowish-green precipitate is formed, changing to dark green.
- D. Dissolve about 5 mg in 1 M *hydrochloric acid* and dilute to 10 ml with the same solvent. Dilute 1 ml of this solution to 10 ml with *water R*. The solution, without further acidification, gives the reaction of primary aromatic amines (2.3.1).

TESTS

Appearance of solution. Dissolve 1.0 g in a mixture of 5 ml of *dilute sodium hydroxide solution R* and 5 ml of *water R*. The solution is not more intensely coloured than reference solution Y₅, BY₅ or GY₅ (2.2.2, *Method II*).

Acidity. To 1.25 g add 25 ml of *carbon dioxide-free water R* and heat at 70 °C for 5 min. Cool for about 15 min in iced water and filter. To 20 ml of the filtrate add 0.1 ml of *bromothymol blue solution R1*. Not more than 0.5 ml of 0.1 M *sodium hydroxide* is required to change the colour of the indicator.

Related substances. Examine by thin-layer chromatography (2.2.27), using *silica gel GF₂₅₄ R* as the coating substance.

Test solution (a). Dissolve 0.30 g of the substance to be examined in *acetone R* and dilute to 10 ml with the same solvent.

Test solution (b). Dilute 1 ml of test solution (a) to 10 ml with *acetone R*.

Reference solution (a). Dissolve 30 mg of *sulfamethizole CRS* in *acetone R* and dilute to 10 ml with the same solvent.

Reference solution (b). Dilute 1 ml of test solution (b) to 20 ml with *acetone R*.

Apply to the plate 2 µl of each solution. Develop over a path of 15 cm using a mixture of 15 volumes of *methanol R* and 80 volumes of *chloroform R*. Dry the plate at 100 °C to 105 °C and examine in ultraviolet light at 254 nm. Any spot in the chromatogram obtained with test solution (a), apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.5 per cent).

Heavy metals (2.4.8). 1.0 g complies with limit test D for heavy metals (20 ppm). Prepare the standard using 2 ml of *lead standard solution (10 ppm Pb) R*.

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Carry out the determination of primary aromatic amino-nitrogen (2.5.8), using 0.2500 g and determining the end-point electrometrically.

1 ml of 0.1 M *sodium nitrite* is equivalent to 27.03 mg of C₉H₁₀N₄O₂S₂.

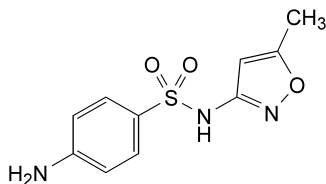
STORAGE

Store protected from light.

01/2005:0108

SULFAMETHOXAZOLE

Sulfamethoxazolum



C₁₀H₁₁N₃O₃S

*M*_r 253.3

DEFINITION

4-Amino-*N*-(5-methylisoxazol-3-yl)benzenesulphonamide.

Content: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

Appearance: white or almost white, crystalline powder.

Solubility: practically insoluble in water, freely soluble in acetone, sparingly soluble in ethanol (96 per cent). It dissolves in dilute solutions of sodium hydroxide and in dilute acids.

IDENTIFICATION

First identification: A, B.

Second identification: A, C, D.

A. Melting point (2.2.14): 169 °C to 172 °C.

B. Infrared absorption spectrophotometry (2.2.24).

Comparison: *sulfamethoxazole CRS*.

C. Thin-layer chromatography (2.2.27).

Test solution. Dissolve 20 mg of the substance to be examined in 3 ml of a mixture of 2 volumes of *concentrated ammonia R* and 48 volumes of *methanol R* and dilute to 5 ml with the same mixture of solvents.

Reference solution. Dissolve 20 mg of *sulfamethoxazole CRS* in 3 ml of a mixture of 2 volumes of *concentrated ammonia R* and 48 volumes of *methanol R* and dilute to 5 ml with the same mixture of solvents.

Plate: *TLC silica gel F₂₅₄ plate R*.

Mobile phase: *dilute ammonia R1, water R, nitromethane R, dioxan R* (3:5:41:51 V/V/V/V).

Application: 5 µl.

Development: over 3/4 of the plate.

Drying: at 100-105 °C.

Detection: examine in ultraviolet light at 254 nm.

Results: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with the reference solution.

D. Dissolve about 5 mg in 10 ml of 1 M *hydrochloric acid*.

Dilute 1 ml of the solution to 10 ml with *water R*. The solution, without further acidification, gives the reaction of primary aromatic amines (2.3.1).

TESTS

Appearance of solution. The solution is clear (2.2.1) and not more intensely coloured than reference solution Y₅, BY₅ or GY₅ (2.2.2, *Method II*).

Dissolve 1.0 g in a mixture of 5 ml of *dilute sodium hydroxide solution R* and 5 ml of *water R*.

Acidity. To 1.25 g, finely powdered, add 25 ml of *water R*. Heat at 70 °C for 5 min. Cool in iced water for about 15 min and filter. To 20 ml of the filtrate add 0.1 ml of *bromothymol blue solution R1*. Not more than 0.3 ml of 0.1 M *sodium hydroxide* is required to change the colour of the indicator.

Related substances. Liquid chromatography (2.2.29).

Test solution. Dissolve 50.0 mg of the substance to be examined in 45 ml of the mobile phase, sonicate at about 45 °C for 10 min, cool and dilute to 50.0 ml with the mobile phase.

Reference solution (a). Dilute 1.0 ml of the test solution to 10.0 ml with the mobile phase. Dilute 1.0 ml of this solution to 100.0 ml with the mobile phase.

Reference solution (b). Dissolve 1 mg of the substance to be examined and 1 mg of *sulfamethoxazole impurity A CRS* in the mobile phase and dilute to 10.0 ml with the mobile phase.

Reference solution (c). Dissolve 1.0 mg of *sulfamethoxazole impurity F CRS* in the mobile phase and dilute to 10.0 ml with the mobile phase. Dilute 1.0 ml of this solution to 100.0 ml with the mobile phase.

Column:

- **size:** *l* = 0.25 m, Ø = 4.0 mm,
- **stationary phase:** *octylsilyl silica gel for chromatography R* (5 µm),
- **temperature:** 30 °C.

Mobile phase: mix 35 volumes of *methanol R2* and 65 volumes of a 13.6 g/l solution of *potassium dihydrogen phosphate R*, then adjust to pH 6.0 with a 100 g/l solution of *potassium hydroxide R*.

Flow rate: 0.9 ml/min.

Detection: spectrophotometer at 210 nm.

Injection: 20 µl.

Run time: 3 times the retention time of sulfamethoxazole.

Relative retentions with reference to sulfamethoxazole (retention time = about 10 min): impurity D = about 0.3; impurity E = about 0.35; impurity F = about 0.45; impurity C = about 0.5; impurity A = about 1.2; impurity B = about 2.0.

System suitability: reference solution (b):

- **resolution:** minimum 3.5 between the peaks due to sulfamethoxazole and impurity A.

Limits:

- **impurities A, B, C, D, E:** for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent),
- **impurity F:** not more than the area of the principal peak in the chromatogram obtained with reference solution (c) (0.1 per cent),
- **any other impurity:** for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent),
- **total:** not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.3 per cent),
- **disregard limit:** 0.25 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.025 per cent).

Heavy metals (2.4.8): maximum 20 ppm.

1.0 g complies with limit test D. Prepare the reference solution using 2 ml of *lead standard solution (10 ppm Pb) R*.

Loss on drying (2.2.32): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 100–105 °C.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Carry out the assay of primary aromatic amino-nitrogen (2.5.8), using 0.200 g and determining the end-point electrometrically.

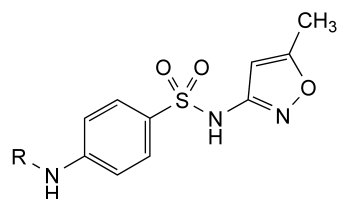
1 ml of 0.1 M *sodium nitrite* is equivalent to 25.33 mg of $C_{10}H_{11}N_4O_3S$.

STORAGE

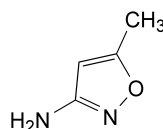
Protected from light.

IMPURITIES

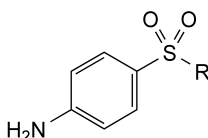
Specified impurities: A, B, C, D, E, F.



- A. R = CO-CH₃: N-[4-[(5-methylisoxazol-3-yl)sulphamoyl]phenyl]acetamide,
- B. R = SO₂-C₆H₄-p-NH₂: 4-[[[(4-aminophenyl)sulphonyl]amino]-N-(5-methylisoxazol-3-yl)benzenesulphonamide,

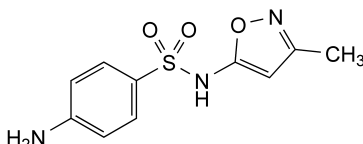


C. 5-methylisoxazol-3-amine,



D. R = OH: 4-aminobenzenesulphonic acid (sulphanilic acid),

E. R = NH₂: 4-aminobenzenesulphonamide (sulphanilamide),

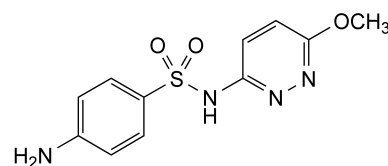


F. 4-amino-N-(3-methylisoxazol-5-yl)benzenesulphonamide.

01/2005:0638

SULFAMETHOXPYRIDAZINE FOR VETERINARY USE

Sulfamethoxypyridazinum ad usum veterinarium



$C_{11}H_{12}N_4O_3S$

M_r 280.3

DEFINITION

Sulfamethoxypyridazine for veterinary use contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of 4-amino-N-(6-methoxypyridazin-3-yl)benzenesulphonamide, calculated with reference to the dried substance.

CHARACTERS

A white or slightly yellowish, crystalline powder, colouring slowly on exposure to light, practically insoluble in water, sparingly soluble in acetone, slightly soluble in alcohol, very slightly soluble in methylene chloride. It dissolves in solutions of alkali hydroxides and in dilute mineral acids. It melts at about 180 °C, with decomposition.

IDENTIFICATION

First identification: A, B.

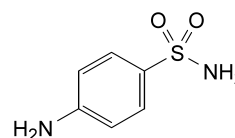
Second identification: B, C, D.

- A. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *sulfamethoxypyridazine CRS*. Examine the substances prepared as discs.
- B. Examine the chromatograms obtained in the test for related substances. The principal spot in the chromatogram obtained with test solution (b) is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a).

01/2005:1571

SULFANILAMIDE

Sulfanilamidum

 $C_6H_8N_2O_2S$ M_r 172.2

DEFINITION

Sulfanilamide contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of 4-aminobenzenesulphonamide, calculated with reference to the dried substance.

CHARACTERS

White or yellowish-white crystals or fine powder, slightly soluble in water, freely soluble in acetone, sparingly soluble in alcohol, practically insoluble in methylene chloride. It dissolves in solutions of alkali hydroxides and in dilute mineral acids.

IDENTIFICATION

First identification: B.

Second identification: A, C, D.

A. Melting point (2.2.14): 164.5 °C to 166.0 °C.

B. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *sulfanilamide CRS*. Examine the substances prepared as discs.

C. Examine the chromatograms obtained in the test for related substances. The principal spot in the chromatogram obtained with test solution (a) is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a).

D. Dissolve about 5 mg in 10 ml of 1 M hydrochloric acid. Dilute 1 ml of the solution to 10 ml with *water R*. The solution, without further acidification, gives the reaction of primary aromatic amines (2.3.1).

TESTS

Solution S. To 2.5 g add 50 ml of *carbon dioxide-free water R*. Heat at about 70 °C for about 5 min. Cool in iced water for about 15 min and filter.

Acidity. To 20 ml of solution S add 0.1 ml of *bromothymol blue solution R1*. Not more than 0.2 ml of 0.1 M sodium hydroxide is required to change the colour of the indicator.

Related substances. Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel F₂₅₄ plate R*.

Test solution (a). Dissolve 20 mg of the substance to be examined in 3 ml of a mixture of 2 volumes of *concentrated ammonia R* and 48 volumes of *methanol R* and dilute to 5 ml with the same mixture of solvents.

Test solution (b). Dissolve 0.10 g of the substance to be examined in 0.5 ml of *concentrated ammonia R* and dilute to 5 ml with *methanol R*. If the solution is not clear, heat gently until dissolution is complete.

Reference solution (a). Dissolve 20 mg of *sulfanilamide CRS* in 3 ml of a mixture of 2 volumes of *concentrated ammonia R* and 48 volumes of *methanol R* and dilute to 5 ml with the same mixture of solvents.

C. Dissolve 0.5 g in 1 ml of a 40 per cent V/V solution of *sulphuric acid R*, heating gently. Continue heating until a crystalline precipitate appears (about 2 min). Cool and add 10 ml of *dilute sodium hydroxide solution R*. Cool again, add 25 ml of *ether R* and shake the solution for 5 min. Separate the ether layer, dry over *anhydrous sodium sulphate R* and filter. Evaporate the ether by heating in a water-bath. An oily residue is obtained which becomes crystalline on cooling; if necessary, scratch the wall of the container with a glass rod. The residue melts (2.2.14) at 102 °C to 106 °C.

D. Dissolve about 5 mg in 10 ml of 1 M hydrochloric acid. Dilute 1 ml of the solution to 10 ml with *water R*. The solution, without further acidification, gives the reaction of primary aromatic amines (2.3.1).

TESTS

Appearance of solution. Dissolve 1.0 g in a mixture of 10 ml of 1 M sodium hydroxide and 15 ml of *water R*. The solution is clear (2.2.1) and not more intensely coloured than reference solution Y₄ or BY₄ (2.2.2, *Method II*).

Acidity. To 1.25 g, finely powdered, add 25 ml of *carbon dioxide-free water R*. Heat at 70 °C for 5 min. Cool in iced water for about 15 min and filter. To 20 ml of the filtrate add 0.1 ml of *bromothymol blue solution R1*. Not more than 0.5 ml of 0.1 M sodium hydroxide is required to change the colour of the indicator.

Related substances. Examine by thin layer chromatography (2.2.27), using *TLC silica gel GF₂₅₄ plate R*.

Test solution (a). Dissolve 0.10 g of the substance to be examined in *acetone R* and dilute to 5 ml with the same solvent.

Test solution (b). Dilute 1 ml of test solution (a) to 10 ml with *acetone R*.

Reference solution (a). Dissolve 20 mg of *sulfamethoxyypyridazine CRS* in *acetone R* and dilute to 10 ml with the same solvent.

Reference solution (b). Dilute 2.5 ml of test solution (b) to 50 ml with *acetone R*.

Apply separately to the plate 5 µl of each solution. Develop over a path of 15 cm using a mixture of 1 volume of *dilute ammonia R1*, 9 volumes of *water R*, 30 volumes of *2-propanol R* and 50 volumes of *ethyl acetate R*. Dry the plate at 100-105 °C and examine in ultraviolet light at 254 nm. Any spot in the chromatogram obtained with test solution (a), apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.5 per cent).

Heavy metals (2.4.8). 1.0 g complies with limit test D for heavy metals (20 ppm). Prepare the standard using 2 ml of *lead standard solution (10 ppm Pb) R*.

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 100-105 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Carry out the assay of primary aromatic amino-nitrogen (2.5.8), using 0.2500 g, determining the end-point electrometrically.

1 ml of 0.1 M sodium nitrite is equivalent to 28.03 mg of C₁₁H₁₂N₄O₃S.

STORAGE

Protected from light.

Reference solution (b). Dilute 1.25 ml of test solution (a) to 50 ml with a mixture of 2 volumes of *concentrated ammonia R* and 48 volumes of *methanol R*.

Reference solution (c). Dissolve 20 mg of the substance to be examined and 20 mg of *sulfamerazine CRS* in 3 ml of a mixture of 2 volumes of *concentrated ammonia R* and 48 volumes of *methanol R* and dilute to 5 ml with the same mixture of solvents.

Apply to the plate 5 µl of each solution. Develop over a path corresponding to two-thirds of the plate height using a mixture of 3 volumes of *dilute ammonia R1*, 5 volumes of *water R*, 40 volumes of *nitromethane R* and 50 volumes of *dioxan R*. Dry the plate at 100 °C to 105 °C and examine in ultraviolet light at 254 nm. Any spot in the chromatogram obtained with test solution (b), apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.5 per cent). The test is not valid unless the chromatogram obtained with reference solution (c) shows two clearly separated principal spots.

Heavy metals (2.4.8). 12 ml of solution S complies with limit test A for heavy metals (20 ppm). Prepare the standard using *lead standard solution (1 ppm Pb) R*.

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Carry out the determination of primary aromatic amino-nitrogen (2.5.8), using 0.140 g and determining the end-point electrometrically.

1 ml of 0.1 M *sodium nitrite* is equivalent to 17.22 mg of C₁₈H₁₄N₄O₅S.

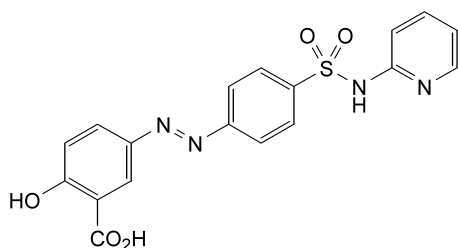
STORAGE

Store protected from light.

01/2005:0863

SULFASALAZINE

Sulfasalazinum



C₁₈H₁₄N₄O₅S

*M*_r 398.4

DEFINITION

Sulfasalazine contains not less than 97.0 per cent and not more than the equivalent of 101.5 per cent of 2-hydroxy-5-[2-[4-(pyridin-2-ylsulphamoyl)phenyl]diazenyl]benzoic acid, calculated with reference to the dried substance.

CHARACTERS

A bright yellow or brownish-yellow, fine powder, practically insoluble in water, very slightly soluble in alcohol, practically insoluble in methylene chloride. It dissolves in dilute solutions of alkali hydroxides.

IDENTIFICATION

Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *sulfasalazine CRS*. Examine the substances prepared as discs.

TESTS

Related substances. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 25.0 mg of the substance to be examined in *dilute ammonia R3* and dilute to 25.0 ml with the same solvent.

Reference solution (a). Dilute 1.0 ml of the test solution to 100.0 ml with *dilute ammonia R3*.

Reference solution (b). Dissolve 1.0 mg of *sulfasalazine derivative for resolution CRS* in 10.0 ml of reference solution (a). Dilute 1.0 ml of this solution to 10.0 ml with reference solution (a).

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4.6 mm in internal diameter, packed with *octadecylsilyl silica gel for chromatography R* (5 µm),
- as mobile phase at a flow rate of 1 ml/min:

Mobile phase A. In a 1000 ml volumetric flask dissolve 1.13 g of *sodium dihydrogen phosphate R* and 2.5 g of *sodium acetate R* in 900 ml of *water R*. Adjust to pH 4.8 with *glacial acetic acid R* and adjust the volume to 1000 ml with *water R*,

Mobile phase B. Mix 1 volume of mobile phase A with 4 volumes of *methanol R*,

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)	Comment
0 - 15	60 → 45	40 → 55	linear gradient
15 - 25	45	55	isocratic
25 - 60	45 → 0	55 → 100	linear gradient
60 - 65	0	100	isocratic
65 - 67	0 → 60	100 → 40	switch to initial composition
67 - 77	60	40	re-equilibration

- as detector a spectrophotometer set at 320 nm.

Inject 20 µl of reference solution (a). Adjust the sensitivity of the detector so that the height of the principal peak in the chromatogram obtained is at least 50 per cent of the full scale of the recorder. Inject 20 µl of reference solution (b). The test is not valid unless the resolution between the peaks corresponding to sulfasalazine and sulfasalazine derivative for resolution is at least 3.0.

Inject 20 µl of the test solution and 20 µl of reference solution (a). When the chromatograms are recorded in the prescribed conditions, the approximate retention times relative to sulfasalazine are the following: impurity A = 2.00; impurity B = 1.85; impurity C = 0.80; impurity D = 1.90; impurity E = 1.63; impurity F = 0.85; impurity G = 1.39; impurity H = 0.16; impurity I = 0.28.

In the chromatogram obtained with the test solution: the area of any peak, apart from the principal peak, is not greater than the area of the principal peak in the chromatogram obtained with reference solution (a) (1 per cent); the sum of the areas of all peaks, apart from the principal peak is not greater than four times the area of the principal peak in the chromatogram obtained with reference solution (a) (4 per cent). Disregard any peak with a retention time shorter than 6 min (corresponding to salicylic acid and sulfapyridine) and

any peak with an area less than 0.05 times the area of the principal peak in the chromatogram obtained with reference solution (a).

Salicylic acid and sulfapyridine. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 25.0 mg of the substance to be examined in *dilute ammonia R3* and dilute to 25.0 ml with the same solvent.

Reference solution (a). Dissolve 5.0 mg of *salicylic acid R* and 5.0 mg of *sulfapyridine CRS* in *dilute ammonia R3* and dilute to 10.0 ml with the same solvent.

Reference solution (b). Dilute 2.0 ml of *reference solution (a)* to 100.0 ml with *dilute ammonia R3*.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4.6 mm in internal diameter, packed with *octadecylsilyl silica gel for chromatography R* (5 µm),
- as mobile phase at a flow rate of 1 ml/min a mixture of 70 volumes of mobile phase A (described in the test for related substances) and 30 volumes of mobile phase B (described in the test for related substances),
- as detector a spectrophotometer set at 300 nm.

Inject 20 µl of reference solution (b). Adjust the sensitivity of the system so that the height of the principal peaks in the chromatogram obtained is at least 50 per cent of the full scale of the recorder. When the chromatogram is recorded in the prescribed conditions, the retention times are: salicylic acid about 6 min and sulfapyridine about 7 min. The test is not valid unless the resolution between the peaks corresponding to salicylic acid and sulfapyridine is at least 2.

Inject 20 µl of the test solution and 20 µl of reference solution (b). Continue the chromatography for 10 min. In the chromatogram obtained with the test solution the area of the peak corresponding to salicylic acid is not greater than 0.5 times the area of the first peak in the chromatogram obtained with reference solution (b) (0.5 per cent) and the peak corresponding to sulfapyridine is not greater than 0.5 times the area of the second peak in the chromatogram obtained with reference solution (b) (0.5 per cent). Disregard any peak with an area less than 0.05 times the area of the principal peak in the chromatogram obtained with reference solution (b).

Chlorides (2.4.4). To 1.25 g add 50 ml of *distilled water R*. Heat at about 70 °C for 5 min. Cool and filter. To 20 ml of the filtrate add 1 ml of *nitric acid R*, allow to stand for 5 min and filter to obtain a clear solution. 15 ml of the filtrate complies with the limit test for chlorides (140 ppm).

Sulphates (2.4.13). To 20 ml of the filtrate prepared for the test for chlorides add 1 ml of *dilute hydrochloric acid R*, allow to stand for 5 min and filter. 15 ml of the filtrate complies with the limit test for sulphates (400 ppm).

Heavy metals (2.4.8). 2.0 g complies with limit test D for heavy metals (10 ppm). Prepare the standard using 2 ml of *lead standard solution (10 ppm Pb) R*.

Loss on drying (2.2.32). Not more than 1.0 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C for 2 h.

Sulphated ash (2.4.14). Not more than 0.5 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.150 g in 0.1 M *sodium hydroxide* and dilute to 100.0 ml with the same solvent. Transfer 5.0 ml of this solution to a 1000 ml volumetric flask containing about 750 ml of *water R*. Add 20.0 ml of 0.1 M *acetic acid* and

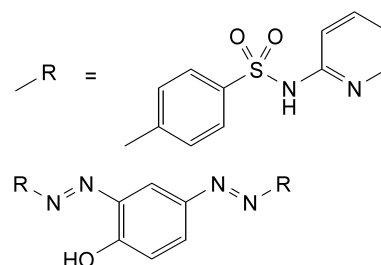
dilute to 1000.0 ml with *water R*. Prepare a standard solution at the same time and in the same manner using 0.150 g of *sulfasalazine CRS*. Measure the absorbance (2.2.25) of the two solutions at the maximum at 359 nm.

Calculate the content of $C_{18}H_{14}N_4O_5S$ from the absorbances measured and the concentration of the solutions.

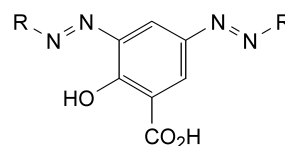
STORAGE

Store protected from light.

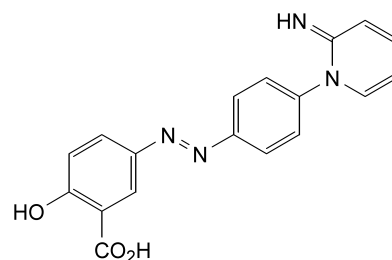
IMPURITIES



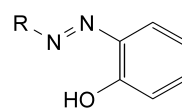
A. 4,4'-[(4-hydroxy-1,3-phenylene)bis(diazenediyl)]bis[*N*-(pyridin-2-yl)benzenesulphonamide],



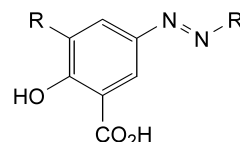
B. 2-hydroxy-3,5-bis[2-[4-(pyridin-2-ylsulphamoyl)phenyl]diazenyl]benzoic acid,



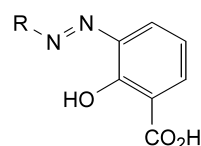
C. 2-hydroxy-5-[2-[4-(2-aminopyridin-1(2*H*)-yl)phenyl]diazenyl]benzoic acid,



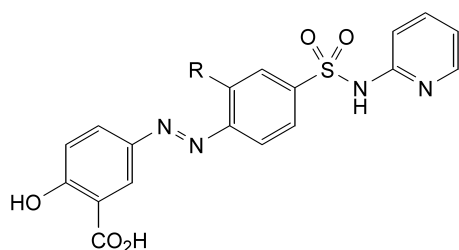
D. 4-[2-(2-hydroxyphenyl)diazenyl]-*N*-(pyridin-2-yl)benzenesulphonamide,



E. 2-hydroxy-4'-(pyridin-2-ylsulphamoyl)-5-[2-[4-(pyridin-2-ylsulphamoyl)phenyl]diazenyl]biphenyl-3-carboxylic acid,

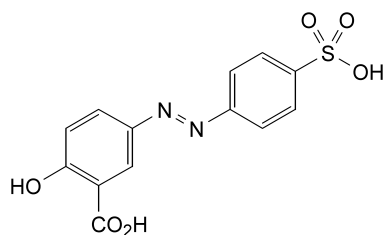


F. 2-hydroxy-3-[2-[4-(pyridin-2-ylsulphamoyl)phenyl]diazenyl]benzoic acid,



G. 5-[2-[4',5-bis(pyridin-2-ylsulphamoyl)biphenyl-2-yl]diazenyl]-2-hydroxybenzoic acid,

H. salicylic acid,

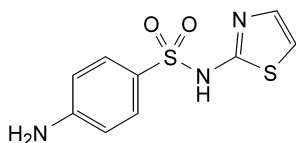


I. 2-hydroxy-5-[2-(4-sulphophenyl)diazenyl]benzoic acid.

01/2005:0742

SULFATHIAZOLE

Sulfathiazolum



$C_9H_9N_3O_2S_2$

M_r 255.3

DEFINITION

Sulfathiazole contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of 4-amino-*N*-(thiazol-2-yl)benzenesulphonamide, calculated with reference to the dried substance.

CHARACTERS

A white or slightly yellowish, crystalline powder, practically insoluble in water, slightly soluble in alcohol, practically insoluble in methylene chloride. It dissolves in dilute solutions of alkali hydroxides and in dilute mineral acids.

IDENTIFICATION

First identification: A, B.

Second identification: A, C, D, E.

- Melting point (2.2.14): 200 °C to 203 °C. Melting may occur at about 175 °C, followed by solidification and a second melting between 200 °C and 203 °C.
- Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *sulfathiazole CRS*. Examine the substances prepared as discs. If the spectra obtained show differences, dissolve the substance to be examined and the reference substance separately in *alcohol R*, evaporate to dryness *in vacuo* and record the spectra again using the residues.
- Examine the chromatograms obtained in the test for related substances. The principal spot in the chromatogram obtained with test solution (b) is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).

D. Dissolve about 10 mg in a mixture of 10 ml of *water R* and 2 ml of 0.1 *M sodium hydroxide* and add 0.5 ml of *copper sulphate solution R*. A greyish-blue or purple precipitate is formed.

E. Dissolve about 5 mg in 10 ml of 1 *M hydrochloric acid*. Dilute 1 ml of the solution to 10 ml with *water R*. The solution, without further addition of acid, gives the reaction of primary aromatic amines (2.3.1).

TESTS

Appearance of solution. Dissolve 1.0 g in 10 ml of 1 *M sodium hydroxide*. The solution is clear (2.2.1) and not more intensely coloured than reference solution GY₄ (2.2.2, *Method II*).

Acidity. To 1.0 g add 50 ml of *carbon dioxide-free water R*. Heat to 70 °C for 5 min. Cool rapidly to 20 °C and filter. To 25 ml of the filtrate add 0.1 ml of *bromothymol blue solution R1*. Not more than 0.1 ml of 0.1 *M sodium hydroxide* is required to change the colour of the indicator.

Related substances. Examine by thin-layer chromatography (2.2.27), using *silica gel H R* as the coating substance.

Test solution (a). Dissolve 0.10 g of the substance to be examined in a mixture of 1 volume of *concentrated ammonia R* and 9 volumes of *alcohol R* and dilute to 10 ml with the same mixture of solvents.

Test solution (b). Dilute 1 ml of test solution (a) to 5 ml with a mixture of 1 volume of *concentrated ammonia R* and 9 volumes of *alcohol R*.

Reference solution (a). Dissolve 20 mg of *sulfathiazole CRS* in a mixture of 1 volume of *concentrated ammonia R* and 9 volumes of *alcohol R* and dilute to 10 ml with the same mixture of solvents.

Reference solution (b). Dissolve 50 mg of *sulfanilamide R* in a mixture of 1 volume of *concentrated ammonia R* and 9 volumes of *alcohol R* and dilute to 100 ml with the same mixture of solvents. Dilute 1 ml of this solution to 10 ml with the same mixture of solvents.

Apply to the plate 10 µl of each solution. Develop over a path of 15 cm using a mixture of 18 volumes of *ammonia R* and 90 volumes of *butanol R*. Dry the plate at 100 °C to 105 °C for 10 min and spray with a 1 g/l solution of *dimethylaminobenzaldehyde R* in *alcohol R* containing 1 per cent *V/V* of *hydrochloric acid R*. Any spot in the chromatogram obtained with test solution (a), apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.5 per cent).

Heavy metals (2.4.8). 1.0 g complies with limit test C for heavy metals (20 ppm). Prepare the standard using 2 ml of *lead standard solution (10 ppm Pb) R*.

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Carry out the determination of primary aromatic amino-nitrogen (2.5.8), using 0.200 g, determining the end-point electrometrically.

1 ml of 0.1 *M sodium nitrite* is equivalent to 25.53 mg of $C_9H_9N_3O_2S_2$.

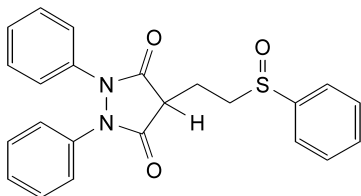
STORAGE

Store protected from light.

01/2005:0790

SULFINPYRAZONE

Sulfinpyrazonum

 $C_{23}H_{20}N_2O_3S$ M_r 404.5

DEFINITION

Sulfinpyrazone contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of 1,2-diphenyl-4-[2-(phenylsulphonyl)ethyl]pyrazolidine-3,5-dione, calculated with reference to the dried substance.

CHARACTERS

A white or almost white powder, very slightly soluble in water, sparingly soluble in alcohol. It dissolves in dilute solutions of alkali hydroxides.

IDENTIFICATION

First identification: A, C.

Second identification: A, B, D.

- A. Melting point (2.2.16): 131 °C to 135 °C.
- B. Dissolve 30.0 mg in 0.01 M sodium hydroxide and dilute to 100.0 ml with the same alkaline solution. Dilute 1.0 ml of this solution to 20.0 ml with 0.01 M sodium hydroxide. Examined between 230 nm and 350 nm (2.2.25), the solution shows an absorption maximum at 260 nm. The specific absorbance at the maximum is 530 to 580.
- C. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *sulfinpyrazone CRS*. Examine the substances prepared as discs.
- D. Dissolve about 10 mg in 3 ml of *acetone R* and add a mixture of 0.2 ml of *ferric chloride solution R2* and 3 ml of *water R*. A red to violet colour develops.

TESTS

Appearance of solution in acetone. Dissolve 1.25 g in *acetone R* and dilute to 25 ml with the same solvent. The solution is clear (2.2.1). The absorbance (2.2.25) of the solution, measured at 420 nm with a path-length of 4 cm, is not greater than 0.10.

Appearance of solution in 1 M sodium hydroxide. Dissolve 1.25 g, heating gently if necessary, in 25 ml of 1 M sodium hydroxide. The solution is clear (2.2.1). The absorbance (2.2.25) of the solution, measured at 420 nm with a path-length of 4 cm, is not greater than 0.15.

Related substances. Examine by thin-layer chromatography (2.2.27), using a suitable silica gel with a fluorescent indicator having an optimal intensity at 254 nm. Expose the plate, in a tank, to the vapour from *glacial acetic acid R*. After 10 min, remove the plate and heat at 60 °C for 40 min. Cool and pass a current of nitrogen over the plate for 10 min. Prepare the solutions immediately before use.

Test solution. Dissolve 0.25 g of the substance to be examined in *acetone R* and dilute to 5 ml with the same solvent.

Reference solution (a). Dilute 2 ml of the test solution to 100 ml with *acetone R*. Dilute 1 ml of this solution to 10 ml with *acetone R*.

Reference solution (b). Dissolve 10 mg of *sulfinpyrazone impurity A CRS* in *acetone R* and dilute to 10 ml with the same solvent.

Reference solution (c). Dilute 5 ml of reference solution (b) to 10 ml with *acetone R*.

Reference solution (d). Dissolve 10 mg of *sulfinpyrazone impurity B CRS* in *acetone R* and dilute to 10 ml with the same solvent.

Reference solution (e). Dilute 5 ml of reference solution (d) to 10 ml with *acetone R*.

Apply to the plate, under an atmosphere of nitrogen, 2 µl of each solution. Develop over a path of 15 cm using a mixture of 20 volumes of *glacial acetic acid R* and 80 volumes of *chloroform R*. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. In the chromatogram obtained with the test solution: any spots corresponding to sulfinpyrazone impurity A and sulfinpyrazone impurity B are not more intense than the spots in the chromatograms obtained with reference solutions (b) (2.0 per cent) and (d) (2.0 per cent), respectively, and at most one of the spots is more intense than the corresponding spot in the chromatogram obtained with reference solution (c) (1.0 per cent) or (e) (1.0 per cent); any spot apart from the principal spot and the two spots previously cited is not more intense than the spot in the chromatogram obtained with reference solution (a) (0.2 per cent).

Heavy metals (2.4.8). 1.0 g complies with limit test C for heavy metals (10 ppm). Prepare the standard using 1 ml of *lead standard solution (10 ppm Pb) R*.

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

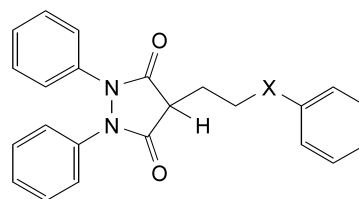
Dissolve 0.300 g in 25 ml of *acetone R*. Add 0.5 ml of *bromothymol blue solution R1*. Titrate with 0.1 M sodium hydroxide until the colour changes from yellow to blue.

1 ml of 0.1 M sodium hydroxide is equivalent to 40.45 mg of $C_{23}H_{20}N_2O_3S$.

STORAGE

Store protected from light.

IMPURITIES

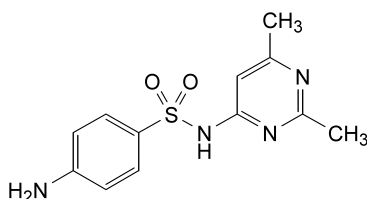


- A. X = SO₂: 1,2-diphenyl-4-[2-(phenylsulphonyl)ethyl]pyrazolidine-3,5-dione,
- B. X = S: 1,2-diphenyl-4-[2-(phenylsulphanyl)ethyl]pyrazolidine-3,5-dione.

01/2005:0639 *Test solution (b).* Dilute 1 ml of test solution (a) to 5 ml with a mixture of 2 volumes of *concentrated ammonia R* and 48 volumes of *methanol R*.

SULFISOMIDINE

Sulfisomidinum



$C_{12}H_{14}N_4O_2S$

M_r 278.3

DEFINITION

Sulfisomidine contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of 4-amino-*N*-(2,6-dimethylpyrimidin-4-yl)benzenesulphonamide, calculated with reference to the dried substance.

CHARACTERS

White or yellowish-white powder or crystals, very slightly soluble in water, slightly soluble in acetone and in alcohol. It dissolves in dilute solutions of the alkali hydroxides and in dilute mineral acids.

It melts at about 240 °C with decomposition.

IDENTIFICATION

First identification: A, B.

Second identification: B, C, D.

- Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *sulfisomidine CRS*. Examine the substances prepared as discs.
- Examine the chromatograms obtained in the test for related substances. The principal spot in the chromatogram obtained with test solution (b) is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a).
- Dissolve 50 mg with heating in 4 ml of *methanol R*. Cool and filter. Add to the filtrate 0.2 ml of a 40 g/l solution of *copper acetate R*. The solution becomes yellowish-green.
- Dissolve about 5 mg in 10 ml of 1 *M hydrochloric acid*. Dilute 1 ml of the solution to 10 ml with *water R*. The solution, without further acidification, gives the reaction of primary aromatic amines (2.3.1).

TESTS

Appearance of solution. Dissolve 1.0 g in a mixture of 5 ml of *dilute sodium hydroxide solution R* and 5 ml of *water R*. The solution is not more intensely coloured than reference solution Y₅, BY₅ or GY₅ (2.2.2, *Method II*).

Acidity. To 1.25 g, finely powdered, add 25 ml of *carbon dioxide-free water R*. Heat at 70 °C for 5 min. Cool in a bath of iced water for about 15 min and filter. To 20 ml of the filtrate add 0.1 ml of *bromothymol blue solution R1*. Not more than 0.2 ml of 0.1 *M sodium hydroxide* is required to change the colour of the indicator.

Related substances. Examine by thin-layer chromatography (2.2.27), using *silica gel GF₂₅₄ R* as the coating substance.

Test solution (a). Dissolve 0.10 g of the substance to be examined in 3 ml of a mixture of 2 volumes of *concentrated ammonia R* and 48 volumes of *methanol R* and dilute to 5 ml with the same mixture of solvents.

Reference solution (a). Dissolve 20 mg of *sulfisomidine CRS* in 3 ml of a mixture of 2 volumes of *concentrated ammonia R* and 48 volumes of *methanol R* and dilute to 5 ml with the same mixture of solvents.

Reference solution (b). Dilute 1.25 ml of test solution (b) to 50 ml with a mixture of 2 volumes of *concentrated ammonia R* and 48 volumes of *methanol R*.

Apply to the plate 5 µl of each solution. Develop over a path of 15 cm using a mixture of 3 volumes of *dilute ammonia R1*, 5 volumes of *water R*, 40 volumes of *nitromethane R* and 50 volumes of *dioxan R*. Dry the plate at 100 °C to 105 °C and examine in ultraviolet light at 254 nm. Any spot in the chromatogram obtained with test solution (a), apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.5 per cent).

Heavy metals (2.4.8). 1.0 g complies with limit test D for heavy metals (20 ppm). Prepare the standard using 2 ml of *lead standard solution (10 ppm Pb) R*.

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Carry out the determination of primary aromatic amino-nitrogen (2.5.8), using 0.2500 g and determining the end-point electrometrically.

1 ml of 0.1 *M sodium nitrite* is equivalent to 27.83 mg of $C_{12}H_{14}N_4O_2S$.

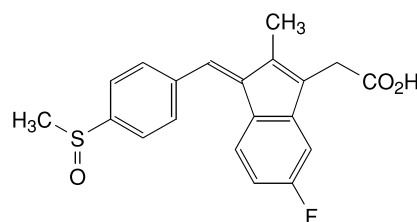
STORAGE

Store protected from light.

01/2005:0864

SULINDAC

Sulindacum



$C_{20}H_{17}FO_3S$

M_r 356.4

DEFINITION

Sulindac contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of (*Z*)-[5-fluoro-2-methyl-1-[4-(methylsulphonyl)benzylidene]-1*H*-inden-3-yl]acetic acid, calculated with reference to the dried substance.

CHARACTERS

A yellow, crystalline powder, very slightly soluble in water, soluble in methylene chloride, sparingly soluble in alcohol. It dissolves in dilute solutions of alkali hydroxides.

It shows polymorphism.

IDENTIFICATION

First identification: C.

Second identification: A, B, D, E.

- A. Melting point (2.2.14): 182 °C to 186 °C.
- B. Dissolve 50 mg in a 0.3 per cent V/V solution of *hydrochloric acid R* in *methanol R* and dilute to 100 ml with the same acid solution. Dilute 2 ml of the solution to 50 ml with a 0.3 per cent V/V solution of *hydrochloric acid R* in *methanol R*. Examined between 230 nm and 350 nm (2.2.25), the solution shows 2 absorption maxima, at 284 nm and 327 nm, and a shoulder at about 258 nm. The ratio of the absorbance measured at the maximum at 284 nm to that measured at the maximum at 327 nm is 1.10 to 1.20.
- C. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *sulindac CRS*. Examine the substances prepared as discs. If the spectra obtained show differences, dissolve the substance to be examined and the reference substance separately in the minimum volume of hot *methanol R*, evaporate to dryness and record new spectra using the residues.
- D. Examine by thin-layer chromatography (2.2.27), using *silica gel GF₂₅₄ R* as the coating substance.
- Test solution.* Dissolve 10 mg of the substance to be examined in *methylene chloride R* and dilute to 10 ml with the same solvent.

Reference solution (a). Dissolve 10 mg of *sulindac CRS* in *methylene chloride R* and dilute to 10 ml with the same solvent.

Reference solution (b). Dissolve 10 mg of *diflunisal CRS* in *methylene chloride R* and dilute to 10 ml with the same solvent. Dilute 1 ml of the solution to 2 ml with reference solution (a).

Apply to the plate 5 µl of each solution. Develop over a path of 15 cm using a mixture of 1 volume of *glacial acetic acid R*, 49 volumes of *methylene chloride R* and 50 volumes of *acetone R*. Dry the plate in a current of warm air and examine in ultraviolet light at 254 nm. The principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a). The test is not valid unless the chromatogram obtained with reference solution (b) shows 2 clearly separated principal spots.

- E. Mix about 5 mg with 45 mg of *heavy magnesium oxide R* and ignite in a crucible until an almost white residue is obtained (usually less than 5 min). Allow to cool, add 1 ml of *water R*, 0.05 ml of *phenolphthalein solution R1* and about 1 ml of *dilute hydrochloric acid R* to render the solution colourless. Filter. Add 1.0 ml of the filtrate to a freshly prepared mixture of 0.1 ml of *alizarin S solution R* and 0.1 ml of *zirconyl nitrate solution R*. Mix, allow to stand for 5 min and compare the colour of the solution with that of a blank prepared in the same manner. The test solution is yellow and the blank is red.

TESTS

Related substances. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 0.10 g of the substance to be examined in the mobile phase and dilute to 50.0 ml with the mobile phase.

Reference solution (a). Dilute 1.0 ml of the test solution to 100.0 ml with the mobile phase. Dilute 5.0 ml to 10.0 ml with the mobile phase.

Reference solution (b). Dissolve 20.0 mg of *sulindac CRS* (which has an assigned content of *E*-isomer) in the mobile phase and dilute to 10.0 ml with the mobile phase.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4.6 mm in internal diameter packed with *silica gel for chromatography R* (10 µm),
- as mobile phase at a flow rate of 2 ml/min a mixture of 1 volume of *glacial acetic acid R*, 4 volumes of *alcohol R*, 100 volumes of *ethyl acetate R* and 400 volumes of *ethanol-free chloroform R*,
- as detector a spectrophotometer set at 280 nm.

Inject 20 µl of reference solution (a) and adjust the sensitivity of the system so that the height of the principal peak in the chromatogram obtained is at least 50 per cent of the full scale of the recorder. Inject 20 µl of reference solution (b) and continue the chromatography for twice the retention time of the principal peak. The chromatogram obtained shows a principal peak corresponding to sulindac and a peak corresponding to the *E*-isomer, with a retention time relative to sulindac of about 1.75.

Inject 20 µl of the test solution, 20 µl of reference solution (a) and 20 µl of reference solution (b). From the chromatograms obtained with the test solution and reference solution (b), determine the percentage content of *E*-isomer, taking into account the assigned content of this isomer in *sulindac CRS*: the value found is not more than 0.5 per cent. In the chromatogram obtained with the test solution: the area of any peak, apart from the principal peak and any peak corresponding to *E*-isomer, is not greater than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent); the sum of the areas of all the peaks, apart from the principal peak, is not greater than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (1 per cent).

Heavy metals (2.4.8). 2.0 g complies with limit test D (10 ppm). Prepare the standard using 2 ml of *lead standard solution* (10 ppm Pb) R.

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 100–105 °C at a pressure not exceeding 700 Pa.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

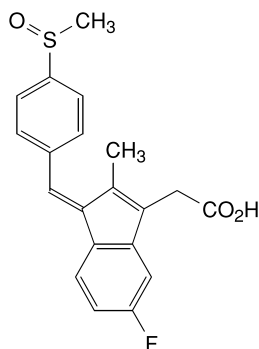
Dissolve 0.300 g in 50 ml of *methanol R*. Titrate with 0.1 M *sodium hydroxide*, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *sodium hydroxide* is equivalent to 35.64 mg of C₂₀H₁₇FO₃S.

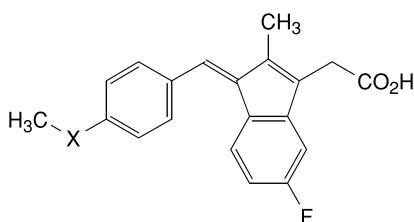
STORAGE

Protected from light.

IMPURITIES



- A. (*E*)-[5-fluoro-2-methyl-1-[4-(methylsulphonyl)benzylidene]-1*H*-inden-3-yl]acetic acid,



- B. X = SO₂: (*Z*)-[5-fluoro-2-methyl-1-[4-(methylsulphonyl)benzylidene]-1*H*-inden-3-yl]acetic acid,
C. X = S: (*Z*)-[5-fluoro-2-methyl-1-[4-(methylsulphanyl)benzylidene]-1*H*-inden-3-yl]acetic acid.

01/2005:0953

SULPHUR FOR EXTERNAL USE

Sulfur ad usum externum

S

A, 32.07

DEFINITION

Sulphur contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of S.

CHARACTERS

A yellow powder, practically insoluble in water, soluble in carbon disulphide, slightly soluble in vegetable oils. The size of most of the particles is not greater than 20 µm and that of almost all the particles is not greater than 40 µm.

It melts at about 120 °C.

IDENTIFICATION

- A. Heated in the presence of air, it burns with a blue flame, emitting sulphur dioxide which changes the colour of moistened *blue litmus paper R* to red.
B. Heat 0.1 g with 0.5 ml of *bromine water R* until decolourised. Add 5 ml of *water R* and filter. The solution gives reaction (a) of sulphates (2.3.1).

TESTS

Solution S. To 5 g add 50 ml of *carbon dioxide-free water R* prepared from *distilled water R*. Allow to stand for 30 min with frequent shaking and filter.

Appearance of solution. Solution S is colourless (2.2.2, *Method II*).

Odour (2.3.4). The substance to be examined has no perceptible odour of hydrogen sulphide.

Acidity or alkalinity. To 5 ml of solution S add 0.1 ml of *phenolphthalein solution R1*. The solution is colourless. Add 0.2 ml of 0.01 *M sodium hydroxide*. The solution is red. Add 0.3 ml of 0.01 *M hydrochloric acid*. The solution is colourless. Add 0.15 ml of *methyl red solution R*. The solution is orange-red.

Chlorides (2.4.4). Dilute 5 ml of solution S to 15 ml with *water R*. The solution complies with the limit test for chlorides (100 ppm).

Sulphates (2.4.13). 15 ml of solution S complies with the limit test for sulphates (100 ppm).

Sulphides. To 10 ml of solution S add 2 ml of *buffer solution pH 3.5 R* and 1 ml of a freshly prepared 1.6 g/l solution of *lead nitrate R* in *carbon dioxide-free water R*. Shake. After 1 min any colour in the solution is not more intense than that in a reference solution prepared at the same time using 1 ml of *lead standard solution (10 ppm Pb) R*, 9 ml of *carbon dioxide-free water R*, 2 ml of *buffer solution pH 3.5 R* and 1.2 ml of *thioacetamide reagent R*.

Sulphated ash (2.4.14). Not more than 0.2 per cent, determined on 1.0 g.

ASSAY

Carry out the oxygen-flask method (2.5.10), using 60.0 mg of the substance to be examined in a 1000 ml combustion flask. Absorb the combustion products in a mixture of 5 ml of *dilute hydrogen peroxide solution R* and 10 ml of *water R*. Heat to boiling, boil gently for 2 min and cool. Using 0.2 ml of *phenolphthalein solution R* as indicator, titrate with 0.1 *M sodium hydroxide* until the colour changes from colourless to red. Carry out a blank titration under the same conditions.

1 ml of 0.1 *M sodium hydroxide* is equivalent to 1.603 mg of S.

STORAGE

Store protected from light.

01/2005:1572

SULPHURIC ACID

Acidum sulfuricum

H₂SO₄M_r 98.1

DEFINITION

Sulphuric acid contains not less than 95.0 per cent *m/m* and not more than 100.5 per cent *m/m* of H₂SO₄.

CHARACTERS

A colourless, oily liquid, very hygroscopic, miscible with water and with alcohol producing intense heat.

The relative density is about 1.84.

IDENTIFICATION

- A. Carefully add 1 ml to 100 ml of *water R*. The solution is strongly acid (2.2.4).
B. The solution obtained in identification test A gives reaction (a) of sulphates (2.3.1).

TESTS

Appearance of solution. Carefully pour, while cooling, 5 ml into 30 ml of *water R* and dilute to 50 ml with the same solvent. The solution is clear (2.2.1) and colourless (2.2.2, *Method II*).

Chlorides (2.4.4). Mix carefully, while cooling, 3.3 g with 30 ml of *water R*. Neutralise with *ammonia R* and dilute to 50 ml with *water R*. 15 ml of the solution complies with the limit test for chlorides (50 ppm).

Nitrates. Add 5 ml to 5 ml of *water R*. Cool to room temperature and add 0.5 ml of *indigo carmine solution R*. The blue colour persists for at least 1 min.

Arsenic (2.4.2). Mix, while cooling, 1 g with 20 ml of *water R* and dilute to 25 ml with the same solvent. The solution complies with limit test A for arsenic (1 ppm).

Iron (2.4.9). Cautiously evaporate 10.0 g and ignite to dull redness. Dissolve the ignition residue in 1 ml of *dilute hydrochloric acid R* with gentle heating and dilute to 25 ml with *water R*. Dilute 1 ml of the solution to 10 ml with *water R*. The solution complies with the limit test for iron (25 ppm).

Heavy metals (2.4.8). 4.0 g complies with limit test F for heavy metals (5 ppm). Prepare the standard using 2 ml of *lead standard solution (10 ppm Pb) R*.

ASSAY

Weigh accurately a ground-glass-stoppered flask containing 30 ml of *water R*. Introduce 0.2 ml of the acid, cool and weigh again. Titrate with 1 M *sodium hydroxide*, determining the end-point potentiometrically (2.2.20).

1 ml of 1 M *sodium hydroxide* is equivalent to 49.04 mg of H_2SO_4 .

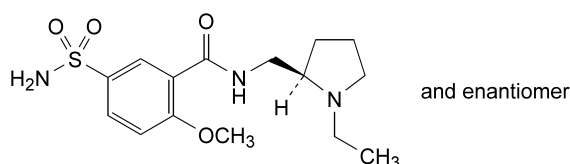
STORAGE

Store in an airtight container.

01/2005:1045

SULPIRIDE

Sulpiridum



$\text{C}_{15}\text{H}_{23}\text{N}_3\text{O}_4\text{S}$

M_r 341.4

DEFINITION

Sulpiride contains not less than 98.5 per cent and not more than the equivalent of 101.0 per cent of (*RS*)-*N*[(1-ethylpyrrolidin-2-yl)methyl]-2-methoxy-5-sulphamoylbenzamide, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, crystalline powder, practically insoluble in water, sparingly soluble in methanol, slightly soluble in alcohol and in methylene chloride. It dissolves in dilute solutions of mineral acids and alkali hydroxides.

IDENTIFICATION

First identification: A, B.

Second identification: A, C, D.

A. Melting point (2.2.14): 177 °C to 181 °C.

B. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *sulpiride CRS*. Examine the substances prepared as discs.

C. Examine the chromatograms obtained in test A for related substances (see Tests) in ultraviolet light at 254 nm. The principal spot in the chromatogram obtained with test solution (b) is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a).

D. To about 1 mg in a porcelain dish, add 0.5 ml of *sulphuric acid R* and 0.05 ml of *formaldehyde solution R*. Examined in ultraviolet light at 365 nm, the solution shows blue fluorescence.

TESTS

Appearance of solution. Dissolve 1.0 g in *dilute acetic acid R* and dilute to 10 ml with the same acid. The solution is clear (2.2.1) and not more intensely coloured than reference solution Y_6 (2.2.2, *Method I*).

Related substances

A. Examine by thin-layer chromatography (2.2.27), using *silica gel HF₂₅₄ R* as the coating substance.

Test solution (a). Dissolve 0.20 g in *methanol R* and dilute to 10 ml with the same solvent.

Test solution (b). Dilute 1 ml of test solution (a) to 10 ml with *methanol R*.

Reference solution (a). Dissolve 20 mg of *sulpiride CRS* in *methanol R* and dilute to 10 ml with the same solvent.

Reference solution (b). Dissolve 5 mg of *sulpiride impurity A CRS* in *methanol R* and dilute to 25 ml with the same solvent.

Reference solution (c). Dilute 1.0 ml of reference solution (b) to 10 ml with *methanol R*.

Apply to the plate 10 µl of each solution. Develop over a path of 10 cm using a mixture of 2 volumes of *concentrated ammonia R*, 10 volumes of *dioxan R*, 14 volumes of *methanol R* and 90 volumes of *methylene chloride R*. Allow the plate to dry in air. Examine in ultraviolet light at 254 nm for identification test C and then spray with *ninhydrin solution R*. Heat at 100-105 °C for 15 min. Examine in daylight. Any spot in the chromatogram obtained with test solution (a) corresponding to the principal spot in the chromatogram obtained with reference solution (b) is not more intense than the spot in the chromatogram obtained with reference solution (c) (0.1 per cent).

B. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 0.100 g in the mobile phase and dilute to 100.0 ml with the mobile phase.

Reference solution (a). Dilute 3.0 ml of the test solution to 100.0 ml with the mobile phase. Dilute 1.0 ml of the solution to 10.0 ml with the mobile phase.

Reference solution (b). Dissolve 10 mg of *sulpiride CRS* and 10 mg of *sulpiride impurity B CRS* in the mobile phase and dilute to 100.0 ml with the same solvent.

The chromatography may be carried out using:

- a column 0.25 m long and 4.6 mm in internal diameter, packed with *octylsilyl silica gel for chromatography R* (5 µm) in spherical micro-particles,
- as mobile phase at a flow rate of 1.5 ml/min a mixture of 10 volumes of *acetonitrile R*, 10 volumes of *methanol R* and 80 volumes of a solution containing 6.8 g/l of *potassium dihydrogen phosphate R* and 1 g/l of *sodium octanesulphonate R*, adjusted to pH 3.3 using *phosphoric acid R*,
- as detector a spectrophotometer set at 240 nm,
- a loop injector.

Adjust the sensitivity of the detector so that the height of the principal peak in the chromatogram obtained with reference solution (a) is not less than 5 per cent of the full scale of the recorder. Inject 10 µl of reference solution (b). The test is not valid unless in the chromatogram obtained the resolution between the two principal peaks is at least 2.5. Inject separately 10 µl of the test solution and 10 µl of reference solution (a). Continue the chromatography for 2.5 times the retention time of sulpiride. In the chromatogram obtained with the test solution, the sum of the areas of any peaks, apart from the principal peak, is not greater than the area of the peak in the chromatogram obtained with reference solution (a) (0.3 per cent).

Chlorides (2.4.4). Shake 1.0 g with 20 ml of *water R*. Filter through a sintered-glass filter (40). To 10 ml of the solution add 5 ml of *water R*. The solution complies with the limit test for chlorides (100 ppm).

Iron (2.4.9). Ignite 1.0 g in a silica crucible. To the residue add 1 ml of 1 M *hydrochloric acid*, 3 ml of *water R* and 0.1 ml of *nitric acid R*. Heat on a water-bath for a few minutes. Place the solution in a test-tube. Rinse the crucible with 4 ml of *water R*. Collect the rinsings in the test-tube and dilute to 10 ml with *water R*. The solution complies with the limit test for iron (10 ppm).

Heavy metals (2.4.8). 1.0 g complies with limit test C for heavy metals (10 ppm). Prepare the standard using 1 ml of *lead standard solution* (10 ppm Pb) *R*.

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 100–105 °C.

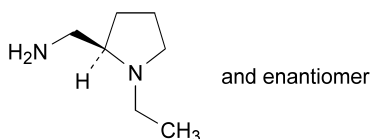
Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

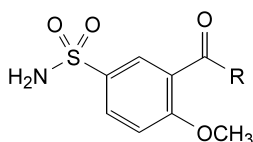
Dissolve 0.250 g in 80 ml of *anhydrous acetic acid R*. Titrate with 0.1 M *perchloric acid*, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *perchloric acid* is equivalent to 34.14 mg of C₁₅H₂₃N₃O₄S.

IMPURITIES



A. [(2*RS*)-1-ethylpyrrolidin-2-yl]methanamine,

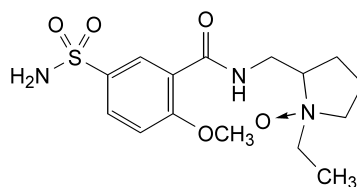


B. R = O-CH₃: methyl 2-methoxy-5-sulphamoylbenzoate,

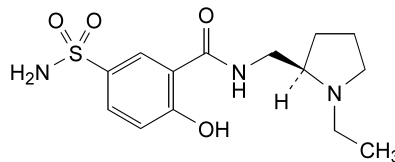
C. R = O-C₂H₅: ethyl 2-methoxy-5-sulphamoylbenzoate,

D. R = OH: 2-methoxy-5-sulphamoylbenzoic acid,

E. R = NH₂: 2-methoxy-5-sulphamoylbenzamide,



F. 1-ethyl-2-[[[(2-methoxy-5-sulphamoylbenzoyl)amino]methyl]pyrrolidine 1-oxide,



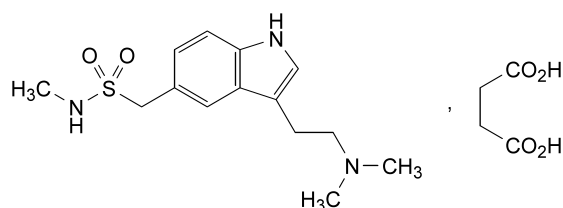
and enantiomer

G. (*RS*)-*N*-[(1-ethylpyrrolidin-2-yl)methyl]-2-hydroxy-5-sulphamoylbenzamide.

01/2005:1573

SUMATRIPTAN SUCCINATE

Sumatriptani succinas



C₁₈H₂₇N₃O₆S

*M*_r 413.5

DEFINITION

[3-[2-(Dimethylamino)ethyl]-1*H*-indol-5-yl]-*N*-methylmethanesulphonamide hydrogen butanedioate.

Content: 97.5 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

Appearance: white to almost white powder.

Solubility: freely soluble in water, sparingly soluble in methanol, practically insoluble in methylene chloride.

IDENTIFICATION

Infrared absorption spectrophotometry (2.2.24).

Preparation: discs.

Comparison: *sumatriptan succinate CRS*.

TESTS

Solution S. Dissolve 1.0 g in *carbon dioxide-free water R* and dilute to 25.0 ml with the same solvent.

pH (2.2.3): 4.5 to 5.3.

Dilute 2.5 ml of solution S to 10 ml with *carbon dioxide-free water R*.

Absorbance (2.2.25): maximum 0.10, measured at 440 nm on solution S.

Impurity A and impurity H. Liquid chromatography (2.2.29).

Test solution. Dissolve 30.0 mg of the substance to be examined in the mobile phase and dilute to 10.0 ml with the mobile phase.

Reference solution (a). Dissolve 2 mg of *sumatriptan impurity A CRS* in the mobile phase, add 1 ml of the test solution and dilute to 100 ml with the mobile phase. Dilute 1 ml to 10 ml with the mobile phase.

Reference solution (b). Dilute 1.0 ml of the test solution to 100.0 ml with the mobile phase. Dilute 1.0 ml to 10.0 ml with the mobile phase.

Reference solution (c). Dissolve 3 mg of *sumatriptan for system suitability CRS* in the mobile phase and dilute to 1 ml with the mobile phase.

Column:

- size: $l = 0.25$ m, $\varnothing = 4.6$ mm,
- stationary phase: *silica gel for chromatography R* (5 μ m).

Mobile phase: mix 10 volumes of a 771 g/l solution of *ammonium acetate R* and 90 volumes of *methanol R*.

Flow rate: 2.0 ml/min.

Detection: spectrophotometer at 282 nm.

Injection: 20 μ l.

Run time: 5 times the retention time of sumatriptan.

System suitability:

- resolution: minimum of 1.5 between the peaks due to sumatriptan and to impurity A in the chromatogram obtained with reference solution (a),
- the chromatogram obtained with reference solution (c) is similar to the chromatogram provided with *sumatriptan for system suitability CRS*.

Limits:

- *impurity A*: not more than 6 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.6 per cent),
- *impurity H*: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.3 per cent).

Related substances. Liquid chromatography (2.2.29).

Solution A. Dissolve 2.925 g of *sodium dihydrogen phosphate R* in 600 ml of *water R*, adjust to pH 6.5 with *strong sodium hydroxide solution R*, dilute to 750 ml with *water R*, add 250 ml of *acetonitrile R* and mix.

Test solution (a). Dissolve 30.0 mg of the substance to be examined in the mobile phase and dilute to 10.0 ml with the mobile phase.

Test solution (b). Dissolve 15.0 mg of the substance to be examined in solution A and dilute to 100.0 ml with solution A.

Reference solution (a). Dissolve 2 mg of *sumatriptan impurity C CRS* in the mobile phase, add 1 ml of the test solution and dilute to 100 ml with the mobile phase. Dilute 1 ml to 10 ml with the mobile phase.

Reference solution (b). Dilute 1.0 ml of the test solution to 100.0 ml with the mobile phase. Dilute 1.0 ml to 10.0 ml with the mobile phase.

Reference solution (c). Dissolve 30 mg of *sumatriptan impurity mixture CRS* in the mobile phase and dilute to 10 ml with the mobile phase.

Reference solution (d). Dissolve 2 mg of *sumatriptan impurity D CRS* in the mobile phase and dilute to 100 ml with the mobile phase. Dilute 1 ml to 10 ml with the mobile phase.

Reference solution (e). Dissolve 15.0 mg of *sumatriptan succinate CRS* in solution A and dilute to 100.0 ml with solution A.

Reference solution (f). Dissolve 3 mg of *sumatriptan impurity C CRS* in solution A, add 40 ml of reference solution (e) and dilute to 100 ml with solution A.

Column:

- size: $l = 0.25$ m, $\varnothing = 4$ mm,
- stationary phase: *octadecylsilyl silica gel for chromatography R* (5 μ m).

Mobile phase: mix 25 volumes of *acetonitrile R* with 75 volumes of a solution prepared as follows: dissolve 0.970 g of *dibutylamine R*, 0.735 g of *phosphoric acid R* and 2.93 g of *sodium dihydrogen phosphate R* in 750 ml of *water R*, adjust to pH 6.5 with *strong sodium hydroxide solution R* and dilute to 1000 ml with *water R*.

Flow rate: 1.5 ml/min.

Detection: spectrophotometer at 282 nm.

Injection: 10 μ l; inject test solution (a) and reference solutions (a), (b), (c) and (d).

Run time: 4 times the retention time of sumatriptan.

System suitability:

- resolution: minimum of 1.5 between the peaks due to sumatriptan and to impurity C in the chromatogram obtained with reference solution (a),
- the chromatogram obtained with reference solution (c) shows 5 clearly separated peaks.

Limits:

The chromatogram obtained with reference solution (c) shows 5 peaks: 1 major peak due to sumatriptan, 3 peaks of about equal area due to impurities B, C and D and 1 peak due to impurity E with an area of about twice the area of the peaks due to impurities B, C or D.

Determine the retention times of impurities B, C, D and E using the chromatograms obtained with reference solutions (a), (c) and (d).

- *impurities B, C, D*: for each impurity, not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent),
- *impurity E or any other impurity*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent),
- *total*: not more than 6 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.6 per cent),
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

Water (2.5.12): maximum 1.0 per cent, determined on 0.500 g.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (2.2.29) as described in the test for related substances.

Injection: 10 μ l; inject test solution (b) and reference solutions (e) and (f).

System suitability: reference solution (f):

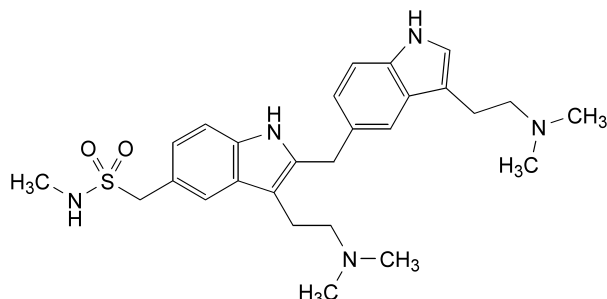
- resolution: minimum of 1.5 between the peaks due to sumatriptan and to impurity C.

Calculate the percentage content of sumatriptan succinate using the chromatogram obtained with reference solution (e).

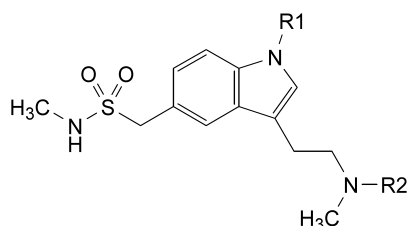
STORAGE

Protected from light.

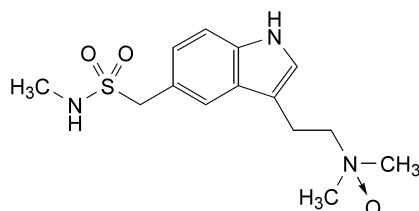
IMPURITIES



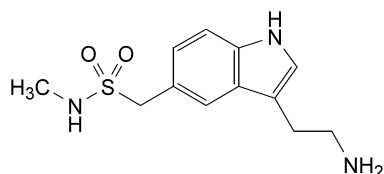
- A. [3-[2-(dimethylamino)ethyl]-2-[[3-[2-(dimethylamino)ethyl]-1*H*-indol-5-yl]methyl]-1*H*-indol-5-yl]-*N*-methylmethanesulphonamide,



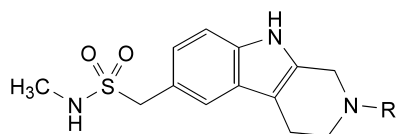
- B. R1 = R2 = H: *N*-methyl[3-[2-(methylamino)ethyl]-1*H*-indol-5-yl]methanesulphonamide,
 C. R1 = CH₂-OH, R2 = CH₃: [3-[2-(dimethylamino)ethyl]-1-(hydroxymethyl)-1*H*-indol-5-yl]-*N*-methylmethanesulphonamide,



- D. *N,N*-dimethyl-2-[5-[(methylsulphamoyl)methyl]-1*H*-indol-3-yl]ethanamine *N*-oxide,

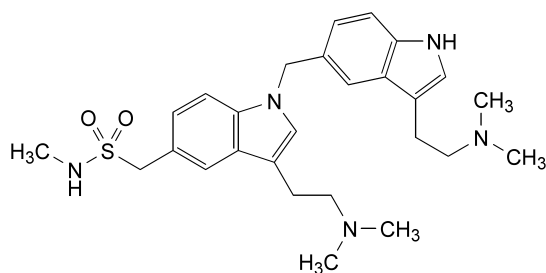


- E. [3-(2-aminoethyl)-1*H*-indol-5-yl]-*N*-methylmethanesulphonamide,



- F. R = H: *N*-methyl(2,3,4,9-tetrahydro-1*H*-pyrido[3,4-*b*]indol-6-yl)methanesulphonamide,

- G. R = CH₃: *N*-methyl(2-methyl-2,3,4,9-tetrahydro-1*H*-pyrido[3,4-*b*]indol-6-yl)methanesulphonamide,



- H. [3-[2-(dimethylamino)ethyl]-1-[[3-[2-(dimethylamino)ethyl]-1*H*-indol-5-yl]methyl]-1*H*-indol-5-yl]-*N*-methylmethanesulphonamide.

01/2005:1371

SUNFLOWER OIL, REFINED

Helianthi annui oleum raffinatum

DEFINITION

Sunflower oil is the fatty oil obtained from the seeds of *Helianthus annuus* L. by mechanical expression or by extraction. It is then refined. A suitable antioxidant may be added.

CHARACTERS

A clear, light yellow liquid, practically insoluble in water and in alcohol, miscible with light petroleum (bp: 40 °C to 60 °C).

It has a relative density of about 0.921 and a refractive index of about 1.474.

IDENTIFICATION

Carry out the identification of fatty oils by thin-layer chromatography (2.3.2). The chromatogram obtained is similar to the typical chromatogram for sunflower oil.

TESTS

Acid value (2.5.1). Not more than 0.5, determined on 10.0 g.

Peroxide value (2.5.5). Not more than 10.0.

Unsaponifiable matter (2.5.7). Not more than 1.5 per cent, determined on 5.0 g.

Alkaline impurities (2.4.19). It complies with the test for alkaline impurities in fatty oils.

Composition of fatty acids (2.4.22, *Method A*). The fatty-acid fraction of the oil has the following composition:

- *palmitic acid*: 4.0 per cent to 9.0 per cent,
- *stearic acid*: 1.0 per cent to 7.0 per cent,
- *oleic acid*: 14.0 per cent to 40.0 per cent,
- *linoleic acid*: 48.0 per cent to 74.0 per cent.

STORAGE

Store in an airtight, well-filled container, protected from light.

LABELLING

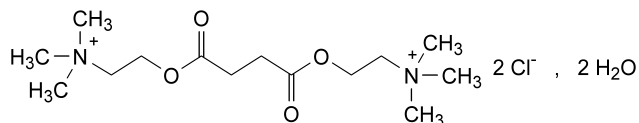
The label states:

- the name and concentration of any added antioxidant,
- whether the oil is obtained by mechanical expression or by extraction.

01/2005:0248

SUXAMETHONIUM CHLORIDE

Suxamethonii chloridum

 $C_{14}H_{30}Cl_2N_2O_4 \cdot 2H_2O$ M_r 397.3

DEFINITION

Suxamethonium chloride contains not less than 98.0 per cent and not more than the equivalent of 102.0 per cent of 2,2'-[butanedioylbis(oxy)]bis(*N,N,N*-trimethylethanaminium) dichloride, calculated with reference to the anhydrous substance.

CHARACTERS

A white or almost white, crystalline powder, hygroscopic, freely soluble in water, slightly soluble in alcohol.

It melts at about 160 °C, determined without previous drying.

IDENTIFICATION

First identification: A, D.

Second identification: B, C, D.

- Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *suxamethonium chloride CRS*. Examine the substances prepared as discs.
- To 1 ml of solution S (see Tests) add 9 ml of *water R*, 10 ml of *dilute sulphuric acid R* and 30 ml of *ammonium reineckate solution R*. A pink precipitate is formed. Allow to stand for 30 min, filter, wash with *water R*, with *alcohol R* and then with *ether R* and dry at 80 °C. The melting point (2.2.14) of the precipitate is 180 °C to 185 °C.
- Dissolve about 25 mg in 1 ml of *water R* and add 0.1 ml of a 10 g/l solution of *cobalt chloride R* and 0.1 ml of *potassium ferrocyanide solution R*. A green colour is produced.
- About 20 mg gives reaction (a) of chlorides (2.3.1).

TESTS

Solution S. Dissolve 1.0 g in *carbon dioxide-free water R* and dilute to 20 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1). Dilute 4 ml of solution S to 10 ml with *water R*. The solution is colourless (2.2.2, *Method II*).

pH (2.2.3). Dilute 1 ml of solution S to 10 ml with *carbon dioxide-free water R*. The pH of the solution is 4.0 to 5.0.

Choline chloride. Examine by thin-layer chromatography (2.2.27), using *cellulose for chromatography R1* as the coating substance.

Test solution. Dissolve 0.4 g of the substance to be examined in *methanol R* and dilute to 10 ml with the same solvent.

Reference solution. Dissolve 0.4 g of *suxamethonium chloride CRS* and 2 mg of *choline chloride R* in *methanol R* and dilute to 10 ml with the same solvent.

Apply to the plate 5 µl of each solution. Prepare the mobile phase as follows: shake together for 10 min, 10 volumes of *anhydrous formic acid R*, 40 volumes of *water R* and 50 volumes of *butanol R*; allow to stand and use the upper layer. Develop over a path of 15 cm. Dry the plate in a

current of air and spray with *potassium iodobismuthate solution R*. Any spot in the chromatogram obtained with the test solution, apart from the principal spot, is not more intense than the spot corresponding to choline chloride in the chromatogram obtained with the reference solution (0.5 per cent). The test is not valid unless the chromatogram obtained with the reference solution shows two clearly separated spots.

Water (2.5.12). 8.0 per cent to 10.0 per cent, determined on 0.30 g by the semi-micro determination of water.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.150 g in 50 ml of *acetic anhydride R*. Titrate with 0.1 M *perchloric acid*, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *perchloric acid* is equivalent to 18.07 mg of $C_{14}H_{30}Cl_2N_2O_4$.

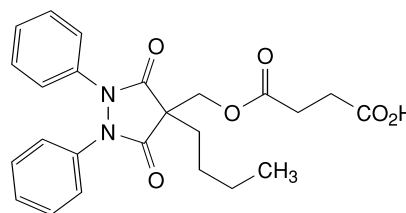
STORAGE

Store in an airtight container, protected from light.

01/2005:1574

SUXIBUZONE

Suxibuzonium

 $C_{24}H_{26}N_2O_6$ M_r 438.5

DEFINITION

4-[(4-Butyl-3,5-dioxo-1,2-diphenylpyrazolidin-4-yl)methoxy]-4-oxobutanoic acid.

Content: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

Appearance: white, crystalline powder.

Solubility: practically insoluble in water, freely soluble in acetone, soluble in alcohol, practically insoluble in cyclohexane.

IDENTIFICATION

Infrared absorption spectrophotometry (2.2.24).

Comparison: *suxibuzone CRS*.

TESTS

Appearance of solution. The solution is clear (2.2.1) and colourless (2.2.2, *Method II*).

Dissolve 1 g in *ethanol R* and dilute to 20 ml with the same solvent.

Related substances. Liquid chromatography (2.2.29).

Test solution. Dissolve 0.10 g of the substance to be examined in *acetonitrile R* and dilute to 25.0 ml with the same solvent.

Reference solution (a). Dissolve 2.8 mg of *phenylbutazone CRS* (impurity A of *suxibuzone*), 2.8 mg of *suxibuzone impurity B CRS* and 2.8 mg of

suxibuzone impurity C CRS in *acetonitrile R* and dilute to 10.0 ml with the same solvent. Dilute 1.0 ml of the solution to 10.0 ml with *acetonitrile R*.

Reference solution (b). Dissolve 4 mg of *phenylbutazone CRS* (impurity A of *suxibuzone*) in *acetonitrile R* and dilute to 100.0 ml with the same solvent. Dilute 1.0 ml of the solution to 10.0 ml with *acetonitrile R*.

Reference solution (c). Dissolve 10 mg of *phenylbutazone CRS* (impurity A of *suxibuzone*) in *acetonitrile R* and dilute to 25.0 ml with the same solvent. Mix 10.0 ml of this solution with 1.0 ml of the test solution and dilute the mixture to 25.0 ml with *acetonitrile R*.

Column:

- **size:** $l = 0.125$ m, $\varnothing = 4.0$ mm,
- **stationary phase:** *octadecylsilyl silica gel for chromatography R* (5 μ m).

Mobile phase: mix 44 volumes of *acetonitrile R* and 56 volumes of a solution prepared as follows: dissolve 6.7 g of *citric acid R* and 2.4 g of *tris(hydroxymethyl)aminomethane R* in 950 ml of *water R*, adjust to pH 3.0 with *citric acid R* and dilute to 1000 ml with *water R*.

Flow rate: 1 ml/min.

Detection: spectrophotometer at 250 nm.

Injection: 10 μ l.

Relative retention with reference to *suxibuzone* (retention time = about 7 min): impurity C = 0.7; impurity A = 1.4; impurity B = 3.3.

System suitability: reference solution (c):

- **resolution:** minimum of 2.0 between the peaks due to *suxibuzone* and to impurity A.

Limits:

- **impurity A:** not more than the area of the corresponding peak in the chromatogram obtained with reference solution (a) (0.7 per cent),
- **impurity B:** not more than the area of the corresponding peak in the chromatogram obtained with reference solution (a) (0.7 per cent),
- **impurity C:** not more than the area of the corresponding peak in the chromatogram obtained with reference solution (a) (0.7 per cent),
- **any other impurity:** not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent),
- **total:** not more than 10 times the area of the principal peak in the chromatogram obtained with reference solution (b) (1.0 per cent),
- **disregard limit:** 0.1 times the area of the peak due to impurity A in the chromatogram obtained with reference solution (a) (0.07 per cent).

Heavy metals (2.4.8): maximum 10 ppm.

2.0 g complies with limit test C. Prepare the standard using 2 mg of *lead standard solution* (10 ppm Pb) *R*.

Loss on drying (2.2.32): maximum 0.5 per cent, determined on 1.000 g by drying in an oven *in vacuo* at 60 °C.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.400 g in previously neutralised *ethanol R* and dilute to 10 ml with the same solvent. Carry out a potentiometric titration (2.2.20) using 0.1 M *sodium hydroxide*.

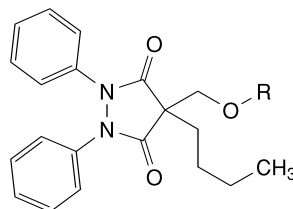
1 ml of 0.1 M *sodium hydroxide* is equivalent to 43.85 mg of $C_{24}H_{26}N_2O_6$.

STORAGE

Protected from light.

IMPURITIES

A. *phenylbutazone*,



B. R = $CO-CH_2-CH_2-CO-O-CH_2-CH_3$: (4-butyl-3,5-dioxo-1,2-diphenylpyrazolidin-4-yl)methyl ethyl butanedioate,

C. R = H: 4-butyl-4-(hydroxymethyl)-1,2-diphenyl-1,2-dihydro-4H-pyrazole-3,5-dione.

01/2005:1811

SWEET ORANGE OIL

Aurantii dulcis aetheroleum

DEFINITION

Essential oil obtained without heating, by suitable mechanical treatment from the fresh peel of the fruit of *Citrus sinensis* (L.) Osbeck (*Citrus aurantium* L. var. *dulcis* L.). A suitable antioxidant may be added.

CHARACTERS

Appearance: clear, pale yellow to orange, mobile liquid, which may become cloudy when chilled.

It has a characteristic odour of fresh orange peel.

IDENTIFICATION

First identification: B.

Second identification: A.

A. Thin-layer chromatography (2.2.27).

Examine the chromatograms obtained in the test for bergapten.

Results A: see below the sequence of the zones present in the chromatograms obtained with the reference solution and the test solution.

Top of the plate	
Bergapten: a greenish-yellow fluorescent zone	
	Many blue fluorescent zones
Reference solution	Test solution

Results B: see below the sequence of the zones present in the chromatograms obtained with the reference solution and the test solution.

Top of the plate	
Linalyl acetate: a brownish-orange fluorescent zone	A brown fluorescent zone
	A faint brownish-orange fluorescent zone (linalyl acetate)
	Many orange fluorescent zones
Linalol: a brownish-orange fluorescent zone	A brownish-orange fluorescent zone (linalol)
Bergaptene: a faint greenish-yellow fluorescent zone	Many brownish-orange fluorescent zones
	Many blue fluorescent zones
Reference solution	Test solution

B. Examine the chromatograms obtained in the test for chromatographic profile.

Results: the characteristic peaks in the chromatogram obtained with the test solution are similar in retention time to those in the chromatogram obtained with the reference solution.

TESTS

Relative density (2.2.5): 0.842 to 0.850.

Refractive index (2.2.6): 1.470 to 1.476.

Optical rotation (2.2.7): + 94° to + 99°.

Peroxide value (2.5.5, Method B): maximum 20.

Fatty oils and resinified essential oils (2.8.7). It complies with the test for fatty oils and resinified essential oils.

Bergaptene. Thin-layer chromatography (2.2.27).

Test solution. Dilute 0.2 ml of the substance to be examined in 1 ml of alcohol R.

Reference solution. Dissolve 2 mg of bergaptene R, 10 µl of linalol R and 20 µl of linalyl acetate R in 10 ml of alcohol R.

Plate: TLC silica gel plate R.

Mobile phase: ethyl acetate R, toluene R (15:85 V/V).

Application: 10 µl, as bands.

Development: over a path of 15 cm.

Drying: in air.

Detection A: examine in ultraviolet light at 365 nm.

Results A: the chromatogram obtained with the test solution shows no greenish-yellow fluorescent zone present in the chromatogram obtained with the reference solution.

Detection B: spray with anisaldehyde solution R and heat at 100-105 °C for 10 min; examine the plate in ultraviolet light at 365 nm.

Chromatographic profile. Gas chromatography (2.2.28): use the normalisation procedure.

Test solution. Dilute 300 µl of the substance to be examined to 1 ml with acetone R.

Reference solution (a). Dilute 10 µl of α-pinene R, 10 µl of β-pinene R, 10 µl of sabinene R, 20 µl of β-myrcene R, 800 µl of limonene R, 10 µl of octanal, 10 µl of decanal R, 10 µl of linalol R, 10 µl of citral R (composed of neral and geranial) and 10 µl of valencene R in 1 ml of acetone R.

Reference solution (b). Dissolve 5 µl of β-pinene R in 10 ml of acetone R. Dilute 0.5 ml to 10 ml with acetone R.

Column:

- **material:** fused silica,
- **size:** $l = 30$ m, $\varnothing = 0.53$ mm,
- **stationary phase:** macrogol 20 000 R (film thickness 1 µm).

Carrier gas: helium for chromatography R.

Flow rate: 1 ml/min.

Split ratio: 1:100.

Temperature:

	Time (min)	Temperature (°C)
Column	0 - 6	50
	6 - 31	50 → 150
	31 - 41	150 → 180
	41 - 55	180
Injection port		250
Detector		250

Detection: flame ionisation.

Injection: 0.5 µl.

Elution order: order indicated in the composition of reference solution (a). Record the retention times of these substances.

System suitability: reference solution (a)

- **resolution:** minimum 3.9 between the peaks due to β-pinene and sabinene and minimum 1.5 between the peaks due to valencene and geranial.

Using the retention times determined from the chromatogram obtained with reference solution (a), locate the components of reference solution (a) in the chromatogram obtained with the test solution.

Determine the percentage content of these components. The limits are within the following ranges:

- α-pinene: 0.4 per cent to 0.6 per cent,
- β-pinene: 0.02 per cent to 0.3 per cent,
- sabinene: 0.2 per cent to 1.1 per cent,
- β-myrcene: 1.7 per cent to 2.5 per cent,
- limonene: 92.0 per cent to 97.0 per cent,
- octanal: 0.1 per cent to 0.4 per cent,
- decanal: 0.1 per cent to 0.4 per cent,
- linalol: 0.2 per cent to 0.7 per cent,
- neral: 0.02 per cent to 0.10 per cent,
- valencene: 0.02 per cent to 0.5 per cent,
- geranial: 0.03 per cent to 0.20 per cent.

Disregard limit: area of the peak in the chromatogram obtained with reference solution (b) (0.01 per cent).

Residue on evaporation: 1.0 per cent to 5.0 per cent.

Evaporate 5.0 g to dryness on a water-bath and dry at 100-105 °C for 4 h.

STORAGE

In a well-filled, airtight container, protected from light, at a temperature not exceeding 25 °C.

LABELLING

The label states the name and concentration of any added antioxidant.

01/2005:0438

TALC**Talcum****DEFINITION**

Talc is a powdered, selected, natural, hydrated magnesium silicate. Pure talc has the formula $[\text{Mg}_3\text{Si}_4\text{O}_{10}(\text{OH})_2]$; M_r 379.3]. It may contain variable amounts of associated minerals among which chlorites (hydrated aluminium and magnesium silicates), magnesite (magnesium carbonate), calcite (calcium carbonate) and dolomite (calcium and magnesium carbonate) are predominant.

PRODUCTION

Talc derived from deposits that are known to contain associated asbestos is not suitable for pharmaceutical use. The manufacturer is responsible for demonstrating by the test for amphiboles and serpentines that the product is free from asbestos. The presence of amphiboles and of serpentines is revealed by X-ray diffraction or by infrared spectrophotometry (see A and B). If detected, the specific morphological criteria of asbestos are investigated by a suitable method of optical microscopy to determine whether tremolite asbestos or chrysotile is present, as described below.

A. Examine by infrared spectrophotometry (2.2.24). In the range 740 cm^{-1} to 760 cm^{-1} using scale expansion, any absorption band at $758 \pm 1\text{ cm}^{-1}$ may indicate the presence of tremolite or of chlorite. If the absorption band remains after ignition of the substance at $850\text{ }^\circ\text{C}$ for at least 30 min, it indicates the presence of the tremolite. In the range 600 cm^{-1} to 650 cm^{-1} using scale expansion, any absorption band or shoulder may indicate the presence of serpentines. Examine the substance prepared as discs using *potassium bromide R*.

B. Examine by X-ray diffraction employing the following conditions:

- Cu K α monochromatic 40 kV radiation, 24 mA to 30 mA,
- incident slit: 1° ,
- detection slit: 0.2° ,
- goniometer speed: $1/10^\circ$ 20/min,
- scanning range: 10° to 13° 2 θ and 24° to 26° 2 θ ,
- the sample is not oriented.

Place the sample on the sample holder; pack and smooth its surface with a polished glass microscope slide.

Record the diffractograms.

The presence of amphiboles is detected by a diffraction peak at $10.5 \pm 0.1^\circ$ 2 θ , the presence of serpentines is detected by diffraction peaks at $24.3 \pm 0.1^\circ$ 2 θ and at $12.1 \pm 0.1^\circ$ 2 θ .

If, by one of the 2 methods, amphiboles and/or serpentine are detected, examine by a suitable method of optical microscopy to determine the asbestos character.

Examined by optical microscopy, the presence of asbestos is shown if the following criteria are met:

- a range of length to width ratios of 20:1 to 100:1, or higher for fibres longer than 5 μm ,
 - capability of splitting into very thin fibrils,
- and if 2 or more of the following 4 criteria are met:
- parallel fibres occurring in bundles,

- fibre bundles displaying frayed ends,
- fibres in the form of thin needles,
- matted masses of individual fibres and/or fibres showing curvature.

CHARACTERS

A light, homogeneous, white or almost white powder, greasy to the touch (non abrasive), practically insoluble in water, in alcohol and in dilute solutions of acids and alkali hydroxides.

IDENTIFICATION

First identification: A.

Second identification: B, C.

- A. Examine by infrared absorption spectrophotometry (2.2.24). The spectrum shows absorption bands at $3677 \pm 2\text{ cm}^{-1}$, at $1018 \pm 2\text{ cm}^{-1}$ and at $669 \pm 2\text{ cm}^{-1}$. Examine the substance as discs prepared using *potassium bromide R*.
- B. In a platinum crucible, melt a mixture of 0.2 g of *anhydrous sodium carbonate R* and 2.0 g of *potassium carbonate R*. To the melted mass add 0.1 g of the substance to be examined and heat until the mixture is completely melted. Allow to cool and transfer the melted mass into an evaporating dish with 50 ml of hot *water R*. Add *hydrochloric acid R* until effervescence ceases. Add 10 ml of *hydrochloric acid R* and evaporate to dryness on a water-bath. Allow to cool. Add 20 ml of *water R*, heat to boiling and filter. (The residue is used for identification test C). To 5 ml of the filtrate add 1 ml of *ammonia R* and 1 ml of *ammonium chloride solution R* and filter. To the filtrate add 1 ml of *disodium hydrogen phosphate solution R*. A white, crystalline precipitate is formed.
- C. The residue obtained in identification test B gives the reaction of silicates (2.3.1).

TESTS

Solution S1. Weigh 10.0 g of the substance to be examined into a conical flask fitted with a reflux condenser, add 50 ml of 0.5 M *hydrochloric acid* gradually while stirring and heat on a water-bath for 30 min. Allow to cool. Transfer the mixture to a beaker and allow the undissolved material to settle. Filter the supernatant through medium-speed filter paper into a 100 ml volumetric flask, retaining as much as possible of the insoluble material in the beaker. Wash the residue and the beaker with 3 quantities, each of 10 ml, of hot *water R*. Wash the filter with 15 ml of hot *water R*, allow the filtrate to cool and dilute to 100.0 ml with the same solvent.

Solution S2. Weigh 0.5 g of the substance to be examined in a 100 ml polytetrafluoroethylene dish, add 5 ml of *hydrochloric acid R*, 5 ml of *lead-free nitric acid R* and 5 ml of *perchloric acid R*. Stir gently then add 35 ml of *hydrofluoric acid R* and evaporate slowly to dryness on a hot plate. To the residue, add 5 ml of *hydrochloric acid R*, cover with a watch-glass, heat to boiling and allow to cool. Rinse the watch-glass and the dish with *water R*. Transfer into a volumetric flask, rinse the dish with *water R* and dilute to 50.0 ml with the same solvent.

pH (2.2.3). The pH of the filtrate obtained in the test for water-soluble substances is 7.0 to 9.0. Read the pH 1 min after inserting the electrode.

Water-soluble substances. To 10.0 g add 50 ml of *carbon dioxide-free water R*, heat to boiling and maintain boiling under a reflux condenser for 30 min. Allow to cool, filter through a medium-speed filter paper and dilute to 50.0 ml with *carbon dioxide-free water R*. Take 25.0 ml of the filtrate, evaporate to dryness and heat at $105\text{ }^\circ\text{C}$ for 1 h. The residue weighs not more than 10 mg (0.2 per cent).

Aluminium. Not more than 2.0 per cent of Al, determined by atomic absorption spectrometry (2.2.23, *Method I*).

Test solution. To 5.0 ml of solution S2 add 10 ml of a 25.34 g/l solution of *caesium chloride R*, 10.0 ml of *hydrochloric acid R* and dilute to 100.0 ml with *water R*.

Reference solutions. Into 4 identical volumetric flasks, each containing 10.0 ml of *hydrochloric acid R* and 10 ml of a 25.34 g/l solution of *caesium chloride R*, introduce respectively 5.0 ml, 10.0 ml, 15.0 ml and 20.0 ml of *aluminium standard solution (100 ppm Al) R* and dilute to 100.0 ml with *water R*.

Measure the absorbance at 309.3 nm, using an aluminium hollow-cathode lamp as the radiation source and a nitrous oxide-acetylene flame.

Calcium. Not more than 0.9 per cent of Ca, determined by atomic absorption spectrometry (2.2.23, *Method I*).

Test solution. To 5.0 ml of solution S2 add 10.0 ml of *hydrochloric acid R*, 10 ml of *lanthanum chloride solution R* and dilute to 100.0 ml with *water R*.

Reference solutions. Into 4 identical volumetric flasks, each containing 10.0 ml of *hydrochloric acid R* and 10 ml of *lanthanum chloride solution R*, introduce respectively 1.0 ml, 2.0 ml, 3.0 ml and 4.0 ml of *calcium standard solution (100 ppm Ca) R1* and dilute to 100.0 ml with *water R*.

Measure the absorbance at 422.7 nm using a calcium hollow-cathode lamp as the radiation source and a nitrous oxide-acetylene flame.

Iron. Not more than 0.25 per cent of Fe, determined by atomic absorption spectrometry (2.2.23, *Method I*).

Test solution. To 2.5 ml of solution S1, add 50.0 ml of 0.5 M *hydrochloric acid* and dilute to 100.0 ml with *water R*.

Reference solutions. Into 4 identical volumetric flasks, each containing 50.0 ml of 0.5 M *hydrochloric acid*, introduce respectively 2.0 ml, 2.5 ml, 3.0 ml and 4.0 ml of *iron standard solution (250 ppm Fe) R* and dilute to 100.0 ml with *water R*.

Measure the absorbance at 248.3 nm using an iron hollow-cathode lamp as the radiation source and an air-acetylene flame. Make a correction using a deuterium lamp.

Lead. Not more than 10 ppm of Pb, determined by atomic absorption spectrometry (2.2.23, *Method I*).

Test solution. Use solution S1.

Reference solutions. Into 4 identical volumetric flasks, each containing 50.0 ml of 0.5 M *hydrochloric acid*, introduce respectively 5.0 ml, 7.5 ml, 10.0 ml and 12.5 ml of *lead standard solution (10 ppm Pb) R1* and dilute to 100.0 ml with *water R*.

Measure the absorbance at 217.0 nm using a lead hollow-cathode lamp as the radiation source and an air-acetylene flame.

Magnesium. 17.0 per cent to 19.5 per cent of Mg, determined by atomic absorption spectrometry (2.2.23, *Method I*).

Test solution. Dilute 0.5 ml of solution S2 to 100.0 ml with *water R*. To 4.0 ml of the solution, add 10.0 ml of *hydrochloric acid R*, 10 ml of *lanthanum chloride solution R* and dilute to 100.0 ml with *water R*.

Reference solutions. Into 4 identical volumetric flasks, each containing 10.0 ml of *hydrochloric acid R* and 10 ml of *lanthanum chloride solution R*, introduce respectively 2.5 ml, 3.0 ml, 4.0 ml and 5.0 ml of *magnesium standard solution (10 ppm Mg) R1* and dilute to 100.0 ml with *water R*.

Measure the absorbance at 285.2 nm using a magnesium hollow-cathode lamp as the radiation source and an air-acetylene flame.

Loss on ignition. Not more than 7.0 per cent, determined on 1.00 g by ignition to constant weight at 1050-1100 °C.

Microbial contamination. If intended for topical administration, the total viable aerobic count (2.6.12) is not more than a total of 10² aerobic bacteria and fungi per gram. If intended for oral administration, the total viable aerobic count (2.6.12) is not more than a total of 10³ aerobic bacteria and not more than 10² fungi per gram.

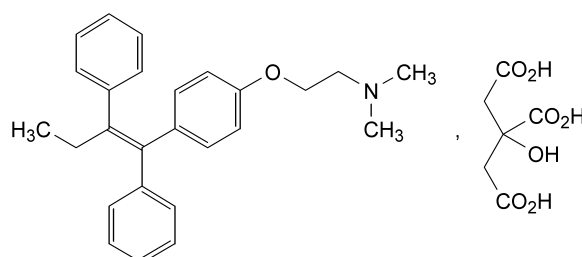
LABELLING

The label states, where applicable, that the substance is suitable for oral or topical administration.

01/2005:1046
corrected

TAMOXIFEN CITRATE

Tamoxifeni citras



C₃₂H₃₇NO₈

M_r 563.6

DEFINITION

Tamoxifen citrate contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of 2-[4-[(Z)-1,2-diphenylbut-1-enyl]phenoxy]-N,N-dimethylethanamine dihydrogen 2-hydroxypropane-1,2,3-tricarboxylate, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, crystalline powder, slightly soluble in water, soluble in methanol, slightly soluble in acetone.

It shows polymorphism.

IDENTIFICATION

First identification: B.

Second identification: A, C, D.

- Dissolve 20 mg in *methanol R* and dilute to 50.0 ml with the same solvent. Dilute 5.0 ml of the solution to 100.0 ml with *methanol R*. Examined between 220 nm and 350 nm (2.2.25), the solution shows two absorption maxima, at 237 nm and 275 nm. The ratio of the absorbance measured at the maximum at 237 nm to that measured at the maximum at 275 nm is 1.45 to 1.65.
- Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *tamoxifen citrate CRS*. Examine the substances prepared as discs. If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in *acetone R*, evaporate to dryness and record new spectra using the residues.
- Examine by thin-layer chromatography (2.2.27), using as the coating substance a suitable silica gel with a fluorescent indicator having an optimal intensity at 254 nm.

Test solution. Dissolve 10 mg of the substance to be examined in *methanol R* and dilute to 10 ml with the same solvent.

Reference solution (a). Dissolve 10 mg of *tamoxifen citrate CRS* in *methanol R* and dilute to 10 ml with the same solvent.

Reference solution (b). Dissolve 10 mg of *tamoxifen citrate CRS* and 10 mg of *clomifene citrate CRS* in *methanol R* and dilute to 10 ml with the same solvent.

Apply separately to the plate 5 µl of each solution. Develop over a path of 15 cm using a mixture of 10 volumes of *triethylamine R* and 90 volumes of *toluene R*. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. The principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a). The test is not valid unless the chromatogram obtained with reference solution (b) shows two clearly separated spots.

- D. Dissolve about 10 mg in 4 ml of *pyridine R* with shaking and add 2 ml of *acetic anhydride R*. A yellow colour is produced. Heat on a water-bath for 2 min. A pink to red colour is produced.

TESTS

***E*-isomer and related substances.** Examine by liquid chromatography (2.2.29). Prepare the solutions immediately before use and protect from light.

Test solution. Dissolve 15.0 mg of the substance to be examined in the mobile phase and dilute to 10.0 ml with the mobile phase.

Reference solution (a). Dissolve 15.0 mg of *tamoxifen citrate for performance test CRS* in the mobile phase and dilute to 10.0 ml with the mobile phase.

Reference solution (b). Dilute 1.0 ml of the test solution to 100.0 ml with the mobile phase.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4.6 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (5 µm),
- as mobile phase at a flow rate of 1.2 ml/min a mixture of 40 volumes of *acetonitrile R* and 60 volumes of *water R* containing 0.9 g/l of *sodium dihydrogen phosphate R* and 4.8 g/l of *N,N*-dimethyloctylamine *R*, adjusted to pH 3.0 with *phosphoric acid R*,
- as detector a spectrophotometer set at 240 nm.

Equilibrate the column with the mobile phase at a flow rate of 1.2 ml/min for about 30 min.

Adjust the sensitivity of the system so that the height of the peak due to the *E*-isomer in the chromatogram obtained with 10 µl of reference solution (a) is not less than 40 per cent of the full scale of the recorder. The test is not valid unless the chromatogram obtained resembles that of the specimen chromatogram provided with the *tamoxifen citrate for performance test CRS* in that the resolution between the peaks due to the *E*-isomer and to tamoxifen impurity F is at least three and there is base-line separation of the peak due to tamoxifen impurity F from the following peak of the principal component.

Inject separately 10 µl of the test solution and 10 µl of reference solution (b). Continue the chromatography for twice the retention time of the tamoxifen peak. In the chromatogram obtained with the test solution: the area of any peak, apart from the principal peak, is not greater than 0.3 times that of the principal peak in the chromatogram obtained with reference solution (b) (0.3 per cent); the sum of the areas of all the peaks, apart from the principal peak and any peak due to the *E*-isomer, is not greater than 0.5 times the area of the principal peak in the chromatogram of reference solution (b) (0.5 per cent). Disregard any peak

due to the citrate (retention time = about 2.5 min) and any peak with an area less than 0.05 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying *in vacuo* at 65 °C for 4 h.

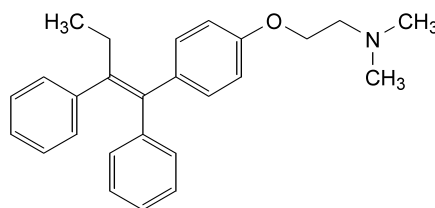
Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

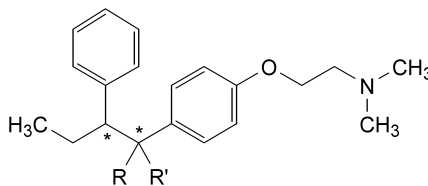
Dissolve 0.400 g in 75 ml of *anhydrous acetic acid R*. Titrate with 0.1 M *perchloric acid* using 0.1 ml of *naphtholbenzein solution R* as indicator.

1 ml of 0.1 M *perchloric acid* is equivalent to 56.36 mg of C₃₂H₃₇NO₈.

IMPURITIES

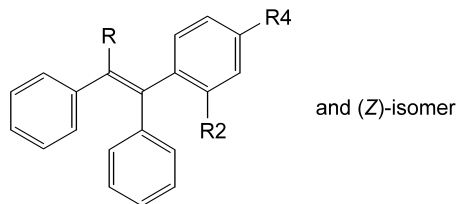


- A. 2-[4-[(*E*)-1,2-diphenylbut-1-enyl]phenoxy]-*N,N*-dimethylethanamine,



- B. R = OH, R' = C₆H₅: 1-[4-[2-(dimethylamino)ethoxy]phenyl]-1,2-diphenylbutan-1-ol,

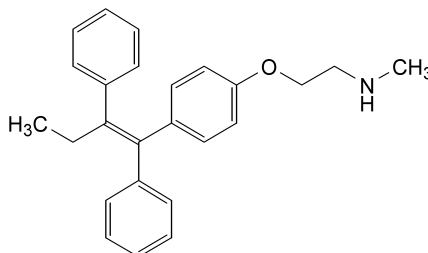
- G. R + R' = O: (2*RS*)-1-[4-[2-(dimethylamino)ethoxy]phenyl]-2-phenylbutan-1-one,



- C. R = R₂ = H, R₄ = O-CH₂-CH₂-N(CH₃)₂: 2-[4-[(*EZ*)-1,2-diphenylethenyl]phenoxy]-*N,N*-dimethylethanamine,

- D. R = CH₃, R₂ = H, R₄ = O-CH₂-CH₂-N(CH₃)₂: 2-[4-[(*EZ*)-1,2-diphenylprop-1-enyl]phenoxy]-*N,N*-dimethylethanamine,

- E. R = C₂H₅, R₂ = O-CH₂-CH₂-N(CH₃)₂, R₄ = H: 2-[2-[(*EZ*)-1,2-diphenylbut-1-enyl]phenoxy]-*N,N*-dimethylethanamine,



- F. 2-[4-[(*Z*)-1,2-diphenylbut-1-enyl]phenoxy]-*N,N*-methylethanamine.

01/2005:1477 IDENTIFICATION

TANNIC ACID**Tanninum****DEFINITION**

Tannic acid is a mixture of esters of glucose with gallic acid and 3-galloylgallic acid.

CHARACTERS

A yellowish-white or slightly brown amorphous light powder or shiny plates, very soluble in water, freely soluble in acetone, in alcohol and in glycerol (85 per cent), practically insoluble in methylene chloride.

IDENTIFICATION

- A. Dilute 0.1 ml of solution S (see Tests) to 5 ml with *water R*. Add 0.1 ml of *ferric chloride solution R1*. A blackish-blue colour is produced which becomes green on the addition of 1 ml of *dilute sulphuric acid R*.
- B. To 1 ml of solution S, add 3 ml of a 1 g/l solution of *gelatin R*. The mixture becomes turbid and a flocculent precipitate is formed.
- C. Dilute 0.1 ml of solution S to 5 ml with *water R*. Add 0.3 ml of *barium hydroxide solution R*. A greenish-blue precipitate is formed.

TESTS

Solution S. Dissolve 4.0 g in *carbon dioxide-free water R* and dilute to 20 ml with the same solvent.

Appearance of solution. Solution S is not more opalescent than reference suspension II (2.2.1).

Dextrins, gum, salts, sugars. To 2 ml of solution S, add 2 ml of *alcohol R*. The solution is clear. Add 1 ml of *ether R*. The solution remains clear for at least 10 min.

Resins. To 5 ml of solution S, add 5 ml of *water R*. The mixture remains clear for at least 15 min (2.2.1).

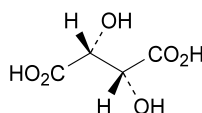
Loss on drying (2.2.32). Not more than 12.0 per cent, determined on 0.200 g by drying at 100 °C to 105 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

STORAGE

Store protected from light.

01/2005:0460

TARTARIC ACID**Acidum tartaricum**C₄H₆O₆M_r 150.1**DEFINITION**

Tartaric acid contains not less than 99.5 per cent and not more than the equivalent of 101.0 per cent of (2R,3R)-2,3-dihydroxybutanedioic acid, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, crystalline powder or colourless crystals, very soluble in water, freely soluble in alcohol.

IDENTIFICATION

- A. Solution S (see Tests) is strongly acid (2.2.4).
- B. It gives the reactions of tartrates (2.3.1).

TESTS

Solution S. Dissolve 5.0 g in *distilled water R* and dilute to 50 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and not more intensely coloured than reference solution Y₆ (2.2.2, *Method II*).

Specific optical rotation (2.2.7). Dissolve 5.00 g in *water R* and dilute to 25.0 ml with the same solvent. The specific optical rotation is + 12.0 to + 12.8.

Oxalic acid. Dissolve 0.80 g in 4 ml of *water R*. Add 3 ml of *hydrochloric acid R* and 1 g of *zinc R* in granules and boil for 1 min. Allow to stand for 2 min. Collect the liquid in a test-tube containing 0.25 ml of a 10 g/l solution of *phenylhydrazine hydrochloride R* and heat to boiling. Cool rapidly, transfer to a graduated cylinder and add an equal volume of *hydrochloric acid R* and 0.25 ml of a 50 g/l solution of *potassium ferricyanide R*. Shake and allow to stand for 30 min. Any pink colour in the solution is not more intense than that in a standard prepared at the same time in the same manner using 4 ml of a 0.1 g/l solution of *oxalic acid R* (350 ppm calculated as anhydrous oxalic acid).

Chlorides (2.4.4). 5 ml of solution S diluted to 15 ml with *water R* complies with the limit test for chlorides (100 ppm).

Sulphates (2.4.13). 10 ml of solution S diluted to 15 ml with *distilled water R* complies with the limit test for sulphates (150 ppm).

Calcium (2.4.3). To 5 ml of solution S add 10 ml of a 50 g/l solution of *sodium acetate R* in *distilled water R*. The solution complies with the limit test for calcium (200 ppm).

Heavy metals (2.4.8). 2.0 g complies with limit test C for heavy metals (10 ppm). Prepare the standard using 2 ml of *lead standard solution (10 ppm Pb) R*.

Loss on drying (2.2.32). Not more than 0.2 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.650 g in 25 ml of *water R*. Titrate with 1 M *sodium hydroxide* using 0.5 ml of *phenolphthalein solution R* as indicator, until a pink colour is obtained.

1 ml of 1 M *sodium hydroxide* is equivalent to 75.05 mg of C₄H₆O₆.

01/2005:1837

TEA TREE OIL**Melaleuca aetheroleum****DEFINITION**

Essential oil obtained by steam distillation from the foliage and terminal branchlets of *Melaleuca alternifolia* (Maiden and Betch) Cheel, *M. linariifolia* Smith, *M. dissitiflora* F. Mueller and/or other species of *Melaleuca*.

CHARACTERS

Appearance: clear, mobile, colourless to pale yellow liquid with a characteristic odour.

IDENTIFICATION

First identification: B.

Second identification: A.

A. Thin-layer chromatography (2.2.27).

Test solution. Dissolve 0.1 ml of the substance to be examined in 5 ml of *heptane R*.

Reference solution. Dissolve 30 µl of *cineole R*, 60 µl of *terpinen-4-ol R* and 10 mg of *α-terpineol R* in 10 ml of *heptane R*.

Plate: TLC silica gel plate *R*.

Mobile phase: *ethyl acetate R*, *heptane R* (20:80 V/V).

Application: 10 µl, as bands.

Development: over a path of 10 cm.

Drying: in air.

Detection: spray with *anisaldehyde solution R*. Heat at 100-105 °C for 5-10 min while observing. Examine in daylight.

Results: see below the sequence of the zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other zones are present in the chromatogram obtained with the test solution.

Top of the plate	
Cineole: a violet-brown zone	A violet-brown zone, less intense (cineole)
Terpinen-4-ol: a brownish-violet zone	A brownish-violet zone (terpinen-4-ol)
α-terpineol: a violet or brownish-violet zone	A violet or brownish-violet zone (α-terpineol)
Reference solution	Test solution

B. Examine the chromatograms obtained in the test for chromatographic profile.

Results: the characteristic peaks in the chromatogram obtained with the test solution are similar in retention time to those in the chromatogram obtained with the reference solution.

TESTS

Relative density (2.2.5): 0.885 to 0.906.

Refractive index (2.2.6): 1.475 to 1.482.

Optical rotation (2.2.7): + 5° to + 15°.

Chromatographic profile. Gas chromatography (2.2.28): use the normalisation procedure.

Test solution. Dissolve 0.15 ml of the substance to be examined in 10 ml of *hexane R*.

Reference solution. Dissolve 5 µl of *α-pinene R*, 5 µl of *sabinene R*, 15 µl of *α-terpinene R*, 5 µl of *limonene R*, 5 µl of *cineole R*, 30 µl of *γ-terpinene R*, 5 µl of *p-cymene R*, 5 µl of *terpinolene R*, 60 µl of *terpinen-4-ol R*, 5 µl of *aromadendrene R* and 5 mg of *α-terpineol R* in 10 ml of *hexane R*.

Column:

- **material:** fused silica,
- **size:** *l* = 30 m (a film thickness of 1 µm may be used) to 60 m (a film thickness of 0.2 µm may be used), Ø = 0.25-0.53 mm,
- **stationary phase:** *macrogol 20 000 R*.

Carrier gas: *helium for chromatography R*.

Flow rate: 1.3 ml/min.

Split ratio: 1:50.

Temperature:

	Time (min)	Temperature (°C)
Column	0 - 1	50
	1 - 37	50 → 230
	37 - 45	230
Injection port		240
Detector		240

Detection: flame ionisation.

Injection: 1 µl.

Elution order: order indicated in the composition of the reference solution. Record the retention times of these substances.

System suitability: reference solution:

- **resolution:** minimum 2.7 between the peaks due to terpinen-4-ol and aromadendrene.

Using the retention times determined from the chromatogram obtained with the reference solution, locate the components of the reference solution in the chromatogram obtained with the test solution. Disregard the peak due to hexane.

Determine the percentage content of these components. The percentages are within the following ranges:

- *α-pinene*: 1.0 per cent to 6.0 per cent,
- *sabinene*: less than 3.5 per cent,
- *α-terpinene*: 5.0 per cent to 13.0 per cent,
- *limonene*: 0.5 per cent to 4.0 per cent,
- *cineole*: less than 15.0 per cent,
- *γ-terpinene*: 10.0 per cent to 28.0 per cent,
- *p-cymene*: 0.5 per cent to 12.0 per cent,
- *terpinolene*: 1.5 per cent to 5.0 per cent,
- *terpinen-4-ol*: minimum 30.0 per cent,
- *aromadendrene*: less than 7.0 per cent,
- *α-terpineol*: 1.5 per cent to 8.0 per cent.

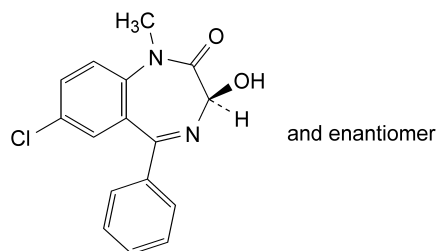
STORAGE

In an airtight, well-filled container, protected from light, at a temperature not exceeding 25 °C.

01/2005:0954

TEMAZEPAM

Temazepamum



C₁₆H₁₃ClN₂O₂

*M*_r 300.7

DEFINITION

(3*RS*)-7-Chloro-3-hydroxy-1-methyl-5-phenyl-1,3-dihydro-2*H*-1,4-benzodiazepin-2-one.

Content: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

Appearance: white or almost white, crystalline powder.

Solubility: practically insoluble in water, freely soluble in methylene chloride, sparingly soluble in alcohol.

IDENTIFICATION

Infrared absorption spectrophotometry (2.2.24).

Comparison: *Ph. Eur. reference spectrum of temazepam.*

TESTS

Impurity A: maximum 0.05 per cent.

Dissolve 0.400 g in *methylene chloride R* and dilute to 20.0 ml with the same solvent. The absorbance (2.2.25) is maximum 0.30 at 409 nm.

Related substances. Liquid chromatography (2.2.29).

Test solution. Dissolve 10.0 mg of the substance to be examined in a mixture of 1 volume of *water R* and 9 volumes of *methanol R* and dilute to 50.0 ml with the same mixture of solvents.

Reference solution (a). Dilute 1.0 ml of the test solution to 100.0 ml with a mixture of 1 volume of *water R* and 9 volumes of *methanol R*. Dilute 2.0 ml of the solution to 10.0 ml with a mixture of 1 volume of *water R* and 9 volumes of *methanol R*.

Reference solution (b). Dissolve 1 mg of *oxazepam R*, 1 mg of *temazepam impurity F CRS* and 1 mg of *temazepam impurity G CRS* in a mixture of 1 volume of *water R* and 9 volumes of *methanol R* and dilute to 25 ml with the same mixture of solvents.

Reference solution (c). Dissolve 1 mg of *temazepam impurity C CRS* and 1 mg of *temazepam impurity D CRS* with a mixture of 1 volume of *water R* and 9 volumes of *methanol R* and dilute to 25 ml with the same mixture of solvents.

Column:

- **size:** $l = 0.15$ m, $\varnothing = 4.6$ mm,
- **stationary phase:** *end-capped octadecylsilyl silica gel for chromatography R* (3.5 μ m).

Mobile phase:

- **mobile phase A:** solution containing 4.9 g/l of *sodium dihydrogen phosphate R* and 0.63 g/l of *disodium hydrogen phosphate R* (pH 5.6),
- **mobile phase B:** *methanol R*,
- **mobile phase C:** *acetonitrile R*,

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)	Mobile phase C (per cent V/V)
0 - 18	54	39	7
18 - 25	54 → 22	39 → 63	7 → 15
25 - 31	22	63	15
31 - 37	22 → 54	63 → 39	15 → 7

Flow rate: 1.5 ml/min.

Detection: spectrophotometer at 230 nm.

Injection: 20 μ l.

Relative retention with reference to temazepam (retention time = about 16 min): impurity E = about 0.55; impurity F = about 0.67; impurity G = about 0.73; impurity B = about 0.8; impurity D = about 1.2; impurity C = about 1.3; impurity A = about 1.5.

System suitability: reference solution (b):

- **resolution:** minimum 1.5 between the peaks due to impurity F and impurity G,

- **peak-to-valley ratio:** minimum 1.7, where H_p = height above the baseline of the peak due to impurity G and H_v = height above the baseline of the lowest point of the curve separating this peak from the peak due to impurity B.

Limits:

- **correction factors:** for the calculation of contents, multiply the peak areas of the following impurities by the corresponding correction factor: impurity F = 3.2; impurity G = 3.1;
- **impurities B, C, D, E, F, G:** for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent);
- **any other impurity:** for each impurity, not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent);
- **total:** not more than 2.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent);
- **disregard limit:** 0.25 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

Loss on drying (2.2.32): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 100-105 °C for 4 h.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.250 g in 50 ml of *nitroethane R*. Titrate with 0.1 M *perchloric acid*, determining the end-point potentiometrically (2.2.20).

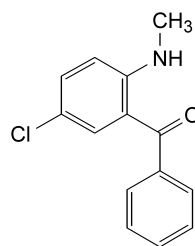
1 ml of 0.1 M *perchloric acid* is equivalent to 30.07 mg of $C_{16}H_{13}ClN_2O_2$.

STORAGE

Protected from light.

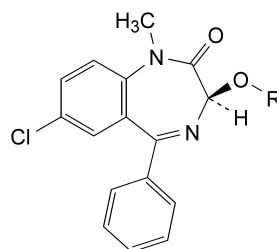
IMPURITIES

Specified impurities: A, B, C, D, E, F, G.



A. [5-chloro-2-(methylamino)phenyl]phenylmethanone,

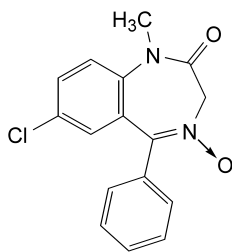
B. oxazepam,



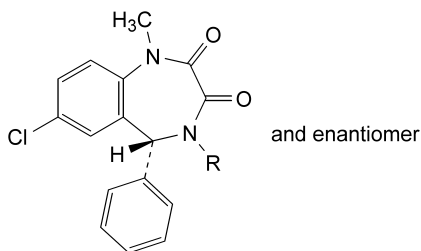
and enantiomer

C. R = CO-CH₃: (3*RS*)-7-chloro-1-methyl-2-oxo-5-phenyl-2,3-dihydro-1*H*-1,4-benzodiazepin-3-yl acetate,

D. R = CH₃: (3*RS*)-7-chloro-3-methoxy-1-methyl-5-phenyl-1,3-dihydro-2*H*-1,4-benzodiazepin-2-one,



E. 7-chloro-1-methyl-5-phenyl-1,3-dihydro-2H-1,4-benzodiazepin-2-one 4-oxide,



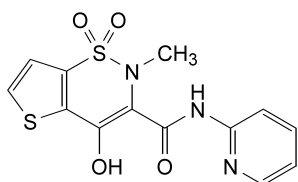
F. R = H: (5*RS*)-7-chloro-1-methyl-5-phenyl-4,5-dihydro-1*H*-1,4-benzodiazepine-2,3-dione,

G. R = CH₃: (5*RS*)-7-chloro-1,4-dimethyl-5-phenyl-4,5-dihydro-1*H*-1,4-benzodiazepine-2,3-dione.

01/2005:1156

TENOXICAM

Tenoxicamum



C₁₃H₁₁N₃O₄S₂

*M*_r 337.4

DEFINITION

Tenoxicam contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of 4-hydroxy-2-methyl-*N*-(pyridin-2-yl)-2*H*-thieno[2,3-*e*]1,2-thiazine-3-carboxamide 1,1-dioxide, calculated with reference to the anhydrous substance.

CHARACTERS

A yellow, crystalline powder, practically insoluble in water, sparingly soluble in methylene chloride, very slightly soluble in ethanol. It dissolves in solutions of acids and alkalis.

It shows polymorphism.

IDENTIFICATION

Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *tenoxicam CRS*. If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in the minimum quantity of *methylene chloride R*, evaporate to dryness on a water-bath and record new spectra using the residues.

TESTS

Appearance of solution. Dissolve 0.10 g in 20 ml of *methylene chloride R*. The solution is clear (2.2.1).

Related substances. Examine by thin-layer chromatography (2.2.27), using *silica gel GF₂₅₄ R* as the coating substance.

Test solution. Dissolve 0.4 g in a mixture of 4 volumes of *concentrated ammonia R* and 96 volumes of *methanol R* and dilute to 5 ml with the same mixture of solvents.

Reference solution (a). Dilute 1 ml of the test solution to 20 ml with a mixture of 4 volumes of *concentrated ammonia R* and 96 volumes of *methanol R*. Dilute 1 ml of the solution to 20 ml with the same mixture of solvents.

Reference solution (b). Dissolve 20 mg of *tenoxicam CRS* and 20 mg of *salicylic acid CRS* in a mixture of 4 volumes of *concentrated ammonia R* and 96 volumes of *methanol R* and dilute to 5 ml with the same mixture of solvents.

Reference solution (c). Dissolve 20 mg of *pyridin-2-amine R* in a mixture of 4 volumes of *concentrated ammonia R* and 96 volumes of *methanol R* and dilute to 5 ml with the same mixture of solvents. Dilute 2 ml of the solution to 50 ml with the same mixture of solvents.

Apply separately to the plate 10 µl of each solution. Develop over a path of 15 cm with a mixture of 5 volumes of *anhydrous formic acid R*, 5 volumes of *methanol R*, 20 volumes of *acetone R* and 70 volumes of *methylene chloride R*. Allow the plate to dry in air. Examine in ultraviolet light at 254 nm. Any spot corresponding to pyridin-2-amine in the chromatogram obtained with the test solution is not more intense than the spot in the chromatogram obtained with reference solution (c) (0.2 per cent). Any spot in the chromatogram obtained with the test solution apart from the principal spot and any spot corresponding to pyridin-2-amine, is not more intense than the spot in the chromatogram obtained with reference solution (a) (0.25 per cent). The test is not valid unless the chromatogram obtained with reference solution (b) shows two clearly separated spots.

Heavy metals (2.4.8). 0.5 g complies with limit test C for heavy metals (20 ppm). Prepare the standard using 5 ml of *lead standard solution (2 ppm Pb) R*.

Water (2.5.12). Not more than 0.5 per cent, determined on 1.000 g by the semi-micro determination of water.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

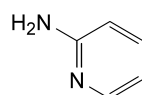
Dissolve 0.250 g in 5 ml of *anhydrous formic acid R*. Add 70 ml of *anhydrous acetic acid R*. Titrate with 0.1 *M perchloric acid*, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 *M perchloric acid* is equivalent to 33.74 mg of C₁₃H₁₁N₃O₄S₂.

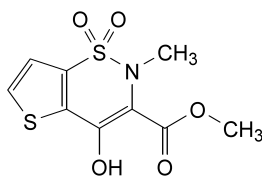
STORAGE

Store protected from light.

IMPURITIES



A. pyridin-2-amine,

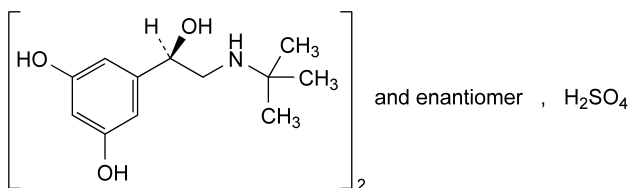


B. methyl 4-hydroxy-2-methyl-2*H*-thieno[2,3-*e*]1,2-thiazine-3-carboxylate 1,1-dioxide.

01/2005:0690

TERBUTALINE SULPHATE

Terbutalini sulfas



$C_{24}H_{40}N_2O_{10}S$

M_r 548.7

DEFINITION

Terbutaline sulphate contains not less than 98.0 per cent and not more than the equivalent of 101.0 per cent of bis[(1*RS*)-1-(3,5-dihydroxyphenyl)-2-[(1,1-dimethylethyl)amino]ethanol] sulphate, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, crystalline powder, freely soluble in water, slightly soluble in alcohol.

It shows polymorphism.

IDENTIFICATION

- Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *terbutaline sulphate CRS*. If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in *aldehyde-free methanol R*, evaporate to dryness and record new spectra using the residues.
- 5 ml of solution S (see Tests) gives reaction (a) of sulphates (2.3.1).

TESTS

Solution S. Dissolve 1.0 g in *carbon dioxide-free water R* and dilute to 50 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1). The absorbance (2.2.25) of solution S measured at 400 nm in a 2 cm cell is not greater than 0.11.

Acidity. To 10 ml of solution S add 0.05 ml of *methyl red solution R*. Not more than 1.2 ml of 0.01 *M sodium hydroxide* is required to change the colour of the indicator to yellow.

Optical rotation (2.2.7). The angle of optical rotation, determined on solution S, is -0.10° to $+0.10^\circ$.

Related substances. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 75.0 mg of the substance to be examined in the mobile phase and dilute to 50.0 ml with the mobile phase.

Reference solution (a). Dissolve 7.5 mg of *terbutaline impurity C CRS* and 22.5 mg of *terbutaline sulphate CRS* in the mobile phase and dilute to 50.0 ml with the mobile phase. Dilute 1.0 ml of the solution to 100.0 ml with the mobile phase.

Reference solution (b). Dilute 1.0 ml of the test solution to 50.0 ml with the mobile phase. Dilute 2.0 ml of the solution to 20.0 ml with the mobile phase.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.15 m long and 4.6 mm in internal diameter packed with *base-deactivated octadecylsilyl silica gel for chromatography R* (5 μ m),
- as mobile phase at a flow rate of 1.0 ml/min a solution prepared as follows: dissolve 4.23 g of *sodium hexanesulphonate R* in 770 ml of 0.050 *M ammonium formate* solution prepared as follows: dissolve 3.15 g of *ammonium formate R* in about 980 ml of *water R*; adjust to pH 3.0 by adding about 8 ml of *anhydrous formic acid R* and dilute to 1000 ml with *water R*; then add 230 ml of *methanol R*,
- as detector a spectrophotometer set at 276 nm.

Adjust the sensitivity of the system so that the height of the principal peak in the chromatogram obtained with reference solution (b) is at least 50 per cent of the full scale of the recorder.

Inject 20 μ l of reference solution (a). When the chromatograms are recorded under the prescribed conditions, the retention times are: *impurity C* about 9 min and *terbutaline sulphate* about 11 min. The test is not valid unless the resolution between the 2 peaks corresponding to *terbutaline sulphate* and *impurity C* is at least 2.0. If necessary adjust the composition of the mobile phase. Decrease the content of *methanol* to increase the retention time.

Inject 20 μ l of the test solution, 20 μ l of reference solution (a) and 20 μ l of reference solution (b). Continue the chromatography for 6 times the retention time of *terbutaline sulphate*. In the chromatogram obtained with the test solution, the area of any peak corresponding to *impurity C* is not greater than twice that of the corresponding peak in the chromatogram obtained with reference solution (a) (0.2 per cent); the area of any other peak, apart from the principal peak, is not greater than that of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent); the sum of the areas of all the peaks, apart from the principal peak and the peak corresponding to *impurity C*, is not greater than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (0.4 per cent). Disregard any peak due to the mobile phase and any peak with an area less than 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (b).

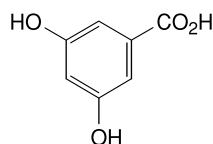
Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 100-105 $^\circ$ C for 3 h.

ASSAY

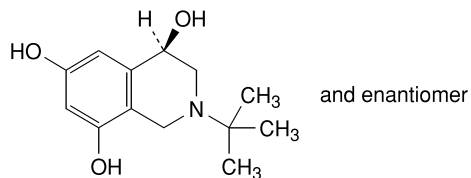
Dissolve 0.400 g in 70 ml of *anhydrous acetic acid R* with heating. Titrate with 0.1 *M perchloric acid*, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 *M perchloric acid* is equivalent to 54.87 mg of $C_{24}H_{40}N_2O_{10}S$.

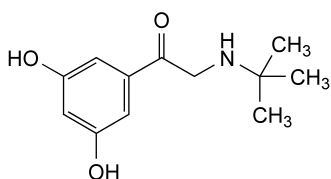
IMPURITIES



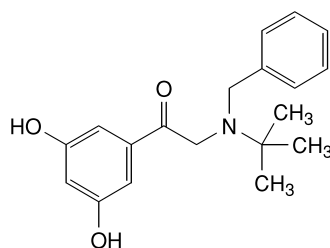
- A. 3,5-dihydroxybenzoic acid (α -resorcylic acid),



- B. (4*RS*)-2-(1,1-dimethylethyl)-1,2,3,4-tetrahydroisoquinoline-4,6,8-triol,



- C. 1-(3,5-dihydroxyphenyl)-2-[(1,1-dimethylethyl)amino]ethanone,

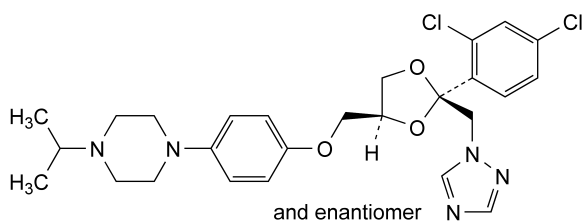


- D. 2-[benzyl-(1,1-dimethylethyl)amino]-1-(3,5-dihydroxyphenyl)ethanone.

01/2005:1270

TERCONAZOLE

Terconazolum

C₂₆H₃₁Cl₂N₅O₃M_r 532.5

DEFINITION

Terconazole contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of 1-[4-[(2*RS*,4*SR*)-2-(2,4-dichlorophenyl)-2-[(1*H*-1,2,4-triazol-1-yl)methyl]-1,3-dioxolan-4-yl]methoxy]phenyl]-4-(1-methylethyl)piperazine, calculated with reference to the dried substance.

CHARACTERS

A white or almost white powder, practically insoluble in water, freely soluble in methylene chloride, soluble in acetone, sparingly soluble in alcohol.

It shows polymorphism.

IDENTIFICATION

First identification: A.

Second identification: B, C.

- A. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *terconazole CRS*. If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in the minimum volume of *acetone R*, evaporate to dryness in a current of air and record new spectra using the residues.

- B. Examine by thin-layer chromatography (2.2.27), using as the coating substance a suitable octadecylsilyl silica gel.

Test solution. Dissolve 30 mg of the substance to be examined in *methanol R* and dilute to 5 ml with the same solvent.

Reference solution (a). Dissolve 30 mg of *terconazole CRS* in *methanol R* and dilute to 5 ml with the same solvent.

Reference solution (b). Dissolve 30 mg of *terconazole CRS* and 30 mg of *ketoconazole CRS* in *methanol R* and dilute to 5 ml with the same solvent.

Apply separately to the plate 5 μ l of each solution. Develop in an unsaturated tank over a path of 10 cm using a mixture of 20 volumes of *ammonium acetate solution R*, 40 volumes of *dioxan R* and 40 volumes of *methanol R*. Dry the plate in a current of warm air for 15 min and expose it to iodine vapour until the spots appear. Examine in daylight. The principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a). The test is not valid unless the chromatogram obtained with reference solution (b) shows two clearly separated spots.

- C. To 30 mg in a porcelain crucible add 0.3 g of *anhydrous sodium carbonate R*. Heat over an open flame for 10 min. Allow to cool. Take up the residue with 5 ml of *dilute nitric acid R* and filter. To 1 ml of the filtrate add 1 ml of *water R*. The solution gives reaction (a) of chlorides (2.3.1).

TESTS

Optical rotation (2.2.7). Dissolve 1.0 g in *methylene chloride R* and dilute to 10 ml with the same solvent. The angle of optical rotation is -0.10° to $+0.10^\circ$.

Related substances. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 0.100 g of the substance to be examined in *methanol R* and dilute to 10.0 ml with the same solvent.

Reference solution (a). Dissolve 2.5 mg of *terconazole CRS* and 2.0 mg of *ketoconazole CRS* in *methanol R* and dilute to 100.0 ml with the same solvent.

Reference solution (b). Dilute 1.0 ml of the test solution to 100.0 ml with *methanol R*. Dilute 5.0 ml of this solution to 20.0 ml with *methanol R*.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.1 m long and 4.6 mm in internal diameter packed with *base-deactivated octadecylsilyl silica gel for chromatography R* (3 μ m),
- as mobile phase at a flow rate of 2 ml/min:

Mobile phase A. A 3.4 g/l solution of *tetrabutylammonium hydrogen sulphate R*,

Mobile phase B. *Acetonitrile R*,

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)	Comment
0 - 10	95 → 50	5 → 50	linear gradient
10 - 15	50	50	isocratic elution
15 - 20	95	5	switch to initial eluent composition
20 = 0	95	5	restart gradient

— as detector a spectrophotometer set at 220 nm.

Equilibrate the column for at least 30 min with *acetonitrile R* at a flow rate of 2 ml/min and then equilibrate at the initial elution composition for at least 5 min.

Adjust the sensitivity of the system so that the height of the principal peak in the chromatogram obtained with 10 µl of reference solution (b) is at least 50 per cent of the full scale of the recorder.

Inject 10 µl of reference solution (a). When the chromatograms are recorded in the prescribed conditions, the retention times are: ketoconazole about 6 min and terconazole about 7.5 min. The test is not valid unless the resolution between the peaks corresponding to ketoconazole and terconazole is at least 13. If necessary, adjust the concentration of acetonitrile in the mobile phase or adjust the time programme for the linear gradient elution.

Inject separately 10 µl of *methanol R* as a blank, 10 µl of the test solution and 10 µl of reference solution (b). In the chromatogram obtained with the test solution: the area of any peak, apart from the principal peak, is not greater than that of the principal peak in the chromatogram obtained with reference solution (b) (0.25 per cent); the sum of the areas of all the peaks, apart from the principal peak, is not greater than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent). Disregard any peak obtained with the blank run and any peak with an area less than 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (b).

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 100-105 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

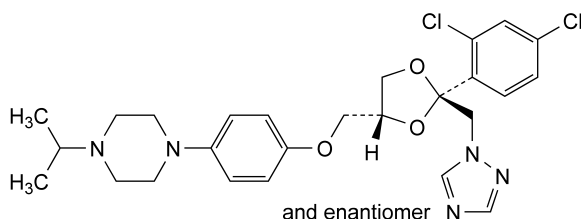
Dissolve 0.150 g in 70 ml of a mixture of 1 volume of *anhydrous acetic acid R* and 7 volumes of *methyl ethyl ketone R*. Titrate with 0.1 M *perchloric acid* determining the end-point potentiometrically at the second point of inflexion (2.2.20).

1 ml of 0.1 M *perchloric acid* is equivalent to 17.75 mg of C₂₆H₃₁Cl₂N₅O₃.

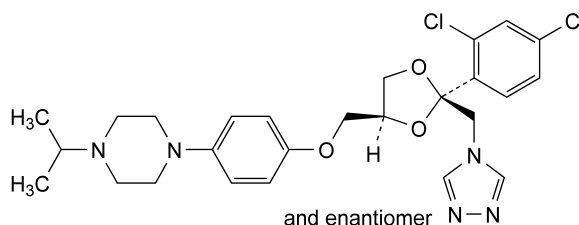
STORAGE

Store protected from light.

IMPURITIES



- A. 1-[4-[(2*RS*,4*RS*)-2-(2,4-dichlorophenyl)-2-[(1*H*-1,2,4-triazol-1-yl)methyl]-1,3-dioxolan-4-yl]methoxy]phenyl]-4-(1-methylethyl)piperazine,

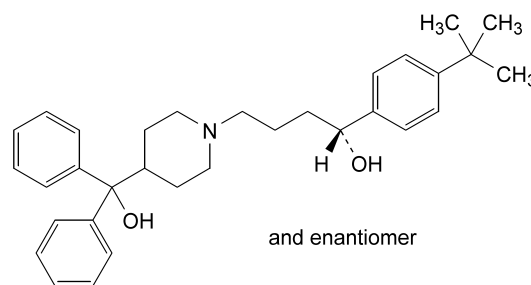


- B. 1-[4-[(2*RS*,4*SR*)-2-(2,4-dichlorophenyl)-2-[(4*H*-1,2,4-triazol-4-yl)methyl]-1,3-dioxolan-4-yl]methoxy]phenyl]-4-(1-methylethyl)piperazine.

01/2005:0955

TERFENADINE

Terfenadinum



C₃₂H₄₁NO₂

M_r 471.7

DEFINITION

Terfenadine contains not less than 98.5 per cent and not more than the equivalent of 101.0 per cent of (1*RS*)-1-[4-(1,1-dimethylethyl)phenyl]-4-[4-(hydroxydiphenylmethyl)piperidin-1-yl]butan-1-ol, calculated with reference to the dried substance.

CHARACTERS

A white, crystalline powder, very slightly soluble in water and in dilute hydrochloric acid, freely soluble in methylene chloride, soluble in methanol.

It shows polymorphism.

IDENTIFICATION

First identification: C.

Second identification: A, B, D.

- A. Melting point (2.2.14): 146 °C to 152 °C.
- B. Dissolve 50.0 mg in *methanol R* and dilute to 100.0 ml with the same solvent. Examined between 230 nm and 350 nm (2.2.25), the solution shows an absorption maximum at 259 nm and shoulders at 253 nm and 270 nm. The specific absorbance at the maximum is 13.5 to 14.9.
- C. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *terfenadine CRS*.
- D. Examine by thin-layer chromatography (2.2.27), using *silica gel HF₂₅₄ R* as the coating substance.
- Test solution.* Dissolve 50 mg of the substance to be examined in *methylene chloride R* and dilute to 10 ml with the same solvent.
- Reference solution.* Dissolve 50 mg of *terfenadine CRS* in *methylene chloride R* and dilute to 10 ml with the same solvent.

Apply to the plate 10 µl of each solution. Develop over a path of 15 cm using a mixture of 10 volumes of *methanol R* and 90 volumes of *methylene chloride R*.

Allow the plate to dry in air and examine in ultraviolet light at 254 nm. The principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with the reference solution.

TESTS

Related substances. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 15 mg of the substance to be examined in the mobile phase and dilute to 10.0 ml with the mobile phase.

Reference solution (a). Dilute 1.0 ml of the test solution to 10.0 ml with the mobile phase. Dilute 1.0 ml of this solution to 20.0 ml with the mobile phase.

Reference solution (b). Dissolve 15 mg of *terfenadine impurity A CRS* in the mobile phase and dilute to 10.0 ml with the mobile phase. To 5.0 ml of the solution, add 5.0 ml of the test solution and dilute to 50.0 ml with the mobile phase.

Reference solution (c). Dilute 10.0 ml of reference solution (a) to 25.0 ml with the mobile phase.

Reference solution (d). Dissolve 0.1 g of *potassium iodide R* in the mobile phase and dilute to 100 ml with the mobile phase. Dilute 1 ml to 100 ml with the mobile phase.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4.6 mm in internal diameter packed with *octylsilyl silica gel for chromatography R* (5 µm),
- as mobile phase at a flow rate of 1 ml/min a mixture prepared as follows: dilute 600 ml of *acetonitrile R* to 1 litre with *diethylammonium phosphate buffer solution pH 6.0 R*,
- as detector a spectrophotometer set at 217 nm,
- a loop injector.

Inject 20 µl of each solution and continue the chromatography for 5 times the retention time of terfenadine. The test is not valid unless, in the chromatogram obtained with reference solution (b), the resolution between the peaks corresponding to terfenadine and impurity A is greater than 5.0 and the mass distribution ratio of the peak corresponding to terfenadine is greater than 2.0. Determine the mass distribution ratio using, as unretained compound, *potassium iodide R*. In the chromatogram obtained with the test solution, the area of any peak apart from the principal peak is not greater than that of the peak in the chromatogram obtained with reference solution (c) (0.2 per cent) and the sum of the areas of all the peaks apart from the principal peak is not greater than the area of the peak in the chromatogram obtained with reference solution (a) (0.5 per cent). Disregard any peak with an area less than 2.5 per cent of that of the peak in the chromatogram obtained with reference solution (c).

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying at 60 °C under a pressure not exceeding 0.5 kPa.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

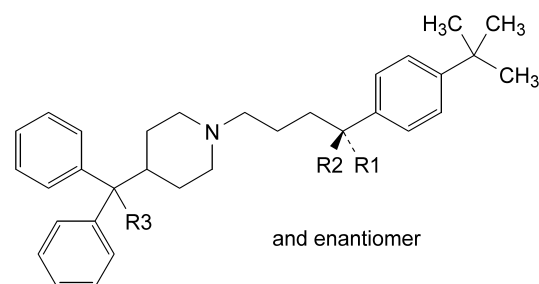
Dissolve 0.400 g in 50 ml of *anhydrous acetic acid R*. Titrate with 0.1 M *perchloric acid*, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *perchloric acid* is equivalent to 47.17 mg of C₃₂H₄₁NO₂.

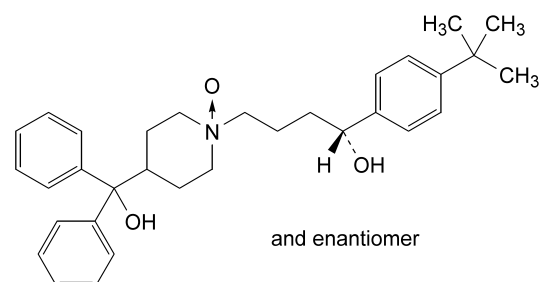
STORAGE

Protected from light.

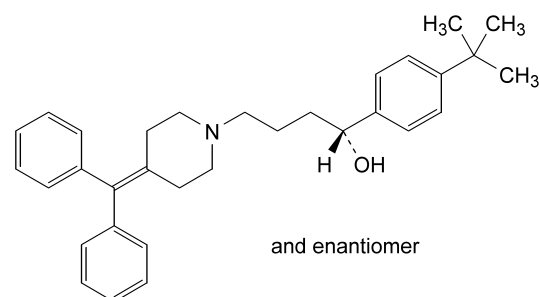
IMPURITIES



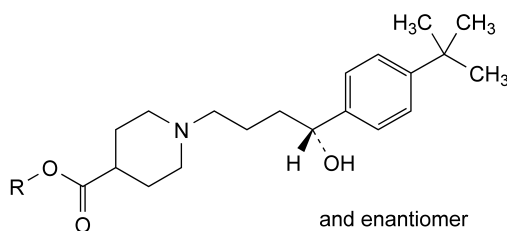
- A. R1 + R2 = O, R3 = OH: 1-[4-(1,1-dimethylethyl)phenyl]-4-[4-(hydroxydiphenylmethyl)piperidin-1-yl]butan-1-one,
- B. R1 = OH, R2 = R3 = H: (1*RS*)-1-[4-(1,1-dimethylethyl)phenyl]-4-[4-(diphenylmethyl)piperidin-1-yl]butan-1-ol,
- H. R1 = R2 = H, R3 = OH: [1-[4-[4-(1,1-dimethylethyl)phenyl]butyl]piperidin-4-yl]diphenylmethanol,



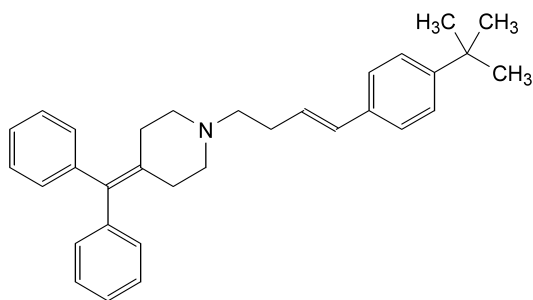
- C. 1-[(4*RS*)-4-[4-(1,1-dimethylethyl)phenyl]-4-hydroxybutyl]-4-(hydroxydiphenylmethyl)piperidine 1-oxide,



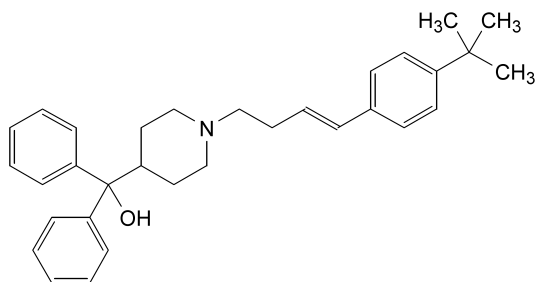
- D. (1*RS*)-1-[4-(1,1-dimethylethyl)phenyl]-4-[4-(diphenylmethylene)piperidin-1-yl]butan-1-ol,



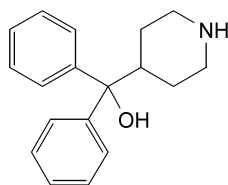
- E. R = H: 1-[(4*RS*)-4-[4-(1,1-dimethylethyl)phenyl]-4-hydroxybutyl]piperidine-4-carboxylic acid,
- J. R = C₂H₅: ethyl 1-[(4*RS*)-4-[4-(1,1-dimethylethyl)phenyl]-4-hydroxybutyl]piperidine-4-carboxylate,



F. 1-[4-[4-(1,1-dimethylethyl)phenyl]but-3-enyl]-4-(diphenylmethylene)piperidine,



G. [1-[4-[4-(1,1-dimethylethyl)phenyl]but-3-enyl]piperidin-4-yl]diphenylmethanol,

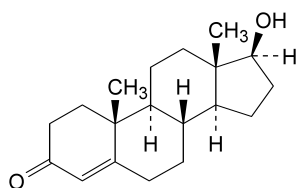


I. diphenyl(piperidin-4-yl)methanol.

01/2005:1373

TESTOSTERONE

Testosteronum



$C_{19}H_{28}O_2$

M_r 288.4

DEFINITION

17β-Hydroxyandrost-4-en-3-one.

Content: 97.0 per cent to 103.0 per cent (dried substance).

CHARACTERS

Appearance: white crystalline powder, or colourless or yellowish-white crystals.

Solubility: practically insoluble in water, freely soluble in alcohol and in methylene chloride, practically insoluble in fatty oils.

mp: about 155 °C.

IDENTIFICATION

Infrared absorption spectrophotometry (2.2.24).

Comparison: testosterone CRS.

TESTS

Specific optical rotation (2.2.7): + 106 to + 114 (dried substance).

Dissolve 0.250 g in *ethanol R* and dilute to 25.0 ml with the same solvent.

Impurities D and F. Thin-layer chromatography (2.2.27).

Test solution. Dissolve 0.100 g of the substance to be examined in *methanol R* and dilute to 10 ml with the same solvent.

Reference solution (a). Dissolve 1 mg of *stanolone R* in *methanol R* and dilute to 10 ml with the same solvent. In 1 ml of this solution, dissolve 10 mg of *testosterone for impurity D identification CRS* (testosterone spiked with about 1 per cent of impurity D).

Reference solution (b). Dilute 1.0 ml of the test solution to 100.0 ml with *methanol R*.

Reference solution (c). Dilute 2.0 ml of reference solution (b) to 10.0 ml with *methanol R*.

Reference solution (d). Dilute 1.0 ml of reference solution (b) to 10.0 ml with *methanol R*.

Plate: TLC silica gel F_{254} plate *R* (6–8 µm).

Preconditioning (in the dark): add about 5 g of powdered *silver nitrate R* to 100 ml of *methanol R*. Stir the suspension for 30 min. Filter or decant the suspension and immerse the plate in the silver nitrate solution for at least 30 min. Dry at 75 °C for 30 min.

A pre-conditioned plate can be stored in the dark for 5–7 days.

Mobile phase: *acetic acid R*, *ethanol R*, *dioxan R*, *methylene chloride R* (1:2:10:90 V/V/V/V).

Application: 2 µl.

Development: in a saturated tank over 3/4 of the plate.

Drying: allow to stand at room temperature and protected from light for 30 min.

Detection: spray with a 200 g/l solution of *toluenesulphonic acid R* in *ethanol R* and heat at 105 °C for 10 min. Examine in ultraviolet light at 365 nm.

System suitability: the chromatogram obtained with reference solution (a) shows 3 clearly separated spots; impurity D R_f = about 0.5; testosterone R_f = about 0.65; impurity F R_f = about 0.7.

Limits:

- *impurity D*: any spot due to impurity D is not more intense than the spot in the chromatogram obtained with reference solution (c) (0.2 per cent),
- *impurity F*: any spot due to impurity F is not more intense than the spot in the chromatogram obtained with reference solution (d) (0.1 per cent).

Related substances. Liquid chromatography (2.2.29).

Test solution. Dissolve 0.100 g of the substance to be examined in *methanol R* and dilute to 10.0 ml with the same solvent.

Reference solution (a). Dissolve 10 mg of *testosterone for system suitability CRS* (containing impurities C and I) in 1 ml of *methanol R*.

Reference solution (b). Dilute 1.0 ml of the test solution to 20.0 ml with *methanol R*. Dilute 1.0 ml of this solution to 10.0 ml with *methanol R*.

Reference solution (c). Dilute 2.0 ml of reference solution (b) to 10.0 ml with *methanol R*.

Column:

- *size*: l = 0.25 m, $\varnothing$ = 4.6 mm,

- *stationary phase*: spherical *end-capped octadecylsilyl silica gel for chromatography R* (5 µm) with a pore size of 15 nm,
- *temperature*: 40 °C.

Mobile phase:

- *mobile phase A*: water for chromatography *R*, methanol *R* (45:55 V/V),
- *mobile phase B*: methanol *R*,

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)
0 - 4	100	0
4 - 24	100 → 60	0 → 40
24 - 53	60 → 0	40 → 100
53 - 55	0	100
55 - 56	0 → 100	100 → 0
56 - 75	100	0

Flow rate: 1.0 ml/min.

Detection: spectrophotometer at 254 nm.

Injection: 20 µl.

Relative retention with reference to testosterone (retention time = about 18 min): impurity G = about 0.6; impurity H = about 0.8; impurity A = about 0.9; impurity I = about 0.95; impurity C = about 1.2; impurity E = about 1.7; impurity J = about 2.1; impurity B = about 2.5.

System suitability: reference solution (a):

- *resolution*: minimum baseline separation between the peaks due to impurity I and testosterone.

Limits: use the chromatogram obtained with reference solution (a) to identify the peaks due to impurities C and I:

- *correction factor*: for the calculation of content, multiply the peak area of impurity I by 2.9,
- *impurity C*: not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent),
- *impurity I*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (c) (0.2 per cent),
- *impurities A, B, E, G, H, J*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (c) (0.1 per cent),
- *any other impurity*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (c) (0.1 per cent),
- *total*: not more than 1.2 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.6 per cent),
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.05 per cent).

Loss on drying (2.2.32): maximum 1.0 per cent, determined on 0.500 g by drying in an oven at 100-105 °C for 2 h.

ASSAY

Dissolve 50.0 mg in *alcohol R* and dilute to 100.0 ml with the same solvent. Dilute 2.0 ml to 100.0 ml with *alcohol R*. Measure the absorbance (2.2.25) at the absorption maximum at 241 nm.

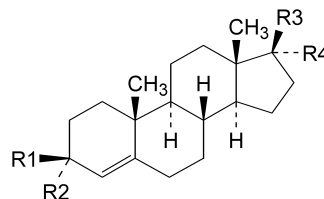
Calculate the content of C₁₉H₂₈O₂ taking the specific absorbance to be 569.

STORAGE

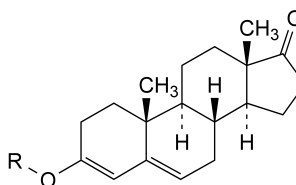
Protected from light.

IMPURITIES

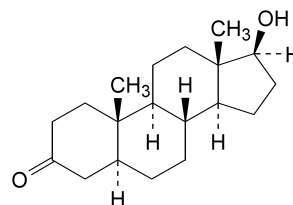
Specified impurities: A, B, C, D, E, F, G, H, I, J.



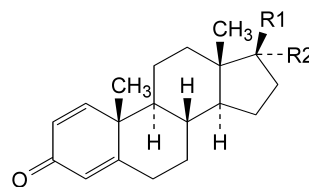
- A. R1 + R2 = R3 + R4 = O: androst-4-ene-3,17-dione (androstenedione),
- C. R1 + R2 = O, R3 = H, R4 = OH: 17α-hydroxyandrost-4-en-3-one (epitestosterone),
- D. R1 = R3 = OH, R2 = R4 = H: androst-4-ene-3β,17β-diol (Δ4-androstenediol),
- E. R1 + R2 = O, R3 = O-CO-CH₃, R4 = H: 3-oxoandrost-4-en-17β-yl acetate (testosterone acetate),



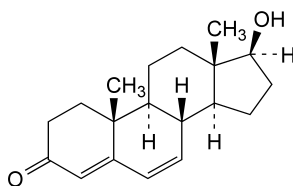
- B. R = C₂H₅: 3-ethoxyandrost-3,5-dien-17-one (androstenedione ethylenelether),
- J. R = CH₃: 3-methoxyandrost-3,5-dien-17-one (androstenedione methylenelether),



- F. 17β-hydroxy-5α-androstan-3-one (androstanolone, stanolone),



- G. R1 + R2 = O: androsta-1,4-diene-3,17-dione (androstadienedione).
- H. R1 = OH, R2 = H: 17β-hydroxyandrost-1,4-dien-3-one (boldenone),

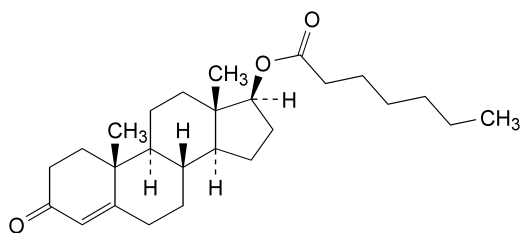


- I. 17β-hydroxyandrost-4,6-dien-3-one (Δ6-testosterone).

01/2005:1048

TESTOSTERONE ENANTATE

Testosteroni enantas

 $C_{26}H_{40}O_3$ M_r 400.6

DEFINITION

Testosterone enantate contains not less than 97.0 per cent and not more than the equivalent of 103.0 per cent of 3-oxoandrost-4-en-17 β -yl heptanoate, calculated with reference to the dried substance.

CHARACTERS

A white or yellowish-white, crystalline powder, practically insoluble in water, very soluble in ethanol, freely soluble in fatty oils.

IDENTIFICATION

First identification: B.

Second identification: A, C, D.

- A. Melting point (2.2.14): 34 °C to 39 °C.
- B. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *testosterone enantate CRS*.
- C. Examine by thin-layer chromatography (2.2.27), using as the coating substance a suitable octadecylsilyl silica gel with a fluorescent indicator having an optimal intensity at 254 nm.

Test solution. Dissolve 5 mg of the substance to be examined in a mixture of 1 volume of *methanol R* and 9 volumes of *methylene chloride R* and dilute to 10 ml with the same mixture of solvents.

Reference solution (a). Dissolve 5 mg of *testosterone enantate CRS* in a mixture of 1 volume of *methanol R* and 9 volumes of *methylene chloride R* and dilute to 10 ml with the same mixture of solvents.

Reference solution (b). Dissolve 5 mg of *testosterone enantate CRS*, 5 mg of *testosterone decanoate CRS* and 5 mg of *testosterone isocaproate CRS* in a mixture of 1 volume of *methanol R* and 9 volumes of *methylene chloride R* and dilute to 10 ml with the same mixture of solvents.

Apply separately to the plate 5 μ l of each solution. Develop over a path of 15 cm using a mixture of 20 volumes of *water R*, 40 volumes of *acetonitrile R* and 60 volumes of *2-propanol R*. Allow the plate to dry in air and heat at 100 °C for 10 min. Allow to cool and examine in ultraviolet light at 254 nm. The principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a). Spray the plate with *alcoholic solution of sulphuric acid R*. Heat at 120 °C for 10 min. Allow to cool and examine in daylight. The principal spot in the chromatogram obtained with the test solution is green and is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a).

The test is not valid unless the chromatogram obtained with reference solution (b) shows three clearly separated principal spots by each method of visualisation.

- D. To about 25 mg add 2 ml of a 10 g/l solution of *potassium hydroxide R* in *methanol R* and boil under a reflux condenser for 1 h. Cool. Add 10 ml of *water R*. Acidify with *dilute hydrochloric acid R* until *blue litmus paper R* turns red. Filter and wash the precipitate with a small quantity of *water R*. The residue, after drying at 60 °C at a pressure not exceeding 0.7 kPa for 3 h, melts (2.2.14) at 150 °C to 153 °C.

TESTS

Specific optical rotation (2.2.7). Dissolve 0.250 g in *dioxan R* and dilute to 25.0 ml with the same solvent. The specific optical rotation is + 77 to + 82, calculated with reference to the dried substance.

Related substances. Examine by thin-layer chromatography (2.2.27), using *silica gel G R* as the coating substance.

Test solution. Dissolve 0.20 g of the substance to be examined in a mixture of 1 volume of *methanol R* and 9 volumes of *methylene chloride R* and dilute to 10 ml with the same mixture of solvents.

Reference solution (a). Dilute 1 ml of the test solution to 100 ml with a mixture of 1 volume of *methanol R* and 9 volumes of *methylene chloride R*.

Reference solution (b). Dissolve 20 mg of *testosterone propionate CRS* in a mixture of 1 volume of *methanol R* and 9 volumes of *methylene chloride R*, add 1 ml of the test solution and dilute to 100 ml with the same mixture of solvents.

Apply separately to the plate 5 μ l of each solution. Develop over a path of 15 cm using a mixture of 20 volumes of *ethyl acetate R* and 80 volumes of *toluene R*. Allow the plate to dry in air. Spray with *alcoholic solution of sulphuric acid R* and heat at 120 °C for 10 min. Any spot in the chromatogram obtained with the test solution, apart from the principal spot, is not more intense than the principal spot in the chromatogram obtained with reference solution (a) (1.0 per cent). The test is not valid unless the chromatogram obtained with reference solution (b) shows two clearly separated principal spots.

Testosterone caproate. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 50.0 mg of the substance to be examined in the mobile phase and dilute to 50.0 ml with the mobile phase.

Reference solution (a). Dissolve 10.0 mg of *testosterone caproate CRS* in the mobile phase and dilute to 20.0 ml with the mobile phase. Dilute 1.0 ml to 50.0 ml with the mobile phase.

Reference solution (b). Dilute 1.0 ml of the test solution to 100.0 ml with the mobile phase. To 1 ml of this solution add 1 ml of reference solution (a).

The chromatographic procedure may be carried out using:

- a stainless steel column 0.12 m long and 4 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (5 μ m),
- as mobile phase at a flow rate of 1.0 ml/min a mixture of 30 volumes of *water R* and 70 volumes of *acetonitrile R*,
- as detector a spectrophotometer set at 240 nm.

Inject 20 μ l of reference solution (b). When using a recorder, adjust the sensitivity of the system so that the height of the two peaks in the chromatogram obtained with reference solution (b) is at least 50 per cent of the full scale of the recorder. The test is not valid unless in the chromatogram

01/2005:0297

obtained the peak corresponding to testosterone caproate has a retention time of about 0.7 relative to the second peak, which corresponds to testosterone enantate.

Inject 20 µl of the test solution and 20 µl of reference solution (a). In the chromatogram obtained with the test solution, the area of any peak corresponding to testosterone caproate is not greater than the area of the peak in the chromatogram obtained with reference solution (a) (1.0 per cent).

Heptanoic acid. Dissolve 0.50 g of the substance to be examined in 10 ml of *alcohol R* previously neutralised to *bromothymol blue solution R3*. Titrate immediately with 0.01 M sodium hydroxide. Not more than 0.6 ml of 0.01 M sodium hydroxide is required to change the colour of the indicator to blue (0.16 per cent).

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in a desiccator over *diphosphorus pentoxide R* at a pressure not exceeding 0.7 kPa.

ASSAY

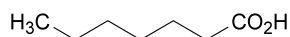
Dissolve 50.0 mg in *ethanol R* and dilute to 100.0 ml with the same solvent. Dilute 2.0 ml to 100.0 ml with *ethanol R*. Measure the absorbance (2.2.25) at the maximum at 241 nm.

Calculate the content of $C_{26}H_{40}O_3$ taking the specific absorbance to be 422.

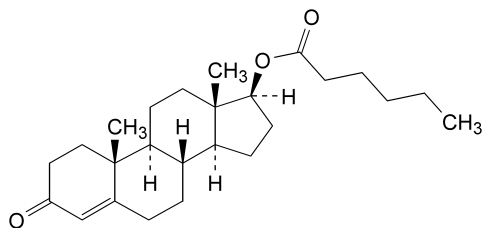
STORAGE

Store protected from light at a temperature of 2 °C to 8 °C.

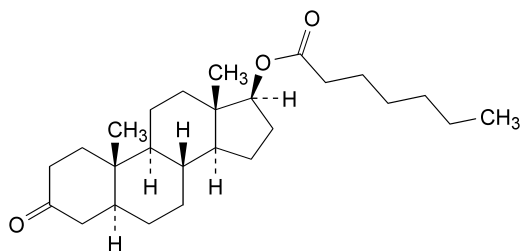
IMPURITIES



A. heptanoic acid,



B. 3-oxoandrost-4-en-17β-yl hexanoate (testosterone caproate),

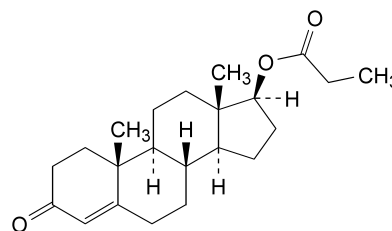


C. 3-oxo-5α-androstan-17β-yl heptanoate,

D. 17β-hydroxyandrost-4-en-3-one (testosterone).

TESTOSTERONE PROPIONATE

Testosteroni propionas


 $C_{22}H_{32}O_3$
 M_r 344.5

DEFINITION

3-Oxoandrost-4-en-17β-yl propanoate.

Content: 97.0 per cent to 103.0 per cent (dried substance).

CHARACTERS

Appearance: white or almost white powder or colourless crystals.

Solubility: practically insoluble in water, freely soluble in acetone and in alcohol, soluble in fatty oils.

IDENTIFICATION

Infrared absorption spectrophotometry (2.2.24).

Comparison: *Ph. Eur. reference spectrum of testosterone propionate.*

TESTS

Specific optical rotation (2.2.7): + 84 to + 90 (dried substance).

Dissolve 0.250 g in *ethanol R* and dilute to 25.0 ml with the same solvent.

Related substances. Liquid chromatography (2.2.29).

Test solution. Dissolve 50.0 mg of the substance to be examined in *methanol R* and dilute to 50.0 ml with the same solvent.

Reference solution (a). Dissolve 2 mg of the substance to be examined and 2 mg of *testosterone acetate CRS* in *methanol R* and dilute to 50.0 ml with the same solvent.

Reference solution (b). Dilute 1.0 ml of the test solution to 100.0 ml with *methanol R*.

Column:

- **size:** $l = 0.25$ m, $\varnothing = 4.6$ mm,
- **stationary phase:** octadecylsilyl silica gel for chromatography *R* (5 µm).

Mobile phase: water *R*, *methanol R* (20:80 V/V).

Flow rate: 1.5 ml/min.

Detection: spectrophotometer at 254 nm.

Injection: 20 µl.

Run time: twice the retention time of testosterone propionate.

Relative retention with reference to testosterone propionate (retention time = about 9 min): impurity C = about 0.5; impurity A = about 0.7; impurity D = about 0.8; impurity B = about 1.4.

System suitability: reference solution (a):

- **resolution:** minimum 4.0 between the peaks due to testosterone propionate and to impurity A.

Limits:

- *any impurity*: not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent),
- *total*: not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (1.0 per cent),
- *disregard limit*: 0.05 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

Loss on drying (2.2.32): maximum 0.5 per cent, determined on 0.500 g by drying in an oven at 100–105 °C for 2 h.

ASSAY

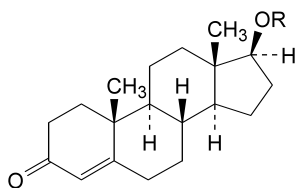
Dissolve 25.0 mg in *ethanol R* and dilute to 250.0 ml with the same solvent. Dilute 10.0 ml of the solution to 100.0 ml with *ethanol R*. Measure the absorbance (2.2.25) at the maximum at 240 nm.

Calculate the content of $C_{22}H_{32}O_3$ taking the specific absorbance to be 490.

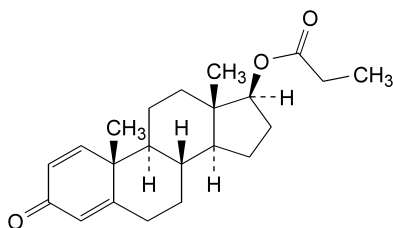
IMPURITIES

Specified impurities: A, B, C, D.

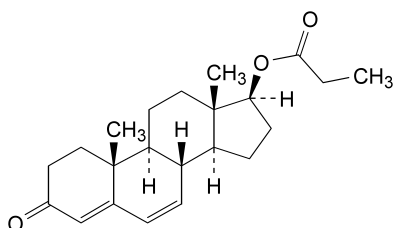
Other detectable impurities: E.



- A. R = CO-CH₃: 3-oxoandrost-4-en-17β-yl acetate (testosterone acetate),
- B. R = CO-CH(CH₃)₂: 3-oxoandrost-4-en-17β-yl 2-methylpropanoate (testosterone isobutyrate),
- C. R = H: testosterone,



- D. 3-oxoandrost-1,4-dien-17β-yl propanoate,

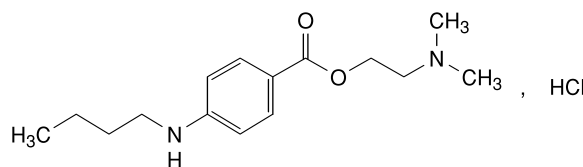


- E. 3-oxoandrost-4,6-dien-17β-yl propanoate.

01/2005:0057

TETRACAINE HYDROCHLORIDE

Tetracaini hydrochloridum

 $C_{15}H_{25}ClN_2O_2$ M_r 300.8

DEFINITION

Tetracaine hydrochloride contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of 2-(dimethylamino)ethyl 4-(butylamino)benzoate hydrochloride, calculated with reference to the dried substance.

CHARACTERS

A white, crystalline powder, slightly hygroscopic, freely soluble in water, soluble in alcohol.

It melts at about 148 °C or it may occur in either of 2 other crystalline forms which melt respectively at about 134 °C and 139 °C. Mixtures of these forms melt within the range 134 °C to 147 °C.

IDENTIFICATION

First identification: A, B, D.

Second identification: B, C, D.

- A. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *tetracaine hydrochloride CRS*.
- B. To 10 ml of solution S (see Tests) add 1 ml of *ammonium thiocyanate solution R*. A white, crystalline precipitate is formed which, after recrystallisation from *water R* and drying at 80 °C for 2 h, melts (2.2.14) at about 131 °C.
- C. To about 5 mg add 0.5 ml of *fuming nitric acid R*. Evaporate to dryness on a water-bath, allow to cool and dissolve the residue in 5 ml of *acetone R*. Add 1 ml of 0.1 M *alcoholic potassium hydroxide*. A violet colour develops.
- D. Solution S gives reaction (a) of chlorides (2.3.1).

TESTS

Solution S. Dissolve 5.0 g in *carbon dioxide-free water R* and dilute to 50 ml with the same solvent.

Appearance of solution. Dilute 2 ml of solution S to 10 ml with *water R*. The solution is clear (2.2.1) and colourless (2.2.2, *Method II*).

pH (2.2.3). Dilute 1 ml of solution S to 10 ml with *carbon dioxide-free water R*. The pH of the solution is 4.5 to 6.5.

Related substances. Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel GF₂₅₄ plate R*. Carry out a preliminary development over a path of 12 cm using a mixture of 4 volumes of *glacial acetic acid R*, 16 volumes of *hexane R* and 80 volumes of *dibutyl ether R*. Remove the plate and dry it in a current of warm air for a few minutes. Allow the plate to cool before use.

Test solution. Dissolve 1.0 g of the substance to be examined in *water R* and dilute to 10 ml with the same solvent.

Reference solution. Dissolve 50 mg of 4-aminobenzoic acid *R* in *water R* and dilute to 100 ml with the same solvent. Dilute 1 ml of the solution to 10 ml with *water R*.

Apply to the plate 5 µl of each solution. Develop over a path of 10 cm using a mixture of 4 volumes of *glacial acetic acid R*, 16 volumes of *hexane R* and 80 volumes of *dibutyl ether R*. Dry the plate at 100 °C to 105 °C for 10 min and examine in ultraviolet light at 254 nm. Any spot in the chromatogram obtained with the test solution, apart from the principal spot, is not more intense than the spot in the chromatogram obtained with the reference solution (0.05 per cent). The principal spot in the chromatogram obtained with the test solution remains at the starting point.

Heavy metals (2.4.8). 12 ml of solution S complies with limit test A for heavy metals (10 ppm). Prepare the standard using *lead standard solution (1 ppm Pb R)*.

Loss on drying (2.2.32). Not more than 1.0 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.250 g in 50 ml of *alcohol R* and add 5.0 ml of 0.01 M *hydrochloric acid*. Carry out a potentiometric titration (2.2.20), using 0.1 M *sodium hydroxide*. Read the volume added between the 2 points of inflexion.

1 ml of 0.1 M *sodium hydroxide* is equivalent to 30.08 mg of C₁₅H₂₅ClN₂O₂.

STORAGE

Store protected from light.

01/2005:0644

TETRACOSACTIDE

Tetracosactidum

H-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-
Lys-Pro-Val-Gly-Lys-Lys-Arg-Arg-Pro-Val-
Lys-Val-Tyr-Pro-OH

C₁₃₆H₂₁₀N₄₀O₃₁S

M_r 2933

DEFINITION

Tetracosactide is a synthetic tetracosapeptide in which the sequence of amino acids is the same as that of the first twenty-four residues of human corticotropin. It is available as an acetate and contains water. It increases the rate at which corticoid hormones are secreted by the adrenal glands. The potency is not less than 800 International Units per milligram, calculated with reference to the anhydrous, acetic acid-free substance.

CHARACTERS

A white or yellow, amorphous powder, sparingly soluble in water.

IDENTIFICATION

- It increases the amount of corticosterone produced by isolated rat adrenal cells in the conditions of the assay.
- Examine by electrophoresis (2.2.31) and thin-layer chromatography (2.2.27) to obtain a two-dimensional separation using two plates with *cellulose for chromatography R1* as the coating substance.
Test solution. Dissolve 1 mg of the substance to be examined in 0.2 ml of a 15.4 g/l solution of *ammonium acetate R* adjusted to pH 8.2 with *dilute ammonia R2*. Add 10 µl of a 2 g/l solution of *trypsin R*, maintain the mixture at 37 °C to 38 °C for 40 min, heat on a water-bath

for 3 min and add 5 µl of *glacial acetic acid R*. Evaporate to dryness at 40 °C at a pressure not exceeding 3 kPa, dry the glassy residue at 40 °C for 1 h and dissolve in 0.1 ml of *glacial acetic acid R*. Dry the solution from the frozen state, dissolve the residue in 0.1 ml of *water R* and dry again from the frozen state. Dry the final residue at 45 °C for 1 h at a pressure not exceeding 3 kPa and dissolve in 50 µl of *water R*.

Reference solution. Prepare at the same time and in the same manner as the test solution, using *tetracosactide CRS* instead of the substance to be examined.

Spray the plates with the electrolyte solution which consists of a solution containing 0.2 per cent V/V of *glacial acetic acid R* and 0.2 per cent V/V of *pyridine R*. Place the filter paper tongues to connect the plates with the appropriate compartment of each trough so that each tongue covers an area 1.5 cm wide at one end of the plate. Close the tank and allow to stand for 30 min. Apply the solutions on the anodic side. Apply to the first plate, at a point about 2.5 cm from each of two adjacent edges, 4 µl of the test solution. Apply to the second plate, at a similar position, 4 µl of the reference solution. Apply to both plates a potential of 280 V for a plate 200 mm long and allow electrophoresis to proceed for 90 min. Allow the plates to dry in air for 30 min and then dry in a current of air at 30 °C for 30 min. Carry out a second separation on each plate by thin-layer chromatography. Develop at right angles to the direction of electrophoresis over a path of 15 cm using a mixture of 8 volumes of *glacial acetic acid R*, 24 volumes of *pyridine R*, 30 volumes of *water R* and 38 volumes of *butanol R*. Dry the plates in a current of air and spray with *ninhydrin solution R1*. The principal spots in the chromatogram obtained with the test solution are similar in position to those in the chromatogram obtained with the reference solution, but their intensity may differ.

TESTS

Specific optical rotation (2.2.7). Dissolve 10.0 mg in 1.0 ml of a mixture of 1 volume of *glacial acetic acid R* and 99 volumes of *water R*. The specific optical rotation is -99 to -109, calculated with reference to the anhydrous, acetic acid-free substance.

Absorbance (2.2.25). Dissolve 1.0 mg in 0.1 M *hydrochloric acid* and dilute to 5.0 ml with the same acid. Examined between 240 nm and 280 nm, the solution shows an absorption maximum at 276 nm. The absorbance at the maximum is 0.51 to 0.61, calculated with reference to the anhydrous, acetic acid-free substance. The ratio of the absorbance at the maximum at 276 nm to the absorbance at 248 nm is 2.4 to 2.9.

Amino acids. Examine by means of an amino-acid analyser. Standardise the apparatus with a mixture containing equimolar amounts of ammonia, glycine and the L-form of the following amino acids:

Lysine	Threonine	Alanine	Leucine
Histidine	Serine	Valine	Tyrosine
Arginine	Glutamic acid	Methionine	Phenylalanine
Aspartic acid	Proline	Isoleucine	

together with half the equimolar amount of L-cystine. For the validation of the method, an appropriate internal standard, such as *DL-norleucine R*, is used.

Test solution. Place 1.0 mg of the substance to be examined in a rigorously cleaned hard-glass tube, 100 mm long and 6 mm in internal diameter. Add a suitable amount of a

50 per cent V/V solution of *hydrochloric acid R*. Immerse the tube in a freezing mixture at -5°C , reduce the pressure to below 133 Pa and seal. Heat at 110°C to 115°C for 16 h. Cool, open the tube, transfer the contents to a 10 ml flask with the aid of five quantities, each of 0.2 ml, of *water R* and evaporate to dryness over *potassium hydroxide R* under reduced pressure. Take up the residue in *water R* and evaporate to dryness over *potassium hydroxide R* under reduced pressure; repeat these operations once. Take up the residue in a buffer solution suitable for the amino-acid analyser used and dilute to a suitable volume with the same buffer solution. Apply a suitable volume to the amino-acid analyser.

Express the content of each amino acid in moles. Calculate the relative proportions of the amino acids, taking that for valine to be equivalent to three. The values fall within the following limits: lysine 3.5 to 4.7; histidine 0.9 to 1.1; arginine 2.7 to 3.3; serine 1.1 to 2.2; glutamic acid 0.9 to 1.1; proline 2.5 to 3.5; glycine 1.8 to 2.2; methionine 0.9 to 1.1; tyrosine 1.7 to 2.2; phenylalanine 0.9 to 1.1. Not more than traces of other amino acids are present, with the exception of tryptophan.

Related peptides

A. Examine by liquid chromatography (2.2.29). Use degassed solvents.

Test solution. Dissolve 1.0 mg of the substance to be examined in 1 ml of *water R*.

Reference solution (a). Dissolve 1.0 mg of the substance to be examined in 1 ml of a 1 per cent V/V solution of *glacial acetic acid R* and add 50 μl of a mixture of 1 volume of *strong hydrogen peroxide solution R* and 999 volumes of *water R*. Allow to stand for 2 h.

Reference solution (b). Dissolve 1.0 mg of *tetracosactide CRS* in 1 ml of *water R*.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4.6 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (10 μm),
- as mobile phase at a flow rate of 2.0 ml/min, a mixture of 365 ml of *acetonitrile R*, 10.0 ml of *glacial acetic acid R* and 10.0 g of *ammonium sulphate R*, diluted to 2000 ml with *water R*,
- as detector a spectrophotometer set at 280 nm.

Inject 20 μl of each solution ensuring that the syringe used to inject the test solution is not contaminated with peroxide. The chromatogram obtained with reference solution (a) shows a peak due to tetracosactide corresponding to the principal peak in the chromatogram obtained with the test solution and a peak with a lower retention time, due to tetracosactide sulfoxide, of significantly greater area than any corresponding peak in the chromatogram obtained with the test solution. The test is not valid unless in the chromatogram obtained with reference solution (a), the resolution between the peaks corresponding to tetracosactide and tetracosactide sulfoxide is at least 7. In the chromatogram obtained with the test solution, the area of the peak corresponding to tetracosactide sulfoxide is not greater than 4 per cent of the sum of the areas of all the peaks, disregarding any peaks due to the solvent and the mobile phase.

B. Examine by thin-layer chromatography (2.2.27), using *cellulose for chromatography R* as the coating substance.

Test solution. Dissolve 3.0 mg of the substance to be examined in 1.5 ml of a mixture of equal volumes of *dilute acetic acid R* and *water R*.

Reference solution (a). Dilute 0.5 ml of the test solution to 10 ml with a mixture of equal volumes of *dilute acetic acid R* and *water R*.

Reference solution (b). Dilute 5 ml of reference solution (a) to 10 ml with a mixture of equal volumes of *dilute acetic acid R* and *water R*.

Reference solution (c). To 0.5 ml of the test solution add 50 μl of a mixture of 1 volume of *strong hydrogen peroxide solution R* and 999 volumes of *water R*. Allow to stand for 2 h.

Apply to the plate as 1 cm bands 10 μl of each solution. Develop over a path of 15 cm using a mixture of 4 volumes of *glacial acetic acid R*, 24 volumes of *pyridine R*, 30 volumes of *water R* and 42 volumes of *butanol R*. Dry the plate in a current of air and spray with *ninhydrin solution R1*. In the chromatogram obtained with reference solution (c), the band corresponding in position to the principal band in the chromatogram obtained with the test solution is reduced in intensity, and a prominent band, due to tetracosactide sulfoxide, of lower R_f is present. In the chromatogram obtained with the test solution, any band, apart from the principal band and any band due to tetracosactide sulfoxide, is not more intense than the band in the chromatogram obtained with reference solution (a) (5.0 per cent) and at most one such band is more intense than the band in the chromatogram obtained with reference solution (b) (2.5 per cent).

Peptide. Not less than 85.0 per cent of peptide, expressed as $\text{C}_{136}\text{H}_{210}\text{N}_{40}\text{O}_{31}\text{S}$, calculated with reference to the anhydrous, acetic acid-free substance.

Examine the chromatograms obtained in test A for related peptides. Calculate the content of $\text{C}_{136}\text{H}_{210}\text{N}_{40}\text{O}_{31}\text{S}$ from the peak heights or areas in the chromatograms obtained with the test solution and reference solution (b), and the declared content of $\text{C}_{136}\text{H}_{210}\text{N}_{40}\text{O}_{31}\text{S}$ in *tetracosactide CRS*.

Acetic acid (2.5.34): 8.0 per cent to 13.0 per cent.

Test solution. Dissolve 10.0 mg of the substance to be examined in a mixture of 5 volumes of mobile phase B and 95 volumes of mobile phase A and dilute to 10.0 ml with the same mixture of solvents.

Water (2.5.12): 5.0 per cent to 16.0 per cent, determined on 80.0 mg by the semi-micro determination of water.

ASSAY

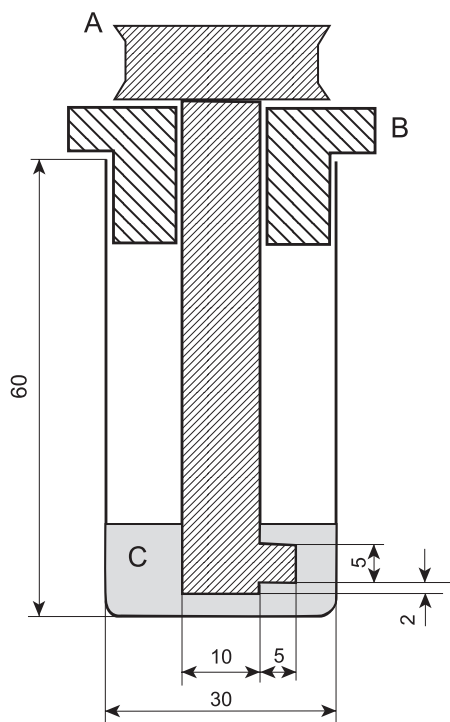
The potency of tetracosactide is estimated by comparing in given conditions its activity in increasing the amount of corticosterone produced by isolated rat adrenal cells with that of the International Reference Preparation of tetracosactide or a reference preparation calibrated in International Units.

The International Unit is the activity contained in a stated amount of the International Reference Preparation which consists of a quantity of synthetic tetracosactide with mannitol. The equivalence in International Units of the International Reference Preparation is stated by the World Health Organisation.

The estimated potency is not less than 80 per cent and not more than 125 per cent of the stated potency. The confidence limits ($P = 0.95$) of the estimated potency are not less than 64 per cent and not more than 156 per cent of the stated potency.

Use siliconised glassware and rinse well with *water R* before use. Kill four male rats, each weighing between 200 g and 400 g, by exsanguination. Remove the adrenal glands, carefully free them of adhering fat and immerse in solution B maintained at 4°C . Cut each gland into four equal pieces and transfer to a suitable plastic stirring apparatus (see

Figure 0644.-1) containing 5 ml of solution C maintained at 37 °C. Disperse the adrenal cells by stirring the mixture at 500 r/min. After 20 min, remove the supernatant liquid, cool to 4 °C, add 5 ml of solution C and repeat the dispersal procedure. Repeat the operation a further three times. Combine the five supernatant liquids, centrifuge at 4 °C in a polyethylene test-tube for 30 min after slow acceleration to 100 *g*. Suspend the resulting pellet in 8 ml of solution D and centrifuge for 30 min at 100 *g*. Again suspend the residue in 8 ml of solution D and filter the mixture through nylon gauze with 100 µm pores into a polyethylene beaker and add a suitable volume of solution D to the filtrate (a total volume of 65 ml to 105 ml has been found suitable). Maintain the resulting suspension at 4 °C.



A - pulley and stirring paddle,

B - bearing,

C - incubation medium.

Figure 0644.-1. – *Stirring Apparatus*
Dimensions in millimetres

Prepare four independent dilutions, with two-fold dose intervals, from solutions of suitable concentrations of the substance to be examined and of the reference preparation using solution E as diluent. Pipette 0.1 ml of each dilution into each of four polystyrene test-tubes and add 1.0 ml of the cell suspension prepared above to each tube. Incubate at 37 °C for 2 h and then cool to 4 °C. Transfer 1 ml of the contents of each tube to a glass tube containing 1.4 ml of *methylene chloride R*, mix in a vortex mixer for 10 s and centrifuge at 3000 *g* for 5 min. Transfer 1 ml of each of the methylene chloride layers, avoiding taking up aqueous phase, to a glass tube containing 0.6 ml of a mixture of 15 volumes of *alcohol R* and 35 volumes of *sulphuric acid R*. Mix in a vortex mixer for 10 s and centrifuge at 1500 *g* for 5 min. Allow each tube to stand for 30 min. Examine by fluorimetry (2.2.21), irradiating the lower layer with an excitant beam of suitable wavelength such as 436 nm or 470 nm and measuring the fluorescence at the maximum between 530 nm and 545 nm. If the solutions are not transferred to spectrophotometric measurement cells, select glass tubes that give fluorescence values for a standard

corticosterone which do not differ from each other by more than 5 per cent. Calculate the result of the assay by the usual statistical methods using the linear portion of the log dose-response curve.

Solution A

<i>Sodium chloride R</i>	6.60 g
<i>Potassium chloride R</i>	0.353 g
<i>Sodium hydrogen carbonate R</i>	0.840 g
<i>Potassium dihydrogen phosphate R</i>	0.161 g
<i>Magnesium sulphate R</i>	0.291 g
<i>Calcium chloride R</i>	0.373 g
<i>2-[4-(2-Hydroxyethyl)piperazin-1-yl]ethane sulphonic acid R</i>	4.77 g

Dissolve the above ingredients in about 950 ml of *water R*, adjust to pH 7.4 with 1 *M sodium hydroxide* and add 60 mg of *benzylpenicillin sodium R* and a quantity of *streptomycin sulphate R* equivalent to 100 mg of streptomycin. Dilute to 1000 ml with *water R*.

Solution B

Add 2 g/l of *glucose R* to solution A.

Solution C

To solution B add 1 g/l of a preparation of collagenase obtained from *Clostridium histolyticum* and of a grade suitable for the preparation of dispersed cells.

Solution D

Add 5 g/l of *bovine albumin R* to solution B.

Solution E

A 9 g/l sterile solution of *sodium chloride R* containing 1 g/l of *bovine albumin R* and adjusted to pH 2.0 with 1 *M hydrochloric acid*.

STORAGE

Store under nitrogen, protected from light, at a temperature between 2 °C and 8 °C.

LABELLING

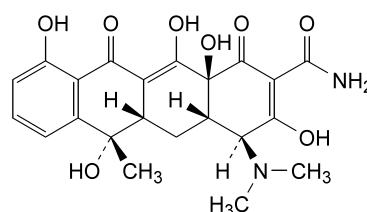
The label states:

- the potency in International Units per milligram,
- the peptide content per container,
- the storage conditions.

01/2005:0211

TETRACYCLINE

Tetracyclinum



$C_{22}H_{24}N_2O_8$

M_r 444.4

DEFINITION

(4*S*,4*aS*,5*aS*,6*S*,12*aS*)-4-(Dimethylamino)-3,6,10,12,12*a*-pentahydroxy-6-methyl-1,11-dioxo-1,4,4*a*,5,5*a*,6,11,12*a*-octahydrotetracene-2-carboxamide.

Content: 88.0 per cent to 102.0 per cent (dried substance).

CHARACTERS

Appearance: yellow, crystalline powder.

Solubility: very slightly soluble in water, soluble in alcohol and in methanol, sparingly soluble in acetone. It dissolves in dilute acid and alkaline solutions.

IDENTIFICATION

A. Thin-layer chromatography (2.2.27).

Test solution. Dissolve 5 mg of the substance to be examined in *methanol R* and dilute to 10 ml with the same solvent.

Reference solution (a). Dissolve 5 mg of *tetracycline hydrochloride CRS* in *methanol R* and dilute to 10 ml with the same solvent.

Reference solution (b). Dissolve 5 mg of *tetracycline hydrochloride CRS*, 5 mg of *demeclocycline hydrochloride R* and 5 mg of *oxytetracycline hydrochloride R* in *methanol R* and dilute to 10 ml with the same solvent.

Plate: TLC octadecylsilyl silica gel F_{254} plate *R*.

Mobile phase: mix 20 volumes of *acetonitrile R*, 20 volumes of *methanol R* and 60 volumes of a 63 g/l solution of *oxalic acid R* previously adjusted to pH 2 with *concentrated ammonia R*.

Application: 1 µl.

Development: over 3/4 of the plate.

Drying: in air.

Detection: examine in ultraviolet light at 254 nm.

System suitability: the chromatogram obtained with reference solution (b) shows 3 clearly separated spots.

Results: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a).

B. To about 2 mg add 5 ml of *sulphuric acid R*. A violet-red colour develops. Add the solution to 2.5 ml of *water R*. The colour becomes yellow.C. Dissolve about 10 mg in a mixture of 1 ml of *dilute nitric acid R* and 5 ml of *water R*. Shake and add 1 ml of *silver nitrate solution R2*. Any opalescence in the solution is not more intense than that in a mixture of 1 ml of *dilute nitric acid R*, 5 ml of *water R* and 1 ml of *silver nitrate solution R2*.

TESTS

pH (2.2.3): 3.5 to 6.0.

Suspend 0.1 g in 10 ml of *carbon dioxide-free water R*.

Specific optical rotation (2.2.7): –260 to –280 (dried substance).

Dissolve 0.250 g in 0.1 M *hydrochloric acid* and dilute to 50.0 ml with the same acid.

Related substances. Liquid chromatography (2.2.29).

Test solution. Dissolve 25.0 mg of the substance to be examined in 0.01 M *hydrochloric acid* and dilute to 25.0 ml with the same acid.

Reference solution (a). Dissolve 25.0 mg of *tetracycline hydrochloride CRS* in 0.01 M *hydrochloric acid* and dilute to 25.0 ml with the same acid.

Reference solution (b). Dissolve 12.5 mg of *4-epitetracycline hydrochloride CRS* in 0.01 M *hydrochloric acid* and dilute to 50.0 ml with the same acid.

Reference solution (c). Dissolve 10.0 mg of *anhydrotetracycline hydrochloride CRS* in 0.01 M *hydrochloric acid* and dilute to 100.0 ml with the same acid.

Reference solution (d). Dissolve 10.0 mg of *4-epianhydrotetracycline hydrochloride CRS* in 0.01 M *hydrochloric acid* and dilute to 50.0 ml with the same acid.

Reference solution (e). Mix 1.0 ml of reference solution (a), 2.0 ml of reference solution (b) and 5.0 ml of reference solution (d) and dilute to 25.0 ml with 0.01 M *hydrochloric acid*.

Reference solution (f). Mix 40.0 ml of reference solution (b), 20.0 ml of reference solution (c) and 5.0 ml of reference solution (d) and dilute to 200.0 ml with 0.01 M *hydrochloric acid*.

Reference solution (g). Dilute 1.0 ml of reference solution (c) to 50.0 ml with 0.01 M *hydrochloric acid*.

Column:

- size: $l = 0.25$ m, $\varnothing = 4.6$ mm,
- stationary phase: *styrene-divinylbenzene copolymer R* (8 µm),
- temperature: 60 °C.

Mobile phase: weigh 80.0 g of *2-methyl-2-propanol R* and transfer to a 1000 ml volumetric flask with the aid of 200 ml of *water R*; add 100 ml of a 35 g/l solution of *dipotassium hydrogen phosphate R* adjusted to pH 9.0 with *dilute phosphoric acid R*, 200 ml of a 10 g/l solution of *tetrabutylammonium hydrogen sulphate R* adjusted to pH 9.0 with *dilute sodium hydroxide solution R* and 10 ml of a 40 g/l solution of *sodium edetate R* adjusted to pH 9.0 with *dilute sodium hydroxide solution R*; dilute to 1000.0 ml with *water R*.

Flow rate: 1.0 ml/min.

Detection: spectrophotometer at 254 nm.

Injection: 20 µl; inject the test solution and reference solutions (e), (f) and (g).

System suitability:

- resolution: minimum 2.5 between the peaks due to impurity A (1st peak) and tetracycline (2nd peak) and minimum 8.0 between the peaks due to tetracycline and impurity D (3rd peak) in the chromatogram obtained with reference solution (e); if necessary, adjust the concentration of 2-methyl-2-propanol in the mobile phase,
- signal-to-noise ratio: minimum 3 for the principal peak in the chromatogram obtained with reference solution (g),
- symmetry factor: maximum 1.25 for the peak due to tetracycline in the chromatogram obtained with reference solution (e).

Limits:

- impurity A: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (f) (5.0 per cent),
- impurity B (eluting on the tail of the principal peak): not more than 0.4 times the area of the peak due to impurity A in the chromatogram obtained with reference solution (f) (2.0 per cent),
- impurity C: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (f) (1.0 per cent),
- impurity D: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (f) (0.5 per cent).

Heavy metals (2.4.8): maximum 50 ppm.

0.5 g complies with limit test C. Prepare the standard using 2.5 ml of *lead standard solution (10 ppm Pb) R*.

Loss on drying (2.2.32): maximum 13.0 per cent, determined on 1.000 g by drying in an oven at 100–105 °C.

Sulphated ash (2.4.14): maximum 0.5 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (2.2.29) as described in the test for related substances with the following modification.

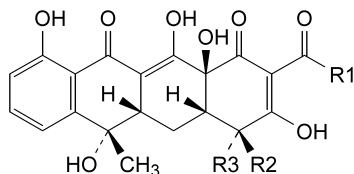
Injection: test solution and reference solution (a).

Calculate the percentage content of $C_{22}H_{24}N_2O_8$.

STORAGE

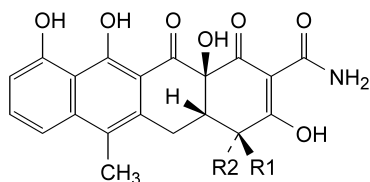
Protected from light.

IMPURITIES



A. $R_1 = NH_2$, $R_2 = H$, $R_3 = N(CH_3)_2$: (4*R*,4*aS*,5*aS*,6*S*,12*aS*)-4-(dimethylamino)-3,6,10,12,12*a*-pentahydroxy-6-methyl-1,11-dioxo-1,4,4*a*,5,5*a*,6,11,12*a*-octahydrotetracene-2-carboxamide (4-epitetracycline),

B. $R_1 = CH_3$, $R_2 = N(CH_3)_2$, $R_3 = H$: (4*S*,4*aS*,5*aS*,6*S*,12*aS*)-2-acetyl-4-(dimethylamino)-3,6,10,12,12*a*-pentahydroxy-6-methyl-4*a*,5*a*,6,12*a*-tetrahydrotetracene-1,11(4*H*,5*H*)-dione (2-acetyl-2-decarbamoylepianhydrotetracycline),



C. $R_1 = N(CH_3)_2$, $R_2 = H$: (4*S*,4*aS*,12*aS*)-4-(dimethylamino)-3,10,11,12*a*-tetrahydroxy-6-methyl-1,12-dioxo-1,4,4*a*,5,12,12*a*-hexahydrotetracene-2-carboxamide (anhydrotetracycline),

D. $R_1 = H$, $R_2 = N(CH_3)_2$: (4*R*,4*aS*,12*aS*)-4-(dimethylamino)-3,10,11,12*a*-tetrahydroxy-6-methyl-1,12-dioxo-1,4,4*a*,5,12,12*a*-hexahydrotetracene-2-carboxamide (4-epianhydrotetracycline).

CHARACTERS

Appearance: yellow, crystalline powder.

Solubility: soluble in water, slightly soluble in alcohol, practically insoluble in acetone. It dissolves in solutions of alkali hydroxides and carbonates. Solutions in water become turbid on standing, owing to the precipitation of tetracycline.

IDENTIFICATION

A. Thin-layer chromatography (2.2.27).

Test solution. Dissolve 5 mg of the substance to be examined in *methanol R* and dilute to 10 ml with the same solvent.

Reference solution (a). Dissolve 5 mg of *tetracycline hydrochloride CRS* in *methanol R* and dilute to 10 ml with the same solvent.

Reference solution (b). Dissolve 5 mg of *tetracycline hydrochloride CRS*, 5 mg of *demeclocycline hydrochloride R* and 5 mg of *oxytetracycline hydrochloride R* in *methanol R* and dilute to 10 ml with the same solvent.

Plate: *TLC octadecylsilyl silica gel F₂₅₄ plate R*.

Mobile phase: mix 20 volumes of *acetonitrile R*, 20 volumes of *methanol R* and 60 volumes of a 63 g/l solution of *oxalic acid R* previously adjusted to pH 2 with *concentrated ammonia R*.

Application: 1 µl.

Development: over 3/4 of the plate.

Drying: in air.

Detection: examine in ultraviolet light at 254 nm.

System suitability: the chromatogram obtained with reference solution (b) shows 3 clearly separated spots.

Results: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a).

B. To about 2 mg add 5 ml of *sulphuric acid R*. A violet-red colour develops. Add the solution to 2.5 ml of *water R*. The colour becomes yellow.

C. It gives reaction (a) of chlorides (2.3.1).

TESTS

pH (2.2.3): 1.8 to 2.8.

Dissolve 0.1 g in 10 ml of *carbon dioxide-free water R*.

Specific optical rotation (2.2.7): –240 to –255 (dried substance).

Dissolve 0.250 g in 0.1 *M hydrochloric acid* and dilute to 25.0 ml with the same acid.

Related substances. Liquid chromatography (2.2.29).

Test solution. Dissolve 25.0 mg of the substance to be examined in 0.01 *M hydrochloric acid* and dilute to 25.0 ml with the same acid.

Reference solution (a). Dissolve 25.0 mg of *tetracycline hydrochloride CRS* in 0.01 *M hydrochloric acid* and dilute to 25.0 ml with the same acid.

Reference solution (b). Dissolve 15.0 mg of 4-epitetracycline hydrochloride CRS in 0.01 *M hydrochloric acid* and dilute to 50.0 ml with the same acid.

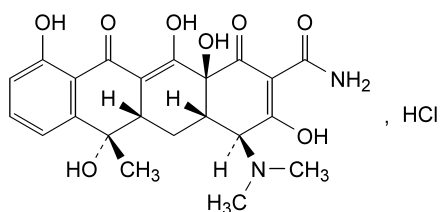
Reference solution (c). Dissolve 10.0 mg of anhydrotetracycline hydrochloride CRS in 0.01 *M hydrochloric acid* and dilute to 100.0 ml with the same acid.

Reference solution (d). Dissolve 10.0 mg of 4-epianhydrotetracycline hydrochloride CRS in 0.01 *M hydrochloric acid* and dilute to 50.0 ml with the same acid.

01/2005:0210

TETRACYCLINE HYDROCHLORIDE

Tetracyclini hydrochloridum



$C_{22}H_{25}ClN_2O_8$

M_r 480.9

DEFINITION

(4*S*,4*aS*,5*aS*,6*S*,12*aS*)-4-(Dimethylamino)-3,6,10,12,12*a*-pentahydroxy-6-methyl-1,11-dioxo-1,4,4*a*,5,5*a*,6,11,12*a*-octahydrotetracene-2-carboxamide hydrochloride.

Content: 95.0 per cent to 102.0 per cent (dried substance).

Reference solution (e). Mix 1.0 ml of reference solution (a), 2.0 ml of reference solution (b) and 5.0 ml of reference solution (d) and dilute to 25.0 ml with 0.01 M hydrochloric acid.

Reference solution (f). Mix 20.0 ml of reference solution (b), 10.0 ml of reference solution (c) and 5.0 ml of reference solution (d) and dilute to 200.0 ml using 0.01 M hydrochloric acid.

Reference solution (g). Dilute 1.0 ml of reference solution (c) to 50.0 ml with 0.01 M hydrochloric acid.

Column:

- **size:** $l = 0.25$ m, $\varnothing = 4.6$ mm,
- **stationary phase:** styrene-divinylbenzene copolymer R (8 μ m),
- **temperature:** 60 °C.

Mobile phase: weigh 80.0 g of 2-methyl-2-propanol R and transfer to a 1000 ml volumetric flask with the aid of 200 ml of water R; add 100 ml of a 35 g/l solution of dipotassium hydrogen phosphate R adjusted to pH 9.0 with dilute phosphoric acid R, 200 ml of a 10 g/l solution of tetrabutylammonium hydrogen sulphate R adjusted to pH 9.0 with dilute sodium hydroxide solution R and 10 ml of a 40 g/l solution of sodium edetate R adjusted to pH 9.0 with dilute sodium hydroxide solution R; dilute to 1000.0 ml with water R.

Flow rate: 1.0 ml/min.

Detection: spectrophotometer at 254 nm.

Injection: 20 μ l; inject the test solution and reference solutions (e), (f) and (g).

System suitability:

- **resolution:** minimum 2.5 between the peaks due to impurity A (1st peak) and tetracycline (2nd peak) and minimum 8.0 between the peaks due to tetracycline and impurity D (3rd peak) in the chromatogram obtained with reference solution (e); if necessary, adjust the concentration of 2-methyl-2-propanol in the mobile phase,
- **signal-to-noise ratio:** minimum 3 for the principal peak in the chromatogram obtained with reference solution (g),
- **symmetry factor:** maximum 1.25 for the peak due to tetracycline in the chromatogram obtained with reference solution (e).

Limits:

- **impurity A:** not more than the area of the corresponding peak in the chromatogram obtained with reference solution (f) (3.0 per cent),
- **impurity B** (eluting on the tail of the principal peak): not more than half the area of the peak due to impurity A in the chromatogram obtained with reference solution (f) (1.5 per cent),
- **impurity C:** not more than the area of the corresponding peak in the chromatogram obtained with reference solution (f) (0.5 per cent),
- **impurity D:** not more than the area of the corresponding peak in the chromatogram obtained with reference solution (f) (0.5 per cent).

Heavy metals (2.4.8): maximum 50 ppm.

0.5 g complies with limit test C. Prepare the standard using 2.5 ml of lead standard solution (10 ppm Pb) R.

Loss on drying (2.2.32): maximum 2.0 per cent, determined on 1.000 g by drying at 60 °C over diphosphorus pentoxide R at a pressure not exceeding 670 Pa for 3 h.

Sulphated ash (2.4.14): maximum 0.5 per cent, determined on 1.0 g.

Bacterial endotoxins (2.6.14): less than 0.5 IU/mg, if intended for use in the manufacture of parenteral dosage forms without a further appropriate procedure for the removal of bacterial endotoxins.

ASSAY

Liquid chromatography (2.2.29) as described in the test for related substances with the following modification.

Injection: test solution and reference solution (a).

Calculate the percentage content of $C_{22}H_{25}ClN_2O_8$.

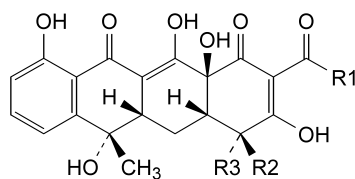
STORAGE

Protected from light. If the substance is sterile, store in a sterile, tamper-proof container.

LABELLING

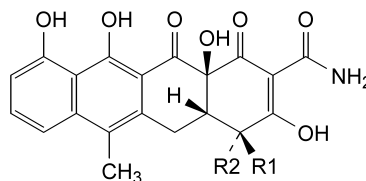
The label states, where applicable, that the substance is free from bacterial endotoxins.

IMPURITIES



A. R1 = NH₂, R2 = H, R3 = N(CH₃)₂: (4R,4aS,5aS,6S,12aS)-4-(dimethylamino)-3,6,10,12,12a-pentahydroxy-6-methyl-1,11-dioxo-1,4,4a,5,5a,6,11,12a-octahydrotetracene-2-carboxamide (4-epitetracycline),

B. R1 = CH₃, R2 = N(CH₃)₂, R3 = H: (4S,4aS,5aS,6S,12aS)-2-acetyl-4-(dimethylamino)-3,6,10,12,12a-pentahydroxy-6-methyl-4a,5a,6,12a-tetrahydrotetracene-1,11(4H,5H)-dione (2-acetyl-2-decarbamoyletetracycline),



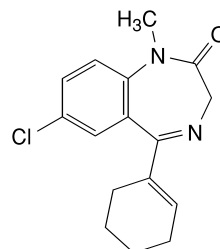
C. R1 = N(CH₃)₂, R2 = H: (4S,4aS,12aS)-4-(dimethylamino)-3,10,11,12a-tetrahydroxy-6-methyl-1,12-dioxo-1,4,4a,5,12,12a-hexahydrotetracene-2-carboxamide (anhydrotetracycline),

D. R1 = H, R2 = N(CH₃)₂: (4R,4aS,12aS)-4-(dimethylamino)-3,10,11,12a-tetrahydroxy-6-methyl-1,12-dioxo-1,4,4a,5,12,12a-hexahydrotetracene-2-carboxamide (4-epianhydrotetracycline).

01/2005:1738

TETRAZEPAM

Tetrazepamum



$C_{16}H_{17}ClN_2O$

M_r 288.8

DEFINITION

7-Chloro-5-(cyclohex-1-enyl)-1-methyl-1,3-dihydro-2H-1,4-benzodiazepin-2-one.

Content: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

Appearance: light yellow or yellow crystalline powder.

Solubility: practically insoluble in water, freely soluble in methylene chloride, soluble in acetonitrile.

IDENTIFICATION

Infrared absorption spectrophotometry (2.2.24).

Comparison: Ph. Eur. reference spectrum of tetrazepam.

TESTS

Related substances. Liquid chromatography (2.2.29).

Test solution. Dissolve 25.0 mg of the substance to be examined in *acetonitrile R* and dilute to 25.0 ml with the same solvent.

Reference solution (a). Dissolve 5.0 mg of the substance to be examined and 5.0 mg of *tetrazepam impurity C CRS* in *acetonitrile R* and dilute to 10.0 ml with the same solvent. Dilute 1.0 ml of the solution to 10.0 ml with *acetonitrile R*.

Reference solution (b). Dilute 1.0 ml of the test solution to 50.0 ml with *acetonitrile R*. Dilute 1.0 ml of this solution to 10.0 ml with *acetonitrile R*.

Column:

- *size*: $l = 0.25$ m, $\varnothing = 4.6$ mm,
- *stationary phase*: octadecylsilyl silica gel for chromatography *R* (5 μ m).

Mobile phase:

- *mobile phase A*: mix 40 volumes of *acetonitrile R* and 60 volumes of a 3.4 g/l solution of *potassium dihydrogen phosphate R*,
- *mobile phase B*: *acetonitrile R*,

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)
0 - 35	100	0
35 - 40	100 → 55	0 → 45
40 - 50	55	45
50 - 60	55 → 100	45 → 0

Flow rate: 1.5 ml/min.

Detection: a spectrophotometer at 229 nm.

Injection: 20 μ l.

System suitability: reference solution (a):

- *resolution*: minimum 2.0 between the peaks due to tetrazepam and to impurity C.

Limits:

- *any impurity*: not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent),
- *total*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (1.0 per cent),
- *disregard limit*: 0.25 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

Chlorides (2.4.4): maximum 100 ppm.

Dissolve 0.750 g in 10 ml of *methylene chloride R* and add 15 ml of *water R*. Shake and separate the 2 layers. Dilute 10 ml of the aqueous layer to 15 ml with *water R*. The solution obtained complies with the limit test for chlorides.

Loss on drying (2.2.32): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 100-105 °C.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

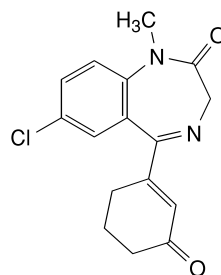
Dissolve 0.230 g in 50.0 ml of *anhydrous acetic acid R*. Titrate with 0.1 M *perchloric acid*, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *perchloric acid* is equivalent to 28.88 mg of $C_{16}H_{17}ClN_2O$.

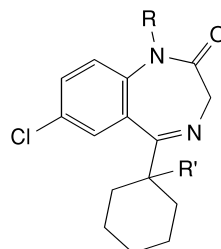
STORAGE

Protected from light.

IMPURITIES



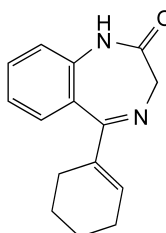
A. 7-chloro-5-(3-oxocyclohex-1-enyl)-1-methyl-1,3-dihydro-2H-1,4-benzodiazepin-2-one,



B. R = R' = H: 7-chloro-5-cyclohexyl-1,3-dihydro-2H-1,4-benzodiazepin-2-one,

C. R = CH₃, R' = H: 7-chloro-5-cyclohexyl-1-methyl-1,3-dihydro-2H-1,4-benzodiazepin-2-one,

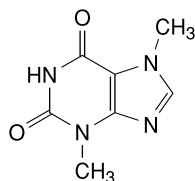
D. R = CH₃, R' = Cl: 7-chloro-5-(1-chlorocyclohexyl)-1-methyl-1,3-dihydro-2H-1,4-benzodiazepin-2-one,



E. 5-(cyclohex-1-enyl)-1,3-dihydro-2H-1,4-benzodiazepin-2-one.

THEOBROMINE

Theobrominum

C₇H₈N₄O₂*M_r* 180.2

DEFINITION

Theobromine contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of 3,7-dimethyl-3,7-dihydro-1*H*-purine-2,6-dione, calculated with reference to the dried substance.

CHARACTERS

A white powder, very slightly soluble in water and in ethanol, slightly soluble in ammonia. It dissolves in dilute solutions of alkali hydroxides and in mineral acids.

IDENTIFICATION

First identification: A, C.

Second identification: B, C.

- A. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *theobromine CRS*.
- B. Dissolve about 20 mg in 2 ml of *dilute ammonia R1*, warming slightly, and cool. Add 2 ml of *silver nitrate solution R2*. The solution remains clear. Boil the solution for a few minutes. A white, crystalline precipitate is formed.
- C. It gives the reaction of xanthines (2.3.1).

TESTS

Acidity. To 0.4 g add 20 ml of boiling *water R* and boil for 1 min. Allow to cool and filter. Add 0.05 ml of *bromothymol blue solution R1*. The solution is yellow or yellowish-green. Not more than 0.2 ml of 0.01 *M sodium hydroxide* is required to change the colour of the indicator to blue.

Related substances. Examine by thin-layer chromatography (2.2.27), using *silica gel GF₂₅₄ R* as the coating substance.

Test solution. To 0.2 g of the finely powdered substance to be examined add 10 ml of a mixture of 4 volumes of *methanol R* and 6 volumes of *chloroform R*. Heat under a reflux condenser on a water-bath for 15 min, shaking occasionally. Cool and filter.

Reference solution. Dissolve 5 mg of *theobromine CRS* in a mixture of 4 volumes of *methanol R* and 6 volumes of *chloroform R* and dilute to 50 ml with the same mixture of solvents.

Apply separately to the plate 10 µl of each solution. Develop over a path of 15 cm using a mixture of 10 volumes of *concentrated ammonia R*, 30 volumes of *acetone R*, 30 volumes of *chloroform R* and 40 volumes of *butanol R*. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. Any spot in the chromatogram obtained with the test solution, apart from the principal spot, is not more intense than the spot in the chromatogram obtained with the reference solution (0.5 per cent).

01/2005:0298 Heavy metals (2.4.8). 1.0 g complies with limit test C for heavy metals (20 ppm). Prepare the standard using 2 ml of *lead standard solution (10 ppm Pb) R*.

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

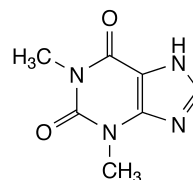
Dissolve 0.150 g in 125 ml of boiling *water R*, cool to 50 °C to 60 °C and add 25 ml of 0.1 *M silver nitrate*. Using 1 ml of *phenolphthalein solution R* as indicator, titrate with 0.1 *M sodium hydroxide* until a pink colour is obtained.

1 ml of 0.1 *M sodium hydroxide* is equivalent to 18.02 mg of C₇H₈N₄O₂.

01/2005:0299

THEOPHYLLINE

Theophyllinum

C₇H₈N₄O₂*M_r* 180.2

DEFINITION

1,3-Dimethyl-3,7-dihydro-1*H*-purine-2,6-dione.

Content: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

Appearance: white, crystalline powder.

Solubility: slightly soluble in water, sparingly soluble in ethanol. It dissolves in solutions of alkali hydroxides, in ammonia and in mineral acids.

IDENTIFICATION

First identification: B, D.

Second identification: A, C, D, E.

- A. Melting point (2.2.14): 270 °C to 274 °C, determined after drying at 100-105 °C.
- B. Infrared absorption spectrophotometry (2.2.24).
Comparison: *Ph. Eur. reference spectrum of theophylline*.
- C. Heat 10 mg with 1.0 ml of a 360 g/l solution of *potassium hydroxide R* in a water-bath at 90 °C for 3 min, then add 1.0 ml of *diazotised sulphanilic acid solution R*. A red colour slowly develops. Carry out a blank test.
- D. It complies with the test for loss on drying (see Tests).
- E. It gives the reaction of xanthines (2.3.1).

TESTS

Solution S. Dissolve 0.5 g with heating in *carbon dioxide-free water R*, cool and dilute to 75 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and colourless (2.2.2, *Method II*).

Acidity. To 50 ml of solution S add 0.1 ml of *methyl red solution R*. The solution is red. Not more than 1.0 ml of 0.01 *M sodium hydroxide* is required to change the colour of the indicator to yellow.

Related substances. Liquid chromatography (2.2.29).

Test solution. Dissolve 40.0 mg of the substance to be examined in the mobile phase and dilute to 20.0 ml with the mobile phase.

Reference solution (a). Dilute 1.0 ml of the test solution to 100.0 ml with the mobile phase. Dilute 1.0 ml of this solution to 10.0 ml with the mobile phase.

Reference solution (b). Dissolve 10 mg of *theobromine R* in the mobile phase, add 5 ml of the test solution and dilute to 100 ml with the mobile phase. Dilute 5 ml of this solution to 50 ml with the mobile phase.

Column:

- *size:* $l = 0.25$ m, $\varnothing = 4$ mm,
- *stationary phase:* octadecylsilyl silica gel for chromatography *R* (7 μ m).

Mobile phase: mix 7 volumes of acetonitrile for chromatography *R* and 93 volumes of a 1.36 g/l solution of sodium acetate *R* containing 5.0 ml/l of glacial acetic acid *R*.

Flow rate: 2.0 ml/min.

Detection: spectrophotometer at 272 nm.

Injection: 20 μ l.

Run time: 3.5 times the retention time of theophylline.

Relative retention with reference to theophylline (retention time = about 6 min): impurity C = about 0.3; impurity B = about 0.4; impurity D = about 0.5; impurity A = about 2.5.

System suitability: reference solution (b):

- *resolution:* minimum 2.0 between the peaks due to theobromine and theophylline.

Limits:

- *impurities A, B, C, D:* for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent),
- *any other impurity:* for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent),
- *total:* not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent),
- *disregard limit:* 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

Heavy metals (2.4.8): maximum 20 ppm.

1.0 g complies with limit test C. Prepare the standard using 2 ml of lead standard solution (10 ppm Pb) *R*.

Loss on drying (2.2.32): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 100–105 °C.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.150 g in 100 ml of water *R*, add 20 ml of 0.1 M silver nitrate and shake. Add 1 ml of bromothymol blue solution *R1*. Titrate with 0.1 M sodium hydroxide.

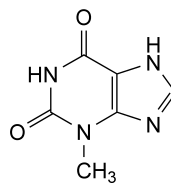
1 ml of 0.1 M sodium hydroxide is equivalent to 18.02 mg of $C_7H_8N_4O_2$.

IMPURITIES

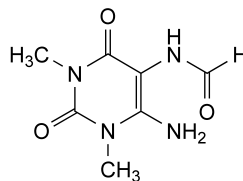
Specified impurities: A, B, C, D.

Other detectable impurities: E, F.

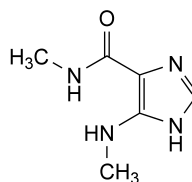
A. caffeine,



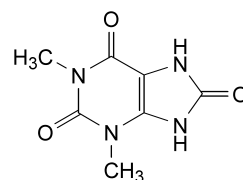
B. 3-methyl-3,7-dihydro-1H-purine-2,6-dione,



C. N-(6-amino-1,3-dimethyl-2,4-dioxo-1,2,3,4-tetrahydropyrimidin-5-yl)formamide,



D. N-methyl-5-(methylamino)-1H-imidazole-4-carboxamide,



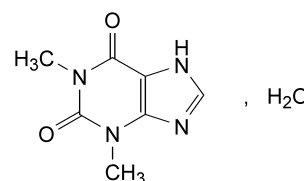
E. 1,3-dimethyl-7,9-dihydro-1H-purine-2,6,8(3H)-trione,

F. etofylline.

01/2005:0302

THEOPHYLLINE MONOHYDRATE

Theophyllinum monohydricum



$C_7H_8N_4O_2 \cdot H_2O$

M_r 198.2

DEFINITION

1,3-Dimethyl-3,7-dihydro-1H-purine-2,6-dione monohydrate.

Content: 99.0 per cent to 101.0 per cent (anhydrous substance).

CHARACTERS

Appearance: white, crystalline powder.

Solubility: slightly soluble in water, sparingly soluble in ethanol. It dissolves in solutions of alkali hydroxides, in ammonia and in mineral acids.

IDENTIFICATION

First identification: B, D.

Second identification: A, C, D, E.

A. Melting point (2.2.14): 270 °C to 274 °C, determined after drying at 100–105 °C.

B. Infrared absorption spectrophotometry (2.2.24).

Preparation: dry the substance to be examined at 100-105 °C before use.

Comparison: *Ph. Eur. reference spectrum of theophylline.*

C. Heat 10 mg with 1.0 ml of a 360 g/l solution of *potassium hydroxide R* in a water-bath at 90 °C for 3 min, then add 1.0 ml of *diazotised sulphanilic acid solution R*. A red colour slowly develops. Carry out a blank test.

D. It complies with the test for water (see Tests).

E. It gives the reaction of xanthines (2.3.1).

TESTS

Solution S. Dissolve 0.5 g with heating in *carbon dioxide-free water R*, cool and dilute to 75 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and colourless (2.2.2, *Method II*).

Acidity. To 50 ml of solution S add 0.1 ml of *methyl red solution R*. The solution is red. Not more than 1.0 ml of 0.01 M *sodium hydroxide* is required to change the colour of the indicator to yellow.

Related substances. Liquid chromatography (2.2.29).

Test solution. Dissolve 40.0 mg of the substance to be examined in the mobile phase and dilute to 20.0 ml with the mobile phase.

Reference solution (a). Dilute 1.0 ml of the test solution to 100.0 ml with the mobile phase. Dilute 1.0 ml of this solution to 10.0 ml with the mobile phase.

Reference solution (b). Dissolve 10 mg of *theobromine R* in the mobile phase, add 5 ml of the test solution and dilute to 100 ml with the mobile phase. Dilute 5 ml of this solution to 50 ml with the mobile phase.

Column:

- size: $l = 0.25$ m, $\varnothing = 4$ mm,
- stationary phase: *octadecylsilyl silica gel for chromatography R* (7 μ m).

Mobile phase: mix 7 volumes of *acetonitrile for chromatography R* and 93 volumes of a 1.36 g/l solution of *sodium acetate R* containing 5.0 ml/l of *glacial acetic acid R*.

Flow rate: 2.0 ml/min.

Detection: spectrophotometer at 272 nm.

Injection: 20 μ l.

Run time: 3.5 times the retention time of theophylline.

Relative retention with reference to theophylline (retention time = about 6 min): impurity C = about 0.3; impurity B = about 0.4; impurity D = about 0.5; impurity A = about 2.5.

System suitability: reference solution (b):

- resolution: minimum 2.0 between the peaks due to theobromine and theophylline.

Limits:

- impurities A, B, C, D: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent),
- any other impurity: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent),

- total: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent),
- disregard limit: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

Heavy metals (2.4.8): maximum 20 ppm.

1.0 g complies with limit test C. Prepare the standard using 2 ml of *lead standard solution (10 ppm Pb) R*.

Water (2.5.12): 8.0 per cent to 9.5 per cent, determined on 0.20 g.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.160 g in 100 ml of *water R*, add 20 ml of 0.1 M *silver nitrate* and shake. Add 1 ml of *bromothymol blue solution R1*. Titrate with 0.1 M *sodium hydroxide*.

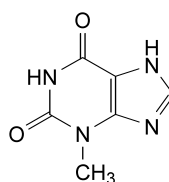
1 ml of 0.1 M *sodium hydroxide* is equivalent to 18.02 mg of $C_7H_8N_4O_2$.

IMPURITIES

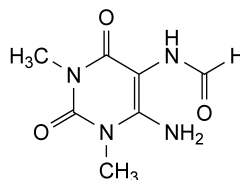
Specified impurities: A, B, C, D.

Other detectable impurities: E, F.

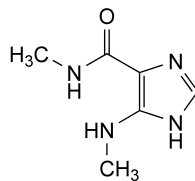
A. caffeine,



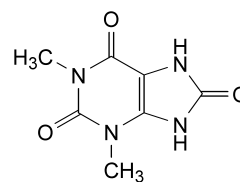
B. 3-methyl-3,7-dihydro-1H-purine-2,6-dione,



C. *N*-(6-amino-1,3-dimethyl-2,4-dioxo-1,2,3,4-tetrahydropyrimidin-5-yl)formamide,



D. *N*-methyl-5-(methylamino)-1H-imidazole-4-carboxamide,



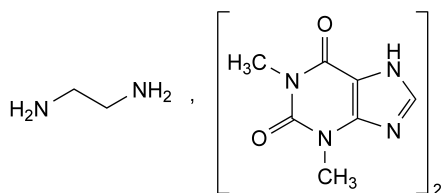
E. 1,3-dimethyl-7,9-dihydro-1H-purine-2,6,8(3H)-trione,

F. etofylline.

01/2005:0300

THEOPHYLLINE-ETHYLENEDIAMINE

Theophyllinum et ethylenediaminum

 $C_{16}H_{24}N_{10}O_4$ M_r 420.4

DEFINITION

Theophylline-ethylenediamine contains not less than 84.0 per cent and not more than the equivalent of 87.4 per cent of theophylline ($C_7H_8N_4O_2$; M_r 180.2) and not less than 13.5 per cent and not more than the equivalent of 15.0 per cent of ethylenediamine ($C_2H_8N_2$; M_r 60.1), both calculated with reference to the anhydrous substance.

CHARACTERS

A white or slightly yellowish powder, sometimes granular, freely soluble in water (the solution becomes cloudy through absorption of carbon dioxide), practically insoluble in ethanol.

IDENTIFICATION

First identification: B, C, E.

Second identification: A, C, D, E, F.

Dissolve 1.0 g in 10 ml of *water R* and add 2 ml of *dilute hydrochloric acid R* dropwise with shaking. Filter. Use the precipitate for identification tests A, B, D and F and the filtrate for identification test C.

- The precipitate, washed with *water R* and dried at 100 °C to 105 °C, melts (2.2.14) at 270 °C to 274 °C.
- Examine the precipitate, washed with *water R* and dried at 100 °C to 105 °C, by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *theophylline CRS*.
- To the filtrate add 0.2 ml of *benzoyl chloride R*, make alkaline with *dilute sodium hydroxide solution R* and shake vigorously. Filter the precipitate, wash with 10 ml of *water R*, dissolve in 5 ml of hot *alcohol R* and add 5 ml of *water R*. A precipitate is formed, which when washed and dried at 100 °C to 105 °C, melts (2.2.14) at 248 °C to 252 °C.
- Heat about 10 mg of the precipitate with 1.0 ml of a 360 g/l solution of *potassium hydroxide R* in a water-bath at 90 °C for 3 min, then add 1.0 ml of *diazotised sulphanilic acid solution R*. A red colour slowly develops. Carry out a blank test.
- It complies with the test for water (see Tests).
- The precipitate gives the reaction of xanthines (2.3.1).

TESTS

Appearance of solution. Dissolve 0.5 g with gentle warming in 10 ml of *carbon dioxide-free water R*. The solution is not more opalescent than reference suspension II (2.2.1) and not more intensely coloured than reference solution GY₆ (2.2.2, *Method II*).

Related substances. Examine by thin-layer chromatography (2.2.27), using as the coating substance a suitable silica gel with a fluorescent indicator having an optimal intensity at 254 nm.

Test solution. Dissolve 0.2 g of the substance to be examined in 2 ml of *water R* with heating and dilute to 10 ml with *methanol R*.

Reference solution. Dilute 0.5 ml of the test solution to 100 ml with *methanol R*.

Apply to the plate 10 µl of each solution. Develop over a path of 15 cm using a mixture of 10 volumes of *concentrated ammonia R*, 30 volumes of *acetone R*, 30 volumes of *chloroform R* and 40 volumes of *butanol R*. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. Any spot in the chromatogram obtained with the test solution, apart from the principal spot, is not more intense than the spot in the chromatogram obtained with the reference solution (0.5 per cent).

Heavy metals (2.4.8). 1.0 g complies with limit test C for heavy metals (20 ppm). Prepare the standard using 2 ml of *lead standard solution (10 ppm Pb) R*.

Water (2.5.12). Not more than 1.5 per cent, determined on 2.00 g dissolved in 20 ml of *anhydrous pyridine R*, by the semi-micro determination of water.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Ethylenediamine. Dissolve 0.250 g in 30 ml of *water R*. Add 0.1 ml of *bromocresol green solution R*. Titrate with 0.1 M *hydrochloric acid* until a green colour is obtained.

1 ml of 0.1 M *hydrochloric acid* is equivalent to 3.005 mg of $C_2H_8N_2$.

Theophylline. Heat 0.200 g to constant mass in an oven at 135 °C. Dissolve the residue with heating in 100 ml of *water R*, allow to cool, add 20 ml of 0.1 M *silver nitrate* and shake. Add 1 ml of *bromothymol blue solution R1*. Titrate with 0.1 M *sodium hydroxide*.

1 ml of 0.1 M *sodium hydroxide* is equivalent to 18.02 mg of $C_7H_8N_4O_2$.

STORAGE

Store in airtight container, protected from light.

01/2005:0301

THEOPHYLLINE-ETHYLENEDIAMINE
HYDRATETheophyllinum et ethylenediaminum
hydricum

DEFINITION

Theophylline-ethylenediamine hydrate contains not less than 84.0 per cent and not more than the equivalent of 87.4 per cent of theophylline ($C_7H_8N_4O_2$; M_r 180.2) and not less than 13.5 per cent and not more than the equivalent of 15.0 per cent of ethylenediamine ($C_2H_8N_2$; M_r 60.1), both calculated with reference to the anhydrous substance.

CHARACTERS

A white or slightly yellowish powder, sometimes granular, freely soluble in water (the solution becomes cloudy through absorption of carbon dioxide), practically insoluble in ethanol.

IDENTIFICATION

First identification: B, C, E.

Second identification: A, C, D, E, F.

Dissolve 1.0 g in 10 ml of *water R* and add 2 ml of *dilute hydrochloric acid R* dropwise with shaking. Filter. Use the precipitate for identification tests A, B, D and F and the filtrate for identification test C.

- A. The precipitate, washed with *water R* and dried at 100 °C to 105 °C, melts (2.2.14) at 270 °C to 274 °C.
- B. Examine the precipitate, washed with *water R* and dried at 100 °C to 105 °C, by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *theophylline CRS*.
- C. To the filtrate add 0.2 ml of *benzoyl chloride R*, make alkaline with *dilute sodium hydroxide solution R* and shake vigorously. Filter the precipitate, wash with 10 ml of *water R*, dissolve in 5 ml of hot *alcohol R* and add 5 ml of *water R*. A precipitate is formed, which when washed and dried at 100 °C to 105 °C, melts (2.2.14) at 248 °C to 252 °C.
- D. Heat about 10 mg of the precipitate with 1.0 ml of a 360 g/l solution of *potassium hydroxide R* in a water-bath at 90 °C for 3 min, then add 1.0 ml of *diazotised sulphanilic acid solution R*. A red colour slowly develops. Carry out a blank test.
- E. It contains 3.0 per cent to 8.0 per cent of water (see Tests).
- F. The precipitate gives the reaction of xanthines (2.3.1).

TESTS

Appearance of solution. Dissolve 0.5 g with gentle warming in 10 ml of *carbon dioxide-free water R*. The solution is not more opalescent than reference suspension II (2.2.1) and not more intensely coloured than reference solution GY₆ (2.2.2, *Method II*).

Related substances. Examine by thin-layer chromatography (2.2.27), using as the coating substance a suitable silica gel with a fluorescent indicator having an optimal intensity at 254 nm.

Test solution. Dissolve 0.2 g of the substance to be examined in 2 ml of *water R* with heating and dilute to 10 ml with *methanol R*.

Reference solution. Dilute 0.5 ml of the test solution to 100 ml with *methanol R*.

Apply separately to the plate 10 µl of each solution. Develop over a path of 15 cm using a mixture of 10 volumes of *concentrated ammonia R*, 30 volumes of *acetone R*, 30 volumes of *chloroform R* and 40 volumes of *butanol R*. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. Any spot in the chromatogram obtained with the test solution, apart from the principal spot, is not more intense than the spot in the chromatogram obtained with the reference solution (0.5 per cent).

Heavy metals (2.4.8). 1.0 g complies with limit test C for heavy metals (20 ppm). Prepare the standard using 2 ml of *lead standard solution* (10 ppm Pb) *R*.

Water (2.5.12): 3.0 per cent to 8.0 per cent, determined on 0.50 g dissolved in 20 ml of *pyridine R*, by the semi-micro determination of water.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Ethylenediamine. Dissolve 0.250 g in 30 ml of *water R*. Add 0.1 ml of *bromocresol green solution R*. Titrate with 0.1 M *hydrochloric acid* until a green colour is obtained.

1 ml of 0.1 M *hydrochloric acid* is equivalent to 3.005 mg of C₂H₈N₂.

Theophylline. Heat 0.200 g to constant mass in an oven at 135 °C. Dissolve the residue with heating in 100 ml of *water R*, allow to cool, add 20 ml of 0.1 M *silver nitrate* and shake. Add 1 ml of *bromothymol blue solution R1*. Titrate with 0.1 M *sodium hydroxide*.

1 ml of 0.1 M *sodium hydroxide* is equivalent to 18.02 mg of C₇H₈N₄O₂.

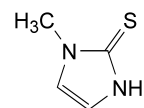
STORAGE

Store in a well-filled airtight container, protected from light.

01/2005:1706

THIAMAZOLE

Thiamazolum



C₄H₆N₂S

*M*_r 114.2

DEFINITION

1-Methyl-1,3-dihydro-2H-imidazole-2-thione.

Content: 98.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

Appearance: white or pale brown, crystalline powder.

Solubility: freely soluble in water, freely soluble in methylene chloride, freely soluble or soluble in ethanol (96 per cent).

IDENTIFICATION

First identification: A, C.

Second identification: A, B, D.

- A. Melting point (2.2.14): 143 °C to 146 °C.
- B. Dissolve 25 mg in 10 ml of a 0.28 per cent V/V solution of *sulphuric acid R* and dilute to 50.0 ml with the same solution. Dilute 1.0 ml of this solution to 100.0 ml with a 0.28 per cent V/V solution of *sulphuric acid R*. Examined between 200 nm and 300 nm (2.2.25), the solution shows 2 absorption maxima, at 211 nm and 251 nm. The ratio of the absorbance measured at the absorption maximum at 251 nm to that measured at the absorption maximum at 211 nm is 2.5 to 2.7.
- C. Infrared absorption spectrophotometry (2.2.24).
Preparation: discs.
Comparison: *thiamazole CRS*.
- D. Thin-layer chromatography (2.2.27).
Test solution. Dissolve 5.0 mg of the substance to be examined in *methanol R* and dilute to 5.0 ml with the same solvent.
Reference solution (a). Dissolve 5.0 mg of *thiamazole CRS* in *methanol R* and dilute to 5.0 ml with the same solvent.
Reference solution (b). Dissolve 5.0 mg of *2-methylimidazole R* in *methanol R* and dilute to 5.0 ml with the same solvent. Dilute 1.0 ml of this solution to 2.0 ml with the test solution.
Plate: TLC silica gel F₂₅₄ plate *R*.

Mobile phase: concentrated ammonia R1, 2-propanol R, toluene R (1:24:75 V/V/V).

Application: 10 µl.

Development: over 2/3 of the plate.

Drying: in air.

Detection: examine in ultraviolet light at 254 nm.

System suitability: reference solution (b):

- expose the plate to iodine vapour for 30 min; the chromatogram shows 2 clearly separated spots.

Results: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a).

TESTS

Solution S. Dissolve 2.0 g in water R and dilute to 20.0 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and not more intensely coloured than reference solution B₆ (2.2.2, Method II).

Related substances. Gas chromatography (2.2.28).

Test solution. Dissolve 0.100 g of the substance to be examined in chloroform R and dilute to 10.0 ml with the same solvent.

Reference solution (a). Dilute 1.0 ml of the test solution to 100.0 ml with chloroform R. Dilute 1.0 ml of this solution to 10.0 ml with chloroform R.

Reference solution (b). Dissolve 5.0 mg of thiamazole impurity A CRS, 5.0 mg of 1-methylimidazole R1 and 5.0 mg of thiamazole impurity C CRS in chloroform R and dilute to 50.0 ml with the same solvent. Dilute 1.0 ml of this solution to 10.0 ml with chloroform R.

Column:

- **material:** fused silica,
- **size:** *l* = 30.0 m, Ø = 0.25 mm,
- **stationary phase:** poly(dimethyl)(diphenyl)siloxane R with special deactivation for basic compounds (film thickness 0.5 µm).

Carrier gas: helium for chromatography R.

Flow rate: 1.5 ml/min.

Split ratio: 3:20.

Temperature:

	Time (min)	Temperature (°C)
Column	0 - 2	100
	2 - 7	100 → 250
	7 - 22	250
Injection port		150
Detector		250

Detection: flame ionisation.

Injection: 1 µl.

Relative retention with reference to thiamazole (retention time = about 6.5 min): impurity A = about 0.3; impurity B = about 0.4; impurity C = about 0.7.

System suitability: reference solution (b):

- **resolution:** minimum 1.5 between the peaks due to impurity A and impurity B.

Limits:

- **impurities A, B, C:** for each impurity, not more than the area of the corresponding peak in the chromatogram obtained with reference solution (b) (0.1 per cent),

- **any other impurity:** for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent),
- **total:** not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent),
- **disregard limit:** 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.02 per cent).

Heavy metals (2.4.8): maximum 10 ppm.

12 ml of solution S complies with limit test A. Prepare the reference solution using lead standard solution (1 ppm Pb) R.

Loss on drying (2.2.32): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 100-105 °C for 2 h.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

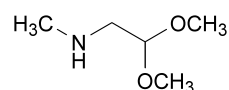
ASSAY

Dissolve 0.250 g in 75 ml of water R. Add 15.0 ml of 0.1 M sodium hydroxide, mix and add with stirring, about 30 ml of 0.1 M silver nitrate. Continue the titration with 0.1 M sodium hydroxide, determining the end-point potentiometrically (2.2.20).

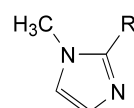
1 ml of 0.1 M sodium hydroxide is equivalent to 11.42 mg of C₄H₆N₂S.

IMPURITIES

Specified impurities: A, B, C.



A. 2,2-dimethoxy-N-methylethanamine,



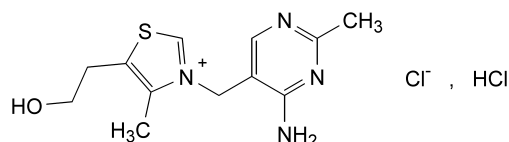
B. R = H: 1-methyl-1H-imidazole,

C. R = SCH₃: 1-methyl-2-(methylsulphanyl)-1H-imidazole.

01/2005:0303
corrected

THIAMINE HYDROCHLORIDE

Thiaini hydrochloridum



C₁₂H₁₈Cl₂N₄OS

*M*_r 337.3

DEFINITION

3-[(4-Amino-2-methylpyrimidin-5-yl)methyl]-5-(2-hydroxyethyl)-4-methylthiazolium chloride hydrochloride.

Content: 98.5 per cent to 101.0 per cent (anhydrous substance).

CHARACTERS

Appearance: white or almost white, crystalline powder or colourless crystals.

Solubility: freely soluble in water, soluble in glycerol, slightly soluble in alcohol.

IDENTIFICATION

First identification: A, C.

Second identification: B, C.

A. Infrared absorption spectrophotometry (2.2.24).

Comparison: thiamine hydrochloride CRS.

B. Dissolve about 20 mg in 10 ml of *water R*, add 1 ml of *dilute acetic acid R* and 1.6 ml of *1 M sodium hydroxide*, heat on a water-bath for 30 min and allow to cool. Add 5 ml of *dilute sodium hydroxide solution R*, 10 ml of *potassium ferricyanide solution R* and 10 ml of *butanol R* and shake vigorously for 2 min. The upper alcoholic layer shows an intense light-blue fluorescence, especially in ultraviolet light at 365 nm. Repeat the test using 0.9 ml of *1 M sodium hydroxide* and 0.2 g of *sodium sulphite R* instead of 1.6 ml of *1 M sodium hydroxide*. Practically no fluorescence is seen.

C. It gives reaction (a) of chlorides (2.3.1).

TESTS

Solution S. Dissolve 2.5 g in *distilled water R* and dilute to 25 ml with the same solvent.

Appearance of solution. The solution is clear (2.2.1) and not more intensely coloured than reference solution Y₇ or GY₇ (2.2.2, *Method II*).

Dilute 2.5 ml of solution S to 5 ml with *water R*.

pH (2.2.3): 2.7 to 3.3.

Dilute 2.5 ml of solution S to 10 ml with *water R*.

Related substances. Liquid chromatography (2.2.29).

Solution A. Add 5 volumes of *glacial acetic acid R* to 95 volumes of *water R* and mix.

Test solution. Dissolve 0.35 g of the substance to be examined in 15.0 ml of solution A and dilute to 100.0 ml with *water R*.

Reference solution (a). Dissolve 5 mg of the substance to be examined and 5 mg of *thiamine impurity E CRS* in 4 ml of solution A and dilute to 25.0 ml with *water R*. Dilute 5.0 ml of the solution to 25.0 ml with *water R*.

Reference solution (b). Dilute 1.0 ml of the test solution to 50.0 ml with *water R*. Dilute 5.0 ml of this solution to 25.0 ml with *water R*.

Column:

- **size:** $l = 0.25$ m, $\varnothing = 4.0$ mm,
- **stationary phase:** spherical *end-capped octadecylsilyl silica gel for chromatography R* (5 μ m) with a specific surface area of 350 m²/g and a pore size of 10 nm,
- **temperature:** 45 °C.

Mobile phase:

- **mobile phase A:** 3.764 g/l solution of *sodium hexanesulphonate R* adjusted to pH 3.1 with *phosphoric acid R*,
- **mobile phase B:** *methanol R*,

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)
0 - 25	90 → 70	10 → 30
25 - 33	70 → 50	30 → 50
33 - 40	50	50
40 - 45	50 → 90	50 → 10

Flow rate: 1.0 ml/min.

Detection: spectrophotometer at 248 nm.

Injection: 25 μ l.

Relative retention with reference to thiamine (retention time = about 30 min): impurity A = about 0.3; impurity B = about 0.9; impurity C = about 1.2.

System suitability: reference solution (a):

- **resolution:** minimum 1.6 between the peaks due to impurity E and to thiamine.

Limits:

- **any impurity:** not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.4 per cent),
- **total:** not more than 2.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (1.0 per cent),
- **disregard limit:** 0.125 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

Sulphates (2.4.13): maximum 300 ppm.

5 ml of solution S diluted to 15 ml with *distilled water R* complies with the limit test for sulphates.

Heavy metals (2.4.8): maximum 20 ppm.

12 ml of solution S complies with limit test A. Prepare the standard using *lead standard solution (2 ppm Pb) R*.

Water (2.5.12): maximum 5.0 per cent, determined on 0.40 g.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.110 g in 5 ml of *anhydrous formic acid R* and add 50 ml of *acetic anhydride R*. Titrate immediately with *0.1 M perchloric acid*, determining the end-point potentiometrically (2.2.20) and carrying out the titration within 2 min. Carry out a blank titration.

1 ml of *0.1 M perchloric acid* is equivalent to 16.86 mg of C₁₂H₁₈Cl₂N₄OS.

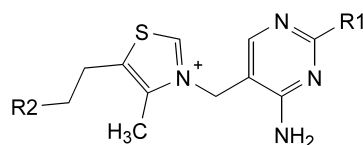
STORAGE

In a non-metallic container, protected from light.

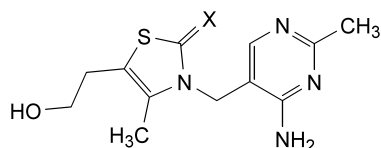
IMPURITIES

Specified impurities: A, B, C.

Other detectable impurities: D, E, F, G, H.

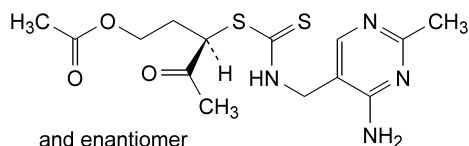


- A. R₁ = CH₃, R₂ = O-SO₃⁻: 3-[(4-amino-2-methylpyrimidin-5-yl)methyl]-4-methyl-5-[2-(sulphonatoxy)ethyl]thiazolium (thiamine sulphate ester),
- B. R₁ = H, R₂ = OH: 3-[(4-aminopyrimidin-5-yl)methyl]-5-(2-hydroxyethyl)-4-methylthiazolium (desmethylthiamine),
- C. R₁ = CH₃, R₂ = Cl: 3-[(4-amino-2-methylpyrimidin-5-yl)methyl]-5-(2-chloroethyl)-4-methylthiazolium (chlorothiamine),
- F. R₁ = C₂H₅, R₂ = OH: 3-[(4-amino-2-ethylpyrimidin-5-yl)methyl]-5-(2-hydroxyethyl)-4-methylthiazolium (ethylthiamine),
- G. R₁ = CH₃, R₂ = O-CO-CH₃: 5-[2-(acetyloxy)ethyl]-3-[(4-amino-2-methylpyrimidin-5-yl)methyl]-4-methylthiazolium (acetylthiamine),



D. X = O: 3-[(4-amino-2-methylpyrimidin-5-yl)methyl]-5-(2-hydroxyethyl)-4-methylthiazol-2(3H)-one (oxothiamine),

E. X = S: 3-[(4-amino-2-methylpyrimidin-5-yl)methyl]-5-(2-hydroxyethyl)-4-methylthiazol-2(3H)-thione (thioxothiamine),

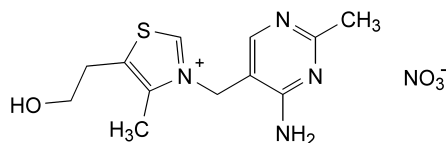


H. (3*RS*)-3-[[[(4-amino-2-methylpyrimidin-5-yl)methyl]thiocarbamoyl]sulphonyl]-4-oxopentyl acetate (ketodithiocarbamate).

01/2005:0531

THIAMINE NITRATE

Thiaini nitras


 $C_{12}H_{17}N_5O_4S$
 M_r 327.4

DEFINITION

3-[(4-Amino-2-methylpyrimidin-5-yl)methyl]-5-(2-hydroxyethyl)-4-methylthiazolium nitrate.

Content: 98.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

Appearance: white or almost white, crystalline powder or small, colourless crystals.

Solubility: sparingly soluble in water, freely soluble in boiling water, slightly soluble in alcohol and in methanol.

IDENTIFICATION

First identification: A, C.

Second identification: B, C.

A. Infrared absorption spectrophotometry (2.2.24).

Comparison: Ph. Eur. reference spectrum of thiamine nitrate.

B. Dissolve about 20 mg in 10 ml of *water R*, add 1 ml of *dilute acetic acid R* and 1.6 ml of 1 *M* sodium hydroxide, heat on a water-bath for 30 min and allow to cool. Add 5 ml of *dilute sodium hydroxide solution R*, 10 ml of *potassium ferricyanide solution R* and 10 ml of *butanol R* and shake vigorously for 2 min. The upper alcoholic layer shows an intense light-blue fluorescence, especially in ultraviolet light at 365 nm. Repeat the test using 0.9 ml of 1 *M* sodium hydroxide and 0.2 g of sodium sulphite *R* instead of 1.6 ml of 1 *M* sodium hydroxide. Practically no fluorescence is produced.

C. About 5 mg gives the reaction of nitrates (2.3.1).

TESTS

Solution S. Dissolve 1.0 g in *carbon dioxide-free water R* and dilute to 50 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and not more intensely coloured than reference solution Y₇ (2.2.2, *Method II*).

pH (2.2.3): 6.8 to 7.6 for solution S.

Related substances. Liquid chromatography (2.2.29).

Solution A. Add 5 volumes of *glacial acetic acid R* to 95 volumes of *water R* and mix.

Test solution. Dissolve 0.35 g of the substance to be examined in 15.0 ml of solution A and dilute to 100.0 ml with *water R*.

Reference solution (a). Dissolve 5 mg of the substance to be examined and 5 mg of *thiamine impurity E CRS* in 4 ml of solution A and dilute to 25.0 ml with *water R*. Dilute 5.0 ml of the solution to 25.0 ml with *water R*.

Reference solution (b). Dilute 1.0 ml of the test solution to 100.0 ml with *water R*.

Column:

– *size*: $l = 0.25$ m, $\varnothing = 4.0$ mm,

– *stationary phase*: spherical *end-capped octadecylsilyl silica gel for chromatography R* (4 μ m) with a specific surface area of 350 m²/g and a pore size of 10 nm,

– *temperature*: 45 °C.

Mobile phase:

– *mobile phase A*: 3.764 g/l solution of *sodium hexanesulphonate R* adjusted to pH 3.1 with *phosphoric acid R*,

– *mobile phase B*: *methanol R2*,

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)
0 - 25	90 → 70	10 → 30
25 - 33	70 → 50	30 → 50
33 - 40	50	50
40 - 45	50 → 90	50 → 10

Flow rate: 1.0 ml/min.

Detection: spectrophotometer at 248 nm.

Injection: 25 μ l.

Relative retention with reference to thiamine (retention time = about 30 min): impurity A = about 0.3; impurity B = about 0.9; impurity C = about 1.2.

System suitability: reference solution (a):

– *resolution*: minimum 1.6 between the peaks due to impurity E and to thiamine.

Limits:

– *any impurity*: not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (1.0 per cent),

– *total*: not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (1.5 per cent),

– *disregard limit*: 0.05 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

Heavy metals (2.4.8): maximum 20 ppm.

1.0 g complies with limit test D. Prepare the standard using 2 ml of *lead standard solution (10 ppm Pb) R*.

Loss on drying (2.2.32): maximum 1.0 per cent, determined on 1.000 g by drying in an oven at 100-105 °C.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

01/2005:0109

ASSAY

Dissolve 0.140 g in 5 ml of *anhydrous formic acid R* and add 50 ml of *acetic anhydride R*. Titrate immediately with 0.1 M *perchloric acid*, determining the end-point potentiometrically (2.2.20) and carrying out the titration within 2 min. Carry out a blank titration.

1.0 ml of 0.1 M *perchloric acid* is equivalent to 16.37 mg of $C_{12}H_{17}N_5O_4S$.

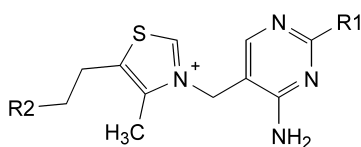
STORAGE

In a non-metallic container, protected from light.

IMPURITIES

Specified impurities: A, B, C.

Other detectable impurities: D, E, F, G, H.



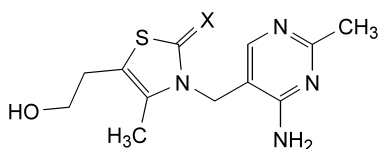
A. $R_1 = CH_3$, $R_2 = O-SO_3^-$: 3-[(4-amino-2-methylpyrimidin-5-yl)methyl]-4-methyl-5-[2-(sulphonatooxy)ethyl]thiazolium (thiamine sulphate ester),

B. $R_1 = H$, $R_2 = OH$: 3-[(4-aminopyrimidin-5-yl)methyl]-5-(2-hydroxyethyl)-4-methylthiazolium (desmethylthiamine),

C. $R_1 = CH_3$, $R_2 = Cl$: 3-[(4-amino-2-methylpyrimidin-5-yl)methyl]-5-(2-chloroethyl)-4-methylthiazolium (chlorothiamine),

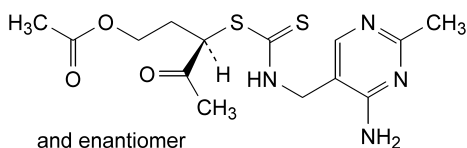
F. $R_1 = C_2H_5$, $R_2 = OH$: 3-[(4-amino-2-ethylpyrimidin-5-yl)methyl]-5-(2-hydroxyethyl)-4-methylthiazolium (ethylthiamine),

G. $R_1 = CH_3$, $R_2 = O-CO-CH_3$: 5-[2-(acetyloxy)ethyl]-3-[(4-amino-2-methylpyrimidin-5-yl)methyl]-4-methylthiazolium (acetylthiamine),



D. $X = O$: 3-[(4-amino-2-methylpyrimidin-5-yl)methyl]-5-(2-hydroxyethyl)-4-methylthiazol-2(3H)-one (oxothiamine),

E. $X = S$: 3-[(4-amino-2-methylpyrimidin-5-yl)methyl]-5-(2-hydroxyethyl)-4-methylthiazol-2(3H)-thione (thioxothiamine),

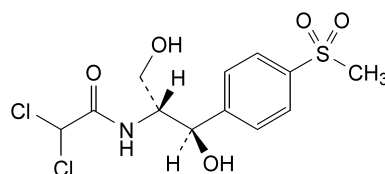


and enantiomer

H. (3*RS*)-3-[[[(4-amino-2-methylpyrimidin-5-yl)methyl]thiocarbamoyl]sulphonyl]-4-oxopentyl acetate (ketodithiocarbamate).

THIAMPHENICOL

Thiamphenicolum


 $C_{12}H_{15}Cl_2NO_5S$
 M_r 356.2

DEFINITION

Thiamphenicol contains not less than 98.0 per cent and not more than the equivalent of 100.5 per cent of 2,2-dichloro-*N*[(1*R*,2*R*)-2-hydroxy-1-(hydroxymethyl)-2-[4-(methylsulphonyl)phenyl]ethyl]acetamide, calculated with reference to the dried substance.

CHARACTERS

A fine, white or yellowish-white, crystalline powder or crystals, slightly soluble in water and in ethyl acetate, very soluble in dimethylacetamide, freely soluble in acetonitrile and in dimethylformamide, soluble in methanol, sparingly soluble in acetone and in ethanol.

A solution in ethanol is dextrorotatory and a solution in dimethylformamide is laevorotatory.

IDENTIFICATION

A. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *thiamphenicol CRS*. Dry the substances at 100 °C to 105 °C for 2 h and examine in the form of discs prepared using *potassium bromide R*.

B. Examine by thin-layer chromatography (2.2.27), using *silica gel GF₂₅₄ R* as the coating substance.

Test solution. Dissolve 0.1 g of the substance to be examined in *methanol R* and dilute to 10 ml with the same solvent.

Reference solution. Dissolve 0.1 g of *thiamphenicol CRS* in *methanol R* and dilute to 10 ml with the same solvent.

Apply separately to the plate 5 µl of each solution. Develop over a path of 10 cm using a mixture of 3 volumes of *methanol R* and 97 volumes of *ethyl acetate R*. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. The principal spot in the chromatogram obtained with the test solution is similar in position and size to the spot in the chromatogram obtained with the reference solution.

C. To 50 mg in a porcelain crucible add 0.5 g of *anhydrous sodium carbonate R*. Heat over an open flame for 10 min. Allow to cool. Take up the residue with 5 ml of *dilute nitric acid R* and filter. To 1 ml of the filtrate add 1 ml of *water R*. The solution gives reaction (a) of chlorides (2.3.1).

TESTS

Acidity or alkalinity. Shake 0.1 g with 20 ml of *carbon dioxide-free water R* and add 0.1 ml of *bromothymol blue solution R1*. Not more than 0.1 ml of 0.02 M *hydrochloric acid* or 0.02 M *sodium hydroxide* is required to change the colour of the indicator.

Specific optical rotation (2.2.7). Dissolve 1.25 g in *dimethylformamide R* and dilute to 25.0 ml with the same solvent. The specific optical rotation is -21 to -24 , calculated with reference to the dried substance.

Melting point (2.2.14). $163\text{ }^{\circ}\text{C}$ to $167\text{ }^{\circ}\text{C}$.

Absorbance (2.2.25). Dissolve 20 mg in *water R*, heating to about $40\text{ }^{\circ}\text{C}$, and dilute to 100.0 ml with the same solvent. Examined between 240 nm and 300 nm, the solution shows 2 absorption maxima, at 266 nm and 273 nm. The specific absorbances at these maxima are 25 to 28 and 21.5 to 23.5, respectively. Dilute 2.5 ml of the solution to 50.0 ml with *water R*. Examined between 200 nm and 240 nm, the solution shows an absorption maximum at 224 nm. The specific absorbance at this maximum is 370 to 400.

Chlorides (2.4.4). Shake 0.5 g with 30 ml of *water R* for 5 min and filter. 15 ml of the filtrate complies with the limit test for chlorides (200 ppm).

Heavy metals (2.4.8). 1.0 g complies with limit test C for heavy metals (10 ppm). Prepare the standard using 1 ml of *lead standard solution (10 ppm Pb R)*.

Loss on drying (2.2.32). Not more than 1.0 per cent, determined on 1.00 g by drying in an oven at $100\text{ }^{\circ}\text{C}$ to $105\text{ }^{\circ}\text{C}$.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 2.0 g.

ASSAY

Dissolve 0.300 g in 30 ml of *alcohol R*, add 20 ml of a 500 g/l solution of *potassium hydroxide R*, mix and heat under a reflux condenser for 4 h. Cool, add 100 ml of *water R*, neutralise with *dilute nitric acid R* and add 5 ml in excess. Titrate with 0.1 M *silver nitrate*, determining the end-point potentiometrically (2.2.20), using a silver indicator electrode and a mercurous sulphate reference electrode or any other appropriate electrode. Carry out a blank test.

1 ml of 0.1 M *silver nitrate* is equivalent to 17.81 mg of $\text{C}_{12}\text{H}_{15}\text{Cl}_2\text{NO}_5\text{S}$.

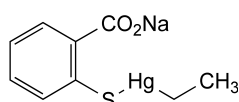
STORAGE

Store in an airtight container, protected from light.

01/2005:1625

THIOMERSAL

Thiomersalum



$\text{C}_9\text{H}_9\text{HgNaO}_2\text{S}$

M_r 404.8

DEFINITION

Sodium ethyl[2-sulphanylbenzoato(2-)-O,S]mercurate(1-).
Content: 97.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

Appearance: white or almost white, crystalline powder.

Solubility: freely soluble in water, sparingly soluble or soluble in alcohol, practically insoluble in methylene chloride.

IDENTIFICATION

First identification: B, D.

Second identification: A, C, D.

A. Melting point of the derivative (2.2.14): $103\text{ }^{\circ}\text{C}$ to $108\text{ }^{\circ}\text{C}$.

Dissolve 0.5 g in *water R* and dilute to 10 ml with the same solvent. Add 2 ml of *dilute hydrochloric acid R*. A white precipitate is formed. Wash the precipitate with *water R* and dry over *diphosphorus pentoxide R* at a pressure not exceeding 0.7 kPa.

B. Infrared absorption spectrophotometry (2.2.24).

Comparison: *thiomersal CRS*.

C. Treat 50 mg by the oxygen-flask method (2.5.10). Use a mixture of 1 ml of *strong hydrogen peroxide solution R* and 50 ml of *water R* to absorb the combustion products. To the solution add 5 ml of *dilute nitric acid R*. 0.1 ml of this solution gives reaction (a) of mercury (2.3.1). To the remaining part of the solution add 10 ml of *dilute hydrochloric acid R* and filter. 5 ml of the filtrate, without further addition of acid, gives reaction (a) of sulphates (2.3.1).

D. Solution S (see Tests) gives reaction (a) of sodium (2.3.1).

TESTS

Solution S. Dissolve 2.0 g in *carbon dioxide-free water R* and dilute to 25 ml with the same solvent.

Appearance of solution. Solution S is not more opalescent than reference suspension II (2.2.1) and not more intensely coloured than reference solution B₆ (2.2.2, *Method II*).

pH (2.2.3): 6.0 to 8.0.

Dilute 5 ml of solution S to 50 ml with *carbon dioxide-free water R*.

Inorganic mercury compounds: maximum 0.70 per cent.

Protect the solutions from light throughout the procedure.

Test solution. Dissolve 25 mg of the substance to be examined in *water R* and dilute to 25.0 ml with the same solvent.

Reference solution. Dissolve 95.0 mg of *mercuric chloride R* in *water R* and dilute to 50.0 ml with the same solvent.

Dilute 1.0 ml of the solution to 20.0 ml with *water R*.

Test reference and blank preparations. Label five 10 ml volumetric flasks A, B, C, D and E. Place 5 ml of the test solution in flasks A, B, C and D. To each of the flasks C and D add 0.5 ml of the reference solution. Dilute the contents of flasks A and C to 10 ml with *water R* (blank preparations A and C). Dilute the contents of flasks B and D to 10 ml with a freshly prepared 332 g/l solution of *potassium iodide R* (test preparation B and reference preparation D). Place 5 ml of a 332 g/l solution of *potassium iodide R* in flask E. Dilute to 10 ml with *water R* (blank preparation E).

Measure the absorbance (2.2.25) of each solution (A_a , A_b , A_c , A_d and A_e) at 323 nm using *water R* as the compensation liquid. Calculate the content of inorganic mercury compounds, expressed as Hg from the expression:

$$\frac{(A_b - A_a - A_e) \times m_R \times 0.1847}{(A_d - A_c - A_b + A_a) \times m_T}$$

m_R = mass of mercuric chloride in the reference solution in milligrams,

m_T = mass of the substance to be examined in milligrams.

Loss on drying (2.2.32): maximum 0.5 per cent, determined on 1.000 g by drying in a desiccator over *diphosphorus pentoxide R* at a pressure not exceeding 0.7 kPa for 24 h.

ASSAY

Place 0.5 g in a 100 ml long-necked combustion flask, add 5 ml of *sulphuric acid R* and heat gently until charring occurs, continue to heat and add dropwise *strong hydrogen*

peroxide solution R until the mixture is colourless. Dilute with *water R*, evaporate until slight fuming occurs, dilute to 10 ml with *water R*, cool down and titrate with 0.1 M ammonium thiocyanate using ferric ammonium sulphate solution R2 as indicator.

1 ml of 0.1 M ammonium thiocyanate is equivalent to 20.24 mg of $C_9H_9HgNaO_2S$.

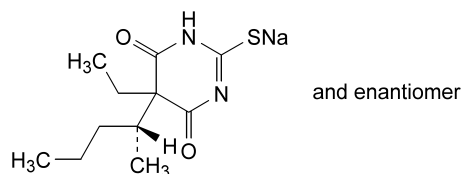
STORAGE

Protected from light.

01/2005:0212

THIOPENTAL SODIUM AND SODIUM CARBONATE

Thiopentalum natricum et natrii carbonas



DEFINITION

Thiopental sodium and sodium carbonate is a mixture of the sodium derivative of 5-ethyl-5-[(1*RS*)-1-methylbutyl]-2-thioxo-2,3-dihydropyrimidine-4,6-(1*H*,5*H*)-dione ($C_{11}H_{17}N_2NaO_2S$; M_r 264.3) and anhydrous sodium carbonate, containing the equivalent of not less than 84.0 per cent and not more than 87.0 per cent of thiopental and not less than 10.2 per cent and not more than 11.2 per cent of Na, both calculated with reference to the dried substance.

CHARACTERS

A yellowish-white powder, hygroscopic, freely soluble in water, partly soluble in ethanol.

IDENTIFICATION

First identification: A, B, E.

Second identification: A, C, D, E.

A. Acidify 10 ml of solution S (see Tests) with *dilute hydrochloric acid R*. An effervescence is produced. Shake with 20 ml of *ether R*. Separate the ether layer, wash with 10 ml of *water R*, dry over *anhydrous sodium sulphate R* and filter. Evaporate the filtrate to dryness and dry the residue at 100 °C to 105 °C (test residue). Determine the melting point (2.2.14) of the test residue. Mix equal parts of the test residue and *thiopental CRS* and determine the melting point of the mixture. The difference between the melting points (which are about 160 °C) is not greater than 2 °C.

B. Examine by infrared absorption spectrophotometry (2.2.24), comparing the test residue (see identification test A) with the spectrum obtained with *thiopental CRS*.

C. Examine by thin-layer chromatography (2.2.27), using *silica gel GF₂₅₄ R* as the coating substance.

Test solution. Dissolve 0.1 g of the substance to be examined in *water R* and dilute to 100 ml with the same solvent.

Reference solution. Dissolve 85 mg of *thiopental CRS* in 10 ml of *dilute sodium hydroxide solution R* and dilute to 100 ml with *water R*.

Apply separately to the plate 10 µl of each solution. Develop over a path of 18 cm using the lower layer of a mixture of 5 volumes of *concentrated ammonia R*,

15 volumes of *alcohol R* and 80 volumes of *chloroform R*. Examine immediately in ultraviolet light at 254 nm. The principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with the reference solution.

D. It gives the reaction of non-nitrogen substituted barbiturates (2.3.1).

E. It gives reaction (a) of sodium (2.3.1).

TESTS

Solution S. Dissolve 5.0 g in *carbon dioxide-free water R* and dilute to 50 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and not more intensely coloured than reference solution GY₃ (2.2.2, *Method II*).

Related substances. Examine by thin-layer chromatography (2.2.27), using *silica gel GF₂₅₄ R* as the coating substance.

Test solution. Dissolve 1.0 g of the substance to be examined in *water R* and dilute to 100 ml with the same solvent. Disregard any slight residue.

Reference solution. Dilute 0.5 ml of the test solution to 100 ml with *water R*.

Apply separately to the plate 20 µl of each solution. Develop over a path of 15 cm using the lower layer of a mixture of 5 volumes of *concentrated ammonia R*, 15 volumes of *alcohol R* and 80 volumes of *chloroform R*. Examine immediately in ultraviolet light at 254 nm. Any spot in the chromatogram obtained with the test solution, apart from the principal spot, is not more intense than the spot in the chromatogram obtained with the reference solution (0.5 per cent). Disregard any spot at the starting-point.

Chlorides (2.4.4). To 5 ml of solution S add 35 ml of *water R* and 10 ml of *dilute nitric acid R*. Shake with three quantities, each of 25 ml, of *ether R* and discard the ether layers. Eliminate the ether from the aqueous layer by heating on a water-bath. 15 ml of the aqueous layer complies with the limit test for chlorides (330 ppm).

Loss on drying (2.2.32). Not more than 2.5 per cent, determined on 0.50 g by drying in vacuo at 100 °C for 4 h.

ASSAY

Sodium. Dissolve 0.400 g in 30 ml of *water R*. Add 0.1 ml of *methyl red solution R* and titrate with 0.1 M *hydrochloric acid* until a red colour is obtained. Boil gently for 2 min. Cool and, if necessary, continue the titration with 0.1 M *hydrochloric acid* until the red colour is again obtained.

1 ml of 0.1 M *hydrochloric acid* is equivalent to 2.299 mg of Na.

Thiopental. Dissolve 0.150 g in 5 ml of *water R*. Add 2 ml of *dilute sulphuric acid R* and shake with four quantities, each of 10 ml, of *chloroform R*. Combine the chloroform layers, filter and evaporate the filtrate to dryness on a water-bath. Dissolve the residue in 30 ml of previously neutralised *dimethylformamide R* and add 0.1 ml of a 2 g/l solution of *thymol blue R* in *methanol R*. Titrate immediately with 0.1 M *lithium methoxide* until a blue colour is obtained. Protect the solution from atmospheric carbon dioxide during the titration.

1 ml of 0.1 M *lithium methoxide* is equivalent to 24.23 mg of $C_{11}H_{18}N_2O_2S$.

STORAGE

Store in an airtight container, protected from light.

01/2005:2005 *Reference solution (c).* Dilute 5.0 ml of reference solution (b) to 10.0 ml with a mixture of 2 volumes of *concentrated ammonia R* and 98 volumes of *methanol R*.

Plate: TLC silica gel R.

Mobile phase: *concentrated ammonia R*, *2-propanol R*, *methylene chloride R* (1:25:74 V/V/V).

Application: 5 µl; apply the test solution and reference solutions (b) and (c).

Development: over 2/3 of the plate.

Drying: in air.

Detection: spray with a freshly prepared mixture of 1 volume of *potassium iodobismuthate solution R* and 10 volumes of *dilute acetic acid R* and then with *dilute hydrogen peroxide solution R*. Immediately cover the plate with a glass plate.

Limits:

- *any impurity:* any spot, apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.2 per cent); not more than 2 such spots are more intense than the spot in the chromatogram obtained with reference solution (c) (0.1 per cent).

Heavy metals (2.4.8): maximum 20 ppm.

1.0 g complies with limit test C. Prepare the standard using 2 ml of *lead standard solution (10 ppm Pb) R*.

Loss on drying (2.2.32): maximum 0.5 per cent, determined on 1.000 g at 50 °C *in vacuo* for 4 h.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

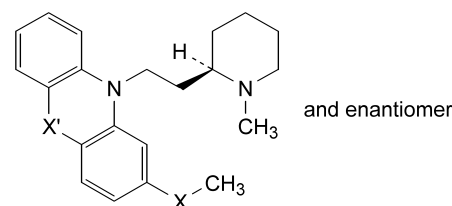
Dissolve 0.300 g in 60 ml of *anhydrous acetic acid R*. Titrate with 0.1 M *perchloric acid*, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *perchloric acid* is equivalent to 37.06 mg of C₂₁H₂₆N₂S₂.

STORAGE

Protected from light.

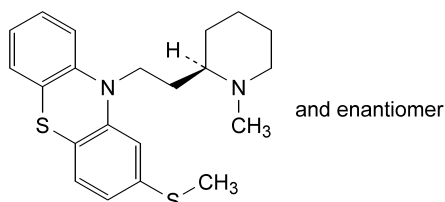
IMPURITIES



- X = X' = SO₂: 10-[2-[(2RS)-1-methylpiperidin-2-yl]ethyl]-2-(methylsulphonyl)-10H-phenothiazine 5,5-dioxide,
- X = SO, X' = S: 10-[2-[(2RS)-1-methylpiperidin-2-yl]ethyl]-2-(methylsulphinyl)-10H-phenothiazine,
- X = S, X' = SO: 10-[2-[(2RS)-1-methylpiperidin-2-yl]ethyl]-2-(methylsulphanyl)-10H-phenothiazine 5-oxide,
- X = X' = SO: 10-[2-[(2RS)-1-methylpiperidin-2-yl]ethyl]-2-(methylsulfinyl)-10H-phenothiazine 5-oxide,
- X = SO₂, X' = S: 10-[2-[(2RS)-1-methylpiperidin-2-yl]ethyl]-2-(methylsulphonyl)-10H-phenothiazine,

THIORIDAZINE

Thioridazinum



C₂₁H₂₆N₂S₂

M_r 370.6

DEFINITION

10-[2-[(2RS)-1-Methylpiperidin-2-yl]ethyl]-2-(methylsulphonyl)-10H-phenothiazine

Content: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

Appearance: white or almost white powder.

Solubilities: practically insoluble in water, very soluble in methylene chloride, freely soluble in methanol, soluble in alcohol.

IDENTIFICATION

First identification: A.

Second identification: B, C, D.

A. Infrared absorption spectrophotometry (2.2.24).

Comparison: Ph. Eur. reference spectrum of thioridazine.

B. It complies with the identification of phenothiazines by thin-layer chromatography (2.3.3).

C. Dissolve 5 mg in 2 ml of *sulphuric acid R*. A blue colour is produced.

D. Dissolve 20 mg in a mixture of 2 ml of *water R* and 0.2 ml of 1 M *sulphuric acid*. Add 1 ml of a 50 g/l solution of *silver nitrate R*. No precipitate is produced.

TESTS

Solution S. Dissolve 1.25 g in *methanol R* and dilute to 25 ml with the same solvent.

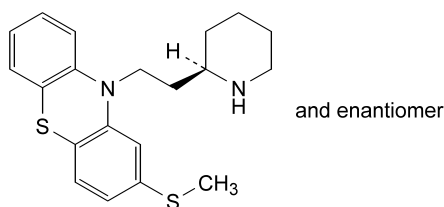
Appearance of solution. Solution S is clear (2.2.1) and not more intensely coloured than intensity 6 of the range of reference solutions of the most appropriate colour (2.2.2, *Method II*).

Related substances. Thin-layer chromatography (2.2.27). Carry out the test as quickly as possible and protected from light. Apply the test solution last.

Test solution. Dissolve 0.2 g of the substance to be examined in a mixture of 2 volumes of *concentrated ammonia R* and 98 volumes of *methanol R* and dilute to 10.0 ml with the same mixture of solvents.

Reference solution (a). Dilute 1.0 ml of the test solution to 100.0 ml with a mixture of 2 volumes of *concentrated ammonia R* and 98 volumes of *methanol R*. Dilute 5.0 ml of this solution to 10.0 ml with a mixture of 2 volumes of *concentrated ammonia R* and 98 volumes of *methanol R*.

Reference solution (b). Dilute 4.0 ml of reference solution (a) to 10.0 ml with a mixture of 2 volumes of *concentrated ammonia R* and 98 volumes of *methanol R*.

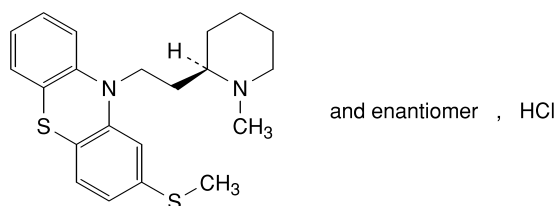


F. 2-(methylsulphanyl)-10-[2-[(2*RS*)-piperidin-2-yl]ethyl]-10*H*-phenothiazine.

01/2005:0586

THIORIDAZINE HYDROCHLORIDE

Thioridazini hydrochloridum



$C_{21}H_{27}ClN_2S_2$

M_r 407.0

DEFINITION

Thioridazine hydrochloride contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of 10-[2-[(2*RS*)-1-methylpiperidin-2-yl]ethyl]-2-(methylsulphanyl)-10*H*-phenothiazine hydrochloride, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, crystalline powder, freely soluble in water and in methanol, soluble in alcohol.

IDENTIFICATION

First identification: A, C.

Second identification: B, C.

- Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *thioridazine hydrochloride CRS*.
- It complies with the identification of phenothiazines by thin-layer chromatography (2.3.3).
- 0.2 g gives reaction (b) of chlorides (2.3.1).

TESTS

Carry out all operations protected from light.

Appearance of solution. Dissolve 1.0 g in *methanol R* and dilute to 20 ml with the same solvent. The solution is clear (2.2.1) and not more intensely coloured than intensity 6 of the range of reference solutions of the most appropriate colour (2.2.2, *Method II*).

pH (2.2.3). Dissolve 0.20 g in 20 ml of *carbon dioxide-free water R*. The pH of the solution is 4.2 to 5.2.

Related substances. Examine by thin-layer chromatography (2.2.27), using *silica gel G R* as the coating substance. Carry out all operations as rapidly as possible, protected from light. Apply the test solution last.

Test solution. Dissolve 0.2 g in a mixture of 2 volumes of *concentrated ammonia R* and 98 volumes of *methanol R* and dilute to 10 ml with the same mixture of solvents.

Reference solution (a). Dilute 2 ml of the test solution to 100 ml with a mixture of 2 volumes of *concentrated ammonia R* and 98 volumes of *methanol R*. Dilute 10 ml of this solution to 100 ml with a mixture of 2 volumes of *concentrated ammonia R* and 98 volumes of *methanol R*.

Reference solution (b). Dilute 1 ml of the test solution to 100 ml with a mixture of 2 volumes of *concentrated ammonia R* and 98 volumes of *methanol R*. Dilute 10 ml of this solution to 100 ml with a mixture of 2 volumes of *concentrated ammonia R* and 98 volumes of *methanol R*.

Apply separately to the plate 5 µl of each solution. Develop over a path of 15 cm using a mixture of 1 volume of *concentrated ammonia R*, 25 volumes of *2-propanol R* and 74 volumes of *chloroform R*. Allow the plate to dry in air. Spray first with a freshly prepared mixture of 1 volume of *potassium iodobismuthate solution R* and 10 volumes of *dilute acetic acid R* and then with freshly prepared *dilute hydrogen peroxide solution R*. Cover the plate immediately with a glass plate of the same size. Any spot in the chromatogram obtained with the test solution, apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (a) (0.2 per cent) and at most two such spots are more intense than the spot in the chromatogram obtained with the reference solution (b) (0.1 per cent). The test is not valid unless the spot in the chromatogram obtained with reference solution (b) is clearly visible.

Heavy metals (2.4.8). 1.0 g complies with the limit test C for heavy metals (20 ppm). Prepare the standard using 2 ml of *lead standard solution (10 ppm Pb) R*.

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C for 4 h.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.300 g in a mixture of 10 ml of *anhydrous acetic acid R* and 60 ml of *acetic anhydride R*. Titrate with 0.1 *M perchloric acid*, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 *M perchloric acid* is equivalent to 40.70 mg of $C_{21}H_{27}ClN_2S_2$.

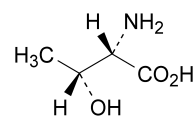
STORAGE

Store protected from light.

01/2005:1049

THREONINE

Threoninum



$C_4H_9NO_3$

M_r 119.1

DEFINITION

Threonine contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of (2*S*,3*R*)-2-amino-3-hydroxybutanoic acid, calculated with reference to the dried substance.

CHARACTERS

A white, crystalline powder or colourless crystals, soluble in water, practically insoluble in alcohol.

IDENTIFICATION

First identification: A, B.

Second identification: A, C, D.

- A. It complies with the test for specific optical rotation (see Tests).
- B. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *threonine CRS*. Examine the substances prepared as discs.
- C. Examine the chromatograms obtained in the test for ninhydrin-positive substances. The principal spot in the chromatogram obtained with test solution (b) is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).
- D. Mix 1 ml of a 2 g/l solution of the substance to be examined with 1 ml of a 20 g/l solution of *sodium periodate R*. Add 0.2 ml of *piperidine R* and 0.1 ml of a 25 g/l solution of *sodium nitroprusside R*. A blue colour develops that changes to yellow after a few minutes.

TESTS

Solution S. Dissolve 2.5 g in *carbon dioxide-free water R* and dilute to 100 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and colourless (2.2.2, *Method II*).

pH (2.2.3). The pH of solution S is 5.0 to 6.5.

Specific optical rotation (2.2.7). Dissolve 1.50 g in *water R* and dilute to 25.0 ml with the same solvent. The specific optical rotation is -27.6 to -29.0 , calculated with reference to the dried substance.

Ninhydrin-positive substances. Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel plate R*.

Test solution (a). Dissolve 0.10 g of the substance to be examined in *dilute hydrochloric acid R* and dilute to 10 ml with the same acid.

Test solution (b). Dilute 1 ml of test solution (a) to 50 ml with *water R*.

Reference solution (a). Dissolve 10 mg of *threonine CRS* in a 1 per cent V/V solution of *hydrochloric acid R* and dilute to 50 ml with the same acid solution.

Reference solution (b). Dilute 5 ml of test solution (b) to 20 ml with *water R*.

Reference solution (c). Dissolve 10 mg of *threonine CRS* and 10 mg of *proline CRS* in a 1 per cent V/V solution of *hydrochloric acid R* and dilute to 25 ml with the same acid solution.

Apply to the plate 5 µl of each solution. Allow the plate to dry in air. Develop over a path of 15 cm using a mixture of 20 volumes of *glacial acetic acid R*, 20 volumes of *water R* and 60 volumes of *butanol R*. Allow the plate to dry in air, spray with *ninhydrin solution R* and heat at 100 °C to 105 °C for 15 min. Any spot in the chromatogram obtained with test solution (a), apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.5 per cent). The test is not valid unless the chromatogram obtained with reference solution (c) shows two clearly separated principal spots.

Chlorides (2.4.4). Dilute 10 ml of solution S to 15 ml with *water R*. The solution complies with the limit test for chlorides (200 ppm).

Sulphates (2.4.13). Dissolve 0.5 g in *distilled water R* and dilute to 15 ml with the same solvent. The solution complies with the limit test for sulphates (300 ppm).

Ammonium (2.4.1). 0.10 g complies with limit test B for ammonium (200 ppm). Prepare the standard using 0.2 ml of *ammonium standard solution (100 ppm NH₄) R*.

Iron (2.4.9). In a separating funnel, dissolve 1.0 g in 10 ml of *dilute hydrochloric acid R*. Shake with three quantities, each of 10 ml, of *methyl isobutyl ketone R1*, shaking for 3 min each time. To the combined organic layers add 10 ml of *water R* and shake for 3 min. The aqueous layer complies with the limit test for iron (10 ppm).

Heavy metals (2.4.8). 2.0 g complies with limit test C for heavy metals (10 ppm). Prepare the standard using 2 ml of *lead standard solution (10 ppm Pb) R*.

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.100 g in 5 ml of *anhydrous formic acid R*. Add 30 ml of *anhydrous acetic acid R*. Titrate with 0.1 M *perchloric acid*, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *perchloric acid* is equivalent to 11.91 mg of C₄H₉NO₃.

STORAGE

Store protected from light.

01/2005:0865

THYME

Thymi herba

DEFINITION

Whole leaves and flowers separated from the previously dried stems of *Thymus vulgaris* L. or *Thymus zygis* L. or a mixture of both species.

Content: minimum 12 ml/kg of essential oil of which a minimum of 40 per cent is thymol and carvacrol (both C₁₀H₁₄O; M_r 150.2) (anhydrous drug).

CHARACTERS

Strong aromatic odour reminiscent of thymol.

Macroscopic and microscopic characters described under identification tests A and B.

IDENTIFICATION

- A. The leaf of *Thymus vulgaris* is usually 4 mm to 12 mm long and up to 3 mm wide, sessile or with a very short petiole. The lamina is tough, entire, lanceolate to ovate, covered on both surfaces by a grey or greenish-grey indumentum; the edges are markedly rolled up towards the abaxial surface. The midrib is depressed on the adaxial surface and is very prominent on the abaxial surface. The calyx is green, often with violet spots and is tubular; at the end are 2 lips of which the upper one is bent back and at the end has 3 lobes, the lower is longer and has 2 hairy teeth. After flowering, the calyx tube is closed by a crown of long, stiff hairs. The corolla, about twice as long as the calyx, is usually brownish in the dry state and is slightly bilabiate.

The leaf of *Thymus zygis* is usually 1.7 mm to 6.5 mm long and 0.4 mm to 1.2 mm wide; it is acicular to linear-lanceolate and the edges are markedly rolled towards the abaxial surface. Both surfaces of the lamina are green to greenish-grey and the midrib is sometimes violet; the edges, in particular at the base, have long, white hairs. The dried flowers are very similar to those of *Thymus vulgaris*.

B. Reduce to a powder (355). The powder of both species is greyish-green or greenish-brown. Examine under a microscope using *chloral hydrate solution R*. The epidermises of the leaves have cells with anticlinal walls which are sinuous and beaded and the stomata are diacytic (2.8.3); numerous secretory trichomes made up of 12 secretory cells, the cuticle of which is generally raised by the secretion to form a globular to ovoid bladder-like covering; the glandular trichomes have a unicellular stalk and a globular to ovoid head; the covering trichomes of the adaxial surface are common to both species; they have warty walls and are shaped as pointed teeth; the warty covering trichomes of the abaxial surface are of many types: unicellular, straight or slightly curved, and bicellular or tricellular, and often elbow-shaped (*Thymus vulgaris*); bicellular or tricellular, more or less straight (*Thymus zygis*). Fragments of calyx are covered by numerous, uniseriate trichomes with 5 or 6 cells and with a weakly striated cuticle. Fragments of the corolla have numerous uniseriate covering trichomes, often collapsed, and secretory trichomes generally with 12 cells. Pollen grains are relatively rare, spherical and smooth with 6 germinal slit-like pores, measuring about 35 µm in diameter. The powder of *Thymus zygis* also contains numerous thick bundles of fibres from the main veins and from fragments of stems.

C. Thin-layer chromatography (2.2.27).

Test solution. To 1.0 g of the powdered drug (355) add 5 ml of *methylene chloride R* and shake for 3 min, filter through about 2 g of *anhydrous sodium sulphate R*.

Reference solution. Dissolve 5 mg of *thymol R* and 10 µl of *carvacrol R* in 10 ml of *methylene chloride R*.

Plate: TLC silica gel *F*₂₅₄ plate *R*.

Mobile phase: *methylene chloride R*.

Application: 20 µl, as bands.

Development: over a path of 15 cm.

Drying: in air.

Detection A: examine in ultraviolet light at 254 nm.

Results A: see below the sequence of the zones present in the chromatograms obtained with the reference solution and the test solution.

Top of the plate	
Thymol: a quenching zone	A prominent quenching zone A quenching zone (thymol)
	Quenching zones
Reference solution	Test solution

Detection B: spray with *anisaldehyde solution R* using 10 ml for a plate 200 mm square and heat at 100-105 °C for 10 min.

Results B: see below the sequence of the zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other zones are present in the lower third of the chromatogram obtained with the test solution. The intensity of the zones due to thymol and carvacrol depends upon the species examined.

Top of the plate	
Thymol: a brownish-pink zone Carvacrol: a pale violet zone	A brownish-pink zone (thymol) A pale violet zone (carvacrol)
	A greyish-pink zone A violet zone (cineole and linalol) A greyish-brown zone (borneol) A violet-blue zone An intense violet zone
Reference solution	Test solution

D. Examine the chromatograms obtained in the assay for thymol and carvacrol.

Results: the characteristic peaks in the chromatogram obtained with the test solution are similar in retention time to those in the chromatogram obtained with the reference solution.

TESTS

Foreign matter (2.8.2): maximum 10 per cent of stems and maximum 2 per cent of other foreign matter. Stems must not be more than 1 mm in diameter and 15 mm in length. Leaves with long trichomes at their base and with weakly pubescent other parts (*Thymus serpyllum L.*) are absent.

Water (2.2.13): maximum 100 ml/kg, determined on 20.0 g of the powdered drug (355).

Total ash (2.4.16): maximum 15.0 per cent.

Ash insoluble in hydrochloric acid (2.8.1): maximum 3.0 per cent.

ASSAY

Essential oil (2.8.12). Use 30.0 g of the drug, a 1000 ml round-bottomed flask and 400 ml of *water R* as the distillation liquid. Distil at a rate of 2-3 ml/min for 2 h without *xylene R* in the graduated tube.

Thymol and carvacrol. Gas chromatography (2.2.28): use the normalisation procedure.

Test solution. Filter the essential oil obtained in the determination of essential oil over a small amount of *anhydrous sodium sulphate R* and dilute to 5.0 ml with *hexane R* by rinsing the apparatus and the anhydrous sodium sulphate.

Reference solution. Dissolve 0.20 g of *thymol R* and 50 mg of *carvacrol R* in *hexane R* and dilute to 5.0 ml with the same solvent.

Column:

- **material:** fused silica,
- **size:** *l* = 30-60 m, Ø = 0.25 mm,
- **stationary phase:** *macrogol 20 000 R* (film thickness 0.25 µm).

Carrier gas: *nitrogen for chromatography R* or *helium for chromatography R*.

Flow rate: 1-2 ml/min.

Split ratio: 1:100.

Temperature:

	Time (min)	Temperature (°C)
Column	0 - 45	40 → 220
Injection port		190
Detector		210

Detection: flame ionisation.

Injection: 0.2 µl.

Elution order: order indicated in the composition of the reference solution. Record the retention times of these substances.

System suitability: reference solution:

- resolution: minimum 1.5 between the peaks due to thymol and carvacrol.

Using the retention times determined from the chromatogram obtained with the reference solution, locate the components of the reference solution in the chromatogram obtained with the test solution.

Determine the percentage content of thymol and carvacrol. Disregard the peak due to hexane.

01/2005:1374

THYME OIL

Thymi aetheroleum

DEFINITION

Essential oil obtained by steam distillation from the fresh flowering aerial parts of *Thymus vulgaris* L., *T. zygis* Loeffl. ex L. or a mixture of both species.

CHARACTERS

Appearance: clear, yellow or very dark reddish-brown, mobile liquid with a characteristic, aromatic, spicy odour, reminiscent of thymol.

Solubility: miscible with ethanol and with light petroleum.

IDENTIFICATION

First identification: B.

Second identification: A.

A. Thin-layer chromatography (2.2.27).

Test solution. Dissolve 0.2 g of the substance to be examined in pentane R and dilute to 10 ml with the same solvent.

Reference solution. Dissolve 0.15 g of thymol R, 25 mg of α-terpineol R, 40 µl of linalol R and 10 µl of carvacrol R in pentane R and dilute to 10 ml with the same solvent.

Plate: TLC silica gel plate R.

Mobile phase: ethyl acetate R, toluene R (5:95 V/V).

Application: 20 µl, as bands.

Development: over a path of 15 cm.

Drying: in air.

Detection: spray with anisaldehyde solution R and heat the plate at 100-105 °C for 5-10 min while observing. Examine in daylight.

Results: see below the sequence of the zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other bands may be present in the chromatogram obtained with the test solution.

Top of the plate	
	A large violet zone (hydrocarbons) (at the solvent front)
Thymol: a brownish-pink zone	A brownish-pink zone (thymol)
Carvacrol: a pale violet zone	A pale violet zone (carvacrol)
Linalol: a violet zone	A violet zone (linalol)
α-Terpineol: a violet zone	A violet zone (α-terpineol)
Reference solution	Test solution

B. Examine the chromatograms obtained in the test for chromatographic profile.

Results: the characteristic peaks in the chromatogram obtained with the test solution are similar in retention time to those in the chromatogram obtained with the reference solution.

TESTS

Relative density (2.2.5): 0.915 to 0.935.

Refractive index (2.2.6): 1.490 to 1.505.

Chromatographic profile. Gas chromatography (2.2.28): use the normalisation procedure.

Test solution. The substance to be examined.

Reference solution. Dissolve 0.15 g of β-myrcene R, 0.1 g of γ-terpinene R, 0.1 g of p-cymene R, 0.1 g of linalol R, 0.2 g of terpinen-4-ol R, 0.2 g of thymol R and 50 mg of carvacrol R in 5 ml of hexane R.

Column:

- material: fused silica,
- size: *l* = 30 m (a film thickness of 1 µm may be used) to 60 m (a film thickness of 0.2 µm may be used), Ø = 0.25-0.53 mm,
- stationary phase: macrogol 20 000 R.

Carrier gas: helium for chromatography R.

Split ratio: 1:100.

Temperature:

	Time (min)	Temperature (°C)
Column	0 - 15 15 - 55	60 60→180
Injection port		200
Detector		220

Detection: flame ionisation.

Injection: 0.2 µl.

Elution order: order indicated in the composition of the reference solution. Record the retention times of these substances.

System suitability: reference solution:

- resolution: minimum 1.5 between the peaks due to thymol and carvacrol,
- number of theoretical plates: minimum 30 000, calculated for the peak due to p-cymene at 80 °C.

Using the retention times determined from the chromatogram obtained with the reference solution, locate the components of the reference solution on the chromatogram obtained with the test solution. Disregard the peak due to hexane.

Determine the percentage content of these components. The limits are within the following ranges:

- β-myrcene: 1.0 per cent to 3.0 per cent,
- γ-terpinene: 5.0 per cent to 10.0 per cent,
- p-cymene: 15.0 per cent to 28.0 per cent,

- *linalol*: 4.0 per cent to 6.5 per cent,
- *terpinen-4-ol*: 0.2 per cent to 2.5 per cent,
- *thymol*: 36.0 per cent to 55.0 per cent,
- *carvacrol*: 1.0 per cent to 4.0 per cent.

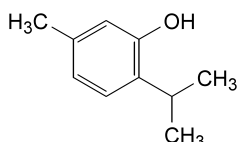
STORAGE

In a well-filled, airtight container, protected from light, at a temperature not exceeding 25 °C.

01/2005:0791

THYMOL

Thymolum

C₁₀H₁₄OM_r 150.2

DEFINITION

Thymol is 5-methyl-2-(methylethyl)phenol.

CHARACTERS

Colourless crystals, very slightly soluble in water, very soluble in alcohol, freely soluble in essential oils and in fatty oils, sparingly soluble in glycerol. It dissolves in dilute solutions of alkali hydroxides.

IDENTIFICATION

First identification: B.

Second identification: A, C, D.

- Melting point (2.2.14): 48 °C to 52 °C.
- Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *thymol CRS*.
- Dissolve 0.2 g with heating in 2 ml of *dilute sodium hydroxide solution R* and add 0.2 ml of *chloroform R*. Heat on a water-bath. A violet colour develops.
- Dissolve about 2 mg in 1 ml of *anhydrous acetic acid R*. Add 0.15 ml of *sulphuric acid R* and 0.05 ml of *nitric acid R*. A bluish-green colour develops.

TESTS

Appearance of solution. Dissolve 1.0 g in 10 ml of *dilute sodium hydroxide solution R*. The solution is not more opalescent than reference suspension IV (2.2.1) and not more intensely coloured than reference solution R₆ (2.2.2, *Method II*).

Acidity. To 1.0 g in a 100 ml glass-stoppered conical flask add 20 ml of *water R*. Boil until dissolution is complete, cool and stopper the flask. Shake vigorously for 1 min. Add a few crystals of the substance to be examined to initiate crystallisation. Shake vigorously for 1 min and filter. To 5 ml of the filtrate, add 0.05 ml of *methyl red solution R* and 0.05 ml of 0.01 M *sodium hydroxide*. The solution is yellow.

Related substances. Examine by gas chromatography (2.2.28).

Test solution. Dissolve 0.100 g in *alcohol R* and dilute to 10.0 ml with the same solvent.

Reference solution (a). Dilute 1 ml of the test solution to 100 ml with *alcohol R*.

Reference solution (b). Dilute 1 ml of reference solution (a) to 10 ml with *alcohol R*.

Reference solution (c). Dilute 5 ml of reference solution (b) to 10 ml with *alcohol R*.

The chromatography may be carried out using:

- a glass or steel column 4 m long and 2 mm in internal diameter packed with *diatomaceous earth for gas chromatography R*, impregnated with a mixture suitable for the separation of free fatty acids,
- *nitrogen for chromatography R* as the carrier gas at a flow rate of 30 ml/min,
- a flame-ionisation detector,

maintaining the temperature of the column at 80 °C, that of the injection port at 250 °C and that of the detector at 300 °C.

Inject 1 µl of each solution and, after 2 min, increase the temperature of the column to 240 °C at a rate of 8 °C/min and maintain at this temperature for 15 min. The test is not valid unless the peak in the chromatogram obtained with reference solution (b) has a signal-to-noise ratio not less than five. In the chromatogram obtained with the test solution, the sum of the areas of the peaks, apart from the principal peak, is not greater than the area of the principal peak in the chromatogram obtained with reference solution (a) (1.0 per cent). Disregard any peak with an area less than that of the principal peak in the chromatogram obtained with reference solution (c).

Residue on evaporation. Evaporate 2.00 g on a water-bath and heat in an oven at 100 °C to 105 °C for 1 h. The residue weighs not more than 1.0 mg (0.05 per cent).

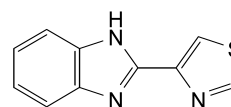
STORAGE

Store protected from light.

01/2005:0866

TIABENDAZOLE

Tiabendazolum

C₁₀H₇N₃SM_r 201.2

DEFINITION

Tiabendazole contains not less than 98.0 per cent and not more than the equivalent of 101.0 per cent of 2-(thiazol-4-yl)-1H-benzimidazole, calculated with reference to the anhydrous substance.

CHARACTERS

A white or almost white, crystalline powder, practically insoluble in water, slightly soluble in alcohol and in methylene chloride. It dissolves in dilute mineral acids. It melts at about 300 °C.

IDENTIFICATION

First identification: B.

Second identification: A, C, D.

- Dissolve 25 mg in 0.1 M *hydrochloric acid* and dilute to 100.0 ml with the same acid. Dilute 2.0 ml of the solution to 100.0 ml with 0.1 M *hydrochloric acid*. Examined between 230 nm and 350 nm (2.2.25), the solution shows two absorption maxima, at 243 nm and 302 nm. The ratio of the absorbance measured at the maximum at 302 nm to that measured at the maximum at 243 nm is 1.8 to 2.1.

- B. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *tiabendazole CRS*. Examine the substances prepared as discs.
- C. Examine the chromatograms obtained in the test for related substances in ultraviolet light at 254 nm. The principal spot in the chromatogram obtained with test solution (b) is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a).
- D. Dissolve about 5 mg in 0.1 M hydrochloric acid and dilute to 5 ml with the same acid. Add 3 mg of *p*-phenylenediamine dihydrochloride *R* and shake until dissolved. Add 0.1 g of zinc powder *R*, mix, allow to stand for 2 min and add 5 ml of ferric ammonium sulphate solution *R2*. A bluish-violet colour develops.

TESTS

Related substances. Examine by thin-layer chromatography (2.2.27), using silica gel HF_{254} *R* as the coating substance.

Test solution (a). Dissolve 0.10 g of the substance to be examined in methanol *R* and dilute to 10 ml with the same solvent.

Test solution (b). Dilute 2 ml of test solution (a) to 20 ml with methanol *R*.

Reference solution (a). Dissolve 20 mg of *tiabendazole CRS* in methanol *R* and dilute to 20 ml with the same solvent.

Reference solution (b). Dilute 1 ml of test solution (b) to 10 ml with methanol *R*.

Reference solution (c). Dilute 1 ml of test solution (b) to 25 ml with methanol *R*.

Apply separately to the plate 20 µl of each solution. Develop over a path of 15 cm using a mixture of 2.5 volumes of water *R*, 10 volumes of acetone *R*, 25 volumes of glacial acetic acid *R* and 62.5 volumes of toluene *R*. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. Any spot in the chromatogram obtained with test solution (a), apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (b) (1.0 per cent) and at most one such spot is more intense than the spot in the chromatogram obtained with reference solution (c) (0.4 per cent).

***o*-Phenylenediamine.** To 5.0 g in a flask fitted with a ground-glass stopper, add 25 ml of a mixture of 1 volume of methanol *R* and 2 volumes of water *R*. Shake for 3 min. Filter through a sintered-glass filter (16) under reduced pressure. To 10 ml of the filtrate add 0.5 ml of hydrochloric acid *R* and 0.5 ml of acetylacetone *R* and shake until the solution is clear. The solution is not more intensely coloured than reference solution *R*₇ (2.2.2, Method I) (10 ppm).

Heavy metals (2.4.8). 1.0 g complies with limit test D for heavy metals (20 ppm). Prepare the standard using 2 ml of lead standard solution (10 ppm Pb) *R*.

Water (2.5.12). Not more than 0.5 per cent, determined on 1.00 g by the semi-micro determination of water.

Sulphated ash (2.4.14). Not more than 0.2 per cent, determined on 1.0 g.

ASSAY

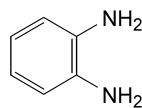
Dissolve 0.150 g in 30 ml of anhydrous acetic acid *R*. Titrate with 0.1 M perchloric acid, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M perchloric acid is equivalent to 20.12 mg of C₂₈H₄₇N₄O₄S.

STORAGE

Store protected from light.

IMPURITIES

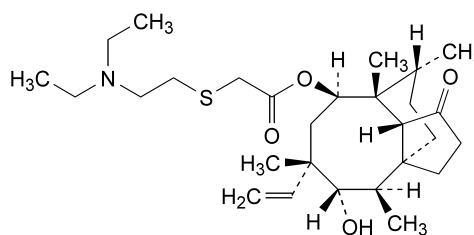


A. benzene-1,2-diamine.

01/2005:1660

TIAMULIN FOR VETERINARY USE

Tiamulinum ad usum veterinarium



C₂₈H₄₇N₄O₄S

*M*_r 493.8

DEFINITION

(3*a*S,4*R*,5*S*,6*S*,8*R*,9*R*,9*a**R*,10*R*)-6-Ethenyl-5-hydroxy-4,6,9,10-tetramethyl-1-oxodecahydro-3*a*,9-propano-3*a**H*-cyclopentacycloocten-8-yl [[2-(diethylamino)ethyl]sulphonyl]acetate.

Content: 96.5 per cent to 102.0 per cent (dried substance).

CHARACTERS

Appearance: sticky, translucent yellowish mass, slightly hygroscopic.

Solubility: practically insoluble in water, very soluble in methylene chloride, freely soluble in anhydrous ethanol.

IDENTIFICATION

Infrared absorption spectrophotometry (2.2.24).

Comparison: *Ph. Eur.* reference spectrum of tiamulin.

TESTS

Appearance of solution. The solution is clear (2.2.1) and its absorbance (2.2.25) at 420 nm is maximum 0.050.

Dissolve 2.5 g in 50 ml of methanol *R*.

Related substances. Liquid chromatography (2.2.29).

Ammonium carbonate buffer solution pH 10.0. Dissolve 10.0 g of ammonium carbonate *R* in water *R*, add 22 ml of perchloric acid solution *R* and dilute to 1000.0 ml with water *R*. Adjust to pH 10.0 with concentrated ammonia *R1*.
Solvent mixture: ammonium carbonate buffer solution pH 10.0, acetonitrile *R1* (50:50 V/V).

Test solution. Dissolve 0.200 g of the substance to be examined in the solvent mixture and dilute to 50.0 ml with the solvent mixture.

Reference solution (a). Dissolve 0.250 g of tiamulin hydrogen fumarate *CRS* in the solvent mixture and dilute to 50.0 ml with the solvent mixture.

Reference solution (b). Dilute 1.0 ml of the test solution to 100.0 ml with the solvent mixture.

Reference solution (c). Dilute 0.1 ml of toluene *R* to 100 ml with acetonitrile *R*. Dilute 0.1 ml of this solution to 100.0 ml with the solvent mixture.

Column:

- size: $l = 0.15$ m, $\varnothing = 4.6$ mm,
- stationary phase: end-capped octadecylsilyl silica gel for chromatography R (5 μ m),
- temperature: 30 °C.

Mobile phase: acetonitrile R1, ammonium carbonate buffer solution pH 10.0, methanol R1 (21:30:49 V/V/V).

Flow rate: 1.0 ml/min.

Detection: spectrophotometer at 212 nm.

Injection: 20 μ l.

Run time: 3 times the retention time of tiamulin.

Relative retention with reference to tiamulin (retention time = about 18 min): impurity A = about 0.22; impurity B = about 0.5; impurity C = about 0.66; impurity D = about 1.1; impurity F = about 1.6; impurity E = about 2.4.

System suitability: reference solution (a):

- baseline separation between the peaks due to tiamulin and impurity D.

Limits:

- impurities A, B, C, D, E, F: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (1.0 per cent),
- any other impurity: for each impurity, not more than 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent),
- total: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (b) (3.0 per cent),
- disregard limit: 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent); disregard any peak present in the chromatogram obtained with reference solution (c).

Loss on drying (2.2.32): maximum 1.0 per cent, determined on 1.000 g by drying in an oven at 80 °C.

Bacterial endotoxins (2.6.14, Method D): less than 0.4 IU/mg, determined in a 1 mg/ml solution in anhydrous ethanol R (endotoxin free) diluted 1:40 with water for bacterial endotoxins test.

ASSAY

Liquid chromatography (2.2.29) as described in the test for related substances with the following modification.

Injection: test solution and reference solution (a).

Calculate the percentage content of $C_{28}H_{47}NO_4S$, from the declared content of tiamulin hydrogen fumarate CRS.

STORAGE

Protected from light.

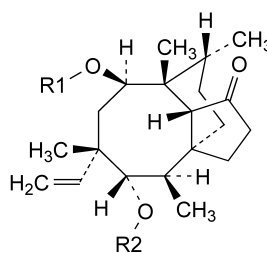
LABELLING

The label states where appropriate that the substance is free from bacterial endotoxins.

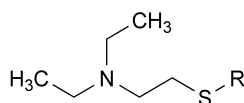
IMPURITIES

Specified impurities: A, B, C, D, E, F.

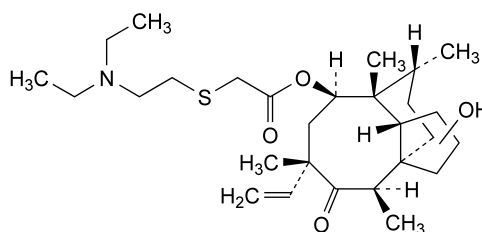
Other detectable impurities: G, H, I, J, K, L, M, N, O, P, Q, R.



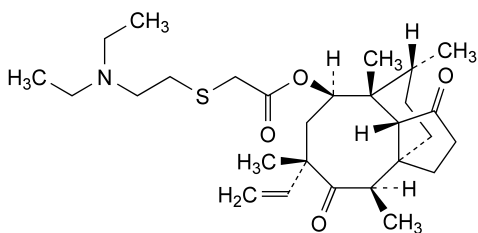
- A. $R_1 = R_2 = H$: (3a*S*,4*R*,5*S*,6*S*,8*R*,9*R*,9a*R*,10*R*)-6-ethenyl-5,8-dihydroxy-4,6,9,10-tetramethyloctahydro-3a,9-propano-3a*H*-cyclopentacycloocten-1(4*H*)-one (mutilin),
- G. $R_1 = CO-CH_2OH$, $R_2 = H$: (3a*S*,4*R*,5*S*,6*S*,8*R*,9*R*,9a*R*,10*R*)-6-ethenyl-5-hydroxy-4,6,9,10-tetramethyl-1-oxodecahydro-3a,9-propano-3a*H*-cyclopentacycloocten-8-yl hydroxyacetate (pleuromutilin),
- J. $R_1 = CO-CH_3$, $R_2 = H$: (3a*S*,4*R*,5*S*,6*S*,8*R*,9*R*,9a*R*,10*R*)-6-ethenyl-5-hydroxy-4,6,9,10-tetramethyl-1-oxodecahydro-3a,9-propano-3a*H*-cyclopentacycloocten-8-yl acetate (mutilin 14-acetate),
- K. $R_1 = H$, $R_2 = CO-CH_3$: (3a*S*,4*R*,5*S*,6*S*,8*R*,9*R*,9a*R*,10*R*)-6-ethenyl-8-hydroxy-4,6,9,10-tetramethyl-1-oxodecahydro-3a,9-propano-3a*H*-cyclopentacycloocten-5-yl acetate (mutilin 11-acetate),
- L. $R_1 = CO-CH_2-O-SO_2-C_6H_4-pCH_3$, $R_2 = H$: (3a*S*,4*R*,5*S*,6*S*,8*R*,9*R*,9a*R*,10*R*)-6-ethenyl-5-hydroxy-4,6,9,10-tetramethyl-1-oxodecahydro-3a,9-propano-3a*H*-cyclopentacycloocten-8-yl [[(4-methylphenyl)sulphonyl]oxy]acetate (pleuromutilin 22-tosylate),
- M. $R_1 = R_2 = CO-CH_3$: (3a*S*,4*R*,5*S*,6*S*,8*R*,9*R*,9a*R*,10*R*)-6-ethenyl-4,6,9,10-tetramethyl-1-oxodecahydro-3a,9-propano-3a*H*-cyclopentacycloocten-5,8-diyl diacetate (mutilin 11,14-diacetate),
- P. $R_1 = CO-CH_2-O-SO_2-C_6H_5$, $R_2 = H$: (3a*S*,4*R*,5*S*,6*S*,8*R*,9*R*,9a*R*,10*R*)-6-ethenyl-5-hydroxy-4,6,9,10-tetramethyl-1-oxodecahydro-3a,9-propano-3a*H*-cyclopentacycloocten-8-yl [(phenylsulphonyl)oxy]acetate,



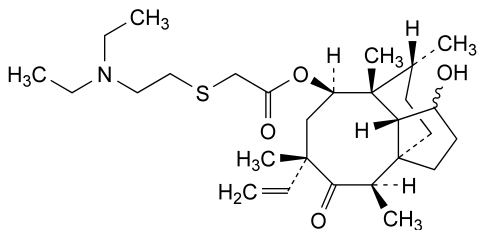
- B. $R = CH_2-C_6H_5$: 2-(benzylsulphanyl)-*N,N*-diethylethanamine,
- C. $R = S-CH_2-CH_2-N(C_2H_5)_2$: 2,2'-(disulphane-1,2-diyl)bis(*N,N*-diethylethanamine),
- O. $R = H$: 2-(diethylamino)ethanethiol,



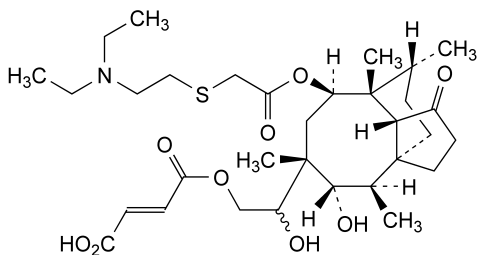
- D. (3a*R*,4*R*,6*S*,8*R*,9*R*,9a*R*,10*R*)-6-ethenylhydroxy-4,6,9,10-tetramethyl-5-oxodecahydro-3a,9-propano-3a*H*-cyclopentacycloocten-8-yl [[2-(diethylamino)ethyl]sulphonyl]acetate,



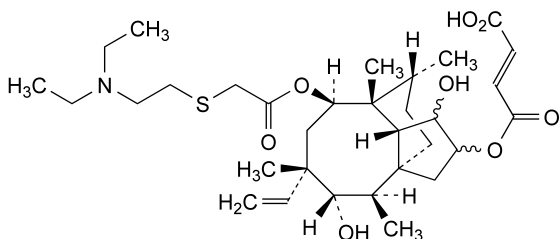
- E. (3a*S*,4*R*,6*S*,8*R*,9*R*,9a*R*,10*R*)-6-ethenyl-4,6,9,10-tetramethyl-1,5-dioxodecahydro-3a,9-propano-3a*H*-cyclopentacycloocten-8-yl [[2-(diethylamino)ethyl]sulphonyl]acetate (11-oxotiamulin),



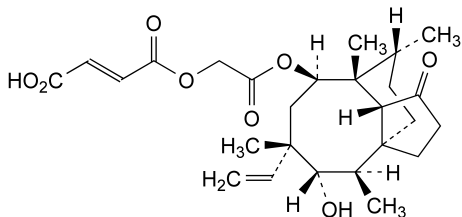
- F. (1*R*,3a*R*,4*R*,6*S*,8*R*,9*R*,9a*R*,10*R*)-6-ethenyl-1-hydroxy-4,6,9,10-tetramethyl-5-oxodecahydro-3a,9-propano-3a*H*-cyclopentacycloocten-8-yl [[2-(diethylamino)ethyl]sulphonyl]acetate (1-hydroxy-11-oxotiamulin),



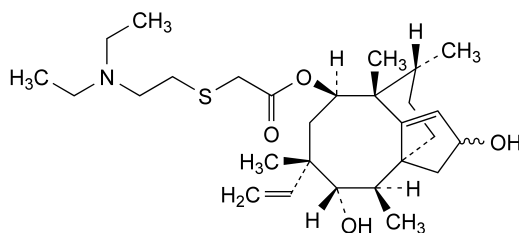
- H. (2*E*)-4-[[2-(3a*S*,4*R*,5*S*,6*R*,8*R*,9*R*,9a*R*,10*R*)-8-[[[2-(diethylamino)ethyl]sulphonyl]acetyl]oxy]-5-hydroxy-4,6,9,10-tetramethyl-1-oxodecahydro-3a,9-propano-3a*H*-cyclopentacycloocten-6-yl]-2-hydroxyethoxy]-4-oxobut-2-enoic acid (19,20-dihydroxytiamulin 20-fumarate),



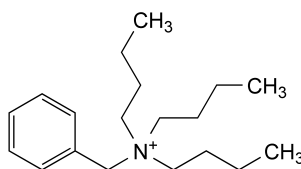
- I. (2*E*)-4-[[2-(3a*S*,4*R*,5*S*,6*S*,8*R*,9*R*,9a*R*,10*R*)-8-[[[2-(diethylamino)ethyl]sulphonyl]acetyl]oxy]-6-ethenyl-1,5-dihydroxy-4,6,9,10-tetramethyldecahydro-3a,9-propano-3a*H*-cyclopentacycloocten-2-yl]oxy]-4-oxobut-2-enoic acid (2,3-dihydroxytiamulin 2-fumarate),



- N. (2*E*)-4-[[2-[(3a*S*,4*R*,5*S*,6*S*,8*R*,9*R*,9a*R*,10*R*)-6-ethenyl-5-hydroxy-4,6,9,10-tetramethyl-1-oxodecahydro-3a,9-propano-3a*H*-cyclopentacycloocten-8-yl]oxy]-2-oxoethoxy]-4-oxobut-2-enoic acid (pleuromutilin 22-fumarate),



- Q. (3a*S*,4*R*,5*S*,6*S*,8*R*,9*R*,10*R*)-6-ethenyl-2,5-dihydroxy-4,6,9,10-tetramethyl-2,3,4,5,6,7,8,9-octahydro-3a,9-propano-3a*H*-cyclopentacycloocten-8-yl [[2-(diethylamino)ethyl]sulphonyl]acetate (3,4-didehydro-2-hydroxytiamulin),

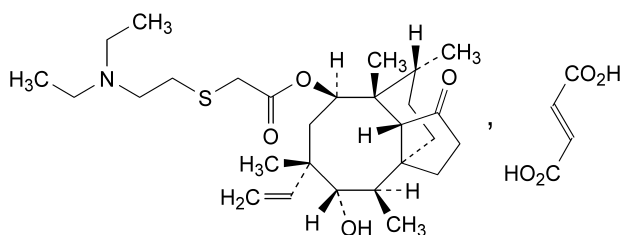


- R. *N*-benzyl-*N,N*-dibutylbutan-1-aminium.

01/2005:1659

TIAMULIN HYDROGEN FUMARATE FOR VETERINARY USE

Tiamulini hydrogenofumaras ad usum
veterinarium

 $C_{32}H_{51}NO_8S$ M_r 610

DEFINITION

(3a*S*,4*R*,5*S*,6*S*,8*R*,9*R*,9a*R*,10*R*)-6-Ethenyl-5-hydroxy-4,6,9,10-tetramethyl-1-oxodecahydro-3a,9-propano-3a*H*-cyclopentacycloocten-8-yl [[2-(diethylamino)ethyl]sulphonyl]acetate hydrogen (*E*)-but-2-enedioate.

Content: 96.5 per cent to 102.0 per cent (dried substance).

CHARACTERS

Appearance: white or light yellow, crystalline powder.

Solubility: soluble in water, freely soluble in anhydrous ethanol and soluble in methanol.

IDENTIFICATION

Infrared absorption spectrophotometry (2.2.24).

Comparison: tiamulin hydrogen fumarate CRS.

TESTS

pH (2.2.3): 3.1 to 4.1.

Dissolve 0.5 g in carbon dioxide-free water *R* and dilute to 50 ml with the same solvent.

Related substances. Liquid chromatography (2.2.29).

Ammonium carbonate buffer solution pH 10.0. Dissolve 10.0 g of ammonium carbonate *R* in water *R*, add 22 ml of perchloric acid solution *R* and dilute to 1000.0 ml with water *R*. Adjust to pH 10.0 with concentrated ammonia *R1*.

Solvent mixture: ammonium carbonate buffer solution pH 10.0, *acetonitrile R1* (50:50 V/V).

Test solution. Dissolve 0.200 g of the substance to be examined in the solvent mixture and dilute to 50.0 ml with the solvent mixture.

Reference solution (a). Dissolve 0.200 g of *tiamulin hydrogen fumarate CRS* in the solvent mixture and dilute to 50.0 ml with the solvent mixture.

Reference solution (b). Dilute 1.0 ml of the test solution to 100.0 ml with the solvent mixture.

Reference solution (c). Dissolve 40.0 mg of *fumaric acid R* in the solvent mixture and dilute to 50.0 ml with the solvent mixture.

Reference solution (d). Dissolve 4 mg of *tiamulin for peak identification CRS* (tiamulin hydrogen fumarate containing impurities B, C, D, F, H and I) in the solvent mixture and dilute to 1 ml with the solvent mixture.

Column:

- size: $l = 0.15$ m, $\varnothing = 4.6$ mm,
- stationary phase: end-capped octadecylsilyl silica gel for chromatography *R* (5 μ m),
- temperature: 30 °C.

Mobile phase: *acetonitrile R1*, ammonium carbonate buffer solution pH 10.0, *methanol R1* (21:30:49 V/V/V).

Flow rate: 1.0 ml/min.

Detection: spectrophotometer at 212 nm.

Injection: 20 μ l.

Run time: 3 times the retention time of tiamulin.

Identification of impurities: use the chromatogram supplied with *tiamulin for peak identification CRS* and the chromatogram obtained with reference solution (d) to identify the peaks due to impurities B and H.

Relative retention with reference to tiamulin (retention time = about 18 min): impurity G = about 0.2; impurity A = about 0.22; impurity H = about 0.23; impurity I = about 0.3; impurity J = about 0.4; impurity K = about 0.45; impurity B = about 0.5; impurity L = about 0.65; impurity C = about 0.66; impurity F = about 0.8; impurity M = about 0.85; impurity D = about 1.1; impurity S = about 1.4; impurity T = about 1.6; impurity E = 2.4.

System suitability: reference solution (a):

- baseline separation between the peaks due to tiamulin and impurity D.

Limits:

- **impurities B, H:** for each impurity, not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (1.5 per cent),
- **impurities A, C, D, E, F, G, I, J, K, L, M, S, T:** for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (1.0 per cent),
- **any other impurity:** for each impurity, not more than 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent),
- **total:** not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (b) (3.0 per cent),
- **disregard limit:** 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent); disregard any peak present in reference solution (c).

Loss on drying (2.2.32): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 100–105 °C.

ASSAY

Liquid chromatography (2.2.29) as described in the test for related substances with the following modification.

Injection: test solution and reference solution (a).

Calculate the percentage content of $C_{32}H_{51}NO_8S$ from the declared content of *tiamulin hydrogen fumarate CRS*.

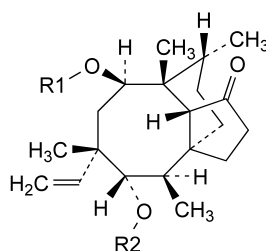
STORAGE

Protected from light.

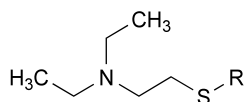
IMPURITIES

Specified impurities: A, B, C, D, E, F, G, H, I, J, K, L, M, S, T.

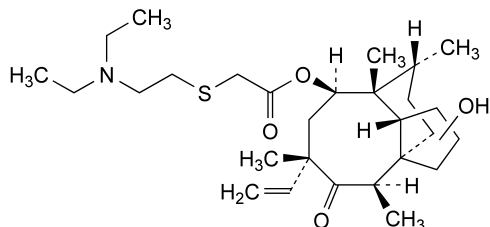
Other detectable impurities: N, O, P, Q, R.



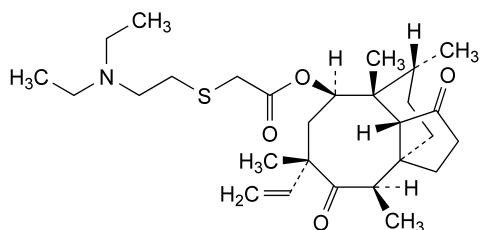
- A. $R_1 = R_2 = H$: (3a*S*,4*R*,5*S*,6*S*,8*R*,9*R*,9a*R*,10*R*)-6-ethenyl-5,8-dihydroxy-4,6,9,10-tetramethyloctahydro-3a,9-propano-3a*H*-cyclopentacycloocten-1(4*H*)-one (mutilin),
- G. $R_1 = CO-CH_2OH$, $R_2 = H$: (3a*S*,4*R*,5*S*,6*S*,8*R*,9*R*,9a*R*,10*R*)-6-ethenyl-5-hydroxy-4,6,9,10-tetramethyl-1-oxodecahydro-3a,9-propano-3a*H*-cyclopentacycloocten-8-yl hydroxyacetate (pleuromutilin),
- J. $R_1 = CO-CH_3$, $R_2 = H$: (3a*S*,4*R*,5*S*,6*S*,8*R*,9*R*,9a*R*,10*R*)-6-ethenyl-5-hydroxy-4,6,9,10-tetramethyl-1-oxodecahydro-3a,9-propano-3a*H*-cyclopentacycloocten-8-yl acetate (mutilin 14-acetate),
- K. $R_1 = H$, $R_2 = CO-CH_3$: (3a*S*,4*R*,5*S*,6*S*,8*R*,9*R*,9a*R*,10*R*)-6-ethenyl-8-hydroxy-4,6,9,10-tetramethyl-1-oxodecahydro-3a,9-propano-3a*H*-cyclopentacycloocten-5-yl acetate (mutilin 11-acetate),
- L. $R_1 = CO-CH_2-O-SO_2-C_6H_4-pCH_3$, $R_2 = H$: (3a*S*,4*R*,5*S*,6*S*,8*R*,9*R*,9a*R*,10*R*)-6-ethenyl-5-hydroxy-4,6,9,10-tetramethyl-1-oxodecahydro-3a,9-propano-3a*H*-cyclopentacycloocten-8-yl [[(4-methylphenyl)sulphonyl]oxy]acetate (pleuromutilin 22-tosylate),
- M. $R_1 = R_2 = CO-CH_3$: (3a*S*,4*R*,5*S*,6*S*,8*R*,9*R*,9a*R*,10*R*)-6-ethenyl-4,6,9,10-tetramethyl-1-oxodecahydro-3a,9-propano-3a*H*-cyclopentacycloocten-5,8-diyl diacetate (mutilin 11,14-diacetate),
- P. $R_1 = CO-CH_2-O-SO_2-C_6H_5$, $R_2 = H$: (3a*S*,4*R*,5*S*,6*S*,8*R*,9*R*,9a*R*,10*R*)-6-ethenyl-5-hydroxy-4,6,9,10-tetramethyl-1-oxodecahydro-3a,9-propano-3a*H*-cyclopentacycloocten-8-yl [(phenylsulphonyl)oxy]acetate,
- T. $R_1 = CO-CH_2-[S-CH_2-CH_2]_2N(C_2H_5)_2$, $R_2 = H$: (3a*S*,4*R*,5*S*,6*S*,8*R*,9*R*,9a*R*,10*R*)-6-ethenyl-5-hydroxy-4,6,9,10-tetramethyl-1-oxodecahydro-3a,9-propano-3a*H*-cyclopentacycloocten-8-yl [[2-[[2-(diethylamino)ethyl]sulphonyl]ethyl]sulphonyl]acetate,



- B. R = CH₂-C₆H₅: 2-(benzylsulphonyl)-*N,N*-diethylethanamine,
 C. R = S-CH₂-CH₂-N(C₂H₅)₂: 2,2'-(disulphane-1,2-diyl)bis(*N,N*-diethylethanamine),
 O. R = H: 2-(diethylamino)ethanethiol,

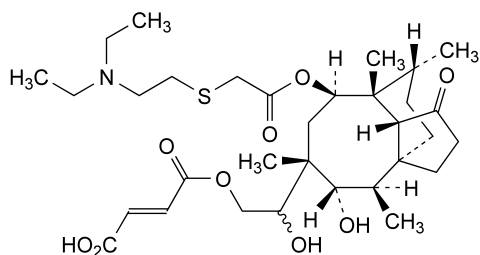


- D. (3a*R*,4*R*,6*S*,8*R*,9*R*,9a*R*,10*R*)-6-ethenylhydroxy-4,6,9,10-tetramethyl-5-oxodecahydro-3a,9-propano-3a*H*-cyclopentacycloocten-8-yl [[2-(diethylamino)ethyl]sulphonyl]acetate,

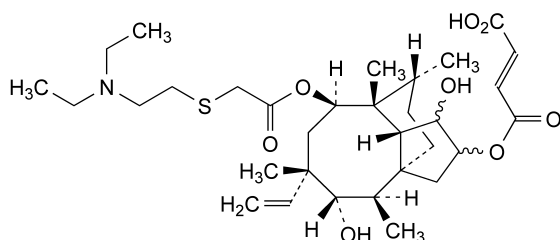


- E. (3a*S*,4*R*,6*S*,8*R*,9*R*,9a*R*,10*R*)-6-ethenyl-4,6,9,10-tetramethyl-1,5-dioxodecahydro-3a,9-propano-3a*H*-cyclopentacycloocten-8-yl [[2-(diethylamino)ethyl]sulphonyl]acetate (11-oxotiamulin),

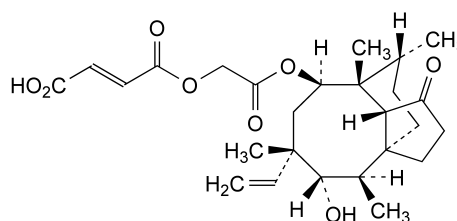
- F. impurity of unknown structure with a relative retention of about 0.8,



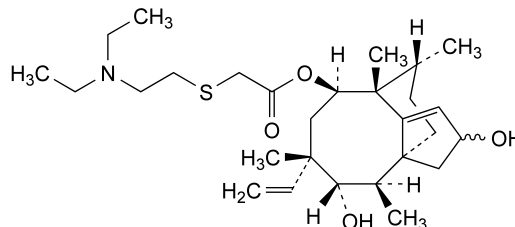
- H. (2*E*)-4-[(2*RS*)-2-[(3a*S*,4*R*,5*S*,6*R*,8*R*,9*R*,9a*R*,10*R*)-8-[[[2-(diethylamino)ethyl]sulphonyl]acetyl]oxy]-5-hydroxy-4,6,9,10-tetramethyl-1-oxodecahydro-3a,9-propano-3a*H*-cyclopentacycloocten-6-yl]-2-hydroxyethoxy]-4-oxobut-2-enoic acid (19,20-dihydroxytiamulin 20-fumarate),



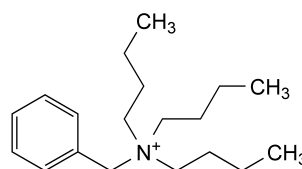
- I. (2*E*)-4-[[[(3a*S*,4*R*,5*S*,6*S*,8*R*,9*R*,9a*R*,10*R*)-8-[[[2-(diethylamino)ethyl]sulphonyl]acetyl]oxy]-6-ethenyl-1,5-dihydroxy-4,6,9,10-tetramethyldecahydro-3a,9-propano-3a*H*-cyclopentacycloocten-2-yl]oxy]-4-oxobut-2-enoic acid (2,3-dihydroxytiamulin 2-fumarate),



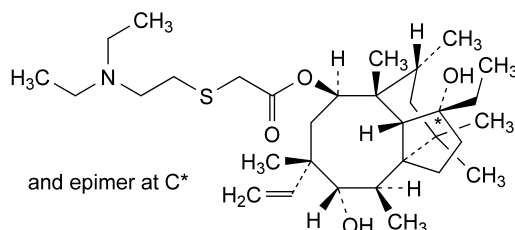
- N. (2*E*)-4-[2-[[[(3a*S*,4*R*,5*S*,6*S*,8*R*,9*R*,9a*R*,10*R*)-6-ethenyl-5-hydroxy-4,6,9,10-tetramethyl-1-oxodecahydro-3a,9-propano-3a*H*-cyclopentacycloocten-8-yl]oxy]-2-oxoethoxy]-4-oxobut-2-enoic acid (pleuromutilin 22-fumarate),



- Q. (3a*S*,4*R*,5*S*,6*S*,8*R*,9*R*,10*R*)-6-ethenyl-2,5-dihydroxy-4,6,9,10-tetramethyl-2,3,4,5,6,7,8,9-octahydro-3a,9-propano-3a*H*-cyclopentacycloocten-8-yl [[2-(diethylamino)ethyl]sulphonyl]acetate (3,4-didehydro-2-hydroxytiamulin),



- R. *N*-benzyl-*N,N*-dibutylbutan-1-aminium,

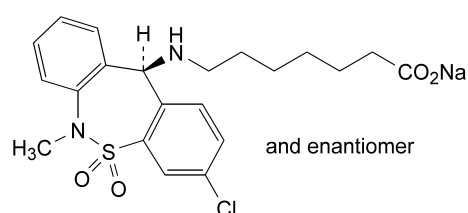


- S. (1*RS*,3a*R*,4*R*,5*S*,6*S*,8*R*,9*R*,9a*R*,10*R*)-6-ethenyl-1-ethyl-1,5-dihydroxy-4,6,9,10,12,12-hexamethyldecahydro-3a,9-propano-3a*H*-cyclopentacycloocten-8-yl [[2-(diethylamino)ethyl]sulphonyl]acetate.

01/2005:2022

TIANEPTINE SODIUM

Tianeptinum natricum

C₂₁H₂₄ClN₂NaO₄S*M_r* 458.9

DEFINITION

Sodium 7-[[[(11*RS*)-3-chloro-6-methyl-6,11-dihydrodibenzo[*c,f*][1,2]thiazepin-11-yl]amino]heptanoate *S,S*-dioxide.

Content: 99.0 per cent to 101.0 per cent (anhydrous substance).

CHARACTERS

Appearance: white or yellowish powder, very hygroscopic.

Solubility: freely soluble in water, in methanol and in methylene chloride.

IDENTIFICATION

A. Infrared absorption spectrophotometry (2.2.24).

Comparison: Ph. Eur. reference spectrum of tianeptine sodium.

B. It gives reaction (a) of sodium (2.3.1).

TESTS

Impurity A. Gas chromatography (2.2.28).

Internal standard solution. Dilute 1 ml of *ethyl 5-bromovalerate R* in *ethanol R* and dilute to 100.0 ml with the same solvent. Dilute 1.0 ml of the solution to 250.0 ml with *ethanol R*.

Test solution. Dissolve 0.1000 g of the substance to be examined in the internal standard solution and dilute to 2.0 ml with the same solution.

Reference solution. Dissolve 10.0 mg of *tianeptine impurity A CRS* in the internal standard solution and dilute to 200.0 ml with the same solution.

Column:

- **material:** fused silica,
- **size:** $l = 25$ m, $\varnothing = 0.25$ mm,
- **stationary phase:** *poly(cyanopropyl)siloxane R* (film thickness 0.2 μ m).

Carrier gas: *helium for chromatography R*.

Linear velocity: 26 cm/s.

Split ratio: 1:100.

Temperature:

- **column:** 150 °C,
- **injection port and detector:** 210 °C.

Detection: flame ionisation.

Injection: 1 μ l.

Run time: twice the retention time of *ethyl 5-bromovalerate*.

System suitability: reference solution:

- **elution order:** ethanol, *ethyl 5-bromovalerate*, impurity A,
- **resolution:** minimum 10 between the peaks due to *ethyl 5-bromovalerate* and impurity A,
- **signal-to-noise ratio:** minimum 20 for the peak due to impurity A.

Limit:

- **impurity A:** not more than the area of the corresponding peak in the chromatogram obtained with the reference solution (0.1 per cent).

Related substances. Liquid chromatography (2.2.29).

Solvent mixture. Mix 50 volumes of *methanol R* and 50 volumes of *water for chromatography R*.

Test solution. Dissolve 50.0 mg of the substance to be examined in the solvent mixture and dilute to 50.0 ml with the solvent mixture.

Reference solution (a). Dilute 1.0 ml of the test solution to 100.0 ml with the solvent mixture. Dilute 1.0 ml of this solution to 20.0 ml with the solvent mixture.

Reference solution (b). Dissolve 20.0 mg of *sodium tianeptine for system suitability CRS* in the solvent mixture and dilute to 200.0 ml with the solvent mixture.

Column:

- **size:** $l = 0.15$ m, $\varnothing = 4.6$ mm,
- **stationary phase:** *octadecylsilyl silica gel for chromatography R* (3 μ m) with a pore size of 0.01 μ m,
- **temperature:** 30 °C.

Mobile phase:

- **mobile phase A:** mix 21 volumes of *methanol R1*, 31.5 volumes of *acetonitrile R1* and 47.5 volumes of a 2 g/l solution of *sodium laurilsulfate R*, adjusted to pH 2.5 with *phosphoric acid R*,
- **mobile phase B:** mix 20 volumes of *methanol R1*, 20 volumes of a 2 g/l solution of *sodium laurilsulfate R*, adjusted to pH 2.5 with *phosphoric acid R* and 60 volumes of *acetonitrile R1*,

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)
0 - 35	100	0
35 - 45	100 → 40	0 → 60
45 - 60	40	60
60 - 70	40 → 100	60 → 0

Flow rate: 1 ml/min.

Detection: spectrophotometer at 220 nm.

Injection: 10 μ l.

Relative retention with reference to tianeptine (retention time = about 30 min): impurity C = about 0.4; impurity D1 = about 0.6; impurity D2 = about 0.8; impurity E = about 1.1; impurity B = about 1.7.

System suitability: reference solution (b):

- **resolution:** minimum 2.5 between the peaks due to tianeptine and impurity E.

Limits:

- **any impurity:** not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent),
- **total:** not more than 8 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.4 per cent),
- **disregard limit:** area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

Water (2.5.12): maximum 5.0 per cent, determined on 0.100 g.

ASSAY

Dissolve 0.165 g in 50 ml of *anhydrous acetic acid R*. Titrate with 0.1 M *perchloric acid*, determining the end-point potentiometrically (2.2.20).

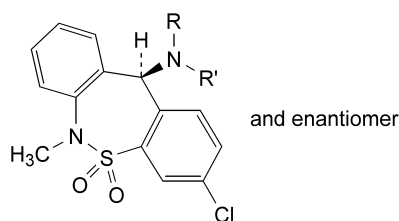
1 ml of 0.1 M *perchloric acid* is equivalent to 22.95 mg of $C_{21}H_{24}ClN_2NaO_4S$.

STORAGE

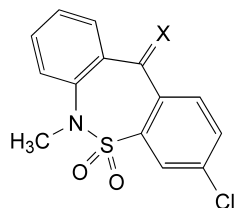
In an airtight container.

IMPURITIES

A. $Br-[CH_2]_6-CO-O-C_2H_5$: *ethyl 7-bromoheptanoate*,



- B. $R = H$, $R' = [CH_2]_6-CO-O-C_2H_5$: ethyl 7-[(11*RS*)-3-chloro-6-methyl-6,11-dihydrodibenzo[*c,f*][1,2]thiazepin-11-yl]amino]heptanoate *S,S*-dioxide,
- E. $R = R' = [CH_2]_6-CO_2H$: 7,7'-[(11*RS*)-3-chloro-6-methyl-6,11-dihydrodibenzo[*c,f*][1,2]thiazepin-11-yl]imino]diheptanoic acid *S,S*-dioxide,

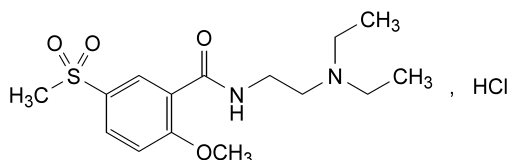


- C. $X = O$: 3-chloro-6-methyldibenzo[*c,f*][1,2]thiazepin-11(6*H*)-one *S,S*-dioxide,
- D. $X = N-[CH_2]_6-CO_2H$: 7-[(11*RS*)-3-chloro-6-methyldibenzo[*c,f*][1,2]thiazepin-11(6*H*)-ylidene]amino]heptanoic acid *S,S*-dioxide.

01/2005:1575

TIAPRIDE HYDROCHLORIDE

Tiapridi hydrochloridum

 $C_{15}H_{25}ClN_2O_4S$ M_r 364.9

DEFINITION

Tiapride hydrochloride contains not less than 98.5 per cent and not more than the equivalent of 101.0 per cent of *N*-[2-(diethylamino)ethyl]-2-methoxy-5-(methylsulphonyl)benzamide hydrochloride, calculated with reference to the dried substance.

CHARACTERS

A white or almost white crystalline powder, very soluble in water, soluble in methanol, slightly soluble in ethanol.

IDENTIFICATION

- A. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *tiapride hydrochloride CRS*. Examine the substances prepared as discs.
- B. Solution S (see Tests) gives reaction (a) of chlorides (2.3.1).

TESTS

Solution S. Dissolve 2.5 g in *carbon dioxide-free water R* and dilute to 50.0 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1). The absorbance (2.2.25) of solution S measured at 450 nm is not more than 0.030.

pH (2.2.3). The pH of solution S is 4.0 to 6.0.

Impurity C. Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel G plate R*.

Test solution. Dissolve 0.400 g of the substance to be examined in *methanol R* and dilute to 10 ml with the same solvent.

Reference solution. Dissolve 20.0 mg of *metoclopramide impurity E CRS* (tiapride impurity C) in *methanol R* and dilute to 50 ml with the same solvent. Dilute 2.0 ml of this solution to 20 ml with *methanol R*.

Apply to the plate 10 µl of the test solution and 10 µl of the reference solution. Develop over a path of 12 cm using a mixture of 2 volumes of *concentrated ammonia R*, 10 volumes of *dioxan R*, 14 volumes of *methanol R* and 90 volumes of *methylene chloride R*. Allow the plate to dry in air. Spray with a 2 g/l solution of *ninhydrin R* in *butanol R* and heat at 100 °C for 15 min. Any spot corresponding to impurity C in the chromatogram obtained with the test solution is not more intense than the spot in the chromatogram obtained with the reference solution (0.1 per cent).

Related substances. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 0.100 g of the substance to be examined in the mobile phase and dilute to 100.0 ml with the mobile phase.

Reference solution (a). Dilute 1.0 ml of the test solution to 10.0 ml with the mobile phase. Dilute 1.0 ml of this solution to 100.0 ml with the mobile phase.

Reference solution (b). Dissolve 5.0 mg of *tiapride hydrochloride CRS* and 5.0 mg of *tiapride N-oxide CRS* in the mobile phase and dilute to 100.0 ml with the mobile phase.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4.6 mm in internal diameter packed with *octylsilyl silica gel for chromatography R* (5 µm),
- as mobile phase at a flow rate of 1.5 ml/min a solution prepared as follows: dissolve 5.44 g of *potassium dihydrogen phosphate R* and 0.08 g of *sodium octanesulphonate R* in 780 ml of *water R*, adjust the pH to 2.7 using *phosphoric acid R* and dilute to 800 ml with *water R*; add 150 ml of *methanol R* and 50 ml of *acetonitrile R* and mix,
- as detector a spectrophotometer set at 240 nm, maintaining the temperature of the column at 40 °C.

Inject 10 µl of reference solution (b). The test is not valid unless the resolution between the peaks corresponding to tiapride (retention time of about 9 min) and tiapride *N-oxide* (retention time of about 13 min) is at least 4.0.

Inject 10 µl of the test solution. Continue the chromatography for 3 times the retention time of tiapride. In the chromatogram obtained with the test solution: the area of any peak, apart from the principal peak, is not greater than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent) and the sum of the areas of any such peaks is not greater than 3 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.3 per cent). Disregard any peak with an area less than half the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

Heavy metals (2.4.8). Dissolve 2.0 g in *water R* and dilute to 20 ml with the same solvent. 12 ml of the solution complies with limit test A for heavy metals (20 ppm). Prepare the standard using *lead standard solution (2 ppm Pb) R*.

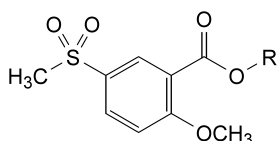
Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

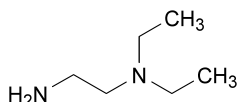
Dissolve 0.300 g in 20 ml of *anhydrous acetic acid R*. Add 20 ml of *acetic anhydride R*. Titrate with 0.1 M *perchloric acid*, determining the end-point potentiometrically (2.2.20). 1 ml of 0.1 M *perchloric acid* is equivalent to 36.49 mg of $C_{15}H_{25}ClN_2O_4S$.

IMPURITIES



A. R = CH₃: methyl 2-methoxy-5-(methylsulphonyl)benzoate,

B. R = H: 2-methoxy-5-(methylsulphonyl)benzoic acid,

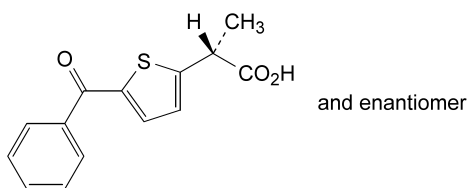


C. *N,N*-diethylethane-1,2-diamine.

01/2005:1157

TIAPROFENIC ACID

Acidum tiaprofenicum



and enantiomer

$C_{14}H_{12}O_3S$

M_r 260.3

DEFINITION

Tiaprofenic acid contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of (2*RS*)-2-(5-benzoylthiophen-2-yl)propanoic acid, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, crystalline powder, practically insoluble in water, freely soluble in acetone, in alcohol and in methylene chloride.

IDENTIFICATION

First identification: C.

Second identification: A, B, D.

A. Melting point (2.2.14): 95 °C to 99 °C.

B. Dissolve 25.0 mg in *ethanolic hydrochloric acid R* and dilute to 50.0 ml with the same solvent. Dilute 1.0 ml of the solution to 50.0 ml with *ethanolic hydrochloric*

acid R. Examined between 220 nm and 350 nm (2.2.25), the solution shows a shoulder at 262 nm and an absorption maximum at 305 nm. The specific absorbance at the maximum is 550 to 590.

C. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *tiaprofenic acid CRS*.

D. Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel F₂₅₄ plate R*.

Test solution. Dissolve 10 mg of the substance to be examined in *methylene chloride R* and dilute to 10 ml with the same solvent.

Reference solution (a). Dissolve 10 mg of *tiaprofenic acid CRS* in *methylene chloride R* and dilute to 10 ml with the same solvent.

Reference solution (b). Dissolve 10 mg of *ketoprofen CRS* in *methylene chloride R* and dilute to 10 ml with the same solvent. Dilute 1 ml of the solution to 2 ml with reference solution (a).

Apply to the plate 10 µl of each solution. Develop over a path of 15 cm using a mixture of 1 volume of *acetic acid R*, 20 volumes of *methylene chloride R* and 80 volumes of *acetone R*. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. The principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a). The identification is not valid unless the chromatogram obtained with reference solution (b) shows two clearly separated principal spots.

TESTS

Appearance of solution. Dissolve 2.0 g in *alcohol R* and dilute to 20 ml with the same solvent. The solution is clear (2.2.1) and not more intensely coloured than reference solution Y₆ (2.2.2, *Method II*).

Optical rotation (2.2.7). Dissolve 0.50 g in *ethyl acetate R* and dilute to 10.0 ml with the same solvent. The angle of optical rotation is −0.10° to +0.10°.

Related substances. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 20.0 mg of the substance to be examined in the mobile phase and dilute to 20.0 ml with the mobile phase.

Reference solution (a). Dilute 1.0 ml of the test solution to 50.0 ml with the mobile phase. Dilute 1.0 ml of the solution to 10.0 ml with the mobile phase.

Reference solution (b). Dilute 5.0 ml of reference solution (a) to 10.0 ml with the mobile phase.

Reference solution (c). Dissolve 10.0 mg of *tiaprofenic acid impurity C CRS* in the mobile phase and dilute to 100.0 ml with the mobile phase. Dilute 1.0 ml of the solution to 50.0 ml with the mobile phase.

Reference solution (d). Dilute 1.0 ml of reference solution (a) to 2.0 ml with reference solution (c).

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4.6 mm in internal diameter packed with *silica gel for chromatography R* (5 µm),
- as mobile phase at a flow rate of 1 ml/min a mixture of 0.25 volumes of *water R*, 20 volumes of *glacial acetic acid R*, 500 volumes of *hexane R* and 500 volumes of *methylene chloride R*. Add the water to the acetic acid, then hexane and methylene chloride. Sonicate the mixture for 2 min. Do not degas with helium during analysis,

– as detector a spectrophotometer set at 250 nm.

Inject 20 µl of reference solution (d). When the chromatograms are recorded in the prescribed conditions the retention times relative to tiaprofenic acid are: impurity A 0.19, impurity B 0.43, impurity C 0.86.

Adjust the sensitivity of the system so that the heights of the principal peaks in the chromatogram obtained with reference solution (d) are at least 50 per cent of the full scale of the recorder. The test is not valid unless in the chromatogram obtained, the resolution between the peaks corresponding to tiaprofenic acid and impurity C is at least 3.0.

Inject 20 µl of the test solution, 20 µl of reference solution (a), 20 µl of reference solution (b) and 20 µl of reference solution (c). Continue the chromatography of the test solution for twice the retention time of tiaprofenic acid. In the chromatogram obtained with the test solution: the area of any peak corresponding to impurity C is not greater than the area of the corresponding peak in the chromatogram obtained with reference solution (c) (0.2 per cent); the area of any peak, apart from the principal peak and any peak corresponding to impurity C, is not greater than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent); the sum of the areas of all the peaks, apart from the principal peak and any peak corresponding to impurity C, is not greater than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.3 per cent). Disregard any peak with an area less than half the area of the principal peak in the chromatogram obtained with the reference solution (b).

Heavy metals (2.4.8). 2.0 g complies with limit test C for heavy metals (10 ppm). Prepare the standard using 2 ml of *lead standard solution* (10 ppm Pb) R.

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying at 60 °C in an oven at a pressure not exceeding 0.7 kPa for 3 h.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

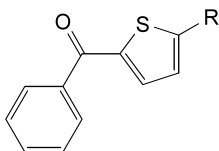
Dissolve 0.250 g in 25 ml of *alcohol R*. Add 25 ml of *water R* and 0.5 ml of *phenolphthalein solution R*. Titrate with 0.1 M *sodium hydroxide*.

1 ml of 0.1 M *sodium hydroxide* is equivalent to 26.03 mg of C₁₅H₁₄N₂O₆S₂.

STORAGE

Store protected from light.

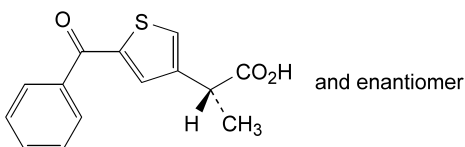
IMPURITIES



A. R = C₂H₅: (5-ethylthiophen-2-yl)phenylmethanone,

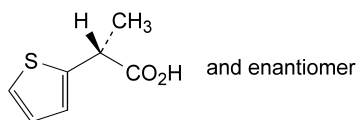
B. R = CO-CH₃: 1-(5-benzoylthiophen-2-yl)ethanone,

F. R = Br: (5-bromothiophen-2-yl)phenylmethanone,



C. (2*RS*)-2-(5-benzoylthiophen-3-yl)propanoic acid,

D. benzoic acid,

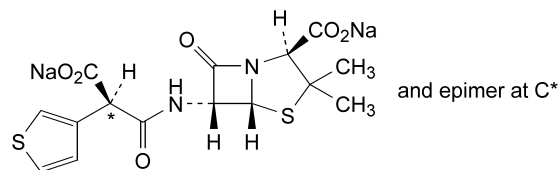


E. (2*RS*)-2-(thiophen-2-yl)propanoic acid.

01/2005:0956

TICARCILLIN SODIUM

Ticarcillinum natricum



C₁₅H₁₄N₂Na₂O₆S₂

M_r 428.4

DEFINITION

Ticarcillin sodium contains not less than 89.0 per cent and not more than the equivalent of 100.5 per cent of disodium (2*S*,5*R*,6*R*)-6-[(2*RS*)-2-carboxylato-2-(thiophen-3-yl)acetyl]amino]-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylate, calculated with reference to the anhydrous substance.

CHARACTERS

A white or slightly yellow powder, hygroscopic, freely soluble in water, soluble in methanol.

IDENTIFICATION

First identification: A, D.

Second identification: B, C, D.

A. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *ticarcillin sodium CRS*.

B. Examine by thin-layer chromatography (2.2.27), using *silanised silica gel H R* as the coating substance.

Test solution. Dissolve 25 mg of the substance to be examined in *methanol R* and dilute to 5 ml with the same solvent.

Reference solution (a). Dissolve 25 mg of *ticarcillin sodium CRS* in *methanol R* and dilute to 5 ml with the same solvent.

Reference solution (b). Dissolve 25 mg of *carbenicillin sodium CRS* and 25 mg of *ticarcillin sodium CRS* in *methanol R* and dilute to 5 ml with the same solvent.

Apply to the plate 1 µl of each solution. Develop over a path of 12 cm using a mixture of 10 volumes of *acetone R* and 90 volumes of a 154 g/l solution of *ammonium acetate R*, adjusted to pH 5.0 with *glacial acetic acid R*. Allow the plate to dry in a current of hot air and expose it to iodine vapour. The principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a). The test is not valid unless the chromatogram obtained with reference solution (b) shows two clearly separated spots.

C. Place about 2 mg in a test-tube about 15 cm long and 15 mm in diameter. Moisten with 0.05 ml of *water R* and add 2 ml of *sulphuric acid-formaldehyde reagent R*.

Mix the contents of the tube by swirling; the solution is brown. Place the test-tube in a water-bath for 1 min; a dark reddish-brown colour develops.

D. It gives reaction (a) of sodium (2.3.1).

TESTS

Solution S. Dissolve 2.50 g in *carbon dioxide-free water R* and dilute to 50 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and not more intensely coloured than reference solution Y₅ (2.2.2, *Method II*).

pH (2.2.3). The pH of solution S is 5.5 to 7.5.

Specific optical rotation (2.2.7). Dissolve 0.250 g in *water R* and dilute to 25.0 ml with the same solvent. The specific optical rotation is + 172 to + 187, calculated with reference to the anhydrous substance.

Related substances. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 25.0 mg of the substance to be examined in mobile phase A and dilute to 25.0 ml with the same mobile phase.

Reference solution (a). Dissolve 20.0 mg of *ticarcillin impurity A CRS* in mobile phase A and dilute to 100.0 ml with the same mobile phase. Dilute 5.0 ml of this solution to 50.0 ml with mobile phase A.

Reference solution (b). Dilute 1 ml of the test solution to 50 ml with mobile phase A.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (5 µm),
- as mobile phase at a flow rate of 1.0 ml/min:

Mobile phase A. A 1.3 g/l solution of *ammonium phosphate R* adjusted to pH 7.0 with *phosphoric acid R*,

Mobile phase B. A mixture of equal volumes of mobile phase A and *methanol R*,

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)	Comment
0 – 30	100 → 30	0 → 70	linear gradient
30 – 40	30	70	isocratic
40 – 45	100	0	equilibration

- as detector a spectrophotometer at 220 nm.

Inject 20 µl of reference solution (b). Adjust the sensitivity of the system so that the height of the two principal peaks are at least 50 per cent of the full scale of the recorder. The test is not valid unless the resolution between the two principal peaks (diastereoisomers) is at least 2.0. Inject 20 µl of the test solution and 20 µl of reference solution (a). In the chromatogram obtained with the test solution: the area of any peak corresponding to ticarcillin impurity A is not greater than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (4 per cent); and the area of any peak apart from the two principal peaks and any peak corresponding to ticarcillin impurity A, is not greater than 1.25 times the area of the principal peak in the chromatogram obtained with the reference solution (a) (2.5 per cent).

N,N-Dimethylaniline (2.4.26, *Method B*). Not more than 20 ppm.

2-Ethylhexanoic acid (2.4.28). Not more than 0.5 per cent *m/m*.

Water (2.5.12). Not more than 5.5 per cent, determined on 0.150 g by the semi-micro determination of water.

Bacterial endotoxins (2.6.14): less than 0.05 IU/mg, if intended for use in the manufacture of parenteral dosage forms without a further appropriate procedure for the removal of bacterial endotoxins.

ASSAY

Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 50.0 mg of the substance to be examined in the mobile phase and dilute to 100.0 ml with the same solvent. Dilute 10.0 ml of the solution to 50.0 ml with the mobile phase.

Reference solution. Dissolve 50.0 mg of *ticarcillin sodium CRS* in the mobile phase and dilute to 100.0 ml with the same solvent. Dilute 10.0 ml of the solution to 50.0 ml with the mobile phase.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (5 µm),
- as mobile phase at a flow rate of 1 ml/min a mixture of 20 volumes of *methanol R* and 80 volumes of a 1.3 g/l solution of *ammonium phosphate R* (adjusted to pH 7.0 with *phosphoric acid R*),
- as detector a spectrophotometer set at 220 nm.

Inject 20 µl of the reference solution. Adjust the sensitivity of the system so that the heights of the two principal peaks are at least 50 per cent of the full scale of the recorder. The test is not valid unless the resolution between the two principal peaks is at least 2.5. Inject the reference solution six times. The test is not valid unless the relative standard deviation of the two peak areas for ticarcillin is at most 1.0 per cent. Inject alternately the test solution and the reference solution. Calculate the percentage content of ticarcillin sodium as the sum of the two peaks.

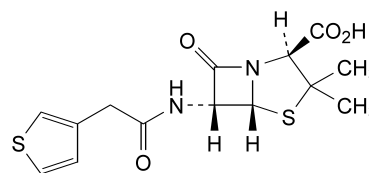
STORAGE

Store in an airtight container, at a temperature of 2 °C to 8 °C. If the substance is sterile, store in a sterile, airtight, tamper-proof container.

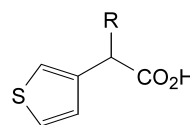
LABELLING

The label states, where applicable, that the substance is free from bacterial endotoxins.

IMPURITIES

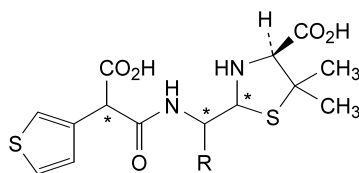


A. (2*S*,5*R*,6*R*)-3,3-dimethyl-7-oxo-6-[(thiophen-3-yl)acetyl]amino-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid (decarboxyticarcillin),



B. R = H: (thiophen-3-yl)acetic acid,

C. R = CO₂H: 2-(thiophen-3-yl)propanedioic acid (3-thienylmalonic acid),

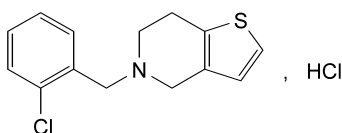


- D. R = CO₂H: (4S)-2-[carboxy[[2-carboxy-2-(thiophen-3-yl)acetyl]amino]methyl]-5,5-dimethylthiazolidine-4-carboxylic acid (penicilloic acids of ticarcillin),
- E. R = H: (4S)-2-[[[2-carboxy-2-(thiophen-3-yl)acetyl]amino]methyl]-5,5-dimethylthiazolidine-4-carboxylic acid (penilloic acids of ticarcillin).

01/2005:1050
corrected

TICLOPIDINE HYDROCHLORIDE

Ticlopidini hydrochloridum



C₁₄H₁₅Cl₂NS

M_r 300.2

DEFINITION

Ticlopidine hydrochloride contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of 5-(2-chlorobenzyl)-4,5,6,7-tetrahydrothieno[3,2-c]pyridine hydrochloride, calculated with reference to the anhydrous substance.

CHARACTERS

A white or almost white, crystalline powder, sparingly soluble in water and in ethanol, very slightly soluble in ethyl acetate.

IDENTIFICATION

First identification: B, D.

Second identification: A, C, D.

- A. Dissolve 40 mg in *water R* and dilute to 100.0 ml with the same solvent (solution A). Dilute 5.0 ml of solution A to 100.0 ml with *water R* (solution B). Examined between 200 nm and 350 nm (2.2.25), solution B shows two absorption maxima, at 214 nm and 232 nm. Examined between 250 nm and 350 nm, solution A shows two absorption maxima, at 268 nm and 275 nm. The ratio of the absorbance measured at the maximum at 268 nm to that measured at the maximum at 275 nm is 1.1 to 1.2.
- B. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *ticlopidine hydrochloride CRS*. Examine the substances prepared as discs.
- C. Mix about 6 mg of *citric acid R* and 0.3 ml of *acetic anhydride R*. Add about 5 mg of the substance to be examined and heat in a water-bath at 80 °C. A red colour develops.
- D. About 20 mg gives reaction (a) of chlorides (2.3.1).

TESTS

Appearance of solution. Dissolve 0.5 g in a 1 per cent V/V solution of *hydrochloric acid R* and dilute to 20 ml with the same acid solution. The solution is clear (2.2.1) and colourless (2.2.2, *Method II*).

pH (2.2.3). Dissolve 0.5 g in *carbon dioxide-free water R* and dilute to 20 ml with the same solvent. The pH of the solution is 3.5 to 4.0.

Related substances. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 0.250 g of the substance to be examined in a mixture of 20 volumes of mobile phase B and 80 volumes of mobile phase A. Dilute to 50.0 ml with the same mixture of solvents.

Reference solution. Dissolve 5.0 mg of *ticlopidine impurity F CRS* in a mixture of 20 volumes of mobile phase B and 80 volumes of mobile phase A. Add 1.00 ml of the test solution and dilute to 100.0 ml with a mixture of 20 volumes of mobile phase B and 80 volumes of mobile phase A. Dilute 1.0 ml of this solution to 10.0 ml with a mixture of 20 volumes of mobile phase B and 80 volumes of mobile phase A.

The chromatographic procedure may be carried out using:

- a stainless steel column, 0.15 m long and 4.6 mm in internal diameter, packed with *octadecylsilyl silica gel for chromatography R* (5 µm),
- as mobile phase at a flow rate of 1.3 ml/min:

Mobile phase A. A 0.95 g/l solution of *sodium pentanesulphonate monohydrate R*, adjusted to pH 3.4 with a 50 per cent V/V solution of *phosphoric acid R*,

Mobile phase B. *Methanol R*,

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)	Comment
0 - 45	80 → 20	20 → 80	linear gradient
45 - 50	20	80	isocratic
50 - 55	20 → 80	80 → 20	linear gradient

- as detector a spectrophotometer set at 220 nm, maintaining the temperature of the column at 40 °C.

Inject 10 µl of a mixture of 20 volumes of mobile phase B and 80 volumes of mobile phase A (blank). Inject 10 µl of the reference solution. When the chromatogram is recorded in the prescribed conditions the retention time for ticlopidine is about 15 min.

Adjust the sensitivity of the system so that, in the chromatogram obtained with the reference solution, the height of the peak due to ticlopidine is at least 5 per cent of the full scale of the recorder. The test is not valid unless in the chromatogram obtained with the reference solution: the resolution between the peaks corresponding to ticlopidine and impurity F is at least 2.0 (modify the pH of mobile phase A if necessary); the signal-to-noise ratio of the peak due to ticlopidine is at least 50.

Inject 10 µl of the test solution and 10 µl of the reference solution. In the chromatogram obtained with the test solution: the area of the peak corresponding to impurity F is not greater than half the area of the corresponding peak in the chromatogram obtained with the reference solution (0.05 per cent); the area of any peak, apart from the principal peak and the peak due to impurity F, is not greater than half the area of the principal peak in the chromatogram obtained with the reference solution (0.05 per cent); the sum of the areas of all the peaks apart from the peak corresponding to ticlopidine, is not greater than the area of the peak corresponding to ticlopidine in the chromatogram obtained with the reference solution (0.1 per cent). Disregard any peak with an area less than 0.1 times that of ticlopidine in the chromatogram obtained with the reference solution.

Formaldehyde. Dissolve 0.200 g in 4.0 ml of *water R*. Add 0.4 ml of *dilute sodium hydroxide solution R*. Centrifuge, filter the supernatant liquid through cotton previously impregnated with *water R* and dilute to 5.0 ml with *water R*. Transfer to a test-tube. Add 5.0 ml of *acetylacetone reagent R1*. Place the test-tube in a water-bath at 40 °C for 40 min. The test solution is not more intensely coloured than a standard prepared at the same time and in the same manner using 5.0 ml of a 0.8 ppm solution of formaldehyde (CH₂O), obtained by dilution of *formaldehyde standard solution (5 ppm CH₂O) R* with *water R* (20 ppm). Examine the tubes down their vertical axis.

Heavy metals (2.4.8). Dissolve 2.0 g in a 85 per cent V/V solution of *methanol R* and dilute to 20.0 ml with the same solvent. 12 ml of the solution complies with limit test B for heavy metals (10 ppm). Prepare the standard using 10 ml of *lead standard solution (1 ppm Pb) R*.

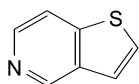
Water (2.5.12). Not more than 0.5 per cent, determined on 0.500 g by the semi-micro determination of water.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

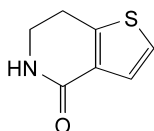
ASSAY

Dissolve 0.150 g in 15 ml of *anhydrous acetic acid R*. Add 35 ml of *acetic anhydride R*. Titrate with 0.1 M *perchloric acid*, determining the end-point potentiometrically (2.2.20). 1 ml of 0.1 M *perchloric acid* is equivalent to 30.02 mg of C₁₄H₁₅Cl₂NS.

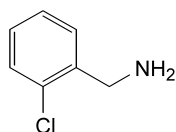
IMPURITIES



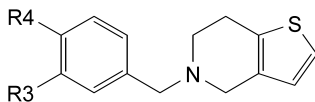
A. thieno[3,2-c]pyridine,



B. 6,7-dihydrothieno[3,2-c]pyridin-4(5*H*)-one,



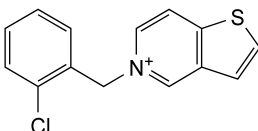
C. (2-chlorophenyl)methanamine,



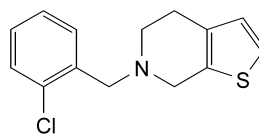
D. R₃ = R₄ = H: 5-benzyl-4,5,6,7-tetrahydrothieno[3,2-c]pyridine,

G. R₃ = Cl, R₄ = H: 5-(3-chlorobenzyl)-4,5,6,7-tetrahydrothieno[3,2-c]pyridine,

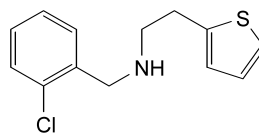
H. R₃ = H, R₄ = Cl: 5-(4-chlorobenzyl)-4,5,6,7-tetrahydrothieno[3,2-c]pyridine,



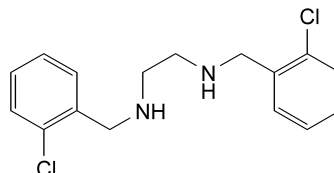
E. 5-(2-chlorobenzyl)thieno[3,2-c]pyridinium,



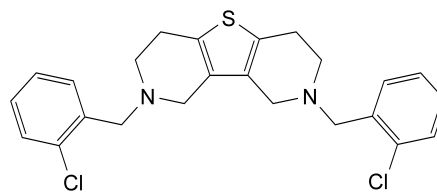
F. 6-(2-chlorobenzyl)-4,5,6,7-tetrahydrothieno[2,3-c]pyridine,



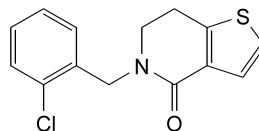
I. *N*-(2-chlorobenzyl)-2-(thiophen-2-yl)ethanamine,



J. *N,N'*-bis(2-chlorobenzyl)ethane-1,2-diamine,



K. 2,8-bis(2-chlorobenzyl)-1,2,3,4,6,7,8,9-octahydrothieno[3,2-*c'*]dipyridine (bis-ticlopidine),

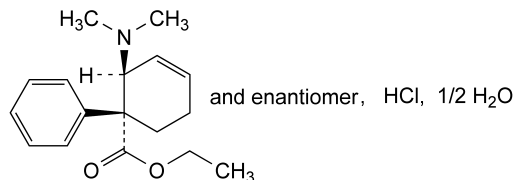


L. 5-(2-chlorobenzyl)-6,7-dihydrothieno[3,2-c]pyridin-4(5*H*)-one.

01/2005:1767

TILIDINE HYDROCHLORIDE HEMIHYDRATE

Tilidini hydrochloridum hemihydricum



C₁₇H₂₄ClNO₂ · 1/2 H₂O

*M*_r 318.9

DEFINITION

Ethyl (1*R*,2*S*)-2-(dimethylamino)-1-phenylcyclohex-3-enecarboxylate hydrochloride hemihydrate.

Content: 99.0 per cent to 101.0 per cent (anhydrous substance).

A suitable antioxidant may be added.

CHARACTERS

Appearance: white or almost white, crystalline powder.

Solubility: freely soluble in water, very soluble in methylene chloride, freely soluble in alcohol.

IDENTIFICATION

A. Infrared absorption spectrophotometry (2.2.24).

Comparison: Ph. Eur. reference spectrum of tilidine hydrochloride hemihydrate.

B. It gives reaction (a) of chlorides (2.3.1).

TESTS

Solution S. Dissolve 1.0 g in *carbon dioxide-free water R* and dilute to 20 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and not more intensely coloured than reference solution Y₇ (2.2.2, Method II).

Acidity or alkalinity. To 20 ml of solution S add 0.2 ml of 0.01 M sodium hydroxide. The pH is not less than 4.1. Add 0.4 ml of 0.01 M hydrochloric acid. The pH is not more than 4.3.

Related substances. Liquid chromatography (2.2.29).

Test solution. Dissolve 50.0 mg of the substance to be examined in *water R* and dilute to 50.0 ml with the same solvent.

Reference solution (a). Dilute 0.5 ml of the test solution to 100.0 ml with *water R*.

Reference solution (b). Dilute 2.0 ml of reference solution (a) to 10.0 ml with *water R*.

Precolumn:

- size: $l = 4$ mm, $\varnothing = 4.0$ mm,
- stationary phase: spherical octadecylsilyl silica gel for chromatography R (5 μ m).

Column:

- size: $l = 0.125$ m, $\varnothing = 4.0$ mm,
- stationary phase: spherical octadecylsilyl silica gel for chromatography R (5 μ m).

Mobile phase: mix equal volumes of acetonitrile R and a 0.98 g/l solution of ammonium carbonate R.

Flow rate: 0.8 ml/min.

Detection: spectrophotometer at 220 nm.

Injection: 10 μ l.

Run time: twice the retention time of tilidine.

Relative retention with reference to tilidine (retention time = about 11 min): impurity D = about 0.4; impurity C = about 0.5; impurity B = about 0.7; impurity A = about 1.5.

Limits:

- **impurities A, B, C:** for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent),
- **any other impurity:** for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent),
- **total:** not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent),
- **disregard limit:** 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

Heavy metals (2.4.8): maximum 20 ppm.

Dissolve 2.0 g in 20 ml of *water R*. 12 ml of the solution complies with limit test A. Prepare the standard using *lead standard solution* (2 ppm Pb) R.

Water (2.5.12): 2.5 per cent to 3.1 per cent, determined on 0.300 g.

Bacterial endotoxins (2.6.14): less than 0.25 IU/mg, if intended for use in the manufacture of parenteral dosage forms without a further appropriate procedure for the removal of bacterial endotoxins.

ASSAY

Dissolve 0.250 g in a mixture of 10 ml of *anhydrous acetic acid R* and 50 ml of *acetic anhydride R*. Titrate with 0.1 M perchloric acid, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M perchloric acid is equivalent to 30.99 mg of C₁₇H₂₄ClNO₂.

STORAGE

Protected from light.

LABELLING

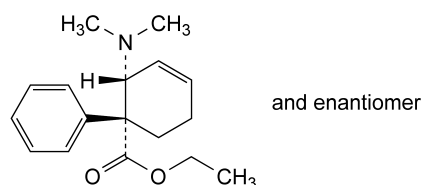
The label states:

- where applicable, that the substance is free from bacterial endotoxins,
- where applicable, the name and concentration of any added antioxidant.

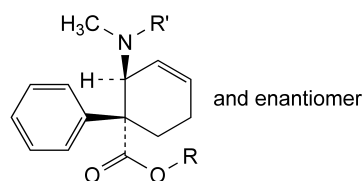
IMPURITIES

Specified impurities: A, B, C.

Other detectable impurities: D.

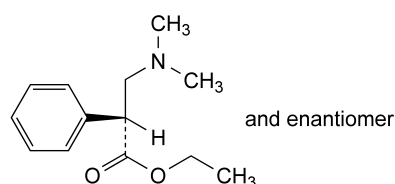


A. ethyl (1*RS*,2*RS*)-2-(dimethylamino)-1-phenylcyclohex-3-enecarboxylate,



B. R = R' = CH₃: methyl (1*RS*,2*SR*)-2-(dimethylamino)-1-phenylcyclohex-3-enecarboxylate,

C. R = C₂H₅, R' = H: ethyl (1*RS*,2*SR*)-2-(methylamino)-1-phenylcyclohex-3-enecarboxylate,

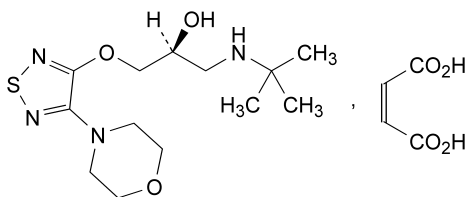


D. ethyl (2*RS*)-3-dimethylamino-2-phenylpropanoate.

01/2005:0572 **Enantiomeric purity.** Examine by liquid chromatography (2.2.29). Carry out the test protected from actinic light.

TIMOLOL MALEATE

Timololi maleas



$C_{17}H_{28}N_4O_7S$

M_r 432.5

DEFINITION

Timolol maleate contains not less than 98.5 per cent and not more than the equivalent of 101.0 per cent of (2S)-1-[(1,1-dimethylethyl)amino]-3-[[4-(morpholin-4-yl)-1,2,5-thiadiazol-3-yl]oxy]propan-2-ol (*Z*)-butenedioate, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, crystalline powder or colourless crystals, soluble in water and in alcohol.

It melts at about 199 °C, with decomposition.

IDENTIFICATION

First identification: A, B.

Second identification: A, C, D.

- Dissolve 1.000 g in 1 M hydrochloric acid and dilute to 10.0 ml with the same acid. The specific optical rotation (2.2.7) is -5.7 to -6.2 .
- Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with timolol maleate CRS.
- Examine the chromatograms obtained in the test for related substances after exposure to iodine vapour. The principal spot in the chromatogram obtained with test solution (b) is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).
- Triturate 0.1 g with a mixture of 1 ml of dilute sodium hydroxide solution R and 3 ml of water R. Shake with three quantities, each of 5 ml, of ether R. To 0.1 ml of the aqueous layer add a solution of 10 mg of resorcinol R in 3 ml of sulphuric acid R. Heat on a water-bath for 15 min. No violet-red colour develops. Neutralise the remainder of the aqueous layer with dilute sulphuric acid R and add 1 ml of bromine water R. Heat on a water-bath for 15 min, then heat to boiling and cool. To 0.2 ml of this solution add a solution of 10 mg of resorcinol R in 3 ml of sulphuric acid R. Heat on a water-bath for 15 min; a violet-red colour develops. Add 0.2 ml of a 100 g/l solution of potassium bromide R and heat for 5 min on a water-bath. The colour becomes violet-blue.

TESTS

Solution S. Dissolve 0.5 g in carbon dioxide-free water R and dilute to 25 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and not more intensely coloured than reference solution B_s (2.2.2, Method II).

pH (2.2.3). The pH of solution S is 3.8 to 4.3.

Enantiomeric purity. Examine by liquid chromatography (2.2.29). Carry out the test protected from actinic light.

Test solution. Dissolve 30.0 mg of the substance to be examined in a mixture of 1 volume of methylene chloride R and 3 volumes of 2-propanol R and dilute to 10.0 ml with the same mixture of solvents.

Reference solution (a). Dissolve 30 mg of timolol maleate CRS in a mixture of 1 volume of methylene chloride R and 3 volumes of 2-propanol R and dilute to 10 ml with the same mixture of solvents.

Reference solution (b). Dissolve 15.0 mg of (*R*)-timolol maleate CRS in a mixture of 1 volume of methylene chloride R and 3 volumes of 2-propanol R and dilute to 10.0 ml with the same mixture of solvents. Dilute 1.0 ml of the solution to 50.0 ml with a mixture of 1 volume of methylene chloride R and 3 volumes of 2-propanol R.

Reference solution (c). Dilute 1 ml of reference solution (a) to 100 ml with a mixture of 1 volume of methylene chloride R and 3 volumes of 2-propanol R. Mix 1 ml of this solution with 1 ml of reference solution (b).

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4.6 mm in internal diameter packed with silica gel OD for chiral separations R (5 µm),
- as mobile phase at a flow rate of 1 ml/min a mixture of 2 ml of diethylamine R, 40 ml of 2-propanol R and 960 ml of hexane R,
- as detector a spectrophotometer set at 297 nm.

Under these conditions the peak corresponding to the (*R*)-isomer appears first.

Inject 5 µl of reference solution (b). Adjust the sensitivity of the system so that the height of the principal peak in the chromatogram obtained is at least 50 per cent of the full scale of the recorder. Inject 5 µl of each solution. The test is not valid unless: in the chromatogram obtained with reference solution (c), the resolution between the peaks corresponding to the (*R*)-enantiomer and to the (*S*)-enantiomer is at least 4.0; the retention times of the principal peaks (corresponding to the (*S*)-enantiomer) in the chromatograms obtained with the test solution and reference solution (a) are identical. In the chromatogram obtained with the test solution, the area of any peak corresponding to the (*R*)-enantiomer is not greater than the area of the principal peak in the chromatogram obtained with reference solution (b) (1 per cent).

Related substances. Examine by thin-layer chromatography (2.2.27), using a TLC silica gel plate GF₂₅₄ R.

Test solution (a). Dissolve 0.50 g of the substance to be examined in methanol R and dilute to 10 ml with the same solvent.

Test solution (b). Dilute 1 ml of test solution (a) to 50 ml with methanol R.

Reference solution (a). Dissolve 10 mg of timolol maleate CRS in methanol R and dilute to 10 ml with the same solvent.

Reference solution (b). Dilute 10 ml of test solution (b) to 50 ml with methanol R.

Apply to the plate 10 µl of each solution. Develop over a path of 15 cm using a mixture of 1 volume of concentrated ammonia R, 20 volumes of methanol R and 80 volumes of methylene chloride R. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. Any spot in the chromatogram obtained with test solution (a), apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.4 per cent). Disregard the spot remaining at the starting point.

Expose the plate to iodine vapour for 2 h. Any spot in the chromatogram obtained with test solution (a), apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.4 per cent). Disregard the spot remaining at the starting point.

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

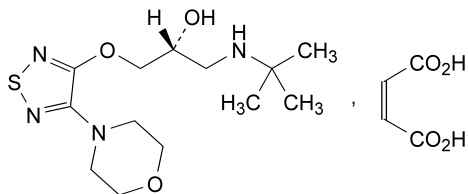
Dissolve 0.350 g in 60 ml of *anhydrous acetic acid R*. Titrate with 0.1 M *perchloric acid* determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *perchloric acid* is equivalent to 43.25 mg of $C_{17}H_{28}N_4O_7S$.

STORAGE

Store protected from light.

IMPURITIES

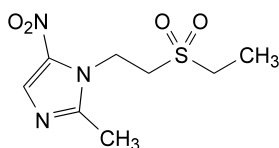


- A. (2R)-1-[(1,1-dimethylethyl)amino]-3-[[4-(morpholin-4-yl)-1,2,5-thiadiazol-3-yl]oxy]propan-2-ol (Z)-butenedioate.

01/2005:1051

TINIDAZOLE

Tinidazolum



$C_8H_{13}N_3O_4S$

M_r 247.3

DEFINITION

Tinidazole contains not less than 98.0 per cent and not more than the equivalent of 101.0 per cent of 1-[2-(ethylsulphonyl)ethyl]-2-methyl-5-nitro-1*H*-imidazole, calculated with reference to the dried substance.

CHARACTERS

An almost white or pale yellow, crystalline powder, practically insoluble in water, soluble in acetone and in methylene chloride, sparingly soluble in methanol.

IDENTIFICATION

First identification: A, C.

Second identification: A, B, D, E.

- A. Melting point (2.2.14): 125 °C to 128 °C.
- B. Dissolve 10.0 mg in *methanol R* and dilute to 100.0 ml with the same solvent. Dilute 1.0 ml of the solution to 10.0 ml with *methanol R*. Examined between 220 nm and 350 nm (2.2.25), the solution shows an absorption maximum at 310 nm. The specific absorbance at the maximum is 340 to 360.

- C. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *tinidazole CRS*. Examine the substances prepared as discs.

- D. Examine the chromatograms obtained in the test for related substances. The principal spot in the chromatogram obtained with test solution (b) is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a).

- E. To about 10 mg add about 10 mg of *zinc powder R*, 0.3 ml of *hydrochloric acid R* and 1 ml of *water R*. Heat in a water-bath for 5 min and cool. The solution gives the reaction of primary aromatic amines (2.3.1).

TESTS

Appearance of solution. Dissolve 1.0 g in *acetone R* and dilute to 20 ml with the same solvent. The solution is clear (2.2.1) and not more intensely coloured than reference solution Y_5 (2.2.2, *Method II*).

Related substances. Examine by thin-layer chromatography (2.2.27), using *silica gel GF₂₅₄ R* as the coating substance.

Test solution (a). Dissolve 0.20 g of the substance to be examined in *methanol R* with the aid of ultrasound and dilute to 10 ml with the same solvent.

Test solution (b). Dilute 1.0 ml of test solution (a) to 10 ml with *methanol R*.

Reference solution (a). Dissolve 20 mg of *tinidazole CRS* in *methanol R* and dilute to 10 ml with the same solvent.

Reference solution (b). Dilute 1.0 ml of test solution (b) to 20 ml with *methanol R*.

Reference solution (c). Dilute 4 ml of reference solution (b) to 10 ml with *methanol R*.

Reference solution (d). Dissolve 10 mg of *2-methyl-5-nitroimidazole R* (impurity A) in *methanol R* and dilute to 100 ml with the same solvent.

Reference solution (e). Dissolve 10 mg of *tinidazole impurity B CRS* in *methanol R* and dilute to 100 ml with the same solvent.

Heat the plate at 110 °C for 1 h and allow to cool.

Apply separately to the plate 10 µl of each solution. Develop over a path of 15 cm using a mixture of 25 volumes of *butanol R* and 75 volumes of *ethyl acetate R*. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. Any spots corresponding to tinidazole impurity A and tinidazole impurity B in the chromatogram obtained with test solution (a) are not more intense than the corresponding spots in the chromatogram obtained with reference solutions (d) and (e) (0.5 per cent), respectively. Any spot, apart from the principal spot and any spots corresponding to tinidazole impurity A and tinidazole impurity B in the chromatogram obtained with test solution (a), is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.5 per cent) and at most one such spot is more intense than the spot in the chromatogram obtained with reference solution (c) (0.2 per cent).

Heavy metals (2.4.8). 1.0 g complies with limit test D for heavy metals (20 ppm). Prepare the standard using 2 ml of *lead standard solution (10 ppm Pb) R*.

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

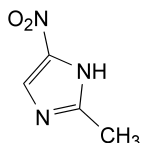
Dissolve 0.150 g in 25 ml of *anhydrous acetic acid R*. Titrate with 0.1 M perchloric acid, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M perchloric acid is equivalent to 24.73 mg of $C_8H_{13}N_3O_4S$.

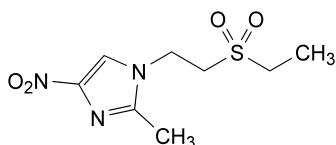
STORAGE

Store protected from light.

IMPURITIES



A. 2-methyl-5-nitro-1*H*-imidazole,

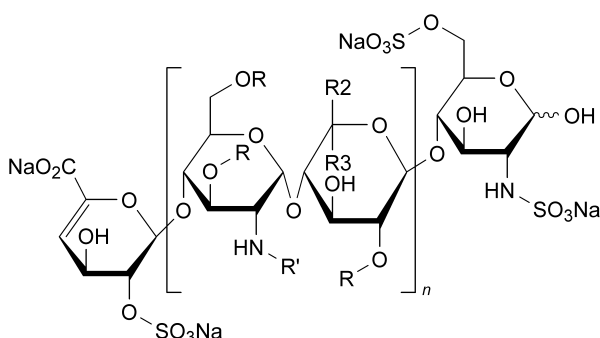


B. 1-[2-(ethylsulphonyl)ethyl]-2-methyl-4-nitro-1*H*-imidazole.

01/2005:1271

TINZAPARIN SODIUM

Tinzaparinum natricum



$n = 1$ to 25, $R = H$ or SO_3Na , $R' = H$ or SO_3Na or $CO-CH_3$
 $R_2 = H$ and $R_3 = CO_2Na$ or $R_2 = CO_2Na$ and $R_3 = H$

DEFINITION

Tinzaparin sodium is the sodium salt of a low-molecular-mass heparin that is obtained by controlled enzymatic depolymerisation of heparin from porcine intestinal mucosa using heparinase from *Flavobacterium heparinum*. The majority of the components have a 2-*O*-sulpho-4-enepyranosuronic acid structure at the non-reducing end and a 2-*N*,6-*O*-disulpho-D-glucosamine structure at the reducing end of their chain.

Tinzaparin sodium complies with the monograph on Low-molecular-mass heparins (0828) with the modifications and additional requirements below.

The mass-average molecular mass ranges between 5500 and 7500 with a characteristic value of about 6500.

The degree of sulphatation is 1.8 to 2.5 per disaccharide unit.

The potency is not less than 70 IU and not more than 120 IU of anti-factor Xa activity per milligram calculated with reference to the dried substance. The ratio of the anti-factor Xa activity to anti-factor IIa activity is between 1.5 and 2.5.

IDENTIFICATION

Carry out identification test C as described in the monograph for *Low-molecular-mass heparins (0828)*. The following requirements apply.

The mass-average molecular mass ranges between 5500 and 7500. The mass percentage of chains lower than 2000 is not more than 10.0 per cent. The mass percentage of chains between 2000 and 8000 ranges between 60.0 and 72.0 per cent. The mass percentage of chains above 8000 ranges between 22.0 and 36.0 per cent.

TESTS

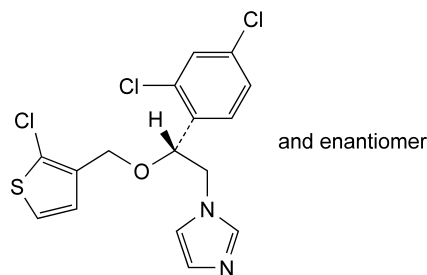
Appearance of solution. Dissolve 1.0 g in 10 ml of *water R*. The solution is clear (2.2.1) and not more intensely coloured than intensity 5 of the range of reference solutions of the most appropriate colour (2.2.2, *Method II*)

Absorbance (2.2.25). Dissolve 50.0 mg in 100 ml of 0.01 M hydrochloric acid. The specific absorbance, measured at 231 nm and calculated with reference to the dried substance, is 8.0 to 12.5.

01/2005:2074

TIOCONAZOLE

Tioconazolum

 $C_{16}H_{13}Cl_3N_2OS$ M_r 387.7

DEFINITION

1-[(2*RS*)-2-[(2-Chlorothiophen-3-yl)methoxy]-2-(2,4-dichlorophenyl)ethyl]-1*H*-imidazole.

Content: 99.0 per cent to 101.0 per cent (anhydrous substance).

CHARACTERS

Appearance: white or almost white, crystalline powder.

Solubility: very slightly soluble in water, very soluble in methylene chloride, freely soluble in alcohol.

IDENTIFICATION

Infrared absorption spectrophotometry (2.2.24).

Comparison: *Ph. Eur. reference spectrum of tioconazole.*

TESTS

Related substances. Liquid chromatography (2.2.29).

Test solution. Dissolve 20.0 mg of the substance to be examined in the mobile phase and dilute to 10.0 ml with the mobile phase.

Reference solution (a). Dilute 1.0 ml of the test solution to 100.0 ml with the mobile phase. Dilute 2.0 ml of this solution to 10.0 ml with the mobile phase.

Reference solution (b). Dissolve 5 mg of *tioconazole for system suitability CRS* in the mobile phase and dilute to 2.5 ml with the mobile phase.

Column:

— *size:* $l = 0.25$ m, $\varnothing = 4.6$ mm,

- *stationary phase*: end-capped octadecylsilyl silica gel for chromatography *R* (5 µm) with a specific surface area of 170 m²/g, a pore size of 12 nm and a carbon loading of 10 per cent.

Mobile phase: mix 1 volume of a 1.7 g/l solution of tetrabutylammonium dihydrogen phosphate *R* previously adjusted to pH 7.4 with dilute ammonia *R*2 and 3 volumes of methanol *R*.

Flow rate: 1 ml/min.

Detection: spectrophotometer at 218 nm.

Injection: 20 µl.

Run time: 2.5 times the retention time of tioconazole.

System suitability: reference solution (b):

- *resolution*: minimum 1.0 between the peaks due to impurity B and impurity C (locate impurities A, B and C by comparison with the chromatogram provided with tioconazole for system suitability CRS).

Limits:

- *correction factors*: for the calculation of contents, multiply the peak areas of the following impurities by the corresponding correction factor: impurity B = 1.7; impurity C = 1.7.
- *impurities A, B, C*: for each impurity, not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.3 per cent),
- *any other impurity*: for each impurity, not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent),
- *total*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (1.0 per cent),
- *disregard limit*: 0.25 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

Water (2.5.12): maximum 0.5 per cent, determined on 1.00 g.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.300 g in 50 ml of anhydrous acetic acid *R*. Titrate with 0.1 M perchloric acid, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M perchloric acid is equivalent to 38.77 mg of C₁₆H₁₃Cl₃N₂OS.

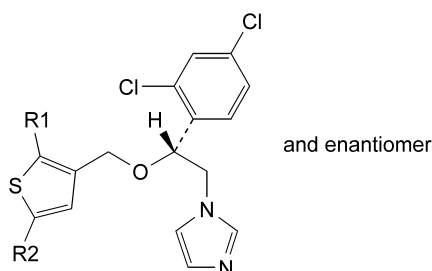
STORAGE

Protected from light.

IMPURITIES

Specified impurities: A, B, C.

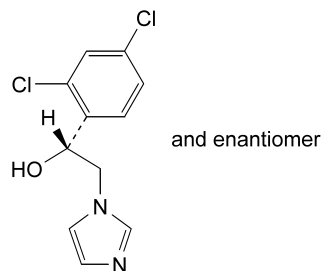
Other detectable impurities: D.



- A. R1 = R2 = H: 1-[(2RS)-2-(2,4-dichlorophenyl)-2-[(thiophen-3-yl)methoxy]ethyl]-1H-imidazole,

- B. R1 = R2 = Cl: 1-[(2RS)-2-(2,4-dichlorophenyl)-2-[(2,5-dichlorothiophen-3-yl)methoxy]ethyl]-1H-imidazole,

- C. R1 = Cl, R2 = Br: 1-[(2RS)-2-[(5-bromo-2-chlorothiophen-3-yl)methoxy]-2-(2,4-dichlorophenyl)ethyl]-1H-imidazole,



- D. (1RS)-1-(2,4-dichlorophenyl)-2-(1H-imidazol-1-yl)ethanol.

01/2005:0150

TITANIUM DIOXIDE

Titanii dioxidum

TiO₂

M_r 79.9

DEFINITION

Titanium dioxide contains not less than 98.0 per cent and not more than the equivalent of 100.5 per cent of TiO₂.

CHARACTERS

A white or almost white powder, practically insoluble in water. It does not dissolve in dilute mineral acids but dissolves slowly in hot concentrated sulphuric acid.

IDENTIFICATION

- When strongly heated, it becomes pale yellow; the colour disappears on cooling.
- To 5 ml of solution S2 (see Tests) add 0.1 ml of *strong hydrogen peroxide solution R*. An orange-red colour appears.
- To 5 ml of solution S2 add 0.5 g of *zinc R* in granules. After 45 min, the mixture has a violet-blue colour.

TESTS

Solution S1. Shake 20.0 g with 30 ml of *hydrochloric acid R* for 1 min. Add 100 ml of *distilled water R* and heat the mixture to boiling. Filter the hot mixture through a hardened filter paper until a clear filtrate is obtained. Wash the filter with 60 ml of *distilled water R* and dilute the combined filtrate and washings to 200 ml with *distilled water R*.

Solution S2. Mix 0.500 g (*m* g) with 5 g of *anhydrous sodium sulphate R* in a 300 ml long-necked combustion flask. Add 10 ml of *water R* and mix. Add 10 ml of *sulphuric acid R* and boil vigorously, with the usual precautions, until a clear solution is obtained. Cool, add slowly a cooled mixture of 30 ml of *water R* and 10 ml of *sulphuric acid R*, cool again and dilute to 100.0 ml with *water R*.

Appearance of solution. Solution S2 is not more opalescent than reference suspension II (2.2.1) and is colourless (2.2.2, *Method II*).

Acidity or alkalinity. Shake 5.0 g with 50 ml of *carbon dioxide-free water R* for 5 min. Centrifuge or filter until a clear solution is obtained. To 10 ml of the solution add 0.1 ml of *bromothymol blue solution R1*. Not more than 1.0 ml of 0.01 M *hydrochloric acid* or 0.01 M *sodium hydroxide* is required to change the colour of the indicator.

Water-soluble substances. To 10.0 g add a solution of 0.5 g of *ammonium sulphate R* in 150 ml of *water R* and boil for 5 min. Cool, dilute to 200 ml with *water R* and filter until a clear solution is obtained. Evaporate 100 ml of the solution to dryness in a tared evaporating dish and ignite. The residue weighs not more than 25 mg (0.5 per cent).

Antimony. To 10 ml of solution S2 add 10 ml of *hydrochloric acid R* and 10 ml of *water R*. Cool to 20 °C, if necessary, and add 0.15 ml of *sodium nitrite solution R*. After 5 min, add 5 ml of a 10 g/l solution of *hydroxylamine hydrochloride R* and 10 ml of a freshly prepared 0.1 g/l solution of *rhodamine B R*. Mix thoroughly after each addition. Shake vigorously with 10.0 ml of *toluene R* for 1 min. Allow to separate and centrifuge for 2 min if necessary. Any pink colour in the toluene phase is not more intense than that in the toluene phase of a standard prepared at the same time in the same manner using a mixture of 5.0 ml of *antimony standard solution (1 ppm Sb) R*, 10 ml of *hydrochloric acid R* and 15 ml of a solution containing 0.5 g of *anhydrous sodium sulphate R* and 2 ml of *sulphuric acid R* instead of the mixture of 10 ml of solution S2, 10 ml of *hydrochloric acid R* and 10 ml of *water R* (100 ppm).

Arsenic (2.4.2). Place 0.50 g in a 250 ml round-bottomed flask, fitted with a thermometer, a funnel with stopcock and a vapour-outlet tube connected to a flask containing 30 ml of *water R*. Add 50 ml of *water R*, 0.5 g of *hydrazine sulphate R*, 0.5 g of *potassium bromide R* and 20 g of *sodium chloride R*. Through the funnel, add dropwise 25 ml of *sulphuric acid R*, heat and maintain the temperature of the liquid at 110 °C to 115 °C for 20 min. Collect the vapour in the flask containing 30 ml of *water R*. Dilute to 50 ml with *water R*. 20 ml of the solution complies with limit test A for arsenic (5 ppm).

Barium. To 10 ml of solution S1 add 1 ml of *dilute sulphuric acid R*. After 30 min, any opalescence in the solution is not more intense than that in a mixture of 10 ml of solution S1 and 1 ml of *distilled water R*.

Heavy metals (2.4.8). Dilute 10 ml of solution S1 to 20 ml with *water R*. 12 ml of the solution complies with limit test A for heavy metals (20 ppm). Prepare the standard using *lead standard solution (1 ppm Pb) R*.

Iron. To 8 ml of solution S2 add 4 ml of *water R*. Mix and add 0.05 ml of *bromine water R*. Allow to stand for 5 min and remove the excess of bromine with a current of air. Add 3 ml of *potassium thiocyanate solution R*. Any colour in the solution is not more intense than that in a standard prepared at the same time in the same manner using a mixture of 4 ml of *iron standard solution (2 ppm Fe) R* and 8 ml of a 200 g/l solution of *sulphuric acid R* (200 ppm).

ASSAY

To 300 g of *zinc R* in granules (710) add 300 ml of a 20 g/l solution of *mercuric nitrate R* and 2 ml of *nitric acid R*, shake for 10 min and wash with *water R*. Pack the amalgamated zinc into a glass tube about 400 mm long and about 20 mm in diameter fitted with a tap and a filter plate. Pass through the column 100 ml of *dilute sulphuric acid R* followed by 100 ml of *water R*, making sure that the amalgam is always covered with liquid. Pass slowly at a rate of about 3 ml/min through the column a mixture of 100 ml of *dilute sulphuric acid R* and 100 ml of *water R* followed by 100 ml of *water R*. Collect the eluate in a 500 ml conical flask containing 50.0 ml of a 150 g/l solution of *ferric ammonium sulphate R* in a mixture of 1 volume of *sulphuric acid R* and 3 volumes of *water R*. Add 0.1 ml of *ferroin R* and titrate immediately with 0.1 M *ammonium and cerium nitrate* until a greenish colour is obtained

(n_1 ml). Pass slowly at a rate of about 3 ml/min through the column a mixture of 50 ml of *dilute sulphuric acid R* and 50 ml of *water R*, followed by 20.0 ml of solution S2, a mixture of 50 ml of *dilute sulphuric acid R* and 50 ml of *water R* and finally 100 ml of *water R*. Collect the eluate in a 500 ml conical flask containing 50.0 ml of a 150 g/l solution of *ferric ammonium sulphate R* in a mixture of 1 volume of *sulphuric acid R* and 3 volumes of *water R*. Rinse the lower end of the column with *water R*, add 0.1 ml of *ferroin R* and titrate immediately with 0.1 M *ammonium and cerium nitrate* until a greenish colour is obtained (n_2 ml).

Calculate the percentage content of TiO_2 from the expression:

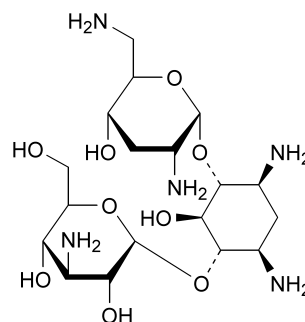
$$\frac{3.99 \times (n_2 - n_1)}{m}$$

m = mass in grams of the substance to be examined used for the preparation of solution S2.

01/2005:0645

TOBRAMYCIN

Tobramycinum


 $\text{C}_{18}\text{H}_{37}\text{N}_5\text{O}_9$
 M_r 467.5

DEFINITION

4-O-(3-Amino-2-deoxy-α-D-glucopyranosyl)-2-deoxy-6-O-(2,6-diamino-2,3,6-trideoxy-α-D-ribohexopyranosyl)-L-streptomine.

Substance produced by *Streptomyces tenebrarius* or obtained by any other means.

Content: 97.0 per cent to 102.0 per cent (anhydrous and 2-methyl-1-propanol-free substance).

PRODUCTION

It is produced by methods of manufacture designed to eliminate or minimise substances lowering blood pressure.

CHARACTERS

Appearance: white or almost white powder.

Solubility: freely soluble in water, very slightly soluble in alcohol.

IDENTIFICATION

First identification: A.

Second identification: B, C.

A. Nuclear magnetic resonance spectrometry (2.2.33).

Preparation: 100 g/l solution in *deuterium oxide R*.

Comparison: 100 g/l solution of *tobramycin CRS* in *deuterium oxide R*.

B. Thin-layer chromatography (2.2.27).

Test solution. Dissolve 20 mg of the substance to be examined in *water R* and dilute to 5 ml with the same solvent.

Reference solution (a). Dissolve 20 mg of *tobramycin CRS* in *water R* and dilute to 5 ml with the same solvent.

Reference solution (b). Dissolve 4 mg of *neomycin sulphate CRS* and 4 mg of *kanamycin monosulphate CRS* in 1 ml of reference solution (a).

Plate: *TLC silica gel plate R*.

Mobile phase: *methylene chloride R*, *concentrated ammonia R*, *methanol R* (17:33:50 *V/V/V*).

Application: 5 µl.

Development: over 2/3 of the plate.

Drying: in a current of warm air.

Detection: spray with a mixture of equal volumes of a 2 g/l solution of *1,3-dihydroxynaphthalene R* in *alcohol R* and a 460 g/l solution of *sulphuric acid R*. Heat at 105 °C for 5-10 min.

System suitability: the chromatogram obtained with reference solution (b) shows 3 major spots which are clearly separated.

Results: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).

- C. Dissolve about 5 mg in 5 ml of *water R*. Add 5 ml of a 1 g/l solution of *ninhydrin R* in *alcohol R* and heat in a water-bath for 3 min. A violet-blue colour develops.

TESTS

pH (2.2.3): 9.0 to 11.0.

Dissolve 1.0 g in 10 ml of *carbon dioxide-free water R*.

Specific optical rotation (2.2.7): + 138 to + 148 (anhydrous and 2-methyl-1-propanol-free substance).

Dissolve 1.00 g in *water R* and dilute to 25.0 ml with the same solvent.

Related substances. Liquid chromatography (2.2.29).

Test solution (a). Dissolve 25.0 mg of the substance to be examined in *water R* and dilute to 25.0 ml with the same solvent.

Test solution (b). Dilute 10.0 ml of test solution (a) to 100.0 ml with *water R*.

Reference solution (a). Dissolve 25.0 mg of *tobramycin CRS* in *water R* and dilute to 100.0 ml with the same solvent.

Reference solution (b). Dilute 1.0 ml of reference solution (a) to 100.0 ml with *water R*.

Reference solution (c). Dilute 1.0 ml of reference solution (a) to 50.0 ml with *water R*.

Reference solution (d). Dissolve 10.0 mg of *kanamycin B CRS* in 20.0 ml of *water R*. To 1.0 ml, add 2.0 ml of reference solution (a) and dilute to 10.0 ml with *water R*.

Reference solution (e). Dilute 10.0 ml of reference solution (a) to 25.0 ml with *water R*.

Column:

- **size:** $l = 0.25$ m, $\varnothing = 4.6$ mm,
- **stationary phase:** *styrene-divinylbenzene copolymer R* (8 µm) with a pore size of 100 nm,
- **temperature:** 55 °C.

Mobile phase: a mixture prepared with *carbon dioxide-free water R* containing 52 g/l of *anhydrous sodium sulphate R*, 1.5 g/l of *sodium octanesulphonate R*, 3 ml/l of *tetrahydrofuran R*, 50 ml/l of 0.2 M *potassium dihydrogen phosphate R* previously adjusted to pH 3.0 with *dilute phosphoric acid R*. Degas.

Flow rate: 1.0 ml/min.

Post-column solution: *carbonate-free sodium hydroxide solution R* diluted 1 to 25 with *carbon dioxide-free water R*, which is added pulseless to the column effluent using a 375 µl polymeric mixing coil.

Flow rate: 0.3 ml/min.

Detection: pulsed amperometric detector or equivalent with a gold working electrode, a silver-silver chloride reference electrode and a stainless steel auxiliary electrode which is the cell body, held at respectively + 0.05 V detection, + 0.75 V oxidation and – 0.15 V reduction potentials, with pulse durations according to the instrument used. The temperature of the detector is set at 35 °C.

Injection: 20 µl; inject test solution (a) and reference solutions (b), (c) and (d).

Run time: 1.5 times the retention time of tobramycin.

Relative retention with reference to tobramycin (retention time = about 18 min): impurity C = about 0.35; impurity B = about 0.40, impurity A = about 0.70.

System suitability:

- **resolution:** minimum of 3.0 between the peaks due to impurity A and to tobramycin in the chromatogram obtained with reference solution (d). If necessary, adjust the concentration of sodium octanesulphonate in the mobile phase;
- **signal-to-noise ratio:** minimum of 10 for the principal peak in the chromatogram obtained with reference solution (b).

Limits:

- **any impurity:** not more than twice the area of the principal peak in the chromatogram obtained with reference solution (c) (1.0 per cent) and not more than 1 such peak has an area greater than the area of the principal peak in the chromatogram obtained with reference solution (c) (0.5 per cent),
- **total:** not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (c) (1.5 per cent),
- **disregard limit:** the area of the principal peak in the chromatogram obtained with reference solution (b) (0.25 per cent).

2-Methyl-1-propanol (2.4.24, *System B*): maximum 1.0 per cent *m/m*.

Water (2.5.12): maximum 8.0 per cent, determined on 0.30 g.

Sulphated ash (2.4.14): maximum 0.3 per cent, determined on 1.0 g.

Bacterial endotoxins (2.6.14): less than 2.0 IU/mg, if intended for use in the manufacture of parenteral dosage forms without a further appropriate procedure for the removal of bacterial endotoxins.

ASSAY

Liquid chromatography (2.2.29) as described in the test for related substances with the following modifications.

Injection: test solution (b) and reference solution (e).

Calculate the percentage content of tobramycin.

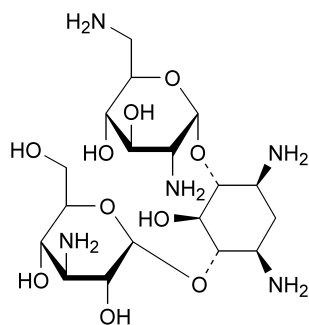
STORAGE

If the substance is sterile, store in a sterile, airtight, tamper-proof container.

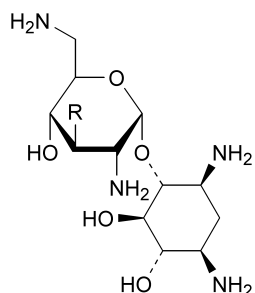
LABELLING

The label states, where applicable, that the substance is free from bacterial endotoxins.

IMPURITIES

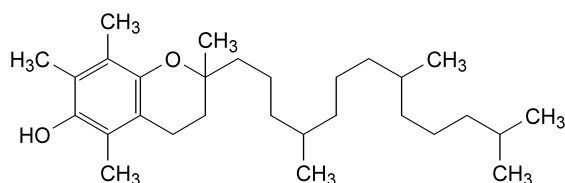


- A. 4-O-(3-amino-3-deoxy- α -D-glucopyranosyl)-2-deoxy-6-O-(2,6-diamino-2,6-dideoxy- α -D-glucopyranosyl)-L-streptamine (kanamycin B),



- B. R = H: 2-deoxy-4-O-(2,6-diamino-2,3,6-trideoxy- α -D-ribohexopyranosyl)-D-streptamine (nebramine),
C. R = OH: 2-deoxy-4-O-(2,6-diamino-2,6-dideoxy- α -D-glucopyranosyl)-D-streptamine (neamine).

01/2005:0692

all-*rac*- α -TOCOPHEROLint-*rac*- α -Tocopherolum $C_{29}H_{50}O_2$ M_r 430.7

DEFINITION

all-*rac*-2,5,7,8-Tetramethyl-2-(4,8,12-trimethyltridecyl)-3,4-dihydro-2*H*-1-benzopyran-6-ol.

Content: 96.0 per cent to 101.5 per cent.

CHARACTERS

Appearance: clear, colourless or yellowish-brown, viscous, oily liquid.

Solubility: practically insoluble in water, freely soluble in acetone, in ethanol, in methylene chloride and in fatty oils.

IDENTIFICATION

First identification: A, B.

Second identification: A, C.

- A. Optical rotation (2.2.7): -0.01° to $+0.01^\circ$.

Dissolve 2.50 g in *ethanol R* and dilute to 25.0 ml with the same solvent.

- B. Infrared absorption spectrophotometry (2.2.24).

Comparison: α -tocopherol CRS.

- C. Thin-layer chromatography (2.2.27).

Test solution. Dissolve 10 mg of the substance to be examined in 2 ml of *cyclohexane R*.

Reference solution. Dissolve 10 mg of α -tocopherol CRS in 2 ml of *cyclohexane R*.

Plate: TLC silica gel F_{254} plate *R*.

Mobile phase: *ether R*, *cyclohexane R* (20:80 V/V).

Application: 10 μ l.

Development: over 2/3 of the plate.

Drying: in a current of air.

Detection: examine in ultraviolet light at 254 nm.

Results: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with the reference solution.

TESTS

Related substances. Gas chromatography (2.2.28): use the normalisation procedure.

Internal standard solution. Dissolve 1.0 g of *squalane R* in *cyclohexane R* and dilute to 100.0 ml with the same solvent.

Test solution (a). Dissolve 0.100 g of the substance to be examined in 10.0 ml of the internal standard solution.

Test solution (b). Dissolve 0.100 g of the substance to be examined in 10 ml of *cyclohexane R*.

Reference solution (a). Dissolve 0.100 g of α -tocopherol CRS in 10.0 ml of the internal standard solution.

Reference solution (b). Dissolve 10 mg of the substance to be examined and 10 mg of α -tocopheryl acetate *R* in *cyclohexane R* and dilute to 100.0 ml with the same solvent.

Reference solution (c). Dissolve 10 mg of all-*rac*- α -tocopherol for peak identification CRS in *cyclohexane R* and dilute to 1 ml with the same solvent.

Reference solution (d). Dilute 1.0 ml of test solution (b) to 100.0 ml with *cyclohexane R*. Dilute 1.0 ml of this solution to 10.0 ml with *cyclohexane R*.

Column:

- **material:** fused silica,
- **size:** $l = 30$ m, $\varnothing = 0.25$ mm,
- **stationary phase:** poly(dimethyl)siloxane *R* (film thickness 0.25 μ m).

Carrier gas: helium for chromatography *R*.

Flow rate: 1 ml/min.

Split ratio: 1:100.

Temperature:

- **column:** 280 $^\circ$ C,
- **injection port and detector:** 290 $^\circ$ C.

Detection: flame ionisation.

Injection: 1 μ l of test solution (b) and reference solutions (b), (c) and (d).

Run time: twice the retention time of all-*rac*- α -tocopherol.

Relative retention with reference to all-*rac*- α -tocopherol (retention time = about 13 min): *squalane* = about 0.5; impurity A = about 0.7; impurity B = about 0.8. Use the chromatogram supplied with the CRS and the chromatogram obtained with reference solution (c) to identify the peaks due to impurities A and B.

System suitability: reference solution (b):

- **resolution:** minimum 3.5 between the peaks due to all-*rac*- α -tocopherol and α -tocopheryl acetate.

Limits:

- **impurity A:** maximum 0.5 per cent,

- *impurity B*: maximum 1.5 per cent,
- *any other impurity*: for each impurity, maximum 0.25 per cent,
- *total*: maximum 2.5 per cent,
- *disregard limit*: the area of the principal peak in the chromatogram obtained with reference solution (d) (0.1 per cent).

ASSAY

Gas chromatography (2.2.28) as described in the test for related substances with the following modification.

Injection: test solution (a) and reference solution (a).

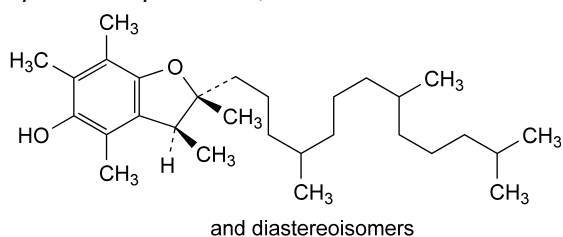
Calculate the percentage content of $C_{29}H_{50}O_2$ taking into account the declared content of α -tocopherol CRS.

STORAGE

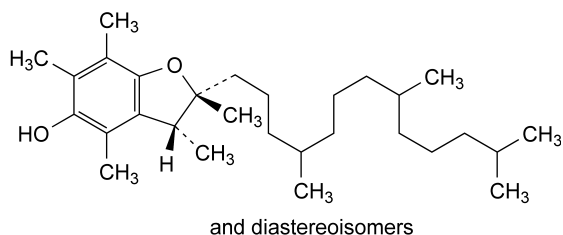
Under an inert gas, protected from light.

IMPURITIES

Specified impurities: A, B.



- A. all-*rac-trans*-2,3,4,6,7-pentamethyl-2-(4,8,12-trimethyltridecyl)-2,3-dihydrobenzofuran-5-ol,

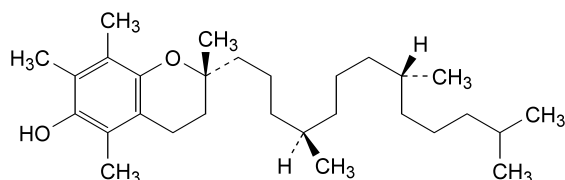


- B. all-*rac-cis*-2,3,4,6,7-pentamethyl-2-(4,8,12-trimethyltridecyl)-2,3-dihydrobenzofuran-5-ol.

01/2005:1256

RRR- α -TOCOPHEROL

RRR- α -Tocopherolum



$C_{29}H_{50}O_2$

M_r 430.7

DEFINITION

RRR- α -Tocopherol contains not less than 96.0 per cent and not more than the equivalent of 102.0 per cent of (2*R*)-2,5,7,8-tetramethyl-2-[(4*R*,8*R*)-4,8,12-trimethyltridecyl]-3,4-dihydro-2*H*-1-benzopyran-6-ol.

CHARACTERS

A clear, colourless or yellowish-brown, viscous, oily liquid, practically insoluble in water, freely soluble in acetone, in ethanol, in methylene chloride and in fatty oils.

IDENTIFICATION

First identification: B, D.

Second identification: A, C, D.

A. It complies with the test for absorbance (see Tests).

B. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with α -tocopherol CRS.

C. Examine by thin-layer chromatography (2.2.27), using a TLC silica gel F_{254} plate R.

Test solution. Dissolve 10 mg of the substance to be examined in 2 ml of cyclohexane R.

Reference solution. Dissolve 10 mg of α -tocopherol CRS in 2 ml of cyclohexane R.

Apply separately to the plate 10 μ l of each solution.

Develop over a path of 15 cm using a mixture of 20 volumes of ether R and 80 volumes of cyclohexane R. Dry the plate in a current of air and examine in ultraviolet light at 254 nm. The principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with the reference solution. Spray the plate with a mixture of 10 volumes of hydrochloric acid R, 40 volumes of a 2.5 g/l solution of ferric chloride R in alcohol R and 40 volumes of a 10 g/l solution of phenanthroline hydrochloride R in alcohol R. After 1 h to 2 h, the principal spots are orange.

D. RRR- α -tocopherol is dextrorotatory (2.2.7). The specific optical rotation after oxidation to the quinone form is not less than + 24.

Dissolve 1.0 g of the substance to be examined in 50 ml of ether R. To the solution add 20 ml of a 100 g/l solution of potassium ferricyanide R in an 8 g/l solution of sodium hydroxide R and shake for 3 min. Wash the ether solution with four quantities, each of 50 ml, of water R, discard the washings and dry the ether layer over anhydrous sodium sulphate R. Evaporate the ether on a water-bath under reduced pressure or in an atmosphere of nitrogen until a few millilitres remain, then complete the evaporation removing the last traces of ether without the application of heat. Immediately dissolve the residue in 5.0 ml of trimethylpentane R and determine the optical rotation.

Calculate the specific optical rotation of the substance in the test solution using as *c* the number of grams of RRR- α -tocopherol in 1000 ml.

TESTS

Absorbance (2.2.25). Dissolve 0.100 g in ethanol R and dilute to 100 ml with the same solvent (solution A). Dilute 10.0 ml of solution A to 100.0 ml with ethanol R (solution B). Measure the absorbance of solution B at the maximum at 292 nm and of solution A at the minimum at 255 nm. The specific absorbance at the maximum is 72.0 to 76.0 and that at the minimum is 5.5 to 8.0.

Acid value (2.5.1). Not more than 2.0, determined on 2.00 g.

Heavy metals (2.4.8). 0.50 g complies with limit test D for heavy metals (20 ppm). Prepare the standard using 1 ml of lead standard solution (10 ppm Pb) R.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g. Use sulphuric acid R instead of dilute sulphuric acid R.

ASSAY

Examine by gas chromatography (2.2.28), using dotriacontane R as the internal standard.

Internal standard solution. Dissolve 0.300 g of dotriacontane *R* in hexane *R* and dilute to 100.0 ml with the same solvent.

Test solution. Dissolve 0.100 g of the substance to be examined in 10.0 ml of the internal standard solution, dilute to 50.0 ml with hexane *R* and mix.

Reference solution. Dissolve 0.100 g of α -tocopherol CRS in 10.0 ml of the internal standard solution, dilute to 50.0 ml with hexane *R* and mix.

The chromatographic procedure may be carried out using:

- a fused-silica column, 15 m long and 0.32 mm in internal diameter coated with a 0.25 μ m layer of poly(dimethyl)siloxane *R*,
- helium for chromatography *R* as the carrier gas at a flow rate of 3 ml/min to 6 ml/min,
- a flame-ionisation detector,

maintaining the temperature of the injection port at 300 °C and that of the detector at 330 °C. Adjust the split ratio between 1 to 10 and 1 to 20. Set the temperature of the column at 200 °C, then raise the temperature at a rate of 5 °C per minute to 250 °C and maintain at 250 °C for 10 min.

Inject directly onto the column or via a glass-lined injection port using an automatic injection device or some other reproducible injection method. Measure the peak areas by electronic integration. The test is not valid unless in the chromatogram obtained with the reference solution, the resolution between the peaks corresponding to dotriacontane and α -tocopherol is at least 9.0.

Interference test. Dissolve 0.100 g of the substance to be examined in hexane *R* and dilute to 50.0 ml with the same solvent. Inject 1 μ l of the solution and record the chromatogram. If a peak is detected with the same t_R value as for dotriacontane, calculate the area of this peak relative to the peak area of α -tocopherol. If the relative peak area is greater than 0.5 per cent, use the corrected peak area $S'_{D(\text{corr})}$ for the final calculation.

$$S'_{D(\text{corr})} = S'_D - \frac{S_I \times S'_T}{S_{TI}}$$

- S'_D = area of the peak corresponding to the internal standard in the chromatogram obtained with the test solution,
- S_I = area of the interfering peak (same t_R value as that of the internal standard) in the chromatogram obtained in the interference test,
- S'_T = area of the peak corresponding to α -tocopherol in the chromatogram obtained with the test solution,
- S_{TI} = area of the peak corresponding to α -tocopherol in the chromatogram obtained in the interference test.

Once the system suitability has been established, inject 1 μ l of the reference solution and record the chromatogram. Measure the areas of the peaks corresponding to α -tocopherol (S_T) and dotriacontane (S_D) and determine the response factor (RF) as described below.

Determine the response factor (RF) for α -tocopherol in the chromatogram obtained with the reference solution from the area of the peak corresponding to α -tocopherol and the peak corresponding to dotriacontane using the expression:

$$\text{RF} = \frac{S_D \times m_T}{S_T \times m_D}$$

Inject 1 μ l of the test solution in the same manner. Measure the areas of the peaks corresponding to α -tocopherol (S'_T) and dotriacontane (S'_D).

Calculate the percentage content of *RRR*- α -tocopherol using the expression:

$$\frac{100 (S'_T \times m_D \times \text{RF})}{S'_{D(\text{corr})} \times m}$$

- S_D = area of the peak corresponding to the internal standard in the chromatogram obtained with the reference solution,
- $S'_{D(\text{corr})}$ = corrected area of the peak corresponding to the internal standard in the chromatogram obtained with the test solution,
- S_T = area of the peak corresponding to α -tocopherol CRS in the chromatogram obtained with the reference solution,
- S'_T = area of the peak corresponding to α -tocopherol in the chromatogram obtained with the test solution,
- m_D = mass of the internal standard in the test solution and in the reference solution, in milligrams,
- m_T = mass of α -tocopherol CRS in the reference solution, in milligrams,
- m = mass of the substance to be examined in the test solution, in milligrams.

STORAGE

Store in an airtight container, under an inert gas, protected from light.

01/2005:0691

α -TOCOPHEROL ACETATE CONCENTRATE (POWDER FORM)

α -Tocopheroli acetatis pulvis

DEFINITION

α -Tocopherol acetate concentrate (powder form) is prepared either by finely dispersing α -Tocopherol acetate (0439) in a suitable carrier of suitable quality (for example gelatin, acacia, carbohydrates, lactoproteins or a mixture thereof) or by adsorbing α -Tocopherol acetate (0439) on silicic acid of suitable quality.

The declared content of α -tocopherol acetate is not less than 25 g per 100 g of concentrate. The concentrate contains not less than 90.0 per cent and not more than 115.0 per cent of the content stated on the label.

CHARACTERS

Almost white, yellowish or light-brown small particles which, depending on their formulation, may be practically insoluble in water or may swell or form a dispersion.

IDENTIFICATION

Examine by thin-layer chromatography (2.2.27), using silica gel HF₂₅₄ *R* as the coating substance.

Test solution. To a quantity of concentrate corresponding to 50 mg of α -tocopherol acetate add 5 ml of 0.01 *M* hydrochloric acid and treat with ultrasound at 60 °C. Add 5 ml of ethanol *R* and 10 ml of cyclohexane *R*, shake for 1 min and centrifuge for 5 min. Use the upper layer.

Reference solution. Dissolve 50 mg of α -tocopherol acetate CRS in cyclohexane R and dilute to 10 ml with the same solvent.

Apply separately to the plate 10 μ l of each solution. Develop over a path of 15 cm using a mixture of 20 volumes of ether R and 80 volumes of cyclohexane R. Dry the plate in a current of air and examine in ultraviolet light at 254 nm. The principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with the reference solution.

ASSAY

Examine by gas chromatography (2.2.28), using dotriacontane R as the internal standard.

Internal standard solution. Dissolve 0.20 g of dotriacontane R in hexane R and dilute to 100.0 ml with the same solvent.

Test solution. Weigh accurately a quantity of the preparation to be examined corresponding to about 0.100 g of α -tocopherol acetate in a 250 ml conical flask. Add 20 ml of 1 M hydrochloric acid and treat with ultrasound at 70 °C for 20 min. Add 50 ml of ethanol R and 50.0 ml of the internal standard solution and thoroughly mix the two phases for 30 min. Allow to separate, and use the upper layer.

Reference solution. Dissolve 0.100 g of α -tocopherol acetate CRS in the internal standard solution and dilute to 50.0 ml with the internal standard solution.

The chromatographic procedure may be carried out using:

- a silanised glass column 2.0 m to 3.0 m long and 2.2 mm to 4.0 mm in internal diameter packed with *diatomaceous earth for gas chromatography* R (125 μ m to 150 μ m or 150 μ m to 180 μ m), silanised with dimethyldichlorosilane and impregnated with 1 per cent *m/m* to 5 per cent *m/m* of *poly(dimethyl)siloxane* R; a plug of silanised glass wool is placed at each end of the column,
- *nitrogen for chromatography* R as the carrier gas at a flow rate of 25-90 ml/min,
- a flame-ionisation detector,

maintaining the column at a constant temperature between 245 °C and 280 °C and the injection port and the detector each at a constant temperature between 270 °C and 320 °C. Set the temperature of the column and the flow rate of the carrier gas in such a manner that the required resolution is achieved.

Make the injections directly onto the column or via an injection port (preferably glass-lined) using an automatic injection device or some other reproducible injection method. Measure the peak areas by electronic integration.

Resolution. Inject 1 μ l of the reference solution. Repeat this operation until the response factor (RF) determined as described below is constant to within ± 2 per cent. The resolution (R_s) between the dotriacontane peak and the α -tocopherol acetate peak is at least 1.4.

Interference test. Weigh accurately a quantity of the substance to be examined corresponding to about 0.100 g of α -tocopherol acetate in a 250 ml conical flask. Add 20 ml of 1 M hydrochloric acid and treat with ultrasound at 70 °C for 20 min. Add 50 ml of ethanol R and 50 ml of hexane R and thoroughly mix the two phases for 30 min. Allow to separate. Inject 1 μ l of the upper layer and record the chromatogram, choosing an attenuation such that the height of the peak corresponding to α -tocopherol acetate is greater than 50 per cent of the maximum recorder response; during the recording, change the attenuation so that any peak appearing with the same t_R value as for dotriacontane

is recorded with a sensitivity at least eight times greater than for the α -tocopherol acetate peak. If a peak with a height of at least 5 mm for a recorder paper width of 250 mm is detected with the same t_R value as for dotriacontane, use the corrected peak area $S'_{D(\text{corr.})}$ for the final calculation.

$$S'_{D(\text{corr.})} = S'_D - \frac{S_I \times S'_T}{f \times S_{TI}}$$

S'_D = area of the peak corresponding to the internal standard in the chromatogram obtained with the test solution,

S_I = area of the interfering peak (same t_R value as that of the internal standard) in the chromatogram obtained in the interference test,

S'_T = area of the peak corresponding to α -tocopherol acetate in the chromatogram obtained with the test solution,

S_{TI} = area of the peak corresponding to α -tocopherol acetate in the chromatogram obtained in the interference test,

f = factor by which the attenuation was changed.

Inject 1 μ l of the reference solution and record the chromatogram, choosing an attenuation such that the peak corresponding to α -tocopherol acetate is greater than 50 per cent of the maximum recorder response. Measure the areas of the peaks corresponding to α -tocopherol acetate (S_T) and to dotriacontane (S_D) and determine the response factor (RF) as described below. Inject 1 μ l of the test solution in the same manner. Measure the areas of the peaks corresponding to α -tocopherol acetate (S'_T) and to dotriacontane (S'_D).

Determine the response factor (RF) for α -tocopherol acetate in the chromatogram obtained with the reference solution from the areas of the peak corresponding to α -tocopherol acetate and the peak corresponding to dotriacontane using the expression:

$$\frac{S_D \times m_T}{S_T \times m_D}$$

Calculate the percentage content of α -tocopherol acetate using the expression:

$$\frac{100 \times S'_T \times m_D \times \text{RF}}{S'_{D(\text{corr.})} \times m}$$

S_D = area of the peak corresponding to the internal standard in the chromatogram obtained with the reference solution,

$S'_{D(\text{corr.})}$ = corrected area of the peak corresponding to the internal standard in the chromatogram obtained with the test solution,

S_T = area of the peak corresponding to α -tocopherol acetate CRS in the chromatogram obtained with the reference solution,

S'_T = area of the peak corresponding to α -tocopherol acetate in the chromatogram obtained with the test solution,

m_D = mass of the internal standard in the test solution and in the reference solution in milligrams,

m_T = mass of α -tocopherol acetate CRS in the reference solution in milligrams,

m = mass of the substance to be examined in the test solution in milligrams.

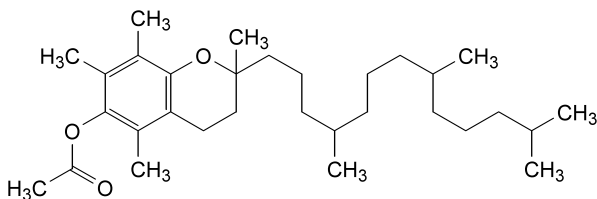
STORAGE

Store in an airtight, well-filled container, protected from light.

LABELLING

The label states the content of α -tocopherol acetate, expressed in grams per 100 g of concentrate.

01/2005:0439

all-*rac*- α -TOCOPHERYL ACETATEint-*rac*- α -Tocopherylis acetas $C_{31}H_{52}O_3$ M_r 472.7

DEFINITION

all-*rac*-2,5,7,8-Tetramethyl-2-(4,8,12-trimethyltridecyl)-3,4-dihydro-2*H*-1-benzopyran-6-yl acetate.

Content: 96.5 per cent to 101.0 per cent.

CHARACTERS

Appearance: clear, colourless or slightly greenish-yellow, viscous, oily liquid.

Solubility: practically insoluble in water, freely soluble in acetone, in ethanol and in fatty oils.

IDENTIFICATION

First identification: A, B.

Second identification: A, C.

A. Optical rotation (2.2.7): -0.01° to $+0.01^\circ$.

Dissolve 2.50 g in *ethanol R* and dilute to 25.0 ml with the same solvent.

B. Infrared absorption spectrophotometry (2.2.24).

Comparison: α -tocopheryl acetate CRS.

C. Thin-layer chromatography (2.2.27).

Test solution. Dissolve about 10 mg of the substance to be examined in 2 ml of *cyclohexane R*.

Reference solution. Dissolve about 10 mg of α -tocopheryl acetate CRS in 2 ml of *cyclohexane R*.

Plate: TLC silica gel F_{254} plate *R*.

Mobile phase: *ether R*, *cyclohexane R* (20:80 V/V).

Application: 10 μ l.

Development: over 2/3 of the plate.

Drying: in a current of air.

Detection: examine in ultraviolet light at 254 nm.

Results: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with the reference solution.

TESTS

Related substances. Gas chromatography (2.2.28): use the normalisation procedure.

Internal standard solution. Dissolve 1.0 g of *squalane R* in *cyclohexane R* and dilute to 100.0 ml with the same solvent.

Test solution (a). Dissolve 0.100 g of the substance to be examined in 10.0 ml of the internal standard solution.

Test solution (b). Dissolve 0.100 g of the substance to be examined in 10 ml of *cyclohexane R*.

Reference solution (a). Dissolve 0.100 g of α -tocopheryl acetate CRS in 10.0 ml of the internal standard solution.

Reference solution (b). Dissolve 10 mg of the substance to be examined and 10 mg of α -tocopherol *R* in *cyclohexane R* and dilute to 100.0 ml with the same solvent.

Reference solution (c). Dissolve 10 mg of all-*rac*- α -tocopheryl acetate for peak identification CRS in *cyclohexane R* and dilute to 1 ml with the same solvent.

Reference solution (d). Dilute 1.0 ml of test solution (b) to 100.0 ml with *cyclohexane R*. Dilute 1.0 ml of this solution to 10.0 ml with *cyclohexane R*.

Column:

- **material:** fused silica,
- **size:** $l = 30$ m, $\varnothing = 0.25$ mm,
- **stationary phase:** poly(dimethyl)siloxane *R* (film thickness 0.25 μ m).

Carrier gas: helium for chromatography *R*.

Flow rate: 1 ml/min.

Split ratio: 1:100.

Temperature:

- **column:** 280 $^\circ$ C,
- **injection port and detector:** 290 $^\circ$ C.

Detection: flame ionisation.

Injection: 1 μ l of test solution (b) and reference solutions (a), (b), (c) and (d).

Run time: twice the retention time of all-*rac*- α -tocopheryl acetate.

Relative retention with reference to all-*rac*- α -tocopheryl acetate (retention time = about 15 min): squalane = about 0.4; impurity A = about 0.7; impurity B = about 0.8; impurity C = about 0.9; use the chromatogram supplied with the CRS to identify the peaks due to impurities A and B in the chromatogram obtained with reference solution (c).

System suitability:

- **resolution:** minimum 3.5 between the peaks due to impurity C and all-*rac*- α -tocopheryl acetate in the chromatogram obtained with reference solution (b),
- in the chromatogram obtained with reference solution (a), the area of the peak due to impurity C is not greater than 0.2 per cent of the area of the peak due to all-*rac*- α -tocopheryl acetate.

Limits:

- **impurities A, C:** for each impurity, maximum 0.5 per cent,
- **impurity B:** maximum 1.5 per cent,
- **any other impurity:** for each impurity, maximum 0.25 per cent,
- **total:** maximum 2.5 per cent,
- **disregard limit:** the area of the principal peak in the chromatogram obtained with reference solution (d) (0.1 per cent).

ASSAY

Gas chromatography (2.2.28) as described in the test for related substances with the following modification.

Injection: test solution (a) and reference solution (a).

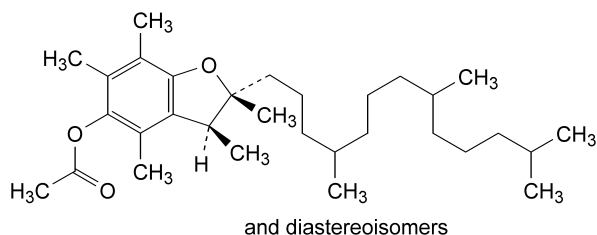
Calculate the percentage content of $C_{31}H_{52}O_3$ taking into account the declared content of α -tocopheryl acetate CRS.

STORAGE

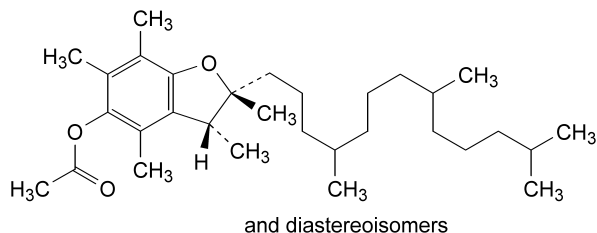
Protected from light.

IMPURITIES

Specified impurities: A, B, C.

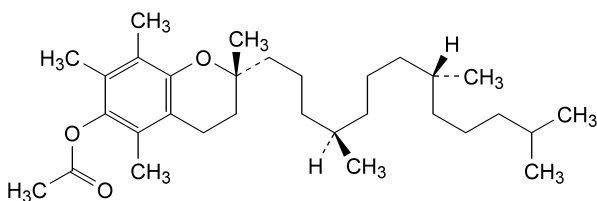


- A. all-*rac-trans*-2,3,4,6,7-pentamethyl-2-(4,8,12-trimethyltridecyl)-2,3-dihydrobenzofuran-5-yl acetate,



- B. all-*rac-cis*-2,3,4,6,7-pentamethyl-2-(4,8,12-trimethyltridecyl)-2,3-dihydrobenzofuran-5-yl acetate,
C. all-*rac*- α -tocopherol.

01/2005:1257

RRR- α -TOCOPHERYL ACETATE*RRR- α -Tocopherylis acetas*C₃₁H₅₂O₃M_r 472.7

DEFINITION

RRR- α -Tocopheryl acetate contains not less than 96.0 per cent and not more than the equivalent of 102.0 per cent of (2*R*)-2,5,7,8-tetramethyl-2-[(4*R*,8*R*)-4,8,12-trimethyltridecyl]-3,4-dihydro-2*H*-1-benzopyran-6-yl acetate.

CHARACTERS

A clear, pale greenish-yellow, viscous, oily liquid, practically insoluble in water, freely soluble in acetone, in ethanol and in fatty oils, soluble in alcohol.

IDENTIFICATION

First identification: B, D.

Second identification: A, C, D.

- A. It complies with the test for absorbance (see Tests).
B. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with α -tocopheryl acetate CRS.
C. Examine by thin-layer chromatography (2.2.27), using a TLC silica gel F₂₅₄ plate R.

Test solution (a). Dissolve 10 mg of the substance to be examined in 2 ml of cyclohexane R.

Test solution (b). In a ground glass-stoppered tube, dissolve 10 mg of the substance to be examined in 2 ml of 2.5 M alcoholic sulphuric acid R. Heat on a water-bath for 5 min. Cool and add 2 ml of water R and 2 ml of cyclohexane R. Shake for 1 min. Use the upper layer.

Reference solution (a). Dissolve 10 mg of α -tocopheryl acetate CRS in 2 ml of cyclohexane R.

Reference solution (b). Prepare as described for test solution (b), using α -tocopheryl acetate CRS instead of the substance to be examined.

Apply separately to the plate 10 μ l of each solution. Develop over a path of 15 cm using a mixture of 20 volumes of ether R and 80 volumes of cyclohexane R. Dry the plate in a current of air and examine in ultraviolet light at 254 nm. The principal spot in the chromatogram obtained with test solution (a) is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a). In the chromatograms obtained with test solution (b) and reference solution (b), there are two spots: the spot with the higher *R_f* value is due to α -tocopheryl acetate and corresponds to the spot in the chromatogram obtained with reference solution (a); the spot with the lower *R_f* value is due to α -tocopherol. Spray the plate with a mixture of 10 volumes of hydrochloric acid R, 40 volumes of a 2.5 g/l solution of ferric chloride R in alcohol R and 40 volumes of a 10 g/l solution of phenanthroline hydrochloride R in alcohol R. In the chromatograms obtained with test solution (b) and reference solution (b), the spot due to α -tocopherol is orange.

- D. After saponification, the resulting RRR- α -tocopherol is dextrorotatory (2.2.7). The specific optical rotation after oxidation to the quinone form is at least + 24.

Carry out the test avoiding exposure to actinic light. Transfer 1.0 g of the substance to be examined to a 250 ml round-bottomed flask with a ground-glass stopper; dissolve in 30 ml of ethanol R and heat under reflux for 3 min. While the solution is boiling, add, through the condenser, 20 ml of 2 M alcoholic potassium hydroxide solution R. Continue heating under reflux for 20 min and, without cooling, add 4.0 ml of hydrochloric acid R dropwise through the condenser. Cool, rinse the condenser with 10 ml of ethanol R, transfer the contents of the flask to a 500 ml separating funnel. Rinse the flask with four quantities, each of 25 ml, of water R and four quantities, each of 25 ml, of ether R. Add the rinsings to the separating funnel. Shake vigorously for 2 min, allow the layers to separate and collect each of the two layers in individual separating funnels. Shake the aqueous layer with two quantities, each of 50 ml, of ether R and add these extracts to the main ether extract. Wash the combined ether extracts with four quantities, each of 100 ml, of water R and discard the washings.

To the ether solution add 40 ml of a 100 g/l solution of potassium ferricyanide R in an 8 g/l solution of sodium hydroxide R and shake for 3 min. Wash the ether solution with four quantities, each of 50 ml, of water R, discard the washings and dry the ether layer over anhydrous sodium sulphate R. Evaporate the ether on a water-bath under reduced pressure or in an atmosphere of nitrogen until a few millilitres remain, then complete the evaporation removing the last traces of ether without the application of heat. Immediately dissolve the residue in 25.0 ml of trimethylpentane R and determine the optical rotation.

Calculate the specific optical rotation of the substance in the test solution using as c the number of grams equivalent to RRR- α -tocopherol (factor 0.911) in 1000 ml.

TESTS

Absorbance (2.2.25). Dissolve 0.150 g in *ethanol R* and dilute to 100 ml with the same solvent. Dilute 10.0 ml of the solution to 100.0 ml with *ethanol R* (solution A). Dilute 20.0 ml of the initial solution to 50.0 ml with *ethanol R* (solution B). Measure the absorbance of solution A at the maximum at 284 nm and the absorbance of solution B at the minimum at 254 nm. The specific absorbance at the maximum is 42.0 to 45.0 and that at the minimum is 7.0 to 9.0.

Acid value (2.5.1). Not more than 2.0, determined on 2.00 g.

Free tocopherol. Not more than 1.0 per cent. Dissolve 0.500 g in 100 ml of 0.25 M *alcoholic sulphuric acid R*. Add 20 ml of *water R* and 0.1 ml of a 2.5 g/l solution of *diphenylamine R* in *sulphuric acid R*. Titrate with 0.01 M *ammonium and cerium sulphate* until a blue colour is obtained which persists for at least 5 s. Carry out a blank titration.

1 ml of 0.01 M *ammonium and cerium sulphate* is equivalent to 2.154 mg of free tocopherol.

Heavy metals (2.4.8). 0.5 g complies with limit test D for heavy metals (20 ppm). Prepare the standard using 1 ml of *lead standard solution (10 ppm Pb) R*.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g. Use *sulphuric acid R* instead of *dilute sulphuric acid R*.

ASSAY

Examine by gas chromatography (2.2.28), using *dotriacontane R* as the internal standard.

Internal standard solution. Dissolve 0.300 g of *dotriacontane R* in *hexane R* and dilute to 100.0 ml with the same solvent.

Test solution. Dissolve 0.100 g of the substance to be examined in 10.0 ml of the internal standard solution, dilute to 50.0 ml with *hexane R* and mix.

Reference solution. Dissolve 0.100 g of α -tocopheryl acetate CRS in 10.0 ml of the internal standard solution, dilute to 50.0 ml with *hexane R* and mix.

The chromatographic procedure may be carried out using:

- a fused-silica column 15 m long and 0.32 mm in internal diameter coated with a 0.25 μ m layer of *poly(dimethyl)siloxane R*,
- *helium for chromatography R* as the carrier gas at a flow rate of 3 ml/min to 6 ml/min,
- a flame-ionisation detector,

maintaining the temperature of the injection port at 300 °C and that of the detector at 330 °C. Adjust the split ratio between 1 to 10 and 1 to 20. Set the temperature of the column at 200 °C, then raise the temperature at a rate of 5 °C per minute to 250 °C and maintain at 250 °C for 10 min.

Inject directly onto the column or via a glass-lined injection port using an automatic injection device or some other reproducible injection method. Measure the peak areas by electronic integration. The test is not valid unless: in the chromatogram obtained with the reference solution, the resolution between the peaks corresponding to dotriacontane and α -tocopheryl acetate is at least 4.0.

Interference test. Dissolve 0.100 g of the substance to be examined in *hexane R* and dilute to 50.0 ml with the same solvent. Inject 1 μ l of the solution and record the

chromatogram. If a peak is detected with the same t_R value as for dotriacontane, calculate the area of this peak relative to the peak area of α -tocopheryl acetate. If the relative peak area is greater than 0.5 per cent, use the corrected peak area $S'_{D(\text{corr})}$ for the final calculation.

$$S'_{D(\text{corr})} = S'_D - \frac{S_I \times S'_T}{S_{TI}}$$

S'_D = area of the peak corresponding to the internal standard in the chromatogram obtained with the test solution,

S_I = area of the interfering peak (same t_R value as that of the internal standard) in the chromatogram obtained in the interference test,

S'_T = area of the peak corresponding to α -tocopheryl acetate in the chromatogram obtained with the test solution,

S_{TI} = area of the peak corresponding to α -tocopheryl acetate in the chromatogram obtained in the interference test.

After the system suitability has been established, inject 1 μ l of the reference solution and record the chromatogram. Measure the areas of the peaks corresponding to α -tocopheryl acetate (S_T) and dotriacontane (S_D) and determine the response factor (RF) as described below.

Determine the response factor (RF) for α -tocopheryl acetate in the chromatogram obtained with the reference solution from the areas of the peak corresponding to α -tocopheryl acetate and the peak corresponding to dotriacontane using the expression:

$$\text{RF} = \frac{S_D \times m_T}{S_T \times m_D}$$

Inject 1 μ l of the test solution in the same manner. Measure the areas of the peaks corresponding to α -tocopheryl acetate (S'_T) and dotriacontane (S'_D).

Calculate the percentage content of RRR- α -tocopheryl acetate using the expression:

$$\frac{100 (S'_T \times m_D \times \text{RF})}{S'_{D(\text{corr})} \times m}$$

S_D = area of the peak corresponding to the internal standard in the chromatogram obtained with the reference solution,

$S'_{D(\text{corr})}$ = corrected area of the peak corresponding to the internal standard in the chromatogram obtained with the test solution,

S_T = area of the peak corresponding to α -tocopheryl acetate CRS in the chromatogram obtained with the reference solution,

S'_T = area of the peak corresponding to α -tocopheryl acetate in the chromatogram obtained with the test solution,

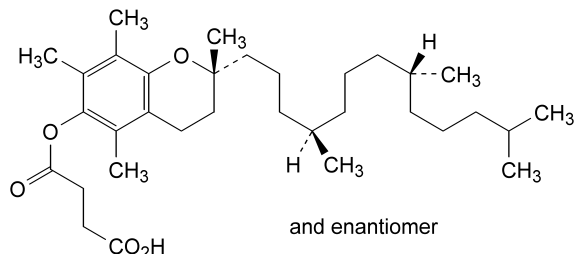
m_D = mass of the internal standard in the test solution and in the reference solution, in milligrams,

m_T = mass of α -tocopheryl acetate CRS in the reference solution, in milligrams,

m = mass of the substance to be examined in the test solution, in milligrams.

STORAGE

Store protected from light.

01/2005:1258
corrected**DL- α -TOCOPHERYL HYDROGEN
SUCCINATE**DL- α -Tocopherylis hydrogenosuccinas $C_{33}H_{54}O_5$ M_r 530.8**DEFINITION**

DL- α -Tocopheryl hydrogen succinate contains not less than 96.0 per cent and not more than the equivalent of 102.0 per cent of (2*RS*)-2,5,7,8-tetramethyl-2-[(4*RS*,8*RS*)-4,8,12-trimethyltridecyl]-3,4-dihydro-2*H*-1-benzopyran-6-yl hydrogen succinate.

CHARACTERS

A white or almost white, crystalline powder, practically insoluble in water, soluble in acetone and in ethanol, very soluble in methylene chloride.

IDENTIFICATION

First identification: B, D.

Second identification: A, C, D.

- A. It complies with the test for absorbance (see Tests).
 B. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *RRR- α -tocopheryl hydrogen succinate CRS*.
 C. Examine by thin-layer chromatography (2.2.27), using silica gel HF_{254} *R* as the coating substance.

Test solution (a). Dissolve 10 mg of the substance to be examined in 2 ml of *cyclohexane R*.

Test solution (b). In a ground glass-stoppered tube, dissolve 10 mg of the substance to be examined in 2 ml of 2.5 *M* alcoholic sulphuric acid *R*. Heat on a water-bath for 5 min. Cool and add 2 ml of *water R* and 2 ml of *cyclohexane R*. Shake for 1 min. Use the upper layer.

Reference solution (a). Dissolve 10 mg of *RRR- α -tocopheryl hydrogen succinate CRS* in 2 ml of *cyclohexane R*.

Reference solution (b). Prepare as described for test solution (b), using *RRR- α -tocopheryl hydrogen succinate CRS* instead of the substance to be examined.

Apply to the plate 10 μ l of each solution. Develop over a path of 15 cm using a mixture of 0.2 volumes of *glacial acetic acid R*, 20 volumes of *ether R* and 80 volumes of *cyclohexane R*. Dry the plate in a current of air and examine in ultraviolet light at 254 nm. The principal spot in the chromatogram obtained with test solution (a) is similar in position and size to the principal spot in the chromatogram obtained with the reference solution (a). In the chromatograms obtained with test solution (b) and reference solution (b), there are two spots: the spot with the higher R_f value is due to α -tocopherol, the spot with the lower R_f value is due to α -tocopheryl acid succinate and corresponds to the spot obtained with reference

solution (a). Depending on the degree of hydrolysis, the lower spot may be weak or even absent. Spray the plate with a mixture of 10 volumes of *hydrochloric acid R*, 40 volumes of a 2.5 g/l solution of *ferric chloride R* in *alcohol R* and 40 volumes of a 10 g/l solution of *phenanthroline hydrochloride R* in *alcohol R*. In the chromatograms obtained with test solution (b) and reference solution (b), the spot due to α -tocopherol is orange.

D. It complies with the test for optical rotation (see Tests).

TESTS

Optical rotation (2.2.7). Dissolve 2.50 g in *ethanol R* and dilute to 25.0 ml with the same solvent. The angle of optical rotation is -0.01° to $+0.01^\circ$.

Absorbance (2.2.25). Dissolve 0.150 g in *ethanol R* and dilute to 100 ml with the same solvent. Dilute 10.0 ml of the solution to 100.0 ml with *ethanol R* (solution a). Dilute 20.0 ml of the initial solution to 50.0 ml with *ethanol R* (solution b). Measure the absorbance of solution (a) at the maximum at 284 nm and the absorbance of solution (b) at the minimum at 254 nm. The specific absorbance at the maximum is 35 to 38 and that at the minimum is 6.0 to 8.0.

Acid value (2.5.1). Between 101 and 108, determined on 1.00 g.

Free tocopherol. Not more than 1.0 per cent. Dissolve 0.500 g in 100 ml of 0.25 *M* alcoholic sulphuric acid *R*. Add 20 ml of *water R* and 0.1 ml of a 2.5 g/l solution of *diphenylamine R* in *sulphuric acid R*. Titrate with 0.01 *M* ammonium and cerium sulphate until a blue colour is obtained which persists for at least 5 s. Carry out a blank titration.

1 ml of 0.01 *M* ammonium and cerium sulphate is equivalent to 2.154 mg of free tocopherol.

Heavy metals (2.4.8). 0.50 g complies with limit test D for heavy metals (20 ppm). Prepare the reference solution using 1 ml of *lead standard solution (10 ppm Pb) R*.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g. Use *sulphuric acid R* instead of *dilute sulphuric acid R*.

ASSAY

Examine by gas chromatography (2.2.28), using *dotriacontane R* as the internal standard.

Internal standard solution. Dissolve 0.300 g of *dotriacontane R* in *hexane R* and dilute to 100.0 ml with the same solvent.

Test solution. Weigh 30.0 mg of the substance to be examined into a 20 ml vial. Pipette 2.0 ml of *methanol R*, 1.0 ml of *dimethoxypropane R* and 0.1 ml of *hydrochloric acid R* into the vial. Cap tightly and sonicate the sample. Allow to stand in the dark for 1 h ($\pm$ 5 min). Remove the sample from the dark, uncap and evaporate just to dryness on a steam bath with the aid of a stream of nitrogen. Pipette 10.0 ml of the internal standard solution into the sample vial. Vortex into solution.

Reference solution. Weigh 30.0 mg of *RRR- α -tocopheryl hydrogen succinate CRS* into a 20 ml vial, weigh to the nearest 0.01 mg. Pipette 2.0 ml of *methanol R*, 1.0 ml of *dimethoxypropane R* and 0.1 ml of *hydrochloric acid R* into the vial. Cap tightly and sonicate the reference solution. Allow to stand in the dark for 1 h ($\pm$ 5 min). Remove the reference solution from the dark, uncap and evaporate just to dryness on a steam bath with the aid of a stream of nitrogen. Pipette 10.0 ml of the internal standard solution into the reference solution vial. Vortex into solution.

The chromatographic procedure may be carried out using:

- a fused-silica open tubular capillary column 15 m long and 0.32 mm in internal diameter coated with a 0.25 µm layer of *poly(dimethyl)siloxane R*,
- *helium for chromatography R* as the carrier gas at a flow rate of 3 ml/min to 6 ml/min,
- a flame-ionisation detector,

maintaining the temperature of the injection port at 300 °C and that of the detector at 330 °C. Adjust the split ratio between 1 to 10 and 1 to 20. Set the temperature of the column at 200 °C, then raise the temperature at a rate of 5 °C per min to 250 °C and maintain at 250 °C for 10 min.

Inject directly onto the column or via a glass-lined injection port using an automatic injection device or some other reproducible injection method. Measure the peak areas by electronic integration. The test is not valid unless: in the chromatogram obtained with the reference solution, the resolution between the peaks corresponding to dotriacontane and α-tocopheryl hydrogen succinate is at least 12.0.

Interference test. Dissolve 0.100 g of the substance to be examined in *hexane R* and dilute to 50.0 ml with the same solvent. Inject 1 µl of the solution and record the chromatogram. If a peak is detected with the same t_R value as for dotriacontane, calculate the area of this peak relative to the peak area of the substance to be examined. If the relative peak area is greater than 0.5 per cent, use the corrected peak area $S'_{D(\text{corr})}$ for the final calculation.

$$S'_{D(\text{corr})} = S'_D - \frac{S_I \times S'_T}{S_{TI}}$$

- S'_D = area of the peak corresponding to the internal standard in the chromatogram obtained with the test solution,
- S_I = area of the interfering peak (same t_R value as that of the internal standard) in the chromatogram obtained in the interference test,
- S'_T = area of the peak corresponding to α-tocopheryl hydrogen succinate in the chromatogram obtained with the test solution,
- S_{TI} = area of the peak corresponding to α-tocopheryl hydrogen succinate in the chromatogram obtained in the interference test.

After the system suitability has been established, inject 1 µl of the reference solution and record the chromatogram. Measure the areas of the peaks corresponding to α-tocopheryl hydrogen succinate (S_T) and dotriacontane (S_D) and determine the response factor (RF) as described below.

Determine the response factor (RF) for α-tocopheryl hydrogen succinate in the chromatogram obtained with the reference solution from the area of the peak corresponding to α-tocopheryl hydrogen succinate and the peak corresponding to dotriacontane using the expression:

$$\text{RF} = \frac{S_D \times m_T}{S_T \times m_D}$$

Inject 1 µl of the test solution in the same manner. Measure the areas of the peaks corresponding to α-tocopheryl hydrogen succinate (S'_T) and dotriacontane (S'_D).

Calculate the percentage content of α-tocopheryl acid succinate using the expression:

$$\frac{100 (S'_T \times m_D \times \text{RF})}{S'_{D(\text{corr})} \times m}$$

- S_D = area of the peak corresponding to the internal standard in the chromatogram obtained with the reference solution,
- $S'_{D(\text{corr})}$ = corrected area of the peak corresponding to the internal standard in the chromatogram obtained with the test solution,
- S_T = area of the peak corresponding to *RRR-α-tocopheryl hydrogen succinate CRS* in the chromatogram obtained with the reference solution,
- S'_T = area of the peak corresponding to *DL-α-tocopheryl hydrogen succinate* in the chromatogram obtained with the test solution,
- m_D = mass of the internal standard in the test solution and in the reference solution in milligrams,
- m_T = mass of *RRR-α-tocopheryl hydrogen succinate CRS* in the reference solution, in milligrams,
- m = mass of the substance to be examined in the test solution, in milligrams.

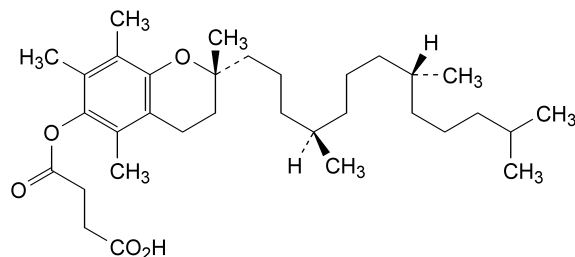
STORAGE

Store protected from light.

01/2005:1259
corrected

RRR-α-TOCOPHERYL HYDROGEN SUCCINATE

RRR-α-Tocopherylis hydrogenosuccinas



$C_{33}H_{54}O_5$

M_r 530.8

DEFINITION

RRR-α-Tocopheryl hydrogen succinate contains not less than 96.0 per cent and not more than the equivalent of 102.0 per cent of (2*R*)-2,5,7,8-tetramethyl-2-[(4*R*,8*R*)-4,8,12-trimethyltridecyl]-3,4-dihydro-2*H*-1-benzopyran-6-yl hydrogen succinate.

CHARACTERS

A white or almost white, crystalline powder, practically insoluble in water, soluble in acetone and in ethanol, very soluble in methylene chloride.

IDENTIFICATION

First identification: B, D.

Second identification: A, C, D.

- It complies with the test for absorbance (see Tests).
- Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *RRR-α-tocopheryl hydrogen succinate CRS*.
- Examine by thin-layer chromatography (2.2.27), using *silica gel HF₂₅₄ R* as the coating substance.

Test solution (a). Dissolve 10 mg of the substance to be examined in 2 ml of *cyclohexane R*.

Test solution (b). In a ground-glass-stoppered tube, dissolve 10 mg of the substance to be examined in 2 ml of *2.5 M alcoholic sulphuric acid R*. Heat on a water-bath for 5 min. Cool and add 2 ml of *water R* and 2 ml of *cyclohexane R*. Shake for 1 min. Use the upper layer.

Reference solution (a). Dissolve 10 mg of *RRR- α -tocopheryl hydrogen succinate CRS* in 2 ml of *cyclohexane R*.

Reference solution (b). Prepare as described for test solution (b), using *RRR- α -tocopheryl hydrogen succinate CRS* instead of the substance to be examined.

Apply separately to the plate 10 μ l of each solution. Develop over a path of 15 cm using a mixture of 0.2 volumes of *glacial acetic acid R*, 20 volumes of *ether R* and 80 volumes of *cyclohexane R*. Dry the plate in a current of air and examine in ultraviolet light at 254 nm. The principal spot in the chromatogram obtained with test solution (a) is similar in position and size to the principal spot in the chromatogram obtained with the reference solution (a). In the chromatograms obtained with test solution (b) and reference solution (b), there are two spots: the spot with the higher R_f value is due to α -tocopherol, the spot with the lower R_f value is due to α -tocopheryl hydrogen succinate and corresponds to the spot obtained with reference solution (a). Depending on the degree of hydrolysis, the lower spot may be weak or even absent. Spray the plate with a mixture of 10 volumes of *hydrochloric acid R*, 40 volumes of a 2.5 g/l solution of *ferric chloride R* in *alcohol R* and 40 volumes of a 10 g/l solution of *phenanthroline hydrochloride R* in *alcohol R*. In the chromatograms obtained with test solution (b) and reference solution (b), the spot due to α -tocopherol is orange.

- D. After saponification, the resulting *RRR- α -tocopherol* is dextrorotatory (2.2.7). The specific optical rotation after oxidation to the quinone form is at least + 24.

Carry out the test avoiding exposure to actinic light. Transfer 1.0 g of the substance to be examined to a round bottomed, ground-glass-stoppered, 250 ml flask and dissolve in 30 ml of *ethanol R* and heat under reflux for 3 min. While the solution is boiling, add, through the condenser, 20 ml of *2 M alcoholic potassium hydroxide solution R*. Continue heating under reflux for 20 min and, without cooling, add 4.0 ml of *hydrochloric acid R* dropwise through the condenser. Cool, rinse the condenser with 10 ml of *ethanol R*, transfer the contents of the flask to a 500 ml separating funnel, rinse the flask with four quantities, each of 25 ml, of *water R* and four quantities, each of 25 ml, of *ether R*. Add the rinsings to the separating funnel. Shake vigorously for 2 min, allow the layers to separate and collect each of the two layers in individual separating funnels. Shake the aqueous layer with two quantities, each of 50 ml, of *ether R* and add these extracts to the main ether extract. Wash the combined ether extracts with four quantities, each of 100 ml, of *water R* and discard the washings.

To the ether solution add 40 ml of a 100 g/l solution of *potassium ferricyanide R* in an 8 g/l solution of *sodium hydroxide R* and shake for 3 min. Wash the ether solution with four quantities, each of 50 ml, of *water R*, discard the washings and dry the ether layer over *anhydrous sodium sulphate R*. Evaporate the ether on a water-bath under reduced pressure or in an atmosphere of nitrogen until a few millilitres remain, then complete the evaporation

removing the last traces of ether without the application of heat. Immediately dissolve the residue in 25.0 ml of *trimethylpentane R* and determine the optical rotation.

Calculate the specific optical rotation of the substance in the test solution using as c the number of grams equivalent to α -tocopherol (factor 0.811) in 1000 ml.

TESTS

Absorbance (2.2.25). Dissolve 0.150 g in *ethanol R* and dilute to 100 ml with the same solvent. Dilute 10.0 ml of the solution to 100.0 ml with *ethanol R* (solution a). Dilute 20.0 ml of the initial solution to 50.0 ml with *ethanol R* (solution b). Measure the absorbance of solution (a) at the maximum at 284 nm and the absorbance of solution (b) at the minimum at 254 nm. The specific absorbance at the maximum is 35 to 38 and that at the minimum is 6.0 to 8.0.

Acid value (2.5.1). Between 101 and 108, determined on 1.00 g.

Free tocopherol. Not more than 1.0 per cent. Dissolve 0.500 g in 100 ml of *0.25 M alcoholic sulphuric acid R*. Add 20 ml of *water R* and 0.1 ml of a 2.5 g/l solution of *diphenylamine R* in *sulphuric acid R*. Titrate with *0.01 M ammonium and cerium sulphate* until a blue colour is obtained which persists for at least 5 s. Carry out a blank titration.

1 ml of *0.01 M ammonium and cerium sulphate* is equivalent to 2.154 mg of free tocopherol.

Heavy metals (2.4.8). 0.50 g complies with limit test D for heavy metals (20 ppm). Prepare the reference solution using 1 ml of *lead standard solution (10 ppm Pb) R*.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g. Use *sulphuric acid R* instead of *dilute sulphuric acid R*.

ASSAY

Examine by gas chromatography (2.2.28), using *dotriacontane R* as the internal standard.

Internal standard solution. Dissolve 0.300 g of *dotriacontane R* in *hexane R* and dilute to 100.0 ml with the same solvent.

Test solution. Weigh 30.0 mg of the substance to be examined into a 20 ml vial. Pipette 2.0 ml of *methanol R*, 1.0 ml of *dimethoxypropane R* and 0.1 ml of *hydrochloric acid R* into the vial. Cap tightly and sonicate the sample. Allow to stand in the dark for 1 h ($\pm$ 5 min). Remove the sample from the dark, uncap and evaporate just to dryness on a steam bath with the aid of a stream of nitrogen. Pipette 10.0 ml of the internal standard solution into the sample vial. Vortex into solution.

Reference solution. Weigh 30.0 mg of *RRR- α -tocopheryl hydrogen succinate CRS* into a 20 ml vial. Pipette 2.0 ml of *methanol R*, 1.0 ml of *dimethoxypropane R* and 0.1 ml of *hydrochloric acid R* into the vial. Cap tightly and sonicate the reference solution. Allow to stand in the dark for 1 h ($\pm$ 5 min). Remove the reference solution from the dark, uncap and evaporate just to dryness on a steam bath with the aid of a stream of nitrogen. Pipette 10.0 ml of the internal standard solution into the reference solution vial. Vortex into solution.

The chromatographic procedure may be carried out using:

- a fused-silica open tubular capillary column 15 m long and 0.32 mm in internal diameter coated with a 0.25 μ m layer of *poly(dimethyl)siloxane R*,
- *helium for chromatography R* as the carrier gas at a flow rate of 3 ml/min to 6 ml/min,
- a flame-ionisation detector,

maintaining the temperature of the injection port at 300 °C and that of the detector at 330 °C. Adjust the split ratio between 1 to 10 and 1 to 20. Set the temperature of the column at 200 °C, then raise the temperature at a rate of 5 °C per min to 250 °C and maintain at 250 °C for 10 min.

Inject directly onto the column or via a glass-lined injection port using an automatic injection device or some other reproducible injection method. Measure the peak areas by electronic integration. The test is not valid unless: in the chromatogram obtained with the reference solution, the resolution between the peaks corresponding to dotriacontane and α -tocopheryl hydrogen succinate is at least 12.0.

Interference test. Dissolve 0.100 g of the solution to be examined in *hexane R* and dilute to 50.0 ml with the same solvent. Inject 1 μ l of the solution and record the chromatogram. If a peak is detected with the same t_R value as for dotriacontane, calculate the area of this peak relative to the peak area of α -tocopheryl succinate. If the relative peak area is greater than 0.5 per cent, use the corrected peak area $S'_{D(\text{corr})}$ for the final calculation.

$$S'_{D(\text{corr})} = S'_D - \frac{S_I \times S'_T}{S_{TI}}$$

S'_D = area of the peak corresponding to the internal standard in the chromatogram obtained with the test solution,

S_I = area of the interfering peak (same t_R value as that of the internal standard) in the chromatogram obtained in the interference test,

S'_T = area of the peak corresponding to α -tocopheryl hydrogen succinate in the chromatogram obtained with the test solution,

S_{TI} = area of the peak corresponding to α -tocopheryl hydrogen succinate in the chromatogram obtained in the interference test.

After the system suitability has been established, inject 1 μ l of the reference solution and record the chromatogram. Measure the areas of the peaks corresponding to the α -tocopheryl hydrogen succinate (S_T) and dotriacontane (S_D) and determine the response factor (RF) as described below.

Determine the response factor (RF) for α -tocopheryl hydrogen succinate in the chromatogram obtained with the reference solution from the area of the peak corresponding to α -tocopheryl hydrogen succinate and the peak corresponding to dotriacontane using the expression:

$$\text{RF} = \frac{S_D \times m_T}{S_T \times m_D}$$

Inject 1 μ l of the test solution in the same manner. Measure the areas of the peaks corresponding to α -tocopheryl hydrogen succinate (S'_T) and dotriacontane (S'_D).

Calculate the percentage content of α -tocopheryl hydrogen succinate using the expression:

$$\frac{100 (S'_T \times m_D \times \text{RF})}{S'_{D(\text{corr})} \times m}$$

S_D = area of the peak corresponding to the internal standard in the chromatogram obtained with the reference solution,

$S'_{D(\text{corr})}$ = corrected area of the peak corresponding to the internal standard in the chromatogram obtained with the test solution,

S_T = area of the peak corresponding to *RRR*- α -tocopheryl hydrogen succinate CRS in the chromatogram obtained with the reference solution,

S'_T = area of the peak corresponding to α -tocopheryl hydrogen succinate in the chromatogram obtained with the test solution,

m_D = mass of the internal standard in the test solution and in the reference solution, in milligrams,

m_T = mass of *RRR*- α -tocopheryl hydrogen succinate CRS in the reference solution in milligrams,

m = mass of the substance to be examined in the test solution, in milligrams.

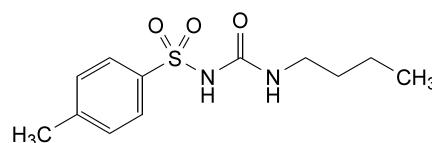
STORAGE

Store protected from light.

01/2005:0304

TOLBUTAMIDE

Tolbutamidum



$C_{12}H_{18}N_2O_3S$

M_r 270.3

DEFINITION

Tolbutamide contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of 1-butyl-3-[(4-methylphenyl)sulfonyl]urea, calculated with reference to the dried substance.

CHARACTERS

A white, crystalline powder, practically insoluble in water, soluble in acetone and in alcohol. It dissolves in dilute solutions of alkali hydroxides.

IDENTIFICATION

First identification: A, C.

Second identification: A, B, D.

A. Melting point (2.2.14): 126 °C to 130 °C.

B. Dissolve 25.0 mg in *methanol R* and dilute to 100.0 ml with the same solvent. Examined between 245 nm and 300 nm (2.2.25), the solution shows three absorption maxima, at 258 nm, 263 nm and 275 nm, and a shoulder at 268 nm. Dilute 10.0 ml of the solution to 250.0 ml with *methanol R*. Examined between 220 nm and 235 nm, the solution shows a single absorption maximum, at 228 nm. The specific absorbance at this maximum is 480 to 520.

C. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *tolbutamide CRS*.

D. To 0.2 g add 8 ml of a 500 g/l solution of *sulphuric acid R* and heat under a reflux condenser for 30 min. Allow to cool. Crystals are formed which, after recrystallisation from hot *water R* and drying at 100 °C to 105 °C, melt (2.2.14) at 135 °C to 140 °C.

TESTS

Appearance of solution. Dissolve 0.2 g in 5 ml of *dilute sodium hydroxide solution R* and add 5 ml of *water R*. The solution is clear (2.2.1) and colourless (2.2.2, *Method II*).

pH (2.2.3). To 2.0 g add 50 ml of *carbon dioxide-free water R* and heat at 70 °C for 5 min. Cool rapidly and filter. The pH of the filtrate is 4.5 to 5.5.

Related substances. Examine by thin-layer chromatography (2.2.27), using *silica gel G R* as the coating substance.

Test solution. Dissolve 0.50 g of the substance to be examined in *acetone R* and dilute to 10 ml with the same solvent.

Reference solution (a). Dissolve 15 mg of *toluenesulphonamide R* in *acetone R* and dilute to 100 ml with the same solvent.

Reference solution (b). To 5 ml of the test solution add 5 ml of reference solution (a).

Apply to the plate 5 µl of the test solution and of reference solution (a) and 10 µl of reference solution (b). Develop over a path of 15 cm using a mixture of 2 volumes of *anhydrous formic acid R*, 8 volumes of *methanol R* and 90 volumes of *chloroform R*. Dry the plate in a current of warm air and heat at 110 °C for 10 min. At the bottom of a chromatography tank, place an evaporating dish containing a 50 g/l solution of *potassium permanganate R* and add an equal volume of *hydrochloric acid R*. Place the plate whilst still hot in the tank and close the tank. Leave the plate in contact with the chlorine vapour for 2 min. Withdraw the plate and place it in a current of cold air until the excess of chlorine is removed and an area of coating below the points of application gives at most a very faint blue colour with a drop of *potassium iodide and starch solution R*; avoid prolonged exposure to cold air. Spray with *potassium iodide and starch solution R* and allow to stand for 5 min. Any spot in the chromatogram obtained with the test solution, apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (a) (0.3 per cent). The test is not valid unless the chromatogram obtained with reference solution (b) shows two clearly separated spots.

Heavy metals (2.4.8). Dissolve 1.0 g in a mixture of 15 volumes of *water R* and 85 volumes of *acetone R* and dilute to 20 ml with the same mixture of solvents. 12 ml of the solution complies with limit test B for heavy metals (20 ppm). Prepare the standard using lead standard solution (1 ppm Pb) obtained by diluting *lead standard solution (100 ppm Pb) R* with a mixture of 15 volumes of *water R* and 85 volumes of *acetone R*.

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

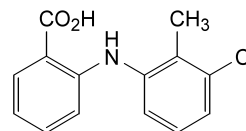
ASSAY

Dissolve 0.2500 g in a mixture of 20 ml of *water R* and 40 ml of *alcohol R*. Titrate with 0.1 M *sodium hydroxide*, using 1 ml of *phenolphthalein solution R* as indicator.

1 ml of 0.1 M *sodium hydroxide* is equivalent to 27.03 mg of C₁₄H₁₂N₂O₃S.

TOLFENAMIC ACID

Acidum tolfenamicum

C₁₄H₁₂ClNO₂M_r 261.7

DEFINITION

2-[(3-Chloro-2-methylphenyl)amino]benzoic acid.

Content: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

Appearance: white or slightly yellow, crystalline powder.

Solubility: practically insoluble in water, soluble in dimethylformamide, sparingly soluble in ethanol and in methylene chloride. It dissolves in dilute solutions of alkali hydroxides.

mp: about 213 °C.

IDENTIFICATION

First identification: B.

Second identification: A, C.

A. Dissolve 20 mg in a mixture of 1 volume of 1 M *hydrochloric acid* and 99 volumes of *methanol R* and dilute to 100 ml with the same mixture of solvents. Dilute 5.0 ml of the solution to 50 ml with a mixture of 1 volume of 1 M *hydrochloric acid* and 99 volumes of *methanol R*. Examined between 250 nm and 380 nm (2.2.25), the solution shows 2 absorption maxima, at 286 nm and 345 nm. The ratio of the absorbance measured at the maximum at 286 nm to that measured at the maximum at 345 nm is 1.2 to 1.4.

B. Infrared absorption spectrophotometry (2.2.24).

Comparison: *tolfenamic acid CRS*.

C. Thin-layer chromatography (2.2.27).

Test solution. Dissolve 25 mg of the substance to be examined in a mixture of 1 volume of *methanol R* and 3 volumes of *methylene chloride R* and dilute to 10 ml with the same mixture of solvents.

Reference solution. Dissolve 25 mg of *tolfenamic acid CRS* in a mixture of 1 volume of *methanol R* and 3 volumes of *methylene chloride R* and dilute to 10 ml with the same mixture of solvents.

Plate: TLC silica gel GF₂₅₄ plate R.

Mobile phase: *glacial acetic acid R*, *dioxan R*, *toluene R* (1:25:90 V/V/V).

Application: 10 µl.

Development: over 2/3 of the plate.

Drying: in a current of warm air.

Detection: ultraviolet light at 254 nm.

Results: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

TESTS

Related substances. Liquid chromatography (2.2.29).

Test solution. Dissolve 50.0 mg of the substance to be examined in 5 ml of *ethanol R* and dilute to 50.0 ml with the mobile phase.

Reference solution (a). Dissolve 25 mg of 2-chlorobenzoic acid *R* and 25 mg of 3-chloro-2-methylaniline *R* in 5 ml of ethanol *R* and dilute to 50.0 ml with the mobile phase. Dilute 1.0 ml of the solution to 50.0 ml with the mobile phase. Dilute 1.0 ml of this solution to 10.0 ml with the mobile phase.

Reference solution (b). Dilute 1.0 ml of the test solution to 10.0 ml with the mobile phase. Dilute 1.0 ml of this solution to 100.0 ml with the mobile phase.

Column:

- **size:** $l = 0.25$ m, $\varnothing = 4.6$ mm,
- **stationary phase:** spherical end-capped octadecylsilyl silica gel for chromatography *R* (5 μ m) with a specific surface area of 450 m²/g and a pore size of 8 nm.

Mobile phase: glacial acetic acid *R*, water *R*, ethanol *R* (2:350:650 V/V/V).

Flow rate: 0.8 ml/min.

Detection: spectrophotometer at 232 nm.

Injection: 20 μ l.

Run time: 3 times the retention time of tolafenamic acid.

Relative retention with reference to tolafenamic acid (retention time = about 15 min): impurity A = about 0.25; impurity B = about 0.34.

System suitability: reference solution (a):

- **resolution:** minimum 2.5 between the peaks due to impurity A and to impurity B.

Limits:

- **impurity A:** not more than the area of the corresponding peak in the chromatogram obtained with reference solution (a) (0.1 per cent),
- **impurity B:** not more than half the area of the corresponding peak in the chromatogram obtained with reference solution (a) (0.05 per cent),
- **any other impurity:** not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent),
- **total:** not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent),
- **disregard limit:** 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.01 per cent).

Copper: maximum 10 ppm.

Atomic absorption spectrometry (2.2.23, *Method I*).

Test solution. Place 1.00 g of the substance to be examined in a silica crucible, moisten with sulphuric acid *R*, heat cautiously on a flame for 30 min and then progressively to about 650 °C. Continue ignition until all black particles have disappeared. Allow to cool, dissolve the residue in 0.1 M hydrochloric acid and dilute to 25.0 ml with the same acid.

Reference solutions. Prepare the reference solutions using copper standard solution (0.1 per cent Cu) *R*, diluted as necessary using 0.1 M nitric acid.

Source: copper hollow-cathode lamp.

Wavelength: 324.8 nm.

Flame: air-acetylene.

Loss on drying (2.2.32): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 100–105 °C.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

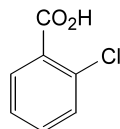
Dissolve 0.200 g with the aid of ultrasound in 100 ml of ethanol *R*. Add 0.1 ml of phenol red solution *R* and titrate with 0.1 M sodium hydroxide.

1 ml of 0.1 M sodium hydroxide is equivalent to 26.17 mg of C₁₄H₁₂ClNO₂.

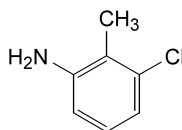
STORAGE

Protected from light.

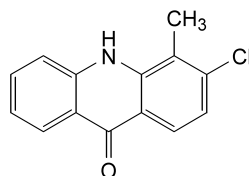
IMPURITIES



A. 2-chlorobenzoic acid,



B. 3-chloro-2-methylaniline,

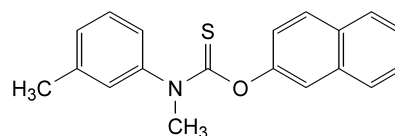


C. 3-chloro-4-methyl-9-oxo-9,10-dihydroacridine.

01/2005:1158

TOLNAFTATE

Tolnaftatum



C₁₉H₁₇NOS

*M*_r 307.4

DEFINITION

Tolnaftate contains not less than 97.0 per cent and not more than the equivalent of 103.0 per cent of *O*-naphthalen-2-yl methyl(3-methylphenyl)thiocarbamate, calculated with reference to the dried substance.

CHARACTERS

A white or yellowish-white powder, practically insoluble in water, freely soluble in acetone and in methylene chloride, very slightly soluble in alcohol.

IDENTIFICATION

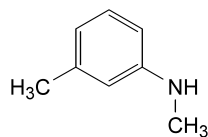
First identification: B.

Second identification: A, C, D.

A. Melting point (2.2.14): 109 °C to 112 °C.

B. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with tolinaftate CRS. Examine the substances prepared as discs.

- C. Examine the chromatograms obtained in the test for related substances. The principal spot in the chromatogram obtained with test solution (b) is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a).
- D. Mix about 1 mg with 0.5 ml of *sulphuric acid R*. Add 0.05 ml of *formaldehyde solution R*. A greenish-blue colour develops.

D. *N*,3-dimethylaniline.

TESTS

Appearance of solution. Dissolve 0.5 g in 10 ml of *acetone R*. The solution is clear (2.2.1) and not more intensely coloured than reference solution Y₆ (2.2.2, *Method II*).

Related substances. Examine by thin-layer chromatography (2.2.27), using *silica gel GF₂₅₄ R* as the coating substance.

Test solution (a). Dissolve 0.10 g of the substance to be examined in *acetone R* and dilute to 2 ml with the same solvent.

Test solution (b). Dilute 0.5 ml of test solution (a) to 10 ml with *acetone R*.

Reference solution (a). Dissolve 25 mg of *tolnaftate CRS* in *acetone R* and dilute to 10 ml with the same solvent.

Reference solution (b). Dilute 1 ml of test solution (b) to 10 ml with *acetone R*.

Reference solution (c). Dissolve 50 mg of *β-naphthol R* in 1 ml of test solution (a) and dilute to 10 ml with *acetone R*. Apply separately to the plate 5 µl of each solution. Develop over a path of 12 cm using *toluene R*. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. Any spot in the chromatogram obtained with test solution (a), apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.5 per cent). The test is not valid unless the chromatogram obtained with reference solution (c) shows two clearly separated spots.

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying at 60 °C at a pressure not exceeding 0.7 kPa for 3 h.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

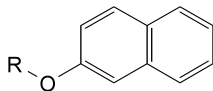
ASSAY

Dissolve 50.0 mg in *methanol R* and dilute to 250.0 ml with the same solvent. Dilute 2.0 ml to 50.0 ml with *methanol R*. Measure the absorbance (2.2.25) at the maximum at 257 nm. Calculate the content of C₁₉H₁₇NOS taking the specific absorbance to be 720.

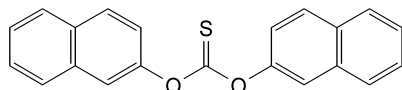
STORAGE

Store protected from light.

IMPURITIES



- A. R = H: naphthalen-2-ol (β-naphthol),
- C. R = CS-Cl: *O*-naphthalen-2-yl chlorothioformate,



- B. *O,O*-dinaphthalen-2-yl thiocarbonate,

TOLU BALSAM

Balsamum tolutanum

01/2005:1596

DEFINITION

Oleo-resin obtained from the trunk of *Myroxylon balsamum* (L.) Harms var. *balsamum*.

Content: 25.0 per cent to 50.0 per cent of free or combined acids, expressed as cinnamic acid (C₉H₈O₂; *M_r* 148.2) (dried drug).

CHARACTERS

Appearance: hard, friable, brownish to reddish-brown mass; thin fragments are brownish-yellow when examined against the light.

Reminiscent odour of vanillin.

Solubility: practically insoluble in water, very soluble to freely soluble in alcohol, practically insoluble in light petroleum.

IDENTIFICATION

Thin-layer chromatography (2.2.27).

Test solution. Stir 0.40 g of the fragmented drug with 10 ml of *methylene chloride R* for 5 min and filter.

Reference solution. Dissolve 50 mg of *benzyl cinnamate R* in *methylene chloride R*, add 50 µl of *benzyl benzoate R* and dilute to 10 ml with *methylene chloride R*.

Plate: *TLC silica gel G plate R*.

Mobile phase: *light petroleum R*, *toluene R* (5:95 V/V).

Application: 20 µl, as bands.

Development: over a path of 15 cm.

Drying: in air.

Detection: spray with *vanillin reagent R* and heat at 100–105 °C for 5 min. Examine in daylight.

Results: see below the sequence of the zones present in the chromatograms obtained with the test and reference solutions. Furthermore, other coloured zones are present in the chromatogram obtained with the test solution.

Top of the plate	
Benzyl benzoate: a greyish-blue zone	a greyish-blue zone
Benzyl cinnamate: a greyish-green zone	a greyish-green zone
Reference solution	Test solution

TESTS

Acid value: 100 to 160.

Dissolve 0.5 g of the fragmented drug in 50 ml of *alcohol R*. Add 0.5 ml of *acid blue 93 solution R* and 5.0 ml of 0.5 *M alcoholic potassium hydroxide*. Stir vigorously and titrate with 0.5 *M hydrochloric acid* until the colour changes from brownish-red to blackish-green (*n*₁ ml of 0.5 *M hydrochloric acid*). Carry out a blank test in the same manner (*n*₂ ml of 0.5 *M hydrochloric acid*). Calculate the acid value in the same manner as the saponification value (2.5.6).

Matter insoluble in alcohol: maximum 5 per cent.

Boil 2.0 g of the fragmented drug with 25 ml of *alcohol* (90 per cent V/V) *R* and filter. Wash the residue with *alcohol* (90 per cent V/V) *R*, boiling until completely extracted, then dry the residue at 100-105 °C. Weigh the residue.

Loss on drying (2.2.32): maximum 5.0 per cent, determined on 2.000 g of the fragmented drug by spreading on a flat evaporating dish 9 cm in diameter and allowing to dry *in vacuo* for 4 h.

Total ash (2.4.16): maximum 0.3 per cent.

ASSAY

Boil 1.500 g under a reflux condenser with 25 ml of 0.5 *M* *alcoholic potassium hydroxide* for 1 h. Evaporate the ethanol and heat the residue with 50 ml of *water R* until the substance is homogeneously distributed. After cooling, add 80 ml of *water R* and a solution of 1.5 g of *magnesium sulphate R* in 50 ml of *water R*. Mix, and allow to stand for 10 min. Filter through a pleated filter paper and wash the residue with 20 ml of *water R*. Combine the filtrate and the washings, acidify with *hydrochloric acid R* and extract with 4 quantities, each of 40 ml, of *ether R*. Discard the aqueous layer. Combine the organic extracts and wash with 2 quantities, each of 20 ml, and with 3 quantities, each of 10 ml, of a 50 g/l solution of *sodium bicarbonate R*. Discard the ether layer. Combine the aqueous extracts, acidify with *hydrochloric acid R* and stir once with 30 ml, twice with 20 ml and once with 10 ml of *methylene chloride R*. Dry the combined methylene chloride extracts over *anhydrous sodium sulphate R*. Filter through a pleated filter and wash the residue with 10 ml of *methylene chloride R*. Reduce the combined methylene chloride extracts to 10 ml by distillation and eliminate the remaining methylene chloride in a current of air. Dissolve the residue with heating in 10 ml of *alcohol R* previously neutralised to *phenol red solution R*. After cooling, titrate with 0.1 *M* *sodium hydroxide*, using the same indicator.

1 ml of 0.1 *M* *sodium hydroxide* is equivalent to 14.82 mg of total acids, expressed as cinnamic acid.

STORAGE

Do not store in powdered form.

01/2005:1478

TORMENTIL

Tormentillae rhizoma

DEFINITION

Whole or cut, dried rhizome, freed from the roots, of *Potentilla erecta* (L.) Raeusch. (*P. tormentilla* Stokes).

Content: minimum 7 per cent of tannins, expressed as pyrogallol (C₆ H₆ O₃; *M_r* 126.1) (dried drug).

CHARACTERS

Macroscopic and microscopic characters described under identification tests A and B.

IDENTIFICATION

- A. The rhizome is cylindrically spindle-shaped, with a very irregular appearance, often forming, twisted, knotty tubers, up to 10 cm long and 1 cm to 2 cm thick, very hard and scarcely branched. The surface is brown to reddish-brown, rugose and has remains of roots and

transversely elongated depressed whitish scars from the stems. At the top of the rhizome the remains of numerous aerial stems may be present. The fracture is short and granular, dark red to brownish-yellow.

- B. Reduce to a powder (355). The powder is reddish-brown. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters: coarsely serrate cluster crystals of calcium oxalate, up to 60 µm in diameter; fragments of thin-walled parenchyma containing reddish-brown tannin; groups of narrow, bordered-pitted vessels with lateral pores; thick-walled and pitted, polygonal parenchyma; groups and fragments of sclerenchymatous thick-walled fibres; occasional fragments of cork with thin-walled, brown, tabular cells. Examine under a microscope using a 50 per cent V/V solution of *glycerol R*. The powder shows spherical or elliptical starch granules, up to about 20 µm in length.
- C. Thin-layer chromatography (2.2.27).

Test solution. To 0.5 g of the powdered drug (355) add 10 ml of *water R*, shake for 10 min and filter. Shake the filtrate with 2 quantities, each of 10 ml, of *ethyl acetate R* and filter the combined upper phases over 6 g of *anhydrous sodium sulphate R*. Evaporate the filtrate to dryness under reduced pressure and dissolve the residue in 1.0 ml of *ethyl acetate R*.

Reference solution. Dissolve 1.0 mg of *catechin R* in 1.0 ml of *methanol R*.

Plate: TLC silica gel plate *R*.

Mobile phase: *glacial acetic acid R*, *ether R*, *hexane R*, *ethyl acetate R* (20:20:20:40 V/V/V/V).

Application: 10 µl, as bands.

Development: over a path of 10 cm.

Drying: in air for 10-15 min.

Detection: spray with a freshly prepared 5 g/l solution of *fast blue B salt R*. Reddish zones appear. Expose the plate to ammonia vapour, the zones become more intense turning reddish-brown. Examine in daylight.

Results: see below the sequence of the zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other fainter zones are present in the chromatogram obtained with the test solution.

Top of the plate	
Catechin: an intense reddish-brown zone	A more intense reddish-brown zone (catechin)
	A fainter zone
	An intense zone
	Fainter zones
Reference solution	Test solution

TESTS

Foreign matter (2.8.2): maximum 3 per cent of root and stems as well as rhizomes with black fracture and maximum 2 per cent of other foreign matter.

Loss on drying (2.8.32): maximum 12.0 per cent, determined on 1.000 g of the powdered drug (355) by drying in an oven at 100-105 °C for 2 h.

Total ash (2.4.16): maximum 5.0 per cent.

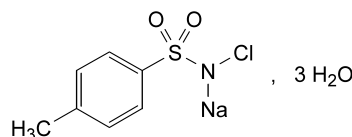
ASSAY

Carry out the determination of tannins in herbal drugs (2.8.14). Use 0.500 g of the powdered drug (180).

01/2005:0381

TOSYLCHLORAMIDE SODIUM

Tosylchloramidum natricum

C₇H₇ClNNaO₂S·3H₂OM_r 281.7

TORMENTIL TINCTURE

Tormentillae tinctura

DEFINITION

Tincture produced from *Tormentil* (1478).

Content: minimum 1.5 per cent *m/m* of tannins, expressed as pyrogallol (C₆H₆O₃; M_r 126.1).

PRODUCTION

The tincture is produced from 1 part of comminuted drug and 5 parts of ethanol (70 per cent *V/V*) by a suitable procedure.

CHARACTERS

Red or reddish-brown liquid.

IDENTIFICATION

Thin-layer chromatography (2.2.27).

Test solution. Mix 1.0 ml of the tincture to be examined with 1.0 ml of alcohol (70 per cent *V/V*) *R*.

Reference solution. Dissolve 1.0 mg of catechin *R* in 1.0 ml of methanol *R*.

Plate: TLC silica gel plate *R*.

Mobile phase: ether *R*, glacial acetic acid *R*, hexane *R*, ethyl acetate *R* (20:20:20:40 *V/V/V/V*).

Application: 10 µl, as bands.

Development: over a path of 10 cm.

Drying: in air for 10-15 min.

Detection: spray with a freshly prepared 5 g/l solution of fast blue B salt *R*. Reddish zones appear. Expose the plate to ammonia vapour, the zones become more intense, turning reddish-brown. Examine in daylight.

Results: see below the sequence of the zones present in the chromatograms obtained with the reference solution and the test solution.

Top of the plate	
Catechin: an intense zone	An intense zone (catechin)
	A fainter zone
	An intense zone
	Fainter zones
Reference solution	Test solution

TESTS

Ethanol content (2.9.10): 64 per cent *V/V* to 69 per cent *V/V*.

Methanol and 2-propanol (2.9.11): maximum 0.05 per cent *V/V* of methanol and maximum 0.05 per cent *V/V* of 2-propanol.

ASSAY

Carry out the determination of tannins in herbal drugs (2.8.14). Use 2.50 g of the tincture to be examined.

DEFINITION

Tosylchloramide sodium contains not less than 98.0 per cent and not more than the equivalent of 103.0 per cent of sodium *N*-chloro-4-methylbenzene-sulphonimide trihydrate.

CHARACTERS

A white or slightly yellow, crystalline powder, freely soluble in water, soluble in alcohol.

IDENTIFICATION

- Solution S (see Tests) turns red litmus paper *R* blue and then bleaches it.
- To 10 ml of solution S add 10 ml of dilute hydrogen peroxide solution *R*. A white precipitate is formed which dissolves on heating. Filter the hot solution and allow to cool. White crystals are formed which, when washed and dried at 100 °C to 105 °C, melt (2.2.14) at 137 °C to 140 °C.
- Ignite 1 g (cautiously, because of the risk of deflagration). Dissolve the residue in 10 ml of water *R*. The solution gives reaction (a) of chlorides (2.3.1).
- The solution prepared for identification test C gives reaction (a) of sulphates (2.3.1).
- The solution prepared for identification test C gives reaction (b) of sodium (2.3.1).

TESTS

Solution S. Dissolve 1.0 g in carbon dioxide-free water *R* and dilute to 20 ml with the same solvent.

Appearance of solution. Solution S is not more opalescent than reference suspension II (2.2.1) and is colourless (2.2.2, Method II).

pH (2.2.3). The pH of solution S is 8.0 to 10.0.

Ortho compound. To 2 g add 10 ml of water *R*, mix, add 1 g of sodium metabisulphite *R* and heat to boiling. Cool to 0 °C, filter rapidly and wash with three quantities, each of 5 ml, of iced water *R*. The precipitate, dried over diphosphorus pentoxide *R* at a pressure not exceeding 600 Pa melts (2.2.14) at a temperature not lower than 134 °C.

Residue insoluble in ethanol. Shake 1.00 g for 30 min with 20 ml of ethanol *R*, filter on a tared filter, wash any residue with 5 ml of ethanol *R* and dry at 100 °C to 105 °C. The residue weighs not more than 20 mg (2 per cent).

ASSAY

Dissolve 0.125 g in 100 ml of water *R* in a ground-glass-stoppered flask. Add 1 g of potassium iodide *R* and 5 ml of dilute sulphuric acid *R*. Allow to stand for 3 min. Titrate with 0.1 M sodium thiosulphate, using 1 ml of starch solution *R* as indicator.

1 ml of 0.1 M sodium thiosulphate is equivalent to 14.08 mg of C₇H₇ClNNaO₂S·3H₂O.

STORAGE

Store in an airtight container, protected from light, at a temperature of 8 °C to 15 °C.

01/2005:0532

TRAGACANTH

Tragacantha

DEFINITION

Tragacanth is the air-hardened, gummy exudate, flowing naturally or obtained by incision from the trunk and branches of *Astragalus gummifer* Labill. and certain other species of *Astragalus* from western Asia.

CHARACTERS

It has the macroscopic and microscopic characters described under identification test A and B.

IDENTIFICATION

- A. Tragacanth occurs in thin, flattened, ribbon-like, white or pale yellow, translucent strips, about 30 mm long and 10 mm wide and up to 1 mm thick, more or less curved; horny, fracture short; the surface is marked by fine longitudinal striae and concentric transverse ridges. It may also contain pieces similar in shape but somewhat thicker, more opaque and more difficult to fracture.
- B. Reduce to a powder (355). The powder is white or almost white and it forms a mucilaginous gel with about ten times its mass of *water R*. Examine under a microscope using a 50 per cent V/V solution of *glycerol R*. The powder shows in the gummy mass numerous stratified cellular membranes turning slowly violet when treated with *iodinated zinc chloride solution R*. The gummy mass includes starch grains, isolated or in small groups, usually rounded in shape and sometimes deformed, with diameters varying between 4 µm and 10 µm, occasionally up to 20 µm, and a central hilum visible between crossed nicol prisms.
- C. Examine the chromatograms obtained in the test for acacia. The chromatogram obtained with the test solution shows three zones, corresponding to galactose, arabinose and xylose. A faint yellowish zone at the solvent front and a greyish-green zone between the zones due to galactose and arabinose may be present.
- D. Moisten 0.5 g of the powdered drug (355) with 1 ml of *alcohol R* and add gradually, while shaking, 50 ml of *water R* until a homogeneous mucilage is obtained. To 5 ml of the mucilage add 5 ml of *water R* and 2 ml of *barium hydroxide solution R*. A slight flocculent precipitate is formed. Heat on a water-bath for 10 min. An intense yellow colour develops.

TESTS

Acacia. Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel plate R*.

Test solution. To 100 mg of the powdered drug (355) in a thick-walled, centrifuge test tube, add 2 ml of a 100 g/l solution of *trifluoroacetic acid R*, shake vigorously to dissolve the forming gel, stopper the test tube and heat the mixture at 120 °C for 1 h. Centrifuge the hydrolysate, transfer the clear supernatant carefully into a 50 ml flask, add 10 ml of *water R* and evaporate the solution to dryness under reduced pressure. To the resulting clear film add 0.1 ml of *water R* and 0.9 ml of *methanol R*. Centrifuge

to separate the amorphous precipitate and dilute the supernatant if necessary to 1 ml with *methanol R*.

Reference solution. Dissolve 10 mg of *arabinose R*, 10 mg of *galactose R*, 10 mg of *rhamnose R* and 10 mg of *xylose R* in 1 ml of *water R* and dilute to 10 ml with *methanol R*.

Apply to the plate as bands 10 µl of each solution. Develop over a path of 10 cm using a mixture of 10 volumes of a 16 g/l solution of *sodium dihydrogen phosphate R*, 40 volumes of *butanol R* and 50 volumes of *acetone R*. Dry the plate in a current of warm air for a few minutes and again develop over a path of 15 cm using the same mobile phase. Dry the plate at 110 °C for 10 min, spray with *anisaldehyde solution R* and dry again at 110 °C for 10 min. The chromatogram obtained with the reference solution shows four clearly separated coloured zones due to galactose (greyish-green to green), arabinose (yellowish-green), xylose (greenish-grey or yellowish-grey) and rhamnose (yellowish-green), in order of increasing R_f value. The chromatogram obtained with the test solution does not show a yellowish-green zone corresponding to the zone of rhamnose in the chromatogram obtained with the reference solution.

Methylcellulose. Examine the chromatograms obtained in the test for acacia. The chromatogram obtained with the test solution does not show a red zone near the solvent front.

Sterculia gum

- A. Place 0.2 g of the powdered drug (355) in a 10 ml ground-glass-stoppered cylinder graduated in 0.1 ml. Add 10 ml of *alcohol (60 per cent V/V) R* and shake. Any gel formed occupies not more than 1.5 ml.
- B. To 1.0 g of the powdered drug (355) add 100 ml of *water R* and shake. Add 0.1 ml of *methyl red solution R*. Not more than 5.0 ml of 0.01 M *sodium hydroxide* is required to change the colour of the indicator.

Foreign matter. Place 2.0 g of the powdered drug (355) in a 250 ml round-bottomed flask and add 95 ml of *methanol R*. Swirl to moisten the powder and add 60 ml of *hydrochloric acid R1*. Add a few glass beads about 4 mm in diameter and heat on a water-bath under a reflux condenser for 3 h, shaking occasionally. Remove the glass beads and filter the hot suspension *in vacuo* through a sintered-glass filter (160). Rinse the flask with a small quantity of *water R* and pass the rinsings through the filter. Wash the residue on the filter with about 40 ml of *methanol R* and dry to constant mass at 110 °C (about 1 h). Allow to cool in a desiccator and weigh. The residue weighs not more than 20 mg (1.0 per cent).

Flow time. Not less than 10 s or, if the substance to be examined is to be used for the preparation of emulsions, not less than 50 s. Place 1.0 g of the powdered drug (125 to 250) in a 1000 ml round bottomed flask with a ground-glass stopper and add 8.0 ml of *alcohol R* and close the flask. Disperse the suspension over the inner surface of the flask by shaking, taking care not to wet the stopper. Open the flask and add in one portion 72.0 ml of *water R*. Stopper the flask and shake vigorously for 3 min. Allow to stand for 24 h and shake vigorously again for 3 min. Eliminate air bubbles by applying vacuum above the mucilage for 5 min. Transfer the mucilage to a 50 ml cylinder. Dip in the mucilage a piece of glass tubing 200 mm long and 6.0 mm in internal diameter and graduated at 20 mm and 120 mm from the lower end; the tubing must not be rinsed with surface-active substances. When the mucilage has reached the upper mark, close the tube with a finger. Withdraw the closed tube, remove the finger and measure with a stop-watch the time needed for the meniscus to reach the lower graduation. Carry out this operation four times and determine the average value of the last three determinations.

Total ash (2.4.16). Not more than 4.0 per cent.

Microbial contamination. Total viable aerobic count (2.6.12) not more than 10^4 micro-organisms per gram, determined by plate count. It complies with the tests for *Escherichia coli* and *Salmonella* (2.6.13).

STORAGE

Store protected from light.

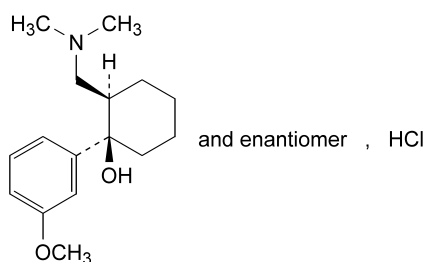
LABELLING

The label states whether or not the contents are suitable for preparing emulsions.

01/2005:1681

TRAMADOL HYDROCHLORIDE

Tramadoli hydrochloridum



$C_{16}H_{26}ClNO_2$

M_r 299.8

DEFINITION

(1*RS*,2*RS*)-2-[(Dimethylamino)methyl]-1-(3-methoxyphenyl)cyclohexanol hydrochloride.

Content: 99.0 per cent to 101.0 per cent (anhydrous substance).

CHARACTERS

Appearance: white, crystalline powder.

Solubility: freely soluble in water and in methanol, very slightly soluble in acetone.

IDENTIFICATION

First identification: B, D.

Second identification: A, C, D.

A. Melting point (2.2.14): 180 °C to 184 °C.

B. Infrared absorption spectrophotometry (2.2.24).

Comparison: tramadol hydrochloride CRS.

C. Chromatograms obtained in the test for impurity E.

Results: the principal spot in the chromatogram obtained with test solution (b) is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a).

D. It gives reaction (a) of chlorides (2.3.1).

TESTS

Solution S. Dissolve 1.0 g in *water R* and dilute to 20 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and colourless (2.2.2, Method II).

Acidity. To 10 ml of solution S, add 0.2 ml of *methyl red solution R* and 0.2 ml of 0.01 M hydrochloric acid. The solution is red. Not more than 0.4 ml of 0.01 M sodium hydroxide is required to change the colour of the indicator to yellow.

Optical rotation (2.2.7): -0.10° to $+0.10^\circ$, determined on solution S.

Impurity E. Thin-layer chromatography (2.2.27).

Test solution (a). Dissolve 0.10 g in *methanol R* and dilute to 2 ml with the same solvent.

Test solution (b). Dilute 1 ml of test solution (a) to 10 ml with *methanol R*.

Reference solution (a). Dissolve 25 mg of tramadol hydrochloride CRS in *methanol R* and dilute to 5 ml with the same solvent.

Reference solution (b). Dissolve 5 mg of tramadol impurity E CRS in 5 ml of *methanol R*. Dilute 1 ml of the solution to 10 ml with *methanol R*.

Reference solution (c). Dissolve 5 mg of tramadol impurity A CRS in 1 ml of reference solution (a).

Plate: TLC silica gel F_{254} plate R, prewashed with *methanol R*.

Mobile phase: concentrated ammonia R, 2-propanol R, toluene R (1:19:80 V/V/V).

Application: 10 μ l.

Development: over 2/3 of the plate. Saturate the plate for 20 min with concentrated ammonia R. For this, add concentrated ammonia R to one trough of a twin trough tank. Just before developing, add the mobile phase to the other trough. Place the plate in the chromatographic tank, ensuring that the layer of silica gel is orientated towards the middle of the tank.

Drying: in air.

Detection: expose the plate to iodine vapour for 1 h, examine in ultraviolet light at 254 nm.

System suitability: the chromatogram obtained with reference solution (c) shows 2 clearly separated spots.

Limit: in the chromatogram obtained with test solution (a):

- **impurity E:** any spot corresponding to impurity E is not more intense and not greater than the spot in the chromatogram obtained with reference solution (b) (0.2 per cent).

Related substances. Liquid chromatography (2.2.29).

Test solution. Dissolve 0.15 g of the substance to be examined in the mobile phase and dilute to 100 ml with the mobile phase.

Reference solution (a). Dilute 2.0 ml of the test solution to 10.0 ml with the mobile phase. Dilute 1.0 ml of this solution to 100 ml with the mobile phase.

Reference solution (b). Dissolve 5 mg of tramadol impurity A CRS in 4.0 ml of the test solution and dilute to 100 ml with the mobile phase.

Column:

- **size:** $l = 0.25$ m, $\varnothing = 4.0$ mm,
- **stationary phase:** base-deactivated end-capped octylsilyl silica gel for chromatography R (5 μ m).

Mobile phase: 295 volumes of acetonitrile R and 705 volumes of a mixture of 0.2 ml of trifluoroacetic acid R and 100 ml of water R.

Flow rate: 1.0 ml/min.

Detection: spectrophotometer at 270 nm.

Injection: 20 μ l.

Run time: 4 times the retention time of tramadol.

Relative retention with reference to tramadol (retention time = about 5 min): impurity A = about 0.85.

System suitability: reference solution (b):

- **resolution:** minimum 2.0 between the peaks due to impurity A and tramadol.

Limits:

- **impurity A:** not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent),
- **any other impurity:** not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent),
- **total:** not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.4 per cent),
- **disregard limit:** 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.02 per cent).

Heavy metals (2.4.8): maximum 20 ppm.

Dissolve 2.0 g in *water R* and dilute to 20 ml with the same solvent. 12 ml of this solution complies with limit test A. Prepare the standard using *lead standard solution* (2 ppm Pb) *R*.

Water (2.5.12): maximum 0.5 per cent, determined on 1.000 g.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

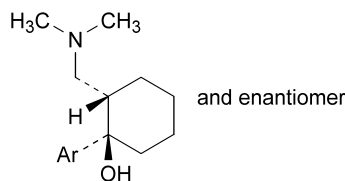
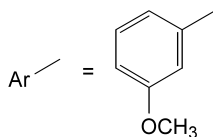
ASSAY

Dissolve 0.180 g in 25 ml of *anhydrous acetic acid R* and add 10 ml of *acetic anhydride R*. Titrate with 0.1 M *perchloric acid*, determining the end-point potentiometrically (2.2.20).

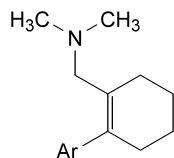
1 ml of 0.1 M *perchloric acid* is equivalent to 29.98 mg of $C_{16}H_{26}ClNO_2$.

STORAGE

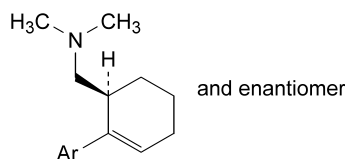
Protected from light.

IMPURITIES

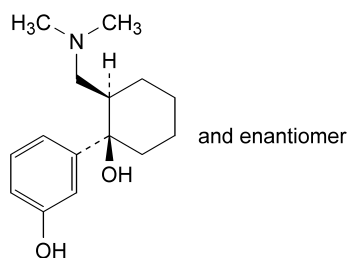
- A. (1*RS*,2*SR*)-2-[(dimethylamino)methyl]-1-(3-methoxyphenyl)cyclohexanol,



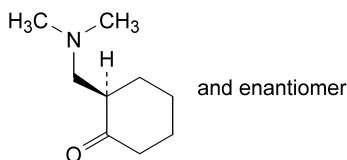
- B. [2-(3-methoxyphenyl)cyclohex-1-en-1-yl]-*N,N*-dimethylmethanamine,



- C. (1*RS*)-[2-(3-methoxyphenyl)cyclohex-2-en-1-yl]-*N,N*-dimethylmethanamine,



- D. (1*RS*,2*RS*)-2-[(dimethylamino)methyl]-1-(3-hydroxyphenyl)cyclohexanol,

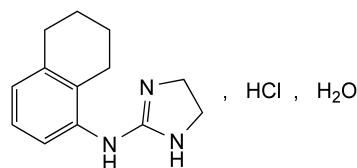


- E. (2*RS*)-2-[(dimethylamino)methyl]cyclohexanone.

01/2005:1597
corrected

TRAMAZOLINE HYDROCHLORIDE MONOHYDRATE

Tramazolini hydrochloridum
monohydricum



$C_{13}H_{18}ClN_3 \cdot H_2O$

M_r 269.8

DEFINITION

N-(5,6,7,8-Tetrahydronaphthalen-1-yl)-4,5-dihydro-1*H*-imidazol-2-amine hydrochloride monohydrate.

Content: 98.5 per cent to 101.5 per cent (anhydrous substance).

CHARACTERS

Appearance: white or almost white, crystalline powder.

Solubility: soluble in water and in alcohol.

IDENTIFICATION

- A. Infrared absorption spectrophotometry (2.2.24).

Comparison: tramazoline hydrochloride monohydrate CRS.

- B. It gives reaction (a) of chlorides (2.3.1).

TESTS

Solution S. Dissolve 2.5 g in *carbon dioxide-free water R* and dilute to 50 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and not more intensely coloured than reference solution Y₆ (2.2.2, Method II).

pH (2.2.3): 4.9 to 6.3 for solution S.

Related substances. Liquid chromatography (2.2.29).

Test solution. Dissolve 50.0 mg of the substance to be examined in a mixture of 50 volumes of *acetonitrile R* and 50 volumes of *water R* and dilute to 50.0 ml with the same mixture of solvents.

Reference solution (a). Dissolve 10.0 mg of tramazoline impurity A CRS and 10.0 mg of tramazoline impurity B CRS in 10 ml of a mixture of 50 volumes of acetonitrile R and 50 volumes of water R and add 10 ml of the test solution.

Reference solution (b). Dilute 0.2 ml of reference solution (a) to 100 ml with a mixture of 50 volumes of acetonitrile R and 50 volumes of water R.

Column:

- size: $l = 0.125$ m, $\varnothing = 4$ mm,
- stationary phase: octadecylsilyl silica gel for chromatography R (5 μ m).

Mobile phase: 2.0 g/l solution of sodium dodecyl sulphate R in a mixture of 6 volumes of 2-propanol R, 42 volumes of acetonitrile R and 52 volumes of water R.

Flow rate: 1.2 ml/min.

Detection: spectrophotometer at 215 nm.

Injection: 5 μ l.

Run time: 3 times the retention time of tramazoline.

Relative retentions with reference to tramazoline (retention time = about 6.5 min): impurity A = about 0.71; impurity B = about 0.86.

System suitability: reference solution (a):

- the chromatogram obtained shows 3 clearly separated peaks,
- resolution: minimum 1.5 between tramazoline and impurity B.

Limits:

- impurity A: not more than 3 times the area of the corresponding peak in the chromatogram obtained with reference solution (b) (0.3 per cent),
- impurity B: not more than 3 times the area of the corresponding peak in the chromatogram obtained with reference solution (b) (0.3 per cent),
- any other impurity: not more than the area of the peak due to impurity B in the chromatogram obtained with reference solution (b) (0.1 per cent),
- total of other impurities: not more than 2 times the area of the peak due to impurity B in the chromatogram obtained with reference solution (b) (0.2 per cent),
- disregard limit: 0.2 times the area of the peak due to impurity B in the chromatogram obtained with reference solution (b) (0.02 per cent).

Water (2.5.12): 6.2 per cent to 7.2 per cent, determined on 0.500 g.

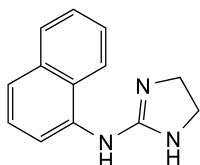
Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

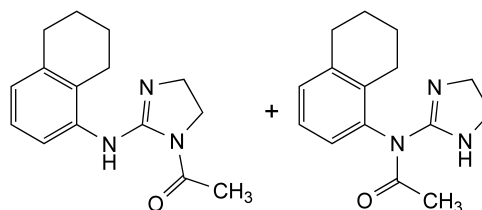
Dissolve 2.000 g in a mixture of 5 ml of 0.1 M hydrochloric acid and 75 ml of alcohol R. Carry out a potentiometric titration (2.2.20) using 1 M sodium hydroxide. Read the volume added between the 2 points of inflexion.

1 ml of 1 M sodium hydroxide is equivalent to 251.8 mg of $C_{13}H_{18}ClN_3$.

IMPURITIES



A. *N*-(naphthalen-1-yl)-4,5-dihydro-1*H*-imidazol-2-amine,

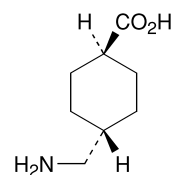


B. mixture of 1-acetyl-2-[(5,6,7,8-tetrahydronaphthalen-1-yl)amino]-4,5-dihydro-1*H*-imidazole and *N*-(4,5-dihydro-1*H*-imidazol-2-yl)-*N*-(5,6,7,8-tetrahydronaphthalen-1-yl)acetamide.

01/2005:0875

TRANEXAMIC ACID

Acidum tranexamicum



$C_8H_{15}NO_2$

M_r 157.2

DEFINITION

Trans-4-(aminomethyl)cyclohexanecarboxylic acid.

Content: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

Appearance: white, crystalline powder.

Solubility: freely soluble in water and in glacial acetic acid, practically insoluble in acetone and in alcohol.

IDENTIFICATION

Infrared absorption spectrophotometry (2.2.24).

Preparation: discs.

Comparison: tranexamic acid CRS.

TESTS

pH (2.2.3): 7.0 to 8.0.

Dissolve 2.5 g in carbon dioxide-free water R and dilute to 50 ml with the same solvent.

Related substances. Liquid chromatography (2.2.29).

Test solution. Dissolve 0.20 g of the substance to be examined in water R and dilute to 20.0 ml with the same solvent.

Reference solution (a). Dilute 5.0 ml of the test solution to 100.0 ml with water R. Dilute 1.0 ml of this solution to 10.0 ml with water R.

Reference solution (b). Dissolve 5 mg of tranexamic acid impurity C CRS in water R and dilute to 50.0 ml with the same solvent. To 1.0 ml of this solution add 1.0 ml of the test solution and dilute to 50.0 ml with water R.

Column:

- size: $l = 0.25$ m, $\varnothing = 4.6$ mm or $l = 0.25$ m, $\varnothing = 6.0$ mm,
- stationary phase: octadecylsilyl silica gel for chromatography R (5 μ m).

Mobile phase: dissolve 11.0 g of anhydrous sodium dihydrogen phosphate R in 500 ml of water R, add 5 ml of triethylamine R and 1.4 g of sodium laurilsulfate R. Adjust to pH 2.5 with dilute phosphoric acid R and dilute to 600 ml with water R. Add 400 ml of methanol R and mix.

Flow rate: 0.9 ml/min.

Detection: spectrophotometer at 220 nm.

Injection: 20 µl.

Run time: 3 times the retention time of tranexamic acid.

Relative retentions with reference to tranexamic acid (retention time = about 13 min): impurity C = about 1.1; impurity D = about 1.3; impurity B = about 1.5; impurity A = about 2.1.

System suitability: reference solution (b):

- **resolution:** minimum of 2.0 between the peaks due to tranexamic acid and to impurity C.

Limits:

- **correction factors:** for the calculation of contents, multiply the peak areas of the following impurities by the corresponding correction factor: impurity B = 1.2; impurity C = 0.005; impurity D = 0.006;
- **impurity A:** not more than 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent);
- **impurity B:** not more than 0.4 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent);
- **any other impurity:** not more than 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent);
- **total of other impurities:** not more than 0.4 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent);
- **disregard limit:** 0.05 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.025 per cent).

Halides expressed as chlorides (2.4.4): maximum 140 ppm.

Dissolve 1.2 g in *water R* and dilute to 50 ml with the same solvent. 15 ml of this solution complies with the limit test for chlorides.

Heavy metals (2.4.8): maximum 10 ppm.

Dissolve 2.0 g in *water R* and dilute to 20 ml with the same solvent. 12 ml of this solution complies with limit test A. Prepare the standard using *lead standard solution (1 ppm Pb) R*.

Loss on drying (2.2.32): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 100–105 °C for 2 h.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

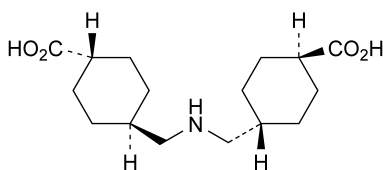
Dissolve 0.140 g in 20 ml of *anhydrous acetic acid R*. Titrate with 0.1 M *perchloric acid*, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *perchloric acid* is equivalent to 15.72 mg of C₈H₁₅NO₂.

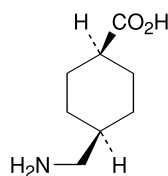
IMPURITIES

Specified impurities: A, B.

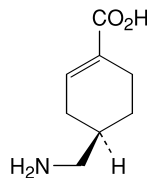
Other detectable impurities: C, D.



A. *trans,trans*-4,4'-(iminodimethylene)di(cyclohexanecarboxylic) acid,

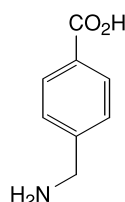


B. *cis*-4-(aminomethyl)cyclohexanecarboxylic acid,



and enantiomer

C. (*RS*)-4-(aminomethyl)cyclohex-1-enecarboxylic acid,

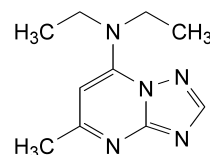


D. 4-aminomethylbenzoic acid.

01/2005:1576

TRAPIDIL

Trapidilum



C₁₀H₁₅N₅

M_r 205.3

DEFINITION

N,N-Diethyl-5-methyl-[1,2,4]triazolo[1,5-*a*]pyrimidin-7-amine.

Content: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

Appearance: white or almost white, crystalline powder.

Solubility: freely soluble in water, soluble in ethanol and in methylene chloride.

mp: about 102 °C.

IDENTIFICATION

Infrared absorption spectrophotometry (2.2.24).

Comparison: *trapidil CRS*.

TESTS

Solution S. Dissolve 2.0 g in *carbon dioxide-free water R* and dilute to 100 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and colourless (2.2.2, *Method II*).

Acidity or alkalinity. To 10 ml of solution S add 0.2 ml of *methyl red solution R* and 0.2 ml of 0.01 M *hydrochloric acid*. The solution is red. Add 0.4 ml of 0.01 M *sodium hydroxide*. The solution is yellow.

Related substances. Liquid chromatography (2.2.29).

Test solution. Dissolve 20.0 mg of the substance to be examined in the mobile phase and dilute to 10.0 ml with the mobile phase.

Reference solution (a). Dissolve 5.0 mg of *trapidil impurity A CRS* in the mobile phase and dilute to 50.0 ml with the mobile phase. Dilute 1.0 ml of the solution to 50.0 ml with the mobile phase.

Reference solution (b). Dissolve 5.0 mg of *trapidil impurity B CRS* in the mobile phase and dilute to 50.0 ml with the mobile phase. Dilute 1.0 ml of the solution to 50.0 ml with the mobile phase.

Reference solution (c). Mix equal volumes of reference solution (a) and reference solution (b).

Column:

- **size:** $l = 0.125$ m, $\varnothing = 4.0$ mm,
- **stationary phase:** base-deactivated octadecylsilyl silica gel for chromatography R (5 μ m),

Mobile phase: 50 ml of *methanol R*, 75 ml of *acetonitrile R* and 800 ml of a 1.7 g/l solution of *potassium dihydrogen phosphate R* adjusted to pH 2.45 with *phosphoric acid R*; dilute to 1000 ml with *water R*.

Flow rate: 1.0 ml/min.

Detection: spectrophotometer at 205 nm.

Injection: 10 μ l.

Run time: 3 times the retention time of *trapidil*.

System suitability:

- **resolution:** minimum of 4.0 between the peaks due to impurity A and impurity B in the chromatogram obtained with reference solution (c).

Limits:

- **impurity A:** not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent),
- **impurity B:** not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent),
- **any other impurity:** not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent),
- **total:** not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent).
- **disregard limit:** 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.01 per cent).

Chlorides (2.4.4): maximum 100 ppm.

Dissolve 0.25 g in 10 ml of *water R* and dilute to 15 ml with *water R*. The solution complies with the limit test for chlorides. Prepare the standard using 5 ml of *chloride standard solution (5 ppm Cl) R*.

Ammonium (2.4.1): maximum 20 ppm.

0.50 g complies with limit test A. Prepare the standard using 0.1 ml of *ammonium standard solution (100 ppm NH₄) R*.

Heavy metals (2.4.8): maximum 10 ppm.

Dissolve 2.0 g in 20 ml of *water R*. 12 ml of the solution complies with limit test A. Prepare the standard using 1 ml of *lead standard solution (10 ppm Pb) R*.

Loss on drying (2.2.32): maximum 0.5 per cent, determined on 1.000 g by drying *in vacuo* at 60 °C for 3 h.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

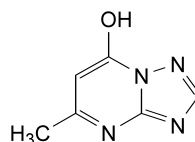
Dissolve 0.180 g in 50 ml of *anhydrous acetic acid R*. Titrate with 0.1 M *perchloric acid*, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *perchloric acid* is equivalent to 20.53 mg of C₁₀H₁₅N₅.

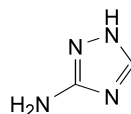
STORAGE

Protected from light.

IMPURITIES



A. 5-methyl-[1,2,4]triazolo[1,5-a]pyrimidin-7-ol,

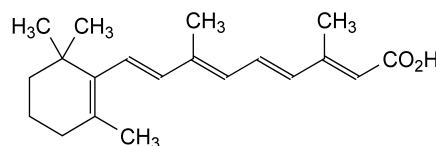


B. 1,2,4-triazol-3-amine.

01/2005:0693

TRETINOIN

Tretinoinum



C₂₀H₂₈O₂

*M*_r 300.4

DEFINITION

Tretinoin contains not less than 98.0 per cent and not more than the equivalent of 102.0 per cent of (2*E*,4*E*,6*E*,8*E*)-3,7-dimethyl-9-(2,6,6-trimethylcyclohex-1-enyl)nona-2,4,6,8-tetraenoic acid, calculated with reference to the dried substance.

CHARACTERS

A yellow or light orange, crystalline powder, practically insoluble in water, soluble in methylene chloride, slightly soluble in alcohol. It is sensitive to air, heat and light, especially in solution.

It melts at about 182 °C, with decomposition.

Carry out all operations as rapidly as possible and avoid exposure to actinic light; use freshly prepared solutions.

IDENTIFICATION

First identification: A, B.

Second identification: A, C, D.

A. Dissolve 75.0 mg in 5 ml of *methylene chloride R* and dilute immediately to 100.0 ml with acidified 2-propanol (prepared by diluting 1 ml of 0.01 M *hydrochloric acid* to 1000 ml with 2-propanol R). Dilute 5.0 ml of this solution to 100.0 ml with the acidified 2-propanol. Dilute 5.0 ml of this latter solution to 50.0 ml with the acidified 2-propanol. Examined between 300 nm and 400 nm (2.2.25), the solution shows a single maximum, at 353 nm. The specific absorbance at the maximum is 1455 to 1545.

B. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *tretinoin CRS*. Examine the substances prepared as discs.

C. Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel GF₂₅₄ plate R*.

Test solution. Dissolve 10 mg of the substance to be examined in *methylene chloride R* and dilute to 10 ml with the same solvent.

Reference solution. Dissolve 10 mg of *tretinoin CRS* in *methylene chloride R* and dilute to 10 ml with the same solvent.

Apply separately to the plate 5 µl of each solution.

Develop over a path of 15 cm using a mixture of 2 volumes of *glacial acetic acid R*, 4 volumes of *acetone R*, 40 volumes of *peroxide-free ether R* and 54 volumes of *cyclohexane R*. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. The principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with the reference solution.

D. Dissolve about 5 mg in 2 ml of *antimony trichloride solution R*. An intense red colour develops and later becomes violet.

TESTS

Related substances. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 0.100 g of the substance to be examined in *methanol R* and dilute to 50.0 ml with the same solvent.

Reference solution (a). Dissolve 10.0 mg of *isotretinoin CRS* in *methanol R* and dilute to 10.0 ml with the same solvent.

Reference solution (b). Dilute 1.0 ml of reference solution (a) to 25.0 ml with *methanol R*.

Reference solution (c). Mix 1.0 ml of reference solution (a) with 0.5 ml of the test solution and dilute to 25.0 ml with *methanol R*.

Reference solution (d). Dilute 0.5 ml of the test solution to 100.0 ml with *methanol R*.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.15 m long and 4.6 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (3 µm),
- as mobile phase at a flow rate of 1.0 ml/min a mixture of 5 volumes of *glacial acetic acid R*, 225 volumes of *water R* and 770 volumes of *methanol R*,
- as detector a spectrophotometer set at 355 nm.

Inject separately 10 µl of reference solutions (b), (c) and (d) and of the test solution. Adjust the sensitivity of the detector so that the height of the principal peak in the chromatogram obtained with reference solution (b) is about 70 per cent of the full scale of the recorder. The test is not valid unless the resolution between isotretinoin and tretinoin in the chromatogram obtained with reference solution (c) is at least 2.0. In the chromatogram obtained with the test solution, the area of the peak due to isotretinoin is not greater than the area of the principal peak in the chromatogram obtained with reference solution (b) (2.0 per cent) and the sum of the areas of any peaks, apart from the principal peak and any peak due to isotretinoin, is not greater than the area of the principal peak in the chromatogram obtained with reference solution (d) (0.5 per cent).

Heavy metals (2.4.8). 0.5 g complies with limit test D for heavy metals (20 ppm). Prepare the standard using 1 ml of *lead standard solution* (10 ppm Pb) *R*.

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.200 g in 70 ml of *acetone R*. Titrate with 0.1 M *tetrabutylammonium hydroxide* determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *tetrabutylammonium hydroxide* is equivalent to 30.04 mg of C₂₀H₂₈O₂.

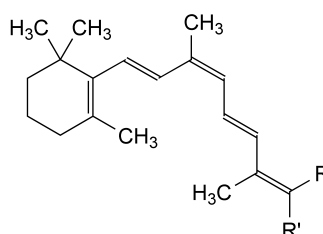
STORAGE

Store in an airtight container, protected from light, at a temperature not exceeding 25 °C.

It is recommended that the contents of an opened container be used as soon as possible and any unused part be protected by an atmosphere of an inert gas.

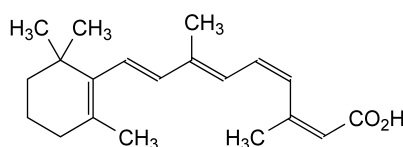
IMPURITIES

A. isotretinoin,



B. R = CO₂H, R' = H: (2*Z*,4*E*,6*Z*,8*E*)-3,7-dimethyl-9-(2,6,6-trimethylcyclohex-1-enyl)nona-2,4,6,8-tetraenoic acid (9,13-di-*cis*-retinoic acid),

D. R = H, R' = CO₂H: (2*E*,4*E*,6*Z*,8*E*)-3,7-dimethyl-9-(2,6,6-trimethylcyclohex-1-enyl)nona-2,4,6,8-tetraenoic acid (9-*cis*-retinoic acid),



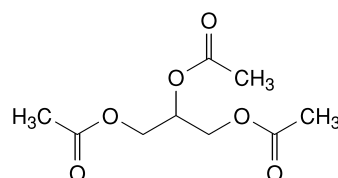
C. (2*Z*,4*Z*,6*E*,8*E*)-3,7-dimethyl-9-(2,6,6-trimethylcyclohex-1-enyl)nona-2,4,6,8-tetraenoic acid (11,13-di-*cis*-retinoic acid),

E. oxidation products of tretinoin.

01/2005:1106
corrected

TRIACETIN

Triacetinum



C₉H₁₄O₆

M_r 218.2

DEFINITION

Triacetin contains not less than 97.0 per cent and not more than the equivalent of 100.5 per cent of propane-1,2,3-triyl triacetate, calculated with reference to the anhydrous substance.

CHARACTERS

A clear, colourless, slightly viscous oily liquid. It is soluble in water, miscible with ethanol and toluene. It boils at about 260 °C.

IDENTIFICATION

Examine by infrared absorption spectrophotometry (2.2.24), comparing with the *Ph. Eur. reference spectrum of triacetin*.

TESTS

Appearance. It is clear (2.2.1) and not more intensely coloured than reference solution Y₆ (2.2.2, *Method II*).

Acidity. Dissolve 5.00 g in 25 ml of *ethanol R*, previously neutralised to 0.2 ml of *phenolphthalein solution R* and add 0.20 ml of 0.1 M *sodium hydroxide*. The pink colour of the mixture persists for 15 s.

Relative density (2.2.5): 1.159 to 1.164.

Refractive index (2.2.6): 1.429 to 1.432.

Water (2.5.12). Not more than 0.2 per cent, determined on 5.00 g by the semi-micro determination of water.

ASSAY

Introduce 0.300 g of the substance to be examined into a 250 ml borosilicate glass flask fitted with a reflux condenser. Add 25.0 ml of 0.5 M *alcoholic potassium hydroxide* and a few glass beads. Attach the condenser and heat under reflux for 30 min. Add 1 ml of *phenolphthalein solution R1* and titrate immediately with 0.5 M *hydrochloric acid*. Carry out a blank test under the same conditions. Calculate the content from the difference in consumption of alkali in the main and the blank procedure.

1 ml of 0.5 M *alcoholic potassium hydroxide* is equivalent to 36.37 mg of C₉H₁₄O₆.

STORAGE

Store in a well-filled container.

CHARACTERS

Appearance: white or almost white, crystalline powder.

Solubility: practically insoluble in water, slightly soluble in methanol, practically insoluble in methylene chloride.

It shows polymorphism.

IDENTIFICATION

A. Infrared absorption spectrophotometry (2.2.24).

Comparison: triamcinolone CRS.

If the spectra obtained show differences, dissolve the substance to be examined and the reference substance separately in *methanol R*, evaporate to dryness, dry the residues at 60 °C at a pressure not exceeding 0.7 kPa and record new spectra using the residues.

B. Thin-layer chromatography (2.2.27). *Prepare the solutions immediately before use and protect from light. Examine the plate under ultraviolet light immediately after development.*

Test solution. Dissolve 10 mg of the substance to be examined in *methanol R* and dilute to 10 ml with the same solvent.

Reference solution (a). Dissolve 20 mg of *triamcinolone CRS* in *methanol R* and dilute to 20 ml with the same solvent.

Reference solution (b). Dissolve 10 mg of *dexamethasone CRS* in reference solution (a) and dilute to 10 ml with the same solution.

Plate: TLC silica gel F₂₅₄ plate *R*.

Mobile phase: add a mixture of 1.2 volumes of *water R* and 8 volumes of *methanol R* to a mixture of 15 volumes of *ether R* and 77 volumes of *methylene chloride R*.

Application: 5 µl.

Development: over a path of 15 cm.

Drying: in air.

Detection: examine in ultraviolet light at 254 nm.

System suitability: the chromatogram obtained with reference solution (b) shows 2 clearly separated spots.

Results: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a).

TESTS

Specific optical rotation (2.2.7): + 65 to + 72 (anhydrous substance).

Dissolve 0.100 g in *dimethylformamide R* and dilute to 10.0 ml with the same solvent.

Related substances. Liquid chromatography (2.2.29).

Prepare the solutions immediately before use and protect from light.

Test solution. Dissolve 25.0 mg of the substance to be examined in a mixture of equal volumes of *methanol R* and *water R* and dilute to 10.0 ml with the same mixture of solvents.

Reference solution (a). Dissolve 2 mg of *triamcinolone CRS* and 2 mg of *triamcinolone impurity C CRS* in a mixture of equal volumes of *methanol R* and *water R* and dilute to 100.0 ml with the same mixture of solvents.

Reference solution (b). Dilute 1.0 ml of the test solution to 100.0 ml with a mixture of equal volumes of *methanol R* and *water R*.

Blank: *methanol R*.

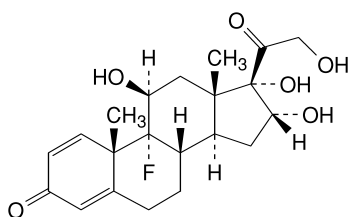
Column:

— size: *l* = 0.25 m, Ø = 4.6 mm,

01/2005:1376

TRIAMCINOLONE

Triamcinolonum

C₂₁H₂₇FO₆M_r 394.4

DEFINITION

9-Fluoro-11β,16α,17,21-tetrahydroxypregna-1,4-diene-3,20-dione.

Content: 97.0 per cent to 103.0 per cent (anhydrous substance).

- *stationary phase*: base-deactivated end-capped octadecylsilyl silica gel for chromatography *R* (5 µm).

Mobile phase: a mixture prepared as follows: in a 1000 ml volumetric flask mix 525 ml of *methanol R* with 400 ml of *water R* and allow to equilibrate; adjust the volume to 1000 ml with *water R* and mix again.

Flow rate: 1 ml/min.

Detection: spectrophotometer set at 238 nm.

Injection: 20 µl.

Run time: 4.5 times the retention time of triamcinolone.

Retention time: triamcinolone = about 11 min.

System suitability: reference solution (a):

- *resolution*: minimum of 1.8 between the peaks due to triamcinolone and to impurity C.

Limits:

- *any impurity*: not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (1 per cent) and not more than 2 such peaks have an area greater than half the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent),
- *total*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (2 per cent),
- *disregard limit*: 0.05 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

Water (2.5.12): maximum 1.0 per cent, determined on 0.500 g.

ASSAY

Prepare the solutions immediately before use and protect from light.

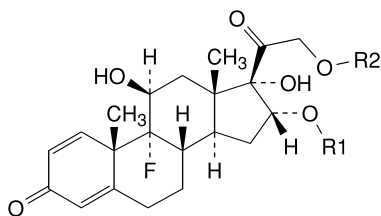
Dissolve 50.0 mg in *alcohol R* and dilute to 50.0 ml with the same solvent. Dilute 2.0 ml of the solution to 100.0 ml with *alcohol R*. Measure the absorbance (2.2.25) at the maximum at 238 nm.

Calculate the content of $C_{21}H_{27}FO_6$ taking the specific absorbance to be 389.

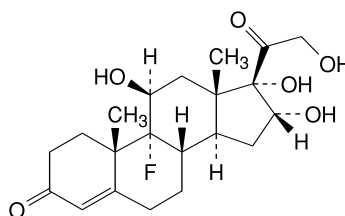
STORAGE

Protected from light.

IMPURITIES



- A. $R_1 = R_2 = CO-CH_3$: 9-fluoro-11β,17-dihydroxy-3,20-dioxopregna-1,4-diene-16α,21-diyl diacetate (triamcinolone 16,21-diacetate),
- B. $R_1 = H, R_2 = CO-CH_3$: 9-fluoro-11β,16α,17-trihydroxy-3,20-dioxopregna-1,4-dien-21-yl acetate (triamcinolone 21-acetate),

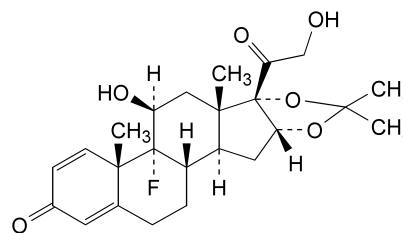


C. 9-fluoro-11β,16α,17,21-tetrahydroxypregna-4-ene-3,20-dione (pretriamcinolone).

01/2005:0533

TRIAMCINOLONE ACETONIDE

Triamcinoloni acetonidum



$C_{24}H_{31}FO_6$

M_r 434.5

DEFINITION

Triamcinolone acetonide contains not less than 97.0 per cent and not more than the equivalent of 103.0 per cent of 9-fluoro-11β,21-dihydroxy-16α,17-(1-methylethylidenedioxy)pregna-1,4-diene-3,20-dione, calculated with reference to the anhydrous substance.

CHARACTERS

A white or almost white, crystalline powder, practically insoluble in water, sparingly soluble in alcohol.

It shows polymorphism.

IDENTIFICATION

First identification: A, B.

Second identification: C, D.

- A. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *triamcinolone acetonide CRS*. If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in the minimum volume of *methanol R* and evaporate to dryness. Using the residues, prepare halogen salt discs or mulls in *liquid paraffin R* and record the spectra again.
- B. Examine by thin-layer chromatography (2.2.27), using as the coating substance a suitable silica gel with a fluorescent indicator having an optimal intensity at 254 nm. *Prepare the solutions immediately before use and protect from light. Examine the plate in ultraviolet light immediately after development.*

Test solution. Dissolve 10 mg of the substance to be examined in *methanol R* and dilute to 10 ml with the same solvent.

Reference solution (a). Dissolve 20 mg of *triamcinolone acetonide CRS* in *methanol R* and dilute to 20 ml with the same solvent.

Reference solution (b). Dissolve 10 mg of *triamcinolone hexacetone CRS* in reference solution (a) and dilute to 10 ml with the same solution.

Apply to the plate 5 µl of each solution. Prepare the mobile phase by adding a mixture of 1.2 volumes of *water R* and 8 volumes of *methanol R* to a mixture of 15 volumes of *ether R* and 77 volumes of *methylene chloride R*. Develop over a path of 15 cm. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. The principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a). The test is not valid unless the chromatogram obtained with reference solution (b) shows two clearly separated spots.

- C. Examine by thin-layer chromatography (2.2.27), using as the coating substance a suitable silica gel with a fluorescent indicator having an optimal intensity at 254 nm. Prepare the solutions immediately before use and protect from light. Examine the plate in ultraviolet light immediately after development.

Test solution (a). Dissolve 10 mg of the substance to be examined in *methanol R* and dilute to 10 ml with the same solvent.

Test solution (b). In a separating funnel, dissolve 10 mg of the substance to be examined in 1.5 ml of *glacial acetic acid R*, add 0.5 ml of a 20 g/l solution of *chromium trioxide R* and allow to stand for 60 min. Add 5 ml of *water R*, 2 ml of *methylene chloride R* and shake vigorously for 2 min. Allow to separate and use the lower layer.

Reference solution (a). Dissolve 10 mg of *triamcinolone acetonide CRS* in *methanol R* and dilute to 10 ml with the same solvent.

Reference solution (b). In a separating funnel, dissolve 10 mg of *triamcinolone acetonide CRS* in 1.5 ml of *glacial acetic acid R*, add 0.5 ml of a 20 g/l solution of *chromium trioxide R* and allow to stand for 60 min. Add 5 ml of *water R*, 2 ml of *methylene chloride R* and shake vigorously for 2 min. Allow to separate and use the lower layer.

Apply separately to the plate 5 µl of each solution. Prepare the mobile phase by adding a mixture of 1.2 volumes of *water R* and 8 volumes of *methanol R* to a mixture of 15 volumes of *ether R* and 77 volumes of *methylene chloride R*. Develop over a path of 15 cm. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. The principal spot in each of the chromatograms obtained with the test solutions is similar in position and size to the principal spot in the chromatogram obtained with the corresponding reference solution. The principal spots in the chromatograms obtained with test solution (b) and reference solution (b) have an R_f value distinctly higher than that of the principal spots in the chromatograms obtained with test solution (a) and reference solution (a).

- D. Mix about 5 mg with 45 mg of *heavy magnesium oxide R* and ignite in a crucible until an almost white residue is obtained (usually less than 5 min). Allow to cool, add 1 ml of *water R*, 0.05 ml of *phenolphthalein solution R1* and about 1 ml of *dilute hydrochloric acid R* to render the solution colourless. Filter. To a freshly prepared mixture of 0.1 ml of *alizarin S solution R* and 0.1 ml of *zirconyl nitrate solution R*, add 1.0 ml of the filtrate. Mix, allow to stand for 5 min and compare the colour of the solution with that of a blank prepared in the same manner. The test solution is yellow and the blank is red.

TESTS

Specific optical rotation (2.2.7). Dissolve 0.100 g in *dioxan R* and dilute to 10.0 ml with the same solvent. The specific optical rotation is + 100 to + 107, calculated with reference to the anhydrous substance.

Related substances. Examine by liquid chromatography (2.2.29). Carry out the test protected from light.

Test solution. Dissolve 25.0 mg of the substance to be examined in 7 ml of *methanol R* and dilute to 10.0 ml with *water R*.

Reference solution (a). Dissolve 2 mg of *triamcinolone acetonide CRS* and 2 mg of *triamcinolone R* in the mobile phase and dilute to 100.0 ml with the mobile phase.

Reference solution (b). Dilute 1.0 ml of the test solution to 100.0 ml with the mobile phase.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4.6 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (5 µm),
- as mobile phase at a flow rate of 1.5 ml/min a mixture prepared as follows: in a 1000 ml volumetric flask mix 525 ml of *methanol R* with 400 ml of *water R* and allow to equilibrate; adjust the volume to 1000 ml with *water R* and mix again,
- as detector a spectrophotometer set at 254 nm.

Equilibrate the column with the mobile phase at a flow rate of 1.5 ml/min for about 10 min.

Adjust the sensitivity of the system so that the height of the principal peak in the chromatogram obtained with 20 µl of reference solution (b) is at least 50 per cent of the full scale of the recorder.

Inject 20 µl of reference solution (a). When the chromatograms are recorded in the prescribed conditions, the retention times are: triamcinolone about 5 min and triamcinolone acetonide about 17 min. The test is not valid unless the resolution between the peaks corresponding to triamcinolone and triamcinolone acetonide is at least 15; if necessary, adjust the concentration of methanol in the mobile phase.

Inject separately 20 µl of the test solution and 20 µl of reference solution (b). Continue the chromatography for 3.5 times the retention time of the principal peak in the chromatogram obtained with the test solution. In the chromatogram obtained with the test solution: the area of any peak, apart from the principal peak, is not greater than 0.25 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.25 per cent); the sum of the areas of all the peaks, apart from the principal peak, is not greater than half the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent). Disregard any peak due to the solvent and any peak with an area less than 0.05 times the area of the principal peak in the chromatogram obtained with reference solution (b).

Water (2.5.12). Not more than 2.0 per cent, determined on 0.500 g by the semi-micro determination of water.

ASSAY

Protect the solutions from light throughout the assay.

Dissolve 50.0 mg in *alcohol R* and dilute to 50.0 ml with the same solvent. Dilute 2.0 ml of this solution to 100.0 ml with *alcohol R*. Measure the absorbance (2.2.25) at the maximum at 238.5 nm.

Calculate the content of $C_{24}H_{31}FO_6$ taking the specific absorbance to be 355.

STORAGE

Store protected from light.

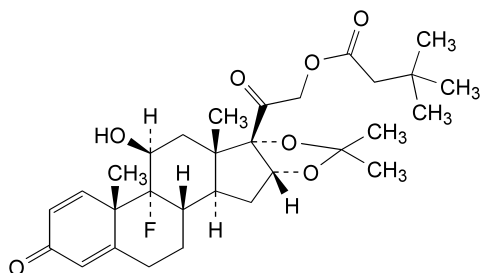
IMPURITIES

A. triamcinolone.

01/2005:0867

TRIAMCINOLONE HEXACETONIDE

Triamcinoloni hexacetonidum



$C_{30}H_{41}FO_7$

M_r 532.6

DEFINITION

Triamcinolone hexacetonide contains not less than 97.0 per cent and not more than the equivalent of 103.0 per cent of 9-fluoro-11β-hydroxy-16α,17-(1-methylethylidenedioxy)-3,20-dioxopregna-1,4-diene-21-yl 3,3-dimethylbutanoate, calculated with reference to the anhydrous substance.

CHARACTERS

A white or almost white, crystalline powder, practically insoluble in water, sparingly soluble in ethanol and in methanol.

IDENTIFICATION

- A. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *triamcinolone hexacetonide CRS*.
- B. Examine by thin-layer chromatography (2.2.27), using as the coating substance a suitable silica gel with a fluorescent indicator having an optimal intensity at 254 nm. *Prepare the solutions immediately before use and protect from light. Examine the plate in ultraviolet light immediately after development.*

Test solution. Dissolve 10 mg of the substance to be examined in *methanol R* and dilute to 10 ml with the same solvent.

Reference solution (a). Dissolve 20 mg of *triamcinolone hexacetonide CRS* in *methanol R* and dilute to 20 ml with the same solvent.

Reference solution (b). Dissolve 10 mg of *triamcinolone acetonide CRS* in reference solution (a) and dilute to 10 ml with the same solution.

Apply separately to the plate 5 µl of each solution. Prepare the mobile phase by adding a mixture of 1.2 volumes of *water R* and 8 volumes of *methanol R* to a mixture of 15 volumes of *ether R* and 77 volumes of *methylene chloride R*. Develop over a path of 15 cm. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. The principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a). The test is not valid unless the chromatogram obtained with reference solution (b) shows two clearly separated spots.

TESTS

Specific optical rotation (2.2.7). Dissolve 0.100 g in *methylene chloride R* and dilute to 10.0 ml with the same solvent. The specific optical rotation is + 92 to + 98, calculated with reference to the anhydrous substance.

Related substances. Examine by liquid chromatography (2.2.29). *Carry out the test protected from light.*

Test solution. Dissolve 25.0 mg of the substance to be examined in *methanol R* and dilute to 10.0 ml with the same solvent.

Reference solution (a). Dissolve 2 mg of *triamcinolone hexacetonide CRS* and 2 mg of *triamcinolone acetonide CRS* in the mobile phase and dilute to 100.0 ml with the mobile phase.

Reference solution (b). Dilute 1.0 ml of the test solution to 100.0 ml with the mobile phase.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4.6 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (5 µm),
- as mobile phase at a flow rate of 2 ml/min a mixture prepared as follows: in a 1000 ml volumetric flask mix 750 ml of *methanol R* with 200 ml of *water R* and allow to equilibrate; adjust the volume to 1000 ml with *water R* and mix again,
- as detector a spectrophotometer set at 254 nm.

Equilibrate the column with the mobile phase at a flow rate of 2 ml/min for about 10 min.

Adjust the sensitivity of the system so that the height of the principal peak in the chromatogram obtained with 20 µl of reference solution (b) is at least 50 per cent of the full scale of the recorder.

Inject 20 µl of reference solution (a). When the chromatograms are recorded in the prescribed conditions, the retention times are: triamcinolone acetonide about 3 min and triamcinolone hexacetonide about 12 min. The test is not valid unless the resolution between the peaks corresponding to triamcinolone acetonide and triamcinolone hexacetonide is at least 20.0; if necessary, adjust the concentration of methanol in the mobile phase.

Inject separately 20 µl of the test solution and 20 µl of reference solution (b). Continue the chromatography for three times the retention time of the principal peak in the chromatogram obtained with the test solution. In the chromatogram obtained with the test solution: the area of any peak, apart from the principal peak, is not greater than half the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent); the sum of the areas of all the peaks, apart from the principal peak, is not greater than the area of the principal peak in the chromatogram obtained with reference solution (b) (1 per cent). Disregard any peak due to the solvent and any peak with an area less than 0.05 times the area of the principal peak in the chromatogram obtained with reference solution (b).

Water (2.5.12). Not more than 2.0 per cent, determined on 0.50 g by the semi-micro determination of water.

ASSAY

Dissolve 50.0 mg in *alcohol R* and dilute to 50.0 ml with the same solvent. Dilute 2.0 ml of this solution to 100.0 ml with *alcohol R*. Measure the absorbance (2.2.25) at the maximum at 238 nm.

Calculate the content of $C_{30}H_{41}FO_7$ taking the specific absorbance to be 291.

STORAGE

Store protected from light.

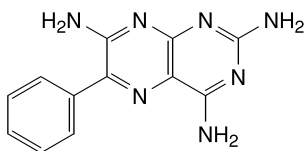
IMPURITIES

A. triamcinolone acetonide.

01/2005:0058

TRIAMTERENE

Triamterenum


 $C_{12}H_{11}N_7$
 M_r 253.3

DEFINITION

Triamterene contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of 6-phenylpteridine-2,4,7-triamine, calculated with reference to the dried substance.

CHARACTERS

A yellow, crystalline powder, very slightly soluble in water and in alcohol.

IDENTIFICATION

- A. Dissolve 0.1 g in a 10 per cent *V/V* solution of 1 *M* hydrochloric acid in ethanol *R* and dilute to 100 ml with the same acid mixture. Dilute 1 ml of this solution to 100 ml with a 10 per cent *V/V* solution of 1 *M* hydrochloric acid in ethanol *R*. Examined between 255 nm and 380 nm, the solution shows 2 absorption maxima (2.2.25), at 262 nm and 360 nm, and a shoulder at 285 nm.
- B. Examined in ultraviolet light at 365 nm, acid solutions, especially a 1 g/l solution in anhydrous formic acid *R*, show an intense blue fluorescence.

TESTS

Acidity. Boil 1.0 g with 20 ml of water *R* for 5 min, cool, filter and wash the filter with 3 quantities, each of 10 ml, of water *R*. Combine the filtrate and washings and add 0.3 ml of phenolphthalein solution *R*. Not more than 1.5 ml of 0.01 *M* sodium hydroxide is required to change the colour of the indicator.

Nitrosotriaminopyrimidine. Examine by thin-layer chromatography (2.2.27), using silica gel HF_{254} *R* as the coating substance.

Test solution. Dissolve 0.2 g of the substance to be examined in 5 ml of anhydrous formic acid *R*, stirring with a glass rod if necessary. Prepare immediately before use.

Reference solution. Dissolve 4 mg of nitrosotriaminopyrimidine *CRS* in anhydrous formic acid *R* and dilute to 100 ml with the same acid.

Apply separately to the plate as bands 1.5 cm long, 20 µl of each solution as 2 applications of 10 µl each, drying in a current of air after each application. Develop over a path of 5 cm using ether *R*. Allow the plate to dry in air and develop over a path of 10 cm using a mixture of 10 volumes of glacial acetic acid *R*, 10 volumes of methanol *R* and 80 volumes of ethyl acetate *R*, the mixture containing 0.5 g/l of sodium fluoresceinate *R*. Dry the plate in a current of air and expose to ammonia vapour for a few seconds. Examine in ultraviolet

light at 254 nm and at 365 nm. Any band corresponding to nitrosotriaminopyrimidine in the chromatogram obtained with the test solution is not more intense than the band in the chromatogram obtained with the reference solution (0.1 per cent).

Related substances. Examine by thin-layer chromatography (2.2.27), using silica gel *G R* as the coating substance.

Test solution. Dissolve 0.1 g of the substance to be examined in 20 ml of dimethyl sulphoxide *R*. Dilute 2 ml of the solution to 50 ml with methanol *R*.

Reference solution. Dilute 1 ml of the test solution to 200 ml with methanol *R*.

Apply separately to the plate 5 µl of each solution. Develop over a path of 15 cm using a mixture of 10 volumes of concentrated ammonia *R1*, 10 volumes of methanol *R* and 90 volumes of ethyl acetate *R*. Allow the plate to dry in air until the solvents have evaporated and examine in ultraviolet light at 365 nm. Any spot in the chromatogram obtained with the test solution, apart from the principal spot, is not more intense than the spot in the chromatogram obtained with the reference solution (0.5 per cent).

Loss on drying (2.2.32). Not more than 1.0 per cent, determined on 1.00 g by drying in an oven at 100 °C to 105 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.150 g in 5 ml of anhydrous formic acid *R* and add 100 ml of anhydrous acetic acid *R*. Titrate with 0.1 *M* perchloric acid determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 *M* perchloric acid is equivalent to 25.33 mg of $C_{12}H_{11}N_7$.

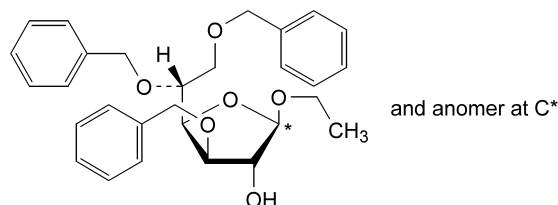
STORAGE

Store protected from light.

01/2005:1740

TRIBENOSIDE

Tribenosidum


 $C_{29}H_{34}O_6$
 M_r 478.6

DEFINITION

Mixture of α - and β -anomers of ethyl 3,5,6-tri-*O*-benzyl-D-glucofuranoside.

Content: 96.0 per cent to 102.0 per cent.

CHARACTERS

Appearance: yellowish to pale yellow, clear, viscous liquid.

Solubility: practically insoluble in water, very soluble in acetone, in methanol and in methylene chloride.

IDENTIFICATION

Infrared absorption spectrophotometry (2.2.24).

Preparation: discs.

Comparison: tribenoside CRS.

TESTS

Solution S. Dissolve 4.00 g in *methanol R* and dilute to 20 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and its absorbance (2.2.25) at 420 nm has a maximum of 0.10.

Specific optical rotation (2.2.7): –31.0 to –40.0.

Dilute 2.0 ml of solution S to 20.0 ml with *methanol R*.

Related substances. Liquid chromatography (2.2.29).

Test solution (a). Dissolve 1.000 g of the substance to be examined in a mixture of 5 volumes of *water R* and 95 volumes of *acetonitrile R* and dilute to 25.0 ml with the same mixture of solvents.

Test solution (b). Dissolve 50.0 mg of the substance to be examined in a mixture of 5 volumes of *water R* and 95 volumes of *acetonitrile R* and dilute to 50.0 ml with the same mixture of solvents.

Reference solution (a). Dilute 25.0 mg of *benzaldehyde R* and 30.0 mg of *tribenoside impurity A CRS* to 100.0 ml with *acetonitrile R*. Introduce 20.0 ml of this solution into a 50 ml volumetric flask, add 2.5 ml of *water R* and dilute to 50.0 ml with *acetonitrile R*.

Reference solution (b). Dissolve 50.0 mg of *tribenoside CRS* in a mixture of 5 volumes of *water R* and 95 volumes of *acetonitrile R* and dilute to 50.0 ml with the same mixture of solvents.

Reference solution (c). Dissolve 12.0 mg of *benzyl ether R* in a mixture of 5 volumes of *water R* and 95 volumes of *acetonitrile R* and dilute to 100.0 ml with the same mixture of solvents.

Column:

- size: $l = 0.15$ m, $\varnothing = 4.6$ mm,
- stationary phase: octadecylsilyl silica gel for chromatography *R* (3 μ m).

Mobile phase:

- mobile phase A: 0.1 per cent V/V solution of *phosphoric acid R*,
- mobile phase B: *acetonitrile R*,

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)
0 - 40	55 $\rightarrow$ 10	45 $\rightarrow$ 90
40 - 55	10	90
55 - 56	10 $\rightarrow$ 55	90 $\rightarrow$ 45
56 - 60	55	45

Flow rate: 1.3 ml/min.

Detection: spectrophotometer at 254 nm.

Injection: 20 μ l; inject test solution (a) and reference solutions (a), (b) and (c).

Relative retentions with reference to the β -anomer of tribenoside (retention time = about 18 min): α -anomer = about 1.1; impurity C = about 0.2; impurity B = about 0.6; impurity D = about 0.8; impurity A = about 1.4.

System suitability: reference solution (b):

- resolution: minimum 3.0 between the peaks due to the α -anomer and to the β -anomer of tribenoside.

Limits:

- **impurity A:** not more than 1.7 times the area of the corresponding peak in the chromatogram obtained with reference solution (a) (0.5 per cent),
- **impurity C:** not more than twice the area of the corresponding peak in the chromatogram obtained with reference solution (a) (0.5 per cent); if the area of the peak due to impurity C in the chromatogram obtained with the test solution is greater than the area of the corresponding peak in the chromatogram obtained with reference solution (a) (0.25 per cent), dilute the test solution to obtain an area equal to or smaller than the area of the peak in the chromatogram obtained with reference solution (a); calculate the content of impurity C taking into account the dilution factor;
- **impurity D:** not more than the area of the principal peak in the chromatogram obtained with reference solution (c) (0.3 per cent),
- **any other impurity:** not more than the area of the peak due to impurity A in the chromatogram obtained with reference solution (a) (0.3 per cent),
- **total:** not more than 6.7 times the area of the peak due to impurity A in the chromatogram obtained with reference solution (a) (2.0 per cent),
- **disregard limit:** 0.17 times the area of the peak due to impurity A in the chromatogram obtained with reference solution (a) (0.05 per cent).

Heavy metals (2.4.8): maximum 20 ppm.

Dilute 5.0 ml of solution S to 20.0 ml with *methanol R*. 12 ml of the solution complies with limit test B. Prepare the standard using lead standard solution (1 ppm Pb) obtained by diluting *lead standard solution (100 ppm Pb) R* with *methanol R*.

ASSAY

Liquid chromatography (2.2.29) as described in the test for related substances with the following modification.

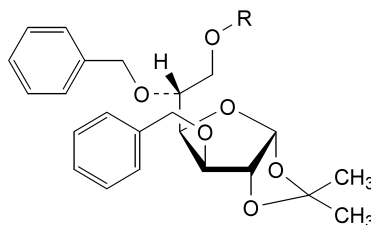
Injection: test solution (b) and reference solution (b).

Calculate the sum of the percentage contents of the α -anomer and the β -anomer of tribenoside.

STORAGE

Under nitrogen, in an airtight container.

IMPURITIES



A. $R = \text{CH}_2\text{-C}_6\text{H}_5$: 3,5,6-tri-*O*-benzyl-1,2-*O*-(1-methylethylidene)- α -D-glucofuranose,

B. $R = \text{H}$: 3,5-di-*O*-benzyl-1,2-*O*-(1-methylethylidene)- α -D-glucofuranose,

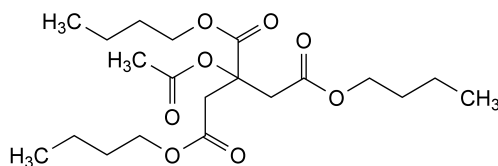
C. $\text{C}_6\text{H}_5\text{-CHO}$: benzaldehyde,

D. $\text{C}_6\text{H}_5\text{-CH}_2\text{-O-CH}_2\text{-C}_6\text{H}_5$: dibenzyl ether.

01/2005:1770 – injection port and detector: 250 °C.

TRIBUTYL ACETYLCITRATE

Tributylis acetylcitras

 $C_{20}H_{34}O_8$ M_r 402.5

DEFINITION

Tributyl 2-(acetyloxy)propane-1,2,3-tricarboxylate.

Content: 99.0 per cent to 101.0 per cent (anhydrous substance).

CHARACTERS

Appearance: clear, oily liquid.

Solubility: not miscible with water, miscible with alcohol and with methylene chloride.

IDENTIFICATION

Infrared absorption spectrophotometry (2.2.24).

Comparison: Ph. Eur. reference spectrum of tributyl acetylcitrate.

TESTS

Appearance. The substance to be examined is clear (2.2.1) and not more intensely coloured than reference solution BY₆ (2.2.2, Method II).**Acidity.** Dilute 10 g with 10 ml of previously neutralised alcohol R, add 0.5 ml of bromothymol blue solution R2. Not more than 0.3 ml of 0.1 M sodium hydroxide is required to change the colour of the indicator to blue.**Refractive index** (2.2.6): 1.442 to 1.445.**Related substances.** Gas chromatography (2.2.28).**Test solution.** Dissolve 1.0 g of the substance to be examined in methylene chloride R and dilute to 20.0 ml with the same solvent.**Reference solution (a).** Dissolve 50 mg of the substance to be examined and 50 mg of tributyl citrate R in methylene chloride R and dilute to 20.0 ml with the same solvent.**Reference solution (b).** Dilute 1.0 ml of the test solution to 20.0 ml with methylene chloride R. Dilute 1.0 ml of this solution to 25.0 ml with methylene chloride R.

Column:

- material: fused silica,
- size: $l = 30$ m, $\varnothing = 0.53$ mm,
- stationary phase: poly[(cyanopropyl)(methyl)][(phenyl)(methyl)]siloxane R (film thickness 1.0 μ m).

Carrier gas: helium for chromatography R.

Linear velocity: 36 cm/s.

Split ratio: 1:20.

Temperature:

- column: 200 °C,

Detection: flame ionisation.

Injection: 1 μ l.

Run time: twice the retention time of tributyl acetylcitrate.

Relative retention with reference to tributyl acetylcitrate (retention time = about 26 min): impurity B = about 0.83; impurity A = about 0.87.

System suitability: reference solution (a):

- resolution: minimum 2.0 between the peaks due to impurity A and tributyl acetylcitrate.

Limits:

- impurities A, B: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent),
- any other impurity: for each impurity, not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent),
- total: not more than 2.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent),
- disregard limit: 0.25 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

Heavy metals (2.4.8): maximum 10 ppm.

2.0 g complies with limit test F. Prepare the standard using 2 ml of lead standard solution (10 ppm Pb) R.

Water (2.5.12): maximum 0.25 per cent, determined on 2.00 g.**Sulphated ash** (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

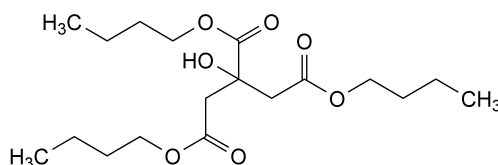
ASSAY

Introduce 1.500 g into a 250 ml borosilicate glass flask. Add 25 ml of 2-propanol R, 50 ml of water R, 25.0 ml of 1 M sodium hydroxide and a few glass beads. Heat under a reflux condenser for 1 h. Allow to cool. Add 1 ml of phenolphthalein solution R1 and titrate with 1 M hydrochloric acid. Carry out a blank titration.

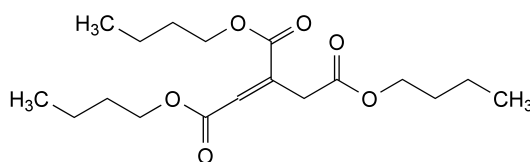
1 ml of 1 M sodium hydroxide is equivalent to 100.6 mg of $C_{20}H_{34}O_8$.

IMPURITIES

Specified impurities: A, B.



A. tributyl 2-hydroxypropane-1,2,3-tricarboxylate (tributyl citrate),



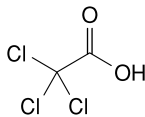
B. tributyl propene-1,2,3-tricarboxylate (tributyl aconitate).

01/2005:1967

01/2005:1479

TRICHLOROACETIC ACID

Acidum trichloroaceticum

 $C_2HCl_3O_2$ M_r 163.4

DEFINITION

2,2,2-Trichloroacetic acid.

Content: 98.0 per cent to 100.5 per cent.

CHARACTERS

Appearance: white, crystalline mass or colourless crystals, very deliquescent.*Solubility*: very soluble in water, in alcohol and in methylene chloride.

IDENTIFICATION

First identification: A.*Second identification*: B, C.

A. Infrared absorption spectrophotometry (2.2.24).

Comparison: Ph. Eur. reference spectrum of trichloroacetic acid.B. To 0.5 ml of solution S (see Tests) add 2 ml of *pyridine R* and 5 ml of *strong sodium hydroxide solution R*. Shake vigorously and heat in a water-bath at 60-70 °C for 5 min. The upper layer shows an intense red colour.

C. Solution S is strongly acidic (2.2.4).

TESTS

Solution S. Dissolve 2.5 g in *water R* and dilute to 25 ml with the same solvent.**Appearance of solution**. Solution S is clear (2.2.1) and not more intensely coloured than reference solution BY₇ (2.2.2, *Method II*).**Chlorides** (2.4.4): maximum 100 ppm.Dilute 5 ml of solution S to 15 ml with *water R*. The solution complies with the limit test for chlorides.**Sulphated ash** (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

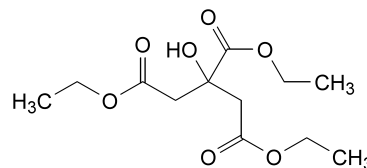
Dissolve 0.150 g in 20 ml of *water R*. Titrate with 0.1 M *sodium hydroxide*, determining the end-point potentiometrically (2.2.20).1 ml of 0.1 M *sodium hydroxide* is equivalent to 16.34 mg of $C_2HCl_3O_2$.

STORAGE

In an airtight container.

TRIETHYL CITRATE

Triethylis citras

 $C_{12}H_{20}O_7$ M_r 276.3

DEFINITION

Triethyl citrate contains not less than 98.5 per cent and not more than the equivalent of 101.0 per cent of triethyl 2-hydroxypropane-1,2,3-tricarboxylate, calculated with reference to the anhydrous substance.

CHARACTERS

A clear, viscous, colourless or almost colourless liquid, hygroscopic, soluble in water, miscible with alcohol, slightly soluble in fatty oils.

IDENTIFICATION

First identification: A, B.*Second identification*: A, C, D.

A. It complies with the test for refractive index (see Tests).

B. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the Ph. Eur. reference spectrum of triethyl citrate.

C. It gives the reaction of esters (2.3.1).

D. To 0.5 ml add 5 ml of *alcohol R* and 4 ml of *dilute sodium hydroxide solution R*. Boil under reflux for about 10 min. 2 ml of the solution gives the reaction of citrates (2.3.1).

TESTS

Appearance. The substance to be examined is clear (2.2.1) and not more intensely coloured than reference solution BY₆ (2.2.2, *Method II*).**Acidity**. Dilute 10 g with 10 ml of previously neutralised *alcohol R*, add 0.5 ml of *bromothymol blue solution R2*. Not more than 0.3 ml of 0.1 M *sodium hydroxide* is required to change the colour of the indicator to blue.**Refractive index** (2.2.6): 1.440 to 1.446.**Related substances**. Examine by gas chromatography (2.2.28).**Test solution**. Dissolve 1.0 ml of the substance to be examined in *methylene chloride R* and dilute to 50.0 ml with the same solvent.**Reference solution (a)**. Dissolve 1.0 ml of the substance to be examined and 0.5 ml of *methyl tridecanoate R* in *methylene chloride R* and dilute to 50.0 ml with the same solvent.

The chromatographic procedure may be carried out using:

- a fused-silica column 30 m long and 0.32 mm in internal diameter coated with *poly(dimethyl)siloxane R* (5 µm),
 - *helium for chromatography R* as the carrier gas with a split ratio of about 1:50 and a linear velocity of about 26 cm/s,
 - a flame-ionisation detector,
- maintaining the temperature of the column at 200 °C and that of the injection port and the detector at 220 °C.

Inject 1.0 µl of each of the solutions. Continue the chromatography for twice the retention time of triethyl citrate which is about 13.6 min.

The test is not valid unless in the chromatogram obtained with reference solution (a), the resolution between the peaks corresponding to triethyl citrate and methyl tridecanoate is at least 1.5.

Calculate the percentage content of related substances from the areas of the peaks in the chromatogram obtained with the test solution by the normalisation procedure, disregarding any peaks with an area less than 0.04 per cent of the area of the principal peak. The content of any related substance is not greater than 0.2 per cent and the sum is not greater than 0.5 per cent.

Heavy metals (2.4.8). Dissolve 4.0 g in 8 ml of *alcohol R* and dilute to 20 ml with *water R*. 12 ml of the solution complies with limit test B for heavy metals (5 ppm). Prepare the standard using lead standard solution (1 ppm) obtained by diluting *lead standard solution (100 ppm Pb) R* with a mixture of equal volumes of *alcohol R* and *water R*.

Water (2.5.12). Not more than 0.25 per cent, determined on 1.000 g by the semi-micro determination of water.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

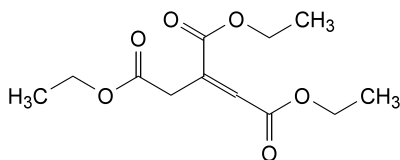
Introduce 1.500 g into a 250 ml borosilicate-glass flask fitted with a reflux condenser. Add 25 ml of *2-propanol R*, 50 ml of *water R*, 25.0 ml of 1 M *sodium hydroxide* and a few glass beads. Heat under a reflux condenser for 1 h. Allow to cool. Add 1 ml of *phenolphthalein solution R1* and titrate with 1 M *hydrochloric acid*. Carry out a blank test under the same conditions.

1 ml of 1 M *sodium hydroxide* is equivalent to 92.1 mg of C₁₂H₂₀O₇.

STORAGE

Store in an airtight container.

IMPURITIES

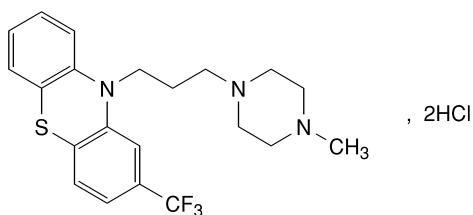


A. triethyl propene-1,2,3-tricarboxylate (triethyl aconitate).

01/2005:0059

TRIFLUOPERAZINE HYDROCHLORIDE

Trifluoperazini hydrochloridum



C₂₁H₂₆Cl₂F₃N₃S

M_r 480.4

DEFINITION

Trifluoperazine hydrochloride contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of 10-[3-(4-methylpiperazin-1-yl)propyl]-2-(trifluoromethyl)-10H-phenothiazine dihydrochloride, calculated with reference to the dried substance.

CHARACTERS

A white to pale yellow, crystalline powder, hygroscopic, freely soluble in water, soluble in alcohol.

It melts at about 242 °C, with decomposition.

IDENTIFICATION

- Protect the solutions from bright light and measure the absorbances immediately.* Dissolve 50 mg in 0.1 M *hydrochloric acid* and dilute to 500 ml with the same acid. Examined between 280 nm and 350 nm, the solution shows an absorption maximum (2.2.25) at 305 nm. Dilute 5 ml of the solution to 100 ml with 0.1 M *hydrochloric acid*. Examined between 230 nm and 280 nm, this solution shows an absorption maximum at 255 nm. The specific absorbance at this maximum is about 650.
- It complies with the identification test for phenothiazines by thin-layer chromatography (2.3.3).
- Place 0.25 g in a 100 ml separating funnel, add 5 ml of *water R* and 2 ml of *dilute sodium hydroxide solution R*. Shake vigorously with 20 ml of *ether R*. Wash the ether layer with 5 ml of *water R*, add 0.15 g of *maleic acid R* and evaporate the ether. The residue, recrystallised from 30 ml of *alcohol R* and dried, melts (2.2.14) at about 192 °C.
- Dissolve about 0.5 mg in 1 ml of *water R*, add 0.1 ml of *bromine water R* and shake for about 1 min. Add dropwise 1 ml of *sulphuric acid R* with constant, vigorous agitation. A red colour develops.
- Dissolve about 50 mg in 5 ml of *water R* and add 2 ml of *nitric acid R*. A dark-red colour develops which turns to pale yellow. The solution gives reaction (a) of chlorides (2.3.1).

TESTS

pH (2.2.3). Dissolve 2.0 g in *carbon dioxide-free water R* and dilute to 20 ml with the same solvent. The pH of the solution is 1.6 to 2.5.

Related substances. Carry out the test protected from bright light.

Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel GF₂₅₄ plate R*.

Test solution. Dissolve 0.2 g of the substance to be examined in a mixture of 5 volumes of *diethylamine R* and 95 volumes of *methanol R* and dilute to 10 ml with the same mixture of solvents. Prepare immediately before use.

Reference solution. Dilute 1 ml of the test solution to 200 ml with a mixture of 5 volumes of *diethylamine R* and 95 volumes of *methanol R*.

Apply to the plate 10 µl of each solution. Develop over a path of 12 cm using a mixture of 10 volumes of *acetone R*, 10 volumes of *diethylamine R* and 80 volumes of *cyclohexane R*. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. Any spot in the chromatogram obtained with the test solution, apart from the principal spot, is not more intense than the spot in the chromatogram obtained with the reference solution (0.5 per cent).

Loss on drying (2.2.32). Not more than 1.5 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.200 g in 50 ml of *alcohol R* and add 5.0 ml of 0.01 M *hydrochloric acid*. Carry out a potentiometric titration (2.2.20), using 0.1 M *sodium hydroxide*. Read the volume added between the 2 points of inflexion.

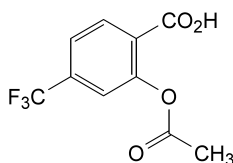
1 ml of 0.1 M *sodium hydroxide* is equivalent to 24.02 mg of $C_{10}H_7F_3O_4$.

STORAGE

Store in an airtight container, protected from light.

TRIFLUSAL

Triflusalum



$C_{10}H_7F_3O_4$

M_r 248.2

DEFINITION

2-(Acetyloxy)-4-(trifluoromethyl)benzoic acid.

Content: 98.5 per cent to 101.5 per cent (dried substance).

CHARACTERS

Appearance: white or almost white, crystalline powder.

Solubility: practically insoluble in water, very soluble in ethanol, freely soluble in methylene chloride.

mp: about 118 °C, with decomposition.

IDENTIFICATION

Infrared absorption spectrophotometry (2.2.24).

Preparation: discs.

Comparison: triflusal CRS.

TESTS

Related substances. Liquid chromatography (2.2.29).

Test solution. Dissolve 0.20 g of the substance to be examined in *acetonitrile R* and dilute to 20.0 ml with the same solvent. *Prepare the solution immediately before use.*

Reference solution (a). Dissolve 5.0 mg of *triflusal impurity B CRS* in *acetonitrile R* and dilute to 10.0 ml with the same solvent.

Reference solution (b). Dilute 1.0 ml of reference solution (a) to 25.0 ml with *acetonitrile R*.

Reference solution (c). Dissolve 2.5 mg of the substance to be examined in *acetonitrile R*, add 5 ml of reference solution (a) and dilute to 10 ml with *acetonitrile R*. *Prepare the solution immediately before use.*

Column:

– size: $l = 0.15$ m, $\varnothing = 4.0$ mm,

– *stationary phase:* octadecylsilyl silica gel for chromatography *R* (4–5 μ m).

Mobile phase:

– *mobile phase A:* acetonitrile *R*,

– *mobile phase B:* 0.5 per cent V/V solution of *phosphoric acid R*,

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)
0 - 20	20 → 70	80 → 30
20 - 25	70	30
25 - 26	70 → 20	30 → 80
26 - 30	20	80

Flow rate: 1.2 ml/min.

Detection: spectrophotometer at 237 nm.

Injection: 10 μ l of the test solution and reference solutions (b) and (c).

Relative retention with reference to triflusal (retention time = about 13 min): *impurity A* = about 0.3; *impurity B* = about 1.2; *impurity C* = about 1.3; *impurity D* = about 1.6.

System suitability: reference solution (c):

– **resolution:** minimum 3.0 between the peaks due to triflusal and *impurity B*.

Limits:

– **impurity B:** not more than 1.5 times the area of the corresponding peak in the chromatogram obtained with reference solution (b) (0.3 per cent),

– **total of impurities other than B:** not more than 0.5 times the area of the peak due to *impurity B* in the chromatogram obtained with reference solution (b) (0.1 per cent),

– **disregard limit:** 0.1 times the area of the peak due to *impurity B* in the chromatogram obtained with reference solution (b) (0.02 per cent).

Heavy metals (2.4.8): maximum 10 ppm.

Dissolve 2.0 g in 12 ml of *alcohol R* and dilute to 20 ml with *water R*. 12 ml complies with limit test B. Prepare the standard using lead standard solution (1 ppm Pb) obtained by diluting *lead standard solution (100 ppm Pb) R* with a mixture of 2 volumes of *water R* and 3 volumes of *alcohol R*.

Loss on drying (2.2.32): maximum 0.5 per cent, determined on 1.000 g by drying in a desiccator *in vacuo* over *diphosphorus pentoxide R*.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g in a platinum crucible.

ASSAY

Dissolve 0.200 g in 50 ml of *ethanol R*. Titrate with 0.1 M *sodium hydroxide*, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *sodium hydroxide* is equivalent to 24.82 mg of $C_{10}H_7F_3O_4$.

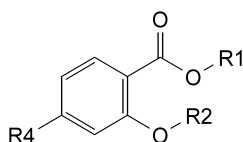
STORAGE

In an airtight container, at a temperature not exceeding 25 °C.

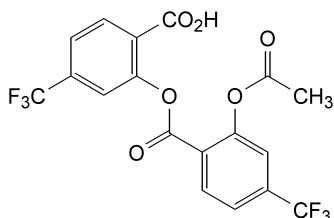
IMPURITIES

Specified impurities: B.

Other detectable impurities: A, C, D.



- A. R1 = H, R2 = CO-CH₃, R4 = CO₂H: 2-(acetyloxy)benzene-1,4-dicarboxylic acid (2-acetoxyterephthalic acid),
- B. R1 = R2 = H, R4 = CF₃: 2-hydroxy-4-(trifluoromethyl)benzoic acid (4-(trifluoromethyl)salicylic acid),
- C. R1 = R2 = CO-CH₃, R4 = CF₃: acetic 2-(acetyloxy)-4-(trifluoromethyl)benzoic anhydride,



- D. 2-[[2-(acetyloxy)-4-(trifluoromethyl)benzoyl]oxy]-4-(trifluoromethyl)benzoic acid.

01/2005:0868

TRIGLYCERIDES, MEDIUM-CHAIN

Triglycerida saturata media

DEFINITION

Mixture of triglycerides of saturated fatty acids, mainly of caprylic acid (octanoic acid, C₈H₁₆O₂) and of capric acid (decanoic acid, C₁₀H₂₀O₂). Medium-chain triglycerides are obtained from the oil extracted from the hard, dried fraction of the endosperm of *Cocos nucifera* L. or from the dried endosperm of *Elaeis guineensis* Jacq.

Content: minimum 95.0 per cent of saturated fatty acids with 8 and 10 carbon atoms.

CHARACTERS

Appearance: colourless or slightly yellowish, oily liquid.

Solubility: practically insoluble in water, miscible with alcohol, with methylene chloride, with light petroleum and with fatty oils.

IDENTIFICATION

First identification: B, C.

Second identification: A, D.

- A. Heat 3.0 g under a reflux condenser for 30 min with 50 ml of a mixture of equal volumes of *alcohol R* and 2 M *alcoholic potassium hydroxide R*. Reserve 10 ml of the mixture for identification test D. To 40 ml of the mixture add 30 ml of *water R*, evaporate the alcohol and acidify the hot solution with 25 ml of *dilute hydrochloric acid R*. After cooling, shake with 50 ml of *peroxide-free ether R*. Wash the ether layer with 3 quantities, each of 10 ml, of *sodium chloride solution R*, dry over *anhydrous sodium sulphate R* and filter. Evaporate the ether and determine the acid value (2.5.1) of the residue, using 0.300 g. The acid value is 350 to 390.
- B. It complies with the test for saponification value (see Tests).
- C. It complies with the test for composition of fatty acids (see Tests).

- D. Evaporate 10 ml of the alcoholic mixture obtained in identification test A to dryness on a water-bath. Transfer the residue into a test-tube, add 0.3 ml of *sulphuric acid R* and close the test-tube with a stopper through which a U-shaped glass tube is inserted. One end of the U-tube is dipped into 3 ml of a 10 g/l solution of *tryptophan R* in a mixture of equal volumes of *sulphuric acid R* and *water R*. Heat the test-tube in a silicone-oil bath at 180 °C for 10 min and collect the liberated fumes in the tryptophan reagent. Heat the tryptophan reagent on a water-bath for 1 min. A violet colour develops.

TESTS

Appearance. The substance to be examined is clear (2.2.1) and not more intensely coloured than reference solution Y₃ (2.2.2, *Method I*).

Alkaline impurities. Dissolve 2.00 g in a mixture of 1.5 ml of *alcohol R* and 3.0 ml of *ether R*. Add 0.05 ml of *bromophenol blue solution R*. Not more than 0.15 ml of 0.01 M *hydrochloric acid* is required to change the colour of the indicator to yellow.

Relative density (2.2.5): 0.93 to 0.96.

Refractive index (2.2.6): 1.440 to 1.452.

Viscosity (2.2.9): 25 mPas to 33 mPas.

Acid value (2.5.1): maximum 0.2.

Hydroxyl value (2.5.3, *Method A*): maximum 10.

Iodine value (2.5.4): maximum 1.0.

Peroxide value (2.5.5, *Method A*): maximum 1.0.

Saponification value (2.5.6): 310 to 360.

Unsaponifiable matter (2.5.7): maximum 0.5 per cent, determined on 5.0 g.

Composition of fatty acids. Gas chromatography (2.4.22, *Method C*).

Column:

- **material:** fused silica,
- **size:** *l* = 30 m, Ø = 0.32 mm,
- **stationary phase:** *macrogol 20 000 R* (film thickness 0.5 µm),

Carrier gas: *helium for chromatography R*.

Flow rate: 1.3 ml/min.

Temperature:

	Time (min)	Temperature (°C)
Column	0 - 1	70
	1 - 35	70 → 240
	35 - 50	240
Injection port		250
Detector		250

Detection: flame ionisation.

Split ratio: 1:100.

Composition of the fatty acid fraction of the substance:

- **caproic acid:** maximum 2.0 per cent,
- **caprylic acid:** 50.0 per cent to 80.0 per cent,
- **capric acid:** 20.0 per cent to 50.0 per cent,
- **lauric acid:** maximum 3.0 per cent,
- **myristic acid:** maximum 1.0 per cent.

Chromium: maximum 0.05 ppm, if intended for use in parenteral nutrition.

Atomic absorption spectrometry (2.2.23, *Method II*).

Test solution. Dissolve 2.0 g of the substance to be examined in *methyl isobutyl ketone R3* and dilute to 10.0 ml with the same solvent.

Solution A. Dilute 0.100 ml of *chromium liposoluble standard solution (1000 ppm Cr) R* to 10.0 ml with *methyl isobutyl ketone R3*.

Stock solution. Dilute 0.100 ml of solution A to 10.0 ml with *methyl isobutyl ketone R3*.

Reference solutions. Prepare 3 reference solutions by dissolving for each 2.0 g of the substance to be examined in the minimum volume of *methyl isobutyl ketone R3*, adding 0.5 ml, 1.0 ml and 2.0 ml, respectively, of stock solution and diluting to 10.0 ml with *methyl isobutyl ketone R3*.

Source: chromium hollow-cathode lamp.

Wavelength: 357.8 nm.

Atomic generator: graphite furnace.

Carrier gas: argon R.

Copper: maximum 0.1 ppm, if intended for use in parenteral nutrition.

Atomic absorption spectrometry (2.2.23, Method II).

Test solution. Dissolve 2.0 g of the substance to be examined in *methyl isobutyl ketone R3* and dilute to 10.0 ml with the same solvent.

Solution A. Dilute 0.100 ml of *copper liposoluble standard solution (1000 ppm Cu) R* to 10.0 ml with *methyl isobutyl ketone R3*.

Stock solution. Dilute 0.100 ml of solution A to 10.0 ml with *methyl isobutyl ketone R3*.

Reference solutions. Prepare 3 reference solutions by dissolving for each 2.0 g of the substance to be examined in the minimum volume of *methyl isobutyl ketone R3*, adding 1.0 ml, 2.0 ml and 4.0 ml, respectively, of stock solution and diluting to 10.0 ml with *methyl isobutyl ketone R3*.

Source: copper hollow-cathode lamp.

Wavelength: 324.7 nm.

Atomic generator: graphite furnace.

Carrier gas: argon R.

Lead: maximum 0.1 ppm, if intended for use in parenteral nutrition.

Atomic absorption spectrometry (2.2.23, Method II).

Test solution. Dissolve 2.0 g of the substance to be examined in *methyl isobutyl ketone R3* and dilute to 10.0 ml with the same solvent.

Solution A. Dilute 0.100 ml of *lead liposoluble standard solution (1000 ppm Pb) R* to 10.0 ml with *methyl isobutyl ketone R3*.

Stock solution. Dilute 0.100 ml of solution A to 10.0 ml with *methyl isobutyl ketone R3*.

Reference solutions. Prepare 3 reference solutions by dissolving for each 2.0 g of the substance to be examined in the minimum volume of *methyl isobutyl ketone R3*, adding 1.0 ml, 2.0 ml and 4.0 ml, respectively, of stock solution and diluting to 10.0 ml with *methyl isobutyl ketone R3*.

Source: lead hollow-cathode lamp.

Wavelength: 283.3 nm.

Atomic generator: graphite furnace coated inside with palladium carbide; calcination is carried out in the presence of oxygen at a temperature below 800 °C.

Carrier gas: argon R.

Nickel: maximum 0.2 ppm, if intended for use in parenteral nutrition.

Atomic absorption spectrometry (2.2.23, Method II).

Test solution. Dissolve 2.0 g of the substance to be examined in *methyl isobutyl ketone R3* and dilute to 10.0 ml with the same solvent.

Solution A. Dilute 0.100 ml of *nickel liposoluble standard solution (1000 ppm Ni) R* to 10.0 ml with *methyl isobutyl ketone R3*.

Stock solution. Dilute 0.100 ml of solution A to 10.0 ml with *methyl isobutyl ketone R3*.

Reference solutions. Prepare 3 reference solutions by dissolving for each 2.0 g of the substance to be examined in the minimum volume of *methyl isobutyl ketone R3*, adding 1.0 ml, 2.0 ml and 4.0 ml, respectively, of stock solution and diluting to 10.0 ml with *methyl isobutyl ketone R3*.

Source: nickel hollow-cathode lamp.

Wavelength: 232 nm.

Atomic generator: graphite furnace.

Carrier gas: argon R.

Tin: maximum 0.1 ppm, if intended for use in parenteral nutrition.

Atomic absorption spectrometry (2.2.23, Method II).

Test solution. Dissolve 2.0 g of the substance to be examined in *methyl isobutyl ketone R3* and dilute to 10.0 ml with the same solvent.

Solution A. Dilute 0.100 ml of *tin liposoluble standard solution (1000 ppm Sn) R* to 10.0 ml with *methyl isobutyl ketone R3*.

Stock solution. Dilute 0.100 ml of solution A to 10.0 ml with *methyl isobutyl ketone R3*.

Reference solutions. Prepare 3 reference solutions by dissolving for each 2.0 g of the substance to be examined in the minimum volume of *methyl isobutyl ketone R3*, adding 1.0 ml, 2.0 ml and 4.0 ml, respectively, of stock solution and diluting to 10.0 ml with *methyl isobutyl ketone R3*.

Source: tin hollow-cathode lamp.

Wavelength: 286.3 nm.

Atomic generator: graphite furnace coated inside with palladium carbide.

Carrier gas: argon R.

Heavy metals (2.4.8): maximum 10 ppm, if intended for use other than parenteral nutrition.

2.0 g complies with limit test D. Prepare the standard using 2 ml of *lead standard solution (10 ppm Pb) R*.

Water (2.5.12): maximum 0.2 per cent, determined on 10.00 g.

Total ash (2.4.16): maximum 0.1 per cent, determined on 2.0 g.

STORAGE

In a well-filled container, protected from light.

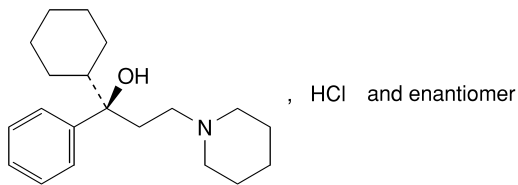
LABELLING

The label states, where applicable, that the substance is intended for use in parenteral nutrition.

01/2005:1626

TRIHEXYPHENIDYL HYDROCHLORIDE

Trihexyphenidyli hydrochloridum

C₂₀H₃₂ClNOM_r 337.9

DEFINITION

(1*RS*)-1-Cyclohexyl-1-phenyl-3-(piperidin-1-yl)propan-1-ol hydrochloride.

Content: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

Appearance: white, crystalline powder.

Solubility: slightly soluble in water, sparingly soluble in alcohol and in methylene chloride.

mp: about 250 °C, with decomposition.

IDENTIFICATION

First identification: A, D.

Second identification: B, C, D.

A. Infrared absorption spectrophotometry (2.2.24).

Comparison: trihexyphenidyl hydrochloride CRS.

B. Thin-layer chromatography (2.2.27).

Test solution. Dissolve 25 mg of the substance to be examined in a mixture of 20 volumes of *methanol R* and 80 volumes of *methylene chloride R* and dilute to 10 ml with the same mixture of solvents.

Reference solution. Dissolve 25 mg of *trihexyphenidyl hydrochloride CRS* in a mixture of 20 volumes of *methanol R* and 80 volumes of *methylene chloride R* and dilute to 10 ml with the same mixture of solvents.

Plate: TLC silica gel G plate R.

Mobile phase: diethylamine R, hexane R (5:95 V/V).

Application: 5 µl.

Development: over 2/3 of the plate.

Drying: in air.

Detection: spray with a 0.1 g/l solution of *chloroplatinic acid R* in *hydrochloric acid R* containing 0.4 per cent V/V of *hydriodic acid R*.

Results: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

C. Dissolve 0.5 g in 5 ml of warm *methanol R* and make just alkaline to *red litmus paper R* with *sodium hydroxide solution R*. A precipitate is formed which, after recrystallisation from *methanol R*, melts (2.2.14) at about 113 °C to 115 °C.

D. It gives reaction (a) of chlorides (2.3.1).

TESTS

pH (2.2.3): 5.2 to 6.2.

Dissolve 0.5 g with heating in 25 ml of *carbon dioxide-free water R*. Cool to room temperature and dilute to 50 ml with *carbon dioxide-free water R*.

Optical rotation (2.2.7): −0.10° to +0.10°.

Dissolve 1.25 g in a mixture of 20 volumes of *methanol R* and 80 volumes of *methylene chloride R* and dilute to 25.0 ml with the same mixture of solvents.

Related substances. Liquid chromatography (2.2.29).

Test solution. Dissolve 20.0 mg of the substance to be examined in the mobile phase and dilute to 10.0 ml with the mobile phase.

Reference solution (a). Dilute 1.0 ml of the test solution to 200.0 ml with the mobile phase. Dilute 10.0 ml to 50.0 ml with the mobile phase.

Reference solution (b). Dissolve 10.0 mg of *trihexyphenidyl impurity A CRS* in the mobile phase and dilute to 10.0 ml with the mobile phase.

Reference solution (c). Dilute 1.0 ml of reference solution (b) to 100.0 ml with the mobile phase.

Reference solution (d). To 1 ml of reference solution (b), add 1 ml of the test solution and dilute to 100 ml with the mobile phase.

Column:

- size: *l* = 0.15 m, Ø = 4.6 mm,
- stationary phase: octadecylsilyl silica gel for chromatography R (5 µm).

Mobile phase: mix 200 ml of *water R* with 0.2 ml of *triethylamine R*. Adjust to pH 4.0 with *phosphoric acid R* and add 800 ml of *acetonitrile R*.

Flow rate: 1.0 ml/min.

Detection: spectrophotometer at 210 nm.

Injection: 20 µl.

Run time: 3 times the retention time of trihexyphenidyl.

System suitability: reference solution (d):

- resolution: minimum 4.0 between the peaks corresponding to trihexyphenidyl and to impurity A.

Limits:

- impurity A: not more than the area of the principal peak in the chromatogram obtained with reference solution (c) (0.5 per cent),
- any other impurity: not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent),
- total: not more than 0.5 per cent,
- disregard limit: 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.02 per cent).

Loss on drying (2.2.32): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 100–105 °C.

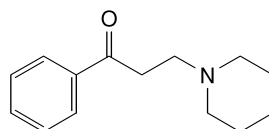
Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.250 g in 50 ml of *alcohol R* and add 5.0 ml of 0.01 M *hydrochloric acid*. Carry out a potentiometric titration (2.2.20), using 0.1 M *sodium hydroxide*. Read the volume added between the 2 points of inflexion.

1 ml of 0.1 M *sodium hydroxide* is equivalent to 33.79 mg of C₂₀H₃₂ClNO.

IMPURITIES

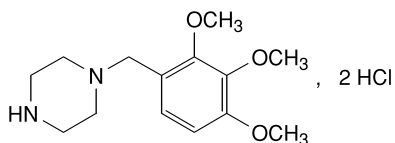


A. 1-phenyl-3-(piperidin-1-yl)propan-1-one.

01/2005:1741 Flow rate: 1.2 ml/min.

TRIMETAZIDINE DIHYDROCHLORIDE

Trimetazidini dihydrochloridum

 $C_{14}H_{24}Cl_2N_2O_3$ M_r 339.3**DEFINITION**

1-(2,3,4-Trimethoxybenzyl)piperazine dihydrochloride.

Content: 98.5 per cent to 101.5 per cent (dried substance).**CHARACTERS***Appearance*: white or almost white, crystalline powder, slightly hygroscopic.*Solubility*: freely soluble in water, sparingly soluble in alcohol.**IDENTIFICATION**

A. Infrared absorption spectrophotometry (2.2.24).

Comparison: Ph. Eur. reference spectrum of trimetazidine dihydrochloride.B. Dissolve 25 mg in 5 ml of *water R*. 2 ml of the solution gives reaction (a) of chlorides (2.3.1).**TESTS****Appearance of solution.** The solution is clear (2.2.1) and not more intensely coloured than reference solution BY₆ (2.2.2, *Method II*).Dissolve 1.0 g in *water R* and dilute to 10 ml with the same solvent.**Related substances.** Liquid chromatography (2.2.29).*Test solution.* Dissolve 0.200 g of the substance to be examined in *water R* and dilute to 50.0 ml with the same solvent.*Reference solution (a).* Dissolve 20.0 mg of trimetazidine for system suitability CRS in *water R* and dilute to 5.0 ml with the same solvent.*Reference solution (b).* Dilute 2.0 ml of the test solution to 100.0 ml with *water R*. Dilute 5.0 ml of this solution to 100.0 ml with *water R*.*Reference solution (c).* Dilute 25.0 ml of reference solution (b) to 50.0 ml with *water R*.*Column*:

- *size*: $l = 0.15$ m, $\varnothing = 4.6$ mm,
- *stationary phase*: spherical octadecylsilyl silica gel for chromatography *R* (5 μ m) with a pore size of 10 nm,
- *temperature*: 30 °C.

Mobile phase:

- *mobile phase A*: mix 357 volumes of *methanol R* and 643 volumes of a 2.87 g/l solution of sodium heptanesulphonate *R* adjusted to pH 3.0 with dilute phosphoric acid *R*,
- *mobile phase B*: *methanol R*,

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)
0 - 50	95 → 75	5 → 25
50 - 52	75 → 95	25 → 5

Detection: spectrophotometer at 240 nm.*Equilibration*: for at least 1 h with the mobile phase at the initial composition.*Injection*: 10 μ l.

Relative retention with reference to trimetazidine (retention time = about 25 min): impurity D = about 0.2; impurity C = about 0.4; impurity H = about 0.6; impurities A and I = about 0.9; impurity E = about 0.95; impurity F = about 1.4; impurity B = about 1.8.

System suitability:

- *peak-to-valley ratio*: minimum 3, where H_p = height above the baseline of the peak due to impurity E and H_v = height above the baseline of the lowest point of the curve separating this peak from the principal peak in the chromatogram obtained with reference solution (a);
- *signal-to-noise ratio*: minimum 10 for the principal peak in the chromatogram obtained with reference solution (c).

Limits:

- *correction factors*: for the calculation of contents, multiply the peak areas of the following impurities by the corresponding correction factor: impurity B = 0.55; impurity C = 0.37; impurity F = 0.71;
- *impurities A, B, C, D, E, F, H, I*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent);
- *any other impurity*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent);
- *total*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent);
- *disregard limit*: area of the principal peak in the chromatogram obtained with reference solution (c) (0.05 per cent).

Impurity G. Thin-layer chromatography (2.2.27).*Test solution.* Dissolve 0.10 g of the substance to be examined in *methanol R* and dilute to 10 ml with the same solvent.*Reference solution.* Dissolve 22.6 mg of piperazine hydrate *R* in *methanol R* and dilute to 100 ml with the same solvent. Dilute 10 ml of the solution to 100 ml with *methanol R*.*Plate*: TLC silica gel plate *R*.*Mobile phase*: concentrated ammonia *R*, alcohol *R* (20:80 V/V).*Application*: 10 μ l.*Development*: over 2/3 of the plate.*Drying*: at 100-105 °C for 30 min.*Detection*: spray with iodoplatinate reagent *R*.*Limit*:

- *impurity G*: any spot due to impurity G is not more intense than the spot in the chromatogram obtained with the reference solution (0.1 per cent, expressed as anhydrous piperazine).

Loss on drying (2.2.32): maximum 2.5 per cent, determined on 1.000 g by drying in an oven at 100-105 °C over diphosphorus pentoxide *R* at a pressure not exceeding 15 kPa.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.120 g in 50.0 ml of *water R*. Add 1 ml of *nitric acid R* and titrate with 0.1 M *silver nitrate*, determining the end-point potentiometrically (2.2.20).

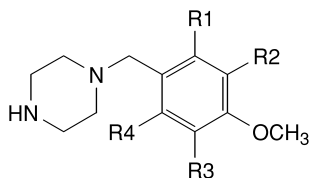
1 ml of 0.1 M *silver nitrate* is equivalent to 16.96 mg of $C_{14}H_{24}Cl_2N_2O_3$.

STORAGE

In an airtight container.

IMPURITIES

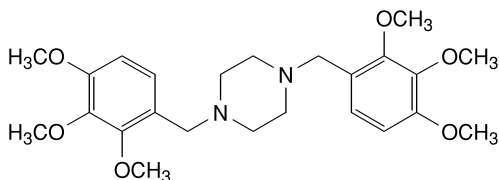
Specified impurities: A, B, C, D, E, F, G, H, I.



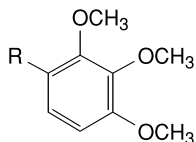
A. $R_1 = R_4 = H$, $R_2 = R_3 = OCH_3$: 1-(3,4,5-trimethoxybenzyl)piperazine,

E. $R_1 = R_3 = OCH_3$, $R_2 = R_4 = H$: 1-(2,4,5-trimethoxybenzyl)piperazine,

F. $R_1 = R_4 = OCH_3$, $R_2 = R_3 = H$: 1-(2,4,6-trimethoxybenzyl)piperazine,



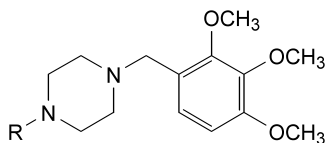
B. 1,4-bis(2,3,4-trimethoxybenzyl)piperazine,



C. $R = CHO$: 2,3,4-trimethoxybenzaldehyde,

D. $R = CH_2OH$: (2,3,4-trimethoxyphenyl)methanol,

G. piperazine,



H. $R = COOC_2H_5$: ethyl 4-(2,3,4-trimethoxybenzyl)piperazine-1-carboxylate,

I. $R = CH_3$: 1-methyl-4-(2,3,4-trimethoxybenzyl)piperazine (*N*-methyltrimetazidine).

DEFINITION

Trimethadione contains not less than 98.0 per cent and not more than the equivalent of 102.0 per cent of 3,5,5-trimethyloxazolidine-2,4-dione, calculated with reference to the dried substance.

CHARACTERS

Colourless or almost colourless crystals, soluble in water, very soluble in alcohol.

IDENTIFICATION

First identification: B.

Second identification: A, C, D.

A. Melting point (2.2.14): 45 °C to 47 °C, determined without previous drying.

B. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *trimethadione CRS*. Examine the substances prepared as discs using 3 mg of the substance to be examined and of the reference substance, respectively, in 0.4 g of *potassium bromide R*.

C. To 2 ml of solution S (see Tests) add 1 ml of *barium hydroxide solution R*. A white precipitate is formed, which dissolves on addition of 1 ml of *dilute hydrochloric acid R*.

D. Dissolve 0.3 g in a mixture of 5 ml of *alcoholic potassium hydroxide solution R* and 5 ml of *alcohol R* and allow to stand for 10 min. Add 0.05 ml of *phenolphthalein solution R1* and neutralise carefully with *hydrochloric acid R*. Evaporate to dryness on a water-bath and shake the residue with four quantities, each of 5 ml, of *ether R*. Filter the combined ether layers and evaporate to dryness. The residue, recrystallised from 5 ml of *toluene R* and dried, melts (2.2.14) at about 80 °C.

TESTS

Solution S. Dissolve 2.0 g in *carbon dioxide-free water R* and dilute to 40 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and colourless (2.2.2, *Method II*).

Acidity or alkalinity. To 10 ml of solution S add 0.1 ml of *methyl red solution R*. Not more than 0.1 ml of 0.01 M *hydrochloric acid* or 0.01 M *sodium hydroxide* is required to change the colour of the indicator.

Heavy metals (2.4.8). 12 ml of solution S complies with limit test A for heavy metals (20 ppm). Prepare the standard using *lead standard solution* (1 ppm Pb) *R*.

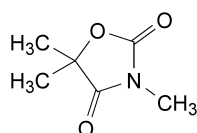
Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.00 g by drying in a desiccator over *anhydrous silica gel R* for 6 h.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

01/2005:0440

TRIMETHADIONE

Trimethadionum



$C_6H_9NO_3$

M_r 143.1

ASSAY

Examine by gas chromatography (2.2.28), using *decanol R* as the internal standard.

Internal standard solution. Dissolve 0.125 g of *decanol R* in *ethanol R* and dilute to 25 ml with the same solvent.

Test solution. Dissolve 0.100 g of the substance to be examined in the internal standard solution and dilute to 10.0 ml with the same solution.

Reference solution. Dissolve 0.100 g of *trimethadione CRS* in the internal standard solution and dilute to 10.0 ml with the same solution.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.75 m long and 3 mm in internal diameter packed with *styrene-divinylbenzene copolymer R* (125 µm to 150 µm),
- *nitrogen for chromatography R* as the carrier gas at a flow rate of 20 ml/min,
- a flame-ionisation detector.

Maintain the temperature of the column at 210 °C, that of the injection port at 240 °C, and that of the detector at 270 °C. Inject 1 µl of each solution.

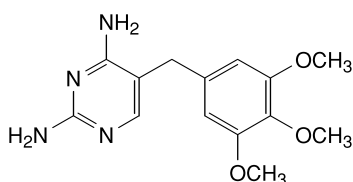
STORAGE

Store protected from light.

01/2005:0060

TRIMETHOPRIM

Trimethoprimum



C₁₄H₁₈N₄O₃

M_r 290.3

DEFINITION

5-(3,4,5-Trimethoxybenzyl)pyrimidine-2,4-diamine.

Content: 98.5 per cent to 101.0 per cent (dried substance).

CHARACTERS

Appearance: white or yellowish-white powder.

Solubility: very slightly soluble in water, slightly soluble in alcohol.

IDENTIFICATION

First identification: C.

Second identification: A, B, D.

- A. Melting point (2.2.14): 199 °C to 203 °C.
- B. Dissolve about 20 mg in 0.1 M sodium hydroxide and dilute to 100.0 ml with the same solvent. Dilute 1.0 ml of this solution to 10.0 ml with 0.1 M sodium hydroxide. Examined between 230 nm and 350 nm (2.2.25), the solution shows an absorption maximum at 287 nm. The specific absorbance at the maximum is 240 to 250.
- C. Infrared absorption spectrophotometry (2.2.24).
Preparation: discs.
Comparison: trimethoprim CRS.
- D. Dissolve about 25 mg, heating if necessary, in 5 ml of 0.005 M sulphuric acid and add 2 ml of a 16 g/l solution of potassium permanganate R in 0.1 M sodium hydroxide. Heat to boiling and add to the hot solution 0.4 ml of formaldehyde R. Mix, add 1 ml of 0.5 M sulphuric acid, mix and heat again to boiling. Cool and filter. To the filtrate add 2 ml of methylene chloride R and shake vigorously. The organic layer, examined in ultraviolet light at 365 nm, shows green fluorescence.

TESTS

Appearance of solution. The solution is not more intensely coloured than reference solution BY₇ (2.2.2, Method II).

Dissolve 0.5 g in 10 ml of a mixture of 1 volume of water R, 4.5 volumes of methanol R and 5 volumes of methylene chloride R.

Related substances

A. Liquid chromatography (2.2.29).

Test solution. Dissolve 25.0 mg of the substance to be examined in the mobile phase and dilute to 25.0 ml with the mobile phase.

Reference solution (a). Dilute 1.0 ml of the test solution to 200.0 ml with the mobile phase.

Reference solution (b). Dissolve 5.0 mg of trimethoprim CRS and 2.5 mg of trimethoprim impurity E CRS in the mobile phase and dilute to 100.0 ml with the mobile phase. Dilute 1.0 ml of the solution to 10.0 ml with the mobile phase.

Column:

- *size*: *l* = 0.250 m, Ø = 4.0 mm,
- *stationary phase*: base-deactivated octadecylsilyl silica gel for chromatography R (5 µm).

Mobile phase: mix 30 volumes of methanol R and 70 volumes of a 1.4 g/l solution of sodium perchlorate R adjusted to pH 3.6 with phosphoric acid R.

Flow rate: 1.3 ml/min.

Detection: spectrophotometer at 280 nm.

Injection: 20 µl loop injector.

Run time: 11 times the retention time of trimethoprim.

Relative retentions with reference to trimethoprim (retention time = about 5 min): impurity C = about 0.8; impurity E = about 0.9; impurity A = about 1.5; impurity D = about 2.0; impurity G = about 2.1; impurity B = about 2.3; impurity J = about 2.7; impurity F = about 4.0.

System suitability: reference solution (b):

- *resolution*: minimum of 2.5 between the peaks.

Limits:

- *correction factors*: for the calculation of contents, multiply the peak areas of the following impurities by the corresponding correction factor: impurity B = 0.43; impurity E = 0.53; impurity J = 0.66;
- *any impurity*: not more than 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent);
- *total*: not more than 0.4 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent);
- *disregard limit*: 0.04 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.02 per cent); disregard any peak corresponding to impurity H (relative retentions = about 10.3).

B. Liquid chromatography (2.2.29).

Test solution. Dissolve 25.0 mg of the substance to be examined in the mobile phase and dilute to 25.0 ml with the mobile phase.

Reference solution (a). Dilute 1.0 ml of the test solution to 200.0 ml with the mobile phase.

Reference solution (b). Dissolve 5.0 mg of trimethoprim CRS and 5.0 mg of trimethoprim impurity B CRS in the mobile phase and dilute to 100.0 ml with the mobile phase.

Column:

- *size*: *l* = 0.250 m, Ø = 4.6 mm,
- *stationary phase*: nitrile silica gel for chromatography R (5 µm) with a specific surface area of 350 m²/g and a pore diameter of 10 nm.

Mobile phase: dissolve 1.14 g of *sodium hexane sulphonate R* in 600 ml of a 13.6 g/l solution of *potassium dihydrogen phosphate R*; adjust to pH 3.1 using *phosphoric acid R* and mix with 400 volumes of *methanol R*.

Flow rate: 0.8 ml/min.

Detection: spectrophotometer at 280 nm.

Injection: 20 µl loop injector.

Run time: 6 times the retention time of trimethoprim.

Relative retentions with reference to trimethoprim (retention time = about 4 min): impurity H = about 1.8; impurity I = about 4.9.

System suitability: reference solution (b):

- **resolution:** minimum of 2.0 between the peaks.

Limits:

- **correction factors:** for the calculation of contents, multiply the peak areas of the following impurities by the corresponding correction factor: impurity H = 0.50; impurity I = 0.28;
- **any impurity:** not more than 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent);
- **total:** not more than 0.4 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent);
- **disregard limit:** 0.04 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.02 per cent); disregard any peak corresponding to impurity B (relative retention = about 1.3).

Impurity K. Gas chromatography (2.2.28).

Test solution. Dissolve 0.500 g of the substance to be examined in 35.0 ml of *citrate buffer solution pH 5.0 R*, add 10.0 ml of *1,1-dimethylethyl methyl ether R*, shake thoroughly and centrifuge for 10 min. Use the upper layer.

Reference solution. Dilute 5.0 ml of *hydrochloric acid R* to 50.0 ml with *water R*. Add 12.5 mg of *aniline R* and shake thoroughly (solution A). To 35.0 ml of *citrate buffer solution pH 5.0 R* add 10.0 µl of solution A and 10.0 ml of *1,1-dimethylethyl methyl ether R*, shake thoroughly and centrifuge for 10 min. Use the upper layer.

Column:

- **material:** fused silica,
- **size:** $l = 30$ m, $\varnothing = 0.53$ mm,
- **stationary phase:** *poly(dimethyl)siloxane R* (film thickness 3 µm).

Carrier gas: *helium for chromatography R*.

Flow rate: 12 ml/min.

Temperature:

- **column:** 80 °C,
- **injection port:** 230 °C,
- **detector:** 270 °C.

Detection: nitrogen-phosphorus detector.

Injection: 3 µl.

Run time: 15 min.

System suitability: reference solution:

- **repeatability:** maximum relative standard deviation of 5.0 per cent after 6 injections.

Limit:

- **impurity K:** not more than the area of the corresponding peak in the chromatogram obtained with the reference solution (5 ppm).

Heavy metals (2.4.8): maximum 20 ppm.

1.0 g complies with limit test C. Prepare the standard using 2 ml of *lead standard solution (10 ppm Pb) R*

Loss on drying (2.2.32): maximum 1.0 per cent, determined on 1.000 g by drying in an oven at 100–105 °C.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.250 g in 50 ml of *anhydrous acetic acid R*. Titrate with 0.1 M *perchloric acid*, determining the end-point potentiometrically (2.2.20).

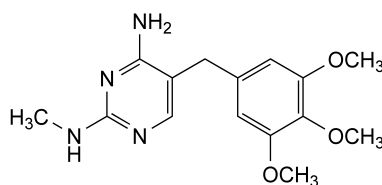
1 ml of 0.1 M *perchloric acid* is equivalent to 29.03 mg of $C_{14}H_{18}N_4O_3$.

IMPURITIES

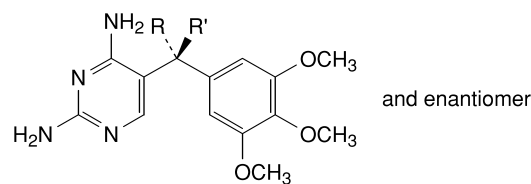
By liquid chromatography A: A, B, C, D, E, F, G, H, I.

By liquid chromatography B: B, H, I.

By gas chromatography: K.



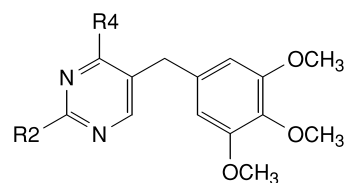
A. *N*²-methyl-5-(3,4,5-trimethoxybenzyl)pyrimidine-2,4-diamine,



and enantiomer

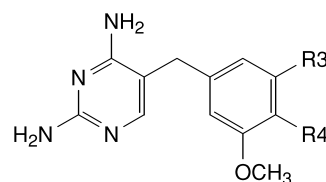
B. $R + R' = O$: (2,4-diaminopyrimidin-5-yl)(3,4,5-trimethoxyphenyl)methanone,

C. $R = OH$, $R' = H$: (*RS*)-(2,4-diaminopyrimidin-5-yl)(3,4,5-trimethoxyphenyl)methanol,



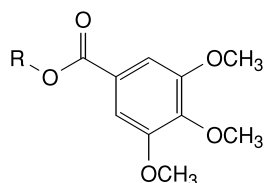
D. $R_2 = NH_2$, $R_4 = OH$: 2-amino-5-(3,4,5-trimethoxybenzyl)pyrimidin-4-ol,

E. $R_2 = OH$, $R_4 = NH_2$: 4-amino-5-(3,4,5-trimethoxybenzyl)pyrimidin-2-ol,



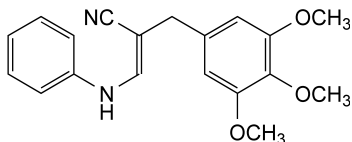
F. $R_3 = Br$, $R_4 = OCH_3$: 5-(3-bromo-4,5-dimethoxybenzyl)pyrimidine-2,4-diamine,

G. $R_3 = OCH_3$, $R_4 = OC_2H_5$: 5-(4-ethoxy-3,5-dimethoxybenzyl)pyrimidine-2,4-diamine,

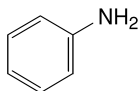


H. R = CH₃: methyl 3,4,5-trimethoxybenzoate,

J. R = H: 3,4,5-trimethoxybenzoic acid,



I. 3-(phenylamino)-2-(3,4,5-trimethoxybenzyl)prop-2-enitrile,

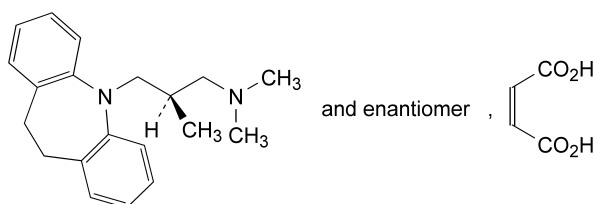


K. aniline.

01/2005:0534

TRIMIPRAMINE MALEATE

Trimipramini maleas



and enantiomer ,

C₂₄H₃₀N₂O₄

M_r 410.5

DEFINITION

Trimipramine maleate contains not less than 98.0 per cent and not more than the equivalent of 101.0 per cent of (2*RS*)-3-(10,11-dihydro-5*H*-dibenzo[*b,f*]azepin-5-yl)-*N,N*,2-trimethylpropan-1-amine (*Z*)-but-2-enedioate, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, crystalline powder, slightly soluble in water and in alcohol.

IDENTIFICATION

First identification: A, C.

Second identification: A, B, D, E.

A. Melting point (2.2.14): 140 °C to 144 °C.

B. Dissolve 40.0 mg in 0.01 *M* hydrochloric acid and dilute to 100.0 ml with the same acid. Dilute 5.0 ml of the solution to 100.0 ml with 0.01 *M* hydrochloric acid. Examined between 230 nm and 350 nm (2.2.25), the solution shows an absorption maximum at 250 nm and a shoulder at 270 nm. The specific absorbance at the maximum is 205 to 235.

C. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with trimipramine maleate CRS.

D. Examine the chromatograms obtained in the test for related substances. The principal spot in the chromatogram obtained with test solution (b) is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).

E. Examine by thin-layer chromatography (2.2.27), using silica gel GF₂₅₄ R as the coating substance.

Test solution. Dissolve 0.20 g of the substance to be examined in methanol R and dilute to 10 ml with the same solvent.

Reference solution. Dissolve 56 mg of maleic acid R in methanol R and dilute to 10 ml with the same solvent.

Apply to the plate as 10 mm bands 5 µl of each solution. Develop over a path of 12 cm using a mixture of 3 volumes of water R, 7 volumes of anhydrous formic acid R and 90 volumes of di-isopropyl ether R. Dry the plate in a current of air for a few minutes and then at 120 °C for 10 min and examine in ultraviolet light at 254 nm. The chromatogram obtained with the test solution shows a zone on the starting line and another zone which is similar in position and size to the principal zone in the chromatogram obtained with the reference solution.

TESTS

Appearance of solution. Dissolve 2.5 g in chloroform R and dilute to 25 ml with the same solvent. The solution is not more intensely coloured than reference solution BY₅ (2.2.2, Method II).

Related substances. Examine by thin-layer chromatography (2.2.27), using silica gel G R as the coating substance.

Test solution (a). Dissolve 0.50 g of the substance to be examined in methanol R and dilute to 10 ml with the same solvent. Prepare immediately before use.

Test solution (b). Dilute 1 ml of test solution (a) to 20 ml with methanol R.

Reference solution (a). Dissolve 25 mg of trimipramine maleate CRS in methanol R and dilute to 10 ml with the same solvent. Prepare immediately before use.

Reference solution (b). Dilute 1 ml of reference solution (a) to 10 ml with methanol R.

Reference solution (c). Dilute 1 ml of reference solution (a) to 25 ml with methanol R.

Reference solution (d). Dissolve 10 mg of iminodibenzyl R in methanol R and dilute to 100 ml with the same solvent. Prepare immediately before use.

Apply separately to the plate 5 µl of each solution. Develop over a path of 15 cm using a mixture of 0.7 volumes of concentrated ammonia R, 10 volumes of ethanol R and 90 volumes of toluene R. Allow the plate to dry in air for 15 min, spray with a 5 g/l solution of potassium dichromate R in a mixture of 1 volume of sulphuric acid R and 4 volumes of water R and examine immediately. In the chromatogram obtained with test solution (a): any spot corresponding to iminodibenzyl is not more intense than the spot in the chromatogram obtained with reference solution (d) (0.2 per cent); any spot, apart from the principal spot and the spot corresponding to iminodibenzyl, is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.5 per cent) and at most three such spots are more intense than the spot in the chromatogram obtained with reference solution (c) (0.2 per cent). Disregard any spot at the starting point.

Heavy metals (2.4.8). 2.0 g complies with limit test C for heavy metals (20 ppm). Prepare the standard using 4 ml of lead standard solution (10 ppm Pb) R.

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.3500 g in 50 ml of *anhydrous acetic acid R*. Titrate with 0.1 M *perchloric acid* determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *perchloric acid* is equivalent to 41.05 mg of C₂₄H₃₀N₂O₄.

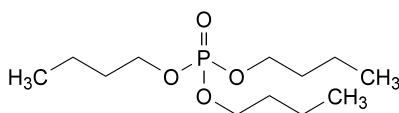
STORAGE

Store protected from light.

01/2005:1682

TRI-*n*-BUTYL PHOSPHATE

Tri-*n*-butylis phosphas



C₁₂H₂₇O₄P

M_r 266.3

CHARACTERS

Appearance: clear, colourless to pale yellow liquid.

Solubility: slightly soluble in water, miscible with alcohol.

bp: about 289 °C, with decomposition.

IDENTIFICATION

Infrared absorption spectrophotometry (2.2.24).

Comparison: *tri-n-butyl phosphate CRS*.

TESTS

Appearance. The substance to be examined is clear (2.2.1) and not more intensely coloured than reference solution Y₆ (2.2.2, *Method II*).

Acidity. Dissolve 50 ml in 50 ml of *alcohol R* previously adjusted with 0.02 M *potassium hydroxide* or 0.02 M *hydrochloric acid* to a bluish-green colour, using 0.5 ml of *bromothymol blue solution R1* as indicator. Titrate with 0.02 M *potassium hydroxide* to (the initial) bluish-green coloration. Not more than 0.8 ml are required.

Related substances. Gas chromatography (2.2.28): use the normalisation procedure.

Test solution. Substance to be examined.

Reference solution. Dissolve 10 mg of the substance to be examined and 10 mg of *methyl myristate R* in *methylene chloride R* and dilute to 10 ml with the same solvent.

Column:

- **material:** fused silica,
- **size:** *l* = 30 m, Ø = 0.32 mm,
- **stationary phase:** *poly(dimethyl)siloxane R* (5 µm).

Carrier gas: *helium for chromatography R*.

Linear velocity: 32 cm/s.

Split-ratio: 65:1.

Temperature:

- **column:** 250 °C,
- **injection port and detector:** 250 °C.

Detector: flame ionisation.

Injection: 1 µl.

Run time: twice the retention time of *tri-n-butyl phosphate*.

System suitability: reference solution:

- **resolution:** minimum 10 between the peaks due to *tri-n-butyl phosphate* and *methyl myristate*.

Limits:

- **any impurity:** maximum 0.1 per cent,
- **total:** maximum 0.3 per cent,
- **disregard limit:** 0.01 per cent.

Chlorides (2.4.4): maximum 200 ppm.

Dissolve 0.25 g in 15 ml of *alcohol (70 per cent V/V) R*. The solution complies with the limit test for chlorides. Prepare the reference solution with 10 ml of *chloride standard solution (5 ppm Cl) R* and 5 ml of *ethanol R*.

Heavy metals (2.4.8): maximum 20 ppm.

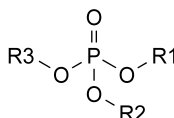
Dissolve 2.0 g in 13 ml of *alcohol R* and dilute to 20.0 ml with *water R*. 12 ml of the solution complies with limit test B. Prepare the standard using *lead standard solution (2 ppm Pb)* prepared by diluting *lead standard solution (100 ppm Pb) R* with a mixture of 5 volumes of *water R* and 13 volumes of *alcohol R*.

Water (2.5.32): maximum 0.1 per cent, determined on 1.0 g.

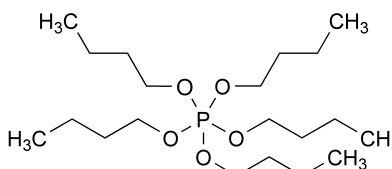
STORAGE

Protected from light.

IMPURITIES



- R1 = R2 = [CH₂]₃-CH₃, R3 = H: dibutyl hydrogen phosphate,
- R1 = [CH₂]₃-CH₃, R2 = R3 = H: butyl dihydrogen phosphate,
- R1 = R2 = [CH₂]₃-CH₃, R3 = CH₃: dibutyl methyl phosphate,
- R1 = R2 = [CH₂]₃-CH₃, R3 = C₂H₅: dibutyl ethyl phosphate,
- R1 = R2 = [CH₂]₃-CH₃, R3 = [CH₂]₂-CH₃: dibutyl propyl phosphate,
- R1 = R2 = [CH₂]₃-CH₃, R3 = CH₂-CH(CH₃)₂: dibutyl 2-methylpropyl phosphate,
- R1 = R2 = [CH₂]₃-CH₃, R3 = [CH₂]₄-CH₃: dibutyl pentyl phosphate,
- H₃C-[CH₂]₃-OH: butan-1-ol,

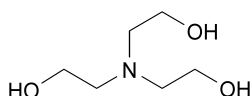


- pentabutyl phosphate.

01/2005:1577 Flow rate: 1 ml/min.

Split ratio: 1:35.

Temperature:

TROLAMINE**Trolaminum** $C_6H_{15}NO_3$ M_r 149.2**DEFINITION**

2,2',2''-Nitrilotriethanol.

Content: 99.0 per cent *m/m* to 103.0 per cent *m/m* of total bases (anhydrous substance).**CHARACTERS***Appearance*: clear, viscous, colourless or slightly yellow liquid, very hygroscopic.*Solubility*: miscible with water and with alcohol, soluble in methylene chloride.**IDENTIFICATION***First identification*: B, C.*Second identification*: A, B, D.

A. Relative density (2.2.5): 1.120 to 1.130.

B. Refractive index (2.2.6): 1.482 to 1.485.

C. Examine the chromatograms obtained in the test for related substances.

Results: the principal peak in the chromatogram obtained with the test solution is similar in retention time and size to the principal peak in the chromatogram obtained with reference solution (a).D. To 1 ml add 0.3 ml of *copper sulphate solution R*. A blue colour develops. Add 2.5 ml of *dilute sodium hydroxide solution R* and heat to boiling. The blue colour remains unchanged.**TESTS****Solution S**. Dissolve 12 g in *water R* and dilute to 20 ml with the same solvent.**Appearance of solution**. Solution S is clear (2.2.1) and not more intensely coloured than reference solution B₆ (2.2.2, *Method II*).**Related substances**. Gas chromatography (2.2.28).*Internal standard solution*. Dissolve 5.0 g of 3-aminopropanol *R* in *water R* and dilute to 100.0 ml with the same solvent.*Test solution*. Dissolve 10.0 g of the substance to be examined in *water R*. Add 1.0 ml of the internal standard solution and dilute to 100.0 ml with *water R*.*Reference solution (a)*. Dissolve 1.0 g of trolamine CRS in *water R* and dilute to 10.0 ml with the same solvent.*Reference solution (b)*. Dissolve 1.0 g of *ethanolamine R*, 5.0 g of *diethanolamine R* and 1.0 g of trolamine CRS in *water R* and dilute to 100.0 ml with the same solvent. To 1.0 ml of the solution add 1.0 ml of the internal standard solution and dilute to 100.0 ml with *water R*.*Column*:

- *material*: fused silica,
- *size*: $l = 25$ m, $\varnothing = 0.25$ mm,
- *stationary phase*: poly(dimethyl)(diphenyl)siloxane *R* (film thickness 0.50 μ m).

Carrier gas: helium for chromatography *R*.

	Time (min)	Temperature (°C)
Column	0	60
	0 - 8.5	60 → 230
	8.5 - 14	230
Injection port		260
Detector		280

Detection: flame ionisation.*Injection*: 2 μ l; if necessary inject a blank.*Elution order*: impurity A, 3-aminopropanol, impurity B, trolamine.*System suitability*: reference solution (b):

- *resolution*: minimum 2 between the peaks due to 3-aminopropanol and impurity A.

Limits:

- *impurity A*: calculate the ratio R1 of the area of the peak due to impurity A to the area of the peak due to the internal standard from the chromatogram obtained with reference solution (b); from the chromatogram obtained with the test solution, calculate the ratio of the area of any peak due to impurity A to the area of the peak due to the internal standard: this ratio is not greater than R1 (0.1 per cent),
- *impurity B*: calculate the ratio R2 of the area of the peak due to impurity B to the area of the peak due to the internal standard from the chromatogram obtained with reference solution (b); from the chromatogram obtained with the test solution, calculate the ratio of the area of any peak due to impurity B to the area of the peak due to the internal standard: this ratio is not greater than R2 (0.5 per cent),
- *total*: calculate the ratio R3 of the area of the peak due to trolamine to the area of the peak due to the internal standard from the chromatogram obtained with reference solution (b); from the chromatogram obtained with the test solution, calculate the ratio of the sum of the areas of any peaks, apart from the principal peak and the peak due to the internal standard, to the area of the peak due to the internal standard: this ratio is not greater than 10 times R3 (1.0 per cent),
- *disregard limit*: 0.5 times the ratio of the area of the peak due to trolamine to the area of the peak due to the internal standard from the chromatogram obtained with reference solution (b) (0.05 per cent).

Impurity C. Gas chromatography (2.2.28). Carry out the test under a ventilated hood, wear gloves and safety glasses.*Test solution*. In a suitable distillation apparatus mix 100.0 g of the substance to be examined and 100.0 ml of *ethylene glycol R* and gently distil 10.0 ml under vacuum at a pressure not exceeding 1.3 kPa. From this distillate, distil 1 ml.*Reference solution*. Dilute 25.0 mg of *N-nitrosodiethanolamine R* to 100.0 ml with *ethylene glycol R*. Dilute 5.0 ml of the solution to 50.0 ml with *ethylene glycol R*. Dilute 5.0 ml of this solution to 50.0 ml with *ethylene glycol R*.*Column*:

- *material*: fused silica,
- *size*: $l = 30$ m, $\varnothing = 0.25$ mm,
- *stationary phase*: macrogol 20 000 *R* (film thickness 0.25 μ m).

Carrier gas: helium for chromatography R.

Flow rate: 0.75 ml/min.

Split ratio: 1:15.

Temperature:

	Time (min)	Temperature (°C)
Column	0 - 2	180
	2 - 8	180 → 240
	8 - 25	240
Injection port		240
Detector		250

Detection: mass selective spectrometer set at 72 m/e or 91 m/e.

Injection: 3 µl; if necessary inject a blank between each injection.

Retention times: impurity C = about 20 min.

Limit:

- **impurity C:** not more than the area of the corresponding peak in the chromatogram obtained with the reference solution (25 ppb).

Heavy metals (2.4.8): maximum 10 ppm.

Dilute 5 ml of solution S to 30 ml with *water R*. The solution complies with limit test A. Prepare the standard using *lead standard solution (1 ppm Pb) R*.

Water (2.5.12): maximum 1.0 per cent, determined on 1.000 g.

Open the titration vessel, introduce the substance to be examined directly into the previously titrated solvent. Stopper the flask immediately.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g. Do not carry out the initial heating on a water-bath.

ASSAY

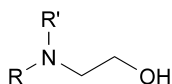
Dissolve 1.200 g in 75 ml of *carbon dioxide-free water R*. Add 0.3 ml of *methyl red solution R*. Titrate with *1 M hydrochloric acid*.

1 ml of *1 M hydrochloric acid* is equivalent to 0.149 g of $C_4H_{11}NO_3$.

STORAGE

In an airtight container, protected from light.

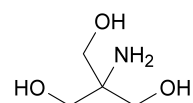
IMPURITIES



- R = R' = H: 2-aminoethanol (ethanolamine),
- R = CH_2-CH_2OH , R' = H: 2,2'-iminodiethanol (diethanolamine),
- R = CH_2-CH_2OH , R' = NO: 2,2'-(nitrosoimino)diethanol (N-nitrosodiethanolamine).

TROMETAMOL

Trometamol



$C_4H_{11}NO_3$

M_r 121.1

DEFINITION

Trometamol contains not less than 99.0 per cent and not more than the equivalent of 100.5 per cent of aminomethylidynetri(methanol), calculated with reference to the dried substance.

CHARACTERS

A white, crystalline powder, or colourless crystals, freely soluble in water, sparingly soluble in alcohol, very slightly soluble in ethyl acetate.

IDENTIFICATION

First identification: B, C.

Second identification: A, B, D.

A. Solution S (see Tests) is strongly alkaline (2.2.4).

B. Melting point (2.2.14): 168 °C to 174 °C.

C. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *trometamol CRS*.

D. Examine the chromatograms obtained in the test for related substances. The principal spot in the chromatogram obtained with test solution (b) is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).

TESTS

Solution S. Dissolve 2.5 g in *carbon dioxide-free water R* and dilute to 50 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and colourless (2.2.2, *Method II*).

pH (2.2.3). The pH of freshly prepared solution S is 10.0 to 11.5.

Related substances. Examine by thin-layer chromatography (2.2.27), using *silica gel G R* as the coating substance. Wash the plate with *methanol R* before applying the solutions.

Test solution (a). Dissolve 0.20 g in 1 ml of *water R*, with heating, and dilute to 10 ml with *methanol R*.

Test solution (b). Dilute 1 ml of test solution (a) to 10 ml with *methanol R*.

Reference solution (a). Dissolve 20 mg of *trometamol CRS* in *methanol R* and dilute to 10 ml with the same solvent.

Reference solution (b). Dilute 1 ml of test solution (a) to 100 ml with *methanol R*.

Apply to the plate 10 µl of each solution. Develop over a path of 10 cm using a mixture of 10 volumes of *dilute ammonia R1* and 90 volumes of *2-propanol R*. Dry the plate at 100 °C to 105 °C. Spray with a 5 g/l solution of *potassium permanganate R* in a 10 g/l solution of *sodium carbonate R*. After about 10 min examine in daylight. Any spot in the chromatogram obtained with test solution (a), apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (b) (1.0 per cent).

Chlorides (2.4.4). To 10 ml of solution S add 2.5 ml of *dilute nitric acid R* and dilute to 15 ml with *water R*. The solution complies with the limit test for chlorides (100 ppm).

Heavy metals (2.4.8). Dissolve 2.0 g in 10 ml of *water R*. Neutralise the solution with *hydrochloric acid R1* and dilute to 20 ml with *water R*. 12 ml of the solution complies with limit test A for heavy metals (10 ppm). Prepare the standard using *lead standard solution (1 ppm Pb) R*.

Iron (2.4.9). Dissolve 1.0 g in *water R* and dilute to 10 ml with the same solvent. The solution complies with the limit test for iron (10 ppm).

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

Bacterial endotoxins (2.6.14): less than 0.03 IU/mg, if intended for use in the manufacture of parenteral dosage forms without a further appropriate procedure for the removal of bacterial endotoxins.

ASSAY

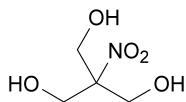
Dissolve 0.100 g in 20 ml of *water R*. Add 0.2 ml of *methyl red solution R*. Titrate with 0.1 M *hydrochloric acid* until the colour changes from yellow to red.

1 ml of 0.1 M *hydrochloric acid* is equivalent to 12.11 mg of $C_{17}H_{20}NO_2$.

LABELLING

The label states, where applicable, that the substance is free from bacterial endotoxins.

IMPURITIES

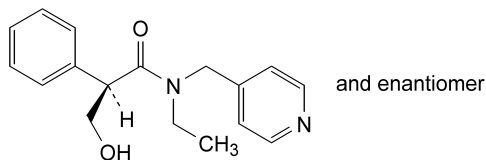


A. nitromethylidynetri(methanol).

01/2005:1159

TROPICAMIDE

Tropicamidum



$C_{17}H_{20}N_2O_2$

M_r 284.4

DEFINITION

Tropicamide contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of (2*RS*)-*N*-ethyl-3-hydroxy-2-phenyl-*N*-(pyridin-4-ylmethyl)propanamide, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, crystalline powder, slightly soluble in water, freely soluble in alcohol and in methylene chloride.

IDENTIFICATION

First identification: C.

Second identification: A, B, D, E.

A. Melting point (2.2.14): 95 °C to 98 °C.

B. Dissolve 20.0 mg in 0.1 M *hydrochloric acid* and dilute to 50.0 ml with the same acid. Dilute 2.0 ml of the solution to 20.0 ml with 0.1 M *hydrochloric acid*. Examined between 230 nm and 350 nm (2.2.25), the solution shows an absorption maximum at 254 nm. The specific absorbance at the maximum is 170 to 190.

C. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *tropicamide CRS*. Examine the substances prepared as discs.

D. Examine the chromatograms obtained in the test for related substances. The principal spot in the chromatogram obtained with test solution (b) is similar in position and size to the spot in the chromatogram obtained with reference solution (a).

E. Dissolve about 5 mg in 3 ml of a mixture of 9 ml of *acetic anhydride R*, 1 ml of *acetic acid R* and 0.1 g of *citric acid R*. Heat on a water-bath for 5 min to 10 min. A reddish-yellow colour is produced.

TESTS

Appearance of solution. Dissolve 0.1 g in *alcohol R* and dilute to 10 ml with the same solvent. The solution is clear (2.2.1) and colourless (2.2.2, *Method II*).

Optical rotation (2.2.7). Dissolve 2.5 g in *ethanol R* and dilute to 25.0 ml with the same solvent. The angle of optical rotation is -0.1° to $+0.1^\circ$.

Related substances. Examine by thin-layer chromatography (2.2.27), using as the coating substance a suitable silica gel with a fluorescent indicator having an optimal intensity at 254 nm.

Test solution (a). Dissolve 0.10 g of the substance to be examined in *methylene chloride R* and dilute to 5 ml with the same solvent.

Test solution (b). Dilute 1 ml of test solution (a) to 20 ml with *methylene chloride R*.

Reference solution (a). Dissolve 10 mg of *tropicamide CRS* in *methylene chloride R* and dilute to 10 ml with the same solvent.

Reference solution (b). Dilute 1 ml of test solution (b) to 10 ml with *methylene chloride R*.

Reference solution (c). Dilute 2 ml of reference solution (b) to 5 ml with *methylene chloride R*.

Reference solution (d). Dissolve 20 mg of 4-[(ethylamino)methyl]pyridine *R* in *methylene chloride R* and dilute to 20 ml with the same solvent. Dilute 1 ml of the solution and 1 ml of reference solution (a) to 10 ml with *methylene chloride R*.

Apply separately to the plate 10 µl of each solution. Develop over a path of 15 cm using a mixture of 0.5 volumes of *concentrated ammonia R*, 5 volumes of *methanol R* and 95 volumes of *methylene chloride R*. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. Any spot in the chromatogram obtained with test solution (a), apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.5 per cent) and at most one such spot is more intense than the spot in the chromatogram obtained with reference solution (c) (0.2 per cent). The test is not valid unless the chromatogram obtained with reference solution (d) shows two clearly separated spots.

Tropic acid. To 10.0 mg add 5 mg of *disodium tetraborate R* and 0.35 ml of a freshly prepared 100 g/l solution of *dimethylaminobenzaldehyde R* in a mixture of 1 volume

of *water R* and 9 volumes of *sulphuric acid R*. Heat on a water-bath for 3 min. Cool in ice water and add 5 ml of *acetic anhydride R*. No violet-red colour develops (0.05 per cent).

Chlorides (2.4.4). Dissolve 1.0 g with heating in 8 ml of *acetic acid R*, cool and dilute to 10 ml with the same acid. Dilute 5 ml of the solution to 15 ml with *water R*. The solution complies with the limit test for chlorides (100 ppm).

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 80 °C at a pressure not exceeding 0.7 kPa for 4 h.

Sulphated ash (2.2.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

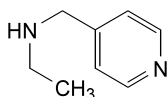
Dissolve 0.200 g in 50 ml of *anhydrous acetic acid R*. Add 0.2 ml of *naphtholbenzein solution R* and titrate with 0.1 M *perchloric acid* until the colour changes from orange to green.

1 ml of 0.1 M *perchloric acid* is equivalent to 28.44 mg of $C_{17}H_{20}N_2O_2$.

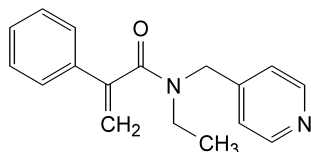
STORAGE

Store protected from light.

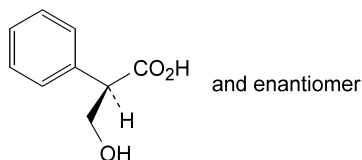
IMPURITIES



A. *N*-(pyridin-4-ylmethyl)ethanamine,



B. *N*-ethyl-2-phenyl-*N*-(pyridin-4-ylmethyl)propenamide,



C. (2*RS*)-3-hydroxy-2-phenylpropanoic acid (tropic acid).

01/2005:0694

TRYPSIN

Trypsinum

DEFINITION

Trypsin is a proteolytic enzyme obtained by the activation of trypsinogen extracted from the pancreas of healthy mammals. It has an activity not less than 0.5 microkatal per milligram, calculated with reference to the dried substance. In solution, it has maximum enzymic activity at pH 8; the activity is reversibly inhibited at pH 3, at which pH it is most stable.

PRODUCTION

The animals from which trypsin is derived must fulfil the requirements for the health of animals suitable for human consumption to the satisfaction of the competent authority.

It must have been shown to what extent the method of production allows inactivation or removal of any contamination by viruses or other infectious agents.

The method of manufacture is validated to demonstrate that the product, if tested, would comply with the following test:

Histamine (2.6.10). Not more than 1 µg of histamine base per 0.2 microkatal of trypsin activity. Use a 10 g/l solution of the substance to be examined in 0.0015 M *borate buffer solution pH 8.0 R* inactivated by heating on a water-bath for 30 min. Carry out dilutions with a 9 g/l solution of *sodium chloride R*.

CHARACTERS

A white or almost white, crystalline or amorphous powder, sparingly soluble in water. The amorphous form is hygroscopic.

IDENTIFICATION

- Dilute 1 ml of solution S (see Tests) to 100 ml with *water R*. In a depression in a white spot-plate, mix 0.1 ml of this solution with 0.2 ml of *tosylarginine methyl ester hydrochloride solution R*. A reddish-violet colour develops within 3 min.
- Dilute 0.5 ml of solution S to 5 ml with *water R*. Add 0.1 ml of a 20 g/l solution of *tosyl-lysyl-chloromethane hydrochloride R*. Adjust to pH 7.0, shake for 2 h and dilute to 50 ml with *water R*. In one of the depressions of a white spot-plate, mix 0.1 ml of this solution with 0.2 ml of *tosylarginine methyl ester hydrochloride solution R*. No reddish-violet colour develops within 3 min.

TESTS

Solution S. Dissolve 0.10 g in *carbon dioxide-free water R* and dilute to 10.0 ml with the same solvent.

Appearance of solution. Solution S is not more opalescent than reference suspension III (2.2.1).

pH (2.2.3). The pH of solution S is 3.0 to 6.0.

Absorbance (2.2.25). Dissolve 30.0 mg in 0.001 M *hydrochloric acid* and dilute to 100.0 ml with the same acid. The solution shows an absorption maximum at 280 nm and a minimum at 250 nm. The specific absorbance at the maximum is 13.5 to 16.5 and that at the minimum is not greater than 7.0.

Chymotrypsin. To 1.8 ml of *buffer solution pH 8.0 R* add 7.4 ml of *water R* and 0.5 ml of 0.2 M *acetyltyrosine ethyl ester R*. While shaking the solution, add 0.3 ml of solution S and start a stop-watch. After exactly 5 min, measure the pH (2.2.3) (test solution). Prepare a reference solution in the same manner, replacing solution S by 0.3 ml of a 0.5 g/l solution of *chymotrypsin BRP* and measure the pH (2.2.3) exactly 5 min after adding the chymotrypsin. The pH of the test solution is higher than that of the reference solution.

Loss on drying (2.2.32). Not more than 5.0 per cent, determined on 0.500 g by drying at 60 °C at a pressure not exceeding 670 Pa for 2 h.

Microbial contamination. Total viable aerobic count (2.6.12) not more than 10⁴ micro-organisms per gram, determined by plate count. It complies with the tests for *Escherichia coli* and *Salmonella* (2.6.13).

ASSAY

The activity of trypsin is determined by comparing the rate at which it hydrolyses *benzoylarginine ethyl ester hydrochloride R* with the rate at which *trypsin BRP* hydrolyses the same substrate in the same conditions.

Apparatus. Use a reaction vessel of about 30 ml capacity provided with:

01/2005:1272

- a device that will maintain a temperature of 25.0 ± 0.1 °C,
- a stirring device (for example, a magnetic stirrer),
- a lid with holes for the insertion of electrodes, the tip of a burette, a tube for the admission of nitrogen and the introduction of reagents.

An automatic or manual titration device may be used. For the latter, the burette is graduated in 0.005 ml and the pH meter is provided with a wide-range scale and glass-calomel electrodes.

Test solution. Dissolve sufficient of the substance to be examined in 0.001 M hydrochloric acid and dilute to 25.0 ml with the same acid in order to obtain a solution containing approximately 700 nanokatal per millilitre.

Reference solution. Dissolve 25.0 mg of *trypsin BRP* in 0.001 M hydrochloric acid and dilute to 25.0 ml with the same acid.

Store the solutions at 0 °C to 5 °C. Warm 1 ml of each solution to about 25 °C over 15 min and use 50 µl of each solution for each titration. Carry out the titration in an atmosphere of nitrogen. Transfer 10.0 ml of 0.0015 M borate buffer solution pH 8.0 R to the reaction vessel and, while stirring, add 1.0 ml of a freshly prepared 6.86 g/l solution of *benzoylarginine ethyl ester hydrochloride R*. When the temperature is steady at 25.0 ± 0.1 °C (after about 5 min) adjust the pH to exactly 8.0 with 0.1 M sodium hydroxide. Add 50 µl of the test solution and start a timer. Maintain the pH at 8.0 by the addition of 0.1 M sodium hydroxide the tip of the microburette being immersed in the solution; note the volume added every 30 s. Follow the reaction for 8 min. Calculate the volume of 0.1 M sodium hydroxide used per second. Carry out a titration in the same manner using the reference solution and calculate the volume of 0.1 M sodium hydroxide used per second.

Calculate the activity in microkatal per milligram using the expression:

$$\frac{m' \times V}{m \times V'} \times A$$

- m* = mass of the substance to be examined, in milligrams,
m' = mass of *trypsin BRP*, in milligrams,
V = volume of 0.1 M sodium hydroxide used per second by the test solution,
V' = volume of 0.1 M sodium hydroxide used per second by the reference solution,
A = activity of *trypsin BRP*, in microkatal per milligram.

STORAGE

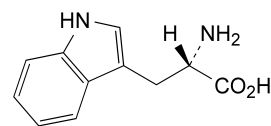
Store in an airtight container, protected from light, at a temperature of 2 °C to 8 °C.

LABELLING

The label states the activity in microkatal per milligram.

TRYPTOPHAN

Tryptophanum

C₁₁H₁₂N₂O₂M_r 204.2

DEFINITION

Tryptophan contains not less than 98.5 per cent and not more than the equivalent of 101.0 per cent of (S)-2-amino-3-(1H-indol-3-yl)propanoic acid, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, crystalline or amorphous powder, sparingly soluble in water, slightly soluble in alcohol. It dissolves in dilute mineral acids and in dilute solutions of alkali hydroxides.

IDENTIFICATION

First identification: A, B.

Second identification: A, C, D.

- It complies with the test for specific optical rotation (see Tests).
- Examine the substance by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *tryptophan CRS*. Examine the substances prepared as discs.
- Examine the chromatograms obtained in the test for ninhydrin-positive substances. The principal spot in the chromatogram obtained with test solution (b) is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).
- Dissolve about 20 mg in 10 ml of *water R*. Add 5 ml of *dimethylaminobenzaldehyde solution R6* and 2 ml of *hydrochloric acid R1*. Heat on a water-bath. A purple-blue colour develops.

TESTS

Appearance of solution. Dissolve 0.1 g in 1 M hydrochloric acid and dilute to 10 ml with the same acid. The solution is clear (2.2.1) and not more intensely coloured than reference solution BY₆ (2.2.2, Method II).

Specific optical rotation (2.2.7). Dissolve 0.25 g in *water R*, heating on a water-bath if necessary, and dilute to 25.0 ml with the same solvent. The specific optical rotation is –30.0 to –33.0, calculated with reference to the dried substance.

Ninhydrin-positive substances. Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel plate R*.

Test solution (a). Dissolve 0.10 g of the substance to be examined in a mixture of equal volumes of *glacial acetic acid R* and *water R* and dilute to 10 ml with the same mixture of solvents.

Test solution (b). Dilute 1 ml of test solution (a) to 50 ml with a mixture of equal volumes of *glacial acetic acid R* and *water R*.

Reference solution (a). Dissolve 10 mg of *tryptophan CRS* in a mixture of equal volumes of *glacial acetic acid R* and *water R* and dilute to 50 ml with the same mixture of solvents.

Reference solution (b). Dilute 5 ml of test solution (b) to 20 ml with a mixture of equal volumes of *glacial acetic acid R* and *water R*.

Reference solution (c). Dissolve 10 mg of *tryptophan CRS* and 10 mg of *tyrosine CRS* in a mixture of equal volumes of *glacial acetic acid R* and *water R* and dilute to 25 ml with the same mixture of solvents.

Apply separately to the plate 5 µl of each solution. Develop over a path of 15 cm using a mixture of 20 volumes of *glacial acetic acid R*, 20 volumes of *water R* and 60 volumes of *butanol R*. Allow the plate to dry in air. Spray with *ninhydrin solution R* and heat at 100 °C to 105 °C for 15 min. Any spot in the chromatogram obtained with test solution (a), apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.5 per cent). The test is not valid unless the chromatogram obtained with reference solution (c) shows two clearly separated spots.

1,1'-Ethylidenebistryptophan and other related substances. Examine by liquid chromatography (2.2.29).

Buffer solution pH 2.3. Dissolve 3.90 g of *sodium dihydrogen phosphate R* in 1000 ml of *water R*. Add about 700 ml of a 2.9 g/l solution of *phosphoric acid R* and adjust to pH 2.3 with the same acidic solution.

Prepare the solutions immediately before use.

Standard solution. Dissolve 10.0 mg of *N-acetyltryptophan R* in a mixture of 10 volumes of *acetonitrile R* and 90 volumes of *water R* and dilute to 100.0 ml with the same mixture of solvents. Dilute 2.0 ml of the solution to 100.0 ml with the same mixture of solvents.

Test solution (a). Dissolve 0.10 g of the substance to be examined in a mixture of 10 volumes of *acetonitrile R* and 90 volumes of *water R* and dilute to 10.0 ml the same mixture of solvents.

Test solution (b). Dissolve 0.10 g of the substance to be examined in the standard solution and dilute to 10.0 ml with the same solution.

Reference solution (a). Dissolve 1.0 mg of *1,1'-ethylidenebistryptophan CRS* in a mixture of 10 volumes of *acetonitrile R* and 90 volumes of *water R* and dilute to 100.0 ml with the same mixture of solvents.

Reference solution (b). Dilute 10.0 ml of reference solution (a) to 50.0 ml with the standard solution.

Reference solution (c). Dilute 10.0 ml of reference solution (a) to 50.0 ml with a mixture of 10 volumes of *acetonitrile R* and 90 volumes of *water R*.

Reference solution (d). Dissolve 0.10 g of the substance to be examined in reference solution (c) and dilute to 10.0 ml with the same solution.

Reference solution (e). Dilute 1.0 ml of reference solution (c) to 10.0 ml with a mixture of 10 volumes of *acetonitrile R* and 90 volumes of *water R*.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4.6 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (5 µm),
- as mobile phase at a flow rate of 0.7 ml/min the following solutions:

Mobile phase A. A mixture of 115 volumes of *acetonitrile R* and 885 volumes of buffer solution pH 2.3,

Mobile phase B. A mixture of 350 volumes of *acetonitrile R* and 650 volumes of buffer solution pH 2.3,

- a gradient programme using the following conditions:

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)	Comment
0 - 10	100	0	isocratic
10 - 45	100 → 0	0 → 100	linear gradient
45 - 65	0	100	isocratic
65 - 66	0 → 100	100 → 0	linear gradient
66 - 80	100	0	re-equilibration

- as detector a spectrophotometer set at 220 nm, maintaining the temperature of the column at 40 °C.

Inject 20 µl of reference solution (b), 20 µl of reference solution (d) and 20 µl of reference solution (e).

When the chromatograms are recorded in the prescribed conditions, the retention times are about 8 min for tryptophan, about 29 min for *N-acetyltryptophan* and about 34 min for *1,1'-ethylidenebistryptophan*. Adjust the sensitivity of the system so that the height of the peak due to *N-acetyltryptophan* in the chromatogram obtained with reference solution (b) is at least 50 per cent of the full scale of the recorder.

The test is not valid unless:

- in the chromatogram obtained with reference solution (b), the resolution between the peaks corresponding to *N-acetyltryptophan* and *1,1'-ethylidenebistryptophan* is at least 8.0. If necessary, adjust the time programme for the elution gradient. An increase in the duration of elution with mobile phase A produces longer retention times and better resolution;
- the chromatogram obtained with reference solution (e) has a signal-to-noise ratio of at least 15.

Inject 20 µl of test solution (a) and 20 µl of test solution (b). Check that in the chromatogram obtained with test solution (a) there is no peak with the same retention time as *N-acetyltryptophan* (in such case correct the area of the *N-acetyltryptophan* peak). In the chromatogram obtained with test solution (b): the area of any peak corresponding to *1,1'-ethylidenebistryptophan* is not greater than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (e) (10 ppm); the sum of the areas of any peaks with retention times less than that of tryptophan is not greater than 0.6 times the area of the peak corresponding to *N-acetyltryptophan* in the chromatogram obtained with reference solution (b) (100 ppm); the sum of the areas of any peaks with a retention time greater than that of tryptophan, apart from *N-acetyltryptophan*, and up to 1.8 times the retention time of *N-acetyltryptophan*, is not greater than 1.9 times the area of the peak due to *N-acetyltryptophan* in the chromatogram obtained with reference solution (b) (300 ppm). Disregard any peak with an area less than 0.02 times that of the peak due to *N-acetyltryptophan* in the chromatogram obtained with reference solution (b).

Chlorides (2.4.4). Dissolve 0.25 g in 3 ml of *dilute nitric acid R* and dilute to 15 ml with *water R*. The solution without any further addition of nitric acid (200 ppm), complies with the limit test for chlorides.

Sulphates (2.4.13). Dissolve 0.5 g in a mixture of 5 volumes of *dilute hydrochloric acid R* and 25 volumes of *distilled water R* and dilute to 15 ml with the same mixture of solvents. The solution complies with the limit test for sulphates (300 ppm).

Ammonium (2.4.1). 0.10 g complies with limit test B for ammonium (200 ppm). Prepare the standard using 0.2 ml of *ammonium standard solution (100 ppm NH₄) R*.

Iron (2.4.9). In a separating funnel, dissolve 0.50 g in 10 ml of *dilute hydrochloric acid R*. Shake with three quantities, each of 10 ml, of *methyl isobutyl ketone RI*, shaking for 3 min each time. To the combined organic layers add 10 ml of *water R* and shake for 3 min. The aqueous layer complies with the limit test for iron (20 ppm).

Heavy metals (2.4.8). 2.0 g complies with limit test D for heavy metals (10 ppm). Prepare the standard using 2 ml of *lead standard solution (10 ppm Pb) R*.

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

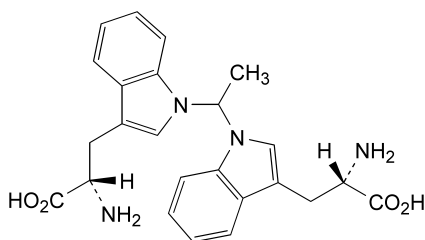
Dissolve 0.150 g in 3 ml of *anhydrous formic acid R*. Add 30 ml of *anhydrous acetic acid R*. Titrate with 0.1 M *perchloric acid*, using 0.1 ml of *naphtholbenzein solution R* as indicator.

1 ml of 0.1 M *perchloric acid* is equivalent to 20.42 mg of $C_{11}H_{12}N_2O_2$.

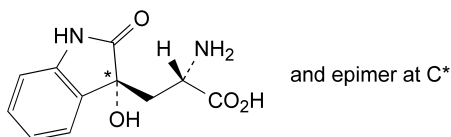
STORAGE

Store protected from light.

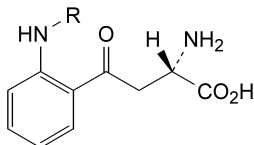
IMPURITIES



- A. 3,3'-[ethylidenebis(1*H*-indole-1,3-diyl)]bis[(2*S*)-2-aminopropanoic] acid (1,1'-ethylidenebistryptophan),

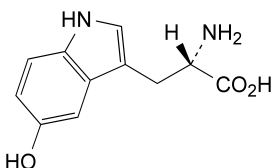


- B. (*S*)-2-amino-3-[(3*RS*)-3-hydroxy-2-oxo-2,3-dihydro-1*H*-indol-3-yl]propanoic acid (dioxindolylalanine),

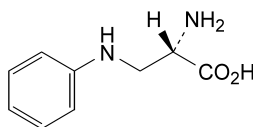


- C. R = H: (*S*)-2-amino-4-(2-aminophenyl)-4-oxobutanoic acid (kynurenine),

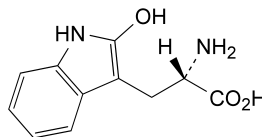
- E. R = CHO: (*S*)-2-amino-4-[2-(formylamino)phenyl]-4-oxobutanoic acid (*N*-formylkynurenine),



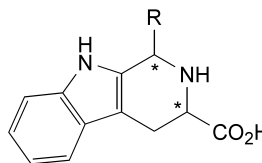
- D. (*S*)-2-amino-3-(5-hydroxy-1*H*-indol-3-yl)propanoic acid (5-hydroxytryptophan),



- F. (*S*)-2-amino-3-(phenylamino)propanoic acid (3-phenylaminoalanine),

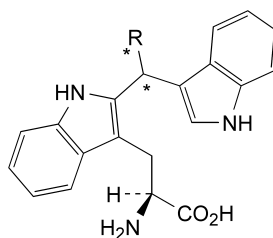


- G. (*S*)-2-amino-3-(2-hydroxy-1*H*-indol-3-yl)propanoic acid (2-hydroxytryptophan),



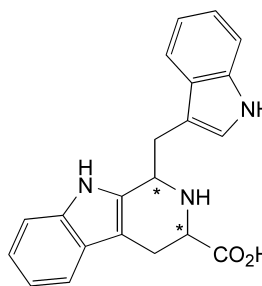
- H. R = H: (3*RS*)-1,2,3,4-tetrahydro-9*H*-β-carboline-3-carboxylic acid,

- I. R = CH₃: 1-methyl-1,2,3,4-tetrahydro-9*H*-β-carboline-3-carboxylic acid,



- J. R = CHOH-CH₂-OH: (*S*)-2-amino-3-[2-[2,3-dihydroxy-1-(1*H*-indol-3-yl)propyl]-1*H*-indol-3-yl]propanoic acid,

- K. R = H: (*S*)-2-amino-3-[2-(1*H*-indol-3-ylmethyl)-1*H*-indol-3-yl]propanoic acid,



- L. 1-(1*H*-indol-3-ylmethyl)-1,2,3,4-tetrahydro-9*H*-β-carboline-3-carboxylic acid.

01/2005:0152

TUBERCULIN FOR HUMAN USE, OLD

Tuberculinum pristinum ad usum humanum

DEFINITION

Old tuberculin for human use consists of a filtrate, concentrated by heating, containing the soluble products of the culture and lysis of one or more strains of *Mycobacterium*

bovis and/or *Mycobacterium tuberculosis* that is capable of demonstrating a delayed hypersensitivity in an animal sensitised to micro-organisms of the same species.

Old tuberculin for human use in concentrated form is a transparent, viscous, yellow or brown liquid.

PRODUCTION

GENERAL PROVISIONS

The production of old tuberculin is based on a seed-lot system. The production method shall have been shown to yield consistently old tuberculin of adequate potency and safety in man. A batch of old tuberculin, calibrated in International Units by the method described under Assay and for which adequate clinical information is available as to its activity in man, is set aside to serve as a reference preparation.

The International Unit is the activity of a stated quantity of the International Standard. The equivalence in International Units of the International Standard is stated by the World Health Organisation.

SEED LOTS

The strains of mycobacteria used shall be identified by historical records that include information on their origin and subsequent manipulation.

The working seed lots used to inoculate the media for the production of a concentrated harvest shall not have undergone more than 4 subcultures from the master seed lot.

Only seed lots that comply with the following requirements may be used for propagation.

Identification. The species of mycobacterium of the master and working seed lots is identified.

Bacterial and fungal contamination. Carry out the test for sterility (2.6.1), using 10 ml for each medium. The working seed lot complies with the test for sterility except for the presence of mycobacteria.

PROPAGATION AND HARVEST

The bacteria are grown in a liquid medium which may be a glycerolated broth or a synthetic medium. Growth must be typical for the strain. The culture is inactivated by a suitable method, such as treatment in an autoclave (121 °C for not less than 30 min) or in flowing steam at a temperature not less than 100 °C for at least 1 h. The culture liquid, from which the micro-organisms may or may not have been separated by filtration, is concentrated by evaporation, usually to one-tenth of its initial volume. The preparation is free from live mycobacteria. The concentrated harvest is shown to comply with the test for mycobacteria (2.6.2) before addition of any antimicrobial preservative or other substance that might interfere with the test. Phenol (5 g/l) or another suitable antimicrobial preservative that does not give rise to false positive reactions may be added.

Only a concentrated harvest that complies with the following requirements may be used in the preparation of the final bulk tuberculin.

pH (2.2.3). The pH of the concentrated harvest is 6.5 to 8.

Glycerol. Where applicable, determine the glycerol content of the concentrated harvest. The amount is within the limits approved for the particular product.

Antimicrobial preservative. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical or physico-chemical method. The content is not less than 85 per cent and not more than 115 per cent of the intended amount. If phenol has been used in the preparation, the concentration is not more than 5 g/l (2.5.15).

Sensitisation. Carry out the test described under Tests.

Sterility (2.6.1). The concentrated harvest complies with the test for sterility, carried out using 10 ml for each medium.

Potency. Determine the potency as described under Assay.

FINAL BULK TUBERCULIN

The concentrated harvest is diluted aseptically.

Only a final bulk tuberculin that complies with the following requirement may be used in the preparation of the final lot.

Sterility (2.6.1). The final bulk tuberculin complies with the test for sterility, carried out using 10 ml for each medium.

FINAL LOT

The final bulk tuberculin is distributed aseptically into sterile containers which are then closed so as to prevent contamination.

Only a final lot that is satisfactory with respect to each of the requirements given below under Identification, Tests and Assay may be released for use.

The following tests may be omitted on the final lot if they have been carried out at the stages indicated:

- live mycobacteria: concentrated harvest,
- sensitisation: concentrated harvest,
- toxicity: concentrated harvest or final bulk tuberculin,
- antimicrobial preservative: final bulk tuberculin.

IDENTIFICATION

Inject increasing doses of the preparation to be examined intradermally into healthy, white or pale-coloured guinea-pigs, specifically sensitised (for example, as described under Assay). A reaction varying from erythema to necrosis is produced at the site of the injection. Similar injections administered to non-sensitised guinea-pigs do not stimulate a reaction. The assay may also serve as identification.

TESTS

Old tuberculin for human use in concentrated form (≥ 100 000 IU/ml) complies with each of the tests prescribed below; the diluted product complies with the tests for antimicrobial preservative and sterility.

Toxicity. Inject a quantity equivalent to 50 000 IU subcutaneously into each of two healthy guinea-pigs weighing 250 g to 350 g and which have not been subjected to any treatment likely to interfere with the test. Observe the animals for 7 days. No adverse effect is produced.

Sensitisation. Use 3 guinea-pigs that have not been subjected to any treatment likely to interfere with the test. On 3 occasions at intervals of 5 days, inject intradermally into each guinea-pig about 500 IU of the preparation to be examined in a volume of 0.1 ml. 2 to 3 weeks after the third injection, administer the same dose intradermally to the same animals and to a control group of 3 guinea-pigs of the same mass that have not previously received injections of tuberculin. After 24 h to 72 h, the reactions in the 2 groups of animals are not substantially different.

Antimicrobial preservative. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical or physico-chemical method. The content is not less than the minimum amount shown to be effective and not more than 115 per cent of the amount stated on the label. If phenol has been used in the preparation, the concentration is not more than 5 g/l (2.5.15).

Live mycobacteria (2.6.2). It complies with the test for mycobacteria.

Sterility (2.6.1). It complies with the test for sterility.

ASSAY

The potency of old tuberculin is determined by comparing the reactions produced by the intradermal injection of increasing doses of the preparation to be examined into sensitised guinea-pigs with the reactions produced by known concentrations of the reference preparation.

Prepare a suspension containing a suitable amount (0.1 mg to 0.4 mg/ml) of heat-inactivated, dried mycobacteria in mineral oil with or without emulsifier; use mycobacteria of a strain of the same species as that used in the preparation to be examined. Sensitise not fewer than 6 pale-coloured guinea-pigs weighing not less than 300 g by injecting intramuscularly or intradermally a total of about 0.5 ml of the suspension, divided between several sites if necessary. Carry out the test after the period of time required for optimal sensitisation which is usually 4 to 8 weeks after sensitisation. Depilate the flanks of the animals so that it is possible to make at least three injections on each side and not more than a total of 12 injection points per animal. Use at least three different doses of the reference preparation and at least 3 different doses of the preparation to be examined. For both preparations, use doses such that the highest dose is about 10 times the lowest dose. Choose the doses such that when they are injected the lesions produced have a diameter of not less than 8 mm and not more than 25 mm. In any given test, the order of the dilutions injected at each point is chosen at random in a Latin square design. Inject each dose intradermally in a constant volume of 0.1 ml or 0.2 ml. Measure the diameters of the lesions 24 h to 48 h later and calculate the results of the test by the usual statistical methods, assuming that the diameters of the lesions are directly proportional to the logarithm of the concentration of the preparation.

The estimated potency is not less than 80 per cent and not more than 125 per cent of the stated potency. The confidence limits ($P = 0.95$) are not less than 64 per cent and not more than 156 per cent of the stated potency.

STORAGE

Store protected from light.

LABELLING

The label states:

- the number of International Units per millilitre,
- the species of mycobacterium used to prepare the product,
- the name and quantity of any antimicrobial preservative or other substance added to the preparation,
- the expiry date,
- where applicable, that old tuberculin is not to be injected in its concentrated form but diluted so as to administer not more than 100 IU per dose.

01/2005:0535

TUBERCULIN PURIFIED PROTEIN DERIVATIVE, AVIAN

Tuberculini aviarii derivatum proteinosum purificatum

DEFINITION

Tuberculin purified protein derivative, avian (Tuberculin PPD, avian) is a preparation obtained from the heat-treated products of growth and lysis of *Mycobacterium avium* capable of revealing a delayed hypersensitivity in an animal sensitised to micro-organisms of the same species.

PRODUCTION

It is obtained from the water-soluble fractions prepared by heating in free-flowing steam and subsequently filtering cultures of *M. avium* grown in a liquid synthetic medium. The active fraction of the filtrate, consisting mainly of protein, is isolated by precipitation, washed and re-dissolved. An antimicrobial preservative that does not give rise to false positive reactions, such as phenol, may be added. The final sterile preparation, free from mycobacteria, is distributed aseptically into sterile tamper-proof glass containers which are then closed so as to prevent contamination. The preparation may be freeze-dried.

The identification, the tests and the determination of potency apply to the liquid form and to the freeze-dried form after reconstitution as stated on the label.

IDENTIFICATION

Inject a range of graded doses intradermally at different sites into suitably sensitised albino guinea-pigs, each weighing not less than 250 g. After 24 h to 28 h, reactions appear in the form of oedematous swellings with erythema with or without necrosis at the points of injection. The size and severity of the reactions vary according to the dose. Unsensitised guinea-pigs show no reactions to similar injections.

TESTS

pH (2.2.3). The pH is 6.5 to 7.5.

Phenol (2.5.15). If the preparation to be examined contains phenol, its concentration is not more than 5 g/l.

Sensitising effect. Use a group of three guinea-pigs that have not been treated with any material which will interfere with the test. On three occasions at intervals of 5 days inject intradermally into each guinea-pig a dose of the preparation to be examined equivalent to 500 IU in 0.1 ml. Fifteen to twenty-one days after the third injection, inject the same dose (500 IU) intradermally into these animals and into a control group of three guinea-pigs of the same mass and which have not previously received injections of tuberculin. 24 h to 28 h after the last injections, the reactions of the two groups are not significantly different.

Toxicity. Use two guinea-pigs, each weighing not less than 250 g and which have not previously been treated with any material which will interfere with the test. Inject subcutaneously into each guinea-pig 0.5 ml of the preparation to be examined. Observe the animals for 7 days. No abnormal effects occur during the observation period.

Sterility. It complies with the test for sterility prescribed in the monograph on *Vaccines for veterinary use (0062)*.

POTENCY

The potency of tuberculin purified protein derivative, avian is determined by comparing the reactions produced in sensitised guinea-pigs by the intradermal injection of a series of dilutions of the preparation to be examined with those produced by known concentrations of a reference preparation of tuberculin purified protein derivative, avian calibrated in International Units.

The International Unit is the activity contained in a stated amount of the International Standard. The equivalence in International Units of the International Standard is stated by the World Health Organisation.

Sensitise not fewer than nine albino guinea-pigs, each weighing 400 g to 600 g, by the deep intramuscular injection of a suitable dose of inactivated or live *M. avium*. Not less than four weeks after the sensitisation of the guinea-pigs, shave their flanks to provide space for not more than four injection sites on each side. Prepare dilutions of the

preparation to be examined and of the reference preparation using isotonic phosphate-buffered saline (pH 6.5 to 7.5) containing 0.005 g/l of *polysorbate 80 R*. Use not fewer than three doses of the reference preparation and not fewer than three doses of the preparation to be examined. Choose the doses such that the lesions produced have a diameter of not less than 8 mm and not more than 25 mm. Allocate the dilutions randomly to the sites using a Latin square design. Inject each dose intradermally in a constant volume of 0.1 ml or 0.2 ml. Measure the diameters of the lesions after 24 h to 28 h and calculate the result of the test using the usual statistical methods and assuming that the diameters of the lesions are directly proportional to the logarithm of the concentration of the tuberculin.

The test is not valid unless the confidence limits ($P = 0.95$) are not less than 50 per cent and not more than 200 per cent of the estimated potency. The estimated potency is not less than 75 per cent and not more than 133 per cent of the stated potency. The stated potency is not less than 20 000 IU/ml.

STORAGE

Store protected from light, at a temperature of 5 ± 3 °C.

LABELLING

The label states:

- the potency in International Units per millilitre.
- the name and quantity of any added substance,
- for freeze-dried preparations:
- the name and volume of the reconstituting liquid to be added,
- that the product should be used immediately after reconstitution.

01/2005:0536

TUBERCULIN PURIFIED PROTEIN DERIVATIVE, BOVINE

Tuberculini bovini derivatum proteinosum purificatum

DEFINITION

Tuberculin purified protein derivative, bovine (Tuberculin PPD, bovine) is a preparation obtained from the heat-treated products of growth and lysis of *Mycobacterium bovis* capable of revealing a delayed hypersensitivity in an animal sensitised to micro-organisms of the same species.

PRODUCTION

It is obtained from the water-soluble fractions prepared by heating in free-flowing steam and subsequently filtering cultures of *M. bovis* grown in a liquid synthetic medium. The active fraction of the filtrate, consisting mainly of protein, is isolated by precipitation, washed and re-dissolved. An antimicrobial preservative that does not give rise to false positive reactions, such as phenol, may be added. The final sterile preparation, free from mycobacteria, is distributed aseptically into sterile, tamper-proof glass containers which are then closed so as to prevent contamination. The preparation may be freeze-dried.

The identification, the tests and the determination of potency apply to the liquid form and to the freeze-dried form after reconstitution as stated on the label.

IDENTIFICATION

Inject a range of graded doses intradermally at different sites into suitably sensitised albino guinea-pigs, each weighing not less than 250 g. After 24 h to 28 h, reactions appear in the form of oedematous swellings with erythema with or without necrosis at the points of injection. The size and severity of the reactions vary according to the dose. Unsensitised guinea-pigs show no reactions to similar injections.

TESTS

pH (2.2.3). The pH is 6.5 to 7.5.

Phenol (2.5.15). If the preparation to be examined contains phenol, its concentration is not more than 5 g/l.

Sensitising effect. Use a group of 3 guinea-pigs that have not been treated with any material which will interfere with the test. On 3 occasions at intervals of 5 days inject intradermally into each guinea-pig a dose of the preparation to be examined equivalent to 500 IU in 0.1 ml. 15 to 21 days after the third injection inject the same dose (500 IU) intradermally into these animals and into a control group of 3 guinea-pigs of the same mass and which have not previously received injections of tuberculin. 24 h to 28 h after the last injections, the reactions of the 2 groups are not significantly different.

Toxicity. Use 2 guinea-pigs, each weighing not less than 250 g and which have not previously been treated with any material which will interfere with the test. Inject subcutaneously into each guinea-pig 0.5 ml of the preparation to be examined. Observe the animals for 7 days. No abnormal effects occur during the observation period.

Sterility. It complies with the test for sterility prescribed in the monograph on *Vaccines for veterinary use* (0062).

POTENCY

The potency of tuberculin purified protein derivative, bovine is determined by comparing the reactions produced in sensitised guinea-pigs by the intradermal injection of a series of dilutions of the preparation to be examined with those produced by known concentrations of a reference preparation calibrated in International Units.

Sensitise not fewer than 9 albino guinea-pigs, each weighing 400 g to 600 g, by the deep intramuscular injection of 0.0001 mg of wet mass of living *M. bovis* of strain AN5 suspended in 0.5 ml of a 9 g/l solution of *sodium chloride R*. Not less than 4 weeks after the sensitisation of the guinea-pigs, shave their flanks to provide space for not more than 4 injection sites on each side. Prepare dilutions of the preparation to be examined and of the reference preparation using isotonic phosphate-buffered saline (pH 6.5-7.5) containing 0.005 g/l of *polysorbate 80 R*. Use not fewer than 3 doses of the reference preparation and not fewer than 3 doses of the preparation to be examined. Choose the doses such that the lesions produced have a diameter of not less than 8 mm and not more than 25 mm. Allocate the dilutions randomly to the sites using a Latin square design. Inject each dose intradermally in a constant volume of 0.1 ml or 0.2 ml. Measure the diameters of the lesions after 24 h to 28 h and calculate the result of the test using the usual statistical methods and assuming that the diameters of the lesions are directly proportional to the logarithm of the concentration of the tuberculin.

The test is not valid unless the confidence limits ($P = 0.95$) are not less than 50 per cent and not more than 200 per cent of the estimated potency. The estimated potency is not less than 66 per cent and not more than 150 per cent of the stated potency. The stated potency is not less than 20 000 IU/ml.

STORAGE

Protected from light, at a temperature of 5 ± 3 °C.

LABELLING

The label states:

- the potency in International Units per millilitre,
- the name and quantity of any added substance,
- for freeze-dried preparations:
 - the name and volume of the reconstituting liquid to be added,
 - that the product should be used immediately after reconstitution.

01/2005:0151

TUBERCULIN PURIFIED PROTEIN DERIVATIVE FOR HUMAN USE

Tuberculini derivatum proteinosum purificatum ad usum humanum

DEFINITION

Tuberculin purified protein derivative (tuberculin PPD) for human use is a preparation obtained by precipitation from the heated products of the culture and lysis of *Mycobacterium bovis* and/or *Mycobacterium tuberculosis* and capable of demonstrating a delayed hypersensitivity in an animal sensitised to micro-organisms of the same species.

Tuberculin PPD is a colourless or pale-yellow liquid; the diluted preparation may be a freeze-dried powder which upon dissolution gives a colourless or pale-yellow liquid.

PRODUCTION

GENERAL PROVISIONS

The production of tuberculin PPD is based on a seed-lot system. The production method shall have been shown to yield consistently tuberculin PPD of adequate potency and safety in man. A batch of tuberculin PPD, calibrated in International Units by method A described under Assay and for which adequate clinical information is available as to its activity in man, is set aside to serve as a reference preparation.

The International Unit is the activity of a stated quantity of the International Standard. The equivalence in International Units of the International Standard is stated by the World Health Organisation.

SEED LOTS

The strains of mycobacteria used shall be identified by historical records that include information on their origin and subsequent manipulation.

The working seed lots used to inoculate the media for production of a concentrated harvest shall not have undergone more than 4 subcultures from the master seed lot.

Only seed lots that comply with the following requirements may be used for propagation.

Identification. The species of mycobacterium of the master and working seed lots is identified.

Bacterial and fungal contamination. Carry out the test for sterility (2.6.1), using 10 ml for each medium. The working seed lot complies with the test for sterility except for the presence of mycobacteria.

PROPAGATION AND HARVEST

The bacteria are grown in a liquid synthetic medium. Growth must be typical for the strain. The culture is inactivated by a suitable method such as treatment in an autoclave (121 °C for not less than 30 min) or in flowing steam at a temperature not less than 100 °C for at least 1 h and filtered. The active fraction of the filtrate, consisting mainly of protein, is isolated by precipitation, washed and re-dissolved. The preparation is free from mycobacteria. The concentrated harvest is shown to comply with the test for mycobacteria (2.6.2) before addition of any antimicrobial preservative or other substance that might interfere with the test. Phenol (5 g/l) or another suitable antimicrobial preservative that does not give rise to false positive reactions may be added; a suitable stabiliser intended to prevent adsorption on glass or plastic surfaces may be added. The concentrated harvest may be freeze-dried. Phenol is not added to preparations that are to be freeze-dried.

Only a concentrated harvest that complies with the following requirements may be used in the preparation of the final bulk tuberculin PPD.

Antimicrobial preservative. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical or physico-chemical method. The content is not less than 85 per cent and not more than 115 per cent of the intended amount. If phenol has been used in the preparation, the concentration is not more than 5 g/l (2.5.15).

Sensitisation. Carry out the test described under Tests.

Sterility (2.6.1). The concentrated harvest, reconstituted if necessary, complies with the test for sterility, carried out using 10 ml for each medium.

Potency. Determine the potency as described under Assay.

FINAL BULK TUBERCULIN PPD

The concentrated harvest is diluted aseptically, after reconstitution if necessary.

Only a final bulk tuberculin PPD that complies with the following requirement may be used in the preparation of the final lot.

Sterility (2.6.1). The final bulk tuberculin PPD complies with the test for sterility, carried out using 10 ml for each medium.

FINAL LOT

The final bulk tuberculin PPD is distributed aseptically into sterile containers which are then closed so as to prevent contamination. It may be freeze-dried.

Only a final lot that is satisfactory with respect to each of the requirements given below under Identification, Tests and Assay may be released for use.

The following tests may be omitted on the final lot if they have been carried out at the stages indicated:

- live mycobacteria: concentrated harvest
- sensitisation: concentrated harvest
- toxicity: concentrated harvest or final bulk tuberculin PPD
- antimicrobial preservative: final bulk tuberculin PPD.

IDENTIFICATION

Inject increasing doses of the preparation to be examined intradermally into healthy, white or pale-coloured guinea-pigs, specifically sensitised (for example as described under Assay). A reaction varying from erythema to necrosis is produced at the site of the injection. Similar injections administered to non-sensitised guinea-pigs do not stimulate a reaction. The assay may also serve as identification.

TESTS

Tuberculin purified protein derivative for human use in concentrated form ($\geq 100\,000$ IU/ml) complies with each of the tests prescribed below; the diluted product complies with the tests for pH, antimicrobial preservative and sterility.

pH (2.2.3). The pH of the preparation, reconstituted if necessary as stated on the label, is 6.5 to 7.5.

Toxicity. Inject subcutaneously 50 000 IU of the preparation to be examined into each of two healthy guinea-pigs weighing 250 g to 350 g and which have not been subjected to any treatment likely to interfere with the test. Observe the animals for 7 days. No adverse effect is produced.

Sensitisation. Use 3 guinea-pigs that have not been subjected to any treatment likely to interfere with the test. On 3 occasions at intervals of 5 days, inject intradermally into each guinea-pig about 500 IU of the preparation to be examined in a volume of 0.1 ml. 2 to 3 weeks after the third injection, administer the same dose intradermally to the same animals and to a control group of three guinea-pigs of the same mass that have not previously received injections of tuberculin. After 24 h to 72 h, the reactions in the 2 groups of animals are not substantially different.

Antimicrobial preservative. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical or physico-chemical method. The content is not less than the minimum amount shown to be effective and not more than 115 per cent of the amount stated on the label. If phenol has been used in the preparation, the concentration is not more than 5 g/l (2.5.15).

Live mycobacteria (2.6.2). It complies with the test for mycobacteria.

Sterility (2.6.1). It complies with the test for sterility.

ASSAY

Use method A or, where the preparation contains 1 IU to 2 IU, use method B.

METHOD A

The potency of tuberculin PPD is determined by comparing the reactions produced by the intradermal injection of increasing doses of the preparation to be examined into sensitised guinea-pigs with the reactions produced by known concentrations of the reference preparation.

Prepare a suspension containing a suitable amount (0.1 mg/ml to 0.4 mg/ml) of heat-inactivated, dried mycobacteria in mineral oil with or without emulsifier; use mycobacteria of a strain of the same species as that used in the preparation to be examined. Sensitise not fewer than six pale-coloured guinea-pigs weighing not less than 300 g by injecting intramuscularly or intradermally a total of about 0.5 ml of the suspension, divided between several sites if necessary. Carry out the test after the period of time required for optimal sensitisation which is usually 4 to 8 weeks after sensitisation. Depilate the flanks of the animals so that it is possible to make at least 3 injections on each side but not more than a total of 12 injection points per animal. Prepare dilutions of the preparation to be examined and of the reference preparation using isotonic phosphate-buffered saline (pH 6.5 to 7.5) containing 50 mg/l of *polysorbate 80 R*. If the preparation to be examined is freeze-dried and does not contain a stabiliser, reconstitute it using the liquid described above. Use at least 3 different doses of the reference preparation and at least 3 different doses of the preparation to be examined. For both preparations, use doses such that the highest dose is about 10 times the lowest dose. Choose the doses such that

when they are injected the lesions produced have a diameter of not less than 8 mm and not more than 25 mm. In any given test, the order of the dilutions injected at each point is chosen at random in a Latin square design. Inject each dose intradermally in a constant volume of 0.1 ml or 0.2 ml. Measure the diameters of the lesions 24 h to 48 h later and calculate the results of the test by the usual statistical methods, assuming that the diameters of the lesions are directly proportional to the logarithm of the concentration of the preparation.

The estimated potency is not less than 80 per cent and not more than 125 per cent of the stated potency. The confidence limits ($P = 0.95$) are not less than 64 per cent and not more than 156 per cent of the stated potency.

METHOD B

The potency of tuberculin PPD is determined by comparing the reactions produced by the intradermal injection of the preparation to be examined into sensitised guinea-pigs with the reactions produced by known concentrations of the reference preparation.

Prepare a suspension in mineral oil with or without emulsifier and containing a suitable amount (0.1 mg/ml to 0.4 mg/ml) of heat-inactivated, dried mycobacteria; use mycobacteria of a strain of the same species as that used in the preparation to be examined. Sensitise not fewer than 6 pale-coloured guinea-pigs weighing not less than 300 g by injecting intramuscularly or intradermally a total of about 0.5 ml of the suspension, divided between several sites if necessary. Carry out the test after the period of time required for optimal sensitisation which is usually 4 to 8 weeks after sensitisation. Depilate the flanks of the animals so that it is possible to make at least 3 injections on each side but not more than a total of 12 injection points per animal. Prepare dilutions of the reference preparation using isotonic phosphate-buffered saline (pH 6.5 to 7.5) containing 50 mg/l of *polysorbate 80 R*. Use at least 3 different doses of the reference preparation such that the highest dose is about 10 times the lowest dose and the median dose is the same as that of the preparation to be examined. In any given test, the order of the dilutions injected at each point is chosen at random in a Latin square design. Inject the preparation to be examined and each dilution of the reference preparation intradermally in a constant volume of 0.1 ml or 0.2 ml. Measure the diameters of the lesions 24 h to 48 h later and calculate the results of the test by the usual statistical methods, assuming that the areas of the lesions are directly proportional to the logarithm of the concentration of the preparation to be examined. (This dose relationship applies to this assay and not necessarily to other test systems.)

The estimated potency is not less than 80 per cent and not more than 125 per cent of the stated potency. The confidence limits ($P = 0.95$) are not less than 64 per cent and not more than 156 per cent of the stated potency.

STORAGE

Store protected from light.

LABELLING

The label states:

- the number of International Units per container,
- the species of mycobacteria used to prepare the product,
- the name and quantity of any antimicrobial preservative or other substance added to the preparation,
- the expiry date,
- for freeze-dried products, a statement that the product is to be reconstituted using the liquid provided by the manufacturer,

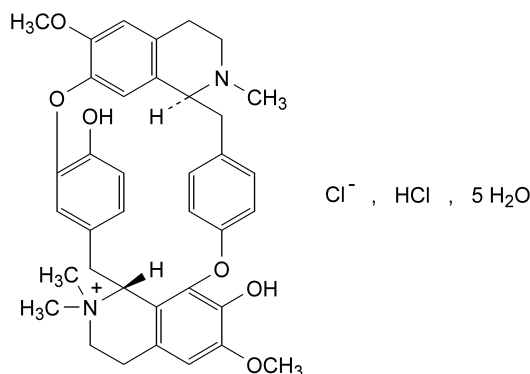
- where applicable, that tuberculin PPD is not to be injected in its concentrated form but diluted so as to administer not more than 100 IU per dose.

If the package does not contain a leaflet warning that the inhalation of concentrated tuberculin PPD may produce toxic effects, this warning must be shown on the label on the container together with a statement that the powder must be handled with care.

01/2005:0305

TUBOCURARINE CHLORIDE

Tubocurarini chloridum

 $\text{C}_{37}\text{H}_{42}\text{Cl}_2\text{N}_2\text{O}_6 \cdot 5\text{H}_2\text{O}$ M_r 772

DEFINITION

Tubocurarine chloride contains not less than 98.0 per cent and not more than the equivalent of 102.0 per cent of (3a*R*,16a*S*)-8,23-dihydroxy-24,29-dimethoxy-3,3,16-trimethyl-2,3,3a,4,15,16,16a,17-octahydro-1*H*-11,13:18,21-dietheno-5,9-metheno-14*H*-pyrido[3',2':14,15][1,11]dioxacycloicosino[2,3,4-*i*]isoquinolinium chloride hydrochloride, calculated with reference to the anhydrous substance.

CHARACTERS

A white or slightly yellowish, crystalline powder, soluble in water and in alcohol, practically insoluble in acetone. It dissolves in solutions of alkali hydroxides.

It melts at about 270 °C, with decomposition.

IDENTIFICATION

First identification: B, E.

Second identification: A, C, D, E, F.

- Dissolve 50.0 mg in *water R* and dilute to 1000.0 ml with the same solvent. Examined between 230 nm and 350 nm (2.2.25), the solution shows an absorption maximum at 280 nm and a minimum at 255 nm. The specific absorbance at the maximum is 113 to 123, calculated with reference to the anhydrous substance.
- Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *tubocurarine chloride CRS*.
- Dissolve about 25 mg in 1 ml of *water R*. Add 0.2 ml of *ferric chloride solution R2* and heat in a water-bath for 1 min. The solution is green. Carry out a blank test; after heating in a water-bath, the colour is brown.
- To 1 ml of solution S (see Tests) add 1 ml of *nitric acid solution of mercury R*. A red colour develops slowly.

E. It gives reaction (a) of chlorides (2.3.1).

F. It gives the reaction of alkaloids (2.3.1).

TESTS

Solution S. Dissolve 0.25 g in *carbon dioxide-free water R* and dilute to 25.0 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and not more intensely coloured than reference solution Y_6 (2.2.2, *Method II*).

pH (2.2.3). The pH of solution S is 4.0 to 6.0.

Specific optical rotation (2.2.7): + 210 to + 222, determined on solution S 3 h after preparation and calculated with reference to the anhydrous substance.

Chloroform-soluble substances. Dissolve 0.25 g in 150 ml of *water R*. Add 5 ml of a saturated solution of *sodium bicarbonate R* and shake with three quantities, each of 20 ml, of *chloroform R*. Wash the combined chloroform layers with 10 ml of *water R*, filter the chloroform solution into a tared beaker and wash the filter with two quantities, each of 5 ml, of *chloroform R*. Add the washings to the filtrate. Evaporate to dryness on a water-bath and dry at 100–105 °C for 1 h. The residue weighs not more than 5 mg. The residue does not dissolve in 10 ml of *water R*, but dissolves on the addition of 1 ml of *dilute hydrochloric acid R*.

Related substances. Examine by thin-layer chromatography (2.2.27), using *silica gel G R* as the coating substance.

Test solution. Dissolve 0.25 g of the substance to be examined in *water R* and dilute to 10 ml with the same solvent.

Reference solution (a). Dilute 1.5 ml of the test solution to 100 ml with *water R*.

Reference solution (b). Dilute 5 ml of reference solution (a) to 10 ml with *water R*.

Apply to the plate 5 µl of each solution. Develop in a non-saturated tank over a path of 15 cm using the lower layer of a mixture of equal volumes of *chloroform R*, *methanol R* and a 125 g/l solution of *trichloroacetic acid R*. Dry the plate in a current of cold air. Spray with a mixture of 1 volume of *potassium ferricyanide solution R*, 1 volume of *water R* and 2 volumes of *ferric chloride solution R1*, prepared immediately before use. Any spot in the chromatogram obtained with the test solution, apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (a) (1.5 per cent) and at most one such spot is more intense than the spot in the chromatogram obtained with reference solution (b) (0.75 per cent).

Water (2.5.12): 9.0 per cent to 12.0 per cent, determined on 0.30 g by the semi-micro determination of water.

Sulphated ash (2.4.14). Not more than 0.25 per cent, determined on 0.2 g.

ASSAY

Dissolve 25.0 mg in *water R* and dilute to 500.0 ml with the same solvent. In the same manner, prepare a reference solution using 25.0 mg of *tubocurarine chloride CRS*. Measure the absorbance (2.2.25) at the maximum at 280 nm.

Calculate the content of $\text{C}_{37}\text{H}_{42}\text{Cl}_2\text{N}_2\text{O}_6$ from the absorbances measured, the concentrations of the solutions and the declared content of anhydrous tubocurarine chloride in *tubocurarine chloride CRS*.

STORAGE

Store in an airtight container.

01/2005:1441

TURMERIC, JAVANESE**Curcumae xanthorrhizae rhizoma****DEFINITION**

Javanese turmeric consists of the dried rhizome, cut in slices, of *Curcuma xanthorrhiza* Roxb. (*C. xanthorrhiza* D. Dietrich). It contains not less than 50 ml/kg of essential oil and not less than 1.0 per cent of dicinnamoyl methane derivatives expressed as curcumin ($C_{21}H_{20}O_6$; M_r 368.4), both calculated with reference to the anhydrous drug.

CHARACTERS

Javanese turmeric has an aromatic odour.

It has the macroscopic and microscopic characters described under identification tests A and B.

IDENTIFICATION

- A. The drug consists of orange-yellow to yellowish-brown or greyish-brown slices, mostly peeled 1.5 mm to 6 mm thick and 15 mm to 50 mm, more rarely up to 70 mm, in diameter. Fragments of the brownish-grey cork are sporadically present. The transverse surface is yellow with dark spots in the paler centre. The fracture is short and finely grained.
- B. Reduce to a powder (355). The powder is reddish-brown. Examine under a microscope, using *chloral hydrate solution R*. The powder shows fragments of colourless parenchyma with orange-yellow to yellowish-brown secretory cells; fragments of reticulate and other vessels; rare fragments of cork and epidermis and fragments of thick-walled unicellular acute trichomes. Examine under a microscope using a 50 per cent *V/V* solution of *glycerol R*. The powder shows numerous stratified, ovoid to irregular starch granules, about 30 µm to 50 µm long and about 10 µm to 30 µm wide, with an eccentric hilum and marked, concentric striations.
- C. Examine by thin-layer chromatography as described in the test for *Curcuma domestica* until the words "Allow the plate to dry in the air". Spray the plate with a freshly prepared 0.4 g/l solution of *dichloroquinonechlorimide R* in *2-propanol R*. Expose the plate to ammonia vapour until the thymol zone becomes bluish-violet. The chromatogram obtained with the reference solution shows almost in the middle a bluish-violet zone (thymol) and in the lower part a yellow zone (fluorescein). The chromatogram obtained with the test solution shows a blue zone (xanthorrhizol) slightly above the zone due to thymol in the chromatogram obtained with the reference solution and two yellowish-brown to brown zones (curcumin and demethoxycurcumin) between the zones due to thymol and fluorescein in the chromatogram obtained with the reference solution.

TESTS

Foreign matter (2.8.2). It complies with the test for foreign matter.

Curcuma domestica. Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel plate R*.

Test solution. Shake 0.5 g of the freshly powdered drug (500) with 5 ml of *methanol R* for 30 min and filter.

Reference solution. Dissolve 5 mg of *fluorescein R* and 10 mg of *thymol R* in 10 ml of *methanol R*.

Apply separately to the plate as bands 10 µl of each solution. Develop over a path of 10 cm using a mixture of 20 volumes of *glacial acetic acid R* and 80 volumes of *toluene R*. Allow

the plate to dry in air. Spray the plate with a mixture of 1 volume of *sulphuric acid R* and 9 volumes of *acetic anhydride R*. Examine the plate in ultraviolet light at 365 nm. In the chromatogram obtained with the test solution, no yellowish-red fluorescent zone (bisdemethoxycurcumin) appears slightly above the greenish-blue fluorescent zone of fluorescein in the chromatogram obtained with the reference solution.

Water (2.2.13). Not more than 120 ml/kg, determined by distillation on 20.0 g of powdered drug (500).

Total ash (2.4.16). Not more than 8.0 per cent.

ASSAY

Essential oil. Carry out the determination of essential oils in vegetable drugs (2.8.12). Use a 500 ml round-bottomed flask, 200 ml of *water R* as the distillation liquid and 0.5 ml of *xylene R* in the graduated tube. Reduce the drug to a powder (500) and immediately use 5.0 g for the determination. Distil at a rate of 3 ml/min to 4 ml/min for 3 h.

Dicinnamoyl methane derivatives. To 0.100 g of the powdered drug (180) add 60 ml of *glacial acetic acid R* and heat in a water-bath at 90 °C for 60 min. Add 2.0 g of *boric acid R* and 2.0 g of *oxalic acid R* and heat in a water-bath at 90 °C for 10 min. Allow to cool, dilute with *glacial acetic acid R* to 100.0 ml and shake. Dilute 5.0 ml of the clear supernatant with *glacial acetic acid R* to 50.0 ml. Measure the absorbance (2.2.25) at 530 nm, using *glacial acetic acid R* as the compensation liquid.

Calculate the percentage content of dicinnamoyl methane derivatives, expressed as curcumin, from the expression:

$$\frac{A \times 0.426}{m}$$

i.e. taking the specific absorbance to be 2350.

A = absorbance at 530 nm,

m = mass of the drug to be examined, in grams.

STORAGE

Store protected from light.

01/2005:1627

TURPENTINE OIL, PINUS PINASTER TYPE**Terebinthini aetheroleum ab pinum pinastrum****DEFINITION**

Essential oil obtained by steam distillation, followed by rectification at a temperature below 180 °C, from the oleoresin obtained by tapping *Pinus pinaster* Aiton. A suitable antioxidant may be added.

CHARACTERS

Appearance: clear, colourless or pale yellow liquid.

It has a characteristic odour.

IDENTIFICATION

First identification: B.

Second identification: A.

A. Thin-layer chromatography (2.2.27).

Test solution. Mix 1 ml of the substance to be examined with *toluene R* and dilute to 10 ml with the same solvent.

Reference solution. Mix 10 µl of *β-pinene R* and 10 µl of *linalol R* with *toluene R* and dilute to 10 ml with the same solvent.

Plate: *TLC silica gel plate R*.

Mobile phase: *ethyl acetate R, toluene R* (5:95 V/V).

Application: 10 µl, as bands.

Development: over a path of 15 cm.

Drying: in air.

Detection: spray with *anisaldehyde solution R* and heat at 100-105 °C for 5-10 min. Examine in daylight.

Results: see below the sequence of the zones present in the chromatograms obtained with the reference solution and the test solution.

Top of the plate	
β-Pinene: a pink zone	A pink zone (β-pinene)
	A pink zone
Linalol: a pinkish-grey zone	3 Faint violet zones
	A faint yellow zone
Reference solution	Test solution

B. Examine the chromatograms obtained in the test for chromatographic profile.

Results: the peaks in the chromatogram obtained with the test solution are similar in retention time to those in the chromatogram obtained with the reference solution.

TESTS

Relative density (2.2.5): 0.856 to 0.872.

Refractive index (2.2.6): 1.465 to 1.475.

Optical rotation (2.2.7): −40° to −28°.

Acid value (2.5.1): maximum 1.0.

Peroxide value (2.5.5, *Method B*): maximum 20.

Fatty oils and resinified essential oils (2.8.7). It complies with the test for fatty oils and resinified essential oils.

Chromatographic profile. Gas chromatography (2.2.28): use the normalisation procedure.

Test solution. The substance to be examined.

Reference solution (a). Dissolve 30 µl of *α-pinene R*, 10 mg of *camphene R*, 20 µl of *β-pinene R*, 10 µl of *car-3-ene R*, 10 µl of *β-myrcene R*, 20 µl of *limonene R*, 10 µl of *longifolene R*, 10 µl of *β-caryophyllene R* and 10 mg of *caryophyllene oxide R* in 1 ml of *hexane R*.

Reference solution (b). Dissolve 5 µl of *β-caryophyllene R* in *hexane R* and dilute to 1 ml with the same solvent. Dilute 0.1 ml to 1 ml with *hexane R*.

Column:

– *material:* fused silica,

– *size:* *l* = 60 m, Ø = 0.25 mm,

– *stationary phase:* *macrogol 20 000 R* (film thickness 0.25 µm).

Carrier gas: *helium for chromatography R*.

Flow rate: 1.0 ml/min.

Split ratio: 1:63.

Temperature:

	Time (min)	Temperature (°C)
Column	0 - 10	60
	10 - 80	60 → 200
	80 - 120	200
Injection port		200
Detector		250

Detection: flame ionisation.

Injection: 0.5 µl.

Elution order: order indicated in the composition of the reference solution (a); record the retention times of these substances.

System suitability:

– *resolution:* minimum 1.5 between the peaks due to *car-3-ene* and *β-myrcene* in the chromatogram obtained with reference solution (a).

Using the retention times determined from the chromatogram obtained with reference solution (a), locate the components of reference solution (a) in the chromatogram obtained with the test solution.

Determine the percentage content of these components. The limits are within the following ranges:

– *α-pinene:* 70.0 per cent to 85.0 per cent,

– *camphene:* 0.5 per cent to 1.5 per cent,

– *β-pinene:* 11.0 per cent to 20.0 per cent,

– *car-3-ene:* maximum 1.0 per cent,

– *β-myrcene:* 0.4 per cent to 1.5 per cent,

– *limonene:* 1.0 per cent to 7.0 per cent,

– *longifolene:* 0.2 per cent to 2.5 per cent,

– *β-caryophyllene:* 0.1 per cent to 3.0 per cent,

– *caryophyllene oxide:* maximum 1.0 per cent.

– *disregard limit:* area of the peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

Residue on evaporation: maximum 2.5 per cent.

STORAGE

In a well-filled, airtight container, protected from light and at a temperature not exceeding 25 °C.

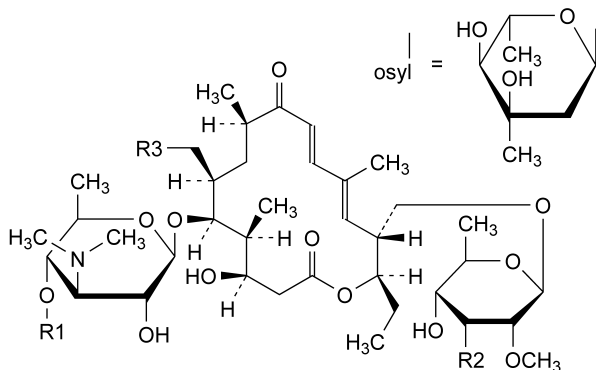
LABELLING

The label states the name and concentration of any added antioxidant.

01/2005:1273

TYLOSIN FOR VETERINARY USE

Tylosinum ad usum veterinarium



Name	Mol. Formula	R1	R2	R3
tylosin A	C ₄₆ H ₇₇ NO ₁₇	osyl	OCH ₃	CHO
tylosin B	C ₃₉ H ₆₅ NO ₁₄	H	OCH ₃	CHO
tylosin C	C ₄₅ H ₇₅ NO ₁₇	osyl	OH	CHO
tylosin D	C ₄₆ H ₇₉ NO ₁₇	osyl	OCH ₃	CH ₂ OH

DEFINITION

Tylosin for veterinary use is a mixture of macrolide antibiotics produced by a strain of *Streptomyces fradiae* or by any other means. The main component of the mixture is (4*R*,5*S*,6*S*,7*R*,9*R*,11*E*,13*E*,15*R*,16*R*)-15-[[[(6-deoxy-2,3-di-*O*-methyl-β-D-allopyranosyl)oxy]methyl]-6-[[[3,6-dideoxy-4-*O*-(2,6-dideoxy-3-*C*-methyl-α-*L*-ribo-hexopyranosyl)-3-(dimethylamino)-β-D-glucopyranosyl]oxy]-16-ethyl-4-hydroxy-5,9,13-trimethyl-7-(2-oxoethyl)oxacyclohexadeca-11,13-diene-2,10-dione (tylosin A, *M_r* 916). Tylosin B (desmycosin, *M_r* 772), tylosin C (macrocin, *M_r* 902) and tylosin D (relomycin, *M_r* 918) may also be present. They contribute to the potency of the substance to be examined, which is not less than 900 IU/mg, calculated with reference to the dried substance.

CHARACTERS

An almost white or slightly yellow powder, slightly soluble in water, freely soluble in ethanol and in methylene chloride. It dissolves in dilute solutions of mineral acids.

IDENTIFICATION

- Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *tylosin CRS*.
- Examine the chromatograms obtained in the test for composition. The retention time and size of the principal peak in the chromatogram obtained with the test solution are the same as those of the principal peak in the chromatogram obtained with reference solution (a).
- Dissolve about 30 mg in a mixture of 0.15 ml of *water R*, 2.5 ml of *acetic anhydride R* and 7.5 ml of *pyridine R*. Allow to stand for about 10 min. No green colour develops.

TESTS

pH (2.2.3). Suspend 0.25 g in 10 ml of *carbon dioxide-free water R*. The pH of the suspension is 8.5 to 10.5.

Composition. Examine by liquid chromatography (2.2.29). Prepare the solutions immediately before use.

The content of tylosin A is not less than 80.0 per cent and the sum of the contents of tylosin A, tylosin B, tylosin C and tylosin D is not less than 95.0 per cent.

Test solution. Dissolve 20.0 mg of the substance to be examined in a mixture of equal volumes of *acetonitrile R* and *water R* and dilute to 100.0 ml with the same mixture of solvents.

Reference solution (a). Dissolve 20.0 mg of *tylosin CRS* in a mixture of equal volumes of *acetonitrile R* and *water R* and dilute to 100.0 ml with the same mixture of solvents.

Reference solution (b). Dissolve 2 mg of *tylosin CRS* and 2 mg of *tylosin D CRS* in a mixture of equal volumes of *acetonitrile R* and *water R* and dilute to 10 ml with the same mixture of solvents.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.20 m long and 4.6 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (5 µm),
- as mobile phase at a flow rate of 1.0 ml/min a mixture of 40 volumes of *acetonitrile R* and 60 volumes of a 200 g/l solution of *sodium perchlorate R* previously adjusted to pH 2.5 using 1 *M* *hydrochloric acid*,
- as detector a spectrophotometer set at 290 nm, maintaining the temperature of the column at 35 °C.

Inject 20 µl of reference solution (b). When the chromatograms are recorded in the prescribed conditions, the retention time of tylosin A is about 12 min. The test is not valid unless, in the chromatogram obtained, the resolution between the peaks corresponding to tylosin A and tylosin D is at least 2.0. Inject 20 µl of the test solution and 20 µl of reference solution (a). Calculate the percentage content of the constituents from the areas of the peaks in the chromatogram obtained with the test solution by the normalisation procedure.

Tyramine. In a 25.0 ml volumetric flask, dissolve 50.0 mg of the substance to be examined in 5.0 ml of a 3.4 g/l solution of *phosphoric acid R*. Add 1.0 ml of *pyridine R* and 2.0 ml of a saturated solution of *ninhydrin R* (about 40 g/l). Close the flask with a piece of aluminium foil and heat in a water-bath at 85 °C for 30 min. Cool the solution rapidly and dilute to 25.0 ml with *water R*. Mix and measure immediately the absorbance (2.2.25) of the solution at 570 nm using a blank solution as the compensation liquid. The absorbance is not greater than that of a standard prepared at the same time and in the same manner using 5.0 ml of a 35 mg/l solution of *tyramine R* in a 3.4 g/l solution of *phosphoric acid R* (0.35 per cent). If intended for use in the manufacture of parenteral dosage forms, the absorbance is not greater than that of a standard prepared at the same time and in the same manner using 5.0 ml of a 15 mg/l solution of *tyramine R* in a 3.4 g/l solution of *phosphoric acid R* (0.15 per cent).

Loss on drying (2.2.32). Not more than 5.0 per cent, determined on 1.000 g by drying in an oven at 60 °C at a pressure not exceeding 0.7 kPa for 3 h.

Sulphated ash (2.4.14). Not more than 3.0 per cent, determined on 1.0 g.

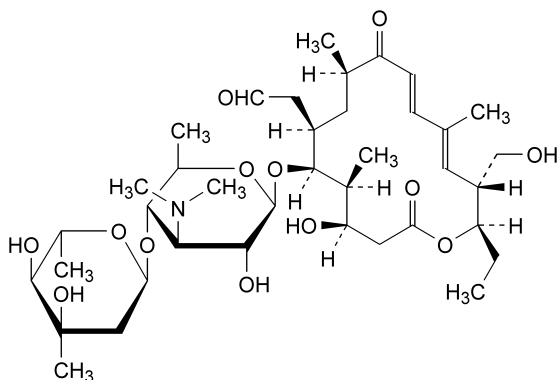
ASSAY

Carry out the microbiological assay of antibiotics (2.7.2). Use *tylosin CRS* as the reference substance.

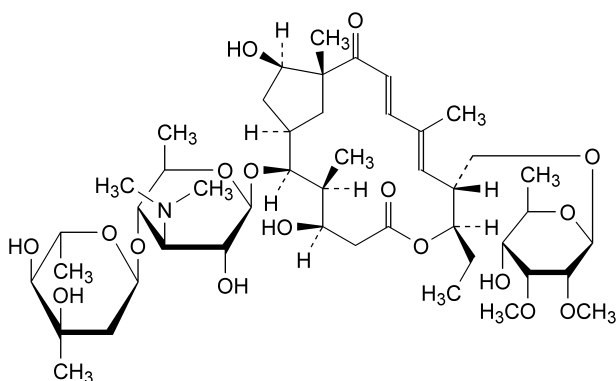
STORAGE

Store protected from light.

IMPURITIES



A. desmycosyltylosin,

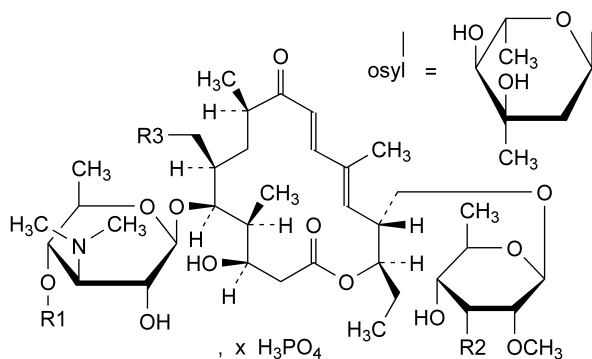


B. tylosin A aldol.

01/2005:1661

TYLOSIN PHOSPHATE BULK SOLUTION FOR VETERINARY USE

Tylosini phosphatis solutio ad usum
veterinarium



Tylosin	R1	R2	R3	Mol. Formula	<i>M_r</i>
A	osyl	OCH ₃	CHO	C ₄₆ H ₇₇ NO ₁₇	916
B	H	OCH ₃	CHO	C ₃₉ H ₆₅ NO ₁₄	772
C	osyl	OH	CHO	C ₄₅ H ₇₅ NO ₁₇	902
D	osyl	OCH ₃	CH ₂ OH	C ₄₆ H ₇₉ NO ₁₇	918

DEFINITION

Solution of the dihydrogen phosphate of a mixture of macrolide antibiotics produced by a strain of *Streptomyces fradiae* or by any other means.

The main component is the phosphate of (4*R*,5*S*,6*S*,7*R*,9*R*,11*E*,13*E*,15*R*,16*R*)-15-[[[(6-deoxy-2,3-di-*O*-methyl-β-*D*-allopyranosyl)oxy]methyl]-6-[[[3,6-dideoxy-4-*O*-(2,6-dideoxy-3-*C*-methyl-α-*L*-ribo-hexopyranosyl)-3-(dimethylamino)-β-*D*-glucopyranosyl]oxy]-16-ethyl-4-hydroxy-5,9,13-trimethyl-7-(2-oxoethyl)oxacyclohexadeca-11,13-diene-2,10-dione (tylosin A phosphate). The phosphates of tylosin B (desmycosin phosphate), tylosin C (macrocin phosphate) and tylosin D (relomycin phosphate) may also be present. The solution also contains sodium dihydrogen phosphate.

Potency: minimum 800 IU/mg of dry residue. Tylosins A, B, C and D contribute to the potency.

CHARACTERS

Appearance: yellow or brownish-yellow, viscous liquid.

Solubility: miscible with water.

IDENTIFICATION

A. Dilute an amount of the preparation to be examined equivalent to 400 000 IU of tylosin phosphate to 100.0 ml with *water R*. Dilute 1.0 ml of this solution to 100.0 ml with *water R*. Examined between 230 nm and 350 nm (2.2.25), the solution shows an absorption maximum at 290 nm. The absorbance at the maximum is not less than 0.70.

B. Examine the chromatograms obtained in the test for composition.

Results: the principal peak in the chromatogram obtained with the test solution is similar in retention time and size to the principal peak in the chromatogram obtained with reference solution (a).

C. Dilute an amount of the preparation to be examined equivalent to 400 000 IU of tylosin phosphate in 10 ml of *water R*. The solution gives reaction (a) of phosphates (2.3.1).

TESTS

pH (2.2.3): 5.5 to 6.5.

Dilute 1.0 g in 10 ml of *carbon dioxide-free water R*.

Composition. Liquid chromatography (2.2.29): use the normalisation procedure.

Prepare the solutions immediately before use.

Test solution. Dilute an amount of the preparation to be examined equivalent to 50 000 IU of tylosin phosphate to 200 ml with a mixture of equal volumes of *acetonitrile R* and *water R*.

Reference solution (a). Dissolve 20 mg of *tylosin CRS* in a mixture of equal volumes of *acetonitrile R* and *water R* and dilute to 100 ml with the same mixture of solvents.

Reference solution (b). Dissolve 2 mg of *tylosin CRS* and 2 mg of *tylosin D CRS* in a mixture of equal volumes of *acetonitrile R* and *water R* and dilute to 10 ml with the same mixture of solvents.

Reference solution (c). Dilute 1.0 ml of reference solution (a) to 100.0 ml with a mixture of equal volumes of *acetonitrile R* and *water R*. Dilute 1.0 ml of this solution to 10.0 ml with the same mixture of solvents.

Column:

- **size:** *l* = 0.20 m, Ø = 4.6 mm;
- **stationary phase:** octadecylsilyl silica gel for chromatography *R* (5 µm);
- **temperature:** 35 °C.

Mobile phase: mix 40 volumes of *acetonitrile R* and 60 volumes of a 200 g/l solution of *sodium perchlorate R* previously adjusted to pH 2.5 using a 36.5 g/l solution of *hydrochloric acid R*.

Flow rate: 1.0 ml/min.

Detection: spectrophotometer at 290 nm.

Injection: 20 µl.

Run time: 1.8 times the retention time of tylosin A.

Relative retention with reference to tylosin A (retention time = about 12 min): impurity A = about 0.35; tylosin C = about 0.5; tylosin B = about 0.6; tylosin D = about 0.85; impurity B = about 0.9 (use the chromatogram supplied with *tylosin CRS* to identify the peaks due to tylosin B and tylosin C).

System suitability: reference solution (b):

- **resolution:** minimum 2.0 between the peaks due to tylosin D and tylosin A.

Limits:

- **tylosin A:** minimum 80.0 per cent;
- **sum of the contents of tylosin A, tylosin B, tylosin C and tylosin D:** minimum 95.0 per cent;
- **disregard limit:** area of the principal peak in the chromatogram obtained with reference solution (c).

Tyramine. In a 25.0 ml volumetric flask, dissolve an amount of the preparation to be examined equivalent to 50 000 IU of tylosin phosphate in 5.0 ml of a 3.4 g/l solution of *phosphoric acid R*. Add 1.0 ml of *pyridine R* and 2.0 ml of a saturated solution of *ninhydrin R* (about 40 g/l). Close the flask with aluminium foil and heat in a water-bath at 85 °C for 20-30 min. Cool the solution rapidly and dilute to 25.0 ml with *water R*. Mix and measure immediately the absorbance (2.2.25) of the solution at 570 nm using a blank solution as the compensation liquid.

The absorbance is not greater than that of a standard prepared at the same time and in the same manner using 5.0 ml of a 35 mg/l solution of *tyramine R* in a 3.4 g/l solution of *phosphoric acid R*.

Phosphate: 8.5 per cent to 10.0 per cent of PO₄, calculated with reference to the dry residue (see Assay).

Test solution. Dissolve an amount of the preparation to be examined equivalent to 200 000 IU of tylosin phosphate in 50 ml of *water R*. Add 5.0 ml of *dilute sulphuric acid R* and dilute to 100.0 ml with *water R*. To 2.0 ml of this solution add successively, mixing after each addition, 10.0 ml of *water R*, 5.0 ml of *ammonium molybdate reagent R2*, 1.0 ml of *hydroquinone solution R* and 1.0 ml of a 200 g/l solution of *sodium metabisulphite R*. Allow to stand for at least 20 min and dilute to 50.0 ml with *water R*. Mix thoroughly.

Reference solution (a). To 1.0 ml of a standard solution containing 0.430 g/l of *potassium dihydrogen phosphate R* (corresponds to 300 ppm of PO₄) add successively, mixing after each addition, 10.0 ml of *water R*, 5.0 ml of *ammonium molybdate reagent R2*, 1.0 ml of *hydroquinone solution R* and 1.0 ml of a 200 g/l solution of *sodium metabisulphite R*. Allow to stand for at least 20 min and dilute to 50.0 ml with *water R*. Mix thoroughly.

Reference solution (b). Prepare as reference solution (a) but using 2.0 ml of the standard solution.

Reference solution (c). Prepare as reference solution (a) but using 5.0 ml of the standard solution.

Compensation liquid. Prepare as reference solution (a) but omitting the standard solution.

Measure the absorbance (2.2.25) of the test solution and of the reference solutions at 650 nm.

Draw a calibration curve with the absorbances of the 3 reference solutions as a function of the quantity of phosphate in the solutions and read from the curve the quantity of phosphate in the test solution.

Determine the percentage content of PO₄, calculated with reference to the dry residue (see Assay).

ASSAY

Carry out the microbiological assay of antibiotics (2.7.2).

Use *tylosin CRS* as the reference substance. Calculate the potency from the mass of the dry residue and the activity of the solution.

Dry residue. Dry 3.0 g of the preparation to be examined *in vacuo* at 60 °C for 3 h and weigh.

STORAGE

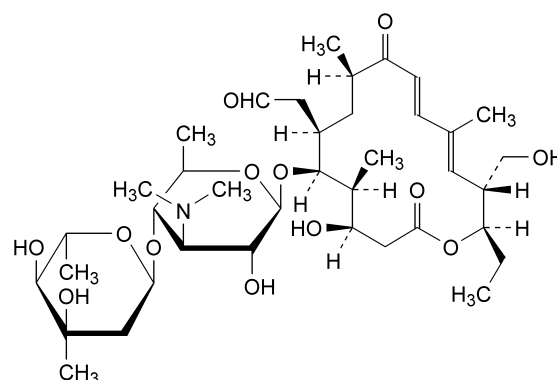
Protected from light, at a temperature of 2 °C to 8 °C.

LABELLING

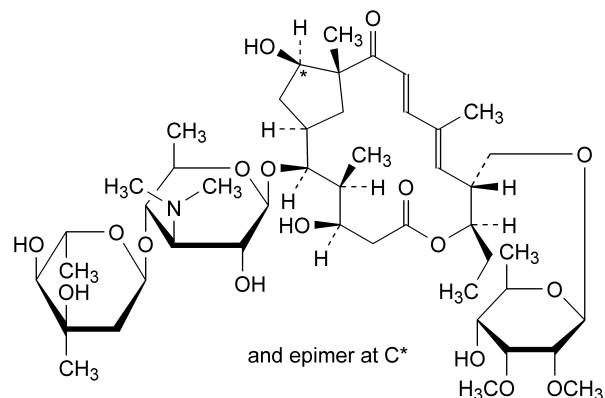
The label states:

- the concentration of the solution in International Units per milligram of preparation.

IMPURITIES



A. desmycinosyltylosin A,

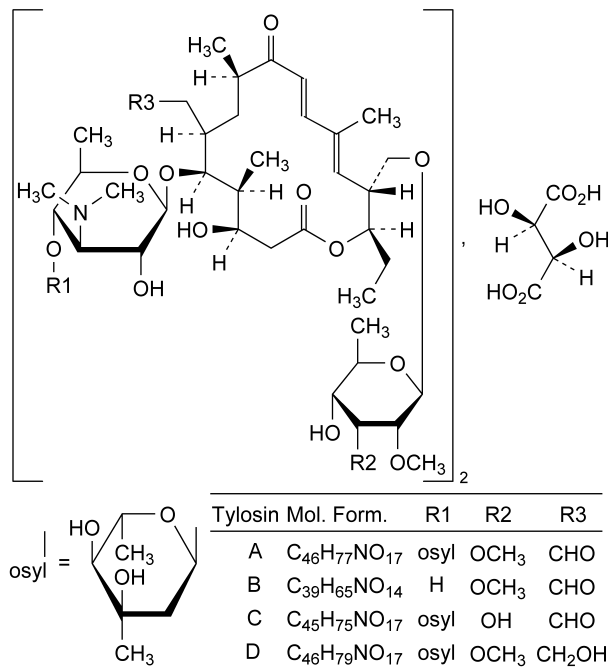


B. (1R,2S,3S,4R,8R,9R,10E,12E,15R,16RS)-9-[[[(6-deoxy-2,3-di-O-methyl-β-D-allopyranosyl)oxy]methyl]-2-[[[3,6-dideoxy-4-O-(2,6-dideoxy-3-C-methyl-α-L-ribo-hexopyranosyl)-3-(dimethylamino)-β-D-glucopyranosyl]oxy]-8-ethyl-4,16-dihydroxy-3,11,15-trimethyl-7-oxabicyclo[13.2.1]octadeca-10,12-diene-6,14-dione (tylosin A aldol).

01/2005:1274 TESTS

TYLOSIN TARTRATE FOR VETERINARY USE

Tylosini tartras ad usum veterinarium



DEFINITION

Tylosin tartrate for veterinary use is a tartrate of a mixture of macrolide antibiotics produced by a strain of *Streptomyces fradiae* or by any other means. The main component of the mixture is (4*R*,5*S*,6*S*,7*R*,9*R*,11*E*,13*E*,15*R*,16*R*)-15-[[[(6-deoxy-2,3-di-*O*-methyl-β-*D*-allopyranosyl)oxy]methyl]-6-[[[3,6-dideoxy-4-*O*-(2,6-dideoxy-3-*C*-methyl-α-*L*-ribo-hexopyranosyl)-3-(dimethylamino)-β-*D*-glucopyranosyl]oxy]-16-ethyl-4-hydroxy-5,9,13-trimethyl-7-(2-oxoethyl)oxacyclohexadeca-11,13-diene-2,10-dione (tylosin A, tartrate *M_r* 1982). Tylosin B (desmycosin, tartrate *M_r* 1694), tylosin C (macrocin, tartrate *M_r* 1954) and tylosin D (relomycin, tartrate *M_r* 1986) may also be present. They contribute to the potency of the substance to be examined, which is not less than 800 IU/mg, calculated with reference to the dried substance.

CHARACTERS

An almost white or slightly yellow, hygroscopic powder, freely soluble in water and in methylene chloride, slightly soluble in ethanol. It dissolves in dilute solutions of mineral acids.

IDENTIFICATION

- Examine by infrared absorption spectrophotometry (2.2.24), comparing with the *Ph. Eur. reference spectrum of tylosin tartrate*.
- Examine the chromatograms obtained in the test for composition. The retention time and size of the principal peak in the chromatogram obtained with the test solution are the same as those of the principal peak in the chromatogram obtained with reference solution (a).
- Dissolve about 30 mg in a mixture of 0.15 ml of *water R*, 2.5 ml of *acetic anhydride R* and 7.5 ml of *pyridine R*. Allow to stand for about 10 min. A green colour is produced.

pH (2.2.3). Dissolve 0.25 g in 10 ml of *carbon dioxide-free water R*. The pH of the solution is 5.0 to 7.2.

Composition. Examine by liquid chromatography (2.2.29). Prepare the solutions immediately before use.

The content of tylosin A is not less than 80.0 per cent and the sum of the contents of tylosin A, tylosin B, tylosin C and tylosin D is not less than 95.0 per cent.

Test solution. Dissolve 20.0 mg of the substance to be examined in a mixture of equal volumes of *acetonitrile R* and *water R* and dilute to 100.0 ml with the same mixture of solvents.

Reference solution (a). Dissolve 20.0 mg of *tylosin CRS* in a mixture of equal volumes of *acetonitrile R* and *water R* and dilute to 100.0 ml with the same mixture of solvents.

Reference solution (b). Dissolve 2 mg of *tylosin CRS* and 2 mg of *tylosin D CRS* in a mixture of equal volumes of *acetonitrile R* and *water R* and dilute to 10 ml with the same mixture of solvents.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.20 m long and 4.6 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (5 μm),
- as mobile phase at a flow rate of 1.0 ml/min a mixture of 40 volumes of *acetonitrile R* and 60 volumes of a 200 g/l solution of *sodium perchlorate R* previously adjusted to pH 2.5 using 1 *M hydrochloric acid*,
- as detector a spectrophotometer set at 290 nm, maintaining the temperature of the column at 35 °C.

Inject 20 μl of reference solution (b). When the chromatograms are recorded in the prescribed conditions, the retention time of tylosin A is about 12 min. The test is not valid unless, in the chromatogram obtained, the resolution between the peaks corresponding to tylosin A and tylosin D is at least 2.0. Inject 20 μl of the test solution and 20 μl of reference solution (a). Calculate the percentage content of the constituents from the areas of the peaks in the chromatogram obtained with the test solution by the normalisation procedure.

Tyramine. In a 25.0 ml volumetric flask, dissolve 50.0 mg of the substance to be examined in 5.0 ml of a 3.4 g/l solution of *phosphoric acid R*. Add 1.0 ml of *pyridine R* and 2.0 ml of a saturated solution of *ninhydrin R* (about 40 g/l). Close the flask with a piece of aluminium foil and heat in a water-bath at 85 °C for 30 min. Cool the solution rapidly and dilute to 25.0 ml with *water R*. Mix and measure immediately the absorbance (2.2.25) of the solution at 570 nm using a blank solution as the compensation liquid. The absorbance is not greater than that of a standard prepared at the same time and in the same manner using 5.0 ml of a 35 mg/l solution of *tyramine R* in a 3.4 g/l solution of *phosphoric acid R* (0.35 per cent). If intended for use in the manufacture of parenteral dosage forms, the absorbance is not greater than that of a standard prepared at the same time and in the same manner using 5.0 ml of a 15 mg/l solution of *tyramine R* in a 3.4 g/l solution of *phosphoric acid R* (0.15 per cent).

Loss on drying (2.2.32). Not more than 4.5 per cent, determined on 1.000 g by drying at 60 °C at a pressure not exceeding 0.7 kPa for 3 h.

Sulphated ash (2.4.14). Not more than 2.5 per cent, determined on 1.0 g.

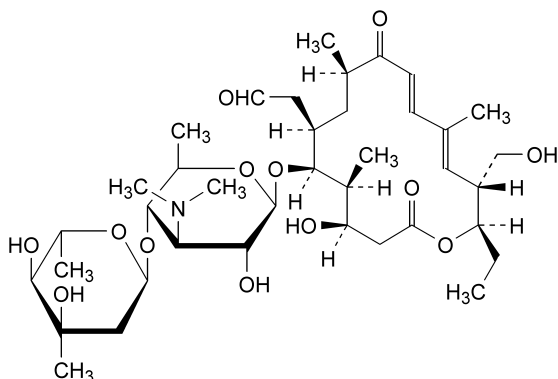
ASSAY

Carry out the microbiological assay of antibiotics (2.7.2). Use *tylosin CRS* as the reference substance.

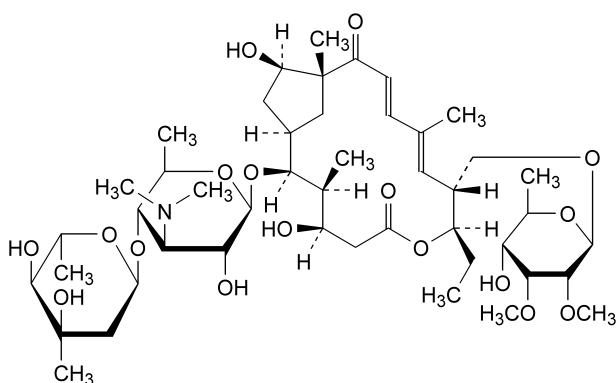
STORAGE

Store in an airtight container, protected from light.

IMPURITIES



A. desmynosyltylosin,

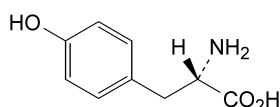


B. tylosin A aldol.

01/2005:1161

TYROSINE

Tyrosinum

 $C_9H_{11}NO_3$ M_r 181.2

DEFINITION

Tyrosine contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of (S)-2-amino-3-(4-hydroxyphenyl)propanoic acid, calculated with reference to the dried substance.

CHARACTERS

A white crystalline powder or colourless crystals, very slightly soluble in water, practically insoluble in alcohol. It dissolves in dilute mineral acids and in dilute solutions of alkali hydroxides.

IDENTIFICATION

First identification: A, B.

Second identification: A, C, D, E.

- A. It complies with the test for specific optical rotation (see Tests).
- B. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with tyrosine CRS. Examine the substances prepared as discs.

- C. Examine the chromatograms obtained in the test for ninhydrin-positive substances. The principal spot in the chromatogram obtained with test solution (b) is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).
- D. To about 50 mg add 1 ml of *dilute nitric acid R*. A dark red colour is produced within 15 min.
- E. Dissolve about 30 mg in 2 ml of *dilute sodium hydroxide solution R*. Add 3 ml of a freshly prepared mixture of equal volumes of a 100 g/l solution of *sodium nitrite R* and a solution of 0.5 g of *sulphanilic acid R* in a mixture of 6 ml of *hydrochloric acid R1* and 94 ml of *water R*. An orange-red colour is produced.

TESTS

Appearance of solution. Dissolve 0.5 g in *dilute hydrochloric acid R* and dilute to 20 ml with the same acid. The solution is clear (2.2.1) and not more intensely coloured than reference solution Y₇ (2.2.2, *Method II*).

Specific optical rotation (2.2.7). Dissolve 1.25 g in a mixture of equal volumes of *dilute hydrochloric acid R* and *water R* and dilute to 25.0 ml with the same mixture of solvents. The specific optical rotation is –11.0 to –12.3, calculated with reference to the dried substance.

Ninhydrin-positive substances. Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel plate R*.

Test solution (a). Dissolve 0.10 g of the substance to be examined in *dilute ammonia R2* and dilute to 10 ml with the same solvent.

Test solution (b). Dilute 1 ml of test solution (a) to 50 ml with *water R*.

Reference solution (a). Dissolve 10 mg of *tyrosine CRS* in 1 ml of *dilute ammonia R2* and dilute to 50 ml with *water R*.

Reference solution (b). Dilute 5 ml of test solution (b) to 20 ml with *water R*.

Reference solution (c). Dissolve 10 mg of *tyrosine CRS* and 10 mg of *phenylalanine CRS* in 1 ml of *dilute ammonia R2* and dilute to 25 ml with *water R*.

Apply to the plate 5 µl of each solution. Develop over a path of 15 cm using a mixture of 30 volumes of *concentrated ammonia R1* and 70 volumes of *propanol R*. Allow the plate to dry in air, spray with *ninhydrin solution R* and heat at 100 °C to 105 °C for 15 min. Any spot in the chromatogram obtained with test solution (a), apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.5 per cent). The test is not valid unless the chromatogram obtained with reference solution (c) shows two clearly separated spots.

Chlorides (2.4.4). Dissolve 0.25 g in 3 ml of *dilute nitric acid R* and dilute to 15 ml with *water R*. The solution complies with the limit test for chlorides, without any further addition of nitric acid (200 ppm).

Sulphates (2.4.13). Dissolve with gentle heating, 0.5 g in 5 ml of *dilute hydrochloric acid R* and dilute to 15 ml with *distilled water R*. The solution complies with the limit test for sulphates (300 ppm).

Ammonium (2.4.1). 0.10 g complies with limit test B for ammonium (200 ppm). Prepare the standard using 0.2 ml of *ammonium standard solution (100 ppm NH₄) R*. Replace the *heavy magnesium oxide R* by 2.0 ml of *strong sodium hydroxide solution R*.

Iron (2.4.9). In a separating funnel, dissolve 1.0 g in 10 ml of *dilute hydrochloric acid R*. Shake with three quantities, each of 10 ml, of *methyl isobutyl ketone R1*, shaking for

3 min each time. To the combined organic layers add 10 ml of *water R* and shake for 3 min. The aqueous layer complies with the limit test for iron (10 ppm).

Heavy metals (2.4.8). 2.0 g complies with limit test C for heavy metals (10 ppm). Prepare the standard using 2 ml of *lead standard solution (10 ppm Pb R)*.

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.150 g in 5 ml of *anhydrous formic acid R*. Add 30 ml of *anhydrous acetic acid R*. Titrate with 0.1 M *perchloric acid*, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *perchloric acid* is equivalent to 18.12 mg of C₉H₁₁NO₃.

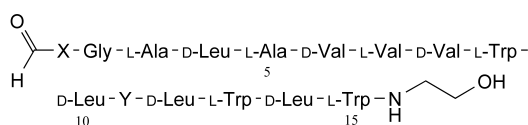
STORAGE

Store protected from light.

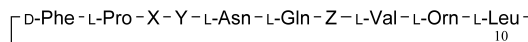
01/2005:1662

TYROTHRICIN

Tyrothricinum



Gramicidin	Mol. formula	<i>M_r</i>	X	Y
A1	C ₉₉ H ₁₄₀ N ₂₀ O ₁₇	1882	L-Val	L-Trp
A2	C ₁₀₀ H ₁₄₂ N ₂₀ O ₁₇	1896	L-Ile	L-Trp
C1	C ₉₇ H ₁₃₉ N ₁₉ O ₁₈	1859	L-Val	L-Tyr
C2	C ₉₈ H ₁₄₁ N ₁₉ O ₁₈	1873	L-Ile	L-Tyr



Tyrocidin	Mol. formula	<i>M_r</i>	X	Y	Z
A	C ₆₆ H ₈₈ N ₁₃ O ₁₃	1271	L-Phe	D-Phe	L-Tyr
B	C ₆₈ H ₈₉ N ₁₄ O ₁₃	1311	L-Trp	D-Phe	L-Tyr
C	C ₇₀ H ₉₀ N ₁₅ O ₁₃	1350	L-Trp	D-Trp	L-Tyr
D	C ₇₂ H ₉₁ N ₁₆ O ₁₂	1373	L-Trp	D-Trp	L-Trp
E	C ₆₆ H ₈₈ N ₁₃ O ₁₂	1255	L-Phe	D-Phe	L-Phe

DEFINITION

Mixture of antimicrobial linear and cyclic polypeptides, isolated from the fermentation broth of *Brevibacillus brevis* Dubos. It consists mainly of gramicidins and tyrocidins as described above; other related compounds may be present in smaller amounts.

Potency: 180 IU/mg to 280 IU/mg (dried substance).

CHARACTERS

Appearance: white or almost white powder.

Solubility: practically insoluble in water, soluble in alcohol and in methanol.

IDENTIFICATION

First identification: B.

Second identification: A.

A. Thin-layer chromatography (2.2.27).

Test solution. Dissolve 5 mg of the substance to be examined in 4.0 ml of *alcohol R*.

Reference solution. Dissolve 5 mg of *tyrothricin CRS* in 4.0 ml of *alcohol R*.

Plate: TLC silica gel *F₂₅₄* plate *R*.

Mobile phase: *methanol R*, *butanol R*, *water R*, *acetic acid R*, *butyl acetate R* (2.5:7.5:12:20:40 V/V/V/V/V).

Application: 1 µl.

Development: over 2/3 of the plate.

Drying: in a current of warm air.

Detection A: examine in ultraviolet light at 254 nm.

Results A: the principal spots or groups of principal spots in the chromatogram obtained with the test solution are similar in position and size to the principal spots or groups of principal spots in the chromatogram obtained with the reference solution. The upper group corresponds to gramicidins, the lower group to tyrocidins.

Detection B: spray with *dimethylaminobenzaldehyde solution R2*. Heat the plate in a current of warm air until the spots appear.

System suitability: reference solution:

- the chromatogram shows 2 clearly separated spots or groups of spots.

Results B: the principal spots or groups of principal spots in the chromatogram obtained with the test solution are similar in position, colour and size to the principal spots or groups of principal spots in the chromatogram obtained with the reference solution. The upper group corresponds to gramicidins, the lower group to tyrocidins.

B. It complies with the test for composition (see Tests).

TESTS

Composition. Liquid chromatography (2.2.29): use the normalisation procedure. *Prepare the solutions immediately before use.*

Test solution. Dissolve 25 mg of the substance to be examined in 10 ml of *methanol R* and dilute to 25.0 ml with the mobile phase.

Reference solution (a). Dissolve 25 mg of *tyrothricin CRS* in 10 ml of *methanol R* and dilute to 25.0 ml with the mobile phase.

Reference solution (b). Dilute 1.0 ml of reference solution (a) to 50.0 ml with the mobile phase.

Column:

- *size:* *l* = 0.25 m, Ø = 4.6 mm,
- *stationary phase:* octadecylsilyl silica gel for chromatography *R* (5 µm),
- *temperature:* 60 °C.

Mobile phase: 0.79 g/l solution of *ammonium sulphate R*, *methanol R* (25:75 V/V).

Flow rate: 1.2 ml/min.

Detection: spectrophotometer at 280 nm.

Injection: 25 µl.

Run time: 6 times the retention time of gramicidin A1. Use the chromatogram obtained with reference solution (a) and the chromatogram supplied with *tyrothricin CRS* to identify the peaks due to gramicidin A1, gramicidin A2 and the tyrocidins.

Relative retention with reference to gramicidin A1 (retention time = about 10 min): gramicidin C1 = about 0.8; gramicidin C2 = about 0.9; gramicidin A2 = about 1.1; tyrocidins = about 1.5 to 6.

System suitability: reference solution (a):

- **resolution:** minimum 1.5 between the peaks due to gramicidin A1 and gramicidin A2.

Composition:

- **sum of gramicidins:** 25 per cent to 50 per cent,
- **sum of tyrocidins:** 50 per cent to 70 per cent,
- **total:** minimum 85 per cent,

- **disregard limit:** the sum of the areas of the peaks due to gramicidins in the chromatogram obtained with reference solution (b).

Loss on drying (2.2.32): maximum 4.0 per cent, determined on 1.000 g by drying under high vacuum at 60 °C for 3 h.

Sulphated ash (2.4.14): maximum 1.5 per cent, determined on 1.0 g.

ASSAY

Carry out the microbiological assay of antibiotics (2.7.2) using the turbidimetric method. Use *gramicidin CRS* as the reference substance.

Test solution. Prepare a solution of tyrothricin containing about the same amount of gramicidin as the corresponding solution of *gramicidin CRS* i.e. 5 times more concentrated.

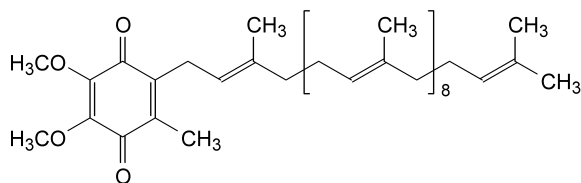
STORAGE

In an airtight container, protected from light.

01/2005:1578 *Relative retention* with reference to ubidecarenone (retention time = about 12 min): impurity D = about 0.67.

UBIDECARENONE

Ubidecarenonum



$C_{59}H_{90}O_4$

M_r 863

DEFINITION

2-[(all-*E*)-3,7,11,15,19,23,27,31,35,39-Decamethyltetracont-2,6,10,14,18,22,26,30,34,38-decaenyl]-5,6-dimethoxy-3-methylbenzene-1,4-dione.

Content: 97.0 per cent to 103.0 per cent.

CHARACTERS

Appearance: yellow or orange, crystalline powder.

Solubility: practically insoluble in water, soluble in acetone, very slightly soluble in ethanol.

It gradually decomposes and darkens on exposure to light.
mp: about 48 °C.

Carry out all operations avoiding exposure to light.

IDENTIFICATION

A. Infrared absorption spectrophotometry (2.2.24).

Preparation: discs.

Comparison: ubidecarenone CRS.

B. Examine the chromatograms obtained in the test for related substances.

Results: the retention time of the principal peak in the chromatogram obtained with the test solution is similar to that of the principal peak in the chromatogram obtained with reference solution (a).

TESTS

Related substances. Liquid chromatography (2.2.29).

Test solution. Dissolve 25.0 mg of the substance to be examined in 25.0 ml of *ethanol R* by heating at about 50 °C for 2 min. Allow to cool.

Reference solution (a). Dissolve 5 mg of ubidecarenone CRS in 5 ml of *ethanol R* by heating at about 50 °C for 2 min. Allow to cool.

Reference solution (b). Dissolve 2 mg of ubidecarenone impurity D CRS in 2 ml of the test solution by heating at about 50 °C for 2 min. Allow to cool. Dilute 1 ml to 50 ml with *ethanol R*.

Reference solution (c). Dilute 1.0 ml of the test solution to 100.0 ml with *ethanol R*.

Column:

- size: $l = 0.15$ m, $\varnothing = 4.6$ mm,
- stationary phase: octadecylsilyl silica gel for chromatography R (5 μ m).

Mobile phase: *ethanol R*, *methanol R2* (20:80 V/V).

Flow rate: 2 ml/min.

Detection: spectrophotometer at 275 nm.

Injection: 10 μ l.

Run time: 2 times the retention time of ubidecarenone.

System suitability: reference solution (b):

- *resolution*: minimum 6.5 between the peaks due to impurity D and to ubidecarenone.

Limits:

- *any impurity*: not more than half the area of the principal peak in the chromatogram obtained with reference solution (c) (0.5 per cent),
- *total*: not more than the area of the principal peak in the chromatogram obtained with reference solution (c) (1.0 per cent),
- *disregard limit*: 0.05 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.05 per cent).

Impurity F. Liquid chromatography (2.2.29).

Test solution. Dissolve 25.0 mg of the substance to be examined in 25.0 ml of *hexane R*.

Reference solution (a). Dissolve 10.0 mg of ubidecarenone for system suitability CRS in 10.0 ml of *hexane R*.

Reference solution (b). Dilute 1 ml of the test solution to 100.0 ml with *hexane R*.

Column:

- size: $l = 0.25$ m, $\varnothing = 4.0$ mm,
- stationary phase: silica gel for chromatography R (7 μ m).

Mobile phase: *ethyl acetate R*, *hexane R* (3:97 V/V).

Flow rate: 2 ml/min.

Detection: spectrophotometer at 275 nm.

Injection: 20 μ l.

Run time: 1.2 times the retention time of ubidecarenone.

Relative retention with reference to ubidecarenone (retention time = about 10 min): impurity F = about 0.85.

System suitability: reference solution (a):

- *resolution*: minimum 1.5 between the peaks due to impurity F and to ubidecarenone.

Limit:

- *impurity F*: not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent).

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

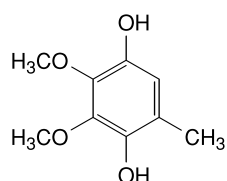
Dissolve 50.0 mg in 1.0 ml of *hexane R* and dilute to 50.0 ml with *ethanol R*. Dilute 2.0 ml of the solution to 50.0 ml with *ethanol R*. Measure the absorbance (2.2.25) at the maximum at 275 nm. Calculate the content of $C_{59}H_{90}O_4$ taking the specific absorbance to be 169.

STORAGE

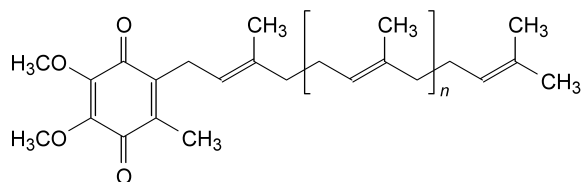
Store in an airtight container, protected from light.

IMPURITIES

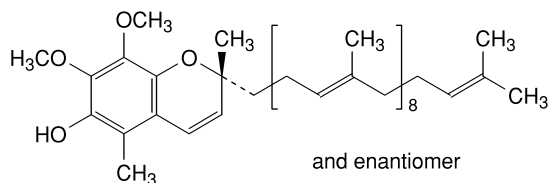
Specified impurities: A, B, C, D, E, F.



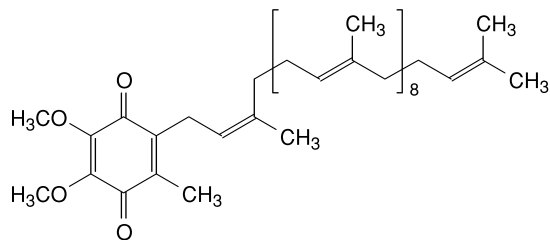
A. 2,3-dimethoxy-5-methylbenzene-1,4-diol,



- B. $n = 5$: 2-[(all-*E*)-3,7,11,15,19,23,27-heptamethyloctadocosa-2,6,10,14,18,22,26-heptaenyl]-5,6-dimethoxy-3-methylbenzene-1,4-dione (ubiquinone-7),
- C. $n = 6$: 5,6-dimethoxy-3-methyl-2-[(all-*E*)-3,7,11,15,19,23,27,31-octamethyldotriaconta-2,6,10,14,18,22,26,30-octaenyl]benzene-1,4-dione (ubiquinone-8),
- D. $n = 7$: 5,6-dimethoxy-3-methyl-2-[(all-*E*)-3,7,11,15,19,23,27,31,35-nonamethylhexatriaconta-2,6,10,14,18,22,26,30,34-nonaenyl]benzene-1,4-dione (ubiquinone-9),



- E. (2*RS*)-7,8-dimethoxy-2,5-dimethyl-2-[(all-*E*)-4,8,12,16,20,24,28,32,36-nonamethylheptatriaconta-3,7,11,15,19,23,27,31,35-nonaenyl]-2*H*-1-benzopyran-6-ol (ubiquinol-9),

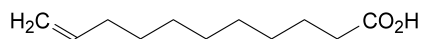


- F. 2-[(2*Z*,6*E*,10*E*,14*E*,18*E*,22*E*,26*E*,30*E*,34*E*,38*E*)-3,7,11,15,19,23,27,31,35,39-decamethyl-2,6,10,14,18,22,26,30,34,38-tetracontadecaenyl]-5,6-dimethoxy-3-methylbenzene-1,4-dione (ubidecarenone (*Z*)-isomer).

01/2005:0461

UNDECYLENIC ACID

Acidum undecylenicum

 $C_{11}H_{20}O_2$ M_r 184.3

DEFINITION

Undecylenic acid contains not less than 97.0 per cent and not more than the equivalent of 102.0 per cent of undec-10-enoic acid.

CHARACTERS

A white or very pale yellow, crystalline mass or colourless or pale yellow liquid, practically insoluble in water, freely soluble in alcohol and in fatty and essential oils.

IDENTIFICATION

- A. Refractive index (2.2.6): 1.447 to 1.450, determined at 25 ± 0.5 °C.
- B. Freezing point (2.2.18): 21 °C to 24 °C.

- C. To 2.0 g add 2 ml of freshly distilled *aniline R* and boil under a reflux condenser for 10 min. Allow to cool and add 30 ml of *ether R*. Shake with three quantities, each of 20 ml, of *dilute hydrochloric acid R* and then with 20 ml of *water R*. Evaporate the organic layer to dryness on a water-bath. The residue, after recrystallisation twice from *alcohol (70 per cent V/V) R* and drying *in vacuo* for 3 h, melts (2.2.14) at 66 °C to 68 °C.
- D. Dissolve 0.1 g in a mixture of 2 ml of *dilute sulphuric acid R* and 5 ml of *glacial acetic acid R*. Add dropwise 0.25 ml of *potassium permanganate solution R*. The colour of the potassium permanganate is discharged.

TESTS

Peroxide value (2.5.5). Not more than 10.

Fixed and mineral oils. To 1.0 g add 5 ml of *sodium carbonate solution R* and 25 ml of *water R* and boil for 3 min. The hot solution is not more opalescent than reference suspension II (2.2.1).

Water-soluble acids. To 1.0 g add 20 ml of *water R* heated to 35 °C to 45 °C and shake for 2 min. Cool and filter the aqueous layer through a moistened filter. To 10 ml of the filtrate add 0.1 ml of *phenolphthalein solution R*. Not more than 0.1 ml of 0.1 *M sodium hydroxide* is required to change the colour of the indicator.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 0.50 g.

Degree of unsaturation. Dissolve 85.0 mg in a mixture of 5 ml of *dilute hydrochloric acid R* and 30 ml of *glacial acetic acid R*. Using 0.05 ml of *indigo carmine solution R1*, added towards the end of the titration, as indicator, titrate with 0.0167 *M bromide-bromate* until the colour changes from blue to yellow. 8.9 ml to 9.4 ml of 0.0167 *M bromide-bromate* is required. Carry out a blank titration.

ASSAY

Dissolve 0.750 g in 10 ml of *alcohol R*. Using 0.1 ml of *phenolphthalein solution R* as indicator, titrate with 0.5 *M sodium hydroxide* until a pink colour is obtained.

1 ml of 0.5 *M sodium hydroxide* is equivalent to 92.14 mg of $C_{11}H_{20}O_2$.

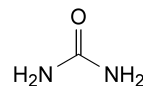
STORAGE

Store in a non-metallic container, protected from light.

01/2005:0743

UREA

Ureum

 CH_4N_2O M_r 60.1

DEFINITION

Carbamide.

Content: 98.5 per cent to 101.5 per cent (dried substance).

CHARACTERS

Appearance: white, crystalline powder or transparent crystals, slightly hygroscopic.

Solubility: very soluble in water, soluble in alcohol, practically insoluble in methylene chloride.

IDENTIFICATION

First identification: A, B.

Second identification: A, C, D.

A. Melting point (2.2.14): 132 °C to 135 °C.

B. Infrared absorption spectrophotometry (2.2.24).

Preparation: discs.

Comparison: urea CRS.

C. Dissolve 0.1 g in 1 ml of *water R*. Add 1 ml of *nitric acid R*. A white, crystalline precipitate is formed.

D. Heat 0.5 g in a test tube until it liquefies and the liquid becomes turbid. Cool, dissolve in a mixture of 1 ml of *dilute sodium hydroxide solution R* and 10 ml of *water R* and add 0.05 ml of *copper sulphate solution R*. A reddish-violet colour is produced.

TESTS

Solution S. Dissolve 10.0 g in *water R* and dilute to 50 ml with the same solvent.

Appearance of solution. The solution is clear (2.2.1) and colourless (2.2.2, *Method II*).

To 2.5 ml of solution S add 7.5 ml of *water R*.

Alkalinity. To 2.5 ml of solution S add 7.5 ml of *water R*, 0.1 ml of *methyl red solution R* and 0.4 ml of 0.01 M *hydrochloric acid*. The solution is red to orange.

Biuret: maximum 0.1 per cent.

To 10 ml of solution S add 5 ml of *water R*, 0.5 ml of a 5 g/l solution of *copper sulphate R* and 0.5 ml of *strong sodium hydroxide solution R*. Allow to stand for 5 min. Any reddish-violet colour in the solution is not more intense than that in a standard prepared at the same time in the same manner using 10 ml of a 0.2 g/l solution of *biuret R*.

Ammonium (2.4.1): maximum 500 ppm, determined on 0.1 ml of solution S.

Heavy metals (2.4.8): maximum 10 ppm.

Dilute 10 ml of solution S to 20 ml with *water R*. 12 ml of the solution complies with limit test A. Prepare the standard using *lead standard solution (1 ppm Pb) R*.

Loss on drying (2.2.32): maximum 1.0 per cent, determined on 1.000 g by drying in an oven at 100–105 °C for 1 h.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.2000 g in *water R* and dilute to 50.0 ml with the same solvent. Introduce 1.0 ml of this solution into a combustion flask. Add 4 g of a powdered mixture of 100 g of *dipotassium sulphate R*, 5 g of *copper sulphate R* and 2.5 g of *selenium R*, and 3 glass beads. Wash any adhering particles from the neck into the flask with 5 ml of *sulphuric acid R*, allowing it to run down the sides of the flask, and mix the contents by rotation. Close the mouth of the flask loosely, for example by means of a glass bulb with a short stem, to avoid excessive loss of sulphuric acid. Heat gradually at first, then increase the temperature until there is vigorous boiling with condensation of sulphuric acid in the neck of the flask; take precautions to prevent the upper part of the flask from becoming overheated. Continue the heating for 30 min. Cool, dissolve the solid material by cautiously adding to the mixture 25 ml of *water R*, cool again and place in a steam-distillation apparatus. Add 30 ml of *strong sodium hydroxide solution R* and distil immediately by passing steam through the mixture. Collect the distillate in 15 ml of a 40 g/l solution of *boric acid R* to which has been added 0.2 ml of *methyl red mixed solution R* and enough *water R* to cover the tip of the condenser. Towards

the end of the distillation, lower the receiver so that the tip of the condenser is above the surface of the acid. Take precautions to prevent any water on the outer surface of the condenser from reaching the contents of the receiver. Titrate the distillate with 0.01 M *sulphuric acid*.

1 ml of 0.01 M *sulphuric acid* is equivalent to 0.6006 mg of CH₄N₂O.

STORAGE

In an airtight container.

01/2005:0958

UROFOLLITROPIN

Urofollitropinum

DEFINITION

Urofollitropin is a dry preparation containing menopausal gonadotrophin obtained from the urine of post-menopausal women. It has follicle-stimulating activity and no or virtually no luteinising activity. The potency is not less than 90 International Units of follicle-stimulating hormone (hFSH) per milligram. The ratio of units of luteinising hormone (interstitial-cell-stimulating hormone) [hLH(ICSH)] to units of follicle-stimulating hormone is not more than 1/60.

PRODUCTION

It may be prepared by a suitable fractionation procedure followed by immunoaffinity chromatography. It is prepared in conditions designed to minimise microbial contamination. The manufacturing process must have been shown to reduce any viral contamination such as hepatitis virus or HIV by appropriate validated methods.

CHARACTERS

Appearance: almost white or slightly yellowish powder.

Solubility: soluble in water.

IDENTIFICATION

When administered as prescribed in the assay it causes enlargement of the ovaries of immature female rats.

TESTS

Hepatitis virus antigens. Examined by a suitably sensitive immunochemical method (2.7.1), hepatitis virus antigens are not detected.

HIV antigen. Examined by a suitably sensitive immunochemical method (2.7.1), HIV antigen is not detected.

Residual luteinising activity. The International Units of FSH and LH are the activities contained in stated amounts of the International Standard of human urinary follicle-stimulating hormone and luteinising hormone (interstitial-cell-stimulating hormone) which consists of a mixture of a freeze-dried extract of urine of post-menopausal women with lactose. The equivalence in International Units of the International Standard is stated by the World Health Organisation. Use immature female rats approximately 21 days old and having masses such that the difference between the heaviest and the lightest rat is not more than 10 g. Assign the rats at random to 4 equal groups of at least 6 animals. If sets of 4 litter mates are available, assign one litter mate at random from each set to each group and mark according to litter.

Inject subcutaneously into each rat 50 IU of *serum gonadotrophin R* on the first day and 25 IU of *chorionic gonadotrophin R* on the fourth day, each in 0.5 ml of *phosphate-albumin buffered saline pH 7.2 R*.

Choose 3 doses of the reference preparation such that the smallest dose produces a depletion of the ovarian ascorbic acid content in all the rats and the largest dose does not produce a maximal depletion in all the rats. Use doses in geometric progression; as an initial approximation, total doses of 0.5 IU, 1.0 IU and 2.0 IU may be tried although the dose to be used will depend on the sensitivity of the animals.

Choose a dose of the preparation to be examined expected to contain 60X IU of follicle-stimulating hormone (hFSH), in which X = the number of IU of hLH in the middle dose of the reference preparation.

Dissolve separately the total quantities of the preparation to be examined and of the reference preparation in 1.0 ml of *phosphate-albumin buffered saline pH 7.2 R*. Inject into a tail vein to each separate group of rats 6 days after the injection of chorionic gonadotrophin. Exactly 4 h after the injection, kill the rats and remove the ovaries from each animal. Remove any extraneous fluid and tissue from the ovaries and weigh the ovaries immediately.

Treat the combined ovaries from each rat separately, as follows. Crush and homogenise within 2 min in a freshly prepared 25 g/l solution of *metaphosphoric acid R* at a temperature of 4 °C and dilute to 7 ml with the same solution. Allow the homogenate to stand for 30 min at 4 °C and centrifuge at 4 °C at approximately 2500 g. Filter the supernatant liquid, if necessary, through a 0.22 µm filter.

Prepare a fresh solution consisting of a mixture of 2 ml of a 45.3 g/l solution of *sodium acetate R* adjusted to pH 7 with *acetic acid R*, 3 ml of *water R* and 2 ml of *dichlorophenolindophenol standard solution R*. Mix 2 ml of this solution with 2 ml of the clear supernatant liquid. 30 s after mixing, measure the absorbance (2.2.25) of the solution at the maximum at about 520 nm. Use as reference a solution with a known content of *ascorbic acid CRS* in a 25 g/l solution of *metaphosphoric acid R*, treated by the same process.

Calculate the amount of ascorbic acid from the ascorbic acid standard curve obtained and express in milligrams per 0.1 g of ovary to obtain the ascorbic acid content of the ovaries. Calculate the mean and its variance of the ascorbic acid content of the ovaries of the rats treated with the preparation to be examined.

For each dose-group of the reference preparation, plot the mean ascorbic acid content of the ovaries as a function of the logarithm of the dose and analyse the regression of the ascorbic acid content on the logarithm of the dose injected, using standard methods of analysis (the method of least squares).

The test is not valid unless:

- the slope constant *b* is significant at the 5 per cent level of significance,
- for the groups treated with the reference preparation, the sum of squares due to linear regression is equal to at least 95 per cent of the total sum of squares of the ascorbic acid content,
- the within-group variance of the ascorbic acid content of the group receiving the preparation to be examined is not significantly different at the 5 per cent level of significance from the within-group variance of the ascorbic acid content of the groups receiving the reference preparation.

The mean ascorbic acid content of the ovaries of the rats treated with the preparation to be examined is not significantly lower than that of the rats treated with the middle dose of the reference preparation (calculated from the regression equation) at the 5 per cent level of significance.

Water (2.5.32): maximum 5.0 per cent.

Bacterial endotoxins (2.6.14, *Method C*): less than 0.40 IU per IU of urofollitropin, if intended for use in the manufacture of parenteral dosage forms without a further appropriate procedure for the removal of bacterial endotoxins.

ASSAY

The follicle-stimulating activity of urofollitropin is estimated by comparing under given conditions its effect in enlarging the ovaries of immature rats treated with chorionic gonadotrophin with the same effect of the International Standard preparation of human urinary follicle-stimulating hormone and luteinising hormone or of a reference preparation calibrated in International Units. The International Units of FSH and LH are the activities contained in stated amounts of the International Standard of human urinary follicle-stimulating hormone and luteinising hormone (interstitial-cell-stimulating hormone) which consists of a mixture of a freeze-dried extract of urine of post-menopausal women with lactose. The equivalence in International Units of the International Standard is stated by the World Health Organisation.

Use immature female rats of the same strain, 19 to 28 days old, differing in age by not more than 3 days and having masses such that the difference between the heaviest and the lightest rat is not more than 10 g. Assign the rats at random to 6 equal groups of at least 5 animals. If sets of 6 litter mates are available, assign one litter mate from each set to each group and mark according to litter.

Choose 3 doses of the reference preparation and 3 doses of the preparation to be examined such that the smallest dose produces a positive response in some of the rats and the largest dose does not produce a maximal response in all the rats. Use doses in geometric progression and as an initial approximation total doses of 1.5 IU, 3.0 IU and 6.0 IU may be tried although the dose will depend on the sensitivity of the animals used, which may vary widely.

Dissolve separately the total quantities of the preparation to be examined and of the reference preparation corresponding to the daily doses to be used in sufficient *phosphate-albumin buffered saline pH 7.2 R* such that the daily dose is administered in a volume of about 0.5 ml. The buffer solution shall contain in the daily dose not less than 14 IU of chorionic gonadotrophin to ensure complete luteinisation. Add a suitable antimicrobial preservative such as 4 g/l of phenol or 0.02 g/l of thiomersal. Store the solutions at 5 ± 3 °C.

Inject subcutaneously into each rat the daily dose allocated to its group. Repeat the injection of each dose 24 h and 48 h after the first injection. About 24 h after the last injection, kill the rats and remove the ovaries from each animal. Remove any extraneous fluid and tissue from the ovaries and weigh the 2 combined ovaries of each animal immediately. Calculate the results by the usual statistical methods, using the mass of the 2 combined ovaries as the response. (The precision of the assay may be improved by a suitable correction of the organ mass with reference to the mass of the animal from which it was taken; an analysis of covariance may be used).

The estimated potency is not less than 80 per cent and not more than 125 per cent of the stated potency. The confidence limits (*P* = 0.95) of the estimated potency are not less than 64 per cent and not more than 156 per cent of the stated potency.

STORAGE

In an airtight, tamper-proof container, protected from light, at a temperature of 2 °C to 8 °C. If the substance is sterile, store in a sterile, airtight, tamper-proof container.

LABELLING

The label states:

- the activity expressed in International Units of follicle-stimulating hormone per container,
- the potency expressed in International Units of follicle-stimulating hormone per milligram,
- where applicable, that the substance is free from bacterial endotoxins.

01/2005:0695

UROKINASE

Urokinasum

DEFINITION

Urokinase is an enzyme, obtained from human urine, that activates plasminogen. It consists of a mixture of low-molecular-mass (LMM) (M_r 33 000) and high-molecular-mass (HMM) (M_r 54 000) forms, the high-molecular-mass form being predominant. The potency is not less than 70 000 International Units per milligram of protein.

PRODUCTION

It is produced by validated methods of manufacturing designed to minimise or eliminate microbial and viral contamination and vasoactive substances; in particular, adequate measures to inactivate viruses are taken, such as heating of the substance in solution at 60 °C for 10 h.

CHARACTERS

A white or almost white, amorphous powder, soluble in water.

IDENTIFICATION

- Place separately in two haemolysis tubes 0.5 ml of citrated human plasma and 0.5 ml of citrated bovine plasma and maintain in a water-bath at 37 °C. To each tube add 0.1 ml of a solution containing a quantity of the substance to be examined equivalent to 1000 IU/ml in *phosphate buffer solution pH 7.4 R* and 0.1 ml of a solution containing a quantity of *humanthrombin R* equivalent to 20 IU/ml in *phosphate buffer solution pH 7.4 R*. Shake immediately. In both tubes, a clot forms and lyses within 30 min.
- Carry out identification by a suitable immunodiffusion test.

TESTS

Appearance of solution. Dissolve 10 mg in 10 ml of *water R*. The solution is clear (2.2.1) and colourless (2.2.2, *Method II*).

Hepatitis B surface antigen. Examine by a suitably sensitive method such as radio-immunoassay. Hepatitis B surface antigen is not detected.

Thromboplastic contaminants

Test solutions. Dissolve suitable quantities of the substance to be examined in *barbital buffer solution pH 7.4 R* to obtain solutions with activities of 5000 IU/ml, 2500 IU/ml, 1250 IU/ml, 625 IU/ml and 312 IU/ml.

To each of six haemolysis tubes 1 cm in internal diameter add 0.1 ml of *citrated rabbit plasma R*. Allocate the test solutions one to each of five of the tubes; add to each tube 0.1 ml of the solution allocated to it and to the sixth tube add 0.1 ml of *barbital buffer solution pH 7.4 R* (blank). Incubate the tubes at 25 ± 0.5 °C for 5 min and add 0.1 ml of a 3.675 g/l solution of *calcium chloride R*. Measure with a stop-watch

the coagulation time for each tube. Plot the shortening of the recalcification time (clotting time of the blank minus clotting time measured) against log concentration in International Units. Extrapolate the best-fitting straight line through the five points until it reaches the log-concentration axis. The urokinase activity at the intersection point, which represents the limit concentration for coagulant activity (zero coagulant activity), is not less than 150 IU/ml.

Molecular fractions. Examine by size-exclusion chromatography (2.2.30).

Test solution. Dissolve about 1 mg of the substance to be examined in 1.0 ml of *0.02 M phosphate buffer pH 8.0 R*. Prepare immediately before use.

The chromatographic procedure may be carried out using:

- a column 0.9 m long and 16 mm in internal diameter packed with *cross-linked dextran for chromatography R3*,
- as mobile phase at a flow rate of 6 ml/h a 17.5 g/l solution of *sodium chloride R* in *0.02 M phosphate buffer solution pH 8.0 R*,

equilibrating the column and operating at 5 °C. Apply the test solution to the head of the column rinsing twice with 0.5 ml portions of the buffer and carry out the elution. The eluate may be collected in fractions of 1 ml. Measure the absorbance (2.2.25) of the eluate at the maximum at 280 nm and plot the individual values on a graph. Draw perpendicular lines towards the axis of the abscissae from the minima before the HMM peak, between the HMM and the LMM peaks, and after the LMM peak, thus identifying the fractions to be considered in calculating the HMM/LMM activity ratio. Pool the HMM fractions and, separately, the LMM fractions. Determine separately the urokinase activity in International Units of each of the fraction pools by the method prescribed under Assay. The ratio of the urokinase activity in the HMM fraction pool to that in the LMM fraction pool is not less than 2.0.

Total protein. Determine the nitrogen content, using 10 mg, by the method of sulphuric acid digestion (2.5.9) and calculate the quantity of protein by multiplying by 6.25.

Pyrogens (2.6.8). If intended for use in the manufacture of parenteral dosage forms without a further appropriate procedure for the removal of pyrogen, it complies with the test for pyrogens. Inject per kilogram of the rabbit's mass 1.0 ml of a sterile 9 g/l solution of *sodium chloride R* containing a quantity of the substance to be examined equivalent to 20 000 IU/ml.

ASSAY

The potency of urokinase is determined by comparing its capacity to activate plasminogen to form plasmin with the same capacity of a reference preparation of urokinase calibrated in International Units; the formation of plasmin is measured by the determination of the lysis time of a fibrin clot in given conditions.

The International Unit is the activity contained in a stated amount of the International Reference Preparation, which consists of freeze-dried urokinase with lactose. The equivalence in International Units of the International Reference Preparation is stated by the World Health Organisation.

Unless otherwise prescribed, use *phosphate buffer solution pH 7.4 R* containing 30 g/l of *bovine albumin R* for the preparation of the solutions and dilutions used in the assay.

Test solution. Prepare a solution of the substance to be examined expected to have an activity of 1000 IU/ml.

Reference solution. Prepare a solution of a reference preparation having an activity of 1000 IU/ml.

Keep the test solution and the reference solution in iced water and use within 6 h. Prepare three serial 1.5-fold dilutions of the reference preparation such that the longest clot-lysis time is less than 20 min and the shortest clot-lysis time is greater than 3 min. Prepare three similar dilutions of the test solution. Keep the solutions in iced water and use within 1 h. Use twenty-four tubes 8 mm in diameter. Label the tubes T_1 , T_2 and T_3 for the dilutions of the test solution and S_1 , S_2 and S_3 for the dilutions of the reference solution, allocating four tubes to each dilution. Place the tubes in iced water. Into each tube, introduce 0.2 ml of the appropriate dilution, 0.2 ml of *phosphate buffer solution pH 7.4 R* containing 30 g/l of *bovine albumin R* and 0.1 ml of a solution of *humanthrombin R* having an activity of not less than 20 IU/ml. Place the tubes in a water-bath at 37 °C and allow to stand for 2 min to attain temperature equilibrium. Using an automatic pipette, introduce into the bottom of the first tube 0.5 ml of a 10 g/l solution of *bovine euglobulins R*, ensuring mixing. At intervals of 5 s, introduce successively into the remaining tubes 0.5 ml of a 10 g/l solution of *bovine euglobulins R*. Using a stop-watch, measure for each tube the time in seconds that elapses between the addition of the euglobulins solution and the lysis of the clot. Plot the logarithms of the lysis times for the substance to be examined and for the reference preparation against the logarithms of the concentration and calculate the activity of the substance to be examined using the usual statistical methods.

The estimated potency is not less than 90 per cent and not more than 111 per cent of the stated potency. The confidence limits ($P = 0.95$) of the estimated potency are not less than 80 per cent and not more than 125 per cent of the stated potency.

STORAGE

Store in an airtight container, protected from light, at a temperature not exceeding 8 °C. If the substance is sterile, store in a sterile, airtight, tamper-proof container.

LABELLING

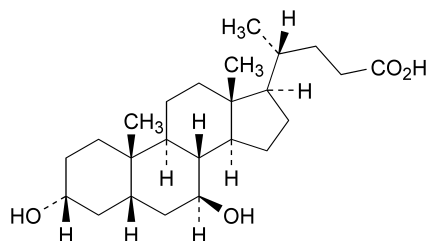
The label states:

- the potency in International Units per milligram of protein,
- where applicable, that the contents are apyrogenic.

01/2005:1275

URSODEOXYCHOLIC ACID

Acidum ursodeoxycholicum



$C_{24}H_{40}O_4$

M_r 392.6

DEFINITION

Ursodeoxycholic acid contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of 3 α ,7 β -dihydroxy-5 β -cholan-24-oic acid, calculated with reference to the dried substance.

CHARACTERS

A white or almost white powder, very slightly soluble in water, freely soluble in alcohol, slightly soluble in acetone and in methylene chloride.

It melts at about 202 °C.

IDENTIFICATION

First identification: A.

Second identification: B, C.

- Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *ursodeoxycholic acid CRS*. Examine the substances as discs prepared using *potassium bromide R*.
- Examine the chromatograms obtained in the test for related substances. The principal spot in the chromatogram obtained with test solution (b) is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).
- Dissolve about 10 mg in 1 ml of *sulphuric acid R*. Add 0.1 ml of *formaldehyde solution R* and allow to stand for 5 min. Add 5 ml of *water R*. The suspension obtained is greenish-blue.

TESTS

Specific optical rotation (2.2.7). Dissolve 0.500 g in *ethanol R* and dilute to 25.0 ml with the same solvent. The specific optical rotation is + 58.0 to + 62.0, calculated with reference to the dried substance.

Related substances. Examine by thin-layer chromatography (2.2.27), using a suitable silica gel as the coating substance.

Test solution (a). Dissolve 0.40 g of the substance to be examined in a mixture of 1 volume of *water R* and 9 volumes of *acetone R* and dilute to 10 ml with the same mixture of solvents.

Test solution (b). Dilute 1 ml of test solution (a) to 10 ml with a mixture of 1 volume of *water R* and 9 volumes of *acetone R*.

Reference solution (a). Dissolve 40 mg of *ursodeoxycholic acid CRS* in a mixture of 1 volume of *water R* and 9 volumes of *acetone R* and dilute to 10 ml with the same mixture of solvents.

Reference solution (b). Dissolve 20 mg of *lithocholic acid CRS* in a mixture of 1 volume of *water R* and 9 volumes of *acetone R* and dilute to 10 ml with the same mixture of solvents. Dilute 2 ml of the solution to 100 ml with a mixture of 1 volume of *water R* and 9 volumes of *acetone R*.

Reference solution (c). Dissolve 20 mg of *chenodeoxycholic acid CRS* in a mixture of 1 volume of *water R* and 9 volumes of *acetone R* and dilute to 50 ml with the same mixture of solvents.

Reference solution (d). Dissolve 20 mg of *cholic acid CRS* in a mixture of 1 volume of *water R* and 9 volumes of *acetone R* and dilute to 100 ml with the same mixture of solvents.

Reference solution (e). Dilute 0.5 ml of test solution (a) to 20 ml with a mixture of 1 volume of *water R* and 9 volumes of *acetone R*. Dilute 1 ml of the solution to 10 ml with a mixture of 1 volume of *water R* and 9 volumes of *acetone R*.

Reference solution (f). Dissolve 10 mg of *ursodeoxycholic acid CRS* in reference solution (c) and dilute to 25 ml with the same solution.

Apply to the plate 5 μ l of each solution. Develop in an unsaturated tank over a path of 15 cm using a mixture of 1 volume of *glacial acetic acid R*, 30 volumes of *acetone R* and 60 volumes of *methylene chloride R*. Dry the plate at 120 °C for 10 min. Spray the plate immediately with a 47.6 g/l solution of *phosphomolybdic acid R* in a mixture

of 1 volume of *sulphuric acid R* and 20 volumes of *glacial acetic acid R* and heat again at 120 °C until blue spots appear on a lighter background. In the chromatogram obtained with test solution (a): any spot corresponding to lithocholic acid is not more intense than the principal spot in the chromatogram obtained with reference solution (b) (0.1 per cent); any spot corresponding to chenodeoxycholic acid is not more intense than the principal spot in the chromatogram obtained with reference solution (c) (1 per cent); any spot corresponding to cholic acid is not more intense than the principal spot in the chromatogram obtained with reference solution (d) (0.5 per cent); any spot, apart from the principal spot and any spots corresponding to lithocholic acid, chenodeoxycholic acid and cholic acid, is not more intense than the principal spot in the chromatogram obtained with reference solution (e) (0.25 per cent). The test is not valid unless the chromatogram obtained with reference solution (f) shows two clearly separated principal spots.

Heavy metals (2.4.8). 1.0 g complies with limit test C for heavy metals (20 ppm). Prepare the standard using 2 ml of *lead standard solution (10 ppm Pb) R*.

Loss on drying (2.2.32). Not more than 1.0 per cent, determined on 1.000 g by drying in an oven at 100-105 °C.

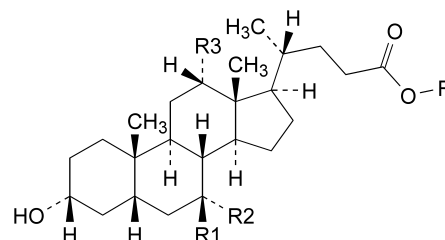
Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.350 g in 50 ml of *alcohol R*, previously neutralised to 0.2 ml of *phenolphthalein solution R*. Add 50 ml of *water R* and titrate with 0.1 M *sodium hydroxide* until a pink colour is obtained.

1 ml of 0.1 M *sodium hydroxide* is equivalent to 39.26 mg of $C_{24}H_{40}O_4$.

IMPURITIES



- A. R = R₁ = R₃ = H, R₂ = OH: chenodeoxycholic acid,
- B. R = R₁ = H, R₂ = R₃ = OH: 3 α ,7 α ,12 α -trihydroxy-5 β -cholan-24-oic acid (cholic acid),
- C. R = R₁ = R₂ = R₃ = H: 3 α -hydroxy-5 β -cholan-24-oic acid (lithocholic acid),
- D. R = R₂ = H, R₁ = R₃ = OH: 3 α ,7 β ,12 α -trihydroxy-5 β -cholan-24-oic acid (ursocholic acid),
- E. R = R₁ = R₂ = H, R₃ = OH: 3 α ,12 α -dihydroxy-5 β -cholan-24-oic acid (deoxycholic acid),
- F. R = R₃ = H, R₁+R₂ = O: 3 α -hydroxy-7-oxo-5 β -cholan-24-oic acid,
- G. R = CH₃, R₁ = OH, R₂ = R₃ = H: methyl 3 α ,7 β -dihydroxy-5 β -cholan-24-oate.

01/2005:0453

VALERIAN ROOT

Valerianae radix

DEFINITION

Valerian root consists of the dried, whole or fragmented underground parts of *Valeriana officinalis* L. *s.l.*, including the rhizome surrounded by the roots and stolons. It contains not less than 5 ml/kg of essential oil for the whole drug and not less than 3 ml/kg of essential oil for the cut drug, both calculated with reference to the dried drug and not less than 0.17 per cent of sesquiterpenic acids expressed as valerenic acid ($C_{15}H_{22}O_2$; M_r 234), calculated with reference to the dried drug.

CHARACTERS

Valerian root has a characteristic odour.

It has the macroscopic and microscopic characters described under identification tests A and B.

IDENTIFICATION

- A. The rhizome is yellowish-grey to pale brownish-grey, obconical to cylindrical, up to about 50 mm long and 30 mm in diameter; the base is elongated or compressed, usually entirely covered by numerous roots. The apex usually exhibits a cup-shaped scar from the aerial parts; stem bases are rarely present. When cut longitudinally, the pith exhibits a central cavity transversed by septa. The roots are numerous, almost cylindrical, of the same colour as the rhizome, 1 mm to 3 mm in diameter and sometimes more than 100 mm long. A few filiform fragile secondary roots are present. The fracture is short. The stolons show prominent nodes separated by longitudinally striated internodes, each 20 mm to 50 mm long, with a fibrous fracture.
- B. Reduce to a powder (355). The powder is pale yellowish-grey to pale greyish-brown. Examine under a microscope using *chloral hydrate solution R*. The powder shows cells containing a pale brown resin or droplets of essential oil; isolated rectangular sclereids with pitted walls 5 µm to 15 µm thick; reticulately-thickened xylem vessels; occasional fragments of cork cells and epidermal cells, some with root hairs. Examine under a microscope using a 50 per cent V/V solution of *glycerol R*. The powder shows numerous fragments of parenchyma with cells, containing single or compound starch granules; the single granules are rounded or elongated, 5 µm to 15 µm in diameter and have sometimes a cleft or radiate hilum; the compound granules have two to six components with an overall diameter of up to 20 µm.
- C. Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel plate R*.

Test solution. Shake 1.0 g of the powdered drug (355) with 6.0 ml of *methanol R* in a 25 ml flask for 15 min and filter. Rinse the flask and the filter with the aid of a small portion of *methanol R* to obtain 5 ml of filtrate. Evaporate the filtrate to about 2 ml and add 3 ml of a 100 g/l solution of *potassium hydroxide R*. Shake with two quantities, each of 5 ml, of *methylene chloride R*. Allow to separate and discard the lower layer. Heat the aqueous layer in a water-bath at 40 °C for 10 min. Cool and add *dilute hydrochloric acid R* until it gives an acid reaction. Shake with two quantities, each of 5 ml, of *methylene chloride R*. Filter the combined lower

layers over *anhydrous sodium sulphate R*. Evaporate the filtrate to dryness. Dissolve the residue in 1.0 ml of *methylene chloride R*.

Reference solution. Dissolve 5 mg of *fluorescein R* and 5 mg of *Sudan red G R* in 20.0 ml of *methanol R*.

Apply to the plate as bands 20 µl of each solution. Develop over a path of 10 cm using a mixture of 0.5 volumes of *glacial acetic acid R*, 35 volumes of *ethyl acetate R* and 65 volumes of *hexane R*. Allow the plate to dry in air. Examine in daylight. The chromatogram obtained with the reference solution shows in its middle part the red zone of Sudan red G and in its lower part the greenish-yellow zone of fluorescein. Spray the plate with *anisaldehyde solution R*. Examine in daylight while heating at 100 °C to 105 °C for 5 min to 10 min. The chromatogram obtained with the test solution shows a violet-blue zone corresponding to hydroxyvalerenic acid at about the same height as the zone of fluorescein in the chromatogram obtained with the reference solution and also shows a violet zone corresponding to valerenic acid at about the same height as that of the zone due to Sudan red G in the chromatogram obtained with the reference solution. The chromatogram obtained with the test solution shows in the upper half other, mostly less intense, pink to violet zones.

TESTS

Foreign matter (2.8.2). Not more than 5 per cent of stem bases and not more than 2 per cent of other foreign matter.

Loss on drying (2.2.32). Not more than 12.0 per cent, determined on 1.000 g of well homogenised powdered drug (355) by drying in an oven at 100 °C to 105 °C, for 2 h.

Total ash (2.4.16). Not more than 12.0 per cent.

Ash insoluble in hydrochloric acid (2.8.1). Not more than 5.0 per cent.

ASSAY

Essential oil. Carry out the determination of essential oils in vegetable drugs (2.8.12). Use 40.0 g of freshly powdered drug (500), a 2000 ml flask, 500 ml of *water R* as the distillation liquid and 0.50 ml of *xylene R* in the graduated tube. Distil at a rate of 3 ml/min to 4 ml/min for 4 h.

Sesquiterpenic acids. Examine by liquid chromatography (2.2.29).

Test solution. Place 1.50 g of the powdered drug (710) in a 100 ml round-bottomed flask with a ground-glass neck. Add 20 ml of *anhydrous methanol R*. Mix and heat on a water-bath under a reflux condenser for 30 min. Allow to cool and filter. Place the filter with the residue in the 100 ml round-bottomed flask. Add 20 ml of *anhydrous methanol R* and heat on a water-bath under the reflux condenser for 15 min. Allow to cool and filter. Combine the filtrates and dilute to 50.0 ml with *anhydrous methanol R* rinsing the round-bottomed flask and the filter.

Reference solution. Prepare the solution immediately before use, protected from bright light. Dissolve 30 mg of *dantrol R* in *anhydrous methanol R* and dilute to 100.0 ml with the same solvent. Dilute 5.0 ml of the solution to 50.0 ml with *anhydrous methanol R*.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (5 µm),
- as mobile phase at a flow rate of 1.5 ml/min:
Mobile phase A. Mix 20 volumes of *acetonitrile R* and 80 volumes of a 5 g/l solution of *phosphoric acid R*,

Mobile phase B. Mix 80 volumes of *acetonitrile R* and 20 volumes of a 5 g/l solution of *phosphoric acid R*,

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)	Comment
0 - 5	55	45	isocratic
5 - 18	55 → 20	45 → 80	linear gradient
18 - 20	20	80	isocratic
20 - 22	20 → 55	80 → 45	linear gradient

– as detector a spectrophotometer set at 220 nm,

– a 20 µl loop injector.

Inject the test solution and the reference solution.

When the chromatograms are recorded in the prescribed conditions, the relative retention times (to dantron) are: acetoxyvalerenic acid about 0.7 and valerenic acid about 1.2.

Calculate the percentage content of acetoxyvalerenic acid using the expression:

$$\frac{A_2 \times m_1 \times 11.51}{A_1 \times m_2}$$

Calculate the percentage content of valerenic acid using the expression:

$$\frac{A_3 \times m_1 \times 8.09}{A_1 \times m_2}$$

Or calculate the percentage content of both sesquiterpenic acids using the expression:

$$\frac{\left[\frac{A_2 \times 11.51}{A_1} + \frac{A_3 \times 8.09}{A_1} \right] \times m_1}{m_2}$$

A_1 = area of the peak due to dantron in the chromatogram obtained with the reference solution,

A_2 = area of the peak due to acetoxyvalerenic acid in the chromatogram obtained with the test solution,

A_3 = area of the peak due to valerenic acid in the chromatogram obtained with the test solution,

m_1 = mass of dantron, in grams, used to prepare the reference solution,

m_2 = mass of the drug to be examined, in grams.

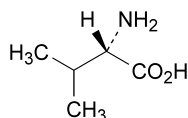
STORAGE

Store protected from light.

01/2005:0796

VALINE

Valinum



$C_5H_{11}NO_2$

M_r 117.1

DEFINITION

Valine contains not less than 98.5 per cent and not more than the equivalent of 101.0 per cent of (S)-2-amino-3-methylbutanoic acid, calculated with reference to the dried substance.

CHARACTERS

White or almost white, crystalline powder or colourless crystals, soluble in water, very slightly soluble in alcohol.

IDENTIFICATION

First identification: A, B.

Second identification: A, C.

A. It complies with the test for specific optical rotation (see Tests).

B. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *valine CRS*. Examine the substances prepared as discs.

C. Examine the chromatograms obtained in the test for ninhydrin-positive substances. The principal spot in the chromatogram obtained with test solution (b) is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).

TESTS

Solution S. Dissolve 2.5 g in *water R* and dilute to 100 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and not more intensely coloured than reference solution BY₆ (2.2.2, Method II).

Specific optical rotation (2.2.7). Dissolve 2.00 g in *hydrochloric acid R1* and dilute to 25.0 ml with the same acid. The specific optical rotation is + 26.5 to + 29.0, calculated with reference to the dried substance.

Ninhydrin-positive substances. Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel plate R*.

Test solution (a). Dissolve 0.10 g of the substance to be examined in *dilute hydrochloric acid R* and dilute to 10 ml with the same acid.

Test solution (b). Dilute 1 ml of test solution (a) to 50 ml with *water R*.

Reference solution (a). Dissolve 10 mg of *valine CRS* in 0.1 M *hydrochloric acid* and dilute to 50 ml with the same acid.

Reference solution (b). Dilute 5 ml of test solution (b) to 20 ml with *water R*.

Reference solution (c). Dissolve 10 mg of *phenylalanine CRS* and 10 mg of *valine CRS* in 0.1 M *hydrochloric acid* and dilute to 25 ml with the same acid.

Apply to the plate 5 µl of each solution. Develop over a path of 15 cm using a mixture of 20 volumes of *glacial acetic acid R*, 20 volumes of *water R* and 60 volumes of *butanol R*. Allow the plate to dry in air, spray with *ninhydrin solution R* and heat at 100 °C to 105 °C for 15 min. Any spot in the chromatogram obtained with test solution (a), apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.5 per cent). The test is not valid unless the chromatogram obtained with reference solution (c) shows two clearly separated spots.

Chlorides (2.4.4). Dilute 10 ml of solution S to 15 ml with *water R*. The solution complies with the limit test for chlorides (200 ppm).

Sulphates (2.4.13). Dissolve 0.5 g in *distilled water R* and dilute to 15 ml with the same solvent. The solution complies with the limit test for sulphates (300 ppm).

Ammonium (2.4.1). 50 mg complies with limit test B for ammonium (200 ppm). Prepare the standard using 0.1 ml of *ammonium standard solution (100 ppm NH₄) R*.

Iron (2.4.9). In a separating funnel, dissolve 1.0 g in 10 ml of *dilute hydrochloric acid R*. Shake with three quantities, each of 10 ml, of *methyl isobutyl ketone R1*, shaking for

3 min each time. To the combined organic layers add 10 ml of *water R* and shake for 3 min. The aqueous layer complies with the limit test for iron (10 ppm).

Heavy metals (2.4.8). 2.0 g complies with limit test D for heavy metals (10 ppm). Prepare the standard using 2 ml of *lead standard solution (10 ppm Pb) R*.

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.100 g in 3 ml of *anhydrous formic acid R*. Add 30 ml of *anhydrous acetic acid R*. Using 0.1 ml of *naphtholbenzein solution R* as indicator, titrate with 0.1 M *perchloric acid* until the colour changes from brownish-yellow to green.

1 ml of 0.1 M *perchloric acid* is equivalent to 11.71 mg of $C_8H_{16}O_2$.

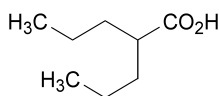
STORAGE

Store protected from light.

01/2005:1378

VALPROIC ACID

Acidum valproicum



$C_8H_{16}O_2$

M_r 144.2

DEFINITION

Valproic acid contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of 2-propylpentanoic acid.

CHARACTERS

A colourless or very slightly yellow, clear liquid, slightly viscous, very slightly soluble in water, miscible with alcohol and with methylene chloride. It dissolves in dilute solutions of alkali hydroxides.

IDENTIFICATION

First identification: B.

Second identification: A, C, D.

A. Refractive index (2.2.5): 1.422 to 1.425.

B. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *valproic acid CRS*.

C. Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel plate R*.

Test solution. Dissolve 50 mg of the substance to be examined in *methanol R* and dilute to 5 ml with the same solvent.

Reference solution. Dissolve 50 mg of *valproic acid CRS* in *methanol R* and dilute to 5 ml with the same solvent.

Apply to the plate 2 µl of each solution. Develop over a path of 15 cm using a mixture of equal volumes of *ether R* and *methylene chloride R*. Allow the plate to dry in air.

Spray with *bromocresol green solution R*. The principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

D. To 1 ml add 3 ml of *dilute sodium hydroxide solution R*. Add 3 ml of *water R* and 1 ml of a 100 g/l solution of *cobalt nitrate R*. A violet precipitate is formed. Filter; the precipitate dissolves in *methylene chloride R*.

TESTS

Appearance of solution. Dissolve 2.0 g in *dilute sodium hydroxide solution R* and dilute to 10 ml with the same alkaline solution. The solution is clear (2.2.1) and not more intensely coloured than reference solution Y_5 (2.2.2, *Method II*).

Related substances. Examine by gas chromatography (2.2.28), using *butyric acid R* as the internal standard.

Internal standard solution. Dissolve 10 mg of *butyric acid R* in *heptane R* and dilute to 200 ml with the same solvent.

Test solution. Dissolve 0.250 g of the substance to be examined in the internal standard solution and dilute to 5.0 ml with the same solution. Dilute 1.0 ml of the solution to 10.0 ml with *heptane R*.

Reference solution. Dissolve 20 mg of the substance to be examined and 20 mg of 2-(1-methylethyl)pentanoic acid *CRS* in *heptane R* and dilute to 10 ml with the same solvent. Dilute 1 ml of the solution to 10 ml with *heptane R*.

The chromatographic procedure may be carried out using:

- a wide-bore fused-silica column 30 m long and 0.53 mm in internal diameter coated with *macrogol 20 000 2-nitrotetraphthalate R* (film thickness 0.5 µm),
 - *helium for chromatography R* as the carrier gas at a flow rate of 8 ml/min,
 - a flame-ionisation detector,
- with the following temperature programme:

	Time (min)	Temperature (°C)	Rate (°C/min)	Comment
Column	0 - 10	130	–	isothermal
	10 - 30	130 → 190	3	linear gradient
Injection port		220		
Detector		220		

Inject 1 µl of each solution. The test is not valid unless, in the chromatogram obtained with the reference solution, the resolution between the peaks corresponding to 2-(1-methylethyl)pentanoic acid and valproic acid is at least 3.0. In the chromatogram obtained with the test solution: the sum of the areas of the peaks, apart from the principal peak, is not greater than three times the area of the peak due to the internal standard (0.3 per cent); none of the peaks, apart from the principal peak, has an area greater than that of the peak due to the internal standard (0.1 per cent). Disregard any peak with an area less than 0.1 times that of the peak due to the internal standard.

Heavy metals (2.4.8). Dissolve 2.0 g in *alcohol (80 per cent V/V) R* and dilute to 20 ml with the same solvent. 12 ml of the solution complies with limit test B for heavy metals (20 ppm). Prepare the standard using lead standard solution (2 ppm Pb) obtained by diluting *lead standard solution (100 ppm Pb) R* with *alcohol (80 per cent V/V) R*.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

01/2005:1058
corrected

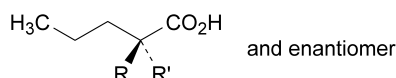
Dissolve 0.100 g in 25 ml of *alcohol R*. Add 2 ml of *water R*. Titrate with 0.1 M sodium hydroxide, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M sodium hydroxide is equivalent to 14.42 mg of $C_{86}H_{76}Cl_3N_9O_{24}$.

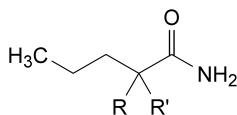
STORAGE

Store in an airtight container.

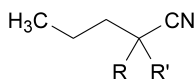
IMPURITIES



- A. $R = R' = H$: pentanoic acid (valeric acid),
- B. $R = H, R' = CH_2-CH_3$: (2*RS*)-2-ethylpentanoic acid,
- C. $R = H, R' = CH(CH_3)_2$: (2*RS*)-2-(1-methylethyl)pentanoic acid,
- D. $R = R' = CH_2-CH_2-CH_3$: 2,2-dipropylpentanoic acid,



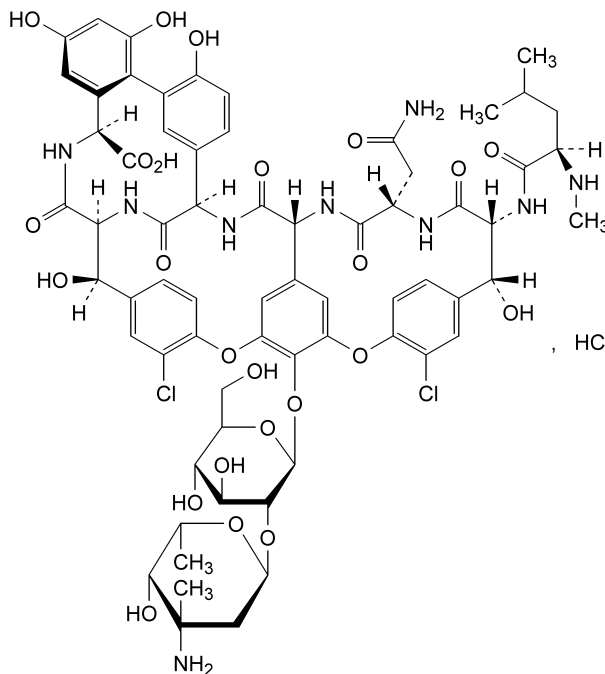
- E. $R = R' = H$: pentanamide (valeramide),
- F. $R = H, R' = CH_2-CH_2-CH_3$: 2-propylpentanamide,
- G. $R = R' = CH_2-CH_2-CH_3$: 2,2-dipropylpentanamide,



- H. $R = R' = H$: pentanenitrile (valeronitrile),
- I. $R = H, R' = CH_2-CH_2-CH_3$: 2-propylpentanenitrile,
- J. $R = R' = CH_2-CH_2-CH_3$: 2,2-dipropylpentanenitrile.

VANCOMYCIN HYDROCHLORIDE

Vancomycini hydrochloridum

 $C_{66}H_{76}Cl_3N_9O_{24}$ M_r 1486

DEFINITION

Vancomycin hydrochloride is the hydrochloride of a mixture of related glycopeptides, consisting principally of the monohydrochloride of (3*S*,6*R*,7*R*,22*R*,23*S*,26*S*,*aS*,36*R*,38*aR*)-3-(2-amino-2-oxoethyl)-44-[[2-*O*-(3-amino-2,3,6-trideoxy-3-*C*-methyl- α -*L*-xylo-hexopyranosyl)- β -D-glucopyranosyl]oxy]-10,19-dichloro-7,22,28,30,32-pentahydroxy-6-[[2*R*]-4-methyl-2-(methylamino)pentanoyl]amino]-2,5,24,38,39-pentaoxo-2,3,4,5,6,7,23,24,25,26,36,37,38,38*a*-tetradecahydro-22*H*-8,11:18,21-dietheno-23,36-(iminomethano)-13,16:31,35-dimetheno-1*H*,13*H*-[1,6,9]oxadiazacyclohexadecino[4,5-*m*][10,2,16]benzoxadiazacyclotetracosine-26-carboxylic acid (vancomycin B), a substance produced by certain strains of *Amycolatopsis orientalis* or obtained by any other means. The potency is not less than 1050 IU/mg, calculated with reference to the anhydrous substance.

CHARACTERS

A white or almost white powder, hygroscopic, freely soluble in water, slightly soluble in alcohol.

IDENTIFICATION

- A. Examine the chromatograms obtained in the test for vancomycin B. The retention time of the principal peak in the chromatogram obtained with test solution (a) is similar to that of the principal peak in the chromatogram obtained with the reference solution.
- B. It gives reaction (a) of chlorides (2.3.1).

TESTS

Appearance of solution. Dissolve 2.50 g in *water R* and dilute to 25.0 ml with the same solvent. The solution is clear (2.2.1). The absorbance (2.2.25) of the solution measured at 450 nm is not greater than 0.10.

pH (2.2.3). Dissolve 0.50 g in *carbon dioxide-free water R* and dilute to 10 ml with the same solvent. The pH of the solution is 2.5 to 4.5.

Vancomycin B. Not less than 93.0 per cent, determined by liquid chromatography (2.2.29).

Test solution (a). Dissolve 10.0 mg of the substance to be examined in mobile phase A and dilute to 5.0 ml with mobile phase A.

Test solution (b). Dilute 2.0 ml of test solution (a) to 50.0 ml with mobile phase A.

Test solution (c). Dilute 0.5 ml of test solution (b) to 20.0 ml with mobile phase A.

Reference solution. Dissolve the contents of a vial of *vancomycin hydrochloride CRS* in *water R* and dilute with the same solvent to obtain a solution containing 0.5 mg/ml. Heat at 65 °C for 24 h. Allow to cool.

Use the solutions within 4 h of preparation.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4.6 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (5 µm),
- as mobile phases at a flow rate of 1.0 ml/min the following solutions:

Mobile phase A. To 4 ml of *triethylamine R* add 1996 ml of *water R* and adjust to pH 3.2 with *phosphoric acid R*; to 920 ml of this solution add 10 ml of *tetrahydrofuran R* and 70 ml of *acetonitrile R*,

Mobile phase B. To 4 ml of *triethylamine R* add 1996 ml of *water R* and adjust to pH 3.2 with *phosphoric acid R*; to 700 ml of this solution add 10 ml of *tetrahydrofuran R* and 290 ml of *acetonitrile R*,

- as detector a spectrophotometer set at 280 nm,
- a 20 µl loop injector.

Record the chromatograms under the following conditions. Elute initially with mobile phase A. After 13 min, use gradient elution increasing the concentration of mobile phase B by 11 per cent *V/V* per minute. Finally, elute for 4 min using mobile phase B.

Inject test solution (c). The test is not valid unless the principal peak in the chromatogram obtained has a signal-to-noise ratio of at least five. Inject test solution (b). The test is not valid unless the symmetry factor of the vancomycin peak is at most 1.6. Inject the reference solution. The test is not valid unless the resolution between the two principal peaks is at least 5.0. Inject test solution (a).

Calculate the percentage content of vancomycin B hydrochloride using the expression:

$$\frac{A_b \times 100}{A_b + \left(\frac{A_t}{25}\right)}$$

A_b = area of the peak corresponding to vancomycin B in the chromatogram obtained with test solution (b),

A_t = sum of the areas of the peaks corresponding to impurities in the chromatogram obtained with test solution (a).

Related substances. Examine by liquid chromatography (2.2.29), as described under the test for vancomycin B.

Inject separately test solution (a), test solution (b) and test solution (c). Calculate the percentage content of each impurity using the expression:

$$\frac{\left(\frac{A_i}{25}\right) \times 100}{A_b + \left(\frac{A_t}{25}\right)}$$

A_i = area of an impurity peak in the chromatogram obtained with test solution (a),

A_b = area of the peak corresponding to vancomycin B in the chromatogram obtained with test solution (b),

A_t = sum of the areas of the peaks corresponding to impurities in the chromatogram obtained with test solution (a).

The content of no impurity is greater than 4.0 per cent and the sum of the contents of impurities is not greater than 7.0 per cent. Disregard any peak with an area less than that of the principal peak in the chromatogram obtained with test solution (c).

Heavy metals (2.4.8). 1.0 g complies with limit test C for heavy metals (30 ppm). Prepare the standard using 3.0 ml of *lead standard solution (10 ppm Pb) R*.

Water (2.5.12). Not more than 5.0 per cent, determined on 0.500 g by the semi-micro determination of water.

Sulphated ash (2.4.14). Not more than 1.0 per cent, determined on 1.00 g.

Bacterial endotoxins (2.6.14): less than 0.25 IU/mg, if intended for use in the manufacture of parenteral dosage forms without a further appropriate procedure for the removal of bacterial endotoxins.

ASSAY

Carry out the microbiological assay of antibiotics (2.7.2). Use *vancomycin hydrochloride CRS* as the reference substance.

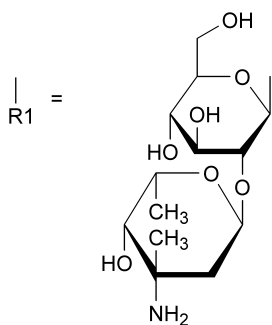
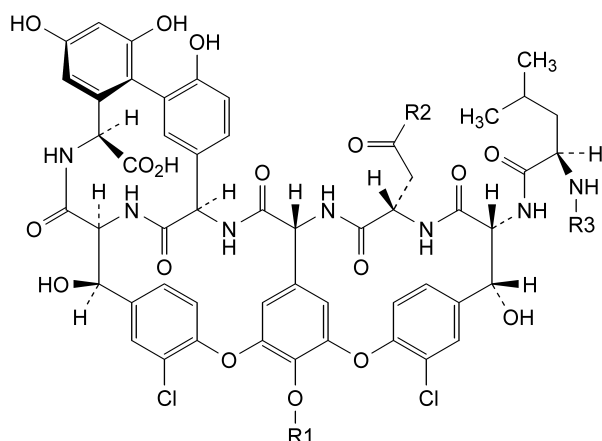
STORAGE

Store in an airtight container, protected from light. If the substance is sterile, store in a sterile, airtight, tamper-proof container.

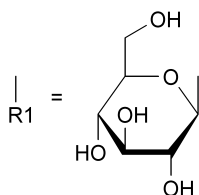
LABELLING

The label states, where applicable, that the substance is free from bacterial endotoxins.

IMPURITIES



- A. R2 = NH₂, R3 = H: *N*-demethylvancomycin B,
 B. R2 = OH, R3 = CH₃: desamidovancomycin B,
 C. R1 = H, R2 = NH₂, R3 = CH₃: aglucovancomycin B,

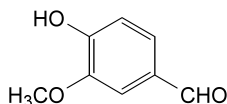


- D. R2 = NH₂, R3 = CH₃: desvancosaminyivancomycin B.

01/2005:0747

VANILLIN

Vanillinum

C₈H₈O₃*M*_r 152.1

DEFINITION

Vanillin contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of 4-hydroxy-3-methoxybenzaldehyde, calculated with reference to the dried substance.

CHARACTERS

White or slightly yellowish, crystalline powder or needles, slightly soluble in water, freely soluble in alcohol and in methanol. It dissolves in dilute solutions of alkali hydroxides.

IDENTIFICATION

First identification: B.

Second identification: A, C, D.

- A. Melting point (2.2.14): 81 °C to 84 °C.
 B. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *vanillin CRS*. Examine the substances prepared as discs.
 C. Examine the chromatograms obtained in the test for related substances in daylight after spraying. The principal spot in the chromatogram obtained with test solution (b) is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).
 D. To 5 ml of a saturated solution of the substance to be examined add 0.2 ml of *ferric chloride solution R1*. A blue colour is produced. Heat to 80 °C. The solution becomes brown. On cooling, a white precipitate is formed.

TESTS

Appearance of solution. Dissolve 1.0 g in *alcohol R* and dilute to 20 ml with the same solvent. The solution is clear (2.2.1) and not more intensely coloured than reference solution B₆ (2.2.2, *Method II*).

Related substances. Examine by thin-layer chromatography (2.2.27), using *silica gel GF₂₅₄ R* as the coating substance.

Test solution (a). Dissolve 0.1 g of the substance to be examined in *methanol R* and dilute to 5 ml with the same solvent.

Test solution (b). Dilute 1 ml of test solution (a) to 10 ml with *methanol R*.

Reference solution (a). Dissolve 10 mg of *vanillin CRS* in *methanol R* and dilute to 5 ml with the same solvent.

Reference solution (b). Dilute 0.5 ml of test solution (a) to 100 ml with *methanol R*.

Apply to the plate 5 µl of each solution. Develop in an unsaturated tank over a path of 10 cm using a mixture of 0.5 volumes of *anhydrous acetic acid R*, 1 volume of *methanol R* and 98.5 volumes of *methylene chloride R*. Dry the plate in a current of cold air. Examine in ultraviolet light at 254 nm. Any spot in the chromatogram obtained with test solution (a), apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.5 per cent). Spray with *dinitrophenylhydrazine-aceto-hydrochloric solution R* and examine in daylight. Any spot in the chromatogram obtained with test solution (a), apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.5 per cent).

Reaction with sulphuric acid. Dissolve 50 mg in 5 ml of *sulphuric acid R*. After 5 min, the solution is not more intensely coloured than a mixture of 4.9 ml of yellow primary solution and 0.1 ml of red primary solution or a mixture of 4.9 ml of yellow primary solution and 0.1 ml of blue primary solution (2.2.2, *Method I*).

Loss on drying (2.2.32). Not more than 1.0 per cent, determined on 1.000 g by drying in a desiccator for 4 h.

Sulphated ash (2.4.14). Not more than 0.05 per cent, determined on 2.0 g.

ASSAY

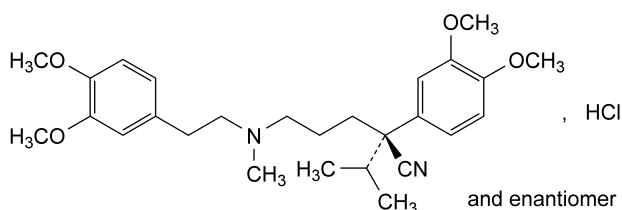
Dissolve 0.120 g in 20 ml of *alcohol R* and add 60 ml of *carbon dioxide-free water R*. Titrate with 0.1 M *sodium hydroxide*, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M *sodium hydroxide* is equivalent to 15.21 mg of C₈H₈O₃.

STORAGE

Store protected from light.

01/2005:0573 D. It gives reaction (b) of chlorides (2.3.1).

VERAPAMIL HYDROCHLORIDE**Verapamili hydrochloridum** $C_{27}H_{39}ClN_2O_4$ M_r 491.1**DEFINITION**

Verapamil hydrochloride contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of (2*RS*)-2-(3,4-dimethoxyphenyl)-5-[[2-(3,4-dimethoxyphenyl)ethyl](methyl)amino]-2-(1-methylethyl)pentanenitrile hydrochloride, calculated with reference to the dried substance.

CHARACTERS

A white, crystalline powder, soluble in water, freely soluble in methanol, sparingly soluble in alcohol.

It melts at about 144 °C.

IDENTIFICATION

First identification: B, D.

Second identification: A, C, D.

- A. Dissolve 20.0 mg in 0.01 *M* hydrochloric acid and dilute to 100.0 ml with the same acid. Dilute 5.0 ml of this solution to 50.0 ml with 0.01 *M* hydrochloric acid. Examined between 210 nm and 340 nm (2.2.25), the solution shows 2 absorption maxima, at 229 nm and 278 nm, and a shoulder at 282 nm. The ratio of the absorbance at the maximum at 278 nm to that at the maximum at 229 nm is 0.35 to 0.39.
- B. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with verapamil hydrochloride CRS. Examine the substances prepared as discs.
- C. Examine by thin-layer chromatography (2.2.27), using as the coating substance a suitable silica gel with a fluorescent indicator having an optimal intensity at 254 nm.

Test solution. Dissolve 10 mg of the substance to be examined in methylene chloride *R* and dilute to 5 ml with the same solvent.

Reference solution (a). Dissolve 20 mg of verapamil hydrochloride CRS in methylene chloride *R* and dilute to 10 ml with the same solvent.

Reference solution (b). Dissolve 5 mg of papaverine hydrochloride CRS in reference solution (a) and dilute to 5 ml with the same solution.

Apply separately to the plate 5 µl of each solution. Develop over a path of 15 cm using a mixture of 15 volumes of diethylamine *R* and 85 volumes of cyclohexane *R*. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. The principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a). The test is not valid unless the chromatogram obtained with reference solution (b) shows 2 clearly separated principal spots.

TESTS

Solution S. Dissolve 1.0 g in carbon dioxide-free water *R* while gently heating and dilute to 20.0 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and colourless (2.2.2, Method II).

pH (2.2.3). The pH of solution S is 4.5 to 6.0.

Optical rotation (2.2.7). The angle of optical rotation, determined on solution S, is -0.10° to $+0.10^\circ$.

Related substances. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 25.0 mg of the substance to be examined in the initial mobile phase composition and dilute to 10.0 ml with the same mobile phase.

Reference solution (a). Dissolve 5 mg of verapamil hydrochloride CRS, 5 mg of verapamil impurity I CRS and 5 mg of verapamil impurity M CRS in the initial mobile phase composition and dilute to 20 ml with the same mobile phase. Dilute 1 ml of this solution to 10 ml with the initial mobile phase composition.

Reference solution (b). Dilute 1.0 ml of the test solution to 100.0 ml with the initial mobile phase composition. Dilute 1.0 ml of this solution to 10.0 ml with the same mobile phase.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4.6 mm in internal diameter packed with end-capped palmitamidopropylsilyl silica gel for chromatography *R* (5 µm),
- as mobile phase at a flow rate of 1.5 ml/min a double isocratic programme using the following conditions:
Mobile phase A. A 6.97 g/l solution of dipotassium hydrogen phosphate *R* adjusted to pH 7.20 with phosphoric acid *R*,
Mobile phase B. Acetonitrile *R*,

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)	Comment
0 - 22	63	37	first isocratic step
22 - 27	63 → 35	37 → 65	switch to second isocratic step
27 - 35	35	65	second isocratic step
35 - 36	35 → 63	65 → 37	switch to initial mobile phase
36 - 50	63	37	equilibration

- as detector a spectrophotometer set at 278 nm.

Equilibrate the column with the initial mobile phase composition for about 60 min.

Inject 10 µl of reference solution (a). When the chromatogram is recorded in the prescribed conditions the retention times are: verapamil, about 16 min, verapamil impurity I, about 21 min and verapamil impurity M about 32 min, eluting as a doublet. The test is not valid unless the resolution between the peaks corresponding to verapamil and verapamil impurity I is at least 5.0 and verapamil impurity M elutes from the column.

Adjust the sensitivity of the system so that the height of principal peak in the chromatogram obtained with 10 µl of the reference solution (b) is at least 15 per cent of the full scale of the recorder.

Inject separately 10 µl of the test solution and 10 µl of reference solution (b). In the chromatogram obtained with the test solution: the area of any peak, apart from the principal peak, is not greater than the area of the

principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent); the sum of the areas of the peaks, apart from the principal peak, is not greater than 3 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.3 per cent). Disregard any peak with an area less than 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (b).

Heavy metals (2.4.8). 1.0 g complies with limit test C for heavy metals (10 ppm). Prepare the standard using 1 ml of lead standard solution (10 ppm Pb) R.

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

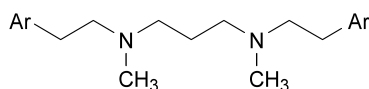
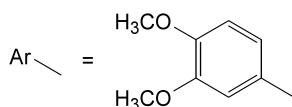
Dissolve 0.400 g in 50 ml of *ethanol* R and add 5.0 ml of 0.01 M hydrochloric acid. Titrate with 0.1 M sodium hydroxide, determining the end-point potentiometrically (2.2.20). Measure the volume of titrant required between the 2 points of inflexion.

1 ml of 0.1 M sodium hydroxide is equivalent to 49.11 mg of $C_{27}H_{39}ClN_2O_4$.

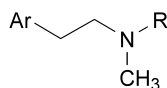
STORAGE

Store protected from light.

IMPURITIES



A. *N,N'*-bis[2-(3,4-dimethoxyphenyl)ethyl]-*N,N'*-dimethylpropane-1,3-diamine,

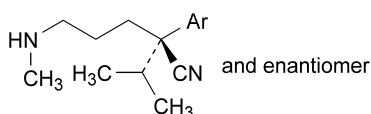


B. R = H: 2-(3,4-dimethoxyphenyl)-*N*-methylethanamine,

C. R = CH₃: 2-(3,4-dimethoxyphenyl)-*N,N*-dimethylethanamine,

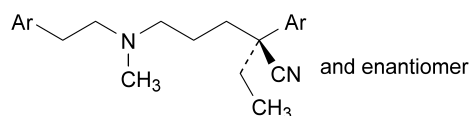
D. R = CH₂-CH₂-CH₂-Cl: 3-chloro-*N*-[2-(3,4-dimethoxyphenyl)ethyl]-*N*-methylpropan-1-amine,

E. Ar-CH₂OH: (3,4-dimethoxyphenyl)methanol,

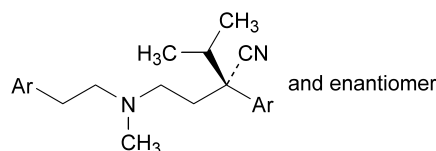


F. (2*RS*)-2-(3,4-dimethoxyphenyl)-5-(methylamino)-2-(1-methylethyl)pentanenitrile,

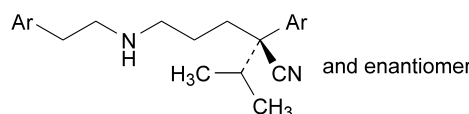
G. Ar-CHO: 3,4-dimethoxybenzaldehyde,



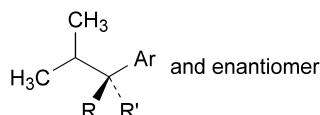
H. (2*RS*)-2-(3,4-dimethoxyphenyl)-5-[[2-(3,4-dimethoxyphenyl)ethyl](methylamino)-2-ethylpentanenitrile,



I. (2*RS*)-2-(3,4-dimethoxyphenyl)-2-[2-[[2-(3,4-dimethoxyphenyl)ethyl](methylamino)ethyl]-3-methylbutanenitrile,

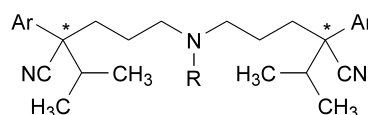


J. (2*RS*)-2-(3,4-dimethoxyphenyl)-5-[[2-(3,4-dimethoxyphenyl)ethyl]amino]-2-(1-methylethyl)pentanenitrile (*N*-norverapamil),



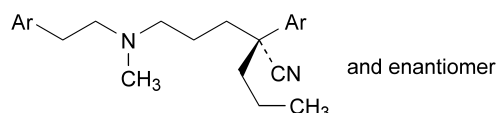
K. R = H, R' = CN: (2*RS*)-2-(3,4-dimethoxyphenyl)-3-methylbutanenitrile,

L. R + R' = O: 1-(3,4-dimethoxyphenyl)-2-methylpropan-1-one,

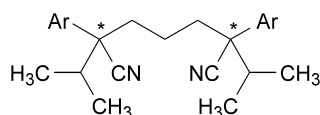


M. R = CH₂-CH₂-Ar: 5,5'-[[2-(3,4-dimethoxyphenyl)ethyl]imino]bis[2-(3,4-dimethoxyphenyl)-2-(1-methylethyl)pentanenitrile],

N. R = CH₃: 5,5'-(methylimino)bis[2-(3,4-dimethoxyphenyl)-2-(1-methylethyl)pentanenitrile],



O. (2*RS*)-2-(3,4-dimethoxyphenyl)-5-[[2-(3,4-dimethoxyphenyl)ethyl](methylamino)-2-propylpentanenitrile,



P. 2,6-bis(3,4-dimethoxyphenyl)-2,6-bis(1-methylethyl)-heptane-1,7-dinitrile.

01/2005:0748 ASSAY

corrected

Examine by liquid chromatography (2.2.29).

*Keep the solutions in iced water before use.**Test solution.* Dilute 1.0 ml of solution S (see Tests) to 5.0 ml with *water R*.*Reference solution (a).* Dissolve the contents of a vial of *vinblastine sulphate CRS* in 5.0 ml of *water R* to obtain a concentration of 1.0 mg/ml.*Reference solution (b).* Dissolve 1.0 mg of *vincristine sulphate CRS* in 1.0 ml of reference solution (a).*Reference solution (c).* Dilute 1.0 ml of reference solution (a) to 50.0 ml with *water R*.*Reference solution (d).* Dilute 1.0 ml of reference solution (c) to 20.0 ml with *water R*.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4.6 mm in internal diameter packed with *octylsilyl silica gel for chromatography R* (5 µm). Place between the injector and the column a guard column packed with suitable silica gel,
- as mobile phase at a flow rate of 1.0 ml/min a mixture of 38 volumes of a 1.5 per cent V/V solution of *diethylamine R* adjusted to pH 7.5 with *phosphoric acid R*, 12 volumes of *acetonitrile R* and 50 volumes of *methanol R*,
- as detector a spectrophotometer set at 262 nm,
- a loop injector.

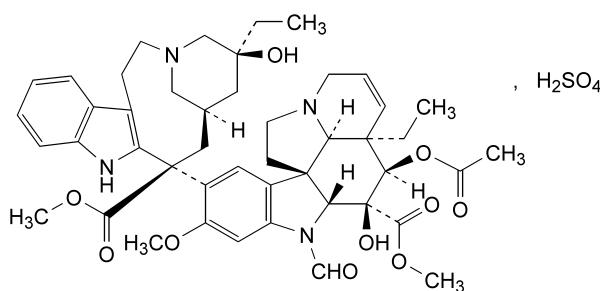
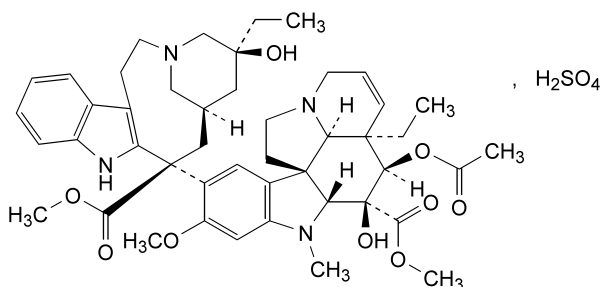
Inject 10 µl of each solution and record the chromatograms for three times the retention time of the peak corresponding to vinblastine. The assay is not valid unless: in the chromatogram obtained with reference solution (b) the resolution between the peaks corresponding to vincristine and vinblastine is not less than four; the peak in the chromatogram obtained with reference solution (d) has a signal-to-noise ratio not less than five. Calculate the percentage content of $C_{46}H_{60}N_4O_{13}S$ from the area of the principal peak in each of the chromatograms obtained with the test solution and reference solution (a) and from the declared content of *vinblastine sulphate CRS*.

STORAGE

Store in an airtight, glass container, protected from light, at a temperature not exceeding –20 °C. If the substance is sterile, store in an sterile, airtight, tamper-proof glass container.

01/2005:0749

corrected

VINCRIStINE SULPHATE**Vincristini sulfas** $C_{46}H_{58}N_4O_{14}S$ M_r 923**VINBLASTINE SULPHATE****Vinblastini sulfas** $C_{46}H_{60}N_4O_{13}S$ M_r 909**DEFINITION**

Vinblastine sulphate contains not less than 95.0 per cent and not more than the equivalent of 104.0 per cent of methyl (3a*R*,4*R*,5*S*,5a*R*,10b*R*,13a*R*)-4-(acetyloxy)-3a-ethyl-9-[(5*S*,7*R*,9*S*)-5-ethyl-5-hydroxy-9-(methoxycarbonyl)-1,4,5,6,7,8,9,10-octahydro-2*H*-3,7-methanoazacycloundecino[5,4-*b*]indol-9-yl]-5-hydroxy-8-methoxy-6-methyl-3a,4,5,5a,6,11,12,13a-octahydro-1*H*-indolizino[8,1-*cd*]carbazole-5-carboxylate sulphate, calculated with reference to the dried substance.

CHARACTERS

A white or slightly yellowish, crystalline powder, very hygroscopic, freely soluble in water, practically insoluble in alcohol.

IDENTIFICATION

- Examine by infrared absorption spectrophotometry (2.2.24), comparing with the *Ph. Eur. reference spectrum of vinblastine sulphate*.
- Examine the chromatograms obtained in the assay. The principal peak in the chromatogram obtained with the test solution is similar in position and approximate size to the principal peak in the chromatogram obtained with reference solution (a).

TESTS

Solution S. Dissolve 50.0 mg in *carbon dioxide-free water R* and dilute to 10.0 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and not more intensely coloured than reference solution Y_7 (2.2.2, *Method I*).

pH (2.2.3). Dilute 3 ml of solution S to 10 ml with *carbon dioxide-free water R*. The pH of this solution is 3.5 to 5.0.

Related substances. Examine the chromatograms obtained in the assay. In the chromatogram obtained with the test solution, the area of any peak apart from the principal peak is not greater than the area of the principal peak in the chromatogram obtained with reference solution (c) (2.0 per cent) and the sum of the areas of any such peaks is not greater than 2.5 times the area of the principal peak in the chromatogram obtained with reference solution (c) (5.0 per cent). Disregard any peak with an area less than that of the peak in the chromatogram obtained with reference solution (d).

Loss on drying. Not more than 15.0 per cent, determined on 3 mg by thermogravimetry (2.2.34). Heat to 200 °C at a rate of 5 °C/min, under a stream of *nitrogen for chromatography R*, at a flow rate of 40 ml/min.

DEFINITION

Methyl (3a*R*,4*R*,5*S*,5a*R*,10b*R*,13a*R*)-4-(acetyloxy)-3a-ethyl-9-[(5*S*,7*R*,9*S*)-5-ethyl-5-hydroxy-9-(methoxycarbonyl)-1,4,5,6,7,8,9,10-octahydro-2*H*-3,7-methanoazacycloundecino[5,4-*b*]indol-9-yl]-6-formyl-5-hydroxy-8-methoxy-3a,4,5,5a,6,11,12,13a-octahydro-1*H*-indolizino[8,1-*cd*]carbazole-5-carboxylate sulphate.

Content: 95.0 per cent to 104.0 per cent (dried substance).

CHARACTERS

Appearance: white or slightly yellowish, crystalline powder, very hygroscopic.

Solubility: freely soluble in water, slightly soluble in alcohol.

IDENTIFICATION

Infrared absorption spectrophotometry (2.2.24).

Comparison: *Ph. Eur. reference spectrum of vincristine sulphate*.

TESTS

Solution S. Dissolve 50.0 mg in *carbon dioxide-free water R* and dilute to 10.0 ml with the same solvent. *Keep the solution in iced water to carry out the test for related substances.*

Appearance of solution. Solution S is clear (2.2.1) and not more intensely coloured than reference solution Y₇ (2.2.2, *Method I*).

pH (2.2.3): 3.5 to 4.5.

Dilute 2 ml of solution S to 10 ml with *carbon dioxide-free water R*.

Related substances. Liquid chromatography (2.2.29). *Keep the solutions in iced water before use.*

Test solution. Dilute 1.0 ml of solution S to 5.0 ml with *water R*.

Reference solution (a). Dissolve the contents of a vial of *vincristine sulphate CRS* in 5.0 ml of *water R* to obtain a concentration of 1.0 mg/ml.

Reference solution (b). Dissolve 1.0 mg of *vinblastine sulphate CRS* in 1.0 ml of reference solution (a).

Reference solution (c). Dilute 1.0 ml of the test solution to 50.0 ml with *water R*.

Reference solution (d). Dilute 1.0 ml of reference solution (c) to 20.0 ml with *water R*.

Precolumn:

- *stationary phase*: octylsilyl silica gel for chromatography *R*.

Column:

- *size*: *l* = 0.25 m, Ø = 4.6 mm,
- *stationary phase*: octylsilyl silica gel for chromatography *R* (5 µm).

Mobile phase:

- *mobile phase A*: 1.5 per cent V/V solution of *diethylamine R* adjusted to pH 7.5 with *phosphoric acid R*,
- *mobile phase B*: *methanol R*,

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)
0 - 12	38	62
12 - 27	38 → 8	62 → 92
27 - 29	8 → 38	92 → 62
29 - 34	38	62

Flow rate: 2 ml/min.

Detection: spectrophotometer at 297 nm.

Injection: 20 µl.

System suitability: reference solution (b):

- *resolution*: minimum 4 between the peaks due to vincristine and vinblastine.

Limits:

- *any impurity*: not more than the area of the principal peak in the chromatogram obtained with reference solution (c) (2.0 per cent),
- *total*: not more than 2.5 times the area of the principal peak in the chromatogram obtained with reference solution (c) (5.0 per cent),
- *disregard limit*: area of the peak in the chromatogram obtained with reference solution (d) (0.1 per cent).

Loss on drying: maximum 12.0 per cent, determined on 3 mg by thermogravimetry (2.2.34). Heat the substance to be examined to 200 °C increasing the temperature by 5 °C/min, under a current of *nitrogen for chromatography R*, at a flow rate of 40 ml/min.

ASSAY

Liquid chromatography (2.2.29) as described in the test for related substances, with the following modifications.

Mobile phase: mix 30 volumes of a 1.5 per cent V/V solution of *diethylamine R* adjusted to pH 7.5 with *phosphoric acid R* and 70 volumes of *methanol R*.

Flow rate: 1.0 ml/min.

Calculate the percentage content of C₄₆H₅₈N₄O₁₄S using the chromatogram obtained with reference solution (a) and the declared content of *vincristine sulphate CRS*.

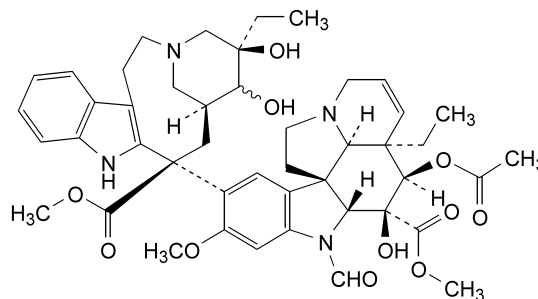
STORAGE

In an airtight, glass container, protected from light, at a temperature not exceeding –20 °C. If the substance is sterile, store in a sterile, airtight, tamper-proof glass container.

IMPURITIES

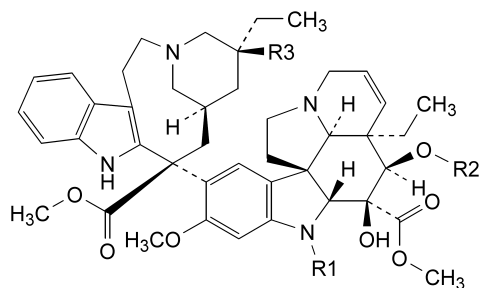
Specified impurities: A, B, C, D, H.

Other detectable impurities: E, F, G.

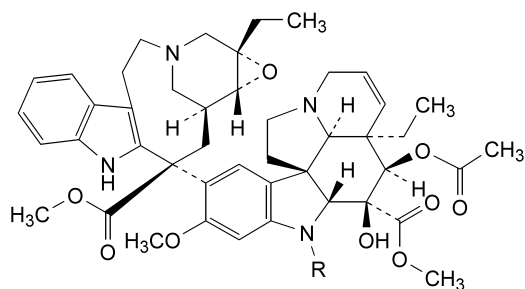


A. methyl (3a*R*,4*R*,5*S*,5a*R*,10b*R*,13a*R*)-4-(acetyloxy)-3a-ethyl-9-[(5*R*,7*S*,9*S*)-5-ethyl-5,6-dihydroxy-9-(methoxycarbonyl)-1,4,5,6,7,8,9,10-octahydro-2*H*-3,7-methanoazacycloundecino[5,4-*b*]indol-9-yl]-6-formyl-5-hydroxy-8-methoxy-3a,4,5,5a,6,11,12,13a-octahydro-1*H*-indolizino[8,1-*cd*]carbazole-5-carboxylate (3'-hydroxy-VCR),

01/2005:1276



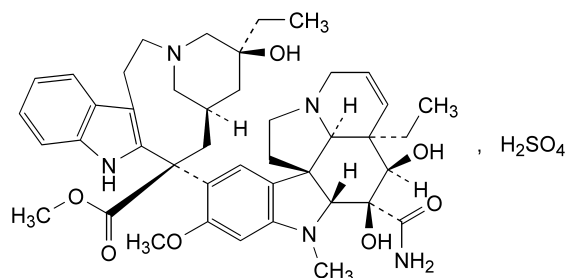
- B. R1 = CHO, R2 = CO-CH₃, R3 = H: methyl (3a*R*,4*R*,5*S*,5a*R*,10b*R*,13a*R*)-4-(acetyloxy)-3a-ethyl-9-[(5*S*,7*S*,9*S*)-5-ethyl-9-(methoxycarbonyl)-1,4,5,6,7,8,9,10-octahydro-2*H*-3,7-methanoazacycloundecino[5,4-*b*]indol-9-yl]-6-formyl-5-hydroxy-8-methoxy-3a,4,5,5a,6,11,12,13a-octahydro-1*H*-indolizino[8,1-*cd*]carbazole-5-carboxylate (4'-deoxyvincristine),
- C. R1 = H, R2 = CO-CH₃, R3 = OH: methyl (3a*R*,4*R*,5*S*,5a*R*,10b*S*,13a*R*)-4-(acetyloxy)-3a-ethyl-9-[(5*S*,7*R*,9*S*)-5-ethyl-5-hydroxy-9-(methoxycarbonyl)-1,4,5,6,7,8,9,10-octahydro-2*H*-3,7-methanoazacycloundecino[5,4-*b*]indol-9-yl]-5-hydroxy-8-methoxy-3a,4,5,5a,6,11,12,13a-octahydro-1*H*-indolizino[8,1-*cd*]carbazole-5-carboxylate (*N*-desmethylvinblastine),
- D. R1 = CHO, R2 = H, R3 = OH: methyl (3a*R*,4*R*,5*S*,5a*R*,10b*R*,13a*R*)-3a-ethyl-9-[(5*S*,7*R*,9*S*)-5-ethyl-5-hydroxy-9-(methoxycarbonyl)-1,4,5,6,7,8,9,10-octahydro-2*H*-3,7-methanoazacycloundecino[5,4-*b*]indol-9-yl]-6-formyl-4,5-dihydroxy-8-methoxy-3a,4,5,5a,6,11,12,13a-octahydro-1*H*-indolizino[8,1-*cd*]carbazole-5-carboxylate (deacetylvincristine),
- E. R1 = CH₃, R2 = H, R3 = OH: methyl (3a*R*,4*R*,5*S*,5a*R*,10b*R*,13a*R*)-3a-ethyl-9-[(5*S*,7*R*,9*S*)-5-ethyl-5-hydroxy-9-(methoxycarbonyl)-1,4,5,6,7,8,9,10-octahydro-2*H*-3,7-methanoazacycloundecino[5,4-*b*]indol-9-yl]-4,5-dihydroxy-8-methoxy-6-methyl-3a,4,5,5a,6,11,12,13a-octahydro-1*H*-indolizino[8,1-*cd*]carbazole-5-carboxylate (deacetylvinblastine),
- H. R1 = CH₃, R2 = CO-CH₃, R3 = OH: vinblastine,



- F. R = CH₃: methyl (3a*R*,4*R*,5*S*,5a*R*,10b*R*,13a*R*)-4-(acetyloxy)-3a-ethyl-9-[(1a*S*,11*S*,13*S*,13a*R*)-1a-ethyl-11-(methoxycarbonyl)-1a,4,5,10,11,12,13,13a-octahydro-2*H*-3,13-methano-oxireno[9,10]azacycloundecino[5,4-*b*]indol-11-yl]-5-hydroxy-8-methoxy-6-methyl-3a,4,5,5a,6,11,12,13a-octahydro-1*H*-indolizino[8,1-*cd*]carbazole-5-carboxylate (leurosine),
- G. R = CHO: methyl (3a*R*,4*R*,5*S*,5a*R*,10b*R*,13a*R*)-4-(acetyloxy)-3a-ethyl-9-[(1a*S*,11*S*,13*S*,13a*R*)-1a-ethyl-11-(methoxycarbonyl)-1a,4,5,10,11,12,13,13a-octahydro-2*H*-3,13-methano-oxireno[9,10]azacycloundecino[5,4-*b*]indol-11-yl]-6-formyl-5-hydroxy-8-methoxy-3a,4,5,5a,6,11,12,13a-octahydro-1*H*-indolizino[8,1-*cd*]carbazole-5-carboxylate (formylleurosine).

VINDESINE SULPHATE

Vindesini sulfas

C₄₃H₅₇N₅O₁₁SM_r 852

DEFINITION

Vindesine sulphate contains not less than 96.0 per cent and not more than the equivalent of 103.0 per cent of methyl (5*S*,7*R*,9*S*)-9-[(3a*R*,4*R*,5*S*,5a*R*,10b*R*,13a*R*)-5-carbamoyl-3a-ethyl-4,5-dihydroxy-8-methoxy-6-methyl-3a,4,5,5a,6,11,12,13a-octahydro-1*H*-indolizino[8,1-*cd*]carbazol-9-yl]-5-ethyl-5-hydroxy-1,4,5,6,7,8,9,10-octahydro-2*H*-3,7-methanoazacycloundecino[4,5-*b*]indole-9-carboxylate sulphate, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, amorphous substance, hygroscopic, freely soluble in water and in methanol, practically insoluble in cyclohexane.

IDENTIFICATION

Examine by infrared absorption spectrophotometry (2.2.24), comparing with the *Ph. Eur. reference spectrum of vindesine sulphate*.

TESTS

Solution S. Dissolve 50 mg in *carbon dioxide-free water R* and dilute to 10 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and not more intensely coloured than reference solution Y₇ (2.2.2, *Method I*).

pH (2.2.3). The pH of solution S is 3.5 to 5.5.

Related substances. Examine by liquid chromatography (2.2.29). *Keep the solutions in iced water before use.*

Test solution. Dissolve 10.0 mg of the substance to be examined in *water R* and dilute to 10.0 ml with the same solvent.

Reference solution (a). Dilute 1.0 ml of the test solution to 50.0 ml with *water R*.

Reference solution (b). Dissolve 1.0 mg of *desacetylvinblastine CRS* in *water R*, add 1.0 ml of the test solution and dilute to 50.0 ml with *water R*.

Reference solution (c). Dilute 1.0 ml of reference solution (a) to 200.0 ml with *water R*.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.15 m long and 4.6 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (5 µm),
- as mobile phase at a flow rate of 2 ml/min:
Mobile phase A. A 1.5 per cent V/V solution of *diethylamine R* adjusted to pH 7.4 with *phosphoric acid R*,
Mobile phase B. *Methanol R*,

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)	Comment
	49	51	equilibration
0 - 40	49	51	isocratic
40 - 49	49 → 30	51 → 70	linear gradient
49 - end	30	70	isocratic

— as detector a spectrophotometer set at 270 nm.

Inject separately 200 µl of the test solution, 200 µl of reference solution (a), 200 µl of reference solution (b) and 200 µl of reference solution (c). Maintain the final concentration of the mobile phase until the total run time is twice the retention time of the principal peak in the chromatogram obtained with the test solution. The test is not valid unless: in the chromatogram obtained with reference solution (b) the retention time of vindesine is less than 40 min; the symmetry factor of the vindesine peak is not more than 2.0; and the resolution of the vindesine and desacetylvinblastine peaks is at least 2.0. In the chromatogram obtained with the test solution: the area of any peak apart from the principal peak is not greater than half of the area of the principal peak in the chromatogram obtained with reference solution (a) (1 per cent); and the sum of the areas of any such peaks is not greater than the area of the principal peak in the chromatogram obtained with reference solution (a) (2 per cent). Disregard any peak with an area less than the area of the principal peak in the chromatogram obtained with reference solution (c).

Acetonitrile. Not more than 1.5 per cent *m/m* of acetonitrile, determined by gas chromatography (2.2.28).

Internal standard solution (a). Dilute 0.500 g of *propanol R* to 100 ml with *water R*.

Internal standard solution (b). Dilute 10.0 ml of internal standard solution (a) to 50.0 ml with *water R*.

Reference solution. Dilute 10.0 g of *acetonitrile R* to 1000 ml with *water R*. To 3.0 ml of this solution add 10.0 ml of internal standard solution (a) and dilute to 50.0 ml with *water R*.

Test solution. Dissolve 40 mg of the substance to be examined in 1.0 ml of internal standard solution (b).

The chromatographic procedure may be carried out using:

- a glass column 1.25 m long and 3 mm in internal diameter packed with *ethylvinylbenzene-divinylbenzene copolymer R*,
- *helium for chromatography R* as the carrier gas at a flow rate of 60 ml/min,
- a flame-ionisation detector,

maintaining the temperature of the column at 170 °C and that of the injection port and of the detector at 250 °C.

Inject 3 µl of the reference solution. The test is not valid unless the resolution between acetonitrile and propanol is greater than 1.5 and the symmetry factor for the acetonitrile peak is not greater than 1.6.

Inject 3 µl of the reference solution and 3 µl of the test solution.

Loss on drying. Not more than 10.0 per cent, determined on 9.00 mg by thermogravimetry (2.2.34). Heat to 200 °C at a rate of 5 °C/min, under a current of *nitrogen for chromatography R* at a flow rate of 40 ml/min.

ASSAY

Examine by liquid chromatography (2.2.29). *Keep the solutions in iced water before use.*

Test solution. Dissolve 5.0 mg of the substance to be examined in *water R* and dilute to 10.0 ml with the same solvent.

Reference solution (a). Dissolve and dilute the entire contents of a vial of *vindesine sulphate CRS* with *water R* to yield a concentration of approximately 0.50 mg/ml.

Reference solution (b). Add 1.0 mg of *desacetylvinblastine CRS* to 2.0 ml of reference solution (a).

The chromatographic procedure may be carried out using:

- a stainless steel column 0.15 m long and 4.6 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (5 µm),
- as mobile phase at a flow rate of 1 ml/min a mixture of 38 volumes of a 1.5 per cent *V/V* solution of *diethylamine R*, previously adjusted to pH 7.4 with *phosphoric acid R*, and 62 volumes of *methanol R*,
- as detector a spectrophotometer set at 270 nm.

Inject 20 µl of reference solution (b) five times. The test is not valid unless: the resolution between the peaks corresponding to vindesine sulphate and desacetylvinblastine sulphate is greater than 1.5; the symmetry factor of the vindesine peak is not more than 2.0; and the coefficient of variation of the area of the vindesine peak, calculated on the five injections is not greater than 1.5 per cent.

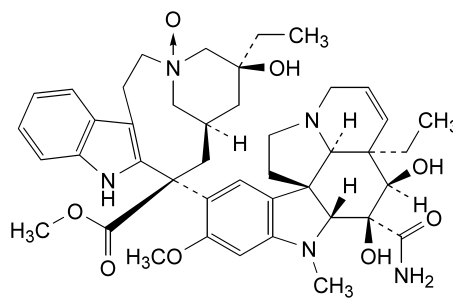
Inject separately 20 µl of the test solution and 20 µl of reference solution (a).

Calculate the content of vindesine sulphate ($C_{43}H_{57}N_5O_{11}S$) using the stated content of *vindesine sulphate CRS*.

STORAGE

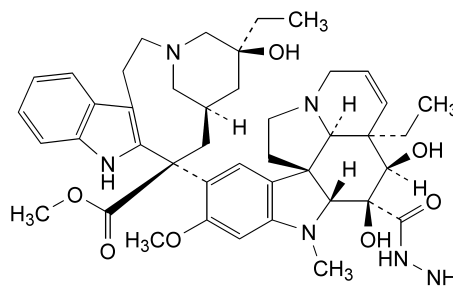
Store in an airtight polypropylene container with a polypropylene cap, at a temperature not exceeding –50 °C. If the substance is sterile, store in a sterile, airtight, tamper-proof container.

IMPURITIES



A. vindesine 3'-N-oxide,

B. vinblastine,

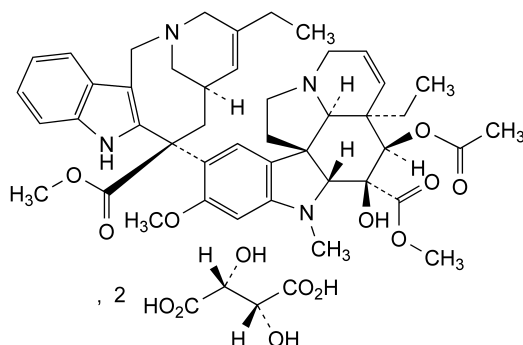


C. desacetylvinblastine hydrazide.

01/2005:2107

VINORELBINE TARTRATE

Vinorelbini tartras

 $C_{53}H_{66}N_4O_{20}$ M_r 1079

DEFINITION

Methyl (3a*R*,4*R*,5*S*,5a*R*,10b*R*,13a*R*)-4-(acetyloxy)-3a-ethyl-9-[(6*R*,8*S*)-4-ethyl-8-(methoxycarbonyl)-1,3,6,7,8,9-hexahydro-2,6-methano-2*H*-azacyclodecino[4,3-*b*]indol-8-yl]-5-hydroxy-8-methoxy-6-methyl-3a,4,5,5a,6,11,12,13a-octahydro-1*H*-indolizino[8,1-*cd*]carbazole-5-carboxylate dihydrogen bis[(2*R*,3*R*)-2,3-dihydroxybutanedioate].

Content: 98.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

Appearance: white or almost white powder, hygroscopic.

Solubility: freely soluble in water and in methanol, practically insoluble in hexane.

IDENTIFICATION

A. Infrared absorption spectrophotometry (2.2.24).

Preparation: dissolve 10 mg in 5 ml of *water R*. Add 0.5 ml of *sodium hydroxide solution R*. Extract with 5 ml of *methylene chloride R*. Dry the organic layer over *anhydrous sodium sulphate R*, filter and reduce its volume to about 0.5 ml by evaporation and apply to a disc of *potassium bromide R*. Evaporate and record the spectrum.

Comparison: *vinorelbine tartrate CRS*, treated as described above.

B. It gives reaction (b) of tartrates (2.3.1).

TESTS

Solution S. Dissolve a quantity equivalent to 140.0 mg of the anhydrous substance in *water R* and dilute to 10.0 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and its absorbance (2.2.25) at 420 nm is maximum 0.030.

pH (2.2.3): 3.3 to 3.8 for solution S.

Related substances. Liquid chromatography (2.2.29): use the normalisation procedure.

Test solution. Dissolve 35.0 mg of the substance to be examined in the mobile phase and dilute to 25 ml with the mobile phase.

Reference solution (a). Dissolve 7 mg of *vinorelbine impurity B CRS* in *water R* and dilute to 50 ml with the same solvent. To 1 ml of this solution add 14 mg of *vinorelbine tartrate CRS*, dissolve in *water R* and dilute to 10 ml with the same solvent. Expose this solution for 1 h to a xenon

lamp apparatus at a wavelength of 310-880 nm, supplying a dose of 1600 kJ/m² at a fluence rate of 500 W/m² in order to generate impurity A.

Reference solution (b). Dilute 1.0 ml of the test solution to 20.0 ml with *water R*. Dilute 1.0 ml of this solution to 100.0 ml with the mobile phase.

Column:

- **size:** $l = 0.15$ m, $\varnothing = 3.9$ mm,
- **stationary phase:** spherical *end-capped octadecylsilyl silica gel for chromatography R* (5 μ m) with a specific surface area of 125 m²/g, a pore size of 30 nm and a carbon loading of 7 per cent,
- **temperature:** 35 ± 5 °C.

Mobile phase: dissolve 1.22 g of *sodium decanesulphonate R* in 620 ml of *methanol R* and add 380 ml of a 7.80 g/l solution of *sodium dihydrogen phosphate R* previously adjusted to pH 4.2 with *dilute phosphoric acid R*.

Flow rate: 1.0 ml/min.

Detection: spectrophotometer at 267 nm.

Injection: 20 μ l.

Run time: twice the retention time of vinorelbine.

Relative retention with reference to vinorelbine (retention time = about 14 min): impurity A = about 0.8; impurity B = about 1.2.

System suitability:

- **peak-to-valley ratio:** minimum 4, where H_p = height above the baseline of the peak due to impurity B and H_v = height above the baseline of the lowest point of the curve separating this peak from the peak due to vinorelbine in the chromatogram obtained with reference solution (a),
- **signal-to-noise ratio:** minimum 10 for the principal peak in the chromatogram obtained with reference solution (b).

Limits:

- **impurity A:** maximum 0.3 per cent,
- **any other impurity:** for each impurity, maximum 0.2 per cent,
- **total of impurities other than A:** maximum 0.7 per cent,
- **disregard limit:** the area of the principal peak in the chromatogram obtained with reference solution (b).

Boron: maximum 50 ppm.

Test solution. Dissolve 0.10 g of the substance to be examined in 2 ml of *water R*. Slowly add 10.0 ml of *sulphuric acid R* while cooling in iced water. Stir and allow to warm to room temperature. Add 10.0 ml of a 0.5 g/l solution of *carminic acid R* in *sulphuric acid R*.

Reference solution. Dilute 2.5 ml of a 0.572 g/l solution of *boric acid R* to 100.0 ml with *water R*. To 2.0 ml of this solution slowly add 10.0 ml of *sulphuric acid R* while cooling in iced water. Stir and allow to warm to room temperature. Add 10.0 ml of a 0.5 g/l solution of *carminic acid R* in *sulphuric acid R*.

Blank solution. To 2.0 ml of *water R* slowly add 10.0 ml of *sulphuric acid R* while cooling in iced water. Stir and allow to warm to room temperature. Add 10.0 ml of a 0.5 g/l solution of *carminic acid R* in *sulphuric acid R*.

After 45 min, measure the absorbance (2.2.25) of the test solution and the reference solution, between 560 nm and 650 nm, using the blank solution as compensation liquid. The maximum absorbance value of the test solution is not greater than that of the reference solution.

Fluorides: maximum 50 ppm.

Potentiometry (2.2.36, *Method I*) using a fluoride-selective indicator electrode and a silver-silver chloride reference electrode.

Test solution. Dissolve 0.19 g of the substance to be examined in 20 ml of *water R*. Add 5.0 ml of *total-ionic-strength-adjustment buffer R* and dilute to 50 ml with *water R*.

Reference solutions. To 0.6 ml, 0.8 ml, 1.0 ml, 1.2 ml and 1.4 ml of *fluoride standard solution (10 ppm F) R*, add 5.0 ml of *total-ionic-strength-adjustment buffer R* and dilute to 50 ml with *water R*.

Introduce the electrodes into the reference solutions and allow to stand for 5 min. Determine the potential difference between the electrodes after 1 min of stabilisation. Using semi-logarithmic paper plot the potential difference obtained for each reference solution as a function of concentration of fluoride. Using exactly the same conditions, determine the potential difference obtained with the test solution and calculate the content of fluoride.

Silver: maximum 5 ppm.

Atomic absorption spectrometry (2.2.23, *Method I*).

Test solution. Dissolve 0.500 g of the substance to be examined in 10.0 ml of *water R*.

Reference solutions. Prepare the reference solutions using *silver standard solution (5 ppm Ag) R* and diluting with a 6.5 per cent *V/V* solution of *lead-free nitric acid R*.

Source: silver hollow-cathode lamp.

Wavelength: 328.1 nm.

Atomisation device: air-acetylene flame.

Water (2.5.12): maximum 4.0 per cent, determined on 0.250 g.

Bacterial endotoxins (2.6.14): less than 2 IU/mg (expressed as vinorelbine base), if intended for use in the manufacture of parenteral dosage forms without a further appropriate procedure for the removal of bacterial endotoxins.

ASSAY

Dissolve 0.350 g in 40 ml of *glacial acetic acid R*. Titrate with 0.1 *M* *perchloric acid*, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 *M* *perchloric acid* is equivalent to 53.96 mg of $C_{53}H_{66}N_4O_{20}$.

STORAGE

Under an inert gas, protected from light, at a temperature not exceeding -15°C .

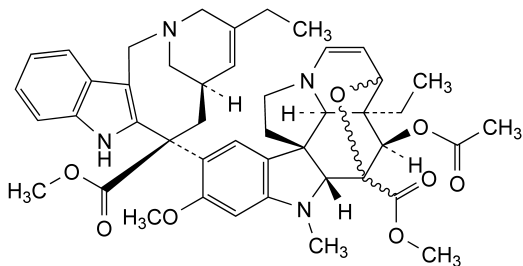
LABELLING

The label states, where applicable, that the substance is free from bacterial endotoxins.

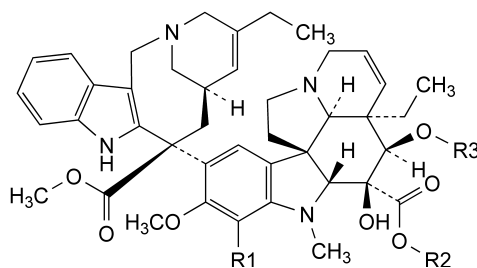
IMPURITIES

Specified impurities: A.

Other detectable impurities: B, C, D, E, F, G, H, I, J.



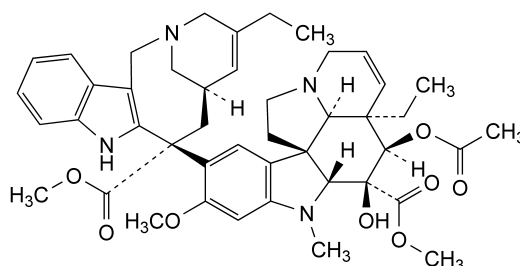
A. methyl (3aS,4R,5aR,10bR,13aR)-4-(acetyloxy)-3,5-epoxy-3a-ethyl-9-[(6R,8S)-4-ethyl-8-(methoxycarbonyl)-1,3,6,7,8,9-hexahydro-2,6-methano-2H-azacyclodecino[4,3-b]indol-8-yl]-8-methoxy-6-methyl-3a,4,5,5a,6,11,12,13a-octahydro-3H-indolizino[8,1-cd]carbazole-5-carboxylate,



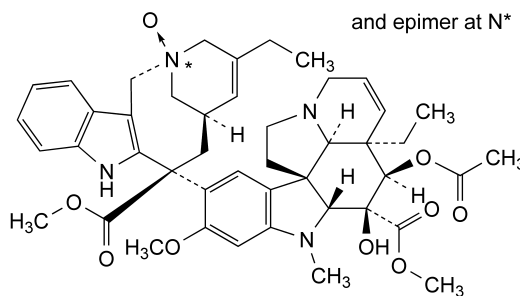
B. R1 = R3 = H, R2 = CH₃: methyl (3aR,4R,5S,5aR,10bR,13aR)-3a-ethyl-9-[(6R,8S)-4-ethyl-8-(methoxycarbonyl)-1,3,6,7,8,9-hexahydro-2,6-methano-2H-azacyclodecino[4,3-b]indol-8-yl]-4,5-dihydroxy-8-methoxy-6-methyl-3a,4,5,5a,6,11,12,13a-octahydro-1H-indolizino[8,1-cd]carbazole-5-carboxylate,

H. R1 = R2 = H, R3 = CO-CH₃: (3aR,4R,5S,5aR,10bR,13aR)-4-(acetyloxy)-3a-ethyl-9-[(6R,8S)-4-ethyl-8-(methoxycarbonyl)-1,3,6,7,8,9-hexahydro-2,6-methano-2H-azacyclodecino[4,3-b]indol-8-yl]-5-hydroxy-8-methoxy-6-methyl-3a,4,5,5a,6,11,12,13a-octahydro-1H-indolizino[8,1-cd]carbazole-5-carboxylic acid,

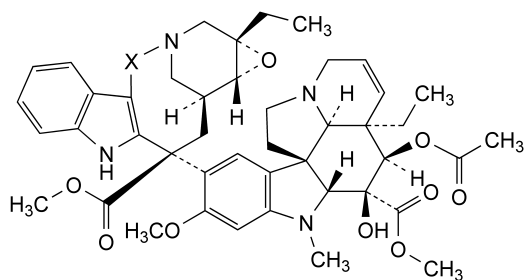
I. R1 = Br, R2 = CH₃, R3 = CO-CH₃: methyl (3aR,4R,5S,5aR,10bR,13aR)-4-(acetyloxy)-7-bromo-3a-ethyl-9-[(6R,8S)-4-ethyl-8-(methoxycarbonyl)-1,3,6,7,8,9-hexahydro-2,6-methano-2H-azacyclodecino[4,3-b]indol-8-yl]-5-hydroxy-8-methoxy-6-methyl-3a,4,5,5a,6,11,12,13a-octahydro-1H-indolizino[8,1-cd]carbazole-5-carboxylate,



C. methyl (3aR,4R,5S,5aR,10bR,13aR)-4-(acetyloxy)-3a-ethyl-9-[(6R,8R)-4-ethyl-8-(methoxycarbonyl)-1,3,6,7,8,9-hexahydro-2,6-methano-2H-azacyclodecino[4,3-b]indol-8-yl]-5-hydroxy-8-methoxy-6-methyl-3a,4,5,5a,6,11,12,13a-octahydro-1H-indolizino[8,1-cd]carbazole-5-carboxylate,

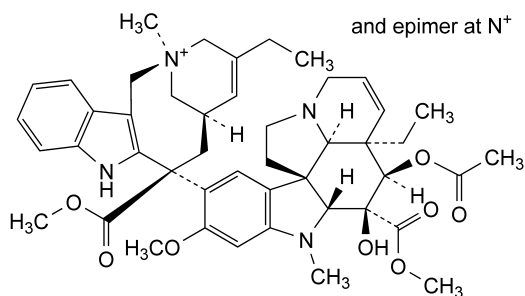


D. methyl (3aR,4R,5S,5aR,10bR,13aR)-4-(acetyloxy)-3a-ethyl-9-[(2RS,6R,8S)-4-ethyl-8-(methoxycarbonyl)-2-oxido-1,3,6,7,8,9-hexahydro-2,6-methano-2H-azacyclodecino[4,3-b]indol-8-yl]-5-hydroxy-8-methoxy-6-methyl-3a,4,5,5a,6,11,12,13a-octahydro-1H-indolizino[8,1-cd]carbazole-5-carboxylate,

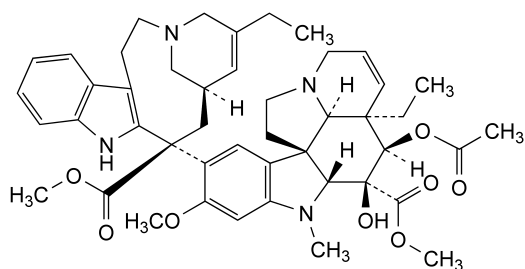


E. X = CH₂-CH₂: methyl (1aS,11S,13S,13aR)-11-[(3aR,4R,5S,5aR,10bR,13aR)-4-(acetyloxy)-3a-ethyl-5-hydroxy-8-methoxy-5-(methoxycarbonyl)-6-methyl-3a,4,5,5a,6,11,12,13a-octahydro-1H-indolizino[8,1-cd]carbazol-9-yl]-1a-ethyl-1a,4,5,10,11,12,13,13a-octahydro-2H-3,13-methanooxireno[9,10]azacycloundecino[5,4-b]indole-11-carboxylate (leurosine),

G. X = CH₂: methyl (1aS,10S,12S,12aR)-10-[(3aR,4R,5S,5aR,10bR,13aR)-4-(acetyloxy)-3a-ethyl-5-hydroxy-8-methoxy-5-(methoxycarbonyl)-6-methyl-3a,4,5,5a,6,11,12,13a-octahydro-1H-indolizino[8,1-cd]carbazol-9-yl]-1a-ethyl-1a,2,4,9,10,11,12,12a-octahydro-3,12-methano-3H-oxireno[8,9]azacyclodecino[4,3-b]indole-10-carboxylate,



F. (2RS,6R,8S)-8-[(3aR,4R,5S,5aR,10bR,13aR)-4-(acetyloxy)-3a-ethyl-5-hydroxy-8-methoxy-5-(methoxycarbonyl)-6-methyl-3a,4,5,5a,6,11,12,13a-octahydro-1H-indolizino[8,1-cd]carbazol-9-yl]-4-ethyl-8-(methoxycarbonyl)-2-methyl-1,3,6,7,8,9-hexahydro-2,6-methano-2H-azacyclodecino[4,3-b]indolium,



J. methyl (3aR,4R,5S,5aR,10bR,13aR)-4-(acetyloxy)-3a-ethyl-9-[(7R,9S)-5-ethyl-9-(methoxycarbonyl)-1,4,7,8,9,10-hexahydro-2H-3,7-methanoazacycloundecino[5,4-b]indol-9-yl]-5-hydroxy-8-methoxy-6-methyl-3a,4,5,5a,6,11,12,13a-octahydro-1H-indolizino[8,1-cd]carbazole-5-carboxylate.

01/2005:0034

VISCOSE WADDING, ABSORBENT

Lanugo cellulosi absorbens

DEFINITION

Absorbent viscose wadding consists of bleached, carefully carded, new fibres of regenerated cellulose obtained by the viscose process, with or without the addition of titanium

dioxide, of linear density 1.0 dtex to 8.9 dtex (dtex = mass of 10 000 m of fibre, expressed in grams) and cut to a suitable staple length. It does not contain any compensatory colouring matter.

CHARACTERS

It is white or very slightly yellow, has a lustrous or matt appearance, and is soft to the touch.

IDENTIFICATION

A. Viscose rayon fibres may be solid or hollow; hollow fibres may have a continuous lumen or be compartmented. The fibres have an average length of 25 mm to 80 mm and when examined under a microscope in the dry state, or when mounted in *alcohol R* and *water R*, the following characters are observed. They are usually of a more or less uniform width, with many longitudinal parallel lines distributed unequally over the width. The ends are cut more or less straight. Matt fibres contain numerous granular particles of approximately 1 µm average diameter.

Solid fibres. In longitudinal view, the surface of the fibres may be uneven or crenate. Fibres having an approximately circular or elliptical cross section have a diameter of about 10 µm to 20 µm and those that are flattened and twisted ribbons vary in width from 15 µm to 20 µm as the twisting of the filament reveals first the major axis and then the minor axis. They are about 4 µm in thickness. Other solid cross sections are Y-shaped and have protruding limbs with the major axis 5 µm to 25 µm in length and the minor axis 2 µm to 8 µm wide.

Hollow fibres. Fibres with a continuous, hollow lumen have a diameter of up to about 30 µm; they are thin-walled, with a wall thickness of about 5 µm. When mounted in *alcohol R* and *water R*, the lumen is clearly indicated in many fibres by the presence of many entrapped air bubbles.

Compartmented fibres. These fibres may have a diameter of up to 80 µm; they are hollow, having a central lumen which is divided up into several compartments. Individual compartments vary in size but typically may be up to about 60 µm in length and there may be more than one compartment across the width of each fibre. Some compartments show entrapped air bubbles when the fibres are mounted in *alcohol R* and *water R*.

- B. When treated with *iodinated zinc chloride solution R*, the fibres become violet.
- C. To 0.1 g add 10 ml of *zinc chloride-formic acid solution R*. Heat to 40 °C and allow to stand for 2 h 30 min, shaking occasionally. It dissolves completely except for the matt variety where titanium dioxide particles remain.
- D. Dissolve the residue obtained in the test for sulphated ash by warming gently with 5 ml of *sulphuric acid R*. Allow to cool and add 0.2 ml of *dilute hydrogen peroxide solution R*. The solution obtained from the lustrous variety undergoes no change in colour; that from the matt variety shows an orange-yellow colour, the intensity of which depends on the quantity of titanium dioxide present.

TESTS

Solution S. Place 15.0 g in a suitable vessel, add 150 ml of *water R*, close the vessel and allow to macerate for 2 h. Decant the solution, squeeze the residual liquid carefully from the sample with a glass rod and mix. Reserve 10 ml of the solution for the test for surface-active substances and filter the remainder.

Acidity or alkalinity. To 25 ml of solution S add 0.1 ml of *phenolphthalein solution R* and to another 25 ml add 0.05 ml of *methyl orange solution R*. Neither solution is pink.

Foreign fibres. Examined under a microscope, it is seen to consist exclusively of viscose fibres, except that occasionally a few isolated foreign fibres may be present.

Fluorescence. Examine a layer about 5 mm in thickness under ultraviolet light at 365 nm. It displays only a slight brownish-violet fluorescence. It shows no intense blue fluorescence, apart from that which may be shown by a few isolated fibres.

Absorbency

Apparatus. A dry cylindrical copper-wire basket 8.0 cm high and 5.0 cm in diameter. The wire of which the basket is constructed is about 0.4 mm in diameter, the mesh is 1.5 cm to 2.0 cm wide and the mass of the basket is 2.7 ± 0.3 g.

Sinking time. Not more than 10 s. Weigh the basket to the nearest centigram (m_1). Take a total of 5.00 g in approximately equal quantities from 5 different places in the product to be examined, place loosely in the basket and weigh the filled basket to the nearest centigram (m_2). Fill a beaker 11 cm to 12 cm in diameter to a depth of 10 cm with water at about 20 °C. Hold the basket horizontally and drop it from a height of about 10 mm into the water. Measure with a stopwatch the time taken for the basket to sink below the surface of the water. Calculate the result as the average of 3 tests.

Water-holding capacity. Not less than 18.0 g of water per gram. After the sinking time has been measured, remove the basket from the water, allow it to drain for exactly 30 s suspended in a horizontal position over the beaker, transfer it to a tared beaker (m_3) and weigh to the nearest centigram (m_4). Calculate the water-holding capacity per gram of absorbent viscose wadding using the following expression:

$$\frac{m_4 - (m_2 + m_3)}{m_2 - m_1}$$

Calculate the result as the average of 3 tests.

Ether-soluble substances. Not more than 0.30 per cent. In an extraction apparatus, extract 5.00 g with *ether R* for 4 h at a rate of at least 4 extractions per hour. Evaporate the ether extract and dry the residue to constant mass at 100 °C to 105 °C.

Extractable colouring matter. In a narrow percolator, slowly extract 10.0 g with *alcohol R* until 50 ml of extract is obtained. The liquid obtained is not more intensely coloured (2.2.2, *Method II*) than reference solution Y₅, GY₆ or a reference solution prepared as follows: to 3.0 ml of blue primary solution add 7.0 ml of hydrochloric acid (10 g/l HCl) and dilute 0.5 ml of this solution to 10.0 ml with hydrochloric acid (10 g/l HCl).

Surface-active substances. Introduce the 10 ml portion of solution S reserved before filtration into a 25 ml graduated ground-glass-stoppered cylinder with an external diameter of 20 mm and a wall thickness of not greater than 1.5 mm, previously rinsed 3 times with *sulphuric acid R* and then with *water R*. Shake vigorously 30 times in 10 s, allow to stand for 1 min and repeat the shaking. After 5 min, any foam present does not cover the entire surface of the liquid.

Water-soluble substances. Not more than 0.70 per cent. Boil 5.00 g in 500 ml of *water R* for 30 min, stirring frequently. Replace the water lost by evaporation. Decant the liquid, squeeze the residual liquid carefully from the sample with a glass rod and mix. Filter the liquid whilst hot. Evaporate

400 ml of the filtrate (corresponding to 4/5 of the mass of the sample taken) and dry the residue to constant mass at 100 °C to 105 °C.

Hydrogen sulphide. To 10 ml of solution S add 1.9 ml of *water R*, 0.15 ml of *dilute acetic acid R* and 1 ml of *lead acetate solution R*. After 2 min, the solution is not more intensely coloured than a reference solution prepared at the same time using 0.15 ml of *dilute acetic acid R*, 1.2 ml of *thioacetamide reagent R*, 1.7 ml of *lead standard solution (10 ppm Pb) R* and 10 ml of solution S.

Loss on drying (2.2.32). Not more than 13.0 per cent, determined on 5.000 g by drying in an oven at 100 °C to 105 °C.

Sulphated ash (2.4.14). Not more than 0.45 per cent for the lustrous variety and not more than 1.7 per cent for the matt variety. Introduce 5.00 g into a previously heated and cooled, tared crucible. Heat cautiously over a naked flame and then carefully to dull redness at 600 °C. Allow to cool, add a few drops of *dilute sulphuric acid R*, then heat and incinerate until all the black particles have disappeared. Allow to cool. Add a few drops of *ammonium carbonate solution R*. Evaporate and incinerate carefully, allow to cool and weigh again. Repeat the incineration for periods of 5 min to constant mass.

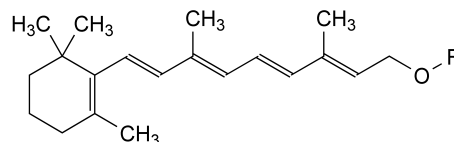
STORAGE

Store in a dust-proof package in a dry place.

01/2005:0217

VITAMIN A

Vitaminum A



Substance	R	Molecular formula	M_r
all-(E)-retinol	R = H	C ₂₀ H ₃₀ O	286.5
all-(E)-retinol acetate	CO-CH ₃	C ₂₂ H ₃₂ O ₂	328.5
all-(E)-retinol propionate	CO-C ₂ H ₅	C ₂₃ H ₃₄ O ₂	342.5
all-(E)-retinol palmitate	CO-C ₁₅ H ₃₁	C ₃₆ H ₆₀ O ₂	524.9

DEFINITION

Vitamin A refers to a number of substances of very similar structure (including (Z)-isomers) found in animal tissues and possessing similar activity. The principal and biologically most active substance is all-(E)-retinol (all-(E)-3,7-dimethyl-9-(2,6,6-trimethylcyclohex-1-enyl)nona-2,4,6,8-tetraen-1-ol; C₂₀H₃₀O). Vitamin A is generally used in the form of esters such as the acetate, propionate and palmitate.

Synthetic retinol ester refers to an ester of synthetic retinol (acetate, propionate or palmitate) or a mixture of synthetic retinol esters.

The activity of vitamin A is expressed in retinol equivalents (R.E.). 1 mg R.E. corresponds to the activity of 1 mg of all-(E)-retinol. The activity of the other retinol esters is calculated using the stoichiometry, so that 1 mg R.E. of vitamin A corresponds to the activity of:

- 1.147 mg of all-(E)-retinol acetate,
- 1.195 mg of all-(E)-retinol propionate,
- 1.832 mg of all-(E)-retinol palmitate.

International units (IU) are also used. 1 IU of vitamin A is equivalent to the activity of 0.300 µg of all-(*E*)-retinol. The activity of the other retinol esters is calculated using the stoichiometry, so that 1 IU of vitamin A is equivalent to the activity of:

- 0.344 µg of all-(*E*)-retinol acetate,
 - 0.359 µg of all-(*E*)-retinol propionate,
 - 0.550 µg of all-(*E*)-retinol palmitate,
- 1 mg of retinol equivalent is equivalent to 3333 IU.

CHARACTERS

Retinol acetate occurs as pale-yellow crystals (melting point about 60 °C). Once melted retinol acetate tends to yield a supercooled melt.

Retinol propionate occurs as a reddish-brown oily liquid.

Retinol palmitate is a fat-like, light yellow solid or a yellow oily liquid, if melted (melting point about 26 °C).

All retinol esters are practically insoluble in water, soluble or partly soluble in ethanol and miscible with organic solvents. Vitamin A and its esters are very sensitive to the action of air, oxidising agents, acids, light and heat.

Carry out the assay and all tests as rapidly as possible, avoiding exposure to actinic light and air, oxidising agents, oxidation catalysts (e.g. copper, iron), acids and heat; use freshly prepared solutions.

IDENTIFICATION

A. Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel F₂₅₄ plate R*.

Test solution. Prepare a solution containing about 3.3 IU of vitamin A per microlitre in *cyclohexane R* containing 1 g/l of *butylhydroxytoluene R*.

Reference solution. Prepare a 10 mg/ml solution of *retinol esters CRS* (i.e. 3.3 IU of each ester per microlitre) in *cyclohexane R* containing 1 g/l of *butylhydroxytoluene R*.

Apply to the plate 3 µl of each solution. Develop immediately over 2/3 of the plate using a mixture of 20 volumes of *ether R* and 80 volumes of *cyclohexane R*. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. The identification is not valid unless the chromatogram obtained with the reference solution shows the individual spots of the corresponding esters. The elution order from bottom to top is: retinol acetate, retinol propionate and retinol palmitate. The composition of the test solution is confirmed by the correspondence of the principal spot or spots with those obtained with the reference solution.

B. It complies with the test for related substances.

TESTS

Retinol. Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel F₂₅₄ plate R*.

Test solution. Prepare a solution in *cyclohexane R*, stabilised with a 1 g/l solution of *butylhydroxytoluene R*, containing about 330 IU of vitamin A per microlitre.

Reference solution. Shake 1 ml of the test solution with 20 ml of 0.1 M *tetrabutylammonium hydroxide* in 2-propanol for 2 min and dilute to 100 ml with *cyclohexane R*, stabilised with a 1 g/l solution of *butylhydroxytoluene R*.

Apply to the plate 3 µl of each solution. Develop immediately over a path of 15 cm using a mixture of 20 volumes of *ether R* and 80 volumes of *cyclohexane R*. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. In the chromatogram obtained with the reference solution

no or only traces of the retinol ester are seen. Any spot corresponding to retinol in the chromatogram obtained with the test solution is not more intense than the spot in the chromatogram obtained with the reference solution (1.0 per cent).

Related substances. Examine by ultraviolet absorption spectrophotometry (2.2.25). Determine the absorption maximum of the solution for “Activity”. The solution shows an absorption maximum at 325 nm to 327 nm. Measure the absorbances at 300 nm, 350 nm and 370 nm and calculate the ratio A_{λ}/A_{326} for each wavelength. None of the ratios A_{λ}/A_{326} exceed 0.593 at 300 nm, 0.537 at 350 nm, 0.142 at 370 nm.

ACTIVITY

The activity of the substance is determined in order to be taken into account for the production of concentrates.

Examine by ultraviolet absorption spectrophotometry (2.2.25). Dissolve 25 mg to 100 mg, weighed with an accuracy of 0.1 per cent, in 5 ml of *pentane R* and dilute with 2-propanol *R1* to a presumed concentration of 10 IU/ml to 15 IU/ml. This solution is also used for the test for related substances.

Determine the absorbance of the maximum at 326 nm. Calculate the activity of vitamin A in International Units per gram from the expression:

$$\frac{A_{326} \times V \times 1900}{100 \times m}$$

A_{326} = absorbance at 326 nm,

m = mass of the substance to be examined, in grams,

V = total volume to which the substance to be examined is diluted to give 10 IU/ml to 15 IU/ml,

1900 = factor to convert the specific absorbance of esters of retinol into International Units per gram.

STORAGE

Store in an airtight, well-filled container, protected from light.

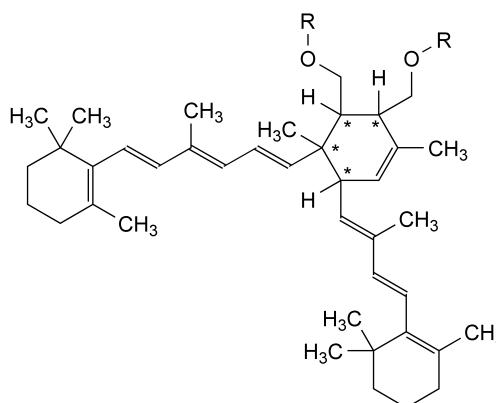
Once the container has been opened, its contents are to be used as soon as possible; any part of the contents not used at once should be protected by an atmosphere of inert gas.

LABELLING

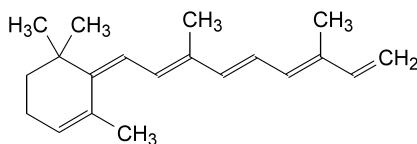
The label states:

- the number of International Units per gram,
- the name of the ester or esters.

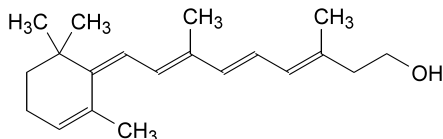
IMPURITIES



A. R = H, CO-CH₃: kitols (Diels-Alder dimers of vitamin A),



- B. (3*E*,5*E*,7*E*)-3,7-dimethyl-9-[(1*Z*)-2,6,6-trimethylcyclohex-2-enylidene]nona-1,3,5,7-tetraene (anhydro-vitamin A),



- C. (3*E*,5*E*,7*E*)-3,7-dimethyl-9-[(1*Z*)-2,6,6-trimethylcyclohex-2-enylidene]nona-3,5,7-trien-1-ol (*retro*-vitamin A),
D. oxidation products of vitamin A.

01/2005:0219

VITAMIN A CONCENTRATE (OILY FORM), SYNTHETIC

Vitaminum A densatum oleosum

DEFINITION

Synthetic vitamin A concentrate (oily form) is prepared from synthetic retinol ester (0217) as is or by dilution with a suitable vegetable fatty oil. The concentrate may contain suitable stabilisers such as antioxidants.

The declared content of vitamin A is not less than 500 000 IU/g and the concentrate contains not less than 95.0 per cent and not more than 110.0 per cent of the content stated on the label.

CHARACTERS

A yellow or brownish-yellow, oily liquid, practically insoluble in water, soluble or partly soluble in ethanol, miscible with organic solvents. Partial crystallisation may occur in highly concentrated solutions.

IDENTIFICATION

Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel F₂₅₄* plate R.

Test solution. Prepare a solution containing about 3.3 IU of vitamin A per microlitre in *cyclohexane R* containing 1 g/l of *butylhydroxytoluene R*.

Reference solution. Prepare a 10 mg/ml solution of *retinol esters CRS* (i.e. 3.3 IU of each ester per microlitre) in *cyclohexane R* containing 1 g/l of *butylhydroxytoluene R*.

Apply to the plate 3 µl of each solution. Develop immediately over a path of 15 cm using a mixture of 20 volumes of *ether R* and 80 volumes of *cyclohexane R*. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. The identification is not valid unless the chromatogram obtained with the reference solution shows the individual spots of the corresponding esters. The elution order from bottom to top is: retinol acetate, retinol propionate and retinol palmitate. The composition of the test solution is confirmed by the correspondence of the principal spot or spots with those obtained with the reference solution.

TESTS

Acid value (2.5.1). Not more than 2.0, determined on 2.0 g.

Peroxide value (2.5.5). Not more than 10.0.

ASSAY

Carry out the assay as rapidly as possible, avoiding exposure to actinic light and air, oxidising agents, oxidation catalysts (e.g. copper, iron), acids and prolonged heat; use freshly prepared solutions. If partial crystallisation has occurred, homogenise the material at a temperature of about 65 °C, but avoid prolonged heating.

Carry out the assay according to Method A. If the assay is not shown to be valid, use Method B.

Method A. Examine by ultraviolet absorption spectrophotometry (2.2.25). Dissolve 25 mg to 100 mg, weighed with an accuracy of 0.1 per cent, in 5 ml of *pentane R* and dilute with *2-propanol R1* to a presumed concentration of 10 IU/ml to 15 IU/ml.

Verify that the absorption maximum of the solution lies between 325 nm and 327 nm and measure the absorbances at 300 nm, 326 nm, 350 nm and 370 nm. Repeat the readings at each wavelength and take the mean values. Calculate the ratio A_{λ}/A_{326} for each wavelength.

If the ratios do not exceed: 0.593 at 300 nm, 0.537 at 350 nm, 0.142 at 370 nm, calculate the content of vitamin A in International Units per gram from the expression:

$$\frac{A_{326} \times V \times 1900}{100 \times m}$$

A_{326} = absorbance at 326 nm,

m = mass of the preparation to be examined, in grams,

V = total volume to which the preparation to be examined is diluted to give 10 IU/ml to 15 IU/ml,

1900 = factor to convert the specific absorbance of esters of retinol into International Units per gram.

If one or more of the ratios A_{λ}/A_{326} exceeds the values given, or if the wavelength of the absorption maximum does not lie between 325 nm and 327 nm, use Method B.

Method B. Examine by liquid chromatography (2.2.29).

Test solution (a). Introduce into a 50 ml volumetric flask, an amount of the preparation to be examined, weighed with an accuracy of 0.1 per cent and equivalent to about 120 000 IU of vitamin A and dissolve immediately in 5 ml of *pentane R*. Add 20 mg to 30 mg of *butylhydroxytoluene R* and 20 ml of 0.1 M *tetrabutylammonium hydroxide in 2-propanol*. Swirl gently for 5 min (an ultrasonic bath is recommended). Dilute to 50.0 ml with *2-propanol R* and homogenise carefully to avoid air-bubbles.

Test solution (b). Introduce 20 mg to 30 mg of *butylhydroxytoluene R* into a 50 ml volumetric flask, add 5 ml of *2-propanol R*, 5.0 ml of test solution (a) and dilute to 50.0 ml with *2-propanol R*. Homogenise carefully to avoid air-bubbles.

Reference solution (a). Introduce into a 50 ml volumetric flask about 120 mg of *retinol acetate CRS*, weighed with an accuracy of 0.1 per cent and proceed as described for test solution (a).

Reference solution (b). Introduce 20 mg to 30 mg of *butylhydroxytoluene R* into a 50 ml volumetric flask, add 5 ml of *2-propanol R*, 5.0 ml of reference solution (a) and dilute to 50.0 ml with *2-propanol R*. Homogenise carefully to avoid air-bubbles.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.125 m long and 4 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (5 µm),
- as mobile phase at a flow rate of 1 ml/min a mixture of 5 volumes of *water R* and 95 volumes of *methanol R*,

01/2005:0218

- as detector a spectrophotometer set at 325 nm,
- a loop injector.

The assay is not valid unless:

- the chromatogram obtained with reference solution (b) shows a principal peak corresponding to that of all-(*E*)-retinol, the retention time of all-(*E*)-retinol being about 3 min,
- there is no peak corresponding to the unsaponified retinol acetate in the chromatogram obtained with reference solution (b) at a retention time of about 6 min.

Inject a suitable volume of reference solution (b) in order to obtain an absorbance in the range of 0.5 to 1.0 at 325 nm and record the chromatogram using an attenuation so that the height of the peak corresponding to vitamin A is not less than 50 per cent of the full scale of the recorder.

Make a total of six such injections. The relative standard deviation of the response for reference solution (b) is not greater than 1 per cent.

Inject the same volume of test solution (b) and record the chromatogram in the same manner.

Calculate the content of vitamin A from the expression:

$$\frac{A_1 \times C \times m_2}{A_2 \times m_1}$$

- A_1 = area of the peak corresponding to all-(*E*)-retinol in the chromatogram obtained with test solution (b),
- A_2 = area of the peak corresponding to all-(*E*)-retinol in the chromatogram obtained with reference solution (b),
- C = concentration of *retinol acetate CRS* in International Units per gram, determined by method A; the absorption ratios A_λ/A_{326} must conform,
- m_1 = mass of the substance to be examined in test solution (a), in milligrams,
- m_2 = mass of *retinol acetate CRS* in reference solution (a), in milligrams.

STORAGE

Store in an airtight, well-filled container, protected from light.

Once the container has been opened, its contents are to be used as soon as possible; any part of the contents not used at once should be protected by an atmosphere of inert gas.

LABELLING

The label states:

- the number of International Units per gram,
- the name of the ester or esters,
- the name of any added stabilisers,
- the method of restoring the solution if partial crystallisation has occurred.

VITAMIN A CONCENTRATE (POWDER FORM), SYNTHETIC

Vitaminum A pulvis

DEFINITION

Synthetic vitamin A concentrate (powder form) is obtained by dispersing a synthetic retinol ester (0217) in a matrix of *Gelatin* (0330) or *Acacia* (0307) or other suitable material.

The declared content of vitamin A is not less than 250 000 IU/g and the concentrate contains not less than 95.0 per cent and not more than 115.0 per cent of the content stated on the label. The concentrate may contain suitable stabilisers such as antioxidants.

CHARACTERS

A yellowish powder usually in the form of particles of almost uniform size which, depending on their formulation, may be practically insoluble in water or may swell or form an emulsion.

IDENTIFICATION

Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel F₂₅₄* plate R.

Test solution. Introduce into a 20 ml glass-stoppered test tube a quantity of the preparation to be examined containing about the equivalent of 17 000 IU of vitamin A. Add about 20 mg of *bromelains R*, 2 ml of *water R* and about 150 µl of *2-propanol R*, swirling gently for 2 to 5 min in a water-bath at 60 °C to 65 °C. Cool to below 30 °C and add 5 ml of *2-propanol R* containing 1 g/l of *butylhydroxytoluene R*. Shake vigorously for 1 min, allow to stand for a few minutes and use the supernatant solution.

Reference solution. Prepare a 10 mg/ml solution of *retinol esters CRS* (i.e. 3.3 IU of each ester per microlitre) in *2-propanol R* containing 1 g/l of *butylhydroxytoluene R*.

Apply to the plate 3 µl of each solution. Develop immediately over a path of 15 cm using a mixture of 20 volumes of *ether R* and 80 volumes of *cyclohexane R*. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. The identification is not valid unless the chromatogram obtained with the reference solution shows the individual spots of the corresponding esters. The elution order from bottom to top is: retinol acetate, retinol propionate and retinol palmitate. The composition of the test solution is confirmed by the correspondence of the principal spot or spots with those obtained with the reference solution.

ASSAY

Carry out the assay as rapidly as possible, avoiding exposure to actinic light and air, oxidising agents, oxidation catalysts (e.g. copper, iron), acids and prolonged heat.

Examine by liquid chromatography (2.2.29).

Test solution (a). Introduce into a 50 ml volumetric flask, an amount of the preparation to be examined, weighed with an accuracy of 0.1 per cent and equivalent to about 120 000 IU of vitamin A. Add 20 mg to 30 mg of *bromelains R*, 20 mg to 30 mg of *butylhydroxytoluene R*, 2.0 ml of *water R* and 0.15 ml of *2-propanol R*. Heat gently in a water-bath at 60 °C to 65 °C for 2 to 5 min. Cool to below 30 °C and add 20 ml of 0.1 M *tetrabutylammonium hydroxide in 2-propanol*. Swirl gently for 5 min (an ultrasonic bath is recommended). Dilute to 50.0 ml with *2-propanol R* and homogenise carefully to avoid air-bubbles. Residue of the matrix may cause more or less cloudiness of the solution.

Test solution (b). Introduce 20 mg to 30 mg of *butylhydroxytoluene R* into a 50 ml volumetric flask, add 5 ml of *2-propanol R*, 5.0 ml of test solution (a) and dilute to 50.0 ml with *2-propanol R*. Homogenise carefully to avoid air-bubbles. Filter before injection.

Reference solution (a). Introduce into a 50 ml volumetric flask about 120 mg of *retinol acetate CRS*, weighed with an accuracy of 0.1 per cent and dissolve immediately in 5 ml of *pentane R*. Add 20 mg to 30 mg of *butylhydroxytoluene R* and 20 ml of *0.1 M tetrabutylammonium hydroxide in 2-propanol*. Swirl gently for 5 min (an ultrasonic bath is recommended) and dilute to 50.0 ml with *2-propanol R*. Homogenise carefully to avoid air-bubbles.

Reference solution (b). Place 20 mg to 30 mg of *butylhydroxytoluene R* in a 50 ml volumetric flask, add 5 ml of *2-propanol R*, 5.0 ml of reference solution (a) and dilute to 50.0 ml with *2-propanol R*. Homogenise carefully to avoid air-bubbles.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.125 m long and 4 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (5 µm),
- as mobile phase at a flow rate of 1 ml/min a mixture of 5 volumes of *water R* and 95 volumes of *methanol R*,
- as detector a spectrophotometer set at 325 nm,
- a loop injector.

The assay is not valid unless:

- the chromatogram obtained with reference solution (b) shows a principal peak corresponding to that of all-(*E*)-retinol, the retention time of all-(*E*)-retinol being about 3 min,
- there is no peak corresponding to unsaponified retinol acetate in the chromatogram obtained with reference solution (b) at a retention time of about 6 min.

Inject a suitable volume of reference solution (b) in order to obtain an absorbance in the range of 0.5 to 1.0 at 325 nm and record the chromatogram using an attenuation so that the height of the peak corresponding to vitamin A is not less than 50 per cent of the full scale of the recorder.

Make a total of six injections. The relative standard deviation of the response for reference solution (b) should not be greater than 1 per cent.

Inject the same volume of test solution (b) and record the chromatogram in the same manner.

Calculate the content of vitamin A from the expression:

$$\frac{A_1 \times C \times m_2}{A_2 \times m_1}$$

- A_1 = area of the peak corresponding to all-(*E*)-retinol in the chromatogram obtained with test solution (b),
- A_2 = area of the peak corresponding to all-(*E*)-retinol in the chromatogram obtained with reference solution (b),
- C = concentration of *retinol acetate CRS* in International Units per gram, determined by the method below,
- m_1 = mass of the substance to be examined in test solution (a), in milligrams,
- m_2 = mass of *retinol acetate CRS* in reference solution (a), in milligrams.

The exact concentration of *retinol acetate CRS* is assessed by ultraviolet absorption spectrophotometry (2.2.25). Dissolve 25 mg to 100 mg, weighed with an accuracy of 0.1 per cent, in 5 ml of *pentane R* and dilute with *2-propanol R1* to a presumed concentration of 10 UI/ml to 15 UI/ml.

Verify that the absorption maximum of the solution lies between 325 nm and 327 nm and measure the absorbances at 300 nm, 326 nm, 350 nm and 370 nm. Repeat the readings at each wavelength and take the mean values. Calculate the ratio A_λ/A_{326} for each wavelength.

If the ratios do not exceed: 0.593 at 300 nm, 0.537 at 350 nm, 0.142 at 370 nm, calculate the content of vitamin A in International Units per gram from the expression:

$$\frac{A_{326} \times V \times 1900}{100 \times m}$$

A_{326} = absorbance at 326 nm,

m = mass of the CRS, in grams,

V = total volume to which the CRS is diluted to give 10 UI/ml to 15 UI/ml,

1900 = factor to convert the specific absorbance of esters of retinol into International Units per gram.

The absorption ratios A_λ/A_{326} must conform.

STORAGE

Store in an airtight, well-filled container, protected from light.

Once the container has been opened, its contents are to be used as soon as possible; any part of the contents not used at once should be protected by an atmosphere of inert gas.

LABELLING

The label states:

- the number of International Units per gram,
- the name of the ester or esters,
- the name of the principal excipient or excipients used and the name of any added stabilisers.

01/2005:0220

VITAMIN A CONCENTRATE (SOLUBILISATE/EMULSION), SYNTHETIC

Vitaminum A in aqua dispergibile

DEFINITION

Synthetic Vitamin A concentrate (solubilisate/emulsion) is a liquid form (water is generally used as solvent) of a synthetic retinol ester (0217) and a suitable solubiliser. The declared content of vitamin A is not less than 100 000 UI/g and the concentrate contains not less than 95.0 per cent and not more than 115.0 per cent of the content stated on the label. The concentrate may contain suitable stabilisers such as antimicrobial preservatives and antioxidants.

CHARACTERS

A yellow or yellowish liquid of variable opalescence and viscosity. Highly concentrated solutions may become cloudy at low temperature or take the form of a gel.

A mixture of 1 g with 10 ml of *water R* previously warmed to 50 °C gives after cooling to 20 °C, a uniform, slightly opalescent and slightly yellow dispersion.

IDENTIFICATION

Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel F₂₅₄ plate R*.

Test solution. Introduce into a 20 ml glass-stoppered test-tube a quantity of the preparation to be examined containing about the equivalent of 17 000 IU of vitamin A. Add 5 ml of *2-propanol R* containing 1 g/l of *butylhydroxytoluene R* and mix thoroughly.

Reference solution. Prepare a 10 mg/ml solution of *retinol esters CRS* (i.e. 3.3 IU of each ester per microlitre) in *2-propanol R* containing 1 g/l of *butylhydroxytoluene R*.

Apply to the plate 3 µl of each solution. Develop immediately over a path of 15 cm using a mixture of 20 volumes of *ether R* and 80 volumes of *cyclohexane R*. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. The identification is not valid unless the chromatogram obtained with the reference solution shows the individual spots of the corresponding esters. The elution order from bottom to top is: retinol acetate, retinol propionate and retinol palmitate. The composition of the test solution is confirmed by the correspondence of the principal spot or spots with those obtained with the reference solution.

ASSAY

Carry out the assay as rapidly as possible, avoiding exposure to actinic light and air, oxidising agents, oxidation catalysts (e.g. copper, iron), acids and prolonged heat.

Examine by liquid chromatography (2.2.29).

Test solution (a). Introduce into a 50 ml volumetric flask, an amount of the preparation to be examined, weighed with an accuracy of 0.1 per cent and equivalent to about 120 000 IU of vitamin A and dissolve immediately in 5 ml of *2-propanol R*. Add 20 mg to 30 mg of *butylhydroxytoluene R* and 20 ml of 0.1 M *tetrabutylammonium hydroxide in 2-propanol*. Swirl gently for 5 min (an ultrasonic bath is recommended). Dilute to 50.0 ml with *2-propanol R* and homogenise carefully to avoid air-bubbles.

Test solution (b). Introduce 20 mg to 30 mg of *butylhydroxytoluene R* into a 50 ml volumetric flask, add 5 ml of *2-propanol R*, 5.0 ml of test solution (a) and dilute to 50.0 ml with *2-propanol R*. Homogenise carefully to avoid air-bubbles.

Reference solution (a). Introduce into a 50 ml volumetric flask about 120 mg of *retinol acetate CRS*, weighed with an accuracy of 0.1 per cent and proceed as described for test solution (a).

Reference solution (b). Place 20 mg to 30 mg of *butylhydroxytoluene R* into a 50 ml volumetric flask, add 5 ml of *2-propanol R*, 5.0 ml of reference solution (a) and dilute to 50.0 ml with *2-propanol R*. Homogenise carefully to avoid air-bubbles.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.125 m long and 4 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (5 µm),
- as mobile phase at a flow rate of 1 ml/min a mixture of 5 volumes of *water R* and 95 volumes of *methanol R*,
- as detector a spectrophotometer set at 325 nm,
- a loop injector.

The assay is not valid unless:

- the chromatogram obtained with reference solution (b) shows a principal peak corresponding to that of all-(*E*)-retinol, the retention time of all-(*E*)-retinol being about 3 min,

- there is no peak corresponding to the unsaponified retinol acetate in the chromatogram obtained with reference solution (b) at a retention time of about 6 min.

Inject a suitable volume of reference solution (b) in order to obtain an absorbance in the range of 0.5 to 1.0 at 325 nm and record the chromatogram using an attenuation so that the height of the peak corresponding to vitamin A is not less than 50 per cent of the full scale of the recorder.

Make a total of six injections. The relative standard deviation of the response for reference solution (b) should not be greater than 1 per cent.

Inject the same volume of test solution (b) and record the chromatogram in the same manner.

Calculate the content of vitamin A from the expression:

$$\frac{A_1 \times C \times m_2}{A_2 \times m_1}$$

A_1 = area of the peak corresponding to all-(*E*)-retinol in the chromatogram obtained with test solution (b),

A_2 = area of the peak corresponding to all-(*E*)-retinol in the chromatogram obtained with reference solution (b),

C = concentration of *retinol acetate CRS* in International Units per gram, determined by the method below,

m_1 = mass of the substance to be examined in test solution (a), in milligrams,

m_2 = mass of *retinol acetate CRS* in reference solution (a), in milligrams.

The exact concentration of *retinol acetate CRS* is assessed by ultraviolet absorption spectrophotometry (2.2.25). Dissolve 25 mg to 100 mg, weighed with an accuracy of 0.1 per cent, in 5 ml of *pentane R* and dilute with *2-propanol R1* to a presumed concentration of 10 IU/ml to 15 IU/ml.

Verify that the absorption maximum of the solution lies between 325 nm and 327 nm and measure the absorbances at 300 nm, 326 nm, 350 nm and 370 nm. Repeat the readings at each wavelength and take the mean values. Calculate the ratio A_{λ}/A_{326} for each wavelength.

If the ratios do not exceed: 0.593 at 300 nm, 0.537 at 350 nm, 0.142 at 370 nm, calculate the content of vitamin A in International Units per gram from the expression:

$$\frac{A_{326} \times V \times 1900}{100 \times m}$$

A_{326} = absorbance at 326 nm,

m = mass of the CRS, in grams,

V = total volume to which the CRS is diluted to give 10 UI/ml to 15 IU/ml,

1900 = factor to convert the specific absorbance of esters of retinol into International Units per gram.

The absorption ratios A_{λ}/A_{326} must conform.

STORAGE

Store in an airtight container, protected from light, at the temperature stated on the label.

Once the container has been opened, its contents are to be used as soon as possible; any part of the contents not used at once should be protected by an atmosphere of inert gas.

LABELLING

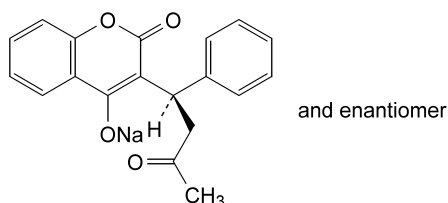
The label states:

- the number of International Units per gram,
- the name of the ester or esters,
- the name of the principal solubiliser or solubilisers used and the name of any added stabilisers,
- the storage temperature.

01/2005:0698 *Test solution (b).* Dilute 2 ml of test solution (a) to 10 ml with *acetone R*.

WARFARIN SODIUM

Warfarinum natricum



$C_{19}H_{15}NaO_4$

M_r 330.3

DEFINITION

Warfarin sodium contains not less than 98.0 per cent and not more than the equivalent of 102.0 per cent of sodium 2-oxo-3-[(1*RS*)-3-oxo-1-phenylbutyl]-2*H*-1-benzopyran-4-olate, calculated with reference to the anhydrous substance.

CHARACTERS

A white powder, hygroscopic, very soluble in water and in alcohol, soluble in acetone, very slightly soluble in methylene chloride.

IDENTIFICATION

First identification: B, D, E.

Second identification: A, C, D, E.

- A. Dissolve 1 g in 25 ml of *water R*, add 2 ml of *dilute hydrochloric acid R* and filter. Reserve the filtrate for identification test E. The precipitate, washed with *water R* and dried at 100 °C to 105 °C, melts (2.2.14) at 159 °C to 163 °C.
- B. Dissolve 1 g in 25 ml of *water R*, add 2 ml of *dilute hydrochloric acid R* and filter. Reserve the filtrate for identification test E. Examine the precipitate by infrared absorption spectrophotometry (2.2.24) comparing with the spectrum obtained with the precipitate prepared in the same manner from *warfarin sodium CRS*.
- C. Examine the chromatograms obtained in the test for related substances. The principal spot in the chromatogram obtained with test solution (b) is similar in position and size to the principal spot in the chromatogram obtained with reference solution (b).
- D. Dissolve 1 g in 10 ml of *water R*, add 5 ml of *nitric acid R* and filter. To the filtrate add 2 ml of *potassium dichromate solution RI* and shake for 5 min. Allow to stand for 20 min. The solution is not greenish-blue when compared with a blank.
- E. The filtrate obtained in identification test A or B gives reaction (b) of sodium (2.3.1).

TESTS

Appearance of solution. Dissolve 1.0 g in *water R* and dilute to 20 ml with the same solvent. The solution is clear (2.2.1) and colourless (2.2.2, *Method II*).

pH (2.2.3). Dissolve 1.0 g in *carbon dioxide-free water R* and dilute to 100 ml with the same solvent. The pH of the solution is 7.6 to 8.6.

Related substances. Examine by thin-layer chromatography (2.2.27), using *silica gel GF₂₅₄ R* as the coating substance.

Test solution (a). Dissolve 0.20 g of the substance to be examined in *acetone R* and dilute to 10 ml with the same solvent.

Reference solution (a). Dilute 1 ml of test solution (b) to 200 ml with *acetone R*.

Reference solution (b). Dissolve 40 mg of *warfarin sodium CRS* in *acetone R* and dilute to 10 ml with the same solvent.

Reference solution (c). Dissolve 10 mg of *acenocoumarol CRS* in *acetone R*, add 1 ml of test solution (a) and dilute to 10 ml with *acetone R*.

Apply separately to the plate 20 µl of each solution. Develop over a path of 15 cm using a mixture of 20 volumes of *glacial acetic acid R*, 50 volumes of *methylene chloride R* and 50 volumes of *cyclohexane R*. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. Any spot in the chromatogram obtained with test solution (a), apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (a) (0.1 per cent). The test is not valid unless the chromatogram obtained with reference solution (c) shows two clearly separated spots and the chromatogram obtained with reference solution (a) shows a clearly visible spot.

Phenolic ketones. Dissolve 1.25 g in a 20 g/l solution of *sodium hydroxide R* and dilute to 10.0 ml with the same solvent. The absorbance (2.2.25), measured at 385 nm within 15 min of preparing the solution, is not greater than 0.20.

Water (2.5.12). Not more than 4.0 per cent, determined on 0.750 g by the semi-micro determination of water.

ASSAY

Dissolve 0.1000 g in 0.01 *M sodium hydroxide* and dilute to 100.0 ml with the same solvent. Dilute 10.0 ml of this solution to 100.0 ml with 0.01 *M sodium hydroxide*. Dilute 10.0 ml of the latter solution to 100.0 ml with 0.01 *M sodium hydroxide*. Measure the absorbance (2.2.25) at the maximum at 308 nm.

Calculate the content of $C_{19}H_{15}NaO_4$ taking the specific absorbance to be 431.

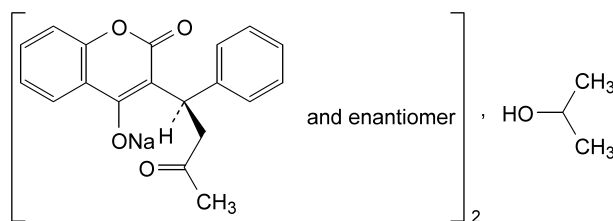
STORAGE

Store in an airtight container, protected from light.

01/2005:0699

WARFARIN SODIUM CLATHRATE

Warfarinum natricum clathratum



DEFINITION

Warfarin sodium clathrate contains not less than 98.0 per cent and not more than the equivalent of 102.0 per cent of sodium 2-oxo-3-[(1*RS*)-3-oxo-1-phenylbutyl]-2*H*-1-benzopyran-4-olate, calculated with reference to the anhydrous, propan-2-ol-free substance. Warfarin sodium clathrate contains approximately 92 per cent of warfarin sodium. It consists of warfarin sodium and propan-2-ol (molecular proportions 2:1) in the form of a clathrate. It contains not less than 8.0 per cent and not more than 8.5 per cent of propan-2-ol.

CHARACTERS

A white powder, very soluble in water, freely soluble in alcohol, soluble in acetone, very slightly soluble in methylene chloride.

IDENTIFICATION

First identification: B, D, E.

Second identification: A, C, D, E.

- A. Dissolve 1 g in 25 ml of *water R*, add 2 ml of *dilute hydrochloric acid R* and filter. Reserve the filtrate for identification test E. The precipitate, washed with *water R* and dried at 100 °C to 105 °C, melts (2.2.14) at 159 °C to 163 °C.
- B. Dissolve 1 g in 25 ml of *water R*, add 2 ml of *dilute hydrochloric acid R* and filter. Reserve the filtrate for identification test E. Examine the precipitate by infrared absorption spectrophotometry (2.2.24) comparing with the spectrum obtained with the precipitate prepared in the same manner from *warfarin sodium CRS*.
- C. Examine the chromatograms obtained in the test for related substances. The principal spot in the chromatogram obtained with test solution (b) is similar in position and size to the principal spot in the chromatogram obtained with reference solution (b).
- D. Dissolve 1 g in 10 ml of *water R*, add 5 ml of *nitric acid R* and filter. To the filtrate add 2 ml of *potassium dichromate solution R1* and shake for 5 min. Allow to stand for 20 min. The solution is greenish-blue when compared with a blank.
- E. The filtrate obtained in identification test A or B gives reaction (b) of sodium (2.3.1).

TESTS

Appearance of solution. Dissolve 1.0 g in *water R* and dilute to 20 ml with the same solvent. The solution is clear (2.2.1) and colourless (2.2.2, *Method II*).

pH (2.2.3). Dissolve 1.0 g in *carbon dioxide-free water R* and dilute to 100 ml with the same solvent. The pH of the solution is 7.6 to 8.6.

Related substances. Examine by thin-layer chromatography (2.2.27), using *silica gel GF₂₅₄ R* as the coating substance.

Test solution (a). Dissolve 0.20 g of the substance to be examined in *acetone R* and dilute to 10 ml with the same solvent.

Test solution (b). Dilute 2 ml of test solution (a) to 10 ml with *acetone R*.

Reference solution (a). Dilute 1 ml of test solution (b) to 200 ml with *acetone R*.

Reference solution (b). Dissolve 40 mg of *warfarin sodium CRS* in *acetone R* and dilute to 10 ml with the same solvent.

Reference solution (c). Dissolve 10 mg of *acenocoumarol CRS* in *acetone R*, add 1 ml of test solution (a) and dilute to 10 ml with *acetone R*.

Apply separately to the plate 20 µl of each solution. Develop over a path of 15 cm using a mixture of 20 volumes of *glacial acetic acid R*, 50 volumes of *methylene chloride R* and 50 volumes of *cyclohexane R*. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. Any spot in the chromatogram obtained with test solution (a), apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (a) (0.1 per cent). The test is not valid unless the chromatogram obtained

with reference solution (c) shows two clearly separated spots and the chromatogram obtained with reference solution (a) shows a clearly visible spot.

Phenolic ketones. Dissolve 1.25 g in a 20 g/l solution of *sodium hydroxide R* and dilute to 10.0 ml with the same solvent. The absorbance (2.2.25), measured at 385 nm within 15 min of preparing the solution, is not greater than 0.20.

Propan-2-ol: 8.0 per cent *m/m* to 8.5 per cent *m/m*, determined by gas chromatography (2.2.28) using *propanol R* as internal standard.

Internal standard solution. Dilute 1.0 ml of *propanol R* to 200.0 ml with *water R*.

Test solution (a). Dissolve 0.250 g of the substance to be examined in *water R* and dilute to 5.0 ml with the same solvent.

Test solution (b). Dissolve 0.50 g of the substance to be examined in the internal standard solution and dilute to 10.0 ml with the internal standard solution.

Reference solution. Dilute 0.50 ml of *propan-2-ol R* to 100.0 ml with the internal standard solution.

The chromatographic procedure may be carried out using:

- a column 1.5 m long and 4 mm in internal diameter packed with *ethylvinylbenzene-divinylbenzene copolymer R* (125 µm to 150 µm),
- *nitrogen for chromatography R* as the carrier gas at a flow rate of 40 ml/min,
- a flame-ionisation detector,

maintaining the temperature of the column at 150 °C, that of the injection port at 180 °C and that of the detector at 200 °C. Inject the selected volumes of the test solutions and the reference solution. Calculate the content of propan-2-ol taking its density at 20 °C to be 0.785 g/ml.

Water (2.5.12). Not more than 0.1 per cent, determined on 2.500 g by the semi-micro determination of water.

ASSAY

Dissolve 0.1000 g in 0.01 *M sodium hydroxide* and dilute to 100.0 ml with the same solvent. Dilute 10.0 ml of this solution to 100.0 ml with 0.01 *M sodium hydroxide*. Dilute 10.0 ml of the latter solution to 100.0 ml with 0.01 *M sodium hydroxide*. Measure the absorbance (2.2.25) at the maximum at 308 nm.

Calculate the content of $C_{19}H_{15}NaO_4$ taking the specific absorbance to be 431.

STORAGE

Store in an airtight container, protected from light.

01/2005:0169
corrected

WATER FOR INJECTIONS

Aqua ad iniectabilia

H₂O

M_r 18.02

DEFINITION

Water for the preparation of medicines for parenteral administration when water is used as vehicle (water for injections in bulk) and for dissolving or diluting substances or preparations for parenteral administration (sterilised water for injections).

Water for injections in bulk

PRODUCTION

Water for injections in bulk is obtained from water that complies with the regulations on water intended for human consumption laid down by the competent authority or from purified water by distillation in an apparatus of which the parts in contact with the water are of neutral glass, quartz or suitable metal and which is fitted with an effective device to prevent the entrainment of droplets. The correct maintenance of the apparatus is essential. The first portion of the distillate obtained when the apparatus begins to function is discarded and the distillate is collected.

During production and subsequent storage, appropriate measures are taken to ensure that the total viable aerobic count is adequately controlled and monitored. Appropriate alert and action limits are set so as to detect adverse trends. Under normal conditions, an appropriate action limit is a total viable aerobic count (2.6.12) of 10 micro-organisms per 100 ml when determined by membrane filtration, using agar medium S, using at least 200 ml of water for injections in bulk and incubating at 30-35 °C for 5 days. For aseptic processing, stricter alert limits may need to be applied.

Total organic carbon (2.2.44): maximum 0.5 mg/l.

Conductivity. Determine the conductivity off-line or in-line under the following conditions.

EQUIPMENT

Conductivity cell:

- electrodes of a suitable material such as stainless steel;
- cell constant: within 2 per cent of the given value determined using a certified reference solution with a conductivity less than 1500 µS·cm⁻¹.

Conductometer: resolution 0.1 µS·cm⁻¹ on the lowest range.

System calibration (conductivity cell and conductometer):

- against one or more suitable certified standard solutions;
- accuracy: within 3 per cent of the measured conductivity plus 0.1 µS·cm⁻¹.

Conductometer calibration: by means of precision resistors or equivalent devices, after disconnecting the conductivity cell, for all ranges used for conductivity measurement and cell calibration (with an accuracy within 0.1 per cent of the stated value, traceable to the official standard).

If in-line conductivity cells cannot be dismantled, system calibration may be performed against a calibrated conductivity cell placed close to the cell to be calibrated in the water flow.

PROCEDURE

Stage 1

1. Measure the conductivity without temperature compensation, recording simultaneously the temperature. Temperature-compensated measurement may be performed after suitable validation.
2. Using Table 0169-1, find the closest temperature value that is not greater than the measured temperature. The corresponding conductivity value is the limit at that temperature.
3. If the measured conductivity is not greater than the value in Table 0169-1, the water to be examined meets the requirements of the test for conductivity. If the conductivity is higher than the value in Table 0169-1, proceed with stage 2.

Table 0169-1. – Stage 1 - Temperature and conductivity requirements (for non-temperature-compensated conductivity measurements)

Temperature (°C)	Conductivity (µS·cm ⁻¹)
0	0.6
5	0.8
10	0.9
15	1.0
20	1.1
25	1.3
30	1.4
35	1.5
40	1.7
45	1.8
50	1.9
55	2.1
60	2.2
65	2.4
70	2.5
75	2.7
80	2.7
85	2.7
90	2.7
95	2.9
100	3.1

Stage 2

4. Transfer a sufficient amount of the water to be examined (100 ml or more) to a suitable container, and stir the test sample. Adjust the temperature, if necessary, and while maintaining it at 25 ± 1 °C, begin vigorously agitating the test sample while periodically observing the conductivity. When the change in conductivity (due to uptake of atmospheric carbon dioxide) is less than 0.1 µS·cm⁻¹ per 5 min, note the conductivity.
5. If the conductivity is not greater than 2.1 µS·cm⁻¹, the water to be examined meets the requirements of the test for conductivity. If the conductivity is greater than 2.1 µS·cm⁻¹, proceed with stage 3.

Stage 3

6. Perform this test within approximately 5 min of the conductivity determination in step 5 under stage 2, while maintaining the sample temperature at 25 ± 1 °C. Add a recently prepared saturated solution of *potassium chloride R* to the test sample (0.3 ml per 100 ml of the test sample), and determine the pH (2.2.3) to the nearest 0.1 pH unit.
7. Using Table 0169-2, determine the conductivity limit at the measured pH value in step 6. If the measured conductivity in step 4 under stage 2 is not greater than the conductivity requirements for the pH determined, the water to be examined meets the requirements of the test for conductivity. If either the measured conductivity is greater than this value or the pH is outside the range of 5.0-7.0, the water to be examined does not meet the requirements of the test for conductivity.

Table 0169.-2. — *Stage 3 - pH and conductivity requirements (for atmosphere and temperature equilibrated samples)*

pH	Conductivity ($\mu\text{S}\cdot\text{cm}^{-1}$)
5.0	4.7
5.1	4.1
5.2	3.6
5.3	3.3
5.4	3.0
5.5	2.8
5.6	2.6
5.7	2.5
5.8	2.4
5.9	2.4
6.0	2.4
6.1	2.4
6.2	2.5
6.3	2.4
6.4	2.3
6.5	2.2
6.6	2.1
6.7	2.6
6.8	3.1
6.9	3.8
7.0	4.6

In order to ensure the appropriate quality of the water, validated procedures and in-process-monitoring of the electrical conductivity and regular microbial monitoring are applied.

Water for injections in bulk is stored and distributed in conditions designed to prevent growth of micro-organisms and to avoid any other contamination.

CHARACTERS

Appearance: clear and colourless liquid.

TESTS

Nitrates: maximum 0.2 ppm.

Place 5 ml in a test-tube immersed in iced water, add 0.4 ml of a 100 g/l solution of *potassium chloride R*, 0.1 ml of *diphenylamine solution R* and, dropwise with shaking, 5 ml of *nitrogen-free sulphuric acid R*. Transfer the tube to a water-bath at 50 °C. After 15 min, any blue colour in the solution is not more intense than that in a reference solution prepared at the same time in the same manner using a mixture of 4.5 ml of *nitrate-free water R* and 0.5 ml of *nitrate standard solution (2 ppm NO₃) R*.

Aluminium (2.4.17): maximum 10 ppb, if intended for use in the manufacture of dialysis solutions.

Prescribed solution. To 400 ml of the water to be examined add 10 ml of *acetate buffer solution pH 6.0 R* and 100 ml of *distilled water R*.

Reference solution. Mix 2 ml of *aluminium standard solution (2 ppm Al) R*, 10 ml of *acetate buffer solution pH 6.0 R* and 98 ml of *distilled water R*.

Blank solution. Mix 10 ml of *acetate buffer solution pH 6.0 R* and 100 ml of *distilled water R*.

Heavy metals (2.4.8): maximum 0.1 ppm.

Heat 200 ml in a glass evaporating dish on a water-bath until the volume is reduced to 20 ml. 12 ml of the concentrated solution complies with limit test A. Prepare the standard using 10 ml of *lead standard solution (1 ppm Pb) R*.

Bacterial endotoxins (2.6.14): less than 0.25 IU/ml.

Sterilised water for injections

DEFINITION

Water for injections in bulk that has been distributed into suitable containers, closed and sterilised by heat in conditions which ensure that the product still complies with the test for bacterial endotoxins. Sterilised water for injections is free from any added substances.

Examined in suitable conditions of visibility, it is clear and colourless.

Each container contains a sufficient quantity of water for injections to permit the nominal volume to be withdrawn.

TESTS

Acidity or alkalinity. To 20 ml add 0.05 ml of *phenol red solution R*. If the solution is yellow, it becomes red on the addition of 0.1 ml of 0.01 M *sodium hydroxide*; if red, it becomes yellow on the addition of 0.15 ml of 0.01 M *hydrochloric acid*.

Conductivity: maximum 25 $\mu\text{S}\cdot\text{cm}^{-1}$ for containers with a nominal volume of 10 ml or less; maximum 5 $\mu\text{S}\cdot\text{cm}^{-1}$ for containers with a nominal volume greater than 10 ml.

Use equipment and the calibration procedure as defined under Water for injections in bulk, maintaining the sample temperature at 25 ± 1 °C.

Oxidisable substances. Boil 100 ml with 10 ml of *dilute sulphuric acid R*. Add 0.2 ml of 0.02 M *potassium permanganate* and boil for 5 min. The solution remains faintly pink.

Chlorides (2.4.4): maximum 0.5 ppm for containers with a nominal volume of 100 ml or less.

15 ml complies with the limit test for chlorides. Prepare the standard using a mixture of 1.5 ml of *chloride standard solution (5 ppm Cl) R* and 13.5 ml of *water R*. Examine the solutions down the vertical axes of the tubes.

For containers with a nominal volume greater than 100 ml, use the following test: to 10 ml add 1 ml of *dilute nitric acid R* and 0.2 ml of *silver nitrate solution R2*. The solution shows no change in appearance for at least 15 min.

Nitrates: maximum 0.2 ppm.

Place 5 ml in a test-tube immersed in iced water, add 0.4 ml of a 100 g/l solution of *potassium chloride R*, 0.1 ml of *diphenylamine solution R* and, dropwise with shaking, 5 ml of *nitrogen-free sulphuric acid R*. Transfer the tube to a water-bath at 50 °C. After 15 min, any blue colour in the solution is not more intense than that in a reference solution prepared at the same time in the same manner using a mixture of 4.5 ml of *nitrate-free water R* and 0.5 ml of *nitrate standard solution (2 ppm NO₃) R*.

Sulphates. To 10 ml add 0.1 ml of *dilute hydrochloric acid R* and 0.1 ml of *barium chloride solution R1*. The solution shows no change in appearance for at least 1 h.

Aluminium (2.4.17): maximum 10 ppb, if intended for use in the manufacture of dialysis solutions.

Prescribed solution. To 400 ml of the water to be examined add 10 ml of *acetate buffer solution pH 6.0 R* and 100 ml of *distilled water R*.

Reference solution. Mix 2 ml of *aluminium standard solution (2 ppm Al) R*, 10 ml of *acetate buffer solution pH 6.0 R* and 98 ml of *distilled water R*.

Blank solution. Mix 10 ml of *acetate buffer solution pH 6.0 R* and 100 ml of *distilled water R*.

Ammonium: maximum 0.2 ppm.

To 20 ml add 1 ml of *alkaline potassium tetraiodomercurate solution R*. After 5 min, examine the solution down the vertical axis of the tube. The solution is not more intensely coloured than a standard prepared at the same time by adding 1 ml of *alkaline potassium tetraiodomercurate solution R* to a mixture of 4 ml of *ammonium standard solution (1 ppm NH₄) R* and 16 ml of *ammonium-free water R*.

Calcium and magnesium. To 100 ml add 2 ml of *ammonium chloride buffer solution pH 10.0 R*, 50 mg of *mordant black 11 triturate R* and 0.5 ml of 0.01 M *sodium edetate*. A pure blue colour is produced.

Heavy metals (2.4.8): maximum 0.1 ppm.

Heat 200 ml in a glass evaporating dish on a water-bath until the volume is reduced to 20 ml. 12 ml of the concentrated solution complies with limit test A. Prepare the standard using 10 ml of *lead standard solution (1 ppm Pb) R*.

Residue on evaporation: maximum 4 mg (0.004 per cent) for containers with a nominal volume of 10 ml or less; maximum 3 mg (0.003 per cent) for containers with a nominal volume greater than 10 ml.

Evaporate 100 ml to dryness on a water-bath and dry in an oven at 100-105 °C.

Particulate contamination: sub-visible particles (2.9.19). It complies with test A or test B, as appropriate.

Sterility (2.6.1). It complies with the test for sterility.

Bacterial endotoxins (2.6.14): less than 0.25 IU/ml.

Current production methods include for example double-pass reverse osmosis coupled with other suitable techniques such as ultrafiltration and deionisation. Correct operation and maintenance of the system is essential.

During production and subsequent storage, appropriate measures are taken to ensure that the total viable aerobic count is adequately controlled and monitored. Appropriate alert and action limits are set so as to detect adverse trends. Under normal conditions, an appropriate action limit is a total viable aerobic count (2.6.12) of 10 micro-organisms per 100 ml when determined by membrane filtration, using agar medium S, at least 200 ml of highly purified water and incubating at 30-35 °C for 5 days.

Total organic carbon (2.2.44): maximum 0.5 mg/l.

Conductivity. Determine the conductivity off-line or in-line under the following conditions.

EQUIPMENT

Conductivity cell:

- electrodes of a suitable material such as stainless steel;
- cell constant: within 2 per cent of the given value determined using a certified reference solution with a conductivity less than 1500 µS·cm⁻¹.

Conductometer: resolution 0.1 µS·cm⁻¹ on the lowest range.

System calibration (conductivity cell and conductometer):

- against one or more suitable certified standard solutions;
- accuracy: within 3 per cent of the measured conductivity plus 0.1 µS·cm⁻¹.

Conductometer calibration: by means of precision resistors or equivalent devices after disconnecting the conductivity cell, for all ranges used for conductivity measurement and cell calibration (with an accuracy within 0.1 per cent of the stated value, traceable to the official standard).

If in-line conductivity cells cannot be dismantled, system calibration may be performed against a calibrated conductivity cell placed close to the cell to be calibrated in the water flow.

PROCEDURE

Stage 1

1. Measure the conductivity without temperature compensation, recording simultaneously the temperature. Temperature-compensated measurement may be performed after suitable validation.
2. Using Table 1927-1, find the closest temperature value that is not greater than the measured temperature. The corresponding conductivity value is the limit at that temperature.
3. If the measured conductivity is not greater than the value in Table 1927-1, the water to be examined meets the requirements of the test for conductivity. If the conductivity is higher than the value in Table 1927-1, proceed with stage 2.

01/2005:1927
corrected

WATER, HIGHLY PURIFIED

Aqua valde purificata

H₂O

M_r 18.02

DEFINITION

Water intended for use in the preparation of medicinal products where water of high biological quality is needed, except where *Water for injections (0169)* is required.

PRODUCTION

Highly purified water is obtained from water that complies with the regulations on water intended for human consumption laid down by the competent authority.

Table 1927-1. – Stage 1 - Temperature and conductivity requirements (for non-temperature-compensated conductivity measurements)

Temperature (°C)	Conductivity ($\mu\text{S}\cdot\text{cm}^{-1}$)
0	0.6
5	0.8
10	0.9
15	1.0
20	1.1
25	1.3
30	1.4
35	1.5
40	1.7
45	1.8
50	1.9
55	2.1
60	2.2
65	2.4
70	2.5
75	2.7
80	2.7
85	2.7
90	2.7
95	2.9
100	3.1

Stage 2

- Transfer a sufficient amount of the water to be examined (100 ml or more) to a suitable container, and stir the test sample. Adjust the temperature, if necessary, and while maintaining it at 25 ± 1 °C, begin vigorously agitating the test sample while periodically observing the conductivity. When the change in conductivity (due to uptake of atmospheric carbon dioxide) is less than $0.1 \mu\text{S}\cdot\text{cm}^{-1}$ per 5 min, note the conductivity.
- If the conductivity is not greater than $2.1 \mu\text{S}\cdot\text{cm}^{-1}$, the water to be examined meets the requirements of the test for conductivity. If the conductivity is greater than $2.1 \mu\text{S}\cdot\text{cm}^{-1}$, proceed with stage 3.

Stage 3

- Perform this test within approximately 5 min of the conductivity determination in step 5 under stage 2, while maintaining the sample temperature at 25 ± 1 °C. Add a recently prepared saturated solution of *potassium chloride R* to the test sample (0.3 ml per 100 ml of the test sample), and determine the pH (2.2.3) to the nearest 0.1 pH unit.
- Using Table 1927-2, determine the conductivity limit at the measured pH value in step 6. If the measured conductivity in step 4 under stage 2 is not greater than the conductivity requirements for the pH determined, the water to be examined meets the requirements of the test for conductivity. If either the measured conductivity is greater than this value or the pH is outside the range of 5.0-7.0, the water to be examined does not meet the requirements of the test for conductivity.

In order to ensure the appropriate quality of the water, validated procedures and in-process monitoring of the electrical conductivity and regular microbial monitoring are applied.

Highly purified water is stored in bulk and distributed in conditions designed to prevent growth of micro-organisms and to avoid any other contamination.

Table 1927-2. – Stage 3 - pH and conductivity requirements (for atmosphere and temperature equilibrated samples)

pH	Conductivity ($\mu\text{S}\cdot\text{cm}^{-1}$)
5.0	4.7
5.1	4.1
5.2	3.6
5.3	3.3
5.4	3.0
5.5	2.8
5.6	2.6
5.7	2.5
5.8	2.4
5.9	2.4
6.0	2.4
6.1	2.4
6.2	2.5
6.3	2.4
6.4	2.3
6.5	2.2
6.6	2.1
6.7	2.6
6.8	3.1
6.9	3.8
7.0	4.6

CHARACTERS

Appearance: clear and colourless liquid.

TESTS

Nitrates: maximum 0.2 ppm.

Place 5 ml in a test-tube immersed in iced water, add 0.4 ml of a 100 g/l solution of *potassium chloride R*, 0.1 ml of *diphenylamine solution R* and, dropwise with shaking, 5 ml of *nitrogen-free sulphuric acid R*. Transfer the tube to a water-bath at 50 °C. After 15 min, any blue colour in the solution is not more intense than that in a reference solution prepared at the same time in the same manner using a mixture of 4.5 ml of *nitrate-free water R* and 0.5 ml of *nitrate standard solution (2 ppm NO₃) R*.

Aluminium (2.4.17): maximum 10 ppb, if intended for use in the manufacture of dialysis solutions.

Prescribed solution. To 400 ml of the water to be examined add 10 ml of *acetate buffer solution pH 6.0 R* and 100 ml of *distilled water R*.

Reference solution. Mix 2 ml of *aluminium standard solution (2 ppm Al) R*, 10 ml of *acetate buffer solution pH 6.0 R* and 98 ml of *distilled water R*.

Blank solution. Mix 10 ml of *acetate buffer solution pH 6.0 R* and 100 ml of *distilled water R*.

Heavy metals (2.4.8): maximum 0.1 ppm.

Heat 200 ml in a glass evaporating dish on a water-bath until the volume is reduced to 20 ml. 12 ml of the concentrated solution complies with limit test A. Prepare the standard using 10 ml of *lead standard solution* (1 ppm Pb) R.

Bacterial endotoxins (2.6.14): less than 0.25 IU/ml.

LABELLING

The label states, where applicable, that the substance is suitable for use in the manufacture of dialysis solutions.

01/2005:0008
corrected

WATER, PURIFIED

Aqua purificata

H₂O *M_r* 18.02

DEFINITION

Water for the preparation of medicines other than those that are required to be both sterile and apyrogenic, unless otherwise justified and authorised.

Purified water in bulk

PRODUCTION

Purified water in bulk is prepared by distillation, by ion exchange, by reverse osmosis or by any other suitable method from water that complies with the regulations on water intended for human consumption laid down by the competent authority.

During production and subsequent storage, appropriate measures are taken to ensure that the total viable aerobic count is adequately controlled and monitored. Appropriate alert and action limits are set so as to detect adverse trends. Under normal conditions, an appropriate action limit is a total viable aerobic count (2.6.12) of 100 micro-organisms per millilitre, determined by membrane filtration, using agar medium S and incubating at 30-35 °C for 5 days. The size of the sample is to be chosen in relation to the expected result.

In addition, the test for total organic carbon (2.2.44) with a limit of 0.5 mg/l or alternatively the following test for oxidisable substances is carried out: to 100 ml add 10 ml of *dilute sulphuric acid R* and 0.1 ml of 0.02 M *potassium permanganate* and boil for 5 min; the solution remains faintly pink.

Conductivity. Determine the conductivity off-line or in-line under the following conditions.

EQUIPMENT

Conductivity cell:

- electrodes of a suitable material such as stainless steel;
- cell constant: within 2 per cent of the given value determined using a certified reference solution with a conductivity less than 1500 µS·cm⁻¹.

Conductometer: resolution 0.1 µS·cm⁻¹ on the lowest range.

System calibration (conductivity cell and conductometer):

- against one or more suitable certified standard solutions;
- accuracy: within 3 per cent of the measured conductivity plus 0.1 µS·cm⁻¹.

Conductometer calibration: by means of precision resistors or equivalent devices, after disconnecting the conductivity cell, for all ranges used for conductivity measurement and cell calibration (with an accuracy within 0.1 per cent of the stated value, traceable to the official standard).

If in-line conductivity cells cannot be dismantled, system calibration may be performed against a calibrated conductivity cell placed close to the cell to be calibrated in the water flow.

PROCEDURE

Measure the conductivity without temperature compensation, recording simultaneously the temperature. Temperature-compensated measurement may be performed after suitable validation.

The water to be examined meets the requirements if the measured conductivity at the recorded temperature is not greater than the value in Table 0008.-1.

Table 0008.-1. – *Temperature and conductivity requirements*

Temperature (°C)	Conductivity (µS·cm ⁻¹)
0	2.4
10	3.6
20	4.3
25	5.1
30	5.4
40	6.5
50	7.1
60	8.1
70	9.1
75	9.7
80	9.7
90	9.7
100	10.2

For temperatures not listed in Table 0008.-1, calculate the maximal permitted conductivity by interpolation between the next lower and next higher data points in the table.

Purified water in bulk is stored and distributed in conditions designed to prevent growth of micro-organisms and to avoid any other contamination.

CHARACTERS

Appearance: clear and colourless liquid.

TESTS

Nitrates: maximum 0.2 ppm.

Place 5 ml in a test-tube immersed in iced water, add 0.4 ml of a 100 g/l solution of *potassium chloride R*, 0.1 ml of *diphenylamine solution R* and, dropwise with shaking, 5 ml of *nitrogen-free sulphuric acid R*. Transfer the tube to a water-bath at 50 °C. After 15 min, any blue colour in the solution is not more intense than that in a reference solution prepared at the same time in the same manner using a mixture of 4.5 ml of *nitrate-free water R* and 0.5 ml of *nitrate standard solution* (2 ppm NO₃) R.

Aluminium (2.4.17): maximum 10 ppb, if intended for use in the manufacture of dialysis solutions.

Prescribed solution. To 400 ml of the water to be examined add 10 ml of *acetate buffer solution pH 6.0 R* and 100 ml of *distilled water R*.

Reference solution. Mix 2 ml of *aluminium standard solution* (2 ppm Al) R, 10 ml of *acetate buffer solution pH 6.0 R* and 98 ml of *distilled water R*.

Blank solution. Mix 10 ml of *acetate buffer solution pH 6.0 R* and 100 ml of *distilled water R*.

Heavy metals (2.4.8): maximum 0.1 ppm.

01/2005:0359

Heat 200 ml in a glass evaporating dish on a water-bath until the volume is reduced to 20 ml. 12 ml of the concentrated solution complies with limit test A. Prepare the standard using 10 ml of *lead standard solution* (1 ppm Pb) R.

Bacterial endotoxins (2.6.14): less than 0.25 IU/ml, if intended for use in the manufacture of dialysis solutions without a further appropriate procedure for removal of bacterial endotoxins.

LABELLING

The label states, where applicable, that the substance is suitable for use in the manufacture of dialysis solutions.

Purified water in containers

DEFINITION

Purified water in bulk that has been filled and stored in conditions designed to assure the required microbiological quality. It is free from any added substances.

CHARACTERS

Appearance: clear and colourless liquid.

TESTS

It complies with the tests prescribed in the section on Purified water in bulk and with the following additional tests.

Acidity or alkalinity. To 10 ml, freshly boiled and cooled in a borosilicate glass flask, add 0.05 ml of *methyle red solution* R. The solution is not coloured red.

To 10 ml add 0.1 ml of *bromothymol blue solution* R1. The solution is not coloured blue.

Oxidisable substances. To 100 ml add 10 ml of *dilute sulphuric acid* R and 0.1 ml of 0.02 M *potassium permanganate* and boil for 5 min. The solution remains faintly pink.

Chlorides. To 10 ml add 1 ml of *dilute nitric acid* R and 0.2 ml of *silver nitrate solution* R2. The solution shows no change in appearance for at least 15 min.

Sulphates. To 10 ml add 0.1 ml of *dilute hydrochloric acid* R and 0.1 ml of *barium chloride solution* R1. The solution shows no change in appearance for at least 1 h.

Ammonium: maximum 0.2 ppm.

To 20 ml add 1 ml of *alkaline potassium tetraiodomercurate solution* R. After 5 min, examine the solution down the vertical axis of the tube. The solution is not more intensely coloured than a standard prepared at the same time by adding 1 ml of *alkaline potassium tetraiodomercurate solution* R to a mixture of 4 ml of *ammonium standard solution* (1 ppm NH₄) R and 16 ml of *ammonium-free water* R.

Calcium and magnesium. To 100 ml add 2 ml of *ammonium chloride buffer solution* pH 10.0 R, 50 mg of *mordant black 11 triturate* R and 0.5 ml of 0.01 M *sodium edetate*. A pure blue colour is produced.

Residue on evaporation: maximum 0.001 per cent.

Evaporate 100 ml on a water-bath and dry in an oven at 100-105 °C. The residue weighs a maximum of 1 mg.

Microbial contamination. Total viable aerobic count (2.6.12) not more than 10² micro-organisms per millilitre, determined by membrane filtration, using agar medium B.

LABELLING

The label states, where applicable, that the substance is suitable for use in the manufacture of dialysis solutions.

WHEAT STARCH

Tritici amylum

DEFINITION

Wheat starch is obtained from the caryopsis of *Triticum aestivum* L. (*T. vulgare* Vill.).

CHARACTERS

Appearance: very fine, white powder which creaks when pressed between the fingers.

Solubility: practically insoluble in cold water and in alcohol.

Wheat starch does not contain starch grains of any other origin. It may contain a minute quantity, if any, of tissue fragments of the original plant.

IDENTIFICATION

- Examined under a microscope using equal volumes of *glycerol* R and *water* R, it presents large and small granules, and, very rarely, intermediate sizes. The large granules, 10 µm to 60 µm in diameter, are discoid or, more rarely, reniform when seen face-on. The central hilum and striations are invisible or barely visible and the granules sometimes show cracks on the edges. Seen in profile, the granules are elliptical and fusiform and the hilum appears as a slit along the main axis. The small granules, rounded or polyhedral, are 2 µm to 10 µm in diameter. Between crossed nicol prisms, the granules show a distinct black cross intersecting at the hilum.
- Suspend 1 g in 50 ml of *water* R, boil for 1 min and cool. A thin, cloudy mucilage is formed.
- To 1 ml of the mucilage obtained in identification test B, add 0.05 ml of *iodine solution* R1. A dark blue colour is produced which disappears on heating.

TESTS

pH (2.2.3): 4.5 to 7.0.

Shake 5.0 g with 25.0 ml of *carbon dioxide-free water* R for 60 s. Allow to stand for 15 min.

Foreign matter. Examined under a microscope using a mixture of equal volumes of *glycerol* R and *water* R, not more than traces of matter other than starch granules are present. No starch grains of any other origin are present.

Total protein: maximum 0.3 per cent of total protein (corresponding to 0.048 per cent N₂, conversion factor: 6.25), determined on 6.0 g by sulphuric acid digestion (2.5.9) modified as follows: wash any adhering particles from the neck into the flask with 25 ml of *sulphuric acid* R; continue the heating until a clear solution is obtained; add 45 ml of *strong sodium hydroxide solution* R.

Oxidising substances (2.5.30): maximum 20 ppm, calculated as H₂O₂.

Sulphur dioxide (2.5.29): maximum 50 ppm.

Iron (2.4.9): maximum 10 ppm.

Shake 1.5 g with 15 ml of *dilute hydrochloric acid* R. Filter. The filtrate complies with the limit test for iron.

Loss on drying (2.2.32): maximum 15.0 per cent, determined on 1.000 g by drying in an oven at 130 °C for 90 min.

Sulphated ash (2.4.14): maximum 0.6 per cent, determined on 1.0 g.

Microbial contamination. Total viable aerobic count (2.6.12) not more than 10^3 bacteria and not more than 10^2 fungi per gram, determined by plate count. It complies with the test for *Escherichia coli* (2.6.13).

01/2005:1379

WHEAT-GERM OIL, REFINED

Tritici aestivi oleum raffinatum

DEFINITION

Fatty oil obtained from the germ of the grain of *Triticum aestivum* L. by cold expression or by other suitable mechanical means and/or by extraction. It is then refined. A suitable antioxidant may be added.

CHARACTERS

Appearance: clear, light yellow liquid.

Solubility: practically insoluble in water and in alcohol, miscible with light petroleum (40 °C to 60 °C).

Relative density: about 0.925.

Refractive index: about 1.475.

IDENTIFICATION

- A. Identification of fatty oils by thin-layer chromatography (2.3.2). The chromatogram obtained is similar to the type chromatogram for wheat-germ oil.
- B. It complies with the test for composition of fatty acids (see Tests).

TESTS

Acid value (2.5.1): maximum 0.9. If intended for use in the manufacture of parenteral dosage forms: maximum 0.3.

Peroxide value (2.5.5): maximum 10.0. If intended for use in the manufacture of parenteral dosage forms: maximum 5.0.

Unsataponifiable matter (2.5.7): maximum 5.0 per cent, determined on 5.0 g.

Alkaline impurities (2.4.19). It complies with the test for alkaline impurities in fatty oils.

Composition of fatty acids. Gas chromatography (2.4.22, Method C). Use the mixture of calibrating substances in Table 2.4.22-3.

Composition of the fatty-acid fraction of the oil:

- *palmitic acid*: 14.0 per cent to 19.0 per cent,
- *stearic acid*: maximum 2.0 per cent,
- *oleic acid*: 12.0 per cent to 23.0 per cent,
- *linoleic acid*: 52.0 per cent to 59.0 per cent,
- *linolenic acid*: 3.0 per cent to 10.0 per cent,
- *eicosenoic acid*: maximum 2.0 per cent.

Brassicasterol (2.4.23): maximum 0.3 per cent of brassicasterol in the sterol fraction of the oil.

Water (2.5.32). If intended for use in the manufacture of parenteral dosage forms: maximum 0.1 per cent, determined on 5.00 g. Use a mixture of equal volumes of *methanol R* and *methylene chloride R* as solvent.

STORAGE

In an airtight, well-filled container, protected from light.

LABELLING

The label states:

- the name and concentration of any added antioxidant,
- where applicable, that the substance is suitable for use in the manufacture of parenteral dosage forms,
- whether the oil is obtained by mechanical means, by extraction or by a combination of the two.

01/2005:1480

WHEAT-GERM OIL, VIRGIN

Tritici aestivi oleum virginale

DEFINITION

Virgin wheat-germ oil is the fatty oil obtained from the germ of the grain of *Triticum aestivum* L. by cold expression or other suitable mechanical means.

CHARACTERS

A clear, light yellow or golden-yellow liquid, practically insoluble in water and in alcohol, miscible with light petroleum (40 °C to 60 °C).

It has a relative density of about 0.925 and a refractive index of about 1.475.

IDENTIFICATION

- A. Carry out the identification of fatty oils by thin-layer chromatography (2.3.2). The chromatogram obtained is comparable with the typical chromatogram for wheat-germ oil.
- B. It complies with test for composition of fatty acids (see Tests).

TESTS

Acid value (2.5.1). Not more than 20.0, determined on 10.0 g.

Peroxide value (2.5.5). Not more than 15.0.

Unsataponifiable matter (2.5.7). Not more than 5.0 per cent, determined on 5.0 g.

Composition of fatty acids (2.4.22, Method C). The fatty-acid fraction of the oil has the following composition:

- *palmitic acid*: 14.0 per cent to 19.0 per cent,
- *stearic acid*: not more than 2.0 per cent,
- *oleic acid*: 12.0 per cent to 23.0 per cent,
- *linoleic acid*: 52.0 per cent to 59.0 per cent,
- *linolenic acid*: 3.0 per cent to 10.0 per cent,
- *eicosenoic acid*: not more than 2.0 per cent.

Brassicasterol (2.4.23). The sterol fraction of the oil contains not more than 0.3 per cent of brassicasterol.

Water (2.5.32). Not more than 0.1 per cent, determined on 5.00 g by the micro-determination of water. Use a mixture of equal volumes of *methanol R* and *methylene chloride R* as solvent.

STORAGE

Store in an airtight, well-filled container, protected from light.

01/2005:1855

WILD PANSY (FLOWERING AERIAL PARTS)

Violae herba cum flore

DEFINITION

Dried flowering aerial parts of *Viola arvensis* Murray and/or *Viola tricolor* L.

Content: minimum 1.5 per cent of flavonoids, expressed as violanthin ($C_{27}H_{30}O_{14}$; M_r 578.5) (dried drug).

CHARACTERS

Macroscopic and microscopic characters described under identification tests A and B.

IDENTIFICATION

A. The stem is angular and hollow. The leaves are oval, petiolate, with a cordate base or elongated and obtuse, with lyrate stipules, divided in the middle. The flowers, with a long peduncle, are zygomorphic, with 5 oval, lanceolate sepals, an appendage pointed outwards and 5 petals of which the lower one bears a spur; in *Viola arvensis*, the petals are shorter than the calyx, the lower petal is cream coloured, with black lines, the 4 upper petals may be cream coloured or violet blue; in *Viola tricolor*, the petals are longer than the calyx and violet coloured, more or less tinged with yellow. The androecium consisting of 5 stamens bears at the apex a membranous connective appendage with 2 spurs. The trilocular ovary shows a short style and globular stigmata. The fruit are navicular capsules, three-lobed, yellowish brown, 5 mm to 10 mm long. The pale yellow, pyriform seeds are about 1 mm long, bearing a caruncle.

B. Reduce to a powder (355). The powder is greenish. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters: fragments of the epidermis of the leaves in surface view with wavy-walled cells and anomocytic stomata (2.8.3); conical unicellular covering trichomes, widened at the base and sharply pointed at the apex, with a striated cuticle; glandular trichomes with a multicellular head, and a short, multicellular stalk in the indentations of the leaf margins; cluster crystals of calcium oxalate, sometimes included in parenchyma; fragments of the corolla with wavy-walled epidermal cells, those from the mid-region papillose and with some extended to form flask or bottle-shaped projections, those from the base of the petals with covering trichomes up to about 300 µm long with characteristic hump-like swellings along their length; spherical or polyhedral pollen grains, 60 µm to 80 µm in diameter, with finely pitted exines and 5 pores (*Viola arvensis*) or 4 pores (*Viola tricolor*); occasional fragments of spiral and reticulate vessels and groups of fibres from the stem.

C. Thin-layer chromatography (2.2.27).

Test solution. Heat in a water-bath at 65 °C for 5 min, with frequent stirring, 2.0 g of the powdered drug (355) in 10 ml of *alcohol (70 per cent V/V) R*. Cool and filter.

Reference solution. Dissolve 2.5 mg of *rutin R*, 2.5 mg of *hyperoside R* and 1.0 mg of *caffeic acid R* in *methanol R* and dilute to 10 ml with the same solvent.

Plate: *TLC silica gel plate R*.

Mobile phase: *anhydrous formic acid R, acetic acid R, water R, ethyl acetate R* (11:11:27:100 V/V/V/V).

Application: 10 µl, as bands.

Development: over a path of 12 cm.

Drying: at 100-105 °C.

Detection: spray with a solution containing 10 g/l of *diphenylboric acid aminoethyl ester R* and 50 g/l of *macrogol 400 R* in *methanol R*. Allow the plate to dry in air for 30 min. Examine in ultraviolet light at 365 nm.

Results: see below the sequence of the zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other zones may be present in the chromatogram obtained with the test solution.

Top of the plate	
Caffeic acid: a greenish-blue to light blue fluorescent zone	A blue fluorescent zone
Hyperoside: a yellowish-brown fluorescent zone	A yellowish-green fluorescent zone
Rutin: a yellowish-brown fluorescent zone	An intense yellowish-brown fluorescent zone (rutin)
	A yellowish-green fluorescent zone
	A yellowish-green fluorescent zone
	A yellowish-green fluorescent zone
Reference solution	Test solution

TESTS

Foreign matter (2.8.2): maximum 3 per cent.

Swelling index (2.8.4): minimum 9, determined on the powdered drug (355).

Loss on drying (2.2.32): maximum 12.0 per cent, determined on 1.000 g of the powdered drug (355) by drying in an oven at 100-105 °C for 2 h.

Total ash (2.4.16): maximum 15.0 per cent.

ASSAY

Stock solution. In a 200 ml flask, introduce 0.300 g of the powdered drug (250) and 40 ml of *alcohol (60 per cent V/V) R*. Heat in a water-bath at 60 °C for 10 min, shaking frequently. Allow to cool and filter through a plug of absorbent cotton into a 100 ml volumetric flask. Transfer the absorbent cotton with the drug residue back into the 200 ml flask, add 40 ml of *alcohol (60 per cent V/V) R* and heat again in a water-bath at 60 °C for 10 min, shaking frequently. Allow to cool and filter into the same 100 ml volumetric flask as used previously. Rinse the 200 ml flask with a further quantity of *alcohol (60 per cent V/V) R*, filter and transfer to the same 100 ml volumetric flask. Dilute to volume with *alcohol (60 per cent V/V) R* and filter.

Test solution. Introduce 5.0 ml of the stock solution into a round-bottomed flask and evaporate to dryness under reduced pressure. Take up the residue with 8 ml of a mixture of 10 volumes of *methanol R* and 100 volumes of *glacial*

acetic acid R and transfer into a 25 ml volumetric flask. Rinse the round-bottomed flask with 3 ml of a mixture of 10 volumes of *methanol R* and 100 volumes of *glacial acetic acid R* and transfer into the same 25 ml volumetric flask as used previously. Add 10.0 ml of a solution containing 25.0 g/l of *boric acid R* and 20.0 g/l of *oxalic acid R* in *anhydrous formic acid R* and dilute to 25.0 ml with *anhydrous acetic acid R*.

Compensation liquid. Introduce 5.0 ml of the stock solution into a round-bottomed flask and evaporate to dryness under reduced pressure. Take up the residue with 8 ml of a mixture of 10 volumes of *methanol R* and 100 volumes of *glacial acetic acid R* and transfer into the same 25 ml volumetric flask as used previously. Add 10.0 ml of *anhydrous formic acid R* and dilute to 25.0 ml with *anhydrous acetic acid R*.

Measure the absorbance (2.2.25) of the test solution at 405 nm after 30 min.

Calculate the percentage content of total flavonoids, expressed as violanthin from the expression:

$$\frac{A \times 1.25}{m}$$

taking the specific absorbance of violanthin to be 400.

A = measured absorbance at 405 nm,

m = mass of the drug to be examined, in grams.

01/2005:1891

WILD THYME

Serpylli herba

DEFINITION

Whole or cut, dried, flowering aerial parts of *Thymus serpyllum* L.s.l.

Content: minimum 3.0 ml/kg of essential oil (dried drug).

CHARACTERS

Macroscopic and microscopic characters described under identification tests A and B.

IDENTIFICATION

A. The stem is much branched, up to about 1.5 mm in diameter, cylindrical or indistinctly quadrangular, green, reddish or purplish, the older stems brown and woody, the younger stems pubescent. The leaves are opposite, 3 mm to 12 mm long and up to 4 mm wide, elliptical to ovate-lanceolate with an obtuse apex, cuneate and shortly petiolate at the base; the margin is entire and markedly ciliate, especially near the base; both surfaces are more or less glabrous but distinctly punctate. The inflorescence is composed of about 6 to 12 flowers in rounded to ovoid, terminal heads. The calyx is tubular, two-lipped with the upper lip dividing to form 3 teeth, the lower lip with 2 teeth, edged with long hairs; inner surfaces strongly pubescent, the hairs forming a closed tube after flowering. The corolla is purplish-violet to red, two-lipped, the lower lip with 3 lobes, upper lip notched, inner surface strongly pubescent; stamens 4, epipetalous, projecting from the corolla tube.

B. Reduce to a powder (355). The powder is greyish-green to brownish-green. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters: fragments of the leaf epidermises with sinuous, slightly thickened anticlinal walls and stomata of the diacytic type (2.8.3); numerous covering trichomes on both epidermises and along the leaf margins, the majority short, conical, unicellular, with thickened and warty walls, fewer long, uniseriate, composed of up to 8 cells, slightly swollen at the joints, with moderately thickened walls; abundant glandular trichomes, mostly multicellular with a small, rounded, unicellular stalk and a large globular head composed of a number of indistinct, radiating cells containing brown secretion, others smaller, capitate, with unicellular stalk and a unicellular, globoid or ovoid head; purplish-violet fragments of the corolla, the outer epidermis with numerous covering and glandular trichomes, inner epidermis papillose; pollen grains spherical to elliptical, 30 µm to 40 µm in diameter, with a finely grained exine and 6 germinal pores.

C. Thin-layer chromatography (2.2.27).

Test solution. To 1.0 g of the powdered drug (355) add 5 ml of *methylene chloride R* and shake for 3 min. Filter through about 2 g of *anhydrous sodium sulphate R*.

Reference solution. Dissolve 5 mg of *thymol R* and 10 µl of *carvacrol R* in 10 ml of *methylene chloride R*.

Plate: TLC silica gel *F₂₅₄* plate *R*.

Mobile phase: *methylene chloride R*.

Application: 20 µl, as bands.

Development: over a path of 15 cm.

Drying: in air.

Detection A: examine in ultraviolet light at 254 nm.

Results A: see below the sequence of the zones present in the chromatograms obtained with the reference solution and the test solution.

Top of the plate	
Thymol: a quenching zone	A prominent quenching zone A quenching zone (thymol)
	Quenching zones
Reference solution	Test solution

Detection B: spray with *anisaldehyde solution R* using 10 ml for a plate 200 mm square and heat at 100-105 °C for 10 min.

Results B: see below the sequence of the zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other zones are present in the lower third of the chromatogram obtained with the test solution. The intensity of the zones due to thymol and carvacrol depends upon the sample examined (chemotypes).

Top of the plate	
Thymol: a brownish-pink zone Carvacrol: a pale violet zone	A brownish-pink zone (thymol) A pale violet zone (carvacrol)
Reference solution	Test solution

TESTS

Foreign matter (2.8.2): maximum 3 per cent, determined on 30 g.

Foreign matter may also consist of acicular to linear-lanceolate leaves with a strongly bent margin, the adaxial surface showing covering trichomes shaped as pointed teeth with warty walls, the abaxial surface showing many types of warty covering trichomes: unicellular, straight or slightly curved, bicellular or tricellular, often elbow-shaped, and bicellular or tricellular, more or less straight (*Thymus vulgaris*, *Thymus zygis*).

Loss on drying (2.2.32): maximum 10.0 per cent, determined on 1.000 g of the powdered drug (355) by drying in an oven at 100–105 °C for 2 h.

Total ash (2.4.16): maximum 10.0 per cent.

Ash insoluble in hydrochloric acid (2.8.1): maximum 3.0 per cent.

ASSAY

Carry out the determination of essential oils in vegetable drugs (2.8.12). Use 50.0 g of the cut drug, a 1000 ml round-bottomed flask and 500 ml of *water R* as the distillation liquid. Distil at a rate of 2–3 ml/min for 2 h without *xylene R* in the graduated tube.

01/2005:1583
corrected

WILLOW BARK

Salicis cortex

DEFINITION

Willow bark consists of the whole or fragmented dried bark of young branches or whole dried pieces of current year twigs of various species of genus *Salix* including *S. purpurea* L., *S. daphnoides* Vill. and *S. fragilis* L. The drug contains not less than 1.5 per cent of total salicylic derivatives, expressed as salicin ($C_{13}H_{18}O_7$; M_r 286.3), calculated with reference to the dried drug.

CHARACTERS

Willow bark is markedly bitter.

It has the macroscopic and microscopic characters described under identification tests A and B.

IDENTIFICATION

- A. The bark is 1 mm to 2 mm thick and occurs in flexible, elongated, quilled or curved pieces. The outer surface is smooth or slightly wrinkled longitudinally and greenish-yellow to brownish-grey. The inner surface is smooth or finely striated longitudinally and white, pale yellow or reddish-brown, depending on the species. The fracture is short in the outer part and coarsely fibrous in the inner region. The diameter of current year twigs is not more than 10 mm. The wood is white or pale yellow.
- B. Reduce to a powder (355). The powder is pale yellow, greenish-yellow or light brown. Examine under a microscope using *chloral hydrate solution R*. The powder shows: bundles of narrow fibres, up to about 600 µm long, with very thick walls and surrounded by a crystal sheath containing prism crystals of calcium oxalate; parenchyma of the cortex with thick, pitted and deeply beaded walls, and containing large cluster crystals of

calcium oxalate; uniseriate medullary rays; thickened and suberised cork cells. Groups of brownish collenchyma from the bud may be present. Twigs show, additionally, fragments of lignified fibres and vessels from the xylem.

- C. Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel plate R*.

Test solution (a). To 1.0 g of the powdered drug (500) add 20 ml of *methanol R*; heat in a water-bath at about 50 °C, with frequent shaking, for 10 min. Cool and filter.

Test solution (b). To 5.0 ml of test solution (a) add 1.0 ml of a 50 g/l solution of *anhydrous sodium carbonate R* and heat in a water-bath at about 60 °C for 10 min. Cool and filter if necessary.

Reference solution. Dissolve 2.0 mg of *salicin R* in 1.0 ml of *methanol R*.

Apply to the plate as bands 20 µl of each. Develop over a path of 15 cm using a mixture of 8 volumes of *water R*, 15 volumes of *methanol R* and 77 volumes of *ethyl acetate R*. Allow the plate to dry in air. Spray with a mixture of 5 volumes of *sulphuric acid R* and 95 volumes of *methanol R*. Heat at 100 °C to 105 °C for 5 min and examine in daylight. The chromatogram obtained with the reference solution shows in the middle third a reddish-violet zone due to salicin. In the chromatogram obtained with test solution (a), the zone due to salicin appears with only slight to moderate intensity. In the chromatogram obtained with test solution (b) the zone due to salicin is clearly more intense and there are, above the zone due to salicin, one (salicortin or 2'-O-acetylsalicortin, or possibly two tremulacin) faint reddish-violet zones. Other blue, yellow or brown zones can occur in both chromatograms.

TESTS

Foreign matter (2.8.2). Not more than 3 per cent of twigs with a diameter greater than 10 mm, and not more than 2 per cent of other foreign matter.

Loss on drying (2.2.32). Not more than 11 per cent, determined on 1.000 g of the powdered drug (355), by drying in an oven at 100 °C to 105 °C for 2 h.

Total ash (2.4.16). Not more than 10 per cent.

ASSAY

Examine by liquid chromatography (2.2.29), using *resorcinol R* as the internal standard.

Internal standard solution. Dissolve 50 mg of *resorcinol R* in 10 ml of *methanol R*.

Test solution. To 0.5 g of the powdered drug (355) add 50 ml of *methanol R* and heat under a reflux condenser for 30 min. Cool and filter. Take up the residue with 50 ml of *methanol R*. Proceed as above. Combine the filtrates and evaporate under reduced pressure. Take up the residue with 5.0 ml of *methanol R*, add 5.0 ml of 0.1 M *sodium hydroxide* and heat in a water-bath at about 60 °C under a reflux condenser, with frequent shaking for about 1 h. After cooling, add 0.5 ml of 1 M *hydrochloric acid*. Dilute the solution to 20.0 ml with a mixture of 50 volumes of *methanol R* and 50 volumes of *water R*. Add 1.0 ml of the internal standard solution to 10.0 ml of this solution. Filter through a membrane filter.

Reference solution (a). Dissolve 18.5 mg of *salicin R* in 10.0 ml of a mixture of 20 volumes of *water R* and 80 volumes of *methanol R* and add 1.0 ml of the internal standard solution.

Reference solution (b). Dissolve 1.0 mg of *picein R* in 1.0 ml of reference solution (a).

The chromatographic procedure may be carried out using:

- a stainless steel column, 0.10 m long and 3 mm or 4 mm in internal diameter, packed with *octadecylsilyl silica gel for chromatography R* (3 µm),
- as mobile phase at a flow rate of 1.0 ml/min a mixture of 1.8 volumes of *tetrahydrofuran R* and 98.2 volumes of *water R*, containing 0.5 per cent V/V of *phosphoric acid R*,
- as detector a spectrophotometer set at 270 nm,
- a loop injector.

Inject 10 µl of reference solution (b). The assay is not valid unless the resolution between the peaks corresponding to salicin and picein and between the peaks corresponding to picein and resorcinol is at least 1.5.

Inject five times 10 µl of reference solution (a).

Inject three times 10 µl of the test solution. Continue the chromatography for four times the retention time of the peak corresponding to salicin.

Calculate the percentage content of total salicylic derivatives, expressed as salicin, from the expression:

$$\frac{S_1 \times S_4 \times m_2 \times p \times 2}{S_2 \times S_3 \times m_1}$$

- S_1 = area of the peak due to salicin in the chromatogram obtained with the test solution,
- S_2 = area of the peak due to resorcinol in the chromatogram obtained with the test solution,
- S_3 = area of the peak due to salicin in the chromatogram obtained with reference solution (a),
- S_4 = area of the peak due to resorcinol in the chromatogram obtained with reference solution (a),
- m_1 = mass of the drug in the test solution, in milligrams,
- m_2 = mass of salicin in reference solution (a), in milligrams,
- p = percentage content of salicin in the reference substance

STORAGE

Store protected from light.

01/2005:0593

WOOL ALCOHOLS

Alcoholes adipis lanae

DEFINITION

Mixture of sterols and higher aliphatic alcohols from wool fat. It may contain not more than 200 ppm of butylhydroxytoluene.

Content: minimum 30.0 per cent of cholesterol.

CHARACTERS

Appearance: pale-yellow or brownish-yellow, brittle mass becoming plastic on heating.

Solubility: practically insoluble in water, soluble in methylene chloride and in boiling ethanol, slightly soluble in alcohol (90 per cent V/V).

IDENTIFICATION

Dissolve 50 mg in 5 ml of *methylene chloride R* and add 1 ml of *acetic anhydride R* and 0.1 ml of *sulphuric acid R*. Within a few seconds, a green colour develops.

TESTS

Appearance of solution. To 1.0 g add 10 ml of *light petroleum R1* and shake while warming in a water-bath. The substance dissolves completely. After cooling, the solution is clear (2.2.1).

Alkalinity. Dissolve 2.0 g in 25 ml of hot *alcohol (90 per cent V/V) R* and add 0.5 ml of *phenolphthalein solution R1*. No red colour develops.

Melting point (2.2.15): minimum 58 °C.

Melt the substance to be examined by heating in a water-bath at a temperature which exceeds the expected melting point by not more than 10 °C; introduce the substance to be examined into the capillary tubes and allow to stand at 15-17 °C for not less than 16 h.

Acid value (2.5.1): maximum 2.0.

If necessary, heat in a water-bath under a reflux condenser to dissolve the substance to be examined.

Hydroxyl value (2.5.3, Method A): 120 to 180.

Peroxide value (2.5.5, Method A): maximum 15.

Take from the substance to be examined wedge-shaped pieces whose base consists of part of the surface. Melt the pieces before carrying out the determination. Before adding 0.5 ml of *saturated potassium iodide solution R*, cool the solution obtained to room temperature.

Saponification value (2.5.6): maximum 12.0, determined on 2.00 g. Heat under reflux for 4 h.

Butylhydroxytoluene. Gas chromatography (2.2.28).

Internal standard solution. Dissolve 0.20 g of *methyl decanoate R* in *carbon disulphide R* and dilute to 100.0 ml with the same solvent. Dilute 1.0 ml of the solution to 10.0 ml with *carbon disulphide R*.

Test solution (a). Dissolve 1.0 g of the substance to be examined in *carbon disulphide R* and dilute to 10.0 ml with the same solvent.

Test solution (b). Dissolve 1.0 g of the substance to be examined in *carbon disulphide R*, add 1.0 ml of the internal standard solution and dilute to 10.0 ml with *carbon disulphide R*.

Reference solution. Dissolve 0.20 g of *butylhydroxytoluene R* in *carbon disulphide R* and dilute to 100.0 ml with the same solvent. Dilute 1.0 ml of the solution to 10.0 ml with *carbon disulphide R*. To 1.0 ml of this solution add 1.0 ml of the internal standard solution and dilute to 10.0 ml with *carbon disulphide R*.

Precolumn: column filled with silanised glass wool.

Column:

- *size:* $l = 1.5$ m, $\varnothing = 4$ mm,
- *stationary phase:* *silanised diatomaceous earth for gas chromatography R* impregnated with 10 per cent *m/m* of *poly(dimethyl)siloxane R*.

Carrier gas: *nitrogen for chromatography R*.

Flow rate: 40 ml/min.

Temperature:

- *column:* 150 °C,
- *injection port:* 180 °C,
- *detector:* 300 °C.

Detection: flame ionisation.

Limit:

– *butylhydroxytoluene*: maximum 200 ppm.

Loss on drying (2.2.32): maximum 0.5 per cent, determined on 2.000 g by drying in an oven at 100–105 °C.

Total ash (2.4.16): maximum 0.1 per cent.

Water-absorption capacity. Place 0.6 g of the substance to be examined and 9.4 g of *white soft paraffin R* in a mortar and melt on a water-bath. Allow to cool and incorporate 20 ml of *water R*, added in portions. Within 24 h no water is released from the almost white, ointment-like emulsion.

ASSAY

Dissolve 0.1000 g in 12 ml of hot *alcohol (90 per cent V/V) R*. Allow to stand for 18 h, filter through a sintered-glass filter (16) and wash the filter with 2 quantities, each of 15 ml, of *alcohol (90 per cent V/V) R*. Combine the filtrate and washings, add 20 ml of a freshly prepared 10 g/l solution of *digitonin R* in *alcohol (90 per cent V/V) R* and heat to about 60 °C. Allow to cool, filter through a sintered-glass filter (16), wash the residue with 10 ml of *alcohol (90 per cent V/V) R* and dry to constant mass at 100–105 °C.

1 g of residue is equivalent to 0.239 g of cholesterol.

STORAGE

In a well-filled container, protected from light.

LABELLING

The label states, where applicable, the concentration of any added butylhydroxytoluene.

01/2005:0134

WOOL FAT**Adeps lanae****DEFINITION**

Purified, anhydrous, waxy substance obtained from the wool of sheep (*Ovis aries*). It may contain not more than 200 ppm of butylhydroxytoluene.

CHARACTERS

Appearance: yellow, unctuous substance. When melted, it is a clear or almost clear, yellow liquid. A solution in light petroleum is opalescent.

Solubility: practically insoluble in water, slightly soluble in boiling ethanol.

It has a characteristic odour.

IDENTIFICATION

- A. In a test-tube, dissolve 0.5 g in 5 ml of *methylene chloride R* and add 1 ml of *acetic anhydride R* and 0.1 ml of *sulphuric acid R*. A green colour develops.
- B. Dissolve 50 mg in 5 ml of *methylene chloride R*, add 5 ml of *sulphuric acid R* and shake. A red colour develops and an intense green fluorescence appears in the lower layer when examined in daylight, with illumination from the rear of the observer.

TESTS

Water-soluble acid or alkaline substances. Melt 5.0 g on a water-bath and shake vigorously for 2 min with 75 ml of *water R* previously heated to 90–95 °C. Allow to cool and filter through filter paper previously rinsed with *water R*. To 60 ml of the filtrate (which may not be clear) add 0.25 ml

of *bromothymol blue solution RI*. Not more than 0.2 ml of 0.02 M *hydrochloric acid* or 0.15 ml of 0.02 M *sodium hydroxide* is required to change the colour of the indicator.

Drop point (2.2.17): 38 °C to 44 °C.

To fill the metal cup, melt the wool fat on a water-bath, cool to about 50 °C, pour into the cup and allow to stand at 15–20 °C for 24 h.

Water-absorption capacity. Place 10 g in a mortar. Add *water R* in portions of 0.2 ml to 0.5 ml from a burette, stirring vigorously after each addition to incorporate the *water R*. The end-point is reached when visible droplets remain which cannot be incorporated. Not less than 20 ml of *water R* is absorbed.

Acid value (2.5.1): maximum 1.0, determined on 5.0 g dissolved in 25 ml of the prescribed mixture of solvents.

Peroxide value (2.5.5, *Method A*): maximum 20.

Before adding 0.5 ml of *saturated potassium iodide solution R*, cool the solution obtained to room temperature.

Saponification value (2.5.6): 90 to 105, determined on 2.00 g while heating under reflux for 4 h.

Water-soluble oxidisable substances. To 10 ml of the filtrate obtained in the test for water-soluble acid or alkaline substances add 1 ml of *dilute sulphuric acid R* and 0.1 ml of 0.02 M *potassium permanganate*. After 10 min, the solution is not completely decolourised.

Butylhydroxytoluene. Gas chromatography (2.2.28).

Internal standard solution. Dissolve 0.2 g of *methyl decanoate R* in *carbon disulphide R* and dilute to 100.0 ml with the same solvent. Dilute 1.0 ml of the solution to 10.0 ml with *carbon disulphide R*.

Test solution (a). Dissolve 1.0 g of the substance to be examined in *carbon disulphide R* and dilute to 10.0 ml with the same solvent.

Test solution (b). Dissolve 1.0 g of the substance to be examined in *carbon disulphide R*, add 1.0 ml of the internal standard solution and dilute to 10.0 ml with *carbon disulphide R*.

Reference solution. Dissolve 0.2 g of *butylhydroxytoluene R* in *carbon disulphide R* and dilute to 100.0 ml with the same solvent. Dilute 1.0 ml of the solution to 10.0 ml with *carbon disulphide R*. To 1.0 ml of this solution add 1.0 ml of the internal standard solution and dilute to 10.0 ml with *carbon disulphide R*.

Column:

- size: $l = 1.5$ m, $\varnothing = 4$ mm,
- stationary phase: *silanised diatomaceous earth for gas chromatography R* impregnated with 10 per cent *m/m* of *poly(dimethyl)siloxane R*.

The column is preceded by a column containing silanised glass wool.

Carrier gas: *nitrogen for chromatography R*.

Flow rate: 40 ml/min.

Temperature:

- column: 150 °C,
- injection port: 180 °C,
- detector: 300 °C.

Detection: flame ionisation.

Limit:

– *butylhydroxytoluene*: maximum 200 ppm.

Paraffins: maximum 1.0 per cent.

The tap and cotton plugs used must be free from grease.

Prepare a column of anhydrous aluminium oxide 0.23 m long and 20 mm in diameter by adding a slurry of *anhydrous*

aluminium oxide R and *light petroleum R1* to a glass tube fitted with a tap and containing *light petroleum R1* (before use, dehydrate the anhydrous aluminium oxide by heating it in an oven at 600 °C for 3 h). Allow to settle and reduce the depth of the layer of solvent above the column to about 40 mm. Dissolve 3.0 g of the substance to be examined in 50 ml of warm *light petroleum R1*, cool, pass the solution through the column at a flow rate of 3 ml/min and wash with 250 ml of *light petroleum R1*. Concentrate the combined eluate and washings to low bulk by distillation, evaporate to dryness on a water-bath and heat the residue at 105 °C for periods of 10 min until 2 successive weighings do not differ by more than 1 mg. The residue weighs a maximum of 30 mg.

Pesticide residues: maximum 0.05 ppm for each organochlorine pesticide, 0.5 ppm for each other pesticide and 1 ppm for the sum of all the pesticides.

All glassware used is thoroughly washed using phosphate-free detergent as follows. The glassware is immersed in a bath of detergent solution (5 per cent in deionised water) and allowed to soak for 24 h. The detergent is washed off with copious amounts of acetone and hexane for pesticide analysis. It is important to keep glassware specifically for pesticide analyses, it must not be mixed up with glassware used for other applications. The glassware used must be free of chlorinated solvents, plastics and rubber materials, in particular phthalate plasticisers, oxygenated compounds and nitrogenated solvents such as acetonitrile. Use hexane, toluene and acetone for pesticide analysis. Use HPLC grade reagents for ethyl acetate, cyclohexane and water.

The test consists of the isolation of pesticide residues by size-exclusion chromatography (2.2.30) followed by solid phase extraction and identification by gas chromatography coupled with an electron capture detector or a thermionic detector.

ISOLATION OF THE PESTICIDE RESIDUES. As detector, use a UV-visible spectrophotometer set at a wavelength of 254 nm to calibrate the chromatographic column for gel permeation.

Calibration is extremely important in gel permeation chromatography to check that the pressure, solvent flow rate, solvent ratio, temperature and column conditions remain constant. The gel permeation column is to be calibrated at regular intervals using a standard mixture prepared as follows: into a 1000 ml volumetric flask, introduce 50.00 g of *maize oil R*, 2.00 g of *di(2-ethylhexyl) phthalate R*, 0.20 g of *methoxychlor R*, 50.0 mg of *perylene R*, 50.0 mg of *naphthalene R*, and 80.0 mg of *sulphur R*. Dilute to 1000.0 ml with a mixture of equal volumes of *cyclohexane R* and *ethyl acetate R*.

To calibrate the column, set the mobile phase at a flow rate of 5 ml/min with a mixture of equal volumes of *cyclohexane R* and *ethyl acetate R*. Inject 5 ml of the standard mixture and record the resulting chromatogram. The retention times for the analytes must not vary by more than ± 5 per cent between calibrations. If the retention time shift is greater than ± 5 per cent, take corrective action. Excessive retention time shifts may be caused by:

- poor laboratory temperature control,
- the pump contains air. This can be verified by measuring the flow rate: collect 25 ml of column eluate in a volumetric flask and record the time (300 ± 5 s),
- a leak in the system.

Changes in pressure, in mobile phase flow rate or in column temperature conditions, as well as column contamination, can affect pesticide retention times and are to be monitored. If the flow rate or column pressure are outside desired bands the guard column or column is to be replaced.

Test solution. In a volumetric flask, dissolve 1 g of wool fat, accurately weighed, in a mixture of 1 volume of *ethyl acetate R* and 7 volumes of *cyclohexane R*. Add 1 ml of an internal standard (2 ppm) either *isodrin R* or *ditalimphos R* and dilute to 20 ml. *The internal standard solutions are used to establish that recoveries of the pesticides from the GPC purification stage, evaporation and solid phase extraction stage are at acceptable levels. Recovery levels of the internal standard solutions from the wool fat are determined by comparing the peak areas of the wool fat extracts with peak areas of solutions of the internal standards.*

Guard column:

- size: $l = 0.075$ m, $\varnothing = 21.2$ mm,
- stationary phase: *styrene-divinylbenzene copolymer R* (5 μ m).

Gel permeation column:

- size: $l = 0.3$ m, $\varnothing = 21.2$ mm,
- stationary phase: *styrene-divinylbenzene copolymer R* (5 μ m).

Mobile phase: *ethyl acetate R*, *cyclohexane R* (1:7 V/V).

Flow rate: 5 ml/min.

Detection: spectrophotometer at 254 nm.

Inject 5 ml of the test solution. Discard the first 95 ml (19 min) of eluate containing the substance to be examined. Collect the next 155 ml of eluate (31 min) containing any pesticide residues in an evaporating vessel.

Place the 155 ml of the eluate collected from the gel permeation chromatography column into an evaporating vessel. Place this vessel in an autoevaporator setting the water-bath temperature at 45 °C and the nitrogen pressure at 55 kPa. Evaporate the eluate down to 0.5 ml.

To prepare the solid phase extraction cartridges take some *magnesium silicate for pesticide residue analysis R* and heat it in a muffle furnace at 700 °C for 4 h to remove moisture and polychlorinated biphenyls. Subsequently allow the magnesium silicate to cool for 2 h and transfer it directly to an oven at 100-105 °C, and allow to stand for 30 min. Transfer the magnesium silicate to a stoppered glass jar and allow to equilibrate for 48 h. This material may be used for up to 2 weeks. After that period the magnesium silicate is to be reactivated, by heating at 600 °C for 2 h in a muffle furnace. Remove the magnesium silicate from the furnace, cool and store in a stoppered glass jar. The magnesium silicate is deactivated by adding 1 per cent of *water R*. After the water has been added, shake the magnesium silicate intermittently over 15 min just prior to use. The deactivated magnesium silicate is suitable for use for up to 1 week. It is essential that only deactivated magnesium silicate is used.

Take a 6 ml empty solid phase extraction cartridge and weigh into the cartridge 1 g of the deactivated magnesium silicate.

At this stage the GPC fraction still contains about 10 per cent of the substance to be examined, so further clean-up is necessary. A separate isolation procedure is carried out a) for organochlorine and synthetic pyrethroid pesticides and b) for organophosphorus pesticides. Place a preconditioned solid phase extraction cartridge containing 1 g of deactivated *magnesium silicate for pesticide residue analysis R* onto a vacuum manifold.

Condition the cartridge by adding 10 ml of *toluene R* and allowing the solvent to elute through the cartridge. Place the 0.5 ml of the solvent fraction from the evaporating vessel on the preconditioned cartridge. Elute the pesticide fractions from the cartridges using 20 ml of either of the 2 different solvent systems shown below:

a) for determination of the organochlorine and synthetic pyrethroid pesticides: *toluene R*. A very small amount of the substance to be examined is co-eluted,

b) for determination of the organophosphorus pesticides: a mixture of 2 volumes of *acetone R* and 98 volumes of *toluene R*. This solvent system is used to elute all the pesticides including the more polar organophosphorus pesticides. Unfortunately some of the substance to be examined is co-eluted with this solvent system, which can interfere with the electron capture detector.

Collect the eluate from the extraction cartridges in 25 ml glass vials. Quantitatively transfer the eluate to an evaporating vessel, washing the vial with 3 quantities, each of 10 ml, of *hexane R*.

Place the evaporating vessel on the autoevaporator and evaporate the solid phase extraction fractions down to 0.5 ml. The water-bath temperature is set at 45 °C and the nitrogen pressure is 55 kPa.

Examine the residues by gas chromatography (2.2.28) using electron capture and thermionic detectors as described below.

Recovery. Calculate the recovery correction factor (R_{cf}) of the internal standards (*ditalimphos R* or *isodrin R*) added to the test solution using the following expression:

$$\frac{\text{peak area of internal standard extracted from the test solution}}{\text{peak area of an internal standard 1 ppm in solution}} \times 100$$

5 ml of the 20 ml test solution containing 1 ml of 2 ppm internal standard concentrated to 0.5 ml is equivalent to 1 ppm of the internal standard in the solution.

If the recovery of the internal standards falls outside the range of 70 per cent to 110 per cent the test is not valid.

Reference solutions. Prepare reference solutions of pesticides using the pesticides standards at a concentration of 0.5 ppm (see composition of reference solutions A to D in Table 0134.-1). Commercially available pesticides may be purchased. The individual standards have a concentration of 10 ppm.

At the same time prepare solutions of pesticides equivalent to the limit of detection of the method (see recommended compositions in Table 0134.-1). These reference solutions are used to optimise the electron capture detector and thermionic detector to achieve the detection limits of the method (reference solutions E and F).

To prepare the reference solutions at the different concentrations use a calibrated pipette and volumetric flasks. To prepare the internal standard solutions G and H use a four-place analytical balance, pipette and volumetric flasks.

IDENTIFICATION AND QUANTIFICATION OF THE PESTICIDE

RESIDUES. To identify any pesticide residues compare the chromatograms obtained with chromatograms obtained with reference solutions A to D.

The identity of the pesticides can be confirmed by spiking samples or overlaying chromatograms using an integration package on a computer. The interpretation of pesticides in trace residue analyses is extremely complex. The detectors, particularly the electron capture detector, are prone to interference, both from the substance to be examined itself, and from solvents, reagents and apparatus used in the extraction. These peaks can easily be misinterpreted or quoted as a false positive. Confirmation of pesticides can be achieved by running samples and standards on different capillary columns (see chromatographic systems A or B described below). The peaks can be identified by using the information in Table 0134.-2.

Table 0134.-1. – Composition of the reference solutions

Reference solution A (0.5 ppm or 0.5 mg/l) (organochlorine and synthetic pyrethroid pesticides)	Reference solution B (0.5 ppm or 0.5 mg/l) (organochlorine and synthetic pyrethroid pesticides)
<i>Cyhalothrin R</i>	<i>Aldrin R</i>
<i>Cypermethrin R</i>	<i>o,p'-DDT R</i>
<i>o,p'-DDE R</i>	<i>o,p'-DDD R</i>
<i>p,p'-DDE R</i>	<i>p,p'-DDD R</i>
<i>p,p'-DDT R</i>	<i>Dieldrin R</i>
<i>Deltamethrin R</i>	<i>α-Endosulphan R</i>
<i>Endrin R</i>	<i>β-Endosulphan R</i>
<i>Heptachlor R</i>	<i>Fenvalerate R</i>
<i>Heptachlor epoxide R</i>	<i>α-Hexachlorocyclohexane R</i>
<i>Hexachlorobenzene R</i>	<i>β-Hexachlorocyclohexane R</i>
<i>Lindane R</i>	<i>δ-Hexachlorocyclohexane R</i>
<i>Tecnazene R</i>	<i>Methoxychlor R</i>
	<i>Permethrin R</i>
Reference solution C (0.5 ppm or 0.5 mg/l) (organophosphorus pesticides)	Reference solution D (0.5 ppm or 0.5 mg/l) (organophosphorus pesticides)
<i>Bromophos-ethyl R</i>	<i>Bromophos R</i>
<i>Carbophenothion R</i>	<i>Chlorpyrifos R</i>
<i>Chlorfenvinphos R</i>	<i>Chlorpyrifos-methyl R</i>
<i>Diazinon R</i>	<i>Coumaphos R</i>
<i>Dichlofenthion R</i>	<i>Phosalone R</i>
<i>Ethion R</i>	<i>Pirimiphos-ethyl R</i>
<i>Fenchlorphos R</i>	<i>Tetrachlorovinphos R</i>
<i>Malathion R</i>	
<i>Propetamphos R</i>	
Reference solution E (electron capture detector calibration mixture)	Reference solution F (thermionic detector calibration mixture)
<i>Aldrin R</i> (0.01 mg/l)	<i>Chlorfenvinphos R</i> (0.05 mg/l)
<i>Cypermethrin R</i> (0.1 mg/l)	<i>Diazinon R</i> (0.05 mg/l)
<i>o,p'-DDD R</i> (0.01 mg/l)	<i>Ethion R</i> (0.05 mg/l)
<i>Deltamethrin R</i> (0.1 mg/l)	<i>Fenchlorphos R</i> (0.05 mg/l)
<i>Endrin R</i> (0.01 mg/l)	<i>Propetamphos R</i> (0.05 mg/l)
<i>β-Hexachlorocyclohexane R</i> (0.01 mg/l)	
Reference solution G (internal standard organo- phosphorus pesticide)	Reference solution H (internal standard organo- chlorine pesticide)
<i>Ditalimphos R</i> (2 ppm or 2.0 mg/l)	<i>Isodrin R</i> (2 ppm or 2.0 mg/l)
<i>Ditalimphos R</i> (1 ppm or 1.0 mg/l)	<i>Isodrin R</i> (1 ppm or 1.0 mg/l)

A knowledge of the different responses the pesticides have with the 2 detectors is useful in identification of unknown peaks.

Once the pesticides have been identified, calculate the content of each pesticide using the following expression:

$$C_P = \frac{P_P \times D \times C_e}{P_e} \times \frac{100}{R_{cf}}$$

- C_P = concentration of identified pesticide (ppm),
 P_P = peak area of the individual pesticide in the test sample obtained,
 C_e = concentration of the individual pesticide in the external standard (ppm),

P_e = peak area of the individual pesticide in the external standard,
 D = dilution factor,
 R_{cf} = recovery correction factor.

The dilution factor (D) can be defined as follows:

$$\text{volume of sample obtained after the 2}^{\text{nd}} \text{ evaporation stage} \\ \text{sample weight} \times \frac{\text{GPC injection volume}}{\text{sample volumetric flask volume}}$$

Table 0134.-2. – *Elution order of the pesticides on chromatographic systems A and B*

Chromatographic system A	Chromatographic system B
Tecnazene	Tecnazene
α -Hexachlorocyclohexane	Hexachlorobenzene
Hexachlorobenzene	α -Hexachlorocyclohexane
β -Hexachlorocyclohexane	Diazinon
Lindane	Lindane
Propetamphos	Propetamphos
δ -Hexachlorocyclohexane	Heptachlor
Diazinon	Dichlofenthion
Dichlofenthion	Aldrin
Chlorpyrifos-methyl	Chlorpyrifos-methyl
Heptachlor	Fenchlorphos
Fenchlorphos	β -Hexachlorocyclohexane
Aldrin	δ -Hexachlorocyclohexane
Malathion	Pirimiphos-ethyl
Chlorpyrifos-ethyl	Chlorpyrifos-ethyl
Bromophos	Bromophos
Pirimiphos-ethyl	Malathion
Heptachlor epoxide	Heptachlor epoxide
Chlorfenvinphos (<i>E</i>)	<i>o,p</i> 'DDE
Chlorfenvinphos (<i>Z</i>)	Chlorfenvinphos (<i>E</i>)
Bromophos-ethyl	α -Endosulphan
<i>o,p</i> 'DDE	Chlorfenvinphos (<i>Z</i>)
α -Endosulphan	Bromophos-ethyl
Tetrachlorvinphos	<i>p,p</i> 'DDE
Dieldrin	Dieldrin
<i>p,p</i> 'DDE	Tetrachlorvinphos
<i>o,p</i> 'DDT	<i>o,p</i> 'DDT
Endrin	Endrin
β -Endosulphan	<i>o,p</i> 'DDD
<i>o,p</i> 'DDD	<i>p,p</i> 'DDD
<i>p,p</i> 'DDD	β -Endosulphan
Ethion	Ethion
Carbophenothion	<i>p,p</i> 'DDT
<i>p,p</i> 'DDT	Carbophenothion
Methoxychlor	Methoxychlor
Phosalone	Cyhalothrin
Cyhalothrin (2 isomers)	<i>cis</i> -Permethrin
<i>cis</i> -Permethrin	Phosalone
<i>trans</i> -Permethrin	<i>trans</i> -Permethrin
Coumaphos	Cypermethrin (4 isomers)
Cypermethrin (4 isomers)	Coumaphos
Fenvalerate (2 isomers)	Fenvalerate (2 isomers)
Deltamethrin	Deltamethrin

Chromatographic system A:

Guard column:

- *material:* deactivated silica,
- *size:* $l = 4.5$ m, $\emptyset = 0.53$ mm.

Column:

- *material:* fused silica,
- *size:* $l = 60$ m, $\emptyset = 0.25$ mm,

– *stationary phase:* poly(dimethyl)(diphenyl)siloxane *R* (film thickness 0.25 μ m).

Carrier gas: helium for chromatography *R*.

Linear velocity: 25 cm/s.

Pressure: 180 kPa.

Temperature:

	Time (min)	Temperature (°C)
Column	0 - 1	75
	1 - 5	75 $\rightarrow$ 175
	5 - 30	175 $\rightarrow$ 275
	30 - 40	275 $\rightarrow$ 285
	40 - 55	285
Injection port		300
Detector		350

Detection: electron capture or thermionic specific detector.

Injection: 2 μ l.

Chromatographic system B which may be used for confirmation analysis:

Guard column:

- *material:* deactivated silica,
- *size:* $l = 4.5$ m, $\emptyset = 0.53$ mm.

Column:

- *material:* fused silica,
- *size:* $l = 60$ m, $\emptyset = 0.25$ mm,
- *stationary phase:* poly(cyanopropyl)(7)(phenyl)(7)(methyl)(86)siloxane *R* (film thickness 0.25 μ m).

Carrier gas: helium for chromatography *R*.

Linear velocity: 25 cm/s.

Pressure: 180 kPa.

Temperature:

	Time (min)	Temperature (°C)
Column	0 - 1	75
	1 - 5	75 $\rightarrow$ 175
	5 - 30	175 $\rightarrow$ 275
	30 - 40	275 $\rightarrow$ 285
	40 - 55	285
Injection port		300
Detector		350

Detection: electron capture or thermionic specific detector.

Injection: 2 μ l.

Chlorides: maximum 150 ppm.

Boil 1.0 g with 20 ml of alcohol (90 per cent V/V) *R* in a round-bottomed flask fitted with a reflux condenser for 5 min. Cool, add 40 ml of water *R* and 0.5 ml of nitric acid *R* and filter. To the filtrate add 0.15 ml of a 10 g/l solution of silver nitrate *R* in alcohol (90 per cent V/V) *R*. Allow to stand for 5 min protected from light. Any opalescence in the solution is not more intense than that in a standard prepared at the same time by adding 0.15 ml of a 10 g/l solution of silver nitrate *R* in alcohol (90 per cent V/V) *R* to a mixture of 0.2 ml of 0.02 M hydrochloric acid, 20 ml of alcohol (90 per cent V/V) *R*, 40 ml of water *R* and 0.5 ml of nitric acid *R*.

Loss on drying (2.2.32): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 100-105 °C for 1 h.

Sulphated ash (2.4.14): maximum 0.15 per cent.

Ignite 5.0 g and use the residue to determine the sulphated ash.

STORAGE

At a temperature not exceeding 25 °C.

LABELLING

The label states, where applicable, the concentration of added butylhydroxytoluene.

Saponification value (2.5.6): maximum 8.0. Heat under reflux for 4 h.

Fatty alcohols and sterols. Gas chromatography (2.2.28).

Test solution. Dissolve 0.25 g of the substance to be examined in 60 ml of *ethanol R* and dilute to 100.0 ml with the same solvent.

Reference solution (a). Dissolve 0.25 g of *hydrogenated wool fat CRS* in 60 ml of *ethanol R* and dilute to 100.0 ml with the same solvent.

Reference solution (b). Dissolve 50 mg of *cetyl alcohol CRS* and 50 mg of *stearyl alcohol CRS* in 60 ml of *ethanol R* and dilute to 100.0 ml with the same solvent.

Column:

- *material*: fused silica,
- *size*: $l = 30$ m, $\varnothing = 0.25$ mm,
- *stationary phase*: *poly(dimethyl)siloxane R* or another non-polar phase (film thickness: 0.25 µm).

Carrier gas: *helium for chromatography R* at a pressure of 100 kPa

Temperature:

	Time (min)	Temperature (°C)
Column	0 - 5	100
	5 - 45	100 → 300
	45 - 60	300
Injection port		325
Detector		350

Detection: flame ionisation.

Injection: 1 µl.

The chromatogram obtained with the test solution does not differ significantly from the chromatogram obtained with reference solution (a) (Figure 0969.-1) and it does not show enhanced peaks with retention times corresponding to cetyl alcohol and stearyl alcohol present in the chromatogram obtained with reference solution (b).

Heavy metals (2.4.8): maximum 10 ppm.

2.0 g complies with limit test C. Prepare the standard using 2 ml of *lead standard solution (10 ppm Pb) R*.

Loss on drying (2.2.32): maximum 3.0 per cent, determined on 2.000 g by drying in an oven at 100-105 °C for 1 h.

Total ash (2.4.16): maximum 0.1 per cent, determined on 5.0 g.

STORAGE

In a well-filled container, protected from light.

01/2005:0969

WOOL FAT, HYDROGENATED

Adeps lanae hydrogenatus

DEFINITION

Mixture of higher aliphatic alcohols and sterols obtained from the direct, high-pressure, high-temperature hydrogenation of *wool fat (0134)* during which the esters and acids present are reduced to the corresponding alcohols. It may contain butylhydroxytoluene.

CHARACTERS

Appearance: white or pale yellow, unctuous substance.

Solubility: practically insoluble in water, soluble in boiling alcohol and in light petroleum.

IDENTIFICATION

First identification: B.

Second identification: A, C.

- A. It complies with the test for melting point (see Tests).
- B. Examine the chromatograms obtained in the test for fatty alcohols and sterols.

Results: the principal peaks in the chromatogram obtained with the test solution are similar in retention time and size to the principal peaks in the chromatogram obtained with reference solution (a).

- C. Dissolve 50 mg in 5 ml of *methylene chloride R* and add 1 ml of *acetic anhydride R* and 0.1 ml of *sulphuric acid R*. A green colour is produced.

TESTS

Melting point (2.2.15): 45 °C to 55 °C. Allow to stand at 20 °C for 16 h.

Acid value (2.5.1): maximum 1.0, determined on 5.0 g.

Hydroxyl value (2.5.3, *Method A*): 140 to 180.

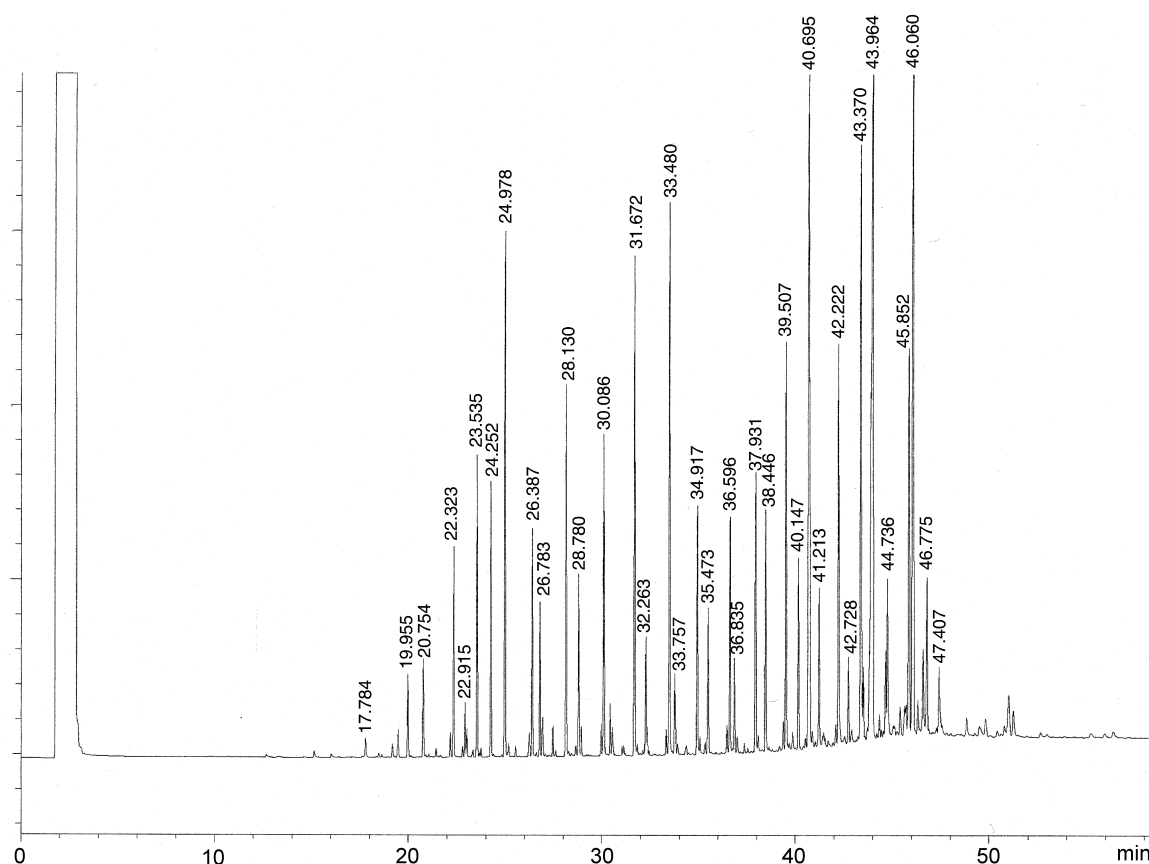


Figure 0969-1. – Chromatogram for the test for fatty alcohols and sterols (reference solution (a)) in hydrogenated wool fat

01/2005:0135

WOOL FAT, HYDROUS

Adeps lanae cum aqua

DEFINITION

Hydrous wool fat is a mixture of 75 per cent *m/m* of wool fat and 25 per cent *m/m* of water. It is obtained by the gradual addition of water to melted wool fat with continuous stirring. It may contain not more than 150 ppm of butylhydroxytoluene.

CHARACTERS

A pale yellow, unctuous substance.

IDENTIFICATION

- In a test-tube, dissolve 0.5 g in 5 ml of *chloroform R* and add 1 ml of *acetic anhydride R* and 0.1 ml of *sulphuric acid R*. A green colour develops.
- Dissolve 50 mg in 5 ml of *chloroform R*, add 5 ml of *sulphuric acid R* and shake. A red colour develops and an intense green fluorescence appears in the lower layer.

TESTS

Water-soluble acid or alkaline substances. Melt 6.7 g on a water-bath and shake vigorously for 2 min with 75 ml of *water R* previously heated to 90 °C to 95 °C. Allow to cool and filter through filter paper previously rinsed with *water R*. To 60 ml of the filtrate (which may not be clear) add 0.25 ml of *bromothymol blue solution R1*. Not more than 0.2 ml of 0.02 M *hydrochloric acid* or 0.15 ml of 0.02 M *sodium hydroxide* is required to change the colour of the indicator.

Drop point (2.2.17): 38 °C to 44 °C, determined on the residue obtained in the test for wool-fat content. To fill the metal cup, melt the residue on a water-bath, cool to about 50 °C, pour into the cup and allow to stand at 15 °C to 20 °C for 24 h.

Water-absorption capacity. Place 10 g of the residue obtained in the test for wool-fat content in a mortar. Add *water R* in portions of 0.2 ml to 0.5 ml from a burette, stirring vigorously after each addition to incorporate the *water R*. The end-point is reached when visible droplets remain which cannot be incorporated. Not less than 20 ml of *water R* is absorbed.

Acid value (2.5.1). Not more than 0.8, determined on 5.0 g dissolved in 25 ml of the prescribed mixture of solvents.

Peroxide value (2.5.5). Not more than 15.

Saponification value (2.5.6): 67 to 79, determined on 2.00 g. Heat under reflux for 4 h.

Water-soluble oxidisable substances. To 10 ml of the filtrate obtained in the test for water-soluble acid or alkaline substances add 1 ml of *dilute sulphuric acid R* and 0.1 ml of 0.02 M *potassium permanganate*. After 10 min, the solution is not completely decolourised.

Butylhydroxytoluene. Not more than 150 ppm, determined by gas chromatography (2.2.28), using *methyl decanoate R* as the internal standard.

Internal standard solution. Dissolve 0.2 g of *methyl decanoate R* in *carbon disulphide R* and dilute to 100.0 ml with the same solvent. Dilute 1.0 ml of this solution to 10.0 ml with *carbon disulphide R*.

Test solution (a). Dissolve 1.0 g of the residue obtained in the test for wool-fat content in *carbon disulphide R* and dilute to 10.0 ml with the same solvent.

01/2005:1380

Test solution (b). Dissolve 1.0 g of the residue obtained in the test for wool-fat content in *carbon disulphide R*, add 1.0 ml of the internal standard solution and dilute to 10.0 ml with *carbon disulphide R*.

Reference solution. Dissolve 0.2 g of *butylhydroxytoluene R* in *carbon disulphide R* and dilute to 100.0 ml with the same solvent. Dilute 1.0 ml of this solution to 10.0 ml with *carbon disulphide R*. To 1.0 ml of this solution add 1.0 ml of the internal standard solution and dilute to 10.0 ml with *carbon disulphide R*.

The chromatography may be carried out using:

- a column 1.5 m long and 4 mm in internal diameter packed with *silanised diatomaceous earth for gas chromatography R* impregnated with 10 per cent *m/m* of *poly(dimethyl)siloxane R*; the column is preceded by a column containing silanised glass wool,
- *nitrogen for chromatography R* as the carrier gas at a flow rate of 40 ml/min,
- a flame-ionisation detector.

Maintain the temperature of the column at 150 °C, that of the injection port at 180 °C and that of the detector at 300 °C. Inject the chosen volumes of test solutions (a) and (b) and of the reference solution.

Paraffins. *The tap and cotton plugs used must be free from grease.* Prepare a column of anhydrous aluminium oxide 230 mm long and 20 mm in diameter by adding a slurry of *anhydrous aluminium oxide R* and *light petroleum R1* to a glass tube fitted with a tap and containing *light petroleum R1*. Allow to settle and reduce the depth of the layer of solvent above the column to about 40 mm. Dissolve 3.0 g of the residue obtained in the test for wool-fat content in 50 ml of warm *light petroleum R1*, cool, pass the solution through the column at a rate of 3 ml/min and wash with 250 ml of *light petroleum R1*. Concentrate the combined eluate and washings to low bulk by distillation, evaporate to dryness on a water-bath and heat the residue at 105 °C for periods of 10 min until 2 successive weighings do not differ by more than 1 mg. The residue weighs not more than 30 mg (1.0 per cent).

Chlorides. Boil 1.3 g with 20 ml of *alcohol (90 per cent V/V) R* under a reflux condenser for 5 min. Cool, add 40 ml of *water R* and 0.5 ml of *nitric acid R* and filter. To the filtrate add 0.15 ml of a 10 g/l solution of *silver nitrate R* in *alcohol (90 per cent V/V) R*. Allow to stand for 5 min, protected from light. Any opalescence in the solution is not more intense than that in a standard prepared at the same time by adding 0.15 ml of a 10 g/l solution of *silver nitrate R* in *alcohol (90 per cent V/V) R* to a mixture of 0.2 ml of 0.02 *M hydrochloric acid*, 20 ml of *alcohol (90 per cent V/V) R*, 40 ml of *water R* and 0.5 ml of *nitric acid R* (115 ppm).

Sulphated ash (2.4.14). Not more than 0.1 per cent. Ignite 5.0 g and use the residue to determine the sulphated ash.

Wool-fat content: 72.5 per cent to 77.5 per cent. In a suitable tared dish containing a glass rod, heat 30.0 g to constant mass on a water-bath, stirring continuously. Weigh the residue.

STORAGE

Store at a temperature not exceeding 25 °C.

LABELLING

The label states, where applicable, the concentration of added butylhydroxytoluene.

WORMWOOD

Absinthii herba

DEFINITION

Wormwood consists of the basal leaves or slightly leafy, flowering tops, or of a mixture of these dried, whole or cut organs of *Artemisia absinthium* L. It contains not less than 2 ml/kg of essential oil, calculated with reference to the dried drug.

CHARACTERS

It has the macroscopic and microscopic characters described under identification tests A and B.

IDENTIFICATION

- A. The leaves are greyish to greenish, densely tomentose on both surfaces. The basal leaves, with long petioles, have triangular to oval bipinnatisect to tripinnatisect lamina, with rounded to lanceolate segments. The cauline leaves are less segmented and the apical leaves are lanceolate. The stem of the flower-bearing region is greenish-grey, tomentose, up to 2.5 mm in diameter and usually with five flattened longitudinal grooves. The capitula are arranged as loose, axillary panicles, inserted at the level of the lanceolate to slightly pinnatisect leaves; they are spherical to flattened hemispherical, 2 mm to 4 mm in diameter and consist of a grey, tomentose involucre, the outer bracts linear, inner layer ovate, blunt at the apices with scarious margins, a receptacle with very long paleae up to 1 mm or more long, numerous yellow, tubular, hermaphroditic florets about 2 mm long and few yellow, ray florets.
- B. Reduce to a powder (355). The powder is greenish-grey. Examine under a microscope using *chloral hydrate solution R*. The powder shows many T-shaped trichomes with a short uniseriate stalk consisting of one to five small cells, perpendicularly capped by a very long, undulating terminal cell tapering at the ends; fragments of epidermises with sinuous to wavy walls, anomocytic stomata (2.8.3) and secretory trichomes each with a short, biseriate, two celled stalk and a biseriate head with two or four cells; fragments of the tubular and ray florets, some containing small cluster crystals of calcium oxalate; numerous paleae each composed of a small cell forming a stalk and a very long, cylindrical and thin-walled terminal cell about 1 mm to 1.5 mm long; spheroidal pollen grains, about 30 µm in diameter, with three pores and a finely warty exine; groups of fibres, small vessels with spiral and annular thickening, larger vessels with bordered pits and parenchyma with moderately thickened and pitted walls, from the stem.
- C. Examine by thin-layer chromatography (2.2.27), using a suitable silica gel as the coating substance.

Test solution. Place 2 g of the powdered drug (355) in 50 ml of boiling *water R* and allow to stand for 5 min, shaking the flask several times. After cooling, add 5 ml of a 100 g/l solution of *lead acetate R*. Mix and filter. Rinse the flask and the residue on the filter with 20 ml of *water R*. Shake the filter with 50 ml of *methylene chloride R*. Separate the organic layer, dry over *anhydrous sodium sulphate R*, filter and evaporate the filtrate to dryness on a water-bath. Dissolve the residue in 0.5 ml of *alcohol R*.

Reference solution. Dissolve 2 mg of *methyl red R* and 2 mg of *resorcinol R* in 10.0 ml of *methanol R*.

Apply to the plate as bands 10 µl of each solution. Develop over a path of 15 cm using a mixture of 10 volumes of *acetone R*, 10 volumes of *glacial acetic acid R*, 30 volumes of *toluene R* and 50 volumes of *methylene chloride R*. Allow the plate to dry in air. Spray the plate with *acetic anhydride-sulphuric acid solution R* and examine in daylight. The chromatogram obtained with the test solution shows the blue zone of artabsin shortly above the red zone of methyl red in the chromatogram obtained with the reference solution. Examine in daylight while heating at 100 °C to 105 °C for 5 min. The chromatogram obtained with the reference solution shows in the middle third the red zone of methyl red and below it the light pink zone of resorcinol. The chromatogram obtained with the test solution shows an intense red to brownish-red zone of absinthin with a similar R_f value to that of the zone due to resorcinol in the chromatogram obtained with the reference solution. Other zones are visible, but less intense than that of absinthin.

TESTS

Foreign matter (2.8.2). Not more than 5 per cent of stems with a diameter greater than 4 mm and 2 per cent of other foreign matter.

Bitterness value. Not less than 10 000. The bitterness value is determined by comparison with quinine hydrochloride, the bitterness value of which is set at 200 000; the bitterness value is the reciprocal of the dilution that still has a bitter taste.

Quinine hydrochloride stock solution. Dissolve 0.100 g of *quinine hydrochloride R* in *water R* and dilute to 100.0 ml with the same solvent. Dilute 1.0 ml of the solution to 100.0 ml with *water R*.

Wormwood extract. To 1.0 g of powdered drug (710) add 1000 ml of boiling *water R*. Heat on a water-bath for 30 min, stirring continuously. Allow to cool and dilute to 1000 ml with *water R*. Shake vigorously and filter, discarding the first 20 ml of filtrate.

Prepare a series of dilutions by placing in the first tube 4.2 ml of quinine hydrochloride stock solution and increasing the volume by 0.2 ml in each subsequent tube to a total of 5.8 ml; dilute the contents of each tube to 10.0 ml with *water R*. Determine as follows the dilution with the lowest concentration that still has a bitter taste. Take 10.0 ml of the weakest solution into the mouth and pass it from side to side over the back of the tongue for 30 s. If the solution is not found to be bitter, spit it out and wait for 1 min. Rinse the mouth with *water R*. After 10 min, use the next dilution in order of increasing concentration.

Calculate the correction factor (k) from the expression:

$$\frac{5.00}{n}$$

n = number of millilitres of quinine hydrochloride stock solution in the dilution of lowest concentration that is bitter.

Dilute 10/ k ml of wormwood extract to 100.0 ml with *water R*. 10.0 ml of this dilution has a bitter taste.

Loss on drying (2.2.32). Not more than 10.0 per cent, determined on 1.000 g of powdered drug (355) by drying in an oven at 100 °C to 105 °C for 2 h.

Total ash (2.4.16). Not more than 12.0 per cent.

Ash insoluble in hydrochloric acid (2.8.1). Not more than 1.0 per cent.

ASSAY

Carry out the determination of essential oil in vegetable drugs (2.8.12). Use 50.0 g of the cut drug, a 1000 ml round-bottomed flask and 500 ml of *water R* as the distillation liquid. Add 0.5 ml of *xylene R* in the graduated tube. Distil at a rate of 2 ml/min to 3 ml/min for not less than 3 h.

STORAGE

Store protected from light.

01/2005:1277

XANTHAN GUM**Xanthani gummi****DEFINITION**

Xanthan gum is a high-molecular-mass anionic polysaccharide produced by fermentation of carbohydrate with *Xanthomonas campestris*. It consists of a principal chain of $\beta(1\rightarrow4)$ -linked D-glucose units with trisaccharide side chains, on alternating anhydroglucose units, consisting of a glucuronic acid unit included between two mannose units. Most of the terminal units contain a pyruvate moiety and the mannose unit adjacent to the principal chain may be acetylated at C-6.

Xanthan gum has a molecular mass of approximately 1×10^6 . It contains not less than 1.5 per cent of pyruvoyl groups ($C_3H_3O_2$; relative mass of the group 71.1), calculated with reference to the dried substance. Xanthan gum exists as the sodium, potassium or calcium salt.

CHARACTERS

A white or yellowish-white, free-flowing powder, soluble in water giving a highly viscous solution, practically insoluble in organic solvents.

IDENTIFICATION

- A. In a flask, suspend 1 g in 15 ml of 0.1 M hydrochloric acid. Close the flask with a fermentation bulb containing barium hydroxide solution R and heat carefully for 5 min. The barium hydroxide solution shows a white turbidity.
- B. To 300 ml of water R, previously heated to 80 °C and stirred rapidly with a mechanical stirrer in a 400 ml beaker, add, at the point of maximum agitation, a dry blend of 1.5 g of carob bean gum R and 1.5 g of the substance to be examined. Stir until the mixture forms a solution, and then continue stirring for 30 min or longer. Do not allow the water temperature to drop below 60 °C during stirring. Discontinue stirring and allow the mixture to stand for at least 2 h. A firm rubbery gel forms after the temperature drops below 40 °C but no such gel forms in a 1 per cent control solution of the sample prepared in the same manner but omitting the carob bean gum.

TESTS

pH (2.2.3). The pH of a 10.0 g/l solution is 6.0 to 8.0.

Viscosity (2.2.10). The viscosity at 24 ± 1 °C is not less than 600 mPa.s. Add 3.0 g within 45 s to 90 s into 250 ml of a 12 g/l solution of potassium chloride R in a 500 ml beaker stirring with a low-pitch propeller-type stirrer rotating at 800 r/min. When adding the substance take care that agglomerates are destroyed. Add an additional quantity of 44 ml of water R, to rinse any adhering residue from the walls of the beaker. Stir the preparation at 800 r/min for 2 h whilst maintaining the temperature at 24 ± 1 °C. Determine the viscosity within 15 min using a rotating viscosimeter set at 60 r/min and equipped with a rotating spindle 12.7 mm in diameter and 1.6 mm high which is attached to a shaft 3.2 mm in diameter. The distance from the top of the cylinder to the lower tip of the shaft being 25.4 mm, and the immersion depth being 50.0 mm.

2-Propanol. Not more than 750 ppm, determined by gas chromatography (2.2.28) using 2-methyl-2-propanol R as the internal standard.

Internal standard solution. Dilute 0.50 g of 2-methyl-2-propanol R to 500 ml with water R.

Test solution. To 200 ml of water R in a 1000 ml round-bottomed flask, add 5.0 g of the substance to be examined and 1 ml of a 10 g/l emulsion of dimeticone R in liquid paraffin R, stopper the flask and shake for 1 h. Distil about 90.0 ml, mix the distillate with 4.0 ml of the internal standard solution and dilute to 100.0 ml with water R.

Reference solution. Dilute a suitable quantity of 2-propanol R, accurately weighed, with water R to obtain a solution having a known concentration of 2-propanol of about 1 mg/ml. To 4.0 ml of this solution add 4.0 ml of the internal standard solution and dilute to 100.0 ml with water R.

The chromatographic procedure may be carried out using:

- a column 1.8 m long and 4.0 mm in internal diameter packed with styrene-divinylbenzene copolymer R,
- helium for chromatography R as the carrier gas at a flow rate of 30 ml/min,
- a flame-ionisation detector,

maintaining the temperature of the column at 165 °C, and that of the injection port and of the detector at 200 °C.

Inject 5 μ l of the test solution and 5 μ l of the reference solution. The retention time of 2-methyl-2-propanol is about 1.5 relative to that of 2-propanol.

Other polysaccharides. Examine by thin-layer chromatography (2.2.27), using a TLC silica gel plate R.

Test solution. To 10 mg in a thick-walled centrifuge test tube add 2 ml of a 230 g/l solution of trifluoroacetic acid R, shake vigorously to dissolve the forming gel, stopper the test tube, and heat the mixture at 120 °C for 1 h. Centrifuge the hydrolysate, transfer the clear supernatant liquid carefully into a 50 ml flask, add 10 ml of water R and evaporate the solution to dryness under reduced pressure. Take up the residue thus obtained in 10 ml of water R and evaporate to dryness under reduced pressure. Wash three times with 20 ml of methanol R and evaporate under reduced pressure. To the resulting clear film which has no odour of acetic acid, add 0.1 ml of water R and 1 ml of methanol R. Centrifuge to separate the amorphous precipitate. Dilute the supernatant liquid, if necessary, to 1 ml with methanol R.

Reference solution. Dissolve 10 mg of glucose R and 10 mg of mannose R in 2 ml of water R and dilute to 10 ml with methanol R.

Apply separately to the plate, as bands, 5 μ l of each solution. Develop over a path of 15 cm using a mixture of 10 volumes of a 16 g/l solution of sodium dihydrogen phosphate R, 40 volumes of butanol R and 50 volumes of acetone R. Spray evenly with a solution of 0.5 g of diphenylamine R in 25 ml of methanol R to which 0.5 ml of aniline R and 2.5 ml of phosphoric acid R have been added. Heat for 5 min at 120 °C. Examine in daylight. The test is not valid unless the chromatogram obtained with the reference solution shows two clearly separated greyish-brown zones due to glucose and mannose in the middle third. The chromatogram obtained with the test solution shows corresponding zones. In addition, one weak reddish and two faint bluish-grey bands may be visible just above the starting line. One or two bluish-grey bands may also be seen in the upper quarter of the chromatogram. No other bands are visible.

Loss on drying (2.2.32). Not more than 15.0 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C for 2.5 h.

Total ash (2.4.16): 6.5 per cent to 16.0 per cent.

Microbial contamination. Total viable aerobic count (2.6.12) not more than 10^3 bacteria and 10^2 fungi per gram, determined by plate count. It complies with the test for *Escherichia coli* (2.6.13).

ASSAY

Test solution. Dissolve a quantity of the substance to be examined corresponding to 120.0 mg of the dried substance in *water R* and dilute to 20.0 ml with the same solvent.

Reference solution. Dissolve 45.0 mg of *pyruvic acid R* in *water R* and dilute to 500.0 ml with the same solvent.

Place 10.0 ml of the test solution in a 50 ml round-bottomed flask, add 20.0 ml of *0.1 M hydrochloric acid* and weigh. Boil on a water-bath under a reflux condenser for 3 h. Weigh and adjust to the initial mass with *water R*. In a separating funnel mix 2.0 ml of the solution with 1.0 ml of *dinitrophenylhydrazine-hydrochloric solution R*. Allow to stand for 5 min and add 5.0 ml of *ethyl acetate R*. Shake and allow the solids to settle. Collect the upper layer and shake with three quantities, each of 5.0 ml, of *sodium carbonate solution R*. Combine the aqueous layers and dilute to 50.0 ml with *sodium carbonate solution R*. Mix. Treat 10.0 ml of the reference solution at the same time and in the same manner as for the test solution.

Immediately measure the absorbance of the two solutions (2.2.25) at 375 nm, using *sodium carbonate solution R* as the compensation liquid.

The absorbance of the test solution is not less than that of the reference solution, which corresponds to a content of pyruvic acid of not less than 1.5 per cent.

pH (2.2.3): 4.0 to 5.5 for solution S.

Impurity A: maximum 100 ppm.

Solution A. Dissolve 0.25 g of the substance to be examined in *methanol R* and dilute to 10 ml with the same solvent. This solution is used to prepare the test solution.

Solution B. Dissolve 50 mg of 2,6-dimethylaniline R in methanol R and dilute to 100 ml with the same solvent. Dilute 1 ml of the solution to 100 ml with methanol R. This solution is used to prepare the reference solution.

Using 2 flat-bottomed tubes with an inner diameter of about 10 mm, place in the first tube 2 ml of solution A, and in the second tube 1 ml of solution B and 1 ml of *methanol R*. To each tube add 1 ml of a freshly prepared 10 g/l solution of *dimethylaminobenzaldehyde R* in *methanol R* and 2 ml of *glacial acetic acid R* and allow to stand at room temperature for 10 min. Compare the colours in diffused daylight, viewing vertically against a white background. Any yellow colour in the test solution is not more intense than that in the reference solution.

Related substances. Liquid chromatography (2.2.29).
Prepare the solutions immediately before use.

Solvent mixture. Mix 8 volumes of *acetonitrile R*, 30 volumes of *methanol R* and 62 volumes of a 2.72 g/l solution of *potassium dihydrogen phosphate R* adjusted to pH 7.2 with *dilute sodium hydroxide solution R*.

Test solution. Dissolve 0.100 g of the substance to be examined in the solvent mixture and dilute to 20.0 ml with the solvent mixture.

Reference solution. Dissolve 5.0 mg of the substance to be examined, 5.0 mg of 2,6-dimethylaniline R, 5.0 mg of xylazine impurity C CRS and 5.0 mg of xylazine impurity E CRS in acetonitrile R and dilute to 100.0 ml with the same solvent. Dilute 1.0 ml of this solution to 10.0 ml with the solvent mixture.

Column:

- size: $l = 0.15$ m, $\varnothing = 3.9$ mm,
- stationary phase: end-capped octylsilyl silica gel for chromatography with polar incorporated groups R (5 μm),
- temperature: 40 °C.

Mobile phase:

- *mobile phase A*: mix 30 volumes of *methanol R* and 70 volumes of a 2.72 g/l solution of *potassium dihydrogen phosphate R* adjusted to pH 7.2 with *dilute sodium hydroxide solution R*,
- *mobile phase B*: *methanol R*, *acetonitrile R* (30:70 V/V).

Time (min)	Mobile phase A (per cent V/V)	Mobile phase B (per cent V/V)
0 - 15	89 $\rightarrow$ 28	11 $\rightarrow$ 72
15 - 21	28	72
21 - 22	28 $\rightarrow$ 89	72 $\rightarrow$ 11
22 - 33	89	11

Flow rate: 1.0 ml/min.

Detection: spectrophotometer at 230 nm.

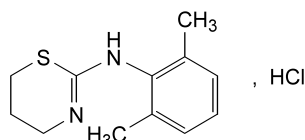
Equilibration: for at least 30 min with a mixture of 28 volumes of mobile phase A and 72 volumes of mobile phase B.

Injection: 20 μ l.

Relative retention with reference to xylazine (retention time = about 7.5 min): impurity A = about 0.8; impurity E = about 1.6; impurity C = about 2.2.

XYLAZINE HYDROCHLORIDE FOR VETERINARY USE

Xylazini hydrochloridum ad usum
veterinarium


$$\text{C}_{12}\text{H}_{17}\text{ClN}_2\text{S}$$
 M_v 256.8

DEFINITION

N-(2,6-Dimethylphenyl)-5,6-dihydro-4*H*-1,3-thiazin-2-amine hydrochloride.

Content: 98.0 per cent to 102.0 per cent (dried substance).

CHARACTERS

Appearance: white or almost white, crystalline powder, hygroscopic.

Solubility: freely soluble in water, very soluble in methanol, freely soluble in methylene chloride.

IDENTIFICATION

A. Infrared absorption spectrophotometry (2.2.24).

Preparation: discs.

Comparison: xylazine hydrochloride CRS.

B. It gives reaction (b) of chlorides (2.3.1).

TESTS

Solution S. Dissolve 5.0 g in carbon dioxide-free water *R* prepared from distilled water *R*, heating at 60 °C if necessary; allow to cool and dilute to 50.0 ml with the same solvent.

Appearance of solution. Solution S is not more opalescent than reference suspension II (2.2.1) and is colourless (2.2.2, *Method II*).

System suitability: reference solution:

- *resolution:* minimum 4.0 between the peaks due to impurity A and xylazine.

Limits:

- *impurities C, E:* for each impurity, not more than twice the area of the corresponding peak in the chromatogram obtained with the reference solution (0.2 per cent),
- *impurities B, D:* for each impurity, not more than twice the area of the peak due to xylazine in the chromatogram obtained with the reference solution (0.2 per cent),
- *any other impurity:* for each impurity, not more than twice the area of the peak due to xylazine in the chromatogram obtained with the reference solution (0.2 per cent),
- *total of impurities other than B, C, D and E:* not more than twice the area of the peak due to xylazine in the chromatogram obtained with the reference solution (0.2 per cent),
- *disregard limit:* 0.5 times the area of the peak due to xylazine in the chromatogram obtained with the reference solution (0.05 per cent); disregard any peak due to the blank.

Heavy metals (2.4.8): maximum 10 ppm.

12 ml of solution S complies with limit test A. Prepare the standard using 10 ml of *lead standard solution* (1 ppm Pb) R.

Loss on drying (2.2.32): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 100–105 °C for 2 h.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.200 g in 25 ml of *alcohol R*. Add 25 ml of *water R*. Titrate with 0.1 M *sodium hydroxide*, determining the end-point potentiometrically (2.2.20).

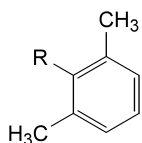
1 ml of 0.1 M *sodium hydroxide* is equivalent to 25.68 mg of C₁₂H₁₇ClN₂S.

STORAGE

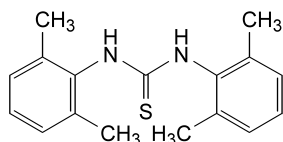
In an airtight container, protected from light.

IMPURITIES

Specified impurities: A, B, C, D, E.



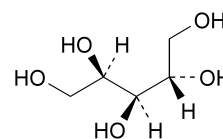
- A. R = NH₂: 2,6-dimethylaniline (2,6-xylidine),
- C. R = N=C=S: 2,6-dimethylphenyl isothiocyanate,
- D. R = NH-CS-NH-[CH₂]₃-OH: *N*-(2,6-dimethylphenyl)-*N'*-(3-hydroxypropyl)thiourea,
- E. R = NH-CS-S-CH₃: methyl (2,6-dimethylphenyl)carbamodithioate,



- B. *N,N'*-bis(2,6-dimethylphenyl)thiourea.

XYLITOL

Xylitolum



C₅H₁₂O₅

*M*_r 152.1

DEFINITION

Xylitol contains not less than 98.0 per cent and not more than the equivalent of 102.0 per cent of *meso*-xylitol, calculated with reference to the anhydrous substance.

CHARACTERS

White crystalline powder or crystals, very soluble in water, sparingly soluble in alcohol.

IDENTIFICATION

First identification: B.

Second identification: A, C.

- A. Melting point (2.2.14): 92 °C to 96 °C.
- B. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *xylitol CRS*. Examine the substance prepared as a mull in *liquid paraffin R*.
- C. Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel G plate R*.

Test solution. Dissolve 25 mg of the substance to be examined in *water R* and dilute to 5.0 ml with the same solvent.

Reference solution (a). Dissolve 25 mg of *xylitol CRS* in *water R* and dilute to 5.0 ml with the same solvent.

Reference solution (b). Dissolve 25 mg of *mannitol CRS* and 25 mg of *xylitol CRS* in *water R* and dilute to 5 ml with the same solvent.

Apply to the plate 2 µl of each solution. Develop over a path of 17 cm using a mixture of 10 volumes of *water R*, 20 volumes of *ethyl acetate R* and 70 volumes of *propanol R*. Allow the plate to dry in air and spray with 4-aminobenzoic acid solution R. Dry the plate in a current of cold air until the acetone is removed. Heat the plate at 100 °C for 15 min. Allow to cool and spray with a 2 g/l solution of *sodium periodate R*. Dry the plate in a current of cold air. Heat the plate at 100 °C for 15 min. The principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a). The test is not valid unless the chromatogram obtained with reference solution (b) shows 2 clearly separated spots.

TESTS

Appearance of solution. Dissolve 2.5 g in *water R* and dilute to 50.0 ml with the same solvent. The solution is not more opalescent than reference suspension IV (2.2.1) and not more intensely coloured than reference solution BY₇ (2.2.2, *Method II*).

Conductivity (2.2.38). Not more than 20 µS·cm⁻¹.

Dissolve 20.0 g in *carbon dioxide-free water R* prepared from *distilled water R* and dilute to 100.0 ml with the same solvent. Measure the conductivity of the solution while gently stirring with a magnetic stirrer.

Reducing sugars. Dissolve 5.0 g in 6 ml of *water R* with the aid of gentle heat. Cool and add 20 ml of *cupri-citric solution R* and a few glass beads. Heat so that boiling begins after 4 min and maintain boiling for 3 min. Cool rapidly and add 100 ml of a 2.4 per cent *V/V* solution of *glacial acetic acid R* and 20.0 ml of 0.025 *M* *iodine*. With continuous shaking, add 25 ml of a mixture of 6 volumes of *hydrochloric acid R* and 94 volumes of *water R* and, when the precipitate has dissolved, titrate the excess of iodine with 0.05 *M* *sodium thiosulphate* using 1 ml of *starch solution R*, added towards the end of the titration, as indicator. Not less than 12.8 ml of 0.05 *M* *sodium thiosulphate* is required (0.2 per cent, calculated as glucose equivalent).

Related products. Examine by gas chromatography (2.2.28) as described under Assay. The sum of the percentage contents of the related products in the chromatogram obtained with the test solution is not greater than 2.0 per cent.

Lead (2.4.10). It complies with the limit test for lead in sugars (0.5 ppm). Dissolve the substance to be examined in 150.0 ml of the prescribed mixture of solvents.

Nickel (2.4.15). It complies with the limit test for nickel in polyols (1 ppm). Dissolve the substance to be examined in 150.0 ml of the prescribed mixture of solvents.

Water (2.5.12). Not more than 1.0 per cent, determined on 1.00 g by the semi-micro determination of water.

Bacterial endotoxins (2.6.14): if intended for use in the manufacture of parenteral dosage forms without a further appropriate procedure for the removal of bacterial endotoxins, less than 4 IU/g for parenteral dosage forms having a concentration of less than 100 g/l of xylitol and less than 2.5 IU/g for parenteral dosage forms having a concentration of 100 g/l or more of xylitol.

ASSAY

Examine by gas chromatography (2.2.28), using erythritol as the internal standard.

Internal standard solution. Dissolve 5 mg of *erythritol R* in *water R* and dilute to 25.0 ml with the same solvent.

Test solution. Dissolve 5.000 g of the substance to be examined in *water R* and dilute to 100.0 ml with the same solvent.

Reference solution. Dissolve 25.0 mg each of *L-arabinitol CRS*, *galactitol CRS*, *mannitol CRS* and *sorbitol CRS* in *water R* and dilute to 100.0 ml with the same solvent. To 10.0 ml of the solution add about 490 mg of *xylitol CRS*, accurately weighed, to obtain a solution having a known concentration of about 49 mg of *xylitol CRS* per millilitre.

The chromatographic procedure may be carried out using:

- a stainless steel column 2 m long and 2 mm in internal diameter packed with acid washed *silanised diatomaceous earth for gas chromatography R* (150–180 µm) and impregnated with 3 per cent *m/m* of *poly[(cyanopropyl)(methyl)](phenyl)(methyl)siloxane R*,
- *nitrogen R* as the carrier gas at a flow rate of 30 ml/min,
- a flame-ionisation detector,

maintaining the temperature of the column at 170 °C for 1 min, then raising the temperature at a rate of 6 °C/min to 200 °C and maintaining the temperature of the injection port and of the detector at 250 °C.

Pipet 1.0 ml of the reference solution and 1.0 ml of the test solution into separate 100 ml round-bottomed flasks. To each flask, add 1.0 ml of the internal standard solution and evaporate the respective mixtures to dryness in a water-bath at 60 °C with the aid of a rotary evaporator. Dissolve each dry residue in 1 ml of *anhydrous pyridine R* and add 1 ml of *acetic anhydride R* to each flask and boil each solution under reflux for 1 h to complete acetylation.

Inject 1 µl each of the solutions obtained from the reference solution and the test solution.

When the chromatograms are recorded in the prescribed conditions, the relative retentions calculated with reference to xylitol are: erythritol about 0.3, arabinitol about 0.7, mannitol about 1.8, galactitol about 2.0 and sorbitol about 2.2.

Calculate the percentage content of C₅H₁₂O₅, taking into account the declared content of *xylitol CRS*, and the percentage content of each related product in the substance to be examined from the expression:

$$100 \times \frac{m_s}{m_u} \times \frac{R_u}{R_s}$$

m_s = mass of the respective component in 1 ml of the reference solution, in milligrams,

m_u = mass of the substance to be examined in 1 ml of the test solution, in milligrams,

R_s = ratio of the surface areas of the derivatised component peak to the derivatised erythritol peak in the chromatogram obtained with the reference solution,

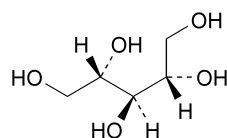
R_u = ratio of the surface areas of the derivatised component peak to the derivatised erythritol peak in the chromatogram obtained with the test solution.

LABELLING

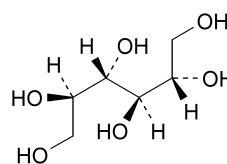
The label states:

- where applicable, the maximum concentration of bacterial endotoxins,
- where applicable, that the substance is suitable for use in the manufacture of parenteral dosage forms.

IMPURITIES



A. *L*-arabinitol,

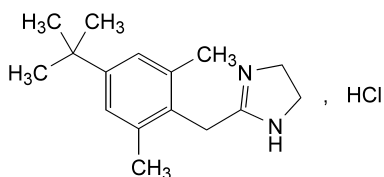


B. *meso*-galactitol,

C. mannitol,

D. sorbitol.

01/2005:1162

XYLOMETAZOLINE HYDROCHLORIDE**Xylometazolini hydrochloridum** $C_{16}H_{25}ClN_2$ M_r 280.8**DEFINITION**

Xylometazoline hydrochloride contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of 2-[4-(1,1-dimethylethyl)-2,6-dimethylbenzyl]-4,5-dihydro-1H-imidazole hydrochloride, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, crystalline powder, freely soluble in water, in alcohol and in methanol.

IDENTIFICATION

First identification: A, E.

Second identification: B, C, D, E.

- Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *xylometazoline hydrochloride CRS*.
- Examine the chromatograms obtained in the test for related substances. The principal spot in the chromatogram obtained with test solution (b) is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).
- Dissolve about 0.5 mg in 1 ml of *methanol R* and add 0.5 ml of a freshly prepared 50 g/l solution of *sodium nitroprusside R* and 0.5 ml of a 20 g/l solution of *sodium hydroxide R*. Allow to stand for 10 min and add 1 ml of an 80 g/l solution of *sodium bicarbonate R*. A violet colour develops.
- Dissolve 0.2 g in 1 ml of *water R*, add 2.5 ml of *alcohol R* and 2 ml of 1 M *sodium hydroxide*. Mix thoroughly and examine in ultraviolet light at 365 nm. The solution shows no fluorescence or at most the same fluorescence as a blank solution prepared in the same manner. The identification is not valid unless a solution prepared in the same manner using *naphazoline hydrochloride CRS* instead of the substance to be examined shows a distinct bluish fluorescence.
- It gives reaction (a) of chlorides (2.3.1).

TESTS

Solution S. Dissolve 2.5 g in *carbon dioxide-free water R* and dilute to 50.0 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and not more intensely coloured than reference solution Y_6 (2.2.2, *Method II*).

Acidity or alkalinity. Dissolve 0.25 g in *water R* and dilute to 25 ml with the same solvent. Add 0.1 ml of *methyl red solution R* and 0.1 ml of 0.01 M *hydrochloric acid*. The solution is red. Not more than 0.2 ml of 0.01 M *sodium hydroxide* is required to change the colour of the indicator to yellow.

Related substances. Examine by thin-layer chromatography (2.2.27), using *silica gel G R* as the coating substance.

Test solution (a). Dissolve 0.20 g of the substance to be examined in *methanol R* and dilute to 5 ml with the same solvent.

Test solution (b). Dilute 1 ml of test solution (a) to 10 ml with *methanol R*.

Reference solution (a). Dissolve 20 mg of *xylometazoline hydrochloride CRS* in *methanol R* and dilute to 5 ml with the same solvent.

Reference solution (b). Dissolve 4 mg of *xylometazoline impurity A CRS* in *methanol R* and dilute to 50 ml with the same solvent.

Reference solution (c). Dilute 1 ml of test solution (b) to 50 ml with *methanol R*.

Apply to the plate 5 µl of each solution. Develop over a path of 10 cm using a mixture of 5 volumes of *concentrated ammonia R* and 100 volumes of *methanol R*. Allow the plate to dry. At the bottom of a chromatography tank place an evaporating dish containing a mixture of 1 volume of *water R*, 1 volume of *hydrochloric acid R1* and 2 volumes of a 15 g/l solution of *potassium permanganate R*. Close the tank and allow to stand for 15 min. Place the dried plate in the tank and reclose the tank. Leave the plate in contact with the chlorine vapour for 5 min. Withdraw the plate and place it in a current of cold air until the excess of chlorine is removed and an area of the coating below the points of application does not give a blue colour with a drop of *potassium iodide and starch solution R*. Spray with *potassium iodide and starch solution R*. Any spot in the chromatogram obtained with test solution (a) corresponding to xylometazoline impurity A, is not more intense than the principle spot in the chromatogram obtained with reference solution (b) (0.2 per cent). Any spot in the chromatogram obtained with test solution (a), apart from the principal spot and the spot corresponding to xylometazoline impurity A is not more intense than the principle spot in the chromatogram obtained with reference solution (c) (0.2 per cent).

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

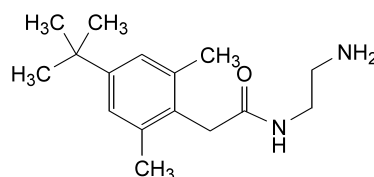
ASSAY

Dissolve 0.200 g in 25 ml of *anhydrous acetic acid R* and add 10 ml of *acetic anhydride R*. Titrate with 0.1 M *perchloric acid*, determining the end-point potentiometrically (2.2.20).

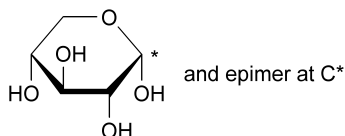
1 ml of 0.1 M *perchloric acid* is equivalent to 28.08 mg of $C_{16}H_{25}ClN_2$.

STORAGE

Store protected from light.

IMPURITIES

- A. *N*-(2-aminomethyl)-2-[4-(1,1-dimethylethyl)-2,6-dimethylphenyl]acetamide.

01/2005:1278
corrected**XYLOSE****Xylosum** $C_5H_{10}O_5$ M_r 150.1**DEFINITION**

Xylose is (+)-D-xylopyranose.

CHARACTERS

A white or almost white, crystalline powder or colourless needles, freely soluble in water, soluble in hot alcohol.

IDENTIFICATION*First identification: A.**Second identification: B, C.*

- A. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *xylose CRS*. Examine the substances prepared as discs.
- B. Examine by thin-layer chromatography (2.2.27), using a suitable silica gel as the coating substance.

Test solution. Dissolve 10 mg of the substance to be examined in a mixture of 2 volumes of *water R* and 3 volumes of *methanol R* and dilute to 20 ml with the same mixture of solvents.

Reference solution (a). Dissolve 10 mg of *xylose CRS* in a mixture of 2 volumes of *water R* and 3 volumes of *methanol R* and dilute to 20 ml with the same mixture of solvents.

Reference solution (b). Dissolve 10 mg of *fructose R*, 10 mg of *glucose R* and 10 mg of *xylose R* in a mixture of 2 volumes of *water R* and 3 volumes of *methanol R* and dilute to 20 ml with the same mixture of solvents.

Apply to the plate 2 µl of each solution and thoroughly dry the starting points. Develop over a path of 15 cm using a mixture of 10 volumes of *water R*, 15 volumes of *methanol R*, 25 volumes of *anhydrous acetic acid R* and 50 volumes of *ethylene chloride R*. The solvents should be measured accurately since a slight excess of water produces cloudiness. Dry the plate in a current of warm air. Spray uniformly with a 5 g/l solution of *thymol R* in a mixture of 5 ml of *sulphuric acid R* and 95 ml of *alcohol R*. Heat in an oven at 130 °C for 10 min. The principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a). The test is not valid unless the chromatogram obtained with reference solution (b) shows three clearly separated spots.

- C. Dissolve 0.1 g in 10 ml of *water R*. Add 3 ml of *cupri-tartaric solution R* and heat. An orange to red precipitate is formed.

TESTS

Solution S. Dissolve 10.0 g in *carbon dioxide-free water R* and dilute to 100 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and colourless (2.2.2, *Method II*).

Acidity or alkalinity. To 50 ml of solution S add 0.3 ml of *phenolphthalein solution R1*. The solution is colourless. Not more than 0.2 ml of 0.1 M *sodium hydroxide* is required to change the colour of the indicator to pink.

Specific optical rotation (2.2.7). Dissolve 10.0 g in 80 ml of *water R*, add 1 ml of *dilute ammonia R2* and dilute to 100.0 ml with *water R*. Allow to stand for 30 min. The specific optical rotation is + 18.5 to + 19.5, calculated with reference to the dried substance.

Chlorides (2.4.4). Dilute 1.5 ml of solution S to 15 ml with *water R*. The solution complies with the limit test for chlorides (330 ppm).

Heavy metals (2.4.8). 12 ml of solution S complies with limit test A for heavy metals (20 ppm). Prepare the standard using *lead standard solution (2 ppm Pb) R*.

Loss on drying (2.2.32). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 100-105 °C at a pressure not exceeding 0.7 kPa.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

01/2005:1382

YARROW

Millefolii herba

DEFINITION

Yarrow consists of the whole or cut, dried flowering tops of *Achillea millefolium* L. It contains not less than 2 ml/kg of essential oil and not less than 0.02 per cent of proazulenes, expressed as chamazulene ($C_{14}H_{16}$; M_r 184.3), both calculated with reference to the dried drug.

CHARACTERS

It has the macroscopic and microscopic characters described under identification tests A and B.

IDENTIFICATION

- A. The leaves are green or greyish-green, faintly pubescent on the upper surface and more pubescent on the lower surface, two to three pinnately divided with linear lobes and a finely pointed whitish tip. The capitula are arranged in a corymb at the end of the stem. Each capitulum, 3 mm to 5 mm in diameter, consists of the receptacle, usually four or five ligulate ray-florets and three to twenty tubular disk-florets. The involucre consists of three rows of imbricate lanceolate, pubescent green bracts arranged with a brownish or whitish, membranous margin. The receptacle is slightly convex and, in the axillae of paleae, bears a ligulate ray floret with a three-lobed, whitish or reddish ligule and tubular disk-florets with a radial, five-lobed, yellowish or light brownish corolla. The pubescent green, partly brown or violet stems are longitudinally furrowed, up to 3 mm thick with a light-coloured medulla.
- B. Reduce to a powder (355). The powder is green or greyish-green. Examine under a microscope, using *chloral hydrate solution R*. The powder shows fragments of the stems, leaves and bracts bearing very rare glandular trichomes with a short stalk and a head formed of two rows of three to five cells enclosed in a bladder-like membrane and uniseriate covering trichomes consisting of four to six small, more or less isodiametric cells at the base and a thick-walled, often somewhat tortuous terminal cell, about 400 μ m to greater than 1000 μ m long; fragments of the ligulate corolla with papillary epidermal cells; small-celled parenchyma from the corolla tubes containing cluster crystals of calcium oxalate; groups of lignified and pitted cells from the bracts; spherical pollen grains, about 30 μ m in diameter, with three germinal pores and spiny exine; groups of sclerenchymatous fibres and small vessels with spiral or annular thickening, from the stem.
- C. Add 2.5 ml of *dimethylaminobenzaldehyde solution R8* to 0.1 ml of solution S (see Tests) and heat on a water-bath for 2 min. Allow to cool. Add 5 ml of *light petroleum R* and shake the mixture vigorously. The aqueous layer shows a blue or greenish-blue colour.
- D. Examine by thin-layer chromatography (2.2.27), using a suitable silica gel as the coating substance.
- Test solution.** Use the solution prepared in identification test C.
- Reference solution.** Dissolve 10 mg of *cineole R* and 10 mg of *guaiazulene R* in 20 ml of *toluene R*. Apply to the plate as bands 20 μ l of each solution. Develop over a path of 10 cm using a mixture of 5 volumes of *ethyl acetate R* and 95 volumes of *toluene R*. Allow

the plate to dry in air. Spray the plate with *anisaldehyde solution R*. Examine in daylight while heating at 100 °C to 105 °C for 5 min to 10 min. The chromatogram obtained with the reference solution shows in the upper part a red zone (guaiazulene) and in the middle part a blue or greyish-blue zone (cineole). The chromatogram obtained with the test solution shows: a violet zone a little above the zone due to guaiazulene in the chromatogram obtained with the reference solution; below this zone a reddish-violet zone; below which, one or two not clearly separated greyish-violet to greyish zones (which changes to greenish-grey after a few hours) and a reddish-violet zone a little above the zone due to cineole in the chromatogram obtained with the reference solution. Further faint zones are present.

TESTS

Solution S. To 2.0 g of the powdered drug (710) add 25 ml of *ethyl acetate R*, shake for 5 min and filter. Evaporate to dryness on a water-bath and dissolve the residue in 0.5 ml of *toluene R*.

Foreign matter (2.8.2). Not more than 5 per cent of stems, with a diameter greater than 3 mm and not more than 2 per cent of other foreign matter.

Loss on drying (2.2.32). Not more than 12.0 per cent determined on 0.500 g of powdered drug (355) by drying in an oven at 100 °C to 105 °C for 2 h.

Total ash (2.4.16). Not more than 10.0 per cent.

Ash insoluble in hydrochloric acid (2.8.1). Not more than 2.5 per cent.

ASSAY

Essential oil. Carry out the determination of essential oil in vegetable drugs (2.8.12). Use 20.0 g of cut drug, a 1000 ml round-bottomed flask and 500 ml of a mixture of 1 volume of *water R* and 9 volumes of *ethylene glycol R* as the distillation liquid. Add 0.2 ml of *xylene R* in the graduated tube. Distil at a rate of 2 ml/min to 3 ml/min for 2 h.

Stop cooling at the end of distillation and continue distilling until the blue, steam-volatile components have reached the lower end of the cooler. Immediately start cooling again, taking care to avoid warming the separation space. Stop the distillation after 5 min. Replace the 1000 ml round-bottomed flask by a 250 ml round-bottomed flask containing a mixture of 0.4 ml of *xylene R* and 50 ml of *water R*. Distil for 15 min. After 10 min read the total volume. To determine the blank value, use 0.2 ml of *xylene R* in the graduated tube and distil a mixture of 0.4 ml of *xylene R* and 50 ml of *water R* for 15 min.

Proazulenes. To ensure that as little water as possible is transferred, transfer the blue essential oil-xylene mixture obtained in the assay of essential oil to a 50 ml volumetric flask with the aid of small portions of *xylene R*, rinsing the graduated tube of the apparatus with *xylene R* and dilute to 50.0 ml with the same solvent. Measure the absorbance (2.2.25) at 608 nm using *xylene R* as the compensation liquid.

Calculate the percentage content of proazulenes, expressed as chamazulene from the expression:

$$\frac{A \times 2.1}{m}$$

i.e. taking the specific absorbance of chamazulene to be 23.8.

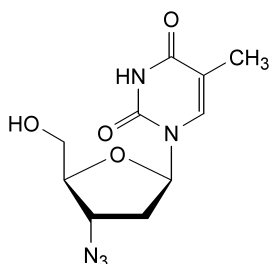
A = absorbance at 608 nm,

m = mass of the drug to be examined in grams.

STORAGE

Store protected from light.

01/2005:1059

ZIDOVUDINE**Zidovudinum** $C_{10}H_{13}N_5O_4$ M_r 267.2**DEFINITION**

Zidovudine contains not less than 97.0 per cent and not more than the equivalent of 102.0 per cent of 1-(3-azido-2,3-dideoxy-β-D-*erythro*-pentofuranosyl)-5-methylpyrimidine-2,4(1*H*,3*H*)-dione, calculated with reference to the dried substance.

CHARACTERS

A white or brownish powder, sparingly soluble in water, soluble in ethanol. It melts at about 124 °C.

It shows polymorphism.

IDENTIFICATION

Examine by infrared absorption spectrophotometry (2.2.24) comparing with the spectrum obtained with *zidovudine CRS*. If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in the minimum quantity of *water R* and evaporate to dryness in a desiccator, under high vacuum over *diphosphorus pentoxide R*. Record new spectra using the residues.

TESTS

Appearance of solution. Dissolve 0.5 g in 50 ml of *water R*, heating if necessary. The solution is not more intensely coloured than reference solution BY₅ (2.2.2, *Method II*).

Specific optical rotation (2.2.7). Dissolve 0.50 g in *ethanol R* and dilute to 50.0 ml with the same solvent. The specific optical rotation, determined at 25 °C is + 60.5 to + 63.0, calculated with reference to the dried substance.

Related substances

A. Examine by thin-layer chromatography (2.2.27), using a plate coated with a suitable silica gel containing a fluorescent indicator having an optimal intensity at 254 nm.

Test solution. Dissolve 0.20 g of the substance to be examined in *methanol R* and dilute to 10 ml with the same solvent.

Reference solution (a). Dissolve 20 mg each of *thymine R*, *zidovudine impurity A CRS* and *triphenylmethanol R* in *methanol R*, add 1.0 ml of the test solution and dilute to 100 ml with *methanol R*.

Reference solution (b). Dilute 5.0 ml of reference solution (a) to 10 ml with *methanol R*.

Apply separately to the plate 10 µl of each solution. Develop over a path of 12 cm using a mixture of 10 volumes of *methanol R* and 90 volumes of *methylene*

chloride R. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. In the chromatogram obtained with the test solution: any spot corresponding to zidovudine impurity A is not more intense than the corresponding spot in the chromatogram obtained with reference solution (b) (0.5 per cent), and any spot apart from the principal spot and any spot corresponding to zidovudine impurity A and thymine (which is limited by liquid chromatography) is not more intense than the spot corresponding to zidovudine in the chromatogram obtained with reference solution (b) (0.5 per cent). Spray the plate with a 10 g/l solution of *vanillin R* in *sulphuric acid R*. In the chromatogram obtained with the test solution, any spot corresponding to triphenylmethanol is not more intense than the corresponding spot in the chromatogram obtained with reference solution (b) (0.5 per cent). The test is not valid unless the chromatogram obtained with reference solution (a) shows four clearly separated spots, corresponding to thymine, zidovudine impurity A, zidovudine and triphenylmethanol, in order of increasing R_f value.

B. Examine by liquid chromatography as described in the assay. Inject separately 10 µl each of test solution (a), reference solution (b), reference solution (d) and reference solution (e). Continue the chromatography for 1.5 times the retention time of the principal peak in the chromatogram obtained with test solution (a). When the chromatograms are recorded in the prescribed conditions, the substances are eluted in the following sequence: thymine, zidovudine and zidovudine impurity B. In the chromatogram obtained with test solution (a): the area of any peak corresponding to thymine is not greater than the area of the peak in the chromatogram obtained with reference solution (b) (2 per cent); the area of any peak corresponding to zidovudine impurity B is not greater than the area of the corresponding peak in the chromatogram obtained with reference solution (d) (1 per cent); the area of any other peak apart from the principal peak is not greater than the area of the peak in the chromatogram obtained with reference solution (e) (0.5 per cent). The total surface area of all the peaks apart from the principal peak obtained in the chromatogram with test solution (a) is not greater than six times the area of the peak obtained with reference solution (e) (3.0 per cent). Disregard any peak with an area less than 10 per cent of the area of the peak in the chromatogram obtained with reference solution (e).

Heavy metals (2.4.8). 1.00 g complies with limit test D for heavy metals (20 ppm). Prepare the standard using 2 ml of *lead standard solution (10 ppm Pb) R*.

Loss on drying (2.2.32). Not more than 1.0 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C.

Sulphated ash (2.4.14). Not more than 0.25 per cent, determined on 1.00 g.

ASSAY

Examine by liquid chromatography (2.2.29).

Test solution (a). Dissolve 50.0 mg of the substance to be examined in the mobile phase and dilute to 50.0 ml with the mobile phase.

Test solution (b). Dilute 10.0 ml of test solution (a) to 50.0 ml with the mobile phase.

Reference solution (a). Dissolve 10.0 mg of *zidovudine CRS* in the mobile phase and dilute to 50.0 ml with the mobile phase.

Reference solution (b). Dissolve 10.0 mg of *thymine R* in *methanol R* and dilute to 50.0 ml with the same solvent. Dilute 5.0 ml of the solution to 50.0 ml with the mobile phase.

Reference solution (c). Dissolve 5 mg of *zidovudine impurity B CRS* in 25.0 ml of reference solution (a) and dilute to 50.0 ml with the mobile phase.

Reference solution (d). Dilute 5.0 ml of reference solution (c) to 50.0 ml with the mobile phase.

Reference solution (e). Dilute 0.25 ml of test solution (a) to 50.0 ml with the mobile phase.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4.6 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (5 µm),
- as mobile phase at a flow rate of 1.2 ml/min a mixture of 20 volumes of *methanol R* and 80 volumes of *water R*,
- as detector a spectrophotometer set at 265 nm.

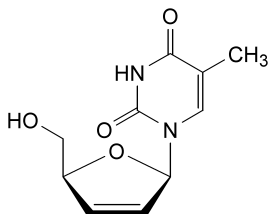
Equilibrate the column with the mobile phase at a flow rate of 1.2 ml per minute for about 45 min. Inject 10 µl of reference solution (c). Adjust the sensitivity of the detector so that the height of the principal peaks in the chromatogram obtained is not less than 70 per cent of the full scale of the recorder. The test is not valid unless the resolution between the peaks due to zidovudine and zidovudine impurity B is at least 1.0. Inject separately 10 µl of test solution (b) and 10 µl of reference solution (a). Adjust the sensitivity of the detector so that the height of the peaks in the chromatograms obtained is not less than 50 per cent of the full scale of the recorder.

Calculate the content of $C_{10}H_{13}N_5O_4$ from the areas of the peaks and the declared content of *zidovudine CRS*.

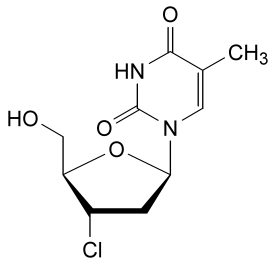
STORAGE

Store protected from light.

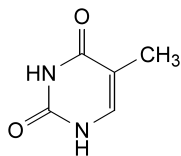
IMPURITIES



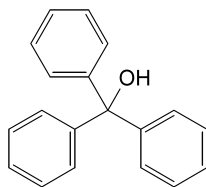
- A. 1-[(2*R*,5*S*)-5-(hydroxymethyl)-2,5-dihydrofuran-2-yl]-5-methylpyrimidine-2,4(1*H*,3*H*)-dione,



- B. 1-(3-chloro-2,3-dideoxy-β-*D*-erythro-pentofuranosyl)-5-methylpyrimidine-2,4(1*H*,3*H*)-dione,



- C. 5-methylpyrimidine-2,4(1*H*,3*H*)-dione (thymine),



- D. triphenylmethanol.

01/2005:1482

ZINC ACETATE DIHYDRATE

Zinci acetas dihydricus

$C_4H_6O_4Zn \cdot 2H_2O$

M_r 219.5

DEFINITION

Zinc acetate dihydrate contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of $C_4H_6O_4Zn \cdot 2H_2O$.

CHARACTERS

A white crystalline powder or leaflets, freely soluble in water, soluble in alcohol.

IDENTIFICATION

- A. It gives reaction (a) of acetate (2.3.1).
B. It gives the reaction of zinc (2.3.1).

TESTS

Solution S. Dissolve 10.0 g in *carbon dioxide-free water R* prepared from *distilled water R* and dilute to 100 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and colourless (2.2.2, *Method II*).

pH (2.2.3). Dilute 10 ml of solution S to 20 ml with *carbon dioxide-free water R*. The pH of the solution is 5.8 to 7.0.

Reducing substances. Boil for 5 min a mixture of 10 ml of solution S, 90 ml of *water R*, 5 ml of *dilute sulphuric acid R* and 1.5 ml of a 0.3 g/l solution of *potassium permanganate R*. The pink colour of the solution remains.

Chlorides (2.4.4). 10 ml of solution S diluted to 15 ml with *water R* complies with the limit test for chlorides (50 ppm).

Sulphates (2.4.13). 15 ml of solution S complies with the limit test for sulphates (100 ppm).

Aluminium. Not more than 5 ppm of Al, determined by atomic absorption spectrometry (2.2.23, *Method I*).

Test solution. Dissolve 2.50 g of the substance to be examined in 20 ml of a 200 g/l solution of *cadmium- and lead-free nitric acid R* and dilute to 25.0 ml with the same acidic solution.

Reference solutions. Prepare the reference solutions using *aluminium standard solution (200 ppm Al) R*, diluted with a 200 g/l solution of *cadmium- and lead-free nitric acid R*.

Measure the absorbance at 309.3 nm, using an aluminium hollow-cathode lamp as a source of radiation and an air-acetylene or an acetylene-nitrous oxide flame.

Arsenic (2.4.2). 0.5 g complies with limit test A for arsenic (2 ppm).

Cadmium. Not more than 2 ppm of Cd, determined by atomic absorption spectrometry (2.2.23, *Method I*).

Test solution. Use the solution described in the test for aluminium.

Reference solutions. Prepare the reference solutions using *cadmium standard solution (0.1 per cent Cd) R*, diluted with a 200 g/l solution of *cadmium- and lead-free nitric acid R*. Measure the absorbance at 228.8 nm, using a cadmium hollow-cathode lamp as a source of radiation and an air-acetylene flame.

Copper. Not more than 50 ppm of Cu, determined by atomic absorption spectrometry (2.2.23, *Method I*).

Test solution. Use the solution described in the test for iron.

Reference solutions. Prepare the reference solutions using *copper standard solution (10 ppm Cu) R*, diluted with a 200 g/l solution of *cadmium- and lead-free nitric acid R*. Measure the absorbance at 324.8 nm, using a copper hollow-cathode lamp as source of radiation and an air-acetylene flame.

Iron. Not more than 50 ppm of Fe, determined by atomic absorption spectrometry (2.2.23, *Method I*).

Test solution. Dissolve 1.25 g of the substance to be examined in 20 ml of a 200 g/l solution of *cadmium- and lead-free nitric acid R* and dilute to 25.0 ml with the same acid solution.

Reference solutions. Prepare the reference solutions using *iron standard solution (20 ppm Fe) R*, diluted with a 200 g/l solution of *cadmium- and lead-free nitric acid R*.

Measure the absorbance at 248.3 nm, using an iron hollow-cathode lamp as a source of radiation and an air-acetylene flame.

Lead. Not more than 10 ppm of Pb, determined by atomic absorption spectrometry (2.2.23, *Method I*).

Test solution. Dissolve 5.00 g of the substance to be examined in 20 ml of a 200 g/l solution of *cadmium- and lead-free nitric acid R* and dilute to 25.0 ml with the same acid solution.

Reference solutions. Prepare the reference solutions using *lead standard solution (0.1 per cent of Pb) R*, diluting with a 200 g/l solution of *cadmium- and lead-free nitric acid R*. Measure the absorbance at 283.3 nm, using a lead hollow-cathode lamp as a source of radiation and an air-acetylene flame.

ASSAY

Dissolve 0.200 g in 5 ml of *dilute acetic acid R*. Carry out the complexometric titration of zinc (2.5.11).

1 ml of 0.1 M sodium edetate is equivalent to 21.95 mg of $C_6H_{12}N_2O_6Zn \cdot 2H_2O$.

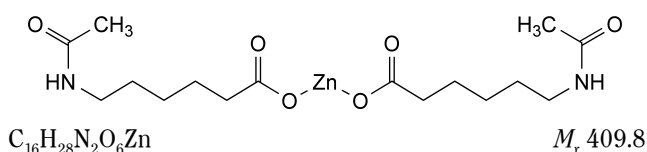
STORAGE

Store in a non-metallic container.

01/2005:1279

ZINC ACEXAMATE

Zinci acexamas



DEFINITION

Zinc acexamate contains not less than 97.5 per cent and not more than the equivalent of 101.0 per cent of zinc 6-(acetylamino)hexanoate, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, crystalline powder, soluble in water, practically insoluble in acetone and in alcohol. It dissolves in dilute nitric acid.

It melts at about 198 °C.

IDENTIFICATION

- Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *zinc acexamate CRS*. Examine the substances prepared as discs.
- 5 ml of solution S (see Tests) gives the reaction of zinc (2.3.1).

TESTS

Solution S. Dissolve 0.5 g in *carbon dioxide-free water R* and dilute to 20 ml with the same solvent.

Appearance of solution. Solution S is not more opalescent than reference suspension IV (2.2.1) and is colourless (2.2.2, *Method II*).

pH (2.2.3). The pH of solution S is 5.0 to 7.0.

6-Aminohexanoic acid. Examine by thin-layer chromatography (2.2.27), using a suitable silica gel as the coating substance.

Test solution. Dissolve 0.30 g of the substance to be examined in *water R* and dilute to 10 ml with the same solvent.

Reference solution. Dissolve 15 mg of 6-aminohexanoic acid *R* in *water R* and dilute to 10 ml with the same solvent. Dilute 1 ml of the solution to 10 ml with *water R*.

Apply to the plate 5 µl of the test solution and 5 µl of the reference solution. Allow the plate to dry in air. Develop over a path of 15 cm using a mixture of 2 volumes of *ammonia R*, 30 volumes of *water R* and 68 volumes of *alcohol R*. Dry the plate in a current of warm air. Spray with *ninhydrin solution R* and heat at 100 °C to 105 °C for 15 min. In the chromatogram obtained with the test solution, any spot corresponding to 6-aminohexanoic acid is not more intense than the spot in the chromatogram obtained with the reference solution (0.5 per cent).

Related substances. Examine by liquid chromatography (2.2.29).

Test solution (a). Dissolve 0.50 g of the substance to be examined in *water R* and dilute to 100.0 ml with the same solvent.

Test solution (b). To 20.0 ml of test solution (a), add 20 ml of the mobile phase, 0.4 ml of a 100 g/l solution of *phosphoric acid R* and dilute to 50.0 ml with the mobile phase.

Reference solution (a). Dissolve 40 mg of *N-acetyl-ε-caprolactam R* in *water R* and dilute to 100.0 ml with the same solvent.

Reference solution (b). Dilute 5.0 ml of reference solution (a) to 100.0 ml with *water R*.

Reference solution (c). Dissolve 20 mg of *zinc acexamate impurity A CRS* in *water R* and dilute to 50.0 ml with the same solvent.

Reference solution (d). Dissolve 40 mg of *ε-caprolactam R* in *water R* and dilute to 100.0 ml with the same solvent. Dilute 5.0 ml of the solution to 100.0 ml with *water R*.

Reference solution (e). To 20.0 ml of test solution (a), add 5.0 ml of reference solution (b), 5.0 ml of reference solution (c), 5.0 ml of reference solution (d) and 0.4 ml of a 100 g/l solution of *phosphoric acid R* and dilute to 50.0 ml with the mobile phase.

Reference solution (f). To 5.0 ml of reference solution (c), add 5.0 ml of reference solution (b), 5.0 ml of reference solution (d) and 0.4 ml of a 100 g/l solution of *phosphoric acid R* and dilute to 50.0 ml with the mobile phase.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4.0 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (5 µm),
- as mobile phase at a flow rate of 1.2 ml/min a mixture of 0.2 volumes of *phosphoric acid R*, 8 volumes of *acetonitrile R* and 92 volumes of *water R*, adjusted to pH 4.5 with *dilute ammonia R1*,
- as detector a spectrophotometer set at 210 nm.

When the chromatograms are recorded in the prescribed conditions, the elution order is: zinc acexamate, ε-caprolactam, zinc acexamate impurity A and *N*-acetyl-ε-caprolactam. Inject 20 µl of reference solution (e). Continue the chromatography for eight times the retention time of zinc acexamate. Adjust the sensitivity of the system so that the height of the peak corresponding to zinc acexamate impurity A is at least 50 per cent of the full scale of the recorder. The test is not valid unless the resolution between the first peak (zinc acexamate) and the second peak (ε-caprolactam) is at least 3.0. If necessary adjust the pH of the mobile phase to 4.7 with *dilute ammonia R1*.

Inject 20 µl of test solution (b) and 20 µl of reference solution (f). In the chromatogram obtained with test solution (b): the area of any peak corresponding to *N*-acetyl-ε-caprolactam is not greater than that of the corresponding peak in the chromatogram obtained with reference solution (f) (0.1 per cent); the area of any peak corresponding to zinc acexamate impurity A is not greater than that of the corresponding peak in the chromatogram obtained with reference solution (f) (2 per cent); the area of any peak corresponding to ε-caprolactam is not greater than that of the corresponding peak in the chromatogram obtained with reference solution (f) (0.1 per cent); the sum of the areas of all the peaks, apart from the principal peak and the peak due to zinc acexamate impurity A is not greater than five times the area of the peak due to *N*-acetyl-ε-caprolactam in the chromatogram obtained with reference solution (f) (0.5 per cent). Disregard any peak with an area less than 0.5 times that of the peak due to *N*-acetyl-ε-caprolactam in the chromatogram obtained with reference solution (f).

Arsenic (2.4.2). 0.5 g complies with limit test A for arsenic (2 ppm).

Cadmium. Not more than 2 ppm of Cd, determined by atomic absorption spectrometry (2.2.23, *Method I*).

Test solution. Dissolve 2.50 g of the substance to be examined in 20 ml of a 200 g/l solution of *cadmium- and lead-free nitric acid R* and dilute to 25.0 ml with the same acidic solution.

Reference solutions. Prepare the reference solutions using *cadmium standard solution (0.1 per cent of Cd) R*, diluted with a 200 g/l solution of *cadmium- and lead-free nitric acid R*.

Measure the absorbance at 228.8 nm, using a cadmium hollow-cathode lamp as a source of radiation and an air-acetylene flame.

Iron. Not more than 50 ppm of Fe, determined by atomic absorption spectrometry (2.2.23, *Method I*).

Test solution. Dissolve 1.25 g of the substance to be examined in 20 ml of a 200 g/l solution of *cadmium- and lead-free nitric acid R* and dilute to 25.0 ml with the same acidic solution.

Reference solutions. Prepare the reference solutions using *iron standard solution (20 ppm Fe) R*, diluted with a 200 g/l solution of *cadmium- and lead-free nitric acid R*.

Measure the absorbance at 248.3 nm, using an iron hollow-cathode lamp as a source of radiation and an air-acetylene flame.

Lead. Not more than 10 ppm of Pb, determined by atomic absorption spectrometry (2.2.23, *Method I*).

Test solution. Dissolve 5.00 g of the substance to be examined in 20 ml of 200 g/l solution of *cadmium- and lead-free nitric acid R* and dilute to 25.0 ml with the same acidic solution.

Reference solutions. Prepare the reference solutions using *lead standard solution (0.1 per cent of Pb) R*, diluting with a 200 g/l solution of *cadmium- and lead-free nitric acid R*. Measure the absorbance at 283.3 nm, using a lead hollow-cathode lamp as a source of radiation and an air-acetylene flame.

Loss on drying (2.2.32). Not more than 1.0 per cent, determined on 1.000 g by drying in an oven at 100 °C to 105 °C.

ASSAY

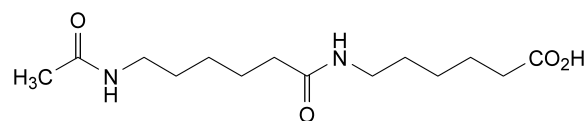
Dissolve 0.400 g in 10 ml of *dilute acetic acid R*. Carry out the complexometric titration of zinc (2.5.11).

1 ml of 0.1 M *sodium edetate* is equivalent to 40.98 mg of C₁₆H₂₈N₂O₆Zn.

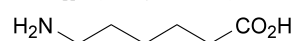
STORAGE

Store in a non-metallic container.

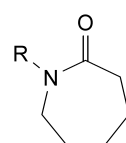
IMPURITIES



A. 6-[[6-(acetylamino)hexanoyl]amino]hexanoic acid,



B. 6-aminohexanoic acid (6-aminocaproic acid),



C. R = CO-CH₃: 1-acetylhexahydro-2H-azepin-2-one (*N*-acetyl-ε-caprolactam),

D. R = H: hexahydro-2H-azepin-2-one (ε-caprolactam).

01/2005:0110

ZINC CHLORIDE

Zinci chloridum

ZnCl₂

M_r 136.3

DEFINITION

Zinc chloride contains not less than 95.0 per cent and not more than the equivalent of 100.5 per cent of ZnCl₂.

CHARACTERS

A white, crystalline powder or cast in white sticks, deliquescent, very soluble in water, freely soluble in alcohol and in glycerol.

IDENTIFICATION

- A. Dissolve 0.5 g in *dilute nitric acid R* and dilute to 10 ml with the same acid. The solution gives reaction (a) of chlorides (2.3.1).
- B. 5 ml of solution S (see Tests) gives the reaction of zinc (2.3.1).

TESTS

Solution S. To 2.0 g add 38 ml of *carbon dioxide-free water R* prepared from *distilled water R* and add *dilute hydrochloric acid R* dropwise until dissolution is complete. Dilute to 40 ml with *carbon dioxide-free water R* prepared from *distilled water R*.

pH (2.2.3). Dissolve 1.0 g in 9 ml of *carbon dioxide-free water R*, ignoring any slight turbidity. The pH of the solution is 4.6 to 5.5.

Oxychlorides. Dissolve 1.5 g in 1.5 ml of *carbon dioxide-free water R*. The solution is not more opalescent than reference suspension II (2.2.1). Add 7.5 ml of *alcohol R*. The solution may become cloudy within 10 min. Any cloudiness disappears on the addition of 0.2 ml of *dilute hydrochloric acid R*.

Sulphates (2.4.13). 5 ml of solution S diluted to 15 ml with *distilled water R* complies with the limit test for sulphates (200 ppm). Prepare the standard using a mixture of 5 ml of *sulphate standard solution (10 ppm SO₄) R* and 10 ml of *distilled water R*.

Aluminium, calcium, heavy metals, iron, magnesium. To 8 ml of solution S add 2 ml of *concentrated ammonia R* and shake. The solution is clear (2.2.1) and colourless (2.2.2, *Method II*). Add 1 ml of *disodium hydrogen phosphate solution R*. The solution remains clear for at least 5 min. Add 0.2 ml of *sodium sulphide solution R*. A white precipitate is formed and the supernatant liquid remains colourless.

Ammonium (2.4.1). 0.5 ml of solution S diluted to 15 ml with *water R* complies with the limit test for ammonium (400 ppm).

ASSAY

Dissolve 0.250 g in 5 ml of *dilute acetic acid R*. Carry out the complexometric titration of zinc (2.5.11).

1 ml of 0.1 M *sodium edetate* is equivalent to 13.63 mg of ZnCl₂.

STORAGE

Store in a non-metallic container.

CHARACTERS

A soft, white or faintly yellowish-white, amorphous powder, free from gritty particles, practically insoluble in water and in alcohol. It dissolves in dilute mineral acids.

IDENTIFICATION

- A. It becomes yellow when strongly heated; the yellow colour disappears on cooling.
- B. Dissolve 0.1 g in 1.5 ml of *dilute hydrochloric acid R* and dilute to 5 ml with *water R*. The solution gives the reaction of zinc (2.3.1).

TESTS

Alkalinity. Shake 1.0 g with 10 ml of boiling *water R*. Add 0.1 ml of *phenolphthalein solution R* and filter. If the filtrate is red, not more than 0.3 ml of 0.1 M *hydrochloric acid* is required to change the colour of the indicator.

Carbonates and substances insoluble in acids. Dissolve 1.0 g in 15 ml of *dilute hydrochloric acid R*. It dissolves without effervescence and the solution is not more opalescent than reference suspension II (2.2.1) and is colourless (2.2.2, *Method II*).

Arsenic (2.4.2). 0.2 g complies with limit test A for arsenic (5 ppm).

Cadmium. Not more than 10 ppm of Cd, determined by atomic absorption spectrometry (2.2.23, *Method II*).

Test solution. Dissolve 2.0 g of the substance to be examined in 14 ml of a mixture of equal volumes of *water R* and *cadmium- and lead-free nitric acid R*, boil for 1 min, cool and dilute to 100.0 ml with *water R*.

Reference solutions. Prepare the reference solutions using *cadmium standard solution (0.1 per cent Cd) R* and diluting with a 3.5 per cent V/V solution of *cadmium- and lead-free nitric acid R*.

Measure the absorbance at 228.8 nm using a cadmium hollow-cathode lamp as source of radiation and an air-acetylene or an air-propane flame.

Iron (2.4.9). Dissolve 50 mg in 1 ml of *dilute hydrochloric acid R* and dilute to 10 ml with *water R*. The solution complies with the limit test for iron (200 ppm). Use in this test 0.5 ml of *thioglycollic acid R*.

Lead. Not more than 50 ppm of Pb, determined by atomic absorption spectrometry (2.2.23, *Method II*).

Test solution. Dissolve 5.0 g in 24 ml of a mixture of equal volumes of *water R* and *cadmium- and lead-free nitric acid R*, boil for 1 min, cool and dilute to 100.0 ml with *water R*.

Reference solutions. Prepare the reference solutions using *lead standard solution (0.1 per cent Pb) R* and diluting with a 3.5 per cent V/V solution of *cadmium- and lead-free nitric acid R*.

Measure the absorbance at 283.3 nm using a lead hollow-cathode lamp as source of radiation and an air-acetylene flame. Depending on the apparatus, the line at 217.0 nm may be used.

Loss on ignition. Not more than 1.0 per cent, determined on 1.00 g at 500 °C.

ASSAY

Dissolve 0.150 g in 10 ml of *dilute acetic acid R*. Carry out the complexometric titration of zinc (2.5.11).

1 ml of 0.1 M *sodium edetate* is equivalent to 8.14 mg of ZnO.

01/2005:0252

ZINC OXIDE

Zinci oxidum

ZnO

M_r 81.4

DEFINITION

Zinc oxide contains not less than 99.0 per cent and not more than the equivalent of 100.5 per cent of ZnO, calculated with reference to the ignited substance.

01/2005:0306

ZINC STEARATE**Zinci stearas****DEFINITION**

Zinc stearate $[(C_{17}H_{35}COO)_2Zn; M_r 632]$ may contain varying proportions of zinc palmitate $[(C_{15}H_{31}COO)_2Zn; M_r 576.2]$ and zinc oleate $[(C_{17}H_{33}COO)_2Zn; M_r 628]$. It contains not less than 10.0 per cent and not more than 12.0 per cent of Zn.

CHARACTERS

A light, white, amorphous powder, free from gritty particles, practically insoluble in water and in ethanol.

IDENTIFICATION

- The residue obtained in the preparation of solution S (see Tests) has a freezing point (2.2.18) not lower than 53 °C.
- Neutralise 5 ml of solution S to *red litmus paper R* with *strong sodium hydroxide solution R*. The solution gives the reaction of zinc (2.3.1).

TESTS

Solution S. To 5.0 g add 50 ml of *ether R* and 40 ml of a 7.5 per cent V/V solution of *cadmium- and lead-free nitric acid R* in *distilled water R*. Heat under a reflux condenser until dissolution is complete. Allow to cool. In a separating funnel, separate the aqueous layer and shake the ether layer with two quantities, each of 4 ml, of *distilled water R*. Combine the aqueous layers, wash with 15 ml of *ether R* and heat on a water-bath until ether is completely eliminated. Allow to cool and dilute to 50.0 ml with *distilled water R* (solution S). Evaporate the ether layer to dryness and dry the residue at 105 °C.

Appearance of solution. Solution S is not more intensely coloured than reference solution Y_6 (2.2.2, *Method II*).

Appearance of solution of fatty acids. Dissolve 0.5 g of the residue obtained in the preparation of solution S in 10 ml of *chloroform R*. The solution is clear (2.2.1) and not more intensely coloured than reference solution Y_5 (2.2.2, *Method II*).

Acidity or alkalinity. Shake 1.0 g with 5 ml of *alcohol R* and add 20 ml of *carbon dioxide-free water R* and 0.1 ml of *phenol red solution R*. Not more than 0.3 ml of 0.1 M *hydrochloric acid* or 0.1 ml of 0.1 M *sodium hydroxide* is required to change the colour of the indicator.

Acid value of the fatty acids (2.5.1). 195 to 210, determined on 0.20 g of the residue obtained in the preparation of solution S, dissolved in 25 ml of the prescribed mixture of solvents.

Chlorides (2.4.4). 2 ml of solution S diluted to 15 ml with *water R* complies with the limit test for chlorides (250 ppm).

Sulphates (2.4.13). Dilute 1 ml of solution S to 50 ml with *distilled water R*. 12.5 ml of the solution diluted to 15 ml with *distilled water R* complies with the limit test for sulphates (0.6 per cent).

Cadmium. Not more than 5 ppm of Cd, determined by atomic absorption spectrometry (2.2.23, *Method II*).

Test solution. Dilute 20.0 ml of solution S to 50.0 ml with a 3.5 per cent V/V solution of *cadmium- and lead-free nitric acid R*.

Reference solutions. Prepare the reference solutions using *cadmium standard solution* (0.1 per cent Cd) *R* and diluting with a 3.5 per cent V/V solution of *cadmium- and lead-free nitric acid R*.

Measure the absorbance at 228.8 nm using a cadmium hollow-cathode lamp as source of radiation and an air-acetylene or an air-propane flame.

Lead. Not more than 25 ppm of Pb, determined by atomic absorption spectrometry (2.2.23, *Method II*).

Test solution. Use solution S.

Reference solutions. Prepare the reference solutions using *lead standard solution* (0.1 per cent Pb) *R* and diluting with a 3.5 per cent V/V solution of *cadmium- and lead-free nitric acid R*.

Measure the absorbance at 283.3 nm using a lead hollow-cathode lamp as source of radiation and an air-acetylene flame. Depending on the apparatus the line at 217.0 nm may be used.

ASSAY

To 1.000 g add 50 ml of *dilute acetic acid R* and boil for at least 10 min or until the layer of fatty acids is clear, adding more *water R* as necessary to maintain the original volume. Cool and filter. Wash the filter and the flask with *water R* until the washings are no longer acid to *blue litmus paper R*. Combine the filtrate and washings. Carry out the complexometric titration of zinc (2.5.11).

1 ml of 0.1 M *sodium edetate* is equivalent to 6.54 mg of Zn.

01/2005:0111

ZINC SULPHATE HEPTAHYDRATE**Zinci sulfas heptahydricus** $ZnSO_4 \cdot 7H_2O$ M_r 287.5**DEFINITION**

Content: 99.0 per cent to 104.0 per cent.

CHARACTERS

Appearance: white, crystalline powder or colourless, transparent crystals, efflorescent.

Solubility: very soluble in water, practically insoluble in alcohol.

IDENTIFICATION

- Solution S (see Tests) gives the reactions of sulphates (2.3.1).
- Solution S gives the reaction of zinc (2.3.1).
- It complies with the limits of the assay.

TESTS

Solution S. Dissolve 2.5 g in *carbon dioxide-free water R* and dilute to 50 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and colourless (2.2.2, *Method II*).

pH (2.2.3): 4.4 to 5.6 for solution S.

Chlorides (2.4.4): maximum 300 ppm.

3.3 ml of solution S diluted to 15 ml with *water R* complies with the limit test for chlorides.

Iron (2.4.9): maximum 100 ppm.

2 ml of solution S diluted to 10 ml with *water R* complies with the limit test for iron. Use in this test 0.5 ml of *thioglycollic acid R*.

ASSAY

Dissolve 0.200 g in 5 ml of *dilute acetic acid R*. Carry out the complexometric titration of zinc (2.5.11).

1 ml of 0.1 M sodium edetate is equivalent to 28.75 mg of $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$.

STORAGE

In a non-metallic, airtight container.

01/2005:1683

ZINC SULPHATE HEXAHYDRATE

Zinci sulfas hexahydricus

$\text{ZnSO}_4 \cdot 6\text{H}_2\text{O}$

M_r 269.5

DEFINITION

Content: 99.0 per cent to 104.0 per cent.

CHARACTERS

Appearance: white, crystalline powder or colourless transparent crystals, efflorescent.

Solubility: very soluble in water, practically insoluble in alcohol.

IDENTIFICATION

- Solution S (see Tests) gives the reactions of sulphates (2.3.1).
- Solution S gives the reaction of zinc (2.3.1).
- It complies with the limits of the assay.

TESTS

Solution S. Dissolve 2.5 g in carbon dioxide-free water R and dilute to 50 ml with the same solvent.

Appearance of solution. Solution S is clear (2.2.1) and colourless (2.2.2, Method II).

pH (2.2.3): 4.4 to 5.6 for solution S.

Chlorides (2.4.4): maximum 300 ppm.

3.3 ml of solution S diluted to 15 ml with water R complies with the limit test for chlorides.

Iron (2.4.9): maximum 100 ppm.

2 ml of solution S diluted to 10 ml with water R complies with the limit test for iron. Use in this test 0.5 ml of thioglycollic acid R.

ASSAY

Dissolve 0.200 g in 5 ml of dilute acetic acid R. Carry out the complexometric titration of zinc (2.5.11).

1 ml of 0.1 M sodium edetate is equivalent to 26.95 mg of $\text{ZnSO}_4 \cdot 6\text{H}_2\text{O}$.

STORAGE

In a non-metallic, airtight container.

DEFINITION

Zinc undecylenate contains not less than 98.0 per cent and not more than the equivalent of 102.0 per cent of zinc di(undec-10-enoate), calculated with reference to the dried substance.

CHARACTERS

A white or almost white, fine powder, practically insoluble in water and in alcohol.

It melts at 116 °C to 121 °C but may leave a slight solid residue.

IDENTIFICATION

- To 2.5 g add 10 ml of water R and 10 ml of dilute sulphuric acid R. Shake with two quantities, each of 10 ml, of ether R. Reserve the aqueous layer for identification test C. Wash the combined ether layers with water R and evaporate to dryness. To the residue add 2 ml of freshly distilled aniline R and boil under a reflux condenser for 10 min. Allow to cool and add 30 ml of ether R. Shake with three quantities, each of 20 ml, of dilute hydrochloric acid R and then with 20 ml of water R. Evaporate the organic layer to dryness on a water-bath. The residue, after recrystallisation twice from alcohol (70 per cent V/V) R and drying *in vacuo* for 3 h, melts (2.2.14) at 66 °C to 68 °C.
- Dissolve 0.1 g in a mixture of 2 ml of dilute sulphuric acid R and 5 ml of glacial acetic acid R. Add dropwise 0.25 ml of potassium permanganate solution R. The colour of the potassium permanganate is discharged.
- A mixture of 1 ml of the aqueous layer obtained in identification test A and 4 ml of water R gives the reaction of zinc (2.3.1).

TESTS

Alkalinity. Mix 1.0 g with 5 ml of alcohol R and 0.5 ml of phenol red solution R. Add 50 ml of carbon dioxide-free water R and examine immediately. No reddish colour appears.

Alkali and alkaline-earth metals. To 1.0 g add 25 ml of water R and 5 ml of hydrochloric acid R and heat to boiling. Filter whilst hot. Wash the filter and the residue with 25 ml of hot water R. Combine the filtrate and washings and add concentrated ammonia R until alkaline. Add 7.5 ml of thioacetamide solution R and heat on a water-bath for 30 min. Filter and wash the precipitate with two quantities, each of 10 ml, of water R. Combine the filtrate and washings, evaporate to dryness on a water-bath and ignite. The residue weighs not more than 20 mg (2 per cent).

Sulphates (2.4.13). To 0.1 g add a mixture of 2 ml of dilute hydrochloric acid R and 10 ml of distilled water R and heat to boiling. Cool, filter and dilute to 15 ml with distilled water R. The solution complies with the limit test for sulphates (500 ppm). Prepare the standard using 5 ml of sulphate standard solution (10 ppm SO_4) R and 10 ml of distilled water R.

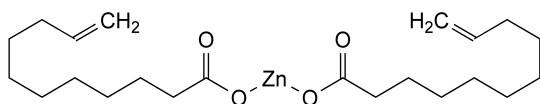
Loss on drying (2.2.32). Not more than 1.5 per cent, determined on 0.500 g by drying in an oven at 100 °C to 105 °C.

Degree of unsaturation. Dissolve 0.100 g in a mixture of 5 ml of dilute hydrochloric acid R and 30 ml of glacial acetic acid R. Using 0.05 ml indigo carmine solution R1, added towards the end of the titration as indicator. Titrate with 0.0167 M bromide-bromate until the colour changes from blue to yellow. 9.1 ml to 9.4 ml of 0.0167 M bromide-bromate is required. Carry out a blank titration.

01/2005:0539

ZINC UNDECYLENATE

Zinci undecylenas



$\text{C}_{22}\text{H}_{38}\text{O}_4\text{Zn}$

M_r 431.9

ASSAY

To 0.350 g add 25 ml of *dilute acetic acid R* and heat to boiling. Carry out the complexometric titration of zinc (2.5.11).

1 ml of 0.1 M sodium edetate is equivalent to 43.19 mg of $C_{22}H_{38}O_4Zn$.

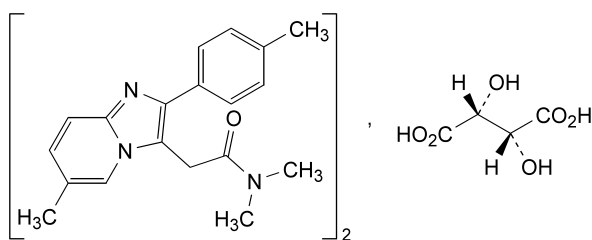
STORAGE

Store protected from light.

01/2005:1280

ZOLPIDEM TARTRATE

Zolpidemi tartras

 $C_{42}H_{48}N_6O_8$ M_r 765

DEFINITION

Zolpidem tartrate contains not less than 98.5 per cent and not more than the equivalent of 101.0 per cent of bis[*N,N*-dimethyl-2-[6-methyl-2-(4-methylphenyl)imidazo[1,2-*a*]pyridin-3-yl]acetamide] (2*R*,3*R*)-2,3-dihydroxybutanedioate, calculated with reference to the anhydrous substance.

CHARACTERS

A white or almost white, crystalline powder, hygroscopic, slightly soluble in water, sparingly soluble in methanol, practically insoluble in methylene chloride.

IDENTIFICATION

First identification: A, C.

Second identification: B, C.

A. Dissolve 0.10 g in 10 ml of 0.1 M hydrochloric acid. Add 10 ml of *water R*. Add dropwise with stirring 1 ml of *dilute ammonia R2*. Filter and collect the resulting precipitate. Wash the precipitate with *water R* and then dry at 100–105 °C for 2 h (test precipitate). Carry out the same operation using *zolpidem tartrate CRS* (reference precipitate). Examine the precipitates by infrared absorption spectrophotometry (2.2.24) comparing the spectra obtained. Examine the precipitates as discs.

B. Examine by thin-layer chromatography (2.2.27), using a *TLC silica gel F₂₅₄ plate R*.

Test solution. Dissolve 50 mg of the substance to be examined in 5 ml of *methanol R*, add 0.1 ml of *diethylamine R* and dilute to 10 ml with *methanol R*.

Reference solution (a). Dissolve 50 mg of *zolpidem tartrate CRS* in 5 ml of *methanol R*, add 0.1 ml of *diethylamine R* and dilute to 10 ml with *methanol R*.

Reference solution (b). Dissolve 50 mg of *flunitrazepam CRS* in 5 ml of *methylene chloride R* and dilute to 10 ml with the same solvent. Mix 1 ml of the solution with 1 ml of reference solution (a).

Apply to the plate 5 µl of each solution. Develop over a path of 12 cm using a mixture of 10 volumes of *diethylamine R*, 45 volumes of *cyclohexane R* and 45 volumes of *ethyl acetate R*. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. The principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a). The test is not valid unless the chromatogram obtained with reference solution (b) shows 2 clearly separated spots.

C. Dissolve about 0.1 g in 1 ml of *methanol R* heating gently. 0.1 ml of the solution gives reaction (b) of tartrates (2.3.1).

TESTS

Appearance of solution. Prepare the solutions protected from light and carry out the test as rapidly as possible. Triturate 0.25 g with 0.125 g of *tartaric acid R*. Dissolve the mixture in 20 ml of *water R* and dilute to 25 ml with the same solvent. The solution is clear (2.2.1) and not more intensely coloured than reference solution Y₆ or BY₆ (2.2.2, Method II).

Related substances. Examine by liquid chromatography (2.2.29).

Test solution. Dissolve 25.0 mg of the substance to be examined in the mobile phase and dilute to 50.0 ml with the mobile phase.

Reference solution (a). Dissolve 5 mg of *zolpidem impurity A CRS* in the mobile phase and dilute to 50 ml with the mobile phase.

Reference solution (b). Dissolve 5 mg of the substance to be examined in the mobile phase and dilute to 50 ml with the mobile phase. To 10 ml of the solution, add 10 ml of reference solution (a).

Reference solution (c). Dilute 2.0 ml of the test solution to 100.0 ml with the mobile phase. Dilute 1.0 ml of the solution to 10.0 ml with the mobile phase.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.15 m long and 3.9 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (4 µm),
- as mobile phase at a flow rate of 1.5 ml/min a mixture of 18 volumes of *acetonitrile R*, 23 volumes of *methanol R* and 59 volumes of a 5.6 g/l solution of *phosphoric acid R* adjusted to pH 5.5 with *triethylamine R*,
- as detector a spectrophotometer set at 254 nm.

Inject 20 µl of reference solution (b). Adjust the sensitivity of the system so that the height of the peak due to zolpidem impurity A is at least 50 per cent of the full scale of the recorder. The test is not valid unless the resolution between the peaks due to zolpidem impurity A and zolpidem tartrate is at least 2.0.

Inject 20 µl of the test solution and 20 µl of reference solution (c). In the chromatogram obtained with the test solution, the sum of the areas of any peaks, apart from the principal peak, is not greater than the area of the principal peak in the chromatogram obtained with reference solution (c) (0.2 per cent). Disregard any peak with an area less than 0.1 times that of the principal peak in the chromatogram obtained with reference solution (c) and any peak (with a relative retention time of 0.16 to the zolpidem peak) corresponding to tartaric acid.

Water (2.5.12). Not more than 3.0 per cent, determined on 0.50 g by the semi-micro determination of water.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

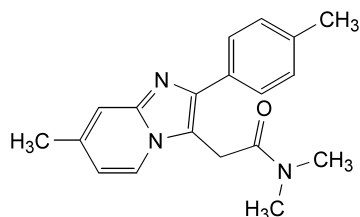
ASSAY

Dissolve 0.300 g in a mixture of 20 ml of *anhydrous acetic acid R* and 20 ml of *acetic anhydride R*. Titrate with 0.1 M *perchloric acid*, determining the end-point potentiometrically (2.2.20). Carry out a blank titration. 1 ml of 0.1 M *perchloric acid* is equivalent to 38.24 mg of $C_{42}H_{48}N_6O_8$.

STORAGE

Store in an airtight container, protected from light.

IMPURITIES

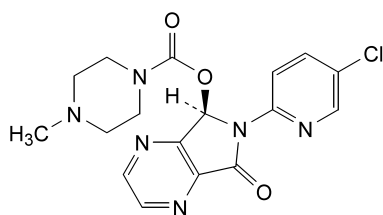


- A. *N,N*-dimethyl-2-[7-methyl-2-(4-methylphenyl)imidazo[1,2-*a*]pyridin-3-yl]acetamide.

01/2005:1060
corrected

ZOPICLONE

Zopiclonum



and enantiomer

$C_{17}H_{17}ClN_6O_3$

M_r 388.8

DEFINITION

Zopiclone contains not less than 98.5 per cent and not more than the equivalent of 100.5 per cent of (5*RS*)-6-(5-chloropyridin-2-yl)-7-oxo-6,7-dihydro-5*H*-pyrrolo[3,4-*b*]pyrazin-5-yl 4-methylpiperazine-1-carboxylate, calculated with reference to the solvent-free substance.

CHARACTERS

A white or slightly yellowish powder, practically insoluble in water, freely soluble in methylene chloride, sparingly soluble in acetone, practically insoluble in alcohol. It dissolves in dilute mineral acids.

It melts at about 177 °C, with decomposition.

IDENTIFICATION

First identification: B.

Second identification: A, C.

- A. Dissolve 50.0 mg in a 3.5 g/l solution of *hydrochloric acid R* and dilute to 100.0 ml with the same solvent. Dilute 2.0 ml of this solution to 100.0 ml with a 3.5 g/l solution of *hydrochloric acid R*. Examined between 220 nm and 350 nm (2.2.25), the solution shows an absorption maximum at 303 nm. The specific absorbance at the maximum is 340 to 380.

- B. Examine by infrared absorption spectrophotometry (2.2.24), comparing with the spectrum obtained with *zopiclone CRS*. Examine the substances prepared as discs.

- C. Examine by thin-layer chromatography (2.2.27), using *silica gel GF₂₅₄ R* as the coating substance.

Test solution. Dissolve 10 mg of the substance to be examined in *methylene chloride R* and dilute to 10 ml with the same solvent.

Reference solution. Dissolve 10 mg of *zopiclone CRS* in *methylene chloride R* and dilute to 10 ml with the same solvent.

Apply to the plate 10 µl of each solution. Develop over a path of 15 cm using a mixture of 2 volumes of *triethylamine R*, 50 volumes of *acetone R* and 50 volumes of *ethyl acetate R*. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. The principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with the reference solution.

TESTS

Solution S. Dissolve 1.0 g in *dimethylformamide R* and dilute to 20.0 ml with the same solvent.

Appearance of solution. Solution S is not more opalescent than reference suspension II (2.2.1) and not more intensely coloured than intensity 5 of the range of reference solutions of the most appropriate colour (2.2.2, *Method II*).

Optical rotation (2.2.7). Dilute 10.0 ml of solution S to 50.0 ml with *dimethylformamide R*. The angle of optical rotation is -0.05° to $+0.05^\circ$.

Related substances. Examine by liquid chromatography (2.2.29). *Prepare the solutions immediately before use.*

Test solution. Dissolve 40.0 mg of the substance to be examined in the mobile phase and dilute to 10.0 ml with the mobile phase.

Reference solution (a). Dilute 3.0 ml of the test solution to 100.0 ml with the mobile phase. Dilute 1.0 ml of this solution to 10.0 ml with the mobile phase.

Reference solution (b). Dilute 1.0 ml of the test solution to 100.0 ml with the mobile phase. Dilute 1.0 ml of this solution to 10.0 ml with the mobile phase.

Reference solution (c). Dissolve 4.0 mg of *zopiclone oxide CRS* in the mobile phase and dilute to 10.0 ml with the mobile phase. To 10.0 ml of this solution, add 1.0 ml of the test solution and dilute to 100.0 ml with the mobile phase.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4.6 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (5 µm),
 - as mobile phase at a flow rate of 1.5 ml/min a mixture of 38 volumes of *acetonitrile R* and 62 volumes of a solution containing 8.1 g/l of *sodium laurilsulfate R* and 1.6 g/l of *sodium dihydrogen phosphate R* adjusted to pH 3.5 with a 10 per cent V/V solution of *phosphoric acid R*,
 - as detector a spectrophotometer set at 303 nm, maintaining the temperature of the column at 30 °C.
- Inject 20 µl of reference solution (c). Adjust the sensitivity of the system so that the height of each of the 2 principal peaks in the chromatogram obtained is at least 30 per cent of the full scale of the recorder. When the chromatograms are recorded in the prescribed conditions, the retention time of zopiclone is 27 min to 31 min. If necessary, adjust the concentration of acetonitrile in the mobile phase (increasing the concentration decreases the retention times and

decreasing the concentration increases the retention times). The test is not valid unless, in the chromatogram obtained with reference solution (c), the resolution between the peaks due to zopiclone oxide and zopiclone is at least 3.0. If the prescribed resolution is not obtained, adjust the mobile phase to pH 4.0 with a 10 per cent *V/V* solution of *phosphoric acid R*.

Inject 20 µl of the test solution, 20 µl of reference solution (a) and 20 µl of reference solution (b). Continue the chromatography for 1.5 times the retention time of zopiclone. In the chromatogram obtained with the test solution: the area of any peak, apart from the principal peak, is not greater than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.3 per cent) and not more than 2 such peaks have an area greater than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent).

2-Propanol. Not more than 0.7 per cent *m/m* of 2-propanol, determined by gas chromatography (2.2.28) using *ethanol R1* as the internal standard.

Internal standard solution. Dilute 5 ml of *ethanol R1* to 100 ml with *ethylene chloride R*. Dilute 1 ml of this solution to 10 ml with *ethylene chloride R*.

Test solution. Dissolve 0.25 g of the substance to be examined in *ethylene chloride R*, add 0.5 ml of the internal standard solution and dilute to 5.0 ml with *ethylene chloride R*.

Reference solution. Dilute 4.5 ml of 2-propanol *R* to 100.0 ml with *ethylene chloride R*. To 1.0 ml of this solution, add 10.0 ml of the internal standard solution and dilute to 100.0 ml with *ethylene chloride R*.

The chromatographic procedure may be carried out using:

- a fused-silica column 10 m long and about 0.53 mm in internal diameter with a 20 µm coating of *styrene-divinylbenzene copolymer R*,
- *helium for chromatography R* as the carrier gas at a flow rate of 4 ml/min,
- a flame-ionisation detector,

with the following temperature programme:

	Time (min)	Temperature (°C)	Rate (°C/min)	Comment
Column	0 - 5	50		isothermal
	5 - 10	50 → 70	4	linear gradient
	10 - 14	70		isothermal
	14 - 20.5	70 → 200	20	linear gradient
	20.5 - 27.5	200		isothermal
Injection port		150		
Detector		250		

Inject 1 µl of the test solution and 1 µl of the reference solution. Calculate the percentage content *m/m* of 2-propanol taking its density to be 0.785 g/ml at 20 °C.

Heavy metals (2.4.8). 1.0 g complies with limit test C for heavy metals (20 ppm). Prepare the standard using 2 ml of *lead standard solution* (10 ppm *Pb*) *R*.

Sulphated ash (2.4.14). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

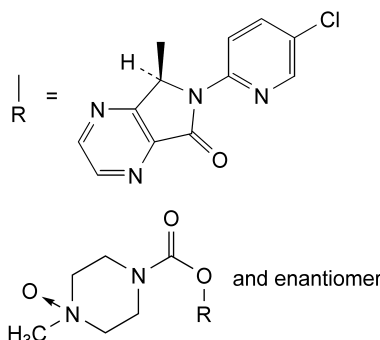
Dissolve 0.300 g in a mixture of 10 ml of *anhydrous acetic acid R* and 40 ml of *acetic anhydride R*. Titrate with 0.1 *M perchloric acid*, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 *M perchloric acid* is equivalent to 38.88 mg of $C_{17}H_{17}ClN_6O_3$.

STORAGE

Store protected from light.

IMPURITIES

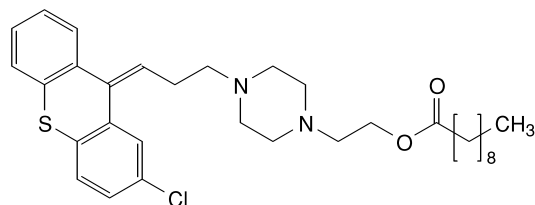


- A. (5*RS*)-6-(5-chloropyridin-2-yl)-7-oxo-6,7-dihydro-5*H*-pyrrolo[3,4-*b*]pyrazin-5-yl 4-methylpiperazine-1-carboxylate 4-oxide (zopiclone oxide),
- B. R-OH and enantiomer: (7*RS*)-6-(5-chloropyridin-2-yl)-7-hydroxy-6,7-dihydro-5*H*-pyrrolo[3,4-*b*]pyrazin-5-one,
- C. R-H: 6-(5-chloropyridin-2-yl)-6,7-dihydro-5*H*-pyrrolo[3,4-*b*]pyrazin-5-one.

01/2005:1707

ZUCLOPENTHIXOL DECANOATE

Zuclopenthixoli decanoas



$C_{32}H_{43}ClN_2O_2S$

M_r 555.2

DEFINITION

2-[4-[3-[(*Z*)-2-chloro-9*H*-thioxanthen-9-ylidene]propyl]piperazin-1-yl]ethyl decanoate.

Content: 98.0 per cent to 102.0 per cent (dried substance).

CHARACTERS

Appearance: yellow, viscous oily liquid.

Solubility: very slightly soluble in water, very soluble in alcohol and in methylene chloride.

IDENTIFICATION

Infrared absorption spectrophotometry (2.2.24).

Comparison: *Ph. Eur. reference spectrum of zuclopenthixol decanoate.*

TESTS

Appearance of solution. The solution is clear (2.2.1).

Using an ultrasonic bath, dissolve 1.0 g in *alcohol R* and dilute to 20.0 ml with the same solvent.

Related substances. Thin-layer chromatography (2.2.27). Carry out the test protected from light and prepare the solutions immediately before use.

Test solution. Dissolve 0.250 g of the substance to be examined in *alcohol R*, using an ultrasonic bath, and dilute to 100.0 ml with the same solvent.

Reference solution (a). Dilute 1.0 ml of the test solution to 100.0 ml with *alcohol R*. Dilute 1.0 ml of this solution to 10.0 ml with *alcohol R*.

Reference solution (b). Dilute 5.0 ml of reference solution (a) to 10.0 ml with *alcohol R*.

Reference solution (c). Dissolve 5.0 mg of *zuclopenthixol impurity B CRS* in *alcohol R* and dilute to 100.0 ml with the same solvent. Dilute 10.0 ml of the solution to 100.0 ml with *alcohol R*.

Reference solution (d). Dissolve 5.0 mg of *zuclopenthixol impurity C CRS* in *alcohol R* and dilute to 100.0 ml with the same solvent. Dilute 2.5 ml of the solution to 20.0 ml with *alcohol R*.

Reference solution (e). Dissolve 5.0 mg of *zuclopenthixol impurity B CRS* and 20.0 mg of the substance to be examined in *alcohol R* and dilute to 100.0 ml with the same solvent.

Plate: TLC silica gel plate *R*.

Mobile phase: diethylamine *R*, propanol *R*, cyclohexane *R* (3:10:90 V/V/V).

Application: 4 µl.

Development: horizontally, over 2/3 of the plate.

Drying: in air.

Detection: spray with a 14 per cent V/V alcoholic solution of sulphuric acid *R* prepared immediately before use; heat at 110 °C for 5 min and examine in ultraviolet light at 365 nm.

System suitability: the chromatogram obtained with reference solution (e) shows 2 clearly separated spots.

Limits:

- **impurity B:** any spot corresponding to impurity B is not more intense than the spot in the chromatogram obtained with reference solution (c) (0.2 per cent),
- **impurity C:** any spot corresponding to impurity C is not more intense than the spot in the chromatogram obtained with reference solution (d) (0.25 per cent),
- **any other impurity:** any spots, apart from the principal spot and any spot corresponding to impurity B or impurity C, are not more intense than the spot in the chromatogram obtained with reference solution (a) (0.1 per cent) and at most 3 such spots are more intense than the spot in the chromatogram obtained with reference solution (b) (0.05 per cent).

Impurity A. Liquid chromatography (2.2.29). Carry out the test protected from light and prepare the solutions immediately before use.

Test solution. Dissolve 40.0 mg of the substance to be examined in *alcohol R* and dilute to 100.0 ml with the same solvent.

Reference solution. Dissolve a quantity of *zuclopenthixol impurity A CRS* corresponding to 5.0 mg of impurity A in *alcohol R* and dilute to 100.0 ml with the same solvent. To 5.0 ml of the solution add 0.6 ml of test solution and dilute to 50.0 ml with *alcohol R*.

Column:

- **size:** $l = 0.15$ m, $\varnothing = 4.6$ mm,
- **stationary phase:** octadecylsilyl silica gel for chromatography *R* (5 µm),
- **temperature:** 40 °C.

Mobile phase: mix 25 volumes of a 8.9 g/l solution of docusate sodium *R* and 75 volumes of *alcohol R*; add 0.1 per cent V/V of phosphoric acid *R*.

Flow rate: 1 ml/min.

Detection: spectrophotometer at 230 nm.

Equilibration: at least 1 h with the mobile phase.

Injection: 20 µl.

System suitability: reference solution:

- **resolution:** minimum of 1.5 between the peaks due to zuclopenthixol decanoate (1st peak) and to impurity A (2nd peak).

Limit:

- **impurity A:** not more than the area of the corresponding peak in the chromatogram obtained with reference solution (1.25 per cent).

Heavy metals (2.4.8): maximum 20 ppm.

1.0 g complies with limit test C. Prepare the standard using 2 ml of lead standard solution (10 ppm Pb) *R*.

Loss on drying (2.2.32): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 60 °C at a pressure not exceeding 0.7 kPa for 3 h.

Sulphated ash (2.4.14): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

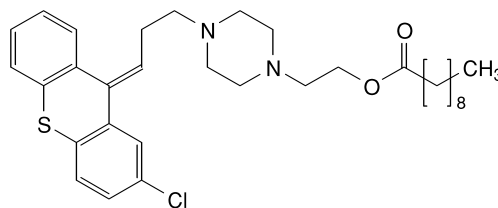
Dissolve 0.250 g in 50 ml of *anhydrous acetic acid R*. Titrate with 0.1 M perchloric acid, determining the end-point potentiometrically (2.2.20).

1 ml of 0.1 M perchloric acid is equivalent to 27.76 mg of $C_{32}H_{43}ClN_2O_2S$.

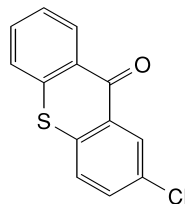
STORAGE

Under an inert gas in an airtight container, protected from light, at a temperature not exceeding –20 °C.

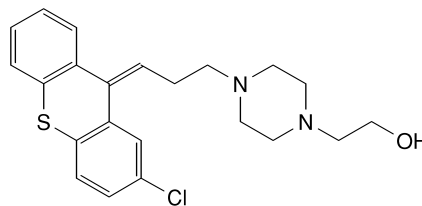
IMPURITIES



A. 2-[4-[3-[(*E*)-2-chloro-9*H*-thioxanthen-9-ylidene]propyl]piperazin-1-yl]ethyl decanoate,



B. 2-chloro-9*H*-thioxanthen-9-one,



C. 2-[4-[3-[(*Z*)-2-chloro-9*H*-thioxanthen-9-ylidene]propyl]piperazin-1-yl]ethanol.

Numerics

1.1. General statements	5	2.2.56. Amino acid analysis	86
1.2. Other provisions applying to general chapters and monographs	5	2.2.5. Relative density	27
1.3. General chapters	6	2.2.6. Refractive index	28
1.4. Monographs	7	2.2.7. Optical rotation	28
1.5. Abbreviations and symbols	9	2.2.8. Viscosity	29
1.6. Units of the International System (SI) used in the Pharmacopoeia and equivalence with other units	10	2.2.9. Capillary viscometer method	29
1.8-Cineole in essential oils, assay of (2.8.11.)	216	2.2. Physical and physicochemical methods	23
1. General notices	5	2.3.1. Identification reactions of ions and functional groups	95
2.1.1. Droppers	17	2.3.2. Identification of fatty oils by thin-layer chromatography	98
2.1.2. Comparative table of porosity of sintered-glass filters	17	2.3.3. Identification of phenothiazines by thin-layer chromatography	99
2.1.3. Ultraviolet ray lamps for analytical purposes	17	2.3.4. Odour	99
2.1.4. Sieves	18	2.3. Identification	95
2.1.5. Tubes for comparative tests	19	2.4.10. Lead in sugars	107
2.1.6. Gas detector tubes	19	2.4.11. Phosphates	108
2.1. Apparatus	17	2.4.12. Potassium	108
2.2.10. Rotating viscometer method	30	2.4.13. Sulphates	108
2.2.11. Distillation range	30	2.4.14. Sulphated ash	108
2.2.12. Boiling point	31	2.4.15. Nickel in polyols	108
2.2.13. Determination of water by distillation	32	2.4.16. Total ash	108
2.2.14. Melting point - capillary method	32	2.4.17. Aluminium	108
2.2.15. Melting point - open capillary method	33	2.4.18. Free formaldehyde	109
2.2.16. Melting point - instantaneous method	33	2.4.19. Alkaline impurities in fatty oils	109
2.2.17. Drop point	33	2.4.1. Ammonium	103
2.2.18. Freezing point	34	2.4.21. Foreign oils in fatty oils by thin-layer chromatography	109
2.2.19. Amperometric titration	34	2.4.22. Composition of fatty acids by gas chromatography	110
2.2.1. Clarity and degree of opalescence of liquids	23	2.4.23. Sterols in fatty oils	111
2.2.20. Potentiometric titration	35	2.4.24. Identification and control of residual solvents	113
2.2.21. Fluorimetry	35	2.4.25. Ethylene oxide and dioxan	118
2.2.22. Atomic emission spectrometry	35	2.4.26. <i>N,N</i> -Dimethylaniline	119
2.2.23. Atomic absorption spectrometry	36	2.4.27. Heavy metals in herbal drugs and fatty oils	119
2.2.24. Absorption spectrophotometry, infrared	37	2.4.28. 2-Ethylhexanoic acid	120
2.2.25. Absorption spectrophotometry, ultraviolet and visible	38	2.4.29. Composition of fatty acids in oils rich in omega-3-acids	121
2.2.26. Paper chromatography	40	2.4.2. Arsenic	103
2.2.27. Thin-layer chromatography	40	2.4.30. Ethylene glycol and diethylene glycol in ethoxylated substances	122
2.2.28. Gas chromatography	42	2.4.3. Calcium	103
2.2.29. Liquid chromatography	43	2.4.4. Chlorides	104
2.2.2. Degree of coloration of liquids	24	2.4.5. Fluorides	104
2.2.30. Size-exclusion chromatography	45	2.4.6. Magnesium	104
2.2.31. Electrophoresis	45	2.4.7. Magnesium and alkaline-earth metals	104
2.2.32. Loss on drying	50	2.4.8. Heavy metals	104
2.2.33. Nuclear magnetic resonance spectrometry	51	2.4.9. Iron	107
2.2.34. Thermal analysis	52	2.4. Limit tests	103
2.2.35. Osmolality	54	2.5.10. Oxygen-flask method	130
2.2.36. Potentiometric determination of ionic concentration using ion-selective electrodes	55	2.5.11. Complexometric titrations	130
2.2.37. X-ray fluorescence spectrometry	56	2.5.12. Water: semi-micro determination	130
2.2.38. Conductivity	56	2.5.13. Aluminium in adsorbed vaccines	131
2.2.39. Molecular mass distribution in dextrans	57	2.5.14. Calcium in adsorbed vaccines	131
2.2.3. Potentiometric determination of pH	26	2.5.15. Phenol in immunosera and vaccines	131
2.2.40. Near-infrared spectrophotometry	59	2.5.16. Protein in polysaccharide vaccines	131
2.2.41. Circular dichroism	63	2.5.17. Nucleic acids in polysaccharide vaccines	132
2.2.42. Density of solids	64	2.5.18. Phosphorus in polysaccharide vaccines	132
2.2.43. Mass spectrometry	65	2.5.19. <i>O</i> -Acetyl in polysaccharide vaccines	132
2.2.44. Total organic carbon in water for pharmaceutical use	68	2.5.1. Acid value	127
2.2.45. Supercritical fluid chromatography	68	2.5.20. Hexosamines in polysaccharide vaccines	132
2.2.46. Chromatographic separation techniques	69	2.5.21. Methylpentoses in polysaccharide vaccines	133
2.2.47. Capillary electrophoresis	74	2.5.22. Uronic acids in polysaccharide vaccines	133
2.2.48. Raman spectrometry	79	2.5.23. Sialic acid in polysaccharide vaccines	133
2.2.49. Falling ball viscometer method	80	2.5.24. Carbon dioxide in gases	134
2.2.4. Relationship between reaction of solution, approximate pH and colour of certain indicators	27	2.5.25. Carbon monoxide in gases	134
2.2.54. Isoelectric focusing	81	2.5.26. Nitrogen monoxide and nitrogen dioxide in gases	135
2.2.55. Peptide mapping	82	2.5.27. Oxygen in gases	136

2.5.28. Water in gases.....	136	2.7.8. Assay of tetanus vaccine (adsorbed).....	198
2.5.29. Sulphur dioxide.....	136	2.7.9. Test for Fc function of immunoglobulin	202
2.5.2. Ester value	127	2.7. Biological assays	187
2.5.30. Oxidising substances.....	137	2.8.10. Solubility in alcohol of essential oils	216
2.5.31. Ribose in polysaccharide vaccines.....	137	2.8.11. Assay of 1,8-cineole in essential oils	216
2.5.32. Water: micro determination	137	2.8.12. Determination of essential oils in vegetable drugs.....	217
2.5.33. Total protein.....	138	2.8.13. Pesticide residues.....	218
2.5.34. Acetic acid in synthetic peptides	141	2.8.14. Determination of tannins in herbal drugs.....	221
2.5.35. Nitrous oxide in gases.....	141	2.8.15. Bitterness value.....	221
2.5.36. Anisidine value	142	2.8.16. Dry residue of extracts.....	222
2.5.3. Hydroxyl value	127	2.8.17. Loss on drying of extracts.....	222
2.5.4. Iodine value	127	2.8.1. Ash insoluble in hydrochloric acid	215
2.5.5. Peroxide value.....	128	2.8.2. Foreign matter	215
2.5.6. Saponification value	129	2.8.3. Stomata and stomatal index	215
2.5.7. Unsaponifiable matter	129	2.8.4. Swelling index.....	215
2.5.8. Determination of primary aromatic amino-nitrogen	129	2.8.5. Water in essential oils.....	216
2.5.9. Determination of nitrogen by sulphuric acid digestion	129	2.8.6. Foreign esters in essential oils	216
2.5. Assays.....	127	2.8.7. Fatty oils and resinified essential oils in essential oils.....	216
2.6.10. Histamine	153	2.8.8. Odour and taste of essential oils.....	216
2.6.11. Depressor substances.....	153	2.8.9. Residue on evaporation of essential oils.....	216
2.6.12. Microbiological examination of non-sterile products (total viable aerobic count).....	154	2.8. Methods in pharmacognosy	215
2.6.13. Microbiological examination of non-sterile products (test for specified micro-organisms).....	156	2.9.10. Ethanol content and alcoholimetric tables	237
2.6.14. Bacterial endotoxins	161	2.9.11. Test for methanol and 2-propanol	239
2.6.15. Prekallikrein activator.....	168	2.9.12. Sieve test.....	239
2.6.16. Tests for extraneous agents in viral vaccines for human use.....	169	2.9.13. Limit test of particle size by microscopy.....	239
2.6.17. Test for anticomplementary activity of immunoglobulin.....	170	2.9.14. Specific surface area by air permeability	239
2.6.18. Test for neurovirulence of live virus vaccines.....	172	2.9.15. Apparent volume	241
2.6.19. Test for neurovirulence of poliomyelitis vaccine (oral)	172	2.9.16. Flowability.....	242
2.6.1. Sterility	145	2.9.17. Test for extractable volume of parenteral preparations.....	243
2.6.20. Anti-A and anti-B haemagglutinins (indirect method)	174	2.9.18. Preparations for inhalation: aerodynamic assessment of fine particles	244
2.6.21. Nucleic acid amplification techniques	174	2.9.19. Particulate contamination: sub-visible particles..	253
2.6.22. Activated coagulation factors.....	177	2.9.1. Disintegration of tablets and capsules.....	225
2.6.24. Avian viral vaccines: tests for extraneous agents in seed lots	177	2.9.20. Particulate contamination: visible particles.....	255
2.6.25. Avian live virus vaccines: tests for extraneous agents in batches of finished product	180	2.9.22. Softening time determination of lipophilic suppositories.....	256
2.6.2. Mycobacteria	149	2.9.23. Pycnometric density of solids.....	257
2.6.7. Mycoplasmas.....	149	2.9.24. Resistance to rupture of suppositories and pessaries	258
2.6.8. Pyrogens.....	152	2.9.25. Chewing gum, medicated, drug release from	260
2.6.9. Abnormal toxicity	153	2.9.26. Specific surface area by gas adsorption.....	260
2.6. Biological tests	145	2.9.27. Uniformity of mass of delivered doses from multidose containers.....	263
2.7.10. Assay of human coagulation factor VII	203	2.9.28. Test for deliverable mass or volume of liquid and semi-solid preparations.....	263
2.7.11. Assay of human coagulation factor IX.....	204	2.9.2. Disintegration of suppositories and pessaries	227
2.7.12. Assay of heparin in coagulation factors	204	2.9.3. Dissolution test for solid dosage forms.....	228
2.7.13. Assay of human anti-D immunoglobulin.....	205	2.9.4. Dissolution test for transdermal patches	231
2.7.14. Assay of hepatitis A vaccine	207	2.9.5. Uniformity of mass of single-dose preparations.....	233
2.7.15. Assay of hepatitis B vaccine (rDNA).....	207	2.9.6. Uniformity of content of single-dose preparations..	234
2.7.16. Assay of pertussis vaccine (acellular).....	208	2.9.7. Friability of uncoated tablets.....	234
2.7.17. Assay of human antithrombin III	209	2.9.8. Resistance to crushing of tablets.....	235
2.7.18. Assay of human coagulation factor II.....	209	2.9.9. Measurement of consistency by penetrometry	235
2.7.19. Assay of human coagulation factor X.....	210	2.9. Pharmaceutical technical procedures	225
2.7.1. Immunochemical methods	187	2-Ethylhexanoic acid (2.4.28.).....	120
2.7.20. <i>In vivo</i> assay of poliomyelitis vaccine (inactivated)	210	2-Propanol and methanol, test for (2.9.11.).....	239
2.7.21. Assay of human von Willebrand factor.....	211	3.1.10. Materials based on non-plasticised poly(vinyl chloride) for containers for non-injectable, aqueous solutions	289
2.7.22. Assay of human coagulation factor XI.....	212	3.1.11. Materials based on non-plasticised poly(vinyl chloride) for containers for dry dosage forms for oral administration	291
2.7.2. Microbiological assay of antibiotics.....	188	3.1.1.1. Materials based on plasticised poly(vinyl chloride) for containers for human blood and blood components.....	269
2.7.4. Assay of human coagulation factor VIII.....	194	3.1.1.2. Materials based on plasticised poly(vinyl chloride) for tubing used in sets for the transfusion of blood and blood components	272
2.7.5. Assay of heparin.....	195		
2.7.6. Assay of diphtheria vaccine (adsorbed)	196		
2.7.7. Assay of pertussis vaccine.....	197		

3.1.13. Plastic additives	293	5.2.7. Evaluation of efficacy of veterinary vaccines	462
3.1.14. Materials based on plasticised poly(vinyl chloride) for containers for aqueous solutions for intravenous infusion	296	5.2.8. Minimising the risk of transmitting animal spongiform encephalopathy agents via human and veterinary medicinal products	463
3.1.15. Polyethylene terephthalate for containers for preparations not for parenteral use	298	5.2. General texts on vaccines	453
3.1.1. Materials for containers for human blood and blood components	269	5.3. Statistical analysis of results of biological assays and tests	475
3.1.3. Polyolefines	274	5.4. Residual solvents	507
3.1.4. Polyethylene without additives for containers for parenteral preparations and for ophthalmic preparations	278	5.5. Alcoholimetric tables	519
3.1.5. Polyethylene with additives for containers for parenteral preparations and for ophthalmic preparations	279	5.6. Assay of interferons	533
3.1.6. Polypropylene for containers and closures for parenteral preparations and ophthalmic preparations	282	5.7. Table of physical characteristics of radionuclides mentioned in the European Pharmacopoeia	539
3.1.7. Poly(ethylene - vinyl acetate) for containers and tubing for total parenteral nutrition preparations	285	5.8. Pharmacopoeial harmonisation	551
3.1.8. Silicone oil used as a lubricant	287	5.9. Polymorphism	555
3.1.9. Silicone elastomer for closures and tubing	288		
3.1. Materials used for the manufacture of containers	269	A	
3.2.1. Glass containers for pharmaceutical use	303	Abbreviations and symbols (1.5.)	9
3.2.2.1. Plastic containers for aqueous solutions for parenteral infusion	309	Abnormal toxicity (2.6.9.)	153
3.2.2. Plastic containers and closures for pharmaceutical use	308	<i>Absinthii herba</i>	2710
3.2.3. Sterile plastic containers for human blood and blood components	309	Absorption spectrophotometry, infrared (2.2.24.)	37
3.2.4. Empty sterile containers of plasticised poly(vinyl chloride) for human blood and blood components	311	Absorption spectrophotometry, ultraviolet and visible (2.2.25.)	38
3.2.5. Sterile containers of plasticised poly(vinyl chloride) for human blood containing anticoagulant solution	312	Acacia	905
3.2.6. Sets for the transfusion of blood and blood components	313	<i>Acaciae gummi</i>	905
3.2.8. Sterile single-use plastic syringes	314	<i>Acaciae gummi dispersione desiccatum</i>	905
3.2.9. Rubber closures for containers for aqueous parenteral preparations, for powders and for freeze-dried powders	316	Acacia, spray-dried	905
3.2. Containers	303	Acamprosate calcium	906
4.1.1. Reagents	321	<i>Acamprosatum calcicum</i>	906
4.1.2. Standard solutions for limit tests	426	Acebutolol hydrochloride	907
4.1.3. Buffer solutions	430	<i>Acebutololi hydrochloridum</i>	907
4.1. Reagents, standard solutions, buffer solutions	321	Aceclofenac	909
4.2.1. Primary standards for volumetric solutions	435	<i>Aceclofenacum</i>	909
4.2.2. Volumetric solutions	435	Acesulfame potassium	911
4.2. Volumetric analysis	435	<i>Acesulfamum kalicum</i>	911
4-Aminobenzoic acid	973	Acetazolamide	912
4. Reagents	321	<i>Acetazolamidum</i>	912
5.10. Control of impurities in substances for pharmaceutical use	559	Acetic acid, glacial	913
5.11. Characters section in monographs	565	Acetic acid in synthetic peptides (2.5.34.)	141
5.1.1. Methods of preparation of sterile products	445	Acetone	913
5.1.2. Biological indicators of sterilisation	447	<i>Acetonum</i>	913
5.1.3. Efficacy of antimicrobial preservation	447	Acetylcholine chloride	914
5.1.4. Microbiological quality of pharmaceutical preparations	449	<i>Acetylcholini chloridum</i>	914
5.1.5. Application of the F_0 concept to steam sterilisation of aqueous preparations	449	Acetylcysteine	915
5.1. General texts on sterility	445	<i>Acetylcysteinum</i>	915
5.2.1. Terminology used in monographs on vaccines	453	Acetylsalicylic acid	917
5.2.2. Chicken flocks free from specified pathogens for the production and quality control of vaccines	453	Acetyltryptophan, <i>N</i> -	918
5.2.3. Cell substrates for the production of vaccines for human use	455	Acetyltyrosine, <i>N</i> -	920
5.2.4. Cell cultures for the production of veterinary vaccines	458	Aciclovir	921
5.2.5. Substances of animal origin for the production of veterinary vaccines	460	<i>Aciclovirum</i>	921
5.2.6. Evaluation of safety of veterinary vaccines	461	<i>Acidum 4-aminobenzoicum</i>	973
		<i>Acidum aceticum glaciale</i>	913
		<i>Acidum acetylsalicylicum</i>	917
		<i>Acidum adipicum</i>	926
		<i>Acidum alginicum</i>	942
		<i>Acidum amidotrizoicum dihydricum</i>	967
		<i>Acidum aminocaproicum</i>	974
		<i>Acidum ascorbicum</i>	1025
		<i>Acidum asparticum</i>	1029
		<i>Acidum benzoicum</i>	1072
		<i>Acidum boricum</i>	1117
		<i>Acidum caprylicum</i>	1172
		<i>Acidum chenodeoxycholicum</i>	1247
		<i>Acidum citricum anhydricum</i>	1306
		<i>Acidum citricum monohydricum</i>	1307
		<i>Acidum edeticum</i>	1494
		<i>Acidum etacrynicum</i>	1542
		<i>Acidum folicum</i>	1630
		<i>Acidum fusidicum</i>	1645
		<i>Acidum glutamicum</i>	1670

<i>Acidum hydrochloridum concentratum</i>	1755	Air, medicinal.....	929
<i>Acidum hydrochloridum dilutum</i>	1756	Air, synthetic medicinal.....	932
<i>Acidum iopanoicum</i>	1824	Alanine.....	933
<i>Acidum iotalamicum</i>	1825	<i>Alaninum</i>	933
<i>Acidum ioxaglicum</i>	1826	Albendazole.....	934
<i>Acidum lacticum</i>	1882	<i>Albendazolum</i>	934
<i>Acidum lactobionicum</i>	1885	<i>Albumini humani solutio</i>	1731
<i>Acidum maleicum</i>	1966	Albumin solution, human.....	1731
<i>Acidum malicum</i>	1966	Alchemilla.....	935
<i>Acidum mefenamicum</i>	1984	<i>Alchemillae herba</i>	935
<i>Acidum methacrylicum et ethylis acrylas polymerisatum</i> 1:1.....	2005	<i>Alcohol benzylicus</i>	1075
<i>Acidum methacrylicum et ethylis acrylas polymerisatum</i> 1:1 dispersio 30 per centum.....	2005	<i>Alcohol cetylicus</i>	1243
<i>Acidum methacrylicum et methylis methacrylas</i> <i>polymerisatum</i> 1:1.....	2006	<i>Alcohol cetylicus et stearylicus</i>	1239
<i>Acidum methacrylicum et methylis methacrylas</i> <i>polymerisatum</i> 1:2.....	2007	<i>Alcohol cetylicus et stearylicus emulsificans A</i>	1239
<i>Acidum nalidixicum</i>	2080	<i>Alcohol cetylicus et stearylicus emulsificans B</i>	1241
<i>Acidum nicotinicum</i>	2097	<i>Alcoholes adipis lanae</i>	2703
<i>Acidum nitricum</i>	2105	Alcoholimetric tables (2.9.10.).....	237
<i>Acidum oleicum</i>	2132	Alcoholimetric tables (5.5.).....	519
<i>Acidum oxolinicum</i>	2165	<i>Alcohol isopropylicus</i>	1841
<i>Acidum palmiticum</i>	2179	<i>Alcohol oleicus</i>	2134
<i>Acidum phosphoricum concentratum</i>	2237	<i>Alcohol stearylicus</i>	2492
<i>Acidum phosphoricum dilutum</i>	2238	<i>Alcuronii chloridum</i>	935
<i>Acidum pipemidicum trihydricum</i>	2249	Alcuronium chloride.....	935
<i>Acidum salicylicum</i>	2395	Alexandrian senna pods.....	2404
<i>Acidum (S)-lacticum</i>	1883	Alfacalcidol.....	937
<i>Acidum sorbicum</i>	2467	<i>Alfacalcidolum</i>	937
<i>Acidum stearicum</i>	2490	Alfadex.....	938
<i>Acidum sulfuricum</i>	2520	<i>Alfadexum</i>	938
<i>Acidum tartaricum</i>	2534	Alfentanil hydrochloride.....	939
<i>Acidum tiaprofenicum</i>	2578	<i>Alfentanili hydrochloridum</i>	939
<i>Acidum tolfenamicum</i>	2601	Alfuzosin hydrochloride.....	941
<i>Acidum tranexamicum</i>	2609	<i>Alfuzosini hydrochloridum</i>	941
<i>Acidum trichloraceticum</i>	2620	Alginic acid.....	942
<i>Acidum undecylenicum</i>	2658	Alkaline-earth metals and magnesium (2.4.7.).....	104
<i>Acidum ursodeoxycholicum</i>	2662	Alkaline impurities in fatty oils (2.4.19.).....	109
<i>Acidum valproicum</i>	2669	Allantoin.....	942
Acid value (2.5.1.).....	127	<i>Allantoinum</i>	942
Acitretin.....	922	Allergen products.....	569
<i>Acitretinum</i>	922	<i>Allii sativi bulbi pulvis</i>	1651
<i>Acriflavinii monochloridum</i>	924	<i>Allium sativum ad praeparationes homoeopathicas</i>	897
Acriflavinium monochloride.....	924	Allopurinol.....	943
Actinobacillosis vaccine (inactivated), porcine.....	784	<i>Allopurinolum</i>	943
Activated charcoal.....	1246	all- <i>rac</i> - α -Tocopherol.....	2590
Activated coagulation factors (2.6.22.).....	177	all- <i>rac</i> - α -Tocopheryl acetate.....	2594
Additives, plastic (3.1.13.).....	293	Almagate.....	945
Adenine.....	924	<i>Almagatum</i>	945
<i>Adeninum</i>	924	Almond oil, refined.....	946
Adenosine.....	925	Almond oil, virgin.....	947
<i>Adenosinum</i>	925	<i>Aloe barbadensis</i>	947
<i>Adeps lanae</i>	2704	<i>Aloe capensis</i>	948
<i>Adeps lanae cum aqua</i>	2709	Aloes, barbados.....	947
<i>Adeps lanae hydrogenatus</i>	2708	Aloes, Cape.....	948
<i>Adeps solidus</i>	1711	Aloes dry extract, standardised.....	949
Adipic acid.....	926	<i>Aloes extractum siccum normatum</i>	949
Adrenaline tartrate.....	927	Alphacyclodextrin.....	938
<i>Adrenalini tartras</i>	927	Alprazolam.....	950
<i>Aer medicinalis</i>	929	<i>Alprazolamum</i>	950
<i>Aer medicinalis artificiosus</i>	932	Alprenolol hydrochloride.....	952
Aerodynamic assessment of fine particles in preparations for inhalation (2.9.18.).....	244	<i>Alprenololi hydrochloridum</i>	952
<i>Aether</i>	1548	Alprostadil.....	953
<i>Aether anaestheticus</i>	1549	<i>Alprostadilum</i>	953
Agar.....	928	Alteplase for injection.....	956
<i>Agrimoniae herba</i>	929	<i>Alteplasmum ad iniectabile</i>	956
Agrimony.....	929	<i>Althaeae folium</i>	1974
		<i>Althaeae radix</i>	1975
		Alum.....	959
		<i>Alumen</i>	959
		<i>Aluminii chloridum hexahydricum</i>	960
		<i>Aluminii hydroxidum hydricum ad adsorptionem</i>	960

<i>Aluminii magnesii silicas</i>	961	<i>Ampicillinum natricum</i>	998
<i>Aluminii oxidum hydricum</i>	962	<i>Ampicillinum trihydricum</i>	1001
<i>Aluminii phosphas hydricus</i>	963	<i>Amygdalae oleum raffinatum</i>	946
<i>Aluminii sulfas</i>	964	<i>Amygdalae oleum virginale</i>	947
Aluminium (2.4.17.)	108	<i>Amylum pregelificatum</i>	2490
Aluminium chloride hexahydrate	960	Anaesthetic ether	1549
Aluminium hydroxide, hydrated, for adsorption	960	Analysis, thermal (2.2.34.)	52
Aluminium in adsorbed vaccines (2.5.13.)	131	<i>Angelicae radix</i>	1003
Aluminium magnesium silicate	961	Angelica root	1003
Aluminium oxide, hydrated	962	Animal anti-T lymphocyte immunoglobulin for human use	1010
Aluminium phosphate, hydrated	963	Animal spongiform encephalopathies, products with risk of transmitting agents of	577
Aluminium sulphate	964	Animal spongiform encephalopathy agents, minimising the risk of transmitting via human and veterinary medicinal products (5.2.8.)	463
Amantadini hydrochloridum	964	Aniseed	1006
Ambroxol hydrochloride	965	Anise oil	1004
<i>Ambroxoli hydrochloridum</i>	965	<i>Anisi aetheroleum</i>	1004
Amfetamine sulphate	966	Anisidine value (2.5.36.)	142
<i>Amfetamini sulfas</i>	966	<i>Anisi fructus</i>	1006
Amidotrizoic acid dihydrate	967	<i>Anisi stellati aetheroleum</i>	2488
Amikacin	968	<i>Anisi stellati fructus</i>	2487
<i>Amikacini sulfas</i>	970	Antazoline hydrochloride	1006
Amikacin sulphate	970	<i>Antazolini hydrochloridum</i>	1006
<i>Amikacinum</i>	968	Anthrax spore vaccine (live) for veterinary use	715
Amiloride hydrochloride	972	Anti-A and anti-B haemagglutinins (indirect method) (2.6.20.)	174
<i>Amiloridi hydrochloridum</i>	972	Antibiotics, microbiological assay of (2.7.2.)	188
Amino acid analysis (2.2.56.)	86	Anticoagulant and preservative solutions for human blood	1007
Aminocaproic acid	974	Anticomplementary activity of immunoglobulin (2.6.17.) ..	170
Aminogluthethimide	975	Anti-D immunoglobulin, human	1732
<i>Aminogluthethimidum</i>	975	Anti-D immunoglobulin, human, assay of (2.7.13.)	205
Amiodarone hydrochloride	977	Anti-D immunoglobulin, human, for intravenous administration	1733
<i>Amiodaroni hydrochloridum</i>	977	Antimicrobial preservation, efficacy of (5.1.3.)	447
Amisulpride	978	Antiserum, European viper venom	806
<i>Amisulpridum</i>	978	Antithrombin III concentrate, human	1733
Amitriptyline hydrochloride	980	Antithrombin III, human, assay of (2.7.17.)	209
<i>Amitriptylini hydrochloridum</i>	980	<i>Antithrombinum III humanum densatum</i>	1733
Amlodipine besilate	981	Anti-T lymphocyte immunoglobulin for human use, animal	1010
<i>Amlodipini besilas</i>	981	<i>Apis mellifera ad praeparationes homoeopathicas</i>	898
Ammonia (¹³ N) injection	817	Apomorphine hydrochloride	1014
<i>Ammoniae (¹³N) solutio iniectionabilis</i>	817	<i>Apomorphini hydrochloridum</i>	1014
<i>Ammoniae solutio concentrata</i>	983	Apparatus (2.1.)	17
Ammonia solution, concentrated	983	Apparent volume (2.9.15.)	241
<i>Ammonii bromidum</i>	985	Application of the <i>F</i> ₀ concept to steam sterilisation of aqueous preparations (5.1.5.)	449
<i>Ammonii chloridum</i>	986	Aprotinin	1015
<i>Ammonii glycyrrhizas</i>	987	Aprotinin concentrated solution	1016
<i>Ammonii hydrogenocarbonas</i>	988	<i>Aprotinini solutio concentrata</i>	1016
Ammonio methacrylate copolymer (type A)	983	<i>Aprotininum</i>	1015
Ammonio methacrylate copolymer (type B)	984	<i>Aqua ad dilutionem solutionum concentratarum ad haemodialysim</i>	1699
<i>Ammonio methacrylatis copolymerum A</i>	983	<i>Aqua ad iniectionabilia</i>	2692
<i>Ammonio methacrylatis copolymerum B</i>	984	<i>Aquae (¹⁵O) solutio iniectionabilis</i>	868
Ammonium (2.4.1.)	103	<i>Aquae trititatae (³H) solutio iniectionabilis</i>	867
Ammonium bromide	985	<i>Aqua purificata</i>	2697
Ammonium chloride	986	<i>Aqua valde purificata</i>	2695
Ammonium glycyrrhizate	987	<i>Arachidis oleum hydrogenatum</i>	1018
Ammonium hydrogen carbonate	988	<i>Arachidis oleum raffinatum</i>	1018
Amobarbital	988	Arachis oil, hydrogenated	1018
Amobarbital sodium	989	Arachis oil, refined	1018
<i>Amobarbitalum</i>	988	<i>Argenti nitras</i>	2412
<i>Amobarbitalum natricum</i>	989	Arginine	1019
Amoxicillin sodium	990	Arginine aspartate	1020
Amoxicillin trihydrate	992	Arginine hydrochloride	1021
<i>Amoxicillinum natricum</i>	990		
<i>Amoxicillinum trihydricum</i>	992		
Amperometric titration (2.2.19.)	34		
Amphotericin B	995		
<i>Amphotericinum B</i>	995		
Ampicillin, anhydrous	996		
Ampicillin sodium	998		
Ampicillin trihydrate	1001		
<i>Ampicillinum anhydricum</i>	996		

<i>Arginini aspartas</i>	1020	Avian viral vaccines: tests for extraneous agents in seed lots (2.6.24.)	177
<i>Arginini hydrochloridum</i>	1021	Azaperone for veterinary use	1036
<i>Argininum</i>	1019	<i>Azaperonum ad usum veterinarium</i>	1036
<i>Arnicae flos</i>	1022	Azathioprine	1037
Arnica flower	1022	<i>Azathioprinum</i>	1037
Arsenic (2.4.2.)	103	Azelastine hydrochloride	1037
<i>Arsenii trioxidum ad praeparationes homoeopathicas</i> ..	895	<i>Azelastini hydrochloridum</i>	1037
Arsenious trioxide for homoeopathic preparations	895	Azithromycin	1039
Articaine hydrochloride	1023	<i>Azithromycinum</i>	1039
<i>Articaini hydrochloridum</i>	1023		
Ascorbic acid	1025	B	
<i>Ascorbylis palmitas</i>	1026	Bacampicillin hydrochloride	1043
Ascorbyl palmitate	1026	<i>Bacampicillini hydrochloridum</i>	1043
Ash insoluble in hydrochloric acid (2.8.1.)	215	Bacitracin	1045
Ash leaf	1026	<i>Bacitracinum</i>	1045
Asparagine monohydrate	1027	<i>Bacitracinum zincum</i>	1047
<i>Asparaginum monohydricum</i>	1027	Bacitracin zinc	1047
Aspartame	1028	Baclofen	1050
<i>Aspartamum</i>	1028	<i>Baclofenum</i>	1050
Aspartic acid	1029	Bacterial endotoxins (2.6.14.)	161
Assay of 1,8-cineole in essential oils (2.8.11.)	216	<i>Ballotae nigrae herba</i>	1113
Assay of diphtheria vaccine (adsorbed) (2.7.6.)	196	<i>Balsamum peruvianum</i>	2215
Assay of heparin (2.7.5.)	195	<i>Balsamum toltutanum</i>	2603
Assay of heparin in coagulation factors (2.7.12.)	204	Bambuterol hydrochloride	1051
Assay of hepatitis A vaccine (2.7.14.)	207	<i>Bambuteroli hydrochloridum</i>	1051
Assay of hepatitis B vaccine (rDNA) (2.7.15.)	207	Barbados aloes	947
Assay of human anti-D immunoglobulin (2.7.13.)	205	Barbital	1052
Assay of human antithrombin III (2.7.17.)	209	<i>Barbitalum</i>	1052
Assay of human coagulation factor II (2.7.18.)	209	<i>Barii sulfas</i>	1053
Assay of human coagulation factor IX (2.7.11.)	204	Barium sulphate	1053
Assay of human coagulation factor VII (2.7.10.)	203	Basic butylated methacrylate copolymer	1053
Assay of human coagulation factor VIII (2.7.4.)	194	<i>BCG ad immunocurationem</i>	635
Assay of human coagulation factor X (2.7.19.)	210	BCG for immunotherapy	635
Assay of human coagulation factor XI (2.7.22.)	212	BCG vaccine, freeze-dried	636
Assay of human von Willebrand factor (2.7.21.)	211	Bearberry leaf	1054
Assay of interferons (5.6.)	533	Beclometasone dipropionate	1055
Assay of pertussis vaccine (2.7.7.)	197	<i>Beclometasoni dipropionas</i>	1055
Assay of pertussis vaccine (acellular) (2.7.16.)	208	Bee for homoeopathic preparations, honey	898
Assay of tetanus vaccine (adsorbed) (2.7.8.)	198	Beeswax, white	1057
Assays (2.5.)	127	Beeswax, yellow	1058
Astemizole	1030	<i>Belladonnae folii extractum siccum normatum</i>	1060
<i>Astemizolum</i>	1030	<i>Belladonnae folii tinctura normata</i>	1061
Atenolol	1032	<i>Belladonnae folium</i>	1058
<i>Atenololum</i>	1032	<i>Belladonnae pulvis normatus</i>	1062
Atomic absorption spectrometry (2.2.23.)	36	Belladonna leaf	1058
Atomic emission spectrometry (2.2.22.)	35	Belladonna leaf dry extract, standardised	1060
Atropine	1033	Belladonna leaf tincture, standardised	1061
Atropine sulphate	1035	Belladonna, prepared	1062
<i>Atropini sulfas</i>	1035	Bendroflumethiazide	1063
<i>Atropinum</i>	1033	<i>Bendroflumethiazidum</i>	1063
Aujeszky's disease vaccine (inactivated) for pigs	715	Benfluorex hydrochloride	1064
Aujeszky's disease vaccine (live) for pigs for parenteral administration, freeze-dried	717	<i>Benfluorexi hydrochloridum</i>	1064
<i>Aurantii amari epicarpium et mesocarpium tinctura</i>	1110	Benperidol	1065
<i>Aurantii amari epicarpium et mesocarpium</i>	1110	<i>Benperidolum</i>	1065
<i>Aurantii amari floris aetheroleum</i>	1112	Benserazide hydrochloride	1067
<i>Aurantii amari flos</i>	1111	<i>Benserazidi hydrochloridum</i>	1067
<i>Aurantii dulcis aetheroleum</i>	2526	Bentonite	1068
<i>Auricularia</i>	601	<i>Bentonitum</i>	1068
Avian infectious bronchitis vaccine (inactivated)	718	<i>Benzalkonii chloridi solutio</i>	1069
Avian infectious bronchitis vaccine (live)	720	<i>Benzalkonii chloridum</i>	1068
Avian infectious bursal disease vaccine (inactivated)	722	Benzalkonium chloride	1068
Avian infectious bursal disease vaccine (live)	723	Benzalkonium chloride solution	1069
Avian infectious encephalomyelitis vaccine (live)	725	Benzathine benzylpenicillin	1077
Avian infectious laryngotracheitis vaccine (live)	727	Benzbromarone	1070
Avian live virus vaccines: tests for extraneous agents in batches of finished product (2.6.25.)	180	<i>Benzbromaronum</i>	1070
Avian paramyxovirus 3 vaccine (inactivated)	728	<i>Benzethonii chloridum</i>	1071
Avian viral tenosynovitis vaccine (live)	729	Benzethonium chloride	1071
		Benzocaine	1072

<i>Benzocainum</i>	1072	Black horehound	1113
Benzoic acid	1072	<i>Bleomycini sulfas</i>	1114
<i>Benzoylis peroxidum cum aqua</i>	1073	Bleomycin sulphate	1114
Benzoyl peroxide, hydrous	1073	Blood and blood components, empty sterile containers of plasticised poly(vinyl chloride) for (3.2.4.)	311
Benzyl alcohol	1075	Blood and blood components, materials for containers for (3.1.1.)	269
Benzyl benzoate	1076	Blood and blood components, sets for the transfusion of (3.2.6.)	313
<i>Benzylis benzoas</i>	1076	Blood and blood components, sterile plastic containers for (3.2.3.)	309
Benzylpenicillin, benzathine	1077	Blood, anticoagulant and preservative solutions for	1007
Benzylpenicillin potassium	1078	Blood, sterile containers of plasticised poly (vinyl chloride) containing anticoagulant solution (3.2.5.)	312
Benzylpenicillin, procaine	1080	Bogbean leaf	1115
Benzylpenicillin sodium	1082	Boiling point (2.2.12.)	31
<i>Benzylpenicillinum benzathinum</i>	1077	<i>Boldi folium</i>	1115
<i>Benzylpenicillinum kalicum</i>	1078	Boldo leaf	1115
<i>Benzylpenicillinum natricum</i>	1082	<i>Borax</i>	1116
<i>Benzylpenicillinum procainum</i>	1080	Boric acid	1117
Betacarotene	1083	Botulinum antitoxin	801
<i>Betacarotenum</i>	1083	Botulinum toxin type A for injection	1117
Betacyclodextrin	1084	Bovine infectious rhinotracheitis vaccine (live), freeze-dried	768
Betadex	1084	Bovine insulin	1797
<i>Betadexum</i>	1084	Bovine leptospirosis vaccine (inactivated)	730
Betahistine mesilate	1086	Bovine parainfluenza virus vaccine (live), freeze-dried	732
<i>Betahistini mesilas</i>	1086	Bovine respiratory syncytial virus vaccine (live), freeze-dried	733
Betamethasone	1087	Bovine viral diarrhoea vaccine (inactivated)	734
Betamethasone acetate	1089	Bromazepam	1119
Betamethasone dipropionate	1090	<i>Bromazepamum</i>	1119
Betamethasone sodium phosphate	1092	Bromhexine hydrochloride	1120
Betamethasone valerate	1094	<i>Bromhexini hydrochloridum</i>	1120
<i>Betamethasoni acetas</i>	1089	Bromocriptine mesilate	1121
<i>Betamethasoni dipropionas</i>	1090	<i>Bromocriptini mesilas</i>	1121
<i>Betamethasoni natrii phosphas</i>	1092	Bromperidol	1124
<i>Betamethasoni valeras</i>	1094	Bromperidol decanoate	1125
<i>Betamethasonum</i>	1087	<i>Bromperidoli decanoas</i>	1125
Betaxolol hydrochloride	1095	<i>Bromperidolum</i>	1124
<i>Betaxololi hydrochloridum</i>	1095	Brompheniramine maleate	1127
<i>Betulae folium</i>	1103	<i>Brompheniramini maleas</i>	1127
Bezafibrate	1096	Brucellosis vaccine (live) (<i>Brucella melitensis</i> Rev. 1 strain), freeze-dried, for veterinary use	735
<i>Bezafibratum</i>	1096	Buccal tablets and sublingual tablets	613
Bifonazole	1098	Budesonide	1128
<i>Bifonazolum</i>	1098	<i>Budesonidum</i>	1128
Bilberry fruit, dried	1099	Bufexamac	1130
Bilberry fruit, fresh	1099	<i>Bufexamacum</i>	1130
Biological assays (2.7.)	187	Buffer solutions (4.1.3.)	430
Biological assays and tests, statistical analysis of (5.3.)	475	Buflomedil hydrochloride	1131
Biological indicators of sterilisation (5.1.2.)	447	<i>Buflomedili hydrochloridum</i>	1131
Biological tests (2.6.)	145	Bumetanide	1132
Biotin	1100	<i>Bumetanidum</i>	1132
<i>Biotinum</i>	1100	Bupivacaine hydrochloride	1133
Biperiden hydrochloride	1101	<i>Bupivacaini hydrochloridum</i>	1133
<i>Biperideni hydrochloridum</i>	1101	Buprenorphine	1135
Biphasic insulin injection	1802	Buprenorphine hydrochloride	1136
Biphasic isophane insulin injection	1803	<i>Buprenorphini hydrochloridum</i>	1136
Birch leaf	1103	<i>Buprenorphinum</i>	1135
Bisacodyl	1104	Buserelin	1137
<i>Bisacodylum</i>	1104	<i>Buserelinum</i>	1137
<i>Bismuthi subcarbonas</i>	1105	Busulfan	1138
<i>Bismuthi subgallas</i>	1106	<i>Busulfanum</i>	1138
<i>Bismuthi subnitras ponderosum</i>	1107	Butcher's broom	1138
<i>Bismuthi subsalicylas</i>	1107	Butylhydroxyanisole	1141
Bismuth subcarbonate	1105	<i>Butylhydroxyanisolum</i>	1141
Bismuth subgallate	1106	Butylhydroxytoluene	1141
Bismuth subnitrate, heavy	1107	<i>Butylhydroxytoluenum</i>	1141
Bismuth subsalicylate	1107		
Bitter fennel	1580		
Bitter-fennel fruit oil	1108		
Bitterness value (2.8.15.)	221		
Bitter-orange epicarp and mesocarp	1110		
Bitter-orange-epicarp and mesocarp tincture	1110		
Bitter-orange flower	1111		
Bitter-orange-flower oil	1112		

<i>Butylis parahydroxybenzoas</i>	1140	Canine leptospirosis vaccine (inactivated).....	740
Butyl parahydroxybenzoate.....	1140	Canine parainfluenza virus vaccine (live).....	742
C		Canine parvovirus vaccine (inactivated).....	743
Cachets.....	601	Canine parvovirus vaccine (live)	744
Caffeine	1145	Cape aloes.....	948
Caffeine monohydrate.....	1146	Capillary electrophoresis (2.2.47.).....	74
Calcifediol.....	1147	Capillary viscometer method (2.2.9.).....	29
<i>Calcifediolum</i>	1147	Caprylic acid.....	1172
<i>Calcii ascorbas</i>	1150	Caprylocaproyl macrogolglycerides.....	1173
<i>Calcii carbonas</i>	1151	<i>Capsici fructus</i>	1174
<i>Calcii chloridum dihydricum</i>	1152	Capsicum.....	1174
<i>Calcii chloridum hexahydricum</i>	1153	<i>Capsulae</i>	599
<i>Calcii dobesilas monohydricum</i>	1153	Capsules.....	599
<i>Calcii folinas</i>	1154	Capsules and tablets, disintegration of (2.9.1.)	225
<i>Calcii glucoheptonas</i>	1156	Capsules, gastro-resistant.....	600
<i>Calcii gluconas</i>	1157	Capsules, hard	600
<i>Calcii gluconas ad iniectionabile</i>	1158	Capsules, modified-release	600
<i>Calcii glycerophosphas</i>	1159	Capsules, oromucosal	613
<i>Calcii hydrogenophosphas anhydricus</i>	1160	Capsules, rectal.....	623
<i>Calcii hydrogenophosphas dihydricus</i>	1160	Capsules, soft.....	600
<i>Calcii hydroxidum</i>	1161	Capsules, vaginal.....	629
<i>Calcii lactas pentahydricus</i>	1162	Captopril	1176
<i>Calcii lactas trihydricus</i>	1162	<i>Captoprilum</i>	1176
<i>Calcii laevulinas dihydricum</i>	1165	Caraway fruit.....	1177
<i>Calcii levofolinas pentahydricus</i>	1163	Carbachol.....	1177
<i>Calcii pantothenas</i>	1166	<i>Carbacholum</i>	1177
<i>Calcii stearas</i>	1167	Carbamazepine	1178
<i>Calcii sulfas dihydricus</i>	1169	<i>Carbamazepinum</i>	1178
Calcitonin (salmon).....	1148	Carbasalate calcium.....	1179
<i>Calcitoninum salmonis</i>	1148	<i>Carbasalatum calcicum</i>	1179
Calcitriol.....	1149	Carbidopa	1180
<i>Calcitriolum</i>	1149	<i>Carbidopum</i>	1180
Calcium (2.4.3.).....	103	Carbimazole	1182
Calcium ascorbate.....	1150	<i>Carbimazolum</i>	1182
Calcium carbonate.....	1151	<i>Carbo activatus</i>	1246
Calcium carboxymethylcellulose	1188	Carbocysteine	1182
Calcium chloride dihydrate.....	1152	<i>Carbocisteinum</i>	1182
Calcium chloride hexahydrate	1153	<i>Carbomera</i>	1183
Calcium dobesilate monohydrate	1153	Carbomers	1183
Calcium folinate	1154	Carbon dioxide	1185
Calcium glucoheptonate.....	1156	Carbon dioxide in gases (2.5.24.)	134
Calcium gluconate.....	1157	<i>Carbonei dioxidum</i>	1185
Calcium gluconate for injection.....	1158	<i>Carbonei monoxidum (¹⁵O)</i>	818
Calcium glycerophosphate.....	1159	Carbon monoxide (¹⁵ O).....	818
Calcium hydrogen phosphate, anhydrous.....	1160	Carbon monoxide in gases (2.5.25.).....	134
Calcium hydrogen phosphate dihydrate.....	1160	Carboplatin.....	1186
Calcium hydroxide	1161	<i>Carboplatinum</i>	1186
Calcium in adsorbed vaccines (2.5.14.)	131	<i>Carboxymethylamylum natricum A</i>	2450
Calcium lactate pentahydrate	1162	<i>Carboxymethylamylum natricum B</i>	2451
Calcium lactate trihydrate.....	1162	<i>Carboxymethylamylum natricum C</i>	2451
Calcium levofolate pentahydrate	1163	Carboxymethylcellulose calcium	1188
Calcium levulinate dihydrate.....	1165	Carboxymethylcellulose sodium	1189
Calcium pantothenate.....	1166	Carboxymethylcellulose sodium, cross-linked.....	1371
Calcium phosphate.....	1167	Carboxymethylcellulose sodium, low-substituted.....	1190
Calcium stearate.....	1167	Carisoprodol.....	1187
Calcium sulphate dihydrate.....	1169	<i>Carisoprodolum</i>	1187
<i>Calendulae flos</i>	1169	Carmellose calcium.....	1188
Calendula flower	1169	Carmellose sodium	1189
Calf coronavirus diarrhoea vaccine (inactivated).....	736	Carmellose sodium, low-substituted	1190
Calf rotavirus diarrhoea vaccine (inactivated).....	737	<i>Carmellosum calcicum</i>	1188
Calicivirus vaccine (inactivated), feline.....	757	<i>Carmellosum natricum</i>	1189
Calicivirus vaccine (live), feline, freeze-dried.....	758	<i>Carmellosum natricum conexum</i>	1371
<i>Camphora racemica</i>	1172	<i>Carmellosum natricum, substitutum humile</i>	1190
Camphor, D-.....	1170	Carmustine	1191
Camphor, racemic.....	1172	<i>Carmustinum</i>	1191
Canine adenovirus vaccine (inactivated)	738	Carnauba wax	1191
Canine adenovirus vaccine (live).....	738	Carteolol hydrochloride.....	1192
Canine distemper vaccine (live), freeze-dried.....	740	<i>Carteololi hydrochloridum</i>	1192
		<i>Carthami oleum raffinatum</i>	2389

Carvedilol.....	1193	<i>Cera flava</i>	1058
<i>Carvedilolum</i>	1193	Cetirizine dihydrochloride.....	1237
<i>Carvi fructus</i>	1177	<i>Cetirizini dihydrochloridum</i>	1237
<i>Caryophylli floris aetheroleum</i>	1335	<i>Cetobemidoni hydrochloridum</i>	1871
<i>Caryophylli flos</i>	1334	Cetostearyl alcohol.....	1239
Cascara.....	1194	Cetostearyl alcohol (type A), emulsifying.....	1239
Cassia oil.....	1196	Cetostearyl alcohol (type B), emulsifying.....	1241
Castor oil, hydrogenated.....	1197	<i>Cetostearyl isononanoas</i>	1242
Castor oil, polyoxyl.....	1949	Cetostearyl isononanoate.....	1242
Castor oil, polyoxyl hydrogenated.....	1948	Cetrimide.....	1243
Castor oil, virgin.....	1197	<i>Cetrimidum</i>	1243
Catgut, sterile.....	873	Cetyl alcohol.....	1243
Catgut, sterile, in distributor for veterinary use.....	885	<i>Cetyl palmitas</i>	1244
Cefaclor.....	1198	Cetyl palmitate.....	1244
<i>Cefaclorum</i>	1198	<i>Cetylpyridinii chloridum</i>	1244
Cefadroxil monohydrate.....	1200	Cetylpyridinium chloride.....	1244
<i>Cefadroxilum monohydricum</i>	1200	Ceylon cinnamon bark oil.....	1295
Cefalexin monohydrate.....	1202	Ceylon cinnamon leaf oil.....	1296
<i>Cefalexinum monohydricum</i>	1202	Chamomile flower, Roman.....	1245
Cefalotin sodium.....	1203	<i>Chamomillae romanae flos</i>	1245
<i>Cefalotinum natricum</i>	1203	Characters section in monographs (5.11.).....	565
Cefamandole nafate.....	1204	Charcoal, activated.....	1246
<i>Cefamandoli nafas</i>	1204	<i>Chelidonii herba</i>	1690
Cefapirin sodium.....	1206	Chenodeoxycholic acid.....	1247
<i>Cefapirinum natricum</i>	1206	Chewing gum, medicated, drug release from (2.9.25.).....	260
Cefatrizine propylene glycol.....	1207	Chewing gums, medicated.....	601
<i>Cefatrizinum propylen glycolum</i>	1207	Chicken flocks free from specified pathogens for the production and quality control of vaccines (5.2.2.).....	453
Cefazolin sodium.....	1209	Chicken infectious anaemia vaccine (live).....	769
<i>Cefazolinum natricum</i>	1209	<i>Chinidini sulfas</i>	2347
Cefixime.....	1211	<i>Chinini hydrochloridum</i>	2348
<i>Cefiximum</i>	1211	<i>Chinini sulfas</i>	2350
Cefoperazone sodium.....	1212	Chitosan hydrochloride.....	1248
<i>Cefoperazonum natricum</i>	1212	<i>Chitosani hydrochloridum</i>	1248
Cefotaxime sodium.....	1214	Chloral hydrate.....	1249
<i>Cefotaximum natricum</i>	1214	<i>Chlorali hydras</i>	1249
Cefoxitin sodium.....	1216	Chlorambucil.....	1250
<i>Cefoxitinum natricum</i>	1216	<i>Chlorambucilum</i>	1250
Cefradine.....	1217	Chloramine.....	2605
<i>Cefradinum</i>	1217	Chloramphenicol.....	1250
Ceftazidime.....	1218	<i>Chloramphenicoli natrii succinas</i>	1252
<i>Ceftazidimum</i>	1218	<i>Chloramphenicoli palmitas</i>	1251
Ceftriaxone sodium.....	1220	Chloramphenicol palmitate.....	1251
<i>Ceftriaxonum natricum</i>	1220	Chloramphenicol sodium succinate.....	1252
Cefuroxime axetil.....	1222	<i>Chloramphenicolum</i>	1250
Cefuroxime sodium.....	1223	Chlorcyclizine hydrochloride.....	1253
<i>Cefuroximum axetili</i>	1222	<i>Chlorcyclizini hydrochloridum</i>	1253
<i>Cefuroximum natricum</i>	1223	Chlordiazepoxide.....	1254
Celiprolol hydrochloride.....	1224	Chlordiazepoxide hydrochloride.....	1255
<i>Celiprololi hydrochloridum</i>	1224	<i>Chlordiazepoxidi hydrochloridum</i>	1255
Cell cultures for the production of veterinary vaccines (5.2.4.).....	458	<i>Chlordiazepoxidum</i>	1254
Cell substrates for the production of vaccines for human use (5.2.3.).....	455	Chlorhexidine diacetate.....	1256
Cellulose acetate.....	1226	Chlorhexidine digluconate solution.....	1258
Cellulose acetate butyrate.....	1227	Chlorhexidine dihydrochloride.....	1259
Cellulose acetate phthalate.....	1227	<i>Chlorhexidini diacetatas</i>	1256
Cellulose, microcrystalline.....	1228	<i>Chlorhexidini digluconatis solutio</i>	1258
Cellulose, powdered.....	1232	<i>Chlorhexidini dihydrochloridum</i>	1259
<i>Cellulosi acetas</i>	1226	Chlorides (2.4.4.).....	104
<i>Cellulosi acetas butyras</i>	1227	Chlorobutanol, anhydrous.....	1261
<i>Cellulosi acetas phthalas</i>	1227	Chlorobutanol hemihydrate.....	1261
<i>Cellulosi pulvis</i>	1232	<i>Chlorobutanolum anhydricum</i>	1261
<i>Cellulosum microcristallinum</i>	1228	<i>Chlorobutanolum hemihydricum</i>	1261
<i>Centaurii herba</i>	1235	Chlorocresol.....	1262
Centaury.....	1235	<i>Chlorocresolum</i>	1262
Centella.....	1236	Chloroquine phosphate.....	1262
<i>Centellae asiaticae herba</i>	1236	Chloroquine sulphate.....	1263
<i>Cera alba</i>	1057	<i>Chloroquini phosphas</i>	1262
<i>Cera carnauba</i>	1191	<i>Chloroquini sulfas</i>	1263
		Chlorothiazide.....	1264

<i>Chlorothiazidum</i>	1264	<i>Cinnarizinum</i>	1297
Chlorphenamine maleate	1265	Ciprofibrate	1299
<i>Chlorphenamini maleas</i>	1265	<i>Ciprofibratum</i>	1299
Chlorpromazine hydrochloride.....	1266	Ciprofloxacin	1300
<i>Chlorpromazini hydrochloridum</i>	1266	Ciprofloxacin hydrochloride.....	1302
Chlorpropamide.....	1266	<i>Ciprofloxacini hydrochloridum</i>	1302
<i>Chlorpropamidum</i>	1266	<i>Ciprofloxacinum</i>	1300
Chlorprothixene hydrochloride	1267	Circular dichroism (2.2.41.)	63
<i>Chlorprothixeni hydrochloridum</i>	1267	Cisapride monohydrate.....	1303
Chlortalidone.....	1269	Cisapride tartrate.....	1304
<i>Chlortalidonum</i>	1269	<i>Cisapridi tartras</i>	1304
Chlortetracycline hydrochloride.....	1270	<i>Cisapridum monohydricum</i>	1303
<i>Chlortetracyclini hydrochloridum</i>	1270	Cisplatin	1306
Cholecalciferol	1272	<i>Cisplatinum</i>	1306
Cholecalciferol concentrate (oily form).....	1273	Citric acid, anhydrous	1306
Cholecalciferol concentrate (powder form).....	1275	Citric acid monohydrate	1307
Cholecalciferol concentrate (water-dispersible form).....	1277	<i>Citronellae aetheroleum</i>	1308
<i>Cholecalciferoli pulvis</i>	1275	Citronella oil.....	1308
<i>Cholecalciferolum</i>	1272	Clarithromycin	1309
<i>Cholecalciferolum densatum oleosum</i>	1273	<i>Clarithromycinum</i>	1309
<i>Cholecalciferolum in aqua dispergibile</i>	1277	Clarity and degree of opalescence of liquids (2.2.1.).....	23
Cholera vaccine	637	Clary sage oil.....	1311
Cholera vaccine, freeze-dried	638	Clazuril for veterinary use	1312
Cholesterol	1279	<i>Clazurilum ad usum veterinarium</i>	1312
<i>Cholesterolum</i>	1279	Clebopride malate	1314
<i>Chorda resorbilis sterilis</i>	873	<i>Clebopridi malas</i>	1314
<i>Chorda resorbilis sterilis in fuso ad usum veterinarium</i> ..	885	Clemastine fumarate	1315
Chromatographic separation techniques (2.2.46.)	69	<i>Clemastini fumaras</i>	1315
Chromatography, gas (2.2.28.)	42	Clenbuterol hydrochloride.....	1317
Chromatography, liquid (2.2.29.)	43	<i>Clenbuteroli hydrochloridum</i>	1317
Chromatography, paper (2.2.26.).....	40	Clindamycin hydrochloride.....	1318
Chromatography, size-exclusion (2.2.30.).....	45	<i>Clindamycini hydrochloridum</i>	1318
Chromatography, supercritical fluid (2.2.45.)	68	<i>Clindamycini phosphas</i>	1319
Chromatography, thin-layer (2.2.27.).....	40	Clindamycin phosphate	1319
<i>Chromii (⁶⁷Cr) edetatis solutio iniectionabilis</i>	819	Clioquinol	1321
Chromium (⁵¹ Cr) edetate injection	819	<i>Clioquinolum</i>	1321
Chymotrypsin.....	1280	Clobazam	1322
<i>Chymotrypsinum</i>	1280	<i>Clobazamum</i>	1322
Ciclopirox.....	1282	Clobetasone butyrate	1323
Ciclopirox olamine.....	1283	<i>Clobetasoni butyras</i>	1323
<i>Ciclopirox olaminum</i>	1283	Clofazimine.....	1324
<i>Ciclopiroxum</i>	1282	<i>Clofaziminum</i>	1324
Ciclosporin	1285	Clofibrate	1325
<i>Ciclosporinum</i>	1285	<i>Clofibratum</i>	1325
Cilastatin sodium	1286	Clomifene citrate	1326
<i>Cilastatinum natricum</i>	1286	<i>Clomifeni citras</i>	1326
Cilazapril.....	1288	Clomipramine hydrochloride.....	1328
<i>Cilazaprilum</i>	1288	<i>Clomipramini hydrochloridum</i>	1328
Cimetidine.....	1289	Clonazepam.....	1330
Cimetidine hydrochloride.....	1290	<i>Clonazepamum</i>	1330
<i>Cimetidini hydrochloridum</i>	1290	Clonidine hydrochloride.....	1331
<i>Cimetidinum</i>	1289	<i>Clonidini hydrochloridum</i>	1331
Cinchocaine hydrochloride	1291	Closantel sodium dihydrate for veterinary use	1331
<i>Cinchocaini hydrochloridum</i>	1291	<i>Closantelum natricum dihydricum</i> ad usum veterinarium	1331
Cinchona bark	1292	Clostridium botulinum vaccine for veterinary use.....	745
<i>Cinchonae cortex</i>	1292	Clostridium chauvoei vaccine for veterinary use.....	745
Cineole.....	1294	Clostridium novyi alpha antitoxin for veterinary use	809
Cineole in essential oils, assay of, 1,8- (2.8.11.).....	216	Clostridium novyi (type B) vaccine for veterinary use.....	746
<i>Cineolum</i>	1294	Clostridium perfringens beta antitoxin for veterinary use	810
<i>Cinnamomi cassiae aetheroleum</i>	1196		810
<i>Cinnamomi cortex</i>	1295	Clostridium perfringens epsilon antitoxin for veterinary use	811
<i>Cinnamomi corticis tinctura</i>	1297		811
<i>Cinnamomi zeylanici folii aetheroleum</i>	1296	Clostridium perfringens vaccine for veterinary use	747
<i>Cinnamomi zeylanicii corticis aetheroleum</i>	1295	Clostridium septicum vaccine for veterinary use.....	749
Cinnamon	1295	Closures and containers for parenteral preparations and ophthalmic preparations, polypropylene for (3.1.6.).....	282
Cinnamon bark oil, Ceylon	1295	Closures and containers for pharmaceutical use, plastic (3.2.2.)	308
Cinnamon leaf oil, Ceylon.....	1296		
Cinnamon tincture.....	1297		
Cinnarizine	1297		

- Closures and tubing, silicone elastomer for (3.1.9.) 288
- Closures for containers for aqueous parenteral preparations, for powders and for freeze-dried powders, rubber (3.2.9.) 316
- Clotrimazole 1333
- Clotrimazolum* 1333
- Clove 1334
- Clove oil 1335
- Cloxacillin sodium 1335
- Cloxacillinum natricum* 1335
- Clozapine 1337
- Clozapinum* 1337
- Coagulation factor II, assay of (2.7.18.) 209
- Coagulation factor IX, assay of (2.7.11.) 204
- Coagulation factor IX, human 1738
- Coagulation factors, activated (2.6.22.) 177
- Coagulation factors, assay of heparin (2.7.12.) 204
- Coagulation factor VII, assay of (2.7.10.) 203
- Coagulation factor VII, human 1734
- Coagulation factor VIII, assay of (2.7.4.) 194
- Coagulation factor VIII, human 1736
- Coagulation factor VIII (rDNA), human 1737
- Coagulation factor X, assay of (2.7.19.) 210
- Coagulation factor XI, assay of (2.7.22.) 212
- Coagulation factor XI, human 1739
- Coated granules 606
- Coated tablets 627
- Cocaine hydrochloride 1338
- Cocaini hydrochloridum* 1338
- Cocois oleum raffinatum* 1339
- Coconut oil, refined 1339
- Cocoyl caprylocaprate 1340
- Cocoylis caprylocapras* 1340
- Codeine 1341
- Codeine hydrochloride dihydrate 1342
- Codeine phosphate hemihydrate 1344
- Codeine phosphate sesquihydrate 1345
- Codeini hydrochloridum dihydricum* 1342
- Codeini phosphas hemihydricus* 1344
- Codeini phosphas sesquihydricus* 1345
- Codeinum* 1341
- Codergocrine mesilate 1347
- Codergocrini mesilas* 1347
- Cod-liver oil (type A) 1348
- Cod-liver oil (type B) 1352
- Coffeinum* 1145
- Coffeinum monohydricum* 1146
- Cola 1356
- Colae semen* 1356
- Colchicine 1357
- Colchicinum* 1357
- Colestyramine 1359
- Colestyraminum* 1359
- Colibacillosis vaccine (inactivated), neonatal piglet 776
- Colibacillosis vaccine (inactivated), neonatal ruminant 778
- Colistimethate sodium 1360
- Colistimethatum natricum* 1360
- Colistini sulfas* 1361
- Colistin sulphate 1361
- Colloidal anhydrous silica 2410
- Colloidal hydrated silica 2411
- Colophonium* 1362
- Colophony 1362
- Coloration of liquids (2.2.2.) 24
- Common stinging nettle for homeopathic preparations .. 895
- Comparative table of porosity of sintered-glass filters (2.1.2.) 17
- Complexometric titrations (2.5.11.) 130
- Composition of fatty acids by gas chromatography (2.4.22.) 110
- Composition of fatty acids in oils rich in omega-3-acids (2.4.29.) 121
- Compressed lozenges 613
- Compressi* 626
- Concentrated solutions for haemodialysis 1700
- Concentrates for injections or infusions 614
- Conductivity (2.2.38.) 56
- Conjugated estrogens 1539
- Containers (3.2.) 303
- Containers and closures for parenteral preparations and ophthalmic preparations, polypropylene for (3.1.6.) 282
- Containers and closures for pharmaceutical use, plastic (3.2.2.) 308
- Containers and tubing for total parenteral nutrition preparations, poly(ethylene - vinyl acetate) for (3.1.7.) ... 285
- Containers for aqueous solutions for intravenous infusion, materials based on plasticised poly(vinyl chloride) for (3.1.14.) 296
- Containers for aqueous solutions for parenteral infusion, plastic (3.2.2.1.) 309
- Containers for dry dosage forms for oral administration, materials based on non-plasticised poly(vinyl chloride) for (3.1.11.) 291
- Containers for human blood and blood components, materials based on plasticised poly(vinyl chloride) for (3.1.1.1.) 269
- Containers for human blood and blood components, materials for (3.1.1.) 269
- Containers for human blood and blood components, plastic, sterile (3.2.3.) 309
- Containers for non-injectable aqueous solutions, materials based on non-plasticised poly(vinyl chloride) for (3.1.10.) 289
- Containers for parenteral preparations and for ophthalmic preparations, polyethylene with additives for (3.1.5.) 279
- Containers for parenteral preparations and for ophthalmic preparations, polyethylene without additives for (3.1.4.) 278
- Containers for pharmaceutical use, glass (3.2.1.) 303
- Containers for preparations not for parenteral use, polyethylene terephthalate for (3.1.15) 298
- Containers of plasticised poly(vinyl chloride) for human blood and blood components, empty sterile (3.2.4.) 311
- Containers of plasticised poly (vinyl chloride) for human blood containing anticoagulant solution, sterile (3.2.5.) .. 312
- Contamination, microbial, test for specified micro-organisms (2.6.13.) 156
- Contamination, microbial, total viable aerobic count (2.6.12.) 154
- Content uniformity of single-dose preparations (2.9.6.) 234
- Control of impurities in substances for pharmaceutical use (5.10.) 559
- Copolymerum methacrylatis butylati basicum* 1053
- Copovidone 1363
- Copovidonum* 1363
- Copper for homeopathic preparations 896
- Copper sulphate, anhydrous 1364
- Copper sulphate pentahydrate 1365
- Coriander 1365
- Coriander oil 1366
- Coriandri aetheroleum* 1366
- Coriandri fructus* 1365
- Corpora ad usum pharmaceuticum* 586
- Cortisone acetate 1367
- Cortisoni acetas* 1367
- Cotton, absorbent 1369
- Cottonseed oil, hydrogenated 1370

Couch grass rhizome	1371	Density of solids (2.2.42.)	64
<i>Crataegi folii cum flore extractum siccum</i>	1714	Density, relative (2.2.5.)	27
<i>Crataegi folium cum flore</i>	1713	Dental type silica	2411
<i>Crataegi fructus</i>	1712	Depressor substances (2.6.11.)	153
Creams	625	Deptropine citrate	1393
Cresol, crude	1371	<i>Deptropini citras</i>	1393
<i>Cresolum crudum</i>	1371	<i>Dequalinii chloridum</i>	1394
<i>Croci stigma ad praeparationes homoeopathicas</i>	900	Dequalinium chloride	1394
Croscarmellose sodium	1371	Desipramine hydrochloride	1395
Crospovidone	1373	<i>Desipramini hydrochloridum</i>	1395
<i>Crospovidonum</i>	1373	Deslanoside	1396
Crotamiton	1374	<i>Deslanosidum</i>	1396
<i>Crotamitonum</i>	1374	Desmopressin	1397
<i>Cupri sulfas anhydricus</i>	1364	<i>Desmopressinum</i>	1397
<i>Cupri sulfas pentahydricus</i>	1365	Desoxycortone acetate	1399
<i>Cuprum ad praeparationes homoeopathicas</i>	896	<i>Desoxycortoni acetas</i>	1399
<i>Curcumae xanthorrhizae rhizoma</i>	2645	Detector tubes, gas (2.1.6.)	19
Cutaneous application, liquid preparations for	607	Determination of essential oils in vegetable drugs (2.8.12.)	217
Cutaneous application, powders for	616	Determination of nitrogen by sulphuric acid digestion (2.5.9.)	129
Cutaneous application, semi-solid preparations for	624	Determination of primary aromatic amino-nitrogen (2.5.8.)	129
Cutaneous application, veterinary liquid preparations for	630	Determination of tannins in herbal drugs (2.8.14.)	221
Cutaneous foams	608	Determination of water by distillation (2.2.13.)	32
<i>Cyamopsidis seminis pulvis</i>	1694	Detomidine hydrochloride for veterinary use	1400
Cyanocobalamin	1375	<i>Detomidini hydrochloridum ad usum veterinarium</i>	1400
Cyanocobalamin (⁵⁷ Co) capsules	819	Devil's claw root	1401
Cyanocobalamin (⁵⁷ Co) solution	820	Dexamethasone	1402
Cyanocobalamin (⁵⁸ Co) capsules	821	Dexamethasone acetate	1403
Cyanocobalamin (⁵⁸ Co) solution	822	Dexamethasone sodium phosphate	1404
<i>Cyanocobalamini (⁵⁷Co) capsulae</i>	819	<i>Dexamethasoni acetas</i>	1403
<i>Cyanocobalamini (⁵⁷Co) solutio</i>	820	<i>Dexamethasoni natrii phosphas</i>	1404
<i>Cyanocobalamini (⁵⁸Co) capsulae</i>	821	<i>Dexamethasonum</i>	1402
<i>Cyanocobalamini (⁵⁸Co) solutio</i>	822	Dexchlorpheniramine maleate	1406
<i>Cyanocobalaminum</i>	1375	<i>Dexchlorpheniramini maleas</i>	1406
Cyclizine hydrochloride	1376	Dexpanthenol	1407
<i>Cyclizini hydrochloridum</i>	1376	<i>Dexpanthenolum</i>	1407
Cyclopentolate hydrochloride	1377	Dextran 1 for injection	1408
<i>Cyclopentolati hydrochloridum</i>	1377	Dextran 40 for injection	1409
Cyclophosphamide	1378	Dextran 60 for injection	1410
<i>Cyclophosphamidum</i>	1378	Dextran 70 for injection	1411
Cyproheptadine hydrochloride	1379	Dextrans, molecular mass distribution in (2.2.39.)	57
<i>Cyproheptadini hydrochloridum</i>	1379	<i>Dextranum 1 ad iniectionem</i>	1408
Cyproterone acetate	1380	<i>Dextranum 40 ad iniectionem</i>	1409
<i>Cyproteroni acetas</i>	1380	<i>Dextranum 60 ad iniectionem</i>	1410
Cysteine hydrochloride monohydrate	1381	<i>Dextranum 70 ad iniectionem</i>	1411
<i>Cysteiini hydrochloridum monohydricum</i>	1381	Dextrin	1412
Cystine	1382	<i>Dextrinum</i>	1412
<i>Cystinum</i>	1382	Dextromethorphan hydrobromide	1412
Cytarabine	1383	<i>Dextromethorphani hydrobromidum</i>	1412
<i>Cytarabinum</i>	1383	Dextromoramide tartrate	1414
D		<i>Dextromoramidi tartras</i>	1414
Dalteparin sodium	1387	Dextropropoxyphene hydrochloride	1414
<i>Dalteparinum natricum</i>	1387	<i>Dextropropoxypheni hydrochloridum</i>	1414
Dapsone	1388	Diazepam	1415
<i>Dapsonum</i>	1388	<i>Diazepamum</i>	1415
Daunorubicin hydrochloride	1389	Diazoxide	1416
<i>Daunorubicini hydrochloridum</i>	1389	<i>Diazoxidum</i>	1416
D-Camphor	1170	<i>Dibutylis phthalas</i>	1417
<i>D-Camphora</i>	1170	Dibutyl phthalate	1417
<i>Decylis oleas</i>	1390	Dichloromethane	2017
Decyl oleate	1390	Diclazuril for veterinary use	1418
Deferoxamine mesilate	1390	<i>Diclazurilum ad usum veterinarium</i>	1418
<i>Deferoxamini mesilas</i>	1390	Diclofenac potassium	1419
Degree of coloration of liquids (2.2.2.)	24	Diclofenac sodium	1420
Deliverable mass or volume of liquid and semi-solid preparations, test for (2.9.28.)	263	<i>Diclofenacum kalicum</i>	1419
Demeclocycline hydrochloride	1392	<i>Diclofenacum natricum</i>	1420
<i>Demeclocyclini hydrochloridum</i>	1392	Dicloxacillin sodium	1422

<i>Dicloxacillinum natricum</i>	1422	Diphenoxylate hydrochloride.....	1455
Dicycloverine hydrochloride.....	1423	<i>Diphenoxylati hydrochloridum</i>	1455
<i>Dicycloverini hydrochloridum</i>	1423	Diphtheria and tetanus vaccine (adsorbed).....	639
Dienestrol.....	1424	Diphtheria and tetanus vaccine (adsorbed) for adults and adolescents.....	639
<i>Dienestrolum</i>	1424	Diphtheria antitoxin.....	801
Diethylcarbamazine citrate.....	1426	Diphtheria, tetanus and hepatitis B (rDNA) vaccine (adsorbed).....	641
<i>Diethylcarbamazini citras</i>	1426	Diphtheria, tetanus and pertussis (acellular, component) vaccine (adsorbed).....	642
Diethylene glycol and ethylene glycol in ethoxylated substances (2.4.30.).....	122	Diphtheria, tetanus and pertussis vaccine (adsorbed).....	643
Diethylene glycol monoethyl ether.....	1426	Diphtheria, tetanus, pertussis (acellular, component) and haemophilus type b conjugate vaccine (adsorbed).....	645
Diethylene glycol monopalmitostearate.....	1428	Diphtheria, tetanus, pertussis (acellular, component) and hepatitis B (rDNA) vaccine (adsorbed).....	647
<i>Diethylen glycoli monoethylicum aetherum</i>	1426	Diphtheria, tetanus, pertussis (acellular, component) and poliomyelitis (inactivated) vaccine (adsorbed).....	648
<i>Diethylen glycoli monopalmitostearas</i>	1428	Diphtheria, tetanus, pertussis (acellular, component), hepatitis B (rDNA), poliomyelitis (inactivated) and haemophilus type b conjugate vaccine (adsorbed).....	650
<i>Diethylis phthalas</i>	1425	Diphtheria, tetanus, pertussis (acellular, component), poliomyelitis (inactivated) and haemophilus type b conjugate vaccine (adsorbed).....	653
Diethyl phthalate.....	1425	Diphtheria, tetanus, pertussis and poliomyelitis (inactivated) vaccine (adsorbed).....	656
Diethylstilbestrol.....	1429	Diphtheria, tetanus, pertussis, poliomyelitis (inactivated) and haemophilus type b conjugate vaccine (adsorbed).....	657
<i>Diethylstilbestrolum</i>	1429	Diphtheria vaccine (adsorbed).....	660
Diflunisal.....	1429	Diphtheria vaccine (adsorbed), assay of (2.7.6.).....	196
<i>Diflunisalum</i>	1429	Diphtheria vaccine (adsorbed) for adults and adolescents.....	661
Digitalis leaf.....	1431	Dipivefrine hydrochloride.....	1456
<i>Digitalis purpureae folium</i>	1431	<i>Dipivefrini hydrochloridum</i>	1456
Digitoxin.....	1432	Dipotassium clorazepate.....	1457
<i>Digitoxinum</i>	1432	Dipotassium phosphate.....	1458
Digoxin.....	1433	Diprophyllyne.....	1459
<i>Digoxinum</i>	1433	<i>Diprophyllynum</i>	1459
Dihydralazine sulphate, hydrated.....	1434	Dipyridamole.....	1460
<i>Dihydralazini sulfas hydricus</i>	1434	<i>Dipyridamolum</i>	1460
Dihydrocodeine hydrogen tartrate.....	1435	Dirithromycin.....	1461
<i>Dihydrocodeini hydrogenotartras</i>	1435	<i>Dirithromycinum</i>	1461
Dihydroergocristine mesilate.....	1437	Disintegration of suppositories and pessaries (2.9.2.).....	227
<i>Dihydroergocristini mesilas</i>	1437	Disintegration of tablets and capsules (2.9.1.).....	225
Dihydroergotamine mesilate.....	1439	Disodium edetate.....	1462
Dihydroergotamine tartrate.....	1440	Disodium phosphate, anhydrous.....	1463
<i>Dihydroergotamini mesilas</i>	1439	Disodium phosphate dihydrate.....	1464
<i>Dihydroergotamini tartras</i>	1440	Disodium phosphate dodecahydrate.....	1464
<i>Dihydrostreptomycini sulfas ad usum veterinarium</i>	1441	Disopyramide.....	1465
Dihydrostreptomycin sulphate for veterinary use.....	1441	Disopyramide phosphate.....	1466
<i>Dikalii clorazepas</i>	1457	<i>Disopyramidi phosphas</i>	1466
<i>Dikalii phosphas</i>	1458	<i>Disopyramidum</i>	1465
Diltiazem hydrochloride.....	1443	Dispersible tablets.....	628
<i>Diltiazemi hydrochloridum</i>	1443	Dissolution test for solid dosage forms (2.9.3.).....	228
Dimenhydrinate.....	1444	Dissolution test for transdermal patches (2.9.4.).....	231
<i>Dimenhydrinatum</i>	1444	Distemper vaccine (live), canine, freeze-dried.....	740
Dimercaprol.....	1445	Distemper vaccine (live) for mustelids, freeze-dried.....	751
<i>Dimercaprolum</i>	1445	Distillation range (2.2.11.).....	30
Dimethylacetamide.....	1446	Disulfiram.....	1467
<i>Dimethylacetamidum</i>	1446	<i>Disulfiramum</i>	1467
Dimethylaniline, <i>N,N</i> -(2.4.26.).....	119	Dithranol.....	1468
<i>Dimethylis sulfoxidum</i>	1445	<i>Dithranolum</i>	1468
Dimethyl sulfoxide.....	1445	DL-Methionine.....	2010
Dimeticone.....	1447	<i>DL-Methioninum</i>	2010
<i>Dimeticonum</i>	1447	DL- α -Tocopheryl hydrogen succinate.....	2597
Dimetindene maleate.....	1448	<i>DL-α-Tocopherylis hydrogenosuccinas</i>	2597
<i>Dimetindeni maleas</i>	1448	Dobutamine hydrochloride.....	1469
<i>Dinatrii edetas</i>	1462	<i>Dobutamini hydrochloridum</i>	1469
<i>Dinatrii phosphas anhydricus</i>	1463	Docusate sodium.....	1471
<i>Dinatrii phosphas dihydricus</i>	1464	Dodecyl gallate.....	1472
<i>Dinatrii phosphas dodecahydricus</i>	1464		
<i>Dinitrogenii oxidum</i>	2110		
Dinoprostone.....	1450		
<i>Dinoprostionum</i>	1450		
Dinoprost trometamol.....	1449		
<i>Dinoprostum trometamolum</i>	1449		
Diosmin.....	1452		
<i>Diosminum</i>	1452		
Dioxan and ethylene oxide (2.4.25.).....	118		
Dip concentrates.....	630		
Diphenhydramine hydrochloride.....	1454		
<i>Diphenhydramini hydrochloridum</i>	1454		

<i>Dodecylis gallas</i>	1472	<i>Enilconazolum ad usum veterinarium</i>	1503
Dog rose.....	1472	Enoxaparin sodium.....	1504
Domperidone.....	1473	<i>Enoxaparinum natricum</i>	1504
Domperidone maleate.....	1475	Enoxolone.....	1505
<i>Domperidoni maleas</i>	1475	<i>Enoxolonum</i>	1505
<i>Domperidonum</i>	1473	Ephedrine, anhydrous.....	1506
Dopamine hydrochloride.....	1476	Ephedrine hemihydrate.....	1507
<i>Dopamini hydrochloridum</i>	1476	Ephedrine hydrochloride.....	1508
Dosulepin hydrochloride.....	1477	Ephedrine hydrochloride, racemic.....	1509
<i>Dosulepini hydrochloridum</i>	1477	<i>Ephedrini hydrochloridum</i>	1508
Doxapram hydrochloride.....	1479	<i>Ephedrini racemici hydrochloridum</i>	1509
<i>Doxaprami hydrochloridum</i>	1479	<i>Ephedrinum anhydricum</i>	1506
Doxepin hydrochloride.....	1480	<i>Ephedrinum hemihydricum</i>	1507
<i>Doxepini hydrochloridum</i>	1480	Epirubicin hydrochloride.....	1509
Doxorubicin hydrochloride.....	1481	<i>Epirubicini hydrochloridum</i>	1509
<i>Doxorubicini hydrochloridum</i>	1481	Equine herpesvirus vaccine (inactivated).....	754
Doxycycline hyclate.....	1482	Equine influenza vaccine (inactivated).....	755
Doxycycline monohydrate.....	1484	<i>Equiseti herba</i>	1511
<i>Doxycyclini hyclas</i>	1482	Equisetum stem.....	1511
<i>Doxycyclinum monohydricum</i>	1484	Ergocalciferol.....	1512
Doxylamine hydrogen succinate.....	1486	<i>Ergocalciferolum</i>	1512
<i>Doxylamini hydrogenosuccinas</i>	1486	Ergometrine maleate.....	1514
Droperidol.....	1487	<i>Ergometrini maleas</i>	1514
<i>Droperidolum</i>	1487	Ergotamine tartrate.....	1515
Droppers (2.1.1.).....	17	<i>Ergotamini tartras</i>	1515
Drop point (2.2.17.).....	33	Erysipelas vaccine (inactivated), swine.....	793
Dry extracts.....	572	Erythritol.....	1516
Dry residue of extracts (2.8.16.).....	222	<i>Erythritolum</i>	1516
Duck viral hepatitis type I vaccine (live).....	751	Erythromycin.....	1518
E			
Ear-drops and sprays.....	602	Erythromycin estolate.....	1520
Ear powders.....	602	Erythromycin ethylsuccinate.....	1521
Ear preparations.....	601	<i>Erythromycini estolas</i>	1520
Ear preparations, semi-solid.....	602	<i>Erythromycini ethylsuccinas</i>	1521
Ear tampons.....	602	<i>Erythromycini lactobionas</i>	1523
Ear washes.....	602	<i>Erythromycini stearas</i>	1526
Ebastine.....	1491	Erythromycin lactobionate.....	1523
<i>Ebastinum</i>	1491	Erythromycin stearate.....	1526
Econazole.....	1492	<i>Erythromycinum</i>	1518
Econazole nitrate.....	1493	Erythropoietin concentrated solution.....	1528
<i>Econazoli nitras</i>	1493	<i>Erythropoietini solutio concentrata</i>	1528
<i>Econazolum</i>	1492	Eserine salicylate.....	2239
Edetic acid.....	1494	Eserine sulphate.....	2240
<i>Edrophonii chloridum</i>	1495	<i>Eserini salicylas</i>	2239
Edrophonium chloride.....	1495	<i>Eserini sulfas</i>	2240
Effervescent granules.....	606	Esketamine hydrochloride.....	1533
Effervescent powders.....	617	<i>Esketamini hydrochloridum</i>	1533
Effervescent tablets.....	627	Essential oils, assay of 1,8-cineole (2.8.11.).....	216
Efficacy of antimicrobial preservation (5.1.3.).....	447	Essential oils, fatty oils and resinified essential oils in (2.8.7.).....	216
Egg drop syndrome '76 vaccine (inactivated).....	753	Essential oils, foreign esters in (2.8.6.).....	216
Elder flower.....	1496	Essential oils in vegetable drugs, determination of (2.8.12.).....	217
Electrophoresis (2.2.31.).....	45	Essential oils, odour and taste (2.8.8.).....	216
Electrophoresis, capillary (2.2.47.).....	74	Essential oils, residue on evaporation (2.8.9.).....	216
<i>Eleutherococci radix</i>	1497	Essential oils, solubility in alcohol (2.8.10.).....	216
Eleutherococcus.....	1497	Essential oils, water in (2.8.5.).....	216
Emetine hydrochloride heptahydrate.....	1499	Ester value (2.5.2.).....	127
Emetine hydrochloride pentahydrate.....	1500	Estradiol benzoate.....	1534
<i>Emetini hydrochloridum heptahydricum</i>	1499	Estradiol hemihydrate.....	1535
<i>Emetini hydrochloridum pentahydricum</i>	1500	<i>Estradioli benzoas</i>	1534
<i>Emplastra transcutanea</i>	616	<i>Estradioli valeras</i>	1536
Empty sterile containers of plasticised poly(vinyl chloride) for human blood and blood components (3.2.4.).....	311	<i>Estradiolum hemihydricum</i>	1535
Emulsifying cetostearyl alcohol (type A).....	1239	Estradiol valerate.....	1536
Emulsifying cetostearyl alcohol (type B).....	1241	Estriol.....	1537
<i>Enalapril maleas</i>	1501	<i>Estriolum</i>	1537
Enalapril maleate.....	1501	<i>Estrogeni coniuncti</i>	1539
Endotoxins, bacterial (2.6.14.).....	161	Estrogens, conjugated.....	1539
Enilconazole for veterinary use.....	1503	Etacrynic acid.....	1542
		Etamsylate.....	1542

<i>Etamsylatum</i>	1542	Extracts, dry residue of (2.8.16.).....	222
Ethacridine lactate monohydrate.....	1543	Extracts, liquid.....	571
<i>Ethacridini lactas monohydricus</i>	1543	Extracts, loss on drying of (2.8.17.).....	222
Ethambutol hydrochloride.....	1544	Extracts, soft	572
<i>Ethambutoli hydrochloridum</i>	1544	Extraneous agents in viral vaccines for human use, tests for (2.6.16.)	169
Ethanol (96 per cent).....	1545	Extraneous agents: tests in batches of finished product of avian live virus vaccines (2.6.25.).....	180
Ethanol, anhydrous.....	1547	Extraneous agents: tests in seed lots of avian viral vaccines (2.6.24.)	177
Ethanol content and alcoholimetric tables (2.9.10.)	237	Eye-drops	603
<i>Ethanolum (96 per centum)</i>	1545	Eye lotions.....	603
<i>Ethanolum anhydricum</i>	1547	Eye preparations	602
Ether	1548	Eye preparations, semi-solid.....	604
Ether, anaesthetic.....	1549		
Ethinylestradiol	1550	F	
<i>Ethinylestradiolum</i>	1550	<i>F₀</i> concept to steam sterilisation of aqueous preparations, application of (5.1.5.)	449
Ethionamide.....	1551	Factor II, human coagulation, assay of (2.7.18.)	209
<i>Ethionamidum</i>	1551	<i>Factor IX coagulationis humanus</i>	1738
Ethosuximide.....	1551	Factor IX, human coagulation	1738
<i>Ethosuximidum</i>	1551	Factor IX, human coagulation, assay of (2.7.11.)	204
Ethoxylated substances, ethylene glycol and diethylene glycol in (2.4.30.)	122	<i>Factor VII coagulationis humanus</i>	1734
Ethyl acetate	1553	Factor VII, human coagulation.....	1734
Ethylcellulose	1555	Factor VII, human coagulation, assay of (2.7.10.)	203
<i>Ethylcellulosum</i>	1555	<i>Factor VIII coagulationis humanus</i>	1736
<i>Ethylendiaminum</i>	1556	<i>Factor VIII coagulationis humanus (ADNr)</i>	1737
Ethylenediamine	1556	Factor VIII, human coagulation	1736
Ethylene glycol and diethylene glycol in ethoxylated substances (2.4.30.).....	122	Factor VIII, human coagulation, assay of (2.7.4.)	194
Ethylene glycol monopalmitostearate.....	1556	Factor VIII (rDNA), human coagulation	1737
Ethylene glycol monostearate.....	1556	Factor X, human coagulation, assay of (2.7.19.)	210
Ethylene oxide and dioxan (2.4.25.)	118	<i>Factor XI coagulationis humanus</i>	1739
<i>Ethylenglycoli monopalmitostearas</i>	1556	Factor XI, human coagulation	1739
Ethylhexanoic acid, 2- (2.4.28.).....	120	Factor XI, human coagulation, assay of (2.7.22.).....	212
<i>Ethylis acetas</i>	1553	Falling ball viscometer method (2.2.49.)	80
<i>Ethylis oleas</i>	1553	Famotidine.....	1575
<i>Ethylis parahydroxybenzoas</i>	1554	<i>Famotidinum</i>	1575
Ethylmorphine hydrochloride	1557	Fatty acids, composition by gas chromatography (2.4.22.)	110
<i>Ethylmorphini hydrochloridum</i>	1557	Fatty oils, alkaline impurities in (2.4.19.)	109
Ethyl oleate	1553	Fatty oils and herbal drugs, heavy metals in (2.4.27.)	119
Ethyl parahydroxybenzoate.....	1554	Fatty oils and resinified essential oils in essential oils (2.8.7.).....	216
Etilefrine hydrochloride	1558	Fatty oils, foreign oils in, by thin-layer chromatography (2.4.21.)	109
<i>Etilefrini hydrochloridum</i>	1558	Fatty oils, identification by thin-layer chromatography (2.3.2.)	98
Etodolac	1560	Fatty oils, sterols in (2.4.23.)	111
<i>Etodolacum</i>	1560	Fc function of immunoglobulin, test for (2.7.9.)	202
Etofenamate	1561	Feline calicivirus vaccine (inactivated)	757
<i>Etofenamatum</i>	1561	Feline calicivirus vaccine (live), freeze-dried	758
Etofylline.....	1563	Feline infectious enteritis (feline panleucopenia) vaccine (inactivated)	759
<i>Etofyllinum</i>	1563	Feline infectious enteritis (feline panleucopenia) vaccine (live)	760
Etomidate	1564	Feline leukaemia vaccine (inactivated).....	761
<i>Etomidatum</i>	1564	Feline panleucopenia vaccine (inactivated).....	759
Etoposide	1565	Feline panleucopenia vaccine (live)	760
<i>Etoposidum</i>	1565	Feline viral rhinotracheitis vaccine (inactivated).....	762
<i>Eucalypti aetheroleum</i>	1570	Feline viral rhinotracheitis vaccine (live), freeze-dried	763
<i>Eucalypti folium</i>	1569	Felodipine	1576
Eucalyptus leaf	1569	<i>Felodipinum</i>	1576
Eucalyptus oil	1570	Fenbendazole for veterinary use	1577
Eugenol	1571	<i>Fenbendazolum ad usum veterinarium</i>	1577
<i>Eugenolum</i>	1571	Fenbufen	1578
European Goldenrod.....	1682	<i>Fenbufenum</i>	1578
European viper venom antiserum.....	806	Fennel, bitter.....	1580
Evaluation of efficacy of veterinary vaccines (5.2.7.)	462	Fennel, sweet.....	1580
Evaluation of safety of veterinary vaccines (5.2.6.)	461	Fenofibrate	1581
<i>Extracta</i>	570		
Extractable volume of parenteral preparations, test for (2.9.17.).....	243		
<i>Extracta fluida</i>	571		
<i>Extracta sicca</i>	572		
<i>Extracta spissa</i>	572		
Extracts	570		
Extracts, dry	572		

<i>Fenofibratum</i>	1581	<i>Fluocinoloni acetonidum</i>	1610
Fenoterol hydrobromide.....	1583	Fluocortolone pivalate.....	1611
<i>Fenoteroli hydrobromidum</i>	1583	<i>Fluocortoloni pivalas</i>	1611
Fentanyl.....	1584	Fluorescein sodium.....	1613
Fentanyl citrate.....	1585	<i>Fluoresceinum natricum</i>	1613
<i>Fentanyl citras</i>	1585	Fluorides (2.4.5.).....	104
<i>Fentanylum</i>	1584	Fluorimetry (2.2.21.)	35
Fenticonazole nitrate	1586	Fluorouracil.....	1614
<i>Fenticonazoli nitras</i>	1586	<i>Fluorouracilum</i>	1614
Fenugreek.....	1588	Fluoxetine hydrochloride.....	1615
Fermentation, products of	576	<i>Fluoxetini hydrochloridum</i>	1615
Ferric chloride hexahydrate	1588	Flupentixol dihydrochloride.....	1617
<i>Ferri chloridum hexahydricum</i>	1588	<i>Flupentixoli dihydrochloridum</i>	1617
<i>Ferrosi fumaras</i>	1589	Fluphenazine decanoate	1619
<i>Ferrosi gluconas</i>	1590	Fluphenazine enantate	1620
<i>Ferrosi sulfas heptahydricus</i>	1591	Fluphenazine hydrochloride	1621
Ferrous fumarate	1589	<i>Fluphenazini decanoas</i>	1619
Ferrous gluconate.....	1590	<i>Fluphenazini enantas</i>	1620
Ferrous sulphate heptahydrate.....	1591	<i>Fluphenazini hydrochloridum</i>	1621
<i>Ferrum ad praeparationes homoeopathicas</i>	899	<i>Flurazepam monohydrochloridum</i>	1622
Feverfew.....	1592	Flurazepam monohydrochloride	1622
<i>Fibrini glutinum</i>	1593	Flurbiprofen	1623
Fibrinogen, human	1740	<i>Flurbiprofenum</i>	1623
<i>Fibrinogenum humanum</i>	1740	Fluspirilene	1625
Fibrin sealant kit.....	1593	<i>Fluspirilenum</i>	1625
<i>Fila non resorbilia sterilia</i>	874	Flutamide.....	1626
<i>Fila non resorbilia sterilia in fuso ad usum</i> <i>veterinarium</i>	888	<i>Flutamidum</i>	1626
<i>Fila resorbilia synthetica monofilamenta sterilia</i>	880	Fluticasone propionate.....	1627
<i>Fila resorbilia synthetica torta sterilia</i>	878	<i>Fluticasoni propionas</i>	1627
<i>Filipendulae ulmariae herba</i>	1980	Flutrimazole.....	1629
<i>Filum bombycis tortum sterile in fuso ad usum</i> <i>veterinarium</i>	887	<i>Flutrimazolum</i>	1629
<i>Filum ethyleni polyterephthalici sterile in fuso ad usum</i> <i>veterinarium</i>	887	Foams, cutaneous	608
<i>Filum lini sterile in fuso ad usum veterinarium</i>	886	Foams, medicated	604
<i>Filum polyamidicum-6/6 sterile in fuso ad usum</i> <i>veterinarium</i>	887	Foams, rectal.....	624
<i>Filum polyamidicum-6 sterile in fuso ad usum</i> <i>veterinarium</i>	886	Foams, vaginal	630
Finasteride.....	1594	<i>Foeniculi amari fructus</i>	1580
<i>Finasteridum</i>	1594	<i>Foeniculi amari fructus aetheroleum</i>	1108
Fish oil, rich in omega-3-acids.....	1595	<i>Foeniculi dulcis fructus</i>	1580
Flecainide acetate	1598	Folic acid.....	1630
<i>Flecainidi acetas</i>	1598	Foot-and-mouth disease (ruminants) vaccine (inactivated)	764
Flowability (2.9.16.).....	242	Foreign esters in essential oils (2.8.6.)	216
Flubendazole	1599	Foreign matter (2.8.2.)	215
<i>Flubendazolum</i>	1599	Foreign oils in fatty oils by thin-layer chromatography (2.4.21.)	109
Flucloxacillin sodium.....	1600	Formaldehyde, free (2.4.18.).....	109
<i>Flucloxacillinum natricum</i>	1600	Formaldehyde solution (35 per cent)	1632
Flucytosine	1602	<i>Formaldehydi solutio (35 per centum)</i>	1632
<i>Flucytosinum</i>	1602	Formoterol fumarate dihydrate	1632
Fludeoxyglucose (¹⁸ F) injection	822	<i>Formoteroli fumaras dihydricus</i>	1632
<i>Fludeoxyglucosi (¹⁸F) solutio iniectabilis</i>	822	Foscarnet sodium hexahydrate.....	1634
Fludrocortisone acetate.....	1603	<i>Foscarnetum natricum hexahydricum</i>	1634
<i>Fludrocortisoni acetas</i>	1603	Fosfomycin calcium	1636
Flumazenil.....	1604	Fosfomycin sodium.....	1637
Flumazenil (N-[¹¹ C]methyl) injection	825	Fosfomycin trometamol.....	1638
<i>Flumazenil (N-[¹¹C]methyl) solutio iniectabilis</i>	825	<i>Fosfomycinum calcicum</i>	1636
<i>Flumazenilum</i>	1604	<i>Fosfomycinum natricum</i>	1637
Flumequine	1605	<i>Fosfomycinum trometamolum</i>	1638
<i>Flumequinum</i>	1605	Fowl-pox vaccine (live)	766
Flumetasone pivalate	1607	<i>Framycetini sulfas</i>	1639
<i>Flumetasoni pivalas</i>	1607	Framycetin sulphate.....	1639
Flunarizine dihydrochloride.....	1608	Frangula bark	1641
<i>Flunarizini dihydrochloridum</i>	1608	Frangula bark dry extract, standardised	1642
Flunitrazepam.....	1609	<i>Frangulae cortex</i>	1641
<i>Flunitrazepamum</i>	1609	<i>Frangulae corticis extractum siccum normatum</i>	1642
Fluocinolone acetonide	1610	<i>Fraxini folium</i>	1026
		Free formaldehyde (2.4.18.).....	109
		Freezing point (2.2.18.).....	34
		Friability of uncoated tablets (2.9.7.).....	234

Fructose	1643	<i>Glucagonum humanum</i>	1665
<i>Fructosum</i>	1643	Glucose, anhydrous	1666
Fucus	1869	Glucose, liquid	1667
<i>Fucus vel Ascophyllum</i>	1869	Glucose, liquid, spray-dried	1668
Functional groups and ions, identification reactions (2.3.1.)	95	Glucose monohydrate	1669
Furosemide	1644	<i>Glucosum anhydricum</i>	1666
<i>Furosemidum</i>	1644	<i>Glucosum liquidum</i>	1667
Furunculosis vaccine (inactivated, oil-adjuvanted, injectable) for salmonids	767	<i>Glucosum liquidum dispersione desiccatum</i>	1668
Fusidic acid	1645	<i>Glucosum monohydricum</i>	1669
G		Glutamic acid	1670
Galactose	1649	Glycerol	1671
<i>Galactosum</i>	1649	Glycerol (85 per cent)	1672
Gallamine triethiodide	1649	Glycerol dibehenate	1673
<i>Gallamini triethiodidum</i>	1649	Glycerol distearate	1674
<i>Gallii</i> (⁶⁷ Ga) <i>citratissolutio iniectionabilis</i>	826	<i>Glyceroli dibehenas</i>	1673
Gallium (⁶⁷ Ga) citrate injection	826	<i>Glyceroli distearas</i>	1674
Gargles	612	<i>Glyceroli monolinoleas</i>	1675
Garlic for homoeopathic preparations	897	<i>Glyceroli mono-oleates</i>	1676
Garlic powder	1651	<i>Glyceroli monostearas 40-55</i>	1677
Gas chromatography (2.2.28.)	42	<i>Glyceroli trinitratissolutio</i>	1678
Gas detector tubes (2.1.6.)	19	Glycerol monolinoleate	1675
Gases, carbon dioxide in (2.5.24.)	134	Glycerol mono-oleates	1676
Gases, carbon monoxide in (2.5.25.)	134	Glycerol monostearate 40-55	1677
Gases, nitrogen monoxide and nitrogen dioxide in (2.5.26.)	135	Glycerol triacetate	2612
Gases, nitrous oxide in (2.5.35.)	141	<i>Glycerolum</i>	1671
Gases, oxygen in (2.5.27.)	136	<i>Glycerolum (85 per centum)</i>	1672
Gases, water in (2.5.28.)	136	Glyceryl trinitrate solution	1678
Gas-gangrene antitoxin, mixed	802	Glycine	1680
Gas-gangrene antitoxin (novyi)	802	<i>Glycinum</i>	1680
Gas-gangrene antitoxin (perfringens)	803	Glycyrrhizate ammonium	987
Gas-gangrene antitoxin (septicum)	804	Goldenrod	1680
Gastro-resistant capsules	600	Goldenrod, European	1682
Gastro-resistant granules	606	Goldenseal rhizome	1683
Gastro-resistant tablets	628	Gonadorelin acetate	1684
Gelatin	1651	<i>Gonadorelini acetas</i>	1684
<i>Gelatina</i>	1651	Gonadotrophin, chorionic	1686
Gels	625	Gonadotrophin, equine serum, for veterinary use	1686
General chapters (1.3.)	6	<i>Gonadotropinum chorionicum</i>	1686
General notices (1.)	5	<i>Gonadotropinum sericum equinum ad usum veterinarium</i>	1686
General statements (1.1.)	5	Goserelin	1687
General texts on sterility (5.1.)	445	<i>Goserelinum</i>	1687
General texts on vaccines (5.2.)	453	<i>Gossypii oleum hydrogenatum</i>	1370
<i>Gentamicini sulfas</i>	1653	Gramicidin	1689
Gentamicin sulphate	1653	<i>Gramicidinum</i>	1689
<i>Gentianae radix</i>	1654	<i>Graminis rhizoma</i>	1371
<i>Gentianae tinctura</i>	1655	<i>Granulata</i>	605
Gentian root	1654	Granules	605
Gentian tincture	1655	Granules, coated	606
Ginger	1656	Granules, effervescent	606
Gingival solutions	612	Granules, gastro-resistant	606
<i>Ginkgo folium</i>	1657	Granules, modified-release	606
Ginkgo leaf	1657	Greater celandine	1690
Ginseng	1658	Griseofulvin	1691
<i>Ginseng radix</i>	1658	<i>Griseofulvinum</i>	1691
Glass containers for pharmaceutical use (3.2.1.)	303	Guaifenesin	1692
Glibenclamide	1659	<i>Guaifenesinum</i>	1692
<i>Glibenclamidum</i>	1659	Guanethidine monosulphate	1694
Gliclazide	1660	<i>Guanethidini monosulfas</i>	1694
<i>Gliclazidum</i>	1660	Guar	1694
Glipizide	1662	Guar galactomannan	1695
<i>Glipizidum</i>	1662	<i>Guar galactomannanum</i>	1695
Glossary (dosage forms)	599	H	
Glucagon	1663	Haemodiafiltration and for haemofiltration, solutions for	1703
Glucagon, human	1665	Haemodialysis, concentrated solutions for	1700
<i>Glucagonum</i>	1663	Haemodialysis solutions, concentrated, water for diluting	1699

Haemodialysis, solutions for.....	1700	<i>Hexamidini diisetionas</i>	1720
Haemofiltration and for haemodiafiltration, solutions for	1703	Hexetidine.....	1721
Haemophilus type b (conjugate), diphtheria, tetanus, pertussis (acellular, component) and poliomyelitis (inactivated) vaccine (adsorbed)	653	<i>Hexetidinum</i>	1721
Haemophilus type b (conjugate), diphtheria, tetanus, pertussis and poliomyelitis (inactivated) vaccine (adsorbed).....	657	Hexobarbital.....	1722
Haemophilus type b conjugate vaccine.....	662	<i>Hexobarbitalum</i>	1722
Halofantrine hydrochloride	1705	Hexosamines in polysaccharide vaccines (2.5.20.)	132
<i>Halofantrini hydrochloridum</i>	1705	Hexylresorcinol.....	1723
Haloperidol.....	1706	<i>Hexylresorcinolum</i>	1723
Haloperidol decanoate.....	1708	<i>Hibisci sabdariffae flos</i>	2376
<i>Haloperidoli decanoas</i>	1708	Highly purified water	2695
<i>Haloperidolum</i>	1706	Histamine (2.6.10.).....	153
Halothane	1709	Histamine dihydrochloride	1724
<i>Halothanum</i>	1709	Histamine phosphate	1725
<i>Hamamelidis folium</i>	1711	<i>Histamini dihydrochloridum</i>	1724
Hamamelis leaf	1711	<i>Histamini phosphas</i>	1725
Hard capsules.....	600	Histidine	1726
Hard fat	1711	Histidine hydrochloride monohydrate	1727
Hard paraffin.....	2186	<i>Histidini hydrochloridum monohydricum</i>	1727
<i>Harpagophyti radix</i>	1401	<i>Histidinum</i>	1726
Hawthorn berries	1712	Homatropine hydrobromide	1728
Hawthorn leaf and flower	1713	Homatropine methylbromide	1729
Hawthorn leaf and flower dry extract	1714	<i>Homatropini hydrobromidum</i>	1728
Heavy bismuth subnitrate.....	1107	<i>Homatropini methylbromidum</i>	1729
Heavy kaolin.....	1869	Homoeopathic preparations.....	893
Heavy magnesium carbonate	1954	Homoeopathic preparations, arsenious trioxide for	895
Heavy magnesium oxide.....	1958	Homoeopathic preparations, common stinging nettle for	895
Heavy metals (2.4.8.)	104	Homoeopathic preparations, copper for	896
Heavy metals in herbal drugs and fatty oils (2.4.27.).....	119	Homoeopathic preparations, garlic for	897
<i>Helianthi annui oleum raffinatum</i>	2524	Homoeopathic preparations, herbal drugs for	893
<i>Heparina massae molecularis minoris</i>	1717	Homoeopathic preparations, honey bee for.....	898
Heparin, assay of (2.7.5.)	195	Homoeopathic preparations, hypericum for	898
Heparin calcium	1715	Homoeopathic preparations, iron for	899
Heparin in coagulation factors, assay of (2.7.12.).....	204	Homoeopathic preparations, mother tinctures for.....	894
Heparins, low-molecular-mass	1717	Homoeopathic preparations, saffron for	900
Heparin sodium	1716	Honey bee for homoeopathic preparations.....	898
<i>Heparinum calcicum</i>	1715	Hop strobile	1730
<i>Heparinum natricum</i>	1716	Human albumin injection, iodinated (¹²⁵ I).....	827
Hepatitis A immunoglobulin, human	1741	Human albumin solution	1731
Hepatitis A (inactivated) and hepatitis B (rDNA) vaccine (adsorbed).....	664	Human anti-D immunoglobulin	1732
Hepatitis A vaccine, assay of (2.7.14.).....	207	Human anti-D immunoglobulin, assay of (2.7.13.).....	205
Hepatitis A vaccine (inactivated, adsorbed)	665	Human anti-D immunoglobulin for intravenous administration	1733
Hepatitis A vaccine (inactivated, virosome)	667	Human antithrombin III, assay of (2.7.17.).....	209
Hepatitis B immunoglobulin for intravenous administration, human	1741	Human antithrombin III concentrate	1733
Hepatitis B immunoglobulin, human	1741	Human coagulation factor II, assay of (2.7.18.).....	209
Hepatitis B (rDNA), diphtheria and tetanus vaccine (adsorbed).....	641	Human coagulation factor IX.....	1738
Hepatitis B (rDNA), diphtheria, tetanus and pertussis (acellular, component) vaccine (adsorbed)	647	Human coagulation factor IX, assay of (2.7.11.).....	204
Hepatitis B vaccine (rDNA).....	670	Human coagulation factor VII	1734
Hepatitis B vaccine (rDNA), assay of (2.7.15.)	207	Human coagulation factor VII, assay of (2.7.10.).....	203
Hepatitis C virus (HCV), validation of nucleic acid amplification techniques for the detection of HCV RNA in plasma pools: Guidelines.....	176	Human coagulation factor VIII.....	1736
Heptaminol hydrochloride.....	1719	Human coagulation factor VIII, assay of (2.7.4.).....	194
<i>Heptaminoli hydrochloridum</i>	1719	Human coagulation factor VIII (rDNA).....	1737
Herbal drug preparations.....	572	Human coagulation factor X, assay of (2.7.19.).....	210
Herbal drugs	572	Human coagulation factor XI.....	1739
Herbal drugs and fatty oils, heavy metals in (2.4.27.).....	119	Human coagulation factor XI, assay of (2.7.22.)	212
Herbal drugs, determination of tannins (2.8.14.)	221	Human fibrinogen.....	1740
Herbal drugs for homoeopathic preparations	893	Human hepatitis A immunoglobulin	1741
Herbal teas.....	573	Human hepatitis B immunoglobulin	1741
Hexamidine diisetionate	1720	Human hepatitis B immunoglobulin for intravenous administration	1741
		Human insulin	1800
		Human measles immunoglobulin.....	1742
		Human normal immunoglobulin.....	1742
		Human normal immunoglobulin for intravenous administration	1744
		Human plasma for fractionation.....	1746
		Human plasma (pooled and treated for virus inactivation)	1747

Human prothrombin complex.....	1748	<i>Hypericum perforatum</i>	
Human rabies immunoglobulin.....	1750	<i>ad praeparationes homoeopathicas</i>	898
Human rubella immunoglobulin.....	1751	Hypromellose.....	1780
Human tetanus immunoglobulin.....	1751	Hypromellose phthalate.....	1781
Human varicella immunoglobulin.....	1752	<i>Hypromellosi phthalas</i>	1781
Human varicella immunoglobulin for intravenous administration.....	1753	<i>Hypromellosum</i>	1780
Human von Willebrand factor, assay of (2.7.21.).....	211	I	
Hyaluronidase.....	1753	Ibuprofen.....	1785
<i>Hyaluronidasum</i>	1753	<i>Ibuprofenum</i>	1785
Hydralazine hydrochloride.....	1754	Iceland moss.....	1787
<i>Hydralazini hydrochloridum</i>	1754	ICH (5.8.).....	551
<i>Hydrargyri dichloridum</i>	1995	Ichthammol.....	1787
<i>Hydrastis rhizoma</i>	1683	<i>Ichthammolum</i>	1787
Hydrochloric acid, concentrated.....	1755	Identification (2.3.).....	95
Hydrochloric acid, dilute.....	1756	Identification and control of residual solvents (2.4.24.)....	113
Hydrochlorothiazide.....	1756	Identification of fatty oils by thin-layer chromatography (2.3.2.).....	98
<i>Hydrochlorothiazidum</i>	1756	Identification of phenothiazines by thin-layer chromatography (2.3.3.).....	99
Hydrocortisone.....	1757	Identification reactions of ions and functional groups (2.3.1.).....	95
Hydrocortisone acetate.....	1759	Idoxuridine.....	1788
Hydrocortisone hydrogen succinate.....	1761	<i>Idoxuridinum</i>	1788
<i>Hydrocortisoni acetas</i>	1759	<i>Iecoris aselli oleum A</i>	1348
<i>Hydrocortisoni hydrogenosuccinas</i>	1761	<i>Iecoris aselli oleum B</i>	1352
<i>Hydrocortisonum</i>	1757	Ifosfamide.....	1789
Hydrogenated arachis oil.....	1018	<i>Ifosfamidum</i>	1789
Hydrogenated castor oil.....	1197	Imipenem.....	1791
Hydrogenated cottonseed oil.....	1370	<i>Imipenemum</i>	1791
Hydrogenated soya-bean oil.....	2475	Imipramine hydrochloride.....	1792
Hydrogenated wool fat.....	2708	<i>Imipramini hydrochloridum</i>	1792
<i>Hydrogenii peroxidum 30 per centum</i>	1763	Immunochemical methods (2.7.1.).....	187
<i>Hydrogenii peroxidum 3 per centum</i>	1762	Immunoglobulin, animal, anti-T lymphocyte, for human use.....	1010
Hydrogen peroxide solution (30 per cent).....	1763	Immunoglobulin for intravenous administration, human hepatitis B.....	1741
Hydrogen peroxide solution (3 per cent).....	1762	Immunoglobulin for intravenous administration, human normal.....	1744
Hydromorphone hydrochloride.....	1763	Immunoglobulin for intravenous administration, human varicella.....	1753
<i>Hydromorphoni hydrochloridum</i>	1763	Immunoglobulin, human anti-D.....	1732
Hydrous wool fat.....	2709	Immunoglobulin, human anti-D, assay of (2.7.13.).....	205
Hydroxocobalamin acetate.....	1765	Immunoglobulin, human anti-D, for intravenous administration.....	1733
Hydroxocobalamin chloride.....	1766	Immunoglobulin, human hepatitis A.....	1741
<i>Hydroxocobalamini acetas</i>	1765	Immunoglobulin, human hepatitis B.....	1741
<i>Hydroxocobalamini chloridum</i>	1766	Immunoglobulin, human measles.....	1742
<i>Hydroxocobalamini sulfas</i>	1767	Immunoglobulin, human normal.....	1742
Hydroxocobalamin sulphate.....	1767	Immunoglobulin, human rabies.....	1750
Hydroxycarbamide.....	1768	Immunoglobulin, human rubella.....	1751
<i>Hydroxycarbamidum</i>	1768	Immunoglobulin, human tetanus.....	1751
Hydroxyethylcellulose.....	1770	Immunoglobulin, human varicella.....	1752
<i>Hydroxyethylcellulosum</i>	1770	Immunoglobulin, test for anticomplementary activity of (2.6.17.).....	170
<i>Hydroxyethylis salicylas</i>	1769	Immunoglobulin, test for Fc function of (2.7.9.).....	202
Hydroxyethylmethylcellulose.....	2018	<i>Immunoglobulinum anti-T lymphocytorum ex animale ad usum humanum</i>	1010
Hydroxyethyl salicylate.....	1769	<i>Immunoglobulinum humanum anti-D</i>	1732
Hydroxyl value (2.5.3.).....	127	<i>Immunoglobulinum humanum anti-D ad usum intravenosum</i>	1733
Hydroxypropylbetadex.....	1771	<i>Immunoglobulinum humanum hepatitis A</i>	1741
<i>Hydroxypropylbetadexum</i>	1771	<i>Immunoglobulinum humanum hepatitis B</i>	1741
Hydroxypropylcellulose.....	1773	<i>Immunoglobulinum humanum hepatitis B ad usum intravenosum</i>	1741
<i>Hydroxypropylcellulosum</i>	1773	<i>Immunoglobulinum humanum morbillicum</i>	1742
Hydroxypropylmethylcellulose.....	1780	<i>Immunoglobulinum humanum normale</i>	1742
Hydroxypropylmethylcellulose phthalate.....	1781	<i>Immunoglobulinum humanum normale ad usum intravenosum</i>	1744
Hydroxyzine hydrochloride.....	1774		
<i>Hydroxyzini hydrochloridum</i>	1774		
Hymecromone.....	1775		
<i>Hymecromonum</i>	1775		
Hyoscine butylbromide.....	1776		
Hyoscine hydrobromide.....	1777		
<i>Hyoscini butylbromidum</i>	1776		
<i>Hyoscini hydrobromidum</i>	1777		
Hyoscyamine sulphate.....	1778		
<i>Hyoscyamini sulfas</i>	1778		
<i>Hyperici herba</i>	2485		
Hypericum.....	2485		
Hypericum for homoeopathic preparations.....	898		

<i>Immunoglobulinum humanum rabicum</i>	1750	Insulin, bovine	1797
<i>Immunoglobulinum humanum rubellae</i>	1751	Insulin, human.....	1800
<i>Immunoglobulinum humanum tetanicum</i>	1751	<i>Insulini biphasici iniectionabilium</i>	1802
<i>Immunoglobulinum humanum varicellae</i>	1752	<i>Insulini isophani biphasici iniectionabilium</i>	1803
<i>Immunoglobulinum humanum varicellae ad usum</i> <i>intravenosum</i>	1753	<i>Insulini isophani iniectionabilium</i>	1803
<i>Immunosera ad usum veterinarium</i>	575	Insulin injection, biphasic	1802
Immunosera and vaccines, phenol in (2.5.15.).....	131	Insulin injection, biphasic isophane	1803
<i>Immunosera ex animale ad usum humanum</i>	573	Insulin injection, isophane.....	1803
Immunosera for human use, animal.....	573	Insulin injection, soluble	1803
Immunosera for veterinary use	575	<i>Insulini solubilis iniectionabilium</i>	1803
<i>Immunoserum botulinicum</i>	801	<i>Insulini zinci amorphi suspensio iniectionabilis</i>	1811
<i>Immunoserum clostridii novyi alpha ad usum</i> <i>veterinarium</i>	809	<i>Insulini zinci cristallini suspensio iniectionabilis</i>	1812
<i>Immunoserum clostridii perfringentis beta ad usum</i> <i>veterinarium</i>	810	<i>Insulini zinci suspensio iniectionabilis</i>	1811
<i>Immunoserum clostridii perfringentis epsilon ad usum</i> <i>veterinarium</i>	811	Insulin lispro	1804
<i>Immunoserum contra venena viperarum</i> <i>europaearum</i>	806	Insulin, porcine.....	1806
<i>Immunoserum diphthericum</i>	801	Insulin preparations, injectable	1808
<i>Immunoserum gangraenicum (Clostridium novyi)</i>	802	<i>Insulinum aspartum</i>	1795
<i>Immunoserum gangraenicum</i> <i>(Clostridium perfringens)</i>	803	<i>Insulinum bovinum</i>	1797
<i>Immunoserum gangraenicum (Clostridium septicum)</i> ..	804	<i>Insulinum humanum</i>	1800
<i>Immunoserum gangraenicum mixtum</i>	802	<i>Insulinum lisprum</i>	1804
<i>Immunoserum tetanicum ad usum humanum</i>	805	<i>Insulinum porcinum</i>	1806
<i>Immunoserum tetanicum ad usum veterinarium</i>	812	Insulin zinc injectable suspension	1811
Implants	614	Insulin zinc injectable suspension (amorphous)	1811
Impurities in substances for pharmaceutical use, control of (5.10.).....	559	Insulin zinc injectable suspension (crystalline)	1812
Indapamide	1793	Interferon alfa-2 concentrated solution	1812
<i>Indapamidum</i>	1793	Interferon gamma-1b concentrated solution	1815
Indicators, relationship between approximate pH and colour (2.2.4.)	27	<i>Interferoni alfa-2 solutio concentrata</i>	1812
<i>Indii (¹¹¹In) chloridi solutio</i>	828	<i>Interferoni gamma-1b solutio concentrata</i>	1815
<i>Indii (¹¹¹In) oxini solutio</i>	829	Interferons, assay of (5.6.).....	533
<i>Indii (¹¹¹In) pentetatis solutio iniectionabilis</i>	830	<i>int-rac-α-Tocopherolum</i>	2590
Indium (¹¹¹ In) chloride solution	828	<i>int-rac-α-Tocopherylis acetate</i>	2594
Indium (¹¹¹ In) oxine solution	829	Intramammary preparations for veterinary use.....	606
Indium (¹¹¹ In) pentetate injection.....	830	Intratumoral devices	607
Indometacin	1794	<i>In vivo</i> assay of poliomyelitis vaccine (inactivated) (2.7.20.)	210
<i>Indometacinum</i>	1794	Iobenguane (¹²³ I) injection.....	831
Infectious bovine rhinotracheitis vaccine (live), freeze-dried.....	768	Iobenguane (¹³¹ I) injection for diagnostic use	832
Infectious bronchitis vaccine (inactivated), avian	718	Iobenguane (¹³¹ I) injection for therapeutic use	833
Infectious bronchitis vaccine (live), avian.....	720	<i>Iobenguani (¹²³I) solutio iniectionabilis</i>	831
Infectious bursal disease vaccine (inactivated), avian.....	722	<i>Iobenguani (¹³¹I) solutio iniectionabilis ad usum</i> <i>diagnosticum</i>	832
Infectious bursal disease vaccine (live), avian	723	<i>Iobenguani (¹³¹I) solutio iniectionabilis ad usum</i> <i>therapeuticum</i>	833
Infectious chicken anaemia vaccine (live)	769	Iodinated (¹²⁵ I) human albumin injection	827
Infectious encephalomyelitis vaccine (live), avian	725	Iodinated povidone	2291
Infectious laryngotracheitis vaccine (live), avian	727	<i>Iodinati (¹²⁵I) humani albumini solutio iniectionabilis</i>	827
Influenza vaccine (split virion, inactivated)	671	Iodine.....	1819
Influenza vaccine (surface antigen, inactivated).....	673	Iodine value (2.5.4.).....	127
Influenza vaccine (surface antigen, inactivated, virosome).....	674	<i>Iodum</i>	1819
Influenza vaccine (whole virion, inactivated)	676	Iohexol.....	1819
Infrared absorption spectrophotometry (2.2.24.)	37	<i>Iohexolum</i>	1819
Infusions	614	Ionic concentration, potentiometric determination of using ion-selective electrodes (2.2.36.).....	55
<i>Inhalanda</i>	618	Ions and functional groups, identification reactions (2.3.1.).....	95
Inhalation gas, krypton (^{81m} Kr).....	833	Ion-selective electrodes, potentiometric determination of ionic concentration (2.2.36.)	55
Inhalation, preparations for.....	618	Iopamidol.....	1822
Inhalation, preparations for: aerodynamic assessment of fine particles (2.9.18.)	244	<i>Iopamidolum</i>	1822
Injections	614	Iopanoic acid.....	1824
Injections or infusions, concentrates for	614	Iotalamic acid.....	1825
Injections or infusions, powders for	614	Ioxaglic acid	1826
Inserts, ophthalmic.....	604	<i>Ipecacuanhae extractum fluidum normatum</i>	1828
Insulin aspart.....	1795	<i>Ipecacuanhae pulvis normatus</i>	1829
		<i>Ipecacuanhae radix</i>	1829
		<i>Ipecacuanhae tinctura normata</i>	1830
		Ipecacuanha liquid extract, standardised	1828
		Ipecacuanha, prepared.....	1829
		Ipecacuanha root	1829

Ipecacuanha tincture, standardised.....	1830	<i>Kalii hydrogenotartras</i>	2282
<i>Ipratropii bromidum</i>	1831	<i>Kalii hydroxidum</i>	2283
Ipratropium bromide.....	1831	<i>Kalii iodidum</i>	2283
Iron (2.4.9.).....	107	<i>Kalii metabisulfis</i>	2284
Iron for homeopathic preparations.....	899	<i>Kalii natrii tartras tetrahydricus</i>	2286
Irrigation, preparations for.....	622	<i>Kalii nitras</i>	2284
Isoconazole.....	1833	<i>Kalii perchloras</i>	2285
Isoconazole nitrate.....	1834	<i>Kalii permanganas</i>	2286
<i>Isoconazoli nitras</i>	1834	<i>Kalii sorbas</i>	2287
<i>Isoconazolum</i>	1833	<i>Kalii sulfas</i>	2288
Isoelectric focusing (2.2.54.).....	81	Kanamycin acid sulphate.....	1867
Isoflurane.....	1835	<i>Kanamycini monosulfas</i>	1868
<i>Isofluranum</i>	1835	<i>Kanamycini sulfas acidus</i>	1867
Isoleucine.....	1836	Kanamycin monosulphate.....	1868
<i>Isoleucinum</i>	1836	Kaolin, heavy.....	1869
Isomalt.....	1837	<i>Kaolinum ponderosum</i>	1869
<i>Isomaltum</i>	1837	Kelp.....	1869
Isoniazid.....	1839	Ketamine hydrochloride.....	1870
<i>Isoniazidum</i>	1839	<i>Ketamini hydrochloridum</i>	1870
Isophane insulin injection.....	1803	Ketobemidone hydrochloride.....	1871
Isoprenaline hydrochloride.....	1839	Ketoconazole.....	1872
Isoprenaline sulphate.....	1840	<i>Ketoconazolum</i>	1872
<i>Isoprenalini hydrochloridum</i>	1839	Ketoprofen.....	1874
<i>Isoprenalini sulfas</i>	1840	<i>Ketoprofenum</i>	1874
Isopropyl alcohol.....	1841	Ketotifen hydrogen fumarate.....	1875
<i>Isopropylis myristas</i>	1842	<i>Ketotifeni hydrogenofumaras</i>	1875
<i>Isopropylis palmitas</i>	1843	Knotgrass.....	1877
Isopropyl myristate.....	1842	Krypton (^{81m} Kr) inhalation gas.....	833
Isopropyl palmitate.....	1843	<i>Kryptonum (^{81m}Kr) ad inhalationem</i>	833
Isosorbide dinitrate, diluted.....	1844		
Isosorbide mononitrate, diluted.....	1845	L	
<i>Isosorbidi dinitras dilutus</i>	1844	Labetalol hydrochloride.....	1881
<i>Isosorbidi mononitras dilutus</i>	1845	<i>Labetaloli hydrochloridum</i>	1881
Isotretinoin.....	1847	<i>Lacca</i>	2409
<i>Isotretinoinum</i>	1847	Lactic acid.....	1882
Isoxsuprine hydrochloride.....	1848	Lactic acid, (S)-.....	1883
<i>Isoxsuprini hydrochloridum</i>	1848	Lactitol monohydrate.....	1883
Ispaghula husk.....	1849	<i>Lactitolum monohydricum</i>	1883
Ispaghula seed.....	1850	Lactobionic acid.....	1885
Isradipine.....	1851	Lactose, anhydrous.....	1886
<i>Isradipinum</i>	1851	Lactose monohydrate.....	1887
Itraconazole.....	1852	<i>Lactosum anhydricum</i>	1886
<i>Itraconazolum</i>	1852	<i>Lactosum monohydricum</i>	1887
<i>Juniperi aetheroleum</i>	1863	Lactulose.....	1888
<i>Juniperi pseudo-fructus</i>	1862	Lactulose, liquid.....	1890
Ivermectin.....	1854	<i>Lactulosum</i>	1888
<i>Ivermectinum</i>	1854	<i>Lactulosum liquidum</i>	1890
		<i>Lanugo cellulosi absorbens</i>	2681
J		<i>Lanugo gossypii absorbens</i>	1369
Javanese turmeric.....	2645	Lauroyl macrogolglycerides.....	1892
Java tea.....	1859	<i>Lavandulae aetheroleum</i>	1894
Josamycin.....	1860	<i>Lavandulae flos</i>	1893
<i>Josamycini propionas</i>	1861	Lavender flower.....	1893
Josamycin propionate.....	1861	Lavender oil.....	1894
<i>Josamycinum</i>	1860	Lead in sugars (2.4.10.).....	107
Juniper.....	1862	Lemon oil.....	1895
Juniper oil.....	1863	<i>Leonuri cardiaca herba</i>	2063
		Leptospirosis vaccine (inactivated), bovine.....	730
K		Leptospirosis vaccine (inactivated), canine.....	740
<i>Kalii acetas</i>	2273	Leucine.....	1897
<i>Kalii bromidum</i>	2273	<i>Leucinum</i>	1897
<i>Kalii carbonas</i>	2274	Leuporelin.....	1898
<i>Kalii chloridum</i>	2275	<i>Leuporelinum</i>	1898
<i>Kalii citras</i>	2275	Levamisole for veterinary use.....	1899
<i>Kalii clavulanas</i>	2276	Levamisole hydrochloride.....	1900
<i>Kalii clavulanas dilutus</i>	2278	<i>Levamisoli hydrochloridum</i>	1900
<i>Kalii dihydrogenophosphas</i>	2280	<i>Levamisolum ad usum veterinarium</i>	1899
<i>Kalii hydrogenoaspartas hemihydricus</i>	2280	<i>Levistici radix</i>	1932
<i>Kalii hydrogenocarbonas</i>	2281	Levocabastine hydrochloride.....	1902

<i>Levocabastini hydrochloridum</i>	1902	<i>Lithii citras</i>	1924
Levocarnitine	1903	Lithium carbonate	1923
<i>Levocarnitinum</i>	1903	Lithium citrate	1924
Levodopa	1904	L-Methionine ([¹¹ C]methyl) injection	834
<i>Levodopum</i>	1904	<i>L-Methionini</i> ([¹¹ C]methyl) solutio iniectabilis	834
Levodropipizine	1905	Lobeline hydrochloride	1925
<i>Levodropipizinum</i>	1905	<i>Lobelini hydrochloridum</i>	1925
Levomenthol	1907	Lomustine	1926
<i>Levomentholum</i>	1907	<i>Lomustinum</i>	1926
Levomepromazine hydrochloride	1908	Loosestrife	1927
Levomepromazine maleate	1908	Loperamide hydrochloride	1928
<i>Levomepromazini hydrochloridum</i>	1908	Loperamide oxide monohydrate	1930
<i>Levomepromazini maleas</i>	1908	<i>Loperamidi hydrochloridum</i>	1928
Levomethadone hydrochloride	1909	<i>Loperamidi oxidum monohydricum</i>	1930
<i>Levomethadoni hydrochloridum</i>	1909	Lorazepam	1931
Levonorgestrel	1911	<i>Lorazepamum</i>	1931
<i>Levonorgestrelum</i>	1911	Loss on drying (2.2.32.)	50
Levothyroxine sodium	1912	Loss on drying of extracts (2.8.17.)	222
<i>Levothyroxinum natricum</i>	1912	Lovage root	1932
<i>Lichen islandicus</i>	1787	Lovastatin	1933
Lidocaine	1913	<i>Lovastatinum</i>	1933
Lidocaine hydrochloride	1914	Low-molecular-mass heparins	1717
<i>Lidocaini hydrochloridum</i>	1914	Lozenges and pastilles	613
<i>Lidocainum</i>	1913	Lozenges, compressed	613
Light liquid paraffin	2186	Lubricant, silicone oil (3.1.8.)	287
Light magnesium carbonate	1954	<i>Lupuli flos</i>	1730
Light magnesium oxide	1958	Lynestrenol	1935
Lime flower	1914	<i>Lynestrenolum</i>	1935
Limit test of particle size by microscopy (2.9.13.)	239	Lysine acetate	1935
Limit tests (2.4.)	103	Lysine hydrochloride	1936
Limit tests, standard solutions for (4.1.2.)	426	<i>Lysini acetas</i>	1935
<i>Limonis aetheroleum</i>	1895	<i>Lysini hydrochloridum</i>	1936
Lincomycin hydrochloride	1915	<i>Lythri herba</i>	1927
<i>Lincomycini hydrochloridum</i>	1915		
Lindane	1916	M	
<i>Lindanum</i>	1916	Macrogol 15 hydroxystearate	1941
Linen thread, sterile, in distributor for veterinary use	886	<i>Macrogol 20 glyceroli monostearas</i>	1942
<i>Lini oleum virginale</i>	1918	Macrogol 20 glycerol monostearate	1942
<i>Lini semen</i>	1918	Macrogol 6 glycerol caprylocaprate	1941
Linoleoyl macrogolglycerides	1917	<i>Macrogol 6 glyceroli caprylocapras</i>	1941
Linseed	1918	<i>Macrogola</i>	1950
Linseed oil, virgin	1918	Macrogol cetostearyl ether	1943
Liothyronine sodium	1919	<i>Macrogolglyceridorum caprylocaprates</i>	1173
<i>Liothyroninum natricum</i>	1919	<i>Macrogolglyceridorum laurates</i>	1892
Liquid and semi-solid preparations, test for deliverable mass or volume (2.9.28.)	263	<i>Macrogolglyceridorum linoleates</i>	1917
Liquid chromatography (2.2.29.)	43	<i>Macrogolglyceridorum oleates</i>	2133
Liquid extracts	571	<i>Macrogolglyceridorum stearates</i>	2491
Liquid glucose	1667	Macrogolglycerol cocoates	1947
Liquid glucose, spray-dried	1668	Macrogolglycerol hydroxystearate	1948
Liquid lactulose	1890	<i>Macrogolglyceroli cocoates</i>	1947
Liquid maltitol	1969	<i>Macrogolglyceroli hydroxystearas</i>	1948
Liquid paraffin	2187	<i>Macrogolglyceroli ricinoleas</i>	1949
Liquid preparations for cutaneous application	607	Macrogolglycerol ricinoleate	1949
Liquid preparations for cutaneous application, veterinary	630	<i>Macrogoli 15 hydroxystearas</i>	1941
Liquid preparations for inhalation	618	<i>Macrogoli aether cetostearylicus</i>	1943
Liquid preparations for oral use	608	<i>Macrogoli aether laurilicum</i>	1944
Liquid sorbitol (crystallising)	2471	<i>Macrogoli aether oleicum</i>	1945
Liquid sorbitol (non-crystallising)	2472	<i>Macrogoli aether stearylicus</i>	1946
Liquid sorbitol, partially dehydrated	2473	<i>Macrogoli oleas</i>	1944
<i>Liquiritiae extractum fluidum</i>		<i>Macrogoli stearas</i>	1946
<i>ethanolicum normatum</i>	1920	Macrogol lauryl ether	1944
<i>Liquiritiae radix</i>	1921	Macrogol oleate	1944
Liquorice ethanolic liquid extract, standardised	1920	Macrogol oleyl ether	1945
Liquorice root	1921	Macrogols	1950
Lisinopril dihydrate	1922	Macrogol stearate	1946
<i>Lisinoprilum dihydricum</i>	1922	Macrogol stearyl ether	1946
<i>Lithii carbonas</i>	1923	Magaldrate	1951
		<i>Magaldratum</i>	1951
		<i>Magnesii acetas tetrahydricus</i>	1952

<i>Magnesii aspartas dihydricus</i>	1953	Materials based on plasticised poly(vinyl chloride) for containers for aqueous solutions for intravenous infusion (3.1.14.)	296
<i>Magnesii chloridum 4.5-hydricum</i>	1955	Materials based on plasticised poly(vinyl chloride) for containers for human blood and blood components (3.1.1.1.)	269
<i>Magnesii chloridum hexahydricum</i>	1956	Materials based on plasticised poly(vinyl chloride) for tubing used in sets for the transfusion of blood and blood components (3.1.1.2.)	272
<i>Magnesii glycerophosphas</i>	1957	Materials for containers for human blood and blood components (3.1.1.)	269
<i>Magnesii hydroxidum</i>	1957	Materials used for the manufacture of containers (3.1.) ..	269
<i>Magnesii oxidum leve</i>	1958	<i>Matricariae aetheroleum</i>	1978
<i>Magnesii oxidum ponderosum</i>	1958	<i>Matricariae extractum fluidum</i>	1977
<i>Magnesii peroxidum</i>	1959	<i>Matricariae flos</i>	1976
<i>Magnesii pidolas</i>	1960	Matricaria flower	1976
<i>Magnesii stearas</i>	1961	Matricaria liquid extract	1977
<i>Magnesii subcarbonas levis</i>	1954	Matricaria oil	1978
<i>Magnesii subcarbonas ponderosus</i>	1954	<i>Maydis amyllum</i>	1964
<i>Magnesii sulfas heptahydricus</i>	1962	<i>Maydis oleum raffinatum</i>	1964
<i>Magnesii trisilicas</i>	1963	Meadowsweet	1980
Magnesium (2.4.6.)	104	Measles immunoglobulin, human	1742
Magnesium acetate tetrahydrate	1952	Measles, mumps and rubella vaccine (live)	678
Magnesium and alkaline-earth metals (2.4.7.)	104	Measles vaccine (live)	679
Magnesium aspartate dihydrate	1953	Measurement of consistency by penetrometry (2.9.9.)	235
Magnesium carbonate, heavy	1954	Mebendazole	1981
Magnesium carbonate, light	1954	<i>Mebendazolum</i>	1981
Magnesium chloride 4.5-hydrate	1955	Meclozine hydrochloride	1982
Magnesium chloride hexahydrate	1956	<i>Meclozini hydrochloridum</i>	1982
Magnesium glycerophosphate	1957	Medicated chewing gum, drug release from (2.9.25.)	260
Magnesium hydroxide	1957	Medicated chewing gums	601
Magnesium oxide, heavy	1958	Medicated feeding stuffs for veterinary use, premixes for ..	617
Magnesium oxide, light	1958	Medicated foams	604
Magnesium peroxide	1959	Medicated plasters	626
Magnesium pidolate	1960	Medicated tampons	628
Magnesium stearate	1961	Medicated vaginal tampons	630
Magnesium sulphate heptahydrate	1962	Medicinal air	929
Magnesium trisilicate	1963	Medicinal air, synthetic	932
Maize oil, refined	1964	Medium-chain triglycerides	2623
Maize starch	1964	Medroxyprogesterone acetate	1983
Malathion	1965	<i>Medroxyprogesteroni acetas</i>	1983
<i>Malathionum</i>	1965	Mefenamic acid	1984
Maleic acid	1966	Mefloquine hydrochloride	1986
Malic acid	1966	<i>Mefloquini hydrochloridum</i>	1986
Mallow flower	1967	Megestrol acetate	1987
Maltitol	1968	<i>Megestrol acetas</i>	1987
Maltitol, liquid	1969	Meglumine	1988
<i>Maltitolum</i>	1968	<i>Megluminum</i>	1988
<i>Maltitolum liquidum</i>	1969	<i>Melaleuca aetheroleum</i>	2534
Maltodextrin	1970	<i>Melissae folium</i>	1989
<i>Maltodextrinum</i>	1970	Melissa leaf	1989
<i>Malvae sylvestris flos</i>	1967	Melting point - capillary method (2.2.14.)	32
Manganese sulphate monohydrate	1971	Melting point - instantaneous method (2.2.16.)	33
<i>Mangani sulfas monohydricus</i>	1971	Melting point - open capillary method (2.2.15.)	33
Mannheimia vaccine (inactivated) for cattle	771	Menadione	1990
Mannheimia vaccine (inactivated) for sheep	772	<i>Menadionum</i>	1990
Mannitol	1971	Meningococcal group C conjugate vaccine	680
<i>Mannitolum</i>	1971	Meningococcal polysaccharide vaccine	682
Maprotiline hydrochloride	1973	<i>Menthae arvensis aetheroleum partim mentholi privum</i>	2050
<i>Maprotilini hydrochloridum</i>	1973	<i>Menthae piperitae aetheroleum</i>	2206
Marek's disease vaccine (live)	774	<i>Menthae piperitae folium</i>	2205
Marshmallow leaf	1974	Menthol, racemic	1991
Marshmallow root	1975	<i>Mentholum racemicum</i>	1991
Mass spectrometry (2.2.43.)	65	<i>Menyanthis trifoliatae folium</i>	1115
Mass uniformity of delivered doses from multidose containers (2.9.27.)	263	Mepivacaine hydrochloride	1992
Mass uniformity of single-dose preparations (2.9.5.)	233	<i>Mepivacaini hydrochloridum</i>	1992
Mastic	1975	Meprobamate	1993
<i>Masticabilia gummis medicata</i>	601	<i>Meprobamatum</i>	1993
<i>Mastix</i>	1975		
Materials based on non-plasticised poly(vinyl chloride) for containers for dry dosage forms for oral administration (3.1.11.)	291		
Materials based on non-plasticised poly(vinyl chloride) for containers for non-injectable, aqueous solutions (3.1.10.)	289		

Mepyramine maleate	1994	<i>Methylthioninii chloridum</i>	2028
<i>Mepyramini maleas</i>	1994	Methylthioninium chloride	2028
Mercaptopurine	1995	Metixene hydrochloride	2029
<i>Mercaptopurinum</i>	1995	<i>Metixeni hydrochloridum</i>	2029
Mercuric chloride	1995	Metoclopramide	2030
Mesalazine	1996	Metoclopramide hydrochloride	2031
<i>Mesalazinum</i>	1996	<i>Metoclopramidi hydrochloridum</i>	2031
Mesna	1998	<i>Metoclopramidum</i>	2030
<i>Mesnum</i>	1998	<i>Metoprololi succinas</i>	2032
Mesterolone	1999	<i>Metoprololi tartras</i>	2034
<i>Mesterolonom</i>	1999	Metoprolol succinate	2032
Mestranol	2000	Metoprolol tartrate	2034
<i>Mestranolum</i>	2000	Metrifonate	2035
Metacresol	2001	<i>Metrifonatum</i>	2035
<i>Metacresolum</i>	2001	Metronidazole	2037
Metamizole sodium	2002	Metronidazole benzoate	2038
<i>Metamizolum natricum</i>	2002	<i>Metronidazoli benzoas</i>	2038
Metformin hydrochloride	2003	<i>Metronidazolum</i>	2037
<i>Metformini hydrochloridum</i>	2003	Mexiletine hydrochloride	2039
Methacrylic acid - ethyl acrylate copolymer (1:1)	2005	<i>Mexiletini hydrochloridum</i>	2039
Methacrylic acid - ethyl acrylate copolymer (1:1) dispersion 30 per cent	2005	Mianserin hydrochloride	2041
Methacrylic acid - methyl methacrylate copolymer (1:1)	2006	<i>Mianserini hydrochloridum</i>	2041
Methacrylic acid - methyl methacrylate copolymer (1:2)	2007	Miconazole	2042
Methadone hydrochloride	2007	Miconazole nitrate	2043
<i>Methadoni hydrochloridum</i>	2007	<i>Miconazoli nitras</i>	2043
Methanol and 2-propanol, test for (2.9.11.)	239	<i>Miconazolum</i>	2042
Methaqualone	2008	Microbiological assay of antibiotics (2.7.2.)	188
<i>Methaqualonum</i>	2008	Microbiological examination of non-sterile products (test for specified micro-organisms) (2.6.13.)	156
Methenamine	2009	Microbiological examination of non-sterile products (total viable aerobic count) (2.6.12.)	154
<i>Methenaminum</i>	2009	Microbiological quality of pharmaceutical preparations (5.1.4.)	449
Methionine	2010	Microcrystalline cellulose	1228
Methionine ([¹⁴ C]methyl) injection, L-	834	Micro determination of water (2.5.32.)	137
Methionine, DL-	2010	Microscopy, limit test of particle size (2.9.13.)	239
<i>Methioninum</i>	2010	Midazolam	2045
Methods in pharmacognosy (2.8.)	215	<i>Midazolamum</i>	2045
Methods of preparation of sterile products (5.1.1.)	445	Milk-thistle fruit	2046
Methotrexate	2011	<i>Millefolii herba</i>	2723
<i>Methotrexatum</i>	2011	Minimising the risk of transmitting animal spongiform encephalopathy agents via human and veterinary medicinal products (5.2.8.)	463
Methylatropine bromide	2014	Minocycline hydrochloride	2047
Methylatropine nitrate	2015	<i>Minocyclini hydrochloridum</i>	2047
<i>Methylatropini bromidum</i>	2014	Minoxidil	2049
<i>Methylatropini nitras</i>	2015	<i>Minoxidilum</i>	2049
Methylcellulose	2016	Mint oil, partly dementholised	2050
<i>Methylcellulosum</i>	2016	Mitomycin	2051
Methyldopa	2016	<i>Mitomycinum</i>	2051
<i>Methyldopum</i>	2016	Mitoxantrone hydrochloride	2053
Methylene blue	2028	<i>Mitoxantroni hydrochloridum</i>	2053
Methylene chloride	2017	Modified-release capsules	600
<i>Methyleni chloridum</i>	2017	Modified-release granules	606
Methylhydroxyethylcellulose	2018	Modified-release tablets	628
<i>Methylhydroxyethylcellulosum</i>	2018	Molecular mass distribution in dextrans (2.2.39.)	57
<i>Methylis parahydroxybenzoas</i>	2013	Molgramostim concentrated solution	2054
<i>Methylis parahydroxybenzoas natricum</i>	2441	<i>Molgramostimi solutio concentrata</i>	2054
<i>Methylis salicylas</i>	2014	Mometasone furoate	2057
Methyl parahydroxybenzoate	2013	<i>Mometasoni furoas</i>	2057
Methylpentoses in polysaccharide vaccines (2.5.21.)	133	Monographs (1.4.)	7
Methylphenobarbital	2019	Morantel hydrogen tartrate for veterinary use	2059
<i>Methylphenobarbitalum</i>	2019	<i>Moranteli hydrogenotartras ad usum veterinarium</i>	2059
Methylprednisolone	2020	Morphine hydrochloride	2060
Methylprednisolone acetate	2022	Morphine sulphate	2061
Methylprednisolone hydrogen succinate	2024	<i>Morphini hydrochloridum</i>	2060
<i>Methylprednisoloni acetas</i>	2022	<i>Morphini sulfas</i>	2061
<i>Methylprednisoloni hydrogenosuccinas</i>	2024	Moss, Iceland	1787
<i>Methylprednisolonum</i>	2020	Mother tinctures for homoeopathic preparations	894
Methylpyrrolidone, N-	2026		
Methyl salicylate	2014		
Methyltestosterone	2027		
<i>Methyltestosteronum</i>	2027		

Motherwort	2063	<i>Natrii chloridum</i>	2428
Mouthwashes	612	<i>Natrii chromatis</i> (⁵¹ Cr) <i>solutio sterilis</i>	840
Moxonidine	2064	<i>Natrii citras</i>	2429
<i>Moxonidinum</i>	2064	<i>Natrii cromoglicas</i>	2429
Mucoadhesive preparations	613	<i>Natrii cyclamas</i>	2430
Mullein flower	2065	<i>Natrii dihydrogenophosphas dihydricus</i>	2431
Multidose containers, uniformity of mass of delivered doses (2.9.27.)	263	<i>Natrii docusas</i>	1471
Mumps vaccine (live)	684	<i>Natrii fluoridi</i> (¹⁸ F) <i>solutio iniectionis</i>	841
Mupirocin	2065	<i>Natrii fluoridum</i>	2432
Mupirocin calcium	2067	<i>Natrii fusidas</i>	2433
<i>Mupirocinum</i>	2065	<i>Natrii glycerophosphas hydricus</i>	2434
<i>Mupirocinum calcicum</i>	2067	<i>Natrii hyaluronas</i>	2434
<i>Musci medicati</i>	604	<i>Natrii hydrogencarbonas</i>	2437
Mycobacteria (2.6.2.)	149	<i>Natrii hydroxidum</i>	2437
Mycoplasmas (2.6.7.)	149	<i>Natrii iodidi</i> (¹²³ I) <i>solutio iniectionis</i>	842
<i>Myristicae fragrantis aetheroleum</i>	2123	<i>Natrii iodidi</i> (¹³¹ I) <i>capsulae ad usum diagnosticum</i>	843
Myrrh	2069	<i>Natrii iodidi</i> (¹³¹ I) <i>solutio</i>	844
<i>Myrrha</i>	2069	<i>Natrii iodidi</i> (¹³¹ I) <i>solutio ad radio-signandum</i>	845
<i>Myrrhae tinctura</i>	2069	<i>Natrii iodidum</i>	2438
Myrrh tincture	2069	<i>Natrii iodohippurati</i> (¹²³ I) <i>solutio iniectionis</i>	845
<i>Myrtilli fructus recens</i>	1099	<i>Natrii iodohippurati</i> (¹³¹ I) <i>solutio iniectionis</i>	846
<i>Myrtilli fructus siccus</i>	1099	<i>Natrii lactatis solutio</i>	2438
Myxomatosis vaccine (live) for rabbits	775	<i>Natrii laurilsulfas</i>	2440
N		<i>Natrii metabisulfis</i>	2441
Nabumetone	2073	<i>Natrii molybdis dihydricus</i>	2442
<i>Nabumetonum</i>	2073	<i>Natrii nitris</i>	2443
<i>N-Acetyltryptophan</i>	918	<i>Natrii nitroprussias</i>	2443
<i>N-Acetyltryptophanum</i>	918	<i>Natrii perboras hydricus</i>	2444
<i>N-Acetyltyrosine</i>	920	<i>Natrii pertechnetatis</i> (^{99m} Tc) <i>fissione formati solutio iniectionis</i>	847
<i>N-Acetyltyrosinum</i>	920	<i>Natrii pertechnetatis</i> (^{99m} Tc) <i>sine fissione formati solutio iniectionis</i>	848
Nadolol	2074	<i>Natrii phosphatis</i> (³² P) <i>solutio iniectionis</i>	849
<i>Nadololum</i>	2074	<i>Natrii picosulfas</i>	2445
Nadroparin calcium	2075	<i>Natrii polystyrenesulfonas</i>	2446
<i>Nadroparinum calcicum</i>	2075	<i>Natrii propionas</i>	2447
Naftidrofuryl hydrogen oxalate	2078	<i>Natrii salicylas</i>	2449
<i>Naftidrofuryli hydrogenooxalas</i>	2078	<i>Natrii selenis pentahydricus</i>	2449
Nalidixic acid	2080	<i>Natrii (S)-lactatis solutio</i>	2439
Naloxone hydrochloride dihydrate	2080	<i>Natrii stearas</i>	2452
<i>Naloxoni hydrochloridum dihydricum</i>	2080	<i>Natrii stearyl is fumaras</i>	2453
Naphazoline hydrochloride	2082	<i>Natrii sulfas anhydricus</i>	2454
Naphazoline nitrate	2083	<i>Natrii sulfas decahydricus</i>	2455
<i>Naphazolini hydrochloridum</i>	2082	<i>Natrii sulfis anhydricus</i>	2455
<i>Naphazolini nitras</i>	2083	<i>Natrii sulfis heptahydricus</i>	2456
Naproxen	2084	<i>Natrii thiosulfas</i>	2456
<i>Naproxenum</i>	2084	<i>Natrii valproas</i>	2457
Nasal drops and liquid nasal sprays	610	Near-infrared spectrophotometry (2.2.40.)	59
<i>Nasalia</i>	610	Neohesperidin-dihydrochalcone	2085
Nasal powders	611	<i>Neohesperidin-dihydrochalconum</i>	2085
Nasal preparations	610	<i>Neomycini sulfas</i>	2086
Nasal preparations, semi-solid	611	Neomycin sulphate	2086
Nasal sticks	611	Neonatal piglet colibacillosis vaccine (inactivated)	776
Nasal washes	611	Neonatal ruminant colibacillosis vaccine (inactivated)	778
<i>Natrii acetat trihydricus</i>	2415	Neostigmine bromide	2088
<i>Natrii acetatis</i> ([¹¹ C]) <i>solutio iniectionis</i>	839	Neostigmine metilsulfate	2089
<i>Natrii alendronas</i>	2416	<i>Neostigmini bromidum</i>	2088
<i>Natrii alginas</i>	2417	<i>Neostigmini metilsulfas</i>	2089
<i>Natrii amidotrizoas</i>	2418	<i>Netilmicini sulfas</i>	2089
<i>Natrii aminosalicylas dihydricus</i>	2419	Netilmicin sulphate	2089
<i>Natrii ascorbas</i>	2420	Neurovirulence test for poliomyelitis vaccine (oral) (2.6.19.)	172
<i>Natrii benzoas</i>	2421	Neurovirulence test of live viral vaccines (2.6.18.)	172
<i>Natrii bromidum</i>	2422	Newcastle disease vaccine (inactivated)	779
<i>Natrii calcii edetas</i>	2422	Newcastle disease vaccine (live)	781
<i>Natrii caprylas</i>	2423	Nicergoline	2091
<i>Natrii carbonas anhydricus</i>	2424	<i>Nicergolinum</i>	2091
<i>Natrii carbonas decahydricus</i>	2425	Nicethamidum	2100
<i>Natrii carbonas monohydricus</i>	2425	Nickel in polyols (2.4.15.)	108
<i>Natrii cetylo- et stearylosulfas</i>	2426		

Niclosamide, anhydrous	2092	<i>Norgestrelum</i>	2119
Niclosamide monohydrate	2093	Normal immunoglobulin for intravenous administration, human	1744
<i>Niclosamidum anhydricum</i>	2092	Normal immunoglobulin, human	1742
<i>Niclosamidum monohydricum</i>	2093	Nortriptyline hydrochloride	2120
Nicotinamide	2094	<i>Nortriptylini hydrochloridum</i>	2120
<i>Nicotinamidum</i>	2094	Noscapine	2121
Nicotine	2095	Noscapine hydrochloride	2122
Nicotine resinate	2096	<i>Noscapini hydrochloridum</i>	2122
Nicotinic acid	2097	<i>Noscapinum</i>	2121
<i>Nicotini resinas</i>	2096	Nuclear magnetic resonance spectrometry (2.2.33.)	51
<i>Nicotinum</i>	2095	Nucleic acid amplification techniques (2.6.21.)	174
Nifedipine	2098	Nucleic acids in polysaccharide vaccines (2.5.17.)	132
<i>Nifedipinum</i>	2098	Nutmeg oil	2123
Nifuroxazide	2099	Nystatin	2124
<i>Nifuroxazidum</i>	2099	<i>Nystatinum</i>	2124
Nikethamide	2100		
Nimesulide	2101	O	
<i>Nimesulidum</i>	2101	<i>O</i> -Acetyl in polysaccharide vaccines (2.5.19.)	132
Nimodipine	2102	Oak bark	2129
<i>Nimodipinum</i>	2102	Octoxinol 10	2129
Nitrazepam	2103	<i>Octoxinolum 10</i>	2129
<i>Nitrazepamum</i>	2103	Octyldodecanol	2130
Nitrendipine	2104	<i>Octyldodecanolum</i>	2130
<i>Nitrendipinum</i>	2104	Octyl gallate	2129
Nitric acid	2105	<i>Octylis gallas</i>	2129
Nitric oxide	2105	Odour (2.3.4.)	99
Nitrofural	2106	Odour and taste of essential oils (2.8.8.)	216
<i>Nitrofuralum</i>	2106	Ofloxacin	2131
Nitrofurantoin	2107	<i>Ofloxacinum</i>	2131
<i>Nitrofurantoinum</i>	2107	Oils rich in omega-3- acids, composition of fatty acids (2.4.29.)	121
Nitrogen	2108	Ointments	625
Nitrogen determination by sulphuric acid digestion (2.5.9.)	129	<i>Oleae folium</i>	2134
Nitrogen determination, primary aromatic amino (2.5.8.)	129	<i>Olea herbaria</i>	595
<i>Nitrogenii oxidum</i>	2105	Oleic acid	2132
<i>Nitrogenium</i>	2108	Oleoyl macroglycerides	2133
<i>Nitrogenium oxygenio depletum</i>	2109	Oleyl alcohol	2134
Nitrogen, low-oxygen	2109	<i>Olivae oleum raffinatum</i>	2135
Nitrogen monoxide and nitrogen dioxide in gases (2.5.26.)	135	<i>Olivae oleum virginale</i>	2136
Nitrous oxide	2110	Olive leaf	2134
Nitrous oxide in gases (2.5.35.)	141	Olive oil, refined	2135
Nizatidine	2111	Olive oil, virgin	2136
<i>Nizatidinum</i>	2111	Olsalazine sodium	2137
<i>N</i> -Methylpyrrolidone	2026	<i>Olsalazinum natricum</i>	2137
<i>N-Methylpyrrolidonum</i>	2026	Omega-3-acid ethyl esters 60	2140
<i>N,N</i> -Dimethylaniline (2.4.26.)	119	Omega-3-acid ethyl esters 90	2142
Nomegestrol acetate	2113	<i>Omega-3 acidorum esteri ethylici 60</i>	2140
<i>Nomegestroli acetas</i>	2113	<i>Omega-3 acidorum esteri ethylici 90</i>	2142
Nonoxinol 9	2114	<i>Omega-3 acidorum triglycerida</i>	2144
<i>Nonoxinolum 9</i>	2114	Omega-3-acids, composition of fatty acids in oils rich in (2.4.29.)	121
Non-sterile products, microbiological examination of (test for specified micro-organisms) (2.6.13.)	156	Omega-3-acids, fish oil rich in	1595
Non-sterile products, microbiological examination of (total viable aerobic count) (2.6.12.)	154	Omega-3-acid triglycerides	2144
Noradrenaline hydrochloride	2114	Omeprazole	2146
Noradrenaline tartrate	2115	Omeprazole sodium	2148
<i>Noradrenalini hydrochloridum</i>	2114	<i>Omeprazolum</i>	2146
<i>Noradrenalini tartras</i>	2115	<i>Omeprazolum natricum</i>	2148
<i>Norcholesteroli iodinati</i> (¹³¹ I) <i>solutio iniectionabilis</i>	836	Ondansetron hydrochloride dihydrate	2149
Norcholesterol injection, iodinated (¹³¹ I)	836	<i>Ondansetroni hydrochloridum dihydricum</i>	2149
Norethisterone	2116	<i>Ononidis radix</i>	2361
Norethisterone acetate	2117	Opalescence of liquids, clarity and degree (2.2.1.)	23
<i>Norethisteroni acetas</i>	2117	<i>Ophthalmica</i>	602
<i>Norethisteronum</i>	2116	Ophthalmic inserts	604
Norfloxacin	2118	<i>Opil pulvis normatus</i>	2151
<i>Norfloxacinum</i>	2118	<i>Opium crudum</i>	2152
Norgestrel	2119	Opium, prepared	2151
		Opium, raw	2152
		Optical rotation (2.2.7.)	28

Oral drops.....	609	<i>Papaveris rhoeados flos</i>	2359
Oral powders.....	617	Paper chromatography (2.2.26.)	40
Oral solutions, emulsions and suspensions	609	Paracetamol	2184
Oral use, liquid preparations for.....	608	<i>Paracetamolum</i>	2184
Orciprenaline sulphate.....	2154	Paraffin, hard	2186
<i>Orciprenalini sulfas</i>	2154	Paraffin, light liquid	2186
Oregano.....	2155	Paraffin, liquid	2187
Organ preservation, solutions for.....	2458	<i>Paraffinum liquidum</i>	2187
<i>Origani herba</i>	2155	<i>Paraffinum perliquidum</i>	2186
Orodispensible tablets.....	628	<i>Paraffinum solidum</i>	2186
Oromucosal capsules	613	Paraffin, white soft	2187
Oromucosal drops, oromucosal sprays and sublingual sprays.....	612	Paraffin, yellow soft.....	2188
Oromucosal preparations.....	611	Parainfluenza virus vaccine (live), bovine, freeze-dried	732
Oromucosal preparations, semi-solid.....	612	Parainfluenza virus vaccine (live), canine	742
Oromucosal solutions and oromucosal suspensions	612	Paraldehyde.....	2189
Orphenadrine citrate	2156	<i>Paraldehydum</i>	2189
Orphenadrine hydrochloride.....	2157	<i>Parenteralia</i>	614
<i>Orphenadrini citras</i>	2156	Parenteral preparations.....	614
<i>Orphenadrini hydrochloridum</i>	2157	Parenteral preparations, test for extractable volume of (2.9.17.)	243
<i>Orthosiphonis folium</i>	1859	Parnaparin sodium	2189
<i>Oryzae amyllum</i>	2369	<i>Parnaparinum natricum</i>	2189
Osmolality (2.2.35.).....	54	Paroxetine hydrochloride hemihydrate.....	2190
Other provisions applying to general chapters and monographs (1.2.).....	5	<i>Paroxetini hydrochloridum hemihydricum</i>	2190
Ouabain	2158	Particles, fine, aerodynamic assessment of in preparations for inhalation (2.9.18.)	244
<i>Ouabainum</i>	2158	Particle size by microscopy, limit test of (2.9.13.)	239
Oxaliplatin	2159	Particulate contamination: sub-visible particles (2.9.19.)	253
<i>Oxaliplatinum</i>	2159	Particulate contamination: visible particles (2.9.20.).....	255
Oxazepam	2162	Parvovirus vaccine (inactivated), canine	743
<i>Oxazepamum</i>	2162	Parvovirus vaccine (inactivated), porcine	787
Oxeladin hydrogen citrate.....	2163	Parvovirus vaccine (live), canine.....	744
<i>Oxeladini hydrogenocitras</i>	2163	<i>Passiflorae herba</i>	2192
Oxfendazole for veterinary use.....	2164	Passion flower	2192
<i>Oxfendazolom ad usum veterinarium</i>	2164	Pastes.....	625
Oxidising substances (2.5.30.).....	137	Pasteurella vaccine (inactivated) for sheep	783
Oxolinic acid.....	2165	Pastilles and lozenges.....	613
Oxprenolol hydrochloride	2166	Patches, transdermal.....	616
<i>Oxprenololi hydrochloridum</i>	2166	Patches, transdermal, dissolution test for (2.9.4.)	231
Oxybuprocaine hydrochloride.....	2167	<i>Pefloxacin mesilas dihydricus</i>	2193
<i>Oxybuprocaini hydrochloridum</i>	2167	Pefloxacin mesilate dihydrate	2193
Oxybutynin hydrochloride	2168	<i>Penbutololi sulfas</i>	2195
<i>Oxybutynini hydrochloridum</i>	2168	Penbutolol sulphate	2195
Oxygen.....	2169	Penetrometry, measurement of consistency (2.9.9.)	235
Oxygen (¹⁵ O).....	837	Penicillamine.....	2196
Oxygen-flask method (2.5.10.).....	130	<i>Penicillaminum</i>	2196
Oxygen in gases (2.5.27.).....	136	<i>Pentaerythrityli tetranitras dilutus</i>	2198
<i>Oxygenium</i>	2169	Pentaerythrityl tetranitrate, diluted	2198
<i>Oxygenium (¹⁵O)</i>	837	Pentamidine diisetonate.....	2199
Oxymetazoline hydrochloride	2170	<i>Pentamidini diisetonas</i>	2199
<i>Oxymetazolini hydrochloridum</i>	2170	Pentazocine	2200
Oxytetracycline dihydrate	2171	Pentazocine hydrochloride.....	2201
Oxytetracycline hydrochloride	2172	<i>Pentazocini hydrochloridum</i>	2201
<i>Oxytetracyclini hydrochloridum</i>	2172	<i>Pentazocinum</i>	2200
<i>Oxytetracyclinum dihydricum</i>	2171	Pentobarbital	2201
Oxytocin	2174	Pentobarbital sodium.....	2202
Oxytocin bulk solution	2175	<i>Pentobarbitalum</i>	2201
<i>Oxytocini solutio</i>	2175	<i>Pentobarbitalum natricum</i>	2202
<i>Oxytocinum</i>	2174	Pentoxifylline	2203
P		<i>Pentoxifyllinum</i>	2203
Palmitic acid.....	2179	Pentoxyverine hydrogen citrate.....	2204
Pancreas powder.....	2179	<i>Pentoxyverini hydrogenocitras</i>	2204
<i>Pancreatis pulvis</i>	2179	Peppermint leaf	2205
<i>Pancuronii bromidum</i>	2182	Peppermint oil	2206
Pancuronium bromide	2182	<i>Pepsini pulvis</i>	2207
Pansy, wild (flowering aerial parts)	2700	Pepsin powder	2207
Papaverine hydrochloride	2183	Peptide mapping (2.2.55.)	82
<i>Papaverini hydrochloridum</i>	2183	Peptides, synthetic, acetic acid in (2.5.34.).....	141

Pergolide mesilate.....	2209	Phenylmercuric nitrate.....	2234
<i>Pergolide mesilas</i>	2209	Phenylpropanolamine hydrochloride.....	2234
Perindopril <i>tert</i> -butylamine.....	2210	<i>Phenylpropanolamini hydrochloridum</i>	2234
Peritoneal dialysis, solutions for.....	2212	Phenytoin.....	2235
Peroxide value (2.5.5.).....	128	Phenytoin sodium.....	2236
Perphenazine.....	2214	<i>Phenytoinum</i>	2235
<i>Perphenazinum</i>	2214	<i>Phenytoinum natricum</i>	2236
Pertussis (acellular, component), diphtheria, tetanus, poliomyelitis (inactivated) and haemophilus type b conjugate vaccine (adsorbed).....	653	Pholcodine.....	2237
Pertussis, diphtheria, tetanus and poliomyelitis (inactivated) vaccine (adsorbed).....	656	<i>Pholcodinum</i>	2237
Pertussis, diphtheria, tetanus, poliomyelitis (inactivated) and haemophilus type b conjugate vaccine (adsorbed).....	657	Phosphates (2.4.11.).....	108
Pertussis vaccine.....	685	Phosphoric acid, concentrated.....	2237
Pertussis vaccine (acellular), assay of (2.7.16.).....	208	Phosphoric acid, dilute.....	2238
Pertussis vaccine (acellular, component, adsorbed).....	686	Phosphorus in polysaccharide vaccines (2.5.18.).....	132
Pertussis vaccine (acellular, co-purified, adsorbed).....	688	pH, potentiometric determination of (2.2.3.).....	26
Pertussis vaccine (adsorbed).....	690	Phthalylsulfathiazole.....	2238
Pertussis vaccine, assay of (2.7.7.).....	197	<i>Phthalylsulfathiazolum</i>	2238
Peru balsam.....	2215	Physical and physicochemical methods (2.2.).....	23
Pessaries.....	629	Physostigmine salicylate.....	2239
Pessaries and suppositories, disintegration of (2.9.2.).....	227	Physostigmine sulphate.....	2240
Pessaries and suppositories, resistance to rupture (2.9.24.).....	258	<i>Physostigmini salicylas</i>	2239
Pesticide residues (2.8.13.).....	218	<i>Physostigmini sulfas</i>	2240
Pethidine hydrochloride.....	2216	Phytomenadione.....	2241
<i>Pethidini hydrochloridum</i>	2216	<i>Phytomenadionum</i>	2241
Pharmaceutical technical procedures (2.9.).....	225	Phytosterol.....	2242
Pharmacognosy, methods in (2.8.).....	215	<i>Phytosterolum</i>	2242
Pharmacopoeial harmonisation (5.8.).....	551	Picotamide monohydrate.....	2243
Phenazone.....	2217	<i>Picotamidum monohydricum</i>	2243
<i>Phenazonum</i>	2217	Pilocarpine hydrochloride.....	2244
Pheniramine maleate.....	2218	Pilocarpine nitrate.....	2246
<i>Pheniramini maleas</i>	2218	<i>Pilocarpini hydrochloridum</i>	2244
Phenobarbital.....	2219	<i>Pilocarpini nitras</i>	2246
Phenobarbital sodium.....	2220	Pimozide.....	2247
<i>Phenobarbitalum</i>	2219	<i>Pimozidum</i>	2247
<i>Phenobarbitalum natricum</i>	2220	Pindolol.....	2248
Phenol.....	2221	<i>Pindololum</i>	2248
Phenol in immunosera and vaccines (2.5.15.).....	131	Pipemidic acid trihydrate.....	2249
Phenolphthalein.....	2222	Piperacillin.....	2250
<i>Phenolphthaleinum</i>	2222	Piperacillin sodium.....	2252
Phenolsulfonphthalein.....	2222	<i>Piperacillinum</i>	2250
<i>Phenolsulfonphthaleinum</i>	2222	<i>Piperacillinum natricum</i>	2252
<i>Phenolum</i>	2221	Piperazine adipate.....	2253
Phenothiazines, identification by thin-layer chromatography (2.3.3.).....	99	Piperazine citrate.....	2254
Phenoxyethanol.....	2223	Piperazine hydrate.....	2255
<i>Phenoxyethanolum</i>	2223	<i>Piperazini adipas</i>	2253
Phenoxymethylpenicillin.....	2224	<i>Piperazini citras</i>	2254
Phenoxymethylpenicillin potassium.....	2226	<i>Piperazinum hydricum</i>	2255
<i>Phenoxymethylpenicillinum</i>	2224	Piracetam.....	2256
<i>Phenoxymethylpenicillinum kalicum</i>	2226	<i>Piracetamum</i>	2256
Phentolamine mesilate.....	2227	Pirenzepine dihydrochloride monohydrate.....	2257
<i>Phentolamini mesilas</i>	2227	<i>Pirenzepini dihydrochloridum monohydricum</i>	2257
Phenylalanine.....	2228	Piretanide.....	2258
<i>Phenylalaninum</i>	2228	<i>Piretanidum</i>	2258
Phenylbutazone.....	2229	Piroxicam.....	2259
<i>Phenylbutazonum</i>	2229	<i>Piroxicamum</i>	2259
Phenylephrine.....	2231	<i>Piscis oleum omega-3 acidis abundans</i>	1595
Phenylephrine hydrochloride.....	2232	Pivampicillin.....	2261
<i>Phenylephrini hydrochloridum</i>	2232	<i>Pivampicillinum</i>	2261
<i>Phenylephrinum</i>	2231	Pivmecillinam hydrochloride.....	2262
<i>Phenylhydrargyri acetas</i>	2232	<i>Pivmecillinami hydrochloridum</i>	2262
<i>Phenylhydrargyri boras</i>	2233	<i>Plantae ad ptisanam</i>	573
<i>Phenylhydrargyri nitras</i>	2234	<i>Plantae medicinales</i>	572
Phenylmercuric acetate.....	2232	<i>Plantae medicinales</i> <i>ad praeparationes homoeopathicas</i>	893
Phenylmercuric borate.....	2233	<i>Plantae medicinales praeparatore</i>	572
		<i>Plantaginis lanceolatae folium</i>	2368
		<i>Plantaginis ovatae semen</i>	1850
		<i>Plantaginis ovatae seminis tegumentum</i>	1849
		Plasma for fractionation, human.....	1746

Plasma, human (pooled and treated for virus inactivation)	1747	Polysorbate 40	2268
<i>Plasma humanum ad separationem</i>	1746	Polysorbate 60	2269
<i>Plasma humanum collectum deinde conditum ad viros extinguendos</i>	1747	Polysorbate 80	2270
Plastic additives (3.1.13.)	293	<i>Polysorbatum 20</i>	2267
Plastic containers and closures for pharmaceutical use (3.2.2.)	308	<i>Polysorbatum 40</i>	2268
Plastic containers for aqueous solutions for parenteral infusion (3.2.2.1.)	309	<i>Polysorbatum 60</i>	2269
Plastic containers for human blood and blood components, sterile (3.2.3.)	309	<i>Polysorbatum 80</i>	2270
Plastic syringes, single-use, sterile (3.2.8.)	314	Poly(vinyl acetate)	2271
Pneumococcal polysaccharide vaccine	691	Poly(vinyl alcohol)	2272
Poliomyelitis (inactivated), diphtheria, tetanus and pertussis (acellular, component) vaccine (adsorbed)	648	Poly(vinyl chloride), non-plasticised, materials based on for containers for dry dosage forms for oral administration (3.1.11.)	291
Poliomyelitis (inactivated), diphtheria, tetanus and pertussis vaccine (adsorbed)	656	Poly(vinyl chloride), non-plasticised, materials based on for containers for non-injectable aqueous solutions (3.1.10.)	289
Poliomyelitis (inactivated), diphtheria, tetanus, pertussis (acellular, component) and haemophilus type b conjugate vaccine (adsorbed)	653	Poly(vinyl chloride), plasticised, empty sterile containers of for human blood and blood components (3.2.4.)	311
Poliomyelitis (inactivated), diphtheria, tetanus, pertussis and haemophilus type b conjugate vaccine (adsorbed)	657	Poly(vinyl chloride), plasticised, materials based on for containers for aqueous solutions for intravenous infusion (3.1.14.)	296
Poliomyelitis vaccine (inactivated)	692	Poly(vinyl chloride), plasticised, materials based on for containers for human blood and blood components (3.1.1.1.)	269
Poliomyelitis vaccine (inactivated), <i>in vivo</i> assay of (2.7.20.)	210	Poly(vinyl chloride), plasticised, materials based on for tubing used in sets for the transfusion of blood and blood components (3.1.1.2.)	272
Poliomyelitis vaccine (oral)	695	Poly (vinyl chloride), plasticised, sterile containers of for human blood containing anticoagulant solution (3.2.5.)	312
Poliomyelitis vaccine (oral), test for neurovirulence (2.6.19.)	172	<i>Poly(vinylis acetat)</i>	2271
<i>Poloxamera</i>	2264	Poppy petals, red	2359
Poloxamers	2264	Porcine actinobacillosis vaccine (inactivated)	784
Polyacrylate dispersion 30 per cent	2265	Porcine influenza vaccine (inactivated)	785
<i>Polyacrylatis dispersio 30 per centum</i>	2265	Porcine insulin	1806
<i>Poly(alcohol vinylicus)</i>	2272	Porcine parvovirus vaccine (inactivated)	787
Polyamide 6/6 suture, sterile, in distributor for veterinary use	887	Porcine progressive atrophic rhinitis vaccine (inactivated)	788
Polyamide 6 suture, sterile, in distributor for veterinary use	886	Porosity of sintered-glass filters (2.1.2.)	17
Polyethyleneglycols	1950	Potassium (2.4.12.)	108
Polyethylene terephthalate for containers for preparations not for parenteral use (3.1.15.)	298	Potassium acetate	2273
Poly(ethylene terephthalate) suture, sterile, in distributor for veterinary use	887	Potassium bromide	2273
Poly(ethylene - vinyl acetate) for containers and tubing for total parenteral nutrition preparations (3.1.7.)	285	Potassium carbonate	2274
Polyethylene with additives for containers for parenteral preparations and for ophthalmic preparations (3.1.5.) ...	279	Potassium chloride	2275
Polyethylene without additives for containers for parenteral preparations and for ophthalmic preparations (3.1.4.) ...	278	Potassium citrate	2275
<i>Polygalae radix</i>	2401	Potassium clavulanate	2276
<i>Polygoni avicularis herba</i>	1877	Potassium clavulanate, diluted	2278
Polymorphism (5.9.)	555	Potassium dihydrogen phosphate	2280
Polymyxin B sulphate	2266	Potassium hydrogen aspartate hemihydrate	2280
<i>Polymyxini B sulfas</i>	2266	Potassium hydrogen carbonate	2281
Polyolefines (3.1.3.)	274	Potassium hydrogen tartrate	2282
Polyoxyl castor oil	1949	Potassium hydroxide	2283
Polyoxyl hydrogenated castor oil	1948	Potassium iodide	2283
Polypropylene for containers and closures for parenteral preparations and ophthalmic preparations (3.1.6.)	282	Potassium metabisulphite	2284
Polysaccharide vaccines, hexosamines in (2.5.20.)	132	Potassium nitrate	2284
Polysaccharide vaccines, methylpentoses in (2.5.21.)	133	Potassium perchlorate	2285
Polysaccharide vaccines, nucleic acids in (2.5.17.)	132	Potassium permanganate	2286
Polysaccharide vaccines, <i>O</i> -acetyl in (2.5.19.)	132	Potassium sodium tartrate tetrahydrate	2286
Polysaccharide vaccines, phosphorus in (2.5.18.)	132	Potassium sorbate	2287
Polysaccharide vaccines, protein in (2.5.16.)	131	Potassium sulphate	2288
Polysaccharide vaccines, ribose in (2.5.31.)	137	Potato starch	2288
Polysaccharide vaccines, sialic acid in (2.5.23.)	133	Potentiometric determination of ionic concentration using ion-selective electrodes (2.2.36.)	55
Polysaccharide vaccines, uronic acids in (2.5.22.)	133	Potentiometric determination of pH (2.2.3.)	26
Polysorbate 20	2267	Potentiometric titration (2.2.20.)	35
		Poultices	625
		Pour-on preparations	631
		Povidone	2289
		Povidone, iodinated	2291
		<i>Povidonum</i>	2289

<i>Povidonum iodinatum</i>	2291	Primula root.....	2310
Powdered cellulose.....	1232	Probenecid.....	2311
Powders and granules for oral solutions and suspensions.....	609	<i>Probenecidum</i>	2311
Powders and granules for syrups.....	610	Procainamide hydrochloride.....	2312
Powders and tablets for rectal solutions and suspensions..	624	<i>Procinamidi hydrochloridum</i>	2312
Powders, ear.....	602	Procaine benzylpenicillin.....	1080
Powders, effervescent.....	617	Procaine hydrochloride.....	2312
Powders for cutaneous application.....	616	<i>Procaini hydrochloridum</i>	2312
Powders for eye-drops and powders for eye lotions.....	603	Prochlorperazine maleate.....	2313
Powders for inhalation.....	619	<i>Prochlorperazini maleas</i>	2313
Powders for injections or infusions.....	614	<i>Producta ab ADN recombinante</i>	584
Powders for oral drops.....	609	<i>Producta ab fermentatione</i>	576
Powders, nasal.....	611	<i>Producta allergenica</i>	569
Powders, oral.....	617	<i>Producta cum possibili transmissione vectorium enkephalopathiarum spongiformium animalium</i>	577
<i>Praeadmixta ad alimenta medicata ad usum veterinarium</i>	617	Products of fermentation.....	576
<i>Praeparationes ad irrigationem</i>	622	Products of recombinant DNA technology.....	584
<i>Praeparationes buccales</i>	611	Products with risk of transmitting agents of animal spongiform encephalopathies.....	577
<i>Praeparationes homoeopathicas</i>	893	Progesterone.....	2314
<i>Praeparationes insulini iniectionabiles</i>	1808	<i>Progesteronum</i>	2314
<i>Praeparationes intramammariae ad usum veterinarium</i>	606	Proguanil hydrochloride.....	2315
<i>Praeparationes intraruminales</i>	607	<i>Proguanili hydrochloridum</i>	2315
<i>Praeparationes liquidae ad usum dermicum</i>	607	Proline.....	2316
<i>Praeparationes liquidae peroraliae</i>	608	<i>Prolinum</i>	2316
<i>Praeparationes liquidae veterinariae ad usum dermicum</i>	630	Promazine hydrochloride.....	2317
<i>Praeparationes molles ad usum dermicum</i>	624	<i>Promazini hydrochloridum</i>	2317
<i>Praeparationes pharmaceuticae in vasis cum pressu</i>	622	Promethazine hydrochloride.....	2318
Pravastatin sodium.....	2292	<i>Promethazini hydrochloridum</i>	2318
<i>Pravastatinum natricum</i>	2292	Propacetamol hydrochloride.....	2319
Prazepam.....	2293	<i>Propacetamoli hydrochloridum</i>	2319
<i>Prazepamum</i>	2293	Propanol.....	2320
Praziquantel.....	2294	<i>Propanolum</i>	2320
<i>Praziquantelum</i>	2294	Propantheline bromide.....	2321
Prazosin hydrochloride.....	2295	<i>Propanthelini bromidum</i>	2321
<i>Prazosini hydrochloridum</i>	2295	Propofol.....	2322
Prednicarbate.....	2297	<i>Propofolum</i>	2322
<i>Prednicarbatum</i>	2297	Propranolol hydrochloride.....	2324
Prednisolone.....	2298	<i>Propranololi hydrochloridum</i>	2324
Prednisolone acetate.....	2299	Propylene glycol.....	2327
Prednisolone pivalate.....	2301	Propylene glycol dicaprylocaprate.....	2327
Prednisolone sodium phosphate.....	2302	Propylene glycol dilaurate.....	2328
<i>Prednisoloni acetas</i>	2299	Propylene glycol monolaurate.....	2329
<i>Prednisoloni natrii phosphas</i>	2302	Propylene glycol monopalmitostearate.....	2330
<i>Prednisoloni pivalas</i>	2301	Propylene glycol monostearate.....	2330
<i>Prednisolonum</i>	2298	<i>Propylenglycoli dicaprylocapras</i>	2327
Prednisone.....	2303	<i>Propylenglycoli dilauras</i>	2328
<i>Prednisonum</i>	2303	<i>Propylenglycoli monolauras</i>	2329
Prekallikrein activator (2.6.15.).....	168	<i>Propylenglycoli monopalmitostearas</i>	2330
Premixes for medicated feeding stuffs for veterinary use ..	617	<i>Propylenglycolum</i>	2327
Preparations for inhalation.....	618	Propyl gallate.....	2325
Preparations for inhalation: aerodynamic assessment of fine particles (2.9.18.).....	244	<i>Propylis gallas</i>	2325
Preparations for irrigation.....	622	<i>Propylis parahydroxybenzoas</i>	2326
Pressurised pharmaceutical preparations.....	622	<i>Propylis parahydroxybenzoas natricum</i>	2448
Prilocaine.....	2305	Propyl parahydroxybenzoate.....	2326
Prilocaine hydrochloride.....	2307	Propylthiouracil.....	2331
<i>Prilocaini hydrochloridum</i>	2307	<i>Propylthiouracilum</i>	2331
<i>Prilocainum</i>	2305	Propyphenazone.....	2332
Primaquine diphosphate.....	2308	<i>Propyphenazonum</i>	2332
<i>Primaquini diphosphas</i>	2308	Protamine hydrochloride.....	2332
Primary aromatic amino-nitrogen, determination of (2.5.8.).....	129	Protamine sulphate.....	2334
Primary standards for volumetric solutions (4.2.1.).....	435	<i>Protamini hydrochloridum</i>	2332
Primidone.....	2309	<i>Protamini sulfas</i>	2334
<i>Primidonum</i>	2309	Protein in polysaccharide vaccines (2.5.16.).....	131
<i>Primulae radix</i>	2310	Protein, total (2.5.33.).....	138
		Prothrombin complex, human.....	1748
		<i>Prothrombinum multiplex humanum</i>	1748
		Protirelin.....	2335
		<i>Protirelinum</i>	2335

- Proxiphylline 2336
Proxiphyllum 2336
Pruni africanae cortex 2339
 Pseudoephedrine hydrochloride 2337
Pseudoephedrini hydrochloridum 2337
Psyllii semen 2338
 Psyllium seed 2338
Pulveres ad usum dermicum 616
Pulveres perorales 617
 Purified water 2697
 Purified water, highly 2695
 Pycnometric density of solids (2.9.23.) 257
 Pygeum africanum bark 2339
 Pyrantel embonate 2339
Pyranteli embonas 2339
 Pyrazinamide 2340
Pyrazinamidum 2340
 Pyridostigmine bromide 2341
Pyridostigmini bromidum 2341
 Pyridoxine hydrochloride 2342
Pyridoxini hydrochloridum 2342
 Pyrimethamine 2343
Pyrimethaminum 2343
 Pyrogens (2.6.8.) 152
- Q**
- Quercus cortex* 2129
 Quinidine sulphate 2347
 Quinine hydrochloride 2348
 Quinine sulphate 2350
- R**
- Rabies immunoglobulin, human 1750
 Rabies vaccine for human use prepared in cell cultures... 699
 Rabies vaccine (inactivated) for veterinary use 790
 Rabies vaccine (live, oral) for foxes 792
 Racemic camphor 1172
 Racemic ephedrine hydrochloride 1509
 Racemic menthol 1991
 Raclopride ($[^{11}\text{C}]$ methoxy) injection 838
Raclopridi ($[^{11}\text{C}]$ methoxy) solutio iniectionabilis 838
 Radionuclides, table of physical characteristics (5.7.) 539
Radiopharmaceutica 578
 Radiopharmaceutical preparations 578
 Raman spectrometry (2.2.48.) 79
 Ramipril 2355
Ramiprilum 2355
 Ranitidine hydrochloride 2357
Ranitidini hydrochloridum 2357
Rapae oleum raffinatum 2359
 Rapeseed oil, refined 2359
Ratanhiae radix 2362
Ratanhiae tinctura 2362
 Reagents (4.) 321
 Reagents (4.1.1.) 321
 Reagents, standard solutions, buffer solutions (4.1.) 321
 Recombinant DNA technology, products of 584
 Rectal capsules 623
 Rectal foams 624
Rectalia 623
 Rectal preparations 623
 Rectal preparations, semi-solid 624
 Rectal solutions, emulsions and suspensions 624
 Rectal tampons 624
 Red poppy petals 2359
 Refractive index (2.2.6.) 28
 Relationship between reaction of solution, approximate pH
 and colour of certain indicators (2.2.4.) 27
 Relative density (2.2.5.) 27
- Reserpine 2360
Reserpinum 2360
 Residual solvents (5.4.) 507
 Residual solvents, identification and control (2.4.24.) 113
 Residue on evaporation of essential oils (2.8.9.) 216
 Resistance to crushing of tablets (2.9.8.) 235
 Resistance to rupture of suppositories and pessaries
 (2.9.24.) 258
 Resorcinol 2360
Resorcinolum 2360
 Restharrow root 2361
Rhamni purshianae cortex 1194
 Rhatany root 2362
 Rhatany tincture 2362
Rhei radix 2363
*Rhenii sulfidi colloidalis et technetii ($^{99\text{m}}\text{Tc}$) solutio
 iniectionabilis* 851
 Rhubarb 2363
 Ribavirin 2364
Ribavirinum 2364
 Riboflavin 2365
Riboflavini natrii phosphas 2366
 Riboflavin sodium phosphate 2366
Riboflavinum 2365
 Ribose in polysaccharide vaccines (2.5.31.) 137
 Ribwort plantain 2368
 Rice starch 2369
Ricini oleum hydrogenatum 1197
Ricini oleum virginale 1197
 Rifabutin 2369
Rifabutinum 2369
 Rifampicin 2371
Rifampicinum 2371
 Rifamycin sodium 2372
Rifamycinum natricum 2372
 Rilmenidine dihydrogen phosphate 2373
Rilmenidini dihydrogenophosphas 2373
 Risperidone 2374
Risperidonum 2374
 Roman chamomile flower 1245
Rosae pseudo-fructus 1472
 Roselle 2376
 Rosemary leaf 2377
 Rosemary oil 2378
Rosmarini aetheroleum 2378
Rosmarini folium 2377
 Rotating viscometer method (2.2.10.) 30
 Roxithromycin 2379
Roxithromycinum 2379
RRR- α -Tocopherol 2591
RRR- α -Tocopherolum 2591
RRR- α -Tocopheryl acetate 2595
RRR- α -Tocopheryl hydrogen succinate 2598
RRR- α -Tocopherylis acetas 2595
RRR- α -Tocopherylis hydrogenosuccinas 2598
 Rubber closures for containers for aqueous parenteral
 preparations, for powders and for freeze-dried powders
 (3.2.9.) 316
 Rubella immunoglobulin, human 1751
 Rubella vaccine (live) 701
Rusci rhizoma 1138
 Rutoside trihydrate 2381
Rutosidum trihydricum 2381
- S**
- Sabalisa serrulatae fructus* 2398
 Saccharin 2387
 Saccharin sodium 2388
Saccharinum 2387

<i>Saccharinum natricum</i>	2388	<i>Silica colloidalis hydrica</i>	2411
<i>Sacchari spheri</i>	2503	Silica, dental type.....	2411
<i>Saccharum</i>	2499	Silicone elastomer for closures and tubing (3.1.9.)	288
Safflower oil, refined.....	2389	Silicone oil used as a lubricant (3.1.8.)	287
Saffron for homeopathic preparations.....	900	Silk suture, sterile, braided, in distributor for veterinary use	887
Sage leaf (<i>salvia officinalis</i>).....	2389		887
Sage leaf, three-lobed.....	2390	Silver nitrate	2412
Sage tincture.....	2391	<i>Silybi mariani fructus</i>	2046
Salbutamol	2391	Simeticone.....	2412
<i>Salbutamoli sulfas</i>	2393	<i>Simeticonum</i>	2412
Salbutamol sulphate	2393	Simvastatin.....	2413
<i>Salbutamololum</i>	2391	<i>Simvastatinum</i>	2413
<i>Salicis cortex</i>	2702	Single-dose preparations, uniformity of content (2.9.6.)... 234	
Salicylic acid.....	2395	Single-dose preparations, uniformity of mass (2.9.5.)..... 233	
<i>Salmonis domestici oleum</i>	2396	Sintered-glass filters (2.1.2.)	17
Salmon oil, farmed.....	2396	Size-exclusion chromatography (2.2.30.).....	45
<i>Salviae officinalis folium</i>	2389	(S)-Lactic acid.....	1883
<i>Salviae sclareae aetheroleum</i>	1311	Sodium acetate ([1- ¹¹ C]) injection.....	839
<i>Salviae tinctura</i>	2391	Sodium acetate trihydrate	2415
<i>Salviae trilobae folium</i>	2390	Sodium alendronate	2416
<i>Sambuci flos</i>	1496	Sodium alginate	2417
Saponification value (2.5.6.).....	129	Sodium amidotrizoate.....	2418
Saw palmetto fruit	2398	Sodium aminosalicylate dihydrate	2419
Scopolamine butylbromide.....	1776	Sodium ascorbate	2420
Scopolamine hydrobromide.....	1777	Sodium benzoate	2421
<i>Scopolamini butylbromidum</i>	1776	Sodium bromide.....	2422
<i>Scopolamini hydrobromidum</i>	1777	Sodium calcium edetate	2422
Selegiline hydrochloride	2400	Sodium caprylate	2423
<i>Selegilini hydrochloridum</i>	2400	Sodium carbonate, anhydrous	2424
<i>Selenii disulfidum</i>	2401	Sodium carbonate decahydrate	2425
Selenium disulphide.....	2401	Sodium carbonate monohydrate	2425
Semi-micro determination of water (2.5.12.)	130	Sodium carboxymethylcellulose	1189
Semi-solid and liquid preparations, test for deliverable mass or volume (2.9.28.)	263	Sodium carboxymethylcellulose, cross-linked	1371
Semi-solid ear preparations	602	Sodium carboxymethylcellulose, low-substituted	1190
Semi-solid eye preparations.....	604	Sodium cetostearyl sulphate	2426
Semi-solid nasal preparations.....	611	Sodium chloride	2428
Semi-solid oromucosal preparations.....	612	Sodium chromate (⁵¹ Cr) sterile solution	840
Semi-solid preparations for cutaneous application	624	Sodium citrate	2429
Semi-solid rectal preparations.....	624	Sodium cromoglicate	2429
Semi-solid vaginal preparations	630	Sodium cyclamate	2430
Senega root	2401	Sodium dihydrogen phosphate dihydrate	2431
<i>Sennae folii extractum siccum normatum</i>	2403	Sodium fluoride	2432
<i>Sennae folium</i>	2402	Sodium fluoride (¹⁸ F) injection	841
<i>Sennae fructus acutifoliae</i>	2404	Sodium fusidate	2433
<i>Sennae fructus angustifoliae</i>	2405	Sodium glycerophosphate, hydrated	2434
Senna leaf	2402	Sodium hyaluronate	2434
Senna leaf dry extract, standardised	2403	Sodium hydrogen carbonate	2437
Senna pods, Alexandrian.....	2404	Sodium hydroxide.....	2437
Senna pods, Tinnevely.....	2405	Sodium iodide.....	2438
Separation techniques, chromatographic (2.2.46.)	69	Sodium iodide (¹²³ I) injection	842
Serine.....	2406	Sodium iodide (¹³¹ I) capsules for diagnostic use.....	843
<i>Serinum</i>	2406	Sodium iodide (¹³¹ I) solution	844
<i>Serpylli herba</i>	2701	Sodium iodide (¹³¹ I) solution for radiolabelling	845
Sertaconazole nitrate.....	2407	Sodium iodohippurate (¹²³ I) injection	845
<i>Sertaconazoli nitras</i>	2407	Sodium iodohippurate (¹³¹ I) injection	846
Sesame oil, refined	2408	Sodium lactate solution.....	2438
<i>Sesami oleum raffinatum</i>	2408	Sodium laurilsulfate	2440
Sets for the transfusion of blood and blood components (3.2.6.)	313	Sodium metabisulphite.....	2441
Shampoos	608	Sodium methyl parahydroxybenzoate.....	2441
Shellac	2409	Sodium molybdate dihydrate	2442
Sialic acid in polysaccharide vaccines (2.5.23.).....	133	Sodium nitrite.....	2443
Sieves (2.1.4.).....	18	Sodium nitroprusside	2443
Sieve test (2.9.12.).....	239	Sodium perborate, hydrated.....	2444
<i>Silica ad usum dentalem</i>	2411	Sodium pertechnetate (^{99m} Tc) injection (fission)	847
Silica, colloidal anhydrous.....	2410	Sodium pertechnetate (^{99m} Tc) injection (non-fission)	848
Silica, colloidal hydrated.....	2411	Sodium phosphate (³² P) injection	849
<i>Silica colloidalis anhydrica</i>	2410	Sodium picosulfate	2445
		Sodium polystyrene sulphonate	2446
		Sodium propionate.....	2447

Sodium propyl parahydroxybenzoate.....	2448	Sorbitol, liquid, partially dehydrated.....	2473
Sodium salicylate.....	2449	<i>Sorbitolum</i>	2470
Sodium selenite pentahydrate.....	2449	<i>Sorbitolum liquidum cristallisabile</i>	2471
Sodium (S)-lactate solution.....	2439	<i>Sorbitolum liquidum non cristallisabile</i>	2472
Sodium starch glycolate (type A).....	2450	<i>Sorbitolum liquidum partim deshydricum</i>	2473
Sodium starch glycolate (type B).....	2451	Sotalol hydrochloride.....	2474
Sodium starch glycolate (type C).....	2451	<i>Sotaloli hydrochloridum</i>	2474
Sodium stearate.....	2452	Soya-bean oil, hydrogenated.....	2475
Sodium stearyl fumarate.....	2453	Soya-bean oil, refined.....	2476
Sodium sulphate, anhydrous.....	2454	Specific surface area by air permeability (2.9.14.).....	239
Sodium sulphate decahydrate.....	2455	Specific surface area by gas adsorption (2.9.26.).....	260
Sodium sulphite, anhydrous.....	2455	Spectinomycin hydrochloride.....	2476
Sodium sulphite heptahydrate.....	2456	<i>Spectinomycini hydrochloridum</i>	2476
Sodium thiosulphate.....	2456	Spectrometry, atomic absorption (2.2.23.).....	36
Sodium valproate.....	2457	Spectrometry, atomic emission (2.2.22.).....	35
Soft capsules.....	600	Spectrometry, mass (2.2.43.).....	65
Softening time determination of lipophilic suppositories (2.9.22.).....	256	Spectrometry, nuclear magnetic resonance (2.2.33.).....	51
Soft extracts.....	572	Spectrometry, Raman (2.2.48.).....	79
<i>Soiae oleum hydrogenatum</i>	2475	Spectrometry, X-ray fluorescence (2.2.37.).....	56
<i>Soiae oleum raffinatum</i>	2476	Spectrophotometry, infrared absorption (2.2.24.).....	37
<i>Solani amyllum</i>	2288	Spectrophotometry, near-infrared (2.2.40.).....	59
<i>Solidaginis herba</i>	1680	Spectrophotometry, ultraviolet and visible absorption (2.2.25.).....	38
<i>Solidaginis virgaureae herba</i>	1682	SPF chicken flocks for production and quality control of vaccines (5.2.2.).....	453
Solid dosage forms, dissolution test for (2.9.3.).....	228	Spiramycin.....	2478
Solids, density of (2.2.42.).....	64	<i>Spiramycinum</i>	2478
Solids, pycnometric density of (2.9.23.).....	257	Spirapril hydrochloride monohydrate.....	2480
Solubility in alcohol of essential oils (2.8.10.).....	216	<i>Spirapрили hydrochloridum monohydricum</i>	2480
Soluble tablets.....	628	Spirolactone.....	2482
<i>Solutiones ad conservationem partium corporis</i>	2458	<i>Spirolactonum</i>	2482
<i>Solutiones ad haemocolaturam</i> <i>haemodiacolaturamque</i>	1703	Spot-on preparations.....	631
<i>Solutiones ad haemodialysim</i>	1700	Sprays.....	631
<i>Solutiones ad peritonealem dialysim</i>	2212	Squalane.....	2483
<i>Solutiones anticoagulantes et sanguinem humanum</i> <i>conservantes</i>	1007	<i>Squalanum</i>	2483
Solutions for haemodialysis.....	1700	Standard solutions for limit tests (4.1.2.).....	426
Solutions for haemodialysis, concentrated, water for diluting.....	1699	<i>Stanni colloidalis et technetii (^{99m}Tc)</i> <i>solutio iniectabilis</i>	853
Solutions for haemofiltration and for haemodiafiltra- tion.....	1703	<i>Stanni pyrophosphatis et technetii (^{99m}Tc) solutio</i> <i>iniectabilis</i>	865
Solutions for organ preservation.....	2458	<i>Stannosi chloridum dihydricum</i>	2486
Solutions for peritoneal dialysis.....	2212	Stannous chloride dihydrate.....	2486
Solvents, residual (5.4.).....	507	Stanozolol.....	2486
Solvents, residual, identification and control (2.4.24.).....	113	<i>Stanozololum</i>	2486
Somatostatin.....	2459	Star anise.....	2487
<i>Somatostatinum</i>	2459	Star anise oil.....	2488
Somatropin.....	2460	Starch glycolate (type A), sodium.....	2450
Somatropin bulk solution.....	2462	Starch glycolate (type B), sodium.....	2451
Somatropin for injection.....	2464	Starch glycolate (type C), sodium.....	2451
<i>Somatropini solutio ad praeparationem</i>	2462	Starch, maize.....	1964
<i>Somatropinum</i>	2460	Starch, potato.....	2288
<i>Somatropinum ad iniectabilium</i>	2464	Starch, pregelatinised.....	2490
Sorbic acid.....	2467	Starch, rice.....	2369
<i>Sorbitani lauras</i>	2467	Starch, wheat.....	2698
<i>Sorbitani oleas</i>	2468	Statistical analysis of results of biological assays and tests (5.3.).....	475
<i>Sorbitani palmitas</i>	2468	Steam sterilisation of aqueous preparations, application of the <i>F</i> ₀ concept (5.1.5.).....	449
<i>Sorbitani sesquioleas</i>	2468	Stearic acid.....	2490
<i>Sorbitani stearas</i>	2469	Stearoyl macroglycerides.....	2491
<i>Sorbitani trioleas</i>	2469	Stearyl alcohol.....	2492
Sorbitan laurate.....	2467	Sterile braided silk suture in distributor for veterinary use.....	887
Sorbitan oleate.....	2468	Sterile catgut.....	873
Sorbitan palmitate.....	2468	Sterile catgut in distributor for veterinary use.....	885
Sorbitan sesquioleate.....	2468	Sterile containers of plasticised poly (vinyl chloride) for human blood containing anticoagulant solution (3.2.5.).....	312
Sorbitan stearate.....	2469	Sterile linen thread in distributor for veterinary use.....	886
Sorbitan trioleate.....	2469		
Sorbitol.....	2470		
Sorbitol, liquid (crystallising).....	2471		
Sorbitol, liquid (non-crystallising).....	2472		

Sterile non-absorbable strands in distributor for veterinary use.....	888	Sulfamethoxypyridazine for veterinary use	2512
Sterile non-absorbable sutures	874	<i>Sulfamethoxypyridazinum ad usum veterinarium</i>	2512
Sterile plastic containers for human blood and blood components (3.2.3.).....	309	Sulfanilamide	2513
Sterile polyamide 6/6 suture in distributor for veterinary use.....	887	<i>Sulfanilamidum</i>	2513
Sterile polyamide 6 suture in distributor for veterinary use.....	886	Sulfasalazine	2514
Sterile poly(ethylene terephthalate) suture in distributor for veterinary use.....	887	<i>Sulfasalazinum</i>	2514
Sterile products, methods of preparation (5.1.1.).....	445	Sulfathiazole	2516
Sterile single-use plastic syringes (3.2.8.).....	314	<i>Sulfathiazolum</i>	2516
Sterile synthetic absorbable braided sutures	878	Sulfinpyrazone	2517
Sterile synthetic absorbable monofilament sutures.....	880	<i>Sulfinpyrazonum</i>	2517
Sterilisation procedures, biological indicators (5.1.2.)	447	Sulfisomidine	2518
Sterility (2.6.1.).....	145	<i>Sulfisomidinum</i>	2518
Sterols in fatty oils (2.4.23.).....	111	<i>Sulfur ad usum externum</i>	2520
Sticks	626	<i>Sulfuris colloidalis et technetii (^{99m}Tc) solutio iniectionabilis</i>	852
Sticks, nasal.....	611	Sulindac	2518
St. John's wort.....	2485	<i>Sulindacum</i>	2518
Stomata and stomatal index (2.8.3.).....	215	Sulphated ash (2.4.14.)	108
<i>Stramonii folium</i>	2492	Sulphates (2.4.13.).....	108
<i>Stramonii pulvis normatus</i>	2494	Sulphur dioxide (2.5.29.).....	136
Stramonium leaf.....	2492	Sulphur for external use	2520
Stramonium, prepared	2494	Sulphuric acid.....	2520
Strands, sterile non-absorbable, in distributor for veterinary use	888	Sulpiride.....	2521
Streptokinase bulk solution	2495	<i>Sulpiridum</i>	2521
<i>Streptokinasi solutio ad praeparationem</i>	2495	<i>Sumatriptani succinas</i>	2522
<i>Streptomycini sulfas</i>	2496	Sumatriptan succinate	2522
Streptomycin sulphate	2496	Sunflower oil, refined	2524
<i>Strontii (⁸⁹Sr) chloridi solutio iniectionabilis</i>	850	Supercritical fluid chromatography (2.2.45.)	68
Strontium (⁸⁹ Sr) chloride injection	850	Suppositories	623
<i>Styli</i>	626	Suppositories and pessaries, disintegration of (2.9.2.).....	227
Sublingual tablets and buccal tablets	613	Suppositories and pessaries, resistance to rupture (2.9.24.)	258
Substances for pharmaceutical use.....	586	Suppositories, lipophilic, softening time determination (2.9.22.)	256
Substances for pharmaceutical use, control of impurities (5.10.).....	559	Sutures, sterile non-absorbable	874
Substances of animal origin for the production of veterinary vaccines (5.2.5.).....	460	Sutures, sterile synthetic absorbable braided	878
Sub-visible particles, particulate contamination (2.9.19.)..	253	Sutures, sterile synthetic absorbable monofilament	880
Succinylsulfathiazole	2498	<i>Suxamethonii chloridum</i>	2525
<i>Succinylsulfathiazolum</i>	2498	Suxamethonium chloride.....	2525
Sucrose.....	2499	Suxibuzone.....	2525
Sufentanil	2501	<i>Suxibuzonum</i>	2525
Sufentanil citrate	2502	Sweet fennel.....	1580
<i>Sufentanili citras</i>	2502	Sweet orange oil.....	2526
<i>Sufentanilum</i>	2501	Swelling index (2.8.4.).....	215
Sugars, lead in (2.4.10.).....	107	Swine erysipelas vaccine (inactivated)	793
Sugar spheres	2503	Swine-fever vaccine (live), classical, freeze-dried	793
Sulfacetamide sodium.....	2504	Symbols and abbreviations (1.5.).....	9
<i>Sulfacetamidum natricum</i>	2504	Synthetic absorbable braided sutures, sterile	878
Sulfadiazine.....	2505	Synthetic absorbable monofilament sutures, sterile.....	880
<i>Sulfadiazinum</i>	2505	Syringes, plastic, sterile single-use (3.2.8.).....	314
Sulfadimidine.....	2506	Syrups.....	609
<i>Sulfadimidinum</i>	2506		
Sulfadoxine.....	2507	T	
<i>Sulfadoxinum</i>	2507	Table of physical characteristics of radionuclides mentioned in the European Pharmacopoeia (5.7.)	539
Sulfafurazole.....	2508	Tablets	626
<i>Sulfafurazolum</i>	2508	Tablets and capsules, disintegration of (2.9.1.).....	225
Sulfaguanidine.....	2508	Tablets, buccal	613
<i>Sulfaguanidinum</i>	2508	Tablets, coated	627
Sulfamerazine.....	2509	Tablets, dispersible	628
<i>Sulfamerazinum</i>	2509	Tablets, effervescent.....	627
Sulfamethizole.....	2510	Tablets for use in the mouth	628
<i>Sulfamethizolum</i>	2510	Tablets for vaginal solutions and suspensions	630
Sulfamethoxazole	2511	Tablets, gastro-resistant.....	628
<i>Sulfamethoxazolum</i>	2511	Tablets, modified-release	628
		Tablets, orodispersible	628
		Tablets, resistance to crushing (2.9.8.)	235
		Tablets, soluble.....	628
		Tablets, sublingual.....	613

Tablets, uncoated	627	Test for methanol and 2-propanol (2.9.11.)	239
Tablets, uncoated, friability of (2.9.7.)	234	Test for neurovirulence of live virus vaccines (2.6.18.)	172
Tablets, vaginal	629	Test for neurovirulence of poliomyelitis vaccine (oral) (2.6.19.)	172
Talc	2531	Testosterone	2542
<i>Talcum</i>	2531	Testosterone enantate	2544
Tamoxifen citrate	2532	Testosterone propionate	2545
<i>Tamoxifeni citras</i>	2532	<i>Testosteroni enantas</i>	2544
<i>Tamponae medicatae</i>	628	<i>Testosteroni propionas</i>	2545
Tampons, ear	602	<i>Testosteronum</i>	2542
Tampons, medicated	628	Tests for extraneous agents in viral vaccines for human use (2.6.16.)	169
Tampons, rectal	624	Tetanus antitoxin for human use	805
Tampons, vaginal, medicated	630	Tetanus antitoxin for veterinary use	812
<i>Tanacetii parthenii herba</i>	1592	Tetanus, diphtheria and hepatitis B (rDNA) vaccine (adsorbed)	641
Tannic acid	2534	Tetanus, diphtheria, pertussis (acellular, component), poliomyelitis (inactivated) and haemophilus type b conjugate vaccine (adsorbed)	653
Tannins in herbal drugs, determination of (2.8.14.)	221	Tetanus, diphtheria, pertussis and poliomyelitis (inactivated) vaccine (adsorbed)	656
<i>Tanninum</i>	2534	Tetanus, diphtheria, pertussis, poliomyelitis (inactivated) and haemophilus type b conjugate vaccine (adsorbed)	657
Tartaric acid	2534	Tetanus immunoglobulin, human	1751
Teat dips	631	Tetanus vaccine (adsorbed)	702
Tea tree oil	2534	Tetanus vaccine (adsorbed), assay of (2.7.8.)	198
Teat sprays	631	Tetanus vaccine for veterinary use	795
<i>Technetii (^{99m}Tc) et etifenini solutio iniectionabilis</i>	853	Tetracaine hydrochloride	2546
<i>Technetii (^{99m}Tc) exametazimi solutio iniectionabilis</i>	854	<i>Tetracaini hydrochloridum</i>	2546
<i>Technetii (^{99m}Tc) gluconatis solutio iniectionabilis</i>	856	Tetracosactide	2547
<i>Technetii (^{99m}Tc) humani albumini solutio iniectionabilis</i>	856	<i>Tetracosactidum</i>	2547
<i>Technetii (^{99m}Tc) macrosalbi suspensio iniectionabilis</i>	858	Tetracycline	2549
<i>Technetii (^{99m}Tc) medronati solutio iniectionabilis</i>	859	Tetracycline hydrochloride	2551
<i>Technetii (^{99m}Tc) mertiatidi solutio iniectionabilis</i>	860	<i>Tetracyclini hydrochloridum</i>	2551
<i>Technetii (^{99m}Tc) microsphaerarum suspensio iniectionabilis</i>	861	<i>Tetracyclinum</i>	2549
<i>Technetii (^{99m}Tc) pentetatis solutio iniectionabilis</i>	862	Tetrazepam	2552
<i>Technetii (^{99m}Tc) sestamibi solutio iniectionabilis</i>	863	<i>Tetrazepamum</i>	2552
<i>Technetii (^{99m}Tc) succimeri solutio iniectionabilis</i>	865	<i>Thallosi (²⁰¹Tl) chloridi solutio iniectionabilis</i>	867
Technetium (^{99m} Tc) colloidal rhenium sulphide injection	851	Thallous (²⁰¹ Tl) chloride injection	867
Technetium (^{99m} Tc) colloidal sulphur injection	852	Theobromine	2554
Technetium (^{99m} Tc) colloidal tin injection	853	<i>Theobrominum</i>	2554
Technetium (^{99m} Tc) etifenin injection	853	Theophylline	2554
Technetium (^{99m} Tc) exametazime injection	854	Theophylline-ethylenediamine	2557
Technetium (^{99m} Tc) gluconate injection	856	Theophylline-ethylenediamine hydrate	2557
Technetium (^{99m} Tc) human albumin injection	856	Theophylline monohydrate	2555
Technetium (^{99m} Tc) macrosalb injection	858	<i>Theophyllinum</i>	2554
Technetium (^{99m} Tc) medronate injection	859	<i>Theophyllinum et ethylenediaminum</i>	2557
Technetium (^{99m} Tc) mertiatide injection	860	<i>Theophyllinum et ethylenediaminum hydricum</i>	2557
Technetium (^{99m} Tc) microspheres injection	861	<i>Theophyllinum monohydricum</i>	2555
Technetium (^{99m} Tc) pentetate injection	862	Thermal analysis (2.2.34.)	52
Technetium (^{99m} Tc) sestamibi injection	863	Thermogravimetry (2.2.34.)	52
Technetium (^{99m} Tc) succimer injection	865	Thiamazole	2558
Technetium (^{99m} Tc) tin pyrophosphate injection	865	<i>Thiamazolum</i>	2558
Temazepam	2535	Thiamine hydrochloride	2559
<i>Temazepamum</i>	2535	Thiamine nitrate	2561
Tenosynovitis avian viral vaccine (live)	729	<i>Thiamini hydrochloridum</i>	2559
Tenoxicam	2537	<i>Thiamini nitras</i>	2561
<i>Tenoxicamum</i>	2537	Thiamphenicol	2562
Terbutaline sulphate	2538	<i>Thiamphenicolum</i>	2562
<i>Terbutalini sulfas</i>	2538	Thin-layer chromatography (2.2.27.)	40
Terconazole	2539	Thiomersal	2563
<i>Terconazolum</i>	2539	<i>Thiomersalum</i>	2563
<i>Terebinthini aetheroleum ab pinum pinastrum</i>	2645	Thiopental sodium and sodium carbonate	2564
Terfenadine	2540	<i>Thiopentalum natricum et natrii carbonas</i>	2564
<i>Terfenadinum</i>	2540	Thioridazine	2565
Terminology used in monographs on vaccines (5.2.1.)	453	Thioridazine hydrochloride	2566
<i>tert-Butylamini perindoprilum</i>	2210	<i>Thioridazini hydrochloridum</i>	2566
Test for anticomplementary activity of immunoglobulin (2.6.17.)	170	<i>Thioridazinum</i>	2565
Test for deliverable mass or volume of liquid and semi-solid preparations (2.9.28.)	263	Three-lobed sage leaf	2390
Test for extractable volume of parenteral preparations (2.9.17.)	243		
Test for Fc function of immunoglobulin (2.7.9.)	202		

Threonine.....	2566	Total organic carbon in water for pharmaceutical use (2.2.44.)	68
<i>Threoninum</i>	2566	Total protein (2.5.33.)	138
Thyme	2567	Toxicity, abnormal (2.6.9.).....	153
Thyme oil	2569	Toxin, botulinum type A for injection.....	1117
Thyme, wild	2701	<i>Toxinum botulinicum typum A ad iniectionabile</i>	1117
<i>Thymi aetheroleum</i>	2569	Tragacanth	2606
<i>Thymi herba</i>	2567	<i>Tragacantha</i>	2606
Thymol.....	2570	Tramadol hydrochloride	2607
<i>Thymolum</i>	2570	<i>Tramadoli hydrochloridum</i>	2607
Tiabendazole	2570	Tramazoline hydrochloride monohydrate	2608
<i>Tiabendazolum</i>	2570	<i>Tramazolini hydrochloridum monohydricum</i>	2608
Tiamulin for veterinary use	2571	Tranexamic acid	2609
Tiamulin hydrogen fumarate for veterinary use	2573	Transdermal patches	616
<i>Tiamulini hydrogenofumaras ad usum veterinarium</i> ..	2573	Transdermal patches, dissolution test for (2.9.4.)	231
<i>Tiamulinum ad usum veterinarium</i>	2571	Trapidil	2610
Tianeptine sodium	2575	<i>Trapidilum</i>	2610
<i>Tianeptinum natricum</i>	2575	Tretinoin	2611
Tiapride hydrochloride	2577	<i>Tretinoinum</i>	2611
<i>Tiapridi hydrochloridum</i>	2577	Triacetin	2612
Tiaprofenic acid	2578	<i>Triacetinum</i>	2612
Ticarcillin sodium.....	2579	Triamcinolone.....	2613
<i>Ticarcillinum natricum</i>	2579	Triamcinolone acetonide	2614
Tick-borne encephalitis vaccine (inactivated).....	703	Triamcinolone hexacetonide	2616
Ticlopidine hydrochloride	2581	<i>Triamcinoloni acetamidum</i>	2614
<i>Ticlopidini hydrochloridum</i>	2581	<i>Triamcinoloni hexacetamidum</i>	2616
<i>Tiliae flos</i>	1914	<i>Triamcinolonum</i>	2613
Tilidine hydrochloride hemihydrate	2582	Triamterene	2617
<i>Tilidini hydrochloridum hemihydricum</i>	2582	<i>Triamterenum</i>	2617
<i>Timololi maleas</i>	2584	Tribenoside	2617
Timolol maleate	2584	<i>Tribenosidum</i>	2617
<i>Tincturae</i>	571	Tributyl acetyl citrate	2619
<i>Tincturae maternas</i> <i>ad praeparationes homoeopaths</i>	894	<i>Tributylis acetyl citras</i>	2619
Tinctures	571	<i>Tricalcii phosphas</i>	1167
Tinidazole	2585	Trichloroacetic acid	2620
<i>Tinidazolum</i>	2585	Triethanolamine	2632
Tinnevely senna pods.....	2405	Triethyl citrate	2620
Tinzaparin sodium	2586	<i>Triethylis citras</i>	2620
<i>Tinzaparinum natricum</i>	2586	Trifluoperazine hydrochloride	2621
Tioconazole	2586	<i>Trifluoperazini hydrochloridum</i>	2621
<i>Tioconazolum</i>	2586	Triflusal	2622
<i>Titanii dioxidum</i>	2587	<i>Triflusalum</i>	2622
Titanium dioxide	2587	<i>Triglycerida saturata media</i>	2623
Titration, amperometric (2.2.19.).....	34	Triglycerides, medium-chain.....	2623
Titration, potentiometric (2.2.20.).....	35	Triglycerides, omega-3-acid.....	2144
Titration, complexometric (2.5.11.)	130	<i>Trigonellae foenugraeci semen</i>	1588
Tobramycin	2588	Trihexyphenidyl hydrochloride	2625
<i>Tobramycinum</i>	2588	<i>Trihexyphenidyli hydrochloridum</i>	2625
α -Tocopherol acetate concentrate (powder form)	2592	Trimetazidine dihydrochloride.....	2626
Tocopherol, all- <i>rac</i> - α	2590	<i>Trimetazidini dihydrochloridum</i>	2626
<i>α-Tocopheroli acetatis pulvis</i>	2592	Trimethadione	2627
Tocopherol, <i>RRR</i> - α	2591	<i>Trimethadionum</i>	2627
Tocopheryl acetate, all- <i>rac</i> - α	2594	Trimethoprim	2628
Tocopheryl acetate, <i>RRR</i> - α	2595	<i>Trimethoprimum</i>	2628
Tocopheryl hydrogen succinate, DL- α	2597	Trimipramine maleate	2630
Tocopheryl hydrogen succinate, <i>RRR</i> - α	2598	<i>Trimipramini maleas</i>	2630
Tolbutamide	2600	<i>Tri-n-butylis phosphas</i>	2631
<i>Tolbutamidum</i>	2600	Tri- <i>n</i> -butyl phosphate	2631
Tolfenamic acid.....	2601	Tritiated (³ H) water injection.....	867
Tolnaftate	2602	<i>Tritici aestivi oleum raffinatum</i>	2699
<i>Tolnaftatum</i>	2602	<i>Tritici aestivi oleum virginale</i>	2699
Tolu balsam	2603	<i>Tritici amyllum</i>	2698
Tormentil	2604	Trolamine.....	2632
<i>Tormentillae rhizoma</i>	2604	<i>Trolaminum</i>	2632
<i>Tormentillae tinctura</i>	2605	Trometamol	2633
Tormentil tincture.....	2605	<i>Trometamolum</i>	2633
Tosylchloramide sodium.....	2605	Tropicamide.....	2634
<i>Tosylchloramidum natricum</i>	2605	<i>Tropicamidum</i>	2634
Total ash (2.4.16.).....	108	Trypsin	2635

<i>Trypsinum</i>	2635	<i>Vaccina ad usum veterinarium</i>	590
Tryptophan.....	2636	Vaccines, adsorbed, aluminium in (2.5.13.).....	131
<i>Tryptophanum</i>	2636	Vaccines, adsorbed, calcium in (2.5.14.).....	131
Tuberculin for human use, old.....	2638	Vaccines and immunosera, phenol in (2.5.15.).....	131
<i>Tuberculini aviarii derivatum proteinosum purificatum</i>	2640	Vaccines for human use.....	588
<i>Tuberculini bovini derivatum proteinosum purificatum</i>	2641	Vaccines for human use, cell substrates for the production of (5.2.3.)	455
<i>Tuberculini derivatum proteinosum purificatum ad usum humanum</i>	2642	Vaccines for human use, viral, extraneous agents in (2.6.16.)	169
Tuberculin purified protein derivative, avian	2640	Vaccines for veterinary use.....	590
Tuberculin purified protein derivative, bovine.....	2641	Vaccines, polysaccharide, hexosamines in (2.5.20.).....	132
Tuberculin purified protein derivative for human use ...	2642	Vaccines, polysaccharide, methylpentoses in (2.5.21.).....	133
<i>Tuberculinum pristinum ad usum humanum</i>	2638	Vaccines, polysaccharide, nucleic acids in (2.5.17.)	132
Tubes for comparative tests (2.1.5.).....	19	Vaccines, polysaccharide, <i>O</i> -acetyl in (2.5.19.).....	132
Tubing and closures, silicone elastomer for (3.1.9.).....	288	Vaccines, polysaccharide, phosphorus in (2.5.18.)	132
Tubing and containers for total parenteral nutrition preparations, poly(ethylene - vinyl acetate) for (3.1.7.) ...	285	Vaccines, polysaccharide, protein in (2.5.16.)	131
Tubing used in sets for the transfusion of blood and blood components, materials based on plasticised poly(vinyl chloride) for (3.1.1.2.)	272	Vaccines, polysaccharide, ribose in (2.5.31.).....	137
Tubocurarine chloride	2644	Vaccines, polysaccharide, sialic acid in (2.5.23.)	133
<i>Tubocurariini chloridum</i>	2644	Vaccines, polysaccharide, uronic acids in (2.5.22.).....	133
Turmeric, javanese.....	2645	Vaccines, SPF chicken flocks for production and quality control of (5.2.2.)	453
Turpentine oil, <i>Pinus pinaster</i> type	2645	Vaccines, terminology (5.2.1.)	453
Tylosin for veterinary use	2647	Vaccines, veterinary, cell cultures for the production of (5.2.4.)	458
<i>Tylosini phosphatis solutio ad usum veterinarium</i>	2648	Vaccines, veterinary, evaluation of efficacy (5.2.7.)	462
<i>Tylosini tartras ad usum veterinarium</i>	2650	Vaccines, veterinary, evaluation of safety (5.2.6.)	461
Tylosin phosphate bulk solution for veterinary use	2648	Vaccines, veterinary, substances of animal origin for the production of (5.2.5.)	460
Tylosin tartrate for veterinary use	2650	Vaccines, viral live, test for neurovirulence (2.6.18.).....	172
<i>Tylosinum ad usum veterinarium</i>	2647	<i>Vaccinum actinobacillosis inactivatum ad suem</i>	784
Typhoid polysaccharide vaccine	705	<i>Vaccinum adenovirocidis caninae vivum</i>	738
Typhoid vaccine.....	707	<i>Vaccinum adenovirocidis caninae inactivatum</i>	738
Typhoid vaccine, freeze-dried.....	707	<i>Vaccinum anaemiae infectivae pulli vivum</i>	769
Typhoid vaccine (live, oral, strain Ty 21a).....	708	<i>Vaccinum anthracis vivum ad usum veterinarium</i>	715
Tyrosine.....	2651	<i>Vaccinum aphtharum epizooticarum inactivatum ad ruminantes</i>	764
<i>Tyrosinum</i>	2651	<i>Vaccinum bronchitidis infectivae aviariae inactivatum</i> ..	718
Tyrothricin.....	2652	<i>Vaccinum bronchitidis infectivae aviariae vivum</i>	720
<i>Tyrothricinum</i>	2652	<i>Vaccinum brucellosis (Brucella melitensis stirpe Rev. 1) vivum cryodesiccatum ad usum veterinarium</i>	735
U		<i>Vaccinum bursitidis infectivae aviariae inactivatum</i>	722
Ubidecarenone.....	2657	<i>Vaccinum bursitidis infectivae aviariae vivum</i>	723
<i>Ubidecarenonum</i>	2657	<i>Vaccinum calicivirocidis felinae inactivatum</i>	757
Udder-washes	631	<i>Vaccinum calicivirocidis felinae vivum cryodesiccatum</i> ..	758
Ultraviolet absorption spectrophotometry (2.2.25.).....	38	<i>Vaccinum cholerae</i>	637
Ultraviolet ray lamps for analytical purposes (2.1.3.).....	17	<i>Vaccinum cholerae cryodesiccatum</i>	638
Uncoated tablets.....	627	<i>Vaccinum clostridii botulini ad usum veterinarium</i>	745
Undecylenic acid	2658	<i>Vaccinum clostridii chauvoei ad usum veterinarium</i>	745
Uniformity of content of single-dose preparations (2.9.6.)	234	<i>Vaccinum clostridii novyi B ad usum veterinarium</i>	746
Uniformity of mass of delivered doses from multidose containers (2.9.27.).....	263	<i>Vaccinum clostridii perfringentis ad usum veterinarium</i>	747
Uniformity of mass of single-dose preparations (2.9.5.)	233	<i>Vaccinum clostridii septici ad usum veterinarium</i>	749
Units of the International System (SI) used in the Pharmacopoeia and equivalence with other units (1.6.) ...	10	<i>Vaccinum colibacillosis fetus a partu recentis inactivatum ad ruminantes</i>	778
Unsaponifiable matter (2.5.7.)	129	<i>Vaccinum colibacillosis fetus a partu recentis inactivatum ad suem</i>	776
Urea.....	2658	<i>Vaccinum diarrhoeae viralis bovinae inactivatum</i>	734
<i>Ureum</i>	2658	<i>Vaccinum diphtheriae adsorbatum</i>	660
Urofollitropin	2659	<i>Vaccinum diphtheriae adulti et adolescentis adsorbatum</i>	661
<i>Urofollitropinum</i>	2659	<i>Vaccinum diphtheriae et tetani adsorbatum</i>	639
Urokinase.....	2661	<i>Vaccinum diphtheriae et tetani adulti et adolescentis adsorbatum</i>	639
<i>Urokinasum</i>	2661	<i>Vaccinum diphtheriae, tetani et hepatitidis B (ADNr) adsorbatum</i>	641
Uronic acids in polysaccharide vaccines (2.5.22.).....	133	<i>Vaccinum diphtheriae, tetani et pertussis adsorbatum</i> ..	643
Ursodeoxycholic acid	2662	<i>Vaccinum diphtheriae, tetani et pertussis sine cellulis ex elementis praeparatum adsorbatum</i>	642
<i>Urtica dioica ad praeparationes homoeopathicas</i>	895		
<i>Uvae ursi folium</i>	1054		
V			
<i>Vaccina ad usum humanum</i>	588		

<i>Vaccinum diphtheriae, tetani, pertussis et poliomyelitidis inactivatum adsorbatum</i>	656	<i>Vaccinum morbi Carrei vivum cryodesiccatum ad canem</i>	740
<i>Vaccinum diphtheriae, tetani, pertussis, poliomyelitidis inactivatum et haemophili stirpe b coniugatum adsorbatum</i>	657	<i>Vaccinum morbi Carrei vivum cryodesiccatum ad mustelidas</i>	751
<i>Vaccinum diphtheriae, tetani, pertussis sine cellulis ex elementis praeparatum et haemophili stirpe b coniugatum adsorbatum</i>	645	<i>Vaccinum morbillorum, parotitidis et rubellae vivum</i>	678
<i>Vaccinum diphtheriae, tetani, pertussis sine cellulis ex elementis praeparatum et hepatitidis B (ADNr) adsorbatum</i>	647	<i>Vaccinum morbillorum vivum</i>	679
<i>Vaccinum diphtheriae, tetani, pertussis sine cellulis ex elementis praeparatum et poliomyelitidis inactivatum adsorbatum</i>	648	<i>Vaccinum morbi Marek vivum</i>	774
<i>Vaccinum diphtheriae, tetani, pertussis sine cellulis ex elementis praeparatum, hepatitidis B (ADNr), poliomyelitidis inactivatum et haemophili stirpe b coniugatum adsorbatum</i>	650	<i>Vaccinum morbi partus diminutionis MCMLXXVI inactivatum ad pullum</i>	753
<i>Vaccinum diphtheriae, tetani, pertussis sine cellulis ex elementis praeparatum poliomyelitidis inactivatum et haemophili stirpe b coniugatum adsorbatum</i>	653	<i>Vaccinum myxomatosis vivum ad cuniculum</i>	775
<i>Vaccinum encephalitis ixodibus advectae inactivatum</i>	703	<i>Vaccinum panleucopeniae felinae infectivae inactivatum</i>	759
<i>Vaccinum encephalomyelitis infectivae aviariae vivum</i>	725	<i>Vaccinum panleucopeniae felinae infectivae vivum</i>	760
<i>Vaccinum erysipelatis suillae inactivatum</i>	793	<i>Vaccinum parainfluenzae viri bovini vivum</i>	732
<i>Vaccinum febris flavae vivum</i>	710	<i>Vaccinum parainfluenzae viri canini vivum</i>	742
<i>Vaccinum febris typhoidi</i>	707	<i>Vaccinum paramyxovirus 3 aviarii inactivatum</i>	728
<i>Vaccinum febris typhoidi cryodesiccatum</i>	707	<i>Vaccinum parotitidis vivum</i>	684
<i>Vaccinum febris typhoidis polysaccharidicum</i>	705	<i>Vaccinum parvovirus caninae inactivatum</i>	743
<i>Vaccinum febris typhoidis vivum perorale (stirpe Ty 21a)</i>	708	<i>Vaccinum parvovirus caninae vivum</i>	744
<i>Vaccinum furunculosis ad salmonidas inactivatum cum adiuvatione oleosa ad iniectionem</i>	767	<i>Vaccinum parvovirus inactivatum ad suem</i>	787
<i>Vaccinum haemophili stirpe b coniugatum</i>	662	<i>Vaccinum pasteurellae inactivatum ad ovem</i>	783
<i>Vaccinum hepatitidis A inactivatum adsorbatum</i>	665	<i>Vaccinum pertussis</i>	685
<i>Vaccinum hepatitidis A inactivatum et hepatitidis B (ADNr) adsorbatum</i>	664	<i>Vaccinum pertussis adsorbatum</i>	690
<i>Vaccinum hepatitidis A inactivatum virosomale</i>	667	<i>Vaccinum pertussis sine cellulis copurificatum adsorbatum</i>	688
<i>Vaccinum hepatitidis B (ADNr)</i>	670	<i>Vaccinum pertussis sine cellulis ex elementis praeparatum adsorbatum</i>	686
<i>Vaccinum hepatitidis viralis anatis stirpe I vivum</i>	751	<i>Vaccinum pestis classicae suillae vivum cryodesiccatum</i>	793
<i>Vaccinum herpesvirus equini inactivatum</i>	754	<i>Vaccinum pneumococcale polysaccharidicum</i>	691
<i>Vaccinum inactivatum diarrhoeae vituli coronaviro illatae</i>	736	<i>Vaccinum poliomyelitis inactivatum</i>	692
<i>Vaccinum inactivatum diarrhoeae vituli rotaviro illatae</i>	737	<i>Vaccinum poliomyelitis perorale</i>	695
<i>Vaccinum influenzae equi inactivatum</i>	755	<i>Vaccinum pseudopestis aviariae inactivatum</i>	779
<i>Vaccinum influenzae inactivatum ad suem</i>	785	<i>Vaccinum pseudopestis aviariae vivum</i>	781
<i>Vaccinum influenzae inactivatum ex corticis antigeniis praeparatum</i>	673	<i>Vaccinum rabiei ex cellulis ad usum humanum</i>	699
<i>Vaccinum influenzae inactivatum ex corticis antigeniis praeparatum virosomale</i>	674	<i>Vaccinum rabiei inactivatum ad usum veterinarium</i>	790
<i>Vaccinum influenzae inactivatum ex viris integris praeparatum</i>	676	<i>Vaccinum rabiei perorale vivum ad vulpem</i>	792
<i>Vaccinum influenzae inactivatum ex virorum fragmentis praeparatum</i>	671	<i>Vaccinum rhinitidis atrophicantis ingravescens suillae inactivatum</i>	788
<i>Vaccinum laryngotracheitis infectivae aviariae vivum</i>	727	<i>Vaccinum rhinotracheitis infectivae bovinae vivum cryodesiccatum</i>	768
<i>Vaccinum leptospirosis bovinae inactivatum</i>	730	<i>Vaccinum rhinotracheitis viralis felinae inactivatum</i>	762
<i>Vaccinum leptospirosis caninae inactivatum</i>	740	<i>Vaccinum rhinotracheitis viralis felinae vivum cryodesiccatum</i>	763
<i>Vaccinum leucosis felinae inactivatum</i>	761	<i>Vaccinum rubellae vivum</i>	701
<i>Vaccinum mannheimiae inactivatum ad bovidas</i>	771	<i>Vaccinum tenosynovitis viralis aviariae vivum</i>	729
<i>Vaccinum mannheimiae inactivatum ad ovem</i>	772	<i>Vaccinum tetani adsorbatum</i>	702
<i>Vaccinum meningococcale classis C coniugatum</i>	680	<i>Vaccinum tetani ad usum veterinarium</i>	795
<i>Vaccinum meningococcale polysaccharidicum</i>	682	<i>Vaccinum tuberculosis (BCG) cryodesiccatum</i>	636
<i>Vaccinum morbi Aujeszkyi ad suem inactivatum</i>	715	<i>Vaccinum varicellae vivum</i>	709
<i>Vaccinum morbi Aujeszkyi ad suem vivum cryodesiccatum ad usum parenterale</i>	717	<i>Vaccinum variolae gallinae vivum</i>	766
		<i>Vaccinum vibriosidis ad salmonidas inactivatum</i>	797
		<i>Vaccinum vibriosidis aquae frigidae inactivatum ad salmonidas</i>	796
		<i>Vaccinum viri syncytialis meatus spiritus bovini vivum cryodesiccatum</i>	733
		Vaginal capsules.....	629
		Vaginal foams.....	630
		Vaginalia.....	629
		Vaginal preparations.....	629
		Vaginal preparations, semi-solid.....	630
		Vaginal solutions and suspensions, tablets for.....	630
		Vaginal solutions, emulsions and suspensions.....	630
		Vaginal tablets.....	629
		Vaginal tampons, medicated.....	630
		Valerianae radix.....	2667
		Valerian root.....	2667

- Validation of nucleic acid amplification techniques for the detection of hepatitis C virus (HCV) RNA in plasma pools: Guidelines.....176
- Valine.....2668
- Valinum*.....2668
- Valproic acid.....2669
- Vancomycin hydrochloride.....2670
- Vancomycini hydrochloridum*.....2670
- Vanillin.....2672
- Vanillinum*.....2672
- Varicella immunoglobulin for intravenous administration, human.....1753
- Varicella immunoglobulin, human.....1752
- Varicella vaccine (live).....709
- Vaselinum album*.....2187
- Vaselinum flavum*.....2188
- Vegetable drugs, determination of essential oils in vegetable drugs (2.8.12.).....217
- Vegetable fatty oils.....595
- Verapamil hydrochloride.....2673
- Verapamili hydrochloridum*.....2673
- Verbasci flos*.....2065
- Veterinary liquid preparations for cutaneous application..630
- Vibriosis (cold-water) vaccine (inactivated) for salmonids..796
- Vibriosis vaccine (inactivated) for salmonids.....797
- VICH (5.8.).....551
- Vinblastine sulphate.....2675
- Vinblastini sulfas*.....2675
- Vincristine sulphate.....2675
- Vincristini sulfas*.....2675
- Vindesine sulphate.....2677
- Vindesini sulfas*.....2677
- Vinorelbine tartrate.....2679
- Vinorelbini tartaras*.....2679
- Violae herba cum flore*.....2700
- Viper venom antiserum, European.....806
- Viscometer method, capillary (2.2.9.).....29
- Viscometer method, falling ball (2.2.49.).....80
- Viscometer method, rotating (2.2.10.).....30
- Viscose wadding, absorbent.....2681
- Viscosity (2.2.8.).....29
- Visible absorption spectrophotometry (2.2.25.).....38
- Visible particles, particulate contamination (2.9.20.).....255
- Vitamin A.....2682
- Vitamin A concentrate (oily form), synthetic.....2684
- Vitamin A concentrate (powder form), synthetic.....2685
- Vitamin A concentrate (solubilisate/emulsion), synthetic.....2686
- Vitaminum A*.....2682
- Vitaminum A densatum oleosum*.....2684
- Vitaminum A in aqua dispergibile*.....2686
- Vitaminum A pulvis*.....2685
- Volumetric analysis (4.2.).....435
- Volumetric solutions (4.2.2.).....435
- Volumetric solutions, primary standards for (4.2.1.).....435
- Von Willebrand factor, human, assay of (2.7.21.).....211
- W**
- Warfarin sodium.....2691
- Warfarin sodium clathrate.....2691
- Warfarinum natricum*.....2691
- Warfarinum natricum clathratum*.....2691
- Water (¹⁵O) injection.....868
- Water, determination by distillation (2.2.13.).....32
- Water for diluting concentrated haemodialysis solutions.....1699
- Water for injections.....2692
- Water for pharmaceutical use, total organic carbon in (2.2.44.).....68
- Water, highly purified.....2695
- Water in essential oils (2.8.5.).....216
- Water in gases (2.5.28.).....136
- Water: micro determination (2.5.32.).....137
- Water, purified.....2697
- Water: semi-micro determination (2.5.12.).....130
- Wheat-germ oil, refined.....2699
- Wheat-germ oil, virgin.....2699
- Wheat starch.....2698
- White beeswax.....1057
- White soft paraffin.....2187
- Wild pansy (flowering aerial parts).....2700
- Wild thyme.....2701
- Willow bark.....2702
- Wool alcohols.....2703
- Wool fat.....2704
- Wool fat, hydrogenated.....2708
- Wool fat, hydrous.....2709
- Wormwood.....2710
- X**
- Xanthan gum.....2715
- Xanthani gummi*.....2715
- Xenon (¹³³Xe) injection.....869
- Xenoni (¹³³Xe) solutio iniectabilis*.....869
- X-ray fluorescence spectrometry (2.2.37.).....56
- Xylazine hydrochloride for veterinary use.....2716
- Xylazini hydrochloridum ad usum veterinarium*.....2716
- Xylitol.....2717
- Xylitolum*.....2717
- Xylometazoline hydrochloride.....2719
- Xylometazolini hydrochloridum*.....2719
- Xylose.....2720
- Xylosum*.....2720
- Y**
- Yarrow.....2723
- Yellow beeswax.....1058
- Yellow fever vaccine (live).....710
- Yellow soft paraffin.....2188
- Z**
- Zidovudine.....2727
- Zidovudinum*.....2727
- Zinc acetate dihydrate.....2728
- Zinc acexamate.....2729
- Zinc chloride.....2730
- Zinci acetate dihydricus*.....2728
- Zinci acexamas*.....2729
- Zinci chloridum*.....2730
- Zinci oxidum*.....2731
- Zinci stearas*.....2732
- Zinci sulfas heptahydricus*.....2732
- Zinci sulfas hexahydricus*.....2733
- Zinci undecylenas*.....2733
- Zinc oxide.....2731
- Zinc stearate.....2732
- Zinc sulphate heptahydrate.....2732
- Zinc sulphate hexahydrate.....2733
- Zinc undecylenate.....2733
- Zingiberis rhizoma*.....1656
- Zolpidemi tartaras*.....2734
- Zolpidem tartrate.....2734
- Zopiclone.....2735
- Zopiclonum*.....2735
- Zuclopenthixol decanoate.....2736
- Zuclopenthixoli decanoas*.....2736

INDEX

To aid users the index includes a reference to the supplement where the latest version of a text can be found.

For example: Acetone.....5.1-2875

means the monograph Acetone can be found on page 2875 of Supplement 5.1.

Note that where no reference for a supplement is made, the text can be found in the principal volume.

Numerics

1.1. General statements	5	2.2.56. Amino acid analysis	86
1.2. Other provisions applying to general chapters and monographs	5	2.2.5. Relative density	27
1.3. General chapters	6	2.2.6. Refractive index	28
1.4. Monographs	7	2.2.7. Optical rotation	28
1.5. Abbreviations and symbols	9	2.2.8. Viscosity	29
1.6. Units of the International System (SI) used in the Pharmacopoeia and equivalence with other units	10	2.2.9. Capillary viscometer method	29
1.8-Cineole in essential oils, assay of (2.8.11.)	216	2.2. Physical and physicochemical methods	23
1. General notices	5	2.3.1. Identification reactions of ions and functional groups	95
2.1.1. Droppers	17	2.3.2. Identification of fatty oils by thin-layer chromatography	98
2.1.2. Comparative table of porosity of sintered-glass filters	17	2.3.3. Identification of phenothiazines by thin-layer chromatography	99
2.1.3. Ultraviolet ray lamps for analytical purposes	17	2.3.4. Odour	99
2.1.4. Sieves	18	2.3. Identification	95
2.1.5. Tubes for comparative tests	19	2.4.10. Lead in sugars	107
2.1.6. Gas detector tubes	19	2.4.11. Phosphates	108
2.1. Apparatus	17	2.4.12. Potassium	108
2.2.10. Rotating viscometer method	30	2.4.13. Sulphates	108
2.2.11. Distillation range	30	2.4.14. Sulphated ash	108
2.2.12. Boiling point	31	2.4.15. Nickel in polyols	108
2.2.13. Determination of water by distillation	32	2.4.16. Total ash	108
2.2.14. Melting point - capillary method	32	2.4.17. Aluminium	108
2.2.15. Melting point - open capillary method	33	2.4.18. Free formaldehyde	109
2.2.16. Melting point - instantaneous method	33	2.4.19. Alkaline impurities in fatty oils	109
2.2.17. Drop point	33	2.4.1. Ammonium	103
2.2.18. Freezing point	34	2.4.21. Foreign oils in fatty oils by thin-layer chromatography	109
2.2.19. Amperometric titration	34	2.4.22. Composition of fatty acids by gas chromatography	110
2.2.1. Clarity and degree of opalescence of liquids	23	2.4.23. Sterols in fatty oils	5.1-2787
2.2.20. Potentiometric titration	35	2.4.24. Identification and control of residual solvents	113
2.2.21. Fluorimetry	35	2.4.25. Ethylene oxide and dioxan	118
2.2.22. Atomic emission spectrometry	35	2.4.26. <i>N,N</i> -Dimethylaniline	119
2.2.23. Atomic absorption spectrometry	36	2.4.27. Heavy metals in herbal drugs and fatty oils	119
2.2.24. Absorption spectrophotometry, infrared	37	2.4.28. 2-Ethylhexanoic acid	120
2.2.25. Absorption spectrophotometry, ultraviolet and visible	38	2.4.29. Composition of fatty acids in oils rich in omega-3-acids	121
2.2.26. Paper chromatography	40	2.4.2. Arsenic	103
2.2.27. Thin-layer chromatography	40	2.4.30. Ethylene glycol and diethylene glycol in ethoxylated substances	122
2.2.28. Gas chromatography	42	2.4.3. Calcium	103
2.2.29. Liquid chromatography	43	2.4.4. Chlorides	104
2.2.2. Degree of coloration of liquids	24	2.4.5. Fluorides	104
2.2.30. Size-exclusion chromatography	45	2.4.6. Magnesium	104
2.2.31. Electrophoresis	45	2.4.7. Magnesium and alkaline-earth metals	104
2.2.32. Loss on drying	50	2.4.8. Heavy metals	104
2.2.33. Nuclear magnetic resonance spectrometry	51	2.4.9. Iron	107
2.2.34. Thermal analysis	52	2.4. Limit tests	103
2.2.35. Osmolality	54	2.5.10. Oxygen-flask method	130
2.2.36. Potentiometric determination of ionic concentration using ion-selective electrodes	55	2.5.11. Complexometric titrations	130
2.2.37. X-ray fluorescence spectrometry	56	2.5.12. Water: semi-micro determination	130
2.2.38. Conductivity	5.1-2783	2.5.13. Aluminium in adsorbed vaccines	131
2.2.39. Molecular mass distribution in dextrans	57	2.5.14. Calcium in adsorbed vaccines	131
2.2.3. Potentiometric determination of pH	26	2.5.15. Phenol in immunosera and vaccines	131
2.2.40. Near-infrared spectrophotometry	59	2.5.16. Protein in polysaccharide vaccines	131
2.2.41. Circular dichroism	63	2.5.17. Nucleic acids in polysaccharide vaccines	132
2.2.42. Density of solids	64	2.5.18. Phosphorus in polysaccharide vaccines	132
2.2.43. Mass spectrometry	65	2.5.19. <i>O</i> -Acetyl in polysaccharide vaccines	132
2.2.44. Total organic carbon in water for pharmaceutical use	68	2.5.1. Acid value	127
2.2.45. Supercritical fluid chromatography	68	2.5.20. Hexosamines in polysaccharide vaccines	132
2.2.46. Chromatographic separation techniques	69	2.5.21. Methylpentoses in polysaccharide vaccines	133
2.2.47. Capillary electrophoresis	74	2.5.22. Uronic acids in polysaccharide vaccines	133
2.2.48. Raman spectrometry	79	2.5.23. Sialic acid in polysaccharide vaccines	133
2.2.49. Falling ball viscometer method	80	2.5.24. Carbon dioxide in gases	134
2.2.4. Relationship between reaction of solution, approximate pH and colour of certain indicators	27	2.5.25. Carbon monoxide in gases	134
2.2.54. Isoelectric focusing	81	2.5.26. Nitrogen monoxide and nitrogen dioxide in gases	135
2.2.55. Peptide mapping	82	2.5.27. Oxygen in gases	136

2.5.28. Water in gases.....	136	2.7.8. Assay of tetanus vaccine (adsorbed).....	5.1-2791
2.5.29. Sulphur dioxide.....	136	2.7.9. Test for Fc function of immunoglobulin	202
2.5.2. Ester value	127	2.7. Biological assays	187
2.5.30. Oxidising substances.....	137	2.8.10. Solubility in alcohol of essential oils	216
2.5.31. Ribose in polysaccharide vaccines.....	137	2.8.11. Assay of 1,8-cineole in essential oils	216
2.5.32. Water: micro determination	137	2.8.12. Determination of essential oils in vegetable drugs.....	217
2.5.33. Total protein.....	138	2.8.13. Pesticide residues.....	218
2.5.34. Acetic acid in synthetic peptides	141	2.8.14. Determination of tannins in herbal drugs.....	221
2.5.35. Nitrous oxide in gases.....	141	2.8.15. Bitterness value.....	221
2.5.36. Anisidine value	142	2.8.16. Dry residue of extracts.....	222
2.5.3. Hydroxyl value	127	2.8.17. Loss on drying of extracts	222
2.5.4. Iodine value	127	2.8.1. Ash insoluble in hydrochloric acid	215
2.5.5. Peroxide value.....	128	2.8.2. Foreign matter	215
2.5.6. Saponification value	129	2.8.3. Stomata and stomatal index	215
2.5.7. Unsaponifiable matter	129	2.8.4. Swelling index.....	215
2.5.8. Determination of primary aromatic amino-nitrogen	129	2.8.5. Water in essential oils.....	216
2.5.9. Determination of nitrogen by sulphuric acid digestion	129	2.8.6. Foreign esters in essential oils	216
2.5. Assays.....	127	2.8.7. Fatty oils and resinified essential oils in essential oils.....	216
2.6.10. Histamine	153	2.8.8. Odour and taste of essential oils.....	216
2.6.11. Depressor substances.....	153	2.8.9. Residue on evaporation of essential oils.....	216
2.6.12. Microbiological examination of non-sterile products (total viable aerobic count).....	154	2.8. Methods in pharmacognosy	215
2.6.13. Microbiological examination of non-sterile products (test for specified micro-organisms).....	156	2.9.10. Ethanol content and alcoholimetric tables	237
2.6.14. Bacterial endotoxins	161	2.9.11. Test for methanol and 2-propanol	239
2.6.15. Prekallikrein activator.....	168	2.9.12. Sieve test.....	239
2.6.16. Tests for extraneous agents in viral vaccines for human use.....	169	2.9.13. Limit test of particle size by microscopy.....	239
2.6.17. Test for anticomplementary activity of immunoglobulin.....	170	2.9.14. Specific surface area by air permeability	239
2.6.18. Test for neurovirulence of live virus vaccines.....	172	2.9.15. Apparent volume	241
2.6.19. Test for neurovirulence of poliomyelitis vaccine (oral)	172	2.9.16. Flowability.....	242
2.6.1. Sterility	145	2.9.17. Test for extractable volume of parenteral preparations.....	243
2.6.20. Anti-A and anti-B haemagglutinins (indirect method)	174	2.9.18. Preparations for inhalation: aerodynamic assessment of fine particles	5.1-2799
2.6.21. Nucleic acid amplification techniques	174	2.9.19. Particulate contamination: sub-visible particles..	253
2.6.22. Activated coagulation factors.....	177	2.9.1. Disintegration of tablets and capsules.....	225
2.6.24. Avian viral vaccines: tests for extraneous agents in seed lots	177	2.9.20. Particulate contamination: visible particles.....	255
2.6.25. Avian live virus vaccines: tests for extraneous agents in batches of finished product	180	2.9.22. Softening time determination of lipophilic suppositories.....	256
2.6.2. Mycobacteria	149	2.9.23. Pycnometric density of solids.....	257
2.6.7. Mycoplasmas.....	149	2.9.24. Resistance to rupture of suppositories and pessaries	258
2.6.8. Pyrogens.....	152	2.9.25. Chewing gum, medicated, drug release from	260
2.6.9. Abnormal toxicity	153	2.9.26. Specific surface area by gas adsorption.....	5.1-2811
2.6. Biological tests	145	2.9.27. Uniformity of mass of delivered doses from multidose containers.....	263
2.7.10. Assay of human coagulation factor VII	203	2.9.28. Test for deliverable mass or volume of liquid and semi-solid preparations.....	263
2.7.11. Assay of human coagulation factor IX.....	204	2.9.2. Disintegration of suppositories and pessaries	227
2.7.12. Assay of heparin in coagulation factors	204	2.9.3. Dissolution test for solid dosage forms.....	228
2.7.13. Assay of human anti-D immunoglobulin.....	205	2.9.4. Dissolution test for transdermal patches	231
2.7.14. Assay of hepatitis A vaccine	5.1-2795	2.9.5. Uniformity of mass of single-dose preparations.....	233
2.7.15. Assay of hepatitis B vaccine (rDNA).....	207	2.9.6. Uniformity of content of single-dose preparations..	234
2.7.16. Assay of pertussis vaccine (acellular).....	208	2.9.7. Friability of uncoated tablets.....	234
2.7.17. Assay of human antithrombin III	209	2.9.8. Resistance to crushing of tablets.....	235
2.7.18. Assay of human coagulation factor II.....	209	2.9.9. Measurement of consistency by penetrometry	235
2.7.19. Assay of human coagulation factor X.....	210	2.9. Pharmaceutical technical procedures	225
2.7.1. Immunochemical methods	187	2-Ethylhexanoic acid (2.4.28.).....	120
2.7.20. <i>In vivo</i> assay of poliomyelitis vaccine (inactivated)	210	2-Propanol and methanol, test for (2.9.11.).....	239
2.7.21. Assay of human von Willebrand factor.....	211	3.1.10. Materials based on non-plasticised poly(vinyl chloride) for containers for non-injectable, aqueous solutions	289
2.7.22. Assay of human coagulation factor XI.....	212	3.1.11. Materials based on non-plasticised poly(vinyl chloride) for containers for dry dosage forms for oral administration	291
2.7.2. Microbiological assay of antibiotics.....	188	3.1.1.1. Materials based on plasticised poly(vinyl chloride) for containers for human blood and blood components.....	269
2.7.4. Assay of human coagulation factor VIII.....	194	3.1.1.2. Materials based on plasticised poly(vinyl chloride) for tubing used in sets for the transfusion of blood and blood components	272
2.7.5. Assay of heparin.....	195		
2.7.6. Assay of diphtheria vaccine (adsorbed)	196		
2.7.7. Assay of pertussis vaccine.....	197		

3.1.13. Plastic additives	293	5.2.5. Substances of animal origin for the production of veterinary vaccines	460
3.1.14. Materials based on plasticised poly(vinyl chloride) for containers for aqueous solutions for intravenous infusion	296	5.2.6. Evaluation of safety of veterinary vaccines and immunosera	5.1-2827
3.1.15. Polyethylene terephthalate for containers for preparations not for parenteral use	298	5.2.7. Evaluation of efficacy of veterinary vaccines and immunosera	5.1-2829
3.1.1. Materials for containers for human blood and blood components	269	5.2.8. Minimising the risk of transmitting animal spongiform encephalopathy agents via human and veterinary medicinal products	463
3.1.3. Polyolefines	274	5.2.9. Evaluation of safety of each batch of veterinary vaccines and immunosera	5.1-2830
3.1.4. Polyethylene without additives for containers for parenteral preparations and for ophthalmic preparations	278	5.2. General texts on vaccines	453
3.1.5. Polyethylene with additives for containers for parenteral preparations and for ophthalmic preparations	279	5.3. Statistical analysis of results of biological assays and tests	475
3.1.6. Polypropylene for containers and closures for parenteral preparations and ophthalmic preparations ...	282	5.4. Residual solvents	507
3.1.7. Poly(ethylene - vinyl acetate) for containers and tubing for total parenteral nutrition preparations	285	5.5. Alcoholimetric tables	519
3.1.8. Silicone oil used as a lubricant	287	5.6. Assay of interferons	533
3.1.9. Silicone elastomer for closures and tubing	288	5.7. Table of physical characteristics of radionuclides mentioned in the European Pharmacopoeia	539
3.1. Materials used for the manufacture of containers	269	5.8. Pharmacopoeial harmonisation	551
3.2.1. Glass containers for pharmaceutical use	303	5.9. Polymorphism	555
3.2.2.1. Plastic containers for aqueous solutions for parenteral infusion	309	A	
3.2.2. Plastic containers and closures for pharmaceutical use	308	Abbreviations and symbols (1.5.)	9
3.2.3. Sterile plastic containers for human blood and blood components	309	Abnormal toxicity (2.6.9.)	153
3.2.4. Empty sterile containers of plasticised poly(vinyl chloride) for human blood and blood components	311	<i>Absinthii herba</i>	2710
3.2.5. Sterile containers of plasticised poly(vinyl chloride) for human blood containing anticoagulant solution	312	Absorption spectrophotometry, infrared (2.2.24.)	37
3.2.6. Sets for the transfusion of blood and blood components	313	Absorption spectrophotometry, ultraviolet and visible (2.2.25.)	38
3.2.8. Sterile single-use plastic syringes	314	Acacia	905
3.2.9. Rubber closures for containers for aqueous parenteral preparations, for powders and for freeze-dried powders ..	316	<i>Acaciae gummi</i>	905
3.2. Containers	303	<i>Acaciae gummi dispersione desiccatum</i>	905
4.1.1. Reagents	321	Acacia, spray-dried	905
4.1.1. Reagents	5.1-2817	Acamprosate calcium	906
4.1.2. Standard solutions for limit tests	426	<i>Acamprosatum calcicum</i>	906
4.1.2. Standard solutions for limit tests	5.1-2818	Acarbose	5.1-2873
4.1.3. Buffer solutions	430	<i>Acarbosum</i>	5.1-2873
4.1. Reagents, standard solutions, buffer solutions	321	Acebutolol hydrochloride	907
4.2.1. Primary standards for volumetric solutions	435	<i>Acebutololi hydrochloridum</i>	907
4.2.2. Volumetric solutions	435	Aceclofenac	909
4.2. Volumetric analysis	435	<i>Aceclofenacum</i>	909
4-Aminobenzoic acid	973	Acesulfame potassium	911
4. Reagents	321	<i>Acesulfamum kalicum</i>	911
5.10. Control of impurities in substances for pharmaceutical use	559	Acetazolamide	912
5.11. Characters section in monographs	565	<i>Acetazolamidum</i>	912
5.1.1. Methods of preparation of sterile products	445	Acetic acid, glacial	913
5.1.2. Biological indicators of sterilisation	447	Acetic acid in synthetic peptides (2.5.34.)	141
5.1.3. Efficacy of antimicrobial preservation	447	Acetone	5.1-2875
5.1.4. Microbiological quality of pharmaceutical preparations	449	<i>Acetonum</i>	5.1-2875
5.1.5. Application of the F_0 concept to steam sterilisation of aqueous preparations	5.1-2821	Acetylcholine chloride	914
5.1. General texts on sterility	445	<i>Acetylcholini chloridum</i>	914
5.2.1. Terminology used in monographs on vaccines	453	Acetylcysteine	915
5.2.2. Chicken flocks free from specified pathogens for the production and quality control of vaccines	5.1-2825	<i>Acetylcysteinum</i>	915
5.2.3. Cell substrates for the production of vaccines for human use	455	Acetylsalicylic acid	917
5.2.4. Cell cultures for the production of veterinary vaccines	458	Acetyltryptophan, <i>N</i> -	918
		Acetyltyrosine, <i>N</i> -	920
		Aciclovir	921
		<i>Aciclovirum</i>	921
		<i>Acidum 4-aminobenzoicum</i>	973
		<i>Acidum aceticum glaciale</i>	913
		<i>Acidum acetylsalicylicum</i>	917
		<i>Acidum adipicum</i>	926
		<i>Acidum alginicum</i>	942
		<i>Acidum amidotrizoicum dihydricum</i>	967
		<i>Acidum aminocaproicum</i>	974
		<i>Acidum ascorbicum</i>	1025
		<i>Acidum asparticum</i>	1029
		<i>Acidum benzoicum</i>	1072
		<i>Acidum boricum</i>	1117

<i>Acidum caprylicum</i>	1172	<i>Aer medicinalis</i>	929
<i>Acidum chenodeoxycholicum</i>	1247	<i>Aer medicinalis artificiosus</i>	932
<i>Acidum citricum anhydricum</i>	1306	Aerodynamic assessment of fine particles in preparations for inhalation (2.9.18.)	5.1-2799
<i>Acidum citricum monohydricum</i>	1307	<i>Aether</i>	1548
<i>Acidum edeticum</i>	1494	<i>Aether anaestheticus</i>	1549
<i>Acidum etacrynicum</i>	1542	Agar	928
<i>Acidum folicum</i>	1630	<i>Agrimoniae herba</i>	929
<i>Acidum fusidicum</i>	1645	Agrimony	929
<i>Acidum glutamicum</i>	1670	Air, medicinal	929
<i>Acidum hydrochloridum concentratum</i>	1755	Air, synthetic medicinal	932
<i>Acidum hydrochloridum dilutum</i>	1756	Alanine	933
<i>Acidum iopanoicum</i>	1824	<i>Alaninum</i>	933
<i>Acidum iotalamicum</i>	1825	Albendazole	934
<i>Acidum ioxaglicum</i>	1826	<i>Albendazolum</i>	934
<i>Acidum lacticum</i>	1882	<i>Albumini humani solutio</i>	1731
<i>Acidum lactobionicum</i>	1885	Albumin solution, human	1731
<i>Acidum maleicum</i>	1966	Alchemilla	935
<i>Acidum malicum</i>	1966	<i>Alchemillae herba</i>	935
<i>Acidum mefenamicum</i>	1984	<i>Alcohol benzylicus</i>	5.1-2886
<i>Acidum methacrylicum et ethylis acrylas polymerisatum</i>	2005	<i>Alcohol cetylicus</i>	1243
<i>Acidum methacrylicum et ethylis acrylas polymerisatum</i>	2005	<i>Alcohol cetylicus et stearylicus</i>	1239
<i>1:1 dispersio 30 per centum</i>	2005	<i>Alcohol cetylicus et stearylicus emulsificans A</i>	1239
<i>Acidum methacrylicum et methylis methacrylas polymerisatum 1:1</i>	2006	<i>Alcohol cetylicus et stearylicus emulsificans B</i>	1241
<i>Acidum methacrylicum et methylis methacrylas polymerisatum 1:2</i>	2007	<i>Alcoholes adipis lanae</i>	2703
<i>Acidum nalidixicum</i>	2080	Alcoholimetric tables (2.9.10.)	237
<i>Acidum nicotinicum</i>	2097	Alcoholimetric tables (5.5.)	519
<i>Acidum nitricum</i>	2105	<i>Alcohol isopropylicus</i>	1841
<i>Acidum oleicum</i>	2132	<i>Alcohol oleicus</i>	2134
<i>Acidum oxolinicum</i>	2165	<i>Alcohol stearylicus</i>	2492
<i>Acidum palmiticum</i>	2179	<i>Alcuronii chloridum</i>	935
<i>Acidum phosphoricum concentratum</i>	2237	Alcuronium chloride	935
<i>Acidum phosphoricum dilutum</i>	2238	Alexandrian senna pods	2404
<i>Acidum pipemidicum trihydricum</i>	2249	Alfacalcidol	937
<i>Acidum salicylicum</i>	5.1-3007	<i>Alfacalcidolum</i>	937
<i>Acidum (S)-lacticum</i>	1883	Alfadex	938
<i>Acidum sorbicum</i>	2467	<i>Alfadexum</i>	938
<i>Acidum stearicum</i>	2490	Alfentanil hydrochloride	939
<i>Acidum sulfuricum</i>	2520	<i>Alfentanili hydrochloridum</i>	939
<i>Acidum tartaricum</i>	2534	Alfuzosin hydrochloride	941
<i>Acidum tiaprofenicum</i>	2578	<i>Alfuzosini hydrochloridum</i>	941
<i>Acidum tolfenamicum</i>	2601	Alginate acid	942
<i>Acidum tranexamicum</i>	2609	Alkaline-earth metals and magnesium (2.4.7.)	104
<i>Acidum trichloroaceticum</i>	2620	Alkaline impurities in fatty oils (2.4.19.)	109
<i>Acidum undecylenicum</i>	2658	Allantoin	942
<i>Acidum ursodeoxycholicum</i>	2662	<i>Allantoinum</i>	942
<i>Acidum valproicum</i>	2669	Allergen products	569
Acid value (2.5.1.)	127	<i>Allii sativi bulbi pulvis</i>	1651
Acitretin	922	<i>Allium sativum ad praeparationes homoeopathicas</i>	897
<i>Acitretinum</i>	922	Allopurinol	943
<i>Acriflavinii monochloridum</i>	924	<i>Allopurinolum</i>	943
Acriflavinium monochloride	924	all- <i>rac</i> - α -Tocopherol	5.1-3023
Actinobacillosis vaccine (inactivated), porcine	784	all- <i>rac</i> - α -Tocopheryl acetate	5.1-3024
Activated charcoal	1246	Almagate	945
Activated coagulation factors (2.6.22.)	177	<i>Almagatum</i>	945
Additives, plastic (3.1.13.)	293	Almond oil, refined	946
Adenine	924	Almond oil, virgin	947
<i>Adeninum</i>	924	<i>Aloe barbadensis</i>	947
Adenosine	925	<i>Aloe capensis</i>	948
<i>Adenosinum</i>	925	Aloes, barbados	947
<i>Adeps lanae</i>	2704	Aloes, Cape	948
<i>Adeps lanae cum aqua</i>	2709	Aloes dry extract, standardised	949
<i>Adeps lanae hydrogenatus</i>	2708	<i>Aloes extractum siccum normatum</i>	949
<i>Adeps solidus</i>	1711	Alphacyclodextrin	938
Adipic acid	926	Alprazolam	950
Adrenaline tartrate	927	<i>Alprazolamum</i>	950
<i>Adrenalini tartras</i>	927	Alprenolol hydrochloride	952
		<i>Alprenololi hydrochloridum</i>	952
		Alprostadiol	953

<i>Alprostadiolum</i>	953	<i>Amoxicillinum natricum</i>	990
Alteplase for injection.....	956	<i>Amoxicillinum trihydricum</i>	992
<i>Alteplasmum ad iniectionabile</i>	956	Amperometric titration (2.2.19.).....	34
<i>Althaeae folium</i>	1974	Amphotericin B.....	995
<i>Althaeae radix</i>	1975	<i>Amphotericinum B</i>	995
Alum.....	959	Ampicillin, anhydrous.....	996
<i>Alumen</i>	959	Ampicillin sodium.....	998
<i>Aluminii chloridum hexahydricum</i>	960	Ampicillin trihydrate.....	1001
<i>Aluminii hydroxidum hydricum ad adsorptionem</i>	960	<i>Ampicillinum anhydricum</i>	996
<i>Aluminii magnesii silicas</i>	961	<i>Ampicillinum natricum</i>	998
<i>Aluminii oxidum hydricum</i>	962	<i>Ampicillinum trihydricum</i>	1001
<i>Aluminii phosphas hydricus</i>	963	<i>Amygdalae oleum raffinatum</i>	946
<i>Aluminii sulfas</i>	964	<i>Amygdalae oleum virginale</i>	947
Aluminium (2.4.17.).....	108	<i>Amylum pregelificatum</i>	2490
Aluminium chloride hexahydrate.....	960	Anaesthetic ether.....	1549
Aluminium hydroxide, hydrated, for adsorption.....	960	Analysis, thermal (2.2.34.).....	52
Aluminium in adsorbed vaccines (2.5.13.).....	131	<i>Angelicae radix</i>	1003
Aluminium magnesium silicate.....	961	Angelica root.....	1003
Aluminium oxide, hydrated.....	962	Animal anti-T lymphocyte immunoglobulin for human use.....	1010
Aluminium phosphate, hydrated.....	963	Animal spongiform encephalopathies, products with risk of transmitting agents of.....	577
Aluminium sulphate.....	964	Animal spongiform encephalopathy agents, minimising the risk of transmitting via human and veterinary medicinal products (5.2.8.).....	463
Amantadine hydrochloride.....	964	Aniseed.....	1006
<i>Amantadini hydrochloridum</i>	964	Anise oil.....	1004
Ambroxol hydrochloride.....	965	<i>Anisi aetheroleum</i>	1004
<i>Ambroxoli hydrochloridum</i>	965	Anisidine value (2.5.36.).....	142
Amfetamine sulphate.....	966	<i>Anisi fructus</i>	1006
<i>Amfetamini sulfas</i>	966	<i>Anisi stellati aetheroleum</i>	2488
Amidotrizoic acid dihydrate.....	967	<i>Anisi stellati fructus</i>	2487
Amikacin.....	968	Antazoline hydrochloride.....	1006
<i>Amikacini sulfas</i>	970	<i>Antazolini hydrochloridum</i>	1006
Amikacin sulphate.....	970	Anthrax spore vaccine (live) for veterinary use.....	715
<i>Amikacinum</i>	968	Anti-A and anti-B haemagglutinins (indirect method) (2.6.20.).....	174
Amiloride hydrochloride.....	972	Antibiotics, microbiological assay of (2.7.2.).....	188
<i>Amiloridi hydrochloridum</i>	972	Anticoagulant and preservative solutions for human blood.....	1007
Amino acid analysis (2.2.56.).....	86	Anticomplementary activity of immunoglobulin (2.6.17.)..	170
Aminocaproic acid.....	974	Anti-D immunoglobulin, human.....	1732
Aminogluthethimide.....	975	Anti-D immunoglobulin, human, assay of (2.7.13.).....	205
<i>Aminogluthethimidum</i>	975	Anti-D immunoglobulin, human, for intravenous administration.....	1733
Amiodarone hydrochloride.....	977	Antimicrobial preservation, efficacy of (5.1.3.).....	447
<i>Amiodaroni hydrochloridum</i>	977	Antiserum, European viper venom.....	806
Amisulpride.....	978	Antithrombin III concentrate, human.....	1733
<i>Amisulpridum</i>	978	Antithrombin III, human, assay of (2.7.17.).....	209
Amitriptyline hydrochloride.....	980	<i>Antithrombinum III humanum densatum</i>	1733
<i>Amitriptylini hydrochloridum</i>	980	Anti-T lymphocyte immunoglobulin for human use, animal.....	1010
Amlodipine besilate.....	981	<i>Apis mellifera ad praeparationes homoeopathicas</i>	898
<i>Amlodipini besilas</i>	981	Apomorphine hydrochloride.....	1014
Ammonia (¹³ N) injection.....	817	<i>Apomorphini hydrochloridum</i>	1014
<i>Ammoniae (¹³N) solutio iniectionabilis</i>	817	Apparatus (2.1.).....	17
<i>Ammoniae solutio concentrata</i>	983	Apparent volume (2.9.15.).....	241
Ammonia solution, concentrated.....	983	Application of the <i>F</i> ₀ concept to steam sterilisation of aqueous preparations (5.1.5.).....	5.1-2821
<i>Ammonii bromidum</i>	985	Aprotinin.....	1015
<i>Ammonii chloridum</i>	986	Aprotinin concentrated solution.....	1016
<i>Ammonii glycyrrhizas</i>	5.1-2876	<i>Aprotinini solutio concentrata</i>	1016
<i>Ammonii hydrogenocarbonas</i>	988	<i>Aprotininum</i>	1015
Ammonio methacrylate copolymer (type A).....	983	<i>Aqua ad dilutionem solutionum concentratarum ad haemodialysim</i>	1699
Ammonio methacrylate copolymer (type B).....	984	<i>Aqua ad iniectionabilia</i>	2692
<i>Ammonio methacrylatis copolymerum A</i>	983	<i>Aquae (¹⁵O) solutio iniectionabilis</i>	868
<i>Ammonio methacrylatis copolymerum B</i>	984	<i>Aquae trititatae (³H) solutio iniectionabilis</i>	867
Ammonium (2.4.1.).....	103	<i>Aqua purificata</i>	2697
Ammonium bromide.....	985		
Ammonium chloride.....	986		
Ammonium glycyrrhizate.....	5.1-2876		
Ammonium hydrogen carbonate.....	988		
Amobarbital.....	988		
Amobarbital sodium.....	989		
<i>Amobarbitalum</i>	988		
<i>Amobarbitalum natricum</i>	989		
Amoxicillin sodium.....	990		
Amoxicillin trihydrate.....	992		

<i>Aqua valde purificata</i>	2695	<i>Auricularia</i>	601
<i>Arachidis oleum hydrogenatum</i>	1018	Avian infectious bronchitis vaccine (inactivated).....	718
<i>Arachidis oleum raffinatum</i>	1018	Avian infectious bronchitis vaccine (live).....	720
Arachis oil, hydrogenated.....	1018	Avian infectious bursal disease vaccine (inactivated).....	722
Arachis oil, refined.....	1018	Avian infectious bursal disease vaccine (live).....	723
<i>Argenti nitras</i>	2412	Avian infectious encephalomyelitis vaccine (live).....	725
Arginine.....	1019	Avian infectious laryngotracheitis vaccine (live).....	727
Arginine aspartate.....	1020	Avian live virus vaccines: tests for extraneous agents in batches of finished product (2.6.25.).....	180
Arginine hydrochloride.....	1021	Avian paramyxovirus 3 vaccine (inactivated).....	728
<i>Arginini aspartas</i>	1020	Avian viral tenosynovitis vaccine (live).....	729
<i>Arginini hydrochloridum</i>	1021	Avian viral vaccines: tests for extraneous agents in seed lots (2.6.24.).....	177
<i>Argininum</i>	1019	Azaperone for veterinary use.....	1036
<i>Arnicae flos</i>	1022	<i>Azaperonum ad usum veterinarium</i>	1036
<i>Arnicae tinctura</i>	5.1-2877	Azathioprine.....	1037
Arnica flower.....	1022	<i>Azathioprinum</i>	1037
Arnica tincture.....	5.1-2877	Azelastine hydrochloride.....	1037
Arsenic (2.4.2.).....	103	<i>Azelastini hydrochloridum</i>	1037
<i>Arsenii trioxidum ad praeparationes homoeopathicas</i> ..	895	Azithromycin.....	1039
Arsenious trioxide for homoeopathic preparations.....	895	<i>Azithromycinum</i>	1039
Articaine hydrochloride.....	1023		
<i>Articaini hydrochloridum</i>	1023	B	
Ascorbic acid.....	1025	Bacampicillin hydrochloride.....	1043
<i>Ascorbylis palmitas</i>	1026	<i>Bacampicillini hydrochloridum</i>	1043
Ascorbyl palmitate.....	1026	Bacitracin.....	1045
Ash insoluble in hydrochloric acid (2.8.1.).....	215	<i>Bacitracinum</i>	1045
Ash leaf.....	1026	<i>Bacitracinum zincum</i>	1047
Asparagine monohydrate.....	1027	Bacitracin zinc.....	1047
<i>Asparaginum monohydricum</i>	1027	Baclofen.....	1050
Aspartame.....	1028	<i>Baclofenum</i>	1050
<i>Aspartamum</i>	1028	Bacterial endotoxins (2.6.14.).....	161
Aspartic acid.....	1029	<i>Ballotae nigrae herba</i>	1113
Assay of 1,8-cineole in essential oils (2.8.11.).....	216	<i>Balsamum peruvianum</i>	2215
Assay of diphtheria vaccine (adsorbed) (2.7.6.).....	196	<i>Balsamum toltanum</i>	2603
Assay of heparin (2.7.5.).....	195	Bambuterol hydrochloride.....	1051
Assay of heparin in coagulation factors (2.7.12.).....	204	<i>Bambuteroli hydrochloridum</i>	1051
Assay of hepatitis A vaccine (2.7.14.).....	5.1-2795	Barbados aloes.....	947
Assay of hepatitis B vaccine (rDNA) (2.7.15.).....	207	Barbital.....	1052
Assay of human anti-D immunoglobulin (2.7.13.).....	205	<i>Barbitalum</i>	1052
Assay of human antithrombin III (2.7.17.).....	209	<i>Barii sulfas</i>	1053
Assay of human coagulation factor II (2.7.18.).....	209	Barium sulphate.....	1053
Assay of human coagulation factor IX (2.7.11.).....	204	Basic butylated methacrylate copolymer.....	1053
Assay of human coagulation factor VII (2.7.10.).....	203	<i>BCG ad immunocurationem</i>	635
Assay of human coagulation factor VIII (2.7.4.).....	194	BCG for immunotherapy.....	635
Assay of human coagulation factor X (2.7.19.).....	210	BCG vaccine, freeze-dried.....	636
Assay of human coagulation factor XI (2.7.22.).....	212	Bearberry leaf.....	1054
Assay of human von Willebrand factor (2.7.21.).....	211	Beclometasone dipropionate, anhydrous.....	5.1-2881
Assay of interferons (5.6.).....	533	Beclometasone dipropionate monohydrate.....	5.1-2883
Assay of pertussis vaccine (2.7.7.).....	197	<i>Beclometasoni dipropionas anhydricus</i>	5.1-2881
Assay of pertussis vaccine (acellular) (2.7.16.).....	208	<i>Beclometasoni dipropionas monohydricus</i>	5.1-2883
Assay of tetanus vaccine (adsorbed) (2.7.8.).....	5.1-2791	Bee for homoeopathic preparations, honey.....	898
Assays (2.5.).....	127	Beeswax, white.....	1057
Astemizole.....	1030	Beeswax, yellow.....	1058
<i>Astemizolum</i>	1030	<i>Belladonnae folii extractum siccum normatum</i>	1060
Atenolol.....	1032	<i>Belladonnae folii tinctura normata</i>	1061
<i>Atenololum</i>	1032	<i>Belladonnae folium</i>	1058
Atomic absorption spectrometry (2.2.23.).....	36	<i>Belladonnae pulvis normatus</i>	1062
Atomic emission spectrometry (2.2.22.).....	35	Belladonna leaf.....	1058
Atropine.....	1033	Belladonna leaf dry extract, standardised.....	1060
Atropine sulphate.....	1035	Belladonna leaf tincture, standardised.....	1061
<i>Atropini sulfas</i>	1035	Belladonna, prepared.....	1062
<i>Atropinum</i>	1033	Bendroflumethiazide.....	5.1-2885
Aujeszký's disease vaccine (inactivated) for pigs.....	715	<i>Bendroflumethiazidum</i>	5.1-2885
Aujeszký's disease vaccine (live) for pigs for parenteral administration, freeze-dried.....	717	Benfluorex hydrochloride.....	1064
<i>Aurantii amari epicarpium et mesocarpium tinctura</i>	1110	<i>Benfluorexi hydrochloridum</i>	1064
<i>Aurantii amari epicarpium et mesocarpium</i>	1110	Benperidol.....	1065
<i>Aurantii amari floris aetheroleum</i>	1112	<i>Benperidolum</i>	1065
<i>Aurantii amari flos</i>	1111	Benserazide hydrochloride.....	1067
<i>Aurantii dulcis aetheroleum</i>	2526		

<i>Benserazidi hydrochloridum</i>	1067	<i>Bismuthi subcarbonas</i>	1105
Bentonite	1068	<i>Bismuthi subgallas</i>	1106
<i>Bentonitum</i>	1068	<i>Bismuthi subnitras ponderosum</i>	1107
<i>Benzalkonii chloridi solutio</i>	1069	<i>Bismuthi subsalicylas</i>	1107
<i>Benzalkonii chloridum</i>	1068	Bismuth subcarbonate	1105
Benzalkonium chloride	1068	Bismuth subgallate	1106
Benzalkonium chloride solution	1069	Bismuth subnitrate, heavy	1107
Benzathine benzylpenicillin	1077	Bismuth subsalicylate	1107
Benzbromarone	1070	Bitter fennel	1580
<i>Benzbromaronum</i>	1070	Bitter-fennel fruit oil	1108
<i>Benzethonii chloridum</i>	1071	Bitterness value (2.8.15.)	221
Benzethonium chloride	1071	Bitter-orange epicarp and mesocarp	1110
Benzocaine	1072	Bitter-orange-epicarp and mesocarp tincture	1110
<i>Benzocainum</i>	1072	Bitter-orange flower	1111
Benzoic acid	1072	Bitter-orange-flower oil	1112
<i>Benzoylis peroxidum cum aqua</i>	1073	Black horehound	1113
Benzoyl peroxide, hydrous	1073	<i>Bleomycini sulfas</i>	1114
Benzyl alcohol	5.1-2886	Bleomycin sulphate	1114
Benzyl benzoate	1076	Blood and blood components, empty sterile containers of plasticised poly(vinyl chloride) for (3.2.4.)	311
<i>Benzylis benzoas</i>	1076	Blood and blood components, materials for containers for (3.1.1.)	269
Benzylpenicillin, benzathine	1077	Blood and blood components, sets for the transfusion of (3.2.6.)	313
Benzylpenicillin potassium	1078	Blood and blood components, sterile plastic containers for (3.2.3.)	309
Benzylpenicillin, procaine	1080	Blood, anticoagulant and preservative solutions for	1007
Benzylpenicillin sodium	1082	Blood, sterile containers of plasticised poly (vinyl chloride) containing anticoagulant solution (3.2.5.)	312
<i>Benzylpenicillinum benzathinum</i>	1077	Bogbean leaf	1115
<i>Benzylpenicillinum kalicum</i>	1078	Boiling point (2.2.12.)	31
<i>Benzylpenicillinum natricum</i>	1082	<i>Boldi folium</i>	5.1-2888
<i>Benzylpenicillinum procainum</i>	1080	Boldo leaf	5.1-2888
Betacarotene	1083	Borage (starflower) oil, refined	5.1-2889
<i>Betacarotenum</i>	1083	<i>Borago officinalis oleum raffinatum</i>	5.1-2889
Betacyclodextrin	1084	Borax	1116
Betadex	1084	Boric acid	1117
<i>Betadexum</i>	1084	Botulinum antitoxin	801
Betahistine dihydrochloride	5.1-2887	Botulinum toxin type A for injection	1117
Betahistine mesilate	1086	Bovine infectious rhinotracheitis vaccine (live), freeze-dried	768
<i>Betahistini dihydrochloridum</i>	5.1-2887	Bovine insulin	1797
<i>Betahistini mesilas</i>	1086	Bovine leptospirosis vaccine (inactivated)	730
Betamethasone	1087	Bovine parainfluenza virus vaccine (live), freeze-dried	732
Betamethasone acetate	1089	Bovine respiratory syncytial virus vaccine (live), freeze-dried	733
Betamethasone dipropionate	1090	Bovine viral diarrhoea vaccine (inactivated)	734
Betamethasone sodium phosphate	1092	Bromazepam	1119
Betamethasone valerate	1094	<i>Bromazepamum</i>	1119
<i>Betamethasoni acetas</i>	1089	Bromhexine hydrochloride	1120
<i>Betamethasoni dipropionas</i>	1090	<i>Bromhexini hydrochloridum</i>	1120
<i>Betamethasoni natrii phosphas</i>	1092	Bromocriptine mesilate	1121
<i>Betamethasoni valeras</i>	1094	<i>Bromocriptini mesilas</i>	1121
<i>Betamethasonum</i>	1087	Bromperidol	1124
Betaxolol hydrochloride	1095	Bromperidol decanoate	1125
<i>Betaxololi hydrochloridum</i>	1095	<i>Bromperidoli decanoas</i>	1125
<i>Betulae folium</i>	1103	<i>Bromperidolum</i>	1124
Bezafibrate	1096	Brompheniramine maleate	1127
<i>Bezafibratum</i>	1096	<i>Brompheniramini maleas</i>	1127
Bifonazole	1098	Brucellosis vaccine (live) (<i>Brucella melitensis</i> Rev. 1 strain), freeze-dried, for veterinary use	5.1-2859
<i>Bifonazolum</i>	1098	Buccal tablets and sublingual tablets	613
Bilberry fruit, dried	1099	Budesonide	1128
Bilberry fruit, fresh	1099	<i>Budesonidum</i>	1128
Biological assays (2.7.)	187	Bufexamac	1130
Biological assays and tests, statistical analysis of (5.3.)	475	<i>Bufexamacum</i>	1130
Biological indicators of sterilisation (5.1.2.)	447	Buffer solutions (4.1.3.)	430
Biological tests (2.6.)	145	Buflomedil hydrochloride	1131
Biotin	1100	<i>Buflomedili hydrochloridum</i>	1131
<i>Biotinum</i>	1100		
Biperiden hydrochloride	1101		
<i>Biperideni hydrochloridum</i>	1101		
Biphasic insulin injection	1802		
Biphasic isophane insulin injection	1803		
Birch leaf	1103		
Bisacodyl	1104		
<i>Bisacodylum</i>	1104		

Bumetanide	1132	Calcium in adsorbed vaccines (2.5.14.)	131
<i>Bumetanidum</i>	1132	Calcium lactate, anhydrous	5.1 -2894
Bupivacaine hydrochloride	1133	Calcium lactate monohydrate	5.1 -2895
<i>Bupivacaini hydrochloridum</i>	1133	Calcium lactate pentahydrate	5.1 -2896
Buprenorphine	1135	Calcium lactate trihydrate	5.1 -2897
Buprenorphine hydrochloride	1136	Calcium levofolinate pentahydrate	1163
<i>Buprenorphini hydrochloridum</i>	1136	Calcium levulinate dihydrate	1165
<i>Buprenorphinum</i>	1135	Calcium pantothenate	1166
Buserelin	1137	Calcium phosphate	1167
<i>Buserelinum</i>	1137	Calcium stearate	1167
Busulfan	1138	Calcium sulphate dihydrate	1169
<i>Busulfanum</i>	1138	<i>Calendulae flos</i>	1169
Butcher's broom	1138	<i>Calendula flower</i>	1169
Butylhydroxyanisole	1141	Calf coronavirus diarrhoea vaccine (inactivated)	736
<i>Butylhydroxyanisolum</i>	1141	Calf rotavirus diarrhoea vaccine (inactivated)	737
Butylhydroxytoluene	1141	Calicivirus vaccine (inactivated), feline	757
<i>Butylhydroxytoluenum</i>	1141	Calicivirus vaccine (live), feline, freeze-dried	758
<i>Butylis parahydroxybenzoas</i>	1140	<i>Camphora racemica</i>	1172
Butyl parahydroxybenzoate	1140	Camphor, D-	1170
C		Camphor, racemic	1172
Cachets	601	Canine adenovirus vaccine (inactivated)	738
Caffeine	1145	Canine adenovirus vaccine (live)	738
Caffeine monohydrate	1146	Canine distemper vaccine (live), freeze-dried	740
Calcifediol	1147	Canine leptospirosis vaccine (inactivated)	740
<i>Calcifediolum</i>	1147	Canine parainfluenza virus vaccine (live)	742
<i>Calcii acetat</i>	5.1 -2893	Canine parvovirus vaccine (inactivated)	743
<i>Calcii ascorbas</i>	1150	Canine parvovirus vaccine (live)	744
<i>Calcii carbonas</i>	5.1 -2894	Cape aloes	948
<i>Calcii chloridum dihydricum</i>	1152	Capillary electrophoresis (2.2.47.)	74
<i>Calcii chloridum hexahydricum</i>	1153	Capillary viscometer method (2.2.9.)	29
<i>Calcii dobesilas monohydricum</i>	1153	Caprylic acid	1172
<i>Calcii folinas</i>	1154	Caprylocaproyl macrogolglycerides	1173
<i>Calcii glucoheptonas</i>	1156	<i>Capsici fructus</i>	1174
<i>Calcii gluconas</i>	1157	Capsicum	1174
<i>Calcii gluconas ad iniectionabile</i>	1158	<i>Capsulae</i>	599
<i>Calcii glycerophosphas</i>	1159	Capsules	599
<i>Calcii hydrogenophosphas anhydricus</i>	1160	Capsules and tablets, disintegration of (2.9.1.)	225
<i>Calcii hydrogenophosphas dihydricus</i>	1160	Capsules, gastro-resistant	600
<i>Calcii hydroxidum</i>	1161	Capsules, hard	600
<i>Calcii lactas anhydricus</i>	5.1 -2894	Capsules, modified-release	600
<i>Calcii lactas monohydricus</i>	5.1 -2895	Capsules, oromucosal	613
<i>Calcii lactas pentahydricus</i>	5.1 -2896	Capsules, rectal	623
<i>Calcii lactas trihydricus</i>	5.1 -2897	Capsules, soft	600
<i>Calcii laevulinas dihydricum</i>	1165	Capsules, vaginal	629
<i>Calcii levofolinas pentahydricus</i>	1163	Captopril	1176
<i>Calcii pantothenas</i>	1166	<i>Captoprilum</i>	1176
<i>Calcii stearas</i>	1167	Caraway fruit	1177
<i>Calcii sulfas dihydricus</i>	1169	Carbachol	1177
Calcitonin (salmon)	1148	<i>Carbacholum</i>	1177
<i>Calcitoninum salmonis</i>	1148	Carbamazepine	1178
Calcitriol	1149	<i>Carbamazepinum</i>	1178
<i>Calcitriolum</i>	1149	Carbasalate calcium	1179
Calcium (2.4.3.)	103	<i>Carbasalatum calcicum</i>	1179
Calcium acetate	5.1 -2893	Carbidopa	1180
Calcium ascorbate	1150	<i>Carbidopum</i>	1180
Calcium carbonate	5.1 -2894	Carbimazole	1182
Calcium carboxymethylcellulose	1188	<i>Carbimazolum</i>	1182
Calcium chloride dihydrate	1152	<i>Carbo activatus</i>	1246
Calcium chloride hexahydrate	1153	Carbocysteine	1182
Calcium dobesilate monohydrate	1153	<i>Carbocisteinum</i>	1182
Calcium folinate	1154	<i>Carbomera</i>	1183
Calcium glucoheptonate	1156	Carbomers	1183
Calcium gluconate	1157	Carbon dioxide	1185
Calcium gluconate for injection	1158	Carbon dioxide in gases (2.5.24.)	134
Calcium glycerophosphate	1159	<i>Carbonei dioxidum</i>	1185
Calcium hydrogen phosphate, anhydrous	1160	<i>Carbonei monoxidum</i> (¹⁵ O)	818
Calcium hydrogen phosphate dihydrate	1160	Carbon monoxide (¹⁵ O)	818
Calcium hydroxide	1161	Carbon monoxide in gases (2.5.25.)	134
		Carboplatin	1186

<i>Carboplatinum</i>	1186	<i>Cefuroximum natricum</i>	1223
<i>Carboxymethylamylum natricum A</i>	2450	Celiprolol hydrochloride.....	1224
<i>Carboxymethylamylum natricum B</i>	2451	<i>Celiprololi hydrochloridum</i>	1224
<i>Carboxymethylamylum natricum C</i>	2451	Cell cultures for the production of veterinary vaccines (5.2.4.)	458
Carboxymethylcellulose calcium	1188	Cell substrates for the production of vaccines for human use (5.2.3.)	455
Carboxymethylcellulose sodium	1189	Cellulose acetate	1226
Carboxymethylcellulose sodium, cross-linked	1371	Cellulose acetate butyrate.....	1227
Carboxymethylcellulose sodium, low-substituted	1190	Cellulose acetate phthalate.....	1227
Carisoprodol.....	1187	Cellulose, microcrystalline.....	1228
<i>Carisoprodolum</i>	1187	Cellulose, powdered	1232
Carmellose calcium.....	1188	<i>Cellulosi acetas</i>	1226
Carmellose sodium	1189	<i>Cellulosi acetas butyras</i>	1227
Carmellose sodium, low-substituted	1190	<i>Cellulosi acetas phthalas</i>	1227
<i>Carmellosum calcicum</i>	1188	<i>Cellulosi pulvis</i>	1232
<i>Carmellosum natricum</i>	1189	<i>Cellulosum microcristallinum</i>	1228
<i>Carmellosum natricum conexum</i>	1371	<i>Centaurii herba</i>	1235
<i>Carmellosum natricum, substitutum humile</i>	1190	Centaury	1235
Carmustine	1191	Centella	1236
<i>Carmustinum</i>	1191	<i>Centellae asiaticae herba</i>	1236
Carnauba wax	1191	<i>Cera alba</i>	1057
Carteolol hydrochloride.....	1192	<i>Cera carnauba</i>	1191
<i>Carteololi hydrochloridum</i>	1192	<i>Cera flava</i>	1058
<i>Carthami oleum raffinatum</i>	2389	Cetirizine dihydrochloride	1237
Carvedilol.....	1193	<i>Cetirizini dihydrochloridum</i>	1237
<i>Carvedilolum</i>	1193	<i>Cetobemidoni hydrochloridum</i>	1871
<i>Carvi fructus</i>	1177	Cetostearyl alcohol.....	1239
<i>Caryophylli floris aetheroleum</i>	1335	Cetostearyl alcohol (type A), emulsifying	1239
<i>Caryophylli flos</i>	1334	Cetostearyl alcohol (type B), emulsifying	1241
Cascara	1194	<i>Cetostearyl isononanoas</i>	1242
Cassia oil	1196	Cetostearyl isononanoate.....	1242
Castor oil, hydrogenated	1197	Cetrimide	1243
Castor oil, polyoxyl	1949	<i>Cetrimidum</i>	1243
Castor oil, polyoxyl hydrogenated.....	1948	Cetyl alcohol	1243
Castor oil, virgin.....	1197	<i>Cetyl palmitas</i>	1244
Catgut, sterile.....	873	Cetyl palmitate.....	1244
Catgut, sterile, in distributor for veterinary use	885	<i>Cetylpyridinii chloridum</i>	1244
Cefaclor	1198	Cetylpyridinium chloride.....	1244
<i>Cefaclorum</i>	1198	Ceylon cinnamon bark oil	1295
Cefadroxil monohydrate	1200	Ceylon cinnamon leaf oil.....	1296
<i>Cefadroxilum monohydricum</i>	1200	Chamomile flower, Roman.....	1245
Cefalexin monohydrate.....	1202	<i>Chamomillae romanae flos</i>	1245
<i>Cefalexinum monohydricum</i>	1202	Characters section in monographs (5.11.).....	565
Cefalotin sodium	1203	Charcoal, activated	1246
<i>Cefalotinum natricum</i>	1203	<i>Chelidonii herba</i>	1690
Cefamandole nafate	1204	Chenodeoxycholic acid	1247
<i>Cefamandoli nafas</i>	1204	Chewing gum, medicated, drug release from (2.9.25.)	260
Cefapirin sodium.....	1206	Chewing gums, medicated	601
<i>Cefapirinum natricum</i>	1206	Chicken flocks free from specified pathogens for the production and quality control of vaccines (5.2.2.)	5.1-2825
Cefatrizine propylene glycol.....	1207	Chicken infectious anaemia vaccine (live).....	769
<i>Cefatrizinum propylen glycolum</i>	1207	<i>Chinidini sulfas</i>	2347
Cefazolin sodium.....	1209	<i>Chinini hydrochloridum</i>	2348
<i>Cefazolinum natricum</i>	1209	<i>Chinini sulfas</i>	2350
Cefixime	1211	Chitosan hydrochloride	1248
<i>Cefiximum</i>	1211	<i>Chitosani hydrochloridum</i>	1248
Cefoperazone sodium	1212	Chloral hydrate.....	1249
<i>Cefoperazonum natricum</i>	1212	<i>Chlorali hydras</i>	1249
Cefotaxime sodium	1214	Chlorambucil.....	1250
<i>Cefotaximum natricum</i>	1214	<i>Chlorambucilum</i>	1250
Cefoxitin sodium	1216	Chloramine	2605
<i>Cefoxitinum natricum</i>	1216	Chloramphenicol.....	1250
Cefradine.....	1217	<i>Chloramphenicoli natrii succinas</i>	1252
<i>Cefradinum</i>	1217	<i>Chloramphenicoli palmitas</i>	1251
Ceftazidime.....	1218	Chloramphenicol palmitate	1251
<i>Ceftazidimum</i>	1218	Chloramphenicol sodium succinate.....	1252
Ceftriaxone sodium.....	1220	<i>Chloramphenicolum</i>	1250
<i>Ceftriaxonum natricum</i>	1220		
Cefuroxime axetil	1222		
Cefuroxime sodium.....	1223		
<i>Cefuroximum axetili</i>	1222		

Chlorcyclizine hydrochloride	1253	<i>Cilastatinum natricum</i>	1286
<i>Chlorcyclizini hydrochloridum</i>	1253	Cilazapril	1288
Chlordiazepoxide	1254	<i>Cilazaprilum</i>	1288
Chlordiazepoxide hydrochloride	1255	Cimetidine	1289
<i>Chlordiazepoxidi hydrochloridum</i>	1255	Cimetidine hydrochloride	1290
<i>Chlordiazepoxidum</i>	1254	<i>Cimetidini hydrochloridum</i>	1290
Chlorhexidine diacetate	1256	<i>Cimetidinum</i>	1289
Chlorhexidine digluconate solution	1258	Cinchocaine hydrochloride	1291
Chlorhexidine dihydrochloride	1259	<i>Cinchocaini hydrochloridum</i>	1291
<i>Chlorhexidini diacetat</i>	1256	Cinchona bark	1292
<i>Chlorhexidini digluconatis solutio</i>	1258	<i>Cinchonae cortex</i>	1292
<i>Chlorhexidini dihydrochloridum</i>	1259	Cineole	1294
Chlorides (2.4.4.)	104	Cineole in essential oils, assay of, 1,8- (2.8.11.)	216
Chlorobutanol, anhydrous	1261	<i>Cineolum</i>	1294
Chlorobutanol hemihydrate	1261	<i>Cinnamomi cassiae aetheroleum</i>	1196
<i>Chlorobutanolum anhydricum</i>	1261	<i>Cinnamomi cortex</i>	1295
<i>Chlorobutanolum hemihydricum</i>	1261	<i>Cinnamomi corticis tinctura</i>	1297
Chlorocresol	1262	<i>Cinnamomi zeylanici folii aetheroleum</i>	1296
<i>Chlorocresolum</i>	1262	<i>Cinnamomi zeylanicii corticis aetheroleum</i>	1295
Chloroquine phosphate	1262	Cinnamon	1295
Chloroquine sulphate	1263	Cinnamon bark oil, Ceylon	1295
<i>Chloroquini phosphas</i>	1262	Cinnamon leaf oil, Ceylon	1296
<i>Chloroquini sulfas</i>	1263	Cinnamon tincture	1297
Chlorothiazide	1264	Cinnarizine	1297
<i>Chlorothiazidum</i>	1264	<i>Cinnarizinum</i>	1297
Chlorphenamine maleate	5.1-2899	Ciprofibrate	1299
<i>Chlorphenamini maleas</i>	5.1-2899	<i>Ciprofibratum</i>	1299
Chlorpromazine hydrochloride	1266	Ciprofloxacin	1300
<i>Chlorpromazini hydrochloridum</i>	1266	Ciprofloxacin hydrochloride	1302
Chlorpropamide	1266	<i>Ciprofloxacin hydrochloridum</i>	1302
<i>Chlorpropamidum</i>	1266	<i>Ciprofloxacinum</i>	1300
Chlorprothixene hydrochloride	1267	Circular dichroism (2.2.41.)	63
<i>Chlorprothixeni hydrochloridum</i>	1267	Cisapride monohydrate	1303
Chlortalidone	1269	Cisapride tartrate	1304
<i>Chlortalidonum</i>	1269	<i>Cisapridi tartras</i>	1304
Chlortetracycline hydrochloride	1270	<i>Cisapridum monohydricum</i>	1303
<i>Chlortetracyclini hydrochloridum</i>	1270	Cisplatin	1306
Cholecalciferol	1272	<i>Cisplatinum</i>	1306
Cholecalciferol concentrate (oily form)	1273	Citric acid, anhydrous	1306
Cholecalciferol concentrate (powder form)	1275	Citric acid monohydrate	1307
Cholecalciferol concentrate (water-dispersible form)	1277	<i>Citronellae aetheroleum</i>	1308
<i>Cholecalciferoli pulvis</i>	1275	Citronella oil	1308
<i>Cholecalciferolum</i>	1272	Clarithromycin	5.1-2900
<i>Cholecalciferolum densatum oleosum</i>	1273	<i>Clarithromycinum</i>	5.1-2900
<i>Cholecalciferolum in aqua dispergibile</i>	1277	Clarity and degree of opalescence of liquids (2.2.1.)	23
Cholera vaccine	637	Clary sage oil	1311
Cholera vaccine, freeze-dried	638	Clazuril for veterinary use	1312
Cholesterol	1279	<i>Clazurilum ad usum veterinarium</i>	1312
<i>Cholesterolum</i>	1279	Clebopride malate	1314
<i>Chorda resorbilis sterilis</i>	873	<i>Clebopridi malas</i>	1314
<i>Chorda resorbilis sterilis in fuso ad usum veterinarium</i> ..	885	Clemastine fumarate	1315
Chromatographic separation techniques (2.2.46.)	69	<i>Clemastini fumaras</i>	1315
Chromatography, gas (2.2.28.)	42	Clenbuterol hydrochloride	5.1-2902
Chromatography, liquid (2.2.29.)	43	<i>Clenbuteroli hydrochloridum</i>	5.1-2902
Chromatography, paper (2.2.26.)	40	Clindamycin hydrochloride	1318
Chromatography, size-exclusion (2.2.30.)	45	<i>Clindamycin hydrochloridum</i>	1318
Chromatography, supercritical fluid (2.2.45.)	68	<i>Clindamycin phosphas</i>	1319
Chromatography, thin-layer (2.2.27.)	40	Clindamycin phosphate	1319
<i>Chromii (⁶⁷Cr) edetatis solutio iniectionabilis</i>	819	Clioquinol	1321
Chromium (⁵¹ Cr) edetate injection	819	<i>Clioquinolum</i>	1321
Chymotrypsin	1280	Clobazam	1322
<i>Chymotrypsinum</i>	1280	<i>Clobazamum</i>	1322
Ciclopirox	1282	Clobetasone butyrate	1323
Ciclopirox olamine	1283	<i>Clobetasoni butyras</i>	1323
<i>Ciclopirox olaminum</i>	1283	Clofazimine	1324
<i>Ciclopiroxum</i>	1282	<i>Clofaziminum</i>	1324
Ciclosporin	1285	Clofibrate	1325
<i>Ciclosporinum</i>	1285	<i>Clofibratum</i>	1325
Cilastatin sodium	1286	Clomifene citrate	1326

<i>Clomifeni citras</i>	1326	Cod-liver oil (type B).....	1352
Clomipramine hydrochloride.....	1328	<i>Coffeinum</i>	1145
<i>Clomipramini hydrochloridum</i>	1328	<i>Coffeinum monohydricum</i>	1146
Clonazepam.....	1330	Cola	1356
<i>Clonazepamum</i>	1330	<i>Colae semen</i>	1356
Clonidine hydrochloride.....	1331	Colchicine	1357
<i>Clonidini hydrochloridum</i>	1331	<i>Colchicinum</i>	1357
Closantel sodium dihydrate for veterinary use	5.1-2903	Colestyramine	1359
<i>Closantelum natricum dihydricum</i> ad usum veterinarium.....	5.1-2903	<i>Colestyraminum</i>	1359
Clostridium botulinum vaccine for veterinary use	745	Colibacillosis vaccine (inactivated), neonatal piglet.....	776
Clostridium chauvoei vaccine for veterinary use.....	745	Colibacillosis vaccine (inactivated), neonatal ruminant....	778
Clostridium novyi alpha antitoxin for veterinary use	5.1-2865	Colistimethate sodium	1360
Clostridium novyi (type B) vaccine for veterinary use.....	746	<i>Colistimethatum natricum</i>	1360
Clostridium perfringens beta antitoxin for veterinary use	5.1-2866	<i>Colistini sulfas</i>	1361
Clostridium perfringens epsilon antitoxin for veterinary use	5.1-2867	Colistin sulphate	1361
Clostridium perfringens vaccine for veterinary use.....	747	Colloidal anhydrous silica	2410
Clostridium septicum vaccine for veterinary use.....	749	Colloidal hydrated silica	2411
Closures and containers for parenteral preparations and ophthalmic preparations, polypropylene for (3.1.6.).....	282	<i>Colophonium</i>	1362
Closures and containers for pharmaceutical use, plastic (3.2.2.)	308	Colophony	1362
Closures and tubing, silicone elastomer for (3.1.9.).....	288	Coloration of liquids (2.2.2.).....	24
Closures for containers for aqueous parenteral preparations, for powders and for freeze-dried powders, rubber (3.2.9.)	316	Common stinging nettle for homoeopathic preparations..	895
Clotrimazole.....	1333	Comparative table of porosity of sintered-glass filters (2.1.2.).....	17
<i>Clotrimazolum</i>	1333	Complexometric titrations (2.5.11.).....	130
Clove	1334	Composition of fatty acids by gas chromatography (2.4.22.)	110
Clove oil	1335	Composition of fatty acids in oils rich in omega-3-acids (2.4.29.)	121
Cloxacillin sodium.....	1335	Compressed lozenges.....	613
<i>Cloxacillinum natricum</i>	1335	<i>Compressi</i>	626
Clozapine	1337	Concentrated solutions for haemodialysis	1700
<i>Clozapinum</i>	1337	Concentrates for injections or infusions.....	5.1-2842
Coagulation factor II, assay of (2.7.18.).....	209	Conductivity (2.2.38.).....	5.1-2783
Coagulation factor IX, assay of (2.7.11.).....	204	Conjugated estrogens	1539
Coagulation factor IX, human.....	1738	Containers (3.2.).....	303
Coagulation factors, activated (2.6.22.).....	177	Containers and closures for parenteral preparations and ophthalmic preparations, polypropylene for (3.1.6.).....	282
Coagulation factors, assay of heparin (2.7.12.)	204	Containers and closures for pharmaceutical use, plastic (3.2.2.)	308
Coagulation factor VII, assay of (2.7.10.).....	203	Containers and tubing for total parenteral nutrition preparations, poly(ethylene - vinyl acetate) for (3.1.7.) ...	285
Coagulation factor VII, human	1734	Containers for aqueous solutions for intravenous infusion, materials based on plasticised poly(vinyl chloride) for (3.1.14.)	296
Coagulation factor VIII, assay of (2.7.4.).....	194	Containers for aqueous solutions for parenteral infusion, plastic (3.2.2.1.)	309
Coagulation factor VIII, human.....	1736	Containers for dry dosage forms for oral administration, materials based on non-plasticised poly(vinyl chloride) for (3.1.11.).....	291
Coagulation factor VIII (rDNA), human	1737	Containers for human blood and blood components, materials based on plasticised poly(vinyl chloride) for (3.1.1.1.)	269
Coagulation factor X, assay of (2.7.19.).....	210	Containers for human blood and blood components, materials for (3.1.1.)	269
Coagulation factor XI, assay of (2.7.22.)	212	Containers for human blood and blood components, plastic, sterile (3.2.3.)	309
Coagulation factor XI, human.....	1739	Containers for non-injectable aqueous solutions, materials based on non-plasticised poly(vinyl chloride) for (3.1.10.)	289
Coated granules	606	Containers for parenteral preparations and for ophthalmic preparations, polyethylene with additives for (3.1.5.)	279
Coated tablets	627	Containers for parenteral preparations and for ophthalmic preparations, polyethylene without additives for (3.1.4.).....	278
Cocaine hydrochloride.....	1338	Containers for pharmaceutical use, glass (3.2.1.).....	303
<i>Cocaini hydrochloridum</i>	1338	Containers for preparations not for parenteral use, polyethylene terephthalate for (3.1.15)	298
<i>Cocois oleum raffinatum</i>	1339	Containers of plasticised poly(vinyl chloride) for human blood and blood components, empty sterile (3.2.4.).....	311
Coconut oil, refined.....	1339		
Cocoyl caprylocaprate.....	1340		
<i>Cocoylis caprylocapras</i>	1340		
Codeine	5.1-2905		
Codeine hydrochloride dihydrate	5.1-2906		
Codeine phosphate hemihydrate.....	5.1-2908		
Codeine phosphate sesquihydrate	5.1-2909		
<i>Codeini hydrochloridum dihydricum</i>	5.1-2906		
<i>Codeini phosphas hemihydricus</i>	5.1-2908		
<i>Codeini phosphas sesquihydricus</i>	5.1-2909		
<i>Codeinum</i>	5.1-2905		
Codergocrine mesilate	1347		
<i>Codergocrini mesilas</i>	1347		
Cod-liver oil (type A).....	1348		

Containers of plasticised poly (vinyl chloride) for human blood containing anticoagulant solution, sterile (3.2.5.)..	312	<i>Cysteini hydrochloridum monohydricum</i>	1381
Contamination, microbial, test for specified micro-organisms (2.6.13.)	156	Cystine	1382
Contamination, microbial, total viable aerobic count (2.6.12.)	154	<i>Cystinum</i>	1382
Content uniformity of single-dose preparations (2.9.6.)....	234	Cytarabine	1383
Control of impurities in substances for pharmaceutical use (5.10.).....	559	<i>Cytarabinum</i>	1383
<i>Copolymerum methacrylatis butylati basicum</i>	1053	D	
Copovidone.....	1363	Dalteparin sodium	1387
<i>Copovidonum</i>	1363	<i>Dalteparinum natricum</i>	1387
Copper for homoeopathic preparations.....	896	Dapsone	1388
Copper sulphate, anhydrous.....	1364	<i>Dapsonum</i>	1388
Copper sulphate pentahydrate.....	1365	Daunorubicin hydrochloride	1389
Coriander	1365	<i>Daunorubicini hydrochloridum</i>	1389
Coriander oil	1366	D-Camphor	1170
<i>Coriandri aetheroleum</i>	1366	<i>D-Camphora</i>	1170
<i>Coriandri fructus</i>	1365	<i>Decylis oleas</i>	1390
<i>Corpora ad usum pharmaceuticum</i>	586	Decyl oleate	1390
Cortisone acetate	1367	Deferoxamine mesilate.....	1390
<i>Cortisoni acetas</i>	1367	<i>Deferoxamini mesilas</i>	1390
Cotton, absorbent	1369	Degree of coloration of liquids (2.2.2.).....	24
Cottonseed oil, hydrogenated	1370	Deliverable mass or volume of liquid and semi-solid preparations, test for (2.9.28.).....	263
Couch grass rhizome	1371	Demeclocycline hydrochloride.....	1392
<i>Crataegi folii cum flore extractum siccum</i>	1714	<i>Demeclocyclini hydrochloridum</i>	1392
<i>Crataegi folium cum flore</i>	1713	Density of solids (2.2.42.).....	64
<i>Crataegi fructus</i>	1712	Density, relative (2.2.5.).....	27
Creams.....	625	Dental type silica.....	2411
Cresol, crude	1371	Depressor substances (2.6.11.).....	153
<i>Cresolum crudum</i>	1371	Deptropine citrate	1393
<i>Croci stigma ad praeparationes homoeopathicas</i>	900	<i>Deptropini citras</i>	1393
Croscarmellose sodium.....	1371	<i>Dequalinii chloridum</i>	1394
Crospovidone	1373	Dequalinium chloride.....	1394
<i>Crospovidonum</i>	1373	Desipramine hydrochloride	1395
Crotamiton	1374	<i>Desipramini hydrochloridum</i>	1395
<i>Crotamitonum</i>	1374	Deslanoside	1396
<i>Cupri sulfas anhydricus</i>	1364	<i>Deslanosidum</i>	1396
<i>Cupri sulfas pentahydricus</i>	1365	Desmopressin.....	1397
<i>Cuprum ad praeparationes homoeopathicas</i>	896	<i>Desmopressinum</i>	1397
<i>Curcumae xanthorrhizae rhizoma</i>	2645	Desoxycortone acetate.....	1399
Cutaneous application, liquid preparations for.....	607	<i>Desoxycortoni acetas</i>	1399
Cutaneous application, powders for	616	Detector tubes, gas (2.1.6.)	19
Cutaneous application, semi-solid preparations for	624	Determination of essential oils in vegetable drugs (2.8.12.)	217
Cutaneous application, veterinary liquid preparations for	630	Determination of nitrogen by sulphuric acid digestion (2.5.9.)	129
Cutaneous foams.....	608	Determination of primary aromatic amino-nitrogen (2.5.8.)	129
<i>Cyamopsidis seminis pulvis</i>	1694	Determination of tannins in herbal drugs (2.8.14.).....	221
Cyanocobalamin	1375	Determination of water by distillation (2.2.13.)	32
Cyanocobalamin (⁵⁷ Co) capsules	819	Detomidine hydrochloride for veterinary use	1400
Cyanocobalamin (⁵⁷ Co) solution	820	<i>Detomidini hydrochloridum ad usum veterinarium</i>	1400
Cyanocobalamin (⁵⁸ Co) capsules	821	Devil's claw root	1401
Cyanocobalamin (⁵⁸ Co) solution	822	Dexamethasone	1402
<i>Cyanocobalamini (⁵⁷Co) capsulae</i>	819	Dexamethasone acetate.....	1403
<i>Cyanocobalamini (⁵⁷Co) solutio</i>	820	Dexamethasone sodium phosphate	1404
<i>Cyanocobalamini (⁵⁸Co) capsulae</i>	821	<i>Dexamethasoni acetas</i>	1403
<i>Cyanocobalamini (⁵⁸Co) solutio</i>	822	<i>Dexamethasoni natrii phosphas</i>	1404
<i>Cyanocobalaminum</i>	1375	<i>Dexamethasonum</i>	1402
Cyclizine hydrochloride	1376	Dexchlorpheniramine maleate	1406
<i>Cyclizini hydrochloridum</i>	1376	<i>Dexchlorpheniramini maleas</i>	1406
Cyclopentolate hydrochloride	1377	Dexpanthenol.....	1407
<i>Cyclopentolati hydrochloridum</i>	1377	<i>Dexpanthenolum</i>	1407
Cyclophosphamide.....	1378	Dextran 1 for injection.....	1408
<i>Cyclophosphamidum</i>	1378	Dextran 40 for injection	1409
Cyproheptadine hydrochloride	1379	Dextran 60 for injection	1410
<i>Cyproheptadini hydrochloridum</i>	1379	Dextran 70 for injection	1411
Cyproterone acetate	1380	Dextrans, molecular mass distribution in (2.2.39.)	57
<i>Cyproteroni acetas</i>	1380	<i>Dextranum 1 ad iniectabile</i>	1408
Cysteine hydrochloride monohydrate	1381	<i>Dextranum 40 ad iniectabile</i>	1409

<i>Dextranum 60 ad iniectionabile</i>	1410	Dimethylacetamide	5.1-2915
<i>Dextranum 70 ad iniectionabile</i>	1411	<i>Dimethylacetamidum</i>	5.1-2915
Dextrin	1412	Dimethylaniline, <i>N,N</i> - (2.4.26.)	119
<i>Dextrinum</i>	1412	<i>Dimethylis sulfoxidum</i>	1445
Dextromethorphan hydrobromide	1412	Dimethyl sulfoxide	1445
<i>Dextromethorphani hydrobromidum</i>	1412	Dimeticone	1447
Dextromoramide tartrate	1414	<i>Dimeticonum</i>	1447
<i>Dextromoramidi tartras</i>	1414	Dimetindene maleate	1448
Dextropropoxyphene hydrochloride	1414	<i>Dimetindeni maleas</i>	1448
<i>Dextropropoxypheni hydrochloridum</i>	1414	<i>Dinatrii edetas</i>	1462
Diazepam	1415	<i>Dinatrii phosphas anhydricus</i>	1463
<i>Diazepamum</i>	1415	<i>Dinatrii phosphas dihydricus</i>	1464
Diazoxide	1416	<i>Dinatrii phosphas dodecahydricus</i>	5.1-2916
<i>Diazoxidum</i>	1416	<i>Dinitrogenii oxidum</i>	5.1-2981
<i>Dibutylis phthalas</i>	1417	Dinoprostone	1450
Dibutyl phthalate	1417	<i>Dinoprostonum</i>	1450
Dichloromethane	2017	Dinoprost trometamol	1449
Diclazuril for veterinary use	1418	<i>Dinoprostum trometamololum</i>	1449
<i>Diclazurilum ad usum veterinarium</i>	1418	Diosmin	1452
Diclofenac potassium	1419	<i>Diosminum</i>	1452
Diclofenac sodium	1420	Dioxan and ethylene oxide (2.4.25.)	118
<i>Diclofenacum kalicum</i>	1419	Dip concentrates	630
<i>Diclofenacum natricum</i>	1420	Diphenhydramine hydrochloride	1454
Dicloxacillin sodium	1422	<i>Diphenhydramini hydrochloridum</i>	1454
<i>Dicloxacillinum natricum</i>	1422	Diphenoxylate hydrochloride	1455
Dicycloverine hydrochloride	1423	<i>Diphenoxylati hydrochloridum</i>	1455
<i>Dicycloverini hydrochloridum</i>	1423	Diphtheria and tetanus vaccine (adsorbed)	639
Dienestrol	1424	Diphtheria and tetanus vaccine (adsorbed) for adults and adolescents	639
<i>Dienestrolum</i>	1424	Diphtheria antitoxin	801
Diethylcarbamazine citrate	1426	Diphtheria, tetanus and hepatitis B (rDNA) vaccine (adsorbed)	641
<i>Diethylcarbamazini citras</i>	1426	Diphtheria, tetanus and pertussis (acellular, component) vaccine (adsorbed)	642
Diethylene glycol and ethylene glycol in ethoxylated substances (2.4.30.)	122	Diphtheria, tetanus and pertussis vaccine (adsorbed)	643
Diethylene glycol monoethyl ether	1426	Diphtheria, tetanus, pertussis (acellular, component) and haemophilus type b conjugate vaccine (adsorbed)	645
Diethylene glycol monopalmitostearate	1428	Diphtheria, tetanus, pertussis (acellular, component) and hepatitis B (rDNA) vaccine (adsorbed)	647
<i>Diethyleneglycoli monoethylicum aetherum</i>	1426	Diphtheria, tetanus, pertussis (acellular, component) and poliomyelitis (inactivated) vaccine (adsorbed)	648
<i>Diethyleneglycoli monopalmitostearas</i>	1428	Diphtheria, tetanus, pertussis (acellular, component), hepatitis B (rDNA), poliomyelitis (inactivated) and haemophilus type b conjugate vaccine (adsorbed)	650
<i>Diethylis phthalas</i>	1425	Diphtheria, tetanus, pertussis and poliomyelitis (inactivated) vaccine (adsorbed)	656
Diethyl phthalate	1425	Diphtheria, tetanus, pertussis, poliomyelitis (inactivated) and haemophilus type b conjugate vaccine (adsorbed)	657
Diethylstilbestrol	1429	Diphtheria vaccine (adsorbed)	660
<i>Diethylstilbestrolum</i>	1429	Diphtheria vaccine (adsorbed), assay of (2.7.6.)	196
Diflunisal	1429	Diphtheria vaccine (adsorbed) for adults and adolescents	661
<i>Diflunisalum</i>	1429	Dipivefrine hydrochloride	1456
Digitalis leaf	1431	<i>Dipivefrini hydrochloridum</i>	1456
<i>Digitalis purpureae folium</i>	1431	Dipotassium clorazepate	1457
Digitoxin	1432	Dipotassium phosphate	1458
<i>Digitoxinum</i>	1432	Diprophylline	1459
Digoxin	1433	<i>Diprophyllinum</i>	1459
<i>Digoxinum</i>	1433	Dipyridamole	1460
Dihydralazine sulphate, hydrated	1434	<i>Dipyridamololum</i>	1460
<i>Dihydralazini sulfas hydricus</i>	1434	Dirithromycin	1461
Dihydrocodeine hydrogen tartrate	1435	<i>Dirithromycinum</i>	1461
<i>Dihydrocodeini hydrogenotartras</i>	1435	Disintegration of suppositories and pessaries (2.9.2.)	227
Dihydroergocristine mesilate	1437	Disintegration of tablets and capsules (2.9.1.)	225
<i>Dihydroergocristini mesilas</i>	1437	Disodium edetate	1462
Dihydroergotamine mesilate	1439	Disodium phosphate, anhydrous	1463
Dihydroergotamine tartrate	1440		
<i>Dihydroergotamini mesilas</i>	1439		
<i>Dihydroergotamini tartras</i>	1440		
<i>Dihydrostreptomycini sulfas ad usum veterinarium</i>	1441		
Dihydrostreptomycin sulphate for veterinary use	1441		
<i>Dikalii clorazepas</i>	1457		
<i>Dikalii phosphas</i>	1458		
Diltiazem hydrochloride	1443		
<i>Diltiazemi hydrochloridum</i>	1443		
Dimenhydrinate	1444		
<i>Dimenhydrinatum</i>	1444		
Dimercaprol	1445		
<i>Dimercaprolum</i>	1445		

Disodium phosphate dihydrate.....	1464	Edrophonium chloride.....	1495
Disodium phosphate dodecahydrate	5.1-2916	Effervescent granules.....	606
Disopyramide.....	1465	Effervescent powders	617
Disopyramide phosphate.....	1466	Effervescent tablets	627
<i>Disopyramidi phosphas</i>	1466	Efficacy of antimicrobial preservation (5.1.3.).....	447
<i>Disopyramidum</i>	1465	Egg drop syndrome '76 vaccine (inactivated).....	753
Dispersible tablets	628	Elder flower.....	1496
Dissolution test for solid dosage forms (2.9.3.).....	228	Electrophoresis (2.2.31.).....	45
Dissolution test for transdermal patches (2.9.4.).....	231	Electrophoresis, capillary (2.2.47.).....	74
Distemper vaccine (live), canine, freeze-dried.....	740	<i>Eleutherococci radix</i>	1497
Distemper vaccine (live) for mustelids, freeze-dried.....	751	Eleutherococcus.....	1497
Distillation range (2.2.11.).....	30	Emetine hydrochloride heptahydrate.....	1499
Disulfiram.....	1467	Emetine hydrochloride pentahydrate.....	1500
<i>Disulfiramum</i>	1467	<i>Emetini hydrochloridum heptahydricum</i>	1499
Dithranol.....	1468	<i>Emetini hydrochloridum pentahydricum</i>	1500
<i>Dithranolum</i>	1468	<i>Emplastra transcutanea</i>	616
DL-Methionine	2010	Empty sterile containers of plasticised poly(vinyl chloride) for human blood and blood components (3.2.4.)	311
<i>DL-Methioninum</i>	2010	Emulsifying cetostearyl alcohol (type A)	1239
DL- α -Tocopheryl hydrogen succinate.....	2597	Emulsifying cetostearyl alcohol (type B).....	1241
<i>DL-α-Tocopherylis hydrogenosuccinas</i>	2597	<i>Enalapril maleas</i>	5.1-2919
Dobutamine hydrochloride.....	1469	Enalapril maleate.....	5.1-2919
<i>Dobutamini hydrochloridum</i>	1469	Endotoxins, bacterial (2.6.14.).....	161
Docusate sodium.....	1471	Enilconazole for veterinary use.....	1503
Dodecyl gallate	1472	<i>Enilconazolum ad usum veterinarium</i>	1503
<i>Dodecylis gallas</i>	1472	Enoxaparin sodium.....	1504
Dog rose.....	1472	<i>Enoxaparinum natricum</i>	1504
Domperidone	1473	Enoxolone.....	1505
Domperidone maleate.....	1475	<i>Enoxolonom</i>	1505
<i>Domperidoni maleas</i>	1475	Ephedrine, anhydrous.....	1506
<i>Domperidonum</i>	1473	Ephedrine hemihydrate.....	1507
Dopamine hydrochloride.....	1476	Ephedrine hydrochloride	1508
<i>Dopamini hydrochloridum</i>	1476	Ephedrine hydrochloride, racemic.....	1509
Dosulepin hydrochloride.....	1477	<i>Ephedrini hydrochloridum</i>	1508
<i>Dosulepini hydrochloridum</i>	1477	<i>Ephedrini racemici hydrochloridum</i>	1509
Doxapram hydrochloride.....	1479	<i>Ephedrinum anhydricum</i>	1506
<i>Doxaprami hydrochloridum</i>	1479	<i>Ephedrinum hemihydricum</i>	1507
Doxepin hydrochloride	1480	Epirubicin hydrochloride.....	1509
<i>Doxepini hydrochloridum</i>	1480	<i>Epirubicini hydrochloridum</i>	1509
Doxorubicin hydrochloride.....	1481	Equine herpesvirus vaccine (inactivated).....	754
<i>Doxorubicini hydrochloridum</i>	1481	Equine influenza vaccine (inactivated)	755
Doxycycline hyclate	1482	<i>Equiseti herba</i>	1511
Doxycycline monohydrate.....	1484	Equisetum stem.....	1511
<i>Doxycyclini hyclas</i>	1482	Ergocalciferol.....	1512
<i>Doxycyclinum monohydricum</i>	1484	<i>Ergocalciferolum</i>	1512
Doxylamine hydrogen succinate.....	1486	Ergometrine maleate.....	1514
<i>Doxylamini hydrogenosuccinas</i>	1486	<i>Ergometrini maleas</i>	1514
Droperidol.....	1487	Ergotamine tartrate.....	1515
<i>Droperidolum</i>	1487	<i>Ergotamini tartas</i>	1515
Droppers (2.1.1.).....	17	Erysipelas vaccine (inactivated), swine	793
Drop point (2.2.17.).....	33	Erythritol.....	1516
Dry extracts.....	572	<i>Erythritolum</i>	1516
Dry residue of extracts (2.8.16.).....	222	Erythromycin	1518
Duck viral hepatitis type I vaccine (live).....	751	Erythromycin estolate.....	1520
E		Erythromycin ethylsuccinate.....	1521
Ear-drops and sprays.....	602	<i>Erythromycini estolas</i>	1520
Ear powders	602	<i>Erythromycini ethylsuccinas</i>	1521
Ear preparations.....	601	<i>Erythromycini lactobionas</i>	1523
Ear preparations, semi-solid	602	<i>Erythromycini stearas</i>	1526
Ear tampons.....	602	Erythromycin lactobionate	1523
Ear washes.....	602	Erythromycin stearate	1526
Ebastine	1491	<i>Erythromycinum</i>	1518
<i>Ebastinum</i>	1491	Erythropoietin concentrated solution.....	1528
Econazole	1492	<i>Erythropoietini solutio concentrata</i>	1528
Econazole nitrate	1493	Eserine salicylate	2239
<i>Econazoli nitras</i>	1493	Eserine sulphate.....	2240
<i>Econazolum</i>	1492	<i>Eserini salicylas</i>	2239
Edetic acid	1494	<i>Eserini sulfas</i>	2240
<i>Edrophonii chloridum</i>	1495	Esketamine hydrochloride.....	1533

<i>Esketamini hydrochloridum</i>	1533	Etofenamate.....	1561
Essential oils, assay of 1,8-cineole (2.8.11.).....	216	<i>Etofenamatum</i>	1561
Essential oils, fatty oils and resinified essential oils in (2.8.7.).....	216	Etofylline.....	1563
Essential oils, foreign esters in (2.8.6.).....	216	<i>Etofyllinum</i>	1563
Essential oils in vegetable drugs, determination of (2.8.12.).....	217	Etomidate.....	1564
Essential oils, odour and taste (2.8.8.).....	216	<i>Etomidatum</i>	1564
Essential oils, residue on evaporation (2.8.9.).....	216	Etoposide.....	1565
Essential oils, solubility in alcohol (2.8.10.).....	216	<i>Etoposidum</i>	1565
Essential oils, water in (2.8.5.).....	216	<i>Eucalypti aetheroleum</i>	1570
Ester value (2.5.2.).....	127	<i>Eucalypti folium</i>	1569
Estradiol benzoate.....	1534	Eucalyptus leaf.....	1569
Estradiol hemihydrate.....	1535	Eucalyptus oil.....	1570
<i>Estradioli benzoas</i>	1534	Eugenol.....	1571
<i>Estradioli valeras</i>	1536	<i>Eugenolum</i>	1571
<i>Estradiolum hemihydricum</i>	1535	European Goldenrod.....	1682
Estradiol valerate.....	1536	European viper venom antiserum.....	806
Estriol.....	1537	Evaluation of efficacy of veterinary vaccines and immunosera (5.2.7.).....	5.1-2829
<i>Estriolum</i>	1537	Evaluation of safety of each batch of veterinary vaccines and immunosera (5.2.9.).....	5.1-2830
<i>Estrogeni coniuncti</i>	1539	Evaluation of safety of veterinary vaccines and immunosera (5.2.6.).....	5.1-2827
Estrogens, conjugated.....	1539	Evening primrose oil, refined.....	5.1-2921
Etacrynic acid.....	1542	<i>Extracta</i>	570
Etamsylate.....	1542	Extractable volume of parenteral preparations, test for (2.9.17.).....	243
<i>Etamsylatum</i>	1542	<i>Extracta fluida</i>	571
Ethacridine lactate monohydrate.....	1543	<i>Extracta sicca</i>	572
<i>Ethacridini lactas monohydricus</i>	1543	<i>Extracta spissa</i>	572
Ethambutol hydrochloride.....	1544	Extracts.....	570
<i>Ethambutoli hydrochloridum</i>	1544	Extracts, dry.....	572
Ethanol (96 per cent).....	1545	Extracts, dry residue of (2.8.16.).....	222
Ethanol, anhydrous.....	1547	Extracts, liquid.....	571
Ethanol content and alcoholimetric tables (2.9.10.).....	237	Extracts, loss on drying of (2.8.17.).....	222
<i>Ethanolum (96 per centum)</i>	1545	Extracts, soft.....	572
<i>Ethanolum anhydricum</i>	1547	Extraneous agents in viral vaccines for human use, tests for (2.6.16.).....	169
Ether.....	1548	Extraneous agents: tests in batches of finished product of avian live virus vaccines (2.6.25.).....	180
Ether, anaesthetic.....	1549	Extraneous agents: tests in seed lots of avian viral vaccines (2.6.24.).....	177
Ethinylestradiol.....	1550	Eye-drops.....	603
<i>Ethinylestradiolum</i>	1550	Eye lotions.....	603
Ethionamide.....	1551	Eye preparations.....	602
<i>Ethionamidum</i>	1551	Eye preparations, semi-solid.....	604
Ethosuximide.....	1551	F	
<i>Ethosuximidum</i>	1551	<i>F</i> ₀ concept to steam sterilisation of aqueous preparations, application of (5.1.5.).....	5.1-2821
Ethoxylated substances, ethylene glycol and diethylene glycol in (2.4.30.).....	122	Factor II, human coagulation, assay of (2.7.18.).....	209
Ethyl acetate.....	1553	<i>Factor IX coagulationis humanus</i>	1738
Ethylcellulose.....	1555	Factor IX, human coagulation.....	1738
<i>Ethylcellulosum</i>	1555	Factor IX, human coagulation, assay of (2.7.11.).....	204
<i>Ethylendiaminum</i>	1556	<i>Factor VII coagulationis humanus</i>	1734
Ethylenediamine.....	1556	Factor VII, human coagulation.....	1734
Ethylene glycol and diethylene glycol in ethoxylated substances (2.4.30.).....	122	Factor VII, human coagulation, assay of (2.7.10.).....	203
Ethylene glycol monopalmitostearate.....	1556	<i>Factor VIII coagulationis humanus</i>	1736
Ethylene glycol monostearate.....	1556	<i>Factor VIII coagulationis humanus (ADNr)</i>	1737
Ethylene oxide and dioxan (2.4.25.).....	118	Factor VIII, human coagulation.....	1736
<i>Ethylenglycoli monopalmitostearas</i>	1556	Factor VIII, human coagulation, assay of (2.7.4.).....	194
Ethylhexanoic acid, 2- (2.4.28.).....	120	Factor VIII (rDNA), human coagulation.....	1737
<i>Ethylis acetas</i>	1553	Factor X, human coagulation, assay of (2.7.19.).....	210
<i>Ethylis oleas</i>	1553	<i>Factor XI coagulationis humanus</i>	1739
<i>Ethylis parahydroxybenzoas</i>	1554	Factor XI, human coagulation.....	1739
<i>Ethylis parahydroxybenzoas natricum</i>	5.1-2920	Factor XI, human coagulation, assay of (2.7.22.).....	212
Ethylmorphine hydrochloride.....	1557	Falling ball viscometer method (2.2.49.).....	80
<i>Ethylmorphini hydrochloridum</i>	1557	Famotidine.....	1575
Ethyl oleate.....	1553	<i>Famotidinum</i>	1575
Ethyl parahydroxybenzoate.....	1554		
Ethyl parahydroxybenzoate sodium.....	5.1-2920		
Etilefrine hydrochloride.....	1558		
<i>Etilefrini hydrochloridum</i>	1558		
Etodolac.....	1560		
<i>Etodolacum</i>	1560		

Fatty acids, composition by gas chromatography (2.4.22.)	110	<i>Filum polyamidicum-6/6 sterile in fuso ad usum veterinarium</i>	887
Fatty oils, alkaline impurities in (2.4.19.)	109	<i>Filum polyamidicum-6 sterile in fuso ad usum veterinarium</i>	886
Fatty oils and herbal drugs, heavy metals in (2.4.27.)	119	Finasteride	1594
Fatty oils and resinified essential oils in essential oils (2.8.7.)	216	<i>Finasteridum</i>	1594
Fatty oils, foreign oils in, by thin-layer chromatography (2.4.21.)	109	Fish oil, rich in omega-3-acids	1595
Fatty oils, identification by thin-layer chromatography (2.3.2.)	98	Flecainide acetate	1598
Fatty oils, sterols in (2.4.23.)	5.1-2787	<i>Flecainidi acetas</i>	1598
Fc function of immunoglobulin, test for (2.7.9.)	202	Flowability (2.9.16.)	242
Feline calcivirosis vaccine (inactivated)	757	Flubendazole	1599
Feline calcivirosis vaccine (live), freeze-dried	758	<i>Flubendazolum</i>	1599
Feline infectious enteritis (feline panleucopenia) vaccine (inactivated)	759	Flucloxacillin sodium	5.1-2925
Feline infectious enteritis (feline panleucopenia) vaccine (live)	760	<i>Flucloxacillinum natricum</i>	5.1-2925
Feline leukaemia vaccine (inactivated)	761	Flucytosine	1602
Feline panleucopenia vaccine (inactivated)	759	<i>Flucytosinum</i>	1602
Feline panleucopenia vaccine (live)	760	Fludarabine phosphate	5.1-2926
Feline viral rhinotracheitis vaccine (inactivated)	762	<i>Fludarabini phosphas</i>	5.1-2926
Feline viral rhinotracheitis vaccine (live), freeze-dried	763	Fludeoxyglucose (¹⁸ F) injection	822
Felodipine	1576	<i>Fludeoxyglucosi (¹⁸F) solutio iniectionabilis</i>	822
<i>Felodipinum</i>	1576	Fludrocortisone acetate	1603
Fenbendazole for veterinary use	1577	<i>Fludrocortisoni acetas</i>	1603
<i>Fenbendazolum ad usum veterinarium</i>	1577	Flumazenil	1604
Fenbufen	1578	Flumazenil (N-[¹¹ C]methyl) injection	825
<i>Fenbufenum</i>	1578	<i>Flumazenil (N-[¹¹C]methyl) solutio iniectionabilis</i>	825
Fennel, bitter	1580	<i>Flumazenilum</i>	1604
Fennel, sweet	1580	Flumequine	1605
Fenofibrate	1581	<i>Flumequinum</i>	1605
<i>Fenofibratum</i>	1581	Flumetasone pivalate	1607
Fenoterol hydrobromide	1583	<i>Flumetasoni pivalas</i>	1607
<i>Fenoteroli hydrobromidum</i>	1583	Flunarizine dihydrochloride	1608
Fentanyl	1584	<i>Flunarizini dihydrochloridum</i>	1608
Fentanyl citrate	1585	Flunitrazepam	1609
<i>Fentanyl citras</i>	1585	<i>Flunitrazepamum</i>	1609
<i>Fentanylum</i>	1584	<i>Flunixin megluminum ad usum veterinarium</i>	5.1-2929
Fenticonazole nitrate	1586	Flunixin meglumine for veterinary use	5.1-2929
<i>Fenticonazoli nitras</i>	1586	Fluocinolone acetonide	1610
Fenugreek	1588	<i>Fluocinoloni acetamidum</i>	1610
Fermentation, products of	576	Fluocortolone pivalate	1611
Ferric chloride hexahydrate	1588	<i>Fluocortoloni pivalas</i>	1611
<i>Ferri chloridum hexahydricum</i>	1588	Fluorescein sodium	1613
<i>Ferrosi fumaras</i>	1589	<i>Fluoresceinum natricum</i>	1613
<i>Ferrosi gluconas</i>	1590	Fluorides (2.4.5.)	104
<i>Ferrosi sulfas heptahydricus</i>	1591	Fluorimetry (2.2.21.)	35
Ferrous fumarate	1589	Fluorouracil	1614
Ferrous gluconate	1590	<i>Fluorouracilum</i>	1614
Ferrous sulphate heptahydrate	1591	Fluoxetine hydrochloride	1615
<i>Ferrum ad praeparationes homoeopathicas</i>	899	<i>Fluoxetini hydrochloridum</i>	1615
Feverfew	1592	Flupentixol dihydrochloride	1617
<i>Fibrini glutinum</i>	1593	<i>Flupentixoli dihydrochloridum</i>	1617
Fibrinogen, human	1740	Fluphenazine decanoate	1619
<i>Fibrinogenum humanum</i>	1740	Fluphenazine enantate	1620
Fibrin sealant kit	1593	Fluphenazine hydrochloride	1621
<i>Fila non resorbilia sterilia</i>	874	<i>Fluphenazini decanoas</i>	1619
<i>Fila non resorbilia sterilia in fuso ad usum veterinarium</i>	888	<i>Fluphenazini enantas</i>	1620
<i>Fila resorbilia synthetica monofilamenta sterilia</i>	880	<i>Fluphenazini hydrochloridum</i>	1621
<i>Fila resorbilia synthetica torta sterilia</i>	878	<i>Flurazepam monohydrochloridum</i>	1622
<i>Filipendulae ulmariae herba</i>	1980	Flurazepam monohydrochloride	1622
<i>Filum bombycis tortum sterile in fuso ad usum veterinarium</i>	887	Flurbiprofen	1623
<i>Filum ethyleni polyterephthalici sterile in fuso ad usum veterinarium</i>	887	<i>Flurbiprofenum</i>	1623
<i>Filum lini sterile in fuso ad usum veterinarium</i>	886	Fluspirilene	1625
		<i>Fluspirilenum</i>	1625
		Flutamide	1626
		<i>Flutamidum</i>	1626
		Fluticasone propionate	1627
		<i>Fluticasoni propionas</i>	1627
		Flutrimazole	1629
		<i>Flutrimazolum</i>	1629
		Foams, cutaneous	608

Foams, medicated	604	Gas-gangrene antitoxin, mixed	802
Foams, rectal	624	Gas-gangrene antitoxin (novyi)	802
Foams, vaginal	630	Gas-gangrene antitoxin (perfringens)	803
<i>Foeniculi amari fructus</i>	1580	Gas-gangrene antitoxin (septicum)	804
<i>Foeniculi amari fructus aetheroleum</i>	1108	Gastro-resistant capsules	600
<i>Foeniculi dulcis fructus</i>	1580	Gastro-resistant granules	606
Folic acid	1630	Gastro-resistant tablets	628
Foot-and-mouth disease (ruminants) vaccine (inactivated)	5.1-2860	Gelatin	1651
Foreign esters in essential oils (2.8.6.)	216	<i>Gelatina</i>	1651
Foreign matter (2.8.2.)	215	Gels	625
Foreign oils in fatty oils by thin-layer chromatography (2.4.21.)	109	Gels for injections	5.1-2843
Formaldehyde, free (2.4.18.)	109	General chapters (1.3.)	6
Formaldehyde solution (35 per cent)	1632	General notices (1.)	5
<i>Formaldehydi solutio (35 per centum)</i>	1632	General statements (1.1.)	5
Formoterol fumarate dihydrate	1632	General texts on sterility (5.1.)	445
<i>Formoteroli fumaras dihydricus</i>	1632	General texts on vaccines (5.2.)	453
Foscarnet sodium hexahydrate	1634	<i>Gentamicini sulfas</i>	1653
<i>Foscarnetum natricum hexahydricum</i>	1634	Gentamicin sulphate	1653
Fosfomycin calcium	1636	<i>Gentianae radix</i>	1654
Fosfomycin sodium	1637	<i>Gentianae tinctura</i>	1655
Fosfomycin trometamol	1638	Gentian root	1654
<i>Fosfomycinum calcicum</i>	1636	Gentian tincture	1655
<i>Fosfomycinum natricum</i>	1637	Ginger	1656
<i>Fosfomycinum trometamolom</i>	1638	Gingival solutions	612
Fowl cholera vaccine (inactivated)	5.1-2861	<i>Ginkgo folium</i>	1657
Fowl-pox vaccine (live)	766	Ginkgo leaf	1657
<i>Framycetini sulfas</i>	1639	Ginseng	5.1-2935
Framycetin sulphate	1639	<i>Ginseng radix</i>	5.1-2935
Frangula bark	1641	Glass containers for pharmaceutical use (3.2.1.)	303
Frangula bark dry extract, standardised	1642	Glibenclamide	1659
<i>Frangulae cortex</i>	1641	<i>Glibenclamidum</i>	1659
<i>Frangulae corticis extractum siccum normatum</i>	1642	Gliclazide	1660
<i>Fraxini folium</i>	1026	<i>Gliclazidum</i>	1660
Free formaldehyde (2.4.18.)	109	Glipizide	1662
Freezing point (2.2.18.)	34	<i>Glipizidum</i>	1662
Friability of uncoated tablets (2.9.7.)	234	Glossary (dosage forms)	599
Fructose	1643	Glucagon	1663
<i>Fructosum</i>	1643	Glucagon, human	1665
Fucus	1869	<i>Glucagonum</i>	1663
<i>Fucus vel Ascophyllum</i>	1869	<i>Glucagonum humanum</i>	1665
Functional groups and ions, identification reactions (2.3.1.)	95	Glucose, anhydrous	1666
Furosemide	5.1-2930	Glucose, liquid	1667
<i>Furosemidum</i>	5.1-2930	Glucose, liquid, spray-dried	1668
Furunculosis vaccine (inactivated, oil-adjuvanted, injectable) for salmonids	767	Glucose monohydrate	1669
Fusidic acid	1645	<i>Glucosum anhydricum</i>	1666
G		<i>Glucosum liquidum</i>	1667
Galactose	1649	<i>Glucosum liquidum dispersione desiccatum</i>	1668
<i>Galactosum</i>	1649	<i>Glucosum monohydricum</i>	1669
Gallamine triethiodide	1649	Glutamic acid	1670
<i>Gallamini triethiodidum</i>	1649	Glutathione	5.1-2936
<i>Gallii (⁶⁷Ga) citratis solutio iniectabilis</i>	826	<i>Glutathionum</i>	5.1-2936
Gallium (⁶⁷ Ga) citrate injection	826	Glycerol	1671
Gargles	612	Glycerol (85 per cent)	1672
Garlic for homoeopathic preparations	897	Glycerol dibehenate	5.1-2938
Garlic powder	1651	Glycerol distearate	1674
Gas chromatography (2.2.28.)	42	<i>Glyceroli dibehenas</i>	5.1-2938
Gas detector tubes (2.1.6.)	19	<i>Glyceroli distearas</i>	1674
Gases, carbon dioxide in (2.5.24.)	134	<i>Glyceroli monolinoleas</i>	1675
Gases, carbon monoxide in (2.5.25.)	134	<i>Glyceroli mono-oleates</i>	1676
Gases, nitrogen monoxide and nitrogen dioxide in (2.5.26.)	135	<i>Glyceroli monostearas 40-55</i>	1677
Gases, nitrous oxide in (2.5.35.)	141	<i>Glyceroli trinitratis solutio</i>	1678
Gases, oxygen in (2.5.27.)	136	Glycerol monolinoleate	1675
Gases, water in (2.5.28.)	136	Glycerol mono-oleates	1676
		Glycerol monostearate 40-55	1677
		Glycerol triacetate	2612
		<i>Glycerolum</i>	1671
		<i>Glycerolum (85 per centum)</i>	1672
		Glyceryl trinitrate solution	1678
		Glycine	1680

<i>Glycinum</i>	1680	Hawthorn leaf and flower dry extract	1714
Glycyrrhizate ammonium	5.1-2876	Heavy bismuth subnitrate	1107
Goldenrod	1680	Heavy kaolin	1869
Goldenrod, European	1682	Heavy magnesium carbonate	1954
Goldenseal rhizome	1683	Heavy magnesium oxide	1958
Gonadorelin acetate	1684	Heavy metals (2.4.8.)	104
<i>Gonadorelini acetat</i>	1684	Heavy metals in herbal drugs and fatty oils (2.4.27.)	119
Gonadotrophin, chorionic	1686	<i>Hederae folium</i>	5.1-2954
Gonadotrophin, equine serum, for veterinary use	1686	<i>Helianthi annui oleum raffinatum</i>	2524
<i>Gonadotropinum chorionicum</i>	1686	<i>Heparina massae molecularis minoris</i>	1717
<i>Gonadotropinum sericum equinum ad usum veterinarium</i>	1686	Heparin, assay of (2.7.5.)	195
Goserelin	1687	Heparin calcium	1715
<i>Goserelinum</i>	1687	Heparin in coagulation factors, assay of (2.7.12.)	204
<i>Gossypii oleum hydrogenatum</i>	1370	Heparins, low-molecular-mass	1717
Gramicidin	1689	Heparin sodium	1716
<i>Gramicidinum</i>	1689	<i>Heparinum calcicum</i>	1715
<i>Graminis rhizoma</i>	1371	<i>Heparinum natricum</i>	1716
Granisetron hydrochloride	5.1-2939	Hepatitis A immunoglobulin, human	1741
<i>Granisetroni hydrochloridum</i>	5.1-2939	Hepatitis A (inactivated) and hepatitis B (rDNA) vaccine (adsorbed)	664
<i>Granulata</i>	605	Hepatitis A vaccine, assay of (2.7.14.)	5.1-2795
Granules	605	Hepatitis A vaccine (inactivated, adsorbed)	665
Granules, coated	606	Hepatitis A vaccine (inactivated, virosome)	667
Granules, effervescent	606	Hepatitis B immunoglobulin for intravenous administration, human	1741
Granules, gastro-resistant	606	Hepatitis B immunoglobulin, human	1741
Granules, modified-release	606	Hepatitis B (rDNA), diphtheria and tetanus vaccine (adsorbed)	641
Greater celandine	1690	Hepatitis B (rDNA), diphtheria, tetanus and pertussis (acellular, component) vaccine (adsorbed)	647
Griseofulvin	1691	Hepatitis B vaccine (rDNA)	670
<i>Griseofulvinum</i>	1691	Hepatitis B vaccine (rDNA), assay of (2.7.15.)	207
Guaifenesin	1692	Hepatitis C virus (HCV), validation of nucleic acid amplification techniques for the detection of HCV RNA in plasma pools: Guidelines	176
<i>Guaifenesinum</i>	1692	Heptaminol hydrochloride	1719
Guanethidine monosulphate	1694	<i>Heptaminoli hydrochloridum</i>	1719
<i>Guanethidini monosulfas</i>	1694	Herbal drug preparations	572
Guar	1694	Herbal drugs	572
Guar galactomannan	1695	Herbal drugs and fatty oils, heavy metals in (2.4.27.)	119
<i>Guar galactomannanum</i>	1695	Herbal drugs, determination of tannins (2.8.14.)	221
H		Herbal drugs for homoeopathic preparations	893
Haemodiafiltration and for haemofiltration, solutions for	1703	Herbal teas	573
Haemodialysis, concentrated solutions for	1700	Hexamidine diisetonate	1720
Haemodialysis solutions, concentrated, water for diluting	1699	<i>Hexamidini diisetonas</i>	1720
Haemodialysis, solutions for	1700	Hexetidine	1721
Haemofiltration and for haemodiafiltration, solutions for	1703	<i>Hexetidinum</i>	1721
Haemophilus type b (conjugate), diphtheria, tetanus, pertussis (acellular, component) and poliomyelitis (inactivated) vaccine (adsorbed)	653	Hexobarbital	1722
Haemophilus type b (conjugate), diphtheria, tetanus, pertussis and poliomyelitis (inactivated) vaccine (adsorbed)	657	<i>Hexobarbitalum</i>	1722
Haemophilus type b conjugate vaccine	662	Hexosamines in polysaccharide vaccines (2.5.20.)	132
Halofantrine hydrochloride	1705	Hexylresorcinol	1723
<i>Halofantrini hydrochloridum</i>	1705	<i>Hexylresorcinolum</i>	1723
Haloperidol	1706	<i>Hibisci sabdariffae flos</i>	2376
Haloperidol decanoate	1708	Highly purified water	2695
<i>Haloperidoli decanoas</i>	1708	Histamine (2.6.10.)	153
<i>Haloperidolum</i>	1706	Histamine dihydrochloride	1724
Halothane	1709	Histamine phosphate	1725
<i>Halothanum</i>	1709	<i>Histamini dihydrochloridum</i>	1724
<i>Hamamelidis folium</i>	1711	<i>Histamini phosphas</i>	1725
Hamamelis leaf	1711	Histidine	5.1-2945
Hard capsules	600	Histidine hydrochloride monohydrate	1727
Hard fat	1711	<i>Histidini hydrochloridum monohydricum</i>	1727
Hard paraffin	2186	<i>Histidinum</i>	5.1-2945
<i>Harpagophyti radix</i>	1401	Homatropine hydrobromide	1728
Hawthorn berries	1712	Homatropine methylbromide	1729
Hawthorn leaf and flower	1713	<i>Homatropini hydrobromidum</i>	1728
		<i>Homatropini methylbromidum</i>	1729
		Homoeopathic preparations	893
		Homoeopathic preparations, arsenious trioxide for	895

Homoeopathic preparations, common stinging nettle for	895	Hydrogenated arachis oil	1018
Homoeopathic preparations, copper for	896	Hydrogenated castor oil	1197
Homoeopathic preparations, garlic for	897	Hydrogenated cottonseed oil	1370
Homoeopathic preparations, herbal drugs for	893	Hydrogenated soya-bean oil	2475
Homoeopathic preparations, honey bee for	898	Hydrogenated wool fat	2708
Homoeopathic preparations, hypericum for	898	<i>Hydrogenii peroxidum 30 per centum</i>	1763
Homoeopathic preparations, iron for	899	<i>Hydrogenii peroxidum 3 per centum</i>	1762
Homoeopathic preparations, mother tinctures for	894	Hydrogen peroxide solution (30 per cent)	1763
Homoeopathic preparations, saffron for	900	Hydrogen peroxide solution (3 per cent)	1762
Honey	5.1-2946	Hydromorphone hydrochloride	1763
Honey bee for homoeopathic preparations	898	<i>Hydromorphoni hydrochloridum</i>	1763
Hop strobile	1730	Hydrous wool fat	2709
Human albumin injection, iodinated (¹²⁵ I)	827	Hydroxocobalamin acetate	1765
Human albumin solution	1731	Hydroxocobalamin chloride	1766
Human anti-D immunoglobulin	1732	<i>Hydroxocobalamini acetat</i>	1765
Human anti-D immunoglobulin, assay of (2.7.13.)	205	<i>Hydroxocobalamini chloridum</i>	1766
Human anti-D immunoglobulin for intravenous administration	1733	<i>Hydroxocobalamini sulfas</i>	1767
Human antithrombin III, assay of (2.7.17.)	209	Hydroxocobalamin sulphate	1767
Human antithrombin III concentrate	1733	Hydroxycarbamide	1768
Human coagulation factor II, assay of (2.7.18.)	209	<i>Hydroxycarbamidum</i>	1768
Human coagulation factor IX	1738	Hydroxyethylcellulose	5.1-2947
Human coagulation factor IX, assay of (2.7.11.)	204	<i>Hydroxyethylcellulosum</i>	5.1-2947
Human coagulation factor VII	1734	<i>Hydroxyethylis salicylas</i>	1769
Human coagulation factor VII, assay of (2.7.10.)	203	Hydroxyethylmethylcellulose	2018
Human coagulation factor VIII	1736	Hydroxyethyl salicylate	1769
Human coagulation factor VIII, assay of (2.7.4.)	194	Hydroxyl value (2.5.3.)	127
Human coagulation factor VIII (rDNA)	1737	Hydroxypropylbetadex	1771
Human coagulation factor X, assay of (2.7.19.)	210	<i>Hydroxypropylbetadexum</i>	1771
Human coagulation factor XI	1739	Hydroxypropylcellulose	1773
Human coagulation factor XI, assay of (2.7.22.)	212	<i>Hydroxypropylcellulosum</i>	1773
Human fibrinogen	1740	Hydroxypropylmethylcellulose	1780
Human hepatitis A immunoglobulin	1741	Hydroxypropylmethylcellulose phthalate	1781
Human hepatitis B immunoglobulin	1741	Hydroxyzine hydrochloride	1774
Human hepatitis B immunoglobulin for intravenous administration	1741	<i>Hydroxyzini hydrochloridum</i>	1774
Human insulin	1800	Hymecromone	1775
Human measles immunoglobulin	1742	<i>Hymecromonium</i>	1775
Human normal immunoglobulin	1742	Hyoscine butylbromide	1776
Human normal immunoglobulin for intravenous administration	1744	Hyoscine hydrobromide	1777
Human plasma for fractionation	1746	<i>Hyoscini butylbromidum</i>	1776
Human plasma (pooled and treated for virus inactivation)	1747	<i>Hyoscini hydrobromidum</i>	1777
Human prothrombin complex	1748	Hyoscyamine sulphate	1778
Human rabies immunoglobulin	1750	<i>Hyoscyamini sulfas</i>	1778
Human rubella immunoglobulin	1751	<i>Hyperici herba</i>	2485
Human tetanus immunoglobulin	1751	Hypericum	2485
Human varicella immunoglobulin	1752	Hypericum for homoeopathic preparations	898
Human varicella immunoglobulin for intravenous administration	1753	<i>Hypericum perforatum</i>	
Human von Willebrand factor, assay of (2.7.21.)	211	<i>ad praeparationes homoeopathicas</i>	898
Hyaluronidase	1753	Hypromellose	1780
<i>Hyaluronidasum</i>	1753	Hypromellose phthalate	1781
Hydralazine hydrochloride	1754	<i>Hypromellosi phthalas</i>	1781
<i>Hydralazini hydrochloridum</i>	1754	<i>Hypromellosum</i>	1780
<i>Hydrargyri dichloridum</i>	1995		
<i>Hydrastis rhizoma</i>	1683	I	
Hydrochloric acid, concentrated	1755	Ibuprofen	1785
Hydrochloric acid, dilute	1756	<i>Ibuprofenum</i>	1785
Hydrochlorothiazide	1756	Iceland moss	1787
<i>Hydrochlorothiazidum</i>	1756	ICH (5.8.)	551
Hydrocortisone	1757	Ichthammol	1787
Hydrocortisone acetate	1759	<i>Ichthammolum</i>	1787
Hydrocortisone hydrogen succinate	1761	Identification (2.3.)	95
<i>Hydrocortisoni acetat</i>	1759	Identification and control of residual solvents (2.4.24.)	113
<i>Hydrocortisoni hydrozenosuccinas</i>	1761	Identification of fatty oils by thin-layer chromatography (2.3.2.)	98
<i>Hydrocortisonum</i>	1757	Identification of phenothiazines by thin-layer chromatography (2.3.3.)	99
		Identification reactions of ions and functional groups (2.3.1.)	95
		Idoxuridine	1788
		<i>Idoxuridinum</i>	1788

<i>Iecoris aselli oleum A</i>	1348	<i>Immunoserum contra venena viperarum</i>	
<i>Iecoris aselli oleum B</i>	1352	<i>europaearum</i>	806
Ifosfamide.....	1789	<i>Immunoserum diphthericum</i>	801
<i>Ifosfamidum</i>	1789	<i>Immunoserum gangraenicum (Clostridium novyi)</i>	802
Imipenem.....	1791	<i>Immunoserum gangraenicum</i>	
<i>Imipenemum</i>	1791	<i>(Clostridium perfringens)</i>	803
Imipramine hydrochloride.....	1792	<i>Immunoserum gangraenicum (Clostridium septicum)</i> ..	804
<i>Imipramini hydrochloridum</i>	1792	<i>Immunoserum gangraenicum mixtum</i>	802
Immunochemical methods (2.7.1.).....	187	<i>Immunoserum tetanicum ad usum humanum</i>	805
Immunoglobulin, animal, anti-T lymphocyte, for human		<i>Immunoserum tetanicum ad usum veterinarium</i> ..	5.1-2868
use.....	1010	Implants.....	5.1-2843
Immunoglobulin for intravenous administration, human		Impurities in substances for pharmaceutical use, control of	
hepatitis B.....	1741	(5.10.).....	559
Immunoglobulin for intravenous administration, human		Indapamide.....	1793
normal.....	1744	<i>Indapamidum</i>	1793
Immunoglobulin for intravenous administration, human		Indicators, relationship between approximate pH and colour	
varicella.....	1753	(2.2.4.).....	27
Immunoglobulin, human anti-D.....	1732	<i>Indii (¹¹¹In) chloridi solutio</i>	828
Immunoglobulin, human anti-D, assay of (2.7.13.).....	205	<i>Indii (¹¹¹In) oxini solutio</i>	829
Immunoglobulin, human anti-D, for intravenous		<i>Indii (¹¹¹In) pentetatis solutio iniectionabilis</i>	830
administration.....	1733	Indium (¹¹¹ In) chloride solution.....	828
Immunoglobulin, human hepatitis A.....	1741	Indium (¹¹¹ In) oxine solution.....	829
Immunoglobulin, human hepatitis B.....	1741	Indium (¹¹¹ In) pentetate injection.....	830
Immunoglobulin, human measles.....	1742	Indometacin.....	1794
Immunoglobulin, human normal.....	1742	<i>Indometacinum</i>	1794
Immunoglobulin, human rabies.....	1750	Infectious bovine rhinotracheitis vaccine (live),	
Immunoglobulin, human rubella.....	1751	freeze-dried.....	768
Immunoglobulin, human tetanus.....	1751	Infectious bronchitis vaccine (inactivated), avian.....	718
Immunoglobulin, human varicella.....	1752	Infectious bronchitis vaccine (live), avian.....	720
Immunoglobulin, test for anticomplementary activity of		Infectious bursal disease vaccine (inactivated), avian.....	722
(2.6.17.).....	170	Infectious bursal disease vaccine (live), avian.....	723
Immunoglobulin, test for Fc function of (2.7.9.).....	202	Infectious chicken anaemia vaccine (live).....	769
<i>Immunoglobulinum anti-T lymphocytorum ex animale ad</i>		Infectious encephalomyelitis vaccine (live), avian.....	725
<i>usum humanum</i>	1010	Infectious laryngotracheitis vaccine (live), avian.....	727
<i>Immunoglobulinum humanum anti-D</i>	1732	Influenza vaccine (split virion, inactivated).....	671
<i>Immunoglobulinum humanum anti-D ad usum</i>		Influenza vaccine (surface antigen, inactivated).....	673
<i>intravenosum</i>	1733	Influenza vaccine (surface antigen, inactivated,	
<i>Immunoglobulinum humanum hepatitis A</i>	1741	virosome).....	674
<i>Immunoglobulinum humanum hepatitis B</i>	1741	Influenza vaccine (whole virion, inactivated).....	676
<i>Immunoglobulinum humanum hepatitis B ad usum</i>		Infrared absorption spectrophotometry (2.2.24.).....	37
<i>intravenosum</i>	1741	Infusions.....	5.1-2842
<i>Immunoglobulinum humanum morbillicum</i>	1742	<i>Inhalanda</i>	5.1-2843
<i>Immunoglobulinum humanum normale</i>	1742	Inhalation gas, krypton (⁸¹ MKr).....	833
<i>Immunoglobulinum humanum normale ad usum</i>		Inhalation, preparations for.....	5.1-2843
<i>intravenosum</i>	1744	Inhalation, preparations for: aerodynamic assessment of fine	
<i>Immunoglobulinum humanum rabicum</i>	1750	particles (2.9.18.).....	5.1-2799
<i>Immunoglobulinum humanum rubellae</i>	1751	Injections.....	5.1-2841
<i>Immunoglobulinum humanum tetanicum</i>	1751	Injections, gels for.....	5.1-2843
<i>Immunoglobulinum humanum varicellae</i>	1752	Injections or infusions, concentrates for.....	5.1-2842
<i>Immunoglobulinum humanum varicellae ad usum</i>		Injections or infusions, powders for.....	5.1-2842
<i>intravenosum</i>	1753	Inserts, ophthalmic.....	604
<i>Immunosera ad usum veterinarium</i>	575	Insulin aspart.....	1795
Immunosera and vaccines, phenol in (2.5.15.).....	131	Insulin, bovine.....	1797
Immunosera and vaccines, veterinary, evaluation of safety		Insulin, human.....	1800
(5.2.6.).....	5.1-2827	<i>Insulini biphasici iniectionabilem</i>	1802
Immunosera and vaccines, veterinary, evaluation of the		<i>Insulini isophani biphasici iniectionabilem</i>	1803
efficacy (5.2.7).....	5.1-2829	<i>Insulini isophani iniectionabilem</i>	1803
Immunosera and vaccines, veterinary, evaluation of the		Insulin injection, biphasic.....	1802
safety of each batch (5.2.9.).....	5.1-2830	Insulin injection, biphasic isophane.....	1803
<i>Immunosera ex animale ad usum humanum</i>	573	Insulin injection, isophane.....	1803
Immunosera for human use, animal.....	573	Insulin injection, soluble.....	1803
Immunosera for veterinary use.....	575	<i>Insulini solubilis iniectionabilem</i>	1803
<i>Immunoserum botulinicum</i>	801	<i>Insulini zinci amorphi suspensio iniectionabilis</i>	1811
<i>Immunoserum clostridii novyi alpha ad usum</i>		<i>Insulini zinci cristallini suspensio iniectionabilis</i>	1812
<i>veterinarium</i>	5.1-2865	<i>Insulini zinci suspensio iniectionabilis</i>	1811
<i>Immunoserum clostridii perfringentis beta ad usum</i>		Insulin lispro.....	1804
<i>veterinarium</i>	5.1-2866	Insulin, porcine.....	1806
<i>Immunoserum clostridii perfringentis epsilon ad usum</i>		Insulin preparations, injectable.....	1808
<i>veterinarium</i>	5.1-2867	<i>Insulinum aspartum</i>	1795

- Insulinum bovinum* 1797
Insulinum humanum 1800
Insulinum lisprum 1804
Insulinum porcinum 1806
Insulin zinc injectable suspension 1811
Insulin zinc injectable suspension (amorphous) 1811
Insulin zinc injectable suspension (crystalline) 1812
Interferon alfa-2 concentrated solution 1812
Interferon gamma-1b concentrated solution 1815
Interferoni alfa-2 solutio concentrata 1812
Interferoni gamma-1b solutio concentrata 1815
Interferons, assay of (5.6.) 533
int-rac- α -Tocopherolum 5.1-3023
int-rac- α -Tocopherylis acetat 5.1-3024
Intramammary preparations for veterinary use 606
Intraruminal devices 607
In vivo assay of poliomyelitis vaccine (inactivated) (2.7.20.) 210
Iobenguane (^{123}I) injection 831
Iobenguane (^{131}I) injection for diagnostic use 832
Iobenguane (^{131}I) injection for therapeutic use 833
Iobenguani (^{123}I) solutio iniectionabilis 831
Iobenguani (^{131}I) solutio iniectionabilis ad usum diagnosticum 832
Iobenguani (^{131}I) solutio iniectionabilis ad usum therapeuticum 833
Iodinated (^{125}I) human albumin injection 827
Iodinated povidone 2291
Iodinati (^{125}I) humani albumini solutio iniectionabilis 827
Iodine 1819
Iodine value (2.5.4.) 127
Iodum 1819
Iohexol 1819
Iohexolum 1819
Ionic concentration, potentiometric determination of using ion-selective electrodes (2.2.36.) 55
Ions and functional groups, identification reactions (2.3.1.) 95
Ion-selective electrodes, potentiometric determination of ionic concentration (2.2.36.) 55
Iopamidol 1822
Iopamidolum 1822
Iopanoic acid 1824
Iotalamic acid 1825
Ioxaglic acid 1826
Ipecacuanhae extractum fluidum normatum 5.1-2951
Ipecacuanhae pulvis normatus 1829
Ipecacuanhae radix 1829
Ipecacuanhae tinctura normata 5.1-2951
Ipecacuanha liquid extract, standardised 5.1-2951
Ipecacuanha, prepared 1829
Ipecacuanha root 1829
Ipecacuanha tincture, standardised 5.1-2951
Ipratropii bromidum 1831
Ipratropium bromide 1831
Iron (2.4.9.) 107
Iron for homeopathic preparations 899
Irrigation, preparations for 622
Isoconazole 1833
Isoconazole nitrate 1834
Isoconazoli nitras 1834
Isoconazolum 1833
Isoelectric focusing (2.2.54.) 81
Isoflurane 1835
Isofluranum 1835
Isoleucine 1836
Isoleucinum 1836
Isomalt 1837
Isomaltum 1837
Isoniazid 1839
Isoniazidum 1839
Isophane insulin injection 1803
Isoprenaline hydrochloride 1839
Isoprenaline sulphate 1840
Isoprenalini hydrochloridum 1839
Isoprenalini sulfas 1840
Isopropyl alcohol 1841
Isopropyliis myristas 5.1-2952
Isopropyliis palmitas 5.1-2953
Isopropyl myristate 5.1-2952
Isopropyl palmitate 5.1-2953
Isosorbide dinitrate, diluted 1844
Isosorbide mononitrate, diluted 1845
Isosorbidi dinitras dilutus 1844
Isosorbidi mononitras dilutus 1845
Isotretinoin 1847
Isotretinoinum 1847
Isoxsuprine hydrochloride 1848
Isoxsuprini hydrochloridum 1848
Ispaghula husk 1849
Ispaghula seed 1850
Isradipine 1851
Isradipinum 1851
Itraconazole 1852
Itraconazolum 1852
Juniperi aetheroleum 1863
Juniperi pseudo-fructus 1862
Ivermectin 1854
Ivermectinum 1854
Ivy leaf 5.1-2954
J
Javanese turmeric 2645
Java tea 1859
Josamycin 1860
Josamycini propionas 1861
Josamycin propionate 1861
Josamycinum 1860
Juniper 1862
Juniper oil 1863
K
Kalii acetat 2273
Kalii bromidum 2273
Kalii carbonas 2274
Kalii chloridum 2275
Kalii citras 2275
Kalii clavulanas 2276
Kalii clavulanas dilutus 2278
Kalii dihydrogenophosphas 2280
Kalii hydrogenoaspartas hemihydricus 2280
Kalii hydrogenocarbonas 2281
Kalii hydrogenotartras 2282
Kalii hydroxidum 2283
Kalii iodidum 2283
Kalii metabisulfis 2284
Kalii natrii tartras tetrahydricus 2286
Kalii nitras 2284
Kalii perchloras 2285
Kalii permanganas 2286
Kalii sorbas 2287
Kalii sulfas 2288
Kanamycin acid sulphate 1867
Kanamycini monosulfas 1868
Kanamycini sulfas acidus 1867
Kanamycin monosulphate 1868
Kaolin, heavy 1869

<i>Kaolinum ponderosum</i>	1869	Levonorgestrel.....	1911
Kelp.....	1869	<i>Levonorgestrelum</i>	1911
Ketamine hydrochloride.....	1870	Levothyroxine sodium.....	1912
<i>Ketamini hydrochloridum</i>	1870	<i>Levothyroxinum natricum</i>	1912
Ketobemidone hydrochloride.....	1871	<i>Lichen islandicus</i>	1787
Ketoconazole.....	1872	Lidocaine.....	1913
<i>Ketoconazolum</i>	1872	Lidocaine hydrochloride.....	1914
Ketoprofen.....	1874	<i>Lidocaini hydrochloridum</i>	1914
<i>Ketoprofenum</i>	1874	<i>Lidocainum</i>	1913
Ketotifen hydrogen fumarate.....	1875	Light liquid paraffin.....	2186
<i>Ketotifeni hydrogenofumaras</i>	1875	Light magnesium carbonate.....	1954
Knotgrass.....	1877	Light magnesium oxide.....	1958
Krypton (^{81m} Kr) inhalation gas.....	833	Lime flower.....	1914
<i>Kryptonum (^{81m}Kr) ad inhalationem</i>	833	Limit test of particle size by microscopy (2.9.13.).....	239
L		Limit tests (2.4.).....	103
Labetalol hydrochloride.....	1881	Limit tests, standard solutions for (4.1.2.).....	426
<i>Labetaloli hydrochloridum</i>	1881	Limit tests, standard solutions for (4.1.2.).....	5.1 -2818
<i>Lacca</i>	2409	<i>Limonis aetheroleum</i>	1895
Lactic acid.....	1882	Lincomycin hydrochloride.....	1915
Lactic acid, (S)-.....	1883	<i>Lincomycini hydrochloridum</i>	1915
Lactitol monohydrate.....	1883	Lindane.....	1916
<i>Lactitolum monohydricum</i>	1883	<i>Lindanum</i>	1916
Lactobionic acid.....	1885	Linen thread, sterile, in distributor for veterinary use.....	886
Lactose, anhydrous.....	1886	<i>Lini oleum virginale</i>	1918
Lactose monohydrate.....	1887	<i>Lini semen</i>	5.1 -2960
<i>Lactosum anhydricum</i>	1886	Linoleoyl macroglycerides.....	1917
<i>Lactosum monohydricum</i>	1887	Linseed.....	5.1 -2960
Lactulose.....	1888	Linseed oil, virgin.....	1918
Lactulose, liquid.....	1890	Liothyronine sodium.....	1919
<i>Lactulosum</i>	1888	<i>Liothyroninum natricum</i>	1919
<i>Lactulosum liquidum</i>	1890	Liquid and semi-solid preparations, test for deliverable mass or volume (2.9.28.).....	263
<i>Lanugo cellulosi absorbens</i>	2681	Liquid chromatography (2.2.29.).....	43
<i>Lanugo gossypii absorbens</i>	1369	Liquid extracts.....	571
Lauroyl macroglycerides.....	1892	Liquid glucose.....	1667
<i>Lavandulae aetheroleum</i>	1894	Liquid glucose, spray-dried.....	1668
<i>Lavandulae flos</i>	1893	Liquid lactulose.....	1890
Lavender flower.....	1893	Liquid maltitol.....	1969
Lavender oil.....	1894	Liquid paraffin.....	2187
Lead in sugars (2.4.10.).....	107	Liquid preparations for cutaneous application.....	607
Lemon oil.....	1895	Liquid preparations for cutaneous application, veterinary.....	630
<i>Leonuri cardiacae herba</i>	2063	Liquid preparations for inhalation.....	5.1 -2843
Leptospirosis vaccine (inactivated), bovine.....	730	Liquid preparations for oral use.....	608
Leptospirosis vaccine (inactivated), canine.....	740	Liquid sorbitol (crystallising).....	2471
Leucine.....	1897	Liquid sorbitol (non-crystallising).....	2472
<i>Leucinum</i>	1897	Liquid sorbitol, partially dehydrated.....	2473
Leuprorelin.....	1898	<i>Liquiritiae extractum fluidum</i> <i>ethanolicum normatum</i>	1920
<i>Leuprorelinum</i>	1898	<i>Liquiritiae radix</i>	1921
Levamisole for veterinary use.....	1899	Liquorice ethanolic liquid extract, standardised.....	1920
Levamisole hydrochloride.....	1900	Liquorice root.....	1921
<i>Levamisoli hydrochloridum</i>	1900	Lisinopril dihydrate.....	1922
<i>Levamisolum ad usum veterinarium</i>	1899	<i>Lisinoprilum dihydricum</i>	1922
<i>Levistici radix</i>	1932	<i>Lithii carbonas</i>	1923
Levocabastine hydrochloride.....	1902	<i>Lithii citras</i>	1924
<i>Levocabastini hydrochloridum</i>	1902	Lithium carbonate.....	1923
Levocarnitine.....	5.1 -2959	Lithium citrate.....	1924
<i>Levocarnitinum</i>	5.1 -2959	L-Methionine ([¹¹ C]methyl) injection.....	834
Levodopa.....	1904	<i>L-Methionini ([¹¹C]methyl) solutio iniectabilis</i>	834
<i>Levodopum</i>	1904	Lobeline hydrochloride.....	1925
Levodropropizine.....	1905	<i>Lobelini hydrochloridum</i>	1925
<i>Levodropropizinum</i>	1905	Lomustine.....	1926
Levomenthol.....	1907	<i>Lomustinum</i>	1926
<i>Levomentholum</i>	1907	Loosestrife.....	1927
Levomepromazine hydrochloride.....	1908	Loperamide hydrochloride.....	1928
Levomepromazine maleate.....	1908	Loperamide oxide monohydrate.....	1930
<i>Levomepromazini hydrochloridum</i>	1908	<i>Loperamidi hydrochloridum</i>	1928
<i>Levomepromazini maleas</i>	1908	<i>Loperamidi oxidum monohydricum</i>	1930
Levomethadone hydrochloride.....	1909		
<i>Levomethadoni hydrochloridum</i>	1909		

Lorazepam.....	1931	Magnesium (2.4.6.)	104
<i>Lorazepamum</i>	1931	Magnesium acetate tetrahydrate	1952
Loss on drying (2.2.32.)	50	Magnesium and alkaline-earth metals (2.4.7.)	104
Loss on drying of extracts (2.8.17.)	222	Magnesium aspartate dihydrate	1953
Lovage root.....	1932	Magnesium carbonate, heavy	1954
Lovastatin	1933	Magnesium carbonate, light	1954
<i>Lovastatinum</i>	1933	Magnesium chloride 4.5-hydrate	1955
Low-molecular-mass heparins	1717	Magnesium chloride hexahydrate	1956
Lozenges and pastilles.....	613	Magnesium glycerophosphate	1957
Lozenges, compressed	613	Magnesium hydroxide.....	1957
Lubricant, silicone oil (3.1.8.).....	287	Magnesium oxide, heavy.....	1958
<i>Lupuli flos</i>	1730	Magnesium oxide, light.....	1958
Lynestrenol.....	1935	Magnesium peroxide	1959
<i>Lynestrenolum</i>	1935	Magnesium pidolate	1960
Lysine acetate	5.1-2960	Magnesium stearate.....	1961
Lysine hydrochloride.....	1936	Magnesium sulphate heptahydrate.....	1962
<i>Lysini acetat</i>	5.1-2960	Magnesium trisilicate	1963
<i>Lysini hydrochloridum</i>	1936	Maize oil, refined.....	1964
<i>Lythri herba</i>	1927	Maize starch	5.1-2965
M		Malathion.....	1965
Macrogol 15 hydroxystearate	1941	<i>Malathionum</i>	1965
<i>Macrogol 20 glyceroli monostearas</i>	1942	Maleic acid	1966
Macrogol 20 glycerol monostearate	1942	Malic acid.....	1966
Macrogol 6 glycerol caprylocaprate.....	1941	Mallow flower.....	1967
<i>Macrogol 6 glyceroli caprylocapras</i>	1941	Maltitol	1968
<i>Macrogola</i>	1950	Maltitol, liquid.....	1969
Macrogol cetostearyl ether	1943	<i>Maltitolum</i>	1968
<i>Macrogolglyceridorum caprylocaprates</i>	1173	<i>Maltitolum liquidum</i>	1969
<i>Macrogolglyceridorum laurates</i>	1892	Maltodextrin	1970
<i>Macrogolglyceridorum linoleates</i>	1917	<i>Maltodextrinum</i>	1970
<i>Macrogolglyceridorum oleates</i>	2133	<i>Malvae sylvestris flos</i>	1967
<i>Macrogolglyceridorum stearates</i>	2491	Manganese sulphate monohydrate	1971
Macrogolglycerol cocoates.....	1947	<i>Mangani sulfas monohydricus</i>	1971
Macrogolglycerol hydroxystearate	1948	Mannheimia vaccine (inactivated) for cattle	771
<i>Macrogolglyceroli cocoates</i>	1947	Mannheimia vaccine (inactivated) for sheep.....	772
<i>Macrogolglyceroli hydroxystearas</i>	1948	Mannitol	1971
<i>Macrogolglyceroli ricinoleas</i>	1949	<i>Mannitolum</i>	1971
Macrogolglycerol ricinoleate	1949	Maprotiline hydrochloride	1973
<i>Macrogoli 15 hydroxystearas</i>	1941	<i>Maprotilini hydrochloridum</i>	1973
<i>Macrogoli aether cetostearylicus</i>	1943	Marek's disease vaccine (live).....	774
<i>Macrogoli aether laurilicum</i>	1944	<i>Marrubii herba</i>	5.1-3039
<i>Macrogoli aether oleicum</i>	1945	Marshmallow leaf	1974
<i>Macrogoli aether stearylicus</i>	1946	Marshmallow root.....	1975
<i>Macrogoli oleas</i>	1944	Mass spectrometry (2.2.43.).....	65
<i>Macrogoli stearas</i>	1946	Mass uniformity of delivered doses from multidose containers (2.9.27.)	263
Macrogol lauryl ether	1944	Mass uniformity of single-dose preparations (2.9.5.)	233
Macrogol oleate	1944	Mastic.....	1975
Macrogol oleyl ether	1945	<i>Masticabilia gummis medicata</i>	601
Macrogols.....	1950	<i>Mastix</i>	1975
Macrogol stearate.....	1946	Materials based on non-plasticised poly(vinyl chloride) for containers for dry dosage forms for oral administration (3.1.11.).....	291
Macrogol stearyl ether.....	1946	Materials based on non-plasticised poly(vinyl chloride) for containers for non-injectable, aqueous solutions (3.1.10.)	289
Magaldrate.....	1951	Materials based on plasticised poly(vinyl chloride) for containers for aqueous solutions for intravenous infusion (3.1.14.)	296
<i>Magaldratum</i>	1951	Materials based on plasticised poly(vinyl chloride) for containers for human blood and blood components (3.1.1.1.)	269
<i>Magnesii acetat tetrahydricus</i>	1952	Materials based on plasticised poly(vinyl chloride) for tubing used in sets for the transfusion of blood and blood components (3.1.1.2.).....	272
<i>Magnesii aspartas dihydricus</i>	1953	Materials for containers for human blood and blood components (3.1.1.)	269
<i>Magnesii chloridum 4.5-hydricum</i>	1955	Materials used for the manufacture of containers (3.1.)	269
<i>Magnesii chloridum hexahydricum</i>	1956	<i>Matricariae aetheroleum</i>	5.1-2967
<i>Magnesii glycerophosphas</i>	1957		
<i>Magnesii hydroxidum</i>	1957		
<i>Magnesii oxidum leve</i>	1958		
<i>Magnesii oxidum ponderosum</i>	1958		
<i>Magnesii peroxidum</i>	1959		
<i>Magnesii pidolas</i>	1960		
<i>Magnesii stearas</i>	1961		
<i>Magnesii subcarbonas levis</i>	1954		
<i>Magnesii subcarbonas ponderosus</i>	1954		
<i>Magnesii sulfas heptahydricus</i>	1962		
<i>Magnesii trisilicas</i>	1963		

<i>Matricariae extractum fluidum</i>	5.1-2966	<i>Mesterololum</i>	1999
<i>Matricariae flos</i>	5.1-2965	Mestranol	2000
Matricaria flower	5.1-2965	<i>Mestranolum</i>	2000
Matricaria liquid extract	5.1-2966	Metacresol	2001
Matricaria oil	5.1-2967	<i>Metacresolum</i>	2001
<i>Maydis amyllum</i>	5.1-2965	Metamizole sodium	2002
<i>Maydis oleum raffinatum</i>	1964	<i>Metamizolum natricum</i>	2002
Meadowsweet	1980	Metformin hydrochloride	2003
Measles immunoglobulin, human	1742	<i>Metformini hydrochloridum</i>	2003
Measles, mumps and rubella vaccine (live)	678	Methacrylic acid - ethyl acrylate copolymer (1:1)	2005
Measles vaccine (live)	679	Methacrylic acid - ethyl acrylate copolymer (1:1) dispersion 30 per cent	2005
Measurement of consistency by penetrometry (2.9.9.)	235	Methacrylic acid - methyl methacrylate copolymer (1:1)	2006
Mebendazole	1981	Methacrylic acid - methyl methacrylate copolymer (1:2)	2007
<i>Mebendazolum</i>	1981	Methadone hydrochloride	2007
Meclozine hydrochloride	1982	<i>Methadoni hydrochloridum</i>	2007
<i>Meclozini hydrochloridum</i>	1982	Methanol and 2-propanol, test for (2.9.11.)	239
Medicated chewing gum, drug release from (2.9.25.)	260	Methaqualone	2008
Medicated chewing gums	601	<i>Methaqualonum</i>	2008
Medicated feeding stuffs for veterinary use, premixes for	617	Methenamine	2009
Medicated foams	604	<i>Methenaminum</i>	2009
Medicated plasters	626	Methionine	2010
Medicated tampons	628	Methionine ([¹¹ C]methyl) injection, L-	834
Medicated vaginal tampons	630	Methionine, DL-	2010
Medicinal air	929	<i>Methioninum</i>	2010
Medicinal air, synthetic	932	Methods in pharmacognosy (2.8.)	215
Medium-chain triglycerides	2623	Methods of preparation of sterile products (5.1.1.)	445
Medroxyprogesterone acetate	1983	Methotrexate	2011
<i>Medroxyprogesteroni acetat</i>	1983	<i>Methotrexatum</i>	2011
Mefenamic acid	1984	Methylatropine bromide	2014
Mefloquine hydrochloride	1986	Methylatropine nitrate	2015
<i>Mefloquini hydrochloridum</i>	1986	<i>Methylatropini bromidum</i>	2014
Megestrol acetate	1987	<i>Methylatropini nitras</i>	2015
<i>Megestrol acetat</i>	1987	Methylcellulose	2016
Meglumine	1988	<i>Methylcellulosum</i>	2016
<i>Megluminum</i>	1988	Methyldopa	2016
<i>Mel</i>	5.1-2946	<i>Methyldopum</i>	2016
<i>Melaleuca aetheroleum</i>	2534	Methylene blue	2028
Melilot	5.1-2970	Methylene chloride	2017
<i>Meliloti herba</i>	5.1-2970	<i>Methyleni chloridum</i>	2017
<i>Melissae folium</i>	1989	Methylhydroxyethylcellulose	2018
Melissa leaf	1989	<i>Methylhydroxyethylcellulosum</i>	2018
Melting point - capillary method (2.2.14.)	32	<i>Methylis nicotinas</i>	5.1-2972
Melting point - instantaneous method (2.2.16.)	33	<i>Methylis parahydroxybenzoas</i>	2013
Melting point - open capillary method (2.2.15.)	33	<i>Methylis parahydroxybenzoas natricum</i>	2441
Menadione	1990	<i>Methylis salicylas</i>	2014
<i>Menadionum</i>	1990	Methyl nicotinate	5.1-2972
Meningococcal group C conjugate vaccine	680	Methyl parahydroxybenzoate	2013
Meningococcal polysaccharide vaccine	682	Methylpentoses in polysaccharide vaccines (2.5.21.)	133
<i>Menthae arvensis aetheroleum partim mentholi privum</i>	2050	Methylphenobarbital	2019
<i>Menthae piperitae aetheroleum</i>	2206	<i>Methylphenobarbitalum</i>	2019
<i>Menthae piperitae folium</i>	2205	Methylprednisolone	2020
Menthol, racemic	1991	Methylprednisolone acetate	2022
<i>Mentholum racemicum</i>	1991	Methylprednisolone hydrogen succinate	2024
<i>Menyanthis trifoliatae folium</i>	1115	<i>Methylprednisoloni acetat</i>	2022
Mepivacaine hydrochloride	1992	<i>Methylprednisoloni hydrogenosuccinas</i>	2024
<i>Mepivacaini hydrochloridum</i>	1992	<i>Methylprednisolonum</i>	2020
Meprobamate	1993	Methylpyrrolidone, N-	2026
<i>Meprobamatum</i>	1993	<i>Methylrosanilinii chloridum</i>	5.1-2973
Mepyramine maleate	1994	Methylrosanilinium chloride	5.1-2973
<i>Mepyramini maleas</i>	1994	Methyl salicylate	2014
Mercaptopurine	1995	Methyltestosterone	2027
<i>Mercaptopurinum</i>	1995	<i>Methyltestosteronum</i>	2027
Mercuric chloride	1995	<i>Methylthioninii chloridum</i>	2028
Mesalazine	1996	Methylthioninium chloride	2028
<i>Mesalazinum</i>	1996	Metixene hydrochloride	2029
Mesna	5.1-2971	<i>Metixeni hydrochloridum</i>	2029
<i>Mesnum</i>	5.1-2971	Metoclopramide	2030
Mesterolone	1999	Metoclopramide hydrochloride	2031

<i>Metoclopramidi hydrochloridum</i>	2031	Multidose containers, uniformity of mass of delivered doses (2.9.27.)	263
<i>Metoclopramidum</i>	2030	Mumps vaccine (live)	684
<i>Metoprololi succinas</i>	2032	Mupirocin.....	2065
<i>Metoprololi tartras</i>	2034	Mupirocin calcium	2067
Metoprolol succinate.....	2032	<i>Mupirocinum</i>	2065
Metoprolol tartrate	2034	<i>Mupirocinum calcicum</i>	2067
Metrifonate	2035	<i>Musci medicati</i>	604
<i>Metrifonatium</i>	2035	Mycobacteria (2.6.2.)	149
Metronidazole	2037	Mycoplasmas (2.6.7.).....	149
Metronidazole benzoate	2038	<i>Myristicae fragrantis aetheroleum</i>	2123
<i>Metronidazoli benzoas</i>	2038	Myrrh	2069
<i>Metronidazolum</i>	2037	<i>Myrrha</i>	2069
Mexiletine hydrochloride.....	2039	<i>Myrrhae tinctura</i>	2069
<i>Mexiletini hydrochloridum</i>	2039	Myrrh tincture	2069
Mianserin hydrochloride	2041	<i>Myrtilli fructus recens</i>	1099
<i>Mianserini hydrochloridum</i>	2041	<i>Myrtilli fructus siccus</i>	1099
Miconazole	2042	Myxomatosis vaccine (live) for rabbits	775
Miconazole nitrate	2043		
<i>Miconazoli nitras</i>	2043	N	
<i>Miconazolum</i>	2042	Nabumetone	2073
Microbiological assay of antibiotics (2.7.2.).....	188	<i>Nabumetonum</i>	2073
Microbiological examination of non-sterile products (test for specified micro-organisms) (2.6.13.).....	156	<i>N</i> -Acetyltryptophan.....	918
Microbiological examination of non-sterile products (total viable aerobic count) (2.6.12.).....	154	<i>N</i> -Acetyltryptophanum.....	918
Microbiological quality of pharmaceutical preparations (5.1.4.).....	449	<i>N</i> -Acetyltyrosine	920
Microcrystalline cellulose.....	1228	<i>N</i> -Acetyltyrosinum	920
Micro determination of water (2.5.32.).....	137	Nadolol	2074
Microscopy, limit test of particle size (2.9.13.).....	239	<i>Nadololum</i>	2074
Midazolam	2045	Nadroparin calcium	2075
<i>Midazolamum</i>	2045	<i>Nadroparinum calcicum</i>	2075
Milk-thistle fruit.....	2046	Naftidrofuryl hydrogen oxalate.....	2078
<i>Millefolii herba</i>	2723	<i>Naftidrofuryli hydrogenooxalas</i>	2078
Minimising the risk of transmitting animal spongiform encephalopathy agents via human and veterinary medicinal products (5.2.8.).....	463	Nalidixic acid.....	2080
Minocycline hydrochloride.....	2047	Naloxone hydrochloride dihydrate.....	2080
<i>Minocyclini hydrochloridum</i>	2047	<i>Naloxoni hydrochloridum dihydricum</i>	2080
Minoxidil	5.1-2974	Naltrexone hydrochloride.....	5.1-2979
<i>Minoxidilum</i>	5.1-2974	<i>Naltrexoni hydrochloridum</i>	5.1-2979
Mint oil, partly dementholised	2050	Naphazoline hydrochloride.....	2082
Mitomycin	2051	Naphazoline nitrate	2083
<i>Mitomycinum</i>	2051	<i>Naphazolini hydrochloridum</i>	2082
Mitoxantrone hydrochloride.....	2053	<i>Naphazolini nitras</i>	2083
<i>Mitoxantroni hydrochloridum</i>	2053	Naproxen	2084
Modified-release capsules.....	600	<i>Naproxenum</i>	2084
Modified-release granules	606	Nasal drops and liquid nasal sprays.....	610
Modified-release tablets	628	<i>Nasalia</i>	610
Molecular mass distribution in dextrans (2.2.39.).....	57	Nasal powders.....	611
Molgramostim concentrated solution	2054	Nasal preparations	610
<i>Molgramostimi solutio concentrata</i>	2054	Nasal preparations, semi-solid.....	611
Mometasone furoate.....	2057	Nasal sticks.....	611
<i>Mometasoni furoas</i>	2057	Nasal washes	611
Monographs (1.4.).....	7	<i>Natrii acetat trihydricus</i>	2415
Morantel hydrogen tartrate for veterinary use	2059	<i>Natrii acetatis ([1-¹¹C]) solutio iniectionabilis</i>	839
<i>Moranteli hydrogenotartras ad usum veterinarium</i>	2059	<i>Natrii alendronas</i>	2416
Morphine hydrochloride.....	2060	<i>Natrii alginas</i>	2417
Morphine sulphate.....	2061	<i>Natrii amidotrizoas</i>	2418
<i>Morphini hydrochloridum</i>	2060	<i>Natrii aminosalicylas dihydricus</i>	2419
<i>Morphini sulfas</i>	2061	<i>Natrii ascorbas</i>	2420
Moss, Iceland	1787	<i>Natrii benzoas</i>	2421
Mother tinctures for homoeopathic preparations	894	<i>Natrii bromidum</i>	2422
Motherwort	2063	<i>Natrii calcii edetas</i>	2422
Mouthwashes	612	<i>Natrii caprylas</i>	2423
Moxonidine	2064	<i>Natrii carbonas anhydricus</i>	5.1-3008
<i>Moxonidinum</i>	2064	<i>Natrii carbonas decahydricus</i>	5.1-3008
Mucoadhesive preparations.....	613	<i>Natrii carbonas monohydricus</i>	5.1-3009
Mullein flower.....	2065	<i>Natrii cetylo- et stearylosulfas</i>	2426
		<i>Natrii chloridum</i>	2428
		<i>Natrii chromatis (⁵¹Cr) solutio sterilis</i>	840
		<i>Natrii citras</i>	2429
		<i>Natrii cromoglicas</i>	2429

<i>Natrii cyclamas</i>	2430	<i>Niclosamidum monohydricum</i>	2093
<i>Natrii dihydrogenophosphas dihydricus</i>	2431	Nicotinamide.....	2094
<i>Natrii docusas</i>	1471	<i>Nicotinamidum</i>	2094
<i>Natrii fluoridi (¹⁸F) solutio iniectionis</i>	841	Nicotine.....	2095
<i>Natrii fluoridum</i>	2432	Nicotine resinate.....	2096
<i>Natrii fusidas</i>	2433	Nicotinic acid.....	2097
<i>Natrii glycerophosphas hydricus</i>	2434	<i>Nicotini resinas</i>	2096
<i>Natrii hyaluronas</i>	2434	<i>Nicotinum</i>	2095
<i>Natrii hydrogenocarbonas</i>	2437	Nifedipine.....	2098
<i>Natrii hydroxidum</i>	2437	<i>Nifedipinum</i>	2098
<i>Natrii iodidi (¹²³I) solutio iniectionis</i>	842	Nifuroxazide.....	2099
<i>Natrii iodidi (¹³¹I) capsulae ad usum diagnosticum</i>	843	<i>Nifuroxazidum</i>	2099
<i>Natrii iodidi (¹³¹I) solutio</i>	844	Nikethamide.....	2100
<i>Natrii iodidi (¹³¹I) solutio ad radio-signandum</i>	845	Nimesulide.....	2101
<i>Natrii iodidum</i>	2438	<i>Nimesulidum</i>	2101
<i>Natrii iodohippurati (¹²³I) solutio iniectionis</i>	845	Nimodipine.....	2102
<i>Natrii iodohippurati (¹³¹I) solutio iniectionis</i>	846	<i>Nimodipinum</i>	2102
<i>Natrii lactatis solutio</i>	2438	Nitrazepam.....	2103
<i>Natrii laurilsulfas</i>	2440	<i>Nitrazepamum</i>	2103
<i>Natrii metabisulfis</i>	2441	Nitrendipine.....	2104
<i>Natrii molybda dihydricus</i>	2442	<i>Nitrendipinum</i>	2104
<i>Natrii nitris</i>	2443	Nitric acid.....	2105
<i>Natrii nitroprussias</i>	2443	Nitric oxide.....	2105
<i>Natrii perboras hydricus</i>	2444	Nitrofur.....	2106
<i>Natrii pertechnetatis (^{99m}Tc) fissionis formati solutio iniectionis</i>	847	<i>Nitrofurum</i>	2106
<i>Natrii pertechnetatis (^{99m}Tc) sine fissionis formati solutio iniectionis</i>	848	Nitrofurantoin.....	2107
<i>Natrii phosphatis (³²P) solutio iniectionis</i>	849	<i>Nitrofurantoinum</i>	2107
<i>Natrii picosulfas</i>	2445	Nitrogen.....	2108
<i>Natrii polystyrenesulfonas</i>	2446	Nitrogen determination by sulphuric acid digestion (2.5.9.).....	129
<i>Natrii propionas</i>	2447	Nitrogen determination, primary aromatic amino (2.5.8.).....	129
<i>Natrii salicylas</i>	2449	<i>Nitrogenii oxidum</i>	2105
<i>Natrii selenis pentahydricus</i>	2449	<i>Nitrogenium</i>	2108
<i>Natrii (S)-lactatis solutio</i>	2439	<i>Nitrogenium oxygenio depletum</i>	2109
<i>Natrii stearas</i>	2452	Nitrogen, low-oxygen.....	2109
<i>Natrii stearyl fumaras</i>	2453	Nitrogen monoxide and nitrogen dioxide in gases (2.5.26.).....	135
<i>Natrii sulfas anhydricus</i>	2454	Nitrous oxide.....	5.1-2981
<i>Natrii sulfas decahydricus</i>	2455	Nitrous oxide in gases (2.5.35.).....	141
<i>Natrii sulfis anhydricus</i>	2455	Nizatidine.....	2111
<i>Natrii sulfis heptahydricus</i>	2456	<i>Nizatidinum</i>	2111
<i>Natrii thiosulfas</i>	5.1-3009	<i>N-Methylpyrrolidone</i>	2026
<i>Natrii valproas</i>	2457	<i>N-Methylpyrrolidonum</i>	2026
Near-infrared spectrophotometry (2.2.40.).....	59	<i>N,N</i> -Dimethylaniline (2.4.26.).....	119
Neohesperidin-dihydrochalcone.....	2085	Nomegestrol acetate.....	2113
<i>Neohesperidin-dihydrochalconum</i>	2085	<i>Nomegestroli acetas</i>	2113
<i>Neomycini sulfas</i>	2086	Nonoxinol 9.....	2114
Neomycin sulphate.....	2086	<i>Nonoxinolum 9</i>	2114
Neonatal piglet colibacillosis vaccine (inactivated).....	776	Non-sterile products, microbiological examination of (test for specified micro-organisms) (2.6.13.).....	156
Neonatal ruminant colibacillosis vaccine (inactivated).....	778	Non-sterile products, microbiological examination of (total viable aerobic count) (2.6.12.).....	154
Neostigmine bromide.....	2088	Noradrenaline hydrochloride.....	2114
Neostigmine metilsulfate.....	2089	Noradrenaline tartrate.....	2115
<i>Neostigmini bromidum</i>	2088	<i>Noradrenalini hydrochloridum</i>	2114
<i>Neostigmini metilsulfas</i>	2089	<i>Noradrenalini tartaras</i>	2115
<i>Netilmicini sulfas</i>	2089	<i>Norcholesteroli iodinati (¹³¹I) solutio iniectionis</i>	836
Netilmicin sulphate.....	2089	Norcholesterol injection, iodinated (¹³¹ I).....	836
Nettle leaf.....	5.1-2980	Norethisterone.....	2116
Neurovirulence test for poliomyelitis vaccine (oral) (2.6.19.).....	172	Norethisterone acetate.....	2117
Neurovirulence test of live viral vaccines (2.6.18.).....	172	<i>Norethisteroni acetas</i>	2117
Newcastle disease vaccine (inactivated).....	779	<i>Norethisteronum</i>	2116
Newcastle disease vaccine (live).....	781	Norfloxacin.....	2118
Nicergoline.....	2091	<i>Norfloxacinum</i>	2118
<i>Nicergolinum</i>	2091	Norgestrel.....	2119
<i>Nicethamidum</i>	2100	<i>Norgestrelum</i>	2119
Nickel in polyols (2.4.15.).....	108	Normal immunoglobulin for intravenous administration, human.....	1744
Niclosamide, anhydrous.....	2092		
Niclosamide monohydrate.....	2093		
<i>Niclosamidum anhydricum</i>	2092		

Normal immunoglobulin, human	1742	Oral solutions, emulsions and suspensions	609
Nortriptyline hydrochloride.....	2120	Oral use, liquid preparations for.....	608
<i>Nortriptylini hydrochloridum</i>	2120	Orciprenaline sulphate.....	2154
Noscapine	2121	<i>Orciprenalini sulfas</i>	2154
Noscapine hydrochloride.....	2122	Oregano.....	2155
<i>Noscapini hydrochloridum</i>	2122	Organ preservation, solutions for.....	2458
<i>Noscapinum</i>	2121	<i>Origani herba</i>	2155
Nuclear magnetic resonance spectrometry (2.2.33.).....	51	Orodispensible tablets.....	628
Nucleic acid amplification techniques (2.6.21.).....	174	Oromucosal capsules	613
Nucleic acids in polysaccharide vaccines (2.5.17.).....	132	Oromucosal drops, oromucosal sprays and sublingual sprays.....	612
Nutmeg oil	2123	Oromucosal preparations.....	611
Nystatin	2124	Oromucosal preparations, semi-solid.....	612
<i>Nystatinum</i>	2124	Oromucosal solutions and oromucosal suspensions	612
O		Orphenadrine citrate.....	2156
<i>O</i> -Acetyl in polysaccharide vaccines (2.5.19.)	132	Orphenadrine hydrochloride.....	2157
Oak bark	2129	<i>Orphenadrini citras</i>	2156
Octoxinol 10	2129	<i>Orphenadrini hydrochloridum</i>	2157
<i>Octoxinolum 10</i>	2129	<i>Orthosiphonis folium</i>	1859
Octyldodecanol.....	2130	<i>Oryzae amyllum</i>	2369
<i>Octyldodecanolum</i>	2130	Osmolality (2.2.35.).....	54
Octyl gallate	2129	Other provisions applying to general chapters and monographs (1.2.).....	5
<i>Octylis gallas</i>	2129	Ouabain.....	2158
Odour (2.3.4.).....	99	<i>Ouabainum</i>	2158
Odour and taste of essential oils (2.8.8.)	216	Oxaliplatin	2159
<i>Oenotherae oleum raffinatum</i>	5.1-2921	<i>Oxaliplatinum</i>	2159
Ofloxacin.....	2131	Oxazepam	2162
<i>Ofloxacinum</i>	2131	<i>Oxazepamum</i>	2162
Oils rich in omega-3- acids, composition of fatty acids (2.4.29.)	121	Oxeladin hydrogen citrate.....	2163
Ointments	625	<i>Oxeladini hydrogenocitras</i>	2163
<i>Oleae folium</i>	2134	Oxfendazole for veterinary use	2164
<i>Olea herbaria</i>	595	<i>Oxfendazolum ad usum veterinarium</i>	2164
Oleic acid	2132	Oxidising substances (2.5.30.).....	137
Oleoyl macroglycerides.....	2133	Oxolinic acid.....	2165
Oleyl alcohol	2134	Oxprenolol hydrochloride	2166
<i>Olivae oleum raffinatum</i>	2135	<i>Oxprenololi hydrochloridum</i>	2166
<i>Olivae oleum virginale</i>	2136	Oxybuprocaine hydrochloride.....	2167
Olive leaf.....	2134	<i>Oxybuprocaini hydrochloridum</i>	2167
Olive oil, refined	2135	Oxybutynin hydrochloride	2168
Olive oil, virgin	2136	<i>Oxybutynini hydrochloridum</i>	2168
Olsalazine sodium.....	2137	Oxycodone hydrochloride	5.1-2987
<i>Olsalazinum natricum</i>	2137	<i>Oxycodoni hydrochloridum</i>	5.1-2987
Omega-3-acid ethyl esters 60.....	2140	Oxygen.....	2169
Omega-3-acid ethyl esters 90.....	2142	Oxygen (¹⁵ O).....	837
<i>Omega-3 acidorum esteri ethylici 60</i>	2140	Oxygen-flask method (2.5.10.).....	130
<i>Omega-3 acidorum esteri ethylici 90</i>	2142	Oxygen in gases (2.5.27.).....	136
<i>Omega-3 acidorum triglycerida</i>	2144	<i>Oxygenium</i>	2169
Omega-3-acids, composition of fatty acids in oils rich in (2.4.29.)	121	<i>Oxygenium (¹⁵O)</i>	837
Omega-3-acids, fish oil rich in	1595	Oxymetazoline hydrochloride	2170
Omega-3-acid triglycerides	2144	<i>Oxymetazolini hydrochloridum</i>	2170
Omeprazole	2146	Oxytetracycline dihydrate	2171
Omeprazole sodium.....	2148	Oxytetracycline hydrochloride	2172
<i>Omeprazolum</i>	2146	<i>Oxytetracyclini hydrochloridum</i>	2172
<i>Omeprazolum natricum</i>	2148	<i>Oxytetracyclinum dihydricum</i>	2171
Ondansetron hydrochloride dihydrate	2149	Oxytocin	2174
<i>Ondansetroni hydrochloridum dihydricum</i>	2149	Oxytocin bulk solution	2175
<i>Ononidis radix</i>	2361	<i>Oxytocini solutio</i>	2175
Opalescence of liquids, clarity and degree (2.2.1.)	23	<i>Oxytocinum</i>	2174
<i>Ophthalmica</i>	602	P	
Ophthalmic inserts	604	Palmitic acid.....	2179
<i>Opii pulvis normatus</i>	2151	Pancreas powder	2179
<i>Opium crudum</i>	2152	<i>Pancreatis pulvis</i>	2179
Opium, prepared.....	2151	<i>Pancuronii bromidum</i>	2182
Opium, raw	2152	Pancuronium bromide	2182
Optical rotation (2.2.7.).....	28	Pansy, wild (flowering aerial parts)	2700
Oral drops	609	Papaverine hydrochloride	2183
Oral powders.....	617	<i>Papaverini hydrochloridum</i>	2183

<i>Papaveris rhoeados flos</i>	2359	Peptide mapping (2.2.55.)	82
Paper chromatography (2.2.26.)	40	Peptides, synthetic, acetic acid in (2.5.34.)	141
Paracetamol	2184	Pergolide mesilate	2209
<i>Paracetamolum</i>	2184	<i>Pergolidi mesilas</i>	2209
Paraffin, hard	2186	Perindopril <i>tert</i> -butylamine	2210
Paraffin, light liquid	2186	Peritoneal dialysis, solutions for	2212
Paraffin, liquid	2187	Peroxide value (2.5.5.)	128
<i>Paraffinum liquidum</i>	2187	Perphenazine	2214
<i>Paraffinum perliquidum</i>	2186	<i>Perphenazinum</i>	2214
<i>Paraffinum solidum</i>	2186	Pertussis (acellular, component), diphtheria, tetanus, poliomyelitis (inactivated) and haemophilus type b conjugate vaccine (adsorbed)	653
Paraffin, white soft	2187	Pertussis, diphtheria, tetanus and poliomyelitis (inactivated) vaccine (adsorbed)	656
Paraffin, yellow soft	2188	Pertussis, diphtheria, tetanus, poliomyelitis (inactivated) and haemophilus type b conjugate vaccine (adsorbed)	657
Parainfluenza virus vaccine (live), bovine, freeze-dried	732	Pertussis vaccine	685
Parainfluenza virus vaccine (live), canine	742	Pertussis vaccine (acellular), assay of (2.7.16.)	208
Paraldehyde	2189	Pertussis vaccine (acellular, component, adsorbed)	686
<i>Paraldehydum</i>	2189	Pertussis vaccine (acellular, co-purified, adsorbed)	688
<i>Parenteralia</i>	5.1-2841	Pertussis vaccine (adsorbed)	690
Parenteral preparations	5.1-2841	Pertussis vaccine, assay of (2.7.7.)	197
Parenteral preparations, test for extractable volume of (2.9.17.)	243	Peru balsam	2215
Parnaparin sodium	2189	Pessaries	629
<i>Parnaparinum natricum</i>	2189	Pessaries and suppositories, disintegration of (2.9.2.)	227
Paroxetine hydrochloride hemihydrate	2190	Pessaries and suppositories, resistance to rupture (2.9.24.)	258
<i>Paroxetini hydrochloridum hemihydricum</i>	2190	Pesticide residues (2.8.13.)	218
Particles, fine, aerodynamic assessment of in preparations for inhalation (2.9.18.)	5.1-2799	Pethidine hydrochloride	2216
Particle size by microscopy, limit test of (2.9.13.)	239	<i>Pethidini hydrochloridum</i>	2216
Particulate contamination: sub-visible particles (2.9.19.)	253	Pharmaceutical technical procedures (2.9.)	225
Particulate contamination: visible particles (2.9.20.)	255	Pharmacognosy, methods in (2.8.)	215
Parvovirus vaccine (inactivated), canine	743	Pharmacopoeial harmonisation (5.8.)	551
Parvovirus vaccine (inactivated), porcine	787	Phenazone	2217
Parvovirus vaccine (live), canine	744	<i>Phenazonum</i>	2217
<i>Passiflorae herba</i>	2192	Pheniramine maleate	2218
Passion flower	2192	<i>Pheniramini maleas</i>	2218
Pastes	625	Phenobarbital	2219
Pasteurella vaccine (inactivated) for sheep	783	Phenobarbital sodium	2220
Pastilles and lozenges	613	<i>Phenobarbitalum</i>	2219
Patches, transdermal	616	<i>Phenobarbitalum natricum</i>	2220
Patches, transdermal, dissolution test for (2.9.4.)	231	Phenol	2221
<i>Pefloxacini mesilas dihydricus</i>	2193	Phenol in immunosera and vaccines (2.5.15.)	131
Pefloxacin mesilate dihydrate	2193	Phenolphthalein	2222
<i>Penbutololi sulfas</i>	2195	<i>Phenolphthaleinum</i>	2222
Penbutolol sulphate	2195	Phenolsulfonphthalein	2222
Penetrometry, measurement of consistency (2.9.9.)	235	<i>Phenolsulfonphthaleinum</i>	2222
Penicillamine	2196	<i>Phenolum</i>	2221
<i>Penicillaminum</i>	2196	Phenothiazines, identification by thin-layer chromatography (2.3.3.)	99
<i>Pentaerythryli tetranitras dilutus</i>	2198	Phenoxyethanol	2223
Pentaerythryl tetranitrate, diluted	2198	<i>Phenoxyethanolum</i>	2223
Pentamidine diisetonate	2199	Phenoxymethylpenicillin	5.1-2992
<i>Pentamidini diisetonas</i>	2199	Phenoxymethylpenicillin potassium	5.1-2994
Pentazocine	5.1-2991	<i>Phenoxymethylpenicillinum</i>	5.1-2992
Pentazocine hydrochloride	5.1-2991	<i>Phenoxymethylpenicillinum kalicum</i>	5.1-2994
Pentazocine lactate	5.1-2992	Phentolamine mesilate	2227
<i>Pentazocini hydrochloridum</i>	5.1-2991	<i>Phentolamini mesilas</i>	2227
<i>Pentazocini lactas</i>	5.1-2992	Phenylalanine	2228
<i>Pentazocinum</i>	5.1-2991	<i>Phenylalaninum</i>	2228
Pentobarbital	2201	Phenylbutazone	2229
Pentobarbital sodium	2202	<i>Phenylbutazonum</i>	2229
<i>Pentobarbitalum</i>	2201	Phenylephrine	2231
<i>Pentobarbitalum natricum</i>	2202	Phenylephrine hydrochloride	2232
Pentoxifylline	2203	<i>Phenylephrini hydrochloridum</i>	2232
<i>Pentoxifyllinum</i>	2203	<i>Phenylephrinum</i>	2231
Pentoxiverine hydrogen citrate	2204	<i>Phenylhydrargyri acetas</i>	2232
<i>Pentoxiverini hydrogenocitras</i>	2204	<i>Phenylhydrargyri boras</i>	2233
Peppermint leaf	2205	<i>Phenylhydrargyri nitras</i>	2234
Peppermint oil	2206		
<i>Pepsini pulvis</i>	2207		
Pepsin powder	2207		

Phenylmercuric acetate	2232	Plasma for fractionation, human	1746
Phenylmercuric borate	2233	Plasma, human (pooled and treated for virus inactivation)	1747
Phenylmercuric nitrate	2234	<i>Plasma humanum ad separationem</i>	1746
Phenylpropanolamine hydrochloride	2234	<i>Plasma humanum collectum deinde conditum ad viros exstinguendos</i>	1747
<i>Phenylpropanolamini hydrochloridum</i>	2234	Plastic additives (3.1.13.)	293
Phenytoin	2235	Plastic containers and closures for pharmaceutical use (3.2.2.)	308
Phenytoin sodium	2236	Plastic containers for aqueous solutions for parenteral infusion (3.2.2.1.)	309
<i>Phenytoinum</i>	2235	Plastic containers for human blood and blood components, sterile (3.2.3.)	309
<i>Phenytoinum natricum</i>	2236	Plastic syringes, single-use, sterile (3.2.8.)	314
Pholcodine	2237	Pneumococcal polysaccharide conjugate vaccine (adsorbed)	5.1-2851
<i>Pholcodinum</i>	2237	Pneumococcal polysaccharide vaccine	691
Phosphates (2.4.11.)	108	Poliomyelitis (inactivated), diphtheria, tetanus and pertussis (acellular, component) vaccine (adsorbed)	648
Phosphoric acid, concentrated	2237	Poliomyelitis (inactivated), diphtheria, tetanus and pertussis vaccine (adsorbed)	656
Phosphoric acid, dilute	2238	Poliomyelitis (inactivated), diphtheria, tetanus, pertussis (acellular, component) and haemophilus type b conjugate vaccine (adsorbed)	653
Phosphorus in polysaccharide vaccines (2.5.18.)	132	Poliomyelitis (inactivated), diphtheria, tetanus, pertussis and haemophilus type b conjugate vaccine (adsorbed)	657
pH, potentiometric determination of (2.2.3.)	26	Poliomyelitis vaccine (inactivated)	692
Phthalylsulfathiazole	2238	Poliomyelitis vaccine (inactivated), <i>in vivo</i> assay of (2.7.20.)	210
<i>Phthalylsulfathiazolum</i>	2238	Poliomyelitis vaccine (oral)	695
Physical and physicochemical methods (2.2.)	23	Poliomyelitis vaccine (oral), test for neurovirulence (2.6.19.)	172
Physostigmine salicylate	2239	<i>Poloxamera</i>	2264
Physostigmine sulphate	2240	Poloxamers	2264
<i>Physostigmini salicylas</i>	2239	Polyacrylate dispersion 30 per cent	2265
<i>Physostigmini sulfas</i>	2240	<i>Polyacrylatis dispersio 30 per centum</i>	2265
Phytomenadione	2241	<i>Poly(alcohol vinylicus)</i>	2272
<i>Phytomenadionum</i>	2241	Polyamide 6/6 suture, sterile, in distributor for veterinary use	887
Phytosterol	2242	Polyamide 6 suture, sterile, in distributor for veterinary use	886
<i>Phytosterolum</i>	2242	Polyethyleneglycols	1950
Picotamide monohydrate	2243	Polyethylene terephthalate for containers for preparations not for parenteral use (3.1.15.)	298
<i>Picotamidum monohydricum</i>	2243	Poly(ethylene terephthalate) suture, sterile, in distributor for veterinary use	887
Pilocarpine hydrochloride	2244	Poly(ethylene - vinyl acetate) for containers and tubing for total parenteral nutrition preparations (3.1.7.)	285
Pilocarpine nitrate	2246	Polyethylene with additives for containers for parenteral preparations and for ophthalmic preparations (3.1.5.) ...	279
<i>Pilocarpini hydrochloridum</i>	2244	Polyethylene without additives for containers for parenteral preparations and for ophthalmic preparations (3.1.4.) ...	278
<i>Pilocarpini nitras</i>	2246	<i>Polygalae radix</i>	2401
Pimozide	2247	<i>Polygoni avicularis herba</i>	1877
<i>Pimozidum</i>	2247	Polymorphism (5.9.)	555
Pindolol	2248	Polymyxin B sulphate	2266
<i>Pindololum</i>	2248	<i>Polymyxini B sulfas</i>	2266
Pipemidic acid trihydrate	2249	Polyolefines (3.1.3.)	274
Piperacillin	2250	Polyoxyl castor oil	1949
Piperacillin sodium	2252	Polyoxyl hydrogenated castor oil	1948
<i>Piperacillinum</i>	2250	Polypropylene for containers and closures for parenteral preparations and ophthalmic preparations (3.1.6.)	282
<i>Piperacillinum natricum</i>	2252	Polysaccharide vaccines, hexosamines in (2.5.20.)	132
Piperazine adipate	2253	Polysaccharide vaccines, methylpentoses in (2.5.21.)	133
Piperazine citrate	2254	Polysaccharide vaccines, nucleic acids in (2.5.17.)	132
Piperazine hydrate	2255	Polysaccharide vaccines, <i>O</i> -acetyl in (2.5.19.)	132
<i>Piperazini adipas</i>	2253	Polysaccharide vaccines, phosphorus in (2.5.18.)	132
<i>Piperazini citras</i>	2254	Polysaccharide vaccines, protein in (2.5.16.)	131
<i>Piperazinum hydricum</i>	2255	Polysaccharide vaccines, ribose in (2.5.31.)	137
Piracetam	2256		
<i>Piracetamum</i>	2256		
Pirenzepine dihydrochloride monohydrate	2257		
<i>Pirenzepini dihydrochloridum monohydricum</i>	2257		
Piretanide	2258		
<i>Piretanidum</i>	2258		
Piroxicam	2259		
<i>Piroxicamum</i>	2259		
<i>Piscis oleum omega-3 acidis abundans</i>	1595		
Pivampicillin	2261		
<i>Pivampicillinum</i>	2261		
Pivmecillinam hydrochloride	2262		
<i>Pivmecillinami hydrochloridum</i>	2262		
<i>Plantae ad ptisanam</i>	573		
<i>Plantae medicinales</i>	572		
<i>Plantae medicinales ad praeparationes homoeopathicas</i>	893		
<i>Plantae medicinales praeparatore</i>	572		
<i>Plantaginis lanceolatae folium</i>	2368		
<i>Plantaginis ovatae semen</i>	1850		
<i>Plantaginis ovatae seminis tegumentum</i>	1849		

Polysaccharide vaccines, sialic acid in (2.5.23.)	133	Povidone	2289
Polysaccharide vaccines, uronic acids in (2.5.22.)	133	Povidone, iodinated	2291
Polysorbate 20	2267	<i>Povidonum</i>	2289
Polysorbate 40	2268	<i>Povidonum iodinatum</i>	2291
Polysorbate 60	2269	Powdered cellulose	1232
Polysorbate 80	2270	Powders and granules for oral solutions and suspensions	609
<i>Polysorbatum 20</i>	2267	Powders and granules for syrups	610
<i>Polysorbatum 40</i>	2268	Powders and tablets for rectal solutions and suspensions	624
<i>Polysorbatum 60</i>	2269	Powders, ear	602
<i>Polysorbatum 80</i>	2270	Powders, effervescent	617
Poly(vinyl acetate)	2271	Powders for cutaneous application	616
Poly(vinyl alcohol)	2272	Powders for eye-drops and powders for eye lotions	603
Poly(vinyl chloride), non-plasticised, materials based on for containers for dry dosage forms for oral administration (3.1.11.)	291	Powders for inhalation	5.1-2845
Poly(vinyl chloride), non-plasticised, materials based on for containers for non-injectable aqueous solutions (3.1.10.)	289	Powders for injections or infusions	5.1-2842
Poly(vinyl chloride), plasticised, empty sterile containers of for human blood and blood components (3.2.4.)	311	Powders for oral drops	609
Poly(vinyl chloride), plasticised, materials based on for containers for aqueous solutions for intravenous infusion (3.1.14.)	296	Powders, nasal	611
Poly(vinyl chloride), plasticised, materials based on for containers for human blood and blood components (3.1.1.1.)	269	Powders, oral	617
Poly(vinyl chloride), plasticised, materials based on for tubing used in sets for the transfusion of blood and blood components (3.1.1.2.)	272	<i>Praeadmixta ad alimenta medicata</i>	
Poly(vinyl chloride), plasticised, sterile containers of for human blood containing anticoagulant solution (3.2.5.)	312	<i>ad usum veterinarium</i>	617
<i>Poly(vinylis acetat)</i>	2271	<i>Praeparationes ad irrigationem</i>	622
Poppy petals, red	2359	<i>Praeparationes buccales</i>	611
Porcine actinobacillosis vaccine (inactivated)	784	<i>Praeparationes homoeopathicas</i>	893
Porcine influenza vaccine (inactivated)	785	<i>Praeparationes insulini injectabiles</i>	1808
Porcine insulin	1806	<i>Praeparationes intramammariae</i>	
Porcine parvovirus vaccine (inactivated)	787	<i>ad usum veterinarium</i>	606
Porcine progressive atrophic rhinitis vaccine (inactivated)	788	<i>Praeparationes intraruminales</i>	607
Porosity of sintered-glass filters (2.1.2.)	17	<i>Praeparationes liquidae ad usum dermicum</i>	607
Potassium (2.4.12.)	108	<i>Praeparationes liquidae peroraliae</i>	608
Potassium acetate	2273	<i>Praeparationes liquidae veterinariae ad usum dermicum</i>	630
Potassium bromide	2273	<i>Praeparationes molles ad usum dermicum</i>	624
Potassium carbonate	2274	<i>Praeparationes pharmaceuticae in vasis cum pressu</i>	622
Potassium chloride	2275	Pravastatin sodium	2292
Potassium citrate	2275	<i>Pravastatinum natricum</i>	2292
Potassium clavulanate	2276	Prazepam	2293
Potassium clavulanate, diluted	2278	<i>Prazepamum</i>	2293
Potassium dihydrogen phosphate	2280	Praziquantel	2294
Potassium hydrogen aspartate hemihydrate	2280	<i>Praziquantelum</i>	2294
Potassium hydrogen carbonate	2281	Prazosin hydrochloride	2295
Potassium hydrogen tartrate	2282	<i>Prazosini hydrochloridum</i>	2295
Potassium hydroxide	2283	Prednicarbate	2297
Potassium iodide	2283	<i>Prednicarbatum</i>	2297
Potassium metabisulphite	2284	Prednisolone	2298
Potassium nitrate	2284	Prednisolone acetate	2299
Potassium perchlorate	2285	Prednisolone pivalate	2301
Potassium permanganate	2286	Prednisolone sodium phosphate	2302
Potassium sodium tartrate tetrahydrate	2286	<i>Prednisoloni acetat</i>	2299
Potassium sorbate	2287	<i>Prednisoloni natrii phosphas</i>	2302
Potassium sulphate	2288	<i>Prednisoloni pivalat</i>	2301
Potato starch	2288	<i>Prednisolonum</i>	2298
Potentiometric determination of ionic concentration using ion-selective electrodes (2.2.36.)	55	Prednisone	2303
Potentiometric determination of pH (2.2.3.)	26	<i>Prednisonum</i>	2303
Potentiometric titration (2.2.20.)	35	Prekallikrein activator (2.6.15.)	168
Poultices	625	Premixes for medicated feeding stuffs for veterinary use	617
Pour-on preparations	631	Preparations for inhalation	5.1-2843
		Preparations for inhalation: aerodynamic assessment of fine particles (2.9.18.)	5.1-2799
		Preparations for irrigation	622
		Pressurised pharmaceutical preparations	622
		Prilocaine	2305
		Prilocaine hydrochloride	2307
		<i>Prilocaini hydrochloridum</i>	2307
		<i>Prilocainum</i>	2305
		Primaquine diphosphate	2308
		<i>Primaquini diphosphas</i>	2308
		Primary aromatic amino-nitrogen, determination of (2.5.8.)	129
		Primary standards for volumetric solutions (4.2.1.)	435

Primidone	2309	Protein, total (2.5.33.)	138
<i>Primidonum</i>	2309	Prothrombin complex, human	1748
<i>Primulae radix</i>	2310	<i>Prothrombinum multiplex humanum</i>	1748
Primula root	2310	Protirelin	2335
Probenecid	2311	<i>Protirelinum</i>	2335
<i>Probenecidum</i>	2311	Proxyphylline	2336
Procainamide hydrochloride	2312	<i>Proxyphyllinum</i>	2336
<i>Procainamidi hydrochloridum</i>	2312	<i>Pruni africanae cortex</i>	2339
Procaine benzylpenicillin	1080	Pseudoephedrine hydrochloride	2337
Procaine hydrochloride	2312	<i>Pseudoephedrini hydrochloridum</i>	2337
<i>Procaini hydrochloridum</i>	2312	<i>Psyllii semen</i>	2338
Prochlorperazine maleate	2313	Psyllium seed	2338
<i>Prochlorperazini maleas</i>	2313	<i>Pulveres ad usum dermicum</i>	616
<i>Producta ab ADN recombinante</i>	584	<i>Pulveres perorales</i>	617
<i>Producta ab fermentatione</i>	576	Purified water	2697
<i>Producta allergenica</i>	569	Purified water, highly	2695
<i>Producta cum possibili transmissione vectorum</i> <i>enkephalopathiarum spongiformium animalium</i>	577	Pycnometric density of solids (2.9.23.)	257
Products of fermentation	576	Pygeum africanum bark	2339
Products of recombinant DNA technology	584	Pyrantel embonate	2339
Products with risk of transmitting agents of animal spongiform encephalopathies	577	<i>Pyranteli embonas</i>	2339
Progesterone	2314	Pyrazinamide	2340
<i>Progesteronum</i>	2314	<i>Pyrazinamidum</i>	2340
Proguanil hydrochloride	2315	Pyridostigmine bromide	2341
<i>Proguanili hydrochloridum</i>	2315	<i>Pyridostigmini bromidum</i>	2341
Proline	2316	Pyridoxine hydrochloride	2342
<i>Prolinum</i>	2316	<i>Pyridoxini hydrochloridum</i>	2342
Promazine hydrochloride	2317	Pyrimethamine	2343
<i>Promazini hydrochloridum</i>	2317	<i>Pyrimethaminum</i>	2343
Promethazine hydrochloride	2318	Pyrogens (2.6.8.)	152
<i>Promethazini hydrochloridum</i>	2318	Q	
Propacetamol hydrochloride	2319	<i>Quercus cortex</i>	2129
<i>Propacetamoli hydrochloridum</i>	2319	Quinidine sulphate	2347
Propafenone hydrochloride	5.1-2996	Quinine hydrochloride	2348
<i>Propafenoni hydrochloridum</i>	5.1-2996	Quinine sulphate	2350
Propanol	2320	R	
<i>Propanolum</i>	2320	Rabies immunoglobulin, human	1750
Propantheline bromide	2321	Rabies vaccine for human use prepared in cell cultures	699
<i>Propanthelini bromidum</i>	2321	Rabies vaccine (inactivated) for veterinary use	790
Propofol	2322	Rabies vaccine (live, oral) for foxes	792
<i>Propofolum</i>	2322	Racemic camphor	1172
Propranolol hydrochloride	2324	Racemic ephedrine hydrochloride	1509
<i>Propranololi hydrochloridum</i>	2324	Racemic menthol	1991
Propylene glycol	2327	Raclopride ([¹⁴ C]methoxy) injection	838
Propylene glycol dicaprylocaprate	2327	<i>Raclopridi ([¹⁴C]methoxy) solutio iniectionabilis</i>	838
Propylene glycol dilaurate	2328	Radionuclides, table of physical characteristics (5.7.)	539
Propylene glycol monolaurate	2329	<i>Radiopharmaceutica</i>	578
Propylene glycol monopalmitostearate	2330	Radiopharmaceutical preparations	578
Propylene glycol monostearate	2330	Raman spectrometry (2.2.48.)	79
<i>Propylenglycoli dicaprylocapras</i>	2327	Ramipril	2355
<i>Propylenglycoli dilauras</i>	2328	<i>Ramiprilum</i>	2355
<i>Propylenglycoli monolauras</i>	2329	Ranitidine hydrochloride	5.1-3001
<i>Propylenglycoli monopalmitostearas</i>	2330	<i>Ranitidini hydrochloridum</i>	5.1-3001
<i>Propylenglycolum</i>	2327	<i>Rapae oleum raffinatum</i>	2359
Propyl gallate	2325	Rapeseed oil, refined	2359
<i>Propylis gallas</i>	2325	<i>Ratanhia radix</i>	2362
<i>Propylis parahydroxybenzoas</i>	2326	<i>Ratanhia tinctura</i>	2362
<i>Propylis parahydroxybenzoas natricum</i>	2448	Reagents (4.)	321
Propyl parahydroxybenzoate	2326	Reagents (4.1.1.)	321
Propylthiouracil	2331	Reagents (4.1.1.)	5.1-2817
<i>Propylthiouracilum</i>	2331	Reagents, standard solutions, buffer solutions (4.1.)	321
Propyphenazone	2332	Recombinant DNA technology, products of	584
<i>Propyphenazonum</i>	2332	Rectal capsules	623
Protamine hydrochloride	2332	Rectal foams	624
Protamine sulphate	2334	<i>Rectalia</i>	623
<i>Protamini hydrochloridum</i>	2332	Rectal preparations	623
<i>Protamini sulfas</i>	2334	Rectal preparations, semi-solid	624
Protein in polysaccharide vaccines (2.5.16.)	131		

Rectal solutions, emulsions and suspensions.....	624	<i>Rutosidum trihydricum</i>	2381
Rectal tampons.....	624	S	
Red poppy petals.....	2359	<i>Sabalis serrulatae fructus</i>	2398
Refractive index (2.2.6.).....	28	Saccharin.....	2387
Relationship between reaction of solution, approximate pH and colour of certain indicators (2.2.4.).....	27	Saccharin sodium.....	2388
Relative density (2.2.5.).....	27	<i>Saccharinum</i>	2387
Reserpine.....	2360	<i>Saccharinum natricum</i>	2388
<i>Reserpinum</i>	2360	<i>Sacchari spheri</i>	2503
Residual solvents (5.4.).....	507	<i>Saccharum</i>	2499
Residual solvents, identification and control (2.4.24.).....	113	Safflower oil, refined.....	2389
Residue on evaporation of essential oils (2.8.9.).....	216	Saffron for homoeopathic preparations.....	900
Resistance to crushing of tablets (2.9.8.).....	235	Sage leaf (<i>salvia officinalis</i>).....	2389
Resistance to rupture of suppositories and pessaries (2.9.24.).....	258	Sage leaf, three-lobed.....	2390
Resorcinol.....	2360	Sage tincture.....	2391
<i>Resorcinolum</i>	2360	Salbutamol.....	2391
Restharrow root.....	2361	<i>Salbutamoli sulfas</i>	2393
<i>Rhamni purshianae cortex</i>	1194	Salbutamol sulphate.....	2393
Rhatany root.....	2362	<i>Salbutamolum</i>	2391
Rhatany tincture.....	2362	<i>Salicis cortex</i>	2702
<i>Rhei radix</i>	2363	Salicylic acid.....	5.1-3007
<i>Rhenii sulfidi colloidalis et technetii (^{99m}Tc) solutio iniectabilis</i>	851	<i>Salmonis domestici oleum</i>	2396
Rhubarb.....	2363	Salmon oil, farmed.....	2396
Ribavirin.....	5.1-3002	<i>Salviae officinalis folium</i>	2389
<i>Ribavirinum</i>	5.1-3002	<i>Salviae sclareae aetheroleum</i>	1311
Riboflavin.....	2365	<i>Salviae tinctura</i>	2391
<i>Riboflavini natrii phosphas</i>	2366	<i>Salviae trilobae folium</i>	2390
Riboflavin sodium phosphate.....	2366	<i>Sambuci flos</i>	1496
<i>Riboflavinum</i>	2365	Saponification value (2.5.6.).....	129
Ribose in polysaccharide vaccines (2.5.31.).....	137	Saw palmetto fruit.....	2398
Ribwort plantain.....	2368	Scopolamine butylbromide.....	1776
Rice starch.....	2369	Scopolamine hydrobromide.....	1777
<i>Ricini oleum hydrogenatum</i>	1197	<i>Scopolamini butylbromidum</i>	1776
<i>Ricini oleum virginale</i>	1197	<i>Scopolamini hydrobromidum</i>	1777
Rifabutin.....	2369	Selegiline hydrochloride.....	2400
<i>Rifabutinum</i>	2369	<i>Selegilini hydrochloridum</i>	2400
Rifampicin.....	2371	<i>Selenii disulfidum</i>	2401
<i>Rifampicinum</i>	2371	Selenium disulphide.....	2401
Rifamycin sodium.....	2372	Semi-micro determination of water (2.5.12.).....	130
<i>Rifamycinum natricum</i>	2372	Semi-solid and liquid preparations, test for deliverable mass or volume (2.9.28.).....	263
Rilmnidine dihydrogen phosphate.....	2373	Semi-solid ear preparations.....	602
<i>Rilmnidini dihydrogenophosphas</i>	2373	Semi-solid eye preparations.....	604
Risperidone.....	2374	Semi-solid nasal preparations.....	611
<i>Risperidonum</i>	2374	Semi-solid oromucosal preparations.....	612
Roman chamomile flower.....	1245	Semi-solid preparations for cutaneous application.....	624
<i>Rosae pseudo-fructus</i>	1472	Semi-solid rectal preparations.....	624
Roselle.....	2376	Semi-solid vaginal preparations.....	630
Rosemary leaf.....	2377	Senega root.....	2401
Rosemary oil.....	2378	<i>Sennae folii extractum siccum normatum</i>	2403
<i>Rosmarini aetheroleum</i>	2378	<i>Sennae folium</i>	2402
<i>Rosmarini folium</i>	2377	<i>Sennae fructus acutifoliae</i>	2404
Rotating viscometer method (2.2.10.).....	30	<i>Sennae fructus angustifoliae</i>	2405
Roxithromycin.....	2379	Senna leaf.....	2402
<i>Roxithromycinum</i>	2379	Senna leaf dry extract, standardised.....	2403
<i>RRR-α-Tocopherol</i>	2591	Senna pods, Alexandrian.....	2404
<i>RRR-α-Tocopherolum</i>	2591	Senna pods, Tinnevely.....	2405
<i>RRR-α-Tocopheryl acetate</i>	2595	Separation techniques, chromatographic (2.2.46.).....	69
<i>RRR-α-Tocopheryl hydrogen succinate</i>	5.1-3026	Serine.....	2406
<i>RRR-α-Tocopherylis acetas</i>	2595	<i>Serinum</i>	2406
<i>RRR-α-Tocopherylis hydrogenosuccinas</i>	5.1-3026	<i>Serpylli herba</i>	2701
Rubber closures for containers for aqueous parenteral preparations, for powders and for freeze-dried powders (3.2.9.).....	316	Sertaconazole nitrate.....	2407
Rubella immunoglobulin, human.....	1751	<i>Sertaconazoli nitras</i>	2407
Rubella vaccine (live).....	701	Sesame oil, refined.....	2408
<i>Rusci rhizoma</i>	1138	<i>Sesami oleum raffinatum</i>	2408
Rutoside trihydrate.....	2381	Sets for the transfusion of blood and blood components (3.2.6.).....	313
		Shampoos.....	608
		Shellac.....	2409

Sialic acid in polysaccharide vaccines (2.5.23.).....	133	Sodium perborate, hydrated.....	2444
Sieves (2.1.4.).....	18	Sodium pertechnetate (^{99m} Tc) injection (fission)	847
Sieve test (2.9.12.).....	239	Sodium pertechnetate (^{99m} Tc) injection (non-fission)	848
<i>Silica ad usum dentalem</i>	2411	Sodium phosphate (³² P) injection	849
Silica, colloidal anhydrous.....	2410	Sodium picosulfate	2445
Silica, colloidal hydrated.....	2411	Sodium polystyrene sulphonate	2446
<i>Silica colloidalis anhydrica</i>	2410	Sodium propionate.....	2447
<i>Silica colloidalis hydrica</i>	2411	Sodium propyl parahydroxybenzoate.....	2448
Silica, dental type.....	2411	Sodium salicylate	2449
Silicone elastomer for closures and tubing (3.1.9.).....	288	Sodium selenite pentahydrate	2449
Silicone oil used as a lubricant (3.1.8.).....	287	Sodium (S)-lactate solution	2439
Silk suture, sterile, braided, in distributor for veterinary use	887	Sodium starch glycolate (type A)	2450
Silver nitrate	2412	Sodium starch glycolate (type B)	2451
<i>Silybi mariani fructus</i>	2046	Sodium starch glycolate (type C)	2451
Simeticone.....	2412	Sodium stearate	2452
<i>Simeticonum</i>	2412	Sodium stearyl fumarate.....	2453
Simvastatin.....	2413	Sodium sulphate, anhydrous.....	2454
<i>Simvastatinum</i>	2413	Sodium sulphate decahydrate.....	2455
Single-dose preparations, uniformity of content (2.9.6.)... 234		Sodium sulphite, anhydrous.....	2455
Single-dose preparations, uniformity of mass (2.9.5.)..... 233		Sodium sulphite heptahydrate.....	2456
Sintered-glass filters (2.1.2.)	17	Sodium thiosulphate.....	5.1-3009
Size-exclusion chromatography (2.2.30.).....	45	Sodium valproate.....	2457
(S)-Lactic acid.....	1883	Soft capsules.....	600
Sodium acetate ([1- ¹³ C]) injection.....	839	Softening time determination of lipophilic suppositories (2.9.22.)	256
Sodium acetate trihydrate	2415	Soft extracts	572
Sodium alendronate	2416	<i>Soiae oleum hydrogenatum</i>	2475
Sodium alginate	2417	<i>Soiae oleum raffinatum</i>	2476
Sodium amidotrizoate.....	2418	<i>Solani amyllum</i>	2288
Sodium aminosaliclylate dihydrate	2419	<i>Solidaginis herba</i>	1680
Sodium ascorbate	2420	<i>Solidaginis virgaureae herba</i>	1682
Sodium benzoate	2421	Solid dosage forms, dissolution test for (2.9.3.).....	228
Sodium bromide.....	2422	Solids, density of (2.2.42.).....	64
Sodium calcium edetate	2422	Solids, pycnometric density of (2.9.23.).....	257
Sodium caprylate	2423	Solubility in alcohol of essential oils (2.8.10.).....	216
Sodium carbonate, anhydrous	5.1-3008	Soluble tablets	628
Sodium carbonate decahydrate	5.1-3008	<i>Solutiones ad conservationem partium corporis</i>	2458
Sodium carbonate monohydrate	5.1-3009	<i>Solutiones ad haemocolaturam</i>	
Sodium carboxymethylcellulose.....	1189	<i>haemodiocolaturamque</i>	1703
Sodium carboxymethylcellulose, cross-linked	1371	<i>Solutiones ad haemodialysim</i>	1700
Sodium carboxymethylcellulose, low-substituted	1190	<i>Solutiones ad peritonealem dialysim</i>	2212
Sodium cetostearyl sulphate	2426	<i>Solutiones anticoagulantes et sanguinem humanum conservantes</i>	1007
Sodium chloride.....	2428	Solutions for haemodialysis.....	1700
Sodium chromate (⁵¹ Cr) sterile solution	840	Solutions for haemodialysis, concentrated, water for diluting.....	1699
Sodium citrate	2429	Solutions for haemofiltration and for haemodiafiltration.....	1703
Sodium cromoglicate	2429	Solutions for organ preservation.....	2458
Sodium cyclamate.....	2430	Solutions for peritoneal dialysis	2212
Sodium dihydrogen phosphate dihydrate	2431	Solvents, residual (5.4.)	507
Sodium fluoride	2432	Solvents, residual, identification and control (2.4.24.)	113
Sodium fluoride (¹⁸ F) injection	841	Somatostatin	2459
Sodium fusidate	2433	<i>Somatostatinum</i>	2459
Sodium glycerophosphate, hydrated	2434	Somatropin.....	2460
Sodium hyaluronate.....	2434	Somatropin bulk solution	2462
Sodium hydrogen carbonate	2437	Somatropin for injection	2464
Sodium hydroxide.....	2437	<i>Somatropini solutio ad praeparationem</i>	2462
Sodium iodide.....	2438	<i>Somatropinum</i>	2460
Sodium iodide (¹²³ I) injection.....	842	<i>Somatropinum ad iniectabilium</i>	2464
Sodium iodide (¹³¹ I) capsules for diagnostic use.....	843	Sorbic acid.....	2467
Sodium iodide (¹³¹ I) solution	844	<i>Sorbitani lauras</i>	2467
Sodium iodide (¹³¹ I) solution for radiolabelling	845	<i>Sorbitani oleas</i>	2468
Sodium iodohippurate (¹²³ I) injection	845	<i>Sorbitani palmitas</i>	2468
Sodium iodohippurate (¹³¹ I) injection	846	<i>Sorbitani sesquioleas</i>	2468
Sodium lactate solution.....	2438	<i>Sorbitani stearas</i>	2469
Sodium laurilsulfate	2440	<i>Sorbitani trioleas</i>	2469
Sodium metabisulphite.....	2441	Sorbitan laurate	2467
Sodium methyl parahydroxybenzoate.....	2441	Sorbitan oleate	2468
Sodium molybdate dihydrate	2442		
Sodium nitrite.....	2443		
Sodium nitroprusside	2443		

Sorbitan palmitate	2468	Stearoyl macroglycerides	2491
Sorbitan sesquioleate.....	2468	Stearyl alcohol.....	2492
Sorbitan stearate.....	2469	Sterile braided silk suture in distributor for veterinary use	887
Sorbitan trioleate	2469	Sterile catgut.....	873
Sorbitol.....	2470	Sterile catgut in distributor for veterinary use	885
Sorbitol, liquid (crystallising).....	2471	Sterile containers of plasticised poly (vinyl chloride) for human blood containing anticoagulant solution (3.2.5.)	312
Sorbitol, liquid (non-crystallising).....	2472	Sterile linen thread in distributor for veterinary use.....	886
Sorbitol, liquid, partially dehydrated.....	2473	Sterile non-absorbable strands in distributor for veterinary use	888
<i>Sorbitolum</i>	2470	Sterile non-absorbable sutures	874
<i>Sorbitolum liquidum cristallisabile</i>	2471	Sterile plastic containers for human blood and blood components (3.2.3.).....	309
<i>Sorbitolum liquidum non cristallisabile</i>	2472	Sterile polyamide 6/6 suture in distributor for veterinary use	887
<i>Sorbitolum liquidum partim deshydricum</i>	2473	Sterile polyamide 6 suture in distributor for veterinary use	886
Sotalol hydrochloride	2474	Sterile poly(ethylene terephthalate) suture in distributor for veterinary use	887
<i>Sotaloli hydrochloridum</i>	2474	Sterile products, methods of preparation (5.1.1.).....	445
Soya-bean oil, hydrogenated.....	2475	Sterile single-use plastic syringes (3.2.8.).....	314
Soya-bean oil, refined.....	2476	Sterile synthetic absorbable braided sutures	878
Specific surface area by air permeability (2.9.14.).....	239	Sterile synthetic absorbable monofilament sutures.....	880
Specific surface area by gas adsorption (2.9.26.)	5.1-2811	Sterilisation procedures, biological indicators (5.1.2.)	447
Spectinomycin hydrochloride.....	2476	Sterility (2.6.1.).....	145
<i>Spectinomycini hydrochloridum</i>	2476	Sterols in fatty oils (2.4.23.).....	5.1-2787
Spectrometry, atomic absorption (2.2.23.).....	36	Sticks	626
Spectrometry, atomic emission (2.2.22.).....	35	Sticks, nasal.....	611
Spectrometry, mass (2.2.43.).....	65	St. John's wort.....	2485
Spectrometry, nuclear magnetic resonance (2.2.33.)	51	Stomata and stomatal index (2.8.3.).....	215
Spectrometry, Raman (2.2.48.).....	79	<i>Stramonii folium</i>	2492
Spectrometry, X-ray fluorescence (2.2.37.).....	56	<i>Stramonii pulvis normatus</i>	2494
Spectrophotometry, infrared absorption (2.2.24.).....	37	Stramonium leaf.....	2492
Spectrophotometry, near-infrared (2.2.40.).....	59	Stramonium, prepared	2494
Spectrophotometry, ultraviolet and visible absorption (2.2.25.).....	38	Strands, sterile non-absorbable, in distributor for veterinary use	888
SPF chicken flocks for the production and quality control of vaccines (5.2.2.).....	5.1-2825	Streptokinase bulk solution	2495
Spiramycin.....	5.1-3010	<i>Streptokinasi solutio ad praeparationem</i>	2495
<i>Spiramycinum</i>	5.1-3010	<i>Streptomycini sulfas</i>	2496
Spirapril hydrochloride monohydrate.....	2480	Streptomycin sulphate	2496
<i>Spiraprii hydrochloridum monohydricum</i>	2480	<i>Strontii (⁸⁹Sr) chloridi solutio iniectabilis</i>	850
Spirolactone	2482	Strontium (⁸⁹ Sr) chloride injection	850
<i>Spirolactonum</i>	2482	<i>Styli</i>	626
Spot-on preparations.....	631	Sublingual tablets and buccal tablets	613
Sprays	631	Substances for pharmaceutical use	586
Squalane	2483	Substances for pharmaceutical use, control of impurities (5.10.).....	559
<i>Squalanum</i>	2483	Substances of animal origin for the production of veterinary vaccines (5.2.5.).....	460
Standard solutions for limit tests (4.1.2.)	426	Sub-visible particles, particulate contamination (2.9.19.) ..	253
Standard solutions for limit tests (4.1.2.)	5.1-2818	Succinylsulfathiazole	2498
<i>Stanni colloidalis et technetii (^{99m}Tc) solutio iniectabilis</i>	853	<i>Succinylsulfathiazolum</i>	2498
<i>Stanni pyrophosphatis et technetii (^{99m}Tc) solutio iniectabilis</i>	865	Sucrose.....	2499
<i>Stannosi chloridum dihydricum</i>	2486	Sufentanil	2501
Stannous chloride dihydrate	2486	Sufentanil citrate	2502
Stanozolol.....	2486	<i>Sufentanili citras</i>	2502
<i>Stanozololum</i>	2486	<i>Sufentanilum</i>	2501
Star anise	2487	Sugars, lead in (2.4.10.).....	107
Star anise oil	2488	Sugar spheres	2503
Starch glycolate (type A), sodium	2450	Sulfacetamide sodium.....	2504
Starch glycolate (type B), sodium	2451	<i>Sulfacetamidum natricum</i>	2504
Starch glycolate (type C), sodium	2451	Sulfadiazine.....	2505
Starch, maize	5.1-2965	<i>Sulfadiazinum</i>	2505
Starch, potato	2288	Sulfadimidine	2506
Starch, pregelatinised	2490	<i>Sulfadimidinum</i>	2506
Starch, rice	2369	Sulfadoxine.....	2507
Starch, wheat.....	2698	<i>Sulfadoxinum</i>	2507
Statistical analysis of results of biological assays and tests (5.3.).....	475		
Stavudine.....	5.1-3012		
<i>Stavudinum</i>	5.1-3012		
Steam sterilisation of aqueous preparations, application of the <i>F</i> ₀ concept (5.1.5.).....	5.1-2821		
Stearic acid.....	2490		

Sulfafurazole.....	2508	Tablets, dispersible.....	628
<i>Sulfafurazolum</i>	2508	Tablets, effervescent.....	627
Sulfaguanidine.....	2508	Tablets for use in the mouth.....	628
<i>Sulfaguanidinum</i>	2508	Tablets for vaginal solutions and suspensions.....	630
Sulfamerazine.....	2509	Tablets, gastro-resistant.....	628
<i>Sulfamerazinum</i>	2509	Tablets, modified-release.....	628
Sulfamethizole.....	2510	Tablets, orodispersible.....	628
<i>Sulfamethizolum</i>	2510	Tablets, resistance to crushing (2.9.8.).....	235
Sulfamethoxazole.....	2511	Tablets, soluble.....	628
<i>Sulfamethoxazolum</i>	2511	Tablets, sublingual.....	613
Sulfamethoxy pyridazine for veterinary use.....	2512	Tablets, uncoated.....	627
<i>Sulfamethoxy pyridazinum ad usum veterinarium</i>	2512	Tablets, uncoated, friability of (2.9.7.).....	234
Sulfanilamide.....	2513	Tablets, vaginal.....	629
<i>Sulfanilamidum</i>	2513	Talc.....	5.1-3017
Sulfasalazine.....	2514	<i>Talcum</i>	5.1-3017
<i>Sulfasalazinum</i>	2514	Tamoxifen citrate.....	5.1-3018
Sulfathiazole.....	2516	<i>Tamoxifeni citras</i>	5.1-3018
<i>Sulfathiazolum</i>	2516	<i>Tamponae medicatae</i>	628
Sulfinpyrazone.....	2517	Tampons, ear.....	602
<i>Sulfinpyrazonum</i>	2517	Tampons, medicated.....	628
Sulfisomidine.....	2518	Tampons, rectal.....	624
<i>Sulfisomidinum</i>	2518	Tampons, vaginal, medicated.....	630
Sulfur ad usum externum.....	2520	<i>Tanacetii parthenii herba</i>	1592
<i>Sulfuris colloidalis et technetii (^{99m}Tc) solutio iniectionabilis</i>	852	Tannic acid.....	2534
Sulindac.....	2518	Tannins in herbal drugs, determination of (2.8.14.).....	221
<i>Sulindacum</i>	2518	<i>Tanninum</i>	2534
Sulphated ash (2.4.14.).....	108	Tartaric acid.....	2534
Sulphates (2.4.13.).....	108	Teat dips.....	631
Sulphur dioxide (2.5.29.).....	136	Tea tree oil.....	2534
Sulphur for external use.....	2520	Teat sprays.....	631
Sulphuric acid.....	2520	<i>Technetii (^{99m}Tc) et etifenini solutio iniectionabilis</i>	853
Sulpiride.....	2521	<i>Technetii (^{99m}Tc) exametazimi solutio iniectionabilis</i>	854
<i>Sulpiridum</i>	2521	<i>Technetii (^{99m}Tc) gluconatis solutio iniectionabilis</i>	856
<i>Sumatriptani succinas</i>	2522	<i>Technetii (^{99m}Tc) humani albumini solutio iniectionabilis</i>	856
Sumatriptan succinate.....	2522	<i>Technetii (^{99m}Tc) macrosalbi suspensio iniectionabilis</i>	858
Sunflower oil, refined.....	2524	<i>Technetii (^{99m}Tc) medronati solutio iniectionabilis</i>	859
Supercritical fluid chromatography (2.2.45.).....	68	<i>Technetii (^{99m}Tc) mertiatidi solutio iniectionabilis</i>	860
Suppositories.....	623	<i>Technetii (^{99m}Tc) microsphaerarum suspensio iniectionabilis</i>	861
Suppositories and pessaries, disintegration of (2.9.2.).....	227	<i>Technetii (^{99m}Tc) pentetatis solutio iniectionabilis</i>	862
Suppositories and pessaries, resistance to rupture (2.9.24.).....	258	<i>Technetii (^{99m}Tc) sestamibi solutio iniectionabilis</i>	863
Suppositories, lipophilic, softening time determination (2.9.22.).....	256	<i>Technetii (^{99m}Tc) succimeri solutio iniectionabilis</i>	865
Sutures, sterile non-absorbable.....	874	Technetium (^{99m} Tc) colloidal rhenium sulphide injection.....	851
Sutures, sterile synthetic absorbable braided.....	878	Technetium (^{99m} Tc) colloidal sulphur injection.....	852
Sutures, sterile synthetic absorbable monofilament.....	880	Technetium (^{99m} Tc) colloidal tin injection.....	853
<i>Suxamethonii chloridum</i>	2525	Technetium (^{99m} Tc) etifenin injection.....	853
Suxamethonium chloride.....	2525	Technetium (^{99m} Tc) exametazime injection.....	854
Suxibuzone.....	2525	Technetium (^{99m} Tc) gluconate injection.....	856
<i>Suxibuzonum</i>	2525	Technetium (^{99m} Tc) human albumin injection.....	856
Sweet fennel.....	1580	Technetium (^{99m} Tc) macrosalb injection.....	858
Sweet orange oil.....	2526	Technetium (^{99m} Tc) medronate injection.....	859
Swelling index (2.8.4.).....	215	Technetium (^{99m} Tc) mertiatide injection.....	860
Swine erysipelas vaccine (inactivated).....	793	Technetium (^{99m} Tc) microspheres injection.....	861
Swine-fever vaccine (live), classical, freeze-dried.....	793	Technetium (^{99m} Tc) pentetate injection.....	862
Symbols and abbreviations (1.5.).....	9	Technetium (^{99m} Tc) sestamibi injection.....	863
Synthetic absorbable braided sutures, sterile.....	878	Technetium (^{99m} Tc) succimer injection.....	865
Synthetic absorbable monofilament sutures, sterile.....	880	Technetium (^{99m} Tc) tin pyrophosphate injection.....	865
Syringes, plastic, sterile single-use (3.2.8.).....	314	Temazepam.....	2535
Syrups.....	609	<i>Temazepamum</i>	2535
T		Tenosynovitis avian viral vaccine (live).....	729
Table of physical characteristics of radionuclides mentioned in the European Pharmacopoeia (5.7.).....	539	Tenoxicam.....	2537
Tablets.....	626	<i>Tenoxicamum</i>	2537
Tablets and capsules, disintegration of (2.9.1.).....	225	Terbutaline sulphate.....	2538
Tablets, buccal.....	613	<i>Terbutalini sulfas</i>	2538
Tablets, coated.....	627	Terconazole.....	2539
		<i>Terconazolum</i>	2539
		<i>Terebinthini aetheroleum ab pinum pinastrum</i>	2645
		Terfenadine.....	2540

<i>Terfenadinum</i>	2540	Thiamphenicol	2562
Terminology used in monographs on vaccines (5.2.1.)	453	<i>Thiamphenicolum</i>	2562
<i>tert-Butylamini perindoprilum</i>	2210	Thin-layer chromatography (2.2.27.).....	40
Test for anticomplementary activity of immunoglobulin (2.6.17.).....	170	Thiomersal.....	5.1-3021
Test for deliverable mass or volume of liquid and semi-solid preparations (2.9.28.).....	263	<i>Thiomersalum</i>	5.1-3021
Test for extractable volume of parenteral preparations (2.9.17.).....	243	Thiopental sodium and sodium carbonate.....	2564
Test for Fc function of immunoglobulin (2.7.9.).....	202	<i>Thiopentalum natricum et natrii carbonas</i>	2564
Test for methanol and 2-propanol (2.9.11.)	239	Thioridazine	2565
Test for neurovirulence of live virus vaccines (2.6.18.)	172	Thioridazine hydrochloride	2566
Test for neurovirulence of poliomyelitis vaccine (oral) (2.6.19.).....	172	<i>Thioridazini hydrochloridum</i>	2566
Testosterone.....	2542	<i>Thioridazinum</i>	2565
Testosterone enantate.....	2544	Three-lobed sage leaf.....	2390
Testosterone propionate.....	2545	Threonine.....	2566
<i>Testosteroni enantas</i>	2544	<i>Threoninum</i>	2566
<i>Testosteroni propionas</i>	2545	Thyme	2567
<i>Testosteronum</i>	2542	Thyme oil	2569
Tests for extraneous agents in viral vaccines for human use (2.6.16.)	169	Thyme, wild	2701
Tetanus antitoxin for human use	805	<i>Thymi aetheroleum</i>	2569
Tetanus antitoxin for veterinary use.....	5.1-2868	<i>Thymi herba</i>	2567
Tetanus, diphtheria and hepatitis B (rDNA) vaccine (adsorbed).....	641	Thymol.....	2570
Tetanus, diphtheria, pertussis (acellular, component), poliomyelitis (inactivated) and haemophilus type b conjugate vaccine (adsorbed).....	653	<i>Thymolum</i>	2570
Tetanus, diphtheria, pertussis and poliomyelitis (inactivated) vaccine (adsorbed).....	656	Tiabendazole.....	2570
Tetanus, diphtheria, pertussis, poliomyelitis (inactivated) and haemophilus type b conjugate vaccine (adsorbed)	657	<i>Tiabendazolum</i>	2570
Tetanus immunoglobulin, human	1751	Tiamulin for veterinary use	2571
Tetanus vaccine (adsorbed)	702	Tiamulin hydrogen fumarate for veterinary use	2573
Tetanus vaccine (adsorbed), assay of (2.7.8.)	5.1-2791	<i>Tiamulini hydrogenofumaras ad usum veterinarium</i> ..	2573
Tetanus vaccine for veterinary use	795	<i>Tiamulinum ad usum veterinarium</i>	2571
Tetracaine hydrochloride	2546	Tianeptine sodium	2575
<i>Tetracaini hydrochloridum</i>	2546	<i>Tianeptinum natricum</i>	2575
Tetracosactide.....	2547	Tiapride hydrochloride	2577
<i>Tetracosactidum</i>	2547	<i>Tiapridi hydrochloridum</i>	2577
Tetracycline	2549	Tiaprofenic acid.....	2578
Tetracycline hydrochloride	2551	Ticarcillin sodium.....	5.1-3021
<i>Tetracyclini hydrochloridum</i>	2551	<i>Ticarcillinum natricum</i>	5.1-3021
<i>Tetracyclinum</i>	2549	Tick-borne encephalitis vaccine (inactivated)	703
Tetrazepam.....	2552	Ticlopidine hydrochloride	2581
<i>Tetrazepamum</i>	2552	<i>Ticlopidini hydrochloridum</i>	2581
Tetryzoline hydrochloride.....	5.1-3020	<i>Tiliae flos</i>	1914
<i>Tetryzolini hydrochloridum</i>	5.1-3020	Tilidine hydrochloride hemihydrate	2582
<i>Thallosi (²⁰¹Tl) chloridi solutio iniectabilis</i>	867	<i>Tilidini hydrochloridum hemihydricum</i>	2582
Thallous (²⁰¹ Tl) chloride injection.....	867	<i>Timololi maleas</i>	2584
Theobromine.....	2554	Timolol maleate.....	2584
<i>Theobrominum</i>	2554	<i>Tincturae</i>	571
Theophylline	2554	<i>Tincturae maternas</i> <i>ad praeparationes homoeopathicas</i>	894
Theophylline-ethylenediamine	2557	Tinctures	571
Theophylline-ethylenediamine hydrate	2557	Tinidazole	2585
Theophylline monohydrate.....	2555	<i>Tinidazolum</i>	2585
<i>Theophyllinum</i>	2554	Tinnevely senna pods.....	2405
<i>Theophyllinum et ethylenediaminum</i>	2557	Tinzaparin sodium	2586
<i>Theophyllinum et ethylenediaminum hydricum</i>	2557	<i>Tinzaparinum natricum</i>	2586
<i>Theophyllinum monohydricum</i>	2555	Tioconazole	2586
Thermal analysis (2.2.34.)	52	<i>Tioconazolum</i>	2586
Thermogravimetry (2.2.34.).....	52	<i>Titanii dioxidum</i>	2587
Thiamazole	2558	Titanium dioxide	2587
<i>Thiamazolum</i>	2558	Titration, amperometric (2.2.19.).....	34
Thiamine hydrochloride	2559	Titration, potentiometric (2.2.20.)	35
Thiamine nitrate.....	2561	Titration, complexometric (2.5.11.)	130
<i>Thiamini hydrochloridum</i>	2559	Tobramycin.....	2588
<i>Thiamini nitras</i>	2561	<i>Tobramycinum</i>	2588
		α -Tocopherol acetate concentrate (powder form)	2592
		Tocopherol, all- <i>rac</i> - α	5.1-3023
		<i>α-Tocopheroli acetatis pulvis</i>	2592
		Tocopherol, <i>RRR</i> - α	2591
		Tocopheryl acetate, all- <i>rac</i> - α	5.1-3024
		Tocopheryl acetate, <i>RRR</i> - α	2595
		Tocopheryl hydrogen succinate, DL- α	2597
		Tocopheryl hydrogen succinate, <i>RRR</i> - α	5.1-3026
		Tolbutamide	2600

<i>Tolbutamidum</i>	2600	Tri- <i>n</i> -butyl phosphate	2631
Tolfenamic acid.....	2601	Tritiated (³ H) water injection.....	867
Tolnaftate	2602	<i>Tritici aestivi oleum raffinatum</i>	2699
<i>Tolnaftatum</i>	2602	<i>Tritici aestivi oleum virginale</i>	2699
Tolu balsam	2603	<i>Tritici amyllum</i>	2698
Tormentil	2604	Trolamine.....	2632
<i>Tormentillae rhizoma</i>	2604	<i>Trolaminum</i>	2632
<i>Tormentillae tinctura</i>	2605	Trometamol	2633
Tormentil tincture.....	2605	<i>Trometamolium</i>	2633
Tosylchloramide sodium.....	2605	Tropicamide.....	2634
<i>Tosylchloramidum natricum</i>	2605	<i>Tropicamidum</i>	2634
Total ash (2.4.16.).....	108	Trypsin	2635
Total organic carbon in water for pharmaceutical use (2.2.44.)	68	<i>Trypsinum</i>	2635
Total protein (2.5.33.)	138	Tryptophan.....	2636
Toxicity, abnormal (2.6.9.).....	153	<i>Tryptophanum</i>	2636
Toxin, botulinum type A for injection.....	1117	Tuberculin for human use, old.....	2638
<i>Toxinum botulinicum typum A ad iniectabile</i>	1117	<i>Tuberculini aviarii derivatum proteinosum</i> <i>purificatum</i>	2640
Tragacanth	2606	<i>Tuberculini bovini derivatum proteinosum</i> <i>purificatum</i>	2641
<i>Tragacantha</i>	2606	<i>Tuberculini derivatum proteinosum purificatum ad usum</i> <i>humanum</i>	2642
Tramadol hydrochloride	2607	Tuberculin purified protein derivative, avian	2640
<i>Tramadoli hydrochloridum</i>	2607	Tuberculin purified protein derivative, bovine.....	2641
Tramazoline hydrochloride monohydrate.....	2608	Tuberculin purified protein derivative for human use	2642
<i>Tramazolini hydrochloridum monohydricum</i>	2608	<i>Tuberculinum pristinum ad usum humanum</i>	2638
Tranexamic acid	2609	Tubes for comparative tests (2.1.5.)	19
Transdermal patches.....	616	Tubing and closures, silicone elastomer for (3.1.9.).....	288
Transdermal patches, dissolution test for (2.9.4.)	231	Tubing and containers for total parenteral nutrition preparations, poly(ethylene - vinyl acetate) for (3.1.7.) ...	285
Trapidil.....	2610	Tubing used in sets for the transfusion of blood and blood components, materials based on plasticised poly(vinyl chloride) for (3.1.1.2.)	272
<i>Trapidilum</i>	2610	Tubocurarine chloride	2644
Tretinoin	2611	<i>Tubocurarini chloridum</i>	2644
<i>Tretinoinum</i>	2611	Turmeric, javanese.....	2645
Triacetin	2612	Turpentine oil, Pinus pinaster type	2645
<i>Triacetinum</i>	2612	Tylosin for veterinary use	2647
Triamcinolone.....	2613	<i>Tylosini phosphatis solutio ad usum veterinarium</i>	2648
Triamcinolone acetoneide.....	2614	<i>Tylosini tartras ad usum veterinarium</i>	2650
Triamcinolone hexacetoneide	2616	Tylosin phosphate bulk solution for veterinary use	2648
<i>Triamcinoloni acetonidum</i>	2614	Tylosin tartrate for veterinary use	2650
<i>Triamcinoloni hexacetoneidum</i>	2616	<i>Tylosinum ad usum veterinarium</i>	2647
<i>Triamcinolonum</i>	2613	Typhoid polysaccharide vaccine	705
Triamterene	2617	Typhoid vaccine.....	707
<i>Triamterenum</i>	2617	Typhoid vaccine, freeze-dried.....	707
Tribenoside.....	2617	Typhoid vaccine (live, oral, strain Ty 21a).....	708
<i>Tribenosidum</i>	2617	Tyrosine.....	2651
Tributyl acetylcitrate	2619	<i>Tyrosinum</i>	2651
<i>Tributylis acetylcitras</i>	2619	Tyrothricin.....	5.1-3028
<i>Tricalcii phosphas</i>	1167	<i>Tyrothricinum</i>	5.1-3028
Trichloroacetic acid	2620		
Triethanolamine	2632		
Triethyl citrate	2620		
<i>Triethylis citras</i>	2620		
Trifluoperazine hydrochloride	2621		
<i>Trifluoperazini hydrochloridum</i>	2621		
Triflusal	2622		
<i>Triflusalum</i>	2622		
<i>Triglycerida saturata media</i>	2623		
Triglycerides, medium-chain.....	2623		
Triglycerides, omega-3-acid.....	2144		
<i>Trigonellae foenugraeci semen</i>	1588		
Trihexyphenidyl hydrochloride.....	2625		
<i>Trihexyphenidyli hydrochloridum</i>	2625		
Trimetazidine dihydrochloride.....	2626		
<i>Trimetazidini dihydrochloridum</i>	2626		
Trimethadione	2627		
<i>Trimethadionum</i>	2627		
Trimethoprim.....	2628		
<i>Trimethoprimum</i>	2628		
Trimipramine maleate	2630		
<i>Trimipramini maleas</i>	2630		
Tri- <i>n</i> -butylis phosphas.....	2631		

U

Ubidecarenone.....	2657
<i>Ubidecarenum</i>	2657
Udder-washes	631
Ultraviolet absorption spectrophotometry (2.2.25.).....	38
Ultraviolet ray lamps for analytical purposes (2.1.3.).....	17
Uncoated tablets.....	627
Undecylenic acid	2658
Uniformity of content of single-dose preparations (2.9.6.)	234
Uniformity of mass of delivered doses from multidose containers (2.9.27.).....	263
Uniformity of mass of single-dose preparations (2.9.5.)	233
Units of the International System (SI) used in the Pharmacopoeia and equivalence with other units (1.6.) ...	10
Unsaponifiable matter (2.5.7.).....	129
Urea.....	2658

<i>Ureum</i>	2658	<i>Vaccinum clostridii septici ad usum veterinarium</i>	749
<i>Urofollitropin</i>	2659	<i>Vaccinum colibacillosis fetus a partu recentis inactivatum ad ruminantes</i>	778
<i>Urofollitropinum</i>	2659	<i>Vaccinum colibacillosis fetus a partu recentis inactivatum ad suem</i>	776
<i>Urokinase</i>	2661	<i>Vaccinum diarrhoeae viralis bovinae inactivatum</i>	734
<i>Uronic acids in polysaccharide vaccines (2.5.22.)</i>	133	<i>Vaccinum diphtheriae adsorbatum</i>	660
<i>Ursodeoxycholic acid</i>	2662	<i>Vaccinum diphtheriae adulti et adolescentis adsorbatum</i>	661
<i>Urtica dioica ad praeparationes homoeopathicas</i>	895	<i>Vaccinum diphtheriae et tetani adsorbatum</i>	639
<i>Urticae folium</i>	5.1-2980	<i>Vaccinum diphtheriae et tetani adulti et adolescentis adsorbatum</i>	639
<i>Uvae ursi folium</i>	1054	<i>Vaccinum diphtheriae, tetani et hepatitis B (ADNr) adsorbatum</i>	641
V		<i>Vaccinum diphtheriae, tetani et pertussis adsorbatum</i> ..	643
<i>Vaccina ad usum humanum</i>	5.1-2835	<i>Vaccinum diphtheriae, tetani et pertussis sine cellulis ex elementis praeparatum adsorbatum</i>	642
<i>Vaccina ad usum veterinarium</i>	590	<i>Vaccinum diphtheriae, tetani, pertussis et poliomyelitidis inactivatum adsorbatum</i>	656
<i>Vaccines, adsorbed, aluminium in (2.5.13.)</i>	131	<i>Vaccinum diphtheriae, tetani, pertussis, poliomyelitidis inactivatum et haemophili stirpe b coniugatum adsorbatum</i>	657
<i>Vaccines, adsorbed, calcium in (2.5.14.)</i>	131	<i>Vaccinum diphtheriae, tetani, pertussis sine cellulis ex elementis praeparatum et haemophili stirpe b coniugatum adsorbatum</i>	645
<i>Vaccines and immunosera, phenol in (2.5.15.)</i>	131	<i>Vaccinum diphtheriae, tetani, pertussis sine cellulis ex elementis praeparatum et hepatitis B (ADNr) adsorbatum</i>	647
<i>Vaccines and immunosera, veterinary, evaluation of efficacy (5.2.7.)</i>	5.1-2829	<i>Vaccinum diphtheriae, tetani, pertussis sine cellulis ex elementis praeparatum et poliomyelitidis inactivatum adsorbatum</i>	648
<i>Vaccines and immunosera, veterinary, evaluation of safety (5.2.6.)</i>	5.1-2827	<i>Vaccinum diphtheriae, tetani, pertussis sine cellulis ex elementis praeparatum, hepatitis B (ADNr), poliomyelitidis inactivatum et haemophili stirpe b coniugatum adsorbatum</i>	650
<i>Vaccines and immunosera, veterinary, evaluation of the safety of each batch (5.2.9.)</i>	5.1-2830	<i>Vaccinum diphtheriae, tetani, pertussis sine cellulis ex elementis praeparatum poliomyelitidis inactivatum et haemophili stirpe b coniugatum adsorbatum</i>	653
<i>Vaccines for human use</i>	5.1-2835	<i>Vaccinum encephalitis ixodibus advectae inactivatum</i>	703
<i>Vaccines for human use, cell substrates for the production of (5.2.3.)</i>	455	<i>Vaccinum encephalomyelitis infectivae aviariae vivum</i>	725
<i>Vaccines for human use, viral, extraneous agents in (2.6.16.)</i>	169	<i>Vaccinum erysipellatis suillae inactivatum</i>	793
<i>Vaccines for veterinary use</i>	590	<i>Vaccinum febris flavae vivum</i>	5.1-2852
<i>Vaccines, polysaccharide, hexosamines in (2.5.20.)</i>	132	<i>Vaccinum febris typhoidi</i>	707
<i>Vaccines, polysaccharide, methylpentoses in (2.5.21.)</i>	133	<i>Vaccinum febris typhoidi cryodesiccatum</i>	707
<i>Vaccines, polysaccharide, nucleic acids in (2.5.17.)</i>	132	<i>Vaccinum febris typhoidis polysaccharidicum</i>	705
<i>Vaccines, polysaccharide, O-acetyl in (2.5.19.)</i>	132	<i>Vaccinum febris typhoidis vivum perorale (stirpe Ty 21a)</i>	708
<i>Vaccines, polysaccharide, phosphorus in (2.5.18.)</i>	132	<i>Vaccinum furunculosis ad salmonidas inactivatum cum adiuvatione oleosa ad iniectionem</i>	767
<i>Vaccines, polysaccharide, protein in (2.5.16.)</i>	131	<i>Vaccinum haemophili stirpe b coniugatum</i>	662
<i>Vaccines, polysaccharide, ribose in (2.5.31.)</i>	137	<i>Vaccinum hepatitis A inactivatum adsorbatum</i>	665
<i>Vaccines, polysaccharide, sialic acid in (2.5.23.)</i>	133	<i>Vaccinum hepatitis A inactivatum et hepatitis B (ADNr) adsorbatum</i>	664
<i>Vaccines, polysaccharide, uronic acids in (2.5.22.)</i>	133	<i>Vaccinum hepatitis A inactivatum virosomale</i>	667
<i>Vaccines, SPF chicken flocks for the production and quality control of (5.2.2.)</i>	5.1-2825	<i>Vaccinum hepatitis B (ADNr)</i>	670
<i>Vaccines, terminology (5.2.1.)</i>	453	<i>Vaccinum hepatitis viralis anatis stirpe I vivum</i>	751
<i>Vaccines, veterinary, cell cultures for the production of (5.2.4.)</i>	458	<i>Vaccinum herpesvirus equini inactivatum</i>	754
<i>Vaccines, veterinary, substances of animal origin for the production of (5.2.5.)</i>	460	<i>Vaccinum inactivatum diarrhoeae vituli coronavirum illatae</i>	736
<i>Vaccines, viral live, test for neurovirulence (2.6.18.)</i>	172	<i>Vaccinum inactivatum diarrhoeae vituli rotavirus illatae</i>	737
<i>Vaccinum actinobacillosis inactivatum ad suem</i>	784	<i>Vaccinum influenzae equi inactivatum</i>	755
<i>Vaccinum adenovirus caninae vivum</i>	738	<i>Vaccinum influenzae inactivatum ad suem</i>	785
<i>Vaccinum adenovirus caninae inactivatum</i>	738	<i>Vaccinum influenzae inactivatum ex corticis antigeniis praeparatum</i>	673
<i>Vaccinum anaemiae infectivae pulli vivum</i>	769	<i>Vaccinum influenzae inactivatum ex corticis antigeniis praeparatum virosomale</i>	674
<i>Vaccinum anthracis vivum ad usum veterinarium</i>	715		
<i>Vaccinum aphtharum epizooticarum inactivatum ad ruminantes</i>	5.1-2860		
<i>Vaccinum bronchitis infectivae aviariae inactivatum</i>	718		
<i>Vaccinum bronchitis infectivae aviariae vivum</i>	720		
<i>Vaccinum brucellosis (Brucella melitensis stirpe Rev. 1) vivum cryodesiccatum ad usum veterinarium</i>	5.1-2859		
<i>Vaccinum bursitis infectivae aviariae inactivatum</i>	722		
<i>Vaccinum bursitis infectivae aviariae vivum</i>	723		
<i>Vaccinum calicivirus felinae inactivatum</i>	757		
<i>Vaccinum calicivirus felinae vivum cryodesiccatum</i> ..	758		
<i>Vaccinum cholerae</i>	637		
<i>Vaccinum cholerae aviariae inactivatum</i>	5.1-2861		
<i>Vaccinum cholerae cryodesiccatum</i>	638		
<i>Vaccinum clostridii botulini ad usum veterinarium</i>	745		
<i>Vaccinum clostridii chauvoei ad usum veterinarium</i>	745		
<i>Vaccinum clostridii novyi B ad usum veterinarium</i>	746		
<i>Vaccinum clostridii perfringentis ad usum veterinarium</i>	747		

<i>Vaccinum influenzae inactivatum ex viris integris praeparatum</i>	676	<i>Vaccinum variolae gallinae vivum</i>	766
<i>Vaccinum influenzae inactivatum ex virorum fragmentis praeparatum</i>	671	<i>Vaccinum vibriosidis ad salmonidas inactivatum</i>	797
<i>Vaccinum laryngotracheitidis infectivae aviariae vivum</i>	727	<i>Vaccinum vibriosidis aquae frigidae inactivatum ad salmonidas</i>	796
<i>Vaccinum leptospirosis boviniae inactivatum</i>	730	<i>Vaccinum viri syncytialis meatus spiritus bovini vivum cryodesiccatum</i>	733
<i>Vaccinum leptospirosis caninae inactivatum</i>	740	Vaginal capsules	629
<i>Vaccinum leucosis felinae inactivatum</i>	761	Vaginal foams	630
<i>Vaccinum mannheimiae inactivatum ad bovidas</i>	771	<i>Vaginalia</i>	629
<i>Vaccinum mannheimiae inactivatum ad ovem</i>	772	Vaginal preparations	629
<i>Vaccinum meningococcale classis C coniugatum</i>	680	Vaginal preparations, semi-solid	630
<i>Vaccinum meningococcale polysaccharidicum</i>	682	Vaginal solutions and suspensions, tablets for.....	630
<i>Vaccinum morbi Aujeszkyi ad suem inactivatum</i>	715	Vaginal solutions, emulsions and suspensions.....	630
<i>Vaccinum morbi Aujeszkyi ad suem vivum cryodesiccatum ad usum parenterale</i>	717	Vaginal tablets	629
<i>Vaccinum morbi Carrei vivum cryodesiccatum ad canem</i>	740	Vaginal tampons, medicated.....	630
<i>Vaccinum morbi Carrei vivum cryodesiccatum ad mustelidas</i>	751	<i>Valerianae radix</i>	2667
<i>Vaccinum morbillorum, parotitidis et rubellae vivum</i>	678	Valerian root.....	2667
<i>Vaccinum morbillorum vivum</i>	679	Validation of nucleic acid amplification techniques for the detection of hepatitis C virus (HCV) RNA in plasma pools: Guidelines.....	176
<i>Vaccinum morbi Marek vivum</i>	774	Valine	2668
<i>Vaccinum morbi partus diminutionis MCMLXXVI inactivatum ad pullum</i>	753	<i>Valinum</i>	2668
<i>Vaccinum myxomatosis vivum ad cuniculum</i>	775	Valproic acid.....	2669
<i>Vaccinum panleucopeniae felinae infectivae inactivatum</i>	759	Vancomycin hydrochloride	2670
<i>Vaccinum panleucopeniae felinae infectivae vivum</i>	760	<i>Vancomycini hydrochloridum</i>	2670
<i>Vaccinum parainfluenzae viri bovini vivum cryodesiccatum</i>	732	Vanillin	2672
<i>Vaccinum parainfluenzae viri canini vivum</i>	742	<i>Vanillinum</i>	2672
<i>Vaccinum paramyxovirus 3 aviarii inactivatum</i>	728	Varicella immunoglobulin for intravenous administration, human	1753
<i>Vaccinum parotitidis vivum</i>	684	Varicella immunoglobulin, human	1752
<i>Vaccinum parvovirus caninae inactivatum</i>	743	Varicella vaccine (live).....	709
<i>Vaccinum parvovirus caninae vivum</i>	744	<i>Vaselinum album</i>	2187
<i>Vaccinum parvovirus inactivatum ad suem</i>	787	<i>Vaselinum flavum</i>	2188
<i>Vaccinum pasteurellae inactivatum ad ovem</i>	783	Vegetable drugs, determination of essential oils in vegetable drugs (2.8.12.)	217
<i>Vaccinum pertussis</i>	685	Vegetable fatty oils.....	595
<i>Vaccinum pertussis adsorbatum</i>	690	Verapamil hydrochloride	2673
<i>Vaccinum pertussis sine cellulis copurificatum adsorbatum</i>	688	<i>Verapamili hydrochloridum</i>	2673
<i>Vaccinum pertussis sine cellulis ex elementis praeparatum adsorbatum</i>	686	<i>Verbasci flos</i>	2065
<i>Vaccinum pestis classicae suillae vivum cryodesiccatum</i>	793	Veterinary liquid preparations for cutaneous application ..	630
<i>Vaccinum pneumococcale polysaccharidicum</i>	691	Vibriosis (cold-water) vaccine (inactivated) for salmonids ..	796
<i>Vaccinum pneumococcale polysaccharidicum coniugatum adsorbatum</i>	5.1-2851	Vibriosis vaccine (inactivated) for salmonids	797
<i>Vaccinum poliomyelitis inactivatum</i>	692	VICH (5.8.).....	551
<i>Vaccinum poliomyelitis perorale</i>	695	Vinblastine sulphate.....	2675
<i>Vaccinum pseudopestis aviariae inactivatum</i>	779	<i>Vinblastini sulfas</i>	2675
<i>Vaccinum pseudopestis aviariae vivum</i>	781	Vincristine sulphate.....	2675
<i>Vaccinum rabiei ex cellulis ad usum humanum</i>	699	<i>Vincristini sulfas</i>	2675
<i>Vaccinum rabiei inactivatum ad usum veterinarium</i>	790	Vindesine sulphate	2677
<i>Vaccinum rabiei perorale vivum ad vulpem</i>	792	<i>Vindesini sulfas</i>	2677
<i>Vaccinum rhinitidis atrophicantis ingravescentis suillae inactivatum</i>	788	Vinorelbine tartrate	5.1-3033
<i>Vaccinum rhinotracheitidis infectivae boviniae vivum cryodesiccatum</i>	768	<i>Vinorelbini tartras</i>	5.1-3033
<i>Vaccinum rhinotracheitidis viralis felinae inactivatum</i> ..	762	<i>Violae herba cum flore</i>	2700
<i>Vaccinum rhinotracheitidis viralis felinae vivum cryodesiccatum</i>	763	Viper venom antiserum, European	806
<i>Vaccinum rubellae vivum</i>	701	Viscometer method, capillary (2.2.9.).....	29
<i>Vaccinum tenosynovitis viralis aviariae vivum</i>	729	Viscometer method, falling ball (2.2.49.).....	80
<i>Vaccinum tetani adsorbatum</i>	702	Viscometer method, rotating (2.2.10.).....	30
<i>Vaccinum tetani ad usum veterinarium</i>	795	Viscose wadding, absorbent	2681
<i>Vaccinum tuberculosis (BCG) cryodesiccatum</i>	636	Viscosity (2.2.8.)	29
<i>Vaccinum varicellae vivum</i>	709	Visible absorption spectrophotometry (2.2.25.).....	38
		Visible particles, particulate contamination (2.9.20.)	255
		Vitamin A	2682
		Vitamin A concentrate (oily form), synthetic	2684
		Vitamin A concentrate (powder form), synthetic.....	2685
		Vitamin A concentrate (solubilisate/emulsion), synthetic	2686
		<i>Vitaminum A</i>	2682
		<i>Vitaminum A densatum oleosum</i>	2684
		<i>Vitaminum A in aqua dispergibile</i>	2686
		<i>Vitaminum A pulvis</i>	2685

Volumetric analysis (4.2.)	435	X-ray fluorescence spectrometry (2.2.37.).....	56
Volumetric solutions (4.2.2.).....	435	Xylazine hydrochloride for veterinary use	2716
Volumetric solutions, primary standards for (4.2.1.)	435	<i>Xylazine hydrochloridum ad usum veterinarium</i>	2716
Von Willebrand factor, human, assay of (2.7.21.).....	211	Xylitol	2717
W		<i>Xylitolum</i>	2717
Warfarin sodium.....	2691	Xylometazoline hydrochloride	2719
Warfarin sodium clathrate	2691	<i>Xylometazolini hydrochloridum</i>	2719
<i>Warfarinum natricum</i>	2691	Xylose.....	2720
<i>Warfarinum natricum clathratum</i>	2691	<i>Xylosum</i>	2720
Water (¹⁵ O) injection.....	868	Y	
Water, determination by distillation (2.2.13.).....	32	Yarrow	2723
Water for diluting concentrated haemodialysis solutions	1699	Yellow beeswax.....	1058
Water for injections.....	2692	Yellow fever vaccine (live)	5.1 -2852
Water for pharmaceutical use, total organic carbon in (2.2.44.).....	68	Yellow soft paraffin.....	2188
Water, highly purified	2695	Z	
Water in essential oils (2.8.5.)	216	Zidovudine.....	5.1 -3043
Water in gases (2.5.28.)	136	<i>Zidovudinum</i>	5.1 -3043
Water: micro determination (2.5.32.).....	137	Zinc acetate dihydrate.....	2728
Water, purified.....	2697	Zinc acexamate	2729
Water: semi-micro determination (2.5.12.).....	130	Zinc chloride	2730
Wheat-germ oil, refined	2699	<i>Zinci acetat dihydricus</i>	2728
Wheat-germ oil, virgin.....	2699	<i>Zinci acexas</i>	2729
Wheat starch	2698	<i>Zinci chloridum</i>	2730
White beeswax.....	1057	<i>Zinci oxidum</i>	2731
White horehound.....	5.1 -3039	<i>Zinci stearas</i>	2732
White soft paraffin.....	2187	<i>Zinci sulfas heptahydricus</i>	2732
Wild pansy (flowering aerial parts).....	2700	<i>Zinci sulfas hexahydricus</i>	2733
Wild thyme	2701	<i>Zinci undecylenas</i>	2733
Willow bark	2702	Zinc oxide.....	2731
Wool alcohols.....	2703	Zinc stearate.....	2732
Wool fat	2704	Zinc sulphate heptahydrate	2732
Wool fat, hydrogenated.....	2708	Zinc sulphate hexahydrate.....	2733
Wool fat, hydrous.....	2709	Zinc undecylenate	2733
Wormwood	2710	<i>Zingiberis rhizoma</i>	1656
X		<i>Zolpidemi tartras</i>	2734
Xanthan gum	2715	Zolpidem tartrate.....	2734
<i>Xanthani gummi</i>	2715	Zopiclone	2735
Xenon (¹³³ Xe) injection.....	869	<i>Zopiclonum</i>	2735
<i>Xenoni (¹³³Xe) solutio iniectabilis</i>	869	Zuclopenthixol decanoate	2736
		<i>Zuclopenthixoli decanoas</i>	2736